Preclinical evaluations of the blend extract of *Souroubea sympetala* and *Platanus occidentalis* in rats: *proposal of a potential novel treatment for post-traumatic stress disorder*

by

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A thesis presented to the Faculty of Graduate and Post Doctoral Affairs in partial fulfillment of the requirements for

Degree

In

Masters of Science in Neuroscience

Carleton University

Ottawa, ON

Dedication

This thesis is dedicated to

My friends and family, for supporting me throughout my studies,

And to the countless people suffering from PTSD:

I’ve gone through it too; know that you are not alone in all of this

Acknowledgements

I would like to thank everyone from Dr. Zul Merali's laboratory for all the assistance and wonderful help they have given me these past few years. I am truly grateful for the mentoring, guidance and knowledge I have acquired through my days here, and will cherish the friendships that I have gained through working with each and every one of my colleagues. I would like to thank Dr. Hymie Anisman for the patience and guidance he has offered me throughout my Masters journey. I would especially like to thank Christian Cayer and Jonathan James for their most appreciated help in data collection and the invaluable guidance they have given me; Marie Claude Audet for her constant wisdom and open door; Eliza Ali for her friendship, Kira Genise for all her help this past summer, as well as Pamela Kent, who has been an incredible source of support and knowledge throughout this experience, I could not have accomplished everything in the last few years without my lab, and will definitely miss everyone as I go on to the next steps of my academic career.

Abstract

Post traumatic stress disorder (PTSD) is characterized by an inability to extinguish fear memories. If left untreated, PTSD typically follows a chronic, unremitting course culminating in significant impairments of vocational, marital and social functioning. Current treatment approaches for PTSD generally yield only symptomatic improvement, possibly owing to the complicated nature of the underlying processes behind the disorder. Due to the less than optimal efficacy of current drug treatments available for PTSD, some attention has also been devoted to exploring the potential of complimentary alternative medicines (CAM). This thesis was aimed at investigating the use of a botanical blend ethanolic extract of a 1:1 *Souroubea sympetala* and *Platanus occidentalis* (Sycamore tree bark) as a potential novel treatment for PTSD, which may act by blocking the reconsolidation of fear memories. During reconsolidation, the formed memories are rendered labile during reactivation, thus providing a window of opportunity for pharmacologically manipulating formed fear memories. In the present thesis, the efficacy of reconsolidation blockade of short-term (4h) as well as long term (24h and 9 days) fear memories through treatment with the plant blend extract (7mg/kg), diazepam (1mg/kg) and vehicle was measured through the Fear Potentiated Startle paradigm.

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List of abbreviations

ACTH	Adrinocorticotropic hormone
AMY	alpha- and beta- amyrin
ANOVA	Analysis of variance
BE	Betulinic acid
CAM	Complimentary alternative medicine
CB1	Cannabinoid type-1
CER	Conditioned emotional response
CNS	Central nervous system
CRF	Corticotrophin releasing factor
CS	Conditioned stimulus
eCB	Endocannabinoid
EMDR	Eye motion desensitization and reprocessing
FPS	Fear potentiated startle
GABA	Gamma-aminobutyric acid
HPA	Hypothalamic, pituitary, adrenal
ITI	Intertrial intervals
PTSD	Post-traumatic stress disorder
SAD	Substance abuse disorder
SNRI	Selective norepinephrine reuptake inhibitor
SPS	Single prolonged stress
SSRI	Selective serotonin reuptake inhibitor
TFCBT	Trauma focused cognitive behavior therapy
US	Unconditioned stimulus

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Introduction

PTSD is a mental illness characterized by the abnormally persistent and excessively strong memory of a traumatic event. It is clinically expressed through manifestations of memory impairment for the context in which the initial trauma occurred, as well as a manifestation of memory intensification for the event (Layton & Krikorian, 2002). In this regard, PTSD can be seen as a form of over-consolidation of a fear memory, intensified by the emotions related to the traumatic event. There exist several pharmacological as well as behavioral treatments for people suffering from PTSD. Pharmacological treatments for PTSD range from broad-spectrum antidepressants to anxiolytics, however many carry heavy side effects alongside low remission rates. Behavioral treatments for PTSD are mainly trauma-focused and can yield generally favorable results, however they can be very stressful for the patient as well as emotionally negative. Current behavioral therapies are mainly centered on the extinction process of fear memory, however recent research suggests that formed memories, once believed to remain relatively stable and permanent, may be susceptible to pharmacological manipulation once reactivated, offering a potentially new avenue for behavioral treatments for PTSD.

Reconsolidation, the process whereby a previously consolidated memory is reactivated, returning it to a labile state, appears to offer a window of opportunity for pharmacological manipulation and has become of great interest with regards to research in PTSD (Duvarci & Nader, 2004). Indeed, the pharmacological blockade of fear memory reconsolidation process might offer a novel treatment option for those afflicted with PTSD. One such pharmacological agent has been studied in recent years for its anxiolytic properties, as well as its benefits as a botanical complimentary alternative medicine: *Sin Susto*. This botanical blend mainly composed of a neotropical vine from South America *Souroubea sympetala,* has a high yield of betulinic

acid (BE), the active ingredient thought to be responsible for its anxiolytic properties. Another botanical compound with a high yield of BE is *Platanus occidentalis*, or sycamore bark, readily found throughout North America. The purpose of the present investigation was to examine the potential effectiveness of the botanical blend extract of *Souroubea sympetala* and *Platanus occidentalis* in blocking fear memory reconsolidation through the use of the fear conditioning paradigm, Fear Potentiated Startle (FPS).

Chapter 1 – Stress and fear memory

Anxiety is an emotion characterized by subjective feelings of tension, cognitions involving apprehension and worry, as well as by heightened autonomic nervous system activity (Kowalski, 2000), whereas fear is an emotional response to a real or a perceived threat (American Psychiatric Association, 2013). From an evolutionary standpoint, anxiety can be considered as being an adaptive response to fear and thus necessary for the survival of many species including humans (Maner & Kenrick, 2010). As adaptive as the "fight or flight" response may be for survival however, feelings of anxiety can become maladaptive if they persist in the prolonged absence of an actual threat, leading to helplessness, immobilization and other mental illnesses such as PTSD.

In the most general sense, the stress response is an adaptive process important in aiding the survival of many species. Physiologically, the stress response is a neurochemical cascade occurring within the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system which becomes activated in response to a real or perceived threat (Axelrod & Reisine, 1984; Selye, 1984). Sympathetic activation causes release of norepinephrine and epinephrine and HPA activation causes the hypothalamic release of corticotrophin-releasing factor (CRF). CRF will then travel through portal blood, causing the release of adrenocorticotropic hormone

(ACTH) from the adrenal cortex into the bloodstream. The neurochemical response to stress serves an important adaptive role in preparing the organism to deal with the threat by mobilizing energy stores, increasing vigilance and cardiovascular function (Greenberg, Carr, & Summers, 2002). Generally, the release of cortisol is self-regulated via a negative feedback loop; the hormone will effectively stop the release of CRF (and ultimately cortisol) thus returning the body to its pre-stressor state. However if a stressor is chronic in nature or abnormally intense, one or more forms of dysregulation of the HPA axis occurs, altering secretion of cortisol with detrimental consequences (Axelrod & Reisine, 1984). In the case of learned fear, the person or animal has developed an association between a certain stressor and the initial physiological feeling of the stress response (fear); therefore due to the learned association, the stress response is triggered each time that specific stressor is encountered. In essence, learned fear in the context of a stress response is an adaptive process (Greenberg et al., 2002; Selye, 1984) that manifests itself both physically, through neurochemical changes in the brain, as well as behaviorally, through responses such as freezing and avoidance; however, it is possible for this process to become maladaptive, leading to the development of anxiety and PTSD.

