Exploration of Cannabinoid and Opioid Agents from Plants Used in Traditional Medicine

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EXPLORATION OF CANNABINOID AND OPIOID AGENTS FROM PLANTS USED IN TRADITIONAL MEDICINE

By:
John Carey Fitzpatrick

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford, MS May 2015

Approved by

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Reader: Dr. Christopher R. McCurdy
I would like to thank the Sally McDonnell Barksdale Honors College for the opportunity to participate in this research project and to write a thesis on my findings. I would also like to thank Dr. Stephen Cutler and Dr. Francisco León for their time and dedication to this project. Without their guidance and leadership this would not have been possible.
ABSTRACT

JOHN CAREY FITZPATRICK: EXPLORATION OF CANNABINOID AND OPIOID AGENTS FROM PLANTS USED IN TRADITIONAL MEDICINE
(Under the direction of Dr. Stephen Cutler)

Ten plants, with known medicinal uses, were gathered to study for their possible production of secondary metabolites that act as opioid and cannabinoid agents. All plants were subjected to in vitro bioassays testing for their affinity for the opioid and cannabinoid receptors. *Banisteriopsis caapi* extracts displayed the greatest affinity for both opioid and cannabinoid receptors. *B. caapi* extracts underwent column fractionation yielding fractions that were subjected to further in vitro studies. We discovered that the ethanolic extract of this plant (foliage) binds to all three opioid receptors (µ, κ and δ) and cannabinoid receptors (CB1 and CB2) with values ranging from 60-90% displacement of the respective radioligands for these receptors. Based on the polarity, we observed that the highest percent of radioligand displacement was noted in the 50:50 hexanes:ethyl acetate fraction on CB1, CB2 and δ-opioid receptors with values of 86.0, 86.9 and 79.3, respectively.
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<tr>
<td>OH</td>
<td>Alcohol</td>
</tr>
<tr>
<td>H</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput Screening</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>THC</td>
<td>Δ⁹-Tetrahydrocannabinol</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid Receptor Type 1</td>
</tr>
<tr>
<td>CB2</td>
<td>Cannabinoid Receptor Type 2</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetone</td>
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<tr>
<td>THH</td>
<td>Tetrahydroharmine</td>
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<tr>
<td>DMT</td>
<td>Dimethyltryptamine</td>
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<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>EtOAc</td>
<td>Ethyl Acetate</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-Layer Chromatography</td>
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<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple-Bond Correlation</td>
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<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Coherence</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>TMS</td>
<td>Trimethylsilane</td>
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<tr>
<td>COSY</td>
<td>Homonuclear Correlation Spectroscopy</td>
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<td>TOCSY</td>
<td>Total Correlation Spectroscopy</td>
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<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Effect Spectroscopy</td>
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<tr>
<td>DEPT</td>
<td>Distortionless Enhancement by Polarization Transfer</td>
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<tr>
<td>HRMS</td>
<td>High-Resolution Mass Spectra</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric Acid</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Correlation</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>Hz</td>
<td>Hetz</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<td>µ</td>
<td>Mu</td>
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<tr>
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<td>δ</td>
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Background

I. Contributions of Bioactive Natural Products to Drug Discovery

Nature has always been a great resource for human advancement. Throughout history, humans have experimented with natural products to produce many different types of goods such as perfumes, spices, and even medicinal remedies. Natural products, or secondary metabolites derived from natural sources, have been a significant resource for the development and production of pharmaceutical compounds (Corrado, 2012). From 1981-2002, 67% of drugs developed worldwide were derived from bioactive natural products or mimics of those products (Cragg, 2006). Within the same time period, 70% of anti-infective drugs were naturally derived, while 67% of anticancer drugs were also naturally derived or inspired from a natural product leads (Cragg, 2006).

Although there is some recent sentiment from the scientific community that the future of medicinal chemistry lies in synthetically produced compounds, there is still much value to be appropriated from natural products. Despite secondary metabolites sharing similar size, structure, functional groups, orientation and stereochemical complexity as synthetic compounds, they alternatively are much more economical to
produce than their synthetic counterparts (Cragg, 1997). It is both economically infeasible and difficult to implement chemical synthesis of compounds that require large-scale multistep reactions to produce synthetic equivalents to the naturally occurring secondary metabolites (Corrado, 2012). Therefore, using the more economical naturally occurring material to conduct the research to isolate and structurally elucidate the metabolites is a better use of financial resources. Additionally, nature provides a wide array of chemical templates that are far beyond the imagination of most organic chemists.

Finding bioactive compounds that can be used for pharmacological and medicinal purposes is the main goal in natural product research. In the past, natural product chemists limited the scope of their work to the isolation and structural elucidation of secondary metabolites due to the lack of communication with biologists studying animal and plant species. For example, biologists have described about 40,000 species of spiders yet less than 1% of their venoms have been investigated (Cragg, 1997). In recent times, the increased communication between biologists and chemists studying natural products has led to the development of the bioassay-guided approach to isolation and structural elucidation of secondary metabolites (Cragg, 2006). This new field has exponentially grown the number of pharmacologically significant metabolites elucidated. Most ancient remedies that have been in use dating back as far as 2800 BC have sense been
investigated for their secondary metabolites. A good example of a discovery resulting from the collaboration between the biologists and chemists are the *Ginkgo biloba* extracts.

The *Ginkgo biloba tree*, which ancient Chinese culture used as a remedy for lethargy, memory loss, mental fuzziness, and vertigo, has been extensively studied and is now known to contain ginkgolides that have shown to be antagonists of platelet aggregation factor (Cragg, 1997). *Ginkgo biloba tree* extracts have shown *in vitro* study results producing neuroprotective effects associated with the extracts’ antioxidant properties of their flavonoid constitutes as well as the extracts’ ability to inhibit nitric oxide-stimulated protein kinase C activity (Bastianetto, 2000). The extracts also exhibited possible uses in neurodegenerative diseases such as Alzheimer’s disease through the inhibition of amyloid-β-induced apoptosis in neuroblastoma cells (Wu, 2006). The major components present in the *Ginkgo biloba* are shown in Figure 1.
Much like the discovery of the *G. biloba* tree extracts, our research targets active compounds from plants used in traditional medicine. Many sources such as plants, marine and microbial organisms have been tested through bioassay-guided fractionation ultimately leading to purification of the active compounds within the samples. Any active compounds that have significant *in vivo* results in animal models may later be used for drug development leading to clinical trials (Cragg, 2006). Although this bioassay guided approach has led to many successful clinical trials the process is tedious and time consuming. To improve the efficiency of the bioassay guided approach highthroughput screening (HTS), where multiple plants are evaluated at the same time, has been adopted.
by most labs to increase the yield of active compounds. Through this extremely sophisticated instrumentation and information technology, laboratories have been able to screen massive amounts of compounds leading to significant drug discoveries. Although this method has historically increased the output of active compounds, it does not hold similarly promising efficacy for the future (Mouhssen, 2013). Over the past few decades HTS has experienced diminishing returns leading to a drug development bottleneck.

