An Ethnobiological Investigation of Q'eqchi' Maya and Cree of Eeyou Istchee Immunomodulatory Therapies

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Abstract

This thesis investigated the phytochemistry and pharmacology of immunomodulatory medicinal plant species used traditionally by the Q'eqchi' Maya Healers Association (QMHA) of Belize, and the Cree of Eeyou Istchee (CEI) of northern Quebec.

Using quantitative ethnobotanical methodology, we identified 107 plant species belonging to 49 families used by Q'eqchi' healers in the treatment of symptoms from 14 usage categories related to inflammation. Regression analysis revealed that the Piperaceae, Araceae, and Begoniaceae are preferentially selected by the Maya. Healer consensus for plant species was high, with 56 species (52%) being used by all the healers, and consensus for usage categories was also high, as informant consensus factor (F_{IC}) values for each category were greater than 0.4.

Fifty-two Belizean species were evaluated for their TNF-α inhibitory activity in an LPS-stimulated THP-1 monocyte model. Twenty-one species (40%) demonstrated significant TNF-α inhibition when assayed at 100 µg/mL, 8 of which had greater than 50% of the activity of the parthenolide positive control (10 µg/mL). Significant regressions were found between the anti-inflammatory activity and total healer frequency of use (F_{use}) and the use reports for 3 usage categories, which indicated that ethnobotanical parameters can in part predict the activity of traditionally used species.

Five sesquiterpene lactones were isolated from the leaves of *Neurolaena lobata*, one of the most active species tested, all of which demonstrated anti-

inflammatory activity greater than that of parthenolide (IC_{50} = 4.79 µM), with IC_{50}s ranging from 0.17-2.32 µM. Lobatin B was the most active isolate tested.

Ethanolic and water extracts of 17 species used by Cree healers were evaluated for their immunomodulatory activity. In general, the average anti-inflammatory activity of ethanolic extracts was 1.8 times greater than that of water extracts, and the pro-inflammatory activity of water extracts was 3.7 times greater than ethanolic extracts. *Picea mariana* and *Pinus banksiana* were the most anti-inflammatory ethanolic and water extracts, while the water extract of *Sarracenia purpurea* was the most pro-inflammatory.

Picea marina cones, the most anti-inflammatory Cree medicine, were subjected to bioassay guided isolation. This led to the isolation of the anti-inflammatory lignan (+)-lariciresinol-9'-*p*-coumarate, which had an IC_{50} of 28.4 µM.

Together, these results validate the traditional knowledge shared by our Q'eqchi' and Cree collaborators, and draw attention to the therapeutic potential of subtropical and boreal plants as culturally appropriate complements to modern medicine.

Résumé

Cette thèse porte sur la phytochimie et la pharmacologie des espèces de plantes médicinales immunomodulatrices utilisées traditionnellement par le Q'eqchi' Maya Healers Association (QMHA) du Belize, et les Cris d'Eeyou Istchee (CEI) du nord du Québec.

En utilisant une méthodologie ethnobotanique quantitative, nous avons identifié 107 espèces végétales appartenant à 49 familles utilisées par les guérisseurs Q'eqchi' dans le traitement de symptômes appartenant à 14 catégories d'utilisation liées à l'inflammation. Une analyse de régression a révélé que les familles Piperaceae, Araceae, et Begoniaceae sont préférentiellement choisis par les Mayas. Le consensus entre guérisseurs pour les espèces végétales était élevé, avec 56 espèces (52%) étant utilisés par tous les guérisseurs, et le consensus pour les catégories d'utilisation était également élevé, car les valeurs de facteur de consensus des informants (F_{IC}) pour chaque catégorie étaient supérieurs à 0,4.

Cinquante-deux espèces du Belize ont été évaluées pour leur activité inhibitrice de TNF-α dans un modèle de THP-1 monocytes stimulés par le LPS. Vingt-et-une espèces (40%) ont montré une inhibition significative de TNF-α lorsque dosés à 100 µg/mL, dont 8 d'entre elles ont démontrées plus de 50% de l'activité du contrôle positif parthénolide (10 µg/mL). Des régressions significatives ont été observées entre l'activité anti-inflammatoire et la fréquence d'utilisation de guérisseurs totale (F_{use}) et les rapports d'utilisation pour 3

catégories d'utilisation, ce qui indique que les paramètres ethnobotaniques peuvent en partie prédire l'activité des espèces traditionnellement utilisées.

Cinq lactones sesquiterpéniques ont été isolés à partir des feuilles de *Neurolaena lobata*, l'une des espèces les plus actives testées, qui a démontré une activité anti-inflammatoire supérieure à celle du parthénolide (CI_{50} = 4,79 µM), avec des CI_{50} allant de 0,17 à 2,32 µM. Lobatin B était l'isolât le plus actif testé.

Des extraits éthanoliques et aqueux de 17 espèces utilisées par les guérisseurs Cris ont été évalués pour leur activité immunomodulatrice. En général, l'activité anti-inflammatoire moyenne des extraits éthanoliques était 1,8 fois supérieure à celle des extraits d'eau, et l'activité pro-inflammatoire des extraits d'eau était de 3,7 fois supérieure à celle des extraits éthanoliques. *Picea mariana* et *Pinus banksiana* étaient les extraits éthanoliques et aqueux avec le plus d'activité anti-inflammatoire, tandis que l'extrait aqueux de *Sarracenia purpurea* était le plus pro-inflammatoire.

Le cône de *Picea marina*, le médicament traditionnelle Cris le plus anti-inflammatoire, a été soumis à une isolation guidée par essais biologiques. Cela a mené à l'isolement du lignane anti-inflammatoire (+)-lariciresinol-9'-*p*-coumarate, qui avait une CI_{50} de 28,4 µM.

Ensemble, ces résultats valident les connaissances traditionnelles partagées par nos collaborateurs Q'eqchi' et Cris, et mettent en évidence le potentiel thérapeutique des plantes subtropicales et boréales comme des compléments à la médecine moderne qui sont culturellement appropriées.

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List of Abbreviations

BITI - Belize Indigenous Training Institute

CEI - Cree of Eeyou Istchee

CIHR - Canadian Institutes of Health Research

EtOH - Ethanol

F_{ic} - Informant consensus factor

F_{use} - Frequency of use

IC_{50} - Half maximal inhibitory concentration

ID_{50} - Half maximal inhibitory dose

LPS - Lipopolysaccharide

N_{taxa} - Number of taxa

N_{ur} - Number of use reports

NHP – Natural Health Product

NMR - Nuclear Magnetic Resonance

QMHA - Q'eqchi' Maya Healers Association

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

rpm - Revolutions Per Minute

SD - Standard Deviation

SE - Standard Error

T2D - Type II Diabetes

TAAM - Team in Aboriginal Anti-diabetic Medicines

TNF-α - Tumor Necrosis Factor alpha

Chapter 1

Introduction and Literature Review

1.1 – General Introduction

Two indigenous communities invited our participation in the evaluation and inclusion of traditional treatments into modern healthcare strategies. The focus of this research was to examine, using what is defined as an ethnobiological approach, traditional therapies with potential immunomodulatory effects. The indigenous communities of interest were the Q'eqchi' Maya of southern Belize, and the Quebec Cree of Eeyou Istchee (CEI). In cooperation with the Q'eqchi' Maya Healers Association (QMHA), this research investigated immunomodulatory botanical therapies (chapters 2-4). The section of this project dealing with the Cree was conducted as a part of the Canadian Institute of Health Research (CIHR) Team in Aboriginal Antidiabetic Medicines (TAAM), and assessed the immunomodulatory activity of CEI anti-diabetic plant treatments (chapters 5-7).

The immune system plays an important role in human health, orchestrating a wide variety of defensive and restorative functions, and is essential in maintaining and re-establishing homeostasis. Inflammation is a crucial tool of the immune system, modulated in response to a wide variety of stimuli. In a healthy individual, inflammation is a beneficial process, however in many disorders, inflammation, or a lack thereof, can cause and/or contribute to the pathology of the condition. As such, immunomodulation, accomplished through numerous pro-

and anti-inflammatory pathways, represents an important target in attempts to understand and treat many categories of illness (Haddad et al., 2005).

1.2 – Collaborating Communities

Q'eqchi' Maya

The Q'eqchi' are descendents of the Ancient Maya whose classic civilization flourished in the first millennium. While the Maya civilization's roots date back over 4000 years, it is unclear how well the tradition has been preserved due to the tumultuous events of history. The late classic period, from approximately AD 600 to 800, was the height of Maya civilization. The classic Maya collapse, beginning around AD 800, and the Spanish conquest beginning in 1528, caused the loss of much Maya culture (Coe, 2002). However, the Maya are a resilient people and are currently enjoying a modern Maya renaissance. Elements of their medicinal traditions persist to this day and enjoy renewed community interest.

There are approximately 30 Q'eqchi' Maya villages located in the Toledo District of southern Belize, and traditional healers provide most of the primary health care needs in these communities. In 1998, with the assistance of international partners and the Belize Indigenous Training Institute (BITI), a local non-governmental organization, several Q'eqchi' healers formed the QMHA with the goals of preserving traditional knowledge, passing on traditional knowledge to younger generations, conserving and domesticating medicinal plant species, and generating income through the sustainable development of natural health products. In addition, the QMHA hope to gain government recognition of the

important role they play in their communities, with the objective of being included in local healthcare delivery strategies. To this end, the QMHA are interested in scientifically validating the safety and efficacy of their traditional medicines.

In collaboration with the QMHA, the University of Ottawa, Cleveland State University, and the Universidad Nacional de Costa Rica, various projects have been implemented to address the conservation of the region's cultural and biological diversity, and to scientifically validate QMHA traditional knowledge. Several studies have been carried out to document the QMHA's traditional knowledge of medicinal plant species (Bourbonnais-Spear et al., 2005; Treyvaud Amiguet et al., 2005; Treyvaud Amiguet et al., 2006), as well as the traditional knowledge of local ecosystems (Pesek et al., 2006, 2009, 2010). In addition, the assessment of the biological activities of QMHA medicinal plant species and the characterization of their phytochemical principles and underlying mechanisms of action provide a pharmacological basis for their use by the QMHA (Awad et al., 2009; Bourbonnais-Spear et al., 2007; Mullally et al., 2011).

Another important objective of the QMHA was the creation of the Itzamma Garden ("home of the Maya God of wisdom, Itzamna"), an ethnobotanical garden managed by the healers association located between the villages of Indian Creek and Golden Stream (Figure 1.1). The Itzamma Garden and Medicinal Plant Project is a collaborative effort at public health promotion and agronomy for sustainable non-timber forest products, built on the premise that traditional ethnobotanical knowledge could be continually utilized as a vehicle for primary healthcare and culturally appropriate development in the context of the

conservation of culture and biodiversity (Arnason et al., 2009; Waldram et al., 2009). The garden combines *in situ* and *ex situ* conservation efforts of species used medicinally by the QMHA, where species already found at the garden have been cultivated alongside species transplanted to the garden from remote regions of the Maya Mountains (Bourbonnais-Spear et al., 2006; Pesek et al., 2007). The plants found at the garden are used in regular practice for community primary health care by the members of the QMHA, and to date, 102 species have been identified (Rojas et al., 2010).

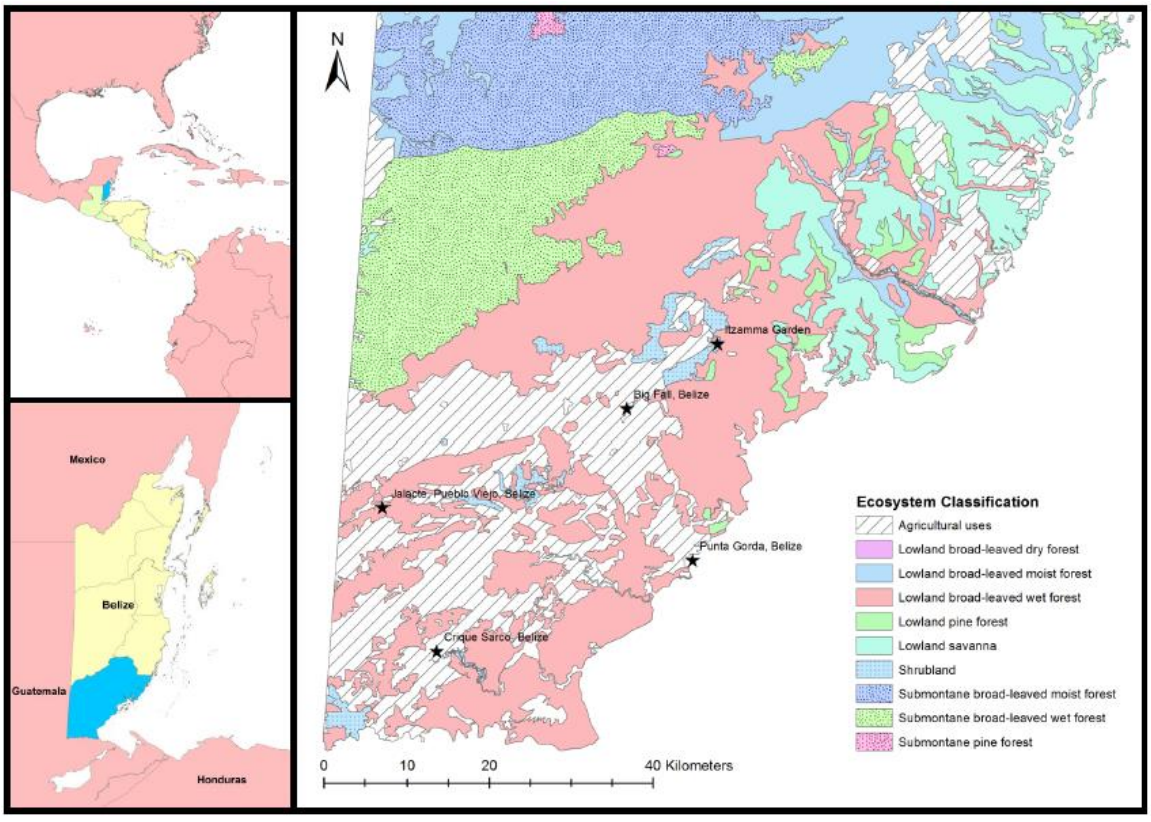

Figure 1.1 - Ecoregions in the Toledo district of southern Belize [1: 1,500,000 scale] based on “Ecosystem classification” data layers (BBMS, 2006). The Itzamma Garden and sites from where plants were collected by the QMHA to be transplanted to Itzamma are indicated by stars along with regional ecological features (adapted from Audet et al., 2013).

The QMHA was originally founded with 10 members, but due to death and departures, there are currently five active Q'eqchi' members (Figure 1.2) of the healers association (as well as an active Mopan Maya healer who is part of the organization): 3 elders and 2 middle-aged men ranging from 33 to 77 years of age, with an average age of 58 years old, from the villages of Jalacte, Big Falls, Indian Creek, and the town of Punta Gorda (Figure 1.1). The five Q'eqchi' members of the QMHA range in experience from 14 to 59 years, with an average of 36 years of experience, with many of the healers having trained and practiced in both Guatemala and Belize. Among the healers are two spiritual leaders. Spirituality plays an important role in Q'eqchi' healing, as is common in many traditional societies, with ceremonial and ritual ethnomedical practices being an integral part of every treatment.

Figure 1.2 – Five members of the Q'eqchi' Maya Healers Association and the manager of the Belize Indigenous Training Institute.

The Toledo District is still home to many remaining areas of primary and secondary semi-evergreen rainforest. A long and intricate association with these forests has provided the Q'eqchi' with a sophisticated botanical tradition. Included within this tradition is much knowledge of plant treatments for symptoms recognized as having an inflammatory component, such as fevers, headaches, stiffness, pain, and swelling (Treyvaud Amiguet et al., 2005). This points towards potential immunomodulatory activity in Q'eqchi' botanical therapies.

Flora of Southern Belize

Over 3400 species of vascular plants have been recorded in Belize (1219 genera and 209 families), with 41 endemic species (1.2% of total flora) (Balick et al., 2001). Southern Belize has a subtropical climate with a short but distinct dry season which gives rise to its semi-evergreen tropical forest. Areas of primary and secondary lowland broad leaf moist forest and scrubland in the region are all used by members of the QMHA to collect medicinal plant species (Figure 1.1). In addition, around the villages, shifting agriculture is practiced, creating "milpas", which are indigenous agriculture areas cleared in the forest for traditional crops intercropped with corn and beans. Following several years of cultivation, plots become fallowed areas which included successional vegetation and secondary forest up to 20 years old. Belize is in the centre for the Mesoamerican biodiversity corridor and is recognized as a biodiversity hotspot in need of conservation (Meyers et al., 2000) based on the high level of endemic species and human impact. For this reason the area is of considerable biological interest and has

high potential for phytochemical and medicinal discovery, but is also an area of concern because of human activities.

The first reports of the flora of Belize were written by the British; they contain a detailed description of the natural history of Belize in the early 19th century. Since then, Morton E. Peck (1870-1959), William A. Schipp (1891-1967), Percival Hildebart Gentle (1890-1958), Cyrus Longworth Lundell (1907-1996), and John M. Dwyer have made important contributions to documenting the flora of Belize. For the purposes of this thesis, the checklist of the Flora of Belize by Balick (2001) was used as the botanical reference. The Flora of Guatemala (Standley and Steyermark, 1946-1977) is the standard key to species for the area.

Cree of Eeyou Istchee

The Cree are Canada's largest aboriginal group, and through their ancient and close relationship with nature, they have developed a strong and diverse botanical tradition. The Cree of Eeyou Istchee (CEI) are a subpopulation of the Cree Nation, and they are located in nine communities spread out between the 49th and 55th parallels in the province of Quebec (Secretariat aux affaires autochtones, 2004) (Figure 1.3).

This study investigating CEI traditional immunomodulatory treatments was conducted as one part of the large collaborative efforts of the CIHR TAAM. The TAAM program is exploring traditionally used CEI anti-diabetic botanicals as a culturally appropriate complement to modern pharmaceuticals in the attempt to

combat high levels of Type II diabetes (T2D) among the Cree. Diabetes is a significant and growing problem worldwide, and is of particular concern among aboriginal populations, including the CEI, where from 1994 to 2004, its prevalence in people aged 20 years or over rose from 6.6% to 17.7% (Legare, 2004; Kuzmina and Dannenbaum, 2004). Research has revealed important connections between diabetes and inflammation (Dandona et al., 2002; Pickup, 2004), hence an immunomodulatory investigation of CEI anti-diabetic botanicals is warranted.

The original ethnobotanical study of the Cree antidiabetic plants was conducted with 60 healers by Alain Cuerrier's group at the Montreal Botanical Garden (Leduc et al., 2006). They developed an innovative quantitative method (syndromic importance value) of assessing the use of plants for diabetic symptoms that allowed a ranking of the most culturally important plants which resulted in the identification of the 17 CEI species studied in this thesis.

Flora of Eeyou Istchee

The flora of CEI territories extends over the northern area of boreal forest in northern Quebec from the southernmost community of Waswanipi to the northernmost community of Whapmagoostui. The climate of this area is represented by long cold winters (6-8 months) and short cool summers. Because of relatively recent glaciation, there are few endemics and the diversity is low. The main families are Pinaceae, Ericaceae, Rosaceae, and Salicaceae. The main floras used in this thesis were the Flore Laurentienne (Marie-Victorin, 1977)

and Flora of Canada (Scoggan, 1978). Although the flora does not have a large diversity of species, and few endemics, the phytochemistry and medicinal activity of plants in this area have not been intensively studied.

Figure 1.3 – Map of the territory of the Cree of Eeyou Istchee, James Bay, Quebec (Adapted from www.ottertooth.com/Native_K/jbcress.htm).

1.3 – Ethnobiology and Immunomodulation

Medical ethnobotany has already proven itself to be very successful in advancing modern knowledge of illness and health, primarily by “discovering” the actions of bioactive plant secondary metabolites often well understood by the indigenous communities in which they are regularly used. Such research has also yielded insight into immunomodulation. In particular, well known botanical immunomodulators such as echinacea, feverfew, ginger, ginseng and goldenseal have long histories of traditional use (Borchers et al., 2000). In addition, the origins of several common anti-inflammatory pharmaceuticals can be traced back to traditional botanical remedies, such as aspirin, an anti-inflammatory COX-inhibitor which is an acetylated form of salicylic acid derived from the naturally occurring salicin found in willow bark. Furthermore, the large number of uninvestigated traditionally used medicinal plant species has been cited by many authors as an argument in favor of continued ethnopharmacological research (Balick and Mendelsohn, 1992; Heinrich, 2000; Shultes, 1991; Sumner, 2000). For these reasons, an exploration of immunomodulatory botanicals used in indigenous communities is likely to bear fruit.

While botanical treatments play a key role in traditional healing, ethnomedical knowledge is not limited to an understanding of the medicinal properties of plants. Traditional concepts of health and disease have their modern correlates, however certain elements that shape the traditional practitioner’s worldview are less familiar in allopathic medicine. Culture bound syndromes, defined as any combination of psychological or somatic symptoms

that constitute a recognizable illness only within a specific culture, are an example of such traditional ethnomedical concepts (American Psychiatric Association, 2000). The Maya recognize a variety of culture-bound syndromes, such as *susto*, *pasmo*, *bilis*, evil eye and evil winds (Bourbonnais-Spear et al., 2007; Kunow, 2003). Without providing an in-depth account of these traditional concepts, they can often be related to emotional, spiritual or supernatural causes. The Maya cosmovision, as well as the Cree cosmovision, sharing similarities with the way many indigenous communities view the world, emphasizes an interconnectedness of all things and a sacred balance that is essential to all life. The Maya tree of life, often depicted as a *kapok* tree (*Ceiba pentandra*, Malvaceae) tree which passes through the underworld, earth, and heaven symbolizes this interconnectedness. In the context of this sacred balance, the Maya view health as being the maintenance of equilibrium within the body, mind and spirit. Illness and disease are believed to be caused by a disruption in this natural harmony and treatments aim to restore natural balance.

An important and widespread concept in Maya ethnomedicine which demonstrates the notion of a sacred balance is the hot/cold dichotomy (Ankli, 1999). Illness and disease are classified as being either hot or cold, as are the plants used to treat them. Cold treatments remedy hot conditions and vice versa, whereas imbalances create problems. While the classification of certain things as being either hot or cold can sometimes follow intuitive logic (hot peppers are hot for example), hot and cold usually refer to intrinsic characteristics rather than degrees of temperature. Even though the hot/cold categorization is not always

intuitive, there is a high degree of agreement in the treatment of illness with either hot or cold plants. In a survey of 11 symptom categories, only three categories had total use reports with less than a 70% agreement in the use of hot or cold plant remedies (Ankli, 1999). The hot/cold dichotomy is clearly an important, widespread and well conserved element of Maya ethnomedicine.

Although the potential of traditional knowledge in applications such drug discovery is widely understood, the fundamental study of this traditional knowledge as an aspect of traditional science and culture is just now being recognized and conserved. Traditional knowledge is disappearing on a global scale (Davis, 1999; Shultes, 1991), and cultural wisdom developed over thousands of years is being lost forever. By recording traditional knowledge, not only is it preserved so that science may benefit, but generations of indigenous communities to come may also learn from their rich cultural heritage. Preserving traditional knowledge also fosters the conservation of biodiversity by providing realistic motives for species conservation and by encouraging a way of life that values nature deeply. Indeed McNeely (2005) argues that the conservation of biodiversity and culture together, termed the “double helix of conservation”, is the only viable option for conservationists.

1.4 – Immunology

Inflammation and Immunomodulation

The immune system can loosely be separated into the innate immune system and the adaptive immune system, both of which contain cellular and

humoral components (Janeway et al., 2005). This research will focus on cellular mediated immunity. Both innate and adaptive immune responses trigger select inflammatory processes in order to protect and repair host cells. Inflammation is a complex set of interactions among a variety of cell types and soluble factors, in response to a wide range of internal and external stimuli (Nathan, 2002). These interactions are controlled by a sophisticated network of integrated pro- and anti-inflammatory pathways, opposing forces that normally keep each other in homeostatic balance. Inflammatory stimuli sufficient to warrant an inflammatory response will result in the simultaneous suppression of anti-inflammatory pathways and the activation of pro-inflammatory pathways. A large number of distinct immunomodulatory stimuli can create a range of different inflammatory responses, specific to the perceived stimulus (Kushner, 1998). Overlapping pathways are differentially modulated in cases of acute, chronic, localized or generalized inflammation, and each of the many inflammatory states has its own specific characteristics.

The immunomodulatory pathways regulate inflammation through a host of inflammatory mediators, most notably cytokines (e.g. IL-1, IL-2, IL-6, IL-12, TNF-α, LTα, LTβ, GM-CSF, etc), small cytokines known as chemokines (i.e. IL-8, MIP-1α, MCP1, RANTES, eotaxin, etc), prostaglandins (i.e. PGI_2, PGE_2, $PGF_{2\alpha}$), leukocyte adhesion molecules (i.e. ICAM, VCAM, E-selectin, etc), reactive oxygen species, and reactive nitrogen species. Cytokines, signal molecules which are predominantly glycoproteins, play an essential role in cell-to-cell communication, and can be produced by a variety of cell types (Kelso, 1998).

Cytokines have been classified according to structure and function, and traditionally, they have also been classified as possessing anti-inflammatory/inhibitory or pro-inflammatory/stimulatory characteristics. However, due to the complexity of the immunomodulatory pathways, such a simplistic classification fails to recognize the true nature of the inflammatory system. For example, Tumor Necrosis Factor-alpha (TNF-α), considered to be the classical pro-inflammatory cytokine which has long been used as a marker for systemic activation of pro-inflammatory mediators, has also been correlated with anti-inflammatory activity in specific circumstances (Opal and DePalo, 2000). Cytokines are involved in an array of parallel and opposing immunomodulatory pathways, exhibiting pleiotropy and redundancy, and the net immunomodulatory effect of cytokines is dependent on a multitude of factors (Opal and DePalo, 2000).

Many immunomodulatory pathways lead to the downstream mediation of nuclear factor κB (NF-κB) and activator protein 1, two transcription factors which play a central and evolutionarily conserved role in regulating the inflammatory response. Of particular interest to this research, NF-κB is a family of transcription factors involved in the expression of numerous immunomodulatory proteins. NF-κB exists in the cytoplasm of many cell types, bound to IκB and rendered inactive. The phosphorylation of IκB is mediated by IKK, which is responsive to a wide variety of immunomodulatory stimuli (Takl and Firestein, 2001). NF-κB is activated in response to various stressors, such as reactive oxygen species (ROS) and UV light, and NF-κB is also activated by a variety of infectious

pathogens. NF-κB activation results in the upregulation of acute phase response proteins, adhesive molecules, and cytokines (Ghosh et al., 1998). NF-κB is also modulated by cytokines, through direct and indirect biofeedback loops (Kishimoto et al., 1994).

Inflammation has been linked to a wide variety of disorders, and NF-κB has been cited as an important therapeutic target (Takl and Firestein, 2001). Many prominent diseases, including atherosclerosis, asthma, diabetes, Alzheimer's disease and multiple sclerosis are thought to have an inflammatory component (Nathan, 2002). Whether inflammation contributes to the cause of these conditions or is produced in response to them is currently not well understood in all cases (Al-Achi et al., 2004). There are several proposed mechanisms offering explanations of how inflammation could contribute to the cause of a condition. In disorders of the immune system, mutations in immunomodulatory pathways can result in an over-stimulation of inflammatory processes causing pathological inflammation, or in an under-stimulation of inflammatory processes causing immunodeficiencies. Defects inhibiting proper phasing of inflammatory sequences have also been suggested as potential causes of inflammatory disorders. In addition to inflammation being a potential cause of many diseases, an immune response to an inflammatory stimulus can strongly contribute to the pathology of numerous illnesses. The inflammatory stimulus can be a xenobiotic, damaged tissue, or a pathogenic microorganism, as in diseases of infectious origin such as tuberculosis and hepatitis C, where inflammation can contribute as much to the pathology as does microbial toxicity. In septic shock, acute local

inflammation leads to a body-wide response which can get out of control and cause organ failure and death. In some cases, inflammation can proceed to a chronic state, associated with diseases such as arthritis and cancer (Nathan, 2002). Inflammation remains one of the main therapeutic targets in many debilitating diseases, and even when the primary pathogenic events are unknown, control of inflammation is often the next best option. Whether it is a pathogenic excess of inflammation, or an immunodeficiency causing an increased risk of acquiring grave infection, research into immunomodulation represents an important avenue of investigation.

Inflammation and Diabetes (Cree Project)

Diabetes mellitus is a metabolic disorder caused by both environmental and genetic factors. Diabetes mellitus Type I is characterized by a loss of insulin secreting pancreatic β-cells. Diabetes mellitus Type II is the result of defective insulin secretion, and/or diminished sensitivity to insulin. Insulin plays an important metabolic role in humans, modulating the cellular uptake of glucose, and controlling the balance between the conversion of glucose to glycogen and the reverse conversion of glycogen to glucose.

Research suggests inflammation plays an important role in diabetes mellitus, and immunomodulatory therapies may provide insight into the treatment of this disorder (Dandona and Aljada, 2002). In Type I diabetes, various diabetogenic factors can cause an increase in the production of ROS, triggering NF-κB activation, and leading to the upregulation of cytokines and chemotactic

agents involved in the inflammatory response (Ho and Bray, 1999). An adaptive immune response ensues, with the recruitment of leukocytes which infiltrate islet-cells and destroy them (Pakala, 1999). T2D is associated with ROS, NF-κB activation, and increasing circulatory concentrations of cytokines, which can contribute to insulin resistance (Nilsson et al., 1998). Research has suggested that a persistent, cytokine-associated acute phase response is closely involved in the pathogenesis of T2D and its associated complications (Pickup, 2004). The established link between diabetes and obesity also appears to have an inflammatory component. Certain cytokines are over expressed in adipose tissue

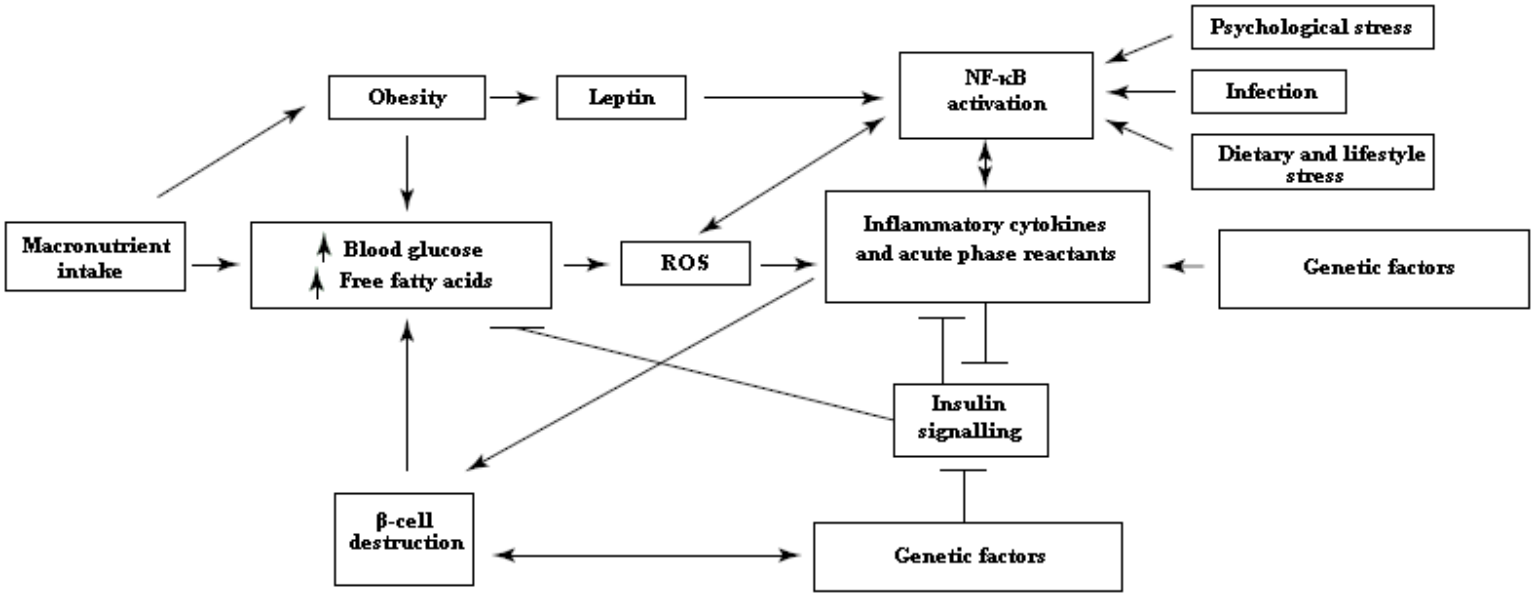

Figure 1.4 - The generation of reactive oxygen species (ROS) and the induction of inflammation (NF-κB activation) by macronutrient intake, obesity, free fatty acids, leptin, infection, dietary and lifestyle stress, psychological stress and genetic factors. Interference with insulin signaling (insulin resistance) leads to hyperglycemia and pro-inflammatory changes. Pro-inflammatory changes (increased pro-inflammatory cytokines and acute phase reactants) also lead to the inhibition of insulin signaling and insulin resistance. Inflammation in β-cells leads to β-cell dysfunction, which in combination with insulin resistance leads to Type II diabetes. Adapted from Dandona and Aljada (2004).

in obesity (Hotamisligil and Spiegelman, 1994), and the production of leptin in obesity can activate NF-κB (Figure 1.4) (Dandona and Aljada, 2004).

Immunomodulatory Botanicals

The diverse and integrated network of pro- and anti-inflammatory pathways provides numerous targets for potential immunomodulatory agents. Many immunomodulatory compounds have been isolated from a variety of plant families and they exhibit an array of immunomodulatory effects through a range of mechanisms of action. In general, the immunomodulatory strategies can be classified in relation to the components of the signaling pathways with which they interact: enzyme inhibitors, antioxidants, nuclear translocation inhibitors and DNA binding blockers. Most plant secondary metabolites are know to modulate inflammation by directly or indirectly associating with: 1) inflammatory mediators such as cytokines, chemokines, and leukocyte adhesion molecules; 2) the production or action of secondary messengers such as cGMP, cAMP and calcium; 3) the expression of immunomodulatory transcription factors such as NF-κB and activator protein 1; 4) pro-inflammatory enzymes such as cyclooxygenase (COX-1 and COX-2), inducible NO synthase (iNOS), and proteases (Calixto, 2003; Calixto, 2004). Of particular interest to this research, the activation of NF-κB can be modulated at several steps within the signaling pathway (Figure 1.5). Pathway specific inhibitors block extracellular stimuli from initiating the inflammatory cascade, antioxidants protect against the formation of intercellular ROS, nuclear translocation inhibitors prevent the translocation of NF-

κB to the nucleus and DNA binding blockers stop the binding of the transcription factor to NF-κB DNA-binding sites thereby inhibiting immunomodulatory gene expression (D'Acquisto, 2002).

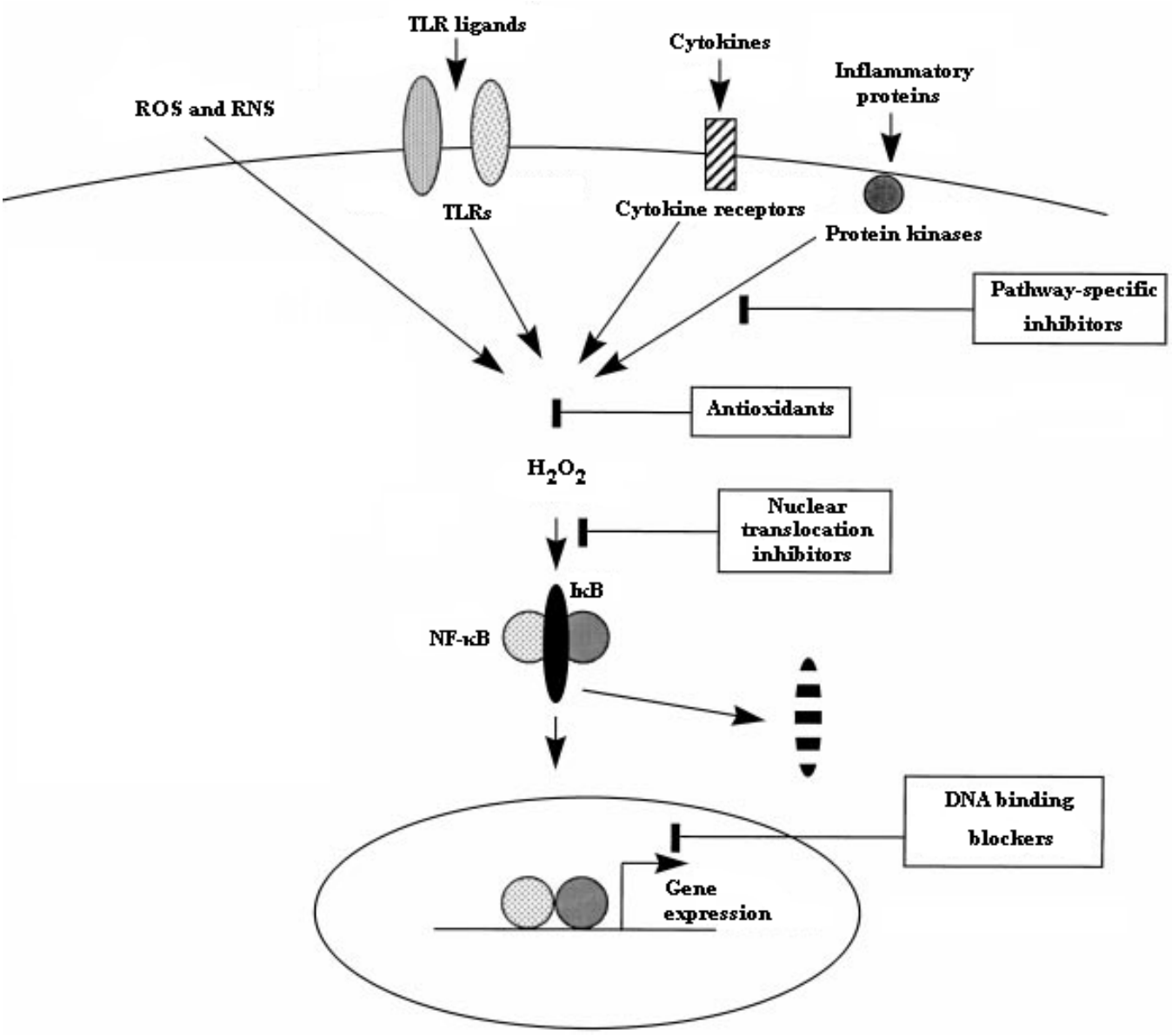

Figure 1.5 – Distinct positions of NF-κB inhibitors in the activation cascade. Diverse stimuli including reactive oxygen species (ROS) and reactive nitrogen species (RNS), Toll-like receptor (TLR) ligands, cytokines and inflammatory proteins initiate the NF-kB cascade. The inhibition of NF-κB mediated gene expression can be controlled by pathway specific inhibitors, antioxidants protecting against H_2O_2 formation, nuclear translocation inhibitors and DNA binding blockers. Adapted from Hehner et al. (1998).

