

THE NEUROPHARMACOLOGICAL ASSESMENT OF *Salvia divinorum* EPLING and
JATIVA-M

By

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To my mother, Ramona Phipps- without her constant dedication and personal sacrifice to ensure I received a good education none of this would be possible.

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LIST OF ABBREVIATIONS

KOR	kappa opioid receptor
p.o.	per os (by mouth)
i.p.	intraperitoneal
SWE	salvia water extract
SHE	salvia hydro-ethanolic extract
SHM	salvia hydro-methanolic extract
TST	tail suspension test
FST	forced swimming test
CG	compulsive gnawing
OFT	open field test
HPLC	high performance liquid chromatography
MS	mass spectrometry
ESI	electrospray ionization

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NEUROPHARMACOLOGICAL ASSESSMENT OF *Salvia divinorum* EPLING & JATIVA-M

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Salvia divinorum (SD) has been used by the native peoples of Oaxaca Mexico as a shamanistic entheogen for numerous generations. First stumbled on scientifically in 1952 when psychoactive mushrooms of the area were being studied, this member of the mint family became an interesting side note in ethnobotanical studies. Over thirty years later the main active ingredient salvinorin A (SA) was identified as the first non-nitrogenous hallucinogen known. After a short time the list of firsts for this plant grew as the pharmacological activity of salvinorin A was defined as a selective kappa opioid receptor (KOR) agonist. In the years to follow certain uses of the plant seemed to contradict the selectivity of salvinorin A. In case reports from Australia *Salvia divinorum* was shown to have antidepressant activity in refractory depression patients when given quids made up of the leaves of *Salvia divinorum*. From numerous studies of KOR agonists showing the depressive effects on monoamine levels in the CNS the use of the plant should cause an exacerbation of symptoms in depressive individuals.

It was with these conclusions in mind that this dissertation project was started. To understand or elicit differences in action of *Salvia divinorum* and salvinorin A when given in formulations that tie in a metabolic element, and more closely mimic extraction methods seen in quids, and the traditional metate preparation of the Mazatec peoples.

The results of this were the characterization of an antidepressant-like activity in the salvia water extract (SWE) when given orally at a concentration of 50mg/kg in the forced swimming test (FST) and tail suspension test (TST). The corollary motor activity test, the open field test (OFT), showed no significant changes in locomotor activity. The main psychoactive compound salvinorin A, when giving i.p., expressed dopaminergic activity in a dose dependent manner that could not be explained via the kappa opioid system. This shows that when testing for pharmacological activity of a plant great care and planning should be taken to characterize activity with formulations that closely resemble those used in human consumption.

CHAPTER 1 INTRODUCTION

Lamiaceae Lindl.

Taxonomic Distribution

The mint family, also known as, Lamiaceae Lindl. (Labiatae Juss.) is a large and widely distributed family. The family is comprised of about 210 genera with 3500 species [1]. Geographically speaking, Lamiaceae members are found through all zones, frigid to the tropics. Mostly comprised of herbs and shrubs, the main distinguishing feature is the tetragonal, or square stem. Other anatomical characteristics of the family are opposite or whorled leaf form, and a dioecious reproductive type. The main mode of pollination is entomophilous with the main orders being hymenoptera, lepidoptera, or diptera. Being a large family, it of course has numerous genera used by humans for food and medicine. Some include: *Mentha* (Mint), *Lavandula* (Lavender), *Ocimum* (Basil), *Organum* (Oregano), *Nepeta* (Catnip), *Rosmarinus* (Rosemary), and *Salvia* (Sage).

Genus *Salvia* L.

Taxonomy

The genus *Salvia* L. (Fig1-1) is comprised of nearly 1000 species, and has undergone marked species radiations in three specific regions: Central and South America (500 spp.), central Asia/Mediterranean (250 spp.), and eastern Asia (90 spp.) [2]. Taxonomically speaking many believe the reasons behind this were the unique stamen number (two stamen vs. four) and, the fusion at the theca which led to a unusual lever-like pollination mechanism (Fig1-2) [3]. As the insect goes to collect nectar from the base of the nectar tube it presses down a pad on the filament as well. This causes the stamen to lower and places the pollen sac onto the insect. For

years this had placed it alone in the tribe Mentheae, but after further analysis of other examples found in the Mentheae tribe proved that *Salvia* is not monophyletic [2], [4].

General ethnobotany

The origins of the name *Salvia* can be traced back to early Rome. Salvias which in Latin means to save is a fitting name for this genus. Anywhere *Salvia* grows there are documented medical uses for the plant. From anti-inflammatory uses, neuroprotective uses, drug withdrawal, to memory enhancement the uses for *Salvia* are as broad in spectrum as the genus is large. For the purposes of this paper *Salvia* species with CNS activity are of interest.

Salvia Miltiorrhiza Bunge. Also known as Red Sage is used widely throughout China as a treatment for insomnia. The compounds of interest are diterpene quinones and of these; miltirone (Fig 1-3) is the most interesting to the medical community. As a partial agonist for gamma-aminobutyric acid (GABA) miltirone produced muscle relaxation and sedation, but without dependency issues that can be seen in full agonists like diazepam. What is interesting as well with this compound is its ability to inhibit the increase in mRNA for the $\alpha 4$ subunit of GABAA commonly seen in alcohol withdrawal. This would make miltirone a novel treatment as a non-habit forming anxiolytic that could be used when treating alcohol abuse [5].

Salvia officinalis L. Also known as Common Sage it is the one species of *Salvia* most people are familiar with due to its use in the culinary arts. Other than being a herb used in cuisine compounds in *Salvia officinalis* have shown activity as sedatives, and memory enhancement as well. Like the diterpene miltirone; carnosol and carnosic acid have been shown to have sedative effects. Their effects however are initiated differently, by binding directly to the chloride channel of the GABA/benzodiazepine complex [6]. But this is not the only CNS activity seen in the common sage.

Salvia officinalis has been touted in many European pharmacopeias as having memory enhancing effects. Because of this, in vitro studies into cholinergic binding properties, and the link to enhanced cognitive function it was thought that the common sage might make a good nutritional supplement for Alzheimer's patients. In a four month clinical study in three different centers in Tehran patients were given an extract of common sage. The results indicated a significantly greater cognitive function versus the placebo [7]. But probably the most interesting *Salvia* species with CNS activity is the only known hallucinogenic sage, the divine seer, SD.

***Salvia divinorum* Epling and Jativa-M**

General ethnobotany

The origins of the ethnobotanical story of SD takes place in Oaxaca Mexico. Gordon Wasson was known for studying psychotropic mushrooms and their role in the religious life of the native peoples of Mexico. In 1953 Wasson [8] began studying the highlands of Southern Mexico and the state of Oaxaca. In the northern most part of the state lie Sierre Mazteca and the Mazatec peoples. While there he was introduced to a plant commonly used by the Mazatecs when mushrooms were not available. This plant was SD or to the Mazatecs *hojas de la Pastora*, *ska Pastora*, or *hojas de María Pastora* which means "leaves of the Shepherdess" or "leaves of the Mary Shepherdess." While *ska Pastora* is the Mazatec word for the plant the addition of *María* most likely comes from the European colonization of the land and so the phrase *hojas de María Pastora* becomes an amalgamation of the pagan views of the Middle American natives with the Christian views brought by early settlers. It seems also noteworthy that other plants from the mint family are held in high regards by the Mazatec people who also regard these plants as family members. *Salvia divinorum* is seen as *la hembra* "the female" *Coleus pumila* is referred

to as the male, El macho, and two forms of *Coleus blumei* are seen as the child and the godson, or el nene and ahijado respectively [8].

In 1961 specimens were collected for identification and sent to the Harvard botanists Schultes and Epling, but all of the specimens provided were not adequate enough for proper determination. Finally in 1962 an adequate specimen was located and identified by Epling as a new *Salvia* species. In a short description of the new plant Epling described the new species as:

allied to *S. cyanea* Lamb. ex. Benth., which is found in central Mexico. The former differs from the latter principally in respect to leaf shape (the attenuation of the blade) and the flattened upper style branch. The bracts of *Salvia divinorum* appear to be tardily deciduous. The species is doubtless striking in its habitat and might possibly be valuable if introduced into horticulture. [9]

A specimen was taken to Mexico City from Huautla de Jimenez by Wasson as well. The specimen grew in open air, and noticed that it did not flower or seed and seemed to reproduce vegetatively from a shoot. It seemed that many of the Mazatec peoples had small private patches of the plant, and no one had seen the plant growing in the wild. With these growing habits it would seem that SD is a cultigen, and to this day most of the SD seen in places outside of Oaxaca come from the Wasson clone.

As far as the hallucinogenic experience both Wasson and Albert Hofmann discuss the experience and the methods used by the Mazatec curandera to administer the plant extract [8], [10]. In Hofmann's case it was actually his wife Anita took his place. Both Wasson and Mrs. Hofmann noted that they saw dancing colors in elaborate and intricate three dimensional designs. Wasson having tried the psychotropic mushrooms as well noted on SD's short onset of action. The Mazatec curandera employed a novel method for creating a crude extract from the plant. Using a metate or stone grinding board the curandera makes a dark paste from the leaves (Fig1-3). Afterwards the metate and the paste is washed with water and strained. The mixture is blessed over resinous incense known as copal and drank.

These first experiences were interesting. Seeing a hallucinogenic member of the mint family was interesting, but it wasn't until later when the psychoactive compound now known as SA was first identified [11] that the interest levels began to peak. SA would label SD as the first plant known with a non-nitrogenous hallucinogen, and stranger still the pharmacological activity of SA was that of a potent, selective KOR agonist [12].

Pharmacology of identified compounds

Naturally occurring compounds: While, there are many novel diterpenoid structures that have been isolated such as the salvinorins (Fig 1-4), the divinatorins, the divnorins, the salvinicins, and the salvidivins the most studied diterpenoid is SA, the main psychoactive compound. SA has been shown to be a potent and highly selective KOR agonist [13] [14]. In a receptorome screening SA was tested on 50 different systems screening specific receptors, ion channels, and transporters. Being a psychoactive compound it was believed that SA would have a similar binding profile to other classical hallucinogens like LSD. This was not the case instead SA should a high affinity for only the KOR. The binding mechanism itself seemed to novel as well. Instead of ionic interactions normally seen it is possible that hydrophobic interactions drive binding and activation of the KOR by SA [15]. Another in vitro study looking at fractions of SD and binding affinity showed that the acetone fraction had the most affinity towards human KOR expressing Chinese hamster ovary cells [16]. The compounds that had the greatest affinity for the KOR were SA, salvinorin B, divinatorin D, and salvinorin G. The order was from greatest affinity to least respectively. There have also been numerous in vivo studies, which have given considerable evidence for the activity SA at the KOR as well. Although, there has been some inconclusive and contradictory evidence in early studies of SA this maybe due to route of administration (i.e. i.p. vs i.v. vs intrathecal vs intracranial), dose range, and vehicle used. SA has a very low solubility in water and due to this the findings from these studies may be altered

depending on the concentration of organic solubilizer used. As an example one study 75% DMSO was used as a vehicle [17]. In terms of KOR agonists in vivo studies usually look at discriminative stimulus effects, locomotor or movement changes, and reduction to pain stimuli (antinociception). For SA this was no different.

Discriminative stimulus refers to a stimulus that is based in reinforcement, and when given allows on to generalize its behavior to the trained response. The general procedure has shown great homogeneity from species to species, with of course a few outliers [18]. Synthetic k-opioids had already been used in discriminative testing in rhesus monkey [19]. There have been three discriminative stimulus effects experiments with SA on two different species of mammals, but they all had similar results. In all three experiments all the drugs tested with SA gave a discriminate response versus the vehicle control and SA showed a generalized response with U-69,593 in monkeys [20], as well as U-69593 [21], U50,488, Salvinorin B ethoxymethyl ether and salvinorin B methoxymethyl ether in rats[22]. In the rhesus monkey study the kappa antagonist GNTI could not complete blocks these effects in one subject, while norBNI the kappa antagonist used in the rat studies did. Bringing into question either binding mechanism differences between the two antagonists or possible species differences within the kappa opioid system.

For the studies of SA and locomotor activity changes there have been two general conclusions. SA either changes locomotor activity when given in acute treatments against a psychostimulant and doesn't change locomotor activity by itself, or in repeated treatments does not effect psychostimulant locomotor activity. As of 2009 numerous studies have been done looking at changes in neurochemistry and locomotor activity. When given with a D2/D3 receptor agonist high of SA 2.0 mg/kg given i.p. potentiated locomotor sensitization while at the lowest

concentration of 0.04 mg/kg when given i.p. attenuated this behavior. The studies' results lead to the conclusion that there is a bidirectional modulation of the kappa opioid/dynorphin system when interacting with the striatal regions [23]. This studies finding were in agreement with earlier studies with SA and the psychostimulant cocaine [24] [25] When given by itself Hooker et al and Carlezon both saw no change in locomotor activity [17] [26].

In antinociception studies SA also exerted its effects via the KOR. In two separate studies SA should an increased in the latency time in the tail-flick test [27] [28]. The max effect was short in duration and peak sharply at 10min returning and then returning steeply to baseline. This effect unfortunately was seen in the psychoactive dose range of SA in man so further studies of SA as a source for pain reduction are limited. But novel compounds have begun to be used in recent studies shifting the scaffolds binding to other opioid receptors.

Semi-synthetics: Recently more and more novel compounds have been produced using the scaffolding of the salvinorins. Herkinorin (See Fig 1-6), which is based on SA scaffolding is an interesting compound. With the addition of the benzene to the C(2) ester of SA the affinity to the KOR was dramatically reduced, while the affinity for the mu-opioid receptor was increase 100 fold [29]. This makes herkinorin a prime candidate for an opiate analgesic. Although it is likely that the mu opoiod side effects like respiratory depression, itching, and nausea will be seen with this molecule, the interesting and highly useful in opioid pain management is that herkinorin, unlike other mu-opioids does not recruit beta-arrestin-2. This means that mu-opioid internalization is halted, and should decrease traditional opiate dependency and tolerance. Although, it should be noted, that after chronic administration, forskolin-stimulated cAMP accumulation was seen in in vitro studies, which is a marker for cellular tolerance [30].

