Anxiety-Reducing Tropical Plants: Phytochemical and Pharmacological Characterization of *Souroubea sympetala* and *Piper amalago*

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ABSTRACT

This thesis investigates the phytochemistry and pharmacology of two neotropical plants used traditionally to treat anxiety and stress, *Souroubea sympetala* (Marcgraviaceae) and *Piper amalago* (Piperaceae). A method of phytochemical analysis was developed to characterize *S. sympetala* extracts, identifying and quantifying four triterpenes, which were present in higher amounts in bark as compared to leaf. Subsequently, a standardized supercritical CO_2 extraction procedure for *S. sympetala* was developed and compared favourably with conventional extraction methods in terms of its anxiety-reducing effects in a behavioural assay of anxiety and content of the active principle, betulinic acid (BA). All of these materials demonstrated anxiolytic properties. The pharmacological mode of action of *S. sympetala* raw plant, extracts and isolated active principle were examined in rodent behavioural models of anxiety. The extracts were shown to have affinity for the γ-amino butyric acid (GABA)a benzodiazepine (GABAa- BZD) receptor of the central nervous system *in vitro*, in a competitive binding assay. Pre-treatment of animals with the GABAa-BZD antagonist flumazenil, followed by plant extract and pure compound extinguished the anxiety-reducing effect, demonstrating that *S. sympetala* and BA act at the GABAa- BZD receptor *in vivo*. The effect of *S. sympetala* in stressed animals, specifically its cortisol-lowering ability was investigated *in vitro* and *in vivo* in rainbow trout. Both leaf extract and BA significantly lowered cortisol in response to an adrenocorticotropic hormone (ACTH) challenge *in vitro* and a standardized net restraint assay *in vivo*. The anxiety-reducing effect of *P. amalago* was examined and the bioactive principle identified by bioassay-guided fractionation. *P. amalago* extract significantly reduced anxiety-like behaviour in rats and demonstrated affinity for the GABAa-BZD receptor *in vitro*. The bioactive molecule was determined to be a furofuran lignan.

Together these results provide a pharmacological basis for the traditional use of *S. sympetala* and *P. amalago* to treat anxiety and elucidate their mode of action and active principles. *S. sympetala* is now thoroughly characterized and represents an excellent candidate plant for development as a natural health product.

RÉSUMÉ

Cette thèse porte sur la phytochimie et pharmacologie de deux plantes néotropicales traditionnellement utilisées pour traiter l'anxiété, *Souroubea sympetala* (Marcgraviaceae) et *Piper amalago* (Piperaceae). Une méthode d'analyse phytochimique a été développée afin de caractériser les extraits de *S. sympetala*, d'identifier et de quantifier quatre triterpènes, qui furent retrouvés en quantités plus élevées dans l'écorce par rapport à la feuille. Par la suite, une procédure standardisée d'extraction au CO_2 supercritique pour *S. sympetala* a été développé et fut comparé favorablement avec les méthodes classiques d'extraction en fonction de sa capacité réductrice d'anxiété dans un bioessai comportemental, et en son contenu du principe actif, l'acide bétulinique. Le mode d'action pharmacologique de matières brutes, d'extraits, et du principe actif de *S. sympetala* ont été examinés chez les rongeurs en utilisant des modèles comportementaux d'anxiété. Tout le matériel testé a démontré un niveau significatif d'anxiolyse . En utilisant un bioessai de liaison compétitive *in vitro,* les extraits ont démontrés un affinité envers les récepteurs GABAa benzodiazépines (GABAa-BZD) du système nerveux central. Le prétraitement des animaux avec flumazénil, un antagoniste du récepteur GABAa-BZD, suivie d'extraits de plantes et de composé pur cancella l'effet anxiolytique, démontrant que *S. sympetala* et BA agissent au niveau du récepteur GABAa-BZD. La capacité de *S. sympetala* à abaisser le niveau de cortisol fut étudié dans un modèle *in vitro* et *in vivo* chez la truite arc-en-ciel. L'extrait de feuilles et BA ont tout deux réduit de façon considérable le niveau de cortisol en réponse à un défi d'hormone adrénocorticotrope (ACTH) *in vitro* et un bioessai standardisé de retenue nette *in vivo.*

L'effet anxiolytique de *P. amalago* a été examinée et le principe bioactifs identifiés par fractionnement guidé avec bioessai. L'extrait de *P. amalago* réduit de façon considérable le comportement d'anxiété des rats et a démontré une affinité pour le récepteur GABAa-BZD *in vitro*. La molécule bioactive fut identifié comme étant un « furofuran lignan ».

Ensemble, ces résultats constituent une base pharmacologique pour l'utilisation traditionnelle de *S. sympetala* et *P. amalago* pour traiter l'anxiété et fournissent un aperçu de leur mode d'action et principes actifs. *S. sympetala*, qui a été entièrement caractérisé, représente un excellent candidat pour le développement d'un produit de santé naturel dérivé de plante.

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LIST OF ABBREVIATIONS

#CAE	number of closed arm entries
#OAE	number of open arm entries
%TCA	percent time spent in closed arms
%TOA	percent time spent in open arms
α-A	α-Amyrin
β-A	β-Amyrin
11β-HSD	11 β-Hydroxysteroid dehydrogenase
^{3}H-Flu	tritiated flunitrazepam
5-HT	5-hydroxytryptamine
ACTH	adrenocorticotropin hormone
APCI	atmospheric chemical ionization
ASE	accelerated solvent extraction
BA	betulinic acid
BZD	benzodiazepine
CAM	complementary and alternative medicine
CER	conditioned emotional response
CNS	central nervous system
CRH	corticotrophin-releasing-hormone
CYP	Cytochrome P450
DAD	diode array detection
Diaz	diazepam
EC_{50}	mean effective concentration
eMEM	enriched minimum essential media
EPM	elevated plus maze
EtOAc	ethylacetate
EtOH	ethanol
FDA	Food and Drug Administration
Flu	flumazenil
FPS	fear potentiated startle
FST	forced swim test
GA	glycyrrhetinic acid
GABA	γ-amino butyric acid
GABA-T	γ-amino butyric acid transaminase
HAM-D	Hamilton depression rating scale
HPA	hypothalamus-pituitary-adrenal
HPI	hypothalamus pituitary interrenal
HPLC	high pressure liquid chromatography
HTS	high throughput screening
IC_{50}	mean inhibitory concentration
Ki	dissociation constant
LDH	lactate dehydrogenase
m/z	mass over charge ratio
MAOB	monoamine oxidase B
MAOI	monoamine oxidase inhibitors

MDD	major depressive disorder
meBA	methyl ester of betulinic acid
MS	mass spectrometry
NCE	new chemical entities
NHP	natural health product
NIH	National Institutes of Health
NMR	nuclear magnetic resonance
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
RIA	radioimmunoassay
SCE	supercritical carbon dioxide extraction
SEM	standard error of the mean
SI	social interaction
SIM	scanning ion mode
SJW	St John's wort
Sox	soxhlet extraction
SSRI	serotonin re-uptake inhibitor
TOA	time in open arms
UA	ursolic acid
UAE	ultrasonic assisted extraction
UNA	Universidad Nacitonal (in Costa Rica)
UPHD	unprotected head dips

CHAPTER 1 – GENERAL INTRODUCTION AND LITERATURE REVIEW

The art of healing comes from nature and not from the physician. Therefore, the physician must start from nature with an open mind. ~ Paracelsus

1.0 Introduction

Plants have been used as medicines for thousands of years (Samuelsson and Bohlin, 2009) and remain a vital source for new drugs today. Medicines initially took the form of teas and knowledge was shared through oral history. Early in the 19th century, the separation and identification of new molecules from medicinal plants began with the isolation of morphine from opium (Samuelsson and Bohlin, 2009). Drug discovery from plants led to isolation of a wide range of molecules that continue to be some of the most important therapeutics available, including vinblastine, vincristine (to treat cancer), quinine (malaria), artemisinin (malaria), and paclitaxel (cancer) (Ojima, 2008). Although the definition of a naturally-derived drug varies, and includes parent compounds, derivatives, analogues, and mimics, an estimated 61% of the 877 small-molecule new chemical entities (NCEs) introduced as drugs worldwide during 1981–2002 can be traced to, or were inspired by, natural products (Newman et al., 2003).

Drug discovery from medicinal plants is a multi-disciplinary science that involves numerous fields of inquiry and analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist, or chemical ecologist who collects and identifies the plant(s) of interest. Collections may involve species with known biological activity for which active compound(s) have not been isolated, such as traditionally used herbal remedies, or may involve random collections for a large screening program, such as that used by the National Institutes of Health (NIH) to identify the cancer drug paclitaxel (taxol) (Cragg and

Newman, 2005). Today there are a variety of dereplication strategies that increase success in identifying novel, bioactive molecules, such as the study of rare plant families. A critical, and emerging issue in this field is the requirement that the intellectual property rights of the country, or indigenous people, where plant collections take place are respected (Baker et al., 1995; Boyd, 1996). Plant extracts are prepared and subjected to biological testing in pharmacological assays that leads to isolation and characterization of the active compound(s) by bioassay-guided fractionation.

In the past three decades, the pharmaceutical industry has abandoned natural product discovery in favour of combinatorial chemistry. Combinatorial chemistry has brought with it the promise of hundreds or thousands of synthetic molecules that can be tested in high through-put screening (HTS) assays and assurances with respect to intellectual property and patent rights. However, despite the promise of combinatorial chemistry and HTS, only one Food and Drug Administration (FDA)-approved drug (sunitinib, for renal carcinoma in 2005) has been developed through HTS of combinatorial chemistry libraries in the past 20 years. Instead, natural products-based drugs are still major entities among FDA-approved drugs, representing 57.7% of all drugs (Ojima, 2008).

For this reason, plants remain a vital source of new molecules for pharmacological targets. While single entity drug discovery has motivated much research in the past, interest in complementary and alternative medicine (CAM) and indigenous traditional medicine has also been a driver for recent research. Regulatory systems have adapted to allow use of traditional crude drugs through the Canadian Natural Health Products, Australian Complementary and Alternative Medicines, US Botanical Drug and EU Phytomedicine regulatory streams.

There are an estimated 250,000 species of higher plants on Earth, only 15% of which have been characterized phytochemically and 6% examined for biological activity (Phillipson, 2004). At the same time, unprecedented rates of habitat loss, particularly in the tropics, where some of the most diverse plants on the planet exist (Brooks et al., 2002; Giam et al., 2010), threaten the permanent loss of plants that may represent sources for novel molecules or treatments for human diseases.

A guiding principle of the research in the Arnason phytochemistry group is the value of characterizing rare, tropical plant species. In collaboration with botanists at the Universidad Nacional (UNA) in Costa Rica, our lab embarked on a natural product discovery study of rare tropical species in the Meso-american corridor, one of the world's hotspots for biodiversity (Myers et al., 2000). There were two purposes of this work. First, to demonstrate the value of tropical ecology through investigation of the phytochemistry and medicinal application of rare tropical species. And, second, to strengthen the argument for protection and preservation of tropical habitat and rare plants.

Several plants were investigated as part of this project, including two rare tropical trees, *Pleodendron costaricense* N. Zamora, Hammel & R. Aguilar (Canellaceae), a new species, and *Ruptiliocarpon caracolito* Hamel and N. Zamora, one of only two members of the Lepidobotryaceae family. *P. costaricense* was phytochemically characterized for the first time and shown to contain molecules with anti-fungal activity (Amiguet et al., 2006). *R. caracolito* yielded several novel spirocaracolitone triterpenoids that demonstrated potent insect anti-feedant properties (Asim et al., 2010). These successes supported the hypothesis that rare tropical plants have novel phytochemistry, or medicinal application, and led to the investigation of a small and unusual plant family not previously described, the Marcgraviaceae.

1.1 Marcgraviaceae and Piperaceae, Tropical Plant Families with CNS Activity

An early survey of the literature in Richard Evan Schultes' seminal work on tropical medicinal plants, *The Healing Forest*, identified the genus *Souroubea* within the Marcgraviaceae as material with an ethnobotanical tradition of use to treat *susto*, or fright (Schultes and Raffauf, 1990). At the same time, an ethnobotanical survey of the Q'eqchi' Maya of Belize identified high use of plants to treat mental illness (Amiguet et al., 2005). The Maya recognize and treat a range of mental illnesses, from epilepsy to *susto*, and have a wide pharmacopeia of tropical plants to treat these illnesses, but the Piperaceae family is significantly over-represented among plants used to treat mental illness (Bourbonnais-Spear et al., 2007). These observations lead to an interest in tropical plants to treat *susto*, a topic that has been examined in some detail by the University of Ottawa natural products group (Awad et al., 2009; Bourbonnais-Spear et al., 2005; Bourbonnais-Spear et al., 2007) and is the topic of this PhD thesis project. The plants investigated in this PhD project were the two putative anxiety-reducing plants, *Souroubea sympetala* Gilg. (Marcgraviaceae) and *Piper amalago* L. (Piperaceae).

1.2 Literature Review

1.2.1 The Marcgraviaceae

The Marcgraviaceae is a neotropical plant family of lianas, climbing shrubs and treelets, arranged in seven genera, *Marcgravia*, *Souroubea*, *Ruyschia*, *Marcgraviastrum*, *Sarcopera*, *Norantea* and *Schwartzia*, and includes approximately 130 species ranging from northern Bolivia and southern Brazil to southern Mexico including the West Indies (Gentry,

1993). The family is characterized by various kinds of sclereids, hypophyllous glands and specific nectary bracts (Dressler, 2004). Several insects, birds and mammals, especially bats, have been suggested or proven as likely pollinators. Five Souroubea species grow in Costa Rica, the two major ones are *S. sympetala* and *S. gilgii* V.A. Richt., they are morphologically very similar and can be difficult to distinguish. The work described in this thesis was done exclusively with *S. sympetala* (Figure 1.1) which is an epiphytic shrub, often vine-like, found in wet forests and disturbed areas and often high up in the forest canopy where they can be difficult to observe. The leaves are alternate, thick and leathery, and epiphytic.

The flowers are red and yellow, radially symmetrical, 0.5 – 0.8 cm long, petals fused, lobes 4 – 5, each flower above a tubular, nectar-producing structure about 1 cm long, with a rounded end, and postulated to be pollinated by butterflies and hawk moths (Lens et al., 2005). Inflorescences are up to 25 cm and unbranched; the flowers bloom in June, November and February. The fruits, which are seen in October, have leathery flesh and few seeds. The plant's range is from Belize to Panama.

1.2.2 *Souroubea sympetala* - A plant to treat susto

Susto is a condition of folk etiology known throughout Latin America and understood to occur following a sudden frightening event that leads to the loss of soul or essence. *Susto* is accompanied by various physiological symptoms including diarrhoea, restlessness, and loss of appetite (Klein, 1978). For diagnostic purposes, *susto* is considered a "culture-bound syndrome" and linked to both anxiety and depression (American Psychiatric Association, 2000; World Health Organization, 2007). In Belize when Q'eqchi' Maya healers were asked about uses for *Souroubea*, four out of ten reported use of *S. sympetala* and *S. gilgii* to prepare

a tea to treat witchcraft, a condition that results in unease, worry, withdrawal, sadness, weight-loss and other symptoms consistent with a folk description of anxiety (Arnason, 2004). This preliminary ethnobotany led to the hypothesis that *S. sympetala* has anxiety-reducing properties and subsequent experiments examined this hypothesis.

Puniani (Puniani, 2004) completed a comprehensive phytochemical characterization of *S. sympetala* and tested the hypothesis that *S. sympetala* had anxiety-reducing properties in animal behavioural assays. The major phyochemicals identified in the leaves were seven pentacyclic triterpenoids: α-amyrin, β-amyrin, ursolic acid, betulinic acid, methyl-2- α hydroxyl ursolic acid, maslinic acid and taraxeryl trans- *p*- hydroxyl cinnamate. The leaves also contain six flavonoids and some other minor contributors. In animal behavioural assays of anxiety, rats fed an ethanolic extract of *S. sympetala* leaf displayed decreased anxiety-like behaviour in the elevated plus maze (EPM) and fear potentiated startle (FPS) paradigms (Puniani, 2004). Bio-assay guided fractionation identified the pentacyclic triterpene betulinic acid (BA) as the principle bioactive molecule. A 1 mg/kg dose of BA significantly reduced anxiety-like behaviour in rats in the EPM, demonstrating that BA has anxiety-reducing properties (Puniani, 2004). Further examinations of the effect of *S. sympetala* on stress demonstrated that rats fed *S. sympetala* extract and a synthetic analog of BA, the methyl ester of BA (meBA), exhibited significantly lower mean plasma corticosterone levels following a restraint stress than vehicle fed animals (Cayer, 2011).

Figure 1.1. 

Souroubea sympetala vine collected in Belize, photograph courtesy of B. Walshe-Rousell.

1.2.3 The Piperaceae

The Piperaceae, commonly called the pepper family, is a large pantropical family of flowering plants consisting of small trees, shrubs or herbs, sometimes epiphytic and often aromatic. The Piperaceae is arranged in five genera, *Verhuellia* Miquel, *Zippellia* Blume, *Manekia* Trelease, *Piper* Linnaeus and *Piperomia* Ruiz & Pavon, with over 3,600 species. The majority of Piperaceae species, 2,000, are members of the genus *Piper*. The *Piper* species are commonly found in the dark understory of lowland tropical rainforests, and are also present in forest gaps and cloud forests. Most *Piper* species are herbs, vines, shrubs or small trees. The fruit of the *Piper* plant, called a peppercorn when it is round and pea-sized, is distributed mainly by birds, with some distribution by bats (Tebbs, 1993).

The *Piper* species are the source of pepper in the worldwide spice markets. The unripened fruit of *P. nigrum* is the source of black pepper and accounts for 35% of the world's total spice trade (Ravindran, 2000). The genus *Piper* L. has long been recognized for its medicinal properties and diversity of biologically active secondary metabolites (Parmar et al., 1997). Consistent with the ethnobotany of the Q'eqchi' Maya, *Piper* species are used throughout the world to treat neurological disorders, in locations including China (Pei, 1983), Nigeria (Abila et al., 1993), Polynesia (Cairney et al., 2002), and Brazil (Cícero Bezerra Felipe et al., 2007). *Piper* species contain unique nitrogen compounds called piperamides, with a broad range of activities, including GABAa receptor agonist actions (Zaugg et al., 2010), CNS-depression (Choi et al., 2007; Li et al., 2007a), insecticidal (De Paula et al., 2000; Scott et al., 2003; Scott et al., 2004) and antioxidant activities (Vijayakumar et al., 2004), and modulation of drug metabolism (Jensen et al., 2006; Koul et al., 2000).

P. methysticum G. Forst (kava) is the most well-characterized anxiolytic herb (Pittler and Ernst, 2003), with significant anxiety-lowering effects observed in clinical trials (Ernst, 2006). Kava's activity is primarily associated with the kava lactones, specifically kavain, which inhibits re-uptake of norepinephrine, and desmethoxyyangonin, a reversible monoamine oxidase B (MAOB) inhibitor (McClatchey et al., 2009). The other kava lactones may have similar mechanisms of action that account for kava's anxiolytic effects.

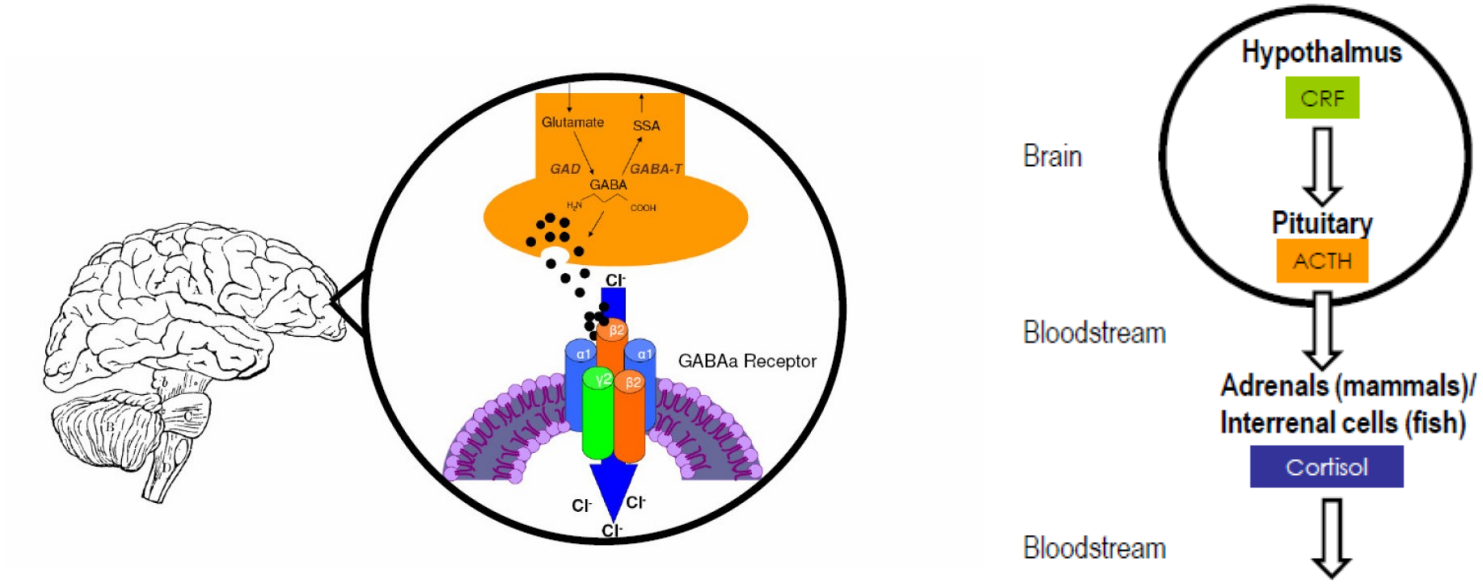
The Q'eqchi' Maya of Belize identified high use of the Piperaceae to treat mental illness (Bourbonnais-Spear et al., 2007). The plants identified to treat mental illness were tested in two *in vitro* pharmacological assays of anxiety and ranked in terms of extract activity and healer consensus (Awad et al., 2009). One of the most active and highly ranked plants in the collection was *Piper amalago*.

P. amalago, commonly called buttonwood or *cordonsillo* (Figure 1.2), is a slender, much branched perennial shrub that grows up to 3 m tall; the leaves are 7–14 cm long and flowers are in greenish or white spikes, each 3 - 7cm long.

Figure 1.2. (A) *Piper amalago* growing in the forest in Belize, **(B)** Detail of flower, photographs courtesy of B. Walshe-Roussel.

1.2.4 Pharmacology of Anxiety and Stress

Anxiety and stress are physiologically distinct phenomena that involve different brain regions, signalling systems, signalling molecules and outcomes. The inhibitory neurotransmitter GABA plays a central role in anxiety. Molecules that target receptors and enzymes in the GABA system are used in the pharmacological treatment of anxiety (Durant et al., 2010; Lydiard, 2003). The hypothalamus-pituitary-adrenal (HPA) axis is a major part of the neuroendocrine system that controls reactions to stress and regulates many body processes. Cortisol (corticosterone in rodents) is the primary signalling molecule in the HPA, as illustrated in Figure 1.3. As discussed above, *S. sympetala* affects both anxiety and stress, and *P. amalgo* has an ethnobotanical tradition in the treatment of *susto*, an anxiety disorder. These observations lead to the proposal that the molecular mechanisms underlying the medicinal use of these plants relate to the anxiety related GABAergic system (*S. sympetala* and *P. amalago*), and the stress system (*S. sympetala*). To this end, two hypotheses were generated and tested regarding the pharmacological basis of activity of these medicinal plants, the anxiety and the stress hypotheses.



	Anxiety	Stress
Physiolgical effect	Reduction of anxiety (anxiolysis)	Reduction of stress response
System Targeted	Central Nervous System	Hypothalamus-Pituitary-Adrenal Axis
Signalling Molecule	GABA	Cortisol
Botanical Tested	*Souroubea sympetala* & *Piper amalago*	*Souroubea sympetala*

Figure 1.3. Schematic representation of two hypotheses regarding pharmacological mode of action investigated, the GABA and the stress hypotheses, highlighting the physiological effect, system targeted key molecules involved in each and the botanical tested in each hypothesis.

1.2.5 GABA and Anxiety

The principal inhibitory neurotransmitter in the central nervous system (CNS), γ – aminobutyric acid (GABA), is central in the pathophysiology of anxiety disorders (Lydiard, 2003; Treiman, 2001). Clinical studies suggesting a role for GABA in the etiology of anxiety include observations that patients with panic disorder show less GABAa receptor binding (Malizia et al., 1998), and lower brain GABA levels than healthy controls (Goddard et al., 2001), and that patients with anxiety disorders exhibit reduced benzodiazepine (BZD) binding relative to controls (Goddard et al., 2004).

The major pharmaceutical target used to treat anxiety disorders is the GABAa receptor, a ligand-gated ion channel receptor composed of five membrane spanning subunits arranged around a central pore. The GABAa receptor is widely distributed throughout the CNS and approximately 30% of all neurons are GABAergic (Purves et al., 2001). As illustrated in Figure 1.4, when GABA binds to the GABAa receptor a conformational change occurs in the receptor that permits influx of chloride ions (Cl^-) into the cell, hyperpolarizing the cell and causing neuronal inhibition. The GABAa receptor contains numerous binding sites, including sites for GABA, barbiturates, benzodiazepines (BZD), picrotoxin and neurosteroids. BZDs are GABAa agonists commonly used in the pharmacological treatment of anxiety disorders. The binding of a BZD to the GABAa receptor at the benzodiazepine binding site elicits an allosteric change in the receptor that increases GABA's affinity for its own binding site, thereby increasing binding of GABA to the GABAa receptor, influx of Cl^- into the cell, neuronal hyperpolarization and neuronal inhibition.

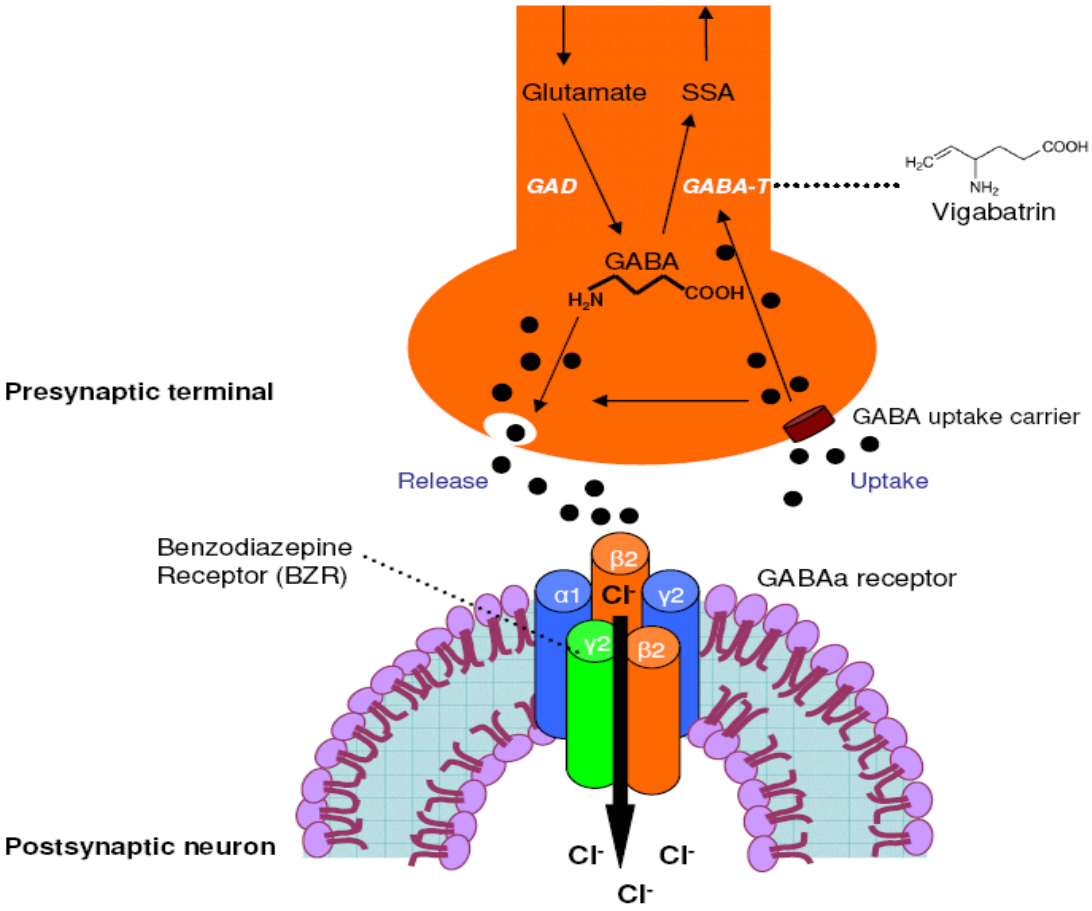


Figure 1.4 Schematic representation of GABA binding to the GABAa receptor. GABA is synthesized from glutamate by glutamate decarboxylase (GAD). GABA is released from the presynaptic terminal into the synaptic cleft where it binds to the GABAa receptor. The GABAa receptor is a ion-gated channel composed of 5 membrane-spanning domains. Binding of GABA to the GABA site on the GABAa receptor elicits an allosteric change in the receptor and results in influx of chloride ions (Cl^-) into the postsynaptic neuron, neuronal hyperpolariaztion and inhition of neuronal signal. Pharmacological agents such as benzodiazepines (BZD) (ex. diazepam, ativan), used to treat anxiety, bind the GABAa receptor at the BZD receptor; this binding causes an allosteric change in the receptor that increases GABA's affinity for its own receptor, thereby increasing Cl^- influx into the postsynaptic neuron, causing increased neuronal inhibition and anxiety reduction. GABA in the synapse is taken back up into the presynaptic neuron via the GABA uptake carrier or into the glial cells and catabolized via GABA-transaminase (GABA-T) to succinic semialdehyde (SSA) which is shunted into the Kreb's cycle. Pharmacological agents to treat anxiety, such as vigabatrin, target the activity of GABA-T. Vigabatrin is an irreversible competitive inhibitor of GABA-T. Schematic adapted from Treiman (2001).

BZDs (including valium, lorazepam and clonazepam) are fast-acting and efficacious pharmaceuticals to treat anxiety. However, these are also associated with deleterious side-effects, including sedation, muscle relaxation, tolerance, memory impairment and potentiation with alcohol and barbiturates (Lader, 2011; Stevens and Pollack, 2005). Further, BZDs are not recommended for chronic use (Ashton and Young, 2003). The side-effects of BZDs have spurred an interest in identifying partial GABA agonists, ligands with a lower affinity for the BZD receptor as BZDs. It is hypothesized that such ligands may elicit a therapeutic effect similar to BZDs without the debilitating side effects.

1.2.6 Anxiety Disorders and Herbal Medicine

Mental illness has a significant economic and social impact on Canadians. Health Canada estimates that 1 in 5 Canadians will suffer from a mental illness during their lifetime, resulting in loss of income, reduced quality of life and increased burden on the medical system. Close to 80% of Canadians are indirectly affected by mental illness, through the illness suffered by family, friends or colleagues. The most common form of mental illness is anxiety (Health Canada, 2002). Anxiety disorders are mental conditions characterized by feelings of fear, concern and apprehension without an identifiable source. Anxiety disorders take several forms and include generalized anxiety disorder, specific phobias, post-traumatic stress disorder, social phobia, obsessive-compulsive disorder and panic disorder, and have high comorbidity with both major depression and substance abuse (Kessler et al., 1997; Kessler et al., 2003).

People with anxiety disorders are high consumers of herbal medicine or complementary and alternative medicine (CAM). An estimated 43% use some form of CAM with the majority using a herbal medicine (Ernst, 2006). Anxiety disorders are also cited as one of the main reasons people try herbal medicines (Wong et al., 1998). There is a growing interest in herbal medicine among consumers; Americans spend US$14 billion a year on complementary and alternative medicines (Ernst, 2002). This interest highlights the commercial importance of ongoing research regarding the efficacy of herbal medicines to treat anxiety.

The most common commercially available herbal anxiolytics are chamomile (*Matricaria recutita* L.), passion flower (*Passiflora incarnata* L.*)*, skullcap (*Scutellaria lateriflora* L.) and kava (*Piper methysticum* G. Forst.). For each of these, *in vitro*, animal and human clinical data support their efficacy to treat anxiety (Sarris et al., 2011) . The bioactivity of chamomile, passion flower and skullcap is associated with flavonoids [apigenin (chamomile), chrysin (passion flower) and baicalin, wogonin and 5,7,2'-trihydroxy-6,8-dimethoxyflavone (skullcap)] that act as GABAa-BZD receptor agonists (Avallone et al., 2000; Dhawan et al., 2001a; Huen et al., 2003a; Huen et al., 2003b; Hui et al., 2002). Kava's bioactivity is associated with the kavalactones, methysticin, dihydromethysticin, kavain, dihydrokavain, demethoxyyangonin, and yangonin. The kavalactones have a variety of pharmacological targets that include, but are not limited to enhanced ligand binding to GABAa receptors. However, unlike BZDs and the other herbal anxiolytic, kavalactones do not bind to the GABAa receptor, but rather activate GABAergic effects by modulation of the GABA channels and GABA binding sites (Gleitz et al., 1996; Jussofie et al., 1994). Kava extracts do not interact with the GABAa-BZD receptor in

rodents (Davies et al., 1992) or *in vitro* and kavalactones do not compete with the GABAa-BZD receptor antagonist flunitrazepam to bind to the GABAa-BZD receptor (Boonen and Häberlein, 1998).

1.2.7 Stress and Cortisol

In animals, stress is perceived and responded to by initiating a cascade of responses in the hypothalamus-pituitary-adrenal (HPA) stress axis. When a stress stimulus is perceived, corticotrophin-releasing-hormone (CRH) is released from the hypothalamus, this causes release of adrenocorticotropin hormone (ACTH) from the pituitary gland. ACTH stimulates the synthesis and release of cortisol from adrenal cells into the blood stream. In animal trials of stress, circulating plasma cortisol (or corticosterone in rodents) is a common biomarker used to confirm that a stimulus exerts significant stress and to distinguish non-stressed from stressed animals. A schematic of the HPA-stress axis is presented in Figure 1.5.

The stress response is adaptive in the short term and required for appropriate responses to stimuli in the environment. However, chronic stress, which can lead to chronically elevated cortisol, hypercortisolism, is maladaptive, and is implicated in disease in both livestock animals and humans. In livestock, elevated stress slows growth and reduces production (Rostagno, 2009). In aquaculture, stress has an overall catabolic effect and causes slowed growth, impaired reproduction and disease (Schreck et al., 2001). In humans, hypercortisolism is associated with a range of illnesses, including Cushing's syndrome, depression, heart disease, obesity and metabolic syndrome (Anagnostis et al., 2009; Carroll

et al., 2007; Gathercole and Stewart, 2010; Smith et al., 2005). In humans, diverse stressful stimuli, including low socioeconomic status, race (Hajat et al., 2010), chronic work stress (Chandola et al., 2006), anxiety and depression (Carroll et al., 2007) all stimulate neuroendocrine responses.