Due to the pleiotropic nature of the inflammatory response and the phytochemical diversity found in medicinal plants, many immunomodulatory botanicals such as *Echinacea* may exhibit both pro- and anti-inflammatory activity. Arabinogalactans found in *Echinacea* water extracts have been shown to have pro- inflammatory activity, while alkylamides found in alcohol extracts of *Echinacea* were shown to be anti-inflammatory (Chicca et al., 2009; Hudson, 2010; Sharma et al., 2006). This is also an example of the phytochemical and pharmacological differences between extraction techniques. Many indigenous communities traditionally prepare their plant medicines by boiling them in water, while common practice in laboratory analysis is to use alcohol or organic solvent extracts.

Maya Immunomodulatory Botanicals

The important link between inflammation and disease can be seen in the proportion of medicinal plants used in the treatment of symptoms strongly related to inflammation compared to the proportion of plants used in the treatment of other symptoms less strongly related to inflammation. For example, Heinrich et al.'s (1998) and Ankli et al.'s (1999) data tell us that of the 320 species used by 2 groups of lowland Maya in the Yucatan peninsula, a large proportion of all mentioned species are associated with uses strongly related to inflammatory conditions: 44% of species recorded are used for gastrointestinal disorders, 43% of species recorded are used for dermatological conditions, and 33% of all species recorded are used for illnesses associated with pain and or fever. With

regards to the total number of use reports, 63% of all use reports were for conditions strongly related to inflammation: 31% of all use reports were for gastrointestinal disorder, 19% of all use reports were for dermatological conditions, and 13% of all use reports were for illnesses associated with pain and or fever. More recently, the ethnobotany of the Q'eqchi' Maya (Treyvaud Amiguet et al., 2005) indicates that four out of the top five reported symptom categories are strongly related to inflammatory conditions: Infections and infestations, digestive system disorders, muscular-skeletal system disorders and skin/subcutaneous cellular tissue disorders. These data collectively indicate that a large proportion of medicinal plant species are used for potential inflammatory conditions and conditions strongly related to inflammation receive the greatest number of use reports. This demonstrates that traditional Maya medicine recognizes an important link between inflammation and disease, even if this link is not explicitly proclaimed by traditional healers.

While a large number of medicinal plant species used by the Maya have been identified as having potential anti-inflammatory properties, relatively little phytochemical or pharmacological investigation of these species has been carried out. There are however several relevant studies which demonstrate that indeed certain Maya medicinal plants do possess important anti-inflammatory properties. In a screening of medicinal plants used by the Maya of the Yucatan, several plant species were found to have noteworthy inhibitory properties of the pro-inflammatory NF-κB pathway in an Electrophoretic Mobility Shift Assay in a HeLa cell line (Ankli et al., 2002). The most potent anti-inflammatory plant tested

was *Crossopetalum gaumeri* (Celastraceae), able to inhibit NF-κB activation at a concentration of 25 mg/mL. However, these anti-inflammatory properties may in fact be due to the toxicity of *C. gaumeri* as the same extract was demonstrated to possess cytotoxic activity against KB cells with a low IC_{50} of 0.7 µg/mL. Additional work on the non-polar fraction of *C. gaumeri* led to the isolation of two compounds: cardenolides-securigenin-3b-0-b-6-deoxyguloside and 19-hydroxy-sarmentogenin-3b-0-b-6-deoxyguloside. Two species of *Diospyros* (Ebenaceae, commonly referred to as the Ebony or Persimmon family) were also tested, *D. anisandra* and *D. cuneata*, and it was revealed that the non-polar fractions of these species possessed important anti-inflammatory activity with relatively lower cyotoxicity values. *D. anisandra* was able to inhibit NF-κB binding at a concentration of 100 µg/mL and *D. cuneata* was inhibitory at 75 µg/mL. While *D. anisandra* demonstrated cytotoxic activity against KB cells with an IC_{50} of 14 µg/mL, and *D. cuneata* was not cytotoxic at the concentrations tested. A literature search did not turn up any subsequent phytochemical or pharmacological investigation of these two species of *Diospyros*, however there has been some more advanced work carried out on *D. leucomelas* which demonstrated anti-inflammatory activity in the carrageenan and serotonin paw oedema tests as well as in the TPA and EPP ear oedema tests (Recio et al., 1995). Bioassay guided fractionation led to the isolation of three triterpenes with anti-inflammatory activity: betulin, betulinic acid, and ursolic acid. These compounds are fairly widespread within the plant kingdom, and it is possible that they also contribute

to the anti-inflammatory activity reported in *D. anisandra* and *D. cuneata*, however further investigation is necessary to confirm this hypothesis.

Another study relevant to a discussion on Maya medicinal plants was carried out by Sosa et al. (2002) in which 7 treatment from 5 Central American species were tested for their anti-inflammatory properties in a Croton-oil induced oedema mouse model: *Aristolochia trilobata* (Aristolochiaceae) leaves and bark, *Bursera simaruba* (Burseraceae) bark, *Hamelia patens* (Rubiaceae) leaves, *Piper amalago* (Piperaceae) leaves and *Syngonium podophyllum* (Araceae) leaves and bark. The author cites these species as being used as folk medicines by the people of Belize and no direct reference is made to the Maya, however these species have been reported to be used by the Maya as topical anti-inflammatory remedies in the treatment of conditions such as rashes, burns and sores by other authors (Avigro and Balick, 1993; Berlin et al., 1974; Dominguez and Alcorn, 1985; Roys, 1976; Treyvaud Amiguet et al., 2005). The data presented in this study demonstrate that indeed most of the treatments tested possess anti-inflammatory activity in the Croton-oil induced oedema mouse model when compared to the non-steroidal anti-inflammatory positive control indomethacin which had an ID_{50} of 93 μg/cm^3. In general, the lipophilic extracts possessed the most anti-inflammatory activity, with the chloroform extract of *A. trilobata* leaves having an ID_{50} of 108 μg/cm^3 and the hexane and chloroform extracts of *B. simaruba* bark possessing an ID_{50} of 221 μg/cm^3 and 143 ug/cm^3. Species belonging to the genus *Aristolochia* were reported to contain aristolochic acids (Hashimoto et al., 1999). These compounds were shown to possess anti-

inflammatory properties as well as inhibitory activity against phospholipase A2, an important enzyme involved in the formation of pro-inflammatory mediators (Vishwanath et al., 1988; Rosenthal et al., 1989). Howerver, aristolochic acids are known to be carcinogenic and nephrotoxic in humans.

Bursera simaruba (red Gumbolimbo) is a tree species of particular interest, as it is one of the most abundant tree species in Belize, Venezuela and other parts of Central America, and is widely used by traditional healers. Tea made from the bark is a commonly used folk remedy for urinary tract infections (Correa and Bernal, 1990) and the Yucatec Maya in Belize use it in the treatment of dermatitis (Arnason et al., 1980). The Huastec Maya use *B. simaruba* for a variety of medicinal and non-medicinal uses purposes including the treatment of burns, headache, nosebleed, fever and stomach-ache as well as an incense and for predicting rain by its flowering (Alcorn, 1984). Another mentioned application for gumbolimbo bark is in the treatment of psoriasis, eczema, insect bites and skin fungus (Gupta, 1995). In addition to the anti-inflammatory properties of *B. simaruba* bark reported by Sosa et al. (2002), hexane extract of leaves has shown to inhibit the adjuvant carrageenan-induced inflammation in rats after oral administration (Abad et al., 1996). Bioassay guided isolation of *B. simaruba* leaf hexane extract led to the isolation of two lignans, methyl-β-peltatin A and methyl-β-peltatin B with methyl-β-peltatin A being a the major active compound in a similar acute phase inflammation induced by carrageenan model (Noguera et al., 2004). The results of the anti-inflammatory effects of these isolated compounds is supported by similar properties reported for other lignans obtained from

traditional botanical therapies which demonstrate that certain lignans can decrease inflammation and promote healthy functioning of the immune system. For example, Clark et al. (1995) demonstrated that Flaxseed lignans are potent inhibitors of platelet-activating factor, an important mediator of inflammation. In addition, lignans obtained from the root of a Korean medicinal plant, *Acanthopanax chiisanensis* (Araliaceae), were demonstrated to possess inhibitory activity against the production of prostaglandin E2 through the direct inhibition of COX activity (Ban et al., 2002).

With regards to the other species discussed in Sosa et al.'s (2002) manuscript, *Hamelia patens, Piper amalago* and *Syngonium podophyllum*, little to no work has be done to further explore their anti-inflammatory properties. There has been some phytochemical research conducted on *H. patens* leading to the isolation of triterpenic and steroidic compounds, flavonoids and alkaloids (Aquino et al., 1990; Chaudhuri and Thakur, 1991), and a variety of piperamides and sesquiterpenes have been isolated from *Piper amalago* (Achenbach et al., 1984; Dominguez et al., 1986; Dyer et al., 2004).

Canadian Immunomodulatory Botanicals

Although the biodiversity of the Canadian flora is limited when compared to tropical biodiversity, several species native to Canada with long histories of traditional use have been extensively investigated for their immunomodulatory properties (Arnason, 1981; Marles, 2001; Tsao and Liu, 2007). One of the most potent anti-inflammatory medicinal species growing in Canada is feverfew

(*Tanacetum parthenium*, Asteraceae). The sesquiterpene lactone parthenolide is the major active constituent of this species, demonstrating potent anti-inflammatory effects *in vitro* and *in vivo* through the alkylating actions of its α-methylene-γ-lactone moiety. Parthenolide works by alkylating a cysteine residue on the activation loop of IκB kinase β, a kinase subunit that plays a critical role in the regulation of cytokine-mediated signalling, and thus preventing the activation of the pro-inflammatory NF-κB pathway (Hehner et al., 1998; Kwok et al., 2001). *Achillea millefolium* is another native Canadian Asteraceae member with well established anti-inflammatory actions attributed to sesquiterpenes it contains (Chandler et al., 1982). In addition, *Heracleum maximum* (Apiaceae) contains anti-inflammatory furanocoumarins used in the treatment of skin conditions such as psoriasis (Neill et al., 2013). Lastly, *Epilobium angustifolium* (Onagraceae) has demonstrated prostaglandin inhibitory effects which have been attributed to the flavonoid glycoside myricetin-3-O-β-*D*-glucoside (Hiermann et al., 2007).

There are also several important native Canadian plants with immunostimulatory properties such as *Echinacea purpurea*, *Panax quinquefolius*, and *Rhodiola rosea*. *Echinacea purpurea* (Asteraceae) has demonstrated pro-inflammatory effects which have been attributed to polysaccharides such as arabinogalactans (Hudson, 2010). Conversely, alklyamides found in *E. purpurea* have also show anti-inflammatory effects (Chicca et al., 2009; Sharma et al., 2006). *Panax quinquefolius* (Araliaceae) is a well studied immunomodulatory species, with a long history of traditional use by Canadian indigenous groups, and now being grown commercially in southern Ontario. Polysaccarides from the

root of *P. quiquefolius* are thought to be the main compounds responsible for the observed immunostimulatory effects of this species (Lui et al., 2012), and several classes of ginsenosides have been identified with several biological activites (Peng et al., 2012). Often described as an adaptogen, *Rhodiola rosea* (Crassulaceae) is a species native to the Canadian artic and is used traditionally for a variety of health promoting effects. *R. rosea* has demonstrated *in vitro* and *in vivo* immunostimulatory effects, and salidroside has been identified as a main active constituent (Lin et al., 2011; Panossian et al., 2010).

1.5 – Objectives

The objectives of this study are guided by the specific goals of each participating community. The first objective of this study was to document anti-inflammatory plant use by the QMHA of Belize, and this ethnobotanical investigation is the focus of Chapter 2. The second objective was to assess the anti-inflammatory activity of medicinal plants used by the QMHA and evaluate if there is a pharmacological basis for plant selection by the QMHA. These objectives are addressed in Chapter 3, which is an ethnopharmacological study of the anti-inflammatory activity of plants used by the QMHA, and Chapter 4, which is a study of the active principles from the QMHA anti-inflammatory medicine, *Neurolaena lobata*.

Regarding the CEI, the first objective of this collaboration was to assess the immunomodulatory activity of plants used in the treatment of Type II diabetes and its associated symptoms, and compare the pharmacological properties of

standard laboratory alcohol extracts to traditionally prepared boiled water extracts of these species. This is the focus of Chapter 5. To fulfill the objective of providing a pharmacological basis for the immunomodulatory activity of CEI medicines, Chapter 6 focuses on the bioassay guided isolation of the active principles from the cones of *Picea mariana*.

Chapter 2

Ethnobotany of Immunomodulatory Treatments Used by the Q'eqchi' Maya of Belize

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Statement of author contribution

BWR and JTA conceived and designed this study. Ethnobotanical interviews and plant collection were conducted by BWR with the assistance of FC who translated interviews and aided in plant collection. Plant species were identified by BWR, MOR and with the assistance of TC for several Araceae species. VC helped coordinate research efforts in Belize. JTA and TP managed and directed the Belize project with the QMHA and contributed to manuscript preparation.

2.1 – Introduction

Maya traditional medicine practiced today has its roots in the classic Maya civilization (Coe, 2002) and various well developed local traditions survive throughout the Mesoamerican area. The Q'eqchi' Maya tradition of Guatemala and Belize is of interest because this culture is one of the more conservative Maya traditions, and because it uses plants from one of the more biodiverse tropical lowland forest regions of the Mesoamerican corridor identified as a hotspot for conservation (Myers et al, 2000).

In a general ethnobotany of the Q'eqchi' Maya Healers Association (QMHA), our research group found that 169 species were being used with a high degree of consensus among healers (Treyvaud Amiguet et al, 2005). Of the 17 usage categories surveyed, infections, digestive system disorders, muscular-skeletal system disorders and skin/subcutaneous cellular tissue disorders were among the top five usage categories, indicating a large number of plant species being used by the members of the QMHA with potential immunomodulatory activity. Since inflammation is now recognized as one of the contributing factors to many debilitating chronic conditions (Haddad et al, 2005), the objective of the present study was to undertake a more detailed examination of Q'eqchi' medicinal plants used for inflammation. Based on ethnobotanical consensus methodology (Heinrich et al, 2009; Moerman, 1991; Trotter and Logan, 1986) emphasizing symptoms readily observed and understood by the healers, the objective of the present study was to identify medicinal plants used in the treatment of inflammatory symptoms. In particular, we identified Q'eqchi' usage

categories related to inflammation, for which the healers identified a large number of medicinal species. These results are analysed by usage categories, Maya concepts of hot and cold plants, healer consensus, taxa used, vegetation and habitat type.

2.2 – Materials and methods

Ethnobotanical interviews

Ethnobotanical interviews were conducted with the Q'eqchi' Maya Healers Association of Belize in collaboration with the Belize Indigenous Training Institute (BITI), a local non-governmental organization that supports the activities of the QMHA. Preliminary interviews were conducted with members of the QMHA to gain insight into the healer's understanding of inflammation and related symptoms recognized and treated with medicinal plants. From these preliminary interviews, a list of 14 Q'eqchi' usage categories were developed with the assistance of Matthew Moher, MD, and reclassified according to the Cook (1995) ethnobotanical standards. Emic (i.e. culture-specific) and etic (i.e. one not based on indigenous concepts) criteria were addressed in developing the list of Q'eqchi' usage categories, as it has been recognized that including both categories of symptoms is important in conducting comprehensive ethnopharmacological field studies (Heinrich, 2009). Open-ended interviews were held with all five members of the QMHA pertaining to plant species used in the treatment of symptoms from these 14 categories. The scope of the interviews included all plant species used by the healers in the treatment of any symptom that fell into one of the 14

Q'eqchi' usage categories, as well as the preparation and dosage used by the healers for each species. This project received ethical approval from the University of Ottawa Research Ethics Board (H 03-07-01) and complies with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans.

Plant material

Plant voucher specimens were collected at three locations in the Toledo district of southern Belize: Jalacte, Punta Gorda, and the Itzamma Ethnobotanical Garden. Jalacte (GPS coordinates: 16º11'18 N, 89º10'54 W) is a small Q'eqchi community of approximately 500 inhabitants in the Maya Mountains along the Guatemala border, at an elevation of approximately 300m. It is surrounded by milpas (indigenous shifting agriculture fields) and is in close proximity to a large area of 1° and 2° broadleaf subtropical moist forest. Classified as lowland broadleaf subtropical moist forest based on "Ecosystem classification" data layers (BBMP, 2006) mapped by Audet (2013), the forests of Jalacte will be termed elevated lowland for the purposes of this study to differentiate them from the much lower elevation forests of the two other collection locations. Punta Gorda (GPS coordinates: 16º05'48 N, 88º49'04 W) is a costal community, and the largest town in the Toledo district, at approximately 10m above sea level. The Q'eqchi' community in Punta Gorda lives on the outskirts of Punta Gorda, known locally as Indianville, which backs on to 2° lowland broadleaf subtropical moist forest. Punta Gorda also hosts a market which sells a small assortment of common medicinal plants that were included in this survey. The Itzamma

Ethnobotanical Garden (GPS coordinates: 16º20'11 N, 88º46'59 W) is a medicinal plant garden managed by the QMHA and BITI in Indian Creek, at approximately 50m above sea level. The garden contains an open yard which grows a host of fruit and medicinal crops, and a large area of managed 2° lowland broadleaf subtropical moist forest where the Q'eqchi' healers cultivate medicinal plants already growing there as well as a wide variety of species transplanted from other areas frequented by the healers (Audet et al, 2013). Collecting and export permits, as well as phytosanitary certificates, were obtained from the Belize Forest Department (Ref. No. CD/60/3/08(33)). Authenticated voucher specimens are deposited at the Juvenal Valerio Rodriguez herbarium (JVR) of the Universidad Nacional de Costa Rica, and the Missouri Botanical Garden herbarium (MO).

Healer consensus

Consensus methodology is used to provide an estimate of the importance of each plant species by quantifying the degree of agreement amongst healers for each specific treatment, as each healer is interviewed independently. The application of these methods to test falsifiable hypotheses concerning human selection and use of plants has been demonstrated by Trotter and Logan (1986), Johns, Kokwaro and Kimanani (1990), Phillips and Gentry (1993) and Johns et al (1994). In this study, the frequency of the mention of a medicinal plant among the healers was used to quantify the degree of confirmation of knowledge of medicinal plants. The frequency of use (F_{use}) (Treyvaud Amiguet et al, 2005) for

each plant was calculated by dividing the number of healers using the plant for any usage category by the total number of healers interviewed (all 5 members of the QMHA). Because the same plant could be used by each healer to treat different symptoms, we also analyzed the consensus in terms of usage categories using the concept of informant consensus originally developed by Trotter and Logan (1986) and readapted by Phillips and Gentry (1993) and more recently Heinrich (2000). This indicates how homogenous the ethnobotanical information is. For each usage category, the data were quantified by adding up the individual reports on the uses of each plant. A taxon may be listed in several of the categories of indigenous uses, however, in terms of use reports, each plant could be considered only once per healer in a single category. This means that if one informant used a plant to treat more than one disease in the same category, we considered it as one use report. We compared then the number of use reports (n_{ur}) to the number of species (n_{taxa}) in each category of use. The informant consensus factor (F_{ic}) was then calculated using the following formula: $F_{IC} = (n_{ur} – n_{taxa}) / (n_{ur} – 1)$. The consensus method helps identify important and interesting species for future cultural and pharmacological research.

Hot-Cold Score

Each healer was asked to rank the plant as hot, cold or neutral which corresponds to their traditional classification system. Hot was scored as +1, cold as -1 and neutral as 0. The sum of each healer's individual hot-cold score was divided by the total number of healers interviewed in this study. These values

were used to develop composite hot-cold score as a culture-specific (emic) criterion.

Regression analysis

Moerman (1991, 1996) developed a method using regression residuals for analyzing ethnobotanical data which identifies plant taxa that are preferentially selected by indigenous healers over what the natural species richness of the taxa would predict. The data recorded during our study were compared to the checklist of vascular plant of Belize (Balick, 2001), the only available flora of Belize, which lists 3426 species belonging to 209 families. The Toledo district of southern Belize, where this study was undertaken, has the greatest species diversity of any district in the country and it contains a large proportion of the species in the checklist. In the present study, the equation which describes the relationship between the number of species in the checklist of the vascular plants of Belize (n_{CL}) and the number of species used medicinally by Q'eqchi' healers (n_{QM}) is: n_{QM} = intercept + slope x log(n_{CL}). At the time, species-area curves were not evaluated, and it is not possible to fully confirm that the collection has reached the asymptote by the method of species-area curve (Cotton, 1996). However, this analysis includes, according to the healers, the majority of Q'eqchi' medicinal species used in the treatment of inflammatory symptoms, and the results are therefore culturally significant and most likely close to the asymptote. The intercept, slope, and residual values were calculated using Prism 5 (GraphPad Software Inc., San Diego, CA, USA), and a p-value less than or equal

to 0.05 was considered significant. Normality was assessed using the Shapiro-Wilk test. Residuals are calculated by subtracting the value predicted by the regression from the observed value, and families or classes and subclasses with positive residuals are taxa used more often than the regression would predict, while those with negative residuals are taxa used less often than predicted.

2.3 – Results

Symptom categories, use reports and medicinal plant species

Preliminary discussions with the members of the Q'eqchi' Maya Healers Association led to the development of 14 usage categories related to immunomodulation which were reclassified into 8 categories according to the Cook (1995) ethnobotanical standards (Table 2.1). The Q'eqchi' usage categories include both emic (i.e. culture-specific) and etic (i.e. one not based on indigenous concepts) criteria, as the inclusion of both was necessary to capture the complete picture of the healer's understanding of inflammation and the relevant plant species used by them. Symptom categories such as fevers, headaches, arthritis and rheumatism, are recognized in a similar fashion by both Q'eqchi' healers and modern medical practitioners as being related to inflammation, and are examples of etic criteria. The emic symptom categories by contrast include indigenous criteria such as "swelling caused by evil spirits" or "swelling caused by subjecting the body to a rapid temperature change" (e.g. jumping into a cool river after getting hot from working). While arthritis and rheumatism, evil spirit swelling, and hot/cold swelling may all share similar

Table 2.1 – Usage categories related to immunomodulation surveyed in ethnobotanical interviews. Q'eqchi' usage categories, Q'eqchi' category names, category description, humoral classification, and Cook (1995) Economic Botany Data Standard classification are presented. Abbreviations for are presented in parentheses.

Q'eqchi' usage category	Q'eqchi' name	Q'eqchi' description	Humoral classification	Cook usage category
Elevated heart rate, breathing rate (**ELE**)	Jump'at i musekak	Fast pulse, fast breathing	Hot	Circulatory System Disorders (**CIR**)
Stomach cramps (**STO**)	Ra muchjej se sa	Cramping in the stomach, intestine and bowels	Cold	Digestive System Disorders (**DIG**)
Ulcers, heartburn (**ULC**)	Ra se a'am	Burning in the stomach	Hot	Digestive System Disorders (**DIG**)
Fever (**FEV**)	Tik	Excess heat and chills	Hot	Infections/Infestations (**INF**)
Arthritis, rheumatism (**ART**)	No specific name	Stiff, swollen, or painful joints caused by manual work and old age	Cold	Muscular-Skeletal System Disorders (**MUS**)
Evil spirit swelling (**EVI**)	Sipok xban maus	Stiff, swollen, or painful joints caused by evil spirits	Hot	Muscular-Skeletal System Disorders (**MUS**)
Hot/cold swelling (**HOT**)	Kwosol	Stiff, swollen, or painful joints caused by fast temperature change	Cold	Muscular-Skeletal System Disorders (**MUS**)
Headache (**HEA**)	Ra se jolom	Aches and pains in the head	Hot	Pain (**PAI**)
Insect bites and stings (**INS**)	Sipok xban kok xul	Insect bites and stings	Hot	Poisonings (**POI**)
Snake bites (**SNA**)	Xku xum kanti	Snake bites	Hot	Poisonings (**POI**)
Allergic rhinitis, hay fever (**ALL**)		Runny nose, sneezing, itchy eyes	Cold	Respiratory System Disorders (**RES**)
Common cold (**COM**)	Ra sa jaj'l	Congestion, coughing, sore throat	Hot	Respiratory System Disorders (**RES**)
Boils (**BOI**)	Saki joj	Boils on skin, caused by infections and impure blood	Hot	Skin/Subcutaneous Cellular Tissue Disorders (**SKI**)
Rash, contact dermatitis (**RAS**)	Ra sa xtyamp l'bak	Redness, bumps, itchiness on skin	Hot	Skin/Subcutaneous Cellular Tissue Disorders (**SKI**)

symptoms, they are recognized by Q'eqchi' healers as having different causes and thus requiring different medicinal plant treatments.

In total, 107 species belonging to 49 families were identified through the ethnobotanical interviews (Table 2.2). The members of the QMHA recognized these 107 species as 110 plants, the increase in number being attributable to the healers distinguishing between three phenotypes of *Piper aequale* Vahl. occurring on limestone cliffs, riverbanks or primary rainforest, and two phenotypes of *Iresine diffusa* Willd., a green phenotype (Biri tak) and a more red phenotype (Kaki biri tak). Interestingly, 5 species collected in the wild do not appear in the checklist of vascular plant of Belize (Balick, 2001): *Anthurium willdenowii* Kunth. (Araceae), *Philodendron radiatum* Schott (Araceae), *Matricaria recutita* L. (Asteraceae), *Miconia gracilis* Triana (Melastomataceae), *Peperomia hirta* C.CD. (Piperaceae). Also of note, two species, *Pectis* sp. (Lamiaceae) and *Ruta graveolens* L. (Rutaceae), were purchased at local markets and two non-wild species, *Cymbopogon citratus* (DC.) Stapf (Poaceae) and *Zingiber officinale* Roscoe (Zingiberaceae), were cultivated by the members of the QMHA at the Itzamma ethnobotanical garden.

Of the 107 species collected, the Q'eqchi' healers used several species interchangeably resulting in 99 unique use profiles which generated a total of 1359 use reports (Figure 2.1). Among the Q'eqchi' usage classifications, headaches, fevers, and stomach cramps generated the most use reports, at 234, 208, and 128 use reports respectively. This represents 97 species used to treat headaches, 87 species used to treat fevers, and 73 species used to treat

stomach cramps. Musculature-skeletal system disorders, pain, and digestive system disorders represented the most use reports in the Cook (1995) classification system, with 309, 234, and 222 use reports respectively, or 75, 97, and 84 species used. The symptom category of allergies generated the fewest use reports, at 6, and the fewest species used, also at 6. This symptom category falls into the Cook (1995) classification of respiratory system disorders, which included the fewest use reports, at 51, and contained the smallest number of species used, at 31. A detailed fact sheet of every species collected which contains the Q'eqchi' use reports for each species can be found in the Handbook of Anti-inflammatory Q'eqchi' Medicinal plants of Belize (Appendix I).

Table 2.2 – Q'eqchi' immunomodulatory medicinal flora. Genus species, Q'eqchi' common name(s), translation of Q'eqchi' name(s), plant part(s) used (B = bark, L = leaf, R = root, S = stem), hot-cold score, frequency of utilization (F_{use}) values, and collection numbers (CN) are presented for 107 species. Medicinal species are grouped by plant family (49 families total). Collection numbers refer to voucher specimens deposited in either the Herbario Juvenal Valerio Rodriguez at the Universidad Nacional in Costa Rica (JVN) or the herbarium of the Missouri Botanical Gardern (MO).

Family	Genus species	Q'eqchi' name(s)	Translation of Q'eqchi' name(s)	Plant part(s) used	Hot-cold score	F_{use}	CN
Acanthaceae	*Aphelandra aurantiaca* (Scheidw.) Lindl.	Jolom chacmut (#1)	Bird's head (Jolom = head; chacmut = a specific bird)	L	-1.00	1	019(JVN)
	Aphelandra scabra (Vahl) Sm.	Jolom chacmut (#2)	See Jolom chacmut (#1)	L	-1.00	1	125(JVR)
	Justicia pectoralis Jacq.	Xucoy'i'kok	Turtle's side (Xucoy = side; i = a; kok = turtle)	L	-1.00	1	032(JVR)
Adiantaceae	*Adiantum latifolium* Lam.	Roq chit cuan (#1)	Black bird's foot (Roq = foot; chit cuan = common black bird)	L	-0.80	1	129(JVR)
	Adiantum petiolatum Desv.	Roq chit cuan (#2)	See Roq chit cuan (#1)	L	-0.80	1	130(JVR)
	Adiantum princeps T. Moore	Roq chit cuan (#3)	See Roq chit cuan (#1)	L	-1.00	1	042(JVR)
	Adiantum tetraphyllum Humb. & Bonpl. ex Willd.	Roq chit cuan (#4)	See Roq chit cuan (#1)	L	-0.80	1	103(JVR)
	Adiantum wilsonii Hook.	Ruj'i'rak'aj'tza	Devil's tongue (Ruj'i'rak = tongue; aj = of; tza = devil)	L	-1.00	1	016(JVR)
Amaranthaceae	*Iresine diffusa* Humb. & Bonpl. ex Willd.	Biri tak;	Go and get a plant that breaks easily (Biri = a plant with nodes that break easily; tak = go and get it);	L	-0.80	1	120(JVR)
		Kaki biri tak	Red Biri tak (Kaki = red)				
Araceae	*Anthurium willdenowii* Kunth	X'chich maus	Devil's sword (X'chich = sword; maus = devil)	L	-0.80	1	054(JVR)

	Monstera acuminata K. Koch	Jol jol	Very loose (Jol = loose)	L	-0.60	1	086(MO)
	Monstera tuberculata Lundell	Letzeb; Sankil pim	A plant that wraps around something; A plant for rotting sores (Sankil = rotting sores; pim = plant)	L	0.50	0.6	070(JVR)
	Philodendron hederaceum (Jacq.) Schott	Kon chi	Bending down like a snake (Kon = bending down; chi = snake-like)	L	0.00	0.8	087(MO)
	Philodendron radiatum Schott	Xilix; Xtonal i uxb	Hand-like; Where the vine roots (Xtonal = base/bottom; i = of; uxb = vine)	L	0.60	0.8	084(JVR)
	Philodendron schottii K. Koch	Kek'ek ux	Very black vine (Kek = black; ek = black; ux = vine)	L	0.25	0.8	099(JVR)
Araliaceae	*Dendropanax arboreus* (L.) Decne. & Planch.	Cojl	A big wooden spoon for stirring pots	L, S	1.00	1	004(JVR)
Aristolochiaceae	*Aristolochia tonduzii* O.C. Schmidt	Sansara kejen; Santa Maria kejen	Medicinal plant that looks like incense burner (Sansara = clay pot for burning incense; kejen = medicinal plant); Saint Mary's medicinal plant (Santa Maria = Saint Mary; kejen = medicinal plant)	L	1.00	1	035(JVR)
Aspleniaceae	*Bolbitis pergamentacea* (Maxon) Ching	Re'quaxiru	For crazy person (Re = for; quaxiru = crazy person)	L	-1.00	0.6	118(JVR)
Asteraceae	*Baccharis trinervis* (Lam.) Pers.	Cherek sak	A specific type of grasshopper (Cherek = large square leg grasshopper; sak = grasshopper	L	-0.60	0.8	100(JVR)
	Matricaria recutita L.	Menseneya (Creole)	Specific name for this plant	L, S	0.25	0.8	107(JVR)

	Mikania leiostachya Benth.	Juruch aj pak	Lizard's back (Jurach = back; aj = of; pak = lizard)	L	-0.60	1	011(JVR)
	Neurolaena lobata (L.) R. Br. ex Cass.	K'a mank	Bitter mango (Ka = bitter; mank = mango)	L	0.00	1	080(JVR)
	Pluchea carolinensis (Jacq.) G. Don	Mai pim (#1)	Pain plant (Mai = pain; pim = plant)	L, S	0.60	1	098(JVR)
	Porophyllum ruderale (Jacq.) Cass.	So'sol pim	Vulture plant (So'sol = vulture; pim = plant)	L	-0.40	0.8	121(JVR)
Begoniaceae	*Begonia glabra* Aubl. var. glabra	Pa'ulul	To make a hole through the brain (Pa = to dig/make a hole through; ulul = brain)	L	-1.00	1	068(JVR)
	Begonia heracleifolia Schltdl. & Cham.	Rutzaj k'opopo'; Xak pek (#1)	Frog cane (Rutzaj = cane plant; k'opopo' = frog); Plant growing on rock (Xak = leaf; pek = rock)	L	-0.40	0.8	133(JVR)
	Begonia nelumbiifolia Schltdl. & Cham.	Xak pek (#2)	See Xak pek (#1)	L	-0.40	1	052(JVR)
	Begonia sericoneura Liebm.	Xak pek (#3)	See Xak pek (#1)	L	-0.40	1	132(JVR)
Bignoniaceae	*Macfadyena unguis-cati* (L.) A.H. Gentry	Rixij tzunun	Hummingbird toenails (Rixij = toenails or fingernails; tzunun = hummingbird)	L, R	-0.67	0.6	071(JVR)
Bromeliaceae	*Pitcairnia punicea* Scheidw.	Kis kim i ha; Mes i ha	Aromatic water palm (Kis = aromatic; kim = palm-like leaf; i = of; ha = water); Water broom (Mes = broom; i = of; ha = water)	L	0.20	0.8	074(JVR)
Burseraceae	*Bursera simaruba* (L.) Sarg.	Kak kajl; Gumbo limbo (Creole)	Peeling Red (Kak = red; kajl = peeling); Specific name for this plant	B	0.50	0.8	077(JVR)

Cactaceae	*Epiphyllum crenatum* (Lindl.) G. Don	Chic'ba'bac (#1)	For joining bones (Chic = to join; ba = of; bac = bone)	L	0.60	1	002-1(JVR)
	Epiphyllum phyllanthus (L.) Haw.	Chic'ba'bac (#2)	See Chic'ba'bac (#1)	L	0.60	1	002-2(JVR)
Celastraceae	*Crossopetalum eucymosum* (Loes. & Pittier) Lundell	Se ruj ajaw chan	Boa constrictor's eye (Se = the; ruj = eye; ajaw chan = boa constrictor)	L	-0.50	0.8	085(JVR)
Chenopodiaceae	*Chenopodium ambrosioides* L.	Isqij'i'pur	Spice for shells (Isqij = aromatic plant for cooking; i = for; pur = shells)	L	0.25	0.8	114(JVR)
Commelinaceae	*Tradescantia spathacea* Sw.	Ton kit	Bloody bottom (Ton = base/bottom; kit = blood)	L	-1.00	0.6	101(JVR)
Convolvulaceae	*Itzaea sericea* (Standl.) Standl. & Steyerm.	Iqbolie pim (#1); Saki iqbolie pim	Snake plant (Iqbolie = a specific type of snake; pim = plant); White iqbolie pim (Saki = white)	L	-1.00	0.8	063(JVR)
Costaceae	*Costus pulverulentus* C. Presl	Kaki chun	Red chun (Kaki = red; chun = this specific plant)	L	-0.60	0.6	078(JVR)
Crassulaceae	*Kalanchoe pinnata* (Lam.) Pers.	No name		L	-1.00	0.6	110(JVR)
Cucurbitaceae	*Gurania makoyana* (Lem.) Cogn.	Kum pim	Pumpkin plant (Kum = pumpkin; pim = plant)	L	-1.00	0.4	051(JVR)
	Momordica charatia L.	Sand'ia cho; Sorosi (Spanish)	Rat's watermelon (Sand'ia wantermelon; cho = rat); Specific name for this plant	L, S	0.60	1	104(JVR)
Davalliaceae	*Nephrolepis biserrata* (Sw.) Schott	Ixqu'oq mo'coch	Cohune palm bending down (Ixqu'oq = bending down; mo'coch = cohune palm)	L	-1.00	1	045(JVR)
Dracaenaceae	*Dracaena americana* Donn. Sm.	Tut	Specific name for this plant	B	0.25	0.6	069(JVR)

Euphorbiaceae	*Croton xalapensis* Kunth	Noq te	Thread tree (Noq = thread; te = tree)	L	0.50	0.8	076(JVR)
Fabaceae	*Acacia cornigera* (L.) Willd.	Subin	Specific name for this plant	L, S	0.25	0.8	072(JVR)
	Desmodium adscendens (Sw.) DC.	Chint pim (#1)	Chint plant (Chint = this specific plant; pim = plant)	L, B	0.20	0.8	093(JVR)
	Desmodium axillare (Sw.) DC. var. *acutifolium* (Kuntze) Urb.	Chint pim (#2)	See Chint pim (#1)	L	0.20	0.8	013(JVR)
	Cojoba graciliflora (S. F. Blake) Britton & Rose	Choql ok te	Cloud bean-pod fruiting tree (Choql = cloud; ok = bean-pod fruit of this tree; te = tree)	L, S	0.67	0.4	003(JVR)
	Mimosa pudica L.	Quare kix	Sleepy kix (Quare = sleepy; kix = this specfic plant)	L, S	-0.60	0.8	127(JVR)
Gesneriaceae	*Besleria laxiflora* Benth.	Jolom masan	Shrimp's head (Jolom = head; masan = shrimp)	L	-1.00	0.6	048(JVR)
	Codonanthe uleana Fritsch	Cacao pim	Cacao plant (Cacao = *Theobroma cacao*; pim = plant)	L	-1.00	0.6	047(JVR)
	Drymonia serrulata (Jacq.) Mart.	Baknel pim	Snake plant (Baknel = a specific type of snake; pim = plant)	L, S	-1.00	1	043(JVR)
Haemodoraceae	*Xiphidium caeruleum* Aubl.	Ixcua'i'kuch	Hawk's food (Ixcua = food; i = of; kuch = hawk)	L, S	-0.60	1	010(JVR)
Lamiaceae	*Cornutia grandifolia* (Schltdl. & Cham.) Schauer	Roq xa'an	Old lady's foot (Roq = foot; xa'an = old lady)	L	-0.20	1	015(JVR)
	Cornutia pyramidata L.	Hob'lob'te	Hollow tree (Hob'lob = hollow; te = tree)	L	0.50	0.8	008(JVR)
	Hyptis capitata Jacq.	Se ruj kaway	Horse's eye (Se = the; ruj = eye; kaway = horse)	L	1.00	1	064(JVR)
	Hyptis verticillata Jacq.	Chu pim	Bad smelling plant (Chu = bad smelling; pim = plant)	L	0.60	1	116(JVR)
	Pectis sp.	Pericón (Spanish)	Specific name for this plant	L, S	0.50	0.4	106(JVR)