The other recent compound of interest is 2-methoxymethyl-salvinorin B (2MOM). Again it is a modification of the C(2) binding moiety (See Fig 1-7). Instead of the proton seen at C2 of salvinorin B 2MOM has a $-\text{CH}_2\text{OCH}_3$ group. This modification has made a highly potent KOR with duration of action of three hours versus 20-30 minutes seen with SA [31]. While no real therapeutic gain can be made from this yet. The understanding of this pharmacophore may aid in treatments for cocaine addiction due to KORs ability to decrease dopamine, weakening the reward mechanism seen with cocaine use.

Whole plant: Though the exact pharmacology is not known it is interesting to note a few case reports from an Australian psychiatrist who stumbled on SD after a patient began to use it [32] [33]. His patient suffered from refractory or treatment resistant depression, which is a special case of major depressive disorder that will be discussed in the next section. It seems after taken low doses of whole leaf quids, or rolled up leave balls that are chewed, the patient scored a remission score on the HAM-D scale. Something that, numerous antidepressant drugs could not do for this patient. This seems at odds with what is characterized at the moment, pharmacologically, with SA. Since SA is a selective KOR agonist what should be seen is the exact opposite in this case report. KOR agonist would cause depressive like effects thus most likely increasing the HAM-D score. To know more about what may be going on in this situation a general understanding of how depressive disorders alter the brain is needed.

Depressive Disorders

Overview

Depression in the United States has been and increasing troubling issue. The current estimates have nearly 7%, or around 15 million, of the adult American population dealing with, or have dealt with, this mental disorder. The National Institute of Health estimates that on

average the economic costs of depression are nearly 83 billion dollars per year in direct and indirect costs. Of these 15 million Americans, nearly 4% lose their lives to suicide every year.

Within the 15 million Americans suffering from depression the incidence and prevalence of the disease is nearly twice as great in women as men. There has been great debate on why this is so, with sociological factors [34], psychological factors [34], and neuroendocrine differences [35] being main argument points.

While there are many classifications or degrees of depressive disorders the main depressive that is discussed throughout this paper will be unipolar or major depressive disorder (MDD). The clinical definition of MDD is: a depressive disorder in which five or more symptoms listed below must be present for at least 2 weeks, but major depression tends to continue for at least 6 months. This list is as follows:

- Trouble sleeping or excessive sleeping
- A dramatic change in appetite, often with weight gain or loss
- Fatigue and lack of energy
- Feelings of worthlessness, self-hate, and inappropriate guilt
- Extreme difficulty concentrating
- Agitation, restlessness, and irritability
- Inactivity and withdrawal from usual activities
- Feelings of hopelessness and helplessness
- Recurring thoughts of death or suicide

Another special subtype of MDD that will be discussed is refractory depression. Refractory depression is described as cases of MDD that do not respond to at least two different antidepressants. The course of action is usually augmentation with nontraditional antidepressant on non-pharmacological treatments discussed later in this chapter.

With such a broad ranging list of symptoms MDD has, in the years, accumulated numerous theories to explain why exactly a person becomes depressed.

Theories of Depression Models

Psychodynamic

The psychodynamic model of depression is one of the oldest models that is still used in psychoanalytical therapy in the present day. First proposed by Freud and his student Abraham this model's basis is around the observation that depressive patients exhibit similar behaviors as someone who is grieving the loss of a loved one [36] (constant weeping, loss of appetite, sleeping difficulties, and general withdrawal). Freud believed that during the normal grieving process the person regresses to the oral stage of development to merge their identity with the loved one who is gone. Eventually in normal people this leads to closure, and allows for the grieving individual to move on. However if resolution is not met during the regression into the oral phase the individual can become depressive through worsening grief and the inability to cope. People who tend to lend themselves to depressive behaviors have been characterized in this model as a person whose needs were under met in the oral development stage. These individuals spend their lives searching for love and approval. This can cause the individual to experience a greater sense of loss [37].

In this theory those who are depressed without suffering a great personally loss are said to have suffered imagined loss. Imagined loss was defined as an unconscious interpretation of a general life event as a severe loss. This in turn causes depression in the individual because they are unable to distinguish between the actual events and the perceived unconscious events.

While there is little empirical data to back up this model it is still a very popular way to define depression among therapists dealing with unipolar depressed individuals.

Behavioral

This model of depression stems from the behavioral psychology model created by psychologists like Pavlov and B.F. Skinner. The main hypothesis of behaviorists is that the human mind and actions are solely based on stimuli from the environment that cause a set and reproducible responses. Reinforcement is a major tool of behavioral psychology and is used to strengthen the likelihood that a certain stimulus will reproduce a set behavior.

In depression behaviorist use the reinforcement to describe depression as well. In 1974 Lewinsohn proposed one of the most visible models [38] which viewed depression being due to a low rate of response-contingent positive reinforcement [39]. In other words the lack of positive interactions from initiating a behavioral response like; starting a conversation with someone new, going to one's favorite art show, driving to the mountains, et cetera causes these behavioral responses to be extinguished. This then deprives the person pleasure, which in turn leads to feelings of dysphoria. The low rate of behavioral responding and the feelings of dysphoria were then driving factors for other symptoms of depression including self-esteem issues and feelings of helplessness. One these symptoms manifest they are reinforced further by social rewarding responses such as sympathy, interest, and concern from others. Because of its reliance on the initiation of behavioral responses this behavioral theory has also been labeled as the social skill deficit theory [40]. Lewinsohn states that as a result of these poor social skills the depressed individual is denied the positive properties of social relationships.

Early studies lended a great deal of empirical evidence for Lewinsohn's model. Some studies concluded that: depressed individuals see themselves as less socially skilled as their non-depressed counterparts [41], [42], [43]. Paralinguistically depressed and non-depressed individuals differ as well. Such paralinguistic behaviors include speech content, facial

expression, gaze, and posture [40]. These are all skills that are used when engaging in positive social relationships.

In 1985 Lewinsohn [44] revised his behavioral model to better incorporate existing knowledge from other models. Once revised the model explained depression's onset as being caused by one or more negative life events. But again the theory focuses on behavioral tools saying that due to inadequate coping and social skills the individual will disrupt positive personal relationships, and begin to focus on negative emotional responses leading to both cognitive and behavioral repercussions. The inclusion of cognitive factors to the behavioral model could stem from the increasing influence cognitive models had in shaping depressive disorder theories.

Cognitive

Cognitive psychology/therapy was originated through the works of Jean Piaget, Albert Ellis and Aaron Beck. The main focus of cognitive psychologists is how people mentally represent image processing through more tangible outlets such as memory, problem solving, and language.

Using these tenets Beck proposed one of the first Cognitive models [45]. Beck's model described depression stemming from a depressive self-schema, which are organized representation of prior experiences. A few examples of such negative self-schemas is "I am worthless", "I can't do my job because I am not smart", or "I have no friends because I am not that special" while these may pop up every so often for a non-depressed individual and quickly diminish due to their infrequency, for a depressed person these schemas tend to be excessive and rigid [38]. Usually stemming from stressors from outside or social arenas once internalized by a depression-vulnerable individual they begin to shape the negative cognitive triad of one's self, the world, and the future. Lastly, negative changes in information processing results in a

distortion or error in reasoning like arbitrary inferences and selective abstraction, which can further fuel these depressive self-schemas.

Empirically this model gained strong support through studies dealing with the negative cognitive triad hypothesis. It had been seen that depressed people do think more negatively about themselves, the world, and the future [46]. Depressed individuals were seen to be more critical of themselves [47] other stimuli [48], and the future [49]. It seems in the case of negative trends in information processing that depressed people are more apt to direct attention to internal (mostly negative) information rather than external stimuli [50].

While Beck's initial theory did increase awareness in negative self views in depression the most influential and widely known cognitive theories of depression, is the learned helplessness model first purposed by Seligman [51]. This model was based on observations of the behavior of laboratory animals which exhibited the lack of escape behavior response after they were unable to control aversive situations, e.g. electric shock, was very similar to the behavior seen in depressed patients. Thus Seligman focused on the depressed person's expectations that they were helpless to control aversive events so their behavior changed to become consistent with these expectations. While this theory has been revised by others [52] it laid the foundation for the attributional based experiments used in studying depression in animal studies like the FST [53] as well as the TST [54] which will be discussed in a later chapter.

Malaise

The Malaise model is the newest model that will be covered in depth. It was first outlined by Charlton in 2000 [55]. What is interesting is that it detaches itself from psychological paradigms of earlier models and instead labels depression as inappropriate sickness behavior generated by abnormalities in cytokines. It goes further labeling current antidepressant therapy's

beneficial effects as analgesic in nature on the core dysphoric emotion of malaise. Sickness behavior was first defined in animal care as a:

physiological and psychological adaptation to acute infective and inflammatory illness in many mammalian species [56]. The characteristic pattern of sickness behavior comprises pyrexia, fatigue, somnolence, psychomotor retardation, anhedonia (lack of ability to experience pleasures such as eating and sex) and impaired cognitive functioning [57].

What is interesting is that the characteristics of sickness behavior listed are nearly identical to the characteristics which define MDDs. The purposed reasoning behind sickness behavior in animals is to initiate a set of actions meant to conserve energy, minimize risk, and activate the immune system, which would be appropriate for a short term all out attack on invading microorganisms [56]. For the major depressive disorder the same characteristics are employed to combat a physical illness as well. This would make the MDD the behavioral response to the somatic manifestation of malaise. Furthermore this would make the mood disorder secondary in nature to the manifestation of malaise. The research behind this theory is not as lengthy as other models since it is fairly new. At the moment though there is an animal model for depression based on the i.v. administration of interferon alpha [58]. Also depression is one of the more highly reported adverse side effects in clinical trials involving interferon and interleukins in the treatment of cancer and viral infections [57]. In recent years there has also been studies outlining the increase of inflammatory markers in depressed patients [59].

Over the years there have been numerous models, more than outlined in this paper, but with all the models, one underlying factor is apparent- the underlying causes and treatments of this disease state are numerous in nature making depression an issue that needs a multifaceted approach to diagnosis and treatment.

Pathophysiology and Treatment

Pathophysiology

As was seen with the numerous models of depression in a psychological construct there are just as many theories in regards to the pathophysiology of depression.

Tying in with the cognitive and behavioral models of depression is the dysregulation in the monoamine neuronal system. The main neurotransmitters involved in MDD are norepinephrine (NE), dopamine (DA), and serotonin (5-HT).

Noradrenergic involvement in depression: neurons containing NE begin in the locus ceruleus (See Fig 1-7) and project to numerous places in the brain including; the cerebral cortex, basal ganglia, the limbic system, hypothalamus, thalamus, and hippocampus. It is understood that the diffuse projection pattern is consistent with NE's role in maintaining arousal and modulating other the function of other neurotransmitters [35] [60].

Dopaminergic involvement in depression: The dopaminergic system arises in four distinct pathways (See Fig 1-8). For the discussion of the involvement of DA in depression two pathways will be discussed. The mesolimbic which begins in the ventral tegmental area (VTA) and projects through the limbic system including the nucleus accumbens, amygdala, hippocampus, and cingulated gyrus modulates emotional regulation, learning, memory, mechanisms for positive reinforcement and thrill seeking [61]. The other important pathway is the mesocortical system which also originates in the VTA and projects to the orbitofrontal cortex, and the prefrontal cortices. This pathway is involved with motivation, attention, and social tasks. Together these two pathways create a behavioral facilitation system [62]. Thus it is believed to contribute to poor concentration, anhedonia, and motivation issues in depression. Newer studies strengthen the behavioral facilitation system argument. A recent study has shown through functional magnetic resonance imaging that the inability to sequester negative thoughts

from the limbic areas is caused by a sharp decrease in activity in the lateral orbitofrontal cortex [63].

Serotonergic involvement in depression: the serotonergic pathway begins in the raphe nuclei of the brainstem and project to numerous parts of the brain including the cerebral cortex, hypothalamus, basal ganglia, and hippocampus [64]. Much like NE, 5-HT projections are numerous and because of this it is believed 5-HT modulates the activity of other neurotransmitters [65] as well being important regulator of libido, appetite, and sleep [66]. It is also suspected that 5-HT regulates circadian rhythms through neuron projecting into the suprachiasmatic nucleus of the hypothalamus where it regulates sleep-wake cycles, hypothalamus-pituitary-adrenal (HPA) axis activation, and body temperature [67]. It is this physiological evidence that allow researchers to link reductions in 5-HT transmission to appetite and sexual behavior problems as well as aggression and suicide in depressed patients [68].

The dysregulation of monoamines has been studied for numerous years and has a solid body of work showing that this is a key pathway in depression pathophysiology, but what caused the change was it one traumatic event, genetic, or in the case of the malaise theory misperceived stressors? The HPA axis has always been secondary to monoamine function when describing depression, but there is more and more evidence that the HPA axis and cytokine proliferation have more of a role than previously thought.

Neuroendocrine and inflammatory markers in depression: The HPA axis, also known as the stress axis is a primary neuroendocrine component of the “fight or flight” response. When a perceived stressor is received by the limbic system the impulse is sent to the hypothalamus which secretes corticotrophin releasing hormone (CRH), which then triggers the pituitary adrenocorticotrophic hormone (ACTH), and finally the ACTH reaches the adrenals where

hormone known as cortisol, a glucocorticoid (GC) steroid, is released. Normally after the stressor dissipates or the negative feedback loop is initiated when cortisol interacts with GC receptors in the hypothalamus causing the hypothalamus to suppress CRH, there is a decrease in circulating levels of cortisol. In depressed patients however this negative feedback loop is muted so in times of chronic stress there are high levels of CRH and cortisol in the brain. This in turn interacts with 5-HT, NE, and DA transmission [38].

There is more and more supporting evidence for the HPA axis's involvement in depression. In rats chronic stress and elevated GC levels alter 5-HT receptors expression in the cortex. There is an increase expression of 5-HT_{2A} and a decrease of 5-HT_{1A}. This is also seen in brains of suicide victims and Cushing syndrome patients, which is, a syndrome marked by hyper secretion of cortisol, and people suffering from this syndrome show a high incidence of depression [69]. Neurogenesis in the hippocampus is also decreased by high levels of GCs in the brain. This is reversed over time by antidepressants and usually correlates with the long period it takes to see increased morale in depressed patients [70].

