Rhodiola rosea L.-An evaluation of safety and efficacy in the context of a neurological disorder, Alzheimer Disease

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ABSTRACT

This thesis examined the safety and efficacy of *Rhodiola rosea* L. (Crassulaceae), a medicinal plant used traditionally by the Inuit of Nunavik, Québec, for the maintenance of mental and physical health. To assess the effects of Nunavik *R. rosea* on the central nervous system, a phytochemically characterized extract was tested in behavioural assays of anxiety with rats. Significant changes in behaviour were observed, particularly in the conditioned emotional response test. *R. rosea* was not a potent modulator of the benzodiazepine site of the $GABA_A$ receptor, indicating possible involvement of other neurotransmitters implicated in the neurobiology of anxiety.

Safety of Nunavik *R. rosea*, its marker phytochemicals, and additional *R. rosea* products was assessed by evaluating the risk of drug interaction potential. Inhibitory capacity was tested on major human drug metabolizing enzymes, the cytochrome P450s. Further, effects on the metabolism of repaglinide, an anti-diabetic drug, were examined in human liver microsomes. While the overall risk of interactions was low, variable impacts of *R. rosea* products on the formation of glucuronide metabolites of repaglinide necessitate caution.

In the TgCRND8 model of Alzheimer disease, *R. rosea* chronic administration led to modest improvements in the survival of male transgenic mice, which exhibit accelerated rates of mortality. Effects on learning and memory performance in the Morris water maze were limited to alterations in the patterns of use of search strategies as determined by our automated scoring algorithm, MWM Visual; changes in escape latencies were not observed.

Nunavik *R. rosea* administration resulted in elevated plasma levels of anandamide (20:1, n-9), a member of the endocannabinoid family, tentatively identified using an untargeted metabolomics approach via ultra-performance liquid chromatography-quadrupole-time-of-flight-

mass spectrometry. *R. rosea* phytochemicals were either eliminated via the renal pathway, or transformed into potential metabolites.

Collectively, the anxiolytic activity of Nunavik *R. rosea* in rats, its protective effects on high background mortality in an aggressive Alzheimer disease model, low risk of inhibition of major enzymes involved in drug metabolism, and its ability to induce detectable changes in *in vivo* metabolic pathways are supportive scientific evidence for the use of this traditional medicine for general well-being by the Inuit.

RÉSUMÉ

Cette thèse a évalué la sûreté et l'efficacité de *Rhodiola rosea* L. (Crassulaceae), une plante médicinale traditionnelle utilisée par le peuple Inuit du Nunavik, Québec, pour le maintien de leur santé mentale et physique. Afin d'évaluer les effets de *R. rosea* sur le système nerveux central, un extrait phytochimiquement caractérisé a été testé par l'entremise de tests de comportement standardisé chez les rats. Des changements significatifs au niveau du comportement furent observés, particulièrement lors de tests de réponses émotionnelles conditionnées. *R. rosea* était un faible modulateur du site benzodiazépine du récepteur $GABA_A$, indiquant l'implication potentielle d'autres réseaux de neurotransmetteurs impliqués dans la neurobiologie de l'anxiété.

La sûreté de *R. rosea* du Nunavik, ses marqueurs phytochimiques, ainsi qu'autres produits naturels contenant *R. rosea* furent évalués en jaugeant leur risque d'interactions potentielles. L'effet inhibitoire de ces extraits furent testés sur les cytochromes P450, communément utilisées dans le métabolisme de drogues, ainsi que sur des enzymes impliquées dans le métabolisme du répaglinide, une drogue antidiabétique, dans des microsomes du foie humain. Quoique le risque d'interactions des produits de *R. rosea* soit minime, l'impact variable des produits de *R. rosea* sur la formation de métabolites glucuronidés du répaglinide nécessite des précautions.

Le modèle TgCRND8 de l'Alzheimer a démontré que l'administration chronique de *R. rosea* a mené à une amélioration modeste de la survie de souris mâles transgéniques, qui exhibent des taux accélérés de mortalité. Les effets sur l'apprentissage et la mémoire dans le labyrinthe de Morris furent limités à des modifications de stratégies de recherche, tel que

déterminé par MWM Visual, notre algorithme automatisé. Des réductions en latences d'échappement n'ont pas été observées.

L'administration de *R. rosea* du Nunavik a entrainé une augmentation d'anandamide (20 :1, n-9), un membre de la famille des endocannabinoïdes, dans le plasma sanguin tel que déterminé par une analyse du métabolome via UPLC-Q-TOF. Les composés phytochimiques de *R. rosea* ont soit été éliminés par la voie rénale, ou transformés en métabolites potentiels. Collectivement, ces activités biologiques apportent un support scientifique en ce qui a trait à l'utilisation de cette plante médicinale traditionnelle pour le bien-être du peuple Inuit.

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of my laboratory findings in the “real world”. My special thanks go to Dr. Alexandra Pettit for undertaking the herculean task of training me on the cryostat, and in immunofluorescence techniques. I would like to thank Jacky Liang and Matthew W. Granger for welcoming me to the Messier lab and guiding me through the process of conversion of video files for the development of MWM Visual. I would also like to thank Christian Cayer for the memorable experience of conducting my first “pharmacokinetic study”.

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endless pep-talks and the 5 AM rides to work. Your unconditional love and prayers have made possible the realization of this dream.

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LIST OF ABBREVIATIONS

4CL	hydroxycinnamate: CoA ligase
4-HPAA	4-hydroxy-phenylacetaldehyde
5-HT	5-hydroxytryptamine
ACE	angiotensin I-converting enzyme
ACN	acetonitrile
AD	Alzheimer Disease
ADME	absorption, distribution, metabolism, excretion
ADRDs	Alzheimer disease and related dementias
AMMC	3-[2-(N, N-diethyl-N-methylammonium)-ethyl]-7-methoxy-4-methylcoumarin
APOE	apolipoprotein E
APP	amyloid precursor protein
ASI	adult social interaction
Aβ	amyloid-β
BACE-1	β-amyloid cleaving enzyme-1
BPSD	behavioural and psychological symptoms of dementia
BZD	benzodiazepine
C4H	cinnamate 4-hydroxylase
CAD	cinnamyl alcohol dehydrogenase
CCR	cinnamyl-CoA reductase
CER	conditioned emotional response
CNS	central nervous system
COX-2	cyclooxygenase-2
$cPLA_2$	cytosolic phospholipase A_2
CRF	corticotropin-releasing factor
CYP	cytochrome P450
DA	dopamine
DAD	diode array detector
DBF	dibenzylfluorescein
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EGCG	epigallocatechin-3-gallate
EPM	elevated-plus maze
EtOH	ethanol
FAAH	fatty acid amide hydrolase
FAS	fatty acid synthase
GABA	γ-aminobutyric acid
GABA-T	GABA-transaminase
GAD	glutamic acid decarboxylase
GAT-1	GABA transporter-1
GC	gas chromatography
GTT	glucose tolerance test
HIF-1α	hypoxia-inducible factor-1α
HLM	human liver microsomes
HPA	hypothalamic-pituitary-adrenal

HPLC	high performance liquid chromatography
IL-1β	interleukin-1β
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
ITI	inter-trial interval
ITT	insulin tolerance test
LC-ESI-Q-TOF	liquid chromatography-electrospray ionization-quadrupole-time-of-flight
LD	light-dark
LTP	long-term potentiation
MeOH	methanol
MS	mass spectrometer
mTOR	mammalian target of rapamycin
MWM	Morris water maze
m/z	mass-to-charge ratio
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NE	norepinephrine
NF-κB	nuclear factor kappa-B
NHP	natural health product
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NonTg	non-transgenic
OF	open-field
OPLS-DA	orthogonal partial least-squares-discriminant analysis
PAL	phenylalanine ammonia lyase
PBS	phosphate-buffered saline
PCA	principal components analysis
p-CD	*p*-coumaric acid decarboxylase
P-gP	P-glycoprotein
PPAR-α/γ/δ	peroxisome proliferator-activated receptor- α/γ/δ
PSEN1/2	presenilin1/2 (gene)
RAGE	receptor for advanced glycation end-products
ROS	reactive oxygen species
SI	social interaction
SNRI	selective serotonin and norepinephrine reuptake inhibitors
$sPLA_2$	secretory phospholipase A_2
SREBP-1	sterol regulatory element binding protein-1
SSRI	selective serotonin re-uptake inhibitors
STZ	streptozotocin
TCM	traditional Chinese medicine
Tg	transgenic
TNF-α	tumour necrosis factor- α
TyrDC	tyrosine decarboxylase
UDPGA	uridine diphosphoglucuronic acid
UPLC-Q-TOF-MS	ultra-performance liquid chromatography-quadrupole-time-of-flight-mass spectrometry
VEGF	vascular endothelial growth factor

CHAPTER 1

General Introduction

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For Section 1.2.4 (Phytochemistry of *Rhodiola rosea*), FA wrote the manuscript, AS reviewed the phytochemical structures, and JTA contributed to the final submission. For section 1.2.5 (Pharmacological activities of *Rhodiola rosea*), FA wrote the manuscript; SALB and JTA contributed to the final submission.

Publications

1.1 INTRODUCTION

"All that man needs for health and healing has been provided by God in nature, the challenge of science is to find it."

-Paracelsus (1493-1541 CE)

The medicinal properties of *Rhodiola rosea* L. (Crassulaceae), particularly for those "aggrieved with headaches," were documented by the Greek physician and botanist, Pedanius Dioscorides, in his pharmacopeia *De Materia Medica*, as early as the 1st century CE (Brown et al., 2002). The "golden root" is considered a sacred healing remedy for nervous disorders and weariness in the folk traditions of the Komi people of Russia; locations where the plant grows in the Ural mountains are known only to a few, and special rituals are associated with their collection (Iljina, 1997). Many cultures in Europe, Asia, and North America, particularly in the sub-Arctic and high temperate regions, have used "roseroot," aptly named for its characteristic rose-like fragrance, as a panacea for numerous ailments, including those of the central nervous system (CNS). Extensive phytochemical and pharmacological investigative efforts on European plant populations pioneered by scientists in Eastern Europe since the 1960s led to the recognition of *R. rosea* as an "adaptogen," similar to *Panax ginseng* C. A. Mey. (Araliaceae), notably for its ability to improve cognitive and physical performance, particularly under stress, to reduce depression, and to act as a stimulant via modulation of multiple CNS pathways (Brown et al., 2002; Panossian et al., 2010a).

In North America, among western Arctic indigenous groups, *R. rosea* is one of the most frequently used medicinal plant species, ranked 8th among a total of 108 plant species (Panchen, 2014). Similar to ethnobotanical uses in Eurasia, *R. rosea* has broad spectrum applications in the treatment of ailments ranging from dermatological, gastrointestinal, and respiratory illnesses to

the maintenance of general wellness and amelioration of mental health issues (Clark and Cuerrier, 2012; Panchen, 2014). Among the Inuit of Nunavik (Northern Québec, Québec) and Nunatsiavut (Northern Labrador, Newfoundland and Labrador), "tullirunaq" (the entire *R. rosea* plant), and "utsuKammak" (*R. rosea* roots), are known to "allow the body to better fight sickness" and to be "good for the brain," respectively. Despite recent evidence of distinct genetics and phytochemistry of Nunavik *R. rosea* populations compared to the better-studied Eurasian ones (Filion et al., 2008; Avula et al., 2009), little is known about the Nunavik populations with respect to their pharmacology in CNS disorders, which is the focus of this thesis.

Traditional and folkloric descriptions of *R. rosea* uses in the context of mental health disorders were further bolstered by a preliminary study indicating evidence of anxiolytic activity of a phytochemically standardized extract of Nunavik *R. rosea in vivo* (Chapter 2; (Cayer et al., 2013)). The intent of this thesis was to explore the efficacy of Nunavik populations of *R. rosea* in the context of Alzheimer disease (AD), a progressive neurodegenerative disorder, and anxiety, a commonly associated neuropsychiatric symptom, in an aggressive mouse model of the disease. Further work evaluating the safety of this plant in terms of potential herb-drug interactions was conducted by examining its inhibitory effects on key human cytochrome P450 (CYP) enzymes and human liver microsome (HLM)-mediated metabolism of a conventional anti-diabetic drug. Bioavailability and metabolism of key *R. rosea* phytochemicals upon oral administration of the whole extract were assessed in a pilot study by implementing a non-targeted metabolomics approach using ultra-performance liquid chromatography-quadrupole-time-of-flight-mass spectrometry (UPLC-Q-TOF-MS) techniques. Overall, the objective of this body of work was to provide platforms for further *in vivo* and clinical research, validation of the traditional knowledge

of the Nunavik Inuit, and to highlight the importance of conservation efforts of this increasingly vulnerable medicinal plant.

1.2 LITERATURE REVIEW

1.2.1 Alzheimer disease: an overview

Since its discovery over a hundred years ago, AD has gained notoriety as the most common etiological subtype of major neurocognitive disorders, or dementia (Sachdev et al., 2014; Alzheimer's Association, 2015). Globally, an estimated 35.6 million people are affected by dementia; 60-70 % of these cases are due to AD (Alzheimer's Disease International, 2009). In Canada, over 300, 000 people were diagnosed with AD in 2008 (Alzheimer Society of Canada, 2010). With the world's population shifting towards an increasingly aging demographic, in the absence of effective new prophylactic and (or) therapeutic interventions, these numbers are projected to triple by 2050, greatly exacerbating the global disease burden (Alzheimer's Disease International, 2009; Alzheimer's Association, 2015).

AD is a debilitating neurodegenerative disorder, characterized by a progressive decline in cognitive and functional abilities, and is ultimately fatal. Memory loss, particularly of recent events, is one of the classic clinical symptoms, accompanied by behavioural and mood changes (Cummings, 2004). As the disease worsens, deterioration of language and communication abilities, deficits in visuo-spatial capacity, impairments in judgement and reasoning, abnormalities in motor and sensory function, and seizures become more commonplace (Cummings, 2004). AD is a leading cause of disability, morbidity and death for those aged 65 and older (U.S. Burden of Disease Collaborators, 2013; National Center for Health Statistics, 2015). AD patients survive approximately 7-10 years after diagnosis, with a steadily declining quality of life (Alzheimer Society of Canada, 2010). The high prevalence of AD, coupled with

the staggering economic and social toll on patients, care-givers, and health care systems, underscores the need for the urgent development of safe and effective therapeutics.

Treatment of AD is further complicated by the multi-factorial nature of the disease, and the long delay between the onset of pathophysiological changes in the brain and the presentation of clinical symptoms. While early-onset (familial) forms of AD occur as a result of mutations in genes responsible for amyloid precursor protein (APP) metabolism and the generation of amyloid-β (Aβ) peptides, namely *APP*, *PSEN1* or *PSEN2* (Bekris et al., 2010), the more common late-onset or sporadic forms of AD (> 95 % of cases) are likely the result of a dynamic interplay between genetic, environmental, and lifestyle factors (Reitz and Mayeux, 2014). Apart from aging, the primary risk factors for AD are: the presence of the apolipoprotein E (*APOE*) *ε4* allele (Raber et al., 2004), poor lifestyle choices including smoking (Rusanen et al., 2011), and the presence of co-morbidities, including midlife obesity (Loef and Walach, 2013), and type 2 diabetes (Gudala et al., 2013). Additionally, social determinants such as fewer years of formal education (Sando et al., 2008; Stern, 2012), and the prior diagnosis of other mental health issues, including anxiety (Burton et al., 2013), post-traumatic stress disorders (Yaffe et al., 2010) and depression (Diniz et al., 2013), increases the likelihood of developing AD.

Currently established pharmacological treatments for AD may delay the progression of the disease by modulating the neurotransmitter imbalance; however, they do not affect the underlying neurodegenerative process. Cholinesterase inhibitors, donepezil (Aricept®, Eisai Inc., Woodcliff Lake, New Jersey), the plant-derived drugs rivastigmine (Exelon®, Novartis Pharmaceuticals Corporation, East Hanover, New Jersey), a semi-synthetic derivative of physostigmine, and galantamine (Razadyne®, Janssen Pharmaceuticals Inc., Titusville, New Jersey), from *Narcissus* spp., are indicated for the treatment of mild to moderate AD and show

modest effects in temporarily stabilizing cognitive decline, functional deterioration and (or) the emergence of new behavioural and psychological symptoms (Birks, 2006). Memantine (Namenda®, Forest Pharmaceuticals, St. Louis, Missouri), an N-methyl-D-aspartate (NMDA) receptor antagonist prescribed for moderate to severe AD cases improves cognition and quality of life of AD patients by ameliorating glutamatergic excitotoxicity either as a monotherapy (Reisberg et al., 2003), or in conjunction with a cholinesterase inhibitor (Tariot et al., 2004). Efficacy and long-term use of these drugs are limited by the presence of adverse side-effects, including nausea, vomiting, diarrhoea, weight loss, insomnia, fatigue, muscle cramps, and bradycardia (Cummings, 2004).

In addition to addressing cognitive symptoms of the disease, successful management of AD must include the mitigation of associated behavioural and psychological symptoms of dementia (BPSD). As many as 80-97 % of AD patients at all stages of the disease exhibit at least one neuropsychiatric symptom (Gauthier et al., 2010), the most prevalent of which are depression, apathy, and anxiety (Steinberg et al., 2008). These co-morbidities greatly contribute to reduced quality of life for AD patients and their care-givers (Banerjee et al., 2006). The US Food and Drug Administration-approved cholinesterase inhibitors may reduce the onset and severity of symptoms, but patients with severe AD often require additional targeted pharmacotherapy, including anxiolytics, anti-depressants, anti-psychotics, and (or) anti-convulsants, thus increasing the risk of poly-pharmacy and the potential for clinically relevant adverse events due to drug-drug interactions (Cummings et al., 2015).

The accessibility and affordability of current AD drugs are restricted in lower and middle-income countries (Suh et al., 2009). By 2025, 75 % of the world's population aged 60 years and over are predicted to be in developing countries, particularly in Asia, Africa and Latin

America, with corresponding increases in the numbers of people suffering from AD (World Health Organization, 2002). Limited options for timely diagnosis, medical interventions, and access to long-term health care facilities, lack of knowledge about AD pathogenesis, and the stigma associated with AD and its behavioural and psychological symptoms are significant barriers to the care and treatment of AD patients in the developing world (World Health Organization, 2002). Traditional healers and medical systems play a vital role in the treatment of illnesses in these countries, including the treatment of multiple CNS disorders (Gureje et al., 2015). Given that two of the most commonly prescribed drugs for AD, galantamine and rivastigmine, are derived from naturally-occurring alkaloids found in traditionally used plants, it is reasonable to hypothesize that ethnobotanically important plants used for the prevention and (or) treatment of neurological disorders may provide a valuable source of safe, effective and affordable alternatives for AD prophylactics.

1.2.2 Alzheimer disease and related dementias among Indigenous Peoples of Canada

Research on AD and related dementias (ADRDs) among indigenous populations in Canada, which comprises of First Nations, Inuit and Métis peoples, is still in its infancy. Considering the rich linguistic and cultural diversity, it is not surprising that traditional and modern perspectives of mental health vary between groups, though underlying commonalities exist. Generally, health in the indigenous worldview is a holistic concept; mental health is inseparable from physical, spiritual, and emotional well-being (Adelson, 2000; Svenson and Lafontaine, 2003). In First Nations and Inuit communities, aging is an important part of the natural life cycle, and elders who continue to actively participate in matters of individual and community interests, and are willing to transmit their knowledge and wisdom to younger

generations, are perceived to be "aging successfully" (Collings, 2001; Assembly of First Nations, 2007; Abonyi and Favel, 2012).

There is a growing realization, however, that fewer First Nations people are aging well, i.e. maintaining the desired level of mental and physical health, engaging in spiritual and social traditions, and retaining a certain level of autonomy (Assembly of First Nations, 2007). While age-related health disorders, including ADRDs, are not new in these communities, the dramatically increased magnitude of incidence and prevalence is a relatively new phenomenon (Henderson and Henderson, 2002; Pace, 2013). In First Nations communities in B.C., Saskatchewan and Ontario, forgetfulness is accepted as a benign and normal part of aging, signifying a return to a child-like stage and closeness to the spirit world (Sutherland, 2007; Hulko et al., 2010; Lanting et al., 2011). However, a distinction is made with respect to dementia and its associated symptoms; these are not considered as "normal" as simple forgetfulness, and often, there is considerable social stigma and embarrassment in these communities (Sutherland, 2007). Moving away from traditional ways of life and losing their connection to the land are reported by contemporary Aboriginal communities to be one of the primary reasons behind the increased prevalence of dementia (Hulko et al., 2010; Lanting et al., 2011; Pace, 2013).

Historical data on the prevalence of ADRDs are limited, but there is evidence of low rates of disease incidence (Henderson and Henderson, 2002; Pace, 2013). Older Aboriginal adults may be more susceptible to the development of ADRDs than their mainstream counterparts due to disparities in health and socioeconomic status (Loppie-Reading and Wein, 2009; Wilson et al., 2010). Due to steadily increasing life expectancies, the numbers of First Nations people aged 60 and older are projected to increase by 3.4 times by 2031 compared to 2006, and consequently, the incidence of age-related disorders, including ADRDs, may increase (Malenfant and Morency,

2011). The presence of three or more chronic health conditions is twice as likely in Aboriginal elderly aged 75 and over (51 %), compared to the non-Aboriginal population (23 %) (Wilson et al., 2010). Indeed, the prevalence of diabetes, hypertension and cardiovascular disease are highly elevated in Aboriginal people compared to non-Aboriginal populations in Canada (Health Canada, 2009). Lower levels of employment and income, and lesser formal education are additional risk factors for higher rates of ADRDs (Loppie-Reading and Wein, 2009). A history of colonial oppression, forced acculturation, and a rapid change in traditional methods of living collectively contributes to overall poor mental health; rates of anxiety, post-traumatic stress disorders and depression among aboriginal people are some of the highest in the country (Cardinal et al., 2004; Government of Canada, 2006).

Prevalence estimates of ADRDs in indigenous populations have been challenging to gauge due to perceptions of symptoms as a normal part of growing old, lack of access to diagnostic health care facilities, shortage of specialized health care providers, particularly those with an understanding of indigenous culture, and lack of culturally-sensitive screening tools (Habjan et al., 2012; Pace, 2013). A community-based survey conducted on a small sample of Cree living on two reserves in Northern Manitoba indicated that rates of AD were lower than in the English-speaking non-Aboriginal elderly, while those of vascular dementia were higher (Hendrie et al., 1993). In British Columbia, Aboriginal people with a registered Indian status showed increasing prevalence of dementia from 1997-2007, with an earlier age of onset than non-Indians, and a higher risk for males compared to females (British Columbia Provincial Health Officer, 2007, 2009; Jacklin et al., 2013), which is in contrast to the general non-Aboriginal trend (Alzheimer Society of Canada, 2010). In 2007, representatives of the First Nations communities of Manitoulin Island, Ontario expressed their concern that rates of ADRDs

were on the rise, and the lack of relevant statistical information on the prevalence of these disorders were creating a challenge for planning an appropriate health response (Sutherland, 2007). In one of the most recent population-based studies in Canada, in First Nations people in Alberta, the age-standardized prevalence of diagnosed dementia from 1998-2009 was 34 % higher than non-First Nations populations (Jacklin et al., 2013). Thus, there is growing evidence of ADRDs as a health problem in these communities.

Jacklin and Warry (2012) noted that apart from elders institutionalized in nursing homes in more urban settings, very few individuals with ADRDs were being treated with prescription medications. Instead, strong family and community networks, and elements of indigenous culture formed the central focus of care and coping mechanisms for patients and caregivers. Some of the preferred ways of treatment and care included traditional medicinal remedies, engaging the patient in speaking the native language, listening to Indian music, and participating in traditional social and spiritual ceremonies (Jacklin and Warry, 2012). Indeed, the protective effects of continuous engagement of the elderly in social and traditional activities, i.e. trapping and fishing (men), and elaborate craftwork (women), have been proposed for the low prevalence of AD in Northern Manitoba (Hendrie et al., 1993). Indigenous communities believe that a balance between traditional Aboriginal and Western approaches will be effective for treating and caring for AD sufferers.

1.2.3 The role of traditional medicine in the context of Alzheimer Disease

While the Western biomedical concept of AD as a progressive neurodegenerative disorder with characteristic clinical and pathological presentation may not be recognized as such, many indigenous cultures worldwide use traditional medicine, including plants, for the prevention and (or) treatment of age-related mental illnesses (Howes and Houghton, 2003;

Adams et al., 2007; Stafford et al., 2008; Perry and Howes, 2011). Symptoms of cognitive decline associated with old age as well as concurrent neuropsychiatric symptoms, including anxiety, are addressed using culture-specific healing practices incorporating herbal remedies that have been passed down via oral traditions or documented in traditional pharmacopeias. In a worldwide survey of ethnobotanical literature by Adams et al. (2007), over 150 plant species were mentioned for age-related cognitive disorders, with the majority of mentions from European herbal pharmacopeias dating back to the 16th and 17th centuries, as well as from Traditional Chinese medicine (TCM) and Ayurvedic systems (Perry and Howes, 2011). In contrast to these well-established written pharmacopeias, only a handful of plants were mentioned for age-related brain disorders from North American folk traditions (Adams et al., 2007). A recent ethnobotanical survey of medicinal plants used by the Q'eqchi' Maya healers of Southern Belize conducted by our group showed that healers recognized many of the classical symptoms of AD, including memory loss, difficulty performing daily tasks, and changes in mood and behaviour, and used plants to treat these symptoms, many of which showed pharmacological activity in pathways relevant to AD pathogenesis (Taylor, 2014). In general, plant uses, either as single herbs or in combination with other plants, ranged from the enhancement of existing cognitive abilities and maintenance of good memory in healthy elderly people, to prevention and (or) treatment of deterioration of memory due to old age, or in the instance of circulatory disorders/stroke (Adams et al., 2007).

Scientific interest and subsequent research on the use of plants for AD has escalated in recent years owing to the unsatisfactory outcomes of existing pharmacotherapies. Several medicinal plants used traditionally for age-related mental illnesses have demonstrated clinical relevance in AD patients. The most well-known of these is *Ginkgo biloba* L. (Ginkgoaceae), not

only a centuries-old TCM remedy for respiratory disorders (Chinese Pharmacopeia Commission, 2005), but also a treatment for memory loss due to circulatory abnormalities in Iran (Ross, 2001), and in Europe since the 1960s (Howes and Houghton, 2003). While studies on a proprietary extract of *G. biloba*, EGb761® are equivocal, recent meta-analyses show that it is superior to placebo in terms of improving cognition, activities of daily living and neuropsychiatric symptoms in dementia patients (Gauthier and Schlaefke, 2014; Tan et al., 2015a).

In addition to *G. biloba*, several other traditional medicinal plants have potential clinical benefits in AD patients. *Melissa officinalis* L. (Lamiaceae), commonly known as lemon balm, was used in Europe to restore memory, promote longevity and treat depression, neuroses, and hysteria (Howes and Houghton, 2009). In a preliminary placebo-controlled clinical trial, treatment with lemon balm extract showed significant improvements in cognition and reduction of agitation in patients with mild to moderate AD (Akhondzadeh et al., 2003a). Plants from the genus *Salvia*, particularly *Salvia officinalis* L. (garden sage), and *Salvia lavandulaefolia* Vahl. (Lamiaceae) (Spanish sage), are reputed to have protective effects against memory disorders in European herbal encyclopaedias (Blumenthal et al., 2000). Administration of *S. officinalis* extract enhanced cognitive outcomes in patients with mild-to-moderate AD (Akhondzadeh et al., 2003b), while *S. lavandulaefolia* essential oil improved cognitive function, reduced behavioural and psychological symptoms, and improved attention (Perry et al., 2003). Thus, an ethnopharmacological approach may be of benefit to identify candidates for AD prophylactics; indeed, in a meta-analysis study of clinical trials using herbal remedies for AD conducted by Man et al. (2008), herbal remedies were at least as effective as conventional interventions for AD therapy and lowered the risk of adverse events arising from use of conventional medicines.

Plants with traditional uses other than those mentioned specifically for "memory loss," particularly those with broad applications for the maintenance of general mental and physical health, have also demonstrated preclinical and clinical relevance in AD models. The most successful of these to date is *Huperzia serrata* (Thunb. ex Murray) Trev. (Huperziaceae), or the toothed club moss, with clinical evidence for improving cognition and quality of life in AD patients with minimal adverse events (Xing et al., 2014). *P. ginseng* (Asian ginseng), used in TCM as a restorative tonic, has been shown to improve cognitive performance in AD patients (Lee et al., 2008). Ayurvedic herbs with "rasayana" effects, i.e. those that promote longevity and freedom from age-related disorders, such as *Withania somnifera* (L.) Dunal, (Indian ginseng), have demonstrated neuroprotective effects in preclinical models of AD (Sehgal et al., 2012). The "built-in" poly-pharmacology of traditional medicinal plants and their phytochemicals at multiple relevant AD drug targets, as acetylcholinesterase inhibitors, antioxidants, anti-inflammatory, and anti-amyloidogenic agents (Frank and Gupta, 2005; Murray et al., 2013; Natarajan et al., 2013; Apetz et al., 2014; Hügel, 2015), may provide some advantage over single-entity drugs, although, further *in vivo* and clinical validation are required.

Among indigenous peoples in Canada, while there are mentions of plant remedies for the treatment of mental illnesses including anxiety, depression, and stress (Davis and Banack, 2012; Uprety et al., 2012), plants used specifically to treat age-related cognitive decline were not encountered frequently. This is similar to trends observed in other geographical regions where historically life expectancies and prevalence of AD are low, and cultural perceptions of memory loss and (or) confusion as an inevitable part of the aging process, or stigma associated with AD and its symptoms prevent disease diagnosis and treatment (Adams et al., 2007; Stafford et al., 2008). Interest in plants that are used for the treatment of AD symptoms is relatively new; as

such, existing ethnobotanical references may exhibit a bias (Adams et al., 2007). Culture-specific explanatory models for the etiology of AD, which may differ considerably from Western understanding of disease pathology, also need to be taken into account. For instance, First Nations people in Ontario, in a manner similar to TCM concepts, believe that senile dementia is caused by reduced blood circulation that causes "chaos and toxicity of the brain" (Yan et al., 2007). Consequently, plants that "thin the blood," such as American ginseng, *Panax quinquefolius* L. (Araliaceae), are recommended for treatment of dementia (Jacklin and Warry, 2012). Additionally, since mental health is inter-woven with physical and emotional well-being in the Aboriginal cosmovision, plants that are classified as general-use tonics for overall mental and physical health are likely to be important in this regard (Black et al., 2008; Clark and Cuerrier, 2012; Panchen, 2014). Among the indigenous groups of the western Arctic, particularly among the Inuit of Nunavik, Northern Québec and of Nunatsiavut, Labrador, *Rhodiola rosea* L. (Crassulaceae), is one of the top-ranked broad use medicinal plants, and uniquely described to be "good for the brain" (Clark and Cuerrier, 2012; Panchen, 2014). While investigations of Nunavik *R. rosea* safety and efficacy in the context of AD and anxiety form the basis of this thesis, the traditional uses, phytochemistry, and pharmacology of the related, but distinct Eurasian populations of *R. rosea* are reviewed in the following sections.

1.2.4 Phytochemistry of *R. rosea*: an overview

R. rosea, commonly known as roseroot, golden root or Arctic root, is a highly valued medicinal plant in the traditional pharmacopeia of certain regions of Europe, particularly Russia, Siberia, the Scandinavian countries, and central Asia, including Northern China and Mongolia (Brown et al., 2002). *R. rosea* has been used for centuries as a remedy for a variety of ailments including nervous system disorders, depression, headaches, fatigue, high altitude sickness,

anemia, impotence, gastrointestinal disorders, infections, and cold and flu symptoms (Brown et al., 2002). The efficacy of *R. rosea* as well as its safety for use in humans had begun to be assessed in preclinical and clinical trials primarily by researchers in Eastern Europe as early as the 1960's, with a dramatic rise in interest among the global research community since the last decade (Panossian et al., 2010a). *R. rosea* has been reported to have antioxidant (Calcabrini et al., 2010), anti-inflammatory (Pooja et al., 2009), neuroprotective (Palumbo et al., 2012), anti-cancer (Liu et al., 2012), antidepressant (Perfumi and Mattioli, 2007), anti-stress (Mattioli et al., 2009), and anti-diabetic (Kwon et al., 2006) activities among many others.

The long-standing traditional use of *R. rosea* and its multiple biological activities have necessitated a closer examination of its phytochemical constituents, especially from its roots and rhizomes which are used most frequently in medicinal preparations. The phytochemical composition of the plant determines its biological activity; variation in the concentration and proportion of bioactive compounds may contribute to the varied pharmacological properties of *R. rosea* observed in the literature. Phytochemicals in *R. rosea* may fluctuate based on several factors, including genotype, geographical location, gender, biotic and abiotic factors, cultivation conditions, method and season of harvest, extraction and storage (Galambosi, 2006; Elameen et al., 2008). In addition, the increased availability and popularity of commercial *Rhodiola* products advertising beneficial health claims make it even more important to accurately identify and profile the phytochemical constituents. Identity markers are crucial for reasons of both safety and efficacy in order to verify that the correct species of *Rhodiola* was used and to prevent substitution by other morphologically similar species. Identification of active principles is needed if standardization of a product with reliable efficacy is to be achieved. In the following sections, the phytochemicals present in Eurasian *R. rosea* and recently discovered Canadian

populations of *R. rosea* are described. The importance of profiling marker compounds via metabolic fingerprinting techniques is also discussed. Additionally, the pharmacological activities of key phytochemicals are briefly mentioned.

1.2.4.1 Phytochemical constituents

Studies on the chemical composition of *R. rosea* were initiated in the 1960s by researchers in Eastern Europe and published mainly in Slavic and Scandinavian languages (Khnykina and Zotova, 1966; Saratikov et al., 1967; Revina et al., 1976; Komar et al., 1980; Kurkin et al., 1986; Dubichev et al., 1991; Furmanowa et al., 1999; Rohloff, 2002). Recent phytochemical work using high-performance liquid chromatography (HPLC) and gas chromatography (GC) coupled with mass spectrometry (MS) techniques have achieved more efficient and rapid separation and identification of known as well as novel minor compounds (Ganzera et al., 2001; Rohloff, 2002; Tolonen et al., 2003a; Tolonen et al., 2003b; Ma et al., 2006; Petsalo et al., 2006; Yousef et al., 2006; Ali et al., 2008; Avula et al., 2009; Ma et al., 2013; Mudge et al., 2013).

Over 140 phytochemicals belonging to several distinct biosynthetic classes have been isolated from *R. rosea* plants, mainly from its roots and (or) rhizomes as well as from the aerial parts (Panossian et al., 2010a). These include phenylethanol derivatives salidroside and *p*-tyrosol, phenylalkanoids, particularly the phenylpropanoid glycosides rosavin, rosarin and rosin (collectively known as the 'rosavins'), terpenes, essential oils, simple phenolics, flavonoids (flavonols, flavonolignans), proanthocyanidins, gallic acid esters, cyanogenic glucosides and tannins. Table 1.2.1 shows a detailed list of *R. rosea* phytochemical compounds grouped by biosynthetic class and Fig. 1.2.1 shows the chemical structures of selected compounds. Of these, salidroside and the rosavins are the most intensively studied compounds.

Table 1.2.1 Phytochemical compounds of *Rhodiola rosea.*

Biosynthetic class	Phytochemicals	References
Phenylmethanoids	Benzyl alcohol O-α-L-arabinopyranosyl-(1→6)-O-β-D-glucopyranoside	(Avula et al., 2009)
	Phenyl methyl O-α-L-arabinofuranosyl-(1→6)-O-β-D-glucopyranoside	(Avula et al., 2009)
	Benzyl-O-β-D-glucopyranoside	(Mudge et al., 2013)
Phenylethanoids	2-phenylethyl O-α-L-arabinopyranosyl-(1→6)-O-β-D-glucopyranoside	(Avula et al., 2009)
	Mongrhoside	(Avula et al., 2009)
	Salidroside: 2-(4-Hydroxyphenyl) ethyl-O-β-D-glucopyranoside	(Troshchenko and Kutikova, 1967)
	Tyrosol: 4-(2-Hydroxyethyl)phenol	(Troshchenko and Kutikova, 1967)
	Viridoside	(Avula et al., 2009)
Phenylpropanoids	Cinnamyl alcohol	(Zapesochnaya and Kurkin, 1982)
	Rosarin (trans-cinnamyl O-(6'-O-α-L-arabinofuranosyl)-β-D-glucopyranoside)	(Zapesochnaya and Kurkin, 1982)
	Rosavin (trans-cinnamyl O-(6'-O-α-L-arabinopyranosyl)-β-D-glucopyranoside)	(Zapesochnaya and Kurkin, 1982)
	Rosin (trans-cinnamyl O-β-D-glucopyranoside)	(Zapesochnaya and Kurkin, 1982)
	Cinnamyl-(6′-O-β-D-xylopyranosyl)-O-β-glucopyranoside	(Tolonen et al., 2003b)
	4-methoxy-cinnamyl-(6'-O-α-arabinopyranosyl)-O-β-glucopyranoside	(Tolonen et al., 2003b)
	Triandrin, Sachaliside 1 (4-hydroxy-cinnamyl-O-β-D-glucopyranoside)	(Kurkin et al., 1991)
	Vimalin (4-methoxy-cinnamyl-O-β-D-glucopyranoside)	(Kurkin et al., 1991)
	(-)-Lariciresinol (lignans)	(Kurkin et al., 1991)
	(-)-Lariciresinol 4-O-β-D-glucopyranoside	(Kurkin et al., 1991)
Phenolic acids	Caffeic acid, Caffeic acid 3-O-β-D-glucopyranoside	(Kurkin et al., 1991)
	Chlorogenic acid	(Kurkin et al., 1991)
	p-Coumaric acid 4-O-β-D-glucopyranoside	(Kurkin et al., 1991)
	p-Coumaric acid 1-O-β-D-glucopyranoside (melilotoside)	(Kurkin et al., 1991)
	Gallic acid esters, methyl gallate	(Kurkin et al., 1984a)

Table 1.2.1 Phytochemical compounds of *Rhodiola rosea* (continued).

Biosynthetic class	Phytochemicals	References
Flavonoids	Rhodionin (herbacetin 7-O-α-rhamnopyranoside)	(Zapesochnaya and Kurkin, 1983)
	Rhodiosin (herbacetin 7-O-(3"-O-β-D-glucopyranosyl-α-L-rhamnopyranoside)	(Zapesochnaya and Kurkin, 1983)
	Rhodiolin	(Zapesochnaya and Kurkin, 1983)
	Tricin	(Brown et al., 2002)
	Kaempferol 3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside	(Avula et al., 2009)
	Kaempferol	(Dubichev et al., 1991)
	8-methylherbacetin	(Kurkin et al., 1984a)
	Acetylrhodalgin	(Kurkin et al., 1984a)
	kaempferol 7-O-α-L-rhamnopyranoside	(Kurkin et al., 1984a)
	Herbacetin	(Jeong et al., 2009)
	Rhodiolinin	(Jeong et al., 2009)
	Rhodionidin (herbacetin-7-O-α-L-rhamnopyranose-8-O-β-D-glucopyranoside)	(Kurkin et al., 1984b)
	Rhodiolgin (gossypetin-7-O-α-L-rhamnopyranoside)	(Kurkin et al., 1984b)
	Rhodiolgidin (gossypetin-7-O-α-L-rhamnopyranose-8-O-β-D-glucopyranoside)	(Kurkin et al., 1984b)
	Rhodalin (herbacetin-8-O-β-D-xylopyranoside)	(Kurkin et al., 1984b)
	Rhodalidin (herbacetin-8-O-β-D-xylopyranose-3-O-β-D-glucopyranoside)	(Kurkin et al., 1984b)
	Gossypetin-di-O-glucoside	(Petsalo et al., 2006)
	OH-gossypetin-7-O-rhamnose-8-O-glucose	(Petsalo et al., 2006)
	Herbacetin-di-O-glucoside	(Petsalo et al., 2006)
	Kaempferol-3-O-glucose-7-O-glucose	(Petsalo et al., 2006)
	Quercetin-3-O-rhamnose-7-O-glucose	(Petsalo et al., 2006)
	Gossypetin-di-O-glucoside or O-diglucoside	(Petsalo et al., 2006)
	Gossypetin-3-O-glucose-7-O-xylose/arabinose	(Petsalo et al., 2006)
	Kaempferol-3-O-rhamnose-7-O-glucose	(Petsalo et al., 2006)
	Herbacetin-3-O-glucose-7-O-xylose/arabinose	(Petsalo et al., 2006)
	Quercetin-3′/4′-rhamnose	(Petsalo et al., 2006)

Table 1.2.1 Phytochemical compounds of *Rhodiola rosea* (continued).

Biosynthetic class	Phytochemicals	References
Oligomeric/ polymeric proanthocyanidins	Prodelphinidin gallates/esters (epigallocatechin gallate dimers)	(Yousef et al., 2006)
Monoterpenes/ glycosides	Rosiridol (3,7-dimethylocta-2,6-diene-1,4-diol)	(Kurkin et al., 1985)
	Rosiridin(e) (3,7-dimethylocta-2,6-diene-1,4-diol 1-O-β-D-glucopyranoside)	(Kurkin et al., 1985)
	Sachalinol A	(Avula et al., 2009)
	Rhodioloside A ((2*E*,6*E*,4*R*)-4,8-dihydroxy-3,7-dimethyl-2,6-octadienyl β-D-glucopyranoside)	(Ma et al., 2006)
	Rhodioloside B ((2*E*,4*R*)-4-hydroxy-3,7-dimethyl-2,6-octadienyl α-D-glucopyranosyl(1→6)-β-D-glucopyranoside)	(Ma et al., 2006)
	Rhodioloside C ((2*E*,4*R*)-4-hydroxy-3,7-dimethyl-2,6-octadienyl β-D-glucopyranosyl(1→3)-β-D-glucopyranoside)	(Ma et al., 2006)
	Rhodioloside D ((2*E*,4*R*)-4,7-dihydroxy-3,7-dimethyl-2-octenyl β-D-glucopyranoside)	(Ma et al., 2006)
	Rhodioloside E ((2*E*)-7-hydroxy-3,7-dimethyl-2-octenyl α-L-arabinopyranosyl(1→6)-β-D-glucopyranoside)	(Ma et al., 2006)
	Rhodioloside F ((2*E*, 4*R*)-4-hydroxy-3,7-dimethyl-2,6-octa-dienyl α-L -arabinopyranosyl(1→6)-β-D-glucopyranoside)	(Ali et al., 2008)
	Geraniol	(Evstatieva et al., 2010)
	Myrtenol	(Evstatieva et al., 2010)
	Geranyl 1-O-α -L-arabinopyranosyl(1→6)-β-D-glucopyranoside	(Mudge et al., 2013)
Triterpenes	Daucosterol	(Kurkin et al., 1985)
	β-sitosterol	(Dubichev et al., 1991)
Cyanogenic glucosides	Lotaustralin	(Akgul et al., 2004)
	Rhodiocyanoside A	(van Diermen et al., 2009)

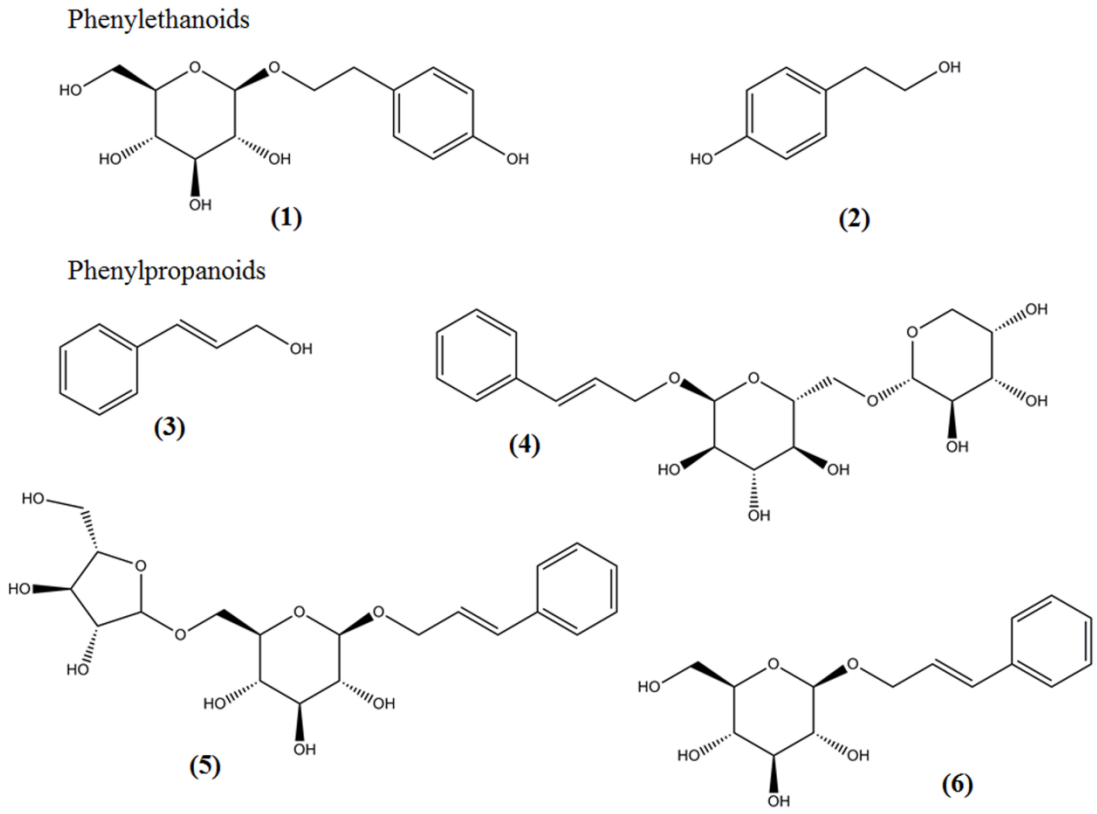

Figure 1.2.1 Chemical structures of selected *Rhodiola rosea* phytochemicals. (**1**) Salidroside, (**2**) *p*-tyrosol, (**3**) Cinnamyl alcohol, (**4**) Rosavin, (**5**) Rosarin, (**6**) Rosin.

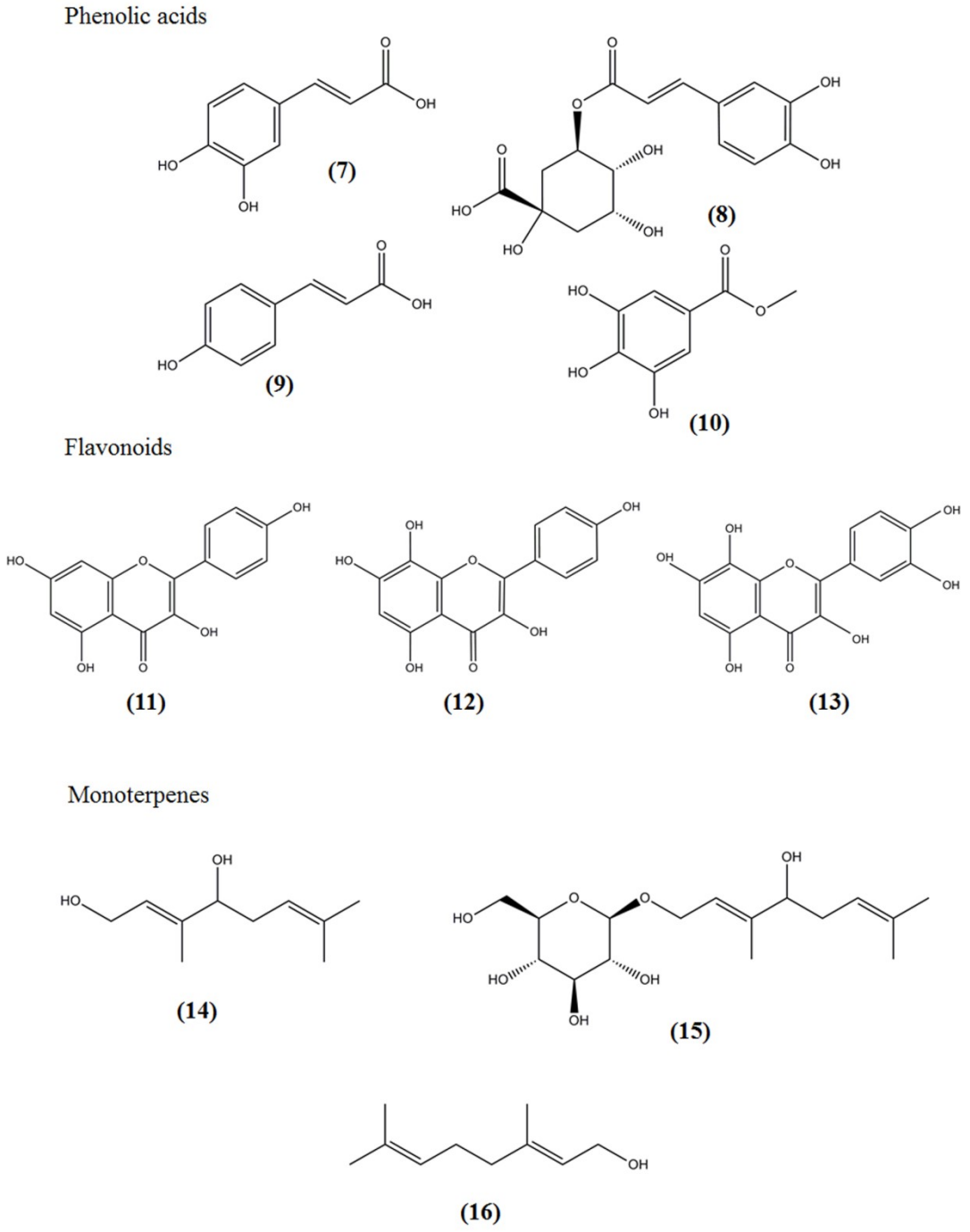

Figure 1.2.1 (continued) Chemical structures of selected *R. rosea* phytochemicals. (**7**) Caffeic acid, (**8**) Chlorogenic acid, (**9**) *p*-Coumaric acid, (**10**) Methyl gallate, (**11**) Kaempferol, (**12**) Herbacetin, (**13**) Gossypetin, (**14**) (-) - Rosiridol, (**15**) Rosiridin, and, (**16**) Geraniol.

1.2.4.1.1 Salidroside

Salidroside **(1)**, the most widely tested compound in pharmacological bioassays, was isolated from *R. rosea* roots along with its aglycone precursor, tyrosol **(2)** and termed 'rhodioloside' (Troshchenko and Kutikova, 1967). Rhodioloside was later re-identified as salidroside (Thieme, 1969) based on its previous isolation from *Salix triandra* L. (Salicaceae) (Brigel and Beguin, 1926; György, 2006). Salidroside was initially the main phytochemical marker compound for the identification and standardization of *R. rosea* extracts (Brown et al., 2002; Wang et al., 2012). However, salidroside has since been isolated from many other *Rhodiola* spp., including *R. sachalinensis* A. Bor (Bi et al., 2009), *R. sexifolia* S. H. Fu, *R. chrysanthemifolia* (H. Léveillé) S. H. Fu, *R. alsia* (Fröderström) S. H. Fu, *R. bupleuroides* (Wallich ex J. D. Hooker & Thomson) S. H. Fu, *R. macrocarpa* (Praeger) S. H. Fu, *R. sacra* (Prain ex Raymond-Hamet) S. H. Fu, *R. kirilowii* (Regel) Maximowicz, *R. sinuata* (Royle ex Edgeworth) S. H. Fu, *R. himalensis* (D. Don) S. H. Fu, *R. coccinea* (Royle) Borissova, *R. crenulata* (J. D. Hooker & Thomson) H. Ohba, *R. tieghemii* (Raymond-Hamet) S. H. Fu, *R. yunnanensis* (Franchet) S. H. Fu, *R. fastigiata* (J. D. Hooker & Thomson) S. H. Fu (Chen et al., 2008b), *R. heterodonta* (J. D. Hooker & Thomson) Borissova (Yousef et al., 2006), and *R. quadrifida* (Pall.) Fisch. et Mey. (Troshchenko and Kutikova, 1967; Wiedenfeld et al., 2007), in varying concentrations. In addition, the presence of salidroside is not restricted to the Crassulaceae family; it has been identified in *Vaccinium vitis-idaea* L. (Ericaceae) (Thieme and Winkler, 1966), *Olea europaea* L. (Oleaceae) (Ryan and Robards, 1998), and *Betula platyphylla* Sukaczev (Betulaceae) (Shen et al., 1999) to name a few. Thus, the presence of salidroside alone is not an adequate phytochemical marker to distinguish *R. rosea* from other *Rhodiola* species.

1.2.4.1.2 Rosavins

Rosavin **(4)**, rosarin **(5)** and rosin **(6)**, collectively known as the 'rosavins', belong to the group of phenylpropanoids, and are glycosides of cinnamyl alcohol **(3)** (Zapesochnaya and Kurkin, 1982). The rosavins are generally used in conjunction with salidroside as diagnostic marker compounds for *R. rosea*. Commercial extracts of *R. rosea* are now standardized to both salidroside (0.8-1 %) and the rosavins (minimum 3 %) in a 3:1 ratio reflecting their approximate concentrations in plant extracts (Brown et al., 2002). Other phenylpropanoids have been identified from *R. rosea* rhizomes and tissue cultures: cinnamyl-(6′-O-β-D-xylopyranosyl)-O-β-glucopyranoside (Tolonen et al., 2003b), sachaliside 1, triandrin (4-hydroxy-cinnamyl-O-β-D-glucopyranoside) (Kurkin et al., 1991), vimalin (4-methoxy-cinnamyl-O-β-D-glucopyranoside) (Kurkin et al., 1991) and 4-methoxy-cinnamyl-(6'-O-α-arabinopyranosyl)-O-β-glucopyranoside (Tolonen et al., 2003b).

1.2.4.1.3 Phenolic compounds

R. rosea contains a large number of simple phenolics, including hydroxycinnamic acids (Brown et al., 2002), caffeic acid **(7)** (Kurkin et al., 1991), chlorogenic acid **(8)** and *p*-coumaric acid **(9)** (Kurkin et al., 1991), gallic acid derivatives **(10)** (Kurkin et al., 1984a), flavonoids and tannins (Pooja et al., 2006). Flavonoids from *R. rosea* are often found as glycosides of kaempferol **(11)**, herbacetin **(12)** and gossypetin **(13)**. The flavonoids include flavonols like rhodiolinin (Jeong et al., 2009), rhodionin, rhodiosin (Zapesochnaya and Kurkin, 1983), flavolignans like rhodiolin (Zapesochnaya and Kurkin, 1983) and proanthocyanidins (Yousef et al., 2006).

The aerial portions of *R. rosea* also contain flavonoids, but not phenylpropanoids. These include glycosides of herbacetin and gossypetin, including rhodionin, rhodionidin, rhodiolgin,

rhodiolgidin, rhodalin, and rhodalidin (Kurkin et al., 1984b). Recently, ten new flavonoids were identified from the leaves and flowers of *R. rosea* by Petsalo et al. (2006) (Table 1.2.1).

1.2.4.1.4 Terpenes

R. rosea roots contain monoterpenes, including rosiridol **(14)** and its glycoside rosiridin **(15)** (Kurkin et al., 1985). In a study by Ma et al. (2006), five new monoterpene glycosides, rhodiolosides A-E were isolated and their structures elucidated. Recently, another new monoterpene glycoside, rhodioloside F, was identified (Ali et al., 2008). Triterpenes daucosterol and β-sitosterol were also identified from *R. rosea* roots (Kurkin et al., 1985; Dubichev et al., 1991).

1.2.4.1.5 Essential oils

The essential oil of *R. rosea* roots contains different mixtures of compounds depending on the source of the plant material as well as the extraction method. *R. rosea* roots from Norway were found to contain around 86 different compounds in the essential oil, consisting mainly of monoterpene hydrocarbons, oxygenated monoterpenes and aliphatic alcohols (Rohloff, 2002). In these samples, n-decanol (30.38 %), geraniol (12.49 %) and 1, 4-*p*-menthadien-7-ol (5.10 %) were the most abundant volatiles detected. Geraniol **(16)** is primarily responsible for the characteristic rose-like fragrance of *R. rosea* roots; other compounds including geranyl formate, geranyl acetate, benzyl alcohol and phenylethyl alcohol play minor roles (Rohloff, 2002). A comparative study of *R. rosea* root essential oils from Bulgaria, China and India showed geraniol to be the primary compound from the former, similar to that observed in roots from Mongolia (Shatar et al., 2007), while phenethylalcohol was the most important compound in the Indian sample (Evstatieva et al., 2010). Finnish populations of *R. rosea* contained myrtenol, trans-pinocarveol, and geraniol as the primary volatiles in the essential oil (Héthelyi et al., 2005).

1.2.4.2 Canadian (Nunavik) populations of *Rhodiola rosea*

Phytochemical comparisons within local Nunavik populations as well as with Eurasian *R. rosea* samples have shown several interesting trends (Filion, 2008; Filion et al., 2008; Avula et al., 2009). HPLC and liquid chromatography-electrospray ionization-time of flight (LC-ESI-TOF) analyses demonstrated that the markers salidroside, rosavin, rosarin and rosin, as well as rosiridin were present in the Nunavik populations, albeit at lower concentrations compared to the Eurasian ones (Filion et al., 2008; Avula et al., 2009). Rhodioloside F, a monoterpene glycoside, was detected in the Eurasian population but only in low amounts in one of the four Nunavik populations analyzed. Rhodioloside D and mongrhoside were detected only in the Eurasian populations but not in the Nunavik ones (Avula et al., 2009), suggesting the potential for these to be distinguishing marker compounds, although more samples are needed to validate these findings. The presence of benzyl alcohol O-α-L-arabinopyranosyl-(1→6)-O-β-D-glucopyranoside, viridoside, and kaempferol 3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside were reported in both the Nunavik and Eurasian populations (Avula et al., 2009).

Within different local Nunavik *R. rosea* populations, the levels of phytochemicals, particularly salidroside, were susceptible to fluctuation, possibly due to environmental factors. Salidroside levels decreased in Nunavik plants upon herbivory by bud mites, indicating that salidroside may be a precursor for plant defence compounds or too expensive to produce by plants already stressed by other environmental stressors to maintain normal state concentrations (Filion, 2008). Gender also seemed to impact salidroside production in Nunavik *R. rosea* populations; male plants contained higher amounts than females (Filion, 2008). In contrast, rosavin concentrations were relatively stable in Nunavik *R. rosea* populations (Filion, 2008).

1.2.4.3 Metabolic profiling of *Rhodiola rosea*

Advances in chromatographic techniques have enabled the rapid detection of phytochemical marker compounds from *R. rosea*. However, since phytochemicals in *R. rosea* populations are subject to high genotypic (Elameen et al., 2008) as well as phenotypic variation, often, more than a few marker compounds are necessary to establish diagnostic phytochemical profiles. To this end, non-targeted fingerprinting methods, including ^{1}H NMR (nuclear magnetic resonance) spectroscopy have recently been applied to separate *R. rosea* populations from different geographical locations harvested at different times based on phytochemical profiles (Ioset et al., 2011). Interestingly, using principal component analyses models based on only the aromatic portion of NMR spectra, the authors reported that the geographical populations clustered separately based on changes in salidroside and rosavin content, while using the entire spectra of all chemical constituents did not separate the populations as well, thus providing validation for the use of these compounds as markers (Ioset et al., 2011). The authors also demonstrated that the time of harvest had an impact on phytochemical content; salidroside and rosavin concentrations increased from May to August, and steadily declined at the end of summer.

Wang et al. (2012) used a fast, sensitive HPLC-UV-based technique to assess the quality of ten different batches of *R. rosea* extracts from different manufacturers in an effort to test whether they could be clustered based on the origin of their raw materials. Indeed, using multivariate analyses, they showed that the extracts separated well based on their province of origin and correlated strongly with the content of rosavin, but not salidroside or tyrosol (Wang et al., 2012), thus demonstrating the importance of using rosavin as a marker for confirming the presence of *R. rosea* in herbal extracts.

1.2.4.4 Biosynthesis of phenylethanol and phenylpropanoid derivatives

1.2.4.4.1 The biosynthesis of salidroside

Salidroside is synthesized from its aglycone precursor, tyrosol, by the enzymatic addition of glucose via UDP-glucosyltransferases (Ma et al., 2007a) or by β-D-glucosidases (Shi et al., 2007). The biosynthetic pathway of salidroside and the rosavins is shown in Fig. 1.2.2. The biosynthesis of tyrosol occurs through the shikimic acid pathway, which produces phenylalanine and tyrosine. There is an ongoing debate in the literature about which amino acid is the precursor of tyrosol (Fig. 1.2.2 paths 1 or 2) (Ma et al., 2008). There are two possible routes to tyrosol production; phenylalanine is de-aminated by phenylalanine ammonium lyase (PAL), eventually forming para-coumaric acid and then tyrosol (Xu and Su, 1997; Li et al., 2005; Ma et al., 2008). This can happen via direct decarboxylation by para-coumaric acid decarboxylase (Liang and Zheng, 1981) or by the conversion of para-coumaric acid into para-coumaryl alcohol by cinnamyl alcohol dehydrogenase (CAD) by a series of enzymatic reactions and then into tyrosol through at least two, as yet uncharacterized steps (Wang et al., 2007b).

The other reigning hypothesis on tyrosol formation is that tyrosine is converted into tyramine by tyrosine decarboxylase (TyrDC). Tyramine is oxidised by tyramine-oxidase to 4-hydroxy-phenylacetaldehyde (4-HPAA), which is then reduced to 4-hydroxy-phenylacetalcohol, also known as tyrosol (Ellis, 1983; Landtag et al., 2002; Ma et al., 2008). There is evidence from biotransformation studies in *Rhodiola* plant callus cultures showing that tyrosine, and not phenylalanine, may be the limiting step for salidroside formation (Ma et al., 2008).

1.2.4.4.2 The biosynthesis of rosavins

The rosavins are cinnamyl alcohol glycosides, and therefore, are products of the shikimic acid pathway derived from the de-amination of phenylalanine by PAL, which forms cinnamic

acid (Fig. 1.2.2). The formation of cinnamyl-CoA ester is catalyzed by hydroxycinnamate: CoA ligase (4CL), which is reduced to cinnamaldehyde by cinnamyl-CoA reductase (CCR). The cinnamaldehyde is further reduced by CAD to cinnamyl alcohol. The enzymes that take part in the formation of the glycosides of cinnamyl alcohol have not yet been elucidated. Rosin is formed by the transfer of one glucose unit to cinnamyl alcohol, and rosarin and rosavin are synthesized by further addition of sugar molecules to rosin (György, 2006).

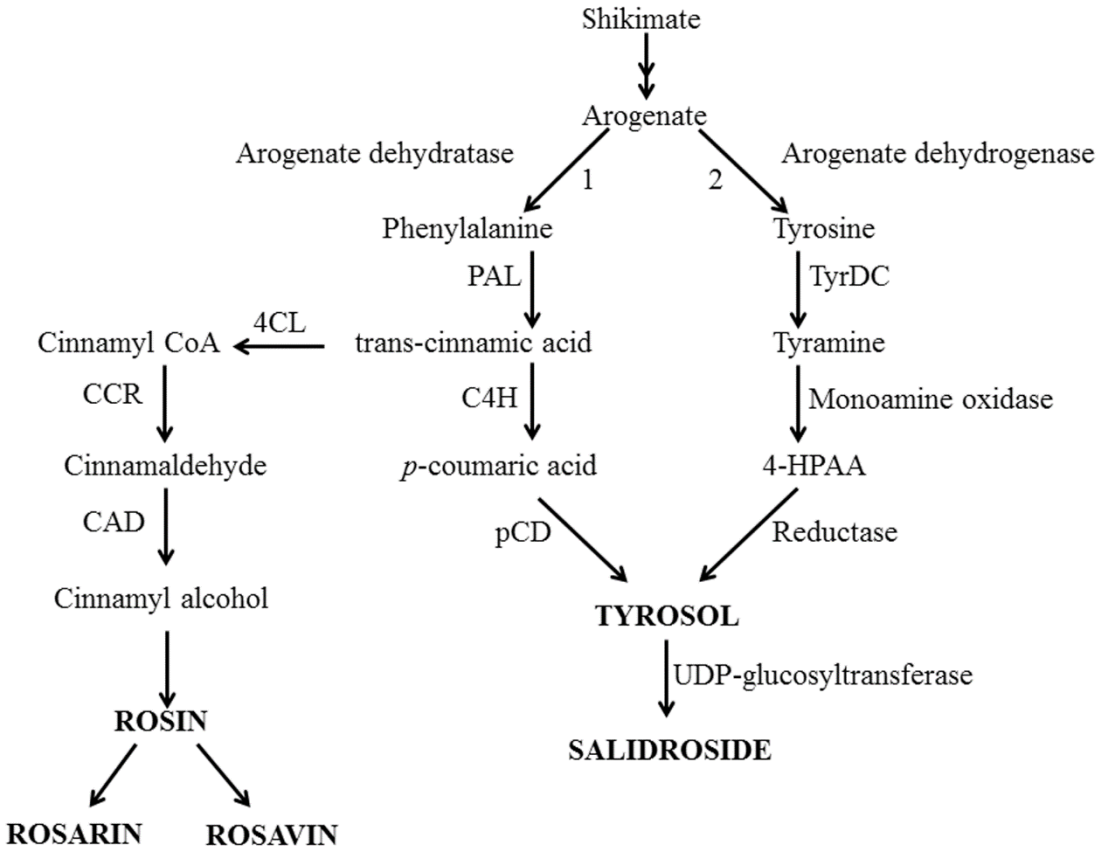

Figure 1.2.2 Schematic of the biosynthesis of key phytochemicals salidroside, tyrosol and the rosavins. See text for pathway description. Salidroside biosynthesis: PAL: Phenylalanine ammonia lyase; TyrDC: tyrosine decarboxylase; C4H: Cinnamate 4-hydroxylase; 4-HPAA: 4-hydroxy-phenylacetaldehyde; pCD: *p*-coumaric acid decarboxylase. Rosavin biosynthesis: 4CL hydroxycinnamate: CoA ligase; CCR: cinnamyl-CoA reductase; CAD: cinnamyl alcohol dehydrogenase.

1.2.4.5 Bioactivity of the phytochemicals of *Rhodiola rosea*

Previous phytochemical investigations of *R. rosea* have led to the isolation and identification of over 140 compounds belonging to different classes of secondary metabolites. However, the biological activity of the *R. rosea* extract has been primarily attributed to a few of these phytochemicals, including salidroside, tyrosol, and rosavin. It is important to keep in mind that other bioactive compounds including phenolics, flavonoids, monoterpenes and triterpenes are also present in *R. rosea,* and that the combination of these compounds in the extract acting synergistically are probably responsible for the sum total of its pharmacological effects.

1.2.4.5.1 Salidroside

Of all the compounds present in *R. rosea*, salidroside is the best studied for its pharmacological effects both *in vitro* and *in vivo.* Salidroside has been reported to have multiple beneficial biological activities, including antioxidant (Chen et al., 2009c), neuroprotective (Chen et al., 2008d), anti-cancer (Hu et al., 2010b), hepatoprotective (Wu et al., 2009), antibacterial (Cybulska et al., 2011), antiviral (Wang et al., 2009b), anti-hypoxic (Tan et al., 2009), anti-inflammatory (Guan et al., 2011b) and anti-hypoglycemic (Yu et al., 2008a) activities.

Cellular oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell. Oxidative stress underlies the pathophysiology of multiple disorders, including neurodegenerative diseases, cardiovascular dysfunction, metabolic syndrome, as well as cancer (Roberts and Sindhu, 2009). Salidroside has been shown to be protective against oxidative stress in many cellular and animal models, commonly induced by hydrogen peroxide (Zhang et al., 2007; Cai et al., 2008; Chen et al., 2009c; Mao et al., 2010; Yu et al., 2010; Guan et al., 2011a; Zhu et al., 2011; Qian et al., 2012; Shi et al., 2012). Some of these underlying mechanisms of action include inhibiting ROS

accumulation, attenuating lipid peroxidation and DNA damage, stabilizing intracellular Ca^{2+} ion levels, restoring the balance of pro- and anti-apoptotic proteins and inhibiting the activity of certain caspases.

In addition to the antioxidant activities of salidroside, it is also a potent neuroprotective agent. Salidroside has been shown to be protective against Aβ *in vitro*, a key peptide involved in the etiology of AD, by the induction of antioxidant enzymes, inhibition of ROS accumulation and reduction of apoptosis (Jang et al., 2003; Zhang et al., 2010b). Aβ oligomers also lead to Ca^{2+} ion dysregulation by over-activation of glutamate receptors, a phenomenon known as glutamate excitotoxicity. Salidroside protects against glutamate excitotoxicity by buffering the excess influx of Ca^{2+} ions and inhibiting the activity of caspase-3 (Cao et al., 2006; Chen et al., 2008d). Salidroside is also protective against *in vitro* and *in vivo* models of Parkinson's disease by attenuating damage to dopaminergic cells by blocking the nitric oxide pathway, inhibiting ROS production (Chen et al., 2007; Li et al., 2011a) and elevating levels of glial-derived neurotrophic factor (Zhang et al., 2006). Salidroside can also reverse apoptosis in neural stem cells isolated from rat hippocampi subjected to streptozotocin (STZ) insult via its antioxidant effects (Qu et al., 2012).

Salidroside protects from ischemic/reperfusion injury in the brain by reducing cerebral edema, reducing markers of lipid peroxidation, increasing levels of antioxidant enzymes and by reducing apoptosis or markers of inflammation including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Song et al., 2006; Zou et al., 2009; Liang et al., 2010; Zhong et al., 2010; Shi et al., 2012). Additionally, salidroside exerts protective effects under hypoxic conditions by upregulating the expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) (Zhang et al., 2009). Attempts have been

made at synthesizing analogues of salidroside and testing their biological activity under conditions of hypoglycemia and serum limitation in PC12 cells (a model of ischemic stroke). Salidroside analogues exhibited equal or greater protective activity by modulating apoptotic gene expression and restoring mitochondrial membrane potential (Guo et al., 2010; Guo et al., 2011; Meng et al., 2011).

Recently, salidroside has shown cytotoxic activity against multiple cancer cell lines, including hormone-sensitive and hormone-resistant breast cancer cells (Hu et al., 2010b; Hu et al., 2010a), gastric cancer, lung cancer (Hu et al., 2010b) and bladder cancer cells (Liu et al., 2012). This compound acts by inducing cell cycle arrest, and subsequently, apoptosis. Salidroside also inhibits metastasis, or the ability of the cancerous mass to migrate to other sites from the primary site by suppressing matrix metalloproteinases (Sun et al., 2012). In addition, salidroside reduces tumour angiogenesis (Skopińska-Rózewska et al., 2008). Clearly, salidroside is a highly active compound with pleiotropic effects on multiple pharmacological targets.

1.2.4.5.2 Tyrosol

Tyrosol has been shown to be protective against adrenaline and $CaCl_2$-induced arrythmia (Maimeskulova and Maslov, 1998). It also protects bone marrow cells from sub-acute lead toxicity by attenuating lipid peroxidation (Pashkevich et al., 2003). It is a better antioxidant/neuroprotective agent against cerebral ischemia compared to salidroside both *in vitro* and *in vivo*; it acts by restoring the balance of pro- and anti-apoptotic proteins (Shi et al., 2012).

