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“Phytochemical profiling and evaluation of the anti-aging and anti-inflammatory potential of cultivated *Eclipta alba* (L.)” **Le Cabec, A.** ; Messaili, S. ; Colas, C. ; Campos, P.-E. ; Destandau, E. *South African Journal of Botany* 2023, 163, 595-608.

“Enhancement of Forskolin Production Using Aeroponic Cultivation of *Coleus forskohlii* and the Impact on the Plant Phytochemistry.” **Le Cabec, A.**; Campos, P.-E.; Yzebe, O.; Pelé, R.; Colas, C.; Destandau, E. *Molecules* 2024, 29, 4215.

Manuscrits en cours de préparation :

“Traditional uses, pharmacological activities and phytochemistry of *Clematis* genus: an updated review” Le Cabec, A. ; Campos, P.-E. ; Destandau, E.

« Multi-informative molecular network including taxonomic metadata for chemical profiling of ten *Clematis* species” Le Cabec, A. ; Campos, P.-E. ; Allard, P-M. ; Brühlhart, E. ; Defossez, E. ; Destandau, E.

“Bioactive Molecular Networking for identifying the anti-inflammatory compounds of *Clematis* species inhibiting the JAKs-STATs pathway” Le Cabec, A. ; Campos, P.-E. ; Allard, P-M.; Destandau, E.

“Phytochemical exploration and biological evaluation of four *Thymus* L. species from Türkiye” Özdemir Nath E., Campos, P.-E., Le Cabec, A.; İpek Gündoğan, G., Ergin, D. ; Çetin A. ; Sönmez, S. ; Destandau E.

Conférences invitées dans un congrès international

“Phytochemical study of ornamental plants cultivated in Centre-Val de Loire, France” Le Cabec, A. ; Colas, C. ; Campos, P.-E. ; Allard, P-M. ; Yzebe, O. ; Destandau, E. 6ème Symposium International de l’AFERP, Juil. 2023 - Orsay.

Communications orales dans un congrès international

“Exploration of the phytochemical diversity of ornamental plants in a co-valorisation objective” **Le Cabec, A.** ; Campos, P.-E. ; Allard, P-M. ; Brühlhart, E. ; Defossez, E. ; Destandau, E. Le Studium conferences : Plant rediscovery with advanced tools for well-being applications, Oct. 2023 - Orléans.

Communications orales dans un congrès national

“Etude métabolomique de plantes ornementales dans un objectif de valorisation” **Le Cabec, A.** ; Campos, P.-E. ; Allard, P-M. ; Brühlhart, E. ; Defossez, E. ; Destandau, E. Biotechnocentre. Oct. 2023 - Nouan-le-Fuzelier.

“Activités antioxydante et anti-collagénase d’*Eclipta alba*” **Le Cabec, A.** ; Messaili, S. ; Colas, C. ; Campos, P.-E. ; Destandau, E. 13e journées du Réseau MétaSP mai 2022 - Saint-Etienne.

Communications par poster dans un congrès international

“From the production of targeted metabolites to the complete valorization of *Coleus forskohlii* Briq. using aeroponic cultivation” **Le Cabec, A.** ; Campos, P.-E. ; Colas, C. ; Yzebe, O. ; Destandau, E. International Congress on Natural Products Research – ICNPR. Juil. 2024 - Krakow (Poland).

“Development of non-targeted metabolomic approaches for the screening of bioactive molecules in *Clematis* species” **Le Cabec, A.** ; Campos, P.-E. ; Allard, P.-M. ; Brühlhart, E. ; Defossez, E. ; Destandau, E. International Congress on Natural Products Research – ICNPR. juil. 2024 - Krakow (Poland).

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“Metabolomics study of ornamental plants in a valorization objective” **Le Cabec, A.** ; Brühlhart, E. ; Campos, P.-E. ; Defossez, E. ; Allard, P.-M. ; Destandau, E. Cosm'ing. Juil. 2023 - St-Malo.

“Phytochemical study of bioactive plants cultivated in Centre-Val de Loire, France” **Le Cabec, A.** ; Colas, C. ; Yzebe, O. ; Campos, P.-E. ; Destandau, E. Trends in Natural Products Research: A PSE Young Scientists' Meeting. Juin 2023 - Paris.

“Phytochemical study of bioactive plants cultivated in Centre-Val de Loire, France” **Le Cabec, A.** ; Colas, C. ; Yzebe, O. ; Campos, P.-E. ; Destandau, E. COSM'INNOV - 5th edition -The International Congress for Cosmetics Research. Mai 2023 - Orléans.

“Anti-aging and anti-inflammatory properties of *Eclipta alba*” **Le Cabec, A.** ; Messaili, S. ; Colas, C. ; Campos, P.-E. ; Destandau, E. Analytics 2022. Sept. 2022 - Nantes.

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“Identification and characterization of anti-collagenase compounds from *Eclipta alba*” **Le Cabec, A.** ; Messaili, S. ; Colas, C. ; Campos, P.-E. ; Destandau, E. École thématique Métabiodivex. Juin 2023 - Aussois.

“Anti-aging and anti-inflammatory properties of *Eclipta alba*” **Le Cabec, A.** ; Messaili, S. ; Colas, C. ; Campos, P.-E. ; Destandau, E. Biotechnocentre. Oct. 2022 - Nouan-le-Fuzelier.

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“Identification et caractérisation de composés anti-collagénase et anti-cyclooxygénase à partir d’*Eclipta alba*” Le Cabec, A. ; Messaili, S. ; Colas, C. ; Campos, P.-E. ; Destandau, E. AFERP Juil. 2022 - e-conference.

Webinaires

“Exploration de la diversité phytochimique du genre *Clematis* à travers l'utilisation d'outils de métabolomique” **Le Cabec, A.** ; Campos, P.-E. ; Allard P.-M. ; Brühlhart E. ; Defossez E. ; Destandau E. AFERP Fév. 2024 – e-conference

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Liste des abréviations

A2780 cells : Human ovarian cancer cells	DMA : Diméthylamine
A375 cells : Human melanoma cells	DNP : Dictionnaire des produits naturels
A549 cells : Human lung cancer cells	DPPH : 2,2-diphenyl-1-picryl-hydrazyl assay
ABTS : Diammonium 2,2'-azinobis[3-ethyl-2,3-dihydrobenzothiazole-6-sulphonate assay	DWL : Demethylwedelolactone
ACN : Acétonitrile	EA : Ethyl Acetate
ACP (PCA) : Analyse en composante principale (Principal component analysis)	EIC : Extracted Ion Chromatogram
AIA : Adjuvant-induced arthritis	ESI : Ionization par électronébulisation (Ionisation Electrospray)
ANOVA : Analysis of variance	EtOH : Ethanol
AP : Aqueous phase	FBMN : Feature Based Molecular Network
APCI : Ionisation chimique à pression atmosphérique	FRAP : Ferric Reducing Antioxidant Power
APPI : Photoionisation à pression atmosphérique	FWHM : Full-Width Half Maximum
BPC : Base Peak Chromatogram	GC : Gaz Chromatography
BV-2 cells : Microglial cells	GES-1 : Normal gastric mucosal epithelial cells lines
CAT : Catalase	GNPS : Global Natural Products Social Network
CCS : Collision Cross Section	GSH : Glutathione
CDHRC : Comité de Développement Horticole de la Région Centre-Val de Loire	HCD : Higher Energy Collisional Dissociation
CHOK1 cells : Ovarian cells	HCT-116 cells : Human colon cancer cells
CIA : Collagen-induced arthritis	HEP-G2 cells : Human liver cancer cells
CID : Dissociation induite par collision	HESI : Heated electrospray
COX-2 : Cyclooxygénase-2	HGC-27 cells : Gastric cancer cells
CPC : Chromatographie de partage centrifuge	HPLC : High Performance Liquid Chromatography
CUPRAC : Cupric ion Reducing Capability	HRBC cells : Human red blood cells
DAD : Diode array detector	HRMS : Spectrométrie de masse haute résolution
DDA : Acquisition dépendante des données	IIMN : Ion identity Molecular Network
DEDL (ELSD) : Détecteur Évaporatif à Diffusion de la Lumière	IL : Interleukin
DIA : Acquisition indépendant des données	JAK3 : Janus Kinase 3

K_D : Coefficient de partage LC : Liquid Chromatography LLE : Liquid/Liquid extraction LPS : Lipopolysaccharide MAPK : Mitogen-activated protein kinases MDA : Malondialdehyde MeOH : Methanol MGC-803 : Gastric cancer cells lines MIC : Minimum inhibitory concentration MMPs : Métalloprotéases matricielles MN : Molecular Network MS : Spectrométrie de masse <i>full scan</i> MS/MS : Spectrométrie de masse en tandem NA : Non-active NO : Nitric oxide NP : Natural product OP : Organic phase ORAC : Oxygen radical Absorbance Capacity assay OT : Orbitrap OVCAR-3 : Human ovarian carcinoma cells lines Ox-LDL : Oxidized low-density lipoprotein PGE2 : Prostaglandin E2 PLS-DA : Partial Least Squares Discriminant Analysis QC : Quality control RCVL (CVLR) : Région Centre-Val de Loire RDB : Ring and Double Bond equivalent RE : Raw Extract	RMN : Résonnance magnétique nucléaire ROS : Espèce réactive à l'oxygène RPLC : Reverse phase liquid chromatography SFC : Supercritical Fluid Chromatography SH-SY5Y : Human neuroblastoma cells SK-OV-3 : Ovarian adenocarcinoma cells lines SM : Spectrométrie de masse SOD : Superoxide Dismutase STAT : Signal Transducers and Activators of Transcription TCM : Médecine Traditionnelle Chinoise TNF-α : Tumor necrosis factor α TOF : Time-Of-Flight analyzer T_R : Temps de rétention t-SNE : t-distributed stochastic neighbor embedding UHPLC : Ultra-High Performance Liquid Chromatographie UV : Ultraviolet VEGF : Vascular endothelial growth factor VIP : Variable importance of projection V_m : Volume de phase mobile V_R : Volume de rétention V_s : Volume de phase stationnaire WE : Water-Ethanol mixture WL : Wedelolactone XO : Xanthine Oxidase

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INTRODUCTION GENERALE

Depuis toujours, l'Homme a utilisé les plantes pour leurs propriétés médicinales. Ces usages, répertoriés dans diverses pharmacopées traditionnelles, constituent la base de médecines comme l'ayurvéda et la médecine traditionnelle chinoise (TCM). Ces médecines traditionnelles, basées sur l'expérience acquise au cours de plusieurs siècles voir millénaires, sont ainsi vues comme une source d'inspiration pour la découverte de molécules bioactives pouvant être utilisées par la médecine moderne^{1,2}. C'est ainsi qu'à partir d'espèces végétales telles que *Papaver somniferum*, *Digitalis purpurea*, *Cinchona officinalis* et *Atropa belladonna*, des composés bioactifs ont été isolés, conduisant respectivement à la découverte de la morphine, de la digitoxine, de la quinine et de l'atropine, des molécules encore largement utilisées en pharmacologie. L'extrait d'*Atropa belladonna*, par exemple, était historiquement utilisée à la Renaissance par les femmes italiennes pour dilater leurs pupilles pour des raisons esthétiques, l'atropine contenu dans l'extrait est encore employée en ophtalmologie de nos jours³. Ces molécules font également partie de la liste des médicaments essentiels publiée par l'OMS (<https://list.essentialmeds.org>).

Les plantes représentent donc une source précieuse de molécules bioactives et ce, pour un large éventail d'applications dans des domaines tels que la pharmaceutique⁴, la cosmétique⁵⁻⁷ ou le bio-contrôle⁸. Ces bioactivités sont, pour la plupart, dues à la présence de **métabolites spécialisés** qui sont généralement des molécules de faible poids moléculaire (< 1000 ou 1500 Da), et principalement produites par des organismes tels que les plantes, les micro-organismes ou les organismes marins en tant que molécules de défense, d'attraction, de communication ou de symbiose^{3,9}. A ce jour, on estime à plus de 700 000 le nombre de molécules naturelles identifiées, mais de nombreux produits naturels restent encore à découvrir, notamment grâce à l'exploration de différents organes, espèces ou genres d'organismes peu ou pas étudiés, ainsi qu'aux avancées technologiques dans plusieurs domaines¹⁰.

La problématique du sourcing des plantes pour l'identification de molécules actives est un sujet complexe¹¹. Tout d'abord, la disponibilité des plantes peut être limitée en raison du déclin de la biodiversité et l'extinction d'espèces. La liste rouge de l'UICN (Union internationale pour la conservation de la nature ; *IUCN* en anglais), référence l'état de conservation de plus de 160 000 espèces, dont plus de 45 000 sont menacées, avec près de 6 000 en danger critique. De plus, l'exploitation excessive de certaines espèces à l'état sauvage peut avoir des risques sur sa disponibilité¹². Par exemple, l'*Aniba rosaeodora*, un arbre que l'on retrouve principalement dans les forêts tropicales du Brésil est fortement exploité pour la production d'huile essentielle de bois de rose, utilisée en parfumerie et cosmétique. Cette espèce est maintenant classée en danger, ce qui a conduit à la mise en place de plantations d'*Aniba rosaeodora*, afin de réduire la pression sur la population sauvage¹³.

La culture de plantes d'intérêt pour la santé et le bien-être, à l'échelle locale permettrait de réduire la pression sur les ressources sauvages tout en garantissant une bonne traçabilité et une diminution de l'impact environnemental dû au transport de ces espèces. Cependant, cultiver des plantes sauvages et encore plus sous un autre climat reste un défi non seulement en termes de production de biomasse mais également en termes de production des métabolites spécialisés d'intérêt. En parallèle, il est également essentiel de valoriser les plantes déjà cultivées sur le territoire, notamment à travers la valorisation de co- ou sous-produit. Cela permet de renforcer l'utilisation de ressources locales déjà disponibles, de réduire la dépendance à l'exploitation d'espèce sauvages menacées et/ou lointaines et de monter des filières d'économie circulaire afin de valoriser l'intégralité de la ressource.

Il est donc crucial de développer et d'appliquer des techniques analytiques nouvelles pour explorer la phytochimie des plantes et maximiser le potentiel de ces ressources naturelles^{4,14}. Afin d'identifier les métabolites spécialisés au sein de mélanges complexes, différents outils analytiques et techniques instrumentales peuvent être employés en fonction des caractéristiques du mélange, d'autant plus que les produits naturels couvrent un large espace chimique^{15,16}.

Le développement des techniques d'analyse chromatographique et spectroscopique permet d'étudier sans cesse une gamme plus large de métabolites. En effet, les progrès de la chromatographie liquide, technique de séparation la plus utilisée dans ce domaine, ont permis la purification de molécules issues de mélanges de plus en plus complexes, tandis que les quantités nécessaires à l'élucidation structurale n'ont cessé de diminuer grâce aux progrès de la spectrométrie de masse (MS) et de la spectroscopie de résonance magnétique nucléaire (RMN).

En pharmacognosie, l'identification de molécules bioactives repose classiquement sur le **fractionnement bio-guidé**. Ce processus consiste à fractionner, par différentes méthodes chromatographiques, des extraits bruts préalablement identifiés comme actifs. Les fractions obtenues sont à nouveau soumises à des tests, et les fractions actives sont purifiées afin d'isoler la ou les molécules responsables de l'activité. Bien que ce procédé puisse être long, il s'avère efficace et a mené à l'identification de molécules actives connues telle que l'artémisinine, un des principaux antipaludiques utilisés à travers le monde actuellement et isolée d'*Artemisia annua*, utilisée en médecine traditionnelle chinoise depuis des millénaires pour ses propriétés antipyrétiques et antipaludiques¹⁷. Cette méthode comporte cependant des limites ; certaines molécules actives peuvent ne pas être identifiées si elles sont présentes en trop faible quantité, si leur action est masquée par d'autres molécules ou si l'effet est dû à une synergie entre plusieurs molécules plutôt qu'à une molécule seule¹⁸. De plus, le nombre

de molécules naturelles décrites augmentant continuellement, la probabilité de purifier à nouveau un composé déjà connu est de plus en plus élevée.

Afin d'accélérer ce processus, les workflows (ou flux de travail) de **déréplication**, c'est-à-dire le fait de différencier les molécules déjà connues des nouvelles, se développent. La déréplication permet ainsi d'identifier des molécules déjà reconnues pour l'activité recherchée, de découvrir une nouvelle activité biologique pour des molécules connues pour une autre activité, ou encore de révéler des molécules nouvelles, qu'elles soient actives ou non. Il est donc indispensable d'avoir une connaissance de ce qui est déjà connu en faisant appel à la littérature et aux bases de données.

Parmi les techniques analytiques rencontrées pour la déréplication, la **résonance magnétique nucléaire** (RMN) et la **spectrométrie de masse** (SM) sont les plus employées. La RMN, technique classiquement utilisée pour l'élucidation structurale de molécules individuelles, permet également, grâce aux innovations techniques et informatiques ces quinze dernières années, l'identification des petites molécules majoritaires au sein de mélanges complexes^{19,20}. La spectrométrie de masse est également une technique largement utilisée pour la déréplication de produits naturels. Cette technique à l'avantage d'être rapide mais surtout très sensible, ce qui permet de travailler sur de petites quantités et d'identifier des composés minoritaires. La possibilité d'ajouter une dimension séparative en amont du spectromètre de masse, avec l'utilisation de la chromatographie en phase liquide, gazeuse ou supercritique en fonction de la polarité et de la volatilité des composés étudiés est également un atout majeur pour l'analyse de mélanges complexes. Malgré ses nombreux avantages, l'utilisation de la spectrométrie de masse a cependant des limites, notamment la variabilité des paramètres d'acquisition, qui rendent difficile la comparaison entre les instruments et l'interprétation des données²¹.

La cosmétique et l'horticulture représentent deux secteurs économiques clés de la Région Centre-Val de Loire, contribuant à faire de cette région un territoire avec un tissu économique dynamique et attractif. L'objectif de cette thèse est le développement d'approches métabolomiques basée sur la spectrométrie de masse et l'utilisation d'outils bio-informatiques afin de caractériser des extraits de plantes originaires d'Asie et d'Inde cultivées à l'échelle régionale pour leurs propriétés pharmacologiques et pour l'identification de molécules présentant des activités antioxydante et anti-inflammatoire.

Le **chapitre I** fait un état de l'art des méthodologies permettant d'accéder à l'identification et l'annotation des molécules. Les instruments et méthodologies mis en place durant cette thèse seront ainsi détaillés. Une première sous-partie se concentrera sur les techniques instrumentales tandis que la deuxième partie détaillera le retraitement de données. Enfin, les outils d'annotation seront détaillés en troisième partie.

Le **chapitre II** s'intéresse à *Eclipta alba* (syn. *Eclipta prostrata*) et *Coleus forskohlii*, deux plantes utilisées dans la médecine ayurvédique, dont le sourcing a été relocalisé en région Centre-Val de Loire (RCVL) au Comité de Développement Horticole de la Région Centre-Val de Loire (CDHRC). Dans le cas de l'étude d'*Eclipta alba*, l'objectif était d'identifier de nouvelles activités biologiques des parties aériennes de la plante pour des applications cosmétiques, et d'identifier les composés majoritaires responsables de ces activités.

Pour l'étude de *Coleus forskohlii*, la culture aéroponique a été mise en place en faisant intervenir différents modes d'élicitation dans le but d'optimiser la production de biomasse notamment des racines et de leur composé bioactif d'intérêt : la forskoline. L'influence des conditions de culture a d'abord été évaluée sur la teneur en forskoline, puis la comparaison des compositions phytochimiques des extraits des parties aériennes et racinaires entre les plantes témoins et élicitées a été menée par l'utilisation d'outils statistiques afin de caractériser les molécules principalement influencées par le traitement.

Enfin, le **chapitre III** s'intéresse au genre *Clematis*, une plante cultivée localement en horticulture (la RCVL est la principale région de France pour sa culture) et dont des usages traditionnels sont répertoriés dans différentes médecines traditionnelles. Une première partie fait état de l'art de l'ethnopharmacologie, des activités biologiques et de la phytochimie des différentes plantes appartenant au genre *Clematis*. Une deuxième partie portera sur une dizaine d'espèces récoltées et analysées en Suisse dans le cadre d'une mobilité, avec l'élaboration d'une méthodologie permettant la déréplication tout en intégrant les données taxonomiques. Enfin, la troisième partie de ce chapitre, s'appuyant sur les connaissances phytochimiques acquises lors de l'étude précédente, se concentrera spécifiquement sur les plantes cultivées en Région Centre-Val de Loire et permettra d'intégrer les données d'activités des extraits testés pour l'identification de potentielles molécules actives.

La mise en place de nouvelles cultures à l'échelle locale, combinée à la valorisation de ressources existantes, contribue à un sourcing plus durable et responsable. Dans ce contexte, des méthodologies analytiques et d'identification de molécules actives ont été développées et adaptées pour explorer diverses sources végétales, répondant ainsi à des problématiques variées. Ces méthodologies ont permis d'enrichir les connaissances sur les plantes d'intérêt, de confirmer la possibilité de les cultiver localement tout en conservant leur potentiel pharmacologique et d'ouvrir de nouvelles perspectives de valorisation.

CHAPITRE I :

ÉTAT DE L'ART – METHODOLOGIE
D'IDENTIFICATION DES MOLECULES
ACTIVES

L'objectif de ce premier chapitre est de contextualiser les différentes méthodologies permettant l'exploration de la phytochimie d'extraits de plantes et l'identification de molécules actives. La première partie de ce chapitre se concentrera sur les techniques instrumentales d'analyse employées dans le cadre de cette thèse, et la deuxième partie abordera le prétraitement des données de spectrométrie de masse en vue d'obtenir une liste de données aussi appelée « *feature list* » la plus représentative possible de l'échantillon et s'intéressera à l'exploitation des données à travers les études statistiques et les réseaux moléculaires. Les outils d'annotation des métabolites spécialisés utilisés au cours de cette thèse seront présentés et expliqués.

Partie I. Techniques instrumentales d'analyse pour l'identification des molécules actives

Différentes techniques chromatographiques telle que la chromatographie en phase liquide (LC), la chromatographie en fluide supercritique (SFC), la chromatographie en phase gazeuse (GC) et la chromatographie de partage centrifuge (CPC), couplables à la spectrométrie de masse (MS), sont utilisées dans le domaine des produits naturels pour la séparation de métabolites à l'échelle analytique et préparative. Dans le cadre de cette thèse, où seule la métabolomique MS a été développée, la majorité des analyses ont été réalisées au sein de l'ICOA sur une chaîne chromatographique UHPLC U3000 RSLC Thermo couplée à un spectromètre de masse maXis Q-TOF Bruker. Des analyses ont également été réalisées à l'Université de Fribourg, dans le cadre d'une mobilité de trois mois, en utilisant une chaîne chromatographique Vanquish Thermo couplée à un spectromètre de masse Orbitrap Q-exactive plus Thermo. Ainsi, dans ce chapitre, seules les techniques séparatives et les spectromètres de masse utilisés au cours de cette thèse sont décrits. Toutefois l'étape de purification des molécules peut s'avérer indispensable afin de valider une activité biologique ou pour effectuer leur caractérisation structurale avec un haut degré de confiance en utilisant la RMN, lorsque ceux-ci ne sont pas connus. Bien que les méthodologies d'analyse par RMN aient fortement évolué ces dernières années avec le développement de workflows et d'outils intégrant l'automatisation et la bio-informatique pour le retraitement des données pour des études métabolomiques^{9,19,22}, celles-ci n'ont pas été approfondies dans cette thèse. C'est pourquoi ce chapitre se concentrera sur les deux techniques séparatives, la CPC et la LC et sur les méthodologies d'analyse par spectrométrie de masse employées durant cette thèse

1. Les techniques séparatives

1.1. La chromatographie de partage centrifuge (CPC)

La CPC est utilisée principalement pour le fractionnement et la purification de composés, en particulier ceux provenant de matrices biologiques²³. C'est une technique basée sur le principe de la chromatographie à contre-courant. Contrairement à la chromatographie liquide, la CPC est une méthode de chromatographie liquide-liquide qui n'utilise pas de phase stationnaire solide mais deux phases liquides non miscibles. La colonne est constituée de disques superposés dans lesquels des cellules reliées entre elles par un capillaire sont gravées. La phase dite stationnaire est maintenue à l'intérieur de la colonne par l'action de la force centrifuge lorsque la colonne est mise en rotation, tandis que la phase mobile est pompée au travers de cette phase stationnaire. La Figure 1 illustre le principe de fonctionnement de la CPC dans ses deux modes possibles : ascendant et descendant.

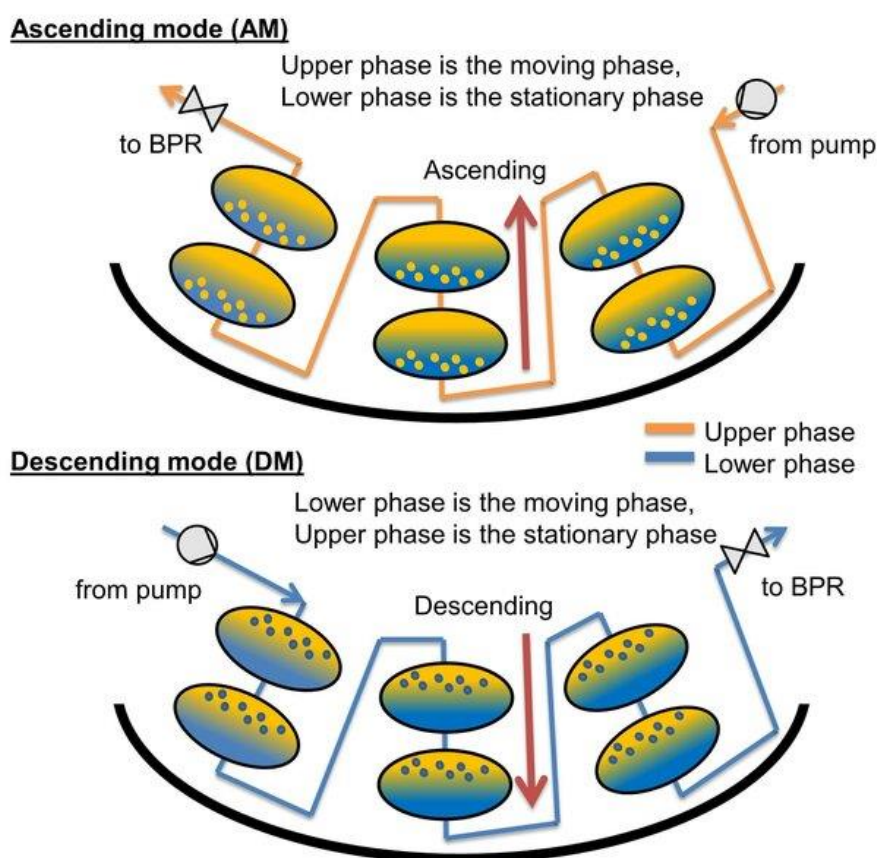


Figure 1 : Schéma du principe de fonctionnement de la CPC en mode ascendant et descendant²⁴

En mode ascendant, la phase stationnaire est plus dense, et la phase mobile plus légère va entrer par le bas de la cellule et entraîner les métabolites à travers la phase stationnaire. En

mode descendant, la phase stationnaire est la phase supérieure, la phase mobile plus dense entre par le haut de la cellule et entraîne ainsi les métabolites.

Les composés cibles sont soumis à un processus de partage continu entre ces deux phases et sont séparés en fonction de leur affinité pour chacune des deux phases représentée par leur coefficient de partage liquide-liquide (K_D)²⁵ comme l'illustre la Figure 2. Le K_D est défini pour un composé par sa concentration dans la phase stationnaire ($[A]_{\text{stat}}$) divisé par sa concentration dans la phase mobile ($[A]_{\text{mob}}$). Le K_D d'un composé est ainsi lié à son volume de rétention (V_r), plus sa valeur est faible, plus son temps de rétention est faible. Un composé avec un K_D égal à 1 a ainsi besoin d'un volume de phase mobile (V_m) égal à un volume de colonne pour être élué.

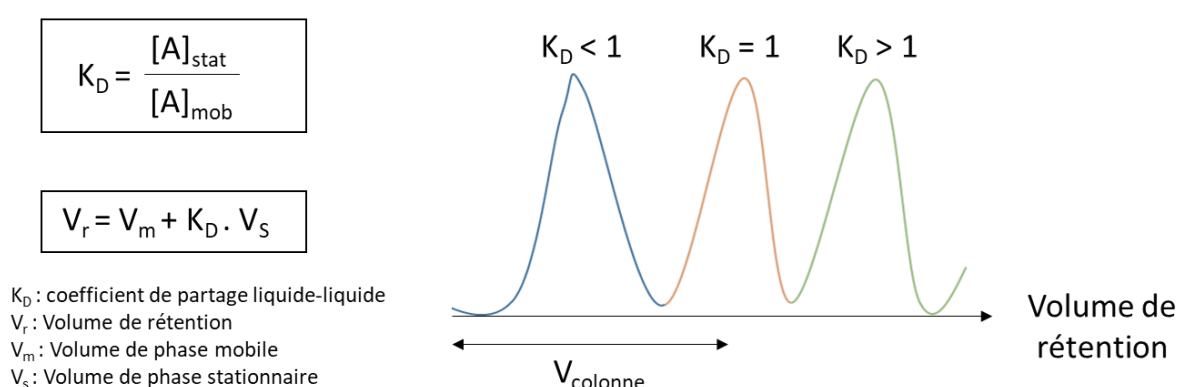


Figure 2 : Principe d'élution des composés en CPC en fonction de leur K_D

Le choix du système biphasique à employer pour la séparation est le paramètre clé à adapter en fonction de l'échantillon à fractionner et de la nature des molécules à séparer. Afin que les métabolites cibles soient séparés dans un temps raisonnable, leur K_D doit être compris entre 0,5 et 5. Les principaux systèmes biphasiques rencontrés sont des systèmes ternaires ou des systèmes quaternaires de type HEMWat (hexane-acétate d'éthyle-méthanol-eau) et Arizona (n-heptane-acétate d'éthyle-méthanol-eau) ; dont la composition et donc la polarité sont adaptées aux objectifs de la séparation²⁵⁻²⁸.

Grâce à la forte proportion de phase stationnaire liquide (entre 50 et 70 % du volume de la colonne), la CPC a l'avantage par rapport à l'HPLC préparative, de pouvoir supporter une quantité plus importante d'échantillon injecté, de ne pas présenter d'adsorption irréversible de l'échantillon et d'avoir un plus faible risque de dénaturation des composés²⁹. De plus, l'extrusion, par le pompage de phase stationnaire fraîche, permet de récupérer les composés qui n'ont pas été élués et ainsi la totalité de l'échantillon injecté peut être récoltée³⁰. Cela fait de la CPC une technique régulièrement utilisée pour le fractionnement et la purification d'extraits de plantes³¹⁻³³.

1.2. La chromatographie liquide (LC)

Les matrices biologiques, notamment les extraits de plantes, sont complexes à analyser en raison de leur grande richesse et de la diversité des molécules qui les composent³⁴. La séparation en chromatographie de partage en phase liquide (LC) repose sur les différences d'affinité des analytes entre la phase stationnaire et la phase mobile. Les analytes sont répartis entre la phase mobile et une couche de solvant organique imprégnant les greffons de phase stationnaire. En fonction de l'affinité des composés avec la phase mobile et la phase stationnaire, leur temps de rétention est impacté. Un composé ayant plus d'affinité avec la phase mobile est élué plus rapidement qu'un composé ayant plus d'affinité avec la phase stationnaire, qui est donc plus retenu.

Deux principaux modes sont utilisés : la phase normale (phase stationnaire polaire) et la phase inverse (phase stationnaire apolaire) ; cette dernière étant la plus couramment employée dans l'étude des produits naturels³⁵. La chromatographie liquide haute performance (HPLC) est ainsi devenue une référence pour l'analyse des produits naturels car elle permet de séparer toutes sortes de métabolites non-volatils de polarité variable allant de molécules très polaires (chromatographie à interaction hydrophile, HILIC) à des composés très apolaires (chromatographie en phase inverse non-aqueuse, NARP). La Figure 3 illustre différentes structures de molécules naturelles de polarités variables classiquement analysées par HPLC.

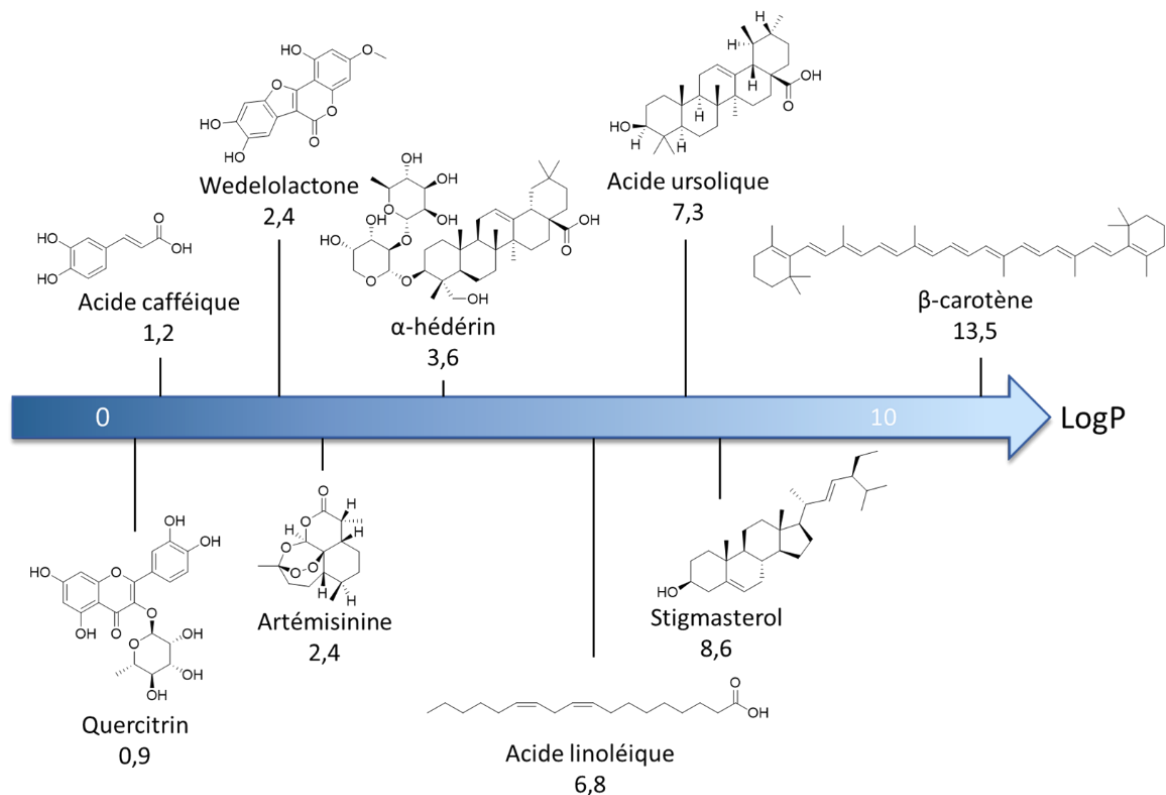


Figure 3 : Exemples de structures de molécules naturelles extraites de plantes de polarités variables classés en fonction de leur LogP³⁶

Les phases stationnaires apolaires sont obtenues par greffage de groupements apolaires, principalement des chaînes carbonées C₁₈ ou C₈. De nos jours, le développement de nouveaux greffages C₁₈ offre la possibilité d'utiliser des gradients avec de l'isopropanol (compatible avec la spectrométrie de masse) tout en commençant l'analyse avec une phase 100% aqueuse permettant ainsi en une même analyse, la rétention et séparation de composés très polaires (LogP proche de 0) et de composés apolaires (LogP supérieur à 10). Plus la densité des chaînes apolaires greffées sur la phase stationnaire est importante, donc plus le taux de greffage est élevé, plus les interactions avec les groupes silanols exposés sur la silice sont limitées ; ce qui améliore ainsi la rétention des analytes apolaires. L'ajout de petits groupes fonctionnels triméthylsilyles (TMS), ou end-capping, limite les interactions avec les silanols résiduels et améliore la symétrie des pics et la séparation. La qualité du greffage et sa structure vont également influencer la résistance au pH d'une colonne. Les phases mobiles couramment employées sur une colonne apolaire sont réalisées à base d'eau et d'un solvant organique polaire miscible comme le méthanol ou l'acétonitrile. Des additifs comme l'acide formique peuvent également y être ajoutés, pour contrôler le pH de la phase mobile, améliorer la sélectivité, la résolution et favoriser l'ionisation en couplage LC-MS⁹.

Lorsque la méthode est adaptée et optimisée, elle peut également séparer les isomères de structure et les diastérisomères, ce qui rend cette méthode utile pour l'étude de mélanges complexes. En revanche, dans le cas des énantiomères, des colonnes spécifiques doivent être employées³⁷. Le développement depuis 20 ans, de colonnes de chromatographie liquide ultra haute performance (UHPLC), avec des particules de taille inférieure à 2 µm et des chaînes chromatographiques permettant de travailler à des pressions supérieures à 600 bars, ont permis de gagner en efficacité ce qui permet une meilleure séparation de structures très proches comme des isomères, et un gain en temps d'analyse^{38,39}. Cependant, ce gain en efficacité a des conséquences sur les analyseurs, qui doivent pouvoir détecter un nombre de points suffisant pour décrire un pic chromatographique.

Il est également possible d'utiliser la chromatographie liquide dans un but préparatif ; afin de purifier ou fractionner un mélange. Dans ce cas, la méthode chromatographique peut être, dans un premier temps, développée sur une colonne analytique HPLC puis transposée sur une colonne dite « préparative » ou « semi-préparative » selon ses dimensions. Comme en chromatographie liquide analytique, l'échantillon est généralement injecté en solution. Idéalement l'échantillon doit être solubilisé dans un faible volume de solvant, et celui-ci ne doit pas être plus éluant que la composition de la phase mobile en condition initiale⁴⁰. Un outil d'aide à la transposition, sous forme de tableur Excel a été développé et est disponible en ligne (<https://farma-unites.unige.ch/fr/rudaz-lab/outils/hplc-calculator>)^{41,42}. A partir des paramètres initiaux d'analyse (débit, temps d'analyse, volume d'injection) et de la géométrie de la colonne (longueur, diamètre, taille des particules), cet outil permet de calculer les

paramètres à modifier, tels que le débit ou le volume d'injection, afin d'adapter la méthode à la géométrie de la colonne sur laquelle elle est transférée.

1.3. Les détecteurs

Les métabolites spécialisés, même lorsque ceux-ci sont présents en petite proportion, peuvent avoir un potentiel actif, comme le cas de la vincristine présente aux alentours de 0,0001% à 0,001% dans la feuille séchée de *Catharanthus roseus*⁴³. C'est pourquoi, afin de les identifier, il est important de développer des méthodologies permettant de travailler sur une large gamme dynamique et de générer un maximum d'information⁴⁴.

A l'échelle analytique tout comme à l'échelle préparative différents analyseurs peuvent être couplés avec la chromatographie en phase liquide. Le **DAD** (*Diode Array Detector*) est un détecteur non-destructif qui donne des informations sur le spectre UV-Visible des molécules, permettant ainsi d'avoir des indications sur la classe chimique du composé et sur sa structure. Ce détecteur est largement utilisé pour la quantification de composés présentant des absorptions notables dans les domaines UV ou visible. Il permet de mesurer l'absorbance à une longueur d'onde spécifique, propre à chaque composé. L'absorbance ainsi mesurée est directement proportionnelle à la concentration du composé dans l'échantillon. Cependant, il n'est pas possible de comparer quantitativement deux composés appartenant à des familles moléculaires différentes, car leurs coefficients d'extinction molaire peuvent varier fortement.

Le **DEDL** (détecteur évaporatif à diffusion de la lumière) est quant à lui un détecteur destructif, dont l'installation à l'échelle préparative nécessite d'avoir un système de *split* entre le détecteur et le collecteur^{40,45}. La détection se déroule en trois étapes : la nébulisation de la phase mobile, son évaporation et la détection. A l'issue de l'évaporation de la phase mobile, les composés non évaporés cristallisés peuvent être détectés grâce à la diffusion de la lumière. A la différence du DAD et de la spectrométrie de masse, le DEDL ne dépend pas de la présence de fonctions chimiques particulières et permet ainsi une détection quasi-universel des composés non-volatils et semi-volatils. Cependant, dans le cas d'une analyse quantitative, la détection étant dépendante de la phase mobile, en mode gradient, il est nécessaire d'avoir un temps de rétention proche pour comparer quantitativement deux analytes. De plus, la réponse du détecteur n'est pas linéaire et peut être décrite par la relation suivante⁴⁶ :

$$A = am^b$$

où A est l'aire du pic chromatographique, a le facteur de réponse, m la masse du composé, et b est l'indice de réponse mesuré à partir de la pente de la courbe $\log A = f(\log m)$. La résultante étant que pour obtenir des courbes linéaires, la double pondération logarithmique suivante est nécessaire⁴⁶ :

$$\text{Log(Aire)} = b \times \text{Log(Concentration)} + \text{Log(a)}$$

Bien que les données de temps de rétention ajoutent de précieuses informations quant à la polarité et la structure des analytes^{9,47,48}, et que le DAD et le DEDL soient particulièrement utilisés et complémentaires dans l'analyse des produits naturels, sans étalon il n'est pas possible d'affiner l'identification des composés détectés. Ainsi, le couplage de la chromatographie liquide à la spectrométrie de masse haute résolution, permet d'avoir accès à davantage d'informations structurales et à une meilleure sensibilité de détection lorsque les composés s'ionisent bien dans les conditions expérimentales, c'est pourquoi le couplage LC-HRMS est particulièrement adapté à la déréplication.

2. La LC-HRMS/MS

En phytochimie, l'utilisation de la spectrométrie de masse est incontournable pour l'identification d'un composé⁴⁹. En effet, la masse exacte est devenue une information essentielle à la description de celui-ci car elle peut donner accès à la formule brute qui vient compléter les informations acquises en RMN. Cependant, au-delà de cette information, la spectrométrie de masse utilisée en mode tandem pour générer des fragments de molécules et associée à des outils de traitement de données permettant l'interprétation des spectres de fragmentation peut devenir une part intégrale de la méthodologie menant à l'identification ou l'annotation de composés⁵⁰.

La spectrométrie de masse permet la mesure d'un rapport masse/charge (m/z) d'un ion et de son abondance. A partir de ces informations, il est possible de construire un spectre, avec en abscisses la valeur m/z et en ordonnées l'abondance relative de l'ion. L'ionisation peut varier en fonction de la structure des molécules, c'est pourquoi il n'est pas possible de comparer quantitativement deux molécules de familles moléculaires différentes. De plus, le choix de la méthode d'ionisation est essentiel, le but étant de pouvoir observer le maximum de composés au sein d'un extrait, ou alors de pouvoir détecter une famille cible de composés.

La structure générale d'un spectromètre de masse se décompose en plusieurs parties : une **source d'ionisation**, un **analyseur** de masse et un **détecteur** (Figure 4). Le signal est ensuite traité pour être interprété. Les parties traitement de données et annotation sont abordées en parties 2 et 3 de ce chapitre.

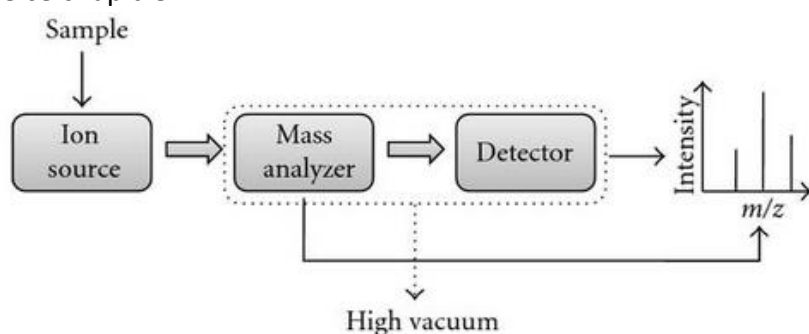


Figure 4 : Principaux éléments d'un spectromètre de masse⁵¹

2.1. La source électrospray (ESI)

En couplage LC-MS le type d'ionisation peut être adapté à la nature des composés analysés. L'ionisation par électronébulisation ou **Electrospray** (ESI), faisant partie des techniques d'ionisation dites « douces », est l'ionisation la plus utilisée dans l'étude des produits naturels⁵². D'autres sources telles que l'ionisation chimique à pression atmosphérique (APCI) ou la photoionisation à pression atmosphérique (APPI) plus adaptées à l'étude de petites molécules plus apolaires peuvent être employées de façon complémentaire (Figure 5)^{53,54}.

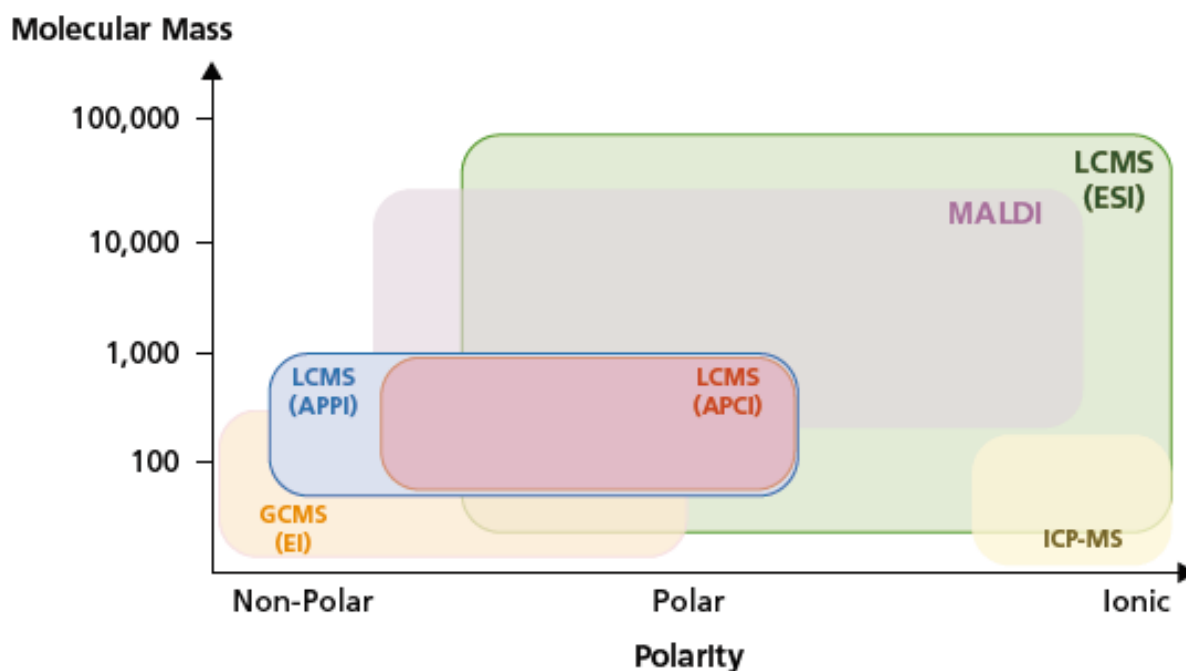


Figure 5 : Techniques courantes d'ionisation (APCI, APPI, EI, ESI, MALDI et ICP) et leur champ d'application en fonction de la polarité et du poids des composés.
(<https://www.shimadzu.com.au>)

La Figure 6 détaille le principe de fonctionnement de l'ESI. L'échantillon est introduit à travers un capillaire au sein de la source. Une différence de potentiel entre le capillaire et une contre-électrode permet d'obtenir un champ électrique, à pression atmosphérique. La tension résultante (2-6 kV), produit des gouttelettes chargées à l'extrémité du capillaire, qui sont pulvérisées dans l'atmosphère par un processus appelé « explosion de Coulomb ». Un gaz neutre est injecté afin de favoriser la nébulisation du spray et de diriger les gouttelettes. Afin de supprimer les dernières molécules de solvant et les molécules neutres, les gouttelettes passent ensuite à travers un rideau de gaz inerte chauffé ou un capillaire chauffé^{21,51,53,55,56}.

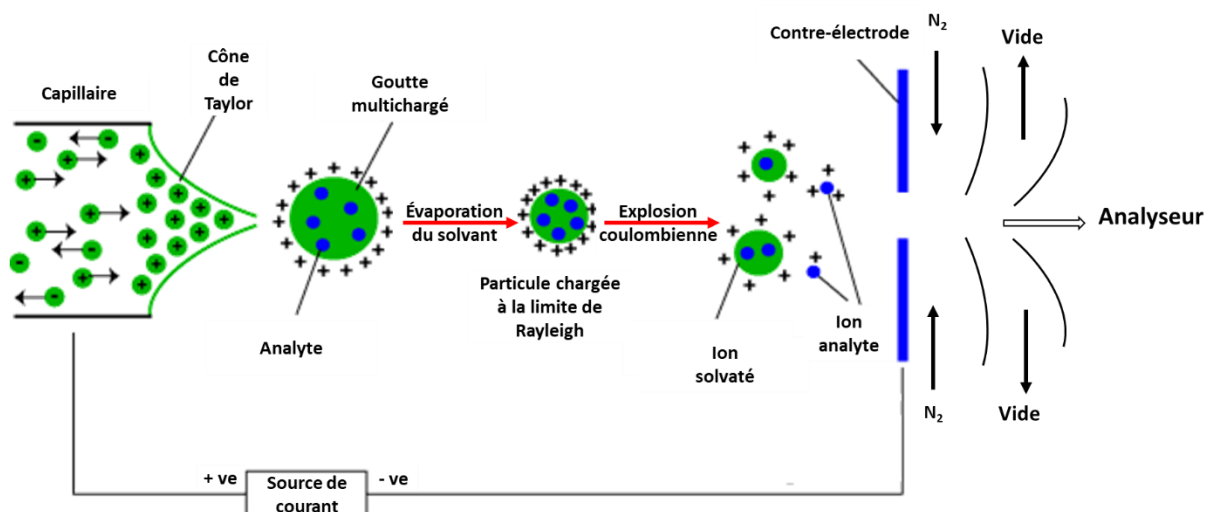
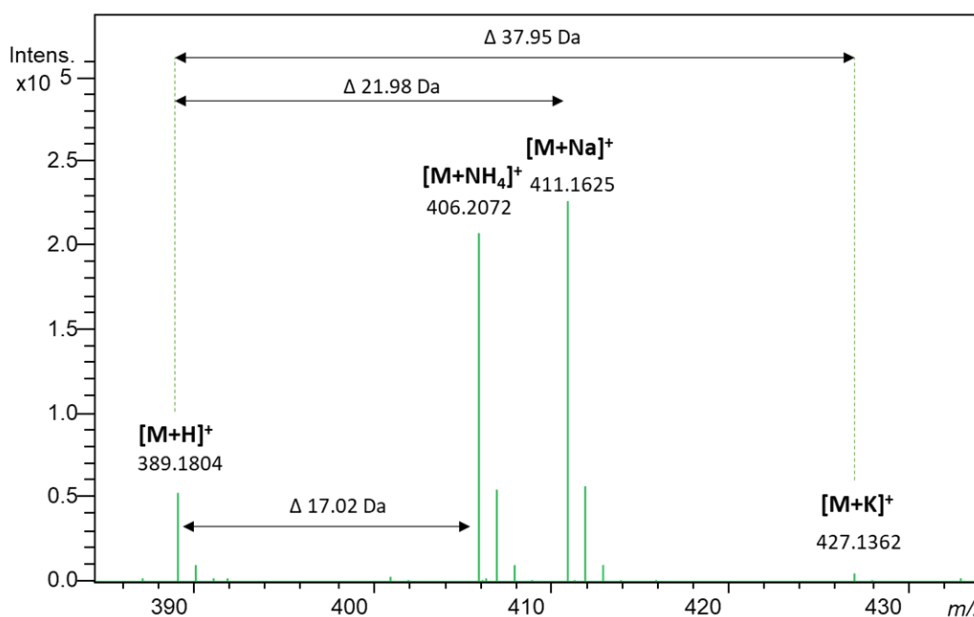


Figure 6 : Schéma de principe d'une source ESI en mode d'ionisation positive (modifié à partir de <https://www.microsaic.com/>).

L'ESI est une source dite « douce » qui génère peu de fragments en source, le pic le plus intense du spectre MS est ainsi généralement l'ion moléculaire ou un adduit. La source ESI permet d'appliquer une différence de potentiel positive ou négative, en fonction de la nature des composés à étudier. En fonction de la polarité de l'ionisation, l'ion moléculaire observé est l'ion de la molécule protonée $[M+H]^+$ ou déprotonée $[M-H]^-$. Dans l'étude des petites molécules (< 1000 Da) les ions produits seront principalement monochargés, bien que l'ESI soit capable de produire des ions multichargés pour les molécules contenant de multiples groupes fonctionnels ou très susceptibles d'être ionisées. Il est possible de vérifier l'état de charge d'une molécule en observant le massif isotopique, ainsi lorsqu'une molécule est dichargée ($z = 2$), entre deux pics du massif isotopique une différence d'environ 0.5 u sera observée alors que lorsqu'il s'agit d'un ion monochargé une différence d'environ 1 u sera observée.

De plus, l'ESI génère également des adduits formés par l'interaction de l'ion moléculaire avec un ou plusieurs atomes ou molécules pour former un ion contenant à la fois l'ion moléculaire et l'atome ou molécule associé. En ionisation positive les adduits majoritairement rencontrés sont : $[M+Na]^+$, $[M+NH_4]^+$, $[M+K]^+$, et $[M+H+CH_3CN]^+$ (si utilisation d'acétonitrile en solvant) alors qu'en ionisation négative les adduits majoritairement rencontrés sont : $[M+Cl]^-$ et $[M+HCOO]^-$ ^{52,57}. Ces adduits sont essentiels lors du traitement de données, car ils permettent de s'assurer que le calcul de formule brute est bien réalisé sur la bonne masse et avec le bon adduit (Figure 7). L'utilisation des deux modes d'ionisation peut s'avérer utile pour repérer avec confiance l'ion moléculaire et ainsi démarrer le travail d'identification. Le Tableau 1 présente les différences de masse entre les adduits et l'ion moléculaire ou les adduits entre eux, fréquemment rencontrés lors de l'utilisation d'une source ESI en modes positif et négatif.

		$[M+Na]^+$	$[M+NH_4]^+$	$[M+K]^+$	$[M+H+CH_3CN]^+$
Positif	$[M+H]^+$	21,9819	17,0265	37,9559	40,9965
	$[M+Na]^+$		4,9554	15,9740	19,0146
	$[M+NH_4]^+$			20,9294	23,9700
	$[M+K]^+$				3,0406
		$[M+Cl]^-$		$[M+HCOO]^-$	
Négatif	$[M-H]^-$	35,9767		46,0054	
	$[M+Cl]^-$			10,0287	

Tableau 1 : Différences de masse entre les adduits fréquemment rencontrés en modes positif et négatif**Figure 7 :** Exemple des principaux adduits détectés sur un spectre de masse avec leur différence de masse par rapport à l'ion $[M+H]^+$ correspondant.

L'ESI est une source sensible à l'effet matrice. La suppression ionique, peut entrainer une disparition de certains composés mineurs ou la diminution de composés majeurs. Cela peut se produire lorsque deux analytes en mélange ont une différence importante d'acidité ou de basicité, l'ion alors observé en mode d'ionisation positive est le meilleur accepteur de proton, alors qu'en mode négatif il s'agira du meilleur donneur de proton. Le couplage UHPLC-HRMS

notamment, grâce à une meilleure séparation chromatographique, limite la suppression d'ion quand la méthode est adaptée à l'échantillon^{21,52,58}. Réaliser des tests de dilution peut permettre de se rendre compte de ces effets matrice, cependant dans le cas de grandes séquences, cela est difficilement réalisable. Lorsque plusieurs échantillons sont analysés à la suite, ou afin d'éviter les effets batch, il peut être intéressant d'ajouter un QC (échantillon de contrôle qualité) et de l'analyser régulièrement au cours de la séquence pour s'assurer de l'absence de variation d'intensité^{52,59}. L'effet matrice et la suppression ionique ainsi que la présence d'adduits peuvent avoir un impact sur la précision d'une quantification. Il est donc nécessaire de s'assurer de la bonne ionisation du composé quantifié, de se référer au même adduit et de réaliser des tests préalables de taux de recouvrement⁵⁹.

Le mode d'ionisation peut également avoir un fort impact sur la détection des métabolites. En effet, en fonction des échantillons, il est possible que les molécules répondent plus favorablement en mode ionisation positive ou négative selon la capacité d'ionisation des composés. Toutefois, dans l'analyse non ciblée le mode positif est généralement préféré car il permet de détecter un plus grand nombre de molécules. Dans la Figure 8, les BPC d'un même extrait de *Rumex usambarensis* en mode ESI positif (A) et négatif (B) montrent des différences d'intensité en fonction du mode d'ionisation et du temps de rétention. De façon générale, les composés de polarité faible, et donc élués avec un pourcentage de phase organique élevé lors d'analyse en RPLC, sont ceux ayant une sensibilité élevée en mode positif⁶⁰.

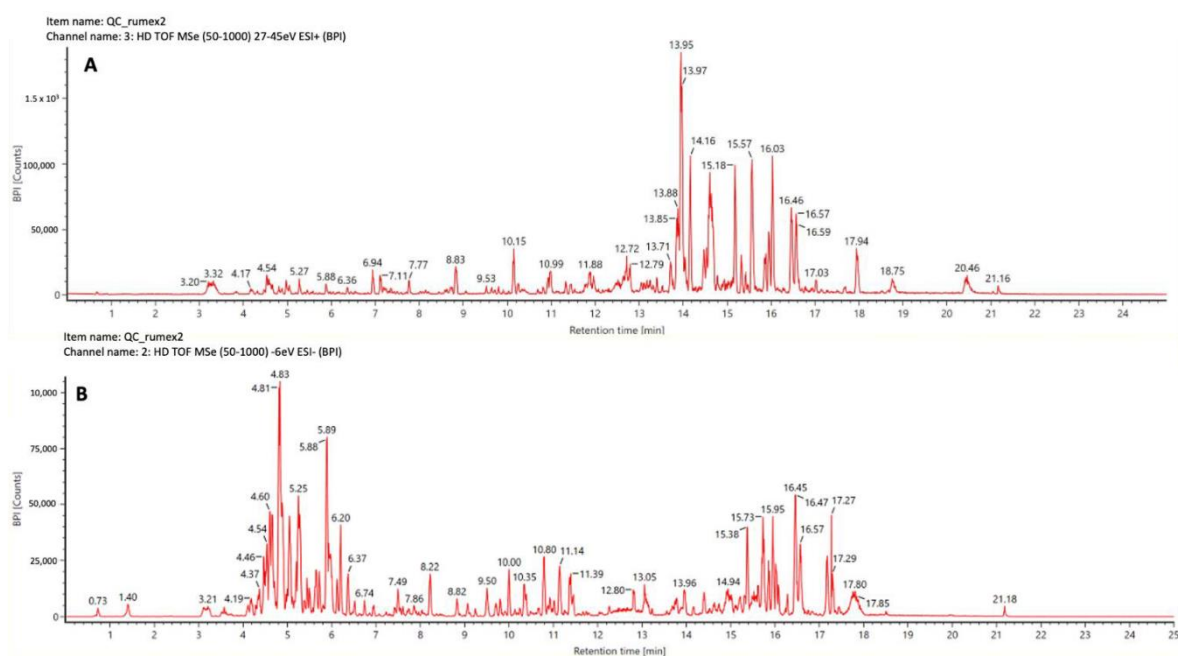


Figure 8 : BPC UHPLC-HRMS/MS d'un mélange d'extraits de *Rumex usambarensis* obtenus en modes positif (A) et négatif (B)⁶¹.

Dans cet exemple le mode négatif permet une meilleure ionisation des composés élués entre 4 et 7 min, montrant qu'en fonction des familles moléculaires et des fonctions chimiques des molécules, un certain mode d'ionisation sera alors plus adapté. Ainsi, lorsque l'on compare un même échantillon dans les deux modes, certaines familles moléculaires peuvent être davantage représentées car mieux ionisées. Souvent, la meilleure sensibilité est obtenue lorsque la forme ionisée de la molécule est favorisée par les conditions de pH *i.e.* lorsqu'un analyte basique est ionisé en utilisant une phase mobile acide et inversement lorsqu'un analyte acide est ionisé en utilisant une phase mobile basique⁶⁰.

Dans l'étude comparant un ensemble de 76 extraits de plantes de la famille de Celastraceae, la représentation des classes chimiques a pu être réalisée sur les analyses en mode positif et négatif (Figure 9). La classe la plus représentée dans les deux cas est la classe des triterpénoïdes, puis dans le cas du mode positif on retrouve les alcaloïdes puis les acides gras, les polycétides et la voie des shikimates et phénylpropanoïdes alors qu'en mode négatif on retrouve en second la voie des shikimates et phénylpropanoïdes, puis les acides aminés et peptides, puis les alcaloïdes et acides gras⁶². Dans le cas des composés phénoliques par exemple, représentés dans la voie des shikimates et phénylpropanoïdes, les molécules comportent des fonctions acides hydroxyl et/ou carboxylique, ces molécules sont mieux détectées sous leur forme déprotonées en ionisation négative⁶³.

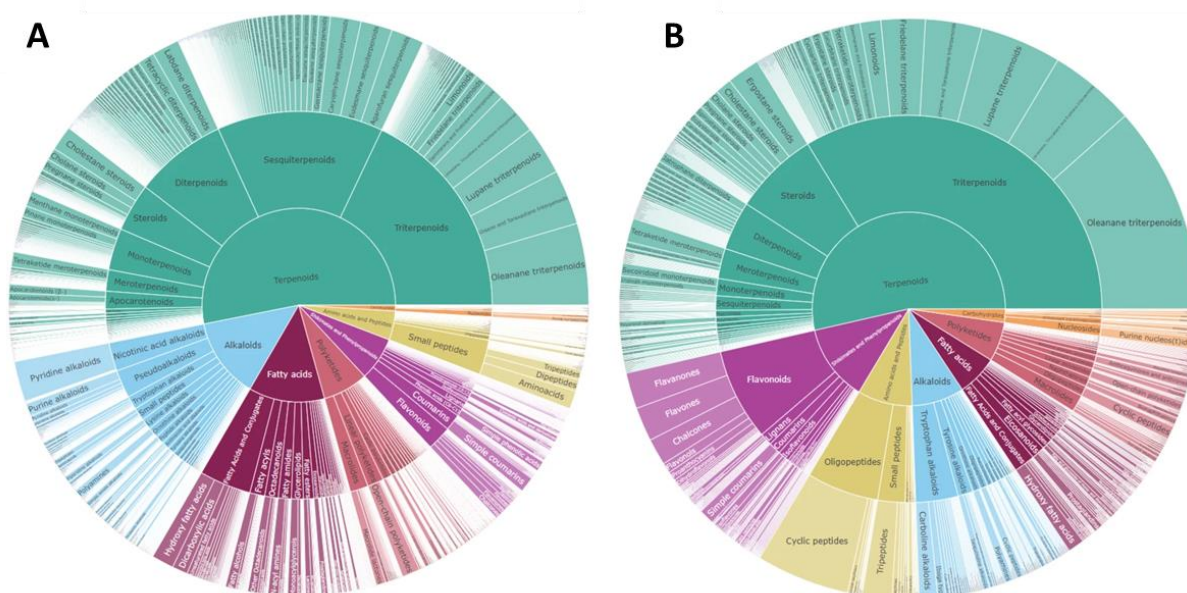


Figure 9 : Diagramme en cercle emboîté de la répartition des différentes classes chimiques proposées par SIRIUS-CANOPUS en modes d'ionisation positif (A) et négatif (B). Chaque couleur correspond à une classe différente.⁶²

2.2. Les analyseurs

Il existe analyseurs peuvent être de basse résolution ou de haute résolution. La spectrométrie de masse à haute résolution (HRMS) peut permettre de déterminer la masse exacte de l'ion moléculaire et des ions fragments dans un échantillon.

Trois types d'instruments permettent de réaliser des analyses de spectrométrie de masse à haute résolution : les spectromètres de masse à résonance cyclotronique ionique (FT-ICR), à temps-de-vol TOF (*Time-of-Flight*) et les Orbitraps (OT). Les deux derniers (TOF et OT) étant les principaux analyseurs rencontrés en métabolomique et dans l'analyse des produits naturels, par la facilité du couplage avec la LC et la réduction du coût des instruments ces dernières années⁶⁴. La vitesse d'acquisition, la gamme de masse, la transmission, la résolution, la précision de mesure de la masse et la justesse de la distribution isotopique sont autant de caractéristiques qui ont un impact sur la qualité des données obtenues^{56,65-67}. La précision de la mesure de la masse tout comme la justesse isotopique, sont indispensables pour permettre une bonne identification du composé. La résolution ($m/\Delta m$) quant à elle permet de distinguer deux composés de masses très similaires (isobares). Elle peut être déterminée à partir de la largeur à mi-hauteur (*Full-Width Half Maximun, FWHM*) d'un pic.

Dans l'analyse non ciblée il est également nécessaire d'acquérir des spectres MS/MS afin d'avoir une information structurale des métabolites présents dans l'échantillon. Pour cela, deux analyseurs successifs sont nécessaires. Le premier permet la sélection de l'ion précurseur, et le second analyse les fragments générés après le passage de l'ion précurseur dans la cellule de collision. Ces fragments vont pouvoir être interprétés pour suggérer l'élucidation structurale du composé⁶⁸. Durant cette thèse, deux appareils ESI-HRMS/MS permettant d'effectuer des expériences de spectrométrie de masse en tandem ont été utilisés, un MaXis Q-TOF Bruker à l'ICOA et une Orbitrap Q-Exactive Plus Thermo Scientific à l'Université de Fribourg, équipés respectivement de sources ESI et HESI (*Heated electrospray*), dont la sonde chauffée a été décrite pour permettre une meilleure désolvatation et améliorer la sensibilité⁴⁹.

a. Le temps de vol

Le Q-TOF (Figure 10) est un instrument hybride combinant un quadripôle et une cellule de collision, avec un analyseur TOF. Le quadripôle opère comme un filtre de masse, il permet de sélectionner la gamme de travail (en m/z). Les ions sélectionnés sont ensuite dirigés vers la cellule de collision, où ils sont accélérés en présence d'un gaz neutre, induisant ainsi la fragmentation suite aux chocs entre les ions et les molécules de gaz : CID (*Collision Induced Dissociation*)^{69,70}.

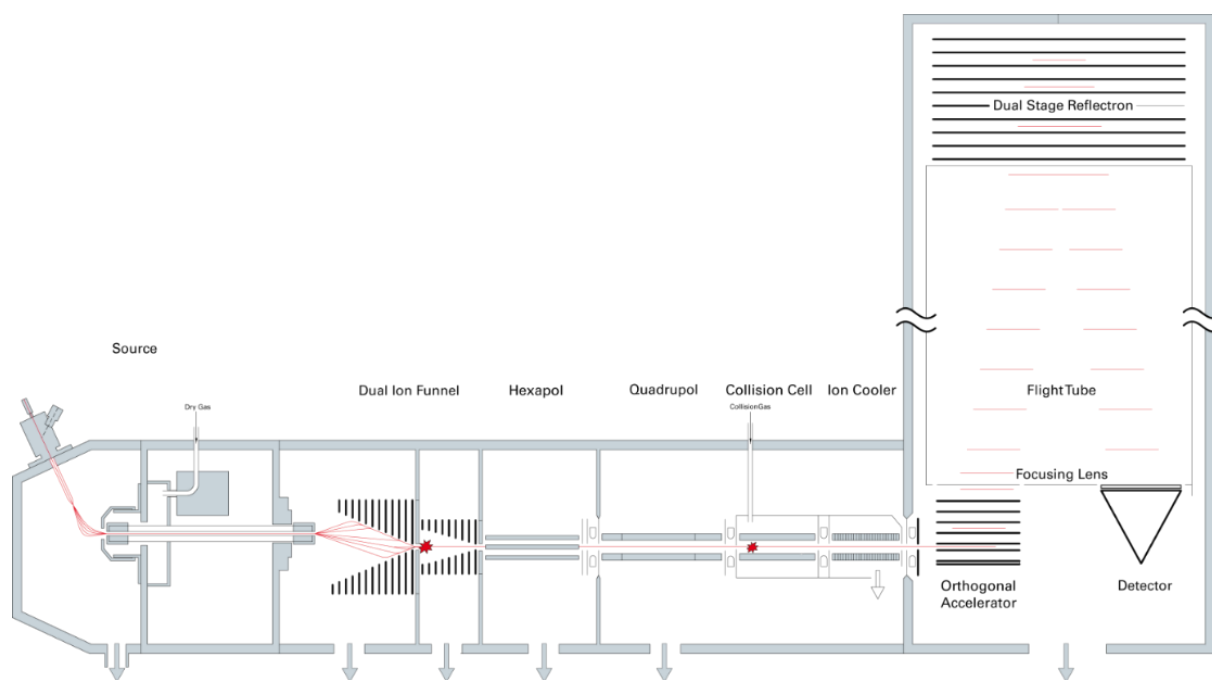


Figure 10 : Schéma du spectromètre de masse maXis Q-TOF Bruker

Il existe deux grands types d'analyseurs TOF : les analyseurs linéaires et les analyseurs avec réflectron. La Figure 11 décrit respectivement la trajectoire des ions à travers le temps de vol dans le cas d'un analyseur linéaire et d'un analyseur avec réflectron (Figures 11A et 11B). L'ajout du réflectron a pour but de limiter la dispersion d'énergie des ions et de les refocaliser ; cela a ainsi permis d'augmenter la résolution de ces spectromètres de masse⁷¹. Dans le cas du Q-TOF Bruker, il s'agit d'un analyseur avec réflectron à déviation orthogonale, comme il est possible de le voir sur la Figure 10. Dans un analyseur à temps de vol, tous les ions reçoivent la même énergie au niveau de la déviation orthogonale. De ce fait, les ions de plus faible m/z arrive en premier au détecteur. Les fragments ainsi formés vont ensuite être accélérés par un champ électrique puis démarrent leur dérive au même moment et en un même point. Lorsque les ions atteignent le détecteur ils vont se séparer en groupe correspondant à leur rapport m/z . Pour une même charge, le groupe le plus léger atteint le détecteur en premier, puis sera suivi par des groupes de masses de plus en plus élevées.

Les TOF sont des analyseurs adaptés pour l'analyse de composés sur une large gamme de masse sans compromettre la sensibilité, possédant une fréquence d'acquisition et une exactitude de masse élevées⁷². La résolution en masse peut aller jusqu'à 40 000 à m/z 1222 dans le cas du maXis Q-TOF Bruker.

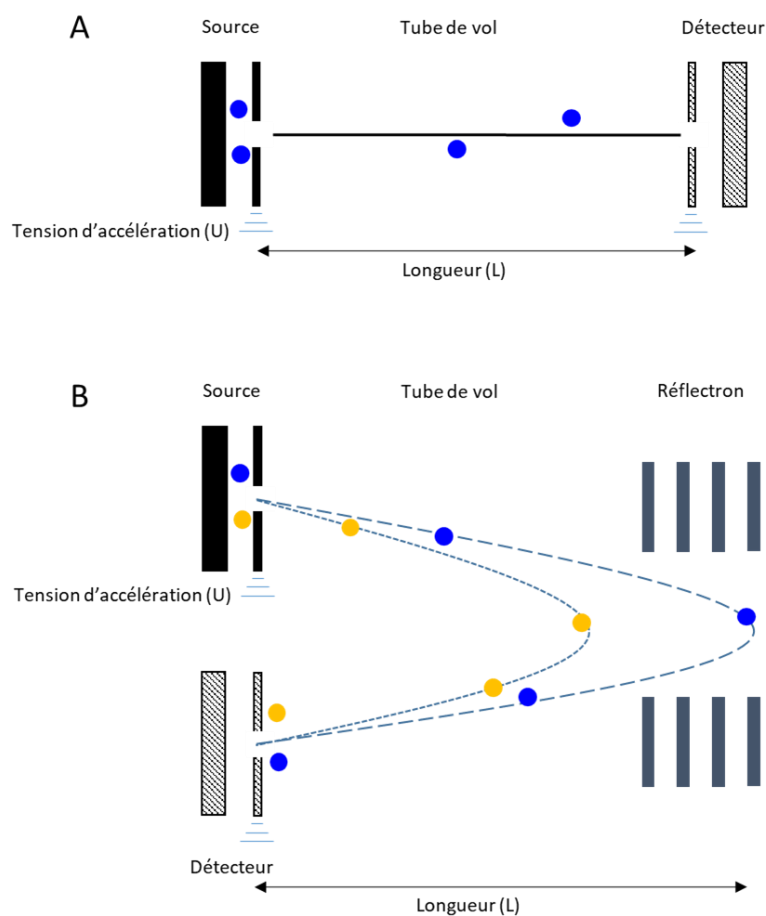


Figure 11 : Schéma d'un analyseur TOF avec (A) un analyseur linéaire ou (B) un analyseur avec réflectron

b. Orbitrap

L'Orbitrap Q-Exactive (Figure 12) possède un quadripôle permettant la sélection de la gamme de masse de travail. Les ions sont ensuite dirigés vers la C-trap (piège ionique) reliée d'un côté à une cellule de collision HCD (*Higher energy Collisional Dissociation*), agissant de façon similaire à une cellule CID, et d'un autre côté à un analyseur Orbitrap. La C-trap permet d'orienter les ions à fragmenter dans la cellule de collision, ceux-ci sont ensuite à nouveau transférés dans la C-trap pour être accumulés et ensuite éjectés dans l'analyseur Orbitrap.

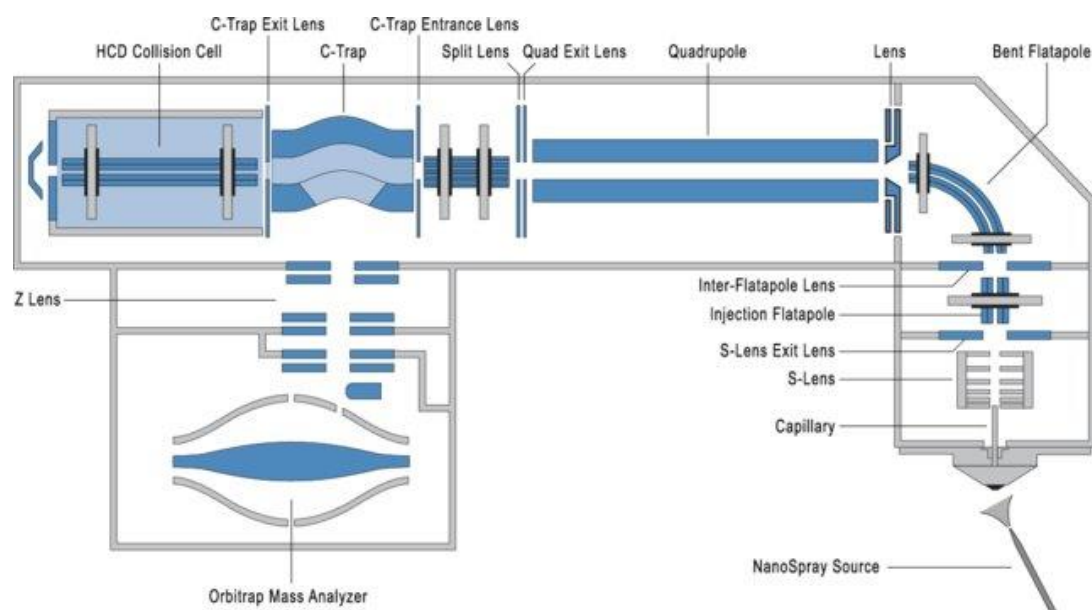


Figure 12 : Schéma du spectromètre de masse Q-Exactive Thermo Scientific

Les **Orbitraps** (Figure 13) sont composés de deux électrodes, une extérieure et une centrale en forme de cylindre. Un champ électrique est créé entre ces deux électrodes et ainsi les ions injectés perpendiculairement à l'axe Z vont se retrouver piégés. Les ions ainsi piégés se mettent à orbiter le long de l'électrode centrale (oscillation radiale sur l'axe des z et oscillation axiale). L'oscillation des ions est détectée par l'électrode extérieure et est convertie par transformée de Fourier pour obtenir un spectre de masse avec les informations de rapport m/z et d'intensité^{64,73}.

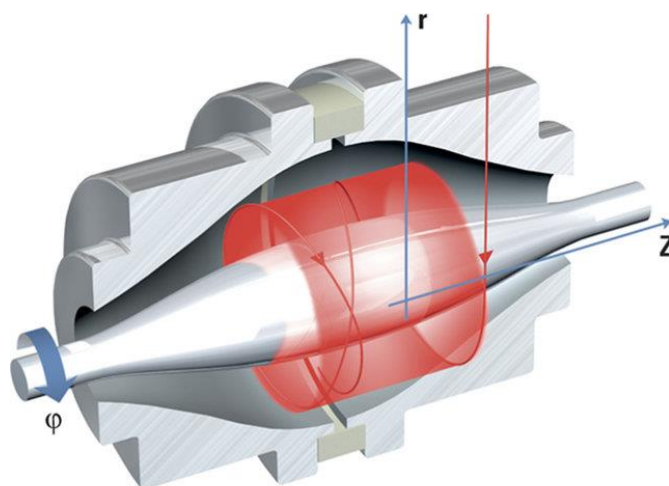


Figure 13 : Schéma d'un analyseur orbitrap⁷⁴.

2.3. La fragmentation

La **fragmentation par collision induite (CID)** permet de produire des ions fragments à différentes énergies de collision. Un ion précurseur est sélectionné pour être fragmenté dans

une cellule de collision dans laquelle est présent un gaz inerte (H_2 , Ar). En fonction de la nature des composés et de leur structure, l'énergie (eV) doit être adaptée afin d'acquérir des informations exploitables.

Les voies de fragmentation vont dépendre de la quantité d'énergie appliquée, qui va conduire au clivage des liaisons les plus faibles^{56,75}. Il est ainsi possible d'interpréter les spectres MS/MS pour déterminer la structure du composé fragmenté. Dans le cas des molécules O-glycosylées par exemple (Figure 14A), des pertes de neutres caractéristiques correspondant aux pertes de sucres sont ainsi faciles à repérer ce qui n'est pas le cas des molécules C-glycosylées pour lesquelles la fragmentation se fait dans le sucre (Figure 14B). Les schémas de fragmentation de certaines familles moléculaires, comme par exemple les flavonoïdes, sont bien décrits dans la littérature permettant l'annotation manuelle de ces composés⁷⁶. Cependant, dans de nombreux cas, la compréhension des spectres de fragmentation reste limitée et requière beaucoup de temps⁵².

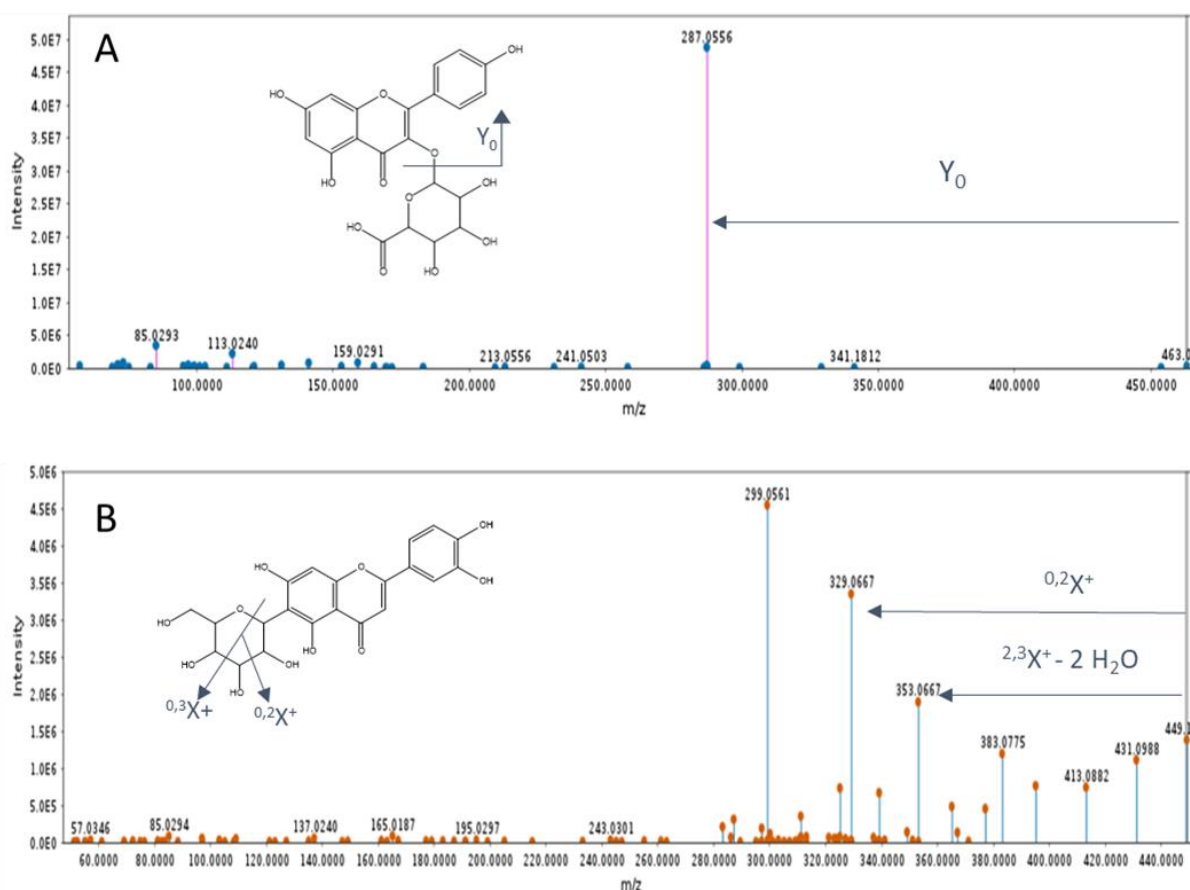


Figure 14 : Spectres MS/MS (A) d'un flavonoïde O-glycosylé (Kampferol-O-glucuronide) et (B) d'un flavonoïde C-glycosylé (Isoorientin)

L'acquisition d'un spectre MS/MS peut se faire à différentes énergies (valeurs discrètes) ou alors en utilisant une rampe d'énergie de collision. Un spectre combiné peut ainsi être obtenu.

Les spectres de masse à faible énergie de collision contiennent en majorité l'ion précurseur et quelques fragments de hauts m/z alors que les spectres obtenus à plus haute énergie de collision contiennent davantage de fragments à de plus faibles m/z . La Figure 15 présente un spectre expérimental MS/MS de la catéchine acquis en mode d'ionisation négatif ESI(-) et comparé à des spectres d'une banque de données à différentes énergies de collision. Il est ainsi facile de se rendre compte que le spectre MS/MS d'un composé va être fortement dépendant de l'énergie de collision appliquée, ce qui peut avoir des conséquences sur l'interprétation et donc l'annotation des métabolites.

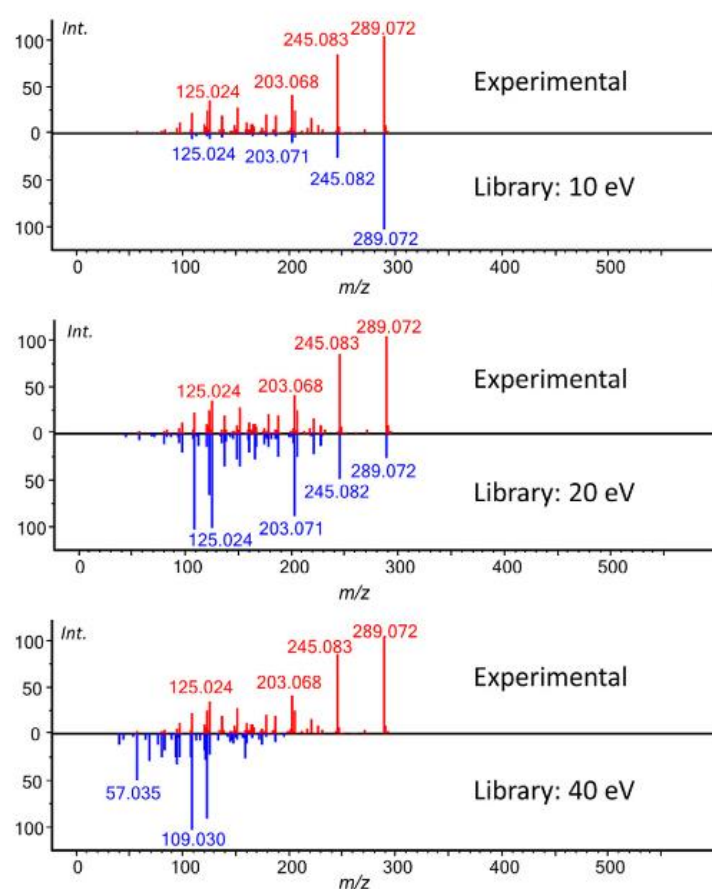


Figure 15 : Comparaison d'un spectre expérimental MS/MS de catéchine vs des spectres d'une bibliothèque à différentes énergies de collision⁷⁷

2.4. L'acquisition d'un spectre LC-HMRS/MS

Deux modes d'acquisition sont utilisés pour la spectrométrie de masse en tandem non ciblée, l'**acquisition indépendante des données** (*Data-independent acquisition*, DIA) et l'**acquisition dépendante des données** (*Data-dependant acquisition*, DDA) (Figure 16).

Dans le mode DIA, un scan de la MS est tout d'abord réalisé et le spectre est découpé en fenêtres d'isolation de taille prédéfinie couvrant la plage de m/z désirée. A l'intérieur de

chaque fenêtre d'isolement, les ions sont ensuite fragmentés puis détectés. Chaque fenêtre produit ainsi un spectre MS/MS de fragmentation à partir de plusieurs précurseurs d'une même gamme de m/z .

Dans le cas du DDA, un ion est sélectionné et fragmenté pour donner un spectre MS/MS s'il répond à certains critères (intensité, état de charge, liste d'exclusion, temps d'exclusion etc.). L'avantage de ce mode d'acquisition est qu'il permet de préserver l'association ions parents/fragments, et ainsi un retraitement simplifié des données⁷⁷⁻⁷⁹.

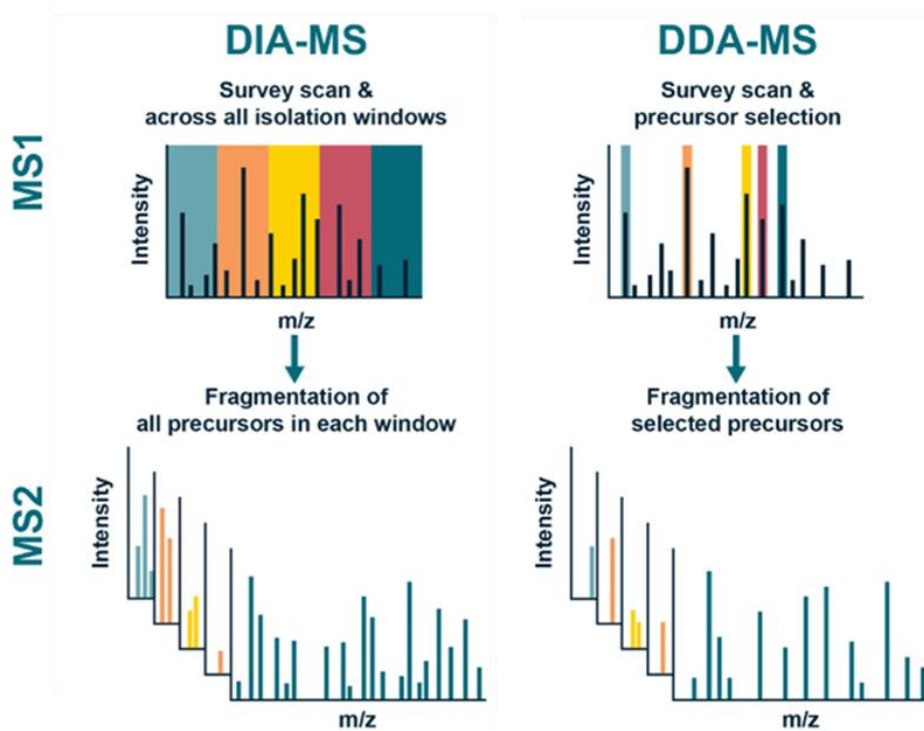


Figure 16 : Principe de l'acquisition indépendante des données (DIA) et de l'acquisition dépendante des données (DDA) (<https://www.crownbio.com>)

Bien que le DIA offre une couverture MS/MS plus importante que le DDA en permettant la fragmentation d'ions qui n'auraient pas atteint les critères pour être fragmentés, il est principalement utilisé dans des approches ciblées avec l'emploi de bibliothèques spectrales. La perte du lien entre les ions fragments et leur ion parent complexifie fortement le retraitement des données et l'étape de déconvolution. Toutefois, de récentes avancées ont permis le développement d'un workflow facilitant la construction de réseaux moléculaires⁸⁰. C'est pourquoi le DDA est le mode le plus couramment utilisé pour des analyses en métabolomique de plantes et de produits naturels, permettant la construction des réseaux moléculaires de manière plus aisée (voir Chap.I2)⁷⁹.

Une des limites de ce mode d'acquisition est le temps pour l'acquisition d'un spectre MS/MS, il est ainsi nécessaire de définir le nombre maximal de précurseurs par cycle MS. Un juste

milieu doit être trouvé entre le nombre de spectres MS (ou *full scan*) à réaliser et le nombre de précurseurs à sélectionner pour être fragmentés. En effet, le développement de l'UHPLC a permis une réduction du temps d'analyse et l'augmentation de la résolution, ce qui se traduit par des pics chromatographiques plus fins, et donc un besoin d'une fréquence d'acquisition du détecteur plus élevée, sans pour autant perdre en sensibilité³⁸. Dans les expériences DDA, une fois le scan MS acquis, l'appareil peut ensuite effectuer l'acquisition de scans MS/MS en fonction des paramètres établis. Plus le nombre de précurseurs à chaque cycle est important plus cela va prendre de temps à l'instrument pour réaliser toutes les acquisitions MS/MS. Il s'agit donc de trouver un compromis entre le temps nécessaire pour réaliser le scan MS sans perdre en sensibilité, et le nombre maximal de précurseurs à fragmenter à chaque scan pour ne pas avoir de cycle trop long, et ainsi avoir toujours assez de points pour décrire un pic chromatographique⁷⁸.

Dans le cas des analyses Q-TOF, si un scan prend 200 ms à être réalisé, et que l'on souhaite fragmenter 4 précurseurs à la même fréquence, alors il faudra au minimum 1 s pour réaliser le full scan et les 4 MS/MS (Figure 17). Ainsi, si le pic est fin et que le nombre de précurseurs est trop élevé, il n'y aura pas un nombre suffisant de MS réalisées pour décrire le pic chromatographique et ainsi de l'information risque d'être manquée. Le Q-TOF possède une résolution similaire en MS et MS/MS, à la différence des Orbitraps pour lesquelles la résolution en MS/MS est souvent sacrifiée afin de gagner en rapidité⁸¹.

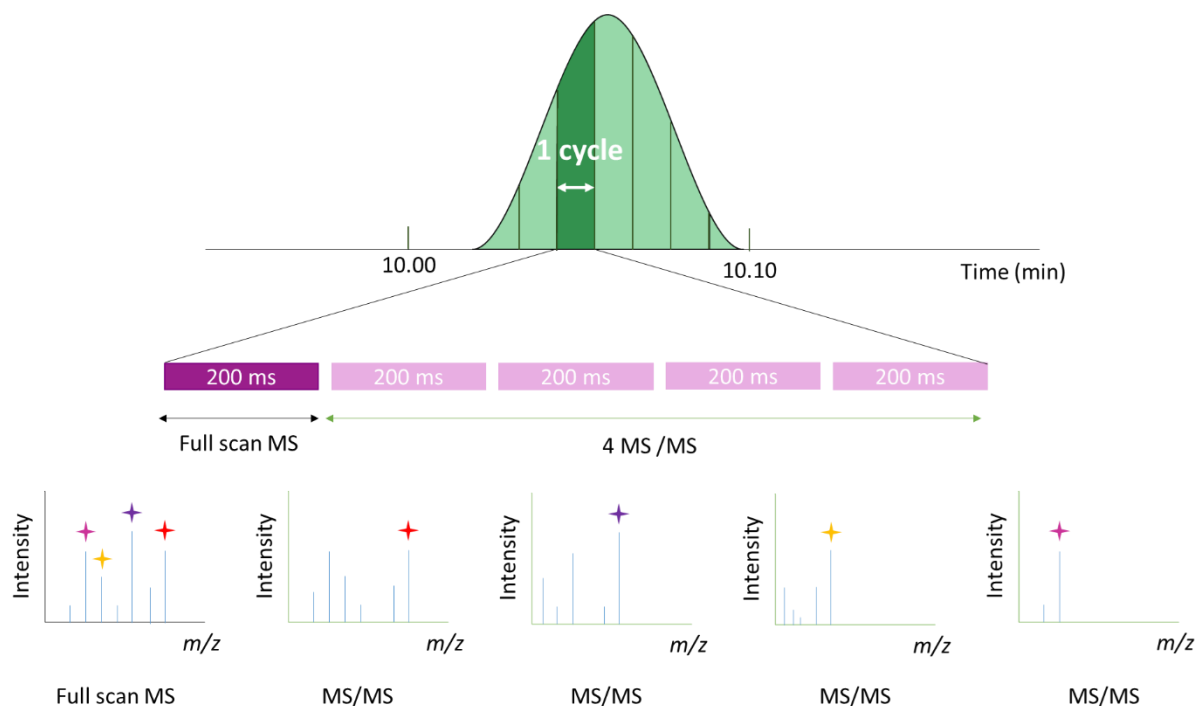


Figure 17 : Principe de fonctionnement du DDA avec 4 précurseurs. Un pic chromatographique est découpé en plusieurs cycles. Chaque cycle comporte 1 spectre full scan (violet foncé) pour lequel sont sélectionnés 4 précurseurs (étoiles de couleur), suivi de 4 scans MS/MS (violet clair) correspondant chacun à un des ions sélectionnés.

