Bioassay-guided isolation and analysis of phytochemicals with endocannabinoid activity in *Achillea millefolium* L.

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Abstract

This thesis used bioassay-guided fractionation to investigate the bioactivity and phytochemistry of *Achillea millefolium* L. (yarrow, *Asteraceae*) roots, flowerheads, and leaves. We also provided a validated analytical high-pressure liquid chromatography with diode array detector (HPLC-DAD) method for detecting and quantitating yarrow metabolites.

A general literature review encompassed the biological, ethnobotanical, and bioactive diversity of *A. millefolium*, but revealed gaps in the knowledge surrounding yarrow's bioactivity with the endocannabinoid system (ECS). Chiefly, we investigated yarrow's potential to inhibit fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), two enzymes which degrade ligands of the ECS.

Crude and liquid extracts of yarrow plant parts Inhibited both FAAH and MAGL *in vitro*, and showed a higher selectivity towards FAAH – a trend upheld throughout bioassays. Organic fractions of roots and flowerheads were sub-fractionated and yielded isolated compounds that inhibited FAAH and MAGL. Root isolates were alkylamides (AKAs), and pellitorine had the lowest half-maximal inhibitory concentrations (IC_{50}) toward FAAH (26.45 µg/mL). Deca-2E,4E,8Z-trienoic acid piperideide was the most potent MAGL inhibitor (IC_{50}=48.92 µg/mL). Flowerhead isolates did not show activity towards FAAH but minimally did so towards MAGL. Dihydroparthenolide inhibited MAGL the most of all isolated compounds (IC_{50} 26.18 µg/mL). The accurate masses of all isolated compounds and the fragmentation patterns for the most inhibitory compounds were acquired using mass spectrometry (MS).

CQA chemical standards were used instead of pursuing their isolation. 1,3-dicaffeoylquinic acid (DCQA) inhibited FAAH and MAGL the most across all tested standards and isolates (54.35% at 5 µg/mL and 45.78% at 10 µg/mL for FAAH and MAGL, respectively).

Using reference standards and isolated compounds, we then validated an analytical HPLC-DAD method developed for detecting and quantitating metabolites in *A. millefolium*. Results showed that roots, flowerheads, and leaves collected in October had more metabolites accumulated than samples from May. Roots mainly contained AKAs, which were unique to them, dihydroparthenolide was only quantified in the flowerheads and was the most abundant of all (44.78 mg/g dry weight), and leaves contained the most chlorogenic acid (30.5 mg/g dry weight). Results showed good linearity, precision, and repeatability of the method.

This thesis provided novel data for the potential of yarrow plant parts, their fractions, and isolated compounds to inhibit FAAH and MAGL, reinforcing their high regard as a medicinal plant in the ethnobotany of North America. Coupled with an analytical method that can be applied to yarrow and other plant species showing similar phytochemistry, it will also help streamline future research into yarrow's metabolites.

Résumé

Cette thèse a utilisé le fractionnement bio-guidé pour étudier la bioactivité et la phytochimie des racines, capitules et feuilles d'*Achillea millefolium* L. (achillée millefeuille, Asteracées). Nous avons également fourni une méthode analytique validée de chromatographie liquide haute pression avec détecteur é réseau de diodes (HPLC-DAD pour la détection et la quantification des métabolites de l'achillée.

Une revue générale de la littérature a couvert la diversité biologique, ethnobotanique et bioactive d'*A. millefolium*, mais a révélé des lacunes dans la connaissance de la bioactivité de l'achillée en lien avec le système endocannabinoïde (SEC). Principalement, nous avons étudié le potentiel de l'achillée à inhiber l'amidase d'acide gras (FAAH) et la lipase monoacylglycérol (MAGL), deux enzymes qui dégradent les ligands du SEC.

Les extraits bruts et liquides des différentes parties de la plante d'achillée ont inhibé à la fois FAAH et MAGL *in vitro*, avec une plus grande sélectivité pour FAAH – une tendance maintenue tout au long des bioessais. Les fractions organiques des racines et des capitules ont été sous-fractionnées et ont produit des composés isolés inhibant FAAH et MAGL. Les isolats des racines étaient des alkylamides (AKAs), et la pellitorine avait la concentration inhibitrice médiane (IC_{50}) la plus faible pour FAAH (26,45 µg/mL). L'acide déca-2E,4E,8Z-triènoïque pipéridéide était l'inhibiteur le plus puissant de MAGL (IC50 = 48.92 µg/mL). Les isolats des capitules n'ont montré aucune activité envers FAAH mais ont légèrement inhibé MAGL. La dihydroparthénolide a montré l'inhibition la plus marquée de MAGL parmi tous les composés isolés (IC50 26,18 µg/mL). Les masses précises de tous les composés isolés et les schémas de fragmentation des composés les plus inhibiteurs ont été obtenus par spectrométrie de masse (MS).

Les standards chimiques d'acides caféoylquiniques (CQA) ont été utilisés au lieu de les isoler. L'acide 1,3-dicaffeoylquinique (DCQA) a inhibé FAAH et MAGL plus que tous les autres standards et isolats testés (54,35 % à 5 µg/mL et 45,78 % à 10 µg/mL pour FAAH et MAGL, respectivement).

En utilisant des standards de référence et des composés isolés, nous avons ensuite validé une méthode analytique HPLC-DAD développée pour la détection et la quantification des métabolites dans *A. millefolium*. Les résultats ont montré que les racines, les capitules et les feuilles collectés en octobre contenaient davantage de métabolites accumulés que les échantillons de mai. Les racines contenaient principalement des AKAs, qui leur étaient uniques, la dihydroparthénolide n'était quantifiée que dans les capitules où elle était la plus abondante de tous les métabolites (44,78 mg/g de poids sec) et les feuilles contenaient le plus d'acide chlorogénique (30,5 mg/g de poids sec). Les résultats ont montré une bonne linéarité, précision et répétabilité de la méthode.

Cette thèse fournit des données inédites sur le potentiel des différentes parties de la plante d'achillée, de leurs fractions et des composés isolés pour inhiber FAAH et MAGL, renforçant leur haute estime en tant que plante médicinale dans l'ethnobotanique de l'Amérique du Nord. Associée à une méthode analytique applicable à l'achillée et à d'autres espèces végétales présentant une phytochimie similaire, elle contribue également à faciliter les futures recherches sur les métabolites de l'achillée.

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Δ9-THC	Δ9-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
AEA	Anandamide
AKA	Alkylamide
AMC-AA	7-amino-4-methyl coumarin-arachidonamide
BBB	Blood-brain barrier
CB	Cannabinoid receptor
CGA	Chlorogenic acid
COX	Cyclooxygenase
CQA	caffeoylquinic acid derivative
DAD	Diode array detector
DCQA	Dicaffeoylquinic acid
DMSO	Dimethyl sulfoxide
ECS	Endocannabinoid system
ES+	Electrospray ionization
FAAH	Fatty acid amide hydrolase
HPLC	High-pressure liquid chromatography
HRes	High resolution
IC_{50}	Half-maximal inhibitory concentration
IL	Interleukin
LOD	Limit of detection
LOQ	Limit of quantitation

LOX	lipoxygenase
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase
MS	Mass spectrometer
m/z	Mass to charge ratio
NMR	Nuclear magnetic resonance
R_t	Retention time
SEM	Standard error of the mean
STL	Sesquiterpene lactone
Subsp.	Subspecies
TFA	Trifluoroacetic acid
TOF	Time-of-flight
Var.	Variety

CHAPTER 1 – GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Achillea millefolium L. (*Asteraceae*), commonly known as yarrow, is a part of the Daisy family of flowering plants, often referred to as the Sunflower or Marigold family, comprised of over 1,620 genera, 23,600 species, and 13 subfamilies, with some of its most well-known species being sunflower, chicory, and lettuce (Tamokou et al. 2017; Nikolić et al. 2015). It is the largest family of vascular plants. Once referred to as the *Compositae* family, since each flower is a composite of smaller flowers, its current scientific name is derived from the Greek word Aster for its star shape. *Achillea* comes from the Greek myth of Achilles, who was said to have used yarrow to help treat his wounds, as did others in ancient Greece (Thieret, 2001), and *millefolium* is the Latin word for "a thousand leaves" due to their fern-like bi- or tripinnately divided leaves.

In North America, the *Asteraceae* family has played a central role in the ethnobotany of many indigenous communities, made evident through the relationship between those communities and the medicinal and cultural uses of the native and introduced flora, including those of the Achillea genus (Moerman 2003; Applequist & Moerman 2011). Yarrow contains a diverse array of bioactive phytochemicals that contribute to its numerous health benefits, such as CQAs, flavonoids, and AKAs found in various quantities or that are unique to the plant's roots, flowerheads, stems, and leaves. The stated classes of secondary metabolites contribute to yarrow's ethnomedicinal uses to treat colds and flu, digestive issues, and wounds, among other symptoms and conditions (Suzuki et al. 2006; Caltagirone 2000; Ku et al. 2013). Over recent decades, some compounds included in yarrow's phytochemistry have shown bioactivity, like anti-inflammatory and neuroprotective potential. Their activity with components of the human ECS, whose receptors and enzymes have been targeted for therapy in recent decades, remains

unknown. A former Master's student from the Harris lab, Alexandra Kachura, found evidence supporting ECS modulation by yarrow roots, flowerheads, and leaves.

This thesis aimed to broaden our knowledge of yarrow's therapeutic potential by testing the inhibition potential extracts of plant material have towards enzymes of the ECS through bioassay-guided fracitonation, and identify some of the most potent metabolites. We also had the goal of validating a developed HPLC-DAD method to detect and quantify secondary metabolites from different plant parts (leaf, flowerhead, root) to provide a reliable and reproducible analysis of plant extracts.

1.2 Literature Review

1.2.1 Biology of Yarrow

Yarrow is a part of the *Anthimideae* tribe, sometimes termed the *Achillea* or *Chrysanthemum* complex, which includes 111 genera and over 1,800 species, mainly spread out across the Mediterranean, Eurasia, Southern Africa, and Central and Eastern Asia (Oberprieler et al. 2007). It is a perennial herb that typically blooms from June to November. Around 200 species of *Achillea* exist worldwide, and three are native to Canada: *A. alpina,* and *A. borealis*, and *A. ptarmica*. *A. millefolium*, studied in this thesis, was introduced from Eurasia (Vascan Canadensys, 2025). This perennial herb is anchored to the ground by several small, horizontal rootstocks (rhizomes). It can reach 0.2 to 1 m in height, with pointed, pedunculate, bipinnate or tripinnate leaves able to reach 5-20 cm in length, and has flowering heads of varying colours, such as white, red (red velvet), or yellow (golden yarrow), with a diameter of 3-5 cm (Ijaz et al. 2020). These flat-topped bunches of flowers emit odours or olfactory queues that attract flies and beetles, allowing yarrow to contribute to pollinator networks (Larue et al. 2016). Additionally,

the plant is highly drought resistant and can grow in dryer or well-developed soil, but requires an optimal temperature of 18 ºC - 24 ºC and sunlight for germination (Lakshmi et al. 2011).

In nature, The *A. millefolium* aggregate has three subspecies (subsp): subsp. *millefolium* (white flowers), subsp. *alpestris* (pinkish flowers) (Wimm & Grab.), and subs. *ceretanum* Sennen. (large white flowers) (Ali et al. 2017). Subsp. *millefolium* was collected and studied in this thesis and has three varieties (var): *A*. *m*. subsp. *m*. var. *millefolium*, *A*. *m*. subsp. *m*. var. *borealis*, and *A*. *m*. subsp. *m*. var. *rubra.* The aggregate is a monophyletic clade with diverse polyploid complexes, leading to plants and populations that vary genetically and morphologically between regions. Varieties from the aggregate can be found in their basal diploid form in Eurasia, and others can reach 4x, 6x, and 8x ploidy levels, resulting from hybridization, polyploidization, and evolution of the species over time in diverse ecological environments (Guo et al. 2004, 2005, 2008, 2012; Ehrendorfer & Guo 2006). Most varieties can be found in grasslands and open forests. The *A. millefolium* aggregate is regarded as the "crown group" of the genus for its most considerable evolution from its ancestral state (apomorphism), polymorphism, polyploidy, and widespread presence (Guo et al. 2005). No wonder many Indigenous peoples have documented the medicinal uses of this plant for generations.

1.2.2 Ethnobotany of Asteraceae and yarrow

American botanist John Harshberger first published the term "ethnobotany" in an 1896 article written for the Botanical Gazette, in which he described it as a way to study the interactions between plants and humans, the distribution of plant species over time and how the knowledge Indigenous groups have of using plants can be of use for manufacturing (Harshberger, 1896). In the present day, ethnobotany has evolved into an interdisciplinary field, weaving botany, anthropology, pharmacology, agriculture, and history (among other fields) together to better understand the complex relationships between people and plants in their environment, from species identification to medicinal applications, thanks to plant knowledge collected over centuries (Balick, 2020). Whereas "manufacturing" and other technological and economic objectives persist, ethnobotanical studies often focus on individual and collective knowledge about plants and their integration into culture and ceremonial customs. While ethnobotanical research usually considers Indigenous Traditional Knowledge and folklore of previous generations, it may explore plant-human relationships within or across cultures and time. According to the First Nations Health Authority in Canada, the traditional use of medicinal plants refers to their health practices, approaches, and knowledge and how they are used in ceremonies, medicines or therapies (FNHA, 2024).

As the largest family of flowering plants in North America, the *Asteraceae* is also the most widely used family of medicinal plants among Indigenous peoples across the continent (Moerman, 1991). Past ethnobotanical meta-analyses of *Asteraceae* showed that it is among the most highly selected families in terms of medicinal uses not only in North America but around the world (Kachura & Harris 2018; Moerman & Pemberton, 1999; Leonti et al. 2003; Phumthum & Barfod, 2019; Hussain, 2024). The *Anthemideae* tribe, like the *Asteraceae* more broadly, is

specifically over-selected for medical uses in North America, particularly *Achillea* and *Artemisia* species, which appear preferentially used for nearly all medicinal uses but mostly markedly for pulmonary and orthopedic uses (Kachura & Harris, 2018). This observation aligns with Applequist & Moerman (2011), who referred to yarrow as a panacea, meaning it can remedy many diseases.

Species of the genus *Achillea* or, simply, the *Achillea* complex, have been used by indigenous communities in North America from coast to coast to coast thanks to its important medicinal properties and spiritual significance (Mulligan & Bassett, 1959; Coxe 1915; Barriga 1992; Bussman 2007). Plants that unrelated indigenous groups use for the stated purposes are more likely than not to be effective since it is statistically unlikely that multiple groups would randomly adopt a medicinal plant (Duke, 1986; Duke & Ayensu, 1985). Some communities, such as the Navajo, consider yarrow a "life medicine" since it can be prepared as an ointment and applied externally on cuts and wounds for its antiseptic properties; it is also used in tea preparations to soothe symptoms of the common cold, gastrointestinal issues, and inflammation (Moerman 1998; Chandler et al. 1982).

Yarrow has also played a spiritual role in the lives of Indigenous peoples. It has been used in ceremonial practices as a protective charm against negative energies, cleansing the spirit and restoring balance to the participants of the ceremony (Moerman, 1998). The strong bond between physical and spiritual healing that yarrow continues to provide for many reflects the knowledge transcending time and underlines yarrow's cultural and holistic significance at the heart of complementary and alternative medicine. Interactions between Indigenous Knowledge holders and scholars have allowed research into yarrow's medicinal uses, phytochemistry and pharmacology.

1.2.3 Phytochemistry and related bioactivity of yarrow

Metabolites produced by plants can vary widely across plant taxa. Still, primary metabolites mainly contribute to species' growth and reproduction. In contrast, secondary metabolites help protect the plant's health, including defence mechanisms against herbivory and pathogens, outcompeting neighbouring plant species for resources, and attracting pollinators (Bocso & Butnariu, 2022). Phytochemical groups may even be organ-specific and appear at distinct plant lifecycle periods. Yarrow produces a variety of secondary metabolites, many of which are bioactive and can contribute to the plant's medicinal effects. Most notably, yarrow produces CQAs, flavonoids, sesquiterpene lactones (STLs), and AKAs.

1.2.3.1 CQAs, flavonoids, and STLs from yarrow

CQA and flavonoids in yarrow are needed for the plant organism's response to environmental stressors. Both are classes of phenolic compounds and are mainly found in the aerial parts of the plant, especially abundant in the flowerheads and leaves (Benetis et al. 2008a, 2008b). Our lab also observed this in yarrow samples harvested at the end of the growing season (October) (Kachura & Harris, 2018).

CQAs comprise caffeic acid and quinic acid esters, including DCQAs formed from the esterification of two caffeic acids and quinic acid. The most common CQA in the plant kingdom and yarrow is chlorogenic acid (CGA), followed by its isomers and derivatives (Woźniak et al. 2021). Beyond CGA, some common DCQA isomers in *A. millefolium* with significant bioactivity are chicoric acid, 1,3-DCQA, 1,5-DCQA, 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA (Innocenti et al. 2007; Vitalini et al. 2011; Benedek et al. 2007) (Table 1-1).

Flavonoids are abundant in the plant kingdom and usually consist of 15 carbon atoms arranged in three rings that can be glycosylated, methylated, or aromatized, which gives them

chemical and functional diversity (Noel et al. 2005). They are commonly found in everyday fruits, vegetables, and plants. Some of the most abundant flavonoids in yarrow include flavonols (quercetin, kaempferol), flavones (apigenin, luteolin), and their glycosides (innocent et al. 2007; Vitalini et al. 2011; Benedek et al. 2007) (Table 1-1).

1.2.3.2 Bioactivity of CQAs and flavonoids *in planta*

Both classes of phenolic compounds are sequestered in plant cell vacuoles and help the plant respond to abiotic and biotic factors (Marrs et al. 1995; Marinova et al. 2007; Li et al. 2019). Their production can be upregulated to help defend plants against abiotic stressors like osmotic stress caused by drought or variable soil salinity, exposure to UV radiation, lipid peroxidation, and low, non-freezing temperatures (Christie et al. 1994; Grace et al. 1998, Tegelberg et al. 2004, Rice-Evans et al. 1997; Tamagnone et al. 1998; Salomon et al. 2021; Landry et al. 1995; Schulz et al. 2016; Havaux & Kloppstech 2001; Agati et al. 2012; Sueishi et al. 2014; Martinez et al. 2016). CQAs and flavonoids also help defend against biotic stressors with their antiparasitic, antibacterial, antifungal, and antioxidant properties, as well as their role in regulating plant tissue senescence (Moglia et al. 2008; Vitalini et al. 2011; Harrison et al. 2003, 2008; Peterson et al. 2005; Candan et al. 2003; Szalma et al. 2005; Cole 1984; Dai & Mumper, 2010; Tamagnone et al. 1998). These phenolic compound groups also promote plant growth by fixing nitrogen through symbiotic relationships with nitrogen-fixing bacteria and have allelopathic potential in influencing the development of the plant host organism and that of neighbouring plants in competition for the same resources (allelopathy) (Niazipoor et al. 2024; Dragoeva et al. 2015; Harborne 1967; Harborne 1976; McClure 1979; Peters et al. 1986). Evidence suggests that CGA and some flavonoids can influence seed germination and

impermeability (Williams & Hoagland 1982; Zhou et al. 2010). Additionally, varying levels of anthocyanins and flavones, subclasses of flavonoids, in the flowers give them different pigmentation, such as red, white, and yellow, that, along with the volatile fragrance metabolites, attract insects as agents for pollination (Narbona et al. 2021). Lastly, similarly to CQAs and flavonoids, STLs can be found in the flowerheads and leaves, where they act as antimicrobial agents to reduce oxidative stress (Aljančić et al. 1999; Hausen et al. 1991; Rauchensteiner et al. 2004; Slobodan et al. 1999). STLs are also antifeedants to chewing insects and birds and can stimulate seed germination (Fraga et al. 2021; de Luque et al. 2000).

Flavonoids and other plant phenolics have garnered much attention over recent decades since they are the most abundant antioxidants in our diet, with pharmacological evidence in favour of their therapeutic potential. As presented in Tables 1-1 and 1-2, the CQAs, flavonoids, and pellitorine (AKA), produced by yarrow possess a range of bioactivities, many of which may contribute to the species medicinal properties and panacea-like traditional use.

1.2.3.3 AKAs from yarrow

Another biologically influential class of secondary metabolites in *Asteraceae*, including *A. millefolium*, are AKAs. Compounds from this class of fatty acid amides are composed of a long-chain fatty acid that can vary in degrees of saturation and length with an amide group (Greger, 1984). The *Achillea* genus contains many AKAs, like the well-known anacycline and pellitorine (Figure 1-1), two isobutylamides with several analogs throughout the Anthemideae. It is among the genera of *Asteraceae* with the most AKAs, along with *Echinacea* (echinacein), *Acmella* (spilanthol), *Heliopsis* (capsaicin), and *Zanthoxylum* (sanshool) (Veryser et al. 2017; Boonen et al. 2012a; Hohmann et al. 2011; Jacobson 1954; Chen et al. 2022; Cheng et al. 2015;

Molina-Torres et al. 1996, 1999; Guetcheung et al. 2018). Different types of AKAs have been identified in yarrow, such as isobutylamides, methylamides, phenylethylamides, piperidides and piperideides (Veryser et al. 2017; Boonen et al. 2012b). Our lab observed the highest AKA content in yarrow roots, followed by flowers, but only towards the end of the growing season (Kachura, 2018).

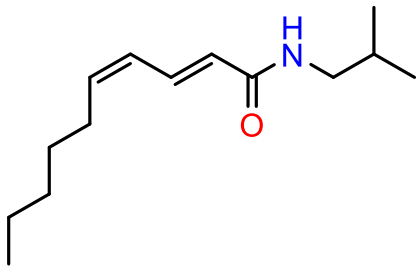

Figure 1-1. Structure of the AKA pellitorine, an isobutylamide produced by yarrow and other *Achillea* species.

1.2.3.4 Bioactivity of AKAs *in planta*

AKAs are primarily found in the roots and help promote plant growth (Ramírez-Chávez et al. 2004). They can act as lipid signal molecules to influence genetic mechanisms involved in signal perception and root system architecture in a dose-dependent manner. This lets them influence primary root and lateral root growth and root hair elongation, as seen in *Arabidopsis thaliana* L. (Brassicaceae) (Ramírez-Chávez et al. 2004). AKAs also play a role in the pathways that influence root growth, like the nitric oxide, cytokinin, and jasmonic acid signalling pathways, and can reduce leaf wilting effects caused by plant pathogens (Ramírez-Chávez et al. 2004; Morquecho-Contreras et al. 2010; Zandonadi et al. 2019; Méndez-Bravo et al. 2011; González-Morales et al. 2015). They can also stimulate the accumulation of plant biomass (Ortíz-Castro et al. 2009) and possess antibacterial, antifungal, antiviral, and insecticidal, activity (Molina-Torres et al. 1999, 2004; Ramírez-Chávez et al. 2004; Hentrich et al. 2013; Simas et al. 2013; López-Bucio et al. 2006, 2007).

1.2.4 Pharmacology of yarrow and its secondary metabolites

The previously listed phytochemical groups found in yarrow, combined or individually, can contribute to yarrow's widely applicable pharmacological profile. Alcohol extracts of yarrow have shown a diverse array of pharmacological activity (Tables 1 to 3). The diversity and quantity of the secondary metabolites mentioned in the previous section increase the potential for bioactivity and interaction among compounds (Mohamed et al. 2018; Li et al. 2023; Dias et al. 2013).

Several species of *Asteraceae* have been investigated for their therapeutic potential based on ethnobotanical data, leading to many well-known and globalized medicinal plants. For

example, *Tanacetum parthenium* L. (feverfew) reduces migraine-related pain intensity, nausea, and vomiting (Palevitch et al. 1988). *Arnica montana* L. (wolf's bane) was just as effective as the non-steroidal anti-inflammatory drug ibuprofen in ameliorating pain and hand function of patients with osteoarthritis of interphalangeal joints of hands. *Echinacea spp*. demonstrated immunomodulatory effects, enhancing immune function when taken as a tea to reduce the severity of respiratory infections or colds (Barrett, 2003).

CQAs and flavonoids have shown a wide range of pharmacological activity, both *in vivo and in vitro*, summarized in Tables 1 and 2 for CQAs and flavonoids, respectively. Some of their activities include anticancer, antidiabetic, and anti-inflammation, and some have even shown the ability to target the integrase of the Human Immunodeficiency Virus to stop it from integrating into the host chromosomes. (Dias et al. 2013; Vitalini et al. 2011; Benedek et al. 2007; Pereira et al. 2018). Pharmacological evidence also supports STLs as having hypolipidemic, anticarcinogenic, and anti-inflammatory potential (Chadwick et al. 2013). Additionally, AKAs from the *Asteraceae* are pharmacologically active, such as those in *Echinacea*, which displayed immunostimulant activity, and those in *Otanthus maritimus* L., *Heliopsis helianthoides*, L., and *Achillea pyrethrum* L. showing anti-rheumatic activity (Gertsch 2004; Hajdu et al. 2014; Ávila-Gálvez et al. 2024; Singh et al. 2020; Cabral et al. 2013;). AKA from certain species like *Zanthoxylum bungeanum* M. (*Rutaceae*) showed neurotrophic mimicking potential (i.e. aiding cell proliferation and differentiation in the nervous system) (Gertsch et al. 2004; Ruiu et al. 2013; Hajdu et al. 2014; Wang et al. 2017).