Loranthaceae	*Phthirusa pyrifolia* (Kunth) Eichler	Neba pim	Orphan plant (Neba = orphan; pim = plant)	L	-0.50	0.8	113(JVR)
Lygodiaceae	*Lygodium heterodoxum* Kunze	Ruxb'i'kaak (#1)	Thunder vine (Ruxb = vine; i = of; kaak = thunder)	L	-1.00	1	026(JVR)
	Lygodium venustum Sw.	Ruxb'i'kaak (#2)	See Ruxb'i'kaak (#1)	L	-1.00	1	049(JVR)
Malvaceae	*Sida rhombifolia* L.	Mes b'eel	Broom for sweeping (Mes = broom; b'eel = for sweeping)	L	-0.60	1	057(JVR)
Marcgraviaceae	*Marcgravia gentlei* Lundell	Rubelsa'i'xul	Snake's belly (Rubelsa = belly; i =of; xul = a specific snake)	L	-1.00	1	050(JVR)
	Souroubea sympetala Gilg	Hub'ub	Specific name for this plant	L	-0.75	0.8	028(JVR)
Melastomataceae	*Arthrostemma ciliatum* Pav. ex D. Don	Selek sak	Grasshopper's leg (Selek = leg; sak = grasshopper)	L	-0.20	0.4	131(JVR)
	Blakea cuneata Standl.	Oxlaho chajom	Thirteen year old teenage boy (Oxlaho = thirteen; chajom = teenage boy)	L	-0.33	0.6	119(JVR)
	Miconia gracilis Triana	Roq muqui	Ground dove's foot (Roq = foot; muqui = ground dove)	L	-0.60	0.8	111(JVR)
Menispermaceae	*Abuta panamensis* (Standl.) Krukoff & Barneby	Raxi chajom kajam	Green teenage boy vine (Raxi = green or blue; chajom = teenage boy; kajam = vine)	L, B	1.00	0.6	139(JVR)
	Disciphania calocarpa Standl.	Roq maus; Xa'ab maus	Devil's foot (Roq = foot; maus = devil); Devil's shoe (Xa'ab = shoe; maus = devil)	L, S	-0.60	1	014(JVR)
Monimiaceae	*Mollinedia guatemalensis* Perkins	Saki kejen	White medicinal plant (Sake = white; kejen = medicinal plant)	L	-0.20	1	027(JVR)
Moraceae	*Dorstenia lindeniana* Bureau	Chacbolie kejen	Tommygoff medicinal plant (Chacbolie = yellow-jaw tommygoff snake; kejen = medicinal plant)	L	-1.00	1	055(JVR)

Ochidaceae	*Oeceoclades maculata* (Lindl.) Lindl.	Iqbolie pim (#2); Kurarin re kitche	See Iqbolie pim (#1); Kurarin re kitche = this specific plant	L	-1.00	1	128(JVR)
Passifloraceae	*Passiflora oerstedii* Mast. var. *choconiana* (S. Watson) Killip	Tu kej kejen	Deer breast medicinal plant (Tu = breast; kej = deer; kejen = medicinal plant)	L	0.60	1	067(JVR)
Piperaceae	*Peperomia hirta* C. DC.	Ixcua'i'xul (#1)	Snake's food (Ixcua = food; i =of; xul = a specific snake)	L, R, S	-1.00	1	040(JVR)
	Peperomia macrostachya (Vahl) A. Dietr.	Mai pim (#2)	See Mai pim (#1)	L, R, S	-1.00	0.8	059(JVR)
	Peperomia obtusifolia (L.) A. Dietr.	Ixcua ajaw chan	Boa constrictor's food (Ixcua = food; ajaw chan = boa constrictor)	L	-1.00	0.8	061(JVR)
	Peperomia urocarpa Fisch. & C.A. Mey.	Ixcua'i'xul (#2)	See Ixcua'i'xul (#1)	L, R, S	-1.00	1	039(JVR)
	Piper aequale Vahl	Kan pom; Pu'chuch remuch kej; Pu'chuch re'tzu'ul	Yellow incense (Kan = yellow; pom = incense); Piper plant for cramps (Pu'chuch = piper plant; re= for; much kej = cramps); Piper plant for mountain (Pu'chuch = piper plant; re= for; tzu'ul = mountain)	L, S	0.40	1	012(JVR)
	Piper amalago L.	Tziritok	Small and fragile	L	1.00	0.8	022(JVR)
	Piper arboreum Aubl.	Tyut'it pu'chuch (#1)	Tied node piper (Tyut = tied; it = node; pu'chuch = piper plant)	L, R, S	1.00	0.6	066-1(JVR)
	Piper auritum Kunth	Ubel	Specific name for this plant	L	1.00	1	060(JVR)
	Piper glabrescens (Miq.) C. DC.	Pu'chuch rekanil	Piper plant for fear (Pu'chuch = piper plant; re= for; kanil = fear)	L, S	0.60	1	138(JVR)

	Piper hispidum Sw.	Rax pu'chuch	Green piper (Rax = Green or blue; pu'chuch = piper plant)	L, S	1.00	1	115(JVR)
	Piper peltatum L.	Saki tyut it; Tyut it	White tied node (Saki = white; tyut = tied; it = node); Tied node (Tyut = tied; it = node)	L, R	-0.40	0.6	081(JVR)
	Piper sanctum (Miq.) Schltdl. ex C. DC.	Tyut'it pu'chuch (#2)	See Tyut'it pu'chuch (#1)	L, S	1.00	0.6	066-2(JVR)
	Piper tuerckheimii C. DC. ex Donn. Sm.	Cux sawi	Specific name for this plant	L	0.20	1	006(JVR)
	Piper yucatanense C. DC.	Che pu'chuch; Pu'chuch rebakel	Piper tree (Pu'chuch = piper plant; che = tree); Piper plant for bones (Pu'chuch = piper plant; re= for; bakel = bones)	L, S	1.00	0.8	088(JVR)
Poaceae	*Cymbopogon citratus* (DC.) Stapf	Kis kim	Aromatic palm (Kis = aromatic; kim = palm-like leaf)	L	0.00	1	126(JVR)
Rhamnaceae	*Gouania polygama* (Jacq.) Urban	Kek xeb	Black wax (Kek = black; xeb = wax)	L	-1.00	1	112(JVR)
Rubiaceae	*Gonzalagunia panamensis* (Cav.) K.Schum.	Tzu'ul che	Mountain tree (Tzu'ul = mountain; che = tree)	L, S	-0.20	1	034(JVR)
	Hamelia patens Jacq.	Chaj max; Jolom'i'posp	Pine tree spider monkey (Chaj = pine tree; max = spider monkey); Matchstick head (Jolom = head, i = of, posp = matchstick)	L	-0.40	1	075(JVR)
	Hoffmannia ghiesbreghtii (Lem.) Hemsl.	Mai pim (#3); Kaki mai pim	See Mai pim (#1); Red mai pim (Kaki = red)	L	-0.60	1	090(JVR)
	Psychotria pleuropoda Donn. Sm.	Kolaras	Beaded Maya necklace	L	-0.33	0.6	097(JVR)
Rutaceae	*Ruta graveolens* L.	Ruda (Spanish)	Rue	L	-1.00	0.4	105(JVR)

Sapindaceae	*Paullinia costata* Schltdl. & Cham.	Korona kix	Crown of thorns (Korona = crown; kix = thorns)	L	0.20	1	030(JVR)
	Serjania mexicana (L.) Willd.	Bolon Yok	Specific name for this plant	L	1.00	0.8	134(JVR)
Selaginellaceae	*Selaginella* sp.	Xquq'i'pek	Hangs off of rock (Xquq = hangs off; i = of; pek = rock)	L	-1.00	1	109(JVR)
	Selaginella umbrosa Lem. ex Hieron	Choql pim	Cloud plant (Choql = cloud; pim = plant)	L	-1.00	1	053(JVR)
Smilacaceae	*Smilax* sp.	Ruchire ak; Sarsafaria (Spanish)	Peccary teeth (Ruchire = teeth; ak = peccary); Specific name for this plant	L	0.00	0.4	046(JVR)
Solanaceae	*Cestrum megalophyllum* Dunal	Ik che	Pepper tree (Ik = pepper; che = tree)	L	-0.40	1	009(JVR)
	Solanum nudum Dunal	Na'i'pajl	Mother of pajl (Na = mother, i = of; pajl = a specific plant)	L	0.40	0.8	089(JVR)
	Solanum torvum Sw.	Pajl	Specific name for this plant	L	0.00	0.8	065(JVR)
Verbenaceae	*Lantana trifolia* L.	Tulush	Dragonfly (Tulush = dragonfly)	L	0.60	1	020(JVR)
	Stachytarpheta frantzii Pol.	Xtye aj pak	Lizard's tail (Xtye = tail; aj = of; pak = lizard)	L, S	-1.00	0.8	056(JVR)
Vitaceae	*Cissus microcarpa* Vahl	Roq ab	Specific name for this plant	L, S	-0.60	1	117(JVR)
Zingiberaceae	*Renealmia aromatica* (Aubl.) Griseb.	Cux tzi	Specific name for this plant	L	0.80	1	038(JVR)
	Zingiber officinale Roscoe	Xan xir	Specific name for this plant	R	1.00	0.8	025(JVR)

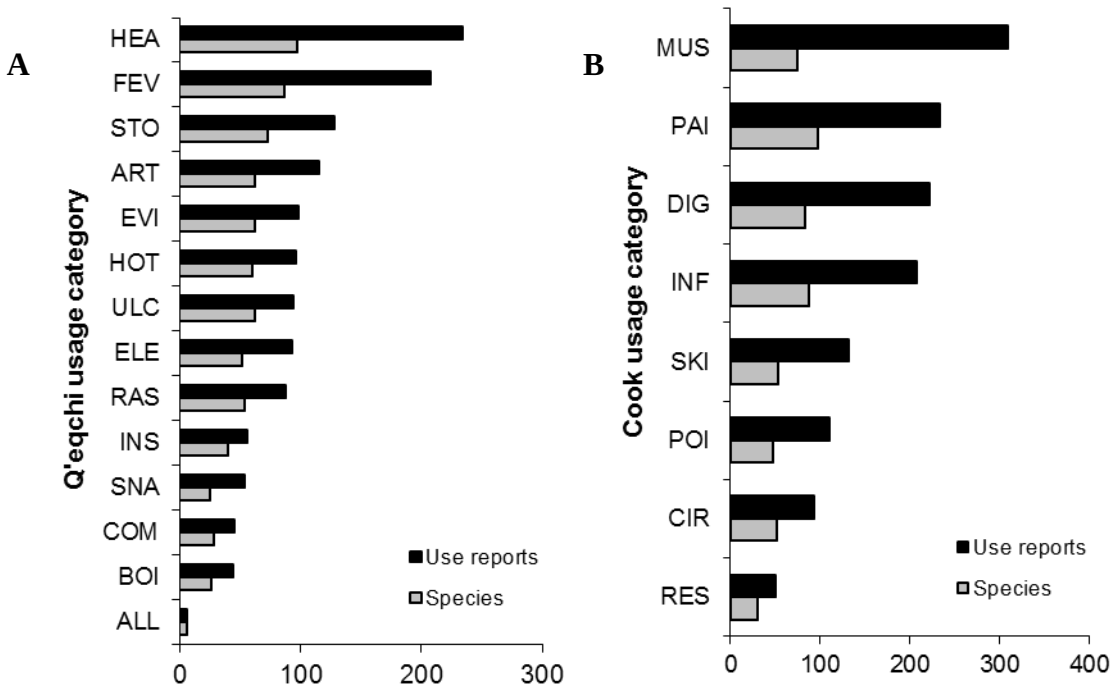

Figure 2.1 – Distribution of use reports and medicinal plant species to Q'eqchi' usage categories (A) and Cook (1995) usage categories (B). Total use reports = 1359, total species = 107. Plant species may appear in more than one category.

Hot-cold classification

The symptom categories recognized by the members of the QMHA as well as the plants used to treat these symptoms are also classified as being with hot, cold, or neutral (Table 1.1, Table 1.2). Of the 14 symptom categories recognized by the healers, 10 are classified as being hot symptoms and 4 are classified as being cold symptoms, indicating inflammation is traditionally classified as a hot condition for the most part. Of the 107 plants used to treat inflammation, 62 species (58%) are classified as cold plants (negative hot-cold score), 40 species (37%) are classified as hot plants (positive hot-cold score), and 5 species (5%) are classified as neutral plants (hot-cold score of 0). As hot symptoms, such as

many of those related to inflammation, are preferentially treated with cold plants, it is not surprising that a majority of plants used by the QMHA in the treatment of inflammatory-related symptoms are classified as cold plants.

Plant families and regression analysis

Of the 49 plant families collected, 13 had three or more species used by the members of the QMHA (Figure 2.2). Piperaceae contained the most species used by the healers in the treatment of immunomodulatory symptoms, who identified 14 species belonging to this family. Six species were used in both

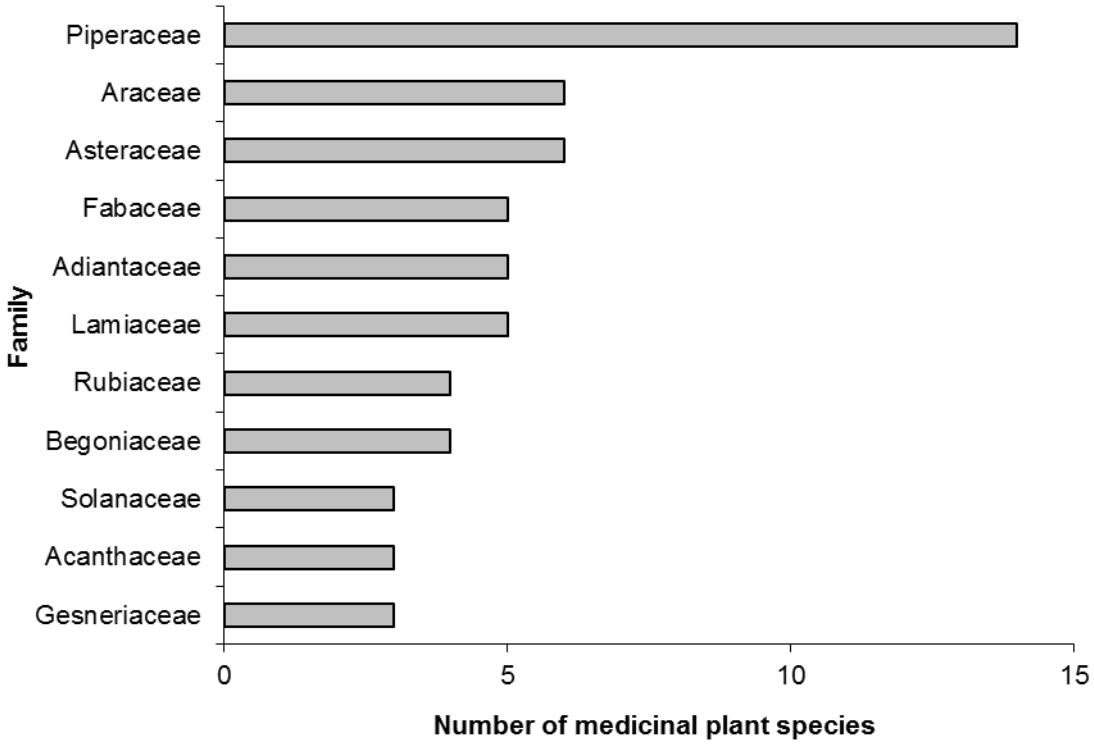

Figure 2.2 – Most frequently used plant families among the 107 collected immunomodulatory plants. Thirteen plant families are presented representing 63 species (number of medicinal plant species used in the family ≥ 3).

Araceae and Asteraceae, and 5 species were indentified as belonging to Adiantaceae and Fabaceae. Many plant families (28) were represented by only one species.

A regression analysis of the families in the Q'eqchi' medicinal flora, which regresses the number of species in each family used medicinally by the QMHA to the total number of species in each family found in Belize, identifies the plant families which are preferentially selected by the healers (Figure 2.3). The standard deviation (SD) of the residuals is 2.1, and there are 5 families with residuals greater than one SD. The Piperaceae is the most preferentially selected

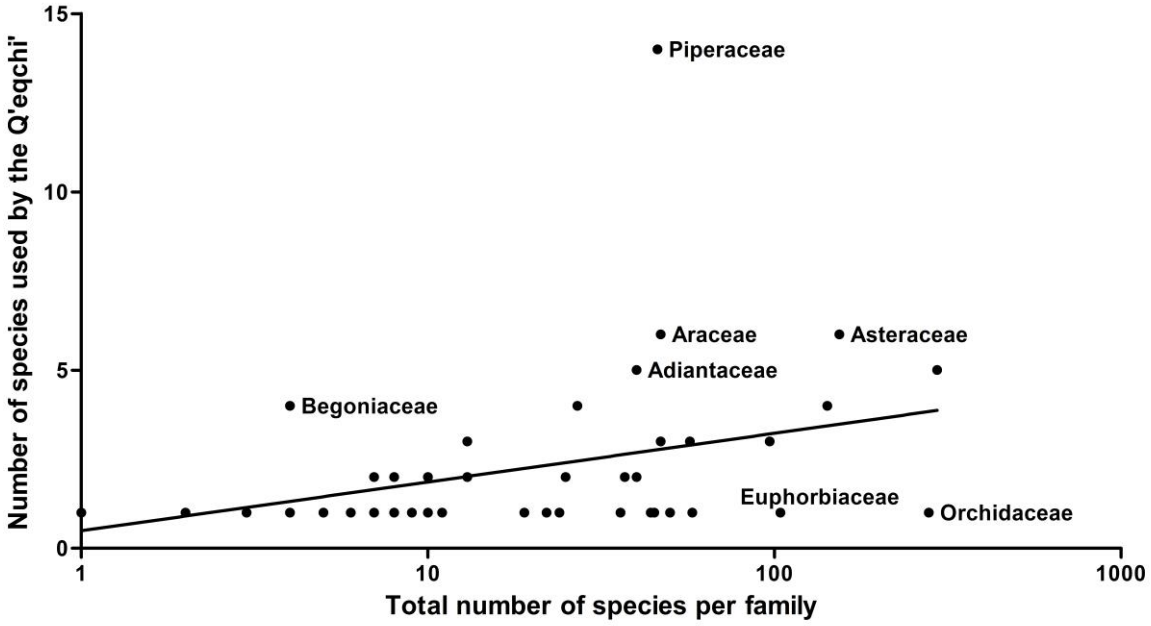

Figure 2.3 – Regression plotting the number of Q'eqchi' immunomodulatory plant species per family versus the total number of species per family in the checklist of vascular plant of Belize (Balick, 2001). Plant families not used by the Q'eqchi' were excluded, plant species collected from commercial or cultivated sources exclusively were excluded, and new species not in the checklist were added to family totals. A semilog line regression is plotted (n_{QM} = 0.47 + 1.39 x log(n_{CL}), df = 45, $p \leq 0.05$, r^2=0.14). Plant families with absolute residual values greater than one standard deviation (SD = 2.1) are labeled.

plant family, with a residual value of 11.2. The Araceae, Begoniaceae, Asteraceae, and Adiantaceae had the next highest residual values, at 3.2, 2.7, 2.5, and 2.3, respectively. The Orchidaceae and Euphorbiaceae had the lowest residual values, at -2.9 and -2.3, respectively. A table of all the residual values extracted from the regression for each plant family can be found in Appendix IIa.

A regression analysis of the classes and subclasses in the Q'eqchi' medicinal flora, which regresses the number of species in each class and subclass used medicinally by the QMHA to the total number of species in each class and subclass found in Belize, identifies the plant class and subclasses which are preferentially selected by the healers (Figure 2.4). The standard

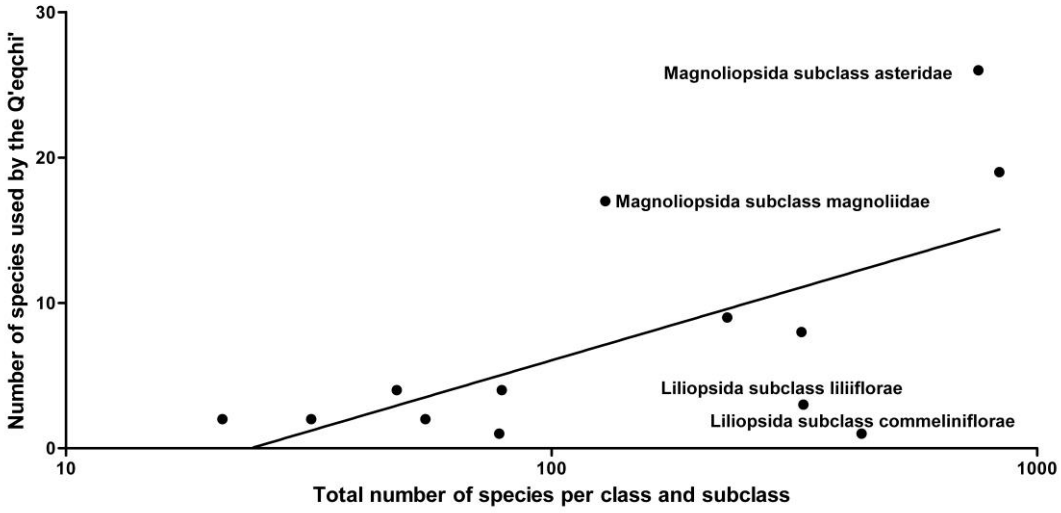

Figure 2.4 – Regression plotting the number of Q'eqchi' immunomodulatory plant species per class and subclass versus the total number of species per class and subclass in the checklist of vascular plant of Belize (Balick, 2001). Classes and subclasses not used by the Q'eqchi' were excluded, plant species collected from commercial or cultivated sources exclusively were excluded, and new species not in the checklist were added to family totals. A semilog line regression is plotted (n_{QM} = -13.5 + 9.8 x log(n_{CL}), df = 11, $p \leq 0.05$, r^2=0.40). Classes and subclasses with absolute residual values greater than one standard deviation (SD = 6.3) are labelled.

deviation (SD) of the residuals is 6.3, and there are 2 classes and subclasses with residuals greater than one SD. The Magnoliopsida subclass Asteridae is the most preferentially selected class and subclass, with a residual value of 11.4, followed by the Magnoliopsida subclass Magnoliidae with a residual value of 9.9. The Liliopsida subclass Commeliniflorae and the Liliopsida subclass Liliiflorae had the lowest residual values, at -11.3 and -8.1, respectively. A table of all the residual values extracted from the regression for each class and subclass can be found in Appendix IIb.

Vegetation types and habitat

The 107 species were of six different vegetation types, with herbaceous and shrub growth forms being the most widely used with 38 and 32 species respectively (Figure 2.5). Vine and liana vegetation types included 11 species each, and the remaining 15 species were either ferns or trees. In total, 10 species were epiphytic, 8 of which were herbs. The majority of plants species, 83 in total, were collected from broadleaf subtropical moist forest, 36 coming from 2° lowland broadleaf subtropical moist forest, 24 from 1° elevated lowland broadleaf subtropical moist forest, and 23 from 2° elevated lowland broadleaf subtropical moist forest (Figure 2.6). Sixteen species were collected from managed open yards, 6 from milpas, and 2 from the market in Punta Gorda. In total, 5 species were collected from riverbanks exclusively. The Q'eqchi' Maya immunomodulatory pharmacopoeia contains species from a wide variety of environments, however the vast majority, 78%, are collected from broadleaf

subtropical moist forest, indicating that the immunomodulatory ethnobotany of the Q'eqchi' Maya is predominately a rainforest ethnobotany.

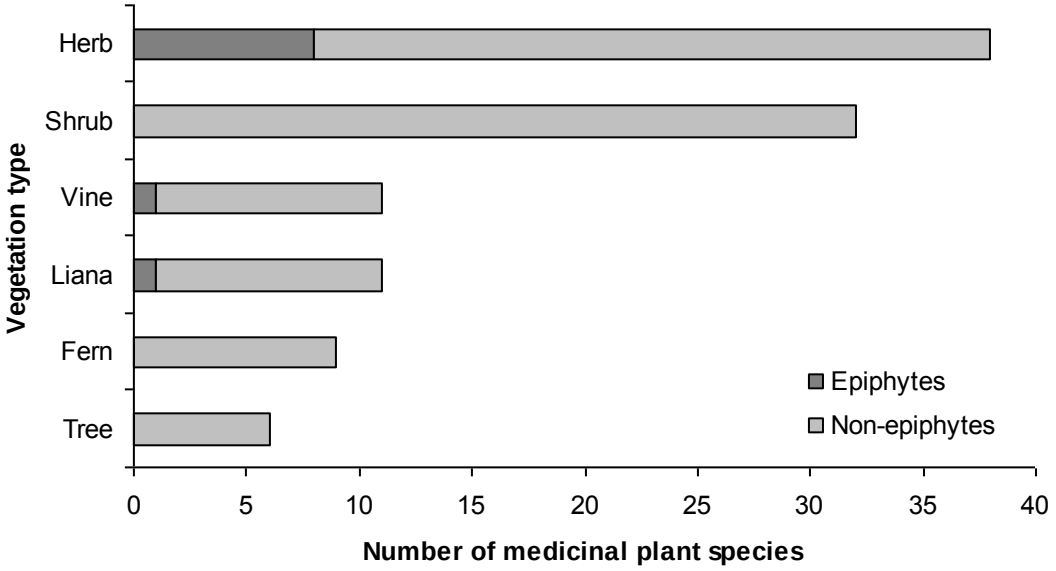

Figure 2.5 – Classification of the 107 immunomodulatory plant species in terms of vegetation type.

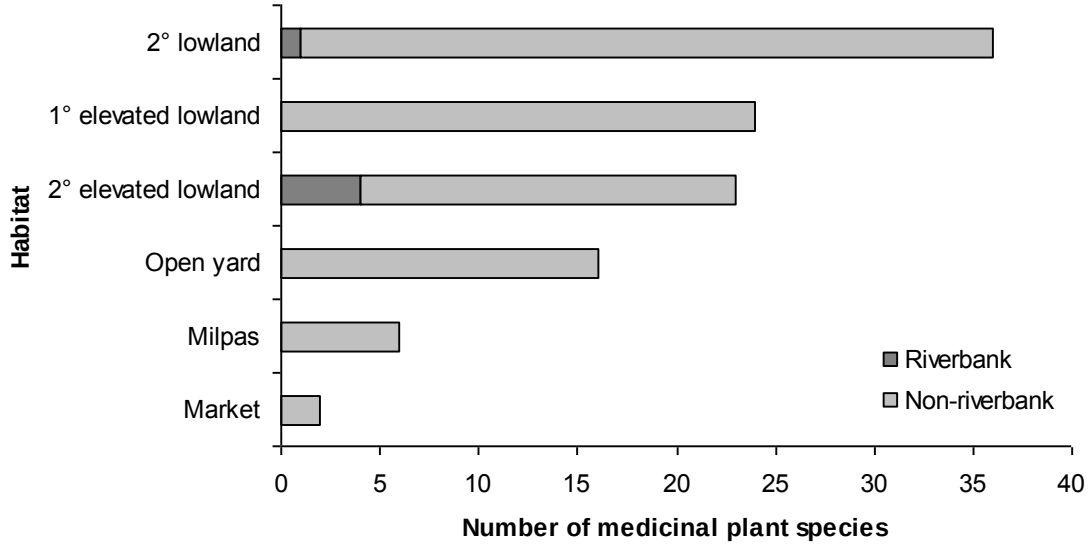

Figure 2.6 – Classification of the 107 immunomodulatory plant species in terms of habitats.

Healer consensus

Frequency of use data for species (F_{use}) showed that 56 species (52% of the total) were used by all healers (Figure 2.7). The F_{use} value was 0.8 for 30 species, 0.6 for 15 species, and 0.4 for 6 species. When considering symptom categories (Table 2.3), the Trotter and Logan informant consensus factor (F_{ic}) was greater than 0.5 for the top three categories of headache, fever, and snake bites. When the Cook classification was used, the F_{ic} values were even higher (all > 0.4) and > 0.6 for the top three categories (Table 2.4). As shown in Table 2.3, the number of taxa and use reports for symptom categories used to calculate the F_{ic} was substantial (N_{taxa} > 25, N_{ur} > 40) with the exception of allergies. Overall, these results show a remarkable consensus on both useful plants and symptom category treatments, especially when we note that the healers were interviewed separately and reported being trained by different individuals in different villages.

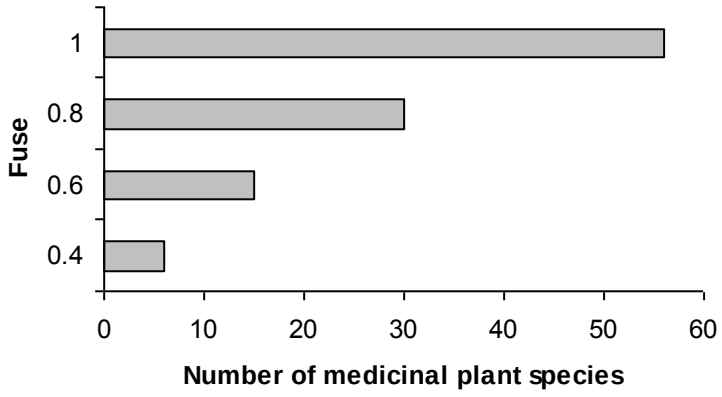

Figure 2.7 – Distribution of the frequency of utilization (F_{use}) of the 107 immunomodulatory plants species among the five healers.

Table 2.3 – Informant consensus factors (F_{ic}) for each Q'eqchi' usage category related to immunomodulation with details of the number of taxa (N_{taxa}) and the number of use report (N_{ur}). A taxon may be listed in several usage categories.

Q'eqchi usage category	N_{taxa}	N_{ur}	F_{ic}
Headache (HEA)	97	234	0.59
Fever (FEV)	87	208	0.58
Snake bites (SNA)	25	54	0.55
Arthritis, rheumatism (ART)	62	115	0.46
Elevated heart rate, breathing rate (ELE)	52	93	0.45
Stomach cramps (STO)	73	128	0.43
Boils (BOI)	26	44	0.42
Rash, contact dermatitis (RAS)	54	88	0.39
Common cold (COM)	28	45	0.39
Hot/cold swelling (HOT)	60	96	0.38
Evil spirit swelling (EVI)	62	98	0.37
Ulcers, heartburn (ULC)	62	94	0.34
Insect bites and stings (INS)	40	56	0.29
Allergic rhinitis, hay fever (ALL)	6	6	0.00
	107	1359	

Table 2.4 – Informant consensus factors (F_{ic}) for each Cook (1995) usage category with details of the number of taxa (N_{taxa}) and the number of use report (N_{ur}). A taxon may be listed in several of usage categories.

Cook usage category	N_{taxa}	N_{ur}	F_{ic}
Muscular-Skeletal System Disorders (MUS)	75	309	0.76
Digestive System Disorders (DIG)	84	222	0.62
Skin/Subcutaneous Cellular Tissue Disorders (SKI)	54	132	0.60
Pain (PAI)	97	234	0.59
Infections/Infestations (INF)	87	208	0.58
Poisonings (POI)	47	110	0.58
Circulatory System Disorders (CIR)	52	93	0.45
Respiratory System Disorders (RES)	31	51	0.40
	107	1359	

2.4 – Discussion

A major finding of this study is that treatment of inflammation is an important focus in Q'eqchi' Maya medicine. In particular, 14 Q'eqchi' usage categories were found and over 100 species used for these symptoms were identified. The most common treatment categories are headaches, fevers and stomach cramps in both use reports and number of taxa used. The most preferred family is Piperaceae, in terms of both number of species used and Moerman regression residual values, while 5 families have 5 or more species represented. Plants from rainforest are clearly preferred and reflect the healer's belief that pristine forests provide more potent medicines. Finally, there is strong consensus between healers for plant species used and within usage categories.

Comparing this to other Mesoamerican Maya ethnobotanical studies, it was found that a large proportion of medicinal plant treatments are classified in usage categories associated with inflammation. Among the Yucatec Maya in southern Mexico, the usage categories of gastrointestinal system disorders, dermatological conditions, and illnesses associated with pain or fever represented 35%, 19%, and 13%, respectively, of the plant species used by Yucatec healers (Ankli et al, 1999). In an ethnobotanical survey of Popoluca and Mixe Maya healers in Mexico, plants used to treat dermatological conditions, gastrointestinal conditions and illnesses associated with pain or fever represented a large proportion of the total medicinal flora used by both of these groups (Leonti et al, 2003). In the case of the Popoluca healers, dermatological conditions, gastrointestinal conditions and illnesses associated with pain or fever

made up 21.7%, 18.5% and 8.5% of the total uses of medicinal flora, respectively. Amongst the Mixe healers, dermatological conditions, gastrointestinal conditions and illnesses associated with pain or fever made up 20%, 20.6% and 16.5% of the total medicinal flora, respectively. A consensus analysis carried out by Heinrich et al (1998) revealed that gastrointestinal system disorders and dermatological conditions had F_{ic} values of 0.71 and 0.52 respectively, indicating a strong degree of consensus for treatments within these usage categories by Yucatec healers in Mexico, and comparable to the F_{ic} values reported in this study. Taken together, these results indicate that various traditional Maya healers in Mexico, as seen in the Q'eqchi Maya of Belize, posses a large proportion of medicinal plant treatments with potential immunomodulatory activity and that the degree of consensus within these groups is high for these usage categories.

In meetings with the QMHA, the healers expressed their sincere desire to see Maya medicine recognized nationally and internationally as an ethical and effective practice. The next step towards that goal is to study the pharmacological properties of the plants and identify the active principles (which is considered in chapters 3 and 4 of this thesis). Ultimately selected plants may be standardized and tested in animal and clinical studies to establish evidence based natural health products.

Chapter 3

Ethnopharmacology of immunomodulatory treatments used by the Q'eqchi' Maya of Belize

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Statement of author contribution

BWR and JTA conceived and designed this study. Plant collection was conducted by BWR with the assistance of FC. Plant species were identified by BWR and MOR. BWR developed the immunomodulatory assays and plant extractions and assays were carried out by BWR with the assistance of ML. VC helped coordinate research efforts in Belize. JTA and TP managed and directed the Belize project with the QMHA and contributed to manuscript.

3.1 – Introduction

Immunomodulatory medicines from major traditional medicine systems like Chinese traditional medicine, Ayurvedic medicine, and European herbal traditions are being intensively studied for their pharmacological action and active principles, leading to evidence based medicines registered as Natural Health Products (NHPs) and phytomedicines, as well as providing leads in the drug development pipeline (Heinrich, 2000). For example, triptolide from *Tripterygium wilfordii* (Celastraceae), the thunder god vine (*lei gong teng*), and curcumin from turmeric (*Curcuma longa*, Zingiberaceae*)*, are promising arthritis treatments (Corson and Crews, 2007), while the aptly named feverfew (*Tanacetum parthenium*, Asteraceae) is a Canadian NHP and European phytomedicine approved as a migraine prophylactic and containing the anti-inflammatory parthenolide (Awang et al, 1991).

Indigenous traditional medicines, including those from the Mesoamerican area, are less well studied for these activities, yet the World Health Organization (1993) estimates that 80% of the populations in developing countries rely on them for primary health care. Our lab has had a continuing collaboration with Q'eqchi' Maya healers of Belize in Central America, who are the main health providers in 30 indigenous villages in the southern Toledo District while modern health care is provided mainly at one regional hospital. In focus group workshops on local health care issues, the healers have clearly stated their desire to have their medicines documented and evaluated for efficacy and safety. They view this

as a step towards formal recognition of Q'eqchi' traditional medicine as an ethical complementary primary health care in remote villages.

In our previous ethnobotanical study of immunomodulatory Q'eqchi medicines (Chapter 2) we identified 14 locally recognized usage categories related to inflammation, that are recognized by healers, which belonged to 8 usage categories as classified by Cook (1995) ethnobotanical standards. A large number of plants (107 species) from 49 families collected predominantly from primary and secondary lowland broadleaf subtropical moist forest were used by the healers to treat symptoms within these usage categories. Plants were classified as hot or cold according to the Q'eqchi' traditional knowledge system, as seen in several other Latin American traditional healing systems such as the Quiché Maya in Guatemala, the Criollos of Argentina, and the Yucatec Maya and Popoluca of Mexico (Ankli et al, 1999; Leonti et al, 2003; Scarpa, 2004; Tedlock, 1987). While some of these plants may have a ritual or spiritual role in healing, our hypothesis was that there is a pharmacological basis to the use of some of these plants. In the present study, we used a THP-1 monocyte bioassay to assess the immunomodulatory activity of plant extracts. While not the only inflammatory target, it is relevant to many conditions and has been widely used as a pilot assay for anti-inflammatory plants. We predicted that plants with greater frequency of ethnobotanical use in some usage categories would have greater anti-inflammatory activity than those with lower frequency of use, and that the variation in activity could be explained by a linear regression. We also predicted that plants classified as cold would be more active than hot plants.

3.2 – Materials and Methods

Ethnobotanical interviews and plant material

Ethnobotanical interviews are described in detail in Chapter 2. Briefly, interviews were conducted with the Q'eqchi' Maya Healers Association (QMHA) pertaining to a variety of inflammatory symptoms and the plants used to treat them. This project received ethical approval from the University of Ottawa Research Ethics Board (H 03-07-01) and complies with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. Plant collecting and export permits were obtained from the Belize Forest Department (Ref. No. CD/60/3/08(33)) and authenticated voucher specimens deposited at the Juvenal Valerio Rodriguez herbarium of the Universidad Nacional de Costa Rica (JVR) and the Missouri Botanical Garden herbarium (MO). Fresh plant material was collected on field excursions with members of the QMHA and immediately preserved in alcohol. Wet plant material was shredded using a blender and extracted twice with EtOH (80% in H2O) at a ratio of 1 g plant material to 10 mL 80% EtOH over 24h at room temperature. The combined extracts were evaporated in vacuo, lyphophillized, and homogenized using a mortar and pestle.

Anti-inflammatory assays

The anti-inflammatory activity of plant extract was assessed by measuring TNF-α reduction in a lipopolysaccharide (LPS) stimulated THP-1 monocyte assay (Zhao et al, 2005). THP-1 cells (human monocyte culture TIB-202, ATCC, Manassas, VA, USA), were cultured in RPMI 1640 media (ATCC, Manassas, VA,

USA) supplemented with 1% 0.05 mM beta-mercaptoethanol, 1% penstrep (Invitrogen, Mississauga, ON, Canada) and 10% fetal bovine serum (Invitrogen, Mississauga, ON, Canada), in a 37°C humidified environment with 5% CO_2. Cells were transferred ($3x10^4$ cells/well) to the wells of a 96-well plate, followed by the addition of plant extract dissolved in 80% EtOH for a final volume of 300 µL/well and a final EtOH concentration 0.5%. Plant extracts were assayed at 10 and 100 µg/mL. Parthenolide (Sigma-Aldrich, St-Louis, MO, USA) was used as a positive control at 1 and 10 µg/mL, and 0.5% EtOH was used as a vehicle control. Extract and controls were assayed in quadruplicate. Following the addition of extracts and controls, cells were incubated for 2 hours, and then stimulated with 1 µg/mL LPS purified from E. coli (Sigma-Aldrich, St-Louis, MO, USA) and allowed to incubate for 20 hours. An unstimulated control containing 0.5% EtOH but no LPS was also assayed. After incubation, cells were centrifuged at 1200 RPM for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. DuoSet® ELISA development kits (R & D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol to measure TNF-α levels in cell culture supernatants. Raw TNF-α values were transformed to a % activity of the parthenolide 10 µg/mL control.

Pro-inflammatory assays

The pro-inflammatory activity of plant extract was assessed by measuring TNF-α production in an LPS stimulated THP-1 monocyte assay. THP-1 cells were cultured as described in the anti-inflammatory assay methodology. Cells

were transferred ($3x10^4$ cells/well) to the wells of a 96-well plate, followed by the addition of plant extract dissolved in 80% EtOH for a final volume of 300 µl/well and a final EtOH concentration 0.5%. Plant extract was assayed at 10 and 100 µg/mL. *Echinacea purpurea* water extract was used as a positive control at 100 µg/mL, and 0.5% EtOH was used as a vehicle control. Extract and controls were assayed in quadruplicate. Following the addition of extracts and controls, cells were incubated for 22 hours, and after incubation, cells were centrifuged at 1200 RPM for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. DuoSet® ELISA development kits (R & D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol to measure TNF-α levels in cell culture supernatants. Raw TNF-α values were transformed to a % activity of the *E. purpurea* 100 µg/mL control.