A technique, reverse pharmacology, aims to reverse the drug development bottleneck. This technique focuses on transdisciplinary exploratory studies and development of drug candidate leads stemming from clinical experiences and experimental observations (Vaidya, 2007). Taking observations from current preclinical and clinical research to better target compound leads in combination with the highthroughput screening could dramatically improve the perceived drug discovery bottleneck (Mouhssen, 2013). The major downside to reverse pharmacology is the risks that are inherent with patient trials being conducted prior to in depth laboratory investigation.

Past discoveries of secondary metabolites have led to very specific assays targeting a single pharmacological use of the natural compound within the samples. There are anticancer, antiviral, genotoxicity, hepatotoxicity, anti-inflammatory,
immunomodulating, gastroprotective, analgesic, and many more types of biological assays (Rahman, 2001). Natural products have been used for several thousand years to produce powerful analgesia by binding to specific targets (Soares-Bezerra, 2013). Certain ATP-gated ion channels within the P2X channel subfamily are providing convincing evidence for their use as pharmacological targets to control both analgesia and inflammation (Soares-Bezerra, 2013). In addition to ligand-gated ion channels such as the P2X purinergic receptors, G protein coupled receptors, shown in Figure 2, such as the opioid and cannabinoid receptors are also providing convincing evidence of powerful analgesic effects by blocking the release of pain propagating neurotransmitters (Cichewicz, 2003). Based on the evidence showing the usefulness of the cannabinoid and opioid receptors as targets for pain modulation, we used cannabinoid and opioid receptor bioassays to evaluate the binding affinity of different plant extracts.
II. Opioid and Cannabinoid Receptors Role in Pain Modulation

For the past 12,000 years there has been documented use of marijuana from areas in Asia to India (Abel, 1979). The hemp plant has been used in many different capacities from clothing to medical treatment. It was used to treat a wide array of ailments including fevers, sleeping disorders, lack of appetite, headache relief and venereal diseases (Mechoulam, 1987). The main psychoactive compound in cannabis is $\Delta^9$-tetrahydrocannabinol (THC), which was isolated and purified in 1965. The chemical structure of THC is shown in Figure 3.
THC’s discovery sparked interest and research dedicated to determining the mechanism by which the compound affected the human body (Welch, 2009). These classes of compounds that encompass THC are referred to as the cannabinoids and they all use the same mechanism that triggers a physiological response. After much research, scientists concluded that there existed neurological receptors infused in some cell types in the human body that are now referred to as the cannabinoid receptors (Welch, 2009). The discovery of the cannabinoid receptors led to further research investigating other ligands that have binding affinities for the cannabinoid receptors. Many compounds, both cannabinoids and noncannabinoids, have been discovered to have significant modulation of the cannabinoid receptors as agonists and antagonists. The cannabinoid receptors are
present in high density in the areas of the brain most associated with emotions, spatial judgment, motor function, and perception of time (Glass, 1997).

The cannabinoid receptors are categorized into two different subtypes, the CB1 and CB2 receptor subclasses. The CB1 receptors are found both in the nervous and non-nervous tissues and is the primary contributor to the central nervous system (CNS) effects caused by the cannabinoids (Mackie, 2008). Although the CB1 is found in various types of tissue it is most prevalent in nervous tissue at the nerve terminals (Mackie, 2008). The CB1 subtype is subject to cannabinoid agonists that contribute to classic symptoms of cannabis usage including: treatment of nausea for certain types of cancer patients, appetite stimulant, relief for ocular swelling associated with glaucoma, and increased motor function in patients with spinal cord ailments (Pertwee, 2006).

The CB2 receptor subclass, which has a lot of homology to the CB1 subclass, is not primarily located in CNS tissue; it is heavily distributed throughout various immune cells (Mackie, 2008). For the past ten years, it was commonly accepted that the CB1 receptor subclasses were the primary contributor to side effects associated with cannabinoid agonists. Recently, a functional presence of CB2 receptors has been studied and new conclusions have been proposed (Onaivi, 2006). The CB2 receptors are now believed to be contributors to depression and drug abuse related to the cannabinoid
agonist. These conclusions have been drawn based on studies showing neuronal CB2 receptors involved in emesis (Onaivi, 2006). A human study was conducted and a CB2 gene type, Q63R polymorphism, which affects the immunomodulating properties of the CB2 receptor, was abundantly present in subjects who were chronically depressed alcoholics (Onaivi, 2006). These new conclusions are shedding light on the possibility that the CB2 receptors play a much larger role in responses to cannabinoid agonists.

Opium poppy, used for its psychotropic affects, has been widespread and its use documented back to antiquity. There is even great speculation amongst anthropologists and historians that the great poet Homer mentioned Helen of Troy in the epic The Odyssey using opium to drug Odysseus’ friends. Opium was utilized in religious ceremonies as a euphoriant, which was traditionally heated and inhaled (Kritikos, 1967). It was not until 1806 that the compound in opium responsible for most of the CNS effects was isolated and named morphine (Brownstein, 1993). Not long after the discovery of morphine, codeine was isolated and structurally characterized from the opium poppy (Brownstein, 1993).

Following the discovery of morphine, an alkaloid and weak base, it began to be manufactured in large amounts for medical purposes, particularly for its analgetic effects. It was found to be a very addictive drug with high abuse. Researchers were then enlisted
to discover a powerful opiate anesthetic such as morphine, but with reduced addictive characteristics. Heroin was then developed as a new, non-addictive and more powerful substitute to morphine (Brownstein, 1993). However, heroin proved to be extremely addictive and was not an adequate replacement for morphine as an anesthetic and analgesic. Figure 4 below shows the similarities in heroin and morphine’s chemical structures and how chemists designed this potent analgesic agent.

![Chemical structure of morphine, heroin, and codeine](image)

Morphine: $R_1 = H$, $R_2 = H$
Heroin: $R_1 = Ac$, $R_2 = Ac$
Codeine C: $R_1 = Me$, $R_2 = H$

Chemical structure of morphine, heroin, and codeine  
**Figure 4**

Scientists continued to search for replacements for morphine both in nature and through chemical synthesis. In 1939, meperidine was synthesized and was the first opiate
discovered to have a different chemical structure to that of morphine (Brownstein, 1993).