While the activity of most AKAs found in yarrow remains unknown, pellitorine has demonstrated antiprotozoal, antibacterial, antithrombotic, anti-cancer and vasoprotective effects (Table 1-2). As observed in our lab by Alexandra Kachura (2018) and Rui Liu (2019) in their

graduate theses, both *A. millefolium* and *Echinacea spp.* extracts containing suspected CQAs and AKAs elicited anti-inflammatory and immunomodulatory potential and inhibited enzymes that degrade signalling lipids of the ECS. Whereas Liu (2019) identified Echinacea CQAs and AKAs as active compounds the active metabolites in yarrow extracts have yet to be characterized (Kachura, 2018). Among more recent pharmacological studies on the signalling pathways of AKAs, evidence continues to show that the interactions between AKAs and the ECS lead to wide-ranging pharmacological activity.

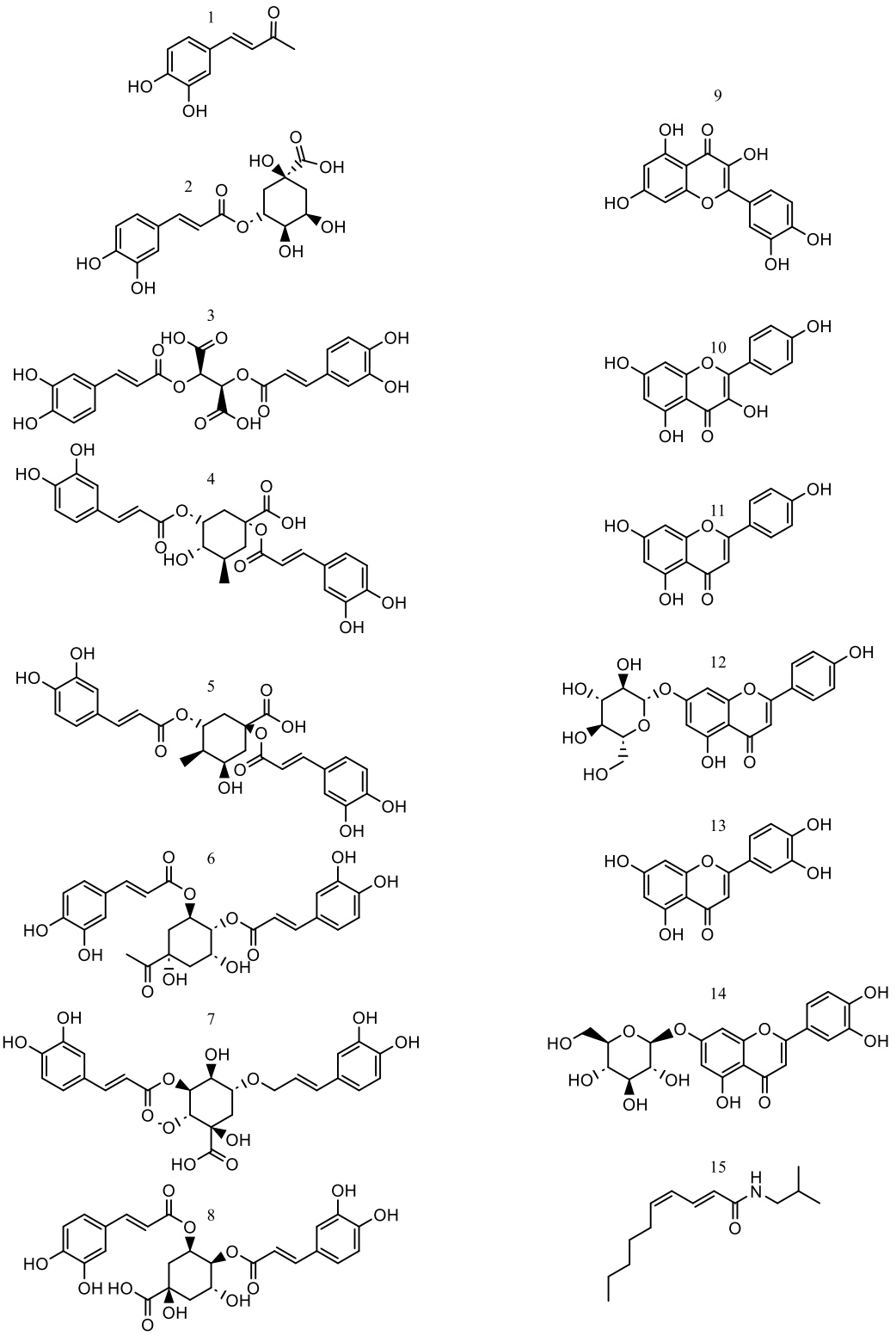

Figure 1-2. CQAs (1-8), flavonoids (9-14), and pellitorine (15) from yarrow. In order: (1) caffeic acid, (2) CGA, (3) chicoric acid, (4) 1,3-DCQA, (5) 1,5-DCQA, (6) 3,4-DCQA, (7) 3,5-DCQA, (8) 4,5-DCQA, (9) quercetin, (10) Kaempferol, (11) apigenin, (12) apigenin-7-*O*-glucoside, (13) luteolin, (14) luteolin-7-*O*-glucoside, (15) pellitorine.

Table 1-1. Pharmacology of caffeic acid and CQAs in *A. millefolium*.

Compound	Oxidation	Inflammation	Cancer	Infection	Mutagenicity	Human immunodeficiency virus
1	Gaspar et al 2009	Li et al 2012; Zhao et al 2008	Kanimozhi & Prasad 2015; Kurata et al 2007	Zhu et al. 2004	Yamada et al. 1996	N/F
2	Venditti et al 2015	Zhao et al 2008	Huang et al. 2019	Zhu et al 2004	Yoshimoto et al 2002	N/F
3	Ma et al 2021	Sadeghabadi et al 2019	Sun et al 2019	Abd-Nikfarjam et al. 2018	N/F	Nobela et al. 2018
4	Danino et al. 2009	Kim et al. 2022	Zhou et al 2020	Zhu et al 2004	Erikel et al. 2019	Slanina et al. 2001
5	Cao et al. 2010	Bezáková et al 2007	Etemadi et al. 2020	Zhu et al 2004	N/F	Robinson et al. 1996
6	Zhang et al. 2014	N/F	Mishima et al. 2005	Zhu et al 2004	Yoshimoto et al 2002	Robinson et al. 1996
7	Hong et al 2015	Hong et al 2015; Park et al. 2022	Mishima et al. 2005	Zhu et al 2004	Yoshimoto et al 2002	Robinson et al. 1996
8	Yang et al 2013	Jang et al. 2021	Mishima et al. 2005	Zhu et al 2004	Yoshimoto et al 2002	Robinson et al. 1996

N/F = Not found

Table 1-2. Pharmacology of flavonoids and the AKA pellitorine in *A. millefolium*.

Compound #	**Oxidation**	**Inflammation**	**Cancer**	**microbial**	**Mutagenicity**	**Human immunodeficiency virus**
9	Hanasaki et al 1994; Lim et al 1998	Manjeet & Gosh 1999	Yang et al. 2017; Hamidullah et al. 2015	Yang et al. 2017; Wang et al 2018	Gupta et al. 2010	Gatto et al. 2002
10	Feng et al 2017; López-Sánchez et al 2007	Park et al 2012	Kashafi et al 2017	Escandón et al. 2016	Choi et al. 1994	Min et al. 2001
11	Zhao et al 2013	Bezáková et al 2007	Silvan et al 2011	Nayaka 2014	Birt et al. 1986	Sanna et al. 2021
12	Wang et al. 2020	Wang et al. 2020	Liu et al. 2020	Sezen et al. 2023	Gulluce et al. 2015	N/F
13	Chen et al 2008	Xagorari et al 2001	Lim et al. 2007	Qian et al. 2020	Choi et al. 1994	Sanna et al. 2021
14	Zang et al. 2016	Bezáková et al. 2007	Hwang et al. 2013	Sezen et al. 2023	Choi et al. 1994	Sanna et al. 2021
15	Singh et al. 2020	Ku et al. 2014	Ee et al. 2010	Althaus et al. 2014	N/F	Rotich et al. 2022

N/F = Not found

1.2.5 The ECS

The ECS has been reported to exist in all Chordata (animals with backbones) (McPartland et al. 2006) and even in primitive invertebrates (De Petrocellis et al. 1999). It spans the body and is critical to mediating pain, inflammation, appetite, mood and growth (McCallum & Russo 2018; Di Marzo et al. 1998). The ECS consists of cannabinoid receptors (CB_1 and CB_2), their interacting endogenous ligands (endocannabinoids), and metabolic enzymes for endocannabinoid synthesis and degradation.

CB_1 is abundant in the central nervous system, and CB_2 is mainly found in immune cells (Herkenham 1991; Kendal & Yudowski 2017; Glass et al. 1997; Howlett et al. 2002; Klein, 2005). Both are G-protein-coupled protein receptors, named after their exogenous ligand, the phytocannabinoid Δ9-tetrahydrocannabinol (Δ9-THC) from *Cannabis sativa* L. (*Cannabaceae*). CB_1s are associated with emotional regulation, stress responsiveness, and behaviour regarding memories, fear, and anxiety (Mackie, 2005; Lutz, 2007; Rubino et al. 2008; Marsicano et al. 2002), helping maintain emotional homeostasis. CB_2s modulate immune and inflammatory responses and mediate analgesic effects in peripheral tissues (Hsieh et al. 2011; Xuc et al. 2014; Buckley et al. 2000). Both types of receptors exert their effects through their endogenous ligands.

Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) are eicosanoids with a long-chain fatty acid arachidonic acid containing an amide, in the case of AEA, or a glycerol, as with 2-AG. They are considered retrograde messengers since, in the nervous system, they are liberated from the postsynaptic dendritic spine, travel across the synaptic cleft, agonize CB_1s on the presynaptic terminal of the axon and can inhibit neurotransmitter release (Südhof & Malenka 2008).

By being a partial (AEA) or full (2-AG) agonist of CBs (Tsuboi et al. 2018), the mediation of their activity exerts their therapeutic effects. AEA and 2-AG have several documented effects across the body. For instance, the lack of local cannabinoid signalling near tumours in bone can increase hyperalgesia and increased sensitivity to pain (Khasabova et al. 2008). Endocannabinoids like AEA are also upregulated to provide antinociception, and blocking CBs can lead to increased pain related to inflammation (Michalski et al. 2007; Nakajima et al., 2006). In autoimmune diseases, 2-AG counteracts inflammatory bowel disorders, significantly reducing systemic and central inflammation (Alhouayek et al. 2011). Given the role of the ECS in energy expenditure homeostasis, ligand degradation by FAAH and MAGL could reduce the ability of the ECS to sense deficient energy balance, gastrointestinal load, and can vary one's incentive to eat (Di Marzo et al. 2014). Also, excessive variation in endocannabinoid levels is associated with energy homeostasis imbalance in the liver, adipose tissue, pancreas, and skeletal muscles (Silvestri et al. 2011). Therefore, ligands and receptors of the ECS play a critical role in managing bodily functions while counteracting many diseases to maintain homeostasis.

When AEA and 2-AG are taken up by the cell, fatty acid-binding proteins carry them to the endoplasmic reticulum. There, they are cleaved by their catabolic enzymes: the serine hydrolases FAAH and MAGL, targeting AEA and 2-AG, respectively (Figure 1-2). Catabolism of AEA and 2-AG yields arachidonic acid (AA) and ethanolamine for AEA or glycerol for 2-AG (2014; Hajdu, 2014). AA is an essential precursor of the eicosanoids of pro-inflammatory pathways, like prostaglandins and leukotrienes, in the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, respectively. The primary source of AA for those pathways comes from LOX,

cytochrome P450, and phospholipid catabolism by phospholipase A2 (Wang et al. 2021; Balsinde et al. 2002).

The pharmacology of AKAs isolated from *A. millefolium* is greatly lacking, except the widely studied pellitorine, an isobutylamide with therapeutic potential presented in Table 2.

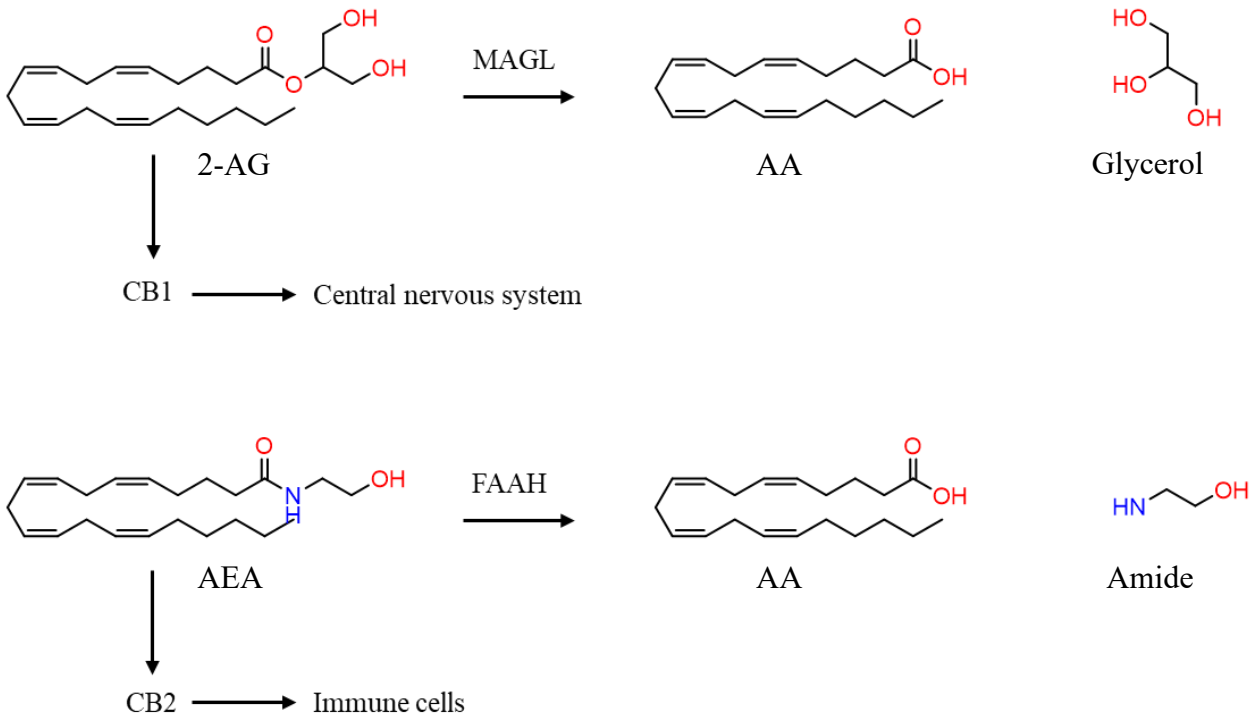

Figure 1-3. 2-AG and AEA degradation by MAGL and FAAH, respectively, and their products.

That being said, AKAs isolated from *Echinacea angustifolia* and *purpurea* have been shown to bind to CB_1s and CB_2s and have a higher affinity for the latter, while most AKAs seemed to interact with the former more (Woelkart et al. 2005; Hohmann et al. 2011). AKAs seem to exert their therapeutic effect upon gene expression, immunomodulation, and anti-inflammation by acting as agonists of CB_2 (Gertsch et al. 2004; Raduner et al. 2006; Chicca et al. 2009; Haller et al. 2010, 2013). AKAs, along with flavonoids, are a part of the "full cannabinoid spectrum,"

capable of modulating the ECS and promoting the "entourage effect," meaning increased therapeutic effects as more than one group is administered together (Cavalli et al. 2021; Ben-Shabat et al. 1998). Kachura (2018) showed that plant parts from yarrow containing CQAs, flavonoids, and AKAs did inhibit FAAH and MAGL and modulated CBs *in vitro*, and these findings were supported by Rui 2019 from plant extracts with similar phytochemicals in echinacea. The inhibitory potential for FAAH and MAGL of certain secondary metabolites from yarrow was further investigated in this work.

1.3 Research Objectives

The main goal of this thesis was to contribute to deepening our knowledge of *A. millefolium*'s bioactive secondary metabolites, to which it owes some of its bioactivity, specifically by testing extracts for their inhibition of FAAH and MAGL.

Chapter 2 investigates yarrow's inhibitory potential using crude extracts of yarrow roots, flowerheads, and leaves, their aqueous and organic liquid fractions and sub-fractions, and finally, their isolated compounds. This chapter also covers tentative compound identification of some isolated compounds from yarrow extracts thanks to high resolution MS.

The third chapter details the development and validation of a novel HPLC method for the detection and quantitation of metabolites from different compound classes in *A. millefolium* that are bioactive. Commercially available standards and isolated compounds were run on HPLC-DAD in tandem with plant extracts for compound identification and quantitation and to ensure the method employed remained rugged and robust throughout replicates. Method validation fortifies the scientific integrity of results and minimizes the risk of errors or inaccuracies other researchers may face and provides them with a tool to do other research

related to plants with a similar phytochemistry to that of yarrow. A flowchart is presented in Figure Z to help visualize the steps and experiments done throughout this project.

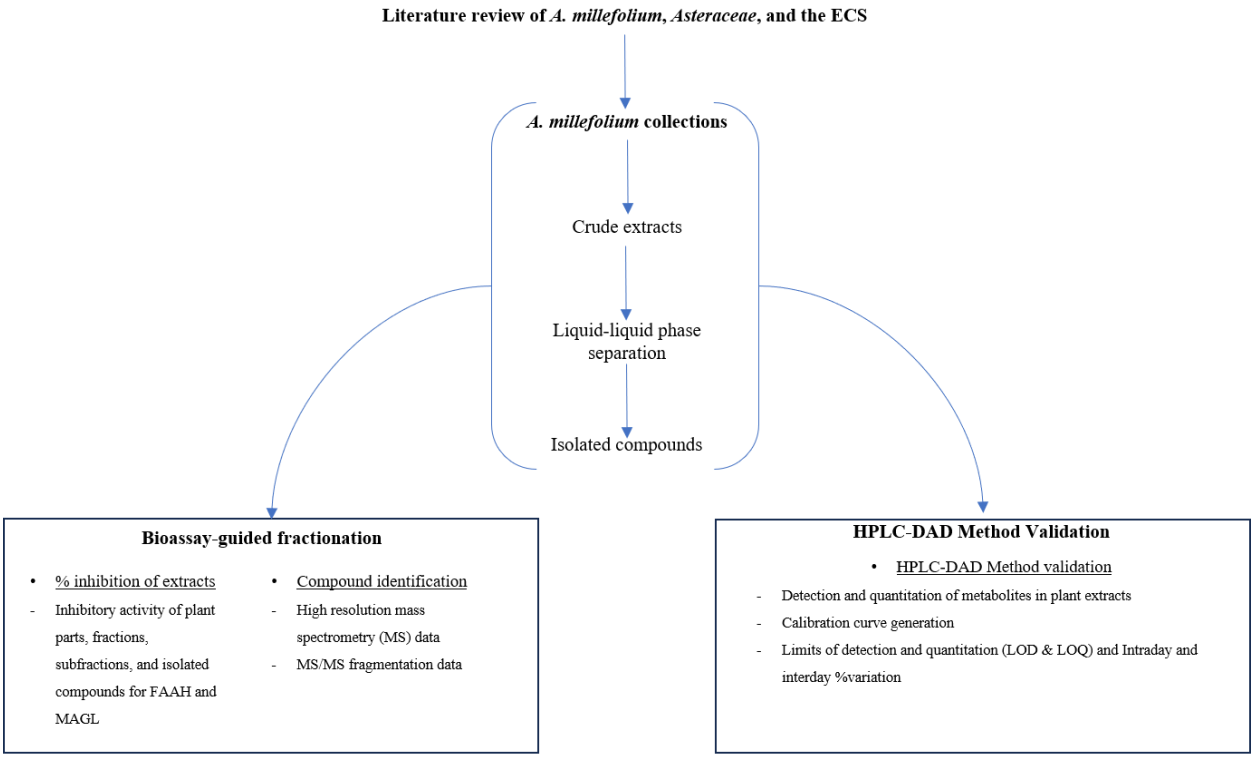

Figure 1-3. Methods and types of research carried out in this thesis. Steps are categorized by analytical and pharmacological research into *A. millefolium*.

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CHAPTER 2: INHIBITORY POTENTIAL OF ACHILLEA MILLEFOLIUM L. ON FAAH AND MAGL

2.1 Abstract

Achillea millefolium L. (yarrow, *Asteraceae*) has an important place in the ethnobotany of North America and is regarded as a panacea for medicinal uses. Recent data show that hhydroethanolic extracts of yarrow root and aerial parts inhibited FAAH and MAGL, which degrade the endocannabinoids AEA and (2-AG), respectively. We used bioassay-guided fractionation to successfully identify active metabolites in yarrow roots, flowerheads, and leaves. Liquid-liquid extraction of crude extracts revealed that organic fractions were more active towards both FAAH and MAGL, and that root and flowerhead was more active than leaf extracts. Sub-fractions of the organic root fraction revealed strong inhibition of FAAH, leading to the isolation of pellitorine (IC_{50} = 26.45 µg/mL) and deca-2E,4E,8Z-trienoic acid piperideide (IC_{50} =28.95 µg/mL). Assay-guided fractionation of the organic flowerhead led to the isolation of dihydroparthenolide, the most potent MAGL inhibitor identified in yarrow (26.18 µg/mL). We confirmed the identities of these active metabolites using HPLC-DAD and time-of-flight mass spectrometry with electrospray ionization (TOF MS ES+). Since all aqueous fractions (root, flowerhead and leaf) inhibited both enzymes to similar degrees and shared similar phytochemistry dominated by DCQAs, we tested commercial standards of DCQAs and, while all compounds inhibited both enzymes dose-dependently, 1,3-DCQA inhibited FAAH and MAGL most potently. Our results support novel ECS modulating potential of whole extracts of yarrow plant parts down to isolated compounds that help deepen our understanding of such a vital medicinal plant that has stood the test of time as a staple in ethnomedicine.

2.2 Introduction

Achillea millefolium L. (*Asteraceae*), commonly known as yarrow, has a rich history as a medicinal plant. It has an important role in the ethnobotany of North America with its uses in the traditional medicine and cultural customs of Indigenous Peoples. Thanks to the knowledge imparted by Indigenous and Traditional knowledge holders with researchers, there are now nearly 400 reported medicinal uses for yarrow, including treatment for respiratory illnesses, digestive problems, swollen tissues, toothache, and skin problems (Moerman, 1998, 2003; Chandler et al. 1982). Accordingly, *A. millefolium* is considered a panacea (Applequist et al. 2011), meaning it can help remedy many diseases.

Yarrow produces a variety of secondary metabolites which contribute to its bioactivity. Most notably, yarrow produces CQAs, flavonoids, and AKAs (Innocenti et al. 2007; Vitalini et al. 2011; Benedek et al. 2007; Veryser et al. 2017; Boonen et al. 2012). *In planta*, the former two are classes of phenolic compounds and help the plant defend against UV radiation and pathogens (Dai & Mumper, 2010). AKAs are generally composed of a long-chain fatty acid and an amide group, and promote primary root growth and root hair elongation (Ramírez-Chávez et al. 2004). When analyzing plant extracts taken at the end of the growing season (toward October), a previous Masters student determined that roots have the highest concentration of AKA, followed by flowerheads and leaves, and CQAs are most abundant in the flowerheads, leaves, then roots(Kachura, 2018). Their research also revealed that hydroethanolic extracts of *A. millefolium* plant parts containing AKAs and CQAs inhibited enzymes that degrade signalling lipids of the ECS, a previously unstudied mechanism potentially mediating the medicinal uses of yarrow.

The ECS exists in all Chordata (McPartland et al. 2006) and even in primitive invertebrates (De Petrocellis et al. 1999), spanning the body and helping maintain homeostasis (McCallum & Russo 2018). CB_1s and CB_2s interact with their endogenous agonist ligands (endocannabinoids), most notably AEA and (2-AG), to modulate pre-synaptic neurotransmitter release and antinociception and anti-inflammation pathways, among other biological responses (Alhouayek et al. 2011). Increased catabolism of AEA and 2-AG by serine hydrolases FAAH and MAGL, respectively, reduces endocannabinoid availability. This can exacerbate inflammation, hyperalgesia, anxiety, and many other conditions, thus reducing the efficiency of the ECS to help maintain homeostasis. (Michalski et al. 2007; Nakajima et al. 2006, Khasabova et al. 2008; Li et al. 2017; Gray et al. 2015). Moreover, the catabolic product (AA) from AEA and 2-AG is a precursor of pro-inflammatory eicosanoids, like prostaglandins and leukotrienes in the COX and LOX pathways (Wang et al. 2021). In her FAAH and MAGL inhibition study, Kachura (2018) observed that root and flowerhead extracts most potently inhibited *in vitro* enzyme activity. AKAs structurally resemble AEA, signifying a potential interaction with FAAH, for instance. Figure 2-1 shows the similarity between the endogenous ECS ligand AEA and the AKA deca−2E,4E,8Z-trienoic acid isobutylamide, found in yarrow, which shares much of the same structure as AEA (Veryser et al. 2017).

This study aimed to identify the active constituents from *A.* millefolium roots, flowerheads, and leaves that underlie the plant's inhibitory potential towards FAAH and MAGL using bioassay-guided fractionation. We started by testing crude root, flowerhead, and leaf extracts for activity, then proceeded to test the activity of their solvent fractions, followed by

sub-fractions, and finally to some of the most active isolated compounds for FAAH and MAGL inhibition in yarrow. Compound identities were confirmed based on high-resolution (HRes) TOF MS ES+ data, followed by TOF MS/MS ES+ to compare previously reported masses and fragmentation patterns of compounds found in *A. millefolium*.