Cytotoxicity

Extract toxicity was established using Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay (Madison, Wisconsin), which examines the release of lactate dehydrogenase as an indicator of cell viability.

Statistics

Statistical analysis was performed with Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Significant differences between treatment and control groups were assessed using one way ANOVAs and a Dunnett's post-hoc test.

Significant differences between all groups were assessed using one way ANOVAs and a Bonferroni post-hoc test. Linear regression was used to model the relationship between the healer consensus for various usage categories (independent variable) and the immunomodulatory activity of plant extracts (dependent variable). A p-value less than or equal to 0.05 was considered statistically significant. Normality was assessed using the Shapiro-Wilk test.

3.3 – Results

Anti-inflammatory activity

The primary data for the LPS-stimulated THP-1 monocyte anti-inflammatory assay (Figure 3.1) illustrate the low TNF-α production in unstimulated cells (12.6 pg/mL), and an elevated production of TNF-α (799.8

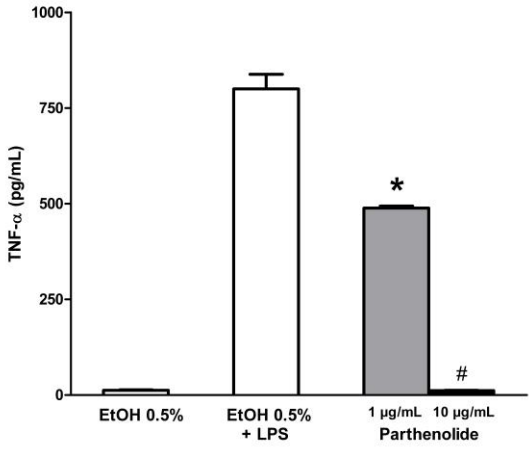

Figure 3.1 – TNF-α production (pg/mL) in an LPS-stimulated THP-1 monocyte anti-inflammatory assay. The average activity + SE, N=4, for the unstimulated vehicle control, the LPS-stimulated vehicle control, and the positive control parthenolide (1µg/mL and 10 µg/mL) is presented. * denotes TNF-α levels that are significantly different ($p \leq 0.05$) from the 0.5% EtOH control and # denotes TNF-α levels that are not significantly different ($p < 0.05$) from the parthenolide control.

pg/mL) after stimulation with LPS. Production of TNF-α is reduced with addition of the positive control parthenolide, and this reduction occurs in a concentration-dependent fashion. In fact, the 10 µg/mL parthenolide treatment returns TNF-α to a level that is not significantly different than the positive control (11.7 pg/mL).

Fifty-two medicinal species used by the Q'eqchi' healers (Table 3.1), selected to represent the 14 recognized usage categories, were extracted in 80% EtOH and assayed at 2 concentrations. In this dataset (Figure 3.2), the primary data have been transformed to represent the % activity of the parthenolide control at 10 µg/mL. Of the 52 species assayed, 21 (40%) displayed significant anti-inflammatory activity relative to the vehicle control at the highest concentration tested (100 µg/mL), and 12 (23%) at the lower concentration tested (10 µg/mL). At the highest concentration tested, 8 species had anti-inflammatory activity greater than 50% of the activity of the positive control, and 4 species did not differ significantly from the activity of parthenolide (*Tradescantia spathacea*, *Lantana trifolia*, *Piper tuerckheimii* and *Mollinedia guatemalensis*).

Of the 21 species assayed that had significant anti-inflammatory activity relative to the vehicle control, only three families had more than one active species, the Araceae (2 species), the Asteraceae (2 species), and the Piperaceae (5 species). This represents 40%, 50%, and 45% of all the species tested in these three families, respectively. The Asteraceae and Piperaceae have a greater proportion of active species when compared to the proportion of all species assayed that showed significant activity (40%). When averaging the anti-inflammatory activity of all species in plant families with at least three species

Table 3.1 - Q'eqchi' immunomodulatory medicinal plants assayed for their anti-inflammatory activity. Genus species, plant part(s) used (B = bark, L = leaf, R = root, S = stem), hot-cold score, and frequency of utilization (F_{use}) values are presented for 52 species arranged by family.

Family	Genus species	Plant part(s) assayed	Hot-cold score	F_{use}
Amaranthaceae	*Iresine diffusa* Humb. & Bonpl. ex Willd.	L	-0.8	1
Araceae	*Anthurium willdenowii* Kunth	L	-0.8	1
Araceae	*Monstera acuminata* K. Koch	L	-0.6	1
Araceae	*Philodendron hederaceum* (Jacq.) Schott	L	0	0.8
Araceae	*Philodendron radiatum* Schott	L	0.6	0.8
Araceae	*Philodendron schottii* K. Koch	L	0.25	0.8
Araliaceae	*Dendropanax arboreus* (L.) Decne. & Planch.	L, S	1	1
Aristolochiaceae	*Aristolochia tonduzii* O.C. Schmidt	L	1	1
Aspleniaceae	*Bolbitis pergamentacea* (Maxon) Ching	L	-1	0.6
Asteraceae	*Baccharis trinervis* (Lam.) Pers.	L	-0.6	0.8
Asteraceae	*Neurolaena lobata* (L.) R. Br. ex Cass.	L	0	1
Asteraceae	*Pluchea carolinensis* (Jacq.) G. Don	L, S	0.6	1
Asteraceae	*Porophyllum ruderale* (Jacq.) Cass.	L	-0.4	0.8
Begoniaceae	*Begonia glabra* Aubl. var. glabra	L	-1	1
Begoniaceae	*Begonia nelumbiifolia* Schltdl. & Cham.	L	-0.4	1
Bromeliaceae	*Pitcairnia punicea* Scheidw.	L	0.2	0.8
Burseraceae	*Bursera simaruba* (L.) Sarg.	B	0.5	0.8
Celastraceae	*Crossopetalum eucymosum* (Loes. & Pittier) Lundell	L	-0.5	0.8
Commelinaceae	*Tradescantia spathacea* Sw.	L	-1	0.6
Costaceae	*Costus pulverulentus* C. Presl	L	-0.6	0.6
Cucurbitaceae	*Gurania makoyana* (Lem.) Cogn.	L	-1	0.4
Dracaenaceae	*Dracaena americana* Donn. Sm.	B	0.25	0.6
Fabaceae	*Acacia cornigera* (L.) Willd.	B	0.25	0.8

Gesneriaceae	*Besleria saxiflora* Benth.	L	-1	0.6
Gesneriaceae	*Codonanthe uleana* Fritsch	L	-1	0.6
Gesneriaceae	*Drymonia serrulata* (Jacq.) Mart.	L, S	-1	1
Haemodoraceae	*Xiphidium caeruleum* Aubl.	L, S	-0.6	1
Lamiaceae	*Hyptis capitata* Jacq.	L	1	1
Lamiaceae	*Hyptis verticillata* Jacq.	L	0.6	1
Malvaceae	*Sida rhombifolia* L.	L	-0.6	1
Melastomataceae	*Arthrostemma ciliatum* Pav. ex D. Don	L	-0.2	0.4
Monimiaceae	*Mollinedia guatemalensis* Perkins	L	-0.2	1
Moraceae	*Dorstenia lindeniana* Bureau	L	-1	1
Orchidaceae	*Oeceoclades maculata* (Lindl.) Lindl.	L	-1	1
Piperaceae	*Peperomia hirta* C.DC.	L, R, S	-1	1
Piperaceae	*Peperomia macrostachya* (Vahl) A. Dietr.	L, R, S	-1	0.8
Piperaceae	*Peperomia obtusifolia* (L.) A. Dietr.	L	-1	0.8
Piperaceae	*Peperomia urocarpa* Fisch. & C.A. Mey.	L, R, S	-1	1
Piperaceae	*Piper aequale* Vahl	L, S	0.4	1
Piperaceae	*Piper auritum* Kunth	L	1	1
Piperaceae	*Piper hispidum* Sw.	L, S	1	1
Piperaceae	*Piper peltatum* L.	L	-0.4	0.6
Piperaceae	*Piper sanctum* (Miq.) Schltdl. ex C.DC.	L, S	1	0.6
Piperaceae	*Piper tuerckheimii* C. DC. ex Donn. Sm.	L	0.2	1
Piperaceae	*Piper yucatanense* C.DC.	L, S	1	0.8
Rhamnaceae	*Gouania polygama* (Jacq.) Urban	L	-1	1
Rubiaceae	*Hamelia patens* Jacq.	L	-0.4	1
Rubiaceae	*Hoffmannia ghiesbreghtii* (Lem.) Hemsl.	L	-0.6	1
Rubiaceae	*Psychotria pleuropoda* Donn. Sm.	L	-0.3	0.6
Solanaceae	*Solanum nudum* Dunal	L	0.4	0.8
Verbenaceae	*Lantana trifolia* L.	L	0.6	1
Zingiberaceae	*Zingiber officinale* Roscoe	R	1	0.8

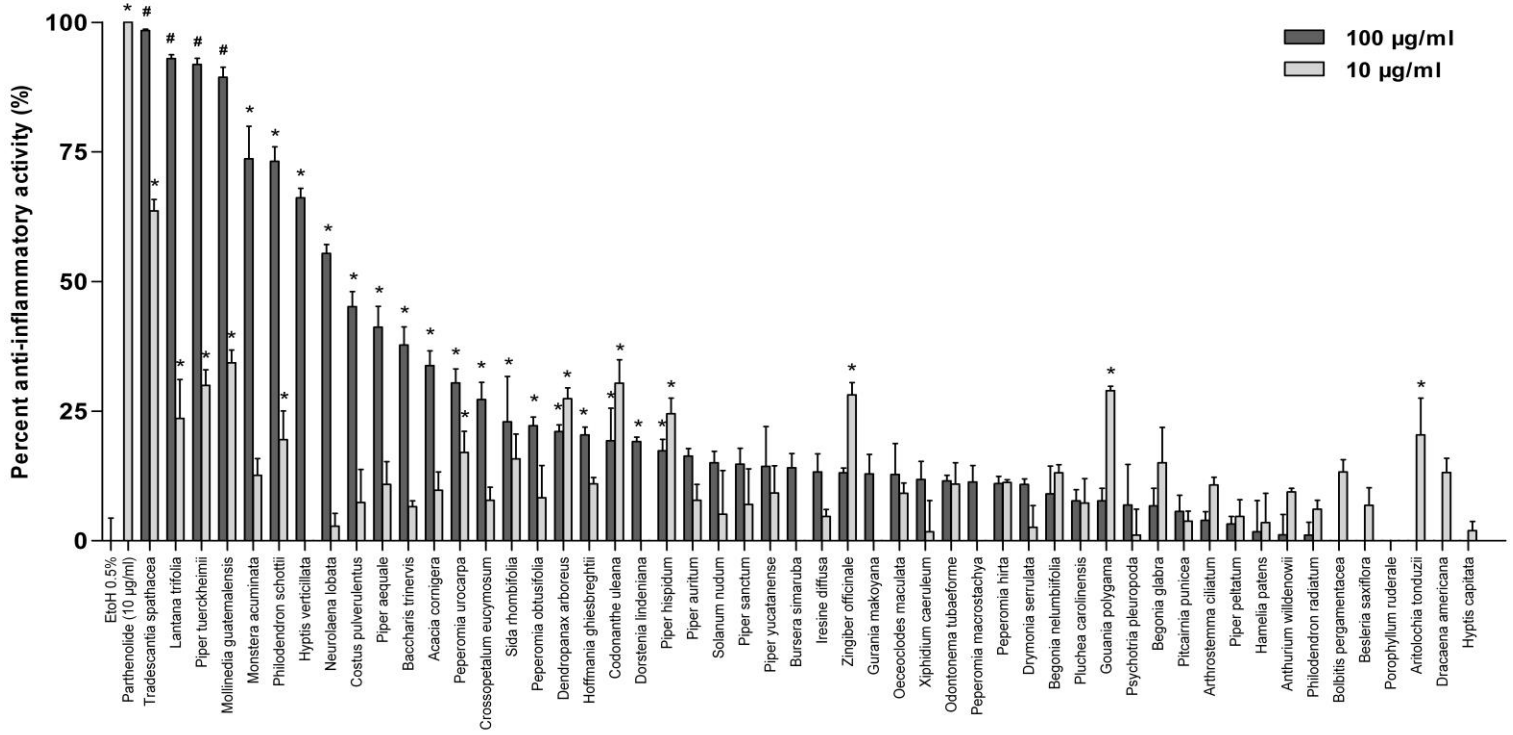

Figure 3.2 – Percent anti-inflammatory activity (%) of 52 Q'eqchi' plants relative to the parthenolide control (10 µg/mL) in LPS-stimulated THP-1 monocytes. Extracts were tested at 10 and 100 µg/mL. The average activity + SE is plotted, N=4. Species are ranked from most to least active at 100 µg/mL. * indicates activity significantly different ($p \leq 0.05$) from the 0.5% EtOH control and # indicates activity not significantly different ($p > 0.05$) from the parthenolide control.

assayed, no significant differences were found between the average activity of each family, although the Araceae, Asteraceae, and Piperaceae displayed 2-3 times the average activity of the Gesneriaceae and Rubiaceae (Figure 3.3). While the Araceae, Asteraceae, and Piperaceae show indications of being more anti-inflammatory than other families tested, it is clear that the activity of species within a family varies greatly.

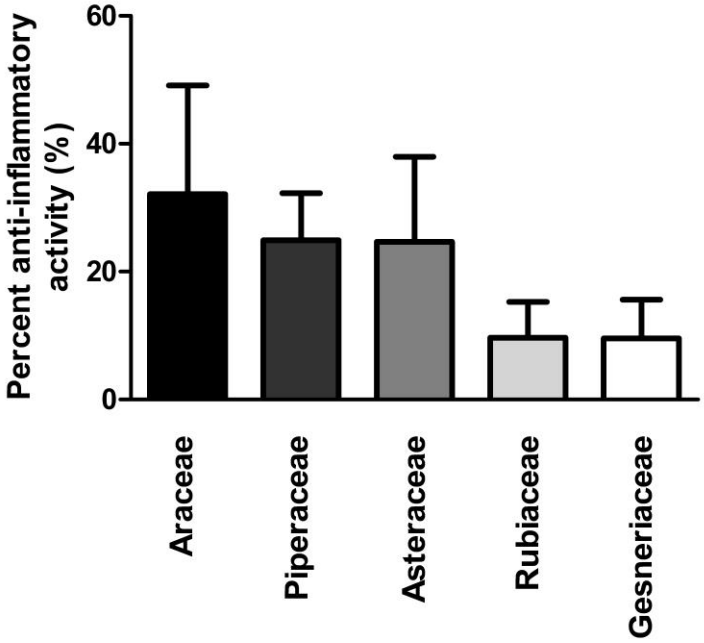

Figure 3.3 - Anti-inflammatory activity of plant families used by the Q'eqchi' healers. The average percent activity of the 10 µg/mL positive control parthenolide for families with N≥3 species assayed (Araceae, N=5; Piperaceae, N=11; Asteraceae, N=4, Rubiaceae, N=3; Gesneriaceae, N=3) is presented, + SE. A 1-way ANOVA followed by Bonferroni's multiple comparison test did not reveal any significant differences between groups ($p > 0.05$).

Pro-inflammatory activity

Since some herbal products such as ginseng and *Echinacea* are known immunostimulants which are often used in the treatment of common colds, a smaller set of plants were tested in a proinflammatory assay at 10 and 100 µg/mL and compared to an *Echinacea purpurea* 100 µg/mL positive control (Figure 3.4). *Echinacea purpurea* significantly increased TNF-α production from 14.2 pg/mL in the vehicle control to 42.9 pg/mL (100% activity). Only 1 of 16 Q'eqchi' medicinal plants assayed displayed significant immunostimulatory activity, *Xiphidium caeruleum*, with 51.1% of the activity of *E. purpurea*. *Drymonia serrulata* and *Peperomia obtusifolia* had 45.6% and 42.1% of the activity of the positive control, respectively, although this activity only approached significance. Although the immunostimulatory activity of *X. caeruleum* is an interesting finding which could be followed up later, the main activity of the plants was anti-inflammatory and the rest of the work follows this activity.

Cytotoxicity

The eight most active anti-inflammatory species (greater than 50% of the activity of the parthenolide positive control at the highest concentration assayed) were tested at 100 µg/mL for cytotoxicity activity (Table 3.2). All of the extracts displayed less than 9% cytotoxicity, suggesting that the TNF-α reduction is not due to cell death, and also suggesting that the plants are not highly toxic to human cell lines.

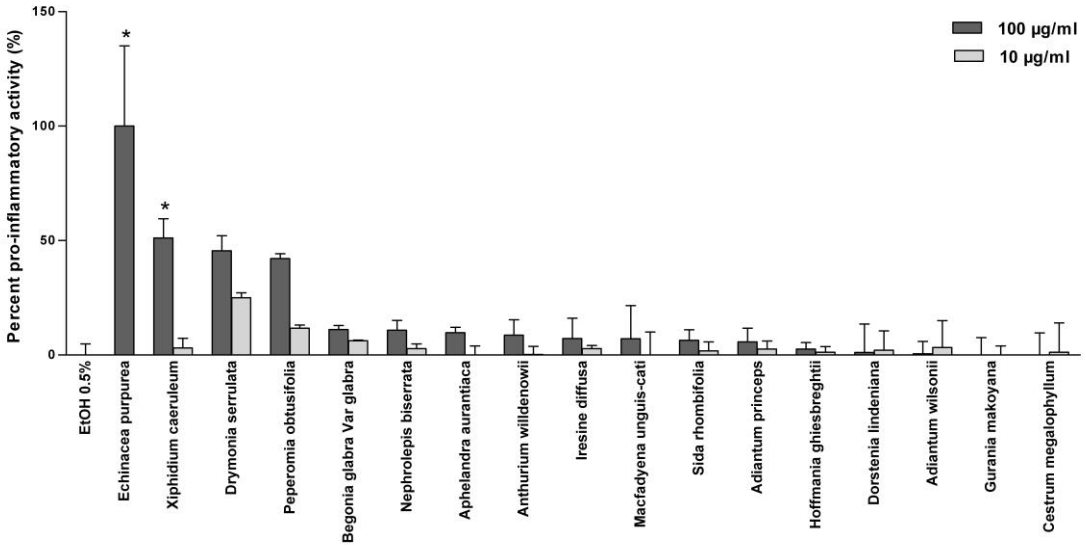

Figure 3.4 – Percent pro-inflammatory activity (%) of 16 Q'eqchi' plants relative to the *Echinacea purpurea* control (100 µg/mL) in THP-1 monocytes. Extracts were tested at 10 and 100 µg/mL, and the average activity ± SE is plotted, N=4. Species are ranked from most to least active at 100 µg/mL. * indicates activity significantly different from the 0.5% EtOH control and # indicates activity not significantly different from the parthenolide control.

Table 3.2 – Percent cytotoxicity (%) to THP-1 monocytes of the eight most anti-inflammatory Q'eqchi' Maya plants (100 µg/mL). Cytotoxicity values are calculated as a percentage (±SE) of LDH released compared to lysed cells. N=3.

Plant	Cytotoxicity (%)	SE
Tradescantia spathacea	8.9	0.9
Mollinedia guatemalensis	-8.4	2.2
Lantana trifolia	8.8	1.1
Piper tuerckheimii	-3.1	0.8
Neurolaena lobata	6.9	1.3
Philodendron schottii	0.6	1.0
Hyptis verticillata	5.3	0.5
Monstera acuminata	8.8	0.5

Humoral classification and ethnobotanical regression

We predicted that plant medicines traditionally classified as cold would be more anti-inflammatory than those classified as hot plants. Although the average anti-inflammatory activity of species classified as cold plants by all the healers (hot/cold score = -1) was almost three times greater than the average anti-inflammatory of species classified as hot plants by all the healers (hot/cold score = 1), the difference did not achieve statistical significance ($p = 0.13$) (Figure 3.4). The linear regression between the anti-inflammatory activity and the hot/cold score of all species assayed was not significant, indicating that the hot/cold score does not reflect anti-inflammatory activity.

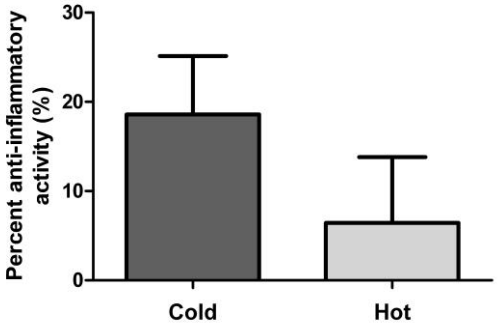

Figure 3.5 – Anti-inflammatory activity of cold plants compared to hot plants. The average percent activity of the 10 µg/mL positive control parthenolide for plants with hot/cold scores of -1 or +1 is presented, + SE. N = 14 for cold plants and N = 8 for hot plants. The difference between groups is not significant ($p = 0.13$).

I tested the prediction that plants with greater frequency of ethnobotanical use in some usage categories would have greater anti-inflammatory activity than those with lower frequency of use, and that the variation in activity could be explained by a positive linear regression. Significant regressions ($p \leq 0.05$), shown in Table 3.3, were found with frequency of use (F_{use}) values ($R^2 = 0.02$), common cold use-reports ($R^2 = 0.32$), and the Cook usage categories of

respiratory system disorders (R^2 = 0.22) and circulatory system disorders (R^2 = 0.26). Regressions with total use-reports and all other Q'eqchi' and Cook usage categories were not significant. No significant regressions were found between the averaged anti-inflammatory activity of plant families and plant class and subclasses and the residual values extracted from the plant family and plant class and subclass Moerman regressions in Chapter 2.

Table 3.3 – Linear regressions of anti-inflammatory activity (100 µg/mL treatment) versus F_{use} and the number of use reports in select Q'eqchi usage category (Q) and Cook usage categories (C). Species with no use-reports for the indicated usage categories were excluded from the regression. Degrees of freedom (DF), R^2 values, and P-values for each regression are presented. Only significant regressions ($P \leq 0.05$) are presented.

Regression	DF	R^2	P
100 µg/mL activity vs. F_{use}	206	0.02	0.048
100 µg/mL activity vs. Common cold (Q)	62	0.32	<0.0001
100 µg/mL activity vs. Respiratory system disorders (C)	66	0.22	<0.0001
100 µg/mL activity vs. Circulatory system disorders (C)	78	0.26	<0.0001

3.4 – Discussion

Q'eqchi' Maya medicinal plants used in the treatment of symptoms related to inflammation showed a high degree of anti-inflammatory activity, with 40% of all species assayed demonstrating significant activity. Eight species showed very high levels of anti-inflammatory activity, demonstrating more than 50% of the activity of the parthenolide positive control, and the activity of 4 of these species did not differ significantly from parthenolide.

Tradescantia spathacea was one of the most active species tested. Previous studies have shown the *in vitro* and *in vivo* anti-inflammatory effects of this species (Bunyapaphatsara, 2000; Perez, 1996), and a crude phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, tannins, simple phenolics, and terpenoids (Parivuguna, 2008), although few compounds have been described and no active anti-inflammatory principles have been isolated. *Lantana trifolia* is also one of the most active species assayed. It has been reported to inhibit carrageenan and histamine-induced rat paw oedema (Silva et al, 2005; Uzcategui et al, 2004), and a variety of flavones and phenylpropanoids have been described in this species (Juiliao et al, 2010). Another one of the most active species is *Piper tuerckheimii*. This is the first report of its anti-inflammatory activity, and no phytochemical studies of this species have been published. However, *Piper* is a very well studied genus, with anti-inflammatory activity being reported in numerous species, including *P. amalago*, *P. betle*, *P. cubeba*, *P. nigrum*, *P. ovatum* and *P. sarmentosum.* In addition, anit-inflammatory piperamides have been isolated from *P. nigrum* (Ahmad et al, 2012; Choi and Hwang, 2003; Ganguly et al, 2007; Rodrigues Silva et al, 2008; Sosa et al, 2002; Zakaria et al, 2010). The fourth species with anti-inflammatory activity not significantly different than that of parthenolide was *Mollinedia guatemalensis*; no phytochemical or pharmacological reports have been published for this species.

Monstera acuminata and *Philodendron schottii* were the fifth and sixth most active species assayed, respectively. There are no reports in the literature

on the biological activities or the phytochemistry of either. In contrast, the seventh most active species, *Hyptis verticillata*, has been extensively studied and reviewed (Picking et al, 2013), with this species demonstrating anti-inflammatory effect when administered topically and internally in mice (Frias et al, 2011). Rosmarinic acid and sideritoflavone have all been isolated from this species and have demonstrated anti-inflammatory activity (Kuhnt et al, 1994 and 1995). *Neurolaena lobata* is the eighth most active species. Anti-inflammatory activity of this species has been observed in a carrageenan-induced mouse paw oedema model (De Las Heras et al., 1998). Previous phytochemical studies have identified 11 sesquiterpene lactones (Borges-Del-Castillo et al., 1982; Manchand and Blount, 1978; Passreiter et al., 1995) and 12 flavonoids in the leaves of this species (Kerr et al., 1981); however the anti-inflammatory activity of these compounds has not been evaluated.

The best evidence for selection of plants by the healers on the basis of pharmacological activity is that provided by the significant regressions of anti-inflammatory activity with quantitative ethnobotanical parameters. These included 4 significant regression found with F_{use}, use-reports for common cold, use-reports for respiratory system disorders, and use-reports for and circulatory system disorders. The R^2 value for the F_{use} regression is only 0.02, indicating that only 2% of the variation in the observed anti-inflammatory activity is explained by the total healer consensus for all usage categories. This regression is likely only significant due to the large sample size. The R^2 values for the 3 significant regressions with usage categories range from 0.22 - 0.32, suggesting that healer

selection for these usage categories predicts 22% - 32% of the observed variance in anti-inflammatory activity. The regression between the number of use reports for common colds and anti-inflammatory activity had the highest R^2 value. Given the important role of viral and bacterial-induced production of TNF-α in modulating the inflammatory response associated with cold and flu like symptoms, the anti-inflammatory activity of plants reportedly used to treat the common cold can be partially explained (Hudson, 2010)

Many other traditionally used Mesoamerican medicinal plants have demonstrated anti-inflammatory activity. In particular, cat's claw, *Uncaria tomentosa* has demonstrated potent TNF-α reduction *in vitro* (Rojas-Duran et al, 2012). Cat's claw is now a registered natural health product (NHP) used for the treatment of anti-inflammatory conditions (Health Canada, 2013). In a screening of the topical anti-inflammatory activity of several plant species from central America, three species of plants used by the QMHA (*Bursera simaruba* bark, *Hamelia patens* leaves, *Piper amalago* leaves) were evaluated for their topical anti-inflammatory activity against the Croton oil-induced ear oedema in mice (Sosa et al, 2002). Most of the chloroform extracts induced a dose-dependent oedema reduction with ID_{50} values ranging between 143 and 498 μg/cm^2. This supports the *in vivo* anti-inflammatory activity of these Q'eqchi' Maya medicinal plants, and helps guide future research on these species.

The results presented in this chapter provide evidence of the potent anti-inflammatory activity of Q'eqchi' Maya medicinal plants, and provide a pharmacological basis for their use as traditional medicines. Also, several

ethnobotanical parameters were significant predictors of the anti-inflammatory activity of QMHA species, further supporting the notion that plant selection by the healers is not random. To continue this work, the most active species should be selected for the elucidation of their active principles. The anti-inflammatory principles of *Neurolaena lobata* will be the focus of Chapter 4, and preliminary results from the bioassay guided isolations of *Monstera acuminata* and *Tradescantia spathacea* are presented in Appendices 3 and 4, respectively. In addition, given the complex nature of the inflammatory response, the anti-inflammatory properties of active species and their isolates should be evaluated in other models of inflammation which measure the effects on other cytokines, chemokines, acute phase reactants, and prostaglandins. This could provide a more complete picture of the properties of these plants and how the entire inflammasome is affected will emerge. Subsequently, the most promising species should be evaluated in appropriate animal and clinical models. In particular, they can be evaluated in a rat ear oedema model, which has replaced traditional models such as rat paw oedema. It is clear that there remains much to be uncovered about the anti-inflammatory nature of several of the most active Q'eqchi' species, as little has been published on their phytochemistry and pharmacology, suggesting that further research into these plants is warranted.

Chapter 4

Potent anti-inflammatory activity of sesquiterpene lactones from *Neurolaena lobata* (L.) R. Br. ex Cass., a Q'eqchi' Maya traditional medicine

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Statement of author contribution

BWR and JTA conceived and designed this study. Plant collection was undertaken by BWR with the assistance of FC. *Neurolaena lobata* was identified by BWR and confirmed by MOR. CC carried out open column chromatography and NMR interpretation with the assistance of MA and TD. BWR completed the isolation with Preparative-scale HPLC and verified isolate purity with analytical-scale HPLC with the assistance of AA. BWR undertook the anti-inflammatory and cytotoxicity assays. VC helped coordinate research efforts in Belize. JTA and TP managed and directed the Belize project with the QMHA and CC, TD and JTA contributed to manuscript preparation.

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4.1 - Introduction

Neurolaena lobata (L.) R. Br. ex Cass. (Asteraceae) is a common medicinal plant found growing in open areas throughout Central America, the Caribbean, and into southern Mexico (Turner, 1982). Known locally by such names as Jackass Bitters (English Creole), *Mano de lagarto* and *Tres puntas* (Spanish), *Kayabim* (Yucatec and Mopan Maya) and *K'a mank* (Q'eqchi' Maya), the bitter tasting leaves of this plant are widely used by indigenous traditional healers in these regions to treat a variety of ailments including malaria, diabetes, skin diseases and cancer (Giron et al., 1991; Hartwell, 1968; Morton, 1981). Among Yucatec Maya, the crushed leaf is used to treat itchy inflammed skin (Arnason et al., 1980). Arvigo and Balick (1993) described a variety of conditions treated with the plant, including ringworm, wounds, sores, and infections, by Mopan Maya and other cultures.

All five members of the Q'eqchi Maya Healers Association report using the leaves of *Neurolaena lobata* for the treatment of inflammation related symptoms, including fevers, headaches, arthritis and rheumatism, insect bites and stings, rashes, stomach cramps, heartburn and ulcers, as well as the common cold. K'a mank, which translates to 'bitter mango', is used internally and externally by Q'eqchi' healers, and the most common preparation method is to boil a maximum of six leaves in approximately 1L of water, half of which is administered to the patient in the morning and the other half is drunk in the afternoon. Conversations with the Q'eqchi' healers reveal that this dilute preparation and dosage reflects the potency of *N. lobata* and the need to use it in moderation. Of the 52 Q'eqchi'

medicines assessed for their activity in chapter 3, *N. lobata* was the 7th most active species tested, demonstrating 73.9% of the activity of the parthenolide 10 µg/ml positive control. The cytotoxicity of the 80% crude *N. lobata* leaf extract was 6.9±1.3%.

Previous phytochemical studies have identified 11 sesquiterpene lactones (Borges-Del-Castillo et al., 1982; Manchand and Blount, 1978; Passreiter et al., 1995) and 12 flavonoids in the leaves (Kerr et al., 1981), and several thymol derivatives in the roots of *N. lobata* (Bohlmann et al., 1979). Manchand and Blount (1978) were the first to isolate the germacranolide sesquiterpene lactones neurolenins A and B (**1**) from the leaves of plants collected in Trinidad. In leaves of *N. lobata* collected in Panama, Borges del Castillo et al (1982) isolated neurolenin B, and identified two novel furanoheliangolide sesquiterpene lactones, lobatins A and B (**4**). Further work by Passreiter et al (1995) identified an additional 7 sesquiterpene lactones from the leaves of plants collected in Guatemala. These include 4 germacranolide sesquiterpene lactones, neurolenins C (**2**), D (**3**), and trace amounts of E and F, as well as 3 furanoheliangolide sesquiterpene lactones, lobatin C in trace amounts and two isovalerates, 9α-hydroxy-8β-isovalerianyloxy-calyculatolide (**5**) and 9α-acetoxy-8β-isovalerianyloxy-calyculatolide, which are the dihydro-derivatives of lobatins B and C respectively.

Several biological activities have been reported for the crude leaf extract of *N. lobata* as well as for some of the isolated sesquiterpene lactones. The leaf extract of *N. lobata* possess several anti-parasitic activities, including anti-

trypanosomal and anti-leishmaniasis activity (Berger et al., 1998; Berger et al., 2001; Muelas-Serrano et al., 2000), and anti-malarial activity against *Plasmodium falciparum*, both *in vitro* and *in vivo* (François et al., 1996; Franssen et al., 1997). In addition, analgesic, anti-ulcer, anti-bacterial, anti-fungal and anti-viral activities of *N. lobata* leaf extracts have been reported (Bedoya et al., 2008; Cáceres et al., 1998; Gracioso et al., 1999; Gracioso et al., 2000; Lentz et al., 1998). Of particular relevance to this study, *N. lobata* leaf extract was anti-inflammatory in carrageenan-induced mouse paw oedema, reducing swelling by 19.5% (De Las Heras et al., 1998). Among the isolated compounds from *N. lobata*, strong anti-malarial activity against *P. falciparum* has been reported for neurolenins A, B, C and D, and to a weaker extent lobatins A and B (François et al., 1996; Blair et al., 2002). Also, neurolenins A and B as well as lobatins A and B have been reported as cytotoxic to human small cell lung carcinoma *in vitro* (François et al., 1996).

The biological activities of sesquiterpene lactones are due to a Michael-type addition reaction on the exocyclic α-methylene-γ-lactone moiety which alkylate sulfhydryl groups. For example, the well known anti-inflammatory agent parthenolide, found in the aptly named traditionally used anti-pyretic feverfew (*Tanacetum parthenium* – Asteraceae), possesses anti-inflammatory activity due to this α-methylene-γ-lactone moiety. Parthenolide works by alkylating a cysteine residue on the activation loop of IκB kinase β, a kinase subunit that plays a critical role in the regulation of cytokine-mediated signalling, and thus preventing the activation of the pro-inflammatory NF-κB pathway (Hehner et al., 1999; Kwok et al., 2001). Since feverfew and Jackass bitters share similar indications in

traditional medicine for migraine headaches, arthritis and fever, and have similar active principles, we examined the anti-inflammatory activity of sesquiterpene lactones isolated from Belizean *N. lobata* leaves. The extent of this anti-inflammatory activity and how it compares to the anti-inflammatory activity of parthenolide is the focus of this study.

4.2 - Materials and Methods

Plant material

Neurolaena lobata (L.) R. Br. Ex Cass. was collected at the Itzamma Ethnobotanical Garden, a medicinal plant garden managed by the QMHA and BITI in Indian Creek, Toledo District, Belize. Collecting and export permits were obtained from the Belize Forest Department (Ref. No. CD/60/3/08(33)). The garden is at an elevation of approximately 30m above sea level. *N. lobata* leaves were collected at flowering stage and immediately preserved in alcohol. Authenticated voucher specimens are deposited at the University of Ottawa herbarium (OTT#17233) and the Juvenal Valerio Rodriguez herbarium of the Universidad Nacional de Costa Rica (JVR#13500).

Extraction and isolation

Wet leaves (477.8 kg) were shredded in a blender and extracted twice in EtOH (80% in H2O; 3.0 L) over 24h at room temperature. The small amount of alcohol used to preserve the bottled leaf material was included in the first extraction. The combined extracts were evaporated in vacuo to yield 41.4 g of

brown residue (crude extract). Dried extract was chromatographed in an open glass column packed with silica gel 60 (70-230 mesh, Merck) eluting with hexanes – EtOAc (100%:0% to 0%:100%) and EtOAc – MeOH (100%:0% to 0%:50%) to yield 14 fractions. TLC analyses were performed on silica gel 60 F254 plates (Merck), and visualization of the plates was carried out using a cerium molybdate stain. Compounds **1**-**5** were found within fractions 6 (980 mg) and 7 (920 mg), which eluted between 40 to 60% EtOAc in hexanes. Fraction 6 resulting from primary fractionation was re-chromatographed in a glass column packed with silica gel eluting with hexanes – EtOAc (100%:0% to 0%:100%) to yield 7 secondary fractions (6-1 to 6-7). Fraction 6-4 (150 mg), eluted with hexanes – EtOAc (60%:40%), was further purified with silica gel eluting with dichlormethane – MeOH (100%:0% to 0%:10%) to yield 7 tertiary fractions (6-4-1 to 6-4-7). Fractions 6-4-1 and 6-4-2 contained compound **1**, which eluted at 1.5% MeOH in DCM. Fraction 6-4-3 contained the isomeric mixtures of compounds **2** and **3**, which eluted at 2% MeOH in DCM. Fraction 6-4-4 contained compound **4**, which eluted at 3% MeOH in DCM. Fraction 7 resulting from primary fractionation was re-chromatographed in a glass column packed with silica gel eluting with hexanes – EtOAc (100%:0% to 0%:100%) to yield 10 secondary fractions (7-1 to 7-10). Fraction 7-5 (277 mg), eluted with hexanes – EtOAc (50%:50%), was further purified with silica gel eluting with dichlormethane – MeOH (100%:0% to 0%:10%) to yield 12 tertiary fractions (7-5-1 to 7-5-12). Fractions 7-5-3 and 7-5-4 contained the same isomeric mixture of compounds **2** and **3**, which eluted at 2%

MeOH in DCM. Fractions 7-5-6 and 7-5-7 contained compound **5**, which eluted at 3% MeOH in DCM.

Final purification and pooling of tertiary fractions was carried out with preparative scale HPLC using a reverse phase Gemini Axia 250 mm × 21.2 column, particle size 10 microns (Phenomenex Inc., Torrance, CA, USA). The Agilent 1200 series preparative HPLC-DAD system (Agilent Technologies, Montreal, QC, Canada) consisted of a binary pump, an autosampler with a 2 ml loop, a diode array detector and a fraction collector. Separation was achieved using a 40 min linear gradient of 40-45% of acetonitrile in water + 0.1% TFA at a flow rate of 31.5 ml/min, with a monitoring wavelength of 210 nm, band width 4, reference off. Isolate purity was verified on an Agilent 1100 series analytical HPLC-DAD system (Agilent Technologies, Montreal, QC, Canada) consisting of an online degasser, a quaternary pump, an auto sampler with a 100 µL built-in injection loop, a column thermostat compartment, and a diode array detector. Separation was achieved on a reverse phase YMC-Pack ODS-A 2.0 mm x 100 mm column, particle size 3 microns (YMC America Inc., Allentown, PA, USA) using a 30 min linear gradient of 5-100% of acetonitrile in water at a flow rate of 0.5 ml/min, and at monitoring wavelengths of 210 nm, 245 nm, 330 nm, and 450 nm, band width 4, reference off. All five isolates eluted between 11.1-14.0 min, with compound **2** eluting at 11.1 min, compound **5** at 11.8 min, compound **3** at 12.0 min, compound **4** at 12.6 min, and compound **1** at 14.0 min. Preparative scale HPLC purification was repeated until isolate purity was 90% or greater, calculated as a percent of the total chromatogram integration at 210 nm. NMR

spectra were recorded on a Bruker Avance 400 spectrometer in either $CDCl_3$ or acetone-d_6, at 400 MHz for ^1H and 100 MHz for ^{13}C.

