Discovery of novel neurologically active phytochemicals in Neotropical Piperaceae: an ethnopharmacological approach

Gabriel Picard

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
In partial fulfillment of the requirements
For the M.Sc. degree in the
Ottawa-Carleton Institute of Biology

Thèse soumise à la
Faculté des études supérieures et postdoctorales
Université d'Ottawa
En vue de l'obtention de la maîtrise en sciences
de l'Institut de biologie Ottawa-Carleton

Résumé

L’objectif premier de ma thèse était de quantifier dans quelle mesure les plantes sont utilisées pour le traitement des maladies mentales et folkloriques telles que *susto* et *mal aire* dans la région néotropicale. Mon second objectif était d'investiguer le potentiel anxiolytique et antiépileptique d’espèces néotropicales des genres *Piper* et *Peperomia* jamais étudiés auparavant.

J’ai d’abord effectué une analyse de la littérature afin d’identifier les familles de plantes qui sont privilégiées pour le traitement des troubles de santé mentale, comportementaux et neurologiques. Cette analyse a identifié la famille des Piperaceae comme étant un groupe d’importance pour le traitement de ces troubles. J’ai ensuite récolté 55 espèces de plantes de la région amazonienne dont 47 espèces de la famille des Piperaceae et 21 espèces traditionnellement utilisées par les Yanesha, un groupe ethnique d'Amazonie Péruvienne, pour le traitement des maladies folkloriques. Afin de cibler les plantes ayant un potentiel anxiolytique et antiépileptique, j’ai effectué une série de bioessais *in vitro* visant à quantifier l’activité des plantes sur système acide γ-aminobutyrique (GABA). Les extraits de plantes ont montré, de façon générale, une forte affinité pour le récepteur GABA-BZD. Elles ont ainsi montré une activité de faible à modéré par rapport à l'inhibition de l'enzyme GABA-T, avec quelques plantes manifestant une activité prometteuse. Les plantes utilisées par les Yanesha ont démontré une activité comparable à celle d'autres plantes de la famille des Piperaceae, avec *Piper cremii* comme étant la plante la plus active dans le bioessai $GABA_A$ et *Drymaria cordata* dans le bioessai GABA-T. Finalement, j’ai présenté l'isolement de quatre composés

phytochimiques chez *Piper tuerckheimii*, une plante considéré comme étant l'un des remède traditionnelle le plus efficace concernant le traitement d'épilepsie et du *susto* par les Maya Q'eqchi' du Belize.

Ma thèse démontrent que la famille des Piperaceae est une source importante de composés neuroactifs et souligne l'importance du savoir traditionnel comme outils de recherche pour mettre en lumière le rôle des produits de santé naturel (PSN) dérivés de plantes pour la médicine alternative.

Abstract

The goal of this thesis was to understand and quantify to what extent plants are used for the treatment of mental and folk illnesses such as *susto* and *mal aire* in the Neotropics and to investigate the anxiolytic and antiepileptic potential of previously unstudied Neotropical members of the genera *Piper* and *Peperomia*.

Firstly, the literature was reviewed and a regression analysis method was used in order to quantitatively determine which plant families are preferred for the treatment of mental, behavioral and neurological health disorders in the Neotropics. This analysis identified Piperaceae, among others, as an important taxonomic group for the treatment of such disorders. Following that lead, a botanical survey was conducted in Peru, where 47 species of Piperaceae and 21 plants traditionally used for folk illnesses by the Yanesha of Peru, an Amazonian ethnic group, were collected. In order to target potential anxiolytic and antiepileptic plants, two high throughput bioassays were used to evaluate the extracts' *in vitro* activity on the γ-aminobutyric acid (GABA) system. Plant extracts in general demonstrated moderate to high affinity to the GABA-BZD receptor. Additionally, extracts demonstrated low to moderate activity in the inhibition of the GABA-T enzyme, with a few plants exhibiting promising activity. Plants selected by the Yanesha showed comparable activity to the other Piperaceae plants with *Piper cremii* being the most active plant in the $GABA_A$ assay, and *Drymaria cordata* in the GABA-T assay. Finally, four phytochemicals from *Piper tuerckheimii*, a plant regarded as one of the most effective traditional remedy for the treatment of epilepsy and *susto* by the Q'eqchi' Maya of Belize presented, were isolated for the first time.

Together, the results presented in this thesis show the significance of Piperaceae as a source for neuroactive compounds and the importance of traditional knowledge and plant derived NHPs as a complementary source of medicine.

Acknowledgements

This work is dedicated to my parents, François Picard and Martine Beaulieu, to my brother, Emmanuel Picard, and to Vashti King, who all contributed to who I am today. I would like to thank my supervisors Dr. Arnason and Dr. Starr for introducing me to the wonderful and breathtakingly unique world of plants. I will forever be grateful for what you have both thought me. The work could not have been completed without the help and contribution of the Yanesha people, the Q'eqchi' Maya healers of the Belize Indigenous Knowledge Institute, and of my Peruvian friends and colleagues Joaquina Castillo-Alban, Dr. Rosario Rojas, and their students. Dr. Céline Valadeau, thank you for introducing me to all the great people of Tsachopen, and for helping me with my research. I would also like to thank the members of my committee, Dr. Naomi Cappuccino, Dr. Charles Darveau, and Dr. Vance Trudeau for your invaluable guidance and feedback given to me throughout my research. Finally, I would like to express my eternal gratitude to all the members of the Arnason lab, past and present, who have been there for me. Most especially, to Martha Mullally and Chieu Anh Ta who have thought me all that I have to know about lab work, and to José Antonio Guerrero, Amar Saleem, and Asim Muhammad, for making me a novice chemist.

Table of Contents

RÉSUMÉ ..... **II**

ABSTRACT ..... **IV**

ACKNOWLEDGEMENTS ..... **VI**

TABLE OF CONTENTS ..... **VII**

CHAPTER 1

INTRODUCTION **1**

1.1 INTRODUCTION TO THE THESIS 2
1.2 BACKGROUND AND LITERATURE REVIEW 3
1.2.1 General ethnobotany of Piperaceae *3*
1.2.2 Folk illness, neuropsychological disorders and Piperaceae *5*
1.2.3 Anxiety, epilepsy, and the GABAergic system *7*
1.3. RATIONALE AND SPECIFIC OBJECTIVES 10

CHAPTER 2

A SYSTEMATIC REVIEW OF THE NEOTROPICAL PSYCHOACTIVE FLORA **13**

2.1 INTRODUCTION 14
2.2 METHODS 17
2.1.1 Selection Criteria *17*
2.2.2 Categorization of Plant Species for Psychotherapeutic Uses *18*
2.2.3 Data Management and Statistical Analyses *18*
2.3 RESULTS 20
2.3.1 Summary of Plant Use *20*
2.3.2 Regression Analysis *22*
2.4 DISCUSSION 32

CHAPTER 3

IN VITRO **PHARMACOLOGICAL EFFECT OF THE NEOTROPICAL PIPERACEAE INCLUDING MEDICINAL PLANTS USED BY THE YANESHA (PERU) IN GABAERGIC BIOASSAYS** **39**

3.1 INTRODUCTION 40
3.2 MATERIALS AND METHODS 43
3.2.1 Plant collection *43*
3.2.2 Plant Extraction and Sample Preparation *47*
3.2.3 $GABA_A$-BZD Receptor Binding Assay *48*
3.2.4 GABA-T Inhibition Assay *49*
3.2.5 Determination of EC_{50} and IC_{50} *50*
3.3 RESULTS AND DISCUSSION 50

CHAPTER 4

BIOASSAY-GUIDED ISOLATION OF THE ANXIOLYTIC AND ANTIEPILEPTIC PRINCIPLE OF *PIPER TUERCKHEIMII*, A PLANT USED BY THE Q'EQCHI' MAYA HEALERS OF BELIZE… **59**

4.1. INTRODUCTION 60
4.2 MATERIALS AND METHODS 62
4.2.1 Plant Material, Extraction and Sample Preparation *62*

4.2.2 Fractionation and Isolation *64*
4.2.3 Compound Identification *64*
4.2.4 $GABA_A$-BZD Receptor Binding Assay *65*
4.2.5 GABA-T Inhibition Assay *66*
4.2.6 Determination of EC_{50} and IC_{50} *67*
4.3 RESULTS AND DISCUSSION 67

CHAPTER 5 75
5.1. OVERVIEW OF ORIGINAL CONTRIBUTIONS TO KNOWLEDGE 76
5.2. PIPERACEAE AS A SOURCE OF NEUROACTIVE PHYTOCHEMICALS 77
5.3. FUTURE DIRECTIONS 80

LITTERATURE CITED........ 83

APPENDIX 1 96

Chapter 1

Introduction

1.1 Introduction to the thesis

There has been a recent increase in the use of plant derived natural health products (NHP's) for the treatment of anxiety and epilepsy disorders (Ekstein and Schachter 2010, Ernst 2006). These herbal remedies are often based on traditional knowledge (Carlini 2003), and exert their effect on the basis of their phytochemical constituents (Awad *et al.* 2009). The Piperaceae family, represented by plants such as black pepper (*Piper nigrum* L., Wattanathorn *et al.* 2008) and Kava (*Piper methysticum* G. Forst., Pittler and Ernst 2000), has been a significant source of alternative medicine for individuals afflicted with mental illness. The perception of mental illness varies strongly depending on the culture; thus, cultural context should not be overlooked, as is often the case, when documenting the traditional knowledge of medicinal plants of a certain group. The link between culture-bound syndromes (i.e. folk illnesses) and mental illness was recently emphasized when plants used for the treatment of ethnopsychiatric disorders by the Q'eqchi' Maya of Belize showed important biological activity in animal behavioral assays (Bourbonnais-Spear *et al.* 2007). Of all the plants available to the Q'eqchi' Maya, Piperaceae species seem to be preferentially selected for the treatment of such conditions (Bourbonnais-Spear *et al.* 2005) and, interestingly, amongst the most active species tested (Awad *et al.* 2009).

The broader goal of this thesis was to elucidate to what extent Piperaceae is used for the treatment of mental and folk illnesses in the Neotropics and to expand on the volume of knowledge pertaining to the biological activity and phytochemistry of Piperaceae. In Chapter 2, results of a systematic review are presented with the objective of compiling the information found in recent and historical ethnobotanical studies regarding the use of plants for the treatment of mental, behavioral and neurological health disorders, with the specific goal of highlighting potential plant families with activity on the γ-aminobutyric acid (GABA) system, a key system linked with epilepsy and anxiety disorders. In Chapter 3, the possible mechanism of action of plants traditionally used for folk illnesses among the Yanesha, an Amazonian Peruvian ethnic group possessing a valuable pharmacopoeia recently documented (Valadeau *et al.* 2010), was determined and the Peruvian Piperaceae flora was compared, in terms of potential anxiolytic and antiepileptic activity, to the Central American species used by the Q'eqchi' Maya. And finally in Chapter 4 focus is put on a representative of the Piperaceae, *Piper tuerckheimii*, and its anxiolytic and antiepileptic activity was investigated.

1.2 Background and literature review

1.2.1 General ethnobotany of Piperaceae

The Piperaceae C. Agardh is a highly diverse, pantropical plant family composed of two primary genera, *Piper* L. (about 2000 species, Fig 1.2 C) and *Peperomia* Ruiz & Pav. (about 1700 species, Fig 1.2 D). In spite of having the greatest diversity occurring in the Neotropics, the majority of the documentation concerning traditional uses of *Piper* comes from Asia due to the well-documented Ayurvedic and Traditional Chinese medicinal knowledge, and where the family is best known as the source of black pepper (*Piper nigrum* L., Srinivasan 2007). The diversity of medicinal uses and secondary metabolites isolated from plants of genus *Piper* is astounding, despite the fact that only a fraction of the total number of known species (4.2 %) has been thoroughly investigated (Parmar *et al.* 1997). Nevertheless, most of the medicinal species mentioned in the literature are from tropical Asia and represent only a portion of the global pepper diversity, suggesting that the true economic and medicinal potential of the family is highly underestimated.

Besides the well-known species of tropical Asia, Piperaceae have a special place in folk medicine all over the Neotropics where they are used for a wide variety of practices. For instance, the significance of Piperaceae as a source of medicine in the Neotropics has been highlighted by Lewis *et al.* (1999) who showed Piperaceae to be the most important medicinal plant family selected by the Aguarunas of Peru. In other parts of the Amazon rainforest, Piperaceae species have been documented in numerous pharmacopoeias and their reported medicinal uses are as diverse as their biodiversity in the region (Schultes and Raffauf 1990). For example, the Shuar of Eastern Ecuador use an assortment of species to sooth inflammation, to reduce fever, to treat diarrhea, headaches, and toothaches and for various other ailments (Bennett *et al.* 2002). In Central America, quantitative analyses have shown comparable results to Lewis *et al.* (1999) pointing out Piperaceae as one of the top ranked families in both the pharmacopoeia of Populaca from Mexico and of the Q'eqchi' Maya from southern Belize, where they are predominantly used to treat dermatological complaints, muscular-skeletal problems, infections, and digestive system disorders (Leonti *et al.* 2003, Amiguet *et al.* 2006). Recent ethnobotanical studies of the Q'eqchi' Maya have also emphasized that many Piperaceae species are also used for the treatment of folk illnesses related to anxiety disorders, and more precisely to treat seizures and epilepsy, disorders which were rarely the focus of previous ethnobotanical studies (Bourbonnais-Spear *et al.* 2005). For the purpose of this thesis focus will be put on the exceptional capability of Piperaceae to interact with the central nervous system as mentioned above.

1.2.2 Folk illness, neuropsychological disorders and Piperaceae

The Piperaceae family is home to one of the most famous of herbal drugs for central nervous system (CNS) activity: Kava. The name Kava (or Kava-Kava) can be applied to both the shrub *Piper methysticum* G. Forst., and the psychoactive beverage that is prepared from its roots. Kava has a long history of being traditionally used, recreationally and spiritually, by Polynesian societies of the South-Pacific and has acquired a cultural importance that extends beyond its relaxing and euphoric properties (Singh 1992). *P. methysticum* has gained popularity in modern medicine for its anxiolytic properties and has been prescribed in European herbal medicine as a sedative, tranquillizer and muscle relaxant (Zou *et al*. 2004). Kavalactones are the active principles, and act at various sites within the CNS. Consequently, kava has been regarded as a highly effective therapeutic alternative for the treatment of anxiety disorders (Singh and Singh 2002, Pittler and Ernst 2000). After commercialization, the use of kava products had increased throughout most of Western countries and was recently one of the most popular, and effective, dietary supplements in the United States (Ernst 2006). This trend was followed by a growing number of controversial cases that surfaced showing evidence of drug interactions and hepatoxicity, resulting in its withdrawal from the Western market even though there is no evidence of toxicity when used in the traditional manner (Teschke *et al.* 2010, Mathews *et al.* 2002). This results in the loss of a key alternative treatment for anxiety disorders, urging the need for research to restore the reputation of a species with a long history of safe use. Consequently, this provides opportunity to investigate the traditional knowledge and to discover novel leads for the treatment of neuropsychological disorders.

Recent ethnobotanical studies have shown that neurological and mental conditions such as epilepsy and the culture-bound syndrome *susto* – a disorder believed to be strongly linked to anxiety, depression based on its symptoms (Rubel 1964, Weller *et al.* 2008) – are well recognized by the Q'eqchi' Maya of Belize (Bourbonnais-Spear *et al.* 2005). Piperaceae species have been identified as highly valuable plants by healers for the treatment of such conditions and have also been shown to exhibit potent anxiolytic properties in animal behavioral assays (Bourbonnais-Spear *et al.* 2007), thus providing biological evidence of the link between *susto* and anxiety. Interestingly, Piperaceae species are not only preferred by traditional healers, but are also amongst the most active species tested based on their effect on the γ-aminobutyric acid (GABA) system, a key pathway involved in anxiety and epilepsy disorders (Awad *et al.* 2007). This highlights the need to focus future pharmacological and phytochemical studies on this unquestionably valuable plant family.

Culture-bound syndromes such as *susto* ("fright sickness") and *mal aire* ("malevolent wind" or "bad air") are found throughout Latin America (Weller *et al.* 2002, Rubel 1964). The distribution of these folk ilnnesses is particularly high in the Andean region of Peru and Ecuador (Cavander and Alban 2009, Bussmann and Sharon 2009, Carey 1993). Now that there is an unquestionable link between these folk illnesses and the Western concept of mental illness, plants of interest throughout the Neotropics should be evaluated for their anxiolytic and antiepileptic activity. Having said that, the task of evaluating an extensive collection of Piperaceae plants found throughout Peru has therefore been undertaken, as well as evaluating those selected for the treatment of folk illness recognized by the Yanesha, an Amazonian Peruvian ethnic group possessing a valuable and recently documented pharmacopoeia (Valadeau *et al.* 2010).

1.2.3 Anxiety, epilepsy, and the GABAergic system

A recent survey has showed that approximately 12 % of the Canadian work force is experiencing mental illness and that 32 % have faced it in the past (Chenier and Thorpe 2011). Globally, it is estimated that more than 450 million people or 7 % of the world's population suffers from anxiety disorders such as phobias, generalized anxiety, obsessive-compulsiveness, and post-traumatic stress amongst others (World Health Organization 2001). The closely related and extremely severe neurological condition of epilepsy has an estimated prevalence of 50 million people worldwide, of which approximately 80% are living in developing countries where diagnosis and treatments are considered privileges (World Health Organization 2001). Briefly, current widely used pharmaceuticals targeting these disorders, such as benzodiazepines (BZD), have adverse side effects including withdrawal symptoms and loss of muscle tone (Lader 2008). As for epilepsy, approximately 35 % of sufferers have drug-resistant seizures (Kwan *et al.* 2010). For these reasons, many people suffering from mental heath disorders are attracted to alternative sources of therapy (Eisenberg *et al.* 1998, Ekstein and Schachter 2010). Recent surveys estimate that 13 % of Canadians suffering from mental health disorders have used alternative health products (Vasiliadis and Tempier 2011), and 43 % of Americans use alternative forms of therapy in order to treat their anxiety (Eisenberg *et al.* 1998). In recent years, there has been a renewed interest in non-allopathic traditional forms of therapies including the use of herbal remedies such as *Passiflora incarnata* L., *Valeriana officinalis* L., and *Piper methysticum* G. Forst. (Ernst 2006). These plants, and their active principles have shown similar clinical effects to conventional pharmaceuticals and have been shown to exhibit their effect through same mechanisms of action, such as by interacting with GABAergic neurotransmission (Appel *et al.* 2011, Awad *et al.* 2009, Boonen and Häberlein 1998).

The common denominator between anxiety and epilepsy disorders seems to be linked to the imbalance in brain levels of γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the mammalian CNS (Chapouthier and Venault 2001). An overview of GABAergic transmission is detailed in Figure 1.1. Abnormalities in GABAergic function and lower levels of GABA in the brain, causing unusual neuronal excitability, are known to occur in both sufferers of seizure disorders and extremely anxious people (Lydiard 2003, Treiman 2001). While each disorder has recommended drugs acting on specific targets, such as $GABA_A$ receptor binding by benzodiazepines (BZD) for anxiety or the inhibition of GABA transaminase (GABA-T) by vigabatrin for epilepsy, there seems to be an overlap with drugs acting both as anxiolytics and anticonvulsants (Ashton and Young 2003). Therefore, it is critical to consider more than one target when screening for anxiolytic and anticonvulsant pharmaceuticals, or in my case, novel compounds of botanical origin.

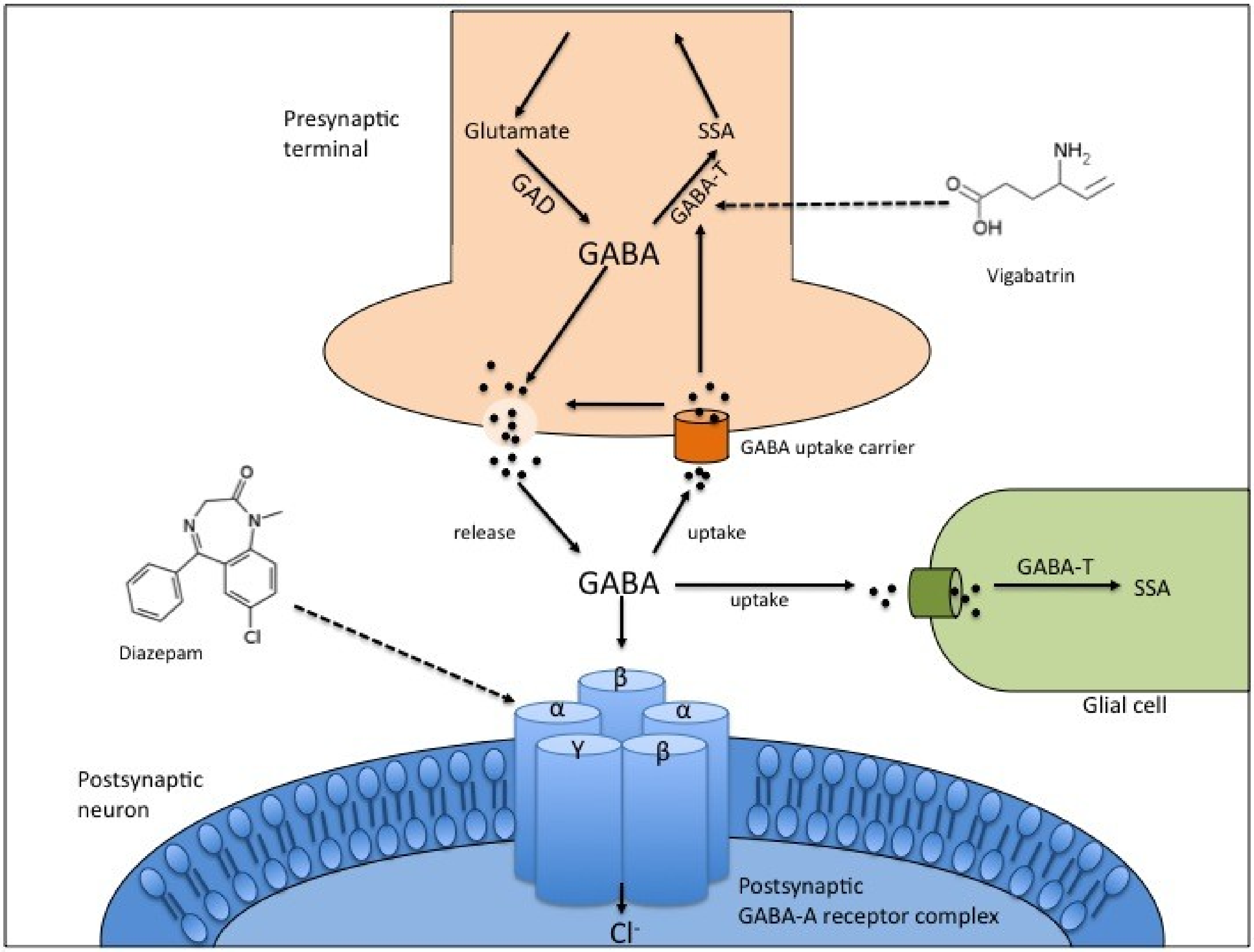


Figure 1.1. Schematic overview of γ-aminobutyric acid (GABA) neurotransmission. GABA is synthesized from glutamate by GAD. GABA is released from the presynaptic terminal into the synaptic cleft where it binds the $GABA_A$ receptor. The $GABA_A$ receptor is an ion-gated channel receptor composed of 5 membrane-spanning domains. Binding of GABA to the GABA site on the $GABA_A$ receptor elicits an allosteric change in the receptor and results in the influx of chloride ions (Cl^-) into the postsynaptic neuron, neuronal hyperpolarization and inhibition of neuronal signal. Pharmacological agents such as benzodiazepines, (e.g., Diazepam, valium, ativan) that are used in the treatment of anxiety bind the $GABA_A$ receptor at the benzodiazepine receptor, this causes an allosteric change in the receptor that increases GABA's affinity for its own receptor, increasing Cl^- influx into the postsynaptic neuron and hence increased neuronal inhibition which causes a reduction in anxiety. GABA in the synapse is taken back up into the presynaptic neuron via the GABA uptake carrier or into the glial cells and catabolized via GABA-T to succinic semialdehyde, which is shunted into the Kreb's cycle. Pharmacological agents used in the treatment of anxiety and epilepsy, such as vigabatrin, target the activity of GABA-T. Vigabatrin is an irreversible suicide inhibitor of GABA-T. Schematic adapted from Suzdak and Jansen 1995).

1.3. Rationale and specific objectives

The first objective of this thesis was to understand and quantify to what extent plants are used for the treatment of mental and folk illnesses in the Neotropics. The second objective was to broaden the scientific knowledge pertaining to the neuroactive activity and phytochemistry of the Piperaceae family by investigating the anxiolytic and antiepileptic potential of previously unstudied Neotropical members of the genera *Piper* and *Peperomia*.

Culture-bound syndromes and other mental health related ailments seem to be understudied and underrepresented in general ethnobotanical studies. Now that we know that there is a strong link between these folk illnesses, such as *susto* and *mal aire*, and western perception of neuropsychological disorders, such as anxiety and epilepsy, it is of interest to investigate the potential of traditionally used medicinal plants to interact with the GABAergic system. In Chapter 2, we expand on the knowledge of the traditional use of neuroactive plants across the Neotropics where such data are lacking, by systematically reviewing the literature with the objective of compiling the information regarding the use of plants for the treatment of mental, behavioral and neurological health disorders, with the specific goal of highlighting potential plant families with GABAergic activity. Using a statistical method adapted by Moerman (1991), we were able to quantitatively determine which plant families are preferred for the treatment of mental illness, and test the idea that plant families with a global tradition of use for such conditions (i.e. Piperaceae) should also have important medicinal value in the Neotropics.