But even newer still is the discussion of the inflammatory immune system, and these new studies in cytokine involvement in depression have led to some interesting conclusions on how this disease state affects the body and vice versa. In recent years it has been shown that cytokines like exogenous stressors can elevate CRH [72]. Cytokines like interleukin-1-beta and tumor necrosis factor-alpha play a role in neural plasticity as well. Through changes in nuclear factor kappa B, mitogen-activated protein kinase and janus kinase/ signal transducer and transcription changes in apoptotic or oxidative mechanism are altered leading to decreased levels of neural growth factors like brain-derived neurotrophic factor. [71]. These factors mimic or may combine

with CRH in altering neuronal growth in key areas for mood and memory like the hippocampus and hypothalamus.

Pharmacological treatment

The monoamine model, for the most part, has been the main model for so long due to the long history of pharmacological agents that have help strengthen it. For example tricyclics like imipramine work by blocking 5-HT and NE reuptake pumps. This presumably allows for longer residing times at the receptor site. MAO inhibitors block degradative pathways allowing for more monoamine neurotransmitters to accumulate in stores, which in turn allows for more of them to be released. Even the newer antidepressant like mirtazipine and nefazodone work on monoamine systems, but instead of increasing neurotransmitter concentrations they work to antagonize 5-HT_{2A} or 5-HT_{2C}. Mirtazipine also antagonize alpha-2 receptors which aids in its therapeutic effects. The antagonism of these receptors is thought to aid in depression symptoms by enhancing stimulation of 5-HT_{1A} receptors [72]

Specific agents for neuroendocrine modulation have been thought of as well. On the forefront of this are the CRH antagonists, specifically CRH sub-1 antagonists. In both clinical studies it was shown that these compounds showed the ability reduce the stress elicited secretion of stress hormones. It was also shown that neither compound impaired the release of ACTH, which was a main worry of drugs from this new class. With these findings, CRH sub-1 antagonists may prove to be a new and novel treatment for stress related disease states, and also may help in the prevention of negative sequelae of severe stressors [73].

Non-Pharmacological treatments

For refractory depression cases pharmacological intervention is not successful so, most physicians begin to weigh non-pharmacological treatments like deep brain stimulation (DBS) and electroconvulsive therapy (ECT).

DBS is a fairly new concept in treating depression, but has shown efficacy in treating refractory depression, and is usually only used after ECT has not been effective. How DBS works is that tiny electrodes are placed into the brain near areas of interest. They are stimulated while the patient is awake to ensure proper placement. From here the patient is placed under anesthesia and the electrodes are hooked up to battery which is implanted into the body near the collarbone, acting like a tiny pacemaker for the brain region. One area targeted by this treatment is the subgenual cingulate region (area 25). Area 25 is high in transporter binding sites with numerous projections to the brain stem, and more importantly the hypothalamus. It also has critical projections to and from the ventral striatum (through the nucleus accumbens) and the limbic system. What is interesting is that area 25 is metabolically overactive in refractory patients. In one study six refractory depression patients were subjected to DBS of the white matter tracts close to area 25. The results were a striking and sustained remission attributed to reduction of cerebral blood flow and downstream changes in limbic and cortical areas as well [74]. Other regions of interest for DBS are the nucleus accumbens, the inferior thalamic peduncle, the rostral cingulate cortex, and the lateral habenula.

ECT is a therapeutic technique that uses pulses of electricity to induce a clonic seizure in patients for around a quarter of a minute. Though the direct mechanism of action is a bit hazy it is still used in psychiatry today. Newer research has shown that ECT has therapeutic effects by increasing brain-derived neurotrophic growth factor (BDGF) [75]. BDGF would stimulate neuronal growth in areas where stress damage has decreased neurogenesis for depression hippocampal neurogenesis for example is what is generally seen after four to six weeks of traditional pharmacological treatment.

Hypothesis and Specific Aims

The study of medicinal plants has yielded numerous novel compounds, and has been used as scaffolding for building a stronger knowledge of medicinal chemistry, pharmacology, and human physiology. But our knowledge of certain disease states is still limited in nature. Mental health disease states cost the United States billions of dollars per year in missed work, have destroyed families, and cause the untimely death of a percentage of people afflicted, but yet our understanding of these disease states are lacking, and have been slowed by negative stigmas towards the mentally ill.

Due to this lack of understanding it is imperative that any claims plants with positive or therapeutic activity be looked at with scientific rigor. After the case reports of SD having activity in refractory patients the goals of this research was to characterize what may be causing this activity- plant or placebo effect. Using this general schematic three specific aims were devised to try to answer if and why this novel hallucinogen would have activity as an antidepressant.

Specific Aim 1

Create an extraction scheme that would optimize the extraction of different categories of compounds (diterpenoids, flavonoids, phenolic acid derivatives, et cetera.) Modeling the original method of ingestion of SD a basic infusion was created. From this starting point two other extraction techniques were created using the solvent polarity constants to give a range of polarities to optimize each extraction method. From here a multistaged analytical scheme can be created to incorporate the quantification of pharmacological activity, but also give insight into structural elucidation as well. Starting with Analytical HPLC each extract will be qualified and quantified for compounds of interest. From here fraction collection will begin using solid phase extract. After this each fraction can be prepared for LC-MS analysis.

Specific Aim 2

Create a panel of behavioral assays as well as possible correlative metabolic studies that allows for a comprehensive look at both SA and the various SD extracts. Assays should consist of antidepressant and any secondary test for qualification of the results; assays such as the Tail Suspension test (TST), Forced Swimming test (FST), the Open Field test (OFT) should be considered.

Specific Aim 3

Get a better understanding of the potential role SA plays on the overall activity of the SD. Using a test for neuroleptic drugs SA will be tested p.o. as well as i.p. to see how it interacts with the dopaminergic pathways via the Compulsive Gnawing test (CG)

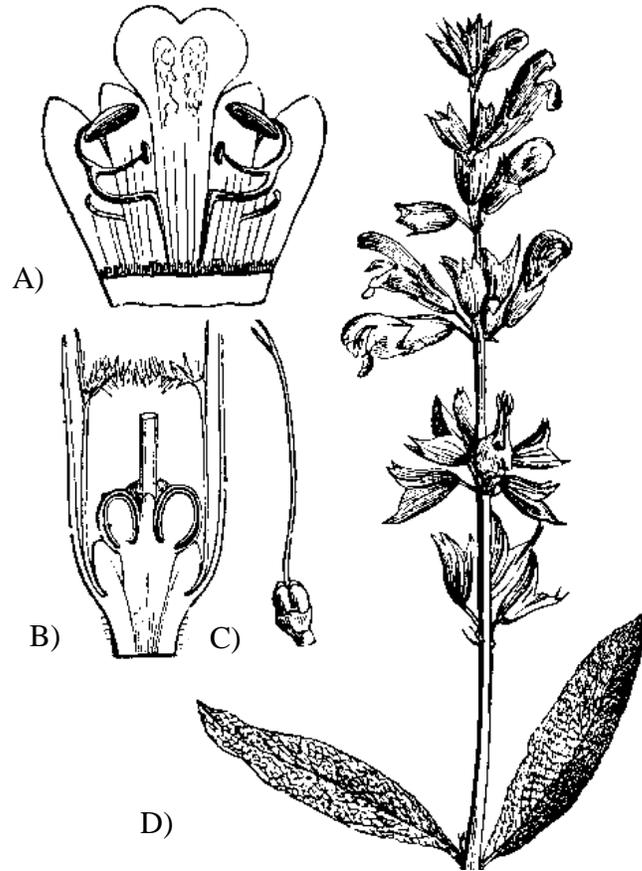


Figure 1-1. A taxonomic sketch of *Salvia officinalis* showing A) the open corolla B) cross section of the pistil and lower flower C) the pistil D) the aerial parts of *Salvia officinalis* [1]



Figure 1-2. A stamen from *Salvia* sp. highlighting the fused theca and lever mechanism for pollination [1]

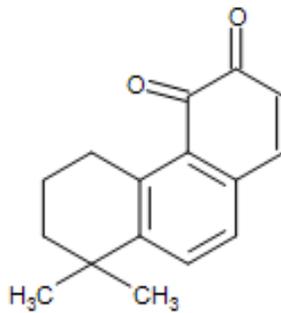
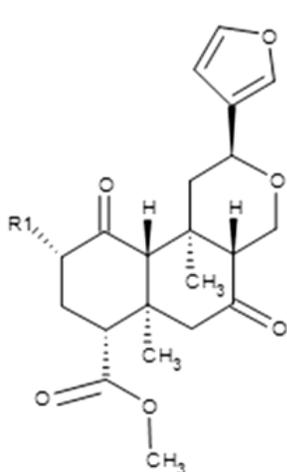


Figure 1-3. Miltirone: the diterpene quinone from *S. miltorrhiza* with CNS activity via the GABA receptor.

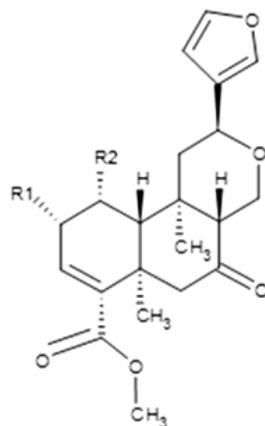


Figure 1-4. A curandera preparing *Salvia divinorum* on a metate [8]



R1: OCOCH₃ = Salvinorin A

R1: H = Salvinorin B



R1: OCOCH₃; R2: OCOCH₃ = Salvinorin C

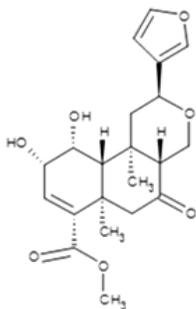
R1: OH; R2: OCOCH₃ = Salvinorin D

R1: OCOCH₃; R2: OH = Salvinorin E

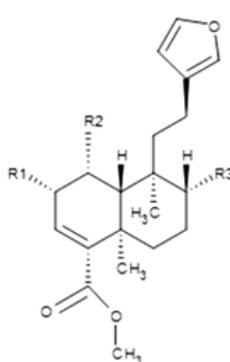
R1: H; R2: OH = Salvinorin F

R1: O; R2: OCOCH₃ = Salvinorin G

R1: OH; R2: OH = Salvinorin H



Salvinorin I

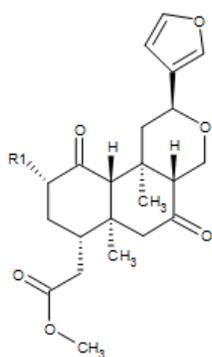


R1:H; R2:OH; R3: CH₃ = Divinatorin A

R1:H; R2:OH; R3: CH₂OH = Divinatorin B

R1:H; R2:H; R3: CH₂OCOCH₃ = Divinatorin C

Figure 1-5. The neoclarodane diterpenoid group known as salvinorin. The psychoactive compound salvinorin A was the first of this group described



R1 = OCH₂OCH₃ = 2MOM
 R1 = OCOC₆H₅ = Herkinorin

Figure 1-6. The semisynthetic structures of herkinorin and 2MOM

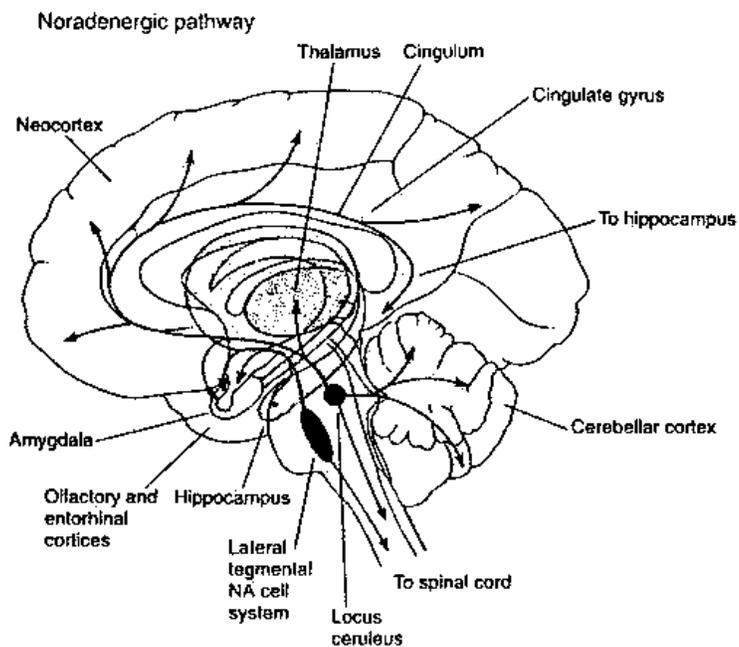


Figure 1-7. The noradrenergic pathway with its main centers located in the Locus cereleus [38]

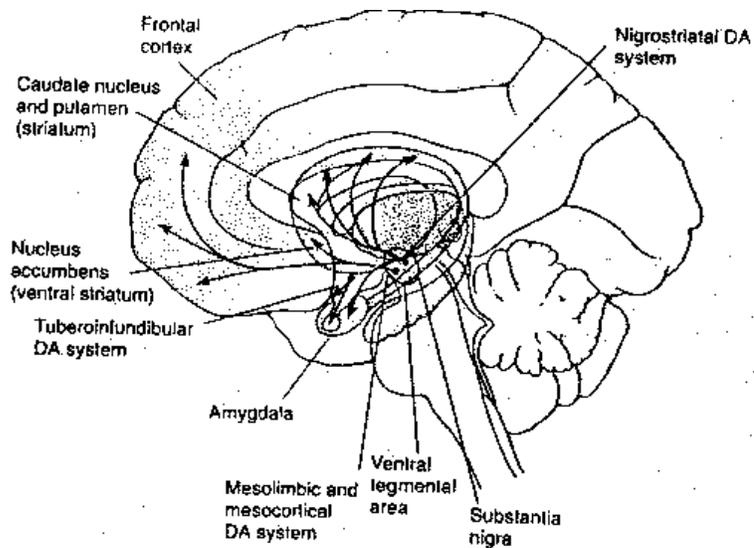


Figure 1-8. The dopaminergic pathways with centers located in four distinct paths. The nigrostriatal originating in the substantia nigra, the tuberoinfundibular originating from the hypothalamus, the mesolimbic originating from the ventral tegmental area, and the mesocortical which also originates in the ventral tegmental area but leads to the prefrontal area of the neocortex [38]

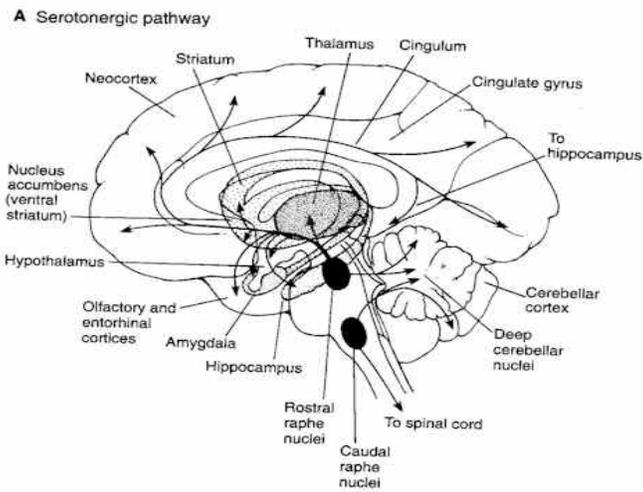


Figure 1-9. Serotonergic pathway arising in the raphe nuclei. In the study of depression the rostral raphe nuclei pathway is the important pathway [38]

CHAPTER 2 EXTRACT PREPARATION AND ANALYSIS OF *Salvia divinorum*

The driving force behind the analysis of SD was the more traditional methods of extraction discussed in chapter one. With this in mind an initial extract method was derived that was based on an infusion of SD in hot, not boiling, water with agitation of the leaf matter via a magnetic stir bar. The salvia water extract (SWE) was allowed to steep for one hour, then freeze dried and stored in a brown glass vial under an argon headspace. SWE was then tested for behavioral activity, which will be discussed in detail in the next chapter. After the initial activity was confirmed other typical hydroalcoholic extracts were created. This chapter will discuss the extraction methodology, qualitative and quantitative high performance liquid chromatography (HPLC) analysis, and finally Mass Spectrometric (MS) analysis.