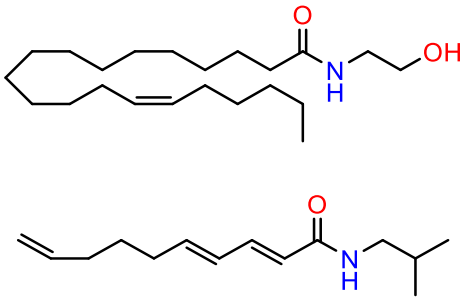


Figure 2-1. The chemical structures of AEA (top), an endocannabinoid, and deca−2E,4E,8Z-trienoic acid isobutylamide (bottom), an AKA from *A. millefolium*. Notice how they are both long-chain fatty acid amides, with an -OH group in the case of AEA.

2.3 Methods

2.3.1 Chemicals and Reagents

HPLC solvents and analytical grade trifluoracetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fischer Scientific (Waltham, MA, USA), respectively. Reference standards were used to identify previously reported yarrow compounds within extracts. Standards of CGA, 1,3-DCQA, 1,4-DCQA, 1,5-DCQA, 3,5-DCQA, and 4,5-DCQA) were purchased from Targetmol (Boston, MA, USA). Standards of the flavonoids luteolin, luteolin 7-*O*-glucoside, apigenin, apigenin-7-*O*-glucoside, and kaempferol were purchased from Cayman Chemical (Ann Arbor, MI, USA). Human recombinant FAAH and MAGL, and the fluorescent probe stubstrates 7-amino-4-methyl coumarin-arachidonamide (AMC-AA) and 4-nitrophenyl acetate (4-NA), were purchased from Cayman Chemical (Ann Arbor, USA). EDTA and Tris were purchased from Sigma-Aldrich (Oakville, Canada).

2.3.2 Collection and extraction of plant material

Achillea millefolium L. (*Asteraceae*) was identified and collected by Dr. Cory Harris and Pierluca Pica in a field beside a footpath (lat 45.418989, long -75.678051), near the corner of Mann Avenue and King Edward Avenue, Ottawa, Ontario, on October 16th, 2022. The voucher specimen was deposited near the University of Ottawa Researcher Herbarium (voucher PP-2024-AM1). Plants were three to four feet tall, and the flowerheads were white-ish brown. Plant parts were separated by hand into roots, leaves and flowerheads (corymb), with stems discarded since the study by Kachura (2018) reported weak FAAH and MAGL inhibition from their extracts. Samples were cleaned to remove soil and debris. Samples were dried in a food dehydrator at 35°C overnight and then milled separately using a Wiley Mill (Thomas Scientific, Swedesboro,

NJ 08085, USA) with a mesh size of 20 (1 mm). Ground plant parts were extracted with 70% ethanol, 10 mL per gram of material, in large Erlenmeyer flasks sealed with aluminum foil and macerated in darkness overnight at 250 rpm. The supernatant was collected, and two more rounds of extraction followed (as described above), pooling the collected supernatants. Liquid extracts were vacuum filtered using a Buchner funnel and filter paper with a pore size of 11 µm. The solvent was removed by roto-evaporating using a Yamato Scientific RE200 Rotary Evaporator with a water bath temperature of 45°C, then lyophilized in a Super Modulyo freeze drier (Thermo Electron, Ottawa, ON, Canada) to remove residual water.

2.3.3 FAAH and MAGL Inhibition Assays

Enzyme assay methods were executed as reported in Liu, 2019, and briefly described below. To confirm previously reported activity in crude extracts of roots, flowerheads, and leaves, each extract was tested at a single concentration of 50 µg/mL for FAAH and 100 µg/mL for MAGL. Aqueous and organic fractions obtained via liquid-liquid fractionation were tested at two concentrations for FAAH (50 and 250 µg/mL) and MAGL (100 and 500 µg/mL). Open-column sub-fractions of root and flowerhead organic fractions were screened at 50 µg/mL and 100 µg/mL for FAAH and MAGL, respectively, and isolated compounds were tested at multiple 6-7 concentrations to observe dose-response and establish their IC_{50} values. Prior to assays, samples (dried extracts, fractions, sub-fractions, purified compounds) were resolubilized in 50% dimethyl sulfoxide (DMSO), vortexed for 30 seconds, and sonicated for five minutes.

2.3.3.1 *FAAH inhibition Assay*

The enzyme was stored at -80°C and thawed immediately before use. Buffer consisted of 125 mM Tris-HCL (pH 9.0) with 1 mM EDTA and 20 µM of AMC-AA. FAAH was diluted 1:40

and incubated with samples at 10 µg/mL. The positive control used was JZL 195 (4.6 µM) (Cayman Chemical, Ann Arbor), and the vehicle control was 2% DMSO. JZL 195 is a potent irreversible inhibitor of FAAH (Bajaj et al. 2022; Sakin et al. 2015). Enzyme activity was analyzed at 37°C for 45 minutes using a Cytation 3 (BioTek) plate reader with excitation at 355 nm and emission at 460 nm.

2.3.3.2 *MAGL Inhibition Assay*

MAGL was stored at -80°C and thawed immediately before use. Buffer consisted of 10 mM Tris-HCL (pH 7.2) with 1 mM EDTA and 236 µM 4-NA. MAGL was diluted 1:20 and incubated with samples at 0.583 µg/mL. The positive control was JZL 184 (5.56 nM) (Cayman Chemical, Ann Arbor), and 2% DMSO was the vehicle control. JZL 184 is a potent irreversible inhibitor of MAGL (Sakin et al. 2015; Ghosh et al. 2013). Enzyme activity was analyzed at room temperature for 15 minutes using a Cytation 3 plate reader at absorbance 405 nm to monitor the reaction.

2.3.3.3 Calculation of %inhibition and IC_{50}

Percent inhibition of FAAH and MAGL activity was calculated by comparing the fluorescence in sample- andvehicle-treated reactions are defined below. Data were analyzed and plotted using GraphPad Prism 10.2.2.

$$[1 - \frac{(Sample\ Enzyme\ End - Sample\ Enzyme\ Start) - (Sample\ Blank\ End - Sample\ Blank\ Start)}{(Vehicle\ Control\ Enzyme\ End - Vehicle\ Control\ Enzyme\ Start) - (Vehicle\ Control\ Blank\ End - Vehcile\ Control\ Blank\ Start)}] * 100$$

IC_{50} values were calculated using GraphPad Prism 10.2.2. The dose-response curves used a nonlinear regression model to plot Log [Inhibitor] vs normalized response – Variable slope (CI ± 95%) (log[concentration] (µg/mL) vs inhibition). Isolated compounds were tested in triplicate at a minimum of six concentrations (12.5 µg/mL-250 µg/mL and 25 µg/mL-500 µg/mL for FAAH

and MAGL, respectively). DCQA standards were tested at 5 µg/mL, 12.5 µg/mL, and 25 µg/mL for FAAH and 10 µg/mL, 25 µg/mL, and 50 µg/mL for MAGL.

2.3.3.4 *Assay-guided fractionation of hydroethanolic extracts*

Fractionation of root, flowerheads, and leaf extracts proceeded in three steps (liquid-liquid fractions, silica column sub-fractions, isolated compounds) as presented in Figure 2-2.

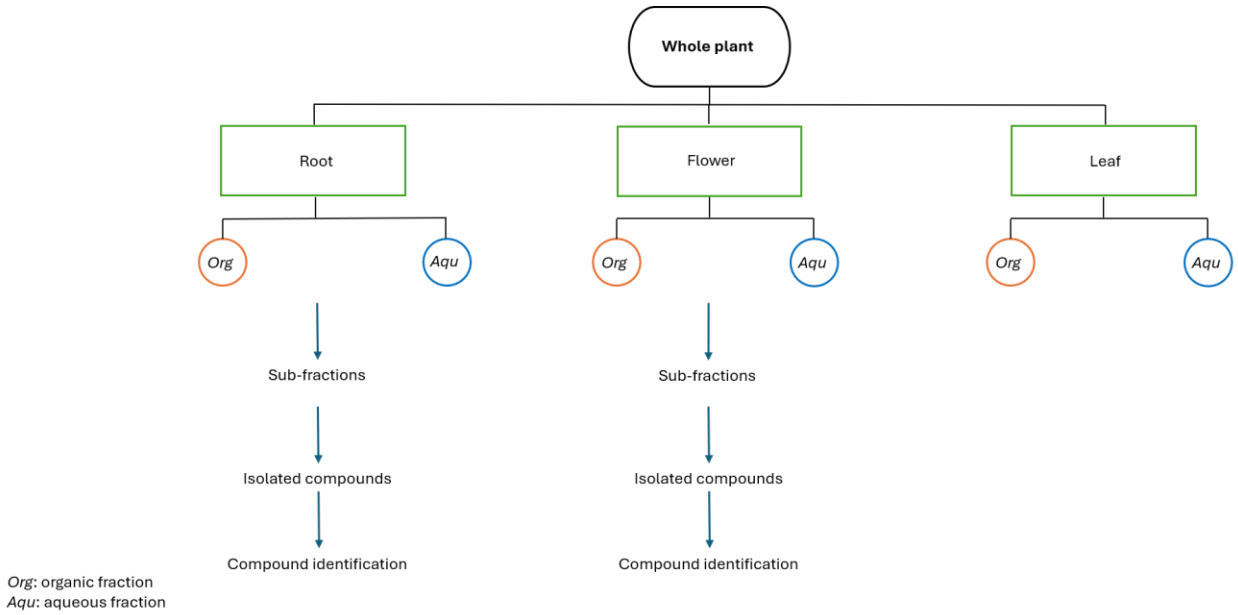


Figure 2-2. Flowchart depicting steps taken for a bioassay-guided fraction of yarrow roots, flowerheads, and leaves. %inhibition of extracts for FAAH and MAGL were assessed from crude extracts to isolated compounds. Crude extracts of each part were separated by liquid phase extraction and then sub-fractionated. The most active sub-fraction from plant parts was used for compound isolation to test metabolites individually for bioactivity.

2.3.4 Liquid-Liquid Phase Separation of Extracts

Aqueous and organic extracts were prepared from crude hydroethanolic extracts of roots, flowerheads, and leaves. Dried extracts were transferred to flasks and mixed with MiliQ water 100 mL:1 g crude extract, then washed in a separatory funnel with 1:2 dichloromethane (DCM) in water. Washes were repeated twice, and both phases were kept. The pooled aqueous phase

was lyophilized, while the pooled organic phase was roto-evaporated to dryness and stored at -20°C until experimental use.

2.3.5 Column Chromatography

The organic fraction of root and flowerhead extracts were fractionated separately using column chromatography with silica gel (60-120 μm particle size) as the stationary phase. The column was washed repeatedly with hexane until the silica gel was saturated with solvent. Extracts were weighed and mixed in flasks 1:1 with cellite, and 300 mL of 100% hexane was added. Once the mixture had homogenized, it was gently poured into the solvent bulb, and fraction collection started while adding 10% DCM in hexane per addition of 300 mL of solvent to 100% DCM. Then, increments of 10% MeOH in DCM were added until 100% MeOH (see Appendix A). Fractions were collected 100 mL at a time.

2.3.6 Thin Layer Chromatography

The phytochemical profiles of collected column fractions were observed using thin-layer chromatography. Silica plates were placed in a jar of 1:1 hexane and ethyl acetate and observed under a UV lamp to look for traces of compounds, and fractions were pooled based on the observed traces. Once the phytochemical profiles of all fractions were assessed, they were combined by grouping them based on observed profiles and roto-evaporated and lyophilized to remove solvent, as described for the extraction of plant material. After weighing the product, fractions were resolubilized with 1:1 DMSO in water.

2.3.7 Compound isolation using HPLC-DAD peak-based fraction collection

The most active root and flowerhead subfractions were each dissolved in DMSO at 50 mg/mL, vortexed for 30 seconds, and sonicated for 5 minutes. Compound isolation was

performed on an HP Agilent 1100 Series Preparative HPLC system. A Phenomenex Luna C18(2) column (5 µm particle size, 250 x 4.6 mm) with an isocratic mobile phase of 80% MeOH in water was used, and the column temperature was maintained at 50°C. The flow rate was 4 mL/min. The automated fraction collector performed peak-based isolation with the DAD set at 268 nm. After repeated runs, each collected peak was pooled and dried, dissolved in 50% DMSO for ready use in bioassays, and analyzed for purity, represented by a single peak, using the purity check function of Agilent ChemStation.

2.3.8 HRes compound identification with TOF MS ES+ and confirmation with TOF MS/MS ES+

Collected peaks from active fractions were analyzed using a Waters Synapt G1 Mass Spectrometer in positive ion mode to identify isolated compounds. Isolates were diluted to 10 µg/mL with acetonitrile (ACN) and injected via a syringe pump at 5 µg/mL, which was rinsed with ACN after each injection. The source temperature was maintained at 80°C, and source conditions for ES+ were 3.5kV had a gas flow rate of 500 mL/min. HRes data and MS/MS data were obtained. Isolate structures were identified based on parent ion mass-to-charge ratios (*m/z*) and product ions. The MS/MS data conditions were 10-3 mbar Argon gas and 20-25 eV collision energy. FooDB version 1.0 was used to compare HRes and MS/MS data obtained to previously reported results. The MS detection scan range was adjusted between *m/z* 50 and 500. The Δ ppm value indicates the mass accuracy of what has been measured by showing the approximation error between the experimental and actual values. The equation is as follows:

$$ppm = \left(theoretical \frac{m}{z} value - \frac{experimental \frac{m}{z} value}{theoretical} \frac{m}{z} value \right) * 10\text{^}6.$$

2.3.9 HPLC analyses of all extracts

HPLC analyses were conducted on an Agilent 1100 series HPLC-DAD system (Agilent Technologies, Santa Clara, California, USA) with ChemStation 3.02B. The column was washed with 5 column volumes of both 100% Milli-Q water:0.1% TFA (mobile phase A) and 100% acetonitrile:0.1% TFA (mobile phase B). Solvents flowed at 400 µL per minute on a Kinetex PS C18 LC column (100 mm x 2.1mm ID, 2.6 µm particle size). The column temperature was maintained at 50°C during a mobile phase gradient of 2.5% acetonitrile in water at 0 min, 23% at 15 min, 7% at 25 min, and 100% between 26 and 30 min, then returned to initial conditions in 0.1 minutes, leaving a total runtime of 30.10 minutes. Prepared extracts and reference standards (CGA (1), 1,3-DCQA (2), 1,4-DCQA (3), 1,5-DCQA (4), 3,5-DCQA (5), 4,5-DCQA (6)) were sonicated for 5 minutes and injected at 1 µL via autosampler in triplicate. Peak identities were confirmed via retention time (R_t) and spectral analyses relative to the reference standards. The peak purity (%purity) of isolated compounds was ≥85%, calculated using the following equation:

$$Peak\ purity = \left(\frac{Peak\ area\ of\ the\ compound}{Total\ area\ of\ all\ observed\ peaks}\right) 100\%$$

2.4 Results

2.4.1 Confirmation of FAAH and MAGL inhibitory activity and HPLC-DAD analyses of yarrow extracts

2.4.1.1 Inhibition by plant part crude extracts

An initial screening of *A. millefolium* root, flowerhead, and leaf crude extracts was executed at 100 µg/mL for FAAH and MAGL to confirm *in vitro* inhibition of both enzymes before pursuing assay-guided fractionation. Results in Figure 2-3 show complete inhibition of

FAAH by all three extracts, whereas their inhibition of MAGL at twice the concentration was relatively weaker (63 to 69%).

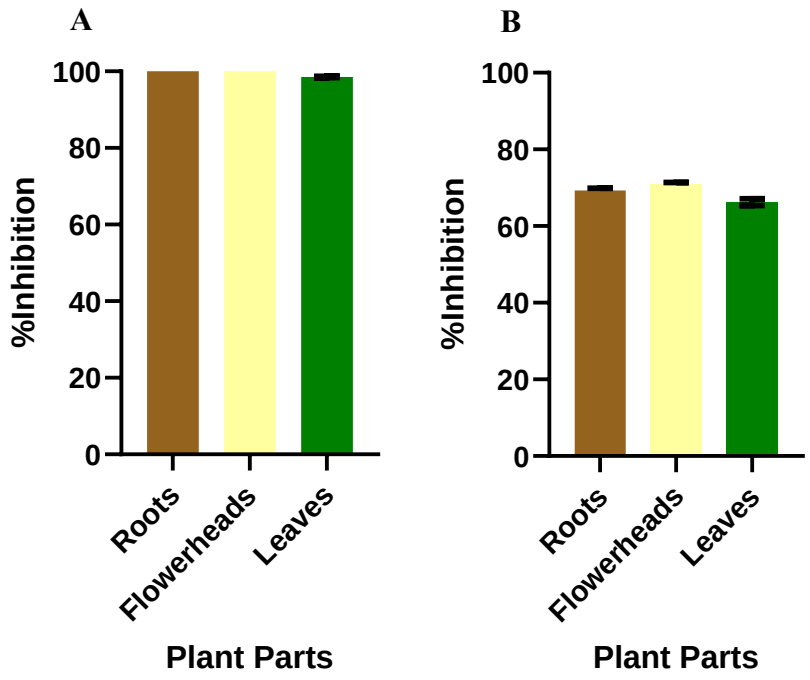


Figure 2-3. Percent inhibition (%) (±SEM) of crude plant extracts (n=3) of AM when incubated with FAAH (A) and MAGL (B). The extract concentration in-well was 100 µg/mL for FAAH and MAGL.

2.4.1.2 Comparison of yarrow crude extracts by HPLC-DAD

HPLC-DAD chromatograms of the roots, flowerheads, and leaves are shown in Figure 2-4, at 268 nm (A-C) to detect AKAs preferentially, and at 330 nm (D-F) to detect CQAs and flavonoids (Chen et al. 2022; Bauer et al., 2007; Mølgaard et al. 2003). The CQA reference standards were seen across plant parts, and helped identify most peaks that were selectively absorbing in the 330 nm spectrum. The flavonoids apigenin, luteolin, and their glucosides, along with kaempferol, were unseen in extracts.

The most phytochemical diversity appeared in the flowerheads (B and E), followed by roots (A and D) and the leaves (C and F) (Figure 2-4). The root spectra showed lower levels of CQAs, but had a series of significant peaks at 268 nm after 20 minutes, which absorbed less in the 330 nm spectra, except for the peak at 24.5 minutes. The two flowerhead spectra (B and E) were very similar, with multiple CQAs, a few compounds selectively detected at 268 nm, and two peaks at 15 and 17 minutes absent from other extracts. Unlike the roots and leaves, flowerheads showed no proper delineation between polar and non-polar compounds between both spectra. Lastly, the leaf extract showed the least chemical diversity among the plant parts (Figure 2-4 C and F) with the main major peak being CGA at approximately 5 minutes.

With confirmation that our crude extracts inhibited both FAAH and MAGL and were chemically distinct, aside from the leaves, we proceeded with liquid-liquid fractionation of each plant part into their respective aqueous and organic fractions for further testing and analyses.

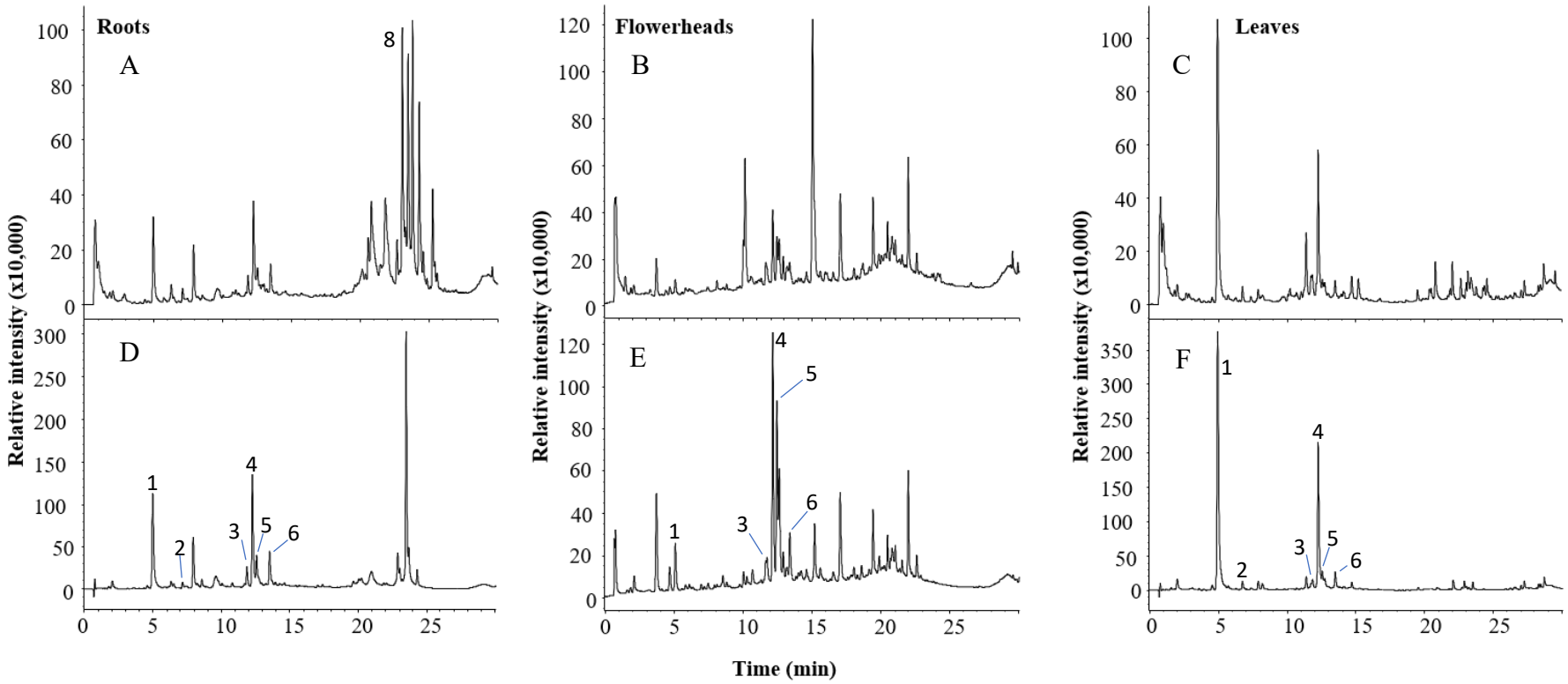


Figure 2-4. **HPLC-DAD chromatograms for root, flowerhead, and leaf crude extracts across time (minutes) at 268 nm (A-C) and 330 nm (D-F)**. Samples were dissolved in MeOH at a concentration of 20 mg/mL. CGA (1), 1,5-DCQA (2), 1,4-DCQA (3), 3,5-DCQA (4), 1,3-DCQA (5), and 4,5-DCQA (6) reference standards were used for peak identification.

2.4.2 Bioactivity of aqueous and organic fraction bioactivity and HPLC-DAD analyses

2.4.2.1 Inhibition by liquid fractions

Upon solvent-solvent fractionation of crude extracts, each plant part's organic and aqueous fractions inhibited FAAH and MAGL activity to varying degrees (Figure 2-5). Notably, the organic fractions of all three plant parts completely inhibited FAAH activity (95%-100%) at both tested concentrations (100 μg/mL and 500 μg/mL in-well). In contrast, results from the aqueous fractions indicated less potent dose-dependent inhibition, with root and leaf fractions performing similarly (18.1 & 55.6%; 14.3% & 57.2%, respectively) and flowerheads displaying stronger activity (45.2% and 91.1%) (Figure 2-5A).

MAGL inhibition by the organic fractions had a similar pattern to FAAH, with both flowerheads and roots eliciting near-complete inhibition (84%-90%) at both tested concentrations. Unlike with FAAH, the leaf organic extract was much weaker. Among aqueous fractions, flowerheads inhibited MAGL more potently than roots and leaves, which demonstrated similar dose-dependent inhibition (Figure 2-5B).