Methadone was later discovered as another opiate with a varying chemical structure to that of morphine (Brownstein, 1993). Later, in 1942, the first ever antagonist to opiates was discovered and named nalorphine. Nalorphine, as seen in Figure 5, and other opiate antagonists were able to reverse the affects of morphine and other opiates while also producing considerable analgesia (Brownstein, 1993).

![Nalorphine Chemical structure](image)

While there were great strides made in the early 20th century in the discovery of various types of opiate agonists and antagonists, the mechanism by which these drugs functioned as well as the receptors they were targeting was still unknown. In 1973,
researchers discovered that there were stereospecific opiate binding sites located in the central nervous system and that these binding sites were not evenly distributed (Brownstein, 1993). The existence of naturally occurring opioid receptors in the human body lead scientists to believe that endogenous opiates, neurotransmitters, were the primary agonists for these binding sites (Brownstein, 1993). Later, scientists studied the neurophysiological and behavioral properties of different opioids observed cross-tolerance, which is the drugs ability to obstruct withdrawal symptoms after the removal of the second drug that the animal was tolerant too, between many of the opiates. From these experiments three different subtypes of opioid receptors were discovered: µ, κ, and δ receptor subtypes.

The µ receptors are located throughout the neuraxis and are most dense in the caudate putamen at the base of the telencephalon in the brain (Dhawan, 1996). The µ receptors when stimulated by opioid agonists inhibit neurotransmitter release, causing muscle contractions. There are two variants of the µ receptors, which differ from each other by the presence of an 8-amino acid sequence within the C terminal portion of the receptor protein, but no molecular biological investigations have validated these conclusions (Dhawan, 1996).
The κ receptor subtypes are located in different areas of the brain depending on what species of animal is being examined. In the guinea pig the κ receptors are found primarily in inner layer of the cerebral cortex, interpeduncular nucleus at the base of the mesencephalon, and the substantia nigra also within the mesencephalon (Dhawan, 1996). In the rat the highest densities are found in the nucleus accumbens in the basal forebrain, dorsal endopiriform nucleus in the piriform cortex, interpeduncular nucleus in the midbrain tegmentum, and the claustrum on the underside of the neocortex (Dhawan, 1996). The function of these receptors includes the regulation of these functions: nociception, diuresis, feeding and neuroendocrine secretions, and also lymphoma cell immune response (Dhawan, 1996).

The δ receptor subtypes distribution throughout the nervous system is much more limited than the other two subtypes. The δ receptor is found in the olfactory bulb in the prosencephalon, neocortex in the cerebral cortex, caudate putamen in the telencephalon, and the nucleus accumbens in the basal forebrain. Research performed at the ultrastructural level provided evidence that δ receptors are responsible for the inhibition of neurotransmitter release from the terminals of primary afferent fibers within the dorsal horn of the spinal cord (Dhawan, 1996). These receptor subtypes play an important role
in mood driven behavior, respiration, cognitive function, gastrointestinal motility, olfaction, motor integration, and analgesia (Dhawan, 1996).

The wide and uneven distribution of opioid receptor subclasses indicates that each subclass may play a distinct role in agonist response (Mansour, 1987).

III.  

*Plants of abuse from abroad and their evaluation for affinity to opioid and cannabinoid receptors*

Many natural products have not yet been evaluated for potential drug discovery leads, which warrant further investigation. In this research project, species of plants were selected for study based on their known use in foreign countries as ancient medicinal remedies as well as being abused for their psychotropic effects. Ten different plant samples were studied and subjected to extraction. The plant with the greatest binding affinity for cannabinoid and opioid receptors based on *in vitro* radioligand bioassays was further fractionated. These fractions were guided by more *in vitro* radioligand bioassays with the ultimate goal of isolating and identifying the active compounds.
a) *Rhamnus purshiana*

*Rhamnus purshiana*, commonly known as Cascara sagrada, belongs to the family Rhamnaceae and has been used for many centuries as a laxative and for blood detoxication (Anon, 1973). It originates in the Pacific Coast region of Canada from British Columbia southward. It also has some prevalence in Southern California (Anon, 1973). The bark of the plant is dried and used as a common traditional medicine. The bark is used extensively for treatment of habitual constipation, but has also been used for the treatment of hemorrhoids and other digestive ailments (Anon, 1973). The plant’s bark can be taken orally in capsule form or as a tea. Due to its terrible taste if taken in its tea
form it can cause acute nausea (Anon, 1973). *R. purshiana* is extremely useful as a laxative because it strengthens the peristaltic muscles that line the intestinal wall allowing for easy bowel evacuation with no need for additional laxatives. The chemical constitutes of the bark that are responsible for stimulating the peristaltic muscles of the colon are hydroxyanthracene derivates particularly anthraquinone glycosides (Anon, 1973). These compounds inhibit the absorption of water and electrolytes in the colon and increase the dilation pressure, which stimulates peristalsis (Anon, 1973).

b) *Arctostaphylos uva-ursi*

*A. uva-ursi* leaves

**Figure 7**
A. uva-ursi, commonly known as bearberry leaf, has been used as an herbal remedy for the treatment of various lower urinary tract infections including inflammatory diseases (Heroutova, 2012). The plant’s leaves have undergone extensive study eventually leading to the discovery of its most prevalent constituent, arbutin. This constituent, when consumed as an herbal tea, is absorbed by the small intestine and then undergoes hepatic conjugation to form hydroquinone (Heroutova, 2012). The use of the herbal tea as a medical treatment is widespread in Germany, Poland, Spain, Lithuania, Slovenia and Estonia.