Chapter 3 involved field work in both the Andean and Amazonian regions of Peru, made possible by collaboration with a group of Peruvian scientists studying the phytochemistry and taxonomy of Piperaceae. This team included Rosario Rojas, (phytochemistry), Gisella Ortega (molecular taxonomy), both of the Universidad Peruana Cayetano Heredia, and Joaquina Alban-Castillo (ethnobotany), of the Museo de Historia Natural in Lima, Peru. This chapter had the objective of comparing the bioactivity of Piperaceae plants used for the treatment of mental and folk illnesses by the Q'eqchi' Maya of Belize to novel species utilized traditionally in the Amazonian basin, yet unstudied. To accomplish this task we conducted a survey of Piperaceae species collected in various regions of Peru, a region where Piperaceae is known for its important biodiversity (Quijano-Abril *et al.* 2006), and collected species traditionally used for folk illnesses among the Yanesha of Tsachopen (Fig. 1.2 A and B), an Amazonian Peruvian ethnic group possessing a valuable pharmacopoeia recently documented (Valadeau *et al.* 2010). More precisely, we investigated whether collected plants would exert their activity on the GABA system by modulating the $GABA_A$ receptor, enhancing the affinity of GABA, or by interacting with the catabolism of GABA, inhibiting the GABA-T enzyme.

Finally, we conducted a bioassay-guided fractionation of *P. tuerckheimii*, recently identified as a plant used with high consensus by Belizean Q'eqchi' healers for the treatment of various ethnopsychiatric neural and mental conditions (Bourbonnais-Spear *et al.* 2005), in order to characterize its phytochemical profile and isolate its active principles (Chapter 4).

Figure 1.2. (A) A panoramic view of the Yanesha community of Tsachopen and the Yanashaga Chemillen National Park, Pasco Region, Oxapampa Province, Peru. (B) The collection of Piperaceae plants with a Yanesha healer. (C) *Piper subflavispicum* C.DC., a typical *Piper* species and (D) *Peperomia sp.,* a typical *Peperomia* species.

Chapter 2

A systematic review of the psychoactive Neotropical flora:

Special focus on mental health and the GABAergic system

Picard G[1], Starr J[1,2], and Arnason JT[1]

[1] Centre for Advanced Research in Environmental Genomics (CAREG), Department of Biology, University of Ottawa, Ottawa, Ontario, Canada
[2] Canadian Museum of Nature, Ottawa, Canada

2.1 Introduction

Each year mental and neurological disorders account for over 1.2 million deaths and contribute to 28% of the total burden of non-communicable diseases, surpassing cancer and cardiovascular diseases (WHO 2005). Despite the wide recognition of these alarming facts by the majority of health specialists, a considerable gap exists between the prevalence of these diseases and investment in treatment and intervention strategies (Saxena *et al.* 2007). In fact, the mental health budgets of the majority of countries constitute less than 1% of their total health expenditures (WHO 2001) and remain a low priority in most low and middle-income countries, where more than 80% of the world population lives (Jacob *et al.* 2007). While recent reports indicate the high prevalence of neuropsychological disorders in low and middle-income countries (Jacob *et al.* 2007), and highlight the scarcity and inequity of mental health resource allocation in these regions (Saxena *et al.* 2007), relatively little is known about the mental health status of indigenous groups throughout the world (WHO 1999).

In addition to environmental and climatic factors rendering indigenous groups more susceptible to various neuropsychiatric conditions (deBittencourt *et al.* 1996a, 1996b), many stressors throughout history, such as marginalization due to colonization, epidemics, conflicts and poverty, contribute to making them subject to higher rates of mental illnesses (WHO 1999). The inadequate distribution of psychiatric aid combined with the scarcity of primary health care forces the majority of indigenous people living in those regions to rely almost entirely on traditional knowledge for maintaining mental well-being. Therefore, similar to other traditional healing in general, mental health treatments by indigenous groups greatly depend on the medicinal flora, combined with spirituality, in order to treat mental, behavioral and neurological disorders (Carlini 2003). This applies to the 30 million indigenous people of tropical America.

The majority of existing data concerning indigenous mental health in the Neotropics comes from historical records and lacks systematic evaluation of the contemporary prevalence of such disorders (Elferink 1999a, 1999b). The study of psychoactive plants used for both ritualistic and/or healing purposes in the Amazonian basin and its surrounding regions has emphasized hallucinogenic plants used for spiritual and curative purposes (Schultes 1969, Schultes 1984, Schultes *et al.* 2001, Bennett 1992). Although the anthropological literature concerning ethnopsychiatry is fairly widespread, few field studies of Neotropical ethnobotany and ethnopharmacology have focused on the role of plants in the treatment of neuropsychiatric disorders (Shepard 1998, Bourbonnais-Spear *et al.* 2005, Rodrigues and Carlini 2006). Nevertheless, Bourbonnais-Spear *et al.* (2005) reported that indigenous cultures have a compelling understanding of plants and their use in various mental and neurological disorders, particularly epilepsy and culture-bound syndromes (i.e. *susto*).

The link between culture-bound syndromes, such as *susto* and *mal aire*, and mental illness was recently emphasized when plants used for the treatment of ethnopsychiatrical and neurological disorders by the Q'eqchi' Maya of Belize showed important biological activity in animal behavioral assays (Bourbonnais-Spear *et al.* 2007), highlighting the importance of traditional plants for the maintenance of well being. However, as mentioned previously, culture-bound syndromes and other mental health related ailments seem to be understudied and underrepresented in ethnobotanical and ethnopharmacological studies. Two reasons may account for this discrepancy: 1) their consideration is disregarded by researchers due to their religious and ritualistic connotation not recognized by modern biomedical symptomatology, or 2) they are absent entirely from the traditional knowledge of the study group in question. To address the state of ethnobotanical information from the present body of literature, the objective of this systematic review is to quantitatively compare the abundance and distribution of plants throughout the Neotropics with their relative usage in the traditional treatment of mental, behavioral and neurological health disorders.

Are medicinal plants selected in a completely random process from the available flora, or are some specific taxa selected more extensively than others? Numerous studies, using a statistical method adapted by Moerman (1991), have shown that there is in fact a taxonomic basis behind the selection of plants for medicine, with certain taxa being overrepresented in traditional pharmacopoeias (Kapur *et al.* 1992, Moerman 1996, Leonti *et al*. 2003, Bourbonnais-Spear *et al.* 2005, Amiguet *et al.* 2006, Douwes *et al.* 2008, Saslis-Lagoudakis *et al.* 2011). Using this simple and straightforward method, we were able to quantitatively identify for the first time plant families that are preferred for the treatment of mental illness in the Neotropics, and examine if plant families with a global tradition of use for such conditions (i.e. Piperaceae) are also represented as having important medicinal value by traditional people of tropical America. Thereafter, special focus was put on certain treatment categories (e.g. culture-bound syndromes, anxiety, epilepsy) in order to target plant taxa that might have the potential to interact with the GABAergic system, a key pathway involved in anxiety and epilepsy disorders.

2.2 METHODS

2.1.1 Selection Criteria

A comprehensive review of scientific literature (from 19XX to 2007) was undertaken to assess the taxonomic composition and prevalence of plants used by Traditional Peoples throughout the Neotropics for the treatment of ethnopsychiatric and (or) neurological disorders. From this review, studies were further selected for meta-analysis based on the criteria that they explicitly provide;

(1) A clear description of the traditional use for each plant recorded in the ethnopharmacopoeia;

(2) A proper taxonomic identification completed by independent professionals. (Preferably to species level); and,

(3) An estimate of their prevalence in treating neuropsychiatric disorders compared to the other therapeutic uses described in the ethnopharmacopoeia.

Accordingly**,** a total of 18 articles and 4 books focusing on ethnobotanical and ethnomedicinal practices in the Neotropics were selected (Table 2.1, Fig. 2.1) whereby a summary of the selected studies and data composition is provided in the Supplementary Information (Table 2.2). Overall, the selected meta-data for the analysis consisted in studies conducted within the boundaries of the tropical regions of America, between the Tropic of Cancer and the Tropic of Capricorn (Smith *et al.* 2003). The meta-data were subsequently divided into 2 primary investigative regions referring to 1) the Amazonian Basin and 2) all neighboring regions encompassing the Andes of Southern Ecuador and Northern Peru, the Atlantic forest of Eastern Brazil, and (to a greater extent) various regions of Central America. Hence, this classification strategy provided the opportunity to assess the data in a systematic manner.

2.2.2 Categorization of Plant Species for Psychotherapeutic Uses

Based on the reported ethnobotanical record, plants were classified as being 'psychoactive' if found to have any potential effect on the mind and brain or general impact toward behavior (Spinella 2001), and further classified under four distinct mutually exclusive therapeutic categories. Following the therapeutic category standards developed by Cook (1995), these sub-classifications included plants used toward (1) mental disorders (MEN), (2) nervous system disorders (NED), and (3) 'religious' and 'ritualistic' purposes (REL). Meanwhile, an additional category, (4) 'culture-bound syndromes' (CUL), was included to account for folk illnesses that are culturally specific (i.e., typically unrecognized by western medicine) yet thought to be strongly linked to mental health (Rubel *et al.* 1984).

2.2.3 Data Management and Statistical Analyses

Statistical regressions adapted by Moerman[1] (1991) were used to derive the functional relationship between the size (total # of species) of plant families and the contribution of psychoactive species to the pharmacopoeia (# of species used for the treatment of mental and neurological disorders within families). The premise underlying Moerman's prevalence analysis is that, if medicinal plants are selected at random, the likelihood of plant medicinal usage should be proportional to its ecological distribution; therefore, larger plant families should inherently have a greater total number of plants

[1] i.e., Regressing the number of species used as medicine per family (MPSPE) on the total number of Neotropical species found in those respective families (FNSPE) -

used by Traditional Peoples than smaller plant families. However, some plant families might be disproportionally represented in the medicinal flora, therefore suggesting that plants might not be selected in a random fashion. The regression analysis seeks to identify a baseline usage relationship, whereby plant families having proportionally higher usage than the baseline could represent groups having a greater cultural importance. For this reason, residual values for each plant family were calculated to determine their relative usage value and then ranked (from largest to smallest) to reveal their therapeutic importance (i.e. higher use than expected). The Flowering Plants of the Neotropics (Smith et al. 2003) was used to verify the total number of taxa per plant family (FNSPE)

All statistical analyses were performed using JMP version 5.0.1 (SAS Institute 2002) for the determination of regression equations, fit (r^2), and signification (p). In total, regression analyses were performed in a hierarchical manner to identify any potential relationships corresponding with each classification category. The first one was done with the complete data set in order to determine the most important plant families for mental health on a broad scale. Two similar analyses were done on subsets of the data based on the two distinct geographical regions in order to detect differences in plant use within regions of Neotropical America. In addition, category specific analyses were done for each of the four therapeutic categories, providing more in-depth information. Finally, in order to target the plant families that have the greatest potential for novel anxiolytic and anti-epileptic herbal drugs, an analysis was done on the ensemble of therapeutic categories that show potential to interact with the GABAergic system. Abnormalities in GABAergic function and lower levels of GABA in the brain, causing unusual neuronal excitability, are known to occur in both sufferers of seizure disorders and extremely anxious people (Lydiard 2003, Treiman 2001). The categories chosen for this includes anxiety related disorders (e.g. nervousness, insomnina, etc.), epilepsy, and various culture-bound syndromes, and were specifically selected based on mechanistic evidence discussed in the literature (Treiman 2001, Lydiard 2003, Bourbonnais-Spear 2007, Spinella 2001). Plants from these categories were selected based on the symptoms traditionally treated that closely reassembled symptoms treated by well-known GABAergic pharmaceuticals (i.e. benzodiazepines, vigabatrin, etc.).

2.3 RESULTS

2.3.1 Summary of Plant Use

Of the total 3858 plant usages documented for ritualistic and/or medicinal purposes, 323 mentioned to be specifically utilized for the treatment of mental health related disorders (Table 2.2). Meanwhile, a total of 271 species constituting 70 families were used for apparent psychoactive properties throughout the Tropics. Notably, 74% of these plants (200 sp.) offer a potential to interact with the GABAergic system based on the symptoms treated, thereby suggesting possible anxiolytic and/or anti-epileptic properties. The total inventory of medicinal plants in the Amazonian Basin was greater than that of the surrounding regions. However, the number of plants used for mental health purposes was two times greater in the surrounding regions than in the Basin (Table 2.2). An important factor, which could potentially explain a fraction of this difference, is the considerable amount of introduced species in the traditional practices of non-amazonian herbal medicine. In fact, only 1.8% of the total plants mentioned in the Amazonian region seemed to be of introduced nature compared to 24.6% for the surrounding regions (Table 2.2). Figures 2.2 and 2.3 illustrate the 10 most represented plant families and genera in terms of their broad use for mental health and more specifically, for their potential to interact with the GABAergic system. A significant overlap in the distribution of the most represented plant families and genera can be observed when comparing both categories; however, it is important to underline the inclusion of the Marcgraviaceae, a small plant family endemic to the Neotropics, in the later category highlighting their importanceas potential GABAergic interactants (Figure 2.3B). A study from our research group by Amiguet *et al.* (2005) was the only study to mention families of seedless plants (i.e. Adiantaceae, Selaginellaceae, etc.), and records were therefore removed from all analysis in case other studies simply failed to consider them. Consequently, 13 species of ferns, mosses and lichens, from seven distinct families were excluded. This, however, provides evidence that these understudied families are of medicinal importance and should not be disregarded in future studies of ethnobotanical nature.

The distribution of plants between the four main usage categories is illustrated in Table 2.3. The grouping with the most mentions is the mental disorders category (130 sp. or 48%) followed by culture-bound syndromes (88 sp.), neurological disorders (41 sp.), and plants with magical or ritualistic uses (39 sp.), which were virtually unique to the Amazonian geographic region. Hallucinogens aside, anxiety related disorders were the highest-ranking sub-category in both geographic regions. Culture-bound syndromes such as “fright illness” (*susto* and *espanto*) and “wind illness” (*mal aire* and *mal viento*) followed closely and accounted for 25% of total mentions. Plants treating *susto* and *espanto* were found in the same ratio in both geographical divisions. Contrarily, the reports of plants used for *mal aire* and *mal viento* seems to be skewed towards the regions neighboring the Amazonian basin. The knowledge of plants treating the neurological disorder of epilepsy is also unevenly distributed between regions, the consequence of one study including 75% of all mentions (Amiguet *et al.* 2005).

2.3.2 Regression Analysis

According to the residuals extracted from the regression analysis (Fig. 2.4A, F_{69} = 24.49, P < 0.0001, r^2 = 0.26) of the Neotropical ethnopharmacopoeia for mental health, the top five ranking plant families are as followed (Table 2.4): Solanaceae, Asteraceae, Lamiaceae, Myristicaceae and Piperaceae. On the other hand, the three plant families with the lowest residuals, therefore the lowest level of usage, are Orchidaceae, Gesneriaceae and Cactaceae. When focusing on the plants with potential to interact with the GABAergic system, the residuals extracted from the regression analysis (Fig. 2.4B, F_{58} = 14.62, P = 0.0003, r^2 = 0.20) showed that the same plant families were the most highly selected with the exception of Fabaceae which replaced Myristicaceae, a family reported to be used ritually as a hallucinogenic snuff in the Amazon region (e.g. *Virola* sp.) (Schultes and Raffauf1990, Bennett *et al.* 2002). The plant families with the highest level of use for mental health were very different when comparing the regression analysis for the two geographical separately (Fig. 2.4C and D, F_{41} = 3.76 P = 0.05, r^2 = 0.08 for the Amazon, and F_{54} = 25.7, P < 0.0001, r^2 = 0.32 for the neighboring regions). The top five ranking plant families (Table 2.5) for the Amazon region are Solanaceae, Myristicaceae, Marcgraviaceae, Acanthaceae, and Annonaceae as opposed to Solanaceae, Asteraceae, Lamiaceae, Myristicaceae, and Piperaceae for the neighboring regions. Only Solanaceae and Myristicaceae are common between the two regions. The most striking difference

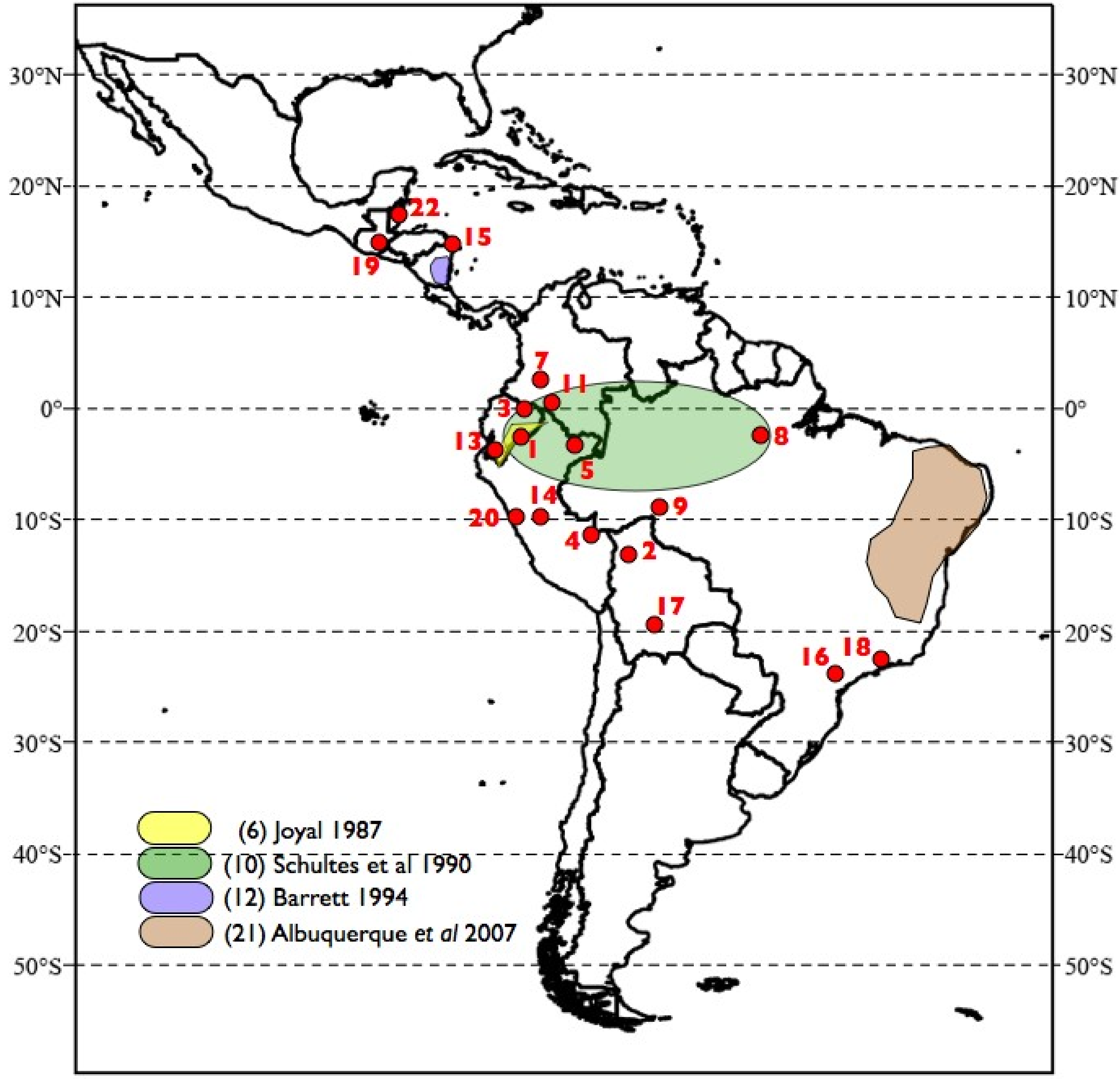


Figure 2.1. Geographic distribution of the selected studies. The numbers correspond to the studies of in Table 2. The map was created using ArcMap 10 (ESRI 2010).

Table 2.1. Brief description of the 22 studies selected.

Study		Country/Region	Ethnic Group(s)	Study Type	Source Type
Amazonian Basin					
(1)	Bennett *et al.* 2002	Eastern Ecuador	Shuar	Book	Original
(2)	Bourdy *et al.* 2000	Bolivian Amazon	Tacana	Article	Original
(3)	Davis *et al.* 1983	Eastern Ecuador	Waorani	Article	Original
(4)	Desmarchelier *et al.* 1996	Peruvian Amazon	Ese'ejas	Article	Original
(5)	Jovel *et al.* 1996.	Peruvian Amazon	Mestizo	Article	Original
(6)	Joyal 1992	Southern and Eastern Ecuador	NA	Article	Compilation of historical collections
(7)	Laferriere 1994	Colombian Amazon	Lowland Inga	Article	Original
(8)	Milliken *et al.* 1992	Brazilian Amazon	Waimiri Atroari	Book	Original
(9)	Prance *et al.* 1977	Brazilian Amazon	Paumari	Article	Original
(10)	Schultes *et al.* 1990	Western Amazon	49 indigenous groups	Book	Compilation
(11)	Vickers *et al.* 1984	Eastern Ecuador	Sioana and Secoya	Book	Original
Surrounding regions					
(12)	Barrett 1994	Atlantic Coast of Nicaragua	6 distinct ethnic groups	Article	Original
(13)	Bussmann *et al.* 2006	Southern Ecuador	Quichua	Article	Original
(14)	De-la-Cruz *et al.* 2007	Eastern Peruvian Andes	Quichua	Article	Original
(15)	Dennis 1988	Eastern Nicaragua	Miskito	Article	Original
(16)	Di Stasi *et al.* 2002	Brazilian Tropical Atlantic Forest	Various rural communities	Article	Original
(17)	Fernandez *et al.* 2003	Bustillo Province, Bolivia	Quichua	Article	Original
(18)	Figueiredo *et al.* 1997	Brazillian Tropical Atlantic Forest	Various coastal communities	Article	Original
(19)	Giron *et al.* 1991	Caribbean Basin of Guatemala	Caribs	Article	Original
(20)	Hammond *et al.* 1998	Eastern Peruvian Andes	Quichua	Article	Original
(21)	de Albuquerque *et al.* 2007	North Eastern Brazil	Various rural and indigenous communities	Article	Review of 21 original studies
(22)	Amiguet *et al.* 2005	Southern Belize	Q'eqchi' Maya	Article	Original

Table 2.2. Distribution of plant used for the treatment of neuropsychological disorders between studies. The total represents the number of mentions, not the number of species.

		Total # of medicinal and/or ritual species	Proportion used for mental health			Potential GABAergic activity		Introduced Species	
Study Location			Total no. use reports	# of species	% of species	# of species	% of species	# of	% of
AMAZON									
	Bennett *et al.* 2002	221	24	22	10.0	14	6.3	2	9.1
	Bourdy *et al.* 2000	150	4	3	2.0	3	2.0	0	0.0
	Davis *et al.* 1983	36	1	1	2.8	0	0.0	0	0.0
	Desmarchelier *et al.* 1996	24	2	2	8.3	0	0.0	0	0.0
	Jovel *et al.* 1996.	60	4	4	6.7	2	3.3	0	0.0
	Joyal 1992	120	4	4	3.3	2	1.7	0	0.0
	Laferriere 1994	46	4	4	8.7	1	2.2	0	0.0
	Milliken *et al.* 1992	59	3	3	5.1	2	3.4	0	0.0
	Prance *et al.* 1977	31	3	2	6.5	1	3.2	0	0.0
	Schultes *et al.* 1990	1516	65	60	4.0	21	1.4	0	0.0
	Vickers *et al.* 1984	53	7	7	13.2	2	3.8	0	0.0
	SUBTOTAL	**2316**	**121**	**112**	**4.8**	**34**	**30.4**	**2**	**1.8**
SURROUNDINGS									
	Barrett 1994	152	30	24	15.8	23	15.1	4	16.7
	Bussmann *et al.* 2006	215	55	43	20.0	40	18.6	13	30.2
	De-la-Cruz *et al.* 2007	87	7	7	8.0	7	8.0	0	0.0
	Dennis 1988	23	4	3	13.0	3	13.0	0	0.0
	Di Stasi *et al.* 2002	114	13	13	11.4	13	11.4	7	53.8
	Fernandez *et al.* 2003	56	7	7	12.5	4	7.1	5	71.4
	Figueiredo *et al.* 1997	35	6	6	17.1	6	17.1	3	50.0
	Giron *et al.* 1991	102	6	6	5.9	6	5.9	4	66.7
	Hammond *et al.* 1998	33	4	4	12.1	1	3.0	3	75.0
	de Albuquerque *et al.* 2007[a]	556	55	47	8.5	41	7.4	13	27.7
	Amiguet *et al.* 2005	169	64	51	30.2	42	24.9	0	0.0
	SUBTOTAL	**1542**	**251**	**211**	**13.7**	**163**	**77.3**	**52**	**24.6**
	TOTAL	**3858**	**372**	**323**	**8.4**	**234**	**72.4**	**54**	**16.7**

[a]A review of 21 studies

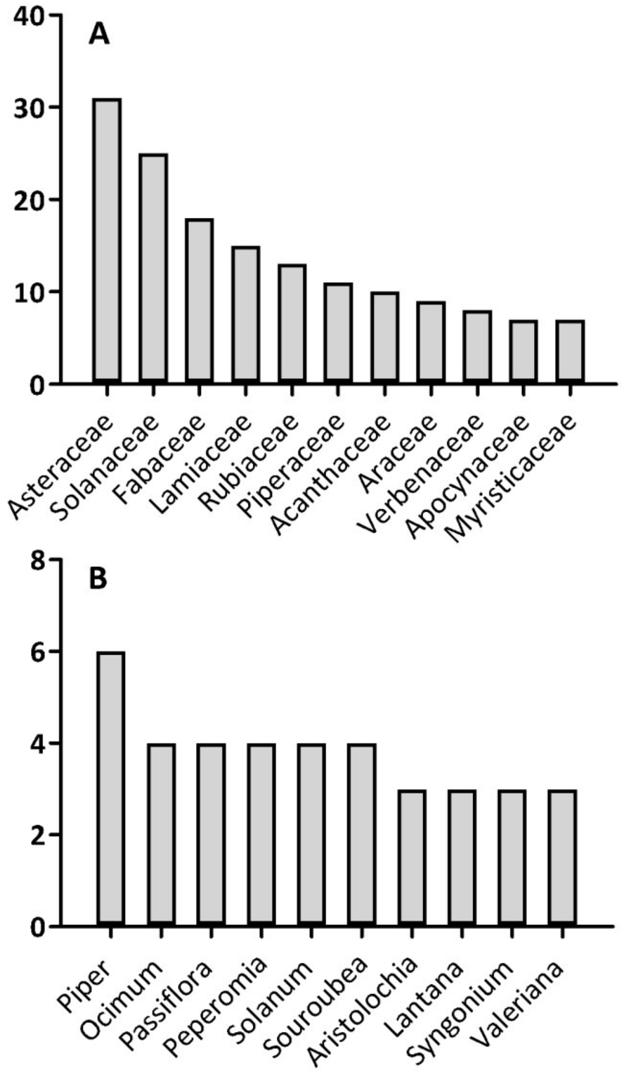


Figure 2.2. Number of species for the 11 most represented plant families (A) and genera (B) for the treatment of neuropsychiatric disorders in the Neotropics.