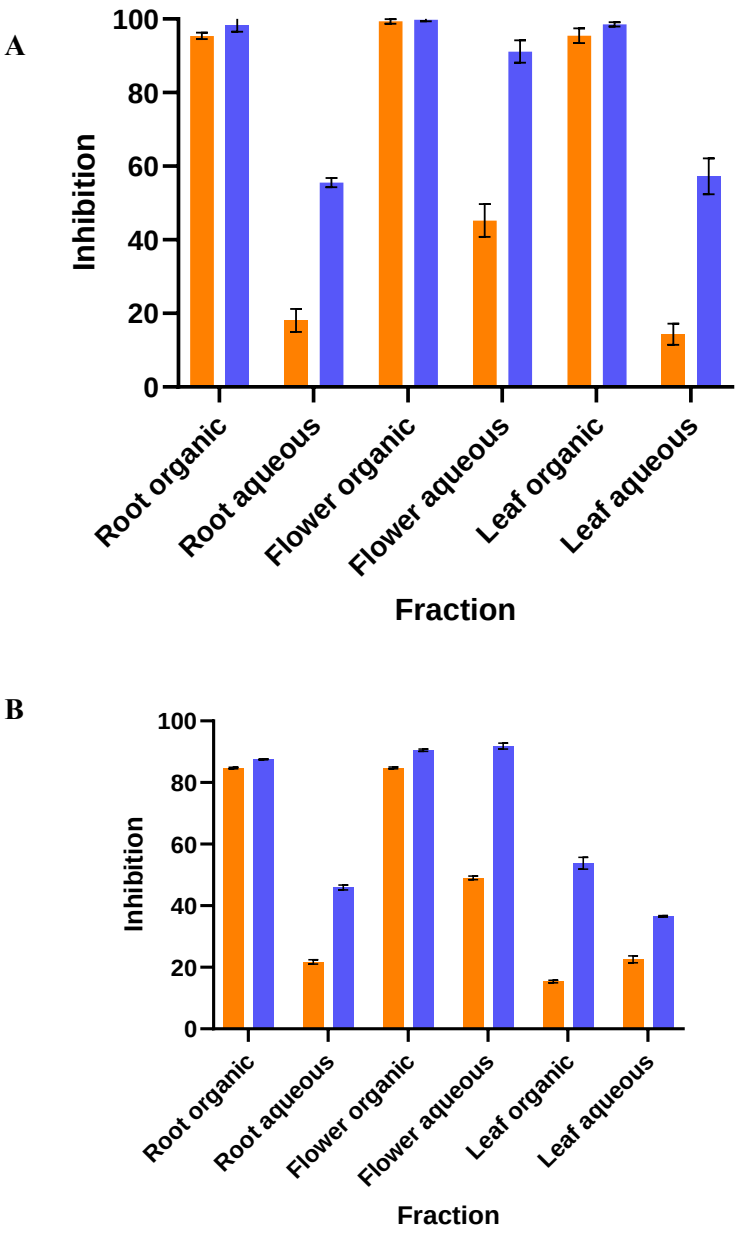


Figure 2-5. Mean percent inhibition (± SEM) of aqueous and organic fractions of roots, flowerheads, and leaves (n=12) for FAAH (A) and MAGL (B). Plant part fractions and their respective concentrations (100 µg/mL (orange) and 500 µg/mL (purple)) are shown.

2.4.2.2 Comparison of liquid fractions by fractions by HPLC-DAD

Trends in the HPLC-DAD profiles of each plant part (Figure 2-6) showed the separation of compounds mostly absorbing at 268 nm (A-C) and 330 nm (D-F) for organic and aqueous

fractions, respectively. The roots (A and B) showed the same phytochemical makeup as the leaves (C and F) before 16 minutes at 330 nm, with peak signals indistinguishable from noise at 268 nm. Liquid fractions of flowerheads (B and E) made phytochemical differences more indistinguishable between spectra compared to the roots and leaves.

While signal peaks of compounds absorbing selectively at 268 nm in organic fractions remained unknown, Figure 2-6 shows that most peaks that absorbed selectively at 330 nm were CQAs. Therefore, we decided to pursue assay-guided fractionation of root and flowerhead organic but, since the leaves had the least bioactivity and contained no unique compounds compared to the roots and flowerheads, they were not pursued for sub-fractionation.

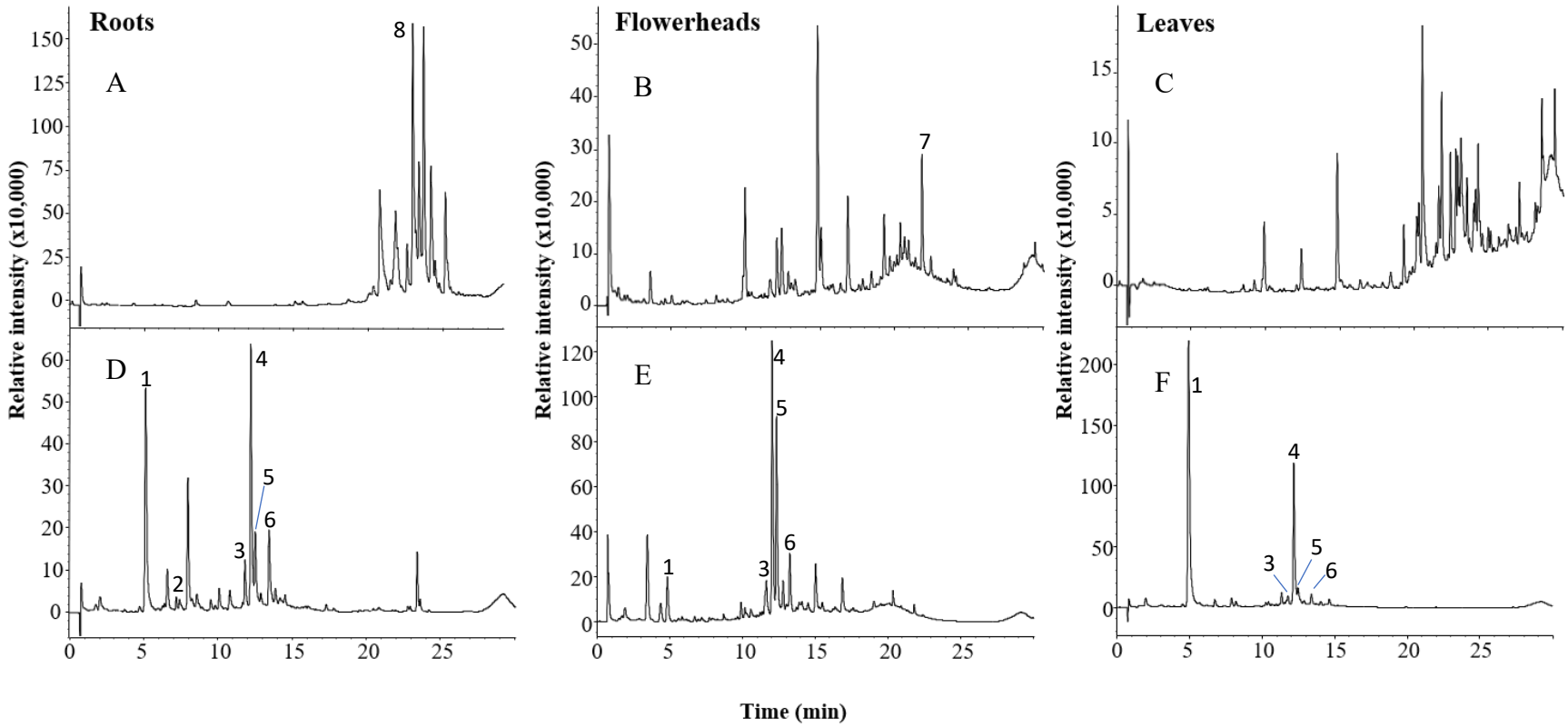


Figure 2-6. HPLC-DAD chromatograms for root, flowerhead, and leaf organic (268 nm, A-C) and aqueous (330 nm, D-F) extracts across time (minutes). Aqueous and organic fractions were dissolved at 10 mg/mL in MeOH and DMSO, respectively. CGA (1), 1,5-DCQA (2), 1,4-DCQA (3), 3,5-DCQA (4), 1,3-DCQA (5), and 4,5-DCQA (6) reference standards were used for peak identification.

2.4.3 Sub-fractionation of roots and flowerheads and their inhibition of FAAH and MAGL

2.4.3.1 Roots

Sub-fraction screening for enzyme inhibition from the organic liquid fraction of yarrow roots showed a higher selectivity for FAAH than MAGL. Results are shown in Figure 2-7. Sub-fraction 6, which eluted between 20%-30% MeOH:DCM, inhibited FAAH most potently (100% inhibition at 100 µg/mL), and was the only inhibitory sub-fraction for MAGL (86.4%). Sub-fractions 1-5, 7-9, and 10-16 inhibited FAAH at 69.7%, 71.8%, and 33.2% but did not inhibit MAGL. Lastly, sub-fractions 17-23, 24-31, and 32-39 were inactive on both assays.

Analyses of subfraction 6 by HPLC-DAD monitored at 268 nm and 330 nm showed seven well-defined peaks, six of which had an optimal absorption at 268 nm and one at 330 nm. All peaks corresponded with major peaks observed in the organic fraction of roots in Figure 2-6, which were subsequently targeted for isolation.

2.4.3.2 Flowerheads

The sub fractionation of the flowerhead organic extract with column chromatography yielded three pooled sub-fractions. Eluted between 30%-35% MeOH: DCM, subfraction 42-53 inhibited FAAH and MAGL the most (100% and 92%, respectively) (Figure 2-7). Subfractions 1-41 and 54-73 had much weaker FAAH inhibition (46.6% and 45.4%, respectively) than MAGL inhibition (85% and 95%, respectively). TLC was conducted to observe the number of distinct compound markers within subfraction 42-53, totalling six. HPLC-DAD analyses revealed six distinct peaks, four of which absorbed light at 268 nm and two at 330 nm. We, therefore, proceeded with compound isolation from subfraction 42-53.

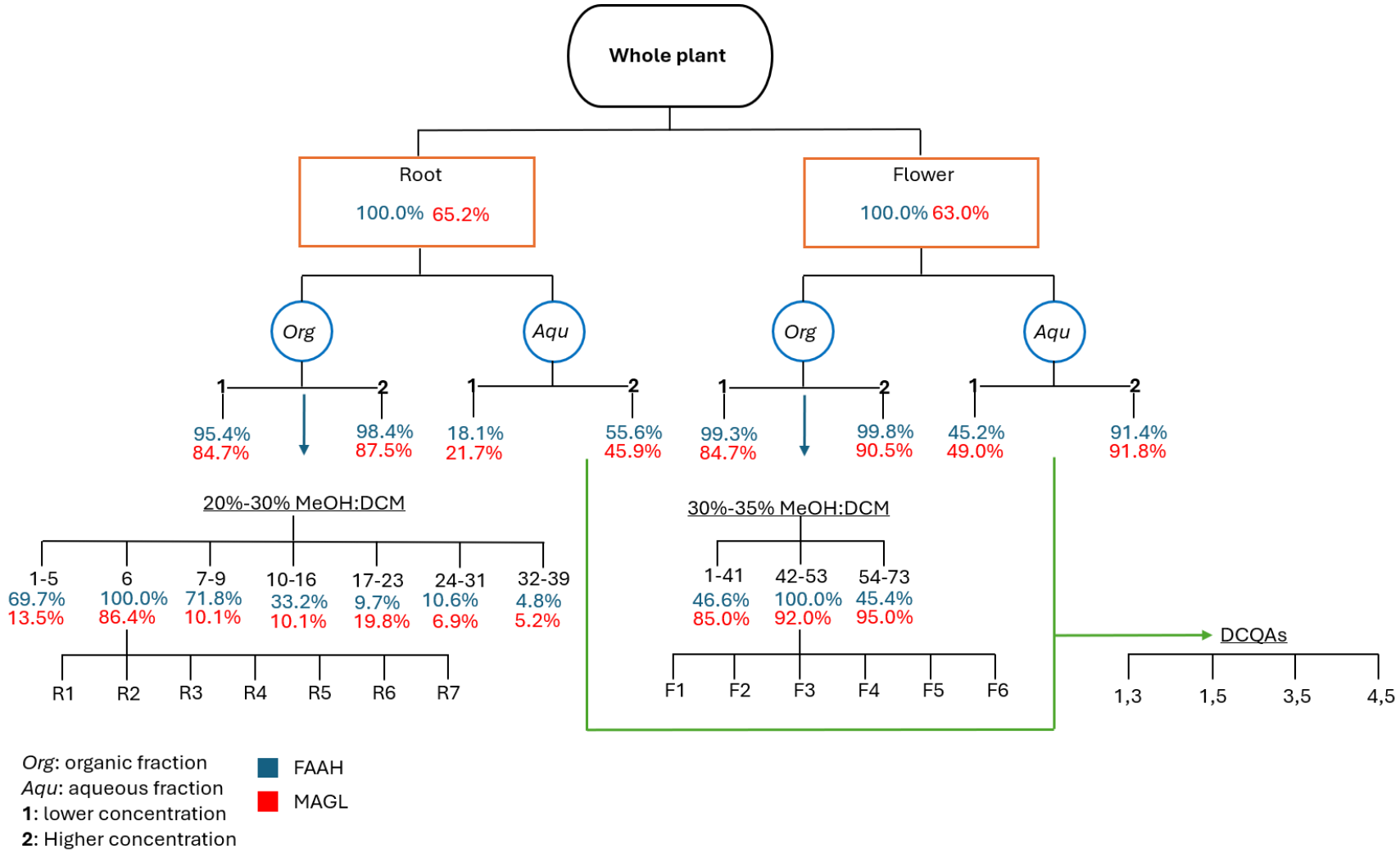


Figure 2-7. Flowchart of bioassay-guided fractionation of *A. millefolium* plant parts. The crude extracts, liquid-liquid fractions, and root and flowerhead organic fraction %inhibitions are shown for FAAH (blue) and MAGL (red). Root (R#) and flowerhead (F#) isolates resulting from their respective sub-fractions and DCQAs tested for their bioactivity are given. Green lines show that DCQAs were observed in the aqueous fractions of plant parts. ±SEM not shown.

2.4.4 Assessment and identification of isolated compounds from the active root sub-fraction

2.4.4.1 Enzyme inhibition by compounds isolated from root sub-fraction 6

The inhibitory activity of the seven isolated compounds from sub-fraction 6 of the organic root extract was assessed for FAAH and MAGL. All compounds inhibited both enzymes dose-dependently. Their IC_{50} values were calculated as shown in Table 2-1 and Figure 2-8. The chromatograms of root isolates overlayed with the chromatogram of the root organic extract are presented in Appendix 2 A-G.

FAAH inhibition data showed that the IC_{50} values for **R2-R6** lay between 26.45 µg/mL-36.51 µg/mL, with the lowest value being that of **R3**, while those for **R1** and **R7** were 89.89 µg/mL and 69.69 µg/mL, respectively.

MAGL inhibition data showed that isolates were overall weaker inhibitors for MAGL than for FAAH. They showed a wider range between IC_{50} values (48.92 µg/mL-64.62 for compounds **R1-R6)**, and **R4** had the lowest value (48.92 µg/mL). **R7** lay outside the stated range.

Table 2-1. The mean percent inhibition (±SEM) and IC50 values (µg/ml)) (±SEM) of FAAH and MAGL among isolated compounds (n=7) from root sub-fraction 6 of sub-fraction 42-45 of *A. millefolium*.

	FAAH IC_{50} (CI ± 95%)	MAGL IC_{50} (CI ± 95%)
R1	89.89 (81.87 to 99.42)	64.62 (56.65 to 73.42)
R2	49.70 (44.82 to 55.60)	52.76 (43.12 to 63.69)
R3	26.45 (21.54 to 32.66)	54.22 (47.81 to 61.89)
R4	28.95 (26.15 to 31.88)	48.92 (38.60 to 60.33)
R5	36.51 (28.13 to 63.80)	64.25 (56.60 to 72.75)
R6	32.69 (23.80 to 70.22)	53.75 (48.48 to 59.37)
R7	69.69 (50.99 to 219.1)	80.65 (73.41 to 90.10)

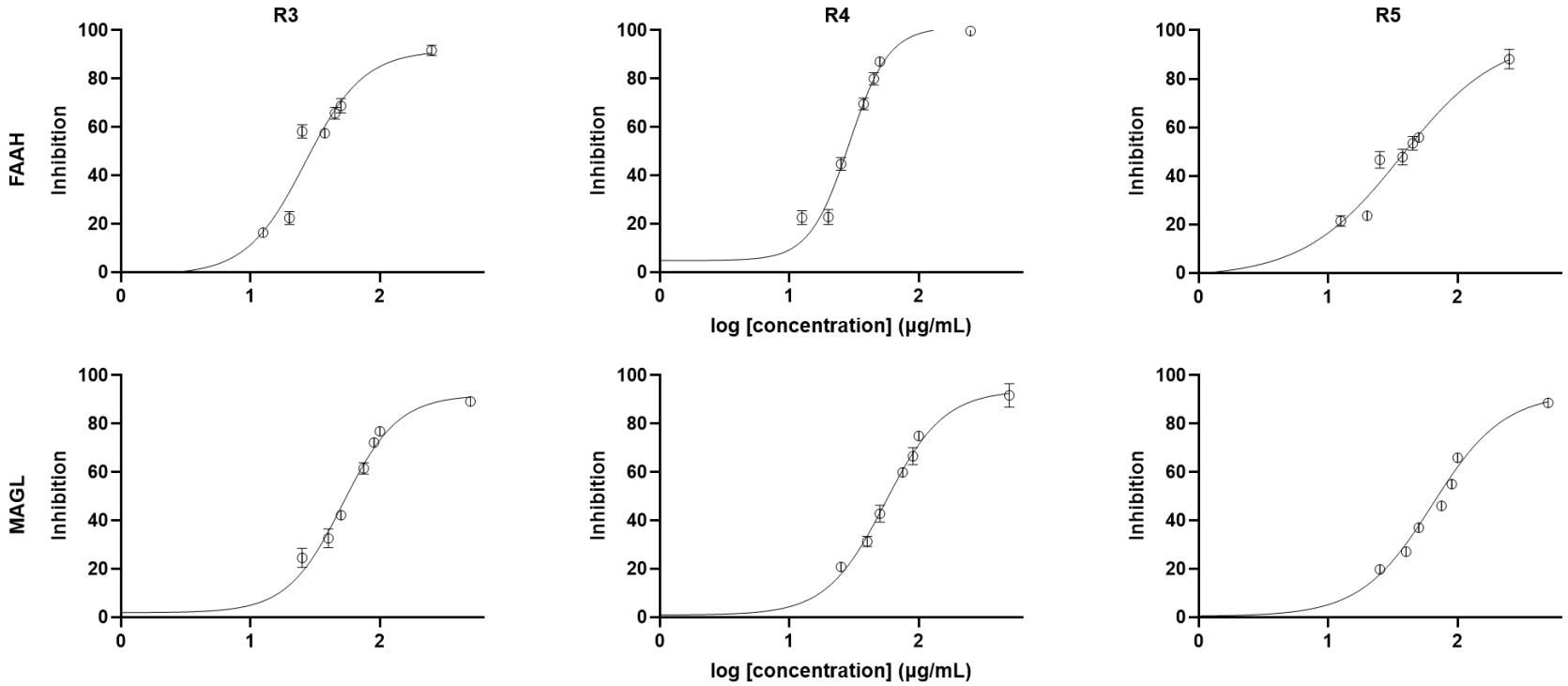


Figure 2-8. Concentration-dependent responses (CI ± 95%) (log[concentration] (µg/mL) vs inhibition) for FAAH and MAGL of root isolated compounds R3, R4, and R5 (n=3, ± SEM).

2.4.4.2 Identification of root isolates

Isolated compounds were tentatively identified based on high resolution TOF-MS ES+ mass-charge ratios compared to reported values for known yarrow metabolites. Table 2-29+-*/ lists compound name and number (in order of elution), structure, chemical -formula, and average molecular weight (MW). Table 2-3 presents observed MS data and +theoretical values utilized to identify the seven compounds, including calculated differences in the theoretical and measured accurate masses (Δ parts per million (ppm)) of isolated compounds from the roots. All Δ ppm values between the theoretical and measured HRes masses are considered very low (±6.1 ppm) (Kind et al. 2018), ranging between -2.0 ppm and 6.1 ppm for root isolates, except for **R6** (-15.9 ppm).

We confirmed the identities of three most active metabolites, **R3**-**R5**, by matching their TOF MS/MS ES+ fragmentation patterns to those in the literature and available on FooDB (2024) (Veryser et al. 2017). The identity of **R3**, pellitorine, was further confirmed via HPLC R_t and spectral analyses relative to a commercial reference standard.The major product ions in MS/MS analyses were the protonated form of **R3**-**R5**. The isobutylamide **R3** displayed a loss of *m/z* 56, corresponding with the loss of the alkyl group directly attached to the amine group and a loss of the amine group (*m/z* 73). Except for **R2**, their losses also corresponded with the loss of the amide group and saturation of a double bond on the alkyl chain (*m/z* 98), followed by the loss of the amide group (*m/z* 101).

R4, a piperideide, and **R5**, a piperidide**,** were differentiated by the unsaturated piperidine ring in **R4**, giving it two less H than **R5**. Both had the product ion *m/z* 149, resulting from a loss of *m/z* 83 and 85, respectively, corresponding with the loss of the amine group typically seen with compounds with a piperidine ring. A cleavage in the fatty acid chain was seen at a loss of

m/z 55 and 59 for **R4** and **R5**, respectively. For **R4**, there was a loss of *m/z* 163 following a hydrogen rearrangement and loss of the carbonyl group, and **R5** saw a loss of *m/z* 70 after cleavage of the fatty acid chain and loss of H.

Like R3-R5, the other four isolated compounds from the roots were AKAs and corresponded to previously reported fatty acid amide molecular masses reported in Veryser et al. (2017) and FooDB. Isolates **R1**, **R2** contained an isobutylamide function (isobutyl group C1-C3 to the right of the amide, like **R3**). **R6** had a 2-methylbutylamide function (methylbutyl group C1-C5 to the right of the amide), and **R7** had a 4-hydroxyphenylethylamide function (hydroxyphenyl group C1-C8 to the right of the amide). Compounds varied in fatty acid chain length (C_{14}, C_{15}, and C_{22}) and contained multiple unsaturated bonds (double and triple bonds).

Checking the %purity of isolated compounds by calculating the area of the peak and taking its percentage from the total chromatogram peak areas with HPLC-DAD, all compounds were ≥85% pure. This allowed us to match some of the characteristic collision-induced dissociation fragmentation patterns related to different parts of each compound.

Table 2-2. The chemical name, optimal absorbance (nm) formula, structure, and average molecular weight (MW) of root isolates. The wavelength each compound absorbed optimally at (either at 268 nm or 330 nm) is shown.

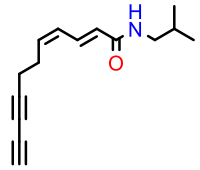
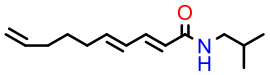
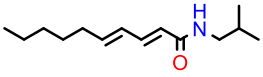
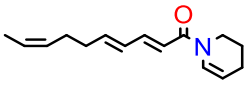
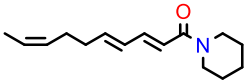
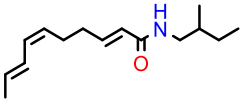
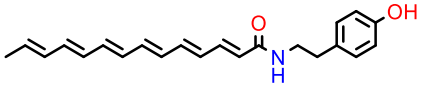
Isolate # and name	Absorbance (nm)	Chemical structure	Average MW (g/mol)
R1: Undeca−2E,4Z-diene−8,10-diynoic acid isobutylamide	268	 $C_{15}H_{19}NO$	229.32
R2: Deca−2E,4E,8Z-trienoic acid isobutylamide	268	 $C_{14}H_{23}NO$	221.34
R3: Deca−2E,4E-dienoic acid isobutylamide (pellitorine)	268	 $C_{14}H_{25}NO$	223.36
R4: Deca-2E,4E,8Z-trienoic acid piperideide	330	 $C_{15}H_{21}NO$	231.34
R5: Deca-2E,4E,8Z-trienoic acid piperidide	268	 $C_{15}H_{23}NO$	233.36
R6: Deca-2E,6Z,8E-trienoic acid 2-methylbutylamide	268	 $C_{15}H_{25}NO$	235.37
R7: Tetradeca-2E,4E-diene-8,10-diynoic acid 4-OH phenylethylamide	268	 $C_{22}H_{25}NO_2$	335.45

Table 2-3. Differences between the measured and theoretical masses in ppm of root isolates. Accurate masses and MS/MS data were determined using TOF-MS-ES+ and TOF MS/MS ES+ respectively, in which the positive adduct was Na^+. Characteristic fragment losses are highlighted in bold.

Isolate	$[M+H]^+$	Monoisotopic mass	Theoretical mass $[M+Na]^+$	Measured mass $[M+Na]^+$	Theoretical and measured mass difference (delta ppm)	Product ions (*m/z*)	Losses (*m/z*)
R1	230	229.1467	252.1359	252.1364	-2.0	Unacquired	Unacquired
R2	222	221.1780	244.1677	244.1663	-5.7	Unacquired	Unacquired
R3	224	223.1936	246.1834	246.1849	6.1	206; 182; 168; 153; 151; 130; 133; 121; 100; 72	-18; -42; **-56**; -71; **-73**; **-98**; **-101**; 91; -124; -152
R4	232	231.1623	254.1521	254.1516	-2.0	177; 165; 149; 110; 107; 69	**-55**; **-83**; -122; -125; -150; **-163**
R5	234	233.1780	256.1677	256.1666	-4.3	175; 164; 149; 110, 84; 81;	**-59**; -70; **-85**; -124; -150; -153
R6	236	235.1936	258.1828	258.1787	-15.9	Unacquired	Unacquired
R7	336	335.1885	358.1781	358.2354	0	Unacquired	Unacquired

2.4.5 Assessment and identification of isolated compounds from the active flowerhead sub-fractions

2.4.5.1 Enzyme inhibition by compounds isolated from flowerhead sub-fraction 42-53

Figure 2-9 shows the observed %inhibition of FAAH and MAGL by compounds isolated from sub-fraction 42-53 of the organic flowerhead fraction. Isolates were initially screened for their activity at 100 µg/mL. Isolates showed negligible or no FAAH inhibition, with **F4** showing the most activity (only 22% inhibition at 100 µg/mL in-well) (Figure 2-9A), and so no additional testing was pursued. Inhibition assays done with MAGL and flowerhead isolates (Figure 2-9 B) showed limited activity as well, will all compounds except **F4** inhibiting MAGL by less than 40%. **F4** was the only compound that inhibited MAGL dose-dependently (Figure 2-10) with an IC_{50} of 26.18 µg/mL and a CI ±95% of 23.12 µg/mL and 29.48 µg/mL. **F1**, **F2**, and **F5** isolates were not bioactive towards FAAH nor MAGL, and **F3** and **F6** were inactive in the FAAH bioassay and weakly inhibited MAGL at 29.6% and 35.9%, respectively.