Partie II. Traitement des données de spectrométrie de masse pour l'annotation et la priorisation

Si, comme cela a été montré ci-dessus, les paramètres d'acquisition des données sont importants, le prétraitement de ces données est une étape cruciale avant de pouvoir en réaliser l'interprétation. Les étapes de traitement de données de spectrométrie de masse réalisées durant cette thèse ont différé en fonction des projets et de leur objectif respectif. Les étapes de traitement des données pour la réalisation d'une *feature list*, de réseaux moléculaires, et d'analyses statistiques sont abordés dans cette seconde partie.

1. Formats des données HRMS/MS

Comme expliqué précédemment, les données exploitées durant cette thèse ont été acquises sur deux instruments possédant deux géométries différentes de deux constructeurs différents. Les grandes étapes du traitement de données sont similaires pour les deux appareils, mais une attention particulière doit être portée au début du processus, particulièrement lors de la conversion des données.

Tout d'abord, afin de suivre les différents workflows de retraitement de données il est souvent nécessaire d'utiliser des données centroïdées et de les convertir dans un format libre « mzXML » ou « mzML »⁸². Les étapes pour parvenir à l'utilisation de données centroïdées et en format libre sont détaillées ci-dessous.

Les données de spectrométrie de masse sont généralement présentées en mode **profile** ou **centroid**. En mode *profile*, un pic est représenté par un grand nombre de points, tandis que dans le mode *centroid*, il est réduit à une seule valeur discrète de m/z . Lors de l'acquisition des données brutes, les spectres de masse sont généralement acquis en mode *profile*, également appelé « continu ». Cependant, il est aussi possible de les acquérir directement en mode *centroid*, ce qui permet de réduire la taille des données mais supprime de l'information, comme la forme du pic. Dans le cas des analyses réalisées sur le Q-TOF, un mode *line* est également disponible. Il s'agit d'un mode spécifique du constructeur Bruker, qui se rapproche du mode *centroid*. Le spectre *line* est un spectre recalculé, il permet également d'alléger la taille des données en prenant la valeur de m/z du barycentre du pic du mode *profile* comme sommet d'un pic gaussien.

Lorsque les données sont en mode *profile*, une étape de centroïdage est donc nécessaire avant le traitement de données. Cette étape consiste à transformer un pic représenté par plusieurs points en un seul pic central. Il est important de garder cette étape en tête car elle peut entraîner un léger décalage m/z comme montré en Figure 18, où le passage du mode *profile* au mode *centroid* a induit un décalage de $0.0004\ m/z$ ⁸³.

Cette étape se réalise généralement au moment de la conversion des données du format constructeur au format libre. En fonction des constructeurs et des appareils utilisés, différentes précautions sont à prendre.

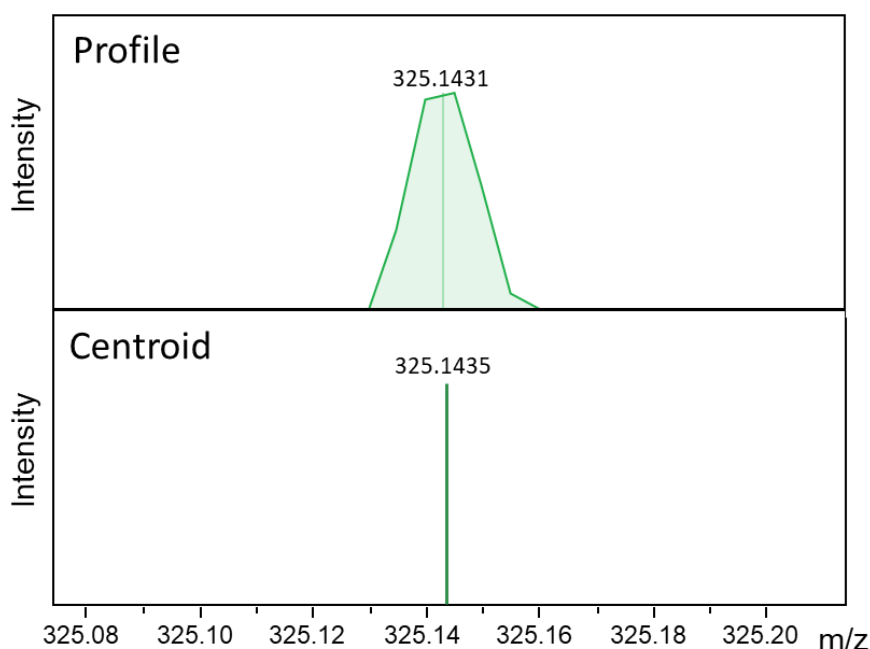


Figure 18 : Principe des modes *profile* et *centroid*

Pour des données brutes (.d) obtenues avec le maXis Q-TOF Bruker il est important de vérifier l'application de la *lock mass*, c'est-à-dire de la correction des valeurs à l'aide d'un calibrant interne, avant conversion des données (ouverture des données sous logiciel constructeur et vérifier que la *lock mass* est appliquée). Les données peuvent ensuite être converties, en ligne de commande, en utilisant l'outil Bruker Compass Xport. En fonction des appareils ou des versions de logiciel, l'ion précurseur n'est pas toujours corrigé avec la *lock mass*, ce qui peut induire une différence de plusieurs dizaines de ppm entre la masse de l'ion parent et sa valeur corrigée. Ce problème a été souligné lors de l'utilisation du maXis Q-TOF Bruker, et du logiciel Bruker DataAnalysis version 4.1 pour l'étude de deux mélanges complexes de deux natures différentes⁸⁴. Ainsi, la valeur de l'ion précurseur des données MS/MS converties sera fausse et ne pourra pas être utilisée pour le calcul de formule brute par exemple. Il est donc important, une fois la conversion des données réalisée, de vérifier que l'ion précurseur indiqué dans le fichier mzXML ou mzML correspond aux données corrigées. Afin de résoudre ce problème, un script disponible en ligne permet de corriger les données lorsque cela est nécessaire (<https://github.com/elnurgar/mzxml-precursor-corrector>)⁸⁵.

Dans le cas de données acquises sur l'Orbitrap de chez Thermo Scientific, les données brutes (raw) sont dans la plupart des cas corrigées en temps réel par rapport à un calibrant lors de l'acquisition. Ces dernières bénéficient donc d'une correction et peuvent ainsi être

directement converties au format mzML ou mzXML en utilisant des outils de conversion libres tels que MS convert de Proteowizard⁸⁶.

2. Obtention d'une *feature table*

Durant cette thèse, deux outils principaux permettant le prétraitement des données ont été utilisés. Dans un premier temps le logiciel constructeur MetaboScape 4.0 avec l'algorithme T-ReX (*Time aligned Region Extraction*), a été employé pour traiter des données de MS acquises sur le maXis Q-TOF Bruker. Les principaux paramètres pouvant être modifiés sont : la gamme de temps de rétention, la gamme de masse et le seuil d'intensité de détection de masse. Il permet un retraitement simple et rapide, et à l'avantage d'utiliser les données au format constructeur, ce qui évite de faire face à de possibles problèmes lors de leur conversion comme expliqué précédemment.

Les données MS/MS ont été traitées par MZmine 3.9 et MZmine 4.2⁸⁷ qui offrent davantage de paramètres de retraitement, mais dont la prise en main peut-être plus longue. MZmine est un logiciel open-source avec une communauté grandissante partageant des workflows ou des conseils. Le logiciel est régulièrement mis à jour avec de nouvelles fonctionnalités pour suivre les avancées technologiques, comme la mobilité ionique ou l'imagerie. Dans le cadre des données MS/MS, le workflow reprenant les grandes étapes de retraitement est détaillé ci-dessous à travers la Figure 19⁸⁸⁻⁹¹.

Grandes étapes du retraitement

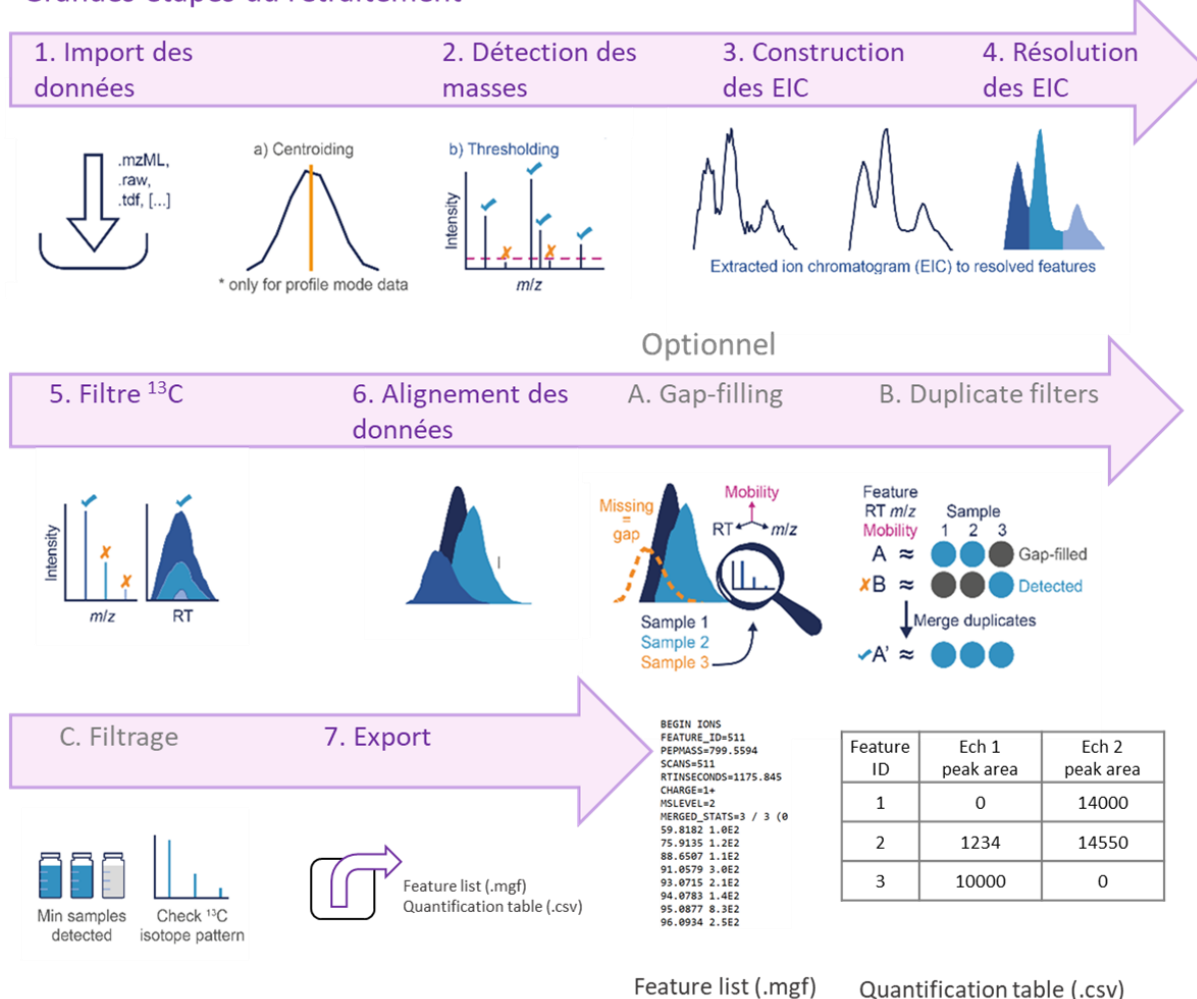


Figure 19 : Etapes nécessaires à l'élaboration d'une *feature list* et de la table de quantification correspondante, comportant 7 grandes étapes écrites en violet (1-7) et 3 étapes optionnelles écrites en gris (A-C).

1. L'import des données : les données préférentiellement converties en format mzML ou mzXML peuvent être importées. Il est possible à ce moment d'avoir une première visualisation des analyses.

2. La détection des pics (*mass detection*) : si les données n'ont pas été précédemment centroidées, il est possible de le réaliser à cette étape. La détection des pics permet de créer une liste de m/z associé à une intensité. Pour cela, il est important de déterminer le seuil d'intensité minimal de détection afin d'éviter que des signaux du bruit de fond, de faible intensité et non pertinents soient considérés comme étant des signaux d'analytes. Ce seuil de bruit de fond va pouvoir être ajusté en fonction de l'appareil et de sa géométrie, des données à analyser, et du retraitement souhaité. Il est possible d'ajuster le seuil limite de bruit de fond en visualisant directement son impact dans une fenêtre. Cette étape a également l'avantage de diminuer la taille des données pour les prochains retraitements. Quand des données MS/MS sont collectées, il faut définir un

seuil pour la MS et un également un seuil pour la MS/MS. Différentes stratégies peuvent être employées, par exemple les seuils pourront être plus bas s'il y a une volonté de détecter les composés les plus minoritaires ou mal ionisés.

3. La reconstruction des chromatogrammes : cette étape permet de construire un EIC (Extract Ion Chromatogram) pour chaque valeur de m/z précédemment détecté.

4. La résolution des EIC : deux principaux algorithmes de résolution peuvent être employés : ADAP resolver et Local minimum resolver. Plusieurs paramètres sont à ajuster, comme le nombre de points minimum pour définir un EIC à partir du seuil minimum établi. Il est important, pendant l'ajustement des paramètres, de visualiser un bon EIC et un mauvais, afin de voir l'impact des paramètres sur la construction des pics chromatographiques et de les adapter afin d'obtenir des EIC de qualité correspondant réellement à des pics.

5. Filtre isotopique ^{13}C : l'application du filtre permet de détecter les pics correspondant aux isotopes ^{13}C . Ce filtre permet de garder uniquement les informations du feature correspondant au pic le plus intense et non lié au pic du ^{13}C . Cela limite la présence de feature correspondant à l'isotope ^{13}C , donc limite les mauvaises propositions d'annotation associées comme présenté Figure 20. De plus, les informations du massif isotopique aide à la détermination de la formule brute.

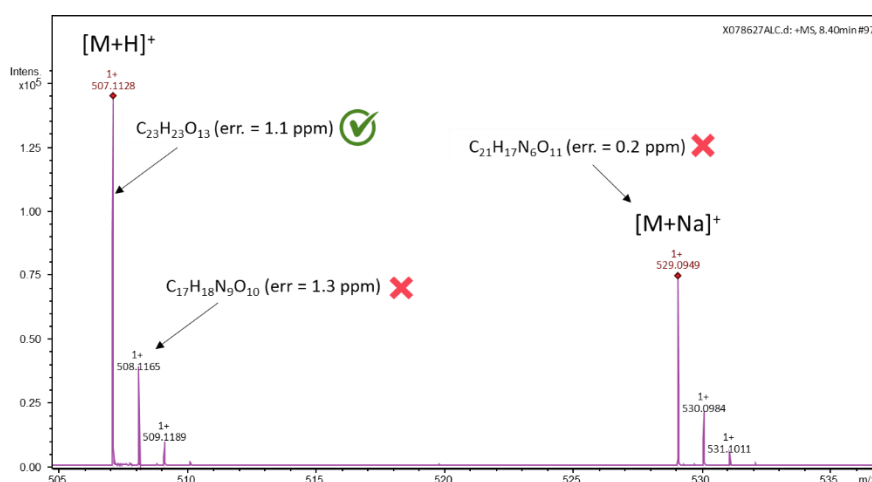


Figure 20 : Exemple de spectre de masse MS de la quercetine-3-acétylglucoside ($\text{C}_{23}\text{H}_{22}\text{O}_{13}$) et propositions de formules brutes associées au $[\text{M}+\text{H}]^+$ à l'isotope ^{13}C et à l'adduit $[\text{M} + \text{Na}]^+$

6. Alignement : cette étape est importante lors de l'analyse de plusieurs échantillons qui serviront à la même étude. Il a pour objectif d'identifier sous une même entité, un même analyte se trouvant dans plusieurs échantillons en prenant en compte la possible légère variation du temps de rétention et de la précision en masse d'une analyse à l'autre. Il s'agit donc d'établir les paramètres optimaux pour tenir compte de ce phénomène, mais sans pour autant avoir sous la même entité deux analytes

différents, comme dans le cas des isomères, qui ne peuvent être distingués que par leur différence de temps de rétention. A la suite de cette étape, une seule *aligned feature table* est obtenue pour l'ensemble d'une série d'analyses servant à la même étude.

7. Export : différents types d'exports sont réalisables en fonction du workflow utilisé par la suite. Les exports consistent généralement en un fichier « mgf (*Mascot Generic Format*) » contenant la liste des *features* et les informations de spectrométrie de masse associées (numéro d'identification, numéro du scan, masse du précurseurs, la charge, masse et intensité/aire des fragments MS/MS), ainsi qu'une table de quantification des *features* « csv » contenant les informations d'intensité ou aire selon l'export choisi. La Figure 21 présente un extrait de *feature list* ainsi que les informations associées.

```

BEGIN IONS
FEATURE_ID=511
PEPMASS=799.5594
SCANS=511
RTINSECONDS=1175.845
CHARGE=1+
MSLEVEL=2
MERGED_STATS=3 / 3 (0 removed due to low quality, 0 removed due to low cosine).
59.8182 1.0E2
75.9135 1.2E2
88.6507 1.1E2
91.0579 3.0E2
93.0715 2.1E2
94.0783 1.4E2
95.0877 8.3E2
96.0934 2.5E2

```

Figure 21 : Extrait d'une *feature list* en mgf obtenue avec un traitement des données par MZmine 3

8. Vérification manuelle : une dernière étape de vérification manuelle est nécessaire afin de s'assurer de la bonne cohérence de la *feature list* avec les chromatogrammes et spectres de masse initiaux.

D'autres étapes optionnelles régulièrement utilisées peuvent être réalisées :

- **A. Le « gap filling » :** cette étape consiste à aller rechercher les *features* de la liste alignée dans tous les échantillons à un seuil de masse inférieur à celui précédemment établi pour venir remplir au maximum la matrice. Cette option permet de vérifier la présence d'un analyte qui n'aurait pas rempli le critère d'intensité dans certains échantillons.
- **B. Duplicate filter :** cette étape peut être nécessaire après l'utilisation du « gap filling » pour éliminer les lignes mal alignées.

- **C. Filtering** : il est possible d'appliquer différents filtres sur la *feature list*. Il est possible de ne garder que les *features* possédant un profil isotopique complet, ou d'éliminer ceux ne possédant pas de MS/MS (pour la création de RM par exemple).

En fonction des objectifs de l'étude, des conditions analytiques et instrumentales les paramètres peuvent varier fortement. Par exemple, les seuils de détection lors de l'utilisation d'un Q-TOF et d'une Orbitrap seront différents, car les Orbitraps produisent généralement une intensité de signal supérieure. Pour ces raisons, il est important d'explorer ses données et d'adapter les seuils et tolérance en fonction. Dans l'étude d'extraits complexes comme les extraits de plantes, il est difficile de réaliser une *feature list* dont les paramètres sont optimaux pour tous les composés, il s'agit donc de faire des compromis afin d'obtenir une vision globale de l'extrait analysé.

3. De la *feature list* à la comparaison d'échantillon

3.1. Les analyses statistiques

Selon la problématique initiale, l'utilisation de méthodes statistiques peut être nécessaire ; notamment lorsque l'on cherche à mettre en avant des marqueurs ou à comparer différents échantillons biologiques. Il existe deux grandes catégories d'analyses statistiques : l'analyse univariée et l'analyse multivariée.

a. Les analyses univariées

L'analyse univariée, comme son nom l'indique, va prendre en compte une seule variable comme par exemple la concentration d'un métabolite spécifique ou une activité biologique dans le cas d'analyse d'extraits de plantes. Il existe différents tests statistiques univariés permettant de comparer les moyennes ou les médianes d'un jeu de données. Les méthodes d'analyse univariée classiquement rencontrées pour les tests paramétriques (homogénéité des variances, normalité, indépendance) sont le test de student (ou t-test), qui permet de comparer les moyennes de deux échantillons ou groupes et l'ANOVA lorsqu'il y a plus de deux groupes⁹².

b. Les analyses multivariées

Lorsque les données à analyser sont des données de LC-MS, chaque ion moléculaire ou adduit va pouvoir être considéré comme une variable. Il est possible de multiplier les tests univariés, pour lequel l'interprétation est plus simple mais dont le risque de faux positif augmente avec le nombre de tests. Une autre approche possible dans le cas des données de métabolomique est de faire appel à des méthodes d'analyses multivariées.

Les méthodes multivariées prennent en compte de deux à plusieurs centaines de variables. Il existe deux principales catégories : les **méthodes non supervisées** et les **méthodes**

supervisées⁹³. Les méthodes non supervisées permettent d'obtenir une première idée de la structure des données sans *a priori*, et de découvrir comment elles se groupent sans indiquer des métadonnées au contraire des méthodes supervisées qui tiennent compte des métadonnées. Les méthodes supervisées sont largement utilisées pour la découverte de biomarqueurs, la classification et la prédiction, ce qui n'est pas possible avec les méthodes non-supervisées⁹⁴. Parmi ces méthodes, l'analyse par composante principale et la régression des moindres carrés partiels discriminante ont pu être employées dans le cadre de cette thèse.

L'analyse par composante principale (ACP) (*Principal Component Analysis, PCA*) est une méthode non supervisée permettant la projection des données dans une dimension réduite en capturant les informations des données autant que possible^{93,95}. L'ACP est principalement utilisée pour trouver des liens entre les individus, sans *a priori* (identification de classe), soit connu par avance soit inconnu. Elle permet également de mettre en évidence les *outliers*, c'est à dire les objets ne rentrant dans aucune classe⁹⁶. Deux principales représentations graphiques sont utilisées en ACP : le graphe des individus (*score plot*) et le cercle des corrélations. Le graphe des individus permet de visualiser la position des individus sur un plan factoriel composé de deux axes, chaque axe étant expliqué par un certain pourcentage de la variance. Le cercle des corrélations permet, quant à lui, d'identifier les variables, expliquant la structuration des données⁹⁷.

De la même façon que l'ACP, la **PLS-DA** (*Partial least squares discriminant analysis*) permet de projeter les données dans une dimension réduite mais en tenant compte des groupes préalablement établis⁹⁸. L'interprétation de données en PLS-DA est particulièrement utilisée pour identifier les variables responsables d'une différence entre les groupes. Tout comme l'ACP, la PLS-DA permet la représentation en graphe des individus et le cercle des corrélations⁹⁷. Une autre caractéristique de la PLS-DA, est son caractère prédictif. Il est ainsi possible de l'utiliser pour prédire le groupe d'appartenance d'un individu en fonction de ses variables. La PLS-DA est particulièrement utilisée dans la recherche de biomarqueurs pour lesquels les groupes sont déjà identifiés⁹³. L'identification des variables impliquées dans la différenciation des groupes peut se faire en utilisant les VIP (*variable of importance in projection*). L'idée de ce score est de mesurer le poids de la contribution de chaque variable. Il est généralement admis qu'une variable peut être considérée comme ayant un impact si sa valeur est supérieure à 1⁹⁹.

Par exemple, dans le cas d'une étude comparative de différents cultivars de d'orge, l'utilisation d'outils de statistique telle que la PLS-DA pour la comparaison d'analyse LC-HRMS d'extrait hydrométhanolique, a permis de mettre en évidence les molécules impliquées dans les différences phytochimique ; leur annotation a ensuite pu être possible (Figure 22)¹⁰⁰.

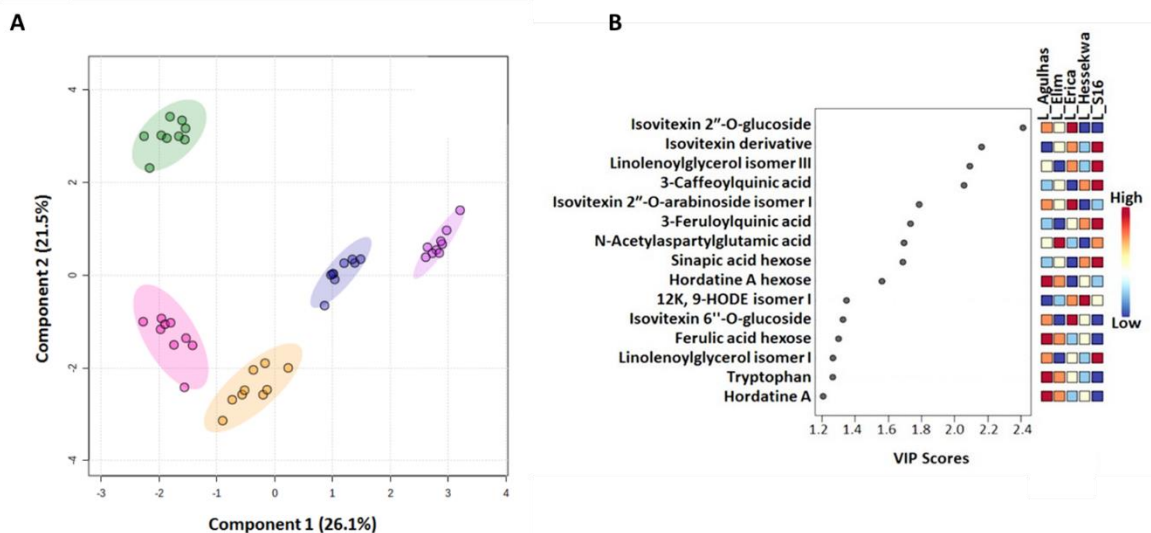


Figure 22 : (A) PLS-DA des extraits de feuilles de cinq cultivars d'orge et (B) métabolites les plus discriminants identifiés par PLS-DA et classés par score d'importance des variables (VIP score)¹⁰⁰

Ces traitements statistiques peuvent se faire en utilisant des langages de programmation comme Python ou R. R est un langage et un environnement développé pour l'analyse statistique et les graphiques alors que Python est un langage plus versatile. La prise en main de langage de programmation, malgré les nombreux tutoriels existant peut-être difficile, c'est pour cela que des interfaces en ligne se sont développées pour rendre plus accessible les outils de statistiques. L'interface en ligne MetaboAnalyst (<https://www.metaboanalyst.ca>) qui utilise le langage R est particulièrement adaptée pour l'analyse statistique de données de chromatographie couplée à de la spectrométrie de masse et permet de faire différents tests comme les ACP et les PLS-DA¹⁰¹.

3.2. Réseaux moléculaires

a. Principe

Les réseaux moléculaires (Figure 23), initialement présentés comme un complément à la déréplication et développés par un laboratoire de l'Université de Californie de San Diego, sont désormais utilisés comme un véritable workflow pour la déréplication¹⁰². Les réseaux moléculaires permettent une visualisation des données MS/MS et de l'espace chimique. Le principe global des réseaux moléculaires est d'organiser les données en fonction de leur similarité spectrale.

Ainsi, les molécules structurellement similaires, qui ont des voies de fragmentation semblables, vont être associées. Pour associer deux molécules, un nombre minimal de fragment et/ou de perte de neutre commun est d'abord fixé, en fonction des énergies de collision employées et de la richesse des spectres MS/MS la valeur est généralement comprise entre 4 et 8. Une fois ce premier critère atteint, un deuxième critère intervient, le **cosine score**. Il permet de relier ou non deux ions entre eux en fonction de cette similarité avec pour la valeur 1, les deux spectres sont superposables et pour la valeur 0, aucune perte de neutre ou d'ion commun. Un seuil minimal de *cosine score*, généralement compris entre 0,6 et 0,8 détermine le seuil à partir duquel les ions vont être reliés. Les clusters vont ainsi se former et les données vont pouvoir s'organiser.

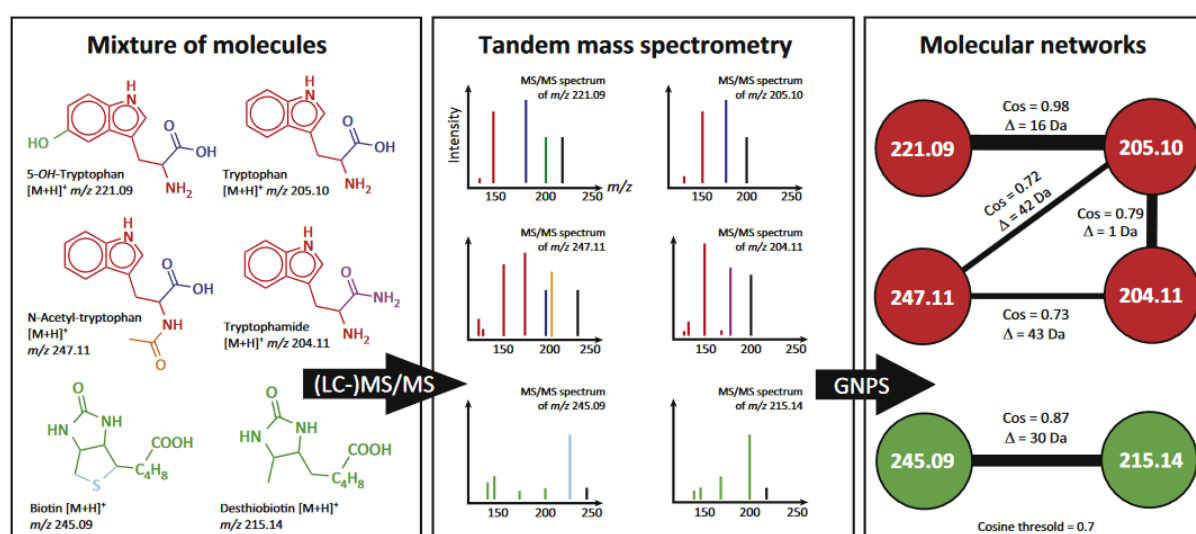


Figure 23 : Principe de fonctionnement des réseaux et exemple d'application avec des dérivés de tryptophan et de biotine¹⁰³. Le mélange de molécules est analysé par LC-MS/MS. Les spectres MS/MS de chacun des composés sont comparés. Les ions des composés analysés sont représentés par des nœuds, et si leur score de similarité (*Cosine score*) dépasse 0,7, ils sont alors reliés pour former un cluster.

Depuis le début des réseaux moléculaires en 2012¹⁰⁴, ils n'ont cessé d'être utilisés et d'évoluer. Cette méthodologie a été rendue accessible par le développement de la plateforme GNPS (<http://gnps.ucsd.edu>)¹⁰⁵ à partir de 2016, permettant de générer en ligne des réseaux moléculaires. Elle s'est depuis répandue et est devenu un outil prisé pour la découverte de nouvelles molécules bioactives¹⁰³.

b. Construction et visualisation des réseaux moléculaires

Les premiers réseaux moléculaires, appelés « **Classical MN** », peuvent être générés directement à partir de données LC-HRMS/MS centroïdées au format mzML, mzXML ou mgf sur la plateforme GNPS et peuvent donc être générés avec ou sans prétraitement des données.

Les “**Feature-Based Molecular Networks**” (FBMN) se sont développés à partir de son introduction en 2017¹⁰⁶ car l'utilisation de cet outil permet de comparer un plus grand nombre d'échantillons, d'avoir une information quantitative à partir des données LC-MS, de favoriser la distinction des isomères et de réduire la redondance d'information. Les FBMN nécessitent cependant un pré-traitement des données comprenant au minimum une détection des *features* et un alignement avant d'être traitées sur la plateforme GNPS. Une table de quantification (csv ou txt) et un document comprenant les informations spectrales (mgf) sont ensuite nécessaires pour l'utilisation de cette méthode. En plus de l'information de la somme d'intensité ou d'aire qui peut être représentée sur le réseau moléculaire, la table de quantification peut être utilisée pour représenter la répartition des ions au sein des échantillons. Dans l'exemple de la Figure 24, issue de l'étude d'un extrait fractionné de *Stellaria alsine*, la somme de l'intensité de l'ion en MS est représentée par rapport à la taille du nœud et la répartition de l'ion entre chacune des fractions est représentée avec une couleur différente ; il est ainsi rapidement visible dans cet exemple que les ions du cluster mis en avant sont majoritairement issus d'une fraction : la fraction acétate d'éthyle¹⁰⁷.

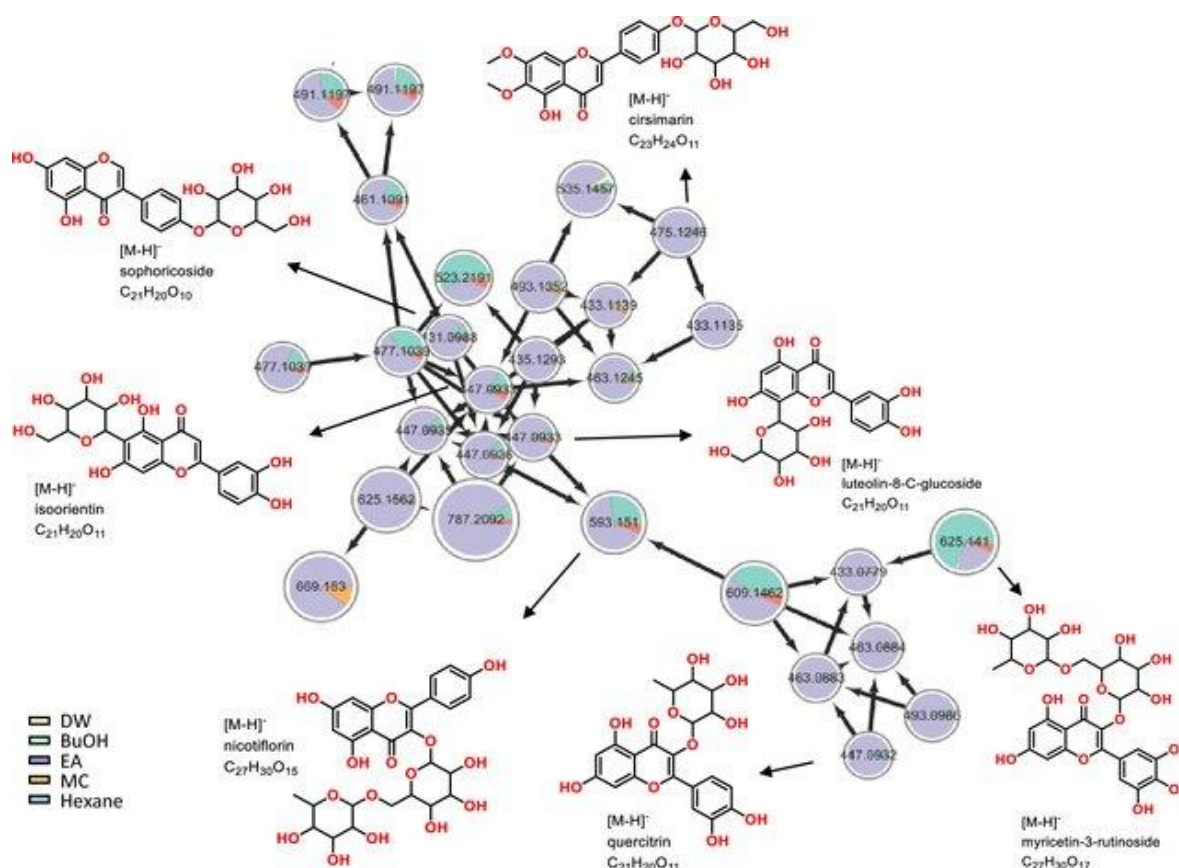


Figure 24 : FBMN des fractions de *Stellaria alsine*¹⁰⁷. Le chiffre indique la valeur m/z de l'ion précurseur et la taille du nœud est proportionnelle à la somme des intensités de l'ion dans les fractions. Les ions sont colorés en fonction des fractions, chaque nœud est ainsi divisé en fonction de sa proportion dans chacune des fractions.

D'autres outils permettant de construire des réseaux moléculaires ont été développés par la communauté scientifique, mais n'ont que peu ou pas été expérimentés durant cette thèse. Parmi eux, l'*Ion Identity Molecular Network* (IIMN) également développé sur la plateforme GNPS permet de relier les adduits d'un même composé et ainsi créer davantage de connexion et éviter la redondance d'information¹⁰⁸.

Il existe également d'autres possibilités ne passant pas par la plateforme GNPS, comme l'utilisation de **MetGem** qui est un logiciel permettant de générer en local des réseaux moléculaires, développé à partir de 2018¹⁰⁹. Le fait de générer des RM en local présente l'avantage de ne pas avoir à déposer ses données sur une plateforme ouverte, ce qui pourrait poser des problèmes quant à la confidentialité des données. De plus, cela assure une indépendance face aux maintenances et aux éventuels problèmes liés à l'utilisation d'un service externe. Cependant, cela nécessite un ordinateur suffisamment performant pour réaliser les calculs requis pour la génération des RM. De plus, MetGem se distingue également car il propose deux visualisations : la première est un réseau moléculaire similaire à celui proposé par GNPS ; la seconde est un graphique basé sur l'algorithme t-SNE (*t-distributed stochastic neighbor embedding*) qui préserve les interactions entre les groupes en les rapprochant dans l'espace. Ainsi, deux clusters chimiquement similaires vont être également à une distance proche lors de la visualisation alors que dans le cas de GNPS, la position des clusters les uns par rapport aux autres dans le réseau moléculaire est seulement liée au nombre de nœuds les composant. Certains RM peuvent être visualisés directement, comme c'est le cas pour les réseaux générés avec MetGem, en revanche, pour les RM établis à l'aide de GNPS, l'utilisation de Cytoscape pour les visualiser est nécessaire¹¹⁰. Enfin, depuis sa dernière version, MZmine 4.0 permet également la construction de réseaux moléculaires, directement à travers son interface.

c. Les réseaux moléculaires multi-informatifs

Les RM qui, comme expliqué précédemment, sont au départ un outil de visualisation de la diversité chimique d'un échantillon, peuvent par l'ajout d'information de différentes origines être utilisés pour la découverte de nouveauté chimique, la comparaison d'échantillon ou la déréplication.

L'utilisation de différentes couches d'information permet d'orienter les études en fonction des objectifs. Un mapping utilisant les données de bioactivité (IC50) et un autre utilisant les données taxonomiques sur un RM construit à partir de 292 extraits d'Euphorbiaceae ont permis de mettre en évidence différentes spécificités. L'étude a ainsi pu être orientée sur un extrait de feuille de *Bocquillona nervosa* dans lequel des composés connus pour leur activité anti-CHIKV ont été identifiés¹¹¹. Dans une autre étude portant sur le même set de données, des clusters spécifiques à chacun des 20 genres des 292 échantillons analysés ont pu être mis

en évidence grâce à un code couleur et les spécificités chimiques de l'espèce *Codiaeum peltatum* sont ressorties, ce qui a mené à l'isolement dans un temps réduit à cinq quinolones tétracycliques monoterpenylées¹¹². Dans un autre temps, la cartographie de l'activité a également permis de prioriser le fractionnement sur 2 clusters seulement qui a mené à l'isolement de 8 composés dont 4 nouveaux. Parmi ces 8 composés, 5 ont montré de fortes activités inhibitrices de la dengue et de zika en test de première intention, 1 composé, l'actephilol B a ensuite montré une activité spécifique pour la cible NS5¹¹³.

Dans le cas des réseaux moléculaires bioactifs, le principe est de coupler les données du RM à un score de bioactivité prédit à partir des niveaux d'activités de chacune des fractions et de l'abondance relative de la molécule à travers les fractions. L'utilisation d'un script disponible en ligne permet d'associer à chaque *feature* le score de bioactivité, qui va ensuite pouvoir être représenté dans le RM (https://github.com/DorresteinLaboratory/Bioactive_Molecular_Networks). Cette méthodologie a ainsi permis de réévaluer l'activité d'un extrait de *Euphorbia dendroides* contre le virus du chikungunya. Grâce à cette approche, une fraction active dont les composés n'avaient pas pu être identifiés par une approche de fractionnement bio-guidée classique ont pu être détectés^{114,115}. Pour confirmer cette méthodologie, les composés mis en avant ont été isolés et testés et deux d'entre eux ont ainsi montré une bonne activité et une sélectivité supérieure à celle des composés identifiés précédemment (Figure 25).

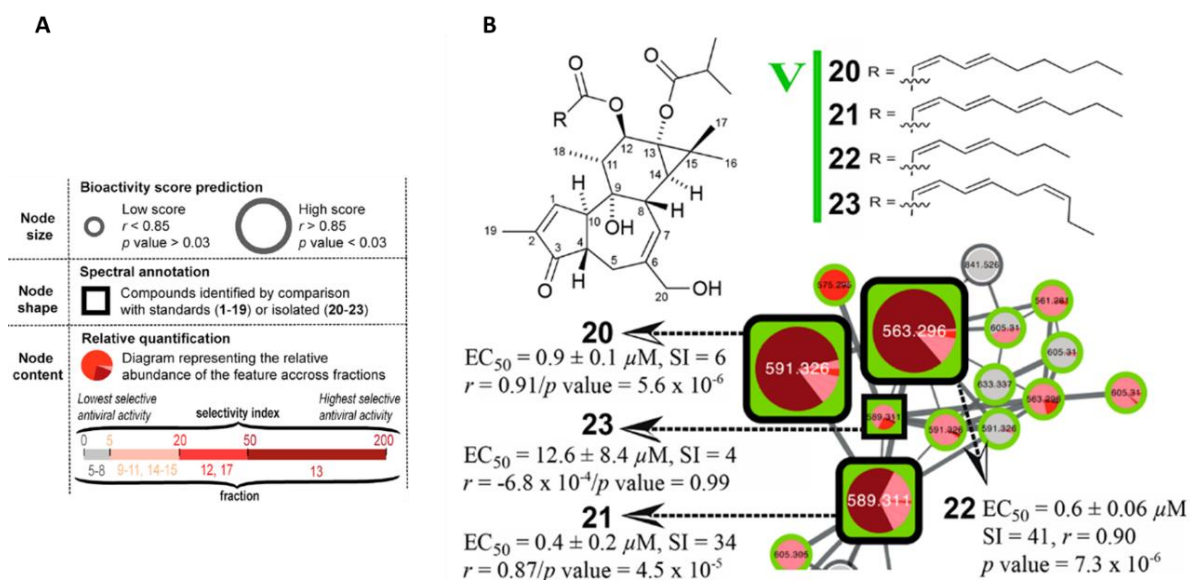


Figure 25 : Zoom sur le cluster avec des scores de bioactivité significatifs du RM d'un extrait de *Euphorbia dendroides*. A) Légende des RM bioactifs. B) Le résultats du réseau bioactif montrent que les molécules 20–22 ont des scores de bioactivité élevés (nœuds larges). Leur isolement a confirmé qu'il s'agit de dérivés de déoxyphorbol esters. L'évaluation de leur activité biologique a révélé que les composés 21 et 22 sont des inhibiteurs sélectifs de la réplication du virus chikungunya. Le composé 23 a également été isolé, mais a montré une activité antivirale inférieure aux composés 20–22.¹¹⁵

Basé sur un le principe d'isolement guidé par les réseaux de bioactivité, quatre composés anti-VRE (*Enterococcus faecium* résistant à la vancomycine), les epicolidines A – C, trois nouveaux acides tétramiques polycycliques et l'antibiotique PF 1052 ont été isolés à partir du champignon *Epicoccum* sp. 1-042¹¹⁶. Il existe ainsi différentes stratégies permettant de visualiser l'activité sur des RM, toutes ayant pour objectif d'orienter le fractionnement.

Il est ainsi possible, en modulant la taille des nœuds, les diagrammes de répartition, les symboles et d'autres fonctionnalités, de représenter plusieurs types d'information (métadonnées) sur un même réseau moléculaire. L'objectif est d'extraire de nouvelles connaissances et de mettre en avant des spécificités, des points communs ou des corrélations entre les informations.

Partie III. L'annotation des données de spectrométrie de masse

L'annotation ou l'identification putative d'un métabolite dans un extrait est souvent sujette à ambiguïté en raison des limitations des méthodes analytiques. Il est ainsi nécessaire de définir et de mettre en place des niveaux de confiance dans le processus d'annotation. Ces niveaux permettent de mieux quantifier la fiabilité de l'identification des métabolites et facilitant la validation des résultats lors d'études métabolomiques. Les différents niveaux d'annotation sont les suivants^{117–119}:

- Le **niveau 0** correspond à une structure isolée dont la structure 3D (configuration absolue) a été confirmée.
- Le **niveau 1** correspond à une **structure confirmée** ; par un standard ou par une méthode orthogonale (LC-MS/MS ou *Collision Cross Section* (CCS, information liée à l'utilisation de la mobilité ionique)).
- Le **niveau 2** correspond à une **structure probable**. Il s'agit de structure dont la correspondance a été trouvée dans la littérature ou des bases de données.
- Le **niveau 3** correspond à une **possible structure ou classe**. Il s'agit de structure pour lequel il existe plusieurs isomères de position par exemple, qu'il est difficile de discerner. Une information supplémentaire doit au moins aller dans le sens de l'hypothèse.
- Le **niveau 4** correspond à l'assignement d'une formule brute.
- Le **niveau 5** correspond à une structure d'intérêt pour laquelle il manque des informations pour déterminer la formule brute et pour laquelle on ne possède que la **masse exacte**.

Identifier des composés au niveau 0 ou 1 peut parfois s'avérer long et compliqué. Pour un composé connu cela peut être car le standard n'est pas disponible commercialement ou alors à un coût très élevé. Pour un composé nouveau, cela peut être en raison de la quantité de matière nécessaire pour l'isolement afin d'effectuer une caractérisation RMN ou encore la complexité de la structure (mobilité des liaisons pour le nombre de conformères, nombre de centre asymétrique etc.) rendant long et difficile la modélisation des spectres de dichroïsme circulaire pour la détermination de la configuration absolue. C'est pourquoi la déréplication s'intéresse à annoter les composés déjà identifiés afin de prioriser les recherches⁴⁸.

Dans le cas de données de spectrométrie de masse, réussir à annoter manuellement les données MS/MS peut s'avérer très laborieux. Bien qu'il existe des bases de données, le manque d'homogénéité (TOF ou OT, énergie de collision, gaz de collision) entraîne des différences dans les spectres MS/MS. Il est donc difficile de comparer des spectres acquis dans différentes conditions. La combinaison des méthodes computationnelles et des paramètres

expérimentaux peuvent aider à la compréhension des mécanismes de fragmentation et aider à une meilleure interprétation des données²¹.

1. L'annotation des features à partir de la MS

1.1. Détermination de la formule brute

La première étape pour aller vers l'annotation ou l'identification de composés d'intérêt est d'identifier les masses d'intérêt, et à partir de ces masses exactes d'être capable de proposer une formule brute. Cela peut s'avérer compliqué, même quand la précision de masse est inférieure à 1 ppm. En effet, plus la masse de l'ion d'intérêt est élevée, plus le nombre de possibilités est élevé et donc plus le risque d'erreur est grand⁵². C'est pourquoi, il est possible d'utiliser différents filtres pour la détermination des formules brutes, les 7 règles d'or établies en 2007 peuvent être résumées¹²⁰ :

1. La restriction des éléments : dans le cas d'annotation de produits naturels et d'extraits de plantes il est possible de réduire le nombre d'hétéroatomes en fonction de la masse étudiée. Il faut également avoir une connaissance des atomes susceptibles d'être présents dans les échantillons en fonction de la source biologique afin de ne pas être trop restrictif et de risquer une mauvaise annotation de formule brute.
2. Règles de Lewis et de Senior : les spectromètres de masse détectent des ions, la masse est donc calculée à partir d'un ion. Cela peut par exemple avoir un impact dans le cas d'une molécule chargée, comme les anthocyanes ou les alcaloïdes, pour lesquelles l'ion visible en ionisation positive ne sera pas le $[M+H]^+$ mais un $[M]^+$, induisant une différence de masse si la recherche est ensuite faite sur une formule brute neutre.
3. Le respect d'un massif isotopique correspondant à une distribution naturelle.
4. Le ratio hydrogène/carbone.
5. Le ratio d'hétéroatome.
6. Probabilité d'hétéroatome.
7. Règle pour la GC-MS (dans le cas où une étape de silylation a été nécessaire).

Lors de la détermination de formules brutes il est important d'avoir une bonne connaissance de ces données pour sélectionner au mieux les critères tels que la tolérance de masse acceptée ou la restriction des atomes.

Pour les données obtenues sur le maXis Q-TOF à l'ICOA, il est possible d'accéder au logiciel SmartFormula (Bruker Daltonics) pour la détermination des formules brutes. Le logiciel permet la prédiction de la composition élémentaire des ions précurseurs et des fragments. De la même façon, MZmine possède un outil intégré permettant la prédiction de formules brutes, qui de la même façon que SmartFormula, respecte les règles heuristiques connues comme les « 7 règles d'or » permettant d'écarter les formules ne correspondant pas dans un contexte de molécules

organiques. Une fois la formule brute de l'ion d'intérêt déterminée, il existe différentes stratégies pour annoter le composé.

1.2. Recherche de formule brute dans les bases de données

Une des premières méthodes pour la déréplication consiste à rechercher dans la littérature et les bases de données de métabolites spécialisés. Il existe de nombreuses bases de données disponibles en ligne en fonction du domaine d'application. Le Tableau 2 répertorie les principales bases de données utiles pour la recherche de formule brute de composés naturels.

Tableau 2 : Principales bases de données pour la recherche de formule brute

	Généraliste ou spécifique	Accessibilité	Nombre de composés	Informations disponibles
CheBI https://www.ebi.ac.uk/chebi/init.do	Petites molécules (naturelles ou synthétiques)	Gratuit	61 400 (complètement annotés)	Chimique
ChEMBL https://www.ebi.ac.uk/chembl/	Molécules bioactives	Gratuit	2 400 000	Bioactivité et génomique
COCONUT ¹²¹ https://steinbeck-lab.github.io/coconut/	Produits naturels	Gratuit	695 000	Relation métabolite-organisme
Le Dictionnaire de Produits Naturels https://dnp.chemnetbase.com	Produits naturels	Accès gratuit limité	340 000	
KnapSack ¹²² http://www.knapsackfamily.com/KNAPSAck_Family	Produits naturels	Gratuit	63 000	Relation métabolite-organisme
LOTUS ¹²³ https://lotus.naturalproducts.net	Produits naturels	Gratuit	750 000	Relation métabolite-organisme
Marinlit https://marinlit.rsc.org	Produits naturels marins		42 000	Relation métabolite-organisme
NPatlas ¹²⁴ https://www.npatlas.org	Produits naturels d'origine microbiennes		32 000	Relation métabolite-organisme
Pubchem ¹²⁵ https://pubchem.ncbi.nlm.nih.gov	Généraliste	Gratuit	119 000 000	Chimique, physique
Reaxys https://www.reaxys.com	Généraliste (composé organique)	Payant	260 000 000	Chimique, physique
Scifinder https://scifinder-n.cas.org	Généraliste (composé organique et inorganique)	Payant	204 000 000	Chimique, physique

Lors de l'étude d'une plante, à partir de l'information de masse exacte et de la formule brute, il est d'abord possible de se concentrer sur la recherche de métabolites déjà connus dans l'espèce, en s'appuyant sur une recherche bibliographique ou en utilisant des bases de données répertoriant les informations taxonomiques. Ensuite, en fonction des résultats obtenus, on peut élargir la recherche au niveau du genre ou de la famille pour accéder à davantage d'informations. L'exploration des bases de données permet un accès rapide aux articles de littérature associés aux molécules décrites. On peut ainsi trouver et comparer des informations telles que les spectres MS/MS, UV-Visible ou RMN avec ses propres données expérimentales. Si aucune information pertinente n'est disponible, il est également possible de consulter des bases de données plus généralistes, bien qu'il existe un risque de rencontrer des produits de synthèse. Lorsque nous utilisons les bases de données ou la littérature pour trouver une annotation coïncidant avec les données expérimentales. Répertorier manuellement tous les composés d'un genre est fastidieux. En effet, bien qu'il soit possible via LOTUS¹²³ par exemple, d'importer des fichiers .SDF via DataWarrior¹²⁶ pour la gestion des informations, les spectres MS/MS de masses ne sont ici pas exploités.

2. L'annotation des features à partir de la MS/MS

Avec le développement des méthodes de déréplication par réseaux moléculaires et l'apport de la bio-informatique dans ce domaine, d'autres outils se sont développés pour la déréplication et l'annotation de métabolites. Il est ainsi possible de venir annoter les composés non pas à partir de la formule mais à partir des données MS/MS. Les cinq principales approches pour la recherche en base de données sont résumées Figure 26 et permettent de comprendre les outils développés ensuite.

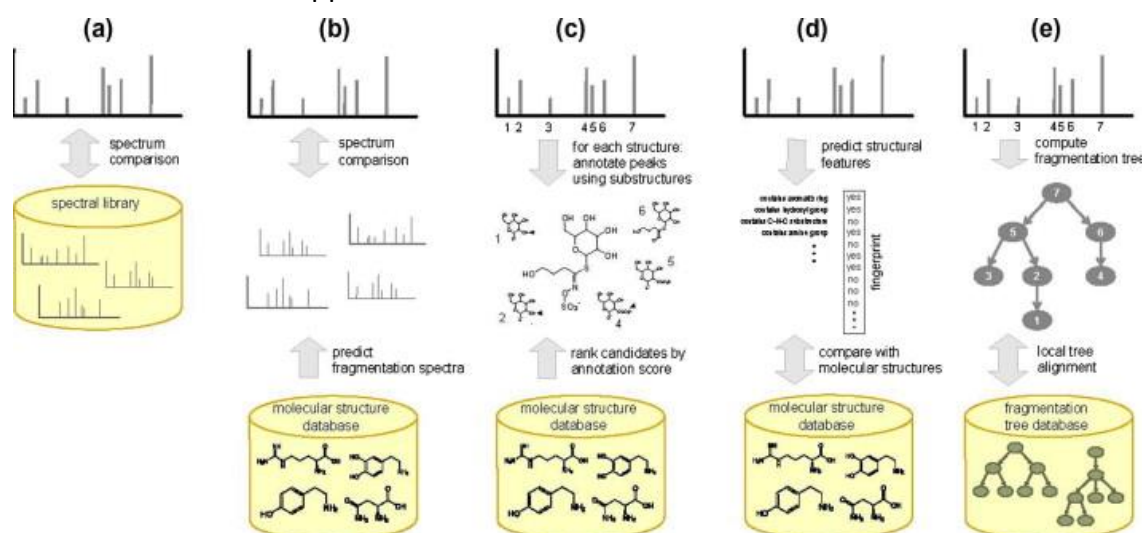


Figure 26 : Les cinq approches de comparaison des données de fragmentation : (a) interrogation de bibliothèques spectrales ; (b) prédiction du spectre de fragmentation ; (c) fragmentation combinatoire ; (d) prédiction des caractéristiques structurales ; et (e) arbres de fragmentation¹²⁷.

2.1. La comparaison de spectre MS/MS

Tout d'abord, il est possible de comparer les spectres expérimentaux avec des spectres contenus dans des bibliothèques. Par exemple, les spectres de masses utilisés pour la réalisation des réseaux moléculaires peuvent être comparés avec la base de données GNPS¹⁰⁵, qui regroupent plusieurs bibliothèques fournies par différents laboratoires internationaux. Les bibliothèques GNPS sont ainsi comparées de la même façon que pour la construction de réseaux moléculaires, en fonction du nombre de fragments communs et d'un cosinus score. Les données expérimentales telles que le type de spectromètres (OT, Q-TOF etc.), le mode d'ionisation ou l'énergie de collision sont généralement disponibles et permettent d'ajouter du poids à l'annotation. MassBank of EU et MassBank of North America (MoNA) permettent toutes deux de charger un spectre de masse (m/z et intensité correspondant) pour comparer avec leur base de données.

MASST est une interface web qui permet de rechercher des spectres MS/MS dans les dépôts de données publiques GNPS/MassIVE¹²⁸. Il vise à faciliter la réutilisation des données de spectrométrie de masse, en uniformisant les formats de données et en traçant l'origine de chaque spectre MS/MS. MASST fournit également un système de reporting pour afficher tous les spectres MS/MS identiques ou similaires trouvés dans les données publiques, avec leurs métadonnées associées. Le développement de MASST a été facilité par l'adoption croissante de formats de données universels et la capacité à connecter toutes les données publiques dans GNPS/MassIVE. Ainsi il est possible de rechercher un spectre MS/MS dans des bases de données publiques pour trouver des spectres identiques ou analogues, y compris des molécules inconnues. Il est ainsi possible de savoir si l'utilisateur, dont les données sont publiques, a également détecté le même composé dans son échantillon et d'accéder potentiellement à ses métadonnées telles que la taxonomie. PlantMasst¹²⁹ a été développé récemment (pré-print) et regroupe des données LC-MS/MS de près de 19 000 extraits de plantes, incluant 246 familles botaniques et près de 1 469 genres. Cette stratégie peut ainsi permettre d'identifier des molécules présentes dans différentes espèces ou genres de plantes, connues ou encore inconnues.

2.2. Les approches computationnelles

Comme expliqué précédemment, un des problèmes majeurs rencontré avec l'utilisation des bases de données c'est qu'il n'est possible d'identifier que les composés déjà préalablement identifiés et dont le spectre MS/MS est répertorié. Les bibliothèques de spectres permettent d'avoir une source fiable, mais celles-ci sont moins complètes que les bases de données de structures moléculaires. C'est pourquoi de nouvelles techniques faisant intervenir des approches computationnelles ont émergé plus récemment afin d'utiliser les informations des bases de données de molécules pour les corrélérer avec les spectres expérimentaux^{127,130,131}.

a. La comparaison avec des spectres prédits

La comparaison de spectre de masse avec des librairies de spectres expérimentaux est limitée par la taille des librairies et la couverture des familles de molécules. Cependant, il est possible d'élargir ses librairies par la prédiction de spectres de masse de composés connus et ainsi de créer des librairies de spectres de fragmentation *in silico*. Ces librairies peuvent ensuite, de la même façon que les librairies expérimentales, être comparées avec des spectres expérimentaux. L'ISDB-DNP¹³² a été construit sur ce principe, par fragmentation *in silico* des structures contenues dans le Dictionnaire des Produits Naturels (DNP) par CFM-ID¹³³. Le DNP étant un produit commercial, une base de données accessible gratuitement a également été générée à partir de la base de données universelle de produits naturels (UNPD-ISDB).

Depuis, l'outil TIMA-R, a intégré les données taxonomiques pour proposer une identification corrélant similarité spectrale et métadonnées taxonomiques¹³⁴. L'outil compare les spectres de masse expérimentaux avec la base de données contenant les informations taxonomiques dans lequel le composé se trouve (espèces, genre, famille etc.). Cette information est comparée avec les métadonnées expérimentales fournies et permet de générer un nouveau score de similarité. Ainsi, si un composé est retrouvé dans le même genre ou la même espèce que l'échantillon analysé, alors le poids de l'identification sera plus important.

b. L'analyse par empreintes moléculaires (*fingerprint*)

Une des approches les plus rencontrées aujourd'hui est l'utilisation des empreintes moléculaires. Les propriétés structurelles, généralement les *fingerprints* sont prédits à partir de spectres expérimentaux et comparés avec des spectres candidats. Les *fingerprints* utilisent le *machine learning* pour prédire un ensemble de propriétés moléculaires du métabolite inconnu à partir de ses données MS/MS¹³⁵. Parmi les approches par empreintes moléculaires, CSI-FingerID intégré à SIRIUS, est parmi les plus performants.

SIRIUS est un logiciel intégrant différents algorithmes et outils et permet d'atteindre un taux de prédiction d'environ 70%^{67,136}. Tout d'abord, à partir de la valeur de m/z mesurée, toutes les formules brutes possibles sont générées, respectant les 7 règles d'or énoncées précédemment. En parallèle, des arbres de fragmentation sont construits à partir des ions fragments observés dans les spectres MS/MS. Le meilleur arbre est ainsi sélectionné à partir de critères comme la correspondance des motifs isotopiques ou la proportion de fragments pouvant être interprétés. Puis CSI:FingerID, basé à la fois sur la prédiction d'une empreinte moléculaire d'un composé interrogé à partir de son arbre de fragmentation et de son spectre¹³⁷, propose des structures 2D issus de bases de données et correspondant aux données expérimentales. Enfin, les molécules d'une base de données chimique sont évaluées en fonction de leur adéquation avec les propositions et une liste de candidat peut être

proposées. Un score de confiance est ainsi associé à chaque identification en fonction de leur adéquation avec le profil estimé.

Les arbres de fragmentation, quant à eux, sont générés de manière computationnelle pour prédire le chemin de fragmentation d'une molécule, ils ont été développés pour l'annotation des spectres MS/MS en représentant chaque pic par un nœud avec une formule moléculaire¹³⁸. Les arbres de fragmentation visent également à identifier les compositions élémentaires des ions fragments et des pertes de neutres pour aider à l'élucidation des structures et sous-structures par fragmentation *in silico* et visent à prédire des empreintes moléculaires et à fournir une stratégie d'identification *de novo*. La Figure 27 détaille le principe de construction des arbres de fragmentation. Chaque nœud représente un fragment du spectre MS/MS et une formule brute est calculée pour chaque fragment.

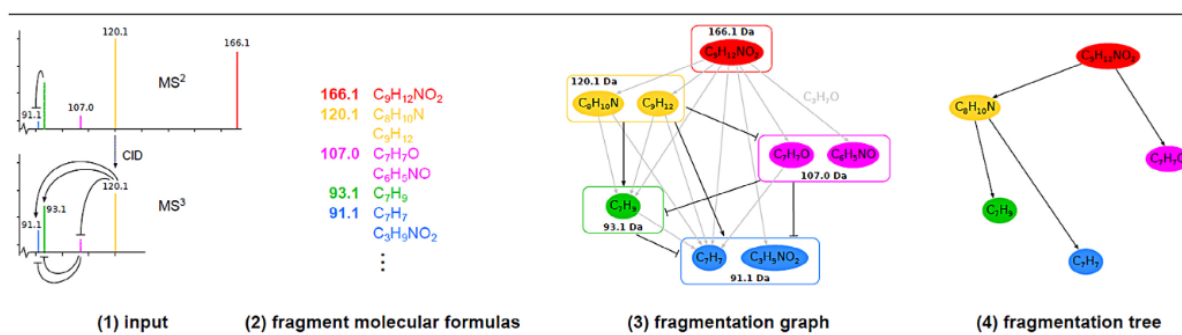


Figure 27 : Schéma du principe de de constructions des arbres de fragmentation à partir de données MSⁿ. Les ions MSⁿ sont assignés par des formules brutes, organisés en un graphique de fragmentation et finalement assemblés en un arbre qui explique au mieux les étapes de fragmentation probables¹³⁸.

Les arbres de fragmentation peuvent être alignés afin d'être comparés et un score est ainsi attribué^{127,138}. Des spectres MS/MS sont introduits en entrée puis les ions MSⁿ sont assignés à des formules moléculaires, organisés dans un graphe de fragmentation et finalement assemblés dans un arbre qui explique au mieux les étapes probables de fragmentation.

Dans l'interface SIRIUS, d'autres outils permettent également d'améliorer l'annotation ou d'accéder à différentes informations. ZODIAC permet par exemple, d'améliorer le rang de prédiction des formules brutes en les comparant aux formules prédites des autres composés d'un même set d'analyses. Cette option n'est à utiliser que si les échantillons ont un lien biologique entre eux.

CANOPUS (*class assignment and ontology prediction using mass spectrometry*) peut également être directement utilisé via l'interface SIRIUS, pour prédire la classe utilisant la classification NPClassifier grâce à l'utilisation de CSI:FingerID sans besoin de chercher dans des bases de données^{136,139,140}. Il permet d'assigner une classe à chaque feature obtenue à partir

d'analyse LC-MS/MS, y compris lorsque qu'aucune proposition de structure n'est enregistrée dans les bases de données ou dans la littérature. CANOPUS utilise les réseaux de neurones profonds pour prédire les classes à partir des spectres MS/MS qui permettent de déterminer un arbre de fragmentation, ce qui va permettre de prédire son empreinte moléculaire qui va pouvoir à ce moment être comparé avec les empreintes moléculaires de composés de classes connues alors même qu'aucune annotation fine ne peut être proposée.

Dans le domaine de la déréplication, une des principales limitations pour l'annotation réside dans la disponibilité des composés dans une base de données. Toutefois, il n'est pas toujours nécessaire d'annoter tous les composés d'un échantillon donné. Parfois, connaître des informations telles que la famille chimique d'un composé peut orienter les études. Différentes stratégies peuvent être mises en œuvre pour déterminer la classe chimique d'un ensemble de composés ou son annotation. En fin de compte, pour une analyse LC-MS comportant près de 20 000 pics, environ 98 % de l'ensemble des données initiales ne sont pas identifiables⁶⁷. Ainsi, les stratégies de priorisation permettant de sélectionner les composés les plus prometteurs pour une étude approfondie, à travers l'utilisation de réseaux moléculaires et en intégrant différentes métadonnées telles que la classe chimique, sa présence au sein du taxon ou l'activité biologique offrent des pistes pour optimiser l'annotation et orienter les recherches. Des outils d'aide à la décision ont d'ailleurs également vu le jour, comme MS2DECIDE pour aider à l'étude des métabolites en comparant les annotations obtenues à partir de différents outils¹⁴¹.

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CHAPITRE II :

DEVELOPPEMENT DE METHODES D'ANALYSES CIBLEES ET NON
CIBLEES POUR LA CULTURE DE DEUX PLANTES D'INTERET EN
REGION CENTRE-VAL DE LOIRE

Introduction

La cosmétique et le secteur horticole constituent deux secteurs économiques majeurs en Région Centre-Val de Loire (RCVL), contribuant à faire de cette région un territoire doté d'un tissu économique attractif.

La filière cosmétique, incluant parfums et produits d'hygiène, compte plus d'une centaine d'unités de production en Centre-Val de Loire, employant plus de 7 500 personnes. Parmi ces établissements, 46 se consacrent à la production de parfums et cosmétiques biologiques ou naturels, témoignant ainsi d'un intérêt croissant pour des solutions plus naturelles¹.

En ce qui concerne la filière horticole en RCVL, plus de 160 entreprises de production horticole (plantes à massif et plantes en pot) et pépinières (ornementale, fruitière) sont dénombrées en 2020, sur une surface totale de 1 126 hectares, représentant ainsi l'équivalent de 1 071 temps pleins. Le Loiret en particulier est le département où ce secteur économique est le plus développé avec 38 % des entreprises régionales^{2,3}

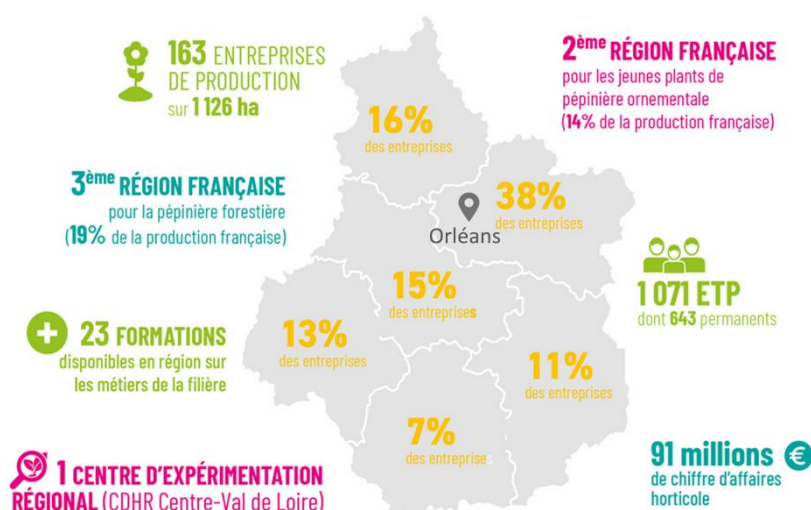


Figure 28 : Répartition des entreprises horticoles en Région Centre-Val de Loire

Afin de répondre à des problématiques d'approvisionnement, de sécurité de la filière et de traçabilité mais également en raison des enjeux environnementaux, la volonté de relocaliser la production de plantes d'intérêt cosmétique en RCVL s'inscrit dans la Stratégie Régionale d'Innovation (DPS 2). Cette initiative repose sur l'expertise disponible en région dans le domaine horticole, notamment grâce au Comité de Développement Horticole de la Région Centre-Val de Loire (CDHRC), station d'expérimentation horticole située à St-Cyr-en-Val (45),

¹ <https://www.ecozoom-centrevaldeloire.fr/filieres/industrie/parfums-et-cosmetiques> (consulté le 15.10.2024)

² <https://www.centre-valdeloire.fr/le-guide-des-aides-de-la-region-centre-val-de-loire/horticulture-pepinieres> (consulté le 15.10.2024)

³ <https://www.cap-filieres.fr/filieres-vegetales/horticulture-pepiniere> (consulté le 15.10.2024)

à quelques kilomètres de l'ICOA. Ainsi le CDHRC mène des programmes expérimentaux de développement d'itinéraires de culture de plantes d'intérêt permettant de favoriser l'implantation et la croissance de la plante et de lutter par des moyens biologiques contre l'attaque de ravageurs ou de maladies. Il transfère ensuite ces bonnes pratiques à ses adhérents capables de développer ces cultures à plus grande échelle. L'intérêt pour filière horticole est principalement une diversification de leur activité et une meilleure utilisation des installations notamment des serres qui peuvent ainsi être utilisées toute l'année.

C'est dans ce contexte que le CDHRC, a entrepris de développer la culture de plantes traditionnellement utilisées en médecine ayurvédique et présentant un intérêt pour la filière cosmétique. Cependant, ces plantes, cultivées hors de leur climat naturel ne sont plus soumises aux mêmes stress et peuvent présenter des profils phytochimiques différents. Les enjeux de cette démarche sont donc de développer les bonnes conditions de culture pour favoriser la croissance de la plante afin d'obtenir une quantité de biomasse suffisante mais également de contrôler sa composition phytochimique en fonction des conditions appliquées afin de vérifier si la phytochimie de la plante reste inchangée par rapport aux plantes cultivées en Asie ou en Inde. En effet, une modification de la composition phytochimique due aux conditions de culture pourrait modifier également les propriétés biologiques revendiquées pour l'utilisation de cette plante.

L'objectif de ce chapitre de thèse est donc de mettre en place différentes méthodes analytiques et métabolomiques appliquées à l'étude de deux ressources végétales issues de la médecine ayurvédique et d'intérêt pour des applications cosmétiques dont les cultures ont été mises en place au CDHRC. En effet, la problématique et les objectifs de valorisation pouvant différer selon les plantes cultivées, il est important de développer une stratégie analytique adaptée à l'étude souhaitée.

Dans une première partie, nous nous sommes intéressés à *Eclipta alba*, une plante de la famille des Astéracées afin de confirmer les activités biologiques pour lesquelles elle est décrite (antioxydante et anti-inflammatoire) et potentiellement d'en identifier de nouvelles. L'étude phytochimique vise à établir un lien entre les activités mises en évidence et la composition de la plante. Dans une seconde partie, nous étudierons *Coleus forskohlii*, une plante de la famille des Lamiacées, principalement connue pour la présence dans ses racines d'une molécule à fort intérêt pharmacologique, nutraceutique et cosmétique : la forskoline. Différents itinéraires de cultures en aéroponie ont été mis en place afin de faciliter l'accès aux racines, et seront comparés à l'aide d'outils statistiques. Enfin la composition phytochimique de ses parties aériennes, sous-produit de la culture de *Coleus forskohlii*, sera comparée à celle des racines afin d'évaluer leur potentiel de valorisation en tant que co-produit.

Partie I. Etude phytochimique et évaluation de l'activité anti-âge d'*Eclipta alba* cultivée en Région Centre-Val de Loire

L'utilisation de ressources naturelles à des fins cosmétiques, notamment en tant qu'actifs, n'est pas nouvelle. Cependant, les avancées dans la compréhension des processus biologiques impliqués dans le vieillissement cutané ont fait évoluer la recherche en cosmétique, vers la recherche de molécules capables d'inhiber des enzymes clés impliquées de ce processus.

Le vieillissement de la peau au cours du temps se traduit par l'apparition de rides et ridules, une perte d'élasticité et de volume, ainsi que par une texture plus rugueuse et une teinte plus pâle⁴. La peau, le plus grand organe du corps humain, est constituée de trois couches superposées : l'épiderme en surface, le derme, puis l'hypoderme, la couche la plus profonde. L'épiderme est principalement constitué de cellules appelées kératinocytes, qui confèrent à l'épiderme sa structure et sa rigidité. Le derme est principalement composé de fibroblastes. Ces derniers produisent les composés de la matrice extracellulaire, très riche en protéines fibreuses comme le collagène et l'élastine, qui assurent la structure et l'élasticité de la peau. Contrairement à l'épiderme, le derme ne subit pas de renouvellement continu, cependant sa matrice extracellulaire peut être désorganisée au cours du processus de vieillissement ou pendant le processus de cicatrisation. Enfin, l'hypoderme est constitué principalement d'adipocytes, cellules dont le rôle principal est le stockage graisseux⁵.

Le processus de vieillissement cutané est divisé en deux catégories : le vieillissement intrinsèque et le vieillissement extrinsèque. Le vieillissement intrinsèque, ou naturel, est causé par les changements d'élasticité de la peau en réponse au temps, tandis que le vieillissement cutané extrinsèque résulte principalement de l'exposition au rayonnement solaire mais également de la pollution, du tabagisme ou encore d'une mauvaise nutrition^{4,6}.

Le stress oxydant est l'un des principaux facteurs responsables du photovieillissement de la peau, il se définit par un déséquilibre entre la production d'espèces pro-oxydantes et les capacités cellulaires antioxydantes. Les espèces réactives à l'oxygène (ROS), des molécules chimiquement instables, dont la production peut être modulée par différents facteurs, jouent un rôle important dans le vieillissement cutané extrinsèque. Les cellules ont des systèmes antioxydants complexes pour réguler l'oxydoréduction cellulaire et les protéger contre les dommages causés par les radicaux libres. Le rôle des antioxydants est donc de neutraliser les

⁴ Papaccio, F.; D'Arino, A.; Caputo, S.; Bellei, B. Focus on the Contribution of Oxidative Stress in Skin Aging. *Antioxidants* **2022**, 11 (6). <https://doi.org/10.3390/antiox11061121>.

⁵ Graham, H. K.; Eckersley, A.; Ozols, M.; Mellody, K. T.; Sherratt, M. J. Human Skin: Composition, Structure and Visualisation Methods. In *Skin Biophysics*; Limbert, G., Ed.; *Studies in Mechanobiology, Tissue Engineering and Biomaterials*; Springer International Publishing: Cham, **2019**; Vol. 22, pp 1–18. https://doi.org/10.1007/978-3-030-13279-8_1

⁶ Tzaphlidou, M. The Role of Collagen and Elastin in Aged Skin: An Image Processing Approach. *Micron* **2004**, 35 (3), 173–177. <https://doi.org/10.1016/j.micron.2003.11.003>.

ROS produites au cours de processus physiologiques afin d'atteindre un niveau d'équilibre, les ROS sont tout de même essentielles dans la signalisation cellulaire et garantissent le maintien de l'homéostasie des cellules. Ce contrôle se produit avec des systèmes enzymatiques et non enzymatiques⁷.

Les superoxydes dismutases, les glutathions peroxydases et la catalase sont trois types d'enzymes impliquées dans la protection contre les ROS. Les Superoxide Dismutases (SODs) catalysent la conversion du radical superoxyde ($O_2^{\bullet}$) en O_2 et H_2O_2 , jouant un rôle clé dans le système de défense antioxydant cellulaire. D'un autre côté, des enzymes sont impliquées dans la production de ROS, c'est le cas de la **xanthine oxydase** (XO) qui catalyse l'oxydation de l'hypoxanthine en xanthine, et de la xanthine en acide urique. Ce processus entraîne la production de ROS, notamment en produisant un radical superoxyde ($O_2^{\bullet}$) et du peroxyde d'hydrogène (H_2O_2) en utilisant l'oxygène moléculaire comme accepteur d'électrons. Les radiations ultraviolettes (UV) induisent une suractivation de cette enzyme, localisée dans les kératinocytes, ce qui contribue ainsi au stress oxydatif dans la peau^{8,9}.

Les ROS ont également la capacité d'activer différentes voies de signalisation dont les kinases de la voie MAPK qui induisent la surexpression des métalloprotéases matricielles (MMPs)¹⁰. Les **collagénases**, qui sont des MMPs, sont des enzymes responsables de la dégradation du collagène. Les MMPs sont également surexprimées par des kératinocytes et des fibroblastes dans les peaux plus âgées alors que la quantité d'inhibiteur reste stable, créant ainsi un déséquilibre et un affaiblissement la structure de la peau. De la même façon, l'**élastase**, une enzyme impliquée dans la dégradation des fibres d'élastine, est surexprimée au cours du vieillissement cutané, entraînant la perte d'élasticité. Ainsi la dégradation du collagène et de l'élastine se traduit par la diminution de la fermeté de la peau et par l'apparition de rides.

D'autres processus physiologiques peuvent également être dérégulés par l'exposition aux UV. C'est le cas notamment de l'inflammation aigüe, réponse physiologique clé du système immunitaire, dont le mécanisme implique une série d'événements dans lesquels le métabolisme de l'acide arachidonique joue un rôle important. Il a été montré que l'exposition aux UV induit l'expression de la **cyclooxygénase-2** (COX-2) dans la peau humaine¹⁰. Les cyclooxygénases (COX) faisant parties des enzymes intervenant dans la voie métabolique de

⁷ Migdal, C.; Serres, M. Espèces Réactives de l'oxygène et Stress Oxydant. *Med Sci (Paris)* **2011**, 27 (4), 405–412.

⁸ Cos, P.; Li, Y.; Calomme, M.; Hu, J. P.; Cimanga, K.; Poel, B. V.; Pieters, L.; Vlietinck, A. J.; Berghe, D. V. Structure-Activity Relationship and Classification of Flavonoids as Inhibitors of Xanthine Oxidase and Superoxide Scavengers. *J. Nat. prod.* **1998**, 61 (1), 71-76

⁹ Deliconstantinos, G.; Villiotou, V.; Stavrides, J. C. Alterations of Nitric Oxide Synthase and Xanthine Oxidase Activities of Human Keratinocytes by Ultraviolet B Radiation. *Biochemical Pharmacology* **1996**, 51 (12), 1727–1738.

¹⁰ Pillai, S.; Oresajo, C.; Hayward, J. Ultraviolet Radiation and Skin Aging: Roles of Reactive Oxygen Species, Inflammation and Protease Activation, and Strategies for Prevention of Inflammation-Induced Matrix Degradation-a Review. *International Journal of Cosmetic Science* **2005** 27, 17–34.

l'acide arachidonique, leur surexpression entraîne une production accrue de médiateurs pro-inflammatoires comme les prostaglandines et les thromboxanes¹¹.

Enfin, d'autres enzymes comme la **tyrosinase**, sont également impliquées dans des processus liés au vieillissement cutané. Une surexpression de la tyrosinase peut induire une surproduction de mélanine et, bien que la mélanine protège la peau des dommages liés aux UV, ce déséquilibre peut être néfaste et induire des troubles cutanés comme des lentigos ou une hyperpigmentation post-inflammatoire¹².

Trouver des molécules capables de moduler l'activité de ces enzymes sans pour autant présenter de caractère cytotoxique est donc un enjeu majeur du secteur cosmétique. Et si des molécules jouant ce rôle peuvent être synthétisées, elles ne répondent alors plus à l'un des autres objectifs du secteur cosmétique, à savoir tendre vers davantage de naturalité. Les métabolites spécialisés des plantes apparaissent alors comme une source naturelle potentielle de ces modulateurs d'activité.

C'est dans ce contexte, qu'*Eclipta alba* (Figure 29), une plante de la famille des astéracées, traditionnellement utilisée en médecine Ayurvédique pour le traitement des troubles digestifs, respiratoires, hépatiques et cutanés ainsi que pour son activité promotrice de la croissance des cheveux a été étudiée.



Figure 29 : Photo des parties aériennes pendant la floraison d'*Eclipta alba* (Gerrit Davidse)

En effet, sa culture a été mise en place au CDHRC, comme preuve de concept de la possibilité de cultiver ce type de plante en RCVL, car différentes activités biologiques intéressantes pour des applications cosmétiques ont déjà été démontrées. L'extrait de feuilles est par exemple décrit comme agent d'entretien de la peau, avec notamment une activité anti-inflammatoire *in vitro* et *in vivo*, ainsi qu'une activité antioxydante. De plus, l'étude de la toxicité d'un extrait

¹¹ Wang, B.; Wu, L.; Chen, J.; Dong, L.; Chen, C.; Wen, Z.; Hu, J.; Fleming, I.; Wang, D. W. Metabolism Pathways of Arachidonic Acids: Mechanisms and Potential Therapeutic Targets. *Signal Transduction and Targeted Therapy* 2021 6:1 **2021**, 6 (1), 1–30.

¹² Ribeiro, A.; Estanqueiro, M.; Oliveira, M.; Sousa Lobo, J. Main Benefits and Applicability of Plant Extracts in Skin Care Products. *Cosmetics* **2015**, 2 (2), 48–65.

éthanolique d'*Eclipta alba* n'a pas montré de toxicité sur fibroblastes de peau humaine, ce qui suggère une bonne tolérance cutanée et un potentiel d'application en cosmétique¹³.

En termes de composition phytochimique, les précédentes études d'*Eclipta alba* ont permis d'identifier des flavonoïdes, des coumarines, des composés sulfatés comme les thiophènes ainsi que de nombreuses saponines triterpéniques, des familles moléculaires d'intérêt en cosmétique^{8,14,15}.

Des travaux ont été initiés durant la thèse du Dr. Souhila Messailli, précédente doctorante au laboratoire de l'ICOA, et ont permis de réaliser un premier profil phytochimique de la plante et de mettre en évidence une activité antioxydante de l'extrait. La culture a été poursuivie au CDHRC et une nouvelle récolte des parties aériennes a été possible en octobre 2021.

Les objectifs sont donc (i) de poursuivre l'étude de la phytochimie de la plante cultivée en Centre-Val de Loire afin de mieux connaître sa composition et de pouvoir la comparer à celle décrite dans la littérature pour les plantes cultivées dans leur habitat naturel, (ii) de réaliser un criblage d'activités d'intérêt cosmétique et d'identifier les molécules responsables de ces activités afin d'élargir les voies de valorisation de la plante en cosmétique.

Pour répondre à ces objectifs une approche de phytochimie classique a été privilégiée. En effet, cette étude ne portait que sur un échantillon (parties aériennes issues des individus d'une seule collecte, avec une seule modalité de culture). Un extrait éthanolique a été réalisé, et le screening d'activités a été effectué avec différents tests antioxydants chimiques (DPPH, CUPRAC et chélation du fer) et enzymatiques (tyrosinase, collagénase, élastase, xanthine oxydase et COX-2) puis une analyse statistique univariée par ANOVA a été menée pour valider la significativité de ces résultats. Parmi ces tests enzymatiques, les quatre premiers étaient déjà en place au laboratoire tandis que le test sur COX-2 permettant d'évaluer l'activité anti-inflammatoire décrite dans la littérature a été mis en place pour répondre au besoin de ce projet. Afin d'identifier les molécules d'intérêt de cet extrait, un fractionnement par extraction liquide-liquide et chromatographie de partage centrifuge a été réalisé et les molécules d'intérêt ont pu être identifiées par HRMS/MS et RMN.

⁸ Cos, P.; Li, Y.; Calomme, M.; Hu, J. P.; Cimanga, K.; Poel, B. V.; Pieters, L.; Vlietinck, A. J.; Berghe, D. V. Structure-Activity Relationship and Classification of Flavonoids as Inhibitors of Xanthine Oxidase and Superoxide Scavengers. *J. Nat. prod.* **1998**, *61* (1), 71-76

¹³ Lee, H. Y. Enhancement of Skin Anti-Inflammatory Activities of *Eclipta Prostrata* L. from the Ultrasonic Extraction Process. *Applied Sciences* **2017**, *7* (12), 1227.

¹⁴ Gendrisch, F.; Esser, P. R.; Schempp, C. M.; Wölfe, U. Luteolin as a Modulator of Skin Aging and Inflammation. *BioFactors* **2021**, *47*, 170–180.

¹⁵ Kim, H. P.; Sin, B. Y. Inhibition of Collagenase by Naturally-Occurring Flavonoids. *Archives of Pharmacol Research* **2005**, *28* (10), 1152–1155.

Phytochemical Profiling and Evaluation of the Anti-aging and Anti-inflammatory Potential of Cultivated *Eclipta alba* (L.)

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Abstract

Natural products are increasingly studied for pharmaceutical and cosmetic applications. In our continuing search for new natural active ingredients for cosmetic uses, we investigated the phytochemistry and studied the bioactivity of *Eclipta alba* (L.) cultivated in Centre-Val de Loire Region (CVLR) in France to develop a new anti-aging active ingredient. This plant is known as the false daisy and is traditionally used in Ayurveda for the treatment of digestive, respiratory, hepatic, and cutaneous disorders and for its activity as a hair growth promoter.

To highlight the compounds involved in different bioactivities, a metabolomic study of the plant was undertaken. The extract of the aerial parts, obtained by ultrasound-assisted extraction in ethanol, was tested for antioxidant activity and for its inhibitory effect on various enzymes involved in skin aging. The raw extract was fractionated using liquid/liquid extraction (LLE) and Centrifugal Partition Chromatography (CPC), then fraction activity was evaluated to target the main active compounds. HRMS/MS and NMR were used to identify the compounds contained in the active fractions.

Some fractions had showed strong collagenase, cyclooxygenase-2 (COX-2) and xanthine oxidase (XO) inhibition. The two most active fractions contained mostly coumestans (demethylwedelolactone (DWL) and wedelolactone (WL)), and other phenolic compounds present in lower amount, which were identified as carriers of the anti-collagenase, anti-cyclooxygenase 2 (COX-2) and anti-xanthine oxidase (XO) activity.

Therefore, this study suggested that *Eclipta alba* grown in CVLR has a phytochemistry in line with the description reported in literature and is highly composed of coumestans, phenolic compounds and saponins. The ethanolic extract has shown anti-inflammatory and antioxidant activity and for the first time an anti-collagenase activity has been demonstrated. These activities have been correlated with the presence of wedelolactone (WL) and demethylwedelolactone (DWL), the two majors' compounds, and other phenolics

compounds. Thus, *Eclipta alba* (L.) cultivated in CVLR could be used as an interesting antioxidant and anti-aging agent for cosmetrical applications.

Key-words: centrifugal partition chromatography, collagenase, cyclooxygenase-2, *Eclipta alba* (L.), phytochemical analysis, UHPLC-HRMS/MS, xanthine oxidase

1. Introduction

Eclipta alba (L.) Hassk. also known as *Eclipta prostrata* (Roxb.) and usually named False daisy or Ink plant in English is a ubiquitous annual herbal plant from the Asteraceae family¹. *E. alba* is a medium-sized branched plant with small white flowers². Commonly encountered in warm humid climates in Asia, South America and Africa, this plant was also identified in rice fields in France in 1991³. Although considered as a common weed by farmers, the plant is used in traditional medicine in different native countries (India, China, Thailand, Brazil, Korea, Ivory Coast)^{1,4}. Among these traditional uses, *E. alba* is used in China, India, and Thailand for the treatment of skin diseases such as atopic dermatitis, a skin disease associated to the inflammatory process and vitiligo⁵. The plant is also widely used to promote hair growth⁶ and to treat hepatitis and jaundice¹. In Eastern Ivory Coast pregnant woman uses the plant to ensure foetal development and facilitate childbirth⁴. It also possesses anti-microbial, anti-hyperlipidemic and anti-diabetic activities². A wide range of secondary metabolites has already been identified in the plant extract of *E. alba* such as saponins, sterols, flavonoids, terpenoids, phenolic acids, thiophenes, polyacetylenes and coumestans^{2,7}.

While the interest of this plant in traditional medicine has led to the study of its anti-inflammatory⁸ and antioxidant^{9,10} properties for pharmaceutical uses, in the field of cosmetics only a few studies have been conducted on its effects to skin health¹¹. The hydroalcoholic extract of *E. alba* showed activity on house dust mite-induced atopic dermatitis¹². Some compounds identified in previous studies, as wedelolactone, were reported to decrease the Reactive Oxygen Species expressions of cells¹³ and to inhibit enzyme involved in the skin-aging or in inflammatory process^{14,15}. Together with the low cell toxicity of *E. alba* extract against human skin fibroblasts (CCD-986sk)¹¹, it suggests a good potential of *E. alba* for its use in cosmetics.

Antioxidant and anti-inflammatory compounds are sought for the use in cosmetic fields to prevent skin damage and inflammation¹⁶, this is why the French Horticultural Development Committee of the Centre Region (Comité de Développement Horticole Région Centre; CDHRC) set up the culture of *E. alba* in CVLR in order to provide a local source of the plant material usable by the French cosmetic industry. To confirm the potential of *E. alba* cultivated in CVLR, we assessed its antioxidant, anti-inflammatory and anti-aging activities through *in vitro* bioactivity assays; the results were correlated with its phytochemical profile. Among antioxidant available assays, 2,2-diphenyl-1-picryl-hydrazyl radical scavenging activity (DPPH),

cupric reducing antioxidant capacity (CUPRAC) and iron chelating are some of the common methods used for antioxidant *in vitro* assays. They are based on hydrogen atom transfer (HAT) or on electron transfer (ET)¹⁷ while xanthine oxidase (XO) inhibition is an enzymatic assay based on the oxidation of xanthine to uric acid producing superoxide radicals¹⁸. The effect of *E. alba* on four more enzymes involved in skin aging and the inflammatory process was also studied through *in vitro* assays: tyrosinase, which catalyses two reactions of melanin synthesis¹⁹; collagenase and elastase, two major structural proteins responsible for the degradation of collagen and elastin respectively²⁰ and COX-2, a pro-inflammatory enzyme of the arachidonic pathways²¹.

As the raw extract of *E. alba* was composed of various compounds, a first step of liquid-liquid extraction (LLE) was used to divide the extract and study the activity of both parts. Then, Centrifugal Partition Chromatography (CPC), a biphasic liquid-liquid separation method²² was used to refine the fractionation of the active part. Each fraction was evaluated for its bioactivity and phytochemical composition through analyses using Ultra High Performance Liquid Chromatography hyphenated to High-Resolution Mass Spectrometry (UHPLC-HRMS/MS).

2. Materials and methods

2.1. Chemicals and plant material

a. Chemicals

The absolute ethanol (>99.7%), 1-butanol, ethyl acetate, acetonitrile, and ethyl acetate (HPLC gradient grade) used for extraction, fractionation and UHPLC-UV-ELSD analysis and formic acid (99-100%) were all purchased from VWR (Fontenay-sous-Bois, France). Ultrapure water was produced with a Milli-Q® EQ 7000 system equipped with a LC-Pak filter from Merck (Darmstadt, Germany). UHPLC-HRMS analyses were performed with water and acetonitrile from Honeywell (Seelze, Germany).

Ammonium acetate, chlorogenic acid, Cu(II), cyclooxygenase-2 kit (MAK399), deuterated methanol, DPPH (2,2-diphenyl-1-picryl-hydrazyl), Iron (III) chloride, ferrozine, glabridin, phosphoric acid, L-dopa (3,4-dihydroxyphenylalanine), sodium hydroxide, neocuproine, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), tyrosinase from mushroom and the xanthine oxidase (19160) kit were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The elastase (E12056) and collagenase (E12055) enzymatic kits were purchased from Thermo-Fischer (Kandel, Germany).

b. Plant material

Plant material was produced by the Comité de Développement Horticole de la Région Centre (CDHRC). Seeds were purchased from Heritage Bio-Natural Systems Pvt. Ltd. (Telangana, India) batch n°HER/ECLI/99 janv 17. Seedlings and crops are sown in a glass greenhouse, with

an aeration temperature of 22°C, shading when radiation exceeds 650 W m⁻² S⁻¹ and controlled irrigation based on the estimating evapotranspiration. The aerial part was collected in October 2021 at the end of the flowering period and then lyophilized with Alpha I-6 (Martin Christ, Osterode am Harz, Germany) before being grounded with the Polymix PX-MFC 90 (Kinematica, Malters, Switzerland).

2.2. Extraction and fractionation

The raw extract was obtained with the ultrasound extractor PEX 1N 1 L (SARL R.E.U.S., Drap, France) in 20 min with a frequency of 20 kHz. Two extractions were conducted by adding respectively 45.28 g and 44.75 g of lyophilized and grounded aerial part to 700 mL of absolute ethanol each. The two extractions were reassembled, the raw extract (RE) was dried under vacuum with Buchi Rotavapor R-114 coupled with Buchi Vac V-100 pump (Buchi, Flawil, Switzerland) and 5.99 g of dried RE was finally obtained.

A LLE was carried out on 2.5 g of RE using 600 mL of a biphasic ethyl acetate/methanol/water (2:1:2, v/v/v) system. The two phases were separated and dried under vacuum. Two phases were obtained, an aqueous phase (AP) and an organic phase (OP) representing 52% and 48% of the RE respectively.

The LLE aqueous phase was fractionated by CPC which was performed with the Gilson CPC 250 PRO and PLC 2250 Purification System (Gilson, Saint-Ave, France). The non-miscible solvent system used was water/methanol/1-butanol (6:1:4, v/v/v). The solvent system (3 L) was equilibrated in a separatory funnel to separate the two phases. The upper solvent phase was used as the stationary phase and the lower one as mobile phase in descending mode. The column was filled in 15 min with a flow rate of 40 mL.min⁻¹ with the upper phase at 500 rpm. Equilibrium with the lower phase was achieved within 10 min at 20 mL.min⁻¹ and 2000 rpm. The equilibrium stage was reached with 62% of retention of the stationary phase in the column. The LLE aqueous phase (225 mg) was dissolved in 10 mL of the CPC biphasic solvent system and injected. Elution with the lower phase was achieved in 30 min at 20 mL.min⁻¹ and 2000 rpm. Then the upper phase was added at 2000 rpm and 20 mL.min⁻¹ for the extrusion step to recover all the compounds retained in the stationary phase. Finally, 60 tubes of 20 mL were collected, evaporated, and analysed by UHPLC-UV-ELSD for reassembly according to their phytochemical profile. Six CPC fractions (FA to FF) were obtained, dried, and weighed. FA and FC were the major fractions representing 31 % and 46 % of the recovered mass respectively, while FB (3%), FD (5%), FE (4%) and FF (11%) were less abundant fractions.

