PHARMACOLOGY AND TOXICLOGY OF *ECHINACEA, SOUROUBEA AND PLATANUS* spp.

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PREFACE

This thesis is submitted for the degree of Doctor of Philosophy at the University of Ottawa. Most of the work presented was conducted in the Department of Biology at the University of Ottawa under the supervision of Professors John T. Arnason and Cory Harris. Field work collecting *Echinacea* spp. samples was carried out with the help of John Arnason, John Baker, Franklin Johnson, and Phil Hinz at Trout Lake Farm, Washington, USA. The collection of *Souroubea* spp. specimens was carried out by Professor Ana Francis Carballo, Pablo Sanchez and Marco Otárola from Escuela de Química, Universidad Nacional Autónoma de Costa Rica and Herbario JVR, Universidad Nacional Autónoma de Costa Rica, Heredia, Costa Rica. The collection of *Platanus* spp. specimens was completed by Dr. Tony Durst from Department of Chemistry and Biomolecular Sciences at the University of Ottawa. The cannabinoid receptor internalization assay was carried with the help of Dr. Lili Wang in the Beijing Institute of Basic Medical Science, Beijing, China. *In vivo* experiments using inflammatory pain models were conducted by Dr. Nadia Caram Salas in Cátedra CONACYT-CICESE. Departamento de Innovación Biomédica (DIB), Ensenada, México. The biochemometric analysis of FAAH inhibition activity relative to detected Echinacea metabolites profile were conducted with the help of Dr Kelly Burkett from Department of Mathematics at the University of Ottawa. The screening of inhibition of alkylamides on intracellular anandamide transporter was conducted in the laboratory of Dr. Dale G Deutsch in Department of Biochemistry and Cell Biology, Stony Brook University, NY, USA. I was the lead or co-lead investigator for areas of phytochemical analysis, pharmacokinetics, hepatic metabolism and *in vitro* cannabinoid activity. Under the guidance of my co-supervisors, I was responsible for the concept formation, data collection and analysis of these studies, as well as most manuscript composition.

Part of this work (Part I of Chapter 1, Chapter 2 and Chapter 3) has been published:

1) Liu R, Ahmed F, Cayer C, Mullally M, Carballo AF, Rojas MO, Garcia M, Baker J, Masic A, Sanchez PE, Poveda L. Merali Z, Durst T, Arnason JT. New Botanical Anxiolytics for Use in Companion Animals and Humans. AAPS J. 2017; 19:1626-31.
2) Liu R, Carballoa AF, Singh R, Saleem A, Rochae M, Cayer C, Mullally M, Rojas MO, Poveda L, Sanchez PE, Garcia M, Baker J, Merali Z, Durst T, Harris CS, Arnason JT. Selective Ion HPLC-APCI-MS Method for the Quantification of Pentacyclic Triterpenes in an Anxiolytic Botanical Dietary Supplement for the Animal Health Market. Nat Prod Commun. 2019; 14:11-14.
3) Liu R, Dobson CC, Foster BC, Durst T, Sanchez P, Arnason JT, Harris CS. Effect of an anxiolytic botanical containing *Souroubea sympetala* and *Platanus occidentalis* on in-vitro diazepam human cytochrome P450-mediated metabolism. J Pharm Pharmacol. 2019; 71:429-437.

Part of the results from Chapter 4 were combined with Dr Zul Merali's work and has been published:

4) Merali Z, Cayer C, Kent P, Liu R, Cal V, Harris CS, Arnason JT. Sacred Maya incense, copal (Protium copal - Burseraceae), has antianxiety effects in animal models. Ethnopharmacol. 2018; 216:63-70. (Appendix 1).

The work described in Chapter 5 was published as a part of a safety evaluation project of natural health product containing *Souroubea* spp and *Platanus* spp in canine models. These results were presented in the following publication:

5) Masric A, Liu R, Simkus K, Wilson J, Baker J, Sanchez P, Saleem A, Harris C, Durst T, Arnason JT. Safety evaluation of a new anxiolytic product containing botanicals *Souroubea* spp and *Platanus* spp in dogs. Can J Vet Res. 2018; 82:3-11. (Appendix 2)

Chapter 6, Chapter 7, and Chapter 8 are under preparation for submission:

6) Liu R, Burkett K, Arnason JT, Johnson F, Hinz P, Baker J, Harris CS. In vitro inhibitory effects of Echinacea spp. chemotypes on fatty acid hydrolase (FAAH).

7) Liu R, Xiong K, Arnason JT, Harris CS. Hepatic Metabolism of alkylamides from Echinacea and effects on in vitro Pharmacological Activity.

8) Liu R, Caram-Salas NL, Deutsch DG, Li W, Wang LL, Arnason JT, Harris CS. Effect of *E. angustifolia* and *E. purpurea* accessions and their active principles on cannabinoid system in vitro and their analgesic affect using a rat chronic inflammatory pain model.

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Many of my colleagues from the Harris and Arnason labs have given me great help during the past five years. Overall, I would like to thank all my colleagues from the Arnason and Harris labs for their company and assistance. Without their support, my graduate life would have been much more stressful.

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ABSTRACT

The research presented in this thesis addressed knowledge gaps for three medicinal plant taxa, Souroubea spp. (Marcgraviaceae) and Platanus (Platanaceae) as well as Echinacea spp. (Asteraceae).

The primary pharmacological mechanism of Souroubea sympetala and Platanus occidentalis were well established, with pentacyclic triterpenes identified as major active principlesMy results indicate that major triterpenoids, and crude plant extracts, selectively inhibited monoacyglycerol lipase (MAGL) activity but not fatty acid amide hydrolase (FAAH) activity. These data suggest a possible secondary anxiolytic mechanism of action through the endocannabinoid system (ECS). My study of herb-drug interactions of Souroubea and Plantanus products showed some potential risk when combined with a classic benzodiazepine class drug, diazepam, and I proposed a mechanism through in vitro CYP450 enzyme inhibition. The pharmacokinetic study revealed the difficulty of detecting betulinic acid in animal blood. To support the development a commercial botanical composed of these medicinal plants, an extraction method and a highly sensitive and selectivity HPLC-APCI-MS based quantification method was successfully developed and validated.

Part II of this thesis focused on the impact of phytochemical variation and hepatic metabolism on the ECS activity of Echinacea spp. and explored the potential for new applications of Echinacea spp. as a natural health product. My research indicated that considerable variability in the content of phenolic and alkylamide (AKA) compounds reflected similar variability in in vitro bioactivity at ECS-related pharmacological targets. Following

biochemometric analysis, several phenolic compounds and AKAs in Echinacea spp. were found to be significant independent variables determining FAAH inhibition and CB receptor activation. Hepatic metabolism was also found to affect the FAAH inhibition of AKA, as increased FAAH inhibitory effects were observed after CYP450-mediated metabolism of both individual AKAs and crude extracts of E angustifolia and E. purpurea, suggesting a “pro-drug” mechanism. Dose dependent activities were observed with oral administration of both E angustifolia and E. purpurea root extract in rat paw model of inflammation and pain. Further tests indicated these activities can be partially blocked by co-administration of CB1 and CB2 receptor antagonists AM251 and AM630, respectively. This evidence suggests activity for peripheral pain was at least partially mediated through the ECS.

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LIST OF ABBREVIATIONS

2-AG:	2-Arachidonoyl glycerol
ADME:	Absorption, Distribution, Metabolism and Excretion
AEA:	Anandamide
AKA:	Alkylamide/alkamide
AMC-AA:	7-amino-4-methyl Coumari-Arachidonamide
AMMC:	3- [2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin
APCI:	Atmospheric pressure chemical ionization
AUC:	Area under the curve
BA:	Betulinic acid
b5:	Cytochrome b5
CAD:	Caffeic acid derivative
CB1:	Cannabinoid receptor 1
CB2:	Cannabinoid receptor 2
CEC:	3-cyano-7-ethoxycoumarin
CER:	Conditioned emotional response
CNS:	Central nerves system
CYP:	Cytochrome P450
DA:	Diazepam
DAD:	Diode array detector
DBF:	Dibenzylfluorescein
DEX:	Dexamethasone
EAR:	*Echinacea angustifolia* root extract
ECS:	Endocannabinoid system
EPM:	Elevated plus maze

EPR:	*Echinacea purpurea* root extract
ESI	Electrospray ionization
FAAH:	Fatty acid amide hydrolase
FABP:	Fatty acid binding protein
FMO:	Flavin-containing monooxygenase
FPS:	Fear potentiated startle
GAD:	Generalized anxiety disorder
GFP	Green fluorescent protein
GPCR:	G-protein-coupled receptor
HLM:	Human liver microsome
HPLC:	High performance liquid chromatography
IC_{50}:	Median inhibition concentration
LOD:	Limit of detection
LOQ:	Limit of quantification
MAGL:	Monoacylglycerol lipase
MFC:	7-methoxy-4-trifluoromethyl-coumarin
MPE:	Maximum possible effect
MS:	Mass spectrometer
NADPH:	Nicotinamide adenine dinucleotide phosphate
NHP:	Natural health product
SNRI:	Serotonin–norepinephrine reuptake inhibitor
SSRI:	Selective serotonin reuptake inhibitor
Pgp:	P-glycoprotein
PK:	Pharmacokinetic
UA:	Ursolic acid
UPLC:	Ultra-performance liquid chromatography

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Natural health products (NHP) are one of the major categories of approved therapeutic products on the Canadian market. According to a survey study by Health Canada in 1999, 26% of respondents reported using NHPs to prevent or treat an illness (Non-Prescription Drug Manufacturers Association of Canada, 1999/2000). However, this number grew to 71% in 2005 (Health Canada, 2005). The majority of NHPs on the market are botanical medicines. Whereas botanical medicines have played an important role in human history, their importance declined in the 20th century with the growing use of conventional single entity drugs produced by the pharmaceutical industry. However, due to concerns over side effects, tolerance, compliance, patient preference and cost of conventional medications, this trend has now reversed direction in developed countries and the use of botanical medicine as NHPs has become a significant factor in personal health care since 1990 (WHO, 2013). The consumption of NHPs is one of the most common form of alternative and complementary healthcare (Kennedy et al., 2009). NHPs differ from other therapeutic products since they are usually not single-active ingredient products but highly complex mixtures of constituents. NHPs, especially herbal products, may contain a large number of natural constituents from multiple biosynthetic classes of phytochemicals. Although they are often considered low technology products, the complex composition makes evaluation of mode of action, pharmacokinetics and safety profiles challenging. The research presented in this thesis focused on these issues in a new herbal medicine based on *Souroubea* and *Platanus* (Part I) and a well-established one, Echinacea (Part II).

1.2 Part I: *Souroubea* spp. and *Platanus* spp. NHP for anxiety.

1.2.1 General introduction to Part I

During the last two decades, our research team at the University of Ottawa and colleagues at the Herbarium at the Universidad Nacional Autonoma of Costa Rica have had an international collaboration to study, preserve, and utilize plant biodiversity for medicinal purposes, especially safe treatments for anxiety. As a discovery strategy, poorly studied plants including Genus *Souroubea* (Figure 1-1) which belongs to a small family of neotropical lianas and shrubs, the Marcgraviaceae, native to Central America, the northern part of South America, and the Caribbean Islands were studied.Two species collected in Costa Rica, *Souroubea gilgii* Gilg. and *S. sympetala* V.A. Richt., were the most promising extracts studied for anxiety treatment in standardized rodent behavioral assays among extracts of many species surveyed and were selected for pharmaceutical development for animal and human health. The phytochemistry of the two species is very similar with triterpenes being the main bioactive molecules (Figure 1-2) (Puniani et al., 2015).

Anxiety disorders are among the most prevalent mental health problems in North America (Kessler et al., 2005). Chronic use of conventional anxiolytic drugs is associated with adverse side effects, including sedation, cognitive impairment, impaired psychomotor coordination, and risk of tolerance, driving the need to find safer alternatives (Ravindran and Stein, 2010). There is a growing body of evidence which shows that complementary and alternative treatment approaches, including traditional herbal remedies, are increasingly used by anxiety patients (Kessler et al., 2001; Barnes et al., 2002 and Van der Watt et al., 2008). Although several botanical species show efficacy in mood disorders, two of the most effective botanicals, St. John's Wort and Kava, have been associated with adverse effects and safe alternatives are needed.

The discovery of anxiolytic effects of *Souroubea* extracts in rodents led to a subsequent investigation of traditional uses of *Souroubea* spp. for mental health. Studies by Puniani et al. (2015) showed that Q'eqchi' Maya healers in Belize used *Souroubea sympetala* for the treatment of witchcraft, the symptoms of which include becoming withdrawn, showing little interest in daily life, and reduced verbal communication. Other ethnobotanical uses of this genus also point to its psychopharmacological properties. The use of *Souroubea guianensis* Aublet var. coralline (Mart) Wittmack by the Kubuyari in the Amazon as a calming agent for symptoms of nervousness in elders has been reported (Schultes and Raffauf, 1990). *Souroubea guianensis* Aublet var. cylindrical Wittmack is used as a tranquilizing medicine by the Karijona and as a treatment for "susto" by the Taiwanos. *Susto,* a culture specific syndrome, has been described as sickness which occurs in response to a frightening event (Puniani et al., 2015). As well, previous investigations of plants used as remedies for the treatment of susto have demonstrated pharmacological activity at targets of anxiolytic drugs (Awad et al., 2009) and reduced anxiety-like behavior in rodents (Bourbonnais-Spear et al., 2007).

1.2.2 Phytochemical constituents and active principles

Five triterpenes (Figure 1-2) were isolated from the active ethyl acetate fraction (Carballo-Arce, 2013). Of these, betulinic acid (BA), a lupane-type triterpene, induced anxiolytic-like activity upon oral administration at 1 mg/kg in the elevated plus maze (EPM) (Durst et al., 2009). Administration of 0.5 mg/kg of BA increased the percentage of time spent in the open arms and the number of unprotected head dips, an indication of increased risk assessment behavior, and significantly decreased the fear potentiated startle (FPS) response compared to the vehicle controls (Cayer, 2011). These findings were bolstered by parallel studies in different strains of mice, including a genetically anxious Balb/c strain, a model of trait anxiety. Intraperitoneal BA administered at 0.25 mg/kg markedly reduced anxiety-like behavior in these mice. Betulinic acid had been previously shown to be pharmacologically active with anti-melanoma and anti-HIV properties (Yogeeswari and Sriram, 2005). Other compounds present in

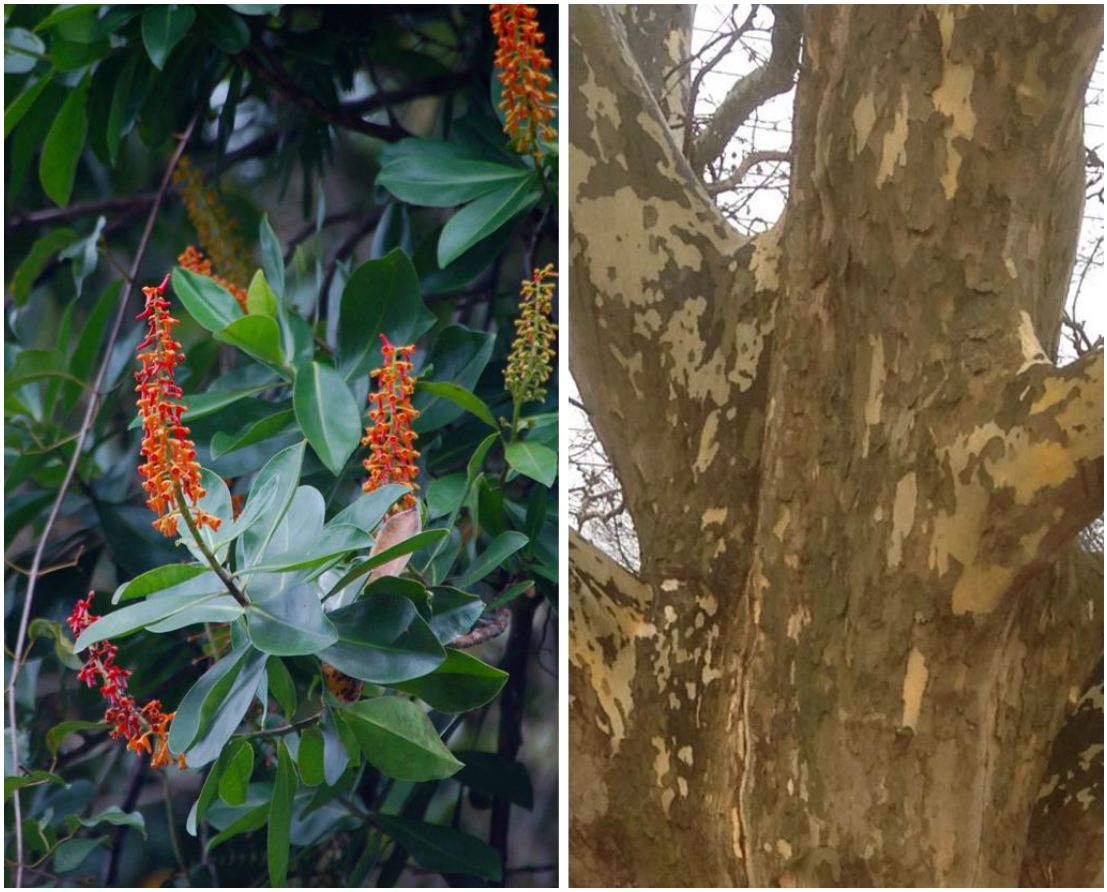


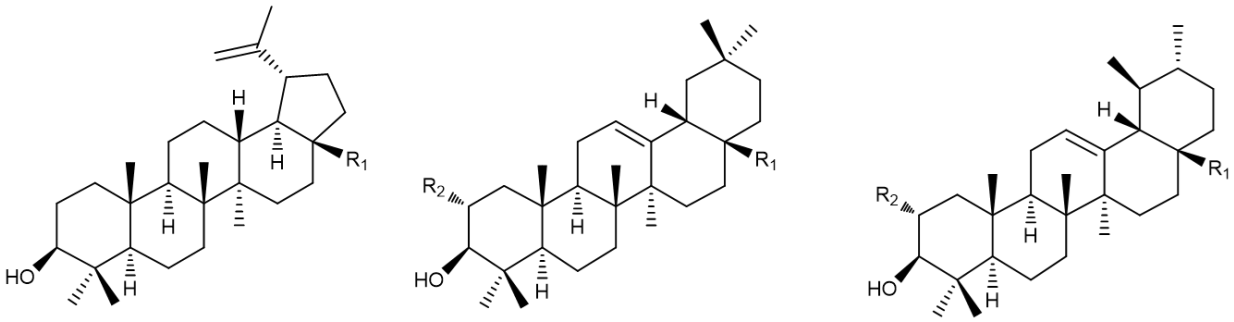
Figure 1-1. *Souroubea* (left) and *Platanus* (right). Imaged by John T. Arnason.

Souroubea spp., including α- and β-amyrins and ursolic acid, were not found to be active in the EPM at the same dose as betulinic acid (Puniani et al., 2015). However, anxiolytic and antidepressant effects have been demonstrated for α- and β-amyrins by others (Aragão et al., 2006). In behavioral assays, the combination of amyrins and betulinic acid was synergistic in promoting anxiolytic effects.

1.2.3 Pharmacology of Souroubea spp.

A series of pre-clinical studies using orally administered *Souroubea* raw plant materials, crude ethanol and ethyl acetate extracts, or supercritical CO_2 extracts have demonstrated anxiolytic potential in rodents using standardized behavioral assays on short-term and long-term treatment regimens (Puniani et al., 2015 and Mullally et al., 2011). Behavioral assays were conducted to elucidate the dose response effects of *Souroubea* spp. ethanol extract in rats in the EPM at 25, 50, 75, and 100 mg/kg. Treatments led to dose-dependent increases in the time spent in the open arms of the EPM, and unprotected head dips, both of which are measures of anxiolytic activity (Puniani et al., 2015). The extract was also demonstrated to significantly reduce the time spent freezing in the conditioned emotional response (CER) test (Cayer, 2011). Different extraction methods showed that supercritical extracts were the most effective with significant effects at 25 mg/kg. Leaf was more effective than stem, and there was little difference between the two species studied (*S. gilgii* and *S. sympetala*), a result consistent with their similar phytochemistry (Mullally et al., 2011).

Bioassay-guided fractionation experiments with sequential hexane, ethyl acetate, and water fractions showed that the active principles were enriched in the ethyl acetate fraction (Mullally et al., 2011). The orally administered ethyl acetate fraction was found to significantly increase time spent in the open arms by rats in the EPM at 50 and 100 mg/kg body weight (Durst et al., 2009, Cayer, 2011). Similarly, at 75 or 100 mg/kg, this fraction reduced freezing time in the contextual CER test (Mullally et al., 2017) and attenuated expression of FPS (Cayer, 2011). The fraction also significantly increased



Triterpene family	Triterpene	R_1	R_2
Lupane	lupeol	CH_3	H
	Betulinic acid	CH_2OH	H
Oleanane	β-amyrin	CH_3	H
Ursane	α-amyrin	CH_3	H
	Ursolic acid	COOH	H

Figure 1-2. Chemical structure of major triterpenes identified in *Souroubea* spp.

total active social interaction at 100 mg/kg (Cayer, 2011). Interestingly, anxiolytic activity of the crude extract in the FPS test was significantly greater than the active ethyl acetate fraction, suggesting that there may be pleiotropic effects at play (Cayer, 2011).

In addition to behavioral modification, *Souroubea* spp. extracts induce physiological changes at targets of anxiety and stress. Evidence of anxiolytic activity of *Souroubea* spp. in unconditioned (EPM, SI) and conditioned (CER, FPS) tests suggested that *Souroubea* extracts and (or) compounds may be modulating anxiety responses by acting as agonists at the GABAA-BZD receptor (Gonzalez et al., 1998; Menard and Treit, 1999 and Rezayat et al., 2005). Mullally et al. (2014) showed that in the presence of a $GABA_A$-BZD receptor-specific antagonist, flumazenil, the anxiolytic activity of extracts of *Souroubea* as well as methyl-BA were markedly attenuated in the EPM, and to a lesser degree, in the CER.

Investigations in *Onchorhyncus mykiss* (rainbow trout) showed that BA was active at the interrenal cells of the hypothalamic-pituitary-interrenal axis in lowering cortisol production (Mullally et al., 2017). Upon *in vivo* administration at 1 mg/kg in acutely stressed fish, BA lowered plasma cortisol but had no effect on plasma cortisol levels in unstressed controls. These cortisol-lowering effects in stressed animals have been observed (Gonzalez et al., 1996) in other species including rats (corticosterone), piglets, dogs, and horses.

Additional mechanisms may contribute to the observed anxiolytic effects that have yet to be tested. Treatment with *Souroubea sympetala* (supercritical CO_2 extract) has been shown to increase the number of unprotected head dips in the EPM, which is a behavioral parameter responsive to 5HT1A (serotonin receptor 1A) ligands (Mullally et al., 2011). Interestingly, response in the SI test appears to also involve serotoninergic receptors (Gonzalez et al., 1996) and there are reported reciprocal interactions between GABA and serotonin circuitry (Andolina et al., 2013 and Ciranna et al., 2006).

Some recent data showed fatty acid amide hydrolase inhibition, implicating the endocannabinoid system.

1.2.4 Development of a low risk veterinary product

In 2010, commercial product for veterinary applications as a canine calming agent for noise aversion were initiated in collaboration with Bioniche Animal Sciences. To provide a cost-effective product, *Souroubea* spp. vine was fortified (55:45 w/w) with sycamore bark (*Platanus occidenta*lis Figure 1-1), a native North American botanical which contains large amounts of betulinic acid (BA) but no amyrins (Durst et al., 2015). Studies of the two raw (unextracted) botanicals, individually or as part of a blend, were undertaken in rodent models and demonstrated that the latter was more active than the individual components. The raw botanical ingredients were formulated for dogs in a 3.5 g chewable tablet containing binder and beef flavoring and 2 g of a 55:45 mixture of *Souroubea* spp. and *Plantanus* spp. standardized to provide the recommended dose of 1 mg/kg betulinic acid for a 10 kg dog. The individual plant components and blend were well tolerated in a pilot dog study in a sub-acute (28 days) pilot study at 8 times recommended dose (Villalobos et al., 2014). Safety was confirmed in a full-scale independent toxicology trial at 5 times dose while monitoring weight, clinical chemistry, and hematology in beagles by an independent study at Kingfisher Inc (Masric et al., 2017). with no significant adverse effects. Studies in dogs at Cancog Technologies in Guleph, Ontario using a thunderstorm model of noise aversion indicated significant dose dependent anxiolytic effects through behavioral modification as well as dose-dependent reduction in plasma cortisol (Masric et al., 2016). A subsequent blinded placebo-controlled study indicated that administration of a single dose of the blend was effective at significantly ($p < 0.05$) lowering cortisol levels in treated animals compared to placebo, 1h after administration.

In 2013–2014, Bioniche sold its veterinary products division. The University of Ottawa license for the technology was then transferred to Souroubea Botanicals Inc. (Ottawa ON), who have marketed the canine tablet through a US distributor (Zentrol™) since June 2016.

1.2.5 Entry into the human market

Based on preliminary studies in rats and dogs, *Souroubea/Platanus* botanical extract is a promising candidate to study for managing anxiety symptoms in humans. Unlike conventional anxiolytics, long-term administration of *Souroubea* active principles does not lead to tolerance or withdrawal effects in rodents. In a study by Cayer (2011), administration of methyl-BA over 30 days resulted in significantly higher percent time spent in the open arms as well as number of unprotected head dips in the EPM compared to controls, i.e., potency and efficacy was sustained over repeated administration. Withdrawal effects of BA and methyl-BA-treated animals assessed during the light and dark cycles using behavioral parameters, food intake, fecal boli output, and body weights were not significantly different from controls (Cayer, 2011).

The Canadian Natural Health Product regulatory framework provides an opportunity for licensing *Souroubea/Platanus* extract as an approved low-risk product based on evidence of safety, quality, and efficacy with a limited treatment claim. Approvals for clinical trials with a *Souroubea/Platanus* extract investigational product (95% ethanol extract) were granted in Dec 2018 and a clinical trial was scheduled for 2019. If successful, the product will be submitted for licencing as an approved NHP. Subsequently, the product could be marketed as a US dietary supplement with a structure-function statement (a statement that describes the role of a dietary ingredient in humans without making a treatment claim). Recent experiments in rodents examined the effects on auditory fear memory reconsolidation elicited by the extract. In the fear-potentiated startle paradigm, significant impairment

of long-term fear memory retention was observed, and no significant effects on short-term memory occurred when the botanical was administered orally directly following memory reactivation. This suggests the potential application in post-traumatic stress disorder in humans. If this can be demonstrated clinically, a standardized extract is a candidate for registration in the US as a botanical drug with evidence-based anxiolytic claims.

1.3 Part II: *Echinacea* spp.

1.3.1 General introduction to Echinacea spp.

Echinacea spp. (Asteraceae), commonly known as purple coneflowers (Figure 1-3), are native to North America (Wichtl, 2004) and were used medicinally for a variety of purposes by the first nations of North America (Shemluck, 1982). For hundreds of years, these plants have been used as an antiseptic or an analgesic to treat poisonous insect and snakebites, toothaches, sore throat, wounds and communicable diseases such as mumps, smallpox, and measles. They were eventually adopted by the early settlers and became popular among the North American eclectic physicians. Alcohol extracts of *Echinacea angustifolia* (DC.) Hell were applied topically for wounds, infections, and poisonous bites and stings as well as orally administered to treat infections. *Echinacea purpurea* (L.) Moench and *Echinacea pallida* were documented but rarely used by the eclectic physicians (Shemluck, 1982). However, *E. purpurea* was brought to Europe by mistake and the European naturopathic doctors and manufacturers found that its efficacy was similar to *E. angustifolia* but was much easier to cultivate. *E. angustifolia* and *E. pallida* were initially considered to be different varieties of the same species until a revision of the genus described them as two separate species in 1968 (McKeown, 1968). Thus, *E. pallida* sometimes was used in Europe as a substitute for *E. angustifolia.*

In modern use, Echinacea-containing products are widely consumed as medicinal products to treat/prevent cold and flu symptoms as well as lung conditions, wound healing and candidiasis (Barrett 2003 and Baum et al., 2006). The pharmacological activity of Echinacea products is based on *in vitro* or *in vivo* studies including immunomodulatory, antimicrobial, anti-inflammatory and anxiolytic activities (Binns et al., 2001; Barrett, 2003; Barnes et al., 2005, Baum et al., 2006; Birt et al., 2008; Guiotto et al., 2008; Chicca et al., 2009; Stanisavljević et al., 2009; Haller et al., 2010&2013; Hu and

Figure 1-3. *Echinacea. angustifolia* (left) and *Echinacea purpurea* (right). *Imaged by John T. Arnason.

Kitts et al., 2014; Sharifi-Rad et al., 2018). Production of Echinacea has increased rapidly due to its great value, and Echinacea-containing products are among the best-selling natural health products (NHP) in North America with a value >$60M/year in the U.S. market alone (Smith et al., 2016) in 2015.

1.3.2 Phytochemistry

The major phytochemicals, identified in *Echinacea* spp. include alkylamides/alkamides (AKA); caffeic acid derivatives (CAD), polysaccharides/glycoproteins and polyenes/polyacetylenes (Binns et al., 2002). At least 20 AKAs have been reported, most of them have been identified as isobutylamides of unsaturated straight-chain fatty acids with one or more olefinic and/or acetylenic bonds (Bauer & Remiger 1989) . Composition of individual AKAs varies by species. For example, isomeric dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (Sloley et al., 2001) is the dominant AKA in the roots and aerial parts of *E. angustifolia* and *E. purpurea*, but it is absent from *E. pallida*. Major isobutylamides from the roots of *E. purpurea* contain acetylenic bonds while those of *E. angustifolia* contain olefinic bond in the fatty acid moeities (Bauer & Remiger 1989). Binns et al (2002) reported the quantitative variation of 19 AKAs in roots and other organs of native plant populations in the *genus Echinacea*. Species-level canonical discriminant analysis (CDA) of roots suggests these phytochemical variables contribute significantly as quantitative phytochemical species markers to discriminate commercial *Echinacea* species. Binns et al (2002A) also reported that these AKAs in root are accumulated with age. The phytochemical composition may also differ between individuals of the same species (Binns et al., 2001).

Caffeic acid derivatives (CADs) such as glycosides and caffeic acid esters are the major phenolic components identified in *Echinacea* spp. Like AKAs, the combination and quantity of CADs varies by Cichoric acid and caftaric acid were found in all *Echinacea* species but are most abundant in the root and aerial parts of *E. purpurea* (Binns et al., 2002A). Chlorogenic acid was found in all *Echinacea*

species as well as a new chemotype (Binns 2002 et al., A, B). Echinacoside is the major component found in the roots of *E. angustifolia* and *E. pallida* (Pietta et al., 1998). However, it is absent from *E. purpurea*. spp. Cynarin was only reported in the roots of *E. angustifolia* (Pietta et al 1998). Verbascoside, 3,4- and 3,5-dicaffeoylquinic acid were also identified in *Echinacea* spp. (Sloley et al., 2001; Barnes et al., 2005).

Polysaccharides and glycoproteins have been reported in roots of *E. angustifolia* and *E. pallida*; roots and aerial parts of *E. purpurea* (Binns 2002 et al., 2002A, B). Bauer (1997) isolated 3 polysaccharides from *E. purpurea:* a methylglucuronoarabinoxylan (MW: 35 kD), an acidic rhamnoarabinogalactan (MW: 450 kD) and a xyloglucan (MW: 79 kD). Blaschek et al (1998) reported a heterogeneous polysaccharide (MW<10kD), an inulin-type fractions (MW: 6 kD) and an acidic arabinogalactan polysaccharide (MW: 70 kD) in *E. purpurea* juice. Classen et al (2000) isolated an arabinogalactanprotein (MW: 120 kD) with a highly branched polysaccharide core in *E. purpurea* juice. Balciunaite et al (2015) also isolated this arabinogalactanprotein in dried *E. purpurea* root with a much lower yield.

Major polyenes/polyacetylenes identified in *Echinacea* spp. including: 8-hydroxytetradeca-9E-ene-11,13-diyn-2-one;, 8-hydroxypentadeca-9E-ene-11,13-diyn-2-one;, tetradeca-8Z-ene-11,13-diyn-2-one, pentadeca-8Z-ene-11,13-diyn-2-one, pentadeca-8Z,13Z-dien-11-yn-2-one; pentadeca-8Z,11E,13Z-trien-2-one and pentadeca-8Z,11Z,13Z-trien-2-one (Binns et al., 2002A). The amounts of these polyenes/polyacetylenes were found higher in *E. pallida* root than in *E. purpurea and E. angustifolia* roots (Barnes et al., 2005).

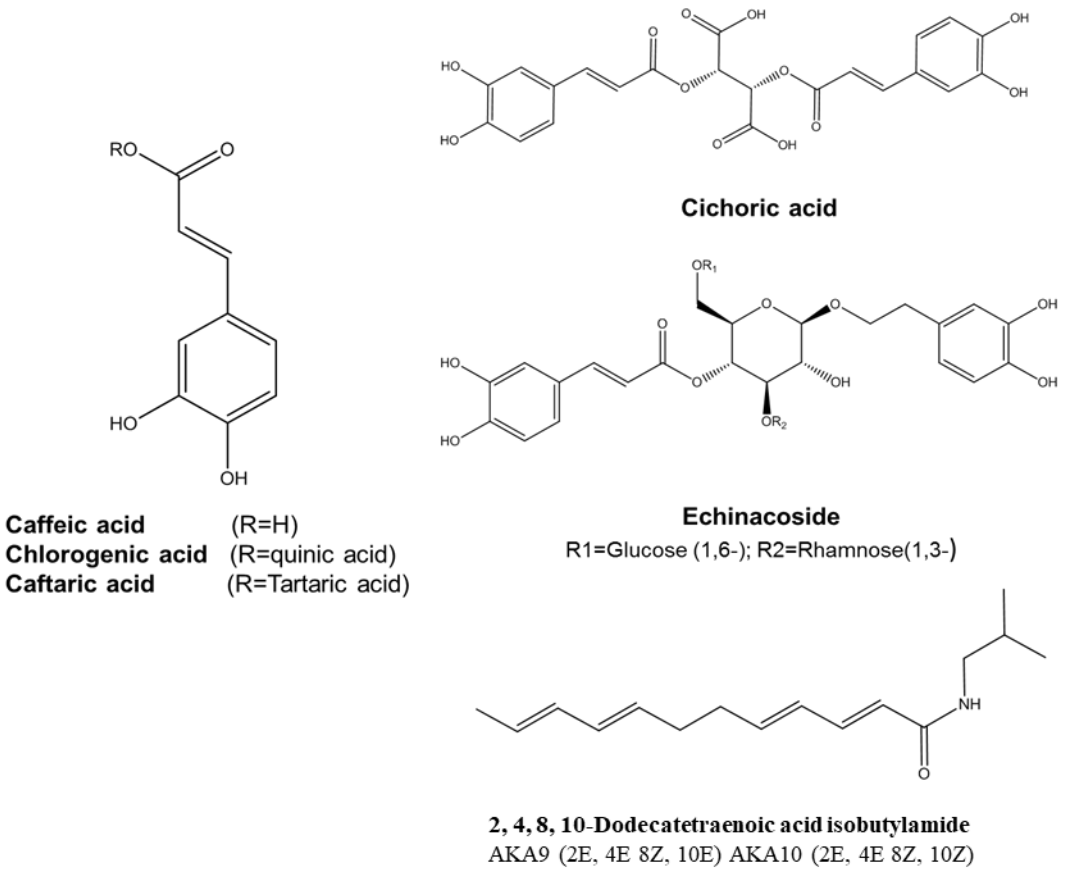


Figure 1-4. Structure of major components of *Echinacea* spp: caffeic acid derivatives (CAD) and alkylamides (AKA).

A series of other plant secondary metabolites including pyrrolizidine-type alkaloids (tussilagine and isotussilagine), flavonoids (quercetin, kaempferol, isorhamnetin and their glycosides), anthocyanins, free phenolic acids (*p*-coumaric, p-hydroxybenzoic and protocatechuic acids) were also reported in *E. purpurea and E. angustifolia* (Bauer 1998; Roder et al 1984; Lin et al 2002; Wichtl 2004).

1.3.3 The endocannabinoid system and related pharmacological targets

The endocannabinoid system (ECS) is an evolutionarily ancient signaling system discovered relatively recently (Pertwee et al., 2006; De Petrocellis et al., 2009) and plays an important role in many physiological processes. Major components and potential pharmacological targets in the system include the cannabinoid receptors, endogenous ligands known as endocannabinoids, reuptake transporter proteins, as well as the enzymes for synthesis and degradation of the endocannabinoids.

Cannabinoid receptors are a type of G-protein-coupled receptor (GPCR). There are currently two known subtypes of cannabinoid receptors (CB1 and CB2) with 44% protein sequence similarity (Svíženská et al., 2008). The CB1 receptor is found throughout the central nervous system but is also expressed in other organs such as lungs, liver and kidneys. The CB2 receptor is expressed mainly in the immune system and in hematopoietic cells, but it can also be found in many peripheral as well as in central nervous system tissues (Pertwee et al., 2006, Kleyer et al., 2012). The role of neuronal CB2 receptors is still unclear. The activation of the CB1 receptor in the central nervous system (CNS) located in the pre-synaptic neuron modulates synaptic plasticity, neuronal excitability, reward and motivation, emotions, learning and memory, locomotion, appetite, and thermoregulation (Kleyer et al., 2012). Activation of the CB2 receptor mainly triggers immunosuppressive effects, which can inhibit inflammation and associated tissue injury in certain pathological conditions. Other peripheral effects of ECS activation include regulation of blood pressure, suppression of gastric and intestinal mobility,

lipogenesis and nociception (Nicolussi and Gertsch, 2015). Other GPCRs such as GPR55 were also found to interact with endocannabinoids.

Endocannabinoids consist of several arachidonic acid derivatives. Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) are considered to be the major endocannabinoids (Pertwee et al., 2010). Unlike the other neurotransmitters which are pre-synthesized and stored in vesicles before release to synapse, the synthesis of endocannabinoids is usually triggered by cellular activation such as calcium influx causing immediate release to the synapse (Nicolussi and Gertsch, 2015). N-acylphosphatidylethanolamine is the main precursor of AEA and the transacylation–phosphodiesterase pathway is considered the major pathway (Figure 1-5). However, there are at least 2 other import pathways for AEA biosynthesis (Muccioli et al., 2010). They are thought to be alternative pathways to compensate the blockage of other pathways under certain conditions. Similar to AEA, 2-AG is also produced by a stimulus such as calcium influx or the activation of Gq/11 coupled receptors (Muccioli et al., 2010). Arachidonic acid-containing diacylglycerol (DAG) are major precursors of 2-AG (Figure 1-5). Alternative pathway including the convertion of phosphatidylcholine and phosphatidic acid by DAG lipase as well as arachidonic acid-containing lysophosphatidic acid by phosphatases (Murataeva et al., 2014).

The degradation of AEA and 2-AG occur mainly through hydrolysis and arachidonic acid is the main deration product. Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are the major enzyme responsible for the hydrolysis of AEA and 2-AG respectively. Other enzymes such as N-acylethanolamine-hydrolyzing acid amidase (NAAA) for AEA and a/b-hydrolase 6 and 12 (ABHD6 and 12) for 2-AG are also involved. Beside hydrolysis, AEA and 2-AG can also be oxidized to PGH2-ethanolamide and PGH2-glyceryl ester by cyclooxygenase 2 respectively (Muccioli et al., 2010). Cytochrome P450 enzymes may also play a role in endocannabinoids degradation (Nicolussi and Gertsch 2015).

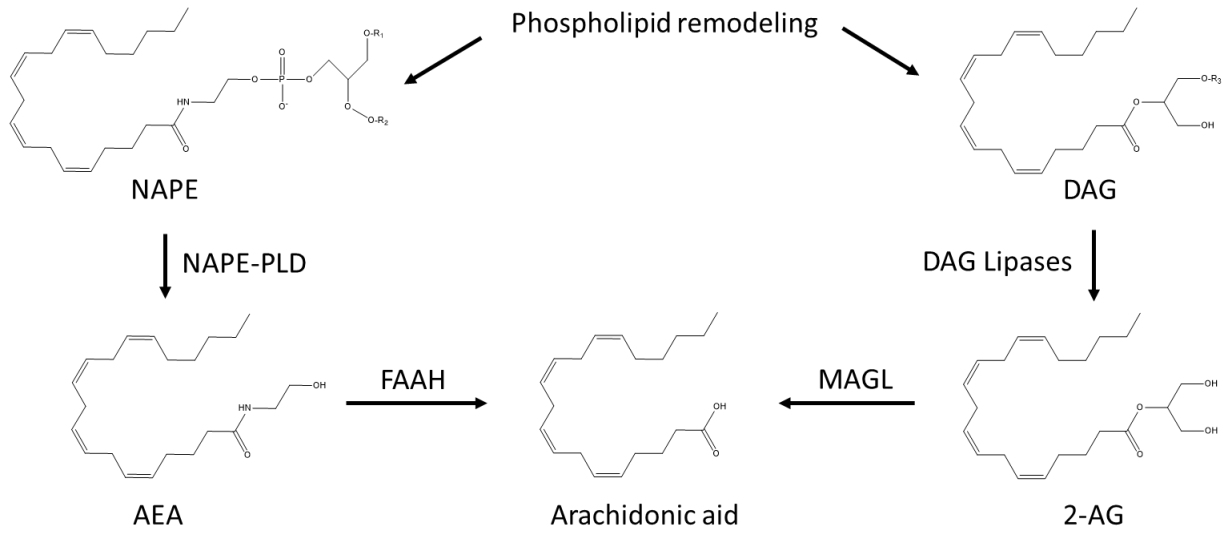


Figure 1-5. Biosynthesis and degradation pathway of major endocannabinoids

AEA: Anandamide; 2-AG: 2-arachidonoyl glycerol; NAPE: N-acetylphosphatidylethanolamine; DAG: Diacylglycerol; NAPE-PLD: N-acetylphosphatidylethanolamine-hydrolysing phospholipase D; DAG-Lipases: Diacylglycerol lipase; FAAH: Fatty acid amide hydrolase; MAGL: Monoacylglycerol lipase.

As the degradation process is mainly intracellular, the re-uptake of endocannabinoids is considered a key factor in the activation of CB receptor. Based on the discovery of the AEA reuptake inhibitor, a membrane transporter was proposed (Beltramo et al., 1997; Battista et al., 2005). However, no transporter protein has been identified so far. Furthermore, many of the of the AEA re-uptake inhibitors were also found to be a FAAH inhibitor which rapidly hydrolyzed the intracellular AEA altering the inward diffusion of AEA. Thus, the pharmacological target of these re-uptake inhibitors may be FAAH instead of a transporter. Additional *in vitro* studies by Dickason-Chesterfield et al. (2006) and Nicolussi et al. (2014) found several reuptake inhibitors (LY2183240 and URB597) that showed low inhibition potency in HeLa or HMC-1 cell lines which lack FAAH activity. On the other hand, indirect evidence suggests the existence of a membrane transporter. Ortega-Gutierrez et al. (2004) experimented the inhibitor UCM707 in FAAH knock out mice and still blocked intracellular AEA accumulation with an IC_{50} around 3μM. While the existence of a membrane transporter is still debated, a series of intracellular transporters of AEA was discovered (Kaczocha et al., 2009; Oddi et al., 2009). Cytoplasmic binding proteins including fatty-acid-binding proteins (FABPs), albumin and 70 kilodalton heat shock proteins (Hsp70) were found to be involved in the intracellular trafficking of AEA. Although 2-AG was found to be hydrolyzed both intra/extra cellularly, and a similar cellular uptake mechanism was also reported. Chicca et al (2012) concluded that 2-AG may compete with the same putative membrane transporter. Furthermore, AEA re-uptake inhibitors AM404 and OMDM-2 were found to inhibit the 2-AG re-uptake. To summarize, the re-uptake of endocannabinoids appears to involve many different mechanisms including facilitated diffusion driven by intracellular enzymatic hydrolysis and interaction with intra/inter cellular binding proteins.

As the involvement of ECS in physiological processes is highly diverse, it is recognized as an important phrenological target (Fonseca et al., 2005). In addition to many synthetic compounds that target the CB receptors, synthesis/degradation enzymes or the re-uptake of endocannabinoids have

been studied for their potential medicinal applications related to anxiety/depression, appetite, Attention deficit hyperactivity disorder (ADHD), Alzheimer's disease, motor dysfunction (Hungtington's disease, Parkinson's disease), multiple sclerosis, pain management, inflammation, atherosclerosis, bone development, nausea/vomiting, and glaucoma, etc.(Fernández-Ruiz, 2009; Tambaro and Bortolato, 2012; Loflin et al., 2014; Whiting et al 2015; Liu et al., 2015; Huang et al., 2016; Häuser et al., 2017; Hasenoehrl et al., 2017; Ho et al., 2017).

Besides endo/synthetic cannabinoids, a number of plant secondary metabolites were found to interact with the ECS system and can also stimulate CB receptors. These phytocannabinoids are not limited to Δ9-tetrahydrocannabinol and related components found in the genus *Cannabis*, with many other classes of plant secondary metabolites now reported to interact with ECS through different mechanisms. For example, salvinorin A from *Salvia divinorum* and falcarinol from *Daucus carota* (carrot) were identified as antagonist and inverse agonist of CB1 receptor, respectively. β-Caryophyllene, found in many plants including *Piper nigrum*, *Melissa officinalis* and *Humulus lupulus*, is a selective CB2 receptor agonist. Macamides from *Lepidium meyenii* and the common flavonoid kaempferol were identified as FAAH inhibitors; Guineensine N-isobutylamide from *Piper nigrum* was also found inhibit the re-uptake of AEA (Russo, 2016).

1.3.4 Alkylamides and CB activity

Early research mainly associated CADs with antioxidant and antimicrobial properties whereas polysaccharides/glycoproteins were associated with the immunomodulatory effects (Barnes 2005). More recent evidence suggests that AKAs may contribute to most of these activities because of their greater bioavailability (Woelkart et al., 2005B). AKAs also interact with the ECS. Woelkart et al (2005A) demonstrated CB binding activity of major AKAs isolated from the root of *E. angustifolia* in a standard receptor binding assay using [3H] CP-55,940 as a radioligand. Although most of the AKAs bind both the CB1 and CB2 receptor, the affinity is more selective toward CB2. Hohmann et al. (2011)

tested CB1 activation activity of major AKAs isolated from the root of *E. purpurea* in a [35S] GTPγS binding experiment performed in rat brain membrane preparations. The results indicated that most AKAs interact with CB1 receptor. However, 7 of 10 tested compounds were recognized as inverse agonists with 2 partial agonists and a neutral compound. Gertsch et al. (2004) reported in an *in vitro* study that Echinacea AKAs may modulate TNF alpha gene expression via CB2 receptor activation. It was then confirmed in an *in vivo* assay with the rodent endocannabinoid system model by Raduner et al. (2006) that the immunomodulatory effects dodeca-2E,4E,8Z,10Z-tetraenoic acid N-isobutyl amide and dodeca-2E,4E-dienoic acid N-isobutyl amide are dependent on a CB2 receptor interaction. Furthermore, Chicca et al. (2006) reported synergistic pharmacological effects on anti-inflammatory and immunomodulatory responses to an *E. purpurea* tincture, as well as a mixture of AKAs, through CB2 receptor activation. In addition, Cech et al. (2006) reported reduced immunomodulatory activity of dodeca-2E,4E,8Z,10Z-tetraenoic acid N-isobutyl amide and *E. purpurea* extract after human Cytochrome P450 enzyme metabolism.

Beyond anti-inflammatory and immune-modulatory activity, anxiolytic activity has also been linked to AKA-ECS interaction. Haller et al. (2010 and 2013) reported *in vivo* studies with rats which demonstrated that both *E. purpurea* and *E. angustifolia* extracts exhibited anxiolytic effects in the elevated plus-maze, social interaction and shock-induced social avoidance tests. Significant anxiolytic results were also been reported in a small clinical trial (Haller et al., 2013) with 33 volunteers. The high dosage group treated with formulated *E. angustifolia* extracts (2 tablets per day) decreased State-Trait Anxiety Inventory (STAI) scores within 3 days in human subjects, an effect that remained stable for the duration up to 2 weeks.

Direct CB receptor binding is not the only mechanism of AKA ECS interaction, AKAs may also enhance the CB receptor activation through the inhibition of FAAH. AKAs were expected to interact with FAAH due to their structural similarity to the AEA. Several AKAs were tested against FAAH

previously. However, the results are controversial. Woelkart et al. (2005B) reported no FAAH inhibitory effects of several AKAs at a concentration of 25 nM in *in vitro* assay with rat brain tissue as enzyme source. Chicca et al. (2006) reported *Echinacea* extract as a partial FAAH inhibitor but failed to establish a significant dose response in an *in vitro* assay with pig brain tissue as enzyme source. The conflicting results may have been caused by different assay condition as well as the micellization of AKAs proposed and tested by Raduner et al. (2007). AKAs aggregate as the concentration increases and from micelles in aqueous solution. Aggregated AKAs are unlikely to access the active site of FAAH. Thus, only the concentration of the AKA monomer is associated with the FAAH inhibitory effect.

1.3.5 Bioavailability

The pharmacological activity of orally administrated herbal medications is usually limited by the bioavailability of the active ingredients. Pharmacokinetics of Echinacea compounds has focused on the caffeic acid derivatives and AKAs.

Matthias et al (2005A) tested the permeability of major Echinacea ingredients through the blood intestinal barrier *in vitro* by using a Caco 2 monolayer. Caffeic acid derivatives (Caftaric acid, cichoric acid and echinacoside) showed poor permeability with no more than 5% diffused through the monolayer in 90 min. On the other hand, 2 of 4 AKAs tested (dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide, dodeca-2E,4E,8Z-trienoic acid isobutylamide, undeca-2E-ene-8,10-diynoic acid isobutylamide and dodeca-2E-ene-8,10-diynoic acid isobutylamide) diffused through the monolayer rapidly with an uptake rate around 80%, 60% 30% and 10 % respectively. A follow up small scale clinical pharmacokinetic study in 9 healthy volunteers has confirmed the *in vitro* results. After oral administration of Echinacea tablets (MediHerb, Australia; each tablet contains *E. angustifolia* root extract 150 mg, containing 2.0mg alkamides, and *E. purpurea* root extract 112.5 mg, containing 2.1mg alkamides), Caffeic acid derivatives were also identified in plasma at any time up to 12 hours after

tablet ingestion. However, all 4 AKAs were detected in plasma 20 minutes after tablet ingestion. The mean maximum plasma concentration (C_{max}) for total AKAs were 336 ± 131ng/ml, time to C_{max} was 2.3 ± 131 hr. Some of the AKAs are detectable in plasma up to 12 hours.

Studies also demonstrated the high bioavailability of AKAs. Dietz et al. (2001) reported that dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide was detectable in blood 1 h after ingestion of a concentrated *E. purpurea* ethanolic extract containing total 4.3 mg isobutylamides in a single healthy volunteer. Woelkart et al. (2006) report dodeca 2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides, the main AKAs in the roots of *E. angustifolia* was detected with a t_{max} of 30.3 minutes and C_{max} at 10.88 ng/mL after a single 2.5-mL dose of *E. angustifolia* extract (containing approximately 2mg of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides) in 11 healthy volunteers. Four other AKAs were also detected in the plasma with a similar t_{max}. Woelkart et al. (2007) conducted similar experiment with *E. purpurea* extract in 10 (8 test, 2 placebo) volunteers with two different dosage form tincture and tablet, both containing the same amount (0.07 mg) of the major alkamides, dodeca-2E,4E,8Z,10E/ Z-tetraenoic acid isobutylamides). Results indicated that *E. purpurea* tincture has a faster absorbance rate (t_{max} at 30 min) and higher C_{max} (0.4 ng/ml) than the tablets (t_{max} at 30 min and C_{max} at 0.12 ng/ml). The overall AUC of tablets are also lower than tinctures suggest a higher bioavailability of tinctures.

Animal studies revealed more details on the distribution of AKAs. Pharmacokinetics and tissue distribution of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides was studied in rats. After an oral dose of 2.5mg/kg of the pure compounds, dodeca-2E,4E,8E,10E/Z tetraenoic acid isobutylamides was detected in plasma, liver and brain including hippocampus, cerebral cortex, striatum and cerebellum in 8 min and reach the maximum concentration at 15 min. C_{max} determined the in plasma, liver, hippocampus, cerebral cortex, striatum and cerebellum are 26.35 ng/ml, 54.65 ng/g, 39.56 ng/g, 36.49 ng/g, 45.96 ng/g and 33.80 ng/g respectively. The concentration decreased slower in brain tissue

compared to liver and plasma. These results indicated that AKAs can cross the blood brain barrier and affect the central nervous system (Veryser et al., 2016).

Although caffeic acid derivatives are usually not detectable in their original form after oral administration, a pharmacokinetic model involving hydrolysis has been proposed. Bel-Rhlid et al. (2012) suggest that cichoric acid can be fully hydrolyzed by esterase produced by gastrointestinal tract microflora to caftaric acid. Caftaric acid can be further hydrolyzed to caffeic acid and tartaric acid which are more bioavailable. The absolute bioavailability of caffeic acid determined in rats is 14.7% (Wang et al, 2014). A similar phenomenon was observed with chlorogenic acid. In both animal and human trails, chlorogenic acid metabolites (caffeic acid, quinic acid and ferulic acid) but not chlorogenic acid were identified in urine; chlorogenic acid conjugate but not chlorogenic acid was found in plasma (Olthof et al., 2001; Gonthier et al., 2003). These findings suggest that future pharmacokinetic study of caffeic acid derivatives should focus more on their metabolites. The permeability of echincoside is very poor in the *in situ* rat intestine model (Shen et al, 2015). However, with the presence of verapamil, an efflux protein inhibitor, the absorption parameters of echinacoside were significant improved. Shen et al (2015) also suggest that essential oil such as clove oil can also improve the absorption of echinacoside in an *in situ* model.

1.4 Rational and Objectives

1.4.1 Souroubea and Platanus

As the botanical blend of *Souroubea sympetala* and *Platanus occidentalis* based product Zentrol™ is well received in the companion animal health market, additional quality safety and efficacy data have been requested by the veterinarians for further adjustment of the formula and dosage. These data would also be valuable for further development of a health product for human use.

Development and validation of a highly sensitive and selective analytical methods for analyzing and fully quantifying of major pentacyclic triterpenes in a variety of *Souroubea* and *Platanus* raw materials, plant extracts and finished blended product are needed. This method is critical for the quality control (QC) of the current products (Zentrol™) on the market as well as the further pharmacological research and future products development. The development of a validated analytical method is presented in chater 2.

The main active ingredient of Zentrol™, betulinic acid, has high permeability but low solubility so its bioavailability is limited to the solvation rate. Although a previous study reported that a single high dose of betulinic acid (>=100mg/kg; Godugu et al., 2014) can be detected in serum after acute administration, the difference in AUC (area under curve) can vary up to 8 fold between formulated and unformulated betulinic acid preparations in a rodent model (Godugu et al., 2014). However, due to the limitation of maximum solubility or the saturation of absorption, distribution, metabolism and excretion (ADME) pathways, the single/repeat oral administration of low betulinic acid concentration products like the Zentrol™ animal product may exhibit a different pharmacokinetic (PK) profile. Thus, the study of the PK profile of Zentrol™ (dose=<5mg/kg betulinic acid content) in beagles was the objective in chapter 5 to guide appropriate dosing of the current products, as well as future products in development.

Plant secondary metabolites are well known to affect the pharmacokinetics ADME of endogenous compounds, drugs and other bioactive substances by altering the activity of certain metabolic enzymes (ie. Cytochrome P450) and transport proteins (ie. P-glycoprotein). These effects may result in the increase/decrease of the bio-availability, efficacy and toxicity of co-administrated health products. The phytochemical studies of *S. sympetala* and *P. occidentalis* (Ibrahim et al., 2009 and Puniani et al., 2014) reveal various pentacyclic triterpenoids and flavonoids. Most of these compounds have not been systemically investigated for the metabolic transformation and inhibitory effect against ADME system. Hence, investigation of the potential risk of NHP-drug interactions related to the concomitant use of this herbal product with conventional medical products, is an important safety study. Consequently, a study in chapter 4 investigated the effect of the *Souroubea-Platanus* extract on the metabolism of the anxiolytic diazepam in an *in vitro* model.

Natural health products are usually categorized as multi-active ingredient medications which could target multiple sites or act together on a single site, additively or synergistically, through direct or indirect interaction. Accordingly, the previously reported synergistic effect on anxiolytic activity observed with the botanical blend of a Souroubea and Platanus extract over activity of single species extracts (Cayer, 2011) could be the result of a secondary anxiolytic mode of action. The endocannabinoid system (ECS) plays an important role in many physiological processes and in mood disorders such as anxiety (Howlett et al., 2002). Several tri-terpenoids have been reported to inhibit the endocannabinoid degradation enzyme Monoacylglycerol lipase (Chicca et al., 2012) including α/β-amyrin, which have also been found in the botanical blend of *S. sympetala* and *P. occidentalis*. This observation suggests that this botanical blend may also exhibit anxiolytic activity by activating the ECS. Currently, there is no evidence to indicate if betulinic acid, the most abundant pentacyclic tri-terpenoid found in botanical blend extract, ursolic acid or lupeol can interact with the ECS. However, due to structure similarity, they may have similar effects on the cannabinoid system as α/β-amyrin.

Thus, these compounds, along with plant extracts were investigated in chapter 4 for possible effects on the endocannabinoid degradation enzymes.