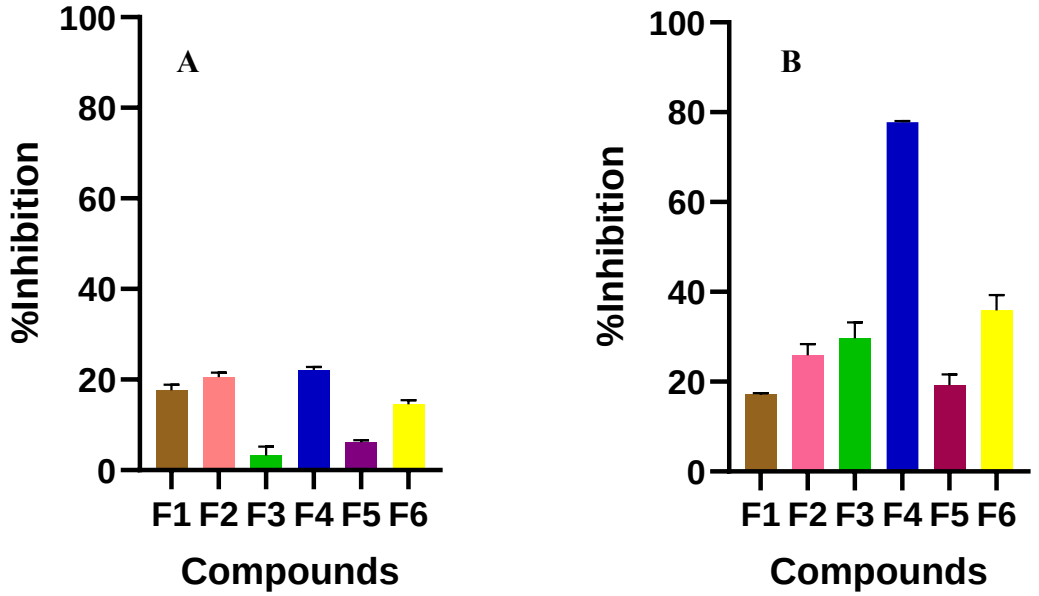


Figure 2-9. Mean percent inhibition (± SEM) of flowerheads isolated compounds (n=6) for FAAH (A) and MAGL (B). F1 (brown), F2 (pink), F3 (green), F4 (blue), F5 (purple), and F6 (yellow) were tested for their inhibition at 100 µg/mL in-well.

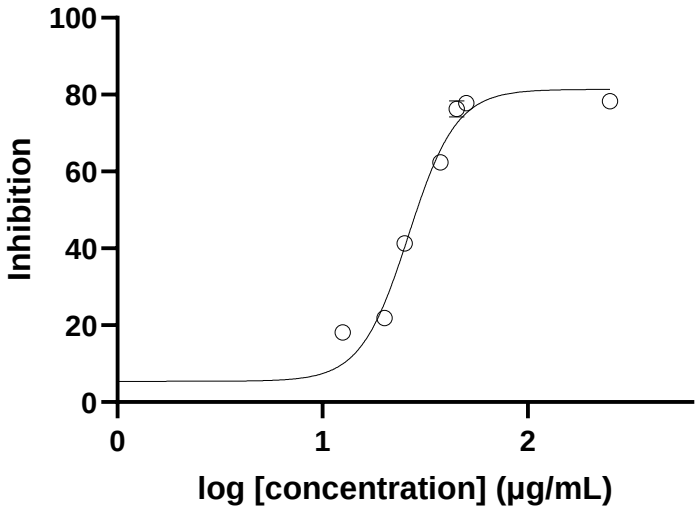


Figure 2-10. Concentration-dependent response of F4 isolated from yarrow flowerhead for MAGL (n=3, ± SEM).

2.4.5.2 Identification of compounds isolated from flowerheads

Table 2-4 shows compound name and number (in order of elution), structure, chemical formula, and average molecular weight (MW) of isolated compounds tentatively identified from the active sub-fraction (42-53) of the organic flowerhead fraction. Table 2-5 presents observed TOF-MS ES+ and MS/MS ES+ data, alongside theoretical values, utilized to identify the six isolated compounds. **F1** was identified as vanillin isobutyrate, an aromatic compound known as a phenol ester, and compounds **F2** and **F4** were identified as the STLs austricin and dihydroparthenolide, respectively. Compound **F3** was tentatively identified as 1-O-isopentyl-3-O-octadec-2-enoyl glycerol, a glyceride known as a 1-alkyl, 3-acylglycerol. Compound **F5** was identified as the dehydrogenated carboxylate product of 9'-hydroxy-α-tocopherol, 9'-carboxy-α-chromanol (FooDB) and **F6** was artemitin, a 7-O-methylated flavonoid confirmed via HPLC R_t and spectral analyses between the reference standard and isolated artemitin from the flowerheads.

While all the observed TOF-MS data matched theoretical values for the assigned parent ion with high accuracy (Δ ppm of -0.5 to 3.9), TOF MS/MS fragmentation results were only obtained for one compound, **F4 (**Table 2-5). The MS/MS spectra for dihydroparthenolide showed the presence of the molecular ion and the protonated ion are *m/z* 250 and 251, respectively. The loss of the carbonyl group corresponded to *m/z* 222, and *m/z* 162, *m/z* 151, and *m/z* 146 related to formation of dienes and dienophiles from the hexene rings in the compound, following a reverse Diels-Alder reaction. Lastly, the lower *m/z* (*m/z* 95, *m/z* 83, and *m/z* 51) can be correlated with fragmentation of the sesquiterpene backbone, resulting in different allylic cations and the oxirane cyclic group (FooDB; Eschenbrenner-Lux & Kumar, 2014; Demarque et al. 2016). Although isolated compounds appeared to be pure on HPLC-DAD (≥85%), there may have been other

isomers of the compound absorbing UV very close to one another and/or degradation between when HPLC analyses were done and when MS/MS data was taken. For this reason, we could only confidently report product ions, and thus confirm identification, for **F4**.

Table 2-4. The chemical name, formula, optimal absorbance (nm), structure, and average molecular weight (MW) of flowerhead isolates.

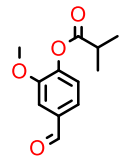
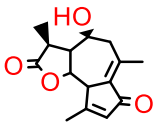
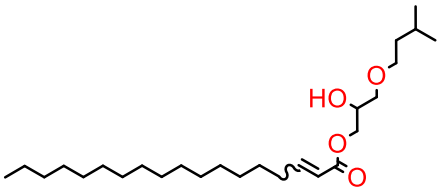
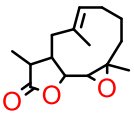
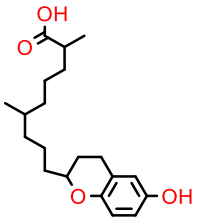
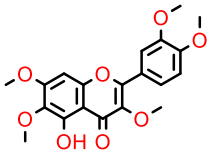
Isolate #	Absorbance (nm)	Formula and structure	Average MW (g/mol)
F1: Vanillin isobutyrate	330	 $C_{12}H_{14}O_4$	222.24
F2: Austricin	268	 $C_{15}H_{18}O_4$	262.30
F3: 1-O-isopentyl-3-o-octadec-2-enoyl glycerol	268	 $C_{26}H_{50}O_4$	426.68
F4: Dihydroparthenolide	268	 $C_{15}H_{22}O_3$	250.33
F5: 9'-carboxy-α-chromanol	330	 $C_{20}H_{30}O_4$	390.56
F6: Artemitin	268	 $C_{20}H_{20}O_8$	388.37

Table 2-5. Differences between the measured and theoretical masses in ppm of flowerhead isolates. Accurate masses and MS/MS data were determined using TOF-MS-ES+ and TOF MS/MS ES+ respectively, in which the positive adduct was Na^+. Characteristic fragment losses are highlighted in bold.

Isolate #	Monoisotopic mass	$[M+H]^+$	Theoretical mass $[M+Na^+]$	Measured mass $[M+Na^+]$	Difference between theoretical and measured values (delta ppm)	Product ions (*m/z*)
F1	222.0892	223	245.0784	245.0791	3	-
F2	262.1205	263	285.1103	285.1114	3.9	-
F3	426.3709	427	449.3601	449.3607	3	-
F4	250.1569	251	273.1461	273.1464	2	251; 250; 222; 221; 219; 162; 151; 146; 162; 95; 83; 51
F5	334.2144	391	413.2662	413.2670	2	-
F6	388.1158	389	411.1056	411.1054	-0.5	-

2.4.6 Enzyme inhibition of aqueous phases and identified DCQAs

Although the aqueous fraction of all tested plant parts were less inhibitory toward both FAAH and MAGL than their corresponding organic fractions (Figure 2-3), the aqueous fractions represented the majority of the hydroethanolic extracts by weight (80-90%) so likely contribute to the bioactivity of crude yarrow preparations. Moreover, DCQAs – which are produced abundantly by roots, flowerheads and leaves, alike (Figure 2-4) – accounted for most major peaks observed in the HPLC chromatograms of all three aqueous phases (Figure 2-6). We therefore decided to test commercial DCQA standards for their FAAH and MAGL inhibition, rather than isolating them from fractions. CGA was found in all plant parts, but had already been tested for inhibition of FAAH by Rui (2019), for which it was a weak inhibitor.

Here, we present inhibition data for four DQCA isomers produced by yarrow, all of which inhibited both enzymes to varying degrees (Figure 2-11). Overall, 1,3-DCQA had the highest %inhibition for FAAH at the 10 µg/mL, and all four surpassed 50% inhihbition at concentrations ≤ 50 µg/mL (Figure 2-11A). Also, 4,5-DCQA nearly had a plateau in activity across concentrations. MAGL bioassay results (Figure 2-11B) showed that 1,3-DCQA and 1,5-DCQA showed the most inhibition at the lowest concentration. 1,3-DCQA also had a plateau between 10 µg/mL and 25 µg/mL.

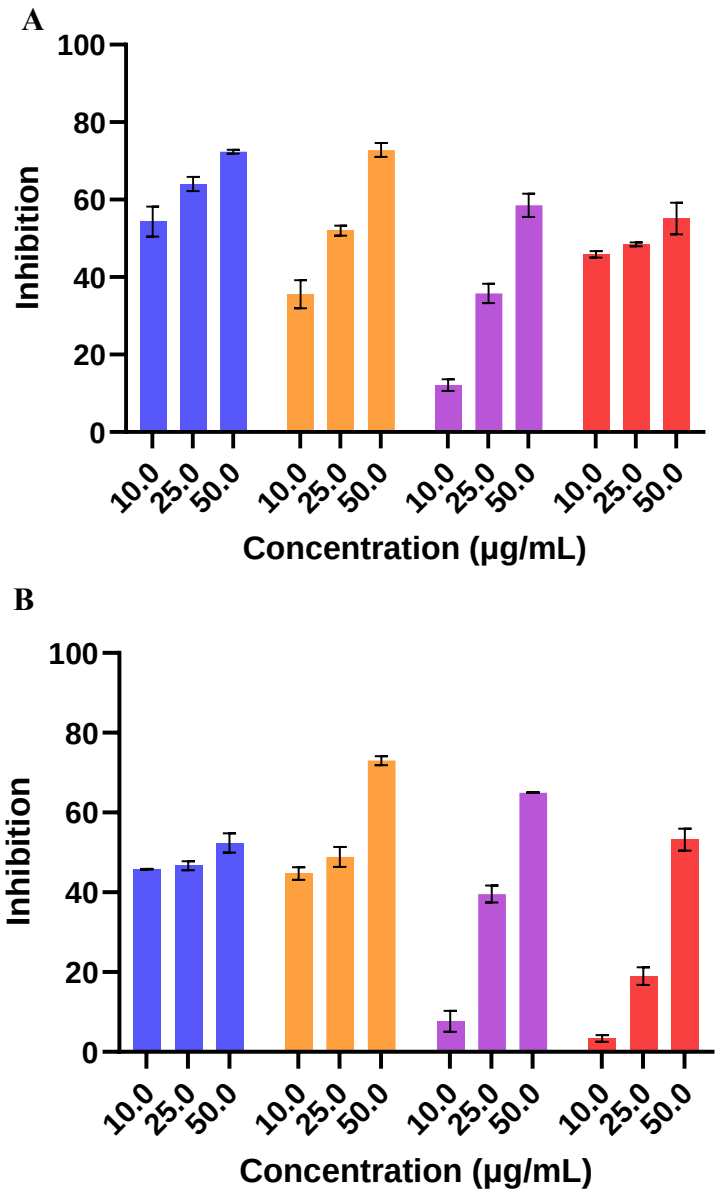


Figure 2-11. The mean percent inhibition (±SEM) of FAAH (A) and MAGL (B) among DCQAs (n=4) found in *A. millefolium*. 1,3-DCQA (blue), 1,5-DCQA (gold), 3,5-DCQA (purple), and 4,5-DCQA (red) were grouped by concentration (µg/mL). Inhibition was analyzed at 10 µg/mL, 25 µg/mL, and 50 µg/mL in-well for FAAH and MAGL.

2.5 Discussion

At the screened concentration to observe the activity of crude extracts for FAAH and MAGL, all plant parts were more selective for the former than the latter - a trend upheld throughout the bioassays in this chapter. In both bioassays, plant parts performed very similarly (near 100% and 63% to 69% for FAAH and MAGL, respectively (Figure 2-3)). Our results agreed with what was reported by Kachura in 2018; the roots and flowerheads inhibited the most, while the leaves followed closely. They also saw that flowerheads were slightly more active than the roots and leaves in the MAGL assay. As for the bioactivity of isolated organic and aqueous fractions (Figure 2-5), while the former almost entirely inhibited both enzymes, the latter was dominated by the inhibition shown by flowerheads (45.2% and 91.1% for FAAH and MAGL, respectively).

The potency of the aerial parts we observed can be linked to yarrow's important role in ethnobotany as a panacea (Applequist & Moerman, 2011). Traditionally, above-ground (aerial) parts were harvested over the roots, and their oily and hydroethanolic extracts were used in balms or as a poultice to be applied topically to open wounds, providing anti-inflammatory and antimicrobial relief (Moerman, 1998; Chandler, 1982; Jalali et al. 2012; Tadić et al. 2017). Likewise, the infusions and decoctions done with yarrow aerial parts in water helped bioactive compounds diffuse into the drink for consumption for the relief of respiratory illness and digestive issues (Moerman, 2003; Arnason et al. 1981; Waugh, 1916; Rousseau, 1945; Mechling, 1959). Data from Asyakina et al. (2021) and Radulović et al. (2015) suggested that a higher amount of phenolics and of elutions from the organic phase tend to contribute the most to yarrow's anti-inflammation potential. Seeing as there were many bioactives in our aqueous and organic fractions in the form of phenolics (CQAs) and non-polar compounds (mainly AKAs), our

data follows those trends in bioactivity. data supports yarrow's therapeutic use in North American ethnobotany, shown by their ability to inhibit both FAAH and MAGL

The chromatograms for liquid phases of each plant part showed CQAs eluting earlier on, compared to non-polar compounds like AKAs – a gradient usually seen in the essential oils of *A. millefolium* (Candan et al. 2003; Rohloff et al. 2000). The gap between phenolics and non-polar compounds is most apparent in the roots, where there is a clear delineation between peak groups before and after 16 minutes in Figure 2-4 (absorbing most at 330 nm and 268 nm, respectively), which were well separated in their respective elution zones in the liquid fractions of Figure 2-6. The relative peak intensities of the roots showed a more intense spectrum in the 268 nm analysis compared to the flowerheads and leaves, whose 330 nm spectrum was much higher.

Most major peaks in plant parts' aqueous phases had been identified using reference standards. Seeing as inhibition data supported organic fractions being most inhibitory, we pursued the sub-fractionation of root and flowerhead organic extracts for compound isolation.

Isolating compounds from bioactive sub-fractions of roots and flowerheads was desired since the non-polar molecules they contained, like AKAs, have shown evidence of bioavailability. Those isolated from *Echinacea* spp showed rapid absorption in the blood 30 minutes after oral administration (Woelkart et al. 2005a, 2006). Matthias et al. (2005) showed that isobutylamides they tested diffused across the blood-intestinal barrier using a Caco 2 monolayer *in vitro*, with up to 80% uptake. In contrast, CQAs showed poor permeability across the barrier, with nearly 5% diffusing the monolayer after 90 minutes. An *in vitro* and *in vivo* study by Veryser et al. (2016) showed that an oral administration of pure AKAs showed high

intestinal absorption and was present in the blood, liver, and brain. It demonstrated a high blood-brain permeation rate, suggesting AKAs can act as modulators of the ECS in the nervous system.

Compound isolation from the roots only yielded AKAs, which, until our study, had not been isolated from *A. millefolium* to be tested for their inhibition of FAAH and MAGL. We found that isolates effectively inhibited enzymes, strengthening their reported bioactivity profile, which is not yet reported in the literature. We provided some of the first evidence of their potential role in the ECS.

Although lacking for yarrow, there is data from *Achillea* spp and *Asteraceae* showing that some of the members of the AKA subgroups we isolated have reported bioactivities. A study by Sasagawa et al. (2006) found that extracts from *E. purpurea* L. (*Asteraceae*) containing isobutylamides, including **R1**, reduced IL-2 production, but not cell viability, between 6.25 µg/mL to 25 µg/mL in human Jurkat T cells, but cytotoxicity increased at concentrations of 50 µg/mL and 100 µg/mL of extract. Vieira et al. (2022) studied isobutylamides, along with methylamides (**R6**) from *Echinacea purpurea*, which effectively lowered inflammation in lipopolysaccharide (LPS)-stimulated THP-1 human macrophages by reducing inflammatory cytokines IL-6 and IL-1β. AKA cytotoxicity of extracts containing AKAs was also unapparent until >200 µg/mL, suggesting those we isolated may be candidates for future enzyme inhibition work in the THP-1 cell line. Overall, however, **R3** would be a better candidate for cell work due to lower chances of being cytotoxic to achieve 50% FAAH inhibition at 26.45 µg/mL. Another study showed that methylamides could inhibit FAAH, MAGL, and even AEA cellular uptake in human U937 cells, reducing ligand degradation (Hajdu et al. 2014). Therefore, *in vitro* cell work should be pursued for those types of AKAs we isolated to study their ECS modulation potential.

Isobutylamides, isolated from *Achillea ptarmica* L. (*Asteraceae*) and *Spilanthes acmella* L. (*Asteraceae*), have also shown antiprotozoal and larvicidal activity. Althaus et al. (2014) reported that pellitorine (**R3**) was the most active against *Plasmodium falciparum* Welch. (Plasmodiidae), a malaria-causing parasite, with an IC_{50} = 3.3 µg/mL; others showed 50% mortality (LD_{50}) towards *Aedes aegypti* (Culicidae) larvae at 6.25 µg/mL in Ramsewak et al. (1999). Seeing as the isobutylamides we isolated, **R1**, **R2**, and **R3**, had higher IC_{50} values against FAAH and MAGL, their antiprotozoal and antiparasitic properties are much more potent, naturally, given their role in plant defence.

Another study investigated COX and LOX inhibition by piperideides (**R4**) and piperidides, including **R4** and **R5**, respectively, which were among the compounds we reported MS/MS data for. Isolated from the organic extracts of *A. millefolium* and *Achillea* spp., Müller-Jakic et al. (1994) reported the inhibition for COX (50 µg/mL) by piperideides as 36%-38%, while that of LOX (11.5 µg/mL) was 6-27%. One Piperidide did not inhibit LOX, while the other showed 64% inhibition. Also, their COX %inhibitions were 21%-25%. Considering that the IC_{50} values we reported for **R4** and **R5** with FAAH were lower than the in-well concentration used to inhibit COX (50 µg/mL), we showed that **R4** and **R5** are more effective at inhibiting FAAH and MAGL than COX and LOX.

Lastly, with evidence of the anti-tumour potential of phenylethylamides like **R7** isolated from *Anacyclus pyrethrum* L. (*Asteraceae*) in a previous study of theirs, Jiang et al. (2020) proposed the synthesis of a synthetic phenylethylamide. Their compound had an anti-neoplastic effect in a cell tumour environment of four human cancer cell lines and was easy to synthesize. The data we provided in this thesis chapter can also guide researchers studying the ECS who want to synthesize potent AKAs and bypass the time- and resource-consuming process of

extracting them from plant material. Synthesizing certain AKAs based on their bioactivity can also optimize their selection process based on the needs of researchers.

Root isolates showed much more potential for FAAH and MAGL inhibition than flowerheads. Metabolites isolated from the latter were largely inactive, and as opposed to the roots, only one potent inhibitor for MAGL showed dose dependence (dihydroparthenolide (**F4**)). Isolates **F1**, **F2**, and **F5** showed no inhibition of FAAH or MAGL and have little to no bioactivity data in the literature. Compound **F1**, vanillin isobutyrate, was inactive. It is derived from vanillin, to which yarrow flowerheads may owe their sweet scent. **F2**, austricin, an STL, was also inactive with FAAH and relatively so with MAGL (20.52% and 25.85%, respectively). Previously isolated from *A. millefolium*, it had an anti-inflammatory role in LPS-stimulated RAW 264.7 mouse macrophage cells, but another study suggested it lacked inhibition of the pro-inflammatory COX-2 pathway – one that the ECS can downregulate (Li et al. 2021; Pandey et al. 2009). Our data suggests that the anti-inflammatory potential of F2 may not be related to its inhibition of FAAH and MAGL since macrophages express CBs and, therefore, enzymes of the ECS enzymes (Zhang & Chen, 2008; Schmidt et al. 2008; Li et al., 2021). **F5**, 9'-carboxy-α-chromanol, a 1- benzopyran, a metabolized version of the vitamin E homologue α-tocopherol, showed no inhibition towards FAAH nor MAGL. Since tocopherols have shown anti-inflammatory potential (Jiang et al. 2001), reference standards could be used to detect them in yarrow samples and test them for FAAH and MAGL inhibition.

F3 is a glycerolipid known as a 1,3-acylglycerol, similar in structure to the endogenous MAGL ligand 2-AG, possibly explaining its favourable interaction with MAGL rather than FAAH, although weak (FooDB, 2024). It is suspected of being among other bioactive glycerolipids within *Punica granatum* (*Lythraceae*) (Fatope et al. 2002; Wu et al. 2017). **F6** was

previously shown to have effective anti-cancer and anti-hypertensive capacity (Vo et al. 2022; De Souza et al. 2011). Although displaying low inhibition compared to the root isolates, **F6** was the second most inhibitory compound towards MAGL of the yarrow flowerhead isolates (35.87%).

F**4**, dihydroparthenolide, an STL, was the only flowerhead isolate for which an IC_{50} could be found for MAGL inhibition (26.18 µg/mL (CI ±95% of 23.12 µg/mL and 29.48 µg/mL)). It has been previously reported in *A. millefolium* aerial parts (Ulubelen et al. 1990). Although no bioactivity data has been reported on dihydroparthenolide until our study, except for data from Marles et al. (1992) showing parthenolide derivatives having the potential to inhibit platelet aggregation, there is data on its dehydrogenated version, parthenolide. Isolated from *Tanacetum vulgare* L. (*Asteraceae*), parthenolide from aerial parts was the most active isolated compound from that species that contributed to anti-inflammation (Schinella et al. 1998). Given **F4**'s lower IC_{50} of 26.18 µg/mL, similar to those of **R3** and **R4,** it is a good candidate for future *in vitro* cell work.

The activity in flowerhead isolates differed greatly from the %inhibitions observed by its mother sub-fraction, 42-53, which showed 100% for FAAH and 92% for MAGL (Figure 2-7). Despite most of the flowerhead isolates being inactive as individual compounds, it is possible that they synergistically inhibited FAAH and MAGL (Azas et al. 2002). It is also likely that we could not see certain bioactive compounds on HPLC-DAD due to them not containing chromophores, and thus not being able to reflect color onto the UV detector. While the sub-fraction we isolated from could be used in studies to test them for their bioactivity, there is a need to explore another analytical technique for the observation of non-light absorbing metabolites. Roots, on the other hand can be studied either in a matrix with the other isolates or separately, making the roots more efficient to survey with FAAH and MAGL.

As we saw in the aqueous extracts of roots, flowerheads, and leaves, CQAs were the outstanding compounds in each solution. 1,3-DCQA, 1,5-DCQA, 3.5-DCQA, and 4,5-DCQA inhibited FAAH and MAGL dose-dependently. The activity we reported reinforces evidence that aqueous solutions of yarrow contain important biomolecules that can inhibit FAAH and MAGL and thus potentially produce a therapeutic. We suggest that they may be responsible for most of the inhibition seen in FAAH and MAGL inhibition in aqueous fractions.

Data for 1,3-DCQA for MAGL and 4,5-DCQA for FAAH may suggest they are allosteric modulators. The activity of 4,5-DCQA across tested concentrations suggests it may be an allosteric antagonist of FAAH. It likely binds to an allosteric site, distinct from the orthosteric site where the substrate (AMC-AA) would bind, and slightly changes the conformation of the orthosteric site, changing the ligand's affinity for it (Price et al. 2005). Given the results, even as the concentration of inhibitor increased, the potential allosteric modulation exerted by 1,3-DCQA and 4,5-DCQA did not change much. This suggestion can be further developed by testing them at higher concentrations. At higher concentrations of inhibitor, if a %inhibition plateau is maintained, the compound may be using an allosteric site. Likewise, if increasing the amount of substrate does not change the %inhibition, then the compound would be exhibiting non-competitive inhibition of the enzyme (Zhang et al. 2018).