Methodology

Extraction: After the behavioral screening of SWE confirmed two new extracts were created; a hydroethanolic extract (SHE) that was a 40:60 mixture of water:ethanol, and a hydromethanolic extract (SHM) that was a 20:80 mixture of water:methanol. The general method of extraction was as follows:

- For each extraction method the weight of leaf matter used was 10g per 200mL of solvent used.
- For the SWE the temperature of infusion was roughly 50 degrees Celsius and constantly agitated using a heat plate/ stirrer (Fisher Scientific). The extract is then placed into a cold ice bath to quickly drop the temperature and allow for a cold protein break.
- For the SHM and SHE the extraction temperature was room temperature (21-24 degrees Celsius). For SHM and SHE the extraction time was also one hour.
- After extraction time is up the extracts are filtered using a Buchner funnel (Fisher Scientific) and the plant water is washed with de-ionized (DI)-water. After plant material is dry the vacuum is reduced on the Buchner funnel. The extract is then collected.
- For SWE the filtered extract is directly placed in the shell freezer and freeze dried. For SHM and SHE the filtered extract is placed in a round bottom flask (RBF) with

considerable head space and placed on a rotary evaporator (Roto-vap) to remove excess alcohol. Afterwards the sample has DI-water added to the RBF and sonicated to remove sample residue from the glass. After which the sample is transferred to the shell freezer and freeze dried.

General HPLC: All extracts are quantified and qualified using a Merck reverse phase C18 column with a C18 pre-column. The HPLC analyses will be performed on a Shimadzu liquid chromatograph (LC-10 AT) with a ternary solvent delivery system, combined with a Diode Array detector (SPD-M 10 A), and a Rheodyne loading valve fitted with a 100 µl sample loop. For HPLC separation of SD extract a LiChroCart RP-select (250mm x 4mm i.d., 5µm particle diameter; Merck) reversed-phase column, with a pre-column (4.6 mm i.d. x 2.5 cm) containing the same packing. Elution was carried out using the following solvents: H₂O (Millipore), Trifluoroacetic acid (TFA) and brought to a pH of 2.8 using the TFA (solvent A) and Methanol, Tetrahydrofuran (THF), TFA in a ratio 98:2 (v:v) brought to a pH of 2.8 using the TFA (solvent B) at a flow rate of 0.8 mL/min. The mobile phase is filtered through a 0.45 µm pore filter (Millipore) and then degassed prior to use. Samples of 10 µl are injected and eluted using a gradient method (Tab 2-1) the detection of the peaks is recorded at 190-400 nm using the photodiode array detector. Chromatography is performed at 35°C temperature is controlled with a column-oven (Shimadzu, CTO-10 AS). The use of THF as a peak resolution aid was kept to a minimum in this study since the diterpenes of SD only shows absorbance at 208nm [76]. The UV cutoff of THF is 240nm which in larger quantities would interfere with the proper quantification of SA.

Qualitative HPLC: Using the methods described in the general HPLC section the qualification of extracts was done using the following parameters:

- After the initial HPLC run each peak from the chromatograph was analyzed for spectrum files of phenolic acids, catechins and flavonoids.

- After a peak's spectrum file is checked candidate compounds are picked to spike into the extract. Much work has been done to identify phenolic compounds in *Salvia* and a review of this was used to determine most likely candidates.
- After candidate screening was done those with a retention time and spectrum file match with a peak of interest were used to quantify the extract.
- After the qualification of the extracts was complete the internal standard apigenin-7-O-glucoside was chosen for the quantification of the candidate compounds

Quantitative HPLC: Using the methods described in the general HPLC section the quantification of extracts was done using the following parameters:

- Each candidate compound that matched in the qualitative screening: caffeic acid, rosmarinic acid and SA were used to create a standard curve to analyze the unknown peak weight of each qualified peak from the extracts.
- The method of quantification of was external standardization with each standard curve extrapolating the mean peak weight from the triplicate run of each extract.
- Linear regression was then used (GraphPad[®]) and each peak's mean concentration was plotted with standard error of the mean.
- Due to only three peaks being qualified and quantified a solid-phase extraction (SPE)-LC/MS-MS method was employed to better understand the water extract behavioral activity.

Solid-phase extraction: Using the SWE chromatograph a primary extraction method was used to split the main extract into three parts based upon increasing methanol concentrations of 0, 55, and 75%. The primary column used was a C-18 column with a 5g bed (Fisher Scientific). From here each sub fraction weighed and subjected to a secondary SPE method using phenyl columns with a 1mg bed (Fisher Scientific). The general procedures were as follows:

- The initial loading solvent of the extracts was equal to the initial concentration of solvent it was collected in. Meaning for primary SPE SWE was diluted in acidic water (AW) with a pH of 2.8 using TFA. For secondary SPE the sub fractions would be in AW, 55:45 methanol:AW, and 75:25 methanol:AW.
- The vacuum pressure on the manifold on each run was set to 15 inches of mercury. And before the sample was placed onto the column. The columns were washed with 10mLs of each solvent used then rinsed with the initial loading solvent.

- For primary SPE the loading concentration was 15mg/mL, for the secondary extraction the loading concentration was set to 5mg/mL. The initial runnings were dried down and scanned via HPLC to ensure no bleed through occurred during loading. The sample was washed with 15mL of solvent for primary SPE, and 5mL of solvent for secondary SPE. The washing were combined and dried.
- For AW samples direct freeze drying of the samples occurred. For hydroalcoholic samples similar measures were used for sample drying. First the samples were subjected to rotary evaporation to eliminate the methanol. From here the residue and excess water was diluted with water and the Roto-vap container was sonicated to loosening the extract residue from the glass.
- The diluted residue was transferred and frozen in the shell freezer, afterwards it was freeze dried.
- Each dried sample was weighed and scanned for peaks of interest using the HPLC method discussed in the general HPLC section.
- From here the new samples were transported with standards to USDA-ARS (Parlier, CA) for quadrupole-time of flight (qtof) MS analysis.

MS analysis: Using the methods highlight in a paper by [77] the samples were ran on a qtof MS by Spencer Walse at the USDA-ARS to determine molecular weight and subsequent molecular candidates for the peaks of interest in SWE. The general method is as follows:

- A Shimadzu system pump, coupled with a ThermoFinnigan UV6000LP LDC photodiode array detector (PDA), a YMC-Pack ODS-AQ analytical column (*l* 250 mm, i.d. 4.6 mm, S 5 μ m), and a Bio-Star qtof mass spectrometer (HPLC-MS) was used.
- Mass spectra were obtained using electrospray ionization (\pm ESI) with a 5 kV spray voltage and a 275 $^{\circ}$ C capillary temperature.
- Sheath and sweep gas flow rates (arb) were 40 and 20, respectively.
- The mobile phase (1 mL/min) was split after the PDA; \sim 10% was directed to the MS and the remainder collected. Eluant composition was (a) 0.1% formic acid in ACN, (b) 10 mM ammonium formate, and (c) 10 mM ammonium formate in 90% ACN. The elution program was isocratic (4a:72b:24c) for 13.5 min, to 4:0:96 over 4.5 min, isocratic (4:0:96) for 17 min.

Results

The results of the extraction method were three distinct looking extracts. SWE was brown in color with mixed shaped and density particles. While SHM and SHE were more uniform in particle shape both had a green tint with SHM being the greener of the two.

From the qualification HPLC work SWE (Fig 2-1a) was shown to have five peaks of interest with a catechin peak at 4.5 mins, two phenolic acid peaks at 10.5 and 14.8 minutes and , a flavone peak at 14.0 minutes, and what looks to be like two diterpenoid peaks at 20.6 and 24.3 minutes. SHE and SHM (Fig 2-1b and Fig 2-1c) show an absence of the catechin peak, a decrease in the first phenolic acid peak, and an increasing diterpenoid/non-polar presence, which was expected from the properties of the different extraction methods. From the compound screening three peaks had positive retention and spectrum matches; the monomeric phenolic acid caffeic acid and the dimer rosmarinic acid, as well as the neocladane diterpenoid SA. The catechin and the flavone failed to get positive retention time matches, but the spectrum files did match. The flavone luteolin-7-O-glucoside came the closest to matching the peak with a retention time difference of roughly 40seconds.

From the quantification studies the three extracts where run in triplicates and the mean peak area was used to determine the amount of CA, RA and SA in each extract. SA was below the limits of quantification in the SWE fraction, and was in SHE and SHM at 19.64 ± 1.21 and 34.7 ± 4.80 respectively; while CA and RA where in SWE, SHE, and SHM at 0.93 ± 0.14 , 0.38 ± 0.02 , 0.31 ± 0.03 for CA, and for RA 2.39 ± 0.23 , 5.75 ± 0.09 , 6.99 ± 0.15 respectively. All values are in mg/g of extract.

In the MS study, caffeic acid and SA where confirmed via MS/MS.. Also, in the 55% fraction are terpenes that were not seen via UV meaning the robustness of this extract can not readily be determine via UV-based methodology. In the fraction with SA was a diterpenoid with

relatively higher ion abundance. From the HPLC qualification studies it is speculated to be SB. SB readily comes from the hydrolysis of SA, and so can have its relative concentration in the extract enhanced by simple manufacturing processes.

General Summary

From the results of extract and analysis section the main conclusions that can be drawn are that UV-HPLC is suitable for discussing the hydroalcoholic extracts like SHE and SHM, but in the case of SWE the sensitivity is not low enough for proper quantification and reliance on LC-MS-MS is needed. Due to sensitivity issues seen with the UV-HPLC method, the SWE was found to be more robust, with numerous compounds of interest available to test, so that further exploration into the activity of SWE discussed in the next chapter can be determined.

Table 2-1. Setup of HPLC method

Minute	H ₂ O (%)	Methanol (%)
0	78	22
7	65	35
23	0	100
30	0	100
32	78	22
35	78	22

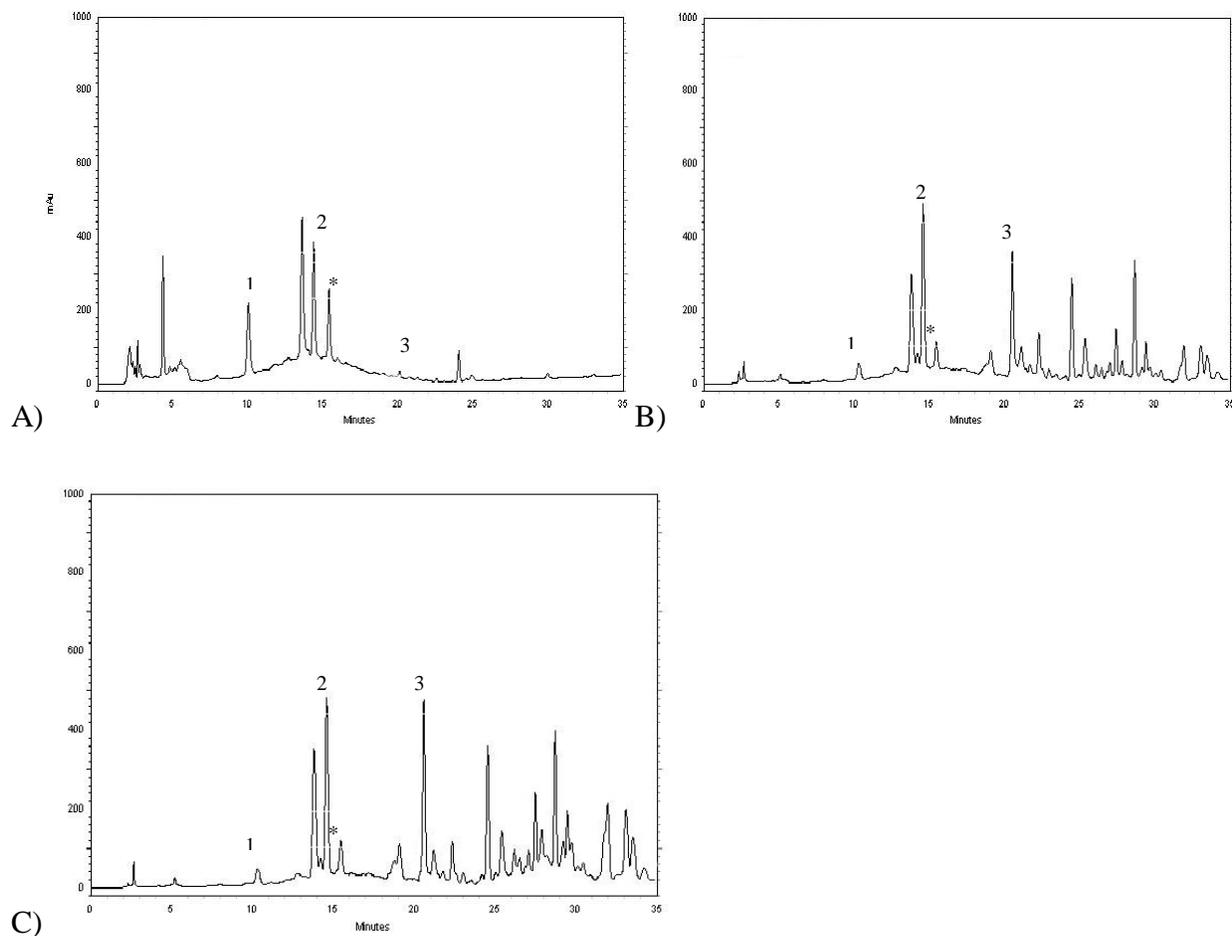


Figure 2-1. The chromatographic readouts for the extracts A) SWE B) SHE and C) SHM at 208nm * = internal standard Apigenin-7-O-glucoside with 1= caffeic acid 2 = rosmarinic acid and 3= salvinorin A