Based on current evdences, I hypothesized that the botanical blend of *Souroubea* and *Platanus* as well as their triterpenoids may affect major drug metabolism enzymes such as the cytochrome P450 family to cause NHP-drug interactions. They may also affect the ECS as a secondary anxiolytic mode of action. Betulinic acid, the main active ingerdent can be measured in serum and used as a marker to evaluate the efficacy of this botanical blend. Therefore, the overall objective of part I was to investigate the pharmacokinetics, safety profile and the alternative pharmacological mechanism of the botanical blend of *S. sympetala* and *P. occidentalis* as well as the development and validation of an analytical method for analyzing and fully quantifying of major pentacyclic triterpenes in a variety of *Souroubea* and *Platanus* preparations. The specific objectives for the study were:

Objective 1: Development and validation of a highly sensitive and selective analytical method for analysing and fully quantifying of major pentacyclic triterpenes in a variety of *Souroubea* and *Platanus* preparations (Chapter 2).

Objective 2: Investigation of the effect of botanical extract containing *S. sympetala* and *P. occidentalis* on major human cytochrome P450 enzymes and diazepam metabolism using an *in-vitro* assay (Chapter 3)

Objective 3: Investigation of the effect of botanical extract containing *S. sympetala* and *P. occidentalis* on the main endocannabinoid degradation enzymes, monoacylglycerol lipase and fatty acid amide hydrolase (Chapter 4)

Objective 4: Investigation of the betulinic acid concentration as well as the possible metabolites in Serum/whole blood of Beagles dogs after oral administration of the blend of *S. sympetala and P. occidentalis* (Chapter 5).

1.4.2 Echinacea spp.

The major phytochemicals identified in *Echinacea* spp. include AKAs; CADs, polysaccharides/ glycoproteins and polyacetylenes (Binns et al., 2002A, B). The phytochemical composition varies not only by species but also among populations in the same species (*E angustifolia*, Binns et al., 2001). Due to the structural similarity to anandamide, AKAs are believed to contribute to Echinacea's anti-inflammatory and anxiolytic activities (Hohmann et al., 2011). Echinacea AKAs are highly bioavailable and known to bind to cannabinoid receptors with selectivity for CB2 (Woelkart et al., 2005A). Individual AKA have been identified with partial agonist, antagonist as well as inverse agonist properties (Hohmann et al., 2011). However, the interaction of AKAs with ECS enzymes such as FAAH and MAGL has not been fully studied. Currently, CADs and polysaccharides appear most relevant for their antimicrobial effects. However, since phenolic compounds have been reported to inhibit FAAH and MAGL (Russo, 2016), the CADs may also affect the endocannabinoids system indirectly.

As the pharmacological effect of *Echinacea* spp. are usually recognized as a combined effect from multiple plant components (Barnes et al., 2005), a new approach was used to determine contributing active metabolites as well as Echinacea chemotypes with strong ECS activity. A collection of commercial plant accessions from a major North American producer, together with isolated Echinacea substances, were studied for their interactions with ECS, including CB receptors, transporter proteins and degradation enzymes using *in vitro* assays. Biochemometric analyses of ECS activity relative to detected metabolite profile are conducted with subsequent evaluation of major identified components to confirm or refute activity.

Oral bioavailability of AKA has been estimated at 60% with a C_{max} of less than 30 min and a half-life of less than 3 hours (Matthias et al., 2005B). These results suggest that *Echiancea*'s AKAs are

rapidly absorbed with a high bioavailability compared to CADs. However, AKAs may also be quickly metabolized by the hepatic enzymes. Echinacea AKAs have been reported to be metabolized through the CYP450 system (Cech et al., 2006) but limited data are available beyond dodeca-2E,4E,8Z,10Z/E-tetraenoic acid isobutylamide with its metabolites tentatively identified by LC-MS. How hepatic metabolism affects the pharmacological effects of AKAs remains unknown. Following CYP450-mediated structural transformation of the substance, the activity of the metabolite may be increased or decreased, or gain new activities, relative to the parent substance. Since most Echinacea products are ingested orally and subjected to first-pass metabolism, investigation of the pharmacological activity of AKA metabolites was undertaken to provide insight into the compounds and mechanisms that contribute to *in vivo* effects.

Although none of the major ingredients' *in vitro* ECS activity are as potent as artificial CB receptor agonist/antagonist or ECS degradation enzyme inhibitors, *Echinacea* spp. extracts showed impressive *in vivo* activity at a relatively low concentration. Haller et al. (2010 &2011) reported orally administered both *E. angustifolia* and *E. purpurea* extract are effective in reducing anxiety in rats using the classic elevated plus maze paradigm at moderately low doses (4-8mg/kg). These results are believed to be mediated by the ECS activity of Echinacea AKAs, most likely through CB1. Hence, *Echinacea* spp. extracts may also be effective in other conditions influenced by the ECS, such as inflammatory pain relief (Barrie and Manolios, 2017).

Based on current data, I hypothesized that the agonist effects on ECS may correlated with the phytochemistry variation of *E purpurea* and *E. angustifolia*, Echinacea genotype with stronger ECS effect maybe used for new application including treatment of periphire pain. Therefore, the overall objective of thesis part II was to investigate the effect of phytochemical variation and hepatic metabolism on ECS related activity of *Echiniacea* spp. components and extracts as well as determine

the pharmacological effect in rodent inflammatory pain model. The specific objectives for the study were:

Objective 5: Investigation of the interaction of Echinacea extracts and isolated substances on endocannabinoid degradation enzymes FAAH and MAGL using *in vitro* assays. Identification of contributing active metabolites as well as Echinacea chemotypes with strong enzyme inhibition activity with biochemometric analysis (Chapter 6).

Objective 6: Characterization of the metabolites formed by human liver enzymatic oxidation of AKAs isolated from Echinacea spp. extract and determination if the hepatic metabolism affects the inhibitory potency of AKAs against FAAH (Chapter 7).

Objective 7: Investigation of the interaction of Echinacea extracts, fractions and isolated substances on endocannabinoid receptor 1 and 2 as well as intracellular anandamide transporter protein (fatty acid binding protein 5) using *in vitro* assays (Chapter 8).

Objective 8: Investigation of the inflammatory pain relief activity of Echinacea extract in a rodent model (Chapter 8).

CHAPTER 2: A SELECTIVE ION HPLC-APCI-MS METHOD FOR THE QUANTIFICATION OF PENTACYCLIC TRITERPENES IN AN ANXIOLYTIC BOTANICAL DIETARY SUPPLEMENT FOR THE ANIMAL HEALTH MARKET

2.1 Introduction

The diverse bioactivities and low toxicity of triterpene rich plant extracts are a good choice for phytopharmaceutical products (Jäger et al., 2009 Laszcyk, 2009). Specially, pentacyclic triterpenes, are well known for their anxiolytic, anti-depressant, antibacterial, anti-inflammatory, anti- HIV and anti-cancer effects (Yogeeswari and Sriram, 2005 and Dressler, 2009). Our collaborative research group in Canada and Costa Rica, has focussed on the anxiolytic properties of several plants containing pentacyclic triterpenes. The genus *Souroubea* (Marcgraviaceae) includes 19 species distributed over much of the neotropics but absent from the Antilles (Aragão et al., 2006). *Souroubea guianensis* Aublet *var cylindrica* Wittm. was used to treat mood and sleep disorders (Shultes and Raffauf ,1990 and Bourbonnais-Spear et al., 2007). In our studies, extracts of leaf and stem of two Central American species, *Souroubea gilgii Gilg* and *Souroubea sympetala* V.A. Richt were found to have significant activity in rat behavioral models of anxiety such as the elevated plus maze (Mullally et al., 2011). Both species have a similar phytochemical profile and anxiolytic activity guided fractionation yielded triterpene rich fractions containing betulinic acid (1), ursolic acid (2), lupeol (3), β-amyrin (4) and α-amyrin (5) (Figure 1-2) (Puniani, 2004). Betulinic acid was found to be the main active principle (Puniani, 2004). Amyrins were also reported to have anxiolytic effect in vivo (Aragão et al., 2006).

Studies in Ontario, Canada identified Sycamore (*Platanus occidentalis* L., Platanaceae) a native North American tree, as an excellent source of betulinic acid from the bark. A proprietary blend of *Souroubea* spp. with *Platanus* spp. herbal ingredients (Zentrol^{TM}) was developed which was found to be more efficacious in animal trials and more cost effective than either botanical alone (Liu et al 2017).

For any new botanical dietary supplement, quality control of active principles is important to be able to compare the eventual product used with clinically tested material. As well, quality control is essential to select the best genotypes for use and preparation of formulated material. A pilot study

described the chromatographic separation and detection of four triterpenes (Figure 1-2) in *Souroubea* spp. (Mullally et al., 2008) but was not applied to *Platanus* spp. or validated for quantitative analytical use. In this chapter, the first objective was the development and validation of an HPLC-APCI-MS method for analyzing and fully quantifying 5 pentacyclic triterpenes in *S. sympetala, P. occidentalis* raw materials and finished blended product. The second objective was to determine amounts of the 5 triterpenes in potential source materials including *S. sympetala., S gilgii, P. occidentalis, Platanus x acerifolia* (Aiton) Willd. and *P. mexicana* Moric.

2.2 Methods and Material

2.2.1 Plant collection

S. sympetala and *S. gilgii* were collected in Costa Rica and vouchers were deposited at the herbarium Juvenal Rodriguez, Universidad Nacional (Table 2-2). Bark of *P. occidentalis*, *P. x acerifolia* and *P. mexicana* was sustainably collected in U.S, Mexico or Canada, from trunks of matures trees > 0.5 m diameter at breast height when it sheds naturally in August-September and vouchers held at the University of Ottawa herbarium.

2.2.2 Extraction

Extracts were prepared by Soxhlet extraction (Mullally et al., 2011). Before the extraction the plant organs or dosage forms were dried at 35 °C in a plant drier for 2hr and ground in a Willey mill with 20 mesh. The ground material (about 0.5 g) was transferred to cellulose thimbles and extracted with a Soxhlet apparatus for 2 hrs (6 cycles / hr) by 250 mL ethyl acetate (1:500). The extracts were concentrated under vacuum on a Yamato Rotary Evaporator RE50 (Yamato Scientific, Japan) with water bath temperature maintained at 40 °C. The extracts were lyophilized in a Super Modulyo freeze drier (Thermo Electron, Ottawa, ON, Canada) and the extract were re-solubilized in methanol (LCMS grade) with the final volume adjusted at 50 ml in a volumetric flask. The extracts were then filter through a 0.22 mm PTFE filter and stored at -20 °C in amber glass vials before HPLC-APCI-MS analysis.

2.2.3 HPLC-APCI-MS analyses

Solvents (LCMS grade) were from Fisher Scientific (Ottawa, ON. Canada). Authentic (>97% purity), betulinic acid, ursolic acid, lupeol, β-amyrin and α-amyrin were from Extrasynthese (Lyon, France). Targeted HPLC-MS analyses were carried out on a 3200 QTRAP (ABSciex, Concord, ON,

Canada) connected to a 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA). Separations were performed at 1mL/min on a Kinetex C18 column, particle size 2.6-micron, 100 mm × 2.1 mm I.D. (Phenomenex, Torrance, CA, USA). Column thermostat was maintained at 55 °C during a linear gradient of 30-100% acetonitrile in water. The column was then washed with 5 column volumes with 100% acetonitrile, returned to the initial conditions in 0.1 min and re-equilibrated for 5 min before the next injection (total run time 30 min). Extracts were sonicated for 5 min and 1μL were injected in triplicate through the auto sampler. The MS was operated in Q1MI negative ionization mode for 1 and 2 and Q1MI positive ionization mode for 3, 4 and 5 (Table 2-1) with a dwell time set at 100 msec. Optimal negative mode Q1MI conditions were: declustering and entrance potentials -60 V, -10 V, nebulizer current -1.0 V, source temperature 500 °C, nebulizer gases 1 and 2 set at 50 psig and 30 pisg respectively. Optimal positive mode Q1MI conditions were: declustering potential, entrance potential and nebulizer current 60 V, 10V and 3V respectively, source temperature 500 °C with ion source gases 1and 2 50 and 55 psig respectively. Curtain gas was set at 20 L/min in both modes.

2.2.4 Quantification and method validation

Linear calibration curves were generated by injecting each compound using an optimized LCMS methodology. Compounds 1, 3-5 were quantified on the basis of area under the peak that bracketed the response obtained from the calibration range of each compound, while 2 was quantified on the basis of peak height. Precision, accuracy, and linearity thresholds were set according to ICH guidelines (ICH-Q2B, 1996). Recovery experiments were undertaken by spiking plant material with pure compounds at 0.1, 0.2, 0.5 and 1 mg/g of betulinic acid and 0.2, 0.4, 1 and 2 mg/g of the other metabolites were spiked; 2, 4, 10, 20 mg/g of betulinic acid were spiked to *P. Occidentalis* due to the high betulinic acid concentration. Spiked and unspiked samples (in triplicate) were extracted following the procedure described above and recovery determined by regression analysis. Calibration curves were prepared at five concentration levels and R2 values obtained for each metabolite. The limits of detection (LOD, 3:1

signal: noise) and limits of quantification (LOQ 10:1 signal: noise) were determined at 6x standard deviation of noise level. The developed and validated method was used to analyze and quantify the amount of the five selected triterpenes in plant samples as well as the Zentrol^{TM} final products

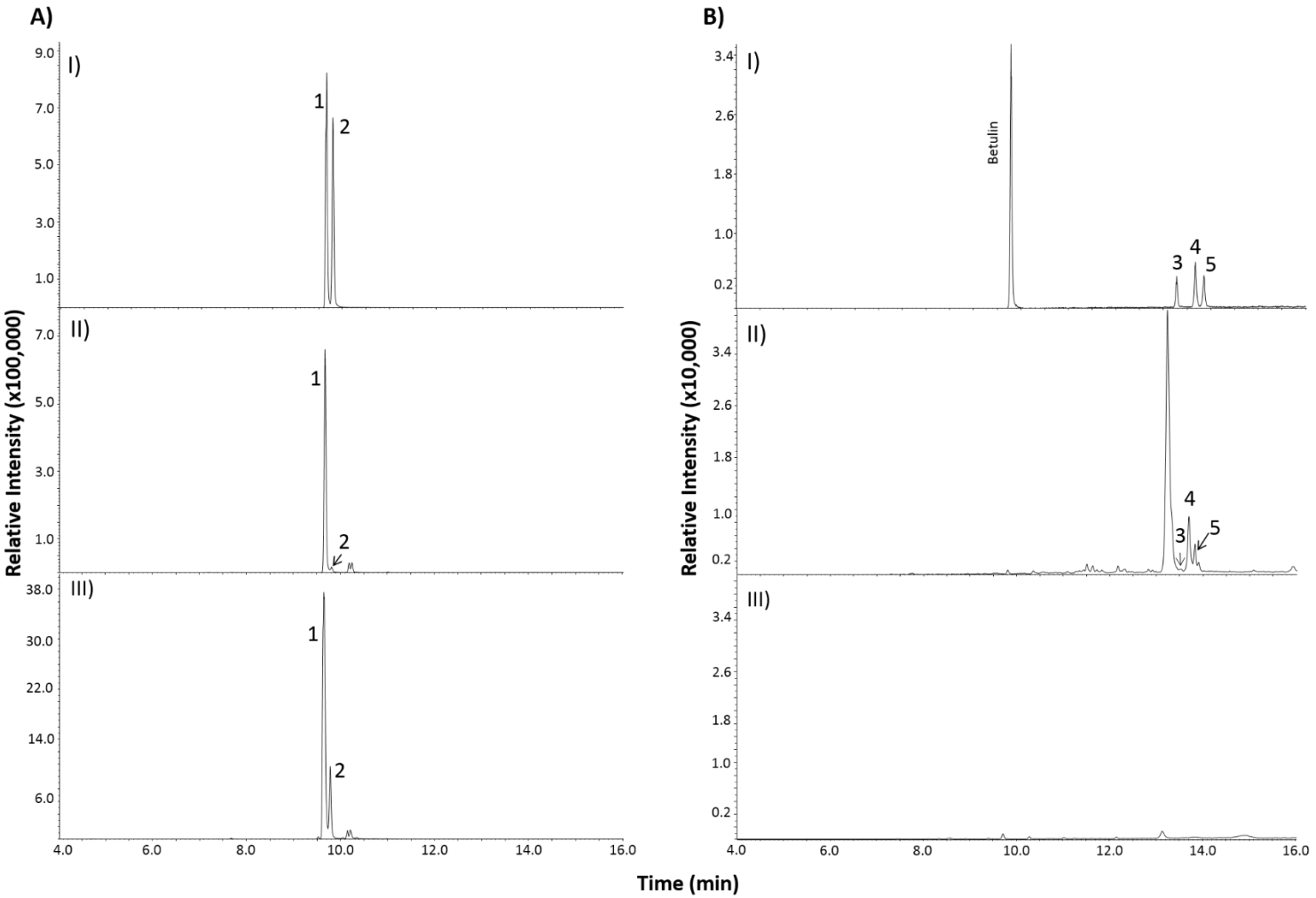


Figure 2-1. Chromatographic separation of triterpenes, betulinic acid (1), ursolic acid (2), lupeol (3), β-amyrin (4) and α-amyrin (5). A) HPLC-APCI (negative ionization mode) Total ion count (TIC) chromatogram and B) HPLC-APCI (positive ionization mode) TIC chromatogram. I): standard compounds mixture, II) *S. sympetala*, III) *P occidentalis*.

2.3 Results and discussion

Table 2-1 shows the detected masses of the target compounds identified from raw materials of *Souroubea* spp, and *Platanus* spp. Compounds 1 and 2 produced two peaks at m/z 455.3 eluting at 9.7 and 9.8 min in negative ionization (Figure 2-1A) and 3, 4, 5 produced three peaks at m/z 425.4 in positive ionization eluting at 13, 14.1 and 14.2 min respectively while betulin eluted at 10.1 min (Figure 2-1B). Since betulin was detected only at trace levels below detection limits in the study materials it was not included in method validation. The developed method enabled us to identify and quantify the markers in plant extracts with a linearity (R2>0.997) for all of the compounds within a range of 0.2-25.0 ng for betulinic acid on column, 0.2-10.0 ng of ursolic acid on column, and 1.0-100.0 ng on column for 3, 4 and 5 (Table 2-2). It is notable that proper integration is very important for authentic quantitation particularly for 1 and 2 with approximately 10% signal overlap and the presence of a semi co-eluting peak with 5. The appearance of additional signals with selected ion monitoring indicates the presence of isomers of amyrins. The sensitivity of the method was assessed by determination of limits of detection and quantification of the compounds under current extraction protocol (Table 2-1) The LOD and LOQ were much lower (pg amounts on column) for 1 and 2 than other compounds 3-5. When recovery analyses of spiked samples were performed for the five triterpenes to determine accuracy, the linear regression of recovered amount versus the spiked were obtained (R2 >0.99) and all the recoveries observed were > 94% (Table 2-1). Based on the results observed for the recoveries, the extraction described in the sample preparation is recommended for targeted analysis. However, in a previous study (Mullally et al., 2011), results by supercritical extraction were similar, and this method could be substituted if desired and the equipment is available. Percentage recovery of 102 % for betulinic acid can be explained by the 1.55 % CV observed during intraday variation study. These results reveal that the method is highly reproducible. The precision of

Table 2-1. Chromatographic, spectrometric and validation data of pentacyclic triterpenes identified from *S. sympetala* by the developed analytical methodology. Limits of detection (LOD, 3:1 signal to noise) and limits of quantification (LOQ, 10:1 signal to noise), of each marker on column (6 x standard deviation) in leaf and stem of *S. sympetala.* R^2 value of linear regressions >0.99. Mean (SEM) are presented for % Intraday variation (n=10), % Interday variation (n=10) and % recovery (n=3).

Triterpene	RT (min)	Ion detected	LOD (ng)	LOQ (ng)	% Intraday variation	% Interday variation	Linearity (R^2)	Range (µg/ml)	% recovery[a]	
									Souroubea	Plantanus
Betulinic acid	9.7	455.3, $[M\text{-}H^+]^-$	0.01	0.03	1.55 (0.4)	4.96 (0.7)	0.998	0.2-25.0	102.3 (2.1)	95.0 (3.0)
Ursolic acid*	9.8	455.3, $[M\text{-}H^+]^-$	6.6	21.9	3.46 (0.5)	5.66 (0.8)	0.997	0.2-10.0	95.1 (2.3)	91.0 (2.2)
Lupeol	13	425.4, $[M+H\text{-}2H]^+$	610	2030	2.71 (0.9)	10.34 (1.4)	0.993	1.0-100.0	96.5 (1.7)	NA
β-amyrin	14.1	425.4, $[M+H\text{-}2H]^+$	363	1210	2.16 (0.2)	5.11 (0.7)	0.998	1.0-100.0	94.6 (1.5)	NA
α-amyrin	14.2	425.4, $[M+H\text{-}2H]^+$	300	998	0.79 (0.2)	5.04 (1.0)	0.998	1.0-100.0	99.8 (1.0)	NA

*Quantified on the basis of peak height

[a] Determined from the slope of regression analysis of recovered quantities insamples spiked with increasing amounts of trierpenes.

the method (Table 2-2), assessed by calculating the coefficient of variation between trials (intraday variation) did not exceed 3.5%. As expected, the interday variation was somewhat higher. It was approximately 5% for all compounds except for 3 (10%). In addition to the soxhlet method here, we previously tested supercritical extraction (SCE), assisted solvent extraction ASE and alcohol extraction (Mullally et al., 2011). Betulinic acid is difficult to fully extract and only SCE gave comparable yields of to the soxhlet method (Carballo, 2014). It can be substituted in the analytical procedure, but it does require a considerable investment in equipment.

As phytochemical variations are quite common intra/inter species from the same family, we applied the validated method to a diversity of biological samples collected in different environments and representing typical botanical raw material for potential use in a natural health product. These included a number of *S. sympetala, S, gilgii, P. occidentalis, P. acerifolia* and *P. mexicanus* collections from North and Central America. During analysis all marker compounds were successfully resolved and quantified from these materials (Table 2-2). Based on the quantitative results obtained, all the selected triterpenes are present in detectable levels in both plant parts of *S. sympetala* and *S. gilgii*. There was a significant variation in the content of this triterpenes according to plant part. Betulinic acid is manly present in the stems. On the other hand, leaf extracts had higher content of α-amyrin and β-amyrin. There was considerable variation in content of the five triterpenes analysed in the samples evaluated. Betulinic acid content ranged from 0.12 to 7.2 mg/g in stem, lupeol and amyrins can vary up to 20 folds in leaves. For the preparation of the animal health product, we decided to use *S. sympetala* plant material from a plantation maintained by the Universidad Nacional, which had a mid-level of triterpenes, but plants were produced in a uniform farm environment to reduce variation.

It is important to mention that *S. sympetala* and *S. gilgii* grow in the same locations and shows the presence of the five triterpenes evaluated in the method in similar amounts. Both species are comparable in anxiolytic activity and show minor difference in phytochemistry and could be used as a

Table 2-2. Quantification (mg of compound/g of dried plant) of five triterpenes in *Souroubea* and *plantanus* samples. Mean (SEM) of concentration are presented.

Code	Location	Year	Plant organ	Sample size	Concentration in plant (mg/g)				
					1	2	3	4	5
SS-01	Barra Colorado, Costa Rica	2009	Stem	3	2.50 (0.02)	0.02 (0.00)	1.36 (0.03)	1.41 (0.03)	1.28 (0.02)
SS-02	Barra Colorado, Costa Rica	2009	Stem	3	2.91 (0.09)	0.06 (0.00)	1.65 (0.02)	1.37 (0.04)	1.75 (0.02)
SS-03	Sarapiqui, Costa Rica	2009	Stem	3	0.82 (0.01)	0.03 (0.00)	0.19 (0.00)	0.24 (0.01)	0.11 (0.00)
SS-04	Sarapiqui, Costa Rica	2009	Stem	3	2.61 (0.04)	0.27 (0.01)	0.81 (0.02)	0.93 (0.03)	0.19 (0.01)
SS-05	Sarapiqui, Costa Rica	2009	Stem	3	2.18 (0.03)	0.02 (0.00)	0.48 (0.01)	0.58 (0.04)	0.70 (0.01)
SS-06	Sarapiqui, Costa Rica	2009	Stem	3	0.12 (0.01)	0.01 (0.00)	0.04 (0.00)	0.03 (0.00)	0.01 (0.00)
SS-07	Sarapiqui, Costa Rica	2009	Stem	3	1.43 (0.00)	0.03 (0.00)	1.18 (0.03)	2.83 (0.09)	0.13 (0.01)
SS-08	Sarapiqui, Costa Rica	2009	Leaf	3	0.11 (0.00)	0.18 (0.01)	0.74 (0.05)	4.71 (0.06)	7.57 (0.09)
SS-09	Sarapiqui, Costa Rica	2009	Leaf	3	0.02 (0.00)	0.11 (0.00)	1.85 (0.07)	5.83 (0.17)	5.31 (0.17)
SS-10	Sarapiqui, Costa Rica	2009	Leaf	3	*	0.05 (0.00)	0.58 (0.01)	2.86 (0.07)	0.47 (0.02)
SS-11	Sarapiqui, Costa Rica	2009	Leaf	3	0.04 (0.00)	0.37 (0.02)	2.57 (0.09)	5.54 (0.09)	8.25 (0.07)
SS-12	Sarapiqui, Costa Rica	2011	Leaf	3	0.03 (0.00)	0.37 (0.01)	3.88 (0.17)	10.14 (0.45)	10.94 (0.6)
SS-13	Tirimbina, Costa Rica	2009	Stem	3	0.83 (0.01)	0.06 (0.00)	0.09 (0.00)	0.14 (0.01)	0.07 (0.00)
SS-14	Tortuguero, Costa Rica	2009	Stem	3	7.22 (0.24)	0.10 (0.00)	1.25 (0.02)	0.79 (0.01)	0.07 (0.00)
SS-15	Tortuguero, Costa Rica	2009	Stem	3	3.70 (0.12)	0.10 (0.00)	0.74 (0.02)	0.97 (0.03)	1.54 (0.04)
SS-16	Tortuguero, Costa Rica	2009	Stem	3	4.92 (0.14)	0.05 (0.00)	0.30 (0.02)	0.45 (0.03)	0.06 (0.00)
SS-17	Tortuguero, Costa Rica	2009	Leaf	3	#	#	0.71 (0.02)	5.12 (0.09)	7.00 (0.21)
SS-18	Tortuguero, Costa Rica	2009	Leaf	3	0.01 (0.00)	0.09 (0.00)	0.18 (0.00)	2.17 (0.02)	0.61 (0.01)
SS-19	Tortuguero, Costa Rica	2009	Leaf	3	0.01 (0.00)	0.45 (0.01)	1.66 (0.06)	6.63 (0.07)	8.12 (0.06)
SS-20	Tortuguero, Costa Rica	2009	Leaf	3	0.00 (0.00)	0.25 (0.01)	1.15 (0.04)	3.18 (0.02)	1.15 (0.04)
SS-21	Tortuguero, Costa Rica	2009	Leaf	3	0.00 (0.00)	0.09 (0.00)	0.66 (0.01)	3.38 (0.04)	4.20 (0.10)
SG-1	Cano Palma, Costa Rica	2010	Leaf	3	0.21 (0.12)	0.02 (0.01)	0.51 (0.02)	3.9 (0.08)	0.48 (0.03)
SG-2	Cano Palma, Costa Rica	2010	Stem	3	1.26 (0.73)	0.02 (0.01)	0.16 (0.00)	0.2 (0.01)	0.23 (0.01)
SG-3	Cano Palma, Costa Rica	2009	stem	3	4.38 (2.53)	0.05 (0.03)	0.83 (0.01)	0.94 (0.01)	0.53 (0.02)
SG-4	Horquetas, Costa Rica	2010	Stem	3	0.58 (0.33)	0.03 (0.02)	0.28 (0.00)	0.52 (0.01)	0.12 (0.00)
SG-5	Horquetas, Costa Rica	2011	Leaf	3	0.05 (0.03)	0.3 (0.18)	0.34 (0.01)	2.37 (0.06)	0.92 (0.01)
SG-6	Sarapiqui, Costa Rica	2010	stem	3	0.85 (0.49)	0.08 (0.05)	0.1 (0.00)	0.15 (0.01)	0.06 (0.00)
SG-7	Sarapiqui, Costa Rica	2009	Stem	3	0.7 (0.4)	0.04 (0.02)	0.36 (0.01)	0.95 (0.03)	0.02 (0.00)

SG-8	Sarapiqui, Costa Rica	2011	Stem	3	1.42 (0.82)	0.03 (0.02)	0.38 (0.01)	0.5 (0.01)	0.19 (0.00)
SG-9	Sarapiqui, Costa Rica	2011	leaf	3	0.18 (0.1)	0.34 (0.19)	1.05 (0.03)	4.74 (0.04)	11.95 (0.12)
SG-10	Sarapiqui, Costa Rica	2011	leaf	3	0.21 (0.12)	1.56 (0.9)	1.02 (0.03)	6.15 (0.08)	7.87 (0.15)
SG-11	Tirimbina, Costa Rica	2009	Leaf	3	0.09 (0.05)	0.01 (0.01)	0.96 (0.04)	4.07 (0.05)	3.45 (0.16)
SG-12	Tirimbina, Costa Rica	2011	Stem	3	1.15 (0.66)	0.06 (0.03)	0.08 (0.00)	0.1 (0.00)	0.13 (0.00)
SG-13	Tirimbina, Costa Rica	2011	Leaf	3	0.01 (0.1)	0.01 (0.01)	0.88 (0.05)	3.57 (0.07)	3.38 (0.06)
SG-14	Tortuguero, Costa Rica	2009	Stem	3	3.09 (1.78)	*	0.26 (0.01)	0.42 (0.01)	0.02 (0.00)
SG-15	Tortuguero, Costa Rica	2009	stem	3	1.13 (0.65)	0.04 (0.02)	0.52 (0.01)	0.44 (0.00)	0.49 (0.00)
SG-16	Tortuguero, Costa Rica	2010	Stem	3	2.04 (1.18)	0.01 (0)	0.73 (0.01)	0.75 (0.01)	0.8 (0.02)
SG-17	Tortuguero, Costa Rica	2010	stem	3	0.84 (0.49)	0.03 (0.02)	0.09 (0.00)	0.09 (0.01)	0.02 (0.00)
SG-18	Tortuguero, Costa Rica	2011	leaf	3	0.74 (0.43)	0.03 (0.02)	0.66 (0.01)	1.14 (0.02)	0.14 (0.01)
SG-19	Tortuguero, Costa Rica	2010	Leaf	3	0.1 (0.06)	0.02 (0.01)	0.54 (0.01)	3.8 (0.06)	2.27 (0.01)
SG-20	Tortuguero, Costa Rica	2010	Leaf	3	0.01 (0)	0.19 (0.11)	0.26 (0.00)	2.01 (0.03)	2.39 (0.06)
SG-21	Tortuguero, Costa Rica	2009	Leaf	3	0.2 (0.12)	0.7 (0.41)	0.81 (0.06)	4.92(0.36)	14.49 (0.56)
SG-22	Tortuguero, Costa Rica	2008	Leaf	3	1.25 (0.72)	0.03 (0.02)	1.71 (0.08)	6.66 (0.15)	4.75 (0.09)
PA-01	Essex-1, Ontario, Canada.	2016	Bark	4	21.16 (5.17)	*	*	*	*
PA-02	Essex-2, Ontario, Canada.	2016	Bark	3	22.13 (2.79)	*	*	*	*
PA-03	Delhi, Ontario, Canada.	2016	Bark	3	16.27 (3.61)	0.26 (0.16)	*	*	*
PA-04	Toronto, Ontario, Canada.	2016	Bark	2	18.53 (7.36)	1.10 (0.07)	*	*	*
PA-05	London, Ontario, Canada.	2016	Bark	1	22.87 (0)	0.25 (0)	*	*	*
PO-01	Experimental Farm-1, Ottawa, Canada.	2016	Bark	3	5.67 (0.35)	3.36 (0.25)	*	*	*
PO-02	Experimental Farm-2, Ottawa, Canada.	2016	Bark	3	10.81 (1.25)	2.00 (0.38)	*	*	*
PO-03	Queen Elizabeth Drive-1, Ottawa, Canada.	2016	Bark	3	9.94 (0.15)	1.36 (0.05)	*	*	*
PO-04	Queen Elizabeth Drive-2, Ottawa, Canada.	2016	Bark	3	11.41 (1.67)	0.82 (0.32)	*	*	*
PO-05	Niagara, Ontario, Canada.	2016	Bark	3	22.12 (3.69)	*	*	*	*
PO-06	London, Ontario, Canada.	2016	Bark	3	6.44 (3.68)	0.58 (0.21)	*	*	*
PO-07	Toronto-1, Ontario, Canada.	2016	Bark	3	6.67 (2.75)	1.08 (0.20)	*	*	*
PO-08	Toronto-2, Ontario, Canada.	2016	Bark	3	14.72 (1.35)	1.21 (0.13)	*	*	*
PO-09	Butlar Farms, Ontario, Canada.	2016	Bark	5	10.12 (1.31)	1.92 (0.36)	*	*	*
PO-10	Butlar Farms, Ontario, Canada.	2016	Bark	1	6.28 (0)	0.60 (0)	*	*	*
PO-11	South Windsor, Ontario, Canada.	2016	Bark	1	11.63 (0)	0.43 (0)	*	*	*

PO-12	South Windsor, Ontario Canada.	2016	Bark	1	9.91 (0)	0.74 (0)	*	*	*
PO-13	Oakland, Tennessee, U.S.	2016	Bark	3	12.85 (9.35)	0.95 (0.50)	*	*	*
PO-14	Cincinatti, Ohio	2016	Bark	3	11.82 (6.36)	1.60 (0.13)	*	*	*
PO-15	Ocean City, Alabama, U.S.	2016	Bark	3	12.08 (3.27)	0.77 (0.18)	*	*	*
PO-16	New, Orleans, Louisiana, U.S.	2016	Bark	3	11.80 (0.21)	0.57 (0.48)	*	*	*
PO-17	Carrollton, Kentucky, U.S.	2016	Bark	3	12.42 (5.79)	0.72 (0.50)	*	*	*
PO-18	Tallahassee, Florida, U.S.	2016	Bark	1	18.34 (0)	0.68 (0)	*	*	*
PO-19	Savannah, Georgia, U.S.	2016	Bark	3	9.22 (1.04)	0.54 (0.21)	*	*	*
PO-20	North of Durham, North Carolina, U.S.	2016	Bark	1	8.02 (0)	0.42 (0)	*	*	*
PO-21	Interstate 81 Welcome Centre, Virginia U.S.	2016	Bark	1	9.53 (0)	1.20 (0)	*	*	*
PM-01	Xalapa, Mexico	2016	Bark	6	16.20 (3.22)	0.41 (0.01)	*	*	*

#: Below limit of quantification
*: Below limit of detection
SS: *Souroubea sympetala*; SG: *Souroubea gilgii*; PA: *Plantanus x Acerifolia* PO: *Plantanus occidentalis* and PM: *Plantanus mexicanus*.

mixed species product as is the case for Hawthorn for which a mixture of *Craetagus monogyna* Jacq and *Craetagus laevigata* (Poir DC) is used (Blumenthal et al., 2003). Among the three species of *Plantanus* collected in 27 sites across North America, betulinic acid is the dominant tritepene accompanied with ursolic acid. Amyrins and lupeol were not detected (Table 2-2). Betulinic acid concentrations varied from 5.7 to 22.1 mg/g in dried bark and ursolic acid concentration ranged from 0 to 3.36 mg/g. Overall, it can be observed that the *P.x acerifolia* exhibited the highest content of betulinic acid, while *P. occidentalis* had the lowest betulinic acid concentration among the three species. This study is the first comparative phytochemical analysis of betulinic acid and ursolic acid in the *Platanus* genus. Due to the diverse biological effects of betulinic acid (anxiolyic, anti-cancer and antiviral effects) (Yogeeswari and Sriram, 2005; Laszcyk et al., 2009), the findings of this study contribute to identifying *Platanus* sources with the highest content of bioactive ingredient. However, *P. occidentalis* is the most readily available species and was the one used in the animal health product.

Finally, the analytical method was applied to determine the quantities of target metabolites in contributing *S. sympetala* herb, and *P. occidentalis* bark, used in the final tablet product intended for canine use (Table 2-3). *P. occidenalis* had the highest concentration of betulinic acid (over 11 mg/ g dry bark) and ursolic acid (over 1.5 mg/ g) while *S. sympetala* was a major contributor of α and β amyrin. With this blend of *S. sympetala* and *P. occidentalis* the final tablet intended for the canine market delivered 11mg of the main active principle 1 and 2.5mg of related triterpenes 2-5. As one tablet is used for a 10kg dog, it provides slightly more than the claimed 1mg/kg dose. In addition, we have completed a stability study of the tablet, and it is highly stable, for at least 1 year (data not shown). Given the results obtained in this study, this validated method is a rapid and dependable procedure to evaluate the triterpene content in different source biological materials and finished product.

CHAPTER 3: EFFECT OF AN ANXIOLYTIC BOTANICAL CONTAINING *SOUROUBEA SYMPETALA* AND *PLATANUS OCCIDENTALIS* ON *IN VITRO* DIAZEPAM HUMAN CYTOCHROME P450 MEDIATED-METABOLISM

3.1 Introduction

Generalized anxiety disorder (GAD) affects health and well-being and is common across North America and abroad. According to the National Institute of Mental Health, approximately 2.7% of American adults suffer from GAD every year with an estimated 5.7% experiencing GAD at some point in their lives (Harvard Medical School, 2017). Due to concerns over side effects, tolerance, compliance, patient preference and the cost of conventional medications, alternative or complementary therapies have received increasing attention from both researchers and consumers in recent years.

A novel anxiolytic herbal medicine containing the dried extracts of *Souroubea sympetala* and *Platanus occidentalis* has been developed for the companion animal market and is currently being developed for clinical evaluation as a treatment for GAD for human (Masic et al., 2018; Durst et al., 2014; Liu et al., 2017). *S. sympetala* is a vine from the Marcgraviaceae family found in Central and South America that was selected for investigation by Costa Rican botanists based on dereplication strategy for poorly studied botanicals (Puniani et al., 2015). *S. sympetala* extract was found to have significant dose dependent anxiolytic effects in animal models including the elevated plus maze and fear potentiated startle. Further analyses identified betulinic acid (BA) as the main active principal in the plant extract, as well as the presence of other pentacyclic triterpenes (Puniani et al., 2015). The pharmacological mode of action was shown to be attributed to agonistic action at the $GABA_A$ receptor which could be abolished with co-administration of the antagonist flumazenil (Mullally et al., 2014).

P. occidentalis has high concentrations of BA in the bark, which was added to the formulation to increase potency of the product (Carballo-Arce, 2013). Although it has only a

weak anxiolytic effect in animal tests, *P. occidentalis* has a synergistic anxiolytic effect when mixed with *S. sympetala* (Villalobos et al., 2014). The blended NHP mixture (Zentrol™) is marketed as an anxiolytic for canine noise aversion. Preliminary safety studies determined that there were no negative effects in canines at ten times the suggested dose (Bertz and Granneman, 1997). These results were confirmed in larger trials with beagles (Masic et al., 2018). However, there are still knowledge gaps in the overall safety of this product.

Metabolic interactions are one of the most common causes of adverse effects when multiple health products are taken concomitantly. Cytochrome P450 (CYP450) is the major Phase I drug-metabolizing enzyme system (Rendic and Di Carlo, 1997; Wanwimolruk and Prachayasittikul, 2014). Many phytochemicals have been identified as substrates, inducers or inhibitors of drug metabolism enzymes, including several CYP isoforms (Brantley et al., 2014). Altered metabolic activity may have significant impact on the plasma concentration and half-life of a medicinal or non-medicinal substance, which can lead to adverse reactions such as overdose or therapeutic failure (Mai et al., 2004). St. John's wort is a well-documented example (Whitten et al., 2006). Clinical studies have also observed the increased CYP3A4 drug metabolism and the reduced absorption of drugs though P-glycoprotein (Pgp) efflux.

The main objective was to evaluate the potential risk of NHP-drug interactions related to the concomitant use of this herbal product with conventional medical products, such as the anxiolytic diazepam (DA). Plant extracts of *S. sympetala* and *P. occidentalis* alone and as a 1:1 w/w mixture, along with their isolated active triterpenes, were tested *in vitro* for inhibitory effects on the activity of various CYP enzymes as well as the production of DA metabolites.

3.2 Materials and methods

3.2.1. Plant extracts and sample preparation

S. sympetala leaves and stem were harvested from a plantation in Costa Rica managed by the Universidad Nacional (UNA) in Sarapiqui (Heredia province). Plants were authenticated to species by UNA botanists (Sanchez, Poveda and Otarola) and the voucher specimens were deposited at the Herbarium Juvenal Rodriguez, UNA and University of Ottawa herbarium (OTT19994). Bark of *Platanus occidentalis* (Plantanaceae) was collected in Guelph Ontario, identified by University of Ottawa botanist Dr. Julian Starr, and prepared as a voucher now deposited at the University of Ottawa herbarium (OTT19995). Dried plant material was ground through 1mm mesh. Ground material (1g samples) of both species were mixed (55:45, w:w) prior to extraction as this method produce a dry powder. The mixed plant material and extracted with 20 mL 95% ethanol by shaking at 250 rpm overnight. The supernatant was collected by vacuum filtration and residues were re-extracted under the same conditions. A third extraction did not significantly increase yield and was not undertaken. The supernatants were then combined and dried by rotatory evaporation at 45°C follow by lyophilization. Dried extracts were stored in -20°C conditions until use.

Standards of betulinic acid (BA), ursolic acid (UA), lupeol, β-amyrin and α-amyrin were purchased from Extrasynthese Lyon, France, and diazepam (DA) solution (1 mg/ml in dissolved in methanol) was obtained from Sigma-Aldrich (Oakville, Ontario). All standard compounds (1 mg/mL) and plant extracts were solubilized in 100% methanol at defined concentrations as a stock solution for experimental use.

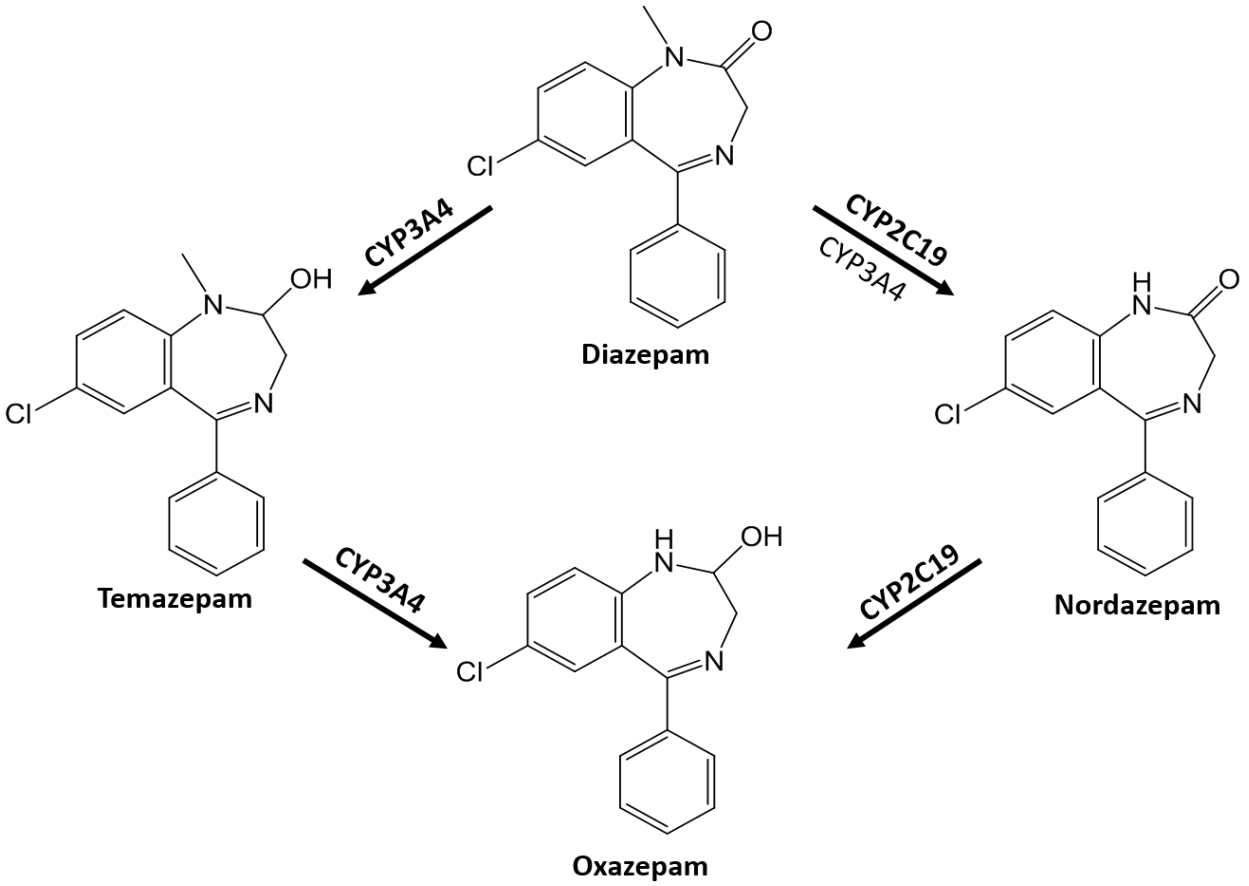


Figure 3-1. Phase I metabolic pathways of diazepam.

3.2.2. Quantification of triterpenes in plant extracts

Phytochemical separation and characterization of the extracts were achieved by a Shimadzu UPLC-MS system (Mandel Scientific Company Inc., Guelph, ON) consisting of LC30AD pumps, a CTO20A column oven, a SIL-30AC auto-sampler, and a LCMS-2020 mass spectrometer. Separation of the marker compounds were performed on a Kinetex 1.7 µm, 150 mm × 2.1 mm i.d. column Phenomenex Inc. The injection volume was 1 µL, column oven temperature was 55°C and flow rate was 0.4 mL / minute. The mobile phases LC-MS grade was: (A) water, (B) acetonitrile with gradient elution: 65-100% B in 7 min, hold 100% B for 8 min, the system was re-equilibrated for 5 min before the next injection. Detection was achieved by using and Atmospheric Pressure Chemical Ionization (APCI) interface operated in positive and negative selective ion monitoring mode (SIM): m/z at 409 ($[M-H2O]^+$) was used to monitor lupeol, α-amyrin and β-amyrin; m/z 455 ($[M-H]^-$) was used to monitor betulinic acid and ursolic acid. Nebulizing gas flow was set at 1.5 L/min and drying gas flow was at 10 L/min. The interface, desolvation line and heat block temperature was set at 350°C, 300°C and 400°C respectively. The scan speed was set at 938 u/sec.

Calibration curves were generated by injecting dilutions (0.5-50 µg/ml) of reference standards spanning the compound concentration in samples. Each sample (0.1-1 mg/mL) was injected in triplicate and peak area was then used to quantify the amount of each marker compound.

3.2.3. Cytochrome P450 assays

A microtiter fluorometric plate assay was used to assess the inhibitory capacities of terpenes and plant extracts toward different CYP450 isoforms. The assays were performed in 96-

Table 3-1. Description of the experimental conditions for the microtiter fluorometric cytochrome P450 (CYP) inhibition studies.

CYP isoform	CYP (nM)	Substrate	Substrate (μM)	Excitation/emission λ (nm)	Incubation time (min)
CYP3A4 + b5	5	DBF	1	490/530	20
CYP2D6	10	AMMC	0.12	410/460	60
CYP2C9 + b5	50	7-MFC	100	410/530	60
CYP2C19 +b5	20	CEC	25	410/460	60

Abbreviations: AMMC, 3- [2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin; b5, cytochrome b5; CEC, 3-cyano-7-ethoxycoumarin; MFC, 7-methoxy-4-trifluoromethyl-coumarin; DBF, dibenzylfluorescein.

well plates under red light to minimize the exposure of fluorescent light to photosensitive material. The fluorescence was measured using a Cytation 3 Cell Imaging Multi-Mode Reader BioTek Instruments Inc., Winooski, VT, USA as outlined in Table 3-1.

Samples were incubated in the presence of CYP450 enzymes with fluorometric substrate and 1.08 mM NADPH in a 0.2 mM phosphate (CYP2C19, CYP2C9 and CYP3A4) or Tris buffer (CYP2C9) with a pH at 7.5 at 37°C for 20-60 min (refer to Table 3-1 for enzyme-specific assay parameters). Ketoconazole 1.9 µM, quinidine 2 µM, sulfaphenazole 100 µM and tranylcypromine 100 µM were used as positive controls for CYP3A4, CYP2D6, CYP2C9 and CYP2C19, respectively. Corning® Supersomes™ Insect Cell Microsomes were used as negative control. These assays were optimized previously (Tam et al., 2015)

IC_{50} values were obtained by using Prism GraphPad version 7.0; log[Inhibitor] vs. normalized response - Variable slope analysis module. Samples were tested in triplicate at a minimum of 5 concentrations.

3.2.4. Diazepam metabolism assay with human liver microsome (HLM)

HLM (Corning Inc.-Life Science, Oneonta, NY) were thawed in a 37°C water bath then placed on ice until required. A 5 µL aliquot of the test extract was incubated with 2 mM NADPH, 2 mg/mL HLM and 3.5 mM DA in 100 mM PBS buffer for 90 min in a shaking incubator set at 37°C and 200 rpm. The reaction was stopped by adding 300 µL of cold methanol. The mixture was then vortexed for 1 min and sonicated for 5 min to neutralize all protein content, and centrifuged at 12,000 rpm for 10 min. The supernatant fluid was filtered through 0.2µ PTFE filters (Chromatographic Specialties Inc., Brockville, ON, Canada) and

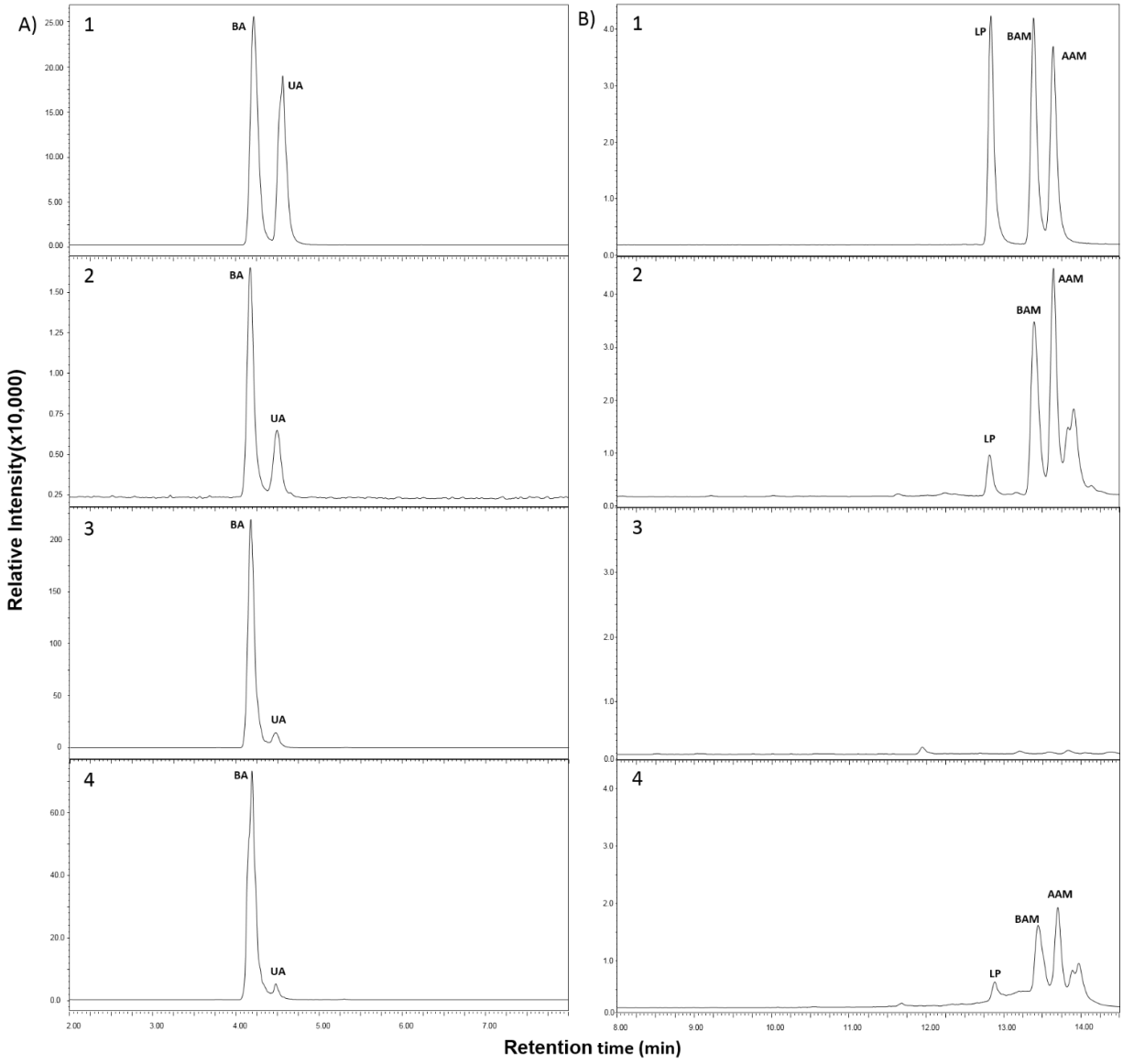


Figure 3-2. UPLC-MS selected ion monitoring (SIM) chromatogram of triterpenes in plant extracts. A) negative ionization SIM monitoring m/z=455 ($[M-H]^-$); B) positive ionization SIM monitoring m/z=409 ($[M-H_2O]^+$). 1, Standard compounds mixture; 2, *Souroubea sympetala* extracts; 3, *Platanus occidentalis* extract; 4, extracts of a combined mixture of the two herbals. BA, betulinic acid. UA, ursolic acid. LP, lupeol, AAM, α-amyrin. BAM, β-amyrin.

stored at -20°C until analysis. Ketoconazole (10 nM), Kava kava extract (0.05 mg/mL), a known CYP450 enzyme inhibitor (Mathews et al., 2002) were used as positive control and 10% methanol was used as vehicle control. All samples were tested with/without HLM in triplicate.

After HLM metabolism, DA and its metabolites (Figure 3-1) were identified by selected ion monitoring (SIM: nordazepam, m/z: 271, oxazepam m/z: 287, and temazepam m/z: 301) using a Shimadzu UPLC-MS system (Mandel Scientific Company Inc., Guelph, ON) consisting of LC30AD pumps, a CTO20A column oven, a SIL-30AC auto-sampler, and a LCMS-2020 mass spectrometer. An Acquity BEH C18 column 100 x 2.1 mm, 1.7 μm particle size (Waters, Mississauga, ON) with an Acquity BEH C18 VanGuard pre-column 5x2.1 mm was used for separation. Mobile phase consisted of H_2O (A) and Acetonitrile (B) with 0.1% formic acid in both. The gradient elution method was initiated at 10% B, then increased to 50% B over 8 min. The column was then washed with 100% B for 4 min and returned to the initial conditions in 2 min. The flow rate was set to 0.5 mL/min with the column temperature set at 50˚C. The mass spectrometer with electrospray ionization ESI interface was operated in positive scan mode. The nebulizing gas flow was set at 1.5 L/min and drying gas flow was at 10 L/min. The desolvation line temperature and heat block temperature was set at 250°C and 400°C respectively. The m/z range of both positive and negative scan is from 150 to 600 with 1500 μ/sec scan speed.

The quantitative analysis of DA metabolites was performed on an Agilent 1200 series HPLC system with quaternary pump G1311A, solvent degasser G1322A, column oven G1316A, and photodiode array detector G1315A. Separation of DA and its metabolites was carried out with a Synergi MAX-RP C12 column 250 x 2 mm, 4 μm particle size (Phenomenex, Mississauga, ON). Mobile phase was H_2O (A) and acetonitrile (B) +0.1% formic acid in both. The isocratic elution method was initialized with 40% B for 10 min. The column was then

washed with 100% B for 5 min, then returned to the initial condition for 1 min. The system was then re-equilibrated for 5 min before the next injection. Flow rate was set to 0.5 mL/min with oven at 55°C. DAD was set to monitoring wavelength 240 nm.

3.2.5. Statistical analysis

Chemstation software Version B 3.02 was used to calculate the peak area of the major metabolites of DA. Percentage inhibition was calculated by comparing the metabolite to the appropriate vehicle control. A 2-way ANOVA followed by both Dunnett and Sidak's multiple comparison test were used to analyze the percentage inhibition of different DA metabolites.

3.3 Results

3.3.1 Quantification of triterpenes in plant extracts

The percentage yield of *P. occidentalis*, *S. sympetala* and mixture extract are 3.34%, 5.23% and 4.32% dry weight of plant material, respectively. All major triterpenes in the plant extract were identified and quantified using UPLC-MS (Figure 3-2, Table 3-2). The *P. occidentalis* extract contained nearly 30% triterpenes by weight, consisting only of betulinic acid (297.9 mg/g) and ursolic acid (20.7 mg/g). The *S. sympetala* extract contained five marker compounds constituting about 15% of the extract. Amyrins were the most abundant terpenes in the extract with 55.2 and 45.6 mg/g for α-amyrin and β-amyrin, respectively. The triterpene content of the mixture was as predicted based on the observed concentrations of these compounds in *P. occidentalis* and *S. sympetala* extract, with the exception of lupeol, which is was higher than expected.

3.3.2 Cytochrome P450 assays

All extracts exhibited strong inhibitory effects >70% on CYP3A4, 2C9 and 2C19 at a concentration at 50 µg/mL (Figure 3-3). The inhibition of CYP2D6 activity was weakest, as only moderate inhibition 40~70% was observed with higher concentrations of 100 µg/mL. Accordingly, the IC_{50} of all plant extracts toward CYP3A4, 2C9 and 2C19 (but not 2D6) were then obtained to further evaluate the inhibition potency (Table 3-3). Similar patterns were observed among all 3 CYP450 enzymes: the inhibition potency of *S. sympetala* extract was 2-4 fold lower than both *P. occidentalis* extract and the mixture, which produced similar IC_{50} values. The high inhibition of CYP 2D6 with the positive control may be due to the fluorescence quenching of quinidine.

