IN VIVO BEHAVIORAL CHARACTERIZATION
OF
ANXIOLYTIC BOTANICALS

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In Partial Fulfillment
of the Requirements for the Degree
of Master of Biology

By
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Abstract

This thesis studied three plants traditionally used for treating a variety of anxiety related conditions. The three species were Roseroot, *Rhodiola rosea* from Nunavik, Cordonsillo, *Piper amalago* from Belize and “Sin Susto”, *Souroubea sympetala* from Costa Rica. The main objective of this research project was to investigate effects on behavior of these traditionally used native plants. It was found that the crude ethanol extracts derived from these plants administered intragastrically had measurable anxiolytic-like effects in male Sprague Dawley rats. Rats treated with extracts of these plants were then tested in several behavioral paradigms: elevated plus maze (EPM), social interaction (SI), conditioned emotional response (CER) and fear potentiated startle FPS. “Sin susto” produced significant anti-anxiety effects in several paradigms. Its active principle, betulinic acid, was significantly active in the EPM and FPS at a dose of 0.5mg/kg. Cordonsillo had strong activity in the SI paradigm and Roseroot in the CER paradigm. The results suggest that traditional use is based on pharmacological activity of the plants.
Table of contents

Abstract ............................................................................................................................................... 2
Table of contents ............................................................................................................................... 3
List of figures and tables .................................................................................................................... 4
List of Abbreviations .......................................................................................................................... 5
CHAPTER 1 INTRODUCTION .................................................................................................................. 6
  1.1 Anxiety ......................................................................................................................................... 7
  1.2 Anatomical and neuropharmacological background of anxiety .............................................. 8
  1.3 Rationale and objectives ........................................................................................................... 12
CHAPTER 2 Anxiolytic properties of *Souroubea sympetala* and Betulinic Acid .......... 14
CHAPTER 3 Fear-reducing properties of Nunavik *Rhodiola rosea* ........................................... 35
CHAPTER 4 *Piper amalago* a social anxiety reducing plant ....................................................... 44
CHAPTER 5 General discussion ......................................................................................................... 53
References .......................................................................................................................................... 61
List of figures and tables

Figure 1 ............................................................................................................................. 11
Figure 2 ............................................................................................................................. 16
Figure 3 ............................................................................................................................. 16
Figure 4 ............................................................................................................................. 26
Figure 5 ............................................................................................................................. 26
Figure 6 ............................................................................................................................. 27
Figure 7 ............................................................................................................................. 28
Figure 8 ............................................................................................................................. 28
Figure 9 ............................................................................................................................. 29
Figure 10 ......................................................................................................................... 30
Figure 11 ......................................................................................................................... 32
Figure 12 ......................................................................................................................... 36
Figure 13 ......................................................................................................................... 37
Figure 14 ......................................................................................................................... 40
Figure 15 ......................................................................................................................... 41
Figure 16 ......................................................................................................................... 42
Figure 17 ......................................................................................................................... 42
Figure 19 ......................................................................................................................... 45
Figure 20 ......................................................................................................................... 48
Figure 21 ......................................................................................................................... 49
Figure 22 ......................................................................................................................... 50
Figure 23 ......................................................................................................................... 51
Figure 24 ......................................................................................................................... 58

Table 1 .............................................................................................................................. 31
Table 2 .............................................................................................................................. 31
Table 3 .............................................................................................................................. 54
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BA</td>
<td>Betulinic Acid</td>
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<tr>
<td>CE</td>
<td>Crude ethanol extract</td>
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<td>CER</td>
<td>Conditioned emotional response</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CS</td>
<td>Conditioned stimulus</td>
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<td>EPM</td>
<td>Elevated plus maze</td>
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<tr>
<td>FPS</td>
<td>Fear potentiated startle</td>
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<tr>
<td>f1</td>
<td>Ethyl acetate soluble fraction of <em>Souroubea spp.</em></td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>ITI</td>
<td>Inter-trial interval</td>
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<tr>
<td>MAOI</td>
<td>Monoamine oxidase inhibitors</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>NHP</td>
<td>Natural Health Products</td>
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<tr>
<td>me-BA</td>
<td>Methyl ester derivative of BA</td>
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<tr>
<td>SI</td>
<td>Social interaction test</td>
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<tr>
<td>SJW</td>
<td>St-John’s Wort</td>
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<tr>
<td>SSRI</td>
<td>Selective serotonin re-uptake inhibitors</td>
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<tr>
<td>TCA</td>
<td>Tricyclic antidepressants</td>
</tr>
<tr>
<td>UH</td>
<td>Unprotected head dip</td>
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<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
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CHAPTER 1 INTRODUCTION

Plants, as a source of medicine, have been used throughout history for treatment of mood disorders (Schmidt et al. 2008) (anxiety, depression, sleeplessness, and physiologically related conditions). Today there are over 10 medicinal plants that are used commercially as regulated Natural Health Products (NHPs), or EU phytomedicines to treat mood disorders related to anxiety (Blumenthal et al. 2000). These are used in North America as over the counter medications by both the general public and diagnosed patients. There is now growing interest in these products among some physicians in North America because patient compliance is high and evidence of efficacy is available. The two most widely used botanicals are St-John’s Wort (SJW) (*Hypericum perforatum*) and Kava Kava (*Piper methysticum*) (Linde 2009; Sarris & Kavanagh 2009b). These medicinal plants have been well studied pharmacologically and phytochemically and there is extensive animal behavior and clinical research supporting their efficacy. Unfortunately, both of these NHPs have come under scrutiny for unrelated toxicology issues (Linde 2009; Sarris & Kavanagh 2009a). SJW has recently been found to have potentially life threatening drug interactions with certain types of drugs, while Kava has been withdrawn from some markets due to idiosyncratic hepatotoxicity in a small number of individuals. Therefore, there is an opportunity and potential need to replace these NHPs. I set out to investigate the efficacy of three traditionally used but poorly studied anxiolytic plants.
1.1 Anxiety

When researchers are observing maladaptive emotional behaviors in an individual, be it human or any other mammal, they are often confronted by an array of overlapping symptoms such as depression and anxiety (Lydiard 1991). This is in part due to the fact that many neuropsychological diseases, most notably anxiety disorders, are in fact almost never expressed exclusively. This is due to the comorbid nature of these diseases or states of behavior.

The prevailing way to characterize anxiety is that its nature is linked to a state of chronic fear that persists in the absence of a direct threat (Coutinho et al. 2010). Although anxiety that occurs when subjected to an aversive external stimulus, it is in essence an adaptive tool for motivating the animal or human to quickly find a way of escaping or terminating this source of initial anxiety, it becomes maladaptive if it persists without reason. In fact, the symptoms associated with anxiety can become quite discomforting and can effectively interfere with a person's ability to function properly.

The prevalence of anxiety disorders is widespread globally and afflicts approximately 12% of the world’s population (Davidson 2009; Somers et al. 2006; Tindle et al. 2005). The strategies employed for treating these anxiety disorders are strongly influenced by local socio-economical factors and traditional practices. In more developed areas of the world, the most common approach for treating anxiety is pharmacotherapy (Cloos & Ferreira 2009; Sheehan & Sheehan 2007b). More specifically, the most widely prescribed drugs for treating anxiety belong to benzodiazepines which
are known to act through the GABAergic system (Dinan 2006; Lader 1984). In more recent decades, busiprone which acts primarily through the serotonergic system is also being used (Dinan 2006). Other pharmacological tools to treat anxiety disorders include monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), and selective serotonin re-uptake inhibitors (SSRI) (Baldwin & Polkinghorn 2005; Sheehan & Sheehan 2007b).