Table 2-2. Primary and secondary SPE weights from 2.89 grams of SWE

Primary	Weight (mg)	Secondary	Weight (mg)	Secondary	Weight (mg)	Secondary	Weight (mg)
0% MeOH	1,890	0% MeOH	1390	50% MeOH	104.5	100% MeOH	8.1
55% MeOH	671.2	0% MeOH	100.5	50% MeOH	433	100% MeOH	32.1
75% MeOH	114	0% MeOH	49	50% MeOH	10.2	100% MeOH	37.6
100% MeOH	15	n/a	n/a	n/a	n/a	n/a	n/a

CHAPTER 3 ANTIDEPRESSANT-LIKE ACTIVITY OF SALVIA WATER EXTRACT

Animal Models

Forced Swimming Test

A widely used behavioural tool for assessing antidepressant activity pre-clinically is the forced swimming test (FST), which was developed by Porsolt and co-workers [53]. This behavioral test was used for producing a state resembling depression in rats. Male CD rats (200 – 250g) were placed into a Plexiglas cylinder (40 x 18 cm i.d.) containing a column of 17 cm of water at $25 \pm 1^\circ\text{C}$. According to Porsolt the rats learn in a pretest session of 15 min that they cannot escape from the cylinder. In the test period, 24h later, the animals were exposed to the experimental conditions for 5 min again [53]. A rat was judged to be immobile whenever it remained floating in the water, in an upright position, making only small movements to keep its head above the water. The FST was recorded digitally using a high-resolution video camera WV-CP244 (Panasonic, Secaucus, NJ, U.S.A). The camera is connected to a computer for automatically recording of the movements (FSTScan[®] software, Cleversys. Inc., Reston, VA). After each test session the animals will be dried for 15 min in a heated enclosure (32°C) and then returned to the home cage. The water in the cylinders will be changed after each animal and its temperature controlled by a thermometer to make sure that the temperature is exactly at $25 \pm 1^\circ\text{C}$. Twenty-four hours after the pre-test session, the animals are exposed again to the conditions outlined above for 5 min (test session)

Tail Suspension Test

The tail suspension test in mice (TST) is widely used for measuring the pharmacological effects of antidepressant drugs or stress-evoked behavior in mice [54]. The TST uses the uncontrollable, inescapable stressor of tail suspension to elicit immobility [54]. The TST

paradigm hangs a mouse by its tail for 5-min. A typical response in this paradigm is struggling alternating with passive immobility. The duration of immobility is accumulated throughout the 5-min period. The procedure is conducted in a sound attenuated room. Automated TST devices are used to measure the duration (sec) of immobility in the tail suspension test. Mice are suspended by the tail with padded clothes pins to the apparatus.

The total duration of the test (5 min) can be divided into periods of agitation and immobility [78]. Antidepressant drugs decrease the duration of immobility, as do psychostimulants and atropine. If coupled with measurement of locomotor activity in different conditions, the test can separate the locomotor stimulant doses from antidepressant doses.

For our study we used an automated analysis of the TST which evaluated the time the animal (Male C57BL/6 mice between 6-12 weeks old and weighing 22–34 g were purchased from Harlan (Indianapolis, IN, U.S.A). Mice were housed in cages of 5 at 20 ± 1 °C in a 12-h light/dark cycle) spent in immobility, struggling, and passive swinging (TailSpusScan[®] software, Cleversys. Inc., Reston, VA). It takes video of behaviors of animal in suspension as input and outputs the states of behaviors. TailSuspScan[®] automates the entire observation process of tail suspension experiment. It automatically detects not only the time animal spent in immobility, but also the time animal is struggling. In addition, TailSuspScan[®] has the capability to differentiate between active struggling mobility and passive swinging mobility. The latter can either be grouped into the immobility category or kept separately for analysis, thus making the results of immobility much more precise. Only the motion caused by active struggling behavior is captured as struggling.

Open Field Test

This method is usually used to evaluate possible sedative or stimulating activities of animals [79]. The test is also used as an indicator for locomotor activity. The open field consists

of a round grey plastic arena measuring 70 cm in diameter surrounded by a grey plastic wall of 34 cm height for rats, or a rectangular box with the dimensions of 33cm X 45cm X 40cm, and is evenly lighted with three 40 W light bulbs. The floor of the arena is divided into several concentric units by black painted lines, dividing the arena into 19 equally sized fields for round arena or 6 for the smaller rectangular arena. Each rat or mouse will be placed in the center of the arena and recorded for 5 min after the FST or TST experiments have concluded. With only those active in these tests being examined. The parameters evaluated in this test include number of field crossings and total distance covered by the animal. We assume that drug effects on the mouse would be present if both number of field crossings and distance are significantly lower in the treatment groups compared to control.

OFTs will be digitally recorded using a high-resolution video camera WV-CP244 (Panasonic, Secaucus, NJ, U.S.A). The analysis of the videos will be performed using TopScan Top View Animal Behavior Analyzing System (version 1.00, Clever Sys Inc. Preston, VA, U.S.A).

Results

FST

The FST was used as a preliminary screening model for the first extraction technique the SWE to see if SD had initial activity. The groups tested were:

- Control; the negative control testing the vehicle which was DI water
- Imipramine; the positive control. Imipramine is a tricyclic antidepressant with known activity in the FST
- All groups were analyzed statistically in the FST and corollary OFT using GraphPad[®] (). A one-way ANOVA was run with a Tukey's comparison post-hoc to determine statistical significance between groups.
- SWE 25mg/kg and 50mg/kg p.o. The infusion of SD the oral dose of 50mg/kg was based on observations from the case report by Jones.

The results (Fig 3-1) were that SWE at 50mg/kg significantly decreased immobility when given orally. The corollary OFT showed no significant changes in locomotor activity when given the active SWE dose of 50mg/kg (Fig 3-2). It was with this result in mind that various extractions of SD were created- discussed in chapter 2, and tested via the TST.

TST

The main screening model used throughout this project. This was the main model used due to the rapid throughput aspect of the test. Since no training period is involved screening extract activity is done more rapidly. The main screening agenda was;

- Multiple doses of each SD extract, SWE, SHE and SHM to look at dose response.
- Pure SA in multiple formulations oral and i.p. as well as multiple doses to explore any differences in activity.
- All oral drugs were given in a vehicle of DI- water except fluoxetine which was solubilized in a 0.5% polyethylene glycol solution.
- For SA 1-5mg/kg 1% ethanol was added. For SA 10mg/kg 2% ethanol was added plus the solubilizer Tween 80[®] also known as polysorbate.
- All groups were analyzed statistically for both the TST and corollary OFT using GraphPad[®]. A one-way ANOVA was run with a Dunnett's comparison post-hoc to using the vehicle as control to determine statistical significance between groups.

The only activity that was statistically significant was the SWE 50mg/kg concentration. This was seen throughout the screening (Figs 3-3, 3-5, and 3-6). There was a slight decrease in immobility at the SWE 25mg/kg, but it was not statistically significant. For the other extracts of SD there was no change in activity from the control across all concentrations of SHE and SHM, which were 12.5, 25, 50, and 100 mg/kg p.o. (See Fig 3-5 and 3-6). SA was also no different than the control both for the p.o. concentrations of 5 and 10 mg/kg, and the i.p. concentrations of 2.5, 5, and 10mg/kg (Fig 3-3 and 3-6). The corollary OFT tests after the TST all were in

agreement with the FST screening. This would equate to the antidepressant-like effects were not based on changes in locomotor activity. So where is this activity coming from? From the previous chapter HPLC chromatographs of SWE showed scant peaks with a few phenolic acids, catechins, and a lone flavone. Since SD has numerous diterpenoids, which do not show up in the chromatographs the SWE was screened for activity using the TRPV3 receptor.

TRPV3 Receptor Screening

Overview

Since SD contains numerous diterpenoid compounds other than SA. It was hypothesized that the antidepressant-like activity seen in SWE might be due in part to these compounds. Since the case reports discussed the efficacy of SD in cases of refractory depression finding a possible alternative mechanism of action not based around the monoamine system was also an interest.

In 2008 the CNS activity of frankincense, or *Boswellia* spp., was characterized via animal studies as well as cell culture [80]. The test compound, Incensole acetate, a cembranoid diterpene was shown to bind to transient receptor potential vanilloid (TRPV) 3 receptors which are receptors used to perceive warmth on the skin. These receptors are located in various regions of the brain as well, and their function there has not been totally understood. When incensole acetate binds to TRPV3 receptors in the CNS it appears to have an antidepressant and anxiolytic-like effect, and it through this mechanism it was shown to have a positive effect on wild type mice when placed in the elevated plus maze and FST while TRPV3 knockouts were not affected.

Methodology

The SWE sample was sent to Max Peters at the Hebrew University. From here images were recorded with an Olympus microscope in combination with Fluoview 100 confocal microscope system. HEK-293 cells were grown under standard conditions, seeded on poly-L-lysine coated cover slips to achieve a confluency of ~60%. 24 hours later the cells were

transfected using TransIT with 3 micrograms of plasmid (containing the TRPV3 channel with a CMV promoter) per 4 mL medium (equal to 3 cover slips). 30 hours later cells were washed with twice with Dulbecco's Modified Eagle Medium (DMEM), transferred to separate Petri dish and incubated with 2 ml of DMEM and 2 mM Fluo-4AM in the presence of 0.0002% pluronic F-127. After 30 min the medium was removed, cells were washed twice with DMEM and incubated for another 30 min in DMEM containing pluronic 0.0002% F-127.

Cells were then transferred to the recording chamber of the confocal microscope and perfused with extracellular solution (NaCl 140, KCl 2.8, MgCl₂ 1, CaCl₂ 2, Glucose 10, Hepes 10) at room temperature (22 degree C) in the presence or absence of the pharmacological agent.

Results

The results of the receptor binding were that SWE had minimal affinity to the TRPV3 cell line. Images from the study show only slight changes in Fluorescence when cell were in the presence of SWE versus control (Fig 3-8a). This compared to the runs with the positive control shows (Fig 3-8b, c and d) the gap in activity between the two substances.

General Summary

From the results discussed in chapter 3 SWE was only active in 50mg/kg when given orally in the FST and TST. Furthermore, the OFT, a test for changes in locomotor activity showed no significant increase in activity for the active concentration of the SWE. Meaning that the activity seen in these two highly regarded animal models for antidepressant activity is based not based on psychostimulant activation and is an antidepressant effect. In an attempt to incorporate the high diterpene presence in SD the active extract was sent to Max Peters to be studied on the TRPV 3 on vitro model. At the one concentration tested there was no significant change in fluorescence activity between the vehicle and the SWE at 0.2mg/mL. With only one

test concentration it should be noted that further work should be done to ensure there is no activity from this fraction on TRPV 3.

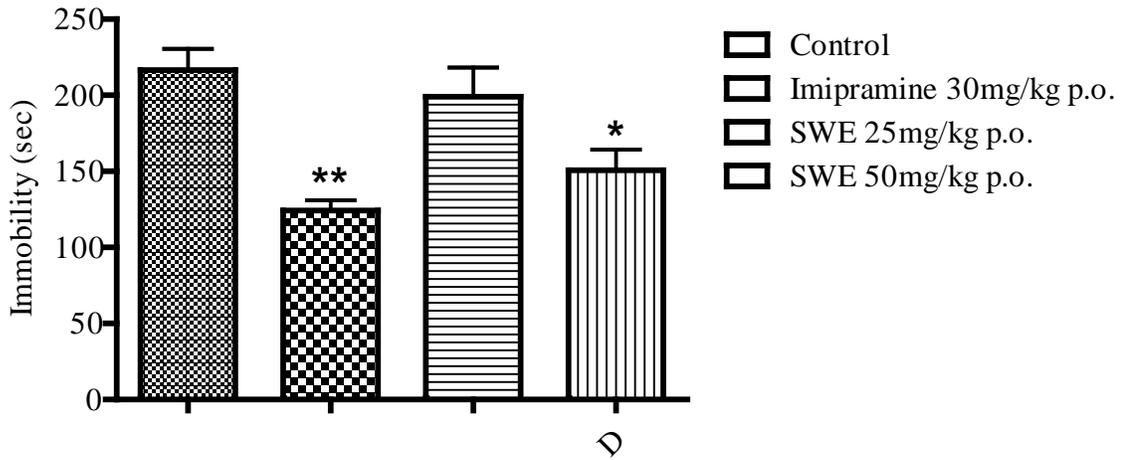


Figure 3-1. Preliminary screening of first SD extract n=9-10 with *= p < 0.05 and **= p < 0.01