Notwithstanding the link between hypercortisolism and a variety of human illnesses, pharmacological treatments for hypercortisolism are still under investigation. The hypercortisolism present in Cushing's syndrome is typically caused by a tumor (adrenal or pituitary) and treated with surgery (Sharma and Nieman, 2011). The interest in the role of hypercortisolism in metabolic syndrome stems from the phenotypic similarities between patients with Cushing's and metabolic syndrome. Both include central obesity, impaired glucose tolerance, insulin resistance, type-two diabetes, increased cardiac risk of mortality, osteoporosis and depression (Gathercole and Stewart, 2010). Normalizing cortisol levels usually reverses the symptoms in Cushing's syndrome (Stewart, 2003).

In the context of metabolic syndrome, inhibition of the enzyme 11β hydroxysteroid dehydrogenase 1 (11- β HSD1) to treat hypercortisolism has been examined (Gathercole and Stewart, 2010). At the pre-receptor level, 11-β HSD1 converts metabolically inactive cortisone to active cortisol (11-dehydrocorticosterone to corticosterone in rodents). In rodent models of metabolic syndrome, inhibition of 11- β HSD1 improves metabolic profile (Gathercole and Stewart, 2010). A recent Phase I clinical trial with a selective 11-β HSD1 inhibitor showed good tolerability, and no activation of the HPA axis in healthy patients (Courtney et al., 2008).

1.2.8 Stress and Herbal Medicine

The CAM term "adaptogen" is typically used to refer to herbal medicines used to treat stress or ameliorate the effects of stress. The concept of adaptogen was originally conceived by Russian physician and scientist Nikolai Lazarev. Lazarev described adaptogens as compounds that increase “the state of non-specific resistance” in stress (Lazarev, 1958; Lazarev et al., 1959). A modern definition of adaptogen is a herbal preparation that reduces stress-induced impairments and disorders related to the neuroendocrine and immune systems, and increases attention and endurance in fatigue (Panossian and Wikman, 2009). The term adaptogen is used in CAM but is not accepted in common pharmacological and pharmaceutical use (European Medicines Agency, 2007).

Among the most common commercially available adaptogens are ginseng (Asian and American, *Panax ginseng* C. A. Meyer and *Panax quinquefolius* L.*)*, eleuthero, or Siberian ginseng (*Eleutherococcus senticosus* Rupr. et Maxim (Maxim)) and rhodiola (*Rhodiola rosea* L.). The bioactive molecules in ginseng are saponin glycosides, referred to as ginsenosides in *P. ginseng* and *P. quinquefolius*, and eleutherosides in *E.senticosus*; the molecules are chemically distinct, but thought to have similar properties (Heinrich et al., 2004). *P. ginseng* extract and ginsenoside Rc significantly reduce corticosterone in restrained mice (Kim et al., 2010; Kim et al., 2003a) and attenuate plasma corticosterone levels induced by direct ACTH administration (Kim et al., 2003a), and red Korean ginseng reduces cortisol in postmenopausal women with climacteric syndromes (Tode et al., 1999). *P. quinquefolius* normalizes plasma corticosterone levels in rats exposed to chronic, unpredictable stress (Rasheed et al., 2008). *E. senticosus* extract lowers corticosterone in rats exposed to chronic stress, but increases basal cortisol levels in endurance athletes (Gaffney et al., 2001). This paradoxical, cortisol-increasing effect under basal, non-stressed, conditions

has also been observed with *P. ginseng*, where the *P. ginseng* extract dose increased circulating cortisol under resting conditions, but lowered it under stress conditions (Kim et al., 2003a).

The adaptogenic properties of *R. rosea* are attributed specifically to p-tyrosol, salidroside, and the phenylpropanoid glycosides that are specific in this plant, called the rosavins (rosin, rosavin, and rosarin) (Iovieno et al., 2011). Rhodiola has a variety of pharmacological targets, including the HPA. *R. rosea* lowers cortisol response to restraint stress in rodents (Panossian and Wikman, 2009) and in humans with stress-related fatigue (Olsson et al., 2009).

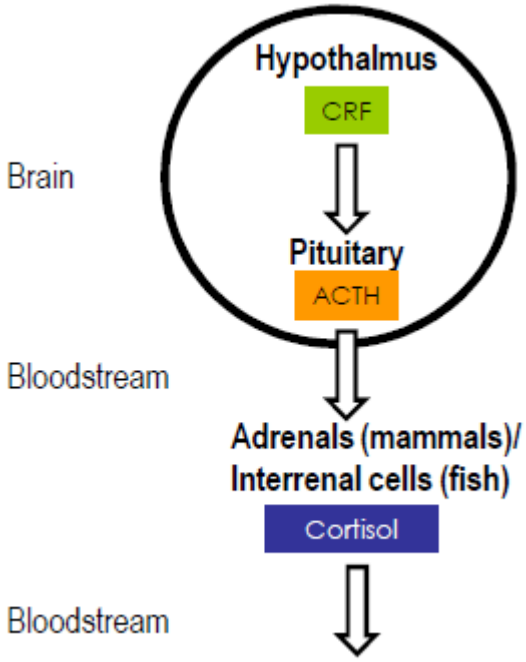


Figure 1.5. Schematic representation of the hypothalamus-pituitary-adrenal stress axis. The perception of a stress stimulus in the environment stimulates the release of corticotrophin-releasing hormone (CRH) from the hypothalamus, which in turn stimulates the release of adrenocorticotropin hormone (ACTH) from the pituitary. ACTH in turn stimulates the release of cortisol from adrenal cells (interrenal cells in trout), which enters the bloodstream. An increase in plasma cortisol levels is the physiological evidence of an elevation in stress.

1. 3 Rationale & Objectives

Ethnobotanical and animal behavioural studies indicate that *S. sympetala* and *P. amalago* reduce anxiety and stress. The fundamental research aspect of this thesis examined the mechanisms of action and active principles of these traditionally used medicines. Further, there is a demand among consumers of herbal products for natural health products (NHPs) to treat anxiety and a need to replace the well-known mental health herbs kava and St John's wort (SJW). The applied aspects of this PhD project examined *S. sympetala* with a view towards its development as a fair trade natural health product from Costa Rica. The project was divided into three parts: plant physiology and phytochemistry, pharmacological mode of action; and ethnopharmacology and natural product discovery.

1.3.1 Plant Physiology and Phytochemistry

In early development of a NHP the principle bioactive must be identified, and analytical methods developed, to quantify it in various plant parts and extracts. Chapter 2 describes the development of a high pressure liquid chromatography (HPLC) atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) (HPLC-APCI-MS) technique to identify and quantify the four primary triterpenes present in *S. sympetala*. The triterpenes in various plant parts, leaves (old and new), bark, wood, fruit and flowers were quantified and the phytochemistry of the plant parts compared.

Objective 1: To develop a HPLC-APCI -MS technique to identify and quantify triterpenes in *S. sympetala.*

Following the development of an analytical technique for *S. sympetala* extracts, a standardized extraction method for sample preparation was developed. Several commonly employed extraction methods were compared to determine the best technique to generate a BA-enriched extract of *S. sympetala* that could be used in subsequent animal experiments. Further, I was interested in examining the application of a new extraction procedure, supercritical CO_2 extraction (SCE), in a NHP context.

> **Objective 2:** To compare extraction techniques of *S. sympetala* to determine the best technique to yield an extract enriched in BA content.

1.3.2 Pharmacological mode of action

The fundamental objective of this PhD project was to investigate the pharmacological mode of action of *S. sympetala* and *P. amalago* to determine how the plants exert anxiolysis (*S. sympetala* and *P. amalago*) and cortisol reduction (*S. sympetala*). Previous experiments demonstrated that *S. sympetala* is anxiolytic. Materials with anxiolytic activity, both mainstream pharmaceuticals and NHPs, are often GABAa agonists, therefore the hypothesis investigated was that *S. sympetala* elicits anxiolysis by binding to the GABAa-BZD receptor. Along with raw plant material and extracts, a more soluble analog of BA, meBA was tested in these assays, and the same hypothesis examined.

> **Hypothesis 1:** Anxiolytic action of *S. sympetala* extracts and meBA is mediated through the GABAa-BZD receptor; *S. sympetala* extracts and meBA are GABAa-BZD receptor agonists.

The testable predictions that follow from this hypothesis are (1) *S. sympetala* and meBA will bind to the GABAa-BZD receptor *in vitro*; and (2) blocking the GABAa-BZD receptor by pre-treating animals with a GABAa-BZD receptor antagonist followed by treatment with *S. sympetala* extract and meBA will result in anxiety-like behaviour in the animals.

The cortisol-lowering effect of *S. sympetala* and BA were examined with *in vitro* and *in vivo* assay assays developed in rainbow trout, *Oncorhynchus mykiss*. The hypothesis that *S. sympetala* and BA act at the site of cortisol synthesis was examined.

> **Hypothesis 2:** *S. sympetala* and BA act at the site of cortisol synthesis (interrenal cells in rainbow trout) to lower cortisol reponse to an ACTH challenge *in vitro* and net restraint *in vivo*.

This hypothesis leads to the following testable predictions: (1) when head kidney cells are preincubated with *S. sympetala* extract or BA, they will release less cortisol following a subsequent ACTH challenge; and (2) fish fed a diet supplemented with BA and then exposed to a physiological stressor will release less cortisol than fish fed an unsupplemented diet.

1.3.3. Ethnopharmacology and Natural Product Discovery

P. amalago is a traditional medicine used to treat *susto*. *P. amalago* extracts interact with two pharmacological targets of the GABA system, the GABAa-BZD receptor and the catabolic enzyme GABA transaminase (GABA-T) (Awad et al., 2009). *P. amalago* was investigated in an animal anxiety behavioural model to determine its anxiolytic activity *in*

vivo to test the hypothesis that *P. amalago* is an anxiolytic plant. Bioassay-guided fractionation of *P. amalago* was conducted with a GABAa-BZD receptor competitive binding assay to identify the bioactive secondary metabolites. Finally, as piperamides are bioactive and have CNS activity, the principle bioactive of *P. amalago* was predicted to be a piperamide.

> **Hypothesis 3:** *P. amalago* is an ethnobotanically identified anxiolytic plant that will have anxiety-reducing properties in an animal model of anxiety. The activity will be associated with a plant secondary metabolite that binds at the GABAa-BZD receptor and can be identified by bioassay-guided fractionation.

This hypothesis led to three testable predictions: (1) rats treated with *P. amalago* extract will display lower anxiety-like behaviour in standardized tests of anxiety than rats fed the vehicle; (2) the bioactive principle of *P. amalago* will be a molecule with high affinity for the GABAa-BZD receptor; and (3) the GABAa-BZD competitive binding assay can be used to distinguish highly bioactive fractions and consequently the bioactive principle of *P. amalago.*

SECTION I

PLANT PHYSIOLOGY & PHYTOCHEMISTRY OF *Souroubea Sympetala*

CHAPTER 2 - CHARACTERIZATION AND QUANTIFICATION OF PHYTOCHEMICAL MARKERS FROM THE NEOTROPICAL MEDICINAL PLANT *SOUROUBEA SYMPETALA* (MARCGRAVIACEAE) BY HPLC-APCI-MS

2.0 Preface

One of the first steps in the development of a NHP is the development of an analytical method to identify and quantify the active secondary metabolites in the material. This chapter addresses the first objective of this PhD project, to develop a HPLC-APCI -MS technique to identify and quantify the major triterpenes present in *S. sympetala* extracts. In this chapter, the method to identify and quantify four triterpenoids in *S. sympetala* is described and the distribution of these marker compounds in various parts of the plant is examined.

2.1 Statement of Author Contributions

The analytical method development was done by me, in collaboration with Kari Kramp and Dr. Ammar Saleem. I prepared all of the extracts and standards. Marco Otorola, Pablo Sanchez, Mario Garcia and Luis Poveda are Costa Rican botanists who collaborated on botanical collection and identification. Tony Durst, Vance. L. Trudeau and John. T. Arnason provided supervision and funding for the work. Special thanks to Linda Kimpe for technical assistance with, and use of, the Dionex ASE 200 Extractor.

Publication:

Mullally, M., Kramp, K., Saleem, A., Otorola Rojas, M., Sanchez Vindas, P., Garcia, M., Poveda Alvarez, L., Durst, T., Trudeau, V.L., Arnason, J.T., 2008. Characterization and Quantification of Triterpenes in the Neotropical Medicinal Plant *Souroubea sympetala* (Marcgraviaceae) by HPLC-APCI-MS. *Natural Product Communications*. 3, 1885-1888.

2.2 Abstract

A rapid, two-solvent, HPLC-APCI-MS method was developed to identify and quantify four pentacyclic triterpenoid marker phytochemicals: betulinic acid; ursolic acid; α-amyrin; and β-amyrin; in extracts of the neotropical medicinal plant *Souroubea sympetala*. Analysis of various plant organs, wood, bark, leaves, fruit and flowers, indicated that the phytochemical distribution and quantity of marker molecules varies across the plant, with betulinic acid and ursolic acid the major constituents in the bark, wood, fruit and flowers and the amyrins the major constituents in the leaves.

2.3 Introduction

The Marcgraviaceae is a neotropical plant family indigenous to tropical America consisting of 5 genera and 130 species (Heywood, 1993) for which the phytochemistry is not well described. The genus *Souroubea* of Marcgraviaceae was identified during a natural product discovery study in Costa Rica as an anxiolytic. *S. guianensis* has also been described in the treatment of *susto* (fright) (Schultes and Raffauf, 1990). *Susto* is a condition of folk etiology known throughout Latin America and understood to occur following a sudden frightening event that leads to the loss of "soul" or essence. The physiological characteristics of *susto* include diarrhea, loss of appetite, and restlessness (Klein, 1978). For diagnostic purposes, *susto* is considered a "culture-bound syndrome" linked to both anxiety and depression (American Psychiatric Association, 2000; World Health Organization, 2007). Preliminary *in vivo* evidence indicates that *S. sympetala* significantly reduces anxiety in rodent behavioural assays of anxiety. Treatment of rats with 1 mg/kg of an ethanolic extract of *S. sympetala* exerted significant anxiolysis in the elevated plus maze (EPM), a standard behavioural assay of anxiety (Puniani, 2004). Further, the ethanolic extract of *S. sympetala* inhibits rat gamma amino butyric acid-transaminase (GABA-T) activity, IC_{50} = 0.6 mg/mL, (Awad et al., 2007), a pharmacological target in the treatment of epilepsy and anxiety (Ashton and Young, 2003; Zwanzger and Rupprecht, 2005).

There are currently no available methods for phytochemical analysis of Marcgraviaceae. The phytochemistry of *S. sympetala* has been fully characterized, and four pentacyclic triterpenoids, betulinic acid (BA), ursolic acid (UA), α-amyrin (α-A) and β-amyrin (β-A) identified as marker phytochemicals for this plant (Figure 2.1) (Puniani, 2004). In this chapter I describe the development of a HPLC-APCI-MS technique for the

identification and quantification of the four triterpenoids in *Souroubea* extracts. Further, I describe the phytochemical variability of the four marker compounds across various plant parts; wood, bark, leaves, fruit and flowers.

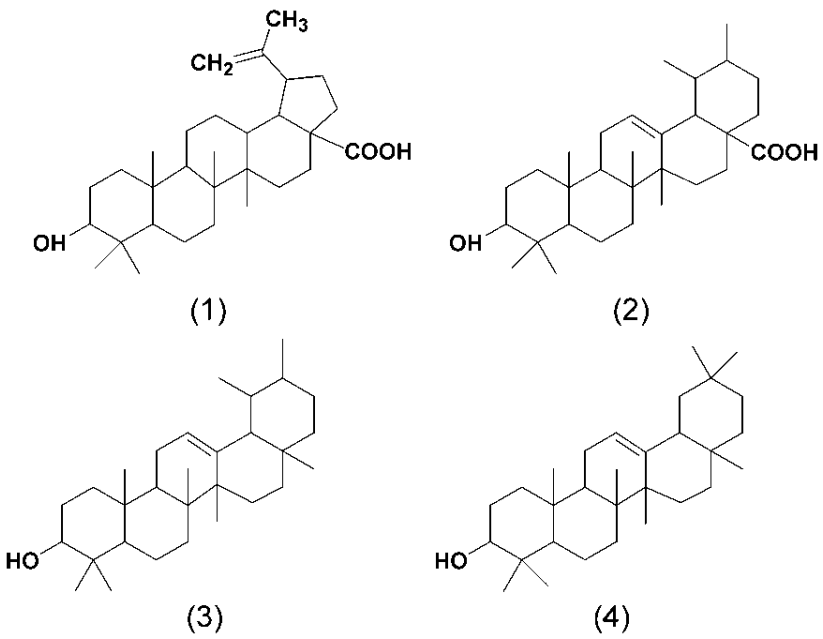


Figure 2.1. The four marker triterpenoids detected and quantified in *S. sympetala*, (1) Betulinic acid, (2) Ursolic acid, (3) α-Amyrin and (4) β – Amyrin.

2.4 Materials & Methods

Analytical grade HPLC solvents were purchased from J.T. Baker (USA). Standards of BA, UA, α-A and β-A were obtained from Sigma (St. Louis, MO).

Sample preparation and extraction

Fresh samples of wild *S. sympetala* wood, bark, early and late foliage (young & old leaves), flowers and fruits were collected in Tortuguero, Costa Rica, and stored in 95% ethanol. Storage ethanol was removed and filtered. Plant material was dried, weighed, coarsely ground via manual blender and extracted via pressurized liquid extraction with an Accelerated Solvent Extraction (ASE) 200 Extractor (Dionex, Sunnydale, USA). The extraction was conducted with 80% ethanol at a temperature of 110ºC, pressure of 120 bar, for two 5 mins static cycles, parameters previously demonstrated to optimize triterpenoid extraction (Zaugg et al., 2006). The ASE extract was combined with the original 95% ethanol extract and dried down via speed vacuum at 40ºC and lyophilized. All extracts were stored in opaque glass vials at 4ºC.

HPLC-APCI-MS analyses

HPLC-APCI-MS analyses were conducted on wood, bark, young leaf, old leaf, flower and fruit extracts. Analyses were performed with a 1100 LC MSD VL APCI system consisting of an autosampler, quaternary pump, photodiode array detector (DAD) and an

online APCI-MS with a mass range of 50 – 15000 Da (Agilent, Palo Alto, CA, USA). A Waters YMC ODS-AM column (100 x 2 mm I.D.; 3 µm particle size, 120 Å), maintained at 45ºC was used at a flow rate of 0.4 mL/min. The elution conditions were optimized with a mobile phase of water (solvent A) and acetonitrile (solvent B) as follows: initial conditions: 70% A: 30% B, linear gradient to 100% B in 10 min, maintained at 100% B for 8 min and returned to 70% A: 30% B in 7 min, post-time 3 min, for a total run time of 28 min. One microlitre of each extract was injected through the autosampler for each run and the elution profiles monitored via MS.

Detection and quantification of triterpenoids was conducted via MS. The mass spectrometer was tuned in positive ion mode at the beginning of all experiments. The optimized spray chamber conditions were: drying gas flow rate of 5.0 L/min; nebulizer pressure of 60 psi; drying gas temperature of 200ºC; vaporizer temperature of 325ºC; capillary voltage of 3200 V; and corona current of 5.0 µA. The MS was operated in scanning ion mode (SIM) and tuned to detect ions with a mass/charge (m/z) ratio of 439.1 (BA), 439.2 (UA) and 409.2 (α-A and β-A) which correspond to the molecular mass of each marker triterpenoid following the loss of a hydroxyl group and hydrogen atom during fragmentation.

Calibration standards

Individual stock solutions of the four standards were dissolved in methanol at a concentration of 2 mg/mL. The stock solutions were diluted through the addition of the appropriate volume of methanol to a range of 1 µg/mL - 1 mg/mL to yield the solutions used to generate the calibration curve. The identities of the marker phytochemicals in the extracts

were determined by comparing the retention times and mass data with those of the calibration standards.

Statistical analysis

All statistical analyses were performed with S-PLUS software version 7.0 (Insightful Corp., Seattle, USA). Tukey multiple mean comparison tests were conducted on log-transformed raw data to compare phytochemical distribution across the plant.

2.5 Results

HPLC method development

Preliminary HPLC study of BA in *S. sympetala* extracts was initiated using diode array detection (DAD) (UV, 205 nm). As Figure 2.2A depicts, DAD sensitivity was low due to poor light absorbance. Detection was enhanced with the use of MS detection versus DAD. The detection method was optimized for all four compounds by selected ion mode (SIM). The gradient was optimized to increase separation. Separation was complicated by the fact that BA and UA have the same molar mass and α-A and β-A are structural isomers. Initial isocractic conditions used caused co-elution of BA and UA. A 10 min gradient followed by 8 min isocratic at 100% acetonitrile eliminated co-elution and resulted in two distinct peaks (Figure 2.2 B).

Chromatographic profiles of *S. sympetala* extracts and compound identification

The chromatograms presented in Figure 2.3 show clear separation of the four triterpenoids in the samples. The phyotchemical profile varies considerably across the plant, with BA and UA detected at highest levels in the bark and wood, and the amyrins detected at highest levels in the leaves. There is a differential distribution of amyrins in the leaves with α-A most prevalent in the young leaves and β-A the major amyrin detected in the old leaves. Lower levels of triterpenoids were detected in the fruit and flowers; in both the major peak detected was BA. The percent yield for each extraction is presented in Table 2.1.

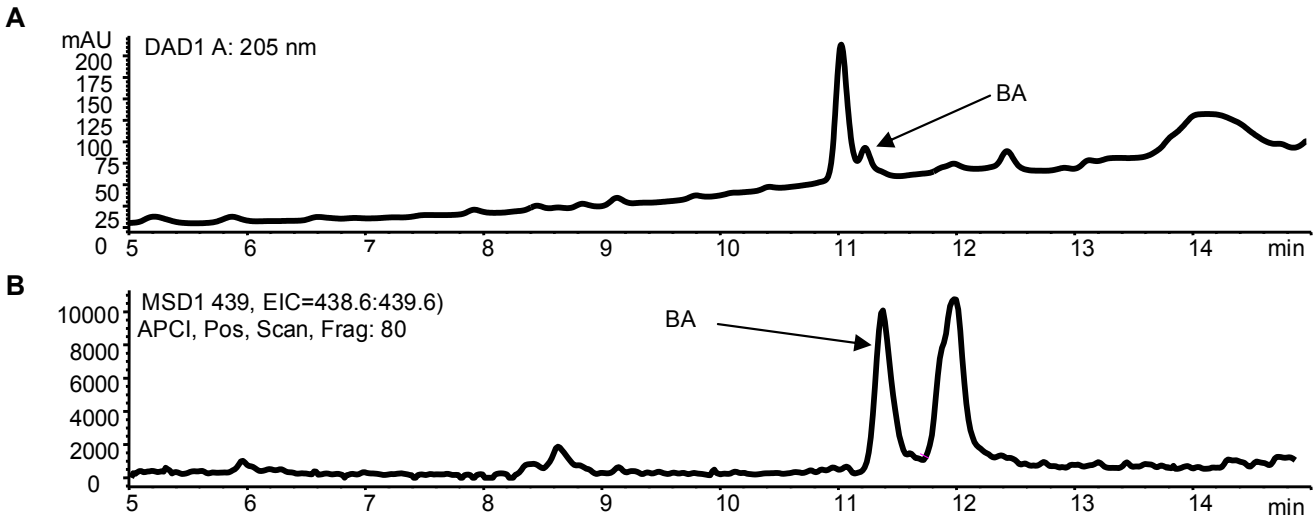


Figure 2.2. HPLC chromatogram of betulinic acid (BA), detected at the retention time of 11.3 min, **(A)** via diode array detection (UV = 205 nm) in an ethanolic extract of *S. sympetala* versus **(B)** the same extract detected via mass spectrometry detection.

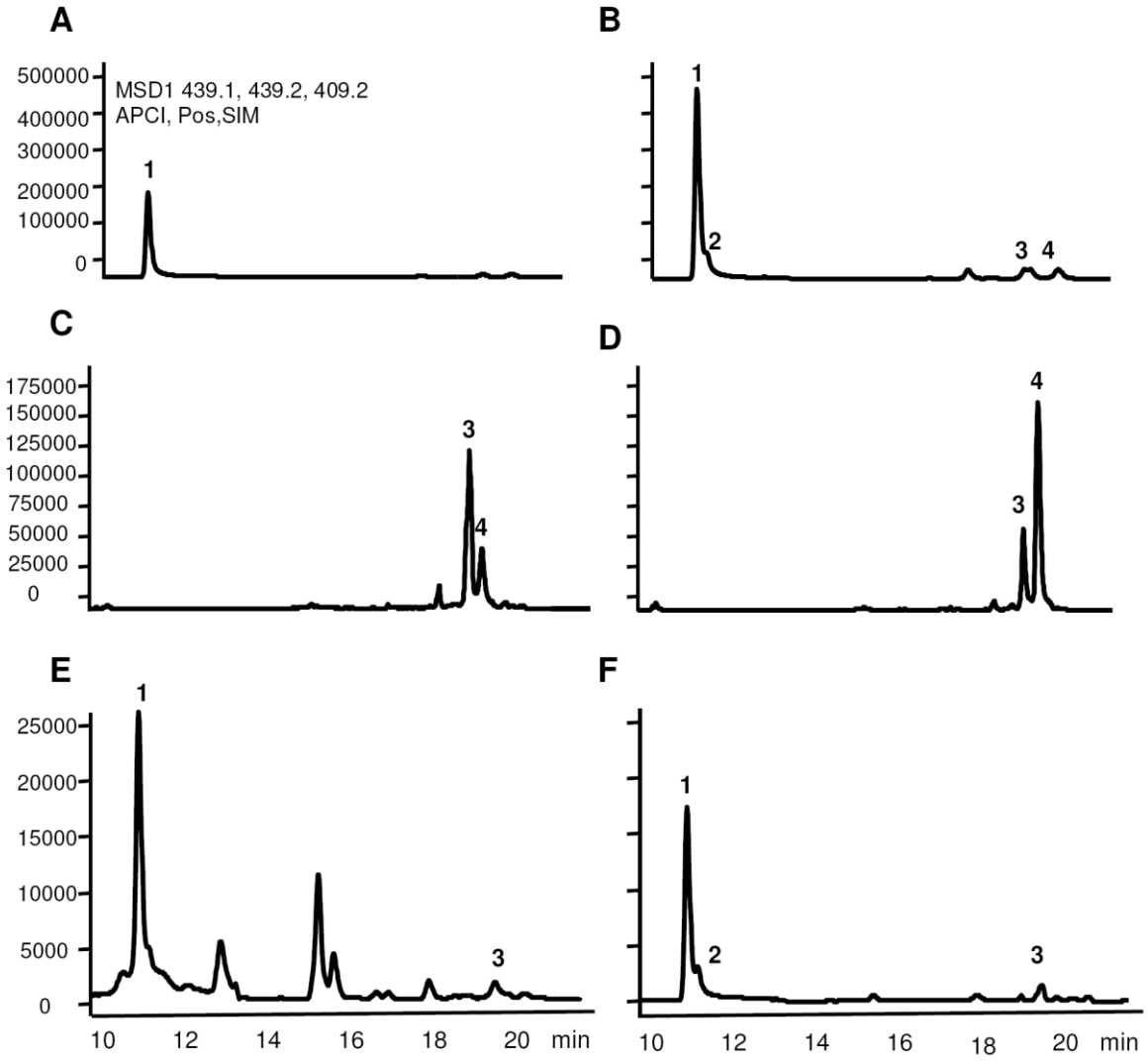


Figure 2.3. HPLC-APCI-MS profiles of *S. sympetala* extracts, (A) wood, **(B)** bark, **(C)** old leaves, **(D)** young leaves, **(E)** flowers and **(F)** fruits, n= 7 for each plant part. Each plant part was extracted via ASE extraction. Marker phytochemicals detected: 1: betulinic acid, 2: ursolic acid, 3: β-amyrin, and 4: α-amyrin. For each sample, 1 µL of a 20 mg/mL extract was injected into the autosampler.

Quantification of triterpenoids in *S. sympetala* extracts

Figure 2.4 presents the quantification of the phytochemicals across the plant parts, calculated as μg/mg extract. Although not statistically significant, there is a trend for more BA in the bark **(**mean: 85.65 ± 30.32 μg/mg extract), than the wood (46.56 ± 11.71 μg/mg). There is significantly more BA in the bark versus the old leaves, young leaves, flowers and fruit ($p < 0.05$), 57, 27 and 16 times more, respectively. The bark contains the greatest amount of UA, 0.49 ± 0.16 μg/mg, significantly higher UA amounts measured in the other plant organs ($p < 0.05$). α-A is the major marker phytochemical present in the old leaves, with a mean value of 11.49 ± 7.71 μg/mg, 3 times that measured in the young leaves, 3.44 ± 3.06 μg/mg. There is also more α-A in the old leaves versus the wood, bark, flowers and fruit, although a significant difference exists only between the α-A content of the old leaves, 11.49 ± 7.71 μg/mg, and that of the fruit, 0.04 ± 0.01 μg/mg ($p < 0.05$). Finally, β-A is the major marker phytochemical in the young leaves, with a mean value of 12.2 ± 6.42 μg/mg versus 6.11 ± 2.99 μg/mg in the young leaves. There is significantly more β-A present in the old leaves versus the flowers and fruit ($p < 0.05$), 27 and 76 times, respectively. There is also significantly more β-A present in the young leaves versus the wood, flowers and fruit ($p < 0.05$), with 55, 55 and 152 times more, respectively.

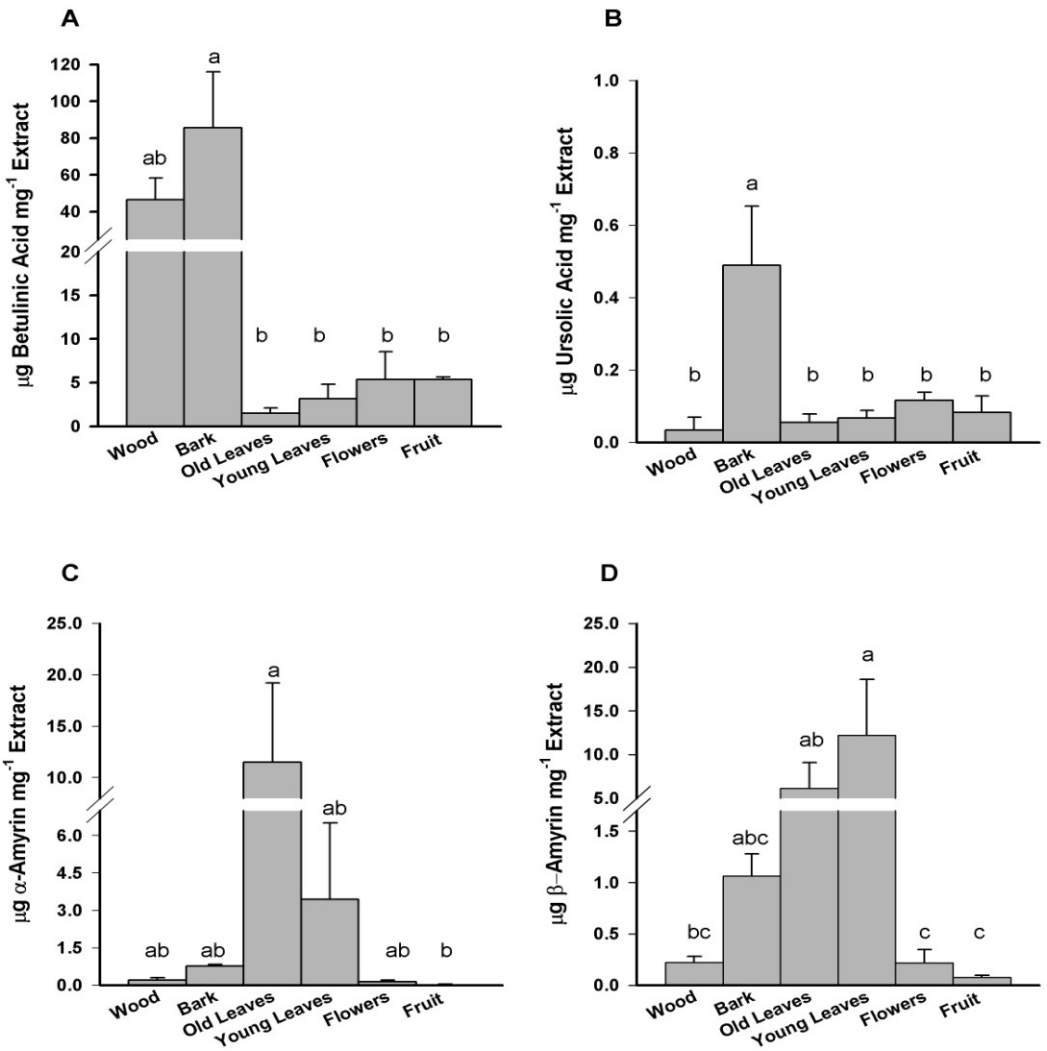


Figure 2.4. Quantitative comparisons of the four marker triterpenoids, betulinic acid **(A)**, ursolic acid **(B)**, α-amyrin **(C)** and β-amyrin **(D)** in wood, bark, old leaf, young leaf, flower and fruit extracts of *S. sympetala*. Letters indicate significant differences, one-way ANOVA, Tukey multiple comparison of means, $p < 0.05$, n = 7 for each plant part. Each plant part was extracted via ASE extraction.

Plant Organ	% Yield
Wood	3.4
Bark	5.0
Old Leaves	16.2
Young Leaves	15.5
Flowers	26.5
Fruit	0.3

Table 2.1. Percent yield for ASE extraction of each of the *S. sympetala* plant organs investigated.

2.6 Discussion

This report provides the first method for identification of *S. sympetala* plant products by HPLC-APCI-MS. The method is straightforward and allows for detection and quantification of the four marker triterpenoids of *S. sympetala*. The extraction method is also rapid and simple, with the added value that ASE extraction methods consume less solvent and are less labour intensive than conventional extraction approaches (Huie, 2002). The HPLC-APCI-MS method is similarly rapid (28 min) and employs a two solvent system that effectively accomplishes the challenging separation of a pair of molecules with identical molecular mass (BA and UA) and a pair of structural isomers (α-A and β-A).

While other methods to separate triterpenoids exist (Schaaf et al., 2000; Zaugg et al., 2006), our method is, to the best of our knowledge, the first to separate this particular combination of molecules. Moreover, we have characterized the phytochemical profile of *S. sympetala* across the plant organs. From this characterization it is clear that the distribution of the triterpenoids varies across the plant, with more BA and UA present in the wood and the bark versus the leaves, whereas the leaves contain more amyrins. This characterization may assist in phytochemical identification of *S. sympetala*. The phytochemical characterization of the various plant parts aids in identification of the plant organs that represent best candidates for BA extraction, further examination for anxiety-lowering properties.

The present study has addressed new strategic priorities in the characterization of *S. sympetala*. Future work will investigate the variability and phytochemical diversity of this species and compare it with other members of the same genus, particularly *S. gilgii*. Finally,

due to the lipophilic nature of these triterpenoids, emerging extraction technologies, specifically supercritical carbon dioxide extract, are under development to optimize complete extraction.