Table. 3-2 Concentration of triterpenes mg/g in *Souroubea sympetala* and *Platanus occidentalis* extracts alone and in a combined mixture (MIX). Quantification results presented as mean ± SEM, n=3.

Extract	Betulinic acid	Ursolic acid	Lupeol	α-amyrin	β-amyrin
P. occidentalis	279.87 ± 18.20	20.67 ± 1.82	ND	ND	ND
S. sympetala	22.29 ± 0.08	0.78 ± 0.02	8.87 ± 0.14	55.19 ± 1.25	45.56 ± 0.95
MIX	159.27 ± 21.14	9.20 ± 1.58	7.21 ± 0.63	22.01 ± 0.82	23.27 ± 0.66

ND: not detected.

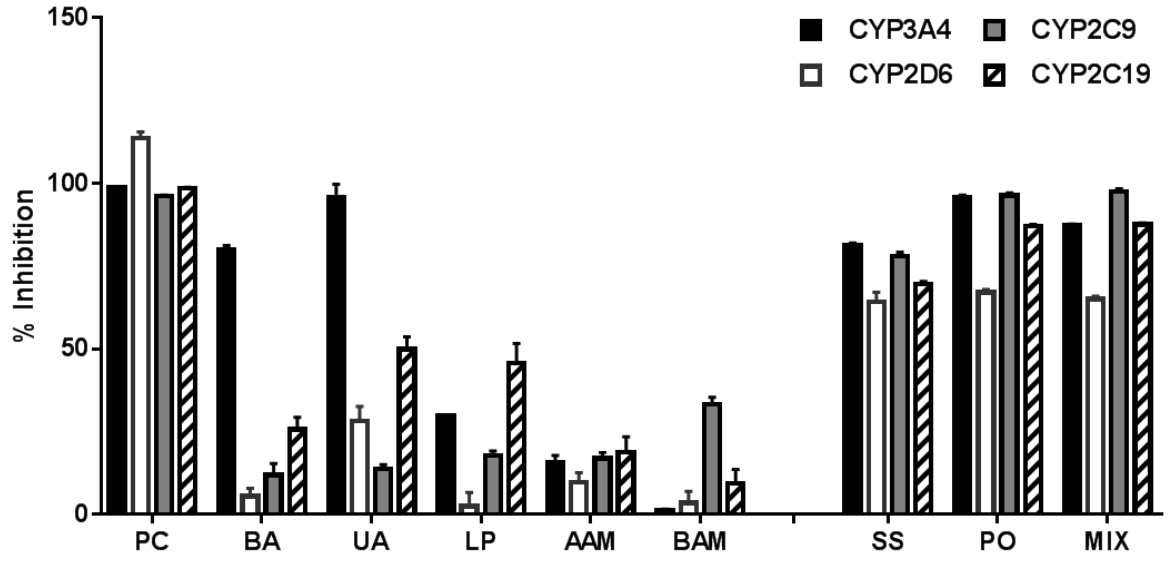


Figure 3-3. Mean percent inhibition of cytochrome P450 3A4, 2D6, 2C9 and 2C19 isoforms by positive controls (left), triterpenes (center) and herbal extracts (right) relative to vehicle control. The triterpenes and *Souroubea sympetala* and *Platanus occidentalis* extracts were tested at 50 µg/mL except with CYP2D6 assay (100 µg/mL). Data presented as mean ± SEM of at least three independent experiments. PC, positive control. BA, betulinic acid. UA, ursolic acid. LP, lupeol, AAM, α-amyrin. BAM, β-amyrin. SS, *S. sympetala*. PO, *P. occidentalis*, and MIX, a combined mixture of the two herbal extracts. Positive control used in the assay were ketoconazole 1.9 µM, quinidine 2 µM, sulfaphenazole 100 µM, and tranylcypromine 100 µM for CYP3A4, CYP2D6, CYP2C9 and CYP2C19 respectively. 20% Methanol was used as vehicle.

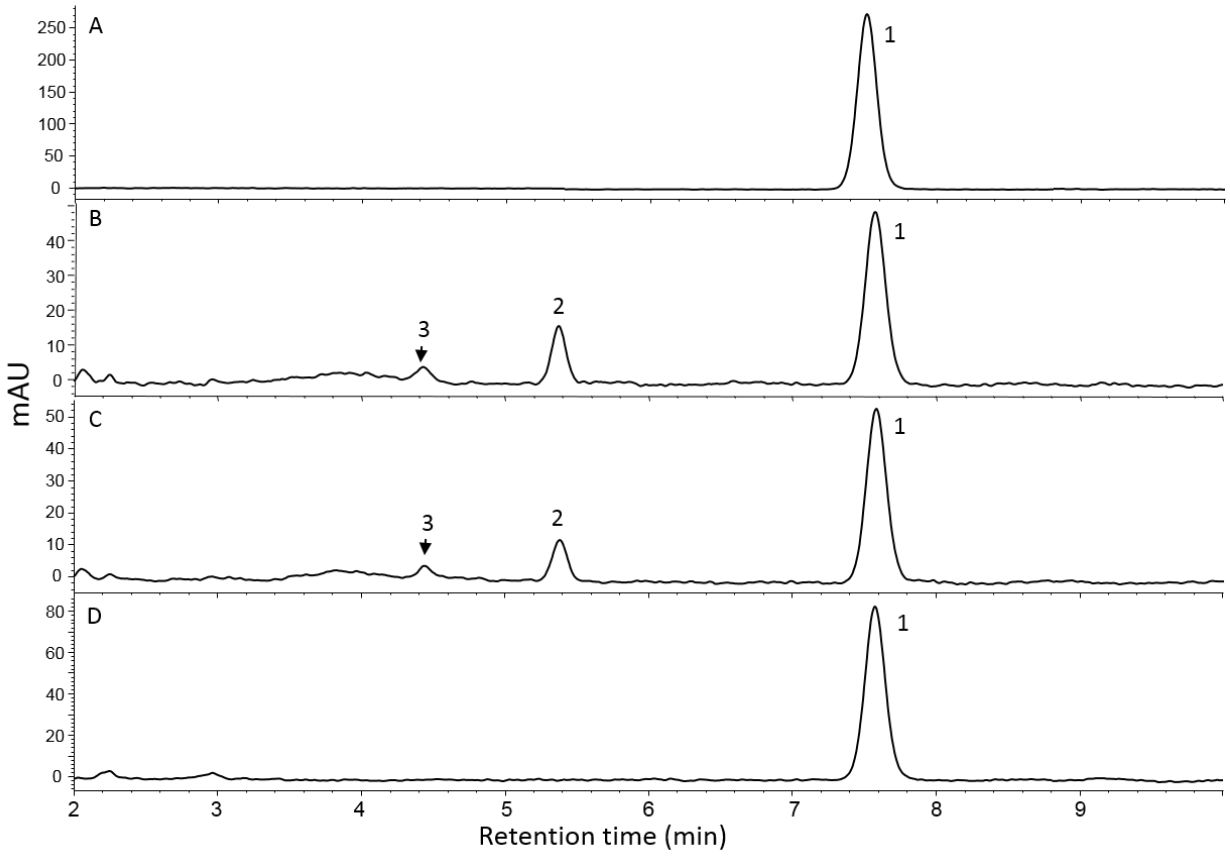


Figure 3-4. HPLC chromatograms of diazepam (DA) metabolite formation under different conditions across time. All chromatograms were monitored at a wavelength of 240 nm. A) DA standard. B) DA incubated with human liver microsomes (HLM) and NADPH. C) DA incubated with HLM, NADPH and mixed plant extract. D) DA incubated with HLM only. 1, diazepam. 2, temazepam and 3, nordazepam.

At 50 µg/mL, individual triterpenes showed selective inhibitory effects on the tested CYP450 enzymes (Figure 3-3). BA strongly inhibited CYP3A4 activity but had very limited effect on CYP2D6, CYP2C9 and CYP2C19. UA exhibited the strongest inhibitory effects on almost all tested CYP450 isoforms, particularly CYP3A4. Lupeol showed only moderate inhibition of CYP2C19 activity, as did β-amyrin, whereas α-amyrin had very limited inhibitory effect on any of the 4 isoforms. β-amyrin also showed highest inhibition on CYP2D6. BA and UA were subsequently tested to obtain IC_{50} for CYP3A4 (Table 3-3).

3.3.3 Diazepam metabolism by human liver microsome (HLM)

In the HLM incubation mixture, all 3 metabolites of DA reported for CYP450-mediated biotransformation were detected by UPLC-MS (Figure 3-4) although oxazepam was present below the limit of quantification. All plant extracts inhibited the formation of DA metabolites, especially at the highest tested concentration, 0.2 mg/mL, which reduced metabolite formation by nearly 50% (Figure 3-5). Of the triterpenes examined, only BA altered the HLM metabolism of DA. In the presence of 25 µg/mL BA, the formation of temazepam and nordazepam were inhibited by 43% and 45%, respectively. LP, AAM, and BAM slightly increased the catalytic activity with respect to the vehicle control, possibly due to hormesis, which is frequently seen in natural product assays.

Two-way ANOVA and Dunnett's multiple comparison test showed that ketoconazole, betulinic acid, and all plant extracts at high concentration (0.2 mg/mL) significantly block the DA metabolism *in vitro*. The 2-way ANOVA and Sidak multiple comparison test indicated percentage inhibition on 2 major DA metabolites formation were not significantly different ($p > 0.05$) than each other in all tested groups.

Table 3.3 Relative potency of cytochrome P450 inhibition elicited by *Souroubea sympetala* and *Platanus occidentalis* plant extracts alone and as a combined mixture (MIX) and five major triterpenes as determined by IC50 concentrations µg/ml (mean ± SEM, n ≥ 3).

	CYP3A4	**CYP2C9**	**CYP2C19**
Betulinic acid	16.98 ± 0.75	>50	>50
Ursolic acid	6.03 ± 0.24	>50	>50
Lupeol	>50	>50	>50
α-amyrin	>50	>50	>50
β-amyrin	NA	>50	>50
S. sympetala	9.92 ± 0.38	13.48 ± 0.94	26.06 ± 0.91
P. accidentalis	3.08 ± 0.14	3.56 ± 0.14	10.19 ± 0.37
MIX	3.98 ± 0.14	3.72 ± 0.13	9.17 ± 0.41

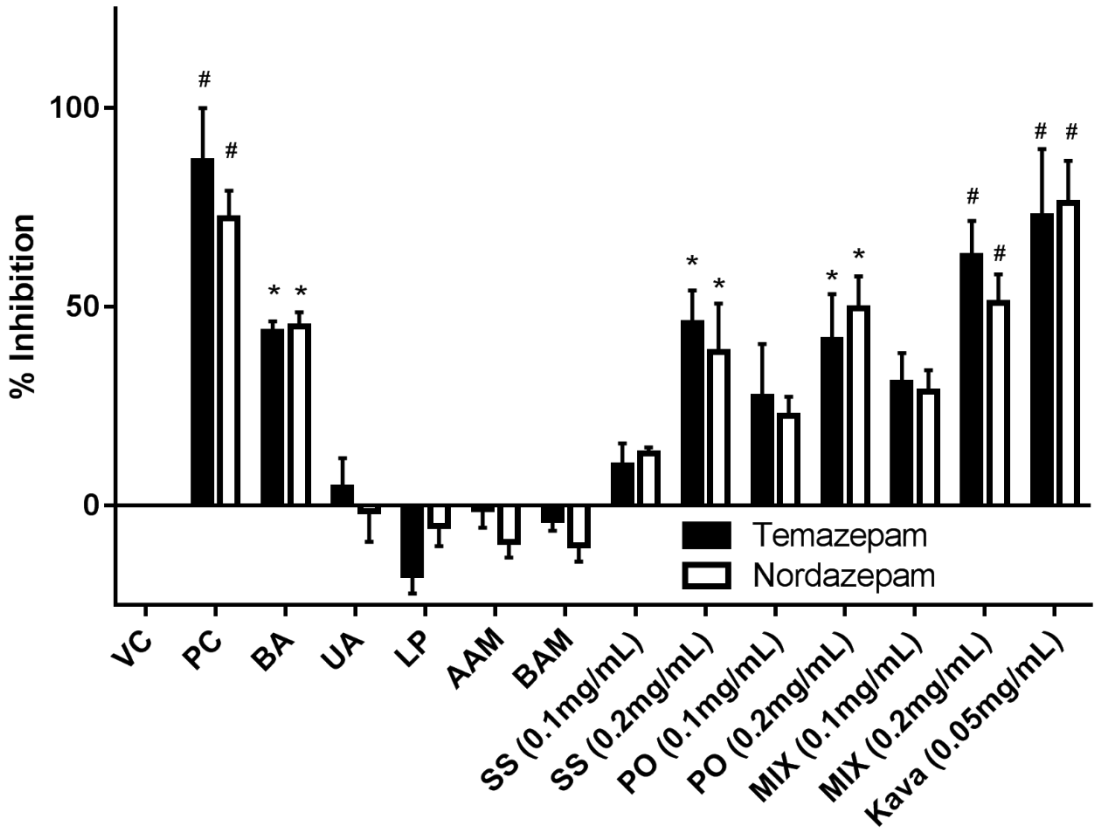


Figure 3-5. Percent inhibition of major diazepam metabolite formation by *Souroubea sympetala* and *Platanus occidentalis* extracts and triterpenes (50 µg/mL) relative to vehicle controls. Mean ± SEM n=3, *BA n=2. 10% methanol is vehicle control (VC). Ketoconazole 10 nM was the positive control PC. BA, betulinic acid. UA, ursolic acid. LP, lupeol, AAM, α-amyrin. BAM, β-amyrin. SS, *S. sympetala*. PO, *P. occidentalis*, and MIX, a combined mixture of the two herbal extracts. The *Kava kava* extract was included as a positive botanical control. A 2 Way Anova with Dunnett's multiple comparisons test were used to compare percentage inhibition to VC. *: $p \leq 0.05$; #: $p \leq 0.01$.

3.4 Discussion

Many common anxiolytic medications may cause serious adverse effects such as benzodiazepine overdose (Greenblatt et al., 1977; Sacre et al., 2017). Accordingly, the administration of these medications is often based on the risk to benefit ratio, which can be narrow in certain if not many populations. Co-administration of other health or dietary products that may inhibit normal CYP-mediated metabolism may increase the rate and potency of adverse effects. Such events may completely alter the risk to benefit ratio of these products.

The inhibitory potential of several active substances found in *S. sympetala* and *P. occidentalis* extracts toward CYP450 isoforms had been previously reported (Seervi et al., 2016; Kim et al., 2014; Moreira et al., 2013), but the plant extracts had not been examined. Our results suggest that both plant extracts and the mixture have potential to inhibit CYP450 enzyme activity. *P. occidentalis* showed 3-4-fold stronger CYP450 inhibition relative to the traditionally used *S. sympetala.* The potency of the mixed extract was similar to that of *P. occidentalis,* suggesting that its addition to *S. sympetala* not only boosts the anxiolytic effect (Liu et al., 2017) but also increases the potential risk of herb-drug interaction when co-administrated with other therapeutic products.

These findings are important as CYP3A4 is one of the major hepatic enzymes that metabolizes many pharmaceuticals in the benzodiazepine class, as well as several selective serotonin reuptake inhibitor (SSRI)/ serotonin–norepinephine reuptake inhibitor (SNRI) medications including escitalopram and buspirone (Rao, 2007; Mahmood et al., 1999). CYP2C19 contributes to the metabolism of many benzodiazepine class medications as an alternative pathway to CYP3A4 (Fukasawa et al., 2007). CYP2D6 is mainly involved in the

metabolism of SSRI/SNRI class medications including duloxetine, paroxetine and fluoxetine (Jin et al., 2016; Hemeryck and Belpaire, 2002). Although many SSRIs such as fluoxetine are metabolized by CYP2D6, they are also inhibitors of CYP2D6. Other studies suggest that, when the CYP2D6 contribution to fluoxetine metabolism diminishes over long-term treatment, CYP2C9, 2C19 and 3A4 may serve as alterative metabolic pathways (Mandrioli et al., 2006). Other human major CYPs such as CYP1A2, CYP2B6, CYP2C8, and CYP2E1 may play a minor role in diazepam metabolism and the inhibitory effects of extract of *Souroubea sympetala* and *Platanus occidentalis* on could be considered in future studies.

Whereas such inhibitory effect increases the risk of NHP-drug interactions, it could also be beneficial when the herbal product is used alone. Several studies indicate that CYP450 isoforms are involved in the metabolism of the pharmacologically active pentacyclic triterpene (Greenblatt et al., 1977; Cichewicz and Kouzi, 2004). CYP450 inhibition may reduce the metabolic elimination of these components, resulting in earlier onset of activity and longer half-life that possibly contribute to the synergetic effect of the 1:1 v/v mixed plant extract observed *in vivo* (Liu et al., 2017).

Compared to the plant extracts, IC_{50} values for individual triterpenes were at least 2-fold higher (except for UA toward CYP3A4). Other minor secondary metabolites reported in *S. sympetala* include: 2-hydroxyursolic acid, taraxenyl *trans*-4-hydroxy-cinnamate, naringenin, methyl ursolate, eriodytiol, methyl 2-α-hydroxyursolate, methyl 2-α-hydroxymaslinate, methyl betulinate, and condrilla sterol (Puniani et al., 2014). Naringenin has been reported to inhibit a number of CYP450 isoforms *in vitro* including CYP1A1, CYP1A2, CYP1B1, CYP2C9 and CYP3A4 (Shimada et al., 2010). Several of these substances are recognized as derivatives of the major triterpenes, and they may process similar CYP450 inhibition effects. This suggests that the

overall composition of the herbal drug needs to be evaluated for its potential for CYP450 inhibition.

DA is a fast-acting benzodiazepine used to treat generalized anxiety disorder with rapid onset action after oral administration and a long half-life from 30 to 100 h (Hoffman et al., 2008). All three major metabolites of DA (Figure 3-1) retain pharmacological activity (Hoffmann-La Roche Ltd, 2018). Like DA, temazepam and oxazepam are full agonists of benzodiazepine site of the $GABA_A$ receptor complex and elicit similar anxiolytic activity. The half-life of temazepam and oxazepam are 8-20 and 8-12 h respectively. Nordazepam is a partial agonist of the benzodiazepine site with reduced pharmacological effect relative to DA and a similar half-life (20-100 h), much longer than temazepam and oxazepam which are more active metabolites (Haddad and Winchester, 1998). Thus, inhibiting DA metabolism or favoring the formation of nordazepam may result in extended half-life. With repeated administration, accumulating plasma levels could eventually cause benzodiazepine overdose and lead to severe adverse effects including central nervous system depression, impaired balance, ataxia, and slurred speech (Greenblatt et al., 1977). At higher concentration, all extracts inhibited the metabolism of DA in the HLM assay, significantly reducing the formation of both temazepam and nordazepam compared to vehicle control. Unlike the CYP inhibitory results obtained using probe substrates, the inhibition of DA metabolism by the extracts were similar. BA is the only triterpene that showed inhibition of DA metabolism. Even though UA showed very potent inhibitory effect on CYP3A4-mediated metabolism of the probe DBF, no inhibitory effects were observed on DA metabolism. This may due to the allosteric behavior or selectivity of CYP3A4. Although still under debate, it is well accepted that multiple substrates can simultaneously bind to CYP3A4 inside or near the active site, affecting catalytic activity (Sevrioukova et al., 2015).

As a result, potency may vary when different substrates are used in the assay (Kenworthy et al., 1999).

The findings in this *in vitro* study suggest that the extracts of *S. sympetala* and *P occidentalis*, alone or in combination, have the potential to affect the efficacy and safety of co-administered DA products, as well as other benzodiazepenes, in certain conditions. The observed inhibition of CYP-mediated metabolism, however, could also be beneficial when the extracts are not consumed with other medications since the pharmacologically active triterpenes are also metabolized though the CYP450 system, possibly increasing their bioavailability and enhancing anxiolytic effect. Finally, clinical study is warranted to investigate whether the results observed here can be applied to humans.

CHAPTER 4: INHIBITION OF MONOACYLGLYCEROL LIPASE BY TRITERPENES FROM ANXIOLYTIC MEDICINAL PLANT EXTRACTS

4.1 Introduction

The neotropical plant family Marcgraviaceae was selected for study of its pharmacological activity by our collaborative Costa Rica-Canada group because it had received little attention in the scientific literature. The Central American species *Souroubea sympetala* was examined for its phytochemistry and anxiety-reducing properties in rodent behavioral models using elevated plus maze (EPM) and fear potentiated startle (FPS) paradigms (Puniani et al., 2014). Leaf and vine extracts were found to be significantly anxiolytic and bioassay guided fractionation indicated that several pentacyclic triterpenoids contributed to this pharmacological activity. Betulinic acid was identified as the main principle bioactive molecule synergized by α and β amyrins. The pharmacological mode of action was shown to be due to *S. sympetala*-mediated activation of $GABA_A$ receptor (Mullallly et al., 2014).

Medicinal plants often have multiple modes of action. In mood disorders such as anxiety, the endocannabinoid system also plays an important role (Howlett 2002). Several tri-terpenoids have been reported to inhibit the endocannabinoid degradation enzyme Monoacylglycerol lipase (MAGL) (King et al., 2009; Chicca et al., 2012). These include α and β -amyrin, major constituents of copal incense (*Protium copal,* Burseraceae), which we have recently shown to elicit CB1-dependent anxiolytic effects in animal models (Merali et al., 2018, Appendix 2). Since *S. sympetala* also contains α and β amyrins, activation of the endocannabinoid system may contribute to the extract's demonstrated anxiolytic activity. Currently, there is no evidence to indicate whether other pentacyclic triterpenoids found in the *S. sympetala* extract, namely betulinic acid, ursolic acid and lupeol, can interfere with MAGL activity. However, due to structural similarity, they may have the same effects as α and β -amyrin. In addition, effects of these triterpenoids on other endocannabinoid degradation enzymes, such as fatty acid amide

hydrolase (FAAH), remains unclear. Thus, the current study investigated the effects of *S. sympetala* triterpenoids as well as a proposed commercial natural health product containing *S. sympetala* and *P. occidentalis* extracts on endocannabinoid degradation enzymes using an *in vitro* assay.

4.2 Method and material

4.2.1 Material

Leaves and stems (herb) of *S. sympetala* were collected from an experimental plantation managed by the Universidad Nacional (UNA) in Sarapiqui (Heredia province) in Costa Rica. *P. occidentalis* bark was collected in Ontario, Canada. Dried plant materials were ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ 08085, USA) to pass through a #20 mesh.

4.2.2 Sample preparation

Plant material (10 grams) was extracted by 25 ml of 95% ethanol overnight on shaker set at 200 rpm. The extract was then filtered; the plant material was extracted again with an additional 25 ml of 95% ethanol. The combined extract was dried under vacuum and lyophilized in a Super Modulyo freeze drier. 50% DMSO was used to dissolve dried extracts for the MAGL inhibition assay.

4.2.3 MAGL inhibition assay

Sample were incubated with 0.583 µg/ml of MAGL (human recombinant, Cayman Chemical), 236 µM of 4 nitrophenyl acetate in 10 mM Tris-HCl buffer with 1 mM EDTA at room temperature for 15 min. A Cytatio3 image reader was used to monitor the reaction at an absorbance of 405 nm. JZL 184 (5.56 nM) was used as positive control. DMSO (2%) was used as vehicle control.

IC_{50} values were obtained by using Prism GraphPad version 7.0; log [Inhibitor] vs. normalized response - Variable slope analysis module. Samples were tested in triplicate at a minimum of 6 concentrations.

4.2.4 FAAH inhibition assay

Sample were incubated with 10 µg/ml of FAAH (human recombinant, Cayman Chemical), 20 µM of AMC-Arachidonoyl Amide in 125 mM Tris-HCl buffer with 1 mM EDTA at 37 ºC for 60 min. A Cytatio3 image reader was used to monitor the reaction. The fluorescence set are as follow: excitation at 355 nm and emission at 460 nm. JZL 195 (4.6 µM) was used as positive control. Ethanol (2%) was used as vehicle control.

4.2.5 Phytochemical analysis

A validated targeted HPLC-MS analysis was used to quantify the triterpenoid in plant extracts. Detailed method was described in Chapter 2. In brief, 1 mg/ml stock solution of dried plant extract in methanol was made and further diluted to 0.1 mg/ml (*S. sympetela*) and 0.01 mg/ml (*P. occidentalis*). One microliter of diluted plant extract was then injected to an Agilent 1200 series HPLC system with a SCIEX 3200 QTRAP with an atmosphere pressure chemical ionization (APCI) source. Separations were performed at 0.4mL/min on a Kinetex C18 column, particle size of 2.6-micron, 100 mm × 2.1 mm I.D. Column thermostat was maintained at 55 °C during a linear gradient of 30-100% acetonitrile in water in 18 min.

Linear calibration curves were generated by injecting each compound using optimized an LC-MS methodology. Compounds were quantified based on area under the peak that bracketed the response obtained from the calibration range of each compound.

4.3 Results

4.3.1 Quantification of triterpenes in plant extract

The phytochemical analyses indicated that the *P. occidentalis* yielded a terpenoid rich extract which contained more than 30% betulinic acid and 2% ursolic acid in the crude extracts. No amyrins were detected in *P. occidentalis* ethnolic extract (Table 4-1, Figure 2-2). The terpene distribution in *S. sympetela* is much more even, all terpenoids were present at a concentration from 1-3% of the total extract (Table 4-1).

4.3.2 Inhibition effect of triterpene and plant extract on MAGL

In the MAGL assay, inhibition was observed with all plant extracts and compounds. Hence, the IC_{50} of all terpenoids and plant extracts against MAGL were further evaluated (Table 4-2). Betulinic acid exhibited the lowest IC_{50} value followed by ursolic acid, β-amyrin, α-amyrin and lupeol. *S. sympetela* extract (476.8 ± 74.3 ng/ml) showed a lower IC_{50} value compared to *P. occidentalis* (746.6 ± 53.9 ng/ml). These values are approximately an order of magnitude greater than the positive control, JZL184 (22 ng/mL).

4.3.3 Inhibition effect of triterpene and plant extract on FAAH

In the FAAH inhibition screening assay, none of the terpenoids showed any inhibition on FAAH activity. However, a 90% increase of the enzyme activity was observed with α-amyrin. A 10-25% increase of FAAH activity was also observed ursolic acid, β-amyrin and lupeol (Figure 4-1). Both plant extract showed about 60% inhibition of FAAH activity relative to the vehicle control.

Table 4-1. Concentration of tri-terpenes in *S. sympetela* whole pant extract and *P. occidentalis* bark extract.

	Concentration (mg/g extract)	
Triterpene	*S. sympetela*	*P. occidentalis*
Lupeol	8.4 ± 0.5	ND
Betulinic acid	32.1 ± 0.3	317.2 ± 22.0
β-amyrin	28.7 ± 1.6	ND
α-amyrin	17.0 ± 1.0	ND
Ursolic acid	1.3 ± 0.01	16.9 ± 1.3

ND: not detected

Table 4-2. MAGL inhibitory potency of pentacyclic triterpenoids and plant extracts.

Sample	IC_{50}	
	(ng/ mL)	(nM)
JZL184 (positive control)	22	42
95% EtOH Extracts		
S. sympetela	481	N/A
P. occidentalis	767	N/A
Triterpenes		
Betulinic acid	405	886
Ursolic acid	413	904
Lupeol	755	1768
α-amyrin	651	1527
β-amyrin	493	1154

N/A: not applicable

4.4 Discussion

This research showed that the main bioactive triterpenoids in *S. sympetala* and *P occidentalis* inhibit the endocannabinoid degrading enzyme MAGL. Accordingly, the previously established anxiolytic effects of these plant extracts may not only be mediated through activation of $GABA_A$ receptors (Mullally et al., 2014), but also through the activation of the cannabinoid receptor system. Betulinic acid, alongside other tested terpenes, exhibited *in vitro* MAGL inhibitory potency that would slow the degradation of endocannabinoids, particularly 2-Arachidonoylglycerol (2-AG). Such activity would lead to a raise of 2-AG levels and the activation of cannabinoid receptor signaling pathways. Several potential pharmacological applications of MAGL inhibitors have been explored by other researchers, including anti-nociceptive, anxiolytic, and anti-emetic responses (Melinda et al., 2013). There are also benefits of MAGL inhibitors in cancer and neurodegeneration (Mulvihill and Nomura, 2013). These studies suggest medicinal potential of terpene-rich herb products.

Other pentacyclic triterpenoids also exhibited potent inhibitory action (Table 4-2) with an IC_{50} at the ng/ml level. Both *S. sympetala* and *P. occidentalis* extract showed MAGL inhibitory effects at comparable potencies. With a similar IC_{50} to pure betulinic acid, the inhibitory effect of *S. sympetala* is most likely a combined effect from all pentacyclic triterpenoids identified in the plant. However, the total amount of these terpenoids is less than 10% of the extract (w/w), which suggests that synergism or other compounds may also contribute to the activity. *P. occidentalis* extract contains 30% betulinic acid which is consistent with the inhibitory effect observed with *P. occident*alis extract.

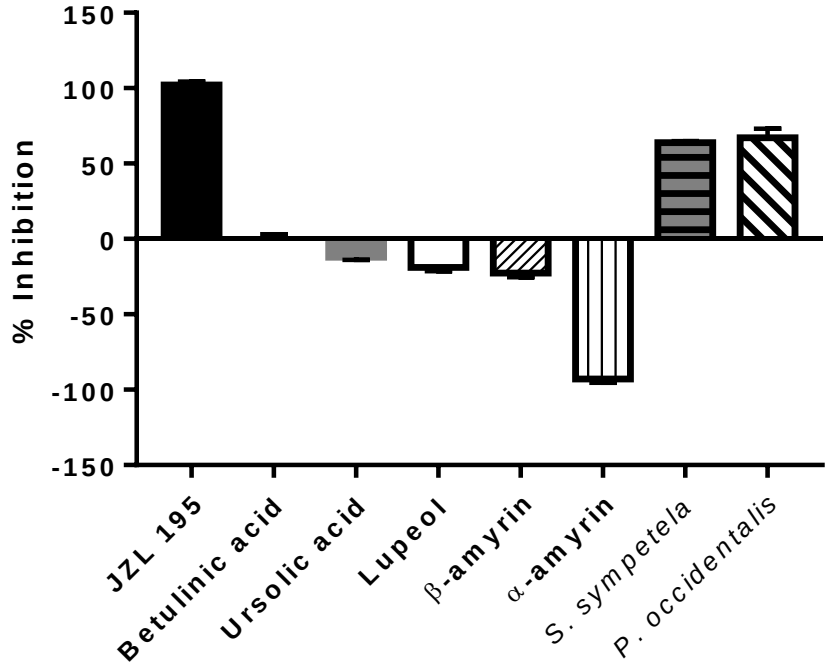


Figure 4-1. FAAH inhibitory potency of pentacyclic triterpenoids and plant extracts. Percent inhibition determined at 50 µg/ml for all compounds and plant extracts relative to vehicle control and expressed as mean +/- SEM (n=3).

Although the experimental methods are different, our results are similar to a previous study by Chicca et al., 2012. Inhibitory effects of identified triterpenoids on endocannabinoid degradation is selective toward MAGL with no impacts on fatty acid amide hydrolase (FAAH). On the contrary, our results reveal that FAAH activities were enhanced by these compounds. A minor increase in enzyme catalytic activity in enzyme inhibition screens are not rare, and these phenomena are usually explained by technical/instrument error or hormesis effect (Calabrese, 2004). However, the 90% enhanced FAAH activity by α-amyrin cannot be ignored. Large direct stimulation of enzyme activity is rarely reported, and it is usually through conformational change by an allosteric modulator. However, there are no allosteric sites in FAAH that have been reported. Rossi et al. (2007) report direct stimulation on FAAH activity via phosphorylation of accessory proteins triggered by follicle-stimulating hormone. However, no further mechanism regarding the involvement of accessory proteins was elucidated. These findings suggest that, although the mechanism is not clear, there are co-factors which may affect the FAAH activity and α-amyrin may enhance the FAAH activity through a similar mechanism as the accessory proteins. In addition, even though pure terpenoids did not inhibit FAAH activity, both plant extracts showed *in vitro* inhibitory effect on FAAH. These findings suggest that non-triterpenoid metabolites may interact with FAAH and potentially contribute to the pharmacology of *S. sympetala* and *P. occidentalis* but additional research into the plants' FAAH inhibiting compounds is required. Collectively, our results reveal a novel mode of action – endocannabinoid system activation – that may contribute to the anxiolytic properties of the studies medicinal plants and the blended commercial product (Zentrol^{TM}).

CHAPTER 5: SERUM/WHOLE BLOOD CONCENTRATION OF BETULINIC ACID, THE MAJOR ACTIVE PRINCIPLE OF A NATURAL HEALTH PRODUCT CONTAINING *SOUROUBEA SYMPETALA* AND *PLATANUS OCCIDENTALIS*. AND ITS METABOLITES IN BEAGLES (RESEARCH NOTE)

5.1 Introduction

A new natural health product (NHP) containing medicinal plants *Souroubea sympetala* and *Platanus occidentalis* has been released on the companion animal health market as "Zentrol^{TM} (Formerly known as Sin susto^{TM}) as a treatment for noise aversion and stress reduction in animals (Liu et al 2017). The product was found to be safe in a 28-day feeding trial in beagles at 5x the recommended dose and effective in reducing behavioural measures of anxiety in a thunderstorm model and blood levels of the stress hormone cortisol (Masic et al., 2017).

The Souroubea- Platanus NHP is well received the in-companion animal health market, but additional information on the pharmacokinetics (PK) of the active principle, betulinic acid (BA) has been requested by the veterinarian. Measure the plasma concentration of active ingredient is the classic approach to investigate the absorption and elimination of a health product. BA, as an anti-cancer agent, has been studied in rats for the pharmacokinetic with a single oral administration at high dose (>=100mg/kg; Godugu et al., 2014). BA was detected with a t_{max} at 2.36 hr. According to the "biopharmaceutics classification system", BA is similar to class II drug with high permeability, low solubility, and the bioavailability of BA is limited by its solvation rate. Although previous studies indicated that BA can be detected in plasma after an acute dose, the area under curve (ACU) is up to 8 fold different between formulated and unformulated BA (Godugu et al., 2014).

Thus, repeated oral administration of Zentrol^{TM} (=<5mg/kg BA content) in beagles may exhibit a very different pharmacokinetic profile. The objective of this study was to measure the concentration of BA in the serum/whole blood samples of beagle dogs as well as identify if potential metabolites were present in the serum/whole blood samples of beagle dogs.

5.2 Method and Material

5.2.1 Animal pharmacokinetic study

Zentrol™ (Previously named Sinsusto™) dog tablets and placebo tablet were obtained from Bioniche Life Science (Belleville, ON.). The target animal safety study was conducted by Kingfisher International Inc., Stouffville, ON. Procedures were designed to avoid or minimize discomfort, distress and pain to the animals in accordance with the principles of U.S. Animal Welfare Act and the guidelines of Canadian Council on Animal Care (CCAC). The CCAC Guide for the Care and Use of Experimental Animals and related policies were regarded as the guidelines to follow. The test facility was a registered research facility under the Animals for Research Act, ON Ministry of Agriculture and Food and was also accredited in Good Animal Practices® by the CCAC. In order to ensure compliance, the protocol was reviewed and approved by test facility's Animal Care Committee before the start of the trial.

Subjects were 18 healthy, intact, male, Beagle dogs, weighing between 7.6 and 12.3 kg and ranging from 7 to 58 months of age. The test subjects were acclimated to study conditions for 8 days prior to dosing. The dogs divided in to 4 groups and were dosed once daily with placebo (10x chewable tablets) or 1x, 5x or 10x of Zentrol tablets at the recommended dose based upon body weight. On Days 1 and 21, blood was collected for plasma determination of plasma levels of BA prior to treatment, and post treatment at 30 minutes, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr and 24 hr. Serum and whole blood samples were stored in -80ºC until analysis.

5.2.2 Sample preparation

To quantify BA in Zentrol^{TM} dog tablets, a 0.5 g sample was extracted in triplicate with 250 mL ethyl acetate using a soxlet apparatus for 2 hrs. The extract was dried by rotatory evaporation at 45 °C, re-dissolved in 20 ml of methanol by sonication for 2 min. The pellet was re-extracted in 20 mL methanol by sonication for 2 min. Total volume was adjusted to 50 mL in a volumetric flask. The extraction was done in triplicate.

To quantify BA serum concentration, a 100 µL of serum sample collected during the safety trial was thawed in an ice bath. Fifty nanogram oleanolic acid (internal standard, solubilized in 100 µL of methanol) was then added to the serum followed by 0.5 mL water (LCMS grade, Fisher Scientific, Nepean, ON) and 3 mL methylene chloride (Optima grade, Fisher Scientific, Nepean, ON) each sample was then well mixed by vortex for 1 min. The mixture was then centrifuged at 2000 rpm for 15 min. Two mL of the organic phase were transferred to another glass tube and dried under N_2 gas. The residue was re-solubilized with 1 mL methanol (LCMS grade, Fisher Scientific, Nepean, ON) with 5 min sonication.

For metabolite identification, 1 mL methanol was added to 100 µL serum. The mixture was vortexed for 1 min then centrifuged at 13400 RCF for 10 min to precipitate the protein. The supernatant was transferred and dried under N_2 gas. The residue was re-solubilized with 0.2 mL methanol with 5 min sonication. A 100 µL whole blood sample repeated - 80ºC freeze thawing for 3 times, then 1 mL methanol was added to the whole blood sample followed by 20 min of sonication. The supernatant was transferred and dried under N_2 gas. The residue was re-solubilized with 0.2 mL methanol by 5 min sonication. All samples were filtered through 0.2 mm PTFE filter before LC-MS analysis.

5.2.3 UPLC-MS analysis

Targeted UPLC-MS analyses were carried out on a Shimazu UPLC-MS (Mandel Scientific Company Inc, Guelph, Ontario, Canada). Separations were performed at 0.35 mL/min on a Kinetex C18 column, particle size 1.7-micron, 150 mm × 2.1 mm I.D. (Phenomenex, Torrance, CA, USA). Column thermostat was maintained at 40ºC during an isocratic elution of 63% acetonitrile in water. The column was then washed with 5 column volumes with 100% acetonitrile, returned to the initial conditions in 0.1 min and re-equilibrated for 3 min before the next injection. The MS was operated in SIM (selected ion monitoring) negative APCI ionization mode monitoring m/z at 455.3 ($[M-H]^+$).

Linear calibration curves were built by injecting dilutions of BA (Extrasynthese, Lyon, France) which bracket the compound concentration in samples. Oleanolic acid was used as internal standard. Each sample was injected in triplicate and the area under each peak was then used to quantify the amount of each marker compound. Recovery experiments were undertaken by spiking serum with pure compounds at 1.97, 7.88, and 19.7 µg/mL. Spiked and unspiked samples were prepared following the procedure described above and recovery determined by regression analysis. Calibration curves were prepared at five concentration levels and R2 values obtained for each metabolite. The limits of detection (LOD, 3:1 signal: noise) and limits of quantification (LOQ 10:1 signal: noise) were determined at 3x standard deviation of noise level.

Non-targeted UPLC-MS analyses were carried out on a Shimazu UPLC-MS (Mandel Scientific Company Inc, Guelph, Ontario, Canada). Separations were performed at 0.4 mL/min on a Kinetex C18 column, particle size 1.7-micron, 150 mm × 2.1 mm I.D. (Phenomenex, Torrance, CA, USA). Column thermostat was maintained at 55ºC during a linear gradient of 30-

100% acetonitrile in water in 18 min. The column was then washed with 5 column volumes with 100% acetonitrile, returned to the initial conditions in 0.1 min and re-equilibrated for 3 min before the next injection. The MS was operated in scan mode at both positive and negative APCI ionization mode scanning m/z from 100 to 900 with a 0.2s scan speed.

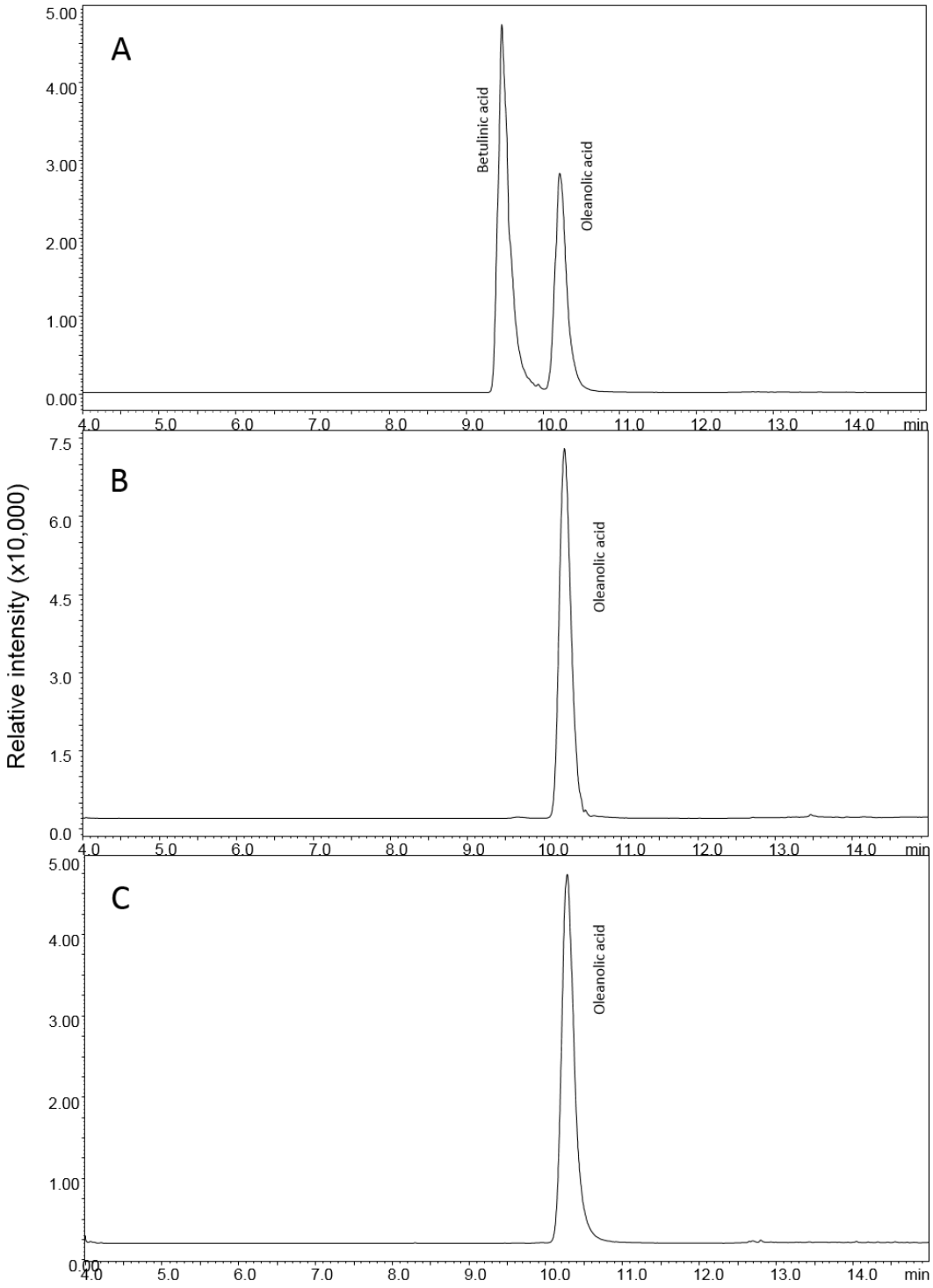


Figure 5-1. UPLC-APCI-MS selected ion chromatogram of A) Standard compound mixture; B) Serum sample collected at 2 hr after oral administration of placebo; C) Serum sample collected at 2 hr after oral administration of formulated product.

5.3 Results

5.3.1 Quantification method vailidation

BA and oleanolic acid (Internal standard) eluted at 9.5 and 10.3 min respectively (Figure 1). The optimized method was able to identify and quantify the BA in serum with a linearity (R^2>0.999) within a range of 0.78-78 pg on column. The LOD and LOQ of BA is 0.111 pg and 0.423 pg on column respectively. Thus, the detection limit and quantification limit of BA in serum is 20 ng/mL and 61 ng/mL respectively. The spike recovery study of BA showed that the linear regression of recovered amount versus the spiked were obtained (R2 >0.99) and all the recoveries observed for was 96.7%.

5.3.2 Detection and quantification of betulinic acid and its predicted metabolite in canine serum and whole blood

The LC/MS quantitative analysis indicated that the Zentrol dog tablet contain a mean (SE) of 5.68±0.14 mg of BA per tablet. No BA was found the placebo tablet at the limit of detection. Thus, the BA dosage for the placebo, 1x, 5x and 10x groups were 0, 5.68±0.139, 28.4±0.695 and 56.8±1.39 mg/dog/day respectively. With the current method condition, BA was not detected in any of the serum samples. Predicted metabolites (Figure 5. 2) of BA were also not detected in both serum and whole blood samples.

5.4 Discussion

Although betulinic acid, the major activity ingredient of Zentrol™ was not detected in the serum and whole blood sample in this study, the efficacy study suggests that BA may have been absorbed but the serum concentration below the LOD of the method used. The bio-availability by oral administration of BA is very poor due to the extremely low aqueous solubility (Jager et al, 2007). Godugu et al (2014) report a maximum plasma concentration of BA at 1.16±0.22 µg/mL with a single oral dose at 100 mg/kg in Sprague Dawley (Female, 6 weeks old). The highest dose (5x tablets) used in our study is only 5.123-7.478 mg/kg/day. Another study has indicated that repeated oral administration of pentacyclic tri-terpenoids also showed very low serum concentration of these compound in C57BL/6 mice (Three-week-old male). All mice had free access to food spiked with 0.5% pentacyclic tri-terpenoids, pentacyclic tri-terpenoids were not detected in the first 4 weeks and no more than 0.61 µg/mL after 8 weeks of treatment. On the other hand, these pentacyclic tri-terpenoids have been found in tissues including brain, heart, liver, kidney, colon and bladder with a much higher concentration (Yin et al, 2012). Udeani et al (1999) also report similar phenomenon in CD-1 Mice with Intraperitoneal injection of BA. High concentrations of BA were detected bladder, fat, lymph node, ovary and spleen.

Although BA has been reported to be chemically stable with metabolic enzymes such as cytochrome P450 monooxidase, several microbial metabolites of BA have been identified (Cichewicz et al, 2004) which suggest BA has the potential to be metabolized by gut microflora. In addition, glucuronidation of BA analog Bevirimat by UDP-glucuronosyl-transferases has been reported. This suggests that BA may also be metabolized by UDP-glucuronosyltransferases (Wen et al 2007). However, none of the predicted metabolites were found in the serum and

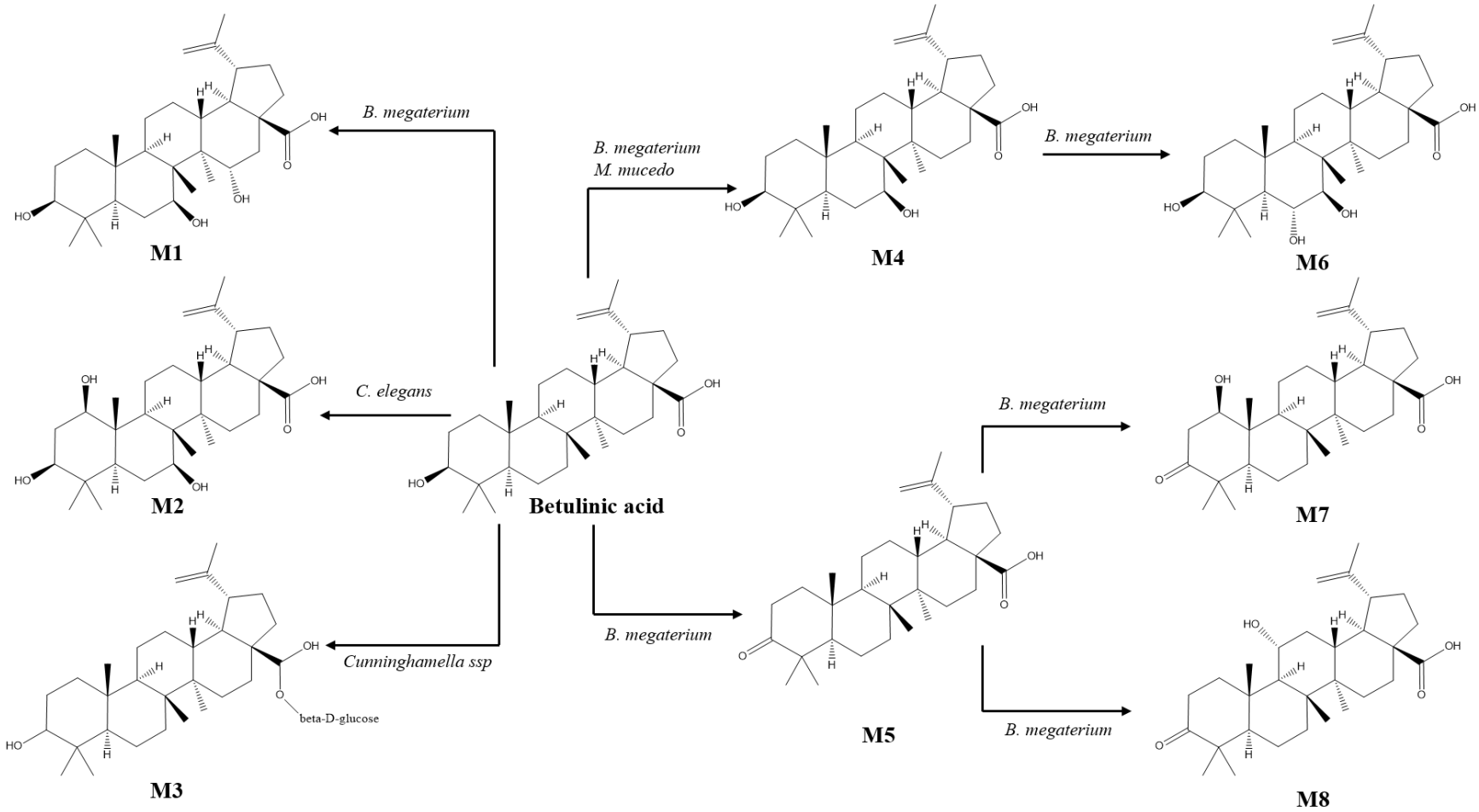


Figure 5-2. Microbial model of betulinic acid metabolic pathways (Cichewicz, et al. 2004).

whole blood of beagle. This may also due to the concentration of these metabolite was below the detection limit.

In summary, with the current dose of Zentrol™, BA cannot be detected in serum samples with our equipment. Due to poor aqueous solubility of this lipophilic compound, BA is more likely to accumulate in tissue such as fat, liver, bladder instead of blood. Therefore, measuring the serum betulinic concentration may not be the best way to investigate the bio-availability of Zentrol™. Fortunately, serum cortisol levels show a robust dose response effect in animals subjected to the simulated thunderstorm stress and provide a clinical biomarker of treatment. Further study focusing on identifying and quantifying BA and its metabolites in the tissue samples such as brain, bladder, liver, fat and kidney in dogs are not ethically feasible with current methods at this time.

CHAPTER 6: IN VITRO INHIBITORY EFFECTS OF *ECHINACEA* SPP. CHEMOTYPES ON FATTY ACID HYDROLASE (FAAH).

6.1 Introduction

Purple coneflowers, *Echinacea* spp (Asteraceae), were traditionally used by North American First Nations to treat upper respiratory infections and wounds (Shemluck, 1982). Today, Echinacea-containing products are among the best-selling natural health products (NHP) in North America (>$60M alone in U.S market, Smith et al., 2015), used mostly for cold and flu management but also for other lung conditions, wound healing and candidiasis (Blumenthal et al., 2003). *Echinacea angustifolia* (DC.) Hell.,. and *Echinacea purpurea* (L.) Moench are the major species used medically at this time (Parsons et al., 2018). Commercial Echinacea products usually contain one or more species.

The major phytochemicals identified in *Echinacea* spp. include alkylamides (AKA), caffeic acid derivatives (CAD) (Figure. 1-4), polysaccharides/glycoproteins and polyacetylenes (Binns et al., 2002). Composition varies by species. For example, echinacoside is absent from *E. purpurea* and most of the major AKAs are absent from *E. pallida*. The root of *E. angustifolia* and the root as well as the aerial parts of *E. purpurea* are the most commonly used material in the NHP industry. Due to the absence of most AKAs, *E. pallida* is not currently favored as an NHP. The phytochemical composition may also differ between populations in one species, for example *E angustifolia*. (Binns et al 2001)

Although Echinacea and related NHP products have been extensively studied for anti-inflammatory, antiviral, antifungal, antibacterial activity (Barrett, 2003), novel neurological activity was documented by Haller et al. (2010 & 2013) who reported *E. angustifolia* and *E. purpurea* root preparations exhibited anxiolytic effects in the elevated plus-maze, social interaction and shock-induced social avoidance tests in rodents.

Pharmacological modes of action of Echinacea include modulation of the endocannabinoid system (ECS) (Chicca et al., 2009), which plays an important role in processes such as appetite, pain, memory and mood, among others. The major pharmacological targets of the ECS are cannabinoid receptors (CB1 & CB2) as well as the enzymes that degrade endocannabinoids. The activation of CB1, distributed primary throughout the central nervous system and some peripheral tissues, can de-polarize excited neurons and inhibit the release of various neurotransmitters including noradrenaline, dopamine, 5-hydroxytryptamine, g-aminobutyric acid and glutamate, which prove beneficial for many CNS-related diseases (Di Marzo et al., 1998). CB2 receptors are expressed mainly on immune cells where they can affect the release of cytokines and modulate the immune response (Guindon and Hohmann, 2008). Furthermore, the activation of CB2 receptors located on inflammatory cells can induce an antinociceptive effect that can be used to relief inflammatory related pain. Endocannabinoid degradation enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), which respectively degrade anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), are also potential pharmacological targets that, blocking the degradation of endocannabinoids, may result in indirect activation of the ECS.

As anandamide analogues, AKAs are believed to contribute to Echinacea's anti-inflammatory and anxiolytic activities (Hohmann et al., 2011). Echinacea AKAs are highly bioavailable and known to bind to cannabinoid receptors with selectivity for CB2 (Woelkart et al., 2005A). Individual AKA have been identified with partial agonist, antagonist as well as Inverse agonist properties (Hohmann et al., 2011). However, the interaction of AKAs with ECS enzymes such as FAAH and MAGL has not been fully studied. Currently, CADs and polysaccharides appear most relevant for antimicrobial effects. However, since phenolic

compounds have been reported to inhibit FAAH and MAGL (Russo, 2016), the CADs may similarly affect ECS signaling.

The objective of this study was to investigate the interaction of Echinacea extracts, fractions and major substances with endocannabinoid degradation enzymes FAAH and MAGL using *in vitro* assays. In an effort to identify contributing active metabolites as well as Echinacea chemotypes with strong enzyme inhibition activity, a collection of commercial accessions from a major North American producer was studied with subsequent biochemometric analysis of FAAH inhibition activity relative to detected metabolite profile.

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6.2 Method and material

6.2.1 Collection of Echinacea accessions

Variable *E. purpurea* and *E. angustifolia* genotypes were selected by plant breeder John Baker in 2015 and 2016 from the diverse germplasm grown at Trout Lake Farm, in the Columbia river baison at Euphrata, WA. Selections were based on traits that reflect good agronomic potential, such as plant height, size and number of leaves, and minimal signs of pests or disease. Plants were harvested whole, with roots intact, then cloned by root division and a root sample taken for analysis. Each accession was maintained in a nursery at the farm site.

6.2.2 Sample preparation

The root samples were dried at 45°C and milled to powder (1 mm mesh). 500 mg of each powdered sample was extracted three times in 15 mL fresh 70% ethanol using ultrasound (5 min) followed by 10 min of centrifugation at 3214 RCF and collection of the supernatant. Supernatant was dried under vacuum. Dried extracts were then re-solubilized in 10 ml of 70% ethanol. One ml of the Supernatant was filtered through 0.22 mm PTFE filter for HPLC-DAD or LC/MS analysis. For enzyme inhibition assay, 25 ml of the extract were dried under vacuum by a Savant speed Vac. The residuals were re-solubilized in 10 ml of 70% ethanol.

6.2.3 Phytochemical analysis

An Agilent HPLC system (model 1100) with Chemstation software (Version B 3.02) were used for phytochemical analysis. The system consisted of an autosampler (G1313A with 100µL loop), a quaternary pump (G1311A), a solvent degasser (G1322A), a column oven (G1316A) and a photodiode array detector (G1315A). Separations were performed on a Phenomenex Luna

(C18 (2) 100X2.1mm, 5um particle size) column obtained from Phenomenex Inc. (Mississauga, Ontario).

The injection volume for standards and samples was 2 µL, and the column oven temperature was set a 45°C. Flow rate was set at 0.4 mL/ minute. Mobile phase A consisted of 0.05% trifluoroacetic acid in water, and mobile phase B consisted of 0.05% trifluoroacetic acid in acetonitrile. The elution gradient was initiated with 10% B held for 1 minute, then increased from 10-100% B in 19 minutes. 100% B was maintained for 5 minutes before returning to initial conditions and re-equilibrating for 5 minutes. A detection wavelength of 268 nm was used for AKAs, and 330 nm for phenolic compounds. Slit width was 4 nm, with spectral data stored at 190-400 nm.

Peak identities were confirmed through relative retention times and spectral analyses compared to authentic standards (Figure 6.1). Quantification results were based on response factors, calculated from injection of known amounts of standard materials.