Since these applied agents do in many cases present adverse effects in patients, a sharp rise in the usage of complementary and alternative medicine (CAM) has been reported in the past decade (Astin 1998; Beaubrun & Gray 2000; Ernst 1999; Ernst 2006; Lader, Tylee, & Donoghue 2009). Medicinal plants are the largest category in these CAM treatments. Rigorous testing of these CAM remedies is essential for their potential future clinical application and to avoid possible herb drug interactions (Izzo & Ernst 2001). It is also important as approximately 40% anxiety suffering patients who take prescribed medication also self administer themselves with CAM remedies (Astin 1998). In addition, in the more rural less developed countries, people rely heavily on traditional knowledge, where phytotherapy is a major component in treating anxiety (Arcury et al. 2006; Grzywacz et al. 2006; Levine & Gaw 1995).

1.2 Anatomical and neuropharmacological background of anxiety

There are many anatomical structures involved in generating, maintaining and dampening a state of anxiety. One example is within the endocrine systems. The
hypothalamic-pituitary adrenal axis (HPA) plays a major role in the acute stress response. If not kept in balance, for example in the case of people suffering from a prolonged state of anxiety, it can over time cause anatomical changes such as enlarged adrenal glands (Kessing, Willer, & Knorr 2011). In the central nervous system (CNS), researchers have identified many sites that are involved with anxiety, however there are three particular structures that stand out, including the amygdala, the hippocampal complex and the prefrontal cortex (Engin & Treit 2007a; Engin & Treit 2007b). All three structures are anatomically and functionally connected.

The amygdaloidal complex is located within the limbic system of the CNS. More specifically within the cortico-temporal area of the brain and is compartmentalised into several distinct nuclei including the basolateral, medial, and central nuclei (Ledoux 2007). The exact functions of each nucleus are not fully understood, but the amygdala has both multiple afferent and efferent connections with most other areas of the brain and does play a major role in the affective evaluation of stressful events (Davis & Whalen 2001). Although for many years researchers have suggested that the hippocampus only plays a peripheral role in the regulation of anxiety, more recent theories have suggested that the hippocampal system may play a more central role than previously envisioned (Lydiard, Brawman-Mintzer, & Ballenger 1996). As with most cortical areas of the brain, the prefrontal cortex has multiple reciprocal connections with other areas of the brain. More specifically, many studies have shown that the medial prefrontal cortex is involved in a variety of anxiety responses (Blanco et al. 2009; Canertas et al. 2010; Etkin 2010). Since all three structures have complex interactions with other parts of the brain
and the neuroendocrine system, it is difficult to focus on a single structure to develop a solution for treating anxiety disorders. This has led to extensive research in the identification of neurotransmitter systems and their respective synaptic receptors utilized by potential anxiolytic compounds (Sheehan & Sheehan 2007b). As well, there are other CNS structures, than the well-studied ones mentioned above, that play a role in the expression of anxiety, such as the nucleus accumbens (Lopes et al. 2007).

The most widely researched target for reducing anxiety is the GABAergic neurotransmitter system. Because GABA is the main inhibitory neurotransmitter in the brain, it has been perceived as having dampening effect in overall brain activity. One of the main targets of this neurotransmitter is the GABA<sub>A</sub> receptor. The GABA<sub>A</sub> receptor is a ligand-gated chloride channel (Fig 1). Binding of GABA in most cases and other positive allosteric modulators such as diazepam to this receptor increases the influx of negatively charged chloride ions. Thus by increasing local synaptic GABA secretion, this usually causes, in most of the synaptic clefts, local hyperpolarizing pulses in the post-synaptic neurons. This in turn implies that the increased GABA presence in the synaptic clefts has thus an overall hyperpolarizing effect on the neurons of the CNS since the GABA<sub>A</sub> receptors are distributed throughout the CNS (Sieghart & Sperk 2002). It is hypothesized that people who are suffering from non-adaptive sustained states of anxiety have unbalanced hyperactive neuronal activity which might be a result of insufficient inhibitory control in the overall CNS neurotransmission. This is the reason that the GABAergic system has been identified as the logical culprit responsible for the dysfunctional state of anxiety.
Both the amygdala and the prefrontal cortex have important roles in emotional processing and expression. These regions play a major role in affective disorders and have been shown repetitively to be involved in anxiety (Davis 1992; Dudai 2003). There is a dense number of GABA<sub>A</sub> receptors in the amygdala (Niehoff, Mashal, & Kuhar 1983). When benzodiazepines, which are GABA<sub>A</sub> specific agonists and are commonly used as psychopharmacological anxiolytics, are administered, a notable reduction in anxiety symptoms is observed. It is thought that by increasing the GABAergic response in the amygdala, abnormal expression of anxiety can be reduced to a “normal” state by altering amygdalar neuronal activity (Pesold & Treit 1995; Shekhar et al. 2003). Neurons from the amygdala have reciprocal projections to various cortical and subcortical regions including the prefrontal cortex (Sah et al. 2003). Deregulations in neuronal activity of any of these regions can produce anxiogenesis. The development of anxiety disorders might partially be related to a dysfunctional feedback mechanism in the amygdala or a dysregulation of response to the HPA axis, which causes an overall deregulation in the secretion of the stress hormone cortisol (Denver 2009).
The serotonergic system is another neurotransmitter system which has been shown more recently to be involved in anxiety. For example, agonists of the 5-HT1A subreceptor induce anti-anxiety effects (Barrett & Vanover 1993). There are several efferent serotonergic projections to the amygdaloid complex and the ventral hippocampus, which target presynaptical GABAergic nerve terminals (Almada et al. 2009). These neuronal inputs have been shown to inhibit the synaptic GABA release, leading to a disinhibition of neuronal activity in the amygdala. Thus by administrating a 5-HT1A agonist, a downstream regulation of amygdaloid GABAergic expression is thus modulated.

Of course, the above explanation only offers a brief summary of the underlying neurobiology implicated in anxiety. There are other neurotransmitter systems like norepinephrine, dopamine, acetylcholine which have shown to be involved in modulating anxiety-like behavior in rodents (Durant, Christmas, & Nutt 2010). In addition, there is evidence that many neuropeptides such as urocortin, neuromedin-B, cholecystokinin and endocannabinoids are involved in anxiety but these systems are less studied than the classic neurotransmitters (Alldredge 2010; Bedard et al. 2007; Merali et al. 2006b; Merali et al. 2006a; Thorsell et al. 1999).

1.3 Rationale and objectives

Given that Kava and SJW, the major anxiolytic botanicals on the market have toxicology issues, this research was initiated to assess the potential of several promising
anxiolytic plants including *Souroubea sympetala* Gilg. (Marcgraviaceae), *Rhodiola rosea* L. (Crassulaceae), and *Piper amalago* L. (Piperaceae). In particular, our collaborative lab group has had a discovery program for medicinal plants in Central America. One plant *Souroubea sympetala*, “sin susto” was identified as a member of a relatively rare and unstudied plant family Marcgraviaceae in collaboration with Costa Rican botanists Pablo Sanchez and Luis Poveda at the Universidad Nacional. It was targeted for study because of South American ethnobotany reports of use for witchcraft, nervousness and “susto” which suggested psychopharmacological activity (Schultes 1990). The second species, *Rhodiola rosea*, “rose root” or “arctic root”, is a well known Eurasian plant used for treatment of stress. The discovery and documentation of a large Canadian population by Vicky Filion (Fillion 2008), led to the second study on Nunavik *Rhodiola rosea*. The third plant, *Piper amalago*, “cordonsillo” was identified in an ethnobotanical study of plants used for mental health by the Q’eqchi’ Maya of Belize.