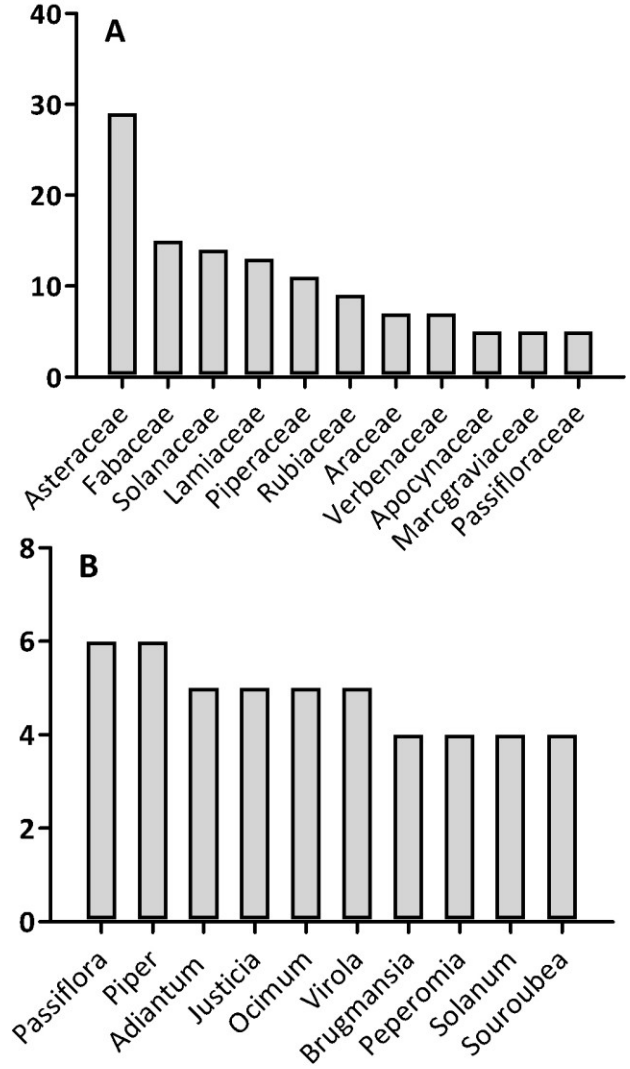


Figure 2.3. Number of species for the 10 most represented plant families (A) and genera (B) that have the potential to interact with the GABAergic system.

Table 2.3. Distribution of plant use between psychotherapeutic usage categories.

Usage Categories		Amazon	Surroundings	Total	
Culture-Bound Syndromes					
	Susto/Espanto	11	31	48	*
	Mal Aire/Mal Viento	5	34	39	*
	Grisi/Crazy Sickness	0	4	4	*
	Others	5	8	11	*
Mental Disorders					
	Anxiety/Nervousness	18	69	87	*
	Insomnia	12	14	26	*
	Madness/Insanity	2	13	15	
	Depression/Sadness	2	5	7	
	Dementia/Cognitive Enhancers	6	1	7	
Neurological Disorders					
	Epilepsy/Seizures	2	27	29	*
	Spasms	1	11	12	
Magical and Ritualistic					
	Hallucinogens	38	1	39	

* Categories with potential to interact with the GABAergic system

is observed when comparing both regions while examining residuals of Asteraceae, which is the family with the lowest usage level in the Amazon and the most highly used in the neighboring regions.

In order to have a more exhaustive understanding of the traditional knowledge concerning ethnopsychiatry in the Neotropics and the plant families which are most highly selected in the treatment of mental disorders and related afflictions, similar regression analysis were done for each specific usage category. The regression analysis for culture-bound syndromes (Fig. 2.5A, $F_{39} = 16.21$, $P < 0.0003$, $r^2 = 0.29$) and for mental disorders (Fig. 2.5B, $F_{46} = 4.83$, $P = 0.003$, $r^2 = 0.09$) yielded comparable results regarding the top five plant families with the highest use, with the exception of Fabaceae and Marcgraviaceae replacing Piperaceae and Zingiberaceae in the later category (Table 2.5). More precisely, the difference between these two categories is not found in the top ranking families but in the order in which they appear in the list, reflecting the symptomatic similarity between these two categories and the possible overlap of medicinal plants selected to treat such afflictions. On the other hand, the residuals extracted from the regression analysis of plant families for neurological disorders (Fig. 2.5C, $F_{17} = 9.09$, $P = 0.0082$, $r^2 = 0.36$) and for magical or ritualistic uses (Fig. 2.5D, $F_{13} = 0.48$, $P = 0.49$, $r^2 = 0.03$) display very distinct use profiles from the other categories. The list of the top and bottom ranking plant families for each category is represented in Table 2.4 and 2.5.

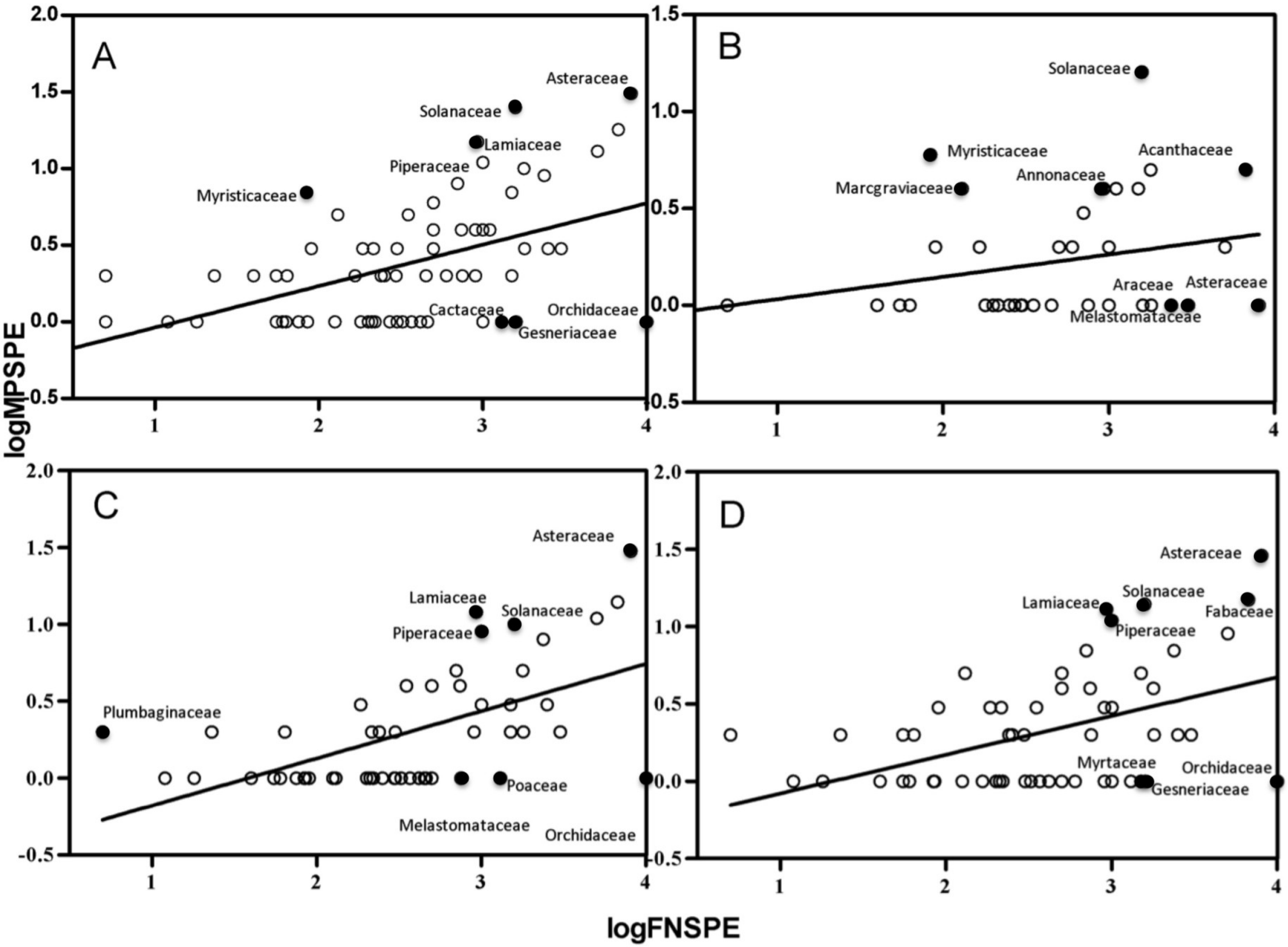


Figure 2.4. Regression plot of the recorded medicinal plant species (logMPSPE) for mental health in the Neotropics (A), for the Amazon (B), for the Surroundings (C) and with the potential to interact with GABA (D), versus the approximate number of species in the flora of the Neotropics (logFSNPE). Filled dots represent the 5 first- and the 3 last-ranking plant families.

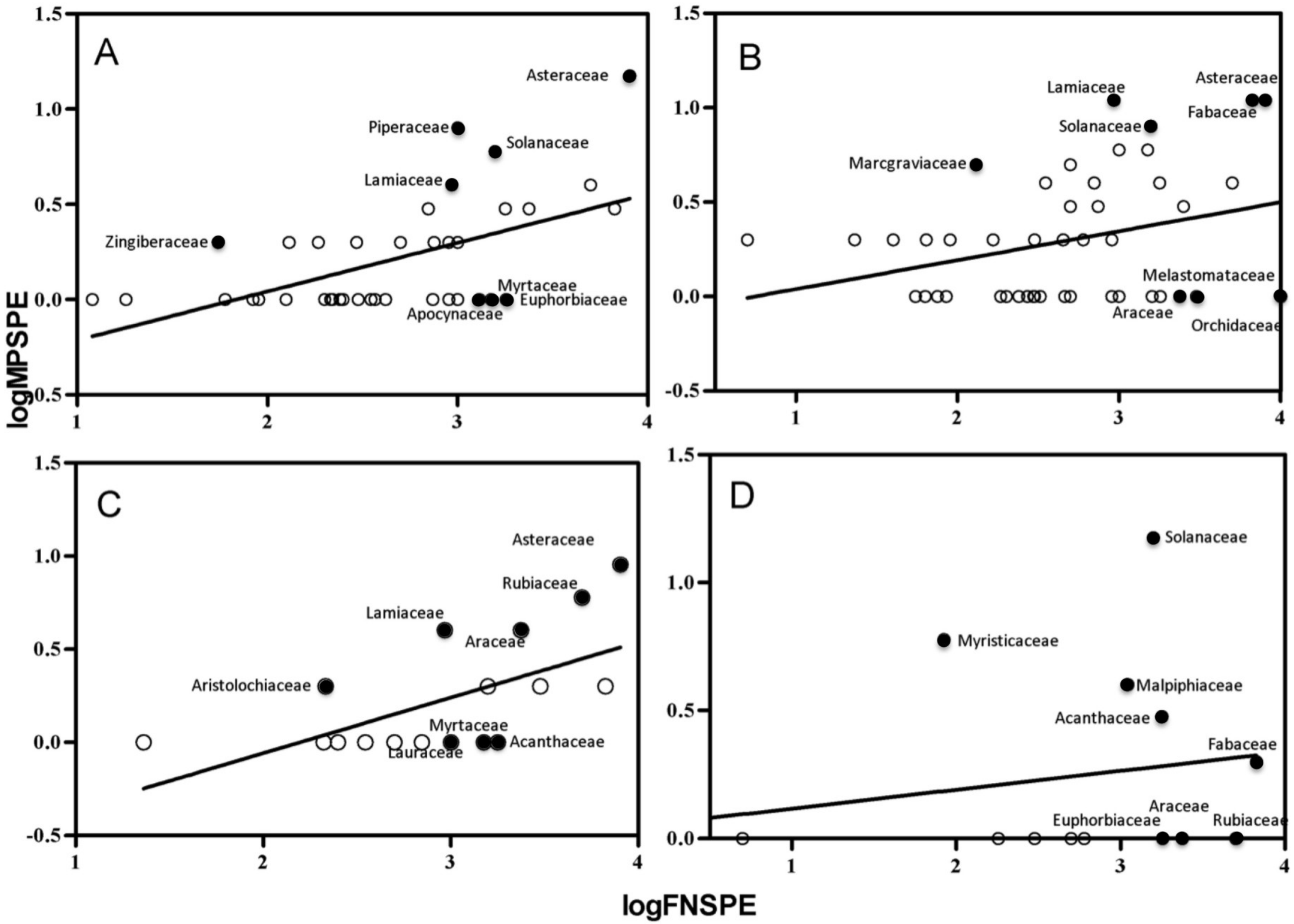


Figure 2.5. Regression plot of the recorded medicinal plant species (logMPSPE) for culture-bound syndromes (A), mental disorders (B), neurological disorders (C) and magic/ritual (D), versus the approximate number of species in the flora of the Neotropics (logFSNPE). Filled dots represent the 5 first- and the 3 last-ranking plant families.

Table 2.4. The 5 first- and the 3 last-ranking families of plants for the treatment of neuropsychiatric disorders in the Neotropics, the Amazon, the surrounding regions, and with the potential to interact with the GABAergic system.

	Neotropics		GABA		Amazon		Surroundings	
	Family	**Residual**	**Family**	**Residual**	**Family**	**Residual**	**Family**	**Residual**
Top Ranking	Solanaceae	0.84	Asteraceae	0.81	Solanaceae	0.91	Asteraceae	0.76
	Asteraceae	0.74	Lamiaceae	0.69	Myristicaceae	0.63	Lamiaceae	0.65
	Lamiaceae	0.68	Solanaceae	0.67	Marcgravia-ceae	0.44	Plumbaginac-eae	0.57
	Myristicaceae	0.63	Piperaceae	0.61	Acanthaceae	0.40	Piperaceae	0.51
	Piperaceae	0.53	Fabaceae	0.54	Annonaceae	0.34	Solanaceae	0.50
Low Ranking	Orchidaceae	-0.77	Orchidaceae	-0.67	Asteraceae	-0.36	Orchidaceae	-0.74
	Gesneriaceae	-0.55	Gesneriaceae	-0.47	Melastomata-ceae	-0.31	Cactaceae	-0.47
	Cactaceae	-0.53	Myrtaceae	-0.46	Araceae	-0.30	Scrophulariac-eae	0.38

Table 2.5. The 5 first- and the 3 last-ranking families in the 4 psychotherapeutic usage categories

	Culture-bound syndromes		Mental disorders		Neurological disorders		Magical	
	Family	**Residual**	**Family**	**Residual**	**Family**	**Residual**	**Family**	**Residual**
Top Ranking	Asteraceae	0.64	Lamiaceae	0.69	Asteraceae	0.44	Solanaceae	0.89
	Piperaceae	0.60	Fabaceae	0.56	Lamiaceae	0.37	Myristicaceae	0.59
	Solanaceae	0.42	Asteraceae	0.55	Rubiaceae	0.32	Malpighiaceae	0.33
	Zingiberaceae	0.32	Solanaceae	0.52	Aristolochia-ceae	0.25	Acanthaceae	0.19
	Lamiaceae	0.31	Marcgraviac-eae	0.48	Araceae	0.25	Fabaceae	-0.02
Low Ranking	Euphorbiaceae	-0.36	Orchidaceae	-0.50	Acanthaceae	-0.31	Rubiaceae	-0.31
	Myrtaceae	-0.34	Melastomata-ceae	-0.42	Myrtaceae	-0.29	Araceae	-0.29
	Apocynaceae	-0.34	Araceae	-0.40	Lauraceae	-0.24	Euphorbiaceae	-0.31

2.4 DISCUSSION

This systematic review has revealed a large traditional knowledge base of indigenous people throughout Tropical America for use of plants for mental health. In particular, many cultures are currently utilizing plants for treatment of neuropsychological disorders, with results varying from 2% to 30% of the total plant analyzed in the analysis (Table 2). Furthermore, by dividing the usage distribution into four psychotherapeutic categories and 12 sub-categories, five sub-categories were identified as being potentially active with the GABAergic system. Hereafter, emphasis is placed on the specific plant families having possible interaction with the GABAergic system.

The distribution profile of top-ranking plant families selected for disorders associated to the GABA system is very similar to the profile encompassing all plants (Fig. 2.4A and B). Not surprisingly, 74% of total species fell within one of the seven GABA related sub-categories. Based on the residuals extracted from the regression analysis, the five top-ranking plant families that are the most likely to interact with the GABAergic system are: Piperaceae, Solanaceae, Fabaceae, Lamiaceae, and Asteraceae. The Piperaceae are well known for their medicinal usage throughout the world having a wide selection of traditionally used species shown to exhibit potent activity on the nervous system (Parmar *et al.* 1997); these include two culturally and economically important species: *Piper nigrum*, black pepper (Srinivasan 2007), and *Piper methysticum*, Kava (Singh 1992). Some traditionally used Neotropical species have also shown to exhibit potent anxiolytic and antiepileptic properties in animal behavioural models (Bourbonnais-Spear *et al*. 2007) based on their effect on the γ-aminobutyric acid (GABA) system (Awad *et al.* 2007). The present study identified 11 potential candidates from the Piperaceae (six species of *Piper*, four of *Peperomia* and one of *Potomorphe*). To our knowledge, only one of these 11 species has been tested for its effect on the nervous system, and none concerning their effect on the GABAergic system (Felipe *et al.* 2007). *Piper tuberculatum*, the species in question, was mentioned to be used in Nicaragua for treatment against *susto* (Barrett 1994), and is also reported to be used by the Krahô Indians of eastern Brazil (Rodrigues and Carlini 2005). Solanaceae, on the other hand, is a widely distributed plant family famous for their psychoactive tropane alkaloids (Schultes *et al.* 2001). People from all corners of the world have used members of the family in a religious or spiritual context, such as the mandrake (*Mandragora officinarum*) and belladonna (*Atropa belladonna*) in Europe, tobacco (*Nicotiana tabacum*) in the Americas and the 'Trumpets of the angels' (*Brugmansia* sp.) in the Amazonian basin (Schultes *et al.* 2001). This review has targeted 10 species (out of the 25) that are used as sedatives, calmatives and anticonvulsants, thus having the potential to interact with the GABAergic system based on our selection criteria. With the exception of a recent study demonstrating anticonvulsant effect of the potato (*Solanum tuberosum*) in mice, through putative binding to GABA receptors, no other plants have been investigated for their potential activity on the GABAergic system (Muceniece *et al.* 2008). Verbenaceae has also shown many promising species, such as a Brazilian folk medicine derived from *Lippia alba* with CNS activity (Zétola *et al.* 2002). Lastly, the Asteraceae and Lamiaceae each have a worldwide distribution, many taxa, and a long history of usage among pharmacopoeias across the globe (Lewis and Elvin-Lewis 2003). In the present study, I have shown that 7 of 29 Asteraceae and 8 of 15 Lamiaceae species have the potential to interact with GABA. These cosmopolitan species have been introduced to Neotropics due to their usage as commercial remedies and culinary herbs, namely: *Matricaria recutita*, *Artemisia vulgaris*, *Tanacetum parthenium*, *Rosmarinus officinalis*, *Mentha* sp., *Cinnamomum zeylanicum,* and *Melissa officinalis*. Most species from the five top-ranking families have unknown phytochemical profiles. For instance, Parmar *et al.* (1997) reviewed the phytochemistry of 84 species of *Piper*, revealing that only approximately 4.2% of the genus has been more or less thoroughly investigated. To my knowledge, little is known regarding the phytochemistry of the genus *Peperomia*. The isolation of active compounds from these targeted plants could potentially offer novel leads to the development of safer and more accessible treatment strategies for neuropsychological disorders such as anxiety and epilepsy.

When comparing the psychoactive flora of the Amazonian basin to its neighboring regions, we can observe a notable difference in the distribution of plant uses (Fig. 2.4C and D). This striking difference in family preference is clearly manifested when observing the residual ranking of the Asteraceae, which seems to be the lowest ranking family in the Amazon and the highest in the surrounding regions. Besides the many diverging ecological factors (climate, altitude, different floristic diversity, etc.) between these regions, some anthropological factors could potentially explain a fraction of this difference. First, the use of narcotic and hallucinogenic plants seems to be distinct to the indigenous groups of the Amazon, where 38 of the 39 species were found, notably rainforest specific species of the genus *Banisteriopsis, Brugmansia* and *Virola*. Second, the weight of introduced plants in the traditional pharmacopoeia is much more important in the Andes and Atlantic coast of Brazil than in the Amazon, where only 2% of the plants were of introduced nature compared to 20% for the neighboring regions. Introduced plants are now an important source of food and are said to provide some of the most important herbal remedies for indigenous groups throughout South America (Bennett and Prance 2000); however, this could reflect the continent's colonial past which could account for a loss of traditional knowledge, particularly the traditional use of 'magical' plants in a non-Christian ritual context in regions such as the Andes, whereas this knowledge seems to be relatively well preserved in shamanistic societies of the Amazon. That being said, the Neotropics could be further divided into multiple geographic regions, taking into account the factors mentioned previously, thereby providing a more extensive survey. As a result, medicinally important and promising regional and cultural specific plant families, such as the Marcgraviaceae, which is know to interact with the GABAergic system (Mullally *et al.* 2011), will not be overlooked.

The most surprising result when examining the distribution of plant use between GABA related sub-categories is the rarity of plants used for the neurological disorder of epilepsy. Compared to other tropical regions of the world, the Neotropical ethnobotanical data available suggests very poor knowledge of anti-epileptic plants (Noumi and Fozi 2003, Stafford *et al.* 2008, Pedersen *et al.* 2009). To be more precise, 29 plants were mentioned for epilepsy; however, this considerable number is distributed unevenly between regions and is skewed by a single study that included 22 of the total species (Amiguet *et al.* 2005). This is surprising considering the dramatic impact of epileptic attacks and the fact that their incidence is increased in regions exposed to high levels of parasitic diseases (deBittencourt *et al.* 1996a, 1996b). Neuropsychiatric disorders such as epilepsy have always been surrounded by a powerful social stigma and have been strongly connected to witchcraft by many indigenous groups, which claim that spiritual intervention is the best treatment (Kufer *et al.* 2005). Since psychoactive plants are strongly linked to the spiritual realm, further studies should not disregard plants reported having religious and ritualistic uses as opposed to evident medicinal uses. Culture-bound syndromes on the other hand seem to be a significant source of mental illness in most regions, having an important number of plants selected for their respective treatment (32% of total species). Fright related illnesses such as *susto,* culturally described as '*soul loss',* are believed to be closely linked to anxiety-related disorders due to their causes and symptoms (Rubel *et al.* 1984, Carey 1993, Weller *et al.* 2002). The plant families preferentially selected for culture-bound syndromes and mental disorders are very comparable (Fig. 2.5A and B), suggesting overlap between these conditions. However, due to their elusive meaning and cultural significance, folk illnesses should be documented and studied separately from widely recognized biomedical diseases. The fact that culture-bound syndromes and mental illness in general are directly related to the supernatural renders them greatly sensitive to the 'westernization' of tropical America and could potentially be the first of the ethnomedicinal knowledge to disappear. For instance, a recent study by Sanz-Biset *et al.* (2009) states that '*vegetalistas*', Peruvian healers specialized in the spiritual, are nearly extinct today, highlighting the urgency to document and conserve traditional knowledge and showing that folk illnesses should not be disregarded when conducting any ethnobotanical related study.

In conclusion, the present study has provided some quantitative insights into the traditional usage of the potentially psychoactive plants of the Neotropics. The diversity of indigenous groups in the Neotropics truly rivals its biodiversity. In the Amazon, the latest estimate states that there are approximately 200 distinct groups present, most of which have never or poorly been investigated (Bennett 1992). The majority of the Neotropical ethnobotanical literature is very hard to acquire and is often found in obscure journals, unpublished theses and out-of-print books. In addition, due to intellectual property issues, many studies will not share the complete detailed lists of plants used (Milliken 1996, 1997, Rodrigues and Carlini 2006). The regression analysis method also has certain drawbacks. Both Bennet and Husby (2008) and Weckerle *et al.* (2011) have pointed out that the method is biased towards larger plant families and does not provide any measure of hypothesis testing, and have suggested more rigorous statistical methods as alternatives (i.e. binomial and Bayesian approaches). The issues mentioned above limit such analysis and certainly affect the outcome. Nevertheless, the current analysis includes the majority of rigorous ethnobotanical studies available, and provides quantitative evidence of plant selectivity for potential psychoactive properties. Folk illnesses have been widely discussed in the ethnopsychological and anthropological literature (Rubel *et al.* 1984, Carey 1993, Weller *et al.* 2002,), whereas the investigation of the ethnopharmacological activities of plants used for their treatment is limited to very few studies (Bourbonnais-Spear *et al.* 2007). The perception of mental illness varies strongly depending on the culture; thus, cultural context should not be overlooked, as is often the case, when documenting the traditional knowledge of medicinal plants of a certain group. The present analysis highlights plants families of special interest, such as the little known Neotropical family Marcgraviaceae, and the pantropical family Piperaceae, used for the treatment of *susto* throughout the Neotropics, and offers promising new leads for ethnopharmacological studies investigating the effects of plants on the GABAergic system.