CHAPTER 3 - ANXIOLYTIC ACTIVITY OF A SUPERCRITICAL CARBON DIOXIDE EXTRACT OF *SOUROUBEA SYMPETALA* (MARCGRAVIACEAE)

3.0 Preface

After developing an analytical method for quality control and quantification of marker phytochemicals, the next step in the development of a NHP is determining the best method of extraction. The experiments in this chapter were designed to address the second objective of this PhD project, to compare extraction techniques of *S. sympetala* to determine the best technique to yield an extract enriched in BA content. Specifically in this chapter I describe the application of a new extraction method, super critical carbon dioxide extraction (SCE) to *S. sympetala*. I compared the phytochemical content, specifically betulinic acid content, of the SCE extract to that of four other conventional extractions of *S. sympetala.* Further, I compared the *in vivo* activity of the SCE extract to a conventional solvent extract, done with ethyl acetate (EtOAc), in the EPM.

3.1 Statement of Author Contributions

I developed the extraction approaches and conducted the extractions for the Sox, UAE, ASE and EtOAc extracts, analysed them and completed the data analysis. Dr. Ammar Saleem provided technical support for sample analysis. I conducted the behavioural assays with Chris Cayer, in Dr. Zul Merali's lab and conducted the data analysis. Kari Kramp developed the SCE extraction method and prepared the SCE extracts. Kari Kramp and I wrote the manuscript. Calum McRae, and Andrew Goulah provided technical assistance for the SCE extractions, John Baker and Bioniche Life Sciences Incorporated provided funds for

the experiments, Marco Otorola, Pablo Sanchez, Mario Garcia, Luis Poveda assisted with plant collection and identification in Costa Rica, Tony Durst, Vance L. Trudeau John Thor Arnason provided funds and supervision for the experiments. Special thanks to Linda Kimpe for use of the Dionex ASE 200 Extractor and Sylvia Edmond for assistance with animal care.

Publication:

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, V.L., Arnason, J.T., 2011. Anxiolytic activity of a supercritical carbon dioxide extract of *Souroubea sympetala* (Marcgraviaceae). *Phytotherapy Research*. 25, 264-270.

3.2 Abstract

The purpose of this work was to develop an extraction technique to yield a betulinic acid-(BA) enriched extract of the traditional anti-anxiety plant *Souroubea sympetala* Gilg (Marcgraviaceae). Five extraction techniques were compared: supercritical CO_2 extraction (SCE), conventional solvent extraction with ethyl acetate (EtOAc), accelerated solvent extraction (ASE), ultrasonic assisted extraction (UAE) and soxhlet extraction (Sox). The EtOAc and SCE extraction methods resulted in BA-enriched extracts, with BA concentrations of 6.78 ± 0.2 and 5.54 ± 0.2 mg/g extract, respectively, as determined by HPLC-APCI-MS. The bioactivity of the BA-enriched extracts was compared in the elevated plus maze (EPM), a validated rodent anxiety behaviour assay. Rats orally administered a 75 mg/kg dose of SCE extract exhibited less anxiety as compared with vehicle controls. The SCE extract demonstrated a dose-response in the EPM, with a trend toward decreased anxiety at 25 mg/kg, although significant anxiolysis was only observed at 75 mg/kg dose. This study demonstrates that SCE can be used to generate a betulinic acid- enriched extract with significant anxiolytic activity *in vivo*. Further, this study provides a scientific basis for the ethnobotanical use of this traditional medicine and a promising lead for a natural health product to treat anxiety.

3.3 Introduction

About 14% of the global burden of disease has been attributed to mental illness (Prince et al., 2007). Among the most common mental illnesses are anxiety disorders, including generalized anxiety, panic disorders and phobias. Anxiety disorders are also commonly comorbid with other mental illnesses, including depression, bipolar disorder and addiction (Kessler et al., 1997; Kessler et al., 2003). Treatments for anxiety disorders include cognitive behaviour therapy and pharmacological interventions, but only 60% of patients are responsive to treatment (Bystritsky, 2006) and the common pharmaceuticals used to treat anxiety, benzodiazepines (BZDs), are associated with serious side effects and not recommended for chronic use (Stevens and Pollack, 2005). Anxiety disorder patients are considerable consumers of alternative treatments; 43% use herbal products to treat their anxiety (Eisenberg et al., 1998). These factors highlight a need for additional anxiolytic plants to be identified and investigated as phytomedicines to treat anxiety. As part of a natural product investigation to identify anxiolytic plants, we identified the genus *Souroubea.*

Souroubea is a group of woody vines belonging to the neotropical family Marcgraviaceae with a tradition of use in both Belize and the Amazon to treat *susto* (fear), a folk-illness associated with anxiety (Schultes and Raffauf, 1990). We have established a link between *susto* and anxiety and fear, and demonstrated that plants used by Belize healers to treat *susto* reduce anxiety and fear in rodents (Bourbonnais-Spear et al., 2007). The work described in this chapter extends this line of investigation by examining the anxiety-reducing properties of *Souroubea sympetala* Gilg (Marcgraviaceae). Initial investigations of *Souroubea* identified a triterpene-enriched fraction that reduced anxiety-like behaviour in rodents in a dose-responsive manner and compared favourably with the anti-anxiety drug

diazepam (Durst et al., 2009). Bioassay guided fractionation identified betulinic acid (BA) as the active principle. BA is a lupane-type triterpene common in the plant kingdom with demonstrated anti-cancer, anti-HIV and anti-malaria activity (Cichewicz and Kouzi, 2004; De Sá et al., 2009; Kessler et al., 2007). The purpose of the work described here was to develop a BA-enriched extract of *S. sympetala* to test *in vivo* for anxiolysis. I compared five extraction approaches, conventional solvent extraction with ethyl acetate (EtOAc), supercritical carbon dioxide extraction (SCE), accelerated solvent extraction (ASE), ultrasonic assisted extraction (UAE) and soxhlet extraction (Sox). The yields and BA content of each extract were compared to identify a method to selectively generate a BA-enriched extract. The bioactivity of the extracts with highest BA content were subsequently compared in the elevated plus maze (EPM), a validated rodent anxiety behaviour assay.

3.4 Materials and Methods

Analytical grade HPLC solvents were purchased from J.T. Baker (USA). Pure betulinic acid (BA) was obtained from Sigma (St. Louis, MO) for use as a standard. Extraction grade solvents (ethyl acetate, 85% ethanol) were purchased from Fisher Scientific (Ottawa, ON Canada).

Plant material

Fresh leaf samples of wild *Souroubea sympetala* (Marcgraviaceae) were collected under permit in Tortuguero, Costa Rica. Samples were dried overnight in a commercial plant drier at 35ºC and ground to 2 mm mesh. Voucher specimens were identified by two of us (L.P. and P.S.) and deposited in the JVR Herbariun, Universidad Nacional Costa Rica, and the University of Ottawa Herbarium (OH No. 19915).

Extractions

A side-by-side comparison of the extraction methods is presented in Table 3.1. A brief description of each method is presented below.

Conventional solvent extraction

Ground samples (2 mm mesh, 2 g) were incubated, with shaking, in 40 mL (1:20 weight: volume) ethyl acetate (EtOAc) for 12 – 15 h at room temperature (RT). The solvent was filtered (Whatman #1) and filter cake re-extracted, twice, as above, with half as much EtOAc (1:10 and 1:5). The total solvent from the three extractions were combined for an exhaustive extraction. For all extracts where solvent was used, the solvent was vigorously removed via rotary evaporation with a Yamato Rotary Evaporator RE50 (Yamato Scientific, Japan) at 40ºC, lyophilized (Super Modulyo, Thermo Electron, USA) and stored in opaque glass vials at 4ºC. This method was optimized for triterpene extraction (Puniani, 2004).

Soxhlet extraction

Ground samples (2 mm mesh, 0.5 g) were loaded into the soxhlet thimble and extracted with 150 mL (1:300 weight: volume) 85% ethanol in a round bottom flask for 5 h.

Ultrasonic assisted extraction

Ground samples (2 mm mesh, 0.5 g) were sonicated in a Branson 1200 ultrasonic bath (Branson Ultrasonics, Danbury, CT) for 10 min with 10 mL (1: 20, weight: volume) 85% ethanol and centrifuged at 5500 rpm for 5 min. The supernatant was filtered (Whatman #41), and the filter cake re-extracted, twice, as above. The supernatants from the three extractions were combined. This method was adapted from Shen and Shao (Shen and Shao, 2005).

Accelerated solvent extraction

Ground samples (2 mm mesh, 1.0 g) were packed into a 60 mL extraction cell and extracted via accelerated solvent extraction (ASE) with an Accelerated Solvent Extraction 200 Extractor (Dionex, Sunnydale, USA). The extraction was conducted with 85% ethanol at a temperature of 110ºC, pressure of 120 bar (12 MPa), for two 5 min static cycles. This method was optimized for triterpene extraction (Zaugg et al., 2006).

Supercritical CO_2 extraction

SCE extractions were performed with a SFT-250 extractor equipped with a 100 mL vessel (Supercritical Fluid Tech., Newark, DE). Ground samples (2 mm mesh, 20 g) were extracted at 80˚C, 600 bar (60 MPa), flow rate of 3 L/min until a 450 g volume of CO_2 was consumed (25:1solvent: biomass) Extraction efficiency was monitored at 5 min intervals. We choose high density conditions (80˚C, 60 MPa, 0.9-0.925 g/mL CO_2 density) to ensure increased solubility of BA and increased extraction efficiency. In preliminary trials, these conditions resulted in high BA yields, as compared to the EtOAc, extracts that had the highest yield of BA, so these conditions were used throughout the experiment.

HPLC-APCI-MS analysis

HPLC-APCI-MS analyses were conducted as previously described (Chapter 2, (Mullally et al., 2008)). Briefly, extracts were dissolved in methanol, to a final concentration of 10 mg/mL, and filtered with a 0.2 µm PTFE filter. One µL of each extract was injected through the autosampler for each run and the elution profiles monitored via MS. A calibration curve was prepared by dissolving BA in methanol at a concentration of 2 mg/mL and diluted to a range of 1 µg/mL - 1000 µg/mL. BA was identified in the extracts by comparing the retention time and mass data with the calibration standard.

Animals

The behavioural experiments were conducted with male Sprague-Dawley rats (225 - 250 g body mass; Charles River Laboratories Inc., St. Constant, Quebec). Rats were individually housed and maintained under standard animal room conditions (clear plexiglass cages, 24 x 30 x 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 University of Ottawa Animal Care Protocol Review Committee and met the guidelines set out by the Canadian Council on Animal Care for the use of animals in research and teaching. Rats were handled for 7 days prior to the experiment to acclimatize to the experimenter and were orally administered a 50% solution of Eagle Brand sweetened condensed milk each day to familiarize them with the feeding procedure.

Drug and plant extract administration

Anxiety-like behaviour of animals treated with the BA-enriched SCE and EtOAc extracts were compared to animals treated with diazepam (valium, positive control) and untreated animals (vehicle control) in the EPM. The plant extract was frozen at -80ºC, pulverized with an ice-cold mortar and pestle and mixed with 50% sweetened, condensed milk to a final concentration of 75 mg of plant extract /kg animal, and stored at 4ºC. Vehicle and extract-treated animals were orally administered their respective treatments daily for three consecutive days (between 10:00 - 2:00 for two days prior to testing, 60 min prior to testing). The animals were randomly assigned to one of four treatment groups: diazepam (5 mg/kg, dissolved in 40% propylene glycol, 10% ethanol, 50% distilled water), vehicle

control (2 mL/kg 50% sweetened condensed milk), EtOAc and SCE leaf extracts (75 mg extract /kg animal). To generate a dose-response curve for the SCE extract, animals in this treatment group were administered two other doses: 8 and 25 mg/kg SCE extract.

Anxiety behaviour assay: Elevated plus maze

The EPM consists of two open arms (50 x 10 cm), two perpendicular arms enclosed by 40 cm high walls, placed 50 cm above the ground, and is based on the conflict between the animal's instinct to explore its environment and its fear of exposed areas and heights. The EPM test is commonly used to assess anxiety-like behaviour in laboratory rodents (File, 1992; Pellow et al., 1985). A video camera was mounted above the arena to permit remote monitoring and recording. Rats (n = 19 for SCE, n = 12 for EtOAc, n = 24 for vehicle, n = 11 for diazepam) were individually placed in the testing room for 1 h acclimatization. Each rat was then placed onto the open central platform of the EPM (facing a closed arm). The behaviour was monitored for 5 min and scored as follows: (1) frequency of entries onto the open arms, (2) percentage of time spent on the open arms (time open/300 x 100), (3) frequency of entries in the closed arms, and (4) risk assessment behaviour (unprotected head dips; head protruding over the edge of an open arm and down toward the floor). Between tests, the EPM was cleaned with 70% isopropanol. The percent of time in the open arms, frequency of open arm entries, and unprotected head dips are all validated measures of anxiety-like behaviour in the EPM. Increases in these measures indicate reduced anxiety-like behaviour, conversely, decreases in these parameters indicates increases in anxiety-like behaviour (File, 1992). The frequency of closed arm entries is considered an index of

general motor activity of the animal and important in establishing the sedative effect of a material (Cruz et al., 1994).

Statistical analysis

One and multi-way analyses of variance (ANOVA) with Bonferonni studentized range tests were performed for mean comparisons (Zar, 1999). Kolmogorov-Schmirnoff and Levene's tests were used to verify the normality of distribution and the homogeneity of residual variance, respectively. All of the Fisher statistics (F), degrees of freedom (df), and p-value estimates were calculated with S-PLUS software version 7.0 (Insightful Corp., Seattle, USA). Data are reported as means ± S.E.M and the level of significance was set at $p < 0.05$.

3.5 Results

Extractions

Of the five extraction methods compared, SCE and EtOAc extraction had the highest BA concentration, 5.54 ± 0.2 and 6.78 ± 0.2 mg/g extract, respectively, values that were not significantly different from each other, but significantly higher than the BA concentration of ASE, UAE and Sox (2.57 ± 0.3, 3.16 ± 0.3 and 2.37 ± 0.3 mg/g, respectively, $F(5, 47)= 28.82$, $p < 0.001$) (Figure 3.1A). The SCE and EtOAc extracts had significantly lower yields, 5.10 ± 0.1 and 5.82 ± 0.6 %, than ASE and Sox, 15.69 ± 0.4, 15.25 ± 0.6, respectively, with no significant difference in yield from UAE, 4.82 ± 0.4 % (Figure 3.1B).

An extraction efficiency curve for the SCE extract was generated by measuring percent yield at 5 min intervals throughout the 75 min extraction. After 35 min, at a flow rate of 3 L/min, the extraction reached 91% completion and after 75 min no additional extract was generated (Figure 3.2A). The concentration of BA through the course of the SCE extraction was determined at 20 min intervals (Figure 3.2B). BA concentration increased from 4.37 mg/g extract in the first fraction to 6.32 mg/g and 8.12 mg/g in the second and third fractions respectively. At 30 min 91% of the extract was collected, however, based on the BA tracking values, at 30 min, the BA concentration was approximately 5.35 mg/g, only 65.9% of the BA concentration in the 60 min fraction. This indicates that for maximum BA to be extracted the extraction conditions need to be continued until 450 g of CO_2 are consumed.

Elevated Plus Maze

The 75 mg/kg dose of SCE extract was effective in several parameters of the EPM as compared to the vehicle control (*i.e.* percent time spent in the open arms: $F(4, 61) = 4.48$, $p < 0.01$; total time in the open arms: $F(4,61) = 4.48$, $p < 0.01$; percent time in the closed arms: $F(4,61) = 2.95$, $p < 0.05$) (Table 3.2). SCE dosed animals had a 50% increase in percent time in the open arms as compared to vehicle controls (Figure 3.3A), spent 50% more total time in the open arms, had 73% more unprotected head dips and a 42% decrease in percent time spent in the closed arms. These animals also had a significant increase in unprotected head dips as compared to EtOAc treated animals (75 mg/kg) and vehicle controls: $F(4,61) = 10.45$, $p < 0.01$ (Figure 3.3B). No differences were observed in any of the parameters of the

EPM between the positive control, diazepam (5 mg/kg), and the 75 mg/kg dose of the EtOAc extract. No differences were observed in the number of closed arm entries for animals treated with the plant extracts versus the vehicle controls.

Increased doses of SCE extract (0, 25 and 75 mg/kg) changed parameters in the EPM in a dose responsive manner; higher SCE extract concentrations resulted in less anxiety-like behaviour, as indicated by increased percent time spent in the open arms. The higher SCE dose (75 mg/kg) significantly increased percent time spent in the open arms as compared to vehicle controls, ($F(4,61) = 4.48$, $p < 0.01$) but not as compared to the 25 mg/kg dose (Figure 3.3C). The higher SCE dose significantly increased the number of unprotected head dips as compared to the vehicle control and the 25 mg/kg dose ($F(4, 61) = 10.45$, $p < 0.01$) (Figure 3.3D).

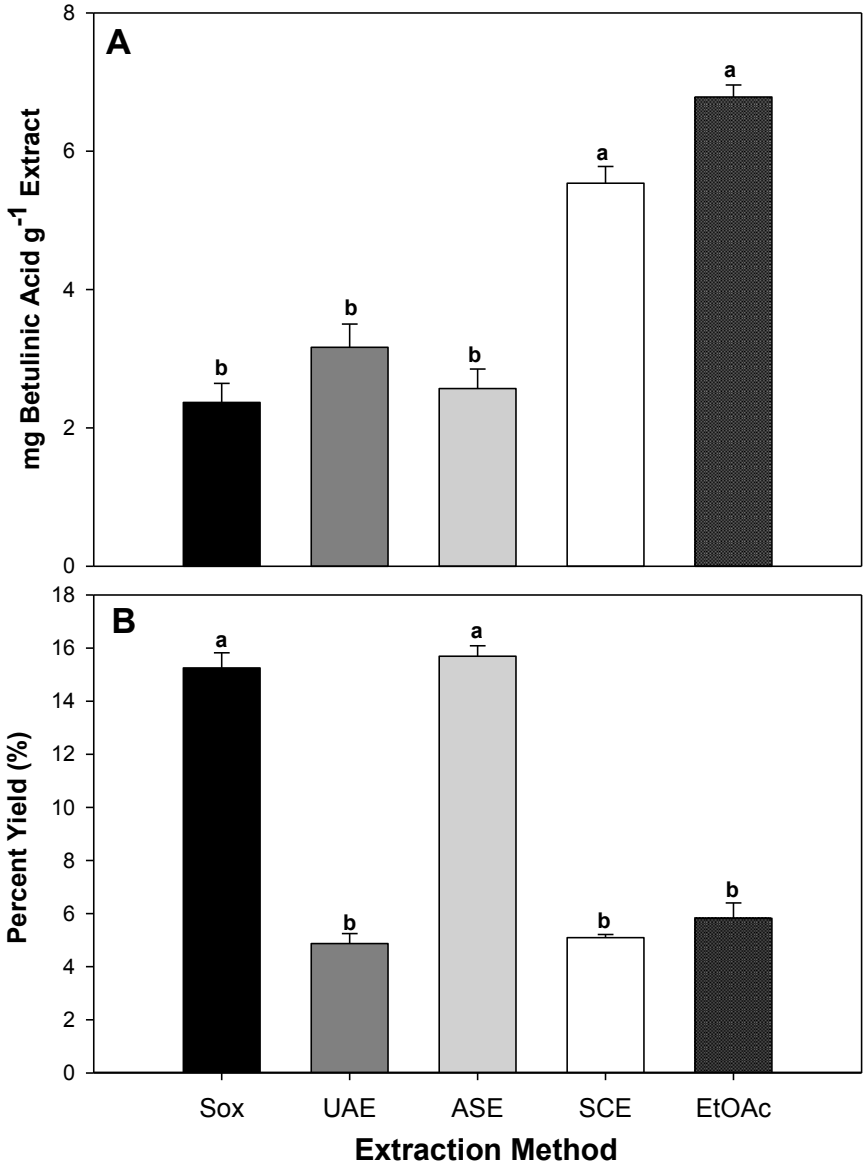


Figure 3.1. Mean betulinic acid content and percent yield for S. sympetala extracts. (A) Mean betulinic acid content in *S. sympetala* leaf extracts generated via Soxhlet Extraction (Sox), Ultrasonic Assisted Extraction (UAE), Accelerated Solvent Extraction (ASE), Supercritical CO_2 extraction (SCE) and conventional ethylacetate extract (EtOAc). **(B)** Comparison of percent yield for *S. sympetala* leaves extracted with the five methods compared. All values represent the group mean ± S.E.M. Different letters indicate statistically significant differences, Bonferroni mean comparison, $p < 0.05$ (n = 3 per extraction approach).

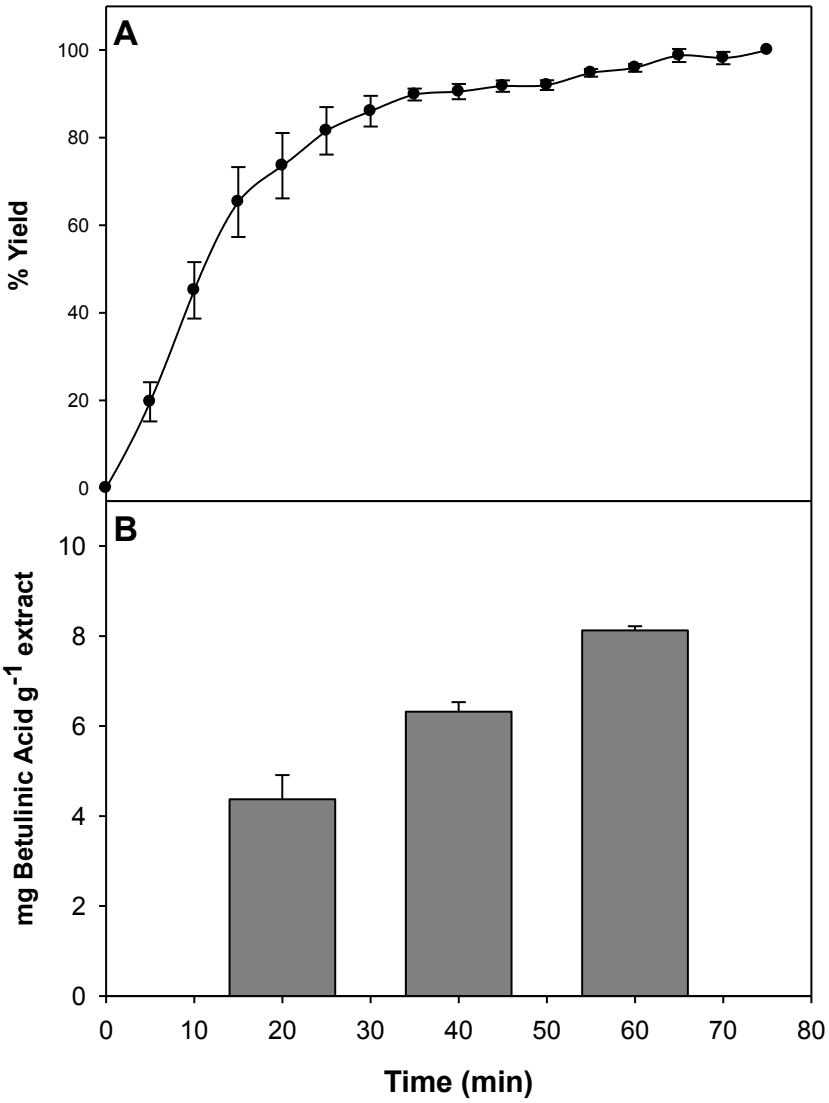


Figure 3.2. Betulinic acid extraction efficiency curve for S. sympetala supercritical CO2 extract. **(A)** Extraction efficiency curve for supercritical CO_2 (SCE) extraction of *S. sympetala.* Each point represents mean mass of extract collected at 5 min intervals throughout the course of the extraction ± S.E.M; **(B)** Betulinic acid content in extracts, mg/g extract, collected at three time points in the SCE extraction (n = 3, mass of starting material = 20.0 g ± 0.03, extracted at 80°C, 600 bar (60 MPa), flow rate: 3 L min^1, 450 g CO_2 consumed).

Table 3.1. Summary of the five extraction methods used to generate a BA-enriched extract of *S. sympetala*.

Extraction Method	Petrochemical Solvent	Mass of Biomass (g)	Solvent: Biomass Ratio	Temperature	Pressure	Extraction Time	Rotary Evaporation & Lyophilization?	Equipment
Conventional	ethylacetate	2.0	20:1	78 ºC	Atmospheric Pressure	3 days	Yes	Benchtop shaker
Soxhlet	ethanol (85 %)	0.5	300:1	23 ºC[†]	Atmospheric Pressure	5 hours	Yes	Soxhlet thimble
Ultrasonic Assisted	ethanol (85 %)	0.5	20:1	20 ºC	Atmospheric Pressure	45 min	Yes	Branson 1200 Ultrasonic Bath
Accelerated Solvent	ethanol (85 %)	1.0	4:1	110 ºC	12 MPa	10 min	Yes	Dionex ASE 200 Accelerated Solvent Extraction System
Supercritical Carbon Dioxide	none	20	25:1	80 ºC	60 MPa	75 min	No	SFT-250 Supercritical Fluid Extractor

Table 3.2. Comparison between the two *S. sympetala* extraction types (EtOAc and SCE), vehicle control (50% sweetened, condensed milk) and positive control (diazepam) in selected parameters of the elevated plus maze paradigm, after a 1 h post-drug interval (n = 7 – 24)

Treatment	T.O.A.	#O.A.E.	U.P.H.D.	% T.O.A.	#C.A.E.	% T.C.A.
Vehicle control n = 24	55.5 ± 7.8	4.5 ± 0.6	3.8 ± 0.6	18.5 ± 2.6	10.6 ± 0.6	52.2 ± 3.6
Diazepam, positive control 5 mg/kg, n = 11	102.3 ± 15.5**	4.7 ± 0.8	13.6 ± 3.1$^{**, \#}$	34.1 ± 5.2**	9.9 ± 1.9	39.4 ± 5.5
Ethyl acetate extract 75 mg/kg, n = 12	72.5 ± 13.9	5.5 ± 0.6	6.8 ± 1.3	24.2 ± 4.6	10.8 ± 0.5	42.0 ± 3.2
Supercritical CO_2 extract 25 mg/kg, n = 7	75.3 ± 19.7	6.3 ± 1.4	6.4 ± 1.9	25.1 ± 6. 6	12.0 ± 0.97	43.37 ± 4.0
Supercritical CO_2 extract 75 mg/kg, n = 12	110.3 ± 6.3$^{*, **}$	8.1 ± 0.4	14.3 ± 1.25$^{**, \#, \#\#}$	36.8 ± 2.09**	9.3 ± 0.72	36.83 ± 1.93*

T.O.A.: time spent in the open arms (min) **#O.A.E.:** number of open arm entries **U.P.H.D.:** number of unprotected head dips
% T.O.A.: percentage of time spent in the open arms **#C.A.E.:** number of closed arm entries **% T.C.A:** percent time in closed arms

** p-value < 0.01 vs. vehicle control, one-way ANOVA
* p-value < 0.05 vs. control, one-way ANOVA
$^{\#}$ p-value < 0.01 vs. ethyl acetate extract, one-way ANOVA
$^{\#\#}$ p-value < 0.01 vs. SCE extract 25 mg/kg, one-way ANOVA

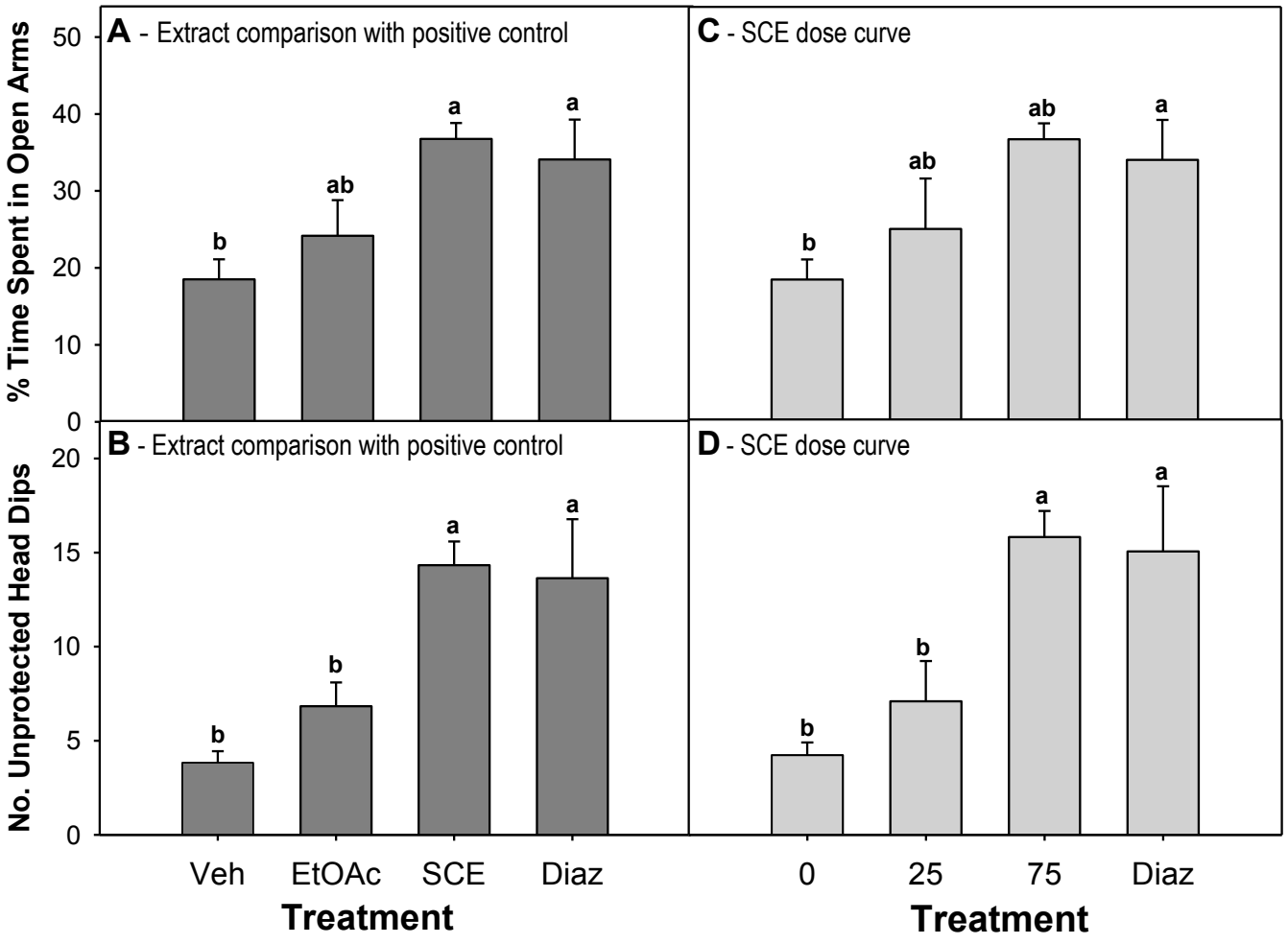


Figure 3.3. Elevated Plus Maze results for S. sympetala extracts. Panel (A) Percent time spent in open arms of the EPM for different treatments; and **(B)** Number of unprotected head dips in the EPM for different treatments (doses: *S. sympetala* EtOAc and SCE extracts: 75 mg/kg, diazepam: 5 mg/kg) after a 1 h post-drug interval (n = 24 for vehicle, n = 12 for EtOAc, n = 12 for SCE, n = 11 for diazepam). **(C)** Dose curve for SCE extract of *S. sympetala* in the EPM. Percent time spent in open arms of the EPM, $R^2 = 0.32$, $p < 0.001$; and **(D)** Number of unprotected head dips for the three doses of the SCE extract (0, 25 and 75 mg/kg) and positive control (diazepam, 5 mg/kg) after a 1 h post-drug interval (n = 7 - 24), $R^2 = 0.60$, $p < 0.001$. All values represent the group mean ± S.E.M.; veh: vehicle control (50% sweetened, condensed milk), letters indicate significant differences, Bonferroni mean comparison, $p < 0.05$.

3.6 Discussion

The objective of this study was to develop a standardized extraction procedure to produce a BA-enriched extract of *S. sympetala* leaves. The results demonstrate that both conventional solvent extraction and SCE extraction generate extracts with highest BA content, although both approaches yield lower amounts of extract as compared to the other extraction methods investigated. When the two BA-enriched extracts, SCE and EtOAc, were compared for their effect on anxiety, only the SCE had a significant anxiety-lowering effect in the EPM.

Each of the extraction methods used, with the exception of Sox and SCE, were previously optimized for triterpene extraction (Shen and Shao, 2005; Zaugg et al., 2006). The Sox extracts had a percent yield and BA concentration comparable to the optimized ASE method, demonstrating that under the conditions used, Sox is efficient at extracting triterpenes from *S. sympetala* leaves. Representative chromatograms can be viewed in previously published work (Mullally et al., 2008); Chapter 2); in all cases the major differences between the extracts was in the amount of BA present. Despite a total lower yield, the higher BA content of SCE and EtOAc extracts demonstrate selectivity of these methods for the extraction of triterpenes. The SCE findings corroborate previous ones reported in the literature, that SCE is an effective method for triterpene extraction (Cossuta et al., 2008). Further, compared to the EtOAc extract, SCE had considerably reduced extraction time, 75 min versus 3 days (Table 3.1). SCE is a method particularly well suited to natural health product extraction because CO_2 is non-toxic, non-flammable, available in high purity (food grade) and easily evaporated from the extract leaving no residue. Further, the tunable solvent properties of CO_2 permit selective, targeted extraction of specific

chemical compound families, so it can be adjusted to selectively extract triterpeniods, like BA (Hamburger et al., 2004; Mukhopadhyay, 2000).

These results indicate that the SCE extract has a tendency to elicit greater anxiolysis than the EtOAc extract, rodents administered the SCE extract exhibited greater risk assessment behaviour, unprotected head dips, than those dosed with the EtOAc extract. This selective effect on risk assessment behaviour suggests that the SCE extract may act via 5-hydroxytryptamine (5-HT) neurotransmission, as this metric of the EPM has been shown to respond specifically to ligands that bind the $5HT_{1A}$ receptor (Griebel et al., 1997). Previous reports of anxiolytic plant extracts in the EPM have required moderate to high doses to elicit significant anxiolysis, from 100 – 500 mg/kg for well characterized herbs, including passion flower (*Passiflora incarnata*, 375 mg/kg (Grundmann et al., 2008), blue skullcap (*Scutellaria lateriflora*, 100 mg/kg (Awad et al., 2003)), and kava (*Piper methysticum*, 120 – 240 mg/kg (Rex et al., 2002)). In light of these, a 75 mg/kg oral dose of the SCE extract of *S. sympetala* is moderate and suggests high and selective anti-anxiety activity.

An important observation is that the SCE extract was more palatable than the EtOAc one. The SCE extracts had a very mild odour and the animals readily consumed it. In contrast, the EtOAc extracts had a strong odour, even after vigorous vacuum removal of solvent, and in early animal trials there were difficulties getting the animals to consume the extract. I opted not to use orogastric gavage to deliver the extract because rats are known to have a stress response to gavage (Balcombe et al., 2004), which might have affected the anxiety-like behaviours monitored in the EPM. For this reason, an alternate extraction method that would yield an extract with similar BA content to the EtOAc extract but that the animals would readily eat was sought. This was the case with the SCE extract, the animals

ate it readily. The increased palatability of the SCE may be due to the residue free, tasteless, and odourless properties of CO_2. Increased palatability is a benefit associated with SCE extracts used in animal trials and would also be considered beneficial in the preparation of *S. sympetala* extracts for veterinary application or human consumption.