Cell culture and anti-inflammatory assay

The anti-inflammatory activity of isolates was assessed by measuring TNF-alpha reduction in a LPS stimulated THP-1 monocyte assay adapted from Zhao et al (2005). THP-1 cells (human monocyte culture TIB-202, ATCC, Manassas, VA, USA), were cultured in RPMI 1640 media (ATCC, Manassas, VA, USA) supplemented with 1% 0.05 mM beta-mercaptoethanol, 1% penstrep (Invitrogen, Mississauga, ON, Canada) and 10% fetal bovine serum (Invitrogen, Mississauga, ON, Canada), in a 37°C humidified environment with 5% CO_2. Cells were transferred ($3x10^4$ cells/well) in a 96-well plate, followed by the addition pure compounds dissolved in 80% EtOH for a final volume of 300 µl/well and a final EtOH concentration 0.5%. The positive control parthenolide (≥98% purity) (Sigma-Aldrich, St-Louis, MO, USA) and the *N. lobata* isolates were assayed at 0.4, 4, 10, and 40 µM in quadruplicate. Following the addition of isolates and controls, cells were incubated for 2 hours, and then stimulated with 1 µg/ml LPS purified from E. coli (Sigma-Aldrich, St-Louis, MO, USA) and allowed to incubate for 20 hours. An unstimulated control containing 0.5% EtOH but no LPS was also assayed. After incubation, cells were centrifuged at 2000 rpm for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. DuoSet® ELISA development kits (R & D Systems,

Minneapolis, MN, USA) were used according to the manufacturer's protocol to measure TNF-alpha levels in cell culture supernatants.

Cytotoxicity

Extract toxicity was established using Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay (Madison, Wisconsin), which examines the release of lactate dehydrogenase as an indicator of cell viability.

Statistics

Statistical analysis was performed with Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Significant differences between treatment and control groups were assessed using one way ANOVAs and a Dunnett's post-hoc test. IC_{50} values were extrapolated from a nonlinear straight line semi-logarithmic regression (TNF-α vs. log concentration). A p value less than or equal to 0.05 was considered significant. Normality was assessed using the Shapiro-Wilk test.

4.3 – Results and Discussion

Silica gel column chromatography and final purification with preparative HPLC resulted in the isolation of five sesquiterpene lactones from the crude 80% leaf extract (**1**-**5** – Figure 4.1). Compounds **1**-**3** are germacranolide sesquiterpene lactones: neurolenin B (**1** – 0.17% of 80% EtOH crude extract), neurolenin C (**2** – 0.21% of 80% EtOH crude extract), and neurolenin D (**3** – 0.28% of 80% EtOH crude extract). It was not possible to separate neurolenin C

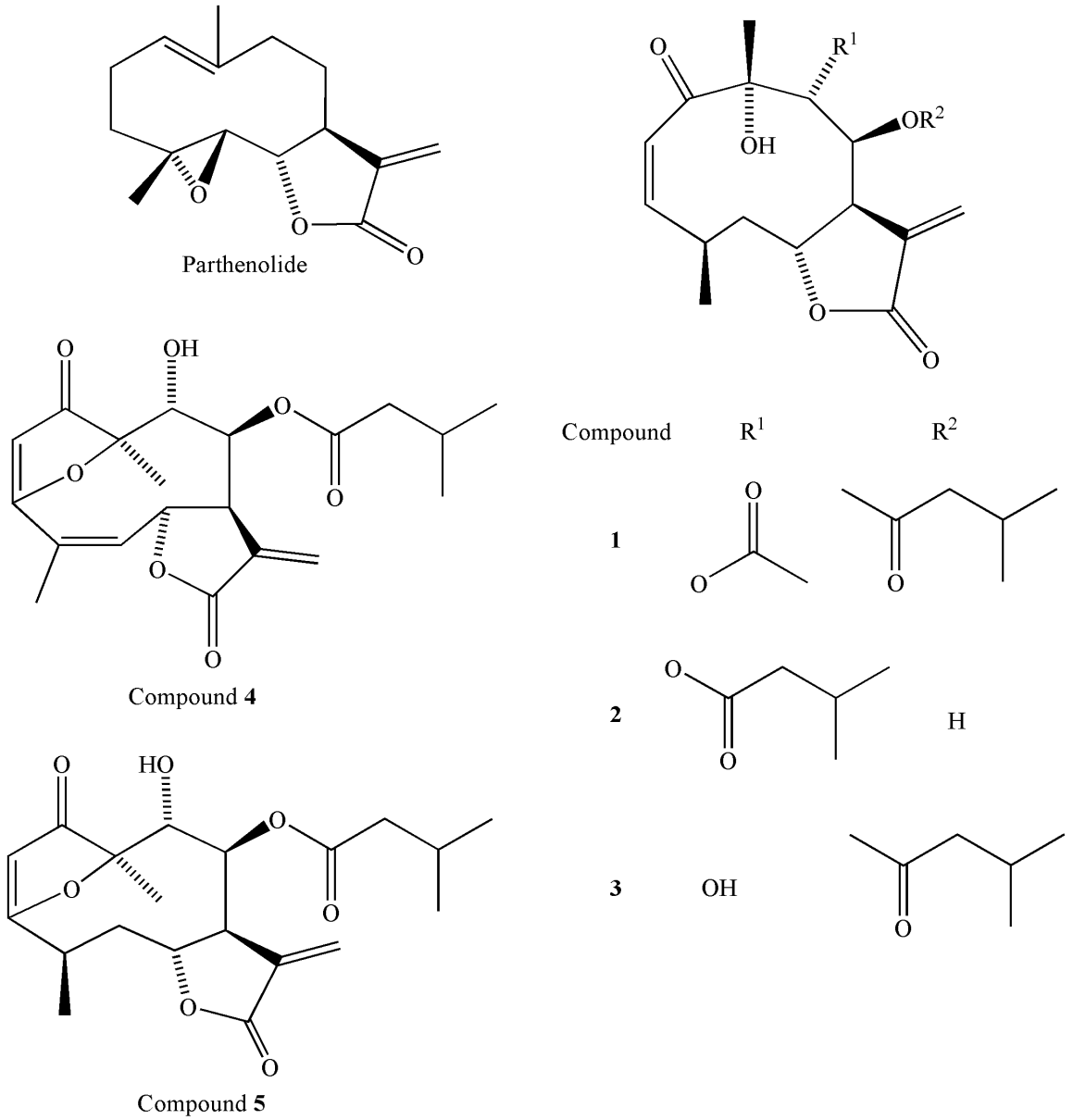

Figure 4.1 – Chemical structures of parthenolide and compounds **1**-**5** isolated from the leaves of *Neurolaena lobata*. Compounds **1**-**3** are germacranolide sesquiterpene lactones: neurolenin B (**1**), neurolenin C (**2**), and neurolenin D (**3**). Compounds **4**-**5** are furanoheliangolide sesquiterpene lactones: lobatin B (**4**) and 9α-hydroxy-8β-isovalerianyloxy-calyculatolide (**5**).

from neurolenin D as they are isomeric isovaleryl esters which differ only in the position of the ester group. As such, further experiments were carried out on the mixture of these two compounds which are present in a 0.75:1 neurolenin C:D ratio as measured by the relative integration of NMR signals. Compounds **4**-**5** are furanoheliangolide sesquiterpene lactones: lobatin B (**4** – 0.24% of 80% EtOH crude extract) and 9α-hydroxy-8β-isovalerianyloxy-calyculatolide (**5** – 0.12% of 80% EtOH crude extract). The spectroscopic properties agreed with those previously described by Manchand and Blount (1978) and Passreiter (1995), and details can be found in Chapter 2 of C. Choueiri's PhD thesis (2013).

Parthenolide and the five isolated sesquiterpene lactones displayed dose-dependent anti-inflammatory activity in LPS-stimulated monocytes (Figure 4.2). At 0.4 μM, the lowest concentration tested, lobatin B demonstrated the greatest reduction in TNF-α production relative to the stimulated vehicle control, lowering levels from 292.9±48.5 pg/ml to 140.4±17.1 pg/ml. Neurolenins C+D and compound **5** also displayed significant TNF-α reduction relative to the stimulated vehicle control. At 4 μM, all five *N. lobata* isolates demonstrated significant reductions ($p < 0.05$) in TNF-α production relative to the stimulated vehicle control, with resulting TNF-α levels ranging from 182.2±22.7 pg/ml in the 4 μM compound **5** treatment to 31.1±8.2 pg/ml in the 4 μM lobatin B treatment. The TNF-α levels in the 4 μM lobatin B treatment were not significantly different from the unstimulated control. At 10 and 40 μM, all compounds tested produced TNF-α levels that were not significantly different from the unstimulated control. At 10 μM, TNF-α levels ranged from 48.8±10.3 pg/ml in the compound **5** treated

monocytes to 9.8±0.9 pg/ml in the lobatin B treated monocytes. At 40 μM, TNF-α levels ranged from 8.3±0.4 pg/ml in the neurolenin C+D treated monocytes to 5.5±0.2 pg/ml in the compound **5** treated monocytes.

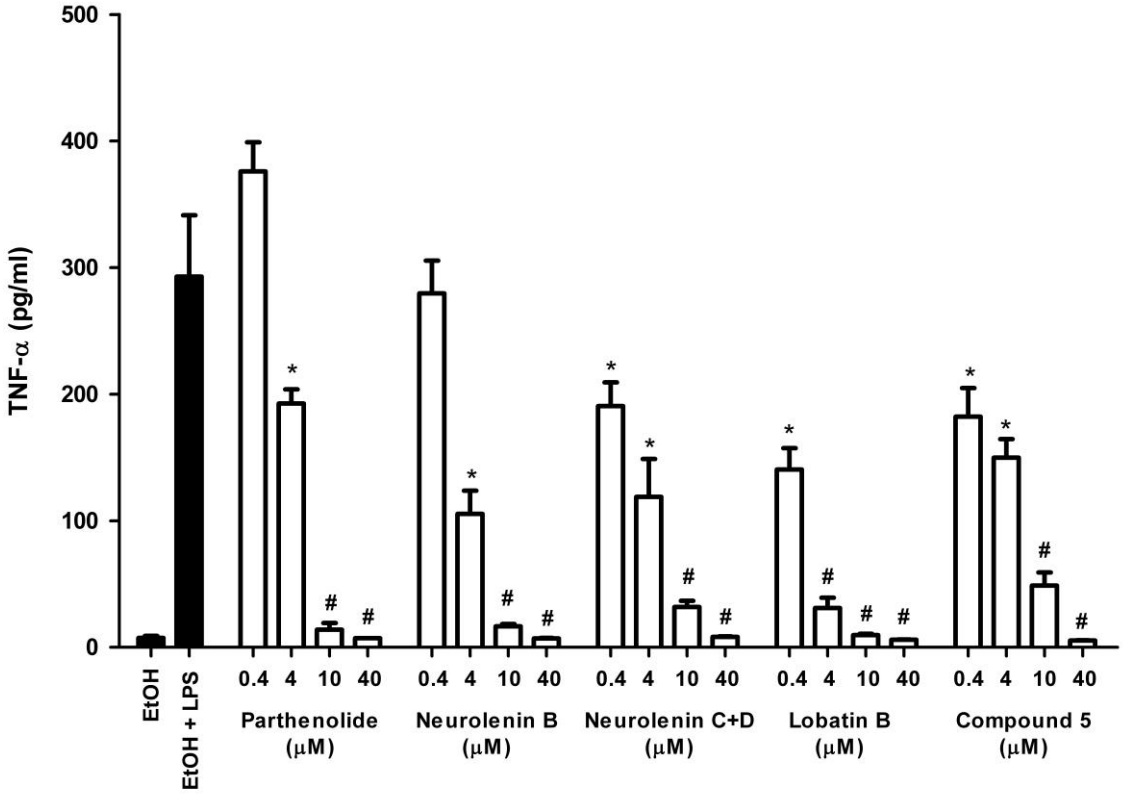

Figure 4.2 – Anti-inflammatory activity of sesquiterpene lactones isolated from *Neurolaena lobata* leaf extract in LPS-stimulated THP-1 monocytes. EtOH 0.5% was used as a vehicle in both unstimulated and LPS-stimulated controls and parthenolide was used as a positive control. Means plus standard error of four replicates are presented. Compound **5** is 9α-hydroxy-8β-isovalerianyloxy-calyculatolide. * denotes TNF-α levels that are significantly different ($p \leq 0.05$) from the 0.5% EtOH control and # denotes TNF-α levels that are not significantly different ($p > 0.05$) from the parthenolide control.

All five *N. lobata* isolates had TNF-α IC_{50} values which were lower than that of parthenolide in LPS-stimulated monocytes (table 4.1). The IC_{50} for parthenolide was 4.79 μM, while the IC_{50}s for the isolates ranged from 2.32 μM for neurolenin B to 0.17 μM for lobatin B. The production of TNF-α is regulated by the pro-inflammatory NF-κB pathway, and numerous natural products have been identified as inhibitors of this pathway (Bremner and Heinrich, 2002 and 2005; Hehner et al., 1999; Nam, 2006). Sesquiterpene lactones are among the most potent inhibitors of this pathway, and several studies have demonstrated the TNF-α inhibition of these α-methylene-γ-lactone containing compounds (Cho et al., 1998; Choi et al., 2012; Ferrari et al., 2012; Jin et al., 2004). While not all studies use parthenolide as a positive control, hence making comparisons between the relative potency of different sesquiterpene lactones difficult, it is clear that the compounds isolated from *Neurolaena lobata* possess strong anti-inflammatory activity.

Parthenolide and all five isolates all displayed moderate cytotoxic effects towards THP-1 monocytes at 40 μM, ranging from 9.6±1.2% for parthenolide to 12.4±2.0% for lobatin B (table 4.1). Although the IC_{50} values for cytotoxicity were not evaluated, it is clear that the therapeutic index for *Neurolaena* compounds is not large, but is larger than parthenolide. Although *N. lobata* is a widely used traditional remedy, and its mode of action is comparable to feverfew, a registered Natural Health Product in Canada and other jurisdictions, its internal use and dose should be approached with caution because of cytotoxicity. The careful use of *N. lobata* by Q'eqchi' Maya healers of Belize underscores this approach.

Table 4.1 – IC_{50}, cytotoxicity of parthenolide and compounds isolated from the leaves of *Neurolaena lobata*. Yields of isolates are also included.

Compound name	IC_{50}[a] (μM)	Cytotoxicity[b] ± S.E. (%)	Yield[c] (%)
Parthenolide	4.79	9.6±1.2	-
Neurolenin B	2.32	11.2±1.7	0.17
Neurolenin C+D	1.10	10.1±1.5	0.49
Lobatin B	0.17	12.4±2.0	0.24
Compound **5**	1.30	10.6±1.9	0.12

Compound **5**: 9α-hydroxy-8β-isovalerianyloxy-calyculatolide.

[a] IC_{50} as extrapolated from a nonlinear straight line semi-logarithmic regression (TNF-α vs. log compound concentration).

[b] Cytotoxicity of compounds to THP-1 monocytes at 40 μM as assessed the release of lactate dehydrogenase.

[c] Percent yield in 80% EtOH crude extract.

The sesquiterpene lactones isolated from the leaves of *N. lobata* are potent inhibitors of TNF-α production in LPS-stimulated THP-1 monocytes, demonstrating greater anti-inflammatory activity than that of parthenolide. Lobatin B is a particularly powerful anti-inflammatory agent as its TNF-α inhibition is an order of magnitude greater than that of parthenolide. The use of *Neurolaena lobata* by the Q'eqchi' Maya healers of Belize as well as its widespread use throughout Central America and the Caribbean as a traditional medicine for inflammatory conditions appears to have a pharmacological and phytochemical

basis given the strong anti-inflammatory activity its leaf extract and isolated sesquiterpene lactones display *in vitro*.

Chapter 5

Immunomodulatory activity of medicinal plants used by the Cree of Eeyou Istchee

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Statement of author contribution

BWR and JTA conceived and designed this study. Plant identification and collection was undertaken by AC. Plant extraction and immunomodulatory assays were carried out by BWR. Total phenolics and total polysaccharide assays were completed by BWR and SW. PSH is the manager off the CIHR TAAM project, and JTA contributed to manuscript preparation.

5.1 – Introduction

The Cree of Eeyou Istchee (CEI) from the James Bay region in northern Quebec have a long history of using medicinal plant species in the treatment of illness. In more recent history, traditional healers in these communities have also been using these remedies to treat Type II diabetes (T2D) and its associated symptoms. Diabetes is a significant and growing problem worldwide, and is of particular concern among aboriginal populations, including the CEI, where from 1994 to 2004 its prevalence in people aged 20 years or over rose from 6.6% to 17.7% (Legare, 2004; Kuzmina and Dannenbaum, 2004).

In a collaboration between the CEI and researchers from the University de Montréal, McGill University, and the University of Ottawa, the anti-diabetic plant preparations being used by Cree healers have been investigated for their anti-diabetic properties using a variety of phytochemical and pharmacological techniques. In this project, considerable emphasis was placed on primary antidiabetic assays such as glucose uptake, insulin sensitivity, adipogensis, and animal models of T2D and obesity (Haddad et al, 2011). Recently, evidence has accumulated that inflammation is a contributing factor to the development and progression of T2D (Dandona and Aljada, 2002). Therefore, the role of traditional medicinal plants in modulating inflammation was an important area to investigate in the assessment of Cree traditional medicine. The area is complicated somewhat by recent research findings which clearly indicated that medicinal plants from other traditions than the Cree can be highly anti-inflammatory due to the presence of secondary metabolites including terpenes, phenolics and

alkaloids (feverfew, cat's claw, devils claw, thundergod vine, tumeric) while others can be proinflammatory (Ginseng and *Echinacea* polysaccharide or polysaccharide-rich glycoprotein derived fractions). For this reason the objective of the present study was to investigate the top 17 Cree plants identified by Leduc et al (2006) for their immunomodulatory properties. Species were tested for both their anti- and pro-inflammatory activity in a THP-1 monocyte line. Two extracts were compared: alcohol extracts rich in secondary metabolites and water extracts which contain additional polysaccharides and glycoproteins. An attempt was made to understand the contribution of plant extract phenolic and polysaccharide content to the immunomodulatory activity of each species.

5.2 – Materials and Methods

Plant material

Plant material was collected by collaborating ethnobotanists at the University de Montréal and air dried using a plant dehydrator at 40°C. Collection data and voucher numbers are provided in Leduc et al (2006). Plant material was ground using a Wiley Mill with a mesh with a 1 mm pore size. Alcohol extracts were prepared by extracting plant material at a ratio of 1g plant material to 10 mL of 80% ethanol, in triplicate. The extract was separated from the plant residue via vacuum filtration using Whatman qualitative 1 filter paper with an 11 µm pore size (Whatman plc, Kent, UK). Filtered extracts were combined and ethanol was evaporated using a rotary evaporator at 40°C and then freeze dried to remove the remaining water. Water extracts were prepared by gently boiling plant

material at a ratio of 1g plant material to 10 mL distilled H2O for 1 hour. Water extract was separated from the plant residue via vacuum filtration using Whatman qualitative 1 filter paper with an 11 µm pore size (Whatman plc, Kent, UK). Filtered extracts were dried using a spray dryer. Alcohol and water extracts were homogenized using a motar and pestle.

Anti-inflammatory assays

The anti-inflammatory activity of plant extracts was assessed by measuring TNF-alpha reduction in a LPS stimulated THP-1 monocyte assay. THP-1 cells (human monocyte culture TIB-202, ATCC, Manassas, VA, USA), were cultured in RPMI 1640 media (ATCC, Manassas, VA, USA) supplemented with 1% 0.05 mM beta-mercaptoethanol, 1% penstrep (Invitrogen, Mississauga, ON, Canada) and 10% fetal bovine serum (Invitrogen, Mississauga, ON, Canada), in a 37°C humidified environment with 5% CO^2. Cells were transferred ($3x10^4$ cells/well) in 96-well plate, followed by the addition of plant extract dissolved in 80% EtOH for a final volume of 300 µl/well and a final EtOH concentration 0.5%. Plant extract was assayed at 10 and 100 µg/ml, parthenolide (≥98% purity) (Sigma-Aldrich, St-Louis, MO, USA) was used as a positive control at 1 and 10µg/ml, and 0.5% EtOH was used as a vehicle control. Extract and controls were assayed in quadruplicate. Following the addition of extracts and controls, cells were incubated for 2 hours, and then stimulated with 1 µg/ml LPS purified from E. coli (Sigma-Aldrich, St-Louis, MO, USA) and allowed to incubate for 20 hours. An unstimulated control containing 0.5% EtOH but no LPS was also

assayed. After incubation, cells were centrifuged at 2000 rpm for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. DuoSet® ELISA development kits (R & D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol to measure TNF-alpha levels in cell culture supernatants. Raw TNF-alpha values were transformed to a % activity of the parthenolide 10µg/ml control.

Pro-inflammatory assays

The pro-inflammatory activity of plant extract was assessed by measuring TNF-alpha production in a LPS stimulated THP-1 monocyte assay. THP-1 cells were cultured as described in the anti-inflammatory assay methodology. Cells were transferred ($3x10^4$ cells/well) to a 96-well plate, followed by the addition of plant extract dissolved in 80% EtOH for a final volume of 300 µl/well and a final EtOH concentration 0.5%. Plant extract was assayed at 10 and 100 µg/ml, *Echinacea purpurea* water extract was used as a positive control at 100µg/ml, and 0.5% EtOH was used as a vehicle control. Extract and controls were assayed in quadruplicate. Following the addition of extracts and controls, cells were incubated for 22 hours, and after incubation, cells were centrifuged at 2000 rpm for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. DuoSet® ELISA development kits (R & D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol to measure TNF-alpha levels in cell culture supernatants.

Total Phenolics

The concentration of total phenolics in the extracts was measured using the Folin-Ciocalteau colorimetric reaction as described in Farsi and Lee (2008). Aliquots of 250 µL of extract (1 mg/mL and 4 mg/mL) were added to test tubes containing 1.25 mL of Folin-Ciocalteau (FC) reagent (Sigma Chemical Company, St Louis, Missouri) and mixed by vortex. After 8 min, 1.0 mL of 7.5% sodium carbonate anhydrate reagent (Fisher, Ottawa, ON) was added. Samples were then incubated in darkness at room temperature for 2 h and transferred to clear 96-well plates for spectrophotometric analysis at 725 nm with a SpectraMax M5 microplate reader. For quantitative purposes, concurrently analysed standards of quercetin (25, 50, 100 and 200 µg/mL) were prepared and the absorbances converted to milligrams of quercetin equivalents/g extract.

Total Polysaccharides

Total polysaccharide content was determined by the crude polysaccharide gravimetric assay as described by Brovelli et al (2005). A crude extract was prepared by adding 40 mL of water to 5 g of plant material and incubating this suspension at 90 °C for 1 h in a water bath. Each sample was cooled to room temperature and then centrifuged at 4000 rpm for 15 min. The supernatant was filtered and the volume adjusted to 20 mL with water. Polysaccharide precipitation was induced by the addition of 30 mL of 99% ethanol followed by vortexing and refrigeration for 15 min. The sample was then centrifuged for 15 min at 4000 rpm, and the supernatant was discarded. The pellet was then

resuspended in 8 mL of water. After the addition of 32 mL of 99% ethanol, the sample was vortexed and refrigerated for 15 min. The supernatant was discarded, and the pellet was dried to constant mass at 65 °C. The dry mass was recorded as the crude polysaccharide fraction and expressed in percentage.

Statistics

Statistical analysis was performed with Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Significant differences between treatment and control groups were assessed using one way ANOVAs and a Dunnett's post-hoc test. Significant difference between the mean immunomodulatory activity of two groups was assessed using a Student's t-test. Linear regression was used to model the relationships between total phenolic and total polysaccharide content (independent variables) and the immunomodulatory activity of plant extracts (dependent variable). A p value less than or equal to 0.05 was considered statistically significant. Normality was assessed using the Shapiro-Wilk test.

5.3 – Results

Seventeen plant species belonging to 7 plant families (Table 5.1) were tested for their anti- and pro- inflammatory activity in THP-1 monocytes. The alcohol extracts exhibited dose-dependent anti-inflammatory activity across the 17 species tested (Figure 5.1). The most active species tested were *P. mariana*, *P. banksiana*, and *L. laricina*, with 91.7%, 79.4% and 51.9% of the activity of the 10 µg/mL parthenolide positive control at 100 µg/mL. Fourteen species

demonstrated significant ($p \leq 0.05$) anti-inflammatory activity relative to the stimulated vehicle control at 100 µg/mL, 8 species had significant activity at 10 µg/mL, and the activity of one species, *P. mariana*, was not significantly different

Table 5.1 – Seventeen anti-diabetic plant species used by the Cree of Eeyou Istchee.

Plant family	Plant species	Plant part	Cree Name
Betulaceae	*Alnus incana* (L.) Moench subsp. *rugosa* (Du Roi) R.T. Clausen	bark	Atushpi
Ericaceae	*Gaultheria hispidula* (L.) Muhl.	leaf	Pieuminaan
	Kalmia angustifolia L.	leaf	Uishipukw
	Rhododendron groenlandicum (Oeder) Kron & Judd	leaf (old)	Kachichepukw
	Rhododendron tomentosum (Stokes) Harmaja subsp. *subarticum* (Harmaja) G.D. Wallace	leaf	N/A
	Vaccinium vitis-idaea L.	fruit	Wishichimna
Lycopodiaceae	*Lycopodium clavatum* L.	whole plant	Pashtnahoagin
Pinaceae	*Abies balsamea* (L.) Mill.	bark	Inaasht
	Juniperus communis L.	female cone	N/A
	Larix laricina K. Koch	bark	Watnagan
	Picea glauca (Moench) Voss	leaf	Minhikw
	Picea mariana (Mill.) BSP	female cone (opened and closed)	Inaahtikw
	Pinus banksiana Lamb.	female cone (closed)	Ushchishk
Rosaceae	*Sorbus decora* C.K. Schneid.	bark	Mushkuminanatikw
Salicaceae	*Populus balsamifera* Lamb.	bark	N/A
	Salix planifolia Pursh subsp. *planifolia*	bark	Pieutikw
Sarraceniaceae	*Sarracenia purpurea* L.	leaf	Ayigadash

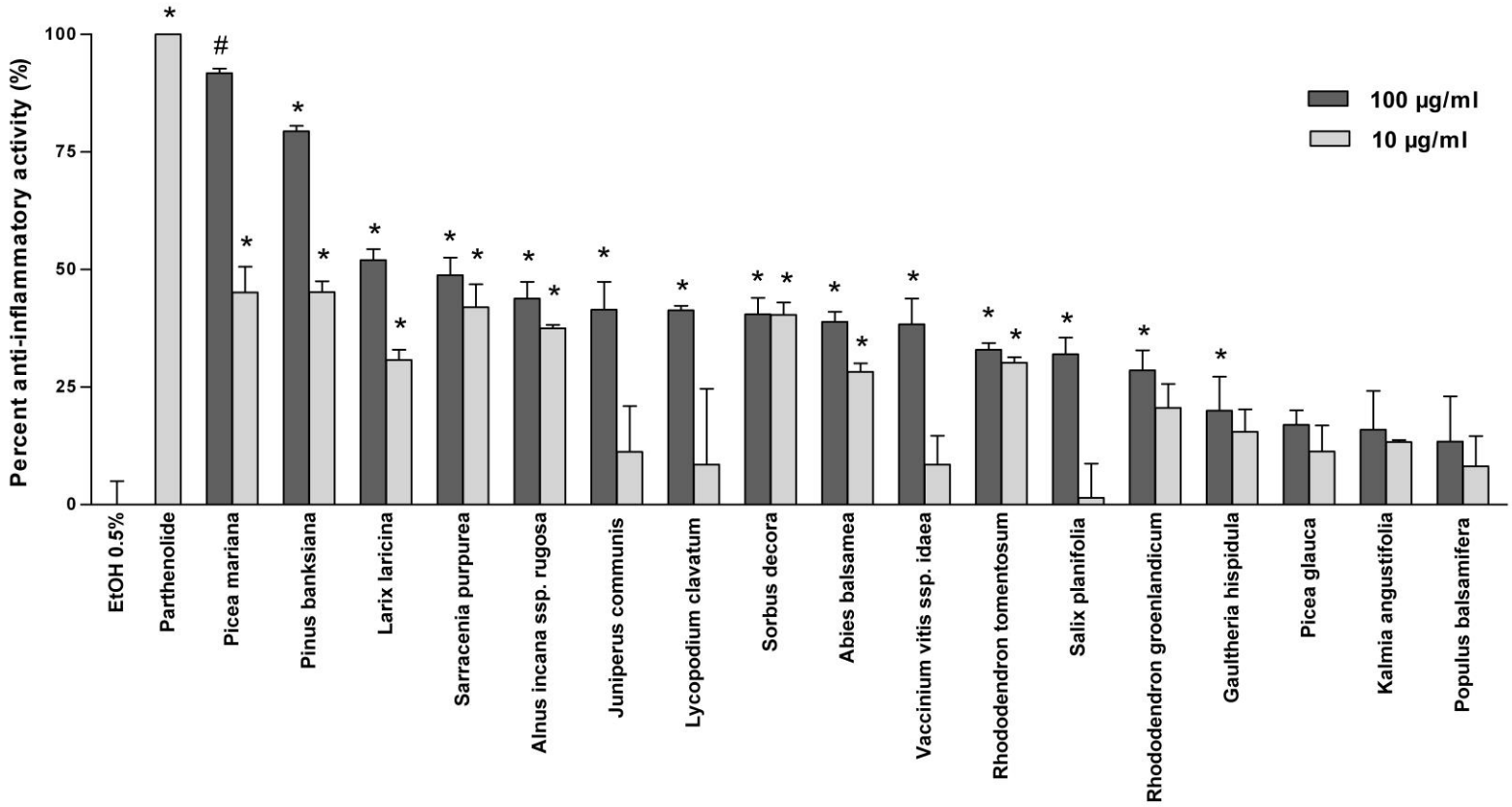

Figure 5.1 – Percent anti-inflammatory activity (%) of ethanol (80%) extracts of anti-diabetic plant species used by the Cree of Eeyou Istchee relative to the parthenolide control (10 µg/mL) in LPS-stimulated THP-1 monocytes. Extracts were tested at 10 and 100 µg/mL. The average activity + SE is plotted, N=4. Species are ranked from most to least active at 100 µg/mL. * indicates activity significantly different ($p \leq 0.05$) from the 0.5% EtOH control and # indicates activity not significantly different ($p > 0.05$) from the parthenolide control.

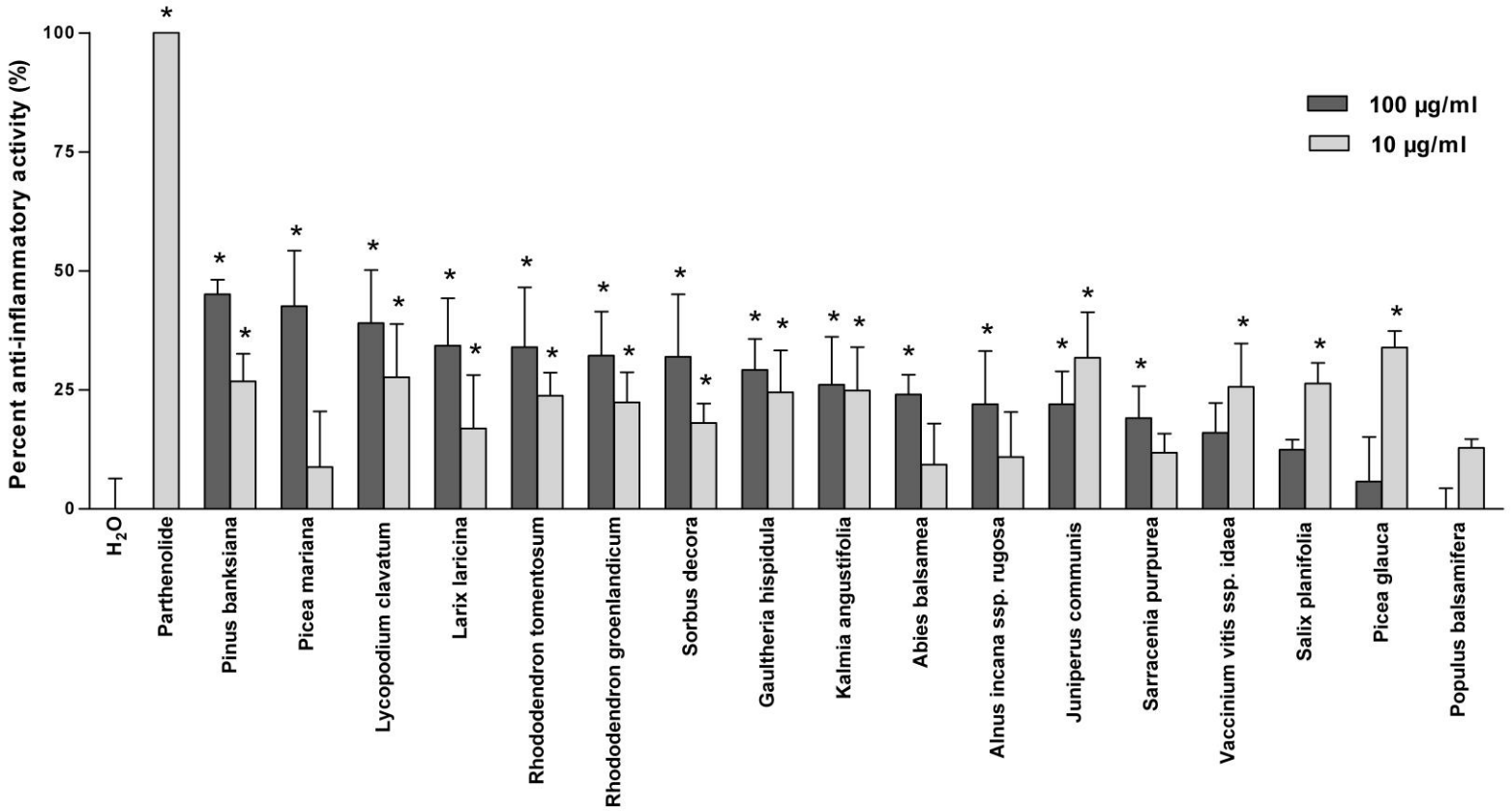

Figure 5.2 – Percent anti-inflammatory activity (%) of water extracts of anti-diabetic plant species used by the Cree of Eeyou Istchee relative to the parthenolide control (10 µg/mL) in LPS-stimulated THP-1 monocytes. Extracts were tested at 10 and 100 µg/mL. The average activity + SE is plotted, N=4. Species are ranked from most to least active at 100 µg/mL. * indicates activity significantly different ($p \leq 0.05$) from the 0.5% H_2O control and # indicates activity not significantly different ($p > 0.05$) from the parthenolide control.

($p > 0.05$) from the parthenolide control. The least active species were *P. glauca*, *K. angustifolia*, and *P. balsamifera*, with 17.0%, 15.9%, and 13.4% of the activity of the parthenolide positive control, respectively. The anti-inflammatory activity of the water extracts was also evaluated, with the three most active species being *P. banksiana*, *P. mariana*, and *L. clavatum*, with 45.1%, 42.6%, and 39.0% of the 10 µg/mL parthenolide positive control at 100 µg/mL, respectively (Fig. 5.2). Thirteen species assayed demonstrated significant anti-inflammatory activity relative to the stimulated vehicle control at 100 µg/mL, and 12 species had significant activity at 10 µg/mL. The least active anti-inflammatory species were *S. planifolia*, *P. glauca*, and *P. balsamifera*, with 12.5%, 5.8%, and 0% of the activity of the positive control, respectively. When comparing the average activity of the alcohol extracts to the average activity of the water extracts, the averaged anti-inflammatory activity is not significantly different between the two preparations at 10 µg/mL concentrations, but when comparing the averaged activity at 100 µg/mL concentrations, the activity of the alcohol extacts (53.4%) is significantly greater ($p \leq 0.05$) than the activity of the water extracts (29.0%).

The alcohol and water extracts of the 17 species were also assayed for their pro-inflammatory activity in THP-1 monocytes. The alcohol extracts exhibited only moderate pro-inflammatory activity (Figure 5.3). The most active species assayed were *L. laricina*, *P. clavatum*, and *S. decora*, demonstrating 50.2, 44.3, and 35.3 % of the activity of the 100 µg/mL *Echinacea purpurea* positive control, respectively. Only the activity of *L. laricina* was significantly

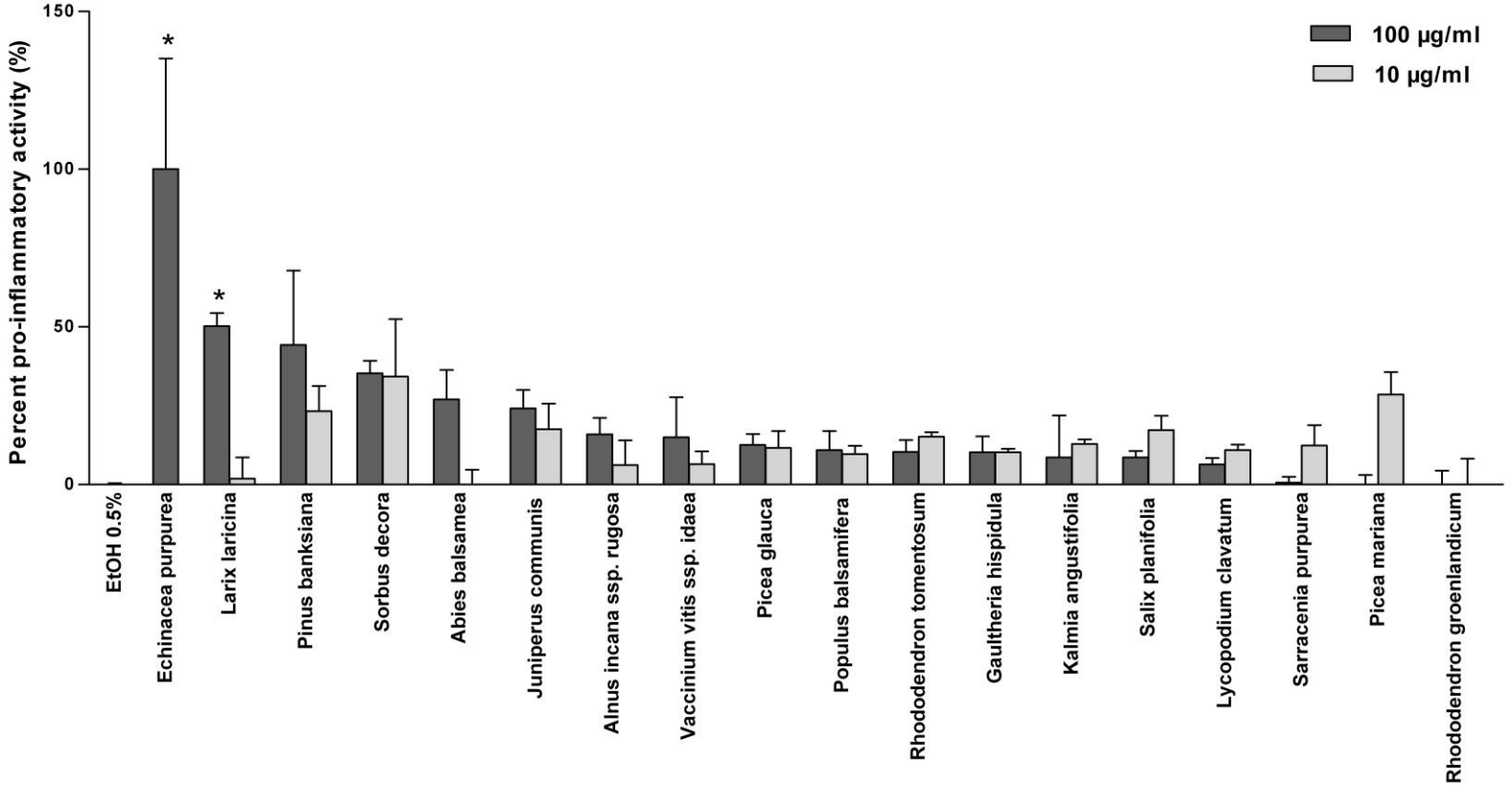

Figure 5.3 – Percent pro-inflammatory activity (%) of ethanol (80%) extracts of anti-diabetic plant species used by the Cree of Eeyou Istchee relative to the *Echinacea purpurea* water extract control (100 µg/mL) in THP-1 monocytes. Extracts were tested at 10 and 100 µg/mL. The average activity + SE is plotted, N=4. Species are ranked from most to least active at 100 µg/mL. * indicates activity significantly different ($p \leq 0.05$) from the 0.5% EtOH control.