1.2 – Post-traumatic stress disorder

Post-traumatic stress disorder (PTSD) is a life altering disorder effecting 37% of individuals that experience a traumatic event (Van Ameringen, Mancini, Patterson, & Boyle, 2008). Until 2013, the American Psychiatric Association considered PTSD as an anxiety disorder. In the latest version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), it is considered as a "trauma- and stressor-related disorder" (American Psychiatric Association, 2013). PTSD is differentiated from other disorders in that category by the fact that it persists for longer than 1 month, and in some cases persists for the individuals' lifetime (American

Psychiatric Association, 2013). The three main symptom clusters for clinical PTSD are re-experiencing, avoidance and increased arousal (Alexander, 2012). The re-experiencing cluster of symptoms refers to intrusive recurrent re-experiencing of the traumatic event, through wakefulness as well as nightmares, in some cases leading the patient to believe that they are actually reliving the event. The avoidance cluster of symptoms refers to the conscious avoidance of certain stimuli related to the traumatic event; such stimuli can include anything from people, to feelings, to objects or places. The final main symptom cluster for PTSD is that of increased arousal, which is often manifested as difficulty concentrating, as well as falling and staying asleep. They also may at times exhibit outbursts of anger along with an exaggerated startle response. Although individuals suffering from PTSD have all experienced or witnessed a traumatic event, the clinical manifestations vary from person to person. In some cases, PTSD can be expressed as periods of anhedonia or dysphoria, while other cases are expressed through arousal and reactive-externalizing; however, PTSD is best known publicly through its dissociative symptoms, as well as fear-related re-living of events and emotional or behavioral symptoms (American Psychiatric Association, 2013).

PTSD can also be expressed as a clinical manifestation of memory impairment for the context in which the initial trauma occurred, as well as a manifestation of memory intensification for the event (Layton & Krikorian, 2002). In this regard, PTSD can be seen as a form of over-consolidation of a fear memory, intensified by the emotions related to the traumatic experience; leading researchers to believe that both the amygdala as well as the hippocampus play roles in the onset and maintenance of PTSD symptoms in patients given the role of these limbic structures in fear memory formation and expression (Baldi & Bucherelli, n.d.). The amygdala specifically plays an important role in emotional processing, and has therefore been thought to

enhance the connections of emotionally arousing stimuli (Grossman, Buchsbaum, & Yehuda, 2002). Closely associated with the amygdala, the hippocampus is known to have important functions with regards to declarative memory, such as facts and events, along with associating temporal and contextual significance to those events (Squire & Zola-Morgan, 1991).

On average, women experience a higher lifetime prevalence of PTSD than men (10% as compared to 5% for men) and will experience symptoms of PTSD for longer periods of time than would men (American Psychiatric Association, 2013). Other risk factors for developing PTSD or influencing its severity include environmental factors, physiological factors as well as temperamental factors (in children) (American Psychiatric Association, 2013).

Currently, there are limited treatment options available for those suffering from PTSD. The most effective behavioral treatments include trauma-focused cognitive behavioral therapy (TFCBT) and eye movement desensitisation and reprocessing (EMDR) (Roberts, Roberts, Jones, & Bisson, 2015). TFCBT is a category of therapy that encompasses different forms of behavioral therapy focused on trauma experienced by the patient. Exposure therapy is a form of TFCBT whereby the patient is made to re-experience certain aspects of the trauma in a safe therapeutic environment, in order to allow for fear memory extinction to occur (Cukor, Spitalnick, Difede, Rizzo, & Rothbaum, 2009). New forms of exposure therapy, specifically for soldiers returning from war, have incorporated virtual reality in order to better appeal to the younger generation of soldiers (Cukor et al., 2009). Theoretically, this form of therapy should be successful with regards to treating PTSD; however many people suffering from PTSD fear stigmatization and thus do not seek any form of treatment, while others cannot properly engage in the therapy due to their emotional numbness associated with the traumatic event (Cukor et al., 2009). Another form of therapy currently being used to treat PTSD is EMDR. EMDR aims to

help patients reprocess the traumatic event at the center of their PTSD. The patient is made to recount memories, images and sensations of the event, while being guided by the therapist to perform side to side eye movements (Bisson, Roberts, Andrew, Cooper, & Lewis, 1996). Many studies have compared the efficacy of TFCBT to EMDR, and have determined that they are equally effective in treating PTSD (Bisson et al., 1996; Pitman et al., 1996). The efficacy of both forms of behavioral treatment greatly depends on the ability of the patients to follow through with the treatment; which can be difficult for those suffering with PTSD due to their emotional avoidance.

Current psychopharmacological treatments are centered around the use of anti-depressants such as selective serotonin reuptake inhibitors (SSRI's), selective-norepinephrine reuptake inhibitors (SNRI's) (Bernardy & Friedman, 2015) as well as anxiolytics such as benzodiazepines which act through the GABAergic pathway (Dinan, 2006). Although many medications are prescribed in order to treat symptoms of PTSD as well as comorbid mental disorders such as anxiety and depression, only two medications are currently approved by the FDA for treatment of PTSD; sertraline (*Zoloft*) and paroxetine HCL (*Paxil*). Sertraline, a SNRI, has shown promising effects on the reduction of arousal type symptoms. One randomized control trial of sertraline investigated its effects on PTSD symptoms of moderate to marked severity in 187 outpatients, meeting the criteria of having a baseline PTSD severity score of at least 50 on the Clinician Administered PTSD Scale Part 2 (Brady et al., 2000). The study found a general decrease in severity in 15 out of 17 studied symptoms; however it was noted that insomnia was significantly more present in patients from the sertraline group than from the placebo group. Paroxetine, a SSRI, has been shown to reduce severity of PTSD symptoms from all three main symptom clusters, along with being proposed as an adjunct therapy with current behavioral

therapy. For example, a randomized control trial investigated the effects of paroxetine as an adjunct therapy to prolonged exposure therapy on adult survivors of the World Trade Center attacks of 2001, meeting the clinical criteria of PTSD, found that patients from the paroxetine/exposure therapy group experienced significantly greater improvement of PTSD and higher remission than that of the placebo group (Schneier et al., 2012). Physical symptoms, however, were not assessed during the trial and moderate side-effects such as headaches and constipation have previously been reported with chronic paroxetine treatment (Ballús et al., 2000). SSRI's such as paroxetine are associated with an average response rate of 60% short-term, however only 20-30% of treated patients achieve full remission long-term (Alexander, 2012).

As previously mentioned, although only two drugs are currently approved by the FDA for specific treatment of PTSD, many other drugs are used to treat its symptoms and comorbid mental disorders, including benzodiazepines. One clinical study looked at the effects of diazepam, a benzodiazepine, on memory acquisition and consolidation in humans (J C Scaife, 2007). They found that diazepam was able to disrupt the association of a conditioned stimulus with an un-conditioned stimulus, making diazepam an interesting avenue to explore with regards to PTSD research. The behavioral therapies currently used to treat PTSD have been shown to be fairly effective; however the nature of the therapy has made it difficult for the patient to complete the treatment, due to the associated emotional difficulties. Many of the pharmacological treatments for PTSD carry heavy side-effects along with their use, and therefore may cause patients to self-medicate, leading to comorbid substance-abuse disorder (SAD) (Cohen & Hien, 2006; Roberts et al., 2015). Due to the current limited treatment options and their variable outcomes, more research is needed at both the basic and clinical level in order to gain insight into

the neurochemical and behavioral mechanisms that underlie PTSD and to develop novel treatment approaches that may benefit these individuals.

1.3 –Animal models of PTSD

Animal research models are employed in studies involving practices which would be unethical as of yet to investigate in human participants. Furthermore, animal models allow for the study of new treatment methodologies, as well as the study of the underlying causes of behavior, such as those associated with the stress response. There are several different animal stress models currently being used to investigate the causes and mechanisms of mental disorders such as PTSD. One of the newer animal models used to study PTSD is single-prolonged stress (SPS). In this paradigm, animals are initially exposed to three consecutive stressors (2 hour restraint, followed by a 20 minute forced swim test, and finally anesthetised using ether), then returned to their home cages and left undisturbed for a period of 7-14 days (Liberzon, Krstov, & Young, 1997). SPS induced symptoms consistent with PTSD including anxiety-like behavior, hyperarousal (i.e. increased acoustic startle response) and enhanced glucocorticoid negative feedback (Khan & Liberzon, 2004; Serova et al., 2013; Yamamoto et al., 2007, 2009). The SPS model has been used to evaluate current (e.g. paroxetine; Takahashi, Morinobu, Iwamoto, & Yamawaki, 2006) and novel treatment approaches (e.g. intranasal neuropeptide Y delivery; Sabban, Serova, Alaluf, Laukova, & Peddu, n.d.).