Selected Echinacea extracts were also analyzed on Shimadzu UPLC-MS system (Mandel scientific company Inc, Guelph, Ontario) which contains LC30AD pumps, a CTO20A column oven, a SIL-30AC autosampler and a LCMS-2020 mass spectrometer. Elution condition were the same as pervious described, the mass spectrometer with electrospary ionization (ESI) interface was operating in positive and negative scan mode, the nebulizing gas flow was set at 1.5 L/min and drying gas flow was at 10 L/min. The desolvation line temperature and heat block temperature was set at 300°C and 450°C respectively. The m/z range of both positive and negative scan is from 100 to 1000 with 938 u/sec scan speed.

6.2.4 FAAH inhibition assay

Sample were incubated with 10 µg/ml of FAAH (human recombinant, Cayman Chemical), 20 µM of AMC-Arachidonoyl Amide in 125 mM Tris-HCl buffer with 1 mM EDTA at 37 ºC for 60 min. A Cytatio3 image reader was used to monitor the reaction. The fluorescence set are as follow: excitation at 355 nm and emission at 460 nm. JZL 195 (4.6 µM) was used as positive control. Ethanol (1.75% for extracts or 2.5% for pure compounds) was used as vehicle control. IC_{50} values were obtained by using Prism GraphPad version 7.0; log [Inhibitor] vs. normalized response - Variable slope analysis module. Samples were tested in triplicate at a minimum of 6 concentrations.

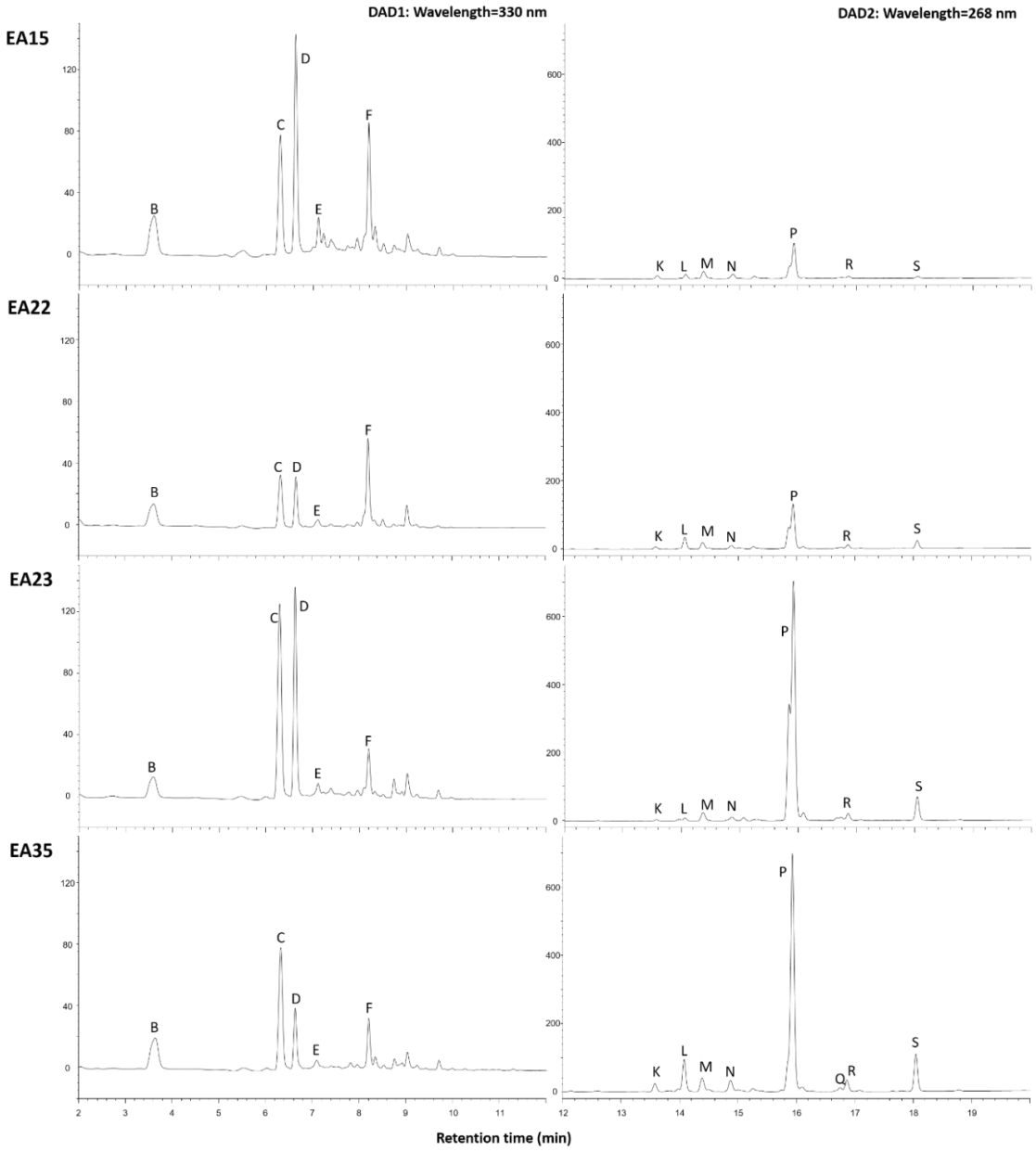
6.2.5 Statistical analysis

Activity and major compound concentrations were measured for two Echinacea species and 40 different genotypes within each species. Due to the presence of unidentified compounds, a relative concentration (log transformed peak area with molecular weight adjustment) was used in the simple linear regression. The relative concentration of 13 and 14 compounds were measured in *E. angustifolia* and *E. purpurea*, respectively. Analysis was done separately for the two species.

Since some of the detected metabolites may be co-regulated by common biosynthetic pathways, we first performed cluster analysis to identify and group co-varying compounds. Clusters of compounds were generated by Hierarchical clustering (UPGMA) using the pvclust R package. Distance between metabolites was measured with one minus the correlation between concentration values.

Simple and multiple linear regression modelling was performed on the observed concentrations of individual compounds and compound clusters (summed concentrations) relative to inhibitory effects on FAAH activities (percentage inhibition) of each Echinacea genotype.

A)


DAD1: Wavelength=330 nm
DAD2: Wavelength=268 nm
EA15
EA22
EA23
EA35
B
C
D
E
F
K
L
M
N
P
Q
R
S
Retention time (min)

B)

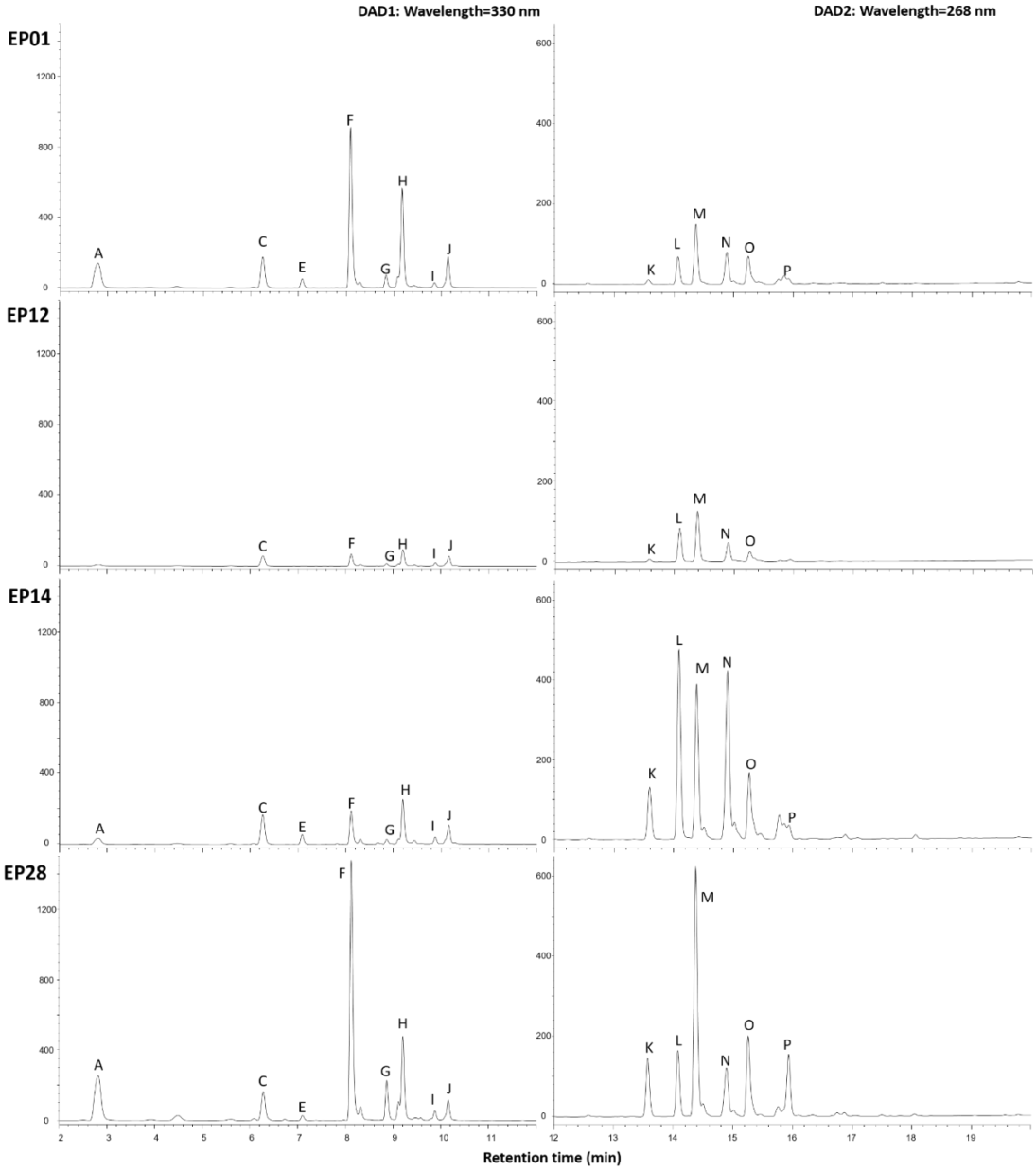
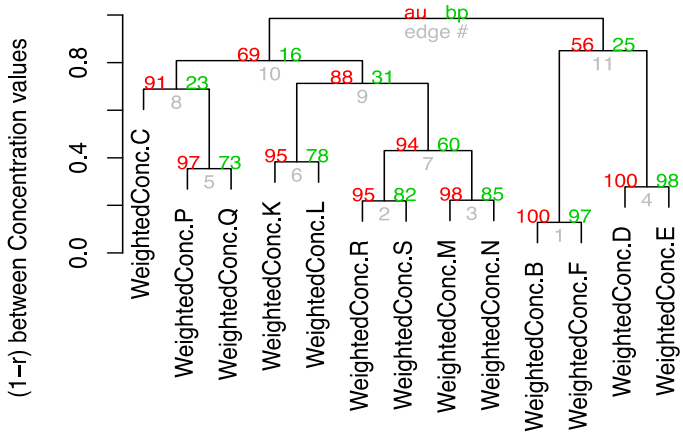


Figure 6-1. HPLC-DAD chromatogram of representative *E. angustifolia* (A) and *E. purpurea* (B) extracts. CADs were monitor at wavelength 330 nm and AKAs were monitored at wave length 268 nm. A: Caftaric acid; B: Chologenic acid; C: Unidentified phenolics 1; D: Echinacoside; E: Unidentified phenolics 2; F: Cichoric acid; G-J: Unidentified phenolics 4-6; K: Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide; L: Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide; M: Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide; N: Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide; O: Dodeca-2E,4Z-diene-8,10-diynioc acid 2-methylbutylamide: P: Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide + Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide; Q-S: Unidentified AKAs 1-3.

A)



B)

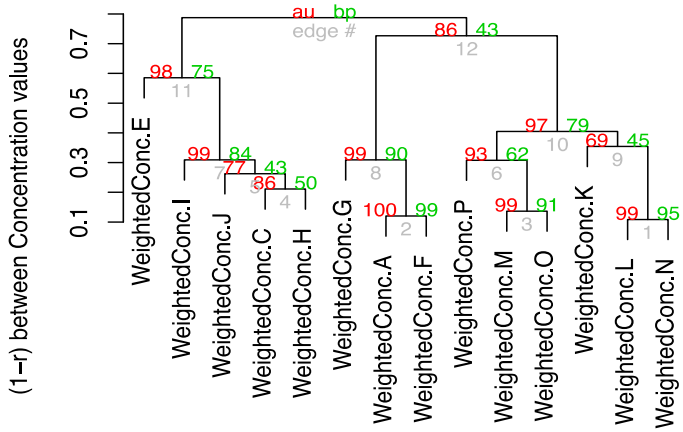


Figure 6-2. Dendogram from hierarchical clustering of *E. angustifolia* (A) and *E. purpurea* (B) metabolite relative to concentration across plant extract. Distance between metabolites was one minus the correlation between the variables.

6.3 Results

6.3.1 Phytochemcal analysis

A total 40 genotypes of each species were analyzed by HPLC-DAD and UPLC-ESI-MS. Representative HPLC-DAD chromatograms of both *E. angustifolia*, and *E. purpurea* are shown in Figure 6-1. The major phytochemicals identified were AKAs and CADs. These included 6 major AKAs, as well as caftaric acid, cichoric acid, chlorogenic acid and Echinacoside. They were identified by comparison of retention time, UV spectrum and mass to charge ratio (m/z) to the commercial or isolated standard compounds. Six peaks are tentatively identified as unknown phenolic compounds since they showed similar UV spectra to chlorogenic acid. Three peaks are also tentatively identified as AKAs based on UV spectrum. Mass to charge ratio of all major peaks were obtained (Table 6-1). The distribution of these compounds is quite uneven among all genotypes. AKA concentrations were found to vary from 9 to 33 fold and 7 to 72 fold in *E purpurea* and *E. angustifolia* respectively. CAD levels also varied greatly with 10 to 20 fold and 5 to 60 fold variation in concentration in *E purpurea* and *E. angustifolia*, respectively.

6.3.2 FAAH inhibition by Echinacea spp. extracts

Echinacea spp. extracts were screened for FAAH inhibition at a standard concentration equal to 25 mg dried plant material per ml of 70% alcohol (Table 6-3). Like phytochemistry, bioactivity results varied by genotype. The FAAH inhibition of 40 *E. angustifolia* genotypes ranged from 33.9% to 71.3% (Table 6-2) and from 32.6% to 85.8% for 40 *E. purpurea* genotypes.

Table 6-1. Percentage inhibition of *Echinacea purpurea* extract on FAAH activity and relative concentration of marker compound in each extract.

Sample code	FAAH % Inhibition	Relative concentration of marker compound in plant extract*																		
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
EP01	68.711	6.388	ND	3.370	ND	0.777	8.645	0.701	5.079	0.295	1.665	0.221	1.255	2.719	1.506	1.312	0.840	ND	ND	ND
EP02	64.874	1.797	ND	2.717	ND	0.250	2.990	0.475	2.000	0.249	0.416	0.204	3.507	6.675	4.882	1.709	1.265	ND	ND	ND
EP03	75.754	3.451	ND	2.390	ND	1.129	5.092	0.671	2.612	0.292	0.565	0.160	1.772	4.563	1.969	1.459	0.875	ND	ND	ND
EP04	67.600	3.150	ND	5.047	ND	0.116	6.606	0.456	4.015	0.292	2.121	0.756	4.574	11.676	5.700	3.945	2.200	ND	ND	ND
EP05	71.679	1.734	ND	1.996	ND	0.340	3.466	0.552	2.176	0.359	0.788	0.789	3.419	4.018	3.175	1.769	2.336	ND	ND	ND
EP06	69.341	5.761	ND	3.160	ND	1.294	7.821	0.724	4.408	0.331	0.803	0.693	3.262	3.872	2.889	2.623	2.193	ND	ND	ND
EP07	86.919	6.383	ND	4.132	ND	1.551	9.643	0.905	5.511	0.615	1.274	0.605	2.427	6.435	3.721	3.503	2.428	ND	ND	ND
EP08	81.296	5.835	ND	3.892	ND	1.447	7.908	0.782	4.811	0.530	1.187	0.800	2.802	6.964	4.128	3.541	2.367	ND	ND	ND
EP09	85.249	6.388	ND	9.945	ND	1.528	6.938	1.080	9.430	1.143	3.547	0.173	2.657	7.084	3.138	2.222	1.093	ND	ND	ND
EP10	80.929	4.243	ND	2.267	ND	0.311	9.006	0.670	2.554	0.130	0.407	0.332	2.423	4.791	3.076	1.871	1.908	ND	ND	ND
EP11	79.829	5.015	ND	3.680	ND	0.790	7.334	0.762	3.332	0.336	0.686	0.637	2.514	5.632	3.002	2.146	2.971	ND	ND	ND
EP12	32.576	0.324	ND	1.084	ND	0.000	0.672	0.155	0.886	0.196	0.593	0.120	1.543	2.235	0.904	0.623	0.235	ND	ND	ND
EP14	50.139	1.527	ND	3.098	ND	0.805	1.834	0.284	2.311	0.390	1.098	2.598	8.754	6.882	8.170	3.315	2.591	ND	ND	ND
EP16	81.418	1.673	ND	4.984	ND	0.979	2.411	0.455	3.926	0.669	2.111	3.118	8.639	7.114	7.608	2.961	2.343	ND	ND	ND
EP17	67.196	1.182	ND	3.325	ND	1.883	1.575	0.212	2.336	0.310	0.827	0.704	1.215	1.959	1.436	0.918	0.754	ND	ND	ND
EP22	57.702	1.294	ND	2.656	ND	0.196	0.989	0.251	1.529	0.399	1.139	0.094	0.519	1.200	0.696	0.372	0.364	ND	ND	ND
EP23	64.588	1.453	ND	1.134	ND	0.169	3.453	0.822	1.250	0.167	0.325	0.184	2.453	2.926	3.715	0.787	0.504	ND	ND	ND
EP24	73.227	1.248	ND	4.240	ND	1.560	1.535	0.350	3.387	0.868	3.071	0.682	2.731	3.746	3.923	1.576	1.134	ND	ND	ND
EP25	76.895	2.019	ND	2.563	ND	0.201	4.250	1.025	2.818	0.443	0.970	0.240	1.855	2.485	2.060	0.844	0.591	ND	ND	ND
EP26	80.532	3.090	ND	3.074	ND	0.253	7.396	1.311	2.671	0.467	0.947	0.397	2.762	2.553	2.247	0.886	0.827	ND	ND	ND
EP27	58.163	1.887	ND	3.655	ND	0.532	3.297	0.438	2.889	0.287	1.199	1.520	2.816	4.137	3.250	1.735	1.716	ND	ND	ND
EP28	76.962	8.091	ND	3.151	ND	0.480	13.968	2.252	4.430	0.679	1.296	2.755	2.999	10.780	2.526	3.669	3.525	ND	ND	ND
EP29	68.319	2.670	ND	2.821	ND	0.689	4.987	0.448	3.383	0.292	1.090	0.605	1.946	5.476	1.906	1.754	1.073	ND	ND	ND
EP30	83.731	3.811	ND	5.185	ND	0.177	6.851	0.482	5.576	0.455	2.453	1.703	5.947	8.472	3.107	2.721	2.287	ND	ND	ND
EP31	62.856	4.334	ND	1.358	ND	0.380	5.922	0.850	0.505	0.165	0.333	0.461	2.822	4.720	2.917	1.699	3.237	ND	ND	ND
EP32	79.495	3.803	ND	1.697	ND	0.494	6.882	0.910	2.301	0.344	0.785	0.917	3.388	3.680	3.206	2.189	3.581	ND	ND	ND
EP33	51.855	1.485	ND	2.987	ND	0.489	3.621	0.986	4.320	0.985	1.673	0.762	1.189	2.275	1.483	0.755	1.459	ND	ND	ND
EP34	59.980	2.080	ND	1.565	ND	0.333	4.968	0.551	2.177	0.173	0.668	0.213	1.395	2.848	1.841	1.234	0.837	ND	ND	ND
EP35	69.064	3.011	ND	2.777	ND	1.773	7.459	0.562	5.690	0.391	1.802	1.820	2.134	5.336	1.878	1.903	1.345	ND	ND	ND
EP36	79.450	4.748	ND	1.336	ND	1.045	13.493	1.181	2.511	0.185	0.585	1.080	5.147	8.069	3.971	2.077	2.010	ND	ND	ND
EP38	61.740	1.132	ND	1.657	ND	0.228	1.617	0.237	1.196	0.191	0.883	0.394	1.210	2.104	0.718	0.548	0.568	ND	ND	ND
EP39	63.677	1.736	ND	1.797	ND	0.521	3.019	0.455	1.641	0.183	0.760	0.231	1.545	2.564	1.818	1.160	0.604	ND	ND	ND
EP42	71.654	2.764	ND	2.767	ND	0.717	4.115	0.347	1.824	0.118	0.844	0.251	1.025	2.859	1.408	0.949	0.638	ND	ND	ND
EP43	79.495	1.913	ND	2.026	ND	0.795	4.826	0.308	2.849	0.140	0.628	1.017	1.432	5.979	1.695	1.514	1.192	ND	ND	ND
EP44	81.929	3.219	ND	4.925	ND	1.178	3.919	0.948	3.033	0.804	1.131	0.232	3.138	4.128	2.263	1.333	1.387	ND	ND	ND
EP46	71.384	1.449	ND	1.886	ND	0.599	3.307	0.825	3.490	0.628	1.662	0.108	0.972	1.852	1.199	0.644	0.357	ND	ND	ND
EP47	53.116	1.178	ND	1.072	ND	0.534	3.055	0.411	1.906	0.313	1.174	0.528	2.692	4.165	2.343	1.362	1.182	ND	ND	ND
EP48	85.804	4.922	ND	3.892	ND	1.462	8.204	0.731	6.184	0.427	1.223	0.271	2.875	4.283	2.632	1.539	1.680	ND	ND	ND
EP49	78.819	4.372	ND	1.804	ND	0.894	6.589	0.499	3.508	0.312	1.117	0.979	2.615	4.377	2.483	2.198	1.075	ND	ND	ND
EP50	65.300	1.407	ND	3.088	ND	0.264	1.871	0.356	2.375	0.342	1.808	0.923	1.565	5.638	2.471	1.638	2.013	ND	ND	ND

*relative concentration was calculated based on peak area with molecular weight adjustment

Table 6-2. Percentage inhibition of *Echinacea angustifolia* extract on FAAH activity and relative concentration of marker compound in each extract.

Sample code	FAAH % Inhibition	Relative concentration of marker compound in plant extract (Peak area with molecular weight adjustment)																		
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
EA 01	56.164	ND	0.155	1.386	0.504	0.307	0.218	ND	ND	ND	ND	0.121	0.311	0.481	0.439	ND	8.776	0.168	0.407	0.694
EA 06	58.734	ND	0.371	1.356	0.435	0.266	0.420	ND	ND	ND	ND	0.047	0.132	0.298	0.238	ND	2.064	0.022	0.190	0.281
EA 07	37.939	ND	0.257	0.411	0.389	0.199	0.163	ND	ND	ND	ND	0.299	0.868	0.255	0.142	ND	13.190	0.221	0.333	0.929
EA 08	55.141	ND	1.418	0.492	0.514	0.296	0.705	ND	ND	ND	ND	0.108	0.241	0.449	0.698	ND	3.408	0.078	0.372	0.535
EA 09	54.715	ND	0.280	1.496	0.508	0.452	0.727	ND	ND	ND	ND	0.216	0.475	0.897	0.482	ND	10.044	0.127	0.655	0.554
EA 10	47.678	ND	0.152	0.460	0.249	0.168	0.092	ND	ND	ND	ND	0.031	0.263	0.283	0.088	ND	4.148	0.080	0.035	0.037
EA 11	58.264	ND	0.733	2.437	0.500	0.253	0.524	ND	ND	ND	ND	0.054	0.072	0.121	0.116	ND	5.613	0.376	0.182	0.213
EA 12	56.886	ND	1.125	1.722	0.517	0.376	1.054	ND	ND	ND	ND	0.058	0.170	0.294	0.173	ND	12.867	0.345	0.359	0.638
EA 13	49.627	ND	1.649	1.645	0.288	0.291	1.239	ND	ND	ND	ND	0.275	3.696	0.847	0.462	ND	14.893	0.473	0.120	0.110
EA 14	59.539	ND	0.306	0.822	1.285	0.649	0.404	ND	ND	ND	ND	0.124	0.444	0.661	0.442	ND	11.964	0.288	0.298	0.786
EA 15	47.719	ND	0.840	1.464	0.882	0.392	0.984	ND	ND	ND	ND	0.178	0.237	0.459	0.284	ND	2.460	0.067	0.095	0.082
EA 16	53.870	ND	0.048	0.688	0.423	0.134	0.112	ND	ND	ND	ND	0.333	0.333	0.537	0.417	ND	8.308	0.064	0.580	1.271
EA 17	56.219	ND	0.158	1.047	0.453	0.211	0.214	ND	ND	ND	ND	0.132	0.334	0.540	0.312	ND	10.756	0.131	0.262	0.900
EA 19	56.633	ND	0.127	0.560	0.510	0.360	0.370	ND	ND	ND	ND	0.290	2.068	1.560	0.893	ND	5.754	0.057	0.371	0.605
EA 20	47.982	ND	0.155	1.627	0.367	0.349	0.149	ND	ND	ND	ND	0.221	0.457	0.842	0.332	ND	8.232	0.137	0.211	0.355
EA 21	47.319	ND	0.154	0.664	0.226	0.236	0.283	ND	ND	ND	ND	0.108	0.264	0.448	0.392	ND	7.486	0.100	0.597	0.593
EA 22	51.907	ND	0.474	0.642	0.204	0.146	0.647	ND	ND	ND	ND	0.129	0.686	0.381	0.221	ND	3.414	0.087	0.167	0.344
EA 23	79.519	ND	0.449	2.335	0.818	0.251	0.350	ND	ND	ND	ND	0.079	0.175	0.542	0.279	ND	17.841	0.149	0.315	1.037
EA 24	69.596	ND	0.650	2.540	0.859	0.346	0.778	ND	ND	ND	ND	0.104	0.278	0.802	0.563	ND	7.115	0.144	0.290	0.725
EA 25	53.750	ND	0.346	1.690	1.067	0.318	0.617	ND	ND	ND	ND	0.089	0.102	0.463	0.284	ND	5.645	0.088	0.274	0.525
EA 27	41.316	ND	0.212	1.014	0.523	0.426	0.288	ND	ND	ND	ND	0.123	0.313	0.467	0.257	ND	5.707	0.165	0.408	0.664
EA 28	62.300	ND	0.192	1.394	0.920	0.482	0.498	ND	ND	ND	ND	0.287	0.408	0.767	0.426	ND	6.808	0.209	0.320	0.765
EA 29	71.310	ND	0.102	0.881	0.671	0.507	0.141	ND	ND	ND	ND	0.087	0.141	0.423	0.462	ND	13.127	0.263	0.389	1.071
EA 30	53.738	ND	0.276	0.069	0.333	0.193	0.354	ND	ND	ND	ND	0.099	0.221	0.259	0.336	ND	7.122	0.126	0.198	0.581
EA 31	63.896	ND	2.880	1.674	0.448	0.319	2.833	ND	ND	ND	ND	0.023	0.119	0.054	0.023	ND	1.895	0.083	0.027	0.056
EA 32	47.339	ND	0.168	0.652	0.514	0.194	0.147	ND	ND	ND	ND	0.072	0.117	0.105	0.169	ND	1.407	0.048	0.271	0.294
EA 33	55.695	ND	0.416	0.568	0.400	0.256	0.483	ND	ND	ND	ND	0.092	0.118	0.377	0.212	ND	7.513	0.124	0.196	0.581
EA 34	62.247	ND	0.471	1.469	0.631	0.271	0.333	ND	ND	ND	ND	0.091	0.289	0.336	0.587	ND	4.126	0.054	0.402	0.585
EA 35	52.105	ND	0.657	1.467	0.263	0.224	0.363	ND	ND	ND	ND	0.470	1.775	0.805	0.675	ND	13.490	0.209	0.501	1.630
EA 37	33.904	ND	0.159	0.407	0.310	0.203	0.109	ND	ND	ND	ND	0.077	0.779	0.068	0.317	ND	3.086	0.059	0.073	0.108
EA 38	68.008	ND	0.347	1.612	0.864	0.247	0.425	ND	ND	ND	ND	0.160	0.462	1.276	1.476	ND	7.659	0.159	0.949	1.814
EA 39	68.118	ND	0.917	1.151	1.242	0.494	0.794	ND	ND	ND	ND	0.081	0.229	0.690	0.356	ND	6.602	0.078	0.374	0.376
EA 40	57.872	ND	0.191	1.437	0.469	0.352	0.245	ND	ND	ND	ND	0.127	0.226	0.306	0.206	ND	6.853	0.170	0.336	0.870
EA 41	62.423	ND	0.175	0.630	0.176	0.162	0.304	ND	ND	ND	ND	0.120	0.295	0.521	0.561	ND	6.699	0.107	0.314	0.762
EA 44	67.634	ND	0.531	1.042	0.913	0.647	1.285	ND	ND	ND	ND	0.104	0.273	0.301	0.273	ND	6.452	0.196	0.426	1.313
EA 45	54.332	ND	1.110	0.538	0.417	0.279	0.330	ND	ND	ND	ND	0.356	0.857	0.205	0.219	ND	2.296	0.061	0.121	0.151
EA 47	34.803	ND	0.195	0.673	0.399	0.279	0.255	ND	ND	ND	ND	0.056	0.051	0.454	0.135	ND	7.466	0.124	0.228	0.535
EA 48	64.182	ND	0.232	0.586	0.909	0.402	0.350	ND	ND	ND	ND	0.032	0.124	0.398	0.308	ND	6.106	0.183	0.242	0.532
EA 49	60.004	ND	1.065	0.374	0.883	0.529	0.679	ND	ND	ND	ND	0.047	0.165	0.208	0.083	ND	4.236	0.127	0.129	0.299
EA 50	61.522	ND	0.103	1.240	0.470	0.268	0.149	ND	ND	ND	ND	0.029	0.052	0.122	0.070	ND	3.990	0.080	0.083	0.202

*relative concentration was calculated based on peak area with molecular weight adjustment

6.3.3 Biochemometric analysis

Simple linear regression of FAAH inhibition across all 40 genotypes of *E. angustifolia* relative to individual compound concentrations identified that FAAH inhibition was significantly predicted by echnicoside, cichoric acid and 2 unknown phenolic compounds (Table 6-3, compounds C-F). No individual AKAs were significantly predictive of activity. In *E. purpurea*, cichoric acid showed the strongest prediction of the FAAH inhibition. Caftaric acid and several other unidentified phenolic compounds were also significant independent variables. For AKAs: dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide and dodeca-2E,4Z-diene-8,10-diynioc acid 2-methylbutylamide showed strongest positive correlation (Table 6-4). The R2 values show that compounds C, D, E and F explain 11.7 to 25.7 percent of variation in inhibition in *E. angustifolia*; compound A, C, D, E, F, G, H, M, N, O and P explain 10.5 to 53.3 percent of variation in inhibition in *E. purpurea*.

To account for potential false-positive identifications resulting from inactive metabolites that co-vary in concentration with active metabolites, hierarchal clustering (Figure 6-2) provided information about which compounds distribute in similar patterns across genotypes. For *E. angustifolia*, compounds were merged into six clusters. This level of merging combines mostly the closest pairs; the clusters were defined as BF, DE, C, KL, MNRS and PQ. Similarly, for *E. purpurea*, compounds were merged into five clusters: E, CHIJ, AFG, MPO and KLN.

The simple linear regression with clustered compounds as independent variables (Table 6-4) showed that E, CHIJ, AFG and MPO in *E. purpurea* significantly ($p< 0.05$) predicted inhibition. In *E. Angustifolia*, significant ($p< 0.05$) independent variables were limited to DE and C.

Table 6-3. Simple linear regression of compound concentration on FAAH Inhibition by *E. angustifolia* and *E. purpurea* extracts. Significant variables are denoted with * (p<0.05), ** (p<0.01), *** (p<0.001).

		E. angustifolia			*E. purpurea*		
Code	Compound	Slope	R^2	*p*	Slope	R^2	*p*
A	Caftaric acid	NA	NA	NA	12.661	0.533	<0.001***
B	Chologenic acid	2.198	0.041	0.211	NA	NA	NA
C	Unidentified phenolics 1	5.267	0.137	0.019*	11.622	0.236	<0.001***
D	Echinacoside	9.945	0.257	0.001**	NA	NA	NA
E	Unidentified phenolics 2	8.529	0.117	0.031*	4.221	0.105	0.045
F	Cichoric acid	4.373	0.118	0.03*	11.279	0.454	<0.001
G	Unidentified phenolics 3	NA	NA	NA	11.707	0.310	<0.001***
H	Unidentified phenolics 4	NA	NA	NA	12.001	0.338	<0.001***
I	Unidentified phenolics 5	NA	NA	NA	5.808	0.080	0.077
J	Unidentified phenolics 6	NA	NA	NA	3.997	0.038	0.230
K	Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide	2.531	0.036	0.238	2.170	0.029	0.290
L	Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	2.198	0.044	0.194	5.758	0.082	0.073
M	Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	2.606	0.039	0.223	9.357	0.172	0.008**
N	Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide	2.057	0.026	0.317	7.015	0.110	0.036*
O	Dodeca-2E,4Z-diene-8,10-diynioc acid 2-methylbutylamide	NA	NA	NA	9.227	0.201	0.004**
P	Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	2.874	0.032	0.269	7.118	0.172	0.008**
Q	Unidentified AKAs 1	2.075	0.018	0.409	NA	NA	NA
R	Unidentified AKAs 2	2.702	0.042	0.202	NA	NA	NA
S	Unidentified AKAs 3	3.347	0.096	0.051	NA	NA	NA

Table 6-4. Simple linear regression of the sum concentration of compound clusters relative to FAAH inhibition by *E. angustifolia* and *E. purpurea* extracts. Significant variables are denoted with * (p<0.05), ** (p<0.01), *** (p<0.001)

E. angustifolia				*E. purpurea*			
Compound code	Slope	R-squared	P-value	Compound code	Slope	R-squared	P-value
B, F	1.069	0.046	0.185	E	4.882	0.178	0.007**
D, E	2.422	0.216	0.003**	C, H, I, J	1.348	0.172	0.008**
C	4.196	0.188	0.005**	A, F, G	2.495	0.349	<0.001***
K, L	-1.227	0.052	0.157	M, O, P	1.617	0.146	0.015**
M, N, R, S	0.822	0.084	0.069	K, L, N	0.624	0.021	0.367
P, Q	0.833	0.024	0.336				

A: Caftaric acid; B: Chologenic acid; C: Unidentified phenolics 1; D: Echinacoside; E: Unidentified phenolics 2; F: Cichoric acid; G-JUnidentified phenolics 4-6; K: Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide; L: Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide; M: Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide; N: Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide; O: Dodeca-2E,4Z-diene-8,10-diynioc acid 2-methylbutylamide: P: Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide + Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide; Q-S: Unidentified AKAs 1-3.

Given that both CADs and AKAs appeared to contribute to FAAH inhibition in one or both species, individual compounds were evaluated for activity. Six of the major AKAs showed inhibitory effects on FAAH activity (Figure 6-3); whereas most elicited weak to moderate inhibition, dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide which showed strong inhibitory effect. Interestingly, its stereoisomer 2E,4E,8Z,10Z-tetraenoic acid isobutylamide only showed weak activity. In contrast, all major CADs were also found to inhibit FAAH activity. The following IC_{50} test determined cichoric acid exhibited the strongest FAAH inhibition potency among all identified Echinacea components followed by 2E,4E,8Z,10E-tetraenoic acid isobutylamide, echinacoside, caffeic acid, chlorogenic acid and caftaric acid (Table 6-5).

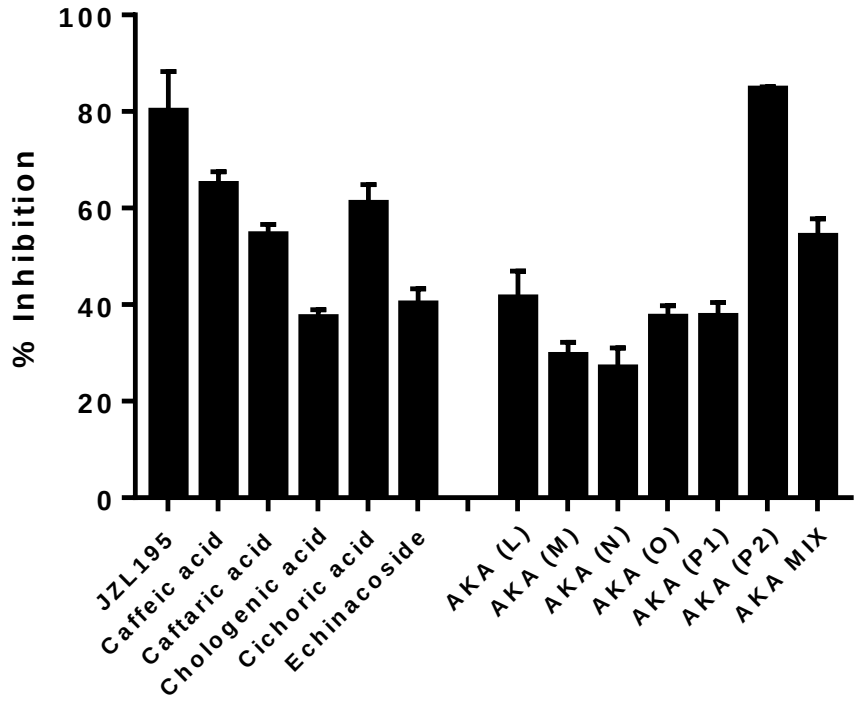


Figure 6-3. Inhibitory effects of major caffeic acid derivatives and alkylamides (AKA) from Echinacea on human recombinant FAAH enzyme activity. Percent inhibition was determined at 25 µg/ml for all compounds, calculated relative to vehicle control and expressed as mean +/- SEM (n=3).

6.4 Discussion

An important result is that root extracts of both *Echinacea* spp inhibited FAAH activity and that both CADs and AKAs appear to contribute to activity (Figure 6-3). Several studies have investigated the FAAH inhibiting potential of Echinacea extracts and AKAs with inconsistent results. Whereas AKAs were expected to interact with FAAH due to their structural similarity to the endogenous substrate anandamide, Woelkart et al (2005) reported no FAAH inhibitory effects for K, M, O and P at a concentration of 25 nM in *in vitro* assay with rat brain tissue as enzyme source. Chicca et al (2006) reported P as a partial FAAH inhibitor but failed to establish a significant concentration-response in an *in vitro* assay with pig brain tissue as enzyme source. In our assay, a purified cloned Human enzyme was used, which provides a more selective and sensitive system specific to human use, albeit more simplified relative to *in vivo*. In this paradigm, all tested AKAs tested showed some activity and, of particular interest, dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide exhibited much stronger FAAH inhibition than its stereoisomer dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide. It is well known that stereoisomers may bind to receptors or enzymes with different affinities. To date, due to the difficulty in separating these two AKAs, their bioactivity is most often evaluated as a racemic mixture. Our study demonstrates that AKA stereoisomerism can impact interactions with FAAH and suggests that a similar phenomenon may also apply to cannabinoid receptor interactions.

Micellization of AKAs in aqueous environments is believed to be another reason for the contradictory results of other authors (Raduner et al., 2007) as AKAs will aggregate and form micelles with increasing concentration. However, since aggregated AKAs are not likely to interact with FAAH, only the monomer concentration is associated with the FAAH inhibitory

Table 6-5. IC_{50} values of major Echinacea components toward FAAH enzyme activity (Mean ± SEM, n=3).

	IC_{50} (μM)
Caffeic acid	116 ± 8
Caftaric acid	143 ± 5
Chlorogenic acid	130 ± 8
Cichoric acid	45 ± 4
Echinacoside	106 ± 6
Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	54 ± 2

effect. Thus, the FAAH inhibitory effect can only be measured below the critical micellization concentration.

The FAAH inhibiting potential of Echinacea CADs is reported here for the first time. Kamfaerol is the only other reported plant phenolic with reported FAAH inhibition activity (Russo, 2016). However, this result is not unexpected because Echinacea CADs are known to inhibiting a range of enzymes including α-amylase, α-glucosidase, angiotensin-converting enzyme and several Cytochrome P450 enzymes (1A2, 2C9 and 3A4) (Oboh et al., 2015; Chiou et al., 2017; Rastogi and Jana 2014). Thus, broad-spectrum activities on other enzymes are not surprising. Notably, due to the catechol moiety, CADs are structurally related to molecules identified as pan assay interference compounds (Baell, 2016). Therefore, although CADs showed higher inhibition on FAAH than AKAs in an *in vitro* system, the *in vivo* effect of these components may differ and also depends on bio-availability. The reported pharmacokinetics studies show that AKAs are highly bio-available (60%) and reach blood circulation after oral administration in a very short time (Woelkart et al., 2005B). On the other hand, the bio-availability of CADs in blood is much lower (1-4%) and they are also less prone to cross the blood-brain barrier. Therefore, AKAs may be more important *in vivo*, particularly with respect to CNS effects. Although caffeic acid was not directly detected in the plant extracts, it is reported that it can be formed through gut-microflora mediated metabolism of other CADs (Bel-Rhlid et al., 2012). Absolute bioavailability of caffeic acid determined in rat is 14.7%, which is much better than other CADs (Wang et al, 2014). Hence, the FAAH inhibition potency of caffeic acid may be more critical in the context of human oral consumption.

The distribution and quantity of plant secondary metabolites is quite variable even in the same species. The commercial breeding material used here were all cultivated in a similar

environment in a plant nursery. Thus, the genotype may be the dominate factor for the phytochemistry variables. It is generally understood that no constituent is solely responsible for the bioactivities of Echinacea. Hence, the individual components with the greatest activity when administered alone may not contribute much to in the activity of complex crude extracts. Our analysis of 40 genotypes from each species with considerable variability in phenolic and AKA contents as well as associated FAAH inhibition represents a viable and valuable approach to identifying active substances within complex chemical mixtures. In contrast to traditional assay-guided fractionation, which is not only time and resource intensive but also limited in its capacity to deal with potential synergies, the executed chemometric approach leverages the natural variability of phytochemical production within the biosynthetically limited metabolome of a single species.

Linear regression was used to identify compounds that were the best independent variables explaining FAAH inhibition. However, our statistical analysis across extracts, though successful in identifying previously unknown actives, did not mirror the test results from individual compounds. Although dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide is the strongest FAAH inhibitor among all AKAs and the dominant AKA in *E. angustifolia* extract, it was not determined to be a significant independent variable. This finding suggests that other mechanisms may be involved. Following single and multiple regression of clusters, compounds C in *E. angustifolia* extract and compound E in *E. purpurea* extract are significant independent variables with the highest slope values. These compounds are not among commonly reported CADs in Echinacea so further studies are needed to identify their structures and potential role in Echinacea pharmacology

The inhibition of FAAH by Echinacea extracts and compounds suggests that Echinacea-derived products may enhance endocannabinoid ligand levels *in vivo* with possible therapeutic effects on inflammation, anxiety and peripheral pain. Further animal and clinical studies are needed. In particular, animal models such as anxiety (EPM, for CNS effect) or pain (Collagen/Adjuvant -Induced Arthritis model, for peripheral effect) where the ECS play a role should be studied. Co-administration of CB receptor antagonist with Echinacea extracts or pure compounds such as cichoric acid or AKAs in animal trials would provide more insight into the contributions of the ECS to therapeutic effects. As Echinacea extracts or pure compounds may act on the CB receptor and FAAH simultaneously, measuring the concentration of endocannabinoid ligands, especially anandamide, in serum as well as in brain tissue may help to determine if the indirect activation of CB receptors through FAAH inhibition is significant.

From an industry perspective, product development and quality control (QC) practices may benefit from our results, which reveal that specific phenolics and AKAs are both potentially acting on endocannabinoid system targets.

CHAPTER 7: HEPATIC METABOLISM OF ALKYLAMIDES FROM ECHINACEA AND EFFECTS ON *IN VITRO* PHARMACOLOGICAL ACTIVITY

7.1 Introduction

Echinacea spp. (Asteraceae), commonly known as Echinacea or purple coneflowers, were traditionally used to treat upper respiratory infections and wounds by the First Nations of North America (Shemluck, 1982). In modern use, Echinacea-containing products are widely used as natural health products to treat cold and flu symptoms as well as lung conditions, wound healing and candidiasis (Parsons et al., 2018). Based on *in vitro* or i*n vivo* studies, the pharmacological effects of Echinacea products include immunomodulatory (Bauer, 1999), antimicrobial (Binns et al., 2000), anti-inflammatory (Aarland et al., 2017) and anxiolytic activities (Haller et al., 2010 &2013).

The major phytochemicals identified in the most commonly used species, *E purpurea* L. (Moench)and *E. angustifolia* (DC.) Hell, include alkylamides (AKA), caffeic acid derivatives, as well as polysaccharides/glycoproteins and polyacetylenes (Binns et al., 2002). In general, the anti-inflammatory and anxiolytic activities of Echinacea extracts are believed to be triggered through the activation of the cannabinoid receptors (Hohmann et al., 2011 and 2009). Previous study has indicated that both phenolics and AKAs inhibit Fatty Acid Amide Hydrolase (FAAH) which metabolizes the endocannabinoid anandamide (Chapter 6). However, Caco-2 monolayer permeability studies suggested that AKAs have much better bio-availability than caffeic acid derivatives (Matthias et al., 2005A). Woelkart et al, (2005A) estimated the absolute bioavailability of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides was 60% in rats. These findings have also been confirmed in human clinical trials (Matthias et al., 2005). An oral dose of AKA was detected in human serum with a C_{max} of less than 30 min and a half-life of less than 3 hours (Matthias et al., 2005). These results suggest that Echinacea's AKAs may be more clinically significant than the caffeic acid derivatives.

Echinacea AKAs have been reported to be metabolized through the CYP450 system (Cech et al., 2006) but limited data are available beyond dodeca-2E,4E,8Z,10Z/E-tetraenoic acid isobutylamide (AKA9/10), the most abundant and well-studied AKA across *Echinacea* spp. Several metabolites of AKA 9/10 have been reported and the structures of these metabolites have been tentatively identified using LC-MS. How hepatic metabolism affects other AKAs remains unknown as does the bioactivity of the metabolites.

In most cases, with CYP450-mediated structural transformation of xenobiotic substrates the activity of the metabolites is usually decreased. However, certain substances may yield metabolites with unchanged or even higher activity. Since most Echinacea products are ingested orally and subject to first-pass metabolism, investigating the pharmacological activity of AKA metabolites should provide insight into the compounds and mechanisms that contribute to *in vivo* effects. For examples, piceatannol is the metabolite of resveratrol through human CYP1A2 (Kim et al., 2009), and it exhibited stronger anti-oxidant activity than its parent compound resveratrol (Ovesná et al., 2006). Thus, I hypothesized that, due to their highly lipophilic structure, the bioactivity of AKAs will increase with CYP-mediated transformation. Using an *in vitro* model of endocannabinoid metabolism, I predicted that the inhibitory effects of AKAs following hepatic microsomal metabolism will be greater than the activity of untreated AKAs. The specific objectives of this study were 1) to characterize the metabolites formed by Human liver enzymatic metabolism of AKAs isolated from *E. purpurea* and *E angustifolia* and 2) to investigate if hepatic metabolism affects the inhibitory effects of AKAs on FAAH.

7.2 Methods and materials

7.2.1 Microsomes, Chemicals and Reagents

Human liver microsome (UltraPool™ HLM 150 Mixed Gender Pooled Cytosol) was purchased from Corning life science (Tewksbury, USA). Fatty acid amide hydrolase and 7-amino-4-methyl CoumariArachidonamide (AMC-AA) was purchased from Cayman Chemicals (Ann Arbor, USA). Nicotinamide adenine dinucleotide phosphate (NADPH), potassium phosphate (mono/dibasic), EDTA and Tris were purchased from Sigma-Aldrich (Oakville, Canada).

Analytical standards of caffeic acid derivative were obtained from Sigma-Aldrich. All solvent used in HPLC and UPLC/MS analysis were optima LC/MS grade solvent purchased from Fisher scientific (Ottawa, Canada).

7.2.2 Echinacea Sample Preparation

Dried root samples were brushed gently to remove soil. The root sample was milled in a Wiley mill to fine powder, passing through a 1mm mesh. One gram of powdered root sample extracted three times with 15 ml fresh 70% ethanol by ultrasound for 10 min. The sample was centrifuged, and the supernatant was collected, combined, and dried under vacuum.

7.2.3 Isolation of pure AKA by using preparative HPLC

Ten gram of dried *E. purpurea* root extract was sonicated with 1L 99% ethanol for 10 min, followed by vacuum filtration. The supernatant was dried under vacuum and the dried residual was re-solubilized in DMSO at 200mg/ml.

The isolation of AKA was performed on an Agilent 1260 Infinity Preparative-scale Purification System with an automate fraction collector. A Phenomenex Gemini C18 column (10 µm practice size, 250 x 21.2 mm) was used for the separation of AKA with an isocratic mobile phase set at 47.5% acetonitrile in water. The flow rate was 42 ml/min. The DAD was set at 268 nm and a peak-based collection program was executed by automated fraction collector. Fractions were pooled and dried under vacuum. HPLC-DAD was used to check the purity of isolated compound. In brief, peak of the target compound was first check by "purity check" function of Agilent Chemstation (B3.02) to investigate if the target peak represents a single compound. The % purity was then estimated by comparing the area of the target peak and total peak areas of the impurities.

7.2.4 Human Liver Microsome Metabolism Assay:

Samples (5 µl of 0.5mg/ml purified AKAs or 20mg/ml of plant extracts) were incubated with 40 µg/ml of HLM and 2 mM NADPH in a 0.2 M PBS buffer at 37 ºC for 180 min in a shaking incubator set at 250 rpm. The total volume of reaction system was 300 µl. Two additional control groups were included with NADPH and HLM respectively replaced by PBS in the reaction matrix.

After incubation, 1 mL of ice-cold methanol was added to terminate the reaction. Protein content were neutralized and precipitated by 30 s vortex and 10 min centrifugation at 13400 RCF. The supernatant was collected. Methanol was evaporated using a centrifugal evaporator. Samples were reconstituted in 200 uL of methanol then sonicated for 5 minutes twice. The supernatant was collected and filtered through a 0.22 µm PTFE membrane prior to injecting 2 µL into the HPLC system.

7.2.5 FAAH Inhibition Assay:

FAAH inhibition assay were previous described in Chapter 4. MeOH was used as vehicle control.

7.2.6 HPLC Analysis and Quantification

An Agilent HPLC system (model 1100) and a Phenomenex lunaclumn (C18 (2) previously described in Chapter 6 was used for phytochemistry analysis.

The injection volume was 2 µL, and the column oven temperature was set a 45°C. Flow rate was set at 0.4 mL/ minute. Mobile phase A consisted of 0.05% Trifluoroacetic acid in water, and mobile phase B consisted of 0.05% trifluoroacetic acid in acetonitrile. The gradient elution procedure was initiated with 10% B in 1 minute, then increased to 10-100% B in 19 minutes. 100% B was held for 5 minutes. We went back to initial conditions, and re-equilibrated for 5 minutes. A detection wavelength of 268nm was used for AKAs, and 330nm for phenolic compounds. Slit width was 4nm, with spectral data stored at 190-400nm.

7.2.7 UPLC/MS Analysis of AKA Metabolites:

The reaction matrix was analyzed on a Shimadzu UPLC-MS system and an Acquity CSH C18 column previously described in Chapter 5 was used for analysis of the AKA metabolites.

The flow rate was set at 0.6 ml/min with the column temperate at 50˚C. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The gradient elution method was initialed with 10% B, then increased to 100% B in 10 min. The column was then washed with 100% B for 5 min and change back to the initial condition in 1 min.

The mass spectrometer with electrospray ionization (ESI) interface was operated in positive and negative scan mode, the nebulizing gas flow was set at 1.5 L/min and drying gas flow was at 10 L/min. The desolvation line temperature and heat block temperature was set at 300°C and 450°C respectively. The m/z range of both positive and negative scan is from 100 to 500 with 938 u/sec scan speed.

7.2.8 Statistical Analysis:

A one-way anova followed by Sidak's multiple comparisons test was used to compare on the FAAH inhibition of each sample (AKA2, AKA3, AKA9, AKA10, EA and EP) incubated with or without NADPH. GraphPad (version 7.0) was used to perform the statistical analysis.

7.3 Results

7.3.1 Isolation of AKA by preparative HPLC

A total of 10 fractions were collected from the preparative HPLC. Each fraction was further analyzed by LC/MS. Tentative identification of the compounds was performed by comparing the mass to charge ratio (m/z) and relative retention time (Rt) to literature values (Table 7.1). Several fractions (F2: AKA 2, F3:AKA3, F6: AKA6; F8: AKA8, F9: AKA9 and F10: AKA10) contained one pure substance (>95% based on UV) in sufficient quantity for further work. AKA2, AKA3, AKA6, AKA8, AKA9 AKA10 were identified as Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide, Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide, Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide, Dodeca-2E,4Z-diene-8,10-diynioc acid 2-methylbutylamide, Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide and Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide respectively (Figure 7-1).

7.3.2 Identification of AKA metabolites after HLM treatment

After the metabolism of the pure AKA compounds, EA root extract, and EP root extract by the human liver microsome (HLM), HPLC-DAD analysis was completed, and chromatograms obtained (Figure 7-2). Several new metabolites were observed following metabolism by the human liver microsome preparation. In Figure 7-2, panels A, B, C and D show the chromatograms of AKA2, AKA3, AKA9 and AKA10 metabolism, respectively. It was found that all of the pure AKAs were metabolized by the human liver microsomes (>90%) except AKA 2 (<50%).

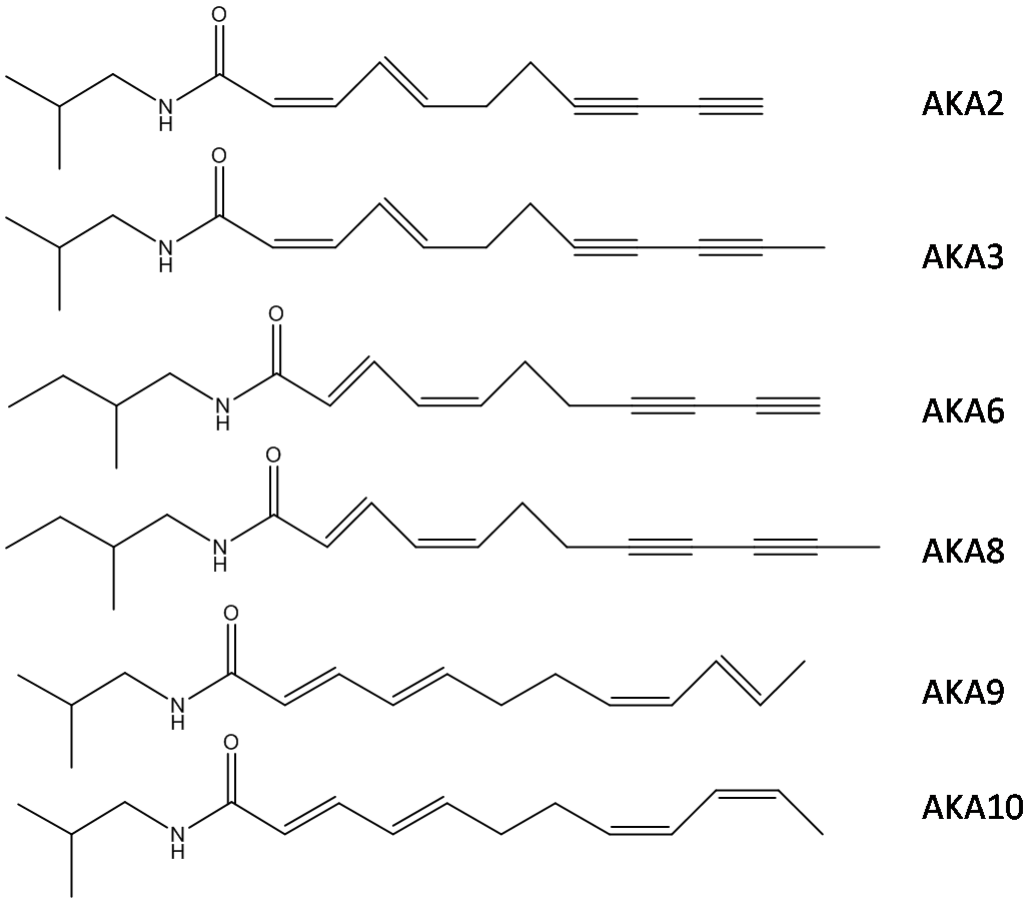


Figure 7-1. Structure of Echinacea alkylamides.