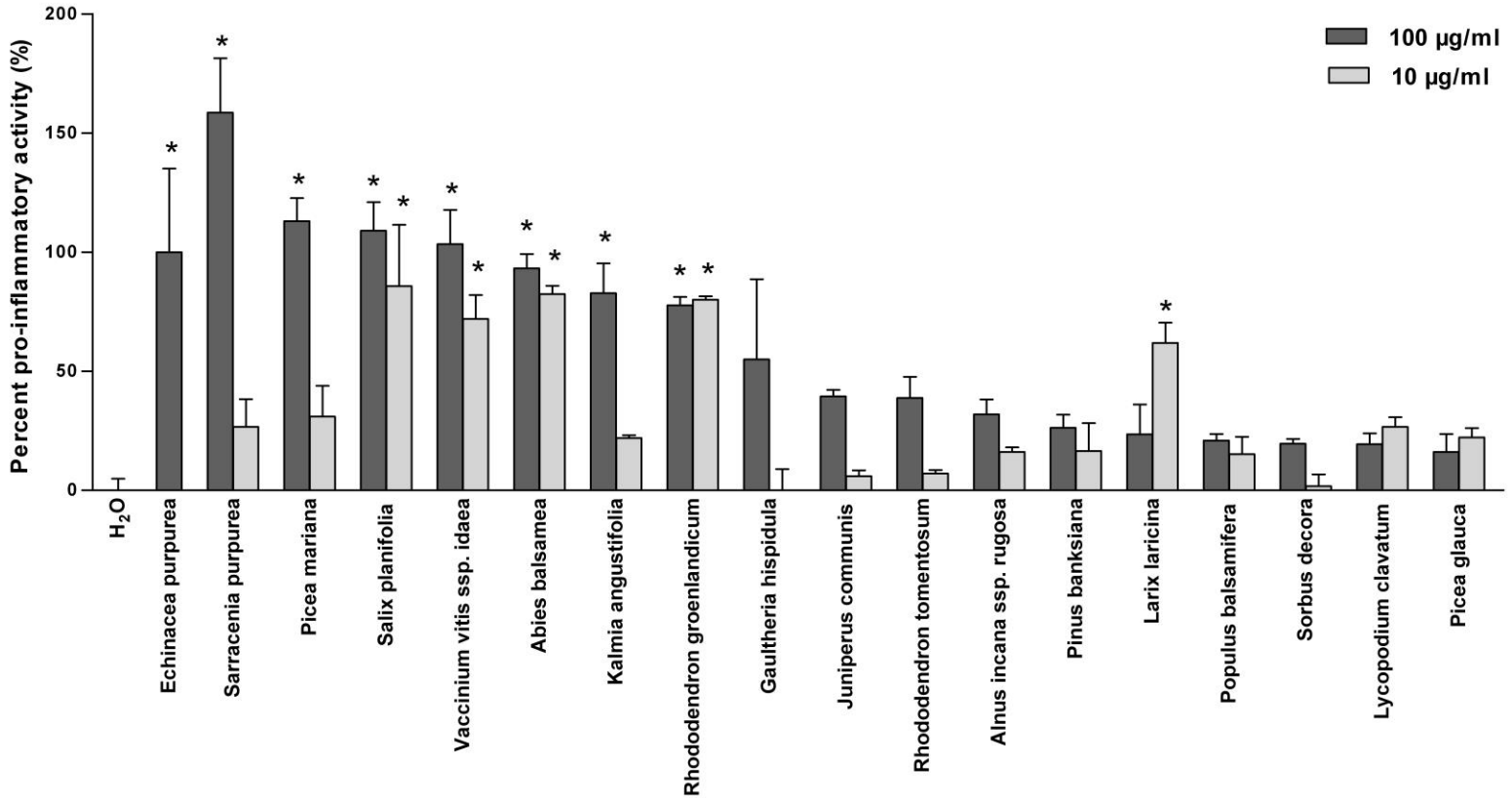

Figure 5.4 – Percent pro-inflammatory activity (%) of water extracts of anti-diabetic plant species used by the Cree of Eeyou Istchee relative to the *Echinacea purpurea* water extract control (100 µg/mL) in THP-1 monocytes. Extracts were tested at 10 and 100 µg/mL. The average activity + SE is plotted, N=4. Species are ranked from most to least active at 100 µg/mL. * indicates activity significantly different ($p \leq 0.05$) from the H_2O control.

different from the vehicle control ($p \leq 0.05$). The least active species tested were *S. purpurea*, *R. groenlandicum*, and *P. mariana*, which did not have any significant activity at 100 µg/mL relative to the vehicle control. The pro-inflammatory activity of the water extracts was also evaluated, with the three most active species being *S. purpurea*, *P. mariana*, and *S. planifolia*, with 158.5%, 113.1%, and 109.0% of the 100 µg/mL *Echinacea* positive control at 100 µg/mL, respectively (Fig. 5.4). Seven species demonstrated significant ($p \leq 0.05$) pro-inflammatory activity at the highest concentration tested, and 5 at the lowest concentration tested. The least active pro-inflammatory species were *S. decora*, *L. clavatum*, and *P. glauca*, with 19.6%, 19.4%, and 16.1% of the activity of the positive control, respectively. When comparing the average activity of the alcohol extracts to the average activity of the water extracts, the activity of the water extracts is significantly greater ($p \leq 0.05$) than the alcohol extracts at concentrations of 10 and 100 µg/mL. Water extracts tested at 10 µg/mL have an average activity of 33.7% compared to the alcohol extracts at 12.8%, and at 100 µg/mL, at water extracts had an average activity of 60.5% compared to 16.5% for the alcohol extracts. The pro-inflammatory activity of polysaccharide fractions from the 17 Cree plants was also evaluated (Fig 5.5). The most active polysaccharide fractions were *S. purpurea*, *J. communis*, and *L. clavatum*, which displayed 34.5%, 31.6% and 21.0% of the activity of the *E. purpurea* positive control, respectively, when assayed at 100 µg/mL. The activity of these three species was significantly different from the vehicle control ($p \leq 0.05$).

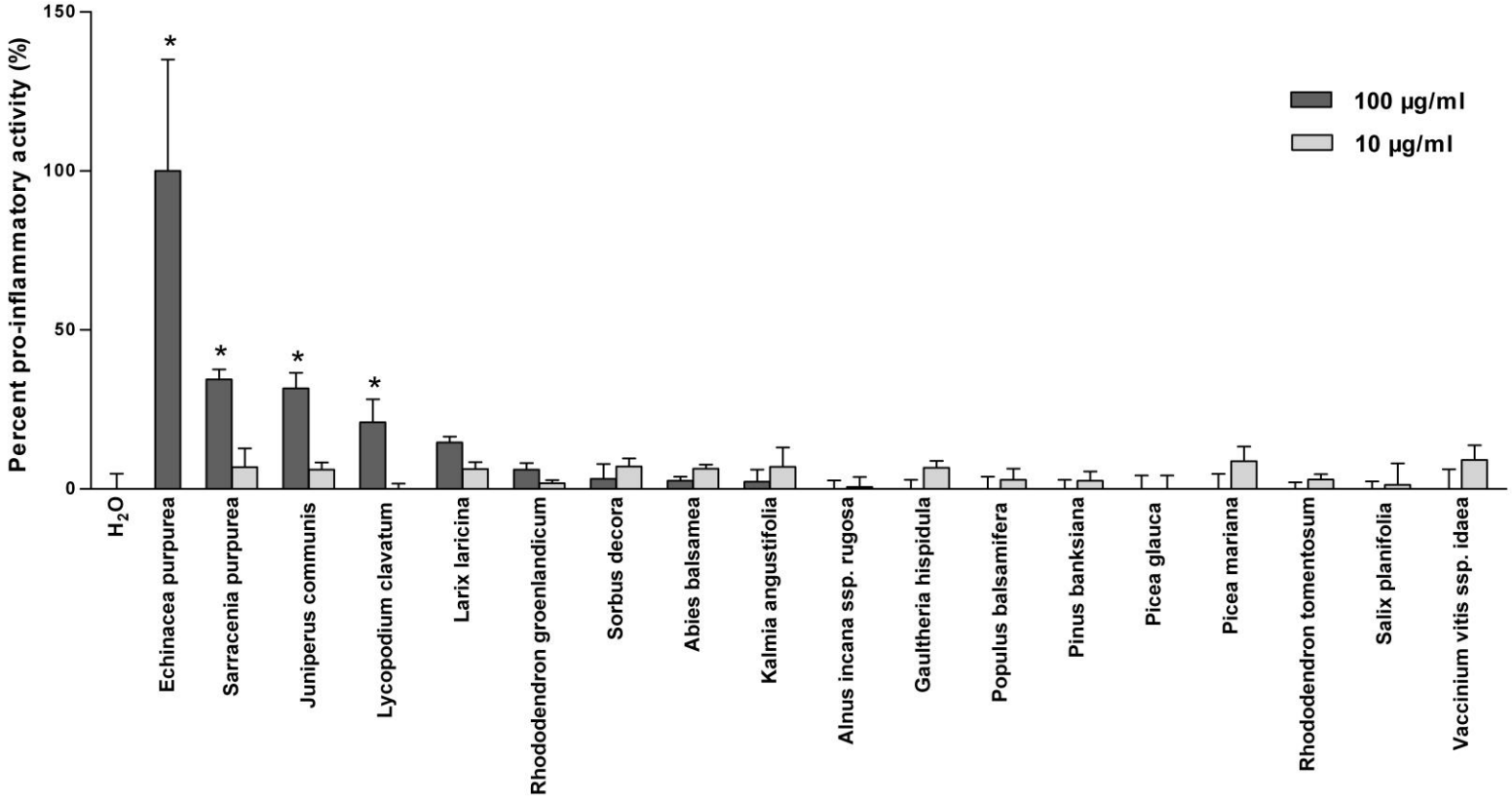

Figure 5.5 – Percent pro-inflammatory activity (%) of polysaccharide extracts of anti-diabetic plant species used by the Cree of Eeyou Istchee relative to the *Echinacea purpurea* water extract control (100 µg/mL) in THP-1 monocytes. Extracts were tested at 10 and 100 µg/mL. The average activity + SE is plotted, N=4. Species are ranked from most to least active at 100 µg/mL. * indicates activity significantly different ($p \leq 0.05$) from the H_2O control.

The total phenolic content and the polysaccharide content of the seventeen Cree species were measured as a crude metric of overall phytochemical composition. Regarding the total phenolic content, the average phenolic content of the alcohol extracts (319.9 µg/g) was significantly greater ($p \leq 0.05$) than the average phenolic content of the water extracts (141.4 µg/g). *P. mariana*, *A. incana*, and *G. hispidula* had the highest phenolic content in the alcohol extracts, and *P. mariana*, *S. planifolia*, and *K. angustifolia* had the greatest phenolic content for the water extracts. The three species with the lowest phenolic content for the water and alcohol extracts were the same, *V. vitis-idaea*, *L. clavatum*, and *J. communis*. The polysaccharide content was highest in *L. laricina* (14.9%), followed by *P. banksiana* (8.9%), and *P. balsamifera* (7.1%), and lowest in *R. groenlandicum* (2.0%), *K. angustifolia* (2.0%), and *P. glauca* (1.5%).

In order to assess the extent to which plant extract phenolic content and polysaccharide content can explain the immunomodulatory activity of CEI anti-diabetic therapies, a linear regression analysis was conducted between the observed anti- and pro-inflammatory activity of alcohol and water extracts and their respective crude phytochemical metrics. A regression between anti-inflammatory activity at 100 µg/mL and the total phenolic content of both alcohol and water extracts was significant, which indicates a positive linear relationship between the phenolic content of each extract and the observed anti-inflammatory activity (Table 5.2). When examining the relationship between the anti-inflammatory activity of alcohol and water extracts and their phenolic content

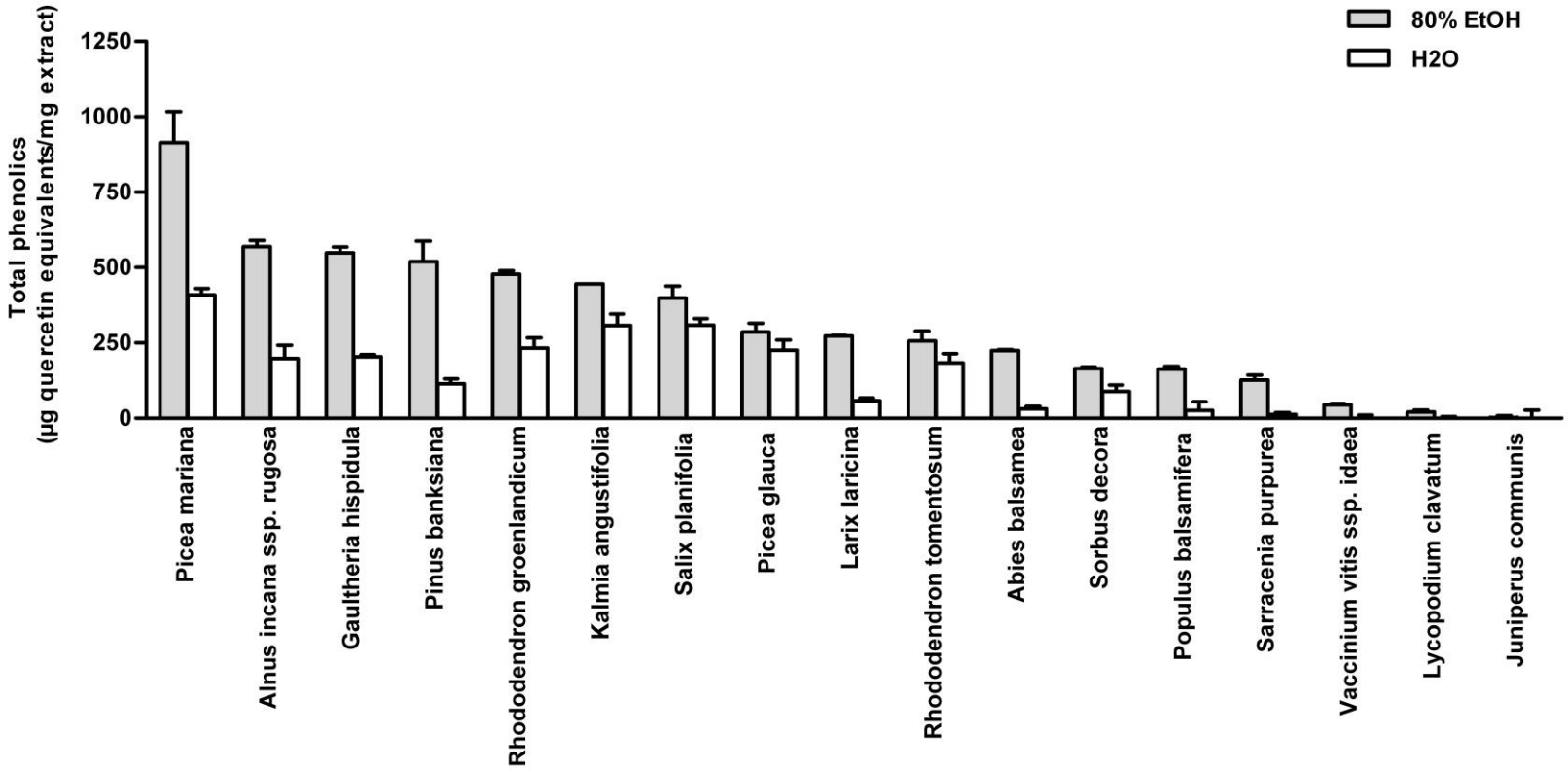

Figure 5.6 – Total phenolic content of ethanol (80%) and water extracts of anti-diabetic plant species used by the Cree of Eeyou Istchee. Average phenolic content (ug quercetin equivalents / mg extracts) + SE are presented, N=3. Plant species are ranked by their phenolic content in ethanol extracts.

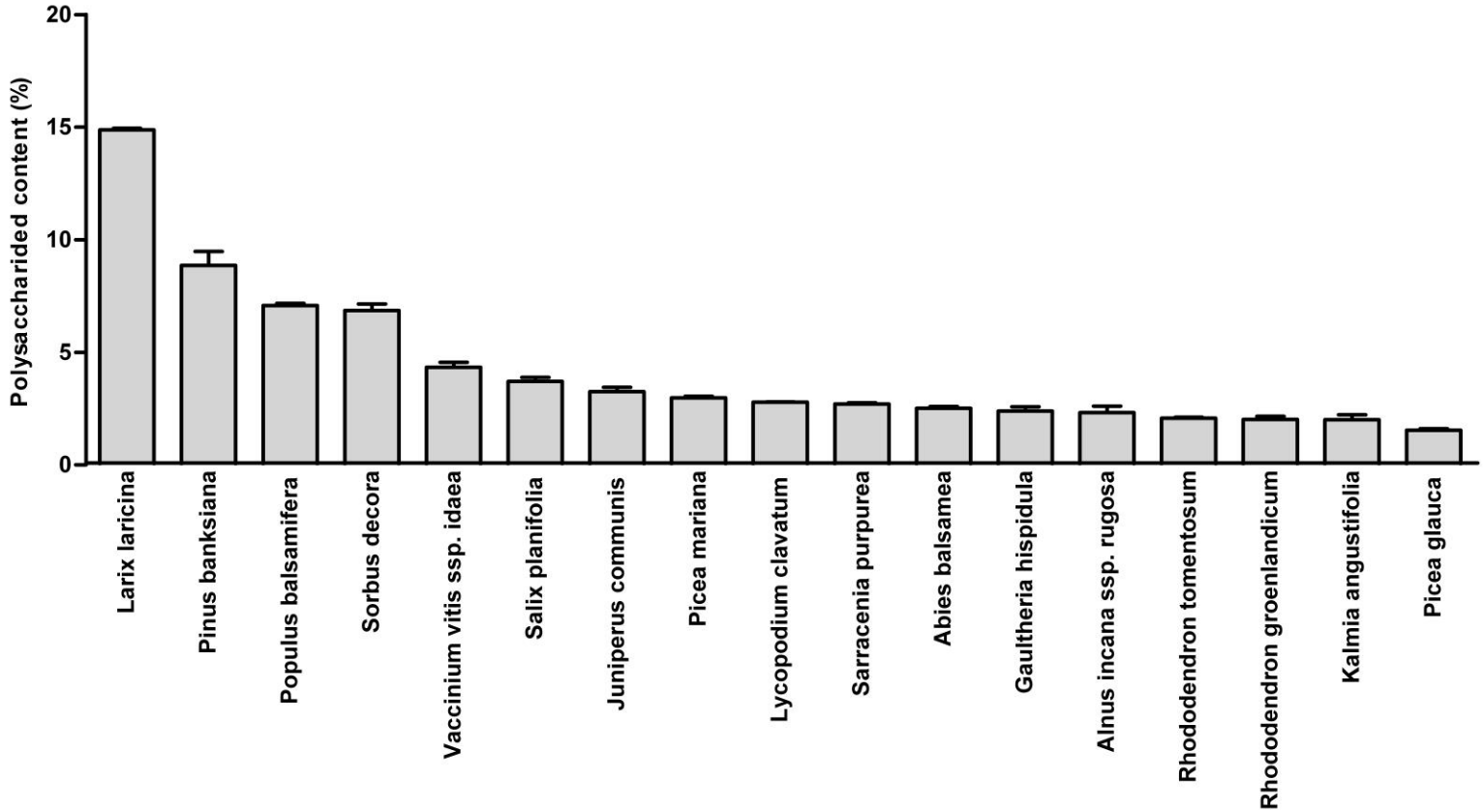

Figure 5.7 – Polysaccharide content in water extracts of anti-diabetic plant species used by the Cree of Eeyou Istchee. Average percent polysaccharide content (%) + SE are presented, N=3. Plant species are ranked by their polysaccharide content.

separately, only the alcohol extract regression was significant. Significant regressions were found between the anti-inflammatory activity of both alcohol and water extracts and the total phenolic content of Pinaceae species. The regression between the anti-inflammatory activity of alcohol extracts of Pinaceae and their phenolic content was significant, however the regression with the water extracts of Pinaceae was not. No significant positive regressions with the phenolic content in Ericaceae species were found. Taken together, and given the large R^2 value (0.60) for the Pinaceae alcohol extract regression, the relationship between the anti-inflammatory activity of Cree extracts and their phenolic content is primarily due to the strong relationship seen in the Pinaceae alcohol extracts. Regressions between pro-inflammatory activity and the total polysaccharide content as well as the pro-inflammatory activity of the polysaccharide fractions were not significant.

Table 5.2 – Linear regressions of anti-inflammatory activity (100 µg/mL treatment) versus total phenolic content. R^2 values, and P-values for each regression are presented. Significant regressions ($p \leq 0.05$) are in bold. No significant positive regressions with the phenolic content in Ericaceae species were found.

Regression	R^2	P
100 µg/mL activity vs. total phenolics (EtOH and H_2O extracts)	0.17	**< 0.001**
100 µg/mL activity vs. total phenolics (EtOH extracts)	0.14	**0.0016**
100 µg/mL activity vs. total phenolics (H_2O extracts)	0.02	0.27
100 µg/mL activity vs. total phenolics (Pinaceae EtOH and H_2O extracts)	0.52	**< 0.0001**
100 µg/mL activity vs. total phenolics (Pinaceae EtOH)	0.60	**< 0.0001**
100 µg/mL activity vs. total phenolics (Pinaceae H_2O extracts)	0.03	0.39

5.4 – Discussion

The most anti-inflammatory species used by the Cree of Eeyou Istchee were the female cones of *Picea mariana* and *Pinus banksiana*, in both alcoholic and water preparations. This is the first report of the potent anti-inflammatory activity of the cones of these two species. Several species used by the CEI have been shown to possess anti-inflammatory activity in previous studies. *Lycopodium clavatum* has demonstrated anti-inflammatory activity in mice, with an alkaloid-rich fraction high in lycopodine being the most active fraction tested (Orhan et al, 2007). This species displayed anti-inflammatory activity in both 80% EtOH and water extracts, and was the third most active water extract assayed. The results presented in this chapter demonstrate that the alcohol and water extracts of *Rhododendron groenlandicum* and *Rhododendron tomentosum* possess significant anti-inflammatory activity. *R. groenlandicum* was previously shown to have a moderate inhibition of nitric oxide production in LPS-stimulated RAW 264.7 macrophages (Dufour et al, 2007) and a review of *R. tomentosum* concludes that this species possess anti-inflammatory activity in vitro and in vivo which is partially due to its rich polyphenolic content (Dampc and Luczkiewicz, 2013). In addition, the female cones of *Juniperus communis* are anti-inflammatory in carrageenan-induced mice paw edema (Akkol et al, 2009). Taken together, these results are comparable to the anti-inflammatory actions demonstrated by the CEI species tested in this study.

The high phenolic content of extracts from medicines used traditionally by the Cree partially explains the anti-inflammatory activity of these species. This

relationship is especially strong with members of the Pinaceae, however little research in to the anti-inflammatory principles of these species has been carried out. Numerous phenolics have been reported in species used by the CEI, with much of the phytochemical analysis focusing on members of the Ericaceae. Although no significant relationship between the anti-inflammatory activity and the total phenolic content of these species was observed, individual phenolic constituents with well established anti-inflammatory activities may contribute to their activity. In particular, (+)-catechin, (-)-epicatechin, chlorogenic acid, quercetin deriviatives such as quercetin-3-O-glucoside and quercetin-3-O-galactoside, *p*-coumaric acid, and procyanidins A1 and A2 are major phenolic constituents of Ericaceae species such as *Gaultheria hispidula*, *Kalmia angustifolia*, *Rhododendron groenlandicum*, *Rhododendron tomentosum*, and *Vaccinium vitis-idaea* (Black et al, 2011; Beaulieu et al, 2010; Saleem et al, 2010). All of these phenolic compounds have reported anti-inflammatory activity in various models of inflammation (Hamalainen et al, 2007; Wang and Mazza, 2002). Also, *Salix planifolia* is reported to contain salicylic acid, the natural precursor to acetylsalicylic acid (aspirin) and is a well know anti-inflammatory agent (Drummond et al, 2013; Yin et al, 1998).

The pro-inflammatory activity of boreal plant species has received less attention in the literature. The most pro-inflammatory alcohol extract was *Larix laricina*, and the most pro-inflammatory water extracts were *Sarracenia purpurea*, *Picea mariana*, and *Salix planifolia*. This is the first report of the pro-inflammatory activity of these three water extracts, however the pro-inflammatory activities of

L. laricina have been reported previously, although its immunostimulatory properties have been attributed to highly water soluble arabinogalactans which are most likely present in smaller amounts in the 80% EtOH extracts. The polysaccharide fraction of *L. laricina* was the most pro-inflammatory Cree plant polysaccharide fraction, which is supported by previous reports of the immunomodulatory properties of *L. laricina* polysaccharides such as arabinogalactans (Kelly, 1999; Kim et al, 2002).

When comparing the activity of 80% EtOH and water extracts, 80% EtOH extracts generally display more anti-inflammatory activity than their aqueous counterparts, and the water extracts generally display more pro-inflammatory activity than alcohol extracts. This is not surprising given the potent anti-inflammatory activities of phenolics which are present in greater quantities in alcoholic extracts and the well know pro-inflammatory activities of polysaccharides found in water extracts. However, as the regressions between immunomodulatory activity and crude phytochemical metrics (total phenolic content and total polysaccharide content) were only strongly predictive for the relationship between the anti-inflammatory activity of 80% EtOH Pinaceae extracts and their phenolic content, and didn't explain much of the immunomodulatory activity observed elsewhere, it is clear that much of the activitiy of the 17 Cree species cannot simply be explained by a single class of compounds. The pharmacodynamics of the competing and synergistic properties of anti- and pro-inflammatory compounds found in the same crude extract are no

doubt complex and need to be studied in more detail to elucidate the contribution of various phytochemicals to the overall observed immunomodulatory effect.

Given the results presented in this chapter, and taking into account the traditional use of these species in the treatment of T2D, using alcoholic formulations of Cree species, although not necessarily a practical solution, may be useful to increase the anti-inflammatory action of these species in vivo. As T2D has an important inflammatory component that is a relevant target in the treatment of this chronic illness, increasing the anti-inflammatory activity of these species may have a therapeutic benefit. Additionally, while the immunostimulatory properties of CEI water extracts may be beneficial in the treatment of colds and flues, it is unclear how pro-inflammatory actions of medicinal plants would affect the pathophysiology of T2D, and species with potent pro-inflammatory activity should be used with caution. Ideally, more research into the in vivo effects of anti- and pro-inflammatory plant extracts is required to better understand the role of immunomodulation in Type-II diabetes.

Elucidating the active immunomodulatory principles, through bioassay guided isolation, in CEI medicinal species is the first step in further research into the ethnopharmacology of Cree medicines, and the anti-inflammatory compounds found in the cones of *Picea mariana*, the most anti-inflammatory alcoholic extract tested, will be the focus of the next chapter (Chapter 6).

Chapter 6

Bioassay guided isolation of anti-inflammatory compounds from an anti-diabetic Cree medicine: *Picea mariana* (Mill.) BSP.

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Statement of author contribution

BWR and JTA conceived and designed this study. Plant identification and collection was undertaken by AC. Extraction and open column chromatography were carried out by BWR and BR. BWR completed the isolation with Preparative-scale HPLC and verified isolate purity with analytical-scale HPLC with the assistance of AA. JAG identified isolated compounds by NMR. BWR and BR undertook the anti-inflammatory, total phenolics, and cytotoxicity assays. PSH is the manager off the CIHR TAAM project, and BR and JTA contributed to manuscript preparation.

6.1 - Introduction

In a previous study, 17 anti-diabetic Cree plants were assessed for their anti-inflammatory activity in an LPS stimulated THP-1 monocyte assay of TNF alpha production (Chapter 5). The most active traditional medicine was *Picea mariana* (Pinaceae) cones, with the 80% ethanol extract tested at 100 µg/ml displaying 92.4% of the anti-inflammatory activity of the 10 µg/ml parthenolide positive control. Black spruce (called Inaahtikw by the Cree of Eeyou Istchee) is traditionally prepared as an infusion of cones and is consumed as a medicinal tea. In an ethnobotanical survey of anti-diabetic plants used by the Cree of Eeyou Istchee, *P. mariana* cones were used by 8 out of 34 traditional healers surveyed in the treatment of 5 of 15 Type II diabetic symptoms (Leduc, 2006). Black spruce cones were ranked 4th out of the 17 anti-diabetic plants described in this survey.

Among other aboriginal groups, an infusion of black spruce branch tips are used for “healing the insides” by the Algonquin (Mishinabeg First Nations), and needles or pitch used as a topical treatment for skin condition by woodlands Cree (Moerman, 1998). The cones are also reportedly used by the woodlands Cree in the treatment of sore mouth, sore throats, and toothaches. Black spruce is also one of the possible plants used by the St. Lawrence Iroquois to treat Cartier’s men for scurvy during the winter of 1535-1536, owing to its high vitamin C content (Arnason et al, 1981) although some sources identify other conifers such as white spruce (*Picea glauca*) and northern white cedar (*Thuja occidentalis*).

Most of the published literature on *Picea mariana* has focused on the phytochemistry of the wood, bark and leaves of this plant, and its anti-oxidant activity. Black spruce wood extracts contain primarily monoterpenes and diterpenes, with the sum of α-pinene, manool, neoabienol and (2)-abienol making up 74.6% and 82.3% of the composition of heartwood and sapwood hexane extracts, respectively (Pichette et al, 1998). The phytochemical composition of *P. mariana* bark is complex, with stilbenes, tannins, and proanthocyanidins reported as major secondary metabolites in this tissue (Anderson and Pigman, 1947; Manners and Swan, 1971; Diouf et al, 2009). Hot water extracts of black spruce bark rich in proanthocyanidins displayed strong anti-oxidant activity *in vitro* (Diouf et al, 2009). Saponified *P. mariana* needle wax contains more than 12 fatty acids (Beri and Lemon, 1970), and essential oil extracted from leaf material contains a mixture of terpenoids and phenylpropane derivatives, with bornyl acetate (41.2%) and camphene (24.9%) being its two main constituents (Hajji, 1984). The essential oil of this species is used by modern day herbalists and aromatherapists for treatment of various skin conditions, coughing, and depression (Chartier, 2009; Lawless, 1995). Although there are a number of publications of *P. mariana* phytochemistry, there is little published on the phytochemistry of its cones. It is interesting that ethnobotany has directed attention to this otherwise little studied plant part. The objective of the present study was to isolate the anti-inflammatory principles of *P. mariana* cones using a bioassay assay guided approach, employing open column chromatography,

preparative-scale and analytical-scale HPLC, and NMR to identify the anti-inflammatory phytochemicals.

6.2 - Materials and Methods

Plant Extraction

Picea mariana cones (1778 g) were ground in a Wiley mill using a mesh with a 1 mm pore size and extracted in 10 L of 80% ethanol in triplicate. The extract was separated from the plant residue via vacuum filtration using Whatman qualitative 1 filter paper with an 11 µm pore size (Whatman plc, Kent, UK). Filtered extracts were combined and ethanol was evaporated using a rotary evaporator at 40°C and then freeze dried to remove the remaining water. Lyophilized extract was homogenized using a motor and pestle, yielding 394.3 g of extract for a percent yield of 22.2%.

Open column fractionation

Open column chromatography was conducted using a 3 L glass column packed with deactivated silica (SiO_2) and loaded with 250 g of homogenized extract. Normal phase column chromatography was carried out using the following solvent gradient: Hex:EtOAc (1:1, 4:6, 3:7, 2:8, 1:9), EtOAc, EtOAc:MeOH (95:5, 9:1, 8:2, 7:3, 6:4, 1:1), MeOH and MeOH:H_2O (9:1; 8:2). Two full column volumes were added for every solvent gradient for a total of 6 L of solvent per phase. Primary fractions were collected in 300 mL aliquots from MeOH:H_2O 1:1 to EtOAc:MeOH 8:2, and then in 500 mL aliquots from EtOAc:MeOH 7:3 to MeOH:H_2O 8:2, yielding 180 fractions that were subjected to

thin layer chromatography (TLC) and HPLC-DAD analysis in order to pool those which exhibited similar phytochemical profiles to generate 14 pooled primary fractions. Pooled primary fractions were dried and homogenized using a motar and pestle.

HPLC-DAD Analysis

The HPLC-DAD analyses were performed on an Agilent 1100 Series system consisting of an autosampler with a 100 µL built in loop, a quaternary pump with a maximum pressure of 400 bars, a column thermostat and a diode array detector. All solvents were HPLC grade quality (Fisher Scientific, CA, USA). For HPLC-DAD analysis the solvents were sonicated at 5 min/L prior to analysis and the column (LUNA C8, 250 × 4.6 mm, particle size 5 micron, Phenomenex, Torrance, CA, USA) was equilibrated for 15 min with the starting conditions prior to analysis. The mobile phase consisted of A = water and B = acetonitrile. The optimal elution conditions for *Picea mariana* cone extract and its fractions were a linear gradient of 15–65% B in 15 min followed by increasing B up to 100% in 0.1 min and maintaining isocratic conditions for 4.9 min. The column was brought back to initial conditions in 0.1 min and maintained at isocratic conditions for 10 min, followed by an 8 min equilibration period prior to subsequent injections (total run time 38 min). The column operated at a flow rate of 1.5 mL/min and column temperature was maintained at 55 °C. One mL of *P. mariana* cone extract and its fractions was filtered though 0.22 µm pore size PTFE syringe tip filters and 1 µL of each extract were injected into the HPLC system though the autosampler.

Monitoring wavelengths of 210 nm, 280 nm, 320 nm and 410 nm with a bandwidth kept at 4 were used for storing the spectra of the extract and the fractions. After the analysis the integration parameters were optimized to obtain reliable baseline separation and peak selection. Five spectra per peak were recorded at the up-slope, apex and down-slope of each peak within the UV wavelength range of 200–400 nm, step size 0.2 sec and a threshold of 1 mAU.

To identify peaks in the extract and fractions, chromatograms were compared with that of 22 plant secondary metabolites, selected following a literature review, of which a 1 µL of a 1 mg/mL solution of each individual standard was chromatographed using the same method as the fractions. The UV spectrum of each standard was used to create a UV library in order to perform peak matches with black spruce extract and fraction peaks eluting at similar retention times. The matching criterion was based on a multiple search choices option. The spectral matching conditions were set so that the threshold for each peak was based on its individual signal to noise ratio. For matching unknown spectra to a reference spectrum the threshold to classify a peak as being a match was set at 990 (corresponds to 99% similarity). The peak purity level was calculated with average spectra and a similarity curve was used to confirm the purity with a threshold to classify a peak as pure set at 990 (99% purity).

Preparative-scale HPLC-DAD isolation

The preparative scale HPLC purification of isolates was undertaken on a reverse phase Gemini Axia column 250 mm × 21.2 mm internal diameter, particle

size 10 microns (Phenomenex Inc., Torrance, CA, USA). The Agilent 1200 Series preparative HPLC system (Agilent Technologies, Montreal, QC, Canada) consisted of a binary pump (flow rate range 5–100 ml/min), an autosampler with a 2 mL loop, a diode array detector with a flow cell (path length 3 mm and maximum pressure limit 120 bars) and a fraction collector (40 mL collection tubes). IR spectra were recorded using a Shimadzu 8400-S FT/IR spectrometer. Optical rotations were assessed on a Perkin-Elmer 241 digital polarimeter. Peak isolation was carried out using a 40 min linear gradient of 40-45% of acetonitrile in water + 0.1% TFA at a flow rate of 31.5 mL/min, and at a monitoring wavelength of 210 nm, band width 4, reference off. Isolate purity was verified on an analytical scale HPLC-DAD using the same conditions described in *7.2.3*. Preparative scale HPLC purification was repeated until isolate purity was 90% or greater, calculated as a percent of the total chromatogram integration at 210 nm.

NMR compound structure elucidation

NMR spectra were recorded on a Bruker Avance 400 spectrometer in deuterated chloroform ($CDCl_3$), at 400 MHz for ^{1}H and 100 MHz for ^{13}C.

Total Phenolics

The total phenolics assay was carried out as described previously (Al-Farsi and Lee, 2008). Briefly, 80 µL of each fraction was transferred into 1.5 mL eppendorf tubes which contained 400 µL of Folin-Ciocalteu reagent (Sigma Chemical Company, St. Louis, Missouri) to obtain concentrations of 50, 100, 200,

and 400 µg/mL. A six point quercetin (Sigma Chemical Company, St. Louis, Missouri) standard curve was generated using concentrations of 50, 100, 200, 300, 400 µg/mL, and a solvent blank. After 5 minutes of incubation at room temperature 270 µL of 7.5% $NaHCO_3$ (Fisher, Ottawa, ON) was added. Subsequently, the mixture was incubated in the dark for 2h at room temperature and transferred to clear 96-well plates for spectrophotometric analysis using a microplate reader measuring the absorbance at 725 nm.

Cell culture

THP-1 cells, ATCC cell culture TIB-202, (ATCC, Manassas, VA), an human immortal monocyte line, were cultured in RPMI 1640 media (ATCC, Manassas, VA) with 1% 0.05 mM beta-mercaptoethanol, 1% penstrep (Invitrogen, Mississauga, Ontario) and 10% fetal bovine serum (Invitrogen, Mississauga, Ontario), in a 37°C humidified environment with 5% CO_2.