1.2.4.5.3 Rosavin

Rosavin shows antibacterial activity against resistant strains of *Neisseria gonorrhoeae* when tested in disk diffusion assays (Cybulska et al., 2011). Daily oral administration of rosavin reduces angiogenesis in L-1 sarcoma cells grafted onto the skin of BALB/c mice, providing a

possible mechanism of action for the observed anti-cancer effects of *R. rosea* (Skopińska-Rózewska et al., 2008). Rosavin exhibits antidepressant activity *in vivo* in rats in the forced swim test by significantly decreasing freezing time and increasing swim duration compared to untreated controls (Kurkin et al., 2006). Rosavin was found to be the most potent among several synthesized phenylpropanoid glycosides in terms of inhibiting acetylcholinesterase activity (Li et al., 2011b), an enzyme which degrades the neurotransmitter acetylcholine, a key drug target in AD.

1.2.4.5.4 Other bioactive compounds

Apart from salidroside, tyrosol and rosavin, other compounds isolated from *R. rosea* roots also have potent biological activity. Flavonols isolated from the roots of *R. rosea*, particularly kaempferol showed strong inhibitory activity against two influenza viruses (Jeong et al., 2009). Bioassay-guided fractionation of *R. rosea* roots using monoamine oxidase A and B inhibitory activity led to the identification of rosiridin, rhodioloside B and C isomers, cinnamyl alcohol, triandrin and epigallocatechin-3-gallate (EGCG) dimers as the most active compounds; in contrast, salidroside or the rosavins possessed no significant activity (van Diermen et al., 2009). Gossypetin-7-O-L-rhamnopyranoside and rhodioflavonoside from the stems of *R. rosea* showed strong inhibitory activity against *Staphylococcus aureus* (Ming et al., 2005). These two compounds were cytotoxic against prostate cancer cells (Ming et al., 2005) and inhibited acetylcholinesterase activity (Hillhouse et al., 2004).

1.2.4.6 Trends in *Rhodiola rosea* phytochemistry

The phytochemistry of *R. rosea*, particularly of several European and Chinese populations has been assessed extensively, leading to the isolation and identification of over a hundred different compounds. Salidroside, despite its widespread prevalence among many

Rhodiola species and several other plant families, continues to be used as a marker compound for the standardization of *R. rosea* extracts (Brown et al., 2002). In light of recent metabolic profiling studies which demonstrate that rosavin, and not salidroside, is a better factor for distinguishing different *R. rosea* populations, these practices might be reconsidered (Ioset et al., 2011; Wang et al., 2012). The biological activity of *R. rosea* is attributed primarily to the presence of salidroside in the published literature, even though the diverse activities of its other constituents have been well established (Kurkin, 2003; van Diermen et al., 2009). In addition, numerous studies report the biological activity of high concentrations of chemically synthesized, commercially available forms of salidroside; these may not reflect the actual levels of salidroside present in the *R. rosea* extract nor the synergistic effect of salidroside with the other phytochemical constituents present in the *R. rosea* extract.

Rapid, sensitive, and cost-effective HPLC-based techniques to profile herbal extracts are crucial, and are beginning to be utilized for *R. rosea* not only for the accurate distinction of morphologically similar *Rhodiola* species and (or) different geographical populations of the same species (Wang et al., 2012), but also for the quantification of key phytochemicals which fluctuate depending on genetic (Elameen et al., 2008) and environmental factors (Galambosi, 2006). An important consideration in the case of phytochemical analyses of *R. rosea* crude extracts is the high concentration of tannins; Yousef et al. (2006) reported that oligomeric and polymeric proanthocyanidins account for approximately 30 % of the dry weight of the acetone extract of *R. rosea*. These tannins may hinder the detection of other compounds, destabilize extracts in solution and interfere with protein-based bioassays (Wang et al., 2012). An additional tannin removal step, usually by polyamide filtration, may be performed prior to phytochemical analyses (Wang et al., 2012).

The beneficial health benefits of *R. rosea* and its phytochemicals have led to the overexploitation of wild populations; in fact, *R. rosea* is classified as an endangered or threatened species in several regions (Galambosi, 2006). Apart from cultivation, biotechnological tools such as plant callus aggregate cultures are used to overexpress enzymes in the biosynthetic pathway to improve salidroside or rosavin production, although these are dependent on culture conditions such as pH, media, and sugar molecules provided as substrate (Xu et al., 1998). Tissue culture techniques have led to the synthesis of entirely new sets of compounds not present in the original roots/rhizomes of the plant, or low amounts of known bioactive compounds (Kurkin, 2003).

R. rosea phytochemicals have been combined with active ingredients from other adaptogenic plants. One such herbal formulation is ADAPT-232®, manufactured by the Swedish Herbal Institute which consists of a fixed combination of dried extracts from roots of *R. rosea*, berries of *Schizandra chinensis* (Turcz.) Baill. (Schisandraceae) and roots of *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Araliaceae), and is standardized with respect to salidroside (0.32%), rosavin, (0.5%), tyrosol (0.05%) schizandrin (0.37%), γ-schizandrin (0.24%), and eleutherosides B and E (0.15%) (Aslanyan et al., 2010). These mixtures of adaptogenic plants have been shown to improve cognitive function via synergistic effects, particularly under stressful situations (Aslanyan et al., 2010).

Thus, the study of the phytochemistry of *R. rosea* provides insight into its biological activity, as well as underlines the importance of establishing appropriate marker compounds. Phytochemical content of *R. rosea* varies from population to population. Geographical location, biotic and abiotic stressors, and gender are important contributors to this variation in phytochemistry, and consequently, biological activity. Fingerprinting and non-targeted profiling

using HPLC or NMR-based techniques will gain prominence in terms of extract standardization and quality control in the near future.

1.2.5 Pharmacological activities of *R. rosea*: an overview

R. rosea is classified as an "adaptogen," placing it in the same category as *P. ginseng*, *E. senticosus*, *S. chinensis*, and *W. somnifera*. The term *adaptogen*, initially introduced by Russian pharmacologist N.V. Lazarev in 1947, is used to describe plants, herbal mixtures, and (or) compounds that offer non-specific resistance against a wide variety of physical, chemical, and biological stressors (Panossian and Wikman, 2010). Adaptogens are hypothesized to act as metabolic regulators, restoring the homeostasis of physiological systems altered in disease or under stressful conditions and thus allowing the organism to adapt (Panossian et al., 1999; Panossian, 2013). In addition, they are also proposed to have a stimulatory effect on physical and mental capabilities, particularly under stressful situations (Panossian and Wagner, 2005). Apart from their good safety and tolerability profiles, adaptogens are characterized by their pleiotropic modes of action and therapeutic effects upon single (Panossian and Wagner, 2005) as well as repeated administration (Panossian et al., 2009a).

Research on the adaptogenic properties of *R. rosea* began in the former Soviet Union in the 1960s, but has come to prominence in the "Western" scientific literature only in the last decade or so (Panossian et al., 2010a). *R. rosea* extracts, particularly from the roots and (or) rhizomes, have been tested *in vitro* as well as *in vivo* in many cellular and animal models of human diseases. It has been reported to have broad-spectrum pharmacological activities, including neuroprotective, antidepressant, anxiolytic, anti-stress, antioxidant, cardioprotective, and fatigue-reducing effects among others. In this section, the biological effects of *R. rosea* in preclinical and clinical studies are described, as well as the underlying modes of action. Table

1.2.2 reviews selected pharmacological activities of *R. rosea* and the model systems/bioassays that were used in these studies.

Table 1.2.2 Summary of selected pharmacological activities of *Rhodiola rosea.*

Biological Activity	Model system/Bioassay	References
Antidepressant	*In vivo*: male Sprague-Dawley rats exposed to chronic stress	(Chen et al., 2008a; Chen et al., 2009b)
	In vivo: male Wistar rats; Porsolt behavioural despair assay	(Panossian et al., 2008)
	In vivo: female Wistar rats exposed to chronic mild stress paradigm	(Mattioli et al., 2009)
Anti-stress	*In vivo:* male Wistar rats exposed to physical and corticotropin-releasing factor (CRF)-induced stress	(Mattioli and Perfumi, 2007)
	In vivo: male Chinchilla rabbits subjected to immobilization	(Panossian et al., 2007)
Antidepressant, adaptogenic, anxiolytic	*In vivo*: female BALB/c mice; forced swim test	(Panossian et al., 2009a)
	In vivo: male CD-1 mice; forced swim test (antidepressant) swimming to exhaustion (adaptogenic) light-dark, open-field tests (anxiety)	(Perfumi and Mattioli, 2007)
Anxiolytic	*In vivo*: male ICR mice; hole-board, elevated-plus maze tests (anxiety)	(Montiel-Ruiz et al., 2012)

Table 1.2.2 Summary of selected pharmacological activities of *Rhodiola rosea* (continued).

Biological Activity	Model system/Bioassay	References
Neuroprotective	*In vitro*: acetylcholinesterase inhibition	(Hillhouse et al., 2004; Wang et al., 2007a)
	In vitro: monoamine oxidase inhibition	(van Diermen et al., 2009)
	In vivo: male Sprague-Dawley rats subjected to intra-cerebroventricular STZ insult; Morris Water Maze test	(Qu et al., 2009)
	In vitro: HCN 1-A cell line exposed to hydrogen peroxide and glutamate	(Palumbo et al., 2012)
Drug (morphine) addiction recovery	*In vivo*: male CD-1 mice; morphine-induced conditioned place preference test	(Mattioli and Perfumi, 2011; Mattioli et al., 2012)
Antioxidant	*In vitro*: human erythrocytes exposed to hypochlorous acid	(De Sanctis et al., 2004)
	In vitro: singlet oxygen, H_2O_2 scavenging; ferric reducing, ferrous chelating and protein thiol protection	(Chen et al., 2008c)
	In vitro: xanthine oxidase, lipoxygenase, tyrosinase inhibition	(Chen et al., 2009a)
	In vitro: xanthine oxidase inhibition, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging	(Horng et al., 2010)
Protection against oxidative stress without antioxidant effects	*In vitro*: human osteosarcoma-derived 143-B , human diploid fibroblast IMR-90, and human neuroblastoma IMR-32 cells exposed to UV, paraquat and H_2O_2	(Schriner et al., 2009a)

Table 1.2.2 Summary of selected pharmacological activities of *Rhodiola rosea* (continued).

Biological Activity	Model system/Bioassay	References
Anti-hyperglycemic	*In vitro*: α-amylase inhibition (ddY strain mouse plasma)	(Kobayashi et al., 2003)
	In vitro: inhibition of porcine pancreatic α-amylase; α-glucosidase, angiotensin I-converting enzyme (ACE) from rabbit lung	(Kwon et al., 2006)
	In vivo: C57BL/Ks db/db diabetic mice	(Sung et al., 2006)
	In vitro: lipase inhibition (cardiac blood of ddY mice)	(Kobayashi et al., 2004)
	In vitro: insulin-stimulated glucose uptake; pRB-deficient mouse embryonic fibroblasts (ME3); 3T3-L1 pre-adipocyte cells	(Christensen et al., 2009)
Anti-fatigue	*In vivo*: Sprague-Dawley rats; exhaustive swimming	(Abidov et al., 2003)
	In vivo: mice; forced swim test	(Kurkin et al., 2006)
	In vivo: male Wistar rats; weight-loaded forced swimming test	(Lee et al., 2009a)
Anti-cancer	*In vivo*: animals with transplanted tumors, Ehrlich adenocarcinoma, Lewis lung carcinoma	(Gol'dberg et al., 2004)
	In vitro: promyelocytic leukemia cells of the HL-60 line	(Majewska et al., 2006)
	In vitro: bladder cancer UMUC3 cells	(Liu et al., 2012)
Protection against ischemia/reperfusion injury	*In vitro*: isolated hearts of male Wistar rats	(Afanas'ev et al., 1997)
	In vivo: male Wistar rats	(Maslov et al., 2009)
Anti-arrhythmia (cardioprotective)	*In vivo*: male Wistar rats exposed to epinephrine-induced arrhythmia	(Maslov et al., 1998)
Cardioprotective/anti-hypoxia	*In vivo*: male albino mice; hypobaric hypoxia	(Arbuzov et al., 2006)
	In vivo: Wistar rats; coronary occlusion	

1.2.5.2 Effects of *R. rosea* on the central nervous system

A number of preclinical and clinical studies have investigated the neurological activities of *R. rosea.* Many of these are in Slavic and Scandinavian languages or in Chinese, and thus inaccessible for general evaluation. Within English language scientific literature, *R. rosea* affects the CNS, as evidenced by its antidepressant, neuroprotective, anxiolytic and anti-stress properties reviewed below. These effects are purported to be mediated by the action of *R. rosea* extracts and (or) its phytochemicals on the neuroendocrine system via modulation of key neurotransmitter systems, the hypothalamic-pituitary-adrenal (HPA) axis, and the sympatho-adrenal system.

1.2.5.2.1 Antidepressant activity of *R. rosea*

Preclinical studies in rats and mice conducted using several different models of depression have demonstrated antidepressant activity of *R. rosea*. In animal models of depression induced by chronic mild stress, *R. rosea* extracts have been reported to significantly improve both behavioural and physiological measures of depression including enhanced response to rewarding stimuli, increased body weight gain, and increased exploratory behaviour compared to untreated controls (Chen et al., 2008a; Chen et al., 2009b; Mattioli et al., 2009). Treatment with *R. rosea* extracts also reduced immobilization times and increased swimming duration of rodents in the forced swim test, a classical paradigm of depression (Perfumi and Mattioli, 2007; Panossian et al., 2008). In humans, Darbinyan et al. (2007) first reported significant improvement of mild to moderate depression symptoms in subjects upon treatment with SHR-5®, a standardized formulation of *R. rosea* in a randomized double-blind placebo-controlled clinical trial.

The antidepressant effects of *R. rosea* seem to be mediated through several neurotransmitter systems. Chen et al. (2009b) demonstrated that treatment with *R. rosea* not only boosted levels of serotonin in rat hippocampi but also induced proliferation of neural stem cells. Similarly, an increase in the diencephalic serotonin levels and the expression of serotonin receptor 1A (5-HT_{1A}) in *R. rosea*-treated rats alleviated depressive symptoms induced by nicotine withdrawal compared to untreated controls (Mannucci et al., 2012). In a diet-induced model of obesity, *R. rosea* in combination with *Citrus aurantium* L. (Rutaceae) led to increased norepinephrine levels in the hypothalamus and dopamine in the frontal cortex, likely due to the modulatory effects on the central monoaminergic system (Verpeut et al., 2013). In fact, *R. rosea* water and methanol extracts showed inhibition of monoamine oxidases A and B *in vitro* (van Diermen et al., 2009). These enzymes play key roles in the metabolism of biogenic amines such as epinephrine, norepinephrine, and dopamine, and are thus are targets for the treatment of depression and neurodegenerative diseases. The antidepressant effects of *R. rosea* may also be enhanced by its inhibitory actions on markers of oxidative stress (Calcabrini et al., 2010) or its anti-inflammatory effects (Pooja et al., 2009).

1.2.5.2.2 Effects of *R. rosea* on cognitive function and mental performance

R. rosea has been traditionally used to enhance memory and concentration (Panossian et al., 2010a). *R. rosea* dietary supplements under the European Food Safety Authority claim that *R. rosea* may be used for "optimal mental and cognitive function" (Panossian et al., 2010a). Although several studies show promising indications of the potential of *R. rosea* as a neuroprotective agent, these are mostly *in vitro* studies; further preclinical and clinical assessments are required to validate these effects.

R. rosea extracts, as well as their constituent phytochemicals, have been shown to inhibit the *in vitro* and *in vivo* activity of acetylcholinesterase (Hillhouse et al., 2004; Wang et al., 2007a; Zhang et al., 2013a), an enzyme responsible for the degradation of the neurotransmitter acetylcholine, the levels of which are reduced in neurodegenerative diseases including AD. Salidroside, an important bioactive compound of *R. rosea*, protects cells against Aβ toxicity *in vitro* through the induction of antioxidant enzymes and inhibition of ROS accumulation (Jang et al., 2003; Zhang et al., 2010b). *R. rosea* extracts, as well as salidroside, protect against glutamate excitotoxicity by modulation of intracellular Ca^{2+} levels (Cao et al., 2006; Palumbo et al., 2012). In addition, *R. rosea* extracts inhibit important enzymes that regulate inflammatory cascades, including members of the phospholipase A_2 superfamily (Pooja et al., 2009), which generate lipid second messenger molecules that can further contribute to AD pathogenesis (Ryan et al., 2009).

In a study by Qu et al. (2009), pre-treatment with *R. rosea* significantly improved spatial learning and memory in cognitively impaired rats. *R. rosea* attenuated neuronal injury via reduction of markers of oxidative stress, including malondialdehyde and boosted the levels of glutathione reductase and reduced glutathione in the hippocampus (Qu et al., 2009). *R. rosea* improved performance of scopolamine-impaired rats in the passive avoidance task, a fear-motivated learning and memory test (Getova and Mihaylova, 2013). In addition to *R. rosea* extracts, salidroside administration improved spatial learning and memory performance of cognitively impaired rats in the Morris water maze task (Zhang et al., 2013a). Salidroside acts via multiple mechanisms, including inhibition of expression of inflammatory markers nuclear factor kappa-B (NF-κB), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and receptor for advanced glycation end-products (RAGE) in the hippocampus (Zhang et al.,

2013a). Salidroside also reduces apoptosis in neural stem cells obtained from rat hippocampi subjected to STZ insult via its antioxidant effects (Qu et al., 2012).

In humans, clinical trials suggest that *R. rosea* treatment as the standardized formulation SHR-5® (standardized to salidroside content) improves mental performance involving complex cognitive tasks, short-term memory and concentration under conditions of stress-induced fatigue in healthy adults (Darbinyan et al., 2000). In separate studies, *R. rosea* extract as the proprietary supplement Vigo dana® also significantly ameliorated cognitive deficiencies including forgetfulness, memory loss and problems in concentration (Fintelmann and Gruenwald, 2007). While promising, some of these clinical studies lack rigorous placebo-controlled comparisons, thus limiting the conclusions that could be drawn.

1.2.5.2.3 Anti-stress (adaptogenic) effects of *R. rosea*

R. rosea exerts its anti-stress effects via simultaneous action on different arms of the stress response system, including the HPA axis and the sympathetic-adrenal gland axis (Lishmanov Iu et al., 1987; Panossian and Wagner, 2005; Mattioli and Perfumi, 2007). In a study by Mattioli and Perfumi (2007), *R. rosea* selectively blocked hypophagia induced by both prolonged restraint (physical stress), as well as intracerebroventricular injection of corticotropin releasing factor (CRF) (physiological stress) in rats. *R. rosea* extract SHR-5® and salidroside significantly reduced circulating levels of phosphorylated stress-activated protein kinase, nitric oxide, and cortisol in rabbits subjected to restraint stress compared to placebo controls (Panossian et al., 2007). *R. rosea*, in combination with other adaptogens in a standardized proprietary herbal formulation ADAPT-232®, exerts stress resistance effects via increasing the expression of molecular chaperones, including heat shock protein hsp 72, and stimulating the release of neuropeptide Y, a key mediator of stress (Panossian et al., 2009a, 2012). Further

preclinical studies assessing the impacts of *R. rosea* monotherapy directly on glucocorticoid levels are warranted. Clinical trials show that *R. rosea* (SHR-5® formulation) decreases salivary cortisol, improves symptoms of stress-induced fatigue and enhances mental and physical performance under stressful conditions consistent with HPA modulation (Darbinyan et al., 2000; Olsson et al., 2009).

1.2.5.2.4 Anxiolytic activity of *R. rosea*

Only a handful of studies have assessed the anxiolytic effects of *R. rosea*. Perfumi and Mattioli (2007) tested the anxiolytic effects of *R. rosea* in the light-dark and open-field behavioural tests in mice. They reported that mice treated with *R. rosea* spent significantly more time in the exposed, unprotected areas of the maze, indicating fear reduction compared to controls. These results were further supported by a recent study by Montiel-Ruiz et al. (2012), which showed that *R. rosea* treatment decreased the number of head dips in the hole-board test similar to a positive control, clonazepam, a $GABA_A$-benzodiazepine receptor agonist. This study also examined the behaviour of mice in the elevated plus-maze test, one of the most commonly used behavioural measures of anxiety in rodents. Although there was a trend towards increased time spent in the open arms of the maze by *R. rosea*-treated mice, the anxiolytic effect was not statistically significant (Montiel-Ruiz et al., 2012). In terms of clinical studies, Bystritsky et al. (2008) conducted a small, pilot study assessing the effects of a proprietary form of *R. rosea* (Rhodax®) for generalized anxiety disorders and showed a significant decrease in the Hamilton anxiety rating scale. However, due to the small sample size, lack of placebo controls, and the open-label design of this study, the results are preliminary.

1.2.5.3 Antioxidant effects of *R. rosea*

The antioxidant effects of *R. rosea* underlie many of its other biological properties. In a comparative study of *R. rosea*, *E. senticosus*, and *Emblica officinalis* Gaertn. (Phyllanthaceae), *R. rosea* was the most potent antioxidant in terms of singlet oxygen and hydrogen peroxide scavenging, and iron-chelating abilities (Chen et al., 2008c). Moreover, antioxidant potential was directly proportional to *R. rosea* polyphenol content (Chen et al., 2008c). *R. rosea* administration protects erythrocytes from oxidative stress induced by hypochlorous acid by preventing depletion of reduced glutathione, inactivation of glyceraldehyde-3-phosphate dehydrogenase, and hemolysis (De Sanctis et al., 2004). Similarly, Calcabrini et al. (2010) demonstrated that *R. rosea* protects against several different oxidative stressors in human keratinocytes by improving reduced glutathione levels, levels of antioxidant enzymes superoxide dismutase and catalase, and reducing intracellular accumulation of ROS. *R. rosea* also inhibits the activity of xanthine oxidase, tyrosinase and lipoxygenase, enzymes that are involved in generation of ROS and in inflammatory pathways (Chen et al., 2009a; Horng et al., 2010). In rats subjected to $A\beta_{1\text{-}40}$ challenge, salidroside has been shown to induce protective effects against cognitive deficits via attenuation of levels of lipid peroxidation product malondialdehyde and enhancement of the activity of superoxide dismutase and glutathione peroxidase in the hippocampus (Zhang et al., 2013a). Other studies have shown that protection against oxidative stressors can be signalled independently of changes in antioxidant enzymes or through the activation of antioxidant response element (Schriner et al., 2009a). In humans, *R. rosea* supplementation in professional rowers significantly increased plasma antioxidant capacity, but did not relieve oxidative stress after exhaustive exercise (Skarpanska-Stejnborn et al., 2009).

1.2.5.4 Effects of *R. rosea* on physical endurance

R. rosea may also improve physical work capacity and endurance, although studies so far show conflicting results. Abidov et al. (2003) showed that oral administration of *R. rosea* in rats increased the duration of exhaustive swimming by stimulating the synthesis of ATP in mitochondria of skeletal muscles. In humans, however, no changes were observed in muscle phosphate levels in trained athletes during or after exercise (Walker et al., 2007). Similarly, *R. rosea* supplementation increased physical activity in mice in the forced swim test (Kurkin et al., 2006). Chronic supplementation in rats showed that *R. rosea* extract not only increased liver glycogen content, and upregulated lipogenic enzyme expression (sterol regulatory element binding protein-1 (SREBP-1), fatty acid synthase (FAS)), heat shock protein 70 expression, Bcl-2/Bax ratio, and oxygen content before swimming, but also facilitated recovery by reducing biomarkers of fatigue including blood urea nitrogen and lactate dehydrogenase (Lee et al., 2009a). These data are supported by microarray analyses examining the effects of *R. rosea* in an herbal preparation, AdMax™ in cultured human fibroblasts, where expression of genes involved in energy metabolism were affected (Antoshechkin et al., 2008).

Studies on humans have been more variable. In clinical studies in healthy untrained individuals, supplementation with Rhodax® significantly decreased inflammatory markers in the blood, including C-reactive protein and creatinine kinase, notably after exhaustive exercise (Abidov et al., 2004; Parisi et al., 2010). Small beneficial changes in time taken to reach exhaustion and maximal oxygen consumption (VO_2 max) have also been reported following acute (De Bock et al., 2004), but not chronic treatment. The acute effect has also been replicated in recreationally active women where *R. rosea* supplementation decreased the perception of effort required during exercise (Noreen et al., 2013). Other studies report that time to exhaustion

and VO_2 max are not altered by *R. rosea* (Earnest et al., 2004; Colson et al., 2005; Walker et al., 2007). Taken together, it remains to be determined whether *R. rosea* enhances physical endurance in humans. Certainly, these differences could be explained by the variation in the type of extracts under study, duration of supplementation and monitoring, choice of subjects, levels of physical training of subjects, etc. A rigorous series of controlled clinical studies are warranted to address these concerns.

1.2.5.5 Anti-diabetic effects of *R. rosea*

R. rosea extracts have been reported to have inhibitory activities on enzymes important for carbohydrate digestion and therefore, a potential preventive effect on the subsequent rise in blood glucose levels. Several studies have shown that *R. rosea* extracts inhibit pancreatic α-amylase (Kobayashi et al., 2003; Kwon et al., 2006), α-glucosidase activities (Kwon et al., 2006), and reduce post-prandial hyperglycemia (Etxeberria et al., 2012). Yet to be identified compounds in *R. rosea* function as partial agonists for the peroxisome proliferator-activated receptor-γ (PPAR-γ), a key target of insulin-sensitizing drugs, thereby increasing insulin-stimulated glucose uptake (Christensen et al., 2009). The methanol extracts of *R. rosea* inhibited lipase activity *in vitro* as well as *in vivo*, and oral administration of rhodionin and rhodiosin isolated from *R. rosea* significantly inhibited post-prandial rise in triglyceride levels (Kobayashi et al., 2004). In diabetic mice treated with *R. rosea*, fasting blood glucose levels were significantly lower, levels of antioxidant enzymes were higher, and lipid peroxidation in hepatic tissue was lower compared to untreated controls (Sung et al., 2006). In STZ-induced diabetic rats, *R. rosea* did not restore blood glucose or insulin levels, but attenuated cardiac dysfunction via upregulation of PPAR-δ (Cheng et al., 2012).

1.2.5.6 Other biological activities of *R. rosea*

R. rosea is has been shown to possess cardioprotective effects against hypoxic/reperfusion damage (Afanas'ev et al., 1997; Arbuzov et al., 2006; Maslov et al., 2009), likely through its effects on the sympathetic nervous system (Maslov et al., 1998). Moreover, *R. rosea* inhibits the division of HL-60 leukemia cells by causing apoptosis and necrosis and inducing cell cycle arrest at the G2/M phase (Majewska et al., 2006). After transplantation of spontaneously metastasizing Lewis lung carcinoma cells into mice, it was shown by Gol'dberg et al. (2004) that *R. rosea* inhibited metastasis by stimulation of immune system cells and decreasing the levels of glucocorticoids. In patients who underwent chemotherapy for ovarian cancer, administration of AdMax™ led to increased levels of lymphocytes and immunoglobulins IgG and IgM (Kormosh et al., 2006). The anti-cancer effects of *R. rosea* may be attributed to its bioactive constituent, salidroside, which exhibits cytotoxic action on multiple cancer cell lines (Hu et al., 2010b). Salidroside as well as *R. rosea* extract was found to be selectively cytotoxic to bladder cancer cell line UMUC3 via inhibition of the mammalian target of rapamycin (mTOR) protein and the induction of autophagy (Liu et al., 2012).

1.2.5.8 Trends in *Rhodiola rosea* pharmacology

There is a wide range of pharmacological activities of *R. rosea*, extending from its effects in the CNS to antibacterial activities, reflecting the heterogeneous traditional uses of this plant. Most studies show a positive effect of *R. rosea* extract in the model being studied, as well as indicate its safety, which is encouraging in terms of its development as a multi-modal therapeutic. At the same time, however, it is important to remember that many of these studies were conducted *in vitro*, an essential starting point for the elucidation of bioactivity as well as identification of active principles, but requiring further validation by well-designed preclinical

and clinical trials. Also, caution should be applied while evaluating results from a wide variety of studies using plant material from different sources, different methods of extraction resulting in varying concentrations of phytochemicals, different dosage and administration regimes in test animals, etc. Several studies report the activities of *R. rosea* in standardized, but complex mixtures of multiple herbs, the individual effects of which are difficult to isolate. In gene expression profiling studies by Panossian et al. (2013), *R. rosea* and its compounds, salidroside, tyrosol, and triandrin led to differential regulation of genes associated with nervous system development and function, neurological disease, and lipid metabolism in a neuroglial cell model. Not surprisingly, the profile and number of target genes affected were altered due to synergistic and (or) antagonistic interactions when *R. rosea* was administered in a mixture of adaptogens as compared to a single extract. Certainly, more studies using *R. rosea* as a monotherapy are warranted.

Of some concern is the inhibitory and (or) inductive activity of *R. rosea* on CYP enzymes (Scott et al., 2006; Brandin et al., 2007; Hellum et al., 2010; Spanakis et al., 2013), highlighting the potential ability of this plant to interfere with the metabolism of co-administered drugs. More studies are clearly needed on the metabolism of *R. rosea* as well as on the bioavailability of its phenolic constituents upon oral administration of the crude extract; pharmacokinetic parameters of active compounds delivered in isolation are different than when administered as an extract (Li et al., 2006; Chang et al., 2007; Zhang et al., 2008; Guo et al., 2012). These studies may provide more insight into which compounds or metabolites are actually responsible for activity.

Thus, there is supporting evidence for the pleiotropic pharmacological activities of *R. rosea.* Future research will need to move towards validation of these activities in more

sophisticated animal and human trials as well as determination of the pharmacokinetic parameters for bioavailability, safety, and efficacy.

1.3 RESEARCH RATIONALE

Discovery of new populations of *R. rosea*, an already prominent medicinal plant in Europe and Asia, bordering the Ungava bay region of Northern Québec, Canada, and reports of their traditional uses by the Inuit peoples of the region as food and medicine led to the initiation of a collaborative project between Dr. Alain Cuerrier (Institut de recherche en biologie végétale, Université de Montréal, Québec, Canada), Dr. John T. Arnason (Department of Biology, University of Ottawa, Ottawa, Canada), and the Makivik Corporation and its subsidiary, Nunavik BioSciences (Québec, Canada), a representative organization of the Nunavik and other Inuit in Québec. Ethnobotanical interviews conducted with the Inuit elders of Nunavik (Cuerrier and Elders of Kangiqsualujjuaq, 2011) and Nunatsiavut, Labrador (Clark and Cuerrier, 2012), revealed *R. rosea* to be one of the most important medicinal plant species with adaptogenic uses, and notably, one of the only medicinal plants specifically recommended to be "good for the brain". Methods of administration were modified depending on the ailment being treated; roots and (or) rhizomes may be prepared with seal oil (Blondeau et al., 2010; Cuerrier and Elders of Kangiqsualujjuaq, 2011), used raw in poultices for topical infections, or drank as infusions or decoctions for better mental and physical health, to name a few (Clark and Cuerrier, 2012). Elders also identified specific collection sites and soil substrates optimal for *R. rosea* growth, and appreciated the risk of over-harvesting, especially since the roots or the whole plant are used medicinally (Clark and Cuerrier, 2012; Cuerrier et al., 2014a).

This research project stemmed from an effort to preserve and validate Inuit traditional knowledge, as well as to develop sustainable micro-economic ventures for the local Inuit communities via development of Nunavik *R. rosea* as a natural health product. To this end, the first surveys of *R. rosea* geographical distribution and ecology were commissioned in 2006 (Filion, 2008). Phytochemical characterization of Nunavik *R. rosea* and comparisons with Eurasian populations showed the presence of diagnostic marker phytochemicals in the Nunavik populations, albeit at lower concentrations compared to their Eurasian counterparts, but with interesting differences apparent in the presence or absence of other phytochemicals (Filion et al., 2008; Avula et al., 2009). Even within different local Nunavik *R. rosea* populations, there was considerable variation in the levels of salidroside due to biotic stressors (Filion, 2008). Differences between Nunavik *R. rosea* and populations from Russia and Central Europe were further corroborated by the presence of two gene duplications in the plastid *trnL-F* region of the former, but not the latter (Cuerrier et al., 2014b). Distinct genetic and phytochemical profiles of Nunavik *R. rosea* may result in novel biological activities, the assessment of which, particularly in the context of AD and its associated behavioural co-morbidity, anxiety, was the focus of this thesis. Given the highly complex pathophysiology of AD, a comprehensive review of all the implicated mechanisms is beyond the scope of this current work. The core mechanisms pertinent to this thesis, namely, the amyloid cascade hypothesis, are discussed below.

Extracellular Aβ plaques and intra-neuronal neurofibrillary tangles composed of hyperphosphorylated tau protein characterize the neuropathological phenotype of AD (Duyckaerts et al., 2009). While the exact pathogenetic mechanisms of AD and their timelines are unclear, several hypotheses have been proposed to explain AD etiology (Barage and Sonawane, 2015). Of these, the “amyloid cascade hypothesis” (Fig. 1.3.1), which postulates that

the abnormal cerebral accumulation and subsequent deposition of Aβ peptides is the primary trigger for the chain of events leading to synaptic injury, neurodegeneration, and dementia (Hardy and Higgins, 1992; Hardy and Selkoe, 2002), has been the overarching focus of AD research, and the cornerstone of guiding the search for disease-modifying therapies (Schenk et al., 2012; Chiang and Koo, 2014). Recent developments suggest that Aβ oligomerization and amyloid deposition maybe part of a larger web of inter-connected events that lead to the disease state (Hardy, 2009; Herrup, 2010; Reitz, 2012; Armstrong, 2014).

Aβ peptides (39-43 amino acid residues) are derived from the transmembrane APP protein by sequential proteolysis by two aspartyl proteases, β and γ-secretases (Selkoe, 2011). Initial cleavage of APP by β-secretase (β-amyloid cleaving enzyme-1, BACE-1) results in the N-terminal ectodomain to be released into the luminal and extracellular fluid, leaving behind a resultant membrane bound C-terminal stub (Cai et al., 2001). Within the membrane, this stub is further cleaved by γ-secretase, a multi-unit protease complex consisting of presenilins at the active site, causing Aβ to be released. Depending on the exact point of cleavage by γ-secretase, Aβ isoforms of various lengths are produced. The $A\beta_{1-42}$ isoform is longer and more hydrophobic in nature than the more abundantly produced $A\beta_{1-40}$, and thus more prone to oligomerization (Selkoe, 2011). Aβ oligomerization is known to precipitate synapse-specific effects including altered plasticity due to inhibition of long-term potentiation (LTP) and (or) facilitation of long-term depression, changes in neurotransmitter levels, cytoskeletal abnormalities, and synapse loss as well as general effects on neuronal dysfunction, including Ca^{2+} dyshomeostasis, mitochondrial damage and oxidative stress, proteasome inhibition, tau hyperphosphorylation and accumulation, endoplasmic reticulum stress, and eventually cell death (Ferreira and Klein, 2011).

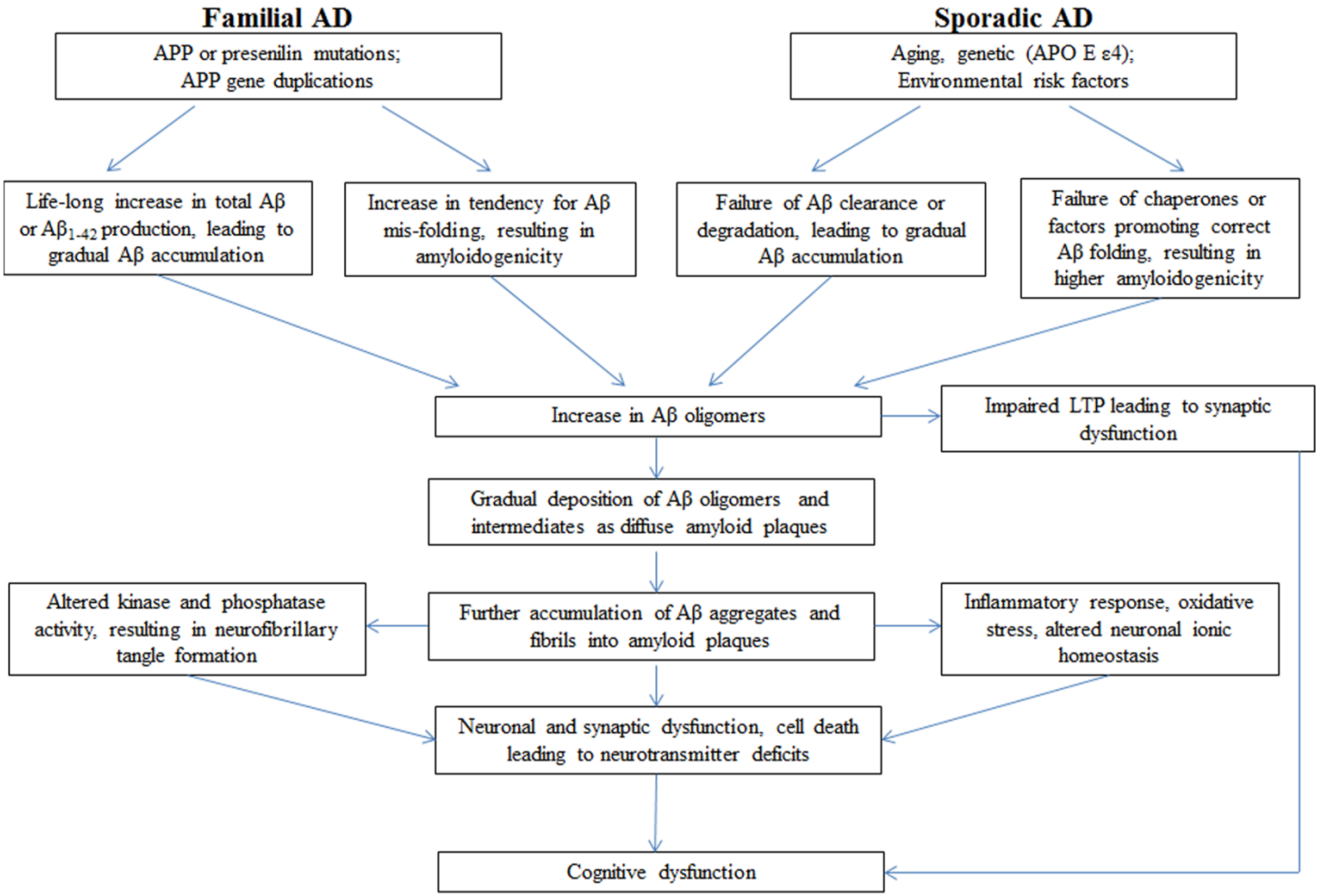

Figure 1.3.1 Amyloid cascade hypothesis (adapted from (Blennow et al., 2010)).

Transgenic mouse models recapitulate certain aspects of AD pathology, and have been invaluable tools for developing insights into disease biology and for designing therapeutics (LaFerla and Green, 2012). The TgCRND8 mouse line, which was used as a model of AD in this thesis, was initially developed on a C57BL/6 x C3H/HeJ genetic background by Chishti et al. (2001). Transgenic mice overexpress human APP with Swedish (KM670/671NL) and Indiana (V717F) mutations under the control of the hamster prion promoter, resulting in accelerated Aβ plaque pathology, impaired learning and memory, and abnormal anxiety profiles compared to wild-type controls (Ma and McLaurin, 2014). Amelioration of Aβ deposition and plaque burden has been shown to reduce cognitive dysfunction in this mouse model, providing a target for the testing of prophylactics (Janus et al., 2000; Grossi et al., 2013).

1.4 OBJECTIVES

The primary objective of this thesis was to evaluate the efficacy of Nunavik populations of *R. rosea* in the context of cognitive and behavioural correlates of AD as well as to ascertain its safety by assessing its potential for causing herb-drug interactions. The underlying hypothesis guiding this research was that *R. rosea*, a medicinal plant used traditionally by the Inuit for general health, with specific references to neurological function, is protective against cognitive and behavioural dysfunction, particularly anxiety, in preclinical models of AD. Specifically, it is predicted that treatment with Nunavik *R. rosea* will ameliorate behavioural measures of anxiety in the TgCRND8 model of AD. It is further predicted that deficits in indices of learning and memory of transgenic animals in the Morris water maze task will be mitigated by *R. rosea*. Additionally, based on the safe long-term traditional use in humans, it is predicted that inhibitory effects of Nunavik *R. rosea* on drug metabolism enzymes will be negligible.

Specific objective 1: To characterize the anxiolytic activity of Nunavik R. rosea

The holistic concept of mental and physical health in Inuit culture is reflected in their use of a sizable number of plants for the maintenance of overall well-being (Clark and Cuerrier, 2012). Plants described for the maintenance of general health may have anxiolytic and (or) anti-stress activities (Black et al., 2008). In collaboration with Christian Cayer (School of Psychology, University of Ottawa, Ottawa, Canada), the first step towards elucidating the pharmacological activities of Nunavik *R. rosea* in the CNS involved examining the *in vivo* effects of a phytochemically standardized extract in several behavioural assays of anxiety in rats. In order to investigate mode of action of the extract as well as its key phenolic marker compounds, their binding ability to the $GABA_A$-Benzodiazepine (BZD) receptor, a pertinent target of anti-anxiety drugs, was also assessed (Chapter 2).

Specific objective 2: To assess the herb-drug interaction potential of Nunavik R. rosea

Despite the long-standing traditional use of Nunavik *R. rosea*, very little is known about its safety, particularly when co-administered with other natural products or conventional drugs. Inhibition or induction of key metabolic enzymes implicated in drug metabolism, including the CYPs, by pharmacologically active natural products may lead to potentially increased risk of adverse events or diminished therapeutic efficacy by altering the plasma concentrations of drugs (Hanley et al., 2011; Goey et al., 2014). For this purpose, the safety of Nunavik *R. rosea* and its marker compounds was assessed against individual enzyme isoforms accounting for the metabolism of majority of xenobiotics, namely CYP3A4, CYP3A5 and CYP2D6, as well as on the metabolism of a commonly prescribed conventional anti-diabetic drug repaglinide in a more biologically relevant HLM system (Chapter 3).

Specific objective 3: To assess the efficacy of Nunavik R. rosea in a transgenic mouse model of AD

Based on its ethnobotanical usage as an adaptogen, and particularly for strengthening neurological function, the activity of Nunavik *R. rosea* was evaluated in the TgCRND8 mouse model of AD (Chapter 5). Transgenic mice exhibit some of the salient pathological features of AD, especially amyloid deposition and cognitive deficits, at an earlier stage than other contemporary mouse models, allowing for testing of interventions within shorter time frames (Chishti et al., 2001). Effects of *R. rosea* on basal anxiety levels, and learning and memory performance in standardized behavioural paradigms were assessed. Assessment of the use patterns of platform search strategies in the Morris Water Maze task by *R. rosea*-treated mice was automated by the development of a neural network-based application, MWM Visual, in collaboration with Dr. Martine Bertrand (Carleton University, Ottawa, Canada) and Graeme P. Taylor (University of Ottawa, Ottawa, Canada), with an eye toward enhancing data extraction and optimizing efficiency of analyses (Chapter 4). Additionally, based on the beneficial effects of European *R. rosea* on longevity (Wiegant et al., 2009; Schriner et al., 2013), the plant was investigated for its effects on the high background mortality in the males of the TgCRND8 model.

Specific objective 4: To assess the effects of Nunavik R. rosea on rat plasma and urine metabolomes in a pilot study

For the pharmacological studies in this thesis, Nunavik *R. rosea* was administered to animals using a unique oral delivery method, i.e. as a suspension in sweetened condensed milk (Cayer et al., 2013), partly in order to render the extract more palatable, and to reduce stress that conventional gavage could potentially introduce in the behavioural investigations. This then led

us to question which *R. rosea* metabolites were being absorbed into the plasma, and thus, potentially contributing to the biological activities in the above studies. Additionally, it was important to determine whether levels of endogenous metabolites were being affected by *R. rosea* treatment, and if so, to identify the key players. Further, the fate of known *R. rosea* marker compounds needed to be mapped to identify their contribution, if any, to possible biological activity. As a preliminary step towards addressing these questions, a non-targeted approach using UPLC-Q-TOF techniques was adopted to evaluate the effects of *R. rosea* administration on the global plasma and urine metabolome of rats (Chapter 6).

CHAPTER 2

Characterization of the anxiolytic activity of Nunavik *Rhodiola rosea*

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Preface

Semi-structured ethnobotanical interviews conducted with Inuit elders revealed *R. rosea* to be among the most important traditional medicinal plants in Nunavik, Québec, and Nunatsiavut, Northern Labrador, for mental health. As a precursor to conducting long-term investigations of an unstudied *R. rosea* chemotype in an advanced model of AD, preliminary tests were conducted to assess whether a phytochemically characterized ethanolic extract of Nunavik *R. rosea* was biologically active in the CNS upon oral administration. To achieve this objective, in collaboration with Christian Cayer (School of Psychology, University of Ottawa, Ottawa, Canada), we examined the potential impacts of short-term Nunavik *R. rosea* administration on anxiety-like behaviour in rats in several behavioural assays, including the elevated-plus maze, social interaction, and conditioned emotional response tests. While the link between AD and anxiety is still under investigation, anxiety is one of the most prevalent behavioural and psychological symptoms of AD (Steinberg et al., 2008), and severity of anxiety increases the likelihood of patients with mild cognitive impairment to phenoconvert to AD (Mah et al., 2015). Thus, evidence of anxiolytic activity would be an important first step towards establishing the efficacy of *R. rosea* in the context of AD. Finally, we sought to explore the mechanism of action of anxiolytic activity of Nunavik *R. rosea* using competitive radioligand binding assays, measuring the binding ability of the extract and its well-studied phytochemicals at the $GABA_A$-BZD receptor.

Statement of author contributions

FA, CC, and JTA conceived and designed this study. Plant identification and collection was undertaken by AC. Phytochemical analysis and characterization of plant extract was performed by FA with the assistance of AS. Behavioural tests were performed by CC. *In vitro* $GABA_A$-BZD assays were carried out by FA. FA, CC and JTA wrote the manuscript, with AC, AS, MA, GR, and ZM contributing to final manuscript submission.

Publication

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2.1 INTRODUCTION

Anxiety disorders are among the most commonly occurring mental health problems, affecting 28.8% of the adult U.S. population (Kessler et al., 2005). Anxiolytic drugs currently in use act via modulation of one or multiple neurotransmitter systems. Potentiation of the γ-amino butyric acid (GABA) receptors in the brain, particularly via binding of agonists to the benzodiazepine (BZD) site, has been a key target in the development of novel anxiolytics (Atack, 2005; Trincavelli et al., 2012). Benzodiazepines, the class of pharmaceuticals that bind to this site, have been a constant theme in anxiety treatment since the 1960s. However, the occurrence of adverse side effects with chronic use, particularly sedation, cognitive impairment, impaired psychomotor coordination, and risk of tolerance, encourages the need for finding safer alternatives (Ravindran and Stein, 2010).

Complementary and alternative approaches, including traditional herbal remedies are preferred over conventional therapies by anxiety patients (Kessler et al., 2001; van der Watt et al., 2008). Medicinal plants with a history of use in mental health disorders are effective agonists at the $GABA_A$-BZD receptor site (Awad et al., 2009) and have demonstrated anxiolytic potential in animal models of anxiety (Bourbonnais-Spear et al., 2007; Mullally et al., 2011).

Rhodiola rosea L. (Crassulaceae) is a medicinal plant distributed throughout the sub-Arctic and high altitude areas of Eurasia and Eastern North America (Panossian et al., 2010a; Cuerrier and Hermanutz, 2012). *R. rosea* is reputed to have "adaptogenic" properties, i.e., the ability to offer resistance against various non-specific stressors (Panossian et al., 2010a). Of particular interest are the traditional uses of this plant for neurological disorders, particularly in improving memory and concentration, alleviating depression, and improving mental performance under stressful conditions (Panossian et al., 2010a). The Eurasian variety

of *R. rosea* has neuroprotective (Qu et al., 2009; Palumbo et al., 2012), antidepressant (Chen et al., 2009b), and stress-reducing activities (Olsson et al., 2009).

Recently discovered populations of *R. rosea* in Nunavik, Northern Québec, Canada, are used by the Inuit to promote mental and physical health (Filion, 2008; Cuerrier and Elders of Kangiqsualujjuaq, 2011). Phytochemical work on this local roseroot variety has shown the presence of important marker compounds present in the Eurasian populations (Filion et al., 2008; Avula et al., 2009). The purpose of the present study was 1) to characterize the anxiolytic properties of Nunavik *R. rosea* using standardized animal behavioural tests, and 2) to explore mechanism of anxiolytic activity using the $GABA_A$-BZD receptor site as the target.

2.2 EXPERIMENTAL

2.2.1 Plant material

R. rosea roots were collected near Kuujjuaq, Nunavik, Québec, Canada, and identified by Alain Cuerrier (Université de Montréal, Canada). A voucher specimen was deposited in the University of Ottawa Herbarium (UOH# 19847).

2.2.2 Plant extraction

R. rosea roots were dried in a commercial plant dryer at 35°C and ground using a Wiley Mill (mesh size = 2 mm). Roots were extracted with 90% ethanol (10 × weight/volume) and vacuum filtered through Whatmann no. 1 filter paper. The residue was re-extracted with an additional 90% ethanol (5 × weight/volume) twice and filtered. The filtrates were combined, solvent was roto-evaporated at 40°C and the extract lyophilized (percent yield = 7%). The extract was stored at 4°C protected from light for experimental purposes.

2.2.3 HPLC-DAD-MS analyses

HPLC-DAD analyses were performed using previously validated methods (Filion et al., 2008). Briefly, *R. rosea* extract was reconstituted in 90% ethanol at 50 mg/mL, sonicated and filtered using 0.2-µm PTFE filters. Analysis of target compounds was undertaken on an Agilent 1100 series system (Agilent Technologies, Inc.). Separation was achieved on a Luna C18 column, 150 × 4.60 mm I.D., particle size 5 µm (Phenomenex, Inc.). The mobile phase was A (water + 0.1% formic acid); B (methanol). The separation gradient was 5-100% B in 25 minutes at 1 mL/min at a column temperature of 40°C. Phytochemical markers, salidroside, tyrosol, rosarin, rosavin, and rosin (> 98 % purity) (Chromadex, Irvine, CA, USA) were identified in the extract based on comparison to retention times of pure standards. Identification was further confirmed by matching MS fragmentation patterns using electrospray ionization in the negative ionization mode. Quantification was based on the area under the peak and calculated based on linear calibration curves (Filion et al., 2008).

2.2.4 Animals

Male Sprague-Dawley rats (Charles River Laboratories) weighing 250-275 g were used for experiments. Animals were housed individually in a temperature and humidity controlled environment on a 12-hour light/dark cycle (lights on at 07:00 hours). Animals had access to food (Purina Rat Chow) and water *ad libitum* and were allowed one week to acclimatize to the vivarium prior to experiments. All experiments were conducted in accordance with the Canadian Council of Animal Care and were approved by the animal care committee of the University of Ottawa, Canada.

2.2.5 Drug administration

R. rosea extract was frozen at -80°C, pulverized in an ice-cold mortar and pestle and suspended in 50% sweetened condensed milk (vehicle). Sweetened condensed milk was administered for one week prior to the start of treatments. This method of oral administration provides advantages over gavage procedures since the animals voluntarily consume their treatment, thus preventing the need for restraint. Rats were randomly assigned to one of four treatment groups: vehicle (2 mL/kg) and three *R. rosea* extract doses; low dose (8 mg/kg), medium dose (25 mg/kg), and high dose (75 mg/kg). All rats were orally administered their respective treatments for three consecutive days prior to testing; the last dose was administered an hour prior to testing. The positive control diazepam (Sandoz Canada; original concentration 5 mg/mL in 40% propylene glycol, 10% alcohol, and 50% water) was reconstituted in 1% methylcellulose and administered via intraperitoneal injection at 2 mg/kg body weight 20 min prior to testing.

2.2.6 Behavioural paradigms

Elevated-Plus Maze (EPM) test: The EPM test assesses anxiety-like behaviour in rodents based on the conflict between an animal's instinct to explore its environment and its fear of exposed areas (Pellow et al., 1985; File, 1992). The test was performed as described previously (Mullally et al., 2011). Briefly, rats (n = 7-15 per group) were placed in the testing room for 1 hour to allow acclimatization, and then each rat was placed onto the central platform of the maze (facing a closed arm). The rat's behaviour was monitored for 5 minutes and scored as follows: (1) percentage of time spent in the open arms, and (2) risk assessment behaviour (quantified by number of unprotected head dips – head protruding down over the edge of an open arm towards the floor). Increases in the open arm time and unprotected head dip measures are indicative of

reduced anxiety (File, 1992), while increased closed arm entries reflect increased general activity (Cruz et al., 1994).

Social Interaction (SI) test: SI experiments were done under semi-aversive (high illumination, familiar environment) conditions. SI was assessed in a rectangular gray Perspex arena (60 cm × 60 cm; 30-cm high walls), illuminated by a bright light source (300 lux) located directly above the arena. A camera linked to a video recorder in an adjacent room was located directly above the arenas to permit remote monitoring/scoring.

The SI test was conducted over a period of three days, between 10:00 A.M. and 2:00 P.M. On day 1, rats along with their test day partner (paired based on body weight; weights did not differ by more than 10 g), were placed in the arena for 5 minutes. On day 2, rats were individually placed in the arena for a period of 5 minutes. On test day (third day), both rats of each pair were given their respective treatments (n = 7-10 per group) 60 minutes prior to being placed into the arena for a 7-minute period. Time spent in active social interaction was determined by the sum of the following behaviours: sniffing, climbing over and under each other, following, and self-grooming; scoring was done by observers blind to the treatment. An increase in total active social interaction is indicative of reduced anxiety (File and Seth, 2003). Locomotion was monitored by counting total squares crossed in the SI box. Between trials, the arena was cleaned with 70% isopropanol.

Conditioned Emotional Response (CER) test: The conditioning chamber (Coulbourn Instruments) for assessment of CER measured 31 cm × 25 cm × 30 cm. The front and back walls were made of clear Plexiglas, and the sidewalls were made of stainless steel panels. The floor was composed of 16 stainless steel rods (4 mm diameter, 1.4 cm apart), which were connected to a shock generator (Coulbourn Instruments, model H13-16) that delivered constant current.

All subjects completed one day of training followed by a day of testing 24 hours later. During the contextual training phase, subjects (n = 7-17 per group) were placed in the conditioning chamber where they received 6 foot shocks (1.0 mA; 1 second in duration) with an average inter-trial interval (ITI) of 1 min. On test days, contextual fear was assessed over a 15-minute period by placing them in the conditioning chamber where they had previously been shocked. Freezing behaviour, as defined by the absence of movement excluding involuntary respiratory activity, was assessed. Evaluations were conducted by trained experimenters blind to the treatment group. Between each training and testing session, cages were cleaned with 70% ethanol.

2.2.7 $GABA_A$-BZD receptor assay

The $GABA_A$-BZD receptor assay was performed using homogenized rat brain membrane preparations according to published protocols (Awad et al., 2009). Briefly, *R. rosea* extract and compounds were reconstituted in 90% ethanol, filtered using 0.2 µm PTFE filters and diluted to achieve final concentrations of 0.03, 0.1, and 1 mg/mL (extract) and 10 µg/mL (compounds). Total binding wells (negative controls) were prepared by adding 20 nM of [^{3}H]-flunitrazepam ($GABA_A$ receptor agonist) (Perkin Elmer). Non-specific binding wells (positive controls) were prepared by adding 20 µM of Ro-15-1788 (Flumazenil) (Sigma-Aldrich), a displacer ($GABA_A$ receptor antagonist). The percent specific binding of [^{3}H]-flunitrazepam for each sample was standardized to the vehicle (0.09% ethanol) and calculated as follows

$$\% \text{ binding} = \left(\frac{\text{Total binding - non-specific binding} \times 100\%}{\text{Total binding}_{\text{control}} - \text{non-specific binding}_{\text{control}}}\right) \quad [1]$$

$$\% \text{ displacement} = 100 - \% \text{ binding} \quad [2]$$

Low % binding values indicate higher ability of the sample to displace the radioligand from the BZD site.

2.2.8 Data analysis

Statistical analyses were performed using Prism software version 6.0 (GraphPad Software, Inc.). Results were analyzed using t-tests (vehicle and diazepam positive control), as well as one-way and two-way analysis of variance (ANOVA) tests for comparison between vehicle controls and the various *R. rosea* treatments depending on the experiment conducted. Post hoc t-tests were conducted with the level of significance set at $p < 0.05$. Data are presented as means ± SEM for behavioural tests and means ± SD for $GABA_A$-BZD assays.

2.3 RESULTS

Phytochemical profiling of Nunavik *R. rosea* extract by HPLC-DAD-MS indicated the presence of all five standards, including phenylethanol derivatives, salidroside (1) and tyrosol (2), and the phenylpropanoids, rosarin (3), rosavin (4), and rosin (5) (Fig. 2.1). Table 2.1 shows the quantification of these compounds in the extract.

R. rosea extract was administered to rats, and their performance was assessed in the EPM. There was a significant increase in percent time spent in the open arms by rats treated with 75 mg/kg *R. rosea* [$F(4, 49) = 10.54$, $p < 0.0001$] (Fig. 2.2A). The number of unprotected head dips was also increased by treatment with *R. rosea* at 25 and 75 mg/kg [$F(4, 49) = 9.861$, $p < 0.0001$] but not at the lowest dose (Fig. 2.2C). Rats treated with the positive control diazepam showed a significant increase in both time spent in open arms (Fig. 2.2A) as well as the number of unprotected head dips (Fig. 2.2C). There were no differences in either the time

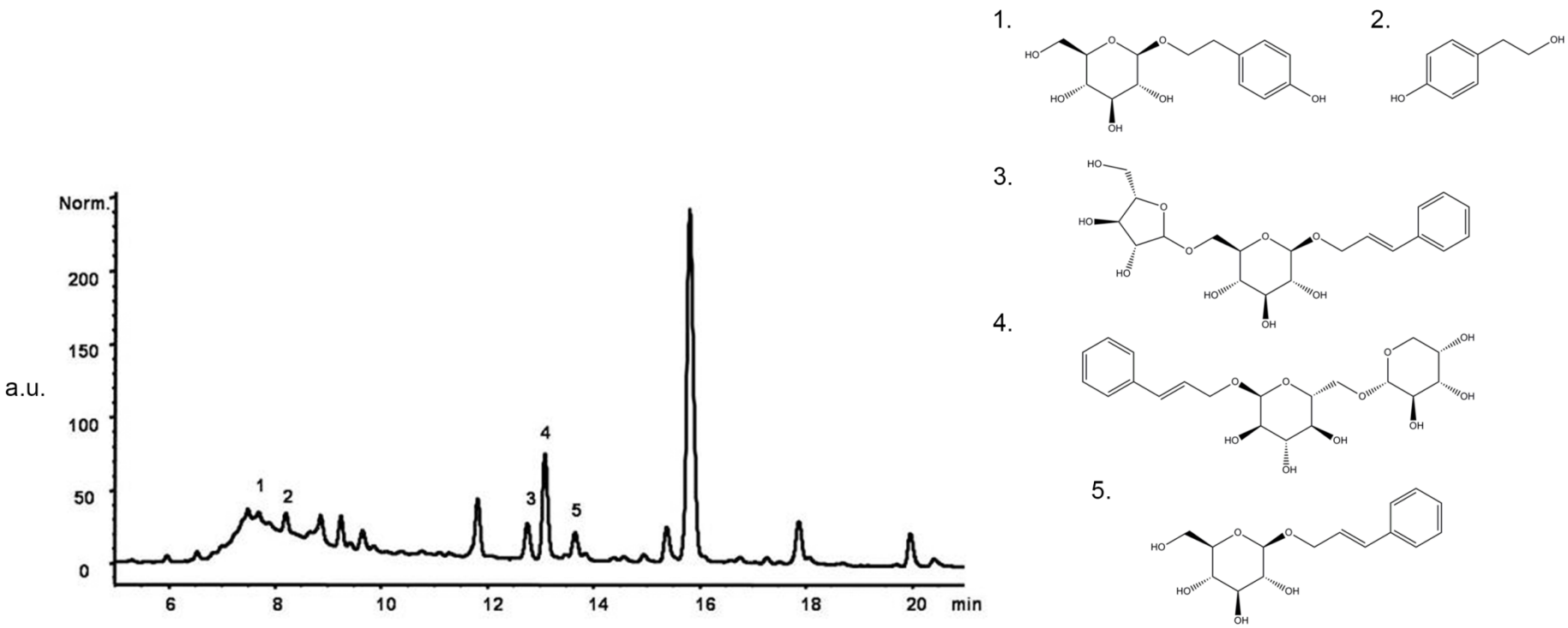


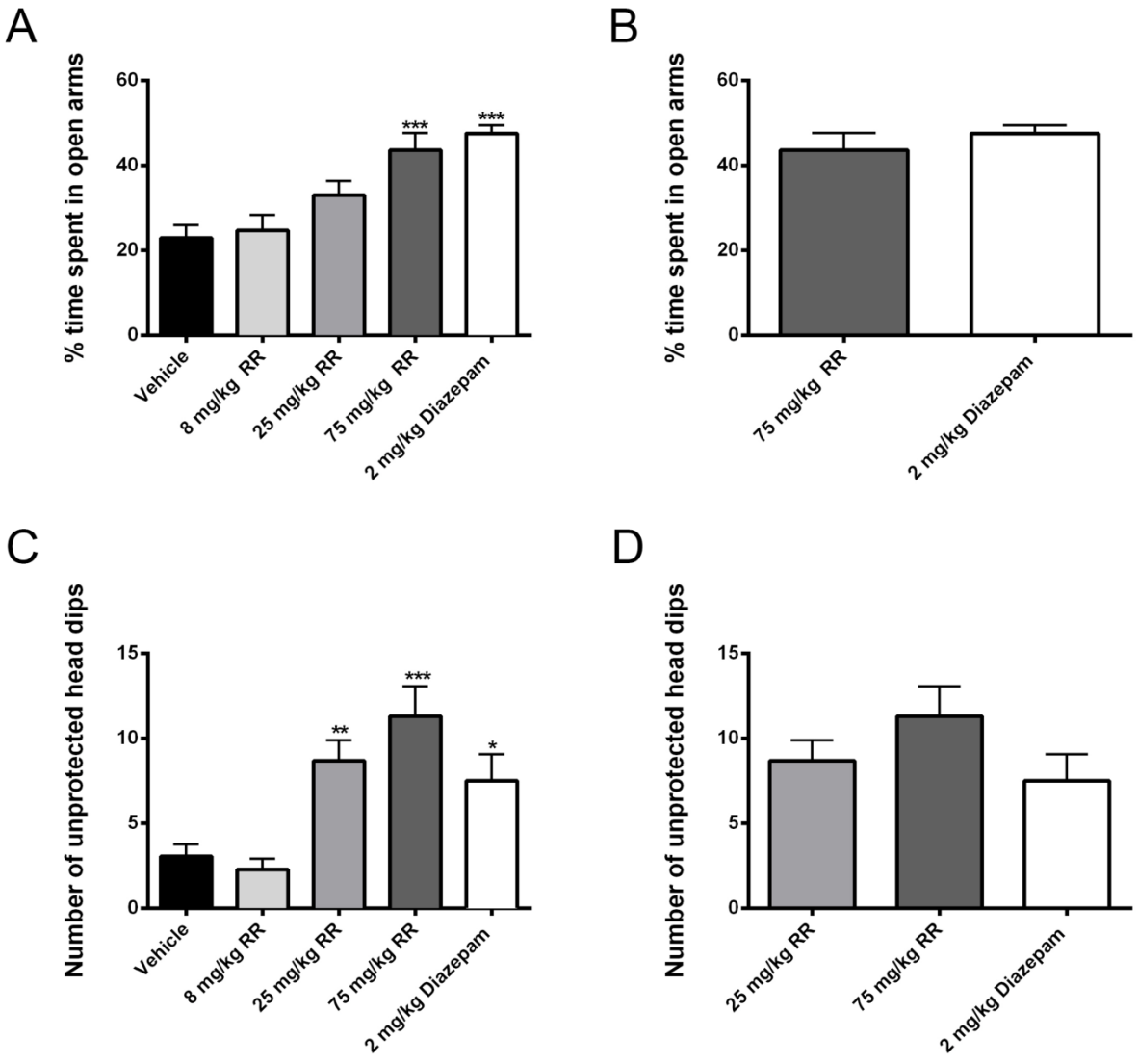
Figure 2.1 HPLC chromatogram of Nunavik *R. rosea* 90% ethanol root extract at 260 nm wavelength. Chemical structures of marker phytochemicals: 1. Salidroside; 2. Tyrosol; 3. Rosarin; 4. Rosavin; 5. Rosin.

Table 2.1 Quantification of phytochemicals in Nunavik *R. rosea* roots using HPLC-DAD-MS analyses (based on triplicate runs).

Compound	Retention time (minutes)	Major fragment (m/z ratio)	Amount (mg/g dry weight roots ± SD[a])
Salidroside (**1**)	7.70	121.0652	1.127 ± 0.022
Tyrosol (**2**)	8.21	126.9166	1.362 ± 0.040
Rosarin (**3**)	12.76	117.0703	0.535 ± 0.011
Rosavin (**4**)	13.10	117.0704	1.309 ± 0.044
Rosin (**5**)	13.66	117.0704	0.308 ± 0.021[b]

[a] SD refers to standard deviation.
[b]The coefficient of variation (COV) [calculated as standard deviation/mean x 100%] was below 5 % for all compounds except rosin, which had a COV value of 6.9 %.

Figure 2.2 Performance of rats treated with vehicle (50 % sweetened condensed milk) and three doses of *Rhodiola rosea* (abbreviated as RR) (8, 25, 75 mg/kg) in the elevated plus maze test. Diazepam (2 mg/kg) was used as a positive control. A) Percent time (±SEM) spent in the open arms (n = 15 for vehicle, n = 7 for 8 mg/kg RR, n = 10 for 25 mg/kg RR, n = 10 for 75 mg/kg RR, n = 8 for diazepam; one-way ANOVA, post hoc Dunnett's test, ***$p < 0.001$ compared to vehicle). B) Percent time (±SEM) spent in the open arms (n = 10 for 75 mg/kg RR, n = 8 for diazepam; two-tailed t-test). C) Number (±SEM) of unprotected head dips on the elevated plus maze (n = 15 for vehicle, n = 7 for 8 mg/kg RR, n = 10 for 25 mg/kg RR, n = 10 for 75 mg/kg RR, n = 8 for diazepam; one-way ANOVA, post hoc Dunnett's test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vehicle). D) Number (±SEM) of unprotected head dips (n = 10 for 25 mg/kg *R. rosea*, n = 10 for 75 mg/kg RR, n = 8 for diazepam; one-way ANOVA).

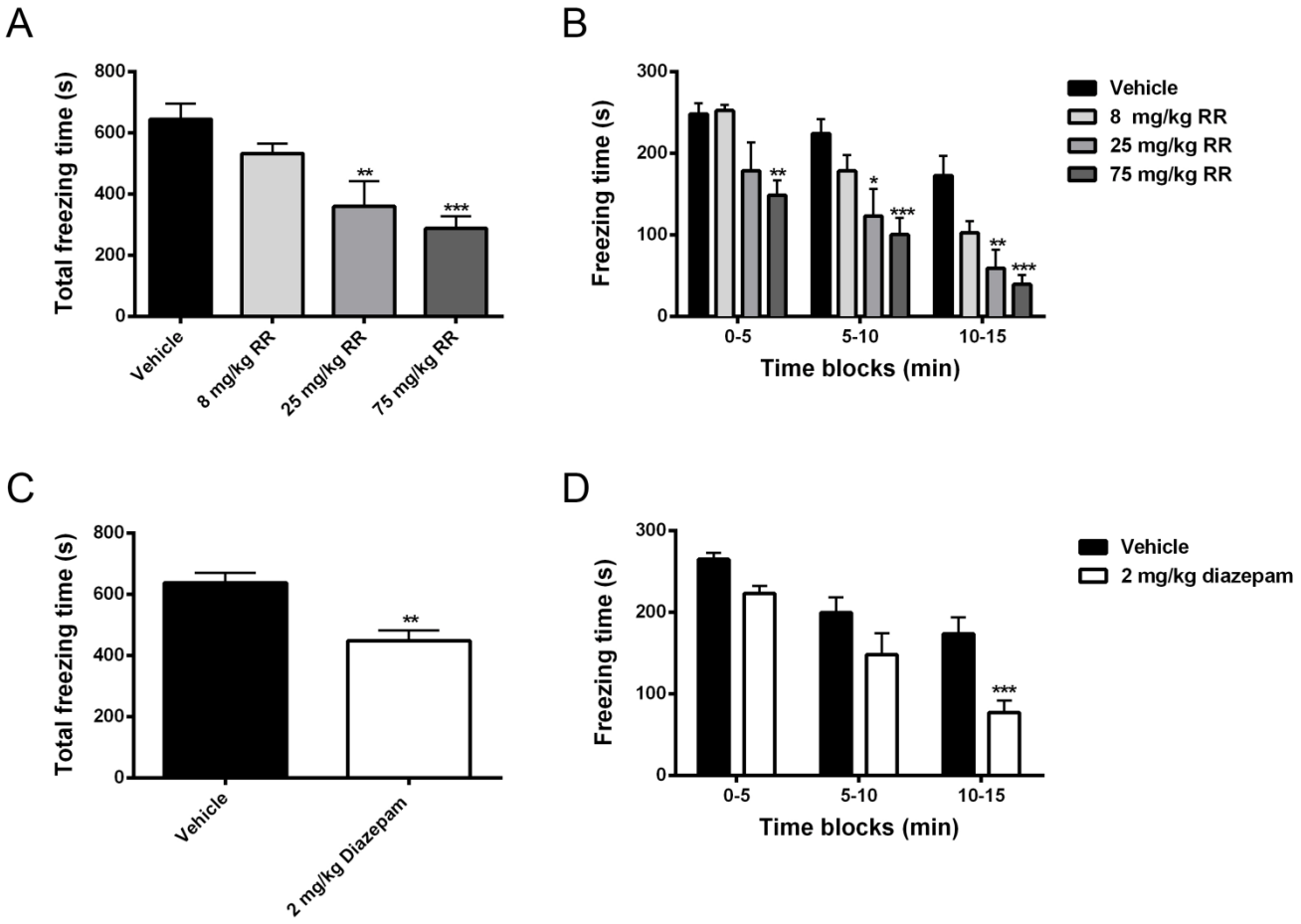

A
% time spent in open arms
60
40
20
0
***
***
Vehicle
8 mg/kg RR
25 mg/kg RR
75 mg/kg RR
2 mg/kg Diazepam
B
% time spent in open arms
60
40
20
0
75 mg/kg RR
2 mg/kg Diazepam
C
Number of unprotected head dips
15
10
5
0
**
***
*
Vehicle
8 mg/kg RR
25 mg/kg RR
75 mg/kg RR
2 mg/kg Diazepam
D
Number of unprotected head dips
15
10
5
0
25 mg/kg RR
75 mg/kg RR
2 mg/kg Diazepam

spent in open arms ($p = 0.3927$) (Fig. 2.2B) or in the number of unprotected head dips between diazepam and the active doses of *R. rosea* ($p = 0.2237$) (Fig. 2.2D). There was no difference in the frequency of closed arm entries between groups (data not shown), indicating the absence of any non-specific locomotory effects of *R. rosea*.