2.3. Phytochemical analysis

a. UHPLC-UV-ELSD analysis

UHPLC was performed with a Thermo Scientific Ultimate 3000 RSLC system with a binary pump and automatic sampler, a thermostated column compartment, DAD and an ELSD

detector. The column was a Macherey-Nagel Nucleodur HTec C18 (100 x 2 mm, 1.8 μm). Gradient mode was used to separate the compounds. The mobile phase was consisted of water (A) and acetonitrile (B), both acidified with 0.1% formic acid. The flow rate was fixed at 0.5 mL.min⁻¹. Elution started with 5% of B during 0.1 min. The amount of B increased up to 75% from 0.1 to 10.5 min and to 90% from 10.5 min to 11 min. From 11 to 12.5 min the amount of B was maintained and then decreased during 0.5 min to the initial conditions. The column was re-equilibrated during 6 min with 5% of B and the temperature was fixed at 40°C. Samples were prepared at a concentration of 5 mg.mL⁻¹ in an EtOH/water mixture (80/20, v/v) and the injection volume was 5 μL .

b. UHPLC-HRMS/MS

The UHPLC system, column and elution method were the same as those used previously (a) Samples were prepared with a concentration of 5 mg.mL⁻¹ in an EtOH/water mixture (80/20, v/v), and the injection volume was 1 μL for RE, AP and OP and 0.3 μL for CPC fractions (FA, FB, FC, FD, FE, FF).

HRMS/MS experiments were performed on a maXis UHR-Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Analyses were acquired in positive electrospray ionization (ESI+) mode. The capillary voltage was maintained at 4.5 kV. Nitrogen was used as drying gas at a flow rate of 9 L.min⁻¹ heated at 200°C and nebulizing gas at a pressure of 2 bar at 2.5 Hz for MS and MS/MS. Mass spectra were recorded in data dependent acquisition (DDA) mode in the m/z range 50-1650. MS/MS was performed on two precursor ions per cycle and fragmented with a variable collision energy of 15-35 eV. Data Analysis 4.4 software was used to process all the MS data and generate molecular formula.

c. NMR analysis

¹H NMR and ¹³C NMR spectra were recorded on a Bruker Avance III HD – 400 MHz spectrometer equipped with a 5 mm BBFO probe at 293 K. Chemical shifts (δ) were referenced using the solvent signals (δ_{H} 3.31, δ_{C} 49.00 for CD₃OD). Spectra were processed using MestReNova software (MNova 14.1.1 Mestrelab Research). Samples were prepared in CD₃OD at 10 mg.mL⁻¹.

2.4. Bioactivity assays

The tests were carried out in triplicate in 96-well microplates. The absorbance or fluorescence were read with the CLARIOstar® Plus microplate reader (BMG LABTECH). RE, AP, OP and CPC fractions were tested. RE and OP were dissolved in ethanol and AP and CPC fractions were dissolved in an EtOH/water mixture (80/20, v/v). Ethanol and buffer were used as blank control for chemical antioxidant assays and enzymatic assays respectively. Concentrations expressed are the final concentrations in the wells.

a. Chemical antioxidant assays

The free radical scavenging activity was determined using DPPH ²³. The DPPH solution was prepared in ethanol at 0.2 mM. 190 μ L of DPPH solution was incubated with 10 μ L of samples (RE, AP, OP, and ethanol for the blank) with final concentrations in the well of 250 μ g.mL⁻¹ and 500 μ g.mL⁻¹. A calibration range (2 to 50 μ g.mL⁻¹ in final wells) of Trolox was prepared in ethanol. After 30 min of incubation in the dark, the absorbance was measured at 516 nm. The percentage of free radical scavenging activity was calculated using Eq. (1):

$$Activity (\%) = \left(\frac{A_{blank} - A_{sample}}{A_{blank}} \right) * 100\% \quad \text{Eq. (1)}$$

with A_{sample} , the absorbance of DPPH solution in presence of the sample and A_{blank} the absorbance of DPPH solution in presence of ethanol only.

The cupric ion reducing capability (CUPRAC) was determined using a solution composed of Cu (II) (3.33 mM), neocuproine (2.5 mM) in ammonium acetate buffer at 0.66 M ²⁴. Samples (RE, AP, OP, and ethanol for the blank) or trolox solution (10 μ L) were reacted with 190 μ L of the cupric solution during 30 min in the dark. After incubation, the measurement of the absorbance of the cupric complex formed was read at 450 nm. Samples were tested at final concentrations of 25 μ g.mL⁻¹ and 50 μ g.mL⁻¹. Trolox solution prepared with a final concentration of 50 μ g.mL⁻¹ in the well induced a maximum of ion reducing capability. The percentage of the cupric capability of samples compared to the maximum of cupric capability of the trolox was calculated using Eq. (2):

$$Cupric\ capability (\%) = \left(\frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \right) * 100\% \quad \text{Eq. (2)}$$

with A_{sample} , the absorbance of the sample, A_{blank} the absorbance of the blank and $A_{control}$ the absorbance with trolox.

The chelating iron inhibition was determined using the method of ²⁵. Samples (RE, AP, OP, ethanol for the blank) or trolox (10 μ L) were reacted with 190 μ L of a solution of ferrozine (0.14 mM) and FeCl₃ (0.475 mM). After 30 min of incubation in the dark, the measurement of the absorbance of the complex Fe²⁺-Ferrozine formed was read at 562 nm. Trolox solution prepared with a final concentration of 100 μ g.mL⁻¹ induced the maximum ion reducing capability. The percentage of iron chelating capability was calculated by comparing tested samples with the maximum iron capability of the trolox using Eq (3):

$$Iron\ chelating\ capability (\%) = \left(\frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \right) * 100\% \quad \text{Eq. (3)}$$

with A_{sample} , the absorbance of Ferrozine-FeCl₃ solution with sample, A_{blank} the absorbance with ethanol and $A_{control}$ the absorbance with trolox.

b. Elastase inhibitor assay

The elastase kit E12056 from ThermoFischer was used to determine the inhibition percentage of the extracts. Briefly, 10 μL of samples were first prepared to obtained final concentrations in the well of 250 $\mu\text{g.mL}^{-1}$ and 125 $\mu\text{g.mL}^{-1}$, and were mixed with 40 μL of 1X buffer (0.1 M Tris-HCl, 0.2 mM sodium azide, pH 8). Elastase enzyme 100 μL at 0.5 U.mL^{-1} was added to the sample wells and 100 μL of buffer to the blank sample and blank control wells and incubated for 5 min. N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone at 50 μM was used as control inhibitor. Lastly, 50 μL of substrate was added to all the wells before an incubation of 2 H at room temperature and a reading of the fluorescence ($\lambda_{\text{excitation}}$ 477 nm - $\lambda_{\text{emission}}$ 525 nm). The inhibition percentage of elastase was calculated according to the following formula Eq. (4):

$$\% \text{ inhibition} = \frac{(F_{\text{control}} - F_{\text{blank control}}) - (F_{\text{sample}} - F_{\text{blank sample}})}{(F_{\text{control}} - F_{\text{blank control}})} * 100\% \quad \text{Eq. (4)}$$

with F_{control} the fluorescence of the enzyme and substrate, $F_{\text{blank control}}$ the fluorescence of the substrate, F_{sample} the fluorescence of the substrate and the enzyme with sample, and $F_{\text{blank sample}}$ the natural fluorescence of the sample.

c. Tyrosinase inhibitor assay

The tyrosinase inhibition was determined according to the protocol of Lim et al. (2009). Briefly, the buffer was prepared with 50 mL of water, 200 μL of NaOH 2 M and 290 μL of H_3PO_4 1 M (pH 6.8). Enzyme was prepared at 125 U.mL^{-1} ; the L-Dopa substrate was prepared at 8.3 mM and the inhibitor at 1000 μM . Samples (10 μL) were first prepared to obtained final concentrations in the well of 500 $\mu\text{g.mL}^{-1}$ and 100 $\mu\text{g.mL}^{-1}$, were first mixed with 70 μL of buffer. Then 40 μL of tyrosinase enzyme (125 U.mL^{-1}) was added to the sample wells or 40 μL of buffer to the blank sample and blank control wells and incubated for 5 min. Glabridin was used at 500 μM as control inhibitor. Lastly, 120 μL of substrate (8.3 mM) was added to all the wells before an incubation of 30 min at room temperature and a reading of the absorbance at 490 nm. The inhibition percentage of tyrosinase was calculated according to the following formula Eq. (5):

$$\% \text{ inhibition} = \frac{(A_{\text{control}} - A_{\text{blank control}}) - (A_{\text{sample}} - A_{\text{blank sample}})}{(A_{\text{control}} - A_{\text{blank control}})} * 100\% \quad \text{Eq. (5)}$$

with A_{control} the fluorescence of the enzyme and substrate, $A_{\text{blank control}}$ the fluorescence of the substrate, A_{sample} the fluorescence of the substrate and the enzyme with sample, and $A_{\text{blank sample}}$ the natural fluorescence of the sample.

d. Collagenase inhibitor assay

The collagenase inhibition was determined using the E12055 kit from ThermoFischer. Samples were prepared to obtain the following concentrations after dilution in the wells: AP, OP and RE at 400 $\mu\text{g.mL}^{-1}$, 200 $\mu\text{g.mL}^{-1}$ and 20 $\mu\text{g.mL}^{-1}$; CPC fractions at 250 $\mu\text{g.mL}^{-1}$ and 50 $\mu\text{g.mL}^{-1}$.

Briefly, 10 μL of samples, was first mixed with 70 μL of reaction buffer 1X (0.05 M Tris-HCl, 0.15 M NaCl 5 mM CaCl_2 , 0.2 mM sodium azide, pH 7.6). Then 100 μL of collagenase enzyme (0.4 $\text{U}\cdot\text{mL}^{-1}$) was added to the sample well or 100 μL of buffer to the blank sample and blank control wells and incubated for 5 min. 1,10-phenanthroline monohydrate at 20 μM was used as control inhibitor. Lastly, 20 μL of substrate was added to all the wells before an incubation of 2 H at room temperature and a reading of the fluorescence ($\lambda_{\text{excitation}}$ 483 - $\lambda_{\text{emission}}$ 530 nm). The inhibition percentage of collagenase was calculated according to the following formula Eq. (6):

$$\% \text{ inhibition} = \frac{(F_{\text{control}} - F_{\text{blank control}}) - (F_{\text{sample}} - F_{\text{blank sample}})}{(F_{\text{control}} - F_{\text{blank control}})} * 100\% \quad \text{Eq. (6)}$$

with F_{control} the fluorescence of the enzyme and substrate, $F_{\text{blank control}}$ the fluorescence of the substrate, F_{sample} the fluorescence of the substrate and the enzyme with sample, and $F_{\text{blank sample}}$ the natural fluorescence of the sample.

e. COX-2 inhibitor assay

The COX-2 inhibition was determined using the MAK399 kit from Sigma-Aldrich. Briefly, for each well a 70 μL mixture was prepared according to the following indication: 66 μL of assay buffer, 1 μL of probe, 2 μL of Cofactor previously diluted 200-fold with assay buffer and 1 μL of COX-2 enzyme. Samples were prepared to obtain the following concentrations after dilution in the wells: AP, OP and RE at 100 $\mu\text{g}\cdot\text{mL}^{-1}$ and 50 $\mu\text{g}\cdot\text{mL}^{-1}$; CPC fractions tested at 5 $\mu\text{g}\cdot\text{mL}^{-1}$ and 2.5 $\mu\text{g}\cdot\text{mL}^{-1}$. Samples (10 μL), or ethanol for the control, were added to the well. Then, 20 μL of arachidonic acid/NaOH solution (arachidonic acid diluted twofold with NaOH solution and diluted tenfold with purified water) was added to each well to initiate reactions. Celecoxib solution prepared according to the supplier's protocol was used as the control inhibitor. Fluorescence ($\lambda_{\text{excitation}} = 535 \text{ nm}$ - $\lambda_{\text{emission}} = 587 \text{ nm}$) was measured immediately after adding the substrate. The inhibition percentage of COX-2 was calculated according to the following formula Eq. (7):

$$\% \text{ inhibition} = \left(\frac{a_{\text{control}} - a_{\text{sample}}}{a_{\text{control}}} \right) \times 100\% \quad \text{Eq. (7)}$$

with a_{control} , the slope of the linear part of the kinetic response of the control measured by fluorescence and a_{sample} the slope of the linear part of the kinetic response of the sample measured by fluorescence.

f. Antioxidant enzymatic assay

The enzymatic antioxidant assay was performed with the 19160 kit from Sigma-Aldrich. Samples (AP, OP, RE and CPC fractions) were prepared to be tested at 2.5 $\mu\text{g}\cdot\text{mL}^{-1}$ and 1 $\mu\text{g}\cdot\text{mL}^{-1}$ after dilution in the wells. Briefly, 10 μL of samples, or control inhibitor were mixed with 200 μL of WST working solution previously prepared with 1 mL of WST solution in 19 mL of buffer solution. Then 20 μL was added to the sample well or 20 μL of buffer to the blank sample and

blank control wells and incubated for 5 min. Chlorogenic acid solution prepared with a final well concentration of 2 μM ($0.71 \mu\text{g.mL}^{-1}$) was used as the control inhibitor. Lastly, 20 μL of substrate was added to all the wells before 20 min incubation at room temperature and a reading of the absorbance at 450 nm. The inhibition percentage of the XO was calculated according to Eq. (8):

$$\% \text{ inhibition} = \frac{(A_{\text{control}} - A_{\text{blank control}}) - (A_{\text{sample}} - A_{\text{blank sample}})}{(A_{\text{control}} - A_{\text{blank control}})} * 100\% \quad \text{Eq. (8)}$$

with A_{control} the absorbance with enzyme, $A_{\text{blank control}}$ the absorbance of the blank control, A_{sample} the absorbance of the sample with enzyme, and $A_{\text{blank sample}}$ the natural absorbance of the sample.

2.5. Data processing

a. Activity assays

Data are presented as means $\pm$ standard deviation (SD). All activity assays were performed in triplicate ($n = 3$). The differences between the different samples were analysed using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference with $p \leq 0.05$. The statistical analysis was performed using XLSTAT 2017.

b. HRMS/MS

UHPLC-HRMS/MS data were processed using DataAnalysis 4.4 software. Molecular formulae were generated using the SmartFormula algorithm with an elemental composition of C, H, O, N < 5 and S < 5 with a mass accuracy < 5 ppm. According to the molecular formula and the MS/MS spectrum a suggestion of structure can be proposed based on the literature of *E. alba*. For the formulae previously non-identified in *E. alba* the MS/MS data were processed with SIRIUS 5.5.7 software²⁶. MS/MS data were first converted to MZxml format and computed. The molecular formulae were compared with the formulae calculated with the DataAnalysis 4.4 software. Fragmentation trees were obtained and compared to the MS/MS spectrum. This enabled a chemical structure to be proposed according to similarity with the literature, online databases (Pubchem, SciFinder, Lotus²⁷, GNPS^{28,29} and interpretation of the spectra.

c. Heatmap

Extraction Ion Chromatograms (EIC) were produced using DataAnalysis 4.4 software from the corresponding molecular formula. EIC were integrated automatically, and peak areas were exported to a matrix. The percentage of a compound in a fraction was calculated according to the example given in Eq. (9) and the heatmap was generated using Excel 2016.

$$\text{Compound proportion \%} = \frac{\text{Area of compound 1 in fraction A}}{\text{Total area of compound 1 in all fractions}} * 100\% \quad \text{Eq. (9)}$$

3. Results and discussion

This study aimed to highlight the bioactive potential of *E. alba* cultivated in Centre-Val de Loire Region (CVLR), France. An ethanolic extract was first prepared using ultrasound-assisted extraction (UAE) on the dried powder of the aerial part of *E. alba* to obtain a large range of molecules. The raw extract was obtained with an extraction yield of 6.7 %. Then, in order to simplify the crude extract and target the most active compounds, the raw extract was divided into two phases with a LLE using the biphasic ethyl acetate/methanol/water (2:1:2, v/v/v) system. The aqueous phase (AP) contained 52% of the initial weight while 48% was in the organic phase (OP). According to UHPLC-DAD-ELSD analysis (data not shown), the AP contained mainly polar compounds such as sugars, phenolic acids or flavonoids, while the OP contained mainly less polar compounds such as carotenoids, fatty acids and chlorophylls.

3.1. Antioxidant and enzymatic activity of *E. alba*

a. Crude extract and LLE fraction evaluation

Antioxidant compounds found in plants are of great interest as they are able to limit the proliferation of reactive oxygen (ROS) and reactive nitrogen (RNS) species involved in skin aging (Mukherjee et al., 2011; Wootton-Beard et al., 2011). Several classes of substances may present antioxidant activity; therefore, antioxidant activity assays require different approaches³⁰. The antioxidant potential of *E. Alba* extract was evaluated through three complementary antioxidant assays (Table 3): DPPH was used to measure the free scavenging activity, CUPRAC to demonstrate the ability to reduce cupric ion, and the iron chelating assay to measure de chelation capability. As a first step, the antioxidant activity of raw extract (RE), aqueous phase (AP) and organic phase (OP) at two concentrations was evaluated.

Table 3: Antioxidant activity of RE, AP, OP on DPPH, CUPRAC and Iron Chelating assays.

	DPPH Inhibition (%)		CUPRAC Reduction capability (%)		Iron chelating Chelation capability (%)	
Sample concentration ($\mu\text{g.mL}^{-1}$)	500	250	50	25	50	25
Raw extract	52.87 $\pm$ 1.13 ^b	62.10 $\pm$ 0.68 ^{ab}	66.35 $\pm$ 3.54 ^a	35.33 $\pm$ 1.05 ^b	101.64 $\pm$ 1.94 ^a	89.71 $\pm$ 2.84 ^b
Aqueous phase	58.11 $\pm$ 2.12 ^{ab}	67.19 $\pm$ 0.98 ^a	88.41 $\pm$ 0.88 ^c	48.28 $\pm$ 4.52 ^d	108.65 $\pm$ 1.66 ^a	89.82 $\pm$ 1.40 ^b
Organic phase	32.11 $\pm$ 12.29 ^c	29.33 $\pm$ 4.36 ^c	20.43 $\pm$ 0.47 ^e	10.18 $\pm$ 0.35 ^f	46.14 $\pm$ 6.50 ^c	25.03 $\pm$ 1.76 ^d

Data are presented as mean $\pm$ SD (n = 3). For the same antioxidant test, means followed by different letter are significantly different at p<0.05.

For the scavenging free radical test, the degree of decolourization of DPPH from purple to light yellow indicated the scavenging efficiency of the samples. The antioxidant potential of the extracts was evaluated and compared to the trolox standard. The raw extract and the aqueous phase at both concentrations showed the highest scavenging activity with 52.87 $\pm$ 1.13% and

62.10 ± 0.68% of inhibition for RE and 58.11 ± 2.12% and 67.19 ± 0.98% for AP at 500 µg.mL⁻¹ and 250 µg.mL⁻¹ respectively. These results were similar to the inhibition of trolox at 10 µg.mL⁻¹ (64.13 ± 0.94%). OP exhibited a significantly lower inhibition at both concentrations with 32.11 ± 12.29% and 29.33 ± 4.36% at 500 µg.mL⁻¹ and 250 µg.mL⁻¹. For the CUPRAC test, the colour was due to the Cu(II)-neocuproine complex formed. The highest ion reducing antioxidant capacity was observed for the aqueous phase at 50 µg.mL⁻¹ with 88.41 ± 0.88 %. RE presented a good reduction capability of 66.35 ± 3.54% at 50 µg.mL⁻¹ whereas OP presented a percentage of only 20.43 ± 0.47% at the same concentration. The results obtained for a concentration of 25 µg.mL⁻¹ were twice as low, indicating a dose response effect. Lastly, the iron chelating capability showed a similar tendency, with a weaker chelation capability of OP, 46.17 ± 6.50% and 25.03 ± 1.76% at 50 µg.mL⁻¹ and 25 µg.mL⁻¹ compared to RE and the AP.

This study showed a higher antioxidant activity for RE and AP for the three assays while OP systematically presented a lower activity (p<0.05). A high reduction and chelating capability of the extract and AP was observed at concentrations of 50 µg.mL⁻¹ and 25 µg.mL⁻¹ while hydrogen atom transfer through the DPPH scavenging assay required a higher concentration²⁴. Previously, Yadav et al.,³¹ had shown the radical scavenging activity of ethanolic extract with IC₅₀ = 136.57 ± 6.83 µg.mL⁻¹ through the DPPH assay, a result that is consistent with an activity of 62.10 ± 0.68 at 250 µg.mL⁻¹. The antioxidant effect of a water extract of *E. alba* has also been reported and chlorogenic acid was identified as a major component responsible for this activity³². These two studies corroborated our results and seem to confirm that AP contains phenolic compounds.

Even if *in-vitro* assays do not reflect the *in-vivo* mechanisms, using antioxidant assays based on different principles gives preliminary results that enable the antioxidant potential of a natural extract to be selected and the molecular family of the compounds involved in the activity to be determined³³. These preview results highlight AP as the most antioxidant part of the extract of *E. alba*.

In order to evaluate the potential of *E. alba* more fully, *in-vitro* enzymatic assays were undertaken on several enzymatic processes that are also involved in skin aging. Xanthine oxidase (XO) is involved in the oxidation mechanism as it produces hydrogen peroxide and superoxide anions during the oxidation of hypoxanthine to xanthine and then uric acid^{18,34}. Elastase and collagenase are two enzymes with a negative effect on the structural skin protein elastin and collagen³⁵. Tyrosinase is involved in the skin pigmentation that leads to the formation of brown spots on the skin with age. COX-2 is involved during the proinflammatory process. The raw extract (RE) and the two LLE phases (AP, OP) were evaluated for their inhibitory activity on these five enzymes. Table 2 shows the inhibition activity at two or three concentrations of sample on each enzyme.

Table 4: Collagenase, COX-2, Elastase, Tyrosinase and Xanthine Oxidase (XO), enzyme inhibitory activity of RE, AP and OP at two or three concentrations.

						Inhibition (%)					
		Collagenase		COX-2		Elastase		Tyrosinase		XO	
Concentration (µg.mL ⁻¹)	400	200	20	100	50	250	125	500	100	2.5	1
Raw extract	101.30 ± 0.36 ^a	102.77 ± 2.44 ^a	NA	95.51 ± 0.62 ^a	85.34 ± 1.16 ^a	23.55 ± 2.93 ^b	NA	18.45 ± 1.04 ^b	NA	72.15 ± 2.46 ^b	40.13 ± 3.96 ^d
Aqueous phase	100.10 ± 0.68 ^{ab}	99.74 ± 4.86 ^{ab}	53.13 ± 0.85 ^c	98.27 ± 1.52 ^a	90.81 ± 1.33 ^a	19.29 ± 2.32 ^b	NA	14.01 ± 1.29 ^b	NA	88.32 ± 2.44 ^a	66.47 ± 0.92 ^b
Organic phase	NA	NA	NA	89.32 ± 0.70 ^a	42.94 ± 17.00 ^b	NA	NA	14.06 ± 3.53 ^b	NA	35.21 ± 1.44 ^{de}	29.69 ± 0.90 ^e
Inhibitor		1,10-phenanthroline monohydrate		Celecoxib		N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone		Glabridin		Chlorogenic acid	
Concentration (µg.mL ⁻¹)		3.96		according to kit protocol		25.14		162.20		0.71	
		80.57 ± 4.85 ^b		89.67 ± 1.11 ^a		89.83 ± 1.39 ^a		43.77 ± 0.83 ^a		51.49 ± 2.71 ^c	

Data are presented as mean ± SD (n = 3). For the same enzyme tested, means followed by different letter are significantly different at p<0.05. Any inhibition values below 10% are considered non-active (NA).

Results show a low activity of *E. alba* extract on the tyrosinase assay at 500 µg.mL⁻¹ and 100 µg.mL⁻¹ with a maximum of inhibition of 18.45 ± 1.04% for the RE. Fractionation did not improve tyrosinase inhibitory activity since both AP and OP also showed a low inhibition with an activity of the same order at 14.01 ± 1.29% and 14.06 ± 3.53%. Concerning elastase inhibition, the raw extract showed a maximum inhibition of 23.55 ± 2.93% at 250 µg.mL⁻¹. AP was similar to the extract while OP did not exhibit any activity. The *E. alba* ethanolic extract had no significant activity on tyrosinase and elastase. This result indicates a weak potential of *E. alba* as a whitening agent to reduce hyperpigmentation and a weak ability to protect elastin fibers and skin elasticity.

Collagenase is involved in skin aging as it degrades collagen, a structural skin protein²⁰. The collagenase activity was determined at 20 µg.mL⁻¹, 200 µg.mL⁻¹ and 400 µg.mL⁻¹ of samples. At 400 µg.mL⁻¹ and 200 µg.mL⁻¹ the raw extract and the aqueous phase both presented a high activity with no significant differences, but at 20 µg.mL⁻¹ the aqueous phase presented the

highest inhibition with $53.13 \pm 0.85\%$ ($p < 0.05$). The organic phase did not exhibit significant inhibition whatever the concentration tested. To the best of our knowledge, this is the first time that the inhibition of collagenase was reported for *E. alba* and these results showed a good inhibition effect of the RE with a high involvement of AP in the activity.

For the XO assay, plant extracts were first tested at higher concentrations ($50 \mu\text{g.mL}^{-1}$, $10 \mu\text{g.mL}^{-1}$, and $5 \mu\text{g.mL}^{-1}$). As very high inhibition without a dose-dependent response was observed, lower concentrations were tested ($2.5 \mu\text{g.mL}^{-1}$ and $1 \mu\text{g.mL}^{-1}$). Chlorogenic acid was used as control inhibitor with a concentration of $0.71 \mu\text{g.mL}^{-1}$ and showed $51.49 \pm 2.71\%$ of inhibition. Aqueous phase compared to raw extract and organic phase at the same concentration showed the highest inhibition with $88.32 \pm 2.44\%$ against $72.15 \pm 2.46\%$ and $35.21 \pm 1.44\%$ at $2.5 \mu\text{g.mL}^{-1}$ respectively ($p < 0.05$). At $1 \mu\text{g.mL}^{-1}$ the higher inhibition effect of the AP was still observed with $66.47 \pm 0.92\%$ ($p < 0.05$). Those results confirm the antioxidant potential observed through the chemical antioxidant assays previously presented.

Lastly, COX-2 inhibition was tested with a concentration of $100 \mu\text{g.mL}^{-1}$ and $50 \mu\text{g.mL}^{-1}$ of crude extract and LLE phases and showed a good inhibitory effect of the samples. At $100 \mu\text{g.mL}^{-1}$ the three samples did not exhibit any significant differences. At the lowest concentration, the inhibition of COX-2 by RE and AP also presented a non-significant difference with $85.34 \pm 1.16\%$ and $90.81 \pm 1.33\%$. Although OP presented a good activity at $100 \mu\text{g.mL}^{-1}$ with $89.32 \pm 0.70\%$, its inhibition effect decreased significantly at $50 \mu\text{g.mL}^{-1}$ and was almost halved, whereas the inhibition effect of crude extract and AP remained higher than 85%. The anti-inflammatory activity of *E. alba* was evaluated using COX-2 and showed a high activity of the raw extract, highlighting a major implication of the aqueous phase and polar compounds. The anti-inflammatory properties of *E. alba* were already reported by measuring the inhibition of the production of Prostaglandin E_2 (PGE_2) from human skin fibroblasts (CCD-986sk) (Lee, 2017) and using carrageenan-induced rat paw oedema method³⁶.

The preliminary work highlighted the potential of *E. alba* ethanolic extract in reducing skin aging through the inhibitory activity on collagenase, XO and COX-2. Whatever the enzyme tested, aqueous phase showed the highest activity and seemed to concentrate the molecules of interest. The AP presented an activity that was similar to or higher than that of RE whereas the OP presented no, or a lower, activity. Thus, to refine the identification of bioactive compounds the aqueous phase was fractionated by CPC and fractions were tested again.

b. CPC fractions evaluation

The AP was fractionated using CPC with a biphasic solvent system in descending mode. Several biphasic systems involving different solvents and percentages of solvent were tested and the best separation was obtained with the water/methanol/butanol system. After collecting the tubes, 6 fractions (FA, FB, FC, FD, FE, FF) were finally obtained. FA and FC together represented almost 80% of the mass part of the AP. In this part, only the XO, collagenase, and COX-2 inhibition potentials were investigated and presented on Figure 30 due to the results obtained previously (3.1.1.).

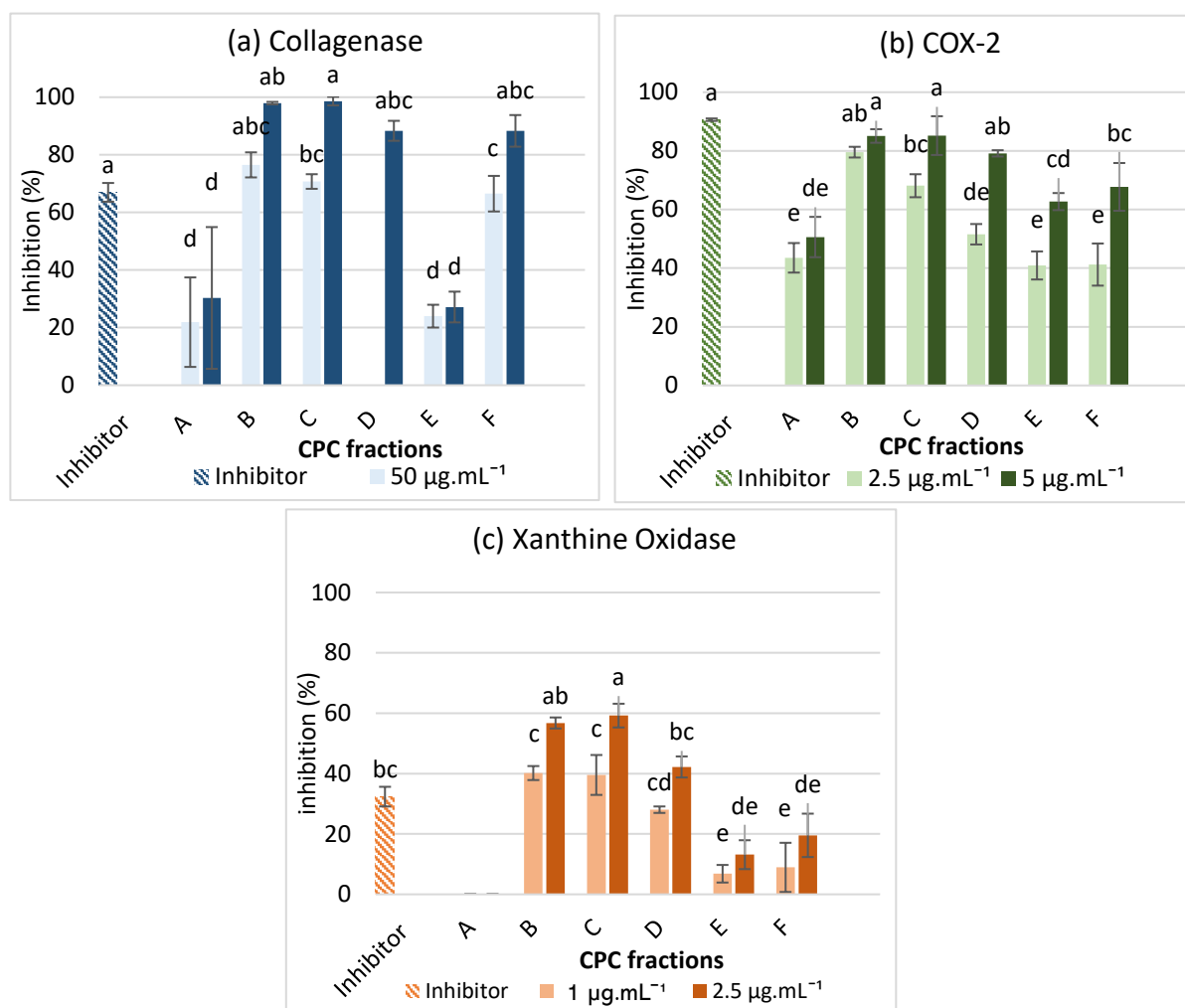


Figure 30: Inhibitory effect of CPC fractions (a) Collagenase, (b) COX-2, (c) Xanthine Oxidase (XO). Data are represented as mean $\pm$ SD ($n = 3$). Any negative inhibition values are considered as 0%. For the same enzyme tested, means followed by the different letter are significantly different at $p < 0.05$. Reference inhibitors are respectively 1.10-phenanthroline monohydrate (a), celecoxib (b) and chlorogenic acid (c).

For collagenase, taking into account the two concentrations ($250 \mu\text{g.mL}^{-1}$ and $50 \mu\text{g.mL}^{-1}$), FB, FC, and FF possessed the highest inhibitory effect with $76.51 \pm 4.35\%$, $70.73 \pm 2.54\%$ and $66.50 \pm 6.18\%$ respectively at $50 \mu\text{g.mL}^{-1}$. The collagenase inhibitory effect of *E. alba* has not been previously reported, but the results obtained previously (3.1.1.) on AP at $200 \mu\text{g.mL}^{-1}$ and the

inhibition of FB, FC and FF at 50 $\mu\text{g.mL}^{-1}$ suggest that *E. alba* has a good potential as an anti-collagenase extract.

Concerning XO, fractions were tested at 1 $\mu\text{g.mL}^{-1}$ and 2.5 $\mu\text{g.mL}^{-1}$. FB and FC presented inhibition percentages of $56.78 \pm 1.83\%$ and $59.23 \pm 3.93\%$ respectively at 2.5 $\mu\text{g.mL}^{-1}$. Both inhibitions are significantly higher than those of FA, FE and FF and FC was also significantly higher than FD ($p < 0.05$). At 1 $\mu\text{g.mL}^{-1}$, FB and FC had a higher inhibition percentage ($p < 0.05$); the inhibition effect of FD was not significantly different. The results obtained for FB, FC and FD were similar to the effect observed with the reference inhibitor chlorogenic acid at 0.71 $\mu\text{g.mL}^{-1}$.

Concerning COX-2, the 5 $\mu\text{g.mL}^{-1}$ FA had a lower inhibition effect as on all enzymes ($p < 0.05$). FC and FB had the highest inhibition effects and were both significantly different from FE, FF and FA. FD had an intermediate inhibition effect. At 2.5 $\mu\text{g.mL}^{-1}$ of fractions, FB and FC also showed the highest inhibition of the enzyme and were both significantly different from the other fractions ($p < 0.05$).

Lastly, FA containing molecules eluted first in descending mode did not show any significant inhibition on collagenase and XO at either of the concentrations tested and only a low activity on COX-2. The inhibition effect of FE and FF was observed for at least one concentration depending on the enzyme, but their effect was lower. FD was active depending on the concentration and the enzyme, but FB and FC were the most active fractions on the three enzymes, exhibiting a high inhibitory effect on XO and COX-2 with an inhibition above 60% at a concentration below 5 $\mu\text{g.mL}^{-1}$.

3.2. Characterization of active fractions

a. Characterization of the two major compounds

FB and FC from the AP of *E. alba* extract were identified as fractions of interest as they always possessed activity and had the most inhibitory effect against XO and COX-2. The UHPLC-UV-ELSD (result not shown) highlighted the presence of a major compound in each fraction. In order to identify the major compounds potentially involved in the inhibition effect, NMR and HRMS analyses of fractions B and C were undertaken. The molecular formula $\text{C}_{15}\text{H}_8\text{O}_7$ was deduced from (+)-ESI-HRMS (m/z 301.0341 $[\text{M}+\text{H}]^+$, err. 4.0 ppm) for the main compound of fraction B and the molecular formula $\text{C}_{16}\text{H}_{10}\text{O}_7$ (m/z 315.0504 $[\text{M}+\text{H}]^+$, err. -1.6 ppm) for the major compound of fraction C. Analysis of the 1D and 2D ^1H and ^{13}C NMR data (CD_3OD) (Table 5) was used to determine their structure. The major compound of FC differed from that of FB by the presence of the methoxy group C-16 (δC 55.45, δH 3.79) instead of a hydroxy group. Using Small Molecule Accurate Recognition Technology (SMART)³⁷, which compares ^1H - ^{13}C HSQC correlations against a library of more than 100,000 natural products, the major compound of FC was identified as wedelolactone (cosine score: 0.97). Based on this result and

in accordance with the literature, the compounds identified in FB and FC were DWL and WL (Figure 31), two coumestans commonly found in *E. alba*¹³.

Table 5: ¹H NMR and ¹³C NMR data of major compounds of fraction B and fraction C (CD₃OD, 400 MHz)^a (spectrum Figure S1 and S2)

	Demethylwedelolactone (DWL)				Wedelolactone (WL)			
	Exp (CD ₃ OD)		Literature (DMSO- <i>d</i> ₆) (Seo et al., 2020)		Exp (CD ₃ OD)		Literature (DMSO- <i>d</i> ₆) (Seo et al., 2020)	
	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H
1			154.36				154.85	
2	100.3	6.33 (1H, d, <i>J</i> =2.0)	99.06	6.35 (1H, d, <i>J</i> =2.1)	99.3	6.37 (1H, d, <i>J</i> =2.3)	98.13	6.45 (1H, d, <i>J</i> =2.3)
3			161.07		163.4		162.24	
4	95.8	6.38 (1H, d, <i>J</i> =1.7)	94.89	6.39 (1H, d, <i>J</i> =2.1)	92.5	6.47 (1H, d, <i>J</i> =2.3)	93.21	6.62 (1H, d, <i>J</i> =2.3)
5			155.05		156.1		157.79	
6			159.36				158.92	
7			100.90		101.1		101.69	
8			113.82		114.4		113.73	
9	105.8	7.31 (1H, s)	104.51	7.21 (1H, s)	105.1	7.31 (1H, s)	104.53	7.24 (1H, s)
10			145.16		145.4		145.42	
11			144.21		144.4		144.34	
12	99.3	7.14 (1H, s)	98.89	7.14 (1H, s)	99.0	7.13 (1H, s)	98.89	7.16 (1H, s)
13			148.67		150.0		148.86	
14			157.88				155.28	
15			95.41		98.11		96.71	
16					55.44	3.85 (3H, s)	55.73	3.81 (3H, s)

^a ¹³C NMR chemical shifts were determined from the indirect dimension of ¹H–¹³C HSQC and ¹H–¹³C HMBC experiments.

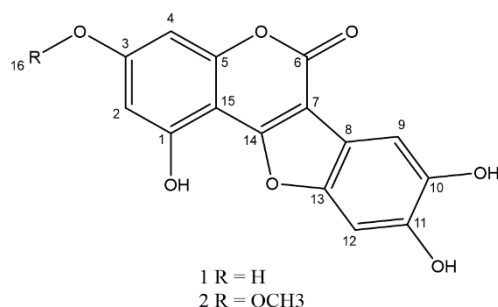


Figure 31: Chemical structure of demethylwedelolactone (1) and wedelolactone (2)

DWL and WL were identified as the two major compounds of FB and FC respectively and were also the two major compounds of AP. UHPLC-ELSD analysis (Figure S3) showed that DWL represented 58% of FB and WL 65% of FC. WL is known to be a major bioactive compound of *E. alba* in traditional Ayurveda medicine³⁸ and was previously identified for its anti-inflammatory and antioxidant properties^{39,40}.

The protective effect of WL was studied on a mouse model irradiated with UVB and showed the decline in the reactive oxygen species and inflammation on the mouse treated with WL⁴¹. Another study showed the inhibition of the production of nitric oxide (NO) and PGE₂ (prostaglandin E₂) as well as their enzymes iNOS and COX-2 in LPS-induced RAW 264.7 cells with no effect on cell viability at concentrations from 0.1 μ M to 100 μ M of WL. This previous study also indicates that WL exerted anti-inflammatory effects by suppressing the NF- κ B pathway⁴⁰. In addition, reactive oxygen species are also implying in the activation of hyaluronidase, elastase, and collagenase enzymes in skin⁴², for this reason inhibiting their activities will also limit the degradation of hyaluronic acid, elastin, and collagen respectively. Therefore, according to our results concerning the inhibitory effect of FB and FC on collagenase, COX-2 and XO, the two coumestans seem to be highly involved. Furthermore, to the best of our knowledge this is the first time that DWL, present in FB, has been highlighted to be active on such target enzyme and the first time that WL has been highlighted to directly inhibited collagenase. Even if, the coumestan are the two major compounds present in the AP, other molecules eluted in FD-FF and present in AP can interact and also have inhibition properties.

b. Characterization of other AP compounds

Therefore, an analysis of AP by UHPLC-ESI-HRMS/MS was undertaken (Figure 32) in order to identify the other compounds. A positive ionization was selected as it gave better ionisation of *E. alba* compounds. Table 6 presents the identifications proposed for the compounds detected in AP.

Identification of the ions was conducted using the SmartFormula algorithm of the DataAnalysis 4.4 software. Molecular formulae were determined using the exact mass of

molecular ions, together with UV information and fragmentation, a structure was proposed for each compound in agreement with the literature and databases. For compounds previously undescribed in *E. alba*, molecular formulae were submitted to databases and the Sirius software was used to obtain a structural proposition.

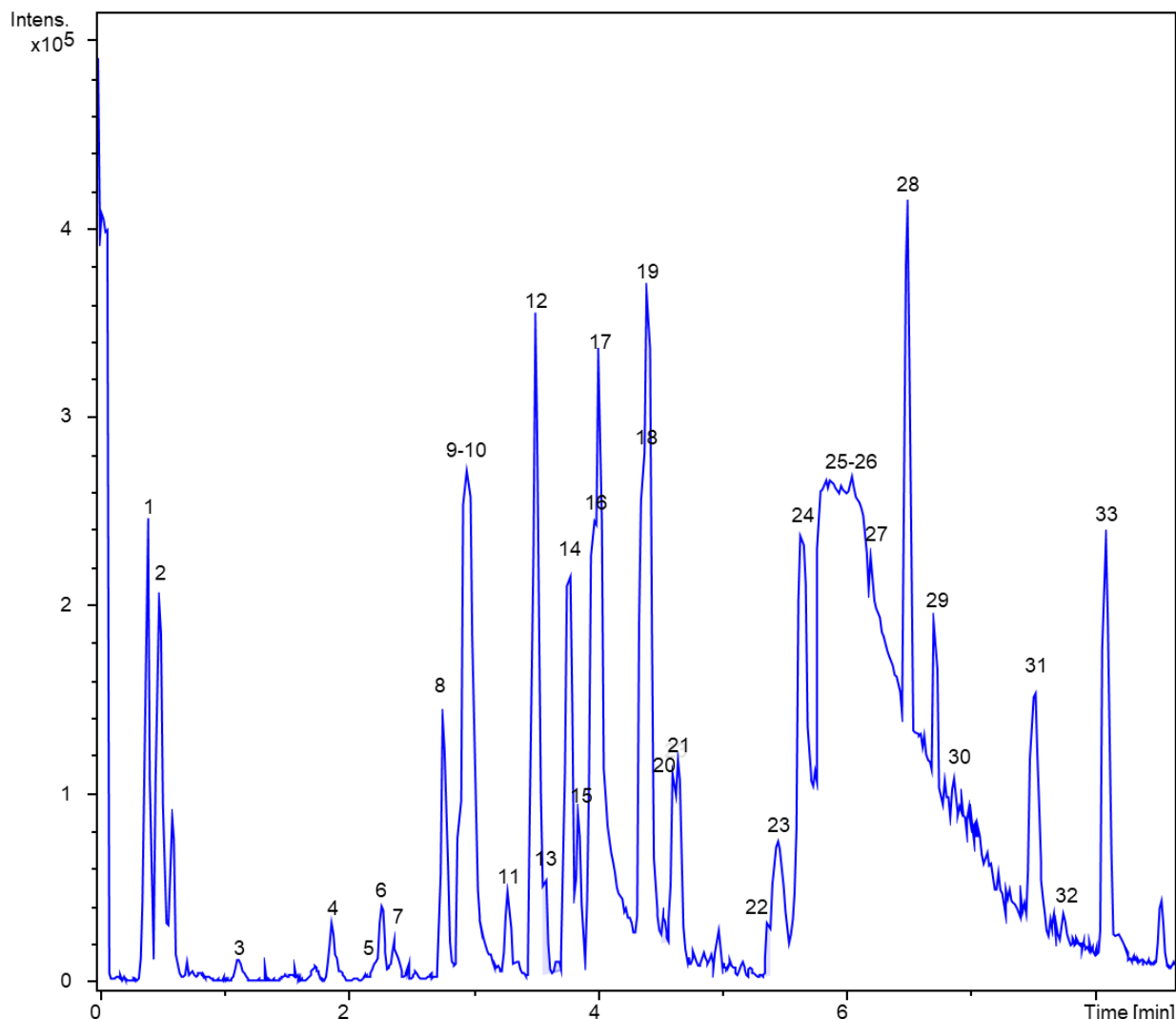


Figure 32: Base Peak Chromatogram (BPC) of the aqueous phase (AP) of *E. alba* by (+)-ESI-HRMS/MS. Separation was performed on a Macherey-Nagel Nucleodur HTec C18 (100 x 2 mm, 1.8 μ m) with gradient elution composed of water (A) and acetonitrile (B), both acidified with 0.1% formic acid and a flow rate fixed at 0.5 mL.min⁻¹.

- *Identification of amino acids and sugars*

Compound **1**, with parent ion $[M+H]^+$ at m/z 343.1231, corresponded to the molecular formula $C_{12}H_{22}O_{11}$. The ion m/z 145.0491 corresponded to the loss of a sugar moiety and H_2O group. Compound **1** was identified as a disaccharide with two hexose units. Compound **2**, with a parent ion $[M+H]^+$ at m/z 268.1039, corresponded to the molecular formula $C_{10}H_{13}N_5O_4$. The ion m/z 136.0624 suggested the loss of a pentose moiety. The compound was annotated as adenosine. Compound **3**, with a parent ion $[M+H]^+$ at m/z 166.0863, corresponded to the

molecular formula $C_9H_{11}NO_2$ and was tentatively identified as phenyl-alanine. Compound **4**, with a parent ion $[M+H]^+$ at m/z 205.0975, corresponded to the molecular formula $C_{11}H_{12}N_2O_2$. The ion m/z 188.0709 on the MS/MS spectrum could be due to the loss of an amino function and the ion m/z 118.0627 corresponded to the indole structure after the loss of the amino and the carboxylic acid groups. Therefore, compound **4** was tentatively identified as tryptophan.

- *Identification of glycoside flavonoids*

According to the literature, the fragmentation of O-glycosylated flavonoids mainly results from the loss of the glycoside part. Compound **5**, $[M+H]^+$ at m/z 451.1587, matched the molecular formula of $C_{21}H_{22}O_{11}$. The MS/MS spectrum presented an ion at m/z 289.0735 resulting from the loss of the hexose part (loss of 162 u) and an ion at m/z 179.0337 resulting from the loss of $C_6H_6O_2$. The aglycone part corresponding to the ion m/z 289.0735 was consistent with eriodictyol, previously identified in the plant⁴³. The UV spectrum with a maximum around 290 nm concurred with the maximum of absorbance of a flavanone⁴⁴ and therefore compound **5** was identified as eriodictyol-*O*-glycoside. Compound **12**, $[M+H]^+$ at m/z 481.0972, corresponded to the molecular formula $C_{21}H_{20}O_{13}$. The MS/MS spectrum indicated the loss of a hexose resulting in an ion at m/z 319.0447 that corresponds to the hexahydroxyflavone skeleton. Myricetin and quercetagenin both matched. As myricetin-glucoside had previously been identified in the plant⁴⁵, compound **12** was associated to myricetin-*O*-glucoside, which is in accordance with the UV spectrum. Compound **13** $[M+H]^+$ at m/z 465.1021 corresponded to the molecular formula $C_{21}H_{20}O_{12}$. The MS/MS spectrum indicated the loss of a hexose resulting in an ion at m/z 303.0491 corresponding to quercetin aglycone. Since quercetin-glucoside had been previously identified⁴⁵, compound **13** and compound **15** with the same fragmentation pattern were both identified as two quercetin-*O*-hexose derivatives. Compound **14**, $[M+H]^+$ at m/z 467.1177, corresponded to the formula $C_{21}H_{22}O_{12}$. The MS/MS spectrum indicated the loss of the hexose, resulting in an ion at m/z 305.0657 followed by the loss of H_2O leading to a fragment ion at m/z 287.0549. Finally, the opening of the cycle forms a fragment ion at m/z 137.0236. Compound **14** is suggested to be dihydroquercetin-hexose. Compound **17**, $[M+H]^+$ at m/z 449.1074, corresponded to the molecular formula $C_{21}H_{20}O_{11}$. The MS/MS spectrum indicated the loss of the hexose resulting in an ion at m/z 287.0551 that matched either luteolin or orobol aglycone. The UV spectrum of compound **17** with λ_{max} 320 nm does not correspond to luteolin but corresponds to an isoflavone such as orobol. Compound **17** was thus identified as orobol-*O*-hexose and orobol-*O*-glucoside was previously described in the plant⁴⁶. Compound **19**, $[M+H]^+$ at m/z 433.1124, corresponded to the molecular formula $C_{21}H_{20}O_{10}$. The MS/MS spectrum indicated the loss of the hexose, resulting in an ion at m/z 271.0599 corresponding to apigenin aglycone, previously described in the plant⁴⁷. The proposed identification for Compound **19** was apigenin-*O*-

hexose. Compound **22**, with a parent ion $[M+H]^+$ at m/z 479.1176, corresponded to the molecular formula $C_{22}H_{22}O_{12}$. The MS/MS spectrum indicated an ion at m/z 317.0651 corresponding to methylquercetin aglycone, suggesting that compound **22** was an O-methylated flavonol hexose derivative such as tamarixin or rhamnetin-O-hexose. Compound **25**, with a parent ion $[M+H]^+$ at m/z 317.0675 corresponding to the molecular formula $C_{16}H_{12}O_7$ was annotated as a flavonoid such as rhamnetin or an O-methyl-isoflavone based on the SIRIUS computation. MS/MS ion at m/z 209.0454 obtained, after the loss of the diphenol ring, corresponds to the formula $C_{10}H_8O_5$. The ion at m/z 209.0454 corresponds to the opening of the pyran cycle in the flavonoid skeleton, hence the ion at m/z 123.0441 corresponds to the $C_7H_7O_2$ ring. The UV spectrum at λ_{max} 320 nm supported the hypothesis of the rhamnetin structure.

- *Identification of phenolic acids*

Compound **6** gave parent ions $[M+H]^+$ at m/z 355.1029 in positive ion mode, resulting in the molecular formula $C_{16}H_{18}O_9$. The ion m/z 163.0392 corresponding to caffeic acid suggested the loss of a $C_7H_{12}O_6$ group, which is consistent with quinic acid. The ions m/z 135.0440 and m/z 117.0326 corresponded to the loss of $-CO$ and $-OH$ functions successively from the caffeic acid. The fragmentation pattern, the parent ions and literature data⁴⁶ indicated a chlorogenic acid derivative. Compounds **8** and **9** with same parent ions and fragmentation pattern were both identified as chlorogenic acid derivatives. Compound **10**, with a parent ion $[M+H]^+$ at m/z 291.0495, corresponded to the molecular formula $C_{14}H_{10}O_7$. The ion at m/z 137.0244 of the MS/MS spectrum was consistent with a hydroxybenzoic acid derivative. No compound has been previously identified with this molecular formula in the plant, but the database suggests that compound **10** is a dihydroxybenzoyloxy-hydroxybenzoic acid. Compounds **18** and **20** gave parent ions $[M+H]^+$ at m/z 517.1330 resulting in a similar molecular formula $C_{25}H_{24}O_{12}$. The ions m/z 499.1228 and m/z 499.1247 suggested the loss of a $-H_2O$ group. The ion m/z 163.0392 was identified as caffeic acid. According to the fragmentation pattern and literature data⁴⁵, both compounds **18** and **20** correspond to dicaffeoylquinic acids.

- *Identification of coumestans*

Compounds **16** and **26** showed parent ions $[M+H]^+$ at m/z 301.0341 and m/z 315.0504, resulting in molecular formulae of $C_{15}H_8O_7$ and $C_{16}H_{10}O_7$ respectively. In accordance with the NMR analysis and the literature, compounds **16** and **26** were identified as demethylwedelolactone and wedelolactone.

- *Identification of triterpene glycosides*

Triterpene glycosides (compounds **27**, **28**, **29**, **30**, **31**, **32** and **33**) were not detected using UV. $[M+NH_4]^+$ ions were mainly detected, except for compounds **31** and **33**. Triterpene glycosides had ions at m/z 455 and m/z 437 (Table 6), both indicating a dihydroxylated triterpenic acid

aglycone with a molecular formula of $C_{30}H_{47}O_3$ that corresponds to the echinocystic acid-type aglycone (m/z 455)⁴⁸ followed by the loss of a H_2O (m/z 437). Compound **27**, with a parent ion $[M+NH_4]^+$ at m/z 814.4929, resulted in the molecular formula $C_{42}H_{68}O_{14}$. The literature suggests the structure of eclalbasaponin I or eclalbasaponin IV⁴⁹. Compounds **28**, **29**, **30** shared similar parent ions $[M+NH_4]^+$ at m/z 900.493 (Table 6), suggesting a similar molecular formula $C_{45}H_{71}O_{17}$ of eclalbasaponin derivatives. Compound **32** with a parent ion $[M+NH_4]^+$ at m/z 652.4413 suggested a molecular formula $C_{36}H_{58}O_9$ corresponding to isoechinocystic acid glucoside. Compound **31** with a parent ion $[M+H]^+$ at m/z 703.4038 suggested the molecular formula $C_{36}H_{62}O_{11}S$. The loss of an H_2O group resulted in the ion m/z 685.3934, followed by the loss of the sugar moiety and the sulphated group at m/z 455.3517 and corresponded to eclalbasaponin IX or eclalbasaponin X⁵⁰. Compound **33** with the same fragmentation pattern can also correspond to eclalbasaponin IX or eclalbasaponin X.

- *Identification of other compounds*

Compound **7**, with a parent ion $[M+H]^+$ at m/z 341.2542, corresponds to the molecular formula $C_{17}H_{32}N_4O_3$. The SIRIUS software with a SIRIUS similarity score of 61.48% suggested a structure corresponding to N-[6-(methylamino)-6-oxohexyl]-6-[-5-methyl-2-oxoimidazolidin-4-yl]-hexanamide. The molecule with a LogP of 0.8 also corresponds in terms of retention time. Compound **11**, with a parent ion $[M+H]^+$ at m/z 387.2004, corresponds to the molecular formula $C_{19}H_{30}O_8$. Junipeionolose and euodinoside A were previously identified in *E. alba*. Their molecular formula and their fragmentation pattern are similar to those of compound **11** with ions m/z 225.1483 and m/z 207.1391 corresponding respectively to the loss of the sugar moiety and the loss of H_2O . It was therefore assumed that compound **11** was Junipeionolose or euodinoside A. Compound **21**, with a parent ion $[M+NH_4]^+$ at m/z 470.2588, corresponded to the molecular formula $C_{20}H_{36}O_{11}$. The MS/MS spectrum with ion m/z 325.1128 corresponding to two sugar moieties (162 u) indicated the loss of the aglycone whose formulae is $C_8H_{16}O$ (128 u). The ion m/z 163.0592 confirmed the presence of the hexose moiety (162 u) and the ion m/z 111.1171 corresponded to the aglycone formula with the loss of H_2O . Calaliukiuenoside previously identified in the plant is consistent with this fragmentation pattern and was tentatively identified as compound **21**. Compound **23**, with a parent ion $[M+H]^+$ at m/z 629.0914, corresponded to the molecular formula $C_{32}H_{20}O_{14}$. This formula does not correspond to a known structure in *E. alba*. The Pubchem proposition with this molecular formula and SIRUS computation suggested that compound **23** was a biflavonoid derivative with 58.47% of similarity. Compound **24**, with a parent ion $[M+H]^+$ at m/z 313.0330, corresponded to the molecular formula $C_{16}H_8O_7$. The fragment ion m/z 161.0224 corresponded to the $C_9H_5O_3$ tri-cycle structure. SIRIUS computation suggested the tetrahydroxybenzonaphthofurandione compound.

Table 6: UHPLC-HR-ESI-MS data of *E. alba* aqueous phase extract

	Retention time (t _R) (min)	Lambda max (nm)	Measured m/z	Adduct	Molecular formula [M]	Error (ppm)	Ring and double bond (RDB)	MS/MS ion fragments	Putative identification	Ref.
1	0.51	-	343.1231	[M+ H] ⁺	C ₁₂ H ₂₂ O ₁₁	0.8	2	145.0491 / 127.0383 / 109.0273	Hexose disaccharide	
2	0.62	204	268.1039	[M+ H] ⁺	C ₁₀ H ₁₃ N ₅ O ₄	-0.5	7	136.0624	Adenosine	
3	1.15	-	166.0863	[M+ H] ⁺	C ₉ H ₁₁ NO ₂	-0.6	5		Phenyl alanine	
4	1.9	-	205.0975	[M+ H] ⁺	C ₁₁ H ₁₂ N ₂ O ₂	-1.7	7	188.0709 / 146.0606 / 118.0627 / 115.0579	L-tryptophan	
5	2.25	245(sh)/290	451.1587	[M+ H] ⁺	C ₂₁ H ₂₂ O ₁₁	-1.5	11	289.0735 / 179.0337	Eriodictyol-hexose	43
6	2.31	256(sh)/318	355.1029	[M+ H] ⁺	C ₁₆ H ₁₉ O ₉	-1.5	8	163.0392 / 135.0440 / 117.0326	Chlorogenic acid derivatives	46
7	2.39		341.2542	[M+ H] ⁺	C ₁₇ H ₃₂ N ₄ O ₃	2.6	4	299.2932	N-[6-(methylamino)-6-oxohexyl]-6-[(4R,5S)-5-methyl-2-oxoimidazolidin-4-yl]hexanamide	
8	2.79	246/322	355.1016	[M+ H] ⁺	C ₁₆ H ₁₈ O ₉	0.5	8	163.0387 / 135.0447 / 117.0355	Chlorogenic acid derivative	46
9	2.96	250/322	355.1012	[M+ H] ⁺	C ₁₆ H ₁₈ O ₉	3.4	8	163.0391 / 135.0438 / 117.0296	Chlorogenic acid derivative	46
10	3.03	218/290(sh)	291.0495	[M+ H] ⁺	C ₁₄ H ₁₀ O ₇	1.5	10	137.0244	Dihydroxybenzoyloxy-hydroxybenzoic acid	
11	3.31	-	387.2004	[M+ H] ⁺	C ₁₉ H ₃₀ O ₈	0	5	225.1483 / 207.1391	Euodioside A / junipeionoloxide	51
12	3.53	260/355	481.0972	[M+ H] ⁺	C ₂₁ H ₂₀ O ₁₃	0.6	12	319.0447	Myricetin-O-hexose	45

PARTIE I. ETUDE PHYTOCHIMIQUE ET EVALUATION DE L'ACTIVITE ANTI-AGE D'E. ALBA CULTIVEE EN RCVL

13	3.6	260/342	465.1021	[M+ H] ⁺	C ₂₁ H ₂₀ O ₁₂	1.1	12	303.0491	Quercetin- <i>O</i> -hexose derivative	45
14	3.79	209/292	467.1177	[M+ H] ⁺	C ₂₁ H ₂₂ O ₁₂	1.6	11	449.1073/305.0657/ 287.0549 / 137.0236	Dihydroquercetin- <i>O</i> -hexose	
15	3.93	250/340	465.1024	[M+ H] ⁺	C ₂₁ H ₂₀ O ₁₂	0.8	12	303.0498/229.0444/ 153.0190	Quercetin- <i>O</i> -hexose derivative	45
16	4.00		301.0341	[M+ H] ⁺	C ₁₅ H ₈ O ₇	4.0	12	283.0226 / 255.0279 / 153.018	Demethylwedelolactone	52
17	4.04	216/320	449.1074	[M+ H] ⁺	C ₂₁ H ₂₀ O ₁₁	0.7	12	287.0551	Orobol- <i>O</i> -hexose	53
18	4.4	220/320	517.1330	[M+ H] ⁺	C ₂₅ H ₂₄ O ₁₂	2	14	499.1228 / 163.0392	Dicaffeoyl quinic acid	45
19	4.46	ND	433.1124	[M+ H] ⁺	C ₂₁ H ₂₀ O ₁₀	1.3	12	271.0599	Apigenin- <i>O</i> -hexose	
20	4.63	221/325	517.1330	[M+ H] ⁺	C ₂₅ H ₂₄ O ₁₂	2	14	499.1247 / 163.0390	Dicaffeoyl quinic acid	45
21	4.67	326(sh)/295 /	470.2588	[M+NH ₄] ⁺	C ₂₀ H ₃₆ O ₁₁	1.8	2	325.1128/163.0592/ 145.0494 / 111.1171	Calaliukiuenoside	43
22	5.4	ND	479.1176	[M+ H] ⁺	C ₂₂ H ₂₂ O ₁₂	3.2	12	317.0651	Tamarixin / rhamnetin- <i>O</i> -hexose	
23	5.47	-	629.0914	[M+ H] ⁺	C ₃₂ H ₂₀ O ₁₄	4.9	18	599.0789/573.1032/ 489.0414 / 429.0242 / 167.0341	Biflavonoid derivative	
24	5.66	250/350	313.033	[M+ H] ⁺	C ₁₆ H ₈ O ₇	-0.3	13	161.0211	naphthofurandione derivative	
25	6.06	220/320	317.0675	[M+ H] ⁺	C ₁₆ H ₁₂ O ₇	-0.3	11	243.0653 /167.0343/123.0441	Rhamnetin	
26	6.1	220/320	315.0504	[M+ H] ⁺	C ₁₆ H ₁₀ O ₇	-1.6	12	209.0454/167.0343/ 123.0443	Wedelolactone	52
27	6.22	323/580	814.4929	[M+NH ₄] ⁺	C ₄₂ H ₆₈ O ₁₄	2.3	8	455.3510/437.3406/ 409.3444 / 191.1811	Eclalbasaponin I / IV	49
28	6.5	-	900.4931	[M+NH ₄] ⁺	C ₄₅ H ₇₁ O ₁₇	0	10	721.4142/703.4056/ 455.3515 /437.3409	Eclalbasaponin glycoside	
29	6.72	-	900.4932	[M+NH ₄] ⁺	C ₄₅ H ₇₁ O ₁₇	0.7	10	721.4142/455.3510 /437.3407	Eclalbasaponin glycoside	
30	6.88	-	900.4935	[M+NH ₄] ⁺	C ₄₅ H ₇₁ O ₁₇	1.5	10	703.4056/455.3512/ 437.3407	Eclalbasaponin glycoside	
31	7.53	-	703.4038	[M+ H] ⁺	C ₃₆ H ₆₂ O ₁₁ S	2.9	6	685.3934/455.3517/437.3415	Eclalbasaponin IX / eclalbasaponin X	50

CHAP II. DEVELOPPEMENT DE METHODES D'ANALYSES CIBLEES ET NON CIBLEES POUR LA CULTURE DE DEUX PLANTES D'INTERET EN RCVL

32	7.75	-	652.4413	[M+NH ₄] ⁺	C ₃₆ H ₅₈ O ₉	1	7	455.3508/437.3412	Isoechinocystic acid glucoside	52
33	8.1	-	703.4048	[M+ H] ⁺	C ₃₆ H ₆₂ O ₁₁ S	0.3	9	685.3934/455.3532/437.3532	Eclalbasaponin IX / eclalbasaponin X	50

3.3. Compound distribution and bioactivity

A heat map built from the peak areas of the EIC of each identified compound and presented on Figure 33 shows the distribution of each compound in the different CPC fractions, making it possible to visualize and to cluster the compounds.

FA contained, in addition to sugar (**1**), eriodictiol-hexose (**5**) and caffeoylquinic acids (**6**, **8** and **9**) in lesser amounts (ELSD analysis), resulting in a fraction that was inactive on XO and only weakly active on collagenase and COX-2.

FB enabled the inhibition of collagenase, COX-2 and XO (Figure 30). NMR and MS analysis identified the demethylwedelolactone (DWL) (**16**) as a major compound of FB and previously described as a bioactive compound of the coumestan family. Compounds **22** and **25**, two O-methyl-isoflavone derivatives, are highly distributed in FB and not in other fractions. Rhamnetin (**25**) was previously suggested to be involved in some anti-inflammatory activity⁵⁴ and may contribute to the activity in addition to the major coumestan. Compounds **18**, **20**, **23**, **24** and **26** were also identified in FB but they are not characteristic as they are also distributed in FC, FD and FE. In addition to the coumestan activity described above, dicaffeoylquinic acid (**18**, **20**) was previously studied for its antioxidant and anti-inflammatory activity^{18,55}, therefore potentially contributing to the activity of the fraction.

FC presented a similar activity to that of FB. WL (**26**), identified by NMR analysis as the major compound of the fraction, has already been described as an anti-inflammatory⁵¹ and antioxidant compound. Compounds **18**, **20**, **23** and **24**, were also distributed in FC. As for FB, dicaffeoylquinic acid (**18**, **20**) could contribute to the activity of the fraction^{18,55}.

FF was also one of the most active fractions especially on collagenase and COX-2. Its chemical composition was markedly different from that of the other active fractions (FB and FC), meaning that different compounds are involved in the bioactivity of the raw extract and the aqueous phase. This can be explained by the presence of most of the glycosylated flavonoids in this fraction, especially flavonols such as myricetin-glycoside (**12**) and quercetin derivatives (**13** and **15**) that previously showed a higher inhibition of collagenase than other flavonoids⁵⁶.

Triterpene glycosides were mostly identified in FD, FE and FF. As FD and FE are weakly active, triterpene glycosides did not appear to be involved in the inhibition of the target enzymes.

The activity of WL, a major compound of FC, has been extensively described while that of WDL, belonging to same molecular family and a major compound of FB, is less well described. Wedelolactone (WL) and demethylwedelolactone (DWL), were previously reported to decrease the Reactive Oxygen Species expression of cells (Mouse Podocyte Clone-5 cells)¹³. Wedelolactone was also identified, along with orobol and isoflavone, to have an anti-inflammatory activity due to the inhibition of nitric oxidase (NO) and prostaglandin E₂ (PGE₂) release⁵¹. Furthermore, wedelolactone exhibited an inhibitory effect on two enzymes

implicated in the inflammatory process, namely 5-lipoxygenase and cyclooxygenase-2 (COX-2)¹⁵. Several other families of compounds such as dicaffeoylquinic acids and flavonoid glycosides also seem to contribute to inhibiting collagenase, COX-2 and XO.

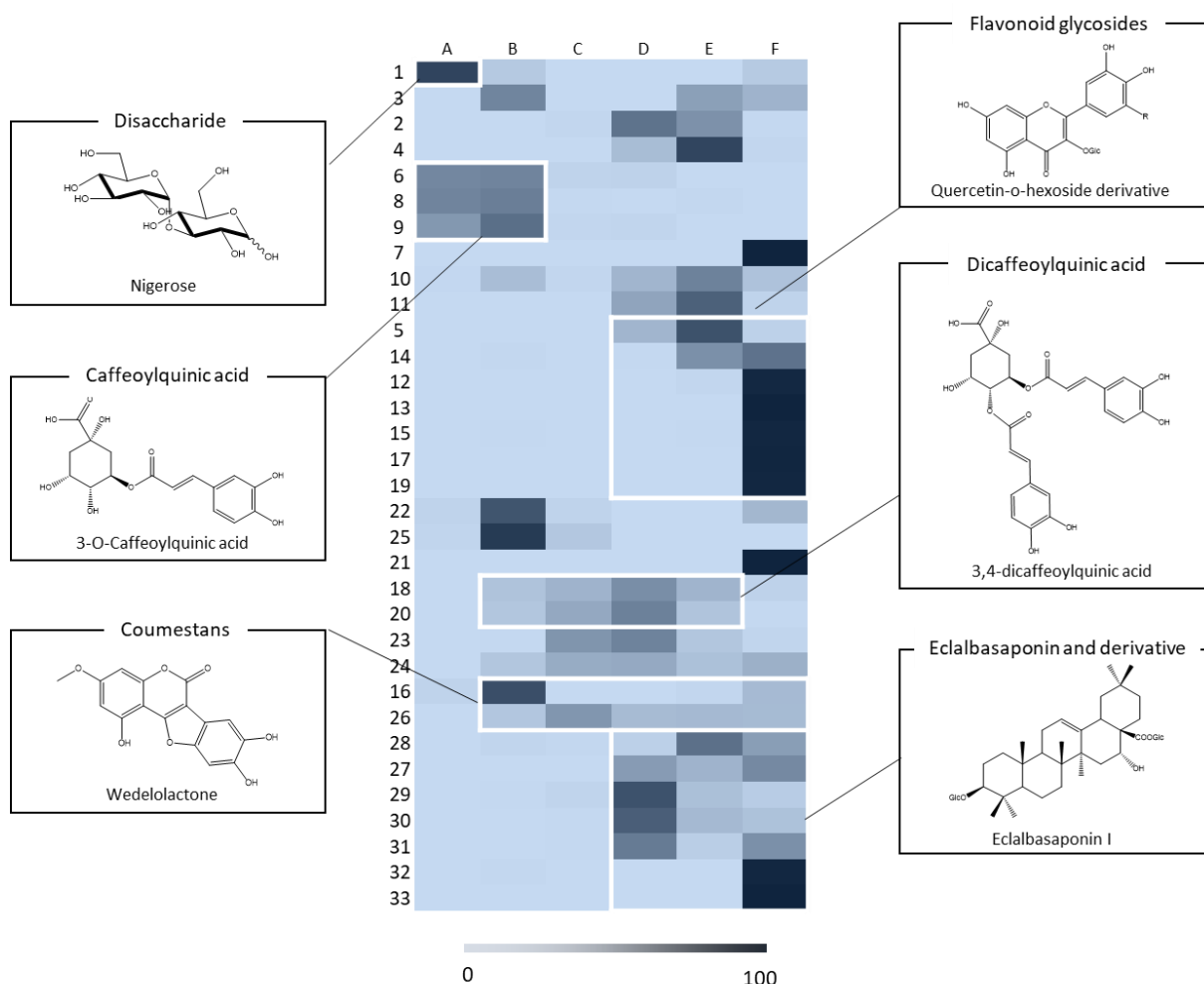


Figure 33: Heat map of the distribution of the compounds (vertical) in the different CPC fractions (horizontal). Each cell indicates the percentage of the abundance of corresponding compound in the fraction compared to its abundance in the others fraction, from 0% to 100%.

4. Conclusion

This study explored the phytochemical and the cosmetical potential of the Ayurveda traditional plant *E. alba* cultivated in CVLR, France. The ethanolic extract of aerial part obtained by ultrasound has demonstrated anti-inflammatory and antioxidant activity in line with the literature on wild plants. Furthermore, to the best of our knowledge this is the first work to report the anti-collagenase activity of *E. alba*.

The fractionation of the ethanolic extract by CPC has made it possible to target fractions and molecules involved in the various activities. The UHPLC-HRMS/MS and NMR analysis have shown that the cultivated plant possesses a similar phytochemical profile to the wild plant

with the identification of the two coumestan (WL and DWL). *In vitro* tests (enzymatic and chemical) showed the most active fractions, that are mainly composed of the two coumestans (WL and DWL), flavonoid glycosides and caffeoylquinic acids, which appear to be responsible for the antioxidant and anti-inflammatory activities.

Previous studies indicated that WL exerted anti-inflammatory effects by suppressing the NF- κ B pathway, declining the reactive oxygen species, and having a photoprotective effect. Our results had shown a direct inhibition of COX-2 by the *E. alba* extract, which suggests inhibition of the pro-inflammatory reaction at different levels (by suppressing NF- κ B pathway and inhibiting COX-2).

The anti-collagenase activity together with antioxidant and anti-inflammatory activity led to a high potential of the plant to act as an anti-aging ingredient. In this way, we have demonstrated that *E. alba* grown in the CVLR (France) has real cosmetic potential and could consider as an interesting secure and traceable local supply for the development of "made in France" ingredients. Future research could be led by carrying out tests on skin models to confirm the potential of *E. alba* extract in cosmetics and focus on the two coumestans that seems highly active.

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Partie II. Culture aéroponique pour une meilleure production de forskolin et valorisation de *Coleus forskohlii*

Dans cette seconde partie de chapitre, nous nous intéressons à *Coleus forskohlii*, dont la culture a été mise en œuvre en région Centre-Val de Loire par le CDHR dans le cadre du projet AERO'DIV. L'objectif principal de ce projet est d'expérimenter des cultures hors sol pour des plantes d'intérêt afin de diversifier, pendant la période estivale, l'utilisation des serres qui sont alors moins exploitées pour la production de plantes ornementales. Ainsi d'autres types de plantes, destinées à d'autres usages, notamment des cultures de plantes tropicales, dont la culture pleine terre n'est pas adaptée à nos conditions climatiques, peuvent y trouver leur place.

La culture hors-sol comme l'aéroponie (Figure 34), présente de nombreux avantages dont notamment une grande flexibilité des conditions de culture avec un apport facilité en solutions nutritives de compositions différentes, l'application de stress et surtout une accessibilité à la partie racinaire de la plante sans impacter sa croissance à la différence de la culture en plein sol. Toutefois la culture d'une plante dans d'autres conditions que ses conditions d'origine et notamment hors sol, donc sans interaction avec les micro-organismes du sol, peut impacter la production de métabolites, c'est pourquoi il y a un travail de recherche pour trouver les bonnes conditions de culture permettant à la fois la production d'une quantité suffisante de biomasse et des métabolites d'intérêt à des pourcentages intéressants.

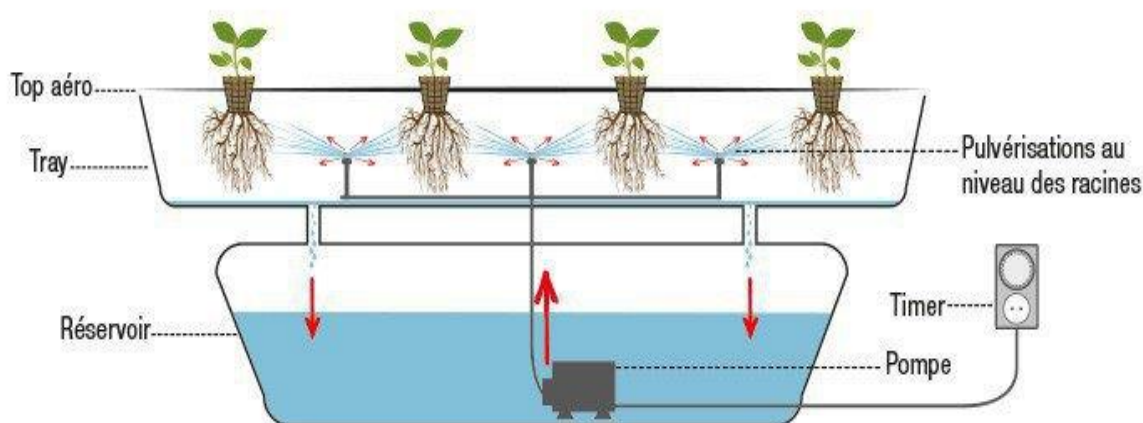


Figure 34 : Schéma de principe d'une culture en aéroponie

C. forskohlii (syn. *C. barbatus* var. *barbatus*) est une plante aromatique vivace de la famille des Lamiacées, originaire des régions tropicales et subtropicales. Dans la médecine traditionnelle hindoue et ayurvédique, *C. forskohlii* est utilisé pour traiter diverses affections, telles que les maladies cardiaques, les coliques abdominales, les troubles respiratoires, l'insomnie et les

convulsions. *C. forskohlii* est déjà bien connu de l'industrie cosmétique¹ pour sa racine riche en forskoline, qui est utilisée dans les gélules amincissantes grâce à ses propriétés brûle-graisse. L'extrait de racines est également intégré dans des produits pour la peau en tant qu'émollient, humectant et agent d'entretien, ainsi que pour la pigmentation². Cette plante a ainsi été sélectionnée par le CDHR pour développer et optimiser un itinéraire de culture visant à produire une biomasse importante de racines et favorisant la production de forskoline.

L'étude visant tout d'abord à déterminer, parmi les conditions de culture aéroponique testées, celle qui permet de produire la plus grande quantité de forskoline, son dosage dans les racines a été effectué afin d'identifier le mode de culture le plus favorable à sa production. Cependant, afin d'avoir une vision plus complète de la composition phytochimique de la plante, la composition des racines et des parties aériennes a été comparée à travers l'utilisation des réseaux moléculaires. Puis, les parties aériennes et racinaires, produites avec la condition de culture améliorant le rendement en forskoline, ont ensuite pu être comparées à celles produites avec la culture aéroponique témoin sans élicitation. L'utilisation d'outils statistiques multivariés a ainsi mis en évidence les molécules dont la production a été stimulée et celles dont la production a été inhibée. Cette meilleure connaissance de la composition des parties aériennes, peu décrites dans la littérature, vise à faciliter la valorisation de la plante entière lors de sa récolte en fin de saison. En effet, si les racines, dont la teneur en forskoline a pu être améliorée, est l'intérêt premier de la production de la plante, les parties aériennes peuvent présenter un intérêt et être valorisées en tant que co-produits pour d'autres applications cosmétiques ou dans d'autres secteurs d'activité.

¹ Meybeck A., Bonte F., Dumas M., André P., Redziniak G., (1995) Utilisation d'un extrait de coleus pour la pigmentation de la peau (Brevet Européen n° EP0486595B1) <https://patents.google.com/patent/EP0486595B1/fr>

² <https://incibeauty.com/ingredients/1306-coleus-forskohlii-root-extract> (consulté le 15/10/2024)

Enhancement of Forskolin Production Using Aeroponic Cultivation of *Coleus forskohlii* and the Impact on the Plant Phytochemistry

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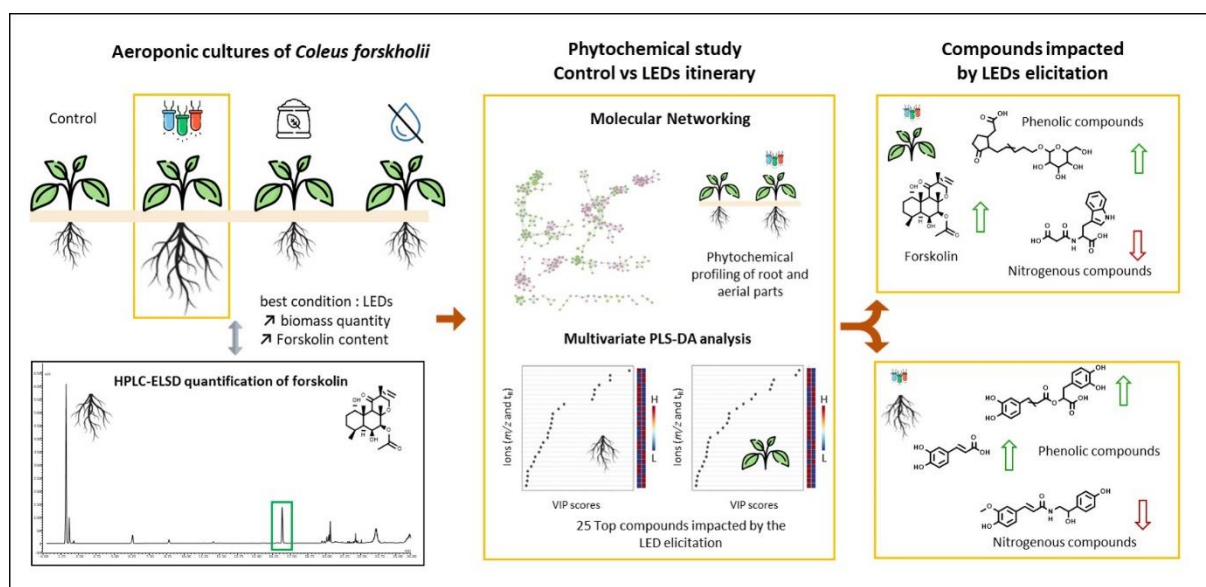
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Graphical abstract



Abstract

Accessing plant resources to extract compounds of interest can sometimes be challenging. To facilitate access and limit the environmental impact, innovative cultivation strategies can be developed. Forskolin is a molecule of high interest, mainly found in the roots of *Coleus forskohlii*. The aim of this study was to develop aeroponic cultivation methods to provide a local source of *Coleus forskohlii* and to study the impact of abiotic stress on forskolin and bioactive metabolite production. Three cultivation itineraries (LED lighting, biostimulant, and hydric stress) along with a control itinerary were established. The forskolin content in the plant roots was quantified using HPLC-ELSD, and the results showed that LED treatment proved to be the most promising, increasing root biomass and the total forskolin content recovered at the end of the cultivation period threefold (710.1 ± 21.3 mg vs. 229.9 ± 17.7 mg). Statistical

analysis comparing the LED itinerary to the control itinerary identified stress-affected metabolites, showing that LEDs positively influence mainly the concentration of phenolic compounds in the roots and diterpenes in the aerial parts of *Coleus forskohlii*. Moreover, to better define the phytochemical composition of *Coleus forskohlii* cultivated in France using aeroponic cultivation, an untargeted metabolomic analysis was conducted using UHPLC-HRMS/MS analysis and molecular networks on both the root and aerial parts. This study demonstrates that aeroponic cultivation, especially with the application of an LED treatment, could be a very promising alternative for a local source of *Coleus forskohlii* leading to easy access to the roots and aerial parts rich in forskolin and other bioactive compounds.

Keywords: aeroponic cultivation; mass spectrometry; metabolomics; molecular network

1. Introduction

Coleus forskohlii (syn. *C. barbatus* var. *barbatus*) is an aromatic perennial plant belonging to the Lamiaceae family, native to tropical and subtropical regions including Brazil, East Africa, India, Myanmar, Nepal, Pakistan, and Sri Lanka^{1,2}. In traditional Hindu and Ayurvedic medicine, *C. forskohlii* has been used to treat a variety of conditions such as heart disease, abdominal colic, respiratory disorders, insomnia, and convulsions³.

The taxonomic classification of *C. forskohlii* has been ambiguous for decades and was only updated in 2019⁴ when the genus *Plectranthus* was divided into three distinct genera: *Plectranthus* sensu stricto, *Coleus*, and *Equilabium*, a decision further supported by the diversity of diterpenoids within these groups⁵. Studies between 1962 and 2019, which included the species *Plectranthus barbatus* as a synonym for *C. forskohlii*, should be reconsidered in light of this update⁴.

Forskolin, discovered in the year 1974, is the main labdane diterpenoid identified in *C. forskohlii*. Its precursor, (13R)-manoyl oxide, was localized in the root cork, which explains why forskolin is mainly localized there^{6,7}. Some studies, however, also mention the presence of forskolin in the aerial parts⁸. Forskolin is mainly known for its role as an activator of hormone-sensitive adenylate cyclase in intact cells or tissues, making it valuable for research on understanding cyclic AMP (adenosine monophosphate)-dependent physiological processes⁶. Forskolin is also used as a dietary supplement for fat loss as it increases cyclic AMP, enhances the use of body fat, and stimulates the basal metabolism and has been shown to be a lipolytic agent in topical application^{9,10}. A large number of studies have investigated forskolin's bioactivities and demonstrated anti-inflammatory properties in vitro and in vivo¹¹. Forskolin could also be used as an anti-inflammatory agent to treat inflammation associated with obesity by inhibiting the expression of NFκB (nuclear factor-kappa B)-dependent genes¹².

Further studies on the root phytochemistry were carried out, and various diterpenoids, mainly forskolin derivatives, were identified¹³. One study compared the phytochemical profile of the genera *Coleus* and *Plectranthus* and revealed the predominant presence in the *Coleus* species of abietanes oxygenated at C-14, with formic acid or acetic acid as acyl groups, while in *Plectranthus*, isovaleric, senecioic, or hydroxybenzoic acid groups can also be found⁵. Other studies, focusing on the aerial parts of *P. barbatus* prior to the division of the genus, identified various diterpenoids^{14,15} and some phenolic compounds with antioxidant properties¹⁶. However, characterization of the phytochemical profile of the aerial parts is very limited.

As forskolin and its derivatives are molecules of pharmaceutical, nutraceutical (sports nutrition, weight management supplement), and cosmetic (lipolytic agent) interest, the demand for *Coleus forskohlii* on the health market has increased, further enhanced by a growing consumer preference for natural products^{10,17}. Therefore, the search for efficient methods for producing high-quality extract, rich in bioactive compounds, or powder of *Coleus forskohlii* is of great interest¹⁸. To meet growing market demand, the development of off-ground cultivation methods that provide easy access to the roots without plant sacrifice, thus allowing several harvests from the same plant, has emerged. While forskolin can be produced from hairy roots or callus cell cultures, these production methods, which require biological engineering, are considerably removed from the intact plant and can result in different metabolomic profiles^{19–21}.

The aim of this study was to develop aeroponic cultivation of *C. forskohlii* to provide a local, easy, and sustainable sourcing of *C. forskohlii*. In aeroponic conditions, plants grow in the air, out of the soil, giving easy access to the roots, and a nutrient solution is sprayed directly onto the roots to promote biomass development^{22,23}. Aeroponic cultivation reduces the consumption of water, fertilizers, pesticides, and herbicides compared to hydroponics or geaponics, but as there is no solid support culture to absorb the excess of nutrients, developing the appropriate system parameters for plant growth is a challenge²⁴.

To achieve this objective, the French Horticultural Development Committee of the Centre-Val de Loire region (Comité de Développement Horticole de la Région Centre-Val de Loire; CDHRC) carried out, for the first time, different cultivation itineraries, involving different stresses or elicitations to assess their impact on plant biomass and metabolite production. Three different aeroponic culture itineraries, artificial lighting (LEDs), biostimulant, and hydric stress, in addition to the control itinerary were evaluated in this way. First, to assess the impact on the production of forskolin, as it is the main valuable compound targeted in *C. forskohlii*, root extracts were analyzed using HPLC-ELSD, and the forskolin content was determined using a calibration curve. Then, to gain greater insight into the plant composition under these growing conditions and therefore to produce a controlled quality extract, the phytochemical profile of the roots and aerial parts of *C. forskohlii* cultivated aeroponically in Région Centre-Val de Loire

was investigated. Many health products (e.g., dietary supplements, cosmetics) on the market today contain *C. forskohlii* root extracts; therefore, it is important to have a good knowledge of the chemical profile of the extract to produce roots rich in forskolin and other bioactive compounds with interesting biological activities. Furthermore, as the aerial parts have been less explored than roots and are produced with aeroponic cultivation, this study could direct future valorization studies to transform this by-product into a co-product. The phytochemical profile was established using a molecular network that associates, in the same cluster, molecules presenting the same fragmentation pathway and that should belong to the same molecular family. This mapping associated with a database is very helpful for compound identification and extract comparison.

Lastly, to better assess the impact of artificial lighting (LEDs), which proved to be the most promising mode of forskolin production, on both forskolin and the other compounds, the roots and aerial extracts obtained with these conditions were further investigated using UHPLC-HRMS analysis with untargeted metabolomic analysis and were compared to the control modality. Statistical data treatments of the UHPLC-HRMS analysis of each part of the plant of the two modalities were performed to highlight potential differences between the two phytochemical profiles and identify compounds potentially affected by the growing conditions.

2. Results and Discussion

The main objectives of this study were to implement aeroponic culture conditions facilitating root harvesting, to develop a local and efficient sourcing of *C. forskohlii* and its main metabolite of interest, namely, forskolin, which is present mainly in the roots. To better qualify and valorize the cultivated plant and highlight the impact of the use of abiotic stresses during cultivation on the production of plant metabolites, the phytochemistry of the root and aerial parts was investigated.

2.1. Forskolin Quantification

The forskolin content in roots from the four cultivation modalities was quantified and compared. The ethanolic extract of *C. forskohlii* was obtained by ultrasonic extraction and then dried to be solubilized at a known concentration of 5 mg/mL. A calibration curve (Figure S4) of forskolin was prepared between 25 and 400 µg/mL. The samples and a calibration curve were analyzed using HPLC-ELSD, and the forskolin content was calculated. Figure 1 presents the forskolin content per gram of *C. forskohlii* roots according to the time (4 and 18 weeks) and cultivation modality (A: control; B: LED; C: biostimulant; D: hydric stress). After 4 weeks of cultivation, the control showed the best result in terms of forskolin content with 1.50 ± 0.04 mg/g of dry roots, while the LED, biostimulant, and hydric stress did not have any positive impact on the concentration of forskolin. The amount of forskolin in cultivated *C. forskohlii* is

comparable to that obtained in plants collected in natural habitats. For instance, the forskolin content of plants obtained from different locations in the Vidarbha region (Maharashtra, India) showed between 0.66 and 10.24 mg/g of forskolin in the dried plant²⁵. After a few weeks of cultivation, the content of forskolin decreased in the roots, especially for the control modality, with a decrease of 49% in forskolin production. A seasonal impact on the content in labdane diterpenes was also recorded for *C. forskohlii* growth in an experimental field²⁶. After 18 weeks, group A reached the same level as group D, which, after 4 weeks, was the one with the lowest percentage of forskolin. The use of a biostimulant (group C) slightly limited the drop in forskolin content (minus 32% after 18 weeks) and led to a higher content than group D after 18 weeks. The itinerary using LEDs was the most promising one, as forskolin production dropped by only 12%. The forskolin content of 1.03 ± 0.03 mg/g of dry roots after 18 weeks of cultivation under lighting compared to 0.78 ± 0.08 mg/g without suggests that the drop in forskolin is linked to the photoperiod.

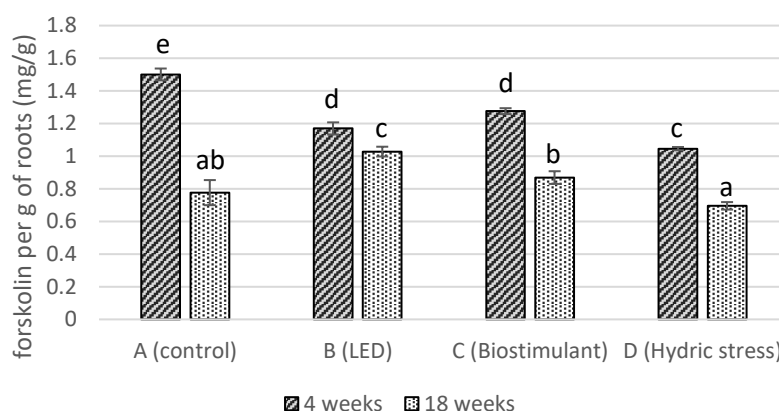


Figure 35: Comparison of the forskolin content per gram of *C. forskohlii* roots according to the time of cultivation and the modality ($n = 3$). Different letters indicate that means are significantly different at $p < 0.05$.

After 18 weeks of cultivation, the total root biomass was collected, weighed, and the total quantity of forskolin produced was calculated. Table 7 compares the quantity of root biomass harvested and the total amount of forskolin recovered relative to the biomass produced. Neither hydric stress nor the biostimulant improved the quantity of biomass and forskolin compared to the control, while LEDs helped to multiply the biomass production by a factor of two, with 296.0 g of dry biomass obtained for itinerary A (control) and 690.8 g obtained for itinerary B (LEDs). By combining the amount of forskolin per gram of biomass (Figure 35) with the quantity of biomass produced, the itinerary using LEDs showed a significantly higher forskolin production with 710.1 mg of forskolin recovered, while the control condition led to the production of 229.9 mg, which triples the final quantity. Figure 36 shows plants of the two itineraries after 18 weeks of cultivation, demonstrating the better root development using LEDs (Figure 36b). The application of supplementary lighting with a photoperiod of 16 h

increased the biomass production after a few weeks of cultivation. The biomass collected at week 4 in itinerary B was smaller than the one collected in itinerary A. This could be related to the natural photoperiod, which was longer in July when harvesting occurred after 4 weeks, compared to October when harvesting occurred after 18 weeks. Therefore, adding LEDs extended the natural photoperiod with a higher impact in October than in July and thus contributed to the increase in root biomass. Light influences root productivity, as shown with *Panax ginseng*, a plant from the Araliaceae family, where the beneficial effect of white LED lighting in a hydroponic cultivation system was demonstrated²⁷.

Table 7: Total biomass of *C. forskohlii* roots and forskolin production according to the cultivation itinerary after 18 weeks of cultivation. Percent decrease or increase in the value compared to itinerary A (control). For quantity of forskolin produced, data are presented as mean SD ($n = 3$); different letters indicate that means are significantly different at $p < 0.05$.

Cultivation Itinerary	A (Control)	B (LEDs)	C (Biostimulant)	D (Hydric Stress)
Dried root biomass (g)	296.0	690.8	264.0	305.2
(Relative to the control)	-	(+133%)	(-11%)	(+3%)
Quantity of forskolin produced (mg)	229.9 ± 17.7 ^a	710.1 ± 21.3 ^b	229.4 ± 8.9 ^a	212.4 ± 4.9 ^a
(Relative to the control)	-	(+209%)	(±0%)	(-8%)



(a)



(b)

Figure 36: Aeroponic system at 18 weeks of cultivation with (a) itinerary A (control) and (b) itinerary B (LEDs).