Chapter 3

In vitro pharmacological effect of the Neotropical Piperaceae including medicinal plants used by the Yanesha (Peru) in GABAergic bioassays.

Picard G[1], Valadeau C[2], Albán–Castillo J[3], Rojas R[4], Starr J[1,5] and Arnason JT[1]

Contribution of co-authors:

The multiple expeditions done throughout Peru in order to collect Piperaceae specimen were accomplished with the help of Joaquina Albán–Castillo, Rosario Rojas, and their students. Plants traditionally utilized by the Yanesha were collected with the help of Céline Valadeau while working on her PhD thesis entitled "*De l'ethnobotanique à l'articulation du soin : Approche du système nosologique chez les Yanesha de Haute Amazonie péruvienne*". Plants were stored and extracts were prepared in Rosario Rojas laboratory.

[1] Centre for Advanced Research in Environmental Genomics (CAREG), Department of Biology, University of Ottawa, Ottawa, Ontario, Canada
[2] Centre National de la Recherche Scientifique (CNRS)/Centre d'Enseignement et de Recherche en Ethnologie Amérindienne (EREA) de l'UMR 7186. Paris Nanterre
[3] Museo de Historia Natural, Universidad Nacional Mayor de San Marcos (USM), Lima, Peru
[4] Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia
[5] Canadian Museum of Nature, Ottawa, Canada

3.1 Introduction

Each year, worldwide, mental and neurological disorders account for over 1.2 million deaths and contribute to 28% of the total burden of non-communicable diseases, surpassing cancer and cardiovascular diseases (WHO 2005). Epilepsy, one of the most serious neurological disorders, is more prevalent in the Neotropics than in other more developed countries (deBittencourt *et al.* 1996). Unfortunately, the inadequate distribution of psychiatric aid, combined with the scarcity of primary health care obliges the majority of indigenous people living in those regions to rely almost entirely on the medicinal flora, combined with superstitions and spirituality, in order to treat mental, behavioural and neurological disorders.

In Peru, in addition to the Quechua people of the Andes, over 300 000 people belonging to 59 different indigenous ethnic groups inhabit the Amazonian region (IBC 2006). The traditional medicinal use of plants by several of these Peruvian indigenous groups has been documented (Schultes and Raffauf 1990); however, with the exception of a few studies, information explicitly focusing on the use of plants for mental and neurological disorders is rather rare (Bussmann *et al.* 2010, De Feo 2003, Shepard 1998). The lack of plants recorded in Peruvian pharmacopoeias to treat disorders such as anxiety or epilepsy could potentially be explained by the fact that the traditional classification of these illnesses, often categorized as "culture-bound syndromes" or folk illnesses, does not conform to conventional medicine and have therefore been overlooked in classic ethnobotanical studies. Nonetheless, a recent ethnobotanical survey documenting plants used by an indigenous people of the Chazuta Valley, considered to be one of the largest of Peruvian Amazonian groups, has identified numerous plants used to treat folk illnesses associated with mental health disorders, such as *susto* ("fright sickness") and *mal aire* ("malevolent wind" or "bad air"), highlighting the importance of including culturally significant terminology in studies (Uzzell 1974, Sanz-Biset *et al.* 2009). Indeed, several outstanding descriptions of what are currently termed culture-specific syndromes of mental disorder (Kennedy, 1973) or "folk" illnesses have been done (Fabrega 1971). According to Rubel (1964), *susto* is a complex illness and its interpretation depends directly on several social factors. The Spanish term of *mal aire* is perhaps the most frequent Spanish-American explanation for illness and its exact nature has an elusive quality (Foster 1953).

Among the Yanesha, an indigenous group of Amazonian Peru, *yoreñets* is a generic term that the informants define as *susto* or in other cases as *mal aire* (Valadeau *et al.* 2010). There does not seem to be a clear difference between those two nominative illness terms. The symptoms appear because of a fright or an attack caused by a "walking shadow soul", *choyeshe'mats*, or "wind living being", *morranesha'*. Those two types of spiritual beings can be seen, *gacha'teñets* illness, or just simply heard, *macatsteñets* illness. Both these illnesses can lead to several symptomatic problems described as depression, weakness and apathy (Valadeau *et al.* 2010). In the present study, I attempt to determine a pharmacological mechanism of action for plants traditionally used for folk illnesses among the Yanesha, recently documented by Bourdy *et al.* (2008).

Our study group has previously reported that neurological and mental conditions such as epilepsy and *susto*, are well recognized by the Q'eqchi' Maya of Belize (Bourbonnais-Spear *et al.* 2005). The Maya are a similar culture to the Yanesha, and both groups believe that culture-bound syndromes are serious illnesses leading to severe conditions if ignored, and therefore use a variety of medicinal plants to treat them. Our study group has also reported that some of the species used by the Q'eqchi' Maya exhibit potent anxiolytic properties in animal behavioural models (Bourbonnais-Spear *et al.* 2007) and have been shown to act on the γ-aminobutyric acid (GABA) system (Awad *et al.* 2009), a key pathway involved in anxiety related disorders (Lydiard 2003). Out of all the plants available to the Q'eqchi' Maya, Piperaceae species seem to be preferentially selected for the treatment of such conditions and, interestingly, amongst the most active species tested (Awad *et al.* 2009). The Yanesha pharmacopeia also includes many Piperaceae species (16.9% of collected plants, Valadeau *et al.* 2010) and are seen as important medicine for folk illnesses like *mal aire* and *susto*. For that reason, this report focuses on the bioactivity of these plants.

Piperaceae, best known as the source of black pepper (*Piper nigrum* L.) is a highly diverse, pantropical plant family composed of two primary genera, *Piper* L. (about 2000 species) and *Peperomia* Ruiz & Pav. (about 1700 species), with the greatest diversity occurring in the Neotropics. The Amazonian Lowlands, as well as the eastern slopes of the Andean Cordillera in the upper Amazon, where the Yanesha live, are considered as one of the areas with greatest *Piper* species richness (Quijano-Abril *et al.* 2006) and can be found in numerous documented pharmacopoeias of the region, such as the Ashaninka, Ashéninka and shipibo'ones (Luziatelli 2010, Leanearts 2006, Tournon 2006). However, to our knowledge, only a small fraction of the Neotropical species have been studied in an ethnopharmacological context and very few species have been studied for their effect on the CNS (Felipe *et al.* 2007). Thus, we have undertaken the evaluation of an extensive collection of Piperaceae plants found throughout Peru as well as those selected by the Yanesha.

The evaluation of potential anxiolytic and antiepileptic properties of the crude extracts from the plants collected in the field was accomplished using two well-established high-throughput bioassays: the $GABA_A$-BZD Receptor Binding Assay and the GABA-T Inhibition Assay (Awad *et al.* 2009). This approach allowed us to assess the anxiolytic and antiepileptic activity of plants in relation to the two main targets in the GABAergic system (Lydiard 2003, Treiman 2001). Therefore the objective of the research was to determine whether plant extracts exert their activity through the mode of action of binding to the BZD site of the $GABA_A$ receptor or by inhibiting GABA-T.

3.2 Materials and Methods

3.2.1 Plant collection

A field trip for collection of a wide variety of Peruvian Piperaceae was undertaken from April to July of 2009. While participating in a variety of field expeditions throughout Peru, a total of 55 plants, 47 of which are from Piperaceae family, were collected. The first collection was carried out in Tsachopen (Fig. 3.1, Pasco Region, Oxapampa province). Yanesha plants were collected based on their recorded traditional uses previously reported by Valadeau *et al.* 2010 and compiled in the book; *Yato' Ramuesh: Plantas Medicinales Yaneshas* (Bourdy *et al.* 2008). Plants used to treat epilepsy, anxiety related disorders, and culture bound illnesses such as *susto* or *mal aire* (Table 3.1) were targeted due to their potential interaction with the GABAergic system (Awad *et al.* 2009, Bourbonnais-Spear *et al.* 2007). The remaining plants were collected during various field expeditions throughout Peru with and organized by collaborator Joaquina Alban-Castillo (Universidad Nacional Mayor de San Marcos) (Table 3.2). Plants were collected according to the issued permit by INRENA (Instituto Nacional de Recursos Naturales, Ministerio de Agricultura, Lima, Peru, 124-2011-AG-DGFFS-DGEFFS). Plant vouchers have been deposited at the UMS (Herbarium of the

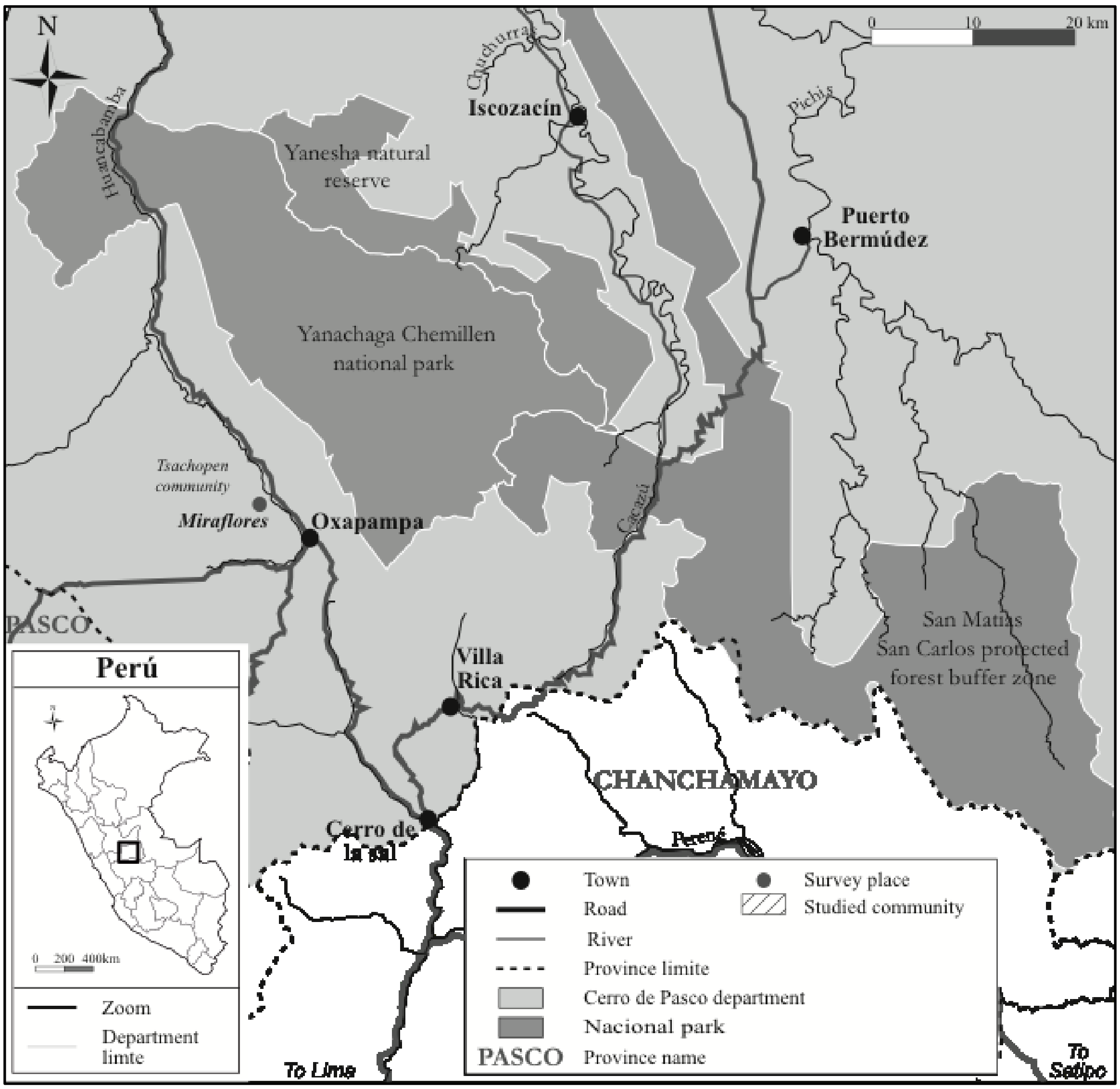


Figure 3.1. Area of study.

Table 3.1. Plants used traditionally by the Yanesha of Tsachopen and identified to have the potential of interacting with the GABAergic system.

Col.#	**Local name**	**Family**	**Species**	**Location**	**Latitude**	**Longitude**	**Alt. (m)**
GP001	Palo Santo (esp.)	Undetermined	Undetermined	Tsachopen, Miraflores	S10.53507	W075.4472	1830
GP002	Senollechap (Yanesha)	Caryophylac-eae	*Drymaria cordata* (L.) Willd. ex Schult. (?)	Tsachopen, Miraflores	S10.53507	W075.4472	1830
GP003	Corarnopan (Yan)	Piperaceae	*Piper subflavispicum* C.DC.	Tsachopen, Miraflores	S10.53507	W075.4472	1830
GP004	Corarnopan (Yan)	Piperaceae	*Piper acutifolium R & P.*	Tsachopen, Miraflores	S10.53531	W075.4464	1793
GP005	Corarnopan (Yan)	Piperaceae	*Piper aff. euriphyllum* Trel.	Tsachopen, Miraflores	S10.53978	W075.4447	1791
GP006	Corarnopan (Yan)	Piperaceae	*Piper cremii* Trel.	Tsachopen, Miraflores	S10.53978	W075.4447	1791
GP007	Sentsopan po'senempan (Yan.)	Piperaceae	*Piper denisii* Trel.	Tsachopen, Miraflores	S10.54645	W075.4472	2049
GP008	Orranapan (Yan), Garponia (esp.)	Piperaceae	*Piper quimirianum* Trel.	Tsachopen, Miraflores	S10.54645	W075.4472	2049
GP009	Popnor, or Antacopa (Yan.), Té de monte (esp.)	Chloranthac-eae	*Hedyosmum* sp. (?)	Tsachopen, Miraflores	S10.54645	W075.4472	2049
GP012	Mecha'tentsopar, (yan.), matico (esp.)	Piperaceae	*Piper sp.*	Tsachopen, Miraflores	S10.54633	W075.4475	2071
GP013	Pashenorren (Yan.)	Piperaceae	*Piper longifolium* R & P..	Tsachopen, Miraflores	S10.54633	W075.4475	2071
GP014	Muecho'tpar (Yan.)	Elaphoglossa-eae	*Elaphoglossum* sp. (?)	Tsachopen, Miraflores	S10.54633	W075.4475	2071
GP016	Puesen (Yan.)	Piperaceae	*Piper cf. adreptum* Trel.	Tsachopen, Miraflores	S10.54505	W075.4478	2157
GP018	Acenacapar (Yan.), Flor de picaflor torsido (esp.)	Undetermined	Undetermined	Tsachopen, Sipizu	S10.53301	W075.4466	1803
GP019	Tepeshpan (Yan.)	Verbenaceae	*Lantana camara* L. (?)	Tsachopen, Sipizu	S10.52714	W075.4462	1786
GP020	Puetse'llompar (yan.)	Undetermined	Undetermined	Tsachopen, Sipizu	S10.52826	W075.4529	1832
GP021	Ollocharetspar (yan.)	Piperaceae	*Peperomia pertomentella* (?)	Tsachopen, Sipizu	S10.53969	W075.4520	2047
GP023	Orranapan pashenorrer (Yan.)	Piperaceae	*Piper carpunya* R & P.	Tsachopen, Sipizu	S10.32.31	W075.26.88	1895
GP024	Corarnopan (Yan.)	Piperaceae	*Piper aduncum* L.	San Ramon	S11.1118	W075.4029	1518
GP045	Corarnopan (Yan.)	Piperaceae	*Piper peltatum* L.	Puerto Moldenado	S12.49611	W069.2121	169
GP062	Muentsopar (Yan.)	Fabaceae	*Mimosa pudica* L.	Reserva IIAP, Iquitos	S3.97194	W073.4211	124

Table 3.2. Plants from the Piperaceae family collected in Peru for the screening of their activity within the GABAergic system

Species	Col.#	Date collected	Location	Latitude	Longitude	Altitude (m)
Piper subflavispicum C.DC.	GP003	05/20/09	Tsachopen, Sector Miraflores	S10.53507	W075.4472	1830.9
Piper acutifolium R & P.	GP004	05/20/09	Tsachopen, Sector Miraflores	S10.53531	W075.44645	1793.6
Piper aff. euriphyllum Trel.	GP005	05/20/09	Tsachopen, Sector Miraflores	S10.53978	W075.44472	1791.9
Piper cremii Trel.	GP006	05/20/09	Tsachopen, Sector Miraflores	S10.53978	W075.44472	1791.9
Piper denisii Trel.	GP007	05/22/09	Tsachopen, Sector Miraflores	S10.54645	W075.44728	2049.8
Piper quimirianum Trel.	GP008	05/22/09	Tsachopen, Sector Miraflores	S10.54645	W075.44728	2049.8
Peperomia sp.	GP010	05/22/09	Tsachopen, Sector Miraflores	S10.54645	W075.44728	2049.8
Piper sp.	GP012	05/22/09	Tsachopen, Sector Miraflores	S10.54633	W075.4475	2071.4
Piper longifolium R & P.	GP013	05/22/09	Tsachopen, Sector Miraflores	S10.54633	W075.4475	2071.4
Peperomia sp.	GP015	05/22/09	Tsachopen, Sector Miraflores	S10.54505	W075.44788	2157.0
Piper cf. adreptum Trel.	GP016	05/22/09	Tsachopen, Sector Miraflores	S10.54505	W075.44788	2157.0
Peperomia sp.	GP017	05/22/09	Tsachopen, Sector Miraflores	S10.54505	W075.44788	2157.0
Peperomia pertomentella (?)	GP021	05/23/09	Tsachopen, Sector Sipizu	S10.53969	W075.45206	2047.1
Piper sp.	GP022	05/23/09	Tsachopen, Sector Sipizu	S10.32.314	W075.26.882	
Piper carpunya R & P.	GP023	05/23/09	Tsachopen, Sector Sipizu	S10.32.314	W075.26882	1895
Piper aduncum L.	GP024	05/24/09	San Ramon	S11.1118	W075.40294	1518.0
Piper ferruginispicum Trel.	GP026	05/24/09	San Ramon	S11.12.130	W075.23333	1794
Peperomia galioides Kunth	GP028	05/30/09	Pamparomas, Ancash	S9.07312	W077.9752	2763.5
Piper aff cupreatum Trel.	GP029	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46262	W069.13781	257.3
Piper dumosum Rudge	GP030	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46454	W069.1352	223.6
Piper pseudoarboreum Yun	GP031	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46454	W069.1352	223.6
Piper margaritanum Trel.	GP032	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46455	W069.13519	257.3
Piper allardii Trel.	GP033	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46455	W069.13519	257.3
Piper propinquum C.DC.	GP034	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46528	W069.13204	223.4
Piper celer Trel.	GP035	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46538	W069.13204	281.5
Piper sp. (nov?)	GP036	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46362	W069.11715	237.8
Piper cf. dilatatum L.C. Rich.	GP037	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46367	W069.11725	273.6
Piper bermudezense Trel.	GP038	06/04/09	Reserva UNAMAD, P.Moldenado	S12.46367	W069.11725	273.6
Piper sp.	GP040	06/05/09	Reserva UNAMAD, P.Moldenado	S12.64651	W069.33395	257.7
Piper pseudoarboreum Yun.	GP041	06/05/09	Reserva UNAMAD, P.Moldenado	S12.64651	W069.33395	257.7
Piper longifilamentosum Trel.	GP042	06/05/09	Reserva UNAMAD, P.Moldenado	S12.64181	W069.33611	223.6
Piper setulosum Trel.	GP043	06/05/09	Reserva UNAMAD, P.Moldenado	S12.39.449	W069.19810	225
Piper peltatum L.	GP045	06/05/09	Puerto Arthuro, P.Moldenado	S12.49611	W069.21216	169.1
Piper callosum R & P.	GP046	06/25/09	Tamshiyacu, Loreto	S4.01115	W073.14061	110.7
Piper sp.	GP047	06/25/09	Tamshiyacu, Loreto	S4.01115	W073.14061	110.7
Piper sp.	GP048	06/25/09	Tamshiyacu, Loreto	S4.01115	W073.14061	110.7
Piper sp.	GP049	06/25/09	Tamshiyacu, Loreto	S4.01115	W073.14061	110.7
Piper sp.	GP050	06/26/09	IIAP, KM2.5 Quinones, Iquitos	S3.7665	W073.27498	91.0
Piper sp.	GP051	06/26/09	Reserva UNAP, Iquitos, Loreto	S3.83155	W073.37358	147.4
Piper loretoanum Trel.	GP052	07/03/09	Reserva IIAP, Iquitos	S3.97071	W073.41995	125.6
Piper soledadense Trel.	GP053	07/03/09	Reserva IIAP, Iquitos	S3.97136	W073.42054	119.1

Table 3.2 (Continued)

Species	Col.#	Date collected	Location	Latitude	Longitude	Altitude (m)
Peperomia sp.	GP055	07/03/09	Reserva IIAP, Iquitos	S3.97161	W073.42038	158.2
Piper barbicuspe Trel.	GP056	07/03/09	Reserva IIAP, Iquitos	S3.97161	W073.42038	158.2
Peperomia sp.	GP057	07/03/09	Reserva IIAP, Iquitos	S3.97161	W073.42038	158.2
Piper leucofuscum Trel.	GP059	07/04/09	Reserva IIAP, Iquitos	S3.97166	W073.42271	147.2
Piper pavonii C.DC.	GP060	07/04/09	Reserva IIAP, Iquitos	S3.97213	W073.42313	96.3
Peperomia pellucida (L.) Kunth	GP061	07/04/09	Reserva IIAP, Iquitos	S3.97194	W073.42119	124.6

Universidad San Marcos) in Lima, Peru, and at the University of Antioquia, Colombia (Appendix 1). Most specimens have been identified to species level by Dr. Ricardo Callejas with the exception of some specimens from the *Peperomia* genus, some damaged or incomplete vouchers, and one particular specimen that could be a novel species. Most of the plants collected in Tsachopen are still awaiting identification by taxonomists at the Museo Nacional de Historia Natural in Lima where they have been deposited.

3.2.2 Plant Extraction and Sample Preparation

Upon collection, plants (leaves) were immediately stored in ethanol for the duration of the fieldwork. The plants were eventually deposited in Dr. Rosario Rojas laboratory at the Universidad Peruana Cayetano Heredia, where they were ground, extracted with approximately 10 x (*m/v*) 95% EtOH for a period of 24 hours at room temperature, then filtered. For each plant, the filtrate was combined with the solvent originally used to store leaves during fieldwork and then roto-evaporated to prepare the solid extract. The resulting material was freeze dried to remove remaining water and stored at 4°C until needed. Prior to experiments, the extract was reconstituted in the desired ratio of EtOH/dH_2O and filtered through a 0.2μm polytetrafluorethylene (PTFE) Chromspec filter.

3.2.3 $GABA_A$-BZD Receptor Binding Assay

The following radioligand binding assay is based on previously described protocols (Snodgrass 1978, Benke and Mohler 1999), which were later adapted by our research group (Awad *et al.* 2009) to be suited for 96 well Multiscreen FB filter plates (1.0/0.65 μm) (Millipor, Billerica, MA, USA).

First, rat brains from male adult Sprague-Dawley obtained by Dr. Zul Merali (Department of Psychology, University of Ottawa) were homogenized in 50mM Tris buffer (50 mM Tris HCl, 50 mM Tris base, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) using a glass homogenizer. The membranes were then isolated by centrifuging the homogenate for 15 min (32000 x g, 4°C), rehomogenizing the pellet in the buffer, and finally collecting the pellet obtained after a second centrifuge round. A standard protein assay was then conducted to determine protein concentration in the pellet (Bradford 1976).

Second, the plates were prepared by adding triplicates of the plant extracts prepared with 95% EtOH to attain a concentration of 10 μg/ml in the well) with the protein (homogenized pellet) and the radioligand ^{3}H-flunitrazepam (PerkinElmer, MA, USA). The total binding is measured by adding the protein in the wells with 20 nM ^{3}H-flunitrazepam. The non-specific binding is measured by adding the protein with 20 nM ^{3}H-flunitrazepam and flumazenil (Sigma-Aldrich, MO, USA), a ligand with greater affinity. The plates were then incubated on ice for 75 min before washing the wells 3 times with the buffer and removing the content under vacuum.