The overall objective of the thesis was to assess the anxiolytic activity of these three promising species in a battery of animal behaviour paradigms. Based on traditional usage of these plants, my hypothesis was that administration of these plants in rats will exhibit some anxiolytic activity in four behavioural models of anxiety. The prediction is that the plant extracts will have significant dose dependent activity in at least one or more paradigms, but since the phytochemistry of each plant is different, it is further predicted that the profile of activity in the 4 tests is species specific.
CHAPTER 2  Anxiolytic properties of *Souroubea sympetala* and Betulinic Acid

*Souroubea sympetala* ("Sin Susto") is a relatively uncommon and poorly studied neotropical vine indigenous to the neotropics. Ethnobotanical investigations in Amazonia suggested that the other species of the genus *Souroubea* belonging to the small family Marcgraviaceae may have psychopharmacological activity (Schultes 1990). The most common way of administering this plant is by crushing the dry or fresh leaves and preparing it as a decoction boiled for 20-30 minutes (50-100 g/litre) (Bourbonnais-Spear et al. 2007).

As previously reported (Bourbonnais-Spear et al. 2007), the term “susto” is a folk illness used to categorize an illness which is symptomatically similar to mental disorders labeled in the Diagnostic and Statistical Manual of Mental Disorders such as general anxiety disorder and posttraumatic stress disorder. To alleviate the symptoms of this culturally bound illness, the Q’eqchi’ healers of Belize who have a rich ethnobotanical tradition using rainforest species are known to use certain plants including *Souroubea sympetala* for treating the (Awad et al. 2009; Quinlan 2010) “susto” illness.

Ethnobotanical investigations suggested that the genus *Souroubea* of the Marcgraviaceae family may contain ingredients with psychopharmacological activity (Awad et al. 2009; Bourbonnais-Spear et al. 2007). Based on preliminary findings using the elevated plus maze behavioural assay, that showed that the crude extract (CE) of *Souroubea sympetala* leaves had anxiolytic activity when administered in rats (Puniani
2001), in this study we further characterized its behavioral effects in other anxiety models. By orally administering different components of the leaves of this plant using validated and standardized animal models of anxiety and fear, we attempted to corroborate our previous findings in a wider experimental context.

As reported earlier, from this plant we isolated and characterized four already known pentacyclic triterpenes as potential bioactive compounds responsible for anxiolysis (Puniani 2001). Preliminary results with the elevated plus maze led us to believe that betulinic acid (BA) was the active ingredient. In order to facilitate potential further application of this bioactive compound we further tested the more soluble methyl ester derivative of BA (me-BA).

In this study, we increased the number of behavioral paradigms to achieve a proper behavioral characterization. In addition to our main hypothesis that Souroubea sympetala and its active ingredients possess anxiolytic activity in rats, we also hypothesized that the proposed active ingredient BA and me-BA showed no deleterious effects after chronic oral administration.
Figure 2. *Souroubea spp.* leaves  Photo by???

Figure 3. Betulinic Acid
Methods & Materials

Plant material

*Souroubea sympetala* leaves were collected under permit in Tortuguero, Costa Rica. Samples were dried overnight in a commercial plant drier at 35°C and ground to 2 mm mesh. A Voucher specimen was deposited in the JVR herbarium, Universidad Nacional Costa Rica, and the University of Ottawa Herbarium (OH No. 19915).

Plant extraction

Plant material was subsequently macerated with EtOH (10mL/g) in a food blender and the resulting dark mixture was filtered under suction in a Buchner funnel through Whatmann no 1 paper, the solids were washed with additional ethanol (5 mL/g); the alcohol and water were then evaporated, using a rotary evaporator and then under vacuum (1mm Hg) to yield the *Souroubea sympetala* crude extract (8 %). The CE was partitioned between the water soluble fraction (f2) ethyl acetate soluble fraction (f1) and to provide a polar and non polar fraction. Also further fractionation of the residue from (f2) was done with EtOH by stirring and heating to reflux for five minutes before filtration and concentration under reduced pressure. The resulting soluble substance was labeled as fraction 3 (f3) and the remaining insoluble fraction was labeled fraction 4 (f4).

Bioassay Guided Isolation

The crude ethanol extracts were obtained by treating the leaves as described above. The CE was further extracted twice by stirring overnight with ethyl acetate. The
ethyl acetate fractions were combined and evaporated to yield a gummy solid. The extract was chromatographed on a silica gel G column (70-200 mesh) eluted with hexane with stepwise increments of ethyl acetate, to produce 4 fractions. In some cases these were rechromatographed to yield pure compounds that were identified by spectroscopically, using a Bruker 500MHz NMR and Kratos high resolution MS. me-BA was prepared semi-synthetically as described previously (Puniani 2001).

**HPLC analysis**

The triterpene LC/MS method utilized an Agilent Series 1100 autosampler, quaternary pump vacuum, degasser, column oven and LC/MS. HPLC analyses were conducted using a YMC HPLC column (2.0 x 100mm, S3µ, 120Å). The mobile phase consisted of solvent A (methanol), solvent B (acetonitrile) and solvent C (0.1% formate with 0.05% ammonium hydroxide in water).

**Animals**

The behavioral 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 Plexiglas 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). Rats were habituated to the non aversive feeding technique, as previously reported (Mullally et al. 2010). 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 plant extracts, betulinic acid and methyl ester derivative, were all suspended in 50% sweetened, condensed milk 1–4 days prior to testing and stored at 4°C. To facilitate the mixing of the crude extract and the different fractions, all were frozen at -80°C, pulverized in an ice-cold mortar and pestle, and mixed with the 50% sweetened condensed milk. All rats were orally administered the respective treatments for three consecutive days (2 days and 60 min prior to testing). CE and fractions were dosed at 100 mg/kg. Three BA doses were given; 0.1, 0.5 and 1mg/kg respectively. Two me-BA doses were given; 0.5 and 1mg/kg respectively.

**Behaviour: Elevated Plus Maze (EPM)**

The EPM is a validated test used to assess anxiety-like behaviour in laboratory rodents (Pellow 1986). 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. The EPM is based on the conflict between the animal’s instinct to explore its environment and its fear of exposed areas and heights. Black curtains 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=7-12/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 rats 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) unprotected head dips (UH); head protruding over the edge of an open arm and down toward the floor, which is an index of risk assessment behaviour. 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 are indicative of reduced anxiety, whereas decreases compared to vehiclessuggest increased anxiety (File 1991). In contrast, the frequency of closed arm entries is an index of general activity (Cruz, Frei, & Graeff 1994).

**Fear potentiated startle (FPS)**

The startle apparatus (Coulbourn Instruments, Whitehall, PA, USA) consisted of a sound attenuated chamber containing two calibrated platforms (18×10 cm) designed to measure the animal’s startle response. Animals were placed in a Teflon cage (18.5×11 cm) positioned atop the platforms. The cage floor consisted of stainless steel rods (4 mm diameter spaced 1.8 cm apart) connected to shock generators (Coulbourn Instruments; H13–16). Force changes produced by the rats’ startle response were measured by the startle sensor platform. The resultant voltage output from the platform transducer was digitized by an analog-to-digital converter card, interfaced with the computer, and recorded using data acquisition software (Coulbourn AASS v3.02). Startle amplitude was defined as the maximum peak-to-peak voltage that occurred during the
first 200 ms after onset of the auditory startle stimulus. A high-frequency speaker, mounted (24 cm) above the platforms, generated white noise, while tones (startle stimulus) were generated by a Sonalert model tone generator (75 kHz; Coulbourn Instruments). The training and testing for FPS spanned 4 days. On day 1, rats (n=8–11/group) were placed inside the startle chamber and exposed to random bursts of white noise (95, 110, and 115 db) for acclimatization and establishment of individual baseline startle amplitudes. On day 2, animals received a conditioning session where a tone (conditioning stimulus; CS) was paired with a shock (unconditioned stimulus; US). Specifically, a 1.0-mA, 0.5 s foot shock (US) was administered during the last 500 ms of the CS (a 4 s tone; 75 KHz). There were seven CS–US trials with an average of 1 min (randomized) intertrial intervals (ITI). Forty-eight hours later (day 4), rats received their respective treatment 60 min before testing for fear potentiation. Twenty trials of 110 db white noise bursts (random 1 min ITI) were followed by five trials of tones paired with noise bursts, and finally, five noise-alone trials. Cages were cleaned with 70% ethanol between testing of each animal. Rats that have learned to associate the CS (tone) with the US (foot shock) typically display a greater startle amplitude in the presence of the CS (Davis 1993b). Administration of anxiolytic compounds decreases the FPS response in rodents(Walker & Davis 2002a).