Itinerary B, which used LEDs, increased the total quantity of forskolin recovered after 18 weeks of cultivation, but forskolin may not be the only metabolite impacted by the stress. Therefore, an untargeted metabolomic study of the phytochemical profile was undertaken not only on roots but also on the aerial parts. As they are directly subjected to the stress, their composition can also be impacted. Furthermore, most previous studies focused on forskolin and therefore investigated only roots, but an investigation of both the roots and aerial parts could be valuable for the overall valorization of the plant.

2.2. Metabolomic Investigation

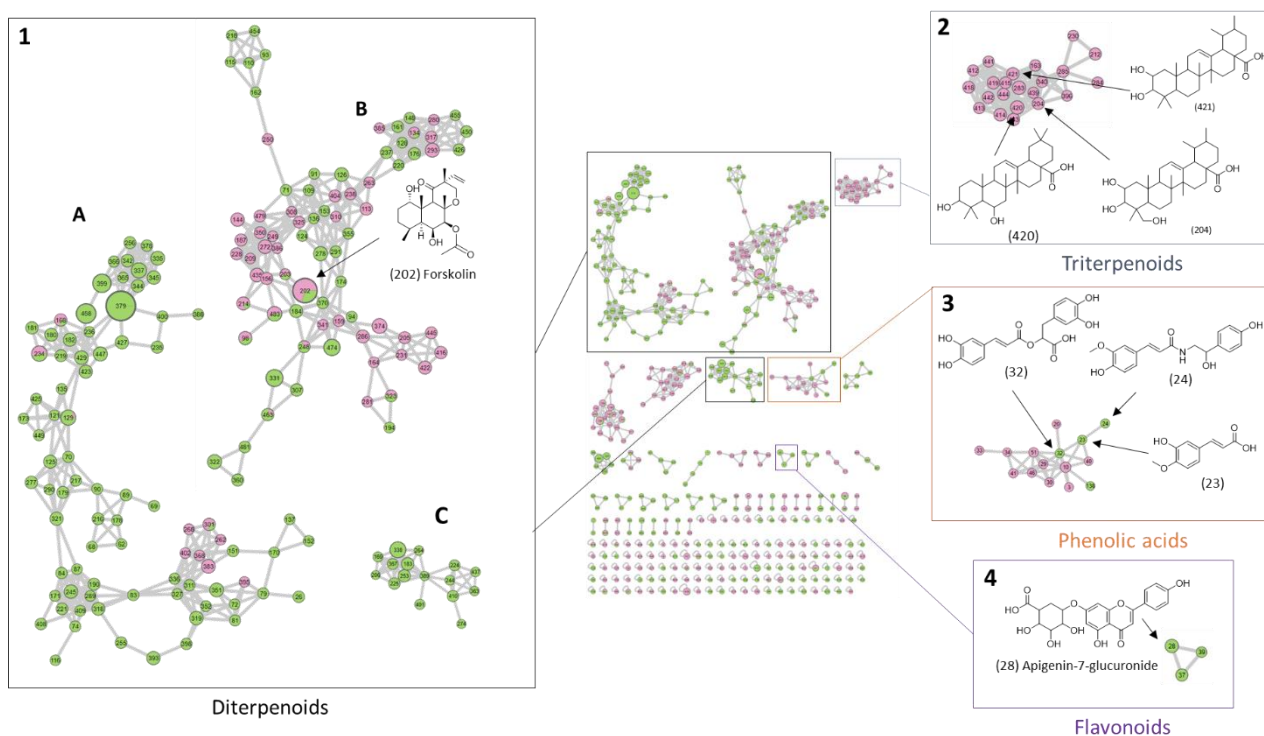
a. Comparison of the Phytochemical Composition of Root and Aerial Parts

The aeroponic cultivation developed in this study under the control condition and LED lighting produced *Coleus forskohlii* with a forskolin content similar to that produced by *C. forskohlii* cultivated in the field in native countries.

As commercial products made from *C. forskohlii* are often root powders or extracts that contain many bioactive molecules and not only forskolin, it is important to determine the global phytochemical composition of the *C. forskohlii* produced in Région Centre-Val de Loire (France) using aeroponic cultivation. As *C. forskohlii* grew in soilless conditions without soil microorganisms and under different climate conditions, it is possible that the metabolomic composition was modified.

Molecular networking is based on the principle that molecules with similar structures tend to have similar mass fragmentation patterns. These patterns can be represented as a graph where nodes represent ions and where edges are drawn between nodes if the similarity score between them exceeds a predefined threshold. Associated nodes define a cluster that should represent molecules belonging to the same molecular family. In molecular networking, several analyses can be joined and therefore compared to help in characterizing the phytochemical profile. The roots of *Coleus forskohlii* have been mainly studied due to their high concentration of forskolin^{28–30}. As the aerial parts have been less studied, building a molecular network using both samples may make it possible to associate molecules of both samples in the same cluster, thus helping in the identification of the aerial parts. Moreover, comparing the composition of the root and aerial parts can reveal common molecules or compound families, facilitating the annotation; it can also highlight the phytochemical specificity of each part of the plant. Figure 37 shows the molecular network obtained on GNPS (global natural products social molecular networking) from the UHPLC-HRMS/MS data in positive ionization mode for QC_R (Quality Control Roots: a mix of root samples from itineraries A and B) and QC_AP (Quality Control Aerial parts: a mix of aerial part samples from itineraries A and B) to be able to detect the main compounds produced in the two conditions (BPC in supplementary data, Figure S5 and S6). Each color corresponds to a part of the plant, with green representing the aerial parts and pink representing the roots. Nodes are divided into two colors when the ion is present in both

samples. The size of the pie chart corresponds to the ion proportion in each sample, while the size of the node represents the sum of the ion intensities in the two samples. In the center of Figure 37, a global overview of the network is shown, with 490 nodes, including the singletons. Three clusters (square 1 on the left part of Figure 37) are dedicated to diterpenoids, with one cluster (1B) including as many molecules from roots as molecules from aerial parts. This cluster includes forskolin (feature 202), which is also present in the aerial parts but in a smaller amount. Although this cluster contains ions from both roots and aerial parts, most of the compounds are found either in the roots or in the aerial parts specifically. This, along with the fact that the other two clusters (1A and 1C) of diterpenes mainly consist of compounds extracted from the aerial parts, indicates that the aerial parts are a source of a wider diversity



of diterpenes, with different skeletons.

Figure 37: Molecular network of QC_R and QC_AP obtained from UHPLC-HRMS/MS data in positive ionization mode using GNPS with visualization performed using Cytoscape 3.10.2. The node size is proportional to the intensity of the ion. The color inside the nodes is a pie chart, with green for ions from aerial parts and pink for ions from roots. The structure of the annotated features characteristic of the compound family was added. Four main groups corresponding to four compound families were identified (1-4), group 1 contains three clusters named 1A, 1B and 1C.

Additionally, a cluster dedicated to triterpenoids was also highlighted for the roots (square 2 on the right of Figure 37). A few phenolic compounds (square 3 on the right of Figure 37), including rosmarinic acid (feature 32), were identified, along with a small cluster of flavonoids containing apigenin-glucuronide (feature 28) (square 4 on the right of Figure 37) in the aerial parts. Phenolic compounds contribute to the antioxidant properties of the plant. Rosmarinic

acid and scutellarein 4'-methyl ether 7-O-glucuronide, both identified in the aerial parts of *P. barbatus*, have been shown to be correlated with the antioxidant activity of a water extract¹⁶. The overall composition of the root and aerial parts of plants grown in an aeroponic system is close to that described in plants grown in more conventional systems, with diterpenoids representing a large part of the composition of *C. forskohlii* and the presence of a few phenolic compounds. Based on this molecular network, a deeper exploration of the phytochemistry and annotation of the top 25 compounds impacted by the use of LEDs during cultivation (Section 2.2.2) was performed.

b. Identification of Compounds Impacted by LEDs

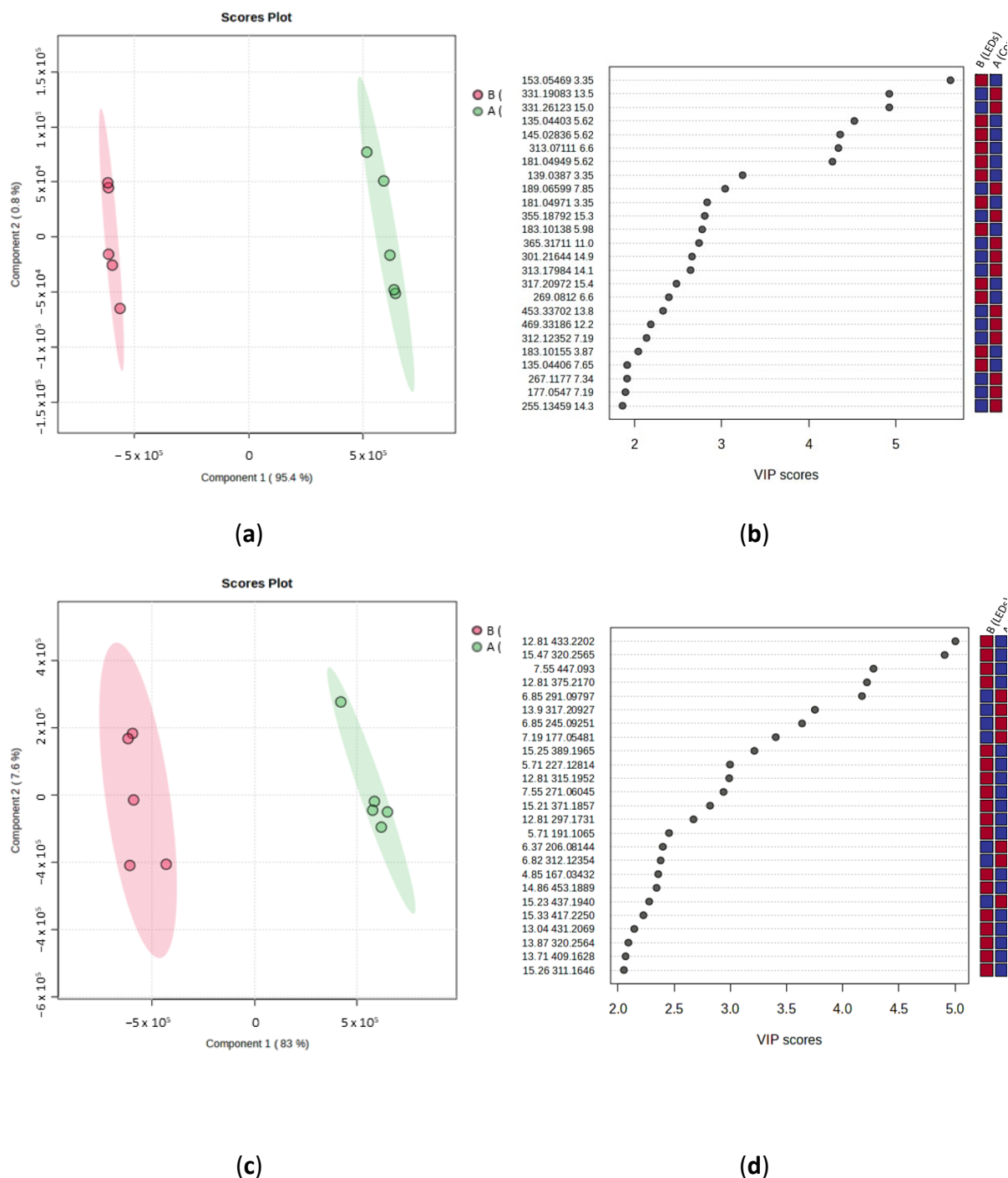
In order to identify which metabolites were the most impacted by the addition of LEDs during cultivation, supervised multivariate analyses were employed. A PLS-DA (Partial Least Squares Discriminant Analysis) was performed using the peak list extracted from MetaboScape, and the top 25 Variables Important in Projection (VIP score > 1) were investigated.

Figure 38a presents the PLS-DA model based on the UHPLC-HRMS analyses in positive ionization mode of the root samples for control and LED conditions after 18 weeks of cultivation. Component 1 describes 95.4% of the variance, while component 2 represents 0.8%. The model achieved a quality prediction of $R^2 = 0.99$ and a predictive ability of $Q^2 = 0.99$. As shown in Figure 38a, two distinct groups can be easily distinguished in component 1, with itinerary A (control) on the right and itinerary B (LEDs) on the left. The samples of each group are separated along component 2 that describes only 0.8% of the variance, indicating the good similarity of samples belonging to the same modality. Figure 38b shows the top 25 VIPs associated with group separation on component 1, with 12 molecules being more intense in the LED cultivation itinerary and 13 more intense in the control condition.

In order to identify the VIPs, the molecular formula was first determined, and MS/MS data were employed to obtain fragmentation information.

Tables 8 and 9 summarize the compound information for the roots and aerial parts, respectively, including chromatographic information, the itinerary in which each compound is more present, the calculated molecular formula, and a putative annotation when possible. To increase the confidence level of the proposed identification, information from HRMS/MS was included; molecular formulae were searched in the LOTUS database from both the *Plectranthus* and *Coleus* genera, and HRMS/MS data were computed using SIRIUS 5.8.

For root samples in Table 2, VIPs 1, 8, and 10 are all in-source fragments corresponding to compound **R1** with a t_R of 3.35 min and $[M+H]^+$ at m/z 199.0598, suggesting a molecular formula of $C_9H_{10}O_5$. The SIRIUS putative annotation suggested vanillylmandelic acid, a phenolic compound found in Lamiaceae³¹. The fragmentation spectrum shows ions at m/z



153.0543 and m/z 135.0434, corresponding to the losses of a carboxylic acidic function (46 u) and of a water molecule (18 u). VIP 2 corresponding to compound **R12** at 13.5 min with a

Figure 38: Supervised multivariate analysis based on untargeted UHPLC-HRMS analyses in positive ionization mode. (a) PLS-DA of itinerary A and B for root extracts and (b) the top 25 associated VIP scores; (c) PLS-DA of itinerary A and B for aerial part extracts and (d) the top 25 associated VIP scores.

$[M+H]^+$ ion at m/z 331.1908 suggests the molecular formula $C_{20}H_{26}O_4$. An abietane diterpenoid derivative, the 14-hydroxytaxodione corresponding to this molecular formula was identified in *Plectranthus grandidentatus* ³².

VIP 3, which is a $[M+Na]^+$ adduct, corresponds to compound **R17** at m/z 331.2612 with a t_R of 15.00 min and indicates a molecular formula of $C_{20}H_{36}O_2$, consistent with a fatty acid derivative. In the molecular network, the corresponding node is a singleton, which does not give any further information on the structure of compound **R17**.

VIPs 4, 5, and 7 are all associated with compound **R3** at a t_R of 5.62 min with a $[M+H]^+$ at m/z 181.0494, which corresponds to the molecular formula $C_9H_8O_4$. The fragmentation spectrum shows ions at m/z 163.0393 and 145.0282, suggesting the successive loss of H_2O (18 u) due to hydroxyl functions, and m/z 135.0438 corresponding to the loss of a carboxylic acid function with another loss of the hydroxyl group with a fragment at m/z 117.0333. The molecular formula and fragmentation pattern suggest that this compound corresponds to caffeic acid, with a SIRIUS score of 100.00%. Caffeic acid is a phenolic compound commonly found in plants, including in the genus *Coleus* ³³. The node corresponding to **R3** is located in cluster 3 (Figure 3) dedicated to phenolic compounds.

VIPs 6 and 17 correspond to compound **R5** at a t_R of 6.60 min with the $[M+H]^+$ at m/z 313.0711, indicating a molecular formula of $C_{17}H_{12}O_6$. No compound in the genus *Plectranthus* or *Coleus* corresponds to this molecular formula, but SIRUS suggested that the compound could be 4-(3,4-Dihydroxyphenyl)-6,7-dihydroxynaphthalene-2-carboxylic acid with a score of 90.26%.

VIP 9 corresponds to **R9** with m/z 189.0659 and a t_R of 7.85 min. No information from the mass spectrum enabled the molecular ion to be identified; therefore, no molecular formula or annotation can be suggested.

VIP 11 corresponds to compound **R18** with m/z 355.1879 and a t_R of 15.30 min. m/z 355.1879 had not been fragmented in MS/MS, which does not allow an annotation. Furthermore, no discriminating adduct that would have enabled the molecular formula to be calculated was found.

VIP 12 corresponds to **R4** at 5.98 min with a $[M+H]^+$ at m/z 183.1015 and $[M+Na]^+$ at m/z 205.0835, suggesting a molecular formula of $C_{10}H_{14}O_3$. The molecular formula corresponds to a small phenolic compound, which is consistent with the retention time, but there is no further information available to suggest an annotation.

VIP 13 corresponds to **R10** with a $[M+H]^+$ m/z 365.3171 and a t_R of 11.00 min. The calculated molecular formula is $C_{22}H_{40}N_2O_2$, a nitrogen compound.

VIP 14 corresponds to compound **R16** at m/z 301.2164 and at t_R of 14.90 min, and the corresponding adduct $[M+Na]^+$ was identified with m/z 323.1981, which indicates a molecular

formula of $C_{20}H_{28}O_2$. In the literature, the two diterpenoids sugiol and barbatusol, corresponding to this molecular formula, were found in the genus *Plectranthus*^{34,35}.

VIP 15 corresponds to **R14** at a t_R of 14.10 min with a $[M+H]^+$ at m/z 313.1798, suggesting the molecular formula to be $C_{20}H_{24}O_3$. The corresponding node is located in a cluster of four nodes mainly from roots, suggesting a skeleton specific to this part of the plant. **R14** has a fragmentation pattern similar to that of diterpenoids and with a retention time consistent with other diterpenoid derivatives.

VIP 16 corresponds to compound **R19** with m/z 317.2097 for $[M+Na]^+$ and a t_R of 15.40 min, which suggests a molecular formula of $C_{18}H_{30}O_3$. According to the retention time and the fragmentation pattern, **R19** may correspond to a fatty acid.

VIP 18 corresponds to compound **R13** with a t_R of 13.80 min and m/z 453.3370. The $[M+H]^+$ and $[M+NH_4]^+$ at 489.3573 and 506.3840 suggested that 453.3370 m/z was $[M - 2H_2O + H]^+$. The molecular formula is $C_{30}H_{48}O_5$, which corresponds to a triterpenoid; moreover, it is located in cluster 2, which is dedicated to triterpenoids. **R13** is annotated as tormentic acid previously identified in the genus *Coleus*³⁶ and in accordance with SIRIUS with a score of 87.45%.

VIPs 20 and 24 correspond to compound **R6** at a t_R of 7.19 min with the $[M - H_2O + H]^+$ at m/z 312.1235 and an in-source fragment at m/z 177.0547. The molecular formula of **R6** is $C_{18}H_{19}NO_5$. SIRIUS suggested the compound to be N-feruloyloctopamine. Ion m/z 177.0540 corresponds to the ferulic derivative part of the molecule, while ion m/z 145.0279 is due to the loss of the methoxy group. Even with the nitrogen part, the node corresponding to **R6** node is located in cluster 3 dedicated to phenolic acids (ferulic derivative).

VIP 21 corresponds to **R2** at a t_R of 3.87 min with $[M+H]^+$ at m/z 183.1015. The fragmentation pattern is similar to that of **R4** with the same molecular formula of $C_{10}H_{14}O_3$.

VIP 22 corresponds to compound **R8** at a t_R of 7.65 min with a fragment at m/z 135.0440 and a parent ion $[M+H]^+$ at m/z 361.0922 and its corresponding adduct $[M+NH_4]^+$ at m/z 378.1188, which corresponds to a molecular formula of $C_{18}H_{16}O_8$. This molecular formula corresponds to rosmarinic acid, with a node located in cluster 3 (phenolic acids), which is in accordance with the SIRIUS annotation with a score of 99.21%. The molecule has already been found many times in the Lamiaceae family including in the genera *Plectranthus*³⁷ and *Coleus*^{16,38,39}.

VIP 23 corresponds to **R7** with m/z 267.1177 and a t_R of 7.34 min. As no information from the mass spectrum made it possible to identify the molecular ion, no molecular formula or annotation can be suggested.

VIP 25 corresponds to **R15** at 14.30 min, and m/z 255.1353 corresponds to a $[M+Na]^+$ adduct with the corresponding $[M+H]^+$ at m/z 233.1533, suggesting a molecular formula of $C_{15}H_{20}O_2$. The retention time and molecular formula are consistent with a terpenoid derivative, but as

the $[M+H]^+$ was not fragmented, no further information was available to suggest an annotation.

Compounds with a retention time in the first part of the chromatogram seem to be more positively impacted by the use of LEDs and vice versa in the second half. LEDs seem to improve the intensity of phenolic compounds such as vanillylmandelic acid (**R1**), caffeic acid (**R3**), or rosmarinic acid (**R8**) in the roots of *C. forskohlii*, whereas they seem to decrease the amount of the most non-polar compounds such as diterpenoids and triterpenoids. As for *Ocimum basilicum*, from the same family as *C. forskohlii*, the artificial LED lighting seems to enhance the plant's phenolic content, as phenolic compounds have UV radiation protection properties⁴⁰. Forskolin, previously quantified in the roots (Section 2.1) and positively influenced by LED lighting, does not appear here in the top 25 VIPs.

Table 8: Identification of the top 25 VIP (VIP score > 1) metabolites discriminating root samples cultivated in control and LED conditions (ordered by t_R).

Compound	t_R	VIP Position	m/z	Adduct or Fragment	Itinerary	Calculated Molecular Formula [M]	Putative Annotation	Molecular Family
R1	3.35	1	153.0546					
		8	139.0387	Fragments	B	$C_9H_{10}O_5$	Vanillylmandelic acid	Phenolic acid
		10	181.0497					
R2	3.87	21	183.1015	$[M+H]^+$	B	$C_{10}H_{14}O_3$	-	-
R3	5.62	7	181.0494	$[M+H]^+$				
		5	145.0283	Fragments	B	$C_9H_8O_4$	Caffeic acid	Phenolic acids
		4	135.0440					
R4	5.98	12	183.1014	$[M+H]^+$	B	$C_{10}H_{14}O_3$	-	-
R5	6.60	6	313.0711	$[M+H]^+$			4-(3,4-Dihydroxyphenyl)-6,7-	
		17	269.0812	Fragment	B	$C_{17}H_{12}O_6$	dihydroxynaphthalene-2-carboxylic	Phenylpropanoids
R6	7.19	20	312.1235	$[M-H_2O+H]$				
		24	177.0547	Fragment	A	$C_{18}H_{19}NO_5$	N-Feruloyloctopamine	Cinnamic acid amides
R7	7.34	23	267.1177	-	A	-	-	-
R8	7.65	22	135.0440	Fragment	B	$C_{18}H_{16}O_8$	Rosmarinic acid	Phenolic acids
R9	7.85	9	189.0659	-	A	-	-	-
R10	11.00	13	365.3171	$[M+H]^+$	A	$C_{22}H_{40}N_2O_2$	-	-
R11	12.20	19	469.3319	$[M+H]^+$	A	-	-	-
R12	13.50	2	331.1908	$[M+H]^+$	A	$C_{20}H_{26}O_4$	14-Hydroxytaxodione (abietane diterpene)	Diterpenes
R13	13.80	18	453.3370	$[M-2H_2O+H]^+$	A	$C_{30}H_{48}O_5$	Tormentic acid	Triterpenes

PARTIE II. CULTURE AEROPONIQUE POUR UNE MEILLEURE PRODUCTION DE FORSKOLINE ET VALORISATION DE
COLEUS FORSKOHLII

R14	14.10	15	313.1798	[M+H] ⁺	A	C ₂₀ H ₂₄ O ₃	Abietane diterpene derivative	Diterpenes
R15	14.30	25	255.1353	[M+Na] ⁺	A	C ₁₅ H ₂₀ O ₂	-	-
R16	14.90	14	301.2164	[M+H] ⁺	A	C ₂₀ H ₂₈ O ₂	<i>Sugiol / barbatusol</i>	Diterpenes
R17	15.00	3	331.2612	[M+Na] ⁺	A	C ₂₀ H ₃₆ O ₂	-	-
R18	15.30	11	355.1879	-	A	-	-	Fatty acids
R19	15.40	16	317.2097	[M+Na] ⁺	B	C ₁₈ H ₃₀ O ₃	Fatty acid derivative	Fatty acids

The same work was performed on the aerial parts of *C. forskohlii* to better understand the impact of LEDs on the whole plant as LEDs illuminate the aerial part. Figure 38a presents the PLS-DA model. Component 1 accounts for 83% of the variance and component 2 for 7.6%, with an R^2 of 0.97 and a predictive ability Q^2 of 0.98. In Figure 38c, two groups can be easily distinguished in component 1, with itinerary A (control) on the right and itinerary B (LEDs) on the left. Figure 4d presents the corresponding top 25 VIP scores, with 18 molecules being more intense for the cultivation itinerary using LEDs and 7 for the control itinerary.

VIPs 1, 4, 11, and 14 are related to **AP10** at a t_R of 12.81 min and correspond to $[M+Na]^+$ at m/z 433.2202, $[M - H_2O + H]^+$ at m/z 375.2170, and two fragments, from the $[M+H]^+$ at m/z 411.2377, m/z 315.1952 and m/z 297.1731. With a molecular formula of $C_{22}H_{34}O_7$, **AP10** can be identified as forskolin.

VIP 2 with m/z 320.2564 and a t_R of 15.47 min, corresponds to compound **AP20**. The calculated molecular formula is $C_{18}H_{35}NO_2$. The fragmentation spectrum of the $[M+H]^+$ at m/z 298.2753 of **AP20** indicates the loss of a molecule of water and the loss of the primary amide function with an ion fragment at m/z 245.2266 corresponding to fatty amide derivatives.

VIPs 3 and 12 correspond to **AP7** at a t_R of 7.55 min with a $[M+H]^+$ at m/z 447.0930 and a molecular formula of $C_{21}H_{18}O_{11}$. The ion at m/z 271.0604 corresponds to aglycone after the loss of the glucuronide moiety (176 u). **AP7** can be annotated as apigenin-glucuronide with a SIRIUS score at 96.74%, which is supported by the GNPS data library. The **AP7** node is located in cluster 4 with only three nodes, meaning that only two other similar flavonoid compounds present the same fragmentation pattern in the aerial parts of *C. forskohlii*.

VIPs 5 and 7 correspond to compound **AP5** at a t_R of 6.85 min with a $[M+H]^+$ at m/z 291.0979, which suggests that the molecular formula of **AP5** is $C_{14}H_{13}N_2O_4$. Fragment m/z 245.0921 corresponds to the loss of a carboxylic acid function (46 u), and fragment m/z 188.0702 corresponds to the loss of the N-malonyl part of the molecule, suggesting that compound **AP5** is N-malonyl-D-tryptophan with a SIRIUS score of 94.46%.

VIP 6 corresponds to **AP14** with a $[M+H]^+$ at m/z 317.2092 and a molecular formula of $C_{20}H_{28}O_3$ associated with a diterpenoid. Several abietane diterpenoids correspond to this formula, such as 11,20-dihydroxysugiol identified in the aerial parts of *Plectranthus cyaneus*, royleanone identified in the whole plant of *Plectranthus grandidentatus*, and 20-Deoxocarnosol isolated from *Coleus barbatus* ^{41–43}.

VIP 8 corresponds to compound **AP6** with a $[M+H]^+$ at m/z 330.1339 and a t_R at 7.19 min. Compound **AP6** shows the same fragmentation pattern as **R6**, and both are located in cluster 3, with an ion source common fragment at m/z 177.0548. The molecular formula is $C_{18}H_{19}NO_5$, and **AP6** is annotated as N-feruloyloctopamine, a hydroxycinnamic amide.

VIPs 9 and 25 correspond to **AP18** with $[M+H]^+$ at m/z 389.1965 and a fragment at m/z 311.1646 at a t_R of 15.25 min, which suggests a molecular formula of $C_{22}H_{28}O_6$. The fragment at m/z 311.1646 indicates the loss of an acetate group together with the loss of a hydroxyl group (78 u). Seven different structures corresponding to abietane diterpenoids, especially abeoabietane diterpenoids or cycloabietane diterpenoids, were found in the genus *Plectranthus* using LOTUS^{44–48}. The location of the corresponding node in cluster 1.A also supports this annotation.

VIPs 10 and 15, which correspond to compound **AP2** at a t_R of 5.71 min, are two fragments at m/z 227.1281 and 191.1065 from the $[M+NH_4]^+$ adduct at m/z 406.2071. The molecular formula of compound **AP2** is $C_{18}H_{28}O_9$. The fragment at m/z 227.1281 corresponds to the loss of a hexose moiety (162 u) with a molecular formula of $C_{12}H_{18}O_4$. The aglycone is annotated as a hydroxyjasmonic acid, which implies that **AP2** is a hydroxyjasmonic hexose, a compound already described in the Lamiaceae family⁴⁹.

VIP 13 corresponding to **AP16** with a $[M+H]^+$ at m/z 371.1857 and a t_R of 15.21 min has a molecular formula of $C_{22}H_{26}O_5$. The node from the molecular network is located in cluster 1.A, which suggests that the compound should be annotated as a diterpenoid.

VIP 16, corresponding to compound **AP3**, is an ion source fragment at m/z 206.0814, itself originating from the $[M+H]^+$ at m/z 252.0866 with a t_R of 6.37 min. The molecular formula of **AP3** is $C_{12}H_{13}NO_5$. The node corresponding to **AP3** is located in the same cluster as **AP5**, suggesting a structural similarity between them. SIRIUS annotated **AP3** with a score of 86.64% as a dipeptide as 2-[(2-carboxyacetyl)amino]-3-phenylpropanoic.

VIP 17 corresponds to compound **AP4** with a t_R of 6.82 min and m/z 312.1235; it is a nitrogen compound with the molecular formula $C_{18}H_{17}NO_4$. The fragment m/z 177.0547 corresponds to the ferulic derivative part of the molecule as described previously for **R6** and **AP6**. SIRIUS suggested that compound **AP4** is a hydroxycinnamic acid amide.

VIP 18, which corresponds to compound **AP1** at a t_R of 4.85 min, is an in-source fragment ion from $[M+H]^+$ at m/z 227.0551 with a molecular formula of $C_{10}H_{10}O_6$. **AP1** corresponds to a phenolic acid with a putative annotation from SIRIUS at 64.38% of 4,6-dimethoxyisophthalic acid.

VIP 19, corresponding to compound **AP15**, is a $[M+Na]^+$ adduct with m/z 453.1889 and a t_R of 14.86 min. The calculated formula of $C_{24}H_{30}O_7$ corresponds to a diterpenoid. A cycloabietane, (2R,2'S,3'R,4'bS,7'R,8'aS,9'S,10'S)-3',10'-Diacetoxy-4'b,5',6',7',8',8'a,9',10'-octahydro-9'-hydroxy-2,4'b,7'-trimethyl-8'-methylidenspiro[cyclopropan-1,2'(1'H)-phenanthren]-1',4'(3'H)-dion, corresponding to this molecular formula was previously identified⁴⁶.

VIP 20, corresponding to **AP17** at m/z 437.1940, is a $[M+Na]^+$ adduct of the $[M+H]^+$ at m/z 415.2117 with a t_R of 15.23 min. The fragmentation pattern of the $[M+H]^+$ corresponds to a

diterpenoid, with a node located in cluster 1.A. The molecular formula of $C_{24}H_{30}O_6$ corresponds to an acetylated derivative of a 6,7-secoabietane diterpene previously identified in the genus *Coleus* ⁵⁰.

VIP 21 corresponds to the $[M+Na]^+$ adduct of the compound **AP19** with a t_R of 15.33 min and m/z 417.2250. The calculated molecular formula of $C_{22}H_{34}O_6$ is associated with different labdane diterpenes previously identified in *Plectranthus* and *Coleus*, including a derivative from forskolin, 9-deoxyforskolin ¹³, a labdane diterpenoid, which is consistent with the molecular network as the node of **AP19** is found in cluster 1.B (Figure 3) where forskolin is located.

VIP 22 corresponds to **AP11** with a $[M+H]^+$ at m/z 431.2069 corresponding to the molecular formula $C_{24}H_{30}O_7$, identical to that of **AP15**, and a t_R of 13.04 min. The node is located in cluster 1.A associated with different diterpenes. As mentioned for **AP15**, a cycloabietane corresponding to this molecular formula was previously identified ⁴⁶.

VIP 23 at a t_R of 13.87 min is a $[M+Na]^+$ adduct with m/z 320.2564 corresponding to **AP13**. The calculated molecular formula is $C_{18}H_{35}NO_2$. As for compound **AP20**, the fragmentation spectrum of the $[M+H]^+$ at m/z 298.2753 indicates the loss of a hydroxyl function and of a primary amide function with an ion fragment at m/z 245.2266, which corresponds to a fatty amide derivative.

VIP 24 corresponds to the $[M+Na]^+$ adduct of compound **AP12** with a $[M+H]^+$ at m/z 387.1810 and a molecular formula of $C_{22}H_{26}O_6$. The fragmentation of the $[M+H]^+$ corresponds to a cycloabietane diterpenoid, coleon Z, as previously reported in *Coleus* spp. ⁵¹.

Itinerary A, with no LED lighting, contains more dipeptide and nitrogen compounds such as cinnamic acid amides, while itinerary B increases the content of almost all listed diterpenes, including forskolin (Compounds **AP10**, **AP11**, **AP12**, **AP16**, **AP17**, **AP18**, and **AP19**), which indicates that LEDs seem to stimulate the biosynthesis of diterpenoids in the aerial parts.

Table 9: Identification of top 25 VIP (VIP score > 1) metabolites discriminating aerial part samples cultivated following control and LED conditions (ordered by t_R).

Compound	t _R (min)	VIP Position	m/z	Adduct or Fragment	Itinerary	Calculated Molecular Formula [M]	Putative Annotation	Molecular Family
AP1	4.85	18	167.0343	Fragment	B	C ₁₀ H ₁₀ O ₆	4,6-Dimethoxyisophtalic acid	Phenolic acid
AP2	5.71	10	227.1281	Fragments	B	C ₁₈ H ₂₈ O ₉	Hydroxyjasmonic acid hexose	Fatty acyl glycoside
		15	191.1065					
AP3	6.37	16	206.0814	Fragment	A	C ₁₂ H ₁₃ NO ₅	2-[(2-Carboxyacetyl)amino]-3-phenylpropanoic	Dipeptides
AP4	6.82	17	312.1235	[M+H] ⁺	A	C ₁₈ H ₁₇ NO ₄	Hydroxycinnamic acid amide derivative	Cinnamic acid amides
AP5	6.85	5	291.0979	[M+H] ⁺	A	C ₁₄ H ₁₃ N ₂ O ₄	N-Malonyl-D-tryptophan	Dipeptides
		7	245.0925	Fragment				
AP6	7.19	8	177.0548	Fragment	A	C ₁₈ H ₁₉ NO ₅	N-Feruloyloctopamine	Cinnamic acid amides
AP7	7.55	3	447.093	[M+H] ⁺	B	C ₂₁ H ₁₈ O ₁₁	Apigenin-glucuronide	Flavonoids
		12	271.0604	Fragment				
AP10	12.81	1	433.2202	[M+Na] ⁺	B	C ₂₂ H ₃₄ O ₇	Forskolin	Diterpenes
		4	375.2170	[M−H ₂ O+H] ⁺				
		11	315.1952	Fragments				
		14	297.1731					
AP11	13.04	22	431.2069	[M+H] ⁺	B	C ₂₄ H ₃₀ O ₇	Cycloabietane derivative	Diterpenes
AP12	13.71	24	409.1628	[M+Na] ⁺	B	C ₂₂ H ₂₆ O ₆	Coleon Z	Diterpenes
AP13	13.87	23	320.2564	[M+Na] ⁺	B	C ₁₈ H ₃₅ NO ₂	N-Acyl amines derivatives	Fatty amides

AP14	13.9	6	317.2092	[M+H] ⁺	A	C ₂₀ H ₂₈ O ₃	11,20-Dihydroxysugiol/ royleanone	Diterpenes
AP15	14.86	19	453.1889	[M+Na] ⁺	B	C ₂₄ H ₃₀ O ₇	Cycloabietane derivative	Diterpenes
AP16	15.21	13	371.1857	[M+H] ⁺	B	C ₂₂ H ₂₆ O ₅	Abetane diterpene derivative	Diterpenes
AP17	15.23	20	437.1940	[M+Na] ⁺	A	C ₂₄ H ₃₀ O ₆	6,7-Secoabietane diterpene derivative	Diterpenes
AP18	15.25	9	389.1965	[M+H] ⁺	B	C ₂₂ H ₂₈ O ₆	Abetane diterpene derivative	Diterpenes
	15.26	25	311.1646	Fragment				
AP19	15.33	21	417.2250	[M+Na] ⁺	B	C ₂₂ H ₃₄ O ₆	9-Deoxyforskolin	Diterpenes
AP20	15.47	2	320.2565	[M+Na] ⁺	B	C ₁₈ H ₃₅ NO ₂	N-Acyl amines derivatives	Fatty amides

To summarize the results obtained by comparing the control and LED modalities on metabolite amount, phenolic compounds appear to be concentrated in the root and aerial parts when using LEDs. This may be explained by the fact that they are generally UV protectors, and a higher light stress could improve their production in order to defend the plant. We also note that nitrogen compounds are more intense in the itineraries without LED stimulation. A possible explanation is that due to a higher biomass production with LED stimulation, amino acids are totally consumed for the production of primary metabolites and are hence less available for the production of other nitrogenous derivatives. Concerning diterpenes, one of the main molecular families of *C. forskohlii*, their concentration is higher in the leaves under LED stimulation, with, in particular, an increase in the forskolin content. The effect of LEDs on the roots is less clear, however. Some of the diterpenes are more concentrated in the roots from itinerary B, while others are more concentrated in the roots from itinerary A. Furthermore, some families of compounds, such as triterpenoids, previously identified in the molecular network, appear to be unaffected.

3. Materials and Methods

3.1. Plant Cultivation

a. Aeroponic System

The aeroponic cultivation of *C. forskohlii* presents several advantages such as a low consumption of water and easy access to the roots during plant growth. The aeroponic system used at the CDHRC consists of a 3 m long, 1.50 m wide cultivation tank. Polystyrene plates are placed on the tray to support the plants.

Cuttings are taken from mother plants grown at the CDHR in plastic pots under greenhouse conditions. Cuttings 5 to 8 cm high are made by cutting the base of the cutting above a node, starting from the stem apex of the aerial parts. Depending on plant development, between 1 and 3 cuttings can be made per stem. Care should be taken to ensure that leaves or leaflets are present at the top of the cutting. To prevent them from drying out, only 2 leaves are left on the cutting; the others are cut off with pruning shears. If the remaining leaves are too large, they are cut in half to prevent the cutting from dying.

The cuttings are then placed in the aeroponic system. Once the plants have roots of over 3 cm, they are placed in cultivation baskets filled with clay balls and then installed on the cultivation device. Each cultivation itinerary consists of 87 *C. forskohlii* plants, with a cultivation surface of 4.50 m² for each itinerary, giving a cultivation density of 19.3 plants/m². The system is housed in a glass greenhouse, which is kept frost-free, and a nutrient solution is diffused for 30 s every 5 min onto the roots using a pump system. A Coic-Lesaint mineral fertilization, based on a set conductivity of 1.5 mS/cm, is carried out. The system was set up on 7 June 2023 with a final collection on 17 October 2023.

b. Cultivation Itinerary

To improve biomass production or the production of compounds of interest, different itineraries were set up: (A) A control itinerary in which no abiotic stress was applied. (B) An itinerary integrating complementary lighting with a photoperiod of 16 h of daylight. The lighting is LED (83% red, 17% blue), with an intensity of 150 $\mu\text{mol}/\text{m}^2/\text{s}$. (C) An itinerary with weekly application of a foliar biostimulant (Laminaveg) at a dose of 2 L/ha. (D) An itinerary with daily application of water stress. The pump is turned off for a period determined by the wilting state of the plant.

c. Harvesting and Drying

During harvesting, the roots of each plant were cut at 10 cm from their base and then dried in a greenhouse. The roots were harvested several times, after 4 weeks of cultivation and at the end after 18 weeks. The aerial parts of the plants were harvested (after 18 weeks) at the end of the cultivation and dried in an oven at 50 °C.

3.2. Phytochemicals

a. Chemicals

The absolute ethanol (>99.7%), acetonitrile (HPLC gradient grade), and formic acid (99–100%) used for extraction and HPLC-ELSD were all purchased from VWR (Fontenay-sous-Bois, France) and were HPLC gradient grade. Ultrapure water was produced with a Milli-Q EQ7000 system equipped with an LC-Pak filter from Merck (Darmstadt, Germany). UHPLC-HRMS analyses were performed with water and LC-MS grade acetonitrile from Honeywell (Seelze, Germany). Forskolin (>99%) was purchased from Extrasynthese (Genay, France), and a stock solution at 1 mg/mL was prepared in methanol.

b. Sample Preparation

An amount of 250 mg of powder of dried roots or aerial parts of *C. forskohlii* was extracted using ultrasound-assisted extraction with 12 mL of solvent during 1 h. The roots were extracted using 100% EtOH, while the aerial parts were extracted using a mixture of EtOH/water (80/20). The extract was then centrifuged during 10 min at 10,700× *g*, and the supernatant was filtered. The final extract was dried under nitrogen flow to obtain the dry crude extract. The dry extract was then dissolved with the same solvent used for extraction at a concentration of 5 mg/mL before further analysis.

All extractions for the quantification of forskolin in the roots were performed in triplicate ($n = 3$), while the extractions for the MS experiments, itineraries A and B of both the roots and aerial parts, were performed 5 times each ($n = 5$). For HRMS/MS, quality control samples were prepared: QC_AP, which is a mix of the supernatants from all aerial parts (itineraries A and B mixed), and QC_R, which is a mix of the supernatants from all roots (itineraries A and B mixed).

3.3. HPLC-ELSD Quantification

HPLC was performed with a Thermo Scientific Ultimate 3000 RSLC system using a binary pump, an automatic sampler, a thermostated column compartment, and an ELSD Sedex 100LT detector (Sedere, Olivet, France) in dynamic gain. The column was a Luna C18(2) (150 × 4.6 mm; 3 µm) (Phenomenex, Le Pecq, France). Gradient mode was used to separate the compounds. The mobile phase consisted of water (A) and acetonitrile (B), both acidified with 0.1% formic acid. The flow rate was fixed at 1 mL/min. Elution started with 10% B during 1.0 min. The amount of B increased up to 74% from 1 to 17.5 min and to 100% from 17.5 min to 18.5 min. The amount of B was maintained at 100% from 18.5 to 24.0 min and then decreased during 1.0 min to the initial conditions until 32 min.

A calibration range of forskolin was prepared in ethanol. Successive dilutions were made to obtain the 5 concentration solutions in the range 25–400 µg/mL (Figure S4).

All extractions for the quantification of forskolin in the roots were performed in triplicate ($n = 3$), and the data are presented as the mean ± standard deviation (SD). The statistical analysis was performed using XLSTAT (2017, Lumivero, Denver, CO, USA); differences between the samples were analyzed using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference with $p \leq 0.05$.

3.4. UHPLC-HRMS and UHPLC-HRMS/MS Analyses

Chromatographic analyses were performed with a Thermo Scientific Ultimate 3000 RSLC system using a binary pump, an automatic sampler, a thermostated column compartment, and a DAD detector (200–800 nm) (Dionex, Germering, Germany).

The column was a Luna Omega C18 (150 × 2.1 mm; 1.6 µm) (Phenomenex, Le Pecq, France). Gradient mode was used to separate the compounds. The mobile phase consisted of water (A) and acetonitrile (B), both acidified with 0.1% formic acid. The flow rate was fixed at 0.5 mL/min. Elution started with 5% B during 0.3 min. The amount of B increased up to 85% from 0.5 to 15 min and to 100% from 15 min to 16 min. From 16 to 21 min, the amount of B was maintained and then decreased during 0.2 min to the initial conditions until 23 min.

The MS and MS/MS experiments were carried out on a maXis UHR-Q-TOF mass spectrometer (Bruker, Bremen, Germany) using the data-dependent acquisition mode (DDA) with an electrospray ion source (ESI) working in positive ionization mode. Nitrogen was used as the drying gas at a flow rate of 9 L/min heated at 200 °C and as the nebulizing gas at a pressure of 2 bar. The mass spectra were recorded in the m/z range 50–1650 at 1.6 Hz for MS and MS/MS. The capillary voltage was set at 4.5 kV. Two precursor ions were selected per cycle, fragmented at two collision energies (15–35 eV), and averaged into one MS/MS spectrum. All extraction replicates were analyzed using MS for statistical analysis, while the QC sample of

the aerial parts (QC_AP) and the QC sample of the roots (QC_R) were analyzed using MS/MS experiments for identification of metabolites (Figures S3 and S4).

3.5. Analyses of Treatments

a. HRMS/MS Treatment

Analyses were converted to mzXML format using CompassXport (Bruker, Bremen, Germany). The mass spectrometry data were first processed with MZmine 3.8⁵². Mass detections were fixed at 1.5×10^4 for the MS1 and 8.0×10^2 for the MS2 level. An ADAP chromatogram builder and resolver were employed, with a minimum of 4 consecutive scans at a threshold of 1.5×10^4 . Alignment was performed based on a m/z tolerance of 0.005 or 8 ppm and a retention time tolerance of 0.8 min. Isotope ^{13}C grouper, gapfilling, and filtering were then performed. A feature list comprising 490 features between 2 and 16 min was finally obtained. A molecular network was then generated using GNPS⁵³ with the Feature-Based Molecular Networking (FBMN) workflow⁵⁴. A molecular network was created in which the edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. The molecular network was then visualized using Cytoscape 3.10.2 software⁵⁵.

The molecular formulae were generated using elemental composition with a mass accuracy ≤ 5 ppm. The mzXML format of the analysis was computed using SIRIUS 5.8⁵⁶ in order to suggest an annotation. Manual interpretation together with the use of databases such as LOTUS⁵⁷ and GNPS⁵³ helped to perform the annotation.

b. Statistical Treatment of HRMS Data

All the MS data were processed using DataAnalysis 4.4 (Bruker, Bremen, Germany). For MS experiments, all extractions of root and aerial parts were performed 5 times ($n = 5$) for a total of 20 samples. MetaboScape 4.0 software (Bruker, Bremen, Germany) was used to build the feature table, a matrix containing the retention times, m/z , and intensity for each ion corresponding to the following parameters: intensity threshold was fixed at 10,000, mass range was fixed from m/z 50 to 1650, and time range between 2.5 and 16 min. The matrix generated contained 1760 variables for root analysis and 1702 variables for aerial part analysis. Then, the MetaboAnalyst 6.0 platform was employed for statistical analysis. A reduced matrix was obtained using MetaboAnalyst 6.0 by conserving only the significant variables (t -test); this treatment allowed the obtainment of a reduced matrix with 371 variables and 441 variables for the roots and aerial parts, respectively. Then, multivariate analysis was performed on the reduced matrix using PLS-DA, and VIP scores were obtained to determine the most discriminant features. The EICs (extracted ion chromatograms) of each of the Top 25 VIPs are reported (Tables S1 and S2) and were further investigated using HRMS/MS data (with itineraries A and B of each part of the plant mixed for HRMS/MS analysis) in order to identify them.

4. Conclusions

This study is the first time that the cultivation of *Coleus forskohlii* using an aeroponic system is reported, allowing a local sustainable production of forskolin. Furthermore, harvesting the roots is easier and does not harm the survival of the plant. The addition of LED lighting during the cultivation process, up to October towards the end of the cultivation period when the natural photoperiod decreases, stimulates the root biomass production of *C. forskohlii* and positively impacts the amount of forskolin recovered, resulting in a forskolin production that is three times higher than under control conditions (710.1 ± 21.3 mg vs. 229.9 ± 17.7 mg).

The global phytochemistry profile of both parts of the plant was evaluated using UHPLC-HRMS/MS data and molecular networking. The results highlighted a high diversity of diterpenoid compounds in both parts of the plant, with forskolin and its derivatives being common. In the aerial parts, a wider variety of diterpenoids was highlighted, represented by several dedicated clusters. Additionally, triterpenoids, phenolic compounds and nitrogenous compounds were present. The overall composition of the roots is similar to the one described in the literature, suggesting that extracts produced from plants using an aeroponic cultivation system will likely have similar bioactivity and, therefore, be valuable in the health market. For aerial parts, which have been less studied than roots, this study provides a first overview of the phytochemical composition.

The aeroponic cultivation method with 16 h LED elicitation allows an increase in the production of *Coleus forskohlii* with an interesting yield of forskolin, making this method a very promising alternative for a local source of forskolin. Moreover, to identify the other metabolites affected by this stress, this study combined the statistical analysis of UHPLC-HRMS data (PLS-DA and VIP scores) with untargeted metabolomics using molecular networking and putative annotation obtained from GNPS, the LOTUS database, and SIRIUS computation of MS/MS data to support the annotation of the top 25 VIPs. These three databases, including experimental spectra, in silico fragmentation, and taxonomic information, improve the annotation of the molecules. The results showed that LEDs increase the phenolic compound composition in the roots, leading to higher amounts of rosmarinic acid or caffeic acid and the biosynthesis of diterpenes in the aerial parts such as forskolin, which are compounds of interest.

A study examining the influence of LEDs with varying parameters (such as duration, photoperiod, and LED color) would be valuable for better understanding their impact on the biosynthetic pathways of forskolin and other bioactive compounds. This research would provide insights into these pathways and help to evaluate the effects of different lighting conditions, ultimately optimizing their use for improved compound production. As the aerial parts of the plant are also rich in diterpenes and phenolic compounds with this culture

method, future research could focus on this part to explore this chemical diversity and include bioactivity assays in order to also valorize this by-product.

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Conclusion générale - les méthodes d'analyse pour la culture de deux plantes d'intérêt en RCVL

D'un point agronomique, ces deux études ont montré la faisabilité de la production de plantes tropicales d'intérêt en Région Centre-Val De Loire tout en préservant leur phytochimie et la teneur en molécules actives d'intérêt cosmétique.

Dans le cas de l'étude d'*Eclipta alba*, la culture a été relocalisée en région Centre-Val de Loire pour répondre à un besoin de traçabilité, de fiabilité d'approvisionnement et afin de s'inscrire dans une démarche éco-responsable de réduction des coûts environnementaux liés au transport. L'objectif principal de l'étude était de contrôler la composition phytochimique par rapport à celle décrite pour les plantes récoltées dans leur habitat naturel et d'explorer les propriétés cosmétiques de cette plante. Cette étude, menée selon une méthodologie conventionnelle de screening d'activité biologique de l'extrait brut, puis de fractionnement afin de cibler les fractions actives, a permis de mettre en avant l'intérêt pour la phase aqueuse (partie polaire) de l'extrait qui a montré les plus fortes inhibitions des enzymes COX-2, Collagénase et XO. L'utilisation de la CPC pour le fractionnement de la phase aqueuse, associée aux tests d'activité, ont permis de mettre en évidence les fractions les plus actives sur ces mêmes enzymes, qui contiennent majoritairement la wedelolactone et la demethylwedelolactone ainsi que des flavonoïdes glycosylés et des acides caffeoylquiniques. La méthode mise en place nous a conduit à l'isolement des 2 molécules majoritaires, la WD et la DWL, déjà bien décrites dans la littérature pour leur activité anti-inflammatoire et antioxydante, mais le screening d'activité sur de nouvelles cibles d'intérêt cosmétique a mis en évidence une activité sur la collagénase permettant d'élargir le potentiel de ces molécules et des extraits d'*Eclipta alba* pour diverses applications dans des produits de soin de la peau.

Dans le cadre d'applications en cosmétique, bien que l'identification des molécules responsables de l'activité soit intéressante pour comprendre les mécanismes d'action du produit et appuyer les revendications d'activité, leur isolement n'est généralement pas effectué car trop long et trop coûteux. L'incorporation de l'extrait brut ou d'un extrait enrichi en molécules d'intérêt est alors favorisé. L'incorporation de la phase aqueuse présente un intérêt, car plus active et plus riche en WL et DWL. De plus, elle ne contient pas de chlorophylles, ce qui apporte un avantage pour une formulation cosmétique non colorée. Il serait également intéressant de travailler davantage le protocole d'extraction afin de mettre en place des extractions plus sélectives et ciblées afin d'accéder plus rapidement à des ratios en composés d'intérêt plus importants.

Dans le cas de l'étude de *Coleus forskohlii*, le contexte est un peu différent. L'objectif premier du projet était d'optimiser la production de la forskoline, une molécule d'intérêt en cosmétique, produite principalement dans les racines et dont l'activité est bien décrite. La

première étape a ainsi consisté à comparer différentes modalités de culture de la plante, afin d'identifier la ou les modalités de culture optimale(s) permettant une production importante de biomasse possédant également une concentration importante de forskoline. La quantification HPLC-DEDL de la forskoline et la mesure de la biomasse récoltée ont ainsi permis de mettre en évidence que l'apport de LED permettait d'augmenter la production totale de forskoline par rapport au témoin.

Par ailleurs, l'étude de la composition phytochimique des parties racinaires et aériennes de *Coleus forskohlii* à travers l'utilisation des réseaux moléculaires a permis de mettre en évidence leur spécificité chimique. Cette approche a permis de mettre en évidence la diversité chimique présente dans la plante et plus particulièrement la richesse en diterpènes des parties aériennes. De plus, l'utilisation des outils de statistiques ont permis de comparer la composition de deux échantillons différents et de mettre en avant les molécules surexprimées lors de l'élicitation des plantes par les LED. Les VIPs, obtenus à partir de la comparaison par PLS-DA des échantillons analysés par LC-HRMS, ont pu être annotés à partir des données MS/MS grâce à l'utilisation de bases de données telles que LOTUS, et d'outils d'annotation comme SIRIUS. Les résultats ont montré que les LEDs augmentaient notamment le contenu en composés phénoliques dans les racines (acide rosmarinique, acide caféique) et en composés diterpéniques dans les parties aériennes.

Cependant, nous avons également été confrontés aux limites de la déréplication lors de cette étude. Cette richesse en diterpènes rend en effet l'utilisation de cette méthodologie complexe pour une identification précise des composés présents. En effet, cette famille de composés, possédant de très nombreux isomères, présente des spectres de fragmentation HRMS/MS extrêmement proches. Dans le cas présent, la prise en compte de la taxonomie de la plante pour l'interrogation des bases de données n'est pas non plus forcément pertinente, étant donné que celle-ci a été révisée en 2019 et que par conséquent, bon nombre de molécules isolées du genre *Coleus* ne sont pas référencées sous le bon genre. En complément de l'étude menée, des approches métabolomiques basées sur la RMN, dont les spectres seront plus discriminants, pourraient aider à l'identification des composés appartenant à la famille des diterpènes. Enfin, compte tenu de la richesse des feuilles en diterpènes, et d'une plus faible utilisation de cette partie de la plante, une valorisation complète de la plante est tout à fait envisageable et différenciante. Différents screenings d'activités pour des activités anti-inflammatoires sur différentes cibles de l'inflammation sont d'ores et déjà planifiées.

Pour conclure, ces deux études, à travers l'application de deux méthodologies différentes adaptées au nombre d'échantillon et aux différents objectifs ont toutes deux permis de valider la faisabilité de relocalisation du sourcing en région Centre-Val de Loire d'*Eclipta alba* et de *Coleus forskohlii* qui peuvent être localement cultivées et valorisées par des fabricants d'actifs cosmétiques.

CHAPITRE III :

DEVELOPPEMENT DE METHODES D'ANALYSES NON CIBLES ET
INTEGRATION D'OUTILS D'ANNOTATION POUR L'EXPLORATION
D'ESPECES DU GENRE *CLEMATIS*

Introduction

La culture de plantes ornementales est particulièrement répandue en Région Centre-Val de Loire et constitue une activité importante pour les entreprises du secteur horticole. Au cours du processus de culture de ces plantes, l'étape de taille, permettant une meilleure floraison et ramification est indispensable, mais génère une grande quantité de déchets verts, considérés comme biodéchets, et non déchets ultimes (articles R.543-225 et R.541-8 du Code de l'environnement), qui depuis 2012 doivent faire l'objet d'une valorisation biologique (article L.541-21-1 du Code de l'environnement). Cette valorisation se limite très souvent à un compostage réalisé directement par les pépinières mais qui paradoxalement n'utilise que très peu ce compost pour les nouvelles cultures de jeunes plants. En effet, les pépinières privilégient bien souvent un amendement organique issu du commerce, dont la composition a été contrôlée afin d'assurer un apport en nutriment régulier aux jeunes plants. Le compost produit est donc bien souvent juste épandu sur les sols alentours. Il est donc important de trouver d'autres voies de valorisation à ces déchets de taille.

Ce dernier chapitre se consacre ainsi à l'étude du genre *Clematis*, appartenant à la famille de Ranunculaceae, un genre de plante présent à l'état sauvage dans la région et également utilisé comme plante ornementale et dont la culture y est fortement répandue avec notamment la présence des deux principales pépinières françaises productrices de Clématites, les pépinières Travers et Javoy, produisant 2,5 millions de jeunes plants à elles deux, dont la moitié de Clématites¹.

La première partie de ce chapitre est une revue littéraire qui vise à dresser un état des lieux des connaissances actuelles sur le genre *Clematis* que ce soit concernant les usages traditionnels, les activités biologiques et la phytochimie des espèces du genre à l'échelle mondiale. Cette partie bibliographique tend à établir une vue d'ensemble des connaissances actuelles sur ces plantes, afin, par la suite, de mieux appréhender l'exploration phytochimique de différentes espèces et de cibler leurs potentielles activités biologiques.

Dans une seconde partie, l'exploration de la composition chimique d'un ensemble de 10 spécimens appartenant à 10 espèces différentes du genre *Clematis*, collectés et analysés en Suisse dans le cadre d'une mobilité, sera faite à travers l'utilisation des réseaux moléculaires. Les feuilles de ces dix spécimens ont été récoltées dans les jardins botaniques de Fribourg et de Neuchâtel, puis soumises à des analyses par LC-HRMS/MS Orbitrap. Lors de la déréplication à l'aide de réseaux moléculaires, l'intégration de divers outils d'annotation tel que SIRIUS et TIMA-R a été mise en place afin d'améliorer la confiance dans les propositions d'identification de molécules faites lors de cette étude.

Enfin, la dernière partie s'intéresse à l'activité anti-inflammatoire d'espèces appartenant au genre *Clematis*. Pour cette dernière étude, les parties aériennes (déchets de taille) de 11 espèces de clématites, dont 9 communes à l'étude des plantes Suisse et cultivées par les Pépinières Travers, situées à St-Cyr-en-Val, à moins de 10 km de l'ICOA ont été extraites et analysées par LC-HMRS/MS. En parallèle, des tests d'activités biologiques ont été réalisés sur

¹ <https://ginco2-centre.mnhn.fr/atlas/espece/190914> (consulté le 22.10.24)

les extraits, notamment l'évaluation de leur capacité à inhiber JAK3, une kinase impliquée dans la voie inflammatoire JAKs/STATs, considérée comme cible prometteuse pour le traitement de diverses pathologies en dermatologie. Cela, dans le but corréler l'activité biologique à la composition phytochimique, à travers l'utilisation des réseaux de bioactivités et ainsi identifier les molécules potentiellement actives directement à partir d'un extrait brut.

Ainsi, la méthodologie, de l'annotation grâce à différents outils déjà mise en place et l'intégration des résultats de bioactivités, seront combinées pour d'identifier des molécules bioactives afin de trouver des voies de valorisation potentielles aux déchets de taille de la filière horticole.

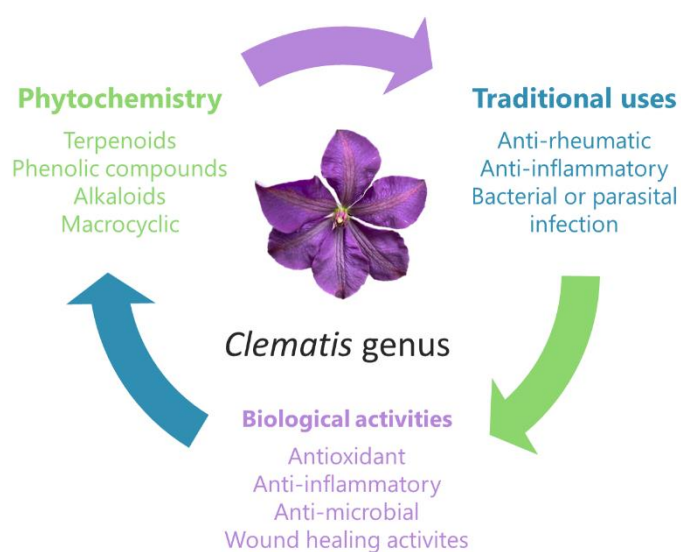
Partie I. Revue de la littérature sur le genre *Clematis* : usages traditionnels,
activités pharmacologiques et phytochimie

Traditional uses, pharmacological activities and phytochemistry of
Clematis genus: an updated review

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Graphical abstract



Abstract

Ethnopharmacological relevance

The genus *Clematis* L. is the third largest genus of the family *Ranunculaceae* and comprises 388 accepted species distributed over most of the world, especially in Asia, North America and Europe. Plants of the *Clematis* genus are used in some countries as food, traditional herbal medicines or for ornamental. Some species are traditionally used to treat arthritis, inflammatory disorders or for antioxidant and anti-microbial activities.

Aim of this review

The latest comprehensive review describing the *Clematis* genus was conducted in 2011. Since then, new species have been reported, along with new identified molecules and bioactivity properties. The review that we propose will, therefore, focus on this recent research to establish an updated reference that can be used as scientific basis for future studies.

Materials and methods

Scientific publication used as references were collected using Google Scholar, Sci-Finder, PubMed, Science Direct, Web of Science and LOTUS: Natural Products Online. World Flora Online was used to confirm the scientific names of the species included in *Clematis* genus.

Results

This review summarizes (I) taxonomic advances, with 27 new validated species reported since 2011, (II) traditional uses of species from *Clematis* genus, (III) Biological activities and cytotoxicity of *Clematis* species extracts. (IV) phytochemical description with more than 500 chemical components (with 121 newly described since 2011) including alkaloids, flavonoids, terpenoids, lignans and coumarins. The pharmacological activities of isolated compounds are also reported. In total, 194 publications dealing with the genus *Clematis* were retrieved in this review.

Conclusions

This review provides a comprehensive summary of recent advancements in research on the genus *Clematis*, with a particular focus on novel insights and developments emerging since 2011 in the realms of ethnopharmacology, phytochemistry, pharmacological activities and related disciplines. Our analysis highlights the significant interest for the genus and unveils a significant number of unstudied species, meaning that *Clematis* genus taxonomy is still in evolution and numerous species are still not studied for their bioactivity or phytochemistry point of view. Furthermore, this review can be used as a valuable reference for future research on the genus *Clematis*.

1. Introduction

The genus *Clematis* L. is the third largest genus of the Ranunculaceae family, comprising 388 accepted species distributed all around the world, especially in Asia, North America and Europe ¹. *Clematis* species are mainly woody or herbaceous vines, though some can also develop as shrubs or upright perennial herbs².

Species from the genus *Clematis* have been used in Traditional Chinese Medicine (TCM), in traditional African medicine, and in various countries worldwide³⁻⁷. *Clematis* species are mainly employed for treating diseases and conditions such as inflammation, rheumatoid arthritis, headaches, or for diuretic and hepatic purposes^{6,8-12}.

Phytochemistry and biological activity studies of *Clematis* have been mainly performed on alcoholic, aqueous and ethyl acetate extracts of both aerial and underground parts¹³⁻¹⁷. Mainly based on traditional uses, these extracts have been evaluated for anti-inflammatory and anti-microbial activities, as well as for antioxidant activity and cytotoxicity.

The compounds isolated from *Clematis* species are mainly terpenoids, phenolic compounds, alkaloids, or compounds as macrocyclic glycosides or anemonin derivatives^{18–22}. Many pharmacological activities such as anti-inflammatory, anti-microbial and cytotoxicity effects have been reported for these compounds and were associated with the biological activity observed for the extracts.

Since 2011, several studies have been conducted on species within the *Clematis* genus. This publication aims to provide an update of the previous comprehensive review on the genus *Clematis*¹⁰, published in 2012 and based on studies conducted before 2011, by gathering the more recent data published after 2011. Hence, this review summarises the most complete knowledge about the traditional uses, pharmacological properties, and phytochemistry of the different *Clematis* species identified thus far.

2. Methodology

Research of scientific publications was carried out using Google Scholar, PubMed, Science Direct, and Web of Science web sites consulted between December 2023 and June 2024. The key search words used included “ethnobotanical uses”, “phytochemistry”, “pharmacological activities” associated to the genus *Clematis*. A particular effort was made to focus on collecting scientific articles published after 2011, since the latest comprehensive review describing the *Clematis* genus was conducted that year¹⁰.

3. Taxonomical and botanical description

The genus *Clematis* L. (Ranunculaceae) comprise 388 accepted species¹. Found on all continents—except Antarctica—and across a diverse range of regions, such as warm-temperate and mountainous environments, *Clematis* genus is one of the most widely distributed genera of flowering plants²³. Species are mostly woody or herbaceous vines but some of them can be shrubs or erect perennial herds².

The phylogeny of the *Clematis* genus is constantly evolving, and several classification systems have been proposed since the 19th century. The last classifications were suggested by Wang and Li, (2005) and Lehtonen et al., (2016). Wang and Li, (2005) suggested 15 sections of *Clematis* had been recognized and discussed according to the morphological characteristic. In 2016, after the reattribution of the genus *Clematis* of two plants in 2014 by the same research team, Lehtonen et al. (2016) suggested the division of the *Clematis* genus into 12 section-level clades (clade A-L) based on their phylogenetic analyses. Subsections could not have been proposed due to the complexity of the phylogeny. In the same study, it is also suggested that *Clematis* genus did not originate solely from the Sino-Himalayan region, but also from the Malagasy, Mediterranean, and Indo-Malayan regions. In 2021, He et al. (2021) analysed 56 species—representing 13 of the 15 sections defined—to better understand the phylogeny and

support this classification. To confirm those hypotheses, more phylogenetic studies involving more species, and specifically from tropical regions, need to be performed.

Phylogenic studies on the *Clematis* genus are also complicated by the fact that, in addition to being already comprised of a very large number of species, the genus is also evolving with the discovery of new species. Indeed, 27 new species have been discovered or accepted since 2011. The repartition of the newly discovered species (*C. aureolata*, *C. austroanatolica*, *C. austrogansuensis*, *C. chaohuensis*, *C. diebuensis*, *C. elobata*, *C. flavidonitida*, *C. gulinensis*, *C. guniuensis*, *C. horripilata*, *C. angiogenesis*, *C. iranica*, *C. jingxiensis*, *C. khuzestanica*, *C. lishanensis*, *C. maguanensis*, *C. melanonema*, *C. morefieldii*, *C. peii*, *C. pettimudiensis*, *C. pseudoconnata*, *C. pseudotubulosa*, *C. udayanii*, *C. vinacea*, *C. wangiana*, *C. wuxiensis*, *C. yuntaishanica*) is presented in Figure 39^{26–45}. 13 of the newly described species have been identified in China, confirming that China is still a centre of high diversity of the genus.

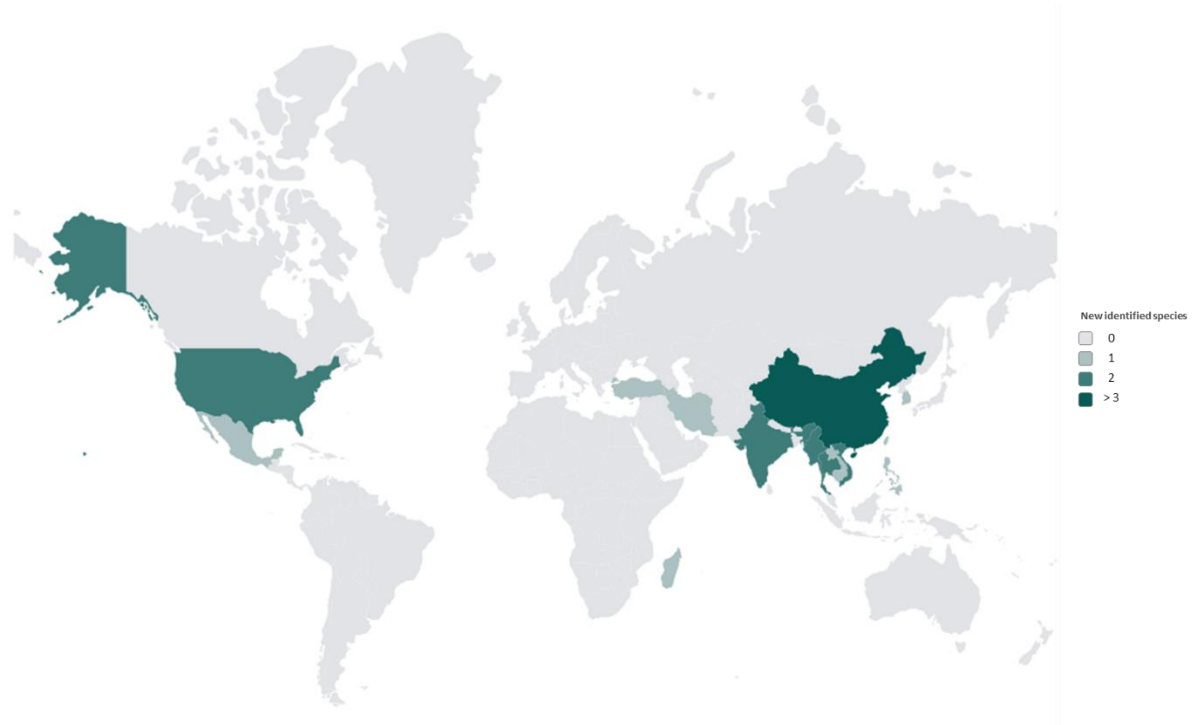


Figure 39: Distribution of the last species of *Clematis* identified since 2011.

4. Traditional uses

Clematis species have been used in medicine to treat different disorders at least since antiquity²³. A large part of the traditional uses previously reported are about inflammatory disorders, diuretic treatments, and infections for the following species: *C. apiifolia*, *C. armandii*, *C. brachiata*, *C. chinensis*, *C. flammula*, *C. glycinoides*, *C. gouriana*, *C. heracleifolia*, *C. hexapetala*, *C. hirsuta*, *C. hirsutissima*, *C. japonica*, *C. microphylla*, *C. montana*, *C. montevidensis*, *C. parviloba*, *C. pickeringii*, *C. recta*, *C. rehderiana*, *C. tangutica*, *C. terniflora* and *C. vitalba*¹⁰.

In some traditional medicine, plant mixtures or specific recipes are reported. Wei-ling-xian also called Clematidis Radix and Rhizome, is comprised of roots of *C. chinensis* Osbeck, *C. hexapetala* or *C. terniflora* var *manshurica* and used in Traditional Chinese medicine (TCM)^{5,12,46,47}. Wei-ling-xian is mainly employed for analgesic, antitumour, rheumatic arthralgia, tendon constriction, inconvenience in flexion, extension and limb numbness^{12,48}. Shu-jing-huo-xie-tang, a mixture containing more than 10 plants and herbal preparations, including Wei-ling-xian, whose main action is the amelioration of muscle and joint pain, is also one of the 10 most used herbal products in TCM and prescribed for stroke patients³ or for acute myocardial infarction⁴⁹. In the Inner Mongolia region, Qingganjiuwei powder, containing nine medicinal plants, including *C. armandii* flowers or stems (Chuan Mu Tong) is a well-known traditional mixture commonly used for its properties on hepatic protection¹¹.

In folk medicine, around 30 species of the genus have been reported to be individually used worldwide (Table 10). The treatment of different inflammatory diseases are the most important traditional uses reported. *C. crassifolia* in China^{4,50}, *C. florida* in Korea, *C. heracleifolia* in both China and Korea¹⁰, and *C. hirsuta* in Africa and South Arabia are all employed as anti-inflammatory agents⁹. The use of *Clematis* species for rheumatoid arthritis, a chronic inflammatory disorder, and arthralgia is well documented. In Algeria, decoction of leaves of *C. cirrhosa* are prepared for the treatment of rheumatic pains⁶. *C. crassifolia*, *C. parviloba* and *C. terniflora* are widely used in addition to Wei-ling-xian in China for the treatment of rheumatoid arthritis¹⁰. *C. terniflora* var. *manshurica* is also used to relieve pain induced by gout, a painful form of arthritis. In Australia, the native species *C. glycinoides*, *C. microphylla* and *C. pickeringii* are used to treat inflammation and pain caused by rheumatism while in Japan, roots of the native *C. japonica* are employed¹⁰. *C. smilacifolia* roots are also reported to be used as an anti-rheumatic in Myanmar⁴. In Algeria, *C. cirrhosa* is reported to be employed for rheumatic pains⁶.

C. apiifolia, *C. florida*, and *C. heracleifolia* are used in Korea as an analgesic. In China, leaves, and roots of *C. crassifolia* and *C. heracleifolia* are employed to relieve pain. *C. brachiata* Thunb., native to the African continent, is employed in South Africa to relieve pain and is reported to be used by foot travellers for its pain relieving properties¹⁰. In Italy, the same traditional properties have been reported for *C. vitalba*, prepared as a refreshing bath for feet⁵¹.

Clematis species are also employed to treat skin inflammation and disorders¹⁰. The implication of *Clematis* genus in various skin diseases have been previously reported; *C. rehderiana* and *C. tangutica* for skin ulcer in Tibet, realisation of *C. microphylla*'s poultice as a counterirritant in Australia, *C. montevidensis* as rubefacient, wound healing and antivenom in South America, treatment of skin burns in India using *C. gouriana* leaves or the use of *C.*

montana in various skin diseases. In China, *C. smilacifolia* has been reported to be used to relieve itch⁴. In traditional African medicine, *C. brachiata* is employed chopped and boiled together with three other plants and used to treat warts and genital sores⁵².

Different uses of *Clematis* have been reported to cure oral and pulmonary inflammation or infection. *C. hirsuta* named Adwe or Langi in Uganda, have been reported for the treatment of oral or dental diseases. Leaves are pound and applied as a paste on a painful tooth or drunk in infusion to treat a toothache or sore throat induced by a bacterial infection⁵³. *C. terniflora* var. *manshurica* roots is also reported for the treatment of toothache and *C. chinensis* and *C. hexapetala* have been used in the treatment of throat inflammation⁴⁸. In China, *C. chinensis*, *C. hexapetala*, and *C. terniflora* var. *manshurica* roots are employed to cure pain and for the treatment of acute tonsillitis⁴⁸. *C. apiifolia* is used in Korea to treat asthma and as an anti-inflammatory agent¹⁰. Fresh leaves of *C. clemensiae* are crushed in water, and the clear solution is taken orally for the treatment of cough in Papua New Guinea⁵⁴. Decoction of aerial part of *C. drummonii* is used for the treatment of cough and flu by the Mayo people, in Mexico⁵⁵. *C. buechananiana*, a plant native to the North East of Pakistan, to South Central China and Indo-China, has been reported to be traditionally used by people of Sikkim (India) to treat various ailments. The roots are commonly used to treat sinusitis (inflammation of the sinus tissues), headache, cold, and nose bleeding⁸. *C. brachiata* employed in Africa for the same utilization, can also be employed in combination with honey for the treatment of sinusitis⁵².

In South Africa, *C. brachiata* has been used against thrush, a common infection caused by *C. albicans*⁵⁶, and in the treatment of schistosomiasis, a parasitic disease¹⁰. Dysentery, an intestinal infection usually caused by *Shigella* spp. and tetanus have been treated in Korea using *C. terniflora* var. *manshurica*. In China, *C. heracleifolia* is reported for antibacterial treatment and in Europe, *C. recta* and *C. vitalba* are reported for antibacterial treatment or antifungal treatment¹⁰.

C. terniflora var. *manshurica* roots are employed in Korea to treat neuralgia⁵⁷. *C. armandii* and *C. montana* are employed in China for the treatment of fever symptoms¹⁰. *C. viridiflora* leaves and roots are used in a preparation called "mucoca" in inhalation to treat headache in Munda (Mozambique)⁵⁸. In Australia, common colds were treated with *C. glycinoides*, *C. microphylla* and *C. pickeringii*¹⁰. In Papua New Guinea, dried leaves of *C. clemensiae* are crushed and sniffed to relieve a headache and clear the nose⁵⁴. In the United States, *C. hirsutissima* is reported to cure headaches and in Europe, *C. recta* is mentioned to cure neuralgic and rheumatic headache¹⁰.

The use of some *Clematis* species for diuretic or hepatic purposes has been reported in Asia and Africa. In China, *C. armandii*, *C. chinensis*, *C. crassifolia*, *C. heracleifolia*, *C. hexapetala*, *C. montana*, *C. parviloba*, and *C. terniflora* are the species identified. In Korea, *C. apiifolia*, *C. florida*, and *C. heracleifolia* are reported. In Tibet and in Japan the use of *C. rehderiana* and

of *C. japonica* roots is reported, respectively¹⁰. In Rwanda, *C. hirsuta*, described for the treatment of liver diseases, is reported to have side effects with risk of abortion and danger for children; which leads traditional health practitioners to reduce the dose for pregnant women and children⁵⁹.

C. armandii, *C. montana*, and *C. parviloba* in China, have been reported for the stimulation of menstrual discharge (emmenagogue property) and lactation. In Korea, *C. terniflora* var. *manshurica* has been reported to stimulate menstrual discharge, leucorrhoea, and menostatis symptoms; and *C. apiifolia* has been used during labour^{10,57}.

Applications that are more anecdotal have also been reported. In Korean traditional medicine, *C. apiifolia*, *C. florida*, and *C. heracleifolia* are described to be used as an antitumour agent. *C. apiifolia* is also described to be used for vitamin B1 deficiency¹⁰.

In Italy, fresh leaves are consumed as food; shoots can also be mixed with *Urtica* and *Smilax* and used in 'frittata' preparation. Shoots of *C. vitalba* are mixed with *Rubus* and *Foeniculum vulgare* Mill. and used to purify milk or as a food integrator for sheep⁵¹. In agriculture, *C. flammula* leaves are reported to prevent the deterioration of stored wheat and corn by insects in Australia¹⁰.

The traditional uses of *Clematis* in different parts of the world have been widely described, various populations have employed native species to cure different disorders. As the genus *Clematis* is highly spread in Asia and traditional medicine is well documented, most of the traditional uses are reported there with most applications of *Clematis* being related to different inflammatory conditions (rheumatoid arthritis, throat inflammation, laryngitis, etc.) and bacterial or parasitic infections. Furthermore, similar uses have been identified all over the world, demonstrating the biological value of these plants.

Table 10: Traditional uses of the genus *Clematis*

<i>Clematis</i> species	Part used	Traditional uses and preparation	Country/region	Ref.
<i>C. apiifolia</i>		Analgesic, anti-inflammatory, anti-tumour, asthma, diuretic, labour, vitamin B1 deficiency	Korea	10 57
<i>C. armandii</i>	Flower or stems (Chuan Mu Tong)	Hepatic protection	Mongolia	10
		Diuretic, fever, lactation promotion, menstrual discharge stimulation	China	11
<i>C. brachiata</i>	Roots	Foot pain, pain reliever, schistosomiasis, thrush (roots cook with salt)	South Africa	56
	Leaves and stem	Coughs, chest pain, and a runny or blocked nose, sinusitis treatment (orally in combination with sugar)	Africa	52
		warts and genital sores (chopped and boiled with three other plants)		
<i>C. buchananiana</i>	Roots	Cold, headache, nose bleeding, sinusitis	India	8
<i>C. chinensis</i>	Roots	Acute tonsillitis, arthralgia, jaundice, numbness in extremities and stiff joints, rheumatic arthritis, throat inflammation, toothache	China	10 48
<i>C. cirrhosa</i>	Leaves	Rheumatic pains (decoction)	Algeria	6
<i>C. clemensiae</i>	Leaves	Nose cleaning (crushed leaves sniffed), cough (clear solution of fresh leaves), headache (dried leaves crushed and sniffed)	Papua New Guinea	54
<i>C. crassifolia</i>	Leaves and roots	Analgesic, anti-inflammatory, diuretic, rheumatoid arthritis, pain reliever	China	50 10
<i>C. drummondii</i>	Aerial part	Cough and flu (decoction)	Mexico	55
<i>C. flammula</i>	Leaves	Insect repellent (prevention of deterioration of stored wheat and corn)	Australia	10
<i>C. florida</i>		Analgesic, anti-inflammatory, anti-tumour, diuretic	Korea	10
<i>C. glycinoides</i>	Leaves	Cold, headache (inhalation of the vapour from freshly crushed leaves), infection, pain, rheumatism	Australia	10
<i>C. gouriana</i>	Leaves	Skin burn (leaf paste)	India	10
<i>C. heracleifolia</i>		Analgesic, anti-bacterial, anti-inflammatory, antitumour, diuretic	China, Korea	10
<i>C. hexapetala</i>	Roots	Acute tonsillitis, jaundice, rheumatic arthritis, throat inflammation, toothache	China	48 10
<i>C. hirsuta</i>	Leaves	Anti-inflammatory	South Arabia	60
		Earache (leaves crushed, squeezed and a small amount of juice is applied through auricular route for two days), respiratory tract problem	Ethiopia	53
		Toothache (pound and apply on painful tooth or drunk as an infusion)	Ugandan	61
<i>C. hirsutissima</i>	Roots	Headache, horse restorative (snuff from the dried and powdered roots),	United States	62
	Whole plant	Itch medicine (infected areas rubbed with boiled plant)	United States	
<i>C. japonica</i>	Roots	Diuretic, rheumatism	Japan	10
<i>C. microphylla</i>		Common colds, counterirritant (poultice), headache, infection, pain, rheumatism	Australia	10
<i>C. montana</i>		Menstrual discharge stimulation, purging properties, skin disease	China	10

<i>Clematis</i> species	Part used	Traditional uses and preparation	Country/region	Ref.
<i>C. montevidensis</i>		Anti-venom, rubefacient, wound healing (wound and external ulcers)	South America	10
<i>C. parviloba</i>		Hydropsy, rheumatism	Australia	10
<i>C. pickeringii</i>		Cold, headache, infection, pain, rheumatism	Australia	10
<i>C. recta</i>		Gonorrhoea, insomnia, neuralgic and rheumatic headache, neuroses of men with testicular pain	Europe	10
<i>C. rehderiana</i>		Diuretic, lump in the abdomen, skin ulcer	Tibet	10
<i>C. smilacifolia</i>	Roots	Rheumatism Relieve itch	Myanmar China	4
<i>C. tangutica</i>		Indigestion, skin ulcer	Tibet	10
<i>C. terniflora</i>				10
<i>C. terniflora</i> var. <i>manshurica</i>	Roots	Rheumatoid arthritis, anti-inflammatory, toothache, pain, dysentery	China, Korea	57
<i>C. terniflora</i> var. <i>robusta</i>				
<i>C. texensis</i>		Fever (In decoction with <i>Baillonella toxisperma</i> , <i>Milicia excels</i> and <i>Opuntia ficus-indica</i>)	Cameroun	63
<i>C. viridiflora</i>	Leaves and roots	Headache (not prepared and inhaled)	Mozambique	58
<i>C. vitalba</i>	Leaves	Anti-bacterial, anti-mycotic	Europe	10
		Food (young, boiled leaves), foot pain relieve (feet bath)	Italy	51
<i>C. zeylanica</i>	Leaves	Cough (decoction, nasal inhalation, topical)	India	64

5. Pharmacological activity of extracts

The genus *Clematis* has been widely described for different traditional uses based on traditional medicine knowledge and from ethnopharmacological inquiry. A large number of pharmacological activities from *Clematis* species has been investigated during the last years to confirm those uses. *In vitro* and *in vivo* experiments have been employed on *Clematis* spp. extracts and fractions. Those pharmacological activities have been summarized in Table 11. As this review focuses on data published after 2011, data published before 2011 were briefly summarized and are accessible in Chawla *et al.* (2012) publication¹⁰.

5.1. Antioxidant activity

Antioxidant activity has been previously reported for the species *C. armandii*, *C. chinensis*, *C. flammula*, *C. rehderiana*, and *C. trichotoma*. *In vitro* studies using DPPH (2,2-diphenyl 1-picrylhydrazyl) assay have been used in most cases on alcoholic (methanol or ethanol), water or chloroform extracts obtained from roots and/or leaves. IC₅₀ values range from 4.6 µg/mL (chloroform extract of leaves of *C. flammula*) to 100 µg/mL for ethanol extraction of roots of *C. chinensis*¹⁰.

Since 2011, the antioxidant properties of *C. brachiata*, *C. connata*, *C. flammula*, *C. gouriana*, *C. graveolens*, *C. hirsuta*, *C. ispanhanica*, *C. orientalis*, and *C. simensis* have been studied through the DPPH radical scavenging assay. The activity of *C. brachiata* leaves using different extraction solvents has been compared⁶⁵. Methanol extract showed the highest activity with IC₅₀ = 180.45 ± 2.4 µg/mL compared to acetone extract with IC₅₀ = 330.40 ± 2.12 µg/mL and water extract with IC₅₀ = 480.25 ± 3.6 µg/mL. The ethyl acetate (EtOAc) fraction of the methanolic (MeOH) extract of aerial part of *C. connata* also showed good activity using the DPPH assay with IC₅₀ = 104 ± 1.5 µg/mL¹⁵. Both methanolic and ethanolic extracts of *C. flammula* leaves have been tested and showed strong activity with IC₅₀ = 90 µg/mL⁶⁶ and IC₅₀ = 8.45 ± 0.063 µg/mL⁶⁷, respectively. The DPPH activity of the ethanol extract and the acetone extract from *C. gouriana* leaves, with IC₅₀ = 129.11 µg/mL and IC₅₀ = 128.77 µg/mL respectively, is similar⁶⁸. The roots of *C. hirsuta* have been investigated for antioxidant activity and the methanolic extract showed a weak activity with IC₅₀ = 599 µg/mL⁶⁹. 63.46% and 66.54% of inhibition was obtained at a dose of 300 µg/mL with the *C. ispanhanica* chloroform extract and *C. orientalis* ethyl acetate extract respectively⁷⁰. Teshome *et al.* explored the methanolic extract of *C. simensis* leaves and obtained a value of IC₅₀ = 1.04 mg/mL⁷¹. In general, moderate activity of the extracts was determined. Alcoholic extracts (ethanol, methanol) yield the best activity, with *C. flammula* appearing to have the highest antioxidant activity using DPPH assay with an IC₅₀ = 8.45 ± 0.063 µg/mL⁶⁷.