Finally, plates were incubated for 24 hours with 25 μL of scintillation fluid (Supermix cocktail, Perkin Elmer, Waltham, MA, USA) in each well. The affinity of the plant extract to bind to the receptor was measured with a microplate scintillation counter (Wallac MicroBeta Trilux, Perkin Elmer, Waltham, MA, USA), and the rate at which ^{3}H-flunitrazepam was displaced was calculated using the formula:

$$\%displacement = 100 - \left(\frac{total\ binding - non\ specific\ binding}{total\ binding_{\ control} - non\ specific\ binding_{\ contriol}} \times 100\right)$$

3.2.4 GABA-T Inhibition Assay

The ability of plant extracts to inhibit GABA-T was evaluated using a high-throughput spectrophotometric *in vitro* enzyme assay (Jung *et al.* 1977) later adapted to be suited for 96-well plates (Awad *et al.* 2007). The plates were prepared by adding triplicates of the plant extracts (prepared with 80% EtOH to attain a concentration of 0.5 mg/ml in the well) with the protein (homogenized pellet) and the buffer (100 mM/L potassium pyrophosphate, 5 mM/L α-ketoglutarate, 4 mM/L nicotinamide adenine dinucleotide, 3.5 mM/L 2-mercaptoethanol, 10 μM/L pyridoxal-5'-phosphate, pH 8.6). Plates were subsequently incubated (15 min, 37°C) before adding 115 mM/L GABA. The rate of the enzymatic reaction (V_{max}) was determined by measuring NADH production at an absorbance of 340 nm at 37^{o}C for 10 minutes within the linear range (Spectramax M5 with SoftMax Pro Software version 4.8, Molecular Devices Corporation, CA, USA). Finally, the percent of GABA-T activity was calculated using the following equation:

$$\%activity = \left(\frac{(T - TB) - (C - CB)}{solvent\ control}\right) \times 100$$

where T is test (enzyme, GABA and extract), TB is test-blank (buffer, GABA and extract), C is control (enzyme, water and extract) and CB is control-blank (buffer, water and extract), and then compared relative to the control value. Gamma-vinyl-GABA (GVG; vigabatrin, Sigma-Aldrich, MO, USA), a well-known GABA-T inhibitor was used as the positive control and completely inhibited the enzyme at 1mM and had an IC_{50} of 84 μM. Some plants could not be tested and were removed from the experiment due to the formation of precipitate in the well interfering with the spectrophotometric part of the assay (Plants GP006, GP026, GP034, GP039, GP040, GP042, GP043)

3.2.5 Determination of EC_{50} and IC_{50}

The crude extract of the most active plants were tested at concentrations (in well) of 1, 3, 10, 30, 100 and 300 μg/ml for the $GABA_A$-BZD Receptor Binding Assay in order to determine the half maximal effective concentration (EC_{50}) and of 0, 0.125, 0.25, 0.5, 0.75 and 1 mg/ml in order to determine the half maximal inhibitory concentration (IC_{50}). Linear regressions were then completed with SigmaPlot (Systat Software Inc., CA, USA) in order to obtain these values.

3.3 Results and Discussion

The bioassay of the crude ethanolic extracts of Peruvian Piperaceae plants and selected plants traditionally used by the Yanesha demonstrated that they possess moderate to high activity in the $GABA_A$-BZD receptor-binding assay (Fig. 3.2A). This bioassay was based on displacement of competing radioligand ^{3}H-flunitrazepam, and is the recognized *in vivo* target for many anxiolytic substances. In addition, the bioassay for the inhibition of the GABA-T enzyme showed low to moderate activity for most plant extracts, with a few plants exhibiting promising activity (Fig. 3.2B). This bioassay is an important pharmacological target for some antiepileptic drugs.

All but four of the 47 Piperaceae plants tested, and 18 out of the 21 plants selected by the Yanesha, were able to inhibit the binding of the radioligand to the $GABA_A$-BZD receptor by more than 50% at the tested concentration. The top four plants and their respective EC_{50} evaluated in a concentration dependent assay (Fig. 3.3) are as follows: *Piper longifilamentosum* Trel. [EC_{50} (± 95% CI)] = 18.3 µg/ml (13.2, 24.6)], *Piper loretoanum* Trel. [EC_{50} (± 95% CI)] = 16.6 µg/ml (10.8, 24.1)], *Piper cremii* Trel. [EC_{50} (± 95 % CI)] = 17.5 µg/ml (10.6, 26.7)], and *Piper barbicuspe* Trel. [EC_{50} (± 95% CI)] = 31.1 µg/ml (23.1, 41.8)]. The most active plants within the Piperaceae family were of the genus *Piper*. However, the activity does not seem to be genus-specific since both active and inactive plants were also found within *Peperomia*. No significant difference was observed when comparing the activity of all plants within genera ($F_{1,45}$ = 1.5035, P = 0.2267). Plants selected by the Yanesha showed comparable activity to the other Piperaceae plants ($F_{1,53}$ = 3.6396, P = 0.0619) with *Piper cremii* Trel. being the third most active plant.

In the second bioassay, only four plants were able to inhibit GABA-T by more than 50% at the concentration tested, with two of them coming from the Yanesha pharmacopoeia. The top four plants and their respective IC_{50} were: *Piper pavonii* C.DC. [IC_{50} (± 95% CI)] = 0.28 mg/ml (0.26, 0.3)], *Piper barbicuspe* Trel. [IC_{50} (± 95% CI)] = 0.28 mg/ml (0.21, 0.35)], *Drymaria cordata* (L.) Willd. ex Schult [IC_{50} (± 95% CI)] =

0.46 mg/ml (0.41, 0.52)], and *Hedyosmum sp.* [IC_{50} (± 95% CI)] = 0.37 mg/ml (0.31, 0.43)] (Fig. 3.4). Similar to the previous assay, the activity does not seem to be genus-specific ($F_{1,40}$ = 0.7712, P = 0.3852). No significant difference was observed when comparing the activity of Yanesha plants to the other Piperaceae species ($F_{1,48}$ = 0.3178, P = 0.5756). Nevertheless, some of the most active species in this assay were plants specifically selected by the Yanesha for the treatment of anxiety and epilepsy related disorders.

In general, the results show comparable activity to plants used by the Q'eqchi Maya of Belize, where *Piper amalago* L., the most active Piperaceae plant had an EC_{50} of 18.6 μg/ml (14.2, 24.1) in the $GABA_A$-BZD receptor binding assay and *Piper tuerckheimii* C.DC. ex Donn. Sm. had an IC_{50} of 0.51 mg/ml (0.40, 0.70) in the GABA-T inhibition assay (Awad *et al.* 2009). These results can also be compared to a study of Danish folk medicine used for epilepsy where *Primula eliator* and *Tanacetum parthenium*, two of their most active plants, respectively had an EC_{50} of 18.45 μg/ml and 40.05 μg/ml in the $GABA_A$-BZD receptor binding assay (Jäger *et al.* 2006).

A thorough review of the published literature suggests that the top ranking *Piper* species mentioned above have never been studied previously. In fact, most Piperaceae collected in this study have never before been investigated in an ethnopharmacological or phytochemical context. There are a few exceptions of course, such as the widespread species *Piper aduncum* L., *Piper acutifolium* R. & P., *Piper peltatum* L., the Andean species *Peperomia galioides* HBK, and the introduced species *Peperomia pellucida* (L.) HBK. Nevertheless, none of the species have been reported to exhibit activity within the GABAergic system. I report here for the first time a pharmacological basis for the ethnopsychiatric use of plants by the Yanesha of Tsachopen, and underline the promising biological activity of the Piperaceae on two specific sites of the GABAergic system.

Plants of the Piperaceae family are well known for the presence of piperamides (Parmar *et al.* 1997), a unique group of nitrogenous secondary metabolites thought to play an important role in the traditional use of these plants for the treatment of various CNS disorders (Pedersen *et al.* 2009, Wattanathorn *et al.* 2008, D'Hooge *et al.* 1996). The recent finding that the amide piperine acts as a positive $GABA_A$-BZD modulator (Zaugg *et al.* 2010), and the observation of *in vivo* anxiolytic activity from such compounds (Yao *et al.* 2009, Felipe *et al.* 2007), highlights the importance of Piperaceae as CNS-depressant plants acting within the GABAergic system. The presence of piperamides in the selection of Neotropical Piperaceae collected could explain their activity. Nevertheless, other classes of secondary metabolites such as naturally occurring flavones and related compounds (i.e. flavonoids) isolated from traditionally used anxiolytic, sedative and anticonvulsant plants have been shown to bind with great affinity to the $GABA_A$-BZD receptor (Medina *et al.* 1997) and pharmacophore models have since been used for the design of potent synthetic molecules (Kahnberg *et al.* 2002), such as triazoloquinazolinediones, a novel class of BZDR ligands (Nilsson *et al.* 2011). Flavonoids from Pipereaceae have been isolated and tested for their antifungal (Lago *et al.* 2004), antiplasmodial (Portet *et al.* 2007), antioxidant (Velozo *et al.* 2009), and antinociceptive properties (Da Silva *et al.* 2010), however, to our knowledge none have been tested for their antiepileptic and anxiolytic properties.

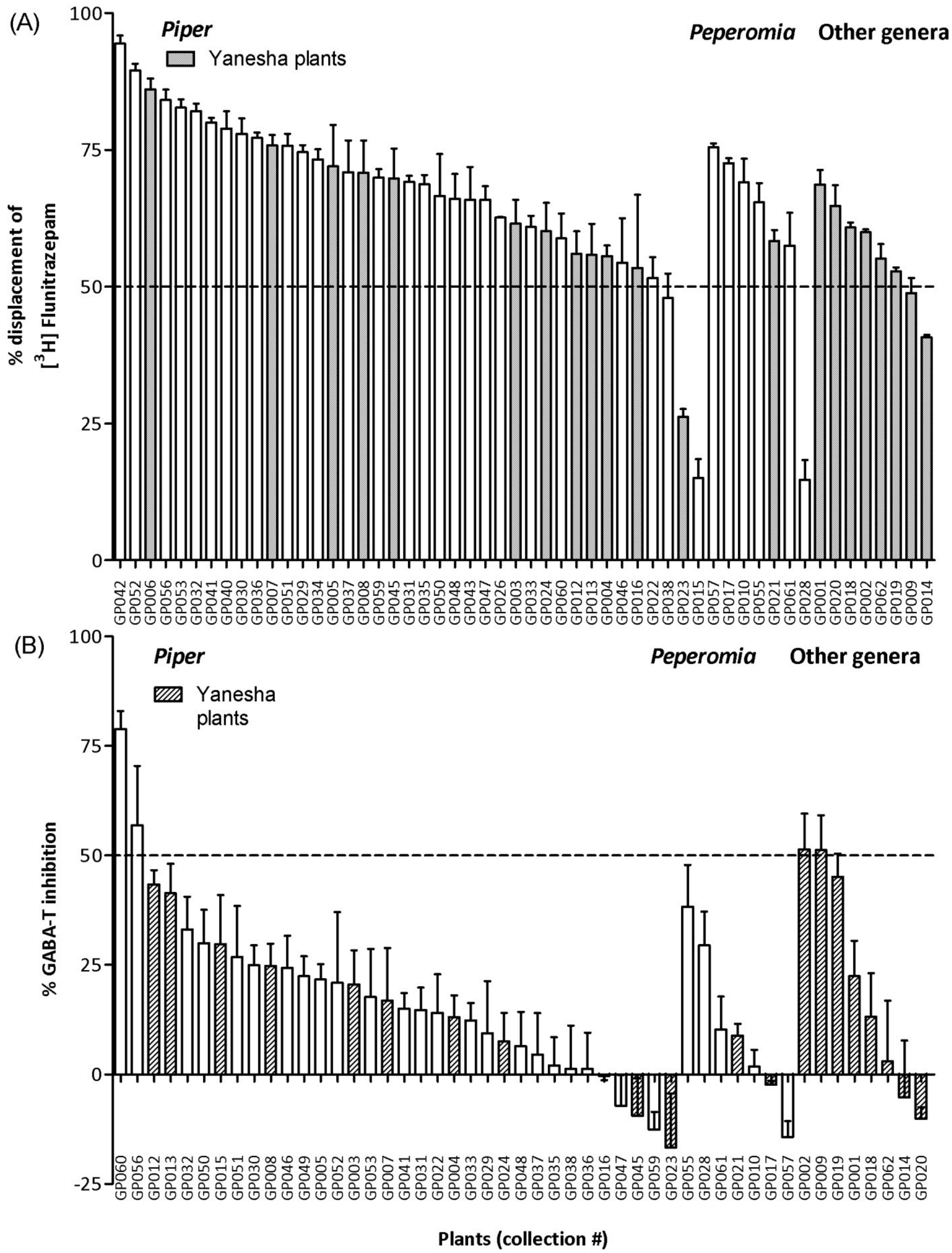


Figure 3.2. The ability of selected species of Piperaceae and plants used by the Yanesha to (A) displace the radioligand [^{3}H]-Flunitrezepam from the GABA$_A$-BZD receptor (±SEM) at 0.1 mg/ml and (B) to inhibit GABA-T (±SEM) at 0.5 mg/ml. For details on collections refer to Table 3.1 and 3.2.

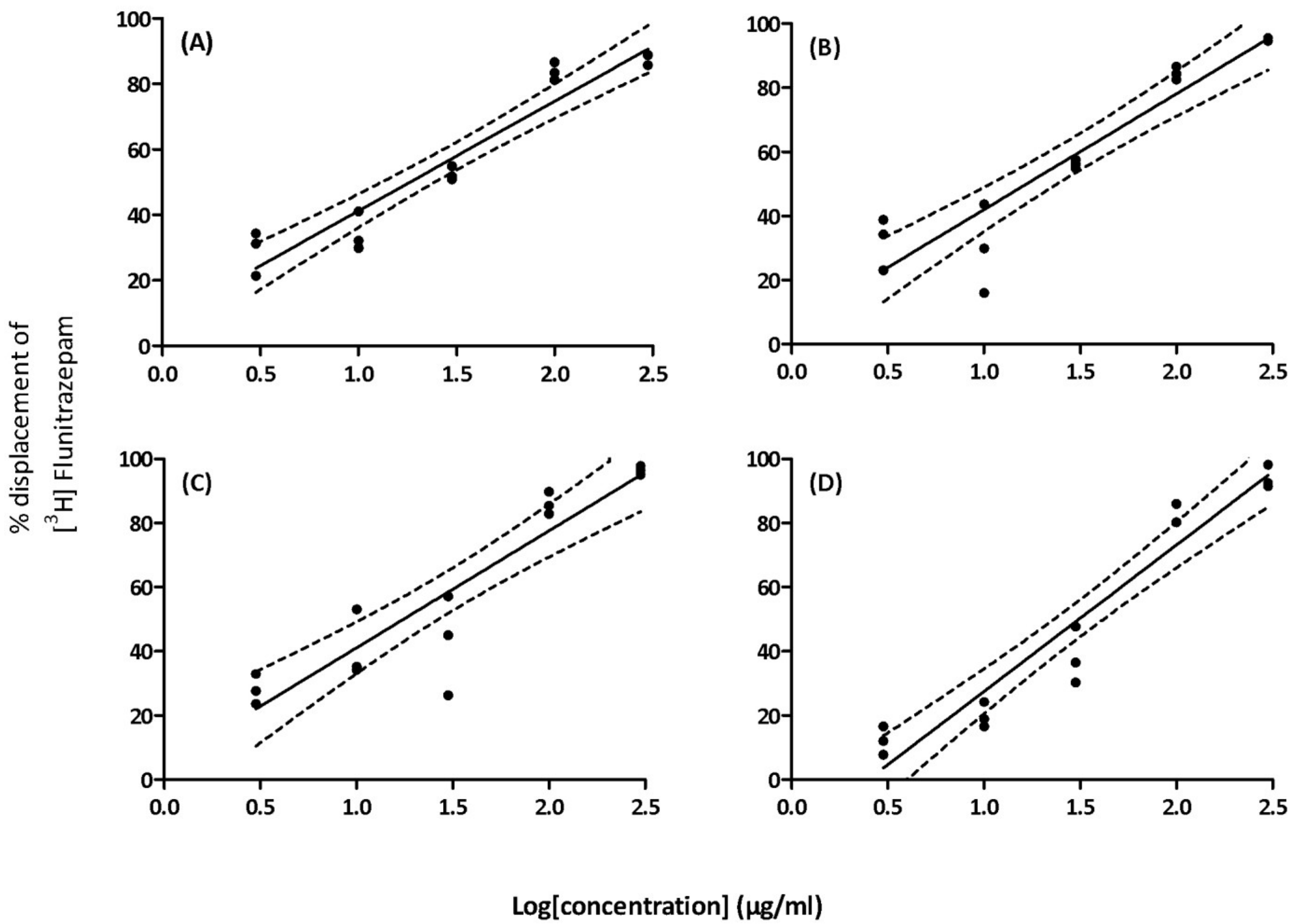


Figure 3.3. Concentration (Log) dependent linear regression analysis (95% CI) of the four most active plant extracts; (A) *Piper longifilamentosum* Trel. (GP042), (B) *Piper loretoanum* Trel. (GP052), (C) *Piper cremii* Trel. (GP006), and (D) *Piper barbicuspe* Trel. (GP0056) in the displacement of [^{3}H] flunitrazepam. Fifty percent effective concentration [EC_{50} (± 95% CI)] = 18.3 μg/ml (13.2, 24.6), 16.6 μg/ml (10.8, 24.1), 17.5 μg/ml (10.6, 26.7) and 31.1 μg/ml (23.1, 41.8) for A, B, C and D respectively (n=3).

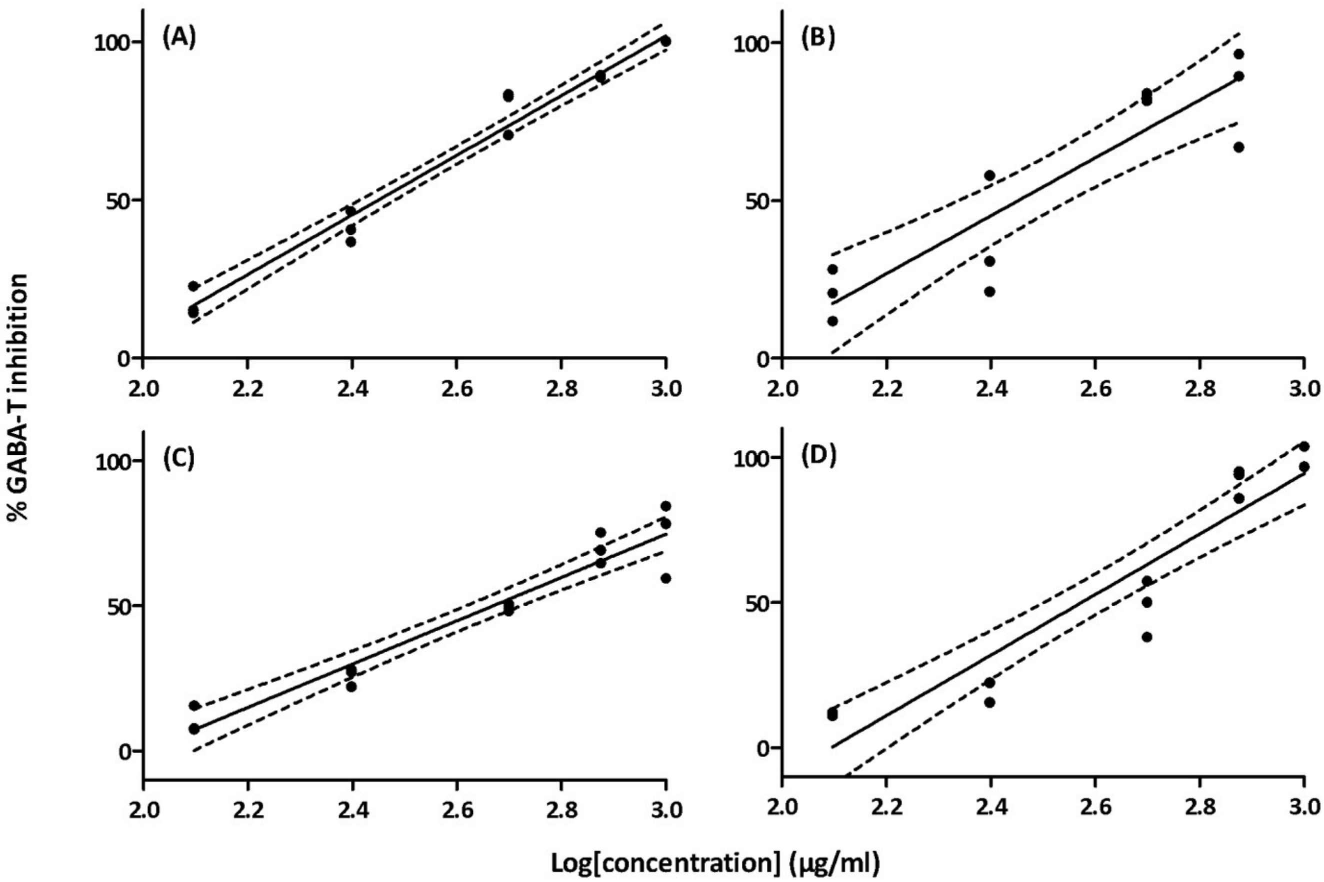


Figure 3.4. Concentration (Log) dependent linear regression analysis (95% CI) of the four most active plant extracts; (A) *Piper pavonii* C.DC. (GP060), (B) *Piper barbicuspe* Trel. (GP056), (C) *Drymaria cordata* (L.) Willd. ex Schult (GP002), and (D) *Hedyosmum sp.* (GP009) and their *in vitro* inhibition of GABA-T. Fifty percent inhibition concentration [IC_{50} (± 95% CI)] = 0.28 mg/ml (0.26, 0.3), 0.28 mg/ml (0.21, 0.35), 0.46 mg/ml (0.41, 0.52) and 0.37 mg/ml (0.31, 0.43) for A, B, C and D respectively (n=3).

The extracts of *Drymaria cordata* (L.) Willd. ex Schult and *Hedyosmum sp*., the most active non-Piperaceae GABA-T inhibitors selected by the Yanesha, offer promising leads for the discovery of anti-epileptic natural compounds. Interestingly, *Drymaria cordata* was the only plant to be specifically chosen for the treatment of epilepsy, whereas most of the other plants were used for anxiety related disorders and culture bound syndromes such as *susto*. A previous study has shown *Drymaria cordata* to exhibit anxiolytic activity in an *in vivo* experiment (Barua *et al.* 2009). On the other hand, the highly aromatic neotropical genus *Hedyosmum* has also been investigated for its effects on the CNS. These effects, analgesic and sedative, have been attributed to the presence of flavonoid glycosides (Cárdenas *et al.* 1993) and sesquiterpene lactones (Tolardo *et al.* 2010) respectively. In addition to the ethnobotanical data available for these two plants and the observed *in vitro* activity, we present here data that show that these plants act on the CNS via the GABAergic system by interacting with GABA-T. It is important to mention that Yanesha plants (i.e. *Elaphoglossum sp.*) that showed weak activity could produce their anxiolytic or antiepileptic effect by possibly having other mechanisms of actions and other targets, such as the glutamate/NMDA receptors, for example (Milton and Jung 2003). Nevertheless, the data presented here provides us with interesting leads for the potential isolation of potent neuroactive plant-derived natural products. The above-mentioned plants of the genus *Piper*, *Drymaria*, and *Hedyosmum* should be collected in bulk and subjected to bioassay-guided fractionation in order to identify the active constituents. Finally, the activity should be confirmed in *in vivo* models.

The purpose of our study was to screen a variety of Neotropical Piperaceae species, and plants used for ethnopsychiatric disorders by the Yanesha people, for their activity on the GABAergic system, thus identifying potential active antiepileptic and anxiolytic plants. Our results show that Piperaceae plants can exhibit great activity in both assays and that most plants used by the Yanesha showed moderate to high activity, especially in form of GABA-T inhibition. The folk illness, or culture-bound syndromes of *susto* (fright) and *mal aire* (malevolent wind) have both been documented as an important and often severe source of ailment throughout the Neotropics (De Feo 2003, Bourbonnais-Spear *et al.* 2005, Sanz-Biset *et al.* 2009). For the Yanesha, being struck by such illness could lead to sadness, depression, anorexia, apathy, insomnia, convulsions, etc (Valladeau *et al.* 2010), all of which could be attributed to anxiety related disorders and even attacks of epilepsy. The presence of *susto* and *mal aire* in Latin America has been extensively documented in an anthropological context, yet plants used for these folk illnesses have rarely been studied for their biological activity in this context. Here, in addition to the work with the Q'Eqchi' Maya of Belize (Awad *et al.* 2009, Bourbonnais-Spear *et al.* 2007), we provide evidence that there is biological basis behind the use of plants in the treatment of *susto* and *mal aire*, and offer the interaction with the GABA-T enzyme and/or the $GABA_A$-BZD receptor as potential mechanisms of action.

Chapter 4

Bioassay-guided isolation of the anxiolytic and antiepileptic principle of *Piper tuerckheimii*, a plant used traditionally by the Q'eqchi' Maya healers of Belize.

Picard G[1], Muhammad A[2], Guerrero JA[1], Walshe-Roussel B[1], Cal V[3], Starr J[1,4] and Arnason JT[1]

Contribution of co-authors:

Brendan Washe-Roussel and Victor Cal of the Belize Indigenous Training Institute collected the bulk material of *P. tuerckheimii*. The bioassay-guided fractionation and HPLC analyses were accomplished with the help of José Antonio Guerrero. Asim Muhammad accomplished the identification of isolated compounds via NMR.

[1] Centre for Advanced Research in Environmental Genomics (CAREG), Department of Biology, University of Ottawa, Ottawa, Ontario, Canada
[2] Ottawa-Carleton Institute of Chemistry, University of Ottawa, Ottawa, Ontario, Canada
[3] Belize Indigenous Training Institute (BITI), Punta Gorda, Toledo District, Belize
[4] Canadian Museum of Nature, Ottawa, Canada

4.1. Introduction

Piper tuerckheimii C. DC. ex Donn. Sm. (Piperaceae), also known as “Cux sawi” by the Q’eqchi’ Maya of Belize, is a small aromatic shrub with a scarce distribution throughout northern Central America. Its range includes Honduras (Bornstein and Coe 2007), Guatemala (Standley and Steyermark 1952), Belize (Balick *et al.* 2000), and the province of Chiapas, Mexico (Breedlove, 1986). The plant is traditionally used as medicine by the Q’eqchi Maya of Guatemala (Michel *et al.* 2007) and of Belize (Amiguet *et al.* 2005). In Livingston Guatemala, the leaves of *P. tuerckheimii* are used to reduce inflammation related to women’s health complaints (Michel *et al.* 2007). In Belize, Q’eqchi’ healers prepare a warm decoction of leaves to be drank for treatment of fever and for various digestive complaints, such as diarrhea and vomiting (Amiguet *et al.* 2005). *P. tuerckheimii* has recently been identified as a plant used with a high level of concensus among Q’eqchi’ healers for the treatment of various ethnopsychiatric neural and mental conditions (Bourbonnais-Spear *et al.* 2005), disease categories often underrepresented in general ethnobotanical studies.