In the SI experiment, *R. rosea* at 25 mg/kg elicited a significant increase in the amount of time spent by rats in active social interaction compared to the vehicle, low (8 mg/kg) or high doses (75 mg/kg) of the extract [$F(3, 34) = 4.908$, $p = 0.0066$] (Fig. 2.1SA, Supplementary information 2.5). Interestingly, there were no differences in locomotory capacity despite the anxiolytic activity demonstrated, indicating the lack of sedative effects at the doses of *R. rosea* tested ($p = 0.4805$) (Fig. 2.1SB, Supplementary information 2.5).

In the contextual CER test, total time spent freezing over the entire 15 min (900 s) test period was significantly lower in *R. rosea*-treated rats compared to vehicle controls at the 25 and 75 mg/kg doses [$F(3, 43) = 10.24$, $p < 0.0001$; (Fig. 2.3A)]. Examining freezing behaviour by 5-minute time blocks (Fig. 2.3B) revealed that during the initial 0- to 5-min time block, only the 75 mg/kg dose reduced freezing significantly ($p < 0.01$). During the second and third time blocks, both the high and medium doses were effective at reducing time spent freezing compared to controls ($p < 0.05$). In addition, treatment with diazepam significantly reduced freezing over the total test duration as expected [($p = 0.0012$; (Fig. 2.3C)]. The reduction of freezing behaviour compared to vehicle controls was prominent in the third time block [$p < 0.001$; (Fig. 2.3D)].

Figure 2.3 Performance of rats in the contextual conditioned emotional response paradigm following consecutive three-day oral administration of *Rhodiola rosea* (abbreviated as RR) (8, 25, 75 mg/kg). A) Total time (±SEM) engaged in freezing over a 900 second testing period (n = 17 for vehicle, n = 10 for 8 mg/kg RR, n = 7 for 25 mg/kg RR, n = 10 for 75 mg/kg RR; one-way ANOVA, post hoc Dunnett's test, ***$p < 0.001$ compared to vehicle). B) Freezing time (±SEM) expressed in 3 time blocks, 0-5, 5-10 and 10-15 minutes (n = 17 for vehicle, n = 10 for 8 mg/kg RR, n = 7 for 25 mg/kg RR, n = 10 for 75 mg/kg RR; two-way repeated measures ANOVA, post hoc Bonferroni test, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, compared to vehicle within a specific time block). C) Total time (±SEM) engaged in freezing over a 900 second-testing period (n = 8 for vehicle, n = 8 for diazepam; two-tailed t-test, **$p < 0.01$). D) Freezing time (±SEM) expressed in 3 time blocks, 0-5, 5-10 and 10-15 minutes (n = 8 for vehicle, n = 8 for diazepam; two-way repeated measures ANOVA, post hoc Bonferroni test, ***$p < 0.001$, compared to vehicle within a specific time block).


A
Total freezing time (s)
800
600
400
200
0
**
***
Vehicle
8 mg/kg RR
25 mg/kg RR
75 mg/kg RR
B
Freezing time (s)
300
200
100
0
**
*
***
**
***
0-5
5-10
10-15
Time blocks (min)
Vehicle
8 mg/kg RR
25 mg/kg RR
75 mg/kg RR
C
Total freezing time (s)
800
600
400
200
0
**
Vehicle
2 mg/kg Diazepam
D
Freezing time (s)
300
200
100
0
***
0-5
5-10
10-15
Time blocks (min)
Vehicle
2 mg/kg diazepam

R. rosea extract was tested for its ability to bind to the $GABA_A$-BZD receptor, an important anxiolytic drug target. In the presence of the crude extract, 72.4% of [^{3}H]-flunitrazepam was able to bind to the receptor, indicating a low displacement activity (27.6%) of the extract at a concentration of 100 µg/mL (Fig. 2.4). The marker compounds showed low individual displacement activity (salidroside = 23.1 ± 17.1%; tyrosol = 8.3 ± 5.5%; rosarin = 16.4 ± 17.7%; rosavin = 9.4 ± 23.1%; rosin = 4.8 ± 21.0%). A linear regression analysis of the dose-response curve of the *R. rosea* extract yielded an IC_{50} (± 95% CI) value of 0.95 mg/mL (0.53, 2.43) ($p = 0.0148$, $R^2 = 0.9995$) (Fig. 2.2S, Supplementary Information 2.5).

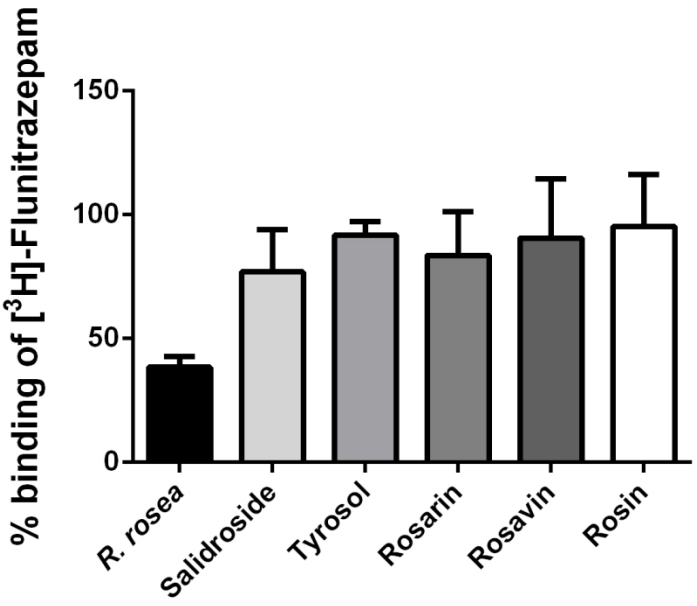


Figure 2.4 Mean percent binding activity (± SD) of [^{3}H]-Flunitrazepam by *R. rosea* extract at 1 mg/mL and five marker phytochemicals at10 µg/mL. Assays were performed in duplicate, with n = 3-4 for each sample per experiment.

2.4 DISCUSSION

The traditional uses of *R. rosea* from Nunavik, Québec, by the indigenous Inuit as a tonic supplement to improve general mental and physical health suggested the possibility of anxiolytic effects in experimental models. In fact, *R. rosea* showed significant dose-dependent activity in two different behavioural paradigms, the EPM and the CER, although not always at the same doses tested, while effects in the SI test were less pronounced.

Studies on the anxiolytic properties of Eurasian populations of *R. rosea* are few. Anxiolytic activity has been previously reported in mice in the light-dark box test (Perfumi and Mattioli, 2007) and in certain parameters of the EPM, although the authors did not observe a significant increase in time spent in the open arms (Montiel-Ruiz et al., 2012). A study of *R. rosea* preparation Rhodax® on humans with generalized anxiety disorders also indicated anxiolytic activity, although the cohort size was too small to draw definite conclusions (Bystritsky et al., 2008).

Pharmacological studies have shown that modulation of specific combinations of subunits of $GABA_A$ receptors is a major factor in mediating anxiolytic responses in the EPM (Menard and Treit, 1999; Rezayat et al., 2005), SI (Gonzalez et al., 1996; Gonzalez et al., 1998; File and Seth, 2003), and fear responses in the CER (Davis et al., 2006). Surprisingly, despite the presence of clear anxiolytic effects in the EPM and CER, the binding affinity of *R. rosea* extract to the $GABA_A$-BZD receptor is low, except at high concentrations, which may not be biologically significant. This weak binding may be responsible, in part, for the lack of non-specific sedative effects observed in the social interaction test, although these effects have to be confirmed using standardized paradigms such as the open-field and the rotarod tests. Well-known biologically active phenylethanol derivatives salidroside and tyrosol, as well as the

phenylpropanoids, rosarin, rosavin, and rosin showed low displacement activity at the $GABA_A$-BZD receptor. *R. rosea* contains flavonoids and terpenes, which may actively modulate the $GABA_A$-BZD receptor complex (Tsang and Xue, 2004; Wang et al., 2005; Vignes et al., 2006; Khom et al., 2010). Flavonoids in *R. rosea* such as kaempferol (Dubichev et al., 1991) have been shown to exert anxiolytic effects through the $GABA_A$ receptor (Grundmann et al., 2009), as have terpenes such as geraniol (Evstatieva et al., 2010), which potentiate the $GABA_A$ receptor response to the GABA neurotransmitter (Aoshima et al., 2006).

Modulation of the $GABA_A$ receptor is not the primary mode of anxiolytic action of *R. rosea*; alternate mechanisms include serotonergic involvement, particularly in mediating "risk assessment" behaviour in EPM including unprotected head dips (Rodgers et al., 1994; Rodgers et al., 1997) and in social interaction (Gonzalez et al., 1998; File and Seth, 2003). Long-term treatment with *R. rosea* restores levels of serotonin in the hippocampus of depressive rats (Chen et al., 2009b) and reduces depression-like symptoms after nicotine withdrawal in dependent rats (Mannucci et al., 2012). *Rhodiola rosea* also inhibits monoamine oxidases (van Diermen et al., 2009), another potential mode of action of anxiolytic drugs (Ravindran and Stein, 2010).

To date, this is the first study of the anxiolytic activity of Nunavik *R. rosea*. We have demonstrated that *Rhodiola rosea* exerts an anxiolytic effect in the learned fear response paradigms and moderate anxiety-inducing environments with less activity in higher anxiety-inducing social interaction contexts. The active dose of the extract seems to be test-specific. The modulation of the $GABA_A$-BZD receptor by *R. rosea* may contribute partly to its anxiolytic activity, but our study does not exclude the potential effects of *Rhodiola rosea* on other

neurotransmitter or peptidergic systems. Further work on active principles and mode of action is warranted.

Our work with *R. rosea* has some interesting parallels to those with plants used for mental health in Central America by Maya healers (Bourbonnais-Spear et al., 2007; Awad et al., 2009). Like the Inuit, Maya healers describe the use of plants for mental health in traditional terms that do not mention Western anxiety concepts as such, yet Maya plants used for mental health clearly have similar pharmacology and behavioural effects to *R. rosea* that are characteristic of anti-anxiety therapeutics in general. An understated role of anxiety treatments may be an important part of traditional medicine used by indigenous peoples.

2.5 SUPPLEMENTARY INFORMATION

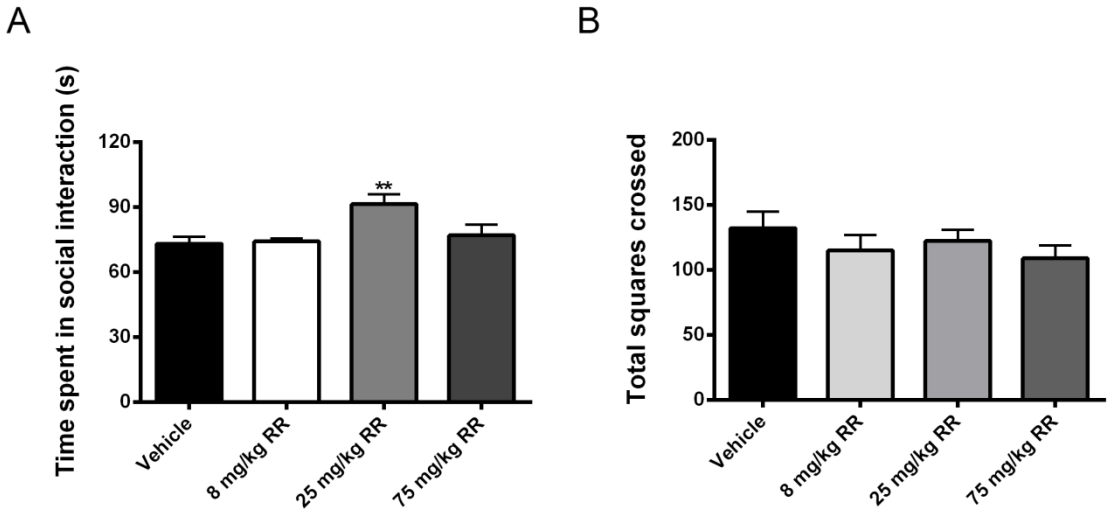


Figure 2.1S Performance of rats in the adult social interaction test following acute administration of *Rhodiola rosea* (abbreviated as RR) (8, 25, 75 mg/kg). A) Time (±SEM) spent in social interaction (n = 10 for vehicle, n = 7 for 8 mg/kg RR, n = 10 for 25 mg/kg RR, n = 8 for 75 mg/kg RR; one-way ANOVA, post hoc Dunnett's test, $^{**}p < 0.01$ compared to vehicle). B) Total squares crossed (±SEM) (n = 10 for vehicle, n = 7 for 8 mg/kg RR, n = 10 for 25 mg/kg RR, n = 8 for 75 mg/kg RR; one-way ANOVA).

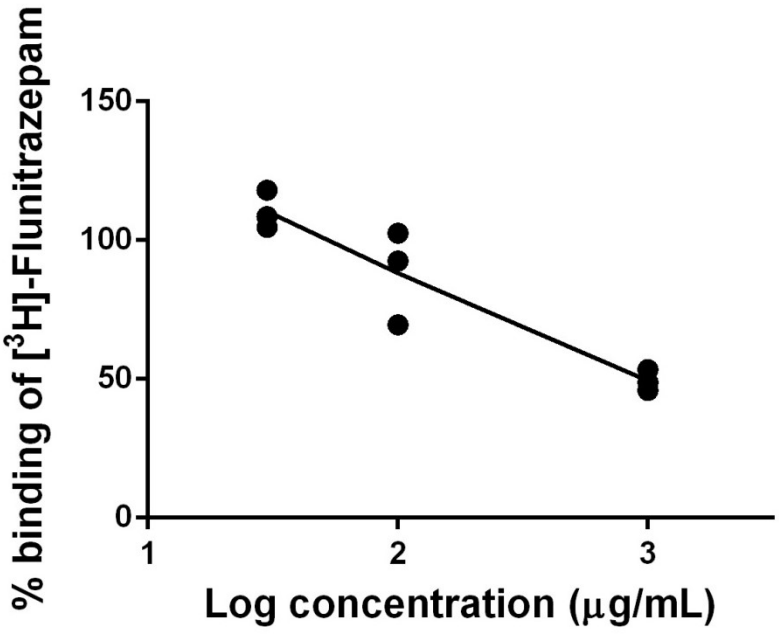


Figure 2.2S Dose response curve of percent binding activity (± SD) of [^{3}H]-Flunitrazepam by *R. rosea* extract at 1 mg/mL, 0.1 mg/mL and 0.03 mg/mL. Linear regression analysis was performed to determine IC_{50} values (± 95 % CI) as 0.95 mg/mL (0.53, 2.43). Assays were performed in duplicate, with n = 3-4 for each sample per experiment.

CHAPTER 3

Effects of *Rhodiola rosea* natural health products on human cytochrome P450-mediated metabolism *in vitro*

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Preface

Following initial evidence of anxiolytic activity (Chapter 2), we proceeded to evaluate the safety of Nunavik *R. rosea* by assessing its ability to participate in herb-drug interactions. For the purposes of this screening study, we focused on the inhibitory potential of Nunavik *R. rosea*, several other wild-harvested and commercial *R. rosea* NHPs, as well as five marker phytochemicals against human CYP3A4/5, and CYP2D6 enzyme isoforms, responsible for metabolizing the majority of clinically relevant drugs. Next, we sought to validate the inhibitory effects measured by fluorometric assays susceptible to interference using an HPLC assay monitoring the metabolism of testosterone by CYP3A4. Furthermore, we assessed the potential of these NHPs to affect the metabolism of repaglinide, a conventional anti-diabetic drug, in a more biologically relevant model, human liver microsomes obtained from a mixed population of donors to better represent the variation in the human population. These studies were particularly relevant in the light of a recent Study of Natural Health Product Adverse Reactions (SONAR) report which stated that certain adverse events, including mania-like symptoms, dizziness, nausea and irritability were reported by a patient using an *R. rosea* NHP in conjunction with prescription depression and anxiety medications.

Statement of author contributions

FA, JTA and BCF conceived and designed this study. Analyses of phytochemical markers were conducted by FA with the assistance of RL. Fluorometric CYP3A4, 3A5 and 2D6 assays were performed by FA. HLM and CYP3A4 HPLC assays were performed by DA with the assistance of RL for metabolite analysis. FA, JTA and BCF contributed to manuscript preparation.

3.1 INTRODUCTION

The use of natural health products (NHPs), including herbal supplements, has steadily increased over the last decade (Lindstrom et al., 2014). General perception of NHPs as "safe" and "natural" as compared to synthetic drugs may lead to prolonged self-administration of these highly complex, biologically active products, often in conjunction with a cocktail of prescription drugs. Nearly 60 % of NHP users in Canada reported the concurrent use of a conventional drug in a national population health survey conducted during 2000-2001 (Singh and Levine, 2006), which may potentially create scenarios for clinically relevant adverse events associated with NHP-drug interactions (Ruschitzka et al., 2000; Piscitelli et al., 2002; Bonetto et al., 2007). Patients taking anti-diabetic and anti-hypertensive medications were estimated to be at particularly high risks of experiencing NHP-drug interactions (Singh and Levine, 2007).

Altered drug disposition via modulation of Phase I oxidative metabolic enzymes, mainly by the CYP isozymes, is one of the key contributing factors underlying adverse drug reactions. Out of 18 human *CYP* gene families, members of the *CYP 1*, *2* and *3* families play important roles in the metabolism of drugs and other xenobiotics (Al Omari and Murry, 2007). Of these, CYP3A4/5, CYP2D6, CYP2C8/9 and CYP2C19 isoforms contribute to the metabolism of about 30.2 %, 20 %, 17.5 %, and 6.8 % of marketed clinical drugs respectively (Zanger and Schwab, 2013). Modulation of these enzyme isoforms by pharmacologically active NHPs have led to clinically significant changes in drug pharmacokinetics. Furanocoumarins from grapefruit juice inhibit enteric CYP3A, leading to significantly higher plasma concentrations of co-administered drugs, which may be of concern especially for drugs with a narrow therapeutic index (Paine et al., 2006; Hanley et al., 2011). Similarly, berberine, a bioactive isoquinoline alkaloid present in *Hydrastis canadensis* L. (Ranunculaceae) (goldenseal), increased the blood concentration of

cyclosporine A, an immunosuppressant drug, in renal transplant recipients via inhibition of CYP3A4 (Wu et al., 2005). On the other hand, induction of CYP3A4 by *Hypericum perforatum* L. (Hypericaceae) (St. John's Wort) leads to lower concentrations of co-administered drugs, which may affect their therapeutic efficacy (Kawaguchi et al., 2004; Portoles et al., 2006; Goey et al., 2014). Thus, characterization of the impacts of NHPs on metabolic enzymes is an important step towards identifying and preventing potential health risks.

The present study focuses on the impact on metabolic enzymes by *Rhodiola rosea* L., which is a widely used commercial medicinal plant, based on well-established traditions of use in the circumpolar regions of Europe, Asia, and North America. It is classified as an "adaptogen," similar to *Panax ginseng* C.A. Mey. (Araliaceae) (Asian ginseng), and *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Araliaceae) (Siberian ginseng), with evidence of pharmacological activity at multiple targets (Panossian et al., 2010a). *R. rosea* supplements abound in the commercial herbal sector, either as single herbal products or in combination with other NHPs, including other adaptogens. The primary types of health claims made by these supplements include the improvement of mental and physical stamina, reduction of work-related stress and fatigue, and (or) maintenance of general well-being (European Food Safety Authority, 2012). With nearly 80 companies involved in the supply and utilization of *R. rosea* products (Ampong-Nyarko et al., 2006), the demand for raw materials has created intense pressure on the existing populations of an already threatened species, leading to further concerns of substitution/adulteration of *Rhodiola* supplements with morphologically similar species (US Pharmacopeial Convention, 2014; Xin et al., 2015)

Very little is known about the effects of these supplements, which may contain extraneous ingredients in addition to *R. rosea* extracts and their related phytochemicals, on CYP-

mediated metabolism. Although generally regarded as safe, previous studies on certain *R. rosea* populations have indicated *in vitro* inhibitory potential on CYP3A4 activity and P-glycoprotein (P-gP)-mediated digoxin transport (Scott et al., 2006; Hellum et al., 2010). Further, patients in clinical studies with several proprietary *R. rosea* extracts have reported side-effects of mild to moderate intensity including nervousness, dizziness and gastrointestinal effects among others (Darbinyan et al., 2007; Edwards et al., 2012; Mao et al., 2015), indicating that in-depth assessment of *R. rosea* products with regard to safety is warranted.

In this study, as a first step towards assessing the likelihood of *R. rosea* NHP-therapeutic drug interactions, we evaluated alcohol and water preparations of selected commercial supplements from North American-sourced companies as well as extracts of wild-collected rhizome samples and prominent marker phytochemicals for their inhibitory potential on selected individual CYPs, including CYP3A4, CYP3A5 and CYP2D6. In addition, we tested these extracts for their ability to affect the metabolism of a representational conventional anti-diabetic drug, repaglinide, by metabolic enzymes present in a 50-donor mixed pool of human liver microsomes. The objective of this study was to identify *R. rosea* formulations with a high risk of modulating CYP metabolism, and to provide recommendations for these products for further advanced *in vivo* and clinical safety evaluations.

3.2 EXPERIMENTAL

3.2.1 Extract preparation

The eight different *R. rosea* NHPs tested in this study are listed in Table 3.1. Each product was assigned a Nutraceutical Research Programme (NRP) #. Samples (NRP# 221-455) were purchased from local commercial sources. NRP# 457 and 458 were 90 % ethanol extracts of *R. rosea* roots and rhizomes from Nunavik, Québec, Canada, and Siberia, Russia, respectively

(supplied by Dr. Alain Cuerrier (Jardin botanique de Montréal, Institut de recherche en biologie végétale, Université de Montréal, Montréal, Canada)). NRP# 459 was kindly provided by the Canadian Phytopharmaceuticals Corp., BC, Canada.

Alcohol (95 % ethanol (NRP# 221-455); 100 % methanol (NRP# 457-459)) and (or) water extracts were prepared at stock concentrations of 50 mg/mL (microtitre fluorometric assays) or 5 mg/mL (HPLC assays) by vigorously vortexing NHP material in 1 mL of the respective solvent for 1 min followed by sonication for 5 min. The extract was subjected to centrifugation for 5 min at 12,000 rpm at room temperature. The supernatant was collected and stored at -20°C prior to testing.

3.2.2 Chemicals and reagents

LC/MS grade methanol (MeOH), water, 95 % ethanol (EtOH), and acetonitrile (ACN) were purchased from Fisher Scientific (Ottawa, ON, Canada). Dibenzylfluorescein (DBF) (Cat# 451750), 3-[2-(N, N-diethyl-N-methylammonium)-ethyl]-7-methoxy-4-methylcoumarin (AMMC) (Cat# 451700), and microsomes derived from baculovirus-infected insect cells expressing CYP3A4 (Cat# 456207), CYP3A5 (Cat# 456235), CYP2D6 (Cat# 456217), insect control (Cat# 456244) and HLM (mixed gender, 50-donor pool) (Cat# 452156) were purchased from Corning Incorporated Life Sciences (Tewksbury, MA, USA). Nicotinamide adenine dinucleotide phosphate, in the reduced form (NADPH), quinidine, repaglinide, and uridine diphosphoglucuronic acid (UDPGA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ketoconazole was purchased from Calbiochem (Gibbstown, NJ, USA). Testosterone and 6-β-hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI, USA).

3.2.3 Phytochemical analyses

Phytochemical analyses were performed on a Shimadzu UPLC-PDA-MS system (Mandel Scientific, Guelph, ON, Canada) with a Waters Acquity UPLC CSH C18 column (2.1 × 100 mm, 1.7 µm particle size). *R. rosea* extracts were prepared as described in section 3.2.1 at 5 mg/mL and filtered using 0.2 µm PTFE filters. The mobile phase was A: water + 0.1 % formic acid and B: acetonitrile + 0.1% formic acid. The separation gradient was from 10 to 95 % B over 8.5 min at a flow rate of 0.6 mL/min and column temperature of 55°C. Phytochemical markers, salidroside, tyrosol, rosarin, rosavin, and rosin (Chromadex, Irvine, CA, USA) were identified in the extract based on comparison to retention times of pure standards. Identification was further confirmed by matching MS fragmentation patterns using electrospray ionization in both the positive (salidroside, tyrosol) and negative (rosarin, rosavin, rosin) ionization modes. Quantification was based on the area under the peak and calculated based on linear calibration curves ($R^2 > 0.998$).

3.2.4 Microtitre fluorometric Assays

Microtitre fluorometric assays were performed to assess the inhibitory potential of *R. rosea* extracts against CYP3A4-, 3A5- and 2D6-mediated metabolism according to previously published protocols (Tam et al., 2009; Cieniak et al., 2013). Briefly, the assays were performed in 96-well plates with white, opaque walls and clear bottoms under red light to minimize the exposure of fluorescent light to photosensitive material. Fluorescence was measured using a Cytofluor 4000 Fluorescence Measurement System (Applied Biosystems, Foster City, CA, USA). For CYP3A4 and 3A5, 10 µL of test extract or compound, 10 nM CYP3A4, 1 µM DBF (dissolved in ACN) and 0.6 mM NADPH were incubated in a 0.19 M phosphate buffer solution (pH 7.4) at a final volume of 200 µL for 20 min. The test samples used active enzyme while the

blank samples used insect controls. The initial and final fluorescence were read at 485 nm excitation and 530 nm emission with a gain of 50. Alcohol extracts were diluted ten-fold prior to testing. The positive inhibitor used was 1.9 μM ketoconazole dissolved in MeOH. A methanol extract of AD-01, or Labrador tea was used as a positive control at a final concentration of 10 μg/mL. For CYP2D6 assays, 10 μL of test extract or compound, 10 nM enzyme, 0.12 μM AMMC (dissolved in ACN) and 0.3 mM NADPH were incubated in a 0.19 M phosphate buffer solution (pH 7.4) at a final volume of 200 μL for 40 min. The initial and final fluorescence were read at 409 nm excitation and 460 nm emission with a gain of 85. Quinidine dissolved in MeOH at 2 μM was the positive inhibitor. A methanol extract of goldenseal was used as a positive control at a final concentration of 10 μg/mL.

3.2.5 CYP3A4-mediated metabolism of testosterone

R. rosea alcohol extracts (250 μg/mL) were incubated with 100 μM CYP3A4 co-expressed with cytochrome b5, 35 μM testosterone, 2.7 mM NADPH and 3.3 mM magnesium chloride ($MgCl_2$) in 0.125M phosphate buffer (total volume of 200 μL) for 30 min at 37°C (Tam et al., 2009). The reaction was stopped by adding 75 μL cold acetonitrile. The mixture was centrifuged at 10,000 rpm and filtered with 0.45 μm PTFE filters. A volume of 10 μL of the reaction mixture was injected into Phenomenex C18 column (4 μm particle size, 250 mm x 2 mm; Torrance, CA, USA) in an Agilent 1100 Series HPLC system with a diode array detector (DAD). Testosterone and its metabolite, 6-β-hydroxytestosterone, were separated by a gradient elution method with an initial ratio of 80:20 water:ACN, followed by a gradient change to 100 % ACN at 10 min, held for 1 min, before returning to initial conditions at 15 min. Flow rate was set at 0.4 mL/min, column temperature at 55°C, and the DAD was set at a wavelength of 245 nm. Ketoconazole (10 μM) was used as a positive control.

3.2.6 HLM-mediated metabolism of repaglinide

HLM were thawed in a 37°C water bath, and then placed on ice. *R. rosea* alcohol extracts at 100 µg/mL were incubated in a 100 mM phosphate buffer solution (pH 7.4) containing 21.6 µM NADPH, 40 µM $MgCl_2$, 44.2 µM repaglinide and 2 mg/mL HLM at a final volume of 500 µL (Cieniak et al., 2013). The reaction mixture was incubated for 90 min in a 37°C shaking incubator at 200 rpm. All reaction mixtures were stopped by the addition of 500 µL of ethyl acetate and the organic layers were extracted twice and combined. The solvents were then evaporated with a SpeedVac and the residues were re-dissolved in 500 µL of MeOH. The samples were filtered into HPLC vials using 0.2 µm PTFE filters and analyzed by HPLC as published previously (Cieniak et al., 2013) (see section 3.2.8).

3.2.7 UDP-glucuronosyltransferase-mediated metabolism

HLM were thawed in a 37°C water bath, and then placed on ice until required. A 10 µL aliquot of extract (5 mg/mL) or control vehicle was added to 50 µL 50 mM Tris buffer (pH 7.5), 12.7 µM alamethicin, 2 mM UDGPA, 22.1 µM repaglinide, 100 µM $MgCl_2$ and 2 mg/mL HLM at a final volume of 500 µL (Cieniak et al., 2013). The reaction mixture was incubated for 40 min in a 37°C shaking incubator set at 200 rpm and stopped with 250 µL of ice cold ACN. The reaction mixture was filtered through a 0.2 µm PTFE filter prior to HPLC analysis (see section 3.2.8).

3.2.8 HPLC-DAD Analyses

A 10 µL aliquot of the prepared samples was injected into a Phenomenex Synergi MaxRP column (4 µm particle size, 250 mm x 2 mm; Phenomenex, Torrance, CA, USA) in an Agilent 1100 Series HPLC system (Cieniak et al., 2013). For the repaglinide assays (including the glucuronidation assays), a gradient elution method with an initial ratio of ACN:0.1% acetic acid

(5:95) and a gradient change to a ratio of ACN:0.1% acetic acid (60:40) at 15 min was used. The column was washed with 100% ACN at 20 min, returned to initial conditions at 22.01 min and re-equilibrated for 3 min. Flow rate was set at 0.4 mL/min, column temperature at 55°C, and the DAD was set at a wavelength of 245 nm.

3.2.9 LC/MS/MS

In order to identify the metabolites of repaglinide, 5 µL of incubation mixture was injected into an Agilent 1200 series HPLC (Agilent Technologies, Santa Ana, CA, USA) with a AB SCIEX 3200 QTRAP® triple quadrupole/ion trap mass spectrometer System (AB SCIEX, Foster City, CA, USA) (Cieniak et al., 2013). The mass spectrometric detection of target compounds was achieved with ESI operating in positive ionization mode within a scanning mass range of 50-600 amu.

3.3 RESULTS

3.3.1 Phytochemical analysis

As part of this study, alcohol and (or) water extracts of eight *R. rosea* NHPs were investigated with respect to their potential ability to interact with other xenobiotics via inhibition of key CYP enzymes or HLM-mediated metabolism of repaglinide. The majority of these NHPs (NRP # 453A, 453B, 454, 455, 459) were commercially available from Canadian herbal product companies while others (NRP #457, 458) were extracted from freshly collected roots and (or) rhizomes (Table 3.1).

Phytochemical analysis of the alcohol and water preparations for each NHP was performed using UPLC-MS with respect to five marker compounds, including salidroside, tyrosol, rosarin, rosavin and rosin (Fig. 3.1). The concentrations of marker compounds differed between alcohol and water extracts, especially, in the case of salidroside, rosarin and rosavin

(Table 3.2). Salidroside content was significantly different ($p < 0.001$) between the alcohol and water preparations for all of the NHPs tested. Rosarin and rosavin concentrations were significantly different for six of the eight NHPs for the different extract types; NRP# 453A and 455 were not different. Tyrosol levels were significantly higher in the water extracts of NRP# 221, 453B and 459 ($p < 0.001$). Similarly, rosin was present at higher concentrations in the water extracts of NRP# 221 and 454 compared to the corresponding alcohol extracts ($p < 0.001$).

Table 3.1 Description of *R. rosea* products (commercial and wild-harvested) tested in the CYP and HLM assays. Product names are listed as stated on the NHP label.

Product name	Source/Supplier	Formulation type
Rhodiola rosea (Lot: Jan 2004)	Trout Lake Farm, WA, USA	Powder
Rhodiola EXTRACT (Lot: 501759)	Natural Factors, Coquitlam, BC, Canada	Capsule (3.5 % total rosavin)
Rhodiola EXTRACT (Lot: 628717)	Natural Factors, Coquitlam, BC, Canada	Capsule (3.5 % total rosavin)
Rhodiola Stress Caps (Lot: 0C200238N)	Sisu, Burnaby, BC, Canada	Capsule (3 % total rosavins)
Rhodiola Rosea (Lot: 590822)	Nature's Way, Markham, ON, Canada	Capsule (3 % rosavins, 1 % salidroside)
Rhodiola rosea root extract	Nunavik, QC, Canada	90 % ethanol extract
Rhodiola rosea root extract	Siberia, Russia	90 % ethanol extract
Rhodiola rosea root extract (Lot: PE20 - 040909)	Canadian Phytopharmaceuticals Corp., Richmond, BC, Canada	75 % USP grade grain alcohol extract

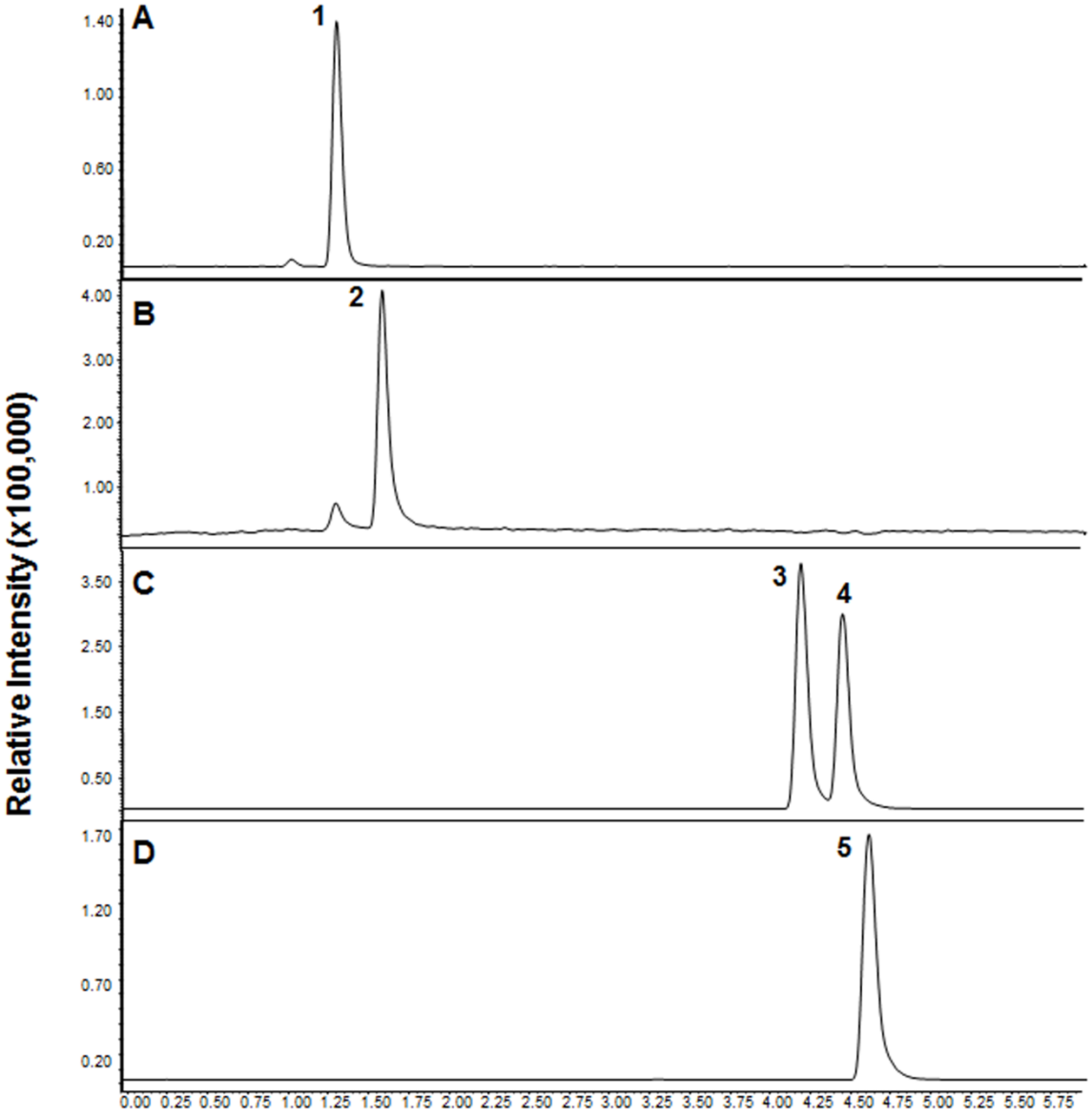


Figure 3.1 UPLC-MS chromatograms of *R. rosea* marker compound standard mix. (A) Salidroside (1); (B) Tyrosol (2); (C) Rosarin (3), Rosavin (4); and (D) Rosin (5).

Table 3.2 Quantification of phytochemical marker compounds in alcohol and water preparations of *R. rosea* NHPs using UPLC-PDA-MS. The concentration of each compound in each individual extract is presented as mg/g extract. Means of three injections ± SEM are presented.

Marker		Salidroside	Tyrosol	Rosarin	Rosavin	Rosin
NRP #	**Alcohol**					
221		8.30 ± 0.24[a]	0.40 ± 0.04[a]	2.33 ± 0.05[a]	7.45 ± 0.13[a]	0.93 ± 0.02[a]
453A		6.69 ± 0.05[a]	0.40 ± 0.02	2.49 ± 0.04	8.42 ± 0.12	1.23 ± 0.04
453B		23.16 ± 0.37[a]	2.32 ± 0.08[a]	2.75 ± 0.02[a]	11.03 ± 0.10[a]	0.98 ± 0.02
454		6.80 ± 0.04[a]	0.32 ± 0.02	2.80 ± 0.01[a]	8.86 ± 0.06[a]	0.97 ± 0.01[a]
455		17.43 ± 0.16[a]	0.41 ± 0.004	2.11 ± 0.06	9.66 ± 0.31	1.01 ± 0.02
457		7.04 ± 0.11[a]	0.73 ± 0.08	2.99 ± 0.01[a]	9.96 ± 0.01[a]	0.89 ± 0.05
458		23.94 ± 0.09[a]	3.14 ± 0.25	13.34 ± 0.04[a]	45.27 ± 0.13[a]	4.65 ± 0.19
459		20.52 ± 0.21[a]	1.35 ± 0.07[a]	3.38 ± 0.02[a]	15.82 ± 0.11[a]	1.82 ± 0.11
NRP #	**Water**					
221		10.00 ± 0.08	0.77 ± 0.02	4.00 ± 0.05	11.46 ± 0.14	1.78 ± 0.08
453A		3.83 ± 0.04	0.52 ± 0.03	2.47 ± 0.03	8.25 ± 0.06	1.21 ± 0.03
453B		12.12 ± 0.25	3.30 ± 0.08	3.11 ± 0.01	12.58 ± 0.03	1.14 ± 0.05
454		8.39 ± 0.11	0.30 ± 0.05	4.32 ± 0.02	16.22 ± 0.10	1.42 ± 0.06
455		8.84 ± 0.004	0.65 ± 0.06	2.02 ± 0.006	9.43 ± 0.03	0.84 ± 0.01
457		6.20 ± 0.07	0.58 ± 0.06	2.47 ± 0.04	8.15 ± 0.09	0.69 ± 0.03
458		21.38 ± 0.13	3.05 ± 0.21	11.89 ± 0.04	41.17 ± 0.38	4.32 ± 0.19
459		30.55 ± 0.32	2.02 ± 0.05	4.63 ± 0.005	21.34 ± 0.03	2.53 ± 0.12

[a] significant difference between alcohol and water extracts using multiple t-tests with false discovery rate (Q) set to 1.0 % ($p < 0.001$)

3.3.2 Inhibition of CYP-mediated metabolism

The alcohol and water extracts of *R. rosea* NHPs were assessed for their effects on individual CYP-mediated metabolism of marker substrates in the microtitre fluorometric assay (Fig. 3.2). The inhibition of CYP3A4 by these extracts at 10 µg/mL was low, ranging from -7.34 % to 21.66 % in contrast to *Rhododendron groenlandicum* (Oeder) Kron & Judd (Ericaceae) (Labrador tea) (AD-01) extract, which inhibited enzyme activity by 76.93 % (Fig. 3.2A). The positive control used, ketoconazole (1.9 µM), completely inhibited enzyme activity. There were no significant differences between alcohol and water extracts in terms of their CYP3A4 inhibitory activity.

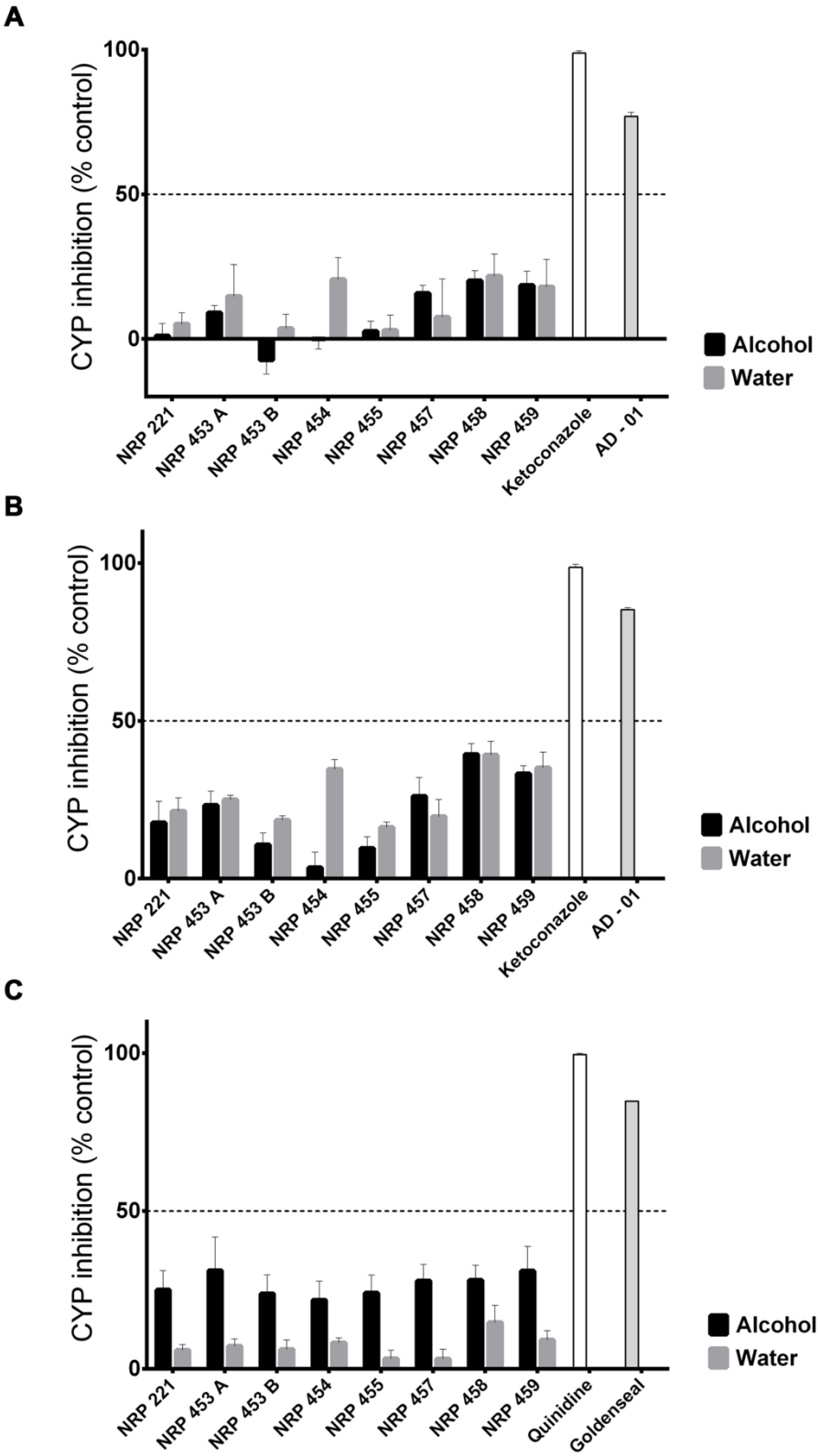
CYP3A5 inhibitory activity among the *R. rosea* NHPs was similarly low, ranging from 3.46 % to 39.39 % when compared to *R. groenlandicum* extract (85.22 %) (Fig. 3.2B). Again, there were no statistically significant differences between alcohol and water extracts in terms of their CYP3A5 inhibitory activity.

The inhibition of CYP2D6 by *R. rosea* NHPs ranged from 3.13 % to 31.13 % (Fig. 3.2C). The alcohol extracts exhibited a trend towards higher inhibitory activity compared to the water extracts; however, this trend was not statistically significant. As a comparison, goldenseal extract inhibited enzyme activity by 84.81 % while the positive control, quinidine (2 µM), completely inhibited enzyme activity.

The five marker phytochemicals, salidroside, tyrosol, rosarin, rosavin and rosin (5 µg/mL) were also tested for their inhibitory potential against CYP3A4, 3A5 and 2D6 (Fig. 3.3). In comparison to the positive control, dillapiol, which showed inhibitory activity at 73.07 %, inhibition among the *R. rosea* markers was low, ranging from 15.51 % to 17.97 %. Similarly, inhibition of CYP3A5 ranged from -14.24 to 6.47 % while that of CYP2D6 ranged from 20.01 %

to 27.75 %. The positive controls for CYP3A5 (ketoconazole) and CYP2D6 (quinidine) completed inhibited the respective enzyme activity.

Figure 3.2 Inhibition of (A) CYP3A4, (B) CYP3A5 and (C) CYP2D6-mediated metabolism of marker substrates by *R. rosea* alcohol and water extracts (10 μg/mL) in a 20 (CYP3A4/5) or 40 minute (CYP2D6) microtitre fluorometric assay. Results are expressed relative to respective vehicle controls as mean CYP inhibition ± SEM (n = 4-5). Positive controls: Ketoconazole (1.9 μM), Quinidine (2 μM), AD-01 (Labrador tea methanol extract) (10 μg/mL), and Goldenseal methanol extract (10 μg/mL).


A
CYP inhibition (% control)
100
50
0
NRP 221
NRP 453 A
NRP 453 B
NRP 454
NRP 455
NRP 457
NRP 458
NRP 459
Ketoconazole
AD - 01
Alcohol
Water
B
CYP inhibition (% control)
100
50
0
NRP 221
NRP 453 A
NRP 453 B
NRP 454
NRP 455
NRP 457
NRP 458
NRP 459
Ketoconazole
AD - 01
Alcohol
Water
C
CYP inhibition (% control)
100
50
0
NRP 221
NRP 453 A
NRP 453 B
NRP 454
NRP 455
NRP 457
NRP 458
NRP 459
Quinidine
Goldenseal
Alcohol
Water

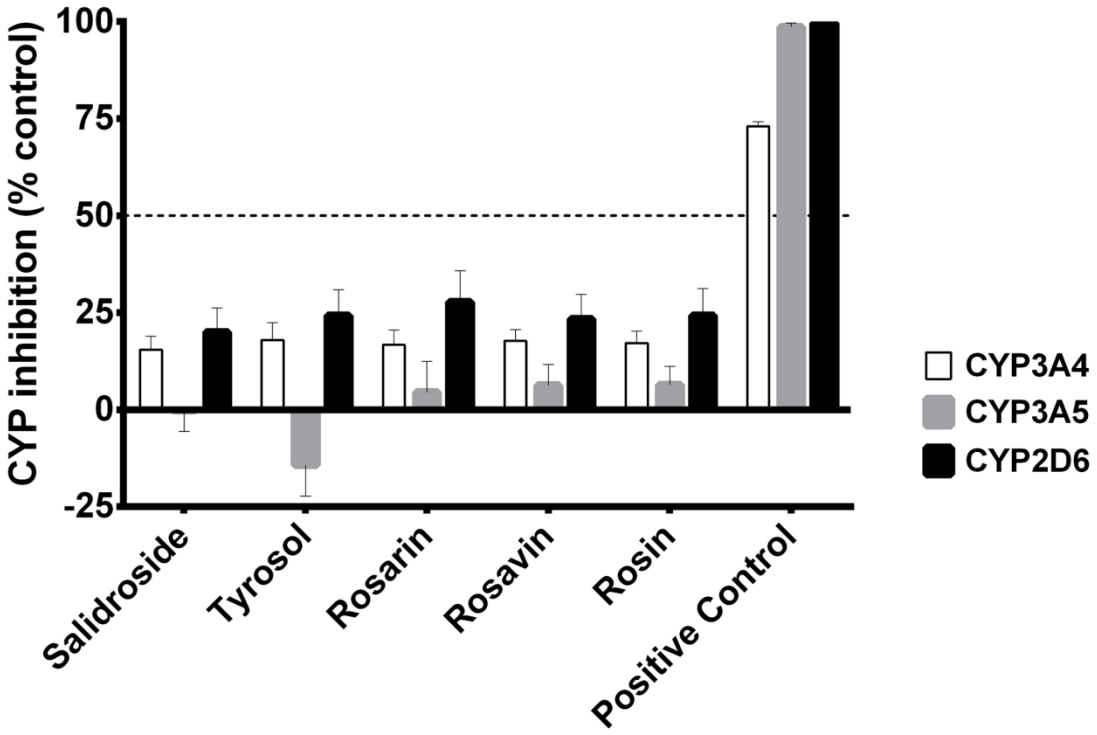


Figure 3.3 Inhibition of CYP3A4, CYP3A5 and CYP2D6-mediated metabolism of marker substrates by *R. rosea* marker phytochemicals (5 µg/mL) in a 20 (CYP3A4/5) or 40 minute (CYP2D6) microtitre fluorometric assay. Results are expressed relative to vehicle control as mean CYP inhibition ± SEM (n = 4-5). Positive controls: CYP3A4: Dillapiol (5 µg/mL); CYP3A5: Ketoconazole (1.9 µM); CYP2D6: Quinidine (2 µM).

Since there were no significant differences between alcohol and water extracts in terms of their CYP inhibitory activity, further experiments were conducted using only alcohol extracts. In order to validate the results from the microtitre fluorometric assay, the extracts were examined for their ability to inhibit CYP3A4-mediated metabolism of testosterone using the HPLC assay (Fig. 3.4). The inhibitory activity of the extracts ranged from -7.41 % to 19.07 %, within a similar range as observed using the fluorometric assay.

3.3.3. HLM-mediated metabolism of repaglinide

R. rosea NHPs co-incubated with the anti-diabetic drug repaglinide (20 µg/mL) in the presence of pooled human liver microsomes exhibited low inhibitory activity on the formation of repaglinide metabolites, except for NRP# 457 which showed moderate activity (59.04 %) (Fig. 3.5). Interestingly, inhibition of glucuronidation of repaglinide by *R. rosea* NHPs encompassed a broad spectrum of variation, ranging from -132.02 % to 86.26 % (Fig. 3.6). NRP# 454-459 were potent inhibitors of glucuronidation with activities ranging from 66.37 % to 86.26 %.

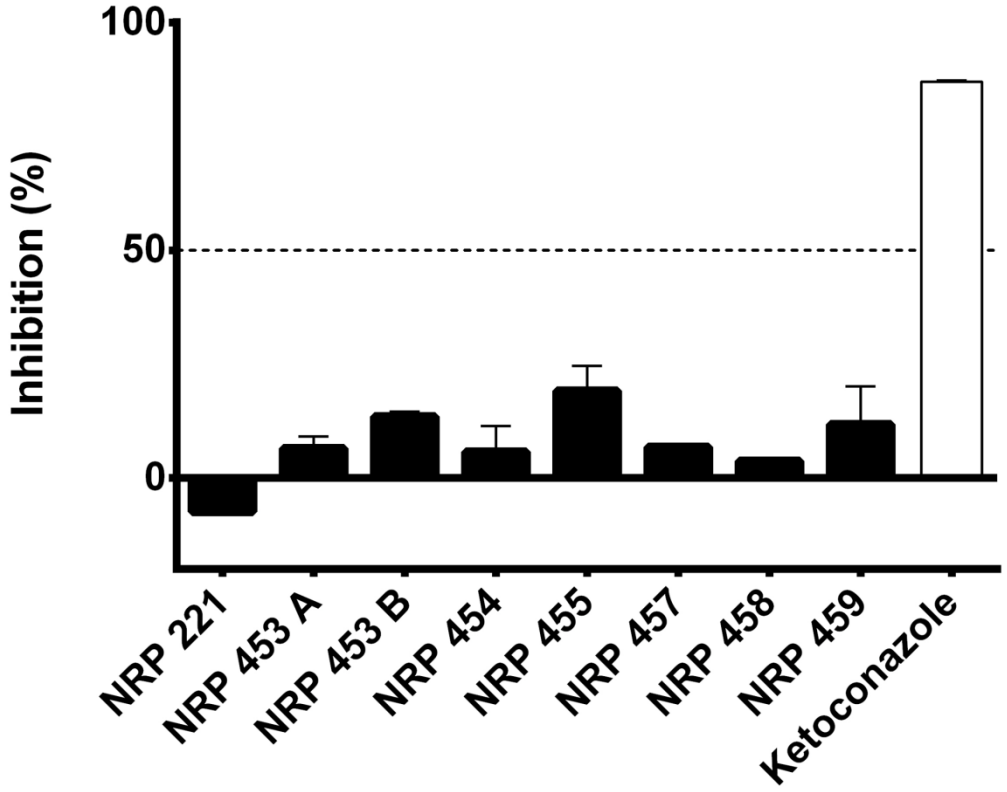


Figure 3.4 Inhibition of CYP3A4-mediated metabolism of testosterone by *R. rosea* alcohol extracts (250 μg/mL) measured using a 30-minute HPLC assay. Results are expressed as mean ± SEM (n = 3). Positive control: Ketoconazole (10 μM).

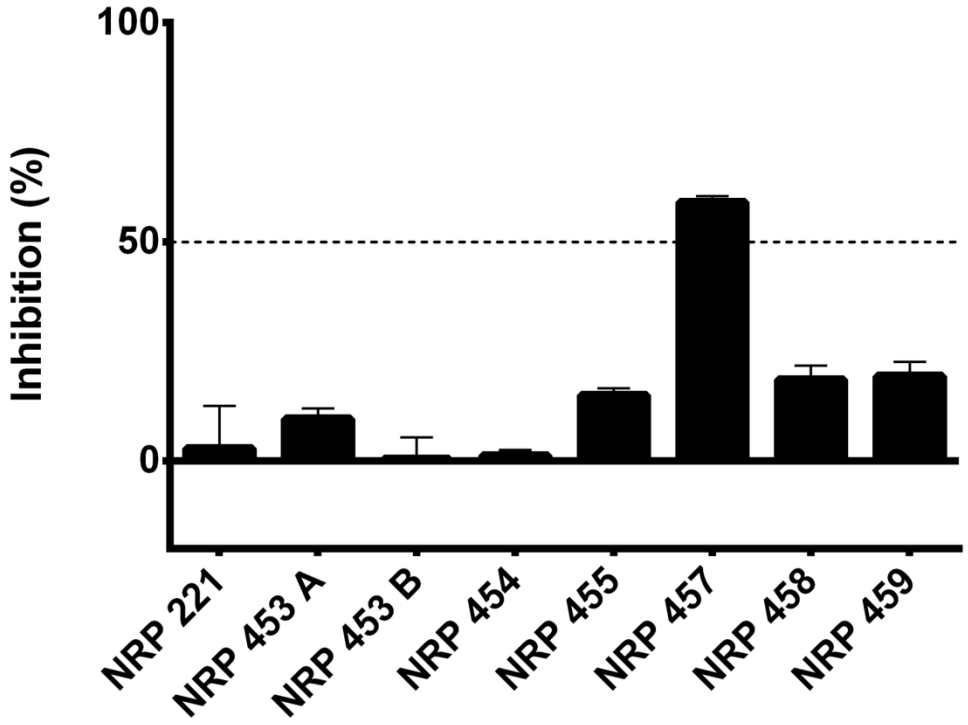


Figure 3.5 Inhibition of repaglinide (20 µg/mL) metabolism following co-incubation with *R. rosea* alcohol extracts (100 µg/mL) by human liver microsomes. Results are expressed as mean ± SEM.

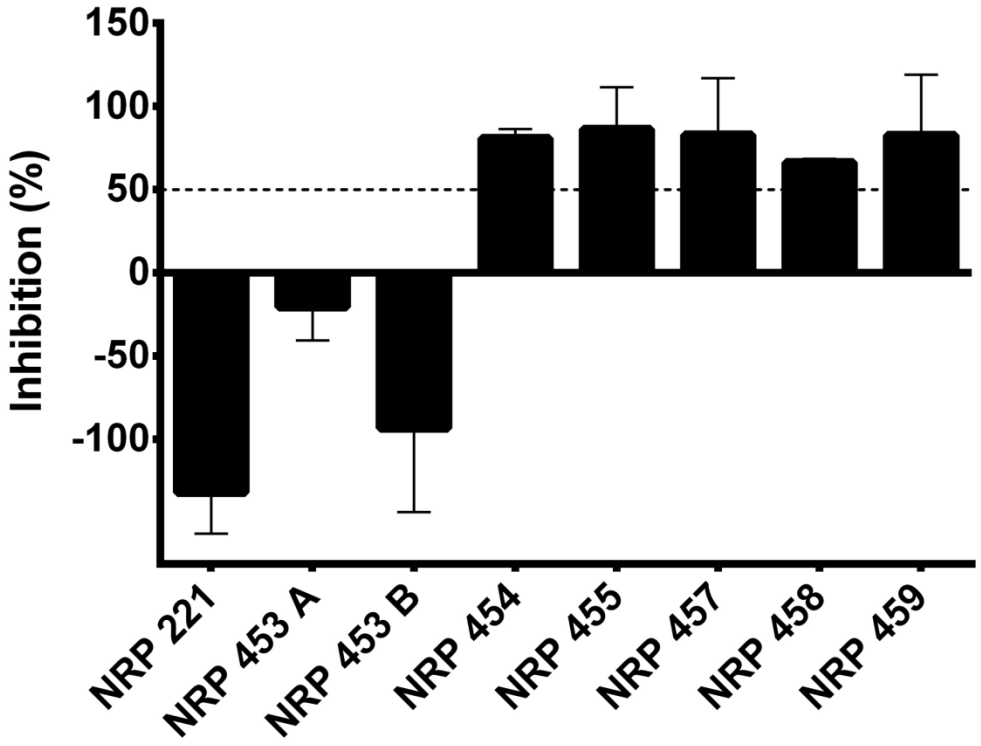


Figure 3.6 Inhibition of UDP-glucuronosyltransferase-mediated metabolism of repaglinide (10 µg/mL) by *R. rosea* alcohol extracts at a concentration of 100 µg/mL. Results are expressed as mean ± SEM.

3.4 DISCUSSION

R. rosea NHPs are used widely to prevent and (or) mitigate a broad range of general health issues. Thus, chances of concomitant use with prescription drugs for diverse health conditions are high, necessitating safety evaluation of these products. As part of this study, alcohol and aqueous preparations of selected commercial and wild-grown *R. rosea* NHPs as well as key marker phytochemicals were assessed for their effects on CYP-mediated metabolism to ascertain the risk of NHP-drug interactions. As a first step, all the products tested in the present study, including the commercial supplements were analyzed by UPLC-MS and found to contain rosavin, rosarin and rosin, the diagnostic phenylpropanoid markers for *R. rosea* species, indicating that the correct herbal entity advertised on the label was present. Not unexpectedly, since there is no consensus on standardization, supplements from different manufacturers varied in the quantities of the marker compounds they contained. A host of intrinsic (raw plant material, organ specificity) and extrinsic (environment and cultivation methods, harvest time, drying and extraction protocols, manufacturing and compounding processes, storage, expiry dates) factors likely contributed to the phytochemical variation between these products (Galambosi et al., 2010; Ampong-Nyarko, 2014).

Traditionally, *R. rosea* has been reported to be administered in alcohol (dried roots steeped in vodka) as well as in aqueous (teas) preparations (Galambosi, 2014); thus it was important to assess the inhibitory profiles of both types of formulations. All marker compounds were detected in both, although phytochemical concentrations were different. In a previous study by Cieniak (2014), alcohol and water extracts of a traditional medicinal plant used by the Cree First Nations to treat symptoms of diabetes showed the presence of similar phenolic compounds in both water and ethanol extracts, although the percent recoveries of individual phytochemicals

were different. For the wild-collected samples (NRP# 457 and 458), a higher concentration of these phenolics was observed in the alcohol as compared to the water preparations, similar to previous studies (Lapornik et al., 2005; Adedapo et al., 2011).

Patterns of abundance for the commercial source NHPs were not as consistent, perhaps due to lack of industry-wide standardization. Hence, differences in the initial raw material used, different standardization procedures between companies, presence of additives, etc. contribute to variation. The present study was limited to testing water preparations of *R. rosea* extracts in capsules, the solvents for which were unspecified on the product labels. True water extractions from raw plant root material may well result in different concentrations of markers as well as overall phytochemical profiles. However, it should be noted that the presence of these five specific compounds, although widely reported in the literature for their biological effects (Cybulska et al., 2011; Qu et al., 2012; Shi et al., 2012), may not necessarily be the primary contributing factors to pharmacological activity and (or) CYP modulation; indeed, despite varying levels of these markers in alcohol and water preparations of individual *R. rosea* NHPs, there were no differences observed in CYP inhibitory activity.

Inhibitory activity was assessed against individual cDNA-expressed human metabolic enzymes associated with major drug metabolism pathways. CYP3A4, the main drug-metabolizing enzyme in humans, is highly expressed in the liver, and to a lesser degree in extra-hepatic tissues and organs such as the small intestine (Danielson, 2002). CYP3A4 is involved in the biotransformation of multiple classes of pharmaceuticals, including anti-cancer drugs, antibiotics, antidepressants, benzodiazepines, angiotensin converting-enzyme inhibitors, calcium channel blockers, statins and several anti-hyperglycemic agents (Zanger and Schwab, 2013). CYP3A5, a closely related isozyme, exhibits lower expression than CYP3A4 in the liver and

intestine, and is mainly associated with extra-hepatic tissues (Danielson, 2002). There is considerable substrate overlap between CYP3A4 and CYP3A5, although there are some notable distinctions (Zanger and Schwab, 2013). *R. rosea* NHPs and phytochemicals generally exhibited weak inhibitory potential against both enzyme isoforms. In comparison, another sub-Arctic plant rich in phenolic compounds, *R. groenlandicum*, a traditional medicinal plant used by the Cree to treat symptoms of diabetes, was a potent CYP3A4/5 inhibitor (Tam et al., 2009). A previous study by Scott et al. (2006) showed much higher inhibitory activity (67 %) of *R. rosea* extract against CYP3A4 in a similar fluorometric assay. The root samples were extracted using 55 % ethanol, which may have a different extraction profile of substances, including a higher concentration of tannins (Downey and Hanlin, 2010), resulting in higher inhibition. It is important to note that in comparison to the extracts from wild-grown rhizomes, the supplements contained non-medicinal ingredients in addition to *R. rosea* extracts, and it is unclear as to the exact proportion of the capsule that is actually *R. rosea* extract. In this instance, these non-medicinals may not be active but yet may not be inert relative to CYP inhibition, which could confound findings and possibly underestimate the inhibitory capacity of *R. rosea*. Hellum et al. (2010) showed potent inhibition of both CYP3A4 activity as well as P-gP transport by Norwegian *R. rosea* clones. Different starting genetic material, environmental factors as well as methodological variations could be contributing to these differences. Interestingly, similar to our findings, these authors also showed that the same set of well-known marker compounds (salidroside, tyrosol and the rosavins), did not predict differences in inhibitory activity − likely other compounds in *R. rosea* were responsible. Other so-called "adaptogenic" plants, including *Panax quinquefolius* L. (Araliaceae) and *E. senticosus* were not inhibitory against CYP3A4 in

similar assay conditions (Budzinski et al., 2000). To our knowledge, this is the first time *R. rosea* products and (or) compounds have been tested against the CYP3A5 isoform.

CYP2D6 expression is not as abundant as CYP3A4 in the liver, but it is responsible for the metabolism of a relatively high number of clinically relevant drugs, including acetylcholinesterase inhibitors, tricyclic anti-depressants, anti-psychotics, β-adrenergic blocking agents, analgesics etc. (Danielson, 2002). Unlike *H. canadensis*, which was previously demonstrated to be a strong CYP2D6 inhibitor (Foster et al., 2003), *R. rosea* NHPs and compounds exhibited low inhibitory capacity. A previous study by Xu et al. (2013) identified rhodiosin and rhodionin, two flavonoids, as potent inhibitors of CYP2D6-mediated metabolism of dextromethorphan in human liver microsomes. Salidroside was not found to be inhibitory, as was observed in the present study. To our knowledge, this is the first time that tyrosol, rosarin, rosavin and rosin were tested against CYP2D6. As *R. rosea* is most likely to be used by patients also taking antidepressants, anti-anxiety drugs, the low inhibition of CYP2D6 may mean a low risk of interactions. Under similar assay conditions, another adaptogen, *E. senticosus* was found to be moderately inhibitory against CYP2D6 (Foster et al., 2003). Future studies assessing the possibility of interactions of *R. rosea* NHPs with commonly used anti-AD drugs in human liver microsomes may be valuable.

R. rosea NHP alcohol preparations were further tested for their ability to affect the metabolism of repaglinide, a commonly used blood glucose-lowering drug, in human liver microsomes, a more biologically complex *in vitro* model of first-pass drug metabolism. Repaglinide was chosen as a representational drug since among the Inuit in Canada who use *R. rosea* as part of their diet/medicine, the prevalence of diabetes is increasing rapidly (Egeland et al., 2011), and consequently, chances of NHP-drug interactions due to co-administration.

Further, the chances of encountering NHP interactions with anti-diabetic drugs are high in Canada according to a national population health survey (Singh and Levine, 2007). Repaglinide is primarily metabolized by the CYP2C8 and CYP3A4 metabolic pathways (Kajosaari et al., 2005). Nunavik *R. rosea* extract (NRP # 457) was the only NHP that demonstrated the potential to moderately inhibit the formation of the metabolite of repaglinide and its glucuronide metabolite, indicating that clinical evaluation of this interaction should be considered. The effects of *R. rosea* NHPs on the formation of the glucuronide metabolite of repaglinide were highly variable, ranging from negative inhibition, this is more metabolite produced than in control conditions, to inhibition. In comparison, Cree anti-diabetic plants are strong inhibitors of repaglinide metabolism, but not of glucuronidation (Cieniak et al., 2013). Varying substrate specificity of different enzyme isoforms in the HLM pool combined with the wide range of physico-chemical properties of crude extracts makes it difficult to identify the underlying mechanism of these non-specific effects. Future studies should include direct testing of *R. rosea* against additional individual metabolic enzymes including UDP-glucuronosyltransferase, as well as other CYP isoforms, including CYP2C8/2C9/2C19 to confirm these effects and gain further mechanistic insight.

In *in vivo* models where multiple drug disposition pathways are in dynamic interplay, results of pharmacokinetic studies on co-administered drugs with *R. rosea* are conflicting. Panossian et al. (2009b) did not report any effects of the Swedish Herbal Institute (SHR-5®) *R. rosea* extract on theophylline and warfarin pharmacokinetics in rats. On the other hand, co-administration of Losartan, an angiotensin II receptor antagonist, and *R. rosea* in rabbits led to a significant increase in plasma levels of the drug metabolite, indicating possible inhibition of CYP2C9 and (or) CYP3A4 isozymes (Spanakis et al., 2013). The inter-species variations in CYP

activities (Martignoni et al., 2006), and differences in research protocols, including sample phytochemical composition may explain these observations.

Taken together, the results of the current study indicate that select *R. rosea* NHPs and compounds may pose a low risk of interactions with conventional drugs; however, non-specific effects on glucuronidation of a co-administered drug in human liver microsomes highlight the need for further testing on individual metabolic enzymes *in vitro*. Evaluation of the *in vitro* CYP inhibitory potential of NHPs enables the prediction of the risk of interactions when taken concurrently with conventional drugs as well as prioritization of products for *in vivo* work and clinical testing. It should be noted that *in vitro* results are often specific to the experimental conditions, and don't necessarily translate to *in vivo* effects on drug pharmacokinetics (Vieira and Huang, 2012). The long-standing traditional uses of *R. rosea* as well as its assessment in several clinical trials in humans have contributed to its general perception as a safe natural product. However, the sheer numbers of available *R. rosea* products with variable botanical source material, standardization protocols, dosages, and formulations make it impossible to test all of them in a clinical setting; thus, preliminary high throughput screening assays as the ones used in this study are recommended. These tests are especially relevant where *R. rosea* is used in combination with other adaptogens; the use of these crude mixtures with multiple phytochemical constituents raises the possibility of unknown NHP-drug interactions.

CHAPTER 4

MWM Visual and MWM Searcher: Automated assessment of learning and memory search strategies in the Morris water maze

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Preface

Despite the widespread use of the Morris water maze (MWM), a behavioural test of learning and memory, and the recent advances in the development of automated tracking software, detailed analyses of search strategies during navigation are not carried out frequently, probably due to the arduous nature of the scoring process, and the possibility of introducing scorer bias. As part of a collaborative endeavor with Dr. Martine Bertrand (Carleton University, Ottawa), and Graeme P. Taylor (Neural Regeneration Laboratory, University of Ottawa, Ottawa), we designed MWM Visual, a feed-forward neural network to allow experimenters to easily visualize and automatically classify mouse swim patterns according to well-established *a priori* categories, thus expanding the scope of MWM analyses beyond conventional measures. The agreement between manual scorers and between manual consensus and MWM Visual scores were quantified by the kappa statistic. This methodology was then applied to experimental tracks generated from vehicle- and *R. rosea*-treated TgCRND8 mice and an analysis of patterns of strategy use was undertaken using the companion Microsoft Excel Macro, MWM Searcher (Chapter 5).

Statement of Author Contributions

FA, MB, CC and SALB conceived and designed the study. FA and CC developed the protocols for discrimination of search strategy categories. MB, under the supervision of SF and GWS, developed the neural network algorithm. GPT developed the excel workbook MWM Searcher for data analysis. FA guided the process of network training by providing agreement scores with manual ratings at every step, assisting with the design of MWM Visual's graphical user interface, and by generating track text files from video recordings provided by CM for validation of MWM Visual for additional pool-platform configurations. Track files for the training cohorts for MWM Visual were provided by FA, MG and MWT. FA, MG, MWT and CC formed the team of trained manual raters. GSVM verified the source code for MWM Visual. FA wrote the manuscript; JTA and SALB provided critical comments.

4.1 INTRODUCTION

The Morris water maze (MWM) task, initially developed by Richard G. Morris, is the most widely used test of hippocampal-dependent spatial learning and reference memory by researchers in behavioural neuroscience (Morris, 1981, 1984; Vorhees and Williams, 2006). The relative simplicity of its design, its cross-species validity, and its ability to assess related types of learning and memory with a few methodological modifications contribute to its widespread popularity in comparison to other learning and memory tasks (Vorhees and Williams, 2006). The MWM test has become an invaluable tool to further the understanding of the effects of anatomical lesions, genetic manipulations, and (or) neuropharmacological interventions on learning and memory in animal models.

The MWM test apparatus consists of a circular pool with a small platform submerged below water level. In the standard version of the test, the position of the platform is kept constant for a number of training (acquisition) days. Animals released into the pool from randomized start locations use distal extra-maze cues to locate the hidden platform to escape from the pool. Procedural differences abound across MWM protocols; variations in the configuration and prominence of extra-maze cues as well as in the ratio of the pool to platform area are important factors that alter the difficulty of the task, and more importantly, affect interpretation of behavioural data (Vorhees and Williams, 2006).