Bearberry leaves have been used since the middle ages in areas such as Central Europe. There is also some literature that supports its use in the “New World” by North American Indians (Heroutova, 2012). The plant’s leaves were used for the treatment of different diseases including: hydrops, lithiasis, diabetes, gonorrhea, and urinary tract diseases. Much of the leaf derivates’ effectiveness as an antimicrobial agent depends the solvent used in extraction. For example a 30% ethanol extract in water of A. uva-ursi leaves inhibited growth of Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens, and Staphylococcus aureus. Alternatively, the 95% ethanol extract showed no inhibition of any microbial bacterial (Heroutova,
2012). The *A. uva-ursi* leaves ability to fight urinary tract infections has been identified as the result of the hydroquinone acting as an antibacterial agent (Heroutova, 2012).

c) *Acacia xanthophloea*

*Acacia xanthophloea*, more commonly known as the yellow fever tree, is a fast-growing tree originating in southern Africa in areas including: Botswana, Kenya, Malawi, Mozambique, Somalia, South Africa, Swaziland, Tanzania, Zambia, and Zimbabwe (Csurhes, 2012). The yellow fever tree name originates from the trees tendency to grow in African swampland where malaria is prevalent, and its yellow tinted bark (Csurhes,
Its uses in Africa have traditionally focused on its aesthetic appeal as it is used as an ornamental piece. In some other parts of Africa it is also used as a source of wood for fire when dried (Csurhes, 2012). Many of the plants in the genus *Acacia* produce a gummy liquid that the tree uses as a mechanism to prevent water loss in the event that the bark of the tree is damaged (Hausfater, 1976). The tree’s healing power was discovered when it treated a dying patient of malaria in early 1630 (Achan, 2011). The tree’s bark and roots extract known as quinine was discovered to be the compound responsible for the plants antimalarial properties (Achan, 2011). Quinine was subsequently exported heavily to Europe to be mixed with sugar and water creating Tonic Water, a historically popular medicinal remedy (Achan, 2011). Quinine is a crystalline alkaloid that is known to have many medicinal properties including antimalarial, antipyretic, analgesic, and anti-inflammatory (Achan, 2011).
d) *Verbascum densiflorum*

*Verbascum densiflorum* belongs to the family Scrophulariaceae, which are herbs commonly found in Europe (Klimek, 2009). The mullein (*V. densiflorum*) plant’s aerial parts have been widely used as an herbal remedy for inflammation of the upper respiratory tract, the common cold, external wounds, bronchitis, and spasmodic cough (Klimek, 2009). The plant’s aerial parts also have been used as a mild diuretic and anti-inflammatory agent for urinary tract infections (Klimek, 2009). The plant also has antibacterial and antiviral uses for the treatment of *Herpes simplex* virus, and influenza viruses (Klimek, 2009). There as been much research studying the chemical compounds
present in *V. densiflorum* that are responsible for its expectorant and antimicrobial effects. The demulcent, emollient, and expectorant capacities are all described by the presence of saponins and mucilage. The antimicrobial, anti-inflammatory, and diuretic capabilities are attributed to the two phenolic compounds, phenylethanoids and flavonoids (Klimek, 2009).

e) *Rubus idaeus*

*Figure 10*

*Rubus idaeus* leaves

*Rubus idaeus* is more commonly known as red raspberry is part of the family Roseaceae that is a native North American species that also grows in northern Asia and Europe (Chevallier, 1996). It is a well-known plant because of its edible fruits
(Baranowska, 2014). *R. idaeus*’ leaves have been utilized for medicinal purposes in multiple different facets including the treatment of diarrhea, menstrual cramps, tonsillitis, mouth sores and inflammations, minor wounds, burns, vomiting, and heart disease (Chevallier, 1996). The plant contains several chemical compounds that contribute to its antimicrobial and antibacterial properties including a number of phenolic compounds such as anthocyanins, ellagitannins, flavonoids, phenolic acids and flavan-3-ols (Baranowska, 2014). *R. idaeus* antimicrobial properties have an inhibitor affect on *Bacillus subtilis, Clostridium sporogenes, Staphylococcus epidermidis, Neisseria meningitides, Moraxella catarrhalis, and Helicobacter pylori* (Baranowska, 2014).
f) *Datura stramonium*

*Datura stramonium*, commonly known as “Jimson weed”, is a toxic weed that is found in the Thirunallar region of Pondicherry, India. The seeds, leaves and stems all contain several alkaloid compounds that are recognized as natural poisons (Sridhar, 2014). The seeds are the most toxic part of the plant that if consumed in a high enough quantity can be fatal. The seeds contain tropane alkaloids that have a strong effect on the central nervous system. These tropane alkaloids include atropine, hyoscyamine, and scopolamine which all affect the nerve cells in the brain and spinal cord. The components within *D. stramonium* can have an effect on breathing, blood circulation, heartbeat, and regulation of internal organs (Sridhar, 2014).
Hyoscyamus niger, commonly known as Henbane, is a plant in the Solanaceae family and can be found throughout areas of Europe and Asia (Zhang, 2014). The seeds of the H. niger have been used historically in areas of China to treat asthma, cough, colic, toothache, inflammation of the pulmonary region, stomach-intestinal pain resulting from worm infestation, diarrhea neuralgia, and Parkinson’s disease. It has known effectiveness as a therapeutic agent, antispasmodic, sedative, analgesic agent, anthelmintic, antitumor, and febrifuge (Zhang, 2014). H. niger’s efficacy as a medicinal remedy stems from its chemical constitutes, which includes the tropane alkaloids such as atropine, scopolamine, anisodamine. There are other chemical constitutes present as well including tyramine alkaloids, withanolides, lignanamides, and flavonoids (Zhang, 2014). The plant can be
toxic if excessive doses are taken which results in respiratory paralysis. The tropane alkaloids affect acetylcholine-mediated signal transduction by acting as agonists on the muscarinic receptors. Being agonists of the muscarinic receptors allows tropane alkaloids to be effective therapeutics for treating nausea, diabetes, and act as an antidote for poisoning by organophosphorus compounds (Zhang, 2014).

\( h \) \hspace{1em} \textit{Nerium oleander}

\textit{N. oleander} aerial part

\textbf{Figure 13}

\textit{Nerium oleander}, commonly known as Kaner, is part of the family Apocynaceae and is native to southern Europe while being naturalized in North America, Europe, and Asia (Fartyal, 2014). The plant’s aerial parts have been attributed with chemical constitutes
that have medicinal properties such as larvicidal, insecticidal, antimitotic, depressant, antifungal, anticancer, antineoplastic, antibacterial, antidiabetic, anti-inflammatory, analgesic, and cardiotonic (Fartyal, 2014). *N. oleander* is useful on the treatment of skin problems, warts, menstrual pain, asthma, seizures, cancer, epilepsy, malaria, leprosy, ringworm, indigestion, and venereal diseases. The antimicrobial properties of the plant can be attributed to its secondary metabolites which include the alkaloids, flavonoids, saponins, tannins and phenols chemical constitutes. The flavonoids are particularly effective as anticancer agents because of their ability as a free radical scavengers that prevents oxidative cell damage (Fartyal, 2014).
i) *Passiflora caerulea*

*Passiflora caerulea*, commonly known as the passionflower of the Passifloraceae family, is native to South America specifically Argentina. The aerial parts of the plant have historically been used as a sedative with no myorelaxant effects. The flavonoid, chrysin, might be responsible for the anxiolytic effects exhibited by the plant (Busilacchi, 2002). The plant also has many other medicinal uses including analgesic, adaptogenic, antiaging, anti-inflammatory, antispasmodic, antistress, antitussive, and hypotensive (Busilacchi, 2002).
Banisteriopsis caapi

**Banisteriopsis caapi** is a hallucinogenic vine originating in the Amazon basin in South America that belongs to the Malpighiaceae family (Gibbons, 2013). It is the main ingredient in a famous religious healing ceremonial drink known as “ayahuasca,” which is a mix of both the *B. caapi* and *Psychotria viridis* (Gibbons, 2013). Although the drink is primary used in South America, its use in North America and Europe has increased in the past few years (Gibbons, 2013). The drink has moved from being the Amazonian
indigenous people’s religious drink to being a key component in the “New Age” rituals around the Western world (Trichter, 2010).