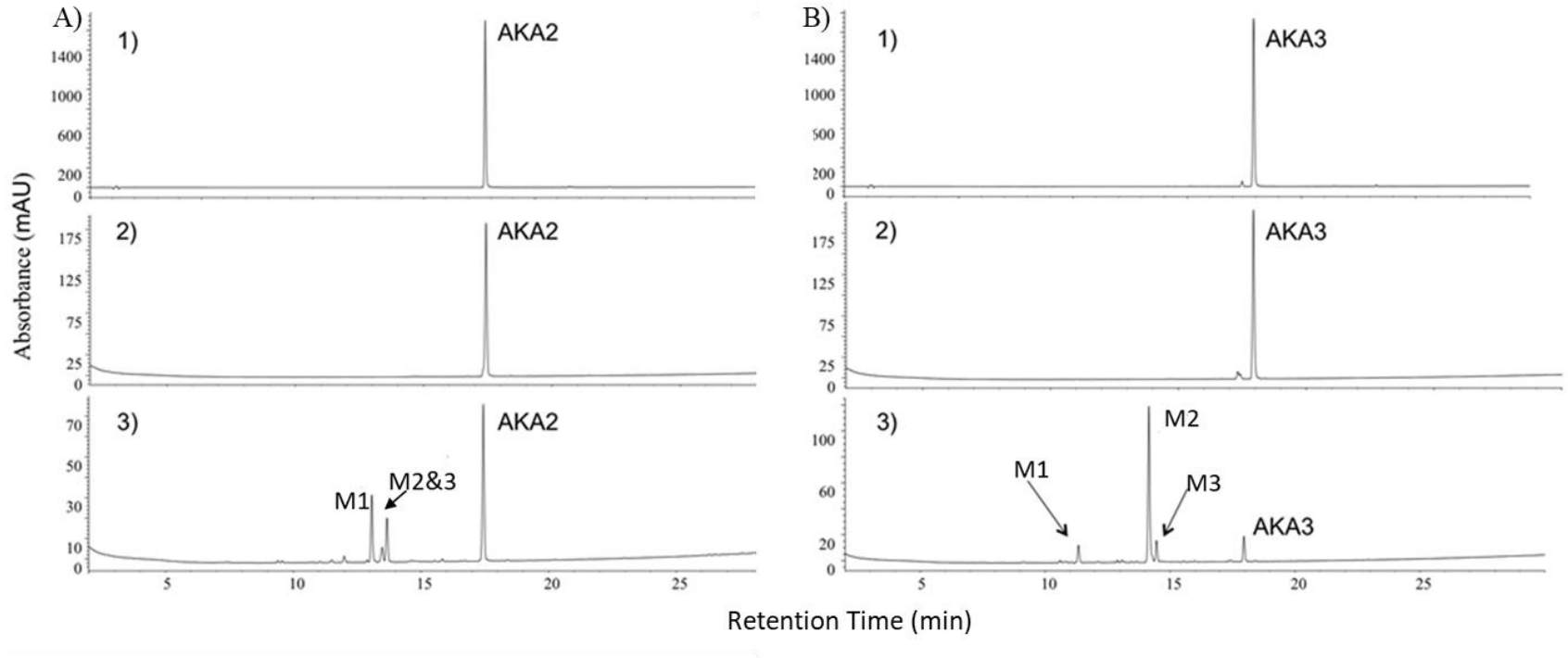
AKA2: Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide; AKA3: Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide; AKAK6: Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide; AKA8: Dodeca-2E,4Z-diene-8,10-diynioc acid 2-methylbutylamide: AKA9: Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide and AKA10: Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide.

Figure 7-3 also shows that the metabolism of the whole Echinacea extracts by liver microsomes produced metabolites. The metabolism of the Echinacea extracts was reduced compared to the metabolism of the pure AKA compounds (Figure 7-2 & 7-3). The major metabolite peaks in extracts corresponded to the metabolites detected in HLM metabolism of single AKAs, but there were also other minor metabolism/ degradation products found in the Echinacea extracts after incubation that were not seen in the metabolite profiles of pure compounds. Parallel to the formation of metabolites in most assays, the levels of parent AKAs were reduced. An exception was AKA2, for which peak area increased by 5-fold in *E angustifolia* and 2-fold in *E. purpurea* extracts.

LC/MS chromatograms were also obtained (data not shown) for pure AKAs incubated in human liver microsome reaction matrices. The chromatograms showed the metabolites formed, and the mass spectrum was used to predict the tentative metabolite structures, based on previous research on metabolism of structural similar compounds such as Echinacea AKAs (Toselli et al., 2010) and arachidonic acid (Needleman et al., 1986). Hepatic metabolism of test AKAs were expected to follow a similar pathway (Figure 7-4). Table 7.1 shows the predicted metabolites of 4 major Echinacea AKAs after HLM metabolism together with characteristic *m/z* signals. Their calculated log P values are also given (Table 7-1) and show that the lipophilicity has been lowered.

7.3.3 FAAH inhibitory of incubation matrix containing AKA metabolites

The results of the fatty acid amide hydrolase (FAAH) inhibition assay are shown in Figure 7-5. An increase in FAAH inhibition by individual compounds and extracts after HLM treatment was observed in all test groups: (compound/extract + HLM + NADPH) was compared to the


A)
1)
AKA2
2)
AKA2
3)
AKA2
M1
M2&3
B)
1)
AKA3
2)
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3)
M2
M1
M3
AKA3
Absorbance (mAU)
Retention Time (min)

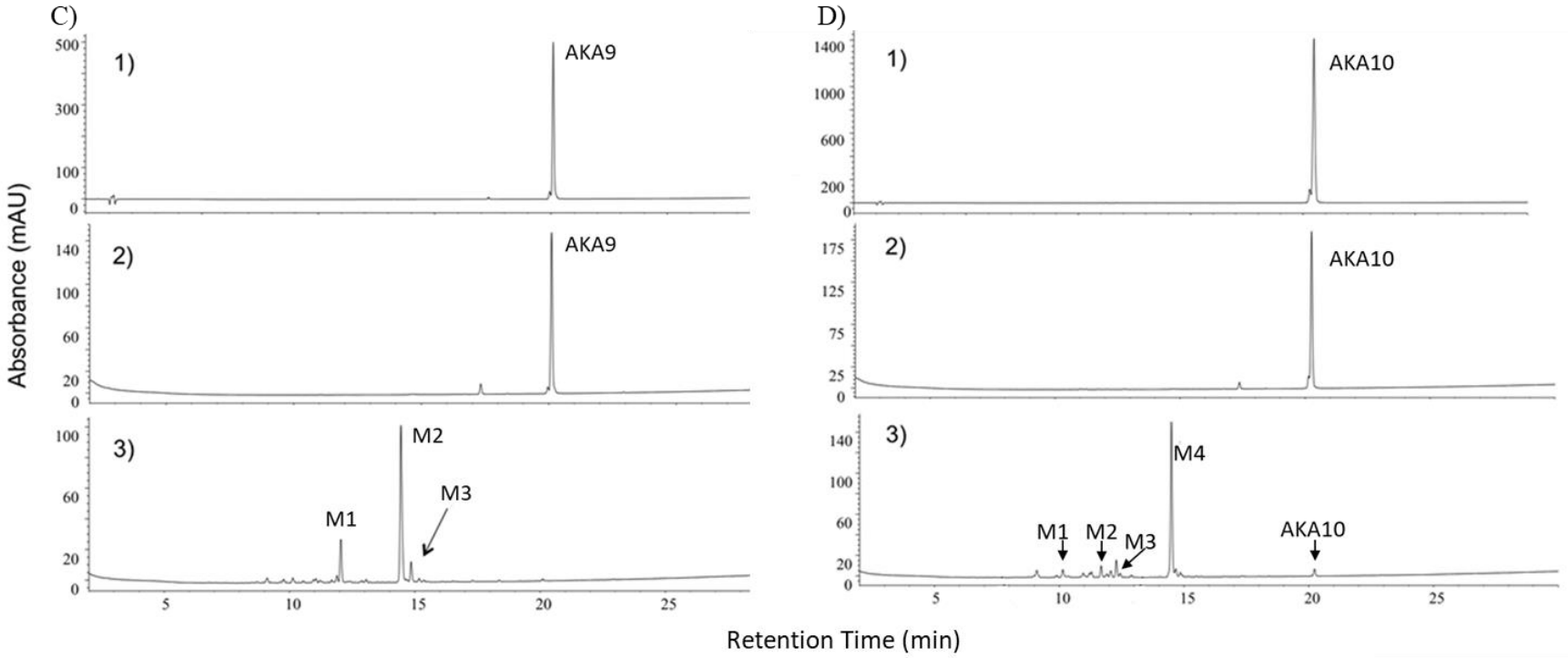

Figure 7-2. HPLC-DAD chromatogram of pure alkylamides AKA2 (A), AKA3 (B), AKA9 (C) and AKA10 (D) before and after metabolism by human liver microsome (HLM) assay. DAD was set at 268 nm. 1) Pure AKA in methanol. 2) Incubation matrix consisted of AKA, HLM but no NADPH. 3) Incubation matrix consisted of AKA, HLM and NADPH. M1-3: metabolite 1-3.

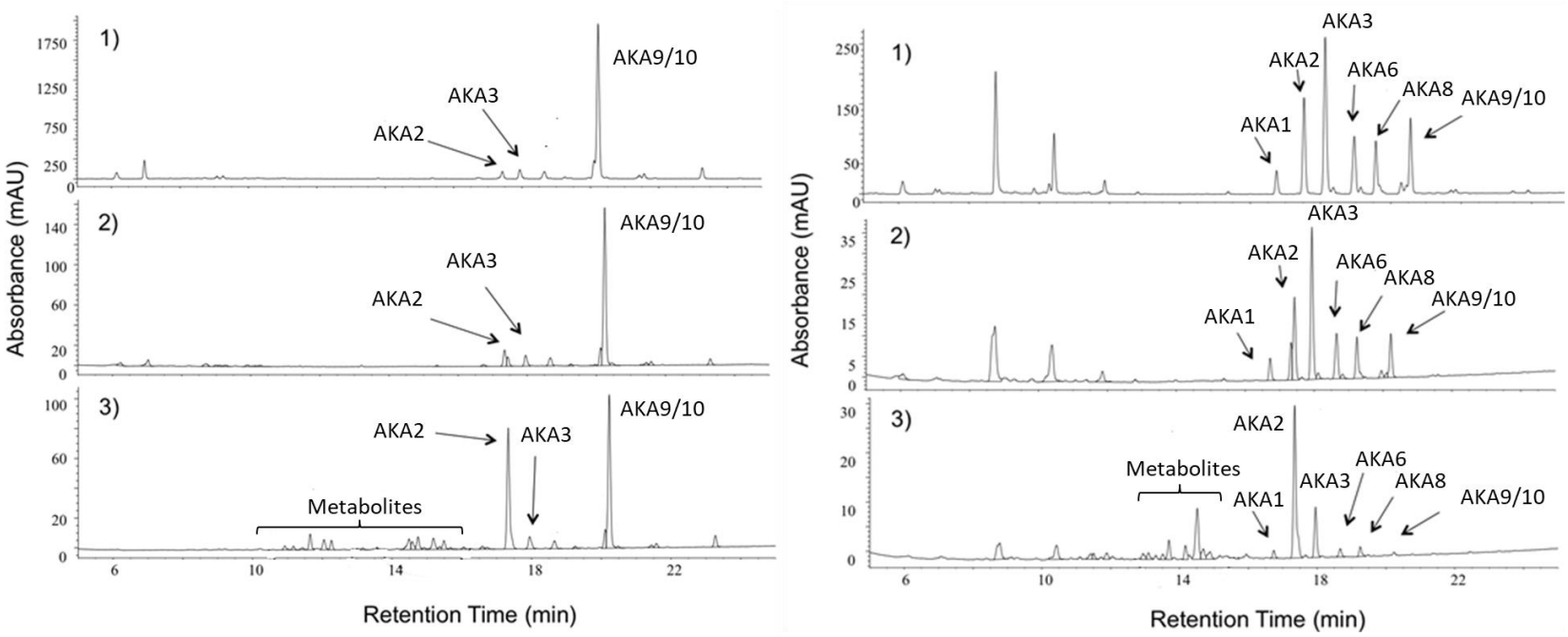

Figure 7-3. HPLC-DAD chromatogram of *E. angustifolia* (left) and *E. purpurea* extract (right) before and after metabolism by human liver microsome (HLM) assay. DAD was set at 268 nm. 1). *Echinacea* spp. extract in methanol. 2). Incubation matrix consisted of extract, HLM but no NADPH. 3). Incubation matrix consisted of extract, HLM and NADPH.

control group (compound/extract + HLM- NADPH). This increase in FAAH inhibitory activity was significant with both plant extracts ($p<0.05$) and all pure compounds ($p<0.01$).

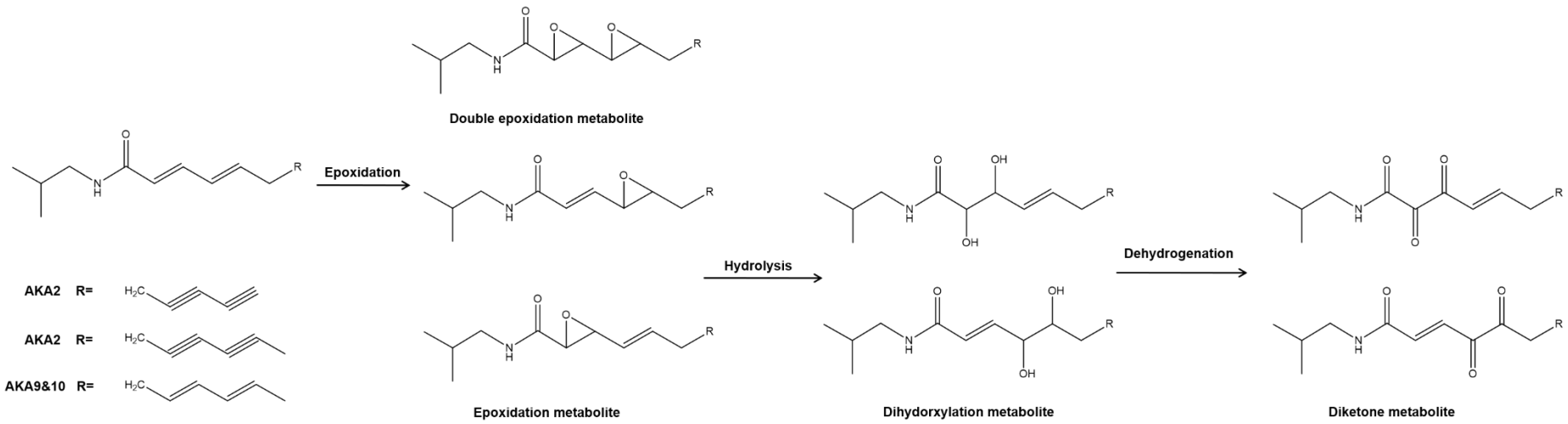

Figure 7-4. Predicted pathway of Echinacea alkylamides metabolism by human Cytochrome P450 enzymes. Structure of the metabolites was predicted based on the mass to charge ratio.

7.4 Discussion

The hypothesis was that liver microsome metabolism of Echinacea compounds and extracts would lead to more polar metabolites and greater FAAH inhibition activity. Decrease in log P (table 7.1) and increase in FAAH inhibition (Figure 7-5) were observed as predicted.

The first objective of this study – to observe and predict the structures of the metabolites formed by human liver enzymatic oxidation of AKAs isolated from *E. purpurea* and *E angustifolia* – was achieved successfully. Metabolites were observed after incubation of all AKAs and Echinacea root extracts with HLM. This indicated that not only the previously reported metabolism of AKA9/10 (Matthias et al., 2005) was repeatable, but also other Echinacea AKAs were metabolized by similar or related hepatic enzymes. None of the metabolites were formed in the absence of NADPH, strongly suggesting that the AKAs and extracts were metabolized by hepatic CYP450 isozymes (Matthias et al., 2005). However, the N-dealkylation metabolite previously reported (Toselli et al., 2010) was not observed in this study. These observations may suggest that the N-dealkylation (by CYP1A1, 1A2 and 1B1) may not be the favorable pathway when other CYPs or FMOs are present.

The metabolite formation in the plant extract assays were lower than with the pure AKAs, suggesting that the presence of other compounds (such as caffeic acid derivatives) may affect the hepatic metabolism of AKAs. Pure constituents and extracts from Echinacea have been reported previously as inhibitors of CYP450 enzymes (Budzinski et al., 2000; Freeman and Spelman, 2008). In particular, caffeic acid has been identified as a potent competitive inhibitor of CYP1A2, 2C9, 2D6 and a weak inhibitor of CYP2C19, CYP3A4 (Rastogi and Jana, 2014). In addition, Matthias and colleagues (2005) also reported that one of the minor AKAs, undeca-2E-

Table 7.1 Mass spectrometric properties and tentative structures of metabolites of 4 major Echinacea alkylamides (AKA).

Alkylamides	Metabolite	M/Z	Predicted Metabolites	LogP*
AKA2	Parent	230.2 $(M+H)^+$	N/A	2.84
	M1	264.2 $(M+H)^+$	Dihydroxylation metabolite	1.25
	M2	246.4 $(M+H)^+$	Epioxidation metabolite 1	1.75
	M3	246.4 $(M+H)^+$	Epioxidation metabolite 2	1.89
AKA3	Parent	244.2 $(M+H)^+$	N/A	3.44
	M1	276.2 $(M+H)^+$	Double epioxidation metabolite	1.39
	M2	260.2 $(M+H)^+$	Epioxidation metabolite 1	2.34
	M3	260.2 $(M+H)^+$	Epioxidation metabolite 2	2.48
AKA9	Parent	248.2 $(M+H)^+$	N/A	3.93
	M1	278.2 $(M+H)^+$	Diketone metabolite 1	1.79
	M2	278.2 $(M+H)^+$	Diketone metabolite 2	2.03
	M3	282.2 $(M+H)^+$	Dihydroxylation metabolite	2.14
AKA10	Parent	248.2 $(M+H)^+$	N/A	3.93
	M1	282.2 $(M+H)^+$	Dihydroxylation metabolite 1	2.14
	M2	282.2 $(M+H)^+$	Dihydroxylation metabolite 2	2.21
	M3	282.2 $(M+H)^+$	Dihydroxylation metabolite 3	2.34
	M4	278.2 $(M+H)^+$	Diketone metabolite	2.67

*****Predicted log P was calculated by Chemdraw 12.0 (CambridgeSoft)

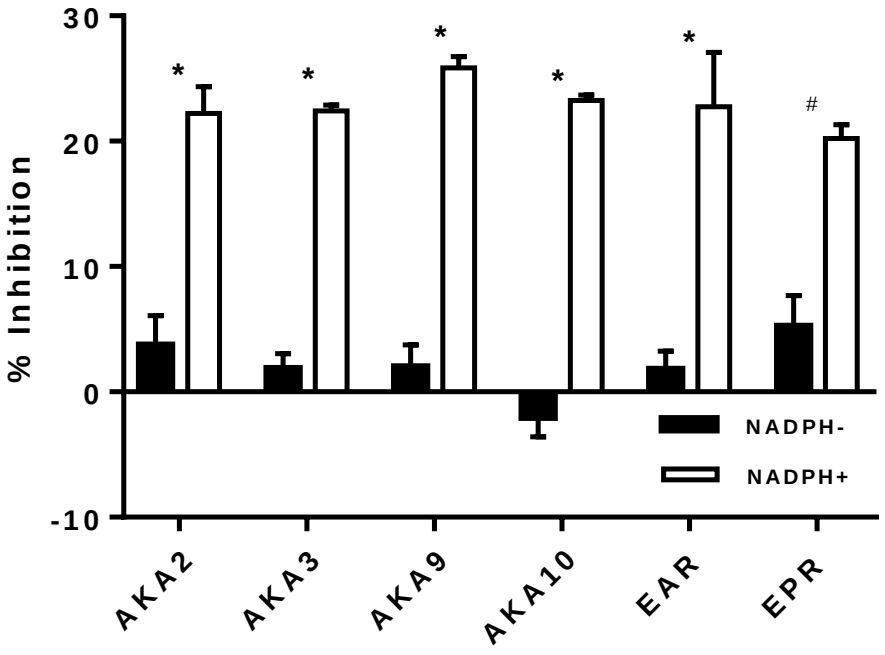

Figure 7-5. FAAH inhibition by different alkylamides (1. 25µg/ml) and Echinacea extracts (50µg/ml) in comparison to inhibition by metabolites following treatment with human liver microsome preparation. EAR: *E. angustifolia* root extract, EPR: *E. purpurea* root extract. A An one way anova followed by Sidak's multiple comparisons test was used to compare each control (NADPH–) and test (NADPH+) sample. *: denotes significant difference between test and control group ($p<0.001$) and #: $p<0.01$.

ene-8,10-diynoic acid isobutyl amide, inhibits the metabolism of AKA9. Hence, the phytochemical complexity in Echinacea may alter the hepatic metabolism of AKAs.

The second objective was to investigate if the hepatic metabolism affects the inhibitory potency of AKAs against FAAH. In general, metabolites of the pharmacologically active parent substances are often found to be less active, but – as is the case for prodrugs (Han and Amidon, 2000) – they can also be more active. A previous study conducted by Cech et al. (2006) indicated that hepatic metabolites of AKA9+10 showed less immunomodulatory effects than the parent compound. However, my data indicates that hepatic metabolites of all 4 individual AKAs tested were more active than the parent compound in the FAAH assay. The FAAH inhibitory activity was significantly increased after the hepatic metabolism. This study is the first to show the potential prodrug mechanism of Echinacea AKAs, and the potential ability of AKA metabolites to act on the endocannabinoid system. Both anxiolytic and immunomodulatory properties of Echinacea extract are thought to be mediated through the activation of CB receptors, directly as agonists or indirectly as FAAH inhibitors, and the metabolism of AKAs may enhance these effects.

The tentative identification of AKA metabolites by LC/MS suggests that the major metabolites are classic oxidation reduction products of the parent compounds, such as epoxidation and dihydroxylation products. Normally these are formed by cytochrome P450s and epoxide hydrolases in microsomes. However, other NADPH dependent enzyme such as flavin-containing monooxygenases might also play a role in the oxidation of AKA, but this was considered unlikely since these enzymes usually favour nucleophilic heteroatom-containing chemicals containing substrates. Further work with selective inhibitors of these enzymes could clarify the potential role of specific hepatic enzymes.

Lipophilicity, usually defined by log P, is considered as an important factor affecting the enzyme binding affinity for potential substrates and inhibitors and is especially useful to predict the pharmacological activity of compound analogs (Pirovano et al., 2012). Whereas, the effect of lipophilicity on FAAH inhibition was not established in this study, Otrubova et al. (2011) concluded that lipophilicity may affect the potency of FAAH inhibition for certain classes of FAAH inhibitors such as trifluoromethyl ketones and oxazolopyridine α-ketoheterocycles with a lipophilic tail. The correlation between the tail's alkyl chain length and FAAH inhibition was parabolic. Further study is needed to determine if the lipophilicity of the AKA metabolites or the position of new moieties (e.g. hydroxyl groups) are important determinants of FAAH inhibition. However, reduced lipophilicity can lower the micellization of AKA metabolites compared to their parent compounds in aqueous environments. Hence, it is another possible explanation of the increased activity. Raduner et al., (2007) proposed that AKAs aggregate as the concentration increases and form micelles in aqueous solutions. AKAs polymers are not likely to interact with FAAH, only the unaggregated AKA monomer concentration is associated with the FAAH inhibitory effect. Following metabolism, the oxygen or hydroxyl group added by CYP450-mediated oxidation of the lipophilic end of the molecular reduces liophilicy. Hence, the metabolites of AKA are less likely to form micelles which will lead to an increase of the monomer concentration. As the concentration increase, even if the metabolite has reduced FAAH inhibitory potency, the overall results may still show an increase in FAAH inhibition in the *in vitro* assay. Furthermore, these metabolites are less likely to cross the blood brain barrier than the parent compound (Kelder et al., 1999). Thus, the hepatic metabolism of Echinacea AKA is more likely to enhance the peripheral pharmacological activity than in the central nervous system.

In conclusion, we suggest that the observed activation of AKAs by CYP mediated metabolism may play a significant role in the *in vivo* activity of Echinacea products. This might be verified in an animal model of anxiety or inflammation by use of a CYP inhibitor such as ketoconazole. If this is validated, it may be worthwhile to isolate and characterize the metabolites by spectroscopic methods and then test the purified compounds *in vitro* and *in vivo*.

CHAPTER 8: INTERACTIONS OF *E. ANGUSTIFOLIA* AND *E. PURPUREA* ACCESSIONS AND THEIR ACTIVE PRINCIPLES WITH THE ENDOCANNABINOID SYSTEM *IN VITRO* AND PERIPHERAL INFLAMMATORY PAIN *IN VIVO*.

8.1 Introduction

Classic research on Echinacea (mostly with *Echinacea purpurea* (L.) Moench and *E. angustifolia* DC (Asteraceae)) has focused on activities such as antimicrobial action, and immunomodulation in relation to traditional uses for colds and flu. (Catanzaro et al 2018). More recent research revealed a new mechanism of Echinacea bioactivity mediated by alkylamides (AKA) acting at the cannabinoid (CB) receptors (Woelkart et al 2005). In addition to binding CB receptors, Echinacea alkylamides (AKA) can modulate endocannabinoid system (ECS) activity through effects on endocannabinoid metabolism and transport (Chicca et al., 2009). Among other physiological and pathophysiological functions, the ECS plays a key role in the regulating acute pain states (Alkaitis et al., 2010) as well as nociceptive pathways in chronic pain (Guindon and Hohmann, 2009 & 2011; Rani Sagar et al., 2012), highlighting their role as endogenous analgesics.

The interaction between Echinacea AKAs and CB receptors appears to selectively activate CB2 receptors, which are abundantly expressed by immune cells, and has been identified as a key mechanism of Echinacea's anti-inflammatory activity (Raduner et al 2006; Chicca et al, 2009). Evidence of CB1 activation, however, is limited and complicated. Gertsch et al (2006) indicated that the binding selectivity of two of the most abundant AKAs, dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (AKA9) and dodeca-2E,4E dienoic acid isobutylamide for CB2 is 30~50-fold higher than for CB1. Hohmann et al (2011) reported that different Echinacea AKAs act as partial agonists, antagonists and reverse agonists of CB1 *in vitro*. How AKAs act in combination, as found in Echinacea extracts, was not evaluated. Interestingly, *in vivo* work has shown that orally administered AKAs at moderately low doses (4-8mg/kg) are effective in reducing anxiety in rats using the classic elevated plus maze paradigm but that activity varied

considerably between different Echinacea products (Haller et al. 2010 &2011). In human trials, oral administration of Echinacea extracts containing AKAs also reduced clinical anxiety scores (Haller et al., 2013). These results are believed to be mediated by the ECS activity of Echinacea AKAs, most likely through CB1.

With evidence of ECS activation through multiple mechanisms, Echinacea products may prove useful in pain management. Whereas research in peripheral pain models is limited, Echinacea was traditionally used by Indigenous North Americans to treat sore throat and toothache (Moerman, 1998). In an uncontrolled human trial, Echinacea extracts were reported to be as effective in reducing sore throat pain as lidocaine (Wonnemann et al., 2007). Notably, the therapeutic potential of CB1 and CB2 receptor agonists, as well as FAAH inhibitors, has been described for neuropathic pain management (Ahn et al., 2009; Rahn and Hohmann, 2009). The potential of Echinacea AKAs in this context thus warrants further study.

Chapter 6 of this thesis described the inhibitory effects of Echinacea extracts and alklyamides on fatty acid amide hydrolase (FAAH), a major enzyme in endocannabinoid degradation. Building on these results, the present study investigated a set of *E. angustifolia* and *E. purpurea* accessions and isolated AKAs for activity as agonists and antagonists in CB1 and CB2 assays. The ability of these extracts and compounds to interfere with fatty acid binding protein-5 activity – a model of endocannabinoid transport, was also tested. Based on the observed CB1 and CB2 agonist activity of many *E angustifolia* accessions, a pooled extract of *E. angustifolia,* and a separate pooled extract of *E. purpurea,* was studied in a well-established animal model for peripheral inflammatory pain. The design was a dose-response experiment with 4 doses and a positive control (dexamethasone).

8.2 Methods

8.2.1 Plant material preparation

Various E. *purpurea* and *E. angustifolia* genotypes were selected by plant breeder John Baker and co-author Arnason in a previous study of germplasm grown at Trout Lake Farms WA. The root samples were dried at 45°C and milled to powder (1 mm mesh). Each powdered sample (500 mg) was extracted three times in 15 mL fresh 70% ethanol using ultrasound (5 min) followed by centrifugation (10 min, 3214 rcf) and collection of the supernatant. Supernatants were dried under vacuum (Speedvac) followed by freeze drying (Super Moduylo).

8.2.2 Phytochemical analysis

An Agilent HPLC system (model 1100) with a Phenomenex Luna (C18, 100X2.1mm, 5um particle size; Phenomenex Inc. Mississauga, Ontario) column was used for phytochemistry analysis obtained from) Detailed method for identification and quantification of targeted compounds were described previously (Chapter 6).

8.2.3 CB receptor Redistribution Assay

Stock solutions of positive controls and samples were prepared by dissolving corresponding compounds/extracts in ethanol and subsequently diluting them with medium to final concentrations of 0.6 and 2.5 mg/ml of plant extracts; 4,12,40, and 120 μg/ml for AKAs, 0.4 and 4μM for Win55,212-2 and 4 μM for rimonabant.

GEFP-CB1 and GEFP-CB2 fusion protein expressed U2OS cell lines were obtained from Thermo Fisher Scientific (Beijing, China) and the assay procedure followed the CB 1/2 Redistribution Assay protocol from Thermo Fisher. In brief, cells were cultured in DMEM (high

glucose) medium with 0.5mg/ml G418 and 10% FBS in a 96 well plate contain DMEM F12 medium with 10 mM HEPES, 1% FBS and 1μM Hoechst (33342) at a concentration of 8,000 cells/100μl/well with 5% CO2 at 37 ºC for 18-24 hours.

For the agonist assay, each well was first washed with 100 μl of medium followed by adding 150 μl of medium and 50 μl of pre-diluted samples or positive control. Cells were then incubated under 5% CO2 at 37 ºC for 120 min. Win55,212-2 (Final concentration at 1 μM) was used as positive control and 0.5 % ethanol was used as vehicle control.

For the antagonist assay, each well was first washed with 100 μl of medium followed by adding 100 μl of medium and 50 μl of per-diluted samples and positive control. Cells were then incubated under 5% CO2 at 37 ºC for 60 mins followed by adding 50 μl medium containing 0.4 μM Win55,212-2. Cells were then incubated for 120 min under the same conditions. Rimonabant (Final concentration = 1 μM) was used as positive control and 0.5 % ethanol was used as vehicle control.

IN Cell Analyzer 1000 Cellular Imaging and Analysis System (GE Healthcare Bio-Sciences Corp) was used to monitor the CB receptor internalization. The excitation/emission was set at 350 /460 nm for Hoechst 33342 and 475 /535 nm for GFP with 300 and 500 ms exposure time respectively. The magnification was set at 20X objectives and 5 photos in different regions of each well were taken. The qualitative analysis of GFP spot formation was done by using an IN-Cell Analyzer 1000 Granularity Analysis Module.

The average of 15 images from triplicate wells per sample was used to calculate the percentage activation or inhibition. The following formulae were used to calculate the % activity (% activation or % inhibition) of Echinacea root extract and AKAs on CB receptors:

$$\% \text{ Activity} = \frac{\text{Endosomes GFP in positive control - Endosomes GFP in sample}}{\text{Endosomes GFP in positive control - Endosomes GFP in vehicle control}} \times 100\%$$

8.2.4 FABP5 binding screens assay

Recombinant human FABP5 was expressed as an N-terminal hexahis-tagged protein using a pET-28a vector (Novagen, Madison, WI, USA). The protein purification, delipidation and FABP5 binding assay procedures were previously described (Kaczocha et al., 2012). In brief, purified human FABP5 (3 μM) was incubated with NBD-stearate (500 nM) in a buffer system contain 30 mM Tris and 100 mM NaCl at pH 7.5. Compounds were then added to the wells and the system were equilibrated at 24°C for 20 minutes in the dark. Loss of fluorescence intensity was monitored with a F5 Filtermax Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Following background subtraction, fluorescence intensity values were normalized to the vehicle control.

8.2.5 Animals preparation

Complete Freund's Adjuvant (CFA; heat-killer M. Tuberculosis) and Dexamethasone were purchased by Sigma-Aldrich, St. Louis, MO, USA. Female Wistar rats were used (CFA 100 μL and weight range 180-220 g). The animals were obtained from our own breeding facilities and had free access to drinking water, but food was withdrawn 12 h before experiments. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann,1983). Additionally, the study was approved by the Institutional Animal Care and Use Committee (Centro de Investigación de Estudios Avanzados Unidad Sur). All compounds and extracts were dissolved in saline and administered orally.

8.2.6 Hargreaves inflammatory pain model

The peripheral pain effect was evaluated using the Hargreaves model of inflammatory pain. Inflammatory pain was induced by injecting a low volume (100 ul) of complete Freund's adjuvant suspended in oil: saline 1:1 emulsion into the right hind paw. The paw withdrawal latency in response to the application of a radiant stimulus onto the plantar surface of both right and left paw was measured using the plantar Analgesia Meter equipment for paw stimulation (IITC Life Science, Woodland Hills, CA, USA) as described in Farrington et al. 2014. The time taken by the animal to respond by licking or flicking its paw was interpreted as positive response (paw withdrawal latency). All animals that presented a baseline response below 15 s prior to the injection of complete Freund's adjuvant were excluded from the study. A cutoff time (20 s) was established at the end of which the heat source shut off auto-matically to avoid tissue damage. Animals were kept (randomized) 1 per cage and staff performing pain experiments were blinded to the content of injections.

We used a n=6 per groups to minimize the biological variability. Rats received vehicle (saline) or increasing doses of *E. angustifolia* (2.5–10 mg/kg), *E. purpurea* (2.5–10 mg/kg), or Dexamethasone (4 mg/kg) by oral administration. All experimental results are expressed as the mean ± SEM for six animals per group. Curves were constructed by plotting the latency of the paw withdrawal as a function of time. An increase of the latency was considered as analgesic effect. Area under the curve (AUC) was calculated by the trapezoidal method to obtain the % on Maximum Possible Effect (%MPE). The analagesic effect was also expressed as the maximum possible effect (%MPE) and calculated as:

$$\%MPE = [(AUC_{Compound} - AUC_{CFA\ injected\ paw}) / (AUC_{normal\ paw} - AUC_{CFA\ injected\ paw})] * 100\%.$$

8.2.7 Statistical analysis

For the peripheral pain effect of the compounds a one-way anova followed by Dunnett's test relative was used to analysis all the data. Simple linear regression modelling was used to analyze potential relationship between log transformed concentration of major component for Echinacea extract and CB1/2 receptor agonist activity. Prism GraphicPad (v.7.0) was used to conduct both analyses.

8.3 Results:

8.3.1 Phytochemistry analysis

Major components from Echinacea spp. were quantified by HPLC-DAD method (Figure 8-1). Echinacoside was the major CADs found in the root extracts of *E. angustifolia* while cichroic acid was the dominant CAD found in *E. purpurea*. dodeca-2E,4E,8Z,10Z/E-tetraenoic acid isobutylamide (AKA9/10) were the dominant AKA in E. angustifolia root extract account for at least 60% of the total AKAs amount and no AKA8 was found in the tested *E. angustifolia* root extracts. The distribution of major AKAs in *E. purpurea* root extract was more even. No single AKA exceed more than 30% of the total AKA amount. Variation of major components was also observed between samples of the same Echinacea species, up to 12 fold for different individual components (Table 8-1).

8.3.2 CB receptor activity

Twelve *E. angustifolia* and ten *E. purpurea* root extracts representing different accessions were evaluated at two concentrations for agonist activity at CB1 (Figure 8-2) and CB2 (Figure 8-4). A one-way ANOVA showed a significant variation in activity by chemotype in high concentration group of E. angustifolia ($F= 7.19$, $p<0.0001$). Eight of the twelve *E. angustifolia* extracts showed positive agonist activity in the CB1 assay and 2 of them at higher concentrations were significant ($p<0.05$) compared to the vehicle control. One chemotype was more active than the positive control (1µM Win55,212-2) at the higher concentration. The ANOVA of the *E. pupurea* extracts showed a significant variation in low concentration group ($F= 7.23$, $p<0.0001$). All *E. purpurea* extracts showed positive agonist activity in the CB1 assay and 4 of them were

Table 8-1. HPLC-DAD quantification of major components in *Echinacea spp* root extract. Mean concentration (n=3) of caffeic acid derivatives and alkylamides (AKA) are presented.

Code	*% Yield*	*Conc in extract (µg/mg)*							
		Chologenic acid	Echinacoside	Cichoric acid	AKA2	AKA3	AKA6	AKA8	AKA9+10
EA01	21.5	0.929	18.245	0.954	0.789	1.416	1.135	N/A	14.464
EA07	36.375	0.913	8.319	0.421	1.301	0.445	0.217	N/A	12.848
EA09	19.875	1.823	19.888	3.446	1.302	2.859	1.350	N/A	17.906
EA13	38.125	5.592	5.872	3.059	5.283	1.408	0.674	N/A	13.842
EA15	31.5	3.446	21.779	2.941	0.411	0.924	0.502	N/A	2.767
EA22	19	3.227	8.341	3.207	1.968	1.269	0.647	N/A	6.366
EA23	42.125	1.377	15.116	0.782	0.226	0.815	0.368	N/A	15.006
EA24	42.875	1.959	15.585	1.708	0.353	1.185	0.730	N/A	5.880
EA25	22.625	1.977	36.693	2.568	0.245	1.296	0.699	N/A	8.841
EA35	30.875	2.752	6.636	1.106	3.134	1.651	1.217	N/A	15.481
EA39	40.875	2.901	23.640	1.829	0.305	1.069	0.485	N/A	5.723
EP01	19	N/A	N/A	42.835	3.598	9.064	4.408	5.258	1.567
EP02	23.375	N/A	N/A	12.041	8.178	18.089	11.619	5.566	1.918
EP05	13.75	N/A	N/A	23.729	13.553	18.512	12.844	9.794	6.019
EP07	20.625	N/A	N/A	44.016	6.413	19.765	10.037	12.930	4.172
EP27	25	N/A	N/A	12.416	6.139	10.484	7.231	5.286	2.432
EP31	21.875	N/A	N/A	25.488	7.031	13.669	7.418	5.913	5.243
EP32	32.625	N/A	N/A	19.859	5.659	7.146	5.467	5.109	3.890
EP36	17.875	N/A	N/A	71.069	15.693	28.595	12.359	8.849	3.984
EP50	28.375	N/A	N/A	6.207	3.005	12.587	4.844	4.396	2.514

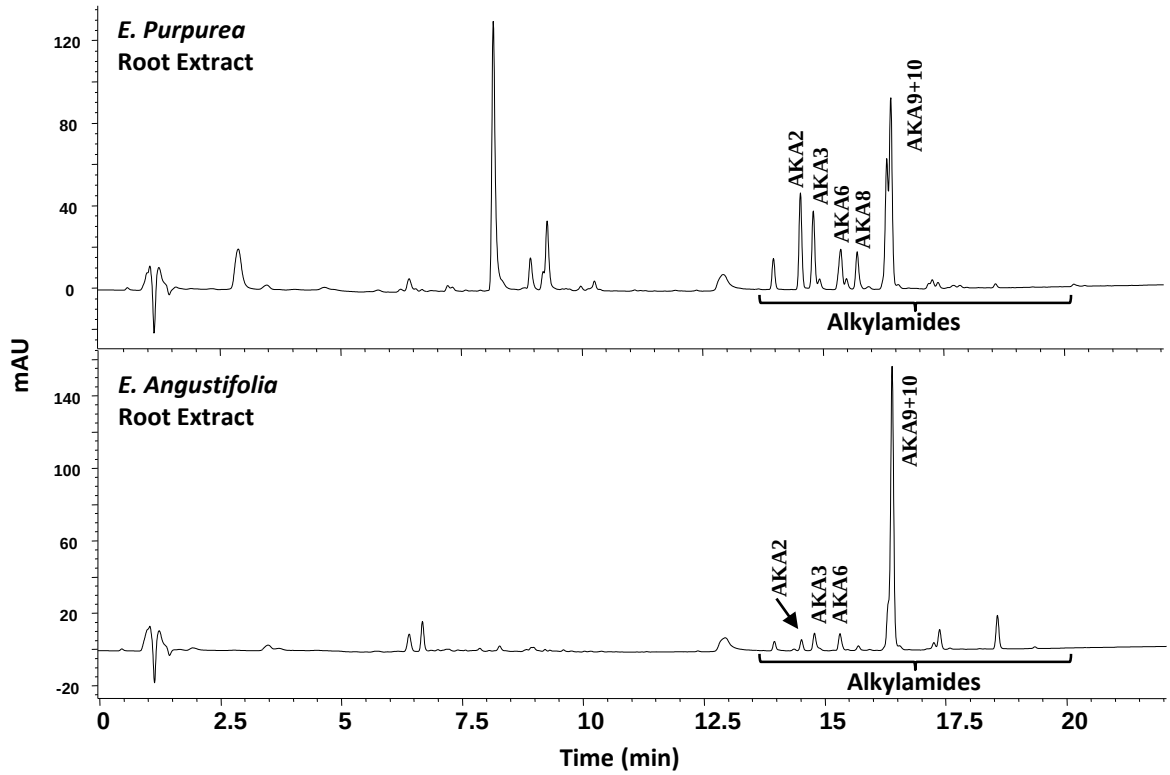

Figure 8-1. HPLC-DAD chromatogram of *E. purpurea* roots and *E. angustifolia* roots at 268 nm. AKA: Alkylamide.

significantly different ($p<0.05$) compared to the vehicle control at lower concentration. In the CB2 assay, one-way ANOVA showed a significant variation of activity by chemotype in low concentration group of *E. angustifolia* ($F= 4.79$, $p<0.001$). and high concentration group of *E. purpurea* ($F= 2.74$, $p<0.05$). Although most of the extracts from both species showed some trend towards an agonist effect, only one *E. angustifolia* extract at both concentrations and one *E. angustifolia* extract at higher concentration showed significant activity ($p<0.05$) compared to the vehicle control. *E. angustifolia* extracts exhibit higher CB2 agonist activity compared to *E. purpurea* extracts. In the CB1 (Figure 8-3) and CB2 (Figure 8-5) antagonist assay, only a few *E. angustifolia* and *E purpurea* accessions showed antagonist effects. However, none of these activities were significant relative to the control group.

Isolated AKAs from Echinacea extracts were tested in the same agonist assays; most compounds were only weak agonists or inactive in the CB1 assay (Figure 8-6A), and only AKA8 at lower concentration (3 µg/mL) showed significant activity. However, the agonist effect of most of the AKAs on CB2 were significant ($p<0.05$) compared to control. An exception was AKA 9, an isomer of 10, which was inactive (Figure 8-6B). The active compounds showed a concentration dependent activity, except for AKA8. In the antagonist assays, most compounds elicited weak or no antagonist effects on both CB1 and CB2 (no significant differences relative to control, Figure 8-7).

8.3.3 CB transporter activity

In in vitro FAPB5 binding assay (Figure 8-8), most of AKAs only showed inhibition effect on FABP5 binding of arachidonic acid at the supra-physiological concentrations. A one-way

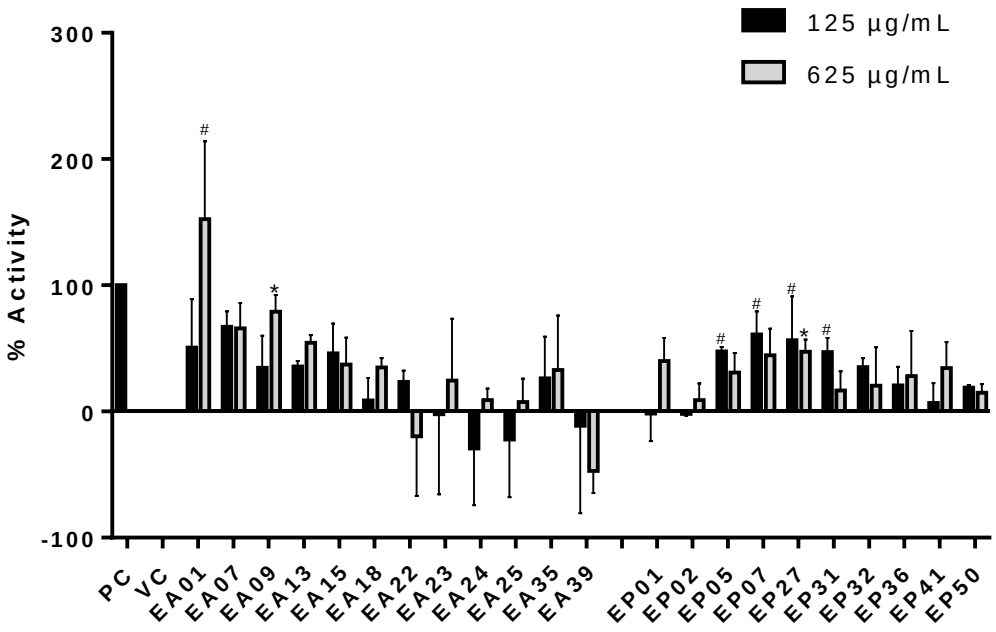

Figure 8-2. Agonist effect of *angustifolia* (EA) and *Echinacea purpurea* (EP) root extract on CB1 receptor in CB receptor redistribution assay. Mean+SEM (n=3) were presented. VC: vehicle control, 0.5 % ethanol; PC: positive control, WIN 55,212-2 (1 µM). A one-way analysis of variance (ANOVA) was performed to evaluate the variance between samples followed by the Dunnett test comparing to the vehicle control. *: $p<0.05$; #: $p<0.01$.

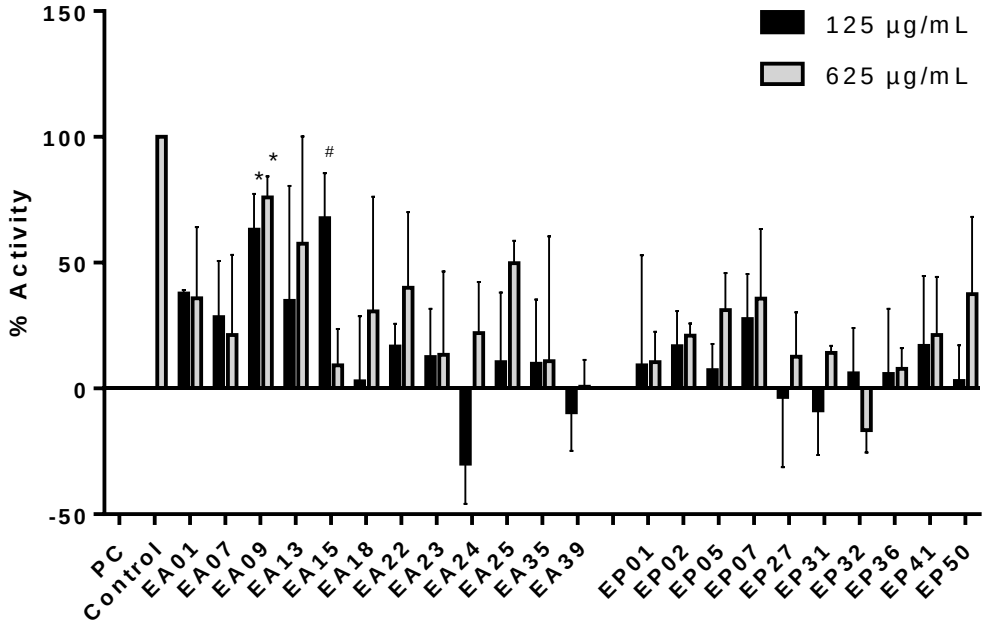

Figure 8-3. Agonist effect of *angustifolia* (EA) and *Echinacea purpurea* (EP) root extract on CB2 receptor in CB receptor redistribution assay. Mean+SEM (n=3) were presented. VC: vehicle control, 0.5 % ethanol; PC: positive control, WIN 55,212-2 (1 µM). A one-way analysis of variance (ANOVA) was performed to evaluate the variance between samples followed by the Dunnett test comparing to the vehicle control. *: $p<0.05$; #: $p<0.01$.

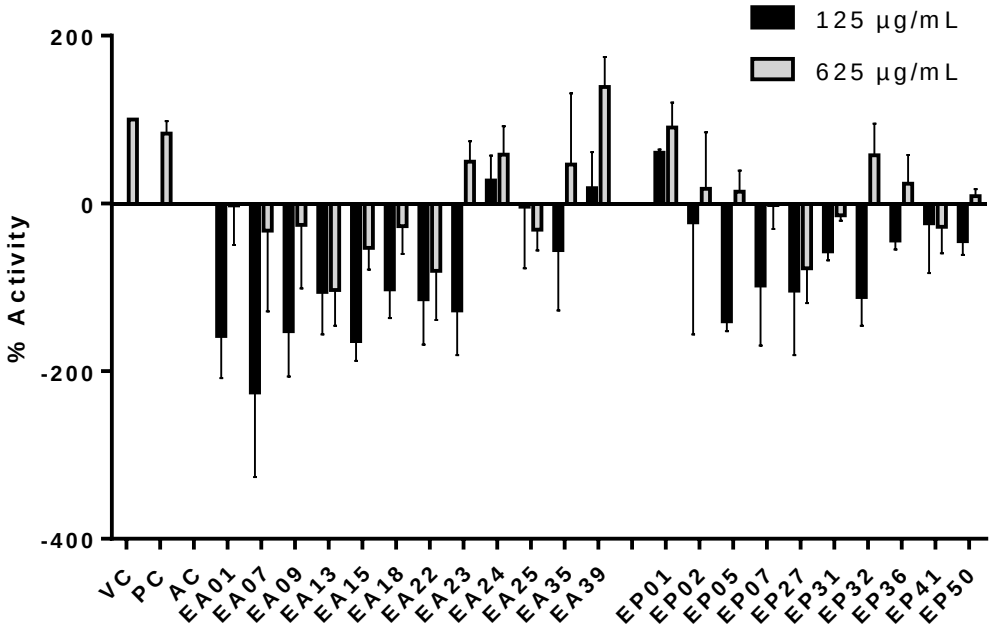

Figure 8-4. Antagonist effect of *angustifolia* (EA) and *Echinacea purpurea* (EP) root extract on CB1 receptor in CB receptor redistribution assay. Mean+SEM (n=3) were presented. VC: vehicle control, 0.5 % ethanol; PC: positive control rimonabant (10 µM), AC: agonist control, WIN 55,212-2 (1 µM). A one-way analysis of variance (ANOVA) was performed to evaluate the variance between samples followed by the Dunnett test comparing to the vehicle control.

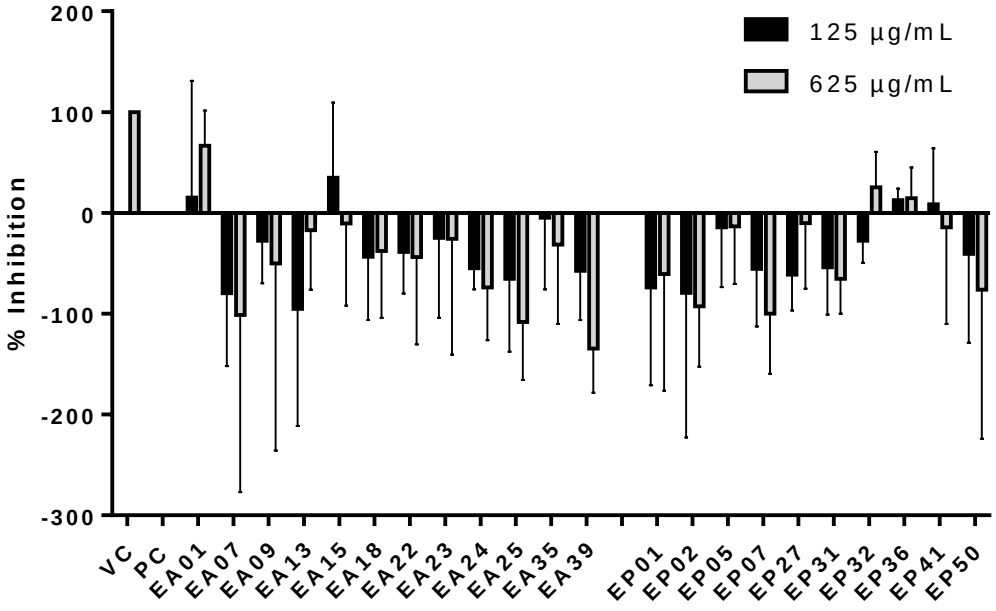

Figure 8-5. Antagonist effect of *angustifolia* (EA) and *Echinacea purpurea* (EP) root extract on CB2 receptor in CB receptor redistribution assay. Mean+SEM (n=3) were presented. VC: vehicle control, 0.5 % ethanol; AC: agonist control, WIN 55,212-2 (1 µM). A one-way analysis of variance (ANOVA) was performed to evaluate the variance between samples followed by the Dunnett test comparing to the vehicle control.

ANOVA analysis followed by Dunnett test was performed to examine the results. All AKAs showed significant ($p<0.05$) inhibitory effect on FABP5 binding of arachidonic acid at the highest concentration tested (100 mM). However, only AKA 3 showed significant inhibition ($p<0.05$) of FAB5 binding activity at 10 mM.

8.3.4 Linear regression analysis of activity of accessions with their phytochemical content.

A simple linear regression of CB2 receptor agonist effect across all 11 chemotypes of *E. angustifolia* extract relative to the measured concentrations of major components showed that, in the high concentration group (0.625 µg/ml), CB2 receptor agonist effect was significantly predicted by total AKA content. Further analysis suggested that the isomers AKA9+10 were the only AKAs significantly correlated to CB2 receptor agonist effects (Table 8-2). CB2 agonist activity did not correlate with identified *E. purpurea* phytochemistry. In terms of CB1 agonist effects, the concentration of AKA2 and AKA9+10 were respectively detected as significant independent variables for agonist effects in the low concentration group (0.125 mg/ml) of both *E. angustifolia* and *E purpurea* extracts (Table 8-1).

8.3.5 Rat chronic inflammatory pain model.

The model involves application of noxious substances to the hind paw, resulting in inflammation (oedema) and measurable nociceptive behavior, including allodynia (heightened responses to non- noxious levels of cutaneous stimulation) and hyperalgesia (heightened responses to noxious levels of cutaneous stimulation), although the underlying signaling mechanisms generating inflammation differ.

Due to the generally positive activity of *Echinacea* ssp. and AKA's in the CB2 agonist assay, a pooled extract of each of *E. angustifolia* roots (EAR) and *E. purpurea* roots (EPR) was

tested in the inflammatory pain model. Oral administration of EAR (Figure 8-9A) and EPR (Figure 8-10A), induced a dose dependent (2.5-10 mg/kg) inhibition of thermal hyperalgesia in the rat. At 10 mg/kg, EAR induced 60% reversal of thermal hyperalgesia (Figure 8-9B) with an overall response duration of 5 hours. Similarly, the administration of EPR using the same dose as EAR induced the maximum analgesic effect, reversing the close to 50% of thermal hyperalgesia (Figure 8-10B). Both Echinacea extracts at the highest dose (10 mg/kg) provided similar results to the positive drug control group treated with dexamethasone (4 mg/kg).

To investigate whether either Echinacea extract suppressed inflammatory pain/ thermal hyperalgesia through the ECS, CB receptor antagonists AM251 (CB1) and AM630 (CB2) were administered in combination with Echinacea extracts in the Hargreaves model. The results indicated that the suppression of thermal hyperalgesia by EAR was significantly reduced when co-administrated with both AM251 and AM630. However, pharmacological response of EPR was only significant reduced by AM630 not AM251. In addition, both CB receptor antagonists showed no activity in this animal model at the tested concentrations (Figure 8-9C; Figure 8-10C).

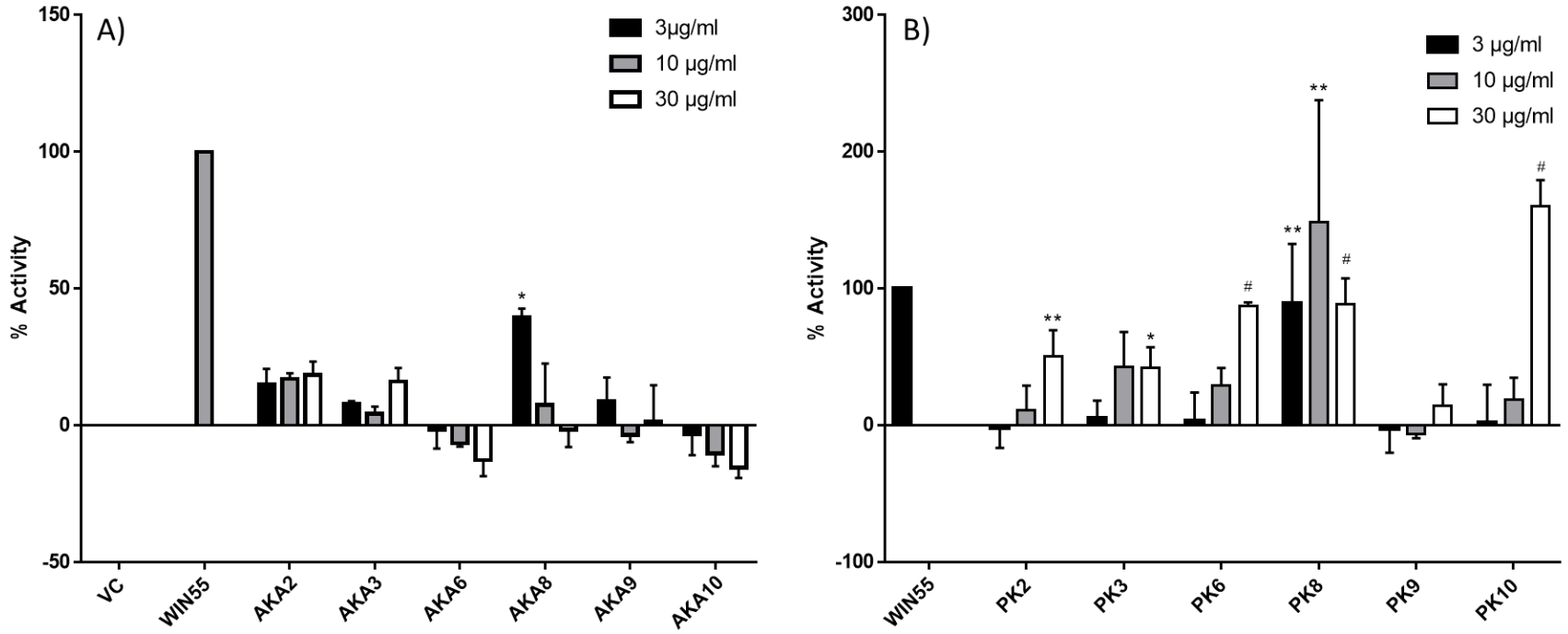

Figure 8-6. Agonist effect of Echinacea alkylamide (AKA) on (A) CB1 or (B) CB2 receptors in CB receptor redistribution assay. Mean+SEM (n=3) were presented. VC: vehicle control, 0.5 % ethanol; PC: positive control, WIN 55,212-2 (1 µM). A one-way analysis of variance (ANOVA) was performed to evaluate the variance between samples followed by the Dunnett test comparing to the vehicle control. *: $p<0.05$; **: $p<0.01$; #: $p<0.001$.