Recent advances in automated tracking software have facilitated the collection of a wealth of positional data over the course of a single MWM trial; however, the development of correspondingly detailed analysis methods has not kept pace. Escape latency, or the time taken to find the platform, and (or) swim path length, remain the most frequently reported measures of learning in the MWM. A progressive decrease in escape latency or path length on acquisition

days is confirmed as evidence of spatial learning. Some studies have gone a step further and integrated positional information into MWM analysis, including cumulative distance to platform or proximity (a measure of the constant distance of the mouse from the platform assessed every five seconds) (Gallagher et al., 1993), or entropy (a measure of how focused the search is in the target area around the platform) (Maei et al., 2009). While highly informative about specific aspects of performance, these single measures are often not sufficient to discriminate between experimental groups, or reveal whether or not observed differences are due to effects on spatial aspects of learning or simply a result of non-cognitive factors, which may play a key role in the learning and memory performance of test subjects (Wolfer et al., 1998; Wolfer and Lipp, 2000).

Analysis of navigational paths over the course of successive acquisition trials can provide deeper insight into the effectiveness of subjects' search strategies in response to treatment or genetic or pharmacological manipulation (Janus, 2004; Brody and Holtzman, 2006; Garthe et al., 2009). The choice of strategy as well as the efficiency of use of a particular strategy may explain the underlying reasons behind patterns observed using conventional measures of learning and memory. An elegant scheme for search strategy analysis in the MWM was developed by Janus (2004), yet this analysis is not routinely conducted due to the labour-intensive process of manually scoring hundreds of trials and the potential for introducing experimenter bias. Attempts have been made to either rapidly visualize (Stover et al., 2012) or automate recognition of *a priori* defined search strategies of rodents in the MWM; but these are not widely accessible to be used by across different pool-platform setups (Graziano et al., 2003; Garthe et al., 2009). As more researchers begin to realize the importance of analyzing search strategy in order to obtain a clearer picture of the entirety of the learning process in animals, development of an easily accessible and intuitive tool to aid in search strategy analysis became a necessity (Stover et al.,

2012; Gil-Mohapel et al., 2013).

To address these needs, the objective here was to develop a fully automated neural network, MWM Visual, that allows researchers to visualize and classify previously defined search strategies from positional data obtained from Ethovision® XT tracking software, and a post-processing data analysis package, MWM Searcher, that enables users to rapidly quantify, plot, and export data tailored to their particular experimental paradigm. In addition, we have used "validation datasets" not used to train the algorithm and compared the double-blinded manual consensus scores to those generated by MWM visual for multiple pool-platform configurations. We have demonstrated that for the current version, there is at least a moderate level of agreement with manual scores for three different pool-platform set-ups, with the potential for greater agreement with the expansion of our internal databases, as well as for adapting the algorithm to more arenas in future versions.

4.2 EXPERIMENTAL

4.2.1 Software requirements for MWM Visual v1.0 and MWM Searcher v1.0

MWM Visual v1.0 is a stand-alone application compatible with Mac OS X 10.9 + and Windows 7. The application can parse files exported from tracking software Ethovision® XT versions 6.0, 7.0 or 8.0 (Noldus Information Technology) saved in ASCII (*.txt*) format. MWM Searcher v1.0 is a data analysis package that operates in Microsoft Excel™ for both Mac and Windows versions with and without macro support. Henceforth, MWM Visual v1.0 and MWM Searcher v1.0 will be referred to as MWM Visual and MWM Searcher, respectively. The suggested workflow for post-processing of MWM data using MWM Visual and Searcher is outlined in Fig. 4.1.

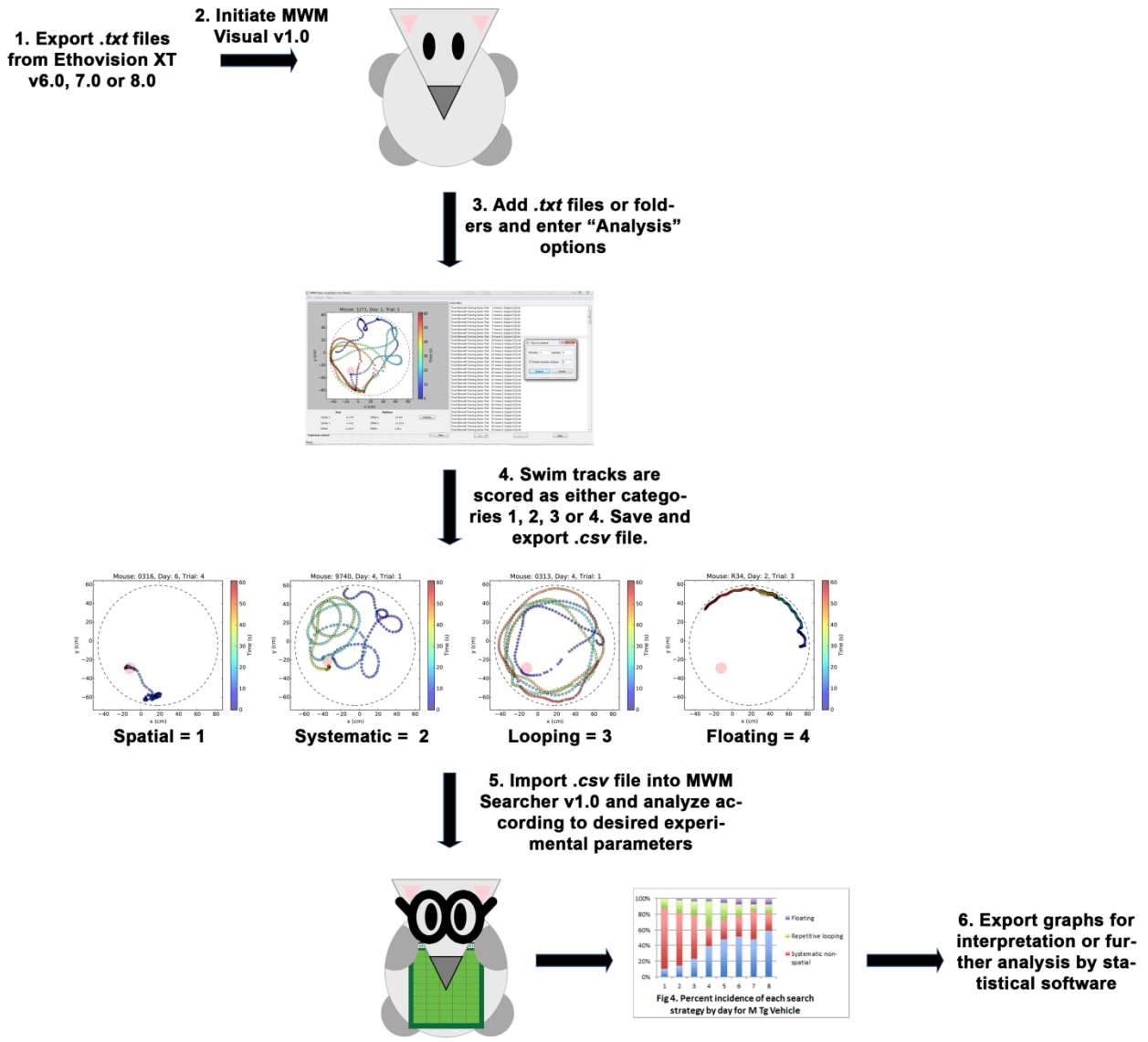


Figure 4.1 Workflow of Morris water maze data processing from tracking software through MWM Visual 1.0 and MWM Searcher 1.0.

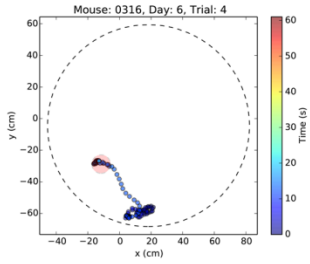
4.2.2 MWM Visual: the neural network

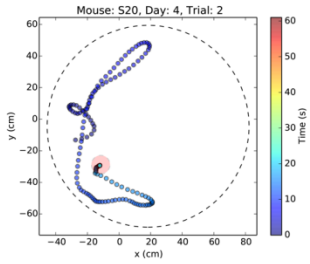
MWM Visual enables the user to visualize individual swim trajectories and automatically classify them into three distinct search strategy categories: (1) spatial, (2) systematic, and (3) looping, adapted according to criteria established by Janus (2004) and Brody and Holtzman (2006). In addition, MWM Visual also recognizes floating, a non-search behaviour that may confound interpretation of MWM results. This enables researchers to pinpoint specific trials where floating occurred and to identify animals that did not exhibit an overall definable navigational strategy for potential exclusion from their other analyses (e.g., escape latency). Representative MWM Visual images of each strategy category as well as floating behaviour in our experimental mice are provided in Fig. 4.2.

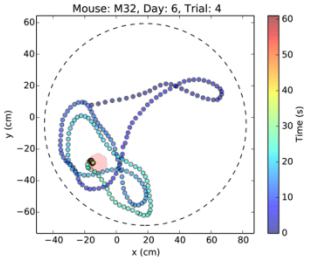
Search strategy classification is performed using a feed-forward neural network implementing fifteen input metrics, which are listed in Appendix A. Briefly, these metrics, weighted based on a "training cohort" of 2399 manually-scored tracks from our internal database, are processed to three output "neurons," one for each of the three categories of search strategies. Floating behavior is identified per trial by two mathematical criteria: velocity < 6 cm/s and escape latency > 50 s per trial. The network was trained with swim trajectory data from MWM trials of wild-type and transgenic TgCRND8 mouse littermates (C57BL/6 X C3H hybrid, backcrossed for 5 generations (N5) to a C57BL/6 lineage). Search strategies in the training dataset were established by two independent investigators, blinded to the identities of the experimental conditions, according to the classification schemes of Brody and Holtzman (2006) and Janus (2004). Additional cohorts not used to train MWM Visual from the same pool set-up as the training sets (Validation cohort #1), or different set-ups (Validation cohorts #2 and 3), were used to challenge the consistency of scoring by the algorithm in comparison to a team of manual scorers.

Figure 4.2 Classification scheme of search strategies and floating behaviour by MWM Visual 1.0. (1) Spatial strategies involve a directed focal search for the platform; they include situations where the subject swims directly to the target, makes at most one complete loop before reaching the target, or concentrates its search in the correct quadrant. (2) Systematic strategies depict a navigational search pattern that encompasses random or scanning behaviour of the entire pool or a focal incorrect strategy wherein a subject explores discrete portions of the pool that do not contain the escape platform before moving to the next quadrant. (3) Looping strategies include behaviours such as circling and chaining with net directional movement but no spatial bias as well as thigmotaxis or "wall hugging." (4) Floating is defined as a non-learning behaviour. Trials in which mice that exhibit prolonged periods of inactivity without forward movement (velocity < 6 cm/s, escape latency > 50 s) are designated as floating trials.

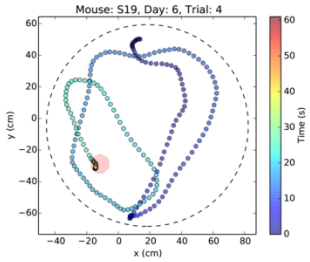
1. Spatial

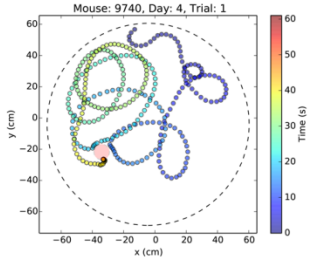


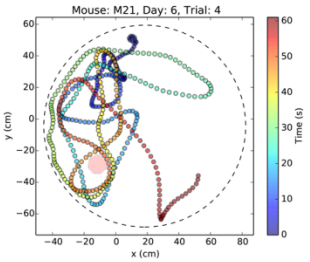




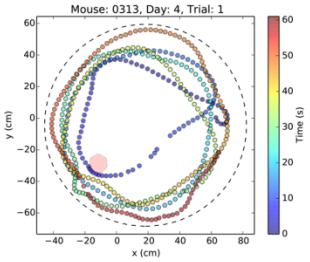
2. Systematic

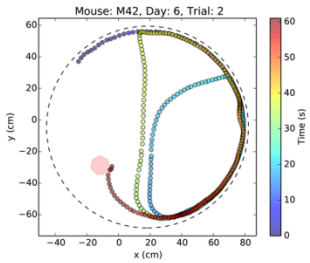


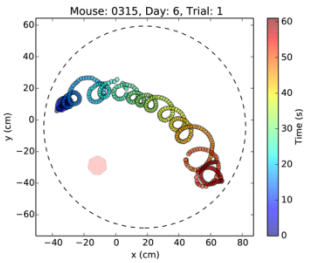




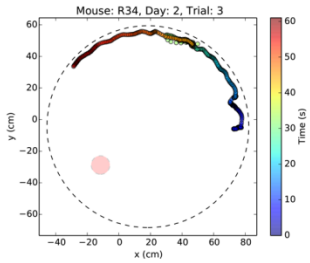
3. Looping

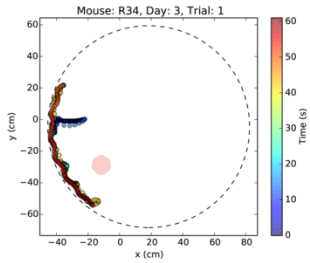


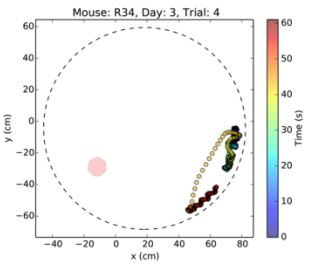




4. Floating







4.2.3 MWM Visual: assignment of strategy scores

In the current version, MWM Visual can be used with MWM apparatus equipped with Ethovision® XT v6.0, 7.0 or 8.0 software. In the graphical user interface (Fig. 4.3), the pool is represented as a two-dimensional circle on an xy-co-ordinate system with a pale pink platform (Fig. 4.3A). After adjusting for camera effects (see section 4.2.5), the dimensions and locations of the pool and platform can be set in the bottom left panel (Fig 4.3B), below the grey track visualization window. Swim trajectories exported from Ethovision® XT in *.txt* format can be uploaded individually or grouped in folders by using the pull-down "+ (Add)" feature and deleted using the "- (Delete)" button (Fig 4.3C). The "Clear" option allows users to reset the loading panel for a new cohort to be analyzed. The "Trajectory control" allows the user to "play" the selected trajectory (Fig 4.3D), with a colour-coded time gradient indicating the temporal movement of the test subject (Fig 4.3E). Once swim trajectories are loaded, the "Analysis" menu (Fig 4.3F) allows the user to enter the number of test days to analyze as well as the threshold for floater exclusion in days. The strategy classifications are saved to a *.csv* file for input into MWM Searcher. The output sheet is organized by animal ID, test day and trial number parsed from the Ethovision® XT *.txt* files. Users have the additional option of saving individual tracks or trajectories from the entire cohort as *.pdf* images for presentation purposes.

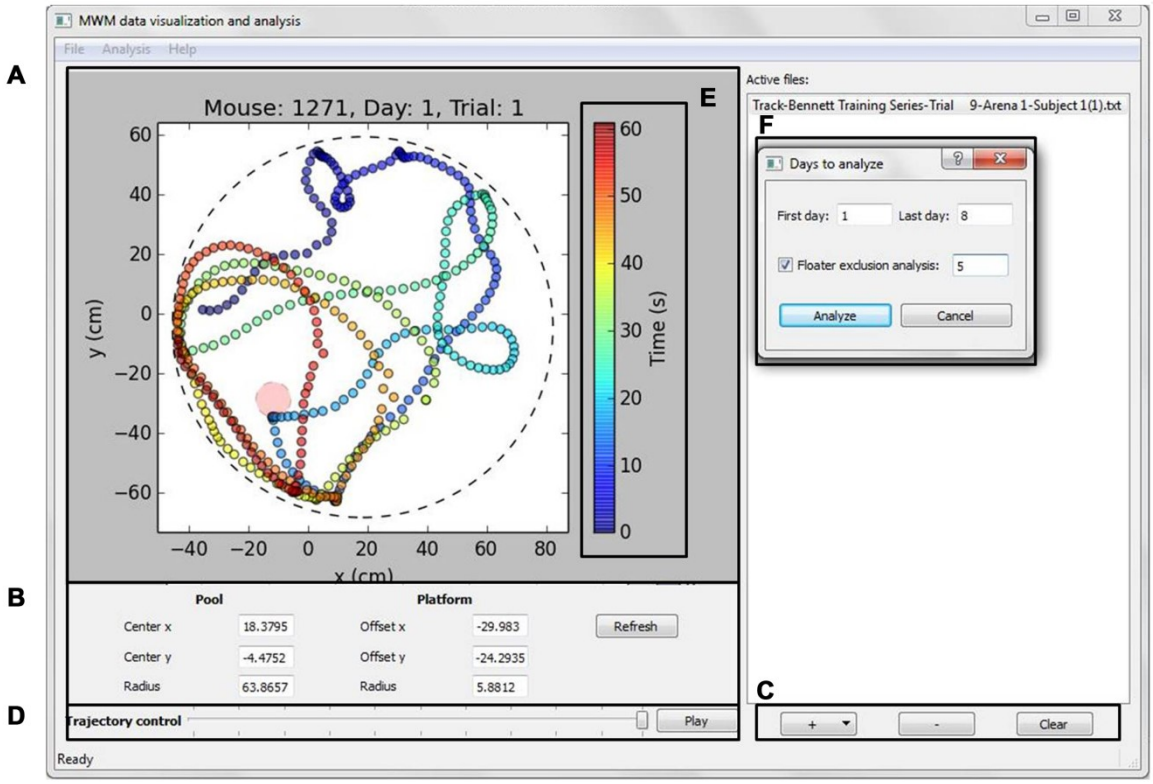


Figure 4.3 MWM Visual 1.0: The graphical user interface. Please see section 4.2.3 for a detailed description.

4.2.4 MWM Searcher: data analysis

MWM Searcher, a Microsoft Excel™ worksheet macro, is used to collate, quantify, and present the raw strategy assignments outputted by MWM Visual in a meaningful and interpretable fashion. MWM Searcher can concurrently process strategy data from 249 subjects. Briefly, raw data (*.csv*) from MWM Visual can either be directly imported into the MWM Searcher worksheets, or copied and pasted into a new tab within MWM Searcher. Users can personalize the data to their specific experimental conditions by entering test subject IDs and condition parameters (e.g. "sex", "genotype", or "treatment"). The user can collate these parameters to form experimental groups (e.g., "wild-type males" vs. "knock-out males"). The "Input" sheet allows users to easily spot any potential mismatches in the expected and actual number of trials for analysis based on the number of acquisition days and the number of trials conducted per test day. MWM Searcher then computes and displays the average, absolute, and percent search strategy incidences for each experimental group as well as plots graphs depicting average values and standard errors. These data can be easily exported and further analyzed using conventional statistical software prior to interpretation.

4.2.5 MWM Visual: Calibration

As part of this study, three different pool and platform configurations were utilized to test the validity of MWM Visual. Prior to search strategy analysis, correct pool and platform parameters must be inputted. Experimental tracks were recorded using Ethovision® XT by means of a video camera with a wide field-of-view lens to record mouse movements using the "centre-point tracking" feature. In Ethovision, the arena was initially calibrated with the actual physically measured pool diameter (127 cm for Training cohorts and Validation cohort # 1; 119.38 cm for Validation cohorts #2 and 3). However, when imported into MWM Visual which

is based on an xy-co-ordinate system centered at (0, 0), the track files that are generated are distorted by a fish-eye effect of the camera lens, or by the camera position being slightly off-center. In order to correct for this, the pool-platform centers, radii and offsets were calculated based on the calibration image file saved in Ethovision using Image J, a freely available image-processing software (http://imagej.nih.gov/ij/download.html). Pool and platform parameters are provided for the datasets used in this study in Table 4.1.

4.2.6 Statistical analyses

In order to quantify the level of agreement between manual raters or between manual scorers and MWM Visual, kappa and weighted kappa scores were computed using the QuickCalcs feature provided by GraphPad Software, Inc. (La Jolla, CA, USA) (http://www.graphpad.com/quickcalcs/kappa1/). The equations for kappa calculation are based on Fleiss et al. (2003), which takes into account the number of agreements between raters that may arise due to chance.

Table 4.1 Pool and platform calibration parameters for MWM Visual calculated using Image J for multiple datasets. Values presented are means of 10 independent measurements.

Dataset	Number of tracks	Pool parameters (cm)			Platform parameters (cm)		
		Center x	**Center y**	**Radius**	**Offset x**	**Offset y**	**Radius**
Training cohort	1856	18.3795	-4.4752	63.8657	-29.983	-24.2935	5.8812
Validation cohort #1	793	18.3795	-4.4752	63.8657	-29.983	-24.2935	5.8812
Validation cohort #2	320	10.3097	-0.6755	60.1196	32.3839	0.7015	5.0906
Validation cohort #3	300	10.2746	0.6485	60.4816	-34.4833	0.4929	5.0598

4.3 RESULTS AND DISCUSSION

The under-utilization of available MWM positional data generated from tracking software, especially in light of the fact that single measures of MWM performance often fall short of capturing the complexity of the learning process, led to the conceptualization and development of MWM Visual. The current version of MWM Visual provides an intuitive, accessible platform to 1) enable time-tagged visualization of the complete swim trajectory during individual MWM trials; and, 2) to perform consistent classification of navigational search strategies based on *a priori* defined categories across several pool-platform configurations. The present version of MWM Visual operates on both Mac (10.9 and higher) as well as PC (Windows 7) operating systems. It is currently capable of analyzing raw swim trajectory files exported as *.txt* files from Ethovision® XT (Noldus Information Technology) versions 6.0, 7.0 and 8.0. MWM Visual employs a classic feed forward neural network to map multiple inputs weighted based on pre-training and computes probabilities for each of the three search strategies per trial; only the predominant strategy is reported for each trial. MWM Visual also incorporates the detection of "floating" phenotypes based on mathematical thresholds (swim velocity < 6 cm/s; escape velocity > 50 s), to further allow researchers the option of flagging non-performers in the MWM test. As part of the MWM data analysis package, we have further included MWM Searcher, a Microsoft Excel™ macro capable of graphing and analyzing the average and daily percent incidences of search strategies reported by MWM Visual.

In order for MWM Visual to be a useful accessory for MWM analysis, it must be able to automatically categorize trajectories with an acceptable degree of consistency and accuracy. To quantify the reliability of MWM Visual scoring, the level of agreement on search strategy classification between teams of independent manual raters (behaviourists trained to score trials

based on established protocols) as well as between the consensus scores achieved by manual raters and corresponding MWM Visual scores was represented by the kappa statistic, κ. Three distinct pool-platform configurations were used in this study (Fig. 4.4). The training cohort and validation cohort 1 data were obtained on Apparatus set-up #1 (Fig. 4.4A), while validation cohorts 2 and 3 data were obtained on Apparatus set-up #2 and 3, respectively (Fig. 4.4B, C). For the current underlying neural net architecture comprising of an input layer with 15 metrics, a hidden layer of 10 neurons and 3 output neurons, inter-rater reliability analysis between MWM Visual and manual consensus scores yielded a kappa value of 0.815 (95% confidence intervals (0.792, 0.838)) for the training dataset, which is slightly lower than the agreement between two manual raters (κ = 0.844; 95 % confidence intervals (0.823, 0.866)) (Table 4.2). Further, the total discrepancy between the manual consensus and MWM Visual rating methods was 11.75 %, and 1.29 % for serious (non-adjacent category) disagreements, respectively (Table 4.1S, Supplementary information, section 4.4).

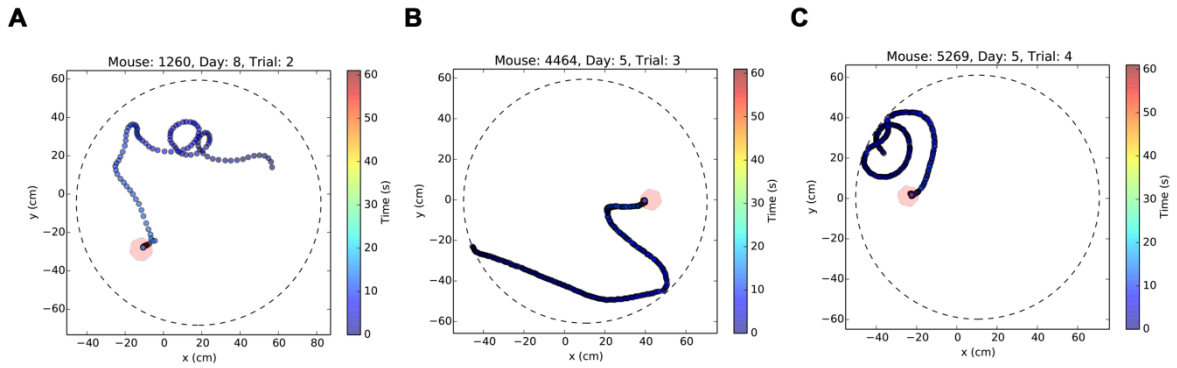


Figure 4.4 MWM Visual representations of the apparatus configurations for (A) training cohort and validation cohort # 1 (Apparatus set-up #1), (B) validation cohort # 2 (Apparatus set-up #2), and, (C) validation cohort # 3 (Apparatus set-up #3) based on calibrated parameters provided in Table 4.1.

Table 4.2 Inter-rater reliability analysis between independent manual raters and MWM Visual and manual consensus. Kappa (κ) coefficients, standard error and 95 % confidence intervals of κ as well as weighted kappa coefficients are presented. Values and qualitative descriptions of strength of agreement were obtained using the kappa calculator provided by GraphPad software (http://www.graphpad.com/quickcalcs/kappa1/). See Methods section 4.2.6 for details.

	Datasets	κ	Standard error of κ	95 % confidence intervals (lower, upper)	Qualitative agreement (κ)	Weighted κ	Qualitative agreement (weighted κ)
Inter-manual							
	Training cohort	0.844	0.011	0.823, 0.866	Very good	0.873	Very good
	Validation cohort #1	0.791	0.020	0.753, 0.830	Good	0.819	Very good
	Validation cohort #2	0.817	0.030	0.758, 0.876	Very good	0.831	Very good
Manual consensus vs MWM Visual							
	Training cohort	0.815	0.012	0.792, 0.838	Very good	0.845	Very good
	Validation cohort #1	0.767	0.021	0.726, 0.802	Good	0.782	Good
	Validation cohort #2	0.753	0.035	0.684, 0.822	Good	0.772	Good

In addition to the training set, we used data from three additional cohorts to gauge the predictive power of MWM Visual for "unseen" datasets. For validation sets # 1 and 2, which have different pool and platform sizes and locations, the magnitude of kappa between MWM Visual and manual consensus agreement were slightly lower compared to that between manual raters, but with overlapping 95 % confidence intervals, similar to the trend observed for the training dataset (Table 4.2). For the kappa statistic, the strength of the agreement between rating methods is often interpreted using a qualitative description assigned based on previously developed benchmark scales (Landis and Koch, 1977; Altman, 1991). Based on these criteria, MWM Visual rating consistently falls into the "good (κ = 0.61-0.80)" to "very good (κ = 0.81-1.00)" range. Weighted kappas (with linear weights) between manual scorers and MWM Visual and manual consensus scores were higher than un-weighted values for the training and validation datasets (Table 4.2, 4.3) since most of the disagreements between rating methods were between adjacent categories (Supplementary Table 1). Validation cohort # 3 offers an interesting case study. For this dataset, there were 4 independent manual raters, and agreements between them were variable, ranging from "moderate" to "very good" (κ = 0.597-0.833), indicating that strategies used by this cohort were particularly challenging to discriminate. Interestingly, the performance of MWM Visual was consistently in the same bracket (κ = 0.718) as with the other cohorts. For each of the datasets, MWM Visual consistently tended to sort more trials in the "spatial" and "systematic" categories and fewer in the "looping" category when compared to manual raters (Table 4.4).

Table 4.3 Inter-rater reliability analysis between independent manual raters and MWM Visual and manual consensus for Validation cohort # 3. Kappa (κ) coefficients, standard error and 95 % confidence intervals of κ as well as weighted kappa coefficients are presented. Values and qualitative descriptions of strength of agreement were obtained using the kappa calculator provided by GraphPad software (http://www.graphpad.com/quickcalcs/kappa1/). See Methods section 4.2.6 for details.

Rater comparisons	κ	Standard error of κ	95 % confidence intervals (lower, upper)	Qualitative agreement (κ)	Weighted κ	Qualitative agreement (weighted κ)
Manual (Rater 1 vs 2)	0.645	0.039	0.569, 0.721	Good	0.688	Good
Manual (Rater 2 vs 3)	0.746	0.036	0.675, 0.816	Good	0.776	Good
Manual (Rater 3 vs 4)	0.749	0.036	0.680, 0.819	Good	0.776	Good
Manual (Rater 1 vs 3)	0.597	0.041	0.516, 0.677	Moderate	0.651	Good
Manual (Rater 1 vs 4)	0.706	0.037	0.634, 0.778	Good	0.743	Good
Manual (Rater 2 vs 4)	0.833	0.031	0.773, 0.894	Very Good	0.851	Very Good
Manual consensus vs MWM Visual	0.718	0.038	0.643, 0.793	Good	0.733	Good

Table 4.4 Percent incidence of strategy and floating behaviour as obtained by Manual and MWM Visual rating methods and the difference (Manual consensus – MWM Visual) within each category for the training and the validation datasets.

Data set	Rating method	Spatial	Systematic	Looping	Floating
Training cohort	Manual consensus	43.97	35.78	17.56	2.69
	MWM Visual	47.90	37.34	12.12	2.64
	Difference	-3.93	-1.56	5.44	0.05
Validation cohort #1	Manual consensus	53.09	36.57	9.96	0.38
	MWM Visual	54.10	39.34	6.18	0.38
	Difference	-1.01	-2.77	3.78	0.00
Validation cohort # 2	Manual consensus	18.13	60.63	21.25	0.00
	MWM Visual	20.63	63.44	15.94	0.00
	Difference	-2.50	-2.81	5.31	0.00
Validation cohort # 3	Manual consensus	11.45	58.25	30.30	0.00
	MWM Visual	14.14	62.63	23.23	0.00
	Difference	-2.69	-4.38	7.07	0.00

Wolfer et al. (2001) initiated the process of automating search strategy recognition; Wintrack, a Windows application, enabled the detection of thigmotaxis and serial search strategy. Graziano et al. (2003) used a different approach to automate recognition of search strategies. They used a parameter-based discriminant function to distinguish between seven search strategy categories. The authors reported high levels of accuracy (97 %) and predictive power (96 %) using this method, although the model is limited to data acquired under the exact same experimental conditions. A MATLAB®-based algorithm for sequential categorization of search strategies provides an interesting alternative solution, although complex strategies were defined by only one or two parameters, and unrecognized strategies had to be scored manually (Garthe et al., 2009; Gil-Mohapel et al., 2013). Recent work by Moreau et al. (2014) used a fuzzy logic sorting system to classify trials into search strategy categories and observed good separation of broad "spatial" or "non-spatial" strategy classes, but was less effective with the distinction of sub-classes.

In this study, we resorted to the multi-layer perceptron, one of the most widely used models of artificial neural networks, to classify search strategies in MWM Visual. The use of neural networks in the field of bioinformatics and medicine is expanding rapidly, with predominant uses in gene identification and prediction, gene interaction, protein secondary structure prediction, microarray analyses etc. (Lancashire et al., 2009; Manning et al., 2014). The flexibility and ease of implementation of neural nets makes it highly advantageous for large datasets with complex, non-linear relationships between variables. The primary strength of neural networks is their ability to "learn" patterns from a given set of example data and to predict results for new, "unseen" datasets. With MWM Visual, we attempted to harness the versatility of neural networks to address the difficulty of automating search strategy recognition across

several MWM apparatus configurations with acceptable levels of consistency. MWM Visual and Searcher were used to analyze experimental MWM data examining the effects of a traditional medicinal plant, *Rhodiola rosea* L., on learning and memory performance by assessing impacts on search strategy use in wild-type and transgenic TgCRND8 mice (see Chapter 5, section 5.3.2).

There are certain limitations to MWM Visual that need to be mentioned. Overall, the agreement between MWM Visual and manual consensus scores was lower than between two manual raters, although MWM Visual was consistent across datasets in contrast to manual raters. While the datasets used in this study for training and validation were different in terms of pool/platform size and (or) location, their calibrated dimensions were still comparable (pool radius, 63.8657 vs 60.1196 cm; platform radius, 5.8812 vs 5.0906 cm for validation cohorts 1 and 2, respectively). Users with very different apparatus, for instance, water mazes for rats which are generally 210-214 cm in diameter (Morris, 1984; Vorhees and Williams, 2006), may have to re-train the network with exemplars from their own dataset. It should be noted that neural networks generally perform better with larger training datasets (Crowther and Cox, 2006); this could be a limitation in situations where sample sizes are small. It is also important to keep in mind that the definitions of search strategy used in MWM Visual are those observed in our mouse strains − different strategies may be observed in other backgrounds, and thus, limit the applicability of MWM Visual. Further, MWM Visual reports the pre-dominant strategy used during a trial, when often, subjects switch from one to another during the course of a trial. In order to counter some of these caveats, we aim to release the training scripts for MWM Visual in version 2.0 to enable researchers to adapt MWM Visual to their particular MWM apparatus for more accurate results.

Despite these limitations, MWM Visual remains a valuable tool for search strategy visualization and classification. We have shown that scoring by MWM Visual is consistent across at least three different pool-platform set-ups, with data acquired using two different cameras. MWM Visual is widely accessible to researchers and available for download at <https://www.med.uottawa.ca/lipidomics/resources.html>, accompanied by a detailed user manual. Future versions will include training scripts for researchers to adapt MWM Visual to their own apparatus. Together with MWM Searcher, MWM Visual is an important contribution towards extending the power of MWM analysis beyond conventional single measures of performance assessment.

4.4 SUPPLEMENTARY INFORMATION

Table 4.1S Discrepancies in scoring between MWM Visual and manual consensus for the training and validation cohorts.

Dataset	Number of trials	Total number of discrepancies	Total discrepancies (%)	Serious discrepancies	[a]Serious discrepancies (%)
Training cohort	1856	218	11.75	24	1.29
Validation cohort #1	793	104	13.11	10	1.26
Validation cohort #2	320	43	13.44	2	0.63
Validation cohort #3	297	46	15.49	3	1.01

a. Serious discrepancies refer to disagreements between rating methods on classifications of non-adjacent categories; for instance, spatial vs looping, systematic vs floating, etc.

CHAPTER 5

Assessment of the efficacy of Nunavik *Rhodiola rosea* in the TgCRND8 model of Alzheimer Disease

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Preface

Given the traditional use of Nunavik *R. rosea* for mental health as well as the potential of the extract and/or certain bioactive phytochemicals to penetrate the blood-brain barrier as demonstrated by its anxiolytic activity (Chapter 2), here we evaluated the neuroprotective properties of *R. rosea* in the context of AD, a progressive neurodegenerative disorder characterized by memory loss and neuropsychiatric symptoms, including anxiety. As part of this study, we assessed the effects of chronic administration of Nunavik *R. rosea* extract in the TgCRND8 mouse model of AD on multiple behavioural indices of cognition and anxiety, as well as on the high background mortality characteristic of this line. *R. rosea* extract was administered orally to two-month old male mice, both wild-type and transgenic, using diluted condensed milk as a vehicle for eight weeks at a dose of 100 mg/kg body weight. Male mice were then subjected to a series of behavioural tests including the Morris water maze test for assessment of spatial learning and memory, as well as the elevated-plus maze, the light-dark box, the open-field and the social interaction tests for anxiety. The objective of this study was to provide proof-of-principle of an interdisciplinary approach combining ethnobotanical-driven drug discovery and hypothesis-driven behavioural testing to identify new potentially restorative agents for AD neurodegeneration.

Statement of author contributions

FA, JTA and SALB conceived and designed this study. Treatment and behavioural paradigms were performed by FA and CC, with the assistance of summer students MWT, MWG, SA and EG. FA, JTA and SALB contributed to manuscript preparation.

5.1 INTRODUCTION

Alzheimer disease (AD) is the most frequently encountered type of dementia, or major neurocognitive disorder. Nearly 15 % of the Canadian population aged 65 or over suffers from AD or related neurocognitive disorders (Alzheimer Society of Canada, 2010). AD is characterized by initial memory loss, progressive impairment of cognitive function, deterioration of language, and loss of motor and sensory abilities at advanced stages (Cummings, 2004). In addition to cognitive impairments, AD patients frequently exhibit neuropsychiatric symptoms, including anxiety, which contribute to deteriorating quality of life (Teri et al., 1999; Porter et al., 2003). Both the cognitive and non-cognitive symptoms of this neurodegenerative disease need to be addressed for effective management of disease progression.

AD is identified histopathologically by two characteristic lesions; plaques composed of Aβ peptides, and intracellular neurofibrillary tangles composed of hyperphosphorylated microtubule-associated tau protein (Querfurth and LaFerla, 2010). The abnormal generation and accumulation of specific isoforms of Aβ is crucial to AD pathogenesis (Hardy and Selkoe, 2002). Aβ peptides are produced by sequential cleavage of APP by β- and γ-secretase enzymes. Mutations in the APP or presenilin genes in the case of familial AD favour the over-production of amyloidogenic Aβ peptides prone to aggregation (Citron et al., 1992; Scheuner et al., 1996). Elevated levels of these Aβ oligomeric species trigger a cascade of cellular changes leading to dysregulation of calcium homeostasis, oxidative stress, activation of microglia, perturbed neurotransmitter signalling, and ultimately, synaptic dysfunction and neuronal death, contributing to the observed symptoms of dementia (Ferreira and Klein, 2011).

Aβ pathology is correlated with learning and memory deficits in transgenic AD mouse models expressing familial AD-linked mutations in APP and (or) presenilin (PS1). In PDAPP

mice overexpressing human APP with the Indiana (V717F) mutation, learning capacity is inversely correlated with hippocampal Aβ plaque burden (Chen et al., 2000). Similarly, in double transgenic APP/PS1 mice, elevated Aβ load, especially in the cortex and hippocampus, is associated with impairments in learning and memory (Gordon et al., 2001; Sadowski et al., 2004; Savonenko et al., 2005). Many of these transgenic mouse models also exhibit perturbations in anxiety-like behaviour, compatible with AD symptoms of apathy, or hyperactivity (Lalonde et al., 2003; Ognibene et al., 2005). Enhanced fear responses and correlated spatial memory deficits are reported in triple transgenic mice expressing APP (K670N/M671L), tau (P301L), and PS1 (M146V) mutations due to an increase in Aβ accumulation in neurons in the basolateral amygdala (España et al., 2010). Thus, interventions that target Aβ biogenesis, aggregation and (or) deposition may be an effective preclinical strategy to offset the learning and memory impairments and behavioural disturbances characteristic of AD.

Current AD treatment paradigms include a cocktail of cholinesterase inhibitors and NMDA receptor antagonists as well as antidepressants, antipsychotics, and anxiolytics for the management of AD-associated neuropsychiatric symptoms (Yiannopoulou and Papageorgiou, 2013). While these drugs provide modest symptomatic relief, they do not alter disease progression. The limited safety, availability and affordability of these drugs necessitate the search for alternatives (Suh et al., 2009). Ethnobotanically selected plant species, particularly in the context of dementia and mood disorders, are promising sources of bioactive secondary metabolites that act at AD drug targets (Howes et al., 2003; Anekonda and Reddy, 2005). Dietary supplementation with phenolic compounds such as resveratrol, grape seed polyphenols, green tea catechins, etc. have been shown to attenuate Aβ pathology and cognitive deficits in

transgenic AD models (Vingtdeux et al., 2008; Lim et al., 2013; Wang et al., 2014) as well as modulate aberrant hyperactivity patterns (Mori et al., 2012; Mori et al., 2013).

Rhodiola rosea L. (Crassulaceae) is well-established in the traditional pharmacopeia of Europe, Asia and certain regions of North America. Of particular interest are ethnobotanical reports of its uses in enhancing cognitive ability, alleviating depression, and improving mental and physical endurance under stress (Khanum et al., 2005; Panossian et al., 2010a). In Canada, *R. rosea* roots and (or) rhizomes are used by the Inuit of Nunavik and Nunatsiavut (Northern Québec) as both food and medicine for boosting overall mental health (Clark and Cuerrier, 2012), and eliminating fatigue (Cuerrier et al., 2014a). Nunavik *R. rosea* is phytochemically distinct from Eurasian populations (Filion et al., 2008; Avula et al., 2009). Phenolics, including the phenylethanol derivative salidroside and the phenylpropanoids rosarin, rosavin and rosin, are the predominant class of secondary metabolites present in the roots, the medicinal part of the plant.

While there is no direct evidence of *R. rosea* activity in the context of AD, research on Eurasian varieties in preclinical models suggest that *R. rosea* may be neuroprotective via its attenuation of Aβ-mediated toxicity, glutamate excitotoxicity, or inflammatory responses. Salidroside exerts protective effects against Aβ toxicity through the induction of antioxidant enzymes and inhibition of ROS accumulation (Zhang et al., 2010b; Zhang et al., 2013a). *R. rosea* extracts, as well as salidroside, protect against glutamate excitotoxicity, a common feature of AD synapses, by modulation of intracellular Ca^{2+} levels (Cao et al., 2006; Palumbo et al., 2012). *R. rosea* extract, as well as rosarin, rosin and salidroside inhibit the expression of pro-inflammatory cytokines in microglial cells upon lipopolysaccharide stimulation (Lee et al., 2013c). In addition, *R. rosea* extracts inhibit enzymes that regulate inflammatory cascades,

including members of the phospholipase A_2 superfamily (Pooja et al., 2009), which generate lipid second messenger molecules that can further contribute to AD pathogenesis (Ryan et al., 2009). *R. rosea*, as well as its constituent phytochemicals inhibit acetylcholinesterase activity, boosting the levels of acetylcholine which are reduced in AD patients (Hillhouse et al., 2004; Wang et al., 2007a).

Based upon the preliminary evidence of activity of *R. rosea* on key targets relevant to AD, the objective of this study was to directly investigate whether a phytochemically standardized extract of Nunavik *R. rosea* affects cognitive (learning and memory) and (or) non-cognitive (anxiety) aspects of AD in TgCRND8 mice, an aggressive early-onset model of AD. In this mouse model, transgenic mice overexpressing human APP with both Swedish and Indiana familial mutations switch to a symptomatic state with observable behavioural deficits in learning and memory and elevated Aβ plaque load by six months of age (Wang et al., 2013a). Nunavik *R. rosea* was previously shown to have anxiolytic and fear-reducing properties in rats (Chapter 2; (Cayer et al., 2013)). Here, *R. rosea* was evaluated for its effects on basal anxiety levels in TgCRND8 mice as well as on survival proportions of these mice which are prone to high rates of mortality (Chishti et al., 2001).

5.2 EXPERIMENTAL

5.2.1 Plant Materials and Preparation

Fresh samples of *R. rosea* roots and rhizomes were collected along the shores of Ungava Bay, Nunavik, Québec, Canada, in 2009 by Dr. Alain Cuerrier (Jardin botanique de Montréal, Institut de recherche en biologie végétale, Université de Montréal, Montréal, Canada). Voucher specimens were deposited in the University of Ottawa Herbarium (UOH# 19847).

Rhizome/root extracts were prepared as described in Cayer et al. (2013). Rhizomes and roots were washed and dried for 12 hours in a commercial plant dryer (NESCO® Food Dehydrator) at 35ºC and ground using a Wiley Mill (mesh size = 2 mm). Plant material was subsequently extracted with 90% ethanol (10 × weight/volume) and filtered under suction in a Büchner funnel through a Whatmann no. 1 filter paper. The residue was re-extracted with additional 90% ethanol (5 × weight/volume) twice and filtered. The filtrates were combined, solvent was roto-evaporated at 40ºC and the extract lyophilized. The percent yield of the extraction was 7 %. The extract was stored at 4°C protected from light for experimental purposes.

5.2.2 Animals

For long-term (chronic) *R. rosea* administration experiments, TgCRND8 mice were used. TgCRND8 mice (C57BL/6 X C3H/HeJ, F1 generation), expressing the Swedish (KM670/671NL) and Indiana (V717F) mutations in the human APP gene under the control of the hamster prion promoter (Chishti et al., 2001) were donated by Dr. Paul Fraser (University of Toronto). These mice were then backcrossed for five generations (N5) to a C57BL/6 lineage (Charles River Laboratories, Wilmington, MA, USA) in the Bennett laboratory to delay the onset of learning and memory impairment, and consequently widen the pre-symptomatic window to enable testing of prophylactic interventions (Wang et al., 2013a; Granger et al., 2015). Heterozygote transgenic (Tg) and congenic wild-type (NonTg) littermates were maintained in our breeding facility at the University of Ottawa. For short-term (acute) dose-response *R. rosea* administration experiments, C57BL/6NCrl mice were obtained from Charles River Laboratories (Wilmington, MA, USA). All animal manipulations were performed in strict accordance with the ethical guidelines for experimentation established by the Canadian Council for Animal Care and

with the approval of the University of Ottawa Animal Care Committee for the ethical treatment of experimental animals.

5.2.3 *R. rosea* administration

For the chronic treatment study, two-month old male Tg and NonTg littermates were treated with *R. rosea* extract at 100 mg/kg body weight/day emulsified in 25% sweetened condensed milk (President's Choice® brand) orally through a 1 mL syringe. This method of administration was adopted to minimize handling stress to the animal caused by conventional gavage techniques (Cayer et al., 2013). Corresponding NonTg and Tg control animals were included which received only vehicle (25 % sweetened condensed milk). Mice were single-housed under a 12 h light/dark cycle and had *ad libitum* access to food (Teklad Global 18 % Protein Rodent Diet, Harlan Laboratories) and water. Following 60 days of daily treatment, the animals underwent a three-week behavioural paradigm (described below) with continuous *R. rosea* or vehicle treatment.

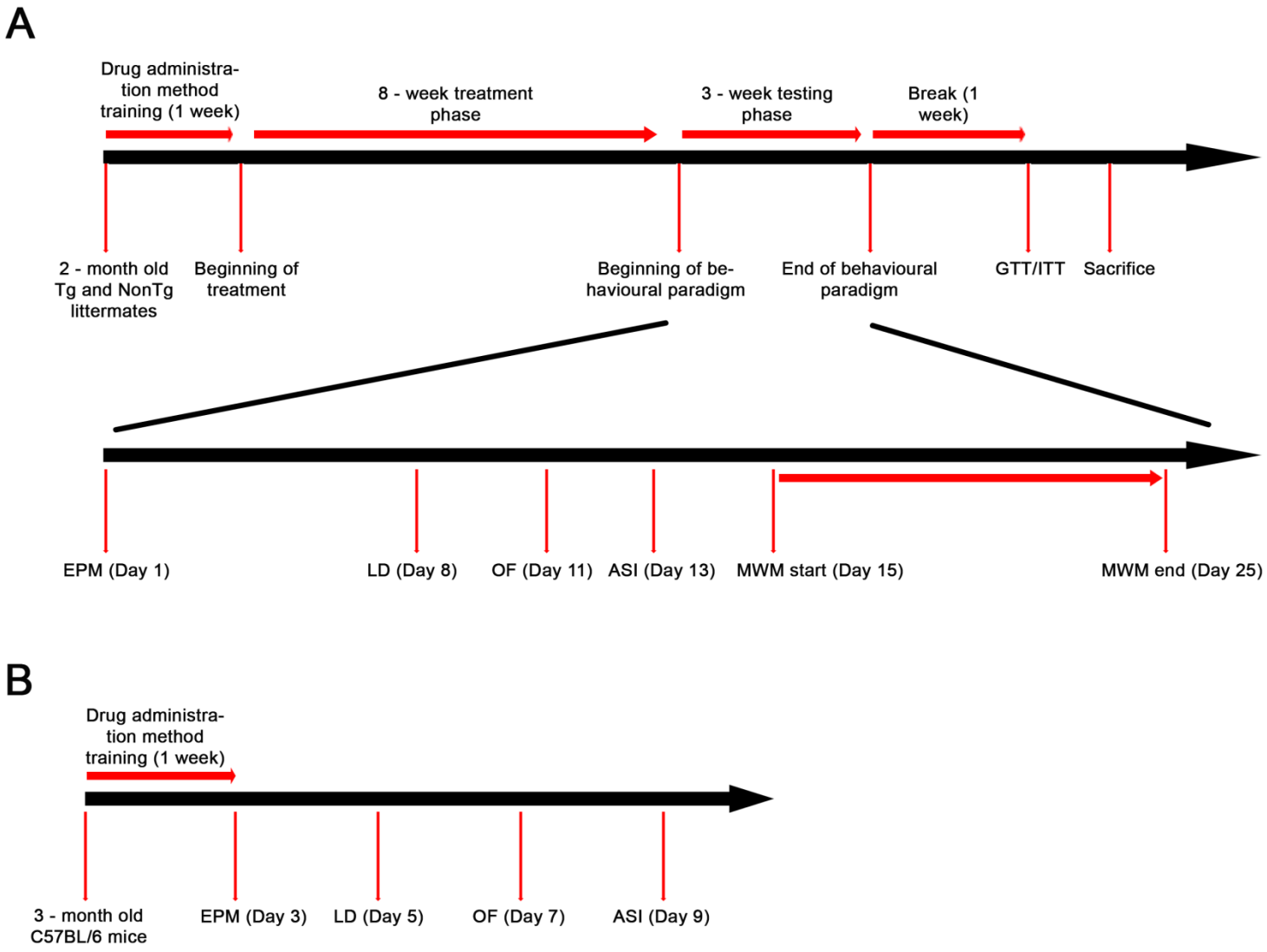
For the acute dose-response study, three-month old male C57BL/6 mice were acclimatized for one week. The mice were then administered *R. rosea* at 30, 100 or 300 mg/kg body weight for a total of 9 days during which time they were subjected to the battery of anxiety assays described below.

5.2.4 Behavioural paradigm

The time-line of behavioural testing for both acute and chronic administration studies is presented in Fig. 5.1. Anxiety indices were measured using the elevated-plus maze (Rodgers and Dalvi, 1997), light-dark exploration (Crawley and Goodwin, 1980), and adult social interaction (File and Seth, 2003; Crawley, 2007) paradigms. Locomotion and anxiety was assessed in the open-field test (Prut and Belzung, 2003). Learning and memory was assessed in the MWM task

(Morris, 1984). Prior to testing, the mice were acclimatized to the testing room for one hour with white noise set at 70 dB (white noise generator- San Diego Instruments, Serial# SDI 000141) to minimize auditory disruptions. The noise was present for the duration of the test.

Figure 5.1 Treatment and behavioural paradigms. (A) For long-term experiments, two-month old male transgenic (Tg) and wild-type (NonTg) littermates were treated with vehicle (25% sweetened condensed milk) or *R. rosea* extract at 100 mg/kg body weight/day following a one-week training period on vehicle alone. Mice were treated for eight weeks followed by a three-week behavioural paradigm while undergoing constant treatment. The behavioural battery consisted of the elevated-plus maze (EPM) test, light-dark exploration (LD) test, open-field (OF) test, adult social interaction (ASI) test and an eleven-day Morris Water Maze (MWM) test. The animals were allowed a one-week break from any further tests before exposure to a glucose tolerance test (GTT). The insulin tolerance test (ITT) was performed on the following day. Animals were sacrificed via euthanyl injections and transcardial perfusion. (B) For short-term dose-response experiments, three-month old male C57BL/6 mice were treated with vehicle (25% sweetened condensed milk) or *R. rosea* extract at 30, 100 or 300 mg/kg body weight/day following a one-week training period on vehicle alone. Untreated mice were included as negative controls. The mice were treated for three days on their respective treatments before commencement of the behavioural sequence which consisted of the EPM, LD, OF and ASI tests with one-day breaks in between individual tests.


A
Drug administra-
tion method
training (1 week)
8 - week treatment
phase
3 - week testing
phase
Break (1
week)
2 - month old
Tg and NonTg
littermates
Beginning of
treatment
Beginning of be-
havioural paradigm
End of behavioural
paradigm
GTT/ITT
Sacrifice
EPM (Day 1)
LD (Day 8)
OF (Day 11)
ASI (Day 13)
MWM start (Day 15)
MWM end (Day 25)
B
Drug administra-
tion method
training (1 week)
3 - month old
C57BL/6 mice
EPM (Day 3)
LD (Day 5)
OF (Day 7)
ASI (Day 9)

Elevated-plus maze

The maze apparatus (Med Associates Inc., ENV-560A) consisted of two arms measuring 5 cm by 60 cm crossed perpendicularly and elevated 1 m off the floor. One arm had open platforms, while the other had opaque walls approximately 14.5 cm high. The arms intersected at a central platform where the light intensity was measured to be 50 lux. Mice were placed in the central platform and allowed to explore for 10 minutes. Movement was recorded with a camera mounted above the maze and tracked using EthoVision® XT 8.0 software (Noldus Information Technology, Leesburg, VA, USA) with infra-red lighting to ensure tracking in the closed arms. Percent time spent in open and closed arms was calculated, as well as the number of open and closed arm entries.

Light-dark exploration test

The test apparatus consisted of an open-field chamber (Med Associates Inc., ENV-510) measuring 20.5 cm × 20.5 cm × 20.5 cm. A dark plastic insert divided the arena into two equal compartments. The front compartment was brightly lit and exposed (light zone), while the other was fully enclosed (dark zone) with an opening for mice to transition between the zones. The light zone was illuminated with two lights at 390 lux and the entire apparatus was enclosed in a sound-attenuating chamber equipped with a fan to ensure adequate ventilation. Mouse movements were tracked using three 16-beam infrared arrays mapping an X-Y co-ordinate system. Post hoc zonal analysis using Activity Monitor software (Med Associates Inc, St. Albans, VT, USA) allowed for the evaluation of percent time spent in each zone and the number of entries into each zone.

Open-field test

The test apparatus consisted of an open-top white plastic box measuring 44 cm × 44 cm × 44 cm (University of Ottawa). The light intensity above the box was measured to be 600 lux to further enhance the anxiety of being in the centre of the box. Mice were placed in a designated corner at the start of the test and allowed to explore freely for a period of 10 minutes. A camera was placed 1.75 m above the box and mouse movements tracked and analyzed by EthoVision® XT 8.0 software (Noldus Information Technology, Leesburg, VA, USA). Anxiety was assessed by the percent time spent in the centre of the box while motor function was measured by distance travelled and velocity.

Adult social interaction test

The apparatus used was a white plastic box (as for the open field test) with a wire-mesh enclosure (5.5 cm × 9.6 cm) aligned along one edge of the box. This allowed for the physical separation of the social target animal while allowing for visual and olfactory cues to be transmitted. The test mouse was first placed in a designated corner on the side opposite to the mesh enclosure in the absence of the target mouse and allowed to explore the arena for five minutes. The mouse was then removed and the social target animal was placed in the mesh enclosure (gender, genotype and age-matched). The test mouse was then placed back in the box and allowed to explore the arena for another five minutes. This test was performed under red light. Mouse movement was recorded through the use of a camera placed above the box and the data tracked and analyzed by EthoVision® XT 8.0 software (Noldus Information Technology, Leesburg, VA, USA). The time spent in nose-nose interaction in the interaction zone in the absence and presence of the social target mouse was assessed.

MWM test

Learning and memory was assessed in the MWM as previously described Wang et al. (2013a). Briefly, the apparatus consisted of a blue plastic pool (Med Associates Inc., ENV-514M-B) placed in a room measuring 2.98 m × 3.97 m × 2.62 m. The pool had an internal diameter of 127 cm and a depth of 42 cm. It was filled with tap water rendered opaque by adding non-toxic white paint to allow for better tracking. The temperature of the water was kept at 21°C. A hidden platform with a diameter of 10 cm was placed 1 cm below the water level in the back-right (acquisition) or the front-left quadrant of the pool (reversal) and served as an escape platform for the mice. The light level in the room was set to 150 lux. Prominent visual cues were provided on two of the walls (black cues on white backgrounds) to spatially orient the mice in the test. A large "X" was placed on the front screen and a square on the left.

Mice were tested over a period of eight days for the initial acquisition phase, followed by a probe test where the platform was removed (retention phase). The probe test was followed by a two-day reversal phase where the position of the platform was changed 180 degrees. For each test day (acquisition and reversal), mice completed four individual trials at 20-min intervals, from 4 randomized starting locations. Performance was recorded with a video camera mounted directly above the pool (Bosch, LTC0355/20; Pentax 3.5-8 mm lens, TS2V314BED). Their swim patterns were tracked and analyzed by EthoVision® XT 8.0 software (Noldus Information Technology, Leesburg, VA, USA). Mice were given a maximum escape latency of 60 seconds to find the submerged platform during each trial. Once they found the platform and stayed on it for 5 seconds, the tracking system stopped the recording and mice were placed back in the cage. However, if a mouse did not find the platform in the 60-second time frame, it was guided to the submerged platform, allowed to orient itself for 5 seconds, and then placed back in its cage. On

probe day, the platform was removed and the mice were allowed to search the pool for the missing platform for a period of 60 seconds.

Escape latency (in seconds) was assessed per day as an average of 4 trials per mouse per treatment group. This measure was used both for the acquisition and reversal phases.

Path efficacy was calculated as a ratio of the direct distance to the platform from the drop-off location over the actual path length taken by the mouse. Daily averages were then calculated and plotted.

Search strategy was assessed using the algorithm developed in Chapter 4. The search strategy implemented by each mouse was determined based on previous work done by Janus (2004) and Brody and Holtzman (2006). Briefly, the swimming track of each mouse for each trial for each day was analyzed using MWM Visual and assigned to one of three major search strategies; spatial, systematic or looping. Spatial strategy describes situations where the mouse swam directly to the platform, or focused its search within the quadrant containing the platform. Systematic strategy was assigned to situations where the mouse scanned the interior portion of the tank without any bias to any particular quadrant or focused on the incorrect quadrant. A trial was classified as repetitive looping if the mouse swam in a circular pattern at a fixed distance from the wall or swam in tightly wound circles. Floaters were classified as mice that had an average velocity of less than 6 cm/s and an escape latency of over 50 s for at least 5 out of the 8 training days.

5.2.5 Glucose tolerance and insulin tolerance tests

At the end of the behavioural paradigm, glucose tolerance (GTT) and insulin tolerance tests (ITT) were performed as described by Fujimoto et al. (2010). For the GTT, following a 16-hour fast, basal glucose concentrations were measured using an Accu-Chek® Nano glucometer

(Roche Diagnostics, Indianapolis, IN, USA). Mice were administered 2 g dextrose/kg body weight intraperitoneally and their glucose levels monitored for 3 hours. For the ITT, basal glucose level was measured following a 4-hour fast after which mice were treated with 0.75 U human recombinant insulin (Sigma-Aldrich, I9278, Lot# 050M8401) per kg body weight. Glucose levels were monitored over 3 hours post-insulin injection.

5.2.6 Survival Analysis

The survival of mice in the treatment and control groups was assessed over the duration of the chronic *R. rosea* treatment experiment. The probability of survival was assessed by the Kaplan-Meier technique using SPSS Statistics software version 22 (IBM), which computes the probability of survival at every occurrence of death. The comparisons of cumulative survival curves between treatment groups were performed using the log-rank, Grehan-Breslow-Wilcoxon and Tarone-Ware tests.

5.2.8 Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA, USA) or SPSS Statistics 22 (IBM Corporation, NY, USA). All data were analyzed using a 2-way ANOVA or 2-way repeated measures ANOVA, with Holm-Sidak post hoc tests. A p-value < 0.05 was considered statistically significant.

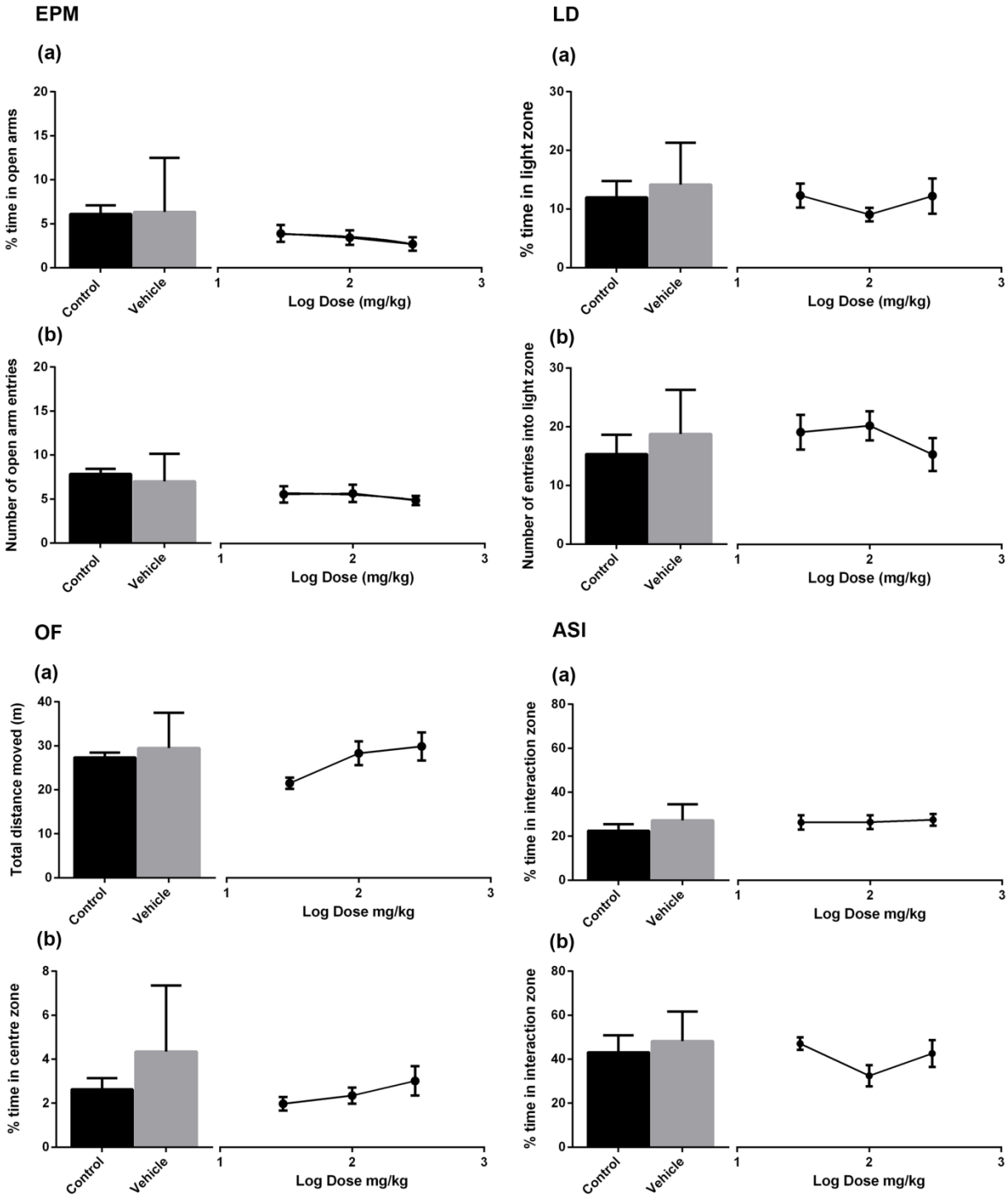
5.3 RESULTS

5.3.1 Effects of acute *R. rosea* treatment on behavioural measures of anxiety

Acute and chronic treatment schedules and behavioural testing timelines are presented in Fig. 5.1. To assess whether *R. rosea* administration induces anxiolytic effects on a short-term basis as observed in rats (Chapter 2), and to establish the effects of daily handling on behavioural

measures of anxiety, three-month old male C57BL/6 mice were treated with either vehicle or *R. rosea* at 30, 100, 300 mg/kg for three days prior to the start of the behavioural assay battery (Fig. 5.1B). Untreated mice were included as negative controls. There were no differences in the parameters measured between untreated controls and vehicle animals ($p > 0.05$) (Fig. 5.2). In each of the four tests of anxiety, no changes were observed in any of the indices of anxiety measured upon treatment with *R. rosea* compared to the controls ($p > 0.05$). In addition, no dose-response effects were observed ($p > 0.05$). Thus, in the short-term experiments, treatment with *R. rosea* did not alter behavioural indices of anxiety at the doses tested.

Figure 5.2 Performance of C57BL/6 control and *R. rosea*-treated mice at 30, 100 and 300 mg/kg body weight in anxiety assays upon short-term treatment. Anxiety in the elevated-plus maze test was assessed by (a) time in the open arms (%), (b) number of entries into the open arms; light-dark exploration test, (a) time in the light zone (%), (b) number of entries into the light zone; open-field test, (a) distance moved, and (b) time in the centre zone (%); and the adult social interaction test, (a) time in the interaction zone without target (%) and (b) time in the interaction zone in the presence of target (%). Data are presented as mean ± SEM. (Control, n = 6; Vehicle, *R. rosea* at 30, 100 and 300 mg/kg, n = 10; t-tests were conducted to determine differences between control and vehicle animals; linear regression analyses were performed to determine whether slope was significantly different from zero).


EPM
(a)
% time in open arms
Control
Vehicle
Log Dose (mg/kg)
(b)
Number of open arm entries
Control
Vehicle
Log Dose (mg/kg)
LD
(a)
% time in light zone
Control
Vehicle
Log Dose (mg/kg)
(b)
Number of entries into light zone
Control
Vehicle
Log Dose (mg/kg)
OF
(a)
Total distance moved (m)
Control
Vehicle
Log Dose mg/kg
(b)
% time in centre zone
Control
Vehicle
Log Dose mg/kg
ASI
(a)
% time in interaction zone
Control
Vehicle
Log Dose mg/kg
(b)
% time in interaction zone
Control
Vehicle
Log Dose mg/kg

5.3.2 Effects of chronic *R. rosea* administration on behavioural measures of anxiety

In the first test of the behavioural battery following chronic *R. rosea* administration, the elevated-plus maze (EPM), Tg mice spent significantly more time in the open arms of the maze irrespective of treatment, indicative of a less anxious phenotype (F (1, 77) = 5.1, p = 0.027) (Fig. 5.3A). Tgs also exhibited more exploratory behaviour by making more entries into the open arms compared to their NonTg counterparts, regardless of treatment (F (1, 77) = 9.6, p = 0.027) (Fig. 5.3B). Further, NonTg mice demonstrated a higher latency to enter into an open arm, suggestive of higher anxiety levels than Tgs, although not statistically significantly (p = 0.056) (Fig. 5.3C).

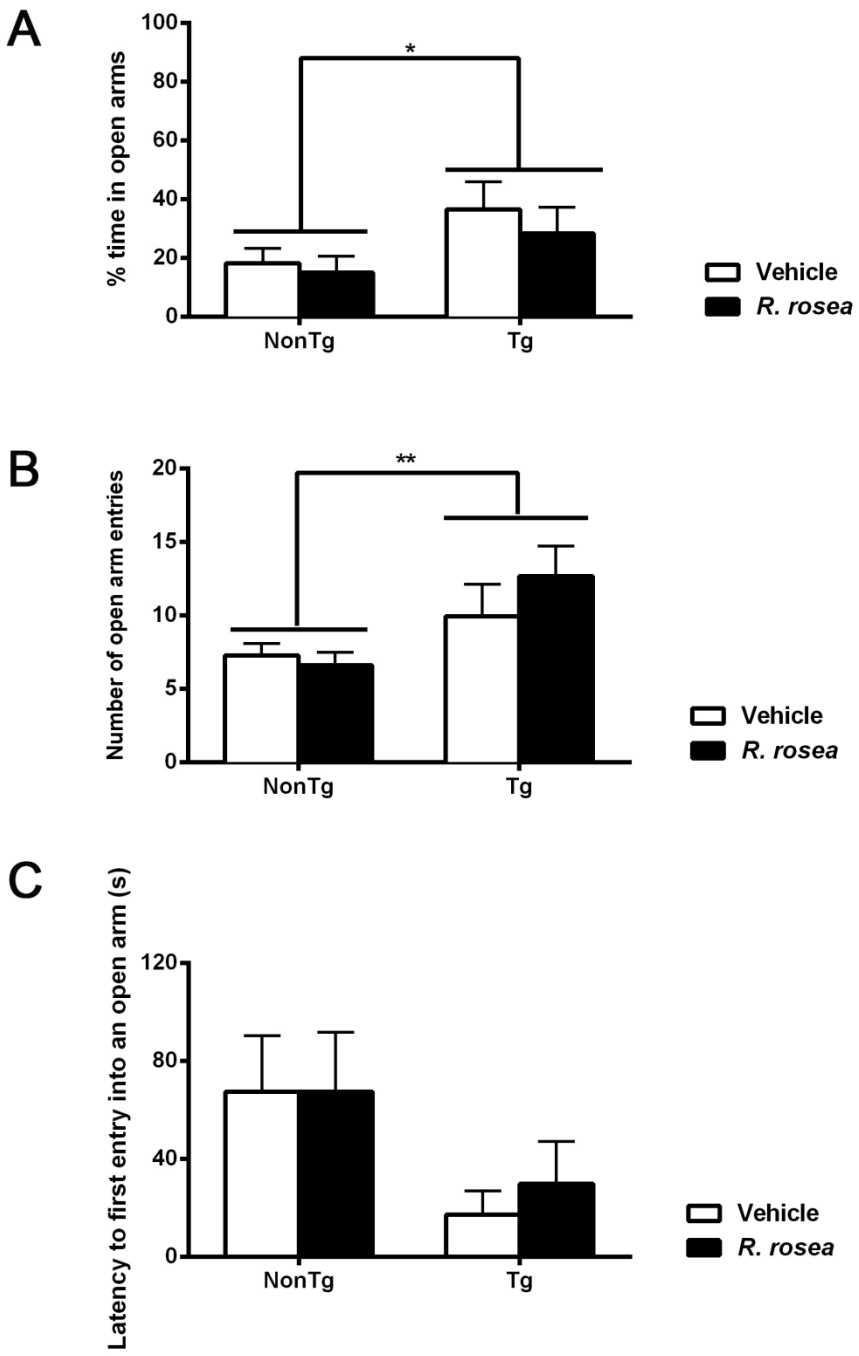


Figure 5.3 Performance of vehicle- and *R. rosea*- treated Tg and NonTg mice in the elevated-plus maze test. Anxiety was assessed by (A) time in the open arms (%), (B) number of entries into the open arms, and (C) latency to first entry into an open arm (s). Data are presented as mean ± SEM. (NonTg vehicle, n = 26; Tg vehicle, n = 16; NonTg *R. rosea*, n = 24; Tg *R. rosea*, n = 15; two-way ANOVA; *$p < 0.05$, **$p < 0.01$).