**Social interaction (SI)**

SI experiments were done under semi-aversive (high illumination, familiar environment) conditions. SI was assessed in a square gray Perspex arena (60 x 60 cm; 30-cm-high walls, divided into 5X5 squares each measuring 12 cm), 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 arenas to permit remote monitoring/scoring and recording of the test sessions. The SI study had a total duration of three days. Rats were randomly assigned to either control or extract treatment groups. Vehicle and drug-treated rats were orally administered their respective solutions daily across three days (at 10:00 a.m. for two days preceding testing, and then 60 min prior to being placed on the SI arena on the test day). The first and second day was used for habituation. On habituation day 1, rats along with their test day partner were placed in the arena for five minutes. On habituation day 2, rats were individually placed in the arena for a period of 5 min. On test day (third day), each rat was allocated to a partner based on body weight, such that members of a pair did not differ by > 10 g. On test day (day 3), both rats of each pair were given either sweetened condensed milk vehicle or their respective treatments (n = 7-12); 60 min prior to being placed into the arena for a 7 min. period. Time spent engaged in active social interaction (including sniffing, climbing over each other, following, allogrooming, playfighting, anogenital sniffing, and grooming) was recorded by an observer blind to drug treatment. Locomotor activity in the arena was assessed by counting the number of squares crossed by the rat. Testing was performed between 10:00 A.M. and 2:00 P.M. in a randomized order. The arena was cleaned with 70% ethanol between each trial.

**Chronic Experiments**

All rats were orally administered the respective treatments for thirty consecutive days until day of testing. Both BA and me-BA treatment groups received a dose of
0.5 mg/kg.

**Locomotor Activity**

Rats were individually housed in their home cages (as described above) while a locomotion monitoring device was placed over the top of each cage. This custom-designed cage top contained an array of six heat-sensitive infrared detectors, which served to track the movement of the animal. The locomotor activity was calculated according to the transitions across six equal “zones” and stored in a computer on a minute-by-minute basis. These movements, or level of activity, can then be compared across groups. Locomotor activity was recorded for one night (4 p.m. to 8 a.m.).

**Withdrawal Symptoms**

Rats were housed in the same standard plastic cages as stated above. Closed circuit cameras were mounted above the cages to monitor behavior during the entire withdrawal period. Red lights (invisible to rodents) were turned on during the dark cycle (7 p.m. to 7 a.m.) to illuminate the cages to facilitate videotaping. After animals finished their last day of testing and received their last drug treatment (day 25 of experiment), they were returned to clean standard cages as stated above. Video recording commenced at 4 p.m. on the same day and continued for 40 hours. Behaviors were scored live from 8 a.m. to 4 p.m. on the day following drug cessation but were scored from videotapes during the evening hours (dark phase). Several behaviors known to be associated with withdrawal symptoms (Aceto et al. 1998; Wei 1973) were monitored, however, due to a low frequency of some behaviors or difficulty scoring via videotape, only four behaviors
of interest were analyzed and presented (exploring, resting, grooming, scratching). All animals were scored for the presence of each behavior 10 times within each hour. Fecal boli output and amount of food consumed during this period were also monitored. Animals were weighed each day prior to drug administration and were weighed again at the end of the withdrawal period to assess if weight loss was a factor in withdrawal.

**Data analysis**

Data obtained from the EPM and SI tests were analyzed using one-way analysis of variance (ANOVA) for each of the behavioral measures with treatment condition as the between-group factor. For the FPS experiment, to obtain an operational measure of fear, data was converted to percent change scores (mean startle amplitude on CS+ noise trails–mean startle amplitude on noise-alone trials/mean startle amplitude on noise-alone trials×100) (Walker & Davis 2002b). The potentiated startle data was then analyzed using a one-way ANOVA with treatment condition as the between-group factor. In some experiments, follow-up analyses were conducted using t tests with a Bonferroni correction to protect the α at 0.05.
Results

Effect of CE and fractions on animal behavior

The extracts were compared in the EPM for their effect on anxiety-like behavior and fear response. As depicted in Fig. 4, both the CE and f1 showed a significant increase in the percentage of time spent in the open arms (F (5, 49) 3.734 = p<0.01). In regards to closed arm entries, no significant differences were apparent in any of the fractions compared to vehicle (data not shown). Administration of both the CE and f1 (Fig. 5) decreased the expression of FPS (F (2, 38) 10.824 =; p< 0.001) compared to vehicles. This was observed without it affecting their baseline startle amplitude noise-alone trials; (data not shown). Also, there was a significant difference in the intensity of the startle response between the CE and the f1 treatment groups (F (2, 38) 10.824 =; p< 0.05). In the SI experiment (Fig. 6), oral treatment of f1 significantly increased levels of total active social interaction of rats (F (1,32) 1.776= p<0.0001 ). The SI experiment also indicated the absence of locomotion perturbations following administration of f1 (data not shown).
Figure 4.  Effects of CE and four different fractions (f1, f2, f3, f4,) on the percentage of time spent on the open arms in rats in the elevated plus maze following oral administration of 100mg /kg of their respective treatments. * $P<0.05$, ** $P<0.005$ indicate significant differences from vehicle.

Figure 5. Effects of the CE and f1 on percent of FPS response in rats following oral administration of 100mg/kg of their respective treatments. † $P<0.01$, †† $P<0.0001$ indicate significant differences from vehicle. * $P<0.05$ indicates a significant difference from CE.
Figure 6. Effects on time spent in SI after the administration of 100 mg/kg of f1 and vehicle. †† $P < 0.0001$ indicates a significant difference from vehicle.

Effect of acute administration of BA on behavior

Administration of both doses (1 and 0.5 mg/kg) of BA increased the percentage of time spent in the open arm ($F(3,36) = 3.306, p<0.05$; Fig. 7a) and the number of UH ($F(3,36) = 3.479, p<0.05$; Fig. 7b). Also, no significant differences were observed when comparing each of the BA doses respectively to the positive control diazepam. The positive control diazepam treatment group differed significantly from the vehicle group in both the percentage time spent in the open arms (fig. 7a) ($F(3,36) = 3.306, p<0.01$) and in the number of UH (fig. 7b) ($F(3,36), 3.479=p<0.05$). Fig. 8 indicates that administration of 0.5mg/kg of BA decreased FPS in relation to the vehicle group ($F(2,39) = 4.223, p<0.01$). No significant difference in the startle response was observed between the vehicle group and the 0.1mg/kg BA group. The baseline startle amplitude was not affected by the administration of BA.
Figure 7A and B. Effects of diazepam and two doses of BA (1 mg/kg & 0.5 mg/kg) on the percentage of time spent on the open arms A) and number of unprotected head dips B) in rats in the elevated plus maze following oral administration of their respective treatments. * $P<0.05$ indicates significant differences from vehicle.

Figure 8. Effects of two doses of BA on percentage of FPS response in rats after being orally administered their respective treatments. † $P<0.01$, indicate significant differences from vehicle.
Effect of me-BA on animal behavior

Similar to the f1, me-BA significantly increased levels of social interaction ($t_{36} = 5.00, p < .00001$); see Fig. 9). Again, as observed with f1, me-BA did not affect locomotor activity (data not shown).

![Figure 9](image)

**Figure 9.** Effects on time spent in social interaction (± SEM) after the administration of 0.5mg/kg me-BA and vehicle ††† $P < 0.00001$ indicate significant differences from vehicle.