Another approach that is widely used to mimic the traumatic events that induce intense and recurrent fear symptoms associated with PTSD in humans is Pavlovian (or classical) fear conditioning. In classical conditioning models of learned fear, an aversive unconditioned stimulus (US) is paired with a neutral conditioned stimulus (CS) such that after repeated pairings, the CS alone acquires the ability to elicit a conditioned response (CR). A conditioned

response in classical conditioning models of learned fear can range from avoidance behavior, to freezing, to eliciting a startle response in the rat, depending on the paradigm of learned fear being used. Two well known animal models of conditioned fear in the realm of PTSD are fear potentiated startle (FPS) (Fig. 1) and conditioned emotional response (CER) (Fig. 2).

Fig. 1 Fear potentiated startle (FPS) paradigm of conditioned fear memory reconsolidation in animals.

Fig. 2 Conditioned emotional response (CER) paradigm of conditioned fear memory reconsolidation in animals.

In the case of CER, a CS, such as a tone or a context, is paired with a US (footshock) and after several pairings the presented CS will elicit a CR (freezing behavior). The CER paradigm has contributed to a wealth of knowledge in the realm of conditioned fear, throughout the memory spectrum. The conditioning principle for FPS is very similar to that of the fear conditioning in CER with the exception that the CR for FPS is an exaggerated startle response; one of the staple symptoms of patients suffering from PTSD. The FPS has two distinct parts to its paradigm; one being the CS (tone) and the other being a white noise. In order to measure the exaggerated startle response, animals are initially put through baseline testing to measure the

stimulus-evoking threshold of the noise blasts for each animal (Daldrup et al., 2015). Once baseline levels of startle response have been measured for each animal, the animals are put through fear conditioning, whereby they learn to associate a shock with a tone (which is distinct from the white noise) through randomly timed intervals. After having paired the US (footshock) to the CS (tone), the animals are then re-exposed to the loud noise bursts followed by the tone; the animals are made to startle from the noise bursts, however they exhibit exaggerated startle responses following the tone, due to the expectation of receiving a shock mimicking the exaggerated stress response of PTSD in humans. The baseline startle response of each animal is then compared to themselves post-conditioning in order to observe the exaggerated startle response created by the conditioned fear.

Animal stress models following the theories of fear conditioning, such as CER and FPS, are often employed in order to learn about the different components of fear memory including acquisition, consolidation, expression, extinction and reconsolidation as well as the neurocircuitry and neurochemical mechanisms involved in these processes (Davis, 1990; Maren, 2001; Paré, Quirk, & Ledoux, 2004). In conditioned fear, memory is initially formed through acquisition which occurs during the initial learning of the CS-US association; while consolidation refers to the stabilization process occurring after acquisition, transforming relatively fragile short-term memories into long-term memories. The expression process demonstrates that the memory initially acquired, has been retained in memory and can be expressed. In conditioned fear the CS is still associated to the US. The GABAergic system appears relevant for fear memory acquisition and expression as rats that received central or systemic injections of midazolam, a well known benzodiazepine, demonstrated amnesia with regards to the initial acquisition and expression of a conditioned fear memory (Harris &

Westbrook, 1998). Fear memory extinction is a form of new learning that acts to suppress or inhibit the original fear memory, as opposed to erasing it (Davis, Ressler, Rothbaum, & Richardson, 2006). This process, whereby animals that go through repeated exposure to the CS in absence of the US, re-learn the memory, in this case learning that the CS is no longer associated to the US and thus should no longer be feared. One study investigated the fear extinction process in mice through the CER paradigm, demonstrating adrenergic involvement (Cain, Blouin, & Barad, 2004). Specifically, systemic pre-treatment with yohimbine, an α_2-adrenergic receptor antagonist, was found to enhance fear memory extinction in both cued and contextual protocols, as compared to controls. Currently, fear memory extinction is being used in TFCBT in patients suffering from PTSD. While somewhat effective as a behavioral treatment for PTSD (Cukor et al., 2009), extinguished memories are not permanently gone, and have the possibility of relapse (Quirk, 2002). In order to offer a more permanent, and effective treatment for PTSD, researchers have begun to study memory reconsolidation.

1.3.1 – Fear memory reconsolidation in PTSD. Reconsolidation of fear memory is "when the reactivation of a previously consolidated fear memory returns it to a labile state, during which it is once again sensitive to protein synthesis inhibition immediately post-reactivation", (Duvarci & Nader, 2004). In the FPS paradigm, the reconsolidation process occurs during the retrieval of a conditioned fear memory through reactivation (Deebiec, 2006; Overeem & Kokkinidis, 2012). The fragility of fear memory during reconsolidation has been well documented (Daldrup et al., 2015; Deebiec, 2006). Recent studies have demonstrated that expression of fear memory can be altered through pharmacological manipulation (Ayers, Missig, Schulkin, & Rosen, 2011; Overeem & Kokkinidis, 2012; Ragen, Seidel, Chollak, Pietrzak, & Neumeister, 2015; Resstel, Corrêa, & Guimarães, 2008), however the reconsolidation process of

fear memory is interesting with regards to PTSD due to its potential ability to permanently alter memory. A preclinical study explored the effects of midazolam on the reconsolidation process using a CER paradigm that showed that the benzodiazepine was able to effectively disrupt the reconsolidation process, with the disruption maintained for at least 11 days (Bustos, Maldonado, & Molina, 2006a). The effects of propranolol, a beta-adrenergic blocker, on reconsolidation blockade have also been examined (Dębiec & Ledoux, 2004). Using a CER paradigm, it was found that when the drug was administered immediately post-reactivation, it successfully blocked the reconsolidation process as demonstrated by decreased freezing levels in the animals. The reconsolidation findings in the animal study were then replicated in a human study through script-driven traumatic imagery induced fear memory reconsolidation blockade (Brunet et al., 2008), however human trials using propranolol in the treatment of PTSD have been largely inconsistent (Brunet et al., 2008; Pitman et al., 2002; Stein, Kerridge, Dimsdale, & Hoyt, 2007). The fragility of fear memory during the reconsolidation process, along with the fact that memory can potentially be manipulated pharmacologically allows for the development of possible novel treatments for fear memory related illnesses such as PTSD. Indeed, a treatment that would disrupt the reconsolidation process could hold clinical relevance as an adjunct to current behavioral therapies; theoretically, the patient would immediately receive a pharmacological treatment post-therapy, disrupting the reconsolidation process for that particular memory, and therefore aid in alleviating the fear associated with the traumatic memory.

Chapter 2 – Complimentary Alternative Medicine

Complimentary alternative medicine (CAM) can be defined as being the “diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole” (Ernst et al., 1995). Studies have shown a rise in the use of CAMs in recent

years for many illnesses including mental illnesses such as PTSD (Beaubrun & Gray, 2000; E. Ernst, 2000; Edzard Ernst, 1998; Lader, Tylee, & Donoghue, 2009). As previously stated, many current treatment options for people suffering from PTSD are ineffective or carry with them negative side-effects causing many to self-medicate, potentially leading to substance-abuse (Cohen & Hien, 2006). Studies have also shown that up to 40% of medicated patients suffering from anxiety also self-medicate using CAM remedies (Astin JA, 1998). Due to the high prevalence of people self-medicating using CAM remedies, as well as the necessity for other treatment options for those suffering of PTSD, there is an urgent need for research into control-tested CAM remedies in order to avoid CAM/drug negative interactions (Izzo & Ernst, 2001).