Similar trends were observed in the light-dark (LD) exploration test. Tgs spent significantly more time in the anxiety-inducing brightly lit light zone (F (1, 77) = 4.7, p = 0.033) (Fig. 5.4A) and correspondingly less time in the sheltered dark zone (F (1, 77) = 4.379, p = 0.040) (Fig. 5.4B). The time spent in the transition zone between the light and the dark zones was not significantly different between any of the treatment groups ($p > 0.05$) (Fig. 5.4C). Interestingly, *R. rosea*-treated Tg mice ventured out more frequently into each of the three zones compared to the vehicle-treated NonTg mice, indicating a greater tendency to explore (Fig. 5.4D-F). Data were analyzed using two-way ANOVAs and showed significant main effects of treatment (F (1, 77) = 4.7, p = 0.033 (light zone), post hoc Holm-Sidak's multiple comparisons test; NonTg vehicle vs Tg *R. rosea*, $p < 0.05$) as well as main effects of treatment and genotype (F (1, 77) = 4.979, p = 0.029, F (1, 77) = 4.152, p = 0.045 (transition zone), post hoc Holm-Sidak's multiple comparisons test; NonTg vehicle vs Tg *R. rosea*, $p < 0.05$; F (1, 77) = 4.639, p = 0.034, F (1, 77) = 4.373, p = 0.040 (dark zone), post hoc Holm-Sidak's multiple comparisons test; NonTg vehicle vs Tg *R. rosea*, $p < 0.05$) (Fig. 5.4D-F).

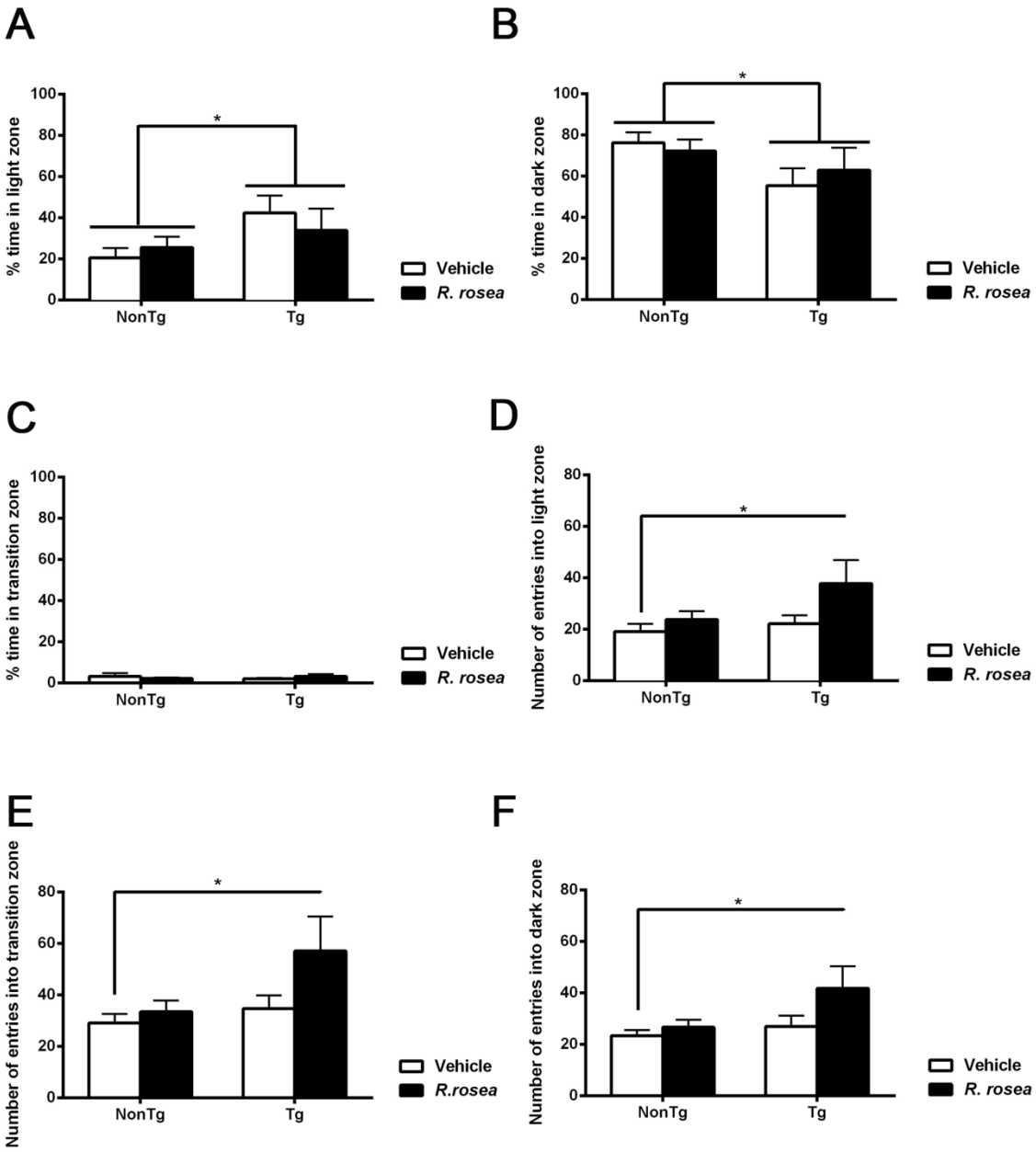


Figure 5.4 Performance of vehicle- and *R. rosea*-treated Tg and NonTg mice in the light-dark exploration test. Anxiety was assessed by (A) time in the light zone (%), (B) time in the dark zone (%), (C) time in the transition zone (%), (D) number of entries into the light zone, (E) number of entries into the transition zone, and (F) number of entries into the dark zone. Data are presented as mean ± SEM. (NonTg vehicle, n = 26; Tg vehicle, n = 16; NonTg *R. rosea*, n = 24; Tg *R. rosea*, n = 15; two-way ANOVA; *$p < 0.05$ comparing NonTg vehicle and Tg *R. rosea*; post hoc Holm-Sidak's tests (D-F)).

The open-field (OF) test was used to assess locomotory ability as well as anxiety. Tg mice move a greater distance over the duration of the test (F (1, 77) = 22.28, $p < 0.0001$) at a greater velocity (F (1, 77) = 22.27, $p < 0.0001$) compared to NonTg mice regardless of treatment (Fig. 5.5A, B). While there was no difference in the time spent in the brightly lit and exposed centre zone between the treatment groups ($p > 0.05$) (Fig. 5.5C), Tg mice spent significantly less time in the corners, indicating reduced anxiety-like behaviour regardless of treatment (F (1, 77) = 8.025, $p = 0.006$) (Fig. 5.5D). Additionally, they made significantly more entries into the exposed centre zone compared to NonTgs further supporting their greater tendency to explore (F (1, 77) = 15.52, $p = 0.0002$) (Fig. 5.5E). The groups did not differ in their latency to enter the centre zone ($p > 0.05$) (Fig. 5.5F).

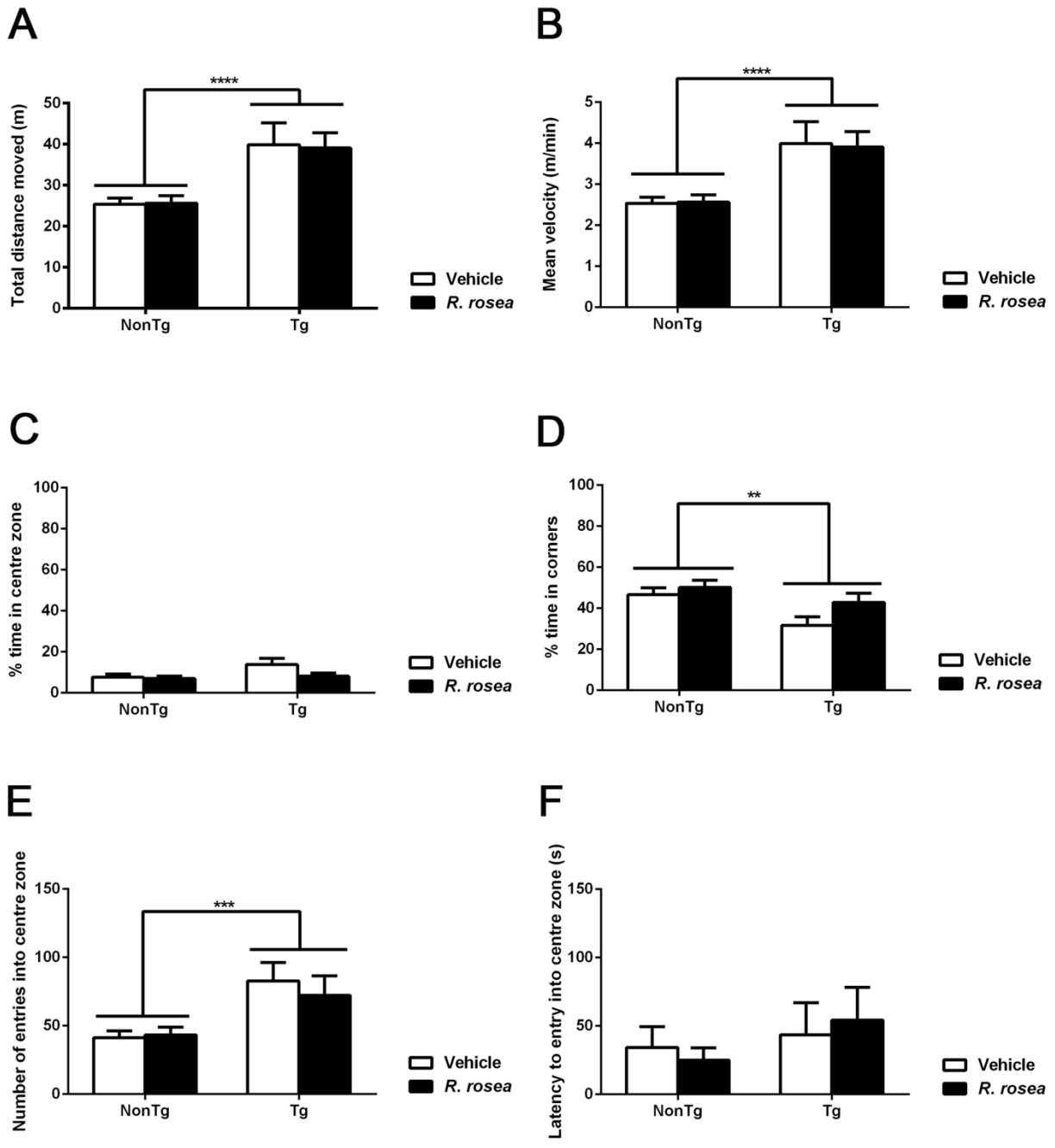


Figure 5.5 Performance of vehicle- and *R. rosea*-treated Tg and NonTg mice in the open-field test. Locomotion was measured by (A) total distance moved (m), and (B) velocity (m/min). Anxiety was assessed by (C) time in the centre zone (%), (D) time in the corners (%), (E) number of entries into the centre zone, and (F) latency to entry into the centre zone (s). Data are presented as mean ± SEM. (NonTg vehicle, n = 26; Tg vehicle, n = 16; NonTg *R. rosea*, n = 24; Tg *R. rosea*, n = 15; two-way ANOVA; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Indices of social anxiety were examined using an adult social interaction (ASI) test. Under both test conditions, i.e., the absence or presence of the social target, there were no differences between the treatment groups in the time spent in the interaction zone around the social target ($p > 0.05$) (Fig. 5.6A, B). The social interaction ratio was calculated as the ratio of the time spent in the interaction zone or in the two-corner zone opposite the interaction zone in the presence compared to the absence of the social target. Tgs exhibited a significantly reduced interaction ratio i.e., they showed less preference for the interaction zone when the target was present than when it was absent compared to the NonTg mice (F (1, 75) = 4.379, p = 0.0398) (Fig. 5.6C). There was no difference in the ratio of time spent in the corner zones opposite the interaction zone in the presence compared to the absence of the social target ($p > 0.05$) (Fig. 5.6D). Overall, Tg mice exhibited reduced anxiety-like behaviour compared to NonTg mice regardless of treatment. Under higher anxiety test conditions, as in the ASI test, Tg mice exhibited lower social interaction capacity in the presence of the social target. In these chronic treatment experiments, *R. rosea* did not exert anxiolytic effects on either genotype, beyond an increased exploration in the LD test.

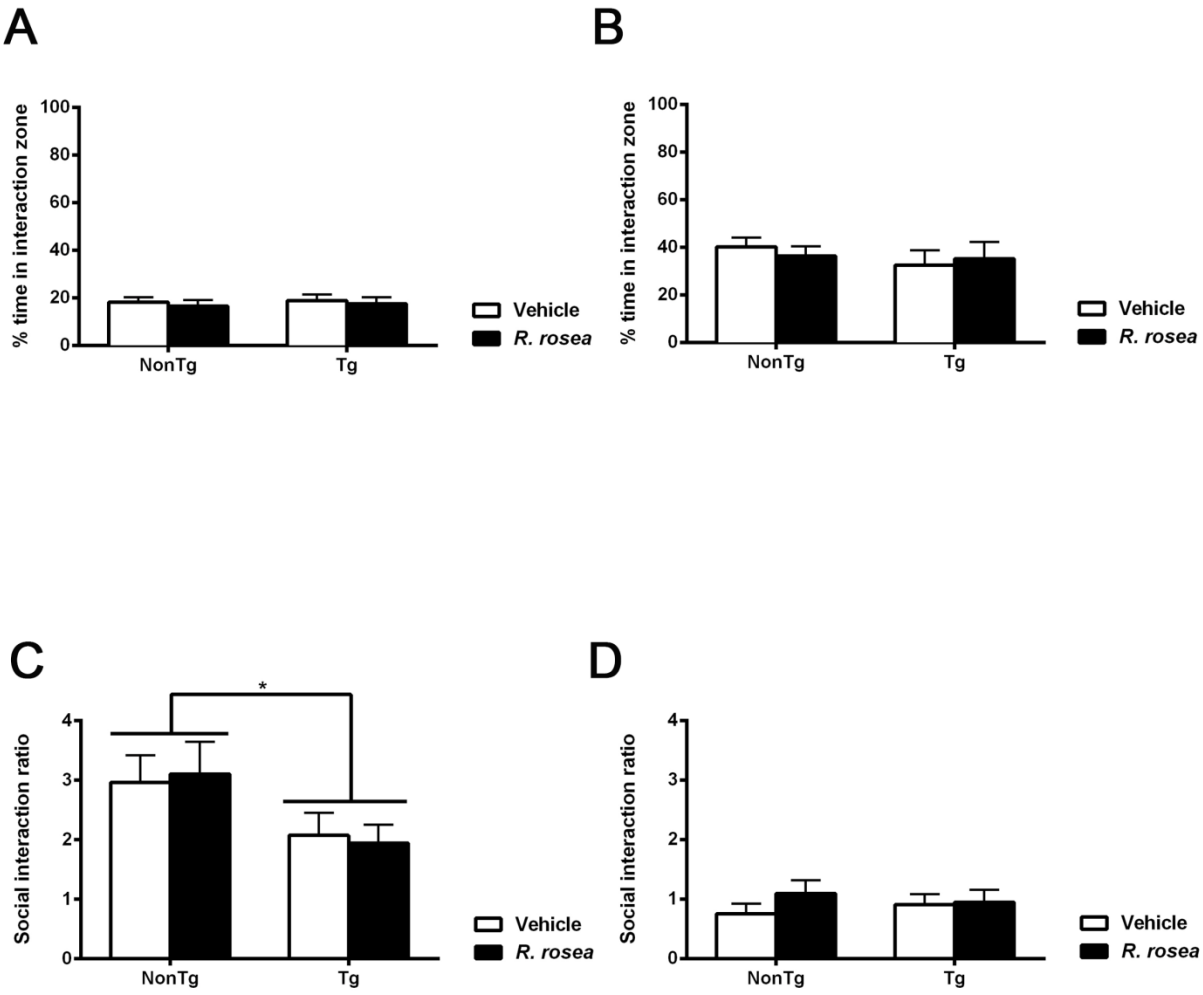


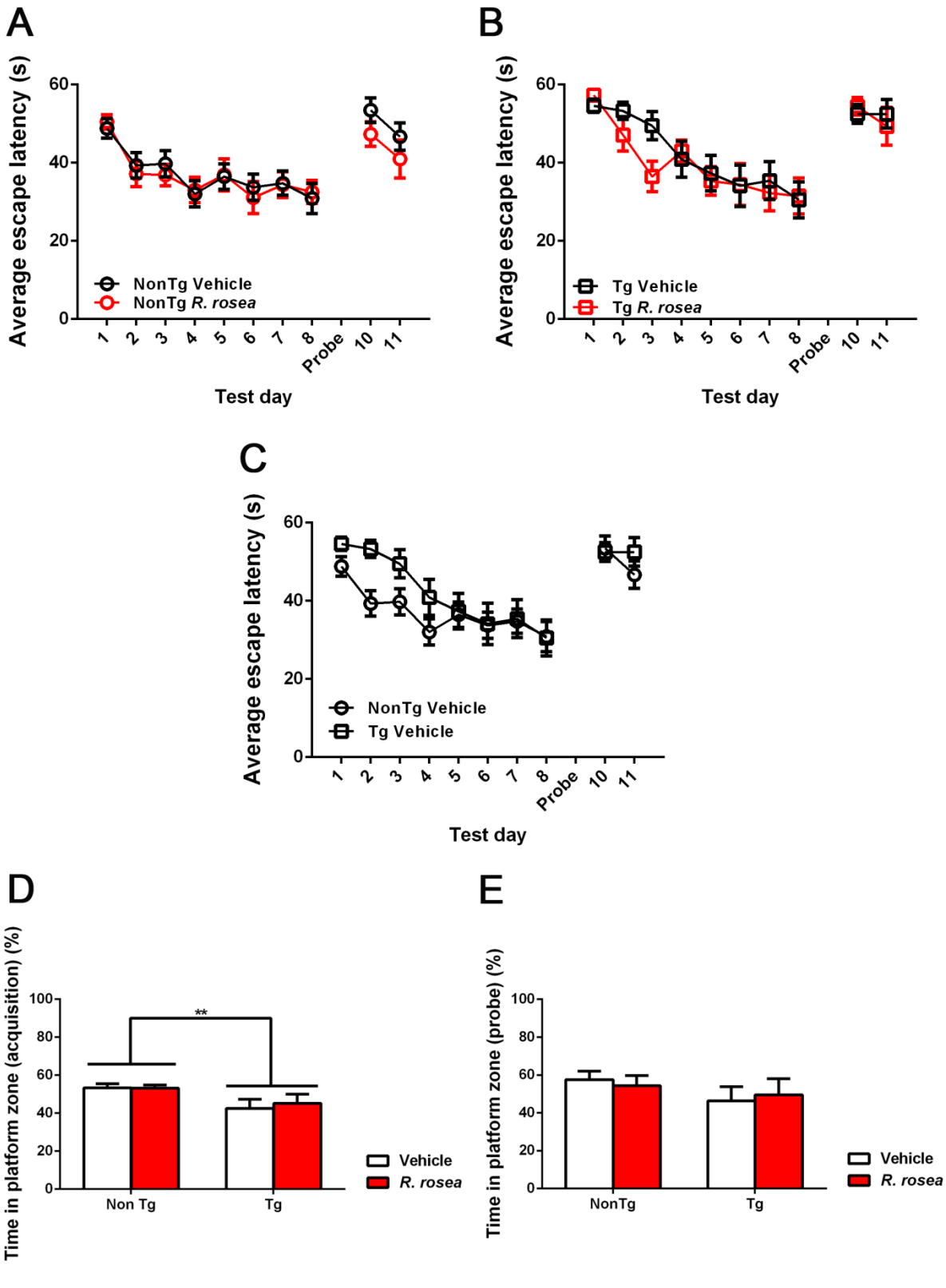
Figure 5.6 Performance of vehicle- and *R. rosea*-treated Tg and NonTg mice in the adult social interaction test. Anxiety was assessed by (A) time in the interaction zone in the absence of a social target (%), (B) time in the interaction zone in the presence of target (%), (C) social interaction ratio, interaction zone (ratio of time in interaction zone in the presence and absence of target), and (D) social interaction ratio, corner zone (ratio of time in corner zone in the presence and absence of target). Data are presented as mean ± SEM. (NonTg vehicle, n = 25; Tg vehicle, n = 16; NonTg *R. rosea*, n = 24; Tg *R. rosea*, n = 15; two-way ANOVA; *$p < 0.05$).

5.3.3 Effects of chronic *R. rosea* treatment on behavioural measures of learning and memory

The MWM test was used to assess the effects of long-term *R. rosea* administration on learning and memory in TgCRND8 mice. Escape latency is one of the most commonly used measures of MWM performance. *R. rosea*-treated NonTg mice did not show any differences in performance compared to vehicle controls over the first eight days of testing (acquisition phase) ($p > 0.05$) (Fig. 5.7A). Similarly, in Tgs, vehicle and *R. rosea*-treated Tg mice escape latencies were comparable except for a slight non-significant improvement in *R. rosea*-treated mice on day 3 ($p > 0.05$) (Fig. 5.7B). While previous studies in our laboratory have shown that male Tg mice at 6 months of age exhibit higher behavioural indices of cognitive reserve and thus only modest impairments in learning and memory (Granger et al., 2015), no statistically significant impairment was observed in vehicle-treated Tg compared to NonTg mice (Fig. 5.7C). Despite the initial differences between their escape latencies (days 1-4), Tg mice showed progressively improved rates of learning while NonTg mice plateaued in their learning ability (days 5-8), consistent with our previous findings of enhanced cognitive reserve (Granger et al., 2015). *R. rosea* did not positively or negatively alter this capacity. This pattern was further echoed in the reversal phase (days 10 and 11) of testing. There were no differences in the ability of mice to readily adapt their learning of finding the platform when its position was altered between NonTg and Tg vehicle mice ($p > 0.05$), and treatment with *R. rosea* did not affect performance in either genotype ($p > 0.05$). While there were no differences in escape latency during the acquisition phase, NonTg mice concentrated their search for the platform during the acquisition phase in the correct quadrant compared to Tg mice, regardless of treatment (F (1, 61) = 8.019, $p = 0.0063$) (Fig. 5.7D). However, this search trend for the platform was not observed in the test of memory

retention represented by the time spent in the platform zone during probe trial; there were no differences between any of the experimental groups ($p > 0.05$) (Fig. 5.7E).

Figure 5.7 Performance of vehicle- and *R. rosea*-treated Tg and NonTg mice in the Morris Water Maze test. Average escape latency (s) comparisons during the acquisition and reversal phases for (A) NonTg vehicle vs NonTg *R. rosea*, (B) Tg vehicle vs Tg *R. rosea* and (C) NonTg vehicle vs Tg vehicle were conducted. The time spent in the platform zone (%) over the eight acquisition days (D) and during probe day (E) are presented. Data are presented as mean ± SEM. (NonTg vehicle, n = 19; Tg vehicle, n = 13; NonTg *R. rosea*, n = 19; Tg *R. rosea*, n = 14; two-way repeated measures ANOVA (A-C) and ordinary two-way ANOVA (D, E); $^{**}p < 0.01$).


A
Average escape latency (s)
60
40
20
0
NonTg Vehicle
NonTg R. rosea
1
2
3
4
5
6
7
8
Probe
10
11
Test day
B
Average escape latency (s)
Tg Vehicle
Tg R. rosea
Test day
C
Average escape latency (s)
NonTg Vehicle
Tg Vehicle
Test day
D
Time in platform zone (acquisition) (%)
100
80
60
40
20
0
**
Non Tg
Tg
Vehicle
R. rosea
E
Time in platform zone (probe) (%)
NonTg
Tg
Vehicle
R. rosea

Path efficacy, or the ratio of the shortest measured distance between the mouse entry point into the pool to the platform over the actual path taken by the mouse was also assessed. Similar to escape latency profiles, initially, NonTg vehicle mice took more direct routes to the platform from their respective drop-off points (days 1-4); however, Tg mice steadily improved and exhibited similar efficacy over days 5-8 ($p > 0.05$) (Fig. 5.8A). *R. rosea* treatment did not affect path efficacy in either NonTg (Fig. 5.8B) or Tg mice (Fig. 5.8C).

As in the OF test, Tg mice swam further (F (1, 61) = 23.31, $p < 0.0001$) (Fig. 5.9A) and and faster (F (1, 61) = 19.56, $p < 0.0001$) than NonTg mice (Fig. 5.9B). Interestingly, Tgs also spent less time in the outer thigmotactic zone compared to the NonTg mice, indicative of a reduced anxiety phenotype (F (1, 68) = 8.119, $p = 0.0058$) (Fig. 5.9C).

Uses of spatial and looping strategies were compared to the frequency of use of systematic strategies. A closer examination of the search strategies employed by the different experimental groups revealed that NonTg vehicle mice predominantly used systematic strategies on day 1, then alternated between spatial and systematic until day 6 when they switched to spatial strategy use (F (7, 336) = 9.616, $p < 0.0001$) (Fig. 5.10A). In Tg vehicle mice however, the use of systematic strategy was predominant until day 4, followed by the alternate use of both spatial and systematic strategies until day 8 when they used predominantly spatial strategy (F (7, 182) = 15.81, $p < 0.0001$) (Fig. 5.10C). These differences likely contributed to the initial phenotype also detected in MWM escape latency between days 1 and 4 between NonTg and Tg vehicle mice. NonTg *R.rosea*-treated mice employed primarily systematic strategies on day 1 similar to the vehicle controls, until day 8 when they used mainly spatial strategies (F (7, 322) = 7.297, $p < 0.0001$) (Fig. 5.10B). Tg *R. rosea* mice alternated between the spatial and systematic strategies and made an earlier transition to predominantly spatial strategy use at day 6 (F (7, 182)

= 23.45, $p < 0.0001$) (Fig. 5.10D). While these subtle differences in strategy use reflect complexity of search behaviour, when comparing spatial or systematic strategy use between treatment groups, there were no overall differences (data not shown).

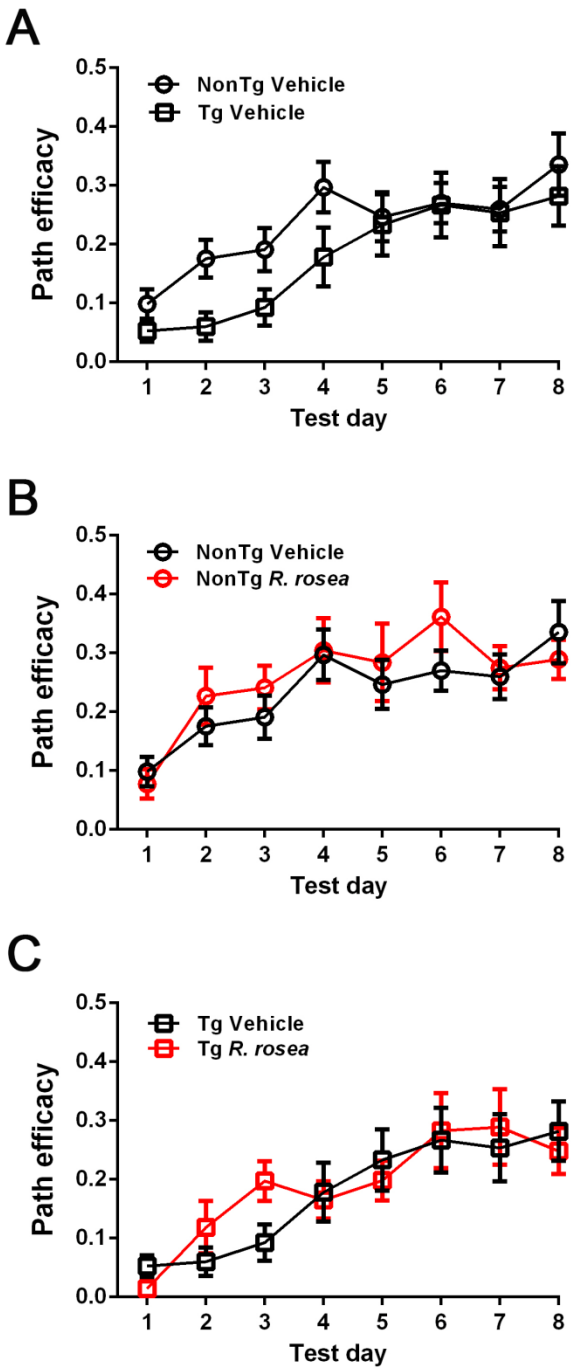


Figure 5.8 Path efficacy, calculated as the ratio of the shortest distance to platform from entry point into pool divided by the actual path length, was compared between (A) NonTg vehicle and Tg vehicle; (B) NonTg vehicle and NonTg *R. rosea*; and (C) Tg vehicle vs Tg *R. rosea*. Data are presented as mean ± SEM. (NonTg vehicle, n = 19; Tg vehicle, n = 13; NonTg *R. rosea*, n = 19; Tg *R. rosea*, n = 14; two-way repeated measures ANOVA).

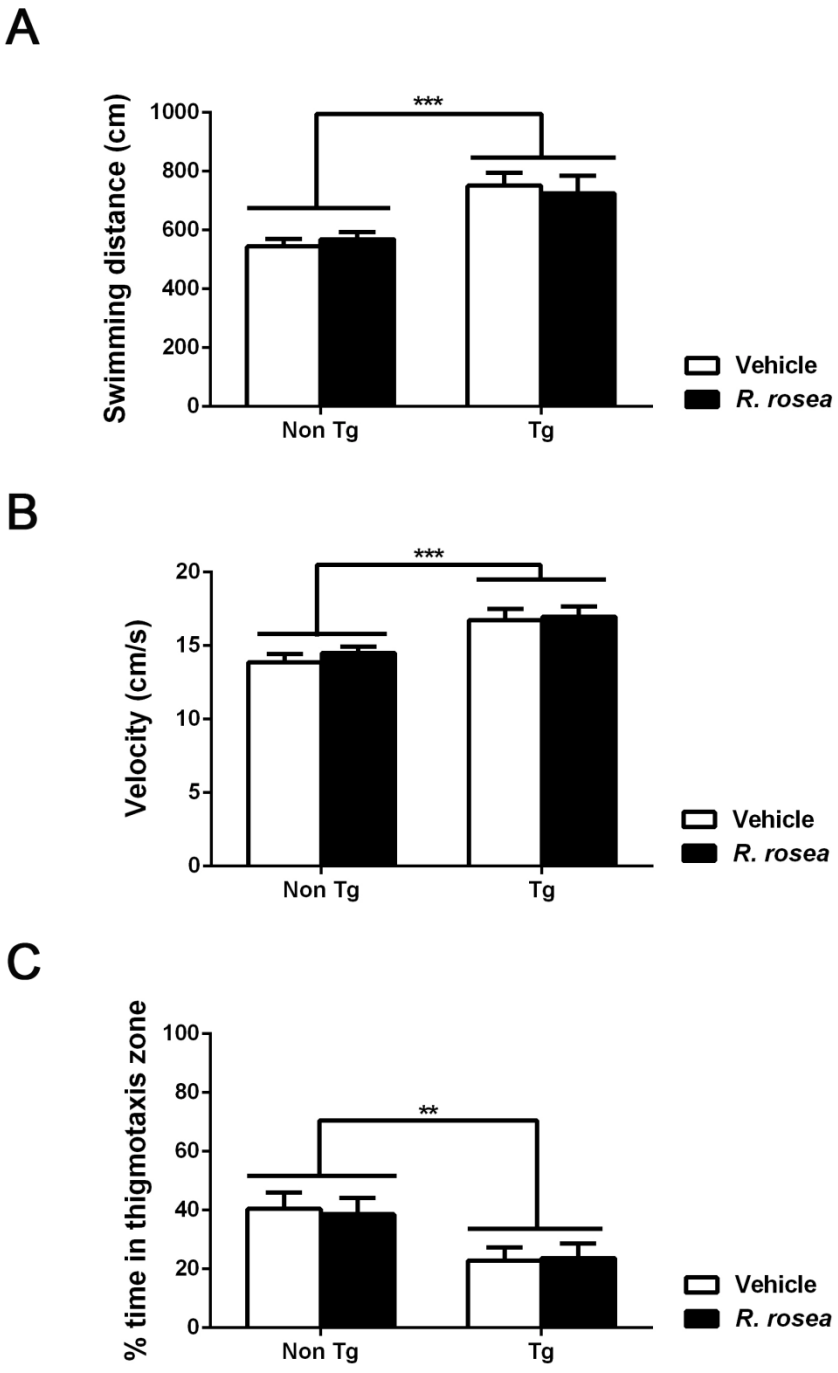


Figure 5.9 Motoric performance of vehicle- and *R. rosea*-treated Tg and NonTg mice in the Morris water maze test. Motoric function was assessed by (A) Swim distance (cm) and (B) velocity (cm/s) while anxiety was assessed using (C) time in thigmotaxis zone (%). Data are presented as mean ± SEM. (NonTg vehicle, n = 19; Tg vehicle, n = 13; NonTg *R. rosea*, n = 19; Tg *R. rosea*, n = 14; two-way ANOVA; $^{**}p < 0.01$, $^{***}p < 0.001$).

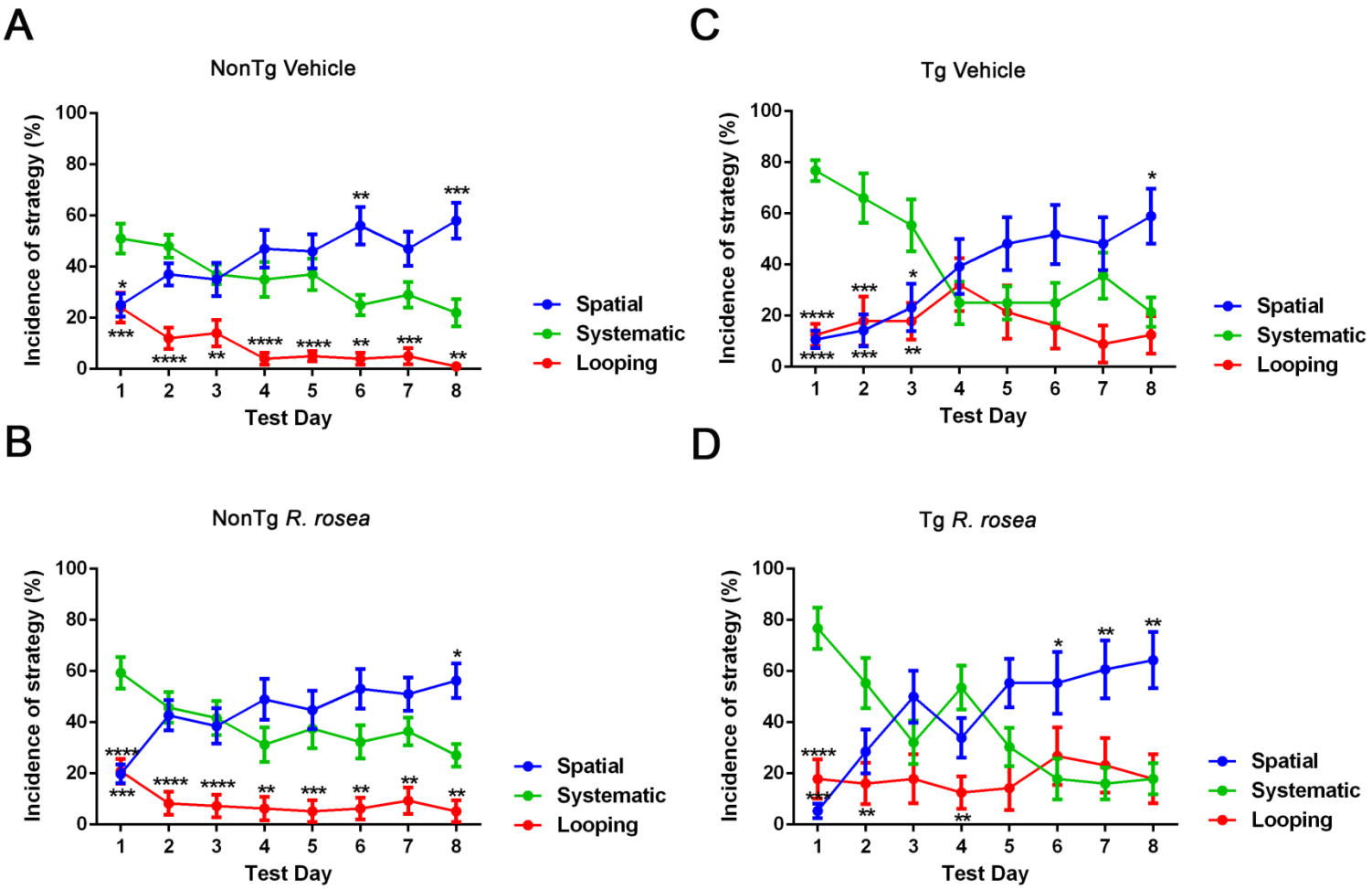


Figure 5.10 Analysis of search strategies of vehicle- and *R. rosea*-treated Tg and NonTg mice over the eight acquisition days of the Morris water maze test using MWM Visual 1.0. Percent incidences of spatial, systematic and looping strategies in (A) NonTg Vehicle, (B) NonTg *R. rosea*, (C) Tg Vehicle, and (D) Tg *R. rosea* mice over the eight test days are presented. Data are presented as mean ± SEM. (NonTg vehicle, n = 25; Tg vehicle, n = 14; NonTg *R. rosea*, n = 24; Tg *R. rosea*, n = 14; two-way repeated measures ANOVA with post hoc Holm-Sidak's t-tests comparing systematic with spatial and looping strategies use within each treatment group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

5.3.4 Effects of *R. rosea* on glucose and insulin tolerance

As part of this study, TgCRND8 mice were exposed to sweetened condensed milk (a high-sugar, high fat vehicle) for prolonged periods of time. To ensure no adverse effects occurred on glucose and insulin sensitivity, glucose and insulin tolerance tests were performed. Tgs were slightly more glucose tolerant as demonstrated by lower area under the curve of their glucose profiles over the 180 minutes of the test (F (1, 72) = 12.43, p = 0.0007) (Fig. 5.11A). There were no differences in insulin tolerance between the groups ($p > 0.05$) (Fig. 5.11B). *R. rosea* did not have any impact on glucose or insulin tolerance in TgCRND8 mice at the dose tested.

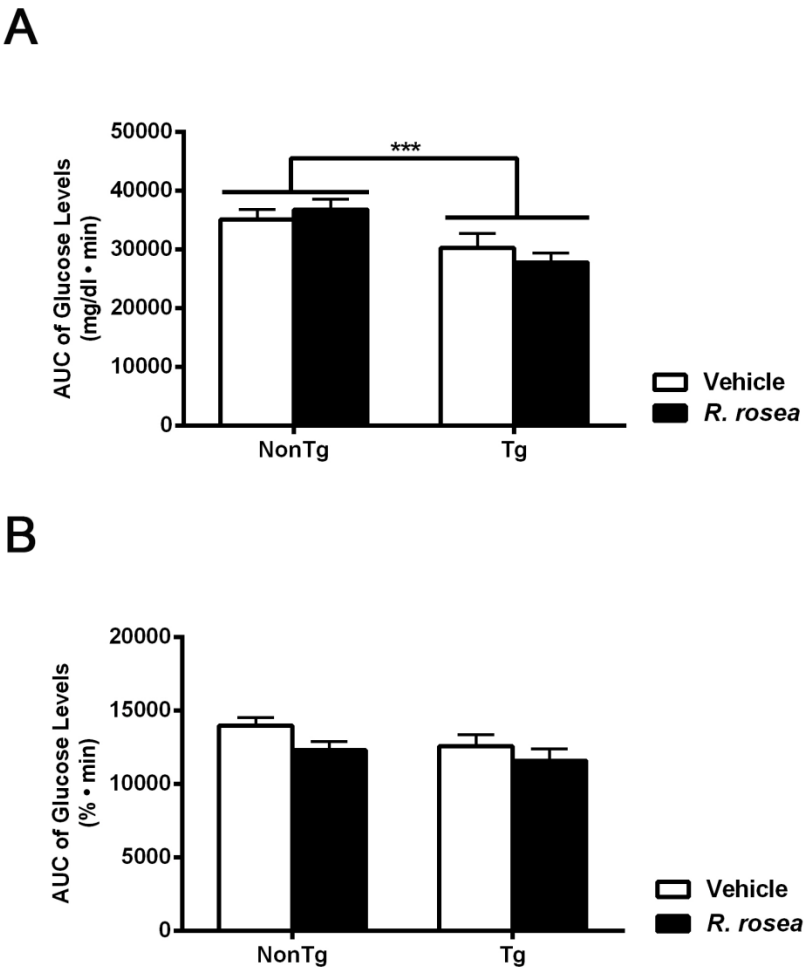


Figure 5.11 Assessment of the effects of long-term vehicle and *R. rosea* treatment on (A) glucose tolerance, and (B) insulin response in TgCRND8 mice. (A) Area under the curve (AUC) of glucose levels (mg/dL • min) following an i.p. injection of dextrose (2 g/kg body weight). (B) AUC of glucose levels following an i.p. injection of 0.71 U recombinant insulin/kg (% • min). Data are presented as mean ± SEM. (NonTg Vehicle, n = 24; NonTg *R. rosea*, n = 23; Tg Vehicle, n = 14; Tg *R. rosea*, n = 14; two-way ANOVA. ***$p < 0.001$).

5.3.5 Effects of *R. rosea* on survival in TgCRND8 mice

Tg vehicle mice exhibited high mortality rates over the course of the study compared to NonTg vehicle mice ($\chi^2 = 15.53$, $p < 0.0001$) (Fig. 5.12A). 53.9 % of Tg vehicle mice survived to the end of the study compared to 100 % survival in the NonTg vehicle group. *R. rosea* treatment in NonTg mice had no adverse effects as survival remained at 100 % until the time of sacrifice. Among Tgs, *R. rosea* mice improved survival, though not significantly, to 71.4% ($\chi^2 = 1.446$, $p = 0.229$) (Fig. 5.12C). Despite no overall survival curve differences, 75 % survivorship was observed at 90 days in vehicle-treated Tg mice and at 124 days in *R. rosea* treated mice. In addition, the hazard ratio, or the rate of survival in the *R. rosea*-treated population was nearly twice (1.805) that of the vehicle-treated Tg mice.

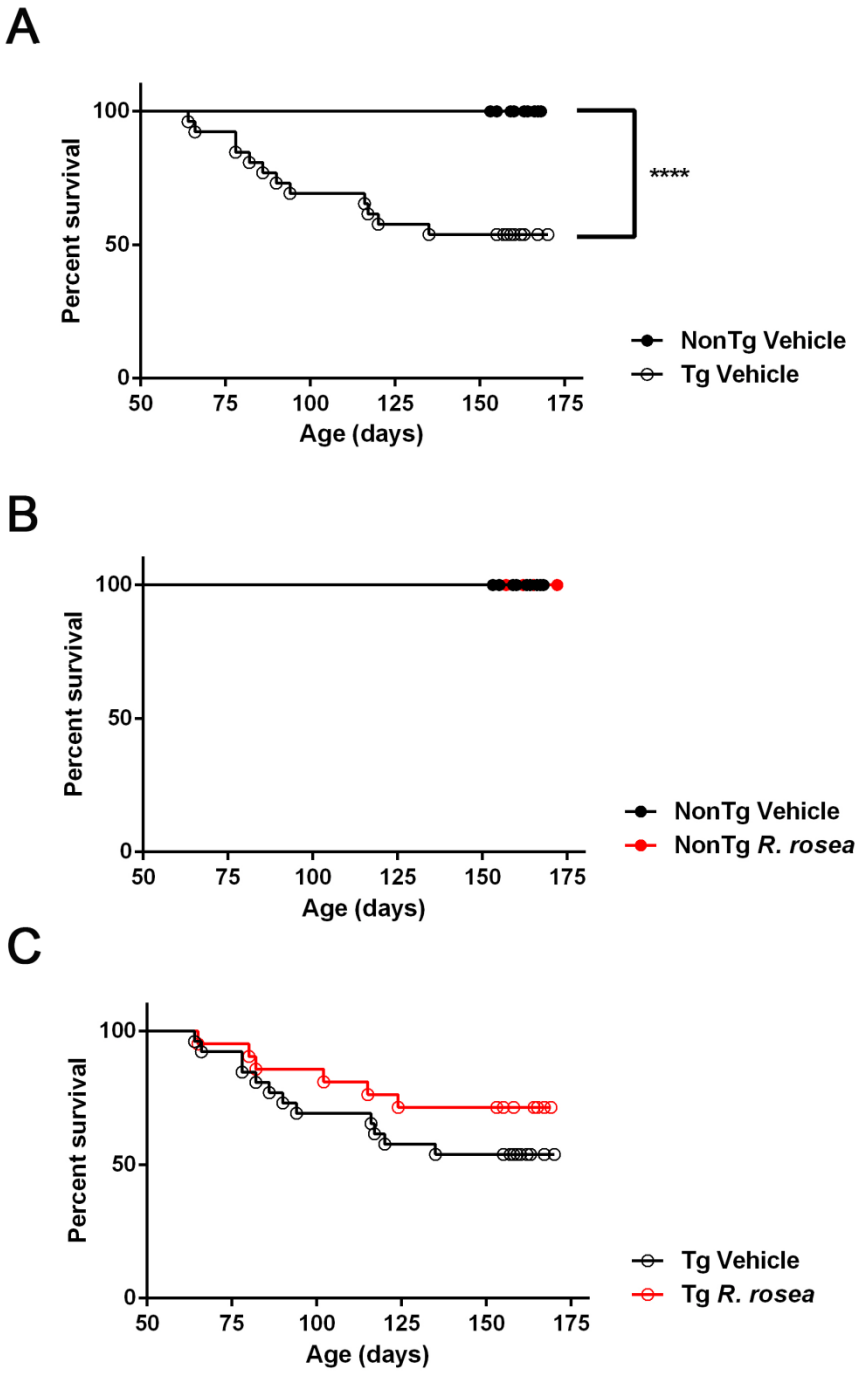


Figure 5.12 Survival curves of vehicle- and *R. rosea*-treated Tg and NonTg mice over the long-term treatment experiment. The Kaplan-Meier method was used to calculate probability of survival at every occurrence of death for (A) NonTg vehicle vs Tg vehicle ; (B) NonTg vehicle vs NonTg *R. rosea* and, (C) Tg vehicle vs Tg *R. rosea*. (NonTg vehicle, n = 26; Tg vehicle, n = 26; NonTg *R. rosea*, n = 24; Tg *R. rosea*, n = 21. Curve analyses were performed using log-rank (Mantel-Cox), Grehan-Breslow-Wilcoxon (A, B, C) and Tarone-Ware tests (C). **** $p < 0.0001$).

5.4 DISCUSSION

In the present study, Nunavik *R. rosea*, a traditional Inuit medicinal plant used for protection against age-related decline in mental and physical capacity, was assessed for its effects on behavioural indices of anxiety and learning and memory. Based on its ethnobotanical uses, as well as its previously demonstrated anxiolytic activity in rats, we hypothesized that *R. rosea* would attenuate non-cognitive and cognitive deficits in TgCRND8 mice when administered prior to the onset of AD-like symptomatology. To our knowledge, this is the first study of the prophylactic effects of *R. rosea* in a mouse model of AD. Collectively, our results demonstrated that chronic oral treatment of *R. rosea* did not affect basal anxiety levels or learning and memory performance in TgCRND8 mice beyond an increase in maze exploration by Tgs in the light dark exploration test. Further, unlike results in Sprague-Dawley rats, acute *R. rosea* treatment at 30, 100, and 300 mg/kg did not change anxiety-like parameters in three-month old C57BL/6 mice. Interestingly, in our study, *R. rosea* treatment partially protected Tg mice from the high mortality rates characteristic of this mouse model; further studies are required to establish whether this protection results from effects on Aβ biogenesis.

Given its previously established anxiolytic activity in rats, as part of this study, *R. rosea* was examined for its ability to affect basal anxiety levels in TgCRND8 mice. In our study, Tg mice showed reduced anxiety-like behaviour compared to their NonTg counterparts in the EPM, LD and OF tests of anxiety. Previous studies in TgCRND8 mice have shown either no difference in anxiety-related behaviour between Tg and NonTg mice (Touma et al., 2004), or reduced anxiety in Tgs in the EPM (Gortz et al., 2008) and OF tests (Ma and McLaurin, 2014). In the ASI test, Tgs showed a significant avoidance of the social interaction zone in the presence of the target as compared to when the target was absent. Similar patterns of reduced Tg social

investigative behaviours were reported in 5 month-old male Tg mice (Lewejohann et al., 2009). Both these observed patterns of hypo- or hyper-anxious behaviours are representative of neuropsychiatric symptoms observed in AD patients who exhibit anxiety as well disinhibitory tendencies (Hart et al., 2003; Lalonde et al., 2012a). Additionally, we observed increased locomotion in Tg mice in the OF test, similar to the restlessness, pacing and (or) wandering behaviour exhibited by AD patients (Lanari et al., 2006). Other studies have reported either hyperactivity in Tg mice (Hyde et al., 2005; Ambree et al., 2009; Walker et al., 2011), or no differences in exploratory activity (Touma et al., 2004; Gortz et al., 2008). These variations are likely due to age, gender, or handling of the test subjects and (or) procedural differences between the behavioural tasks.

While prophylactic interventions including the oral administration of plant-derived phenolics, ferulic acid and tannic acid have been effective in either eliminating these hyperactive tendencies and (or) normalizing decreased-anxiety like behaviour (Mori et al., 2012; Mori et al., 2013; Ma and McLaurin, 2014), oral administration of *R. rosea* at 100 mg/kg did not have any effects on anxiety parameters beyond augmenting explorative behaviour in the Tgs in the LD test. Anxiolytic drugs including classical benzodiazepines, and serotonin 5-HT_A receptor agonists increase explorative activity in tests of anxiety (Bourin and Hascoet, 2003; Prut and Belzung, 2003). Perfumi and Mattioli (2007) demonstrated anxiolytic effects of European *R. rosea* in the light-dark and open-field tests, including increased locomotion. Further, another study by Mattioli and Perfumi (2007) showed that under depressive conditions induced by chronic mild stress in rats, treatment with *R. rosea* was able to rescue decreased exploration which is characteristic of depressed rats. This may be in part due its modulatory effects on key monoaminergic neurotransmitter signalling systems involved in anxiety and depression (van

Diermen et al., 2009; Mannucci et al., 2012; Verpeut et al., 2013). An alternative interpretation, given the general reduced anxiety-like behaviours observed in Tg mice, is that *R. rosea* may be exacerbating the hyperactive phenotype in Tg mice, and this increased locomotion is being observed as anxiolytic behaviour.

In order to evaluate the effects of *R. rosea* on anxiety in mice that do not exhibit this hypo-anxious phenotype, parallel experiments were conducted with C57BL/6 mice. Additionally, it was important to assess whether prolonged handling and social isolation in test mice were obscuring potential pharmacological effects of *R. rosea*. Unlike in Sprague-Dawley rats, Nunavik *R. rosea* did not have any anxiolytic effects in C57BL/6 mice. Genetic background is a major contributor to anxiety phenotypes in rodents. C57BL/6 mice characteristically display low levels of basal anxiety (Griebel et al., 2000); *R. rosea* may not be able to reduce these levels further. The difference in the results between rats and mice could be attributed to an inter-species difference in metabolic rates. Mice have higher metabolic rates than rats, as well as a different complement of cytochrome P450 enzymes, and maybe clearing the extract more efficiently leading to reduced times for biological activity (Lim et al., 1994; Born et al., 2003; Martignoni et al., 2006). Thus, despite our attempts to account for this in the mice by increasing the doses administered compared to those in rats, it is likely that bioactive compounds of *R. rosea* were metabolized. Future studies using shorter interval times between extract administration and behavioural testing may provide additional insight.

As part of this study, the effects of Nunavik *R. rosea* on learning and memory in TgCRND8 mice was assessed by a reference memory version of the MWM test. In the present study, *R. rosea* administration did not influence classical indices of learning and memory in either NonTgs or Tgs compared to their respective vehicle controls. This is in contrast to a

previous study that demonstrated protective effects of *R. rosea* pre-treatment on STZ-induced cognitive deficits in rats, although the variety of *R. rosea* tested was distinct from ours (Qu et al., 2009). Further, it should be noted that in our study, vehicle-treated Tg mice at 5.5 months of age were indistinguishable from NonTg controls in their rates of memory acquisition, retention or adaptability to learning, despite the presence of Aβ plaques. Untreated, naïve male Tg mice exhibit memory impairments at 6 months of age, albeit more subtle when compared to age-matched females (Granger et al., 2015). Vehicle-treated NonTgs, but not Tg mice, that were subjected to prolonged periods of social isolation, daily handling stress and a battery of anxiety tests prior to testing in the water maze showed impaired performance compared to untreated, naive controls (data not shown), likely due to considerably higher rates of thigmotaxis, an indicator of anxiety-like behaviour and (or) decreased motivation, as indicated by the disproportionately high number of floaters. Floating behaviour has been likened to a lack of motivation, or behavioural despair characterized by immobility (Baeta-Corral and Gimenez-Llort, 2015). Interestingly, the *R. rosea*-treated NonTg cohorts had fewer numbers of floaters compared to vehicle controls. This anti-depressive effect was further supported by work from Panossian et al. (2008) who demonstrated that *R. rosea*, as well as its phytochemicals salidroside and tyrosol, can reduce swimming immobility times in rats with depression-like symptoms.

For future studies, cued water maze trials or non-spatial pre-training may be useful in reducing these non-search behaviours that interfere with spatial learning (Vorhees and Williams, 2006). The addition of a more challenging and sensitive test of spatial learning and memory, including the T-maze alternation or the radial arm water maze, or the refinement of current MWM protocols to include a smaller platform size, or changing platform locations on a day-to-

day basis may help to clarify the subtle differences in these mice and the effects of potential prophylactics (Stewart et al., 2011).

Examination of navigational search strategies used by mice during MWM trials reveal interesting patterns that are not reflected by escape latencies. Similar to previous work by Janus (2004), untrained mice initially relied on non-goal oriented random or scanning strategies, and adopted progressively more spatial strategies. Tg mice also demonstrated higher incidences of characteristic looping behaviour (Janus, 2004; Brody and Holtzman, 2006). Vehicle-treated Tg mice showed delayed rates of transition to goal-directed spatial strategies compared to NonTgs. It is interesting to note that Tgs have successfully used these spatially imprecise strategies to exhibit comparable rates of learning to the NonTgs. *R. rosea*-treated Tg mice are able to switch to the use of more spatially-precise strategies earlier than the vehicle controls. Although *R. rosea* may be affecting the choice of strategy, this choice did not translate to better MWM performance. The mechanism by which *R. rosea* does this is not clear. It can be speculated that since hippocampal neurogenesis correlates with the adoption of spatially precise strategies (Gil-Mohapel et al., 2013), *R. rosea* may be promoting neural stem cell proliferation in the hippocampus (Chen et al., 2009b); however, this remains to be confirmed.

Perhaps the most striking effect of *R. rosea* was the increased resistance of Tg mice to mortality. Mortality rates observed in our vehicle-treated Tg group were comparable to those reported previously for mice on a mixed C57/C3H background (Chishti et al., 2001); the risk of death was nearly halved in the *R. rosea*-treated group. We have yet to establish whether *R. rosea* induces any changes in cortical and hippocampal Aβ deposition. High rates of mortality are commonly observed in transgenic APP mice due to cerebrovascular complications resulting from increased Aβ load (Lalonde et al., 2012b). TgCRND8 mice exhibit impaired cortical

microvasculature as a consequence of Aβ accumulation, thus increasing the risk of strokes (Dorr et al., 2012). Treatments that inhibit Aβ oligomerization and (or) fibrillization can attenuate these alterations in the vasculature, and may improve survival. *R. rosea* may be ameliorating vascular Aβ deposition or enhancing clearance, although this needs to be confirmed. Although we only followed the study cohorts until 5.5 months of age, future studies should aim to examine the impacts of *R. rosea* on the actual life-span of mice, both healthy controls and in a diseased state. Previous studies in other eukaryotes including nematodes, flies and yeast have shown that supplementation with *R. rosea* extends life span, although the molecular mechanism of action is still a topic of debate (Schriner et al., 2009b; Wiegant et al., 2009; Gospodaryov et al., 2013b; Gospodaryov et al., 2013a; Schriner et al., 2013). Resistance to stress and (or) reactive oxygen species may play important roles in this regard.

Our study provides an important first look into the effects of *R. rosea* in the context of neurodegenerative disorders as well as their associated behavioural and psychological symptoms. The Inuit value *R. rosea* as both a food and medicine and recognize it to be "good for the brain" (Clark and Cuerrier, 2012). While impacts of *R. rosea* on anxiety and learning and memory in male TgCRND8 mice are subtle, the most promising effect to date lies in its potential ability to improve survival in transgenic mice which are susceptible to high mortality rates, yet exhibit high levels of cognitive reserve. Median survival for AD patients from the onset of dementia to death is around 7.1 years (Fitzpatrick et al., 2005); *R. rosea* may have the potential to mitigate the toxic effects on vascular Aβ over the course of phenoconversion, extending, potentially, the efficacy of cognitive "boosters" including donepezil. Future studies directed towards the effects of *R. rosea* on vascular Aβ burden will help to elucidate possible mechanisms of action.

CHAPTER 6

Nunavik *Rhodiola rosea* administration in rats: effects on plasma and urine metabolomes

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Preface

In an effort to elucidate novel mechanisms of action of Nunavik *R. rosea* as well as to determine the metabolic fate of known *R. rosea* phytochemicals, we used a combination of non-targeted and targeted metabolomics approaches using UPLC-Q-TOF analytical platforms. Untargeted metabolomics strategies were implemented in conjunction with multivariate statistical techniques to examine the global profiles of plasma and urine metabolites from rats upon short-term treatment with vehicle or *R. rosea*. Targeted studies using reference standards were used to monitor phytochemicals in plasma and urine at pre- and post-dose time-points, as well as to track their availability in the brain. Further, the presence of commonly reported Phase I and Phase II biotransformation adducts were monitored in these tissues. The overall objective of this pilot study was to identify new metabolic pathways affected by *R. rosea*, as well as to determine the bioavailability of selected parent compounds and (or) metabolites in rat biofluids using our unique method of administration.

Statement of author contributions

FA and JTA conceived and designed this study. FA performed the pharmacokinetic study with the assistance of CC. UPLC-Q-TOF analyses and multivariate statistical comparisons using MarkerLynx software were performed with the assistance of AS. FA wrote the manuscript; AS and JTA reviewed the manuscript.

6.1 INTRODUCTION

Rhodiola rosea L. (Crassulaceae) is a medicinal plant of great traditional importance in Europe, Asia and the sub-Arctic regions of North America (Brown et al., 2002; Alm, 2004; Blondeau et al., 2010; Cuerrier and Elders of Kangiqsualujjuaq, 2011). Its broad spectrum applications in maintaining and improving mental and physical function, particularly under stressful conditions, has led to a large number of preclinical studies exploring the pharmacological actions of this plant (Ahmed et al., 2014a). Extensive phytochemical investigations of *R. rosea* rhizomes and (or) roots have revealed the presence of several classes of compounds (Ahmed et al., 2014b); yet, pharmacological and bioavailability studies in the literature disproportionately place emphasis on the phenylethanol derivative salidroside, which is not specific to *R. rosea* (Shen et al., 1999), and to a lesser degree, the phenylpropanoids, rosarin, rosavin and rosin.

The complexity of multi-component herbal extracts and the detection and (or) quantification of their phytochemical constituents in equally complex biological matrices makes pharmacokinetic studies especially challenging (Lan et al., 2013). Understanding of the absorption, distribution, metabolism and excretion (ADME) properties of *R. rosea*, or its effects upon levels of endogenous metabolites *in vivo*, particularly upon oral administration of the whole extract is limited. Bioavailability studies have focused on the absorption of individual marker entities, particularly salidroside, upon either intra-gastric or intravenous administration (Chang et al., 2007; Yu et al., 2008b; Guo et al., 2012; Zhang et al., 2013c; Guo et al., 2014a; Guo et al., 2014b). Several analytical methods, including HPLC with UV detection (Mao et al., 2007), HPLC coupled with mass spectrometry (MS) (Yu et al., 2008b), and HPLC with tandem mass spectrometry (MS/MS) (Chang et al., 2007; Guo et al., 2012; Zhang et al., 2013c; Guo et al., 2014a) have been used for the detection and quantification of *R. rosea* compounds in biological

matrices upon administration of pure compounds. However, pharmacokinetic parameters of salidroside are altered by the presence of other compounds when delivered as a complete extract in comparison to its pure form, resulting in different concentrations of compounds absorbed in the plasma, and thus, potentially different pharmacological effects (Li et al., 2006; Panossian et al., 2010b). Further, the metabolism of these compounds *in vivo* or the response of the endogenous metabolome to these xenobiotic interventions is unknown. It is important, therefore, to characterize the effects of the whole extract on the global metabolite pool so as to elucidate the basis for pharmacological activity of *R. rosea*.

Metabolomics, or the study of the set of endogenous small molecules (molecular weight < 1 kDa) in a biological system, and metabonomics, the perturbations in the metabolome of a system in response to an intervention (Holmes et al., 2008), are recently providing insight for many diverse applications, including pharmaceutical (Puchades-Carrasco and Pineda-Lucena, 2015) and traditional medicine research (Zhang et al., 2010a). Although both NMR spectroscopy and MS can be used as analytical platforms for metabolomics (Patti et al., 2012), the high sensitivity, accuracy, and resolving power of UPLC-MS technologies, coupled with TOF detection has proved to be a powerful tool for the analysis of herb-induced changes in endogenous metabolites as well as for elucidating pathways of xenobiotic metabolism (Wang et al., 2011). The simultaneous assessment of hundreds of metabolite features in complex matrices is increasingly being used to characterize and fingerprint plant populations from different regions (Ioset et al., 2011), to conduct poly-pharmacokinetics (Wang et al., 2011), to elucidate bioactive components in complex TCM herbal mixtures and their metabolites in biofluids (Ni et al., 2010; Tao et al., 2013; Yi et al., 2014; Zhang et al., 2014), etc.

As part of this pilot study, a rapid and sensitive UPLC-Q-TOF method was developed to compare the global metabolite profiles of control and Nunavik *R. rosea*-treated rat biofluids, namely plasma and urine, using a non-targeted metabolomics approach in an effort to tentatively identify distinguishing biomarkers upon short-term repeated oral administration. An ethanol extract of the roots of Nunavik *R. rosea* was suspended in sweetened condensed milk and administered orally in keeping with our studies on anxiety behaviour in rats (Chapter 2, section 2.2) as well as learning and memory performance in TgCRND8 mice (Chapter 5, section 5.2). Further, the absorption, excretion and metabolism of five key phenolics from *R. rosea* was tracked by analysis of plasma and urine at various sampling time-points. The objective of this study was to examine the impacts of *R. rosea* treatment on endogenous metabolite profiles in comparison to controls, and to provide preliminary insight into the metabolic fate of key phytochemicals to generate a platform for further analytical investigation into the metabolism of *R. rosea in vivo* as well as the identification of potential pharmacological targets.

6.2 EXPERIMENTAL

6.1.2 Plant material and extraction

Plant collection and extraction protocols are described in Chapter 5, Section 5.2.1.

6.2.2 Animals

Male Sprague-Dawley rats (Charles River Laboratories, St-Constant, Québec) were used for experiments. Animals were housed in groups of two in a temperature and humidity-controlled environment on a 12-hour light/dark cycle (lights on at 07:00 hours). Animals were habituated to a purified AIN-93M diet (TD.94048, Harlan Laboratories Inc.) for one week prior to the initiation of treatments. All experiments were conducted in accordance with the Canadian

Council of Animal Care, and were approved by the animal care committee of the University of Ottawa, Canada.

6.2.3 Extract Administration

Rats were randomly assigned to one of four treatment groups (N = 3 per group): untreated controls, vehicle (2 mL/kg) and two *R. rosea* extract doses; low dose (75 mg/kg body weight), and high dose (250 mg/kg body weight). *R. rosea* extract was suspended in 50% sweetened condensed milk (vehicle) and administered as described in Chapter 2, section 2.2.5; Cayer et al. (2013).

6.2.4 Dosing, sample collection and extraction

Following six days of daily treatments, animals were fasted for 12 hours with *ad libitum* access to water prior to the initiation of dosing and collection of tissue samples at selected time-points (Fig. 6.1). Plasma and urine samples (pooled from 3 animals) were collected before and after their respective treatments; brain tissue was collected at the time of sacrifice. Food was offered at 2 hours after initiation of the pharmacokinetic study.

Blood samples were collected in heparinized tubes by incisions made into the caudal vein, followed by centrifugation at 4000 rpm for 10 minutes at 4°C to obtain plasma. Urine samples were collected by manually expressing the bladder of individual animals onto sterile plastic sheets. At the end of the 8-hour study, animals were sacrificed by decapitation and whole brains were dissected. Samples were flash frozen and stored at -80°C for further analysis.

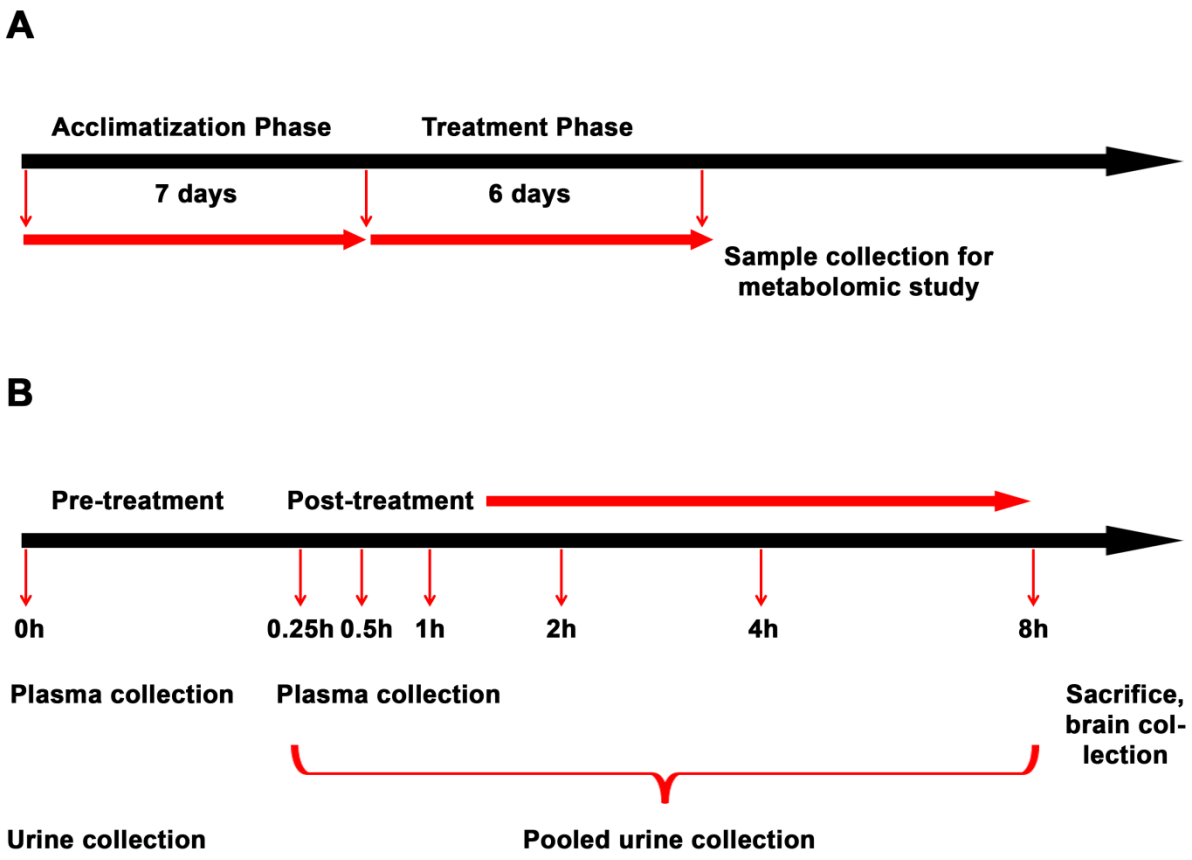


Figure 6.1 Schematic of the (A) treatment, and (B) tissue collection paradigm for the Nunavik *R. rosea* metabolomic study in Sprague-Dawley rats. Rats were orally administered vehicle (50 % sweetened condensed milk), *R. rosea* at a low (75 mg/kg) or high (250 mg/kg) dose for 6 days; an additional untreated control group was included (n = 3 for all groups). Plasma and urine samples from each treatment group (pooled from 3 animals) were collected prior to treatment on day 7 and post-treatment at selected time-points. Brains from individual animals were collected post-sacrifice after 8 hours post-treatment.

Prior to extraction, samples were thawed on ice. Plasma and urine extraction methods were based on protocols used by Yi et al. (2014). Briefly, 1.1 mL of methanol (LC-MS grade) was added to 100 µL of plasma, vortexed for 1 min, and then centrifuged for 10 min at 12, 000 rpm to precipitate proteins. The supernatant was dried using a stream of nitrogen gas, followed by re-constitution in 100 µL of LC-MS grade ACN:MeOH:H_2O (40:40:20) with 0.1 % formic acid. For urine samples, 0.1 mL of sample was mixed with a 10-fold volume of methanol, followed by a similar extraction protocol as described above. Brain tissue (cerebrum, 400 mg) was homogenized in 4 mL of methanol, sonicated for 10 min and stored at -20°C overnight. A 1 mL aliquot of the extract was centrifuged at 12, 000 rpm for 15 minutes, dried using a nitrogen evaporator, and then re-constituted in ACN:MeOH:H_2O (40:40:20) with 0.1 % formic acid.

6.2.6 UPLC-Q-TOF analyses

6.2.6.1. UPLC conditions

A Waters Acquity™ UPLC (consisting of a vacuum degasser, an autosampler, a binary pump, and an oven) was equipped with an ACQUITY UPLC® BEH C18 column (2.1 mm × 50 mm, i.d. 1.7 µm, Waters Corp., Milford, USA). The analytical column was maintained at a temperature of 50 °C and the mobile phase was composed of (A) acetonitrile, and, (B) water, with 0.1 % formic acid added to each. A solvent gradient system was used going from 5-95% A in 5 min, with a flow rate of 0.8 mL/min and an injection volume of 2 µL. The column was washed with 100% A from 5-5.50 min.

6.2.6.1. MS conditions

MS analysis was performed on a Q-TOF analyzer (Xevo-G2, Waters Corporation, Milford, MA, USA) in positive and negative ion modes (positive mode data was used for subsequent multivariate analyses). Low collision energy was set at 6V. The following optimized

parameters were used: scan time 0.08 seconds; sample cone voltage, capillary 3.2 kV for positive ionization and 2.5 kV for negative ionization extraction cone 4.0; 35 V; source temperature, 150 °C; desolvation temperature, 450 °C; desolvation gas flow, 1110 L/h; cone gas flow, 20 L/h. MS data were collected in the full scan mode from m/z 50-1000. All the data were acquired using an independent reference lock mass via the LockSpray™ interface to ensure accuracy and reproducibility during the MS analysis. Leucine enkephalin was used as the reference ion for positive ion mode ($[M+H]^+$ = 556.2771 at 2 ng/μL under a flow rate of 5 μL/min). Data were collected in the centroid mode, and the LockSpray frequency set at 15 s and averaged over 5 scans for correction. Following the UPLC-Q-TOF scan, the low energy (function 1) raw data were analyzed using MassLynx V4.1 SCN918 and MarkerLynx software (Waters Corp.). Peaks were detected within 0.3-5 min retention time window. Marker intensity threshold was set at 100 counts with in a mass window of 0.05 and the noise elimination level was set at 8. The intensity of each ion was normalized with respect to the total ion count to generate a data matrix.