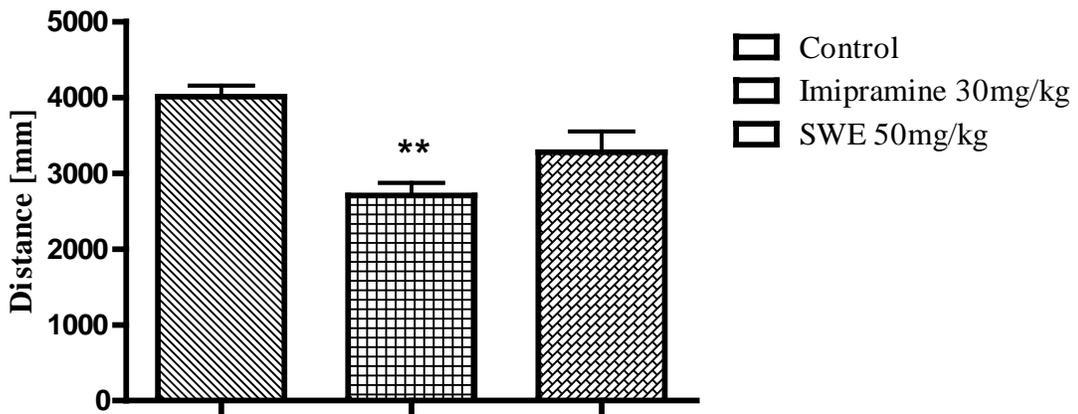


Fig 3-2. Corollary OFT results in mm for the preliminary FST run n=4-6 with *= p < 0.05 and **= p < 0.01

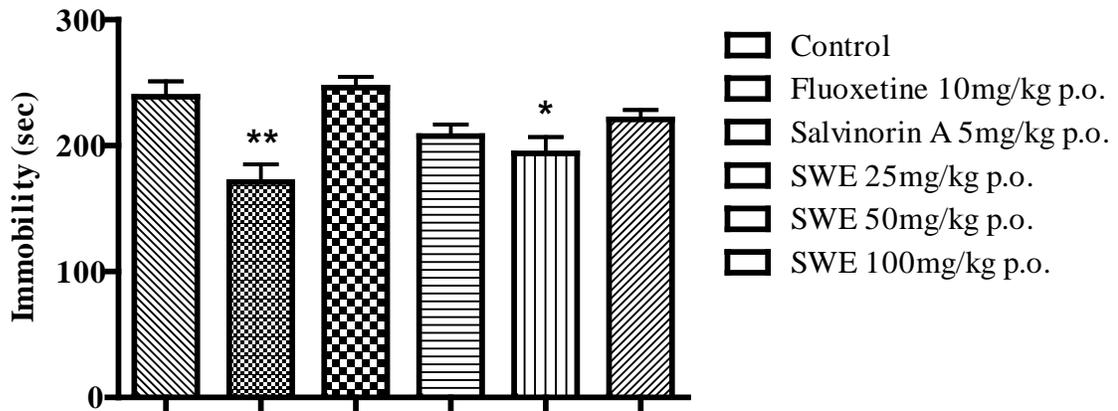


Fig 3-3. TST results screening SWE activity at various concentrations n= 12-14 with *= $p < 0.05$ and **= $p < 0.01$

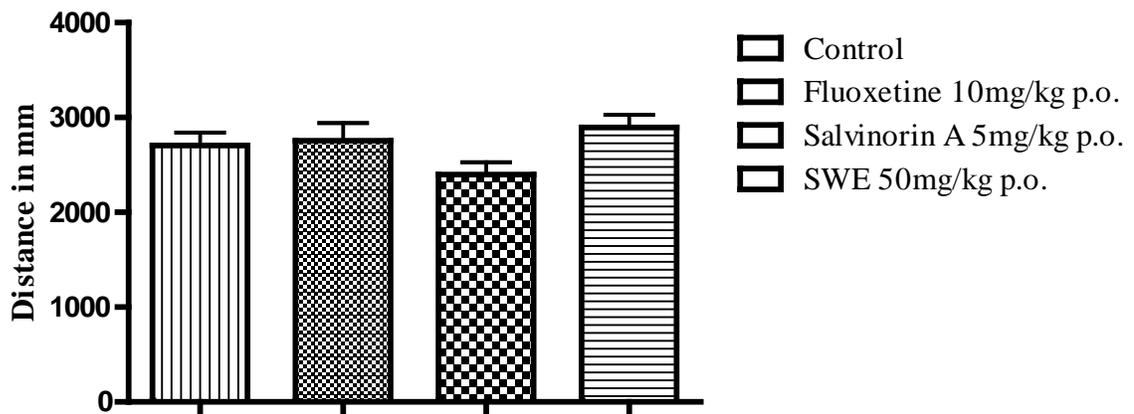


Fig 3-4. OFT paired with the SWE screening experiment n=14-16 with *= $p < 0.05$ and **= $p < 0.01$

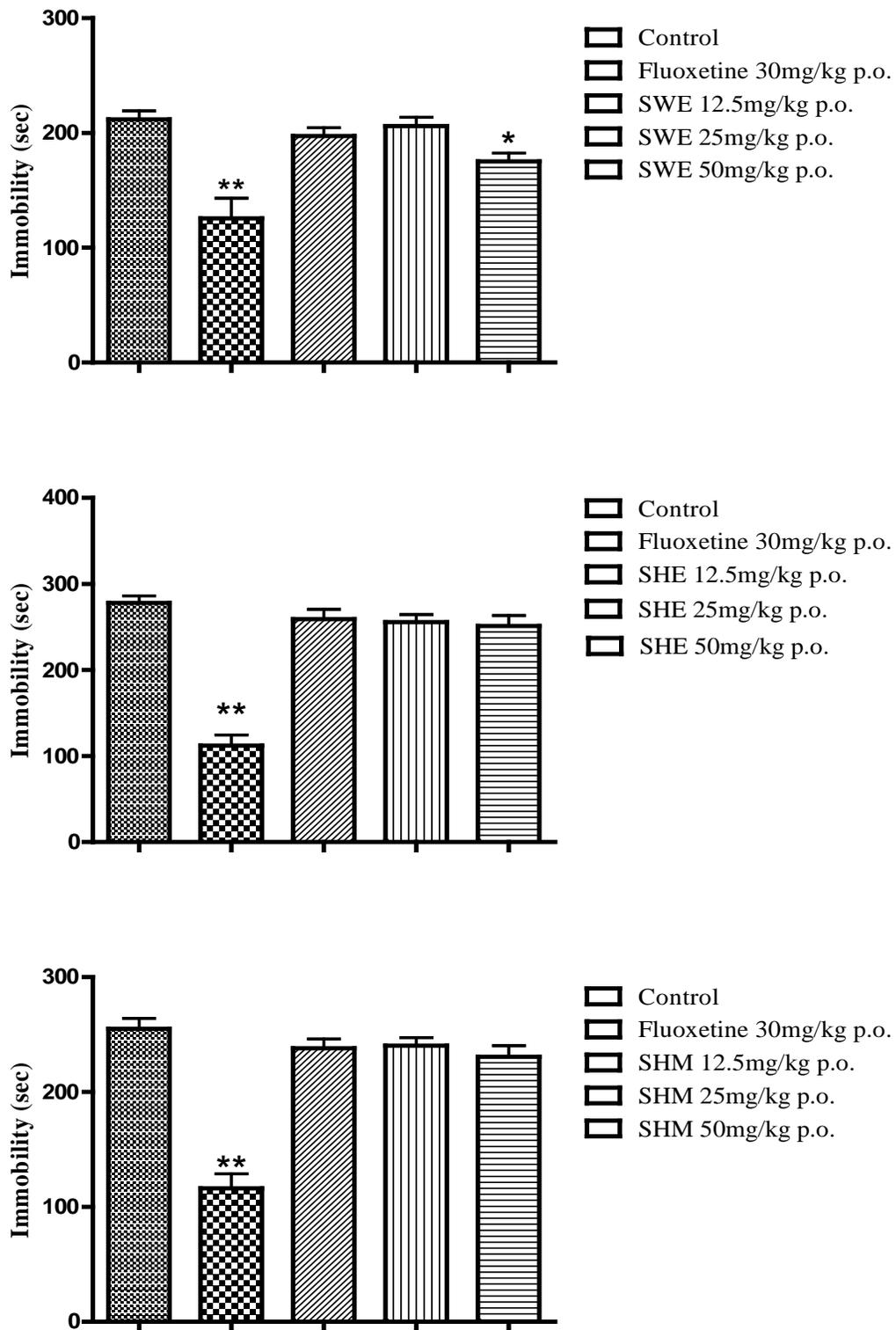


Fig 3-5. Comparison of low dose SD extracts via TST a) SWE b) SHE c) SHM n= 8-21 with *= p < 0.05 and **= p < 0.01

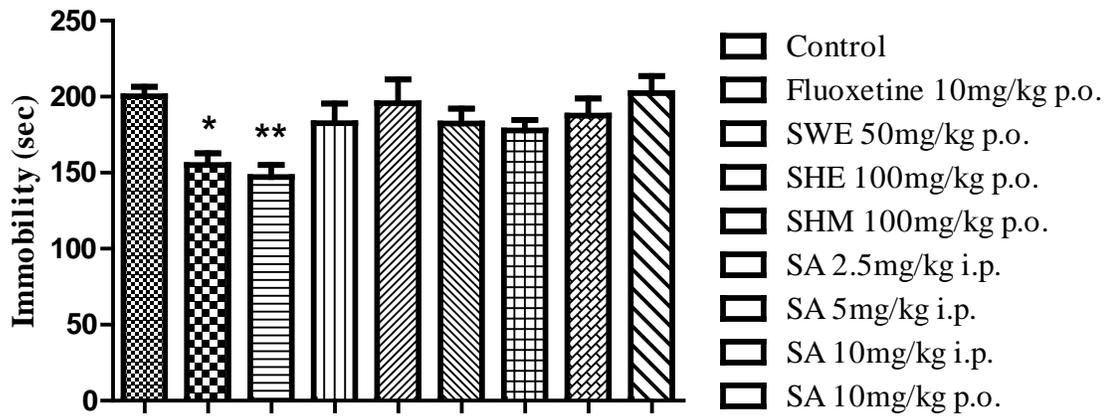


Fig 3-6. TST comparing SA i.p. with high dose SD extracts for SHE and SHM n= 10-11 with *= p < 0.05 and **= p < 0.01

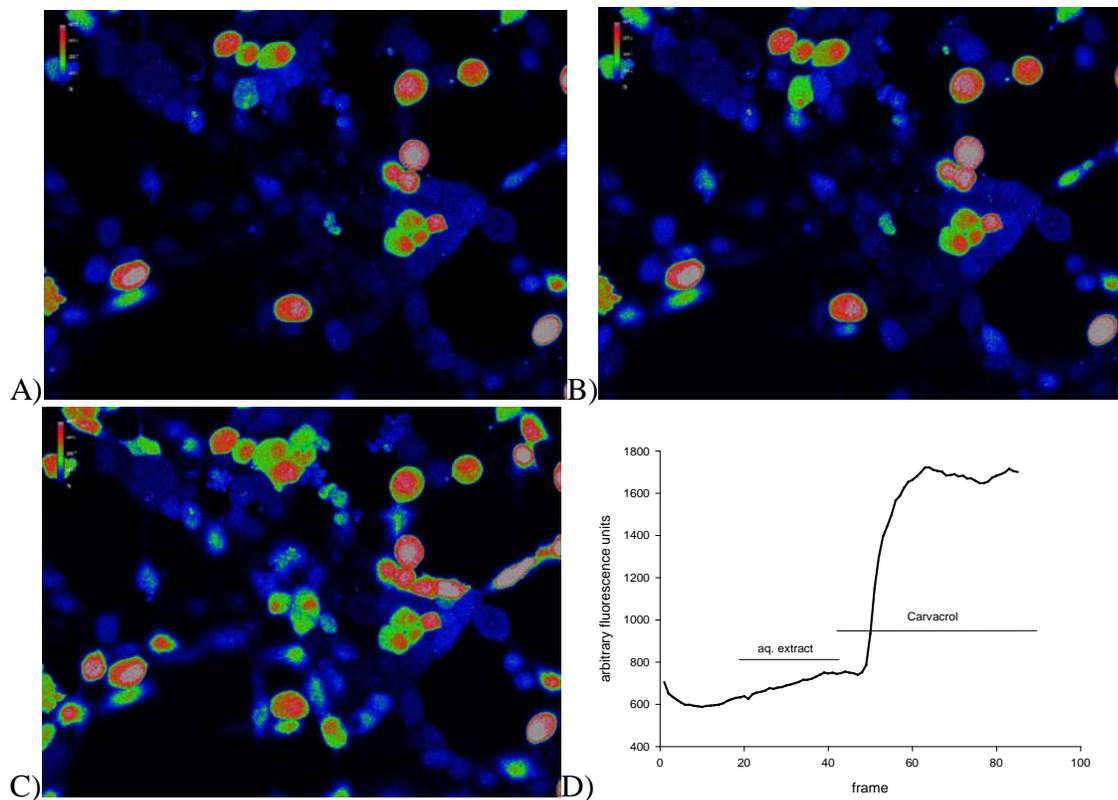


Fig 3-7. Fluorescence recording of TRPV3 activity for A) control B) 0.2 mg SWE C) the TRPV3 agonist carvacrol 500µM and D) the Fluorescence activity per frame of each agent. Images care of Max Peters and the Hebrew University.

CHAPTER 4
DOPAMINERGIC ACTIVITY OF *Salvia divinorum* VIA THE COMPULSIVE GNAWING
TEST

Overview of the Compulsive Gnawing Test

Both oral and motor stereotypy behaviors are based on dopaminergic stimulation in rodents [81], [82]. The main differences lie in brain regions stimulated, and the dopamine receptors that are stimulated. For oral stereotypy behavior the main regions of the brain connected with these are the ventrolateral striatum and the substantia nigra par reticulata [83]. While for locomotor stereotypies the dorsal striatum plays a major role [84], [24]. In both cases it seems that dopamine 1 and dopamine 2 receptors play a substantial role in these stereotypies. For the analysis of these stereotypical behaviors animal models are generally employed. For motor based stereotypies the OFT is used while for oral stereotypies the CG can be used.

The CG was first created to study one specific stereotypical oral behavior exhibited in rodents [85]. When rats are given apomorphine they will compulsively gnaw. But in mice it is seen that giving apomorphine alone will not have an effect on gnawing. One can potentiate this effect though by giving a drug with similar action (other dopaminergics) or enhancing dopaminergic stimulation e.g. large doses of alpha-2 agonists like clonidine [86] and central acting cholinergics concurrently. The gnawing stereotypy can then be quantified using corrugated paper placed in a holding cage after a set time period the damage to the corrugated paper is measured and analyzed for statistical significance.