2.1 – Botanicals: Betulinic acid, *Souroubea sympetala* and *Platanus occidentalis*.

Medicinal botanicals make up the largest part of complimentary alternative medicines, and could be potential substitutes for current pharmacological treatments for mental illnesses including PTSD. One such plant compound that has been studied in recent years is *Souroubea sympetala* (Fig. 3), an uncommon neotropical vine indigenous to South America. *S. sympetala* or "*Sin Susto*", is a genus of vine belonging to the small *Marcgraviaceae* family, potentially possessing anxiolytic properties (Cayer, 2011; Schultes & Raffauf, 1990).

Fig. 3 *Souroubea Sympetala* leaves

The term "*susto*" is used to describe an illness known to the Q'eqchi healers of Belize as the "fright sickness" (Thomas (2009). Similar to the American Psychiatric Association (2013) definition of anxiety disorder, *susto* is therefore a fear memory based illness. *Sin Susto* – translating to "without fear", has been used for generations for its anxiolytic properties by the Q'eqchi healers. The most common way of administering the *Sin Susto* is by crushing the fresh or dry leaves, boiling the mixture in water and drinking as a tea (50-100g/L) (Bourbonnais-Spear et al., 2007). Due to its purported anxiolytic properties, *Sin Susto* was investigated further through ethnobotanical investigation (Bourbonnais-Spear et al., 2007). Preliminary findings using an elevated plus maze paradigm showed that oral administration of the crude extract of *S. sympetala* in rats demonstrated anxiolytic properties (Puniani, 2001). Further investigation led to determining that the likely active ingredient in *S. sympetala* was a known pentacyclic triterpene, betulinic acid (BE) (Puniani, 2001) (Fig. 4). Findings were later replicated using the crude extract of *S. sympetala*, containing 7% BE (Cayer, 2011; Mullally et al., 2011).

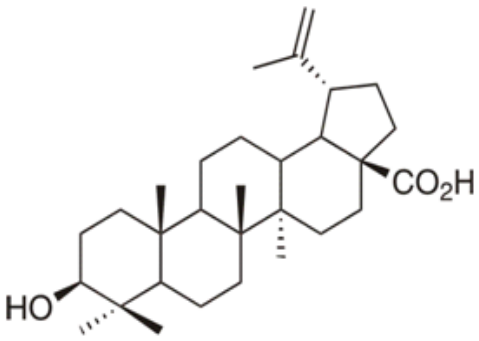

Fig. 4 3b-Hydroxy-lup-20(29)-en-28-oic acid (betulinic acid)

Betulinic acid (BE) is a lupane-type triterpene commonly found in botanicals, and demonstrates anti-malarial, anti-HIV as well as anti-cancer properties (Kessler, Mullauer, de Roo, & Medema, 2007; Mullally et al., 2011). Another botanical containing a high yield of BE is *Platanus occidentalis*, or sycamore tree bark (Fig. 5). *P. occidentalis* is readily found throughout

North America, and can be collected without damage to the environment. Due to its high yield of BE, along with its availability, *P. occidentalis* could be a ready addition to *S. Sympetala* as a potential new CAM treatment.

Fig. 5 *Platanus Occidentalis* bark

Interestingly, preliminary findings from our laboratory have demonstrated that *S. sympetala* combined with *P. occidentalis* exerts anxiolytic effects that exceed those of *S. sympetala* when given alone (Rickaby, 2012). These findings suggest that a botanical blend of *S. sympetala* and *P. occidentalis* extract is an appealing combination for further research.

Chapter 3 – Rationale and Objectives

Currently, those suffering from PTSD are encountering the prospect of insufficient and inefficient treatment options. As previously mentioned, although behavioral therapy is effective in many people with PTSD, the therapy process is itself both emotionally negative as well as stressful for the patient. While it may be effective at weakening the association between the fear and the memory, therapy does not alter it permanently, allowing for the possibility of relapse. Many pharmacological treatments being used to treat PTSD carry heavy side-effects and

relatively low remission rates. Due to a relatively high prevalence in the current use of CAM in those suffering from mental illness, low incidence of side-effects associated with CAM, as well as the negative perceptions regarding current mainstream treatment options, there is an opportunity for research in the discovery and development of CAM for new potential treatments for mental illnesses such as PTSD.

The central purpose of this thesis was to evaluate the effects of a botanical blend extract comprising *S. sympetala* and *P. occidentalis* on reconsolidation of fear memory using the FPS paradigm as a preclinical pharmacological approach to treating PTSD-like symptoms. Specifically, it was hypothesised that a) animals trained in the FPS paradigm will display enhanced fear potentiated startle response, consistent with PTSD-like hyper-arousal, b) administration of the blend extract (*S. sympetala* and *P. occidentalis*) or diazepam (positive control) immediately post-recall will block reconsolidation of fear memory and cause reduced levels of fear potentiation, c) treatment with the blend extract (*S. sympetala* and *P. occidentalis)* like that of diazepam would lead to lower levels of fear potentiation in long-term memory (LTM) but not short-term memory (STM), d) in the absence of reconsolidation, treatment with the blend extract (*S. sympetala* and *P. occidentalis*) would have no effect on general memory.

Methods and Materials

Chapter 4 – Materials

4.1 – Plant material. *S. sympetala* leaves were collected under permit in Tortuguero, Costa Rica. Samples were dried overnight in a commercial plant drier at 35°C and ground to 2mm mesh. A Voucher specimen was deposited in the JVR herbarium, Universidad Nacional Costa Rica, and the University of Ottawa Herbarium (OH No. 19915). *P. occidentalis* bark was collected in Guelph Ontario. Sycamore trees shed their bark yearly, some of which falls to the

ground and gets collected. The bark was cleaned with water, dried overnight in a commercial plant drier at 35°C and ground to 2mm mesh.

4.2 – Plant extraction. Plant material of *S. sympetala* was subsequently macerated with EtOH (10mL/g) in a food blender, the resulting dark mixture then filtered under suction in a Buchner funnel through Whatmann No 1 paper. The remaining solids were washed with additional ethanol (5mL/g); the alcohol and water were then evaporated using a rotary evaporator and then under vacuum (1mm Hg) to yield the *S. sympetala* crude extract (8%). The plant material of *P. occidentalis* underwent the same procedure in order to produce the crude extract at a yield of 13%.

4.3 – Animals. The behavioral experiments were conducted with male Sprague-Dawley rats (225-250g body mass; Charles River Laboratories Inc., St. Constant, Quebec). Rats were pair housed and maintained under standard animal room conditions (clear Plexiglass cages, 24 x 30 x 18cm, 12h light-dark cycle, 21±1°C, 60% humidity, Purina Lab Chow and tap water ad libitum). All experimental procedures were approved by and met the guidelines set out by the Canadian Council on Animal Care (CCAC). Rats were habituated to the non aversive feeding technique, as previously reported (Cayer, 2011; Mullally et al., 2011). All attempts were made to minimize the number of animals used in the study, while maintaining the integrity of the experiments and results.

Chapter 5 – Methods

5.1 Drug administration. The botanical blend extracts of *S. sympetala* and *P. occidentalis* as well as the diazepam were all suspended in 50% sweetened-condensed milk, both were made the day of testing and stored at 4°C. All rats were orally administered the respective treatments immediately after testing (Fear Potentiated Startle). Based on previous findings

(Cayer, 2011) the blend extract was given at a dose of 7 mg/kg, and the diazepam was given at a dose of 1 mg/kg.

5.2 Behavior – Fear Potentiated Startle. The startle apparatus (Coulbourn Instruments, Whitehall, PA, USA) consisted of a sound attenuating chamber containing two calibrated platforms (18 x 10cm) designed to measure the animal's startle response. Animals were placed in a Teflon cage (18.5 x 11cm) positioned atop the platforms. The cage floor consisted of steel rods (4mm diameter spaced 1.8cm apart) connected to shock generators (Coulbourn Instuments; H13-16) located outside of the startle apparatus. Force changes produced by the rats' startle response were measured by the startle sensor platforms. The resulting voltage output from the platform transducers was digitized by an analog-to-digital converter card, interfaced with the computer, and recorded using data acquisition software (Coulbourn AASS v3.02). Startle amplitude was defined as the maximum peak-to-peak voltage that occurred during the first 200ms following onset of the auditory startle stimulus. A high-frequency speaker, mounted (24cm) above the platforms, generated white noise bursts, while tones (startle stimulus) were generated by a Sonalert model tone generator (75kHz; Coulbourn Instruments).