6.2.3 Statistical Analyses

MarkerLynx software (Waters Corp.) was used for multivariate analysis to investigate the differences in metabolite signal intensity between groups of samples. Principal components analysis (PCA) followed by supervised two-class orthogonal partial least-squares discriminant analysis (OPLS-DA) models were utilized for the generation of S-plots, and the prioritization of distinguishing biomarkers. The METLIN metabolite database (Scripps center for metabolomics, https://metlin.scripps.edu) was used for tentative identifications (positive ion mode, 5-12 ppm accuracy range). For the detection of *R. rosea* phytochemicals in plasma, urine and brain tissue, comparisons to retention times, m/z ratios and MS fragmentation patterns of pure reference

standards (Chromadex, Irvine, CA, USA) at a detection limit of 3:1 signal-to-noise ratio were used.

6.3 RESULTS

Analysis of plasma and urine samples by UPLC-Q-TOF yielded approximately 1600 distinct metabolites, annotated by retention time and m/z data. Analysis of the plasma metabolomics data using PCA analysis demonstrated the clear separation of the control and vehicle groups from the *R. rosea*-treated groups (Fig. 6.2). The 250 mg/kg *R. rosea*-treated metabolome was not as closely clustered as the other groups, perhaps indicating a time-sensitive variation in metabolites. A "supervised" two-class OPLS-DA model considering the control and vehicle animals as a single group and the *R. rosea*-treated animals as the other group led to the isolation of eleven distinguishing biomarkers; their exact masses and retention times are presented in Fig. 6.3B. Two of these discriminating biomarkers were elevated in the *R. rosea*-treated animals, including anandamide (20:1, n = 9), belonging to the class of N-acyl ethanolamines (endocannabinoids) (2.5 times higher), and ascorbyl-palmitate (12.4 times higher) (Fig.6.3C).

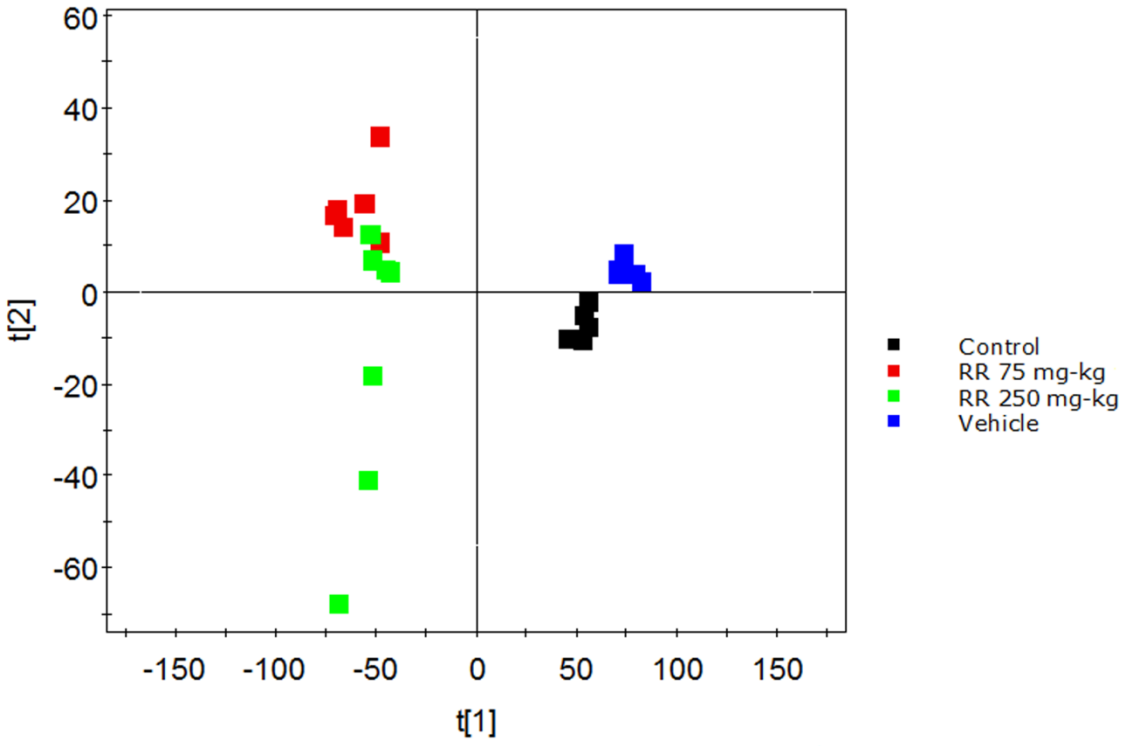
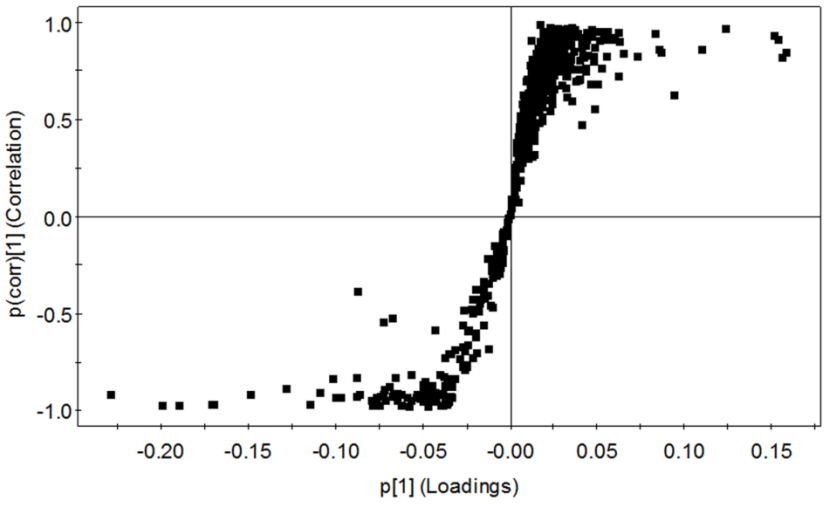


Figure 6.2 Principal components analysis of the metabolome of plasma samples from control (black), vehicle (blue), *R. rosea* (RR) at 75 mg/kg (red) and *R. rosea* (RR) at 250 mg/kg (green) treatment groups. The first two principal components are shown.

Figure 6.3 Tentative identification of discriminating markers between control (untreated and vehicle) and *R. rosea* (75 and 250 mg/kg)-treated rat plasma samples. (A)The co-variance p[1], and the correlation p(corr)[1] loadings from a two class OPLS-DA model (Control + Vehicle vs RR 75 mg/kg + RR 250 mg/kg) shown in S-plot format. Individual data points are exact mass/retention time pairs. (B) Average measured intensities of key discriminating markers (exact mass/retention time pairs) for the control and *R. rosea*-treated groups. (C) Structures of two proposed compounds elevated in *R. rosea*-treated plasma: anandamide (top) (2.83_376.3138, 12 ppm accuracy), and ascorbyl palmitate (bottom) (3.06_415.2678, 2 ppm accuracy).

A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-0.5
-1.0
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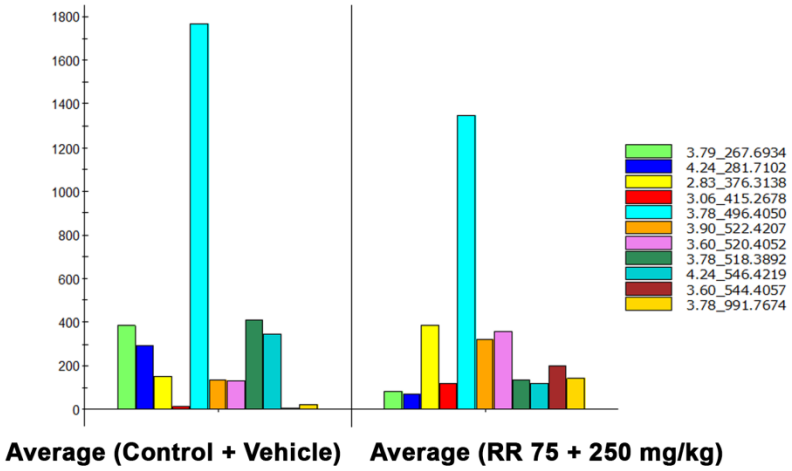
-0.00
0.05
0.10
0.15
p[1] (Loadings)

B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3.60_520.4052
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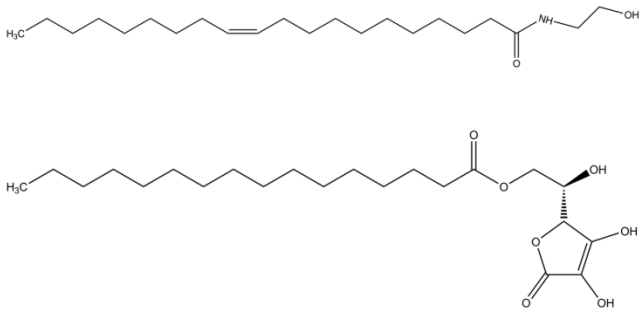
3.78_991.7674
Average (Control + Vehicle)
Average (RR 75 + 250 mg/kg)

C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OH
O
H3C
O
O
OH
O
OH
O
OH

PCA analysis of the urine metabolome did not lead to as clear-cut clusters, likely due to fewer sampling points (Fig.6.4). A supervised two-class OPLS-DA model with controls, vehicles and pre-treated *R. rosea* samples as one group and post-treated *R. rosea* samples as the second group revealed the presence of nine discriminant biomarkers (Fig. 6.5B). Two of these were salidroside and rosin which are species-specific secondary metabolites present in *R. rosea* roots (Fig. 6.5C).

Using a targeted approach using the extracted ion feature of the UPLC-Q-TOF-MS MassLynx^{TM} software, the presence of *R. rosea* reference standards salidroside, tyrosol, rosarin, rosavin, and rosin were evaluated in plasma, urine and brain tissue. The phytochemical markers (in their parent forms) were detected in the *R. rosea*-treated plasma samples, particularly at the high dose, at 2 hours post-treatment, but not at any of the other time-points. This indicates that perhaps maximal concentration is reached at 2 hours post-oral administration, although this remains to be confirmed by a true pharmacokinetic study. Salidroside was detected in both the low dose and high dose *R. rosea*-treated samples, while rosarin/rosavin and rosin were detected in the high dose-treated samples only (Table 6.1).

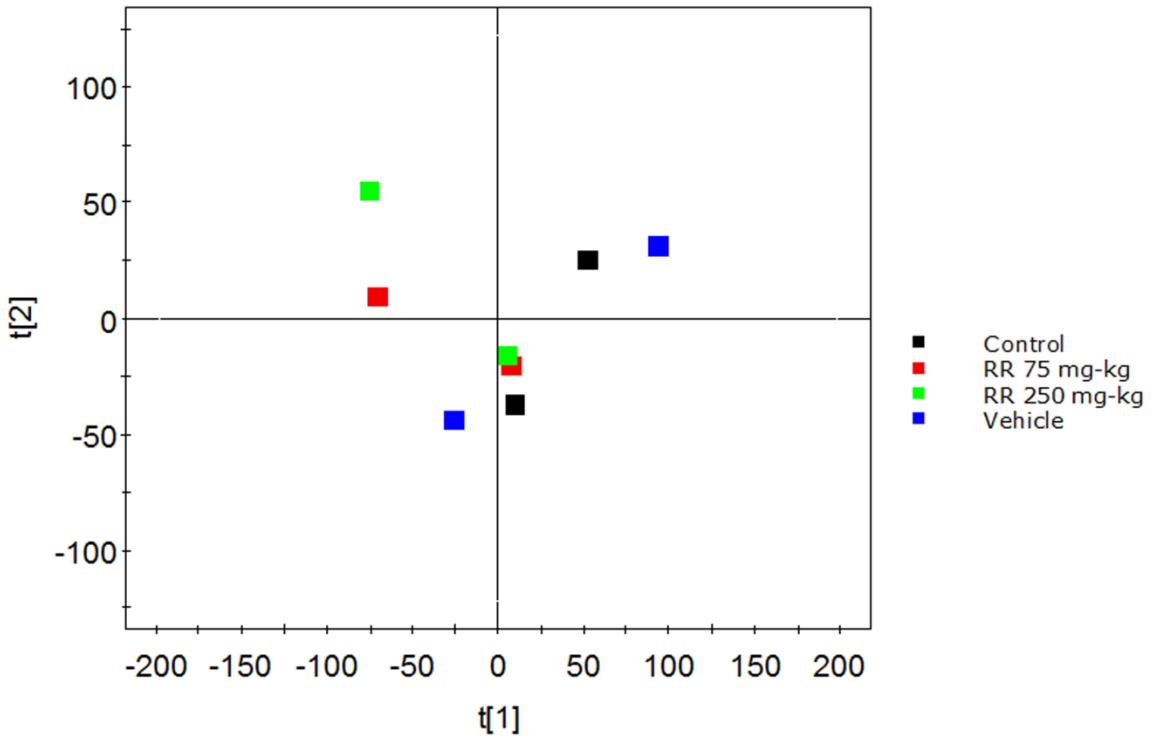
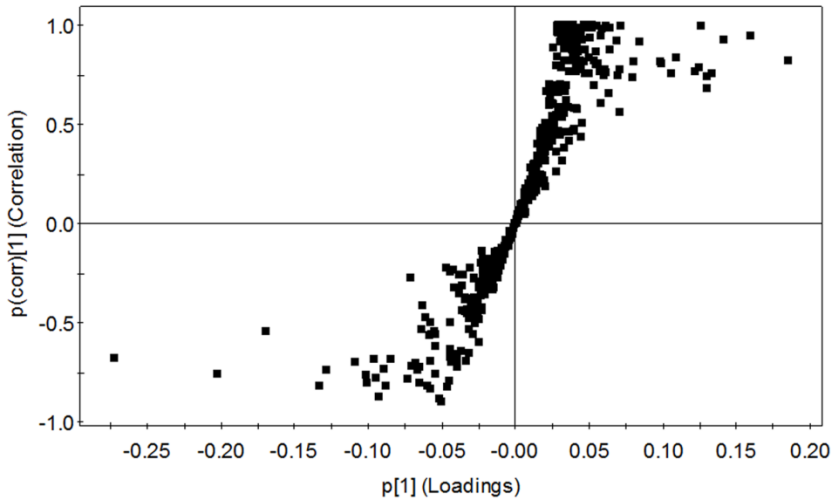


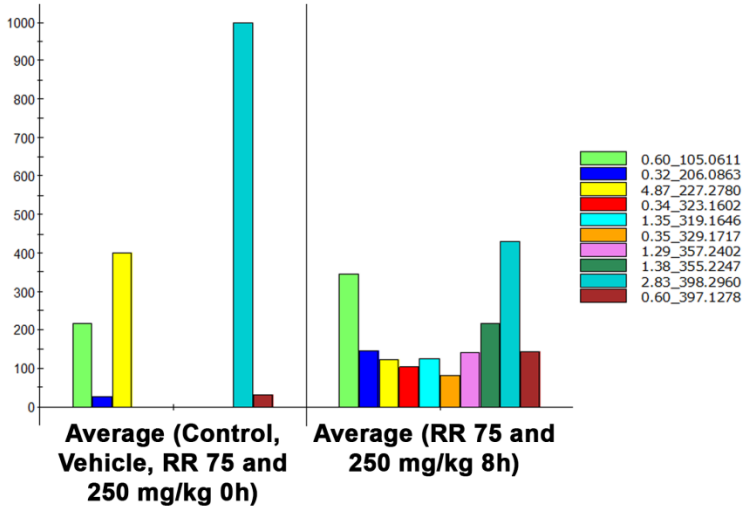
Figure 6.4 Principal components analysis of the metabolome of urine samples from control (black), vehicle (blue), *R. rosea* (RR) at 75 mg/kg (red) and *R. rosea* (RR) at 250 mg/kg (green) treatment groups. The first two principal components are shown.

Figure 6.5 Tentative identification of discriminating markers between control (untreated, vehicle and *R. rosea* pre-treatment 0h) and *R. rosea* (75 and 250 mg/kg, post-treatment 8h)-treated rat urine samples. (A)The co-variance p[1], and the correlation p(corr)[1] loadings from a two class OPLS-DA model (Control + Vehicle + RR 75, 250 mg/kg 0h vs RR 75 mg/kg + RR 250 mg/kg 8h) shown in S-plot format. Individual data points are exact mass/retention time pairs. (B) Average measured intensities of key discriminating markers (exact mass/retention time pairs) for the control and pre-treatment vs *R. rosea*-treated groups. (C) Structures of two compounds elevated in *R. rosea*-treated urine: salidroside (0.34_323.1602) (left), and rosin (1.35_319.1646) (right).

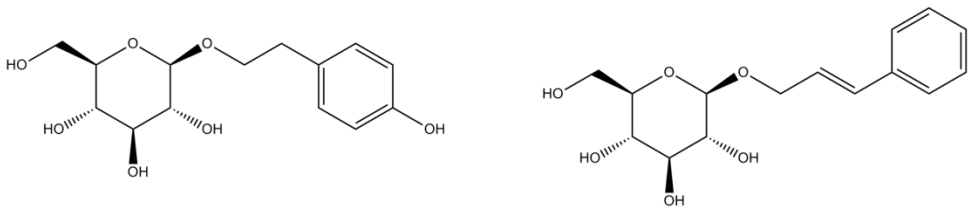
A


p(corr)[1] (Correlation)
1.0
0.5
0.0
-0.5
-1.0
-0.25
-0.20
-0.15
-0.10
-0.05
-0.00
0.05
0.10
0.15
0.20
p[1] (Loadings)

B


1000
900
800
700
600
500
400
300
200
100
0
Average (Control, Vehicle, RR 75 and 250 mg/kg 0h)
Average (RR 75 and 250 mg/kg 8h)
0.60_105.0611
0.32_206.0863
4.87_227.2780
0.34_323.1602
1.35_319.1646
0.35_329.1717
1.29_357.2402
1.38_355.2247
2.83_398.2960
0.60_397.1278

C


HO
O
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OH

Table 6.1 Detection of marker phytochemicals salidroside, tyrosol, rosarin, rosavin and rosin in the plasma of control, vehicle and *R. rosea*-treated rats using the extracted ion feature of UPLC-Q-TOF in the positive ion mode. The plus (+) sign indicates presence of the compound while the minus sign (-) indicates levels present below the limits of detection.

Treatment group	Marker compound	m/z	Time point						
			0h	0.25h	0.5h	1h	2h	4h	8h
Control	Salidroside	323.154	-	-	-	-	-	-	-
	Tyrosol	121.094	-	-	-	-	-	-	-
	Rosarin	451.214	-	-	-	-	-	-	-
	Rosavin	451.214	-	-	-	-	-	-	-
	Rosin	319.163	-	-	-	-	-	-	-
Vehicle	Salidroside	323.154	-	-	-	-	-	-	-
	Tyrosol	121.094	-	-	-	-	-	-	-
	Rosarin	451.214	-	-	-	-	-	-	-
	Rosavin	451.214	-	-	-	-	-	-	-
	Rosin	319.163	-	-	-	-	-	-	-
RR 75 mg/kg	Salidroside	323.154	-	-	-	-	+	-	-
	Tyrosol	121.094	-	-	-	-	-	-	-
	Rosarin	451.214	-	-	-	-	-	-	-
	Rosavin	451.214	-	-	-	-	-	-	-
	Rosin	319.163	-	-	-	-	-	-	-
RR 250 mg/kg	Salidroside	323.154	-	-	-	-	+	-	-
	Tyrosol	121.094	-	-	-	-	-	-	-
	Rosarin	451.214	-	-	-	-	+	-	-
	Rosavin	451.214	-	-	-	-	+	-	-
	Rosin	319.163	-	-	-	-	+	-	-

All of the plant markers, except tyrosol, were excreted in the urine in their parent forms within a time-frame of 8 hours post-oral administration for both the low and high *R. rosea* doses (Table 6.2). Extracted ion chromatograms of post *R. rosea*-treated urine samples clearly revealed the presence of *R. rosea* marker compounds which were not observed in pre-treated samples from the same animals (Appendix B). These compounds were below the limits of detection in brain tissue of *R. rosea*-treated animals (Table 6.3).

In order to determine whether *R. rosea* reference compounds underwent metabolic transformation, the m/z ratios of all the biomarkers detected in plasma and urine of the study subjects were screened for biotransformation adducts resulting from commonly occurring Phase 1 and Phase 2 metabolic reactions. Of these, only a few types of possible adducts were observed, including demethylation, hydroxylation, and desaturation (Phase I) and glycine conjugations (Phase II) (Table 6.4). Interestingly, tyrosol, which was not detected in its parent form in any plasma or urine samples, was the most likely candidate among the *R. rosea* phytochemicals for biotransformation reactions.

Table 6.2 Detection of marker phytochemicals salidroside, tyrosol, rosarin, rosavin and rosin in the urine of control, vehicle and *R. rosea*-treated rats using the extracted ion feature of UPLC-Q-TOF in the positive ion mode. The plus (+) sign indicates presence of the compound while the minus sign (-) indicates levels present below the limits of detection.

Treatment group	Marker compound	m/z	Time point	
			0h	8h
Control	Salidroside	323.154	-	-
	Tyrosol	121.094	-	-
	Rosarin	451.214	-	-
	Rosavin	451.214	-	-
	Rosin	319.163	-	-
Vehicle	Salidroside	323.154	-	-
	Tyrosol	121.094	-	-
	Rosarin	451.214	-	-
	Rosavin	451.214	-	-
	Rosin	319.163	-	-
RR 75 mg/kg	Salidroside	323.154	-	+
	Tyrosol	121.094	-	-
	Rosarin	451.214	-	+
	Rosavin	451.214	-	+
	Rosin	319.163	-	+
RR 250 mg/kg	Salidroside	323.154	-	+
	Tyrosol	121.094	-	-
	Rosarin	451.214	-	+
	Rosavin	451.214	-	+
	Rosin	319.163	-	+

Table 6.3 Detection of marker phytochemicals salidroside, tyrosol, rosarin, rosavin and rosin in the brain tissue of control, vehicle and *R. rosea*-treated rats using the extracted ion feature of UPLC-Q-TOF in the positive ion mode. The plus (+) sign indicates presence of the compound while the minus sign (-) indicates levels present below the limits of detection.

Treatment group	Marker compound	m/z	Presence/absence
Control	Salidroside	323.107	-
	Tyrosol	121.076	-
	Rosarin	451.157	-
	Rosavin	451.156	-
	Rosin	319.115	-
Vehicle	Salidroside	323.107	-
	Tyrosol	121.076	-
	Rosarin	451.157	-
	Rosavin	451.156	-
	Rosin	319.115	-
RR 75 mg/kg	Salidroside	323.107	-
	Tyrosol	121.076	-
	Rosarin	451.157	-
	Rosavin	451.156	-
	Rosin	319.115	-
RR 250 mg/kg	Salidroside	323.107	-
	Tyrosol	121.076	-
	Rosarin	451.157	-
	Rosavin	451.156	-
	Rosin	319.115	-

Table 6.4 Potential biotransformation reactions of reference compounds in plasma and urine of *R. rosea*-treated rats.

Sample	Compound	m/z (biotransformation reaction)	Formula	Description	Metabolism Phase	Measured m/z	Calculated m/z	Error
Plasma	Salidroside	1.9793	CH_2+O	De-methylation + hydroxylation	1	321.1794	321.1747	0.0047
	Tyrosol	57.0215	C_2H_3NO	Glycine conjugation	2	178.1181	178.1155	0.0026
	Rosin	57.0215	C_2H_3NO	Glycine conjugation	2	376.1845	376.1848	-0.0003
Urine	Tyrosol	14.0157	CH_2	De-methylation	1	107.0763	107.0783	-0.002
	Tyrosol	27.9949	CO	De-carbonylation	1	93.097	93.0991	-0.0021
	Tyrosol	13.9793	O-H_2	Hydroxylation + desaturation	1	135.0745	135.0733	0.0012
	Rosin	2.0157	H_2	Desaturation	1	317.148	317.1473	0.0007

6.4 DISCUSSION

In this pilot study, a short-term one-week intervention protocol with *R. rosea* at low (75 mg/kg) and high (250 mg/kg) doses altered the metabolomes of rat plasma and urine. Multivariate analyses revealed several discriminating biomarkers, levels of which were markedly changed in the plasma of *R. rosea*-treated animals compared to controls. Tentative identification using METLIN Scripps database searches demonstrated that levels of N-cis-11 – eicosaenoylethanolamine, or anandamide (20: 1, n-9), belonging to the class of endocannabinoids, and a fat-soluble derivative of ascorbic acid, ascorbyl palmitate, were elevated in the plasma of *R. rosea*-treated animals. In the urine of *R. rosea*-treated animals (post-treatment phase), salidroside and rosin were among several biomarkers that were significantly elevated compared to controls and *R. rosea* pre-treated samples. Analysis of plant reference compounds revealed detectable concentrations at 2 hours post-treatment in the plasma and elimination via the renal pathway. Several biotransformed metabolites of these compounds were potentially identified, including Phase I and II metabolites, although further validation experiments are essential for confirmation.

The present study is unique compared to previously reported metabonomic studies on herbal extracts. For this study, the *R. rosea* extract was suspended in 50 % sweetened condensed milk and delivered orally, as was previously published (Cayer et al., 2013). The vehicle contains a high percentage of lipids; the bioavailability of the constituent compounds may be different than if the extract was solubilized in water, for instance. Further, we used a repeated dosing regimen of *R. rosea* and the patterns of bioavailability may vary based on the type of dosing schedule i.e. single dose or longer-term experiments. While direct comparisons to existing studies are not feasible, in one of the few studies to examine bioavailability upon oral

administration of the entire extract, salidroside and rosavin reached their maximum concentrations in plasma at 1 hour post-administration, and declined within 5-6 hours of administration (Panossian et al., 2010b). In the present study, *R. rosea* markers were excreted in their parent form within 8 hours of treatment; further studies with more intermediate urine collection time-points would enable a more accurate estimation of elimination time. Levels were below the limits of detection in brain tissue; the only other study to report salidroside detection in the brain administered pure salidroside intravenously (Zhang et al., 2013c). This pattern of low bioavailability and rapid elimination is similar to that observed for other dietary polyphenols (Manach et al., 2004).

Repeated daily treatment with Nunavik *R. rosea* led to changes in global plasma metabolite profiles compared to untreated and vehicle controls, albeit in a small group of animals, which could be attributed to the presence of plant compounds, their metabolites and (or) endogenous responses to these xenobiotics. Several discriminating biomarkers were listed by the UPLC-Q-TOF MarkerLynx analysis software; however, identification of these proved to challenging using existing database searches. Two compounds that were elevated in *R. rosea*-treated groups were tentatively identified as anandamide (20: 1, n-9), and ascorbyl palmitate. While the pharmacological activities of anandamide (20:4, n-6) as a neuromodulatory and neuroprotective agent are intensively studied (Luchicchi and Pistis, 2012), less is known about the specific biological activity of this particular endocannabinoid-like ligand. It may act as an agonist of the PPAR-α, similar to other monounsaturated fatty acid ethanolamides, such as oleoylethanolamide, and play roles in lipid metabolism and energy homeostasis (Fu et al., 2003) or exhibit analgesic and anti-inflammatory activities (Suardiaz et al., 2007). Another compound, tentatively identified as ascorbyl palmitate, was also elevated in *R. rosea*-treated plasma samples.

Ascorbic acid (or its fatty acid ester) has not previously been reported in *R. rosea*; it is likely derived from the rat diet formulation (AIN-93M, Harlan Laboratories) which contains a mix of vitamins; administration with *R. rosea* seemingly increased the bioavailability of this antioxidant (given that food intake was similar between treatment groups). However, it is important to note that these are tentative identifications, and thus, further verification and quantification needs to be performed using reference standards.

PCA analyses on the urine control and pre-treatment samples compared to post-treatment samples revealed that two *R. rosea*-markers, salidroside and rosin were highly elevated in the latter group. These phenolics are clearly being processed by the urinary route, although examination of biliary and (or) fecal samples will provide a more complete picture of the elimination process. Ideally, levels of bioactive compounds need to be elevated for sufficient periods of time at specific concentrations for therapeutic effects− *R. rosea* may need to be administered daily for observation of pharmacological effects. Although many common biotransformation adducts were screened for detection of potential metabolites of these common *R. rosea* phenolics, only a few matches were found in the plasma and urine, which would require further verification, possibly with high collision energy experiments. There may be other *R. rosea* compounds in the plasma being absorbed in their intact parent form, as a metabolite or bound to plasma proteins (Manach et al., 2004), the detection of which will form the basis of future studies.

Despite the limitations of this pilot study, which include its small sample sizes and tentative identification of discriminating marker compounds, this first investigation into the effects of Nunavik *R. rosea* on the global plasma and urine metabolomic profiles generates interesting platforms for further exploration. Slow absorption of key phenolics into the plasma as

well as their rapid elimination highlights the importance of conducting pharmacological and (or) pharmacokinetic studies on the whole extract; studies assessing the effects of single compounds may not be representative. Characterization of the complete secondary metabolite profile of *R. rosea*, as well as a detailed study of the bioactive components i.e. those absorbed into the plasma as well as their metabolites are essential next steps for understanding of pharmacology as well as safety for this widely used medicinal plant.

CHAPTER 7

General Discussion

7.1. OVERVIEW OF RESULTS AND CLAIMS TO ORIGINALITY

The work presented in this thesis examines, for the first time, the biological activities and safety of Canadian (Nunavik) populations of *R. rosea*, a traditional Inuit food and medicinal plant. Ethnobotanical uses of Nunavik populations for optimal mental health as well as their distinct phytochemical and genetic attributes compared to Eurasian populations of the same species led to the present investigation of their potency as an anxiolytic and as a neuroprotective agent in a transgenic mouse model of AD, a debilitating neurodegenerative disorder. This thesis further attempted to lay the groundwork in terms of determining the safety of Nunavik *R. rosea*, particularly via assessments of *in vitro* herb-drug interaction potential, a vital aspect of the development of any good quality NHP. The global impact of *R. rosea* on rat plasma and urine metabolomes were evaluated in order to determine not only the fate of known phytochemical markers, but also to elucidate novel metabolites that are altered so as to gain insight into new avenues of biological action of *R. rosea*. Information about pharmacological activity as well as safety would provide supporting evidence for, 1) the validation of traditional Inuit medicinal knowledge, 2) the development of safe, affordable, accessible and culturally relevant phytomedicinal products in these rural communities, and, 3) the establishment of commercial micro-enterprises based on sustainable cultivation and harvesting practices for local economic and social benefit.

The characterization of anxiolytic activities in rats upon short-term administration was the first *in vivo* behavioural study on Canadian populations of *R. rosea* (Chapter 2; (Cayer et al., 2013)). Reduction of anxiety-like traits in the elevated-plus maze and fear conditioned response tests, but not in the social interaction test, indicated specific pharmacological modes of action. There were no previous reports of the activity of this plant on the GABAergic system. A minor

role as a weak agonist at the $GABA_A$-BZD receptor suggested that Nunavik *R. rosea* may be exerting anxiolytic effects via other neurotransmitter systems. Amelioration of anxiety in rats signalled the potential ability of the constituents of a phytochemically characterized extract of this traditional medicine to permeate the blood brain barrier, and the possibility of its use for the management of AD cognitive deficits and neuropsychiatric symptoms.

Following preliminary evidence of activity, Nunavik *R. rosea* as well as a selected number of commercial *R. rosea* NHPs, primarily from North American companies, were assessed for their ability to affect selected human CYP enzymes, as well as Phase I and II enzymes from mixed-donor pooled human liver microsomes (Chapter 3). While *in vitro* data does not necessarily amount to clinical relevance, it does provide an initial idea of possible risk and allows for the selection of potential candidates for further *in vivo* and clinical investigation. To our knowledge, this was the first time that these NHPs, and their phenolic constituents, namely salidroside, tyrosol, rosarin, rosavin and rosin, were tested for their drug interaction potential. Based on these *in vitro* assays, the risk of interaction, assessed by the inhibition of CYP3A4, CYP3A5 and CYP2D6 isoforms, was found to be minimal, especially in comparison to well-known inhibitors goldenseal and Labrador tea. However, the presence of additives in the commercial products may very well have led to a diluted effect, and thus, an underestimation of CYP inhibition by the botanical extract. Alcohol and water preparations of the above products did not vary in their inhibitory potential; however, water extracts prepared from botanical materials may yield different results. Nunavik *R. rosea* was found to moderately affect the human liver microsome-mediated metabolism of repaglinide, a commonly prescribed conventional anti-diabetic drug (Bidstrup et al., 2003), as well as the formation of its glucuronide metabolite. Taken together, the potential of Nunavik *R. rosea* to cause drug interaction events

appears to be low but cannot be completely ruled out, and further tests on other key CYPs as well as *in vivo* studies are warranted.

The next two sections in this thesis dealt with the assessment of the efficacy of Nunavik *R. rosea* in the context of AD, the first study to directly do so on any *R. rosea* variety. Spatial learning and memory of control and *R. rosea*-treated mice in the TgCRND8 mouse model of AD was assessed by the reference memory version of the MWM task. In order to probe the under-utilized positional data generated by Ethovision® XT, the MWM tracking software used at the University of Ottawa Behaviour Core Facility, and to enhance the understanding of the effects of prophylactic interventions on performance behaviour, a novel methodology, a neural network-based algorithm which we termed MWM Visual, was developed and optimized (Chapter 4). This application enabled the automatic visualization and classification of search strategies adopted by mice to search for the hidden platform, providing a way to depict and quantify the entirety of search behaviour. Irrespective of the complexity of the dataset, MWM Visual maintained a consistently good agreement with manual consensus rating, indicating reliable scoring. MWM Visual is currently usable for three different pool-platform settings; future versions will aim towards incorporating additional arena configurations. This tool, along with MWM Searcher, has the potential to greatly reduce experimenter bias and expedite the post-scoring data analysis process.

Contrary to our initial hypothesis, no improvements in learning and memory performance were observed in the MWM task in *R. rosea*-treated mice using conventional escape latency or path efficacy measures. Search strategy analysis using MWM Visual revealed a slightly earlier adoption of goal-oriented spatial strategies by *R. rosea*-treated Tgs, but this did not lead to an overall increase in the use of these more efficient routes or a decrease in escape latency. In

retrospect, the study design itself may have hindered the detection of any subtle effects of *R. rosea* on learning and memory. In particular, the long-term daily handling of test mice, the incorporation of additional behavioural assays prior to MWM testing, as well as the exclusive use of Tg males, which were recently shown to demonstrate behavioural indices of higher cognitive reserve (Granger et al., 2015), may have obscured genotypic differences between the controls, thus making it challenging to assess the impact of *R. rosea* intervention. Future studies should address efficacy in females.

Long-term Nunavik *R. rosea* administration did not lead to any observable effects on basal anxiety levels in this AD model, beyond an increased exploration in the light-dark test, contrary to predictions based on results from Chapter 2. As discussed in Chapter 5, these mice have altered anxiety profiles precipitated by impaired GABAergic inhibition in the hippocampus (Ma and McLaurin, 2014). Although only one pertinent target was investigated in this thesis, the weak activities of *R. rosea* at one of the components of the GABAergic system suggests that *R. rosea* may not modulate the imbalance between excitatory and inhibitory neurotransmission, resulting in higher observed hyperactivity in both vehicle and treatment Tgs compared to wild-types. Attempts to replicate the anxiolytic effects in wild-type C57BL/6 mice were unsuccessful, likely due to species-specific differences in xenobiotic metabolism (Lim et al., 1994; Born et al., 2003; Martignoni et al., 2006), and (or) methodological variations. Interestingly, *R. rosea* reduced the high mortality exhibited by the males of this transgenic line, the first time that this has been demonstrated in a mammalian model, albeit to a modest degree. Examining the effects of *R. rosea* on the levels and fibrillization of neurotoxic soluble Aβ oligomers in the brain, as well as on vascular Aβ burden will be important next steps towards establishing a mode of action of this plant.

The effects of *R. rosea* on the metabolome of rat biofluids were evaluated for the first time using UPLC-Q-TOF techniques, and provided evidence of large-scale global changes in metabolite levels upon oral administration (Chapter 6). Elucidation of these discriminating metabolites may lead to the identification of new targets of action of *R. rosea*, and shed light on the metabolic fate of known markers. Multivariate statistical tools enabled the tentative identification of an endogenous cannabinoid-like ligand, anandamide (20:1, n-9), that was elevated in *R. rosea*-treated samples, with possible roles in lipid metabolism (Fu et al., 2003), and (or) modulation of inflammation (Suardiaz et al., 2007). Phytochemical markers of *R. rosea* were absorbed slowly into the plasma and eliminated relatively rapidly in their native form, or metabolically transformed via Phase I and II enzymatic processes. Markers in their parent form were either below the limits of detection in the brain, present as metabolites, or were unable to cross the blood brain barrier, thus calling into question whether these highly studied compounds are in fact, reaching effective therapeutic concentrations *in vivo*, or if their metabolites and (or) other phytochemicals are responsible for activity. This study emphasizes the need for more pharmacological studies using crude extracts as opposed to those that assess the effects of these compounds in isolation.

Collectively, this set of investigative studies constitutes the first foray into the knowledge about the efficacy of Nunavik *R. rosea* in the context of AD, anxiety and its safety. Several important milestones in the development of a good quality NHP, including evaluation in different animal models, utilization of different administration and dosing regimens, metabolism and safety were covered in this thesis. Preliminary evidence of novel activities suggests that further mechanistic studies on this traditional Inuit remedy are merited.

7.2 COMPARISONS TO LITERATURE

Despite its long-established uses in the context of multiple neurological disorders, including for "enhancing the intellect" (Halldorsson, 1783; Panossian et al., 2010a), surprisingly, studies of *R. rosea* in biologically relevant models of AD are few. *In vitro* studies have shown protective effects of *R. rosea* and (or) its most well-studied phytochemical, salidroside, on pathways implicated in AD pathogenesis, including Aβ-induced cytotoxicity (Zhang et al., 2010b), oxidative stress (Qu et al., 2012), inflammation (Pooja et al., 2009; Gao et al., 2015), cholinergic deficits (Hillhouse et al., 2004; Wang et al., 2007a), and glutamate toxicity (Palumbo et al., 2012). *R. rosea* extract and (or) salidroside reversed cognitive deficits in rats induced by Aβ, streptozotocin or D-galactose insult in the MWM and the passive avoidance task by mitigating elevated levels of oxidative stress and inflammatory markers (Qu et al., 2009; Zhang et al., 2013a; Gao et al., 2015). To date, there are no placebo-controlled clinical trials on the efficacy of *R. rosea* monotherapy for AD patients, although a small-scale study of *R. rosea* in combination with additional vitamins and minerals on adults with cognitive complaints i.e. forgetfulness and deficiencies in concentration, has shown promise (Fintelmann and Gruenwald, 2007).

R. rosea dietary supplementation has been shown to extend life-span in various model organisms, including *Caenorhabditis elegans* (Wiegant et al., 2009), and *Drosophila melanogaster* (Jafari et al., 2007; Schriner et al., 2009b; Schriner et al., 2013), though the mechanisms involved are still a matter of debate. Unlike resveratrol, a plant-derived phenolic with health benefits, which appears to promote longevity by the activation of sirtuins (Sin et al., 2015), a family of NAD+-dependent histone deacetylases (Morris, 2013), *R. rosea* does not seem to act via this pathway, or other pathways involved in caloric restriction-induced life-span

extension, including insulin and insulin-like growth factor signalling, and the target of rapamycin (TOR) (Schriner et al., 2013). Some studies suggest that *R. rosea* may promote longevity by the reduction of mitochondrial ROS levels (Schriner et al., 2009b), or the activation of transcription factors involved in the regulation of genes with roles in stress resistance and detoxification (Wiegant et al., 2009).

Several plants with traditional uses in the treatment of neurological disorders, particularly from TCM, Ayurvedic and European pharmacopeias (Table 7.1) have been assessed in preclinical and clinical trials for their efficacy as potential AD therapeutics (Table 7.2). The advantage of botanicals for the use of AD is apparent in their pleiotropic modes of action− the extracts and their phytochemicals not only attenuate Aβ levels, but also possess other bioactivities relevant to AD, including the modulation of cholinergic, dopaminergic and glutamatergic neurotransmission (Wake et al., 2000; Zhang and Hu, 2001; Park et al., 2002; Choudhary et al., 2005; Wang et al., 2006; Berger et al., 2011; Zhou et al., 2011; Geromichalos et al., 2012; Kehr et al., 2012; Chu et al., 2014; Kim et al., 2014; Li et al., 2014a). Several mechanisms are involved in plant extract or phytochemical-mediated neuroprotection against Aβ, including the amelioration of Aβ-induced oxidative stress, inflammation and apoptosis (Shi et al., 2009; Wan et al., 2014), promotion of APP cleavage via α-secretases (Colciaghi et al., 2004; Durairajan et al., 2011), inhibition of β and γ-secretases (Jia et al., 2004; Kang et al., 2013) and inhibition of Aβ oligomerization and fibrillogenesis (Kumar et al., 2012; Xie et al., 2014), to name a few. The quality of supporting evidence for anti-AD activity differs, ranging from extensive preclinical and multi-center placebo-controlled clinical studies for *Ginkgo biloba* (ginkgo) and *Huperzia serrata* (toothed clubmoss), to *Melissa officinalis* (lemon balm) and *Salvia* spp. (sage) with one or two clinical studies, to *Withania somnifera* and *Centella asiatica*

(gotu kola) with solely preclinical evidence. Active molecules include compounds from all three major classes of plant secondary metabolites, including alkaloids, terpenes and phenolics (Fig. 7.1).

Table 7.1 Traditional uses of selected plants with preclinical and clinical evidence in AD and anxiety

Plant species	Family	Traditional uses (medicinal system)
Centella asiatica (L.) Urban	Apiaceae	To enhance intelligence and memory, promote wound healing (Kapoor, 1990) (TCM, Ayurveda)
Crocus sativus L.	Iridaceae	To treat memory problems (Akhondzadeh, 2007) (Iran)
Ginkgo biloba L.	Ginkgoaceae	To treat memory loss due to circulatory disorders (Ross, 2001; Howes and Houghton, 2003) (Iran, Europe); Remedy for coughs/asthma ((Chinese Pharmacopeia Commission, 2005) (TCM)
Huperzia serrata (Thunb. ex Murray) Trev.	Huperziaceae	To treat schizophrenia, contusions, strains, swelling, rheumatism, myasthenia gravis, and fever (Ma et al., 2007b) (TCM)
Hypericum perforatum L.	Hypericaceae	To treat neurosis, anxiety, depression, sedation, excitability, neuralgia (Barnes et al., 2007) (Europe)
Melissa officinalis L.	Lamiaceae	To treat memory loss, melancholia, neuroses and hysteria, and to promote long life (Howes and Houghton, 2009) (Europe)
Panax ginseng C. A. Mey.	Araliaceae	Acts as a stimulant/tonic, to enhance mental and physical performance, to provide resistance to stress, to prevent exhaustion and disease (Hügel, 2015) (TCM)
Polygala tenufolia Willd.	Polygalaceae	To treat neuropsychological disease (Perry and Howes, 2011); to relieve insomnia, neurasthenia, amnesia, depression, anxiety-related palpitations, restlessness, disorientation, dementia, and memory failure (Huang, 1993) (TCM, Kampo)

Table 7.1 Traditional uses of selected plants with preclinical and clinical evidence in AD and anxiety (continued)

Plant species	Family	Traditional uses (medicinal system)
Salvia officinalis L./ *S. lavandulaefolia* Vahl./ *S. miltiorrhiza* Bunge	Lamiaceae	To treat memory disorders (Blumenthal et al. 2000) (Europe); To promote blood circulation, end blood stagnation, and tranquilize the mind (Chen et al. 2014) (TCM)
Scutellaria lateriflora var. grohii Boivin/ *S. baicalensis* Georgi	Lamiaceae	To treat anxiety and related disorders (Felter and Lloyd, 1905); sedative properties (Zhu, 1998) (TCM, Kampo, North America)
Withania somnifera L. (Dunal)	Solanaceae	To promote long life, youthful vigour, and good intellectual powers (Sivarajan and Balachandran, 1994) (Ayurveda)

Table 7.2 Activities of selected plants and their active molecules in cognitive aspects of AD at the preclinical and clinical level.

Plant species	Active molecule (s)	Anti-Amyloidogenic activity	Alternate bioactivities	Preclinical evidence	Clinical observations	Safety information
Centella asiatica (L.) Urban	-Triterpene saponosides -Caffeoylquinic acids	-Mitigated Aβ-induced cell death, alterations in tau expression and phosphorylation (Gray et al., 2014)	-Inhibited $cPLA_2$ and $sPLA_2$ activities (Defillipo et al., 2012)	-Improved cognitive performance in passive avoidance task in STZ-injected rats (Veerendra Kumar and Gupta, 2003)	-N/A in AD patients -Enhanced working memory in healthy elderly (Wattanathorn et al., 2008).	-*In vitro*: inhibited CYP2C9, CYP2D6 and CYP3A4 activities (Pan et al., 2010); 2C19 (Pan et al., 2011); CYP1A2 (Savai et al., 2015)
Crocus sativus L.	-Carotenoids (crocins) -Terpenes	-Inhibited $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ fibrillogenesis (Papandreou et al., 2006; Ghahghaei et al., 2013)	-Inhibited acetylcholinesterase (Geromichalos et al., 2012)	-Significantly attenuated STZ-induced learning and memory impairment in passive avoidance test (Khalili and Hamzeh, 2010)	-Preliminary evidence of cognitive benefits in mild-to-moderate AD, similar efficacy to donepezil (Akhondzadeh et al., 2010). -Comparable to memantine in reducing cognitive decline in patients with moderate to severe AD (Farokhnia et al., 2014)	-*In vivo*: safe and well-tolerated in healthy volunteers (Modaghegh et al., 2008)
Ginkgo biloba L.	-Flavonoid glycosides -Terpene trilactones (ginkgolide A, B, C, J), bilobalide	-Blocked Aβ-induced apoptosis, ROS accumulation, mitochondrial dysfunction and activation of c-jun N-terminal kinase (JNK) (Shi et al., 2009)	-Increased dopamine and acetylcholine levels in rat prefrontal cortex (Kehr et al., 2012)	-Improved cognitive function in TgCRND8 mice in the Barnes Maze test (Liu et al., 2015); Tg2576 mice in the MWM (Stackman et al., 2003)	-Meta-analyses: Positive effects on cognition, activities of daily living for AD, vascular or mixed dementia with BPSD (Gauthier and Schlaefke, 2014; Tan et al., 2015a; von Gunten et al., 2015)	-Generally regarded as safe; caution advised when administered in conjunction with anti-coagulants (Diamond and Bailey, 2013)

Table 7.2 Activities of selected plants and their active molecules in cognitive aspects of AD at the preclinical and clinical level (continued).

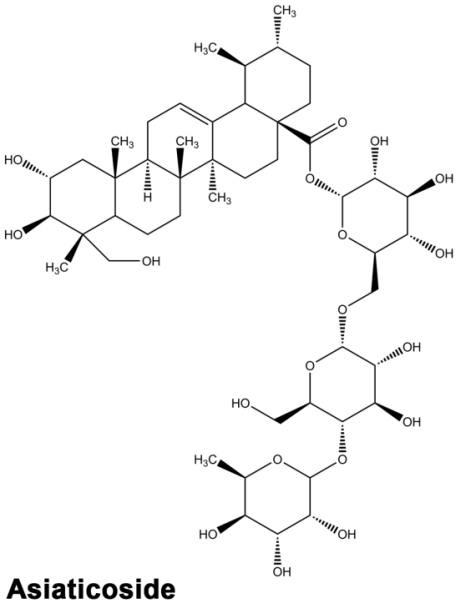
Plant species	Active molecule (s)	Anti-Amyloidogenic activity	Alternate bioactivities	Preclinical evidence	Clinical observations	Safety information
Huperzia serrata (Thunb. ex Murray) Trev.	-Alkaloid (Huperzine A)	-Reduced insoluble and soluble Aβ levels, and amyloid plaque formation in APP/PS1transgenic AD mice via reduction of iron uptake (Huang et al., 2014)	-Potent, reversible inhibitor of acetylcholinesterase (Wang et al., 2006)	-Improved learning and memory in the MWM in triple transgenic AD mice (Ratia et al., 2013)	-Meta-analyses: positive effects on cognition, activities of daily living, behavioural disturbance, and functional performance in patients with AD and vascular dementia, with recommendation for better-designed trials, (Li et al., 2008; Yang et al., 2013; Xing et al., 2014)	-*In vivo*: pro-drug ZT-1 well-tolerated in healthy human volunteers (Jia et al., 2013)
Hypericum perforatum L.	-Flavonoids (hyperoside) -Phloroglucinol derivatives (hyperforin, tetrahydrohyperforin)	-Reduced intra-cerebral soluble $A\beta_{42}$ species by increasing Aβ excretion into the bloodstream via ABCC 1 transporter (Hofrichter et al., 2013)	-Decreased levels of tau hyper-phosphorylation (Inestrosa et al., 2011)	-Restored cognitive function by reducing plaque pathology in APP-Tg mice in the MWM (Inestrosa et al., 2011)	-N/A in AD patients -Did not enhance memory in healthy volunteers ((Ellis et al., 2001; Siepmann et al., 2002)	-*In vitro, in vivo*: Induced cytochrome P450 (CYP) isozymes, such as CYP3A4, CYP2C19, CYP2C9, and the P-glycoprotein (PgP) transporter; not to be taken with immune-suppressants or cardiovascular drugs (Rahimi and Abdollahi, 2012)

Table 7.2 Activities of selected plants and their active molecules in cognitive aspects of AD at the preclinical and clinical level (continued).

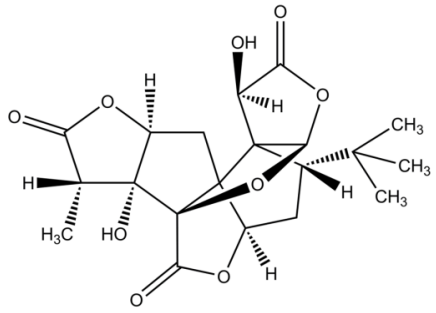
Plant species	Active molecule (s)	Anti-Amyloidogenic activity	Alternate bioactivities	Preclinical evidence	Clinical observations	Safety information
Melissa officinalis L.	-Flavonoids (rosmarinic acid and derivatives) -Monoterpene aldehydes and glycosides	-Protective against Aβ-induced oxidative changes and cell death (Sepand et al., 2013)	-Acetylcholine receptor binding activity, including nicotinic and muscarinic (Wake et al., 2000) -Inhibited acetylcholinesterase activity (Dastmalchi et al., 2009)	-Ameliorated scopolamine-induced learning deficits in rats in the MWM (Soodi et al., 2014)	-Improved cognitive performance in patients with mild-to-moderate AD (Akhondzadeh et al., 2003a)	-Generally regarded as safe
Panax ginseng C. A. Mey.	-Triterpenoid saponins (ginsenosides)	-Lowered Aβ levels by modulating γ-secretase activity via activation of lipid kinase, PI4KIIα (Kang et al., 2013) -Reduced $A\beta_{40}$ and $A\beta_{42}$ levels via enhancing neprilysin gene expression (Yang et al., 2009)	-Regulation of cholinergic neurotransmission (Chu et al., 2014; Kim et al., 2014)	-Attenuated cognitive deficits in the MWM test and passive avoidance test in advanced glycation endproducts-injected rats (Tan et al., 2015b)	-Open label study: improved cognitive scores in AD patients (Lee et al., 2009b) -Meta-analyses: inconclusive; dearth of high quality rigorously controlled studies (Lee et al., 2009b; Geng et al., 2010)	-*In vivo*: Induced CYP3A activity in the liver and possibly the gastrointestinal tract; no effect on PgP activity in healthy volunteers (Malati et al., 2012)
Polygala tenufolia Willd.	-Saponins (tenuifolin) -Oligosaccharide esters -Cinnamic acid derivatives	-Decreased Aβ production via BACE-1 inhibition (Jia et al., 2004)	-Inhibited acetylcholinesterase activity (Park et al., 2002)	-Abolished scopolamine-induced cognitive impairments in rat in the passive avoidance and water maze test (Park et al., 2002)	-None in AD patients -Improved memory in the elderly compared to placebo controls (Shin et al., 2009)	-*In vitro*: Inhibited CYP2E1; no effect on CYP1A2, CYP2A6, CYP2C8/9/19, CYP2D6, CYP3A (Li et al., 2014b)

Table 7.2 Activities of selected plants and their active molecules in cognitive aspects of AD at the preclinical and clinical level (continued).

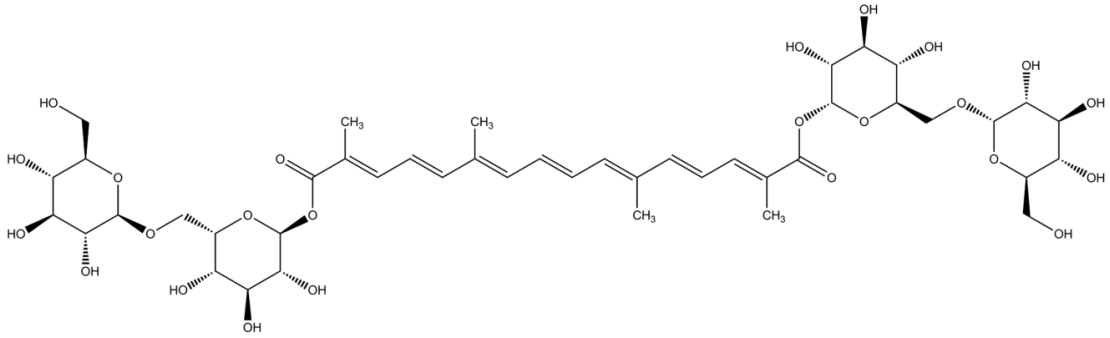
Plant species	Active molecule (s)	Anti-Amyloidogenic activity	Alternate bioactivities	Preclinical evidence	Clinical observations	Safety information
Salvia officinalis L./ *S. lavandulaefolia* Vahl./ *S. miltiorrhiza* Bunge	-Monoterpenes (1,8-cineole, α- and β-pinene -Polyphenols (salvianolic acid B, rosmarinic acid) -Diterpene quinones, (tanshinone II A, cryptotanshinone)	-Inhibited aggregation of Aβ into fibrils and degrades preformed fibrils (Durairajan et al., 2008; Wang et al., 2013b) -Reduced ROS formation, lipid peroxidation, DNA fragmentation, caspase-3 activation, and tau hyperphosphorylation (Iuvone et al., 2006)	-Inhibited acetylcholinesterase (Zhou et al., 2011)	-Improved spatial learning and memory in APP/PS1 mice in MWM (Mei et al., 2009).	-Enhanced cognitive performance, attention in mild to moderate AD patients (Akhondzadeh et al., 2003b; Perry et al., 2003)	-*In vivo*: Induced mouse CYP1A, CYP2C and CYP3A (Kuo et al., 2006)
Scutellaria lateriflora var. grohii Boivin/ *S. baicalensis* Georgi	-Flavonoids (baicalein, baicalin)	-Inhibited Aβ oligomerisation and toxicity (Lu et al., 2011) -Reduced Aβ production by increasing non-amyloidogenic processing of APP (Zhang et al., 2013b)	- Alleviated endoplasmic reticulum stress-induced apoptosis (Choi et al., 2010)	-Reduced learning impairments in the radial arm water maze (Zhang et al., 2013b)	-None in AD patients	-*In vivo*: Inhibited CYP3A4, PgP (Cho et al., 2011); CYP1A2 (Gao et al., 2014) in rats
Withania somnifera L. (Dunal)	-Steroid lactones (Withanolides) -Withanosides -Alkaloids -Sitoindosides	-Inhibited the formation of mature Aβ fibrils (Kumar et al., 2012) -Reduced deposition of Aβ in brain vasculature by enhanced expression of low-density lipoprotein receptor-related protein in the liver (Sehgal et al., 2012)	-Inhibited acetylcholinesterase, butyrylcholinesterase (Choudhary et al., 2005)	-Mitigated behavioural deficits in the radial arm maze in APP/PS1 mice (Sehgal et al., 2012)	-None in AD patients	-*In vitro*: Did not inhibit CYP1A2, 2C9 (Savai et al., 2015) -*In vivo*: Did not effect CYP3A4 (Savai et al., 2013)



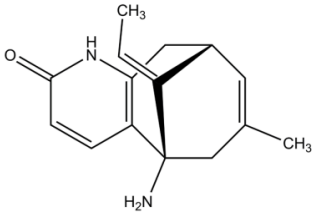
Asiaticoside



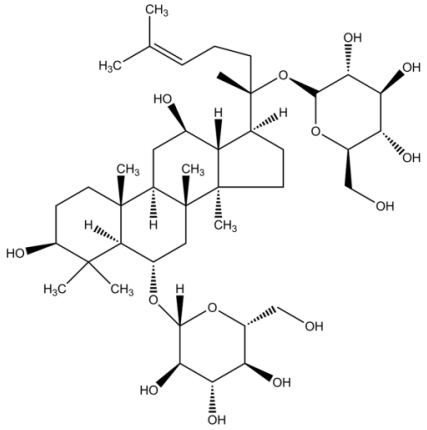
Ginkgolide A



Crocin

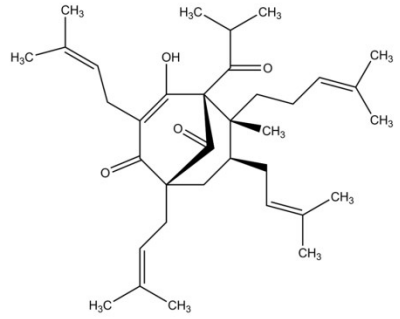


Huperzine A

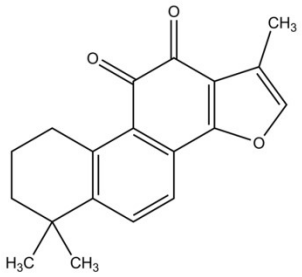


Ginsenoside Rg1

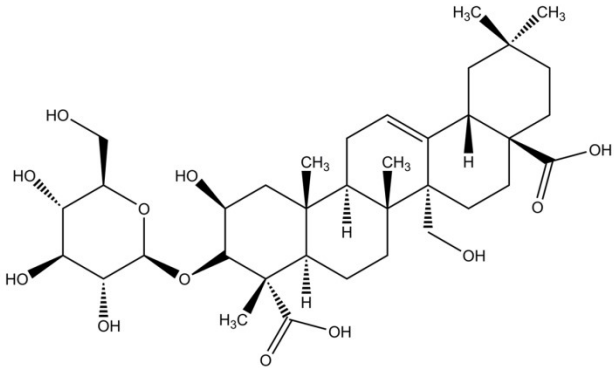
Figure 7.1 Chemical structures of bioactive phytochemicals from botanicals with potential activities in the context of AD.



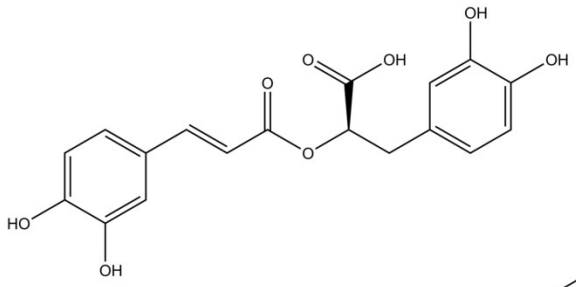
Hyperforin



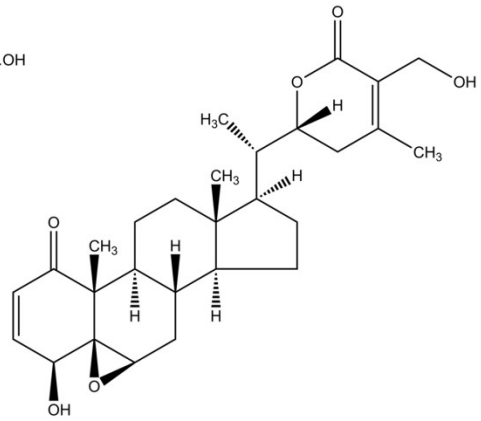
Tanshinone II A



Tenuifolin



Rosmarinic acid



Withaferin A

Figure 7.1 (continued) Chemical structures of bioactive phytochemicals from botanicals with potential activities in the context of AD.

Given the rising popularity of *R. rosea* as an herbal supplement (Smith et al., 2015), studies on safety are surprisingly few, and likely long overdue. *In vitro* studies indicate potent inhibition of CYP3A4 (Scott et al., 2006; Hellum et al., 2010) and P-glycoprotein (Hellum et al., 2010) by *R. rosea* extracts, though this does not necessarily translate to perturbations of co-administered drug pharmacokinetics *in vivo* (Panossian et al., 2009b) or adverse events in clinical trials (Mao et al., 2015). Variations in raw material due to abiotic and biotic factors as well as methods of extract preparation will lead to inevitable differences in phytochemical composition, pharmacological activity and risk of drug interactions (Vieira and Huang, 2012). Thus, effects on CYP inhibition from one particular *R. rosea* NHP cannot be reliably extrapolated to another, and it is recommended that manufacturing companies ensure phytochemical standardization of their products as well as utilize high throughput assays for the determination of any drug interaction risk.

Studies on the safety of anti-AD herbals are variable, ranging from *in vitro* studies only to clinical trials in healthy human volunteers (Table 7.2). Most of these extracts are safe and well tolerated, with mild to moderate side-effects (Gurley et al., 2005; Modaghegh et al., 2008; Wang et al., 2009a; Diamond and Bailey, 2013). However, some phytochemicals, for instance, baicalin from *Scutellaria baicalensis* (skullcap), alters plasma levels of co-administered drugs *in vivo* (Gao et al., 2014). Others, including St. John's wort, *Hypericum perforatum*, are well-known inducers of multiple CYP isoforms and efflux transporters, and are contraindicated for use with immunosuppressant and cardiovascular drugs (Rahimi and Abdollahi, 2012).

Anxiety disorders are among the most common mental illnesses in Canada; nearly 12 % of the population is affected at any given time (Health Canada, 2002). Anxiety is also one of the most prevalent neuropsychiatric symptoms of AD (Steinberg et al., 2008). An estimated 43% of

people suffering from anxiety use some type of complementary therapy, including herbal medicine (Ernst, 2006). Anxiolytic activity of *R. rosea* has been demonstrated previously in mice, though mechanisms of action were not elucidated (Perfumi and Mattioli, 2007; Montiel-Ruiz et al., 2012). The neurobiology of anxiety involves abnormalities of multiple neurotransmitter signalling networks (Nutt et al., 2002), as reflected in the clinical efficacy of selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs), and benzodiazepines (Tyrer and Baldwin, 2006) for treatment. Serotonergic involvement (Chen et al., 2009b) or monoamine oxidase inhibition (van Diermen et al., 2009) may be responsible for the observed anxiolytic activity of *R. rosea*. In humans, there is preliminary evidence of *R. rosea* (Rhodax®) for generalized anxiety disorders, although clinical trials with rigorous placebo controls are needed to establish anxiolytic activity conclusively (Bystritsky et al., 2008).

With the exception of *Huperzia serrata*, plants with evidence of anti-AD activity also possess anxiolytic properties (Table 7.3). While this is by no means an exhaustive compilation of anxiolytic herbs, it is important to note that these plants may have efficacy in mitigating both the cognitive and behavioural aspects of AD. Modulation of GABAergic neurotransmission seems to be the primary mechanism involved in the anxiolytic activity of these plants. Extracts and (or) active molecules act via differential activation of GABA receptors (Candelario et al., 2015), activation of glutamate decarboxylase (GAD) (Lee et al., 2013b), the enzyme responsible for the synthesis of GABA from glutamic acid, inhibition of GABA-Transaminase (GABA-T) (Awad et al., 2007), which degrades GABA, or binding to the $GABA_A$-BZD receptor (Ceremuga et al., 2015).

Table 7.3 Activities of selected plants and their active molecules as anxiolytics in preclinical and clinical studies.

Plant species	Active molecule (s)	Mode of action	Preclinical evidence	Clinical observations
Centella asiatica (L.) Urban	-Asiatic acid -Triterpenoids: (madecassoside and asiaticoside)	-Modulation of BZD site, $GABA_A$ receptor (Ceremuga et al., 2015) -Activation of GAD, GABA-T inhibition (Awad et al., 2007)	-Anxiolytic in rats in the EPM (Ceremuga et al., 2015); mice under chronic immobilization stress in EPM, LD and OF tests (Wanasuntronwong et al., 2012)	-Attenuated the peak acoustic startle response amplitude 30 and 60 minutes after treatment; no effects on self-rated mood (Bradwejn et al., 2000) -Positive benefits on anxiety related disorders, stress and associated depression (Jana et al., 2010)
Crocus sativus L.	-Crocins -Safranal	-$GABA_A$-BZD receptor complex (Hosseinzadeh and Sadeghnia, 2007)	-Anxiolytic effects in rats in LD (Pitsikas et al., 2008); mice in EPM (Hosseinzadeh and Noraei, 2009)	-
Ginkgo biloba L.	-Ginkgolide A -Bilobalide	-Regulated expression of glucocorticoid receptors in hippocampus (Ma et al., 2012) -Normalized stress-induced elevation in brain levels of catecholamines (NE, DA), 5-HT and plasma corticosterone (Shah et al., 2003)	-Anxiolytic in EPM, OF in mice (Kuribara et al., 2003; Ma et al., 2012)	-Reduced neuropsychiatric symptoms, including anxiety in patients with AD/vascular dementia (Scripnikov et al., 2007); MCI (Gavrilova et al., 2014); generalized anxiety disorders upon long term administration; (Woelk et al., 2007)
Hypericum perforatum L.	-Hyperforin derivative	-$GABA_A$-BZD receptor complex -anxiolytic activity blocked by pre-treatment with benzodiazepine antagonist Flumazenil (Vandenbogaerde et al., 2000).	-Anxiolytic in EPM, OF in rats (Can et al., 2011; Husain et al., 2011)	-

Table 7.3 Activities of selected plants and their active molecules as anxiolytics in preclinical and clinical studies (continued).

Plant species	Active molecule (s)	Mode of action	Preclinical evidence	Clinical observations
Melissa officinalis L.	-Rosmarinic acid -Triterpenoids (ursolic acid, oleanolic acid)	-Decreased serum corticosterone levels, boosted GABA levels in mouse dentate gyrus (Yoo et al., 2011) -GABA-T inhibition (Awad et al., 2007)	-Anxiolytic effects in rats in EPM (Taiwo et al., 2012); mice in EPM, not in OF (Ibarra et al., 2010)	-Open-label study: ameliorated anxiety manifestations and anxiety-associated symptoms in stressed volunteers with mild-to-moderate anxiety disorders and sleep disturbances (Cases et al., 2011) -In combination with *Valeriana officinalis*, showed anxiolytic activity and modulated mood in healthy volunteers (Kennedy et al., 2006a) -Increased calmness in healthy volunteers (Kennedy et al., 2002; Kennedy et al., 2003; Kennedy et al., 2004)
Panax ginseng C. A. Mey.	-Ginsenosides (Rb1, Rg1, Rg3, Rg5 and Rk)	-$GABA_A$ receptor activation via the γ_2 subunit, enhanced GABA current (Lee et al., 2013a)	-Anxiolytic in EPM in mice (Cha et al., 2005; Carr et al., 2006; Kim et al., 2009)	-Modest benefits in anxiety symptoms in fibromyalgia patients (Braz et al., 2013)
Polygala tenufolia Willd.	-Polygalasaponins -3,4,5-Trimethoxycinnamic acid (TMCA)	-Increased activation of GAD, expression of γ-subunit of $GABA_A$ receptors in the cerebellar granule cells (Lee et al., 2013b)	-Anxiolytic in mice in hole-board, EPM, OF (Yao et al., 2010)	-

Table 7.3 Activities of selected plants and their active molecules as anxiolytics in preclinical and clinical studies (continued).

Plant species	Active molecule (s)	Mode of action	Preclinical evidence	Clinical observations
Salvia officinalis L./ *S. lavandulaefolia* Vahl./ *S. miltiorrhiza* Bunge	-Terpenes (α-pinene) -Rosmarinic acid	-$GABA_A$-BZD receptor binding (Kavvadias et al., 2003) -Inhibited acetylcholinesterase and butyrylcholinesterase (Kennedy et al., 2006b) -GABA-T inhibition (Awad et al., 2007)	-Anxiolytic in EPM in mice (Satou et al., 2014)	-Reduced anxiety, increased alertness and calmness in healthy volunteers; effects were dose-dependent (Tildesley et al., 2005; Kennedy et al., 2006b)
Scutellaria lateriflora var. grohii Boivin/ *S. baicalensis* Georgi	-Flavonoids (wogonin, baicalein)	-Modulation of the $GABA_A$-BZD receptor (Hui et al., 2002) -GABAergic non-benzodiazepine sites involved, not 5-HT system (de Carvalho et al., 2011)	-Anxiolytic in mice in EPM (de Carvalho et al., 2011); in rats in EPM, OF (Awad et al., 2003)	-No difference in anxiety symptoms compared to placebo; improved overall mood (Brock et al., 2014)
Withania somnifera L. (Dunal)	-Glycowithanolides	-Activated inotropic $GABA_A$ channels; potent agonist of GABAρ1 receptors (Candelario et al., 2015)	- Anxiolytic in rats in EPM, SI (Bhattacharya et al., 2000); social isolation-induced anxiety (Gupta and Rana, 2007)	-Meta-analyses: Better than placebo on anxiety or stress scales, concerns about variation in study design and potential bias (Pratte et al., 2014)

Studies on the bioavailability of *R. rosea* phenolic compounds following repeated oral administration of the crude extract are very limited. However, the bioavailability of salidroside has been studied. It is affected by the presence of other compounds in the *R. rosea* crude extract in comparison to when administered alone, resulting in altered concentrations of compounds in the plasma (Li et al., 2006; Panossian et al., 2010b). Extensive preclinical evidence of the biological activities of other plant-derived polyphenols against pertinent AD targets exists, particularly in the case of green tea catechins (Walker et al., 2015), resveratrol (Porquet et al., 2014), oleuropein (Grossi et al., 2013), and curcumin (Zhang et al., 2015). In general, dietary polyphenols, a large and chemically diverse class of compounds, are poorly absorbed, rapidly eliminated, and extensively metabolized, making studies of bioavailability particularly challenging (Singh et al., 2008). For instance, curcumin, a bioactive curcuminoid from the rhizomes of *Curcuma longa* L. (Zingiberaceae), undergoes extensive hepatic and intestinal metabolism, particularly glucuronidation and sulfation, which leads to low systemic availability following oral administration (Sharma et al., 2007). Similarly, resveratrol, a bioactive phytoalexin, is present in the plasma not as its aglycone, but primarily as glucuronide and sulfate conjugates (Gambini et al., 2015), and concentrations in the brain are less than 1 % of the oral dose administered (Abd El-Mohsen et al., 2006). Repeated administration of grape seed polyphenols, however, did lead to improved bioavailability of epicatechin and catechins in the brain of rats as compared to acute dosing (Ferruzzi et al., 2009). Other strategies to improve the bioavailability of these neuroprotective compounds include co-administration with other natural products such as piperine that inhibit Phase II glucuronidation (Suresh and Srinivasan, 2010) and the use of nanoparticle formulations, liposomes and phospholipid-complexed polyphenols (Anand et al., 2007; Ray et al., 2011).