The drink is prepared by boiling the leaves of *B. caapi* and the leaves of *P. viridis* together with other various native South American plants (Gibbons, 2013). The active components found in *B. caapi* are β-carboline alkaloid derivatives, which are harmine, harmaline, and tetrahydroharmine (THH) (Gibbons, 2013). The average drink of ayahuasca contains 467 mg of harmine, 160 mg of tetrahydroharmine, 41 mg of harmaline, and 60 mg of dimethyltryptamine (DMT). The DMT in the drink is the active constituent of *P. viridis* and is the main psychoactive ingredient of the drink (Gibbons, 2013). The *P. viridis* and other additive plants in the drink make up the principle sources of active ingredients in ayahuasca, while the *B. caapi* acts as a monoamine oxidase inhibitor by way of β-carboline alkaloids, which makes the tryptamines of the additive plants orally active (Winkelman, 2005). The human body naturally produces peripheral monoamine oxidase (MAO), which can break down DMT quickly enough so it doesn’t reach the central nervous system if taken orally rendering the drink inactive as a hallucinogenic. Therefore the ayahuasca drink must be mixed with an MAO inhibitor such as *B. caapi* to allow for the DMT to have the effect above (Gibbons, 2013).
The three β-carboline alkaloid derivatives of the *B. caapi* have promising prospects as medicinal treatments for neurological disorders (Gibbons, 2013). The harmaline and harmine derivatives ability to act as a monoamine oxidase inhibitor (MAO) of both –A and –B isozymes present in the brain, indicates the plant's ability to prevent neuronal cell damage (Gibbons, 2013). Both harmaline and harmine are able to stimulate dopamine release within the CNS (Gibbons, 2013). Many neurodegenerative disorders such as Parkinson’s disease and Alzheimer’s are in part the result of oxidative free radicals damaging neurological cells. Proanthocyanidins found in the *B. caapi* possess antioxidant properties as well as moderate MAO-B inhibitory properties that can protect neurological cells from damage by oxidative free radicals (Gibbons, 2013).
Chemical structures of harmaline and harmine

Figure 17
Materials and Methods:

I. General experimental procedures

$^1$H and $^{13}$C Nuclear Magnetic Resonance (NMR) spectra were obtained on Bruker model AMX 500 NMR and 400 Avance NMR spectrometers with standard pulse sequences, operating at 500 MHz and 400 MHz, respectively in $^1$H and 100 MHz and 125 MHz in $^{13}$C, respectively. The chemical shift values were reported in parts per million units (ppm) from trimethylsilylamine (TMS) using known solvent chemical shifts. Coupling constants were recorded in Hertz (Hz). Standard pulse sequences were used for COSY, HMQC, HMBC, TOCSY, NOESY and DEPT. High-resolution mass spectra (HRMS) were measured on a Micromass Q-Tof Micro mass spectrometer with a lock spray source. Column chromatography was carried out on silica gel (70-230 mesh, Merck), Sephadex LH-20 (Mitsubishi Kagaku, Tokyo, Japan). TLC (silica gel 60 F254) was used to monitor fractions from column chromatography. Visualization of the TLC plates was achieved with a UV lamp ($\lambda = 254$ and 365 nm) and anisaldehyde/acid spray reagent (MeOH-acetic acid-anisaldehyde-sulfuric acid, 85:9:1:5). Solvents were certified grade for Fischer Scientific. All chemicals used were purchased from Sigma-Aldrich (St. Louis,
Mo) with the following exceptions: for the binding experiments, [\(^3\)H]-CP-55,940 (144 Ci/mmol), was purchased from Perkin-Elmer Life Sciences Inc. (Boston, MA, U.S.A.). CP-55,940 was purchased from Tocris Bioscience (Ellisville, Missouri, U.S.A.).

II. Plant material

The plants *Banisteriopsis caapi*, *Rhamnus purshiana*, *Arctostaphylos uva-ursi*, *Acacia xanthophloea*, *Verbascum densiflorum*, and *Rubus idaeus* were all obtained from Bouncing Bear Botanicals Company. The plants *Datura stramonium*, *Hyoscyamus niger*, *Nerium oleander*, and *Passiflora caerulea* were all collected in the Quetta region of Pakistan in July of 2012 and were identified by Dr. Arsala Mansoor from the Department of Biochemistry, Bolan Medical College, Quetta, Pakistan. All plants were separated into 3 parts: the leaves, stem/bark, seeds. The leaves of the *B. caapi* were investigated, the bark of the *R. purshiana* was investigated, the leaves of the *A. uva-ursi* was investigated, the roots of the *A. xanthophloea* were investigated, the leaves and stems of the *V. densiflorum* were investigated, the leaves of the *R. idaeus* were investigated, the seeds of the *D. stramonium* were investigated, the seeds of the *H. niger* were investigated, the leaves and stems of the *N. oleander* were investigated, and the leaves and stems of the *P. caerulea* were investigated.
III. Material Preparation

The plant material was sent to our lab in a variety of forms. Some were intact leaves and aerial parts, while others were broken down into smaller pieces. To allow for a uniform ethanolic extraction all of the plant material was grinded down to a powder. This allowed for the greatest surface area to be achieved leading to better ethanolic extraction. Each sample of plant material was grinded into powder using a KIKA Werke M20 grinder, as pictured below in Figure 18. The grinding yielded approximately 110 grams per plant.

![KIKA Werke M20 Grinder](image-url)

Figure 18
**IV. Extraction and isolation**

The ten plants were each extracted with 450 ml of ethanol after maceration for three days, separately. After removal of the solvent by rotoevaporation a viscous residue (crude extract) remained yielding results shown in Table 1.