Methodology

The general conditions used for the CG model were as such:

- Male C57BL/6 mice were used for all experiments. The weight range was between 18 and 28g, and all mice were allowed to eat and drink *ad libitum* until dosing begins.

- One hour before the experiment the mice were brought to the testing room to allow for adequate adaptation of the new surroundings.
- After being treated with the test compound each group of cage mates were placed in a clear non-ported cage measuring 45x45x20cm with a piece of corrugated paper covering the bottom. A lid is placed on top of the cage and the animals are observed for one hour.
- Afterwards the mice are placed back in their original cages, and transported back into their housing area

The original data analysis conditions seemed outdated, and cumbersome for large experiments. The original parameters to evaluate the bite marks were to place a template upon the corrugated paper. The template has 10 rectangular windows which are further subdivided into 10 more equal areas. This would give 100 areas, and was used to give the data in a percent damaged. The template area analysis idea leaves the possibility for false negatives, as well as subjective analytical practices so a newer data analysis scheme was created to ensure reliable results, decrease subjectivity, and allow for digital archiving of data. The new data analysis method is as follows:

- The corrugated paper is allowed to dry for 24 hours to let urine dry. After dried the paper is photographed using a 4 megapixel camera. The auto function is used, but a macro setting is employed to allow for better focusing on the bite marks.
- The photo is then uploaded to a computer and opened in Adobe Photoshop®. The image is cropped so that only the paper is visible. Then the command auto-contrast is used to remove any contrast differences in the photo. Afterwards the command levels is used and shifted to the first valley to the right to recapture the true color of the corrugated paper.
- Now a new layer is created and a three pixel black brush is used to trace over the “ridged” or “pinched” bite marks (Fig 4-1). Larger holes can be filled in with a larger pixel brush. After all the bite marks are traced. The background picture is made invisible leaving just the layer. A white background gradient is used to create a black and white picture is created of the bite marks (Fig 4-2). The layer is saved as new a new jpeg image.
- From here the new jpeg image is opened in SigmaScan for the preliminary study and Image J® (NIH) in all studies thereafter. The black and white picture is turned into a

binary picture, and analyzed using the “Analyze Particle” function is used to get the pixel area of the black marks, which is the bite marks.

- These values are then imported into GraphPad[®] and analyzed for statistical significance.

Results

In the CG studies conducted, SA was active when given i.p. at the doses of 2.5, 5, and 10 mg/kg while inactive at 1 mg/kg i.p. (Fig 4-5) as well as 5 and 10 mg/kg p.o. (Fig 4-3). When trying to find similar activity in synthetic drugs with known pharmacological activity only the dopamine reuptake inhibitors bupropion and nomifensine showed significant gnawing effects (Figs 4-4 and 4-5). Whereas the KOR agonist U-69,593 and various antidepressants with serotonergic and noradrenergic activity should little potentiating effects in gnawing when co-administered with apomorphine (Fig 4-4). In the antagonism studies only the dopamine antagonist haloperidol attenuated the gnawing effects of SA, nomifensine, and bupropion. The KOR antagonist norBNI should not antagonistic activity at 10 and 20 mg/kg (Fig 4-6). For the treatment groups the formulations were as follows:

- The oral formulation for drugs other than SA, SHM, and SHE was deionized water. For SA, SHM, and SHE 1% ethanol (v/v) was added.
- For drugs injected intraperitoneally the formulation was isotonic saline with 0.02% ascorbic acid (w/v). For SA 1-5mg/kg 1% ethanol was added. For SA 10mg/kg 2% ethanol was added plus the solubilizer Tween 80[®] also known as polysorbate.
- For the vehicle + apomorphine groups, both the oral and intraperitoneal formulations with the highest percentage of ethanol were tested to ensure there were no interactions. Both groups had no significant change from the apomorphine control group.

General Summary

From the results of chapter 4 we find that in the CG test SA 2.5-10mg/kg when given i.p. was as active as the positive controls nomifensine and bupropion. When trying to antagonize this activity the KOR antagonist norBNI was unable to significantly reduce gnawing at both 10 and

20mg/kg i.p. whereas, the dopamine antagonist haloperidol successfully antagonized the activity of the active SA groups in the concentration of 0.6mg/kg i.p. The results here show that SA in high concentrations does not fit with the synthetic KOR agonist U-69,593.



Figure 4-1. The differences between ridging (left) and puncture (right) type bite marks



Figure 4-2. Setup of picture for data analysis A) imported paper from the camera B) Cropped with auto contrast and level adjustments C) Final layer with black bite marks

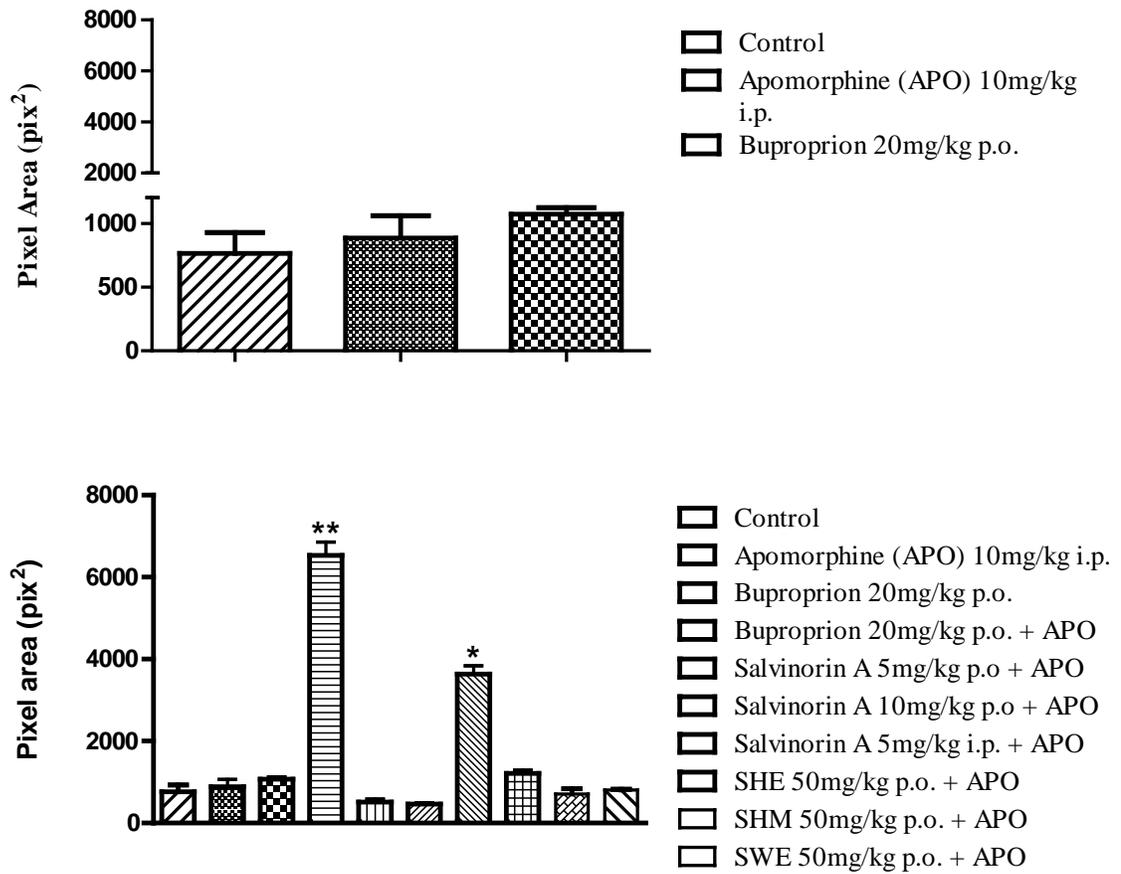


Figure 4-3. Preliminary CG data on salvinorin A and the positive control bupropion. With a) showing no changes between dopaminergic groups and b) the additive activity n=15 with *= p < 0.05 and **= p < 0.01



Figure 4-4. Compulsive gnawing results comparing various drugs with differing CNS activity n=15 with *= p < 0.05 and **= p < 0.01

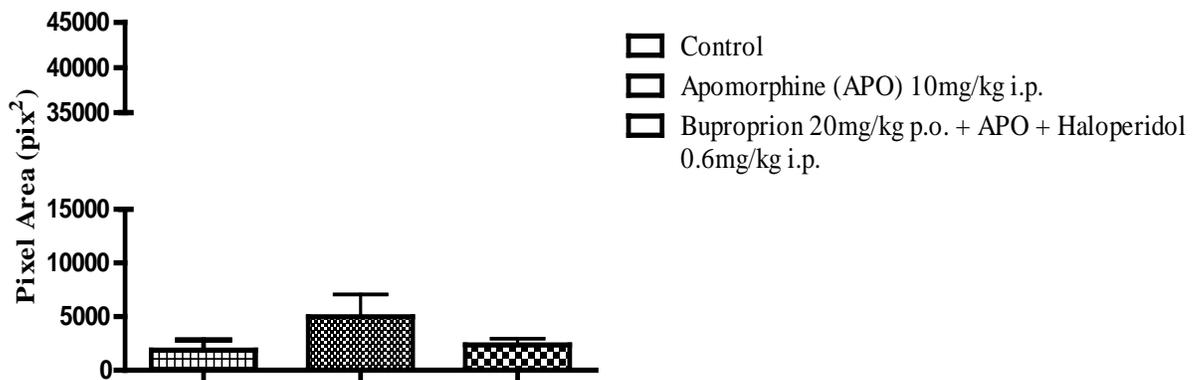


Figure 4-5. Compulsive gnawing results of the antagonism of the active compound bupropion by the nonselective dopamine antagonist haloperidol n=15 with *= p < 0.05 and **= p < 0.01

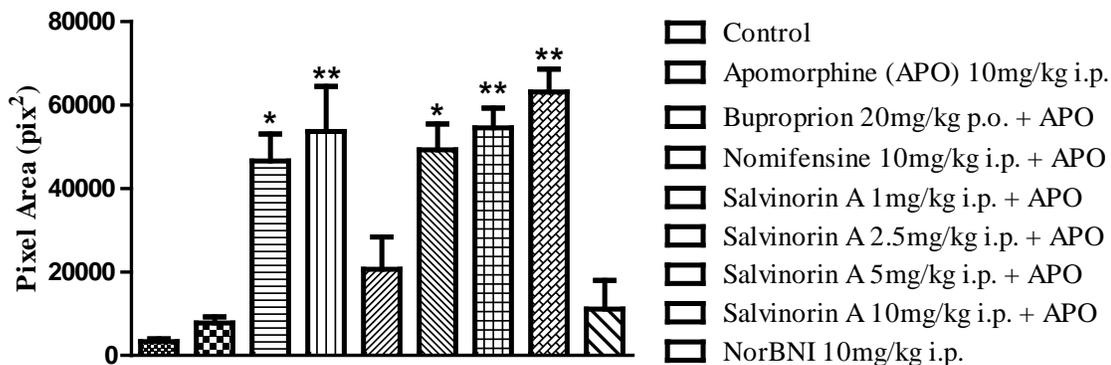


Figure 4-6. Salvinorin A's dose response curve and the positive controls bupropion and nomifensine. n=15-20 with *= p < 0.05 and **= p < 0.01

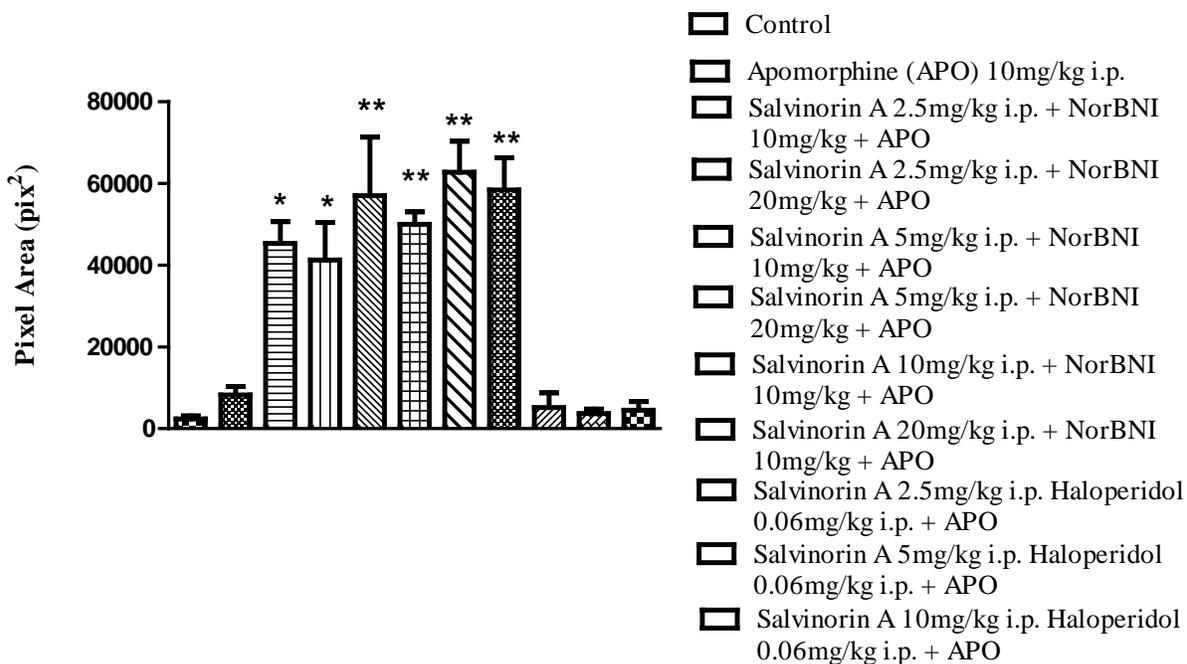


Figure 4-7. Compulsive gnawing results for the antagonism salvinorin A using haloperidol and NorBNI n=15 with *= p < 0.05 and **= p < 0.01

CHAPTER 05 DISCUSSION

In the first specific aim the main goals were to create and analyze various extracts via HPLC and LC-MS-MS. What was seen in the analysis was in line with *Salvia* species [87] caffeic acid derivatives, flavonoids, and diterpenes, also the extracts followed the expected trend of having more diterpenoids present as the concentration of alcohol increased. Another interesting conclusion from the evaluation of the active extract SWE, from the chromatographs of SWE it can be said that it is a relative bare extract, when discussing ultraviolet absorption, when compared to SHM. The UV visible diterpenoid peaks in SWE are very limited with only a scant amount of SA (below LLOQ) and a higher concentration of terpenes where seen via the MS studies. This would coincide with early experiences with SD when using it with the Mazateca peoples. The uses of the Matate board breaks open the glandular trichomes that house the terpenes [88] while the water rinse would only bring low amounts of SA into solution, due to the inability for SA to solublize in water. This shows the vast difference in preparation styles for smoking, quids, and metate preparation. Numerous leaves are need for hallucinogenic activity to be reached when using older methods, which can be seen here in the LC analysis. This brings into question what role the other terpenes have in the CNS activity in both the SWE and SD itself. In earlier studies of SD, SB and other diterpenoids, for example was shown to bind to KORs as well [16], but it has been discussed that SB may have antagonistic properties. Of course more studies are needed to verify this hypothesis.