Table 11: Pharmacological activities of extracts of *Clematis* species (results based on studies published after 2011)

Species	Plant part	Extract or fraction	Pharmacological activity	Model	Bioactivity	Ref.
<i>C. aethusifolia</i>	Whole plant	n-BuOH fraction of 70% EtOH extract	Insecticidal activity	<i>Plutella xylostella</i> (L.) larvae	96.43 ± 3.57% of mortality at 24 h at 10 mg/mL	72
<i>C. apiifolia</i>	Bark	70% EtOH extract	Antioxidant	Benzo[a]pyrene-induced effect on HaCat cells (human aneuploidy immortal keratinocyte)	Reduction of benzo[a]pyrene induced production of ROS (Reactive Oxygen Species) with a reduction effect of benzo[a]pyrene on IL-8 (Interleukin-8)	13
<i>C. armandii</i>	Stem	95% EtOH extract	Anti-neuroinflammatory	LPS-stimulated (lipopolysaccharide) BV-2 cells (microglial)	Inhibition of NO (nitric oxide) production with IC ₅₀ = 129.7 ± 3.5 µg/mL	73
		CH ₂ Cl ₂ fraction of 95% EtOH extract			IC ₅₀ = 72.0 ± 2.4 µg/mL	
<i>C. brachiata</i>	Leaves	DCM:MeOH extract	Antimicrobial	Dilution assay with: <i>S. sanguis</i> (ATCC10566), <i>P. gingivals</i> (ATCC 33277), <i>C. albicans</i> (ATCC 10231), <i>C. glabrata</i> (ATCC 90030), <i>C. krusei</i> (ATCC 14243)	MIC (minimum inhibitory concentration) = 670 µg/mL, NA, MIC = 330 µg/mL, MIC = 330 µg/mL, MIC = 420 µg/mL	56
		Aqueous extract			NA or low value	
	Stems	DCM:MeOH extract			NA or low value	
		Aqueous extract			NA or low value	
	Flowers	DCM:MeOH extract			MIC = 830 µg/mL, NA, MIC = 500 µg/mL, MIC = 420 µg/mL, NA	
	Roots	DCM:MeOH extract			NA, MIC = 500 µg/mL, NA, NA, NA	
	Leaves	Acetone extract	Antioxidant	ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid assay)	IC ₅₀ = 130 ± 1.4 µg/mL	65
		Acetone extract		DPPH	IC ₅₀ = 330.40 ± 2.12 µg/mL	
		MeOH extract		ABTS	IC ₅₀ = 60 ± 0.80 µg/mL	
				DPPH	IC ₅₀ = 180.45 ± 2.4 µg/mL	
		Aqueous extract		ABTS	IC ₅₀ = 170 ± 0.75 µg/mL	
				DPPH	IC ₅₀ = 480.25 ± 3.6 µg/mL	
<i>C. buchananiana</i>	Roots	MeOH extract	Anti-inflammatory	Inhibition of HRBC (human red blood cells) haemolysis Albumin protein denaturation method	Inhibition of 70.7 ± 1.3 % at 3 mg/mL Inhibition of protein denaturation at 59.9 ± 6.8 mg/mL	8
<i>C. burgensis</i>	Leaves	MeOH extract	Antimicrobial	Disc diffusion and dilution assay with <i>C. albicans</i>	MIC = 780 µg/mL	74

Species	Plant part	Extract or fraction	Pharmacological activity	Model	Bioactivity	Ref.
			Wound healing activity	Wistar rats	Faster complete wound closure with topical application	
<i>C. chinensis</i>	Aerial part	Aqueous extract	Anti-diabetic	Diabetes induced male Wistar rats	Effect at 250 mg/kg and 500 mg/kg on renal function index, fasting blood glucose (FBG), blood fat, oxidation index, and pathological kidney	75
	Roots and rhizomes	Huangqin Qingre Qubi Capsule (plant mix)	Anti-inflammatory	AIA (Adjuvant Induced Arthritis) rats	anti-inflammatory and anti-rheumatic effects through the FZD8-Wnt/ β -catenin signalling pathway in AIA rats	76
	Roots and rhizomes	Extract in rice yellow wine with <i>Notopterygium incisum</i>	Anti-inflammatory	AIA rats paw swelling assay	Decrease of pro-inflammatory protein (TNF- α (Tumour Necrosis Factor α), IL-6, VGEF (Vascular Endothelial Growth Factor))	7
	Roots and rhizomes	Chinese rice yellow wine extract	Anti-inflammatory	LPS-induced RAW264.7 (macrophage cells) CIA rats (Collagen Induced Arthritis)	Inhibition of NO production amelioration of rheumatoid arthritis symptoms	46
				AIA rats	Inhibition of pro-inflammatory cytokines (IL-6, IL-1B, TNF- α and VGEF)	
<i>C. cirrhosa</i>	Aerial part	Essential oil	Antioxidant	ABTS DPPH	128.91 $\pm$ 0.40 μ g eq. Trolox/mg IC ₅₀ = 5.10 $\pm$ 0.14 mg/mL	77
<i>C. cirrhosa</i>	Aerial part	MeOH extract	Antioxidant	ABTS DPPH FRAP	236.26 $\pm$ 0.56 μ g eq. Trolox/mg IC ₅₀ = 1.98 $\pm$ 0.08 mg/mL 212.42 $\pm$ 2.63 mg eq. Trolox/g	78
		70% MeOH extract		ABTS DPPH FRAP	237.80 $\pm$ 0.24 μ g eq. Trolox/mg IC ₅₀ = 2.32 $\pm$ 0.29 mg/mL 205.15 $\pm$ 3.22 mg eq. Trolox/g	
		MeOH extract	Enzyme inhibition activity	Inhibition of AChE (acetylcholinesterase) (%) BChE (butyrylcholinesterase) (%) Tyrosinase Amylase Glucosidase	At 2 mg/mL: 69.03 $\pm$ 1.13 % 47.67 $\pm$ 1.86 % 82.34 $\pm$ 0.39 % 36.63 $\pm$ 2.17 % 14.03 $\pm$ 0.91 %	
		70% MeOH extract		Inhibition of AChE (%) BChE (%) Tyrosinase Amylase Glucosidase	At 2 mg/mL: 78.38 $\pm$ 0.36 % 34.22 $\pm$ 1.25 % 86.56 $\pm$ 0.59 % 24.70 $\pm$ 0.83 % 40.93 $\pm$ 4.50 %	

Species	Plant part	Extract or fraction	Pharmacological activity	Model	Bioactivity	Ref.
<i>C. connata</i>	Aerial part	EtOAc fraction of 90% MeOH extract	Antioxidant	DPPH	IC ₅₀ = 104 ± 1.5 µg/mL	15
<i>C. filamentosa</i>	//	Total flavones content (provided by another Institute)	Anti-inflammatory	Oxidized low-density lipoprotein (OxLDL)-induced (Oxidized Low Density Lipoprotein) vascular smooth muscle cells	Protection against OxLDL injury in vascular smooth muscle cells Inhibition of the expression of inflammatory factors and reduction of apoptosis	79
<i>C. flammula</i>	Aerial part	Hydrolat	Anti-cancer	Tumour cell lines HCT-116 (human colon cancer cells) Swiss albino mice LPS-treated	Enhance the apoptosis induced by TRAIL cells (Tumour necrosis factor-related apoptosis-inducing ligand) Reduction of the damage effect of LPS-induced colon carcinoma <i>in vivo</i>	80
	Leaves	EtOH extract	Anti-cancer	HEP-G2 (human liver cancer cells) OVCAR-3 (human ovarian carcinoma cells) A2780 (human ovarian cancer cell)	Dose-dependent cytotoxic effect against with IC ₅₀ = 220 µg/mL at 48 h IC ₅₀ = 151 µg/mL at 72 h IC ₅₀ = 77.0 µg/mL at 72 h	14
			Antioxidant	Superoxide radical scavenging (non-enzymatic) Superoxide radical scavenging (enzymatic) Xanthine oxidase inhibition NADH oxidase inhibition	34.6 ± 1.2 % of inhibition at 50 µg/mL 49.7 ± 1.6 % of inhibition at 100 µg/mL 25.0 ± 2.33 % of inhibition at 100 µg/mL 64.6 ± 4.08% of inhibition at 100 µg/mL	
<i>C. flammula</i>	Leaves	Aqueous fraction from CH ₂ Cl ₂ extract	Anti-cancer	CHL and PLC cells lines (human liver cancer)	IC ₅₀ = 58.5 and 47.3 µg/mL	81
		Aqueous fraction from EtOAc extract	Antioxidant	ABTS ORAC (Oxygen Radical Absorbance Capacity assay)	7.9 mmoles Trolox/ g plant extract 487 mmoles Trolox eq/g of plant	
		Aqueous fraction from CH ₂ Cl ₂ extract		ABTS ORAC	10.5 mmoles Trolox/ g plant extract 387 mmoles Trolox eq/g of plant	
	Leaves	EtOH extract	Anti-inflammatory	COX (cyclooxygenase) inhibition Inhibition of HRBC haemolysis	IC ₅₀ = 223.26 ± 17.60 µg/mL IC ₅₀ = 7.23 ± 0.42 µg/mL	67

Species	Plant part	Extract or fraction	Pharmacological activity	Model	Bioactivity	Ref.
			Antioxidant	DPPH	IC ₅₀ = 8.45 ± 0.063 µg/mL	
				FRAP	IC ₅₀ = 18.55 ± 0.93 µg/mL	
		CH ₂ Cl ₂ fraction from EtOH extract	Anti-inflammatory	Inhibition of HRBC haemolysis	IC ₅₀ = 9.49 ± 0.71 µg/mL	
		EtOAc fraction from EtOH extract	Antimicrobial	Disc diffusion assay - <i>E. Coli</i> and <i>S. Aureus</i>	MIC = 1250 µg/mL, MIC = 78.0 µg/mL	
	Flower	EtOAc extract	Antimicrobial	Disc diffusion and dilution assay - <i>R. Solani</i>	MIC = 700 µg/mL	82
		Hexane extract			MIC = 1560 µg/mL	
		EtOAc extract	Antioxidant	DPPH	IC ₅₀ = 410 ± 0.003 µg/mL	
		MeOH extract			IC ₅₀ = 190 ± 0.01 µg/mL	
		EtOAc extract	Molluscicidal activity	<i>G. Truncatula</i>	LC ₅₀ = 11.65 mg/L after 48 h of exposure	
		Hexane extract			LC ₅₀ = 11.87 mg/L after 48 h of exposure	
	Leaves	EtOAc	Antioxidant	DPPH	IC ₅₀ = 220 ± 0.01 µg/mL	82
		MeOH extract			IC ₅₀ = 120 ± 0.001 µg/mL	
		80% MeOH extract			IC ₅₀ = 90 ± 0.006 µg/mL	
		EtOH extract	Antimicrobial	Dilution assay - <i>C. albicans</i> (5 different strains)	Geometrical mean MIC ₅₀ = 189.43 µg/mL	83
				<i>C. albicans</i> ATCC 10231 biofilm	36.78 ± 1.09% at 500 µg/mL	
		Deep eutectic solvent (Choline chloride-acetic acid (1:2) (mole/mole))	Antimicrobial	Dilution assay: <i>C. albicans</i> methicillin-resistant, <i>S. Aureus</i> , <i>P. aeruginosa</i>	MIC = 390 µg/mL for all strains	84
				ABTS	97.23 ± 1.72% (crude extract, exact concentration not known)	
			Antioxidant	DPPH	94 ± 4.21% (crude extract, exact concentration not known)	
	Aerial part	Polysaccharide precipitate of water extraction from acetone/EtOH mixture	Antioxidant	Myocardial injury induced rat male Wistar	Reduction of DNA fragmentation and increase of antioxidant enzyme activity at dose 100 mg/kg	85
	Leaves	EtOH extract	Gastroprotective	Male mice stress-induced ulcer	<i>In vivo</i> assay at 0.04 – 5.33 mg/mL had shown anti-ulcer activity	86

Species	Plant part	Extract or fraction	Pharmacological activity	Model	Bioactivity	Ref.
<i>C. florida</i>	Roots	n-BuOH fraction of MeOH extract	Anti-inflammatory	AIA rats	Reduction of the paw oedema at dose 200 mg/kg/day reduction of pro-inflammatory mediators (IL-1 β , TNF- α and IL-6) level	17
				LPS-induced RAW264.7 cells	Inhibition of NO production with IC ₅₀ = 16.76 $\pm$ 2.36 μ g/mL	
<i>C. florida</i> var. <i>plena</i>	Roots	n-BuOH fraction of MeOH extract	Anti-inflammatory	Male ICR mice - xylene induced ear swelling	Inhibition of 48.7% at 800 mg/kg	87
<i>C. gouriana</i>	Aerial part	EtOAc fraction of 70% MeOH extract	Anti-inflammatory	LPS-induced RAW264.7 cells	Inhibition of NO production of 77.24 $\pm$ 0.71% at 100 μ g/mL concentration	88
				Carrageenan-induced paw oedema rat model	Reduction of oedema 65.83 $\pm$ 3.07% at 400 mg/kg after 5 h	
	Leaves	Aqueous acetone extract	Antioxidant	ABTS	IC ₅₀ = 31.16 μ g/mL	68
				DPPH	IC ₅₀ = 129.11 μ g/mL	
				FRAP	24.48 $\pm$ 0.13 μ g eq Trolox/mg	
				ABTS	IC ₅₀ = 25.35 μ g/mL	
				DPPH	IC ₅₀ = 128.77 μ g/mL	
				FRAP	16.87 $\pm$ 0.27 μ g eq Trolox/mg	
	Leaves	EtOAc fraction of aqueous acetone extract	Cytotoxicity	C6 cell line (glioma stem cells)	62.34 $\pm$ 0.03 at 100 μ g/mL after 48 h	
				CHOK1 cell lines (Chinese hamster ovary)	64.45 $\pm$ 0.80 at 100 μ g/mL after 48 h	
	Leaves	MeOH extract	Wound healing activity	Wistar rats	Reduction in wound area and faster rate of epithelialization (18.28 $\pm$ 1.17 days vs 24.12 $\pm$ 0.28 days for control)	89
<i>C. grata</i>	Roots	MeOH extract	Antimicrobial	Disc diffusion assay - <i>S. Aureus</i>	16.00 $\pm$ 0.82 mm	90
	Leaves				19.33 $\pm$ 1.18 mm	
	Stem				20.66 $\pm$ 0.54 mm	

Species	Plant part	Extract or fraction	Pharmacological activity	Model	Bioactivity	Ref.
<i>C. graveolens</i>	Roots	MeOH extract	Antioxidant	DPPH	38.73 µg/mL	91
	Leaves				11.55 µg/mL	
	Stem				11.39 µg/mL	
	Leaves	EtOH extract	Antimicrobial	<i>P. Aeruginosa</i> PAO1 biofilm	80% of inhibition (raw extract)	
	Leaves	Essential oil	Antimicrobial	Disc diffusion assay - <i>E. Coli</i> , <i>C. albicans</i> , <i>A. Flavus</i> , <i>A. Niger</i>	Value between 9.8 – 15.66 mm on different tested organisms	
<i>C. graveolens</i>	Leaves	Fixed oil (soxhlet using n-hexane solvent)	Antioxidant	DPPH	IC ₅₀ = 14.06 ± 0.03 µg/mL	92
		Essential oil			IC ₅₀ = 15.83 ± 0.02 µg/mL	
	Roots	Saponins extract obtained from 70% EtOH extract	Anti-inflammatory	CIA rats	Significant anti-arthritic activity in a dose-dependent manner and inhibition of pro-inflammatory cytokine	
<i>C. hirsuta</i>	Leaves	CH ₂ Cl ₂ extract	Antimicrobial	Disc diffusion and dilution assay - <i>P. aeruginosa</i> , <i>S. Typhi</i> , <i>E. Coli</i> , <i>S. Boydii</i> , <i>S. Aureus</i>	Antibacterial properties at 50, 100 and 200 mg/mL	61
		MeOH extract			Antibacterial properties at 50, 100 and 200 mg/mL	
	Leaves	Acetone, CH ₂ Cl ₂ , EtOAc, EtOH, MeOH extracts	Antimicrobial	Disc diffusion and dilution assay - <i>S. aureus</i> (ATCC-25923), <i>E. coli</i> (ATCC-25922), <i>Shigella</i> sp., <i>P. aeruginosa</i> , <i>C. albicans</i> , <i>Penicillium</i> sp., <i>A. niger</i> , <i>A. fumigatus</i>	All extracts have MIC between 7.5 - 60 mg/ml on different tested organisms	93
	Roots	MeOH extract	Antimicrobial	Disc diffusion assay - <i>E. Coli</i>	35 mm at 1.0 mg/mL	69
			Antioxidant	DPPH	IC ₅₀ = 599 µg/mL	
	Roots	n-hexane/EtOAc MeOH	Antioxidant	DPPH	41.7% of inhibition of DPPH at 62.5 µg/mL 56.7% of inhibition of DPPH at 62.5 µg/mL	94
	Leaves	CH ₂ Cl ₂ , EtOH, petroleum ether extracts	Antimicrobial	Disc diffusion assay - <i>S. Aureus</i> , <i>E. Coli</i> , <i>S. Pyogenes</i> , <i>K. Pneumoniae</i>	Zone of inhibition in range 11-19 mm	95
		EtOH extract	Antioxidant	DPPH FRAP	IC ₅₀ = 43.2 µg/mL IC ₅₀ = 118.3 µg/mL	
<i>C. huchouensis</i>	Leaves and stem	Polysaccharide precipitate of water extraction from 95% EtOH extract	Antioxidant	Superoxide radical assay hydroxyl radical assay reducing power assay	IC ₅₀ = 27.1 µg/mL IC ₅₀ = 640 µg/mL 0.499 at 10 mg/mL	96
<i>C. ispananica</i>	Leaves	n-BuOH, CH ₂ Cl ₂ , EtOAc, hexane, water extracts	Antioxidant	DPPH FRAP	Between 48.5 - 63.5% of inhibition at 300 µg/mL	70

Species	Plant part	Extract or fraction	Pharmacological activity	Model	Bioactivity	Ref.
<i>C. lasiandra</i>	Aerial part	70% EtOH extract	Insecticidal activity	Aphicidal activity against <i>Acyrtosiphon pisum</i> Harris	Aphicidal activity with LC ₅₀ value of 9.46 mg/mL	97
		n-BuOH fraction of 70% EtOH extract			Aphicidal activity with LC ₅₀ value of 2.81 mg/mL	
		EtOAc fraction of 70% EtOH extract	Antiviral	Anti-TMV (tobacco mosaic virus) on <i>N. Glutinosa</i> plants	Inactivation, protective and curative effects against TMV with antiviral inhibition activity of 73.96% at 20 mg/mL	
		n-BuOH fraction of 70% EtOH extract			Inactivation, protective and curative effects against TMV with antiviral inhibition activity of 66.31% at 20 mg/mL	
<i>C. longicauda</i>	Leaves	MeOH extract	Antimicrobial	Disc diffusion and dilution assay with <i>C. albicans</i>	MIC = 780 µg/mL	74
			Wound healing activity	Wistar rats	Faster complete wound closure with topical application	
<i>C. napaulensis</i>	Leaves	//	Antioxidant	DPPH	Good potential	98
<i>C. orientalis</i>	Flower	70% EtOH extract	Anti-inflammatory	Formaldehyde induced arthritis Sprague-Dawley rats	Reduction in paw swelling and its diameter at doses of 50, 100 and 200 mg/kg	99
<i>C. orientalis</i>	Leaves	n-BuOH, CH ₂ Cl ₂ , EtOAc, hexane, water extracts	Antioxidant	DPPH - FRAP	Between 52.4 - 66.5% of inhibition at 300 µg/mL	70
<i>C. simensis</i>	Leaves	80% MeOH extract	Anti-inflammatory	Carrageenan induced paw oedema mice	Decrease in oedema after 4 h at 400 mg/kg	71
			Wound healing activity	Excision wound Swiss albino mice	Reduction in healing time and wound size with application of 5% and 10% (w/w) extract	100
			Anti-leishmanial activity	<i>In vitro</i> activity against <i>Leishmania</i> spp	IC ₅₀ values of 46.12 ± 0.03 and 8.18 ± 0.10 µg/mL on the promastigotes of <i>L. aethiopica</i> and <i>L. donovani</i>	
	Roots	CH ₂ Cl ₂ extract	Anti-malarial	Chloroquine sensitive rodent malaria parasite, <i>P. Berghei</i> in Swiss Albino mice	At dose 400 mg/kg and 600 mg/kg decrease in temperature and at 600 mg/kg reduction of the parasitaemia level	101
	Stem bark	CH ₂ Cl ₂ , EtOH, petroleum ether extracts	Antioxidant	DPPH	IC ₅₀ value for DPPH between 42 - 64 µg/mL	102
<i>C. terniflora</i>	Aerial part	Crude herb	Anti-inflammatory	Carrageenan-induced chronic non-bacterial prostatitis rats	Inhibition of the inflammation in rat prostate at dose 15 g/kg	103
	Leaves and stem	70% EtOH eluted fraction of the 70% EtOH extract	Anti-inflammatory	LPS-induced RAW264.7 cells carrageenan-induced rat paw	Inhibition of NO and PGE ₂ at dose of 125 mg/mL and decrease of xylene-induced inflammation and paw oedema	104

Species	Plant part	Extract or fraction	Pharmacological activity	Model	Bioactivity	Ref.
<i>C. terniflora</i> var. <i>manshurica</i>	Aerial part	70% EtOH eluted fraction from 70% EtOH extract	Antinociceptive activity	oedema xylene-induced mice ear swelling	Decrease of the reaction time using hot-plate test at 150 mg/kg and 300 mg/kg	103
			Antinociceptive activity	Mice	Activity at dose 15g/kg for the 70% EtOH eluted fraction from 70% EtOH extract during writhing test	
		50% EtOH extract	Neuroprotective effect	Corticosterone-induced mitochondrial membrane potential (MMP) in rat pheochromocytoma (PC12) cells	Decrease of apoptosis and mitochondrial damage at dose 300 µg/mL and 500 µg/mL	105
	Roots	Aqueous extract	Anti-inflammatory	LPS-induced RAW264.7 cells	Decrease of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, NO and PGE2)	106
				Carrageenan-induced rat paw oedema	Anti-oedema effect	
<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	Triterpenoid saponins extract	Anti-inflammatory	CIA Wister female rats	Decrease of arthritis symptoms	107
<i>C. viticella</i>	Leaves	EtOH extract	Antimicrobial	<i>P. aeruginosa</i> PAO1 biofilm	> 60% of inhibition (raw extract)	108

Other common assays using chemical reactions can also be performed to determine the antioxidant activity. ABTS assay has been employed by Mostafa et al. (2018) on *C. brachiata* leaves. Methanol extract showed the highest activity with $IC_{50} = 60 \pm 0.80 \mu\text{g/mL}$ compared to acetone extract with $IC_{50} = 130 \pm 1.4 \mu\text{g/mL}$ and water extract with $IC_{50} = 170 \pm 0.75 \mu\text{g/mL}$. The trend is the same as with DPPH assay, the methanol extract being the most active, then the acetone extract, and finally the water extract. Both aqueous fractions from ethyl acetate extraction and from chloroform extraction of *C. flammula* have been compared using ABTS and ORAC assays ⁸¹. For ABTS, the aqueous fraction from chloroform extraction with 10.5 mmoles Trolox eq/g plant extract had a higher antioxidant capacity than the aqueous fraction from ethyl acetate with 7.9 mmoles Trolox eq/g plant extract. Whereas using ORAC assay the opposite result was observed with 487 mmoles Trolox eq/g of plant for the aqueous fraction from ethyl acetate and 387 mmoles Trolox eq/g of plant for the aqueous fraction from chloroform extraction (Atmani et al., 2011).

The ethanolic extract of the leaves of *C. flammula* showed $IC_{50} = 18.55 \pm 0.93 \mu\text{g/mL}$ using FRAP assay ⁶⁷. Besides the DPPH assay, ABTS and FRAP assays were conducted on ethanol and acetone extract of *C. gouriana*. IC_{50} value of $31.16 \mu\text{g/mL}$ and IC_{50} of $25.35 \mu\text{g/mL}$ were obtained respectively for ethanol and acetone extracts using ABTS. Respective values of $16.87 \pm 0.27 \mu\text{g eq Trolox/mg of extract}$ and $24.48 \pm 0.13 \mu\text{g eq Trolox/mg of extract}$ were obtained ⁶⁸. In addition to DPPH inhibition by *C. ispanhanica* and *C. orientalis* previously reported, FRAP inhibition was also determined with a value of 62.5% of inhibition at $300 \mu\text{g/mL}$ for *C. ispanhanica* and 64.13% of inhibition at $300 \mu\text{g/mL}$ for *C. orientalis* ⁷⁰.

Zhang et al., (2014) investigated the antioxidant activities of samples of polysaccharide precipitate from the ethanolic extract of *C. huchouensis* leaves' and stems' and demonstrated reducing power and scavenging effects of superoxide and hydroxyl radicals.

To conclude, both leaves and roots of some *Clematis* spp have been tested for antioxidant activity, leaves seem to present stronger antioxidant activities than the roots, especially using alcoholic extracts with ethanol or methanol. *C. flammula* is the species presenting the stronger antioxidant effect with the lower IC_{50} value using DPPH and an IC_{50} using FRAP of $18.55 \pm 0.93 \mu\text{g/mL}$ ⁶⁷.

The data obtained from chemical antioxidant tests, such as those mentioned, offer utility but solely reflect the activity within those conditions. Antioxidant assays employed are mainly chemical models based on proton and electron transfer. A good activity was observed for most of the tested extracts with *C. flammula* having the strongest antioxidant potential. For a more precise evaluation of a sample using chemical *in vitro* antioxidant potential, a comprehensive approach including various assays that target different antioxidant mechanisms is advisable

¹⁰⁹. However, *in vitro* assays using different cell models are needed to delve deeper into the antioxidant potential of those species.

Some *in vitro* enzymatic and cellular assays have been performed to measure the antioxidant capacity of *Clematis* extract. Lee et al. (2019) tested *C. apiifolia* ethanolic bark extract on HaCaT cells (human keratinocyte) and demonstrated the inhibition of ROS production, the inhibition of aryl hydrocarbon receptor activation and the regulation of the antioxidant system and therefore suggested the possible use of this extract as treatment for vitiligo or disorders induced by oxidative stress.

Antioxidant properties of the ethanolic extract of *C. flammula* leaves were also studied and confirmed its scavenging capability of the superoxide radical and the inhibition of xanthine oxidase ¹⁴. Moreover *C. flammula* extract has been reported as a pre-treatment to boost the activity of SOD (superoxide dismutase) and CAT (catalase) ⁸⁶. GSH (Glutathione) levels was also improved, and MDA (malondialdehyde) values declined, indicating its action in favour of the antioxidant system.

One *in vivo* study has been performed on the impact of the water extract of the aerial part of *C. chinensis* on diabetic rats ⁷⁵. The study revealed that *C. chinensis* extract enhanced the antioxidant activity of SOD and GSH-Px (Glutathione-Peroxidase) in dose-dependent manner, and the level of MDA in the renal cortex decreased. These results suggested that *C. chinensis* extract could be used to attenuate the oxidative stress in the kidney and therefore ameliorate kidney damage in diabetic rats.

5.2. Anti-inflammatory

Anti-inflammatory properties of some *Clematis* spp. extracts have been previously reported ¹⁰. As the anti-inflammatory properties of *Clematis* is widely used in traditional medicine and several species are employed according to the region, new studies have been performed more recently and are described below.

The anti-inflammatory activity of extracts from 11 species has been investigated since 2011. An ethanolic extract and its chloroform fraction obtained from *C. armandii* stem showed a moderate inhibition of the NO productions in LPS-stimulated BV-2 cells, primary microglia cells used as an *in vitro* neurodegenerative disease model. Chloroform fraction showed the highest activity with an $IC_{50} = 72.0 \pm 2.4 \mu\text{g/mL}$ and cell viability of $95.3 \pm 3.2 \%$ ¹⁶.

C. buchananiana, widely employed for traditional medicinal purposes, has been explored using *in vitro* assays and the capacity of *C. buchananiana* roots extract to stabilize the HRBC membrane was demonstrated, confirming its anti-inflammatory properties ⁸.

Traditional preparations involving *C. chinensis* roots and rhizomes have been tested using *in vivo* assays with AIA models. Huangqin Qingre Qubi Capsule (HQC), an extract produced in rice yellow wine with the dry roots of *C. chinensis* and other plants as *Notopterygium incisum* have

been shown to decrease pro-inflammatory cytokines as TNF- α , IL-6 and VEGF ^{7,46,47,76}. A clinical study on patients suffering from rheumatoid arthritis showed the reduction of inflammation using the traditional mixture and animal experiments on a rat model showed that HQC inhibits inflammation through the IL-17 signaling pathway ¹¹⁰

The anti-inflammatory properties of the total flavones content of *C. loureiroana* demonstrated a protective effect on oxLDL-induced (oxidized low-density lipoprotein) injury cells; an inhibition of expression of inflammatory factors IL-1 β and TNF- α and a reduction of apoptosis was observed ⁷⁹. Belkhir et al., (2023) compared the ethanolic extract of *C. flammula* leaves and its chloroform fraction. Results revealed a better inhibition of HRBC haemolysis by the ethanolic extract associated with a moderate inhibition of the COX activity with IC₅₀ = 223.26 $\pm$ 17.60 μ g/mL.

The butanol fraction of a methanolic extract of *C. florida* and *C. florida* var. *plena* roots were tested for anti-inflammatory activity and demonstrated the inhibition of NO production in LPS-induced RAW264.7 cells ¹⁷. The study was completed with an *in vivo* assay that showed the reduction of the paw oedema of AIA rats at a dose of 200 mg/kg/day and a reduction of the pro-inflammatory mediators IL-1 β , TNF- α and IL-6. Another *in vivo* assay on mice was performed, with a dose of 800 mg/kg, *C. florida* var. *plena* that allowed the inhibition of 48.7% of xylene-induced ear swelling on mice ⁸⁷. Both studies demonstrated a moderate anti-inflammatory activity of the species *C. florida* and one of its variety *C. florida* var. *plena*.

C. gouriana has been tested both on LPS-induced RAW264.7 cells and using an *in vivo* carrageenan-induced rat paw oedema model. The ethyl acetate fraction obtained from the 70% methanol extract of the aerial part demonstrated an inhibition of NO production at 100 μ g/mL as well as a reduction of 65.83% of oedema at 400 mg/kg after 5 h ⁸⁸. The roots of *C. henryi* Oliv. were extracted targeting triterpenes saponins. This extract demonstrated anti-inflammatory properties with an inhibition of pro-inflammatory cytokines and a significant anti-arthritis activity in a dose-dependent manner in collagen-induced arthritis in rats ⁹². Flowers of *C. orientalis* were extracted in 70% EtOH and tested at different doses on formaldehyde-induced arthritis in rats. Doses in a range of 50 – 200 mg/kg showed a reduction in paw swelling and its diameter ⁹⁹. Methanolic extract of *C. simensis* leaves decreased the swelling of carrageenan-induced paw oedema in mice after 4 h at 400 mg/kg ⁷¹.

C. terniflora leaves and stem were extracted using 70% EtOH and purified using macroporous resin. The 70% EtOH eluted fraction was tested and demonstrated an inhibition of NO and PGE₂ at a dose of 125 mg/mL in LPS-induced RAW264.7 cells completed by a reduction of the xylene-induced ear and carrageenan-induced paw oedema ¹⁰⁴. The *C. terniflora* aerial part has also been tested using carrageenan-induced chronic non-bacterial prostatitis rats to measure the inhibition of the inflammation of the prostate. At 15g/kg, results showed an inhibition of

the inflammation ¹⁰³. *C. terniflora* var. *manshurica* has been more recently tested, a water extract of the roots was realised and a decrease of the pro-inflammatory mediators, TNF- α , IL-1 β , IL-6, NO and PGE₂, were demonstrated in LPS-induced RAW264.7 cells ¹⁰⁶. The anti-oedema effect on the carrageenan-induced rat paw completed the study. ¹⁰⁷ and Li et al., (2017) specifically studied the triterpenoid saponins extract both on CIA rats with an amelioration of the arthritis symptoms.

In a clinical trial of a commercial medicine (SKI306X), a mixture containing a hydro-alcoholic extract of *C. terniflora* var *manshurica* roots was also shown to relieve the pain of osteoarthritis patients ¹⁰.

To conclude, both *in vitro* and *in vivo* assays have been performed to evaluate the anti-inflammatory effect of *Clematis* spp. Most of the *in vitro* assays have been performed using RAW 264.7 cells, a model widely used to measure different inflammatory mediators; while *in vivo* assays have been performed using the induced arthritis rat model. Both aerial part and roots have been tested and have demonstrated a decrease of pro-inflammatory mediators in different *in vitro* assays and a reduction of oedema in different *in vivo* assays. Therefore, those results support the traditional uses of *Clematis* spp. for anti-arthritis properties and the relief of joint pain.

Clematis spp. may have other anti-inflammatory applications such as for rheumatoid arthritis. *C. terniflora* has also shown an inhibition of prostate inflammation ¹⁰³ and *C. armandii* presented a moderate inhibition of NO on LPS-stimulated BV-2 cells, a model of cells employed for neurodegenerative diseases. Those results suggest that *C. terniflora* and *C. armandii* possess anti-inflammatory action with different applications.

5.3. Anti-microbial uses

Some antimicrobial activities of *C. apiifolia*, *C. dioscoreifolia*, *C. ganpiniana*, *C. hirsuta*, *C. papuasica* and *C. vitalba* extracts on different strains have been previously discussed ¹⁰. *C. brachiata*, *C. burgensis*, *C. flammula*, *C. grata*, *C. graveolens*, *C. hirsuta*, *C. lasiandra* and *C. longicauda* extracts have been studied since 2011 for antibacterial and antifungal properties. Agar disk or well diffusion methods are the most employed to determine the activity. Most of the bacteria and fungi species tested are involved in oral or respiratory infection.

It is reported that *C. brachiata*, *C. burgensis*, *C. flammula*, *C. graveolens*, *C. hirsuta* and *C. longicauda* displayed an inhibitory activity against *C. albicans* ^{56,83,84,91,93,112}. *C. brachiata* leaves extract have shown an activity against two other *Candida* spp. with $330 < \text{MIC} < 420 \mu\text{g/mL}$ supporting its traditional use against oral thrush ⁵⁶. The MIC₅₀ value of a *C. flammula* ethanolic extract has been determined on five strains of *C. albicans* with a mean value of $189.43 \mu\text{g/mL}$ ⁸³. The activity of a *C. flammula* leaves extract, produced with a choline chloride

based deep eutectic solvent, has been tested on three bacterial strains including *C. albicans* and a MIC value of 390 µg/mL was found for all strains⁸⁴.

C. burgensis extract displayed an activity against *S. sanguinis* with MIC value of 0.67 mg/mL⁷⁴. Belkhir et al., (2023) evaluated the antibacterial activity of *C. flammula* on *S. aureus* and *E. coli* cell cultures. The ethyl acetate fraction from the ethanolic extract presented a higher activity against *E. coli* with MIC value of 78.0 µg/mL. Methanolic extract of roots, leaves, and stems of *C. grata* have been studied individually by Kumari et al., (2019). Results showed a better inhibition of the stem extract against *S. aureus*. The essential oil of *C. graveolens* has been tested for antimicrobial activity against five different strains (*A. flavus*, *A. niger*, *B. cereus*, *C. albicans*, *E. coli*) using disc diffusion method and showed a diffusion value between 9.8 and 15.66 mm⁹¹.

C. hirsuta has been studied again with different types of extract or different parts of the plant against different microorganisms. Both chloroform and methanol extracts of *C. hirsuta* leaves have been tested and shown antibacterial activities at doses of 50 to 200 mg/mL⁶¹. MIC values of five extracts of leaves (acetone, chloroform, ethyl acetate, ethanol and methanol) have been determined; they showed values in the range of 7.5-60 mg/mL against *A. niger*, *C. albicans*, *E. coli*, *Penicillium* sp., *P. aeruginosa*, *S. aureus* and *Shigella* sp with a global highest activity of the acetone extract⁹³. Growth inhibition of *E. coli* has also been observed using roots extract of *C. hirsuta*⁶⁹. Adane et al., (2023) demonstrated the antibacterial activity (*S. aureus*, *S. pyogenes*, *E. coli* and *K. pneumoniae*) of leaves extracts using chloroform, ethanol, or petroleum ether solvents, with a better inhibition for the chloroform extract.

Ourabah et al., (2020) also determined the antibiofilm activity of *C. flammula* extract with an inhibition rate of biofilm formation of 36.78 ± 1.09 % at 500 µg/mL. The inhibition of *P. aeruginosa* biofilm was compared between ethanol extracts of *C. grata* and *C. viticella*¹⁰⁸ and the best inhibition of biofilm was obtained using *C. grata*.

Even if some studies reported in Table 11 present only diffusion method without any determination of MIC value, complicating the comparative study, most of the species tested presented inhibitory activities against bacteria and fungi involved in oral or respiratory infections. These activities support the traditional uses of *Clematis* spp. against cough, throat infection, or dental disease.

5.4. Miscellaneous enzyme inhibitory activity

Chohra et al., (2020) investigated miscellaneous enzyme inhibition activity of *C. cirrhosa*. Methanol and methanol/water (70/30) extracts were compared. Inhibition of acetylcholinesterase (AChE), amylase, butyrylcholinesterase (BChE), glucosidase and tyrosinase were determined at an extract concentration of 2 mg/mL. The methanol/water

extract showed the highest inhibition activity against AChE, amylase, and glucosidase enzymes with inhibition percentages between 40 and 87%. Further studies need to be performed on the plant, traditionally used in Algeria ⁶ as diuretic agent and for rheumatism pain, to demonstrate its corresponding bioactivity.

5.5. Cytotoxicity on cell lines

C. chinensis roots ethanolic extract, *C. ligustifolia* aerial part methanolic extract and *C. terniflora* var. *manshurica* aerial part ethanolic extract have been previously cited by Chawla et al., (2012) to have anti-cancer activity. More recently, *C. gouriana* and *C. flammula* have also been tested for anti-cancer activity. The hydrolat of the aerial part of *C. flammula* inhibited LPS-induced colon inflammation in Swiss albino mice⁸⁰. They also indicated that the hydrolat is a good sensitizer of TRAIL-induced (tumour necrosis factor-related apoptosis-inducing ligand) apoptosis in HCT-166 cells and suggested the hydrolat is a tumour suppressor. Ethanolic extract of leaves of *C. flammula* have also been studied and tested on different cell lines ¹⁴. The extract showed a moderate cytotoxic effect against HEPG2 and OVCAR-3, and a promising cytotoxic effect against the human ovarian cancer cell line A2780 with IC₅₀ = 77.0 µg/mL at 72 h. The cytotoxicity of the aqueous fraction obtained from chloroform extract of *C. flammula* has also been tested on two cell lines and the extract showed cytotoxicity against CHL and PLC cell lines, two human hepatoma cell lines, with IC₅₀ of 58.8 µg/mL and 47.3 µg/mL respectively ⁸¹. These studies suggest that polar extracts and fractions of *C. flammula* possess significant cytotoxicity against several cancer cell lines.

Cytotoxicity of the ethyl acetate fraction from aqueous acetone extract of *C. gouriana* has been studied and shown to have a moderate activity with inhibition of cell growth at a percentage of 62.34 ± 0.03 at 100 µg/mL and 64.45 ± 0.80 at 100 µg/mL after 48 h on C6 (glioma cell line) and CHOK1 (Chinese Ovarian Hamster) cell lines respectively ⁶⁸.

5.6. Wound healing activity

Clematis spp. is used in folk medicine for skin applications as wound healing for example. *C. montevidensis* particularly, has been identified to be used for the treatment of wounds in South America ¹⁰. This species has not been the subject of biological studies on wound healing, but other species such as *C. burgensis*, *C. longicauda*, *C. simensis* and *C. gouriana* have been studied in this regard ^{71,89,112}. Leaves have been studied using methanol or a mixture of methanol and water for extraction and the extracts have been incorporated into different topical cream formulas to be applied on the wound.

C. longicauda's and *C. burgensis*' *in vivo* activity has been compared using excision wound model and re-sutured incision wound model ¹¹². Both *C. longicauda* and *C. burgensis* tested at 10% (w/w) concentration in an ointment base showed a positive wound healing effect implying a faster epithelialization.

To study the wound healing activity of *C. gouriana*, excision and incision models were treated using a 0.2% (w/w) gel while a dead space wound model was treated orally via suspension with a final administration of 30 mg/kg body-weight⁸⁹. Taken together, results demonstrated a significant reduction in wound area, a faster rate of epithelialization and an increase in the tensile strength using *C. gouriana* topically; and the oral administration of *C. gouriana* showed an increase in the granulation tissue weight. Those results suggest a significant wound healing activity of *C. gouriana*.

To study the wound healing activity of *C. simensis* two models have been employed⁷¹. Ointments were prepared at 5% (w/w) and 10% (w/w) of an 80% methanol extract. Topical application of *C. simensis* showed a significant improvement in wound contraction, epithelization and a decrease in wound surface area; all these results therefore suggest a significant wound healing activity of *C. simensis* in topical applications.

These three studies on wound healing applications all employed methanolic extract of *Clematis* spp. and demonstrated wound healing activity even at a low concentration of 0.2% (w/w) for *C. gouriana*⁸⁹.

5.7. Other activity

Antinociceptive and neuroprotective activity of *C. terniflora* has been explored^{103–105}. *C. terniflora* var. *manshurica*, widely employed in traditional medicine in China and in Korea^{57,113}, was shown to decrease the reaction time of mice using hot-plate test at a dose of 150 mg/kg¹⁰⁴ and to possess a neuroprotective effect in rat pheochromocytoma cells at a dose of 300 µg/mL¹⁰⁵.

Insecticidal activity of *C. aesthusifolia* against *Plutella xylostella* (L.) larvae⁷² and of *C. lasiandra* against *Acyrtosipon pisum* Harris⁹⁷ has been investigated. The ethanolic extract of *C. aesthusifolia* was compared with petroleum ether, EtOAc, n-BuOH and water fractions and the n-BuOH extract induced the highest mortality of *P. xylostella* (L.) larvae with a percentage of $96.43 \pm 3.57\%$ at 24 h⁷². The highest insecticidal activity against *A. pisum* was demonstrated with the n-BuOH fraction from 70% EtOH extract of *C. lasiandra*⁹⁷. A significant aphicide activity of the n-BuOH fraction with a LC₅₀ value of 2.81 mg/mL was determined.

Both studies showed better insecticidal activity of the butanol fraction obtained from a 70% ethanol extract suggesting bioactive compounds with insecticidal activity are mainly present in this fraction. Further studies still need to be performed to suggest *C. aesthusifolia* and *C. lasiandra* compounds as novel biopesticides.

Some *Clematis* spp. can also be traditionally used to protect cultures of *C. flammula* in Australia. The flower of *C. flammula* has been explored for antimicrobial activity against *Rhizoctonia solani*, responsible for a disease affecting potatoes, beetroots, or carrots. A

moderate activity of hexane and EtOAc extracts of the flower, with MIC value of 1560 µg/mL and 700 µg/mL respectively, have been determined ⁸².

One anti-viral activity has been explored and a moderate anti-tabacco mosaic virus activity of *C. lasiandra* was demonstrated ¹¹⁴. Both n-BuOH and EtOAc fractions of the 70% ethanol aerial part extract demonstrated a moderate anti-tabacco mosaic virus activity on *N. glutinosa* plant (less than 74% at 20 mg/mL for both).

The molluscicide activity of *C. flammula* flower has been studied against *G. truncatula* ⁸². A similar LC₅₀ value of 11.87 mg/mL and 11.65 mg/mL for both hexane and EtOAc extracts was determined. These extracts, at LC₅₀, did not show any negative effects on the various species of the associated fauna present in the south-west region of Tunisia after 48 h of exposure.

An *in vivo* anti-malarial study of roots of *C. simensis* has been assessed ¹⁰¹. The chloroform fraction of the hydroalcoholic extract, tested at a dose of 600 mg/kg, suggested a moderate anti-malarial activity of *C. simensis* roots. Further studies are necessary to confirm a potential anti-malarial activity of *C. simensis*, traditionally used in Ethiopian folklore medicine for such application.

In general, both the aerial parts and the roots have shown activities, thus justifying their use in traditional medicine. Indeed, different *Clematis* spp. extracts have been shown to decrease the level of proinflammatory cytokines^{7,46,47,76,106}, preventing chronic inflammation and to present different antimicrobial activities^{56,83,84,91,93,112}. The combination of these two *in vitro* bioactivities can explain one of the most studied *in vivo* activity, wound healing ¹¹⁵. Indeed, even if inflammation is an essential step of the wound healing cascade, it is a rapid and highly orchestrated process, and a prolonged inflammation may result in deregulated stages of the wound healing which may lead to excessive scarring. Antimicrobial activities of extracts may act in synergy with the inflammatory step to help the immune system fight against invading pathogens while the anti-inflammatory activity will help to solve the inflammation process, thus reducing the risk of chronic wounds. These also justify the use of *Clematis* spp. in traditional medicine all over the world for such activities. The more polar extracts and fractions appear to be the most active, suggesting a good potential for polar and medium-polar metabolites contained in those extracts. However, more information regarding potential adverse effects and *in vivo* studies of extracts is needed to confirm some activities.

6. Phytochemistry and pharmacological activities of isolated compound

Preliminary phytochemical studies of some species have been recently performed using chemical reactions to identify families of components present in the plant. The phytochemical composition of *C. hirsute*, widely spread in Ethiopia and used in traditional medicine, has been explored and revealed the presence of alkaloids, saponins, tannins, phenols, and flavonoids in the methanol roots extract; and it was shown to be associated to a moderate antioxidant and

antimicrobial activity but no precise identification has been performed ⁶⁹. Deeper phytochemical studies on the genus *Clematis* have resulted in the isolation of diverse chemical compounds. Specialized metabolites of the genus *Clematis* are diverse and can lead to different biological activities. The main chemical compounds reported from *Clematis* species are terpenoids, phenolic compounds, alkaloids, and compounds such as macrocycles or anemonin derivatives.

Structurally diverse and biologically active compounds have been identified from 28 species of *Clematis*: *C. aethusifolia*, *C. akebioides*, *C. apiifolia*, *C. armandii*, *C. brevicaudata*, *C. chinensis*, *C. florida*, *C. gouriana*, *C. grata*, *C. graveolens*, *C. heracleifolia*, *C. hexapetala*, *C. hirsuta*, *C. intricata*, *C. lasiandra*, *C. ligustifolia*, *C. montana*, *C. orientalis*, *C. parviloba*, *C. puberula*, *C. songarica*, *C. stans*, *C. tangutica*, *C. terniflora*, *C. tibetana*, *C. uncinata*, *C. vitalba* and *C. viticella*. Molecules are grouped in Tables 12 - 15 with their associated bioactivity at the time that it was tested. 538 different compounds are reported, including 121 novel structures.

6.1. Terpenes and terpenoids

Terpenes and terpenoids such as monoterpenes, sesquiterpenes and triterpenes have been isolated from the genus *Clematis* (Table 12). Most of the triterpenes isolated are saponins.

a. Monoterpenoids

Among the monoterpenoids isolated from *Clematis* species, 14 monocyclic monoterpenoids, including 12 monoterpenoids glycosides, were obtained from the n-butanolic fraction of the ethanolic extract of the aerial part of *C. chinensis*¹¹⁶. A new stereoisomeric monoterpene glucoside, (6Z)-9-hydroxylinaloyl glucoside, was identified in the n-butanolic of the methanolic extract of *C. heracleifolia* leaves¹¹⁷. 9-hydroxylinaloyl glucoside, was identified in both *C. akebioides* stems and *C. heracleifolia* leaves ^{117,118}.

b. Sesquiterpenoids

Several glycosylated sesquiterpenoids have been found in *C. chinensis* and *C. hexapetala*, including 5 new structures. (3S,10R)-3-O-β-D-apiofuranosyl-(1->6)-β-D-glucopyranosyloxy-10,11-dihydroxy-3,7,11-trimethyldodeca-1,6-diene and (3S,10R)-3,10-β-D-diglucopyranosyloxy-11-hydroxy-3,7,11-trimethyldodeca-1,6-diene have both been isolated from the butanol subfraction of the ethanolic extract of the aerial part of *C. chinensis*. These two compounds did not show cytotoxicity against MGC6803 and Ishikawa cells ¹¹⁶. Three guaianolide sesquiterpenoids, hexapetol A, 11-epi-hexapetol A and 11-epi-hexapetol A-3-O-β-D-glucopyranoside, have been recently isolated from *C. hexapetala* ¹¹⁹. These molecules were tested for anti-inflammatory activity against LPS stimulated TNF-α and NO release in RAW 264.7 cells and PTP1B inhibitory activity assay, but no activity was determined.

c. Triterpenoids

Of the terpene class, triterpenes are the most common in the *Clematis* genus and most of them are specifically triterpenoid saponins. More than 40 new structures of triterpenoids have been discovered since 2011. Triterpenoids have been isolated from *C. argentea*, *C. tangutica* and *C. uncinata* roots and *C. chinensis*, *C. heracleifolia*, *C. lasiantha*, *C. manshurica* and most of the time, they have been found in the roots and rhizomes part of the plant, suggesting that this part is richer in saponins than the aerial part. The main activities targeted are anti-cancer activity (hepatocellular carcinoma, gastric carcinoma, glioblastoma, colorectal cancer, human leukaemia, etc.), cardioprotective, anti-inflammatory, and insecticidal properties.

The most common skeleton for triterpenoids found in the genus *Clematis* is oleanane type, and more particularly hederagenin and its derivatives. Clematangenin B and huzhangoside B, both isolated for the first time in the species *C. florida* var. *plena*, have demonstrated an anti-inflammatory activity using the xylene-induced ear oedema assay with 54.7% and 50.9% of inhibition, respectively, at a dose of 200 mg/kg⁸⁷. Clematangenin B has also been isolated by Yang et al. (2021) together with huzhangoside C, and both showed the reduction of the pro-inflammatory mediators' level in LPS treated RAW264.7 cells.

AR-6, a triterpenoid saponin isolated from the roots of *C. chinensis*, has been investigated for anti-inflammatory effects and it was shown that treatment using AR-6 reduced TNF- α and IL-1 β level, two inflammatory mediators and the symptoms of arthritis in CIA rats, which could explain the use of the roots of *C. chinensis* for rheumatoid arthritis in Chinese traditional medicine¹²⁰. Nine triterpenoids including four new structures, clematichinenoside H-K, have been isolated from *C. chinensis* and their activity against NO production in LPS-induced RAW264.7 macrophages has been evaluated¹²¹. Among the new structures, clematichinenoside I showed the highest activity with IC₅₀ value of 28.2 $\pm$ 3.7 μ M. Mandshunoside F, mandshunoside G, mandshunoside H and mandshunoside I, four new compounds from the roots and rhizomes of *C. ternstroemia* var. *mandshurica*, have been evaluated for their inhibitory activity against LPS-induced NO production in RAW264.7 macrophages¹²². Mandshunoside G revealed the highest activity with IC₅₀ = 8.3 μ M and a cell viability superior to 90%¹²². The anti-inflammatory properties of clematichinenoside AR extracted from *C. chinensis* roots and rhizomes have been investigated by Xiong et al. (2019) and Yan et al. (2015). Both suggested the effect of clematichinenoside AR to antagonize the inflammatory and cytotoxic activities induced by TNF- α .

Cheng et al. (2018) isolated a hederagenin saponin from *C. ganpiniana* and demonstrated its strong cytotoxicity effect on two breast cancer cell lines and the induction of apoptosis in breast cancer cells via the mitochondrial pathway. Zhao et al. (2014a) tested the cytotoxicity effect of saponin CP4 against different tumour cell lines and showed this compound presented

cytotoxicity on leukaemia HL-60 cells, hepatocellular carcinoma Hep-G2 cells and gastric carcinoma SGC-7901 cells with IC_{50} of $4.13 \pm 0.15 \mu M$, $6.52 \pm 0.53 \mu M$ and $IC_{50} = 5.13 \pm 0.32 \mu M$, respectively. The cytotoxicity of triterpenoids isolated from *C. lasiandra* have also been tested on the three same tumour cell lines and showed an IC_{50} in the range from 1.40 to 19.50 μM for five compounds. The two non-active compounds did not present the free carboxylic acid group at position C-28, suggesting the importance of the carboxylic acid group in the relation between structure and activity ¹²⁷.

3- β -O-[β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl] hederagenin-11,13-dien-28-oic acid and 3- β -O-{ β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)]- β -D-xylopyranosyl} oleanolic acid were isolated for the first time from the butanol fraction of the ethanolic extract of *C. argentea* roots ¹²⁸. Both compounds were tested for cytotoxicity and presented an $IC_{50} = 6.61 \pm 1.40 \mu M$ and $IC_{50} = 3.82 \pm 0.28 \mu M$ against HL-60 cells, an $IC_{50} = 5.30 \pm 1.90 \mu M$ and $IC_{50} = 2.74 \pm 0.34 \mu M$ against Hep-G2 cells, and an $IC_{50} = 25.40 \pm 0.87 \mu M$ and $IC_{50} = 10.35 \pm 1.34 \mu M$ against human glioblastoma U251MG cells. Zhao et al. (2014b) determined comparable values of IC_{50} against U251MG cells with a value of $21.07 \pm 2.05 \mu M$ for 3- β -O-[β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl] hederagenin-11,13-dien-28-oic acid. 3-O- β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl oleanolic acid extracted from the whole *C. lasiandra* plant with 70% ethanol, was tested against HL-60, HepG2 and SGC-7901, with $IC_{50} = 1.40 \pm 0.52 \mu M$, $IC_{50} = 19.50 \pm 5.20 \mu M$ and $IC_{50} = 8.37 \pm 0.17 \mu M$, respectively. Eight new oleanane-type triterpenoid saponins, clematangoticosides A-H, have been isolated from *C. tangutica* together with eight already known structures ¹³⁰. Among the new structures, only clematangoticoside D and F have shown cytotoxicity against SGC-7901 with IC values of $24.14 \pm 2.10 \mu M$ and $21.35 \pm 1.88 \mu M$, respectively. Mandshunoside C, mandshunoside D, and mandshunoside E—three new compounds from the roots and rhizomes of *C. mandshurica*—were evaluated for their cytotoxicity against HCT-116 and HT-29, two human colorectal cancer cell lines. Mandshunoside D had the highest activity on both cells lines with $IC_{50} = 0.6 \mu M$ against HCT-116 and $IC_{50} = 0.9 \mu M$ against HT-29 ¹³¹.

The cytotoxicity of 7 triterpenoids extracted from *C. tangutica*, including the novel compound 3-O- β -D-allopyranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl hederagenin 28-O- pyranosyl ester have been tested against HGC-27 (gastric cancer), SK-OV-3 (ovarian adenocarcinoma), MGC-803 (gastric cancer) and GES-1 (normal gastric mucosal epithelial cells). All the seven compounds tested were inactive to GES-1 and MGC-803. The new compound presented a selective cytotoxicity against HGC-27 with an IC_{50} value of $20.1 \pm 0.5 \mu M$ and hederasaponin B was active against HGC-27, HeLa, and SK-OV-3 with IC_{50} values of $16.4 \pm 0.6 \mu M$, $71.3 \pm 3.9 \mu M$, and $64.4 \pm 3.8 \mu M$, respectively.

The cytotoxicity of nineteen isolated compounds extracted from *C. uncinata* have been tested on CaSki cervical cancer cells ¹³². Among these nineteen triterpenoid saponins, hederasaponin CP4, hederasaponin CP7, and huzhangoside C presented interesting IC₅₀ values of 2.97 ± 1.07 µM, 26.07 ± 1.47 µM, and 9.05 ± 3.61 µM, respectively.

3-O- $\{\beta$ -D-xylopyranosyl-(1→2)- $\{\beta$ -D-glucopyranosyl-(1→4)- α -L-arabinopyranosyl} hederagenin 28-O- α -L-rhamnopyranosyl-(1→4)- β -D-glucopyranosyl-(1→6)- β -D-glucopyranoside, 3-O- β -D-ribopyranosyl oleanolic acid 28-O- α -L-rhamnopyranosyl-(1→4)- β -D-glucopyranosyl-(1→6)- β -D-glucopyranoside and 3-O- β -D-xylopyranosyl-(1→2)- α -L-arabinopyranosyl hederagenin 28-O- α -L-rhamnopyranosyl-(1→4)- β -D-glucopyranosyl-(1→6)- β -D-glucopyranoside were characterized for the first time and were shown to provide a cardioprotective effect by decreasing levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) ¹³³. Ciwujianoside C3, hederacholichiside E, kalopanaxsaponin G, and saponin PJ3—also isolated from *C. tangutica*—participated in the cardioprotective activity of the extract ¹³³. Asperosaponin VI, cauloside D, clematangoside B, leontoside, clematangoside C, kizutasaponin K12, clematibetoside C, hederacholichiside F were isolated from the whole *C. tangutica* plant and demonstrated a protective effect in hypoxia-induced myocardial injury ¹³⁴. A new ursane-type and a new taraxerane-type saponin, two skeletons less frequently found in the genus *Clematis*, have been isolated together with 7 already known triterpenes saponins ¹²⁹.

Other bioactivity has also been investigated for some triterpenoids saponins. Saponin CP4 has been isolated from the ethanolic extract of *C. aethusifolia* and from roots of *C. argentea*. Tian et al., (2021) studied the antifeedant and insecticidal activity of saponins and determined 69.87% of growth inhibition after 48 h at 1500 µg/mL using saponin CP4. Clematoside S and 3-O- β -D-ribopyranosyl-(1 → 3)- α -L-rhamnopyranosyl-(1→2)- $\{\beta$ -D-glucopyranosyl-(1→4)- β -D-xylopyranosyl hederagenin, both isolated from *C. aethusifolia*, demonstrated similar insecticidal activity to saponin CP4 previously described ⁷². Clematograpeolenoside A, characterized for the first time, clematoside S, huzhangoside D, and tomentoside A have been extracted and isolated from *C. graveolens*. The four compounds have been tested for insecticidal activity. Tomentoside A showed the highest activity with LC₅₀ of 1.2 and 0.5 mg/mL after 72 and 96 h against *Aphis craccicova* and LC₅₀ of 0.1 mg/mL after 24 h against termite, *Coptotermis homii* ¹³⁵.

Triterpenoids from *C. lasiandra* have been tested for aphicidal activity against *A. psidium*. All compounds presented a significant aphicidal activity through oral toxicity with LC₅₀ comprised between 0.13 mg/mL and 0.98 mg/mL after 72 h ⁹⁷.

The main triterpenoids identified in the *Clematis* genus are saponins with oleanane-type skeleton and among the compounds tested, triterpenoids saponins extracted from *C. florida*,

C. terniflora, and *C. chinensis* have shown *in vivo* and *in vitro* anti-inflammatory properties which could explain the use of *Clematis* spp in traditional medicine.

d. Steroids

Very few steroids have been identified in the genus *Clematis*, Chawla et al. (2012) mentioned 7 steroids including stigmasterol, 5- α -stigmastane-3- β -6- α -diol, stigmasterol-3-O- β -D-glucopyranoside, 3- β -hydroxy-stigmast-5,22-dien-7-one, daucosterol, β -sitosterol, and β -sitosterol- β -D-glucoside. More recently, ikshusterol-3-O-glucoside has been isolated from the ethyl acetate extract of *C. gouriana* roots and *in vitro* phospholipase A₂ assay for anti-snake venom *Naja naja* (Indian cobra) activity revealed 30% of inhibition at 1000 μ g/mL¹³⁶.

Table 12: Terpenoids isolated from *Clematis* species and their related biological activities (* structures newly described since 2011)

	Compound name	Species	Part of the plant	Extraction	Activity	Biological activity of the isolated compound	Ref.
Mono-terpenoids							
1	(+)-dehydrovomifoliol	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 (human gastric carcinoma) with $IC_{50} = 21.32 \pm 1.71 \mu M$ Ishikawa cells (human endometrial adenocarcinoma) with $IC_{50} = 17.96 \pm 0.82 \mu M$	116
2	(3S,5R,6R,7E,9S)-megastigman-7-ene-3,5,6,9-tetrol 3-O- β -D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 20.35 \pm 2.68 \mu M$ Ishikawa cells with $IC_{50} = 53.32 \pm 1.82 \mu M$	116
3	(6Z)-9-hydroxylinaloyl glucoside*	<i>C. heracleifolia</i>	Leaves	n-BuOH fraction from MeOH extract			117
4	9-hydroxylinaloyl glucoside	<i>C. akebioides</i> , <i>C. heracleifolia</i>	Leaves, stems	n-BuOH fraction from MeOH extract, EtOAc			117 118
5	Alangionoside E	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 11.09 \pm 2.63 \mu M$ Ishikawa cells with $55.06 \pm 1.56 \mu M$	116
6	Byzantionoside B	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 18.88 \pm 3.31 \mu M$ Ishikawa cells with $IC_{50} = 0.02 \pm 0.01 \mu M$	116
7	Citroside A	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 22.56 \pm 2.35 \mu M$ Ishikawa cells with $IC_{50} = 3.77 \pm 1.51 \mu M$	116
8	Corchoionol C	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 20.85 \pm 1.53 \mu M$ Ishikawa cells with $IC_{50} = 10.57 \pm 1.39 \mu M$	116
9	Icariside C3	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract			116
10	Linarionoside A	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract			116
11	Linarionoside C	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 62.37 \pm 2.24 \mu M$ Ishikawa cells with $IC_{50} = 22.95 \pm 2.45 \mu M$	116

12	Myrsinioside A	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 22.03 \pm 1.49 \mu M$ Ishikawa cells with $IC_{50} = 9.28 \pm 1.27 \mu M$	116
13	Platanioside A	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 15.68 \pm 2.14 \mu M$ Ishikawa cells with $IC_{50} = 52.58 \pm 2.58 \mu M$	116
14	Platanioside D	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 7.85 \pm 1.18 \mu M$ Ishikawa cells with $IC_{50} = 1.41 \pm 0.82 \mu M$	116
15	Turpinioside A	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: MGC-803 with $IC_{50} = 13.88 \pm 1.13 \mu M$ Ishikawa cells with $IC_{50} = 48.60 \pm 2.34 \mu M$	116
Sesqui-terpenoids							
16	(3S,10R)-3,10-β-D-diglucoopyranosyloxy-11-hydroxy-3,7,11-trimethyldodeca-1,6-diene*	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract			116
17	(3S,10R)-3-O-β-D-apiofuranosyl-(1'6)-b-D-glucopyranosyloxy-10,11-dihydroxy-3,7,11-trimethyldodeca-1,6-diene*	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract			116
18	11,13-dihydroglucozaluzanin C	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH			119
19	11,13-dihydrozalunin C	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH			119
20	11-epi-hexapetol A*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH			119
21	11-epi-hexapetol α-3-O-beta-d-glucopyranoside*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH			119
22	Hexapetol A*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH			119
Triterpenoids							
23	30-nor arjunolic acid	<i>C. ligusticifolia</i>					20
24	3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]-hederagenin	<i>C. akebioides</i>	Stems, aerial part	EtOAc fraction from 80% EtOH			118
25	3-O-[β-D-xylopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 4)]-α-L-arabinopyranosyl] hederagenin 28-O-α-L-rhamnopyranosyl-(1 → 4)-	<i>C. akebioides</i> , <i>C. tangutica</i>	Whole plant, aerial part, stems	EtOAc fraction from 70 or 80% EtOH	Cardioprotective activity	Weak decrease of the levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in cardiomyocytes anoxia/reoxygenation (A/R) model	133 118

	β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside*						
26	3-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-O- β -D-glucopyranosyl hederagenin	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: U251MG cells (human glioblastoma) with IC ₅₀ = 25.16 $\pm$ 3.78 μ M	129
27	3-O- β -D-allopyranosyl (1 $\rightarrow$ 3)- α -L-rhamnopyranosyl (1 $\rightarrow$ 2)- α -L-arabinopyranosyl hederagenin 28-O-pyranosyl ester*	<i>C. tangutica</i>		n-BuOH extract	Anti-cancer	Cytotoxicity against: HGC-27 (gastric cancer) with IC ₅₀ = 20.1 $\pm$ 0.5 μ M	137
28	3-O- β -D-glucopyranosyl (1 $\rightarrow$ 3)-[α -L-rhamnopyranosyl(1 $\rightarrow$ 6)]- β -D-glucopyranosyl (1 $\rightarrow$ 4)- β -D-glucopyranosyl(1 $\rightarrow$ 4)- β -D-ribopyranosyl(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
29	3-O- β -D-ribopyranosyl- (1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)]- α -L-arabinopyranosyl oleanolic acid 28-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside*	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	NA	129
30	3-O- β -D-ribopyranosyl oleanolic acid 28-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside*	<i>C. tangutica</i>	Whole plant	70% EtOH	Cardioprotective activity	Decrease of the levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in cardiomyocytes anoxia/reoxygenation (A/R) model	133
31	3-O- β -D-ribopyranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl oleanolic acid 28-O- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside*	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	NA	129
32	3-O- β -D-ribopyranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)]- α -L-arabinopyranosyl hederagenin*	<i>C. lasiantha</i>	Whole plant	70% EtOH	Anti-cancer	Cytotoxicity against: HL-60 (tumour human leukaemia) with IC ₅₀ = 7.02 $\pm$ 0.24 μ M Hep-G2 (human hepatocellular carcinoma) with IC ₅₀ = 3.35 $\pm$ 0.17 μ M SGC-7901 (human hepatocellular carcinoma) with IC ₅₀ = 1.96 $\pm$ 0.34 μ M	127

33	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl oleanolic acid 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside	<i>C. chinensis</i> , <i>C. florida</i> , <i>C. florida</i> var. <i>plena</i>	Roots, roots and rhizomes	MeOH, 70% EtOH, n-BuOH fraction from MeOH extract	Anti-inflammatory	NA	121 17 87
34	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl oleanolic acid*	<i>C. apiifolia</i> var. <i>argenteilucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 cells with IC ₅₀ = 2.91 ± 0.24 μM Hep-G2 cells with IC ₅₀ = 2.26 ± 0.18 μM SGC-7901 cells (gastric carcinoma) with IC ₅₀ = 0.87 ± 0.09 μM	129 121
35	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylopyranosyl hederagenin	<i>C. aethusifolia</i>	Whole plant	70% EtOH	Anti-insecticidal activity	Antifeedant activity against 3rd instar larvae of <i>P. xylostella</i> with DC ₅₀ = 744.24 μg/mL at 48 h Insecticidal activity against <i>P. xylostella</i> with LC ₅₀ = 1652.15 μg/mL after 72 h Insect growth inhibitory activity against <i>P. xylostella</i> with 69.58% of growth inhibition after 48 h at 1500 μg/mL	72
36	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylopyranosyl hederagenin*	<i>C. lasiandra</i>	Whole plant	70% EtOH	Anti-cancer	Cytotoxicity against: HL-60 (tumour human leukaemia) with IC ₅₀ = 9.23 ± 0.25 μM Hep-G2 (human hepatocellular carcinoma) IC ₅₀ = 5.62 ± 0.29 μM SGC-7901 (human hepatocellular carcinoma) IC ₅₀ = 3.75 ± 0.39 μM	127
37	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylopyranosyl oleanolic acid	<i>C. apiifolia</i> var. <i>argenteilucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 cells (tumour human leukaemia) with IC ₅₀ = 3.29 ± 0.21 μM Hep-G2 cells (human hepatocellular carcinoma) with IC ₅₀ = 3.79 ± 0.29 μM SGC-7901 cells (gastric carcinoma) with IC ₅₀ = 1.94 ± 0.40 μM	129
38	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl oleanolic acid	<i>C. lasiandra</i>	Whole plant	70% EtOH	Anti-cancer	Cytotoxicity against: HL-60 (tumour human leukaemia) with IC ₅₀ = 3.29 ± 0.21 μM Hep-G2 (human hepatocellular carcinoma) with IC ₅₀ = 6.52 ± 0.53 μM	127

							SGC-7901 (human hepatocellular carcinoma) with IC ₅₀ = 5.13 ± 0.32 µM		
39	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl 3β,21α-dihydroxyolean-12-en-28-oic acid*	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	NA		129	
40	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl hederagenin	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 cells (tumour human leukaemia) with IC ₅₀ = 12.74 ± 0.29 µM Hep-G2 cells (human hepatocellular carcinoma) with IC ₅₀ = 9.97 ± 0.40 µM SGC-7901 cells (gastric carcinoma) with IC ₅₀ = 11.50 ± 0.37 µM		129	
41	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl hederagenin*	<i>C. lasiandra</i>	Whole plant	70% EtOH	Anti-cancer	Cytotoxicity against: HL-60 (tumour human leukaemia) with IC ₅₀ = 12.74 ± 0.29 µM Hep-G2 (human hepatocellular carcinoma) IC ₅₀ = 9.97 ± 0.40 µM SGC-7901 (human hepatocellular carcinoma) with IC ₅₀ = 11.50 ± 0.37 µM		127	
42	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl oleanolic acid	<i>C. apiifolia</i> var. <i>argenteolucida</i> , <i>C. lasiandra</i>	Whole plant, roots	70% EtOH, n-BuOH fraction from 70% EtOH	Anti-cancer	Cytotoxicity against: U251MG cells (human glioblastoma) with IC ₅₀ = 6.95 ± 0.87 µM HL-60 (tumour human leukaemia) IC ₅₀ = 1.40 ± 0.52 µM Hep-G2 (human hepatocellular carcinoma) IC ₅₀ = 19.50 ± 5.20 µM SGC-7901 (human hepatocellular carcinoma) IC ₅₀ = 8.37 ± 0.17 µM		129 127	
43	3-O-β-D-ribosepyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl oleanolic acid 28-O-β-D-glucopyranosyl ester*	<i>C. aethusifolia</i> , <i>C. lasiandra</i>	Whole plant	70% EtOH	Anti-cancer	NA		127 72	
44	3-O-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside*	<i>C. chinensis</i> , <i>C. tangutica</i>	Whole plant, roots and rhizomes	70% EtOH	Anti-inflammatory Cardioprotective	NA Decrease of the levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in cardiomyocytes anoxia/reoxygenation (A/R) model		121 133	

45	3-β-[(α-L-arabinopyranosyl)-oxy]olean-12-en-28-oic acid	<i>C. puberula</i> var. <i>ganpiniana</i>	Roots and rhizomes				18
46	3-β-O-[β-D-ribofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl] hederagenin-11,13-dien-28-oic acid*	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 cells (tumour human leukaemia) with IC ₅₀ = 6.61 ± 1.40 μM Hep-G2 cells (human hepatocellular carcinoma) with IC ₅₀ = 5.30 ± 1.90 μM U251MG cells (human glioblastoma) with IC ₅₀ = 25.40 ± 0.87 μM	128
47	3-β-O-[β-D-ribofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-23-hydroxy-olean-11,13-dien-28-oic acid	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against U251MG (human glioblastoma) with IC ₅₀ = 21.07 ± 2.05 μM	129
48	3-β-O-[β-D-xylofuranosyl-(1→3)-α-L-rhamno pyranosyl-(1→2)-β-D-glucopyranosyl]-28-hydroxy-18α-H-ursan-20-en*	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against U251MG cells (human glioblastoma) with IC ₅₀ = 38.51 ± 5.09 μM	129
49	3-β-O-[β-D-xylofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-12-oleanene-3b,28-diol*	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	NA	22
50	3-β-O-[β-D-xylofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-28-hydroxy-olean-12-en	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: U251MG cells (human glioblastoma) IC ₅₀ = 28.38 ± 3.16 μM	129
51	3-β-O-[β-D-xylofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-28-hydroxy-taraxeran-14-en*	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: U251MG cells (human glioblastoma) with IC ₅₀ = 29.08 ± 4.33 μM	129
52	3-β-O-[β-D-ribofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylofuranosyl] oleanolic acid*	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 cells (tumour human leukaemia) with IC ₅₀ = 3.82 ± 0.28 μM Hep-G2 cells (human hepatocellular carcinoma) with IC ₅₀ = 2.74 ± 0.34 μM U251MG cells (human glioblastoma) with IC ₅₀ = 10.35 ± 1.34 μM	128

53	3-β-O-α-L-rhamnopyranosyl-(1→2)-arabinopyranosyl oleanolic acid	<i>C. puberula</i> var. <i>ganpiniana</i>	Roots and rhizomes				18
54	3-β-O-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside	<i>C. tangutica</i>	Whole plant	Butanol fraction from 75% MeOH extract	Anti-cancer	NA	130
55	Anhuenoside E	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	NA	129
56	Ar-6	<i>C. chinensis</i>	Roots and rhizomes		Anti-inflammatory	Inhibition of production of proinflammatory mediators (TNF-α and IL-1B) and NF-κB p65 sub-units (TNF-α and COX-2) expression in collagen-induced arthritis rat model	120
57	Arjunolic acid	<i>C. ligusticifolia</i> , <i>C. montana</i>					20
58	Asperosaponin VI	<i>C. tangutica</i>	Whole plant	70% EtOH	Cardioprotective activity	Protective effect against LDH and CK-MB in hypoxia-induced myocardial injury model with ED ₅₀ values of 120.33 ± 7.32 μM and 15.17 ± 0.35 μM	134
59	Cauloside D	<i>C. heracleifolia</i> , <i>C. tangutica</i>	Whole plant	70% EtOH, n-BuOH fraction from 75% EtOH extract	Cardioprotective activity	Protective effect against LDH and CK-MB in hypoxia-induced myocardial injury model with ED ₅₀ values of 127.22 ± 8.42 μM and 121.92 ± 8.15 μM	134 138
60	Ciwujianoside C3	<i>C. heracleifolia</i> , <i>C. tangutica</i>	Whole plant	70% EtOH, n-BuOH fraction from 75% EtOH extract	Cardioprotective activity	Decrease of the levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in cardiomyocytes anoxia/reoxygenation (A/R) model	134 138
61	Clemargenoside H	<i>C. apiifolia</i> var. <i>argenteolucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	NA	129
62	Clemastanoside D	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
63	Clematangoside A*	<i>C. tangutica</i>	Whole plant	70% EtOH	Cardioprotective activity	NA	134
64	Clematangoside B*	<i>C. tangutica</i>	Whole plant	70% EtOH	Cardioprotective activity	Protective effect against LDH and CK-MB in hypoxia-induced myocardial injury model with ED ₅₀ values of 114.26 ± 6.24 μM and 110.51 ± 5.57 μM	134
65	Clematangoside C*	<i>C. tangutica</i>	Whole plant	70% EtOH, n-BuOH fraction from 75% MeOH extract	Anti-inflammatory Cardioprotective	Inhibition of COX-2 enzyme with IC ₅₀ value 6.03 ± 0.24 μM Protective effect against LDH and CK-MB in hypoxia-induced myocardial injury model with ED ₅₀ values of 92.44 ± 4.47 μM and 87.39 ± 3.66 μM	139 134

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66	Clematangoside D*	<i>C. tangutica</i>	Whole plant	70% EtOH, n-BuOH fraction from 75% MeOH extract	Anti-inflammatory Cardioprotective	Inhibition of COX-2 enzyme with IC ₅₀ value 12.44 ± 0.15 µM Protective effect against LDH and CK-MB in hypoxia-induced myocardial injury model with ED ₅₀ values of 77.31 ± 3.82 µM and 75.77 ± 2.43 µM	139 134
67	Clematangoticoside A*	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	NA	130
68	Clematangoticoside B*	<i>C. florida</i> , <i>C. florida</i> var. <i>plena</i> , <i>C. tangutica</i>	Roots	MeOH, n-BuOH fraction from MeOH extract	Anti-inflammatory Anti-cancer	Reduction of pro-inflammatory mediators (IL-1β, TNF-α and IL-6) level in AIA rat Inhibition of JAK/stat signalling pathway in LPS treated RAW264.7 cells Male ICR mice - ear oedema induced by xylene – 54.7% of inhibition at 200 mg/mg NA	87 17 130
69	Clematangoticoside C*	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	NA	130
70	Clematangoticoside D*	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	Cytotoxicity against SGC-7901 (human gastric carcinoma) with IC ₅₀ value of 24.14 ± 2.10 µM	130
71	Clematangoticoside E*	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	NA	130
72	Clematangoticoside F*	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	Cytotoxicity against SGC-7901 (human gastric carcinoma) with IC ₅₀ value of 21.35 ± 1.88 µM	130
73	Clematangoticoside G*	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	NA	130
74	Clematangoticoside H*	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	NA	130
75	Clematangoticoside J	<i>C. tangutica</i>		n-BuOH subfraction from 75% MeOH extract	Anti-inflammatory	Inhibition of COX-2 enzyme with IC ₅₀ value 936 ± 0.19 µM	139
76	Clematernoside B	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells of 51.3% at 50 µM and cell viability > 90%	122
77	Clematernoside D	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells of 56.5% at 50 µM and cell viability > 90%	122
78	Clematernoside E	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
79	Clematibetoside A	<i>C. tibetana</i>	Aerial part				140

80	Clematibetoside B	<i>C. tangutica</i> <i>C. tibetana</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	NA	140 130
81	Clematibetoside C	<i>C. tangutica</i> , <i>C. tibetana</i>	Whole plant	70% EtOH	Cardioprotective activity	NA	140 133
82	Clematichinenoside A	<i>C. aethusifolia</i> , <i>C. apiifolia</i> var. <i>argenteilucida</i> , <i>C. chinensis</i> , <i>C. delavayi</i> , <i>C. lasiandra</i>	Whole plant, aerial part, roots	70% EtOH, n-BuOH fraction from 70% EtOH extract	Anti-cancer Anti-inflammatory	NA NA	121 127 72 129 141 142
83	Clematichinenoside AR	<i>C. chinensis</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes		Anti-inflammatory	Inhibition of the proliferation and inflammatory response of FLS (fibroblast-like synoviocytes) Inhibition of TNF- α -stimulated adhesion of monocyte to endothelial cells by inhibition of the expression of two cell adhesion proteins (ICAM- 1 and VCAM-1) Inhibition of NF- κ B activity by inhibiting I κ B kinase activity Antagonization of the inflammatory and cytotoxic activities induced by human TNF- α	111 143 124 123
84	Clematichinenoside AR2	<i>C. chinensis</i>	Roots and rhizomes				141
85	Clematichinenoside B	<i>C. chinensis</i>	Roots and rhizomes				141
86	Clematichinenoside C	<i>C. apiifolia</i> var. <i>argenteilucida</i> , <i>C. terniflora</i> var. <i>manshurica</i> , <i>C. uncinata</i>	Roots and rhizomes	50% EtOH n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HCT-116 with IC ₅₀ = 12.3 μ M HT-29 with IC ₅₀ = 10.3 μ M	131 132 126
87	Clematichinenoside A	<i>C. chinensis</i>	Roots and rhizomes				141
88	Clematichinenoside C	<i>C. chinensis</i>	Roots and rhizomes				141
89	Clematiganoside A	<i>C. aethusifolia</i> , <i>C. apiifolia</i> var. <i>argenteilucida</i> , <i>C. heracleifolia</i> , <i>C. puberula</i> var. <i>ganpiniana</i> , <i>C. terniflora</i> var. <i>manshurica</i> , <i>C. uncinata</i>	Whole plant, roots and rhizomes	50% or 70% EtOH, n-BuOH fraction from 70% or 75% EtOH extract	Anti-cancer	Cytotoxicity against: HCT-116 with IC ₅₀ = 16.1 μ M HT-29 with IC ₅₀ = 12.5 μ M	18 131 132 72 138 22 126
90	Clematis hederagenin saponin (CHS)	<i>C. puberula</i> var. <i>ganpiniana</i>	Roots and rhizomes		Anti-cancer	Cytotoxic effects on MCF-7 and MDA-MB-231 (breast cancer cells) in a dose- and time-dependent manner	125
91	Clematiunicinoside A*	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
92	Clematiunicinoside B*	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132

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93	Clematiunicinoside C*	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
94	Clematiunicinoside D*	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
95	Clematiunicinoside E*	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
96	Clematiunicinoside F*	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
97	Clematiunicinoside G*	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
98	Clematiunicinoside H*	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
99	Clematochinenoside A	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	50% EtOH	Anti-cancer	Cytotoxicity against: HCT-116 with IC ₅₀ = 8.4 µM HCT-116 and HT-29 with IC ₅₀ = 7.3 µM	131
100	Clematochinenoside E	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells of 58.1% at 50 µM and cell viability > 90%	122
101	Clematochinenoside F	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells of 62.1% at 50 µM and cell viability > 90%	122
102	Clematochinenoside H*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 32.3 ± 2.4 µM	121
103	Clematochinenoside I*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 28.2 ± 3.7 µM	121
104	Clematochinenoside J*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 30.1 ± 3.3 µM	121
105	Clematochinenoside K*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	121
106	Clematograeolinoside A*	<i>C. graveolens</i>	Roots and rhizomes	85% EtOH	Insecticidal activity	Cytotoxic activity against <i>Aphis craccivora</i> with LC ₅₀ of 3.2 and 2.6 mg/ml after 72 and 96 h and against <i>Coptotermis homii</i> with LC ₅₀ of 0.1 mg/L after 24 h	135
107	Clematomandshurica saponin C	<i>C. chinensis</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells of 63.2% at 50 µM and cell viability > 90%	122 141
108	Clematoside S	<i>C. aethusifolia</i> , <i>C. apiifolia</i> var. <i>argenteolucida</i> , <i>C. grata</i> , <i>C. graveolens</i> , <i>C. intricata</i> , <i>C. tangutica</i>	Whole plant, roots	70% EtOH, 85% EtOH, n-BuOH fraction from 70% EtOH extract	Anti-cancer Insecticidal activity	Cytotoxicity against: HL-60 cells (tumour human leukaemia) with IC ₅₀ = 8.46 ± 0.35 µM / IC ₅₀ = 5.21 ± 0.52 µM Hep-G2 cells (human hepatocellular carcinoma) with IC ₅₀ = 7.82 ± 0.25 µM / IC ₅₀ = 1.88 ± 0.41 µM SGC-7901 cells (gastric carcinoma) with IC ₅₀ = 6.19 ± 0.18 µM / IC ₅₀ = 8.18 ± 0.53 µM U251MG cells (human glioblastoma) with IC ₅₀ = 17.32 ± 1.25 µM	10 135 144 126 130

						Antifeedant activity against 3rd instar larvae of <i>P. Xylostella</i> with $DC_{50} = 748.28 \mu\text{g/mL}$ at 48 h Insecticidal activity against <i>P. Xylostella</i> with $LC_{50} = 1462.78 \mu\text{g/mL}$ after 72 h Insect growth inhibitory activity against <i>P. Xylostella</i> with 68.25% of growth inhibition after 48 h at 1500 $\mu\text{g/mL}$ Cytotoxic activity against <i>Aphis craccivora</i> with LC_{50} of 2.3 and 1.9 mg/ml after 72 h and 96 h and against <i>Coptotermis homii</i> with LC_{50} of 0.2 mg/L after 24 h	
109	Clemantoside A	<i>C. montana</i>					10
110	Clemantoside B	<i>C. montana</i>					10
111	Clemantoside C	<i>C. montana</i>					10
112	Clemantoside F	<i>C. montana</i>					10
						Cytotoxicity against: HL-60 cells (tumour human leukaemia) with $IC_{50} = 7.34 \pm 0.17 \mu\text{M}$ Hep-G2 cells (human hepatocellular carcinoma) with $IC_{50} = 19.16 \pm 0.39 \mu\text{M}$ SGC-7901 cells (gastric carcinoma) with $IC_{50} = 18.22 \pm 0.18 \mu\text{M}$	128 126
113	Cussonside B	<i>C. apiifolia</i> var. <i>argenteilucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Aphicidal activity against <i>A. pisum</i> with $LC_{50} = 0.98 \text{ mg/mL}$ at 72 h and deterrent activity against <i>A. pisum</i> with DI value = 0.62 at 0.125 mg/mL	140 97
114	Dipsacoside B	<i>C. lasiandra</i> , <i>C. tibetana</i>	Whole plant	70% EtOH	Anti-insecticidal		
115	Flaccidoside II	<i>C. apiifolia</i> var. <i>argenteilucida</i> , <i>C. heracleifolia</i>	Whole plant, roots	n-BuOH fraction from 70% or 75% EtOH extract	Anti-cancer	NA	138 126
116	Flaccidoside III	<i>C. lasiandra</i>	Whole plant	70% EtOH	Anti-insecticidal	Aphicidal activity against <i>A. pisum</i> with $LC_{50} = 0.39 \text{ mg/mL}$ at 72 h and deterrent activity against <i>A. pisum</i> with DI value = 0.72 at 0.125 mg/mL	97
117	Friedelin	<i>C. hexapetala</i>	Roots				145
118	Hederacholichiside E	<i>C. tangutica</i>	Whole plant	70% EtOH	Cardioprotective activity	Weak decrease of the levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in cardiomyocytes anoxia/reoxygenation (A/R) model	133

119	Hederacholichiside F	<i>C. puberula</i> var. <i>ganpiniana</i> , <i>C. tangutica</i>	Whole plant, roots and rhizomes	70% EtOH, Butanol fraction from 75% MeOH extract	Anti-cancer Cardioprotective activity	Cytotoxicity against: HGC-27 with IC ₅₀ = 67.6 ± 2.4 µM SK-OV-3 (ovarian adenocarcinoma) with IC ₅₀ = 87.4 ± 2.7 µM NA	18 137 134 130
120	Hederagenic acid	<i>C. apiifolia</i> , <i>C. chinensis</i>	Aerial part, roots and rhizomes	MeOH extract	Anti-cancer	Cytotoxicity against L1210 (mouse lymphocytic leukaemia cell line) with IC ₅₀ = 19.4 ± 0.7 µg/mL HL-60 (tumour human leukaemia cell line) with IC ₅₀ = 16.7 ± 0.5 µg/mL SK-OV-3 (ovarian cancer cell line) with IC ₅₀ = 18.0 ± 0.4 µg/mL	146 147 141 148
121	Hederagenin	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes		Anti-inflammatory	Inhibition of the production of pro-inflammatory mediators (PGE ₂ , TNF-α, IL-1β and IL-6) in LPS- induced RAW264.7 cells and inhibition of oedema in carrageenan-induced mouse hind paw oedema	146
122	Hederagenin 3-O-α-l- rhamnopyranosyl-(1→2)-β-D- xylopyranoside	<i>C. lasiandra</i>	Whole plant	70% EtOH	Anti-insecticidal	Aphicidal activity against <i>A. pisum</i> with LC ₅₀ = 0.13 mg/mL at 72 h and deterrent activity against <i>A.</i> <i>pisum</i> with DI value = 0.83 at 0.125 mg/mL	97
123	Hederagenin-3-β-O-α-L- arabinopyranoside	<i>C. puberula</i> var. <i>ganpiniana</i>	Roots and rhizomes				18
124	Hederasaponin B	<i>C. chinensis</i> , <i>C. lasiandra</i> , <i>C.</i> <i>tangutica</i> , <i>C. tibetana</i> , <i>C.</i> <i>uncinata</i>	Whole plant	70% EtOH, n-BuOH, n- BuOH fraction from 75% MeOH extract	Anti-inflammatory Anti-insecticidal	Cytotoxicity against: HGC-27, SK-OV-3 and HeLa (cervical cancer) with IC ₅₀ values of 16.4 - 71.3 µM Inhibition of COX-2 enzyme with IC ₅₀ value 2.59 ± 0.11 µM Aphicidal activity against <i>A. pisum</i> with LC ₅₀ = 0.26 mg/mL at 72 h and deterrent activity against <i>A.</i> <i>pisum</i> with DI value = 0.90 at 0.125 mg/mL	140 132 97 141 139 137
125	Hederasaponin C	<i>C. lasiandra</i> , <i>C. tibetana</i>	Whole plant	70% EtOH	Anti-insecticidal	Aphicidal activity against <i>A. pisum</i> with LC ₅₀ = 0.44 mg/mL at 72 h and deterrent activity against <i>A.</i> <i>pisum</i> with DI value = 0.68 at 0.125 mg/mL	140 97

126	Hederasaponin CP4	<i>C. uncinata</i>	Roots		Anti-cancer	Cytotoxicity against CaSki (epidermoid carcinoma) with IC ₅₀ = 2.97 ± 1.07 µM	132
127	Hederasaponin CP6	<i>C. uncinata</i>	Roots		Anti-cancer	NA	132
128	Hederasaponin CP7	<i>C. uncinata</i>	Roots		Anti-cancer	Cytotoxicity against CaSki (epidermoid carcinoma) with IC ₅₀ = 26.07 ± 1.47 µM	132
129	Hederoside H1	<i>C. tangutica</i>		n-BuOH fraction from 75% MeOH extract, n-BuOH extract	Anti-cancer Anti-inflammatory	NA Inhibition of COX-2 enzyme with IC ₅₀ = 78 ± 0.13 µM	139 137
130	Hemslonin A	<i>C. apiifolia</i> var. <i>argenteilucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 cells (tumour human leukaemia) with IC ₅₀ = 6.43 ± 0.17 µM Hep-G2 cells (human hepatocellular carcinoma) with IC ₅₀ = 17.86 ± 0.31 µM SGC-7901 cells (gastric carcinoma) with IC ₅₀ = 19.48 ± 0.25 µM	126
131	Heracleifolioside A*	<i>C. heracleifolia</i>	Whole plant	n-BuOH fraction from 75% EtOH extract			138
132	Heracleifolioside B*	<i>C. heracleifolia</i>	Whole plant	n-BuOH fraction from 75% EtOH extract			138
133	Heracleifolioside C*	<i>C. heracleifolia</i>	Whole plant	n-BuOH fraction from 75% EtOH extract			138
134	HN saponin H	<i>C. tibetana</i>	Aerial part				140 10
135	Huzhangoside B	<i>C. apiifolia</i> var. <i>argenteilucida</i> , <i>C. florida</i> var. <i>plena</i> , <i>C. heracleifolia</i> , <i>C. intricata</i> , <i>C. tibetana</i> , <i>C. uncinata</i>	Roots	MeOH, n-BuOH fraction from 70% or 75% EtOH extract	Anti-cancer Anti-inflammatory	NA Male ICR mice - ear oedema induced by xylene – 50.9% of inhibition at 200 mg/mg	140 132 87 22 126 129 138
136	Huzhangoside C	<i>C. aethusifolia</i> , <i>C. apiifolia</i> var. <i>argenteilucida</i> , <i>C. florida</i> , <i>C. florida</i> var. <i>plena</i> , <i>C. heracleifolia</i> , <i>C. lasiandra</i> , <i>C. uncinata</i>	Whole plant, roots	MeOH, 70% EtOH, n-BuOH fraction from MeOH extract	Anti-cancer Anti-inflammatory	Cytotoxicity against CaSki (epidermoid carcinoma) with IC ₅₀ value of 9.05 ± 3.61 µM Reduction of pro-inflammatory mediators (IL-1β, TNF-α and IL-6) level in AIA rat inhibition of JAK/stat signalling pathway in LPS treated RAW264.7 cells	132 97 87 72 17 138 22 126

					Anti-insecticidal	Aphicidal activity against <i>A. pisum</i> with $LC_{50} = 0.90$ mg/mL at 72 h and deterrent activity against <i>A. pisum</i> with DI value = 0.55 at 0.125 mg/mL	129
137	Huzhangoside D	<i>C. apiifolia</i> var. <i>argentilucida</i> , <i>C. chinensis</i> , <i>C. graveolens</i> , <i>C. tibetana</i> , <i>C. uncinata</i>	Roots, roots and rhizomes	85% EtOH, n-BuOH fraction from 70% EtOH extract	Insecticidal activity	Cytotoxic activity against <i>Coptotermis homii</i> with LC_{50} of 0.2mg/L after 24 h	132 135 141 126 129
138	Huzhongoside B	<i>C. chinensis</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	50% EtOH	Anti-cancer	Cytotoxicity against: HCT-116 with $IC_{50} = 12.9 \mu M$ HT-29 with $IC_{50} = 13.5 \mu M$	131 141
139	Kalopanax saponin A	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	Cytotoxicity against: SGC-7901 (human gastric carcinoma) with $IC_{50} = 9.08 \pm 0.29 \mu M$ Hep-G2 cells (human hepatocellular carcinoma) with $IC_{50} = 7.26 \pm 0.78 \mu M$ HL-60 cells (tumour human leukaemia) with $IC_{50} = 8.72 \pm 0.91 \mu M$ U251MG cells (human glioblastoma) with $IC_{50} = 27.20 \pm 2.78 \mu M$	130
140	Kalopanaxsaponin G	<i>C. tangutica</i>	Whole plant	70% EtOH, n-BuOH, n-BuOH fraction from 75% MeOH extract	Cardioprotective activity Anti-cancer Anti-inflammatory	Decrease of the levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in cardiomyocytes anoxia/reoxygenation (A/R) model NA Inhibition of COX-2 enzyme with $IC_{50} = 90.73 \pm 0.77 \mu M$	139 137 133
141	Kizutasaponin K10	<i>C. tibetana</i>					140
142	Kizutasaponin K12	<i>C. tangutica</i>	Whole plant	70% EtOH, n-BuOH fraction from 75% MeOH extract	Cardioprotective activity Anti-cancer	NA NA	133 130
143	Kizutasaponin K3	<i>C. tibetana</i>					140 10
144	Koelreuteria saponin A	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	Cytotoxicity against: SGC-7901 (human gastric carcinoma) with $IC_{50} = 9.08 \pm 0.29 \mu M$ Hep-G2 cells (human hepatocellular carcinoma) with $IC_{50} = 7.26 \pm 0.78 \mu M$	130

						HL-60 cells (tumour human leukaemia) with IC ₅₀ = 8.72 ± 0.91 µM U251MG cells (human glioblastoma) with IC ₅₀ = 27.20 ± 2.78 µM	
145	Leontoside	<i>C. tangutica</i>	Whole plant	70% EtOH	Cardioprotective activity	NA	134
146	Mandshunoside B	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 12.9 ± 1.2 µM	121
147	Mandshunoside C*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	50% EtOH	Anti-cancer	Cytotoxicity against: HCT-116 with IC ₅₀ = 3.0 µM HT-29 with IC ₅₀ = 2.8 µM	131
148	Mandshunoside D*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	50% EtOH	Anti-cancer	Cytotoxicity against: HCT-116 with IC ₅₀ = 0.6 µM HT-29 with IC ₅₀ = 0.9 µM	131
149	Mandshunoside E*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	50% EtOH	Anti-cancer	Cytotoxicity against: HCT-116 with IC ₅₀ = 2.7 µM HT-29 with IC ₅₀ = 4.2 µM	131
150	Mandshunoside F*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells with IC ₅₀ value of 127 µM and cell viability > 90%	122
151	Mandshunoside G*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells with IC ₅₀ value of 8.3 µM and cell viability > 90%	122
152	Mandshunoside H*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells of 69.2% at 50 µM and cell viability > 90%	122
153	Mandshunoside I*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	95% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-induced RAW264.7 cells of 64.6% at 50 µM and cell viability > 90%	122
154	Neolasiandroside A*	<i>C. lasiandra</i>	Whole plant	70% EtOH	Insecticidal activity	Aphicidal activity against <i>A. pisum</i> with LC ₅₀ = 0.36 mg/mL at 72 h and deterrent activity against <i>A. pisum</i> with DI value = 0.53 at 0.125 mg/mL	97
155	Neolasiandroside B*	<i>C. lasiandra</i>	Whole plant	70% EtOH		Aphicidal activity against <i>A. pisum</i> with LC ₅₀ = 0.30 mg/mL at 72 h and deterrent activity against <i>A. pisum</i> with DI value = 0.57 at 0.125 mg/mL	97
156	Neolasiandroside C*	<i>C. lasiandra</i>	Whole plant	70% EtOH		Aphicidal activity against <i>A. pisum</i> with LC ₅₀ = 0.68 mg/mL at 72 h and deterrent activity against <i>A. pisum</i> with DI value = 0.67 at 0.125 mg/mL	97

157	Oleanolic acid	<i>C. apiifolia</i> , <i>C. armandii</i> , <i>C. chinensis</i>	Aerial part, roots and rhizomes	MeOH extract, 70% EtOH	Anti-cancer	Cytotoxicity against:	149
						L1210 (mouse lymphocytic leukaemia cell line) with $IC_{50} = 25.6 \pm 0.3 \mu\text{g/mL}$	
						HL-60 (tumour human leukaemia cell line) with $IC_{50} = 21.2 \pm 0.4 \mu\text{g/mL}$	
						SK-OV-3 (ovarian cancer cell line) with $IC_{50} = 15.4 \pm 0.3 \mu\text{g/mL}$	
158	Prosapogenin CP11	<i>C. puberula</i> var. <i>ganpiniana</i>	Roots and rhizomes				10
159	Prosaponin 10a	<i>C. chinensis</i>	Roots and rhizomes				18
160	Prosaponin CP0	<i>C. chinensis</i>	Roots and rhizomes				141
161	Prosaponin CP1	<i>C. chinensis</i>	Roots and rhizomes				141
162	Prosaponin CP10	<i>C. chinensis</i>	Roots and rhizomes				141
163	Prosaponin CP2	<i>C. chinensis</i>	Roots and rhizomes				141
164	Prosaponin CP2a	<i>C. chinensis</i>	Roots and rhizomes				141
165	Prosaponin CP2b	<i>C. chinensis</i>	Roots and rhizomes				141
166	Prosaponin CP3	<i>C. chinensis</i>	Roots and rhizomes				141
167	Prosaponin CP3a	<i>C. chinensis</i>	Roots and rhizomes				141
168	Prosaponin CP3b	<i>C. chinensis</i>	Roots and rhizomes				141
169	Prosaponin CP4	<i>C. chinensis</i>	Roots and rhizomes				141
170	Prosaponin CP5	<i>C. chinensis</i>	Roots and rhizomes				141
171	Prosaponin CP6	<i>C. chinensis</i> , <i>C. tibetana</i>	Aerial part, roots and rhizomes				140
172	Prosaponin CP7	<i>C. chinensis</i>	Roots and rhizomes				141
173	Prosaponin CP7a	<i>C. chinensis</i>	Roots and rhizomes				141
174	Prosaponin CP8	<i>C. chinensis</i> , <i>C. tibetana</i>	Aerial part, roots and rhizomes				140
175	Prosaponin CP8a	<i>C. chinensis</i>	Roots and rhizomes				141
176	Prosaponin CP9	<i>C. chinensis</i>	Roots and rhizomes				141
177	Prosaponin CP9a	<i>C. chinensis</i>	Roots and rhizomes				141
178	Quinatic acid	<i>C. ligusticifolia</i>					20
179	Sapindoside B	<i>C. tangutica</i>	Whole plant	n-BuOH fraction from 75% MeOH extract	Anti-cancer	Cytotoxicity against:	130
						SGC-7901 (human gastric carcinoma) with $IC_{50} = 10.32 \pm 0.48 \mu\text{M}$	
						Hep-G2 cells (human hepatocellular carcinoma) with $IC_{50} = 4.24 \pm 0.58 \mu\text{M}$	

						HL-60 cells (tumour human leukaemia) with IC ₅₀ = 6.58 ± 0.42 µM U251MG cells (human glioblastoma) with IC ₅₀ = 15.37 ± 1.18 µM	
180	Saponin 1	<i>C. chinensis</i>	Roots and rhizomes				141
181	Saponin 2	<i>C. chinensis</i>	Roots and rhizomes				141
182	Saponin 3	<i>C. chinensis</i>	Roots and rhizomes				141
183	Saponin 4	<i>C. chinensis</i>	Roots and rhizomes				141
184	Saponin 5	<i>C. chinensis</i>	Roots and rhizomes				141
185	Saponin CP4	<i>C. aethusifolia</i> , <i>C. apiifolia</i> var. <i>argenteilucida</i>	Whole plant, roots	70% EtOH, n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 cells (tumour human leukaemia) with IC ₅₀ = 4.13 ± 0.15 µM Hep-G2 cells (human hepatocellular carcinoma) with IC ₅₀ = 6.52 ± 0.53 µM SGC-7901 cells (gastric carcinoma) with IC ₅₀ = 5.13 ± 0.32 µM	72
					Insecticidal activity	Antifeedant activity against 3rd instar larvae of <i>P. xylostella</i> with DC ₅₀ = 737.19 µg/mL at 48 h, insecticidal activity against <i>P. xylostella</i> with LC ₅₀ = 1785.96 µg/mL after 72 h, Insect growth inhibitory activity against <i>P. xylostella</i> with 69.87% of growth inhibition after 48 h at 1500 µg/mL	126
186	Saponin CP7	<i>C. apiifolia</i> var. <i>argenteilucida</i> , <i>C. chinensis</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 cells (tumour human leukaemia) with IC ₅₀ = 4.99 ± 0.37 µM Hep-G2 cells (human hepatocellular carcinoma) with IC ₅₀ = 7.15 ± 0.26 µM SGC-7901 cells (gastric carcinoma) with IC ₅₀ = 9.36 ± 1.35 µM	126
					Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 4.2 ± 2.1 µM	121
187	Saponin PJ3	<i>C. apiifolia</i> var. <i>argenteilucida</i> , <i>C. tangutica</i>	Whole plant, roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer Cardioprotective activity	NA Decrease of the levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in cardiomyocytes anoxia/reoxygenation (A/R) model	133 129
188	Tanguticoside A	<i>C. akebioides</i>	Aerial part	EtOAc fraction from 80% EtOH extract			118
189	Tanguticoside B	<i>C. tangutica</i>	Unknwon	n-BuOH extract	Anti-cancer	NA	137

PARTIE I. REVUE LITTÉRAIRE SUR LE GENRE CLEMATIS : USAGES TRADITIONNELS, ACTIVITÉS PHARMACOLOGIQUES ET PHYTOCHIMIE

190	Tauroside st-h1	<i>C. tangutica</i>	Unknwon	n-BuOH extract	Anti-cancer	Cytotoxicity against SK-OV-3 with IC ₅₀ value of 48.7 ± 3.9 µM	137
191	Tomentoside A	<i>C. florida</i> , <i>C. florida</i> var. <i>plena</i> , <i>C. graveolens</i>	Roots and rhizomes	MeOH, EtOH, n-BuOH fraction from MeOH extract	Anti-inflammatory Insecticidal activity	NA Cytotoxic activity against <i>Aphis craccivora</i> with LC ₅₀ of 1.2 and 0.5 mg/ml after 72 and 96 h and against <i>Coptotermis homii</i> with LC ₅₀ of 0.1 mg/L after 24 h	135 87 17
192	Ursolic acid	<i>C. apiifolia</i>	Aerial part	MeOH extract	Anti-cancer	Cytotoxicity against: L1210 (mouse lymphocytic leukaemia cell line) with IC ₅₀ = 8.3 ± 0.5 µg/mL HL-60 (tumour human leukaemia cell line) with IC ₅₀ = 12.8 ± 0.4 µg/mL SK-OV-3 (ovarian cancer cell line) with IC ₅₀ = 7.7 ± 0.2 µg/mL	147
193	Vitalboside A	<i>C. vitalba</i>					10
194	Vitalboside B	<i>C. akebioides</i>	Aerial part	EtOAc fraction from 80% EtOH extract			118
195	Vitalboside D	<i>C. vitalba</i>					10
196	Vitalboside F or Songaroside B	<i>C. vitalba</i>					10
197	Vitalboside G	<i>C. vitalba</i>					10
198	Vitalboside H	<i>C. vitalba</i>					10
199	Yemuoside YM32	<i>C. heracleifolia</i>	Whole plant	n-BuOH fraction from 75% EtOH extract			138
201	α-amyrin	<i>C. purpurea</i>					10
200	α-hederin	<i>C. puberula</i> var. <i>ganpiniana</i> , <i>C. tibetana</i>	Roots and rhizomes				10 18 140
203	α-L-rhamnopyranosyl(1-4)-β-D-glucopyranosyl(1-3)-3,23β-dihydroxy-olean-12-en-28-oic acid	<i>C. grata</i>	Roots				150
202	β-amyrin	<i>C. montana</i> , <i>C. purpurea</i>					10
204	β-D-glucopyranosyl(1-4)-β-D-xylopyranosyl(1-3)-α-L-rhamnopyranosyl(1-3)-3β-hydroxy-olean-12-en-28-oic acid	<i>C. grata</i>	Roots				150
205	β-hederin	<i>C. apiifolia</i> var. <i>argenteilucida</i>	Roots	n-BuOH fraction from 70% EtOH extract	Anti-cancer	Cytotoxicity against U251MG cells (human glioblastoma) with IC ₅₀ = 10.46 ± 1.15 µM	129
Steroids							

206	3- β -hydroxy-stigmast-5,22-dien-7-one	<i>C. armandii</i>					151
207	5- α -stigmastane-3- β -6- α -diol	<i>C. armandii</i>					151
208	Daucosterol	<i>C. armandii</i> , <i>C. chinensis</i> , <i>C. delavayi</i> , <i>C. hexapetala</i>	Aerial part				145
							142
							141
							151
209	Ikshusterol-3-O-glucoside	<i>C. gouriana</i>	Roots	EtOAc	Snake antivenom activity	30% of inhibition at 1000 μ g/mL of PLA2 protein against <i>Naja naja</i> (Indian cobra) crude venom	136
210	Stigmasterol	<i>C. apiifolia</i> , <i>C. armandii</i> , <i>C. hirsuta</i>		n-hexane/EtOAc	Antioxidant	DPPH IC ₅₀ = 19.4 μ g/mL	148
							151
							94
211	Stigmasterol-3-O- β -D-glucopyranoside	<i>C. armandii</i>					151
212	β -sitosterol	<i>C. angustifolia</i> , <i>C. apiifolia</i> , <i>C. chinensis</i> , <i>C. hexapetala</i> , <i>C. hirsuta</i> , <i>C. intricata</i> , <i>C. ligustifolia</i> , <i>C. montana</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	n-hexane/EtOAc			10
							145
							20
							141
							152
							148
213	β -sitosterol- β -D-glucoside	<i>C. montana</i>					10

6.2. Phenolic compounds

a. Phenolic acids and glycosides

About fifty phenolic acids and glycosides have been identified in the genus *Clematis* (Table 13). Antioxidant, anti-inflammatory, and different cytotoxicity properties are the main bioactivities investigated. DPPH assay was used to test the antioxidant properties of isolated compounds from the aerial part of *C. tashiroi*; among the phenolic glycosides tested, six of them were new structures. The highest activities have been identified for clematiside G and clematiside A with IC₅₀ values of 13.0 and 13.3 µM, respectively. The most promising compound, clematiside G, has also been tested for anti-inflammatory properties using the inhibition of NO production in RAW 264.7 cells and an interesting value for IC₅₀ of 14.9 µM ± 0.30 µM was obtained ¹⁵³.