Effect of chronic administration me-BA on behavior

To further investigate the potential effects of chronically administering me-BA on rat behavior, we tested the animals after 30 days of daily dosage. Rats that were chronically treated with me-BA spent significantly more time in the open arm area than vehicle treated rats ($F (1,20) 8.016 = p<0.05$; Fig. 10a) and had a significant higher number of unprotected head dips ($F (1,20) 6.485 = p<0.05$; Fig 10b).
Figure 10A and B. Effects of chronic (30 days) oral administration of 0.5mg/kg me-BA on the percentage of time spent on the open arms A) and the number of unprotected head dips B) in rats. * P<0.05 indicates a significant difference from vehicle.

**Locomotion**

Locomotion was not significantly increased or decreased by any of the treated groups. Both the closed arm entries and number of squares crossed in the SI arena were similar between all groups (data not shown).

**Withdrawal effects of BA**

No differences were found between treatment groups on any measure of behavior, however, as expected, light phase differences were found on all measures with the exception of grooming behavior (see Table 1). This phenomenon can be explained by the rat’s innate tendency to sleep during the day and explore during the evening. Fecal counts and grams of food eaten were also analyzed using repeated-measures ANOVA. Again, no group differences were found for either behavior (see Table 2). No significant weight changes were observed throughout the 30 days of treatment (see Fig.11).
Table 1. Withdrawal effects of chronically administered BA (0.5mg/kg) and me-BA over both the light phase and dark phase of the withdrawal period. Mean time ± SE (min) spent in an activity is shown. No significant differences were found between the groups for either phase.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Light Cycle</th>
<th></th>
<th>Dark Cycle</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>BA</td>
<td>me-BA</td>
<td>Control</td>
<td>BA</td>
</tr>
<tr>
<td>Exploring</td>
<td>6.67 ± 1.71</td>
<td>4.83± 2.61</td>
<td>7.167 ± 1.30</td>
<td>22.0 ± 2.54</td>
<td>25.83 ± 2.02</td>
</tr>
<tr>
<td>Resting</td>
<td>30.17 ± 3.45</td>
<td>33.17 ± 3.4</td>
<td>25.83 ± 3.11</td>
<td>19.5 ± 1.84</td>
<td>19.17 ± 2.36</td>
</tr>
<tr>
<td>Grooming</td>
<td>4.0 ± 0.52</td>
<td>3.67 ± 1.73</td>
<td>5.167 ± 0.48</td>
<td>5.5 ± 1.06</td>
<td>3.67 ± 0.56</td>
</tr>
<tr>
<td>Scratching</td>
<td>0</td>
<td>0.17 ± 0.17</td>
<td>0.4 ± 0.25</td>
<td>3.0 ± 0.73</td>
<td>1.33 ± 0.76</td>
</tr>
</tbody>
</table>

Table 2. Withdrawal effects of chronically administered BA (0.5mg/kg) and me-BA (0.5 mg/kg) on fecal production and food consumption (g) over two consecutive days. No significant differences were found between the groups.

<table>
<thead>
<tr>
<th></th>
<th>Day One</th>
<th>Day Two</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>BA</td>
</tr>
<tr>
<td>Feces Boli</td>
<td>35.67 ± 2.69</td>
<td>32.92± 3.10</td>
</tr>
<tr>
<td>Food (g)</td>
<td>27.77 ±1.10</td>
<td>28.08± 1.78</td>
</tr>
</tbody>
</table>
Figure 11. Effects of 0.5mg/kg BA and me-BA on weight gain over time after chronic oral administration. (No significant differences)

Discussion

Both the CE and f1 of *Souroubea sympetala* reduced both innate anxiety and learned fear. As previously shown, one of the active principles is BA. Based on a previous pilot study, we again found that that 100mg/kg of the CE was an effective dose for inducing anxiolytic-like behavior in rats. We thus tested different fractions obtained from the crude extract. A series of behavioral tests was performed to further investigate the anxiolytic effects of the *Souroubea sympetala* CE, its fractions and the proposed active ingredient.

The results of this study indicate that the CE, f1, and BA found in the leaves of *Souroubea sympetala* and the semi-synthetic me-BA alleviated both anxiety and fear responses in the rat. Through standard behavioral models, the measures of both
unconditioned anxiety responses (EPM, SI) and learned fear responses (FPS) indicated a reduction in anxiety compared to control animals. In the EPM, administration of the CE, f1 or BA increased time spent on the open arms and increased the number of unprotected head dips which are indicative of an anti-anxiety effect. EPM results with BA showed a similar anxiolytic effect to those of the positive control diazepam. Findings with BA are consistent with our previous data showing similar effects in preliminary trials (Puniani 2001). Similarly in the SI test, administration of f1 or the semi-synthetic me-BA increased time spent engaged in active social interaction which is a behavioral effect also consistent with a reduction in anxiety levels. It is important to note that increased social interaction and increased time spent on the open arms of the EPM was not accompanied by changes in locomotor activity. Indeed, no sedation in overall motor activity was observed in both the EPM and SI behavioral paradigms, which is a side effect of conventional drugs like diazepam (File 1992).

The effects of the CE, f1 and BA were also tested in a FPS model of learned fear. Administration of these compounds decreased the FPS compared to vehicle treated rats. This effect was apparent in the absence of any differences in startle reactivity in the noise alone trials.

Taken together, these findings show that *Souroubea sympetala* has widespread anxiolytic properties as the effects of the CE and f1 were evident across a variety of different anxiety and fear provoking situations. Furthermore, the findings provide further evidence that BA is one of the active ingredients of this plant.
As for the effect of chronic administration of BA and me-BA in the rats, there was no weight loss or withdrawal symptoms associated with its consumption. It is known that chronic use of TCA’s and MAOI’s has been associated with increased appetite and weight gain (Allison & Casey 2001; Mihara, McCombs, & Williams 2005; Nasrallah 2003; Vanina et al. 2002), while conversely, other studies indicated weight loss during withdrawal from dependence-inducing anxiolytic substances such as benzodiazepines and morphine. Our measurements revealed no differences in body weight between groups at any point during the study, nor were any there any differences in food intake found during the withdrawal period. Feces counts were taken primarily as an indication of normal functioning, but also to look for the presence of diarrhea or constipation during withdrawal. Again, no differences were found in these measures. As shown in the results, the cessation of chronic administration of both BA and me-BA showed no detectable differences in any behavior at any time during the withdrawal period between the rats treated chronically and controls.

Overall it can be concluded that Souroubea sympetala extract reduces both innate anxiety and learned fear and that BA is one of the bioactive ingredients.
Rhodiola rosea L. (Crassulaceae), with common names rose root, arctic root, is a circumpolar species of arctic and alpine regions. Roots of this perennial herbaceous plant have been used medicinally by Chinese, Russian, Scandinavian and Inuit people for centuries. It is especially popular in Eurasia, where it is valued for its reputed “adaptogenic” properties, i.e. that the roots of this plant offer an increase resistance to cold, raise cognitive vigilance and alleviate depression and anxiety symptoms (Panossian, Wikman, & Sarris 2010). One report has shown that oral administration of an ethanol extract of a Eurasian collection induced antidepressive but also anxiolytic activity in mice(Perfumi & Mattioli 2007). Phytochemical markers that are present in the roots of this species are shown in Fig. 12. It is not known if these compounds have any activity in behavioral effects.
Figure 12. Structure of phenolic glycosides from present in *Rhodiola rosea* roots. (Fillion 2008)

Recently our research group discovered a large population of the plant along the eastern shores of Ungava Bay in Nunavik, northern Quebec (Fillion 2008). This local roseroot variety is largely unstudied except that it has been shown to have the same phytochemical markers as seen in the Eurasian variety. This population of plants is of interest to the Inuit of Nunavik, as a potential natural resource. Therefore, the purpose of the present study was to fully characterize extracts of the Nunavik material in well defined behavioral assays for anxiety to assess its potential as a Natural Health Product for treatment of anxiety.
Methods

The same conditions were used as with in *Souroubea sympetala* experiment with the exceptions indicated below.