7.3 FUTURE RESEARCH

Over the course of this project, it became apparent that in the existing ethnobotanical studies of the Inuit, or other First Nations peoples in Canada in general, there was a noticeable lack of information regarding the use of medicinal plants for ADRDs, with *R. rosea* use by the Inuit being an exception (Clark and Cuerrier, 2012). The relatively recent increase in AD prevalence (Pace, 2013), as well as the social stigma surrounding this category of disorders (Sutherland, 2007), may have led to this negative bias in the ethnobotanical literature. The growing awareness of ADRDs in First Nations communities as an illness as opposed to a natural part of the aging process, and the preference for culturally relevant remedies for treatment purposes (Jacklin and Warry, 2012) may encourage further dialogue between ethnobotanists and community healers on remedies used for the treatment of AD symptoms.

The mechanism of the anxiolytic activity of Nunavik *R. rosea* in rats needs to be determined. While the $GABA_A$-BZD receptor remains a key molecular target for anxiolytics, modulation of GABAergic neurotransmission by inhibiting the catabolism of GABA by GABA-T, enhancing synthesis by activation of GAD, regulating its transport by GABA transporter (GAT-1) or inhibiting its re-uptake are some other potential sites of anxiolytic action (Kent et al., 2002). Indeed, in a screening study of anxiolytic botanicals, *M. officinalis* (lemon balm) was shown to inhibit GABA-T, whereas *C. asiatica* (gotu kola) stimulated GAD activity (Awad et al., 2007). Serotonin is also important in the regulation of anxiety, and the 5-$HT1_A$ receptor is particularly important in the context of anxiety disorders (Akimova et al., 2009); in fact, partial agonists of this receptor are well-established anxiolytics (Taylor, 1988). Previous indications of *R. rosea* boosting serotonin levels in the hippocampus of rats under chronic mild stress have been reported (Chen et al., 2009b). Further, amelioration of symptoms of nicotine withdrawal by

R. rosea was abolished by an antagonist of the 5-HT1$_A$ receptor, indicating that this may be a viable target (Mannucci et al., 2012). Given the (tentative) evidence of elevated levels of an endogenous cannabinoid-like molecule in *R. rosea*-treated rat plasma, an interesting new study would be to assess whether *R. rosea* has inhibitory effects on Fatty Acid Amide Hydrolase (FAAH), the enzyme responsible for the intracellular degradation of anandamide (Gaetani et al., 2003). FAAH inhibitors have been shown to possess anxiolytic-like activity in rats (Kathuria et al., 2003). Naturally occurring flavonoids, including kaempferol which has been isolated from *R. rosea* (Dubichev et al., 1991), are potent inhibitors of FAAH (Thors et al., 2008).

An important step towards elucidating the life-span extension mechanism of *R. rosea* in TgCRND8 mice would be to assess the burden of Aβ in cerebral vasculature. Accumulation of Aβ peptides leads to vasoconstriction, vascular degeneration and consequently, a higher risk of haemorrhages and stroke (Perlmutter, 1994; Miao et al., 2005; Tian et al., 2006). The TgCRND8 mouse model exhibits progressive impairments in vascular architecture and function due to the presence of both soluble Aβ oligomers and insoluble amyloid deposits (Dorr et al., 2012). Naturally occurring cyclohexanehexol stereoisomers, including scyllo-inositol, have been shown to reverse changes in vascular Aβ levels and increase survival in these mice (McLaurin et al., 2006). The impact of *R. rosea* on other mechanisms involved in promoting longevity, namely inhibition of markers of oxidative stress (ROS) and inflammation (TNF-α, interleukins) would provide further insight (Si and Liu, 2014). Additionally, the effects of the *R. rosea* extract should be assessed on the natural life-span of wild-type and transgenic AD mice to see whether the protective effects are still valid. In order to clarify the effects of *R. rosea* on learning and memory, the inclusion of female TgCRND8 mice with lower behavioural indices of cognitive reserve in the MWM (Granger et al., 2015), assessment of learning and memory separately,

instead of at the end of a battery of other tasks which may influence performance, and the incorporation of tasks more sensitive to detection of cognitive impairments, including the radial arm water maze (Stewart et al., 2011), may be useful.

Despite the reported safety of *R. rosea* in clinical trials, the issue of phytoequivalence must be kept in mind, i.e. results from phytochemically different extracts in terms of efficacy and safety should not be extrapolated to new NHPs. In a case study of a patient with bipolar disorder and attention deficit hyperactivity disorder, intake of *R. rosea* supplements and other NHPs concomitantly with anti-depressants and anxiolytics led to mania-like symptoms, dizziness, nausea and irritability (Khamba, 2013). Although it is difficult to say which drug, NHP, or combination thereof led to these reactions, further safety testing of *R. rosea* should be implemented. Population studies of the rates of use of *R. rosea* in conjunction with known AD or other conventional drugs are essential for gauging the likelihood of encountering such interactions. Inhibitory effects on other major Phase I enzymes, including CYP2C8/9, CYP1A2, and CYP2C19 isoforms, and Phase II enzymes (UDP-glucuronosyltransferases) should be tested. Co-administration of Nunavik *R. rosea* and donepezil, a conventional anti-AD drug, and a substrate of CYP3A4 and CYP2D6 (Tiseo et al., 1998), followed by the subsequent examination of pharmacokinetic parameters in the plasma would provide a higher level of evidence for the possibility of interactions, and enable planning for further safety and tolerability testing in clinical studies in humans.

REFERENCES

Abd El-Mohsen M, Bayele H, Kuhnle G, Gibson G, Debnam E, Kaila Srai S, Rice-Evans C, Spencer JP (2006) Distribution of [^{3}H]trans-resveratrol in rat tissues following oral administration. The British journal of nutrition 96:62-70.

Abidov M, Grachev S, Seifulla RD, Ziegenfuss TN (2004) Extract of *Rhodiola rosea* radix reduces the level of C-reactive protein and creatinine kinase in the blood. Bulletin of experimental biology and medicine 138:63-64.

Abidov M, Crendal F, Grachev S, Seifulla R, Ziegenfuss T (2003) Effect of extracts from *Rhodiola rosea* and *Rhodiola crenulata* (Crassulaceae) roots on ATP content in mitochondria of skeletal muscles. Bulletin of experimental biology and medicine 136:585-587.

Abonyi S, Favel M (2012) Marie's Story of Aging Well: Toward New Perspectives on the Experience of Aging for Aboriginal Seniors in Canada. Anthropology and Aging Quarterly 33:11-20.

Adams M, Gmunder F, Hamburger M (2007) Plants traditionally used in age related brain disorders--a survey of ethnobotanical literature. Journal of ethnopharmacology 113:363-381.

Adedapo A, Jimoh F, Afolayan A (2011) Comparison of the nutritive value and biological activities of the acetone, methanol and water extracts of the leaves of *Bidens pilosa* and *Chenopodium album*. Acta poloniae pharmaceutica 68:83-92.

Adelson N (2000) Being alive well: health and the politics of Cree well-being. Toronto: University of Toronto Press.

Afanas'ev SA, Lishmanov YB, Lasukova TV, Naumova AV (1997) Effect of *Rhodiola rosea* on the resistance of isolated heart from stressed rats to ischemic and reperfusion damage. Bulletin of experimental biology and medicine 123:447-449.

Ahmed F, Bennett SAL, Arnason JT (2014a) Pharmacological activities of *Rhodiola rosea*. In: Rhodiola rosea (Cuerrier A, Ampong-Nyarko K, eds), pp 189-204. Boca Raton, FL: CRC Press.

Ahmed F, Filion V, Saleem A, Arnason JT (2014b) Phytochemistry of *Rhodiola rosea*. In: Rhodiola rosea (Cuerrier A, Ampong-Nyarko K, eds), pp 65-86. Boca Raton, FL: CRC Press.

Akgul Y, Ferreira D, Abourashed EA, Khan IA (2004) Lotaustralin from *Rhodiola rosea* roots. Fitoterapia 75:612-614.

Akhondzadeh S (2007) Herbal medicine in the treatment of psychiatric and neurological disorders. In: Low-Cost Approaches to Promote Physical and Mental Health: Theory, Research and Practice. (Abate L, ed), pp 119-138. New York, NY, USA: Springer.

Akhondzadeh S, Noroozian M, Mohammadi M, Ohadinia S, Jamshidi AH, Khani M (2003a) *Melissa officinalis* extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomised, placebo controlled trial. Journal of neurology, neurosurgery, and psychiatry 74:863-866.

Akhondzadeh S, Noroozian M, Mohammadi M, Ohadinia S, Jamshidi AH, Khani M (2003b) *Salvia officinalis* extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomized and placebo-controlled trial. Journal of clinical pharmacy and therapeutics 28:53-59.

Akhondzadeh S, Shafiee Sabet M, Harirchian MH, Togha M, Cheraghmakani H, Razeghi S, Hejazi SS, Yousefi MH, Alimardani R, Jamshidi A, Rezazadeh SA, Yousefi A, Zare F, Moradi A, Vossoughi A (2010) A 22-week, multicenter, randomized, double-blind controlled trial of *Crocus sativus* in the treatment of mild-to-moderate Alzheimer's disease. Psychopharmacology 207:637-643.

Akimova E, Lanzenberger R, Kasper S (2009) The serotonin-1A receptor in anxiety disorders. Biological psychiatry 66:627-635.

Al Omari A, Murry DJ (2007) Pharmacogenetics of the Cytochrome P450 Enzyme System: Review of Current Knowledge and Clinical Significance. Journal of Pharmacy Practice 20:206-218.

Ali Z, Fronczek FR, Khan IA (2008) Phenylalkanoids and monoterpene analogues from the roots of *Rhodiola rosea*. Planta medica 74:178-181.

Alm T (2004) Ethnobotany of *Rhodiola rosea* (Crassulaceae) in Norway. Sida, Contributions to Botany 21:324-344.

Altman DG (1991) Practical statistics for medical research. London: Chapman and Hall.

Alzheimer's Association (2015) 2015 Alzheimer's Disease Facts and Figures. Alzheimer's & Dementia 11:332+.

Alzheimer's Disease International (2009) World Alzheimer's Report 2009. In. London: Alzheimer's Disease International.

Alzheimer Society of Canada (2010) Rising Tide: The Impact of Dementia on Canadian Society. In. Ontario: Alzheimer Society of Canada.

Ambree O, Richter H, Sachser N, Lewejohann L, Dere E, de Souza Silva MA, Herring A, Keyvani K, Paulus W, Schabitz WR (2009) Levodopa ameliorates learning and memory deficits in a murine model of Alzheimer's disease. Neurobiology of aging 30:1192-1204.

Ampong-Nyarko K (2014) *Rhodiola rosea* Cultivation in Canada and Alaska. In: Rhodiola rosea (Cuerrier A, Ampong-Nyarko K, eds), pp 125-150. Boca Raton, FL: CRC Press.

Ampong-Nyarko K, Brown J, De Mulder J, Chaudhary N, Zhang Z, Jiao A (2006) Specialty Crop Report: *Rhodiola rosea* commercialization in Alberta. In. Alberta: Alberta Agriculture, Food and Rural Development, Industry Development Sector.

Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Molecular pharmaceutics 4:807-818.

Anekonda TS, Reddy PH (2005) Can herbs provide a new generation of drugs for treating Alzheimer's disease? Brain research Brain research reviews 50:361-376.

Antoshechkin A, Olalde J, Antoshechkina M, Briuzgin V, Platinskiy L (2008) Influence of the plant extract complex "AdMax" on global gene expression levels in cultured human fibroblasts. Journal of dietary supplements 5:293-304.

Aoshima H, Takeda K, Okita Y, Hossain SJ, Koda H, Kiso Y (2006) Effects of beer and hop on ionotropic gamma-aminobutyric acid receptors. Journal of agricultural and food chemistry 54:2514-2519.

Apetz N, Munch G, Govindaraghavan S, Gyengesi E (2014) Natural compounds and plant extracts as therapeutics against chronic inflammation in Alzheimer's disease--a translational perspective. CNS & neurological disorders drug targets 13:1175-1191.

Arbuzov AG, Krylatov AV, Maslov LN, Burkova VN, Naryzhnaya NV (2006) Antihypoxic, cardioprotective, and antifibrillation effects of a combined adaptogenic plant preparation. Bulletin of experimental biology and medicine 142:212-215.

Armstrong RA (2014) A critical analysis of the 'amyloid cascade hypothesis'. Folia neuropathologica / Association of Polish Neuropathologists and Medical Research Centre, Polish Academy of Sciences 52:211-225.

Aslanyan G, Amroyan E, Gabrielyan E, Nylander M, Wikman G, Panossian A (2010) Double-blind, placebo-controlled, randomised study of single dose effects of ADAPT-232 on cognitive functions. Phytomedicine 17:494-499.

Assembly of First Nations (2007) Sustaining the Caregiving Cycle: First Nations People and Aging. In. Ottawa, Ontario: A Report from the Assembly of First Nations to the Special Senate Committee on Aging.

Atack JR (2005) The benzodiazepine binding site of GABA(A) receptors as a target for the development of novel anxiolytics. Expert opinion on investigational drugs 14:601-618.

Avula B, Wang YH, Ali Z, Smillie TJ, Filion V, Cuerrier A, Arnason JT, Khan IA (2009) RP-HPLC determination of phenylalkanoids and monoterpenoids in *Rhodiola rosea* and identification by LC-ESI-TOF. Biomedical chromatography 23:865-872.

Awad R, Levac D, Cybulska P, Merali Z, Trudeau VL, Arnason JT (2007) Effects of traditionally used anxiolytic botanicals on enzymes of the gamma-aminobutyric acid (GABA) system. Canadian Journal of Physiology and Pharmacology 85:933-942.

Awad R, Arnason JT, Trudeau V, Bergeron C, Budzinski JW, Foster BC, Merali Z (2003) Phytochemical and biological analysis of skullcap (*Scutellaria lateriflora* L.): a medicinal plant with anxiolytic properties. Phytomedicine 10:640-649.

Awad R, Ahmed F, Bourbonnais-Spear N, Mullally M, Ta CA, Tang A, Merali Z, Maquin P, Caal F, Cal V, Poveda L, Vindas PS, Trudeau VL, Arnason JT (2009) Ethnopharmacology of Q'eqchi' Maya antiepileptic and anxiolytic plants: effects on the GABAergic system. Journal of ethnopharmacology 125:257-264.

Baeta-Corral R, Gimenez-Llort L (2015) Persistent hyperactivity and distinctive strategy features in the Morris water maze in 3xTg-AD mice at advanced stages of disease. Behavioral neuroscience 129:129-137.

Banerjee S, Smith SC, Lamping DL, Harwood RH, Foley B, Smith P, Murray J, Prince M, Levin E, Mann A, Knapp M (2006) Quality of life in dementia: more than just cognition. An analysis of associations with quality of life in dementia. Journal of neurology, neurosurgery, and psychiatry 77:146-148.

Barage SH, Sonawane KD (2015) Amyloid cascade hypothesis: Pathogenesis and therapeutic strategies in Alzheimer's disease. Neuropeptides 52:1-18.

Barnes J, Anderson LA, Philipson JD (2007) Herbal Medicines, 3rd Edition. London (GB): Pharmaceutical Press.

Bekris LM, Yu CE, Bird TD, Tsuang DW (2010) Genetics of Alzheimer disease. Journal of geriatric psychiatry and neurology 23:213-227.

Berger F, Hensel A, Nieber K (2011) Saffron extract and trans-crocetin inhibit glutamatergic synaptic transmission in rat cortical brain slices. Neuroscience 180:238-247.

Bhattacharya SK, Bhattacharya A, Sairam K, Ghosal S (2000) Anxiolytic-antidepressant activity of *Withania somnifera* glycowithanolides: an experimental study. Phytomedicine 7:463-469.

Bi HM, Zhang SQ, Liu CJ, Wang CZ (2009) High hydrostatic pressure extraction of salidroside from *Rhodiola sachalinensis*. Journal of Food Process Engineering 32:53-63.

Bidstrup TB, Bjornsdottir I, Sidelmann UG, Thomsen MS, Hansen KT (2003) CYP2C8 and CYP3A4 are the principal enzymes involved in the human *in vitro* biotransformation of the insulin secretagogue repaglinide. British journal of clinical pharmacology 56:305-314.

Birks J (2006) Cholinesterase inhibitors for Alzheimer's disease. The Cochrane database of systematic reviews:CD005593.

Black PL, Arnason JT, Cuerrier A (2008) Medicinal Plants used by the Inuit of Qikiqtaaluk (Baffin Island, Nunavut). Botany 86:157-163.

Blennow K, Hampel H, Weiner M, Zetterberg H (2010) Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. Nature reviews Neurology 6:131-144.

Blondeau M, Roy C, Cuerrier A (2010) Plants of the Villages and the Parks of Nunavik. Québec: Éditions MultiMondes.

Blumenthal M, Goldberg A, Brinckmann J (2000) Herbal Medicine. Expanded Commission E monographs. Newton, USA: American Botanical Council.

Bonetto N, Santelli L, Battistin L, Cagnin A (2007) Serotonin syndrome and rhabdomyolysis induced by concomitant use of triptans, fluoxetine and hypericum. Cephalalgia : an international journal of headache 27:1421-1423.

Born SL, Api AM, Ford RA, Lefever FR, Hawkins DR (2003) Comparative metabolism and kinetics of coumarin in mice and rats. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 41:247-258.

Bourbonnais-Spear N, Awad R, Merali Z, Maquin P, Cal V, Arnason JT (2007) Ethnopharmacological investigation of plants used to treat susto, a folk illness. Journal of ethnopharmacology 109:380-387.

Bourin M, Hascoet M (2003) The mouse light/dark box test. European journal of pharmacology 463:55-65.

Bradwejn J, Zhou Y, Koszycki D, Shlik J (2000) A double-blind, placebo-controlled study on the effects of Gotu Kola (*Centella asiatica*) on acoustic startle response in healthy subjects. Journal of clinical psychopharmacology 20:680-684.

Brandin H, Viitanen E, Myrberg O, Arvidsson AK (2007) Effects of herbal medicinal products and food supplements on induction of CYP1A2, CYP3A4 and MDR1 in the human colon carcinoma cell line LS180. Phytotherapy research 21:239-244.

Braz AS, Morais LC, Paula AP, Diniz MF, Almeida RN (2013) Effects of *Panax ginseng* extract in patients with fibromyalgia: a 12-week, randomized, double-blind, placebo-controlled trial. Revista brasileira de psiquiatria 35:21-28.

Brigel M, Beguin C (1926) Isolation of rutoside, asparagines and a new glycoside, hydrolysable by emulsion, salidroside from *Salix triandra* L. . Comptes rendus hebdomadaires des séances del'Académie des sciences 183:321-323.

British Columbia Provincial Health Officer (2007, 2009) Pathways to Healing: 2nd Report on the Health and Well-Being of Aboriginal People in British Columbia. In. Victoria, B. C.: Provincial Health Officer's Annual Report.

Brock C, Whitehouse J, Tewfik I, Towell T (2014) American Skullcap (*Scutellaria lateriflora*): a randomised, double-blind placebo-controlled crossover study of its effects on mood in healthy volunteers. Phytotherapy research 28:692-698.

Brody DL, Holtzman DM (2006) Morris water maze search strategy analysis in PDAPP mice before and after experimental traumatic brain injury. Experimental neurology 197:330-340.

Brown RP, Gerbarg PL, Ramazanov Z (2002) *Rhodiola rosea*: A Phytomedicinal Overview. HerbalGram 56:40-52.

Budzinski JW, Foster BC, Vandenhoek S, Arnason JT (2000) An *in vitro* evaluation of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures. Phytomedicine 7:273-282.

Burton C, Campbell P, Jordan K, Strauss V, Mallen C (2013) The association of anxiety and depression with future dementia diagnosis: a case-control study in primary care. Family practice 30:25-30.

Bystritsky A, Kerwin L, Feusner JD (2008) A pilot study of *Rhodiola rosea* (Rhodax) for generalized anxiety disorder (GAD). Journal of alternative and complementary medicine 14:175-180.

Cai H, Wang Y, McCarthy D, Wen H, Borchelt DR, Price DL, Wong PC (2001) BACE1 is the major beta-secretase for generation of Abeta peptides by neurons. Nature neuroscience 4:233-234.

Cai L, Wang H, Li Q, Qian Y, Yao W (2008) Salidroside inhibits H_2O_2-induced apoptosis in PC12 cells by preventing cytochrome c release and inactivating of caspase cascade. Acta biochimica et biophysica Sinica 40:796-802.

Calcabrini C, De Bellis R, Mancini U, Cucchiarini L, Potenza L, De Sanctis R, Patrone V, Scesa C, Dacha M (2010) *Rhodiola rosea'*s ability to enrich cellular antioxidant defences of cultured human keratinocytes. Archives of dermatological research 302:191-200.

Can OD, Ozturk Y, Ozkay UD (2011) Effects of insulin and St. John's Wort treatments on anxiety, locomotory activity, depression, and active learning parameters of streptozotocin-diabetic rats. Planta medica 77:1970-1976.

Candelario M, Cuellar E, Reyes-Ruiz JM, Darabedian N, Feimeng Z, Miledi R, Russo-Neustadt A, Limon A (2015) Direct evidence for GABAergic activity of *Withania somnifera* on mammalian ionotropic GABAA and GABArho receptors. Journal of ethnopharmacology 171:264-272.

Cao LL, Du GH, Wang MW (2006) The effect of salidroside on cell damage induced by glutamate and intracellular free calcium in PC12 cells. Journal of Asian natural products research 8:159-165.

Cardinal JC, Schopflocher D, Svenson L, Morrison K, Laing L (2004) First Nations in Alberta: a focus on health service use. Edmonton: Alberta Health and Wellness.

Carr MN, Bekku N, Yoshimura H (2006) Identification of anxiolytic ingredients in ginseng root using the elevated plus-maze test in mice. European journal of pharmacology 531:160-165.

Cases J, Ibarra A, Feuillere N, Roller M, Sukkar SG (2011) Pilot trial of *Melissa officinalis* L. leaf extract in the treatment of volunteers suffering from mild-to-moderate anxiety disorders and sleep disturbances. Mediterranean journal of nutrition and metabolism 4:211-218.

Cayer C, Ahmed F, Filion V, Saleem A, Cuerrier A, Allard M, Rochefort G, Merali Z, Arnason JT (2013) Characterization of the anxiolytic activity of Nunavik *Rhodiola rosea*. Planta medica 79:1385-1391.

Ceremuga TE, Valdivieso D, Kenner C, Lucia A, Lathrop K, Stailey O, Bailey H, Criss J, Linton J, Fried J, Taylor A, Padron G, Johnson AD (2015) Evaluation of the anxiolytic and antidepressant effects of asiatic acid, a compound from Gotu kola or *Centella asiatica*, in the male Sprague Dawley rat. American Association of Nurse Anesthetists Journal 83:91-98.

Cha HY, Park JH, Hong JT, Yoo HS, Song S, Hwang BY, Eun JS, Oh KW (2005) Anxiolytic-like effects of ginsenosides on the elevated plus-maze model in mice. Biological & pharmaceutical bulletin 28:1621-1625.

Chang YW, Yao HT, Hsieh SH, Lu TJ, Yeh TK (2007) Quantitative determination of salidroside in rat plasma by on-line solid-phase extraction integrated with high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. Journal of chromatography B, Analytical technologies in the biomedical and life sciences 857:164-169.

Chen CH, Chan HC, Chu YT, Ho HY, Chen PY, Lee TH, Lee CK (2009a) Antioxidant activity of some plant extracts towards xanthine oxidase, lipoxygenase and tyrosinase. Molecules 14:2947-2958.

Chen G, Chen KS, Knox J, Inglis J, Bernard A, Martin SJ, Justice A, McConlogue L, Games D, Freedman SB, Morris RG (2000) A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease. Nature 408:975-979.

Chen H, Chen JZ, Hou RR, Yang AL, Kang XG, Zhang Y, Li YL, Li HL (2007) Inihibition of salidroside on apoptosis of PC12 cells induced by paraquat and its related mechanisms. Chinese Journal of Pharmacology and Toxicology 21:190-196.

Chen QG, Zeng YS, Tang JY, Qin YJ, Chen SJ, Zhong ZQ (2008a) [Effects of *Rhodiola rosea* on body weight and intake of sucrose and water in depressive rats induced by chronic mild stress]. Zhong xi yi jie he xue bao = Journal of Chinese integrative medicine 6:952-955.

Chen QG, Zeng YS, Qu ZQ, Tang JY, Qin YJ, Chung P, Wong R, Hagg U (2009b) The effects of *Rhodiola rosea* extract on 5-HT level, cell proliferation and quantity of neurons at cerebral hippocampus of depressive rats. Phytomedicine 16:830-838.

Chen S, Zhang D, Chen S, Xia T, Gao Q, Duan Y, Zhang F (2008b) Determination of salidroside in medicinal plants belonging to the *Rhodiola* L. genus originating from the Qinghai-Tibet plateau. Chromatographia 68:299-302.

Chen TS, Liou SY, Chang YL (2008c) Antioxidant evaluation of three adaptogen extracts. The American journal of Chinese medicine 36:1209-1217.

Chen X, Zhou SL, Gu XS, Ding F (2008d) Protective effects of salidroside on glutamate induced neurotoxicity in cultured hippocampal neurons. Acta Anatomica Sinica 39:355-359.

Chen X, Zhang Q, Cheng Q, Ding F (2009c) Protective effect of salidroside against H_2O_2-induced cell apoptosis in primary culture of rat hippocampal neurons. Molecular and cellular biochemistry 332:85-93.

Cheng YZ, Chen LJ, Lee WJ, Chen MF, Jung Lin H, Cheng JT (2012) Increase of myocardial performance by *Rhodiola*-ethanol extract in diabetic rats. Journal of ethnopharmacology 144:234-239.

Chiang K, Koo EH (2014) Emerging therapeutics for Alzheimer's disease. Annual review of pharmacology and toxicology 54:381-405.

Chinese Pharmacopeia Commission (2005) Pharmacopeia of the People's Republic of China. China: People's Medical Publishing House.

Chishti MA et al. (2001) Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695. The Journal of biological chemistry 276:21562-21570.

Cho YA, Choi JS, Burm JP (2011) Effects of the antioxidant baicalein on the pharmacokinetics of nimodipine in rats: a possible role of P-glycoprotein and CYP3A4 inhibition by baicalein. Pharmacological reports 63:1066-1073.

Choi JH, Choi AY, Yoon H, Choe W, Yoon KS, Ha J, Yeo EJ, Kang I (2010) Baicalein protects HT22 murine hippocampal neuronal cells against endoplasmic reticulum stress-induced apoptosis through inhibition of reactive oxygen species production and CHOP induction. Experimental & molecular medicine 42:811-822.

Choudhary MI, Nawaz SA, ul-Haq Z, Lodhi MA, Ghayur MN, Jalil S, Riaz N, Yousuf S, Malik A, Gilani AH, ur-Rahman A (2005) Withanolides, a new class of natural cholinesterase inhibitors with calcium antagonistic properties. Biochemical and biophysical research communications 334:276-287.

Christensen KB, Minet A, Svenstrup H, Grevsen K, Zhang H, Schrader E, Rimbach G, Wein S, Wolffram S, Kristiansen K, Christensen LP (2009) Identification of plant extracts with

potential antidiabetic properties: effect on human peroxisome proliferator-activated receptor (PPAR), adipocyte differentiation and insulin-stimulated glucose uptake. Phytotherapy research 23:1316-1325.

Chu S, Gu J, Feng L, Liu J, Zhang M, Jia X, Liu M, Yao D (2014) Ginsenoside Rg5 improves cognitive dysfunction and beta-amyloid deposition in STZ-induced memory impaired rats via attenuating neuroinflammatory responses. International immunopharmacology 19:317-326.

Cieniak C (2014) Evaluation of safety and efficacy of traditional Cree medicinal plants. In. Ottawa: University of Ottawa.

Cieniak C, Liu R, Fottinger A, Smiley SA, Guerrero-Analco JA, Bennett SA, Haddad PS, Cuerrier A, Saleem A, Arnason JT, Foster BC (2013) *In vitro* inhibition of metabolism but not transport of gliclazide and repaglinide by Cree medicinal plant extracts. Journal of ethnopharmacology 150:1087-1095.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360:672-674.

Clark C, Cuerrier A (2012) Inuit ethnobotany and ethnoecology in Nunavik and Nunatsiavut, northeastern Canada In. Montréal: Université de Montréal

Colciaghi F, Borroni B, Zimmermann M, Bellone C, Longhi A, Padovani A, Cattabeni F, Christen Y, Di Luca M (2004) Amyloid precursor protein metabolism is regulated toward alpha-secretase pathway by *Ginkgo biloba* extracts. Neurobiology of disease 16:454-460.

Collings P (2001) "If you've got everything, it's good enough": Perspectives on successful aging in a Canadian Inuit community. Journal of Cross-Cultural Gerontology 16:127-155.

Colson SN, Wyatt FB, Johnston DL, Autrey LD, FitzGerald YL, Earnest CP (2005) *Cordyceps sinensis*- and *Rhodiola rosea*-based supplementation in male cyclists and its effect on muscle tissue oxygen saturation. Journal of strength and conditioning research / National Strength & Conditioning Association 19:358-363.

Crawley J, Goodwin FK (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacology, biochemistry, and behavior 13:167-170.

Crawley JN (2007) What is wrong with my mouse ? Behavioral phenotyping of transgenic and knockout mice, 2nd Edition. New York: Wiley-Liss.

Crowther PS, Cox RJ (2006) Accuracy of Neural Network Classifiers as a Property of the Size of the Data Set. In: Knowledge-Based Intelligent Information and Engineering Systems (Gabrys B, Howlett RJ, Jain LC, eds), pp 1143-1149: Springer Berlin Heidelberg.

Cruz AP, Frei F, Graeff FG (1994) Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacology, biochemistry, and behavior 49:171-176.

Cuerrier A, Elders of Kangiqsualujjuaq (2011) The Botanical Knowledge of the Inuit of Kangiqsualujjuaq, Nunavik. Inukjuak, QC, Canada: Avataq Cultural Institute.

Cuerrier A, Hermanutz L (2012) Our Plants… our Land: Plants of Nain and Torngat Mountains Basecamp & Research Station (Nunatsiavut). Montréal & St-John's: IRBV, MUN.

Cuerrier A, Tendland Y, Rapinski M (2014a) Ethnobotany and Conservation of *Rhodiola* Species. In: Rhodiola rosea, pp 35-64: CRC Press.

Cuerrier A, Archambault M, Rapinski M, Bruneau A (2014b) Taxonomy of *Rhodiola rosea* L., with Special Attention to Molecular Analyses of Nunavik (Québec) Populations. In: Rhodiola rosea L. (Cuerrier A, Ampong-Nyarko K, eds), pp 1-33: CRC Press.

Cummings JL (2004) Alzheimer's disease. The New England journal of medicine 351:56-67.

Cummings JL, Isaacson RS, Schmitt FA, Velting DM (2015) A practical algorithm for managing Alzheimer's disease: what, when, and why? Annals of clinical and translational neurology 2:307-323.

Cybulska P, Thakur SD, Foster BC, Scott IM, Leduc RI, Arnason JT, Dillon JA (2011) Extracts of Canadian first nations medicinal plants, used as natural products, inhibit *Neisseria gonorrhoeae* isolates with different antibiotic resistance profiles. Sexually transmitted diseases 38:667-671.

Danielson PB (2002) The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. Current drug metabolism 3:561-597.

Darbinyan V, Kteyan A, Panossian A, Gabrielian E, Wikman G, Wagner H (2000) *Rhodiola rosea* in stress induced fatigue--a double blind cross-over study of a standardized extract SHR-5 with a repeated low-dose regimen on the mental performance of healthy physicians during night duty. Phytomedicine 7:365-371.

Darbinyan V, Aslanyan G, Amroyan E, Gabrielyan E, Malmstrom C, Panossian A (2007) Clinical trial of *Rhodiola rosea* L. extract SHR-5 in the treatment of mild to moderate depression. Nordic journal of psychiatry 61:343-348.

Dastmalchi K, Ollilainen V, Lackman P, Boije af Gennas G, Dorman HJ, Jarvinen PP, Yli-Kauhaluoma J, Hiltunen R (2009) Acetylcholinesterase inhibitory guided fractionation of *Melissa officinalis* L. Bioorganic & medicinal chemistry 17:867-871.

Davis JD, Banack SA (2012) Ethnobotany of the Kiluhikturmiut Inuinnait of Kugluktuk, Nunavut, Canada. Ethnobiology Letters 3:78-90.

Davis M, Myers KM, Chhatwal J, Ressler KJ (2006) Pharmacological treatments that facilitate extinction of fear: Relevance to psychotherapy. NeuroRx 3: :82-96.

De Bock K, Eijnde BO, Ramaekers M, Hespel P (2004) Acute *Rhodiola rosea* intake can improve endurance exercise performance. International Journal of Sport Nutrition and Exercise Metabolism 14 298-307.

de Carvalho RS, Duarte FS, de Lima TC (2011) Involvement of GABAergic non-benzodiazepine sites in the anxiolytic-like and sedative effects of the flavonoid baicalein in mice. Behavioural brain research 221:75-82.

De Sanctis R, De Bellis R, Scesa C, Mancini U, Cucchiarini L, Dacha M (2004) *In vitro* protective effect of *Rhodiola rosea* extract against hypochlorous acid-induced oxidative damage in human erythrocytes. BioFactors 20:147-159.

Defillipo PP, Raposo AH, Fedoce AG, Ferreira AS, Polonini HC, Gattaz WF, Raposo NR (2012) Inhibition of $cPLA_2$ and $sPLA_2$ activities in primary cultures of rat cortical neurons by *Centella asiatica* water extract. Natural Product Communications 7:841-843.

Diamond BJ, Bailey MR (2013) *Ginkgo biloba*: indications, mechanisms, and safety. The Psychiatric clinics of North America 36:73-83.

Diniz BS, Butters MA, Albert SM, Dew MA, Reynolds CF (2013) Late-life depression and risk of vascular dementia and Alzheimer's disease: systematic review and meta-analysis of community-based cohort studies. The British journal of psychiatry : the journal of mental science 202:329-335.

Dorr A, Sahota B, Chinta LV, Brown ME, Lai AY, Ma K, Hawkes CA, McLaurin J, Stefanovic B (2012) Amyloid-beta-dependent compromise of microvascular structure and function in a model of Alzheimer's disease. Brain : a journal of neurology 135:3039-3050.

Downey MO, Hanlin RL (2010) Comparison of Ethanol and Acetone Mixtures for Extraction of Condensed Tannin from Grape Skin. South African Journal for Enology and Viticulture 31:154-159.

Dubichev AG, Kurkin VA, Zapesochnaya GG, Vorontsov ED (1991) Chemical composition of the rhizomes of *Rhodiola rosea* by the HPLC method. Chemistry of Natural Compounds 27:161-164.

Durairajan SS, Liu LF, Lu JH, Koo I, Maruyama K, Chung SK, Huang JD, Li M (2011) Stimulation of non-amyloidogenic processing of amyloid-beta protein precursor by cryptotanshinone involves activation and translocation of ADAM10 and PKC-alpha. Journal of Alzheimer's disease 25:245-262.

Durairajan SS, Yuan Q, Xie L, Chan WS, Kum WF, Koo I, Liu C, Song Y, Huang JD, Klein WL, Li M (2008) Salvianolic acid B inhibits Abeta fibril formation and disaggregates preformed fibrils and protects against Abeta-induced cytotoxicty. Neurochemistry international 52:741-750.

Duyckaerts C, Delatour B, Potier MC (2009) Classification and basic pathology of Alzheimer disease. Acta neuropathologica 118:5-36.

Earnest CP, Morss GM, Wyatt F, Jordan AN, Colson S, Church TS, Fitzgerald Y, Autrey L, Jurca R, Lucia A (2004) Effects of a commercial herbal-based formula on exercise performance in cyclists. Medicine and science in sports and exercise 36:504-509.

Edwards D, Heufelder A, Zimmermann A (2012) Therapeutic effects and safety of *Rhodiola rosea* extract WS(R) 1375 in subjects with life-stress symptoms--results of an open-label study. Phytotherapy research 26:1220-1225.

Egeland GM, Cao Z, Young TK (2011) Hypertriglyceridemic-waist phenotype and glucose intolerance among Canadian Inuit: the International Polar Year Inuit Health Survey for Adults 2007-2008. Canadian Medical Association journal 183:E553-558.

Elameen A, Klemsdal SS, Dragland S, Fjellheim S, Rognli OA (2008) Genetic diversity in a germplasm collection of roseroot (*Rhodiola rosea*) in Norway studied by AFLP. Biochemical Systematics and Ecology 36:706-715.

Ellis BE (1983) Production of hydroxyphenylethanol glycosides in suspension cultures of *Syringa vulgaris*. Phytochemistry 22:1941-1943.

Ellis KA, Stough C, Vitetta L, Heinrich K, Nathan PJ (2001) An investigation into the acute nootropic effects of *Hypericum perforatum* L. (St. John's Wort) in healthy human volunteers. Behavioural pharmacology 12:173-182.

Ernst E (2006) Herbal remedies for anxiety - a systematic review of controlled clinical trials. Phytomedicine 13:205-208.

España J, Gimenez-Llort L, Valero J, Minano A, Rabano A, Rodriguez-Alvarez J, LaFerla FM, Saura CA (2010) Intraneuronal beta-amyloid accumulation in the amygdala enhances fear and anxiety in Alzheimer's disease transgenic mice. Biological psychiatry 67:513-521.

Etxeberria U, de la Garza AL, Campion J, Martinez JA, Milagro FI (2012) Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic alpha amylase. Expert opinion on therapeutic targets 16:269-297.

European Food Safety Authority (2012) "General function" health claims under Article 13.

Evstatieva L, Todorova M, Antonova D, Staneva J (2010) Chemical composition of the essential oils of *Rhodiola rosea* L. of three different origins. Pharmacognosy magazine 6:256-258.

Farokhnia M, Shafiee Sabet M, Iranpour N, Gougol A, Yekehtaz H, Alimardani R, Farsad F, Kamalipour M, Akhondzadeh S (2014) Comparing the efficacy and safety of *Crocus sativus* L. with memantine in patients with moderate to severe Alzheimer's disease: a double-blind randomized clinical trial. Human psychopharmacology 29:351-359.

Ferreira ST, Klein WL (2011) The Abeta oligomer hypothesis for synapse failure and memory loss in Alzheimer's disease. Neurobiology of learning and memory 96:529-543.

Ferruzzi MG, Lobo JK, Janle EM, Cooper B, Simon JE, Wu QL, Welch C, Ho L, Weaver C, Pasinetti GM (2009) Bioavailability of gallic acid and catechins from grape seed polyphenol extract is improved by repeated dosing in rats: implications for treatment in Alzheimer's disease. Journal of Alzheimer's disease 18:113-124.

File SE (1992) Usefulness of animal models with newer anxiolytics. Clinical neuropharmacology 15 Suppl 1 Pt A:525A-526A.

File SE, Seth P (2003) A review of 25 years of the social interaction test. European journal of pharmacology 463:35-53.

Filion VJ (2008) A novel phytochemical and ecological study of the Nunavik medicinal plant *Rhodiola rosea* L. Ottawa: University of Ottawa.

Filion VJ, Saleem G, Rochefort M, Allard M, Cuerrier A, Arnason JT (2008) Phytochemical analysis of Nunavik *Rhodiola rosea* L. Natural Product Communications 3:721-726.

Fintelmann V, Gruenwald J (2007) Efficacy and tolerability of a *Rhodiola rosea* extract in adults with physical and cognitive deficiencies. Advances in therapy 24:929-939.

Fitzpatrick AL, Kuller LH, Lopez OL, Kawas CH, Jagust W (2005) Survival following dementia onset: Alzheimer's disease and vascular dementia. Journal of the neurological sciences 229-230:43-49.

Fleiss JL, Levin B, Paik MC (2003) Statistical Methods for Rates and Proportions, 3rd Edition. Hoboken, New Jersey: John Wiley and Sons, Inc.

Foster BC, Vandenhoek S, Hana J, Krantis A, Akhtar MH, Bryan M, Budzinski JW, Ramputh A, Arnason JT (2003) *In vitro* inhibition of human cytochrome P450-mediated metabolism of marker substrates by natural products. Phytomedicine 10:334-342.

Frank B, Gupta S (2005) A review of antioxidants and Alzheimer's disease. Annals of clinical psychiatry : official journal of the American Academy of Clinical Psychiatrists 17:269-286.

Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, Piomelli D (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature 425:90-93.

Fujimoto K, Chen Y, Polonsky KS, Dorn GW, 2nd (2010) Targeting cyclophilin D and the mitochondrial permeability transition enhances beta-cell survival and prevents diabetes in Pdx1 deficiency. Proceedings of the National Academy of Sciences of the United States of America 107:10214-10219.

Furmanowa M, Kedzia B, Hartwich M, Kozlowski J, Krajewska-Patan A, Mscisz A, Jankowiak J (1999) Phytochemical and pharmacological properties of *Rhodiola rosea* L. Herba Polonica 45:108-113.

Gaetani S, Cuomo V, Piomelli D (2003) Anandamide hydrolysis: a new target for anti-anxiety drugs? Trends in molecular medicine 9:474-478.

Galambosi B (2006) Demand and availability of *Rhodiola rosea* L. raw material. In: Medicinal and Aromatic Plants (Bogers RJ, E.; CL, Lange D, eds), pp 223-236. Netherlands: Springer.

Galambosi B (2014) *Rhodiola rosea* Cultivation in Europe. In: Rhodiola rosea (Cuerrier A, Ampong-Nyarko K, eds), pp 87-115. Boca Raton, FL: CRC Press.

Galambosi B, Galambosi ZS, Heinonen A, Uusitalo M (2010) Post-harvest procedures of *Rhodiola rosea* in Finland. In: 7th Annual Natural Health Product Research Conference: The Next Wave, pp S1-6: Pharmaceutical Biology.

Gallagher M, Burwell R, Burchinal M (1993) Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. Behavioral neuroscience 107:618-626.

Gambini J, Ingles M, Olaso G, Lopez-Grueso R, Bonet-Costa V, Gimeno-Mallench L, Mas-Bargues C, Abdelaziz KM, Gomez-Cabrera MC, Vina J, Borras C (2015) Properties of Resveratrol: *In Vitro* and *In Vivo* Studies about Metabolism, Bioavailability, and Biological Effects in Animal Models and Humans. Oxidative medicine and cellular longevity 2015:837042.

Ganzera M, Yayla Y, Khan IA (2001) Analysis of the marker compounds of *Rhodiola rosea* L. (golden root) by reversed phase high performance liquid chromatography. Chemical & pharmaceutical bulletin 49:465-467.

Gao J, He H, Jiang W, Chang X, Zhu L, Luo F, Zhou R, Ma C, Yan T (2015) Salidroside ameliorates cognitive impairment in a d-galactose-induced rat model of Alzheimer's disease. Behavioural brain research 293:27-33.

Gao N, Qi B, Liu FJ, Fang Y, Zhou J, Jia LJ, Qiao HL (2014) Inhibition of baicalin on metabolism of phenacetin, a probe of CYP1A2, in human liver microsomes and in rats. PloS one 9:e89752.

Garthe A, Behr J, Kempermann G (2009) Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PloS one 4:e5464.

Gauthier S, Schlaefke S (2014) Efficacy and tolerability of *Ginkgo biloba* extract EGb 761(R) in dementia: a systematic review and meta-analysis of randomized placebo-controlled trials. Clinical interventions in aging 9:2065-2077.

Gauthier S, Cummings J, Ballard C, Brodaty H, Grossberg G, Robert P, Lyketsos C (2010) Management of behavioral problems in Alzheimer's disease. International psychogeriatrics / IPA 22:346-372.

Gavrilova SI, Preuss UW, Wong JW, Hoerr R, Kaschel R, Bachinskaya N, Group GIS (2014) Efficacy and safety of *Ginkgo biloba* extract EGb 761 in mild cognitive impairment with neuropsychiatric symptoms: a randomized, placebo-controlled, double-blind, multi-center trial. International journal of geriatric psychiatry 29:1087-1095.

Geng J, Dong J, Ni H, Lee MS, Wu T, Jiang K, Wang G, Zhou AL, Malouf R (2010) Ginseng for cognition. The Cochrane database of systematic reviews:CD007769.

Geromichalos GD, Lamari FN, Papandreou MA, Trafalis DT, Margarity M, Papageorgiou A, Sinakos Z (2012) Saffron as a source of novel acetylcholinesterase inhibitors: molecular docking and *in vitro* enzymatic studies. Journal of agricultural and food chemistry 60:6131-6138.

Getova DP, Mihaylova AS (2013) Effects of *Rhodiola rosea* extract on passive avoidance tests in rats. Central European Journal of Medicine 8:176-181.

Ghahghaei A, Bathaie SZ, Kheirkhah H, Bahraminejad E (2013) The protective effect of crocin on the amyloid fibril formation of Abeta42 peptide *in vitro*. Cellular & molecular biology letters 18:328-339.

Gil-Mohapel J, Brocardo PS, Choquette W, Gothard R, Simpson JM, Christie BR (2013) Hippocampal neurogenesis levels predict water maze search strategies in the aging brain. PloS one 8:e75125.

Goey AK, Meijerman I, Rosing H, Marchetti S, Mergui-Roelvink M, Keessen M, Burgers JA, Beijnen JH, Schellens JH (2014) The effect of St John's wort on the pharmacokinetics of docetaxel. Clinical pharmacokinetics 53:103-110.

Gol'dberg ED, Amosova EN, Zueva EP, Razina TG, Krylova SG, Reikhart DV (2004) Effects of extracts from medicinal plants on the development of metastatic process. Bulletin of experimental biology and medicine 138:288-294.

Gonzalez LE, Andrews N, File SE (1996) 5-HT1A and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. Brain research 732:145-153.

Gonzalez LE, Ouagazzal AM, File SE (1998) Stimulation of benzodiazepine receptors in the dorsal hippocampus and median raphe reveals differential GABAergic control in two animal tests of anxiety. The European journal of neuroscience 10:3673-3680.

Gordon MN, King DL, Diamond DM, Jantzen PT, Boyett KV, Hope CE, Hatcher JM, DiCarlo G, Gottschall WP, Morgan D, Arendash GW (2001) Correlation between cognitive deficits and Abeta deposits in transgenic APP+PS1 mice. Neurobiology of aging 22:377-385.

Gortz N, Lewejohann L, Tomm M, Ambree O, Keyvani K, Paulus W, Sachser N (2008) Effects of environmental enrichment on exploration, anxiety, and memory in female TgCRND8 Alzheimer mice. Behavioural brain research 191:43-48.

Gospodaryov DV, Yurkevych IS, Lushchak OV, Lushchak VI (2013a) Correction: Lifespan extension and delay of age-related functional decline caused by *Rhodiola rosea* depends on dietary macronutrient balance. Longevity & healthspan 2:12.

Gospodaryov DV, Yurkevych IS, Jafari M, Lushchak VI, Lushchak OV (2013b) Lifespan extension and delay of age-related functional decline caused by *Rhodiola rosea* depends on dietary macronutrient balance. Longevity & healthspan 2:5.

Government of Canada (2006) The mental health and well-being of Aboriginal peoples in Canada. In: The Human Face of Mental Health and Mental Illness in Canada 2006. Ottawa: Government of Canada.

Granger MW, Franko B, Taylor MW, Messier C, Bennett SAL (2015) The TgCRND8 mouse model of Alzheimer's disease exhibits sexual dimorphisms in behavioral indices of cognitive reserve. Journal of Alzheimer's Disease Under review.

Gray NE, Morre J, Kelley J, Maier CS, Stevens JF, Quinn JF, Soumyanath A (2014) Caffeoylquinic acids in *Centella asiatica* protect against amyloid-beta toxicity. Journal of Alzheimer's disease 40:359-373.

Graziano A, Petrosini L, Bartoletti A (2003) Automatic recognition of explorative strategies in the Morris water maze. Journal of neuroscience methods 130:33-44.

Griebel G, Belzung C, Perrault G, Sanger DJ (2000) Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology 148:164-170.

Grossi C, Rigacci S, Ambrosini S, Ed Dami T, Luccarini I, Traini C, Failli P, Berti A, Casamenti F, Stefani M (2013) The polyphenol oleuropein aglycone protects TgCRND8 mice against A-beta plaque pathology. PloS one 8:e71702.

Grundmann O, Nakajima J, Kamata K, Seo S, Butterweck V (2009) Kaempferol from the leaves of *Apocynum venetum* possesses anxiolytic activities in the elevated plus maze test in mice. Phytomedicine 16:295-302.

Guan S, Wang W, Lu J, Qian W, Huang G, Deng X, Wang X (2011a) Salidroside attenuates hydrogen peroxide-induced cell damage through a cAMP-dependent pathway. Molecules 16:3371-3379.

Guan S, Feng H, Song B, Guo W, Xiong Y, Huang G, Zhong W, Huo M, Chen N, Lu J, Deng X (2011b) Salidroside attenuates LPS-induced pro-inflammatory cytokine responses and improves survival in murine endotoxemia. International immunopharmacology 11:2194-2199.

Gudala K, Bansal D, Schifano F, Bhansali A (2013) Diabetes mellitus and risk of dementia: A meta-analysis of prospective observational studies. Journal of diabetes investigation 4:640-650.

Guo N, Ding W, Wang Y, Hu Z, Wang Z, Wang Y (2014a) An LC-MS/MS method for the determination of salidroside and its metabolite p-tyrosol in rat liver tissues. Pharmaceutical biology 52:637-645.

Guo N, Zhu M, Han X, Sui D, Wang Y, Yang Q (2014b) The metabolism of salidroside to its aglycone p-tyrosol in rats following the administration of salidroside. PloS one 9:e103648.

Guo N, Hu Z, Fan X, Zheng J, Zhang D, Xu T, Yu T, Wang Y, Li H (2012) Simultaneous determination of salidroside and its aglycone metabolite p-tyrosol in rat plasma by liquid chromatography-tandem mass spectrometry. Molecules 17:4733-4754.

Guo Y, Zhao Y, Zheng C, Meng Y, Yang Y (2010) Synthesis, biological activity of salidroside and its analogues. Chemical & pharmaceutical bulletin 58:1627-1629.

Guo Y, Li X, Zhao Y, Si Y, Zhu H, Yang Y (2011) Synthesis and biological evaluation of two salidroside analogues in the PC12 cell model exposed to hypoglycemia and serum limitation. Chemical & pharmaceutical bulletin 59:1045-1047.

Gupta GL, Rana AC (2007) Protective effect of *Withania somnifera* (Dunal) root extract against protracted social isolation induced behavior in rats. Indian journal of physiology and pharmacology 51:345-353.

Gureje O, Nortje G, Makanjuola V, Oladeji B, Seedat S, Jenkins R (2015) The role of global traditional and complementary systems of medicine in treating mental health problems. The Lancet Psychiatry 2:168-177.

Gurley BJ, Gardner SF, Hubbard MA, Williams DK, Gentry WB, Cui Y, Ang CY (2005) Clinical assessment of effects of botanical supplementation on cytochrome P450 phenotypes in the elderly: St John's wort, garlic oil, *Panax ginseng* and *Ginkgo biloba*. Drugs & aging 22:525-539.

György Z (2006) Glycoside production by *in vitro Rhodiola rosea* cultures. Finland: University of Oulu.

Habjan S, Prince H, Kelley ML (2012) Caregiving for elders in first nations communities: social system perspective on barriers and challenges. Canadian journal on aging = La revue canadienne du vieillissement 31:209-222.

Halldorsson B (1783) Grasnytjar. Copenhagen.

Hanley MJ, Cancalon P, Widmer WW, Greenblatt DJ (2011) The effect of grapefruit juice on drug disposition. Expert opinion on drug metabolism & toxicology 7:267-286.

Hardy J (2009) The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. Journal of neurochemistry 110:1129-1134.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-356.

Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. Science 256:184-185.

Hart DJ, Craig D, Compton SA, Critchlow S, Kerrigan BM, McIlroy SP, Passmore AP (2003) A retrospective study of the behavioural and psychological symptoms of mid and late phase Alzheimer's disease. International journal of geriatric psychiatry 18:1037-1042.

Health Canada (2002) A report on mental illnesses in Canada. In. Ottawa, Canada: Health Canada.

Health Canada (2009) A Statistical Profile on the Health of First Nations in Canada: Self-rated Health and Selected Conditions, 2002 to 2005. Ottawa: Health Canada.

Hellum BH, Tosse A, Hoybakk K, Thomsen M, Rohloff J, Georg Nilsen O (2010) Potent *in vitro* inhibition of CYP3A4 and P-glycoprotein by *Rhodiola rosea*. Planta medica 76:331-338.

Henderson JN, Henderson LC (2002) Cultural construction of disease: a "supernormal" construct of dementia in an American Indian tribe. Journal of cross-cultural gerontology 17:197-212.

Hendrie HC, Hall KS, Pillay N, Rodgers D, Prince C, Norton J, Brittain H, Nath A, Blue A, Kaufert J, et al. (1993) Alzheimer's disease is rare in Cree. International psychogeriatrics / IPA 5:5-14.

Herrup K (2010) Reimagining Alzheimer's disease--an age-based hypothesis. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:16755-16762.

Héthelyi EB, Korany K, Galambosi B, Domokos J, Palinkas J (2005) Chemical composition of the essential oil from rhizomes of *Rhodiola rosea* L. grown in Finland. Journal of Essential Oil Research 17:628-629.

Hillhouse B, Ming D, French C, Towers GH (2004) Acetylcholine Esterase Inhibitors in *Rhodiola rosea*. Pharmaceutical Biology 42:68-72.

Hofrichter J, Krohn M, Schumacher T, Lange C, Feistel B, Walbroel B, Heinze HJ, Crockett S, Sharbel TF, Pahnke J (2013) Reduced Alzheimer's disease pathology by St. John's Wort treatment is independent of hyperforin and facilitated by ABCC1 and microglia activation in mice. Current Alzheimer research 10:1057-1069.

Holmes E, Wilson ID, Nicholson JK (2008) Metabolic phenotyping in health and disease. Cell 134:714-717.

Horng CT, Liu IM, Kuo DH, Tsai YW, Shieh P (2010) Comparison of xanthine oxidase-inhibiting and free radical-scavenging activities between plant adaptogens of

Eleutherococcus senticosus and *Rhodiola rosea*. Drug Development Research 71:249-252.

Hosseinzadeh H, Sadeghnia HR (2007) Protective effect of safranal on pentylenetetrazol-induced seizures in the rat: involvement of GABAergic and opioids systems. Phytomedicine 14:256-262.

Hosseinzadeh H, Noraei NB (2009) Anxiolytic and hypnotic effect of *Crocus sativus* aqueous extract and its constituents, crocin and safranal, in mice. Phytotherapy research 23:768-774.

Howes MJ, Houghton PJ (2003) Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacology, biochemistry, and behavior 75:513-527.

Howes MJ, Houghton PJ (2009) Traditional medicine for memory enhancement. In: Herbal drugs: Ethnomedicine to modern medicine (Ramawat KG, ed), pp 239-291. New York: Springer.

Howes MJ, Perry NS, Houghton PJ (2003) Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. Phytotherapy research 17:1-18.

Hu X, Zhang X, Qiu S, Yu D, Lin S (2010a) Salidroside induces cell-cycle arrest and apoptosis in human breast cancer cells. Biochemical and biophysical research communications 398:62-67.

Hu X, Lin S, Yu D, Qiu S, Zhang X, Mei R (2010b) A preliminary study: the anti-proliferation effect of salidroside on different human cancer cell lines. Cell biology and toxicology 26:499-507.

Huang KC (1993) The Pharmacology of Chinese Herbs. Boca Raton, FL: CRC Press, Inc.

Huang XT, Qian ZM, He X, Gong Q, Wu KC, Jiang LR, Lu LN, Zhu ZJ, Zhang HY, Yung WH, Ke Y (2014) Reducing iron in the brain: a novel pharmacologic mechanism of huperzine A in the treatment of Alzheimer's disease. Neurobiology of aging 35:1045-1054.

Hügel HM (2015) Brain Food for Alzheimer-Free Ageing: Focus on Herbal Medicines. Advances in experimental medicine and biology 863:95-116.

Hui KM, Huen MS, Wang HY, Zheng H, Sigel E, Baur R, Ren H, Li ZW, Wong JT, Xue H (2002) Anxiolytic effect of wogonin, a benzodiazepine receptor ligand isolated from *Scutellaria baicalensis* Georgi. Biochemical pharmacology 64:1415-1424.

Hulko W, Camille E, Antifeau E, Arnouse M, Bachynski N, Taylor D (2010) Views of First Nation elders on memory loss and memory care in later life. Journal of cross-cultural gerontology 25:317-342.

Husain GM, Chatterjee SS, Singh PN, Kumar V (2011) Beneficial effect of *Hypericum perforatum* on depression and anxiety in a type 2 diabetic rat model. Acta poloniae pharmaceutica 68:913-918.

Hyde LA, Kazdoba TM, Grilli M, Lozza G, Brusa R, Zhang Q, Wong GT, McCool MF, Zhang L, Parker EM, Higgins GA (2005) Age-progressing cognitive impairments and neuropathology in transgenic CRND8 mice. Behavioural brain research 160:344-355.

Ibarra A, Feuillere N, Roller M, Lesburgere E, Beracochea D (2010) Effects of chronic administration of *Melissa officinalis* L. extract on anxiety-like reactivity and on circadian and exploratory activities in mice. Phytomedicine 17:397-403.

Iljina I (1997) Komi Folk Medicine. Syktyvkar, Russia: Komi khisnoje isdatelstvo.

Inestrosa NC, Tapia-Rojas C, Griffith TN, Carvajal FJ, Benito MJ, Rivera-Dictter A, Alvarez AR, Serrano FG, Hancke JL, Burgos PV, Parodi J, Varela-Nallar L (2011) Tetrahydrohyperforin prevents cognitive deficit, Abeta deposition, tau phosphorylation and synaptotoxicity in the APPswe/PSEN1DeltaE9 model of Alzheimer's disease: a possible effect on APP processing. Translational psychiatry 1:e20.

Ioset KN, Nyberg NT, Van Diermen D, Malnoe P, Hostettmann K, Shikov AN, Jaroszewski JW (2011) Metabolic profiling of *Rhodiola rosea* rhizomes by (1)H NMR spectroscopy. Phytochemical analysis 22:158-165.

Iuvone T, De Filippis D, Esposito G, D'Amico A, Izzo AA (2006) The spice sage and its active ingredient rosmarinic acid protect PC12 cells from amyloid-beta peptide-induced neurotoxicity. The Journal of pharmacology and experimental therapeutics 317:1143-1149.

Jacklin KM, Warry W (2012) 2009 American Anthropological Association Meeting, New Orleans, LA, Session on Culture, Health and Aging in Native North American Communities: Forgetting and Forgottten: Dementia in Aboriginal Seniors. Anthropology and Aging Quarterly 33:14.

Jacklin KM, Walker JD, Shawande M (2013) The emergence of dementia as a Health Concern Among First Nations Populations in Alberta, Canada. Canadian Journal of Public Health 104:e39-e44.

Jafari M, Felgner JS, Bussel, II, Hutchili T, Khodayari B, Rose MR, Vince-Cruz C, Mueller LD (2007) *Rhodiola*: a promising anti-aging Chinese herb. Rejuvenation research 10:587-602.

Jana U, Sur TK, Maity LN, Debnath PK, Bhattacharyya D (2010) A clinical study on the management of generalized anxiety disorder with *Centella asiatica*. Nepal Medical College journal 12:8-11.

Jang SI, Pae HO, Choi BM, Oh GS, Jeong S, Lee HJ, Kim HY, Kang KJ, Yun YG, Kim YC, Chung HT (2003) Salidroside from *Rhodiola sachalinensis* protects neuronal PC12 cells

against cytotoxicity induced by amyloid-beta. Immunopharmacology and immunotoxicology 25:295-304.

Janus C (2004) Search strategies used by APP transgenic mice during navigation in the Morris water maze. Learning & memory 11:337-346.

Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, Chishti MA, Horne P, Heslin D, French J, Mount HT, Nixon RA, Mercken M, Bergeron C, Fraser PE, St George-Hyslop P, Westaway D (2000) A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. Nature 408:979-982.

Jeong HJ, Ryu YB, Park SJ, Kim JH, Kwon HJ, Kim JH, Park KH, Rho MC, Lee WS (2009) Neuraminidase inhibitory activities of flavonols isolated from *Rhodiola rosea* roots and their *in vitro* anti-influenza viral activities. Bioorganic & medicinal chemistry 17:6816-6823.

Jia H, Jiang Y, Ruan Y, Zhang Y, Ma X, Zhang J, Beyreuther K, Tu P, Zhang D (2004) Tenuigenin treatment decreases secretion of the Alzheimer's disease amyloid beta-protein in cultured cells. Neuroscience letters 367:123-128.

Jia JY, Zhao QH, Liu Y, Gui YZ, Liu GY, Zhu DY, Yu C, Hong Z (2013) Phase I study on the pharmacokinetics and tolerance of ZT-1, a prodrug of huperzine A, for the treatment of Alzheimer's disease. Acta pharmacologica Sinica 34:976-982.

Kajosaari LI, Laitila J, Neuvonen PJ, Backman JT (2005) Metabolism of repaglinide by CYP2C8 and CYP3A4 *in vitro*: effect of fibrates and rifampicin. Basic & clinical pharmacology & toxicology 97:249-256.

Kang MS, Baek SH, Chun YS, Moore AZ, Landman N, Berman D, Yang HO, Morishima-Kawashima M, Osawa S, Funamoto S, Ihara Y, Di Paolo G, Park JH, Chung S, Kim TW (2013) Modulation of lipid kinase PI4KIIalpha activity and lipid raft association of presenilin 1 underlies gamma-secretase inhibition by ginsenoside (20S)-Rg3. The Journal of biological chemistry 288:20868-20882.

Kapoor LD (1990) Handbook of Ayurvedic Medicinal Plants. Boca Raton, Florida: CRC press.

Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nature medicine 9:76-81.

Kavvadias D, Monschein V, Sand P, Riederer P, Schreier P (2003) Constituents of sage (*Salvia officinalis*) with *in vitro* affinity to human brain benzodiazepine receptor. Planta medica 69:113-117.

Kawaguchi A, Ohmori M, Tsuruoka S, Nishiki K, Harada K, Miyamori I, Yano R, Nakamura T, Masada M, Fujimura A (2004) Drug interaction between St John's Wort and quazepam. British journal of clinical pharmacology 58:403-410.

Kehr J, Yoshitake S, Ijiri S, Koch E, Noldner M, Yoshitake T (2012) Ginkgo biloba leaf extract (EGb 761(R)) and its specific acylated flavonol constituents increase dopamine and acetylcholine levels in the rat medial prefrontal cortex: possible implications for the cognitive enhancing properties of EGb 761(R). International psychogeriatrics / IPA 24 Suppl 1:S25-34.

Kennedy DO, Little W, Scholey AB (2004) Attenuation of laboratory-induced stress in humans after acute administration of *Melissa officinalis* (Lemon Balm). Psychosomatic medicine 66:607-613.

Kennedy DO, Little W, Haskell CF, Scholey AB (2006a) Anxiolytic effects of a combination of *Melissa officinalis* and *Valeriana officinalis* during laboratory induced stress. Phytotherapy research 20:96-102.

Kennedy DO, Scholey AB, Tildesley NT, Perry EK, Wesnes KA (2002) Modulation of mood and cognitive performance following acute administration of *Melissa officinalis* (lemon balm). Pharmacology, biochemistry, and behavior 72:953-964.

Kennedy DO, Pace S, Haskell C, Okello EJ, Milne A, Scholey AB (2006b) Effects of cholinesterase inhibiting sage (*Salvia officinalis*) on mood, anxiety and performance on a psychological stressor battery. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 31:845-852.

Kennedy DO, Wake G, Savelev S, Tildesley NT, Perry EK, Wesnes KA, Scholey AB (2003) Modulation of mood and cognitive performance following acute administration of single doses of *Melissa officinalis* (Lemon balm) with human CNS nicotinic and muscarinic receptor-binding properties. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 28:1871-1881.

Kent JM, Mathew SJ, Gorman JM (2002) Molecular targets in the treatment of anxiety. Biological psychiatry 52:1008-1030.

Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE (2005) Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Archives of general psychiatry 62:593-602.

Kessler RC, Soukup J, Davis RB, Foster DF, Wilkey SA, Van Rompay MI, Eisenberg DM (2001) The use of complementary and alternative therapies to treat anxiety and depression in the United States. The American journal of psychiatry 158:289-294.

Khalili M, Hamzeh F (2010) Effects of active constituents of *Crocus sativus* L., crocin on streptozocin-induced model of sporadic Alzheimer's disease in male rats. Iranian biomedical journal 14:59-65.

Khamba B (2013) Mental Health Study of Natural Health Product Adverse Reactions (SONAR) (interview).

Khanum F, Bawa AS, Singh B (2005) *Rhodiola rosea*: A Versatile Adaptogen. Comprehensive Reviews in Food Science and Food Safety 4:55-62.

Khnykina LA, Zotova MI (1966) To the pharmacognostic study of *Rhodiola rosea*. Aptechnoe Delo 15:34-38.

Khom S, Strommer B, Ramharter J, Schwarz T, Schwarzer C, Erker T, Ecker GF, Mulzer J, Hering S (2010) Valerenic acid derivatives as novel subunit-selective $GABA_A$ receptor ligands - *in vitro* and *in vivo* characterization. British journal of pharmacology 161:65-78.

Kim MS, Yu JM, Kim HJ, Kim HB, Kim ST, Jang SK, Choi YW, Lee do I, Joo SS (2014) Ginsenoside Re and Rd enhance the expression of cholinergic markers and neuronal differentiation in Neuro-2a cells. Biological & pharmaceutical bulletin 37:826-833.

Kim TW, Choi HJ, Kim NJ, Kim DH (2009) Anxiolytic-like effects of ginsenosides Rg3 and Rh2 from red ginseng in the elevated plus-maze model. Planta medica 75:836-839.

Kobayashi K, Takahashi T, Takano F, Fushiya S, Batkhuu J, Sanchir C, Yoshizaki F (2004) Survey of the influence of mongolian plants on lipase activity in mouse plasma and gastrointestinal tube. Natural Medicines 58:204-208.

Kobayashi K, Baba E, Fushiya S, Takano F, Batkhuu J, Dash T, Sanchir C, Yoshizaki F (2003) Screening of Mongolian plants for influence on amylase activity in mouse plasma and gastrointestinal tube. Biological & pharmaceutical bulletin 26:1045-1048.

Komar VV, Karpliuk ZV, Kit SM, Komar LV, Smolins'Ka VO (1980) Macro- and microelement composition of root extracts of *Rhodiola rosea* (golden root). Farmatsevtychnyi Zhurnal 3:58-60.

Kormosh N, Laktionov K, Antoshechkina M (2006) Effect of a combination of extract from several plants on cell-mediated and humoral immunity of patients with advanced ovarian cancer. Phytotherapy research 20:424-425.

Kumar S, Harris RJ, Seal CJ, Okello EJ (2012) An aqueous extract of *Withania somnifera* root inhibits amyloid beta fibril formation *in vitro*. Phytotherapy research 26:113-117.

Kuo YH, Lin YL, Don MJ, Chen RM, Ueng YF (2006) Induction of cytochrome P450-dependent monooxygenase by extracts of the medicinal herb *Salvia miltiorrhiza*. The Journal of pharmacy and pharmacology 58:521-527.

Kuribara H, Weintraub ST, Yoshihama T, Maruyama Y (2003) An anxiolytic-like effect of *Ginkgo biloba* extract and its constituent, ginkgolide-A, in mice. Journal of natural products 66:1333-1337.

Kurkin VA (2003) Phenylpropanoids from medicinal plants: Distribution, classification, structural analysis, and biological activity. Chemistry of Natural Compounds 39:123-153.

Kurkin VA, Zapesochnaya GG, Shchavlinskii AN (1984a) Flavonoids of the rhizomes of *Rhodiola rosea* III. Chemistry of Natural Compounds 20:367-368.

Kurkin VA, Zapesochnaya GG, Shchavlinskii AN (1984b) Flavonoids of the epigeal part of *Rhodiola rosea* I. Chemistry of Natural Compounds 20:623-624.

Kurkin VA, Zapesochnaya GG, Shchavlinskii AN (1985) Terpenoids of the rhizomes of *Rhodiola rosea*. Chemistry of Natural Compounds 21:593-597.

Kurkin VA, Zapesochnaya GG, Gorbunov YN, Nukhimovskii EL, Shreter AI, Shchavlinskii AN (1986) Chemical investigation of some species of the genera *Rhodiola* L. and *Sedum* L. and problems of their chemotaxonomy. Rastitel'nye Resursy 22:310-319.

Kurkin VA, Zapesochnaya GG, Dubichev AG, Vorontsov ED, Aleksandrova IV, Panova RV (1991) Phenylpropanoids of callus culture of *Rhodiola rosea*. Khimiya-Prirodnykh_Soedinenii 4:481-490.

Kurkin VA, Dubishchev AV, Zapesochnaya GG, Titova IN, Braslavskii VB, Pravdivtseva OE, Ezhkov VN, Avdeeva EV, Petrova ES, Klimova IY (2006) Effect of phytopreparations containing phenylproapanoids on the physical activity of animals. Pharmaceutical Chemistry Journal 40:149-150.

Kwon YI, Jang HD, Shetty K (2006) Evaluation of *Rhodiola crenulata* and *Rhodiola rosea* for management of type II diabetes and hypertension. Asia Pacific journal of clinical nutrition 15:425-432.

LaFerla FM, Green KN (2012) Animal models of Alzheimer disease. Cold Spring Harbor perspectives in medicine 2:1-13.

Lalonde R, Fukuchi K, Strazielle C (2012a) APP transgenic mice for modelling behavioural and psychological symptoms of dementia (BPSD). Neuroscience and biobehavioral reviews 36:1357-1375.

Lalonde R, Fukuchi K, Strazielle C (2012b) Neurologic and motor dysfunctions in APP transgenic mice. Reviews in the neurosciences 23:363-379.

Lalonde R, Lewis TL, Strazielle C, Kim H, Fukuchi K (2003) Transgenic mice expressing the betaAPP695SWE mutation: effects on exploratory activity, anxiety, and motor coordination. Brain research 977:38-45.

Lan K, Xie G, Jia W (2013) Towards polypharmacokinetics: pharmacokinetics of multicomponent drugs and herbal medicines using a metabolomics approach. Evidence-based complementary and alternative medicine 2013:1-12.

Lanari A, Amenta F, Silvestrelli G, Tomassoni D, Parnetti L (2006) Neurotransmitter deficits in behavioural and psychological symptoms of Alzheimer's disease. Mechanisms of ageing and development 127:158-165.

Lancashire LJ, Lemetre C, Ball GR (2009) An introduction to artificial neural networks in bioinformatics--application to complex microarray and mass spectrometry datasets in cancer studies. Briefings in bioinformatics 10:315-329.

Landis JR, Koch GG (1977) An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. Biometrics 33:363-374.

Landtag J, Baumert A, Degenkolb T, Schmidt J, Wray V, Scheel D, Strack D, Rosahl S (2002) Accumulation of tyrosol glucoside in transgenic potato plants expressing a parsley tyrosine decarboxylase. Phytochemistry 60:683-689.

Lanting S, Crossley M, Morgan D, Cammer A (2011) Aboriginal experiences of aging and dementia in a context of sociocultural change: qualitative analysis of key informant group interviews with Aboriginal seniors. Journal of cross-cultural gerontology 26:103-117.