While no other research has been done to show ECS modulation by DCQAs, an extensive list of studies detailing their *in vitro* bioactivity exists. 1,3-DCQA, isolated from *Inula viscosa* L. Greuter. (*Asteraceae*), was an effective antioxidative compound in oxidative stress-induced cultured 3T3 and B16 mouse cell lines, which both carry CBs and thus FAAH and MAGL (Danino et al. 2009; Bouaboula et al. 2005; Sailler et al. 2014). This suggests that, as seen in Figure 2-8, 1,3-DCQA may have been a critical contributor in limiting FAAH and MAGL

degradation of the endocannabinoids AEA and 2-AG, thus allowing them to agonize CBs to suppress the production of inflammation-related cytokines like interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) (Parlar et al. 2021). Indeed, that same study found that 1,3-DCQA inhibited LOX dose-dependently, and the evidence we provide for that compound's inhibitory potential can help explain its activity to an extent. Preventing AEA and 2-AG degradation would also reduce the amount of substrate (AA) used in pro-inflammatory and, therefore, oxidative pathways like the LOX pathway. Notably, The primary source of AA for pro-inflammatory pathways comes from membrane phospholipid metabolism, not from that of endocannabinoids (Korotkova & Lundberg, 2014).

As for 1,5-DCQA, it has shown an antihypertensive effect in adult Wistar rats through vasorelaxation, a response typically mediated by CB_1s of the ECS (Hakkou et al. 2017; Lake et al. 1997). A lack of FAAH can contribute to induced hypotension by AEA, so inhibition for FAAH by 1,5-DCQA would likely decrease AEA degradation, allowing it to carry out the observed effect AEA would have been in a higher amount (Pacher et al. 2005).

The two weaker DCQAs tested in this study, 3,5-DCQA and 4,5-DCQA, have shown a variety of bioactivities. Chiefly, in *in vitro* cell models, they reduce oxidative stress by inhibiting LPS-induced expression of NO, along with the pro-inflammatory cytokines (IL-6) like COX-2 (Xu et al. 2024; Jang et al. 2021). Although 3,5-DCQA and 4,5-DCQA inhibited FAAH and MAGL less than 1,3-DCQA and 1,5-DCQA in our assays, they are still bioactive towards the enzymes and may have carried out their *in vitro* activity partly through the ECS. Since all four compounds were active in our bioassays, they should continue to be studied to see how they affect the ECS *in vitro*.

All isolated compound inhibition for FAAH and MAGL we showed in this study is novel. We ultimately discovered that the root isolates from the organic fraction of yarrow, composed entirely of AKAs, were potent inhibitors of FAAH and MAGL. On the other hand, Flowerhead isolates were not, aside from F4, which selectively inhibited MAGL. Lastly, DCQAs, present in roots, flowerheads, and leaves showed potent enzyme inhibition. Therefore, we identified some of the main contributors of FAAH and MAGL inhibition within yarrow that help elucidate its role in the ECS as inhibitors of FAAH and MAGL.

2.6 References

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CHAPTER 3: HPLC-DAD METHOD VALIDATION FOR THE QUANTIFICATION OF MAJOR BIOACTIVE METABOLITES IN *ACHILLEA MILLEFOLIUM* L ROOTS, FLOWERHEADS, AND LEAVES

3.1 Abstract

Achillea millefolium L. (yarrow, *Asteraceae*) is an important plant in North American ethnobotany, used for a wide-range of medicinal purposes and containing a variety of bioactive secondary metabolites. An optimized HPLC-DAD method for the quantification of bioactive compounds in the roots, flowerheads, and leaves of *A. millefolium* plant material was developed and validated. Providing an essential tool for the characterization of yarrow plant material, the method effectively separated and quantified 14 metabolites representing several classes of phytochemicals, including CQAs, flavonoids, AKAs, STLs, glycerolipids, and benzopyrans. This is the first time a single method was developed to separate and identify major peaks representing so many structurally diverse *Achillea* metabolites across different plant parts. We report the quantitation of 1-O-isopentyl-3-o-octadec-2-enoyl glycerol, dihydroparthenolide, 9'-carboxy-α-chromanol, artemitin, pellitorine, deca-2E,4E,8Z-trienoic acid piperideide. deca-2E,4E,8Z-trienoic acid piperidide, and deca-2E,6Z,8E-trienoic acid 2-methylbutylamide. The method showed good linearity of the UV response (between 0.991 and 0.999) through the tested concentration ranges. The limits of detection (LOD) and quantitation (LOQ) were between 0.81 ng and 18.01, and 1.5 and 54.6, respectively. The %intra- and %inter-day variations (0.89 – 3.30 and 0.95 – 9.01, respectively) demonstrated good method repeatability. Metabolites were compared between hydroethanolic extracts of leaf, root and flowerhead samples collected in the early and late growth season, with the later collection time showing the greater concentrations of most phytochemicals across plant parts. The roots presented the most phytochemical diversity, with AKAs, like pellitorine, the most abundant AKA, only detected in the roots. The flowerheads were richest in dihydroparthenolide, the most abundant compound in plant material. The leaves

were dominated by CQAs and contained the most CGA, which was the second most abundant compound across plant parts.

3.2 Introduction

Achillea millefolium L. (yarrow, *Asteraceae*) is a member of the largest family of vascular plants: the Daisy family. Nearly 200 species of *Achillea* exist worldwide, with *A. millefolium* having three subspecies., of which *A. millefolium* subsp. *millefolium*, introduced to North America from Eurasia, has three varieties (var.) (Alie et al. 2017; Guo et al. 2004, 2005). The *A. millefolium* aggregate, regarded as the "crown group" of the *Achillea* genus as it is noted for its apomorphism and widespread presence, has had an important role in the ethnobotany of North America (Guo et al. 2005). Past meta-analyses showed that not only has *Asteraceae* been one of the most widely selected families for medicinal use around the world, but *Achillea* species, along with *Artemisia*, have been over-selected for their medicinal properties as well (Kachura & Harris, 2018; Moerman, 1999; Leonti et al. 2003; Phumthum & Barfod, 2019; Moradifa, 2021; Hussain, 2024). Most notably, *Achillea spp.* has been used to help treat pulmonary and orthopedic conditions, in addition to gastrointestinal issues and inflammation, and has also played a spiritual role in ceremonies to cleanse the spirit from negativity (Moerman, 1998). The knowledge acquired on the multifaceted uses of yarrow has led researchers to describe it as a panacea – properly suggesting it is a highly versatile medicinal plant. In addition to other reported bioactivities, yarrow extracts and metabolites were shown to inhibit endocannabinoid degradation in Chapter 2 of this thesis, revealing the ECS as a potential mechanism underlying yarrow's diverse medicinal uses (Applequist & Moerman, 2011; Coxe, 1915; Barriga, 1992; Bussman, 2007)

A. millefolium produces several secondary metabolites to which it owes its bioactivity: CQAs, flavonoids, sesquiterpenes lactones (STL), and AKAs (Barda et al. 2021; Ali & Gopalakrishnan, 2017). Common phenolics in yarrow include the CQAs CGA and various

DCQAs, and the flavonoids apigenin, luteolin, and kaempferol. They are mainly found in the leaves and flowerheads and are responsible for the plant's response to abiotic and biotic stressors (Vitalini et al. 2011; Benetis et al. 2008a, 2008b), including osmotic stress, antipredation, and antioxidation, and the formation of symbiotic relationships with nitrogen-fixing bacteria (Weidner et al. 2007; Harrison et al. 2008; Niazipoor et al. 2024; Dragoeva et al. 2015). Outside of their roles in plants, their pharmacological activity encompasses anti-inflammation, antimutagenicity, and neuroprotective potential (Lee et al. 2007; Yoshimoto et al. 2002; Kwon et al. 2014; Kim et al. 2005).

Similarly to CQAs and flavonoids, STLs can be found in the flowerheads and leaves, where they act as antimicrobial agents to reduce oxidative stress (Aljančić et al. 1999; Hausen et al. 1991; Rauchensteiner et al. 2004; Slobodan et al. 1999). STLs are also antifeedants to chewing insects and birds and can stimulate seed germination (Fraga et al. 2021; de Luque et al. 2000). Medicinally, they have shown hypolipidemic, anticarcinogenic, and anti-inflammatory potential (Chadwick et al. 2013). Lastly, AKAs are lipid signalling molecules involved in the organism's signal perception, while also influencing primary root and lateral root growth, and root hair elongation (Ramírez-Chávez et al. 2004; Morquecho-Contreras et al. 2011). They also have antimicrobial properties (Molina-Torres et al. 1999, 2004). Pharmacologically, they possess antithrombotic, anticancer, vasoprotective, and, alongside CQAs and flavonoids, posses suspected cannabimimetic properties, the latter of which was supported in Chapter 2 of this thesis (Spelman et al. 2011; Simas et al. 2013; Kachura Master's 2018; Liu et al. 2019).

Given the cosmopolitan distribution and use of this medicinal species, a single HPLC-DAD method was developed and validated for detecting and quantifying secondary metabolites in roots, flowerheads, and leaves. Among yarrow metabolites, phenolics (including flavonoids)

have been analyzed and quantified more extensively compared to its other chemical groups (Walker et al. 2000; Vitalini et al. 2011; Czech et al. 2023; Dias et al. 2013; Benetis et al. 2008b). Detection and quantitation of CQAs and flavonoids in other species of *Achillea*, like *A. Setecea*, *A. arabica*, and *A. cappadocica,* and the *Asteraceae* species *Echinacea purpurea* L., *Artemisia dracunculus* L., and *Espeletia barclayana* L. showed those compound groups to be in high quantity (Raudone et al. 2022; Tajner-Czopek et al. 2020).

As a group of compounds characteristic of the *Asteraceae*, several sesquiterpenes have been previously analyzed in *Achillea* samples as well (Seaman 1982; Montsko et al. 2008; Rauchensteiner et al. 2002; Trifunović et al. 2005; Trendafilova et al. 2006). In Chapter 2 of this thesis, we found that the STL dihydroparthenolide inhibited the serine protease MAGL. Phytochemical profiling of AKAs in *Achillea* spp. has shown a wide range of structural sub-groups, including isobutylamides (pellitorine and anacycline), methylamides, piperidine alkaloids, and phenylethylamides (Veryser et al. 2017; Althaus et al. 2014; Lazarević et al. 2010). Other notable species within *Asteraceae* with AKAs are *Echinacea* spp. (echinacein), *Acmella* spp. (spilanthol), and *Heliopsis* spp. (capsaicin) (Veryser et al. 2017; Boonen et al. 2012; Hohmann et al. 2011; Jacobson 1954; Chen et al. 2022; Cheng et al. 2015; Molina-Torres et al. 1996, 1999; Guetcheung et al. 2018).

In this study, we developed and validated an HPLC-DAD method for phytochemical analysis of *A. millefolium* roots, flowerheads, and leaves in the early and late growth season to accomplish two objectives: i) to separate major secondary metabolites detected in all three plant parts using a single method, and ii) to identify, quantify and compare metabolites in different yarrow plant parts. We successfully identified and quantified six CQAs, one flavonoid, one STL, four AKAs, one benzopyran, and one acyl glycerol in the crude extracts of yarrow roots,

flowerheads, and leaves. Such a validated method, which spans a variety of bioactive phytochemical classes found in different plant tissues, was previously unavailable.

3.3 Materials and Methods

3.3.1 Materials

Analytical grade HPLC solvents and trifluoroacetic acid (TFA) were purchased from MilliporeSigma (Darmstadt, Germany). The CGA, kaempferol, luteolin, luteolin-7-*O*-glucoside, apigenin, apigenin-7-*O*-glucoside, and rutin standards were purchased from Cayman Chemical (Ann Arbor, MI, USA) and standards of 1,3-DCQA; 1,4-DCQA; 1,5-DCQA; 3,5-DCQA; 4,5 DCQA, artemitin, and pellitorine were purchased from TargetMol (Boston, MA, USA). Reference standards of apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, rutin, and their corresponding aglycones were obtained from Extrasynthese (Lyon, France). All standards were solubilized in liquid chromatography-mass spectrometry-grade MeOH. The remaining compounds were isolated in-lab and identified following the HPLC-DAD and MS protocols described in Chapter 2.

3.3.2 Sample collection and extraction

Sample collection and preparation are as described in Chapter 2. Briefly, *A. millefolium* leaf, flowerheads and root samples were collected in May and October from the same population near the University of Ottawa main campus (Ottawa, Ontario). Samples were cleaned, dried using a food dehydrator at 37°C (forced air), and stored in the freezer (-20°C) until experimental use. Prior to extraction, dried material was ground by Wiley Mill (mesh size 20) and 500 mg was extracted twice by sonication (2x15 mins) in 70% ethanol (2x25mL). After centrifugation (10 mins at 3900 rpm), pooled supernatants were titrated to 50 mL in volumetric flasks with 70%

ethanol. One mL of each extract was filtered (PTFE syringe filter) into HPLC vials and stored at -20°C prior to HPLC analysis.

3.3.3 HPLC-DAD analyses

This method represents the final optimized conditions for targeted HPLC-DAD analyses after testing several experimental parameters, including mobile phase ratios, runtime length, UV-detection spectra, HPLC column and oven temperature, and solvent flow rate. Analyses were done on an Agilent 1100 series HPLC-DAD system (Agilent Technologies, Santa Clara, California, USA) using ChemStation 3.02B. The column was washed with 5 column volumes of both 100% Milli-Q water:0.1% TFA (mobile phase A) and 100% acetonitrile:0.1% TFA (mobile phase B). Solvents flowed at 400 µL per minute on a Kinetex PS C18 LC column (100 mm x 2.1mm ID, 2.6 µm particle size). The column temperature was maintained at 50°C during a mobile phase gradient of 2.5% acetonitrile in water at 0 min, 23% at 15 min, 70% at 25 min, and 100% between 26 and 30 min., then returned to initial conditions in 0.1 minutes, leaving a total runtime of 30.10 minutes. Prepared extracts were sonicated for 5 minutes and injected at 1 µL via autosampler in triplicate.

3.3.4 Method validation and quantitation of metabolites

The optimized HPLC-DAD methodology was used to validate the reproducibility and sensitivity of metabolite detection and quantitation and to quantify 14 compounds detected at varying levels in yarrow plant part extracts.

3.3.4.1 Sensitivity

Linear calibration curves were generated by injecting serial dilutions of each compound at concentrations ranging from 0.625 µg/mL to 500 µg/mL, yielding an R^2 value for each compound. The standard error (SE) of the intercept was multiplied by the square root of the

number of concentrations used per compound to yield the standard deviation of the intercept, giving the following equation:

$$SD = SE(\sqrt{N})$$

To obtain the LOD and LOQ, the SD of the intercept was divided by the slope and multiplied by either 3.3 (LOD) or 10 (LOQ), in the following equations:

$$LOD = 3.3\left(\frac{SD}{slope}\right)$$

$$LOQ = 10\left(\frac{SD}{slope}\right)$$

Since some isolated compounds did not have commercially available reference standards, their respective %purity was multiplied by the signal-to-noise ratio of 3.3:1 and 10:1 to obtain their relative LOD and LOQ, respectively. Coefficients of variation were calculated with the mean signal-to-noise ratio per triplicate injection.

3.3.4.2 Repeatability

Method repeatability was assessed by determining the intra and intrer-day variations between same-sample injections to give the coefficient of variation (CV (%)), which expresses the SD as a percentage of the mean. To calculate the intraday variation, the SD of results obtained within the same day were divided by the mean of those results multiplied by 100 to yield a percentage:

$$CV(\%) = \left(\frac{SD}{mean}\right)100$$

The inter-day variation was determined with the same equation by taking the SD of results across different days divided by the mean across those days instead.

3.3.4.3 Compound quantification

Calibration curves were generated for each compound by using their optimal UV absorbance (268 nm or 330 nm) to calculate the area under the curve of each compound in HPLC-DAD chromatograms with concentrations ranging from 0.625 µg/mL – 500 µg/mL. Metabolites which did not have standards available were relatively quantified based on their %purity (calculation described above), multiplied by their respective calibration curve equation to give their corrected quantity in mg/g of dried plant part material. AKAs 11, 13 and 14, 1-O-isopentyl-3-o-octadec-2-enoyl glycerol, and dihydroparthenolide were quantified at 268 nm, while CQAs, artemitin, 9'-carboxy-αchromanol, and deca-2E,4E,8Z-trienoic acid piperideide were quantified at 330 nm. The intraday mean of compound quantities was calculated from triplicate injections (n=1) and the average of those means was used to calculate inter-day variation (n=3).

3.4 Results

This study aimed to develop a single analytical method for the separation, identification and quantitation of major metabolites in different plant parts of *A, millefolium.* Fourteen phytochemicals including six CQAs, one flavonoid, one STL, four AKAs, one benzopyran, and one acyl glycerol alongside ethanolic extracts of leaves, flowerheads and roots, were used for method development and validation.

3.4.1 Method optimization

To separate the 14 structurally diverse target metabolites (Tables 3-1 & 3-2) and accommodate the background chemical complexity of leaf, flowerhead and root extracts, the optimized HPLC method utilized a 30-minute gradient of 0.1% TFA MilliQ water and 0.1% TFA acetonitrile, a Kinetex PS C18 column at 50°C. For optimal sensitivity, UV absorbance was monitored at 268 nm and 330 nm for quantitation, depending on each compound's optimal absorbance wavelength (see Tables 3-1 & 3-2). Since all target compounds absorbed at 280 nm, that wavelength was chosen to visualize them in Figure 3-1. The red overlayed spectrum for the flowerheads makes the difference in phytochemistry between May and October apparent. The figure shows the separation and identification of all fourteen studied metabolites in root, flowerhead and leaf extracts using the optimized method. Compounds were numbered in order of elution based on R_t. Notably, TFA afforded better peak shape than non-acidified mobile phases and acetonitrile afforded superior peak separation and a shorter gradient time than methanol. Due to the structural diversity of target metabolites, baseline separation of all 14 compounds was not achieved with alternative gradients extending as long as 36.1 minutes. The optimized total run time of 30.1 minutes nonetheless achieved reliable and adequate separation of metabolites for UV-based identification and quantitation.

Previously reported flavonoid glycosides apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, and rutin were not detected in samples. Injected reference standards eluted between 8-13 minutes but all major peaks corresponded to DCQAs based on spectral comparison the extract and reference standard peaks. -

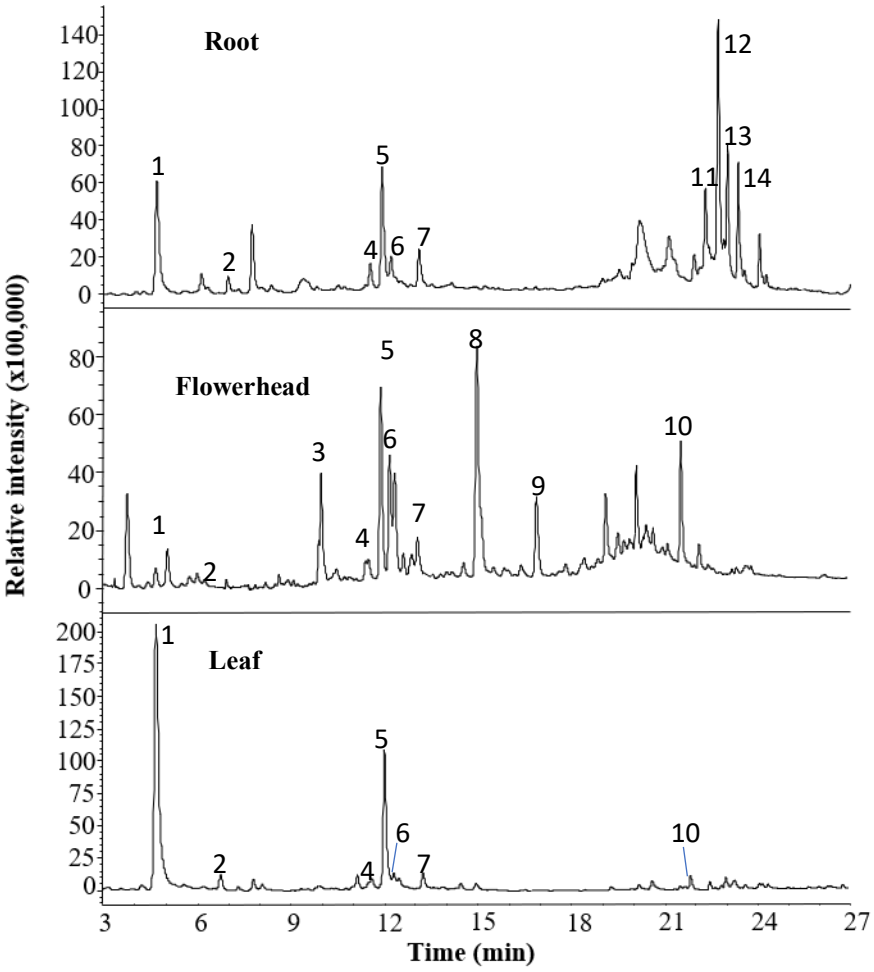

Figure 3-1. Chromatograms of roots, flowerheads, and leaves at 280 nm.

3.4.2 Method validation

3.4.2.1 Sensitivity and precision

Compounds identified in ethanolic extracts of crude yarrow roots, flowerheads, and leaves with purchased standards and compounds isolated in-lab are shown in Tables 3-1 & 3-2, respectively. Analytes were numbered in order of elution, shown by their R_t. Linearity (R^2) of UV response for all compounds was >0.99, signifying a strong positive correlation between concentrations and responses for the tested compound concentration ranges (0.625-500.0 µg/mL).

Method sensitivity was determined through the limits of detection and quantitation of all fourteen metabolites. Standards compounds in Table 3-1 had LODs of ≤ 3.1 ng and LOQs ≤ 10.2 ng. For isolates, the %purity of isolated compounds was calculated and their values were multiplied with their signal-to-noise ratio to obtain their relative LOD and LOQ. Results in Table 3-2 showed that isolates had LODs ≤ 18.01 and LOQs ≤ 54.6 ng, with 13 showing higher values (134 ng (LOD) and 404 ng (LOQ). Compounds consistently had LOD values three times lower than LOQs.

Method precision was assessed by calculating the coefficient of variation between intraday and inter-day experiments, which did not exceed 3.30% (**3**) and 9.01% (**7**), respectively. The higher %variance of the inter-day runs was to be expected, with three analytes varying >6% (**6** (8.99%) **7** (9.01%), and 9 (6.53%).

Table 3-1. HPLC-DAD validation data of reference standards identified in extracts of yarrow roots, flowerheads, and leaves, in order of elution. The optimal UV absorbance (nm) at which validation was done for each compound is given, and compound purity was ≥99%. The R^2 value of linear regression of concentration ranges (µg/mL) and LOD and LOQ values of all identified compounds are presented, along with %intraday and %interday variation (±SEM), n=10).

#	Name	Class	Absorbance (nm)	R_t	Range (µg/mL)	Linearity (R^2)	LOD (ng)	LOQ (ng)	%intraday variation	%interday variation
1	CGA	CQA	330	4.9	0.625-500.0	0.999	1.2	2.9	0.95 (0.5)	1.61 (1.1)
2	1,5-DCQA	CQA	330	7.8	0.625-500.0	0.999	0.81	2.43	1.4 (0.4)	0.95 (0.7)
4	1,4-DCQA	CQA	330	10.3	0.625-500.0	0.999	0.5	1.5	2.83 (0.4)	2.29 (0.2)
5	3,5-DCQA	CQA	330	11.7	0.625-500.0	0.999	3.1	10.2	2.0 (0.5)	4.58 (0.5)
6	1,3-DCQA	CQA	330	12.2	0.625-500.0	0.999	1.3	3.9	1.29 (0.4)	8.99 (0.3)
7	4,5-DCQA	CQA	330	13.4	0.625-500.0	0.991	2.6	8.0	1.25 (0.3)	9.01 (0.5)
10	Artemitin	Flavonoid	330	22.0	0.625-500.0	0.999	1.4	4.4	2.3 (1.2)	4.31 (0.7)
11	Pellitorine	AKA	268	23.01	0.625-500.0	0.993	2.1	6.5	0.9 (0.1)	1.54 (0.4)

Table 3-2. HPLC-DAD validation data of isolated compounds identified in extracts of yarrow roots, flowerheads, and leaves. UV absorbance (nm) at which validation was done for each compound is given, and compound purity was ≥85%. The R^2 value of linear regression of concentration ranges (µg/mL) and LOD and LOQ values of all identified compounds are presented, along with %intraday and %interday variation (±SEM), n=10).