Investigation of *C. aethusifolia* led to the identification of 10 compounds extracted from the aerial part using ethanolic extraction, and four of them have been described for the first time (aethusifolins A-D) ¹⁵⁴. No activity has been identified for these new compounds but Chang et al., (2017) demonstrated the cytotoxicity of 4-O-Caffeoyl-2-C-methyl-D-erythrano-1,4-lactone and 1-O-caffeoyl-β-D-glucopyranose, also isolated from this plant, against a human melanoma cell line with IC₅₀ of 17.7 ± 1.4 µM and 15.3 ± 2.6 µM, respectively.

The cytotoxicity against human neuroblastoma cell lines (SH-SY) and human melanoma cell lines (A375) has also been identified for the 1-O-caffeoyl-β-D-glucopyranose isolated from *C. aethusifolia* and *C. chinensis*. 4-O-β-D-galactopyranosyl-ethyl-E-caffeate and 4-O-β-D-galactopyranosyl-3-hydroxyl-phenylethene, two new compounds isolated from *C. lasiandra*, have also been identified to possess IC₅₀ on HL-60, HepG2, and SGC-7901 cell lines in the range of 9.73 – 22.31 µM ¹⁵⁵.

Several phenolic acids or glycosides have been investigated for anti-inflammatory properties. Among the compounds tested, eleven compounds were revealed to possess anti-inflammatory properties (inhibition of NO production in LPS-stimulated RAW 264.7 cells or inhibition of the production of pro-inflammatory mediators) ^{119,156–160} and only clematiside G has been extracted from the aerial part ¹⁵³. Those studies highlight the interest of the phenolic compounds from the underground part of the plant for having anti-inflammatory activities. Some species have only been studied on the underground part, which can lead to a lack of information on the aerial part and therefore a lack of knowledge of its bioactivity.

b. Flavonoids

Some flavones have been isolated from *Clematis* spp. as apigenin derivatives ^{66,161} from *C. aethusifolia* leaves and from *C. flammula* flowers. Apigenin-7-O-β-[4''-O-E-p-coumaroyl glucoside] and apigenin-7-O-β-[6''-O-E-p-coumaroyl glucoside]—extracted using ethyl acetate and methanol, respectively—have shown antioxidant activity, especially for apigenin-7-O-β-

[6''-O-E-*p*-coumaroyl glucoside] with $IC_{50} = 20 \pm 0.01 \mu\text{g/mL}$ using DPPH assay ⁶⁶. Apigenin aglycone and derivatives have been tested for cytotoxicity against diverse tumour cell lines. 3'-O-methyl-apigenin-7-O- β -D-glucopyranoside presented the highest activity against SK-OV-3 (ovarian cell line) and A549 (human lung cancer cell line) with IC_{50} value of $28.5 \mu\text{M}$ and $40.7 \mu\text{M}$ ¹⁶¹ while apigenin-7-O- β -D-glucopyranoside had the highest activity against SH-SY5Y (human neuroblastoma cells) with IC_{50} value of $46.9 \mu\text{M}$. Some C-glucoside derivatives of apigenin, such as isovitexin and isovitexin 6''-O-E-*p*-coumarate, have also been isolated from *C. lasiandra* and a significant activity against tobacco mosaic virus has been highlighted for the *p*-coumarate derivative at $500 \mu\text{g/mL}$ ¹⁶².

Flavonols, such as quercetin and kaempferol derivatives, have been isolated from *C. aesthusifolia*, *C. flammula*, *C. lasiandra*, and from some cultivars such as *Clematis* cultivar 'Madame Julia Correvon' ^{14,161–163}. Eleven flavonols have been extracted from *C. aesthusifolia* and have been tested for cytotoxic activity against SK-OV-3 but none of them showed any interesting activity ¹⁶¹. Kaempferol 3-O-[(6-O-caffeoyl)-glucosyl(1 $\rightarrow$ 2)]-(6-O-caffeoyl)-glucoside-7-O-rhamnoside is the only new flavonol structure identified and it was found in *C. flammula* leaves but has not been associated with any biological activity ¹⁴. Y. Li et al. (2021) identified two kaempferol glycoside compounds and a quercetin glycoside with antiviral activity against tobacco mosaic virus on *N. glutinosa* plants. Kaempferol 3-O- α -L-rhamnopyranoside and quercetin 3-O- β -D-glucopyranuronide presented inactivation, protective, and curative efficacies at concentrations of $500 \mu\text{g/mL}$. Saiginol E and K are among the phenylacetylated flavonols identified in *C. flammula*.

Some anthocyanidins have also been isolated from *Clematis* spp. The study of the flower of some cultivars has led to the isolation of some cyanidin and delphinidin glycosides but without any related biological activity ^{163,164}. Only a few studies have been made on the flowers of *Clematis* spp., which explains the lack of knowledge on their composition in anthocyanidins.

c. Lignans

Lignans and neolignans, which constitute a significant subset of natural phenolic compounds, are synthesized through the combination of two or more phenylpropanoid units. About fifty lignans and neolignans have been isolated from the aerial part and from roots and rhizomes of *Clematis* spp., mostly using the ethanolic extraction ^{16,73,119,131,155,165–170};

One new structure, (7R,8S)-9-Acetyl-dehydrodiconiferyl alcohol, has been isolated from the dichloromethane subfraction of the ethanolic extract of *C. armandii* ¹⁶. Four isolated compounds have been tested for inhibitory effects on NO production in LPS-stimulated BV-2 cells. (7R,8S)-9-Acetyl-dehydrodiconiferyl alcohol and (7R,8S,8'S)-3,3'-dimethoxy-7,9'-epoxylignane-4,4',9-triol presented the highest inhibition with $IC_{50} = 9.3 \pm 0.7 \mu\text{M}$ and $IC_{50} = 3.9 \pm 0.5 \mu\text{M}$, respectively ¹⁶. Among the lignans isolated from *C. manshurica* roots, the two new structures clemomanshurinane C and clemomanshurinane D as well as (+)-isolariciresinol

3- α -O- β -D-glucopyranoside and (+)-pinoresinol have shown anti-inflammatory activity by inhibiting lipoxygenase. (+)-isolariciresinol 3- α -O- β -D-glucopyranoside showed the highest activity with 41.9 ± 6.2 % of inhibition at $50 \mu\text{M}$ ¹⁶⁵.

(7R,8S)-dihydrodehydrodiconiferyl alcohol, isolated from the butanol fraction of the ethanolic extract of *C. chinensis*, has shown an interesting cytotoxicity against Ishikawa cells with IC_{50} values of $10.1 \pm 1.1 \text{ mM}$ ¹⁷⁰. (+)-lariciresinol-4-O- β -D-glucopyranoside and secoisolariciresinol 9-O- β -D-glucopyranoside, both isolated from *C. hexapetala*, possessed respectively 48.40% and 58.60% of inhibition ratio against PTP1B ¹¹⁹. Tian et al. (2014) isolated four lignans from *C. lasiandra* including one new structure, the (8R)-3,3'-dimethoxy-4,4',9,9'-tetrahydroxy-5',8-lignan 3'-O- β -D-glucopyranoside was found to have a cytotoxic property against HepG2 cells with $\text{IC}_{50} = 93.52 \pm 0.13 \mu\text{M}$. Lasiandranin A, a new isolated compound, and pinnatifidanin BV—both extracted from *C. lasiandra*—have shown antibacterial activity against *R. solanacearum*, a plant pathogenic bacteria ¹⁶⁶. Cytotoxic activity against ECA-109 of eleutheroside E, isolated from *C. terniflora*, has been tested and an IC_{50} value of $3.26 \mu\text{M/mL}$ was determined ¹⁶⁷.

d. Coumarins

Among the coumarin isolated from *Clematis* spp., only two have shown biological activity ^{117,154,160,170}. Aesculetine, extracted from the aerial part of *C. aesthusifolia*, possessed $\text{IC}_{50} = 15.3 \pm 3.8 \mu\text{M}$ against tumour cells A375 ¹⁵⁴ and isoscopolactin, extracted from the roots of *C. florida*, presented anti-inflammatory activity by inhibiting NO production on LPS-induced RAW264.7 cells ($\text{IC}_{50} = 22.88 \mu\text{M}$) ¹⁶⁰

Table 13: Phenolic compounds isolated from *Clematis* species and their related biological activities (* structure newly described since 2011)

	Compound name	Species	Part of the plant	Extraction	Activity	Biological activity of the isolated compound	Ref.
Phenolic compounds							
214	(-)-4-(3,4-dihydroxyphenyl)-5-hydroxy-2(5H)-furanone	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154
215	(E)-4-hydroxy-dodec-2-enedioic acid-12-O-methylester	<i>C. florida</i>	Roots	MeOH	Anti-inflammatory	NA	160
216	1,4-dimethyl (2R,3R)-2,3-bis[[(2E)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl] oxy] butanedioate	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity	Moderate aphicidal activity against <i>A. pisum</i> with LC ₅₀ value of 129.36 µg/mL at 72 h, moderate repellent activity against <i>A. pisum</i> at 24 h	171
217	1-feruloyl-β-D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract			170
218	1-O-(trans-caffeoyl)-β-D-glucopyranose	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	DPPH assay with ED ₅₀ of 29.1 ± 0.32 µM	153
219	1-O-caffeoyl-β-D-glucopyranose	<i>C. aethusifolia</i> , <i>C. chinensis</i>	Aerial part	95% EtOH, n-BuOH fraction from EtOH extract	Anti-cancer	Cytotoxicity against: A375 (human melanoma cells) with IC ₅₀ = 15.3 ± 2.6 µM SH-SY5Y (human neuroblastoma cells) with IC ₅₀ = 20.0 ± 0.9 µM	154 170
220	1-O-caffeoyl-β-D-glucopyranoside	<i>C. terniflora</i>	Aerial part	70% EtOH			167
221	2,3-O-dicaffeoyltartaric acid	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH			171
222	2-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol	<i>C. akebioides</i>	Aerial part	EtOAc fraction from 80% EtOH extract			118
223	3,4-dihydroxyacetophenone	<i>C. akebioides</i>	Whole plant	AcOEt fraction from EtOH extract			172
224	3-O-(β-D-glucopyranosyl)-1-(3',5'-dimethoxy-4'-hydroxyphenyl)-1-propanone	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract			170
225	4-hydroxy-3-methoxyphenyl-β-D-glucopyranoside	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
226	4-O-Caffeoyl-2-C-methyl-D-erythrano-1,4-lactone	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	Cytotoxicity against A375 with IC ₅₀ = 17.7 ± 1.4 µM	154

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227	4-oxo-5-(O-β-D-glucopyranosyl)-pentanoic acid-1-O-methyl ester	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154
228	4-O-β-D-galactopyranosyl-ethyl-E-caffeate*	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 with IC ₅₀ = 20.56 ± 0.32 μM HepG2 with IC ₅₀ = 9.73 ± 0.47 μM SGC-7901 with IC ₅₀ = 15.12 ± 0.39 μM	155
229	4-O-β-D-galactopyranosyl-3-hydroxyl-phenylethene*	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Anti-cancer	Cytotoxicity against: HL-60 with IC ₅₀ = 22.31 ± 0.54 μM HepG2 with IC ₅₀ = 12.24 ± 1.21 μM SGC-7901 with IC ₅₀ = 18.72 ± 0.33 μM	155
230	5-O-feruloyl-2-deoxy-D-ribo-nog-lactone	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells by 35.1 % at 50 μM, cell viability > 90%	158
231	5-O-isoferuloyl-2-deoxy-D-ribo-nog-lactone	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells by 29.3 % at 50 μM, cell viability > 90%	158
232	5-O-isoferuloyl-2-deoxy-D-ribo-nog-lacton*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots	MeOH	Anti-inflammatory	Decreased production of pro-inflammatory mediators (NO and PGE2) and downregulation of COX-2 in LPS-induced BV2 (microglial) cells	156
233	6-O-(trans-caffeoyl)-D-glucopyranose	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	NA	153
234	6-O-trans-p-coumaroyl- D-glucopyranose	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	NA	153
235	9-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl isoferuloyl ester*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells by 36.2 % at 50 μM, cell viability > 90%	158
236	Aethusifolin A*	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154
237	Aethusifolin B*	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154
238	Aethusifolin C*	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154
239	Aethusifolin D*	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154
240	Benzylalcohol α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH			154
241	Benzyl-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154

242	Brevicaudatoside A*	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity	Moderate aphicidal activity against <i>A. pisum</i> with LC ₅₀ value of 77.86 µg/mL at 72 h, repellent activity against <i>A. pisum</i> at 24 h	171
243	Brevicaudatoside B*	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity		171
244	Caffeic acid	<i>C. aethusifolia</i> , <i>C. akebioides</i> , <i>C. brevicauda</i> , <i>C. heracleifolia</i> , <i>C. montana</i> , <i>C. viticella</i>	Aerial part, leaves	95% EtOH, n-BuOH fraction from MeOH extract	Anti-cancer Anti-inflammatory	NA Significant anti-inflammatory activity at 100 µM by reducing the release of NO in LPS-stimulated macrophages	154 117 171 174 175
245	Caffeic acid ethyl ester	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity		171
246	Caffeoyl-glycolic acid methyl ester	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity		171
247	Calceolarioside B	<i>C. crassifolia</i>					50
248	Clematiside A*	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	DPPH assay with ED ₅₀ of 13.3 ± 0.37 µM	153
249	Clematiside B*	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	DPPH assay with ED ₅₀ of 87.0 ± 0.28 µM	153
250	Clematiside C*	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	NA	153
251	Clematiside D*	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	DPPH assay with ED ₅₀ of 47.0 ± 0.28 µM	153
252	Clematiside E*	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	DPPH assay with ED ₅₀ of 39.9 ± 1.06 µM	153
253	Clematiside F*	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	NA	153
254	Clematiside G*	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant Anti-inflammatory	DPPH assay with ED ₅₀ of 13.0 ± 0.38 µM Inhibition of the production of NO with IC ₅₀ = 14.9 ± 0.30 µM	153
255	Clematiside H*	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	DPPH assay with ED ₅₀ of 17.0 ± 0.23 µM	153
256	Clematisphenoside A*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
257	Clematisphenoside B*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
258	Clematisphenoside C*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
259	Curcusinol	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	Inhibition effect against TNF-α release with IC ₅₀ = 3.419 µM PTP1B enzymatic inhibition of 86% and IC ₅₀ = 4.623 µM	119
260	Ethylene glycol 1-O-(6-O-caffeoyl)-β-D-glucopyranoside	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity		171
261	Ferulic acid	<i>C. akebioides</i> , <i>C. heracleifolia</i>	Leaves	n-BuOH fraction from MeOH extract			117 175
262	Fumalic acid	<i>C. intricata</i>					10

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263	Glucosyringic acid	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
264	Hexapetoside A*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	NA	119
265	Hexapetoside B*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	NA	119
266	Hexapetoside C*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	NA	119
267	Hexapetoside D*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	NA	119
268	Hexapetoside E*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	NA	119
269	Hexapetoside F*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	NA	119
270	Hydroxytyrosol	<i>C. viticella</i>	Aerial part	EtOH	Anti-inflammatory	Significant anti-inflammatory activity at 100 µM by reducing the release of NO in LPS-stimulated macrophages and inhibition of the production of TNF-α	174
271	Hyuganoside iiii	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract			170
272	Ibotanolide B	<i>C. crassifolia</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells by 38.7 % at 50 µM, cell viability > 90%	158 50
273	Kinsenoside	<i>C. florida</i>	Roots	MeOH	Anti-inflammatory	NA	160
274	Mandshurica B*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW 264.7 macrophages in dose-dependent manner and of the pro-inflammatory cytokine TNF-α at 2.5 µM	159
275	Mandshusica C*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH	Anti-inflammatory	Significant inhibitory effect on NO production at different concentrations 2.5 µM – 10 µM and significant inhibitory effects on TNF-α content in LPS-induced RAW 264.7 cells at 5 µM	176
276	Mandshusica D*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH	Anti-inflammatory	Significant inhibitory effect on NO production at different concentrations 2.5 µM – 10 µM	176
277	Mandshusica E*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH	Anti-inflammatory	Significant inhibitory effect on NO production at different concentrations 2.5 µM – 10 µM and significant inhibitory effects on TNF-α content in LPS-induced RAW 264.7 cells at 10 µM	176
278	Methyl 6-(4-Hydroxy-3-Methoxy Phenyl) hexanoate	<i>C. florida</i>	Roots	MeOH	Anti-inflammatory	Inhibition of the production of NO on LPS-induced RAW264.7 cells with IC ₅₀ = 6.32 µM	160
279	Methyl caffeate	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity	Moderate aphicidal activity against <i>A. pisum</i> with LC ₅₀ value of 96.67 µg/mL at 72 h, repellent activity against <i>A. pisum</i> at 24 h	171

280	p-coumaric acid	<i>C. akebioides</i> , <i>C. viticella</i>	Whole plant, aerial part	EtOAc fraction from 70% EtOH extract			172 174 175
281	p-hydroxybenzoic acid	<i>C. akebioides</i> , <i>C. viticella</i>	Whole plant, aerial part	EtOAc fraction from 70% EtOH extract			172 174
282	Protocatechuic acid	<i>C. akebioides</i> , <i>C. heracleifolia</i>	Whole plant, leaves	EtOAc fraction from 70% EtOH extract, n-BuOH fraction MeOH extract			172 117
283	Protocatechuic aldehyde	<i>C. akebioides</i>	Whole plant	EtOAc fraction from 70% EtOH extract			172
284	Pseudolaroside B	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
285	Salicylic acid	<i>C. intricata</i>					10
286	Trans ferulic acid	<i>C. florida</i>	Roots	MeOH	Anti-inflammatory	NA	160
287	Trans isoferulic acid	<i>C. florida</i> , <i>C. florida</i> var. <i>plena</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots	MeOH	Anti-inflammatory	Inhibition of the production of pro-inflammatory mediators (PGE2 and NO) in LPS-induced BV2 cells (microglial) and decrease of the expression of iNOS and COX-2 genes	156 87 160
288	Trans-caffeic acid 4-O-β-D-glucopyranoside	<i>C. tashiroi</i>	Aerial part	95% EtOH	Antioxidant	DPPH assay with ED ₅₀ = 175.4 ± 0.70 μM	153
289	Vanillic acid	<i>C. akebioides</i>	Whole plant	EtOAc fraction from 70% EtOH extract			172
290	α-viniferin	<i>C. hexapetala</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	60% EtOH, 90% EtOH	Anti-inflammatory	Significant inhibitory effect on NO production at concentrations 2.5 – 10 μM	119 176
291	β-hydro-xypropiovanillone-3-O-β-D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract			170
Flavonoids							
292	3,3',7-trihydroxy-4',5-dimethoxyflavone	<i>C. delavayi</i>	Aerial part				142
293	3',4',5,7-tetrahydroxy-6-C-glucopyranosyl flavone	<i>C. crassifolia</i>					50
294	3,4,6,7,8,3',4'-heptamethoxyflavone	<i>C. hexapetala</i>					10
295	3'-O-methyl-apigenin-7-O-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	Cytotoxic activity against: SK-OV-3 (ovarian cancer cell line) with IC ₅₀ = 28.5 μM A549 (human lung cancer cell line) with IC ₅₀ = 40.7 μM	161

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296	3'-O-methyluteolin	<i>C. viornae</i>					10
297	4',7-dimethoxykaempferol	<i>C. purpurea</i>					10
298	6-hydroxybiochain A	<i>C. hexapetala</i>					177
299	Apigenin	<i>C. aethusifolia</i> , <i>C. terniflora</i> , <i>C. trichotoma</i> Nakain, <i>C. viornae</i>	Aerial part	95% EtOH	Anti-cancer	Cytotoxic activity against: A375 with IC ₅₀ = 57.2 µM SH-SY5Y with IC ₅₀ = 20.0 µM	10 161
300	Apigenin 8-C-neohesperidoside	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity	Moderate aphicidal activity against <i>A. pisum</i> with LC ₅₀ value of 171.15 µg/mL at 72 h, repellent activity against <i>A. pisum</i> at 24 h	171
301	Apigenin-6,8-di-C-β-D-glucoside	<i>C. rehderiana</i>					10
302	Apigenin-7-O-(6''-E-p-coumaroyl)- β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	Cytotoxicity against SK-OV-3 (ovarian cancer cell line) with IC ₅₀ = 70.2 µM	161
303	Apigenin-7-O-(6''-acetyl)-β-D- glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	161
304	Apigenin-7-O-β-[4''-O-E-p- coumaroyl glucoside]	<i>C. flammula</i>	Flowers	EtOAc extract	Antioxidant	DPPH IC ₅₀ = 80 ± 0.02 µg/mL Total antioxidant activity: 445 ± 0.01 mg/g	66
305	Apigenin-7-O-β-[6''-O-E-p- coumaroyl glucoside]	<i>C. flammula</i>	Flowers	MeOH extract	Antioxidant	DPPH IC ₅₀ = 20 ± 0.01 µg/mL Total antioxidant activity: 480 ± 0.01 mg/g	66
306	Apigenin-7-O-β-D-(-6''-p- coumaroyl)-glucopyranoside	<i>C. tangutica</i>	Aerial part	70% EtOH	Intestinal anti- oedema Cardioprotective activity	Attenuation of intestinal ischemia-reperfusion injury by decreasing ROS generation and Fe2+ accumulation Protective effect against ischemia/reperfusion injury in primary myocardial cells and in mice hearts in dose-dependent manner Amelioration of myocardial ischemia/reperfusion injury by activating the AMPK signalling pathway and relieving endoplasmic reticulum stress	178 179 180
307	Apigenin-7-O-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	Cytotoxicity against SH-SY5Y with IC ₅₀ = 46.9 µM	161
308	Apigenin-7-O-β-D-glucoside	<i>C. viornae</i>					10
309	Chrysoeriol 7-O-β-D- glucopyranoside	<i>C. viticella</i>	Aerial part	EtOH			174
310	Clematine	<i>C. armandii</i>	Aerial part	EtOH			181

311	Cyanidin 3-O-β-(2''-E-caffeoylglucopyranosyl)-(1→2)-O-β-(6''-malonylgalactopyranoside)	<i>Clematis cultivar 'Niobe'</i>	Flowers	MeOH with formic acid (2%)	163
312	Cyanidin 3-O-β-(2''-E-caffeoylglucopyranosyl)-(1→2)-O-β-(6''-succinylgalactopyranoside)	<i>Clematis cultivar 'Niobe'</i>	Flowers	MeOH with formic acid (2%)	163
313	Cyanidin 3-O-β-(2''-E-caffeoylglucopyranosyl)-(1→2)-O-β-(6''-malonylgalactopyranoside)-3'-O-β-glucuronopyranoside	<i>Clematis cultivar 'Madame Julia Correvon'</i>	Flowers	MeOH with formic acid (2%)	163
314	Cyanidin 3-O-β-(2''-E-caffeoylglucopyranosyl)-(1→2)-O-β-galactopyranoside	<i>Clematis cultivar 'Niobe'</i>	Flowers	MeOH with formic acid (2%)	163
315	Cyanidin 3-O-β-(2''-E-caffeoylglucopyranosyl)-(1→2)-O-β-galactopyranoside-3'-O-β-glucuronopyranoside	<i>Clematis cultivar 'Madame Julia Correvon'</i>	Flowers	MeOH with formic acid (2%)	163
316	Cyanidin 3-O-β-(2''-E-feruloylglucopyranosyl)-(1→2)-O-β-(6''-malonylgalactoside)-3'-O-β-glucuronopyranoside	<i>Clematis cultivar 'Madame Julia Correvon'</i>	Flowers	MeOH with formic acid (2%)	163
317	Daidzein	<i>C. hexapetala</i>			177
318	Delphinidin 3-O-β-[(2''-trans-caffeoyl-glucopyranosyl)-(1→2)-(6''-trans-caffeoyl- tartaroyl-malonyl-galactopyranoside)]-3'-O-β-glucuronopyranoside*	<i>Clematis cultivars 'Jackmanii Superba'</i>	Flowers	MeOH with formic acid (2%)	163
319	Delphinidin 3-O-β-[(2''-trans-caffeoylglucopyranosyl)-(1→2)-(6''-trans-caffeoyl-tartaroyl-malonylgalactopyranoside)]-7-O-β-glucopyranoside*	<i>Clematis cultivars 'Jackmanii Superba'</i>	Flowers	MeOH with formic acid (2%)	164
320	Delphinidin 3-O-β-[(2''-trans-caffeoylglucopyranosyl)-(1→2)-(6''-succinylgalactopyranoside)]-7-O-β-glucopyranoside	<i>Clematis cultivar 'Fujimusume'</i>	Flowers	MeOH with formic acid (2%)	163
321	Formononetin	<i>C. hexapetala</i>			177
322	Genistein	<i>C. hexapetala</i>			177

PARTIE I. REVUE LITTÉRAIRE SUR LE GENRE CLEMATIS : USAGES TRADITIONNELS, ACTIVITÉS PHARMACOLOGIQUES ET PHYTOCHIMIE

323	Hesperetin	<i>C. hexapetala</i>					177
324	Hirsutrin	<i>C. trichotoma</i>					10
325	Hyperin (hyperoside)	<i>C. hexapetala</i>					177
326	Isokaempferide	<i>C. purpurea</i>					10
327	Isoorientin	<i>C. rehderiana, C. viticella</i>	Aerial part	EtOH			174
328	Isoorientin 3'-O-methyl ether	<i>C. viticella</i>	Aerial part	EtOH			174
329	Isoquercetin	<i>C. stans</i>					
330	Isovitexin	<i>C. lasiandra, C. viornae</i>	Aerial part	70% EtOH extract	Antimicrobial	NA	10 162
331	Isovitexin 6-O-E-p-coumarate	<i>C. lasiandra, C. rehderiana</i>	Aerial part	70% EtOH extract	Antimicrobial	Significant antiviral activity against TMV (tobacco mosaic virus) on <i>Nicotiana glutinosa</i> including inactivation ($74.66 \pm 1.88\%$), protective ($59.00 \pm 3.78\%$) and curative ($43.92 \pm 1.41\%$) effects at 500 µg/mL	162
332	Kaempferol	<i>C. brevicaudata, C. hexapetala, C. intricata, C. purpurea</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity	Moderate aphicidal activity against <i>A. pisum</i> with LC ₅₀ value of 313.77 µg/mL at 72 h, repellent activity against <i>A. pisum</i> at 24 h	10 171
333	Kaempferol 3,7-di-O- β -L-rhamnopyranoside	<i>C. lasiandra</i>	Aerial part	70% EtOH extract	Antimicrobial	NA	162
334	Kaempferol 3-O-(2''-benzoyl)- β -D-glucopyranosyl-7-O- β -L-rhamnopyranoside*	<i>C. lasiandra</i>	Aerial part	70% EtOH extract	Antimicrobial	Weak antiviral activity against TMV (tobacco mosaic virus) with <i>Nicotiana glutinosa</i> inactivation, protective and curative efficacies with effect in the range 14 - 23 % at 500 µg/mL	162
335	Kaempferol 3-O-[(6-O-caffeoyl)-glucosyl(1 $\rightarrow$ 2)]-(6-O-caffeoyl)-glucoside-7-O-rhamnoside*	<i>C. flammula</i>	Leaves	EtOH			14
336	Kaempferol 3-O- β -L-rhamnopyranoside	<i>C. lasiandra</i>	Aerial part	Shfu 70% EtOH extract	Antimicrobial	Significant antiviral activity against TMV (tobacco mosaic virus) <i>Nicotiana glutinosa</i> including inactivation ($64.62 \pm 2.86\%$), protective ($57.44 \pm 0.95\%$) and curative ($41.17 \pm 3.8\%$) effects at 500 µg/mL	162
337	Kaempferol-3,7-O-dirhamnoside	<i>C. terniflora</i>					10
338	Kaempferol-3-O-[α -L-rhamnopyranosyl (1 $\rightarrow$ 6)]- β -D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	161

339	Kaempferol-3-O-[β-D-glucopyranosyl (1→2)]-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	161
340	Kaempferol-3-O-galactoside-7-O-rhamnoside	<i>C. flammula</i>	Leaves	EtOH			14
341	Kaempferol-3-O-glucoside	<i>C. purpurea</i> , <i>C. terniflora</i> , <i>C. trichotoma</i> Nakai, <i>Clematis</i> cultivar 'Madame Julia Correvon'	Flowers	MeOH with formic acid (2%)			10
342	Kaempferol-3-O-glucoside-7-O-rhamnoside	<i>C. flammula</i>	Leaves	EtOH			14
343	Kaempferol-3-O-rutinoside	<i>C. terniflora</i> , <i>Clematis</i> cultivar 'Madame Julia Correvon'	Flowers	MeOH with formic acid (2%)			10
344	Kaempferol-7-O-glucoside	<i>C. purpurea</i>					10
345	Linarin	<i>C. hexapetala</i>					177
346	Liquiritigenin	<i>C. hexapetala</i>					177
347	Liquiritigenin-7-O-β-D-glucopyranoside H	<i>C. hexapetala</i>					177
348	Luteolin	<i>C. brevicaudata</i> , <i>C. terniflora</i> , <i>C. trichotoma</i> , <i>C. viornae</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity	Moderate aphicidal activity against <i>A. pisum</i> with LC ₅₀ value of 92.54 µg/mL at 72 h, repellent activity against <i>A. pisum</i> at 24 h	10 171
349	Luteolin-6-C-glucoside	<i>C. viornae</i>					10
350	Luteolin-7-O-glucoside	<i>C. viornae</i>					10
351	Luteolin-7-O-β-D-glucuronide	<i>C. viornae</i>					10
352	Manghaslin	<i>C. viticella</i>	Aerial part	EtOH			174
353	Naringenin	<i>C. hexapetala</i>					177
354	Nobiletin	<i>C. hexapetala</i>					177
355	Puerarin	<i>C. hexapetala</i>					177
356	Quercetin	<i>C. brevicaudata</i> , <i>C. intricata</i> , <i>C. lasiandra</i> , <i>C. purpurea</i> , <i>C. trichotoma</i>	Aerial part	70% EtOH extract, CH ₂ Cl ₂	Insecticidal activity	Aphicidal activity against <i>A. pisum</i> with LC ₅₀ value of 83.33 µg/mL at 72 h, repellent activity against <i>A. pisum</i> at 24 h,	10 171
357	Quercetin 3,7-di-O-α-L-rhamnopyranoside	<i>C. viticella</i>	Aerial part	EtOH			174
358	Quercetin 3-O-β-D-glucopyranoside	<i>C. lasiandra</i>	Aerial part	70% EtOH extract	Antimicrobial	NA	162

359	Quercetin 3-O-β-D-glucopyranuronide	<i>C. lasiandra</i>	Aerial part	70% EtOH extract	Antimicrobial	Significant antiviral activity against TMV (tobacco mosaic virus) <i>Nicotiana glutinosa</i> including inactivation ($82.54 \pm 1.87\%$), protective ($34.92 \pm 2.68\%$) and curative ($32.37 \pm 1.30\%$) effects at 500 µg/mL	162
360	Quercetin 3-O-galactoside	<i>Clematis cultivar 'Madame Julia Correvon'</i>	Flowers	MeOH with formic acid (2%)			163
361	Quercetin 3-O-glucoside	<i>Clematis cultivar 'Madame Julia Correvon'</i>	Flowers	MeOH with formic acid (2%)			163
362	Quercetin 3-O-rutinoside	<i>Clematis cultivar 'Madame Julia Correvon'</i>	Flowers	MeOH with formic acid (2%)			163
363	Quercetin 3-O-rutinoside-7-O-β-D-glucopyranoside	<i>C. lasiandra</i>	Aerial part	70% EtOH extract	Antimicrobial	NA	162
364	Quercetin 3-O-β-D-glucuronide	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity	Moderate aphicidal activity against <i>A. pisum</i> with LC ₅₀ value of 111.42 µg/mL at 72 h, repellent activity against <i>A. pisum</i> at 24 h	171
365	Quercetin 3-O-β-D-glucuronide 6"-methyl ester	<i>C. brevicaudata</i>	Aerial part	CH ₂ Cl ₂ /MeOH	Insecticidal activity	NA	171
366	Quercetin 7-O-α-L-rhamnopyranoside	<i>C. viticella</i>	Aerial part	EtOH			174
367	Quercetin-3,7-O-diglucoside	<i>C. purpurea</i>					10
368	Quercetin-3'-O-methyl-3-O-[α-L-rhamnopyranosyl (1→6)]-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	161
369	Quercetin-3-O-[α-L-rhamnopyranosyl(1→6)]-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	161
370	Quercetin-3-O-[β-D-glucopyranosyl(1→2)]-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	161
371	Quercetin-3-O-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside	<i>C. lasiandra</i>	Aerial part	70% EtOH extract	Antimicrobial	NA	162
372	Quercetin-3-O-galactoside-7-O-rhamnoside	<i>C. flammula</i>	Leaves	EtOH			14

373	Quercetin-3-O-glucoside-7-O-rhamnoside	<i>C. flammula</i>	Leaves	EtOH			14
374	Quercetin-3-O-β-D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	161
375	Quercetin-3-O-β-D-glucopyranosyl	<i>C. rehderiana, C. stans</i>					10
376	Resokaempferol	<i>C. terniflora</i>					10
377	Rutin	<i>C. stans, C. terniflora</i>					10
378	Saiginol E	<i>C. flammula</i>	Leaves	EtOH			14
379	Saiginol K	<i>C. flammula</i>	Leaves	EtOH			14
380	Tangeritin	<i>C. hexapetala</i>					177
381	Tectoridin	<i>C. hexapetala</i>					177
382	Terniflonoside A	<i>C. terniflora</i>					10
383	Terniflorin	<i>C. terniflora</i> var. <i>robusta</i>					10
384	Vitaboside	<i>C. vitalba</i>					10
Lignans							
385	(-)-7'R*-O-methyl-7-epi-tanegool	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH			176
386	(-)-episyngaresinol	<i>C. parviloba</i>					10
387	(-)-lariciresinol	<i>C. parviloba</i>					10
388	(-)-syngaresinol	<i>C. parviloba</i>					10
389	(-)-syngaresinol-4'-O-β-D-glucopyranoside	<i>C. parviloba</i>					10
390	(+)-clemomanshurinane A	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes				10
391	(+)-clemomanshurinane B	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes				10
392	(+)-dihydrodehydrodiconiferyl alcohol	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes				10
393	(+)-epipinoresinol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA	16
394	(+)-erythro-1,2-bis(4-hydroxy-3-methoxyphenyl)-1,3-propandiol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA	16
395	(+)-isolariciresinol 3-α-O-β-D-glucopyranoside	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	41.9 ± 6.2 % of inhibition at 50 μM of lipoxygenase type I-B	165
396	(+)-lariciresinol	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH			159
397	(+)-lariciresinol 4-O-β-D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Cytotoxic activity against	NA	170

three cancer cell lines (HeLa, MGC-803, Ishikawa)						
398	(+)-lariciresinol-4,4'-O-bis-β-D-glucopyranoside	<i>C. armandii</i> , <i>C. stans</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes			182 10
399	(+)-lariciresinol-4'-O-β-D-glucopyranoside	<i>C. armandii</i> , <i>C. stans</i>				182 10
400	(+)-lariciresinol-4-O-β-D-glucopyranoside	<i>C. armandii</i> , <i>C. hexapetala</i> , <i>C. stans</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	PTP1B enzymatic inhibition of 48.30%
401	(+)-medioresinol	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH		159
402	(+)-medioresinol-4'-O-β-D-glucopyranoside	<i>C. parviloba</i>				10
403	(+)-pinoresinol	<i>C. armandii</i> , <i>C. chinensis</i> , <i>C. parviloba</i> , <i>C. terniflora</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Stems, aerial part; roots and rhizomes	EtOH, 90% EtOH, CH ₂ Cl ₂	Anti-neuroinflammatory	165
				fraction from 95% EtOH extract,		183
				n-BuOH fraction from EtOH extract		170
						159
						16
404	(+)-pinoresinol-4,4'-O-bis glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells
405	(+)-pinoresinol-4,4'-O-bis-β-D-glucopyranoside	<i>C. armandii</i> , <i>C. parviloba</i> , <i>C. stans</i>				182
						184
406	(+)-pinoresinol-4'-O-β-D-glucopyranoside	<i>C. armandii</i> , <i>C. parviloba</i>				10
407	(+)-syringaresinol	<i>C. armandii</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Stems, roots and rhizomes	90% EtOH, CH ₂ Cl ₂ fraction from 95% EtOH extract		182
						142
408	(+)-syringaresinol-4'-O-β-D-glucopyranoside	<i>C. armandii</i> , <i>C. delavayi</i> , <i>C. parviloba</i>	Aerial part			184
409	(2R,3R)-secoisolariciresinol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA
410	(7R, 8S, 8'S, 7'S)-4, 4', 7', 9-tetrahydroxy-3, 3'-dimethoxy-7-O-9'-lignan	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH		16
						176

411	(7R,8S)-9-Acetyl-dehydrodiconiferyl alcohol*	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-inflammatory	Inhibition of NO production with IC ₅₀ = 9.3 ± 0.7 µM and inhibition of TNF-α at 25 µM in LPS-stimulated BV-2 cells Inhibition of production of proinflammatory mediators by suppressing JNK/NF-κB signalling in LPS-stimulated RAW264.7 cells decrease of LPS-mediated Src/FAK axis activation and macrophage migration	16 168
412	(7R,8S)-dehydrodiconiferyl alcohol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	Inhibition of NO production in LPS-stimulated BV-2 cells with IC ₅₀ = 3.9 ± 0.5 µM	16
413	(7R,8S)-dihydrodehydrodiconiferyl alcohol	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	IC ₅₀ values of 10.1 ± 1.1 µM against Ishikawa cells No cytotoxicity against HeLa and MGC-803	170
414	(7R,8S,8'S)-3,3'-dimethoxy-7,9'-epoxylignane-4,4',9'-triol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA	16
415	(7R,8S,8'S)-3,3'-dimethoxy-7,9'-epoxylignane-4,4',9'-triol (-)-lariciresinol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA	16
416	(7S,8R)-3,3'-dimethoxy-4,9,9'-trihydroxy-4',7'-epoxy-5',8'-lignan 9-O-β-D-glucopyranoside	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	In vitro cytotoxicity by the MTT assay against HL-60, HepG2, and SGC-7901	Cytotoxicity against: HL-60 with IC ₅₀ = 87.46 ± 0.65 µM HepG2 with IC ₅₀ = 56.12 ± 0.73 µM SGC-7901 with IC ₅₀ = 81.47 ± 0.43 µM	155
417	(7S,8R)-3,3',5'-trimethoxy-4,9,9'-trihydroxy-4',7'-epoxy-5',8'-lignan 4-O-β-D-glucopyranoside	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	In vitro cytotoxicity by the MTT assay against HL-60, HepG2, and SGC-7901	Cytotoxicity against: HL-60 with IC ₅₀ = 87.32 ± 0.74 µM HepG2 with IC ₅₀ = 52.71 ± 0.45 µM SGC-7901 with IC ₅₀ = 68.27 ± 0.55 µM	155
418	(7S,8R)-3,3'-dimethoxy-4,9,9'-trihydroxy-4',7'-epoxy-5',8'-lignan 9'-O-β-D-glucopyranoside	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	In vitro cytotoxicity by the MTT assay against HL-60, HepG2, and SGC-7901	Cytotoxicity against: HL-60 with IC ₅₀ = 90.58 ± 0.82 µM HepG2 with IC ₅₀ = 62.31 ± 1.45 µM SGC-7901 with IC ₅₀ = 78.92 ± 0.95 µM	155
419	(7S,8S)-dihydrodehydrodiconiferyl alcohol 9-O-β-D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803 and Ishikawa cells	170
420	(8R)-3,3'-dimethoxy-4,4',9,9'-tetrahydroxy-5',8'-lignan 3'-O-β-D-glucopyranoside*	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Anti-cancer	Cytotoxicity against HepG2 with IC ₅₀ = 93.52 ± 0.13 µM No cytotoxicity against HL-60 and SGC-7901	155

421	7S,8R-erythro-4,7,9-trihydroxy-3,3'-dimethoxy-8-O-4'-neolignan-9'-O-β-D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
422	7S,8R-erythro-7,9,9'-trihydroxy-3,3'-dimethoxy-8-O-4'-neolignan-4-O-β-D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
423	Armandiside	<i>C. armandii</i>					182
424	Boehmenan	<i>C. armandii</i> , <i>C. hirsuta</i>	Stems, roots	CH ₂ Cl ₂ fraction from 95% EtOH extract, n-hexane/EtOAc	Anti-cancer	Inhibition of A431 cells (human epidermoid carcinoma) proliferation with inhibition of STAT3	
						Cytotoxic effect against: A549 NSCLC cells (non-small-cell lung cancer) with IC ₅₀ = 6.48 μM	169
						Impairment of growth and induction of apoptosis and modulation of EGFR signalling pathway	7
					Antioxidant	DPPH IC ₅₀ = 2.1 μg/mL	16
425	Clemaphenol A	<i>C. chinensis</i>	Roots				94
426	Clemastanin A	<i>C. terniflora</i> var. <i>manshurica</i> , <i>C. stans</i>	Roots and rhizomes	EtOH	Anti-inflammatory	18.5 ± 5.3 % of inhibition at 50 μM of lipoxygenase type I-B	141
427	Clemastanin B	<i>C. stans</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH			165
428	Clemomanshurinane C*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	33.6 ± 4.4 % of inhibition at 50 μM of lipoxygenase type I-B	10
429	Clemomanshurinane D*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	39.2 ± 5.7 % of inhibition at 50 μM of lipoxygenase type I-B	176
430	Dihydrodehydrodiconiferyl alcohol 4'-O-β-D-glucoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	165
431	ecdysanol B	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH	Anti-inflammatory	Significant inhibitory effect on NO production at 2.5-10 μM	170
432	Eleutheroside E	<i>C. terniflora</i>	Aerial part	70% EtOH	Anti-cancer	Cytotoxicity against human ECA-109 with IC ₅₀ = 3.26 μM/mL	176
433	Erythro-(7S,8R)-guaiaacylglycerol-β-O-4'-dihydroconiferyl ether	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	167

434	Erythro-2,3-bis(4-hydroxy-3-methoxy-phenyl)-3-methoxypropanol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA	16
435	Erythro-carolignan E	<i>C. armandii</i> , <i>C. hirsuta</i>	Stems, roots	CH ₂ Cl ₂ fraction from 95% EtOH extract, n-hexane/EtOAc	Anti-neuroinflammatory Antioxidant	NA DPPH IC ₅₀ = 7.4 µg/mL	16 94
436	Erythro-guaiacylglycerol-β-coniferyl ether	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	Inhibition of NO production in LPS-stimulated BV-2 cells with IC ₅₀ = 31.4 ± 1.3 µM	16
437	Hexapetoside G*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	NA	119
438	Hexapetoside I*	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	NA	119
439	Isolariciresinol	<i>C. hexapetala</i> , <i>C. stans</i>					177
440	Justiciresinol	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH			176
441	Lanicepside C*	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
442	Lanicepside D*	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
443	Lasiandranin A*	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Antimicrobial	MIC Value of 25 µg/mL against <i>R. solanacearum</i> , a plant pathogenic bacterium	166
444	Lasiandranin B*	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Antimicrobial	NA	166
445	Lasiandranin C*	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Antimicrobial	NA	166
446	Lasiandranin D*	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Antimicrobial	NA	166
447	Liriodendrin	<i>C. armandii</i> , <i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	182 170
448	Mandshurica A*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW 264.7 macrophage in dose-dependent manner by range 2.5 - 10 µM	159
449	Mandshusica F*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	90% EtOH			176
450	Matairesinol	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
451	Matairesinol 4'-O-β-D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
452	Pinnatifidanin BV	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Antimicrobial	MIC Value of 25 µg/mL against <i>R. solanacearum</i> , a plant pathogenic bacterium	166
453	Pinnatifidanin BVI	<i>C. lasiandra</i>	Whole plant	70% EtOH extract	Antimicrobial	NA	166
454	Salvadoraside	<i>C. armandii</i>					10

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455	Secoisolariciresinol 9-O-β-D-glucopyranoside	<i>C. hexapetala</i>	Roots and rhizomes	60% EtOH	Anti-inflammatory	PTP1B enzymatic inhibition of 58.60%	119
456	Syringaresinol	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
457	Syringaresinol 4'-O-β-D-glucopyranoside	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
458	Threo-1,2-bis(4-hydroxy-3-methoxyphenyl)-1,3-propandiol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA	16
459	Threo-2,3-bis(4-hydroxy-3-methoxyphenyl)-3-methoxypropanol	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA	16
460	Threo-carolignan E	<i>C. armandii</i> , <i>C. hirsuta</i>	Stems, roots	CH ₂ Cl ₂ fraction from 95% EtOH extract, n-hexane/EtOAc	Anti-neuroinflammatory	NA	16 94
461	Threo-carolignan H	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	NA	16
462	Threo-guaiacylglycerol-β-coniferyl ether	<i>C. armandii</i>	Stems	CH ₂ Cl ₂ fraction from 95% EtOH extract	Anti-neuroinflammatory	Inhibition of NO production in LPS-stimulated BV-2 cells with IC ₅₀ = 17.8 ± 0.9 μM	16
Coumarins							
463	1-O-p-coumaroyl-D-glucopyranose	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
464	4,6,7-trimethoxy-5-methyl-coumarin	<i>C. delavayi</i>	Aerial part				142
465	4,7-dimethoxy-5-methyl-coumarin	<i>C. delavayi</i>	Aerial part				142
466	7-hydroxy-4,6-dimethoxy-5-methyl coumarin	<i>C. delavayi</i>	Aerial part				142
467	Aesculetine	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	Cytotoxicity against A375 (human melanoma cell line) with IC ₅₀ = 15.3 ± 3.8 μM	154
468	Aesculin	<i>C. heracleifolia</i>	Leaves	n-BuOH fraction from MeOH extract			117
469	Isoscopolactin	<i>C. florida</i>	Roots	MeOH	Anti-inflammatory	Inhibition of the production of NO on LPS-induced RAW264.7 with IC ₅₀ = 22.88 μM	160
470	Lanicepside E*	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
471	Scopoletin	<i>C. intricata</i>					10

472	Scopolin	<i>C. chinensis</i>	Aerial part	n-BuOH fraction from EtOH extract	Anti-cancer	No cytotoxicity against HeLa, MGC-803, and Ishikawa cells	170
473	Siderin	<i>C. ligusticifolia</i>					185

Table 14: Alkaloids isolated from *Clematis* species and their related biological activities (* structure newly described since 2011)

	Compound name	Species	Part of the plant	Extraction	Activity	Biological activity of the isolated compound	Ref.
474	(3S)-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
475	(6-O- β -D-glucopyranosyl-1H-indol-3-yl) carboxylic acid methyl ester*	<i>C. chinensis</i> , <i>C. terniflora</i>	Aerial part, roots and rhizomes	70% EtOH	Anti-inflammatory Anti-cancer	NA Cytotoxicity against ECA-109 with IC ₅₀ = 1.85 μ M/mL	173 167
476	1H-indole-3-carboxaldehyde	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	20.60% of inhibition of ear swelling using croton oil-induced ear oedema in Kunming mice	173
477	1-ribityl-2,3-diketo-1, 2, 3, 4-tetrahydro-6,7-dimethyl-quinoxaline	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
478	2-ethyl-3-methylmaleimide-N- β -D-glucopyranoside	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154
479	2-methylamino-benzoic acid	<i>C. intricata</i>					10
480	5-O-3-carboxy-indole-2-deoxy-D-ribono-g-lactone*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 12.4 μ M, cell viability > 90%	158
481	5-O-3-carboxy-indole-3-O-(β -D-glucopyranosyl)-2-deoxy-D-ribono-g-lactone*	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 8.3 μ M, cell viability > 90%	158
482	7-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl 3-indolecarbonate	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 9.1 μ M, cell viability > 90%	158
483	Anabellamide	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
484	Choline	<i>C. purpurea</i> Plena <i>elegans</i>					10
485	Clemaïne	<i>C. purpurea</i> Plena <i>elegans</i>					10
486	Clematisindolin A*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of TNF- α in LPS-induced RAW 264.7 cells (macrophage) with IC ₅₀ = 2.63 μ M 14.60% of inhibition of ear swelling using croton oil-induced ear oedema in Kunming mice	173
487	Clematisindolside A*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173

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488	Clematisindolside B*	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
489	Corytuberine	<i>C. recta</i>					10
490	Dictamnine	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of TNF- α in LPS-induced RAW 264.7 cells (macrophage) with IC ₅₀ = 6.75 μ M 10.40% of inhibition of ear swelling using croton oil-induced ear oedema in Kunming mice	173
491	Magnoflorine	<i>C. terniflora</i> var. <i>manshurica</i>			Anti-inflammatory	Decrease of the proliferation, migration, invasion, and reactive oxygen species in IL-1 β -treated MH7A (rheumatoid fibroblast-like synovocyte) cells and decrease of the symptoms in AIA rat models	186
492	Methyl 1H-indole-3-carboxylate	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of TNF- α in LPS-induced RAW 264.7 cells (macrophage) with IC ₅₀ = 7.80 μ M 24.90% of inhibition of ear swelling using croton oil-induced ear oedema in Kunming mice	173
493	Methyl 6-ethoxy-1H-indole-3-carboxylate	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of TNF- α in LPS-induced RAW 264.7 cells (macrophage) with IC ₅₀ = 6.59 μ M 9.10% of inhibition of ear swelling using croton oil-induced ear oedema in Kunming mice	173
494	Methyl 6-hydroxy-1H-indole-3-carboxylate	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	Inhibition of TNF- α in LPS-induced RAW 264.7 cells (macrophage) with IC ₅₀ = 3.99 μ M and inhibition of the IL-6 expression at 20 μ M 20.20% of inhibition of ear swelling using croton oil-induced ear oedema in Kunming mice	173
495	Methyl 6-methoxy-1H-indole-3-carboxylate	<i>C. chinensis</i>	Roots and rhizomes	70% EtOH	Anti-inflammatory	NA	173
496	Methyl 6-methoxy-3-indolecarbonate	<i>C. florida</i> var. <i>plena</i>	Roots	MeOH	Anti-inflammatory	NA	87
497	Methyl 6-O- α -L- rhamnopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl 3-indolecarbonate	<i>C. florida</i> var. <i>plena</i>	Roots	MeOH	Anti-inflammatory	NA	87
498	Methyl-7-ethoxy-3-indolecarbonate	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes				10
499	Methyl-7-methoxy-3-indolecarbonate	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes				10

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500	Methyl-7-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl-3-indolecarbonate	<i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes				10
501	Plenane A*	<i>C. florida</i>	Roots	MeOH	Anti-inflammatory	Inhibition of the production of NO on LPS-induced RAW264.7 cells with IC ₅₀ = 40.92 μ M	160
502	Scopoline	<i>C. aethusifolia</i>	Aerial part	95% EtOH	Anti-cancer	NA	154
503	α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl 6-methoxy-3-indolecarbonate*	<i>C. florida</i> var. <i>plena</i>	Roots	MeOH	Anti-inflammatory	Male ICR mice - ear oedema induced by xylene – 27.0% of inhibition at 200 mg/kg	87
504	α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl-3-indolecarbonate	<i>C. florida</i> var. <i>plena</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes	MeOH, EtOH	Anti-inflammatory	Inhibition of the production of pro-inflammatory mediators (PGE2 and NO) in LPS-induced BV-2 cells and decrease of the expression of iNOS and COX-2 genes	156
							87
						Inhibition of NO production in LPS-stimulated RAW264.7 cells with IC ₅₀ = 7.7 μ M, cell viability > 90%	158
505	α -magnoflorine	<i>C. parviloba</i> , <i>C. recta</i>					10
506	β -magnoflorine	<i>C. parviloba</i> , <i>C. recta</i> , <i>C. terniflora</i> var. <i>manshurica</i>	Roots and rhizomes				10

Table 15: Miscellaneous compounds isolated from *Clematis* species and their related biological activities (* structure newly described since 2011)

	Compound name	Species	Part of the plant	Extraction	Activity	Biological activity of the isolated compound	Ref.
Anemonin derivatives							
507	Anemonin	<i>C. angustifolia</i> , <i>C. crassifolia</i> , <i>C. flammula</i> , <i>C. hexapetala</i> , <i>C. montana</i> , <i>C. recta</i>	Whole plant, flower	precipitation of the essential oil and extraction with chloroform	Molluscicide activity	Activity against <i>G. truncatula</i> LC ₅₀ = 9.64 mg/mL after 48 h	187 50 82 141 10
508	Protoanemonin	<i>C. apiifolia</i> , <i>C. flammula</i>					188
509	Ranunculin	<i>C. flammula</i>					10
Macrocylic							
510	Berchemolide	<i>C. armandii</i>					189
511	Clemahexapetoside A	<i>C. hexapetala</i>	Roots and rhizomes				187 190
512	Clemahexapetoside B	<i>C. hexapetala</i>	Roots and rhizomes				187
513	Clemoarmanoside A	<i>C. armandii</i>					189
514	Clemoarmanoside B	<i>C. armandii</i>					189
515	Clemochinenoside A	<i>C. chinensis</i> , <i>C. hexapetala</i> , <i>C. terniflora</i> var. <i>mandshurica</i>	Roots and rhizomes				10 187
516	Clemochinenoside B	<i>C. hexapetala</i>	Roots and rhizomes				187 190
517	Clemochinenoside C*	<i>C. chinensis</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibitory activity against LPS-induced TNF- α production in RAW246.7 cells with IC ₅₀ = 12.9 $\pm$ 2.3 μ M	191
518	Clemochinenoside D*	<i>C. chinensis</i>	Roots and rhizomes	EtOH	Anti-inflammatory	Inhibitory activity against LPS-induced TNF- α production in RAW246.7 cells with IC ₅₀ = 18.4 $\pm$ 2.7 μ M	191
Volatils							
519	9,12,15-Octadecatrienoic acid ethyl ester	<i>C. cirrhosa</i>	Aerial part	Essential oil			77
520	9,12-Octadecadienoic acid ethyl ester	<i>C. cirrhosa</i>	Aerial part	Essential oil			77
521	Coniferaldehyde	<i>C. montana</i>					10
522	Hexadecanoic acid ethyl ester	<i>C. cirrhosa</i>	Aerial part	Essential oil			77

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523	Hexadecanoic acid methyl ester	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
524	Hexahydrofarnesyl acetone	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
525	Juniper Camphor	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
526	Myristicinether oxyde	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
527	Nonanol	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
528	Octanol	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
529	Palmitic acid	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
530	Phytol	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
531	Thymol	<i>C. cirrhosa</i>	Aerial part	Essential oil	77
532	Vanillin	<i>C. montana</i>			10
Others					
533	2-phenethyl-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside	<i>C. akebioides</i>	Aerial part	EtOAc fraction from 80% EtOH extract	118
534	Aurantiamide acetate	<i>C. terniflora</i>			167
535	D-galactopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\leftrightarrow$ 1)- β -D-glucopyranoside	<i>C. flammula</i>	Leaves	MeOH Antioxidant	DPPH IC ₅₀ = 70 $\pm$ 0.005 μ g/mL Total antioxidant activity: 462 $\pm$ 0.02 mg/g 66
536	Mannitol	<i>C. intricata</i>			10
537	n-octacosanol	<i>C. intricata</i>			10
538	Oleic acid	<i>C. hirsuta</i>	Roots	n-hexane/EtOAc	94

6.3. Alkaloids

Several alkaloids have been identified in the genus *Clematis* (Table 14). Scopolin has been found in the aerial part of *C. aesthusifolia*¹⁵⁴. Thirteen indole alkaloids have been isolated from the roots and rhizomes of *C. chinensis*¹⁷³. Clematisindolin A presented anti-inflammatory properties against LPS-induced TNF- α and NO release in RAW 264.7 cells with IC₅₀ value of 2.63 μ M and 14.60% of inhibition of the ear swelling. Dictamnine, methyl 1H-indole-3-carboxylate, methyl 6-ethoxy-1H-indole-3-carboxylate, methyl 6-hydroxy-1H-indole-3-carboxylate, and 1H-indole-3-carboxaldehyde have also been reported to have an anti-inflammatory activity¹⁷³. Plenane A, a newly isolated alkaloid from *C. florida* roots also presented *in vivo* anti-inflammatory properties with IC₅₀ value of 40.92 μ M using ear oedema induced by xylene assay¹⁶⁰. α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl 6-methoxy-3-indolecarbonate, another new alkaloid compound isolated from the roots of *C. florida* var. *plena*, presented a 27.0% reduction of swelling at 200 mg/kg of the ear oedema induced by xylene assay⁸⁷.

Two new alkaloids with antioxidant activity have also been isolated from roots of *C. terniflora* var. *manshurica*¹⁵⁸. 5-O-3-carboxy-indole-2-deoxy-D-ribo-n-g-lactone and 5-O-3-carboxy-indole-3-O-(β -D-glucopyranosyl)-2-deoxy-D-ribo-n-g-lactone have shown potent inhibitory activity against LPS-induced nitric oxide (NO) production in RAW 246.7 macrophages with IC₅₀ = 12.4 μ M and IC₅₀ = 8.3 μ M and cell viability above 90%. (6-O- β -D-glucopyranosyl-1H-indol-3-yl) carboxylic acid methyl ester extracted for the first time, showed cytotoxic activity against human ECA-109 with IC₅₀ value of 1.85 μ M¹⁶⁷.

All the alkaloids identified in the genus *Clematis* have been extracted from the roots or rhizomes and have been mainly tested for anti-inflammatory activity.

6.4. Miscellaneous compounds

a. Anemonin derivatives

Ranunculin is the glycoside precursor of protoanemonin, a toxin present in fresh plants of the *Ranunculaceae* family including *Clematis* spp^{192–194}. During drying of the plant, the protoanemonin is dimerized to an inactive form, the anemonin. Protoanemonin is widely described to cause extreme irritation of the skin and intense inflammation.

These compounds can also present other activity, as anemonin extracted from the flower of *C. flammula* has presented a molluscicidal activity against *G. truncatula* with LC₅₀ value of 9.64 mg/mL after 48 h⁸².

b. Macrocyclic compounds

Seven macrocyclic compounds have been extracted from *C. armandii*, *C. chinensis*, *C. hexapetala*, and *C. terniflora* var. *manshurica* (Table 15). Clemochinenoside C and clemochinenoside D have been previously reported¹⁰. These are two macrocyclic structures,

isolated for the first time from the roots and rhizomes of *C. chinensis*, which showed IC_{50} values of $12.9 \pm 2.3 \mu M$ and $18.4 \pm 2.7 \mu M$ against LPS-induced TNF- α production in RAW246.7 cells, respectively ¹⁹¹. These results suggest the anti-inflammatory potential of macrocyclic compounds from *Clematis* spp.

7. Conclusion

A total of 32 species have been reported in the traditional uses worldwide for treating diseases or conditions such as inflammation, rheumatoid arthritis, headaches, or for diuretic and hepatic purposes. Most of these species, employed in traditional medicine and for pharmacological purposes, have been evaluated on several biological models to demonstrate their bioactivity.

In general, both aerial parts and roots have shown activities, justifying their use in traditional medicine. Anti-microbial and anti-inflammatory are the two main activities demonstrated *in vitro*, which can be correlated to the wound healing properties of several *Clematis* spp. demonstrated *in vivo* because, to be initiated, the wound healing process requires these two steps. Furthermore, these two main activities are also reflected through other traditional uses such as the use of *Clematis* against toothache, cough, or throat inflammation, where anti-microbial and anti-inflammatory properties are also necessary.

However, *in vivo* studies are extremely limited and more information regarding potential adverse effects of extracts is needed to confirm some activities. The phytochemical composition of some *Clematis* spp. has been studied to correlate bioactivity and phytochemistry. A total of 538 phytochemical compounds, including 121 novel structures since 2011 have been isolated and identified from 36 different species of the genus *Clematis*, including mainly terpenoids, phenolic compounds, alkaloids, and compounds such as macrocyclic or anemonin derivatives. However, a large part of the genus has not been studied yet in terms of phytochemistry. *C. chinensis*, with more than 100 isolated compounds identified, is the most studied species as it is employed in Traditional Chinese Medicine.

This review offers a comprehensive summary of recent available data on the genus *Clematis* in ethnopharmacology, phytochemistry, and pharmacological activities. Some of the traditional medicine applications can be justified with the capability of some *Clematis* extracts to decrease the level of proinflammatory cytokines, preventing chronic inflammation and demonstrating antimicrobial activities.

Our review highlights the significant interest in the genus. Among the 388 species, there is a considerable number of unstudied species, some of them due to their recent identification and others despite their use in traditional medicine. Additionally, this review provides a valuable foundation for future research on the genus *Clematis*.

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Partie II. Réseau moléculaire multi-informatif pour le profilage chimique de dix espèces de *Clematis*

Multi-informative molecular network including taxonomic metadata for chemical profiling of ten *Clematis* species

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Abstract

Clematis species are primarily known for their ornamental uses, making them commonly encountered plants. Some species are also used in traditional medicine, especially in Traditional Chinese Medicine (TCM) where the use of *Clematis* spp. roots are common to treat various inflammatory diseases. However, among the 388 species recorded, only a few have been previously investigated from a chemical point of view. Based on the phytochemical profiles of highly studied species as *C. chinensis*, this article aims to explore the phytochemical profiles of the leaves of ten species. In this study, we employed non targeted UHPLC-MS/MS metabolomic approach for the annotation of specialized metabolites. Molecular networking was combined with three annotation tools to suggest a putative identification of the compounds present in the hydroalcoholic extract. The investigation reveals a high diversity of phenolic compounds with mainly flavonoids derivatives (*O*-glycosylated, *C*-glycosylated, acetylated, substituted by hydroxycinnamic moieties etc.) as well as some lignans. Some of them very specific to one species as the *C*-glycosylated flavonoids just founded in *C. viticella*. Among the other compound classes, carotenoid derivatives and a few alkaloids were annotated. This metabolic profiling provides comprehensive information about shared characteristics and specificities between the ten *Clematis* species, among them *C. koreana* appears to be the species with the highest specific metabolites content. This work opens perspective for bioactivity investigation for mainly anti-inflammatory and antioxidant activities.

1. Introduction

Plants are a rich source of natural products (NPs) and among them there is a broad diversity of specialized metabolites, which are produced to provide protection, attract beneficial organism, or offer a competitive advantage. These metabolites possess diverse chemical structures and biological activities, making them valuable for application in pharmaceuticals, cosmetics and agriculture.

Furthermore, NPs are particularly interesting due to their coverage of a vast chemical space¹. It is therefore crucial to develop and apply innovative analytical techniques to explore plant phytochemistry but the rediscovery of known compound is common in natural product research with more than 700 000 NPs described². Just focusing on the *Clematis* L. genus, the third largest within the Ranunculaceae family, comprising 388 accepted species distributed over most of the world, especially in Asia, North America and Europe³, phytochemical studies of *Clematis* species have led to the identification of over 500 compounds in the whole plant, predominantly terpenoids, phenolic compounds and alkaloids. But around the 388 accepted species, the phytochemistry of only 36 species had been previously described with *C. chinensis* being by far the most studied and explored species. Furthermore, *Clematis* genus is described in the Traditional Chinese Medicine, in Traditional African medicine and in various countries worldwide for the treatment of diseases and conditions such as inflammation, rheumatoid arthritis or headache^{4–9}. Bioactivity assays have been performed on *Clematis* extracts and on isolated compounds, that have demonstrated promising antioxidant and anti-inflammatory properties, supporting the traditional uses of *Clematis* spp. for anti-arthritis properties^{10–13}. Taking together, as a major part of the species of the *Clematis* genus remains unexplored, *Clematis* presenting a promising opportunity for the identification of new NPs.

NP-based drug discovery, analytical and computational approaches have been developed over the last twenty years to better understand the chemical complexity of plant¹⁴. Indeed, metabolomics can help to accelerate dereplication by organizing data and identifying known compounds, it therefore allows a faster highlighting of unknown compounds or a prioritization of NPs of interest for isolation^{15–18}.

Furthermore, the putative identification of a metabolite in an extract can be long and can be erroneous, particularly if they are performed only using one source of annotation, particularly due to source fragmentation, to the difficulty of differentiating isomers or because not all compound had been already described. Molecular Network alone directly do not help to perform annotation, but by connecting nodes according their spectral similarity, this information can be extended to other connected nodes, facilitating the interpretation and the identification of related structure¹⁹. To address this issue, a wide range of computational metabolomics tools have been developed to help in the annotation of compounds, allowing the determination of chemical classes or the suggestion of a potential structure^{14,20–22}. To

enhance the confidence level of annotations different tools can be implemented incorporating additional information and cross-referencing results from different sources.

In this article, the chemical diversity of 10 species of *Clematis* (*C. alpina*, *C. chinensis*, *C. flammula*, *C. heracleifolia*, *C. koreana*, *C. tangutica*, *C. terniflora*, *C. recta*, *C. viticella* and *C. vitalba*) collected in the Botanical Garden of Fribourg and Neufchatel had been explored. Among them, the phytochemistry of some species like *C. chinensis* have already been well explored while the phytochemistry of *C. alpina*, *C. recta* and *C. koreana* have never been explored to date. Studying these ten species in a same dataset allowed, by comparison to well-studied species, a better dereplication of unstudied species. UHPLC-MS/MS analysis had been combined with molecular network and computational annotation tools to generate multi-informative molecular network. First of all, the construction of molecular network using GNPS¹⁹ integrated the research of information from their library using the same cosine score principle for the comparison of the experimental spectrum and the spectrum from the library. Then TIMA-R²³, a tools which used taxonomical information (species, genus and family) coupled to the ISDB-DNP library, scored the annotation considering the biological context of the analysed extract. Finally, SIRIUS²⁴ an *in silico* fragmentation tools was employed and the all resulting annotation were compared to suggest a commune or a consensus annotation. This strategy allowed the compound class annotation and the first exploration of the phytochemistry of the ten *Clematis* species.

2. Materials et methods

2.1. Plant material

Leaves of *C. alpina*, *C. heracleifolia*, *C. recta* and *C. viticella* were collected in the Botanical Garden of Fribourg (Switzerland) and *C. chinensis*, *C. flammula*, *C. koreana*, *C. tangutica*, *C. terniflora* and *C. vitalba* were collected in the Botanical Garden of Neuchatel (Switzerland) during April and May 2023. Plant leaves were collected and immediately put in liquid nitrogen and then stored at -70°C up to being used.

2.2. Extraction and sample preparation

The extraction had been performed according the DBGI protocol (<https://www.dbgi.org/dendron-dbgi/notes/mpboa8wpvhpygf5fw4o9v11/#extraction-method>). Briefly, around 50 mg of lyophilized plant are ground using the Retsch during 2.5 minutes at 25 Hz and then 1.7 mL of a mixture of 80/20 methanol/water with 0.01% formic acid is added to the grind and extraction is carried out under the same condition. Centrifugation at 13 000 rpm and supernatant was then transfer in 2 mL vial to be analyzed.

2.3. UHPLC-HRMS/MS analysis

UHPLC was performed with a Thermo vanquish UHPLC system interfaced to a Q-Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) using a heated electrospray ionization (HESI) source. The column was a Waters BEH C18 (100 × 2.1 mm, 1.7 μm) and gradient mode was used to separate the compounds. The mobile phase consisted of water (A) and acetonitrile (B) both acidified with 0.1% formic acid. Linear gradient started at 2% of B and increased to 100% over 10 minutes and was maintained at 100% B for 2 minutes. Column temperature was fixed at 40 °C, flow rate was fixed at 600 μL·min⁻¹ and volume of injection was 2 μL.

Analyses were acquired in positive mode (HESI+). MS/MS experiments were carried out using data-dependent acquisition mode (DDA). Full scans were acquired at a resolution of 35,000 full width at half maximum (FWHM) (at m/z 200) and MS/MS scans at 17,500 FWHM. After being acquired in a MS/MS scan, parent ions were placed in a dynamic exclusion list for 2.0 seconds. MS/MS events were performed on the 5 most intense ions detected in full-scan MS (Top5 experiment). MS/MS isolation window width was 1 Da, and the stepped normalized collision energy was set to 15, 30, and 45 units. Source voltage was maintained at 3.5 kV; Sheath gas flow rate (N₂), 55 units; auxiliary gas flow rate, 15 units; spare gas flow rate, 3.0; capillary temperature, 275.00°C; and S-Lens RF Level, 50.

2.4. Raw data treatment

Analyses were converted to mzML format using MSConvert software²⁵ and were then processed with MZmine 3.9²⁶. Mass detection levels were fixed at 5×10^4 for the mass spectrum level 1 (MS¹) and 0 for the fragmentation spectrum (MS²). An Automated Data Analysis Pipeline (ADAP) chromatogram builder and resolver were employed for features comprises between 2 and 10 min, with a minimum of 4 consecutive scans at a threshold of 5×10^4 ²⁷. Alignment was performed based on m/z tolerance of 0.008 Da absolute tolerance in MS¹ and a retention time tolerance of 0.2 min. Isotope ¹³C grouper was also performed. Gapfilling and filtering were then performed on the aligned table. A feature list comprising 1779 features was finally obtained.

2.5. Feature-based molecular networking

A molecular network was generated using GNPS¹⁹ with the Feature-Based Molecular Networking (FBMN) workflow²⁸. The MGF and metadata table obtained from pre-processing was transferred through WinSCP to GNPS. A FBMN was created in which the edges were filtered to have a cosine score above 0.7 and more than 6 matched ions and a maximum size of a cluster was set to 100. The molecular network was then visualized using Cytoscape 3.10.2 software²⁹. The FBMN can be found on GNPS platform (ID=287d5a1ce59b41f0830458e49dab59c5).

2.6. Metabolite annotation using computational tools and manual interpretation

Annotation was performed in combination of several annotation tools. First, the FBMN was annotated using GNPS. The library spectra were filtered in the same manner as the FBMN processing, with cosine score above 0.7 and more than 6 matched ions. GNPS provide a score comprise between 0.7 and 1 corresponding to the cosine score between the experimental spectrum and the one provides in the library. Then, TIMA-R v2.9 was performed using the MGF files and the metadata table containing the taxonomical information of the samples. This tool based on R language is able to compared *in silico* spectrum calculated from the LOTUS database^{23,30–32} and to correlated this information with the taxonomical context (species, genus, family etc.) of the analyzed samples. According to the proximity between the experimental sample and the organism from which the annotation was derived, a similarity score is calculated that combines spectral and taxonomic information. This score ranges from 0 to 1, with a score of 1 indicating a well-annotated metabolite found in the higher taxonomical rank. The MGF file was also processed using SIRIUS 5.8, an *in silico* annotation tool. This software combines formula calculation with the prediction of a molecular fingerprint for experimentally detected metabolites based on their fragmentation trees and spectra. The resulting score ranges from 0 (indicating a highly potential candidate) and decreases toward negative infinity. Confidence in annotation from GNPS, is level 2 or 3 and TIMA-R and SIRIUS are level 3 according to the description of the Metabolomics Standards Initiative³³.

3. Results and discussion

3.1. Overview of the molecular networking (MN)

The ten *Clematis* species were analysed using UHPLC-HRMS/MS and the data were processed to obtained a feature list containing 1 779 features corresponding to aligned detected ions. In order to have a better understanding of the similarities and differences among the species, Figure 40 represents the number of unique ions (dark color) for each species as well as the number of common ions (light color), defined as an ion detected in at least two species/samples.

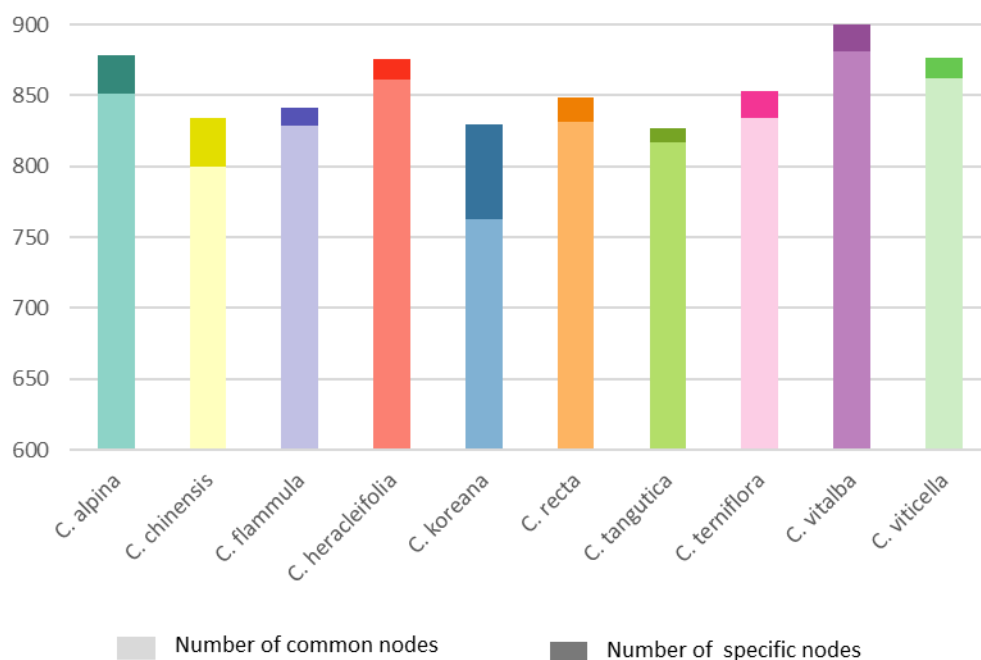


Figure 40: Data processing of the UHPLC-MS/MS analysis of the ten *Clematis* species. Number of detected ions per species commune to at least one other species (light color) and number of specific ions (dark color).

As presenting in Figure 40, the use of the same data processing treatment for all samples led to 830 to 900 detected ions in each sample. Most of them are shared by at least two species, indicating a certain similarity between the samples. This justifies the use of MN for exploration and annotation, as it helps to group identical ions of different samples into a unique feature³⁴. Among the species, *C. koreana* stands out from the other species with 67 nodes specific to this species following by *C. chinensis* with 34 unique nodes.

Feature-based molecular networking was therefore used to explore the phytochemical diversity of the 10 *Clematis* species studied. Figure 41 represents the molecular network, which consisted of 1779 nodes grouped in 146 clusters (with more than 2 nodes connected) and 819 single nodes. The color illustrates the distribution of the ions across the ten *Clematis* species, by using a pie chart, where each color corresponds to a different species. The objective of this step, is to localize specificity or commune ions in the ten species.

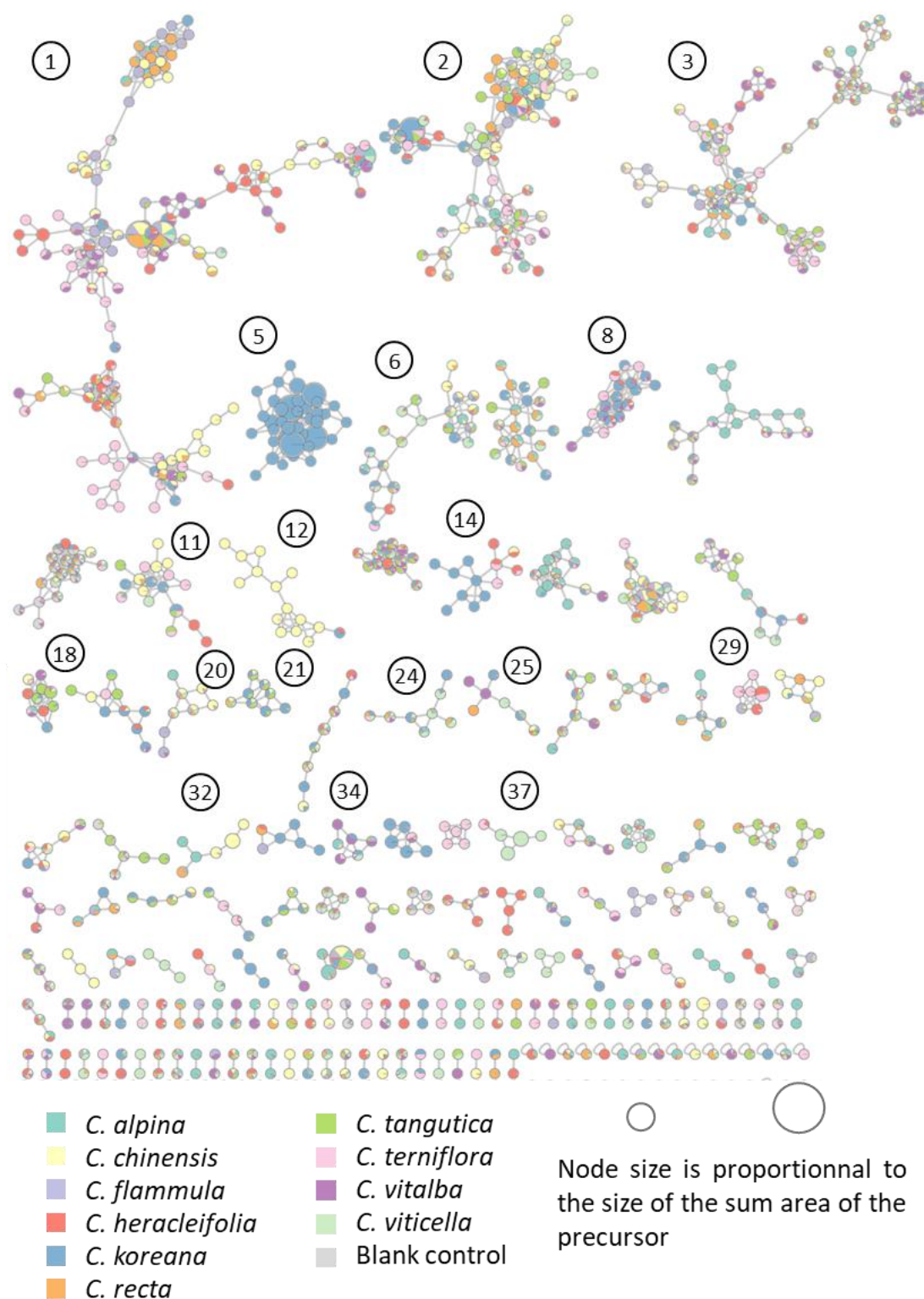


Figure 41: Metabolic profile of 10 *Clematis* species and relative distribution of the precursor ion among the studied species. Feature-based Molecular Network of the (+)-HESI MS/MS spectra, pie charts illustrating the distribution of the feature in the studied *Clematis* species for which each colour is associated to a species. Node size is proportional to the sum area and number of some investigated cluster is mention.

The three most important clusters (1-3), representing 262 nodes, consist of ions from all ten species (Figure 41). As expected, Figure 40 shows that a large portion of the phytochemistry of *Clematis* species is shared and results in numerous molecules of the same family group into large clusters. In contrast, other clusters are specific of a single species; for instance, *C. koreana* stands out among the ten analyzed *Clematis* with unique cluster such as cluster 5. The specificity of *C. chinensis* is also visible with its unique cluster 12. Among the molecular network, it is also possible to see inside a cluster, some specific region, where only one or two species are represented, traducing the presence of a similar compound's family in the different samples but with some slightly modification as in cluster 14, where a part is dedicated for *C. koreana* and the other part is represented by three species.

CANOPUS had been employed using SIRIUS 5.8 to identify the NP chemical pathway of the ion^{21,24}. The annotation of the natural product pathways and their distribution among the samples provide an initial overview of the analyzed species. The sunburst (Figure 42A) represents the different compound class at 2 levels (pathways and superclasses), with a different color attributed to each pathway. In the three important classes of specialized metabolites, the most represented are compounds derived from the shikimate and phenylpropanoid pathways (22%), including flavonoids and phenolic compounds; the second class consists of terpenoids (20%) with mainly apocarotenoids and sesquiterpenoids, and finally alkaloids (15%) (Figure 42A). As expected from the literature, a large number of phenolic compounds were detected, solvent of extraction employed (methanol/water 80/20 with 0.01% FA) favor the extraction of polar and medium-polar components. Studies of the aerial parts or the leaves of *Clematis* species, including *C. chinensis*, *C. terniflora*, *C. heracleifolia* and *C. viticella* spp., four species also studied herein, led to the isolation of a high diversity of coumaric, ferulic and caffeic derivatives³⁵⁻³⁹. Regarding the flavonoids, some glycosylated kaempferol, quercetin and apigenin were previously isolated from *C. flammula* or *C. tangutica*⁴⁰⁻⁴². Concerning the terpenoids, a high amount of triterpenoids saponins was previously described in the literature review of Chawla *et al.* (2012)⁷. However, the specific parts of the plant studied were not always mentioned, although it appears that they are primarily derived from the roots, which is the part of *Clematis chinensis* used in Traditional Chinese Medicine (TCM)^{43,44}. Therefore, the two parts of the plants have different phytochemistry, and it appears that significantly fewer triterpenoid saponins are extracted from the leaves compared to the roots. Finally, regarding the alkaloids, a few indolic derivatives were isolated but only from roots of *C. chinensis* or *C. terniflora* var. *manshurica*^{36,45}. Only scopoline, extracted from of *C. aethusifolia*, was identified in the aerial parts⁴⁶.

Primary metabolites have also been extracted and are present in the plants extracts, with a high proportion of fatty acids such as glycerolipids and glycerolphospholipids, as well as

various minor acids and peptides. In this study we focus on the specialized metabolites, representing 67% of the detected ions.

Additionally, mapping the compound class on the MN can guide the exploration, as certain compound class may be of interest depending the objective. As illustrated in Figure 42B the MN is mapped using the same color as the sunburst for each compound class. A large majority of the largest clusters stand out with consistent colors, indicating coherence in class annotation. The three most important cluster (1-3) are associated with phenolic compounds (cluster 1 and 2) or terpenoids (cluster 3).

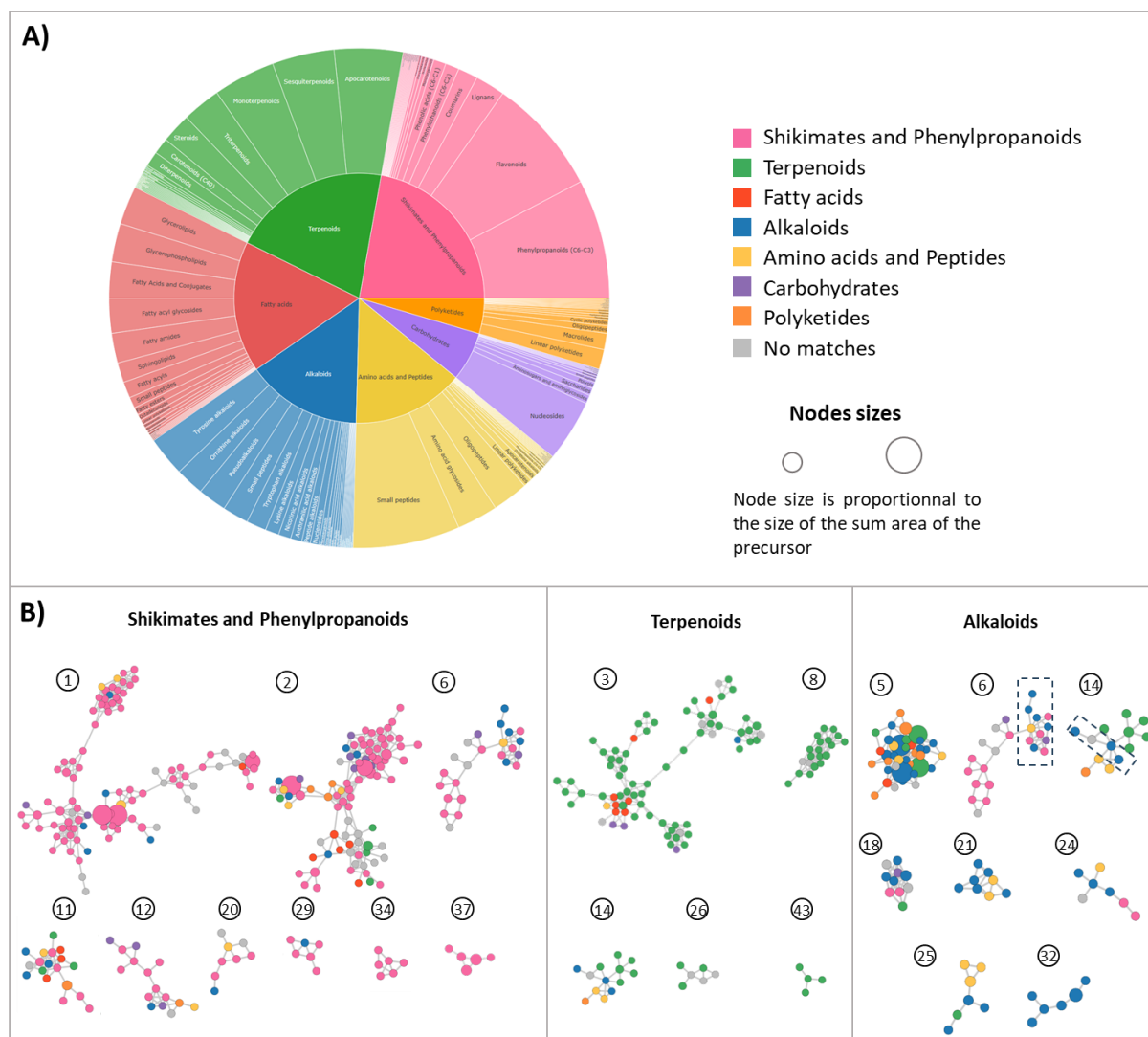


Figure 42: A) Sunburst of the (+)-HESI MS/MS spectra annotated using Canopus Natural Products Classification. B) Main clusters of *Clematis* FBMN in (+)-HESI MS/MS annotated using Canopus Natural products classification. Cluster or part of the cluster (dotted lines) are ranked by pathway and each color represents a different molecular pathway.

However, for some clusters a few nodes of different colors sometimes appear, this is notably the case for clusters where majority of the nodes are identified from the alkaloids class

(Cluster 5, 6 and 14 for example). This may suggest spectral similarity with other molecular families or this could also indicate that the current classification of families is incorrect indicating that some caution should be taken when interpreting these nodes.

In order to have a better understanding of the phytochemistry of the *Clematis* spp. Analysis, several clusters were deeper investigated and a comparison of the annotation obtained from the 3 tools (GNPS, TIMA-R and SIRIUS) was realized. Figures 43-47 present the investigated cluster where nodes are displayed as a pie chart to reflect the distribution of each ion among the 10 species (each species is represented by one color); the size of the node is proportional to the sum of the area of ion for the 10 species and annotated structure of some nodes are displayed. The tables (Table 16-20) include information such as retention time, the detected m/z and the corresponding adduct, the molecular formula and the error in ppm. It also provides the main MS² fragments, the NP class using NP classifier, the annotated compound name and the score from each tool used for annotation. Annotations are taking into account, when possible, of the different tools with the confidence score. For SIRIUS, the CSI:fingerID⁴⁷ score had been employed, the closer the score is to 0, the higher the confidence. For TIMA-R the closer the score is to 1, the better is the confidence in the annotation. The final score considers both chemical similarity and taxonomical information. If a compound has never been described in the genus or in the family, this may decrease the score. Lastly, GNPS dereplication uses the similar principle as molecular construction, relying on a cosine score with GNPS database, where the closer the score is to 1, the higher is the confidence.

3.2. Focus on the shikimates and phenylpropanoids pathway

Compounds from the shikimates and the phenylpropanoids represented around 22% of the compounds in the *Clematis* extracts with 9 mains cluster of different sizes. Among the 9 clusters, clusters 1, 2, 6, 12 and 37 have been deeper explored. The two mains' clusters (1-2) are respectively composed of 100 and 87 nodes each and are not specific to a species, even if some nodes can be presented in only one species. 16 compounds have been annotated accurately from the Cluster 1 using SIRIUS²⁴, TIMA-R²³ and GNPS¹⁹ and are all summarized in Table 16.

Cluster 1, in Figure 43, is specific of flavonoids substituted by hydroxycinnamic acid and derivatives (coumaric acid, caffeic acid and chlorogenic acid and derivatives in blue dot square) and hydroxycinnamic derivatives themselves (orange dot square). As the size of the node is proportional to the sum of precursor area, two nodes stand out, among them, compound 155 was annotated as chlorogenic acid by the three annotated tools with a good confidence (-4.44 for SIRIUS, 0.58 for TIMA-R and 0.97 for GNPS) along with compound 50 annotated as caffeic acid (-1.31 for SIRIUS, 0.80 for TIMA-R and 0.98 for GNPS).