The results described in this chapter demonstrate that SCE can selectively extract triterpenoids and can be used to generate extracts enriched with the bioactive triterpenoid BA. The behavioural data indicate that *S. sympetala* is a significant anxiolytic, and that SCE extracts of this putative natural health product are more effective in reducing rodent anxiety-like behaviour than a conventional solvent extract. Future work on the presence of CNS active ligands, such as serotonin and melatonin in the plant extracts would be valuable.

SECTION II

Pharmacological mode of action of *Souroubea sympetala*

CHAPTER 4 - *SOUROUBEA SYMPETALA* (MARCGRAVIACEAE): A MEDICINAL PLANT THAT EXERTS ANXIOLYSIS THROUGH INTERACTION WITH THE GABAa BENZODIAZEPINE RECEPTOR

4.0 Preface

This chapter addresses the fundamental objective of this PhD project, to investigate the pharmacological mode of action of *S. sympetala*, to determine how it reduces anxiety. The first hypothesis of the PhD project is investigated in the experiments of this chapter, namely that *S. sympetala* extracts and meBA are GABAa-BZD receptor agonists. The predictions tested are: one, that *S. sympetala* and meBA bind to the GABAa-BZD receptor *in vitro*; and two, blocking the GABAa-BZD receptor by pre-treating animals with a GABAa-BZD receptor antagonist will extinguish anxiolysis of *S. sympetala* extracts and meBA.

4.1 Statement of Author Contributions:

I ran the animal assays with the assistance of Chris Cayer and I conducted the data analysis, I ran the *in vitro* receptor binding assays and conducted the data analysis. I wrote the manuscript. Kari Kramp provided the SCE leaf extracts, Marco Otorola, Pablo Sanchez, Mario Garcia and Luis Poveda assisted with plant collection and identification in Costa Rica**,** Tony Durst supplied the meBA, the animal experiments were conducted in Zul Merali's lab at the Royal Ottawa Mental Health Centre, Vance L. Trudeau and John Thor Arnason provided funding and supervision of the experiments.

Publication:

This chapter has been submitted in manuscript form to the *Journal of Ethnopharmacology*.

4.2 Abstract

The anxiolytic plant *Souroubea sympetala* was investigated in anxiety and depression behavioural assays. The raw plant material (leaf and bark), extracts of the plant and analogue of the principle bioactive, methyl ester of betulinic acid (meBA), had significant anxiety-lowering capacity in the elevated plus maze (EPM), social interaction (SI) and conditioned emotional response – context (CER) assays. To test the hypothesis that the plant material and pure compound act at the pharmacologically important GABAa – benzodiazepine (GABAa-BZD) receptor, animals were pre-treated with the GABAa-BZD receptor antagonist flumazenil and then plant extracts or meBA. Pre-treatment with flumazenil eliminated the anxiety-reducing effect, demonstrating that *S. sympetala* acts via an agonist action on the GABAa-BZD receptor. An in vitro GABAa-BZD competitive receptor binding assay also demonstrated that *S. sympetala* extracts have an affinity for the GABAa-BZD receptor, with an EC50 value of 123 µg/mL (EtOAc leaf extract) and 154 µg/mL (SCE extract). Finally, the SCE leaf extract had a significant anti-depressant effect in the forced swim test. These results show the anxiolytic effect of *S. sympetala*, raw plant, extracts and pure compound, the anti-depressant effect of the SCE leaf extract, and demonstrate for the first time that *S. sympetala* acts at the GABAa-BZD receptor to elicit anxiolysis.

4.3 Introduction

Mental illness is a significant and detrimental form of sickness, accounting for an estimated 12% of the world disease burden (Prince et al., 2007). Anxiety disorders are the most common type of mental illness among both Canadians and Americans, affecting 12 – 18% of adults (Health Canada, 2002; Kessler et al., 2005), and an estimated 40% of people with anxiety disorders choose herbal products to treat their illness (Ernst, 2006). In recent years, two of the most popular herbal medicines used to treat mental illness, St. John's wort (SJW, *Hypericum perforatum* L.*)*, for mild depression, and kava (*Piper methysticum)*, for anxiety, have come under scrutiny due to interactions with cytochrome P450 enzymes (SJW), specifically induction of CYP 3A4 (Markowitz et al., 2003), and concerns about hepatotoxicity (kava) (Center for Disease Control and Prevention, 2003; Currie and Clough, 2003; Madabushi et al., 2006; Whitten et al., 2006). Further, the front-line pharmaceuticals to treat anxiety disorders, the benzodiazepines (BZDs), have well established deleterious side-effects including sedation, muscle relaxation and memory impairment (Stevens and Pollack, 2005). These concerns highlight a need for the identification of new and efficacious herbal medicines for mental illness, and specifically anxiety disorders.

The major neurotransmitter implicated in the etiology of anxiety disorders is γ-amino butyric acid (GABA) (Lydiard, 2003; Treiman, 2001). GABA is the major inhibitory neurotransmitter in the central nervous system (CNS), and aberrations in GABA levels and genotypic differences in GABAa receptor configurations are associated with increased propensity to develop anxiety disorders (Goddard et al., 2001; Malizia et al., 1998; Möhler, 2006). The major pharmacological target in the GABA system is the ligand gated ion-channel GABAa receptor, specifically the BZD-binding site of the GABAa receptor. There

is a growing discussion in the literature that natural products containing GABAa –BZD receptor site ligands with lower affinity than pharmaceutical drugs may be promising leads in the treatment of anxiety. The underlying argument is that ligands with lower affinity for this site may retain therapeutic effect without undesirable side-effects (Tsang and Xue, 2004; Wang et al., 2005).

As part of an on-going investigation of ethnobotanically important tropical anxiolytic plants in the natural products group at the University of Ottawa, *S. sympetala* was identified as a promising plant with anxiety reducing properties and the triterpene betulinic acid (BA) identified as the bioactive principal (Durst et al., 2009). BA has anxiolytic activity (Cayer, 2011), but is difficult to work with in both *in vivo* and *in vitro* experiments, due to well described challenges with solubility (Drag-Zalesinska et al., 2009; Gauthier et al., 2010). To address the solubility issues of the pure compound, several analogs of BA were synthesized; of these, the methyl ester of BA (meBA, Figure 4.1) was demonstrated to have a better solubility profile and to retain the anxiolytic effects *in vivo* previously observed with BA (Cayer, 2011). In the experiments described in this chapter, meBA was used as the pure compound assayed.

The objective of the experiments described in this chapter was to characterize the anxiolytic activity of *S. sympetala*, an ethnobotanically identified plant used to treat *susto*, an anxiety disorder (American Psychiatric Association, 2000; World Health Organization, 2007), by investigating the activity and pharmacological mode of action of plant parts (leaves and bark), extracts and meBA. The material was tested in a step-wise manner; first raw plant material, then extracts and finally pure compound. To examine the mode of action of *S. sympetala*, I tested the hypothesis that *S. sympetala* extracts and meBA act at the

GABAa-BZD receptor. To do this, rats were pre-treated with flumazenil (Ro 15-1788), a GABAa-BZD antagonist used to treat benzodiazepine overdose (Thomson et al., 2006; Weinbroum et al., 1996). Flumazenil occupies the GABAa-BZD receptor so that subsequent ligands are unable to access it. Following pre-treatment with flumazenil, rats were dosed with *S. sympetala* and meBA. If these materials act at the GABAa-BZD, pre-treatment with the GABAa antagonist will block their access to the receptor and no reduction of anxiety-like behaviour will be observed. Finally, in humans anxiety is commonly comorbid with depression (Judd et al., 1998) . Previous work has demonstrated that *S. sympetala* has significant anxiety-lowering effects, because of the connection between anxiety and depression, I thought it would be interesting and valuable to assess the leaf extract and meBA in a behavioural model of depression.

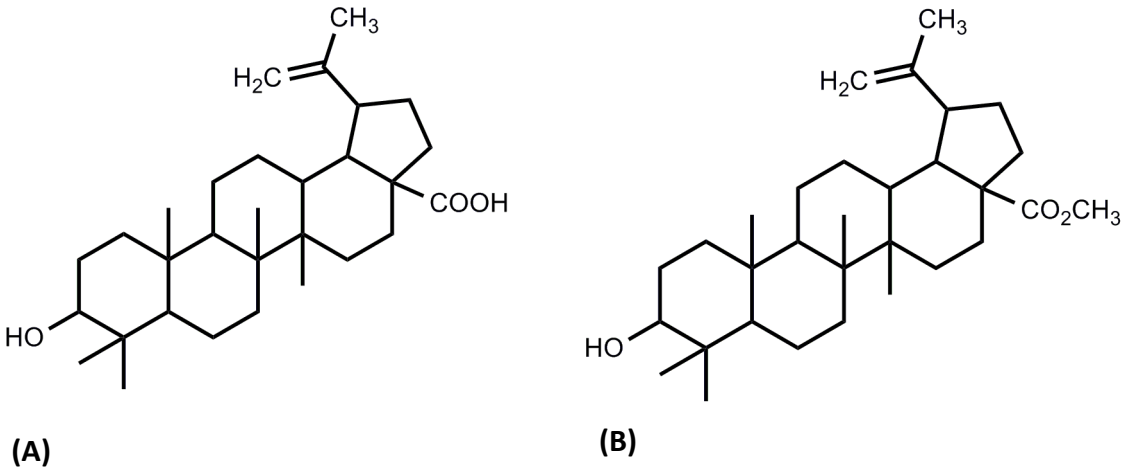


Figure 4.1. Chemical structure of **(A)** betulinic acid and **(B)** methyl ester analogue of betulinic acid.

4.4 Materials and Methods

Extraction grade solvent (ethyl acetate) was purchased from Fisher Scientific (Ottawa, ON Canada). Flumazenil (Ro 15-1788), alpha-cyclodextrin and betulinic acid were purchased from Sigma-Aldrich (Markham, Ont.). The methyl ester of betulinic acid (meBA) was synthesised by Dr. Tony Durst's natural product chemistry group.

Plant material

Fresh samples of wild *Souroubea sympetala* (Marcgraviaceae) were collected under permit in Tortuguero, Costa Rica. Samples were dried overnight in a commercial plant drier at 35ºC and ground to 2 mm mesh. Voucher specimens were identified by local botanists (L.P. and P.S.), then deposited in the JVR Herbarium, Universidad Nacional Costa Rica, and the University of Ottawa Herbarium (OH No. 19915).

Plant Extractions

A conventional ethyl acetate (EtOAc) extraction of ground leaf, and a supercritical carbon dioxide (SCE) extract of ground leaf and bark were prepared as previously described (Mullally et al., 2008). The EtOAc extract was dried by rotary evaporation with a Yamato Rotary Evaporator RE50 (Yamato Scientific, Japan) at 40ºC, lyophilized (Super Modulyo, Thermo Electron, USA) and stored in opaque glass vials at 4ºC.

GABAa-BZD-S Receptor Binding Assay

The GABAa-BZD receptor binding assay was adapted from previously described protocols (Benke, 1999; Snodgrass, 1978) and conducted in a 96-well microplate as described previously (Awad et al., 2009). Plant extracts were dissolved in 99% ethanol and serially diluted to generate a response curve. The final ethanol concentration in each well was 0.99% v/v.

Animals

Experiments were conducted with male Sprague-Dawley rats (225-250 g body mass; Charles River Laboratories Inc., St. Constant, Quebec). Rats were housed individually and maintained under standard animal room conditions (clear plexiglass cages, 24 x 30 x 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 University of Ottawa Animal Care Protocol Review Committee and met the guidelines set out by the Canadian Council on Animal Care for the use of animals in research and teaching. Rats were handled for 7 days prior to the experiment to acclimatize them to the experimenter and orally administered a 50% solution of Eagle Brand sweetened condensed milk each day to familiarize them with the feeding procedure. Animals were rested for seven days between elevated plus maze (EPM) and conditioned emotional response (CER) experiments, during this period daily handling and feeding continued. All attempts were made to minimize the number of animals used in the study, while maintaining the integrity of the experiments and results.

Drug Administration

Whole leaf and bark (0.5 mm mesh) extracts, and meBA were suspended in 50% sweetened, condensed milk and administered daily for three days (between 10:00 - 2:00 for two days prior to testing, 60 min prior to testing). Each dosage day, rats were orally administered either 50% sweetened condensed milk vehicle (2 mL/kg) alone (vehicle control) or a treatment. The botanical treatments were: whole plant (1 g/kg leaf, 0.25 g/kg bark), EtOAc and SCE leaf extract (75 mg/kg), SCE bark extract (2.5 mg/kg) and meBA (5 mg/kg), all of which were suspended in sweetened condensed milk (50%). BA concentrations of each botanical treatment were determined by HPLC-MS- DAD as previously described (Mullally et al., 2008) and are presented in Table 4.1. The drug treatments were flumazenil (Ro 15-1788, dissolved in saline and cyclodextrin (5% w/v)), administrered intra-peritoneally (*i.p.*), and diazepam, (5 mg/kg, dissolved in 40% propylene glycol, 10% ethanol, 50% distilled water), administered orally. Animals were dosed with 1 mg/kg flumazenil 45 min prior to plant extract treatment between 10:00 – 2:00 for two days prior to testing and 60 min before testing. A washout period of 7 days was placed between each behavioural test.

Behavioural assays

To eliminate the effect of one behavioural test on the next one, we started with the least aversive behavioural assay and proceeded to the most aversive one. Behavioural tests were performed in the following order: (1) Social interaction (SI), (2) Elevated plus maze (EPM) and (3) Conditioned emotional response (context) (CER). A separate group of animals were used in the forced swim test.

Social Interaction Test

The social interaction (SI) test examines the amount of time animals spend interacting with an unknown animal in a familiar environment. The amount of time spent interacting is an inverse measure of anxiety, *i.e.* animals that interact more are understood to have less anxiety than those that interact less (File et al., 1978). Total number of squares crossed in the arena is considered a measure of locomotor activity. SI was carried out under semi-aversive (high illumination, familiar environment) conditions. SI was assessed in a rectangular gray Perspex arena (60 x 60 cm; 30 cm-high walls), illuminated by a bright light source (300 lux) located directly above the arena. A camera linked to a video recorder in an adjacent room was located directly above the arena to permit remote monitoring, scoring and recording of the test sessions. Each SI study had a total duration of four days. Rats were randomly assigned to a treatment group and orally dosed their respective solutions once a day for four days. The third day was used for habituation. On habituation day, rats were individually placed in the arena for 4 min. On test day, each rat was assigned to a partner based on body mass (members of a pair did not differ by > 10 g), dosed, and placed in the arena for 7.5 min. Time spent in social interaction (including sniffing, climbing over each other, following, allogrooming (grooming the other animal), fighting, anogenital sniffing, and self-grooming) was recorded. Testing was performed between 10:00 - 14:00, in randomized order. The arena was cleaned with 70% isopropanol between trials.

Elevated Plus Maze

The EPM test assesses anxiety-like behaviour in laboratory rodents (File, 1992; Pellow et al., 1985) and is based on the conflict between the animals' instinct to explore its environment and its fear of exposed areas and heights. Time spent in the open arms is an inverse measure of anxiety, *i.e.* animals that spend more time in the open arms are understood to have less anxiety than those that spend more time in the closed arms. The EPM consists of two open arms (50 x 10 cm), and two perpendicular arms enclosed by 40 cm high walls, and is placed 50 cm above the ground. A black curtain surrounded the chamber to limit the influence of spatial cues and other extraneous stimuli. A video camera was mounted above the arena to permit remote monitoring and recording. Rats (n= 9 / group) were individually placed in the testing room for 1 h acclimatization. Each rat was then placed onto the open central platform of the EPM (facing a closed arm). The rat's behaviour was monitored for 5 min and scored as follows: (1) frequency of entries onto the open arms, (2) percentage of time spent on the open arms (time open/300 × 100), (3) frequency of entries in the closed arms, and (4) risk assessment behaviour (unprotected head dips; head protruding over the edge of an open arm and down toward the floor). Between tests, the EPM was cleaned with 70% isopropanol. The percent of time in the open arms, frequency of open arm entries, and unprotected head dips are all validated measures of anxiety-like behaviour in the EPM (Carobrez and Bertoglio, 2005; Griebel et al., 1997; Pellow et al., 1985). Increases in these measures are indicative of reduced anxiety, whereas decreases suggest increased anxiety (File, 1992). In contrast, the frequency of closed arm entries is an index of general locomotor activity and provides insight into the sedative effects of a material (Cruz et al., 1994).

Conditioned Emotional Response (Context)

The CER test assesses fear in an animal. In this test, animals are placed in an enclosure 24 h prior to testing and randomly administered an aversive stimulus (electrical foot shock). The following day, the animals are placed in the same chamber (context) and freezing behaviour, indicative of avoidance of aversive stimulus, is measured. Drugs that reduce freezing in the CER are considered to have anxiolytic properties.

Subjects completed 1 day of training prior to testing 24 h later. During the contextual training phase, subjects were placed in the conditioning chamber where they received six foot shocks (1.0 mA, 1 s duration) on a random schedule with an average interval of 1 min between each foot shock. The conditioning chamber (Coulbourn Instruments, Whitehall, PA, USA) measured 31 x 25 x 30 cm, with clear plexiglass walls in front and back, stainless steel side panels, and a floor comprised of 16 stainless steel rods (2 mm diameter, 3 cm apart), connected to a constant current shock generator (Coulbourn Instruments; model H13–16). On the test day, contextual fear was assessed over a 15 min period by placing rats (n= 9 / group) in the conditioning chamber where they had previously been shocked. Freezing behaviour, an index of conditioned fear, was measured using the software program ODlog (Macropod Software). Freezing was defined as the absence of movement, excluding involuntary respiratory movements (Blanchard and Blanchard, 1969).

Forced Swim Test

The forced swim test is a widely used behavioural despair paradigm used to evaluate the effectiveness of antidepressant drugs (Porsolt et al., 1977a; Schiller et al., 1992). The

forced swim arena (Stoelting Co., Wood Dale, Illinois) consisted of a clear Plexiglas cylinder (diameter: 20 cm, height: 45 cm, water height: 20 cm) with a center drain and valve at the base of the cylinder for water level adjustment. Two test arenas were set up on a table in a designated testing room and filled with fresh tap water of 25 °C. To allow for remote monitoring and recording, a camera was mounted on a tripod at a sufficient distance to allow both arenas to be monitored at the same time. Hand towels (one per subject) were used to dry subjects after testing and two recovery cages situated under a heat lamp (~ 37 °C) were set up to ensure subjects were kept warm until they were returned to their home cage.

Testing spanned two days and was divided into a habituation session and test session. During the habituation session, cage mates were each placed in a forced swim arena simultaneously for 15 min without behavioural recording. At the end of the session, subjects (n = 10/ group) were dried by hand using a hand towel and placed in a heated recovery cage for 10 min before being returned to the home cage. The water in the cylinders was changed between subjects. The procedure during the test session was identical to that of habituation session except the duration of the test was 5 min and behavioural recording took place. The time spent immobile (no movement in all four limbs and tail, with all limbs under water) by each subject was recorded. Movements made by the subject to maintain its head above the water were not included in time spent immobile. High levels of immobility in the forced swim test are considered to be an index of depressive-like symptoms in animals (Malkesman et al., 2006; Porsolt et al., 1977b; Porsolt et al., 1978).

Data Analysis

GABAaR- BZD Receptor Binding Assay

The GABAaR- BZD receptor binding assay was conducted as described in *Current Protocols in Pharmacology* (Benke & Monner, 1999). Briefly, whole brains were harvested from male rats, flash frozen into liquid nitrogen and stored at -80˚C until the tissue was required. Whole brains were thawed on ice, manually sliced with a spatula and then homogenized with an electronic laboratory tissue homogenizerat high speed for 10 – 30 s in a10X volume of 50mM Tris buffer (pH 7.4). The tissue homogenate was then centrifuged at 32,000 x *g* for 15 min at 4˚C, the supernatant decanted, the pellet resuspended in 25 mL of 50mM Tris buffer (pH 7.4) and centrifuged again at 32,000 x *g* for 15 min at 4˚C. The resulting tissue preparation was diluted to a concentration of 30 μg protein per 100 μL, flash frozen into liquid nitrogen and stored at -80˚C until the assay was completed.

Total binding and nonspecific binding were computed by running the assay in the absence of competitive ligand (total) and with the addition of the 20 nM flumazenil (Ro 15-1788) (non-specific). All plant extracts were compared to a solvent control (0.99% ethanol). Percent specific binding of ^{3}H-flunitrazepam was computed with the following equation: ((Cpm in well – Cpm nonspecific binding)/Cpm solvent well) x 100 = % Specific Binding). Competitive binding assays were attempted with BA and meBA, however owing to well described difficulties with solubility (Drag-Zalesinska et al., 2009; Gauthier et al., 2010; Qian et al., 2009) and the tendency of triterpenoids to precipitate at cold temperatures, the concentration ranges required for the assay could not be achieved. The binding affinity curves were generated with GraphPad Prism 5 (GraphPad Software, La Jolla, USA).

Behavioural Assays

For the SI test, raw values were converted to percentage of time spent interacting. For CER, raw freezing scores were transformed into a percentage of time spent freezing within each 1 min bin. The 15 1 min bins were then collapsed to three 5 min time blocks. In the EPM, both raw time values and percentage time values were computed and compared. The data were compared with one way analyses of variance (ANOVA) with Bonferroni multiple comparison of means test (Zar, 1999). In the case of the unprotected head dips (UPHD) in the EPM for whole leaf and bark treatments, individual pair-wise t-test comparisons were conducted because the presence of zero values for the control group made the data recalcitrant to ANOVA analysis. Kolmogorov-Schmirnoff and Levene's tests were used to verify the normality of distribution and the homogeneity of residual variance, respectively. In cases where the data did not meet assumptions of parametric tests, they were transformed. All of the Fisher statistics (F), degrees of freedom (df), and p-value estimates were calculated with S-PLUS software version 7.0 (Insightful Corp., Seattle, USA). Data are reported as means ± standard error of the mean (S.E.M) and the level of significance was set at $p < 0.05$.

Table 4.1. Betulinic acid (BA) concentration in each botanical treatment fed to the animals in these experiments (mg BA / kg animal). BA quantification was done by HPLC-APCI-MS (see Chapter 2).

Treatment	Final BA Concentration
Raw leaf	0.1
Raw bark	0.5
Supercritical CO_2 leaf extract	5
Supercritical CO_2 bark extract	0.5
EtOAc leaf extract	5
EtOAc bark extract	Extract yield too low to test

4.5 Results

Effect of Raw *S. sympetala* Plant Material on Anxiety-Like Behaviour

In the EPM test, the raw bark and leaf, administered at doses of 0.25 g/kg and 1 g/kg, respectively, had a significant effect on test parameters as compared to vehicle control, but not when compared to each other. Animals treated with bark and leaf spent 72% and 71%, respectively, more time in the open arms than vehicle treated animals (Figure 4.2A, $F(2, 22) = 4.90$, $p < 0.05$). Animals treated with the raw plant material also had significantly more unprotected head dips (U.P.H.D.) than vehicle control animals (bark (mean U.P.H.D $=3.2 \pm 1.0$), $t(8) = 3.18$, $p = 0.01$; and leaf (mean U.P.H.D $= 2.8 \pm 0.7$), $t(8) = -4.19$, $p = 0.003$) (Figure 4.2B). No differences in number of closed arm entries for plant treated animals versus vehicle were observed ($F(2, 22) = 0.13$, $p = 0.88$) (Table 4.2).

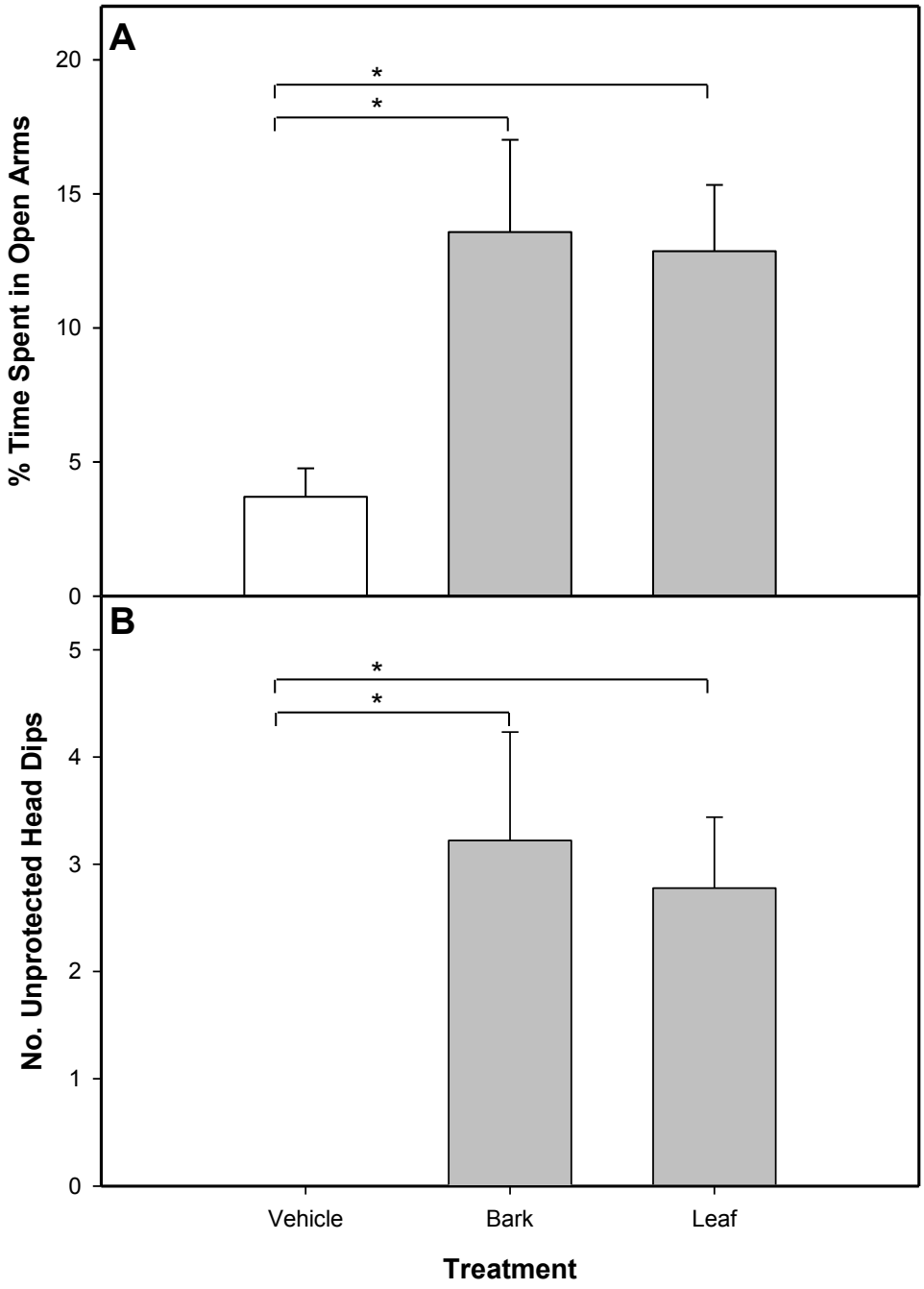


Figure 4.2. Effect of raw S. sympetala bark and leaf on anxiety-like behaviours in rats. **(A)** Percent total time spent in the open arms of the elevated plus maze; and **(B)** Number of unprotected head dips. All values represent the group mean ± S.E.M. of n = 7- 9 per group. **Vehicle:** vehicle control (50% sweetened, condensed milk), **Bark:** dried inner bark of adventitious roots ground to 0.5 mm mesh, 0.25 g/ kg, **Leaf:** dried whole leaves ground to 0.5 mm mesh, 1 g/kg, * Indicates significant difference from vehicle, one-way ANOVA (% TOA) with Dunnett's post-hoc test and paired t-test(UPHD), $p<0.05$.

Table 4.2. Comparison between the two *S. sympetala* raw materials, bark and leaf, and vehicle control (50% sweetened, condensed milk) in selected parameters of the elevated plus maze paradigm, after a 1 h post-drug interval.

Treatment	T.O.A.	#O.A.E.	#C.A.E.	% T.C.A.
Vehicle control				
n = 7	11.1 ± 3.2	1.3 ± 0.4	9.0 ± 1.1	63.2 ± 4.3
Raw bark				
0.25 g/ kg	40.7 ± 10.3	4. 8 ±1.2*	8.4 ± 1.0	50.5 ± 3. 8
n = 9				
Raw leaf				
1 g/ kg	38.6 ± 7.4*	4.1 ± 0.8*	8.3 ± 0.8	47.8 ± 3.1*
n = 9				

T.O.A.: time spent in the open arms (s) **#O.A.E.:** number of open arm entries **#C.A.E.:** number of closed arm entries **% T.C.A:** percent time in closed arms

* Indicates significant difference from vehicle control, one-way ANOVA, $p < 0.05$.

Effect of *S. sympetala* Extracts on Anxiety-Like Behaviour

Social Interaction Assay

In the social interaction assay (SI), both *S. sympetala* leaf extracts (SCE and EtOAc, 75 mg/kg) significantly increased amount of time animals spent interacting as compared to vehicle controls (Figure 4.3A, $F(2, 56) = 24.36$, $p < 0.05$). The two plant extracts had a similar effect; no significant difference was observed between them. No significant effect on locomotion was observed, in that the number of squares crossed in the SI arena for the extract-dosed animals did not differ from that of the vehicle-dosed animals (Figure 4.3B, $F(2, 56) = 1.77$, $p = 0.18$).

Conditioned Emotional Response (CER - Context)

When rats were re-exposed to the context where they had experienced an aversive event (foot shock), treatment with the *S. sympetala* extracts (SCE and EtOAc, 75 mg/kg) significantly decreased percent time freezing in the first 5 min block of the test (Figure 4, $F(2, 42) = 8.31$ $p < 0.05$) as compared to vehicle controls.

Elevated Plus Maze

The SCE leaf extract (75 mg/kg) had a significant effect on percent time spent in the open arms, as compared to vehicle control and SCE bark extract (2.5 mg/kg) (Figure 5A, $F(5, 55) = 10.26$, $p < 0.05$). Treatment of animals with the SCE leaf extract also significantly

increased number of unprotected head dips as compared to both vehicle control and SCE bark extract (Figure 5B, $F(5,55) = 15.06$, $p < 0.01$).

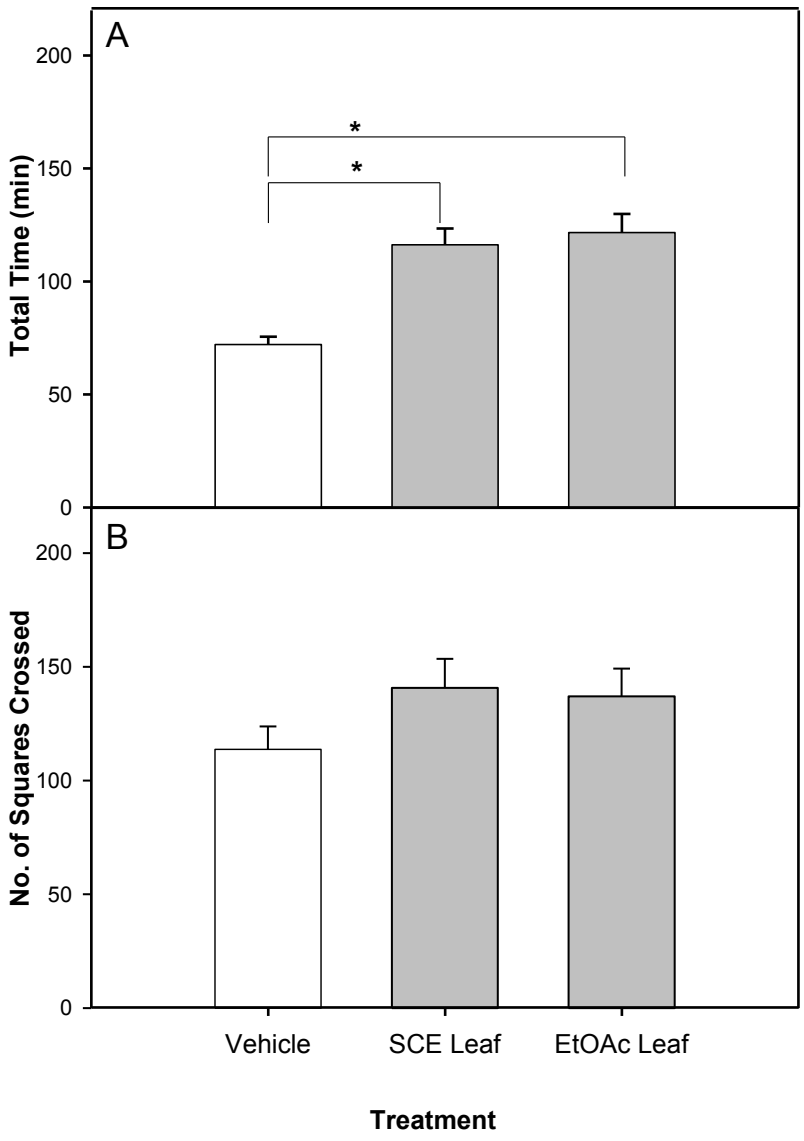


Figure 4.3. Effect of *S. sympetala* plant extracts on social interaction of rats. (A) Total time spent interacting with an unfamiliar animal in the social interaction assay; and **(B)** locomotor activity, as measured by number of squares crossed in open field of animals treated with *S. sympetala* extracts after a 1 h post-drug interval. All values represent the group mean ± S.E.M of n = 12- 24 per group. **Vehicle:** vehicle control (50% sweetened, condensed milk), **SCE:** supercritical CO_2 extract of *S. sympetala* leaves (75 mg/kg), **EtOAc:** ethylacetate extract of *S. sympetala* leaves (75 mg/kg). * Indicates significant difference from vehicle, one-way ANOVA, with Dunnett's post-hoc test $p < 0.05$.