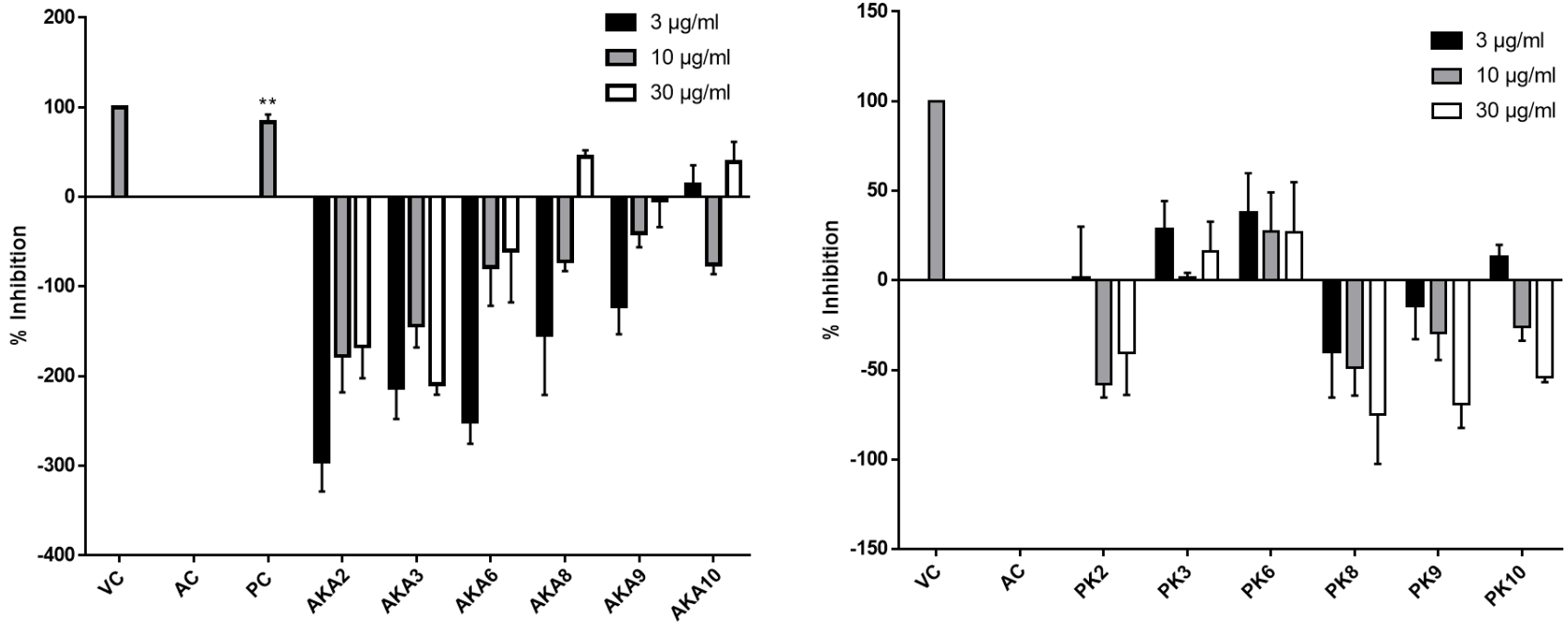

Figure 8-7. Antagonist effect of Echinacea alkylamide on (A) CB1 or (B) CB2 receptors in CB receptor redistribution assay. Mean+SEM (n=3) were presented. VC: vehicle control, 0.5 % ethanol; PC: positive control; rimonabant (10 µM), AC: agonist control, WIN 55,212-2 (1 µM). A one-way analysis of variance (ANOVA) was performed to evaluate the variance between samples followed by the Dunnett test comparing to the agonist control.; **: $p<0.01$

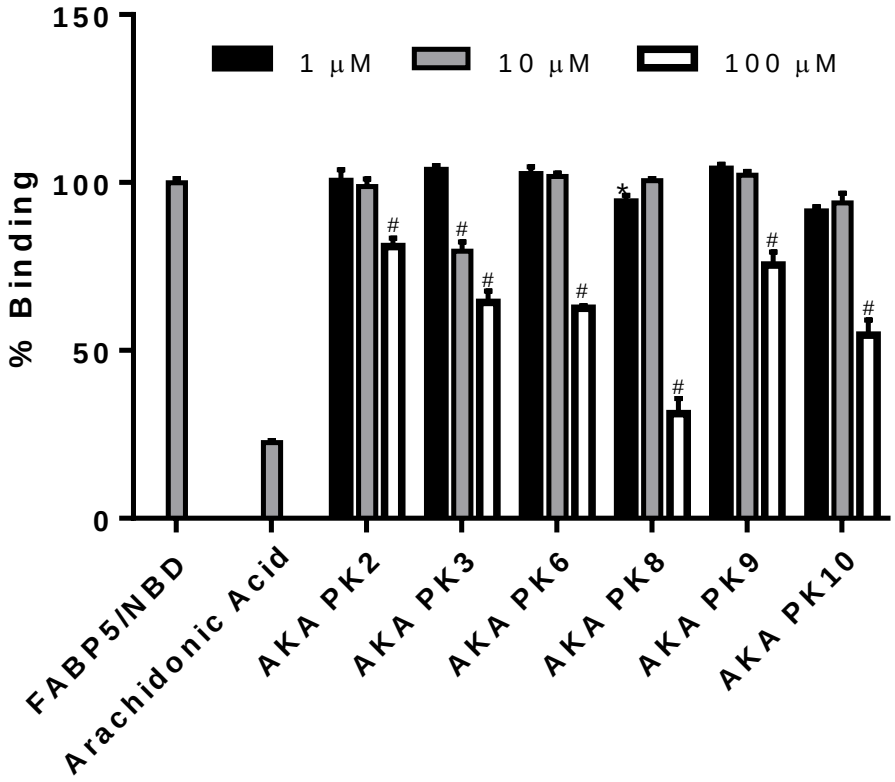

Figure 8-8. Effect of Echinacea alkylamide (AKA) on Fatty acid-binding protein 5 (FABP5) binding activity. AKAs were tested with 3 concentration (1 µM, 10 µM and 100 µM), Mean+sem (N=6) were presented. DMSO was used as Vehicle control and Arachidonic acid (10 µM) were used as positive control. A one-way analysis of variance (ANOVA) was performed to evaluate the variance between samples followed by the Dunnett test comparing to the vehicle control. *:$p<0.05$; **: $p<0.001$

8.4 Discussion

While the activity of AKAs and *Echinacea* spp extracts in receptor assays has been reported previously, this is the first report of activity in the CB redistribution assay, which follows receptor internalization as a measure of agonist or antagonist activity. Our results reconfirm the agonist activity of AKAs and extracts in this new assay and, as previously reported (Woelkart et al., 2006; Raduner et al., 2007), most activity was associated with CB2, particularly with *E. angustifolia* extracts or isolated compounds. *E. purpurea* accessions were generally less active. Extracts of both species, as well as AKAs, were inactive or weakly active in the CB1 agonist assay, and both CB receptor antagonist assays. Our unexpected observation that some extracts and AKAs appear to selectively enhance CB1 receptor internalization in the presence of WIN 55,212-2 deserves further investigation as it suggests that these samples may sensitize CB1 to agonists or, alternatively, accelerate internalization and desensitization in the presence of an agonist.

The FABP5 assay, a model of anadamide transport, showed that several isolated AKAs may interfere with endocannabinoid transport but only at the high concentrations where established micellization of lipid like AKAs (Raduner et al., 2007) likely played a role in the observed results. Previously reported in vitro data demonstrate that Echinacea extracts and AKAs influence endocannabinoid transport (Chicca et al., 2009). FABP5 appears to be an unlikely mechanism given the high concentrations required to impact binding (Figure 8- 9) but supports micellization effects as a potential mechanism, as suggested by Chicca et al. (2009).

The effects of Echinacea components, especially AKAs, are not limited to the CB receptors and transporters, but also on the endocannabinoid's degradation enzymes. Previous research

Table 8-2 Simple linear regression of major components of the *E. angustifolia* and *E. purpurea* extract concentration relative to CB1 receptor agonist effects.

	E. angustifolia						E. purpurea					
	0.125 mg/ml			0.625 mg/ml			0.125 mg/ml			0.625 mg/ml		
	SLOPE	R^2	*P*	SLOPE	R^2	*P*	SLOPE	R^2	*P*	SLOPE	R^2	*P*
AKA2	40.86	0.37	**<0.05***	32.22	0.08	0.40	18.37	0.09	0.37	-3.74	<0.01	0.90
AKA3	-22.77	0.02	0.67	40.95	0.02	0.65	6.39	<0.01	0.90	-0.49	<0.01	0.98
AKA6	-10.31	0.01	0.82	68.99	0.09	0.36	30.53	0.05	0.55	-1.42	<0.01	0.96
AKA8	N/A	N/A	N/A	N/A	N/A	N/A	71.62	0.23	0.19	36.5	0.18	0.26
AKA9+10	30.48	0.06	0.47	109.5	0.28	0.10	82.34	0.48	**<0.05***	-2.71	<0.01	0.92
Total CAD	-81.87	0.24	0.12	-52.76	0.04	0.58	5.29	<0.01	0.85	17.79	0.18	0.25
Total AKA	43.44	0.09	0.36	124.6	0.28	0.09	37.47	0.07	0.49	1.58	<0.01	0.96

CAD: Caffeic acid derivatives; AKA: Alkylamide.

Table 8-3 Simple linear regression of major components of the *E. angustifolia* and *E. purpurea* extract concentration relative to CB2 receptor agonist effects.

	E. angustifolia						E. purpurea					
	0.125 mg/ml			0.625 mg/ml			0.125 mg/ml			0.625 mg/ml		
	SLOPE	R^2	*P*	SLOPE	R^2	*P*	SLOPE	R^2	*P*	SLOPE	R^2	*P*
AKA2	22.61	0.05	0.56	21.68	0.23	0.14	18.37	0.09	0.37	44.99	0.25	0.17
AKA3	27.08	0.04	0.58	50.91	0.23	0.14	18.82	0.11	0.38	-4.26	<0.01	0.88
AKA6	16.26	0.02	0.70	32.50	0.13	0.28	18.98	0.10	0.40	26.11	0.08	0.46
AKA8	N/A	N/A	N/A	N/A	N/A	N/A	37.58	0.32	0.12	38.68	0.13	0.34
AKA9+10	11.12	0.01	0.77	54.93	0.43	**<0.05***	-7.16	0.02	0.73	3.86	<0.01	0.91
Total CAD	11.74	<0.01	0.82	-7.56	<0.01	0.843	8.60	0.07	0.49	-11.83	0.05	0.55
Total AKA	21.36	0.03	0.63	67.60	0.51	0.014	19.67	0.10	0.42	31.58	0.10	0.41

CAD: Caffeic acid derivatives; AKA: Alkylamide.

showed that both CADs and AKAs for *Echinacea* spp inhibit the Fatty acid amide hydrolase (FAAH) in *in vitro* assays (Chapter X). FAAH inhibitors have been reported effectively reducing pain in rodent arthritis models by reduce inflammatory flares (McDougall et al 2015). Furthermore, alkylamides are known to be metabolized by hepatic CYP450 enzymes if consumed orally. Two studies were attempted to evaluate the impacted of hepatic metabolism on AKA activities; Cech et al (2006) reported reduced suppression IL-2 secretion in stimulated T cells by Echinacea alkylamides (AKA9+10) after hepatic oxidation. However, it was found in chapter 7 that metabolism enhanced FAAH inhibitory effects of hepatic metabolites of AKA2, AKA3 AKA9 and AKA10. Together, current evidence suggests the oval all pharmacological outcome of oral consumed Echinacea products is not determined by a single active ingredient but a variety of components and their metabolites which can interact with the endocannabinoids with different mechanisms.

This is also the first attempt to assay germplasm accessions for breeding purposes to determine if selection of high activity genotypes is feasible. Clearly high activity genotypes can be identified, especially in the CB2 receptor agonist assay, and this may be a way to identify elite germplasm for targeted activity for managing pain and inflammation, as demonstrated in the animal model. Correlations to phytochemical characteristics of the extracts were generally insignificant, but this may be a result of the complexity of the extracts and relatively low number of tested accessions (10-11).

A highly positive result is the demonstration that both Echinacea root extracts have dose dependent activity in the rat paw model of peripheral pain. In fact, the results show that the extract is just as effective as dexamethasone (at concentrations 2-3 times higher than this corticosteroid

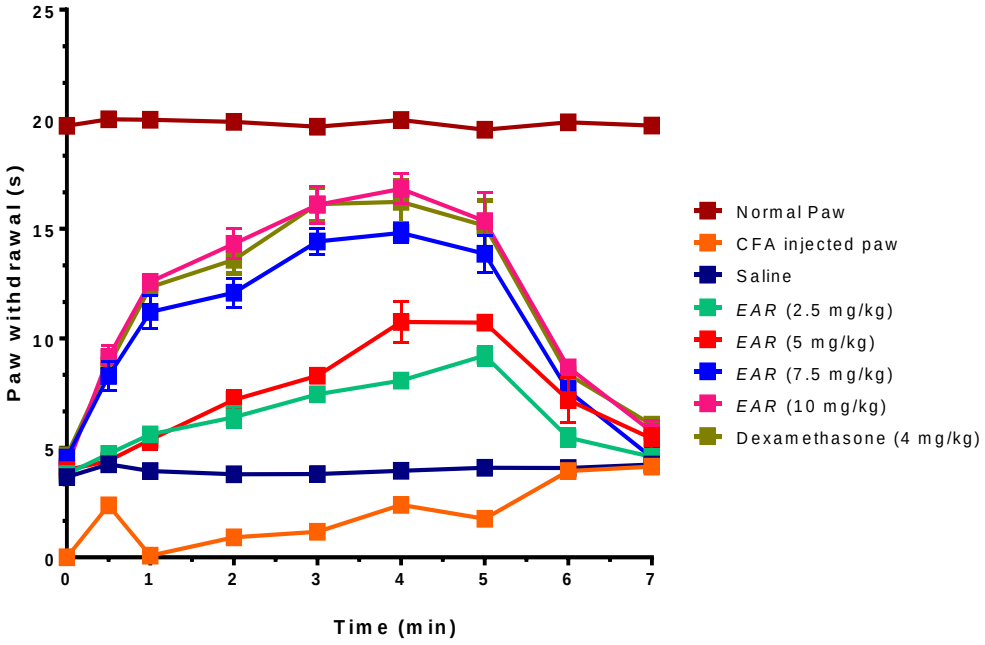

Figure 8-9A. Time course paw withdrawal observed after acute oral administration of *E. angustifolia* root extract (EAR) and dexamethasone in peripheral pain in rats. The Normal Paw group is placed as a reference of the maximum possible effect. Saline solution was used as vehicle control for Complete Freund's Adjuvant (CFA). In all cases, data are presented as mean ± SEM for 6 rats.

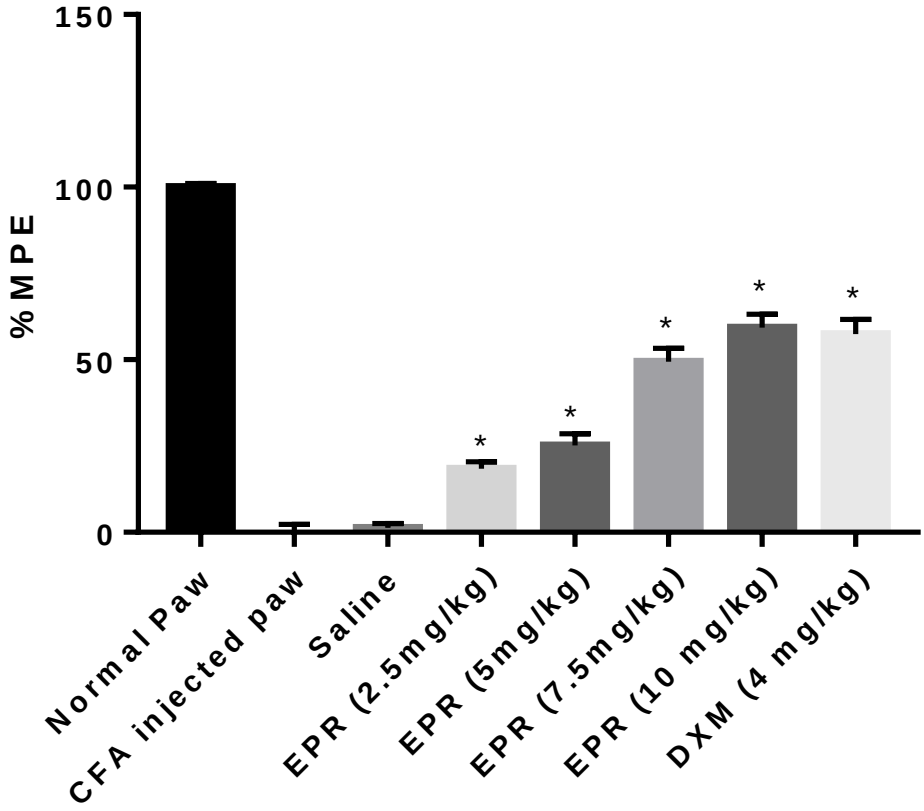

Figure 8-9B. Effect of the oral administration of *E. angustifolia* root extract (EAR) and dexamethasone (DEX) in peripheral pain in rats. Data are presented area under the curve (AUC) of % of maximum possible effect (%MPE) as a function of dose. The Normal Paw group is placed as a reference of the maximum possible effect. Data are the mean ± SEM for 6 animals. *Significantly different from the vehicle (Saline) group ($P < 0.01$), as determined by oneway ANOVA analysis followed by *post hoc* Dunnett's t-test.

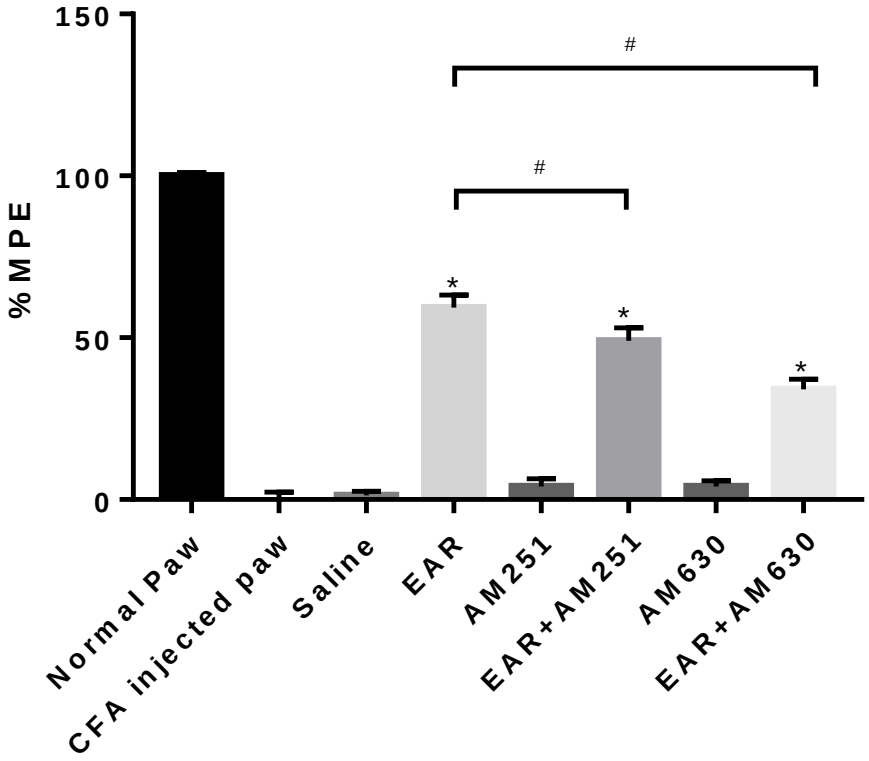

Figure 8-9C. Effect of the oral administration of *E. angustifolia* root extract (EAR) and cannabinoid receptor antagonist AM251 (CB1) and AM630 (CB2) on peripheral pain in rats. Data are presented area under the curve (AUC) of % of maximum possible effect (%MPE) as a function of dose. The Normal Paw group is placed as a reference of the maximum possible effect. Data are the mean ± SEM for 6 animals. *: Significantly different from the vehicle (Saline) group ($P < 0.01$); #: Significantly different from the EAR without antagonist group ($P < 0.01$). as determined by ANOVA analysis followed by *post hoc* Dunnett's t-test.

pharmaceutical. It is a remarkable level of activity for a crude plant extract. Although the animal result is positive, the link to pharmacological mechanism and active principle *in vivo* is not yet clearly demonstrated. The overall anti-inflammatory effect of *Echinacea* spp extracts is considered as a combination effect of several classes of components. The anti-inflammatory of AKAs, CADs and essential oils were all reported through different mechanism *in vivo* through oral administration (Chao et al., 2009; He et al., 2009; Yu et al., 2013; Zhu et al., 2015 and Manayi et al., 2015). Although polysaccharides were also reported as anti-inflammatory, the effects are limited to intravenous and topical application. As the most bio-available components from Echinacea, AKAs' anti-inflammatory potential is also reportedly associated with the COX 1 and 2 inhibition (Clifford et al., 2002) as well as effects on NF-κB expression (Matthias et al., 2008).

The connection between the ECS and inflammation, particularly inflammatory pain, is well established (Nagarkatti et al., 2009) as both CB1 and CB2 agonists provide effective treatment *in vivo* (Clayton et al 2002). Our results are the first to demonstrate the analgesic potential of Echinacea in a peripheral pain model. Suppression of inflammatory pain was partially blocked by CB2 antagonist AM630 for both species, and by CB1 antagonist AM251 for EAR, data that reflect the relative agonist effects observed using receptor internalization assays (Figure 8-2 & 8-6). Whereas CB2 antagonism reduced the effects of both EAR and EPR extracts to a similar degree, activation of CB1 appears to account for the stronger analgesic response to EAR relative to EPR. Accordingly, while activation of peripheral CB2 receptors contributed strongly to the analgesic effects of both extracts (likely by reducing inflammation), *E. angustifolia* also acts on central CB1 receptors and shows greater therapeutic potential. Interestingly, as reported previously for Echinacea's anti-inflammatory activity (Gerstch, 2006), analgesic mechanisms were not limited to the ECS as both extracts elicited responses in the presence of CB antagonists.

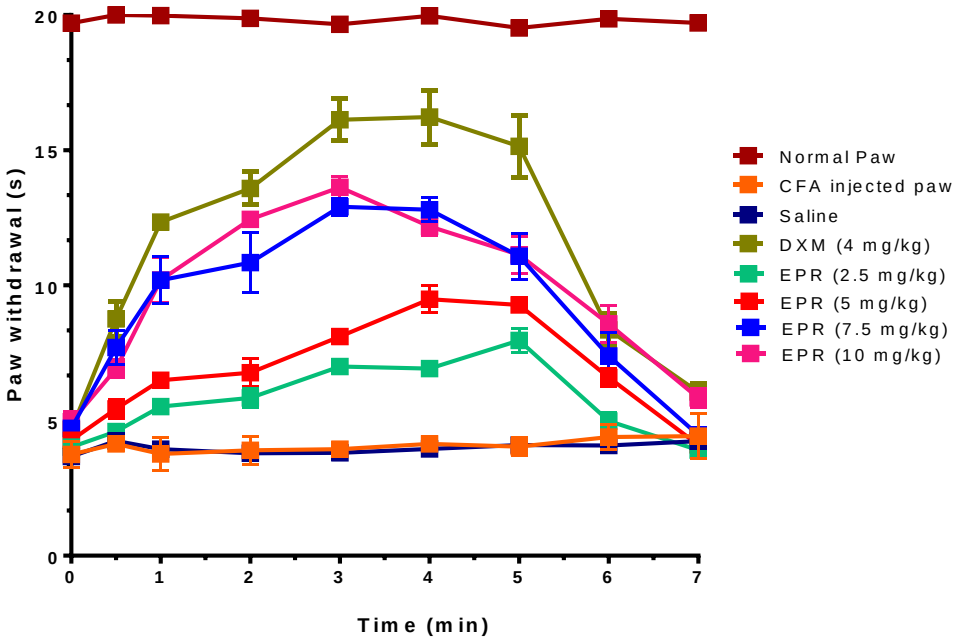

Figure 8-10A. Time course paw withdrawal observed after acute oral administration of *E. purpurea* root extract (EPR) and dexamethasone in rats. The Normal Paw group is placed as a reference of the maximum possible effect. Saline solution was used as vehicle control for Complete Freund's Adjuvant (CFA). In all cases data are presented as mean ± SEM for 6 rats.

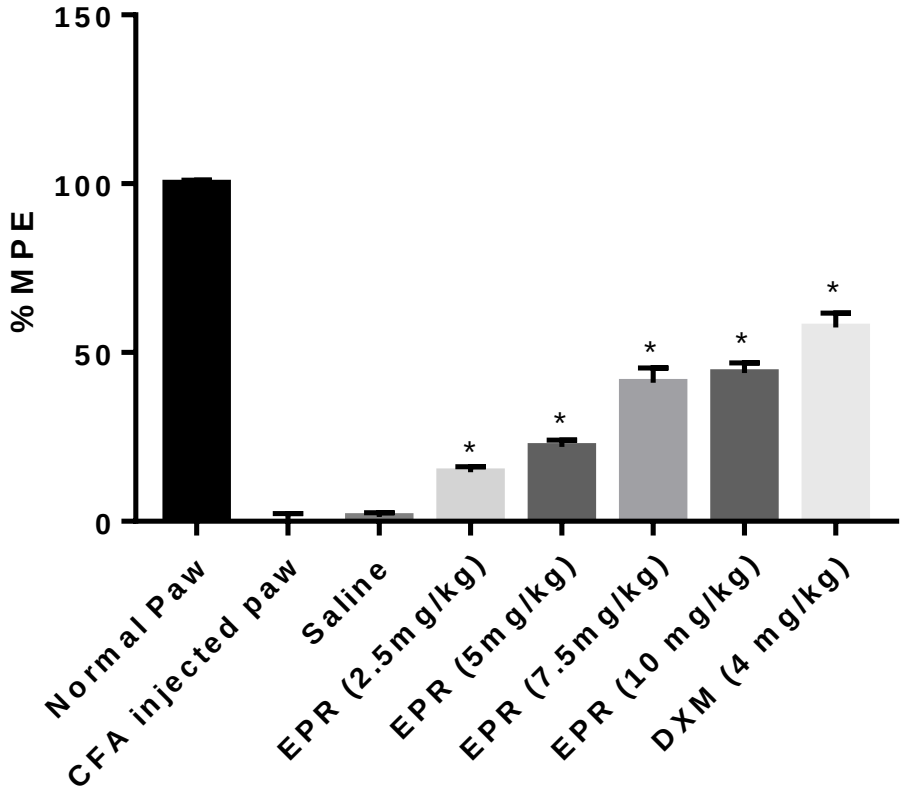

Figure 8-10B Effect of the oral administration of *E. angustifolia* root extract (EAR) and dexamethasone (DEX) in peripheral pain in rats. Data are presented area under the curve (AUC) of % of maximum possible effect (%MPE) as a function of dose. The Normal Paw group is placed as a reference of the maximum possible effect. Data are the mean ± SEM for 6 animals. *Significantly different from the vehicle (Saline) group ($P < 0.01$), as determined by oneway ANOVA analysis followed by *post hoc* Dunnett's t-test.

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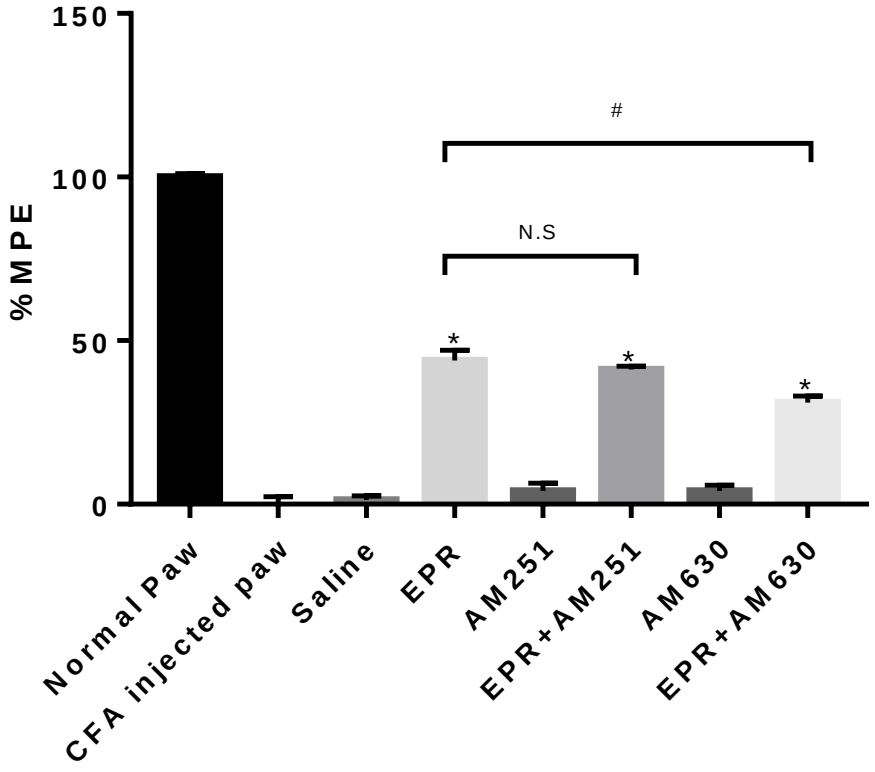

Figure 8-10C. Effect of the oral administration of *E. angustifolia* root extract (EAR) and cannabinoid receptor antagonist AM251 (CB1) and AM630 (CB2) in peripheral pain model in rat. Data are presented area under the curve (AUC) of % of maximum possible effect (%MPE) as a function of dose. The Normal Paw group is placed as a reference of the maximum possible effect. Data are the mean ± SEM for 6 animals. *: Significantly different from the vehicle (Saline) group ($P < 0.01$); #: Significantly different from the EPR without antagonist group ($P < 0.01$); as determined by one-way ANOVA analysis followed by *post hoc* Dunnett's t-test. N.S: not significant ($p>0.05$).

Whether this non-ECS response is mediated by anti-inflammatory or anti-nociceptive mechanisms warrants further investigation.

We know that Echinacea was traditionally used as treatment for sore throat, burns, arthritis, tonsillitis and wounds, among other conditions and symptoms (Binns 2001). The present study suggests it may be active in peripheral pain conditions appropriate for NHP use, such as arthritis, carpel tunnel syndrome etc. In these conditions, inflammation and pain are symptoms that may respond well to *E. angustifolia* treatments. Although *Echinacea* spp. extacts per se have not been investigated previously, the effect and safety of highly standardized Ginger (*Zingiber officinale*) and Echinacea (*Echinacea angustifolia*) extract supplementation on inflammation and chronic pain in NSAIDs poor-responders has shown efficacy in a pilot study in human subjects with knee arthrosis (Rondanelli et al., 2017). Other neuropathic pain conditions such as diabetic and chemotherapy-induced neuropathies could be investigated. Experiments reported by Reyes-García et al., (2002 and 2004) B vitamins complex (B1, B6 and B12) in the same animal model have led to its use as a pain killer (Doloneurobion-merck®) for neuropathic pain in diabetes. Clinical evaluation of the *Echinacea spp.* extracts in human trials for some of these conditions is also warranted, as Echinacea has a good safety profile.

CHAPTER 9: GENERAL DISCUSSION

9.1. *Souroubea* and *Platanus*

Based on previous research conducted by Puniani et al. (2015); Mullally et al. (2011&2014) and Cayer (2011), significant anxiolytic effects with the oral administration of purified betulinic acid, *Souroubea sympetala* and *Platanus occidentalis* extracts and raw material as well as the blend of *Souroubea* spp. and *Platanus* spp. These findings were observed in rodent models such as the elevated plus maze, conditioned emotional response and social interaction paradigms. One pharmacological mode of action has been shown to be agonist activity at the GABAa-BZD receptor (Mullally et al., 2014). However, natural health products usually contain several bioactive compounds with multiple pharmacological targets. My results suggest a second possible anxiolytic mechanism through the interaction with the endocannabinoid system (ECS), specifically through the inhibition on MAGL, one of the major degradation enzyme of endocannabinoid ligands. The *in vitro* enzyme inhibition assay suggested that, in addition to the previously reported activity of α and β amyrins (Chicca et al., 2012), other major triterpenoids including betulinic acid, ursolic acid and lupeol in the herbal blend, as well as the plant extracts, also selectively inhibited MAGL activity but not FAAH activity. Furthermore, another triterpenoid rich product, the Central American sacred incense called copal (*Protium copal* – Burseraceae) was tested in for its anxiolytic effect in similar rodent models. The results revealed significant anxiolytic effects of copal vapour and that this effect was blocked by AM-251, an inverse agonist of CB1 receptor. These data suggest that interaction with the ECS may be a second anxiolytic mode of action of the botanical blend of *S. sympetala* and *P. occidentalis*.

The blend of *S. sympetala* and *P. occidentalis* is well established in the companion animal market (Zentrol^{TM}) and human products are now approved by Health Canada for clinical evaluation. I have developed and validated the extraction and LC-APCI-MS based quantification

methods for the marker compounds with adequate sensitivity and selectivity to ensure the quality of the clinical trial and commercial products. This is a substantive contribution to quality control of the commercial development an an NHP. These methods can also be used in source material selection and development of new product formulation.

The first large safety study in dogs was conducted before the market release of Zentrol^{TM}. Results indicated no adverse effects with a does 5 times higher than the recommend dose. The study also revealed the difficulty of detecting blood levels of BA or metabolites, but cortisol was found to be a good marker of biological response that could be used in place of BA concentration. The results not only assure that Zentrol^{TM} is safe in dogs, but also provided insight for the following application for a clinical evaluation of human products which was approved by Health Canada in Dec 2018.

Although these botanical ingredients might be considered safe based on their long history of use in humans by indigenous healers (Schultes and Raffauf, 1990; Puniani et al., 2015; Arnason et al., 1981; Hamel and Chiltoskey, 1975) as well as the safety evaluation in canines (Villalobos et al., 2014; Masic et al., 2018), adverse effects may be caused by other situations such as herb-drug interactions. Several triterpenoids found in the botanical blend were reported to cause inhibition of CYP450 isoforms (Kim et al., 2004; Moreira et al., 2013; Seervi et al., 2016), which are considered to be the most important Phase I drug-metabolizing enzyme system and a major source of metabolic adverse effects (Bertz et al., 1991; Villalobos., et al 2014). However, the *S. sympetala* and *P. occidentalis* plant extracts had not been examined. The drug interaction study that we conducted (Chapter 4) showed potential risk of interaction with diazepam through CYP450 enzyme inhibition. However, *in vitro* results are not necessarily directly translatable to clinically

significant effects. Thus, further study in a clinical setting and through pharmacovigilance is warranted to assess risk in humans.

To conclude, my hypothesis was upheldin the *in vitro* system, and my study completes critical missing information for approval of *S. sympetala* and *P. occidentalis* for use beyond pre-clinical studies described previously. Botanical blends containing S. *sympetala* and *P. occidentalis* are generally considered to be safe if not used in combination with other drugs, effective and evidence-based for the animal and human NHP market.

9.2 Echinacea

Phytochemical variations in one species are often caused by environment factors such as temperature, insolation, nutrient availability or herbivory (Rapinski et al., 2014). However, my research indicated that considerable phytochemical variation can also be observed within individuals of a population of Echinacea grown in a controlled field environment, representing genetic differences. These features can be inherited but long-term selection will be required to produce stable varieties with different phytochemistry.

As the therapeutic benefits of *Echinacea* ssp. are usually recognized as a combined effect from multiple components, especially the secondary metabolites, the approach taken was to establish the relationship between the pharmacological activities and the phytochemical composition. By analyzing 40 genotypes from each species with considerable variability in phenolic and AKA contents, as well as FAAH inhibition, we evaluated the contribution of components (and clusters) toward the observed bioactivity (and variation in bioactivity). Several phenolic compounds and AKAs in *Echinacea* spp. were found to be significant independent variables explaining the FAAH inhibition by linear regression analysis. While many identified compounds were indeed active, their relative contributions based on modelling differed from their relative potencies when tested as individual compounds. Hence, synergism and antagonism between Echinacea components may play an important role in the overall pharmacological outcome of the whole extracts. My results also indicated for the first time that caffeic acid derivatives may also interact with the ECS by inhibiting FAAH activity. Similar phenomena were also observed with the CB1/2 receptor binding activity with a smaller samples size. In addition, my results also demonstrated that AKAs may block intercellular anandamide transports (Fab5) function at higher concentrations.

Due to the high bioavailability of AKAs compare to CADs, the AKAs are believed to be more clinically significant (Woelkart et al., 2011). However, they also face extensive first pass effect by hepatic metabolic enzymes, especially the CYP450 family (Matthias et al., 2005 and Modarai et al., 2007) after oral administration. Lee et al. (2007) reported only approximately 0.1 – 0.2 % and 7 – 46 % of dodeca-2E,4E,8Z,10E-tetraenoic acid from oral administrated *Echinacea* spp. powder were detected respectively in urinary and fecal samples at 24hrs. Hence, understanding the impact of metabolic transformation of AKA on the pharmacological activities are quite important. A previous study conducted by Cech et al. (2006) indicated that hepatic metabolites of dodeca-2E,4E,8Z,10Z/E-tetraenoic acid showed less immunomodulatory effects than the parent compound. However, my results indicated an increase of FAAH inhibitory after CYP450 mediated metabolism of 4 individual AKAs as well as crude extracts of *E angustifolia* and *E. purpurea.* Based on these findings, I concluded that the observed activation of AKAs by CYP mediated metabolism may play a significant role in the *in vivo* activity of *Echinacea* products.

Echinacea AKAs are reported to have anti-inflammatory and immunomodulatory activities (Barnes et al., 2005) and can be useful to treat related symptoms. AKAs were hypothesized to interact with the ECS to mediate some of these pharmacological effects. My research demonstrated both *E angustifolia* and *E. purpurea* root extract elicit dose dependent analygesic activity in a rat paw oedema model of inflammation pain. Further tests with CB receptor antagonists AM251 and AM680 co-administrated with Echinacea extract demonstrated a significantly reduction of the pharmacological activity. This evidence suggests analgesic activity for peripheral pain was at least partially mediated through the ECS. The reduction of peripheral pain in an animal model by Echinacea is an original finding.

In summary, my hypothesishas been upheld in the *in vitro* and *in vivo* system, and my work extends the discovery of the involvement of the ECS in the mode of action of Echinacea, providing new evidence to support and expand the applications of *Echinacea* ssp. as natural health products.

9.3 Future work

The validated extraction and analytical method established for *S. sympetala* and *P. occidentalis*, allows further work for the improved germplasm selection in *S. sympetala* cultivation, which is now being taken into consideration. As *P. occidentalis* trees are widely distributed from northeastern Mexico to southern Ontario. Significant variation in the terpenes especially betulinic acid, the main active ingredient in the botanical blend were seen. Thus, a large-scale screen is needed to evaluate the most suitable material used in the blend. In addition, as the bio-activity of terpenes are not considered as the main source to cause potential herb-drug integration, the CYP450 inhibition screening assay can also used in the source material selection to reduce the interaction risk without lowering the pharmacological outcome.

Based on the current evidences in hand, a human clinical trial to evaluate the safety and efficacy on generalized anxiety (GAD) are planned. If results are positive in a human clinical trial, similar approaches maybe to evaluate the possibility of application to treat Post-traumatic stress disorder (PTSD).

As one of the most well cultivated herbal products, *Echinacea* ssp. are one of the few herbals that have been through germplasm selection programs to improve the yield or therapeutic outcome. My work introduces new methods for selecting elite species to improve germplasm in *Echinacea* ssp. It may help the industry in product development and quality control (QC) practices, which reveal that specific phenolics and AKAs are both potentially acting on endocannabinoid system targets. However, my work is limited by sample size and pharmacological target selection. As there are other potential pharmacological target in the ECS, such as transient receptor potential cation channel subfamily V member 1 (TRPV1), GPR55 and Monoacylglycerol lipase (MAGL), further studies are needed to study the effect of

phytochemical variation on the other receptors, enzymes and transporter proteins with a larger sample size for more *Echinacea* species. Furthermore, as my work elucidated the impact of human metabolic system on the bioactivity of Echinacea components, pharmacokinetic factors also need to be considered in future studies.

Based on the positive result form the chronic inflammatory pain model in rat and overall good safety profile of *Echinacea* spp., clinical trails of the *Echinacea* spp. extracts in human are warranted to evaluate the efficacy to relief the peripheral pain cause by inflammation, diabetic complications, or even chemotherapy-induced peripheral neuropathy.

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REFERENCES

Aarland RC, Bañuelos-Hernández AE, Fragoso-Serrano M, Sierra-Palacios ED, Díaz de León-Sánchez F, Pérez-Flores LJ, Rivera-Cabrera F, Mendoza-Espinoza JA. Studies on phytochemical, antioxidant, anti-inflammatory, hypoglycaemic and antiproliferative activities of *Echinacea purpurea* and *Echinacea angustifolia* extracts. Pharm Biol. 2017; 55:649-656.

Andolina D, Maran D, Valzania A, Conversi D, Puglisi-Allegra S. Prefrontal/amygdalar system determines stress coping behavior through 5-HT/GABA connection. Neuropsychopharmacology. 2013; 38:2057–67.

Ahn K, Johnson DS, Cravatt BF. Fatty acid amide hydrolase as a potential therapeutic target for the treatment of pain and CNS disorders. Expert Opin Drug Discov. 2009; 4:763-784.

Alkaitis MS, Solorzano C, Landry RP, Piomelli D, DeLeo JA, Romero-Sandoval EA. Evidence for a role of endocannabinoids, astrocytes and p38 phosphorylation in the resolution of postoperative pain. PLoS One. 2010; 5(5): e10891.

Aragão GF, Carneiro LMV, Junior APF, Vieira LC, Bandeira PN, Lemos TL, Viana GS. A possible mechanism for anxiolytic and antidepressant effects of alpha- and beta-amyrin from *Protium heptaphyllum* (Aubl.) march. Pharmacol Biochem Behav. 2006; 85:827–34.

Arnason JT, Hebda RJ, Johns T. Use of plants for food and medicine by Native Peoples of eastern Canada. Can J Bot. 1981; 59: 2189-2325.

Awad R, Ahmed F, Bourbonnais-Spear N, Mullally M, Ta CA, Tang A, Merali Z, Maquin P, Caal F, Cal V, Poveda L, Vindas PS, Trudeau VL, Arnason JT. Ethnopharmacology of Q'eqchi' Maya antiepileptic and anxiolytic plants: effects on the GABAergic system. J Ethnopharmacol. 2009; 125:257–64.

Baell JB. Feeling Nature's PAINS: Natural Products, Natural Product Drugs, and Pan Assay Interference Compounds (PAINS). Nat Prod. 2016; 79:616-28.

Balciunaite G, Juodsnukyte J, Savickas A, Ragazinskiene O, Siatkute L, Zvirblyte G, Mistiniene E, Savickiene N. Fractionation and evaluation of proteins in roots of *Echinacea purpurea* (L.) Moench. Acta Pharm. 2015; 65:473-9.

Barnes PM, Powell-Griner E, McFann K, Nahin RL. Complementary and alternative medicine use among adults: United States, 2002. Adv Data. 2004;1–19.

Barnes J, Anderson LA, Gibbons S, Phillipson JD. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt. *Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. J Pharm Pharmacol. 2005; 57:929-54.

Barrie N, Manolios N. The endocannabinoid system in pain and inflammation: Its relevance to rheumatic disease. Eur J Rheumatol. 2017; 4(3):210-218.

Barrett B. Medicinal properties of *Echinacea*: a critical review. Phytomedicine. 2003; 10:66-86.

Battista N, Gasperi V, Fezza F, Maccarrone M. The anandamide membrane transporter and the therapeutic implications of its inhibition. Therapy. 2005; 2:141–150.

Bauer R, Remiger P. TLC and HPLC Analysis of Alkamides in Echinacea Drugs. Planta Med. 1989; 55:367-71.

Bauer, R. *Echinacea* – pharmazeutische qualität und therapeutischer wert. Z. Phytother. 1997; 18: 207–214.

Bauer, R. *Echinacea*: biological effects and active principles. In: Lawson, L. D., Bauer, R. (eds) Phytomedicines of Europe: chemistry and biological activity. American Chemical Society, Washington DC. 1998; Chapter 12:140–157

Bauer R. Chemistry, analysis and immunological investigations of *Echinacea* phytopharmaceuticals. Immunomodulatory Agents from Plants. 1999. 41-88.

Baum BR, Binns SE, Arnason JT. Integrating recent knowledge about the genus *Echinacea*: morphology, molecular systematics, phytochemistry. HerbalGram. 2006; 72:32–46.

Bel-Rhlid R, Pagé-Zoerkler N, Fumeaux R, Ho-Dac T, Chuat JY, Sauvageat JL, Raab T. Hydrolysis of chicoric and caftaric acids with esterases and Lactobacillus johnsonii in Vitro and in a gastrointestinal model. J Agric Food Chem. 2012; 60:9236-41.

Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, Piomelli D. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science. 1997; 277:1094-1097.

Bertz RJ, Granneman GR. Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. Clin. Pharmacokinet. 1997; 32:210-258.

Birt DF, Widrlechner MP, LaLone CA, Wu L, Bae J, Solco AKS, Kraus GA, Murphy PA, Wurtele ES, Leng Q, Hebert SC, Maury WJ, Price JP. *Echinacea* in infection. Am J Clin Nutr. 2008; 87: 488S–492S.

Blumenthal M, Hall T, Goldberg A, Kunz T, Dinda K (eds). The ABC guide to clinical herbs. American Botanical Council, Austin Texas. 2003; 235-46.

Binns SE, Purgina B, Bergeron C, Smith ML, Ball L, Baum BR, Arnason JT. Light-mediated antifungal activity of *Echinacea* extracts. Planta Med. 2000; 66:241-4.

Binns SE, Livesey JF, Arnason JT, Baum BR. Phytochemical variation in *echinacea* from roots and flowerheads of wild and cultivated populations. J Agric Food Chem. 2002 (A); 50:3673-87.

Binns SE, Livesey JF, Arnason JT, Baum BR. Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). Biochem Syst Ecol. 2002(B); 30: 837-854.

Bourbonnais-Spear N, Awad R, Maquin P, Cal V, Vindas PS, Poveda L, Arnason JT. Plant use by the q'eqchi' maya of belize in ethnopsychiatry and neurological pathology. Econ Bot. 2005; 59:326–336.

Bourbonnais-Spear N, Awad R, Merali Z, Maquin P, Cal V, Arnason JT. Ethnopharmacological investigation of plants used to treat *susto*, a folk illness. J Ethnopharmacol. 2007; 109:380–7.

Blaschek W, Doll M, Franz G. *Echinacea* – polysaccharide. Z Phytother. 1998; 19:255–262.

Bortolato M, Campolongo P, Mangieri RA, Scattoni ML, Frau R, Trezza V, La Rana G, Russo R, Calignano A, Gessa GL, Cuomo V, Piomelli D. Anxiolytic-like properties of the anandamide transport inhibitor AM404. Neuropsychopharmacology. 2006; 31:2652-2659.

Brantley SJ, Argikar AA, Lin YS, Nagar S, Paine MF. Herb-drug interactions: challenges and opportunities for improved predictions. Drug Metab Dispos. 2014; 42:301-17.

Budzinski JW, Foster BC, Vandenhoek S, Arnason JT. An in vitro evaluation of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures. Phytomedicine. 2000; 7:273-782.

Calabrese EJ. Hormesis: a revolution in toxicology, risk assessment and medicine. EMBO Rep. 2004; 5: S37-40.

Cayer C. In vivo Behavioural Characterization of Botanical Anxiolytics M.Sc. thesis, University of Ottawa. Ottawa, Ontario, 2011; http://hdl.handle.net/10393/20473; doi: 10.20381/ruor-5089.

Carballo AF. Phytochemical Investigations of Costa Rican Marcgraviaceae and Development of Insecticide Synergists, PhD thesis, University of Ottawa. 2014; http://hdl.handle.net/

Cech NB, Tutor K, Doty BA, Spelman K, Sasagawa M, Raner GM, Wenner CA. Liver enzyme-mediated oxidation of *Echinacea purpurea* alkylamides: production of novel metabolites and changes in immunomodulatory activity. lanta Med. 2006; 72:1372-1377.

Cichewicz RH, Kouzi SA. Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. Med Res Rev. 2004; 24:90-114.

Ciranna L. Serotonin as a modulator of glutamate- and GABA mediated neurotransmission: implications in physiological functions and in pathology. Curr Neuropharmacol. 2006; 4:101–14.

Chao PC, Hsu CC, Yin MC. Anti-inflammatory and anti-coagulatory activities of caffeic acid and ellagic acid in cardiac tissue of diabetic mice. Nutr Metab (Lond). 2009; 6:33.

He W, Fang T, Tu P. Research progress on pharmacological activities of echinacoside. Zhongguo Zhong Yao Za Zhi. 2009; 34:476-9.

Chicca A, Pellati F, Adinolfi B, Matthias A, Massarelli I, Benvenuti S, Martinotti E, Bianucci AM, Bone K, Lehmann R, Nieri P. Cytotoxic activity of polyacetylenes and polyenes isolated from roots of *Echinacea* pallida. Br J Pharmacol. 2008; 153:879-85.

Chicca A, Raduner S, Pellati F, Strompen T, Altmann KH, Schoop R, Gertsch J. Synergistic immunomopharmacological effects of N-alkylamides in *Echinacea purpurea* herbal extracts. Int Immunopharmacol. 2009; 9:850-858.

Chiou SY, Sung JM, Huang PW, Lin SD. Antioxidant, Antidiabetic, and Antihypertensive Properties of *Echinacea purpurea* Flower Extract and Caffeic Acid Derivatives Using In Vitro Models. J Med Food. 2017; 20:171-179.

Clayton N, Marshall FH, Bountra C, O'Shaughnessy CT. CB1 and CB2 cannabinoid receptors are implicated in inflammatory pain. Pain. 2002; 96:253-60.

Clifford LJ, Nair MG, Rana J, Dewitt DL. Bioactivity of alkamides isolated from *Echinacea purpurea* (L.) Moench. Phytomedicine. 2002; 9:249-53.

De Petrocellis L, Di Marzo V. An introduction to the endocannabinoid system: from the early to the latest concepts. Best Pract Res Clin Endocrinol Metab. 2009; 23:1-15.

DeLeo JA, Yezierski RP. The role of neuroinflammation and neuroimmune activation in persistent pain. Pain. 2001; 90:1-6.

Di Marzo V, Bisogno T, Melck D, Ross R, Brockie H, Stevenson L, Pertwee R, De Petrocellis L. Interactions between synthetic vanilloids and the endogenous cannabinoid system. FEBS Lett. 1998; 436:449-54.

Di Marzo V. New approaches and challenges to targeting the endocannabinoid system. Nat Rev Drug Discov. 2018; 17:623-639.

Dickason-Chesterfield AK, Kidd SR, Moore SA, Schaus JM, Liu B, Nomikos GG, Felder CC. Pharmacological characterization of endocannabinoid transport and fatty acid amide hydrolase inhibitors. Cell Mol Neurobiol. 2006; 26:407-23.

Dietz B, Heilmann J, Bauer R. Absorption of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides after oral application of *Echinacea purpurea* tincture. Planta Med. 2001; 67:863-4.

Dressler, S. Neotropical Marcgraviaceae. In: Milliken, W., Klitgard, B. & Baracat, A. (2009 onwards), Neotropikey - Interactive key and information resources for flowering plants of the Neotropics. 2009. http://www.kew.org/science/tropamerica/neotropikey/families/Marcgraviaceae. htm. March 2013

Durst T, Merali Z, Arnason JT, Sanchez-Vindas PE, Poveda Alvarez LJ. Anxiolytic Marcgraviaceae compositions containing betulinic acid, betulinic acid derivatives, and methods. 2009; US Patent 7488722.

Durst T, Baker J, Arnason JT, Wade M, Merali Z, Cayer C, Mullaly M, Alkamade S, Carballo AF. Plant compositions and methods and uses thereof for treating elevated glucocorticoid related disorders, and anxiety. 2015; US Pat. Appl. US 201502831.90A1.

Fernández-Ruiz J. The endocannabinoid system as a target for the treatment of motor dysfunction. Br J Pharmacol. 2009; 156:1029-40.

Fonseca FRD, Arco ID, Bermudez-Silva FJ, Bilbao A, Cippitelli A, Navarro M. The endocannabinoid system: physiology and pharmacology. Alcohol Alcohol, 2005; 40:2–14.

Fukasawa T, Suzuki A, Otani K. Effects of genetic polymorphism of cytochrome P450 enzymes on the pharmacokinetics of benzodiazepines. J Clin Pharm Ther. 2007; 32:333-341.

Gertsch J, Schoop R, Kuenzle U & Suter A. *Echinacea* alkylamides modulate TNF-α gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. FEBS Letters. 2004; 577:563-569.

Gertsch J, Raduner S, Altmann KH. New natural noncannabinoid ligands for cannabinoid type-2 (CB2) receptors. J Recept Signal Transduct Res. 2006; 26:709-30.

Godugu C, Patel AR, Doddapaneni R, Somagoni J, Singh M. Approaches to improve the oral bioavailability and effects of novel anticancer drugs berberine and betulinic acid. PLoS One. 2014; 9: e89919.

Gonthier MP, Verny MA, Besson C, Rémésy C, Scalbert A. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J Nutr. 2003; 133:1853-9.

Gonzalez LE, Andrews N, File SE. 5-HT(1A) and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. Brain Res. 1996; 732:145–53.

Gonzalez LE, Ouagazzal M, File SE. Stimulation of benzodiazepine receptors in the dorsal hippocampus and median raphe reveals differential GABAergic control in two animal tests of anxiety. Eur J Neurosci. 1998; 10:3673–80.

Greenblatt DJ, Allen MD, Noel BJ, Shader RI. Acute overdosage with benzodiazepine derivatives. Clin. Pharmacol. Ther. 1977; 21: 497-514.

Guimarães FS, Chiaretti TM, Graeff FG, Zuardi AW. Antianxiety effect of cannabidiol in the elevated plus-maze. Psychopharmacology, Psychopharmacology (Berl). 1990; 100:558-559.

Guindon J, Hohmann AG. Cannabinoid CB2 receptors: a therapeutic target for the treatment of inflammatory and neuropathic pain. Br J Pharmacol. 2008; 153(2):319-34.

Guindon J, Hohmann AG. The endocannabinoid system and pain. CNS Neurol Disord Drug Targets. 2009; 8(6):403-21.

Guindon J, Hohmann AG. The endocannabinoid system and cancer: therapeutic implication. Br J Pharmacol. 2011; 163:1447-1463.

Guiotto P, Woelkart K, Grabnar I, Voinovich D, Perissutti B, Invernizzi S, Granzotto M, Bauer R. Pharmacokinetics and immunomodulatory effects of phytotherapeutic lozenges (bonbons) with *Echinacea purpurea* extract. Phytomedicine. 2008; 15:547-54.

Haddad LM. Winchester JF. Clinical Management of Poisoning and Drug Overdose. 3rd ed, 1998. W.B. Saunders Company.

Haller J, Hohmann J, Freund TF. The effect of *Echinacea* preparations in three laboratory tests of anxiety: comparison with chlordiazepoxide. Phytotherapy Research, 24, 1605-1613. Phytother Res. 2010; 24:1605-1613.

Haller J, Freund TF, Pelczer KG, Füredi J, Krecsak L, Zámbori J. The anxiolytic potential and psychotropic side effects of an *Echinacea* preparation in laboratory animals and healthy volunteers. Phytother Res. 2013; 27:54-61.

Hamel P. and Chiltoskey M. Cherokee Plants and Their Uses—A 400 Year History. Herald Publishing Company, Sylva. 1975.

Han HK, Amidon GL. Targeted prodrug design to optimize drug delivery. AAPS PharmSci. 2000; 2: E6.

Harvard Medical School, 2007. National Comorbidity Survey (NCS). (2017, August 21). Retrieved from https://www.hcp.med.harvard.edu/ncs/index.php. Data Table 1: Lifetime prevalence DSM-IV/WMH-CIDI disorders by sex and cohort; Data Table 2: 12-month prevalence DSM-IV/WMH-CIDI disorders by sex and cohort.

Hasenoehrl C, Storr M, Schicho R. Cannabinoids for treating inflammatory bowel diseases: where are we and where do we go? Expert Rev Gastroenterol Hepatol. 2017; 11:329-337.

Häuser W, Fitzcharles MA, Radbruch L, Petzke F. Cannabinoids in Pain Management and Palliative Medicine. Dtsch Arztebl Int. 2017; 114:627-634.

Health Canada. Baseline natural health products survey among consumers. 2005.

Hemeryck A. and Belpaire FM. Selective serotonin reuptake inhibitors and cytochrome P-450 mediated drug-drug interactions: an update. Curr. Drug Metab. 2002; 3: 13-37.

Hoffman EJ, Mathew. Anxiety disorders: a comprehensive review of pharmacotherapies. SJ. Mt Sinai J Med. 2008; 75:248-62.

Hoffmann-La Roche Ltd. Valium Product Monograph. 2018.

Hohmann J, Rédei D, Forgo P, Szabó P, Freund TF, Haller J, Bojnik E, Benyhe S. Alkamides and a neolignan from *Echinacea purpurea* roots and the interaction of alkamides with G-protein-coupled cannabinoid receptors. Phytochemistry. 2011; 72:1848-1853.