<table>
<thead>
<tr>
<th>Part of Plant</th>
<th>Extract Weights (grams)</th>
<th>% Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. caapi</em></td>
<td>leaves</td>
<td>3.2</td>
</tr>
<tr>
<td><em>R. purshiana</em></td>
<td>bark</td>
<td>3.2</td>
</tr>
<tr>
<td><em>A. uva-ursi</em></td>
<td>leaves</td>
<td>4.7</td>
</tr>
<tr>
<td><em>A. xanthophloea</em></td>
<td>roots</td>
<td>4.9</td>
</tr>
<tr>
<td><em>V. densiflorum</em></td>
<td>aerial part</td>
<td>3.6</td>
</tr>
<tr>
<td><em>R. idaeus</em></td>
<td>leaves</td>
<td>2.5</td>
</tr>
<tr>
<td><em>D. stramonium</em></td>
<td>seeds</td>
<td>4.7</td>
</tr>
<tr>
<td><em>H. niger</em></td>
<td>seeds</td>
<td>3.9</td>
</tr>
<tr>
<td><em>N. oleander</em></td>
<td>aerial part</td>
<td>5.8</td>
</tr>
<tr>
<td><em>P. caerulea</em></td>
<td>aerial part</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Ethanolic extraction results

**Table 1**

The ethanolic extracts from the plants were then subjected to bioassay radioligand displacement testing in the cannabinoid and opioid receptor assays. The ethanolic extract...
from the plants showed varying binding affinity through radioligand displacement on cannabinoid receptors (8% - 41%). The extracts radioligand displacement for opioid receptors also showed great variability (.5%-79.1% δ, 21.6%-64.96% κ, 9.5%-73% μ.)

<table>
<thead>
<tr>
<th></th>
<th>CB2 %</th>
<th>δ %</th>
<th>κ %</th>
<th>μ %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition</td>
<td>Inhibition</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td><strong>B. caapi</strong></td>
<td>28.8</td>
<td>79.1</td>
<td>64.9</td>
<td>73.0</td>
</tr>
<tr>
<td><strong>R. purshiana</strong></td>
<td>-</td>
<td>30.0</td>
<td>-</td>
<td>20.9</td>
</tr>
<tr>
<td><strong>A. uva-ursi</strong></td>
<td>8.0</td>
<td>0.5</td>
<td>-</td>
<td>23.9</td>
</tr>
<tr>
<td><strong>A. xanthophloea</strong></td>
<td>-</td>
<td>24.8</td>
<td>-</td>
<td>27.6</td>
</tr>
<tr>
<td><strong>V. densiflorum</strong></td>
<td>41.0</td>
<td>39.7</td>
<td>30.9</td>
<td>42.7</td>
</tr>
<tr>
<td><strong>R. idaeus</strong></td>
<td>27.2</td>
<td>24.9</td>
<td>31.1</td>
<td>-</td>
</tr>
<tr>
<td><strong>D. stramonium</strong></td>
<td>27.1</td>
<td>49.6</td>
<td>21.6</td>
<td>55.5</td>
</tr>
<tr>
<td><strong>H. niger</strong></td>
<td>24.8</td>
<td>27.1</td>
<td>-</td>
<td>9.5</td>
</tr>
<tr>
<td><strong>N. oleander</strong></td>
<td>-</td>
<td>14.5</td>
<td>23.1</td>
<td>-</td>
</tr>
<tr>
<td><strong>P. caerulea</strong></td>
<td>-</td>
<td>58.9</td>
<td>25.3</td>
<td>48.6</td>
</tr>
</tbody>
</table>

Radioligand displacement binding affinity of various subtypes

Table 2
V. Cell culture

HEK-293 cells stably transfected with opioid receptor subtypes µ, δ, and κ were used to perform the opioid receptor binding assays. These cells were maintained at 37 °C and 5% CO₂ in a Dulbecco’s modified Eagle medium (DMEM) nutrient mixture supplemented with 2 mM L-glutamine, 10% fetal bovine serum, penicillin–streptomycin, and either G418 or hygromycin B antibiotic solutions. Membranes for the radioligand binding assays were prepared by scraping the cells in a 50 mM Tris-HCl buffer, followed by homogenization, sonication, and centrifugation for 40 min at 13650 rpm at 4 °C. These were kept at -80 °C until used for bioassays. Protein concentration was determined via Bio-Rad Protein Assay (Bradford, 1976).

VI. Radioligand Binding for Cannabinoids and Opioid Receptor Subtypes

All the extracts, fractions, and pure compounds from B. caapi were run in competition binding assays against both cannabinoid receptor subtypes and all three opioid receptor subtypes (León et al., 2013). Cannabinoid binding took place under the following conditions: 10 µM of each compound was incubated with 0.6 nM [³H] CP 55,940 and 10 µg of CB1 or CB2 membranes for 90 minutes in a silanized 96-well plate. The reaction was terminated via rapid vacuum filtration through GF/B filters presoaked
with 0.3% bovine serum albumin (BSA) using a Perkin-Elmer 96-well Unifilter followed by 10 washes with 50 mM Tris-HCl. Plates were read using a Perkin-Elmer Topcount.

Opioid binding assays were performed under the following conditions: 10 µM of each compound was incubated with [³H]-DAMGO (µ), [³H]-U-69,593 (κ), or [³H]-enkephalin (δ) for 60 min in a 96-well plate. Percent binding was calculated as the average of the triplicate tested at 10 µM. Each sample concentration point of the compounds tested in dose response was in triplicate, and each compound showing activity was tested at least three times. The reaction was terminated via rapid vacuum filtration through GF/B filters presoaked with 0.3% bovine serum albumin (BSA) using a Perkin-Elmer 96-well Unifilter followed by 10 washes with 50 mM Tris-HCl. Plates were read using a Perkin-Elmer Topcount. Total binding was defined as binding in the presence of 1.0% DMSO. Nonspecific binding was the binding observed in the presence of 10µM DAMGO (µ), nor-binaltorphimine (κ), or DPDPE (δ). Specific binding was defined as the difference between total and nonspecific binding. Percent binding was calculated using the following formula: (binding of compound - nonspecific binding) x 100/specific binding.
VII. Animal Model

Adult male Swiss Webster mice (21-24g) were obtained from Harlan Laboratories. All animals were housed five to a cage and received food/water ad lib. The housing facilities were maintained on a 12 h light/dark schedule (lights on at 6:00 am and off at 6:00 pm). Mice were randomly divided into 3 groups (n=10/dose).