Those phenolic compounds are a non-specific class of compound contained in the ten species as well as in a lot of plants genus as they contribute to the defence against ultraviolet or pathogen attacks and play an important role in growth and development⁴⁸.

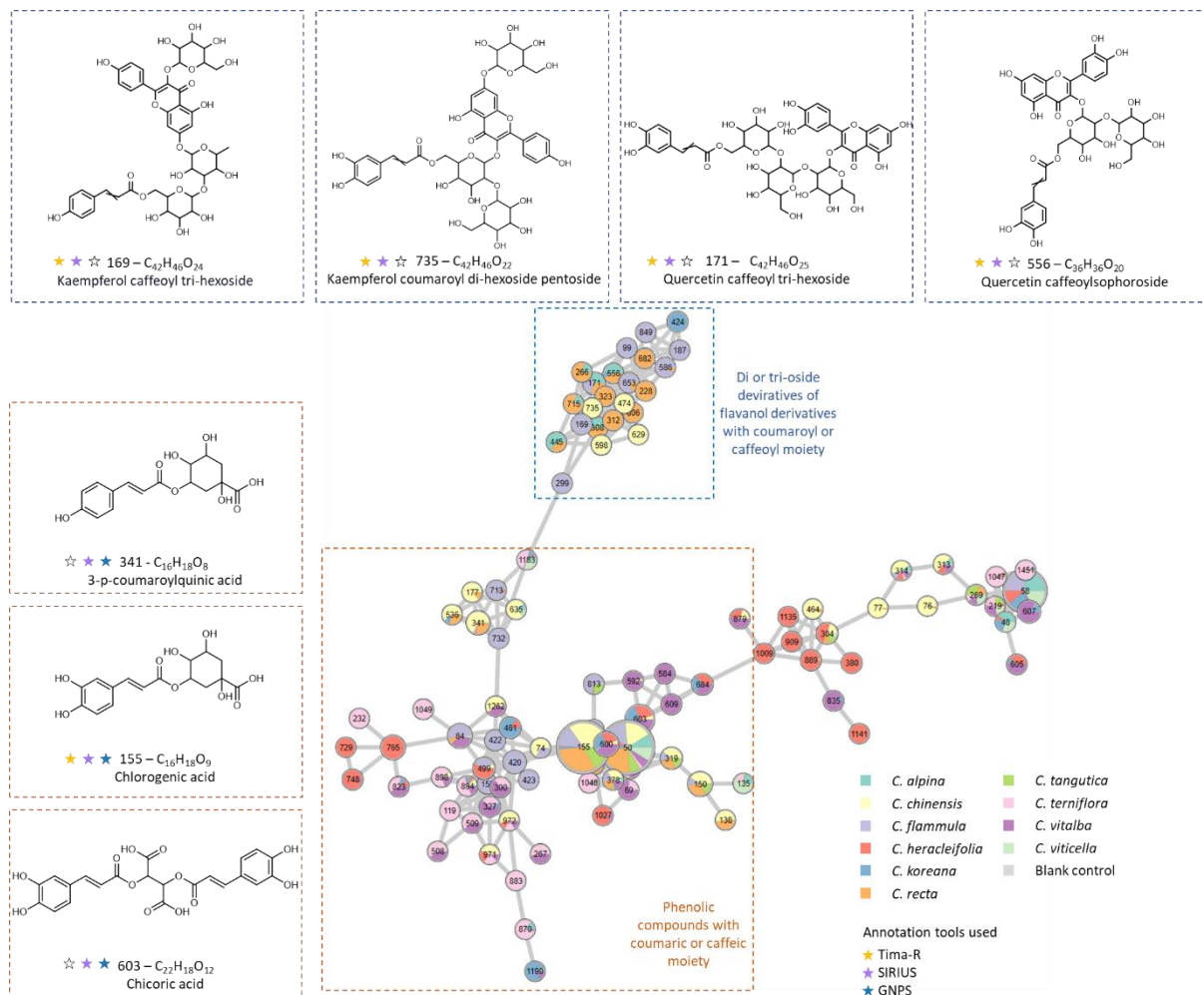


Figure 43: Cluster 1 of the (+)-HESI MS/MS spectra with a focus on the compounds from the shikimate and phenylpropanoid pathway predicted by Canopus. Each color is representing a Clematis species and the size of the node is proportional to the sum area intensity. Dotline square are corresponding to the zone where the ion is identified and some structures are suggested from the annotation tools, for each a colored star indicated the annotation tool able to propose identification.

A region of cluster 1 (blue dot square) in Figure 43, is dedicated to flavonoids with coumaroyl and caffeoyl moieties only that are present in five of the ten species (*C. alpina*, *C. chinensis*, *C. flammula*, *C. koreana* and *C. recta*). Tima-R and Sirius both were able to annotated kaempferol and quercetin derivatives (compounds 169, 735, 171 and 556). The retention time of these compounds ranges between 2.0 and 3.5 min, as they are polar compounds with at least two sugar moieties. These species contain derivatives with either a quercetin or a kaempferol aglycone and various combinations of glycosylation or hydroxycinnamic moieties. Even if part

of the cluster 1 gathers ions from 5 species mainly, each ion is represented in one or maximum two colors indicating that it is specific to one or two species. In *C. flammula*, two apigenin coumaroyl glucoside were isolated previously, and the position of the coumaroyl showed to influence the antioxidant activity of the molecules¹¹. In the same species, kaempferol derivatives with two caffeoyl moieties were isolated from an ethanolic leaves extracts⁴⁰. This previous founding supports the annotation of the compounds.

In global, the annotation of the flavonoid's derivatives is similar between the different tools, even if the Inchikey 2D of the annotations is different. This issue can be explained by the limitations of the technic in identifying the exact position of each substituent on the aglycone, and therefore producing a different Inchikey 2D. However, all annotations indicate the same structural decomposition. When comparing three quercetin-monoglucosides (quercetin-4'-O-glucoside, quercetin-3-O-glucoside and quercetin-7-O-glucoside) any difference had been reported in positive ionization even using MS³ spectra of the aglycone. Additionally, the same study mentioned that no difference have been showed between the two hexose sugars attached to the position 3-O of a flavonoid aglycone⁴⁹. Therefore, two different 2D structures can presented same MS² spectra, for those family of compounds, when two propositions where equivalent, a "consensus" annotation had been suggested (Table 16). The annotation of those derivatives using Tima-R reveals to be with a low score (< 0.60), that can be explained by the fact that those types of derivatives had not been reported yet in the species or in the genus resulting in a weak taxonomical score. Furthermore, different studies highlighted these compounds as strong α -amylase inhibitors, suggesting *Clematis* species containing these compounds, *C. alpina*, *C. chinensis*, *C. flammula*, *C. koreana* and *C. recta*, could have an interesting anti-diabetic activity^{50,51}.

Table 16: Annotation table of the main compounds from the cluster 1 of the shikimates and phenylpropanoids pathway

Feature ID	T _R (min)	Precursor <i>m/z</i>	Adduct	Raw formula (err.)	MS/MS	NP class (CANOPUS)	Compound annotation	SIRIUS	TIMA-R	GNPS
50	2.04	181.0497	[M+H] ⁺	C ₉ H ₈ O ₄ (4.4 ppm)	145.03 135.04	cinnamic acids and derivatives	caffeic acid	-1.31	0.80	0.98
155	2.27	355.1030	[M+H] ⁺	C ₁₆ H ₁₈ O ₉ (1.8 ppm)	163.04 145.03 135.04	cinnamic acids and derivatives	chlorogenic acid	-4.44	0.58	0.97
169	2.30	935.2451	[M+H] ⁺	C ₄₂ H ₄₆ O ₂₄ (2.2 ppm)	449.11 325.09 287.06 163.04	flavonols	kaempferol caffeoyl tri-hexoside	-131.96	0.57	-
171	2.31	951.2417	[M+H] ⁺	C ₄₂ H ₄₆ O ₂₅ (1.7 ppm)	465.11 303.05 163.04	flavonols	quercetin caffeoyl tri-hexoside	-77.15	0.56	-
228	2.43	921.2304	[M+H] ⁺	C ₄₁ H ₄₄ O ₂₄ (0.9 ppm)	465.10 303.05 163.04	-	quercetin trioside	-195.98	0.58	-
308	2.56	935.2460	[M+H] ⁺	C ₄₂ H ₄₆ O ₂₄ (0.9 ppm)	449.11 303.05 163.04	flavonols	quercetin caffeoylsophoroside-rhamnoside	-152.31	0.59	-
312	2.56	905.2363	[M+H] ⁺	C ₄₁ H ₄₄ O ₂₃ (1.9 ppm)	449.11 287.06 163.04	-	kaempferol coumaroyl di-hexoside pentoside	-82.97	0.47	-
341	2.60	339.1078	[M+H] ⁺	C ₁₆ H ₁₈ O ₈ (1 ppm)	147.04 119.05	cinnamic acids and derivatives	3-p-coumaroylquinic acid	-5.79	0.52	0.88
420	2.72	427.1243	[M+H] ⁺	C ₁₉ H ₂₂ O ₁₁ (2.6 ppm)	325.09 181.05 163.04 145.03	cinnamic acids and derivatives	6'-O-caffeoylgoodyeroside	-132.34	0.44	-
445	2.76	919.2498	[M+H] ⁺	C ₄₂ H ₄₆ O ₂₃ (0.1 ppm)	433.11 287.06 163.04	flavonols	kaempferol caffeoyl di-hexoside pentoside	-64.71	0.58	-
556	2.91	789.1864	[M+H] ⁺	C ₃₆ H ₃₆ O ₂₀ (1.1 ppm)	303.05 163.04	flavonols	quercetin caffeoyl di-hexoside	-47.80	0.58	-
586	2.87	1113.2718	[M] ⁺	C ₅₁ H ₅₃ O ₂₈ (0 ppm)	465.10 325.09 303.05 163.04	-	delphinindin hexoside di-caffeoylhexoside	-321.21	0.47	-
598	2.97	933.2317	[M+H] ⁺	C ₄₂ H ₄₄ O ₂₄ (2.3 ppm)	463.09 287.06 163.04	flavonols	kaempferol caffeic or coumaric moiety dioside acetylated	-203.54	-	-
603	2.98	492.1145	[M+NH ₄] ⁺	C ₂₂ H ₁₈ O ₁₂ (1.7 ppm)	295.05 163.04 145.04	cinnamic acids and derivatives	chicoric acid	-13.63	-	0.89
653	3.07	1097.2787	[M] ⁺	C ₅₁ H ₅₃ O ₂₇ (1.7 ppm)	611.14 449.11 325.04 287.06 163.04	-	cyanidin glucoside di-caffeoylglucoside	-206.04	-	-

735	3.19	903.2563	[M+H] ⁺	C ₄₂ H ₄₆ O ₂₂ (1.1 ppm)	325.09 287.06 163.04	-	kaempferol coumaroyl di- hexoside pentoside	-361.66	0.54	-
765	3.25	451.1239	[M+H] ⁺	C ₂₁ H ₂₂ O ₁₁ (0.9 ppm)	325.09 163.04 127.04	cinnamic acids and derivatives	okanin-4'-O-glucoside or derivatives	-60.34	0.46	-
889	3.46	506.1295	[M+NH ₄] ⁺	C ₂₃ H ₂₀ O ₁₂ (0.4 ppm)	309.06 177.05 163.04 145.03	cinnamic acids and derivatives	2-[3-(3,4- dihydroxyphenyl)prop-2- enoyloxy]-3-[3-(4-hydroxy-3- methoxyphenyl)prop-2- enoyloxy]butanedioic acid	-35.71	-	-
1135	3.96	520.1449	[M+NH ₄] ⁺	C ₂₄ H ₂₂ O ₁₂ (0.1 ppm)	309.06 177.06 163.04	cinnamic acids and derivatives	2,3-bis[3-(4-hydroxy-3- methoxyphenyl)prop-2- enoyloxy]butanedioic acid	-96.16	-	-

As mentioned previously, cluster 2 (Figure 44) is also a cluster presenting mainly compounds from the shikimates and phenylpropanoids pathway. An important region, contains flavonol derivatives (blue dot square) with a quercetin or kaempferol aglycone related to a rutinoside, glucuronide or hexoside moiety. Among the compounds, quercetin glucuronide (ID 664) is the major detected compound, presents in 6 out of 10 species. Quercetin-3-O- β -glucuronide was previously isolated from the aerial part of *C. brevicaudata*⁵². Other quercetin or kaempferol derivatives with two sugar moieties or glucuronide moiety were also previously isolated from several species of *Clematis* as *C. aethusifolia*, *C. lasiandra* or *C. flammula*^{40,53,54}.

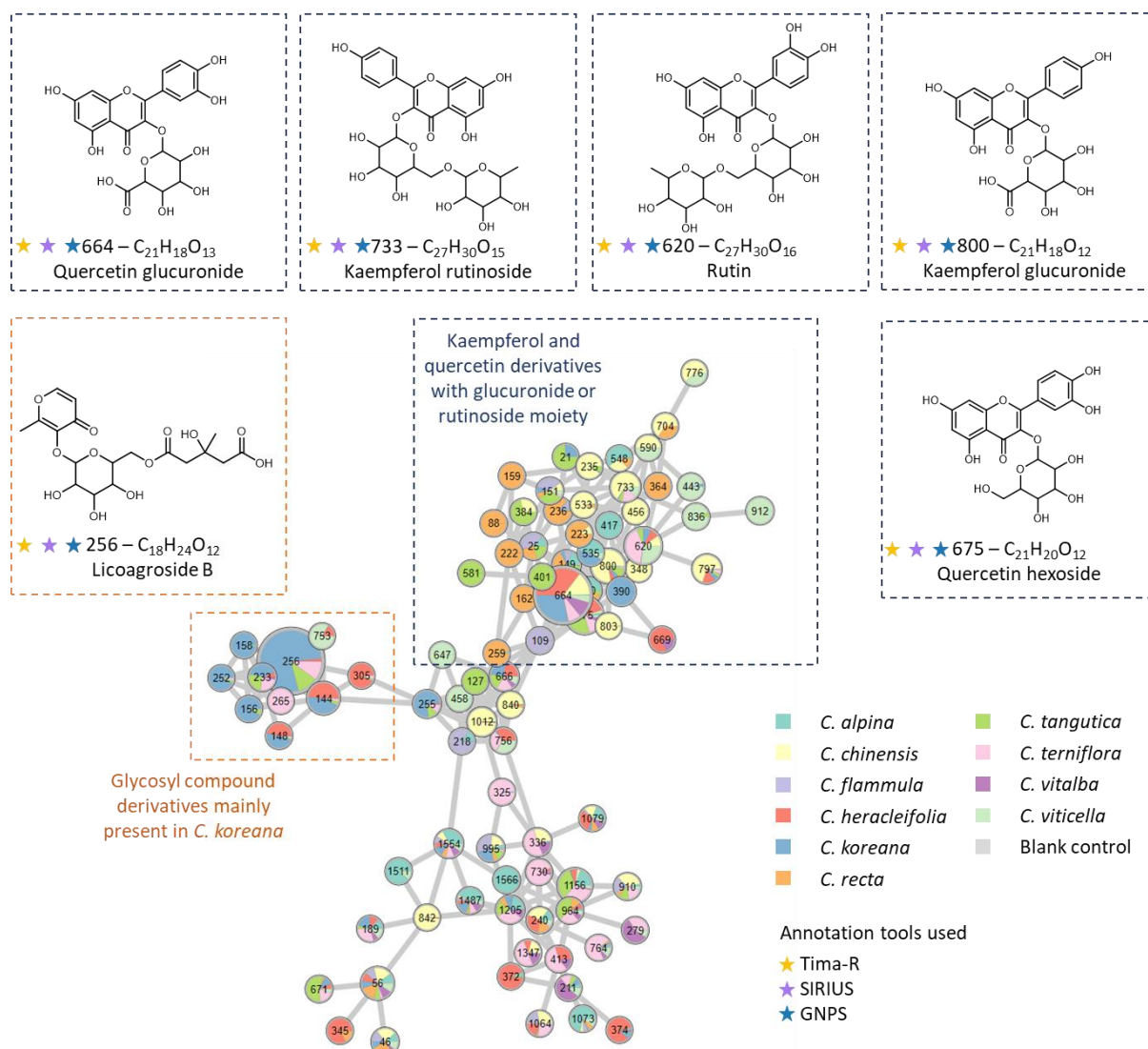


Figure 44: Cluster 2 of the (+)-HESI MS/MS spectra with a focus on the compounds from the shikimate and phenylpropanoid pathway predicted by Canopus. Each color is representing a *Clematis* species and the size of the node is proportional to the sum area intensity. Dotted square are corresponding to the zone where the ion is identified and some structures are suggested from the annotation tools, for each a colored star indicated the annotation tool able to propose identification.

Within this large cluster, a specific area related to *C. koreana* stands out. The major compound was identified using the three annotation tools as licoagroside B, a maltol glycoside derivative. This part of the cluster is only link to the rest of the cluster with two edges with ID 255, which is in fact an in-source fragment. Within the same cluster, depending on its size and clustering parameters, two distant nodes may not belong to the same family but can share common ions. Herein, these maltol glycoside derivatives have the same structure than the glycosylated C ring of flavonol compounds explaining why they share common fragments. Some studies highlighted the antioxidant activities and the anti-inflammatory activities of these compounds⁵⁵⁻⁵⁷.

To further identify flavonoids in the *Clematis* species, two specifics clusters, associated with one species, were highlighted using MN (cluster 12 and 37, Figure 45). Cluster 12 shows 15 nodes, including 14 ions exclusively found in *C. chinensis*, showing a distinct pattern. These compounds were identified as quercetin and kaempferol acetyl derivatives. The plant appears to produce a significant number of acetylated compounds, as seen in cluster 1, where a flavonoid derivative with both hydroxycinnamic moiety and acetylation was also identified (compound 598). Acylation of flavonoids improved their thermostability, and their light resistance⁵⁸.

While the *O*-glycosyl flavonoids had been well described in the MN, *C*-glycosyl flavonoids, that present different fragmentation pattern, had also been found in the *Clematis* genus, and more specifically in *C. viticella* with cluster 37. The difference in the linkage can have an impact on the activity of the compound as the C-C bond is more stable than C-O bond, resulting in difference of the absorption and metabolism *in vivo*⁵⁹.

Table 17: Annotation table of the main compounds from the cluster 2 of the shikimates and phenylpropanoids pathway

Feature ID	RT (min)	Precursor m/z	Adduct	Raw formula (err.)	MS/MS	NP class (CANOPUS)	Compound annotation	SIRIUS	TIMA-R	GNPS
56	2.05	365.0846	[M+Na] ⁺	C ₁₅ H ₁₈ O ₉ (0.7 ppm)	365.08 203.05 185.04	cinnamic acids and derivatives	caffeic acid hexose	-24.26	0.44	-
144	2.25	375.0927	[M+H] ⁺	C ₁₅ H ₁₈ O ₁₁ (1.4 ppm)	375.09 127.04	-	glucuronid derivatives	-193.67	-	-
236	2.45	611.1608	[M+H] ⁺	C ₂₇ H ₃₀ O ₁₆ (0.2 ppm)	465.11 449.11 303.05 287.05	flavonols	quercetin hexoside pentoside	-14.93	0.72	0.87
256	2.47	433.1345	[M+H] ⁺	C ₁₈ H ₂₄ O ₁₂ (1 ppm)	127.04	-	licoagroside b	-28.97	0.37	0.93
390	2.68	641.1327	[M+H] ⁺	C ₂₇ H ₂₈ O ₁₈ (3.3 ppm)	479.08 303.05	flavonols	quercetin hexoside glucuronide	-26.67	0.53	-
620	3.00	611.1617	[M+H] ⁺	C ₂₇ H ₃₀ O ₁₆ (1.7 ppm)	465.11 303.05	flavonols	rutin	-5.74	0.75	0.95
664	3.08	479.0829	[M+H] ⁺	C ₂₁ H ₁₈ O ₁₃ (1.8 ppm)	303.05 85.03	flavonols	quercetin glucuronide	-8.17	0.79	0.9
675	3.10	465.1036	[M+H] ⁺	C ₂₁ H ₂₀ O ₁₂ (1.8 ppm)	303.05 85.03	flavonols	quercetin hexoside	-5.62	0.79	0.96
733	3.18	595.1659	[M+H] ⁺	C ₂₇ H ₃₀ O ₁₅ (0.3 ppm)	449.11 287.06 85.03	flavonols	kaempferol rutinoside	-16.71	0.79	0.96
797	3.30	449.1082	[M+H] ⁺	C ₂₁ H ₂₀ O ₁₁ (0.8 ppm)	303.05 85.03	flavonols	quercetin pentoside	-3.94	0.75	0.95
800	3.31	463.0873	[M+H] ⁺	C ₂₁ H ₁₈ O ₁₂ (0.4 ppm)	287.06 85.03	flavonols	kaempferol glucuronide	-2.71	0.58	0.9
912	3.50	609.1811	[M+H] ⁺	C ₂₈ H ₃₂ O ₁₅ (0.5 ppm)	301.07 286.05	flavones	diosmetin hexoside pentoside	-10.2	0.67	0.91

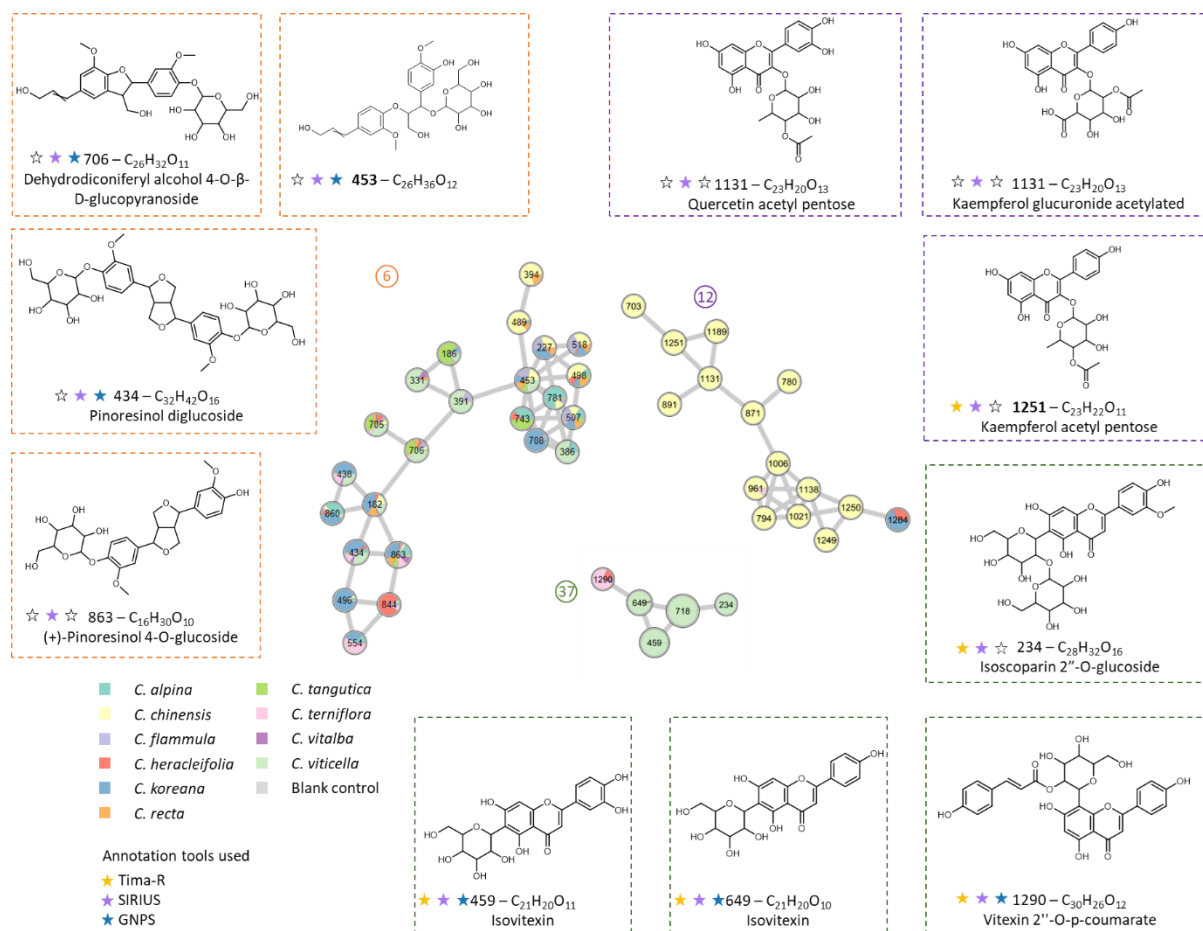


Figure 45: Cluster 6, 12 and 37 of the (+)-HESI MS/MS spectra with a focus on the compounds from the shikimate and phenylpropanoid pathway predicted by Canopus. Each color is representing a *Clematis* species and the size of the node is proportional to the sum area intensity. Dotline square are corresponding to the cluster where the ion is identified and some structures are suggested from the annotation tools, for each a colored star indicated the annotation tool able to propose identification.

Table 18: Annotation table of the main compounds from the clusters 6, 12 and 37 of the shikimates and phenylpropanoids pathway

Feature ID (cluster)	RT (min)	Precursor m/z	Adduct	Raw formula (err.)	MS/MS	NP Class (CANOPUS)	Compound annotation	SIRIUS	TIMA-R	GNPS
518 (6)	2.87	523.2175	[M+H] ⁺	C ₂₆ H ₃₄ O ₁₁ (0.8 ppm)	343.15 291.12 179.07 137.06	-	Glochidioboside	-76.83	-	-
863 (6)	3.41	538.2278	[M+NH ₄] ⁺	C ₂₆ H ₃₂ O ₁₁ (1.8 ppm)	235.10 175.08 137.06	furofuranoids lignans	(+)-pinoresinol 4-O-glucoside	-5.48	-	-
706 (6)	3.14	538.2287	[M+NH ₄] ⁺	C ₂₆ H ₃₂ O ₁₁ (0.9 ppm)	311.13 175.08 137.06	furanoid lignans	dehydronicoferyl alcohol 4-O-β-D-glucopyranoside	-17.71	-	0.83
391 (6)	2.69	556.2398	[M+NH ₄] ⁺	C ₂₆ H ₃₄ O ₁₂ (0.7 ppm)	311.13 161.06 131.05	furanoid lignans	olivil 4'-O-glucoside	-24.75	-	-
453 (6)	2.87	558.2546	[M+NH ₄] ⁺	C ₂₆ H ₃₆ O ₁₂ (0.2 ppm)	331.06 207.10 137.06	neolignans	2-[3-[4-1,3-dihydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-2-yl]oxy-3-methoxyphenyl]propoxy]-6-(hydroxymethyl)oxane-3,4,5-triol	-25.52	-	0.82
498 (6)	2.86	586.2858	[M+H+DM A] ⁺	C ₂₈ H ₄₀ O ₁₂ (0.8 ppm)		neolignans	2-[4-hydroxy-2,3-bis[(4-hydroxy-3,5-dimethoxyphenyl)methyl]butoxy]-6-methyloxane-3,4,5-triol	-129.55	-	-
434 (6)	2.76	700.2811	[M+NH ₄] ⁺	C ₃₂ H ₄₂ O ₁₆ (0 ppm)	341.14 235.10 175.08 137.06	furofuranoids lignans	pinoresinol diglucoside	-3.00	-	0.87
708 (6)	3.15	702.2989	[M+NH ₄] ⁺	C ₃₂ H ₄₄ O ₁₆ (3 ppm)	331.15 287.13 151.08	-	2-(hydroxymethyl)-6-[[5-(3-hydroxypropyl)-7-methoxy-2-[3-methoxy-4-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]-2,3-dihydro-1-benzofuran-3-yl]methoxy]oxane-3,4,5-triol	-65.38	-	-
554 (6)	2.91	760.3018	[M+NH ₄] ⁺	C ₃₄ H ₄₆ O ₁₈ (1.2 ppm)	401.16 265.11 205.09 173.06	furofuranoids lignans	liriodendrin	-13.5	-	0.79

182 (6)	2.33	556.2399	[M+NH ₄] ⁺	C ₂₆ H ₃₄ O ₁₂ (0.9 ppm)	341.14 187.08 137.06	furanoid lignans	2-[4-[4-[hydroxy-(4-hydroxy-3-methoxyphenyl)methyl]-3-(hydroxymethyl)oxolan-2-yl]-2-methoxyphenoxy]-6-(hydroxymethyl)oxane-3,4,5-triol	-18.19	-	0.84
1251 (12)	4.25	475.1235	[M+H] ⁺	C ₂₃ H ₂₂ O ₁₁ (0 ppm)	287.06 189.08 129.06	flavonols	kaempferol acetyl pentoside	-13.83	0.47	-
1131 (12)	3.95	505.0976	[M+H] ⁺	C ₂₃ H ₂₀ O ₁₃ (0.1 ppm)	287.06 159.03 85.03	flavonols	kaempferol glucuronide acetylated	-47.87	0.47	-
1138 (12)	3.98	491.1191	[M+H] ⁺	C ₂₃ H ₂₂ O ₁₂ (1.4 ppm)	303.05 171.07 85.03	flavonols	quercetin acetylated pentoside	-21.75	0.4	-
459 (37)	2.78	449.1079	[M+H] ⁺	C ₂₁ H ₂₀ O ₁₁ (0.1 ppm)	353.07 329.07 299.06	flavones	isoorientin	-7.78	0.84	0.96
649 (37)	3.06	433.1131	[M+H] ⁺	C ₂₁ H ₂₀ O ₁₀ (0.4 ppm)	337.07 313.07 283.06	flavones	isovitexin	-7.39	0.60	0.96
1290 (37)	4.37	579.1497	[M+H] ⁺	C ₃₀ H ₂₆ O ₁₂ (1.1 ppm)	379.08 313.07 147.04	flavones	vitexin 2''-O-p-coumarate	-36.9	0.73	0.84
234 (37)	2.45	625.1777	[M+H] ⁺	C ₂₈ H ₃₂ O ₁₆ (2.2 ppm)	367.08 343.08 313.07	flavones	isoscoparin 2''-O-glucoside	-23.92	0.54	-

Within the shikimates and the phenylpropanoids pathway, flavonoids and phenylpropanoids compounds are not the only NP classes identified. Lignans are also an important part of the pathway. Cluster 6, as shown in Table 18 and Figure 45, is dedicated to lignan and their derivatives, with ten annotated lignans. A few lignans were previously isolated from *Clematis* species. The (7R, 8S)-9-Acetyl-dehydrodiconiferyl alcohol with the same aglycone as compound ID 706 annotated as dehydrodiconiferyl alcohol 4-O- β -D-glucopyranoside, had been previously isolated from the stem of *C. armandii*, and had shown anti-inflammatory properties as well as anti-neuroinflammatory activities^{60,61}.

Lignans are predominantly detected as the $[M+NH_4]^+$ ion adduct, as observed during the analysis of *Shisandra chinensis* using ESI(+)-TOF-MS⁶². However, this predominance of $[M+NH_4]^+$ adduct can lead to complications in their identification by annotation tools. Indeed, depending on the level of detection of the MS1 during data processing, if the $[M+H]^+$ or $[M+Na]^+$ are not enough intense, they may be excluded from the final data. Therefore, this makes it difficult to differentiate the $[M+NH_4]^+$ adduct from a wrong $[M+H]^+$ for example. To ensure accurate identification, manual verification of the adduct was performed. Some dimethylamine adduct $[M+H+DMA]^+$ were therefore identified with a difference of m/z 45 with the $[M+H]^+$. ID 498 from cluster 6 was therefore identified as neolignans. Several ions, as ions ID 434, 453 and 706, were also initially misidentified using SIRIUS, but after a manual correction of the adduct, accurate results were obtained.

Further exploration of the clusters allowed the identification of another cluster dedicated to lignans, which was initially misidentified as being mainly part of the alkaloid's pathway. The automatic treatment of the analysis did not well recognize $[M+NH_4]^+$ adduct and suggested raw formula with nitrogen to respect the nitrogen rules. Cluster 18 (not shown) is therefore composed of glycosylated lignans, not of nitrogen compound as alkaloids, as previously predicted by CANOPUS (Figure 42). These issues underscore the need of a manual verification, even if the tools are more and more performant and continue to be improved.

3.3. Focus of the terpenoids pathway

In the literature on *Clematis*, triterpenoids are the most described class; a wide variety of triterpenoids have been extracted and isolated from the genus. The identified compounds are mostly triterpene saponins, often containing more than 3 sugars moieties but were mostly contained in the roots^{43,63–66}. Figure 46 shows two specific clusters of terpenoids contained in the analyzed *Clematis* species.

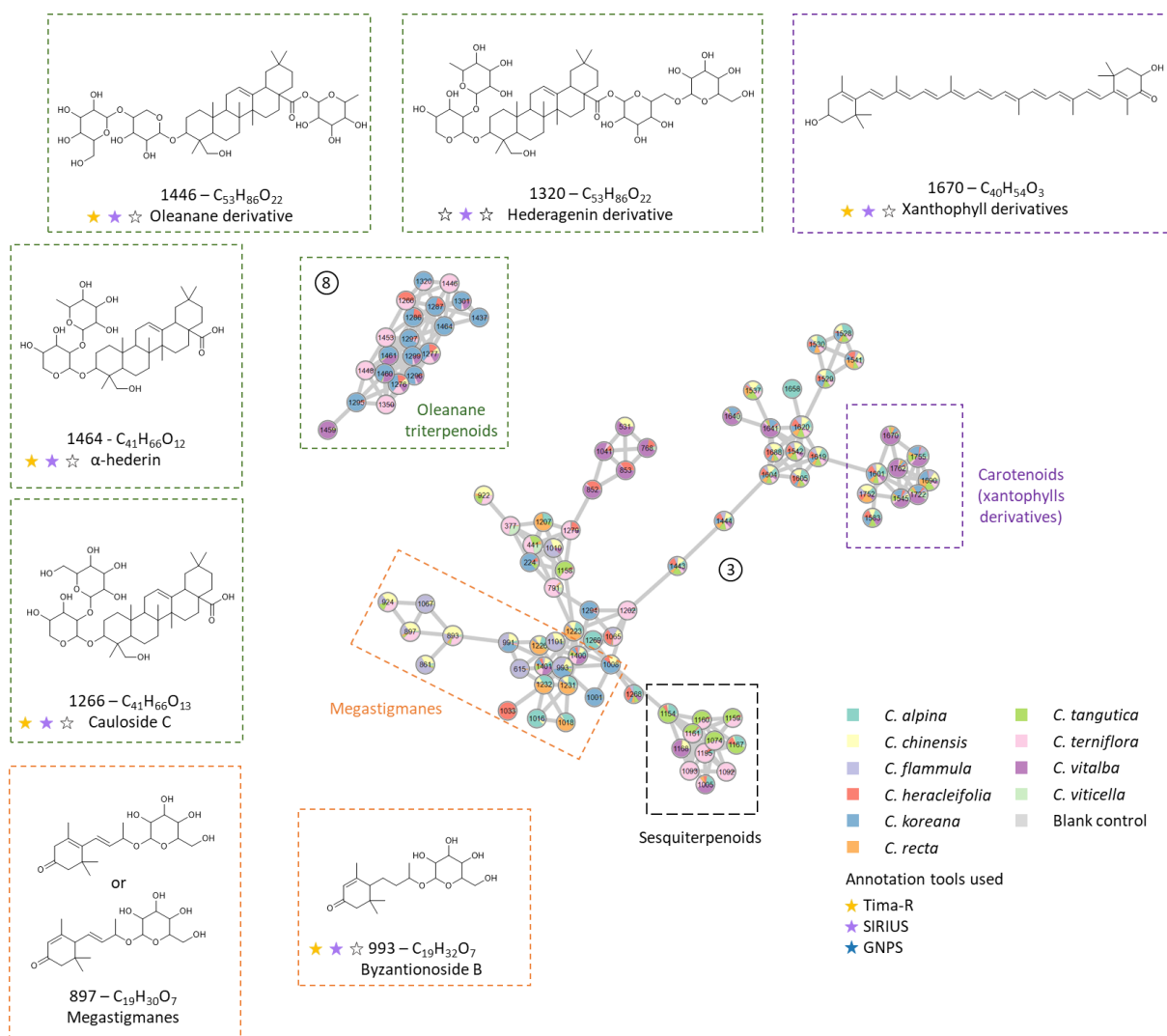


Figure 46: Cluster 3 and 8 of the (+)-HESI MS/MS spectra with a focus on the compounds from the terpenoids pathway predicted by Canopus. Each color is representing a *Clematis* species and the size of the node is proportional to the sum area intensity. Dotted square are corresponding to the zone where the ion is identified and some structures are suggested from the annotation tools, for each a colored star indicated the annotation tool able to propose identification.

Clusters 3 and 8 have both been identified as containing terpenoids using the NP classifier. Cluster 3 is a significant cluster, containing 76 nodes while cluster 8 is smaller with 20 nodes, mainly detected in four species: *C. koreana*, *C. terniflora*, *C. vitalba* and *C. heracleifolia*. Cluster 3 includes several classes of terpenoids with two distinct regions of retention. A first region, with orange frame in Figure 46, with compounds with retention times less than 5 min and a second region, with purple frame, with compounds with retention time of more than 7 min, corresponding respectively to megastigmanes glycoside and carotenoids (C40). The investigation of the cluster reveals a lot of fragments, leading to information redundancy in the MN. Adding a filter such as “metaCorrelate feature grouping” in MZmine and performed Ion Identity Molecular Network allows to reduce this redundancy, however it could also

complexify the exploration⁶⁷. The megastigmanes glycosides are an oxidative product from β -catorenoids with a C13 skeleton. Some megastigmanes glycosides had been previously isolated from the stem of *Clematis parviloba* but no activity have been reported⁶⁸. However, another study on megastigmanes isolated from the silkworm (*Bombyx mori* L.) droppings highlighted their promotion activity on oxygenase-1 and SIRT1 activity, the first one considered as an antioxidant enzyme and the second one, known to downregulate the NF- κ B pathway and so considered as anti-inflammatory enzyme⁶⁹.

CANOPUS identified a region of sesquiterpenoids in cluster 3, contained mainly in *C. terniflora* and *C. tangutica*, but any structure could be associated. Only few sesquiterpenoids were described in the literature including two sesquiterpenoids described in the aerial parts of *C. chinensis* recently⁷⁰.

Cluster 8 is composed of triterpenoids, as shown in Table 19 and Figure 46. Among the species analysed only 4 on 10 species present triterpenoids, meaning they may not contain triterpenoids saponins or maybe under the detection level. Most of the annotated molecules are oleanane triterpenoid saponins with several sugar moieties. Based on the literature, many triterpenoids have been isolated from *Clematis* species, primarily from the roots rather than the leaves or stems. However, a few oleanane derivatives, mainly with hederagenin skeleton, which is an olean-12-en-28-oic acid, have also been identified in the whole plant or in aerial parts specifically. Additionally, other skeleton as ursane and taraxerane skeletons, have been found in the roots once but not in the aerial parts⁷¹. The triterpenoids saponins reported were composed of two to six sugars moieties with either hexose, pentose or aldopentose, such as compound ID 1320 annotated as 3-O- α -L-rhamnopyranosyl (1 $\rightarrow$ 2)- α -L-arabinopyranosyl hederagenin 28-O- β -D-glucopyranosyl (1 $\rightarrow$ 6)- β -D-glucopyranosyl ester. ID 1446, mainly found in the *C. terniflora* extract herein, was identified as 3-O- β -D-glucopyranosyl (1 $\rightarrow$ 4)- α -L-arabinopyranosyl hederagenin 28-O- α -L-rhamnopyranosyl, this compound was previously identified in the dried aerial parts of *C. tangutica*⁷². The study of the aerial parts of *C. akebioides* and the study of the whole plant of *C. tangutica* reported hederagenin triterpene saponins with anti-cancer bioactivity⁷³ and cardioprotective effect^{74,75}.

Furthermore, as for the cluster 3, a lot of compounds detected are not the $[M+H]^+$ but fragments with the redundancy of m/z 437 or 455 at different retention times. The EIC at m/z 437 shows two distinct regions of retention times with dozens of peaks (Figure S7, supplementary data), traducing the presence of the aglycone with different positions or types of glycosylation. Those fragments in the final dataset correspond to fragments produced in source, as O-glycosylation is weak and higher masses corresponding to the real $[M+H]^+$ are therefore not in the molecular network. The method should be modified if targeting that class of compounds. The analytical conditions employed for this study are suitable for several

classes of compounds but not well adapted for glycosylated triterpenoids. In positive mode, it was previously noticed than base peak is the aglycone and not the entire compound; negative mode should here being helpful as $[M+HCOO]^-$ and deprotonated ion $[M-H]^-$ can be usually observed⁷⁶. Regarding the data processing noise level should be also reduced to identified the $[M+H]^+$ or molecular adduct but with the risk to collected ions from background.

3.1. Focus on the nitrogen compounds

Among the CANOPUS annotations, the last one compound class explored is the alkaloids. Here, we also examined amino acids and peptides due to the potential for confusion. Regarding Figure 42, lots of nodes were identified as being alkaloids, but as explained before ammonium adduct is not well detected leading to an over estimation of the amount of nitrogen compounds including alkaloids and amino acids and peptides in the extracts. However, few alkaloids were already identified in some species of the *Clematis* genus but not as much as estimated here first.

Table 20 and Figure 47 resume our founding regarding the annotation of chemical compound of this class in the analyzed species. Cluster 25, is a cluster of compounds with a well distribution in almost all the samples. Half of the cluster was annotated as amino acids while the other part was annotated as alkaloids. Deeper exploration allowed the annotation of one compound, tryptophan, a well-known aromatic amino acid found in plants.

Then, investigation of cluster 32, led to the identification of 4 alkaloids. Magnoflorine has been here annotated in *C. recta*, in with its two isomers (α -magnoflorine and β -magnoflorine) were previously identified in *C. terniflora* var. *manshurica*, *C. florida* var. *plena*, *C. parviloba* and *C. recta*^{7,77}. The action of magnoflorin was investigated and showed anti-inflammatory properties, with the reduction of pro-inflammatory cytokines. The study also showed an anti-rheumatoid arthritis effect, and helped to improve symptoms of rats affected by rheumatoid arthritis⁷⁷.

Table 19: Annotation table of the main compounds from the clusters 3 and 8 of the terpenoids pathway

Feature ID (cluster)	RT (min)	m/z	Adduct	Raw formula (err.)	MS/MS	NP class	Compound annotation	SIRIUS	TIMA-R	GNPS
897 (3)	3.48	371.2064	[M+H] ⁺	C ₁₉ H ₃₀ O ₇ (0.1 ppm)	209.15 191.14 133.10	Megastigmanes	3,5,5-trimethyl-4-[3[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxybut-1-enyl]cyclohex-2-en-1-one (3-Oxo- α -ionol glucoside) or 3,5,5-trimethyl-4-[3-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxybut-1-enyl]cyclohex-3-en-1-one	-48.52	0.45	-
993 (3)	3.61	373.2223	[M+H] ⁺	C ₁₉ H ₃₂ O ₇ (0.6 ppm)	211.17 193.16 135.12	Megastigmanes	Byzantionoside B	-20.66	-	0.89
1620 (3)	8.54	393.2785	[M+H] ⁺	C ₂₇ H ₃₆ O ₂ (0.8 ppm)	145.09 119.09 93.07	Apocarotenoids	4,9,13-trimethyl-15-(2,6,6-trimethylcyclohexen-1-yl)pentadeca-2,4,6,8,10,12,14-heptaenoic acid or 15-(4-hydroxy-2,6,6-trimethylcyclohexen-1-yl)-4,9,13-trimethylpentadeca-2,4,6,8,10,12,14-heptaenal	-80.49	0.48	-
1670 (3)	9.04	583.4151	[M+H] ⁺	C ₄₀ H ₅₄ O ₃ (0.9 ppm)	221.15 119.09	Carotenoids (C40)	Xanthophyll derivatives	-48.42	0.42	-
1690 (3)	9.29	617.4201	[M+H] ⁺	C ₄₀ H ₅₆ O ₅ (0.1 ppm)	299.20 221.15 165.09	Carotenoids (C40)	Xanthophyll derivatives	-41.87	0.38	-
1722 (3)	9.55	615.4414	[M+H] ⁺	C ₄₁ H ₅₈ O ₄ (1 ppm)	221.15 119.09	Carotenoids (C40)	Xanthophyll derivatives	-66.66	0.21	-
1755 (3)	9.84	601.4251	[M+H] ⁺	C ₄₀ H ₅₆ O ₄ (0.1 ppm)	491.36 221.05 165.09	Carotenoids (C40)	Xanthophyll derivatives	-46.9	0.56	-
1266 (8)	4.30	767.4561	[M+H] ⁺	C ₄₁ H ₆₆ O ₁₃ (2 ppm)	455.35 437.34 203.18	Oleanane triterpenoids	Cauloside C	-43.62	0.59	0.70

1320 (8)	4.50	1092.5953	[M+NH ₄] ⁺	C ₅₃ H ₈₆ O ₂₂ (0.4 ppm)	455.35 437.34 85.03	Oleanane triterpenoids	3-O- α -L-rhamnopyranosyl (1->2)- α -L-arabinopyranosyl hederagenin 28-O- β -D-glucopyranosyl (1->6)- β -D-glucopyranosyl ester	-7.73	-	-
1437 (8)	5.88	897.5202	[M+H] ⁺	C ₄₇ H ₇₆ O ₁₆ (0.5 ppm)	455.35 437.34 85.03	Oleanane triterpenoids	-	-33.28	0.53	-
1446 (8)	6.04	913.5155	[M+H] ⁺	C ₄₇ H ₇₆ O ₁₇ (0 ppm)	455.35 437.34 85.03	Oleanane triterpenoids	3-O- β -D-glucopyranosyl (1->4)- α -L-arabinopyranosyl hederagenin 28-O- α -L-rhamnopyranosyl	-64.49	0.61	-
1464 (8)	6.32	751.4623	[M+H] ⁺	C ₄₁ H ₆₆ O ₁₂ (0.5 ppm)	455.35 437.34 85.03	Oleanane triterpenoids	α -hederin	-13.69	0.66	-

Table 20: Annotation table of the main compounds from the compound class of cluster 5, 25 and 32 of the alkaloids and amino acids pathway

Feature ID (Cluster)	T _R (min)	Precursor m/z	Adduct	Raw formula (err.)	MS/MS	NP superclass	Compound annotation	SIRIUS	TIMA-R	GNPS
41 (25)	2.03	205.0973	[M+H] ⁺	C ₁₁ H ₁₂ N ₂ O ₂ (0.15 ppm)	188.07 146.06	Small peptides	Tryptophan	-0.35	0.51	0.99
276 (32)	2.52	300.1596	[M+H] ⁺	C ₁₈ H ₂₁ NO ₃ (1.8 ppm)	269.12 107.05	Tyrosine alkaloids	N-Methylcoclaurine	-28.58	0.68	-
404 (32)	2.71	342.1706	[M] ⁺	C ₂₀ H ₂₃ NO ₄ (0.39 ppm)	297.11 265.09 58.07	Tyrosine alkaloids	Magnoflorine or Isocorydine	-36.37	0.52	-
565 (32)	2.93	314.1767	[M+H] ⁺	C ₁₉ H ₂₃ NO ₃ (2.1 ppm)	283.13 107.05	Tyrosine alkaloids	Armepavine	-14.83	0.54	-
379 (32)	2.67	344.186	[M+H] ⁺	C ₂₀ H ₂₅ NO ₄ (0.49 ppm)	137.06 58.07	Tyrosine alkaloids	Laudanine	-25.47	0.49	-
472 (5)	2.80	282.2063	[M+?] ⁺	C ₁₆ H ₂₇ NO ₃ (3.4 ppm)	130.09 107.09 -	Ornithine alkaloids	-	-	-	-
1308 (5)	4.44	280.2273	[M+?] ⁺	C ₁₇ H ₂₉ NO ₂ (4.5 ppm)	144.10 81.07	Amino acids and derivatives	-	-	-	-
1259 (5)	4.27	266.2111	[M+?] ⁺	C ₁₆ H ₂₇ NO ₂ (1.3 ppm)	130.09 81.07	lysine alkaloids	-	-	-	-

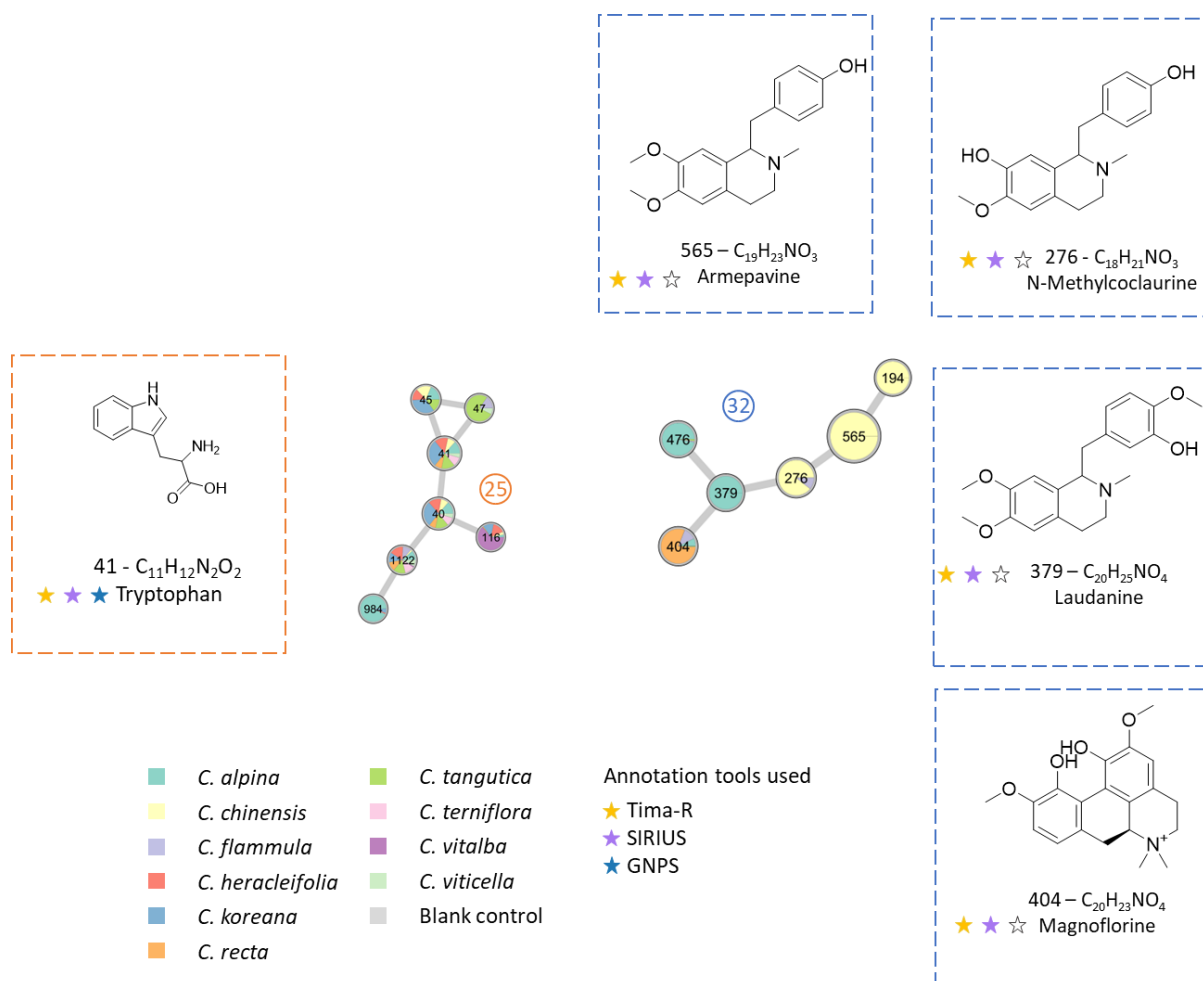


Figure 47: Cluster 25 and 32 of the (+)-HESI MS/MS spectra with a focus on the compounds from the alkaloids pathway predicted by Canopus. Each color is representing a *Clematis* species and the size of the node is proportional to the sum area intensity. Dotline square are corresponding to the zone where the ion is identified and some structures are suggested from the annotation tools, for each a colored star indicated the annotation tool able to propose identification.

4. Conclusion

This phytochemical investigation of the aerial parts of 10 *Clematis* species led to the annotation of around sixty compounds, primarily from the shikimate and phenylpropanoids pathway, specifically glycosylated flavonoids. This study reported herein, a first description of the chemical composition of *C. alpina*, a species for which any phytochemical description is available in the state of the art. In the same idea, just one study is available about the chemical composition of *C. koreana*, where three triterpenoids saponins and five phenolic compounds and flavonoids derivatives were identified⁷⁸; Herein, this study highlighted this species have some chemical specificities in comparison to the 9 other ones and so, a deeper investigation about its phytochemistry should be undertaken. Same reflection can be made about *C. recta* for which only two alkaloids and anemonin derivatives were reported⁷. Furthermore, we report herein the specificity of *C. viticella* which is the only one among the 10 species studied highlight a high content of C-glycosylated flavonoids, that could involve different bioactivity⁵³, as isovitexin, reported herein in *C. viticella* and only reported in *C. lasiandra* previously. The specificity of *C. chinensis* regarding the acetylation of numerous flavonoids derivatives was also highlighted. Among the annotated compounds, the bioactivity of some of them were already previously investigated and are in accordance with some *in vivo* studies made on some *Clematis* species extracts. Therefore, a thorough understanding of the phytochemical composition of a plant can guide future biological investigations.

Furthermore, this study highlights the necessity of manually explore the data, such as verifying the correct adducts in certain cases. Especially concerning some known family, as lignan which has been described to be mostly ionized with the $[M+NH_4]^+$ adduct, therefore when annotated a plant or a specific species, if possible, it is good to have an idea of some expected compound families. Finally, the use of three annotation tools: GNPS, which is based on experimental spectra; TIMA-R, which utilizes an *in silico* taxonomically annotated library; and SIRIUS, which employs fragmentation tree prediction, helps to enhanced the confidence in the results. As SIRIUS is a *in silico* tools based on wide range library, it is able to suggest a formula and a structure or several structure which could correspond to only some fragment or neutral loss of the mass spectrum, therefore it is important to check the consistency of the proposition as it could also annotate non-natural compound for example. This is why, the use of TIMA-R helps to maintain consistency by comparing known compounds in the literature, providing valuable biological context. Further, comparing with experimental spectra as on GNPS is also relevant particularly if data are acquired in similar condition. Among the compound annotated, 17 compounds were consistently annotated by the 3 tools, 37 by two tools, and 11 only by one tool demonstrating the usefulness of combining the three tools for greater confidence.

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Partie III. Réseau moléculaire bioactif pour l'identification de composés inhibiteurs de l'activité JAK3

Bioactive molecular networking for identifying the anti-inflammatory
compounds of *Clematis* species inhibiting the JAKs-STATs pathway

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Abstract

The *Clematis* genus includes over 380 species distributed worldwide and some of them are reported to be traditionally used mainly for anti-inflammatory properties as rheumatoid arthritis treatment or for pain relief. This study aims to investigate the bioactivity of eleven *Clematis* species *C. alpina*, *C. armandii*, *C. flammula*, *C. heracleifolia*, *C. koreana*, *C. macropetala*, *C. recta*, *C. tangutica*, *C. terniflora*, *C. vitalba* and *C. viticella*; and to correlate their activity with their phytochemical profile. Aerial parts of each species were extracted using hydroalcoholic and ethyl acetate solvents. UPLHC-HRMS/MS analysis of the samples was conducted alongside *in vitro* bioactivity assays to evaluate JAK3 inhibition, a Janus Kinase involved in the JAK/STAT pathway, one of the four main inflammatory pathways. Molecular networking was employed to correlate bioactivity assay and potentially active compounds. Our findings highlighted *C. tangutica*, *C. heracleifolia* and *C. recta* as promising species, with six key ions identified, including, lutein and a monogalactosyldiacylglycerol derivative, suggesting their roles in the observed bioactivities.

1. Introduction

The genus *Clematis* is the third largest in the Ranunculaceae family, with 388 identified species distributed over the world and especially in Asia, North America and Europe¹. In various traditional medicine, as the Traditional Chinese medicine, the use of *Clematis* species in mixtures is well-documented, mainly for pain relief or anti-inflammatory purposes^{2,3}. One of the most recognized application is for the relief of rheumatoid arthritis, which has been extensively reported³⁻⁵. In addition, *Clematis* species are traditionally used to treat skin ailments such as skin inflammation, irritation, and sunburn. For example, *C. tangutica* has been reported to treat skin ulcers in Tibet^{3,4}. Beyond skin-related conditions, *C. hirsuta* have been reported to treat oral and pulmonary inflammation in Uganda⁶.

The recurrence of similar traditional uses across different species in different regions suggests the presence of bioactive molecules or at least families of molecules that could be shared by multiple species within the genus. Indeed, some studies on *Clematis* spp. extracts and some isolated compounds have been already reported for promising antioxidant, anti-inflammatory and wound-healing properties^{7–13}. For anti-inflammatory activity, these studies focused on about ten species with primarily examining *C. chinensis* and *C. terniflora* var. *manshurica* and mainly targeting the NF- κ B pathway^{14–21}. And even if this pathway is considered as a key signalling pathway involved in the expression of proinflammatory genes including cytokines and chemokines, nowadays it has been shown that this signalling pathway is not isolated in the regulation of inflammation. There may be direct or indirect regulation with other molecules, which consequently triggers interactions with other signalling pathways as PI3K/AKT pathway, MAPK/ERK pathway or JAKs-STATs pathway. *Clematis* spp. extracts may also act on these pathways, highlighting the need for further investigation to better understand the anti-inflammatory potentials of *Clematis* species.

Among these pathways, targeting JAKs/STATs pathway appears as a possibility for the treatment of different diseases related to inflammation. Indeed, the deregulation of the JAKs-STATs pathway contributes to autoimmune and chronic inflammatory conditions²². JAK-dependent cytokines play a key role in driving these conditions and so, the use of Janus kinases (JAKs) inhibitors, which are an important family of tyrosine kinases including JAK1, JAK2, JAK3 and Tyk2, with important roles transducing cytokine-mediated signals²³, appears as a good solution for treatment. Some inhibitors of these kinases have already been discovered and tested in a wide range of disorders, including eczema, rheumatoid arthritis or inflammatory bowel disease, but the effectiveness of those inhibitors varies between conditions. However, some synthetic JAKs inhibitors have been approved by the FDA, as Tofacitinib, a pan-JAK inhibitor (inhibiting the three JAKs), approved for the treatment of psoriatic arthritis and rheumatoid arthritis, or Upadacitinib, a JAK1 inhibitor, approved for the treatment of psoriatic arthritis, rheumatoid arthritis and atopic dermatitis²⁴. These findings have led to increase research into JAK inhibitors, especially because founding a specific JAK inhibitor rather than a pan-JAK inhibitor can reduce side effect as infections or cytopenias. Therefore, discovering new inhibitors of JAK3 is a major issue for the treatment of some inflammatory diseases, especially as no JAK3-selective inhibitor has reached clinical stages^{23,25}. Specialized metabolites from plants, with their wide chemical space, may appear as an interesting source to find inhibitors of these enzymes. Among them, aerial parts of *Clematis* species could be particularly promising as they were traditionally used to treat rheumatoid arthritis, a disease for which the down regulation of the JAKs/STATs pathway has been shown to be particularly interesting⁵.

Recent developments in metabolomics present new opportunities to explore the phytochemistry of plant and especially with the development of molecular network (MN)

since the creation of the Global Natural Products Social Molecular Network (GNPS) platform, which provides an online open-access to connect MS/MS spectra based on their similarities²⁶. Among these new exploration methods, combining MN with biological assays allows to highlight the compounds potentially responsible for the targeted activity and therefore allows to prioritize isolation and identification of these compounds²⁷.

This work aims to highlight the species that can be used for targeting the JAKs-STATs pathway and to identify compounds or at least compound families responsible for this activity based on the combination of the chemical profiling of extracts with inhibitory assay on JAK3. The chemical profiles of the 11 species of *Clematis*: *C. alpina*, *C. armandii*, *C. flammula*, *C. heracleifolia*, *C. koreana*, *C. macropetala*, *C. recta*, *C. tangutica*, *C. terniflora*, *C. vitalba* and *C. viticella*, have been performed using LC-HMRS/MS of both water/ethanol and ethyl acetate extracts. Multi-informative MN had been generated by integrating the sample taxonomy and the bioactivity. Two strategies have been applied to related the bioactivity of the extract to the MN and the ions or cluster zone highlighted have been investigated deeper to correlate the potential of the extract with its composition.

2. Material et method

2.1. Plant material

C. alpina, *C. armandii*, *C. flammula*, *C. heracleifolia*, *C. koreana*, *C. macropetala*, *C. recta*, *C. tangutica*, *C. terniflora*, *C. vitalba* and *C. viticella* had been cultivated in pot in a greenhouse at Pépinières Travers (Saint-Cyr en Val, France). Aerial parts have been collected between March and May 2024 according to their growth and were stored at -80°C before being used.

2.2. Chemicals

The absolute ethanol (>99.7%) and ethyl acetate was from VWR (Fontenay sous-Bois, France). Ultrapure water was produced with a Milli-Q EQ7000 system equipped with an LC-Pak filter from Merck (Darmstadt, Germany). UHPLC-HRMS analyses were performed with water and LC-MS grade acetonitrile and isopropanol from Honeywell (Seelze, Germany). Chlorogenic acid, coumaric acid, rutin, quercetin-3-O-glucuronide, α -hederin, pinorelinol diglucoside were from Sigma-Aldrich (Saint Quentin Fallavier, France) and chicoric acid, ferulic acid, tiliroside, hyperoside, quercetin-3-acetylglucoside, orientin, homororientin, vitexin-2-O-rhmnoside, α -santorin, quercetin dihydrate, tamarixetin, coniferoyl alcohol, and β -sitosterol were from Extrasynthese (Genay, France).

Two standard solutions were prepared by adding to 1 mL of methanol, 4 μ L of a standard solution at 1 mg/mL (unless otherwise specified). Mix1 contains: chlorogenic acid, coumaric acid, chicoric acid, ferulic acid, tiliroside, rutin, hyperoside, quercetin-3-O-glucuronide, quercetin-3-acetylglucoside, orientin; and Mix2: homororientin vitexin-2-O-rhmnoside, α -

hederin, pinoresinol diglucoside (20 μ L), α -santorin, quercetin dihydrate, tamarixetin, coniferoyl alcohol (20 μ L), β -sitosterol (10 μ L).

2.3. Sample preparation

Aerial parts have been grounded using Kinematica Polymix. 200 mg of dried powder was extracted using ultrasound assisted extraction with 8 mL of either a mixture of ethanol and water (80/20) or with ethyl acetate. The extract was then centrifugated during 10 min at 10 700 g and the supernatant was filtered using PTFE filter. The final extract was dried under nitrogen flow. Dried samples are then resolubilized at 2 mg/mL in MeOH/water (80/20) for hydroalcoholic extract and 100% MeOH for ethyl acetate extract for LC-HMRS/MS analysis or in DMSO for biological assays.

A quality control of the 11 plant extracts were realized for each extraction solvent using 100 μ L of each resolubilized extract (QC_WE and QC_AC for quality control for hydroalcoholic and ethyl acetate extract respectively).

2.4. Analysis

The LC-MS/MS analysis were performed with a Thermo Scientific Ultimate 3000 RSLC system interfaced with a maXis UHR-Q-TOF mass spectrometer (Bruker, Bremen, Germany). Chromatographic system is composed of a binary pump, an automatic sampler, a thermostated column compartment, and a DAD detector (200–800 nm) (Dionex, Germering, Germany). The column was a Luna Omega C18 (100 $\times$ 2.1 mm; 1.6 μ m) (Phenomenex, Le Pecq, France). Gradient mode was used to separate the compounds. The mobile phase consisted of water (A) and acetonitrile/isopropanol 50/50 mixture (B), both acidified with 0.1% formic acid. The flow rate was fixed at 0.5 mL/min. Elution started with 3% B and increase during 12 min until 45%. The amount of B increased up to 70% from 12 to 17 min and is maintained until 20 min. The amount of B then decreased during 0.5 min to the initial conditions until 25 min. The MS/MS experiments were carried out using the data-dependent acquisition mode (DDA) with an electrospray ion source (ESI) working in positive ionization mode. Nitrogen was used as the drying gas at a flow rate of 9 L/min heated at 200°C and as the nebulizing gas at a pressure of 2 bar. The mass spectra were recorded in the m/z range 50–1650 at 1.6 Hz. The capillary voltage was set at 4.5 kV. Four precursor ions were selected per cycle, fragmented at two collision energies (20–45 eV), and averaged into one MS/MS spectrum. Samples were prepared at 5 mg/mL and the volume of injection was fixed at 0.4 μ L. QC samples were analyzed during the batch to guaranty the comparison of the samples.

2.5. Janus Kinase 3 bioactivity assay

Kinase enzymatic activities were assayed in 384-well plates using the ADP-Glo™ assay kit (Promega, Madison, WI) according to the recommendations of the manufacturer. This assay

is a luminescent ADP detection assay (described in Zegzouti et al.²⁸). It provides a homogeneous and high-throughput screening method to measure kinase activity by quantifying the amount of ADP produced during a kinase reaction. The human protein kinase JAK3 was produced as a recombinant, expressed by baculovirus in Sf9 insect cells. Briefly, the reactions were carried out in a final volume of 6 μ L for 30 min at 30°C in kinase buffer: 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 25 mM Tris-HCl pH 7.5, 50 μ g/mL heparin, with peptide JAKtide 94 μ M (GGEEEEYFELVKKKK) as substrate in the presence of 10 μ M ATP. After that, 6 μ L of ADP-GloTM kinase reagent was added to stop the kinase reaction. After an incubation time of 50 min at room temperature (RT), 12 μ L of kinase detection reagent was added for one hour at RT. The transmitted signal was measured using the Envision (PerkinElmer, Waltham, MA) microplate luminometer and expressed in Relative Light Unit (RLU). To validate the kinase assay, Tofacitinib (CP-690550, #S2789, Selleckchem) was used as a specific control inhibitor of JAK3 under the same conditions than the tested compounds. To evaluate the activity of the extracts, two concentrations 50 μ g/mL and 5 μ g/mL were tested. The inhibition was then calculated on the residual activity of the kinase in contact with the extract.

2.6. Raw data processing

Analyses were converted to mzXML format using CompassXport (Bruker, Bremen, Germany) and the mass spectrometry data were processed with MZmine 4.2. Mass detections were fixed at 500 for the MS1 and 100 for the MS2 level. Chromatogram builder in a range time 0.5-22 min, with a minimum of 4 consecutive scans at a threshold of 500 and a minimum absolute height of 1000. Local minimum resolver was then employed with chromatographic threshold 0.95 and a minimum peak top/edge 1.8. Isotope ¹³C grouper was also performed to detected isotope and avoid redundancy. Alignment was performed based on *m/z* tolerance of 0.005 Da or 10 ppm and a retention time tolerance of 0.05 min. Gapfilling and filtering were then performed on the aligned table.

2.7. Molecular Network

A molecular network was generated using GNPS²⁶ with the Feature-Based Molecular Networking (FBMN) workflow²⁹. A molecular network was created in which the edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. The molecular network was then visualized using Cytoscape 3.10.2 software³⁰.

Different representations were used to integrate the bioactivity results on JAK3 in the molecular network mapping. First, three categories have been defined: extracts inhibiting more than 70% (red); extracts inhibiting moderately between 40% and 70% (orange); and extracts considered non-active with an inhibition of less than 40% (pale yellow). Each node is divided using pie-chart repartition of the area of the ion in the samples. Second, the Pearson correlation coefficient between feature intensities and biological activities of the extracts was

based on the protocol using a R script developed by L.-F. Nothias *et al.* in 2018²⁷. A node with correlation score superior to 0.65 with a p value inferior to 0.03 have been considered as potentially active.

3. Results and discussion

3.1. Anti-inflammatory bioactivity assay

The anti-inflammatory properties of species of *Clematis* had been previously investigated based on traditional uses of the plant to treat skin related inflammation or rheumatic pains^{3-5,31}. Ethanolic extracts of the leaves of *C. flammula* have shown an inhibition of COX-2²⁰ with an IC₅₀ of 223.26 ± 17.60 µg/mL and shown an anti-ulcer activity *in vivo*³². Some extracts, as the butanol fraction of the methanolic extract of *C. florida*, the enriched saponins extract from *C. henryi* Oliv or the aqueous extract from roots of *C. terniflora*, have also produced a decrease of the pro-inflammatory cytokines of the NFκB pathway (IL-6, TNF-α, IL-1β). However no activity of *Clematis* had been previously tested on JAK3, a Janus Kinase studied for its proinflammatory activities and mainly described to be involved in multiple chronic inflammatory diseases³³.

The ethyl acetate and ethanol/water (80/20) extracts of the eleven *Clematis* spp. extracts have been tested for their inhibitory ability of JAK3 at 50 and 5 µg/mL in duplicate. The percentage of inhibition at 50 µg/mL on JAK3 are presented Figure 48 (inhibition at 5 µg/mL is presented in supporting data, Figure S8).

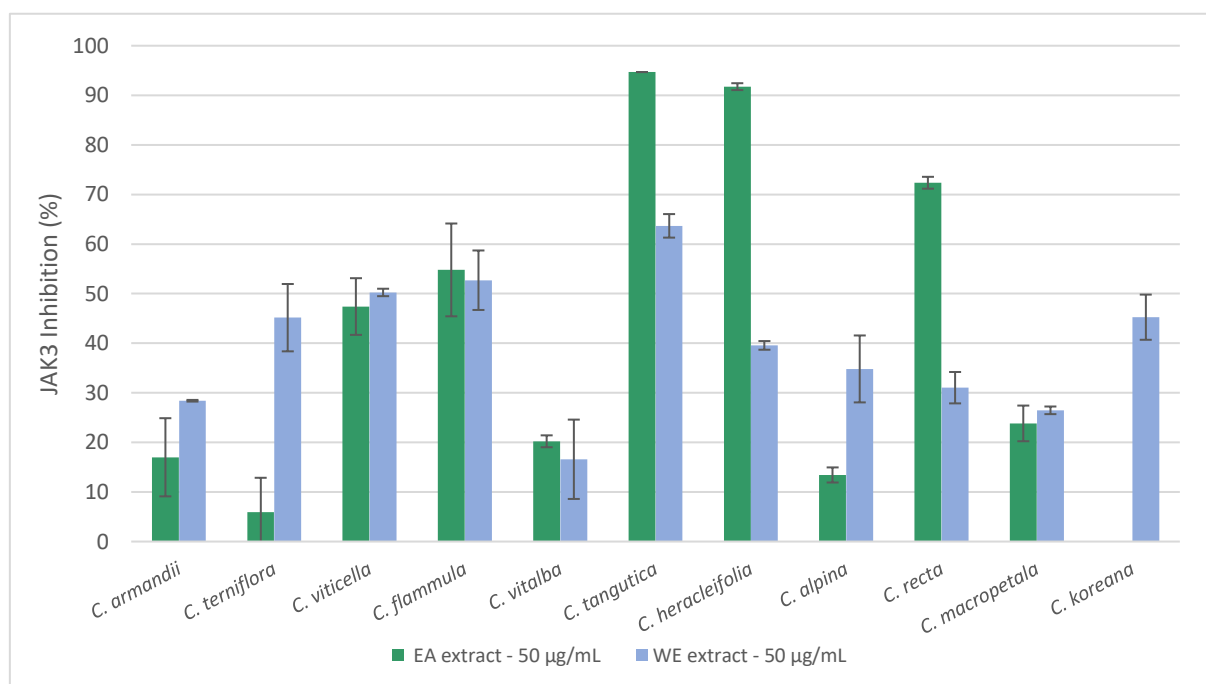


Figure 48: Percentage of inhibition of JAK3 of ethyl acetate (EA) in green, and hydroethanolic (WE) in blue, extracts of the eleven *Clematis* species, tested at 50 µg/mL.

Among the 11 *Clematis* species, only three species, *C. armandii*, *C. vitalba* and *C. macropetala* have shown low inhibition both for hydroalcoholic and ethyl acetate extracts, the 8 other ones showing moderate to high inhibition for at least one extract, these results suggest a good potential of this genus for this biological target. Only two species, *C. viticella* and *C. flammula*, demonstrated a moderate inhibition for both extracts while for three species, *C. terniflora*, *C. alpina* and *C. koreana*, hydroalcoholic extracts showed a moderate inhibition and their ethyl acetate extracts showed a lower inhibition. The last three other species, *C. tangutica*, *C. heracleifolia* and *C. recta*, ethyl acetate extracts exhibited a very promising inhibitory effect with $95 \pm 0\%$, $92 \pm 1\%$ and $72 \pm 1\%$ of inhibition at $50 \mu\text{g/mL}$ while the hydroalcoholic extracts showed a more moderate inhibition. *C. tangutica* is described in traditional uses in Tibet for skin ulcer, which can result from diverse diseases or nondisease phenomena. It included inflammatory ulcer, where inflammation results from a lesion^{3,34}. However, no mechanisms of action or phytochemical studies of the plant have been described to provide a better understanding.

These results suggest that different molecules or molecule families can be involved in the anti-inflammatory activity and may vary depending of the species. The inhibition potential was also determined at $5 \mu\text{g/mL}$ showing a dose-dependant response compared to $50 \mu\text{g/mL}$ and *C. tangutica* ethyl acetate extract remained very active with $76 \pm 1\%$ of inhibition of JAK3 (Figure S8). This value for a raw extract can be considered as very promising since single molecules approved as drugs by FDA to treat rheumatoid arthritis, Tofacitinib and Upadacitinib, showed IC_{50} of 1 nM and 0.7 nM ($3.1\text{E-}4$ and $2.7\text{E-}4 \mu\text{g/mL}$ respectively)³⁵. Twelve compounds, founded in plant and used in traditional Chinese medicines, selected by docking and information of their effect found in literature, have been tested for their ability to inhibit JAK3³⁶. Among the twelve compounds, three compounds demonstrated significant reduction of the activity of JAK3 with an IC_{50} inferior to $100 \mu\text{M}$. Cryptotanshinone, Icaritin, and Indirubin are the most promising compounds with IC_{50} comprise between 20 and $50 \mu\text{M}$ ($7\text{-}13 \mu\text{g/mL}$ according the molecules). These molecules are respectively a abietane diterpenoids, a prenylated flavonoid and a bisindolic alkaloid showing the diversity of compounds leading to a commune activity³⁶. However, this method based on docking is only efficient if the active molecules are already well describe, and can not lead to the discovery of new structure.

Ethyl acetate extracts, showing a larger activity range and higher inhibition percentages compared to hydroethanolic ones, were integrated into the molecular network to identify the molecules potentially involved in this activity.

3.2. Feature based molecular networking investigation of the ethyl acetate extracts

In order to identify molecular family potentially involved in the JAK3 inhibition, a feature-based molecular network (FBMN) using the ethyl acetate extracts was first constructed.

The LC-HRMS/MS analysis have been processed using Mzmine 4.2 and the Feature-Based Molecular Network have been then constructed on GNPS (Figure 49A) where each species are visualized using a different color^{26,29,37}. The molecular network obtained includes 616 nodes which are grouped in 40 clusters (>2 nodes) and 395 singletons. Most of the node are divided in at least two or three species, only few ions of the ethyl acetate are specific of one species. Only two clusters are specific of a species, a cluster constituted of 7 nodes specific of *C. koreana* and another cluster of 3 nodes specific of *C. armandii*.

In order to visualize the commune molecules between the three most active extracts that could be involved in the anti-inflammatory activity, three groups of extracts have been determined according to their activity (Figure 49B). The highly active extract group with *C. tangutica*, *C. heracleifolia*, *C. recta* presenting a JAK3 inhibition superior to 70%; the medium active extract group with inhibition comprised between 40% and 70% with *C. viticella* and *C. flammula*, and the weak active extract group with inhibition lower to 40% with *C. armandii*, *C. terniflora*, *C. vitalba*, *C. alpina*, *C. macropetala* and *C. koreana*. Ions that are detected in highly active extract are colored in red or in orange for moderate active extract. Therefore nodes that appeared mainly colored in red/orange could be associated as a potentially active metabolite, on the contrary nodes represented only in yellow could be associated to non active molecule.

Red ions are found in different clusters, primarily in cluster 1, where they are mainly present in *C. tangutica*. However, a few molecules are also found in clusters 3, 5, as well as in smaller cluster and in single nodes. There is no cluster that consistently appears entirely red; in each case, there are also yellow nodes in the same cluster. This could indicate that it is not an entire family of molecules involved in the activity, but rather specific molecules from different families, as previously described³⁶. Among the red nodes, most of them are relate to one or two active species, but any node is present exclusively in the three most active species and not in the other. It could suggest that among the active extract, different molecules can be involved in the JAK 3 inhibitory effect.

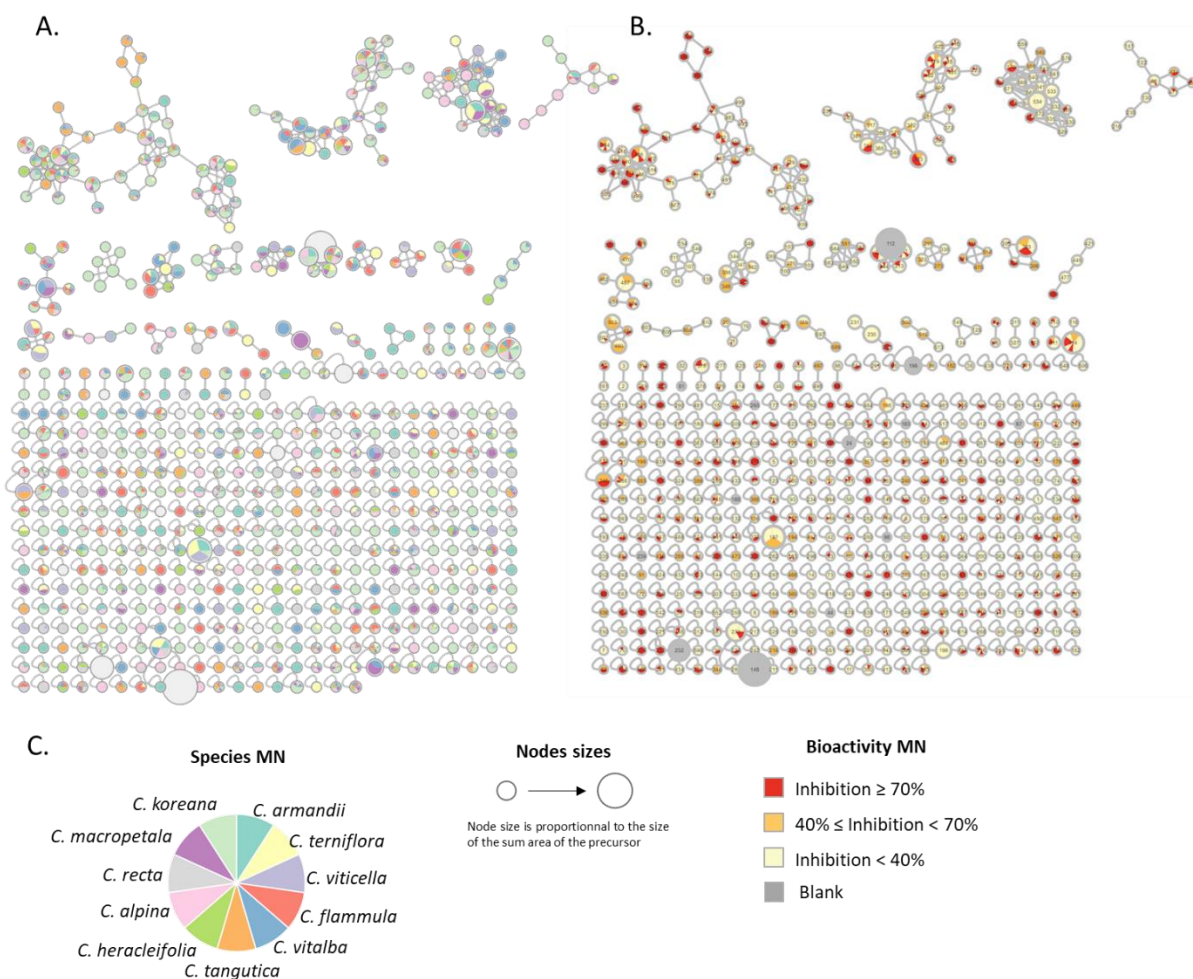


Figure 49: Feature Based Molecular Network (FBMN) of the 11 species of *Clematis* ethyl acetate extracts. A) MN colored with the different species and. B) MN colored with the percentage of inhibition of JAK3. C) Legend associated to each MN with nodes proportional to the sum precursor area.

Another representation based on the bioactivity-based MN was introduced by L.-F. Nothias in 2018. The Pearson correlation (Cor) between the feature intensity in the samples and the associated bioactivity is calculated using R in order to highlight potentially active metabolites. To being able to predict the bioactivity score, samples have to be analyzed and tested at a known concentration with related but different phytochemical profiles, in order to have a different concentration of the metabolites among the samples. It could be fractions, obtained from a same extract as in the proof of concept or also different extracts²⁷.

In our study, we used the ethyl acetate extracts of the 11 *Clematis* species. The border nodes are represented in green for ions with a potentially significantly correlation with bioactivity ($\text{Cor} > 0.65$ and $p \text{ value} < 0.03$). In addition, the previous mapping of the bioactivity of the extracts had been conserved to verified the consistency between the two methods (Figure 50).

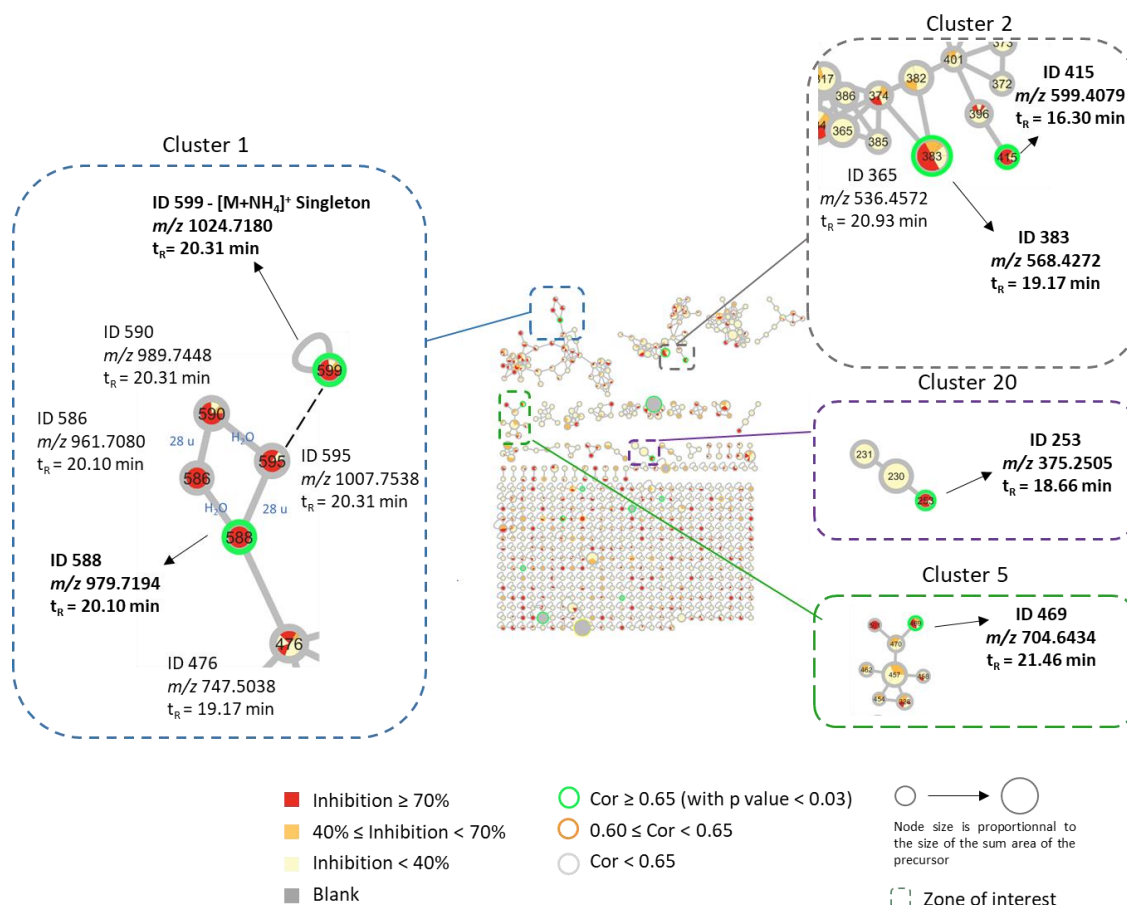


Figure 50: Bioactivity-based Molecular Network of the 11 ethyl acetate extracts of *Clematis* spp. using both color mapping of the active extract and Pearson correlation. Nodes of the MN are colored with the percentage of inhibition of JAK3 and border are colored according the correlation score. Zone of interest with both a red ion and a good correlation score (green border) are detailed.

The use of the two complementary mapping highlights zone of interest in the MN. Among the 616 nodes, 6 nodes are potentially positively correlated to the bioactivity. The two methods show consistency with nodes highlighted in green in the molecular network (with $cor \geq 0.65$ and p value < 0.03) that are in a zone of high bioactivity (red partition). An investigation of the molecular network had been tentatively performed using GNPS and SIRIUS annotation. However, none of the 6 highlighted nodes had been identified using the GNPS and SIRUS. The entire cluster where nodes of interest are highlighted had been therefore explored in order to tentatively identified associated nodes.

In Cluster 1, just one node, with ID 588 at m/z 979.7134 with a $t_R = 20.10$ min, is highlighted in green (with $cor \geq 0.65$ and p value < 0.03). This ion is in an area containing other red ions with the first representation. The investigation of this area led to identify the feature with ID 586 at m/z 961.7080 and a $t_R = 20.10$ min as the $[M-H_2O+H]^+$ fragment of the ID 588. In the same way, the two other features highly represented in red have been linked together with the feature with ID 595 at m/z 1007.7538 and a $t_R = 20.31$ min as the $[M+H]^+$ and the feature

with ID 590 at m/z 989.7448 and a $t_R = 20.31$ min as the $[M-H_2O+H]^+$ fragment of the same compound. A deeper exploration of other compounds highlighted in green in the MN also led to the identification of one singleton with ID 599 at m/z 1024.7810 and a $t_R = 20.31$ min as the $[M+NH_4]^+$ adduct of the same compound, confirming the interest of this area of the network for this biological activity. Thus, leading to two compounds with a difference of 28 u highlighted in this area but unfortunately, any proposition of identification was available for these two compounds through GNPS, TIMA-R or SIRIUS. However, the feature with ID 588 is linked to the feature with ID 476, with m/z 747.5038 and $t_R = 19.17$. This feature has been annotated as the 1-18:3-2-16:3-monogalactosyldiacylglycerol using SIRIUS, suggesting the cluster to be glycosyldiacylglycerols derivatives. Mass fragmentation spectra show commons fragments between ID 588, ID 595 and ID 476 at m/z 585.4502 (fatty acyls chains without the base), 333.2594 and 307.2263 as well as a similar global profile corresponding to the fragmentation of the fatty acyls chain as seen in the mirror plot (Figures S9 and S10). Monogalactosyldiacylglycerols (MGDG) are abundant polar lipids present in thylakoid membranes of plant. The effect of MGDG on several inflammatory cytokines have been evaluated and the results suggest that MGDG has an anti-inflammatory activity in human articular cartilage³⁸, what supports our hypothesis. The difference of 28 u can be potentially explained by a longer carbonyl chain, increasing as well the retention time.

The same work was undertaken for the cluster 2, where two features, ID 383 and ID 415 with m/z 568.4272 and 599.4079 respectively. Any proposition of identification was available for these two compounds through GNPS, TIMA-R or SIRIUS, however the feature with ID 365, with m/z 536.4572 and $t_R = 20.93$ min, linked to the second order to feature with ID 383 through the feature with ID 374, have been annotated using GNPS as β -carotene, under its M^+ molecular ion species as it is predominantly observed for carotenes in ESI(+)³⁹, which is consistent with the research in MassBank, suggesting the cluster to be carotenoids derivatives. A deeper analyzing of the MS/MS spectra of the feature with ID 383 and its comparison with the spectra of carotenoids available in MassBank allowed the annotation of this compound of interest as lutein⁴⁰, another carotenoid. Lutein have been described to attenuates neuroinflammation in LPS-activated BV-2 microglia, suggesting that lutein has a great potential in inflammation-related neurodegenerative disorders^{41–43}.

Among the other nodes highlighted, ID 469 in cluster 5 was examined and found to be a fragment, while ID 253 in cluster 20, is an $[M+Na]^+$ adduct, in the same group as two other non-active ions, therefore no identification or structural hypothesis could be formulated for these two compounds. Although not always possible, the use of information from an identified node to another which is not was useful in order to annotate or propose an annotation hypothesis to three ions of interest. It could be interesting to build another network taking up other information, in order to access new information in the same way.

3.3. Exploration of the hydroalcoholic extracts

However, exploring neighboring nodes or the cluster does not always prove sufficient to annotate the ions of interest. Another strategy has been employed to identify compound present in ethyl acetate extract. As molecules with similar spectrum can be connected using MN; a second MN using both information from the three most active ethyl acetate extracts and their respective hydroalcoholic extract, most described, has been constructed (Figure 51). In parallel, 18 standard compounds, chlorogenic acid, coumaric acid, chicoric acid, ferulic acid, tiliroside, rutin, hyperoside, quercetin-3-O-glucuronide, quercetin-3-acetylglucoside, orientin, homororientin vitexin-2-O-rhamnoside, α -hederin, pinoresinol diglucoside, α -santorin, quercetin dihydrate, tamarixetin, coniferoyl alcohol and β -sitosterol, previously identified in hydroalcoholic *Clematis* extracts and belonging to families of compounds that have already been identified as anti-inflammatory compounds, have been injected and four of them have been successfully identified in this MN (Figure 51), allowing an annotation with a high confidence (level 1 according the MSI⁴⁴).

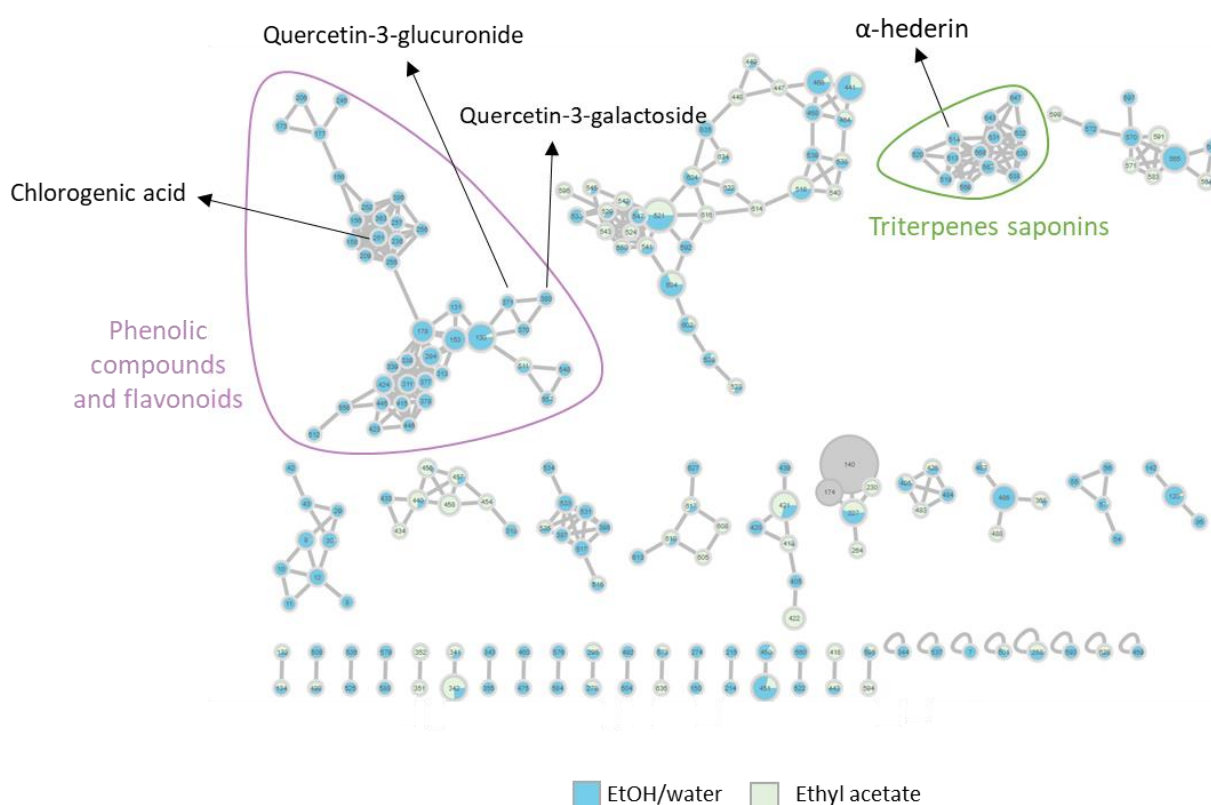


Figure 51: Feature Based Molecular Network (FBMN) of the hydroalcoholic (blue) and ethyl acetate (green) extracts of *C. tangutica*, *C. heracleifolia* and *C. recta*. The four standards identified are localized in the MN.