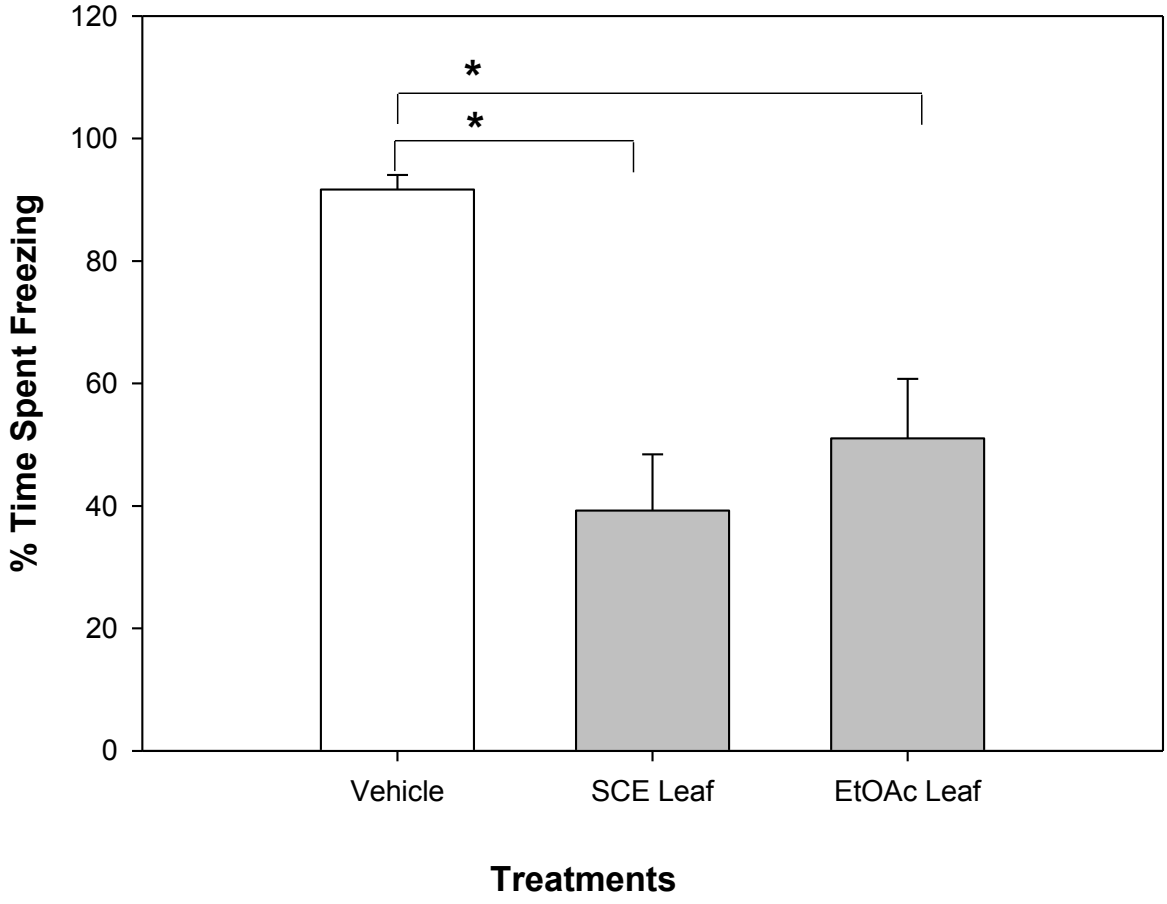


Figure 4.4. Effect of *S. sympetala* leaf extracts on percent time spent freezing in the conditioned emotional response (CER) – context assay after a 1 h post-plant interval (n = 9). All values represent the group mean ± S.E.M. of n = 7- 16 per group; **vehicle**: vehicle control (50% sweetened, condensed milk), **SCE**: supercritical CO_2 extract of *S. sympetala* leaves (75 mg/kg), **EtOAc**: ethylacetate extract of *S. sympetala* leaves (75 mg/kg).
* Indicates significant difference from vehicle, one-way ANOVA, with Dunnett's post-hoc test $p < 0.05$.

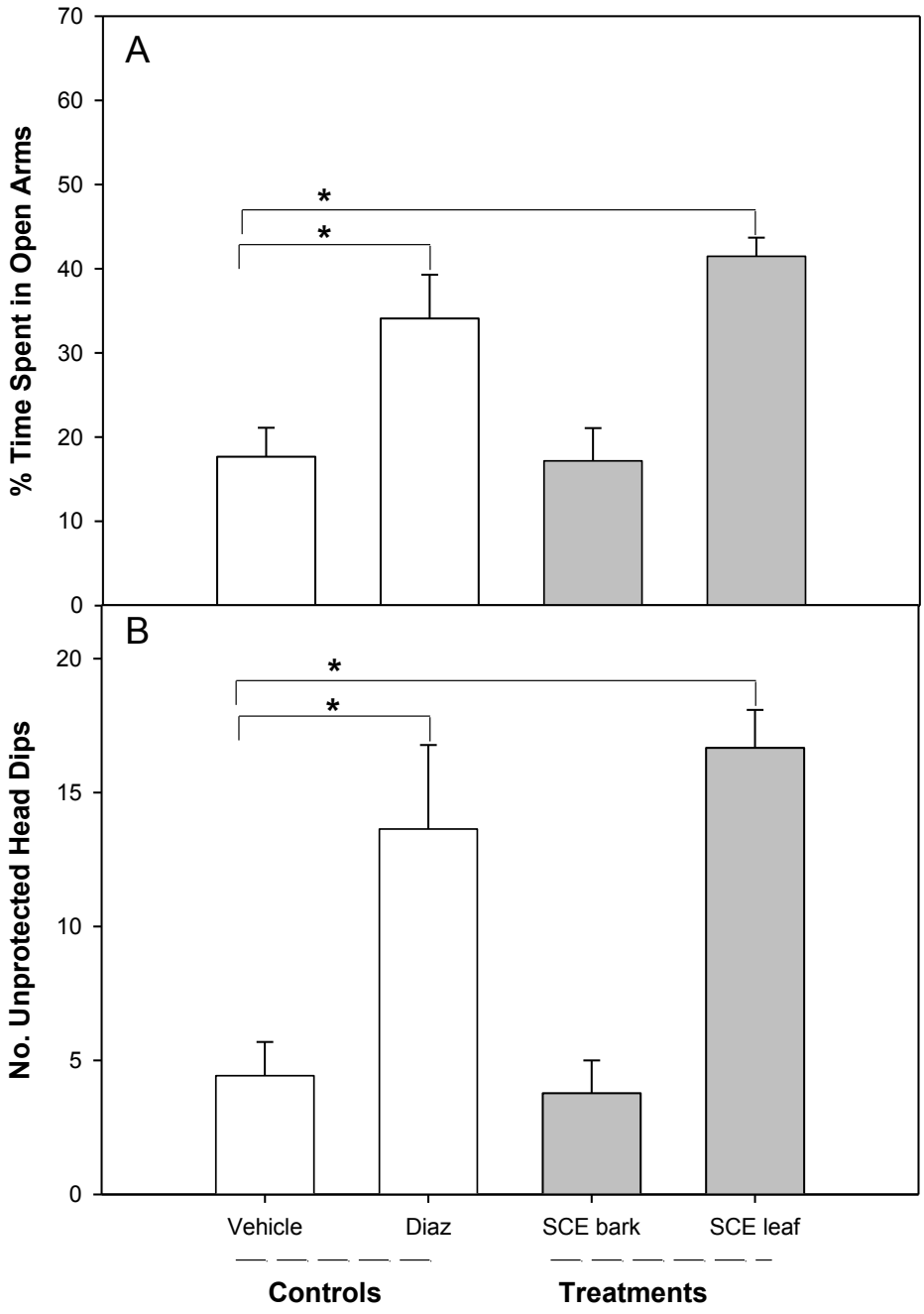


Figure 4.5. Effect of SCE extracts of *S. sympetala* bark and leaf extracts on anxiety-like behaviours in rats in the elevated plus maze (EPM); (A) Percent total time spent in the open arms, and **(B)** Number of unprotected head dips. All values represent the group mean ± S.E.M.; of n = 7- 9 per group. **Vehicle:** vehicle control (50% sweetened, condensed milk), **Diaz:** diazepam, 2 mg/kg, **SCE bark:** supercritical CO_2 extract of inner bark (2.5 mg/kg), **SCE Leaf:** supercritical CO_2 extract (75 mg/kg). *Indicates significant difference from vehicle, one-way ANOVA, with Dunnett's post-hoc test $p < 0.05$.

Effect of Pre-treatment with GABAa-BZD Receptor Antagonist Flumazenil in Behavioural Assays

Elevated Plus Maze

The SCE leaf extract (75 mg/kg) had a significant effect on percent time spent in the open arms, as compared to vehicle control and flumazenil (0.5 mg/kg) (Figure 4.6 $F(5, 55) = 10.26$, $p < 0.05$). Animals pre-treated with flumazenil (0.5 mg/kg) followed by SCE leaf extract spent significantly less time in the open arms, 13.3 ± 1.9 %, as compared to the animals treated with the SCE leaf extract, 41.5 ± 2.2 % (Figure 4.6, $F(5, 55) = 10.26$, $p < 0.05$).

CER - Context

The EtOAc leaf extract (75 mg/kg) had a significant effect on percent time spent freezing, as compared to vehicle control and flumazenil (0.5 mg/kg) (Figure 4.7, $F(4,42) = 8.31$, $p < 0.01$). Pre-treatment with flumazenil (0.5 mg/kg) followed by EtOAc extract did not increase the amount of time spent freezing as compared to EtOAc extract (75.5 ± 10.9% (flu + EtOAc extract) versus 51.0 ± 9.74 % (EtOAc extract)).

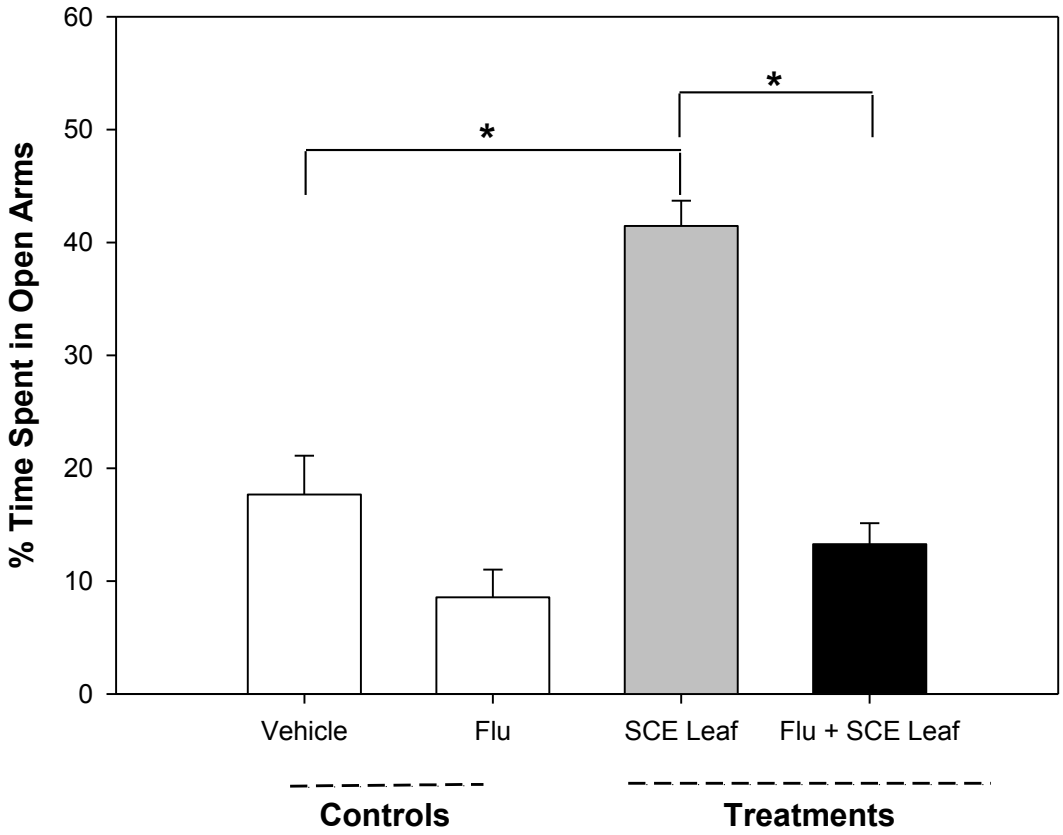


Figure 4.6. Effect of pre-treatment with flumazenil on percent total time spent in the open arms of the elevated plus maze (EPM). All values represent the group mean ± S.E.M. of n = 7 – 16 per group. **Vehicle:** vehicle control (50% sweetened, condensed milk), **Flu:** flumazenil, i.p. (0.5 mg/kg), **SCE leaf:** supercritical CO_2 extract of leaf (75 mg/kg), **Flu + SCE:** flumazenil (0.5 mg/kg) followed by supercritical CO_2 extract of leaf (75 mg/kg) 45 min later. * Indicates significant difference, one-way ANOVA, with Dunnett's post-hoc test $p < 0.05$.

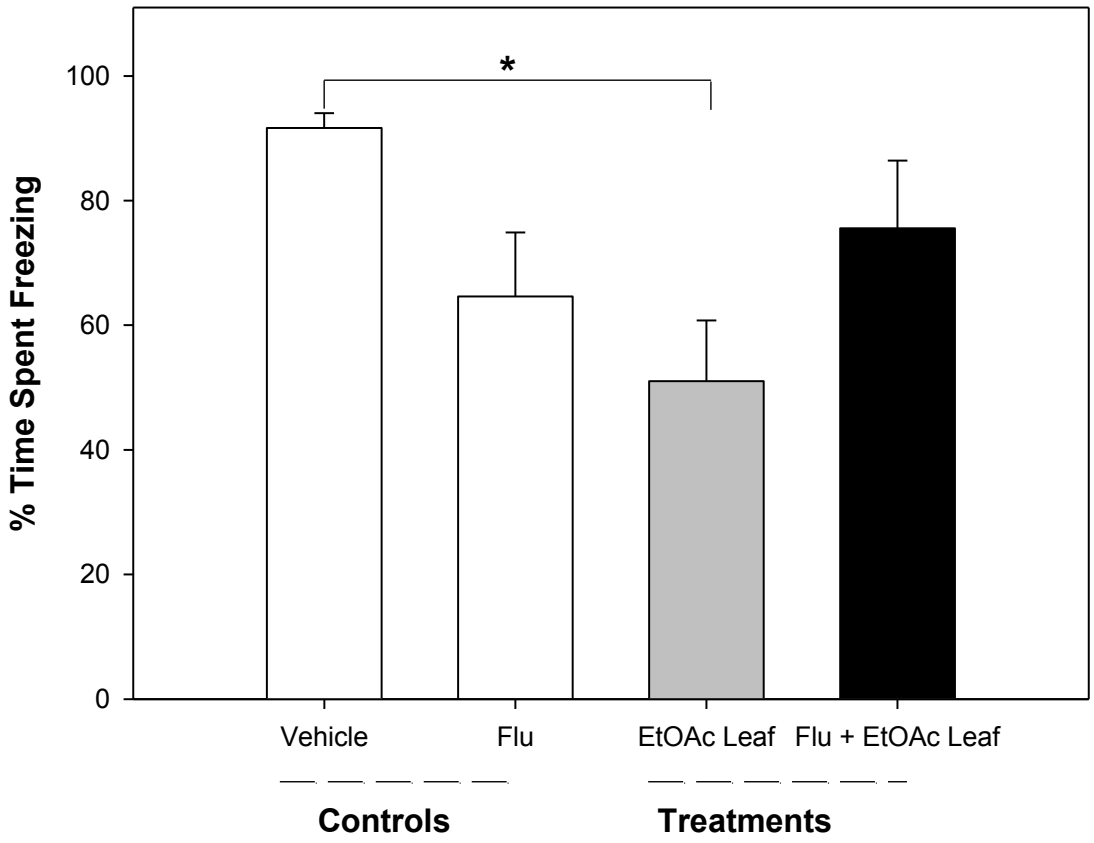


Figure 4.7. Effect of pre-treatment with flumazenil on percent total time spent freezing in the conditioned emotional response (CER) – context assay. All values represent the group mean ± S.E.M. of n = 7 – 16 per group. **Vehicle:** vehicle control (50% sweetened, condensed milk), **Flu:** flumazenil, i.p. (0.5 mg/kg), **EtOAc Leaf:** EtOAc extract of leaf (75 mg/kg), **Flu + EtOAc Leaf:** flumazenil (0.5 mg/kg) followed by ethylacetate extract of *S. sympetala* leaf (75 mg/kg) 45 min later, stars indicate significant difference from vehicle, one-way ANOVA, with Dunnett's post-hoc test $p < 0.05$.

Effect of meBA on Anxiety-like and Depressive Behaviour

Elevated Plus Maze & CER - Context

The methyl ester analog of betulinic acid (meBA, 5 mg/kg) had a significant effect on percent time spent in the open arms of the EPM as compared to vehicle control and animals pre-treated with flumazenil followed by meBA (Figure 4.8A, $F(3, 26) = 3.95$, $p < 0.05$). In the CER, animals treated with meBA (5 mg/kg) spent significantly less time freezing in the first five minutes than vehicle or flumazenil- treated animals (Figure 4.8B, $F(3, 27) = 4.77$, $p < 0.05$).

Forced Swim Test

Animals treated with the SCE leaf extract (75 mg/kg) spent significantly less time immobile in the swim despair assay as compared to vehicle treated animals, (Figure 4.9 $F(3, 34) = 6.27$, $p < 0.05$).

***In vitro* GABAa-BZD Receptor Site Competitive Binding Assay**

Both *S. sympetala* extracts exhibited dose-dependent displacement of ^{3}H-flunitrazepam from the GABA BZD receptor site *in vitro* (Figure 4.10). The two extract types demonstrated similar capacity to displace ^{3}H-flunitrazepam, with 50% of the labeled drug displaced (EC_{50}) at a concentration of 123 µg/mL (EtOAc extract) and 154 µg/mL (SCE extract). Non-linear regressions yielded competitive binding curves for each extract, goodness of fit: R^2 for SCE = 0.77, R^2 for EtOAc = 0.91, and computed dissociation constants (K_i) of $K_i = 5.42 \times 10^{-7}$ M BA (in SCE extract) and $K_i = 2.64 \times 10^{-7}$ M BA (in EtOAc extract).

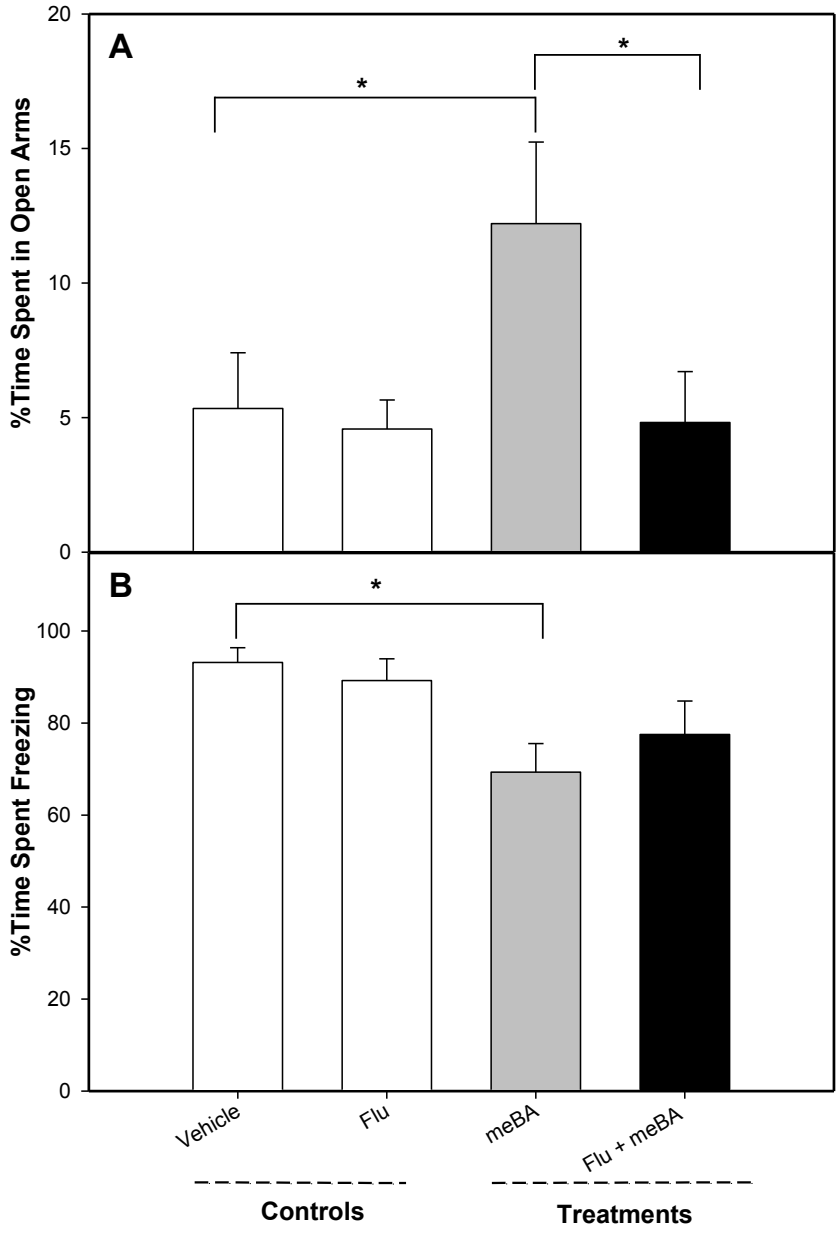


Figure 4.8. Effect of meBA on anxiety-like behaviour of rats. (A) Percent total time spent in the open arms of the elevated plus maze ; and **(B)** percent time spent freezing in the first five minutes of the conditioned emotional response (context) assay for animals treated with vehicle, flumazenil, meBA, or flumazenil + meBA, after a 1 h post-drug interval (n = 12 – 24). All values represent the group mean ± S.E.M., of n = 7 – 16 per group. **Vehicle:** vehicle control (50% sweetened, condensed milk), **Flu:** flumazenil (0.5 mg/kg), **meBA:** methyl ester of betulinic acid, 5 mg/kg, suspended in sweetened, condensed milk, **Flu + meBA:** flumazenil (0.5 mg/kg) followed by meBA, 5 mg/ kg, 45 min later, data analysed with one-way ANOVA, with Dunnett's post-hoc test, stars indicate significant differences, $p < 0.05$.

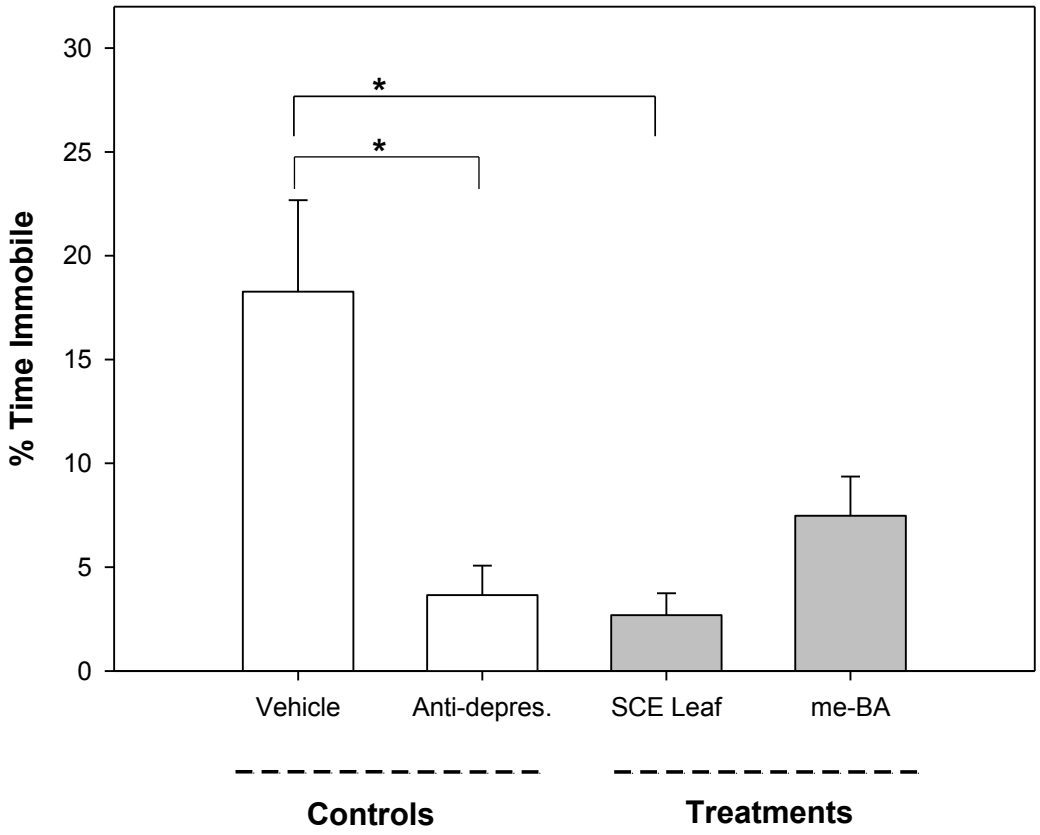
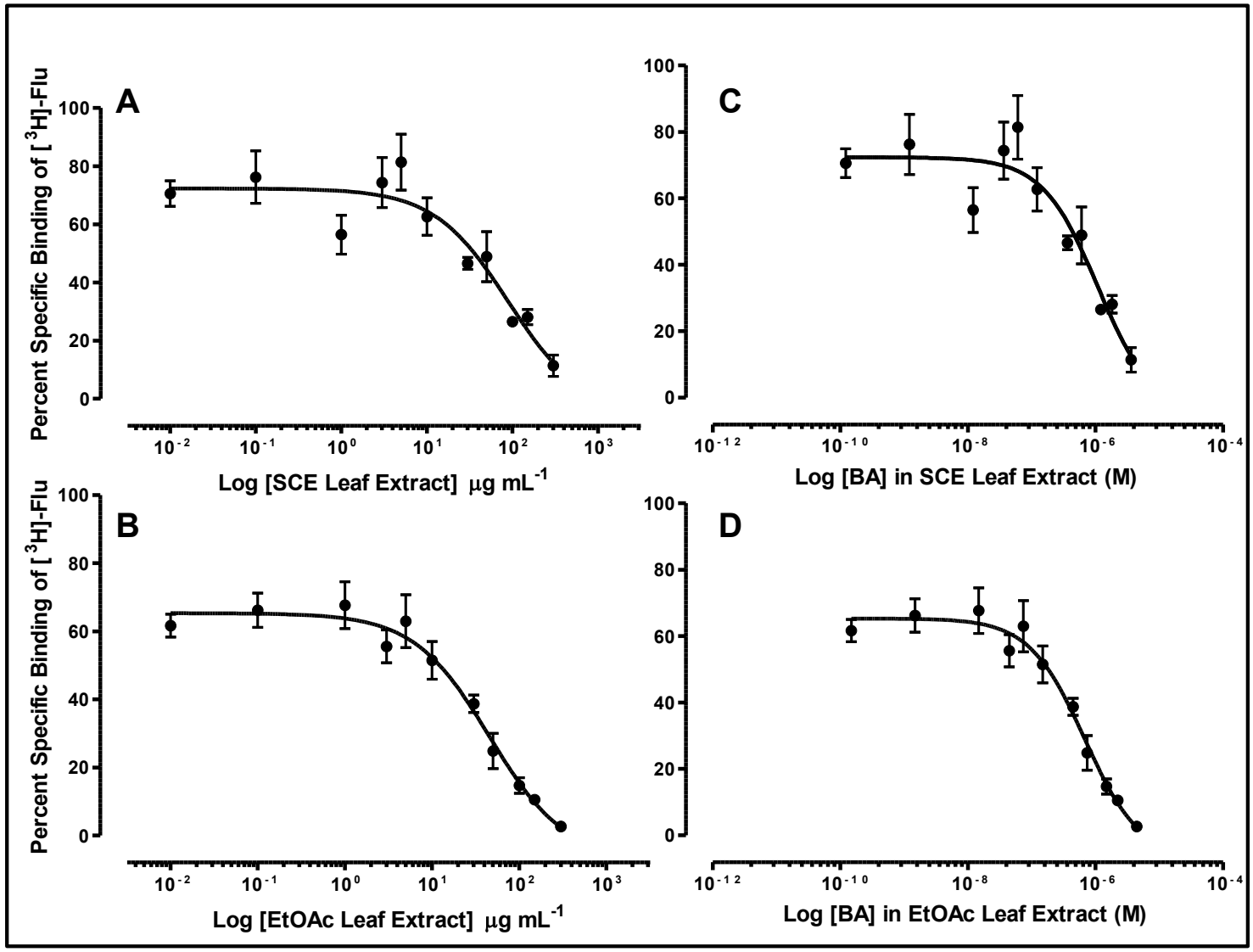


Figure 4.9. Effect of SCE leaf extract and meBA on percent time spent immobile in the forced swimming assay. Percentage of total time spent immobile for animals treated vehicle, anti-depressant, supercritical CO_2 leaf extract and meBA, after a 1 hr post-drug interval. All values represent the group mean ± S.E.M. of n = 10 per group. **Vehicle:** vehicle control (50% sweetened, condensed milk), **Anti-depres.:** antidepressant desipramine, (5 mg/kg), **SCE Leaf:** supercritical CO_2 extract of *S. sympetala* leaves (75 mg/kg) suspended in sweetened, condensed milk, **meBA:** methyl ester of betulinic acid (5 mg/kg), suspended in sweetened, condensed milk. Data analysed with one-way ANOVA, with Dunnett's post-hoc test, stars indicate significant difference from vehicle, $p < 0.05$.

Figure 4.10. Competitive binding curve for S. sympetala extracts, (A) supercritical carbon dioxide (SCE) extract of leaves; **(B)** conventional EtOAc extract of leaves, versus [3H]-flunitrazepam to the GABAa-BZD receptor in the rat cerebral cortex membrane preparations. Non-linear regression yielded competitive binding curve for each extract, goodness of fit: R^2 for SCE = 0.77, R^2 for EtOAc = 0.91. In the panels on the right, the data are presented as percent specific binding versus log concentration of betulinic acid in the **(C)** SCE extract and **(D)** EtOAc extract. Non-linear regressions revealed a competitive binding curve for each extract, R^2 = 0.77 (SCE), R^2 = 0.91 (EtOAc) and computed dissociation constants (K_i) of $K_i = 5.42 \times 10^{-7}$ M (SCE) and $K_i = 2.64 \times 10^{-7}$ M (EtOAc). Each data point represents n = 3 ± S.E.M.


A
B
C
D
Percent Specific Binding of [3H]-Flu
Log [SCE Leaf Extract] μg mL-1
Log [EtOAc Leaf Extract] μg mL-1
Log [BA] in SCE Leaf Extract (M)
Log [BA] in EtOAc Leaf Extract (M)

4.6 Discussion

This study examined the effect of *S. sympetala* raw plant, extracts and analogue of the principle bioactive, meBA, in behavioural models of anxiety and one behavioural model of depression. The hypothesis examined in the study was that the anxiolytic plant *S. sympetala* reduces anxiety by acting at the GABAa-BZD receptor. The observed effects in the EPM, SI and CER assays indicate that the plant material, leaf extract and meBA are anxiolytic, without a sedative effect, at the concentrations examined in these experiments. The *in vitro* assay indicates that the extracts have affinity for the GABAa-BZD receptor. Moreover, pre-treatment of animals with the GABAa-BZD receptor antagonist, flumazenil, blocks the anxiolytic effect of both the SCE leaf extract and meBA, indicating that these act directly at the GABAa-BZD receptor *in vivo*. Pre-treatment with flumazenil had a less pronounced effect in the CER. While it increased time spent freezing after treatment with both leaf extract and meBA, the increase was not significant. The leaf extract had a significant anti-depressive effect, in that animals treated with leaf extract spent more time swimming in the forced swim assay. The results demonstrate for the first time that raw *S. sympetala* plant, extracts and meBA all possess anxiolytic activity *in vivo* and the pharmacological mode of action is by agonist action against the GABAa –BZD receptor.

The finding that *S. sympetala* acts at the GABAa-BZD receptor is aligned with evidence that other well characterized anxiolytic herbs, passionflower (*Passiflora incarnata* L.), skullcap (*Scutellaria baicalensis* Georgi) and valerian root (*Valeriana officinalis* L.), contain molecules that are GABAa-BZD receptor agonists. Pre-treatment with a GABAa-BZD receptor antagonist followed by 375 mg extract/kg (passionflower) or 200 mg extract/kg (skullcap) extinguishes anxiolysis in rodent models (Grundmann et al., 2008; Lolli

et al., 2007). The bioactive principle of *S. sympetala* is the triterpenoid BA, as opposed to passionflower and skullcap, where the bioactivity is associated with flavonoids (woginin from skullcap, and chrysin from passionflower, as well as the indole alkaloids). In valerian root, the bioactivity is associated with the sesquiterpenoid valerenic acid, also a GABAa-BZD receptor agonist (Trauner et al., 2008). Valerenic acid interacts specifically with the β3 subunits of the GABAa-BZD receptor,and mice with wild type β3 subunits treated with valerenic acid displayed significantly reduced anxiety as compared to mice with mutated β3 subunits treated with valerenic acid (Benke et al., 2009).

Both *S. sympetala* raw leaf and bark reduced anxiety in rats. The raw leaf has the lowest BA concentration of all the treatments examined, but an anxiolytic effect was still observed. Further, when SCE bark and leaf were compared, the leaf extract had a significant anxiolytic effect with no effect observed for the SCE bark. The phytochemical profile of the SCE bark extract shows that it is essentially pure BA, with no detectable presence of other triterpenoids. In contrast, both the raw leaf material and SCE leaf extract contain a variety of triterpenoids (as discussed in Chapter 2). This result suggests that the activity of the more phytochemically complex material, *i.e.* raw leaf and leaf extract, may be associated with synergy of the triterpenes present and suggests that raw leaf material could be developed as a NHP, without extraction. Further animal trials with the raw leaf material should be done to determine how robust the anxiety-reducing effect of raw plant material is.

The SCE leaf extract had a significant anti-depressive effect. The moderate dose, 75 mg/kg, and a single dosing period, compare favourably with other herbs used traditionally to treat depression. These herbs, including SJW (500 mg/kg ethanolic extract, oral administration, acute treatment) (Butterweck et al., 2003), the African medicinal plants

Boophone disticha L.f. and *Mondia whitei* Hook.f. (250 mg/kg ethanolic extract, oral administration, 3 times) (Pedersen et al., 2008), Chinese peony (*Paeonia lactiflora* Pall., 500 mg/kg intragastric administration for 7 days) (Mao et al., 2008) and curcumin from *Curcuma longa* (turmeric) (20 mg/kg, *i.p.* for 21 days) significantly decrease time spent immobile in the forced swim assay. The pure compound, meBA, did not have a significant effect in the forced swim assay, despite the fact that the two treatments were matched for BA and meBA content. This result is similar to that observed for the raw plant material above, and suggests a possible synergistic effect for the extract material, perhaps the presence of multiple triterpenoids in the extract accounts for the increase in activity. It is also possible that a different phytochemical, either a triterpenoid or another molecule present in the extract, is responsible for the anti-depressant effect. Pharmacological agents that are effective in the forced swim assay are typically molecules that target serotonin, including tricyclics, monoamine oxidase inhibitors (MAOIs) and atypical antidepressants (Willner and Mitchell, 2002). The UPHD metric of the EPM also responds specifically to ligands that bind the serotonin receptor ($5HT_{1A}$ receptor) (Griebel et al., 1997), and all of the materials tested, with the exception of the SCE bark, had a significant effect on this metric. These observations, taken together, suggest at least two targets for *S. sympetala* in the CNS, the GABAa-BZD receptor and a receptor, enzyme or molecule that targets serotonin and plays a role in depression. Further experiments to examine the effect of *S. sympetala* extract on serotonin re-uptake and metabolism may provide insight to this mechanism. Finally, of interest is the observation that there is a significant correlation between the efficacy of anti-depressants tested in the forced swim assay and potency of molecules in clinical trials, a characteristic not demonstrated for other animal models of depression (Willner, 1985) and one that could mean that *S. sympetala* would be an effective anti-depressant in humans.