The mouse tetrad is a behavioral assay developed by Martin et al. (1994) to characterize the biological effects of cannabinoids using locomotor activity, nociception, changes in body temperature, and catalepsy. The assay has been well documented to indicate that the typical effects of cannabinoids is decreased in locomotion, and increased in cataleptic activity, antinociception, and hypothermia (Pertwee, 2006). Twenty-four hours prior to the start of the experiment, mice were acclimated for 15 minute increments to the cold hotplate container and apparatus. On the experimental day, mice were brought into the experimental room and allowed to acclimate to the room settings for 30 minutes and then to the locomotor chamber for 30 minutes. Baseline readings for antinociception both spinal (tail-flick) and supraspinal (hotplate), catalepsy, and hypothermia were evaluated pre-injection. Animals were injected with either the vehicle (ethanol:cremophor:saline in a 1:1:18 ratio) or the test compound (10 mg/kg – 100
mg/kg). Animals were then allowed to individually quantify in the locomotor chamber (San Diego Instruments) for 30 minutes (expressed as the number of photo-beam breaks). The last 10 minutes of quantifying time was used for data analysis. Hotplate and tail-flick latencies (using cut off time of 45 seconds and 15 seconds, respectively, to reduce the chance of tissue damage), the change in core body temperature (°C) and catalepsy (secs) were recorded at 30 minutes post-injection.

Data was show as mean ± SEM with each group having n = 10 animals. Both hotplate and tail-flick were expressed as percent maximum effect (%MPE = [(post-drug latency-basal latency)/(cutoff latency-basal latency)] X 100 (Martin et. al., 1994).

Statistical analysis was performed using one way ANOVA preceded by the Dunnett’s post hoc test to define significant difference against the vehicle control at p<0.05.
Results and Discussion:

I. Bioassay-guided Fractionation

Based on the radioligand displacement results of the bioassay *B. caapi*, was selected for further evaluation. With the aim to get a greater amount of the *B. caapi* extract, the rest of the available plant material was ground into a powder yielding 64.6 grams. The ground material was then subjected to extraction with 250 ml of ethanol after maceration for three days yielding (1.86 g). The ethanolic extracts of *B. caapi* were all combined obtaining (4.5 g) and then chromatographed on Si gel flash column with stepwise fractions from hexanes to methanol, yielding eight fractions, which are shown with their radioligand displacement binding affinities below in Table 3. The *B. caapi* fractions were then subjected to bioassay radioligand displacement testing. The results showed varying radioligand displacement binding affinity for cannabinoid receptors (22.6% - 86% CB1, 26.8% - 86.9% CB2). The extracts radioligand displacement for opioid receptors also showed great variability (30.3%-79.3% δ, 6.9%-55.8% κ, 1.5%-62.9% μ.)
### Table 3

Radioligand displacement binding affinity of *B. caapi* fractions

<table>
<thead>
<tr>
<th>Fraction Description</th>
<th>CB1 % Inhibition</th>
<th>CB2 % Inhibition</th>
<th>δ % Inhibition</th>
<th>κ % Inhibition</th>
<th>µ % Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexanic fraction (100%; 29mg)</td>
<td>22.6</td>
<td>26.8</td>
<td>-</td>
<td>6.9</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>hex:EtOAc (75:25; 632mg)</strong></td>
<td><strong>37.9</strong></td>
<td><strong>66.7</strong></td>
<td><strong>39.4</strong></td>
<td><strong>29.9</strong></td>
<td><strong>10.5</strong></td>
</tr>
<tr>
<td><strong>hex:EtOAc (50:50; 96mg)</strong></td>
<td><strong>86.0</strong></td>
<td><strong>86.9</strong></td>
<td><strong>79.3</strong></td>
<td><strong>55.8</strong></td>
<td><strong>62.9</strong></td>
</tr>
<tr>
<td>hex:EtOAc (25:75; 64mg)</td>
<td>39.8</td>
<td>64.5</td>
<td>60.8</td>
<td>39.1</td>
<td>35.5</td>
</tr>
<tr>
<td>EtOAc fraction (100%; 58mg)</td>
<td>35.3</td>
<td>54.8</td>
<td>36.3</td>
<td>22.7</td>
<td>17.3</td>
</tr>
<tr>
<td><strong>EtOAc:MeOH (90:10; 484mg)</strong></td>
<td><strong>34.1</strong></td>
<td><strong>57.3</strong></td>
<td><strong>55.6</strong></td>
<td><strong>25.9</strong></td>
<td><strong>28.6</strong></td>
</tr>
<tr>
<td><strong>EtOAc:MeOH (75:25; 2.17g)</strong></td>
<td><strong>47.3</strong></td>
<td><strong>51.1</strong></td>
<td><strong>44.2</strong></td>
<td><strong>34.3</strong></td>
<td><strong>39.8</strong></td>
</tr>
<tr>
<td>MeOH fraction (100%; 3.58g)</td>
<td>64.4</td>
<td>43.8</td>
<td>30.3</td>
<td>15.6</td>
<td>19.5</td>
</tr>
</tbody>
</table>

Based on the radioligand displacement results of the bioassay we selected 100% hexanes, 75% hexanes in EtOAc, 50% hexanes in EtOAc, and 75% EtOAc in hexanes fractions to continue to investigate. The four fractions were submitted to Si gel thin layer chromatography (TLC). Dichloromethane (DCM) and methanol were used to dissolve the extracts. Various solvents were used in different concentrations to create the systems for the TLC plates.
TLC: 80% hexanes in EtOAc (1: 100% Hexanes Extract. 2: 75-25% hex:EtOAc. 3: 50-50% hex:EtOAc. 4: 25-75% hex:EtOAc.)

Figure 19

TLC: 70% hexanes in EtOAc (1: 100% Hexanes Extract. 2: 75-25% hex:EtOAc. 3: 50-50% hex:EtOAc. 4: 25-75% hex:EtOAc.)

Figure 20
TLC: 90% DCM in Acetone (1: 100% Hexanes Extract. 2: 75-25% hex:EtOAc. 3: 50-50% hex:EtOAc. 4: 25-75% hex:EtOAc.)

**Figure 21**

Based on the TLC results we choose fractions 75% hexanes in EtOAc and 50% hexanes in EtOAc for additional studies. The two fractions were subjected to a sephadex column using a solvent system of hexanes, DCM, and MeOH (1:1:1). Thirty-two subfractions were obtained from the 75% hexanes (600 mg) in EtOAc sephadex column and were subsequently combined based on the TLC (DCM:MeOH; 9:1). The 50% hexanes (500 mg) in EtOAc sephadex column yielded twenty-nine fractions, which were subsequently combined based on TLC (DCM:MeOH; 9:1). The subfractions from 75% hexanes in EtOAc were combined into two groups, fractions (BC-C-2(1-5), (BC-C-2(10-
The subfractions from 50% hexane in EtOAc were combined into four groups, fractions (BC-C-3(1-5), (BC-C-3(6-9), (BC-C-3(10-12), and (BC-C-3(13-29).