In this MN, the first cluster is dedicated to phenolic and flavonoid compounds; and the third is dedicated to triterpenoids saponins as it can be suggested by the presence of the different standards. However, any compounds from those families are also present in the ethyl acetate fraction. These compounds could contribute to the moderate JAK3 inhibition of the hydroalcoholic extracts but they could not explain the high activity of the ethyl acetate extracts, indicating that the compounds involved in the anti-inflammatory process through the JAKs/STATs pathway of our study are different than those identified in a recent study on *C. tangutica* where spiraoside (quercetin-4'-O-glucoside), presented a potent anti-inflammatory activity⁴⁵. The comparison of hydroalcoholic extract and ethyl acetate unfortunately provides no additional information among the clusters where ions are shared between the 2 solvents. Our study suggests the anti-inflammatory activities of some *Clematis* species through the JAKs-STATs pathway is less correlated to their composition in polar to medium polar compounds as polyphenols or saponins but seems to be more related to their composition in non-polar compounds as polyunsaturated fatty acids and carotenoids. These families of compounds have already been linked to a modulation of this pathway in some microalgae extracts⁴⁶. The traditional uses and the good *in vivo* anti-inflammatory activities of *Clematis* extracts can therefore be explained by an additivity of action through at least two different inflammatory pathways, polyphenols and saponins inhibiting the NF- κ B pathway and polyunsaturated fatty acids and carotenoids inhibiting the JAKs-STATs pathway.

4. Conclusion

The present study aims to correlate the inhibition of JAK3 activity with the chemical composition of several *Clematis* spp. to identify the molecules potentially involved in the anti-inflammatory activity. This work highlights the good activity of 3 ethyl acetate extracts of *C. tangutica*, *C. heracleifolia* and *C. recta* species, and the good activity of the hydroalcoholic extract of *C. tangutica* species for inhibition of JAK3, which had never been studied before. A molecular network has been created to map the results of the bioactivity assay to highlight relevant ions that may correlate with this activity. The use of a molecular network annotated according to percentage of activity, combined with a bioactivity network using Pearson correlation, enabled us to focus jointly on 6 molecules potentially involved in this activity. Our findings highlight the presence of lutein, and derivatives of monogalactosyldiacylglycerol, both identified as having high potential activity within the molecular network. Further investigations of the extracts are necessary, as not all highlighted ions have been identified. Fractionating the two or three most active extracts could be beneficial, specifically targeting the highlighted compounds for further testing to confirm the bioactivity of the ions. As this method relies on the effective ionization of the molecules in the extract, the richness of the MS/MS libraries, and the capability of *in silico* annotation tools for accurate identification, some bioactive molecules could be overlooked. However, it remains an efficient strategy for prioritizing the bioactivity and phytochemical studies and it also underlines that the good *in*

vivo anti-inflammatory activities of *Clematis* extracts are not due to the presence of one single compound but can be explained by an additivity of action through at least two different inflammatory pathways, polyphenols and saponins inhibiting the NF- κ B pathway as it was previously shown and polyunsaturated fatty acids and carotenoids inhibiting the JAKs-STATs pathway as it was described herein.

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Conclusion Générale - Exploration d'espèce du genre *Clematis*

Ce dernier chapitre s'est intéressé à différentes espèces du genre *Clematis*, une plante largement cultivée en Région Centre-Val de Loire et dont de nombreux sous-produits de taille sont actuellement peu valorisés. Cette matière première, disponible en grande quantité et présentant un fort potentiel de valorisation, répond totalement aux objectifs des travaux entrepris au cours de cette thèse à savoir, la valorisation de ressources végétales présentent localement.

Dans un premier temps, la revue littéraire sur le genre *Clematis* a permis d'obtenir une vue d'ensemble des usages traditionnels des espèces du genre ainsi qu'une connaissance approfondie de la phytochimie de la plante. Elle a également permis d'établir un inventaire des tests d'activités réalisés à la fois sur les extraits et sur des molécules isolées. Tout d'abord, parmi les 388 espèces de *Clematis* répertoriées, 32 d'entre-elles ont été décrites pour un usage traditionnel. Parmi ces utilisations, les principales sont liées à des activités anti-inflammatoires comme pour le soulagement de l'arthrite rhumatoïde. Un total de 538 composés isolés a également pu être répertorié, parmi lesquels de nombreuses saponines triterpéniques, principalement identifiées dans les racines, des composés phénoliques dont de nombreux flavonoïdes, et des dérivées d'anémone. *C. chinensis* et *C. terniflora* var *manshurica* font parties des deux espèces les plus étudiées. Ainsi, une grande proportion des molécules isolées provient généralement de l'une de ces deux espèces. Le genre *Clematis* ne peut être résumé à deux espèces, il est ainsi important de s'intéresser à d'autres espèces du genre. Bien que la composition et l'activité des racines de *C. chinensis*, utilisées en médecine traditionnelle, soit le plus répertoriée, il est également important de noter que les feuilles d'autres espèces, telle que *C. cirrhosa* sont également utilisées pour des propriétés anti-inflammatoires. De plus, quelques tests d'activité réalisés sur des feuilles d'autres espèces vont également de ce sens. Ainsi, il est intéressant d'aller approfondir ces activités dans différentes espèces de *Clematis*, les parties aériennes étant plus facilement accessibles et valorisables en tant que coproduits que les racines.

Dans un second temps, à partir de feuilles récoltées en Suisse, dans le cadre d'une mobilité, une première exploration phytochimique des espèces a été réalisée afin de mettre en avant les spécificités chimiques et points communs des différentes espèces. D'un point de vue méthodologique, cette étude aussi permis l'implémentation du retraitement de données de spectrométrie de masse avec Mzmine 3.9 puis sa version 4.2. Cela a permis la mise en place de l'utilisation de TIMA-R, afin d'associer la taxonomie et phytochimie et à confirmer l'intérêt de SIRIUS 5.8. L'installation des logiciels a été possible grâce à la mise à disposition d'un ordinateur sous Linux dédié, permettant le retraitement d'un plus grand nombre d'analyses. Ainsi, d'un point de vue de la composition phytochimique des plantes, de nombreux flavonoïdes et dérivés ont pu être identifiées, et certaines spécificités en fonction des espèces

ont pu être mises en avant, telles que la présence de flavonoïdes acétylés pour *C. chinensis*, ou encore la présence de flavonoïdes C-glycosylés chez *C. viticella*. Un cluster très spécifique à *C. koreana* a également été mis en avant, cependant aucune hypothèse de structure n'a pu être possible à l'aide des outils d'annotation ou par interprétation manuelle du spectre.

Cependant, les plantes étudiées lors de cette étude provenaient du jardin botanique de Fribourg, où seulement quelques feuilles ont pu être collectées il n'était ainsi pas possible d'approfondir les analyses. Afin de pouvoir étudier plus spécifiquement la composition moléculaire de cette plante, il a fallu cultiver des plantes localement, dédiées à cette étude. Cela a pu être fait avec la collaboration des Pépinières Travers et cela a permis de confirmer, lors de l'analyse de cette même espèce cultivée en Centre-Val de Loire, les particularité phytochimique de celle-ci. Au sein du cluster inconnu de *C. koreana*, deux molécules en particulier ont été ciblées pour être isolées. Cependant, la quantité de biomasse disponible ainsi que le fait que ces composés soient structurellement très proches et présents en petites quantités (analyse LC-DEDL) n'ont pas permis la purification d'une quantité suffisante des composés pour effectuer une caractérisation structurale de ceux-ci. Seule une partie de leur structure, correspondant à un motif geranyl est pour le moment déterminée. De nouvelles étapes d'isolement doivent être menées sur une plus grande quantité de matière végétale afin d'obtenir une quantité suffisante pour une caractérisation par RMN.

Dans une troisième partie, après avoir appliqué l'utilisation des outils d'annotation sur le set Suisse, une collecte de partie aériennes en collaboration avec les Pépinières Travers du Centre-val de Loire a été possible. Cette fois-ci, deux types d'extraits, hydroalcooliques et acétate d'éthyle, ont été réalisés et une cible anti-inflammatoire complémentaire de celles étudiées jusqu'à présent sur des extraits de Clématites a été visée pour la réalisation de test d'activité. Les échantillons ont ainsi été testés sur JAK3, une janus kinase impliquée dans la voie JAKs/STATs, qui est l'une des voies associées au processus inflammatoire et mise en avant récemment comme particulièrement prometteuse pour le développement de diverses pathologies en dermatologie. L'objectif ici a été de corrélérer la composition phytochimique des échantillons avec leur activité biologique afin de cibler les espèces les plus actives et d'identifier les molécules potentiellement responsables de cette activité. Deux méthodologies d'intégration des informations de bioactivités ont été expérimentées et les deux ont convergé vers la mise en avant de composés identiques. L'utilisation des réseaux de bioactivités a ainsi permis de mettre en avant deux dérivés de monogalactosyldiacylglycerol (MGDG) et la lutéine, dont les activités anti-inflammatoires ont été précédemment décrites, ce qui concorde avec notre étude.

Il pourrait également être intéressant d'acquérir le standard de lutéine, de le réinjecter dans le réseau et de le tester afin de confirmer la validité de cette méthodologie. La même démarche peut être entreprise avec le MGDG mais en revanche, il ne semble pas que ce soit

cette molécule qui porte l'activité mais des dérivés. Cela permettrait donc juste de confirmer que les molécules potentiellement actives sont bien des dérivés du MGDG. Dans ce cas-ci, le fractionnement de l'extrait acétate d'éthyle le plus actif (ou présentant la plus grande proportion de ces composés), à savoir l'extrait de *C. tangutica* afin de purifier et caractériser ces molécules semble plus pertinent et est envisagé comme poursuite d'étude. La suite de l'étude se concentrera probablement sur l'exploration d'extraits acétate d'éthyle, un développement chromatographique plus spécifique pourrait être envisagé pour adapter la méthode chromatographique à la polarité de ces molécules et améliorer la séparation (greffages, gradient plus long, pourcentage d'isopropanol plus élevé etc.). L'activité de l'extrait hydroalcoolique de *C. tangutica* étant également importante, les deux extraits pourraient également être comparés, afin d'identifier les molécules communes. Enfin, une extraction ciblée et/ou un fractionnement pourrait être envisagé afin d'obtenir un extrait ou une fraction plus riche en molécules actives.

Pour conclure, ce chapitre présente une étude complète sur différentes espèces de *Clematis*. Le développement d'une méthodologie basée sur des analyses UHPLC-HRMS/MS et l'utilisation des réseaux moléculaires, incluant l'implémentation de divers outils informatiques à chaque étape a ainsi permis de réaliser une étude phytochimique permettant l'annotation de potentielles molécules bioactives avec une plus grande confiance, le tout uniquement à partir d'extraits bruts.

CONCLUSION GENERALE ET PERSPECTIVES

Cette thèse s'inscrit dans un contexte régional où l'industrie cosmétique et le secteur horticole sont fortement implantés. Afin de répondre aux attentes des consommateurs, la filière cosmétique régionale évolue, notamment au travers la mise en place d'un sourcing plus local, afin de garantir une préservation de la biodiversité, d'assurer une bonne traçabilité et de réduire l'impact environnemental lié au transport de matières premières. Cette volonté de valorisation de plantes locales est également une opportunité pour les acteurs de la filière horticole (horticulteurs et pépiniéristes) de participer activement au développement de voies de valorisation de co- ou sous-produits de leur filière limitant ainsi leur production de déchets peu ou non valorisés. De plus, cela leur permet avec l'aide d'organismes tels que le Centre de Développement Horticole de la Région Centre-Val de Loire (CDHRC), de participer à la mise en place de nouveaux itinéraires de cultures pour la production de plantes d'intérêt. Cependant, pour pouvoir valoriser ces plantes dans des secteurs tels que la nutrition, la santé ou le bien-être, il est indispensable de contrôler la qualité de la matière végétale et son contenu en molécules bioactives par la réalisation d'études phytochimiques afin de valider leur potentiel ou d'explorer de nouvelles molécules et/ou activités afin d'ouvrir de nouvelles voies de valorisation.

C'est dans ce contexte que cette thèse avait pour objectif le développement d'approche de métabolomique à travers l'utilisation des réseaux moléculaires et l'intégration d'outils d'annotation pour l'exploration phytochimique de plantes cultivées en Région Centre-Val de Loire. Ainsi, les travaux réalisés ont principalement porté sur la mise en place de méthodologies de retraitement et d'interprétation de données de spectrométrie de masse à partir d'analyses UHPLC-HRMS/MS de différentes plantes dont la culture a été mise en place en Région-Centre Val de Loire et de plantes dont la culture est largement déployée et engendre des sous-produits. Ainsi, différentes stratégies analytiques ont dû être mises en œuvre afin de répondre aux attentes, contraintes et problématiques soulevées par chaque culture.

Dans le premier chapitre, un état de l'art des méthodologies actuelles permettant l'exploration phytochimique d'extraits de plantes et l'identification de molécules par spectrométrie de masse a été réalisé. Cela a permis de mettre en évidence l'intérêt de différents outils en fonction de l'étude souhaitée. Que ce soit l'utilisation d'outils statistiques univariés pour valider la significativité d'activité biologique ou multivariés pour la recherche de marqueurs chimiques entre deux populations distinctes ou encore l'utilisation des réseaux moléculaires pour la dérégulation d'extraits naturels. Concernant cette dernière partie, cela a également permis de mettre en avant les efforts qui sont actuellement mis en place afin de développer des outils d'annotation complémentaires pour améliorer la fiabilité des annotations effectuées, souvent dans le but de prioriser l'identification de composés bioactifs.

Dans le second chapitre, deux études ont été menées sur des plantes tropicales utilisées en médecine traditionnelle Ayurvédique dont la production a pu être réalisée en Région Centre-Val de Loire. Ainsi, il a été possible de montrer la faisabilité d'une telle production en région, tout en conservant les molécules d'intérêt des plantes. Tout d'abord l'étude concernant *Eclipta alba*, visait à examiner son potentiel pour des applications cosmétiques et de contrôler sa composition phytochimique par rapport à celle décrite dans la littérature pour les spécimens récoltés dans leur habitat naturel. L'utilisation d'une méthodologie conventionnelle, associant fractionnement par extraction liquide/liquide et CPC à un screening d'activité biologique à chaque étape, a permis de mettre en évidence pour l'extrait une activité inhibitrice de la collagénase, de la cyclooxygénase-2 et de la xanthine oxydase, trois enzymes impliquées dans le vieillissement cutané. L'activité anti-inflammatoire et antioxydante de la plante étaient déjà décrites, en revanche, l'activité anti-collagénase a été mise en évidence pour la première fois. Celles-ci ont été principalement associées à la wedelolactone et dehydrowedelolactone deux coumestan majoritaires de l'extrait. Une optimisation du procédé d'extraction pourrait donc être envisageable afin de cibler plus spécifiquement ces composés et donc d'augmenter leur teneur au sein de l'extrait. L'utilisation de cet extrait en cosmétique pourrait donc avoir un intérêt multiple, avec notamment une action antioxydante, une action anti-inflammatoire et une action sur la préservation de l'élasticité de la peau.

Par la suite, l'étude de *Coleus forskohlii*, dont la forskoline est extraite des racines pour des applications en nutrition en tant qu'amincissant ou en cosmétique comme agent émoliant, humectant ou pigmentant, a été implanté Comité de Développement Horticole de la Région Centre-Val de Loire par une culture en aéroponie permettant un accès facilité aux racines sans effet délétère sur la plante. L'objectif principal était dans un premier temps d'identifier la modalité de culture optimale (éclairage LED) parmi celles mises en place pour la production de la forskoline. Cette étude a également permis de comparer la composition phytochimique des racines et des parties aériennes de cette modalité avec le témoin correspondant et l'utilisation d'outils statistiques a mis en évidence les molécules sur et sous exprimées. L'ajout de LEDs a tout d'abord permis d'augmenter significativement la quantité de biomasse, qui avec une teneur en forskoline légèrement plus élevée après 18 semaines de culture, permet de multiplier par trois la quantité de forskoline produite finale pour un même nombre de plants. Les LEDs ont également permis d'augmenter la concentration en composés phénoliques dans les racines et en composés diterpéniques dans les parties aériennes.

Ces résultats ont ainsi permis au CDHRC d'orienter leur choix de conditions de culture, guidé par la volonté d'augmenter la quantité de forskoline produite. L'utilisation de fertilisants organiques n'a pas été mentionnée dans ce manuscrit, mais des essais ont été menés. Ceux-ci ont montré que leur utilisation permettait l'augmentation de la teneur en forskoline dans les racines, cependant, la croissance d'une grande majorité des plantes n'a pas pu être

poursuivies en raison de nombreuses carences. Des modalités couplant par exemple, la fertilisation organique et l'utilisation de LEDs, qui permet d'améliorer la biomasse, pourraient être envisagées. D'autres combinaisons pourraient être faites dans ce sens, afin de pouvoir produire des plants ayant une croissance suffisante tout en les stressant convenablement pour augmenter la teneur en forskoline. Aussi, il pourrait être intéressant d'un point de vue de l'échantillonnage de diviser les plants d'une même modalité en sous-groupe et non de tous les rassembler, afin de pouvoir avoir une idée de la variabilité biologique, d'avoir ainsi un plus grand nombre de répétabilité biologiques pour une meilleure significativité statistique.

De plus, cette étude a permis d'initier l'exploration des parties aériennes, pour lesquelles moins d'études sont disponibles en raison du grand intérêt pour la forskoline présente majoritairement dans les racines. Ces premiers résultats ont montré une très grande diversité de composés diterpéniques, cependant, les limites de l'annotation à partir des informations de spectrométrie de masse ont ici été atteintes et il n'a pas été possible d'établir des propositions d'annotation pour beaucoup de composés. La proximité structurale de cette famille n'engendre que peu de différences en termes de fragmentation. L'utilisation d'un autre procédé de fragmentation tel que l'UVPD, avec une méthode dédiée pourrait permettre de mieux les distinguer. La déréplication par RMN en utilisant des outils tels que MixONat ou l'isolement de composés majoritaires pourrait permettre d'établir des propositions ou identifier (au niveau 1) certains composés pour ensuite, à partir de ceux-ci, propager des propositions d'annotations dans le réseau moléculaire. Cependant, cette première étude des parties aériennes, bien que préliminaire, est prometteuse. En effet, cette richesse en diterpénoïdes de type abiétane est très intéressante pour des valorisations futures en tant qu'actifs, cette famille de composés ayant déjà démontré un bon nombre d'activités biologiques notamment des activités anti-inflammatoires, antibactérienne et antifongique. L'élicitation des plants par les LEDs a un impact sur leur composition, ainsi il pourrait également être envisager de suivre la modulation de l'activité des extraits pour identifier les compositions les plus favorables à l'activité biologique. Des valorisations en dermo-cosmétique sont ainsi tout à fait envisageables également pour les parties aériennes et les plants élicités.

Enfin, la dernière partie de cette thèse a été consacrée à l'étude de diverses espèces appartenant au genre *Clematis*. La revue bibliographique a permis d'avoir une vision globale de la phytochimie déjà décrite du genre *Clematis*, des activités, aussi bien *in vitro* que *in vivo* déjà mise en avant sur ces plantes mais également de leurs usages traditionnels. Celle-ci a permis de mettre en évidence que même si c'est un genre abondant et contenant beaucoup d'espèces (environ 300), les études se sont focalisées sur très peu d'entre elles, soulignant ainsi l'intérêt que peut avoir l'étude d'autres espèces appartenant à ce genre, pouvant avoir leur spécificité d'un point de vue chimique. Etant donné que beaucoup de molécules de ce genre ont été isolées (plus de 500), la réalisation d'une base de données *in silico* de spectres

MS/MS serait intéressante afin de pouvoir comparer plus facilement les spectres de fragmentation issus des analyses des différentes espèces acquis dans le cadre de cette étude aux données de la littérature.

Ensuite, l'étude de feuilles récoltées dans les jardins botaniques de Neuchâtel et de Fribourg a confirmé l'intérêt que peut avoir l'étude d'autres espèces appartenant à ce genre, certaines montrant effectivement des spécificités chimiques, tel que *Clematis koreana* ou *Clematis viticella*. Cela a aussi montré l'intérêt de l'utilisation de différents outils d'annotation et de leur combinaison pour améliorer la confiance en l'annotation. Il pourrait être intéressant de poursuivre cette étude en incluant encore davantage d'espèces appartenant à ce genre pour essayer de cartographier au mieux l'espace chimique de ces plantes. La récolte de ces espèces s'inscrit également dans le contexte du Digital Botanical Garden Initiative (DBGI), mise en place par Pierre-Marie Allard et Emmanuel Defossez, dont l'ambition est d'explorer des solutions innovantes pour la collecte, la gestion et le partage d'informations numériques acquises sur des collections botaniques vivantes. Les informations obtenues suite à l'étude approfondie des réseaux moléculaires, pourraient être intégrées également afin d'enrichir les connaissances résultant de ces analyses. Les compétences acquises à travers cette mobilité ont ainsi pu être transférées et poursuivies à l'ICOA, notamment avec l'utilisation de différents outils de retraitement comme MZmine, SIRIUS puis Tima-R. Dans une démarche plus globale, il pourrait être intéressant pour le laboratoire de mettre en place le partage de ses données de spectrométrie de masse et des métadonnées associées sur MassIVE afin d'enrichir la couverture de l'outil plantMASST et ainsi de mettre à disposition de la communauté scientifique les informations disponibles en interne au sein du laboratoire.

Finalement, l'objectif de cette étude étant le même que celui des deux premières plantes, à savoir la valorisation de plantes cultivées en Centre Val de Loire, l'étude de différentes espèces du genre *Clematis* cultivées en région a pu être possible à travers une collaboration avec les Pépinières Travers. Cette étude avait pour objectif l'étude des parties aériennes (feuilles et tiges), dont la ressource est disponible en grande quantité et constitue pour le moment un déchet vert qui est produit lors de la taille des plants. Ainsi 11 espèces dont 9 communes avec l'étude réalisée sur les plantes récoltées en Suisse ont pu être analysées. L'application d'une méthodologie utilisant à nouveaux les réseaux moléculaires mais en les couplant cette fois-ci avec des tests d'activité sur une kinase impliquée dans les processus inflammatoire, JAK3, a permis la mise en évidence des molécules avec un potentiel inhibiteur. Celles-ci ont déjà été décrites dans la littérature pour leur action anti-inflammatoire mais sans études sur leur mode d'action. La mise en place de cette méthodologie de réseaux de bioactivité a ainsi pu être initiée à l'ICOA, celle-ci doit encore être complétée par la réalisation de tests d'activité biologique des molécules mises en évidence ou sur des fractions enrichies en ces molécules, mais les résultats obtenus étant cohérents avec la bibliographie et leur activité précédemment décrite, cette méthodologie semble prometteuse.

Pour conclure, l'ensemble de ces études ont fait ressortir l'intérêt de la déréplication par spectrométrie de masse et de la mise en place d'outils d'annotation et de priorisation pour l'identification de molécules au potentiel bioactif. Les outils statistiques et de métabolomique au sens large peuvent ainsi être combinés pour l'exploration chimique d'extrait. L'étude de différents échantillons au sein d'une même étude permet également d'ajouter des points de comparaison avec la littérature, dans le cas par exemple où un même ion est identifié dans deux espèces et que seule l'une d'entre-elles a été précédemment étudiée. Les réseaux moléculaires peuvent ainsi être utilisés pour comparer les profils de différentes parties d'une même plante, comme avec le réseau construit à partir des analyses UHPLC-HRMS/MS des extraits de racines et de feuilles de *C. forskohlii* ou les profils de différentes espèces dans le cas de l'étude du genre *Clematis*.

Enfin, l'utilisation de ces outils permet également de croiser un grand nombre d'informations, que ce soit au niveau de l'échantillon, comme la source végétale ou son activité biologique, mais également au niveau moléculaire comme la classe du composé ou la proposition de structure avec différents outils. L'utilisation de Canopus par exemple pour l'annotation des classes, bien que les proportions réelles des différentes classes dans l'échantillon puissent être biaisées par une mauvaise détection de l'ion précurseur, offre une belle vue d'ensemble d'un échantillon. Il est dans ces d'autant plus important d'associer d'autres informations que celles fournies par la comme par exemple, le spectre UV des molécules qui permet également d'avoir une idée de la classe chimique, tout comme le temps de rétention qui renseigne sur la polarité. Ces données peuvent être complémentaires, apportant ainsi de la confiance dans le traitement des données MS et permettent d'avoir une meilleure connaissance de l'échantillon analysé. Cela peut être utile tant pour améliorer la connaissance de la composition phytochimique d'un genre, d'une espèce ou même d'un organe d'une plante ou alors pour corréler une activité biologique à la composition phytochimique afin de ne pas passer à côté de molécule pouvant avoir un intérêt pharmacologique.

La méthodologie développée dans cette thèse, est actuellement en cours d'application sur une étude phytochimique de différentes espèces appartenant au genre *Thymus* sur lesquels des tests d'activités biologique ont également été réalisés, cependant, afin de pallier certains problèmes rencontrés au cours de l'étude sur le genre *Clematis* (notamment la reconnaissance des adduits), d'autres outils disponibles sur MZmine4 ont été testés afin de générer des Ion Identity Molecular Network. Cette méthodologie va également être au centre d'un projet structurant du Cancéropôle Grand Ouest afin d'identifier des molécules actives contre l'ostéosarcome à partir d'éponges marines. Les échantillons étant en quantité limités, il est primordial de prioriser l'isolement et la caractérisation des molécules ayant un intérêt thérapeutique. Enfin les résultats prometteurs des extraits d'espèce de *Clematis* sur l'inhibition de JAK3 et le grand intérêt de la Pépinière Travers pour nos travaux encouragent l'équipe à poursuivre cette étude.

ANNEXES

Chapitre II : Développement de méthodes d'analyse ciblées et non ciblées pour la culture de deux plantes d'intérêt en Région Centre-Val de Loire

Figure S1 : Spectres RMN ^1H et HSQC de la wedelolactone

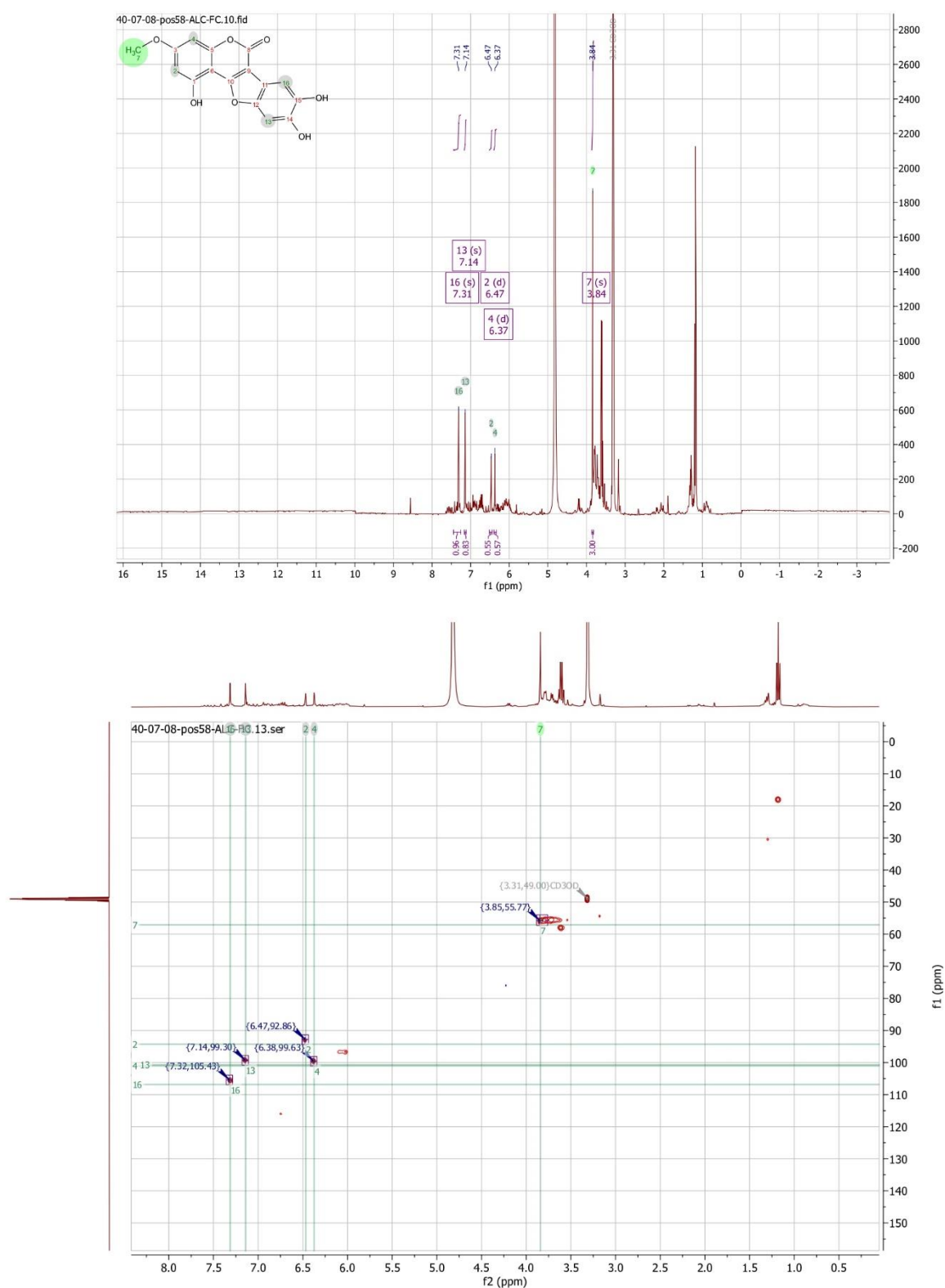


Figure S2 : Spectres RMN ^1H et HSQC de la demethylwedelolactone

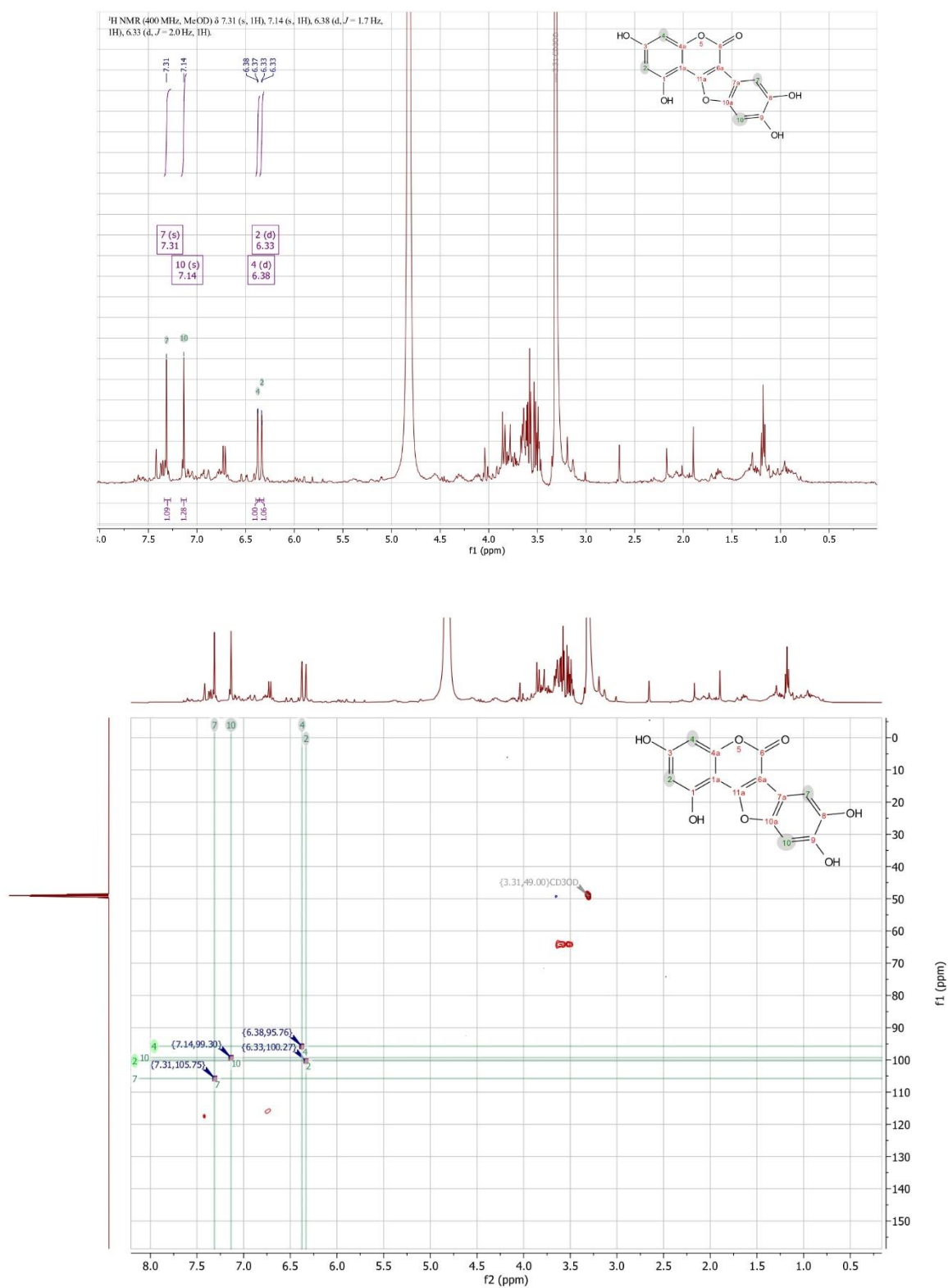


Figure S3: UHPLC-ELSD analysis of FB and FC

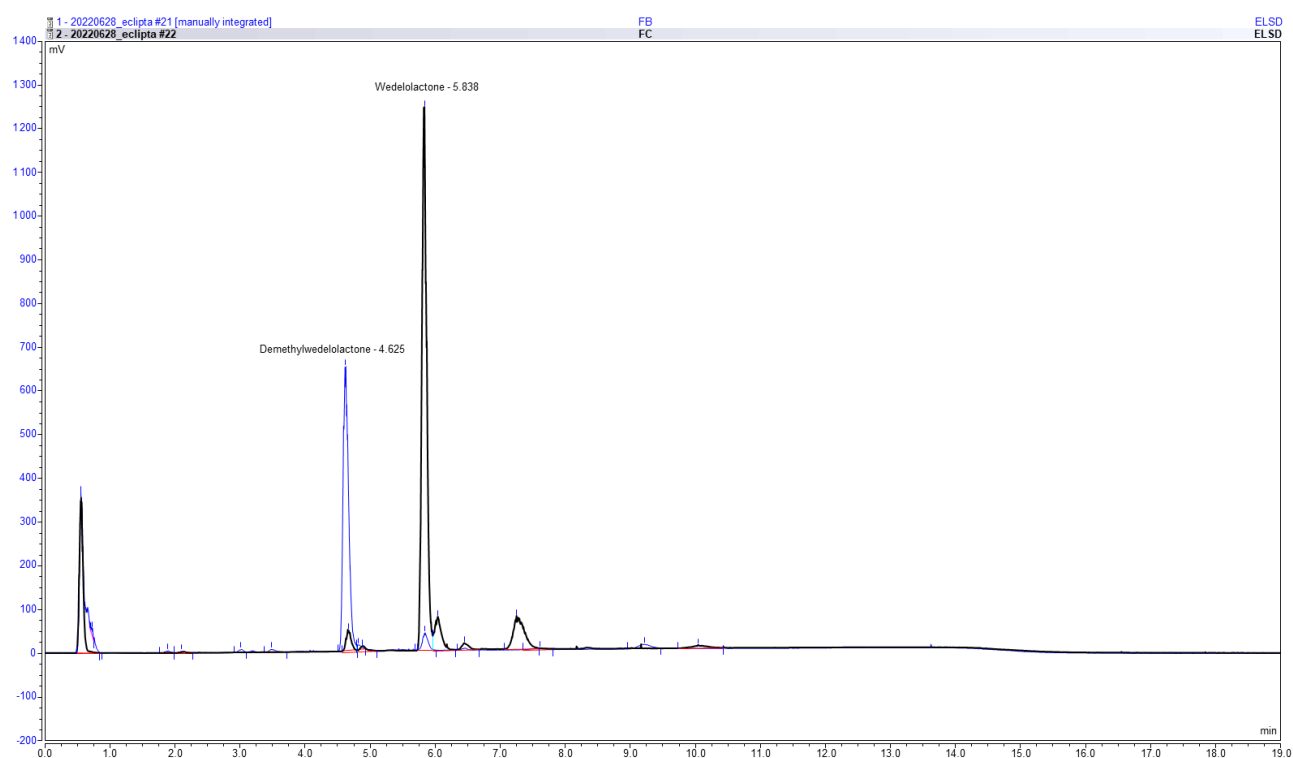


Figure S4: Calibration curve of the forskolin quantification $\text{Log}(A) = f(\text{log}(C))$ with A the area and C the concentration in $\mu\text{g/mL}$

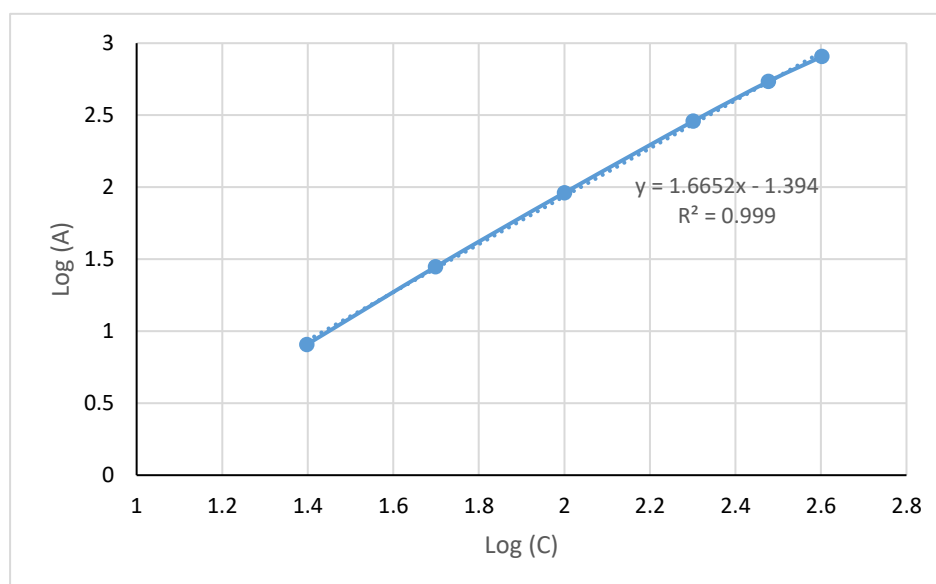


Figure S5: Base peak chromatogram UHPLC-(+)ESI-MS/MS of QC_R (roots extract)

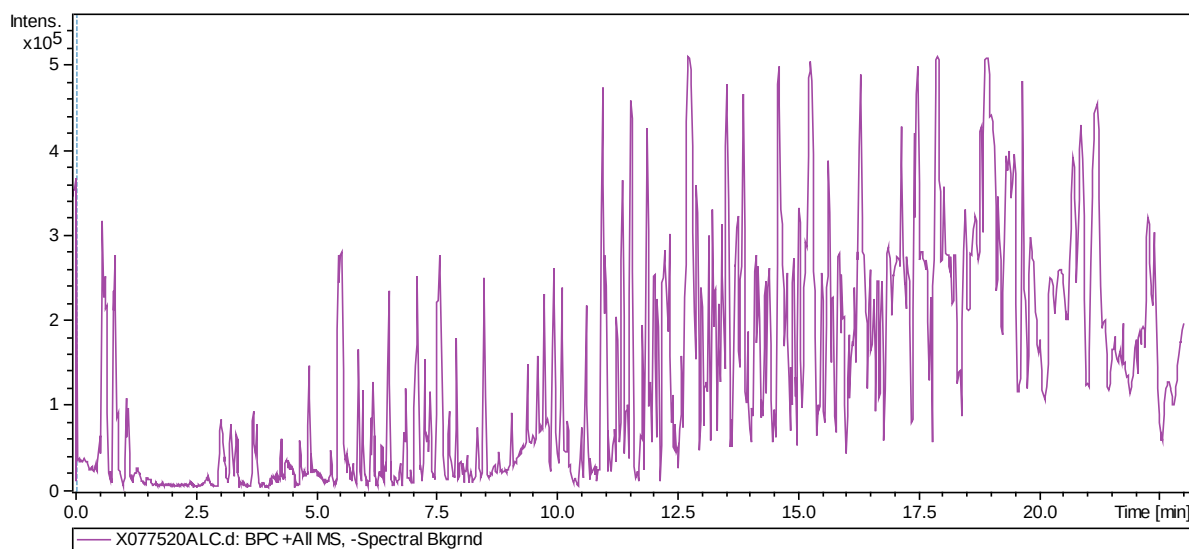


Figure S6: Base peak chromatogram UHPLC-(+)ESI-MS/MS of QC_AP (aerial part extract)

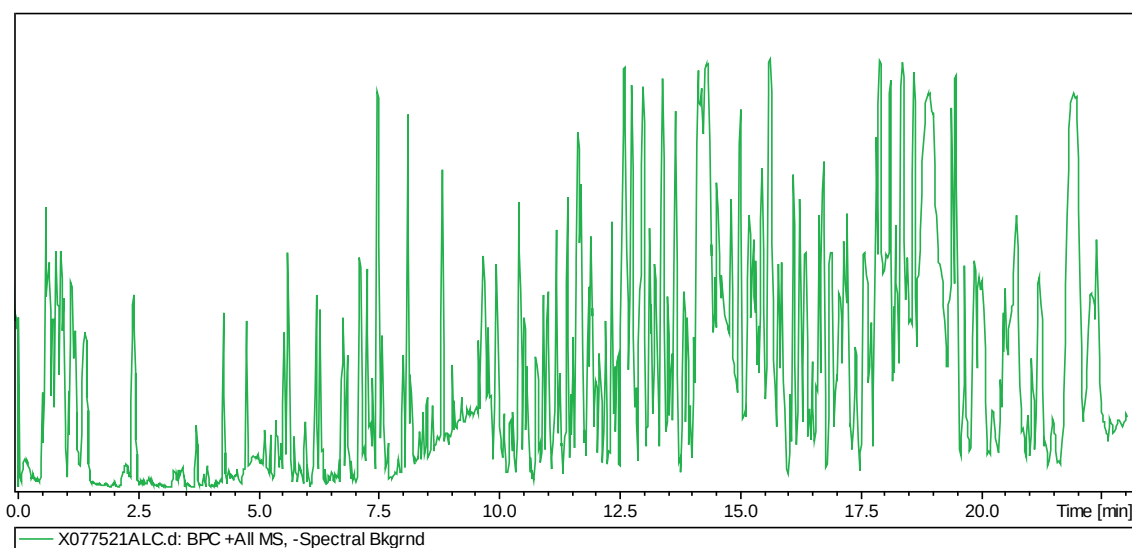
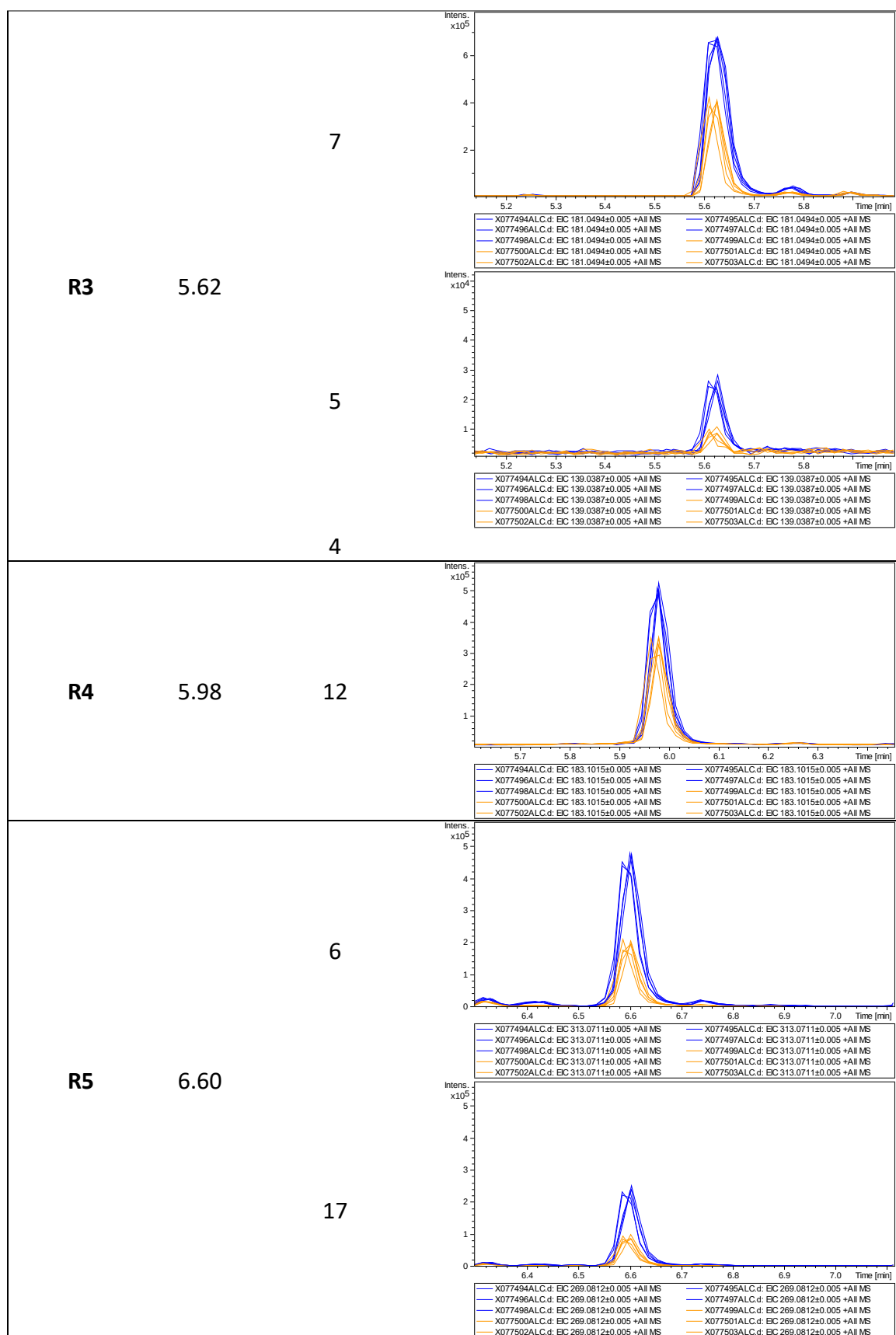
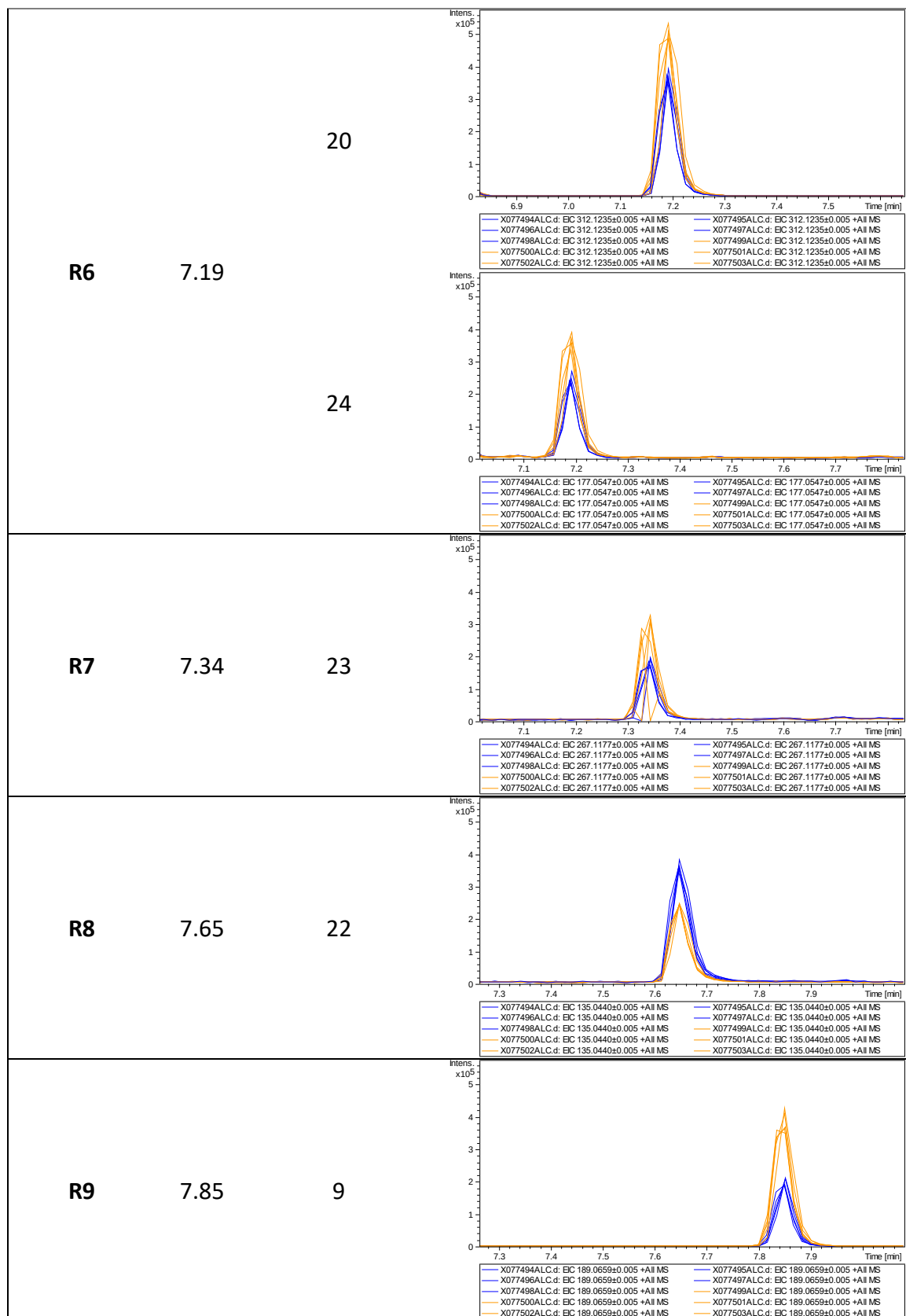
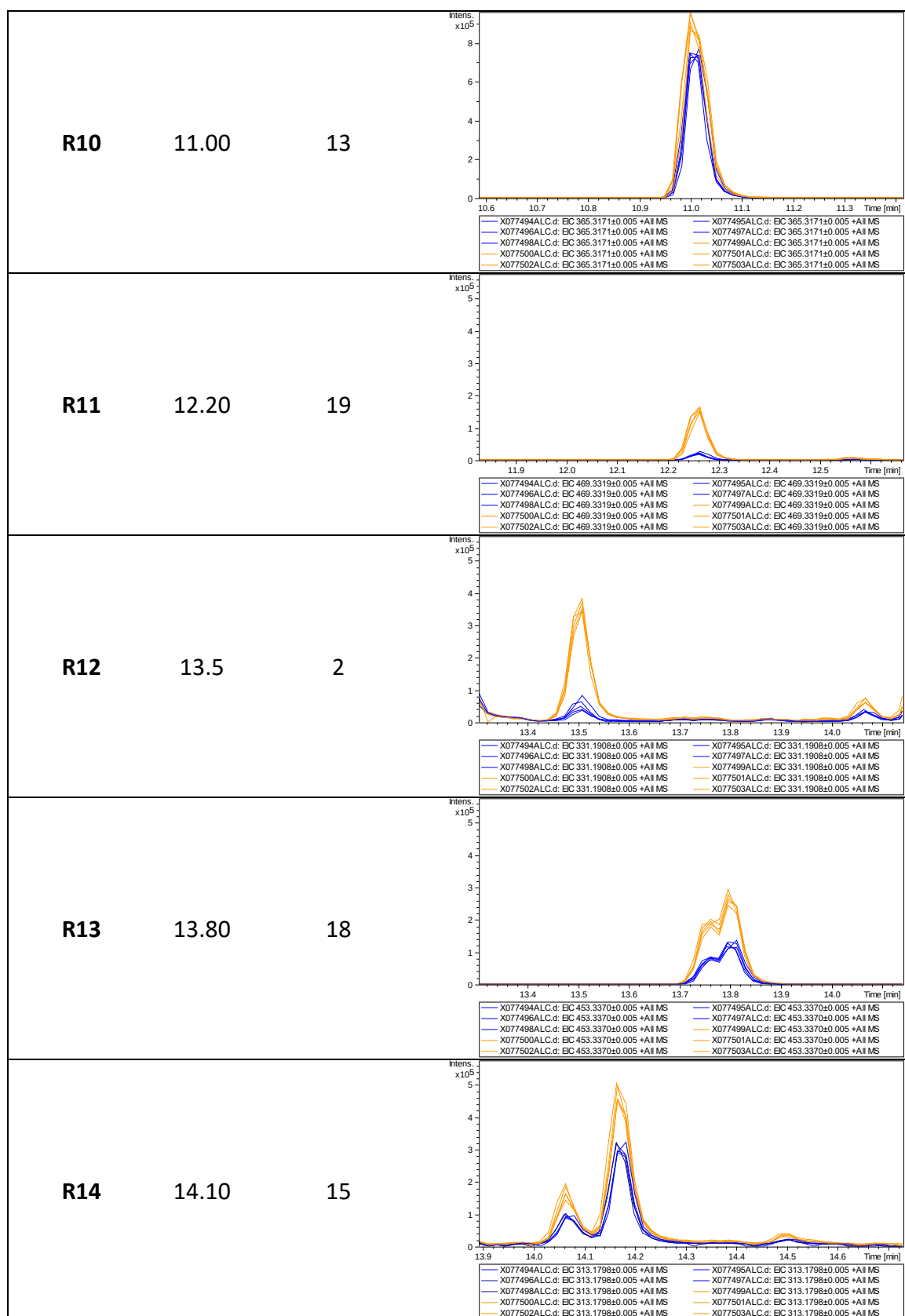


Table S1: Extract ion chromatogram of VIPs compounds in roots (R1-R19) (with yellow samples from itinerary B and blue sample from itinerary A)

Compound	Rt	VIP position	EIC
R1	3.35	1	
		8	
		10	
R2	3.87	21	







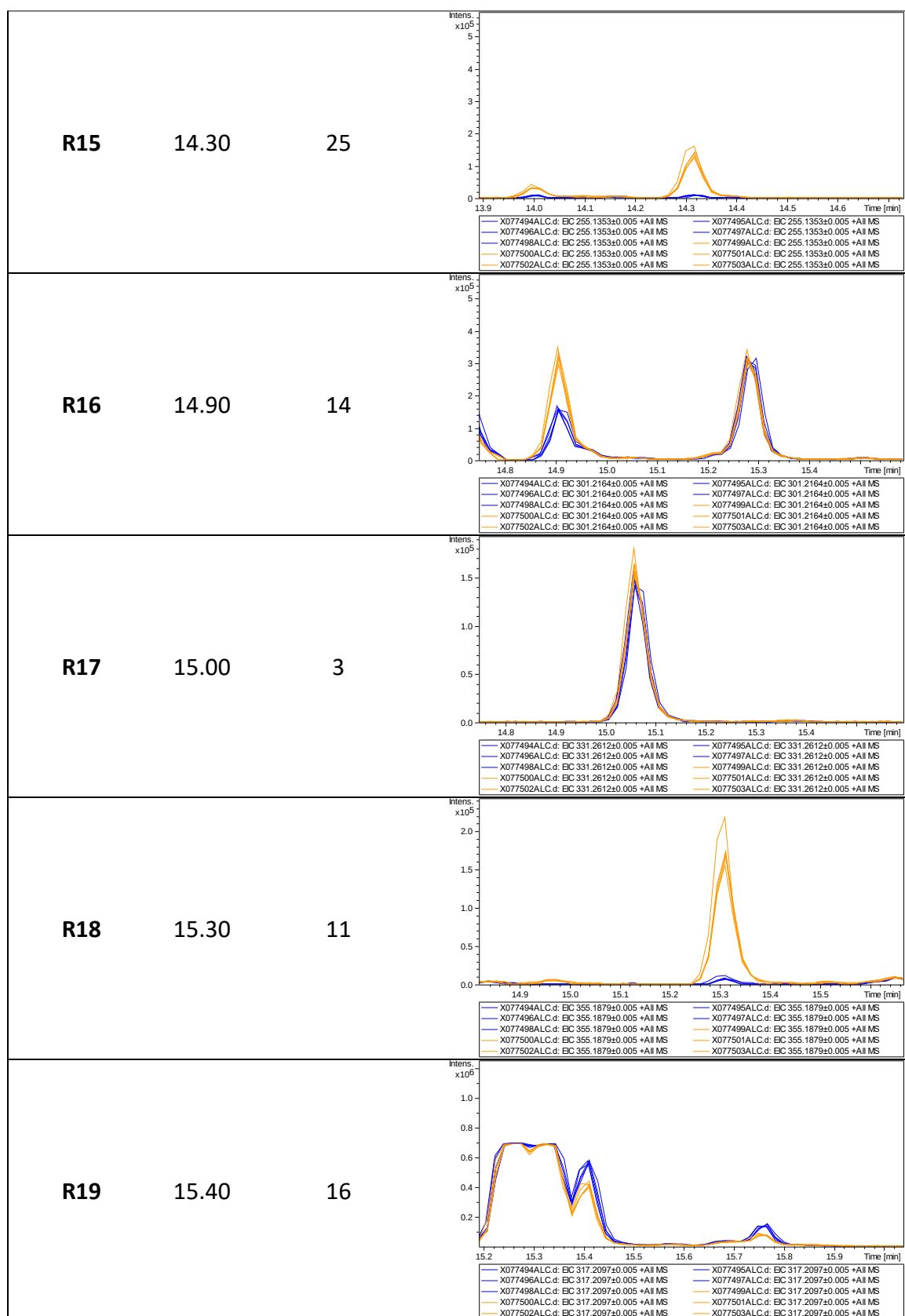
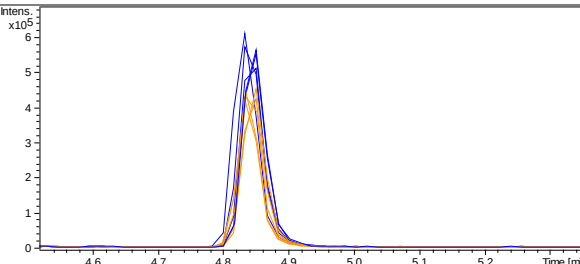
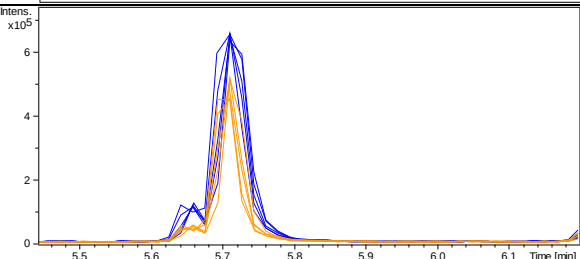
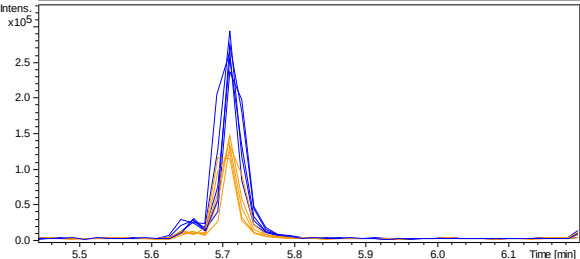
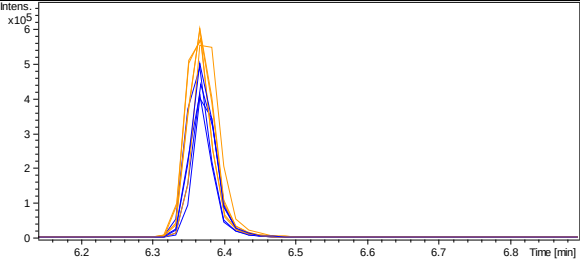
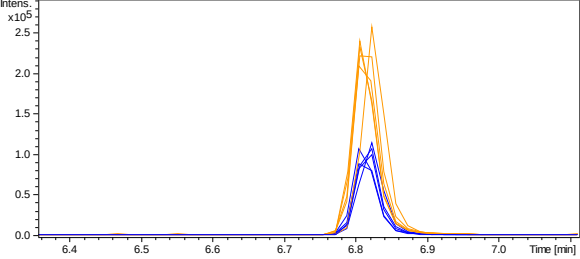
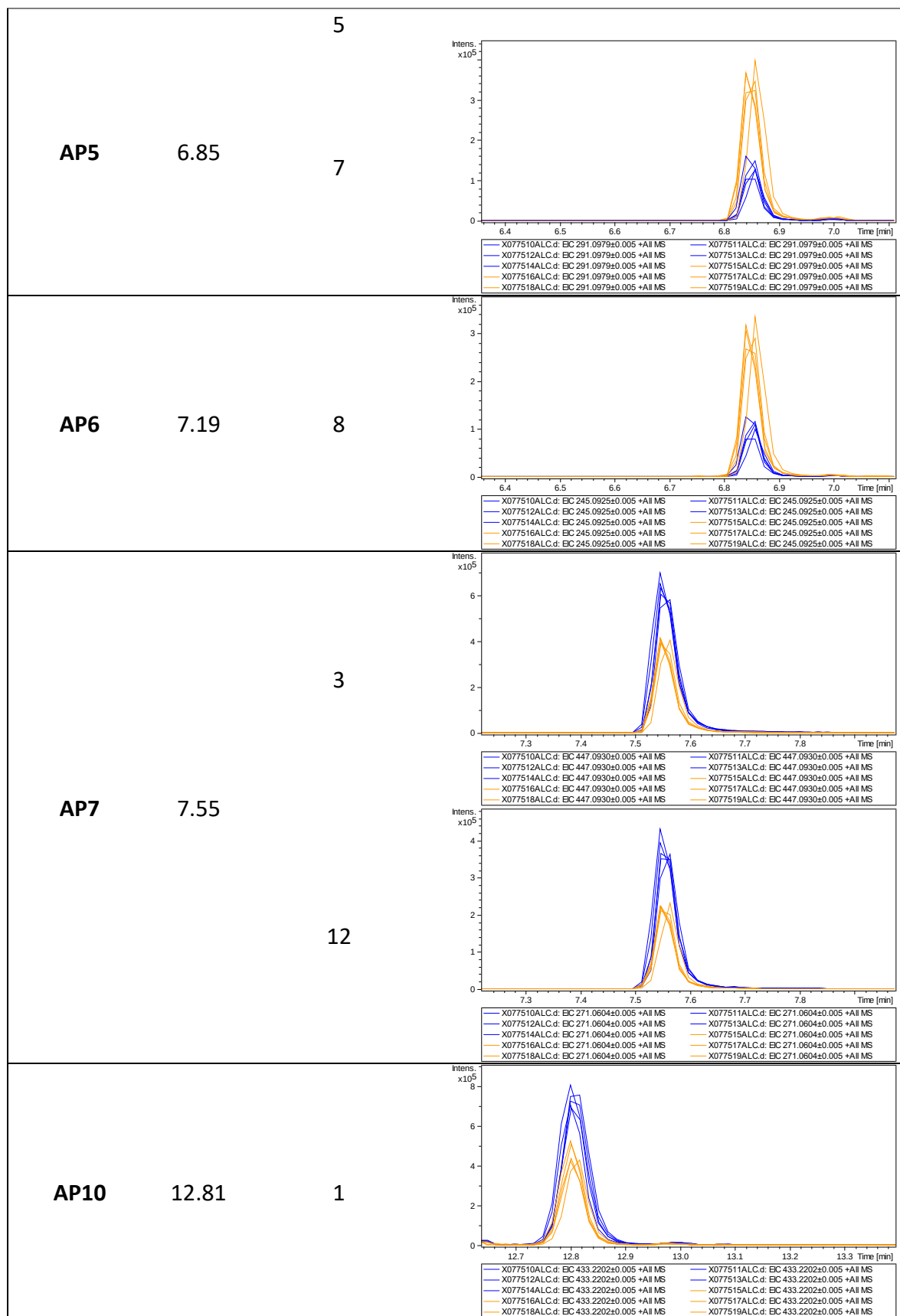
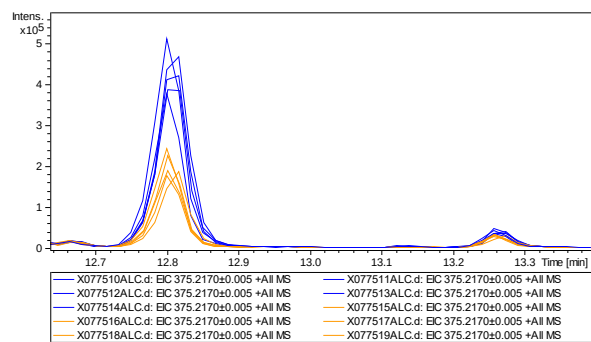


Table S2: Extract ion chromatogram of compound impacted in roots (AP1-AP20) (with yellow samples from itinerary B and blue sample from itinerary A)

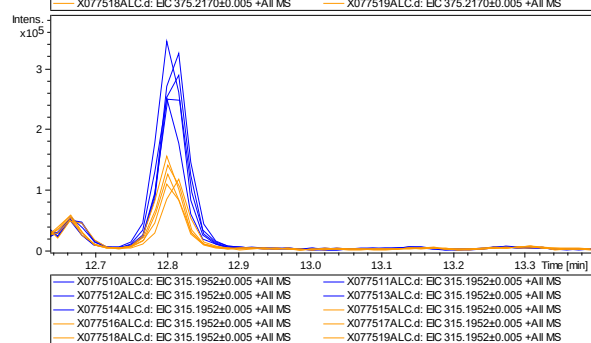
Compound	Rt	VIP position	EIC
AP1	4.85	18	 X077510ALC.d: EIC 167.0343±0.005 +All MS X077512ALC.d: EIC 167.0343±0.005 +All MS X077514ALC.d: EIC 167.0343±0.005 +All MS X077516ALC.d: EIC 167.0343±0.005 +All MS X077518ALC.d: EIC 167.0343±0.005 +All MS X077511ALC.d: EIC 167.0343±0.005 +All MS X077513ALC.d: EIC 167.0343±0.005 +All MS X077515ALC.d: EIC 167.0343±0.005 +All MS X077517ALC.d: EIC 167.0343±0.005 +All MS X077519ALC.d: EIC 167.0343±0.005 +All MS
AP2	5.71	10	 X077510ALC.d: EIC 227.1281±0.005 +All MS X077512ALC.d: EIC 227.1281±0.005 +All MS X077514ALC.d: EIC 227.1281±0.005 +All MS X077516ALC.d: EIC 227.1281±0.005 +All MS X077518ALC.d: EIC 227.1281±0.005 +All MS X077511ALC.d: EIC 227.1281±0.005 +All MS X077513ALC.d: EIC 227.1281±0.005 +All MS X077515ALC.d: EIC 227.1281±0.005 +All MS X077517ALC.d: EIC 227.1281±0.005 +All MS X077519ALC.d: EIC 227.1281±0.005 +All MS
		15	 X077510ALC.d: EIC 191.1065±0.005 +All MS X077512ALC.d: EIC 191.1065±0.005 +All MS X077514ALC.d: EIC 191.1065±0.005 +All MS X077516ALC.d: EIC 191.1065±0.005 +All MS X077518ALC.d: EIC 191.1065±0.005 +All MS X077511ALC.d: EIC 191.1065±0.005 +All MS X077513ALC.d: EIC 191.1065±0.005 +All MS X077515ALC.d: EIC 191.1065±0.005 +All MS X077517ALC.d: EIC 191.1065±0.005 +All MS X077519ALC.d: EIC 191.1065±0.005 +All MS
AP3	6.37	16	 X077510ALC.d: EIC 206.0814±0.005 +All MS X077512ALC.d: EIC 206.0814±0.005 +All MS X077514ALC.d: EIC 206.0814±0.005 +All MS X077516ALC.d: EIC 206.0814±0.005 +All MS X077518ALC.d: EIC 206.0814±0.005 +All MS X077511ALC.d: EIC 206.0814±0.005 +All MS X077513ALC.d: EIC 206.0814±0.005 +All MS X077515ALC.d: EIC 206.0814±0.005 +All MS X077517ALC.d: EIC 206.0814±0.005 +All MS X077519ALC.d: EIC 206.0814±0.005 +All MS
AP4	6.82	17	 X077510ALC.d: EIC 312.1235±0.005 +All MS X077512ALC.d: EIC 312.1235±0.005 +All MS X077514ALC.d: EIC 312.1235±0.005 +All MS X077516ALC.d: EIC 312.1235±0.005 +All MS X077518ALC.d: EIC 312.1235±0.005 +All MS X077511ALC.d: EIC 312.1235±0.005 +All MS X077513ALC.d: EIC 312.1235±0.005 +All MS X077515ALC.d: EIC 312.1235±0.005 +All MS X077517ALC.d: EIC 312.1235±0.005 +All MS X077519ALC.d: EIC 312.1235±0.005 +All MS



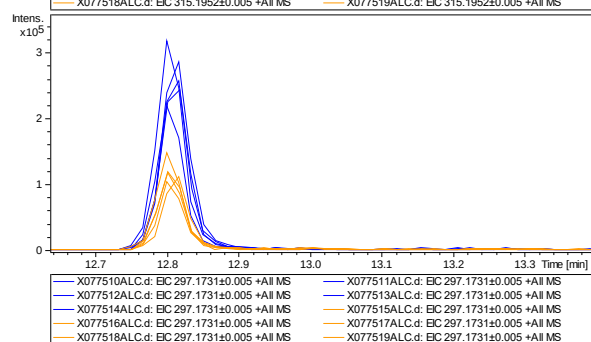
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11



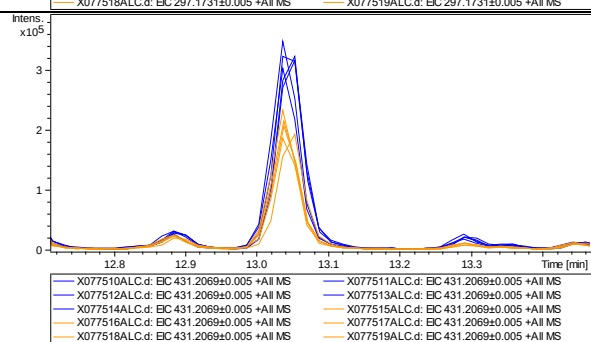
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AP11

13.04

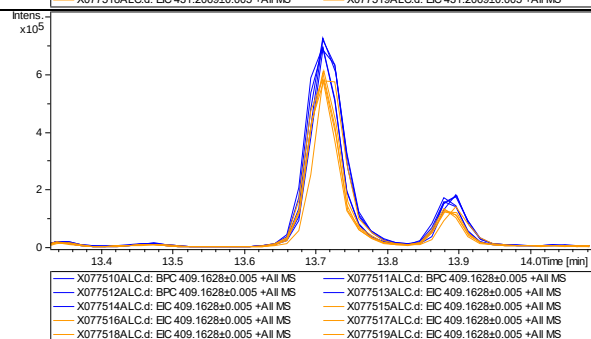
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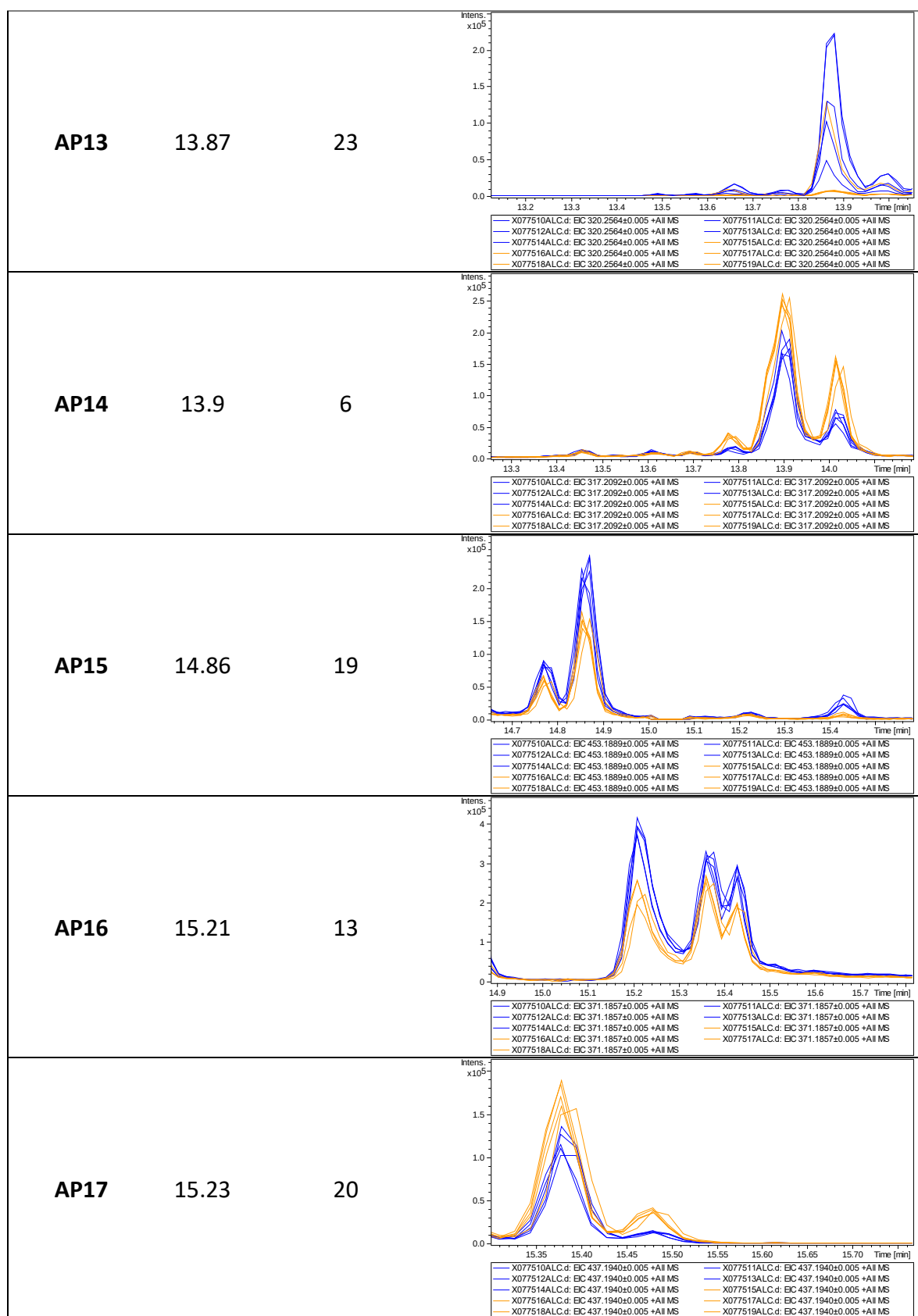


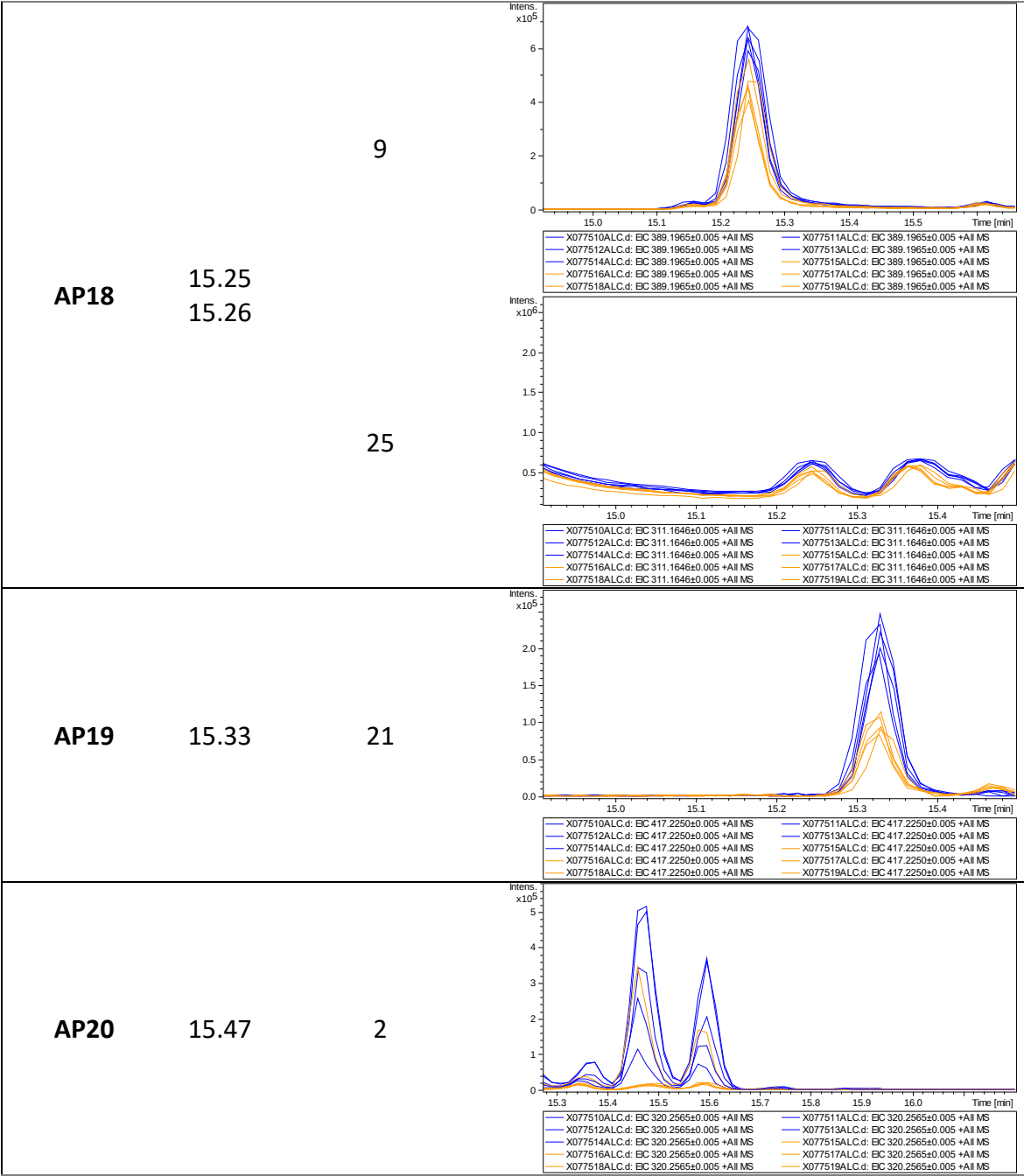
AP12

13.71

24







Chapitre III : Développement de méthodes d'analyses non ciblées et intégration d'outils d'annotation pour l'exploration d'espèces du genre *Clematis*

Figure S7: Extraction Ion Chromatogram of m/z 437.3340 $\pm$ 0.008 Da of (+)-UHR-MS/MS analysis of *C. terniflora*

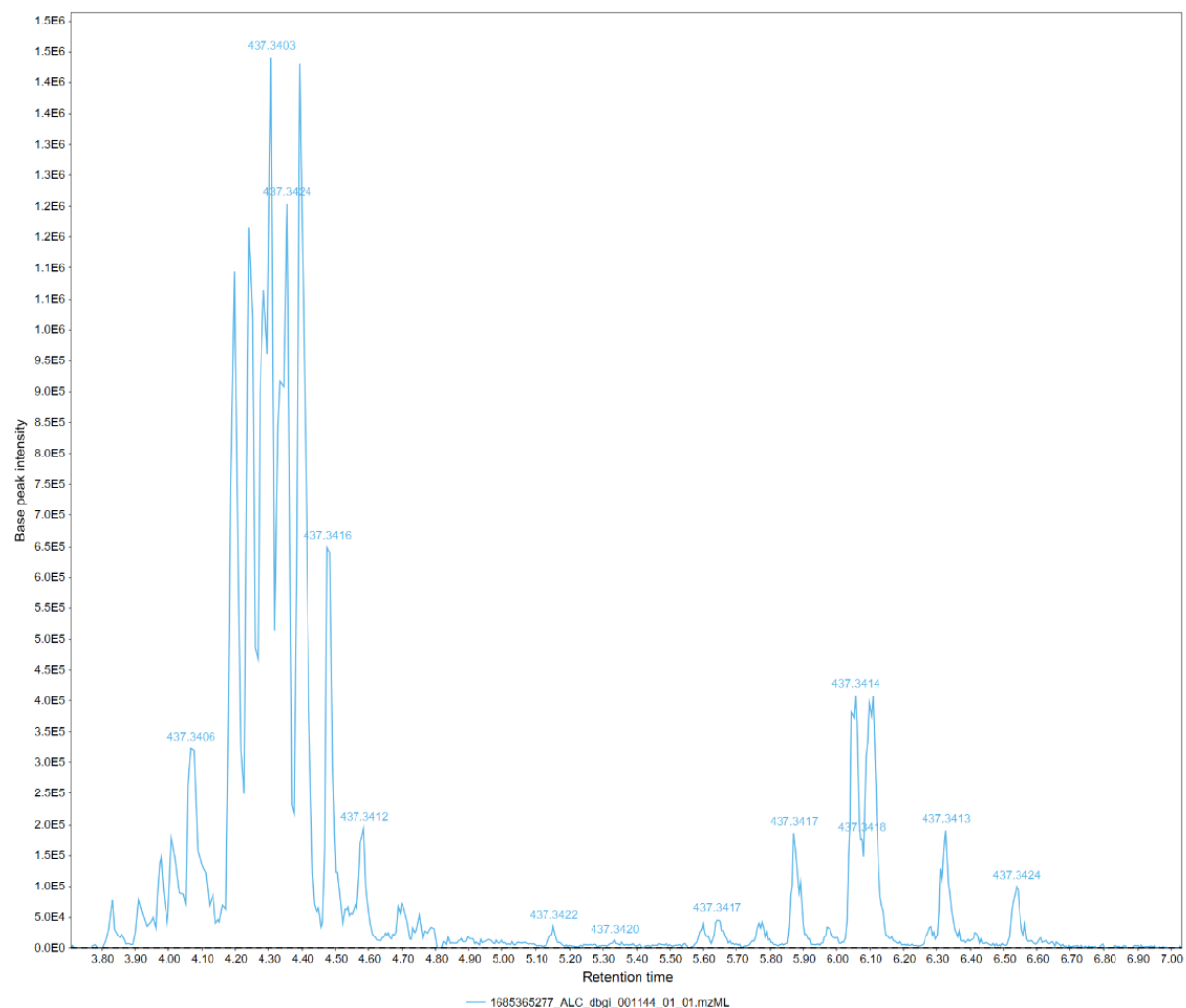


Figure S8: Activities of ethyl acetate (EA) and hydroethanolic (WE) extracts of the 11 *Clematis* species on JAK3 tested at 5 $\mu\text{g/mL}$. The different colors correspond to the two solvent of extraction

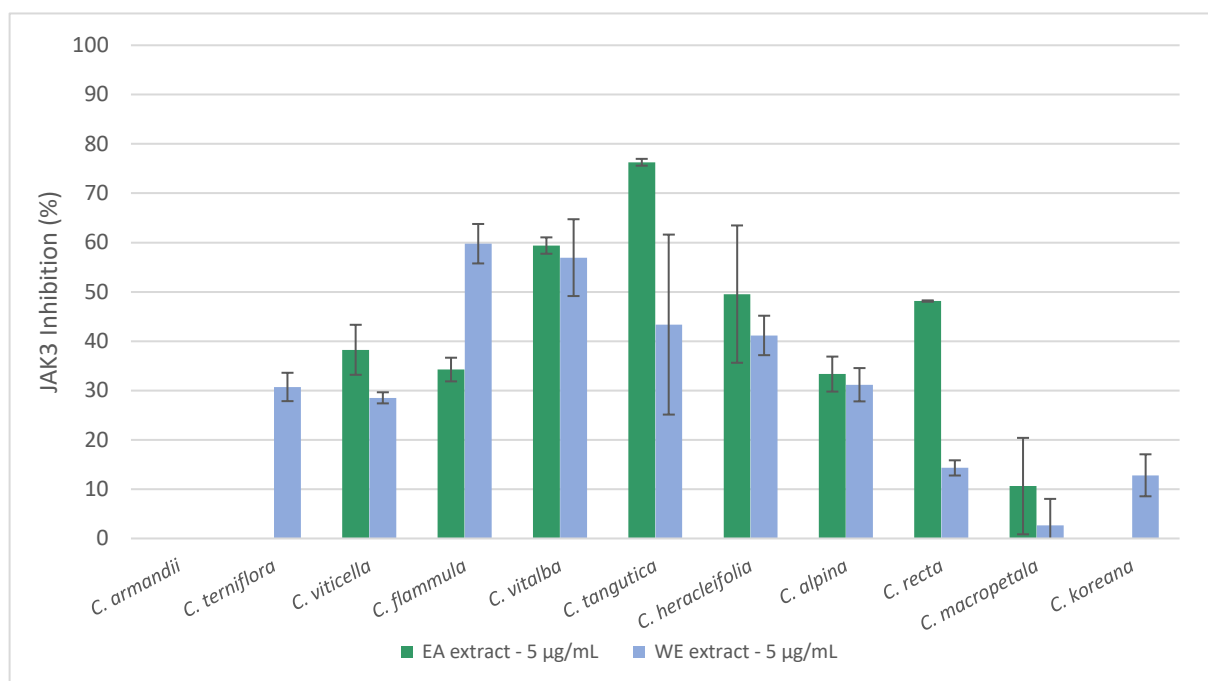


Figure S9: Mirror plot mass spectrum of compound ID 476 and ID 588

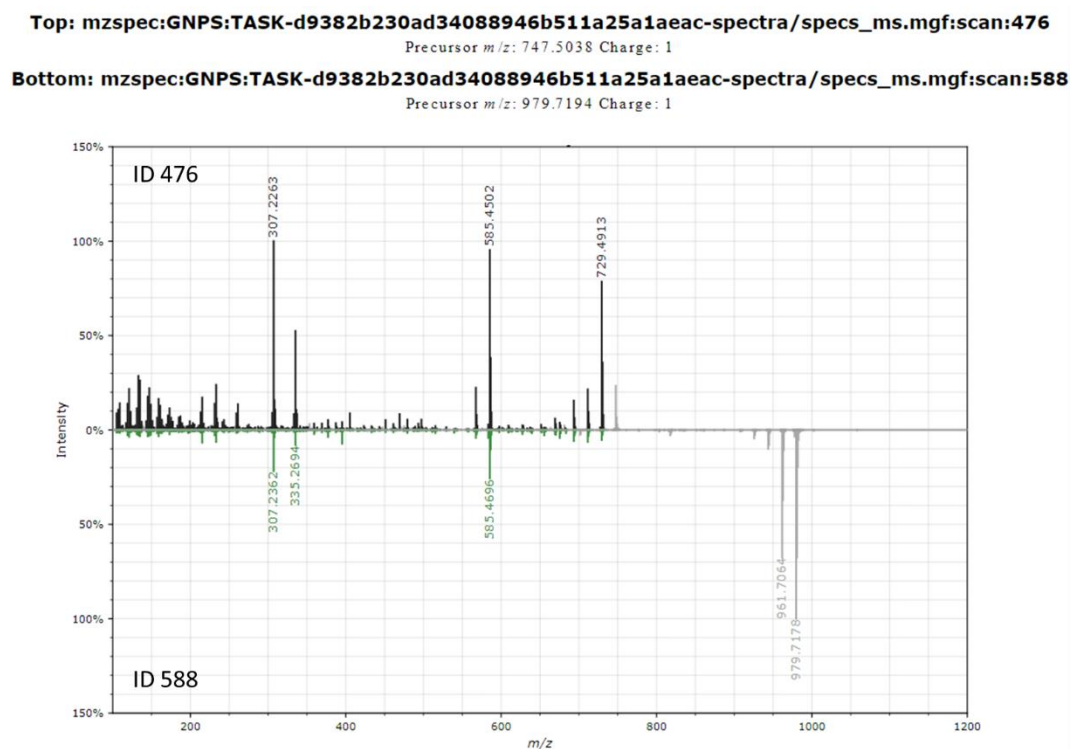


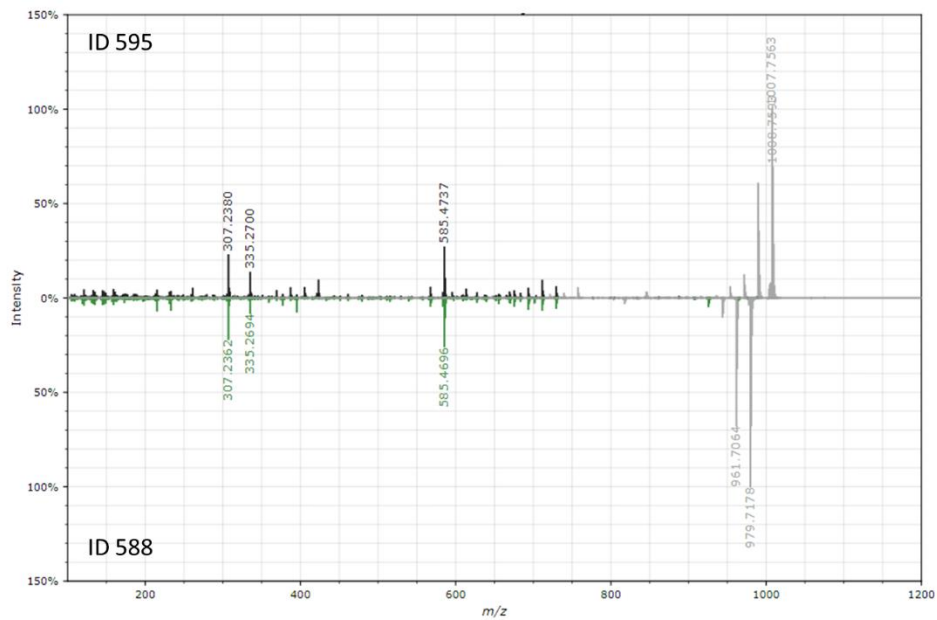
Figure S10: Mirror plot mass spectrum of compound ID 595 and ID 588

Top: mzspect:GNPS:TASK-d9382b230ad34088946b511a25a1aeac-spectra/specs_ms.mgf:scan:595

Precursor m/z : 1007.7538 Charge: 1

Bottom: mzspect:GNPS:TASK-d9382b230ad34088946b511a25a1aeac-spectra/specs_ms.mgf:scan:588

Precursor m/z : 979.7194 Charge: 1



Exploration phytochimique de plantes cultivées en Région Centre-Val de Loire : développement d'approches métabolomiques et intégration d'outils d'annotation

Résumé :

Depuis toujours, les plantes sont une source d'inspiration pour l'Homme qui a su en tirer profit pour la recherche de molécules actives. Les extraits végétaux sont complexes et présentent des activités variées : antioxydante, anti-âge, anti-inflammatoire etc. Cependant, l'identification des molécules impliquées dans ces activités peut être longue et laborieuse. Cette thèse vise à développer des méthodologies basées sur la spectrométrie de masse et le traitement des données pour explorer la phytochimie d'extraits de plantes d'intérêt biologique cultivées à l'échelle régionale.

Tout d'abord, l'étude des parties aériennes d'*Eclipta alba* a permis, grâce à des étapes de fractionnement, d'identifier les molécules actives sur trois enzymes impliquées dans le vieillissement cutané. Ensuite, l'étude des parties aériennes et racinaires de *Coleus forskohlii*, cultivé en aéroponie selon différentes modalités, a souligné l'intérêt de l'ajout de LED pour la production de forskoline et l'utilisation d'outils statistiques a mis en évidence les molécules impactées par cet éclairage. L'analyse des réseaux moléculaires a mis en avant les spécificités chimiques de chaque partie de la plante, soulignant l'intérêt des parties aériennes peu valorisées. Enfin, la mise en place de réseaux moléculaires multi-informatifs, associant différents outils d'annotation, a permis d'étudier la phytochimie de 10 espèces de *Clematis*, espèce ornementale dont la culture est fortement développée en région Centre-Val de Loire, avec un meilleur niveau de confiance. De plus, l'activité anti-inflammatoire déterminée pour certains extraits a pu être associée aux réseaux moléculaires afin de corréler l'activité et la composition phytochimique des extraits et ainsi guider l'identification de molécules actives.

Mots clés : phytochimie, spectrométrie de masse, métabolomique, réseaux moléculaires, ressources végétales locales

Phytochemical exploration of plants cultivated in Centre-Val de Loire region: development of metabolomic approaches and integration of annotation tools

Summary:

Plants have been a source of inspiration for humans, who have learned to benefit from them in the search for active molecules. Plant extracts are complex mixtures that can exhibit different activities: antioxidant, anti-aging, anti-inflammatory, etc. However, identifying the molecules involved in these activities can be time-consuming and laborious. The objective of this thesis is to develop methodologies based on mass spectrometry and data processing, while considering various problematics, to explore the phytochemistry of extracts of biological interest from plant cultivated locally.

First, the study of the aerial parts of *Eclipta alba*, highlighted, through fractionation steps, active molecules on three enzymes involved in skin aging. Then, the study of the aerial and root parts of *Coleus forskohlii*, cultivated in aeroponics according various cultivation conditions, underscored the benefits of adding LEDs for the production of forskolin and the use of statistical tools revealed the expression of molecules impacted by this lighting. The use of molecular network has also highlighted the chemical specificity of each part of the plant and showed the interest of the aerial parts, often under valorized. Finally, the establishment of multi-informative molecular network, combining various tools of annotation, allowed the study of 10 *Clematis* species, an ornamental species highly cultivated in the Centre-Val de Loire region, with a better confidence level. The anti-inflammatory activity determined for certain extracts was then correlated with the molecular networks to link the activity and phytochemical composition, thereby guiding the identification of actives molecules.

Keywords: phytochemistry, mass spectrometry, metabolomics, molecular network, local plant resources