The experiments described in this chapter show for the first time that *S. sympetala* is an anxiolytic plant that targets the pharmacologically important GABAa-BZD receptor to reduce anxiety. *S. sympetala* also has significant anti-depressant effects. *S. sympetala* has no adversive effect on locomotor activity, as compared to the vehicle control, and at the concentrations tested in these expierments. This is an important observation, because anxiolytics and anti-depressives commonly include sedation, muscle relaxation and ataxia as side-effects (Atack, 2003). Taken together, the results from the behavioural assays demonstrate that *S. sympetala* raw plant, extracts and meBA significantly reduce anxiety and depressive behaviour in rodents without any apparent adverse sedative effects after acute administration. Further experiments are planned to examine the applicability of the raw plant material in the treatment of anxiety and the mode of action associated with the observed anti-depressant effect. The data presented in this chapter suggests that *S. sympetala* is a good candidate for further development as a NHP. Finally, the behavioural results corroborate the ethnobotanical identification of *S. sympetala* in the treatment of anxiety and the link between the folk illness *susto* and anxiety.

CONFIDENTIAL

CHAPTER 5 - A BOTANICAL EXTRACT OF *SOUROUBEA SYMPETALA* AND ITS ACTIVE PRINCIPLE, BETULINIC ACID, HAVE CORTISOL LOWERING CAPACITY IN STRESSED RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

5.0 Preface

This chapter further examines the pharmacology of *S. sympetala*, specifically the capacity of *S. sympetala* to have a stress-reducing effect in an animal model. The cortisol-lowering capacity of *S. sympetala* and its active principle, BA, are examined. In experiments conducted in Dr. Merali's neuropsychology lab, meBA reduced circulating plasma corticosterone in restrained rats (Merali, 2004). This led to the hypothesis that *S. sympetala* and BA may act within the hypothalamus-pituitary-adrenal axis (HPA), a major part of the neuroendocrine system that controls the stress response and results in the release of cortisol. In collaboration with the laboratories of two fish physiologists, Dr. Tom Moon and Dr. Kathleen Gilmour, I developed two assays, one *in vitro* and one *in vivo* one with rainbow trout, *Oncorhynchus mykiss*, as a model fish species, to examine this putative cortisol-lowering effect. The second hypothesis of the PhD project is examined in this chapter, that *S. sympetala* and BA act at the site of cortisol synthesis (interrenal cells in rainbow trout) to lower the cortisol reponse to an ACTH challenge *in vitro* and net restraint *in vivo*. Two predictions were tested, (1) that head kidney cells preincubated with *S. sympetala* extract or BA, will release less cortisol following an ACTH challenge; and (2) fish fed a diet supplemented with BA and then exposed to a physiological stressor will release less cortisol than fish fed an unsupplemented diet. The data demonstrate that both *S. sympetala* and BA alter ACTH-induced cortisol secretion and suggest that *S. sympetala* and BA have two paths

of action, in the central nervous system (CNS), the GABAa receptor, as presented and discussed in Chapter 4, and the HPA axis, as presented and discussed in this chapter.

5.1 Statement of Author Contributions

The *in vitro* head kidney cell assay was taught to me by Dr. Caroline Mimeault and conducted in the lab of Dr. Tom Moon. I conducted the *in vivo* experiments in Dr. Kathleen Gilmour's lab in collaboration with Fida Ahmed. Marco Otorola, Pablo Sanchez, Mario Garcia and Luis Poveda are tropical botanists who collected and identified the plants in Costa Rica. Vance. L. Trudeau and John. T. Arnason provided supervision and funding for this work.

Publication:

The data presented in this chapter have been incorporated into a patent application filed by Bioniche Life Sciences Incorporated. When the patent process is completed, the manuscript will be submitted to *Aquaculture*.

5.2 Abstract

The traditional anxiolytic plant *Souroubea sympetala* and its bioactive principle, betulinic acid (BA) were investigated for their cortisol-lowering capacity in rainbow trout, *Oncorhynchus mykiss*. Two assays were employed, an *in vitro* head kidney assay and an *in vivo* standardized net restraint assay. In the *in vitro* assay, head kidney cells incubated with *S. sympetala* extract and BA released significantly less cortisol in response to an ACTH challenge than cells incubated with media, with a 50% reduction in cortisol response at 71.45 μg/mL (extract) and 32.7 μM (BA). In the *in vivo* assay, fish fed a commercial trout pellet diet supplemented by 1 mg/kg BA for three days released significantly less cortisol in response to a physiological net restraint assay than fish fed the unsupplemented diet. The results demonstrate that *S. sympetala* and BA act at the interrenal cells of the hypothalamus-pituitary-interrenal (HPI) axis to significantly alter the stress response of rainbow trout. The potential application of BA in an aquaculture setting and implications of cortisol reduction in humans, are discussed.

5.3 Introduction

In vertebrates, the perception of stress initiates a response cascade involving the hypothalamus-pituitary-adrenal (HPA, in mammals) or interrenal (HPI, in fish) axis. When perceived by hypothalamic neurons, the stressor stimulates the release of corticotrophin-releasing-hormone (CRH) from the hypothalamus which stimulates the release of adrenocorticotropin hormone (ACTH) from the pituitary gland. ACTH, in turn travels to the adrenal cortex (mammals) or interrenal tissue of the head kidney (fish) to stimulate the synthesis and release of cortisol (corticosterone in rats) into the blood. In stress trials, circulating plasma cortisol (corticosterone) is considered a common biomarker to confirm that a stimulus exerts significant stress and to distinguish non-stressed from stressed animals (Gamperl et al., 1994).

Previous experiments demonstrated that juvenile pigs housed in groups, and exposed to housing and aggression-related stress, when fed dried *S. sympetala* leaves (500 mg/kg), or 1.0 mg/kg betulinic acid (BA) had significantly reduced plasma cortisol levels (Pineiro, 2005). Further, experiments with restrained rats demonstrated that a 1 mg/kg dose of the methyl ester of BA (meBA) significantly reduced plasma corticosterone after 30 and 60 min of restraint (Z. Merali, unpublished data). These observations led to the hypothesis that *S. sympetala*, BA and meBA act through the HPA axis to reduce circulating plasma cortisol (or corticosterone) levels in stressed mammals.

Stress is of particular concern in aquaculture. The primary stress response in teleost fishes (mobilization of the catecholamine and glucocorticoid stress hormones) leads to mobilization of energy-rich substrates by depletion of hepatic glycogen stores, elevation of

plasma glucose, changes in circulating free fatty acid levels and general inhibition of protein synthesis. These responses mean that stress has an overall catabolic effect on fish, and in an aquaculture setting elevated cortisol levels adversely affect growth rate, immunity and reproduction (Schreck et al., 2001). The experiments described in this chapter were conducted on rainbow trout, *Oncorhynchus mykiss*. Rainbow trout are a good species to examine the effects of stress because they are known to experience chronic stress in aquaculture situations (Ruyet et al., 2008), and the salmonoids, including salmon, trout and whitefish, are amoung the most farmed fish in the world (FAO, 2007).

Rainbow trout possess a hypothalamic-pituitary-interrenal (HPI) axis which is functionally equivalent to the mammalian HPA axis. Interrenal cells are located within the anterior kidney, or head kidney, of teleost fish where they represent a small proportion of the overall cells in this part of the kidney (Hontela et al., 2008). I tested the hypothesis that *S. sympetala* extract and BA act via the HPI axis to reduce the cortisol response of stressed trout. This hypothesis was examined in two ways, first using an *in vitro* head kidney cell assay, and second using an *in vivo* net restraint assay. For the *in vitro* assay, head kidney cells were incubated with *S. sympetala* extracts and BA and cortisol release in response to ACTH induction assessed. The *in vivo* assay consisted of feeding rainbow trout a standard commercial diet supplemented with BA and then subjecting them to a standardized net restraint. Circulating plasma cortisol levels were then assessed to establish whether a BA-enriched diet changed the cortisol response of a stressed trout.

5.4 Materials and Methods

Chemicals

Analytical grade HPLC solvents were purchased from J.T. Baker (USA). Betulinic acid (BA) was obtained from Sigma-Aldrich (St. Louis, MO). Extraction grade solvent (ethyl acetate) was purchased from Fisher Scientific (Ottawa, ON Canada).

Plant material

Fresh samples of wild *S. sympetala* were collected under permit in Tortuguero, Costa Rica. Samples were dried overnight in a commercial plant drier at 35 ºC and ground to 2 mm mesh. Voucher specimens were identified by two of us (L.P. and P.S.) and deposited in the JVR Herbariun, Universidad Nacional Costa Rica, and the University of Ottawa Herbarium (OH No. 19915).

Conventional solvent extraction

Samples (2 g) were incubated, with shaking, in 40 mL (1:20 weight: volume) ethyl acetate (EtOAc) for 12 – 15 h at room temperature (RT). The solvent was filtered (Whatman #1) and the filter cake re-extracted twice as above, with half as much EtOAc (1:10 and 1:5). The total solvent from the three extractions was combined for an exhaustive extraction. The solvent was removed by rotary evaporation with a Yamato Rotary Evaporator RE50 (Yamato Scientific, Japan) at 40ºC, lyophilized (Super Modulyo, Thermo Electron, USA) and stored

in opaque glass vials at 4ºC. Total BA content of extracts was determined as previously described (Mullally et al., 2008).

Animals

Female juvenile rainbow trout (*Oncorhynchus mykiss*), 75 – 150 g, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). Fish were transported to the University of Ottawa Aquatic Care Facility and maintained in fiberglass holding tanks (110-115 L) continuously supplied with aerated, dechloraminated City of Ottawa tap water at 13 °C. Fish were subjected to a constant 12L:12D photoperiod and fed five times per week with commercial trout pellets (Classic Floating Trout Grower, Martin Mills, Tavistock, ON). All experiments were carried out under protocols approved by the University of Ottawa Protocol Review Committee and adhere to published guidelines of the Canadian Council on Animal Care for the use of animals in teaching and research.

Preparation of Cell Suspensions

Trout head kidney cells were prepared using methods adapted from Leblond *et al.* (Leblond et al., 2001). Briefly, fish (n = 6 - 7, mass = 129 ± 11.8 g) were anesthetized with benzocaine (30 to 35 mg/L), blood was collected by caudal puncture into heparinized syringes and fish were then euthanized by a sharp blow to the head followed by trans-spinal sectioning. The head kidney was removed and placed in a solution of enriched minimum essential medium (eMEM, Sigma-Aldrich) supplemented with collagenase/dispase (2 mg/mL). The tissue was manually disrupted by gently pressing it on the interior walls of the tube with a small spatula and incubated for 1 h at 10 - 13˚C with agitation. The homogenate

was then sequentially filtered (200 µm filter, rinsed with MEM, then a 75 µm filter and rinsed with MEM), centrifuged (260 *g*, 10 - 13˚C, 5 min) and the pellet resuspended in 1 mL MEM.

Stimulation of Head Kidney Cells with *S. sympetala* Extract and Betulinic Acid

Cells were plated in a 96-well microtiter plate with 150 µL containing 50 x 10^6 cells/well. Cells were incubated with BA (1, 3, 10, 30 and 100 µg/mL; 0.46, 1.4, 4.6, 13.7 and 45.7 µMolar) or *S. sympetala* extract (1, 3, 10, 30 or 100 µg/L) dissolved in DMSO (final concentration of DMSO: 0.3% v/v) for 60 min at 10 – 13ºC, then stimulated with 1U/ml procine ACTH (Sigma-Aldrich) and incubated for an additional 60 min. Then cells were collected and transferred to 1.5 mL conical plastic tubes and centrifuged (20,000 x *g*, 2 min); the supernatant was collected and flash frozen in liquid nitrogen for subsequent cortisol assay using a commercially available, standard RIA (MP Biomedicals Ltd., Solon, ON). Cytotoxicity was assessed at the end of the experiment with the lactate dehydrogenase (LDH) assay according to Mommsen and Moon (Mommsen and Moon, 1987). Four control wells were prepared: DMSO blank (0.3% v/v), DMSO plus ACTH (1 U/mL), eMEM blank and eMEM plus ACTH (1 U/mL).

Preparation of fish food supplemented with betulinic acid

BA was added to moistened ground commercial trout pellets (Classic Floating Trout Grower, Martin Mills, Tavistock, ON), which were then re-formed into pellets using a commercial food extruder, and air-dried in a fumehood. Fish (n = 28, mass = 148.7 ± 9.85) randomly selected from a holding tank were placed into separate tanks and habituated to the feeding regimen for five days, during which time they were fed 1% of total body mass per day using normal commercial food. Thereafter, one tank was fed the BA-supplemented trout pellets (1 mg BA/ kg fish) and the other, a control tank, (trout chow, no BA) at 1% of body mass per day once a day for 3 days, between 10:00 – 2:00 pm. The netting stress was conducted on day three, four hours after the last feeding.

Netting Stress Assay

Fish were individually netted in rapid succession into dipnets and the nets were secured to the outside of the experimental tank. The fish were held in the nets for 1 h, then anesthetized with benzocaine and euthanized as above, after removing a caudal blood sample. The blood was centrifuged, and plasma was collected and flash frozen in liquid nitrogen for subsequent cortisol assay by RIA (MP Biomedicals Ltd., Solon, ON).

Statistical Analyses

One-way and two-way analyses of variance (ANOVA) with Bonferonni studentized range tests were performed for mean comparisons (Zar, 1999). Kolmogorov-Schmirnoff and

Levene's tests were used to verify the normality of distribution and the homogeneity of residual variance, respectively. In cases where the data did not meet assumptions of the parametric tests, they were transformed. All of the Fisher statistics (F), degrees of freedom (df), and p-value estimates were calculated with S-PLUS software version 7.0 (Insightful Corp., Seattle, WA). Data are reported as means ± S.E.M and the level of significance was set at $p < 0.05$. The effective concentration at which the cortisol response was reduced to 50% (EC_{50}) was calculated for *S. sympetala* extract and BA by determining the equation of the linear regressions of extract or BA concentration versus cortisol release in the head kidney assay.

5.5 Results

Incubation of Head Kidney Cells with *S. sympetala* Extract

Rainbow trout head kidney cells pre-incubated with *S. sympetala* extract (3, 10, 30 and 100 µg/mL) released less cortisol in response to an ACTH (1 U/mL) challenge than the positive control group (MEM + ACTH, no *S. sympetala* extract) (Figure 5.1). A linear regression was fitted to the log extract concentration, such that cortisol release = -22.45 (log extract concentration) + 91.62, $R^2 = 0.6$, $p < 0.01$. Incubation with *S. sympetala* extract had no significant effect on cell viability (as measured by LDH leakage) in the assay as compared to the negative (un-stimulated) or positive (0, MEM + ACTH) controls.

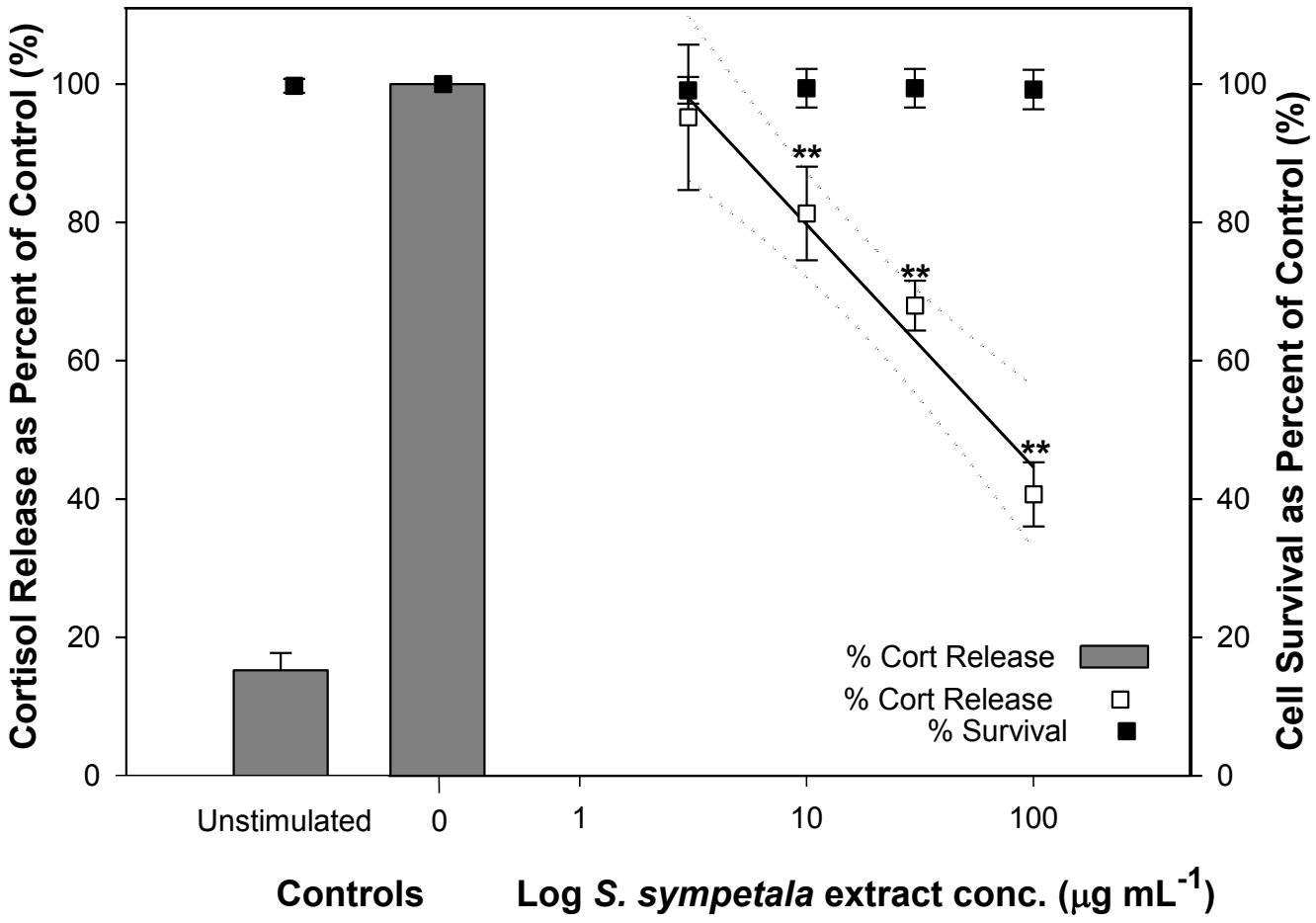


Figure 5.1. Effect of incubation with *S. sympetala* leaf extract on cortisol released from rainbow trout (Oncorhynchus mykiss) head kidney cells with and without challenge with 1 U/mL adrenocorticotropic hormone (ACTH). Open squares represent cortisol release as a percent of the positive control (0); closed squares represent cell viability at each concentration as a percent of positive control (0). Values represent the group mean ± S.E.M of n = 6; dotted line represents the 95% confidence intervals, linear regression equation: y = -22.45(log extract concentration) + 91.62, R^2= 0.60, $p < 0.01$, EC_{50} = 71.45 μg/mL. ** Indicates significant difference from positive control (0), $p < 0.001$, one-way ANOVA, multiple comparison of means, Bonferonni correction. **Un-stimulated**: cells incubated with minimal essential media (MEM); **0:** cells incubated with MEM+1U/mLACTH.

Stimulation of Trout Head Kidney Cells with Betulinic Acid

Rainbow trout head kidney cells pre-incubated with BA (0.46, 1.4, 4.6, 13.7 and 45.7 μMolar) released less cortisol in response to an ACTH (1 U/mL) challenge than cells in the positive control group (MEM + ACTH) (Figure 5.2). A linear regression of log concentration indicated a significant concentration-dependent response to pre-incubation with BA, linear regression equation: cortisol response = -22.5 (log BA concentration) + 84.09, R^2= 0.60 $p < 0.05$, EC_{50} = 5.72 μM. Incubation with BA had no significant effect on cell viability (as measured by LDH leakage) compared to the negative (unstimulated) or positive (0, MEM + ACTH) controls.

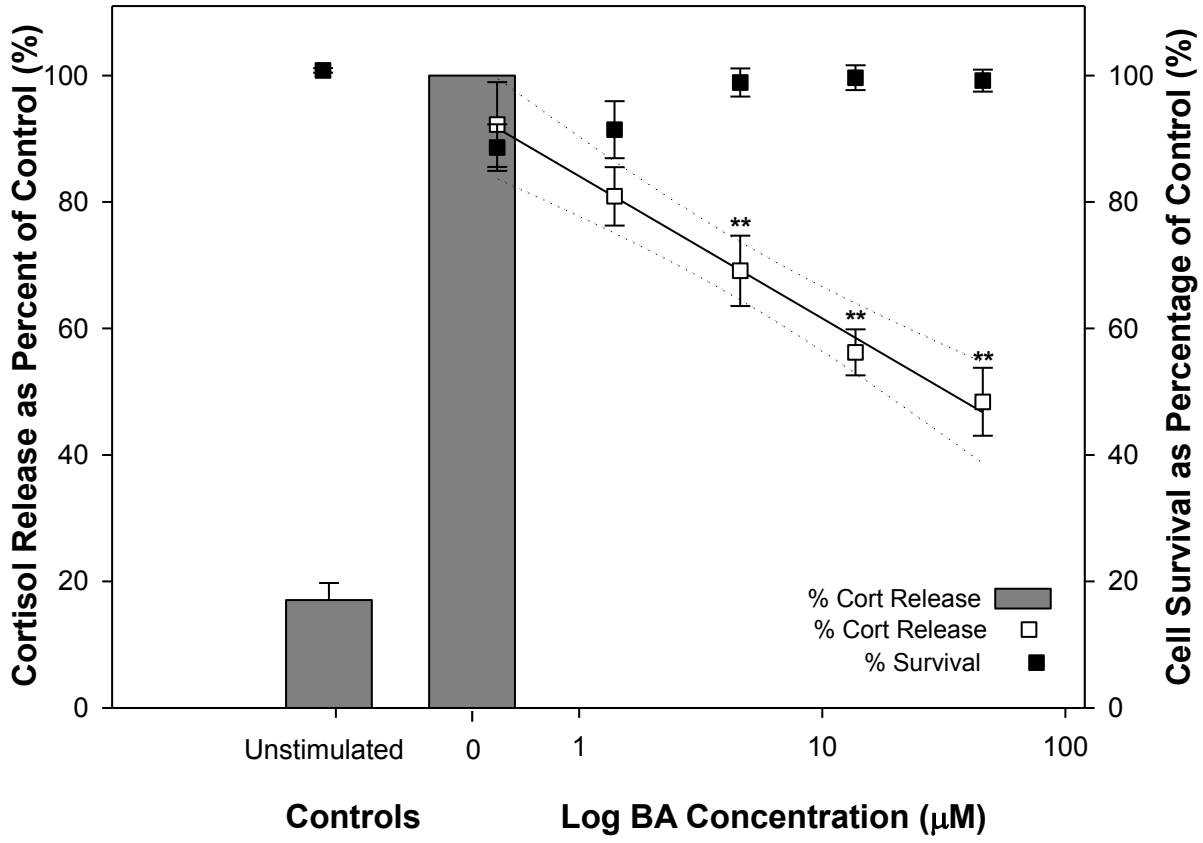


Figure 5.2. Effect of incubation with betulinic acid (BA) on cortisol release from head kidney cells of rainbow trout (*Oncorhynchus mykiss*) after stimulation with 1 U/mL adrenocorticotropic hormone (ACTH). See Figure 1 legend for details. Values represent the group mean ± S.E.M of n = 7; dotted line represents the 95% confidence intervals, linear regression equation: cortisol response = -22.5 (log BA concentration) + 84.09, R^2= 0.60 $p <$ 0.05, EC_{50} = 32.73 μM. ** Indicates significant difference from stimulated control (0), $p <$ 0.001, one-way ANOVA, multiple comparison of means, Bonferonni correction.

Effect of BA-Enriched Diet on Restrained Fish

Circulating cortisol concentration data in the net restrained trout were analysed with a two-way ANOVA. Food (control/BA) and movement (free/restrained) were the factors. There was a significant interaction effect for food and movement, $F(1, 22) = 16.99$, $p = 0.0004$, a significant main effect of movement, $F(1, 22) = 28.93$, $p = 0.00002$, and no main effect of food ($F(1, 22) = 0.41$, $p = 0.52$). The interaction effect indicates that the mean plasma cortisol of fish fed the BA-supplemented diet (1 mg/kg) and restrained, is significantly lower than the mean plasma cortisol levels of fish fed the control diet and restrained (Figure 5.3). That is, restraint does not significantly increase mean plasma cortisol in the presence of BA; there is no significant difference between cortisol levels in fish fed and swimming freely, versus fish fed BA and restrained. There was no significant difference in mean plasma cortisol levels of free swimming fish fed the control food and free swimming fish fed the BA supplemented food. Finally, supplementing the diet with BA had no apparent effect on fish behaviour or health, although no toxicity assays were performed.

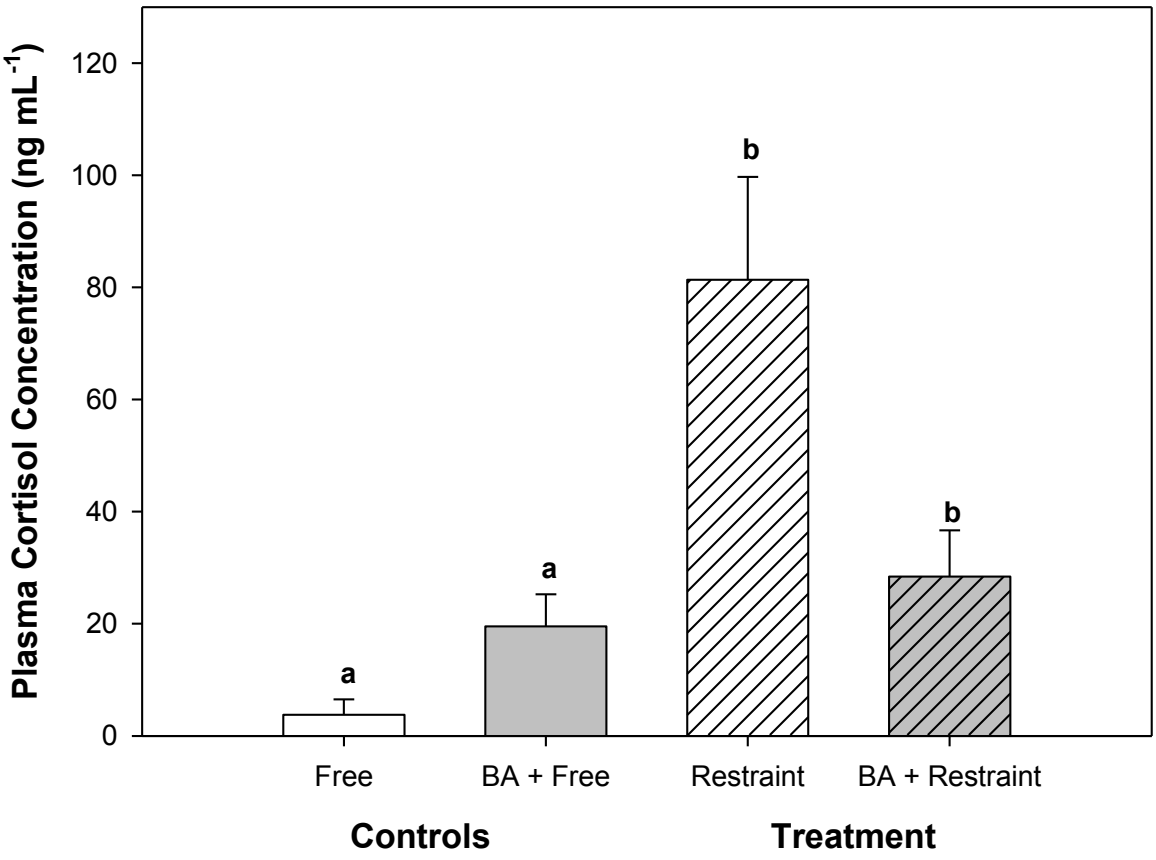


Figure 5.3. Effect of betulinic acid (BA) on the cortisol response to a netting restraint stressor in rainbow trout (*Oncorhynchus mykiss*). Values represent the group mean ± S.E.M. of n = 5-7. Data was analysed by 2 way ANOVA with food (control/BA) and movement (free/restrained) as the factors. There was a significant interaction effect between movement and food, $F(1, 22) = 16.99$, $p = 0.0004$, a significant main effect of movement, $F(1, 22) = 28.93$, $p = 0.00002$, and no main effect of food ($F(1, 22) = 0.41$, $p = 0.52$). Letters indicate significant differences, $p < 0.001$, one-way ANOVA, multiple comparison of means, Bonferonni correction. **Free** control fish diet, free swimming; **BA + Free** fish diet supplemented with 1 mg/kg BA, free swimming; **Restraint,** control fish diet, net restraint for 60 min; **BA + Restraint** fish diet supplemented with 1 mg/kg BA, net restraint for 60 min.

5.6 Discussion

This study examined the putative cortisol lowering capacity of both *S. sympetala* extract and BA using an *in vitro* head kidney assay and an *in vivo* net-restraint assay in rainbow trout, *Oncorhynchus mykiss*. The observed effects were consistent in both the *in vitro* and *in vivo* models employed, and revealed a cortisol lowering effect of both plant extract and BA in response to an ACTH signal *in vitro,* and a cortisol lowering effect of BA during an acute netting stress *in vivo*. The effects of both *S. sympetala* extract and BA appear to be directly on the trout head kidney cells, and presumably the steroidogenic interrenal cells of this tissue. That said, it is not possible to rule out upstream effects on the HPI. BA can cross the blood brain barrier (Udeani et al., 1999), and the results discussed in Chapter 4 indicate that it can act in the CNS. Finally, no toxic effects of *S. sympetala* extracts or BA were observed in our *in vitro* assays, and no apparent changes in the health of fish fed the BA supplemented diet were observed.

The data from these experiments both reinforce the existing literature about the toxicity profile of BA, and suggest a novel cortisol-lowering ability. BA is a lupine-derived triterpene commonly found in the plant kingdom and known for its well-described anti-cancer activity (Kessler et al., 2007; Mullauer et al., 2010). Importantly, BA is demonstrated to be selectively pro-apoptotic to cancer cells with no toxic effect on healthy cells (Pisha et al., 1995). Our observation that incubation with BA did not affect cell viability (as assessed by LDH leakage), is consistent with previous reports (Galgon et al., 2005; Rzeski et al., 2006; Zuco et al., 2002) and further demonstrates the low toxicity profile of BA. The anti-cancer activity of BA occurs via the intrinsic, mitochondrial, apoptosis pathway and involves the mitochondrial permeability transition pore (MPTP) and induction of cytochrome c

release (Mullauer et al., 2010; Rzeski et al., 2006). The mitochondria are also the site of steroid biosynthesis and changes in mitochondrial membrane permeability may account for the reduced mean plasma cortisol levels observed after exposure to BA.

These results suggest that BA could be used in standard aquaculture procedures shown to induce cortisol release in fish, such as transport, handling and grading (Barton, 2000; Ruyet et al., 2008; Schreck et al., 2001; Trenzado et al., 2008). Harikrishnan and others (Harikrishnan et al., 2011) have suggested use of plant products, including medicinal herbals, herbal extracts and active compounds for immunostimulation and enhanced immune response to infection in fish farm settings. BA is a relatively cheap, commercially accessible food additive with low apparent toxicity and is bioavailable in trout, potentially BA could be added to trout food to protect against the deleterious effects of elevated cortisol. Further, a natural product additive like BA may be less likely to raise public concerns with respect to human consumption of farmed fish and dispersal in the local environment.

In Chapter 4, I demonstrated that *S. sympetala*, a plant traditionally used to treat the culture-bound illness *susto*, is an effective anxiolytic in laboratory models of anxiety. In a modern mental health context, *susto* is understood to have links to both anxiety and depression (American Psychiatric Association, 2000; World Health Organization, 2007), post-traumatic stress disorder (PTSD) and stress (Smith et al., 2009; Weller et al., 2008). In light of the results presented here, *S. sympetala* and BA may be beneficial in human mood disorders beyond anxiety, where stress is a major factor. Stress and stress-related illness is widespread, and links between elevated plasma cortisol and heart disease (Smith et al., 2005), obesity (Travison et al., 2007) and depression (Carroll et al., 2007; Parker et al., 2003) have been reported. Aberrations in HPA axis function, including hypercortisolism, are

strongly associated with depression (Gallagher et al., 2008). Drugs that inhibit cortisol synthesis, including ketoconazole, aminoglutethimide and metyrapone, have been examined for their therapeutic potential in treating depression (Kling et al., 2009; Starkman et al., 2001) and have shown some promise in bipolar patients with depressive symptoms (ketoconazole) (Brown et al., 2001), and in patients with major depressive disorder (MDD). In a blind, placebo-controlled study with patients suffering from MDD, co-delivery of metyrapone (an inhibitor of cortisol synthesis by blocking the mitochrondrial steroidogenic enzyme 11-β hydroxylase) and a standard serotonergic antidepressant (nefazodone or fluvoxamine), significantly reduced depression (50% reduction in an index of depression, the HAM-D score, at day 35 of treatment) (Jahn et al., 2004). However, despite the potential of cortisol-lowering drugs to treat depression, they are also associated with serious side-effects (Thomson and Craighead, 2008), including ketoconazole's potential for liver toxicity (Kim et al., 2003b) and strong inhibition of cytochrome P450 3A (Cook et al., 2004), with the consequence that in some clinical trials up to 20% of patients drop out due to the side-effects (Wolkowitz et al., 1999). Given the low toxicity of BA, *S. sympetala* and BA may represent cortisol-lowering entities with a more agreeable side-effect profile. This needs to be investigated in further experiments.

The cortisol lowering effect observed in this experiment may be due to changes in mitochondrinal permeability, as discussed above. The cortisol lowering effect may also be due to inhibition of steroidogenesis enzymes at the interrenal cells by BA or other triterpenes (α-amyrin, β-amyrin and ursolic acid) present in the extract. The triterpenes are structurally similar to cholesterol, the endogenous ligand of the steroidogenic enzymes, as presented in Figure 5.4. The cortisol-lowering effect may also be due to inhibition of 11-β

hydroxysteroid dehydrogenase (11- β HSD), the enzyme that converts metabolically inactive cortisone to active cortisol (11-dehydrocorticosterone to corticosterone in rodents) at the glucocorticoid receptor level. Inhibition of 11- β HSD reduces plasma cortisol (corticosterone) and has been investigated for the pharmacological treatment of hypercortisolism (Gathercole and Stewart, 2010). 11- β HSD is expressed in the head kidney cells of rainbow trout, although its biological function in this tissue is unknown (Kusakabe et al., 2003). Carbenoxolone, a synthetic derivative of glycyrrhetinic acid (GA), derived by hydrolysis of glycyrrhizic acid, a molecule originally extracted from the herb liquorice (*Glycyrrhiza glabra* L.). (Whorwood et al., 1993) is a non-specific 11- β HSD inhibitor, with affinity for both enzyme isoforms, 11- β HSD1 and 11- β HSD2 (Gathercole and Stewart, 2010). Like BA and the other molecules present in the *S. sympetala* extract (see Chapter 2), carbenoxolone is a pentacyclic triterpenoid. Perhaps BA and the other triterpenes in the extract also have an inhibitory effect on 11- β HSD in the trout head kidney cells, reducing the cortisol response.

With its cortisol-reducing activity and demonstrated low toxicity (Pisha et al., 1995), *S. sympetala* and BA may represent promising leads for therapeutics in the area of stress and stress-associated diseases in people. Further experiments to examine the site of action, whether at the interrenal cells (adenal cortex), or other sites in the HPI (HPA), and targets of action (mitochondrial membrane permeability, inhibition of steroidogenesis, or inhibition of 11- β HSD) will serve to clarify the precise site of action of BA. Finally, in our experiments, the cells and animals were pre-treated with BA and then exposed to an acute stress. Further work will examine the extent to which an acute dose of BA can reduce the cortisol response following a stressful event.