Howlett AC. The cannabinoid receptors. Prostaglandins Other Lipid Mediat. 2002; 68-69:619-31.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev. 2002; 54:161-202.

Ho WSV, Kelly MEM. Cannabinoids in the Cardiovascular System. Adv Pharmacol. 2017; 80:329-366.

Hu C, Kitts DD. Studies on the antioxidant activity of *Echinacea* root extract. J Agric Food Chem. 2000; 48:1466-1472.

Huang WJ, Chen WW, Zhang X. Endocannabinoid system: Role in depression, reward and pain control (Review). Mol Med Rep. 2016; 14:2899-903.

Ibrahim MA, Mansoor AA, Gross A, Ashfaq MK, Jacob M, Khan SI, Hamann MT. Methicillin-Resistant Staphylococcus Aureus (MRSA)-Active Metabolites from *Platanus Occidentalis* (American Sycamore). J. Nat. Prod. 2009; 72:2141–2144.

ICH-Q2B. Validation of analytical procedure: Methodology International Conference on harmonization, Geneva 1-11. 1996. URL[http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf] accessed March 2013

Jäger S, Trojan H, Kopp T, Laszczyk MN, Scheffler A. Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent plant extracts. Molecules (Basel, Switzerland). 2009; 14: 2016–2031.

Jin X, Potter B, Luong TL, Nelson J, Vuong C, Potter C, Xie L, Zhang J, Zhang P, Sousa J, Li Q, Pybus BS, Kreishman-Deitrick M, Hickman M, Smith PL, Paris R, Reichard G, Marcsisin SR. Pre-clinical evaluation of CYP 2D6 dependent drug-drug interactions between primaquine and SSRI/SNRI antidepressants. Malar J. 2016; 15:280.

Kaczocha M, Glaser ST, Deutsch DG. Identification of intracellular carriers for the endocannabinoid anandamide. Proc Natl Acad Sci U S A. 2009; 106:6375-80.

Kelder J, Grootenhuis PD, Bayada DM, Delbressine LP & Ploemen JP. Polar Molecular Surface as a Dominating Determinant for Oral Absorption and Brain Penetration of Drugs. Pharm Res. 1999; 16:1514-1519.

Kennedy DA, Hart J, Seely D. Cost effectiveness of natural health products: a systematic review of randomized clinical trials. Evid Based Complement Alternat Med. 2009; 6:297-304.

Kenworthy KE, Bloomer JC, Clarke SE, Houston JB. CYP3A4 drug interactions: correlation of 10 in vitro probe substrates. Br J Clin Pharmacol. 1999; 48:716-27.

Kessler RC, Soukup J, Davis RB, Foster DF, Wilkey SA, Van Rompay MI, et al. The use of complementary and alternative therapies to treat anxiety and depression in the United States. Am J Psychiatry. 2001; 158:289–94.

Kessler RC, Berglund P, Demler O, Jin R, Walters EE. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the national comorbidity survey replication. Arch Gen Psychiatry. 2005; 62:593–602.

Kim TW, Chang SC, Lee JS, Hwang B, Takatsuto S, Yokota T, Kim SK. Cytochrome P450-catalyzed brassinosteroid pathway activation through synthesis of castasterone and brassinolide in Phaseolus vulgaris. Phytochemistry. 2004; 65:679-89.

Kim DH, Ahn T, Jung HC, Pan JG, Yun CH. Generation of the human metabolite piceatannol from the anticancer-preventive agent resveratrol by bacterial cytochrome P450 BM3. Drug Metab Dispos. 2009; 37:932-6.

King AR, Dotsey EY, Lodola A, Jung KM, Ghomian A, Qiu Y, Fu J, Mor M, Piomelli D. Discovery of potent and reversible monoacylglycerol lipase inhibitors. Chem Biol. 2009; 16:1045-52.

Kleyer J, Nicolussi S, Taylor P, Simonelli D, Furger E, Anderle P, Gertsch J. Cannabinoid receptor trafficking in peripheral cells is dynamically regulated by a binary biochemical switch. Biochem Pharmacol. 2012; 83:1393-1412.

LaLone CA, Rizshsky L, Hammer KD, Wu L, Solco AK, Yum M, Nikolau BJ, Wurtele ES, Murphy PA, Kim M, Birt DF. Endogenous levels of *Echinacea* alkylamides and ketones are important contributors to the inhibition of prostaglandin E2 and nitric oxide production in cultured macrophages. J Agric Food Chem. 2009; 57:8820-30.

Laszcyk, M. Pentacyclic tuterpenes of the lupane, oleanane and ursane group as tools in cancer therapy. Planta Med. 2009; 75: 1549–1560.

Lee SO, Wu L, Wurtele ES, Hendrich S. Identification of Bauer alkamide #8 in urine and feces of human subjects ingesting Echinacea root powder. FASEB J. 2007; 21, A111.

Lienert D, Anklam E, Panne U. Gas chromatography‐mass spectral analysis of roots of *Echinacea* species and classification by multivariate data analysis. Phytochem Anal. 1998; 9:88-98.

Liu CS, Chau SA, Ruthirakuhan M, Lanctôt KL, Herrmann N. Cannabinoids for the Treatment of Agitation and Aggression in Alzheimer's Disease. CNS Drugs. 2015; 29:615-23.

Liu J, Yang L, Dong Y, Zhang B, Ma X. Echinacoside, an Inestimable Natural Product in Treatment of Neurological and other Disorders. Molecules. 2018; 23:1213.

Lin L, Qiu S, Lindenmaier M, He X, Featherstone T, Cordell GA. Patuletin-3-O-rutinoside from the aerial parts of *Echinacea angustifolia*. Pharm Biol. 2002; 40: 92–95.

Liu M, Wilairat P, Go ML. Antimalarial alkoxylated and hydroxylated chalcones [corrected]: structure-activity relationship analysis. J Med Chem. 2001; 44:4443-52.

Liu R, Ahmed F, Cayer C, Mullally M, Carballo AF, Rojas MO, Garcia M, Baker J, Masic A, Sanchez PE, Poveda L. Merali Z, Durst T, Arnason JT. New Botanical Anxiolytics for Use in Companion Animals and Humans. AAPS J. 2017; 19:1626-31.

Loflin M, Earleywine M, De Leo J, Hobkirk A. Subtypes of attention deficit-hyperactivity disorder (ADHD) and cannabis use. Subst Use Misuse. 2014; 49:427-34.

Mahmood I, Sahajwalla C. Clinical pharmacokinetics and pharmacodynamics of buspirone, an anxiolytic drug. Clin Pharmacokinet. 1999; 36:277-87.

Mai I, Bauer S, Perloff ES, Johne A, Uehleke B, Frank B, Budde K, Roots I. Hyperforin content determines the magnitude of the St John's wort–cyclosporine drug interaction. Clin Pharmacol Ther. 2004; 76:330-340.

Manayi A, Vazirian M, Saeidnia S. *Echinacea purpurea*: Pharmacology, phytochemistry and analysis methods. Pharmacogn Rev. 2015; 9:63-72.

Mandrioli R, Forti GC, Raggi MA. Fluoxetine metabolism and pharmacological interactions: the role of cytochrome p450. Curr. Drug Metab. 2006; **7**: 127-33.

Martignoni M, Groothuis GM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin Drug Metab Toxicol. 2006; 2:875-94.

Masic A, Landsberg G, Milgram B, Baker J, Simkus K, Dick P, Hand K, de Rivera C, Merali Z, Durst T, Sanchez P, Garcia M, Wilson J, Arnason JT. Efficacy in beagle dogs of Souroubea Botanical™ tablets, a canine calming agent containing *Souroubea* spp. and *Platanus* spp. Can J Vet Res. 2016.

Masric A, Liu R, Simkus K, Wilson J, Baker J, Sanchez P, Saleem A, Harris C, Durst T, Arnason JT. Safety evaluation of a new anxiolytic product containing botanicals *Souroubea* spp and *Platanus* spp in Can J Vet Res. 2018; 82:3-11.

Mathews JM, Etheridge AS, Black SR. Inhibition of human cytochrome P450 activities by kava extract and kavalactones. Drug Metab Dispos. 2002;30(11):1153-7.

Matthias A, Penman KG, Matovic NJ, Bone KM, De Voss JJ, Lehmann RP. Bioavailability of *Echinacea* constituents: Caco-2 monolayers and pharmacokinetics of the alkylamides and caffeic acid conjugates. Molecules. 2005A; 10:1242-51.

Matthias A, Gillam EM, Penman KG, Matovic NJ, Bone KM, De Voss JJ, Lehmann RP. Cytochrome P450 Enzyme-mediated Degradation of *Echinacea* Alkylamides in Human Liver Microsomes. Chem Biol Interact. 2005B; 155(1-2): 62-70.

Matthias A, Banbury L, Bone KM, Leach DN, Lehmann RP. *Echinacea alkylamides* modulate induced immune responses in T-cells. Fitoterapia. 2008; 79:53-58.

Menard J, Treit D. Effects of centrally administered anxiolytic compounds in animal models of anxiety. Neurosci Biobehav Rev. 1999; 23:591–613.

Modarai M, Gertsch J, Suter A, Heinrich M, Kortenkamp A. Cytochrome P450 inhibitory action of Echinacea preparations differs widely and co-varies with alkylamide content. J Pharm Pharmacol. 2007; 59:567-573.

Moerman, D. E. Native american ethnobotany (Vol. 879). Portland, OR: Timber press. 1998.

Moreira Fde L, De Souza GH, Rodrigues IV, Lopes NP, de Oliveira AR. A non-michaelian behavior of the in vitro metabolism of the pentacyclic triterpene alfa and beta amyrins by employing rat liver microsomes. J Pharm Biomed Anal. 2013; 84:14-19.

Muccioli GG. Endocannabinoid biosynthesis and inactivation, from simple to complex. Drug Discov Today. 2010; 15:474-83.

Muccioli GG, Naslain D, Bäckhed F, Reigstad CS, Lambert DM, Delzenne NM, Cani PD. The endocannabinoid system links gut microbiota to adipogenesis. Mol Syst Biol. 2010; 6:392.

Mullally M, Kramp K, Saleem A, Otarola-Rojas M, Vindas P, Garcia M, Alvarez L, Durst T, Trudeau V, Arnason JT. Characterization and quantification of triterpenes in the neotropical medicinal plant Souroubea sympetala (Marcgraviaceae) by HPLC-APCI-MS. Nat Prod Commun. 2008; 3:1885–1888.

Mullally M, Kramp K, Cayer C, Saleem A, Ahmed F, McRae C, Baker J, Goulah A, Otorola M, Sanchez P, Garcia M, Poveda L, Merali Z, Durst T, Trudeau VL, Arnason JT. Anxiolytic activity of a supercritical carbon dioxide extract of *Souroubea sympetala* (Marcgraviaceae). Phyther Res. 2011; 25:264–70.

Mullally M, Cayer C, Kramp K, Otárola Rojas M, Sanchez Vindas P, Garcia M, Poveda Alvarez L, Durst T, Merali Z, Trudeau VL, Arnason JT. *Souroubea sympetala* (Marcgraviaceae): a medicinal plant that exerts anxiolysis through interaction with the $GABA_A$ benzodiazepine receptor. Can J Physiol Pharmacol. 2014; 92:758–64.

Mullally M, Mimeault C, Rojas M, Vindas P, Garcia M, Alvarez L, Moon TW, Gilmoura KM, Trudeau VL, Arnason JT. A botanical extract of *Souroubea sympetala* and its active principle, betulinic acid, attenuate the cortisol response to a stressor in rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 2017; 468(1):26–31.

Mulvihill MM, Nomura DK. Therapeutic potential of monoacylglycerol lipase inhibitors. Life Sci. 2013; 92:492-497.

Murataeva N, Straiker A, Mackie K. Parsing the players: 2-arachidonoylglycerol synthesis and degradation in the CNS. Br J Pharmacol. 2014 Mar;171(6):1379-91.

Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M. Cannabinoids as novel anti-inflammatory drugs. Future Med Chem. 2009; 1:1333-49.

Nicolussi S, Gertsch J. Endocannabinoid transport revisited. Vitam Horm. 2015; 98:441-85.

Nicolussi S, Chicca A, Rau M, Rihs S, Soeberdt M, Abels C, Gertsch J. Correlating FAAH and anandamide cellular uptake inhibition using N-alkylcarbamate inhibitors: from ultrapotent to hyperpotent. Biochem Pharmacol. 2014; 92:669-89.

Oboh G, Agunloye OM, Adefegha SA, Akinyemi AJ, Ademiluyi AO. Caffeic and chlorogenic acids inhibit key enzymes linked to type 2 diabetes (in vitro): a comparative study. J Basic Clin Physiol Pharmacol. 2015; 26:165-70.

Oddi S, Fezza F, Pasquariello N, D'Agostino A, Catanzaro G, De Simone C, Rapino C, Finazzi-Agrò A, Maccarrone M. Molecular identification of albumin and Hsp70 as cytosolic anandamide-binding proteins. Chem Biol. 2009; 16:624-32.

Olthof MR, Hollman PC, Katan MB. Chlorogenic acid and caffeic acid are absorbed in humans. J Nutr. 2001; 131:66-71.

Ortega-Gutiérrez S, Hawkins EG, Viso A, López-Rodríguez ML, Cravatt BF. Comparison of anandamide transport in FAAH wild-type and knockout neurons: evidence for contributions by both FAAH and the CB1 receptor to anandamide uptake. Biochemistry. 2004; 43:8184-8190.

Otrubova K, Ezzili C, Boger DL. The discovery and development of inhibitors of fatty acid amide hydrolase (FAAH). Bioorg Med Chem Lett. 2011; 21:4674-85.

Ovesná Z, Kozics K, Bader Y, Saiko P, Handler N, Erker T, Szekeres T. Antioxidant activity of resveratrol, piceatannol and 3,3',4,4',5,5'-hexahydroxy-trans-stilbene in three leukemia cell lines. Oncol Rep. 2006; 16:617-24.

Parsons JL, Cameron SI, Harris CS, Smith ML. Echinacea biotechnology: advances, commercialization and future considerations. Pharm Biol. 2018;56(1):485-494.

Pertwee, R. G. The pharmacology of cannabinoid receptors and their ligands: an overview. Int J Obes. 2006; 30: S13-S18.

Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, Mechoulam R, Ross RA. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2. Pharmacol Rev. 2010; 62:588-631.

Pirovano A, Huijbregts MA, Ragas AM, Hendriks AJ. Compound lipophilicity as a descriptor to predict binding affinity (1/K(m)) in mammals. Environ Sci Technol. 2012; 46:5168-74.

Pietta P, Mauri P, Bauer R. MEKC analysis of different species. Planta Med. 1998; 64: 649–652.

Price DM, Kindscher K. One Hundred Years of *Echinacea angustifolia* Harvest in the Smoky Hills of Kansas, USA. Econ. Bot. 2007; 61:86–95.

Puniani E. Novel natural product based antianxiety therapy and natural insecticides, PhD thesis University of Ottawa. 2004.

Puniani E, Cayer C, Kent P, Mullally M, Sánchez-Vindas P, Poveda Álvarez L, Cal V, Merali Z, Arnason JT, Durst T. Ethnopharmacology of *Souroubea sympetala* and *Souroubea gilgii* (Marcgraviaceae) and identification of betulinic acid as an anxiolytic principle. Phytochemistry. 2015; 113:73–8.

Raduner S, Majewska A, Chen JZ, Xie XQ, Hamon J, Faller B, Altmann KH, Gertsch J. Alkylamides from *Echinacea* are a new class of cannabinomimetics. Cannabinoid type 2 receptor-dependent and -independent immunomodulatory effects. J Biol Chem. 2006; 281:14192-206.

Rani Sagar D, Burston JJ, Woodhams SG, Chapman V. Dynamic changes to the endocannabinoid system in models of chronic pain. Philos Trans R Soc Lond B Biol Sci. 2012; 367:3300-11.

Rahn EJ, Hohmann AG. Cannabinoids as pharmacotherapies for neuropathic pain: from the bench to the bedside. Neurotherapeutics. 2009; 6:713-37.

Rao N. The clinical pharmacokinetics of escitalopram. Clin Pharmacokinet. 2007; 46:281-90.

Rapinski M, Liu R Saleem A, Arnason JT, Cuerrier A. Environmental trends in the variation of biologically active phenolic compounds in Labrador tea, Rhododendron groenlandicum, from northern Quebec, Canada. Botany. 2014; 92: 783–794.

Rastogi H, Jana S. Evaluation of inhibitory effects of caffeic acid and quercetin on human liver cytochrome p450 activities. Phytother Res. 2014; 28:1873-8.

Ravindran LN, Stein MB. The pharmacologic treatment of anxiety disorders: a review of progress. J Clin Psychiatry. 2010; 71:839–54.

Rendic S, Di Carlo FJ. Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and Inhibitors. Drug Metab. Rev. 1997; 29: 413-580.

Rezayat M, Roohbakhsh A, Zarrindast MR, Massoudi R, Djahanguiri B. Cholecystokinin and GABA interaction in the dorsal hippocampus of rats in the elevated plus-maze test of anxiety. Physiol Behav. 2005; 84:775–82.

Roder E, Wiedenfeld H, Hille T, Britz-Kirstgen R. Pyrrolizidine in *Echinacea angustifolia* DC und *Echinacea purpurea.* M Arzneimittelforschung. 1984; 124: 2316–2317.

Rondanelli M, Riva A, Morazzoni P, Allegrini P, Faliva MA, Naso M, Miccono A, Peroni G, Degli Agosti I, Perna S. The effect and safety of highly standardized Ginger (*Zingiber officinale*) and *Echinacea* (*Echinacea angustifolia*) extract supplementation on inflammation and chronic pain in NSAIDs poor responders. A pilot study in subjects with knee arthrosis. Nat Prod Res. 2017; 31:1309-1313.

Russo EB. Beyond Cannabis: Plants and the Endocannabinoid System. Trends Pharmacol Sci. 2016; 37:594-605.

Sacre L, Ali SM, Villa A, Jouffroy R, Raphalen JH, Garnier R, Baud FJ. Toxicodynetics in nordiazepam and oxazepam overdoses. Ann Pharm Fr. 2017; 75:163-171.

Schultes RE, Raffauf RF. The healing Forest: medicinal and toxic plants of the Northwest Amazonia. Portland: Dioscorides Press; 1990.

Seervi M, Lotankar S, Barbar S, Sathaye S. Assessment of cytochrome P450 inhibition and induction potential of lupeol and betulin in rat liver microsomes. Drug Metab Pers Ther. 2016; 31:115-22.

Sevrioukova IF, Poulos TL. Current Approaches for Investigating and Predicting Cytochrome P450 3A4-Ligand Interactions. Adv Exp Med Biol. 2015; 851:83-105.

Shamloo M, Babawale EA, Furtado A, Henry RJ, Eck PK, Jones PJH. Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments. Sci Rep. 2017; 7:9133.

Sharifi-Rad M, Mnayer D, Morais-Braga MFB, Carneiro JNP, Bezerra CF, Coutinho HDM, Salehi B, Martorell M, Del Mar Contreras M, Soltani-Nejad A, Uribe YAH, Yousaf Z, Iriti M, Sharifi-Rad J. *Echinacea* plants as antioxidant and antibacterial agents: From traditional medicine to biotechnological applications. Phytother Res. 2018; 32:1653-1663.

Shemluck M. Medicinal and other uses of the Compositae by Indians in the United States and Canada. J Ethnopharmacol. 1982; 5:303-58.

Shimada T, Tanaka K, Takenaka S, Murayama N, Martin MV, Foroozesh MK, Yamazaki H, Guengerich FP, Komori M. Structure-function relationships of inhibition of human cytochromes P450 1A1, 1A2, 1B1, 2C9, and 3A4 by 33 flavonoid derivatives. Chem Res Toxicol. 2010; 23:1921-35.

Sloley BD, Urichuk LJ, Tywin C, Coutts RT, Pang PK, Shan JJ. Comparison of chemical components and antioxidants capacity of different *Echinacea* species. J Pharm Pharmacol. 2001; 53:849-57.

Smith T, Kawa K, Eckl V, Johnson J. Sales of Herbal Dietary Supplements in US Increased 7.5% in 2015 Consumers spent $6.92 billion on herbal supplements in 2015, marking the 12th consecutive year of growth. HerbalGram. 2016; 111: 67-73.

Stanisavljević I, Stojičević S, Veličković D, Veljkovića V, Lazića M. antioxidant and antimicrobial activities of *echinacea* (*Echinacea purpurea* L.) extracts obtained by classical and ultrasound extraction. Chin. J. Chem. Eng. 2009; 17:478-483.

Svízenská I, Dubový P, Sulcová A. Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures--a short review. Pharmacol Biochem Behav. 2008; 90:501-511.

Tam TW, Liu R, Arnason JT, Krantis A, Staines WA, Haddad PS, Foster BC. Actions of ethnobotanically selected Cree anti-diabetic plants on human cytochrome P450 isoforms and flavin-containing monooxygenase 3. J Ethnopharmacol. 2009; 126:119-26.

Tambaro S, Bortolato M. Cannabinoid-related agents in the treatment of anxiety disorders: current knowledge and future perspectives. Recent Pat CNS Drug Discov. 2012; 7:25-40.

Toselli F, Matthias A, Bone KM, Gillam EM, Lehmann RP. Watanabe M. Metabolism of the major *Echinacea* alkylamide N-isobutyldodeca-2E,4E,8Z,10Z-tetraenamide by human recombinant cytochrome P450 enzymes and human liver microsomes. Phytother Res. 2010; 24:1195-201.

Udeani GO, Zhao GM, Geun Shin Y, Cooke BP, Graham J, Beecher CW, Kinghorn AD, Pezzuto JM. Pharmacokinetics and tissue distribution of betulinic acid in CD-1 mice. Biopharm Drug Dispos. 1999; 20:379–83.

Van der Watt G, Laugharne J, Janca A. Complementary and alternative medicine in the treatment of anxiety and depression. Curr Opin Psychiatry. 2008; 21:37–42.

Veryser L, Taevernier L, Joshi T, Tatke P, Wynendaele E, Bracke N, Stalmans S, Peremans K, Burvenich C, Risseeuw M, De Spiegeleer B. Mucosal and blood-brain barrier transport kinetics of the plant N-alkylamide spilanthol using in vitro and in vivo models. BMC Complement Altern Med. 2016; 16:177.

Villalobos P, Baker J, Sanchez P, Durst T, Masic A, Arnason JT. Clinical observations and safety profile of oral herbal products, *Souroubea* and *Platanus* spp.: a pilot-toxicology study in dogs. Acta Vet-Beogr. 2014; 64(2):269–75.

Wanwimolruk S, Prachayasittikul V. Cytochrome P450 enzyme mediated herbal drug interactions (Part 1). EXCLI J. 2014; 13:347–391.

Wen Z, Martin DE, Bullock P, Lee KH, Smith PC. Glucuronidation of anti-HIV drug candidate bevirimat: identification of human UDP-glucuronosyltransferases and species differences. Drug Metab Dispos. 2007; 35:440-448.

Wichtl, M. (ed.) Herbal drugs and phytopharmaceuticals. A handbook for practice on a scientific basis, 3rd edn. 2004. Medpharm Scientific Publishers, Stuttgart.

Whiting PF, Wolff RF, Deshpande S, Di Nisio M, Duffy S, Hernandez AV, Keurentjes JC5, Lang S, Misso K, Ryder S, Schmidlkofer S, Westwood M, Kleijnen J. JAMA. Cannabinoids for Medical Use: A Systematic Review and Meta-analysis. 2015; 313:2456-73.

Whitten DL, Myers SP, Hawrelak JA, Wohlmuth H. The effect of St. John‘s wort extracts on CYP3A: A systematic review of prospective clinical trials. Br. J. Clin. Pharmacol. 2006; 62: 512–526.

World Health Organization. WHO Traditional Medicine Strategy 2014-2023. 2013.

Woelkart K, Koidl C, Grisold A, Gangemi JD, Turner RB, Marth E, Bauer R. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia* in humans. J Clin Pharmacol. 2005A; 45:683-9.

Woelkart K, Xu W, Pei Y, Makriyannis A, Picone RP, Bauer R. The endocannabinoid system as a target for alkamides from *Echinacea* angustifolia roots. Planta Med. 2005B; 71:701-5.

Woelkart K, Frye RF, Derendorf H, Bauer R, Butterweck V. Pharmacokinetics and tissue distribution of dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides after oral administration in rats. Planta Med. 2009; 75:1306-13.

Woelkart K, Kollroser M, Magnes C, Sinner F, Frye RF, Derendorf H, Bauer R, Butterweck V. Absolute/relative bioavailability and metabolism of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (tetraenes) after intravenous and oral single doses to rats. Planta Med. 2011; 77(16):1794-9.

Wonnemann M, Helm I, Stauss-Grabo M, Röttger-Luer P, Tran CT, Canenbley R, Donath F, Nowak H, Schug BS, Blume HH. Lidocaine 8 mg sore throat lozenges in the treatment of acute pharyngitis. A new therapeutic option investigated in comparison to placebo treatment. Arzneimittelforschung. 2007; 57:689-97.

Yin MC, Lin MC, Mong MC, Lin CY. Bioavailability, distribution, and antioxidative effects of selected triterpenes in mice. J Agric Food Chem. 2012; 60(31):7697-7701.

Yogeeswari P, Sriram D. Betulinic acid and its derivatives: a review on their biological properties. Curr Med Chem. 2005; 12:657–66.

Yu D, Yuan Y, Jiang L, Tai Y, Yang X, Hu F, Xie Z. Anti-inflammatory effects of essential oil in *Echinacea purpurea* L. Pak J Pharm Sci. 2013; 26:403-8.

Zhang Y, Raap DK, Garcia F, Serres F, Ma Q, Battaglia G, Van de Kar LD. Long-term fluoxetine produces behavioral anxiolytic effects without inhibiting neuroendocrine responses to conditioned stress in rats. Brain Res. 2000; 855:58-66.

APPENDIX 1 SACRED MAYA INCENSE, COPAL (PROTIUM COPAL- BURSERACEAE), HAS ANTIANXIETY EFFECTS IN ANIMAL MODELS.

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Abstract

Ethnopharmacological relevance

The Maya have traditionally used copal, *Protium copal,* as incense during ceremonies since pre-Columbian times. Anecdotally, copal (when burned as incense), is thought to elicit mentally uplifting and calming effects. The main objective of this study was to determine whether the incense elicits anxiolytic-like behavior in animal models using rats. A second objective was to characterize active constituents and discern potential mechanism(s) of action, specifically the involvement of the GABAergic and endocannabinoid (eCB) systems. Despite the extensive Central American use of this resin, there are currently no known scientific behavioral or pharmacological studies done with the incense.

Materials and methods

Quantification of the triterpenes in the copal resin and cold trapped incense was achieved by HPLC MS. Behavioral effects in rats were assessed using the elevated plus maze (EPM), social interaction (SI) test, conditioned emotion response (CER) and Novel object recognition (NOR) paradigms. Rats were exposed to burning copal (200 mg) over 5 min in a smoking chamber apparatus and then immediately tested in each behavioral paradigm. Follow-up SI tests were done using two antagonists flumazenil (1 mg/kg) and AM251 (1 mg/kg) administered systemically. Inhibition of MAGL (monoacylglycerol lipase) was measured by microplate assay with recombinant human enzyme and probe substrate.

Results

Phytochemical analysis revealed that copal resin and incense had high α- and β-amyrins and low lupeol triterpene content. Exposure to *Protium copal*incense significantly reduced

anxiety-like behavior in the SI and CER tests. In contrast, no anxiolytic effects were observed in the EPM. The CER effect was time dependent. Both flumazenil and AM251 blocked the anxiolytic activity of copal revealing the involvement of GABAergic and endocannabinoid systems. Copal, as well as the identified triterpenes, potently inhibited monoacylglycerol lipase (MAGL) activity in vitro ($IC_{50} \leq 811$ ng/mL).

Conclusions

This is the first study to show that copal incense from *Protium copal* elicits anxiolytic-like effects in fear and social interaction models as evidenced by a reduced learned fear behavior and an increase in active social interaction. It's high α and β-amyrin content suggests behavioral effects may be mediated, in part, by the known action of these terpenes at the benzodiazepine receptor. Furthermore, *P. copal*'s observed activity through the eCB system via MAGL offers a new potential mechanism underlying the anxiolytic activity.

1. Introduction

Copal resin (Pom in several Maya languages) harvested from the bark of the lowland tropical tree *Protium copal* L. (Burseraceae) is a sacred essence (Itz) through which the Maya Gods manifest themselves on earth (Schele and Freidel, 1993). Documented in the rich Maya archeological record, copal (mainly but not exclusively *P. copal* resin) has been used as an incense throughout the Maya territory of Mesoamerica (Figure. A1-1), during important religious ceremonies (Case et al., 2003). Today, the incense is still used widely in traditional Maya ceremonies (Fig. 1) such as celebration of the 260 day ceremonial calendar (the Tzolkin), healing ceremonies to purify and restore patients to health, and to bless agricultural crops. Copal represents one of the most important ritual and ceremonial plants of many species which are widely used not only by indigenous peoples of the Americas (Turi and Murch, 2013), but in religious rituals/ceremonies in certain current cultural practices.

When used in ceremonies, copal creates a sacred atmosphere for participants. For those who have participated in Maya ceremonies, the effect of copal incense on mood – at least anecdotally – is often rapid and intense. Clearly, this mood is created, in large part, by the reverence the Maya have for their traditional gatherings and the customary and widespread use of the incense at the start of these ceremonies. In this report we examined whether *P. copal* may also have direct pharmacological effect on neurochemical processes affecting mood, through the rapid absorption of inhaled volatiles.

Protium copal resin is generally characterized by its distinct odor and relatively high melting point. Copal resins have a hard, adhesive, gum-like texture and are typically quite malleable, but can harden into amber through polymerization, oxidation, and the loss of essential

Figure 1A-1. A. Left: Stella (7th century) at Nimli Punit Ceremonial Site showing spiritual leaders placing copal in sacred fire B. modern calendar ceremony at Lubantuun ceremonial site with Maya spiritual leaders placing copal in sacred fire.

oils (Rao et al., 2013). Phytochemical analyses reveal that *P. copal* resins are mainly composed of mixture of terpenoids. The essential oil of *P. copal* is dominated by alpha-pinene, sabinene and limonene (Hernández-Vázquez et al., 2010; Lucero-Gómez et al., 2014). Analysis of the related South American *Protium hepataphyllum* and *P icicariba* by Gas Chromatography-Mass Spectrometry revealed that α-amyrin, and β-amyrin are the major pentacyclic triterpenes in the resin. Aragão et al. (2006) reported anxiolytic and antidepressant effects of bark extracts of Brazilian *P. heptaphyllum*when administered orally or intraperitoneally in some animal models. Examination of the mechanisms responsible for these effects suggested that α- and β-amyrins interact with benzodiazepine-type $GABA_A$ receptors to produce sedative and/or anxiolytic effects but also increase noradrenergic activity to evoke antidepressant action (Aragão et al., 2006). Other researchers (Chicca and Gertsch, 2012), have reported that the isolated triterpene, β-amyrin, is also an inhibitor of monoacylglycerol lipase (MAGL). By blocking endocannabinoid (2-arachidonoylglycerol) degradation, MAGL inhibitors enhance endocannabinoid signaling and elicit antidepressant and anxiolytic effects (Wang et al., 2016). Essential oils and resins from *Protium* species also demonstrate anti-inflammatory activity in both animal- and cell-based experimental models (Siani et al., 1999; Melo et al., 2011)

The behavioral effects of inhaled *Protium copal* incense have not been examined. The purpose of this study was to assess potential anxiolytic and cognitive effects of *P. copal* incense using multiple validated behavioral paradigms. We hypothesized that rats exposed to copal incense will exhibit lower levels of anxiety-like behaviors in the elevated plus-maze (EPM) test, social interaction (SI) test, and conditioned emotional response (CER) paradigms, and could elicit cognitive exploratory behavior (NOR). While *Protium* components interact with

benzodiazepine-type $GABA_A$ receptors, the chemistry and anxiolytic-like activity of *P. copal* and, more pertinently, copal incense was unknown until studied here.

2. Materials and Methods

2.1 Plant material

A sample of *Protium copal* resin was collected in the Toledo district Southern Belize under permit from the Belize forest service, then stored in a dry area at room temperature away from direct sunlight. A voucher specimen was collected and deposited in the University of Ottawa Herbarium (OTT No19199).2.2 Phytochemical analysis

2.2 Phytochemical analysis

A sample of *P. copal* resin was ground through 1 mm mesh. A portion (0.5 g) of ground material was extracted in triplicate with 250 mL ethyl acetate using a soxlet apparatus for 2 h. The extract was dried by rotatory evaporation at 40 °C, and re-dissolved in 20 mL of methanol (Lot# SHBB8243V) by sonication for 2 min. The pellet was re-extracted in 20 mL methanol by sonication for 2 min. Total volume was adjusted to 50 mL in a volumetric flask. A 1.0 mL sample of extract was filtered through 0.22 µm PTFE filter analysis by HPLC-MS-MS (using a Sciex3200 QTRAP). The extract was stored at −20 °C.

To analyze the chemistry of *P. copal* vapor, we vaporized 200 mg of resin as described for animal trials (below) and connected two cold traps in series from the vaporization chamber to a Fisherbrand™ Air Cadet™ Vacuum/Pressure Station (Fisher Scientific, Ottawa). Resin vapor was drawn through fritted glass gas dispersion tubes into consecutive glass cold traps in an ice bath, each filled with 80 mL of methanol. The two methanol extracts were then pooled and dried by rotoevaporation at room temperature then stored at −20 °C. Replicates of the vapor extract were reconstituted in methanol (10 mg/mL) on the day of analysis.

Phytochemical separation of resin and resin vapor extracts was achieved on a Kinetex 2.6 μm, 100 mm × 2.1 mm i.d. column (Phenomenex Inc.). The injection volume was 1 μL, column oven temperature was 55 °C and flow rate was 0.4 mL/min. The mobile phases (LC/MS grade) were: A = water, B = acetonitrile with gradient elution: 30–100% B in 10 min, hold 100% B for 8 min, 100–30% B in 7 min, hold 30% B 3 to equilibrate 8 min. Detection was achieved using Q1M1 negative (betulinic acid and ursolic acid), Q1M1 positive (betulin, lupeol and α-amyrin and β-amyrin) ionization modes. Authentic standards of betuliic acid, betulin, lupeol and α-amyrin and β-amyrin (Sigma) were used to develop a linear standard curve for quantitation and to determine recovery (> 95%) in spike recovery experiments.

2.*3 Animals*

The behavioral experiments were conducted with male Sprague-Dawley rats (225–250 g body mass; Charles River Laboratories Inc., St. Constant, Quebec). Rats weighing approximately 225 g upon arrival were housed individually and maintained under standard animal housing conditions (clear Plexiglas cages, 24 × 30 × 18 cm, 12 h light-dark cycle, 21±1 °C, 60% humidity, Purina Lab Chow and tap water ad libitum). All experimental procedures were approved by the Research Ethics Committee of the University of Ottawa Institute of Mental Health Research and met the guidelines set out by the Canadian Council on Animal Care (CCAC) (ACC-2015–003 at the IMHR). All attempts were made to minimize the number of animals used in the study, while maintaining the integrity of the experiments and results

2.4 Exposure chamber

Prior to each behavioral test, rats were placed in the specially designed exposure chamber for 5 min. The exposure chamber constituted of a standard housing cage with the ceiling sealed

with Parafilm to contain the resin vapor and prevent its escape. The exposure chamber was connected via rubber latex tubing to a vaporizing chamber containing a heating coil and was also connected to an electric pump, which created a gentle vacuum to pull the copal incense through to the exposure chamber. The animals in the control group were subjected to similar conditions, except that the tube from the heating coil was disconnected to allow for the entry of fresh air. Separate exposure chambers and tubing were used for control and experimental groups. Prior to the SI test, rats from each pair were exposed to this phase simultaneously in two separate cages. Between testing, the exposure chambers were cleaned with 70% ethanol and furnished with fresh new bedding.

2.5 Behavioral paradigms

Behavioral paradigms and apparatus used have been described in detail previously in Cayer et al. (2013). Briefly the elevated plus maze (EPM) is a validated test used to assess anxiety-like behavior in laboratory rodents (Pellow and File, 1986). The EPM consists of two open arms (planks; 50 × 10 cm), two perpendicular arms enclosed by 40 cm high walls (alleys) and is placed 50 cm above the ground. The EPM is based on the conflict between the animal's instinct to explore its environment and its fear of more exposed (vulnerable to attack) areas and the height. The percent of time spent in the open arms, and unprotected head dips are validated measures of anxiety-like behavior in the EPM which increases in these measures indicative of reduced anxiety (File, 1995). In contrast, the frequency of arm entries is an index of locomotor activity (Cruz et al., 1994).

The Conditioned emotional response (CER) is a validated measure of fear in animals. In this test, rats that have been previously conditioned by exposure to a mild foot shock, are placed

back in the conditioning chamber (where they had previously been shocked), and contextual fear was assessed by measuring the freezing response over a 20-min period. Freezing behavior was defined by the absence of movement (excluding involuntary respiratory activity). For the CER test, the measure of interest was the percentage of time spent freezing over the 10-min interval (Davis, 1994; Luyten et al., 2011).

Social interaction (SI) was assessed in pairs of animals and was determined by the sum of the following behaviors: sniffing, climbing over and under, following, allogrooming and play fighting. An increase in total active social interaction is indicative of reduced anxiety (File and Seth, 2003).

Novel object recognition (NOR) is cognitive task that assesses working memory, short-term memory and long-term memory depending on the length of the delay between trials. Rats were singly-housed for over a week before the test day. Habituation to the open field box took place the day before the test in order to familiarize the rats with the area once again. This prevented environmental factors (i.e. unfamiliarity) from confounding the animal's interest in the objects. On test day, the NOR task was divided into two phases: acquisition- and test-phases. The acquisition phase consisted of placing the rat in the open arena for 3.5 min with two identical objects (A_1 and A_2) in opposite corners. Following the acquisition phase, there was a 2 h interval before exposing the animals to their respective treatments and entering the test phase. The test phase is similar to the acquisition phase, however, one of the objects (A_1 or A_2), is replaced by a novel object (B). The rat is given 3.5 min to freely explore these objects. Behaviors such as sniffing, licking, and touching the object with forepaws whilst sniffing, were all scored as measures of object exploration. Increased exploration time of the novel object (B) compared to the other object (A_1 or A_2) in the test phase is indicative of better memory function (Carlini et al.,

2012). Objects used in this experiment were constructed from Duplo LEGO pieces. The novel object differed from the other object in colour and shape. Objects were approximately 6 in. tall and wide to ensure rats do not climb over or sit on the objects. Low light levels were used in this experiment to prevent aversive exploratory conditions for the animals.

2. 6 Drug administration of receptor agonists

Both GABAa and CB1 receptor antagonists were administered in a 1 mL/kg volume. Flumazenil (Sigma Aldrich) was dissolved in 0.9% NaCl and, AM251 (Tocris) was dissolved in 5% polyethylene glycol, 5% Tween-80%, and 90% saline. For the smoke control group. 200 mg of the vehicle (0 mg nicotine) was administered.

2.7 MAGL assay

Copal extract, each of the three principle triterpene constituents (α-amyrin, β-amyrin, lupeol) (Sigma Aldrich, Oakville) and the positive control, JZL184 (Cayman Chemical, Ann Arbor) were solubilized and serially diluted in DMSO with an equal part of ddH_2O. As per manufacturer's instructions, aliquots of human recombinant MAGL (Cayman Chemical, Ann Arbor), stored at −80 °C, were thawed immediately prior to use and diluted in 125 mM Tris hydrochloride buffer (Tris-HCl), pH 7.2, with 1 mM EDTA. Enzyme activity was monitored via the fluorescent probe substrate (4-nitrophenyl) 4-[(3-phenoxyphenyl)methyl]piperazine-1-carboxylate (4-NA) using excitation and emission wavelengths of 360 and 460 nm, respectively, over 20 min (Cytation 3, BioTek). Percent inhibition was calculated relative to vehicle control (Vehicle) as follows, correcting for background fluorescence of samples and reagents relative to enzyme-free conditions:

$$\%\ \text{inhibition} = \left[100 - \frac{(\text{Sample}_{T\text{final}} - \text{Sample}_{T0}) - (\text{Sample blank}_{T\text{final}} - \text{Sample blank}_{T0})}{(\text{Vehicle}_{T\text{final}} - \text{Vehicle}_{T0}) - (\text{Vehicle blank}_{T\text{final}} - \text{Vehicle blank}_{T0})} * 100 \right] * 100\%$$

IC_{50} values were obtained by using the prism graph pad (version 7.0) log[Inhibitor] vs. normalized response -- Variable slope analysis module. Samples were tested in triplicate on at least three separate occasions.

2.8 Data analysis

All data represent mean ± S.E.M. values. Data obtained from the EPM, SI and NOR tests were analyzed using one-way analysis of variance (ANOVA) for each of the behavioral measures with treatment condition as the between-group factor. In some experiments, follow-up analyses were conducted using *t*-tests with a Bonferroni correction to protect the α at 0.05. Results from the CER sessions were analyzed using a 2 × 2 repeated-measures ANOVA, with condition as the between-group factor and time (two 5 min time blocks) as the within, or, repeated factor. In the case of a significant interaction in the CER, *post hoc* tests were conducted with Newman-Keuls to account for multiple comparisons ($p < .05$). Effect sizes were calculated for all statistically significant results from the EPM NOR and SI results, as well as for a significant treatment effect in the CER if present. Alpha level was set at 0.05 for all variables. Significant findings are displayed in the figures and tables.

3. Results

3.1 Phytochemical characterization of Protium Copal resin and incense

Analysis of the triterpene content of the *Protium copal* resin used in the behavioral test by HPLC-MS/MS indicated that the resin contained very high concentrations of α-amyrin and β-amyrin, and lower levels of lupeol (Table A1-1). Additional samples of resin showed similar results. The same triterpenes were identified in trapped incense (Figure A1-2).

3.2 Elevated Plus-Maze

A one-way ANOVA was conducted in order to compare the group means of the air - control, smoke-control, and copal conditions against various dependent variables (i.e. open-arm time, closed-arm time, latency to first open-arm entry, and unprotected head dips). For the percentage of time spent in the open arm, the ANOVA test revealed that there were no significant differences between the groups F (2, 21) = 0.06, p = .942. Results are summarized in Figure A1-3. Similarly, no significant effects were found in regards to closed-arm time F (2, 21) = 0.52, p = .602, latency to first open arm F (2, 16) = 0.77, p = .479, or unprotected head dips F (2, 21) = 0.67, p = .522.

3.3 Contextual Fear Conditioning

The repeated-samples ANOVA revealed a significant treatment effect for percentage of time spent in the freezing response during the contextual task, F (1, 18) = 6.76, p = 0.02 (η^2 = 0.75), in that rats exposed to burning copal spent significantly less time freezing in the CER paradigm than controls (see Figure A1-4). These effects were evident across the first two 5 min. time blocks but not the last two five min time blocks. Furthermore, the large effect size

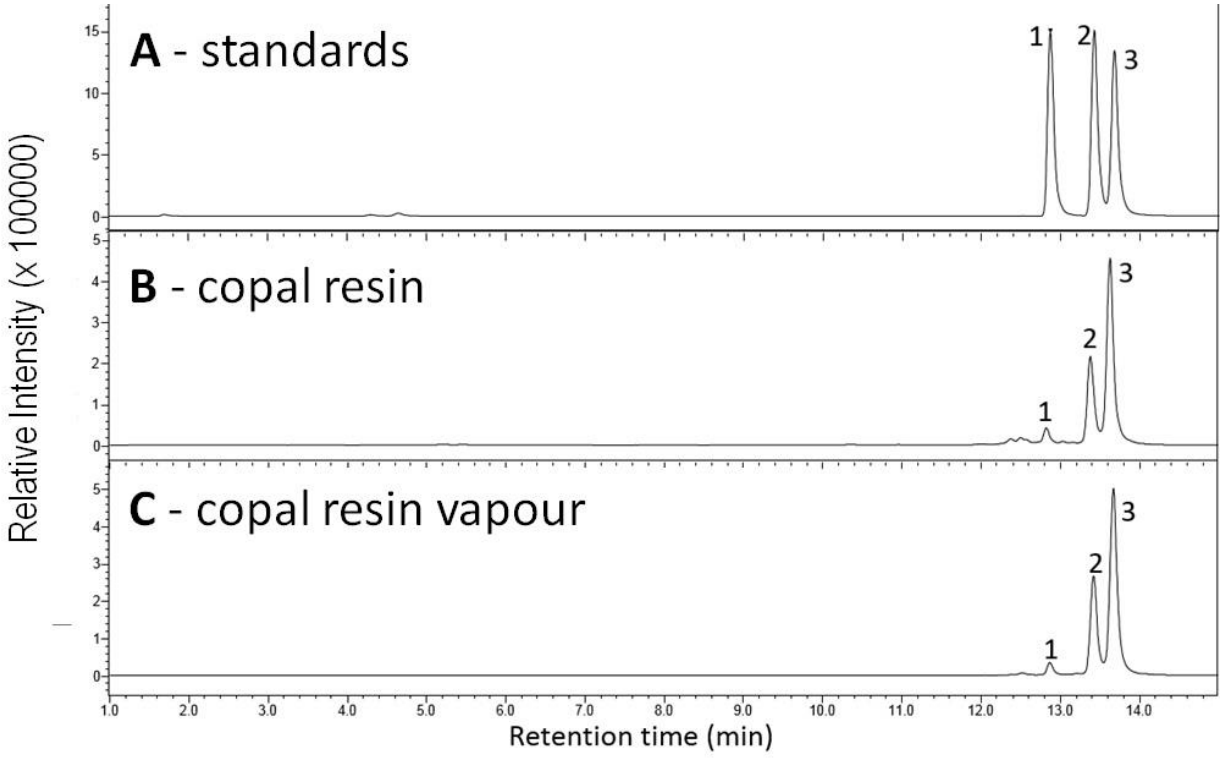

Figure A1-2. UPLC/MS selected ion chromatogram of triterpenes identified in copal resin and resin vapor. (A) Mixture of reference standards in methanol; (B) Copal resin solubilized in methanol; and (C) Copal resin vapor (incense) extract solubilized in methanol.

Table A1-1. Quantification of triterpenes in the sample of *P. copal* resin.

	Triterpene (mg/g) (mean ± SEM, N=3)		
	α-amyrin	β-amyrin	lupeol
*Protium copa*l resin	142.5 ± 4.19	121.4 ± 3.81	20.5 ± 0.86

($\eta^2 = 0.75$) suggested a large treatment effect of copal incense. The analyses also revealed a statistically significant effect of time, irrespective of treatment condition, F (1, 18) = 7.10, p = 0.02, indicating that freezing levels decreased over time, as expected (Blanchard & Blanchard, 1969). There was no significant interaction between treatment condition and time, F (1, 18) = 0.02, $p > 0.05$.

3.4 Social Interaction Test

A one-way ANOVA was completed, comparing the means of total active social interaction of all three groups (air -control, smoke-control, and copal conditions). This test revealed that the group means of total active social interaction were significantly different, F (2, 21) = 17.956, p = 0.0001. The post-hoc Bonferroni test revealed that the Copal group spent significantly more time interacting than both the smoke control rats and the air control rats (p= 0.0001). Results are summarized in Figure A1-5.

3.5 Novel Object Recognition Task

Discrimination Index (DI) values represent a ratio indicating the difference in exploration time of the novel and familiar objects, represented by the equation: $DI = (TN - TF)/ (TN + TF)$, where TN is the exploration time of the novel object and TF is the exploration time of the familiar object. This value ranges between -1 and +1, where a more negative score is associated with spending more time exploring the familiar object and a more positive score is associated with exploring the novel object (Antunes & Biala, 2012).

A one-way ANOVA of the DI values indicated a non-significant difference in group means. Thus, no apparent cognitive effects of copal were observed. However, a one-way ANOVA of the mean total exploration times (i.e. amount of time exploring both the familiar and

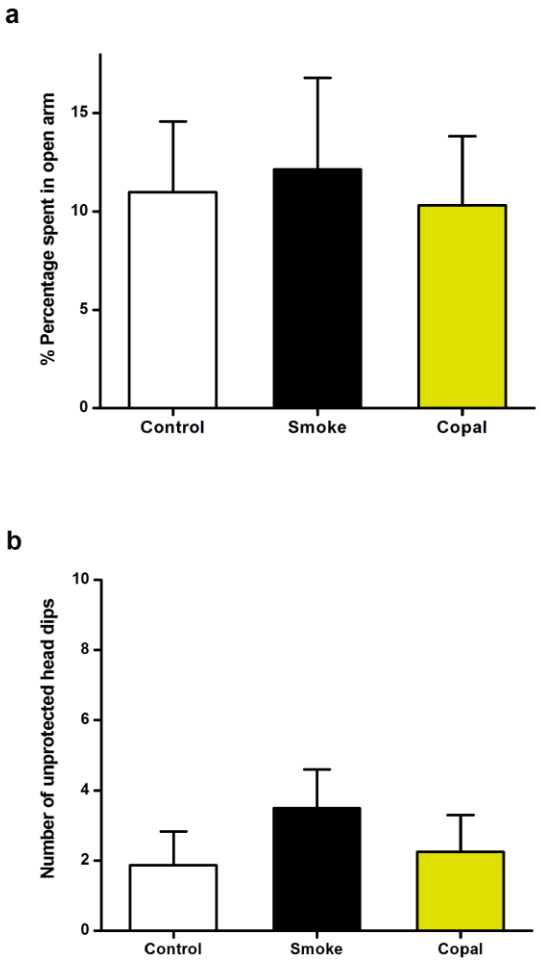

Figure A1-3. Percentage of time spent in the a) open arms in rats and b) the number of unprotected head dips in the elevated plus maze test (EPM) following air-control, smoke-control (200 mg) and copal (200 mg) conditions in the exposure chamber. A one-way ANOVA showed no significant differences between groups.

novel objects) for the copal group (M = 29.95 sec) was significantly higher than the air-control (M = 17.11 sec) and smoke control (M = 20.99 sec) groups F (2, 20) = 3.563, p = .0475. (Figure A1-6). Thus, a measure of locomotor activity was conducted, assessing the number of squares crossed during the test phase of the NOR task. A one-way ANOVA indicated that there was a significant difference between the mean number of squares crossed F (2, 18) = 3.97, p = .037. Tukey's HSD revealed that there was a significant difference between the copal group (M = 54.5) and the air-control group (M = 23.375), $p < .05$, with copal-exposed rats displaying higher levels of locomotor activity than air-control rats (Data not shown).

3.6 Treatment of rats with $GABA_A$ antagonist Flumazenil.

Because the SI paradigm revealed the most robust anxiolytic response and relates directly to copal's traditional use, we proceeded to investigate potential mechanism of action through co-administration of flumazenil, a specific $GABA_A$ receptor antagonist. A one-way ANOVA was conducted, comparing the means of total active social interaction of all four experimental groups. The test revealed a significant Treatment effect F (3, 35) =21.81, $p<0.0001$, $\eta^2=0.65$. Post-hoc Bonferroni tests demonstrated that the rats exposed to Copal smoke spent significantly more time interacting than the control rats, and that pre-treatment with Flumazenil significantly inhibited the effects of the Copal smoke (Figure A1-7AB). Follow up tests also confirmed that Flumazenil did not have an effect on its own t (9) =0.32, p=0.7583 as levels of social interaction for Flumazenil-treated rats were similar to controls. Squares crossed showed no significance in locomotor activity (data not shown).

Table A1-2. Relative potency of MAGL inhibition elicited by P. copal resin and its major triterpenes as determined by IC50 concentrations established using log-linear regression modeling of respective concentration-activity relationships.

Sample	IC_{50} (mean N=3)	r-squared
*Protium copa*l resin	790.6±113.9 ng/mL	0.955
α-amyrin	487.4±64.4 ng/mL (1142±150.9 nM)	0.962
β-amyrin	676.7±50.6 ng/mL (1586±118.6 nM)	0.954
Lupeol	785.4±76.2 ng/mL (1841±178.4 nM)	0.973

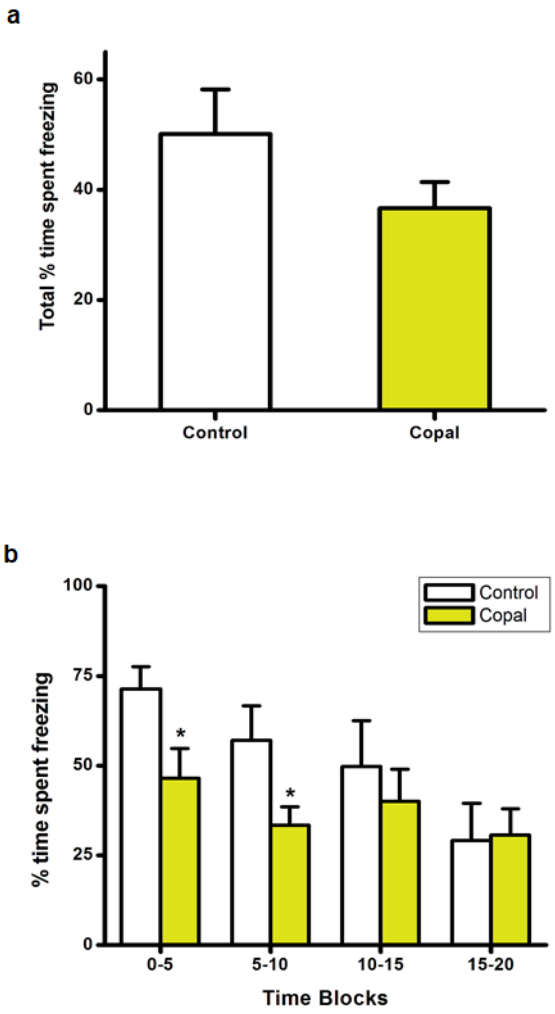

Figure A1-4. Effects of copal on total freezing time (%) in rats in the contextual conditioned emotional response (CER) test following air-control, and copal (200 mg) conditions. b) A breakdown of the (%) freezing time in rats in 5 min time blocks. The repeated measures ANOVA revealed statistically significant effects of group, $F(1, 18) = 6.76$, $p = .02$ ($\eta 2 = 0.75$), and time, $F(1, 18) = 7.10$, $p = .02$. With respect to the group effect, rats exposed to burning copal spent significantly less time freezing during the contextual task of the CER test than controls. This effect was evident across the first and second 5 min time blocks. * = $p<.05$.

3.7 Treatment of rats with MAGL inhibitor, AM251 (Social interaction)

A one-way ANOVA was conducted, comparing the means of total active social interaction of all four experimental groups. The test revealed a significant difference Treatment effect $F(3, 36) = 36.42$, $p < 0.0001$, $\eta^2 = 0.75$. Post-hoc Bonferroni tests demonstrated both that 1) the rats exposed to Copal spent significantly more time interacting than the control rats, and that 2) pre-treatment with AM251 inhibited the effects of the Copal smoke. Follow up tests also confirmed that AM251 did not have an effect on its own $t(9) = -1.62$, $p = 0.1389$ as levels of social interaction for AM251-treated rats were similar to controls. The results are summarized in Figure A1-7B. Locomotor activity in the arena did not reveal a significant effect $F(3, 32) = 0.83$, $p = 0.4851$.

3.8 Inhibition of human MAGL by copal

Solubilized copal resin inhibited the *in vitro* catalytic activity of human recombinant MAGL in a concentration-dependent manner, as did each of the identified triterpenes. While the crude resin was less potent than individual triterpenes, all samples exhibited IC_{50} values in the high nM range (Table A1-2).

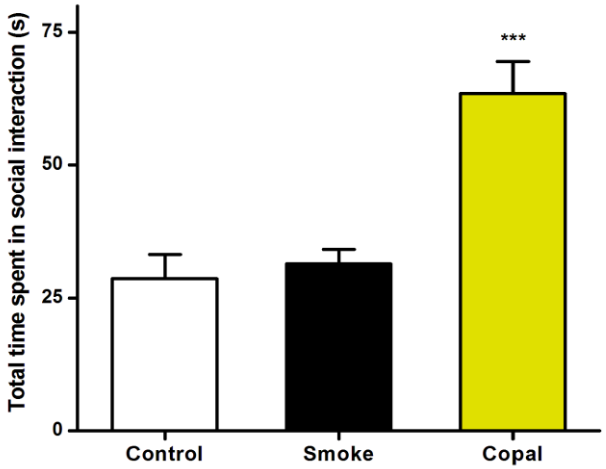

Figure A1-5. Mean duration of total active social interaction activity in SI test following air-control, smoke-control (200 mg) and copal (200 mg) conditions. $F(2, 21) = 17.956$, $p<.0001$ indicates a significant difference from air-control.

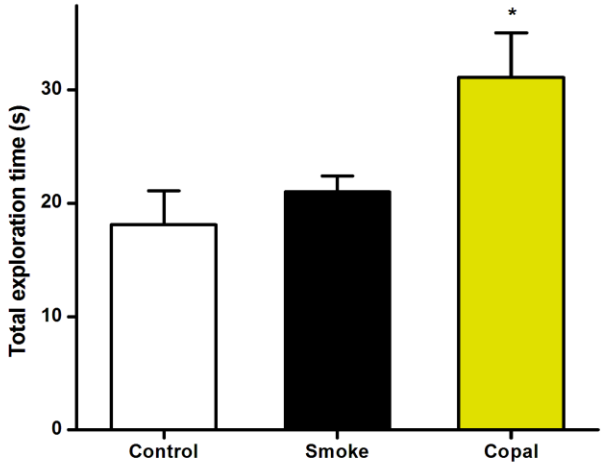

Figure A1-6. Mean duration of total exploration time of the both familiar and novel objects in the test phase (3.5 min) of the NOR task following air-control, smoke-control (200 mg) and copal (200 mg) conditions. F (2, 20) = 3.563, * = $p<.05$ indicates a significant difference from air-control.