5.2.1 – Experiment 1. Rats (N=8/group) were placed into the startle chamber and exposed to 30 random bursts of white noise (95, 110, 115db) for acclimatization and establishment of individual baseline startle amplitudes, lasting approximately 15 minutes. On day 2, rats were put through a conditioning session where a tone (conditioned stimulus; CS) was paired with a shock (unconditioned stimulus; US). More specifically, a 1.0-mA, 0.5 s foot shock (US) was administered during the last 500 ms of the tone (CS; 4s; 75 KHz). There were 7 CS-US trials with an average of 1 min (randomized) intertrial intervals, the total session lasting approximately 8 minutes. Day 3 was a rest day, where the animals were left undisturbed in their

home cages. On day 4, rats were placed in the startle cages and were put through the fear potentiation test. Testing consisted of 20 trials of 110db white noise bursts (random 1 min ITI), followed by 5 trials of tones paired with noise bursts, and finally, five unpaired noise burst trials. Testing on day 4 lasted approximately 30 minutes. Rats received their respective treatment (blend extract, diazepam or vehicle) immediately after testing. Post-reactivated short-term memory (PR-STM) was measured 4h after initial reactivation (Fig. 6a).

Fig. 6a Experimental design timeline for experiment 1. FPS paradigm investigating PR-STM after 4 hours, treatments of blend extract (7mg/kg), diazepam (1mg/kg) and vehicle were immediately given post-recall. Lightning bolt represents shock (US), speaker represents tone (CS); white noise bursts are present on days 1 and 4 (startle stimulus).

5.2.2 – Experiment 2. Post-reactivated long-term memory (PR-LTM) was also measured. Animals were randomly assigned to the three groups (vehicle, blend extract and diazepam) habituated, and trained as in experiment 1, however rats were tested again 24h following initial reactivation on day 4 (Fig. 6b).

Fig. 6b Experimental design timeline for experiment 2. FPS paradigm investigating PR-LTM after 24 hours, treatments of blend extract (7mg/kg), diazepam (1mg/kg) and vehicle were immediately given post-recall on day 4. Lightning bolt represents shock (US), speaker represents tone (CS); white noise bursts are present on days 1, 4 and 5 (startle stimulus).

5.2.3 – Experiment 3. Post-reactivated long-term memory (PR-LTM) was measured after 9 days. Animals were randomly assigned to the three groups (vehicle, blend extract and diazepam) habituated, and trained as in experiment 1, however rats were tested again 9 days following initial reactivation on day 4 (Fig. 6c).

Fig. 6c Experimental design timeline for experiment 3. FPS paradigm investigating PR-LTM after 9 days, treatments of blend extract (7mg/kg), diazepam (1mg/kg) and vehicle were immediately given post-recall on day 4. Lightning bolt represents shock (US), speaker represents tone (CS); white noise bursts are present on days 1, 4 and 13 (startle stimulus).

5.2.4 – Experiment 4. Animals were randomly assigned to the three groups (vehicle, blend extract and diazepam) habituated and trained as in experiment 1, however on day 4 of the protocol, rats received their respective treatments without memory reactivation, thus serving as non-reactivated controls. Fear potentiation was then tested 24h later (day 5) and 9 days later (day 13) (Fig. 6d)

Fig. 6d Experimental design timeline for experiment 4. FPS paradigm investigating drug effects of blend extract (7mg/kg), diazepam (1mg/kg) and vehicle given on day 4, on non-reactivated fear memories after a) 24 hours and b) 9 days. Lightning bolt represents shock (US), speaker represents tone (CS); white noise bursts are present on days 1, 5 and 13 (startle stimulus).

Statistical Analysis

A one-way analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* tests were used to compare potentiation, a measure of retention, of fear-memory across groups. Significance was set as $p<0.05$. In all cases, the independent variable was designated treatment and the dependent variable was percent of potentiation observed during the test session.

Results

Chapter 6.1 – Experiment 1: PR-STM not impaired following post-reactivation administration of blend extract

As depicted in Figure 7b, a one-way ANOVA demonstrated that neither the administration of the blend extract nor the administration of diazepam post-reactivation significantly attenuated the fear potentiated startle response, $F(2,27) =0.856$; $p<0.4363$, 4 hrs after reactivation.

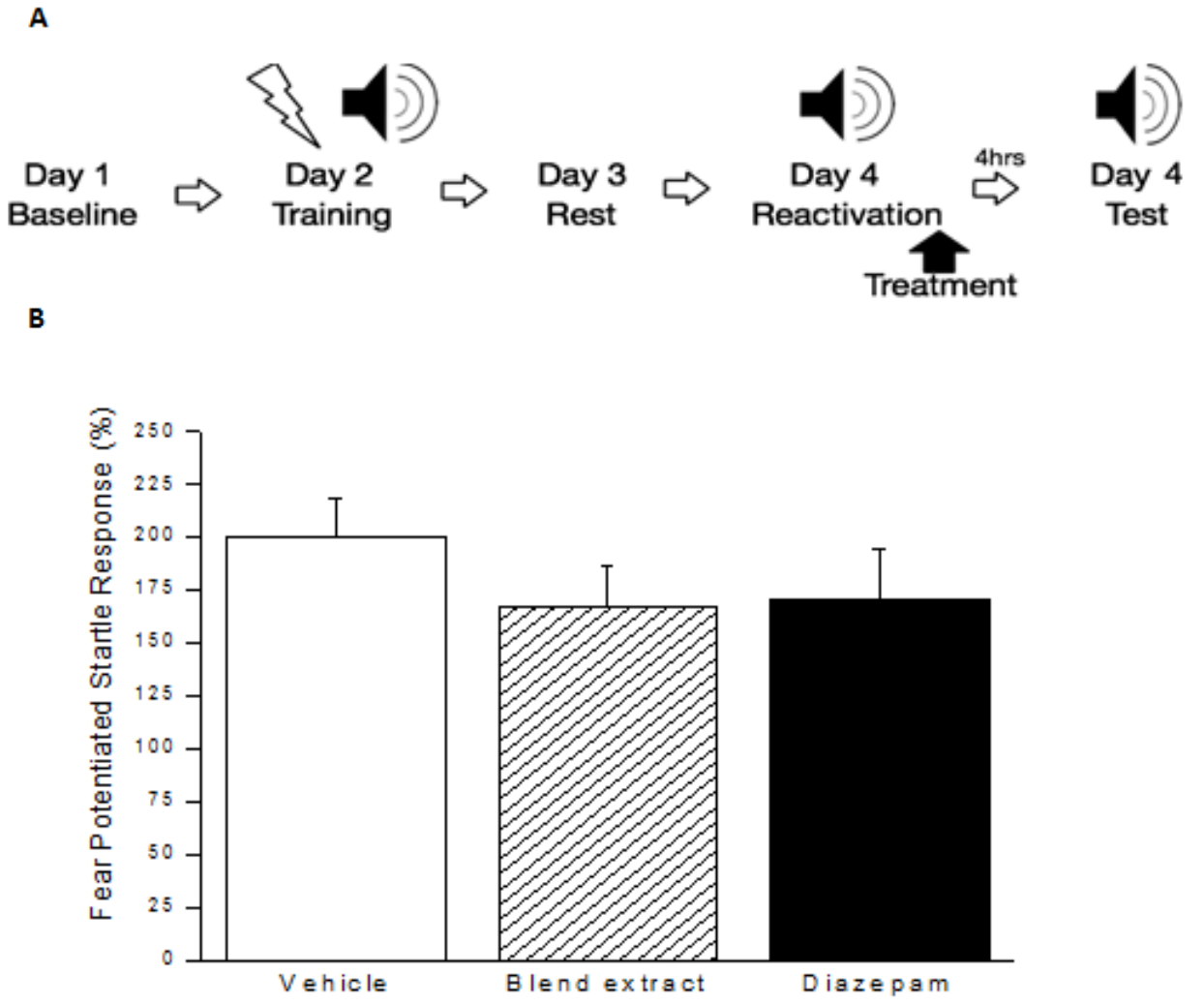

Fig. 7 Effects of post-reactivation oral administration of blend extract (7mg/kg), diazepam (1mg/kg) and vehicle on fear potentiation (%) of auditory fear memory 4hrs post-reactivation in the FPS paradigm. a) Experimental design timeline used with data presented below. Lightning bolt represents shock (US), speaker represent tone (CS); white noise bursts (startle stimulus) are present on days 1, 4. b) Blend extract and diazepam did not significantly effect PR-STM after 4 hours, as compared to vehicle. N=8/group.