Anti-inflammatory assay

The bioassay was carried out as described previously (Zhao, 2005) with several modifications. Briefly, the anti-inflammatory activity of plant extract was assessed by measuring TNF-α reduction in a LPS stimulated THP-1 monocyte assay. 3×10^4 THP-1 monocytes were added to the wells of a V-bottom polystyrene 96-well plate (Corning Incorporated, NY), followed by the addition *P.mariana* extract, fractions, or compounds dissolved in 80% EtOH for a final volume of 300 µl/well and a final EtOH concentration 0.5%. Ethanol was used in

the unstimulated and negative control and parthenolide (≥98% purity) (Sigma-Aldrich, St-Louis, MO, USA) 10 µg/mL (40 µM) was used as a positive control. Plant extract and fractions were assayed at 10 and 100 µg/ml, and pure compounds were assayed at 0.4, 4, 10, and 40 µM. All extracts and controls were assayed in triplicate. Following the addition of samples and controls, cells were incubated for 2 hours, and then stimulated with 1µg/ml LPS purified from *E. coli* (Sigma-Aldrich, St-Louis, MO, USA) and allowed to incubate for 20 hours. An unstimulated control containing 0.5% EtOH but no LPS was also assayed. After incubation, cells were centrifuged at 2000 rpm for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. Human TNF-α DuoSet® ELISA Development kits (R & D Systems, Minneapolis, Minnesota) were used according to the manufacturer's protocol to quantify TNF-α levels in cell culture supernatants from the anti-inflammatory monocyte bioassay. In order to accurately compare the activity of plant extract and fractions across all ELISA plates, raw TNF-α values (pg/ml) were transformed into the percent TNF-α reduction relative to the activity of the 10 µg/mL parthenolide positive control.

Cytotoxicity Assay

CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wisconsin), which examines the release of lactate dehydrogenase as an indicator of cell viability, was used to measure the cytotoxicity of *P. mariana* extract and fractions.

Statistics

Statistical analysis was performed with Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Significant differences between treatment and control groups were assessed using one way ANOVAs and a Dunnett's post-hoc test. IC_{50} values were extrapolated from a nonlinear straight line semi-logarithmic regression (TNF-α vs. log compound concentration). A p value less than or equal to 0.05 was considered statistically significant. Normality was assessed using the Shapiro-Wilk test.

6.3 - Results

The open column chromatography fractionation of *Picea mariana* cone extract generated a total of 180 primary fractions which were combined based on phytochemical similarity to yield 14 pooled primary fractions (F1-F14) (Fig 6.1). Fraction 12 displayed two distinctly different physical phases which were separated into 12a (sticky gum) and 12b (dry powder).

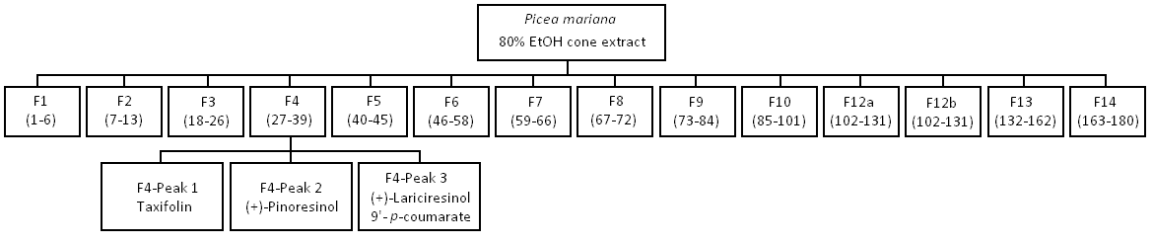


Figure 6.1 – Bioassay guided isolation scheme for *Picea mariana* 80% EtOH cone extract.

The anti-inflammatory activity of cone extract and the pooled primary fractions (10 and 100 µg/mL) was assessed in an LPS-stimulated THP-1 monocyte assay (Fig 6.2). The unfractionated crude cone extract displayed

potent anti-inflammatory activity, with 38.6% and 67.6% of the activity of the 10 µg/mL parthenolide positive control at 10 and 100 µg/mL, respectively. All of the pooled primary fractions demonstrated moderate to very strong dose-dependent anti-inflammatory activity. Of the 15 fractions assayed, 4 (F3, F4, F5, and F7) had activity that was not significantly different ($p > 0.05$) from the 10 µg/mL parthenolide control at the highest concentration tested. The two most active fractions were F4 and F5, which had 91% and 92% of the activity of the 10 µg/mL parthenolide control at 100 µg/mL. F14 had the lowest anti-inflammatory activity, with 36.3% of the activity of the 10 µg/mL positive control at 100 µg/mL.

The cytotoxicity of *P. mariana* cone extract and pooled primary fractions to the THP-1 monocytes was measured at the highest concentration tested in the anti-inflammatory assay (100 µg/mL) (Fig. 6.3). Of the 15 fractions assayed, 11 fractions had less than 10% cytotoxicity and 10 of these had less than or equal to 6% cytotoxicity. The least cytotoxic pooled primary fraction was F3 which had a cytotoxicity of 1.5%. Fractions 1, 11, and 14 had moderate cytotoxic effects of 16.7%, 16.1%, and 16.0%, respectively. The strongest degree of cytotoxicity was observed in F12a (21.0%). There is an elevated level of cytotoxicity in fractions which were eluted with the most polar solvents, as a cytotoxic peak is observed from F11 to F14. Therefore the degree to which these polar fractions were able to inhibit TNF-α production in THP-1 monocytes can in part be attributed to their cytotoxicity. However, the regression between the anti- inflammatory activity of all fractions tested and their cytotoxicity was not significant, indicating that cytotoxicity was not a predictor of the anti-inflammatory activity of the fractions.

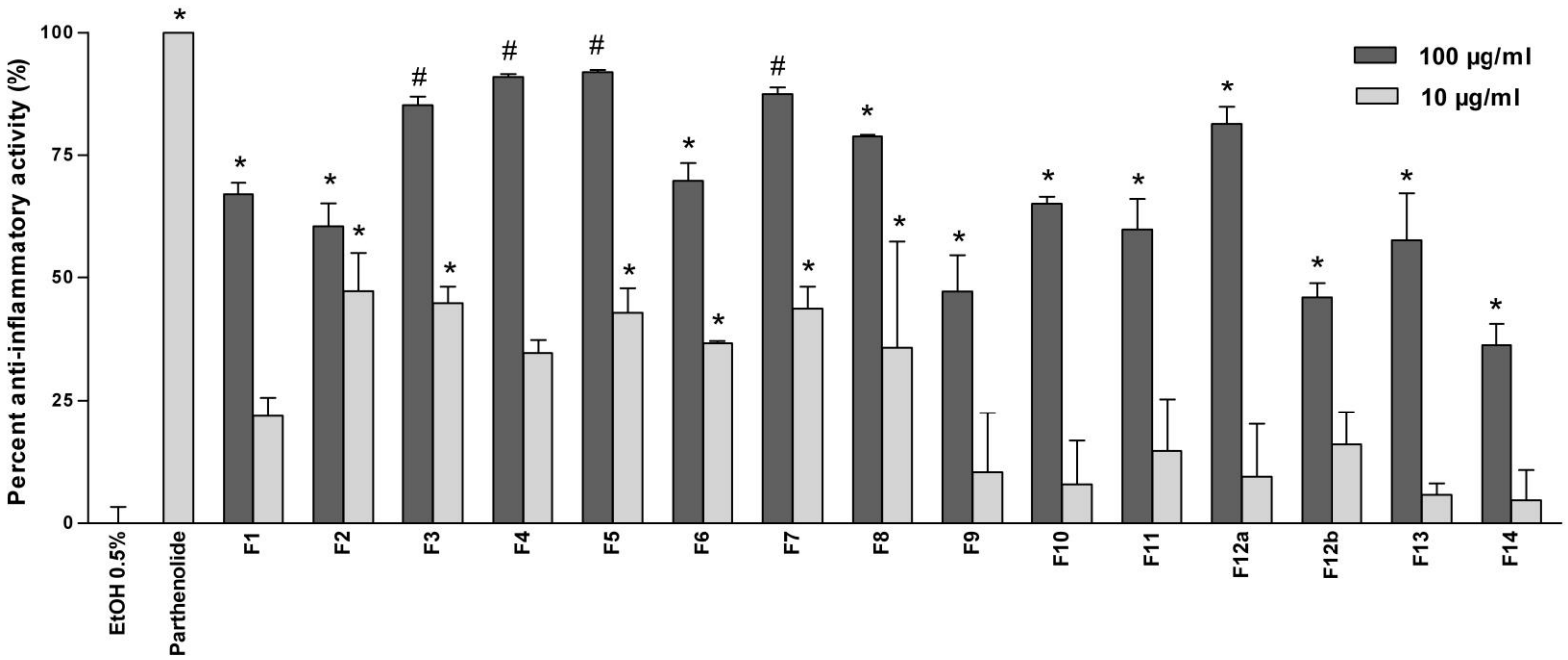


Figure 6.2 – Percent anti-inflammatory activity (%) of *Picea mariana* primary fractions relative to the parthenolide control (10 µg/mL) in LPS-stimulated THP-1 monocytes. Fractions were tested at 10 and 100 µg/mL. The average activity + SE is plotted, N=4. * indicates activity significantly different ($p \leq 0.05$) from the 0.5% EtOH control and # indicates activity not significantly different ($p \geq 0.05$) from the parthenolide control.

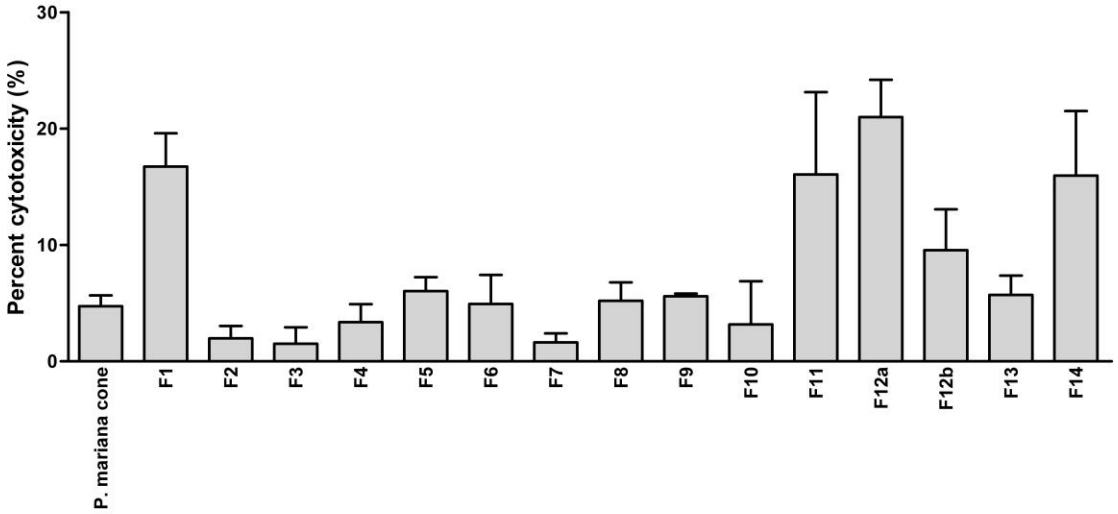


Figure 6.3 – Percent cytotoxicity (%) to THP-1 monocytes of *Picea mariana* primary fractions (100 µg/mL). Cytotoxicity values are calculated as a percentage (±SE) of LDH released compared to lysed cells. N=4.

As phenolics are secondary metabolites found in a wide variety of medicinally important plant species, including members of the Pinaceae, and these compounds may help to control oxidative stress and consequently the inflammatory response, the total phenolic content of the primary pooled fractions was evaluated (Fig 6.4). Overall, the pooled primary fractions were very rich in phenolic content, with 9 of 15 fractions tested containing greater than 500 µg quercetin equivalents / mg fraction. Fractions 11, 12a and 12b displayed greater than 1000µg of quercetin equivalents/ mg fraction. The high phenolic content in these fractions can be attributed to the large amount of polar-soluble phenolics which eluted at the more polar solvent gradients. F2 had the lowest phenolic content (172.4 quercetin equivalents / mg fraction). A regression between the

anti-inflammatory activity of all fractions tested and their phenolic content was not significant, indicating that total phenolic content was not a dominant predictor of the anti-inflammatory activity of the fractions. Similarly, a regression between the cytotoxicity of all fractions tested and their phenolic content was not significant, indicating that total phenolic content was not a dominant predictor of the cytotoxicity of the fractions.

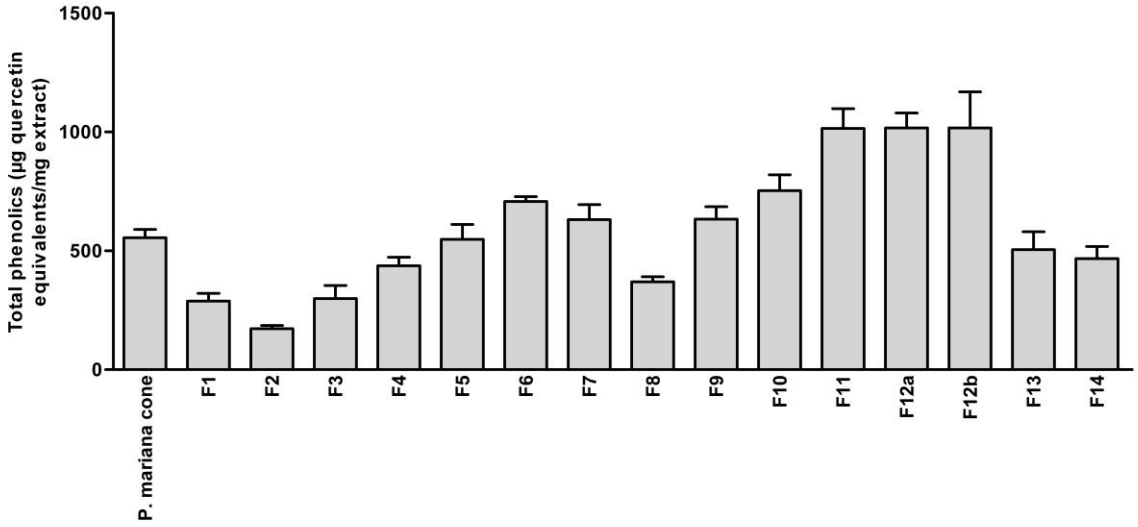


Figure 6.4 - Total phenolic content *Picea mariana* primary fractions. Means (µg quercetin equivalents / mg extracts) plus standard error are presented, N=4.

All pooled primary fractions were profiled using HPLC-DAD to examine the phytochemical complexity of each fraction and determine which of the most active fractions was the best suited for compound isolation and identification. The two most active fractions at the highest concentration tested were F4 and F5, both with greater than 90% of the activity of the 10 µg/mL parthenolide positive control. F4 was selected for isolation of is major constituents as HPLC-DAD

profiling revealed that it was composed of only three major peaks (Fig. 6.5), compared to the much more complex F5. F4 was also only weakly cytotoxic (3.4%) and had one of the lowest total phenolic contents of all the fractions (437.5 µg quercetin equivalents / mg fraction). When comparing the UV spectra of the three major peaks to UV spectral library, the presence of the flavonoid taxifolin (dihydroquercetin) (MW = 304.25 g/mol) (Fig. 6.6) was confirmed on the basis of a 99.9% match with the taxifolin reference spectrum and an identical retention time of 5.50 min compared to the elution of the taxifolin standard. No UV spectral matches were found for the other two major peaks eluting at 9.18 and 12.22 min, hence these peaks were isolated using preparative-scale HPLC and their structures were elucidated using ^{1}H and ^{13}C NMR. The peak at a retention time of 9.18 min was identified as the lignan (+)-pinoresinol (MW = 358.39 g/mol) based on NMR spectra matching previously reported by Xie et al (2003). The ^{13}C NMR spectrum for (+)-pinoresinol can be found in appendix Va. The peak at a retention time of 12.22 min was identified as the lignan (+)-lariciresinol 9'*p*-coumarate (MW = 506.54 g/mol) based on the match of its NMR spectrum with that previously reported by Yang at al (2005). The ^{13}C NMR spectrum for (+)-pinoresinol can be found in appendix Vb.

The anti-inflammatory activity of the three compounds isolated from F4 was assessed at 0.4, 4, 10, and 40 µM (Fig. 6.7). Taxifolin did not demonstrate any significant reduction in TNF-α production relative to the LPS-stimulated vehicle control at any of the concentrations tested. (+)-Pinoresinol only demonstrated significant anti-inflammatory activity relative to the LPS-stimulated

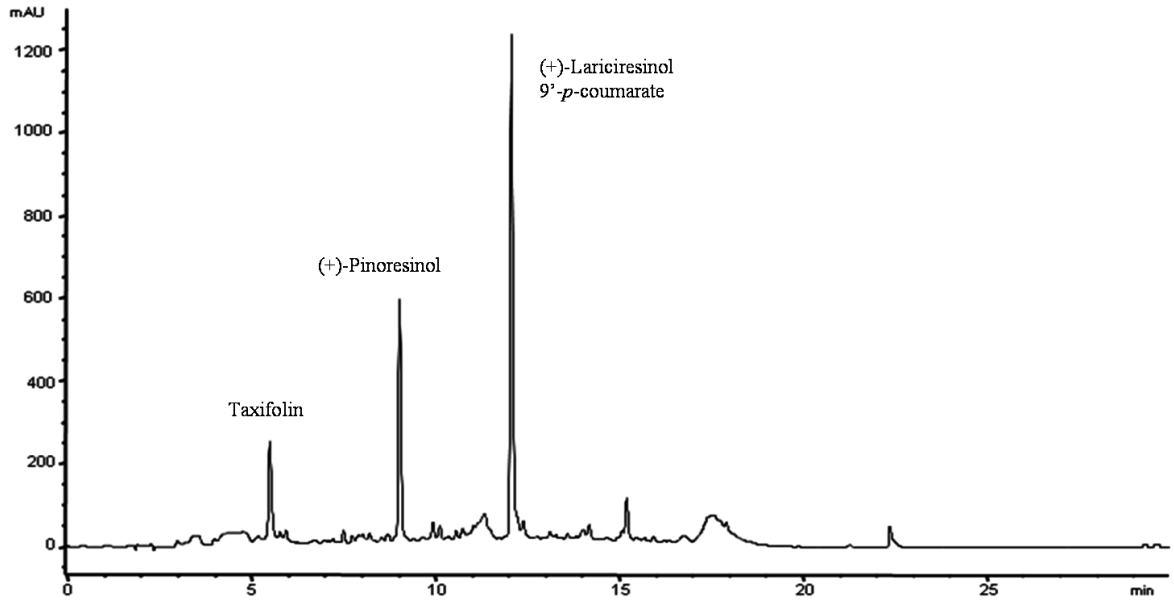


Figure 6.5 - Chromatographic profile of *Picea mariana* cone fraction F4 at 210nm.

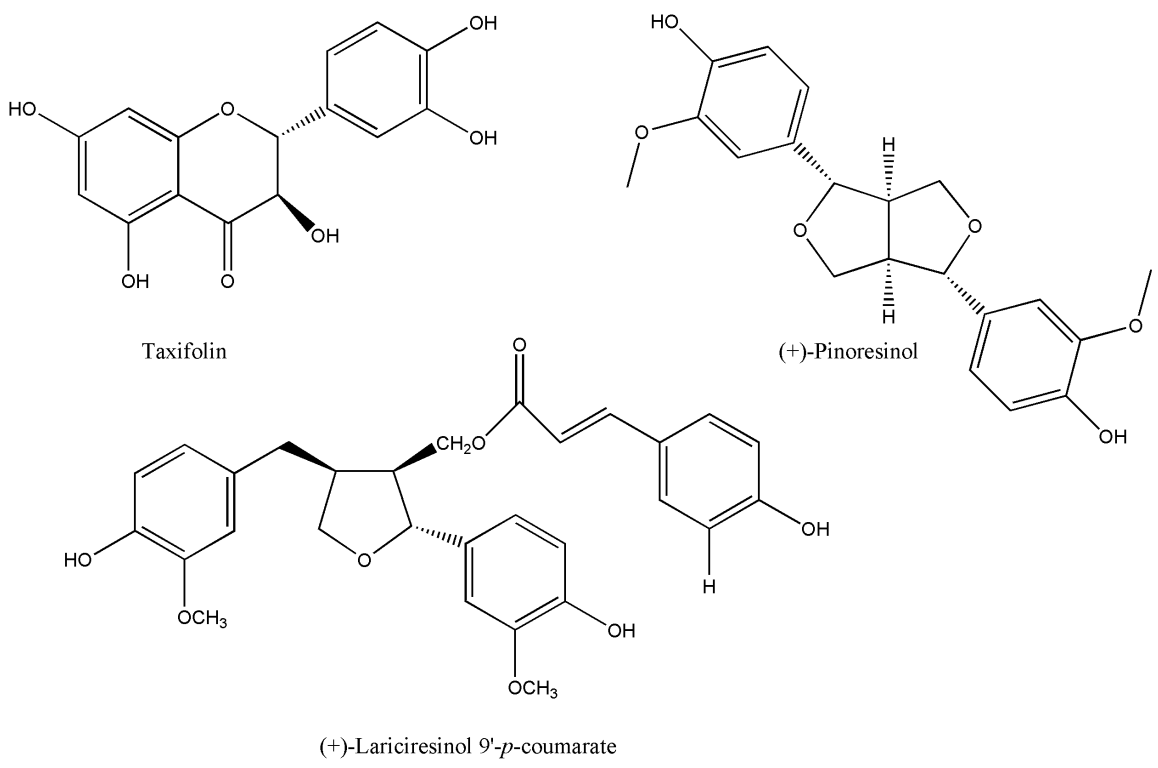


Figure 6.6 – Compounds isolated from *Picea mariana* cone fraction F4.

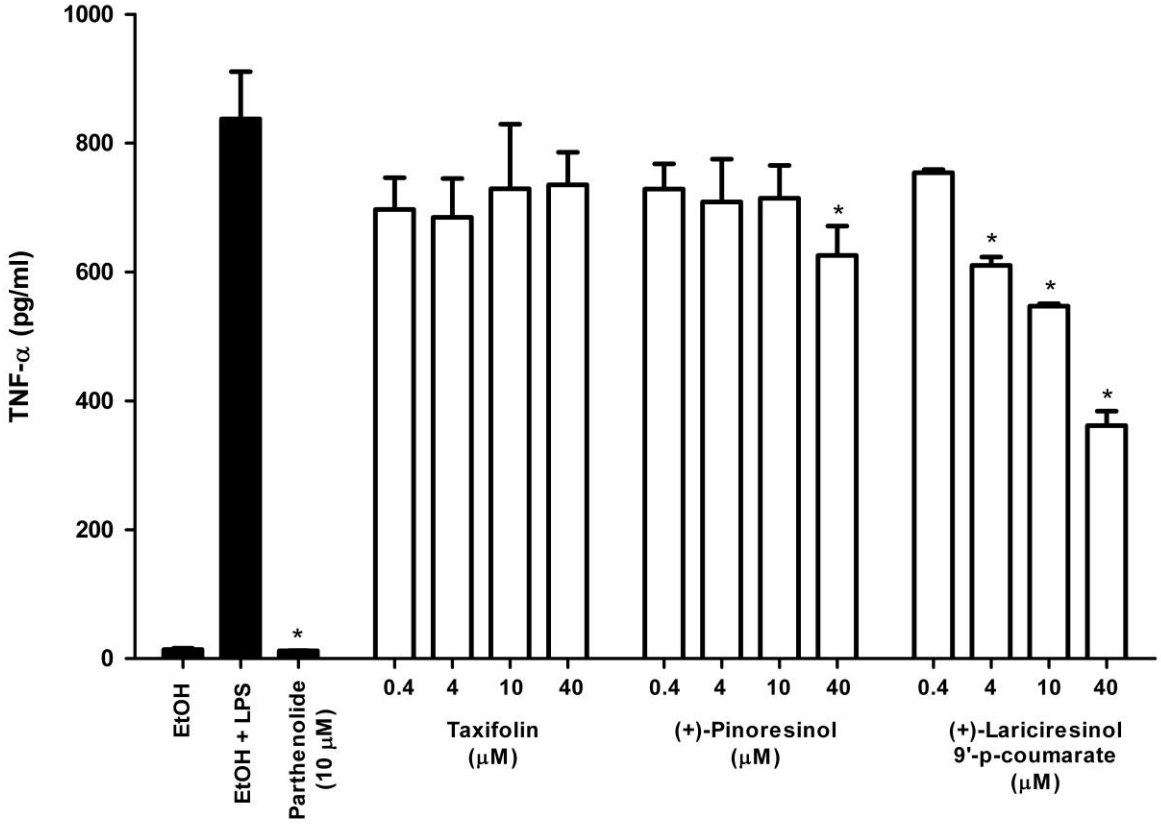


Figure 6.7 - Anti-inflammatory activity of compounds isolated from *Picea mariana* cone extract in LPS-stimulated THP-1 monocytes. EtOH 0.5% was used as a vehicle in both unstimulated and LPS-stimulated controls and parthenolide was used as a positive control. Means plus standard error of four replicates are presented. * denotes TNF-α levels that are significantly different ($p \leq 0.05$) from the stimulated 0.5% EtOH control.

vehicle control at the highest concentration tested (40 μM), reducing TNF-α levels from 837.5 pg/mL to 625.9 pg/mL. (+)-Lariciresinol 9'-*p*-coumarate displayed the strongest anti-inflammatory activity of the three *P. mariana* compounds assayed, lowering TNF- α production to 361.5 pg/mL at 40 μM. It was also the only compound to display dose-dependent anti-inflammatory activity, allowing an IC_{50} of 28.4 μM to be calculated for (+)-lariciresinol 9'-*p*-coumarate.

6.4 – Discussion

While the cones of *Picea mariana* have a long history of use as a traditional medicine amongst the Cree of Eeyou Istchee and other aboriginal groups, little phytochemical or pharmacological research has been directed towards this plant part. In one of the few studies which did examine black spruce cones in comparison to other parts from this species, the cones were highest in both total phenolic content as well as anti-oxidant capacity when compared to the needles, bark, and wood (Zulaica-Villagomez, 2005). Older cones, as well as juvenile cones with the seeds removed, had a much lower total phenolic content and lost over half of their anti-oxidant capacity. In discussion with Cree elders during the course of our work, they did not make a distinction between the age of the cones used in the treatment of Type II diabetic symptoms, and a mixture of both closed and open cones was used in the preparation of medicinal infusions.

To the best of our knowledge, this is the first report of the potent anti-inflammatory of black spruce cones. The anti-inflammatory activity of *Picea*

mariana bark has been previously reported in LPS and IFN-γ stimulated RAW 264.7 cell as measured by a reduction in nitrite production (Diouf, 2009). The bark extract was also reported to be high in phenolic content, with a proanthocyanidin rich fraction being the most anti-inflammatory fraction tested. The present study did not find a significant relationship between the anti-inflammatory activity of the cone fractions and their total phenolic content. High phenolic content was found in all of the primary pooled fractions, indicating that total phenolic content alone is not enough to predict anti-inflammatory activity, and that individual compounds from active fractions must be isolated to explain the anti-inflammatory activity of the most active fractions.

Taxifolin and (+)-pinoresinol did not demonstrate any substantial TNF-α reduction capacity in LPS-stimulated monocytes that would explain the potent anti-inflammatory activity of the cone extract or F4. However, previous studies have shown that the flavonoid taxifolin does posess moderate anti-inflammatory activity in other cell lines (Park et al, 2000; Rhee et al, 2008). The lignan (+)-pinoresinol has been shown to have anti-inflammatory effects in Caco-2 cells (During, 2012), although it only possessed weak anti-inflammatory activity at the highest concentration tested in this study. In contrast, (+)-lariciresinol 9'-*p*-coumarate is clearly an active anti-inflammatory principle of the cone extract and the F4 fraction. It is the first reported anti-inflammatory activity of this compound. (+)-Lariciresinol and *p*-coumaric acid have both demonstrated anti-inflammatory activity *in vitro* (Kupeli et al, 2003; Yen et al, 2010). The IC_{50} of lariciresinol 9'-*p*-

coumarate is 28.5 μM, making it moderately anti-inflammatory when compared to the IC_{50} of parthenolide at 4.79 μM (Chapter 4).

This study has investigated the potent anti-inflammatory activity of black spruce cones and the bioassay guided isolation of cone extract has led to the identification of the anti-inflammatory (+)-lariciresinol 9'-*p*-coumarate. Further work is needed to fully characterize the potent activity of this traditional medicine, and other highly active primary pooled fractions can be subjected to bioassay-guided isolation of their active principles. In addition, active fractions and (+)-lariciresinol 9'-*p*-coumarate can be tested in other models of inflammation to asses their effects on multiple markers of inflammation. Given the potent activity of this plant species, and the strong anti-inflammatory activity of cone fractions, the results presented in this study support the evaluation of this traditional medicine and its active constituents in animal and clinical models.

Chapter 7

General Discussion

7.1 – Summary of results and major findings

Traditional healers recognize the important role of inflammation in health and illness, and worldwide indigenous pharmacopeias contain numerous plants with immunomodulatory activity. This project focused on the immunomodulatory properties of medicinal plants used by two indigenous communities, the Q'eqchi' Maya of Belize and the Cree of Eeyou Istchee (CEI) in northern Quebec. Medicinal plant species used in the treatment of a variety of inflammatory-related symptoms by the Q'eqchi' Maya Healers Association (QMHA) was the focus of Chapters 2-4, and the immunomodulatory activity of medicinal plants used by the CEI in the treatment of Type II diabetes and its associated symptoms was the subject of chapters 5-6.

Q'eqchi' Maya Healers of Belize

The ethnobotanical survey of medicinal plants used by the QMHA in the treatment of 15 inflammatory-related symptoms identified a total of 107 species belonging to 49 families. In total, the five healers interviewed provided a total of 1359 use-reports, with the symptom categories of muscular-skeletal system disorders, digestive system disorders, and skin/subcutaneous cellular tissue disorders generating the most use-reports. Healer consensus on medicinal plant use was high, with more than half of all species identified being used by all 5

members of the QMHA. A regression analysis identified Piperaceae, Araceae and Begoniaceae as having the highest residual values extracted from the regression of the number of medicinal plant species in the family used versus the total number of species in the family, indicating that these plant families are preferentially selected by the healers in the treatment of inflammatory related symptoms. Overall, 78% of species were collected from primary and secondary broadleaf subtropical moist forests, indicating that the immunomodulatory ethnobotany of the Q'eqchi' Maya is predominantly a rainforest ethnobotany. This in the first detailed ethnobotanical survey of the anti-inflammatory medicinal plants used by the Q'eqchi' Maya of Belize. The documentation of 107 anti-inflammatory traditional medicines is an important contribution to the ethnobotanical literature and helps the QMHA achieve its goal of traditional knowledge conservation.

Of the 107 plant species used by the members of the QMHA in the treatment of inflammatory-related symptoms, 52 species were assayed for their anti-inflammatory activity in LPS-stimulated THP-1 monocytes. A majority of the plants assayed, 52%, demonstrated anti-inflammatory activity by significantly reducing TNF-α levels relative to the LPS-stimulated vehicle control. Eight species displayed potent anti-inflammatory activity, with greater than 50% of the activity of the 10 μg/ml parthenolide positive control. Significant regressions were found between the anti-inflammatory activity and the total healer frequency of use (F_{use}) and the use reports for common cold, respiratory system disorders, and circulatory system disorders. This indicates that these symptom categories can in

part predict the anti-inflammatory activity of plants used by the QMHA. The anti-inflammatory activity of traditional Q'eqchi' medicines provides a wealth of ethnopharmacological information that can be used as the starting point for more detailed investigations of active species. Moreover, the data presented provid a pharmacological basis for the use of Q'eqchi' medicinal plants. This supports the QMHA's objective of scientifically validating their traditional medicines in efforts to gain local and governmental acceptance and support for their role in the delivery of primary healthcare in rural villages.

Neurolaena lobata, one of the most anti-inflammatory species used by the Q'eqchi' Maya, was selected for an investigation of its active principles. Known to contain a variety of sesquiterpene lactones, a class of phytochemicals with well documented anti-inflammatory properties, leaf extract of *N. lobata* demonstrated 73.9% of the anti-inflammatory activity of the sesquiterpene lactone parthenolide. Five sesquiterpene lactones were isolated from *N. lobata*, 3 germacranolide sesquiterpene lactones (neurolenin B, neurolenin C, and neurolenin D) and 2 furanoheliangolide sesquiterpene lactones (lobatin B and 9α-hydroxy-8β-isovalerianyloxy-calyculatolide). Neurolenins C and D are isomeric isovaleryl esters which differ only in the position of the ester group and are present as a stable mixture of the two compounds are a ration of 0.75:1 C:D. The IC_{50}s of the isolates ranged from 0.17-2.32 μM, which were all lower than the parthenolide IC_{50} of 4.79 μM. The most active isolate was lobatin B, with an IC_{50} of 0.17 μM. This is the first report of the potent anti-inflammatory activity of sesquiterpene lactones from *Neurolaena lobata*, providing a pharmacological basis for its use as

an anti-inflammatory traditional medicine. The activity of lobatin B, an order of magnitude greater than the activity of parthenolide which is considered the “gold standard” for NF-κB inhibition by a natural product, makes it one of the most potent anti-inflammatory natural products described in the literature. While Q’eqchi’ plants cannot yet be characterized as fully evidence based medicines, as could be said for some natural health products in Canada and phytomedicines in Europe, the journey in this direction has begun.

Cree of Eeyou Istchee

The Cree of Eeyou Istchee (CEI) traditional healers use 17 plant species belonging to 7 plant families in the treatment of Type II diabetes and its related symptoms. Following discussions with members of the CEI, the immunomodulatory activity of standard laboratory ethanolic extracts as well as the immunomodulatory activity of traditionally prepared boiling water extracts was examined in order to compare the phytochemical and pharmacological properties of both types of extracts in the context of Type II diabetes. In general, the ethanolic extracts displayed more anti-inflammatory activity than the water extracts in LPS-stimulated THP-1 monocytes, with the average activity of the ethanolic extracts being 1.8 times greater than that of the water extracts. The most anti-inflammatory Cree species were *Picea mariana* and *Pinus banksiana*, displaying the greatest TNF-α inhibition when both ethanolic and water extracts were assayed. Regarding pro-inflammatory activity, water extracts displayed more pro-inflammatory activity than the ethanolic extracts in THP-1 monocytes,

with the average activity of the water extracts being 3.7 times greater than the average activity of the ethanolic extracts. The most pro-inflammatory ethanolic extract was from *Larix laricina* and the most pro-inflammatory water extract was from *Sarracenia purpurea*.

The total phenolic content was measured and the ethanolic extracts were significantly higher in phenolic content, with the average phenolic content of the ethanolic extracts being 2.3 times higher than the average phenolic content of the water extracts. A regression between the anti-inflammatory and total phenolic content of the extracts was significant, indicating that the total phenolic content partially explains the anti-inflammatory activity observed in the extracts. This regression was also significant when examining the Pinaceae species separately, however it was not significant when examining the Ericaceae species separately, indicating that total phenolic content can only partially explain the anti-inflammatory activity in the Pinaceae but that this is not the case with the Ericaceae.

This is the first comprehensive investigation of the immunomodulatory activity of 17 species used by the Cree in the treatment of Type II diabetes and its associated symptoms. Describing the anti- and pro-inflammatory activity of these species is an important contribution to the CIHR TAAM's objectives of evaluating the safety and efficacy of CEI anti-diabetic botanicals. Comparing the immunomodulatory activity and phytochemistry of ethanolic and boiled water extracts directly addresses the objective of the participating Cree communities to evaluate the traditional preparation methods alongside standard laboratory

practices, and it reveals that extraction method is an important consideration when it comes to *in vitro* anti- and pro-inflammatory activity.

Picea mariana cone extract, the most potent anti-inflammatory traditional medicine used by the CEI healers in the treatment of Type II diabetes and its related symptoms, was selected for bio-assay guided isolation of its active principles. Primary fractionation of cone extract yielded 180 fractions which were pooled to 14 fractions based on phytochemical similarity. One of the most active fractions, F4, was subjected to preparative-scale HPLC and the flavonoid taxifolin, as well as the two lignans (+)-pinoresinol and (+)-lariciresinol-9'-*p*-coumarate, were the major constituents of this fraction. (+)-lariciresinol-9'-*p*-coumarate was the only isolate to demonstrate important anti-inflammatory activity, and had a TNF-α inhibitory IC_{50} of 28.4 μM. This is the first investigation of the anti-inflammatory principles of *Picea mariana* cones, and the first report of the anti-inflammatory activity of (+)-lariciresinol-9'-*p*-coumarate, which partially explains the potent anti-inflammatory medicine of this traditionally used medicine.

7.2 – Comparisons between Q'eqchi Maya immunomodulatory plants, Cree of Eeyou Istchee immunomodulatory plants, and published literature

The investigation of the immunomodulatory activity of Q'eqchi' Maya of Belize and Cree of Eeyou Istchee medicinal plants described the anti-inflammatory activity of 124 species and the pro-inflammatory activity of 33 species. While the pharmacological evaluation of plant species used by both

groups followed a similar methodology, this thesis was not intended as a cross cultural comparison, as the context of each indigenous community's participation guided the direction of the research. The study of Q'eqchi' medicines was an ethnobotanical project which fulfilled the QMHA objectives of documenting and scientifically validating their traditional knowledge. The collaboration with the Cree of Eeyou Istchee through the CIHR TAAM did not have an ethnobotanical mandate, as this research had already been carried out (Leduc, 2006). The objective of this study was to asses the immunomodulatory activity of medicinal plants used in the context of diabetes, and discussions with the CEI led to an emphasis being placed on understanding the pharmacology of traditional medicinal plant preparations compared to standard laboratory preparations. Although the needs of each collaborating community directed the research projects, and the floras used by each group are markedly different, general comparisons between the Q'eqchi' Maya of Belize and the Cree of Eeyou Istchee can be made and insight gleamed from each project can be applied to other contexts.

When comparing the species used as traditional medicines by both communities, the differences in the flora of each region is clear. Of the 17 species belonging to 7 families used by the CEI, 11 (65%) belong to either the Ericaceae or the Pinaceae. While of the 107 species belonging to 49 families used by the QMHA, only 20 (19%) belong to the two most frequently used families, the Piperaceae and the Araceae. The exceptional biodiversity of the Belizean flora is also reflected in the exceptional medicinal plant diversity used by

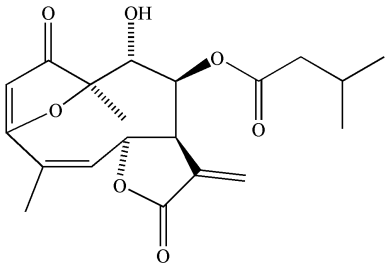
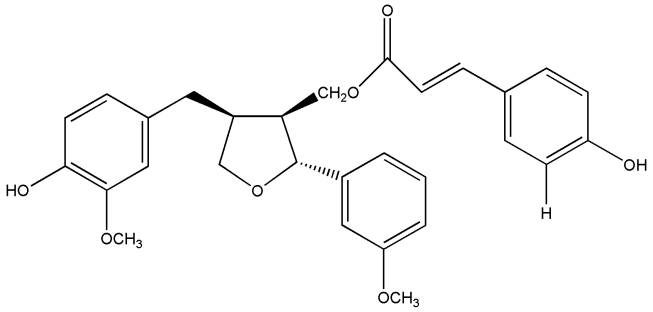
the QMHA. Among both groups of healers, the majority of medicinal plant species are collected for their leaves. Forty-one percent of traditional medicines used by the CEI and 87% of those used by the Q'eqchi' are leaf preparations. However, the CEI use a larger proportion of bark, 35% compared to the 3% of species used for their bark by the QMHA, as tree species are a dominant feature of the boreal forest. In addition, cones are used exclusively by the CEI, collected from two species of Pinaceae. Broadleaf evergreen trees are used by the QMHA for their leaves and bark, but the majority of medicinally used species are herbs and shrubs. Vines, lianas, and ferns, both epiphytic and non-epiphytic, are used exclusively by the QMHA and are characteristic lifeforms of subtropical flora much less represented in boreal regions.