Recent ethnopharmacological studies by our research group have shown that neurological and mental conditions such as epilepsy and the culture-bound syndrome *susto*, (a disorder believed to be strongly linked to anxiety and depression due to its symptoms (Rubel 1964, Weller *et al.* 2008)), are well recognized by the Q’eqchi’ Maya of Belize (Bourbonnais-Spear *et al.* 2005). *Piper tuerckheimii,* along with other species, has been identified as a highly effective plant by healers for the treatment of *susto*, and has been shown to exhibit potent anxiolytic properties in animal behavioural models suggesting that there may be a link between *susto* and anxiety (Bourbonnais-Spear *et al.* 2007).

The common denominator between epilepsy and anxiety-related disorders seems to be linked to the imbalance in brain levels of γ-aminobutyric acid (GABA), the chief inhibitory neurotransmitter in the mammalian central nervous system (Lydiard 2003, Treiman 2001). A recent ethnopharmacological investigation of Q'eqchi' Maya plants used for the treatment of folk ilnesses (i.e. *susto*) and neuropsychological disorders has demonstrated that plants exhibit their antiepileptic and anxiolytic activity by interacting with GABAergic system, either by inhibiting GABA-transaminase (GABA-T) or by binding to the $GABA_A$-benzodiazepine ($GABA_A$-BZD) receptor, two key pharmacological targets for anxiety and epilepsy drugs (Awad *et al.* 2009). The study demonstrated that *P. tuerckheimii* is not only preferred, but has also been found to be amongst the most active species tested exhibiting potent activity at both pharmacological sites (Awad *et al.* 2009), leading to the present study. To our knowledge, the phytochemical profile of *Piper tuerckheimii* has not yet been studied. Following up on previous recommendations (Awad *et al.* 2009), we describe here the phytochemical investigation of *Piper tuerckheimii* through bioassay-guided fractionation and the isolation of constituents responsible for its anxiolytic and antiepileptic activity using two well-established high-throughput bioassays; the $GABA_A$-BZD Receptor Binding Assay and the GABA-T Inhibition Assay.

4.2 Materials and Methods

4.2.1 Plant Material, Extraction and Sample Preparation

This project was accomplished in collaboration with the Belize Indigenous Training Institute (BITI) and was initially reviewed by the Science and Health Science Research Ethics Board of the University of Ottawa (Approval #H03-07-01). A scientific research/collection permit was also issued by the Ministry of Natural Resources and the Environment of Belize (Ref# CD/60/3/08[33]) prior to collection of plant material. Fresh leaves of *Piper tuerckheimii* (Fig. 4.1) were collected by Brendan Walshe-Roussel and Federico Caal in the Maya Mountains just outside of Jalacte, Toledo District, Belize. Herbarium samples were authenticated by Marco Otarola Rojas and deposited at the Universidad Nacional de Costa Rica (Voucher #: Spear 19710)

Upon collection, plants (leaves) were immediately stored in ethanol for the duration of the fieldwork. The plants were then deposited in the laboratory at the University of Ottawa where they were ground, extracted (twice), and then filtered. The first extraction of *Piper tuerckheimii* (0.45 Kg of dried leaves) was achieved by placing the material in approximately 8L of 80% EtOH for a period of 48 hours at room temperature, and the second with similar methods but in 4L of 80% EtOH. The two extracts were combined with the filtered solvent originally used to store leaves during fieldwork and then roto-evaporated to prepare the solid extract. The resulting material was freeze-dried to remove remaining water to yield approximately 80g of dark green residue (crude extract). Prior to experiments, the extract/fractions were reconstituted in the desired ratio of solvents and filtered through a 0.2 μm polytetrafluorethylene (PTFE) Chromspec filter (Chromatographic Specialties Inc., Ontario, Canada).

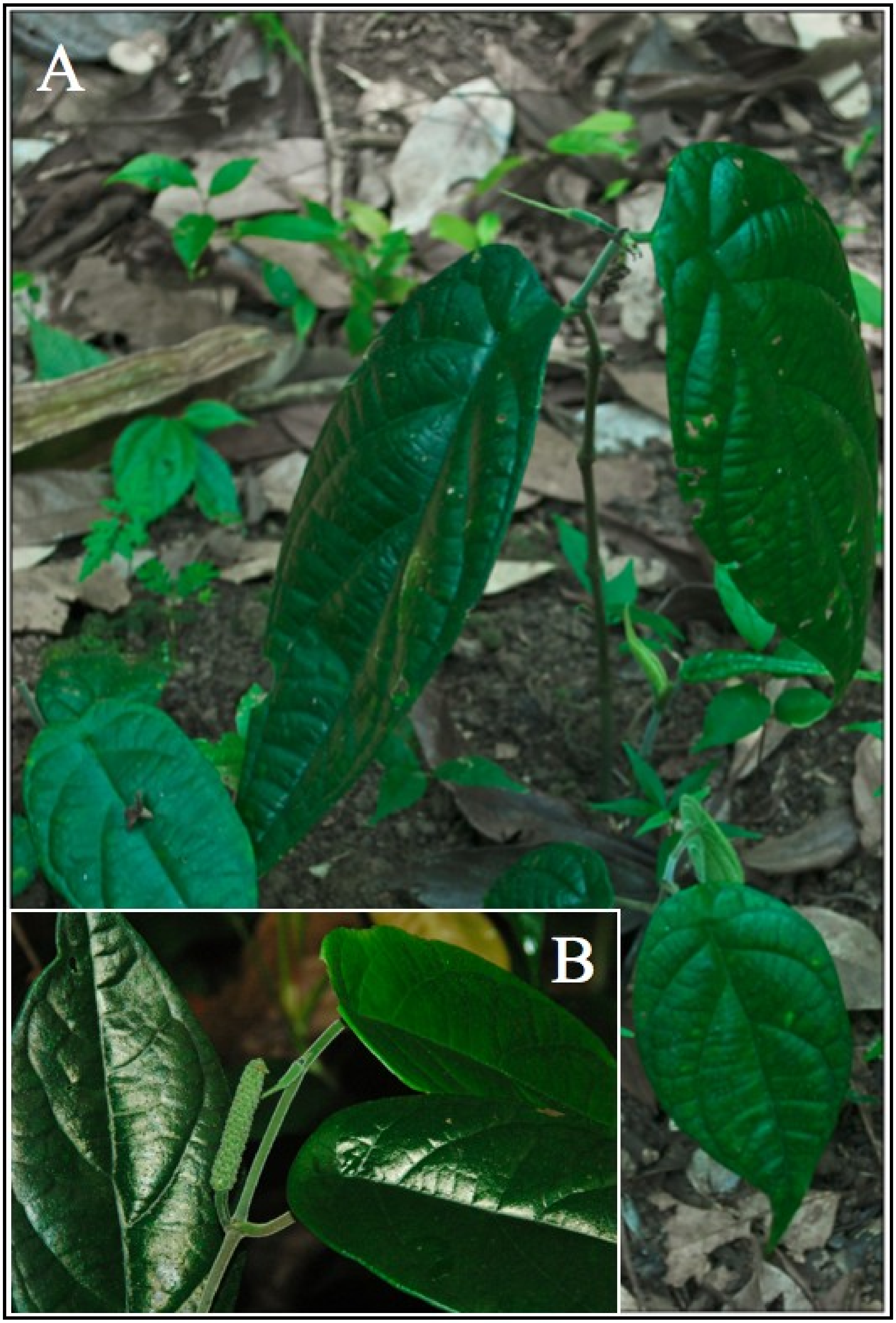


Figure 4.1. Photograph of (A) Cux sawi, *Piper tuerckheimii* C. DC. Ex Donn, Sm. (Piperaceae), taken by Jonathan Ferrier, and (B) its flowering body, taken by Brendan Walshe-Roussel.

4.2.2 Fractionation and Isolation

The primary fractionation of the crude ethanolic extract of *Piper tuerckheimii* was achieved using column chromatography on silica gel. Eighty grams of the extract was chromatographed in a 2 L glass column packed with silica gel 60 (2 Kg, Merck) and eluted with a solvent gradient of different polarities and collected in aliquots of 250 mL. The gradient of the mobile phase consisted of hexanes→ethyl acetate (EtOAc) (1:0→0:1), EtOAc→methanol (MeOH) (1:0→0:1), and MeOH→H_2O (90:10) in 10% increments. A total of 152 aliquots were collected, which after comparing TLC profiles, were pooled based on their similarity to give 10 fractions (PT-F1 – PTF-10).

Fraction PT-F6 (2.2 g), eluted with EtOAc-MeOH (95:5), was the most active in both assays and was therefore selected for further purification. The fraction was separated by preparative scale HPLC-DAD (1200 series, Agilent Technologies Inc., Palo Alto, CA) on a reverse-phase Gemini Axia 250mm x 21.2 mm column, particle size 10 μm (Phenomenex Inc., Torrance, CA). HPLC fractionation was performed using a linear gradient of H_2O→acetonitrile (95:5 to 50:50) in 20 minutes at a flow rate of 31.5 mL/min. Aliquots of 0.4 mL (100 mg/mL) were injected onto the columns 36 times and collections were made based on the detection of peaks at monitory wavelengths if 210, 280 and 330 nm. Collected fractions were pooled to give a total of 23 major peaks (PT-F6-P1 – PT-F6-P23).

4.2.3 Compound Identification

Active compounds isolated from PT-F6 were identified by nuclear magnetic resonance (NMR) spectroscopy. Compounds were dissolved in deuterated MeOH

(CD_3OD) prior to analysis. ^1H and ^{13}C spectra were recorded on a Bruker Avance 400 spectrometer. The identities of compounds were confirmed by comparing the obtained spectra to those of the literature by using SciFinder (CAS, Columbus, OH). See *results and discussion* section for compound structures and associated references.

4.2.4 GABA$_A$-BZD Receptor Binding Assay

The following radioligand binding assay is based on previously described protocols (Snodgrass 1978, Benke and Mohler 1999), which were later adapted by our research group (Awad *et al.* 2009) to be suited for 96 well Multiscreen FB filter plates (1.0/0.65 μm) (Millipor, Billerica, MA, USA). First, rat brains from male adult Sprague-Dawley obtained by Dr. Zul Merali (Department of Psychology, University of Ottawa) were homogenized in 50mM Tris buffer (50 mM Tris HCl, 50 mM Tris base, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) using a glass homogenizer. The membranes were then isolated by centrifuging the homogenate for 15 min (32000 x g, 4°C), rehomogenizing the pellet in the buffer, and finally collecting the pellet obtained after a second centrifuge round. A standard protein assay was then conducted to determine protein concentration of the homogenized pellet (Bradford 1976). Second, the plates were prepared by adding triplicates of the plant extracts (prepared with 95% EtOH to attain a concentration of 10 μg/mL in the well) with the protein (homogenized pellet) and the radioligand ^3H-flunitrazepam (PerkinElmer, MA, USA). The total binding is measured by adding the protein in the wells with 20 nM ^3H-flunitrazepam and the non-specific binding by adding the protein with 20 nM ^3H-flunitrazepam and flumazenil (Sigma-Aldrich, MO, USA), a ligand with greater affinity. The plates were then incubated on ice for 75 min before washing the wells 3 times with the buffer and removing the content under vacuum. Finally, plates were incubated for 24 hours with 25 μL of scintillation fluid (Supermix cocktail, Perkin Elmer, Waltham, MA, USA) in each well. The affinity of the plant extract to bind to the receptor is measured with a microplate scintillation counter (Wallac MicroBeta Trilux, Perkin Elmer, Waltham, MA, USA), and the rate at which ^{3}H-flunitrazepam was displaced was calculated using the formula:

$$\%displacement = 100 - \left(\frac{total\ binding - non\ specific\ binding}{total\ binding\ _{control} - non\ specific\ binding\ _{contriol}} \times 100\right)$$

4.2.5 GABA-T Inhibition Assay

The ability of plant extracts to inhibit GABA-T will be evaluated using a high-throughput spectrophotometric *in vitro* enzyme assay (Jung *et al.* 1977) later adapted to be suited for 96-well plates (Awad *et al.* 2007). The plates were prepared by adding triplicates of the plant extracts (prepared with 80% EtOH to attain a concentration of 0.5 mg/mL in the well) with the protein (homogenized pellet) and the buffer (100 mM/L potassium pyrophosphate, 5 mM/L α-ketoglutarate, 4 mM/L nicotinamide adenine dinucleotide, 3.5 mM/L 2-mercaptoethanol, 10 μM/L pyridoxal-5'-phosphate, pH 8.6). Plates are subsequently preincubated (15 min, 37°C) before adding 115 mM/L GABA. The rate of the enzymatic reaction (V_{max}) is determined by measuring NADH production at an absorbance of 340 nm at 37^{o}C for 10 minutes within the linear range (Spectramax M5 with SoftMax Pro Software version 4.8, Molecular Devices Corporation, CA, USA). Finally, the percent of GABA-T activity is calculated using the following equation:

$$\%activity = \left(\frac{(T - TB) - (C - CB)}{solvent\ control}\right) \times 100$$

where T is test (enzyme, GABA and extract), TB is test-blank (buffer, GABA and extract), C is control (enzyme, water and extract) and CB is control-blank (buffer, water and extract), and then compared relative to the control value. Gamma-vinyl-GABA (GVG; vigabatrin, Sigma-Aldrich, MO, USA), a well-known GABA-T inhibitor was used as the positive control and completely inhibited the enzyme at 1mM.

4.2.6 Determination of EC_{50} and IC_{50}

The crude extract of *Piper tuerckheimii* was tested at concentrations (in well) of 1, 3, 10, 30, 100 and 300 μg/mL for the $GABA_A$-BZD receptor binding assay in order to determine the half maximal effective concentration (EC_{50}) and of 0, 0.125, 0.25, 0.5, 0.75 and 1 mg/mL in order to determine the half maximal GABA-T inhibitory concentration (IC_{50}). Active peaks isolated from PT-F6 were tested at concentrations (in well) of 1, 3, 10, 30, and 100 μg/mL in both assays in order to determine their EC_{50} and IC_{50}. Linear regressions were then completed with SigmaPlot (Systat Software Inc., CA, USA) in order to obtain these values.

4.3 Results and Discussion

Activity of the crude extract of *P turckheimii* was observed in both pharmacological assays (Fig. 4.2), and showed linear concentration dependent activity. The extract had an IC_{50} of 0.64 mg/mL (0.53, 0.82) in the GABA-T assay, and an EC_{50} of 48.3 *μ*g/mL (37.1, 63.09) in the $GABA_A$-BZD receptor binding assay. These *in vitro* results are comparable to *Melissa officinalis* L. (Lemon balm, Awad *et al.* 2009, Jäger *et al.* 2006) and *Passiflora incarnata* L. (Passionflower, Weiss *et al.* 2011), two important

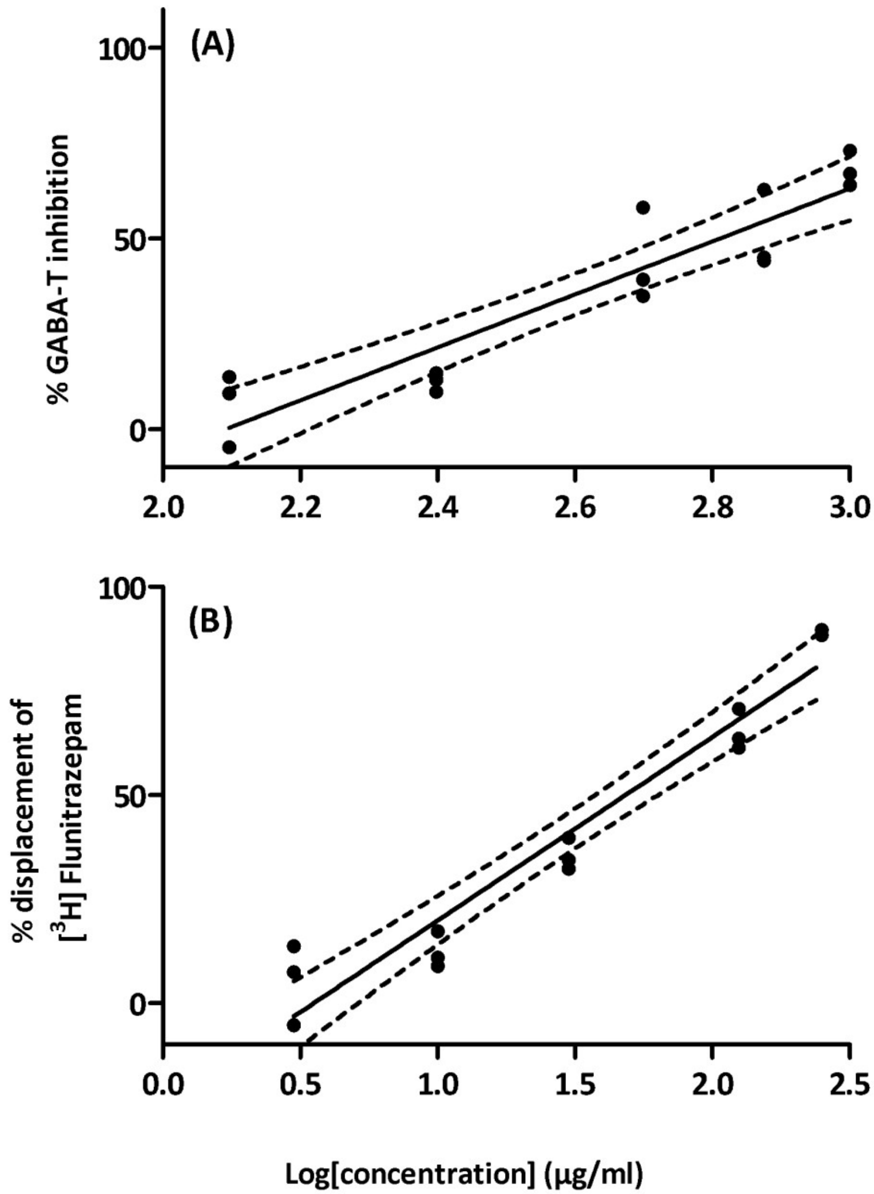


Figure 4.2. Concentration (Log) dependent linear regression analysis (95% CI) of the crude extract of *Piper tuerckheimii* for the (A) *in vitro* inhibition of GABA-T and (B) in the displacement of [^{3}H] Flunitrazepam. (A) Fifty percent inhibition concentration [IC_{50} (± 95% CI)] = 0.64 mg/mL (0.53, 0.82) and (B) Fifty percent effective concentration [EC_{50} (± 95% CI)] = 48.3 μg/mL (37.1, 63.09).

herbal remedies used worldwide for the treatment of anxiety and that have also shown activity *in vivo* (Hwang *et al.* 2011, Grundmann *et al.* 2008).

Bioassay-guided fractionation of the leaves of *Piper tuerckheimii*, the part of the plant traditionally used by the healers, was undertaken in order to identify and isolate the constituents responsible for its activity. A detailed fractionation scheme is illustrated in Figure 4.3. Out of the 11 primary fractions, one showed greater than 25% activity in the GABA-T inhibition assay and four in the $GABA_A$-BZD receptor binding assay. However, only one showed activity greater than 50% at the test concentration (Fig. 4.4). Fraction PT-F6 exhibited the greatest activity in both assays (38.6% ± 5.7 GABA-T inhibition and 64.9% ± 3.6 displacement of the radioligand) and was comparable to the crude extract when tested at the same concentration. For these reasons PT-F6 was chosen for further fractionation and phytochemical characterization.

The sub-fractionation of the most active fraction (PT-F6) using preparative scale HPLC yielded 23 major peaks, 5 of which were found to be responsible for the activity. Four of those peaks were found to be pure compounds (PT-F6-Pk4, Pk8, Pk9, and Pk11), and the other active peak was a mixture (PT-F6-Pk3). Compound **1**, **2**, **3**, and **4** were purified from peak 4, peak 8, peak 9, and peak 11 respectively.

The structures of the compounds (Fig. 4.5) were established by comparing spectroscopic data obtained from 1D and 2D NMR experiments with those reported in the literature (Zhang *et al.* 2009, Ishikawa *et al.* 2002, Matsuura *et al.* 2004). The IC_{50} and EC_{50} of these compounds, determined from dose response curves, are detailed in Table 4.1. The fraction PT-F6-Pk3 exhibited the greatest activity at the highest tested

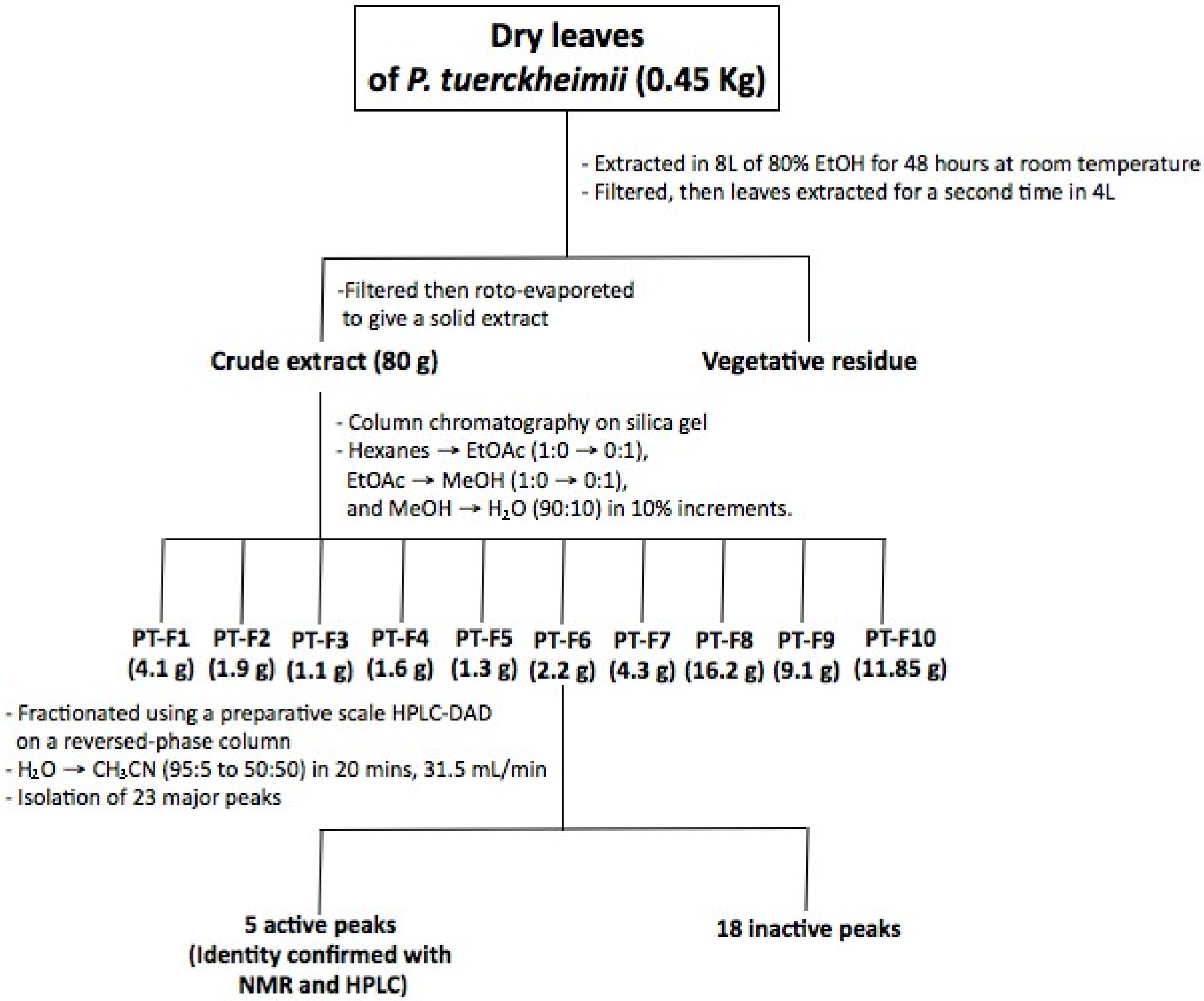


Figure 4.3. Bioassay-guided fractionation scheme of the dry leaves of *Piper tuerckheimii* C. DC. ex Donn. Sm. (Piperaceae).