6.2 – Experiment 2: PR-LTM impaired at 24h following post-reactivation administration of blend extract

A one-way ANOVA revealed that post-reactivation administration of the blend extract or diazepam significantly effected recall of fear memory 24hrs after reactivation, $F(2, 27) = 5.160$; $p<0.0127$ (Figure 8b). *Follow-up* tests indicated that treatment with blend extract or diazepam resulted in significantly decreased ($p<0.05$) potentiation as compared to vehicle controls.

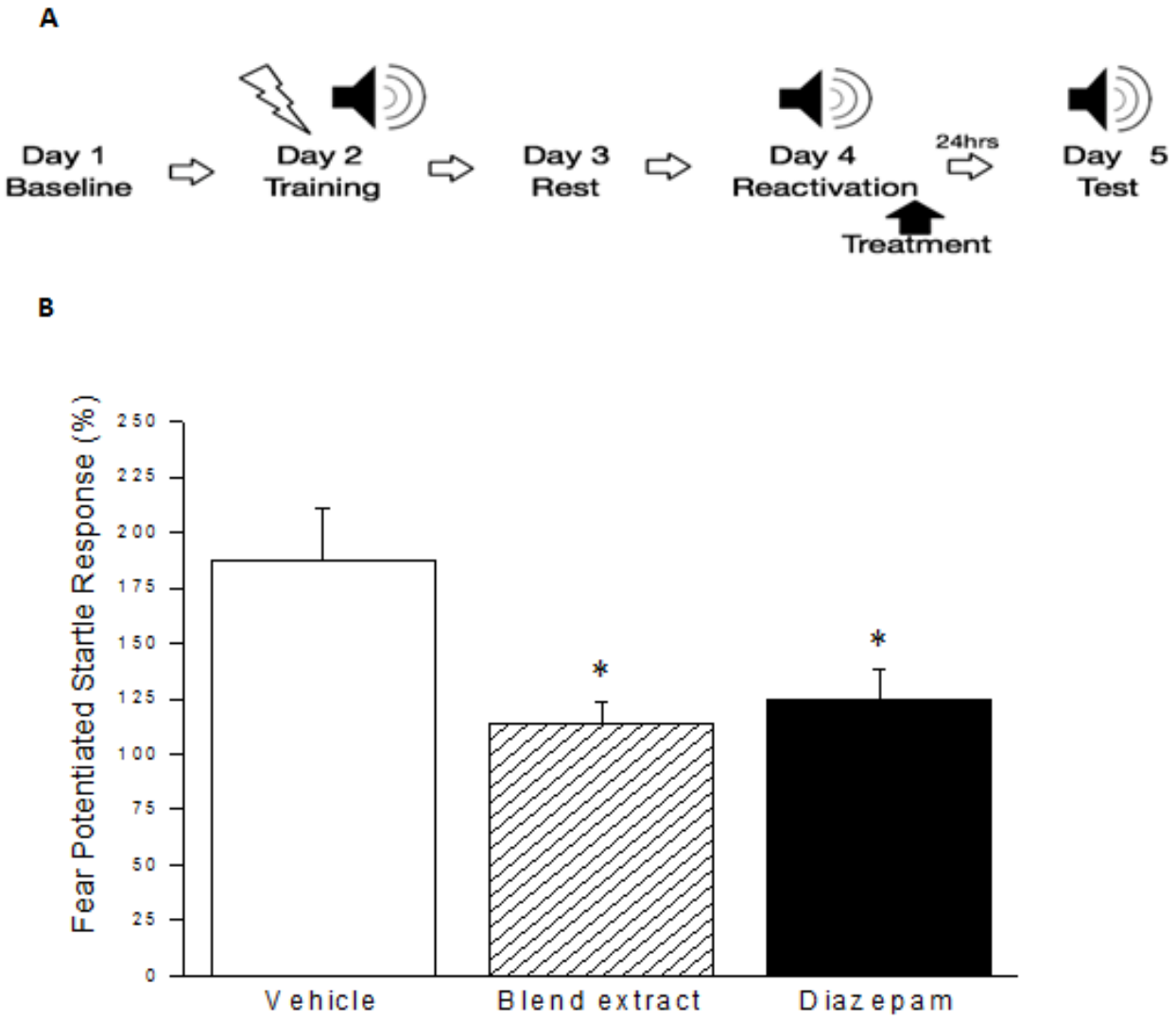

Fig. 8 Effects of post-reactivation oral administration of blend extract (7mg/kg), diazepam (1mg/kg) and vehicle on fear potentiation (%) of auditory fear memory 24hrs post-reactivation in the FPS paradigm. a) Experimental design timeline used with data presented below. Lightning bolt represents shock (US), speaker represents tone (CS); white noise bursts (startle stimulus) are present on days 1, 4 and 5. b) Blend extract and diazepam produce significant reduction in fear potentiation (%) of PR-LTM after 24 hours as compared to vehicle. Blend extract ($p=0.02$*), Diazepam ($p=0.04$*). N=8/group.

6.3 – Experiment 3: PR-LTM impaired at 9 days following post-reactivation administration of blend extract

A one-way ANOVA revealed significant differences in recall of fear memory 9 days after reactivation (Figure 9b) between groups, $F(2,27) = 5.100$; $p<0.0132$. Newman-Keuls tests indicated a significant decrease in fear potentiation in animals that were administered blend extract when compared to vehicle controls ($p<0.05$), but no significant differences between diazepam and vehicle groups.