Lastly, analytically speaking SWE is quite a challenge to older methods of quantification where the implication of UV methodologies is solely used. The trend to create methods in which you can fraction collect and get mass spectra analysis in real time is increasing steadily in the field of natural products. While extracts do need to be standardized based on weight and

percentage of extract for the basis of extract consistency for good manufacturing purposes and to ensure activity. The reliance on UV as the sole method of analysis is slowly coming to an end

From the second aim, the screening of SD in the behavior models for antidepressant action, SWE at the concentration of 50mg/kg when given orally showed significant activity in decreasing immobility times. The OFT showed that locomotor activity was not an influencing factor in SWE's activity. No other extract or, the pure compound, SA when given i.p. or p.o. had any significant influence on immobility times. A literature review for any antidepressant-like activity in the *Salvia* genus came up with monoamine oxidase (MAO) inhibitory effects in *S. miltorrhiza*. One study showed inhibition in human recombinant MAO-A when studying four isolated tashinone diterpenes. The IC₅₀, though, was seen at quite a high concentration of 23mM [89]. Another study using the same plant showed inhibitory effects of an extract of red sage in rat brain homogenate. It has been observed in other plant species that catechins also have MAO inhibitory activity [90]. Even though the screening of TRPV 3 was negative the diterpenoids found in SD may still play a role in the activity of the SWE.

The scant amount of SA in the activity also means that the possibility for SD to still garnish positive pharmacological activity is well welcomed. Since depression is such a wide spread problem in developed nations, further studies into the exact mechanisms behind SWE's activity is a very exciting prospect to think about. Later studies into antagonism should be done to determine these mechanisms. WAY 100135 and SA itself may be good candidates. If any of the diterpenes do antagonize KORs the antidepressant action may be seen partly from this pathway [26], as well as the traditional serotonergic pathways seen in other plants like *Hypericum perforatum* [91][88].

Furthermore, knowing that there is secondary activity other than SA coming from SD is intriguing, and leads to some interesting questions to arise. Do these secondary effects have any bearing on the hallucinatory effects of SD?

Finally the third and final aim was to look at SA's role in modulating the dopaminergic system via the CG. When first screening interactions with apomorphine it was hypothesized that SA would lead to no changes in gnawing. What was interesting is that it showed the opposite in when giving i.p. In CG studies SA was active after i.p. administration in three different doses 2.5, 5, and 10 mg/kg in a dose dependent manner. Only bupropion and nomifensine showed interactions like this when screening drugs with known pharmacological actions. So what does this mean? Does SA bind to KORs- yes the data compiled over the years gives a solid foundation for this, but why is the potentiation of a dopaminergic response to apomorphine when the synthetic U-69593 does not accomplish this? And why is effect not blocked by the kappa opioid antagonist? When trying to antagonize this effect, the kappa opioid antagonist norBNI was unsuccessful in blocking the gnawing effect at both 10 and 20mg/kg. In the introduction oral stereotypy behavior was defined mostly in the striatum and with D1 and D2 receptor stimulation. In previous studies involving SA there was a decrease of dopamine overflow [24]. This was analogous to studies using U-69593 a synthetic KOR agonist. It has also been shown that decreases in dopamine overflow have been attributed to a decrease in dopamine release and an increase in transporter mediated uptake [91]. With findings consistent with Zhang et al. [24] as well as attenuated effects after using norBNI SA has been touted to show inhibitory effects in mesostriatal transmission due to the activation of KORs in the dorsal striatum. In further dopamine related studies with SA [34] and locomotor activity, SA was given concurrently with the D2/D3 agonist quinpirole it showed both an attenuation of locomotor sensitization at the

lowest dose of 0.04mg/kg and a potentiation effect at the highest dose of 2mg/kg. The potentiation effect was comparable to the effects seen when U-69,593 was co-administered with quinpirole. In both studies SA showed a similar effect in locomotor behaviors as the synthetic counter-part U-69593, but when co-administered with apomorphine only SA showed a synergistic increase in the gnawing effect; U-69593 had no bearing on gnawing whatsoever. Furthermore quinpirole itself has shown to increase certain oral stereotypies but not self biting or compulsive gnawing [92] leaving D1 stimulation as a key receptor subtype in psycho-stimulant oral stereotypies that involve gnawing or biting. In the quinpirole study of dopaminergic interaction with the SA that were described as being a bidirectional modulation effect seen with the co-administration of SA, but not U-69593 in locomotor sensitization. While they saw potentiation of locomotor sensitization in both U-69593 and SA only SA at a low dose showed attenuation.

For the locomotor activity most response are localized in the dorsal striatum, while oral stereotypies are found in the ventrolateral striatum. For low doses SA showed no statistical effects on gnawing much like U-69593, but high doses 2.5mg/kg i.p. and greater SA showed gnawing behaviors. The exact opposite of the quinpirole so, does this mean if the bidirectional modulation exist with SA does its effects differ from conventional KOR agonist in the ventrolateral striatum? Since the ventral striatum includes the nucleus accumbens how do high doses of SA in conjunction with dopaminergic activity from other compounds affect this reward center?

There are many questions left to be answered with SD, but one solid answer did come from this project. If anything this project should solidify the importance of study a pharmacologically active plant in a broad to narrow type of experiment scheme. While this process may be more

complex it yields a stronger understanding of the exact nature of the plant when used in different formulations. With the aqueous based methods we see low concentrations of SA in ratio to the phenolic acids, flavonoids. When the threshold concentration of SA is reached for hallucinations there is still this ratio of other constituents that increase as well, which is why Jones's patients still could see antidepressant effects after hallucinating [32] [33]. For recreational use, even with the SA fortified extracts, there is still a ratio of other compounds being inhaled. How do they affect the CNS and the effects of SA? Since these high doses of SA are seen in recreational products do similar results seen in the CG occur? These are merely speculation, but through better planned studies that take into consideration methods in which drug gets into the body, simply incorporating inhalation studies with SD can allow for these can be answered as well.

APPENDIX A
ADDITIONAL TABLES

Chapter 3

Table A-1. Column statistics for FST results

	Control	Imipramine 30mg/kg	SWE 25mg/kg	SWE 50mg/kg
Number of values	9	9	10	9
Mean	216.5	124.2	199.1	150.7
Standard Deviation	42.04	20.13	60.64	40.70
Standard error	14.01	6.710	19.18	13.57

Table A-2. Column statistics for FST paired OFT

	Control	Imipramine 30mg/kg	SWE 50mg/kg
Number of values	6	4	6
Mean	4011	2706	3276
Standard Deviation	357.5	331.9	678.5
Standard error	146.0	165.9	277.0

Table A-3. Column statistics for TST screening various SWE concentrations

	Control	Fluoxetine 10mg/kg	Salvinorin A 5mg/kg	SWE 25mg/kg	SWE 50mg/kg	SWE 100mg/kg
Number of values	13	13	14	12	14	14
Mean	238.9	171.2	246	207.7	193.9	220.9
Standard deviation	43.94	49.68	32.52	32.15	49.16	28.30
Standard error	12.19	13.78	8.69	9.28	13.14	7.56

Table A-4. Column statistics for SWE screening OFT

	Control	Fluoxetine 10mg/kg	Salvinorin A 5mg/kg	SWE 50mg/kg
Number of values	14	16	16	16
Mean	2704	2755	2398	2895
Standard deviation	512	746.9	518.2	533
Standard error	136.8	186.7	129.5	136.8

Table A-5. Column statistics for TST of all extracts

	Control	Fluoxetine 30mg/kg p.o.	SWE 12.5 mg/kg p.o.	SWE 25 mg/kg p.o.	SWE 50 mg/kg p.o.
Number of values	21	12	18	17	13
Mean	211.9	125.5	197.4	205.9	175.3
Standard deviation	34.06	61.48	29.42	32.34	25.28
Standard error	7.432	17.75	6.934	7.844	7.011
	Control	Fluoxetine 30mg/kg p.o.	SHE 12.5 mg/kg p.o.	SHE 25 mg/kg p.o.	SHE 50 mg/kg p.o.
Number of values	12	10	12	12	12
Mean	277.5	112.2	258.9	255.4	258.9
Standard deviation	29.09	38.78	39.28	30.63	39.28
Standard error	8.397	12.26	11.34	8.841	11.34
	Control	Fluoxetine 30mg/kg p.o.	SHM 12.5 mg/kg p.o.	SHM 25 mg/kg p.o.	SHM 50 mg/kg p.o.
Number of values	11	10	11	11	11
Mean	255.1	116.1	238	240.4	230.7
Standard deviation	30.04	40.49	27.21	23.17	31.61
Standard error	9.057	12.8	8.204	6.985	9.53

Table A-6. Column statistics of final TST

	Control	Fluoxetine 10 mg/kg	SWE 50mg/kg	SHE 100mg/kg	SHM 100mg/kg	SA 2.5mg/kg i.p.	SA 5mg/kg i.p.	SA 10mg/kg i.p.	SA 10mg/kg p.o.
Number of values	11	10	10	11	11	11	11	11	10
Mean	200.3	155.1	147.2	182.6	195.7	182.5	177.8	187.6	202.3
Standard deviation	21.01	24.29	24.91	42.47	51.77	32.33	22.52	37.08	35.63
Standard error	6.335	7.680	7.877	12.81	15.61	9.744	6.789	11.17	11.27

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Table A-7. Column statistics for synthetic compound CG test

	Control	Apomorphine 10mg/kg i.p. (APO)	Bupropion 20mg/kg p.o + APO.
Number of values	3	3	3
Mean	1400	2593	36874
Standard deviation	732.4	1190	2134
Standard error	422.8	687.0	1232
	Fluoxetine 10mg/kg p.o +APO	Venlafaxine 20mg/kg p.o. + APO	Mirtazapine 10mg/kg p.o. + APO
Number of values	3	3	3
Mean	4850	3718	2951
Standard deviation	2134	1992	1395
Standard error	1232	1127	805.2
	U-69,593 5mg/kg i.p. + APO	Vehicle p.o. + APO	Vehicle i.p.+ APO.
Number of values	3	3	3
Mean	4061	3592	4604
Standard deviation	4572	1810	780.6
Standard error	2744	1045	450.7

Table A-8. Column statistics for CG test antagonism study

	Control	Apomorphine 10mg/kg	Bupropion 20mg/kg i.p. + APO + Haloperidol 0.6mg/kg i.p.
Number of values	3	3	3
Standard deviation	1973	4977	2370
Standard error	2094	586.2	950.4

Table A-9 Column statistics for CG test salvinorin A study

	Control	Apomorphine 10mg/kg i.p. (APO)	Bupropion 20mg/kg p.o. + APO	Nomifensine 10mg/kg i.p. + APO	SA 1mg/kg i.p. + APO
Number of Values	5	4	4	4	6
Mean	3323	7753	46536	53614	20616
Standar deviation	1490	3014	13043	21576	18930
Standard error	666.5	1507	6521	10788	7728

	SA 2.5mg/kg i.p. + APO	SA 5mg/kg i.p. + APO	SA 10mg/kg i.p. + APO	NorBNI 10mg/kg i.p. + APO
Number of Values	3	3	3	4
Mean	49224	54487	63071	11078
Standar deviation	10801	8264	9568	13783
Standard error	6236	4771	5524	6892

Table A-10. Column statistics for CG test salvinorin A antagonism study

	Control	Apomorphine 10mg/kg i.p. (APO)	SA 2.5mg/kg i.p. + APO + NorBNI 10mg/kg i.p	SA 2.5mg/kg + APO + NorBNI 20mg/kg i.p.
Number of values	3	3	3	3
Mean	8220	2371	45310	41253
Standard deviation	3656	1303	9198	15876
Standard error	2111	752.5	5311	9166
	SA 10mg/kg i.p. + APO + NorBNI 10mg/kg i.p.	SA 10mg/kg i.p. + APO + NorBNI 20mg/kg i.p.	SA 2.5 mg/kg i.p. + APO + Haloperidol 0.6mg/kg i.p.	SA 5 mg/kg i.p. + APO + Haloperidol 0.6mg/kg i.p.
Number of values	3	3	3	3
Mean	49988	3730	62703	58352
Standard deviation	5266	1774	13168	13654
Standard error	3040	1024	7603	7883

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BIOGRAPHICAL SKETCH

Stephen Matthew Phipps, born in South Lake Tahoe California in 1982, moved to Florida after only a few short years. He and his family moved to Oakland where he graduated high school in 2000 from Edgewater High School's magnet program- Engineering, Science and Technology. He was accepted to the University of Central Florida where he studied Biology and planned to become a medical doctor. After taking a class in Ethnobotany he decided to pursue a Botany degree at the University of Florida.

After receiving his A.A. from the University of Central Florida he started his Bachelors of Science degree in 2002 in Botany. While studying he received an internship from USDA-CMAVE and worked under Dr. Eric Schmelz. Working with cow pea () Stephen learned various bioassay and analytical techniques. His work with Dr. Schmelz earned him an award for research excellence and their project was later published in the journal *PNAS*.

Upon graduating in 2004 he began working on his PhD with Dr. Veronika Butterweck, and was a teaching assistant in the College of Pharmacy. In 2008 he was award a travel grant to discuss his poster at the GA meeting in Athens, Greece. Currently he has published on peer-review article, and has been co-author for one article, as well as a review article. His doctoral work focuses on *Salvia divinorum* and its pharmacological activity.

Finally in the spring of 2009 he received his PhD from the University of Florida after completing his work with *Salvia divinorum*