#	Name	Class	Absorbance (nm)	R_t	Range (µg/mL)	Linearity (R^2)	LOD (ng)	LOQ (ng)	%intraday variation	%interday variation
3	1-O-isopentyl-3-o-octadec-2-enoyl glycerol	Glycerolipid	268	10.3	0.625-500.0	0.999	18.01	54.6	3.30 (0.6)	5.83 (0.6)
8	Dihydroparthenolide	STL	268	15.2	0.625-500.0	0.996	12.8	38.9	2.10 (0.4)	4.51 (0.8)
9	9'-carboxy-α-chromanol	1-Benzopyran	330	17.2	0.625-500.0	0.999	4.86	14.7	2.50 (0.2)	6.53 (0.7)
12	Deca-2E,4E,8Z-trienoic acid piperideide	AKA	330	23.5	0.625-500.0	0.996	2.27	6.86	2.05 (0.3)	5.59 (0.6)
13	Deca-2E,4E,8Z-trienoic acid piperidide	AKA	268	23.8	0.625-500.0	0.995	134	404	2.17 (0.9)	4.39 (0.8)
14	Deca-2E,6Z,8E-trienoic acid 2-methylbutylamide	AKA	268	24.2	0.625-500.0	0.995	12.5	37.9	0.89 (0.2)	5.32 (1.0)

3.4.3 Metabolite profiles of early- and late-season yarrow roots, flowerheads and leaves

To apply the developed methods to crude yarrow extracts, a comparison was made between the phytochemistry of yarrow roots, flowerheads, and leaves collected in May, during the late vegetative to early flowering stages, and the late (seeding) growth season of October 2022. Overall, the end-of-season extracts had much more phytochemical diversity and more analytes. Aside from **5,** all metabolites, if initially present in the early season, were less abundant than in extracts of the late season.

3.4.3.1 Roots

The roots had the most phytochemical diversity of the tested plant parts in early- and late-season extracts, with most of the targeted compounds detected (ten and eleven different metabolites observed per timepoint, respectively). The early-season extracts showed CQAs and AKAs only, while the STL dihydroparthenolide (**8)** was detected and quantified in the late season. CGA (**1**) was the main metabolite in the early and late season samples (3.05 mg/g and 8.75 mg/g dried material, respectively) while compounds **1**, **4**, and **12** were the only ones detected at more than 0.1% (>1 mg/g) dried material in May and only **4**, **7**, and **8** were less than 0.1% (<1 mg/g) dried material in October extracts. Compounds **12**, **13**, and **14** were unique to the roots and had higher quantities in the fall, at which time **12** was the predominant AKA. All other metabolites in the roots were in higher quantities in the fall (Table 3-3).

3.4.3.2 Flowerheads

Flowerhead chromatograms differed markedly between May and October collections, with compounds **3**, **6**, and **10** detected in late-season – but not early-season – plant material. 1-O-isopentyl-3-o-octadec-2-enoyl glycerol (**3**) was uniquely detected in late-season flowerhead. DCQA **2** and the AKAs (**11-14**) in not detected in either collection (Table 3-3 and Figure 3-2).

Despite the lower chemical diversity in the early-season, flowerheads were the only plant part to show **8** and had the highest quantity of metabolites **1**, **4**, and **9** among plant parts. 1,4-DCQA (**4**) was the most abundant metabolite in early season flowerheads (6.81 mg/g dried material), decreasing to 3.76 mg/g in the late season. At that point in time, **8** was the most abundant metabolite in flowerheads and across plant parts (44.78 mg/g). Detected levels of other metabolites except for **8** and **9** declined from May to October.

3.4.3.3 Leaves

The leaves had the least phytochemical diversity of plant parts, only showing **1**, **2**, and **4-7** in spring, but did have the most of compounds **1**, **2**, **5**, and **7** compared to the roots and flowerheads. CGA was the most quantified metabolite in the leaves both in the early and late seasons (5.04 mg/g and 30.50 mg/g, respectively). All of its metabolites except for CGA declined in the fall, but they did show newly produced **8**, **9**, and **10** - the latter two being unique to them and the flowerheads.

Table 3-3. The quantification in dried yarrow root (R), flowerhead (F), and leaf (L) material (mg/g) are given for May and October collections, respectively. The optimal UV absorbance for the quantitation of each compound is the same as those presented for compound validation.

	Concentration in dried material (mg/g)					
#	R May	R October	F May	F October	Leaves	Leaves
1	3.05	8.75	5.04	1.41	4.29	30.50
2	0.12	1.71	N/D	N/D	2.06	0.20
3	N/D	N/D	N/D	4.13	N/D	N/D
4	1.99	0.35	6.81	3.76	1.08	0.46
5	0.61	4.34	2.68	2.55	8.87	7.16
6	0.14	1.69	N/D	1.89	1.03	N/D
7	0.24	0.96	0.91	0.49	1.14	0.575
8	N/D	0.71	1.88	44.7	N/D	3.24
9	N/D	N/D	0.34	1.845	N/D	0.46
10	N/D	N/D	N/D	2.15	N/D	0.51
11	0.38	2.05	N/D	N/D	N/D	N/D
12	2.93	8.17	N/D	N/D	N/D	N/D
13	0.33	1.94	N/D	N/D	N/D	N/D
14	0.21	1.34	N/D	N/D	N/D	N/D

N/D = Not detected

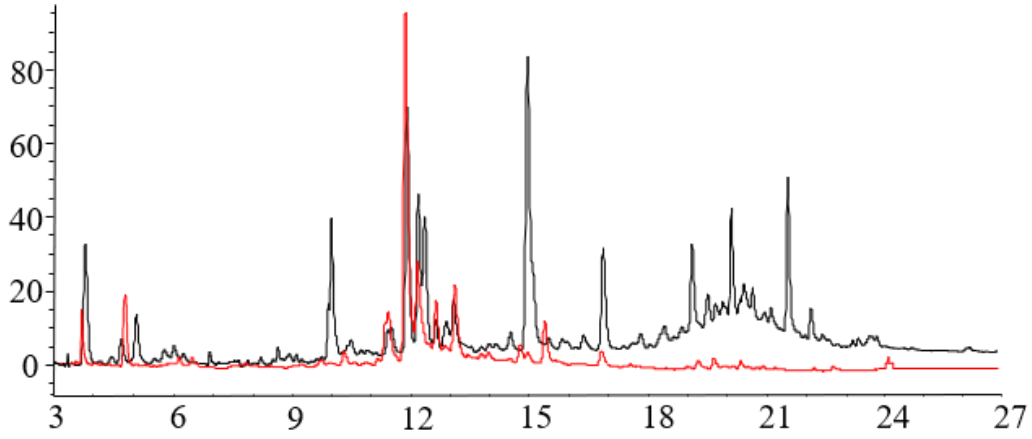

Figure 3-2. Chromatograms of flowerheads at 280 nm. The spectrum in black is from October while the red spectrum is from May.

3.5 Discussion

Method optimization effectively separated all 14 target metabolite peaks observed in yarrow root, flowerhead, and leaf chromatograms, as seen in their shared UV absorption at 280 nm in Figure 3-1. Adequate peak separation signified that compounds could be identified when comparing their R_t to that of the eight reference standards and six isolated compounds. Separation was important since initial attempts (unoptimized) resulted in signal overlap in extracts and among standards, such as **4**, **5**, and **6,** which eluted within two minutes of each other, and **11**-**14**, which eluted within one minute of each other.

The calibration curve of each analyte showed good linearity ($R^2 \geq 0.99$), and LOD and LOQ values for all compounds were within their limits. The method showed high repeatability with %variance of intraday data laying below the inter-day variance, which lay < 10%, indicating method precision. This data suggested that the concentration ranges of analytes given in Tables 3-1 and 3-2 demonstrated the intervals at which the method has an appropriate level of precision, accuracy, and linearity suitable for compound detection and quantitation using HPLC-DAD (Hubert et al. 2004, 2007; Araujo, 2009).

Compounds **3**, **8**, **11**, **13**, and **14** were detected and quantified at 268 nm, in the 260 nm to 270 nm range of absorbance for AKAs, STLs, and glycerolipids (Chen et al. 2022; Jessing et al 2009; Mendoza et al. 2012). For CQAs and artemitin (**11)**, a flavonoid, an optimal wavelength of 330 nm was used. Although not within the typically seen analytical range of 250-280 nm in the literature it allowed for better peak integration (Vitalini et al. 2011; Zheng et al. 2018). For HPLC systems without DAD using a single UV wavelength, 280 nm should be chosen to be able to detect and quantify all major metabolites, albeit with reduced sensitivity (i.e. high LOD and LOQ values). That is why chromatograms in Figure 3-1 are given at 280 nm.

Analyses of CQAs, starting with **1**, showed a LOD and LOQ of 1.2 ng and 2.9 ng, respectively, compared to 460 ng and 1520 ng previously reported by Benetis et al. (2008a) in a homogenized *A. millefolium* mixture, showing that our method is more sensitive to **1**. The method they suggested also starts at a solvent gradient of the mobile phases at 90% H_2O and 10% ACN, leading to a longer analytical runtime. The gradient we employed of 97.5% H_2O and 2.5% can made phenolic compounds elute sooner in the protocol, as seen with the elution of CGA in our protocol (4.9 mins) versus theirs (after eleven minutes). Another paper by Benetis et al. (2008b) showed CGA as having a mean of 8.03 mg/g total in aerial parts (stem, leaves, and flowerheads) during the flowering season – a time of year when we reported 5.04 mg/g of flowerheads. Benedek et al. (2007) reported that the mean of DCQA quantities across seeded wild yarrow populations across Europe was 0.84% of total yarrow content. Since plant collections were performed at unspecified time points, if they were harvested throughout the growth season, our collections yielded 3.7% total DCQA content in dried plant material.

The CQAs **5** and **7** showed 26.02 mg/g dry matter and 10.24 mg/g, respectively, for the flowerheads and upper leaves of wild yarrow in Dias et al. (2013) (unspecified time of year), compared to our calculated 9.71 mg/g and 1.07 mg/g, showing that **5** is produced more than **7** in the tested aerial parts, as we saw as well. The only other phenolic compound that was not a CQA, **10**, a flavonoid, typically seen in the genus *Artemisia* from *Asteraceae* but also found in yarrow, had yet to be quantified in *A. millefolium* until our study (de Souza et al. 2011). Kontogianni et al. (2020) showed that artemitin accounted for 2.12 mg/g of dried, whole *Artemisia annua* L. (*Asteraceae*), the least quantified of eleven total flavonoids from that plant, but comparable to what we quantified (2.66 mg/g).

Pedneault et al. (2013) noted that hydroponically grown yarrow showed flavonoids tended to accumulate 35 and 87 days (vegetative and early flowering stages, respectively) after sowing, while we collected early yarrow samples between those timepoints. Moreover, they reported that flavonoids were in meagre quantities in outdoor yarrow 122 days after sowing. Our late-season collection in October followed that downward trend in flavonoids since no apigenin, luteolin, kaempferol, and their glucosides could be detected and quantified in extracts. Samples should be collected when flavonoids are at higher levels in the future, especially during the June to November flowering season (Radušienė et al. 2023).

As for AKAs, our study is among the first to quantify them within *A. millefolium* and in *Achillea* spp. Since data is scarce, we can compare our results to other *Asteraceae* species. Spelman et al. (2009) analyzed four AKAs from *E. purperea* root using a method 13.1 minutes longer than ours and reported a mean of 1.6 mg/g dried material at 1:11 solute-to-solvent ratio in March, whereas our extracts yielded 3.47 mg/g at 1:10 in the May collection. Since AKAs are signal molecules involved in primary and lateral root growth and root hair elongation (Ramírez-Chávez et al. 2004), it is reasonable to suggest that AKAs would be in higher quantity at the beginning of the growth season within *A. millefolium* roots, while undergoing active growth, as seen in *E. purpuerea* (Stuart & Wills, 2000). In addition, Boonen et al. (2012) noted that AKAs from *Anacyclus pyrethrum* (unspecified collection time) accounted for 4.87% per 100g of dried material, compared to our 3 AKAs totalling 3.13%, and also found that pellitorine was the major AKA. In future, the variation of AKA quantities across the root structure can be looked at by separating the tips of the lateral roots (root apical meristem where primary growth happens) from parts of the roots closer to the stem of the plant. AKAs would be higher in the root tips, rather than parts of the roots closer to the stem of the plant where there is less active growth (Maslova

et al. 2013; Aloni et al. 2006). Additionally, AKA quantities from the roots of individual yarrow organisms can be compared to hypothesize how their respective AKA contents may affect the amount and growth of lateral roots.

As with AKAs, STLs have been detected but not quantified in *A. millefolium*. However, they have been quantified in the *Achillea* genus (Montsko et al. 2008). A study performed on *A. collina* L. and *A. pratensis* L. showed a mean of 0.523 g of STLs per 100 g dry material across 14 compounds from the flowers, while for **8,** we observed 2.24 g/100 g of dried flowerheads in the late season (Glasl et al. 1999). The STL **8** was the most quantified metabolite of all samples, uniquely found in the flowerheads. Dihydroparthenolide is an STL known to stimulate seed germination in *Striga* (witchweed, *Orobanchaceae*) when it was isolated from *Ambrosia artemisiifolia* (common ragweed, *Asteraceae*), and STLs, in general, typically serve as a defence mechanism against herbivory and pathogens, as players in allelopathy for the competition of resources that seeds desperately need to germinate in the coming growth year, and as a response to potential drought or excess UV radiation seeds may encounter which can inhibit their growth (Fischer et al. 1989, 1990; Burnett et al. 1974; Ivie et al. 1975; Macías et al. 1996). October samples had already past reproductive maturity, a process that contributes to the onset of senescence in aerial parts during which the flowers produced seeds, which acted as sinks for resources coming from source organs (stems and leaves) (Vico et al. 2016; Schippers et al. 2015).Therefore, its higher quantity in flower samples, where seeds are found in yarrow, is justified as it would increase seed viability in the coming growth year.

Certain ecological stressors were at play in October compared to May that could have influenced each plant part's phytochemical quantities. The yarrow we collected in October was undergoing senescence – the final growth stage during which the deteriorating organism must

ensure efficient energy use by adapting to several ecological stressors. This includes a shorter photoperiod, compared to May through August, which would see yarrow invest in more stem growth and leaf extension to maximize its sunlight exposure and outcompete neighbours for it. This would have reduced available phytochemicals in the flowerheads, further reducing the already scarce flavonoids due to the plant being less selective of UV radiation reduction at that time of year. Kachura (2018) also saw this trend in their fall collections, with flavonoids making up ≤0.1mg/g dry material, which were otherwise much higher between the end of June and the end of August (between 24 mg/g and 35 mg/g, respectively). This was likely due to yarrow being in the flowering stage of life at the time of collection when flavonoids tend to accumulate the most (Špinarová & Petříková, 2003). There would have also been less competition for sunlight in the summer, and the plant used this to its advantage to continue flowering, which led to more flavonoids being produced to protect against UV radiation. To support the aging and stressed organism, CQAs would have acted as antioxidants spanning the organism over, as we saw, especially in leaves (30.50 mg/g dry material of CGA in October) (Torras-Claveria et al. 2012; Holmes & Smith, 1977; Morgan & Smith 1976, 1978).

Additionally, as a perennial, yarrow must prepare for winter dormancy and remain alive until the next growth season. Given resources like nitrogen and phosphorus were likely scarcer during the fall than in spring, yarrow would have had to invest its energy in more root growth. (Struik, 1965; Maslova et al. 2013). This would explain the higher AKA content seen in later-season extracts as opposed to earlier ones from May - as root growth settled from active to reduced, and the need for AKAs as signals would have decreased.

Finally, **3** and **9** have not been previously detected or quantified in yarrow. Therefore, the validation data provided in Table 3-2 is newly reported data that shows high-accuracy results, allowing us to be confident in our reported data.

The validated HPLC-DAD method we proposed is a valuable tool for researchers to detect and quantify a wide variety of metabolic classes within yarrow, including CQAs, flavonoids, AKAs, STL, glycolipids, and potentially tocopherols (**9** is a precursor to them), all within the same method runtime. It also provides novel detection of compounds **3**, **8**, **9**, and **12** and their quantitation, along with newly discovered quantitation for **10**, **11**, **13**, and **14** in late-season samples of yarrow roots, flowerheads, and leaves. We provided the optimal wavelengths for proper detection and quantitation of all compounds in this study. We also showed that the validated method has good linearity, low LOD and LOQ, and low intraday and interday %variances. All of this was done in a timelier HPLC method runtime than currently published methods for *A. millefolium* while allowing for the broadest variety of analytes in extracts. This method is also transferable to HPLC systems with a single UV detector, and the optimal compound UV absorption wavelengths provided will enhance sensitivity.

With our current data, collections should be done at the end of the growth season at the same location with the same population of yarrow since that is when most of yarrow's bioactive metabolites accumulate. Samples can also be collected more frequently, starting when the frost subsides, until November, to establish more trends in the phytochemistry across that population more efficiently.

3.6 References

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CHAPTER 4 – GENERAL DISCUSSION

4.1 General Discussion

This thesis gave new insights into the bioactive metabolites responsible for some of the medicinal qualities of *A. millefolium* (Asteraceae) – a plant species deeply rooted in the ethnobotany of many regions worldwide. We also reported a validated analytical method developed for detecting and quantitating compounds within yarrow that can also be applied to other species sharing similar phytochemistry.

First, the reader is given a literature review about the many fascinating aspects that make yarrow a tried-and-true medicinal plant.

One of the most unique facets of the *A. millefolium* aggregate, the "crown group" of the Achillea genus, is how diverse it is in nature (Guo et al. 2005). Thanks to its widespread presence from region to region and continent to continent, it has many polyploid complexes that have aided its hybridization and evolution over time (Guo et al. 2004, 2005, 2008, 2012; Ehrendorfer & Guo 2006). Due to its ability to grow across the continent, it has seen extended use in North American ethnobotany. Most Indigenous communities have used it for medicinal and spiritual practices (Moerman 2003). Historically, the *Asteraceae* have been among the most highly selected plant families for medicinal use in North America and worldwide (Moerman 1991*;* Kachura & Harris 2018; Moerman 1999). The Achillea complex has been used to treat ailments by unrelated Indigenous communities from coast to coast to coast in North America, and it would be statistically unlikely that the nearly 400 communities with documented uses of the plant would adopt yarrow randomly (Duke 1986; Duke & Ayensu 1985; *Moerman 1998, 2003; Chandler et al. 1982).*

Yarrow notably produces many secondary metabolites that contribute *in planta* to abiotic and biotic stress responses or act as signal ligands essential to plant growth and survival. Some such classes were studied in this thesis: CQAs, a flavonoid, AKAs, STLs, and others. In the past, our lab reported suspected AKAs in *A. millefolium* and reported that AKAs in *Echinacea spp*. (*Asteraceae*) have shown immunomodulatory potential through the ECS (Kachura, 2018; Liu, 2019). The ever-evolving information on the pharmacological potential of yarrow's bioactives and our understanding of its ethnobotanical history as a panacea drove us to investigate if the plant's activity was partly driven by its interaction with the ECS. While most of the compounds we studied in this thesis had some established bioactivity, none had any with the ECS.

Through its ligands and receptors, the ECS helps maintain homeostasis by binding with its agonists, AEA and 2-AG (Hsieh et al. 2011; Chiurchiù et al. 2014; Haller et al. 2013). Lesser available endocannabinoids, through more degradation by FAAH and MAGL, can cause an imbalance in ECS activity, leading to adverse side effects (Gray et al. 2015; LI et al. 2017). Therefore, fewer endocannabinoids present to interact with CBs means a lesser ability to maintain homeostasis.

We successfully addressed the lack of evidence of compounds within yarrow that have the potential to inhibit FAAH and MAGL, thus contributing to the knowledge of why yarrow has been held in such high regard in ethnobotany. We did so following bioassay-guided fractionation.

We provided a methodology that allowed for the phytochemical analysis and bioassay study of yarrow plant parts, active sub-fractions, and isolated compounds in a single method. We also managed to identify some of the most inhibitory metabolites for FAAH and MAGL thanks to our MS analysis technique. Despite many isolated research articles exploring the bioactivity of

separate yarrow plant parts or phytochemical classes, ours is the first to combine several plant parts and metabolite classes comprehensively.

When separating roots, flowerheads, and leaves into aqueous and organic fractions, we observed the most inhibitory activity in the latter. We focused on fractionating root and flowerhead organic extracts since these showed the most bioactivity at the lowest concentration, and compounds within were lesser known and typically do not have reference standards to help confirm their identities. As we learned, the AKAs of roots were responsible for the most *in vitro* activity in root organic sub-fraction matrices. While all AKAs were bioactive, they were more selective towards FAAH, like pellitorine, **R4**, and **R5** (IC_{50}= 26.45 µg/mL, 28.95 µg/mL, 36.51 µg/mL, respectively). Despite more phytochemical variety in flowerheads than in roots, made evident by the more diverse compound classes we isolated, all isolates were inactive toward FAAH. However, one was active for MAGL: dihydroparthenolide. It was the isolate with the lowest IC_{50} of all isolated compounds (26.18 µg/mL), nearly equalling pellitorine for FAAH. The novel findings detailed for the identified isolates of the organic fraction of yarrow help elucidate their function as FAAH and MAGL inhibitors for the first time.

Another class of compounds confirmed for their enzyme inhibition were the DCQAs we tested. We contributed to the extensive list of bioactivity of the DCQAs we studied by providing evidence for their role in enzyme inhibition in the ECS for the first time. In assays with DCQA standards, the main contributors to the inhibition of both enzymes were 1,3-DCQA and 4,5-DCQA for FAAH and 1,3-DCQA and 1,5-DCQA for MAGL. The compounds were within nearly ±5% of their IC_{50} values at 10 µg/mL for both enzymes. Although more concentrations must be used to ascertain their IC50 values more closely, they are among the most potent compounds identified and tested with both enzymes in this thesis. Despite their potency, isolated compounds

of the DCQAs we tested should be assayed since the activity of refined standards may not necessarily equal that of unrefined compounds (Stermitz et al. 2000).

With our goal of finding some of the most active compounds within yarrow for FAAH and MAGL inhibition met, giving insight into its traditional uses, we proceeded with the validation of our analytical method.

We provided an optimized method for detecting and quantitating yarrow metabolites in the roots, flowerheads, and leaves at two different time points (May and October). We reported good separation of target bioactives promptly (30.1 minutes). We provided optimal wavelengths for compound analysis, including one at which all target compounds absorbed UV (280 nm) for those without an HPLC-DAD system. Our study is the first analytical method to quantify diverse compound classes, some of which are bioactive and have shown inhibition of FAAH and MAGL, like CQAs, AKAs, a flavonoid and an STL.

We gave insight into differences in metabolite accumulation observed in May (early season) and October (late season) collections, which saw a higher accumulation in the latter than the former, including for AKAs, dihydroparthenolide, and DCQAs. Overall, the roots had the most phytochemical diversity, while the flowerheads were the only part to contain dihydroparthenolide in the highest quantity compared to all metabolites (44.7 mg/g dried material). Our data showed that the method showed linearity, precision, and repeatability, essential for method validation. Therefore, our data suggests that our method was proven to be an efficient tool for researchers who want to analyze and quantify metabolites of *A. millefolium* and possibly other species of Achillea and Asteraceae with similar phytochemical composition.

As for DCQAs, in Chapter 2, Figure 2-6 shows that the DCQAs we tested were in the aqueous fractions of the roots, flowerheads, and leaves but mainly in the former two. The %inhibitions of the aqueous fractions they were in were not as high as their individual %inhibitions, indicating a possible antagonistic effect between them and the other metabolites in that matrix. We also cited possible allosteric inhibition for FAAH and MAGL by DCQAs.

Recognizing the need to widen the collection area for yarrow and continue harvesting samples throughout the year, in future, we recommend that plant collections be done across and outside of Ottawa every two weeks from the beginning of April to November to observe trends throughout the blooming season. Also, plant parts should be further separated into sections, like the flower from the flowerhead or apical buds, to observe trends in dihydroparthenolide accumulation or root epidermis from the cortex to see where AKAs tend to accumulate more. This would provide more details on the ecophysiological roles of metabolites as plants age. The knowledge it would contribute over time would let researchers predict trends in metabolite accumulation, allowing them to be more selective in their collection time points to meet their needs.

The proposed collection method would also provide information on geographical variability to observe how populations of *A. millefolium* may differ in the types and amounts of metabolites they produce. Preferred areas would be those experiencing different abiotic factors like drought, soil salinity, and access to light, as well as biotic factors like herbivory and pollinator access. *A. millefolium* is a diverse group within the *Achillea* genus, and expanding collection time points, and locations can gather much unknown data on several species, subspecies, and varieties that can deepen our knowledge of its evolution. It would give more insight into the bioactive compounds of yarrow experienced by many of its users in ethnobotany.

In terms of natural health product (NHP) research and development with yarrow, it would help producers remain up-to-date on trends in bioactives, keeping consumers satisfied and regulators notified on the contents of proposed products. Further strengthening the potential of the analytical method we used to identify the most bioactive metabolites in samples would help achieve more representation of yarrow in the NHP market. Those studying NHPs are also constantly looking for more efficient extraction methods for bioactives.

A worthy alternative to the traditional maceration extraction technique is ultrasonic-assisted extraction. That technique has shown nearly 11.5% more total phenolic content after two rounds of sonication, totalling 30 minutes versus 48 hours of maceration (Oroian et al. 2020). Another advantage over maceration is that the ultrasonic frequency at which the solvent is vibrating during extraction can be adjusted, with frequencies between 20 kHz-40kHz favouring different sizes of bioactives from fruits and vegetables (Guandalini et al. 2019; Corrales 2008). Granted, this extraction technique's most significant limiting factor is the smaller scale at which extraction can occur compared to the hundreds of grams permitted by maceration. It is advantageous for future analytical work since very little plant material is usually required for HPLC or MS analyses.