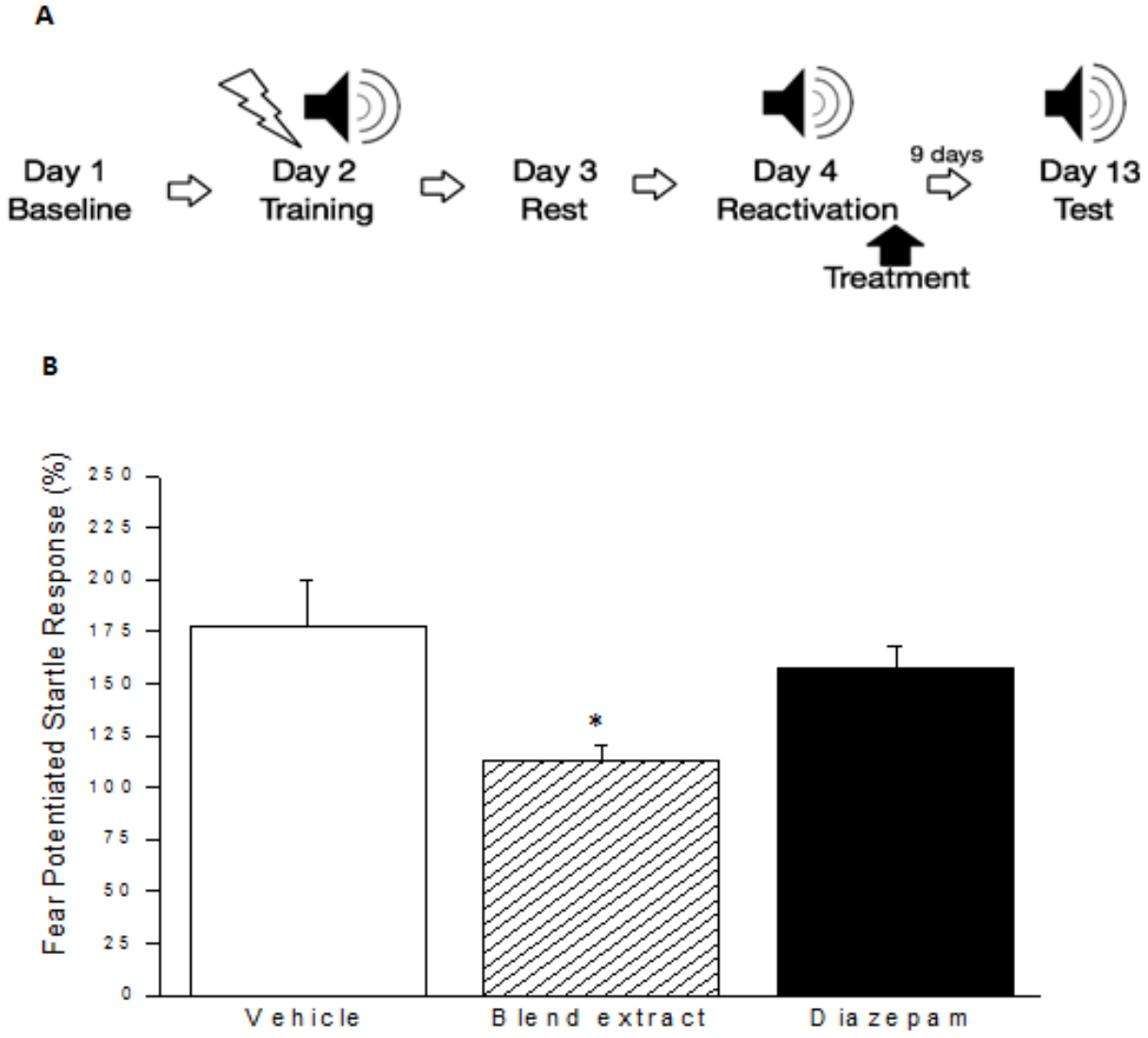

Fig. 9 Effects of post-reactivation oral administration of blend extract (7mg/kg), diazepam (1mg/kg) and vehicle on fear potentiation (%) of auditory fear memory 24hrs post-reactivation in the FPS paradigm. a) Experimental design timeline used with data presented below. Lightning bolt represents shock (US), speaker represents tone (CS); white noise bursts (startle stimulus) are present on days 1, 4 and 13. b) Blend extract produces significant reduction in fear potentiation (%) of PR-LTM after 9 days, as compared to vehicle. Blend extract ($p=0.004$*). N=8/group.

6.4 – Experiment 4: Reconsolidation blockade by blend extract is selective to reactivated fear memories

As displayed in Figure 10b, one-way ANOVA revealed that in the absence of reactivation, administration of the blend extract or diazepam did not significantly effect potentiation of fear memory 24hrs, $F(2,19) = 0.831$; $p < 0.4509$ or 9 days after treatment ($F(2,21) = 1.341$; $p < 0.2830$) (Figure 10c).

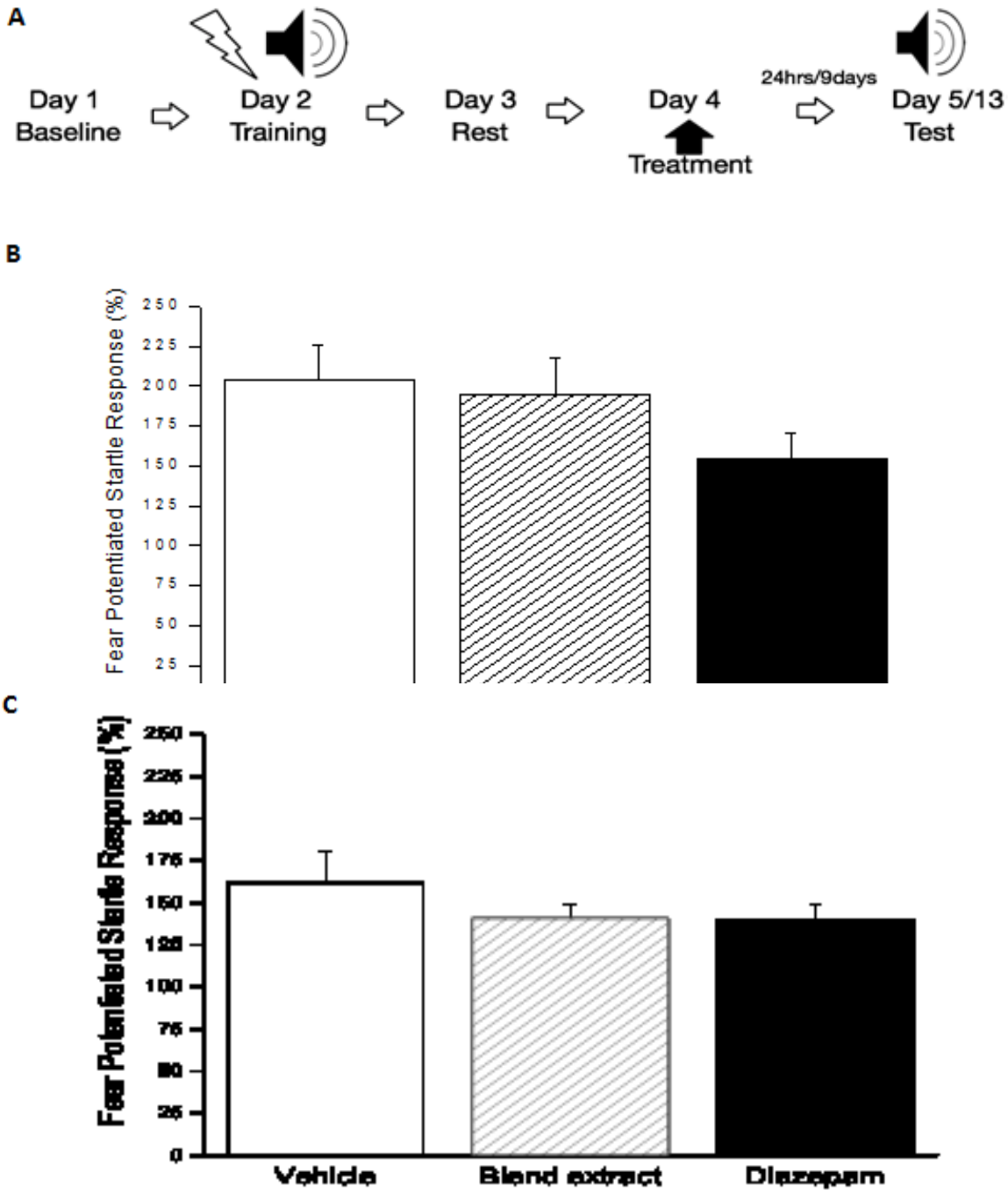

Fig. 10 Effects of oral administration of blend extract (7mg/kg), diazepam (1mg/kg) and vehicle on fear potentiation (%) of non-reactivated auditory fear memory in rats through FPS, b) 24hrs and c) 9 days after treatment. a) Experimental design timeline used with data presented bellow. Lightning bolt represents shock (US), speaker represents tone (CS); white noise bursts (startle stimulus) are present on days 1, 5 and 13. b) Blend extract and diazepam had no effect on non-reactivated fear memory 24 hours and c) 9 days after FPS training. N=8/group.