Lapornik B, Prosek M, Wondra AG (2005) Comparison of extracts prepared from plant by-products using different solvents and extraction time. Journal of Food Engineering 71:214-222.

Lee BH, Kim HJ, Chung L, Nah SY (2013a) Ginsenoside Rg(3) regulates $GABA_A$ receptor channel activity: involvement of interaction with the gamma(2) subunit. European journal of pharmacology 705:119-125.

Lee CI, Han JY, Hong JT, Oh KW (2013b) 3,4,5-Trimethoxycinnamic acid (TMCA), one of the constituents of Polygalae Radix enhances pentobarbital-induced sleeping behaviors via GABAAergic systems in mice. Archives of pharmacal research 36:1244-1251.

Lee FT, Kuo TY, Liou SY, Chien CT (2009a) Chronic *Rhodiola rosea* extract supplementation enforces exhaustive swimming tolerance. The American journal of Chinese medicine 37:557-572.

Lee MS, Yang EJ, Kim JI, Ernst E (2009b) Ginseng for cognitive function in Alzheimer's disease: a systematic review. Journal of Alzheimer's disease 18:339-344.

Lee ST, Chu K, Sim JY, Heo JH, Kim M (2008) *Panax ginseng* enhances cognitive performance in Alzheimer disease. Alzheimer disease and associated disorders 22:222-226.

Lee Y, Jung JC, Jang S, Kim J, Ali Z, Khan IA, Oh S (2013c) Anti-Inflammatory and Neuroprotective Effects of Constituents Isolated from *Rhodiola rosea*. Evidence-based complementary and alternative medicine 2013:1-9.

Lewejohann L, Reefmann N, Widmann P, Ambree O, Herring A, Keyvani K, Paulus W, Sachser N (2009) Transgenic Alzheimer mice in a semi-naturalistic environment: more plaques, yet not compromised in daily life. Behavioural brain research 201:99-102.

Li J, Wu HM, Zhou RL, Liu GJ, Dong BR (2008) Huperzine A for Alzheimer's disease. The Cochrane database of systematic reviews:CD005592.

Li W, Du GS, Huang QN (2005) Salidroside contents and related enzymatic activities in *Rhodiola kirilowii* callus. Acta Botanica Boreali-Occidentalia Sinica 25:1645-1648.

Li X, Ye X, Li X, Sun X, Liang Q, Tao L, Kang X, Chen J (2011a) Salidroside protects against MPP(+)-induced apoptosis in PC12 cells by inhibiting the NO pathway. Brain research 1382:9-18.

Li XD, Kang ST, Li GY, Li X, Wang JH (2011b) Synthesis of some phenylpropanoid glycosides (PPGs) and their acetylcholinesterase/xanthine oxidase inhibitory activities. Molecules 16:3580-3596.

Li Z, Zhu S, Du G (2006) Comparison of the pharmacokinetics of salidroside and salidroside in the extracts of *Rhodiola rosea* L in rats. Asian Journal of Pharmacodynamics and Pharmacokinetics 6:224-226.

Li Z, Liu Y, Wang L, Liu X, Chang Q, Guo Z, Liao Y, Pan R, Fan TP (2014a) Memory-Enhancing Effects of the Crude Extract of *Polygala tenuifolia* on Aged Mice. Evidence-based complementary and alternative medicine 2014:1-10.

Li ZL, Dong XZ, Wang DX, Dong RH, Guo TT, Sun Y, Liu P (2014b) [Effect of oligosaccharide esters and polygalaxanthone Ill from *Polygala tenuifolia* Willd towards cytochrome P450]. China journal of Chinese materia medica 39:4459-4463.

Liang XQ, Xie P, Zhang Y, Shi T, Yan TH, Wang QJ (2010) Effects of salidroside on myocardial Ischemia/Reperfusion injury in rats. Chinese Journal of Natural Medicines 8:127-131.

Liang ZH, Zheng GZ (1981) Secondary metabolism of high plants. Plant Physiology Communications 17:14–21.

Lim CK, Yuan ZX, Lamb JH, White IN, De Matteis F, Smith LL (1994) A comparative study of tamoxifen metabolism in female rat, mouse and human liver microsomes. Carcinogenesis 15:589-593.

Lim HJ, Shim SB, Jee SW, Lee SH, Lim CJ, Hong JT, Sheen YY, Hwang DY (2013) Green tea catechin leads to global improvement among Alzheimer's disease-related phenotypes in NSE/hAPP-C105 Tg mice. The Journal of nutritional biochemistry 24:1302-1313.

Lindstrom A, Ooyen C, Lynch ME, Blumenthal M, Kawa K (2014) Sales of Herbal Dietary Supplements Increase by 7.9% in 2013, Marking a Decade of Rising Sales: Turmeric Supplements Climb to Top Ranking in Natural Channel. HerbalGram:52-56.

Lishmanov Iu B, Trifonova Zh V, Tsibin AN, Maslova LV, Dement'eva LA (1987) [Plasma beta-endorphin and stress hormones in stress and adaptation]. Biulleten' eksperimental'noi biologii i meditsiny 103:422-424.

Liu X, Hao W, Qin Y, Decker Y, Wang X, Burkart M, Schotz K, Menger MD, Fassbender K, Liu Y (2015) Long-term treatment with Ginkgo biloba extract EGb 761 improves

symptoms and pathology in a transgenic mouse model of Alzheimer's disease. Brain, behavior, and immunity 46:121-131.

Liu Z, Li X, Simoneau AR, Jafari M, Zi X (2012) *Rhodiola rosea* extracts and salidroside decrease the growth of bladder cancer cell lines via inhibition of the mTOR pathway and induction of autophagy. Molecular carcinogenesis 51:257-267.

Loef M, Walach H (2013) Midlife obesity and dementia: meta-analysis and adjusted forecast of dementia prevalence in the United States and China. Obesity 21:E51-55.

Loppie-Reading C, Wein F (2009) Health Inequalities and Social Determinants of Aboriginal Peoples' Health. In. Prince George, B. C.: National Collaborating Centre for Aboriginal Health.

Lu JH, Ardah MT, Durairajan SS, Liu LF, Xie LX, Fong WF, Hasan MY, Huang JD, El-Agnaf OM, Li M (2011) Baicalein inhibits formation of alpha-synuclein oligomers within living cells and prevents Abeta peptide fibrillation and oligomerisation. Chembiochem : a European journal of chemical biology 12:615-624.

Luchicchi A, Pistis M (2012) Anandamide and 2-arachidonoylglycerol: pharmacological properties, functional features, and emerging specificities of the two major endocannabinoids. Molecular neurobiology 46:374-392.

Ma C, Hu L, Lou Z, Wang H, Gu X (2013) Preparative separation and purification of four phenylpropanoid glycosides from *Rhodiola rosea* by high-speed counter-current chromatography. Journal of Liquid Chromatography and Related Technologies 36 116-126.

Ma G, Li W, Dou D, Chang X, Bai H, Satou T, Li J, Sun D, Kang T, Nikaido T, Koike K (2006) Rhodiolosides A-E, monoterpene glycosides from *Rhodiola rosea*. Chemical & pharmaceutical bulletin 54:1229-1233.

Ma K, McLaurin J (2014) alpha-Melanocyte stimulating hormone prevents GABAergic neuronal loss and improves cognitive function in Alzheimer's disease. The Journal of neuroscience 34:6736-6745.

Ma L, Wang S, Tai F, Yuan G, Wu R, Liu X, Wei B, Yang X (2012) Effects of bilobalide on anxiety, spatial learning, memory and levels of hippocampal glucocorticoid receptors in male Kunming mice. Phytomedicine 20:89-96.

Ma LQ, Liu BY, Gao DY, Pang XB, Lu SY, Yu HS, Wang H, Yan F, Li ZQ, Li YF, Ye HC (2007a) Molecular cloning and overexpression of a novel UDP-glucosyltransferase elevating salidroside levels in *Rhodiola sachalinensis*. Plant cell reports 26:989-999.

Ma LQ, Gao DY, Wang YN, Wang HH, Zhang JX, Pang XB, Hu TS, Lu SY, Li GF, Ye HC, Li YF, Wang H (2008) Effects of overexpression of endogenous phenylalanine ammonia-lyase (PALrs1) on accumulation of salidroside in *Rhodiola sachalinensis*. Plant biology 10:323-333.

Ma X, Tan C, Zhu D, Gang DR, Xiao P (2007b) Huperzine A from *Huperzia* species--an ethnopharmacolgical review. Journal of ethnopharmacology 113:15-34.

Maei HR, Zaslavsky K, Wang AH, Yiu AP, Teixeira CM, Josselyn SA, Frankland PW (2009) Development and validation of a sensitive entropy-based measure for the water maze. Frontiers in integrative neuroscience 3:1-13.

Mah L, Binns MA, Steffens DC, Alzheimer's Disease Neuroimaging I (2015) Anxiety symptoms in amnestic mild cognitive impairment are associated with medial temporal atrophy and predict conversion to Alzheimer disease. The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry 23:466-476.

Maimeskulova LA, Maslov LN (1998) [The anti-arrhythmia action of an extract of *Rhodiola rosea* and of n-tyrosol in models of experimental arrhythmias]. Eksperimental'naia i klinicheskaia farmakologiia 61:37-40.

Majewska A, Hoser G, Furmanowa M, Urbanska N, Pietrosiuk A, Zobel A, Kuras M (2006) Antiproliferative and antimitotic effect, S phase accumulation and induction of apoptosis and necrosis after treatment of extract from *Rhodiola rosea* rhizomes on HL-60 cells. Journal of ethnopharmacology 103:43-52.

Malati CY, Robertson SM, Hunt JD, Chairez C, Alfaro RM, Kovacs JA, Penzak SR (2012) Influence of *Panax ginseng* on cytochrome P450 (CYP)3A and P-glycoprotein (P-gp) activity in healthy participants. Journal of clinical pharmacology 52:932-939.

Malenfant EC, Morency JD (2011) Population projections by Aboriginal Identity in Canada, 2006 to 2031. In. Ottawa, ON: Statistics Canada, Cat No. 91-552-XWE.

Man SC, Durairajan SS, Kum WF, Lu JH, Huang JD, Cheng CF, Chung V, Xu M, Li M (2008) Systematic review on the efficacy and safety of herbal medicines for Alzheimer's disease. Journal of Alzheimer's disease 14:209-223.

Manach C, Scalbert A, Morand C, Remesy C, Jimenez L (2004) Polyphenols: food sources and bioavailability. The American journal of clinical nutrition 79:727-747.

Manning T, Sleator RD, Walsh P (2014) Biologically inspired intelligent decision making: a commentary on the use of artificial neural networks in bioinformatics. Bioengineered 5:80-95.

Mannucci C, Navarra M, Calzavara E, Caputi AP, Calapai G (2012) Serotonin involvement in *Rhodiola rosea* attenuation of nicotine withdrawal signs in rats. Phytomedicine 19:1117-1124.

Mao GX, Wang Y, Qiu Q, Deng HB, Yuan LG, Li RG, Song DQ, Li YY, Li DD, Wang Z (2010) Salidroside protects human fibroblast cells from premature senescence induced by H_2O_2 partly through modulating oxidative status. Mechanisms of ageing and development 131:723-731.

Mao JJ, Xie SX, Zee J, Soeller I, Li QS, Rockwell K, Amsterdam JD (2015) *Rhodiola rosea* versus sertraline for major depressive disorder: A randomized placebo-controlled trial. Phytomedicine 22:394-399.

Mao Y, Zhang X, Zhang X, Lu G (2007) Development of an HPLC method for the determination of salidroside in beagle dog plasma after administration of salidroside injection: application to a pharmacokinetics study. Journal of separation science 30:3218-3222.

Martignoni M, Groothuis GM, de Kanter R (2006) Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert opinion on drug metabolism & toxicology 2:875-894.

Maslov LN, Lishmanov Iu B, Maimeskulova LA, Krasnov EA (1998) [Mechanism of the anti-arrhythmic effect of *Rhodiola rosea* extract]. Biulleten' eksperimental'noi biologii i meditsiny 125:424-426.

Maslov LN, Lishmanov YB, Arbuzov AG, Krylatov AV, Budankova EV, Konkovskaya YN, Burkova VN, Severova EA (2009) Antiarrhythmic activity of phytoadaptogens in short-term ischemia-reperfusion of the heart and postinfarction cardiosclerosis. Bulletin of experimental biology and medicine 147:331-334.

Mattioli L, Perfumi M (2007) *Rhodiola rosea* L. extract reduces stress- and CRF-induced anorexia in rats. Journal of psychopharmacology 21:742-750.

Mattioli L, Perfumi M (2011) Effects of a *Rhodiola rosea* L. extract on acquisition and expression of morphine tolerance and dependence in mice. Journal of psychopharmacology 25:411-420.

Mattioli L, Funari C, Perfumi M (2009) Effects of *Rhodiola rosea* L. extract on behavioural and physiological alterations induced by chronic mild stress in female rats. Journal of psychopharmacology 23:130-142.

Mattioli L, Titomanlio F, Perfumi M (2012) Effects of a *Rhodiola rosea* L. extract on the acquisition, expression, extinction, and reinstatement of morphine-induced conditioned place preference in mice. Psychopharmacology 221:183-193.

McLaurin J, Kierstead ME, Brown ME, Hawkes CA, Lambermon MH, Phinney AL, Darabie AA, Cousins JE, French JE, Lan MF, Chen F, Wong SS, Mount HT, Fraser PE, Westaway D, St George-Hyslop P (2006) Cyclohexanehexol inhibitors of Abeta aggregation prevent and reverse Alzheimer phenotype in a mouse model. Nature medicine 12:801-808.

Mei Z, Zhang F, Tao L, Zheng W, Cao Y, Wang Z, Tang S, Le K, Chen S, Pi R, Liu P (2009) Cryptotanshinone, a compound from *Salvia miltiorrhiza* modulates amyloid precursor protein metabolism and attenuates beta-amyloid deposition through upregulating alpha-secretase *in vivo* and *in vitro*. Neuroscience letters 452:90-95.

Menard J, Treit D (1999) Effects of centrally administered anxiolytic compounds in animal models of anxiety. Neuroscience and biobehavioral reviews 23:591-613.

Meng Y, Guo Y, Ling Y, Zhao Y, Zhang Q, Zhou X, Ding F, Yang Y (2011) Synthesis and protective effects of aralkyl alcoholic 2-acetamido-2-deoxy-beta-D-pyranosides on hypoglycemia and serum limitation induced apoptosis in PC12 cell. Bioorganic & medicinal chemistry 19:5577-5584.

Miao J, Xu F, Davis J, Otte-Holler I, Verbeek MM, Van Nostrand WE (2005) Cerebral microvascular amyloid beta protein deposition induces vascular degeneration and neuroinflammation in transgenic mice expressing human vasculotropic mutant amyloid beta precursor protein. The American journal of pathology 167:505-515.

Ming DS, Hillhouse BJ, Guns ES, Eberding A, Xie S, Vimalanathan S, Towers GH (2005) Bioactive compounds from *Rhodiola rosea* (Crassulaceae). Phytotherapy research 19:740-743.

Modaghegh MH, Shahabian M, Esmaeili HA, Rajbai O, Hosseinzadeh H (2008) Safety evaluation of saffron (*Crocus sativus*) tablets in healthy volunteers. Phytomedicine 15:1032-1037.

Montiel-Ruiz RM, Roa-Coria JE, Patiño-Camacho SI, Flores-Murrieta FJ, Déciga-Campos M (2012) Neuropharmacological and toxicity evaluations of ethanol extract from *Rhodiola rosea*. Drug Development Research 73:106-113.

Moreau PH, Melis V, Crouch B, Harrington C, Riedel G (2014) New approach to analyse navigational search strategies used by mice during a water maze task. In: Proceedings of measuring behaviour (Spink AJ, Loijens LWS, Woloszynowska-Fraser M, Noldus LPJJ, eds). Wagenningen, The Netherlands.

Mori T, Koyama N, Guillot-Sestier MV, Tan J, Town T (2013) Ferulic acid is a nutraceutical beta-secretase modulator that improves behavioral impairment and alzheimer-like pathology in transgenic mice. PloS one 8:e55774.

Mori T, Rezai-Zadeh K, Koyama N, Arendash GW, Yamaguchi H, Kakuda N, Horikoshi-Sakuraba Y, Tan J, Town T (2012) Tannic acid is a natural beta-secretase inhibitor that prevents cognitive impairment and mitigates Alzheimer-like pathology in transgenic mice. The Journal of biological chemistry 287:6912-6927.

Morris BJ (2013) Seven sirtuins for seven deadly diseases of aging. Free radical biology & medicine 56:133-171.

Morris R (1981) Spatial Localization Does Not Require the Presence of Local Cues Learning and Motivation 12:239-260.

Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. Journal of neuroscience methods 11:47-60.

Mudge E, Lopes-Lutz D, Brown PN, Schieber A (2013) Purification of Phenylalkanoids and monoterpene glycosides from *Rhodiola rosea* L. roots by high-speed counter-current chromatography. Phytochemical analysis 24:129-134.

Mullally M, Kramp K, Cayer C, Saleem A, Ahmed F, McRae C, Baker J, Goulah A, Otorola M, Sanchez P, Garcia M, Poveda L, Merali Z, Durst T, Trudeau VL, Arnason JT (2011) Anxiolytic activity of a supercritical carbon dioxide extract of *Souroubea sympetala* (Marcgraviaceae). Phytotherapy research 25:264-270.

Murray AP, Faraoni MB, Castro MJ, Alza NP, Cavallaro V (2013) Natural AChE Inhibitors from Plants and their Contribution to Alzheimer's Disease Therapy. Current neuropharmacology 11:388-413.

Natarajan S, Shunmugiah KP, Kasi PD (2013) Plants traditionally used in age-related brain disorders (dementia): an ethanopharmacological survey. Pharmaceutical biology 51:492-523.

National Center for Health Statistics (2015) Deaths: Final Data for 2013. National Vital Statistics Report. In. Hyattsville, MD: National Center for Health Statistics.

Ni S, Qian D, Duan JA, Guo J, Shang EX, Shu Y, Xue C (2010) UPLC-QTOF/MS-based screening and identification of the constituents and their metabolites in rat plasma and urine after oral administration of *Glechoma longituba* extract. Journal of chromatography B, Analytical technologies in the biomedical and life sciences 878:2741-2750.

Noreen EE, Buckley JG, Lewis SL, Brandauer J, Stuempfle KJ (2013) The effects of an acute dose of *Rhodiola rosea* on endurance exercise performance. Journal of strength and conditioning research / National Strength & Conditioning Association 27:839-847.

Nutt DJ, Ballenger JC, Sheehan D, Wittchen HU (2002) Generalized anxiety disorder: comorbidity, comparative biology and treatment. The international journal of neuropsychopharmacology 5:315-325.

Ognibene E, Middei S, Daniele S, Adriani W, Ghirardi O, Caprioli A, Laviola G (2005) Aspects of spatial memory and behavioral disinhibition in Tg2576 transgenic mice as a model of Alzheimer's disease. Behavioural brain research 156:225-232.

Olsson EM, von Scheele B, Panossian AG (2009) A randomised, double-blind, placebo-controlled, parallel-group study of the standardised extract SHR-5 of the roots of *Rhodiola rosea* in the treatment of subjects with stress-related fatigue. Planta medica 75:105-112.

Pace JE (2013) Meanings of Memory: Understanding Aging and Dementia in First Nations communities on Manitoulin Island, Ontario. In. Hamilton, Ontario: McMaster University.

Paine MF, Widmer WW, Hart HL, Pusek SN, Beavers KL, Criss AB, Brown SS, Thomas BF, Watkins PB (2006) A furanocoumarin-free grapefruit juice establishes furanocoumarins

as the mediators of the grapefruit juice-felodipine interaction. The American journal of clinical nutrition 83:1097-1105.

Palumbo DR, Occhiuto F, Spadaro F, Circosta C (2012) *Rhodiola rosea* extract protects human cortical neurons against glutamate and hydrogen peroxide-induced cell death through reduction in the accumulation of intracellular calcium. Phytotherapy research 26:878-883.

Pan Y, Abd-Rashid BA, Ismail Z, Ismail R, Mak JW, Pook PC, Er HM, Ong CE (2010) *In vitro* modulatory effects on three major human cytochrome P450 enzymes by multiple active constituents and extracts of *Centella asiatica*. Journal of ethnopharmacology 130:275-283.

Pan Y, Abd-Rashid BA, Ismail Z, Ismail R, Mak JW, Pook PC, Er HM, Ong CE (2011) *In vitro* modulatory effects of *Andrographis paniculata*, *Centella asiatica* and *Orthosiphon stamineus* on cytochrome P450 2C19 (CYP2C19). Journal of ethnopharmacology 133:881-887.

Panchen Z (2014) Arctic ethnobotany: medicinal plant uses of western Arctic indigenous groups. In. Ottawa: Carleton University.

Panossian A, Wagner H (2005) Stimulating effect of adaptogens: an overview with particular reference to their efficacy following single dose administration. Phytotherapy research 19:819-838.

Panossian A, Wikman G (2010) Effects of adaptogens on the central nervous system and the molecular mechanisms associated with their stress - protective activity. Pharmaceuticals 3:188-224.

Panossian A, Wikman G, Wagner H (1999) Plant adaptogens. III. Earlier and more recent aspects and concepts on their mode of action. Phytomedicine 6:287-300.

Panossian A, Wikman G, Sarris J (2010a) Rosenroot (*Rhodiola rosea*): traditional use, chemical composition, pharmacology and clinical efficacy. Phytomedicine 17:481-493.

Panossian A, Hambardzumyan M, Hovhanissyan A, Wikman G (2007) The adaptogens rhodiola and schizandra modify the response to immobilization stress in rabbits by suppressing the increase of phosphorylated stress-activated protein kinase, nitric oxide and cortisol. Drug target insights 2:39-54.

Panossian A, Wikman G, Kaur P, Asea A (2009a) Adaptogens exert a stress-protective effect by modulation of expression of molecular chaperones. Phytomedicine 16:617-622.

Panossian A, Hovhannisyan A, Abrahamyan A, Wikman G (2010b) Pharmacokinetics of active constituents of *Rhodiola rosea* L. special extract SHR-5. In: Comprehensive Bioactive Natural Products Vol 2: Efficacy, Safety and Clinical evaluation (Part-1) (Gupta VK, ed), pp 1-23. Houston, TX: Studium Press LLC.

Panossian A, Wikman G, Kaur P, Asea A (2012) Adaptogens stimulate neuropeptide Y and hsp72 expression and release in neuroglia cells. Frontiers in neuroscience 6:1-12.

Panossian A, Hovhannisyan A, Abrahamyan H, Gabrielyan E, Wikman G (2009b) Pharmacokinetic and pharmacodynamic study of interaction of *Rhodiola rosea* SHR-5 extract with warfarin and theophylline in rats. Phytotherapy research 23:351-357.

Panossian A, Hamm R, Kadioglu O, Wikman G, Efferth T (2013) Synergy and Antagonism of Active Constituents of ADAPT-232 on Transcriptional Level of Metabolic Regulation of Isolated Neuroglial Cells. Frontiers in neuroscience 7:1-17.

Panossian A, Nikoyan N, Ohanyan N, Hovhannisyan A, Abrahamyan H, Gabrielyan E, Wikman G (2008) Comparative study of *Rhodiola* preparations on behavioral despair of rats. Phytomedicine 15:84-91.

Panossian AG (2013) Adaptogens in mental and behavioral disorders. The Psychiatric clinics of North America 36:49-64.

Papandreou MA, Kanakis CD, Polissiou MG, Efthimiopoulos S, Cordopatis P, Margarity M, Lamari FN (2006) Inhibitory activity on amyloid-beta aggregation and antioxidant properties of *Crocus sativus* stigmas extract and its crocin constituents. Journal of agricultural and food chemistry 54:8762-8768.

Parisi A, Tranchita E, Duranti G, Ciminelli E, Quaranta F, Ceci R, Cerulli C, Borrione P, Sabatini S (2010) Effects of chronic *Rhodiola rosea* supplementation on sport performance and antioxidant capacity in trained male: preliminary results. The Journal of sports medicine and physical fitness 50:57-63.

Park CH, Choi SH, Koo JW, Seo JH, Kim HS, Jeong SJ, Suh YH (2002) Novel cognitive improving and neuroprotective activities of *Polygala tenuifolia* Willdenow extract, BT-11. Journal of neuroscience research 70:484-492.

Pashkevich IA, Uspenskaia Iu A, Nefedova VV, Egorova AB (2003) [Comparative evaluation of effects of p-tyrosol and *Rhodiola rosea* extract on bone marrow cells *in vivo*]. Eksperimental'naia i klinicheskaia farmakologiia 66:50-52.

Patti GJ, Yanes O, Siuzdak G (2012) Innovation: Metabolomics: the apogee of the omics trilogy. Nature reviews Molecular cell biology 13:263-269.

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. Journal of neuroscience methods 14:149-167.

Perfumi M, Mattioli L (2007) Adaptogenic and central nervous system effects of single doses of 3% rosavin and 1% salidroside *Rhodiola rosea* L. extract in mice. Phytotherapy research 21:37-43.

Perlmutter LS (1994) Microvascular pathology and vascular basement membrane components in Alzheimer's disease. Molecular neurobiology 9:33-40.

Perry E, Howes MJ (2011) Medicinal plants and dementia therapy: herbal hopes for brain aging? CNS neuroscience & therapeutics 17:683-698.

Perry NS, Bollen C, Perry EK, Ballard C (2003) *Salvia* for dementia therapy: review of pharmacological activity and pilot tolerability clinical trial. Pharmacology, biochemistry, and behavior 75:651-659.

Petsalo A, Jalonen J, Tolonen A (2006) Identification of flavonoids of *Rhodiola rosea* by liquid chromatography-tandem mass spectrometry. Journal of chromatography A 1112:224-231.

Piscitelli SC, Burstein AH, Welden N, Gallicano KD, Falloon J (2002) The effect of garlic supplements on the pharmacokinetics of saquinavir. Clinical infectious diseases 34:234-238.

Pitsikas N, Boultadakis A, Georgiadou G, Tarantilis PA, Sakellaridis N (2008) Effects of the active constituents of *Crocus sativus* L., crocins, in an animal model of anxiety. Phytomedicine 15:1135-1139.

Pooja, Bawa AS, Khanum F (2009) Anti-inflammatory activity of *Rhodiola rosea*--"a second-generation adaptogen". Phytotherapy research 23:1099-1102.

Pooja KRA, Khanum F, Bawa AS (2006) Phytoconstituents and antioxidant potency of *Rhodiola rosea* - A versatile adaptogen. Journal of Food Biochemistry 30 203-214.

Porquet D, Grinan-Ferre C, Ferrer I, Camins A, Sanfeliu C, Del Valle J, Pallas M (2014) Neuroprotective role of trans-resveratrol in a murine model of familial Alzheimer's disease. Journal of Alzheimer's disease 42:1209-1220.

Porter VR, Buxton WG, Fairbanks LA, Strickland T, O'Connor SM, Rosenberg-Thompson S, Cummings JL (2003) Frequency and characteristics of anxiety among patients with Alzheimer's disease and related dementias. The Journal of neuropsychiatry and clinical neurosciences 15:180-186.

Portoles A, Terleira A, Calvo A, Martinez I, Resplandy G (2006) Effects of *Hypericum perforatum* on ivabradine pharmacokinetics in healthy volunteers: an open-label, pharmacokinetic interaction clinical trial. Journal of clinical pharmacology 46:1188-1194.

Pratte MA, Nanavati KB, Young V, Morley CP (2014) An alternative treatment for anxiety: a systematic review of human trial results reported for the Ayurvedic herb ashwagandha (*Withania somnifera*). Journal of alternative and complementary medicine 20:901-908.

Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. European journal of pharmacology 463:3-33.

Puchades-Carrasco L, Pineda-Lucena A (2015) Metabolomics in pharmaceutical research and development. Current opinion in biotechnology 35:73-77.

Qian EW, Ge DT, Kong SK (2012) Salidroside protects human erythrocytes against hydrogen peroxide-induced apoptosis. Journal of natural products 75:531-537.

Qu ZQ, Zhou Y, Zeng YS, Li Y, Chung P (2009) Pretreatment with *Rhodiola rosea* extract reduces cognitive impairment induced by intracerebroventricular streptozotocin in rats: implication of anti-oxidative and neuroprotective effects. Biomedical and environmental sciences 22:318-326.

Qu ZQ, Zhou Y, Zeng YS, Lin YK, Li Y, Zhong ZQ, Chan WY (2012) Protective effects of a *Rhodiola crenulata* extract and salidroside on hippocampal neurogenesis against streptozotocin-induced neural injury in the rat. PloS one 7:e29641.

Querfurth HW, LaFerla FM (2010) Alzheimer's disease. The New England journal of medicine 362:329-344.

Raber J, Huang Y, Ashford JW (2004) ApoE genotype accounts for the vast majority of AD risk and AD pathology. Neurobiology of aging 25:641-650.

Rahimi R, Abdollahi M (2012) An update on the ability of St. John's wort to affect the metabolism of other drugs. Expert opinion on drug metabolism & toxicology 8:691-708.

Ratia M, Gimenez-Llort L, Camps P, Munoz-Torrero D, Perez B, Clos MV, Badia A (2013) Huprine X and huperzine A improve cognition and regulate some neurochemical processes related with Alzheimer's disease in triple transgenic mice (3xTg-AD). Neuro-degenerative diseases 11:129-140.

Ravindran LN, Stein MB (2010) The pharmacologic treatment of anxiety disorders: a review of progress. The Journal of clinical psychiatry 71:839-854.

Ray B, Bisht S, Maitra A, Maitra A, Lahiri DK (2011) Neuroprotective and neurorescue effects of a novel polymeric nanoparticle formulation of curcumin (NanoCurc) in the neuronal cell culture and animal model: implications for Alzheimer's disease. Journal of Alzheimer's disease 23:61-77.

Reisberg B, Doody R, Stoffler A, Schmitt F, Ferris S, Mobius HJ, Memantine Study G (2003) Memantine in moderate-to-severe Alzheimer's disease. The New England journal of medicine 348:1333-1341.

Reitz C (2012) Alzheimer's disease and the amyloid cascade hypothesis: a critical review. International journal of Alzheimer's disease 2012:369808.

Reitz C, Mayeux R (2014) Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. Biochemical pharmacology 88:640-651.

Revina TA, Krasnov EA, Sviridova TP, Stepanyuk GY, Surov YP (1976) Biological characteristics and chemical composition of *Rhodiola rosea* L. grown in Tomsk. Rastitel'nye Resursy 12:355-360.

Rezayat M, Roohbakhsh A, Zarrindast MR, Massoudi R, Djahanguiri B (2005) Cholecystokinin and GABA interaction in the dorsal hippocampus of rats in the elevated plus-maze test of anxiety. Physiology & behavior 84:775-782.

Roberts CK, Sindhu KK (2009) Oxidative stress and metabolic syndrome. Life sciences 84:705-712.

Rodgers RJ, Dalvi A (1997) Anxiety, defence and the elevated plus-maze. Neuroscience and biobehavioral reviews 21:801-810.

Rodgers RJ, Cole JC, Davies A (1994) Antianxiety and behavioral suppressant actions of the novel 5-HT1A receptor agonist, flesinoxan. Pharmacology, biochemistry, and behavior 48:959-963.

Rodgers RJ, Cutler MG, Jackson JE (1997) Behavioural effects in mice of subchronic buspirone, ondansetron and tianeptine. II. The elevated plus-maze. Pharmacology, biochemistry, and behavior 56:295-303.

Rohloff J (2002) Volatiles from rhizomes of *Rhodiola rosea* L. Phytochemistry 59:655-661.

Ross IA (2001) Medicinal plants of the world. Chemical constituents, traditional and modern medicinal uses. Clifton, NJ: Humana Press.

Rusanen M, Kivipelto M, Quesenberry CP, Jr., Zhou J, Whitmer RA (2011) Heavy smoking in midlife and long-term risk of Alzheimer disease and vascular dementia. Archives of internal medicine 171:333-339.

Ruschitzka F, Meier PJ, Turina M, Luscher TF, Noll G (2000) Acute heart transplant rejection due to Saint John's wort. Lancet 355:548-549.

Ryan D, Robards K (1998) Critical review: Phenolic compounds in olives. The Analyst 123:31R-44R.

Ryan SD, Whitehead SN, Swayne LA, Moffat TC, Hou W, Ethier M, Bourgeois AJ, Rashidian J, Blanchard AP, Fraser PE, Park DS, Figeys D, Bennett SA (2009) Amyloid-beta42 signals tau hyperphosphorylation and compromises neuronal viability by disrupting alkylacylglycerophosphocholine metabolism. Proceedings of the National Academy of Sciences of the United States of America 106:20936-20941.

Sachdev PS, Blacker D, Blazer DG, Ganguli M, Jeste DV, Paulsen JS, Petersen RC (2014) Classifying neurocognitive disorders: the DSM-5 approach. Nature reviews Neurology 10:634-642.

Sadowski M, Pankiewicz J, Scholtzova H, Ji Y, Quartermain D, Jensen CH, Duff K, Nixon RA, Gruen RJ, Wisniewski T (2004) Amyloid-beta deposition is associated with decreased hippocampal glucose metabolism and spatial memory impairment in APP/PS1 mice. Journal of neuropathology and experimental neurology 63:418-428.

Sando SB, Melquist S, Cannon A, Hutton M, Sletvold O, Saltvedt I, White LR, Lydersen S, Aasly J (2008) Risk-reducing effect of education in Alzheimer's disease. International journal of geriatric psychiatry 23:1156-1162.

Saratikov AS, Krasnov EA, Khnykina LA, Duvidzon LM (1967) Separation and study of individual biologically active agents from *Rhodiola rosea* and *Rhodiola quadrifida*. Izv Sib Otd Akad Nauk SSSR, Ser Biol-Med Nauk 1:54–60.

Satou T, Kasuya H, Maeda K, Koike K (2014) Daily inhalation of alpha-pinene in mice: effects on behavior and organ accumulation. Phytotherapy research 28:1284-1287.

Savai J, Varghese A, Pandita N (2013) Lack of the cytochrome P450 3A interaction of methanolic extract of *Withania somnifera*, Withaferin A, Withanolide A and Withanoside IV. Journal of Pharmaceutical Negative Results 4:26-32.

Savai J, Varghese A, Pandita N, Chintamaneni M (2015) *In vitro* assessment of CYP1A2 and 2C9 inhibition potential of *Withania somnifera* and *Centella asiatica* in human liver microsomes. Drug metabolism and personalized therapy 30:137-141.

Savonenko A, Xu GM, Melnikova T, Morton JL, Gonzales V, Wong MP, Price DL, Tang F, Markowska AL, Borchelt DR (2005) Episodic-like memory deficits in the APPswe/PS1dE9 mouse model of Alzheimer's disease: relationships to beta-amyloid deposition and neurotransmitter abnormalities. Neurobiology of disease 18:602-617.

Schenk D, Basi GS, Pangalos MN (2012) Treatment strategies targeting amyloid beta-protein. Cold Spring Harbor perspectives in medicine 2:a006387.

Scheuner D et al. (1996) Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nature medicine 2:864-870.

Schriner SE, Avanesian A, Liu Y, Luesch H, Jafari M (2009a) Protection of human cultured cells against oxidative stress by *Rhodiola rosea* without activation of antioxidant defenses. Free radical biology & medicine 47:577-584.

Schriner SE, Abrahamyan A, Avanessian A, Bussel I, Maler S, Gazarian M, Holmbeck MA, Jafari M (2009b) Decreased mitochondrial superoxide levels and enhanced protection against paraquat in *Drosophila melanogaster* supplemented with *Rhodiola rosea*. Free radical research 43:836-843.

Schriner SE, Lee K, Truong S, Salvadora KT, Maler S, Nam A, Lee T, Jafari M (2013) Extension of *Drosophila* lifespan by *Rhodiola rosea* through a mechanism independent from dietary restriction. PloS one 8:e63886.

Scott IM, Leduc RJ, Burt AJ, Marles RJ, Arnason JT, Foster BC (2006) The Inhibition of Human Cytochrome P450 by Ethanol Extracts of North American Botanicals. Pharmaceutical Biology 44:315-327.

Scripnikov A, Khomenko A, Napryeyenko O, Group G-NS (2007) Effects of *Ginkgo biloba* extract EGb 761 on neuropsychiatric symptoms of dementia: findings from a randomised controlled trial. Wiener medizinische Wochenschrift 157:295-300.

Sehgal N, Gupta A, Valli RK, Joshi SD, Mills JT, Hamel E, Khanna P, Jain SC, Thakur SS, Ravindranath V (2012) *Withania somnifera* reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein in liver. Proceedings of the National Academy of Sciences of the United States of America 109:3510-3515.

Selkoe DJ (2011) Alzheimer's disease. Cold Spring Harbor perspectives in biology 3:1-16.

Sepand MR, Soodi M, Hajimehdipoor H, Soleimani M, Sahraei E (2013) Comparison of Neuroprotective Effects of *Melissa officinalis* Total Extract and Its Acidic and Non-Acidic Fractions against A beta-Induced Toxicity. Iranian journal of pharmaceutical research 12:415-423.

Shah ZA, Sharma P, Vohora SB (2003) *Ginkgo biloba* normalises stress-elevated alterations in brain catecholamines, serotonin and plasma corticosterone levels. European neuropsychopharmacology 13:321-325.

Sharma RA, Steward WP, Gescher AJ (2007) Pharmacokinetics and pharmacodynamics of curcumin. Advances in experimental medicine and biology 595:453-470.

Shatar S, Adams RP, Koenig W (2007) Comparative study of the essential oil of *Rhodiola rosea* L. from Mongolia. Journal of Essential Oil Research 19:215-217.

Shen Y, Kojima Y, Terazawa M (1999) Four glucosides of p-hydroxyphenyl derivatives from birch leaves. Journal of Wood Science 45:332-336.

Shi C, Zhao L, Zhu B, Li Q, Yew DT, Yao Z, Xu J (2009) Protective effects of *Ginkgo biloba* extract (EGb761) and its constituents quercetin and ginkgolide B against beta-amyloid peptide-induced toxicity in SH-SY5Y cells. Chemico-biological interactions 181:115-123.

Shi LL, Wang L, Zhang YX, Liu YJ (2007) Approaches to biosynthesis of salidroside and its key metabolic enzymes. Forestry Studies in China 9:295-299.

Shi TY, Feng SF, Xing JH, Wu YM, Li XQ, Zhang N, Tian Z, Liu SB, Zhao MG (2012) Neuroprotective effects of Salidroside and its analogue tyrosol galactoside against focal cerebral ischemia *in vivo* and H_2O_2-induced neurotoxicity *in vitro*. Neurotoxicity research 21:358-367.

Shin KY, Lee JY, Won BY, Jung HY, Chang KA, Koppula S, Suh YH (2009) BT-11 is effective for enhancing cognitive functions in the elderly humans. Neuroscience letters 465:157-159.

Si H, Liu D (2014) Dietary antiaging phytochemicals and mechanisms associated with prolonged survival. The Journal of nutritional biochemistry 25:581-591.

Siepmann M, Krause S, Joraschky P, Muck-Weymann M, Kirch W (2002) The effects of St John's wort extract on heart rate variability, cognitive function and quantitative EEG: a comparison with amitriptyline and placebo in healthy men. British journal of clinical pharmacology 54:277-282.

Sin TK, Yung BY, Siu PM (2015) Modulation of SIRT1-Foxo1 signaling axis by resveratrol: implications in skeletal muscle aging and insulin resistance. Cellular physiology and biochemistry 35:541-552.

Singh M, Arseneault M, Sanderson T, Murthy V, Ramassamy C (2008) Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. Journal of agricultural and food chemistry 56:4855-4873.

Singh SR, Levine MA (2006) Natural health product use in Canada: analysis of the National Population Health Survey. The Canadian journal of clinical pharmacology 13:e240-250.

Singh SR, Levine MA (2007) Potential interactions between pharmaceuticals and natural health products in Canada. Journal of clinical pharmacology 47:249-258.

Skarpanska-Stejnborn A, Pilaczynska-Szczesniak L, Basta P, Deskur-Smielecka E (2009) The influence of supplementation with *Rhodiola rosea* L. extract on selected redox parameters in professional rowers. International Journal of Sport Nutrition and Exercise Metabolism 19:186-199.

Skopińska-Rózewska E, Malinowski M, Wasiutyński A, Sommer E, Furmanowa M, Mazurkiewicz M, Siwicki AK (2008) The influence of *Rhodiola quadrifida* 50% hydro-alcoholic extract and salidroside on tumor-induced angiogenesis in mice. Polish Journal of Veterinary Sciences 11 97-104.

Smith T, Lynch ME, Johnson J, Kawa K, Bauman H, Blumenthal M (2015) Herbal Dietary Supplement Sales in US Increase 6.8% in 2014. HerbalGram 107:52-59.

Song YY, Qi G, Han JT, Li YP (2006) Effect of salidroside on changes of interleukin beta of rats with global cerebral ischemia-reperfusion injury. Chinese Journal of Clinical Rehabilitation 10 30-32.

Soodi M, Naghdi N, Hajimehdipoor H, Choopani S, Sahraei E (2014) Memory-improving activity of *Melissa officinalis* extract in naive and scopolamine-treated rats. Research in pharmaceutical sciences 9:107-114.

Spanakis M, Vizirianakis IS, Batzias G, Niopas I (2013) Pharmacokinetic interaction between losartan and *Rhodiola rosea* in rabbits. Pharmacology 91:112-116.

Stackman RW, Eckenstein F, Frei B, Kulhanek D, Nowlin J, Quinn JF (2003) Prevention of age-related spatial memory deficits in a transgenic mouse model of Alzheimer's disease by chronic *Ginkgo biloba* treatment. Experimental neurology 184:510-520.

Stafford GI, Pedersen ME, van Staden J, Jager AK (2008) Review on plants with CNS-effects used in traditional South African medicine against mental diseases. Journal of ethnopharmacology 119:513-537.

Steinberg M, Shao H, Zandi P, Lyketsos CG, Welsh-Bohmer KA, Norton MC, Breitner JC, Steffens DC, Tschanz JT, Cache County I (2008) Point and 5-year period prevalence of neuropsychiatric symptoms in dementia: the Cache County Study. International journal of geriatric psychiatry 23:170-177.

Stern Y (2012) Cognitive reserve in ageing and Alzheimer's disease. The Lancet Neurology 11:1006-1012.

Stewart S, Cacucci F, Lever C (2011) Which memory task for my mouse? A systematic review of spatial memory performance in the Tg2576 Alzheimer's mouse model. Journal of Alzheimer's disease 26:105-126.

Stover KR, O'Leary TP, Brown RE (2012) A Computer-Based Application for Rapid Unbiased Classification of Swim Paths in the Morris Water Maze. In: Proceedings of Measuring Behaviour (Spink; AJ, Greico; F, Krips; OE, Loijens; LWS, Noldus; LPJJ, Zimmerman. PH, eds), pp 353-357. Utrecht, The Netherlands.

Suardiaz M, Estivill-Torrus G, Goicoechea C, Bilbao A, Rodriguez de Fonseca F (2007) Analgesic properties of oleoylethanolamide (OEA) in visceral and inflammatory pain. Pain 133:99-110.

Suh GH, Wimo A, Gauthier S, O'Connor D, Ikeda M, Homma A, Dominguez J, Yang BM, Mental Health Economics Task Force of the International Psychogeriatric A (2009) International price comparisons of Alzheimer's drugs: a way to close the affordability gap. International psychogeriatrics / IPA 21:1116-1126.

Sun C, Wang Z, Zheng Q, Zhang H (2012) Salidroside inhibits migration and invasion of human fibrosarcoma HT1080 cells. Phytomedicine 19:355-363.

Sung HK, Sun HH, Se YC (2006) Antioxidative effects of *Cinnamomi cassiae* and *Rhodiola rosea* extracts in liver of diabetic mice. BioFactors 26:209-219.

Suresh D, Srinivasan K (2010) Tissue distribution & elimination of capsaicin, piperine & curcumin following oral intake in rats. The Indian journal of medical research 131:682-691.

Sutherland M (2007) New Visions and Understanding: Alzheimer's Disease and Related Dementias with Aboriginal Individuals-Roundtable Forum. In. Sudbury, Ontario.

Svenson KA, Lafontaine C (2003) Chapter six: The search for wellness. In: First Nations and Inuit Regional Health Survey Steering Committee, First Nations and Inuit Regional Health Survey. Ottawa: First Nations and Inuit Regional Health Survey Steering Committee.

Taiwo AE, Leite FB, Lucena GM, Barros M, Silveira D, Silva MV, Ferreira VM (2012) Anxiolytic and antidepressant-like effects of *Melissa officinalis* (lemon balm) extract in rats: Influence of administration and gender. Indian journal of pharmacology 44:189-192.

Tam TW, Liu R, Arnason JT, Krantis A, Staines WA, Haddad PS, Foster BC (2009) Actions of ethnobotanically selected Cree anti-diabetic plants on human cytochrome P450 isoforms and flavin-containing monooxygenase 3. Journal of ethnopharmacology 126:119-126.

Tan CB, Gao M, Xu WR, Yang XY, Zhu XM, Du GH (2009) Protective effects of salidroside on endothelial cell apoptosis induced by cobalt chloride. Biological & pharmaceutical bulletin 32:1359-1363.

Tan MS, Yu JT, Tan CC, Wang HF, Meng XF, Wang C, Jiang T, Zhu XC, Tan L (2015a) Efficacy and adverse effects of *Ginkgo biloba* for cognitive impairment and dementia: a systematic review and meta-analysis. Journal of Alzheimer's disease 43:589-603.

Tan X, Gu J, Zhao B, Wang S, Yuan J, Wang C, Chen J, Liu J, Feng L, Jia X (2015b) Ginseng improves cognitive deficit via the RAGE/NF-kappaB pathway in advanced glycation end product-induced rats. Journal of ginseng research 39:116-124.

Tao Y, Xu H, Wang S, Wang B, Zhang Y, Wang W, Huang B, Wu H, Li D, Zhang Y, Xiao X, Li Y, Yang H, Huang L (2013) Identification of the absorbed constituents after oral administration of Yuanhu Zhitong prescription extract and its pharmacokinetic study by rapid resolution liquid chromatography/quadrupole time-of-flight. Journal of chromatography B, Analytical technologies in the biomedical and life sciences 935:1-9.

Tariot PN, Farlow MR, Grossberg GT, Graham SM, McDonald S, Gergel I, Memantine Study G (2004) Memantine treatment in patients with moderate to severe Alzheimer disease already receiving donepezil: a randomized controlled trial. The Journal of the American Medical Association 291:317-324.

Taylor DP (1988) Buspirone, a new approach to the treatment of anxiety. Federation of American Societies for Experimental Biology journal 2:2445-2452.

Taylor MW (2014) Targeting neurodegeneration in Alzheimer’s disease using natural products derived from Maya traditional medicine. In: Department of Biology. Ottawa: University of Ottawa.

Teri L, Ferretti LE, Gibbons LE, Logsdon RG, McCurry SM, Kukull WA, McCormick WC, Bowen JD, Larson EB (1999) Anxiety of Alzheimer's disease: prevalence, and

comorbidity. The journals of gerontology Series A, Biological sciences and medical sciences 54:M348-352.

Thieme H (1969) On the identity of glucoside rhodioloside and salidroside. Die Pharmazie 24:118-119.

Thieme H, Winkler H (1966) On the occurrence of salidroside in the leaves of the red whortleberry. Die Pharmazie 21:182.

Thors L, Belghiti M, Fowler CJ (2008) Inhibition of fatty acid amide hydrolase by kaempferol and related naturally occurring flavonoids. British journal of pharmacology 155:244-252.

Tian J, Shi J, Smallman R, Iwatsubo T, Mann DM (2006) Relationships in Alzheimer's disease between the extent of Abeta deposition in cerebral blood vessel walls, as cerebral amyloid angiopathy, and the amount of cerebrovascular smooth muscle cells and collagen. Neuropathology and applied neurobiology 32:332-340.

Tildesley NT, Kennedy DO, Perry EK, Ballard CG, Wesnes KA, Scholey AB (2005) Positive modulation of mood and cognitive performance following administration of acute doses of *Salvia lavandulaefolia* essential oil to healthy young volunteers. Physiology & behavior 83:699-709.

Tiseo PJ, Perdomo CA, Friedhoff LT (1998) Concurrent administration of donepezil HCl and ketoconazole: assessment of pharmacokinetic changes following single and multiple doses. British journal of clinical pharmacology 46 Suppl 1:30-34.

Tolonen A, Hohtola A, Jalonen J (2003a) Liquid chromatographic analysis of phenylpropanoids from *Rhodiola rosea* extracts. Chromatographia 57:577-579.

Tolonen A, Pakonen M, Hohtola A, Jalonen J (2003b) Phenylpropanoid glycosides from *Rhodiola rosea*. Chemical & pharmaceutical bulletin 51:467-470.

Touma C, Ambree O, Gortz N, Keyvani K, Lewejohann L, Palme R, Paulus W, Schwarze-Eicker K, Sachser N (2004) Age- and sex-dependent development of adrenocortical hyperactivity in a transgenic mouse model of Alzheimer's disease. Neurobiology of aging 25:893-904.

Trincavelli ML, Da Pozzo E, Daniele S, Martini C (2012) The $GABA_A$-BZR complex as target for the development of anxiolytic drugs. Current topics in medicinal chemistry 12:254-269.

Troshchenko AT, Kutikova GA (1967) Rhodioloside from *Rhodiola rosea* and *Rh. quadrifida*. I. Chemistry of Natural Compounds 3 204-207.

Tsang SY, Xue H (2004) Development of effective therapeutics targeting the $GABA_A$ receptor: naturally occurring alternatives. Current pharmaceutical design 10:1035-1044.

Tyrer P, Baldwin D (2006) Generalised anxiety disorder. Lancet 368:2156-2166.

U.S. Burden of Disease Collaborators (2013) The state of U. S. health, 1990-2010: Burden of diseases, injuries and risk factors. Journal of the Americal Medical Association 310:591-608.

Uprety Y, Asselin H, Dhakal A, Julien N (2012) Traditional use of medicinal plants in the boreal forest of Canada: review and perspectives. Journal of ethnobiology and ethnomedicine 8:1-14.

US Pharmacopeial Convention (2014) *Rhodiola rosea* root and rhizome. In: Herbal Medicines Compendium.

van der Watt G, Laugharne J, Janca A (2008) Complementary and alternative medicine in the treatment of anxiety and depression. Current opinion in psychiatry 21:37-42.

van Diermen D, Marston A, Bravo J, Reist M, Carrupt PA, Hostettmann K (2009) Monoamine oxidase inhibition by *Rhodiola rosea* L. roots. Journal of ethnopharmacology 122:397-401.

Vandenbogaerde A, Zanoli P, Puia G, Truzzi C, Kamuhabwa A, De Witte P, Merlevede W, Baraldi M (2000) Evidence that total extract of *Hypericum perforatum* affects exploratory behavior and exerts anxiolytic effects in rats. Pharmacology, biochemistry, and behavior 65:627-633.

Veerendra Kumar MH, Gupta YK (2003) Effect of *Centella asiatica* on cognition and oxidative stress in an intracerebroventricular streptozotocin model of Alzheimer's disease in rats. Clinical and experimental pharmacology & physiology 30:336-342.

Verpeut JL, Walters AL, Bello NT (2013) *Citrus aurantium* and *Rhodiola rosea* in combination reduce visceral white adipose tissue and increase hypothalamic norepinephrine in a rat model of diet-induced obesity. Nutrition research 33:503-512.

Vieira MT, Huang S (2012) Botanical-Drug Interactions: A scientific perspective. Planta medica 78:1400-1415.

Vignes M, Maurice T, Lante F, Nedjar M, Thethi K, Guiramand J, Recasens M (2006) Anxiolytic properties of green tea polyphenol (-)-epigallocatechin gallate (EGCG). Brain research 1110:102-115.

Vingtdeux V, Dreses-Werringloer U, Zhao H, Davies P, Marambaud P (2008) Therapeutic potential of resveratrol in Alzheimer's disease. BMC neuroscience 9 Suppl 2:S6.

von Gunten A, Schlaefke S, Uberla K (2015) Efficacy of *Ginkgo biloba* extract EGb 761 in dementia with behavioural and psychological symptoms: A systematic review. The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry:1-12.

Vorhees CV, Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nature protocols 1:848-858.

Wake G, Court J, Pickering A, Lewis R, Wilkins R, Perry E (2000) CNS acetylcholine receptor activity in European medicinal plants traditionally used to improve failing memory. Journal of ethnopharmacology 69:105–114.

Walker JM, Fowler SW, Miller DK, Sun AY, Weisman GA, Wood WG, Sun GY, Simonyi A, Schachtman TR (2011) Spatial learning and memory impairment and increased locomotion in a transgenic amyloid precursor protein mouse model of Alzheimer's disease. Behavioural brain research 222:169-175.

Walker JM, Klakotskaia D, Ajit D, Weisman GA, Wood WG, Sun GY, Serfozo P, Simonyi A, Schachtman TR (2015) Beneficial effects of dietary EGCG and voluntary exercise on behavior in an Alzheimer's disease mouse model. Journal of Alzheimer's disease 44:561-572.

Walker TB, Altobelli SA, Caprihan A, Robergs RA (2007) Failure of *Rhodiola rosea* to alter skeletal muscle phosphate kinetics in trained men. Metabolism: clinical and experimental 56:1111-1117.

Wan WB, Cao L, Liu LM, Kalionis B, Chen C, Tai XT, Li YM, Xia SJ (2014) EGb761 provides a protective effect against Abeta1-42 oligomer-induced cell damage and blood-brain barrier disruption in an *in vitro* bEnd.3 endothelial model. PloS one 9:e113126.

Wanasuntronwong A, Tantisira MH, Tantisira B, Watanabe H (2012) Anxiolytic effects of standardized extract of *Centella asiatica* (ECa 233) after chronic immobilization stress in mice. Journal of ethnopharmacology 143:579-585.

Wang BS, Wang H, Wei ZH, Song YY, Zhang L, Chen HZ (2009a) Efficacy and safety of natural acetylcholinesterase inhibitor huperzine A in the treatment of Alzheimer's disease: an updated meta-analysis. Journal of neural transmission 116:457-465.

Wang F, Shing M, Huen Y, Tsang SY, Xue H (2005) Neuroactive flavonoids interacting with $GABA_A$ receptor complex. Current drug targets CNS and neurological disorders 4:575-585.

Wang F, Blanchard AP, Elisma F, Granger M, Xu H, Bennett SA, Figeys D, Zou H (2013a) Phosphoproteome analysis of an early onset mouse model (TgCRND8) of Alzheimer's disease reveals temporal changes in neuronal and glia signaling pathways. Proteomics 13:1292-1305.

Wang H, Zhou G, Gao X, Wang Y, Yao W (2007a) Acetylcholinesterase inhibitory-active components of *Rhodiola rosea* L. Food Chemistry 105:24-27.

Wang H, Ding Y, Zhou J, Sun X, Wang S (2009b) The *in vitro* and *in vivo* antiviral effects of salidroside from *Rhodiola rosea* L. against coxsackievirus B3. Phytomedicine 16:146-155.

Wang J, Bi W, Cheng A, Freire D, Vempati P, Zhao W, Gong B, Janle EM, Chen TY, Ferruzzi MG, Schmeidler J, Ho L, Pasinetti GM (2014) Targeting multiple pathogenic

mechanisms with polyphenols for the treatment of Alzheimer's disease-experimental approach and therapeutic implications. Frontiers in aging neuroscience 6:42.

Wang L, Shi LL, Liu YJ (2007b) Effects of different light treatments on growth and PAL activity of the suspension-cultured cells of *Rhodiola fastigiata*. Scientia Silvae Sinicae 43:49-53.

Wang Q, Yu X, Patal K, Hu R, Chuang S, Zhang G, Zheng J (2013b) Tanshinones inhibit amyloid aggregation by amyloid-beta peptide, disaggregate amyloid fibrils, and protect cultured cells. ACS chemical neuroscience 4:1004-1015.

Wang R, Yan H, Tang XC (2006) Progress in studies of huperzine A, a natural cholinesterase inhibitor from Chinese herbal medicine. Acta pharmacologica Sinica 27:1-26.

Wang X, Sun H, Zhang A, Jiao G, Sun W, Yuan Y (2011) Pharmacokinetics screening for multi-components absorbed in the rat plasma after oral administration traditional Chinese medicine formula Yin-Chen-Hao-Tang by ultra performance liquid chromatography-electrospray ionization/quadrupole-time-of-flight mass spectrometry combined with pattern recognition methods. The Analyst 136:5068-5076.

Wang Z, Hu H, Chen F, Zou L, Yang M, Wang A, Foulsham JE, Lan K (2012) Metabolic profiling assisted quality assessment of *Rhodiola rosea* extracts by high-performance liquid chromatography. Planta medica 78:740-746.

Wattanathorn J, Mator L, Muchimapura S, Tongun T, Pasuriwong O, Piyawatkul N, Yimtae K, Sripanidkulchai B, Singkhoraard J (2008) Positive modulation of cognition and mood in the healthy elderly volunteer following the administration of *Centella asiatica*. Journal of ethnopharmacology 116:325-332.

Wiedenfeld H, Dumaa M, Malinowski M, Furmanowa M, Narantuya S (2007) Phytochemical and analytical studies of extracts from *Rhodiola rosea* and *Rhodiola quadrifida*. Die Pharmazie 62:308-311.

Wiegant FA, Surinova S, Ytsma E, Langelaar-Makkinje M, Wikman G, Post JA (2009) Plant adaptogens increase lifespan and stress resistance in *C. elegans*. Biogerontology 10:27-42.

Wilson K, Rosenberg MW, Abonyi S, Lovelace R (2010) Aging and health: an examination of differences between older Aboriginal and non-Aboriginal people. Canadian journal on aging 29:369-382.

Woelk H, Arnoldt KH, Kieser M, Hoerr R (2007) *Ginkgo biloba* special extract EGb 761 in generalized anxiety disorder and adjustment disorder with anxious mood: a randomized, double-blind, placebo-controlled trial. Journal of psychiatric research 41:472-480.

Wolfer DP, Lipp HP (2000) Dissecting the behaviour of transgenic mice: is it the mutation, the genetic background, or the environment? Experimental physiology 85:627-634.

Wolfer DP, Stagljar-Bozicevic M, Errington ML, Lipp HP (1998) Spatial Memory and Learning in Transgenic Mice: Fact or Artifact? News in physiological sciences 13:118-123.

Wolfer DP, Madani R, Valenti P, Lipp HP (2001) Extended analysis of path data from mutant mice using the public domain software Wintrack. Physiology & behavior 73:745-753.

World Health Organization (2002) Active ageing: a policy framework, 2002 health report. In. Geneva: World Health Organization.

Wu X, Li Q, Xin H, Yu A, Zhong M (2005) Effects of berberine on the blood concentration of cyclosporin A in renal transplanted recipients: clinical and pharmacokinetic study. European journal of clinical pharmacology 61:567-572.

Wu YL, Lian LH, Jiang YZ, Nan JX (2009) Hepatoprotective effects of salidroside on fulminant hepatic failure induced by D-galactosamine and lipopolysaccharide in mice. The Journal of pharmacy and pharmacology 61:1375-1382.

Xie H, Wang JR, Yau LF, Liu Y, Liu L, Han QB, Zhao Z, Jiang ZH (2014) Quantitative analysis of the flavonoid glycosides and terpene trilactones in the extract of *Ginkgo biloba* and evaluation of their inhibitory activity towards fibril formation of beta-amyloid peptide. Molecules 19:4466-4478.

Xin T, Li X, Yao H, Lin Y, Ma X, Cheng R, Song J, Ni L, Fan C, Chen S (2015) Survey of commercial *Rhodiola* products revealed species diversity and potential safety issues. Scientific reports 5:8337.

Xing SH, Zhu CX, Zhang R, An L (2014) Huperzine a in the treatment of Alzheimer's disease and vascular dementia: a meta-analysis. Evidence-based complementary and alternative medicine 2014:1-10.

Xu J, Su Z, Feng P (1998) Suspension culture of compact callus aggregate of *Rhodiola sachalinensis* for improved salidroside production. Enzyme and Microbial Technology 23 20-27.

Xu JF, Su ZG (1997) Regulation of metabolism for improved salidroside production in cell suspension culture of *Rhodiola sachalinensis* A. Bor: the effect of precursors. Natural Product Research and Development 10:8-14.

Xu W, Zhang T, Wang Z, Liu T, Liu Y, Cao Z, Sui Z (2013) Two potent cytochrome P450 2D6 inhibitors found in *Rhodiola rosea*. Die Pharmazie 68:974-976.

Yaffe K, Vittinghoff E, Lindquist K, Barnes D, Covinsky KE, Neylan T, Kluse M, Marmar C (2010) Posttraumatic stress disorder and risk of dementia among US veterans. Archives of general psychiatry 67:608-613.

Yan H, Li L, Tang XC (2007) Treating senile dementia with traditional Chinese medicine. Clinical interventions in aging 2:201-208.

Yang G, Wang Y, Tian J, Liu JP (2013) Huperzine A for Alzheimer's disease: a systematic review and meta-analysis of randomized clinical trials. PloS one 8:e74916.

Yang L, Hao J, Zhang J, Xia W, Dong X, Hu X, Kong F, Cui X (2009) Ginsenoside Rg3 promotes beta-amyloid peptide degradation by enhancing gene expression of neprilysin. The Journal of pharmacy and pharmacology 61:375-380.

Yao Y, Jia M, Wu JG, Zhang H, Sun LN, Chen WS, Rahman K (2010) Anxiolytic and sedative-hypnotic activities of polygalasaponins from *Polygala tenuifolia* in mice. Pharmaceutical biology 48:801-807.

Yi T, Zhu L, Tang YN, Zhang JY, Liang ZT, Xu J, Zhao ZZ, Yu ZL, Bian ZX, Yang ZJ, Chen HB (2014) An integrated strategy based on UPLC-DAD-QTOF-MS for metabolism and pharmacokinetic studies of herbal medicines: Tibetan "Snow Lotus" herb (*Saussurea laniceps*), a case study. Journal of ethnopharmacology 153:701-713.

Yiannopoulou KG, Papageorgiou SG (2013) Current and future treatments for Alzheimer's disease. Therapeutic advances in neurological disorders 6:19-33.

Yoo DY, Choi JH, Kim W, Yoo KY, Lee CH, Yoon YS, Won MH, Hwang IK (2011) Effects of *Melissa officinalis* L. (lemon balm) extract on neurogenesis associated with serum corticosterone and GABA in the mouse dentate gyrus. Neurochemical research 36:250-257.

Yousef GG, Grace MH, Cheng DM, Belolipov IV, Raskin I, Lila MA (2006) Comparative phytochemical characterization of three *Rhodiola* species. Phytochemistry 67:2380-2391.

Yu S, Liu M, Gu X, Ding F (2008a) Neuroprotective effects of salidroside in the PC12 cell model exposed to hypoglycemia and serum limitation. Cellular and molecular neurobiology 28:1067-1078.

Yu S, Shen Y, Liu J, Ding F (2010) Involvement of ERK1/2 pathway in neuroprotection by salidroside against hydrogen peroxide-induced apoptotic cell death. Journal of molecular neuroscience 40:321-331.

Yu S, Liu L, Wen T, Liu Y, Wang D, He Y, Liang Y, Liu X, Xie L, Wang G, Wei W (2008b) Development and validation of a liquid chromatographic/electrospray ionization mass spectrometric method for the determination of salidroside in rat plasma: application to the pharmacokinetics study. Journal of chromatography B, Analytical technologies in the biomedical and life sciences 861:10-15.

Zanger UM, Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacology & therapeutics 138:103-141.

Zapesochnaya GG, Kurkin VA (1982) Glycosides of cinnamyl alcohol from the rhizomes of *Rhodiola rosea*. Chemistry of Natural Compounds 18:685-688.

Zapesochnaya GG, Kurkin VA (1983) The flavonoids of the rhizomes of *Rhodiola rosea*. II. A flavonolignan and glycosides of herbacetin. Chemistry of Natural Compounds 19:21-29.

Zhang A, Sun H, Wang Z, Sun W, Wang P, Wang X (2010a) Metabolomics: towards understanding traditional Chinese medicine. Planta medica 76:2026-2035.

Zhang F, Zhan Q, Gao S, Dong X, Jiang B, Sun L, Tao X, Chen WS (2014) Chemical profile- and pharmacokinetics-based investigation of the synergistic property of platycodonis radix in traditional Chinese medicine formula Shengxian decoction. Journal of ethnopharmacology 152:497-507.

Zhang J, Liu A, Hou R, Zhang J, Jia X, Jiang W, Chen J (2009) Salidroside protects cardiomyocyte against hypoxia-induced death: a HIF-1alpha-activated and VEGF-mediated pathway. European journal of pharmacology 607:6-14.

Zhang J, Chen X, Wang P, Huo L, Shen Z, Guo X, Cheng W, Bi K (2008) LC-MS determination and pharmacokinetic study of salidroside in rat plasma after oral administration of traditional chinese medicinal preparation *Rhodiola crenulata* extract. Chromatographia 67:695-700.

Zhang J, Zhen YF, Pu Bu Ci R, Song LG, Kong WN, Shao TM, Li X, Chai XQ (2013a) Salidroside attenuates beta amyloid-induced cognitive deficits via modulating oxidative stress and inflammatory mediators in rat hippocampus. Behavioural brain research 244:70-81.

Zhang JM, Hu GY (2001) Huperzine A, a nootropic alkaloid, inhibits N-methyl-D-aspartate-induced current in rat dissociated hippocampal neurons. Neuroscience 105:663-669.

Zhang L, Yu H, Zhao X, Lin X, Tan C, Cao G, Wang Z (2010b) Neuroprotective effects of salidroside against beta-amyloid-induced oxidative stress in SH-SY5Y human neuroblastoma cells. Neurochemistry international 57:547-555.

Zhang L, Yu H, Sun Y, Lin X, Chen B, Tan C, Cao G, Wang Z (2007) Protective effects of salidroside on hydrogen peroxide-induced apoptosis in SH-SY5Y human neuroblastoma cells. European journal of pharmacology 564:18-25.

Zhang L, Fang Y, Xu Y, Lian Y, Xie N, Wu T, Zhang H, Sun L, Zhang R, Wang Z (2015) Curcumin Improves Amyloid beta-Peptide (1-42) Induced Spatial Memory Deficits through BDNF-ERK Signaling Pathway. PloS one 10:e0131525.

Zhang SQ, Obregon D, Ehrhart J, Deng J, Tian J, Hou H, Giunta B, Sawmiller D, Tan J (2013b) Baicalein reduces beta-amyloid and promotes nonamyloidogenic amyloid precursor protein processing in an Alzheimer's disease transgenic mouse model. Journal of neuroscience research 91:1239-1246.

Zhang Y, Li L, Lin L, Liu J, Zhang Z, Xu D, Xiang F (2013c) Pharmacokinetics, tissue distribution, and excretion of salidroside in rats. Planta medica 79:1429-1433.

Zhang YH, Chen SD, Li JL, Yang HQ, Zheng R, Zhou HY, Wang G, Lu GQ (2006) Salidroside promotes the expression of GDNF in the MPTP model of Parkinson's disease. Chinese Journal of Neurology 39:540-543.

Zhong H, Xin H, Wu LX, Zhu YZ (2010) Salidroside attenuates apoptosis in ischemic cardiomyocytes: a mechanism through a mitochondria-dependent pathway. Journal of pharmacological sciences 114:399-408.

Zhou Y, Li W, Xu L, Chen L (2011) In *Salvia miltiorrhiza*, phenolic acids possess protective properties against amyloid beta-induced cytotoxicity, and tanshinones act as acetylcholinesterase inhibitors. Environmental toxicology and pharmacology 31:443-452.

Zhu Y, Shi YP, Wu D, Ji YJ, Wang X, Chen HL, Wu SS, Huang DJ, Jiang W (2011) Salidroside protects against hydrogen peroxide-induced injury in cardiac H9c2 cells via PI3K-Akt dependent pathway. DNA and cell biology 30:809-819.

Zou YQ, Cai ZY, Mao YF, Li JB, Deng XM (2009) [Effects of salidroside-pretreatment on neuroethology of rats after global cerebral ischemia-reperfusion]. Journal of Chinese integrative medicine 7:130-134.

APPENDIX A

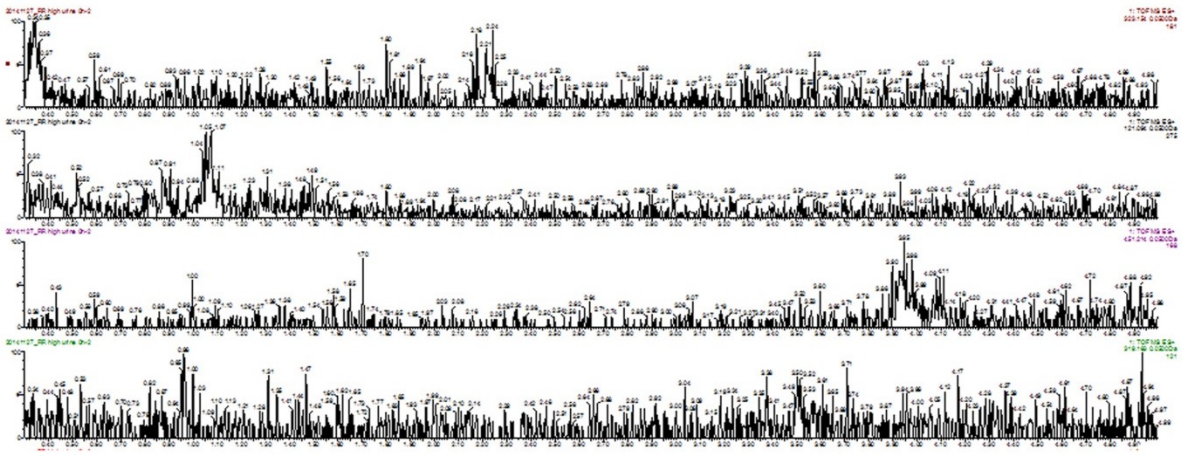
Input metrics for the feed-forward neural network used in MWM Visual

Metric	Reference
Escape latency	(Brody and Holtzman, 2006)
Percentage of time spent in the platform quadrant	(Wolfer et al., 2001)
Longest time the mouse looks at the platform (ratio)	(Wolfer et al., 2001)
Ratio of time spent in the thigmotaxis zone	(Brody and Holtzman, 2006)
Renormalized mean of the squared distance to the geometrical center	(Graziano et al., 2003)
Standard deviation of the distance to the geometrical center	(Graziano et al., 2003)
Standard deviation of the search path around the platform's center	(Graziano et al., 2003)
Anisotropy	N/A
Entropy	(Maei et al., 2009)
Mean angular velocity of position vector	(Graziano et al., 2003)
Mean angular velocity of tangential vector	(Graziano et al., 2003)
Dominant frequency in the tangential vector orientation	N/A
Mean deviation of points with respect to a line connecting geometric center of the path to the platform	(Wolfer et al., 2001)
Mean time spent per visited site	(Wolfer et al., 2001)
Coverage (ratio)	(Wolfer et al., 2001)

APPENDIX B

Extracted ion chromatograms for salidroside, tyrosol, rosarin/rosavin and rosin in the positive ion mode of (A) urine collected from *R. rosea*-treated rats (250 mg/kg) prior to treatment (0h) and (B) post-*R. rosea* treatment (8h).

A



B