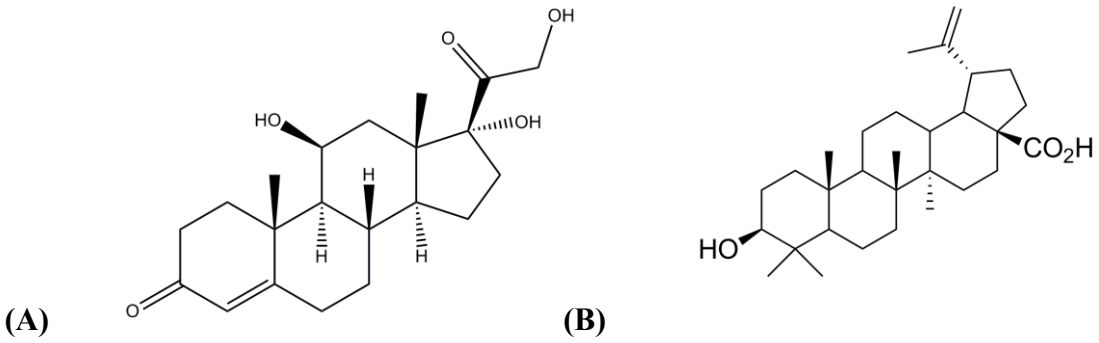


Figure 5.4. Structural comparison of **(A)** cholesterol; and **(B)** betulinic acid.

SECTION III

Ethnopharmacology & Natural Product Discovery

CHAPTER 6: ANXIOLYTIC ACTIVITY OF AN ETHNOBOTANICALLY IDENTIFIED PEPPER, *PIPER AMALAGO*, AND IDENTIFICATION OF A FUROFURAN LIGNAN AS ITS BIOACTIVE PRINCIPLE

6.0 Preface

In the *S. sympetala* project, the bioactive principle was identified well before I joined the project. One of the purposes of the work described in this chapter was for me to conduct a bioassay-guided fractionation from start to finish.

This experimental chapter examines the third hypothesis of the PhD project, that *P. amalago*, an ethnobotanically identified plant used to treat *susto*, has anxiety-reducing properties in an animal model of anxiety. I tested the predictions that: (1) rats treated with *P. amalago* extract will display lower anxiety-like behaviour in standardized tests of anxiety than rats fed the vehicle; (2) the bioactive principle of *P. amalago* will be a molecule with high affinity for the GABAa-BZD receptor; and (3) the GABAa-BZD competitive binding assay can be used to distinguish highly bioactive fractions and consequently the bioactive principle of *P. amalago.*

6.1 Statement of Author Contributions

I conducted the behavioural experiments in collaboration with Chris Cayer, I prepared the large-scale crude extract and I screened all of the fractions for bioactivity. Fractionation of the crude extract and chemical characterization was done by Dr. Muhammad Asim, Fida Ahmed assisted with extract preparation and early characterization of the extract, Brendan Walshe-Roussell collected the plant in Belize; Victor Cal and Francisco Caal of the

Belize Indigenous Training Institute lent their expertise about plant identification and traditional use, Dr. Zul Merali, Dr. Vance. L. Trudeau and Dr. John. T. Arnason provided supervision and funding for the work.

Publication:

This manuscript will be submitted to: *Journal of Natural Products* or *Journal of Ethnopharmacology*

6.2 Abstract

Examination of the traditionally used pepper plant, *Piper amalago* L., demonstrated anxiolytic activity of ethanol extracts in animal behaviour assays *in vivo* and interaction with the neurological GABA system *in vitro.* Treatment of rats with 75 mg/kg of the ethanolic extract significantly increased percent time spent in the open arms of the elevated plus maze (EPM), evidence of reduced anxiety. In an *in vitro* GABAa competitive binding assay, a 66.5 µg/mL concentration of *P. amalago* ethanol extract displaced 50% of the GABAa–BZD receptor ligand flunitrazepam. Bioassay-guided fractionation identified a furofuran lignan, a molecule with structural similarity to yangambin, with high affinity for the GABAa-BZD receptor as the principle bioactive. The results suggest that the ethnobotanical use of this plant to treat epilepsy and the culture bound syndrome, *susto,* has a pharmacological basis.

6.3 Introduction

In an ethnobotanical survey of the Q'ecqhi' Maya of Belize we found high use of plants for neurological disorders including headaches, *susto* (a culture bound illness associated with fright) and epilepsy (Bourbonnais-Spear et al., 2005). Screening of plants used to treat *susto* in animal behavioural assays of anxiety demonstrated the link between *susto* and anxiety. Specifically, *susto* plants significantly reduced anxiety-like behaviour in rodents, suggesting that the neuropathology associated with *susto* is similar to that associated with anxiety (Bourbonnais-Spear et al., 2007).

The neuropathology of anxiety and epilepsy involve the neurotransmitter γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system (CNS). Reduction in CNS inhibition is associated with excessive activity that is experienced as anxiety or in severe cases, epileptic seizures (Treiman, 2001). A therapeutic approach to this neuronal excessive activity is to increase GABA levels in the brain. This can be achieved by inhibiting activity of the catabolic enzyme in the GABA system, GABA-transaminase (GABA-T), or by increasing the affinity of GABA for its receptor, GABAa, facilitated by the binding of GABA agonists, such as benzodiazepines (BZD) to the GABAa-BZD receptor {{168 Mula,M. 2007; 168 Mula,M. 2007; 145 Zwanzger,P. 2005; 119 Yogeeswari, P. 2005; 142 Kinrys, G. 2003}}.

We previously reported on screening plants identified by the Q'ecqh'i healers for their effect on conditions associated with the GABA-system (Awad et al., 2009). The model developed showed a positive correlation between healer consensus, for plant use to treat *susto*, and extract interaction with the GABAa receptor. The results suggest that the selection of a plant to treat *susto* may be linked to the plant's pharmacological interaction with the

GABAa receptor (Awad et al., 2009). One of the most active plants in the GABAa competitive binding assay was *Piper amalago* L. (Piperaceae).

The Piperaceae family is well known for central nervous system (CNS) activity. *Piper* species are used throughout the world to treat neurological disorders, locations including in China (Pei, 1983), Nigeria (Abila et al., 1993), Polynesia (Cairney et al., 2002) and Brazil (Cícero Bezerra Felipe et al., 2007). *Piper* species contain unique nitrogen compounds called piperamides with a broad range of bioactivity including GABAa receptor agonist activity (Zaugg et al., 2010), anti-depressant activity (Lee et al., 2005), CNS depression (Li et al., 2007), insecticidal (De Paula et al., 2000; Scott et al., 2003; Scott et al., 2004), antioxidant (Vijayakumar et al., 2004), and modulation of drug metabolism (Jensen et al., 2006; Koul et al., 2000). Further, *P. methysticum* (kava) is one of the most studied herbal anxiolytics and a Cochrane Systematic Review has found that in clinical trials, kava extract is an effective herbal to treat anxiety (Pittler and Ernst, 2003).

Given the ethnobotanical data, the correlation between GABAergic activity and healer consensus, and the well known high bioactivity of *Piper* species, *P. amalago* is a good candidate for further examination as a tropical anxiolytic. The work described here was done to examine the effect of an ethanolic extract of *P. amalago* in a behavioural assay of anxiety and to identify the secondary metabolite(s) responsible for the activity. The hypothesis tested was that *P. amalago* is an anxiolytic plant that acts as a GABAa-BZD receptor agonist. Bioassay-guided fractionation of the ethanolic extract of *P. amalago* was conducted with an *in vitro* GABAa-receptor binding assay to screen active fractions and identify active molecules. In the screening portion of the experiment, the hypothesis tested was that the principle bioactive molecule of *P. amalago* is a piperamide because of the prevalence of piperamides in *Piper* species and because piperamides interact with the CNS.

6.4 Methods and Materials

Plant material

Fresh samples of wild *Piper amalago* (Piperaceae) were collected under permit from the Itzamma ethnobotanical garden in Toledo district, Belize. Samples were stored in 90% ethanol for shipping. Voucher specimens were identified (by L.P. and P.S.) and deposited in the JVR Herbairun, Universidad Nacional Costa Rica, and the University of Ottawa Herbarium (OH No. 19915). The plants were collected under a permit issued by the Ministry of Natural Resources of the Government of Belize (Ref # CD/6-/9/03). The plants and the traditions associated with them are the intellectual property of the Maya and cannot be developed further without their express permission, as detailed in the Nagoya Protocol on Access and Benefit Sharing (CBD Secretariat, 2011).

Plant Extraction and Fractionation

The plant material was removed from the storage ethanol, dried at room temperature and ground in a Wiley mill (2 mm mesh). The leaves (750 g) were incubated 1:10 (weight: volume) with 80% EtOH and incubated overnight at room temperature with shaking. Samples were filtered, and the filter cake re-extracted twice with decreased 90% EtOH (1:5 and 1:2.5 weight: volume, respectively). The supernatants were combined, solvent removed via rotary evaporation at 40ºC and the extracts lyophilized. The crude extract (CE) was stored in opaque glass vials at 4ºC. A second, smaller scale extraction (100 g leaves, 2 mm mesh) was carried out with ethyl acetate (EtOAc) under the same conditions, 1:10 (weight :

volume), incubated at RT with shaking, solvent removed by rotary evaporation and lyophilisation and extract store in opaque glass vials at 4ºC.

A portion of the crude extract (42.0 g) was separated by open column chromatography (2 kg silica gel) with a step gradient of hexanes and EtOAc (1 – 100% EtOAc), 200 ml aliquots, increased by 10% EtOAc. The flow rate was approximately 20 mL/min. The effluents were combined to 19 fractions (Fr-1 to Fr-19) based on thin layer chromatography (TLC) patterns (detection at 254 nm and in daylight after staining with ceric ammonium nitrate sulfuric acid reagent).

The fractions were then submitted to bioassay. The most active fraction, 6, was sub-fractionated by column chromatography (silica gel) using hexanes and ethyl acetate (5:95–100:0), and separated into 18 sub-fractions (6-1 to 6-18). These fractions were submitted to bioassay. Fractions 6-10, 6-11, 6-15, and 6-16 were most active; 6-11 proved to be a pure compound, whereas 6-10, 6-15 and 6-16 were subjected to further purification by preparative HPLC (Waters Prep LC 4000, equipped with PDA) ODS column (21.2 mm x 250 mm, 10 um), using a gradient of acetonitrile/water (ACN/H_2O) (25:75 to 100:0 in 40 min, flow rate 20 mL/min) to obtain pure compounds (Table 6.2 and 6.3). Structural identity of pure compounds was obtained via NMR.

Animals

Experiments were conducted with male Sprague-Dawley rats (225-250 g body mass; Charles River Laboratories Inc., St. Constant, Quebec). Rats (n = 45) were housed individually and maintained under standard animal room conditions (clear plexiglass cages,

24 x 30 x 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 and met the guidelines set out by the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching. Rats were handled for 7 days prior to the experiment to acclimatize to the experimenter and orally administered a 50% solution of Eagle Brand sweetened condensed milk each day to familiarize them with the feeding procedure. All attempts were made to minimize the number of animals used in the study, while maintaining the integrity of the experiments and results.

Drug Administration

The crude plant extract was suspended in 50% sweetened, condensed milk. Vehicle- and plant extract- treated rats were orally administered treatments daily for three days (between 10:00 - 2:00 pm for two days prior to testing, 1 h prior to testing). Each dosage day, rats were orally administered either 50% sweetened, condensed milk vehicle (2 mL /kg), plant extract (three doses: 8, 25 and 75 mg/kg) or diazepam *i.p.* (5 mg/kg, dissolved in 40% propylene glycol, 10% ethanol, 50% distilled water).

Elevated Plus Maze

The EPM test assesses anxiety-like behaviour in laboratory rodents (File, 1992; Pellow et al., 1985) and is based on the conflict between the animal's instinct to explore its environment and its fear of exposed areas and heights. Time spent in the open arms is an inverse measure of anxiety, *i.e.* animals that spend more time in the open arms are understood to have less anxiety than those that spend more time in the closed arms. The EPM consists of two open arms (50 x 10 cm), two perpendicular arms enclosed by 40 cm high walls, and is placed 50 cm above the ground. A black curtain surrounded the chamber to limit the influence of spatial cues and other extraneous stimuli. A video camera was mounted above the arena to permit remote monitoring and recording. Rats (n = 8-10/group) were individually placed in the testing room for 1 h acclimatization. Each rat was then placed onto the open central platform of the EPM (facing a closed arm). The rat's behaviour was monitored for 5 min and scored as follows: (1) frequency of entries onto the open arms, (2) percentage of time spent on the open arms (time open/300×100), (3) frequency of entries in the closed arms, and (4) risk assessment behaviour (unprotected head dips; head protruding over the edge of an open arm and down toward the floor). Between tests, the EPM was cleaned with 70% isopropanol. The percent of time in the open arms, frequency of open arm entries, and unprotected head dips are all validated measures of anxiety-like behaviour in the EPM (Carobrez and Bertoglio, 2005; Griebel et al., 1997; Pellow et al., 1985). Increases in these measures are indicative of reduced anxiety, whereas decreases suggest increased anxiety (File, 1992). In contrast, the frequency of closed arm entries is an index of general activity and provides insight into the sedation properties of the material tested (Cruz et al., 1994).

GABAa-BZD-S Receptor Binding Assay

The GABAa-BZDS binding assay was adapted from previously described protocols (Benke, 1999; Snodgrass, 1978) and conducted in a 96-well microplate as described previously ((Awad et al., 2009); Chapter 4). Plant crude extracts were dissolved in 99% ethanol and serial diluted to generate a concentration curve. Extract fractions were dissolved in 99% ethanol and assayed at 200 µg/mL. The final ethanol concentration in each well was 0.99% v/v.

Data analysis

GABAaR- BZD Receptor Binding Assay

Total binding and nonspecific binding were computed by running the assay in the absence of competitive ligand (total) and with the addition of 20 nM flumazenil (Ro 15-1788) (non-specific). All plant extracts were compared to a solvent control (0.99% ethanol). Percent specific binding of ^{3}H-flunitrazepam was computed with the following equation: (Cpm in well – Cpm nonspecific binding)/Cpm solvent well x 100 = % Specific Binding.

Elevated Plus Maze

The data were compared with one and multi-way analyses of variance (ANOVA) with Dunnett test comparison of the treatment means with the reference mean (vehicle) (Zar, 1999). Kolmogorov-Schmirnoff and Levene's tests were used to verify the normality of distribution and the homogeneity of residual variance, respectively. All of the Fisher

statistics (F), degrees of freedom (df), and p-value estimates were calculated with S-PLUS software version 7.0 (Insightful Corp., Seattle, USA). Data are reported as means ± standard error of the mean (S.E.M.)

6.5 Results

Elevated Plus Maze

In the EPM test, *P. amalago* crude extract administered at a dose of 75 mg/kg had a significant effect on test parameters as compared to vehicle control; (i.e. total time spent in open arms: $F(4,40) = 4.45$, $p < 0.01$; number of unprotected head dips: $F(4, 40) = 3.16$, $p < 0.05$; percent time spent in open arms: $F(4,40) = 4.45$, $p < 0.05$; and percent time spent in the closed arms: $F(4, 40) = 5.82$, $p < 0.01$) (Table 6.1). Animals treated with the highest dose of *P. amalago* extract (75 mg/kg) spent 36% more time in the open arms and had 20% and more unprotected head dips than vehicle controls (Figure 6.1). The 25 mg/kg dose of extract significantly reduced percent time spent in closed arms (% TCA) as compared to vehicle control ($F(4, 10) = 5.82$, $p = 0.01$). A linear regression of dose versus percent time spent in open arms indicates a significant effect of dose (linear regression equation: %TOA = 0.27 (extract dose) + 20.53, $R^2 = 0.26$, $p < 0.01$), despite the fact that the lower *P. amalago* doses did not have a significant effect in most of the parameters of the EPM.

Bioassay Guided Fractionation

Both the crude ethanolic and ethyl acetate extracts of *P. amalago* exhibited concentration-dependent displacement of ^{3}H-flunitrazepam from the GABA BZD receptor *in*

vitro (Figure 6.2). Both extracts displaced ^{3}H-flunitrazepam (^{3}H-Flu), with 50% displacement (EC_{50}) observed at 41.7µg/mL (EtOH extract) and 33.9 µg/mL (EtOAc extract). Because the EC_{50} values did not differ greatly, the subsequent bulk extraction of *P. amalago* was conducted with EtOH, a solvent that extracts a wide range of secondary metabolites. Following fractionation of the crude EtOH extract, fraction 6 was identified as the most active (Figure 6.3A) with > 95% of ^{3}H-Flu displaced from the GABAa-BZD receptor when incubated with fraction 6. Fraction 6 was then further fractionated to yield fractions 6-1 to 6-18. Each of these was assayed in the GABAa-BZD receptor competitive binding assay, with the fractions in the medium to non-polar range (6-7 to 6-18) showing highest displacement (Figure 6.3B). Fraction 6-11, with 1.29% binding, yielded a pure compound, a furofuran lignan (Table 6.2, **6**). Three of the high activity fractions, 6-10, 6-15 and 6-16 were further fractionated by preparative HPLC, each peak was collected, the structure determined with NMR, and the pure compound assayed. Several furofuran lignans (Table 6.2, **4**, **5**, **8**, **9**) where the structures have been previously elucidated (Seo et al., 2008) and piperamides (Table 6.2, **2**, **3**, **7**, **10**, **11** and **12**) were purified from the most active fractions (Table 6.3), including three novel piperamides which have not been described previously (Compounds **10**, **11** and **12**, NMR data presented in Table 6.4).

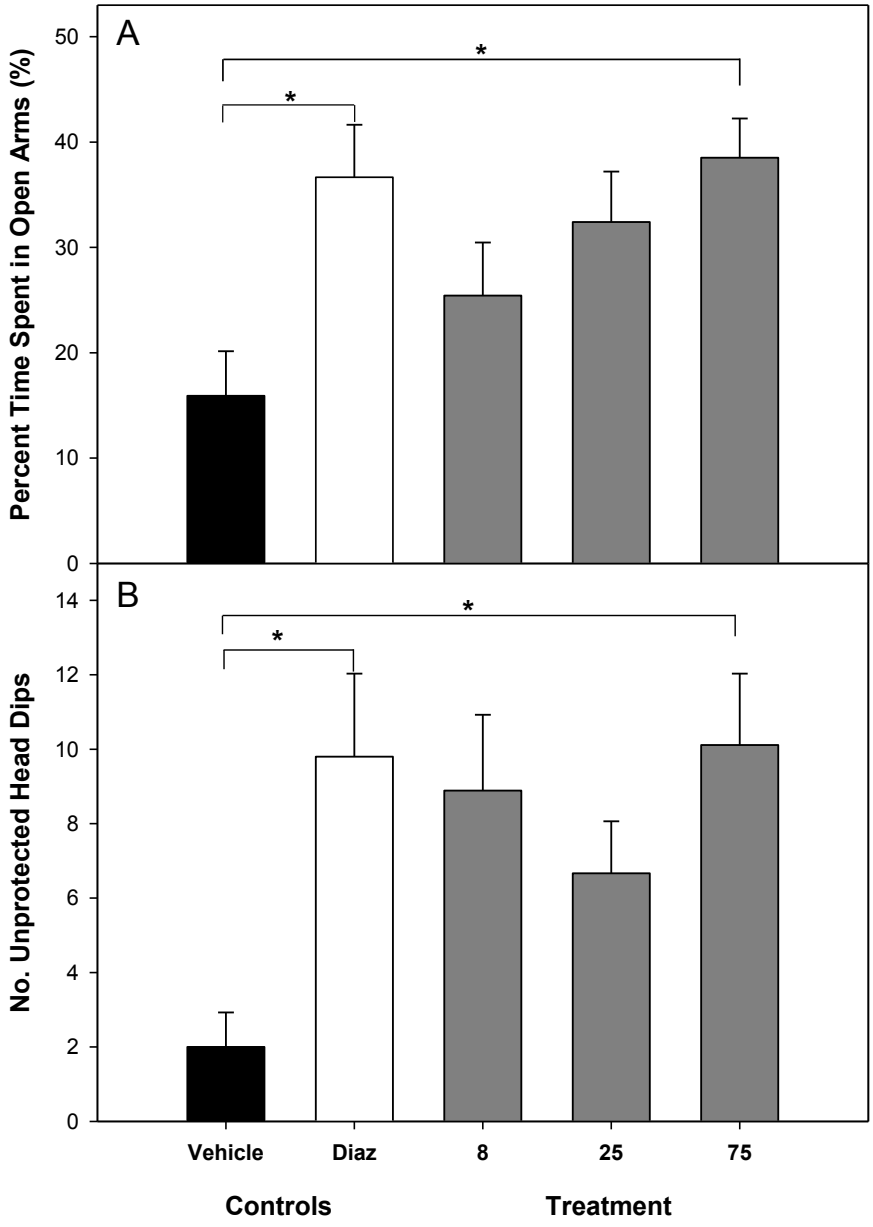


Figure 6.1. Effect of crude ethanolic extract of *P. amalago* on anxiety-like behaviour of rats in the elevated plus maze. (A) Percent total time spent in the open arms of the elevated plus maze; and **(B)** number of unprotected head dips for animals treated with vehicle, diazepam and P. amalago extract (8, 25 and 75 mg/kg), after a 1 h post-drug interval. All values represent the group mean ± S.E.M. of n = 8 – 10. Sstars indicate significant difference from vehicle control, $p < 0.05$, one-way ANOVA with Dunnett's post-hoc test. **Vehicle:** vehicle control (50% sweetened, condensed milk); **Diaz:** diazepam, 5 mg/kg; **8, 25 and 75:** 8, 25 and 75 mg/kg of *P. amalago* extract suspended in sweetened, condensed milk, administered orally.

Table 6.1. Comparison between vehicle control (50% sweetened condensed milk), diazepam (5 mg/kg) and *Piper amalago* extracts (8, 25 and 75 mg/kg, oral dose) in selected parameters of the elevated plus maze test, after a 1 h post-drug interval (n = 8 – 10).

Treatment	T.O.A.	#O.A.E.	#C.A.E.	% T.C.A.
Vehicle control n = 8	41.49 ± 12.56	3.38 ± 1.19	10.25 ± 1.44	52.58 ± 2.29
Diazepam 5 mg kg^{-1}, n = 10	109.98 ± 14.94*	7.20 ± 1.51	13.10 ± 0.72	29.78 ± 4.20$^{\#}$
***P. amalago* extract 8 g kg^{-1}, n = 9**	76.27 ± 15.11	5.89 ± 0.90	11.11 ± 0.56	43.02 ± 3.42
***P. amalago* extract 25 g kg^{-1}, n = 9**	97.21 ± 14.39	5.44 ± 0.94	10.56 ± 0.88	35.64 ± 3.40$^{\#}$
***P. amalago* extract 75 g kg^{-1}, n = 9**	115.52 ± 11.18*	6.78 ± 0.60	10.11 ± 0.51	37.00 ± 3.57$^{\#}$

T.O.A.: time spent in the open arms (min) **#O.A.E.:** number of open arm entries **#C.A.E.:** number of closed arm entries **% T.C.A:** percent time in closed arms

* p-value < 0.05 vs. vehicle control, one-way ANOVA, ** p-value < 0.01 vs. vehicle control, one-way ANOVA, # p-value < 0.001 vs. vehicle control, one-way ANOVA with Dunnett's post-hoc test.

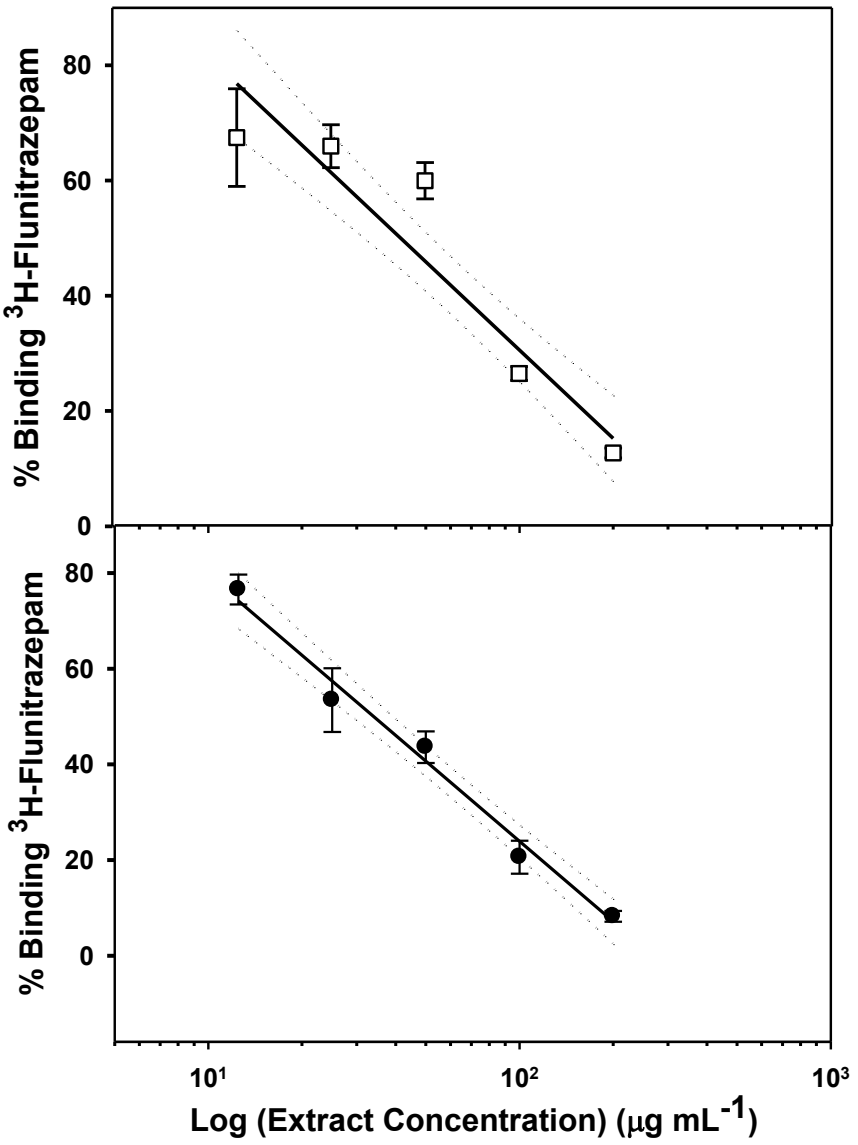


Figure 6.2. GABAa – BZD receptor competitive binding assay, displacement of ^{3}H-flunitrazepam versus log concentration of extract for **(A) ethanolic extract of *P. amalago***, equation of linear regression: % binding = - 50.93 (log (extract concentration)) + 132.49, R^2 = 0.85, $p < 0.001$; and **(B) ethyl acetate extract of *P. amalago***, equation of linear regression: % binding = - 55.52 (log (extract concentration)) + 134.96, R^2 =0.95, $p < 0.001$. All values represent the group mean (n = 3) ± S.E.M. IC_{50} values were calculated from the linear regression equations.

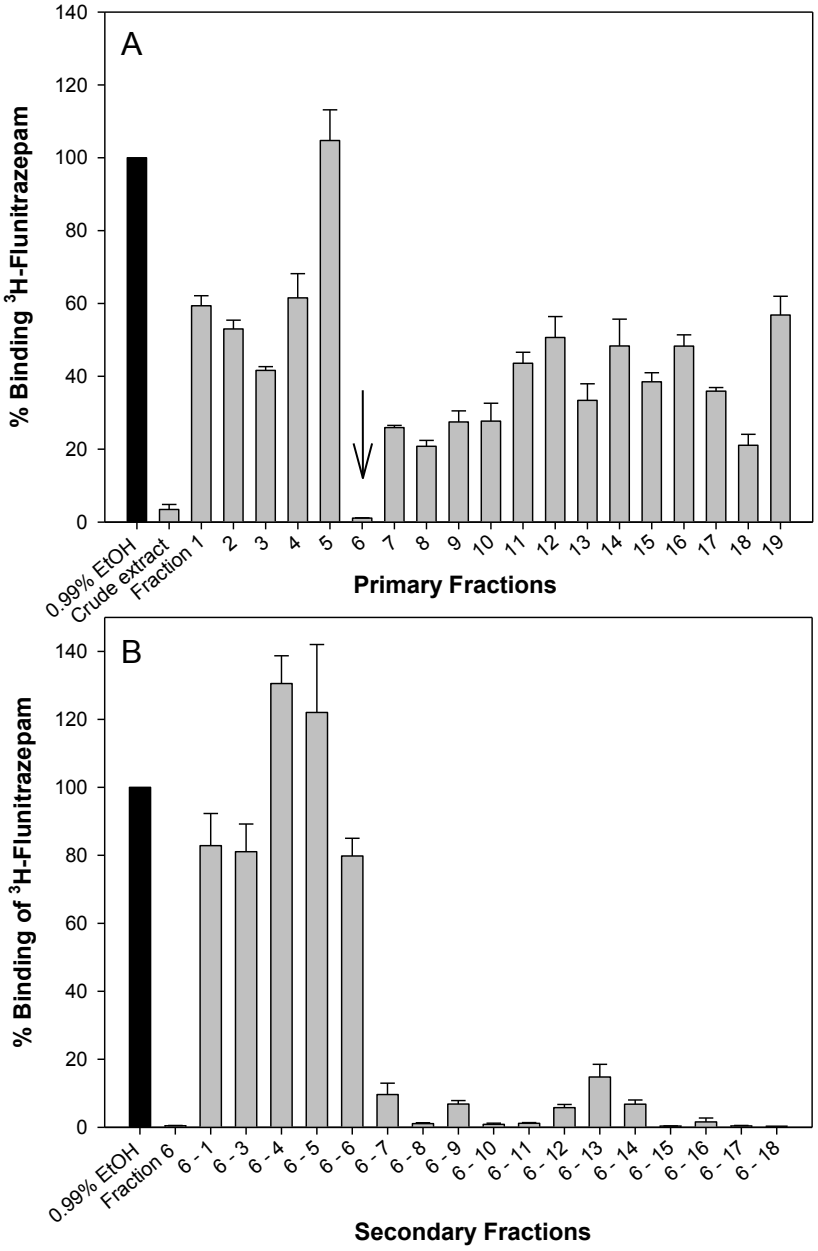


Figure 6.3. (A) Percent binding of ^{3}H-flunitrazepam in GABAa-BZD receptor competitive binding assay when incubated with the primary *P. amalago* fractions. The most active fraction in the bioassay, Fraction 6, is highlighted with an arrow. **(B)** Fraction 6 was identified as one of the most active (0.65% binding, 99.35% displacement of ^{3}H-Flu) and further fractionated to yield fractions 6 – 1 to 6 – 18. Each fraction was tested at a final concentration of 200 µg/mL, all values represent the group mean ± S.E.M. of n = 3.

Table 6.2. Activity of furofuran lignans purified from *P. amalago* fractions in the GABAa-BZD receptor competitive binding assay. Each molecule assayed at 200 µg/mL.

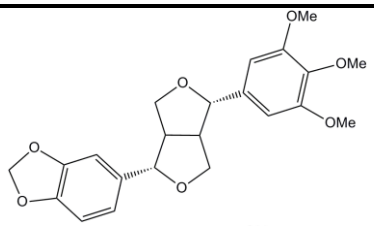
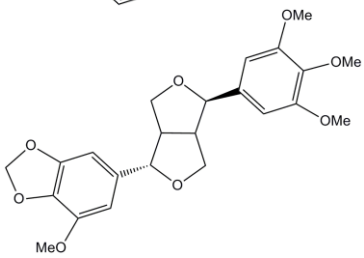
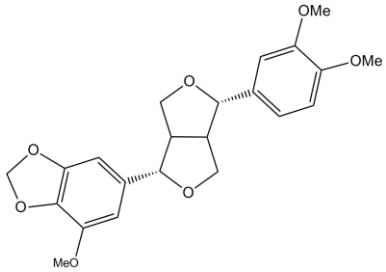
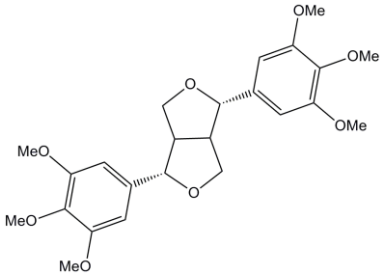
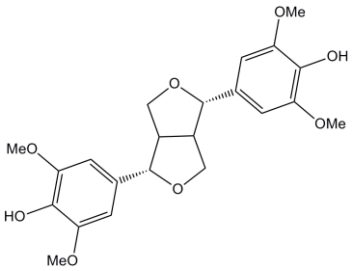
Purified from Fraction	Molecule	Structure	% Flumazenil Binding
6-10	**4**		>100
6-10	**5**		>100
6-11	**6**		1.17 ± 0.18
6-15	**8**		90.83 ± 10.14
6-16	**9**		80.25 ± 4.97

Table 6.3. Activity of other molecules purified from *P. amalago* fractions in the GABAa-BZD competitive binding assay. Each molecule assayed at 200 µg/mL.

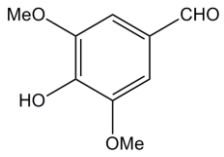
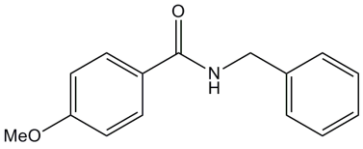
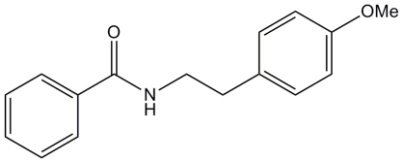
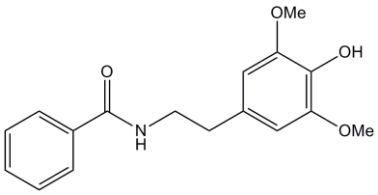
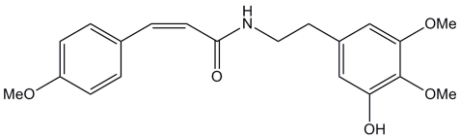
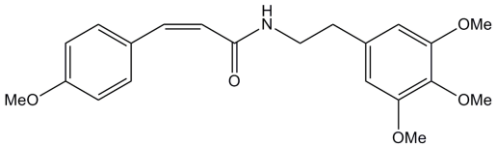
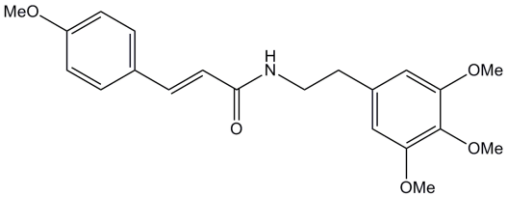
Purified from Fraction	Molecule	Structure	% Binding ^{3}H-Flu
6-10	**1**		69.0 ± 20.3
6-10	**2**		76.4 ± 2.0
6-10	**3**		66.0 ± 2.5
6-10	Mixture of furofuran lignans		50.8 ± 14.3
6-15	**7**		84.9 ± 10.4
6-16	**10**		64.9 ± 3.5
6-16	**11**		76.0 ± 3.6
6-16	**12**		Not tested
6-16	Mixture of piperamides		51.2 ± 3.5

Table 6.4. NMR spectroscopic data of novel piperamide compounds **10**, **11**, and **12** from *P. amalago* in $CDCl_3$.