**Plant material**

*Rhodiola rosea* root material was collected under permit in Nunavik, Canada. Samples were dried overnight in a commercial plant drier at 35°C and ground to 2 mm mesh. A Voucher specimen was deposited in the University of Ottawa Herbarium (OH No. 19847).

**Plant extraction**

Plant material was subsequently macerated with EtOH (10mL/g) in a food blender and the resulting dark mixture was filtered under suction in a Buchner funnel through
Whatmann no 1 paper. The solids were washed with additional ethanol (5 mL/g); the alcohol and water were then evaporated, using a rotary evaporator and then under vacuum (1 mm Hg) to yield the *Rhodiola rosea* crude extract (7%).

**Drug Administration**

Rats were randomly assigned to one of four treatment groups: vehicle (2 ml/kg) and the three plant extract doses; low dose (8 mg/kg), medium dose (25 mg/kg) and high dose (75 mg/kg). Rats received their respective treatments for three consecutive days until testing. They received their last dose 60 minutes prior to testing.

**Behaviour: Elevated Plus Maze (EPM)**

As described in chapter 2.

**Social interaction (SI)**

As described in chapter 2.

**Conditioned emotional response (CER)**

Apparatus: The conditioning chamber (Coulbourn Instruments) measured 31 cm × 25 cm × 30 cm. The front and back walls were made of clear Plexiglas and two side walls made of stainless steel panels. The floor was composed of 16 stainless steel rods (4 mm diameter, 1.4 cm apart), which were connected to a Coulbourn Instruments shock generator (model H13–16) that delivered constant current.
Procedure: All subjects completed 1 day of training followed by a day of testing 24-h later. During the contextual training phase, subjects were laced in the conditioning chamber where they received 6 footshocks (1.0mA; 1-s in duration) with an average intertrial interval (ITI) of 1-min. On the test days, contextual fear was assessed over a 15-min period by placing them in the conditioning chamber where they had previously been shocked. Freezing behavior was determined by the absence of movement excluding involuntary respiratory. The absence or presence of complete immobility was recorded over the course of the test period. Evaluations of freezing were conducted by trained experimenters blind to the drug condition. Between each training and testing session, cages were cleaned with 70% ethanol.

Results

EPM

Different doses of the *Rhodiola rosea* extract were compared in the EPM. As depicted in Figure 14A, there was a dose dependent increase in time spent in the open arms and the number of unprotected head dips. Only the high dose of 75 mg/kg showed a significant increase in the percentage of time spent in the open arms (F (3, 38) 7.389 = p<0.001) while the number of UH (Fig.14B) was significantly higher following administration of both the medium and high doses F (3,38) 7.325 = p<0.001 & p< 0.0001 respectively).
Figure 14 A and B. A) Percentage of time (±SEM) spent in the open arms and B) the number (±SEM) of unprotected head dips on the elevated plus maze following three consecutive daily oral administration of *Rhodiola rosea* (8, 25, 75 mg/kg). † p < 0.001, †† p < .0001 indicate significant differences from vehicle.

SI

In the SI experiment (Figure 15), oral administration of the medium dose of the extract elicited a significant increase in the amount of time spent engaged in active social interaction compared to vehicle or the low or high dose of the extract (F (3,31) 4.908= p<0.01 ). There was no dose dependent increase in total social activity. The SI experiment also indicated the absence of locomotion perturbations (data not shown).
Figure 15. Effects on time spent in social interaction following three consecutive daily administration of *Rhodiola rosea* (8, 25, 75 mg/kg). * p < 0.01 indicate significant differences from vehicle.

CER

In the contextual CER test, total time spent freezing was significantly lower in *Rhodiola rosea* treated rats compared to those that received the vehicle treatment (F 3, 656 = 53.46, p < .0001; Fig. 16). Analysis of the time spent freezing when broken into 3-5 min time blocked revealed a significant treatment x time block interaction; Fig. 17). Both of these results showed clear dose dependence. Follow-up analyses revealed that during the first time block only the high dose of the extract differed from the vehicle animals, during the time block, both high and medium doses significantly differed from controls, while by the third time block, all doses elicited significantly lower levels of freezing compared to vehicle-treated controls. Self grooming behavior was also monitored but showed no significant differences (data not shown).
Figure 16. Total time engaged in freezing over a 900 secs. period (±SEM) following three consecutive daily oral administration of *Rhodiola rosea* (8, 25, 75 mg/kg) in the contextual CER paradigm. † p < 0.001, †† p < .001 indicate significant differences from vehicle.

Figure 17. The breakdown in 3 time blocks of the freezing time (±SEM) expressed as a percentage following three consecutive daily oral administration of *Rhodiola rosea* (8, 25, 75 mg/kg) in the contextual CER paradigm. * p < 0.01, † p < .001, †† p < .0001 indicate significant differences from vehicle.
Discussion

Acute administration of the *Rhodiola rosea* CE (3 consecutive days) mildly increased exploratory open arm behavior at the high dose and total social interaction was only slightly increased at the medium dose (borderline significance). Interestingly, we observed a large magnitude response with the CER test which was highly significant. The conditioned fear response was effectively reduced with all three doses thus indicating a strong fear attenuation effect of the CE of this plant when administered to rats. It would seem the the *Rhodiola rosea* CE is more involved in learned fear responses vs innate anxiety response. Although there is little information on Inuit uses of the plant except for food, the above fear responses are consistent with Icelandic historical use of the plant as a preparation for warriors to reduce fear before battle (Filion, 2008). The active principles in rose root are unconfirmed, but the the rosavin and salidroside phenolics are the candidate phytochemicals.
CHAPTER 4  *Piper amalago* a social anxiety reducing plant

Historically, cultivated pepper plants (*Piper nigrum, P. guianense* and *P. longum*) have been used as a valuable and popular commodity and their widespread usage is prevalent around the globe. *Piper methysticum*, “Kava”, is a widely used commercial phytomedicine indicated for anxiety which is well supported in animal and clinical research (Sarris and Kavanagh, 2009). The Piperaceae family is pantropicaly distributed and there are 2000 species in the neotropics where the family is most abundant. In previous ethnobotanical work by our laboratory, the Piperaceae were identified as being a highly used plant family for mental health conditions (Bourbonnais-Spear et al 2005). *Piper amalago L, “cordonsillo”* the species used for the present study was found to be a high consensus plant for “susto” a culture bound syndrome associated with anxiety.

The phytochemistry of the Piperaceae reveals piperamides, lignans and other secondary metabolites. As for a commercial pepper, the piperamide piperine is predominantly present. A recent study showed that piperine exhibits an anti-depressive effect through the serotoninergic system (Mao et al. 2011). The active anxiolytic principles of kava are kavalactones, which act at the GABAa receptor (Sarris and Kavanagh, 2009). Phytochemical analysis in our laboratory Mullally (2011) has shown that both piperamides and lignans are present in *Piper amalago* (Fig 18.). Bioguided isolation showed that one lignan has high affinity in a GABAa binding assay suggesting that this compound is a candidate active principle.
The hypothesis of the current work was that the ethnobotanical consensus data on susto suggesting that *P. amalago* has anxiolytic properties can be verified through animal trials. It is predicted that these properties will be revealed in significant alteration of behavior in the standardized anxiety paradigms by *P. amalago* extracts. Because of time restraints and low availability of plant material, the experimental conditions with *Piper amalago* deviated slightly from those employed with the two other plants. Rats were given a single administration instead of three at each dose and no familiarization with the arena was undertaken prior to SI testing.
Methods

The same conditions were used as with in *Rhodiola rosea* experiment with the exceptions indicated below.