Figure 22

These six combined groups of subfractions were submitted to bioassay radioligand displacement testing. The results showed varying radioligand displacement binding affinity for cannabinoid receptors (2.5%-71.2% CB1, 11.5%-78.1% CB2). The extracts radioligand displacement for opioid receptors also showed great variability (15.7%-86.7% δ, 11.8%-61.4% κ, 10.2%-45% μ.) The results of the radioligand displacement binding affinity are shown below in Table 4.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>CB1 %</th>
<th>CB2 %</th>
<th>δ %</th>
<th>κ %</th>
<th>μ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-C-2(1-5) (102mg)</td>
<td>71.2</td>
<td>69.7</td>
<td>68.3</td>
<td>47.8</td>
<td>39.1</td>
</tr>
<tr>
<td>BC-C-2(10-13) (21mg)</td>
<td>26.0</td>
<td>30.0</td>
<td>15.7</td>
<td>19.4</td>
<td>-</td>
</tr>
<tr>
<td>BC-C-3(1-5) (18mg)</td>
<td>2.5</td>
<td>26.1</td>
<td>30.7</td>
<td>11.8</td>
<td>10.2</td>
</tr>
<tr>
<td>BC-C-3(6-9) (69mg)</td>
<td>65.6</td>
<td>78.1</td>
<td>86.7</td>
<td>61.4</td>
<td>45.0</td>
</tr>
<tr>
<td>BC-C-3(10-12) (78mg)</td>
<td>38.5</td>
<td>14.1</td>
<td>52.6</td>
<td>-</td>
<td>41.7</td>
</tr>
<tr>
<td>BC-C-3(13-29) (18mg)</td>
<td>28.3</td>
<td>11.5</td>
<td>48.3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Radioligand displacement binding affinity of *B. caapi* fractions
(BC-C-2 and BC-C-3)

**Table 4**
II. Isolation and Purification

The BC-C-3(10-13) subfraction showed in the TLC (Figure 22) the presence of a major compound with some impurities. It was subjected to $^1$H NMR NMR spectroscopy and the results are shown below in Figure 23.

![$^1$H NMR Spectroscopy of BC-C-3(10-13)](image)

**Figure 23**

The BC-C-3(10-13) fraction NMR’s was too crude to conclude any results.

Therefore, the fraction was subjected to additional sephadex column fractionations in an
attempt to isolate a major compound. The sephadex column fractionation isolated a single active fraction that was subjected to further NMR analysis. The sample was labeled BC-C-3(10) (1.2 mg) and was subjected to three NMR tests including $^1$H NMR, $^{13}$C NMR, and DEPT NMR. The results for those respective NMR’s are seen below in Figures 24-27.

![BC-C-3(10) 1H NMR Spectroscopy](image)

$^1$H NMR Spectroscopy of BC-C-3(10)

**Figure 24**
$^{13}$C NMR Spectroscopy of BC-C-3(10)

**Figure 25**
$^{13}$C NMR Spectroscopy of BC-C-3(10) Overlay

Figure 26
DEPT NMR Spectroscopy of BC-C-3(10)

Figure 27
Based on the NMR results for BC-C-3(10) a purified compound, α-Amyrin, was isolated. The chemical structure of α-Amyrin, which is based on the comparison of its $^1$H and $^{13}$C NMR data and the reported literature, is shown in Figure 28 (Wehril, 1988).

Further NMR studies will be performed to elucidate the active compounds in BC-C-2(1-5), BC-C-3(1-5), BC-C-3(6-9), BC-C-3(10-12), and BC-C-3(13-29). Given that the amount of each extract is so small isolating the main component within each subfraction is the main goal. α-Amyrin is the main compound within subfraction BC-C-3(10-13), but there are other possible active compounds within the subfraction that could be isolated given a greater quantity of extract. More plant material will need to be
gathered to further investigate other possible active compounds within subfraction BC-C-3(10-13) as well as the other subfractions.

III. Animal Model

The hex:EtOAc (50-50) fraction from the B. caapi from Table 3 was selected because of its high cannabinoid and opioid receptor binding affinity values and was further subjected to a mouse model study to observe the fractions neurological interaction. The results for locomotive activity, hotplate latency, catalepsy latency, change in rectal temperature, and tail-flick latency testing are shown below in Figures 29-33 respectively.
Locomotive Latency

Figure 29

Hotplate Latency

Figure 30
Catalepsy Latency

Figure 31

Change in Rectal Temperature

Figure 32
The *in vivo* mouse model study showed no promising results. The test examined the fraction’s affect on the central nervous system. Given that the fraction submitted was only tested for activity in the central nervous system, there is possible peripheral nervous system stimulation that will be investigated in a later study.
Conclusion:

Traditional medicine has used secondary metabolites derived from natural products to treat various medical ailments for thousands of years. Natural products have led to numerous drug discoveries and continue to show significant strides towards further applications for pain and addiction modulation therapies. In this research we sought to evaluate ten different plants’, which were historically used as medical remedies, binding affinities for opioid and cannabinoid receptors. Each plant underwent extraction and was subjected to preliminary bioassays to determine which plant had the greatest radioligand displacement. *B. caapi* was determined to have the greatest binding affinity for both cannabinoid and opioid receptors. The *B. caapi* ethanolic extract then underwent silica gel column fractionation yielding fractions that were each individually subjected to further bioassays. From the bioassay results the fraction hex:EtOAc (50-50) had the highest radioligand displacement. A sample of the hex:EtOAc (50-50) fraction was submitted to an *in-vivo* mouse model study to evaluate it’s neurological affects. No significant results could be concluded from the mouse model. The hex:EtOAc (50-50) fraction was then subjected to further bioassay guided sephadex column fractionations.
leading to six fractions with significant radioligand displacement to be isolated. One of these fractions, BC-C-3(10-13) underwent NMR spectroscopy. The results from that NMR led to further sephadex column fractionations, which resulted in a pure compound being isolated. This pure fraction, BC-C-3(10), was submitted to three different NMR spectroscopy studies which revealed the chemical structure of the fraction known by the common name α-Amyrin. The next aim in this study is to investigate the other five sephadex column subfractions for the compound that is responsible for the radioligand displacement and validate its pain and addiction modulation efficacy.
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