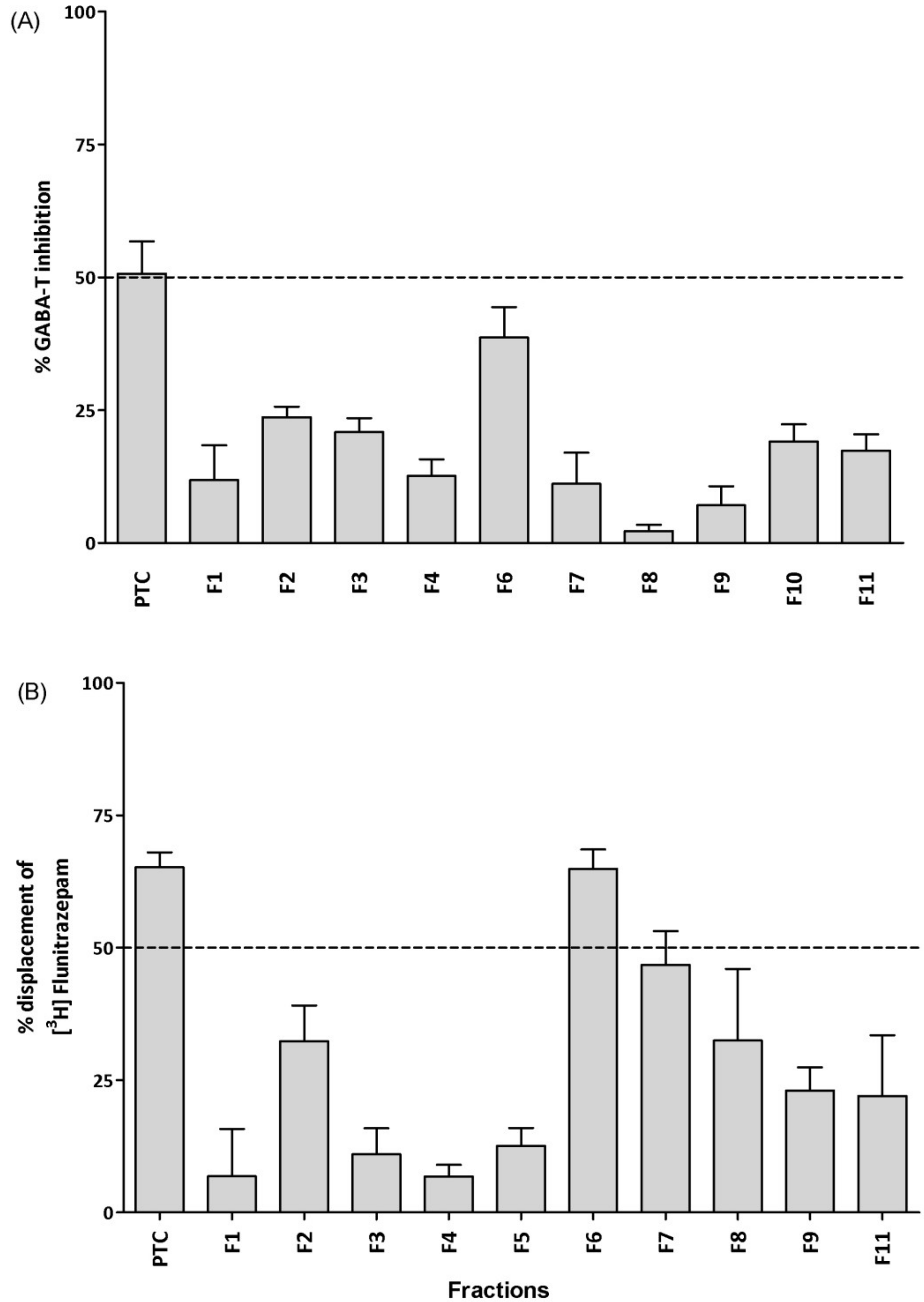
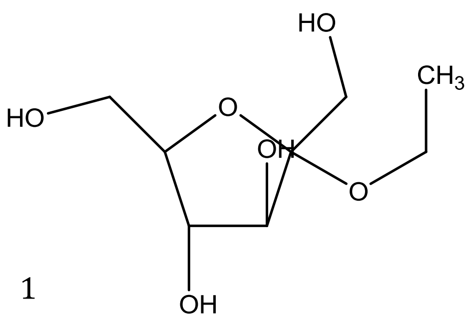
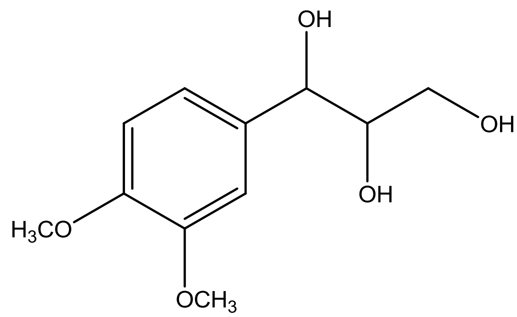


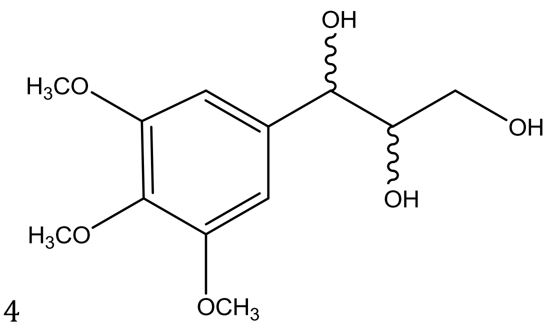
Figure 4.4. The ability of the crude extract (PTC) and primary fractions of *Piper tuerckheimii* to (A) to inhibit GABA-T (±SEM) at 0.5 mg/ml and (B) displace the radioligand [^{3}H]-Flunitrezepam from the $GABA_A$-BZD receptor (±SEM) at 0.1 mg/ml.



1



2



3 and 4

Figure 4.5. Chemical structures of the isolated active compounds from the *Piper tuerckheimii* extract. (1) D-Fructofuranoside, (2) 1,2,3-Propanetriol,1-(3,4-dimethoxyphenyl)- (3 and 4) 1,2,3-Propanetriol, 1-(3,4,5-trimethoxyphenyl)-.

Table 4.1. Inhibitory concentration for 50% *in vitro* inhibition of GABA-T and effective concentration for 50% displacement of [^{3}H] flunitrazepam of the fraction or compounds isolated from PT-F6.

Fraction or Compound	IC_{50}	EC_{50}
Pk3	58.07^a	-
1	456.2^b	210.6^b
2	425.4^b	-
3	-	149.4^b
4	-	441.5^b

$^a\mu$g/mL
$^b\mu$M

concentration in the GABA-T inhibition assay, followed by compound **2** and **1**. In the BZD displacement assay compound **3** exhibited the greatest displacement of ^{3}H-flunitrazepam followed by **2** and **4**. Compound **2** was the only one the show considerable activity at both sites. To our knowledge, this is the first time that biological activity has been reported for any of those compounds.

As previously mentioned, the crude extract of *P. tuerckheimii* showed comparable activity to *M. officinalis* (Awad *et al.* 2009, Jäger *et al.* 2006) and *P. incarnata* (Weiss *et al.* 2011). Additionally, we have found that compounds **1** and **2** showed similar inhibitory activity to rosmarinic acid, an active constituent of Lemon balm (40% GABA-T inhibition at 288 μM and 304 μM compared to 277 μM, Awad *et al.* 2009). On the other hand, compound **1**, **3** and **4** have showed comparable and lower activity in the competitive $GABA_A$-BZD receptor binding assay compared to previously reported benzodiazepine ligands isolated from plants. Compound **3** showed the greatest affinity to the receptor with an EC_{50} of 149.4 μM, where apigenin isolated from *Tanacetum parthenium* (Jäger *et al.* 2009) and the two flavonoids hispidulin and cirsilineol isolated from *Artemisia herba-alba* (Salah *et al.* 2005) had respective EC_{50}'s of 12 μM, 8 μM and 104 μM respectively. The flavonoids, which are also present in Piperaceae (Parmar *et al.* 1997), are known to have great affinity to the $GABA_A$-BZD receptor (Jäger *et al.* 2011, Medina *et al.* 1997), where piperamides, compounds specific to Piperaceae such as piperine, have been found to be important GABA-T inhibitors (Awad, 2008). It would therefore be worthwhile to target these specific compounds when isolating potential anxiolytic and antiepileptic compounds from Piperaceae plants, or if the collection of a greater amount of *P. tuerckheimii* material could be attempted.

In short, we have isolated four compounds from *Piper tuerckheimii* via bioassay-guided fractionation and have demonstrated that they exhibit their anxiolytic and antiepileptic effect by binding to the $GABA_A$-BZD receptor, by inhibiting GABA-T, or both. These results provide a pharmacological basis behind the choice of plants by Q'eqchi' healers.

Chapter 5

General discussion

5.1. Overview of original contributions to knowledge

This thesis presents a thorough review of the use of psychoactive plants in the Neotropics, and highlights the Piperaceae as a key family in the traditional treatment of mental and folk illnesses. The data presented broadens the scientific knowledge pertaining to the Neotropical biodiversity, neuroactivity and phytochemistry of the Piperaceae family.

In Chapter 2, I present the first systematic review pertaining to the use of plants for neuropsychological disorders and their associated folk illnesses by traditional cultures of the Neotropics. A total of 200 species were identified as having the potential to interact with the GABAergic system based on their traditional use and the symptoms treated, thus providing likely candidates for anxiolytic and antiepileptic plants. As suggested, application of the Moerman analysis (1991) revealed the Piperaceae as one of the most highly selected plant families by the various ethnic groups, thus highlighting the pantropical use of Piperaceae species for the treatment of mental illnesses and the maintenance of well-being.

The Andean and Amazonian regions of Peru are known as some of the most culturally and biologically diverse regions of the Neotropics. In fact, around 59 different ethnic groups live in the Amazon region (IBC 2006), which is also one of the most species-rich regions on the planet (Gentry 1988). These regions have a wealth of traditional knowledge on the use of plants for medicine (Schultes and Raffauf 1990) and a rich center of Piperaceae biodiversity (Quijano-Abril *et al.* 2006). However, to our knowledge, only a small fraction of the Neotropical Piperaceae species have been studied in an ethnopharmacological context and, for that matter, very few species have been thoroughly studied for their effect on the CNS (Felipe et al. 2007). In Chapter 3, I present the first comprehensive investigation of the Peruvian Piperaceae diversity for their ability to interact with the GABAergic system, and for the first time, present *in vitro* experimental data demonstrating a pharmacological basis behind the use of plants for mental and folk illnesses by the Yanesha, one of the many ethnic groups of Amazon Basin. Of the 47 Piperaceae tested and the 21 plants used by the Yanesha, many demonstrated the ability to facilitate GABAergic transmission via the GABA receptors, and the ability to inhibit the GABA-T enzyme. The results pertaining to the anxiolytic and antiepileptic properties of Yanesha Piperaceae plants were comparable to those used by the Q'eqchi' Maya of Belize (Awad *et al.* 2009), thus confirming the significance of Piperaceae as a source of important psychotherapeutic herbal alternatives.

Finally, following up on the previous recommendations by Bourbonnais-Spear *et al.* (2005) who first highlighted *Piper tuerckheimii* as one of the preferred plants used by the Q'eqchi' Maya of Belize for the treatment of epilepsy and folk illnesses, and by Awad *et al.* (2009) who first reported on its pharmacological mode of action, I now have identified the active principles responsible for its *in vitro* anxiolytic and antiepileptic activity. This suggests that the Piperaceae may be an important source for neuroactive compounds, and confirms the importance of plant derived NHPs as a complementary source of medicine.

5.2. Piperaceae as a source of neuroactive phytochemicals

A review of the phytochemistry of the genus *Piper* written by Parmar et al. (1997) revealed a rich diversity of compounds (around 600) coming from more than 80 species. Since then more species have been studied, however the total available knowledge on the phytochemistry only encompasses less than 12% of the total diversity (Dyer *et al.* 2004). To my knowledge, even less is known concerning the phytochemistry of the genus *Peperomia*. These reports indicate that there remain approximately 880 *Piper* species (12% of the estimated number of 1000 species), which have never been investigated. The global traditional knowledge around the Piperaceae, with plants such as *Piper nigrum* and *Piper methysticum*, includes great evidence that the family can interact with the CNS. This thesis demonstrates that Neotropical species from the Piperaceae have the potential to interact with the GABAergic system. All things considered, very few studies have actually isolated and illustrated neuroactive compounds from the family. Here I present a review of the known pharmacological literature available and incorporate the compounds isolated from *Piper tuerckheimii* to the list of known phytochemical exerting CNS activity (Table 5.1).

Table 5.1. Review of the pharmacological evidence of neuroactive compounds isolated from Piperaceae.

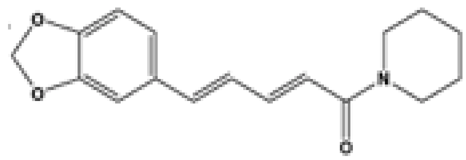
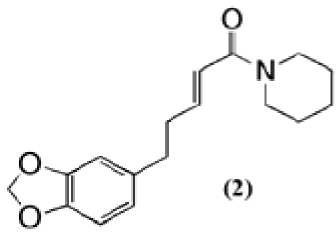
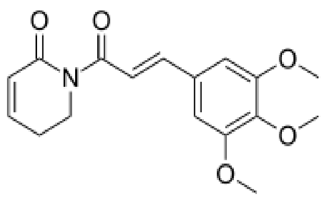
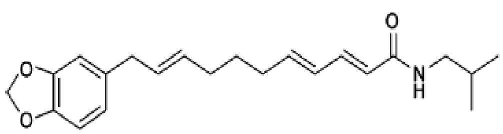
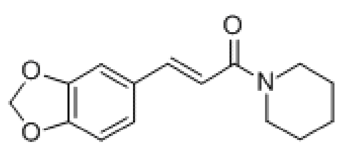
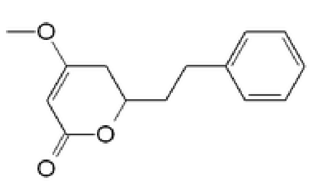
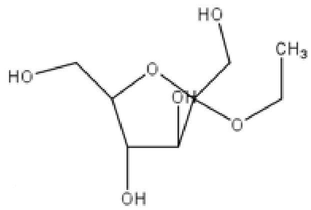
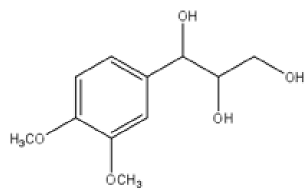
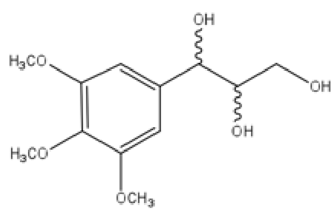
Compound	Structure	Bioactivity	Source
Piperine		Anticonvulsant activity Positive $GABA_A$-BZD Receptor Modulator *in vivo* antidepressant effect GABA-T inhibition	D'Hooge *et al.* 1996 Zaugg *et al.* 2010 Wattanathorn *et al.* 2008 Awad (2008)
4,5-dihydropiperine	 (2)	Positive $GABA_A$-BZD Receptor Modulator	Perdersen *et al.* 2009
Piperlongumine		*In vivo* antidepressant *In vivo* anxiolytic	Felipe *et al.* 2007 Felipe *et al.* 2007
Laetispicine		*In vivo* antidepressant	Yao *et al.* 2009
Antiepilepsirine		*In vivo* antiepileptic Antidepressant *In vitro* GABA-T inhibition	Wanga et al. 1999 Li *et al.* 2007 Awad (2008)
Dihydrokavain		Inhibition of voltage-dependent Na+-channels *In vivo* anxiolytic *In vitro* GABAergic modulation	Magura *et al.* 1997 Smith *et al.* 2001 Yuan *et al.* 2002

Table 1. Continued.

Compound	Structure	Bioactivity	Source
1		*In vitro* GABA-T inhibition	This thesis
2		*In vitro* GABA-T inhibition *In vivo* $GABA_A$-BZD binding affinity	This thesis This thesis
3		*In vivo* $GABA_A$-BZD binding affinity	This thesis

5.3. Future directions

Natural products from plants are a well-known and significant source of novel drugs (Newman and Cragg 2007). The research presented in this thesis highlights the importance of certain Neotropical plant families and specific traditionally used plants as a rich source of neuroactive compounds, especially having the ability to interact with the GABAergic system. More precisely, further ethnopharmacological and phytochemical studies of the plant families highlighted in the systematic review (Chapter 2) – such as Piperaceae and the rare Marcgraviaceae – would be greatly valuable for the discovery of neuroactive natural products. Novel compounds from the most active Yanesha plants –

mainly from *Piper cremii* and *Drymaria cordata* – could potentially be isolated via bioassay-guided fractionation. These targeted plants and their related active principle should also be examined *in vivo* using standardized behavioral models (i.e. the open field test, the elevated plus maze test, and the acoustic startle response) in order to verify and quantify the true efficacy and safety of the plants in animals.

The essential oils of plants have been traditionally used for the treatment of a plethora of ailments (del Carmen Jiménez-Pérez *et al.* 2011), and this knowledge as now been transferred to the increasingly popular branch of complementary and alternative medicine (CAM) known as aromatherapy (Cooke and Ernst 2000). Aromatherapy is now considered to be the most common CAM utilized for the treatment of anxiety and depression and recent systematic reviews have demonstrated the method has being effective (Lee *et al.* 2011, Perry and Perry 2006). Plants of the Piperaceae family are noted as being remarkably aromatic and the body of literature associated with the phytochemical composition and bioactivity of their essential oils is astounding (Sirat *et al.* 2010, Qin *et al.* 2010, etc.). Nevertheless, essential oils of Piperaceae have not yet been investigated for *in vivo* reduction of anxiety. Most Piperaceae plants traditionally used by the Yanesha highlighted in this study (Chater 3) can be administered to the patient by the healers in the form of a vapor or steam bath (Fig. 5.1, Valadeau *et al.* 2010, Bourdy *et al.* 2008). A thorough investigation of the therapeutic properties of the essential oils excreted and absorbed during the process of these vapor baths should be undertaken in the future. They could potentially result in the identification of novel NHPs and ultimately, highligt the significance of quantitative ethnobotanical and ethnopharmacological studies.

Figure 5.1. Photograph of a Yanesha preparation of a "vapor bath" made with leaves of *Piper* species. Photo taken by Céline Valadeau (Bourdy *et al.* 2008).

Litterature Cited

(WHO), W. H. O. (1999). The mental health of indigenous people: an international overview. Geneva, Switzerland: World Health Organization.

(WHO), W. H. O. (2001). Mental health : new understanding, new hope. Geneva, Switzerland: World Health Organization.

(WHO), W. H. O. (2005). Mental health: facing the challenges, building solutions. . Copenhagen, .Denmark: WHO Regional Office for Europe

Amiguet, V. T., Arnason, J. T., Maquin, P., Cal, V., Sanchez-Vindas, P., & Alvarez, L. P. (2006). A regression analysis of Q'eqchi' Maya medicinal plants from Southern Belize. Economic Botany, 60(1), 24-38.

Amiguet, V. T., Arnason, J. T., Maquin, P., Cal, V., Vindas, P. S., & Poveda, L. (2005). A consensus ethnobotany of the Q'eqchi' Maya of Southern Belize. Economic Botany, 59(1), 29-42.

Ashton, H., & Young, A. H. (2003). GABA-ergic drugs: exit stage left, enter stage right. Journal of Psychopharmacology, 17(2), 174–178.

Awad, R. (2008). Ethnopharmacology of medicinal plants used in anxiety and epilepsy: Effects on y-aminobutyric acid (GABA) metabolism. University of Ottawa, Ottawa.

Awad, R., Ahmed, F., Bourbonnais-Spear, N., Mullally, M., Ta, C. A., Tang, A., et al.. (2009). Ethnopharmacology of Q'eqchi' Maya antiepileptic and anxiolytic plants: Effects on the GABAergic system. Journal of Ethnopharmacology, 125(2), 257-264.

Awad, R., Levac, D., Cybulska, P., Merali, Z., Trudeau, V. L., & Arnason, J. T. (2007). Effects of traditionally used anxiolytic botanicals on enzymes of the gamma-aminobutyric acid (GABA) system. Canadian Journal of Physiology and Pharmacology, 85(9), 933-942.

Awad, R., Muhammad, A., Durst, T., Trudeau, V. L., & Arnason, J. T. (2009). Bioassay-guided Fractionation of Lemon Balm (Melissa officinalis L.) using an In Vitro Measure of GABA Transaminase Activity. Phytotherapy Research, 23(8), 1075-1081.

Balick, M. J., H., N. M., & E., A. D. (2000). Checklist of the vascular plants of Belize. (Vol. 85): New York Bot. Gard.

Barrett, B. (1994). Medicinal plants of Nicaragua's Atlantic coast. Economic Botany, 48(1), 8-20.

Barua, C. C., Roy, J. D., Buragohain, B., Barua, A. G., Borah, P., & Lahkar, M. (2009). Anxiolytic effect of hydroethanolic extract of Drymaria cordata L Willd. Indian Journal of Experimental Biology, 47(12), 969-973.

Benke, D., & Mohler, H. (1999). Characterization of Benzodiazepine Binding to GABAA Receptors. In M. W. SJ Enna, JW Ferkany, T Kenakin, P Moser, B Ruggeri (Ed.), Current Protocols in Pharmacology Vol 5: John Wiley & Sons, Inc.

Bennett, B. C. (1992). Hallucinogenic Plants of the Shuar and Related Indigenous Groups in Amazonian Ecuador and Peru. Brittonia, 44(4), 483-493.

Bennett, B. C., Baker, M., & Andrade, P. (2002). Ethnobotany of the Shuar of Eastern Ecuador. (Vol. 14): Advances in Economic Botany.

Bennet, B. C. & Husby, C. E. (2008). Patterns of medicinal plant use: an examination of the Ecuadorian Shuar medininal flora using contingency table and binomial analysis. Journal of Ethnopharmacology. 116, 422-430.

Bennett, B. C., & Prance, G. T. (2000). Introduced plants in the indigenous pharmacopoeia of northern South America. Economic Botany, 54(1), 90-102.

Boonen, G., & Häberlein, H. (1998). Influence of genuine kavapyrone enantiomers on the GABA-A binding site. Planta Medica, 64(6), 504-506.

Bornstein, A., & Coe, F. G. (2007). The genus Piper (Piperaceae) in Honduras. Novon, 17(1), 11–19.

Bourbonnais-Spear, N., Awad, R., Maquin, P., Cal, V., Vindas, P. S., Poveda, L., et al.. (2005). Plant use by the Q'eqchi' Maya of Belize in ethnopsychiatry and neurological pathology. Economic Botany, 59(4), 326-336.

Bourbonnais-Spear, N., Awad, R., Merali, Z., Maquin, P., Cal, V., & Arnason, J. T. (2007). Ethnopharmacological investigation of plants used to treat susto, a folk illness. Journal of Ethnopharmacology, 109(3), 380-387.

Bourdy, G., Valadeau, C., Alban Castillo, J. (2008). Yato’ Ramuesh: Pare’shemats Yanesha, Yato’ Ramuesh: Plantas medicinales Yaneshas. Lima, Peru: PRODAPP, IRD

Bourdy, G., DeWalt, S. J., Chavez de Michel, L. R., Roca, A., Deharo, E., Munoz, V., et al. (2000). Medicinal plants uses of the Tacana, an Amazonian Bolivian ethnic group. Journal of Ethnopharmacology, 70, 87–109.

Bradford, M. M. (1976). Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. Analytical Biochemistry, 72(1-2), 248-254.

Breedlove, D. E. (1986). Flora de Chiapas. In Listados Floríst (Vol. 4). México.

Bussmann, R. W., Glenn, A., & Sharon, D. (2010). Healing the body and soul: Traditional remedies for "magical" ailments, nervous system and psychosomatic disorders in Northern Peru. African Journal of Pharmacy and Pharmacology, 4(9), 580-U587.

Bussmann, R. W., & Sharon, D. (2006). Traditional medicinal plant use in loja province, southern Ecuador. Journal of Ethnobiology and Ethnomedicine, 2.

Bussmann, R. W., & Sharon, D. (2009). Shadows of the colonial past - diverging plant use in Northern Peru and Southern Ecuador. Journal of Ethnobiology and Ethnomedicine, 5.

Cardenas, L. C., Rodriguez, J., Villaverde, M. C., Riguera, R., Cadena, R., & Otero, J. A. (1993). The Analgesic Activity of Hedyosmum-Bonplandianum - Flavonoid Glycosides. Planta Medica, 59(1), 26-27.

Carey, J. W. (1993). Distribution of Culture-Bound Illnesses in the Southern Peruvian Andes. Medical Anthropology Quarterly, 7(3), 281-300.

Carlini, E. A. (2003). Plants and the central nervous system. Pharmacology Biochemistry and Behavior, 75(3), 501-512.

Cavender, A. P., & Alban, M. (2009). The use of magical plants by curanderos in the Ecuador highlands. Journal of Ethnobiology and Ethnomedicine, 5.

Chapouthier, G., & Venault, P. (2001). A pharmacological link between epilepsy and anxiety? TRENDS in Pharmacological Sciences, 22(10), 491-493.

Cook, F. E. M. (1995). Economic Botany Data Collection Standard. Kew: Kew: Royal Botanic Gardens.

Cooke, B., Ernst, E., & . (2000). Aromatherapy: a systematic review. Br. J. Gen. Pract., 50, 493–496.

Da Silva, R. Z., Yunes, R. A., de Souza, M. M., Delle Monache, F., & Cechinel, V. (2010). Antinociceptive properties of conocarpan and orientin obtained from Piper solmsianum C. DC. var. solmsianum (Piperaceae). Journal of Natural Medicines, 64(4), 402-408.

Davis, E. W., & Yost, J. A. (1983). The ethnomedicine of the Waorani of Amazonian Ecuador. Journal of Ethnopharmacology, 9(2-3), 273-297.

de Albuquerque, U. P., de Medeiros, P. M., de Almeida, A. L. S., Monteiro, J. M., de Freitas Lins Neto, E. M., de Melo, J. G., et al. (2007). Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: A quantitative approach. . Journal of Ethnopharmacology, 114(3), 325-354.

De Feo, V. (2003). Ethnomedical field study in northern Peruvian Andes with particular reference to divination practices. Journal of Ethnopharmacology, 85(2-3), 243-256.

De-la-Cruz, H., Vilcapoma, G., & Zevallos, P. A. (2007). Ethnobotanical study of medicinal plants used by the Andean people of Canta, Lima, Peru. . Journal of Ethnopharmacology, 111(2), 284-294.

deBittencourt, P. R. M., Adamolekum, B., Bharucha, N., Carpio, A., Cossio, O. H., Danesi, M. A., et al.. (1996). Epilepsy in the tropics .2. Clinical presentations, pathophysiology, immunologic diagnosis, economics, and therapy. Epilepsia, 37(11), 1128-1137.

deBittencourt, P. R. M., Adamolekum, B., Bharucha, N., Carpio, A., Cossio, O. H., Danesi, M. A., et al.. (1996). Epilepsy in the tropics .1. Epidemiology, socioeconomic risk factors, and etiology. Epilepsia, 37(11), 1121-1127.

del Carmen Jiménez-Pérez, N., Lorea-Hernández, F. G., Jankowski, C. K., & Reyes-Chilpa, R. (2011). Essential Oils in Mexican Bays (Litsea spp., Lauraceae): Taxonomic Assortment and Ethnobotanical Implication. Economic Botany, 65(2), 178-189.

Dennis, P. A. (1988). Herbal medicine among the miskito of eastern Nicaragua. Economic Botany, 42(1), 16-28(1), 16-28.