4. Discussion

The phytochemical characterization of *Protium copal* resin showed high levels of pentacyclic triterpenes. These compounds have also been detected in *P. heptaphyllum* and *P. icicariba* resins as well as putative copal resin from a 15th/16th century Mesoamerican mosaic (Stacey et al., 2006). When previously administered orally to rodents, amyrins are known to have anxiolytic, antinociceptive and anti-inflammatory properties mediated through activation of the GABA and endocannabinoid systems (Aragão et al., 2007; da Silva et al., 2011; Chicca and Gertsch, 2012) but the effect of traditionally used copal incense has never been examined. Further examination of monoterpene and sesquiterpenes may be warranted.

Following a brief, 5-min exposure to the copal incense, distinct responses were elicited in different rodent models of anxiety and cognition (EPM, SI, CER and NOR) (Figures. A1-3 to 7) Behavioral measures obtained using the EPM initially suggested that *P. copal* incense does not produce generalized anxiolytic effects, of which the performance on the EPM is a good indicator, as no differences were observed between treatment groups for both time of permanence and frequency of entries in the open arms, as well as risk assessment behavior (indicated by unprotected head dips). Furthermore, copal incense did not impact overall activity levels in the EPM, as measured by the total number of closed arm entries. By contrast; the observed effects in the CER paradigm supported our initial hypothesis of anxiolytic activity following exposure to copal incense, at least in the context of fear response. The CER paradigm is commonly used to assess conditioned or learned fear, and anxiety (Blanchard and Blanchard, 1969; Davis et al., 1993; Luyten et al., 2011). The significantly reduced freezing response in rats in the copal group suggests that copal incense evokes anxiolytic-like behaviors with respect to learned contextual fear.

In contrast to the lack of discernible effects on the EPM test, *P. copal* incense elicited a robust increase in social interaction, in support of our hypothesis. The differences across test paradigms may be attributable to the specific types of anxiety-like responses assessed. Indeed, the ethological nature of the EPM test is assumed to rely on an innate approach-avoidance conflict between an innate drive to explore the environment and the natural tendency of rats to avoid open (or vulnerable) areas related to anxiety/fear of potential danger (File and Seth, 2003). In the case of the SI test, the total active social interaction serves as an indicator of the social approach or avoidance, reflecting socially anxiogenic or anxiolytic effects (Ramos, 2008). Therefore, the marked increase in total active social interaction in rats exposed to copal incense is indicative of reduced social anxiety (File and Seth, 2003; Walsh and Cummins, 1976), an effect with particular pertinence to copal's traditional ceremonial and socially healing roles.

In addition to the anxiolytic-like effects seen in CER (Figure. A1-4) and NOR (Figure. A1-6) paradigms, the observed differences in social interaction levels during the SI tests provide support for – and insight into – the social role of copal incense in setting the atmosphere in traditional Maya ceremonies. The SI test is based on the assumption that social interactions with others are a source of rewarding and positive experience for humans, and has been demonstrated extensively in a rodent model (Panksepp and Beatty, 1980; Varlinskaya and Spear, 2008). Several studies suggest that the fundamental behaviors assessed by the SI paradigms can provide some insight into the social features (e.g. Panksepp and Beatty, 1980). For instance, Varlinskaya and Beatty (2008) demonstrated a marked increase in total active social interaction in a sample of adolescent rats following a period of social isolation. The social significance of copal among the people of ancient Mesoamerica is clear from historical evidence, and is reflected in the social features involved in its collection and ritual uses (Case et al., 2003). The enhanced level of active

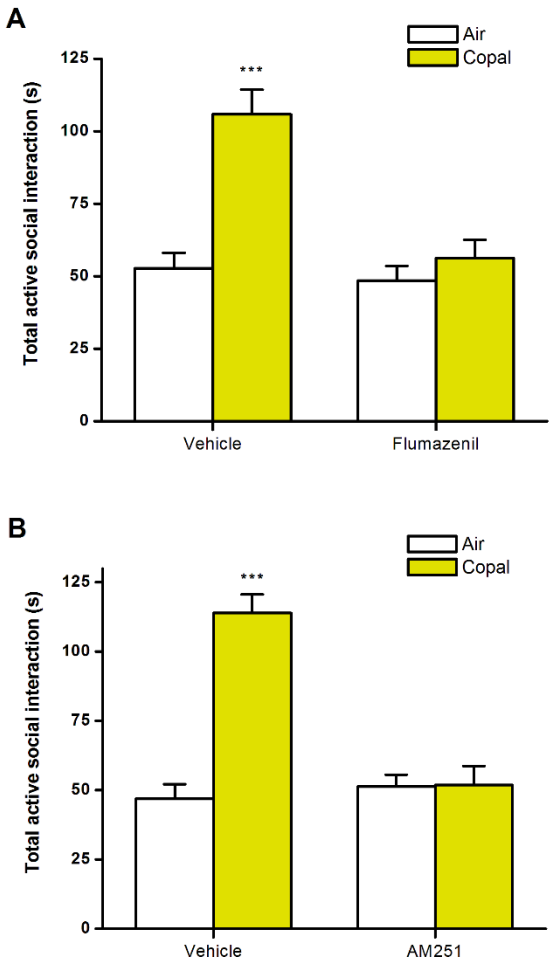

Figure A1-7. Mean duration of total active social interaction activity in SI test with i.p. injection of a) the GABAA receptor antagonist flumazenil (1 mg/kg) and b) the CB1 receptor antagonist AM251 (1 mg/kg) on either air or copal exposure (200 mg). Rats were injected 20 min their respective i.p. treatments 20 min prior to inhalation chamber exposure. Rats were tested immediately after being taken out of the inhalation chamber. A) $F(3, 35) = 21.81$ B) $F(3, 36) = 36.42$, *** = $p<.0001$ indicates a significant difference from air-control.

social interaction evident after exposure to copal incense suggests a facilitative role of copal in social behavior. Future studies should use other behavioral paradigms that specifically measure sociability and social behavior, without the confounding variable of anxiety.

Taken together, the evidence supports the hypothesis that *P. copal* incense possesses anxiolytic qualities in social and fear contexts. However, it should be noted that the behavioral paradigms chosen for this study did not assess a single factor of anxiety, but rather what appeared to be specific sub-types of the larger multi-dimensional construct of anxiety (Luyten et al., 2011; Ramos, 2008). According to this current view, the range of animal models of anxiety reflect a series of interrelated aspects of anxiety-like behaviors that model the distinct pathologies of clinical anxiety (Luyten et al., 2011; Ramos, 2008). Furthermore, there is general agreement in the empirical literature that fear and anxiety are mediated through distinct neuronal systems, in which activation of the central and lateral amygdala have been shown to be responsible for fear and general anxiety-like behaviors, respectively (File and Seth, 2003; Korte and de Boer, 2003). Therefore, the differences in the results of this study reflect alternative indices of the general construct of anxiety, in that exposure to copal incense elicits anxiolytic-like behaviors in a learned-fear model, but not generalized anxiety (Ramos, 2008). While the independent and interacting roles of the GABAergic and endocannabinoid systems in distinct anxiety-like behaviors remains incompletely understood, copal's effects (or lack thereof) across models suggest that these signaling pathways are not universally implicated in all types of anxiety. Further research with additional animal models of anxiety could provide a broader and more comprehensive understanding of the anxiolytic qualities of copal incense.

In regards to the cognitive effects of *P. copal*, the NOR task revealed that *P. copal* treated rats did not perform significantly better on the short-term memory task. However, the data did

show some increase in exploration activity which was observed to be mainly object exploration. The NOR data also shows that no sedative effects were demonstrated.

This is the first empirical investigation of the psychopharmacological effects of copal resins when administered in incense form. Previous work on *Protium heterophyllum* extracts and isolated amyrins strongly had suggested the interaction with benzodiazepine-type $GABA_A$ receptors. The present work is in agreement with this and the effect on MAGL of *Protium copal* crude resin and each of the major triterpenes that collectively account for 30% by weight of the resin used in ceremony strongly suggests interaction with the endocanabinoid system as well. Triterpenes like the amyrins and lupeol are lipophilic, easily vaporized, and quickly absorbed through lipid bilayers. Amyrins and similar triterpenes inhibit MAGL activity by binding into a hydrophobic pocket leading to loss of catalytic activity. Unlike most experimental and drug-pipeline MAGL inhibitors, which are suicide inhibitors, triterpene binding is reversible (King et al., 2009).

Hypothetically, upon exposure to resin vapor, ceremony participants inhale the triterpenes, leading to rapid absorption and good bioavailability with no first-pass metabolism. Once the lipophilic molecules enter the brain, they inhibit MAGL to increase eCB (and/or GABA) signaling and reduce anxiety. After the exposure (i.e. the ceremony ends), triterpenes will dissociate from MAGL and out of brain, reducing the anxiolytic effects and leaving the ceremonial experience imbued with altered mood and cognition. Additional in vivo work is needed to confirm this mechanism.

Future studies should also investigate anxiolytic action of other traditionally used Mesoamerican botanical resins included under the common name “copal”. These include

several *Bursera* spp, *Pinus* spp and *Amyris* spp. which also have significant terpene content (Stacey et al., 2006). These resins were more commonly used in Central Mexico than the Maya lowlands.

In summary, the current study introduced novel evidence of the selective anxiolytic effects following acute exposure to copal incense derived from *Protium copal*. Furthermore, the marked increase in active social interaction among rats exposed to copal incense suggests that copal incense may play an influential role in human social and ritual behaviors. The high importance of copal incense in ancient and modern life of the Maya, indicates that they have long been aware of its sacred value.

Acknowledgements

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Conflict of interest

There is no conflict of interest.

References

Aragão GF, Carneiro LM, Junior AP, Vieira LC, Bandeira PN, Lemos TL, Viana GS. A possible mechanism for anxiolytic and antidepressant effects of alpha- and beta-amyrin from Protium heptaphyllum (Aubl.) March. Pharmacol Biochem Behav. 2006; 85(4):827-34.

Aragão GF, Cunha Pinheiro MC, Nogueira Bandeira P, Gomes Lemos TL, de Barros Viana GS. Analgesic and anti-inflammatory activities of the isomeric mixture of alpha- and beta-amyrin from Protium heptaphyllum (Aubl.) march. J Herb Pharmacother. 2007; 7(2):31-47.

Carlini VP, Machado DG, Buteler F, Ghersi M, Ponzio MF, Martini AC, Schiöth HB, de Cuneo MF, Rodrigues AL, de Barioglio SR. Acute ghrelin administration reverses depressive-like behavior induced by bilateral olfactory bulbectomy in mice. Peptides. 2012; 35(2):160-5.

Cayer C, Ahmed F, Filion V, Saleem A, Cuerrier A, Allard M, Rochefort G, Merali Z, Arnason JT. Characterization of the anxiolytic activity of Nunavik Rhodiola rosea. Planta Med. 2013; 79(15):1385-91.

Case RJ, Tucker AO, Maciarello MJ, Wheeler KA. Chemistry and ethnobotany of commercial incense copals copal blanco, copal oro, and copal negro, of North America. Econ Bot 2003; 57:189–202.

Chicca J, Gertsch J. The antinociceptive triterpene β-amyrin inhibits 2-arachidonoylglycerol (2-AG) hydrolysis without directly targeting cannabinoid receptors. Br J Pharmacol, 2012. 167(8): 1596-1608.

Chirumbolo S. Plant-derived extracts in the neuroscience of anxiety on animal models: biases and comments. Int. J. Neurosci. 2012; 122:177–188.

Cruz AP, Frei F, Graeff FG. Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacol. Biochem. Behav. 1994; 49:171–176.

Davis M. The role of the amygdala in emotional learning. Int. Rev. Neurobiol. 1994: 36:225–266.

File SE. Animal models of different anxiety states. Adv. Biochem. Psychopharmacol. 1995; 48:93–113.

File SE, Seth P. A review of 25 years of the social interaction test. Eur. J. Pharmacol. 2003; 463:35–53.

Gnatta JR, Piason PP, Lopes Cde L, Rogenski NM, Silva MJ. Aromatherapy with ylang ylang for anxiety and self-esteem: a pilot study. Rev Esc Enferm USP. 2014; 48(3):492-9.

Hernández-Vázquez L, Mangas S, Palazón J, Navarro-Ocaña A. Valuable medicinal plants and resins: Commercial phytochemicals with bioactive properties. Ind Crops Prod. 2010; 31: 476–480.

King AR, Dotsey EY, Lodola A, Jung KM, Ghomian A, Qiu Y, Fu J, Mor M, Piomelli D. Discovery of potent and reversible monoacylglycerol lipase inhibitors. Chem Biol. 2009; 16:1045-52.

Köteles F, Babulka P. Role of expectations and pleasantness of essential oils in their acute effects. Acta Physiol Hung. 2014; 101(3):329-40.

Laughlin, R.M., 1975. The Great Tzotzil Dictionary of San Lorenzo Zinacantan - Smithsonian Contributions to Anthropology Number 19. Smithsonian Institution Press, W.

Lillehei AS, Halcon LL. A systematic review of the effect of inhaled essential oils on sleep. J Altern Complement Med 2014; 20:441–451.

Lucero-Gómez P, Mathe C, Vieillescazes C, Bucio L, Belio I, Vega R. Analysis of Mexican reference standards for Bursera spp. resins by Gas Chromatography–Mass Spectrometry and application to archaeological objects. J Archaeol Sci. 2014; 41:679–690.

Luyten L, Vansteenwegen D, van Kuyck K, Gabriëls L, Nuttin B. Contextual conditioning in rats as an animal model for generalized anxiety disorder. Cogn Affect Behav Neurosci. 2011; 11:228–244.

Melo CM, Morais TC, Tomé AR, Brito GA, Chaves MH, Rao VS, Santos FA. Anti-inflammatory effect of α,β-amyrin, a triterpene from Protium heptaphyllum, on cerulein-induced acute pancreatitis in mice. Inflamm Res. 2011; 60(7):673-81.

Pellow S, File S.E. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol. Biochem. Behav. 1986; 24:525–529.

Rao Z, Dong K, Yang X, Lin J, Cui X, Zhou R, Deng Q. Natural amber, copal resin and colophony investigated by UV-VIS, infrared and Raman spectrum. Sci. China Phys. Mech. Astron. 2013; 56:1598–1602.

Sarris J, McIntyre E, Camfield DA. Plant-based medicines for anxiety disorders, part 2: a review of clinical studies with supporting preclinical evidence. CNS Drugs. 2013; 27(4):301-19.

Sarris J, Panossian A, Schweitzer I, Stough C, Scholey A. Herbal medicine for depression, anxiety and insomnia: a review of psychopharmacology and clinical evidence. Eur Neuropsychopharmacol. 2011; 21(12):841-60.

Siani AC, Ramos MF, Menezes-de-Lima O Jr, Ribeiro-dos-Santos R, Fernadez-Ferreira E, Soares RO, Rosas EC, Susunaga GS, Guimarães AC, Zoghbi MG, Henriques MG. Evaluation of anti-inflammatory-related activity of essential oils from the leaves and resin of species of Protium. J Ethnopharmacol. 1999; 66(1):57-69.

Stacey RJ, Cartwright CR, McEWAN C. Chemical characterization of ancient mesoamerican "copal" resins: preliminary results. Archaeometry. 2006; 48:323–340.

Schele, L. Freidel, D., Maya Cosmos, Harper. New York. 1993.

Tedlock, D. Popol Vuh: The Definitive Edition of The Mayan Book of The Dawn Of Life And The Glories Of, 2nd Revised edition edition. ed. Touchstone, New York. 1996.

Wang Y, Gu N, Duan T, Kesner P, Blaskovits F, Liu J, Lu Y, Tong L, Gao F, Harris C, Mackie K, Li J, Tan Q, Hill MN, Yuan Z, Zhang X. Monoacylglycerol lipase inhibitors produce pro- or antidepressant responses via hippocampal CA1 GABAergic synapses. Mol Psychiatry. 2017; 22(2):215-226.

APPENDIX 2: SAFETY EVALUATION OF A NEW ANXIOLYTIC PRODUCT CONTAINING BOTANICALS *SOUROUBEA* SPP. AND *PLATANUS* SPP. IN DOGS

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Abstract

Separation anxiety and noise aversion are common behavioral problems in dogs. They elicit fear responses such as cowering, seeking out the owner, and attempting to escape. This can result in property damage, injury to the dog, and disruption of the owner-pet bond, possibly leading to pet abandonment or euthanasia. A novel botanical anxiolytic product was evaluated for safety in dogs as the target animal species. Its intended use is for the treatment and prevention of anxiety and noise aversion in dogs. It contains a defined mixture of Souroubea spp. vine and Platanus spp. bark, delivering the active principle, betulinic acid, at a recommended dose of 1 mg/kg body weight (BW). In the current target animal safety study, 16 healthy male beagle dogs were administered either a placebo or the newly formulated botanical tablets at 0.5×, 2.5×, or 5× the recommended dose (1 mg/kg BW) over 28 d. The dogs were monitored for occurrence of any systemic or local adverse events. In the investigation presented here, there were no clinically significant adverse effects following treatment, as determined by clinical observations, physical examinations, BW, hematology, clinical biochemistry, and urinalysis. Pharmacokinetic analysis demonstrated that the concentration of betulinic acid in serum was below 0.020 μg/mL in treated animals. Under the conditions of these studies, the formulated blend of S. sympetala and P. occidentalis, when administered up to 5× the intended dose for 28 consecutive d, showed no adverse effects on the health of dogs.

1. Introduction

Separation anxiety and aversion to loud noises such as thunder, are common behavioral problems in companion dogs. These behaviors are also associated with fear responses, such as cowering, freezing, scanning and seeking out the owner, and attempting to escape. In some cases, anxiety responses may lead to property damage, injury to the dog, disruption of the owner-pet bond, and potentially pet abandonment and/or euthanasia (Sherman et al., 2008; Shull-Selcer et al., 1991; Thompson et al., 1998). Anxiety induced stress may also result in negative health effects and decreased lifespan if left untreated (Dreschel, 2010). There are several treatments currently available for anxiety and noise aversion in dogs, including pharmacotherapy [e.g., benzodiazepines, phenothiazines, tricyclic antidepressants (TCAs), and selective serotonin reuptake inhibitors (SSRI)], behavioral management and systematic desensitization, dog-appeasing pheromones, and homeopathic remedies (Sherman et al., 2008).

Based on our discovery research (Puniani et al 2015; Mullally et al., 2011), a novel chewable tablet was developed for canine anxiety, containing plant-derived triterpenes. The new product is currently marketed in the United States under the commercial name Zentrol^{TM} (Pegasus Laboratories, Pensacola, Florida, USA). The plant-based product is defined as a mixture of natural botanical ingredients containing the active principle, betulinic acid, and related triterpenes. The product is a blend of 55:45 w/w *Souroubea* spp. (SSB) and *Platanus* spp. tree bark (PTB). *Souroubea*spp. is defined as a "leaf and stem growth" of the neotropical vines *S. sympetela* Gilg. or *S. gilgii,* or any mixture of both. The 2 species have similar phytochemical ingredients (Carballo-Arce, 2013). *Platanus* spp. tree bark is defined as the bark of *P. occidentalis* (American sycamore) or *P. acerfolia* (Plantanaceae), or any mixture of these. It

contains betulinic acid, while SSB contains betulinic acid as well as alpha and beta amyrins, lupeol, and ursolic acid, which improve the efficacy of the mixture.

Significant anxiolytic effects of orally administered *S. sympetala* raw material, their extracts, or purified betulinic acid, were found in standardized rodent models of anxiety (Mullally et al., 2011; Cayer, 2011). *Souroubea sympetala*extracts and betulinic acid derivatives acted as agonists at the $GABA_A$-benzodiazepine receptor (Mullally et al., 2014), a major central nervous system receptor implicated in the etiology of anxiety and target for many anti-anxiety drugs. The botanical materials and/or betulinic acid also reduce plasma cortisol levels in rats and fish (Mullally et al., 2011). In addition, betulinic acid has been used in studies demonstrating inhibition of HIV replication (Fujioka et al., 1994; Kashiwada et al., 1996; Mayaux et al.,1994), anti-inflammatory action (Mukherjee et al 1997; Recio et al., 1995), selective apoptosis of specific cancers (Pisha et al., 1995; Selzer et al., 2000; Zuco 2002; Fulda et al., 2000; Ramadoss et al., 2000), and treatment of bacterial infections (Ramadoss et al., 2000; Schühly et al., 1999). These studies have suggested minimal toxicity at doses 500× higher than the dose used in the current study. In a study examining betulinic acid's ability to inhibit melanoma tumor growth in mice, there were no signs of acute or chronic toxicities associated with betulinic acid at repeated doses as high as 500 mg/kg body weight (BW) (Pisha et al., 1995). Rats receiving chronic administration of 0.5 mg/kg BW of betulinic acid did not show signs of withdrawal. There were no effects on locomotion, behavior, fecal output, food intake, or weight in any of the rats during the course of the study (Cayer, 2011).

The botanical ingredients in these triterpene anxiolytic tablets have a long history of use by Indigenous healers, suggesting that they are safe (Chandramu et al., 2003; Schultes et al., 1990; Bourbonnais-Spear et al., 2006&2007; Arnason et al., 1981; Hamel and Chiltoskey, 1975).

However, to date, there is no information available on the safety of combined SSB and PTB use in dogs. Limited data from a pilot toxicology study (Villalobos et al., 2014) assessing the safety of SSB and PTB compared to a placebo for 28 d, revealed that the botanical components had no negative effects on the health of dogs and were deemed safe. This enabled the design and execution of the present, larger controlled target safety and toxicology study for the new product. The primary objective in the present study was to assess the safety of a dog tablet containing a blend of SSB and PTB at elevated doses with dogs as the target animal species. A second objective was to determine the half-life of betulinic acid or its predicted metabolites, in serum that could be monitored at multiple time points.

2. Materials and methods

2.1 Botanical material

The leaves and stems of S. sympetala [Universidad Nacional archive (UNA) voucher no. JVR 12823] and S. gilgii (UNA voucher no. JVR 12844 and JVR 12894) samples used in this study were grown in Sarapiqui, Heredia Province, Costa Rica. Plants were identified by the UNA botanists and voucher samples were deposited at the Herbarium Juvenal Rodriguez, Universidad Nacional of Costa Rica and University of Ottawa herbarium (OTT19994). The P. occidentalis and P. acerifolia (voucher no. OTT19608/9) samples were collected in Guelph, Ontario, and vouchers were archived at the University of Ottawa herbarium. Souroubea spp. and PTB were dried at 45°C. The dried herbal material was ground to a fine powder that passed through a 0.25 mm screen. Finally, they were thoroughly blended together at a 55:45 w/w ratio of SSB:PTB. The powdered botanical mixture was formulated into a chewable tablet dosage with binder and a flavoring agent at Canvet (Guelph, Ontario) for Souroubea Botanicals (Ottawa, Ontario).2.2 Tablet preparation

The placebo (Lot# T11L218) formulation contained the microcrystalline cellulose Avicel PH200 (FMC BioPolymer, Philadelphia, Pennsylvania, USA), acacia gum (Pre-Hydrated Gum Arabic FT), FlavorPal (TetraGenx, Saint-Laurent, Quebec), and magnesium stearate. The test product for this study (Lot# T11L217) was formulated as a 2 g chewable tablet containing no less than 5 mg of the primary active ingredient, betulinic acid, derived from a SSB and PTB blend. In addition to the botanical blend, the test product contained acacia gum (Pre-Hydrated Gum Arabic), FlavorPal (TetraGenx), microcrystalline cellulose (Avicel PH101) and magnesium stearate.

Table 2A-1. Summary of the treatment groups and dosing frequency.

Group	Dosage	n	Route	Frequency
Group 1	0	4	Oral	10 tablets, once daily
Group 2	0.5 X	4	Oral	1 tablet, once daily
Group 3	2.5 X	4	Oral	5 tablets, once daily
Group 4	5 X	4	Oral	10 tablets, once daily

recommended label dose for a 10 kg dog is 2 tablets delivering 1 mg/kg BW of betulinic acid. The tablets were analyzed for betulinic acid content using the high-performance liquid chromatography-mass spectrometry (HPLC/MS) method described previously (Carballo-Arce,2013). Briefly, botanical tablets or placebo tablets were ground with a coffee blender. A total of 0.5 g of material was extracted with 250 mL ethyl acetate using a Soxhlet apparatus for 2 h. The extract was dried by rotatory evaporation at 45°C and re-dissolved in 20 mL of methanol by sonication for 2 min. The pellet was re-extracted in 20 mL of methanol by sonication for 2 min. Total volume was adjusted to 50 mL in a volumetric flask. Extracts were further diluted at a 1:10 ratio and used for analysis. All test samples were prepared and tested in triplicates.

2.3 Chemicals

An analytical standard of betulinic and oleanolic acids were purchased from Extrasynthese (Lyon, France). Methanol (Methanol, Optima LC/MS Grade), water (Water, Optima LC/MS Grade), acetonitrile (Acetonitrile, Optima LC/MS Grade) and methylene chloride (Methylene Chloride, Optima LC/MS Grade) were obtained from Fisher Scientific (Nepean, Ontario).

2.4 Experimental study design

This was a single-site clinical laboratory study involving randomization of experimental units to 4 dosing groups, with a repeated dosing regimen at 1 of 4 dosage levels (Table A2-1). The target animal safety study was conducted at the contract research organization Kingfisher International (Stouffville, Ontario). Procedures were designed to avoid or minimize discomfort, distress, and pain to the animals, in accordance with the principles and the guidelines of Canadian Council on Animal Care (CCAC). The test facility was a registered research facility under the Animals for Research Act. The Ontario Ministry of Agriculture, Food and Rural

Table A2-2. Timeline and safety parameter schedule of events for the safety study in beagle dogs.

Period	Day	Body weight	Physical exam	Clinical pathology [a]	Urinalysis	Blocking and assignments	Treatment	PK blood collection [b]	Clinical observations
Acclimation (days -8 to -1)	Day -8		●						
	Day -7				●				
	Day -5			●					
	Day -1	●				●			
Treatment phase (days 0-27)	Day 0							●	
	Day 6				●				Once daily
	Day 7			●					
	Day 13				●		Day 0 to 27: 1x daily		
	Day 14	●	●	●					
	Day 21			●	●			●	
	Day 27				●				
Close out (day 28)	Day 28	●	●	●					

[a] Includes hematology, clinical chemistry and cortisol analysis

[b] Day 0 & Day 21: PK blood collection pre-dose, and then 30 minutes ± 1 min; 1 h ± 1 min; 2 h ± 2 min; 4 h ± 3 min; 8 h ± 5 min; 12 h ± 5 min; and 24 h ± 5 min post treatment.

Affairs and was also accredited for Good Animal Practices by the CCAC. In order to ensure compliance, the experimental protocol (KFI-057-SC-2711) was reviewed and approved by the test facility's Animal Care Committee before the start of the trial.

2.5 Randomization procedures and blinding

The study was placebo-controlled, randomized, and double-blinded. Eighteen intact male beagle dogs entered the 8-day acclimation period. Sixteen were selected for participation in the dosing period based on the study inclusion criteria. On day 1, dogs selected for inclusion in the study were ranked by descending Study Animal ID and randomized to 1 of 2 groups, using a random number generator in Microsoft Excel 2003 (Microsoft Corporation, Redmond, Washington, USA). The veterinarian performing the physical examinations, the laboratory staff, and any staff performing daily clinical observations were blinded to experimental groups.

2.6 Test animals and study schedule

Subjects were 16 healthy, intact, male, beagle dogs, weighing between 7.6 and 13.8 kg and ranging from 7 to 58 mo of age with no clinically significant health abnormalities, based on pre-study examinations [physical examinations, BW, hematology, clinical chemistry, and urinalysis (Table A2-2). The dogs were dosed once daily with either placebo or 0.5×, 2.5×, or 5× the recommended dose (0.5, 2.5, and 5 mg/kg BW, respectively) of triterpene containing botanical tablets based on the BW (Groups 1 to 4, respectively; Table A2-1). Following dosing, dogs were closely monitored for 2 h to ensure that there were no adverse reactions and general clinical health observations were carried out once daily throughout the study. General clinical health observations included assessment of the eyes, mucous membranes, respiration, pulse, neurological condition, fecal consistency, presence of blood in feces, and behavior. Physical

examinations and BW measurements were performed by the study veterinarian on days 14 and 28 (Table A2-2). Blood was collected by jugular venipuncture and the following hematology, hormone, and clinical chemistry parameters were measured: red blood cell (RBC) count, hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, leukocyte count, leukocyte differential, RBC morphology, cortisol, alkaline phosphatase, total bilirubin, unconjugated bilirubin, gamma glutamyl transferase, aspartate aminotransferase, creatine kinase, urea, creatinine, total protein, albumin, globulin and A/G (albumin/globulin) ratio (calculated), glucose, amylase, electrolytes (sodium, potassium, chloride), calcium, phosphorus, and magnesium. Urine was collected by cystocentesis or catheterization and urinalysis was performed for the following parameters: color, turbidity, pH, protein, glucose, ketones, bilirubin, specific gravity, and microscopic analysis. On days 0 and 21, blood was collected for determination of betulinic acid levels in serum before and after treatment at 30 min, and 1, 2, 4, 8, 12, and 24 h (Table I). Plasma samples were stored at −80°C until the end of the study and sent to the University of Ottawa for pharmacokinetic analysis using the HPLC/MS method. All dogs received dry dog food throughout the study, which met the requirements for energy, protein, vitamins, and minerals for the species and age class. Each dog was singly housed in a floor pen and received access to water ad libitum. Dogs were removed from pens for daily cleaning and socialization during the treatment period. Neither medications nor vaccinations were administered to any of the dogs during the course of study.

2.7 Pharmacokinetic analysis of betulinic acid in serum

Analysis of serum betulinic acid concentration was performed using the method of Carballo-Arce et al (Carballo-Arce,2013). To measure the serum betulinic acid concentration, a

100 µL serum sample was used. A 50 ng sample of oleanolic acid (internal standard, solubilized in 100 µL methanol) was added to the serum followed by 0.5 mL of water (LCMS grade) and 3 mL of methylene chloride. Each sample was mixed by vortex for 1 min. The mixture was then centrifuged at 2000 × g for 15 min. A 2-mL aliquot of the organic phase was transferred and dried under nitrogen gas. The residue was re-solubilized with 1 mL of methanol for 5 min of sonication. Targeted UPLC-MS analyses of betulinic acid were carried out on a Shimazu UPLC-MS (Mandel Scientific Company, Guelph, Ontario) as previously described (Carballo-Arce,2013). Separations were performed at a flow rate of 0.35 mL/min on a Kinetex C18 column, particle size 1.7 µm, 150 mm × 2.1 mm ID (Phenomenex, Torrance, California, USA). The column thermostat was maintained at 40°C during an isocratic elution of 63% acetonitrile in water. The column was then washed with 5 column volumes with 100% acetonitrile, returned to the initial conditions in 0.1 min, and re-equilibrated for 3 min before the next injection. The mass spectrometer was operated in atmospheric-pressure chemical ionization mode, monitoring m/z at 455.3 [$(M-H)^+$]. Linear calibration curves were built by injecting dilutions of betulinic acid bracket, the compound concentration in samples. Oleanolic acid was used as an internal standard. Each sample was injected in triplicates and the area under each peak was then used to quantify the amount of each marker compound. Recovery experiments were undertaken by spiking serum with pure compounds at 1.97, 7.88, and 19.7 µg/mL. Spiked and un-spiked samples were prepared following the procedure described above and recovery was determined by regression analysis. Calibration curves were prepared at 5 concentration levels and R2 values were obtained for each metabolite. The limits of detection [(LOD) 3:1, signal: noise] and limits of quantification [(LOQ) 10:1, signal: noise] were determined at 3× standard deviation of noise level.

3. Results

3.1 UPLC/MS analysis of tablets

The quantitative UPLC/MS analysis indicated that the tablets containing a 55:45 w/w blend of SSB and PTB had a mean ± SE of 5.68 ± 0.14 mg betulinic acid per tablet (n = 3), while no betulinic acid was detected in placebo tablets. Hence, the actual dose fits the criteria of the study design. For example, the 0.5× dose (1 tablet) provides 0.568 mg/kg BW for a 10 kg dog. The placebo tablets contained no detectable betulinic acid.

3.2 Product administration and tolerability

Dosing was readily achieved and appeared to be well-tolerated by most dogs. One dog in the placebo group (group 1) was noted to have vomited whole tablets within the hour after treatment on several occasions. The dog was re-dosed with new tablets on all but one occasion without issue. After the first incident of this dog vomiting tablets, it was not re-dosed. In addition, on day 28, 4 dogs were noted to have visible signs of test article or placebo in their feces. These dogs were all in either group 1 or group 4, each having received 10 tablets of product or placebo daily (Table 2A-1). Since this occurred in both the placebo and 5× groups, this was likely caused by the high number of whole tablets dosed and had no impact on the study.

3.3 Clinical observations and adverse events

No clinically significant findings following 28 d of treatment were noted during clinical observations, physical examinations, BW measurements, hematology, cortisol analysis, or urinalysis. All biological indicators were within normal ranges in all experimental groups.

Table A2-3. Summary of adverse events noted during the 28-day target animal safety study.

Treatment groups	Test subjects (n)	Adverse event	Findings and conclusions
0.5 x	4	Epiphora.	1 dog, due to chronic blockage of lachrymal canal that preceded study.
2.5 x	4	Epiphora.	1 dog, due to chronic blockage of lachrymal canal that preceded study.
5 x	4	Undigested tablets in feces.	2 dogs, attributed to high number of tablets administered (n=10).
		Elevated urea and creatine during treatment period and post-treatment period.	1 dog euthanized at end of study. Gross pathology and histopathology revealed normal kidneys with no renal lesions.
		Epiphora.	1 dog, due to chronic blockage of lachrymal canal that preceded study.
Placebo	4	Vomiting tablets, undigested tablets in feces.	1 dog vomited tablets. 1 dog, undigested tablets in feces. – each case was attributed to high number of tablets administered (n=10).
		Intermittent loose feces with frank blood and mucus.	3 dogs, due to pre-existing Giardia infection, known to be endemic in Beagle colony.
		Epiphora.	1 dog, due to chronic blockage of lachrymal canal that preceded study.

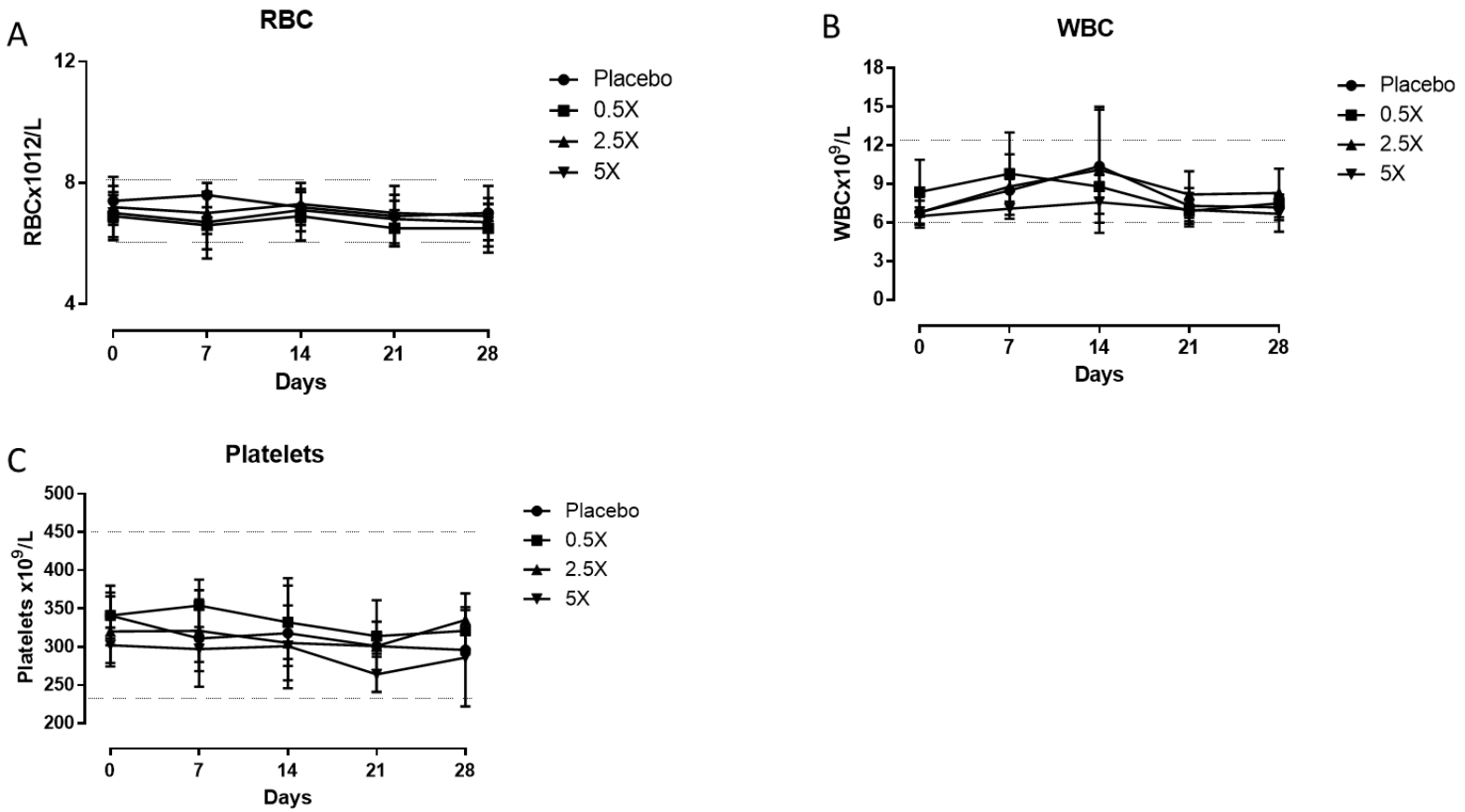

Figure A2-1. Hematology results A: RBC; B: WBC; C: Platelets. Mean 6 standard deviation (n = 4) are presented; normal range was determined by measure a total of 188 health beagles in the facility.

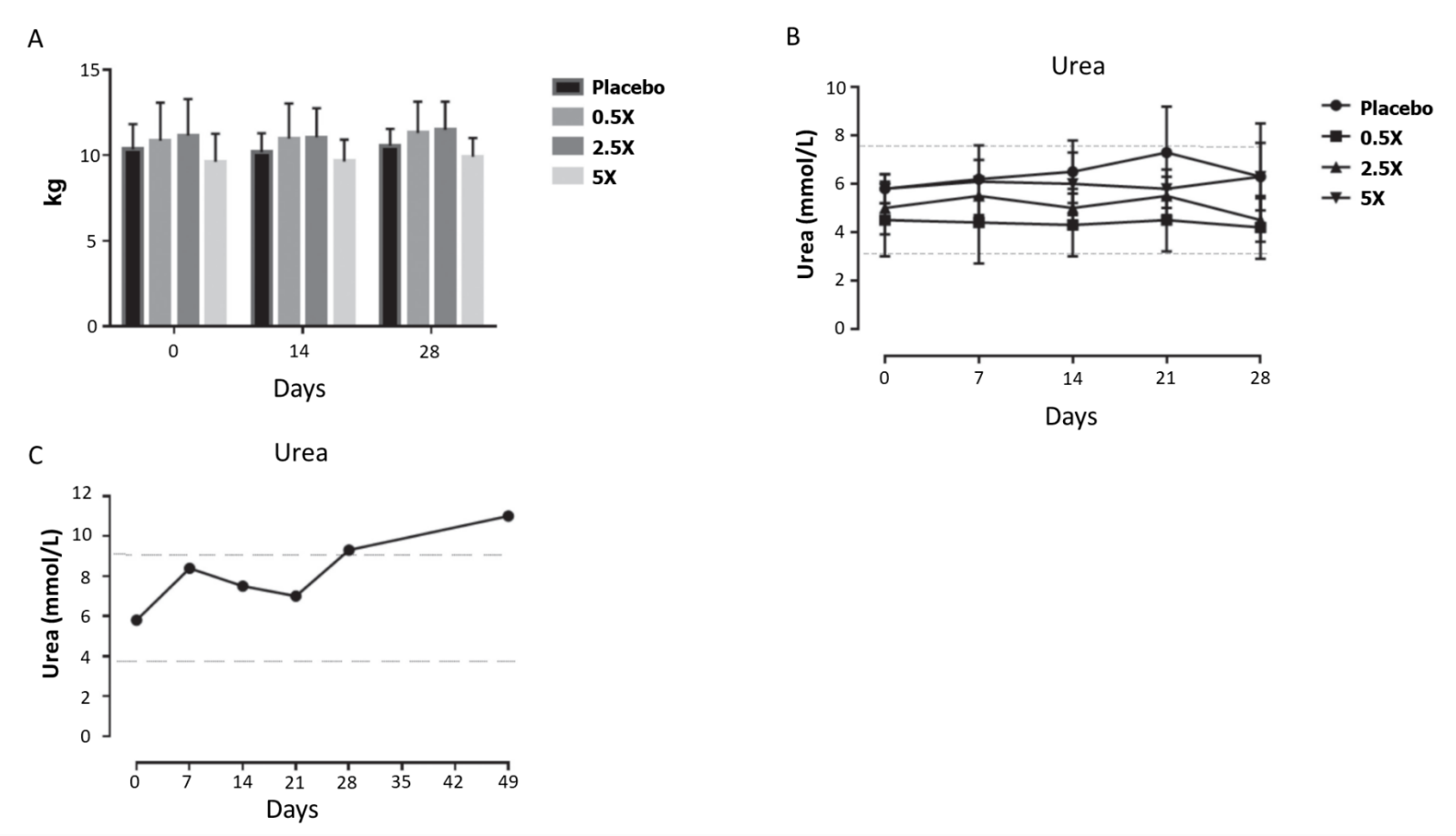

Figure A2-2. A: Body weights in experimental groups during the course of study. B: Mean urea values (mmol/L) for all dogs in each experimental group during the course of study. C: Urea values in one dog from Group 4 during the course of study and 21 days post dosing.

Hematology (Figures A2-1A - A2-1C) and most relevant clinical chemistry (Table A2-4) parameters were within normal species-specific reference ranges. There were no changes in BW (Figure A2-2A) over 28 d of treatment. There were few minor adverse events noted and described (Table A2-3). Epiphora was observed frequently during both acclimation and treatment periods and was considered not to be dose-related. Three dogs on placebo were noted to have intermittent loose feces containing frank blood and often mucus. *Giardia* spp. oocysts were isolated from feces of only one dog by floatation method. No clinical or toxicological significance was attributed to these findings. Only one dog in the 5× group demonstrated mildly elevated urea levels outside normal ranges (Figure A2-2C) during the treatment period. However, no other dogs displayed similar abnormalities and urea levels were within reference range during the course of the study (Figure A2-2B). An additional evaluation of urea and creatinine performed 21 d after the end of the treatment showed that the elevations were sustained, and the dog was euthanized for further testing. Gross pathology and histopathology revealed no renal lesions and found that the kidneys were normal. Therefore, no clinical or toxicological relevance was attributed to these findings, which were considered not to be product-related.

3.4 Pharmacokinetics of betulinic acid in serum

The active component, betulinic acid, or its anticipated metabolites, were below the lower limit of detection (LLOD) in serum following multiple sampling on day 21, as measured by UPLC-MS (Figure 5-1).

Table A2-4. Summary of clinical chemistry results presented as mean 6 standard deviation (n = 4).

	Glucose (mmol/L)	Total protein (g/L)	Creatinine (μmol/L)	ALT (U/L)	AST (U/L)	Urea (mmol/L)
Normal range[a]	3.8-6.2	50-64	49-92	22-61	27-53	3.4-7.9
Placebo group						
Day -5	4.6 ± 0.2	58 ± 4	60 ± 7	37 ± 10	36 ± 5	5.8 ± 0.6
Day 7	4.1 ± 0.5	59 ± 3	58 ± 7	35 ± 7	33 ± 2	6.2 ± 0.8
Day 14	4.7 ± 0.3	59 ± 2	67 ± 21	32 ± 8	32 ± 1	6.5 ± 1.3
Day 21	4.6 ± 0.1	58 ± 5	79 ± 26	32 ± 6	38 ± 5	7.3 ± 1.9
Day 28	4.7 ± 0.3	58 ± 5	62 ± 3	36 ± 6	39 ± 5	6.3 ± 1.4
0.5x dosage group						
Day -5	5.1 ± 0.4	55 ± 5	59 ± 4	33 ± 8	36 ± 7	4.5 ± 1.5
Day 7	5.0 ± 0.7	55 ± 5	57 ± 7	30 ± 7	40 ± 8	4.4 ± 1.7
Day 14	5.2 ± 0.4	57 ± 4	60 ± 5	33 ± 6	36 ± 9	4.3 ± 1.3
Day 21	4.9 ± 0.4	56 ± 4	63 ± 5	31 ± 9	40 ± 10	4.5 ± 1.3
Day 28	5.0 ± 0.6	55 ± 4	60 ± 6	38 ± 6	39 ± 9	4.2 ± 1.3
2.5x dosage group						
Day -5	4.9 ± 0.5	58 ± 3	61 ± 8	36 ± 10	34 ± 6	5.0 ± 1.1
Day 7	4.9 ± 0.3	57 ± 4	60 ± 7	36 ± 6	33 ± 7	5.5 ± 0.8
Day 14	4.9 ± 0.5	58 ± 5	60 ± 6	34 ± 7	34 ± 7	5.0 ± 0.8
Day 21	4.7 ± 0.2	58 ± 2	63 ± 8	36 ± 9	38 ± 7	5.5 ± 0.8
Day 28	4.9 ± 0.4	56 ± 3	62 ± 8	38 ± 8	37 ± 10	4.5 ± 0.9
5x dosage group						
Day -5	4.6 ± 0.7	56 ± 3	65 ± 7	32 ± 5	33 ± 8	5.8 ± 0.6
Day 7	4.3 ± 0.9	53 ± 2	67 ± 17	33 ± 4	32 ± 3	6.1 ± 1.5
Day 14	4.7 ± 0.7	54 ± 1	80 ± 40[b]	27 ± 6	34 ± 11	6.0 ± 1.3
Day 21	4.4 ± 0.5	53 ± 2	69 ± 20	30 ± 4	36 ± 5	5.8 ± 0.8
Day 28	4.4 ± 0.8	54 ± 1	90 ± 56[b]	53 ± 42[b]	34 ± 6	6.3 ± 2.2

ATL- alanine aminotransferase; AST-aspartate aminotransferase.
a Normal range was determined by measure a total of 188 health beagles in the facility.
b Values outside the normal range.

4.4 Discussion

The current study provides additional safety information on the novel botanical anxiolitic product, Zentrol (Pegasus Laboratories), in dogs. Classified as a natural health product, this botanical preparation does not require formal approval from regulatory agencies for registration purposes and consequently, does not require demonstration of safety and efficacy through numerous sets of studies, as is normally the case for veterinary biologics or pharmaceuticals. In order to extend our scientific knowledge and to satisfy both groups of potential clients, veterinarians in practice and pet owners, we conducted a series of well-designed and controlled laboratory and clinical studies to demonstrate both safety and efficacy of our novel anxiolytic product in dogs. The dosing regimen for our product is 1 mg/kg BW of betulinic acid at the time of an anticipated stressful event (thunderstorms, travel, vet visits), on a per-need basis. The current study was designed to provide answers on the safety profile of the product should it be administered at a higher dose than what is typically recommended and for a longer period of time.

The results observed in the present study expand on and are in agreement with previous observations from a pilot safety study (Villalobos et al., 2014), which demonstrated that there were no toxic effects of SSB and PTB botanicals administered separately at elevated doses for 28 d. The present study demonstrated that the botanical blend of both SSB and PTB is safe and well-tolerated in dogs, even when administered at 5× the recommended dose, following prolonged consecutive administration.

There were no clinically significant abnormalities observed in any of the dogs receiving the product multiple times over an extended period of time, as determined by physical examination, hematology, and clinical biochemistry results.

It is interesting to note that all occurrences of frank blood in the stool were noted during the dosing period in 3 placebo-treated dogs. Finding of *Giardia* spp. oocysts in feces from one dog may explain the occurrence of these clinical signs. Giardiasis in colony dogs at research facilities is commonly found and is considered endemic, despite implementation of high biosecurity measures. All 3 of these dogs were in the group receiving the placebo, so there was no possible relationship with the test article in any case. Furthermore, the placebo tablets contained 820 mg of the ingredient Avicel PH200 (microcrystalline cellulose) per tablet, compared to 410 mg of the ingredient Avicel PH101 (microcrystalline cellulose) in the test tablet. This was the only difference in the non-active ingredients between the placebo and the test article, made to compensate for the lack of the active ingredient, the SSB/PPT botanical blend. Whether this has any etiologic significance remains unknown.

An additional minor safety concern was raised in one dog in Group 4 (5×) that showed mildly increased urea values at 21 d post-dosing. The dog was humanely euthanized and subjected to a full gross necropsy and histopathology evaluation by a board-certified pathologist from the Animal Health Laboratory at the Ontario Veterinary College at the end of the study. Each kidney had a similar histologically normal appearance, with no evidence of significant glomerular, tubular, or renal interstitial injury or inflammation. Specifically, there was no significant microscopic evidence of glomerulitis or glomerulopathy in kidney sections stained with hematoxylin and eosin (H&E) stain or with periodic acid-Schiff stain, to highlight glomerular basement membranes. Therefore, kidneys were deemed grossly and

histopathologically normal and no clinical or toxicological relevance was attributed to these findings or to the use of the test article.

The active component, betulinic acid, was below the LLOD in serum following multiple sampling on day 21, as measured by HPLC/MS. This suggests that the betulinic acid was rapidly absorbed and metabolized prior to first sampling (30 min post dosing), making it difficult to be detected by the method used, which is primarily developed and validated for detection of betulinic acid in rodent models. This hypothesis is supported by the data from pilot efficacy and onset determination studies (Internal reports VRI-130-12008-CE and VRI-16048-CE) that demonstrated significant serum cortisol reduction 30 min after dosing and robust anxiolytic effect 1h after dosing. In previous studies, significantly higher doses of betulinic acid were administered before it could be detected in plasma. Godugu et al (Godugu et al., 2014) reported a maximum plasma concentration of betulinic acid of 1.16 ± 0.22 μg/mL with a single oral dose of 100 mg/kg BW in Sprague Dawley rats. Similar low serum levels were found by Udeani et al (Udeani et al., 1999) in mice. However, betulinic acid is highly lipohilic and has been found in tissues including brain, heart, liver, kidney, colon, and bladder with a much higher concentration than serum (Udeani et al., 1999). Our results, with conventional UPLC/MS, suggest that serum levels of betulinic acid may not be a straightforward way to measure the concentration of the active component in the blood stream and therefore, determine possible dose frequency at the clinical level. With the current methodology, despite several modifications to the test protocol, detection of betulinic acid in canine serum was not achieved and was deemed an impractical strategy to monitor treatments. One may argue that the presence of betulinic acid or its metabolites may be detected in feces or other excrements. However, feces and excrement

analysis would have their own set of limitations: undeveloped test method, inconsistent sampling (irregular defecation), and potential degradation of the active ingredient.

Controlled studies demonstrating onset and dose related efficacy of these novel anxiolytic botanical tablets, based on lowering cortisol levels and anxiety parameters in a noise-induced dogs' model, are complete and the manuscript is currently undergoing review. Although the product revealed no apparent toxicity, future efficacy studies in selected and larger populations of pet dogs, would be of value. In addition to this, the botanical product should be evaluated for interaction with commonly prescribed drugs. Studies examining withdrawal effects in dogs after chronic use of the blend should also be conducted. Although, this was not a problem observed in studies with rats (unpublished data).

In summary, under the conditions of this study and based on the results of daily observations, physical examinations, BW, clinical pathology, and urinalysis, the botanical tablets administered up to 5× the intended dose for 28 consecutive d, had no observed negative effects on the health of dogs and as such, have been deemed safe.

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Competing interests

Financial support was provided by Bioniche Life Sciences to Novometrix Research and to Paul Dick and Associates for their work. Since the corporate reorganization of Bioniche Life Sciences, leading to the sale of veterinary technologies, the product has been licensed to Souroubea Botanicals and several of the authors are principals in the new company (Arnason, Baker, Durst, Sanchez). Dr. Masic was employed by Bioniche Life Sciences at the time of study execution.

References

Sherman BL, Mills DS. Canine anxieties and phobias: An update on separation anxiety and noise aversions. Vet Clin North Am Small Anim Pract. 2008; 38:1081–1106.

Shull-Selcer EA, Stagg W. Advances in the understanding and treatment of noise phobias. Vet Clin North Am Small Anim Pract. 1991; 21:353–367.

Thompson SB. Pharmacologic treatment of phobias. In: Dodman NH, Shuster L, editors. Psychopharmacology of Animal Behavior Disorders. Malden, Massachusetts: Blackwell Science; 1998. pp. 141–182.

Dreschel NA. The effects of fear and anxiety on health and lifespan in pet dogs. Appl Anim Behav Sci. 2010; 125:157–162.

Puniani E, Cayer C, Kent P, et al. Ethnopharmacology of Souroubea sympetala and Souroubea gilgii (Marcgraviaceae) and identification of betulinic acid as an anxiolytic principle. Phytochemistry. 2015; 113:73–78.

Mullally M, Kramp K, Cayer C, et al. Anxiolytic activity of a supercritical carbon dioxide extract of Souroubea sympetala. Phytother Res. 2011; 25:264–270.

Carballo-Arce AF. PhD dissertation. Ottawa, Ontario: University of Ottawa; 2013. Phytochemical investigations of Costa Rican Marcgraviaceae and development of insecticide synergists.

Cayer C. MSc dissertation. Ottawa, Ontario: University of Ottawa; 2011. In vivo behavioural characterization of anxiolytic botanicals.

Mullally M, Cayer C, Kramp K, et al. Souroubea sympetala (Marcgraviaceae): A medicinal plant that exerts anxiolysis through interaction with the GABAA benzodiazepine receptor. Can J Physiol Pharmacol. 2014; 92:758–764.

Mullally M. PhD dissertation. Ottawa, Ontario: University of Ottawa; 2011. Anxiety-reducing tropical plants: Phytochemical and pharmacological characterization of Souroubea sympetala and Piper amalago.

Fujioka T, Kashiwada Y, Kilkuskie RE, et al. Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from Syzigium claviflorum, and the anti-HIV activity of structurally related triterpenoids. J Nat Prod. 1994; 57:243–247.

Kashiwada Y, Hashimoto F, Cosentino LM, Chen CH, Garrett PE, Lee KH. Betulinic acid and dihydrobetulinic acid derivatives as potent anti-HIV agents. J Med Chem. 1996; 39:1016–1017.

Mayaux JF, Bousseau A, Pauwels R, et al. Triterpene derivatives that block entry of human immunodeficiency virus type 1 into cells. Proc Natl Acad Sci USA. 1994;91: 3564–3568.

Mukherjee PK, Saha K, Das J, Pal M, Saha BP. Studies on the anti-inflammatory activity of rhizomes of Nelumbo nucifera. Planta Med. 1997; 63:367–369.

Recio MC, Giner RM, Máñez S, et al. Investigations on the steroidal anti-inflammatory activity of triterpenoids from Diospyros leucomelas. Planta Med. 1995; 61:9–12.

Pisha E, Chai H, Lee IS, et al. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med. 1995; 1:1046–1051.

Selzer E, Pimentel E, Wacheck V, et al. Effects of betulinic acid alone and in combination with irradiation in human melanoma cells. J Invest Dermatol. 2000; 114:935–940.

Zuco V, Supino R, Righetti SC, et al. Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. Cancer Lett. 2002; 175:17–25.

Fulda S, Debatin KM. Betulinic acid induces apoptosis through a direct effect on mitochondria in neuroectodermal tumors. Med Pediatr Oncol. 2000; 35:616–618.

Ramadoss S, Jaggi M, Siddiqui MJA. Use of betulinic acid and its derivatives for inhibiting cancer growth and a method of monitoring this. 6048847. U.S. Patent. A filed March 18, 1998 and issued April 11, 2000.

Schühly W, Heilmann J, Calis I, Sticher O. New triterpenoids with antibacterial activity from Zizyphus joazerio. Planta Med. 1999; 65:740–743.

Chandramu C, Manohar RD, Krupadanam DG, Dashavantha RV. Isolation, characterization and biological activity of betulinic acid and ursolic acid from Vitex negundo L. Phytother Res. 2003;17:129–134.

Schultes RE, Raffauf RF. The Healing Forest: Medicinal and Toxic Plants of the Northwest Amazonia. Portland, Oregon: Dioscorides Press; 1990.

Bourbonnais-Spear N, Awad R, Merali Z, Maquin P, Cal V, Arnason JT. Ethnopharmacological investigation of plants used to treat susto, a folk illness. J Ethnopharmacol. 2007; 109:380–387.

Bourbonnais-Spear N, Poissant J, Cal V, Arnason JT. Culturally important plants from southern Belize: Domestication by Q'eqchi' Maya healers and conservation. Ambio. 2006;35: 138–140.

Arnason T, Hebda RJ, Johns T. Use of plants for food and medicine by Native Peoples of eastern Canada. Can J Bot. 1981; 59:2189–2325.

Hamel PB, Chiltoskey MU. Cherokee Plants and Their Uses: A 400 Year History. Sylva, NC: Herald Publishing; 1975. pp. 1–52.

Villalobos P, Baker J, Sanchez Vindas P, Durst T, Masic A, Arnason JT. Clinical observations and safety profile of oral herbal products, Souroubea and Platanus spp: A pilot-toxicology study in dogs. Acta Vet. 2014; 64:269–275.

Godugu C, Patel AR, Doddapaneni R, Somagoni J, Singh M. Approaches to improve the oral bioavailability and effects of novel anticancer drugs berberine and betulinic acid. PLoS ONE. 2014; 9: e89919.

Udeani GO, Zhao GM, Geun Shin Y, et al. Pharmacokinetics and tissue distribution of betulinic acid in CD-1 mice. Biopharm Drug Dispos. 1999; 20:379–383.