**Plant material**

*Piper amalago* leaves were collected under permit in Belize. Samples were dried overnight in a commercial plant drier at 35°C and ground to 2 mm mesh. A Voucher specimen was deposited in the University of Ottawa Herbarium (OH No. #$%#$%).

**Plant extraction**

Plant material was subsequently macerated with EtOH (10mL/g) in a food blender and the resulting dark mixture was filtered under suction in a Buchner funnel through Whatmann no 1 paper, the solids were washed with additional ethanol (5 mL/g); the alcohol and water were then evaporated, using a rotary evaporator and then under vacuum (1mm Hg) to yield the *Piper amalago* crude extract (13 %).

**Drug Administration**

Each treatment group received a single dose (8, 25 and 75mg/ kg) 60 minutes prior to each test.
Behaviour: Elevated Plus Maze (EPM)

As described in chapter 2.

Social interaction (SI)

As described in chapter 2, except with the following details. SI experiments were done under semi-aversive (high illumination, semi- familiar environment) conditions. Rats did not have a second habituation day (familiarization with arena).

Conditioned emotional response (CER)

On the test days, contextual fear was assessed over a 20-min period by placing them in the conditioning chamber where they had previously been shocked.
Results

EPM

Different doses of the *Piper amalago* extract were compared in the EPM and there was a clear dose dependent effect on both time spent in open arms but not the number of unprotected head dips (Fig. 20a. and b). Only the medium and high doses showed a significant increase in the percentage of time spent in the open arms (F (4, 41) 3.996= p<0.05 and p<0.01). The number of UH (Fig.20b) showed significance in both low and high doses (F (4,41) 2.238 = p<0.05). A trend was apparent for the medium dose but no significance was observed. There were no significant differences between the high dose and positive control diazepam in both the % open arm time and the # of unprotected head dips.

![Figure 20 A and B](image_url)

*Figure 20 A and B  A) Percentage time (±SEM) spent in the open arms and B) the number (±SEM) of unprotected head dips on the elevated plus maze following a single oral administration of *Piper amalago* (8, 25, 75 mg/kg).* * p < 0.05 , † p < 0.01 indicate significant differences from vehicle.
SI

In the SI experiment (Fig.21), oral administration of the low and the high doses of the extract significantly increased the amount of time spent engaged in active social interaction compared to vehicle administration (F (3,37) 16.742= p<0.01 ). The significance was more evident with the medium dose (F (3,37) 16.742= p<0.0001 ). The SI experiment also indicated the absence of locomotion perturbations (data not shown).

Figure 21. Effects on time spent in social interaction following a single dose of *Piper amalago* (8, 25, 75 mg/kg). † p <0.01, †† †† P <0.0001 indicate significant differences from vehicle.

CER

In the contextual CER test, total time spent freezing declined with dose and was significantly lower in *Piper amalago* only high dose treated rats compared to those that received the vehicle treatment (F 3, 29 = 2.479, p < .05; Fig. 22). Analysis of the time spent freezing when broken into four time blocks of 5 mins. revealed significance in the
third time block with the high dose only. All doses elicited significantly lower levels of freezing compared to vehicle-treated controls in the fourth time block. Self-grooming behavior was also monitored but showed no significant differences (data not shown).

![Graph showing percentage of time engaged in freezing following a single oral dose of Piper amalago (8, 25, 75 mg/kg) in the contextual CER paradigm. * p <0.05 indicates a significant difference from vehicle.]

Figure 22. Percentage of time engaged in freezing following a single oral dose of *Piper amalago* (8, 25, 75 mg/kg) in the contextual CER paradigm. * p <0.05 indicates a significant difference from vehicle.
**Discussion**

The CE of *Piper amalago* leaves caused a significant increase in open arm exploratory behavior and in total social activity after only a single administration of the CE. The fear extinction process seen in the CER test was effective only at the highest dose. These effects are similar to the effects of kava, *Piper mythisticum*, in promoting social interaction. Thus considering that both *Piper amalago* and *Piper mythisticum* share the same genus, it is possible that the anxiolytic activity observed with these plants is produced by mutual compounds. Ongoing work currently being conducted is using bioassay guided isolation to identify active principles in *Piper amalago*, which appear to
be piperamides and lignans. Studies by Awad and colleagues have shown that the piperamides inhibit GABA transaminase, which is an enzyme metabolizing GABA (Awad et al. 2007). Increased GABA levels in the brain may account for these behavioural effects. Piperine is known to inhibit enzymes that are important in drug metabolism (Atal, Dubey, & Singh 1985). Therefore it is plausible that piperine inhibits catabolism of phytochemicals present in the extract (including itself) and acts as an enhancer of the anxiolytic compounds present in *Piper amalago.*
CHAPTER 5  General discussion

Investigation through behavioral testing revealed that administration of the crude extract (CE) of all three plants resulted in various degrees of anti-anxiety effects in adult male Sprague Dawley rats. The anxiety-like behaviours measured in each test differed in intensity and specificity from one plant to the other.

*Souroubea sympetala* was by far the most extensively studied plant of the three. We investigated not only the CE of the leaves but also several fractions and some isolated pure compounds. Through bioassay guided isolation we have identified an active ingredient: Betulinic Acid. Rats that received BA expressed the same response as the positive control diazepam. Also, we have tested a more soluble and semi-synthetic methyl-ester derivative of BA which induced similar anxiolytic effects to those of BA itself. Verification of potential deleterious consequences from long term oral administration of both the BA and me-BA was also assessed. Currently, there is an ongoing effort both at the academic and industrial level to further our understanding of the pharmacological and endocrine activity plants elicit after oral administration. More behavioral tests are being done to further validate our current findings.

Open arm exploratory behavior, total social interaction and startle response attenuation were all increased after an acute dosage (3 consecutive days) and chronic administration (30 days) of either the *Souroubea sympetala* CE or BA.
As shown in chapter 3, administration of the *Rhodiola rosea* CE elicited anxiolytic activity and was highly effective in attenuating the learned fear response. As shown in chapter 4, administration of the *Piper amalago* CE was also anxiolytic but elicited more activity in the social interaction paradigm.

In conclusion, we can confirm that the hypothesis that the traditional use of these plants is based on a pharmacological effect on behaviour is upheld in the rat model. Also, the predication that variable phytochemistry would lead to a different profile of activity in the behavioural test was supported.

**Table 3. Summary of the behavioral results**

<table>
<thead>
<tr>
<th></th>
<th>Doses</th>
<th>EPM</th>
<th>SI</th>
<th>CER</th>
<th>FPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Souroubea sympatela</em> (CE, f1)</td>
<td>100 mg/kg</td>
<td>↑</td>
<td>↑</td>
<td>*↑</td>
<td>↑</td>
</tr>
<tr>
<td><strong>BA</strong></td>
<td>0.1, 0.5, 1 mg/kg</td>
<td>n/a,↑,↑</td>
<td>*0</td>
<td>n/a</td>
<td>0,↑,n/a</td>
</tr>
<tr>
<td><strong>me-BA</strong></td>
<td>0.5 mg/kg</td>
<td>↑</td>
<td>↑</td>
<td>*↑</td>
<td>0↑</td>
</tr>
<tr>
<td><strong>Piper Amalago</strong></td>
<td>8, 25, 100 mg/kg</td>
<td>0,↑,↑</td>
<td>↑,↑,↑</td>
<td>0,0,↑</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Rhodiola Rosea</strong></td>
<td>8, 25, 100 mg/kg</td>
<td>0,↑,↑</td>
<td>0,↑,0</td>
<td>↑,↑,↑</td>
<td>n/a</td>
</tr>
</tbody>
</table>

* data not shown (collaboration with Martha Mullaly)

↑ Anxiolysis

0 no effect

The methods used in these various behavioral tests provide a toolkit in which researchers can assess the anxiolytic effects of drugs, in our case plant materials. The standardization of these tests has favored rapid communication and replicability of
studies. However, there are no absolute correlations with clinical research results. This
requires us to be prudent when bringing forward an interpretation of results.