	10		**11**		**12**	
C/H no.	**δC**	**δH (*J* in Hz)**	**δC**	**δH (*J* in Hz)**	**δC**	**δH (*J* in Hz)**
1	127.4	-	127.4	-	127.4	-
2	130.8	7.42 d (8.8)	130.8	7.43 d (8.8)	129.3	7.41 d (8.8)
3	113.7	6.82 d (8.8)	113.7	6.81 d (8.8)	114.2	6.86 d (8.8)
4	159.9	-	159.9	-	160.9	-
5	113.7	6.82 d (8.8)	113.7	6.81 d (8.8)	114.2	6.86 d (8.8)
6	130.8	7.42 d (8.8)	130.8	7.43 d (8.8)	129.3	7.41 d (8.8)
7	134.9	6.63 d (12.4)	134.4	6.63 d (12.8)	140.8	7.56 d (15.6)
8	122.5	5.80 d (12.4)	122.3	5.81 d (12.8)	118.0	6.18 d (15.6)
9	167.2	-	167.3	-	166.2	-
1′	136.4	-	136.4	-	134.6	-
2′	104.5	6.20 d (2.0)	105.5	6.30 s	105.6	6.41 s
3′	152.3	-	153.3	-	153.3	-
4′	136.4	-	136.4	-	134.6	-
5′	149.2	-	153.3	-	153.3	-
6′	108.0	6.29 d (2.0)	105.4	6.30 s	105.6	6.41 s
7′	35.4	2.64 t (6.8)	35.7	2.70 t (7.2)	36.0	2.81 t (6.8)
8′	40.3	3.49 q (6.8)	40.5	3.51 q (7.2)	40.7	3.62 q (6.8)
4-OMe	55.2	3.77 s	55.2	3.77 s	55.3	3.77 s
3′-OMe	55.8	3.79 s	56.0	3.77 s	56.1	3.77 s
4′-OMe	60.9	3.84 s	60.8	3.79 s	60.8	3.79 s
5′-OMe	-	-	56.0	3.79 s	56.1	3.79 s

6.6 Discussion

This study examined the effect of a *P. amalgo* extract in a behavioural model of anxiety and isolated and identified the principle bioactive molecule(s). The observed effects in the EPM show that *P.amalago* lowers anxiety-like behaviour in rodents. The bioassay guided fractionation identified the primary bioactive principle as a furofuran lignan (**6**), other furofuran lignans (**8** and **9**) and piperamides (**2**, **3**, **10** and **11**) with modest affinity for the GABAa-BZD receptor. The results taken together demonstrate that the ethnobotanically identified *P. amalago* is an anxiolytic plant. Based on the bioassay guided fractionation, the principle bioactive is assumed to be compound **6**, is a furofuran lignan with binding affinity for the GABAa-BZD receptor.

P. amalago has been ethnobotanically identified as a plant to treat *susto.* Healers in Belize prepare the leaves as a decoction (Bourbonnais-Spear et al., 2007) and use a bath prepared with leaves to treat the spiritual disease *mal vientos* (Arvigo and Balick, 1998), while Nicararguan midwives use a decoction of leaves and root to reduce nervousness during childbirth (Coe, 2008). A recent report has investigated the activity of *P. amalago* in the EPM, where high doses (250 and 420 mg/kg *i.p*) had an anxiogenic (anxiety-increasing) effect; extract dosed animals spent significantly more time in the closed arms of the assay compared to diazepam controls (Lopes et al.,2010). The authors suggest a biphasic dose-response for *P. amalago* in rats, with low dose being anxiolytic, and high ones, anxiogenic. Our finding, that the low oral dose of 75 mg/kg of *P. amalago* has a significant anxiolytic effect in the EPM, could support this hypothesis. An investigation of the toxicity of *P. amalago* reported no significant toxic or mutagenic effects and reported a LD_{50} of 2.55 kg/kg, indicating that *P. amalago* is not a highly toxic plant (Lopes et al.,2010).

The activity in the EPM of *P. amalago* is similar to that of another well characterized anxiolytic in the Piperaceae, *P. mythesticum*, kava, well known for its traditional ethnobotanical use in Polynesia for its calming effect (Stevinson et al., 2002). Kava has been shown to interact with a variety of CNS targets. Its activity may involve some weak GABAa binding (Boonen and Häberlein, 1998) as well as β-adrenergic downregulation, monoamine oxidase- B (MAO-B) inhibition and norepinephrine re-uptake (Jussofie et al., 1994; Magura et al., 1997; Uebelhack et al., 1998). Kava is considered one of the most effective botanical anxiolytics and its activity is supported not only by animal trials similar to those shown here, but also by several human clinical trials where treatment with kava extract significantly decreased anxiety (Pittler and Ernst, 2003). A key difference is that the active principles in kava are kavalactones, which are biosynthetically distinct from the furofuran lignans reported here. Kava is associated with reported cases of idiosyncratic hepatotoxicity, which may be associated with the kavalactones (Center for Disease Control and Prevention, 2003; Humberston et al., 2003). The furofuran lignans may be a safer alternative, because another furofuran with structural similarity to this one, yangambin, has low toxicity (Marques et al., 2003; Monte Neto et al., 2008) but the toxicity of the molecules extracted in this experiment needs to be evaluated.

The principle bioactive compound identified (**6**) is a furofuran lignan that displaces > 95% of ^{3}H-flunitrazepam in the GABAa-BZDR competitive binding assay. This molecule bears structural similarlity to yangambin (Figure 6.4), a furofuran lignan purified from *Magnolia fargesii* (Finet & Gagnep.) W. C. Cheng. Yangambin (25 mg/kg, *i.p.*) reduces spontaneous locomotor activity and isolation-induced aggression in mice (Macre and Towers, 1984) with an overall CNS depressant effect. At higher doses, 50 mg/kg yangambin

i.p. decreases locomotion, decreases swimming in the forced swim test and increases sedation (de Sousa, et al., 2005). In the experiment described here, a moderate oral dose of *P. amalago* extract did not significantly affect locomotion; at the dose tested and as compared to controls, no differences were observed in the number of closed arm entries (Table 6.1), suggesting that compound **6** does not possess the same sedative effects as yangambin. There are two potential explanations for this: one, it could be a dose effect, *i.e.* the 75 mg/kg dose of *P. amalago* extract may have been too low to have a sedative effect; or, two, it may be that **6** has anxiolytic, but not sedative, activity. The potential sedative or CNS depressant effects of **6** merit further examination. The other furofuran ligans isolated (**4**, **5**, **8** and **9**) bear structural similarity to **6**, but are less active. This may reflect molecular size or stereochemistry constraints required for molecules that can interact with the GABAa-BZD receptor.

One of the predictions in the bioassay guided fractionation was that the bioactive principle of *P. amalago* was a piperamide. While the piperamides isolated exhibit modest affinity for the GABAa-BZD receptor, they do not appear to be central to the anxiolysis of *P. amalago*. However, to verify the possible anxiolytic effect of compound **6**, additional animal trials with the pure comound are required. It is possible that the anxiolytic effect observed *in vivo* is due to interaction with the GABAa-BZD receptor or interaction with other aspects of the GABA system, such as the GABA catabolism enzyme GABA-transaminase (GABA-T), as we have previously reported strong GABA-T inhibition by piperamides (Awad, 2008).

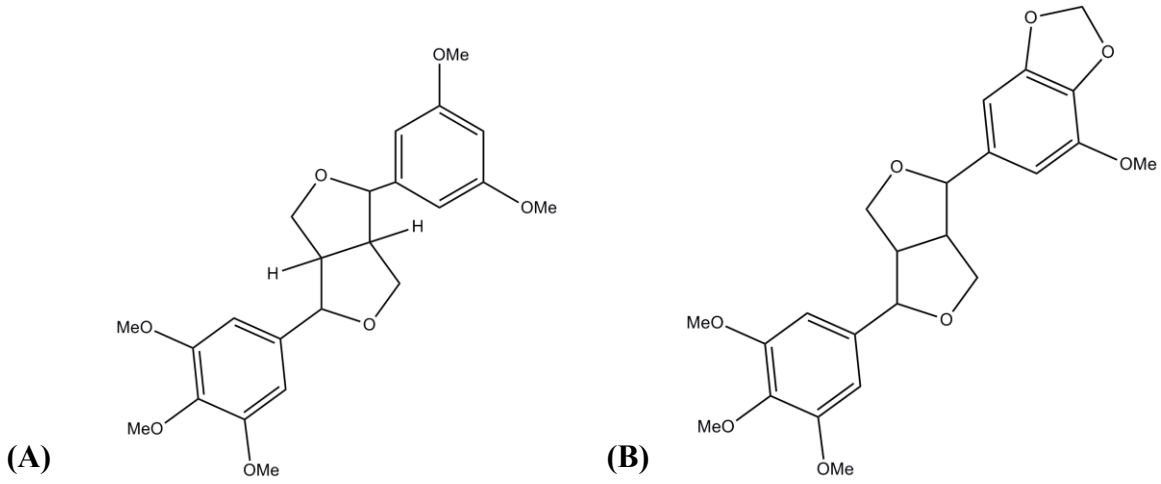


Figure 6.4. Structural comparison of **(A)** yangambin, a CNS active furofuran lignan purified from *Magnolia fargesii,* and **(B)** compound **6**, the most bioactive molecule isolated from *P. amalago*, also a furofuran lignan.

In the experiments described in this chapter, I used modern pharmacological approaches to assess a plant used by the Q'ech'i Maya to treat *susto*. The data demonstrate that *P. amalago* is an anxiolytic plant and the principle bioactive molecule responsible for the activity is a furofuran lignan, compound **6,** that is a GABAa-BZD receptor agonist. The results suggest that the traditional use of the plant has, in part, a pharmacological basis and provides evidence that Mayan traditional medicine has potential value in a complementary medicine system. Future work will include toxicity assays of **6**, as well as behavioural assays with the pure compound, either isolated from the plant or produced synthetically.

CHAPTER 7 - GENERAL DISCUSSION

7.1 Overview of results and novel contributions

The two plants examined in this PhD project were examined based on the rationale that unexamined tropical plants represent a good source of bioactive natural products, which are leads for natural health products, novel drugs, or drugs with novel action. Furthermore, ethnobotany combined with modern pharmacological assays was proposed as an effective way to identify novel tropical plants that merit further examination (*S. sympetala* and *P. amalago*) and a means to understand what pharmacology is (or is not) associated with traditional medicines.

Early work in the *S. sympetala* project determined its anxiolytic effect and identified the bioactive principle responsible, BA. The first study in this thesis addressed a common source of criticism and frustration in the discussion of NHPs: the lack of standardization of phytochemical content of NHPs used in experiments to investigate their mode of action or efficacy (Ernst, 2006; Garrard et al., 2003; Turner, 2009). This weakness makes it difficult to compare observed effects of NHPs, especially across different extract preparations or experimental paradigms. The applied objective set out in Chapter 2 was to develop a HPLC-APCI-MS method to analyse *S. sympetala* material. The development of this method allowed treatments in the subsequent animal experiments to be matched for BA dose (Chapter 4).

The challenge of standardization is present in NHP plant material preparation, as well. The objective of Chapter 3 was to develop an extraction procedure for *S. sympetala* that would yield a BA-enriched extract. Four standard herbal extraction procedures and one

novel approach, SCE extraction, were compared for yield and BA content. The conventional and SCE extractions generated extracts with highest BA concentration. Comparison of these two extracts in the EPM demonstrated that they both reduced anxiety-like behaviour in rats. Chapter 3 represents the first time that extraction methods for *S. sympetala* have been systematically compared and the first time that a SCE extraction method has been developed for *S. sympetala*. The results suggest that SCE is a technique that could be applied to NHPs, and specifically anxiolytic herbals, for animal or human consumption.

Chapters 4 and 5 provide insight into the pharmacological mode of action of *S. sympetala*, BA and me-BA. Chapter 4 tests the hypothesis that *S. sympetala* extracts and meBA are GABA agonists. There is some evidence to support the first testable hypothesis, as both EtOAc and SCE *S. sympetala* extracts displaced ^{3}H-Flu *in vitro* to bind to the GABAa-BZD receptor. This was not observed with the pure compound, likely due to the fact that triterpenes are not very soluble and precipitate in cold temperatures, such as 4˚C at which the assay was run. Pre-treatment of animals with flumazenil, a GABAa-BZD antagonist, followed by both extract and pure compound eliminated reduction of anxiety-like behaviour, providing support for the second hypothesis that the extract material and meBA are GABAa-BZD agonists.

This is the first time that the pharmacological mode of action of *S. sympetala* has been investigated. Importantly, treatment with the extract, but not the pure compound (meBA), had an anti-depressive effect. This result suggests that *S. sympetala* has multiple targets in the CNS, namely the GABAa-BZD receptor, as well as targets associated with depression, potentially serotonin or dopamine (Haenisch and Bönisch, 2011). The results from Chapter 4 also indicate that the dried leaf or bark of *S. sympetala* could be effective as

an NHP, because they reduced anxiety despite low total BA content. If so, this would be a more cost-effective way to develop the material than extracting it.

Chapter 5 investigated whether *S. sympetala* and BA act at the site of cortisol synthesis to lower cortisol reponse in fish *in vitro* and *in vivo*. The results from the head kidney cell experiments support the first testable prediction of this hypothesis. Pre-incubation of head kidney cells with BA and *S. sympetala* extract significantly decreased cortisol release in response to an ACTH challenge. Moreover, the results of the *in vivo* experiment provide support for the second testable prediction of the hypothesis, as fish fed a diet supplemented with BA released less cortisol in response to a physiological stressor than fish fed the unsupplemented diet. These results also confirm that the cortisol-lowering effect occurs in a wide range of animals, as it has now been observed in rats, pigs and fish. Hypercortisolism associated with chronic stress lowers growth rate, increases disease and is detrimental in agriculture and aquaculture settings (Rostagno, 2009; Schreck et al., 2001). These experiments suggest that BA may represent a natural product food supplement that can be added to the diet of production animals to protect against the deleterious effects of chronic stress.

Chapter 6 assessed whether *P. amalago* possesses anxiety-reducing properties that are associated with a plant secondary metabolite and whether this plant secondary metabolite binds the GABAa-BZD receptor. The first testable prediction of the hypothesis is supported by the evidence that *P. amalago* extracts significantly reduce anxiety-like behaviour in the EPM. The second and third testable predictions are supported by the evidence that the molecule with demonstrated *in vitro* affinity for the GABAa-BZD receptor, a furofuran lignan, may be.responsible for the anxiolytic activity observed in the behavioural assays.

The results from Chapter 6 add to the body of evidence that the combination of ethnobotany, modern pharmacological screening and bioassay-guided isolation is a powerful approach to identify novel tropical plants to treat anxiety (Awad et al., 2009; Bourbonnais-Spear et al., 2007).

7.2 The effect of BA on Anxiety and Stress: A Proposal

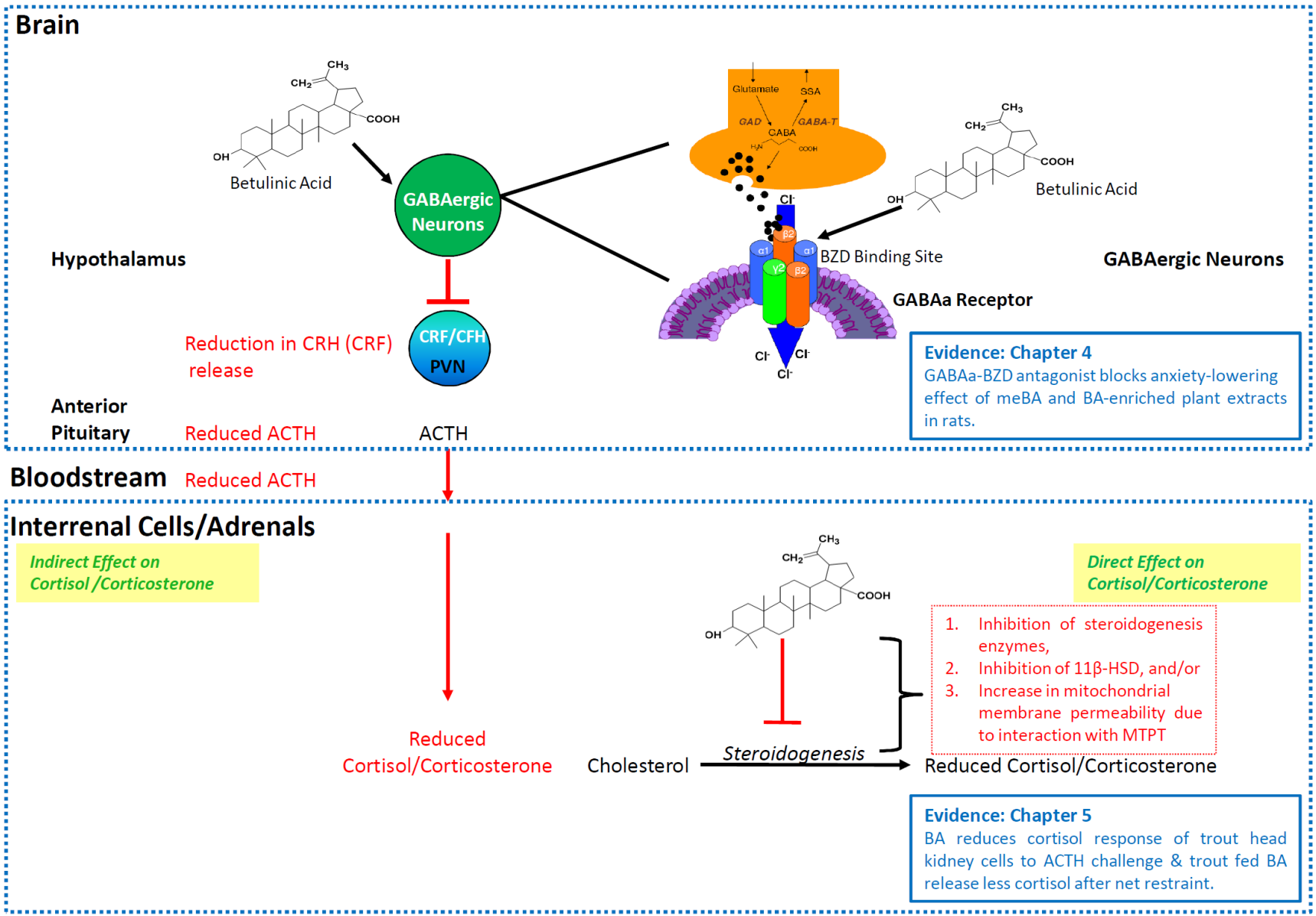
A question that emerges from the pharmacological experiments with *S. sympetala* that has not been experimentally addressed is: How do the GABAergic and cortisol lowering effects interact? Specifically, how and where do these effects overlap or intersect? The following section proposes a potential mechanism for the interaction of the GABAergic and endocrine effects of BA.

Mainstream pharmaceutical BZDs (ex: aloprozalam, diazepam) alter HPA axis function and reduce cortisol. This occurs because the inhibitory effects of GABA antagonise corticotropin-releasing hormone (CRH) release from the paraventricular nucleus (PVN) of the hypothalamus (Heberlein et al., 2008). It is known that activity of the HPA axis is reduced after injection of GABAa agonists (Makara and Stark, 1974), or after peripheral injection of benzodiazepines in animals (Imaki et al., 1995), and that GABA inhibits CRH release from hypothalamic slices *in vitro* (Calogero et al., 1988). Recent evidence points to the fact that GABA is a particularly important neurotransmitter in the PVN of the hypothalamus, the origin of the endocrine response to stress. In experimental animals, GABAa receptors are present in the afferents that terminate on CRH neurons (Kovács et al., 2004), there is direct contact between GABA-containing-terminals and neurosecretory cells

immunoreactive for CRH, and almost half (46%) of all synaptic connections in the PVN are immunoreactive to GABA (Miklós and Kovács, 2002). The high concentration of GABAa receptors in the PVN means that GABA agonists act directly at the hypothalamus to dampen HPA axis activity with the downstream effect being a reduction in plasma cortisol levels.

The experimental results presented in this thesis demonstrate that BA is a GABAa-BZD receptor agonist that lowers cortisol in animal models of stress. Based on the similarities in pharmacological effect between BA and classical BZDs, I propose that BA acts like a classical BZD at the GABAa receptor to reduce anxiety, and to reduce cortisol by acting via GABAa receptors at the PVN to reduce CRH release and consequent cortisol synthesis. However, this proposal does not account for reduced cortisol release after incubating trout head kidney cells with *S. sympetala* extract and BA. As discussed in Chapter 5, BA may reduce cortisol by a number of mechanisms, including: (1) inhibition of steroidogensis (due to structural similarities between BA and cholesterol); (2) inhibition of 11-β HSD, which converts metabolically inactive cortisone to active cortisol (11-dehydrocorticosterone to corticosterone in rodents), because the pharmaceutical carbenoxolone is a triterpene that inhibits 11-β HSD (Gathercole and Stewart, 2010); and/or (3) alteration of the mitochondrial membrane through interaction with the mitochondrial permeability transition pore (Fulda and Kroemer, 2009) with consequent changes to steroidogenesis, which occurs in the mitochondria. A schematic illustrating the proposal of central and adrenal/interrenal action of BA to lower both anxiety and cortisol is presented in Figure 7.1.

Figure 7.1 Proposed mode of action of BA to reduce anxiety and cortisol. The results from Chapter 4 demonstrate that BA has an axiety-lowering effect due to agonism of the GABAa-BZD receptor. I propose that BA acts like a classical BZD to indirectly affect plasma cortisol/corticosterone levels, lowering them by acting at the GABAergic neurons of the paraventricular nucleus (PVN) of the hypothalamus. When BA acts on the PVN of the hypothalamus, the inhibitory effect of GABA reduces corticotrophin releasing factor/corticotrophin releasing hormone (CRF-fish/CRH-mammals). This reduction in CRF/CRH reduces adrenocroticotropin hormone (ACTH) release from the anterior pituitary into the bloodstream. Less ACTH in the bloodstream acts at the interrenal/adrenal cortex and stimulates less cortisol/corticosterone release. This proposal cannot account for the direct effect of BA on trout interrenal cells observed in Chapter 5. In this case, three possible targets of BA action are proposed (1) inhibition of steroidogenesis enzymes; (2) inhibition of 11β-hydroxylase; and (3) changes in mitochondrial membrane permeability due to interaction with the mitochondrial permeability transition pore (MPTP) that alters cortisol synthesis. Sites of action where there is evidence from the experiments in this thesis are highlighted in blue text boxes, sites where the effects of BA are proposed, but there is currently no evidence, are in red.


Brain
Betulinic Acid
GABAergic Neurons
Hypothalamus
Glutamate
SSA
GAD
GABA-T
GABA
Cl-
BZD Binding Site
GABAergic Neurons
Betulinic Acid
GABAa Receptor
Reduction in CRH (CRF) release
CRF/CFH
PVN
Evidence: Chapter 4
GABAa-BZD antagonist blocks anxiety-lowering effect of meBA and BA-enriched plant extracts in rats.
Anterior Pituitary
Reduced ACTH
ACTH
Bloodstream
Reduced ACTH
Interrenal Cells/Adrenals
Indirect Effect on Cortisol /Corticosterone
Direct Effect on Cortisol/Corticosterone
1. Inhibition of steroidogenesis enzymes,
2. Inhibition of 11β-HSD, and/or
3. Increase in mitochondrial membrane permeability due to interaction with MTPT
Reduced Cortisol/Corticosterone
Cholesterol
Steroidogenesis
Reduced Cortisol/Corticosterone
Evidence: Chapter 5
BA reduces cortisol response of trout head kidney cells to ACTH challenge & trout fed BA release less cortisol after net restraint.

7.3 How does *S. sympetala* Compare with other Herbal Anxiolytics?

The two best known and studied herbs for mental illness are Saint John's wort (SJW, *Hypericum perforatum*) for mild to moderate depression, and *P. methysticum* (kava) for anxiety. Both herbs are unique, having a large body of literature which supports their use and efficacy to treat mental illness (Linde et al., 2008; Pittler and Ernst, 2003). However, there are serious challenges associated with each, including interactions with hepatic enzymes (Whitten et al., 2006) (SJW) and hepatotoxicity (kava) (CDC, 2003; Currie and Clough, 2003)). The concerns about SJW and retraction of kava (it has been withdrawn from the Canadian market and several European markets (Ulbricht et al., 2005)), have created an important opportunity to develop new commercial products for people suffering with mood disorders seeking an efficacious NHP.

Patients with mental illness are not compliant in their use of standard pharmaceuticals. Ellen and others (Ellen et al., 2007) report that less than 50% of patients prescribed antidepressants are still taking them after 3 months. Similarly, 25% of patients prescribed citalopram (SSRI, prescribed for depression) discontinued use of the medication before remission (Warden et al., 2007). In contrast, the high reported use of herbals by people with mental illness (Ernst, 2006) and the lower side-effect profiles of NHPs (Freeman et al., 2010) suggest there may be more compliance associated with therapeutic NHP use, but at a minimum indicate that there is a demand for herbals with proven efficacy to treat mental illness. *S. sympetala* is a plant with demonstrated anxiolytic, anti-depressant and cortisol-lowering effects that represents an excellent candidate for the development of a NHP to treat mental illness.

A recent survey regarding Canadians' use of alternative health product (NHPs and vitamins) to treat mental illness reveals concomitant use of SJW and kava by respondents with a prescription for anxiolytics (11% and 3 %), anti-depressants (15% and 3%) and mood stabilizers (17% and 8%). In light of the side effects of both of these NHPs, it is alarming that the authors report that 29% of respondents indicated that the product used was recommended by a health professional, and in more than 50% of the cases the products were recommended by a family doctor or GP (Vasiliadis and Tempier, 2011). This survey demonstrates two important points with respect to the development of a NHP for mental illness. First, not only are people with mental illness high users of NHPs, they use them in combination with mainstream pharmaceuticals. This means that drug-herb interactions need to be investigated as part of the development of an NHP to treat mental illness. Second, because family doctors recommend NHPs for mental illness, they must be apprised of recent evidence regarding drug-herb interactions and side-effects of NHPs. These points must be considered as *S. sympetala* is developed as a NHP.

Overall, as presented in Table 7.1, *S. sympetala* shares several characteristics with other herbals, including GABAa-BZD receptor agonism, and efficacy in animal models of anxiety. *S. sympetala* is distinct in its cortisol-lowering effect and bioactive principle. Several of the other anxiolytic herbals contain bioactive flavonoids, whereas BA is a triterpenoid.

The proposal that BA acts like a classical BZD to lower anxiety and cortisol is both intuitive and attractive given that this mechanism has been investigated intensively in BZDs. This is likely a positive attribute in a NHP, as the mode of action of BA and *S. sympetala* may be rapidly deduced and lead to accelerated adoption of *S. sympetala* as a NHP. This is

analagous to the case of SJW where the herbal product has a pharmacological action, inhibition of serotonin reuptake (Müller, 2003), very similar to an existing, well examined pharmaceutical, the serotonin reuptake inhibitors (SSRIs), such as fluoxetine. An important caveat is that BZDs are associated with side-effects (O'Brien, 2005). In chronic administration to rats, similar side effects (sedation, tolerance) were not observed in animals treated with BA or *S. sympetala* extract (Cayer, 2011) and BA has a low toxicity profile, with no signs of acute or chronic toxicity observed at repeated doses of 500 mg/kg in mice and no significant adverse events were reported or after topical betulin treatment of squamous cell carcinoma (Huyke et al., 2009; Pisha et al., 1995). Moreover, a pharmacokinetic study of bevirimat (3- O – 3', 3' – dimethylsuccinyl-betulinic acid), in healthly volunteers (oral doses from 25 – 250 mg) showed a long elimination half life (60 – 80 h), suggesting that some degree of accumulation may occur after repeated administration. Bevirimat is eliminated *in vitro* primarily by hepatic glucuronidation. It does not undergo oxidative metabolism in human liver microsomes or interact with cytochrome P450 or human glycoprotein. No changes were observed in CYP3A activity between vehicle treated and drug-dosed healthy volunteers after 10 days of treatment (Martin et al., 2007). Despite the apparent low toxicity of BA, further examination of possible BZD-like side-effects of BA and *S. sympetala* is merited.

7.4 Future Research

This thesis provides strong evidence for the efficacy of *S. sympetala* as an anxiety-reducing herbal and sets the groundwork for further investigation. Described below are several new areas that offer promising areas for further research.

The two most common *Souroubea* species that grow in Costa Rica, *S. sympetala* and *S. gilgii*, are phenotypically very similar and difficult to distinguish. An analytical method to distinguish them should be developed, since ground or processed material cannot be identified by taxonomic characters. This could be achieved by the use of genetic markers and DNA bar coding, an approach that is being used with success to identify and authenticate medicinal plants (Gao et al., 2010; Kiran et al., 2010; Moon et al., 2010). Another approach for species authentication is metabolome profiling with mass spectrometry (MS) and nuclear magnetic resonance (NMR) (Gilard et al., 2010; Holmes et al., 2006; Seger and Sturm, 2007). The recent acquisition of a Quantitative Time of Flight Mass Spectrometer (QTOF-MS) by the Arnason lab offers the opportunity to use these emerging technologies to develop a method to discriminate *S. sympetala* and *S. gilgii*.

The proposal for BA's anxiolytic and cortisol-lowering effects discussed above (Section 7.2, Figure 7.1) presents several testable questions. For instance, if the cortisol-lowering effect of BA occurs in part by binding the GABAa receptors at the PVN, it would be expected that animals treated with BA would have lower ACTH and CRH levels in addition to lowered cortisol. This could be tested by repeating the *in vivo* net restraint experiments and measuring plasma ACTH levels and CRH transcript (mRNA) levels in the pituitary.

The ability of BA to inhibit steroidogenesis enzymes could be tested by with the experimental assay developed by Cooke (Cooke, 1991) and adapted by members of Dr. Tom Moon's research group to be used with rainbow trout head kidney cells. Briefly, the assay employs radiolabelled pregnenolone and examines the effect of steroidogenesis inhibitors on the downstream yield of radiolabelled cortisol and other steroids, after incubation with head

kidney microsoms. The change in production of specific steroid molecules, measured by radioactive detection, indicates which steroidogenic enzyme(s) (are) affected.

S. sympetala is an anxiolytic plant which represents an excellent candidate for development as a NHP. Further experiments are planned to look at both the toxicological profile and the anxiolytic effect in companion animals, domestic dogs, with the view to developing *S. sympetala* as a herbal anxiolytic for the veterinary market. Finally, it is hoped that ultimately *S. sympetala* can be developed as a fair trade NHP, from Costa Rica, for the treatment of anxiety in humans.

The experimental approach used to identify *P. amalago* as a new anxiolytic herbal combined ethnobotany and modern pharmacological screening assays. In addition to *P. amalago* several other plants were highly ranked for activity in CNS enzyme assays and healer consensus and remain to be examined. These plants should be followed up with bulk extractions and bioassay guided fractionations. Further experiments with *P. amalago* will test the pure active compound (**6**, furanofuran lignan), either isolated from the plant or synthesized, in rats to determine if it is an effective anxiolytic.

Table 7.1 Comparison of *S. sympetala* with other commercially available anxiolytic herbals.

Herbal	Indication	Mechanisms of action	Active molecule (s)	Animal Evidence	Clinical Evidence	Toxicity	Status in the Marketplace
St John's wort *(Hypericum perforatum*)	Mild to moderate depression	Selective serotonin reuptake inhibition(Knüppel and Linde, 2004)	Hypericin Hyperforin		Effective in treating depression, Cochrane Database of Systematic Reviews (Linde et al., 2008), concerns about heterogeneity in methods and variability in outcomes	Upregulates expn of CYP3A4, resulting in serious effects on other drugs (Whitten et al., 2006)	OTC, labelled with warning, proposed to be behind the counter
Kava (*Piper methysticum*)	Anxiety	Inhibits NE uptake, alters Na^+/K^+ channels, modulation of GABA channels & binding sites (Gleitz et al., 1996; Jussofie et al., 1994; Magura et al., 1997)	Kawain Dihydrokawain		Effective in treating anxiety, Cochrane Review (Pittler and Ernst, 2003)	Reports of hepatotoxicity (Center for Disease Control and Prevention, 2003)	Banned in Canada, Australia, Germany, France & Switzerland
Passionflower (*Passiflora incarnata / P.coerulea*)	Anxiety Insomnia	GABAa-BZDR agonist (Grundmann et al., 2008)	5,7 – dihydroxyflavone (chrysin)	Anxiolysis in rats in EPM (Medina et al., 1990; Wolfman et al., 1994)	Reduction of anxiety in state trait assessment after acute oral dose (Movafegh et al., 2008) Significant reduction in HAMA-A scores after chronic dosing (Akhondzadeh et al., 2001)	One report, full recovery(Fisher et al., 2000)	OTC
Skullcap (Scutellaria lateriflora / S. baicalensis)	Anxiety Insomnia	GABAa-BZDR agonist (Huen et al., 2003a; Huen et al., 2003b; Hui et al., 2002)	2' – trihydroxy-6,8-dimethoxy flavone 5,7- dihydroxy-8-methoxyflavone	Anxiolysis in EPM (Awad et al., 2003; Huen et al., 2003a)	Trend for reduction in self-reports of anxiety, not statistically significant (Wolfson and Hoffmann, 2003)	LD_{50} of *S. baicalensis* = 3.9 g/kg for mice (Huen et al., 2003a)	OTC

Herbal	Indication	Mechanisms of action	Active molecule (s)	Animal Evidence	Clinical Evidence	Toxicity	Status in the Marketplace
Lemon Balm (*Melissa officinalis*)	Anxiety Insomnia	GABA-T inhibition MAO-A inhibition (Awad et al., 2007; Kennedy et al., 2002; Kennedy et al., 2004)	Citranellal Geraniol		Hops + lemonblam, increase in self-reported calmness during lab stress test (Kennedy et al., 2006)	Generally Regarded As Safe (GRAS)	OTC
Gotu kola (*Centella asiatica*)	Anxiety Insomnia	GABA-T inhibition (Awad et al., 2007)	Asiaticoside	Anxiolytic in animals(EPM, open field, social interaction) (Wijeweera et al., 2006)	Acute dose significantly reduced acoustic startle, no effect on mood (Bradwejn et al., 2000)	Hepatic damage in rats after chronic, high dose (Oruganti et al., 2010)	OTC
California poppy (*Eschscholzia californica*)	Anxiety Insomnia Pain	GABAa-BZDR agonist $5HT_{1A}$ binding (Rolland et al., 2001)	Escholidine Cheilanthifoline An aporphine alkaloid	Anxiolysis in animals (familiar environment, anti-conflict test) (Gafner et al., 2006; Rolland et al., 1991)	Significant reduction in HAMA-A scores after chronic dose(Hanus et al., 2004)	No reports	OTC
Chamomile (*Matricaria recutita*)	Anxiety Insomia	Binds GABA receptors (Avallone et al., 2000)	Apigenin		Chronic dose in people with GAD, significant reduction in HAM-A scores(Amsterdam et al., 2009)	GRAS	OTC
Souroubea sympetala	Anxiety (*susto*)	GABAa-BZD receptor agonist Cortisol-lowering	Betulinic acid		No	No evidence for plant or extracts, BA has low toxicity (Cayer, 2011)	Not available

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