Desmarchelier, C., Gurni, A., Ciccia, G., & Giulietti, A. M. (1996). Ritual and medicinal plants of the Ese'ejas of the Amazonian rainforest (Madre de Dios, Perú). . Journal of Ethnopharmacology, 52(1), 45-51.

DHooge, R., Pei, Y. Q., Raes, A., Lebrun, P., vanBogaert, P. P., & DeDeyn, P. P. (1996). Anticonvulsant activity of piperine on seizures induced by excitatory amino acid receptor agonists. Arzneimittel-Forschung-Drug Research, 46(6), 557-560.

Di Stasi, L. C., Oliveira, G. P., Carvalhaes, M. A., Queiroz-Junior, M., Tien, O. S., Kakinami, S. H., et al.. (2002). Medicinal plants popularly used in the Brazilian tropical Atlantic forest. Fitoterapia, 73(1), 69-91.

Douwes, E., Crouch, N. R., Edwards, T. J., Mulholland, D. A. (2008). Regression analyses of southern African ethnomedicinal plants: informing the targeted selection of bioprospecting and pharmacological screening subjects. Journal of Ethnopharmacology. 119, 356-364.

Eisenberg, D. M., Davis, R. B., Ettner, S. L., Appel, S., Wilkey, S., van Rompay, M., et al.. (1998). Trends in alternative medicine use in the United States, 1990-1997 - Results of a follow-up national survey. Jama-Journal of the American Medical Association, 280(18), 1569-1575.

Ekstein, D., & Schachter, S. C. (2010). Natural Products in Epilepsy—the Present Situation and Perspectives for the Future. Pharmaceuticals, 3, 1426-1445.

Elferink, J. G. R. (1999). Epilepsy and its treatment in the ancient cultures of America. Epilepsia, 40(7), 1041-1046.

Elferink, J. G. R. (1999). Mental disorder among the Incas in ancient Peru. History of Psychiatry, 10(39), 303-318.

Ernst, E. (2006). Herbal remedies for anxiety - a systematic review of controlled clinical trials. Phytomedicine, 13(3), 205-208.

Fabrega, H. J. (1971). Medical Anthropology. Biennial Review of Anthropology, 7, 167-229.

Felipe, F. C. B., Sousa, J. T., Souza, L. E. D. O., Silveira, J. A., Uchoa, D. E. D. A., Silveira, E. R., et al.. (2007). Piplartine, an amide alkaloid from Piper tuberculatum, presents anxiolytic and antidepressant effects in mice. Phytomedicine, 14(9), 605-612.

Fernandez, E. C., Sandi, Y. E., & Kokoska, L. (2003). Ethnobotanical inventory of medicinal plants used in the Bustillo province of the Potosi department, Bolivia. Fitoterapia, 74(4), 407-416.

Figueiredo, G. M., Leitao-Filho, H. F., & Begossi, A. (1997). Ethnobotany of Atlantic forest coastal communities: II. diversity of plant uses at Sepetiba bay (SE Brazil). Human Ecology, 25(2), 353-360.

Gentry, A. H. (1988). Tree species richness of upper Amazonian forests. Proceedings of the National Academy of Sciences, 85(156-159).

Giron, L. M., Freire, V., Alonzo, A., & Caceres, A. (1991). Ethnobotanical survey of the medicinal flora used by the caribs of Guatemala. Journal of Ethnopharmacology, 34(2-3), 173-187.

Grundmann, O., Wang, J., McGregor, G. P., & Butterweck, V. (2008). Anxiolytic Activity of a Phytochemically Characterized Passiflora incarnata Extract is Mediated via the GABAergic System. Planta Medica, 74(15), 1769-1773.

Hammond, G. B., Fernandez, I. D., Villegas, L. F., & Vaisberg, A. J. (1998). A survey of traditional medicinal plants from the Callejon de Huaylas, department of Ancash, Peru. Journal of Ethnopharmacology 61(1), 17-30.

Hartshorn, G. S., Nicolait, R., & al, e. (1984). Belize: country environmental profile, a field study.

I.B.C. (2006). Atlas de comunidades nativas de la selva central. Lima, Peru: Instituto del bien commun.

Ishikawa, T., Fujimatu, E., & Kitajima, J. (2002). Water-Soluble Constituents of Anise: New Glucosides of Anethole Glycol and Its Related Compounds. Chem. Pharm. Bull. , 50(11), 1460-1466.

Jacob, K., Sharan, P., Mirza, I., Garrido-Cumbrera, M., Seedat, S., Mari, J., et al.. (2007). Mental health systems in countries: Where are we now? . Lancet, 370(9592), 1061-1077.

Jager, A. K., Gauguin, B., Adsersen, A., & Gudiksen, L. (2006). Screening of plants used in Danish folk medicine to treat epilepsy and convulsions. Journal of Ethnopharmacology, 105(1-2), 294-300.

Jovel, E. M., Cabanillas, J., & Towers, G. H. N. (1996). An ethnobotanical study of the traditional medicine of the mestizo people of Suni Mirano, Loreto, Peru. Journal of Ethnopharmacology, 53(3), 149-156.

Joyal, E. (1987). Ethnobotanical field notes from Ecuador: Camp, prieto, jørgensen, and giler. Economic Botany, 41(2).

Jung, M. J., Lippert, B., Metcalf, B. W., Bohlen, P., & Schechter, P. J. (1977). Gamma-Vinyl Gaba (4-Amino-Hex-5-Enoic Acid), a New Selective Irreversible Inhibitor of Gaba-T - Effects on Brain Gaba Metabolism in Mice. Journal of Neurochemistry, 29(5), 797-802.

Kahnberg, P., Lager, E., Rosenberg, C., Schougaard, J., Camet, L., Sterner, O., et al.. (2002). Refinement and evaluation of a pharmacophore model for flavone derivatives binding to the benzodiazepine site of the GABA(A) receptor. Journal of Medicinal Chemistry, 45(19), 4188-4201.

Kapur, S. K., Shahim A. K., Sarin, Y. K., Moerman, D. E. (1992). The medicinal flora of Majouri-Kirchi forests, India. Journal of Ethnopharmacology. 36, 87-90.

Kennedy, J. G. (1973). Cultural Psychiatry. In J. J. Honigmann (Ed.), Handbook of Social and Cultural Anthropology (Vol. I I I (7-1) I (98)). Chicago.

Kufer, J., Forther, H., Poll, E., & Heinrich, M. (2005). Historical and modern medicinal plant uses - the example of the Ch'orti' Maya and Ladinos in Eastern Guatemala. Journal of Pharmacy and Pharmacology, 57(9), 1127-1152.

Kwan, P., Arzimanoglou, A., Berg, A. T., Brodie, M. J., Hauser, W. A., Mathern, G., et al.. (2010). Definition of drug resistant epilepsy: Consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. Epilepsia, 51(6), 1069-1077.

Lader, M. (2008). Effectiveness of benzodiazepines: do they work or not? Expert Rev. Neurother., 8(8), 1189-1191.

Laferriere, J. E. (1994). Medicinal plants of the lowland Inga people of Colombia. International Journal of Pharmacognosy, 32(1), 90-94.

Lago, J. H. G., Ramos, C. S., Casanova, D. C. C., Morandim, A. D., Bergamo, D. C. B., Cavalheiro, A. J., et al.. (2004). Benzoic acid derivatives from piper species and their fungitoxic activity against Cladosporium cladosporioides and C-sphaerospermum. Journal of Natural Products, 67(11), 1783-1788.

Lee, Y., Wu, Y., Tsang, H. W. H., Leung, A. Y., & Cheung, W. M. (2011). A Systematic Review on the Anxiolytic Effects of Aromatherapy in People with Anxiety Symptoms. The Journal of Alternative and Complementary Medicine 17(2), 101-108.

Lenaerts, M. (2005). Anthropologie des indiens Ashéninka d'Amazonie : nos soeurs Manioc et l'étranger jaguar. Paris: L'Harmattan.

Leonti, M., Ramirez, F., Sticher, O., & Heinrich, M. (2003). Medicinal flora of the Popoluca, Mexico: A botanical systematical perspective. Economic Botany, 57(2), 218-230.

Lewis, W. H., & Elvin-Lewis, P. F. (2003). Medical Botany. Hoboken, NJ: Wiley.

Lewis, W. H., Lamas, G., Vaisberg, A., Corley, D. G., & Sarasara, C. (1999). Peruvian medicinal plant sources of new pharmaceuticals (International Cooperative Biodiversity Group-Peru). Pharmaceutical Biology, 37, 69-83.

Li, S., Wang, C., Li, W., Koike, K., Nikaido, T., & Wang, M. (2007). Antidepressant-like effects of piperine and its derivative, antiepilepsirine. Journal of Asian Natural Products Research, 9(5), 435-444.

Luziatelli, G., Sorensen, M., Theilade, I., & Molgaard, P. (2010). Ashaninka medicinal plants: a case study from the native community of Bajo Quimiriki, Junin, Peru. Journal of Ethnobiology and Ethnomedicine, 6.

Lydiard, R. B. (2003). The role of GABA in anxiety disorders. Journal of Clinical Psychiatry, 64, 21-27.

Magura, E. I., Kopanitsa, M. V., Gleitz, J., & al., e. (1997). Kava extract ingredients, (±)-kavain inhibit voltage-operated Na+-channels in rat CA1 hippocampal neurons. Neuroscience, 81(2), 345-351.

Mathews, J. M., Etheridge, A. S., & Black, S. R. (2002). Inhibition of human cytochrome P450 activities by kava extract and kavalactones. Drug Metabolism and Disposition, 30(11), 1153-1157.

Matsuura, H., Miyazaki, H., Asakawa, C., Amano, M., Yoshihara, T., & Mizutani, J. (2004). Isolation of alpha-glucosidase inhibitors fro hyssop (Hyssopus officinalis). Phytochemistry, 65(2004), 91-97.

Medina, J. H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D., et al.. (1997). Overview - Flavonoids: A new family of benzodiazepine receptor ligands. Neurochemical Research, 22(4), 419-425.

Michel, J., Duarte, R. E., Bolton, J. L., Huang, Y., Caceres, A., Veliz, M., et al.. (2007). Medical potential of plants used by the Q'eqchi Maya of Livingston, Guatemala for the treatment of women's health complaints. Journal of Ethnopharmacology, 114(1), 92-101.

Milliken, W. (1992). The Ethnobotany of the Waimiri Atroari Indians of Brazil. Kew: Royal Botanic Gardens.

Milliken, W., & Albert, B. (1996). The use of medicinal plants by the Yanomami Indians of Brazil. Economic Botany, 50(1), 10-25.

Milliken, W., & Albert, B. (1997). The use of medicinal plants by the Yanomami Indians of Brazil .2. Economic Botany, 51(3), 264-278.

Milton, J., and Jung, P. (2003). Epilepsy as a dynamic disease. NY. USA.: Springer-Verlag.

Moerman, D. E. (1991). The Medicinal Flora of Native North-America - an Analysis. Journal of Ethnopharmacology, 31(1), 1-42.

Muceniece, R., Saleniece, K., Krigere, L., Rumaks, J., Dzirkale, Z., Mezhapuke, R., et al.. (2008). Potato (Solanum tuberosum) juice exerts an anticonvulsant effect in mice through binding to GABA receptors. Planta Medica, 74(5), 491-496.

Mullally, M., Kramp, K., Cayer, C., Saleem, A., Ahmed, F., McRae, C., et al.. (2011). Anxiolytic Activity of a Supercritical Carbon Dioxide Extract of Souroubea sympetala (Marcgraviaceae). Phytotherapy Research, 25, 264-270.

Newman, D. J., & Cragg, G. M. (2007). Natural Products as Sources of New Drugs over the Last 25 Years. Journal of Natural Products, J. Nat. Prod., 2007, 70 (3), pp 461–477(70), 3.

Nilsson, J., Gidlof, R., Nielsen, E. O., Liljefors, T., Nielsen, M., & Sterner, O. (2011). Triazoloquinazolinediones as novel high affinity ligands for the benzodiazepine site of GABA(A) receptors. Bioorganic & Medicinal Chemistry, 19(1), 111-121.

Noumi, E., & Fozi, F. L. (2003). Ethnomedical botany of epilepsy treatment in Fongo-Tongo Village, Western Province, Cameroon. Pharmaceutical Biology, 41(5), 330-339.

Parmar, V. S., Jain, S. C., Bisht, K. S., Jain, R., Taneja, P., Jha, A., et al.. (1997). Phytochemistry of the genus Piper. Phytochemistry, 46(4), 597-673.

Pedersen, M. E., Metzler, B., Stafford, G. I., van Staden, J., Jager, A. K., & Rasmussen, H. B. (2009). Amides from Piper capense with CNS Activity - A Preliminary SAR Analysis. Molecules, 14(9), 3833-3843.

Perry, N., & Perry, E. (2006). Aromatherapy in the management of psychiatric disorders: clinical and neuropharmacological perspectives. CNS Drugs, 20(4), 257–280.

Pittler, M. H., & Ernst, E. (2000). Efficacy of kava extract for treating anxiety: Systematic review and meta-analysis. Journal of Clinical Psychopharmacology, 20(1), 84-89.

Portet, B., Fabre, N., Roumy, V., Gornitzka, H., Bourdy, G., Chevalley, S., et al.. (2007). Activity-guided isolation of antiplasmodial dihydrochalcones and flavanones from Piper hostmannianum var. berbicense. Phytochemistry, 68(9), 1312-1320.

Prance, G. T., Campbell, D. G., & Nelson, B. W. (1977). The ethnobotany of the Paumarí indians. Economic Botany, 31(2), 129-139.

Qin, W., Huang, S., Li, C., Chen, S., & Peng, Z. (2010). Biological activity of the essential oil from the leaves of Piper sarmentosum Roxb. (Piperaceae) and its chemical constituents on Brontispa longissima (Gestro) (Coleoptera: Hispidae) Pesticide Biochemistry and Physiology, 96(3), 132-139.

Quijano-Abril, M. A., Callejas-Posada, R., & Miranda-Esquivel, D. R. (2006). Areas of endemism and distribution patterns for Neotropical Piper species (Piperaceae). Journal of Biogeography, 33(7), 1266-1278.

Rodrigues, E., & Carlini, E. A. (2006). A comparison of plants utilized in ritual healing by two brazilian cultures: Quilombolas and kraho indians. Journal of Psychoactive Drugs, 38(3), 285-295.

Rubel, A. J. (1964). The Epidemiology of a Folk Illness - Susto in Hispanic America. Ethnology, 3(3), 268-283.

Rubel, A. J., O'Nell, C. W., & Collado-Ardon, R. (1984). Susto, a Folk Illness. Berkeley: University of California Press.

Sanz-Biset, J., Campos-de-la-Cruz, J., Epiquien-Rivera, M. A., & Canigueral, S. (2009). A first survey on the medicinal plants of the Chazuta valley (Peruvian Amazon). Journal of Ethnopharmacology, 122(2), 333-362.

Sasli-Lagoudakis, C. H., Williamson, E. M., Savolainen, V., Hawkins, J. A. (2011). Cross-cultural comparaison of three medicinal floras and implications for bioprospecting strategies. Journal of Ethnopharmacology. 135, 476-487.

Saxena, S., Thornicroft, G., Knapp, M., & Whiteford, H. (2007). Resources for mental health: Scarcity, inequity, and inefficiency. Lancet, 370(9590), 878-889.

Schultes, R. E. (1969). Hallucinogens of Plant Origin. Science, 163(3864), 245-&.

Schultes, R. E. (1984). Fifteen years of study of psychoactive snuffs of South America: 1967-1982--a review. Journal of Ethnopharmacology, 11(1), 17-32.

Schultes, R. E., Hofmann, A., & Ratsch, C. (2001). Plants of the Gods: Their Sacred, Healing, and Hallucinogenic Powers. (2nd edition ed.): Healing Arts Press.

Schultes, R. E., & Raffauf, R. F. (1990). The healing forest: Medicinal and toxic plants of the northwest Amazonia. Portland, Or: Dioscorides Press.

Shepard, G. H. (1998). Psychoactive plants and ethnopsychiatric medicines of the matsigenka. Journal of Psychoactive Drugs, 30(4), 321-332.

Singh, Y. N. (1992). Kava - an Overview. Journal of Ethnopharmacology, 37(1), 13-45.

Singh, Y. N., & Singh, N. N. (2002). Therapeutic potential of kava in the treatment of anxiety disorders. Cns Drugs, 16(11), 731-743.

Sirat, H. M., Thai, O. B., & Ahmad, F. (2010). Chemical Composition of the Essential Oil of Piper maingayi Hk. F. Journal of Essential Oil Research, 22, 323-324.

Smith, K. K., Dharmaratne, R. W., Feltenstein, M. W., Broom, S. L., Roach, J. T., Dhammika Nanayakkara, N. P., et al.. (2001). Anxiolytic effects of kava extract and kavalactones in the chick social separation-stress paradigm. Psychopharmacology, 155, 86-90.

Smith, N., Mori, S. A., Henderson, A., Stevenson, D. W., & Heald, S. V. (2003). Flowering Plants of the Neotropics.: Princeton University Press.

Snodgrass, S. R. (1978). Use of Muscimol-H-3 for Gaba Receptor Studies. Nature, 273(5661), 392-394.

Spinella, M. (2001). The psychopharmacology of herbal medicine. Cambridge, Massachusetts: MIT Press.

Srinivasan, K. (2007). Black Pepper and its Pungent Principle-Piperine: A Review of Diverse Physiological Effects. Critical Reviews in Food Science and Nutrition, 47, 735–748.

Stafford, G. I., Jager, A. K., & van Staden, J. (2005). Activity of traditional South African sedative and potentially CNS-acting plants in the GABA-benzodiazepine receptor assay. Journal of Ethnopharmacology, 100(1-2), 210-215.

Standley, P. C., & Steyermark, J. A. (1952). Piperaceae. In Flora of Guatemala - Part III Fieldiana, Bot, 24(3), 228–337

Suzdak, P. D., & Jansen, J. A. (1995). A Review of the Preclinical Pharmacology of Tiagabine: A Potent and Selective Anticonvulsant GABA uptake inhibitor. Epilepsia, 36(6), 612-626.

Teschke, R. (2010). Kava hepatotoxicity. A clinical review. Annals of Hepatology, 9(3), 251-265.

Thorpe, K., & Chenier, L. (2011). Building Mentally Healthy Workplaces: Perspectives of Canadian Workers and Front-Line Managers: The Conference Board of Canada.

Tolardo, R., Zetterman, L., Bitencourtt, D. R., Mora, T. C., de Oliveira, F. L., Biavatti, M. W., et al.. (2010). Evaluation of behavioral and pharmacological effects of Hedyosmum brasiliense and isolated sesquiterpene lactones in rodents. Journal of Ethnopharmacology, 128(1), 63-70.

Tournon, J. (2006). Las Plantas, los Rao y sus espíritus. (Etnobotánica del Ucayali). Ucayali: Gobierno regional de Ucayalio.

Treiman, D. M. (2001). GABAergic mechanisms in epilepsy. Epilepsia, 42, 8-12.

Uzzell, D. (1974). "Susto" Revisited: Illness as Strategic Role. American Ethnologist, 1(2), 369-378.

Valadeau, C. (2010). De l'ethnobotanique à l'articulation du soin : Approche du système nosologique chez les Yanesha de Haute Amazonie péruvienne. Université Paul Sabatier, Toulouse.

Valadeau, C., Castillo, J. A., Sauvain, M., Lorese, A. F., & Bourdy, G. (2010). The rainbow hurts my skin: Medicinal concepts and plants uses among the Yanesha (Amuesha), an Amazonian Peruvian ethnic group. Journal of Ethnopharmacology, 127(1), 175-192.

Vasiliadis, H.-M., & Tempier, R. (2011). Reporting on the prevalence of drug and alternative health product use for mental health reasons: results from a national population survey. J. Popul. Ther. Clin. Pharmacol., 18(1), e33-e43.

Velozo, L. S. M., Ferreira, M. J. P., Santos, M. I. S., Moreira, D. L., Guimaraes, E. F., Emerenciano, V. P., et al.. (2009). C-glycosyl flavones from Peperomia blanda. Fitoterapia, 80(2), 119-122.

Vickers, W. T., & Plowman, T. (1984). Useful plants of the Siona and Secoya Indians of Eastern Ecuador. Fieldiana Botany, 15, 1-63.

Wanga, L., Zhaoa, D., Zhanga, Z., Zuoa, C., Zhangb, Y., Peib, Y. Q., et al.. (1999). Trial of antiepilepsirine (AES) in children with epilepsy. Brain & Development, 21, 36-40.

Wattanathorn, J., Chonpathompikunlert, P., Muchimapura, S., Priprem, A., & Tankamnerdthai, O. (2008). Piperine, the potential functional food for mood and cognitive disorders. Food and Chemical Toxicology, 46(9), 3106-3110.

Weckerle, C. S., Cabras, S., Castellanos, E. M., Leonti, M. (2011). Quantitative methods in ethnobotany and ethnopharmacology: considering the overall-flora – hypothesis testing for over- and underused plant families with the Bayesian approach. Journal of Ethnopharmacology. 137, 837-843.

Weiss, G., Appel, K., Rose, T., Fiebich, B., Kammler, T., & Hoffmann, C. (2011). Modulation of the gamma-Aminobutyric Acid (GABA) System by Passiflora incarnata L. Phytotherapy Research, 25(6), 838-843.

Weller, S. C., Baer, R. D., Garcia, J. G. D., Glazer, M., Trotter, R., Pachter, L., et al.. (2002). Regional variation in Latino descriptions of susto. Culture Medicine and Psychiatry, 26(4), 449-472.

Weller, S. C., Baer, R. D., Garcia, J. G. D., & Rocha, A. L. S. (2008). Susto and nervios: Expressions for stress and depression. Culture Medicine and Psychiatry, 32(3), 406-420.

Yao, C. Y., Wang, J., Dong, D., Qian, F. G., Xie, J., & Pan, S. L. (2009). Laetispicine, an amide alkaloid from Piper laetispicum, presents antidepressant and antinociceptive effects in mice. Phytomedicine, 16(9), 823-829.

Yuan, C. S., Dey, L., Wang, A., Mehendale, S., Xie, J.T., Aung, H.H. & Ang-Lee, M.K. (2002). Kavalactones and dihydrokavain modulate GABAergic activity in a rat gastricbrainstem preparation. . Planta Medica, 68(12), 1092-1096.

Zaugg, J., Baburin, I., Strommer, B., Kim, H. J., Hering, S., & Hamburger, M. (2010). HPLC-Based Activity Profiling: Discovery of Piperine as a Positive GABA(A) Receptor Modulator Targeting a Benzodiazepine-Independent Binding Site. Journal of Natural Products, 73(2), 185-191.

Zetola, M., De Lima, T. C. M., Sonaglio, D., Gonzalez-Ortega, G., Limberger, R. P., Petrovick, P. R., et al.. (2002). CNS activities of liquid and spray-dried extracts from Lippia alba - Verbenaceae (Brazilian false melissa). Journal of Ethnopharmacology, 82(2-3), 207-215.

Zhang, Z., Wang, D., Zhao, Y., Gao, H., Hu, Y., & Hu, J. (2009). Fructose-derived carbohydrates from Alisma orientalis. Natural Products Research, 23(11), 1013-1020.

Zou, L., Henderson, G. L., Harkey, M. R., Sakai, Y., & Li, A. (2004). Effects of Kava (Kava-kava, 'Awa, Yaqona, Piper methysticum) on c-DNA-expressed cytochrome P450 enzymes and human cryopreserved hepatocytes. Phytomedicine, 11(4), 285-294.

Appendix 1

Muestras botánicas (Piperaceae) entregadas por Gabriel Picard (Univ. De Otawa-Canadá)-abril 2011

ESPECIES BOTÁNICAS	Código USM
1. *Piper subflavispicum* C.DC.	243153
2. *Piper acutifolium* R & P.	243152
3. *Piper aff. euriphyllum* Trel.	243154
4. *Piper cremii* Trel.	243151
5. *Piper denisii* Trel.	243162
6. *Piper quimirianum* Trel.	243150
7. *Peperomia* sp.	243149
8. *Piper sp.*	243148
9. *Piper longifolium* R & P.	243163
10. *Peperomia sp.*	243147
11. *Piper cf. adreptum* Trel.	243164
12. *Peperomia* sp.	243146
13. *Piper* sp.	243145
14. *Piper carpunya* R & P.	243142
15. *Piper aduncum* L.	243139
16. *Piper sinuclausum* Trel.	243144
17. *Piper ferruginispicum* Trel.	243138
18. *Piper abditum* Trel.	243173
19. *Peperomia galioides*	243171
20. *Piper aff cupreatum* Trel.	243169
21. *Piper dumosum* Rudge	243168
22. *Piper pseudoarboreum* Yun	243170
23. *Piper margaritanum* Trel.	243181
24. *Piper allardii* Trel.	243167
25. *Piper propinquum* C.DC.	243182
26. *Piper celer* Trel.	243183
27. *Piper* sp. (nov?)	243184
28. *Piper cf. dilatatum* L.C. Rich.	243166
29. *Piper bermudezense* Trel.	243185
30. *Piper secundum* R & P.	243165
31. *Piper sp.*	243160
32. *Piper pseudoarboreum* Yun.	243159
33. *Piper longifilamentosum* Trel.	243161
34. *Piper setulosum* Trel.	243158
35. *Piper peltatum* L.	243157
36. *Piper callosum*	243141
37. *Piper sp.*	243155
38. *Piper sp.*	243156
39. *Piper sp.*	243143
40. *Piper sp.*	243186
41. *Piper sp.*	243172
42. *Piper loretoanum* Trel.	243187
43. *Piper soledadense* Trel.	243179
44. *Peperomia sp.*	243178
45. *Piper barbicuspe* Trel.	243177
46. *Peperomia sp.*	243176
47. *Piper leucofuscum* Trel.	243175
48. *Piper pavonii* C.DC.	243174
49. *Peperomia pellucida*	243180