The EPM test is often used in rodents as a golden standard for preliminary
investigation into possible behavioral modulation. Potential anxiolytic or anxiogenic
compounds are often initially screened by this simple paradigm. It thus seemed logical to
initiate our battery of behavioral tests for each plant with this paradigm. Since this test is
by far the most widely used test in animal anxiety research, there are a lot of theories as
to which CNS systems are involved. Ethologically the contextual contrast between the
open arms and the closed arm areas is meant to measure in each individual rat their
natural propensity for exploratory behavior. Because of the increased risk of predatory
challenge, the open arm area is considered an aversive stimulus, and also because of the
natural fear of rats to open spaces (Pellow 1986). As for which neurotransmitter systems
are involved in the behavioral expression in the EPM, there are still a lot of unknown
variables. But a predominant amount of pharmacological studies with this specific test
have shown that modulation of the GABA\textsubscript{A} receptor via agonists is a major factor in
modulating the anxiety-like response behaviour in the EPM (Gonzalez, Ouagazzal, &
File 1998;Menard & Treit 1999;Rezayat et al. 2005). But there are studies that also
indicate also a serotonergic involvement in mediating EPM anxiety-like behavior
(Grundmann et al. 2007;Rodgers, Cole, & Davies 1994;Rodgers, Cutler, & Jackson
1997).
The SI test uses both lighting and novelty as an aversive stimulus to gauge behavioral response in mice and rats. What is interesting about this test is that a cross-species ethological link can almost be extrapolated between rats exposed to this test and humans suffering from social anxiety disorder. Social anxiety disorder symptoms have clinical similarities to people suffering from general anxiety disorder. The neurochemical circuitries involved seem to overlap in both types of disorders (Ganasen & Stein 2010; Stein & Vythilingum 2007; Stein, Westenberg, & Liebowitz 2002). Also, the SI test seems to differ from the EPM test on a neuropharmacological level as some studies suggest. More specifically, these studies suggest that the CNS circuitries involved in SI act more through the serotonergic receptors than by the GABA$_A$ receptors (Gonzalez, Andrews, & File 1996). One drawback of this paradigm is that testing occurs in pairs. It is thus difficult to examine individual behavioral differences.

As for the CER paradigm, there is strong evidence that the conditioning is highly associated with amygdalar neuronal control (Spevack, Campbell, & Drake 1975). Furthermore, many findings have shown that the freezing response in CER is strongly associated with amygdalar GABAergic activity (Davis et al. 2006).

In the FPS, it has been shown that electrolytic lesions of the amygdala blocks the startle response (Hitchcock 1987). Like CER, the FPS effect is highly dependant on the amygdaloid complex (Davis 1993a; Puniani 2001). Functional anatomy studies show that several nuclei of the amygdala have reciprocal connections to various cortices, including the medial prefrontal cortex (Sah, Faber, Lopez de, & Power 2003). Of the
17 different nuclei of the amygdala that have these two way connections, the basolateral and central nuclei have electrophysiological evidence that show synaptic activation of the GABA_A receptors (Martina, Royer, & Pare 2001). Since the FPS paradigm is associated with loud auditory stimulus, some researchers have brought forward that this test can be the rodent equivalent of post-traumatic stress disorder (Cohen et al. 2006; Morgan, III et al. 1995; Smith et al. 2011). As described above, we chose these particular tests in order to make an attempt at creating a link between animal behavior research and human psychopathology.

The only pure compound presented in this thesis is BA and derivative me-BA. Both did elicit anxiolytic properties when administered to rats. But this does not exclude the presence of other anxiolytic phytochemicals in the studied plant. As shown in figure 5 there is some significant difference between the CE and f1 response to startle potentiation. The CE showed an increased attenuation in the startle response. Thus, it is possible that biochemical phenomena such synergy and pleiotropy might be occurring without our knowledge (Bol et al. 2000; Butterweck & Schmidt 2007). One report suggests such a pleiotropy effect with the plant extract of Cinnamomum cassia (Yu, Lee, & Jang 2007). The high level of anxiolytic like activity of the CE suggests that other components may have an additive or synergistic effect. The presence of other triterpenes and flavonoids such as alpha and beta amyrin and Ursolic acid have been detected through our previous HPLC LC/MS analysis (Puniani 2001). The existence of BA and related triterpenes has been known for many decades. Recently, the discovery that BA
derivatives have a considerable potential in the treatment of cancer and HIV has caused an increased interest in this compound (Yogeeswari & Sriram 2005).

Figure 24. Alpha Amyrin (left) and Ursolic acid (right)

The prevalence of various anxiety related illness throughout the world is a pressing health issue. Currently approximately one fifth of the world adult population will suffer from any anxiety disorder during their lifetime (Filion 2008; Kessler & Wang 2008; Sheehan & Sheehan 2007a). In the past decades, there has been an increase in the globalization of information which has brought a rising awareness of the deleterious side effects linked with classic psychopharmacology. Alternative therapies which are regarded as more appealing to patients are being self administered by afflicted people in western society (Lakhan & Vieira 2010). One major component of this alternative trend is the usage of plant parts which are administered in a variety of ways (Yeung, Gubili, & Cassileth 2008). As mentioned previously, herbal therapy is becoming extensively prevalent throughout the world and not only limited to developing countries. Thus the demand and use of medicinal plant remedies has inevitably risen in western countries over the past decades (Ernst 2006). Although the usage of natural health products has
risen over the past years, the pharmaceutical research interests in this field have declined over the last decade. The main reason for the recent slowdown is that the research in this field is time-consuming, highly complex and has ineffective marketability (Kong, Li, & Zhang 2009). By increasing scientific research into the phytotherapy field, and thus offering plausible scientific explanations based on empirical evidence, this will hopefully result in clinical applications and industrial viability.

Considering the taxing cost of the current healing practices that are being applied in most developed urban areas, it would seem highly inefficient to transpose these “westernized” treatments to poorer and less developed areas of population. In light of current findings, including ours, the contrary might even be a more manageable approach to treating anxiety related symptoms.

But there are also serious medical concerns about potential drug-herb interactions when using plants as treatments (Tarirai, Viljoen, & Hamman 2010; Taylor et al. 2006a). Presently, half of individuals suffering from anxiety disorders using prescribed drugs are concomitantly using CAM (Taylor et al. 2006b). It is thus important to further study the neuropharmacological mechanisms behind the anxiolytic activity of currently used plants.

These plants could also have potential applications in the veterinary sector. They could help alleviate certain anxiogenic factors that are presently influencing the livestock in this industry. Animals raised in large scale industrial farms are housed in an environmentally stressful context and express high levels of glucocorticoids, resulting in
reduced reproductive efficiency and limited yield in overall productivity. Combining anxiolytic plant material with feed might be a viable solution in reducing circulating glucocorticoids in these farm animals.

Future studies will be needed to properly confirm the potential clinical applications of these plant materials, be it their extract or their proposed active compounds. Neuronal membrane receptor binding studies with plant extracts would help us determine their binding affinities to GABA and 5-HT receptors. It would help us also to determine where in the brain these anxiolytic phytochemicals might elicit anti-anxiety effects. As shown by many previous studies, a route of action through the GABAergic system seems highly probable. We are currently investigating the effect of these three plants with GABA<sub>A</sub> receptor binding assays. One plant thus far, *Souroubea sympetala*, has a confirmed active principle in animal trials and in vitro bioassay guided isolation suggested furofuran ligans may be the active principle of *P amalago*. This phytochemical should be studied *in vivo*. A full workup of active principles is needed for *R rosea*.

In conclusion, recognizing that these extracts exhibit significant anxiolytic properties in rats may indicate therapeutic relevance in humans. As evidence accumulates for significant anxiolytic activity of natural health products, it is likely that this avenue of therapy still merit mainstream medical consideration in the future.
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