Discussion

The central purpose of this thesis was to evaluate the effects of a botanical blend extract comprised of *S. sympetala* and *P. occidentalis* on reconsolidation of fear memory using the FPS paradigm as a preclinical pharmacological approach to treating PTSD-like symptoms. It was observed that neither the botanical blend extract nor diazepam produced significant effects on fear memory reconsolidation compared to controls 4 hours post-reactivation. This finding is consistent with previous literature that found protein synthesis in the basolateral amygdala has a time-limited role on memory consolidation, whereby amnesic effects of reconsolidation blockade would not be seen before 6 hours post-reactivation (Nader, Schafe, & Le Doux, 2000). It was also observed that 24 hr post-reactivation both the botanical blend extract and diazepam significantly disrupted fear memory reconsolidation. At 9 days post-reactivation, only the botanical blend extract, and not diazepam, significantly disrupted fear memory reconsolidation. Together these findings show that the botanical blend extract has long term effects on fear memory reconsolidation which could be clinically relevant with regards to a potential treatment option for PTSD. Finally, it was also observed that the effect of the botanical blend extract was selective to the activated memory as opposed to a general amnesic effect, as no memory impairment was observed when the blend extract was administered without prior reactivation. Consistent with the fact that the concept of fear memory reconsolidation relies on the premise that the original fear memory returns to a labile state upon reactivation (Nader et al., 2000), it follows that if a fear memory is not reactivated, no pharmacological manipulation of the memory during recall would be possible.

In summary, the botanical blend extract successfully blocked fear memory reconsolidation in PR-LTM after 24 hours as well as 9 days later, did not have any effect on PR-

STM after 4 hours, and was found to selectively inhibit the memories that had been reactivated prior to treatment administration; thus leading to the conclusion that the botanical blend extract specifically blocked fear memory reconsolidation in this series of experiments. Similar to previous findings, diazepam was also found to block fear memory reconsolidation 24 hours post-reactivation (Bustos et al., 2006; Stern, Gazarini, Takahashi, Guimarães, & Bertoglio, 2012), however it did not block fear memory reconsolidation 9 days post-reactivation; leading to the possibility that it did not completely block reconsolidation, and may have simply had amnesic effects. Interestingly, one study using midazolam which acts via the same $GABA_A$-benzodiazepine receptor subunit as diazepam, reported a significant inhibition of PR-LTM at 24hrs and at 11 days following post-reactivation drug administration (Bustos, Maldonado and Molina, 2006). Another study used midazolam as a control for a cannabinoid type 1 (CB1) receptor antagonist, in order to investigate their effects on fear memory reconsolidation (Stern, Gazarini, Takahashi, Guimarães, & Bertoglio, 2012). Midazolam demonstrated memory reconsolidation blockade 24 hours post-reactivation, however the authors did not investigate reconsolidation blockade at later time point. Discrepancies between those findings and the findings of the present experiments may arise due to the different benzodiazepines analyzed (diazepam *vs* midazolam) or due to the differing methodologies; both studies (Bustos et al., 2006; Stern et al., 2012) utilized CER in order to assess memory retention as opposed to FPS. Thus, while the above evidence suggests a potential for benzodiazepines to block memory reconsolidation when administered post-reactivation, supplementary research is required to confirm whether the effects of benzodiazepines, and in particular diazepam, are experienced long term.

Together these findings suggest that the blend extract successfully disrupts fear memory

reconsolidation, and therefore could be an effective adjunct therapy to current behavioral therapies in the treatment of PTSD. In order to realistically determine its clinical relevance as a potential treatment option, the blend extracts mechanism of action must also be verified. While more research is required in this area, there is some indication that the blend extract acts through GABAergic pathways. In this regard, a phytochemical study determined that raw *S. sympetala* extract and methyl ester BA agonistically binded to $GABA_A$-benzodiazepene receptor subunits, leading to decreased anxiety-like symptoms in rats (Mullally et al., 2011). In addition, when rats were pre-treated with the $GABA_A$ receptor antagonist, flumazenil, the ability of *S. sympetala* to alleviate the anxiety-like symptoms was abolished providing further evidence that the plant is mediating its anti-anxiety effects via a GABAergic mechanism (Mullally et al., 2011).

It is important to recognize that, although BE is the purported central active ingredient of the blend extract, studies on HPLC LC/MS analysis have shown that other pentacyclic triterpenes, such as alpha- and beta- amyrin (AMY) are also present and may synergistically act with BE to mediate effects (Puniani, 2001). Interestingly, a phytochemical study on AMY, found that the triterpene demonstrated anxiolytic effects in the elevated plus maze (EPM) as well as open-field test (Aragão et al., 2006). The authors were subsequently able to block the anxiolytic effects of AMY using flumazenil, providing evidence that AMY may also be working through the GABAergic system. As previously mentioned, the GABAergic system appears to be relevant for memory reconsolidation as midazolam blocked PR-LTM at 24hrs and at 11 days when administered post reactivation during contextual fear conditioning (Bustos, Maldonado and Molina, 2006). Diazepam in the present study also showed some efficacy at attenuating reconsolidation.

Interestingly, recent preliminary findings have suggested another potential mechanism for

the blend extract to mediate its effect on reconsolidation through the endocannabinoid system. A preliminary phytochemical study has determined that triterpenes found in the blend extract inhibit monoacylglycerol lipase (MAGL), the primary enzyme responsible for degrading the endocannabinoid 2-arachidonyl glycerol, one of the two ligands associated with the cannabinoid type-1 (CB1) receptor (Lui et al., 2015). As in the case of GABA, there is also ample evidence to support involvement of the endocannabinoid system in memory reconsolidation (Atsak et al., 2012; Campolongo et al., 2009; Lin, Mao, & Gean, 2006, p. -; Stern et al., 2012). For example, when administered post-reactivation in the CER paradigm, the CB1 receptor antagonist cannabidiol blocked PR-LTM after 24hours and 7 days, while having no effect on general memory in the absence of reactivated fear memories (Stern et al., 2012). The authors were also able to block the effects of cannabidiol through the administration of selective CB1 receptor antagonist AM251, thus confirming cannabidiol's effect on the CB1 receptor.

The current clinical climate is not very favorable for those suffering from PTSD. The available behavioral treatment options are very stressful and emotionally negative for the patients, while the currently used pharmaceutical treatments often carry heavy side effects and do not offer favorable remission rates for the majority of the population. To alleviate the situation, research into potential novel treatments is of utmost importance for the overall health and wellbeing of those suffering from PTSD. Previous evidence, supported by the findings of the current thesis, suggests that the blend extract could eventually be looked at as an adjunct treatment administered in conjunction with behavioral therapy in the treatment of PTSD. The current thesis has discovered that the blend extract blocks fear memory reconsolidation with effects lasting at least 9 days, without having an effect on general memory when administered without memory reactivation. These findings are comparable to the literature on fear memory

reconsolidation; that manipulation of the fear memory process does not seem to have a side-effect on unrelated memory, nor on the learning of new fear memories (Gamache, Pitman, & Nader, 2012). Previous phytochemical research on the blend extract found no detrimental effects of *S. sympetala* in rats (Cayer, 2011), however more research would be needed in order to determine its potential toxicity in humans. The clear preclinical behavioral data from the blend extract, its safety with regards to rats, in addition to its clinical potential as an adjunct treatment for current behavioral treatments, allow for the possibility that the blend extract could become a novel treatment option for those suffering from PTSD in the future.

Findings from the current thesis do come with certain limitations with regards to their real-world applicability. It is well documented throughout literature that women seem to be more affected by PTSD than men; they experience a higher lifetime prevalence (10% as compared to 5% for men) and will experience symptoms of PTSD for longer periods of time (American Psychiatric Association, 2013). The current thesis focused on the effects of the blend extract on fear memory reconsolidation in male rats, as opposed to female rats. This trend does seem to appear throughout PTSD literature as well, and must be addressed by filling the gaps in research on reconsolidation of fear memory in female rats. Future research on the blend extract should focus on its effects in female rats, in order to better determine its applications to the clinical population.

Although findings on the blend extract carry limitations, they also offer the potential of a novel treatment for those suffering from PTSD; a treatment that has the possibility of permanently altering the emotional toll that a traumatic memory can have on the life and wellbeing of someone living with PTSD.

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