Our method allows for a larger-scale extraction of plant material that can be done overnight, which is most suitable for capturing a larger quantity of metabolites in greater variety. However, some smaller-scale extraction methods have shown high-yield potential of metabolites. One such method used natural deep eutectic solvents, hydrogen bond donors and acceptors, called NADES, in an ultrasonic bath extraction protocol using lactic acid-based NADES, which gave a higher yield of polyphenolic compounds in end-of-summer samples (Ivanović et al. 2022). That extraction method also yielded samples that showed more antibacterial and

antifungal activity than ethanolic extracts, positively correlating with their higher number of bioactive compounds. In theory, an ultrasound-assisted extraction step could improve quantities of metabolites. However, this requires more time and bigger ultrasonic apparatuses and can only be done with much smaller amounts than the overnight maceration extraction method we used. In contrast to ultrasound-assisted extraction, using a supercritical anti-solvent (CO_2) method showed a 3-fold increase in the concentration of phenolics compared to an ultrasound-assisted extraction method (Villanueva-Bermejo et al. 2017). Increasing pressure in MPa can yield more plant metabolites with less material. As pressure increases from 10 MPa (where polyphenols seem to be isolated) to 15 MPa, smaller molecules can be isolated, like AKAs. Alternatives in extraction methodology are not the only consideration for future work. Analytical methods must be looked into as well to explain the bioactivity of some of yarrow's flowerhead subfractions.

The lack of observed activity of flowerhead isolates, as opposed to their mother subfraction, could potentially be explained by the activity of certain compounds which do not contain a chromophore (unable to absorb light and reflect a colour) or are more prone to degradation. Since we only isolated one noteworthy inhibitor from the flowerheads, dihydroparthenolide, but sub-fractions were highly active against FAAH and MAGL, the isolation of active components would likely benefit most from using HPLC-ESI/MS in the future. Advantages of this technique over HPLC-DAD include examining a wide range of molecules of varying polarities in the same sample and quantitating them, even at very low concentrations (Pellati et al. 2011). It is also a technique that is mindful of samples with compounds that may degrade more rapidly at room temperature or with exposure to light or air by combining multiple analytical experiments (Bergeron et al. 2002; Biesaga 2011). Lesser degradation could potentially lead to better product ion (*m/z*) results, as we only recorded

MS/MS data for pellitorine, **R4,** R5, and dihydroparthenolide, in Chapter 2, and would make compound structure elucidation with nuclear magnetic resonance (NMR) more viable – a technique that we tried very hard to use in compound identification. The proposed HPLC-ESI/MS technique could streamline the analytical process for bioactive sub-fractions, ultimately saving researchers time and precious plant material. Optimization of the isolation and identification process is needed to properly study the effects of metabolites in an *in vitro* cell model.

Using a cell model, sub-fraction and isolated compound inhibition of FAAH and MAGL and their ECS modulation potential can be evaluated. As Kachura (2018) observed, immunomodulation can be assessed by monitoring the production of the pro-inflammatory cytokines IL-6 and IL-8 in an *in vitro* rhinovirus-infected BEAS-2B human bronchial epithelial cell line. The most potent sub-fractions of roots and flowerheads and the isolates they yielded could be tested at varying concentrations for their cytotoxicity, followed by their apparent FAAH and MAGL inhibition through reduced cytokine production. Their activity can be tested by seeing if their incubation with cells increases or decreases different types of cytokines and chemokines implicated as inflammatory mediators (Sharma et al. 2006). The likeliest candidates for FAAH and MAGL inhibition would be dihydroparthenolide, pellitorine, **R4**, **R5,** 1,3-DCQA, 1,5-DCQA, and 4,5-DCQA. Work with this cell line can also be used to test for the affinity of bioactive isolates for CB_1 and CB_2, compared to natural ligands (AEA and 2-AG), and to test the kinetics of binding (association and dissociation rates) of compounds, along with their potential to act as receptor agonists or antagonists. Results from such a study would provide much-needed evidence of the potential isolates have to modulate the ECS that is currently lacking, which can be further investigated *in silico*.

Finally, a critical study to determine if the active compounds from this thesis could be used as natural health products would be *in silico* modelling of blood-brain barrier (BBB) permeability using molecular simulation with the GROMACS 4 program (Hess et al. 2008). The program makes it possible to calculate the ratio of compounds in the brain versus the blood (log[brain]/[blood], logBB), a gold-standard calculation for BBB permeability. We cited evidence suggesting AKAs can efficiently permeate the BBB in Chapter 2. Simulating a lipid membrane through which molecule diffusion and permeation can be analyzed for compounds we isolated could precede valuable work *in vivo* in mice, for example. It would allow researchers to hypothesize how well certain AKAs would permeate the BBB compared to one another. Using GROMACS 4 can save time, money, and resources and has proven to generate reproducible data, correctly predicting compound permeation and availability in the brain (Carpenter et al. 2014).

Yarrow's biology, ethnobotany, phytochemistry, pharmacology, and interactions with the ECS come together to truly display its rich history as a medicinal plant and how current-day research can be adapted to fill gaps in knowledge regarding some of the biomolecules that are at the forefront of the plant's medicinal properties. The future works we have recommended would help gather evidence on the novel ECS modulators we identified in this thesis. Also, our method validation for detecting and quantitating specific bioactives in yarrow could be applied to other species of the *Asteraceae* with similar phytochemistry. The knowledge we began acquiring here can one day allow researchers to effectively communicate trends in plant metabolite production in crops and their bioactivity with stakeholders in the natural health products industry. This would allow companies to prioritize species with more bioactive potential over others. The novel data presented in this thesis contributes to the need to produce reliable, sustainable, and profitable therapeutic products.

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Appendix A. Solvent systems used as the mobile phase for column chromatography of the root and flower organic phases. Solvent Combinations of 300 mL were added.

Solvent addition	Mobile phase (%)		
	Hexane	Dimethylsulfoxide	Methanol
1	100	-	-
2	90	10	-
3	80	20	-
4	70	30	-
5	60	40	-
6	50	50	-
7	40	60	-
8	30	70	-
9	20	80	-
10	10	90	-
11	-	100	-
12	-	90	10
13	-	80	20
14	-	70	30
15	-	60	40
16	-	50	50
17	-	40	60
18	-	30	70
19	-	20	80
20	-	10	90
21	-	-	100

Appendix B. Root isolate HPLC spectra overlayed with the root organic phase spectrum

A

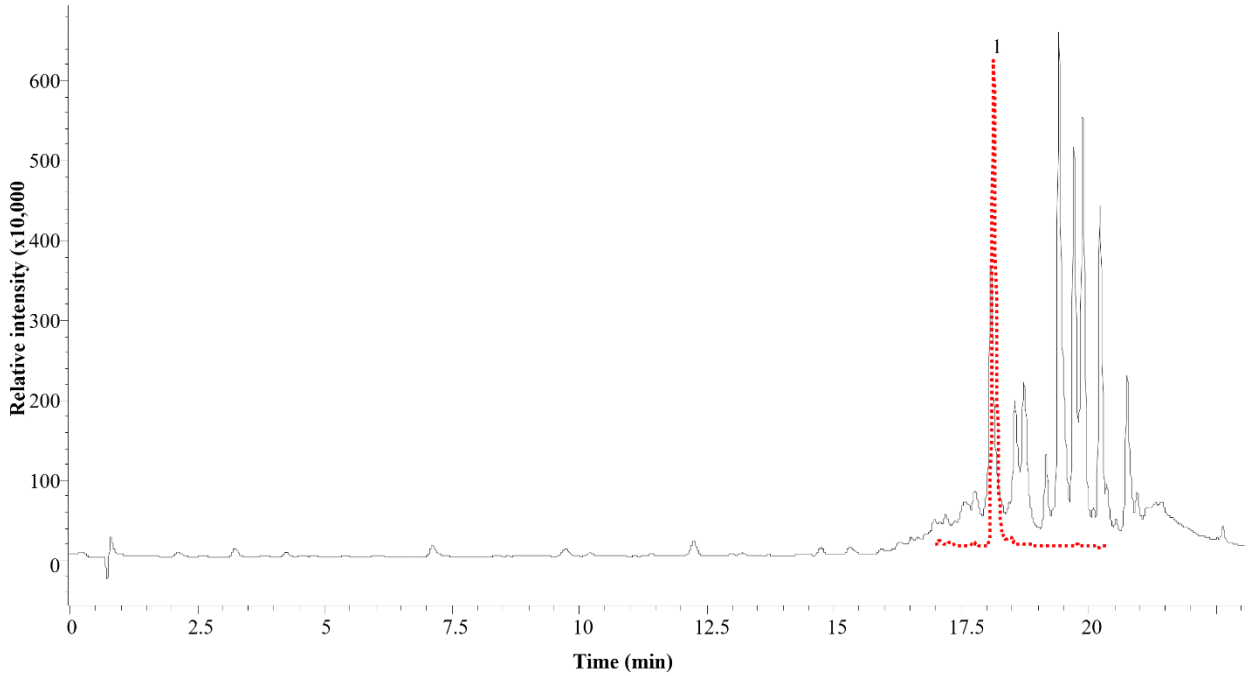

Figure 1. Chromatogram at 268 nm of the root organic extract (black, solid lines) with the overlay of R1 (red, dotted lines).

B

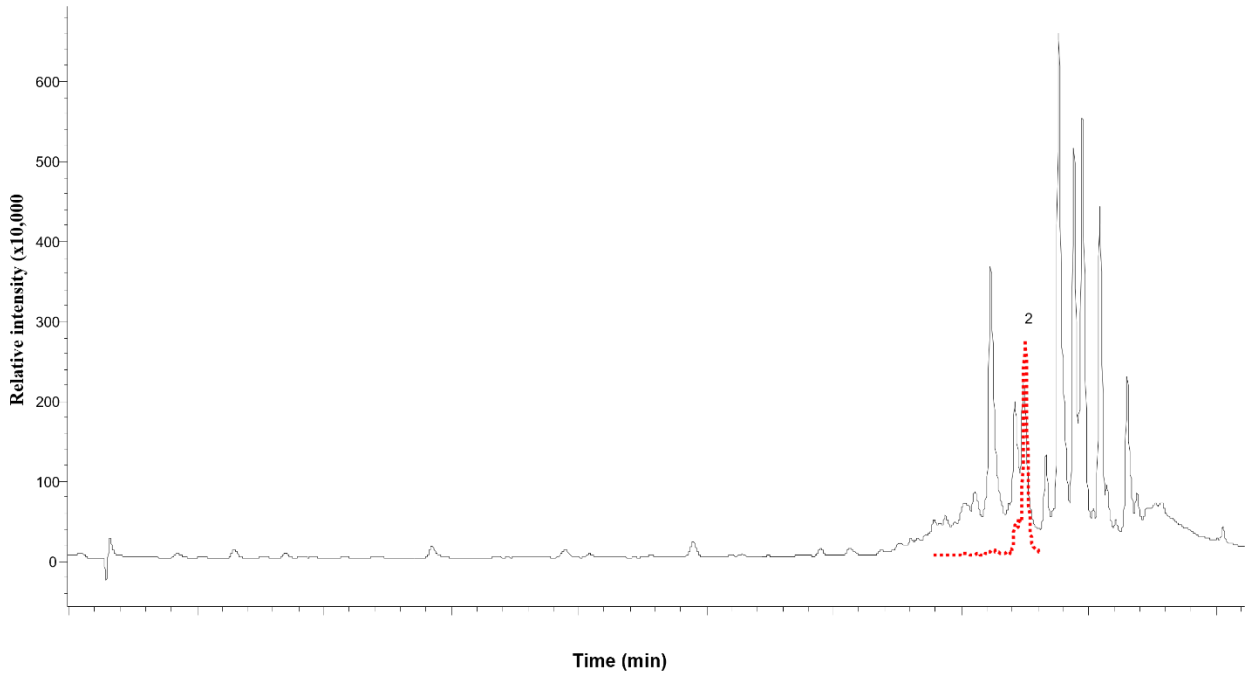

Figure 2. Chromatogram at 268 nm of the root organic extract (black, solid lines) with the overlay of R2 (red, dotted lines).

C

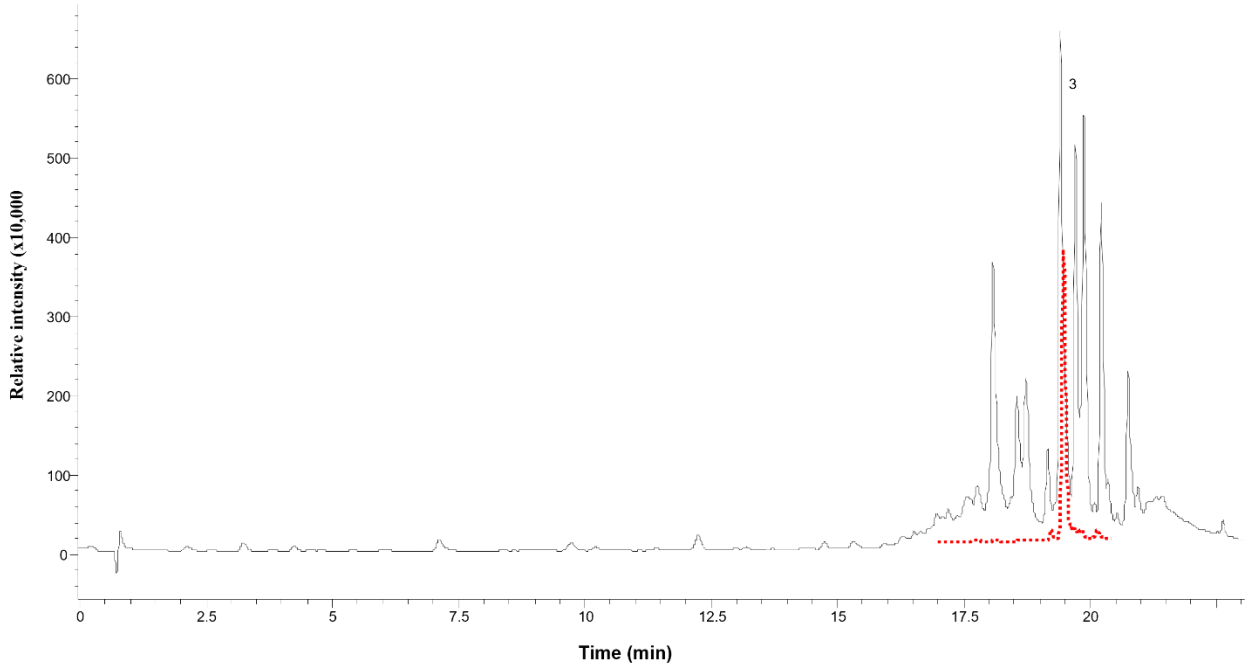

Figure 3. Chromatogram at 268 nm of the root organic extract (black, solid lines) with the overlay of R3 (red, dotted lines).

D

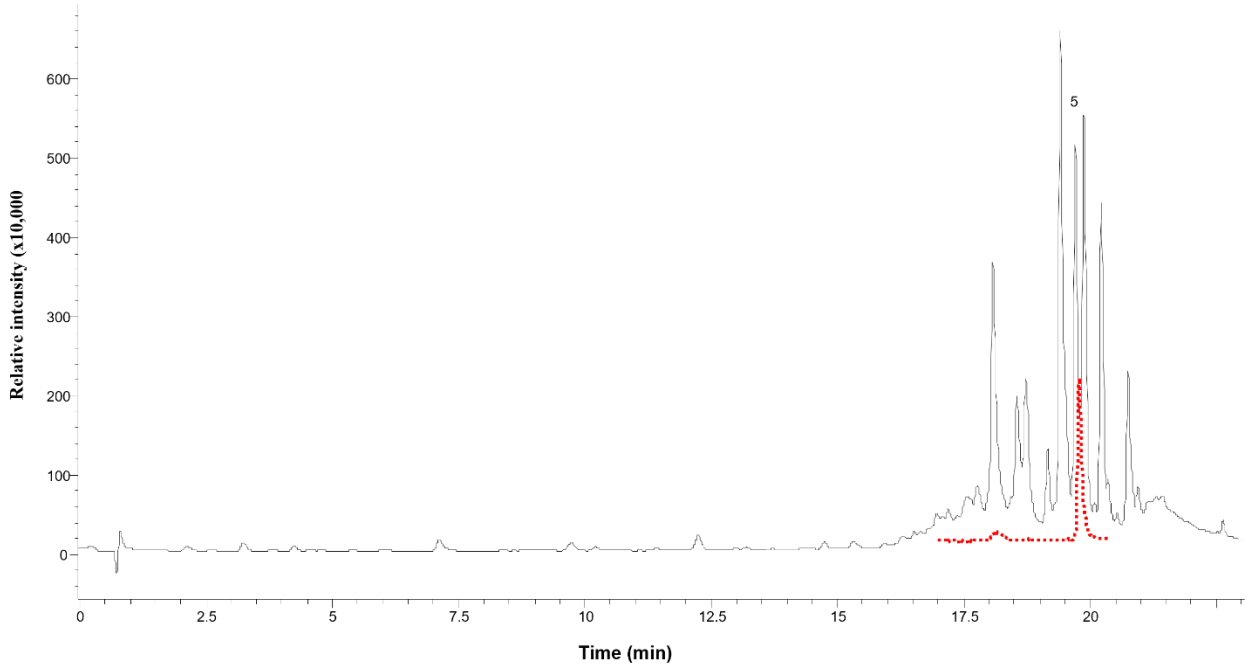

Figure 4. Chromatogram at 268 nm of the root organic extract (black, solid lines) with the overlay of R4 (red, dotted lines).

E

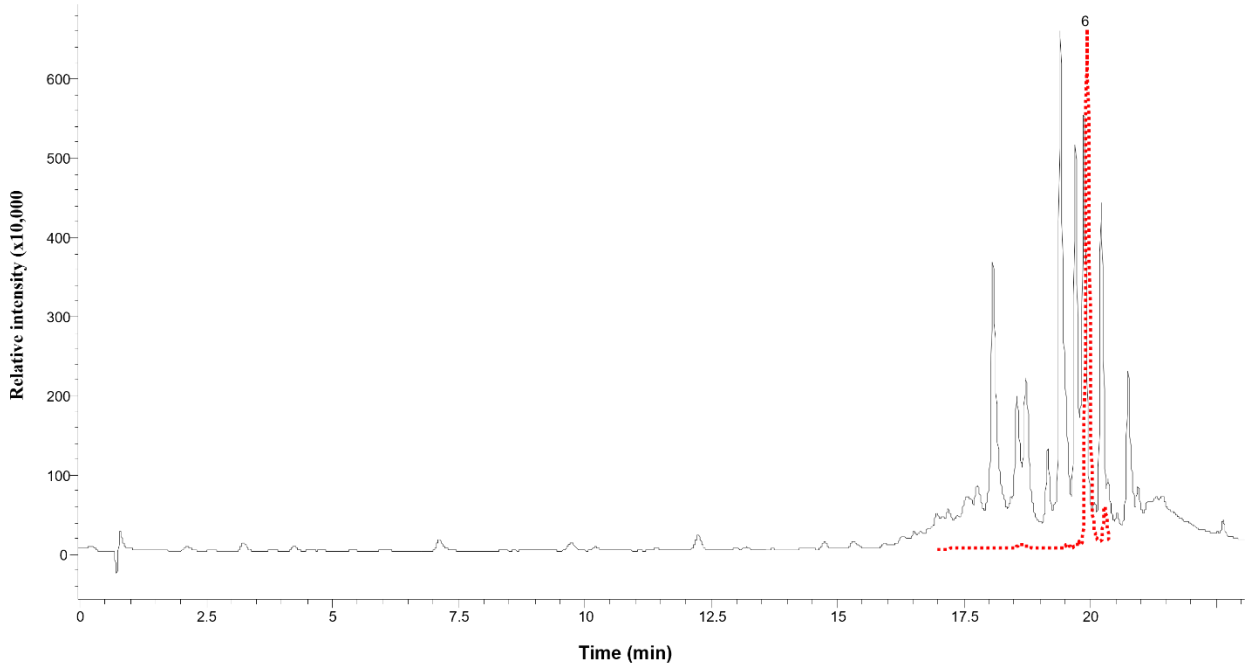

Figure 5. Chromatogram at 268 nm of the root organic extract (black, solid lines) with the overlay of R5 (red, dotted lines).

F

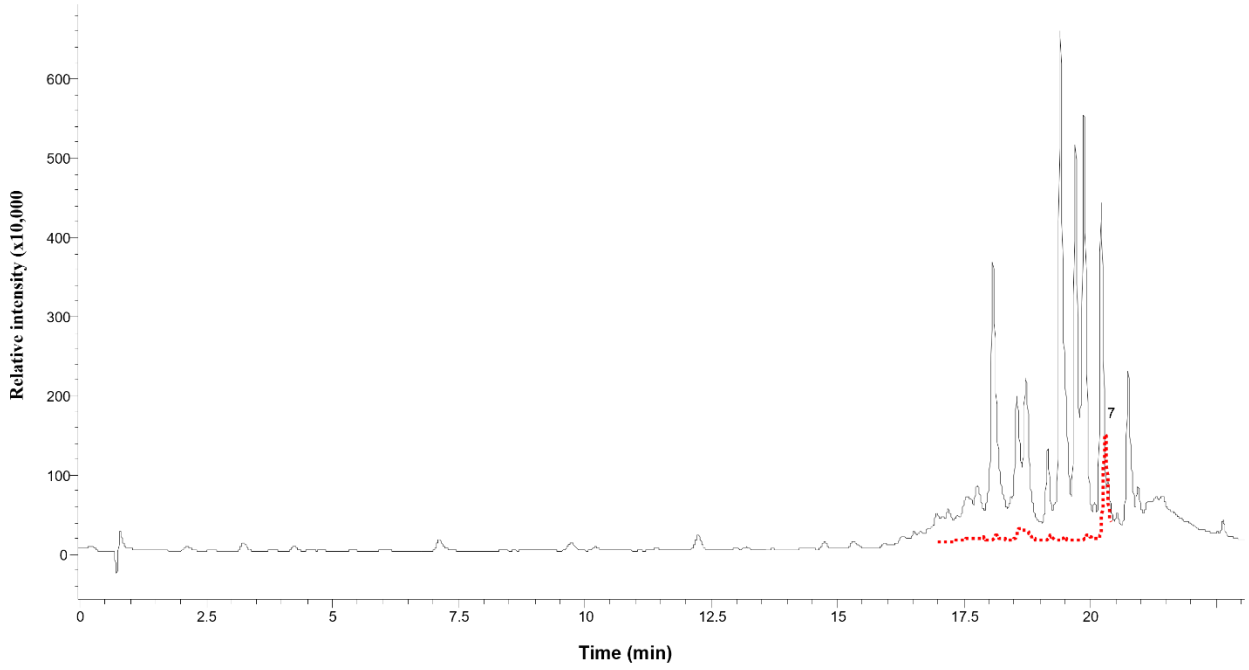

Figure 6. Chromatogram at 268 nm of the root organic extract (black, solid lines) with the overlay of R6 (red, dotted lines).

G

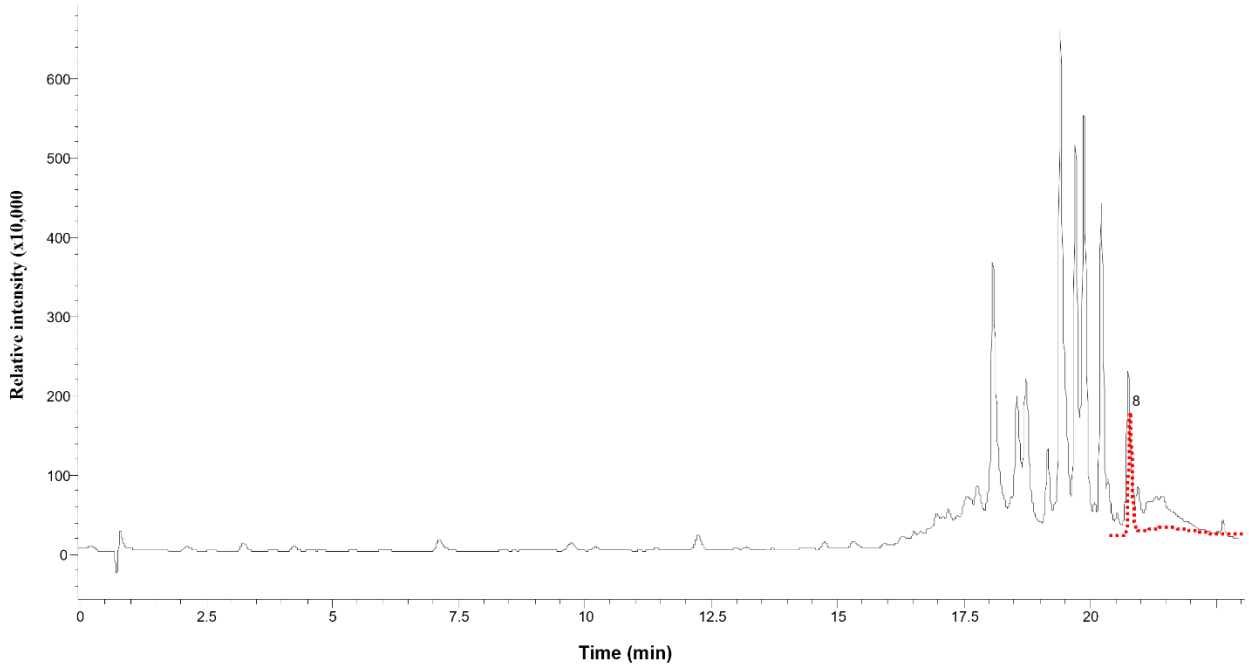

Figure 7. Chromatogram at 268 nm of the root organic extract (black, solid lines) with the overlay of R7 (red, dotted lines).