When comparing the immunomodulatory activity of QMHA and CEI traditional medicines, it is evident that a wide range of medicinal plant species from both indigenous traditions possess pharmacological activity. The average anti-inflammatory activity of CEI plant species (39.8±5.1% the activity of parthenolide 10 µg/mL) is significantly greater ($p \leq 0.05$) than the average anti-inflammatory activity of QMHA plant species (22.7±6.0% the activity of parthenolide 10 µg/mL) at the highest concentration tested. While the average anti-inflammatory activity of CEI plants is higher than that of QMHA plants, the 52 QMHA species from 29 families assayed encompass a much more diverse spectrum of plants than the 17 CEI species from 7 families, 11 of which represent only 2 families. Of the plant families used by the QMHA with the highest average anti-inflammatory activity when tested at 100 µg/mL - the Araceae (34.1±17.0%

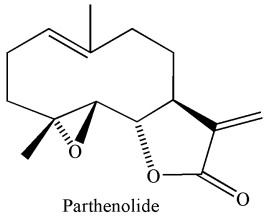
the activity of parthenolide 10 µg/mL), the Piperaceae (24.9±7.4% the activity of parthenolide 10 µg/mL), and the Asteraceae (24.7±13.3% the activity of parthenolide 10 µg/mL) – only the average activity of the Piperaceae differed significantly ($p \leq 0.05$) from the average activity of the Pinaceae (53.4±11.3% the activity of parthenolide 10 µg/mL), the plant family used most by Cree healers. The average activity of the Ericaceae species used in CEI communities (27.2±4.1% the activity of parthenolide 10 µg/mL) did not differ significantly from the average activity of the Pinaceae, or from any of the QMHA used plant families. When examining the anti-inflammatory activity of individual species, 15.4% of QMHA species assayed demonstrated greater than 50% of the activity of the 10 µg/mL parthenolide positive control, which is comparable to the 17.6% of CEI species assayed which demonstrated greater than 50% of the activity of parthenolide. In contrast, the three most active species assayed, which demonstrated TNF-α inhibition that was not significantly different than that of the positive control, were all QMHA species. Although the CEI species assayed demonstrated somewhat greater average anti-inflammatory relative to the QMHA species assayed, the average activity of the most active plant families in both groups did not differ significantly, and the proportion of highly active species is similar in both indigenous communities studied. Taken together, this indicates that the average anti-inflammatory activity in plant species belonging to both communities are similar, however the most active species can be found in the subtropical forests used by the QMHA of Belize.

A comparison of the current finding with anti-inflammatory natural products from well-known traditional medicines was made (Table 7.1), from which it is possible to make some generalizations regarding the phytochemistry of species used by both indigenous groups. Boreal plant species are known to be high in phenolic content, as the anti-oxidant properties of these molecules help them cope with high light levels due to long day length at northern latitudes (Martz et al, 2009; Stark et al, 2008). Also, the phenolic content in CEI species partially explained their observed anti-inflammatory activity. Indeed, the compound isolated here and in part responsible for the anti-inflammatory activity of *Picea mariana* is a lignan, a type of phenolic. In addition to being well known anti-oxidants (Shahidi et al, 1992), phenolics also possess a wide range of anti-inflammatory properties (Hamalainen et al, 2007; Wang and Mazza, 2002). Species used by the CEI have been reported to be rich in a wide range of phenolics, with members of the Ericaceae containing flavonoids such as cathechin, epicathechin and quercetin derivatives and phenolic acids such as chlorogenic acid (Black et al, 2011; Beaulieu et al, 2010; Saleem et al, 2010), and members of the Pinaceae also containing lignans (Castro et al, 1996). Many anti-inflammatory natural products from traditional medicines are phenolics, most notably curcumin from *Curcuma longa* (turmeric) and catechin from *Camellia sinensis* (green tea). Both of these phenolics have a wide range of anti-inflammatory properties, with inhibitory activities in the NF-κB pathway, COX pathway, and nitric oxide pathway (Table 8.1).

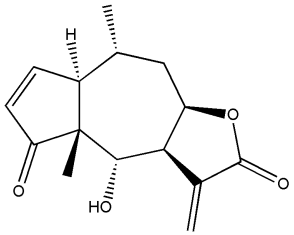
Table 7.1 – Anti-inflammatory medicinal plant species, their traditional uses, phytochemistry, bioactivity, and major active compounds. The two plant species focused on in this thesis (*Neurolaena lobata* and *Picea mariana*) are compared to several well know traditional anti-inflammatory medicines.

Medicinal plant species	Traditional use	Phytochemistry	Anti-inflammatory activity	Active compound (major)
Neurolaena lobata	K'a mank is used by the Q'eqchi Maya to treat fevers, headaches, and various skin and inflammatory conditions. Widespread Caribbean use for skin diseases, malaria, and cancer.	Sesquiterpene lactones and flavonoids have been identified in the leaves. 5 sesquiterpene lactones isolated in this study.	- Crude extract 72.2% parthenolide TNF-α inhibition - Isolates TNF-α inhibition IC_{50}s 0.17-2.32 µM, lobatin B most active - Leaf extract 19.5% swelling reduction in mouse paw oedema	 Lobatin B
Picea mariana	Inaahtikw is used by the Cree of Eeyou Istchee to treat Type II diabetes. Used by other First Nations in the treatment of skin conditions and mouth ailments.	Rich in phenolics including flavonoids, tannins, stilbenes and lignans. Also high in terpenes. Taxifolin (flavonoid) and two lignans identified in this study.	- Crude extract 92.4% parthenolide TNF-α inhibition - (+)-lariciresinol-9'-*p*-coumarate TNF-α inhibition IC_{50} 28.4 µM - No published reports of cone anti-inflammatory activity	 (+)-Lariciresinol 9'-*p*-coumarate

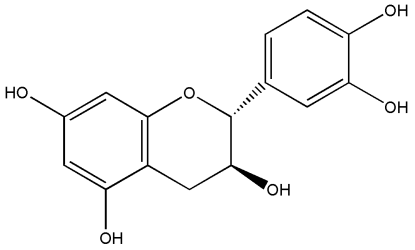
Tanacetum parthenium Refs: 1, 2, 3	Feverfew is used traditionally in the treatment of fevers, headaches, and arthritis. It is an approved natural health product for the prophylaxis of migraines.	Sesquiterpene lactones are active constituents, parthenolide being the major. Also contains flavonoid glycosides, sesquiterpenes and monoterpenes.	- Parthenolide TNF-α inhibion IC_{50} 4.79 µM - Inhibits NF-κB activation and NF-κB-dependent gene expression by inhibiting IκB
Arnica montana Refs: 4, 5, 6	Arnica is traditionally used by Europeans and Native Americans to treat inflammation and pain of sprains, bruises and wounds. Approved for external use only.	Sesquiterpene lactones of the helenanolide type are main active principles, helenalin is the major. Also contains flavonoids, triterpene alcohols, and coumarins.	- Inhibits NF-κB (Helenalin has 100% NF-κB inhibition at 10 µM) - Decreases COX-2 expression
Camellia sinensis Refs: 7, 8, 9	Green tea is used traditionally in China and India as a stimulant, a diuretic, in wound healing, and in the treatment of a variety of chronic ailments.	Contains flavonoids, such as catechin and its derivatives. Also contains sterols, carotenoids, and tocopherols.	- Inhibits NF-κB - Decreases COX-2 expression - Decreases iNOS expression
Curcuma longa Refs: 10, 11, 12	Turmeric is used traditionally in India, Pakistan, and Bangladesh to reduce inflammation, applied externally for skin ailments, and used in the treatment of numerous chronic ailments.	Curcuminoids are the main active principles, curcumin being the major constituent. Also contains essential oil of monoterpenes and sesquiterpenes.	- Inhibits NF-κB - Decreases COX-2 expression - Decreases iNOS expression - Inhibits activator protein 1 activation



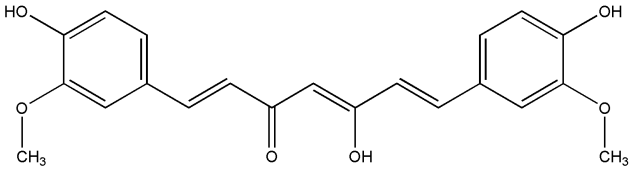
Parthenolide



Helenalin

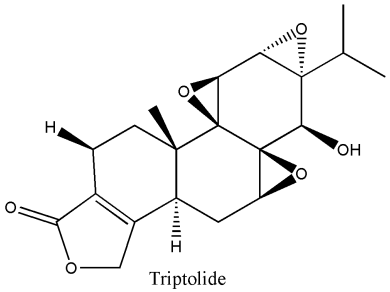


Catechin

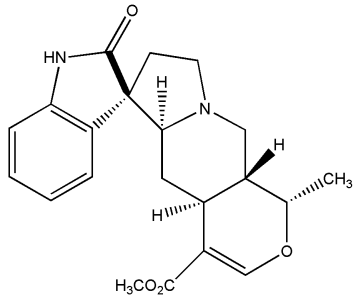


Curcumin

Tripterygium wilfordii Refs: 13, 14, 15	Thunder God vine is a traditional Chinese medicine used to treat swelling, fevers, and pain, as well as auto-immune diseases.	Sesquiterpenes, diterpenes, and triterpenes are the main secondary metabolites. The diterpene triptolide is the major active constituent.	- Inhibits NF-κB - Decreases COX-2 expression - Decreases iNOS expression
Uncaria tomentosum Refs: 16, 17	The woody vine cat's claw is widely used in Latin America to treat a variety of conditions, including inflammatory and digestive system disorders.	Alkaloids are the major secondary metabolites, primarily of the indole and oxindole types. Mitraphylline is the major active constituent.	- Crude extract 80% inhibition of TNF-α in LPS-stimulated THP-1 monocytes - Mitraphylline has anti-inflammatory activity in LPS treated mice



Triptolide



Mitraphylline

References: 1 – Hehner et al, 1999; 2 – Kwok at al, 2001; 3 – Saadane at al, 2007; 4 – Hsieh, 2006; 5 – Lyss at al, 1997; 6 – Lyß et al, 1998; 7 – Paquay et al, 2000; 8 – Seeram et al, 2003; 9 – Velayutham et al, 2008; 10 – Aggarwal, 2009; 11 – Kang et al, 2004; 12 – Xu et al, 1997; 13 – Brinker at al, 2007; 14 – Ma et al, 2007; 15 – Xu et al, 2011; 16 – Heitzman et al, 2005; 17 – Rojas-Duran et al, 2012.

In contrast to boreal plant species, tropical plant species are most well known for their alkaloid and terpene content (Langenheim, 1983). Recent work by Rojas-Duran (2012) led to the isolation of the alkaloid mitraphylline from *Uncaria tomentosum* (cat's claw) which inhibited TNF-α in LPS-stimulated THP-1 monocytes and demonstrated anti-inflammatory activity in LPS-stimulated mice. In addition to the potent anti-inflammatory compounds isolated from *Neurolaena lobata*, terpenes have been isolated from several widely used anti-inflammatory medicinal plants. These include the diterpene triptolide isolated from *Tripterygium wilfordii* (Thunder God vine) and the sesquiterpene lactones parthenolide from *Tanacetum parthenium* (feverfew) and helenalin from *Arnica montana* (Arnica) (Table 8.1). Triptolide has demonstrated NF-κB pathway inhibition, COX-2 inhibitory activity, and nitric oxide pathway inhibitory activity. Parthenolide and helenalin are potent NF-κB pathway inhibitors, both *in vitro* and *in vivo*. Although the anti-inflammatory activity of QMHA and CEI medicinal plants cannot be reduced to a few select compounds, an overview of the results presented in this thesis compared with the literature indicates that different classes of compounds are the principal contributors to the anti-inflammatory activity of QMHA and CEI medicinal species.

Regarding immunostimulation, the average pro-inflammatory activity of ethanolic extracts of CEI plant species (16.5±3.6% the activity of *Ecinacea purpurea* 100 µg/mL), and water extracts (60.5±10.4% the activity of *Ecinacea purpurea* 100 µg/mL) is significantly greater ($p \leq 0.05$) than the average pro-inflammatory activity of QMHA plant species (2.3±6.0% the activity of *Ecinacea*

purpurea 100 µg/mL) at the highest concentration tested. Although there was not a significant relationship between the polysaccharide content of water extracts of CEI medicinal plants and their observed pro-inflammatory activity, polysaccharide fractions from several species demonstrated immunostimulatory properties. Polysaccharides from medicinal plant species are known to possess pro-inflammatory activity, most notably polysaccharides from ginseng and arabinogalactans from *Ecinacea purpurea* and *Larix laricina* (Hudson, 2010; Kelly, 1999; Kim e al 2002; Lui et al 2012). As the results in this study demonstrate, it is clear that not all polysaccharides are equal in terms of their immunostimulatory activity. Although a polysaccharide analysis and an evaluation of the pro-inflammatory activity of water extracts was not a part of the research mandate with the QMHA, it can be expected that Q'eqchi' species may also contain varied levels of polysaccharides, as polysaccharides are ubiquitous plant primary metabolites. Similarly, it may be expected that water extracts of QMHA plants would be more pro-inflammatory than ethanolic extracts from the same species.

7.3 – Concluding remarks and future work

The results presented in this thesis demonstrate that the QMHA and the CEI use a wide variety of medicinal plants with immunomodulatory properties. For the Q'eqchi' healers, there is clear evidence of a strongly conserved elaborate tradition with regards to anti-inflammatory medicinal plants, and that species selected by the healers have a pharmacological basis. For the Cree

healers, plants used in the treatment of Type II diabetes and its symptoms have both anti- and pro-inflammatory properties and are rich in anti-inflammatory phenolics and pro-inflammatory polysaccharides. In both communities, certain plants that healers use had especially potent TNF-α modulatory activity in THP-1 monocytes. Due to the complexity of the inflammatory response, in order to more accurately characterize the immunomodulatory properties of these plants it would be important to measure the effects on a wider range or cytokines, chemokines, and acute phase reactants, as well as on alternative pro-inflammatory mechanisms such as the COX and nitric oxide pathways. By having a more complete picture of the effects of these traditional medicines on the inflammasome, insight into their mechanism of action can be gleamed and appropriate therapeutic targets can be selected for further study. Additionally, it would be fruitful to assess the anti-inflammatory activity of the 55 species used by the QMHA not assessed in this study, as well as to subject other active species from both groups of plants to bioassay guided isolation of their active principles.

With regards to *Neurolaena lobata* and the potent anti-inflammatory agents isolated from its leaves, further characterization of the active phytochemicals in this species is warranted, as only 5 of 11 reported sesquiterpene lactones were isolated from this species. One of the secondary objectives of QMHA with regards to this project is the development a natural health product for local, national, or international markets, so that funds from the sale of such a product could support the medicinal plant conservation and

cultivation activities of the healers association. Given the cytotoxicity associated with sesquiterpene lactones, which are potent alkylating agents, a prudent approach would be to follow the example of *Arnica montana*, which contains the sesquiterpene lactone helenalin and is licensed natural health product for external use (Health Canada, 2013). Appropriate animal and clinical models could be selected for a variety of inflammatory skin conditions, such as those treated by the QMHA which include skin rash and insect bites and stings. Following rigorous safety and efficacy studies, *N. lobata* could also be investigated for is use in the prophylaxis of migraines, an approved indication in Canada and European Union for the sesquiterpene lactone-rich *Tanacetum parthenium* (Blumenthal et al, 2000; Boon, 2003; Health Canada, 2013).

The main objective of the CIHR TAAM collaboration with the CEI was to evaluate the safety and efficacy of plants traditionally used in the treatment of Type II diabetes and its associated symptoms. Although the plants in this thesis were evaluated for their immunomodulatory properties, chronic inflammation is a contributing factor in the pathophysiology of Type II diabetes and plants with anti-inflammatory properties may be useful in slowing progression of this illness. Firstly, more work would need to be done to fully characterize *Picea mariana*, the most anti-inflammatory CEI species assayed, as this phytochemically complex species no doubt contains numerous active principles not isolated in this study. Secondly, appropriate anti-diabetic animal models would be a suitable *in vivo* model to use to more accurately assess the anti-diabetic properties of this species and its immunomodulatory activity in a diabetes-specific context. As well,

the emphasis placed on comparing the phytochemical and immunomodulatory properties of water and ethanol extracts by the CEI should be continue by a more detailed examination of how competing anti- and pro-inflammatory principles in a phytochemically complex crude extract contribute to the overall observed immunomodulatory effect, both *in vitro* and more importantly *in vivo*. Special attention should be placed on plants that exhibit strong immunostimulatory effects to assess the potential negative effects of a pro-inflammatory plant on the already chronically inflamed diabetic state.

Taken together, the results from this thesis demonstrate the immunomodulatory properties of a wide range of traditionally used medicinal plants. This thesis also shows how, using similar scientific methodology, research can adapt to the needs of two indigenous communities with different objectives. Both approaches have provided a potential pharmacological basis for plant selection in the collaborating indigenous communities, and opened the door for much future study of immunomodulatory plants used by the QMHA and the CEI.

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Appendix 1

Handbook of Anti-inflammatory Q'eqchi' Maya Medicinal Plants of Belize

Q'eqchi' Maya Healers Association

Handbook of Anti-inflammatory Q'eqchi' Maya Medicinal Plants of Belize

FIRST DRAFT

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Sponsored by:

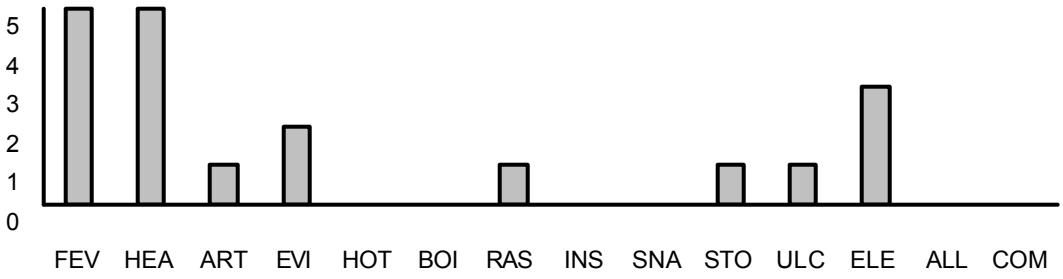
Canadian International Development Agency (CIDA)

Aphelandra aurantiaca (Scheidw.) Lindl.

Q'eqchi' names	Translation
Jolom chacmut (#1)	Bird's head (Jolom = head; chacmut = a specific bird)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
19	8	5



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.
Same uses as Aphelandra scabra.

Anti-inflammatory activity (%)

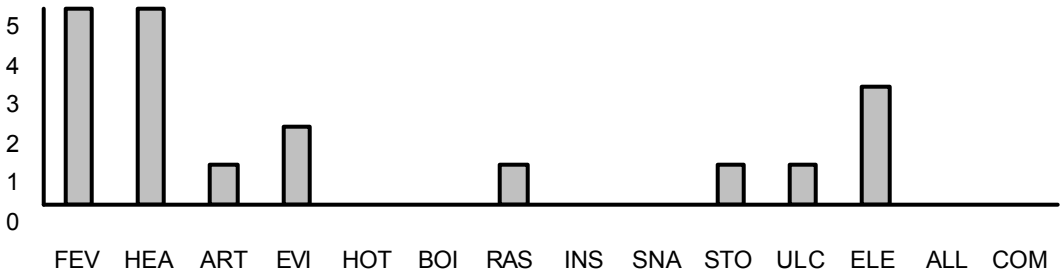
10 µg/mL	100 µg/mL
N.D.	N.D.

Aphelandra scabra (Vahl.) Sm.

Q'eqchi' names	Translation
Jolom chacmut (#2)	Bird's head (Jolom = head; chacmut = a specific bird)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
19	8	5



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.
Same uses as Aphelandra aurantica.

Anti-inflammatory activity (%)

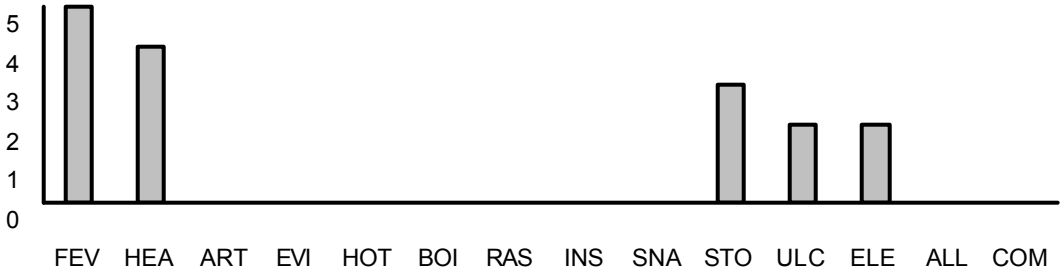
10 µg/mL	100 µg/mL
N.D.	N.D.

Justicia pectoralis Jacq.

Q'eqchi' names	Translation
Xucoy'i'kok	Turtle's side (Xucoy = side; i = a; kok = turtle)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
16	5	4



Preparation

Crush 20 tops of young plants in 1 gal of cold water, drink 1 cup and bathe twice daily. Can add 20 leaves of Lygodium heterodoxum and/or Stachytarpheta frantzii.

Anti-inflammatory activity (%)

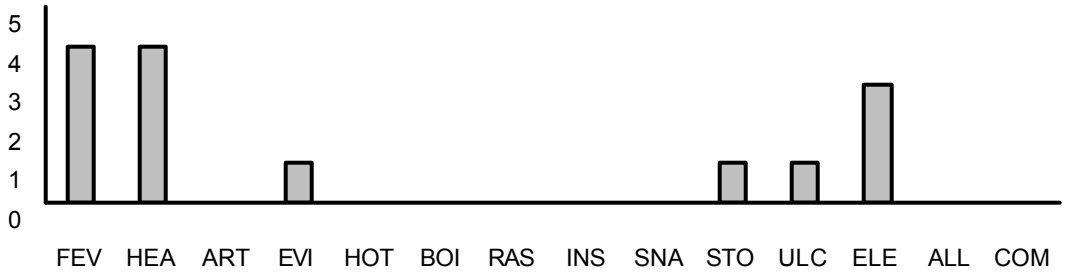
10 µg/mL	100 µg/mL
N.D.	N.D.

Adiantum latifolium Lam.

Q'eqchi' names	Translation
Roq chit cuan (#1)	Black bird's foot (Roq = foot; chit cuan = common black bird)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.80	1

Total use reports	Q'eqchi' categories	Cook categories
14	6	5



Preparation

Crush 13 blades in 1 gal of cold water, drink 1 cup twice daily. Can use Adiantum latifolium, Adiantum petiolatum, Adiantum princeps, and/or Adiantum tetraphyllum, variety is preferable.

Anti-inflammatory activity (%)

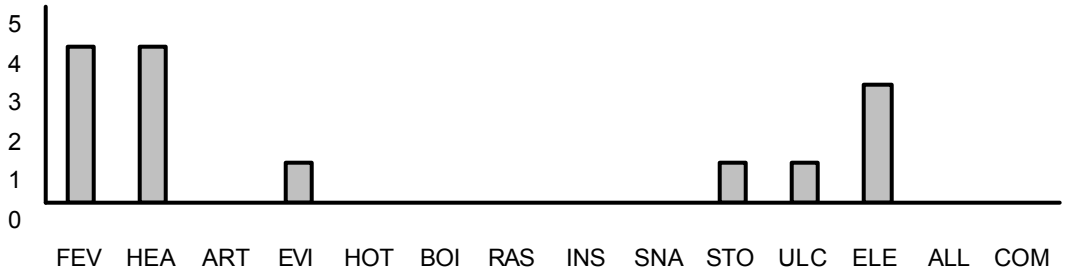
10 µg/mL	100 µg/mL
N.D.	N.D.

Adiantum petiolatum Desv.

Q'eqchi' names	Translation
Roq chit cuan (#2)	Black bird's foot (Roq = foot; chit cuan = common black bird)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.80	1

Total use reports	Q'eqchi' categories	Cook categories
14	6	5



Preparation

Crush 13 blades in 1 gal of cold water, drink 1 cup twice daily. Can use Adiantum latifolium, Adiantum petiolatum, Adiantum princeps, and/or Adiantum tetraphyllum, variety is preferable.

Anti-inflammatory activity (%)

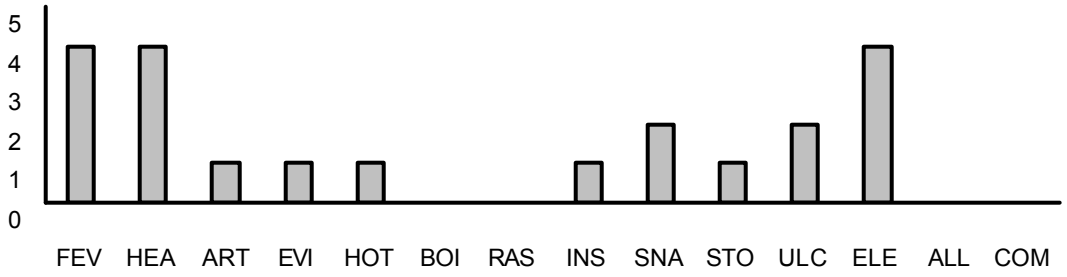
10 µg/mL	100 µg/mL
N.D.	N.D.

Adiantum princeps T. Moore

Q'eqchi' names	Translation
Roq chit cuan (#3)	Black bird's foot (Roq = foot; chit cuan = common black bird)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
21	10	6



Preparation

Crush 13 blades in 1 gal of cold water, drink 1 cup twice daily. Can use Adiantum latifolium, Adiantum petiolatum, Adiantum princeps, and/or Adiantum tetraphyllum, variety is preferable.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

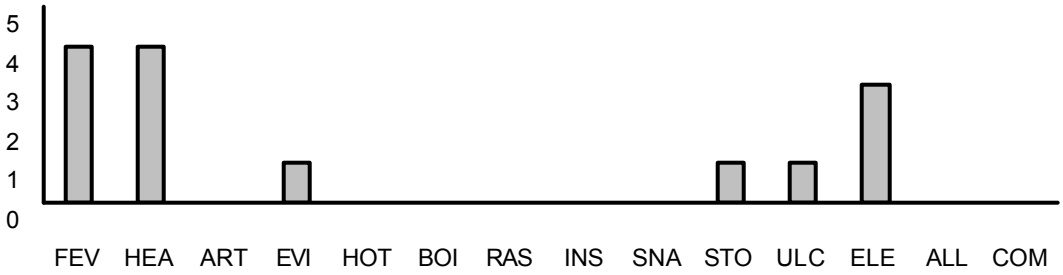
Adiantum tetraphyllum Humb. & Bonpl. ex Willd.

Q'eqchi' names
Roq chit cuan (#4)

Translation
Black bird's foot (Roq = foot; chit cuan = common black bird)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.80	1

Total use reports	Q'eqchi' categories	Cook categories
14	6	5



Preparation
Crush 13 blades in 1 gal of cold water, drink 1 cup twice daily. Can use Adiantum latifolium, Adiantum petiolatum, Adiantum princeps, and/or Adiantum tetraphyllum, variety is preferable.

Anti-inflammatory activity (%)

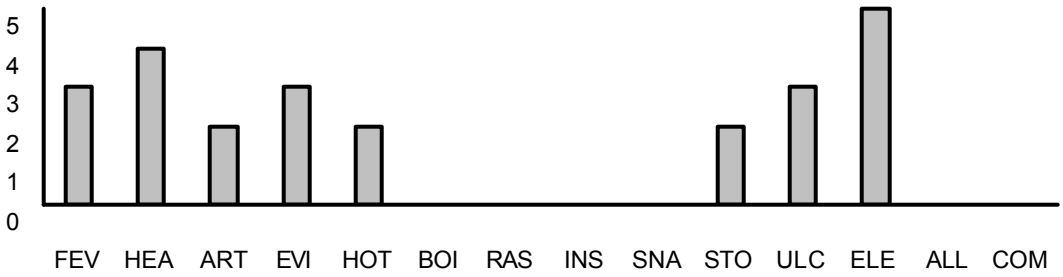
10 µg/mL	100 µg/mL
N.D.	N.D.

Adiantum wilsonii Hook.

Q'eqchi' names	Translation
Ruj'i'rak'aj'tza	Devil's tongue (Ruj'i'rak = tongue; aj = of; tza = devil)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
24	8	5



Preparation

Crush 13 leaves in 1 gal of cold water, drink 1 cup twice daily.

Anti-inflammatory activity (%)

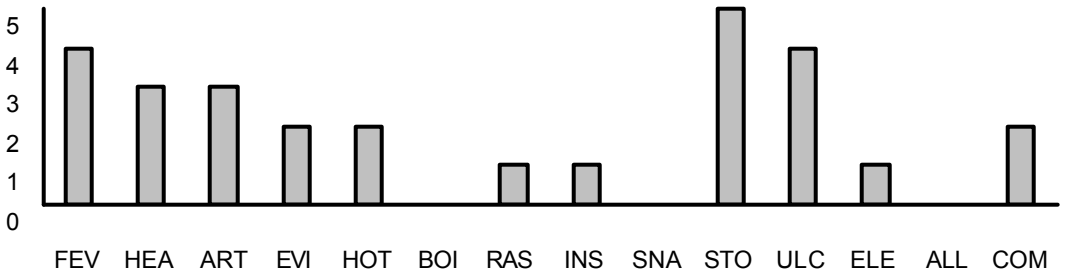
10 µg/mL	100 µg/mL
N.D.	N.D.

Iresine diffusa Willd.

Q'eqchi' names	Translation
Biri tak	Go and get a plant that breaks easily (Biri = a plant with nodes that break easily; tak = go and get it)
Kaki biri tak	Red Biri tak (Kaki = red)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.80	1

Total use reports	Q'eqchi' categories	Cook categories
28	11	8



Preparation

Crush 20 leaves or 13 tops of plants in 1 gal of cold water, drink 1 cup twice daily.

Anti-inflammatory activity (%)

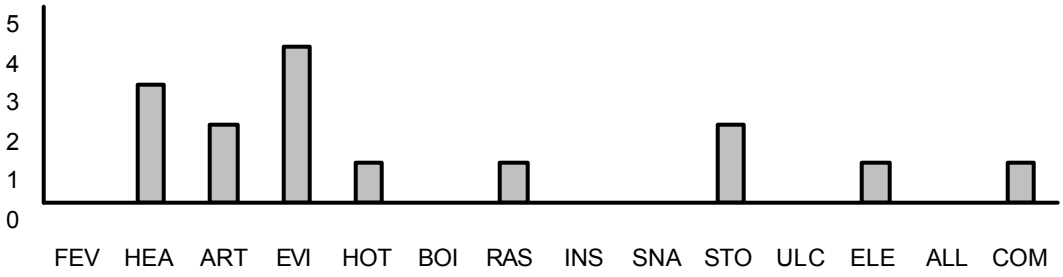
10 µg/mL	100 µg/mL
4.7	13.3

Anthurium willdenowii Kunth.

Q'eqchi' names	Translation
X'chich maus	Devil's sword (X'chich = sword; maus = devil)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.80	1

Total use reports	Q'eqchi' categories	Cook categories
15	8	6



Preparation

Crush 12-13 leaves in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

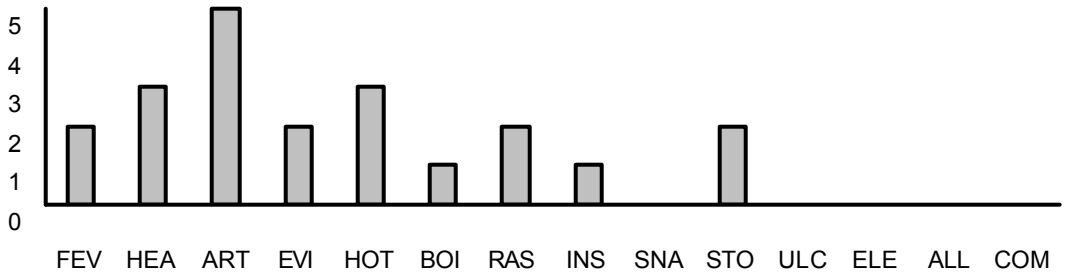
10 µg/mL	100 µg/mL
9.4	1.1

Monstera acuminata K. Koch

Q'eqchi' names	Translation
Jol jol	Very loose (Jol = loose)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.60	1

Total use reports	Q'eqchi' categories	Cook categories
21	9	6



Preparation

Heat 2-3 leaves, apply warm leaves to the affected area and rub into the skin for several minutes. Do this before going to sleep.

Anti-inflammatory activity (%)

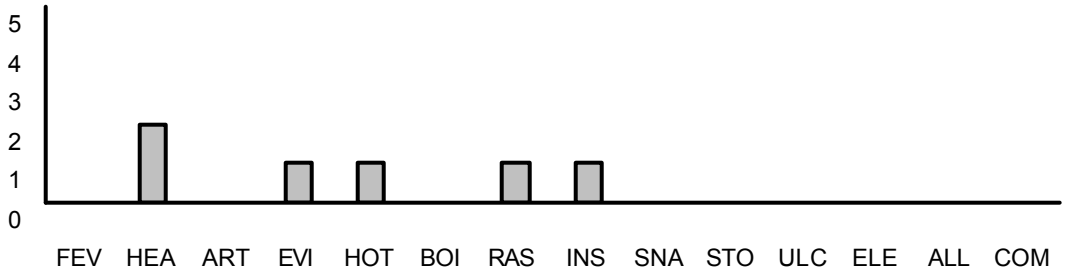
10 µg/mL	100 µg/mL
12.6	73.7

Monstera tuberculata Lundell

Q'eqchi' names	Translation
Letzeb	A plant that wraps around something
Sankil pim	A plant for rotting sores (Sankil = rotting sores; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.50	0.6

Total use reports	Q'eqchi' categories	Cook categories
6	5	4



Preparation

Crush 12-13 leaves in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

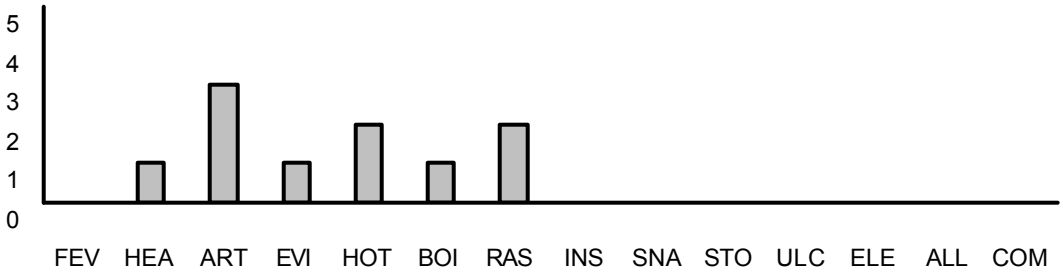
Philodendron hederaceum (Jacq.) Schott

Q'eqchi' names
Kon chi

Translation
Bending down like a snake (Kon = bending down; chi = snake-like)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
10	6	3



Preparation
Heat 2-3 leaves, apply warm leaves to the affected area and rub into the skin for several minutes. Do this before going to sleep.

Anti-inflammatory activity (%)

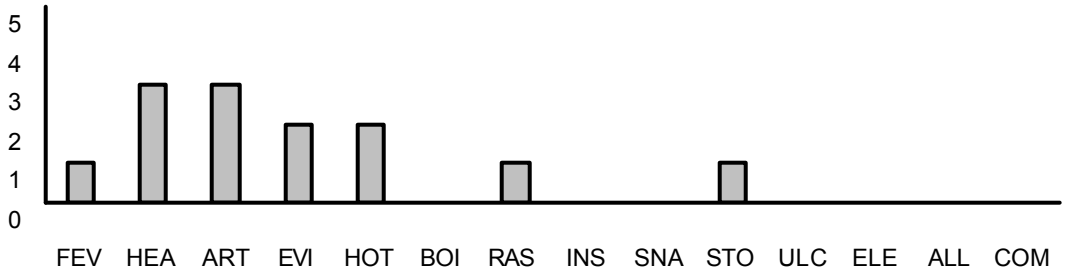
10 µg/mL	100 µg/mL
10.9	11.6

Philodendron radiatum Schott

Q'eqchi' names	Translation
Xilix	Hand-like
Xtonal i uxb	Where the vine roots (Xtonal = base/bottom; i = of; uxb = vine)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.60	0.8

Total use reports	Q'eqchi' categories	Cook categories
13	7	5



Preparation

Heat 2-3 leaves, apply warm leaves to the affected area and rub into the skin for several minutes. Do this before going to sleep.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
6.1	1.1

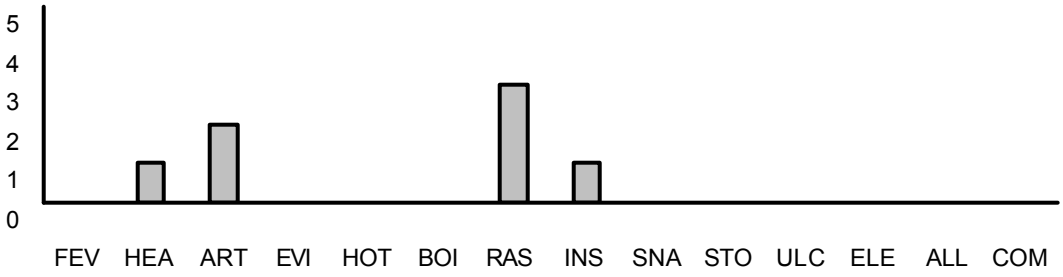
Philodendron schottii K. Koch

Q'eqchi' names
Kek'ek ux

Translation
Very black vine (Kek = black; ek = black; ux = vine)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.25	0.8

Total use reports	Q'eqchi' categories	Cook categories
7	4	4



Preparation
Crush 6-7 leaves in 1 gal of cold water, bathe twice daily. External use only.

Anti-inflammatory activity (%)

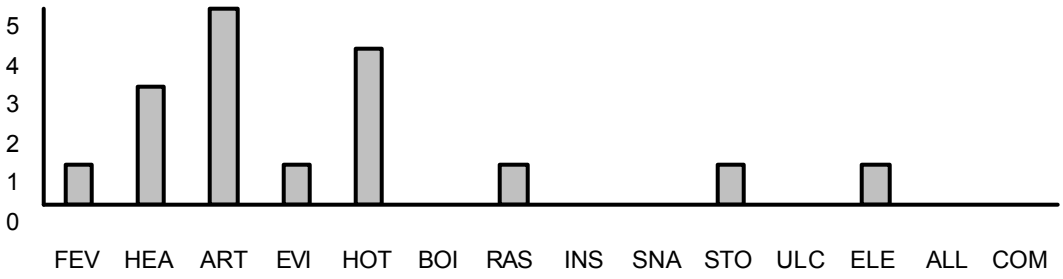
10 µg/mL	100 µg/mL
19.5	73.2

Dendropanax arboreus (L.) Decne & Planch

Q'eqchi' names	Translation
Cojl	A big wooden spoon for stirring pots

Plant part(s) used	Hot/Cold Score	Fuse
L, S	1.00	1

Total use reports	Q'eqchi' categories	Cook categories
17	8	6



Preparation

Boil 20 leaves and stems in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

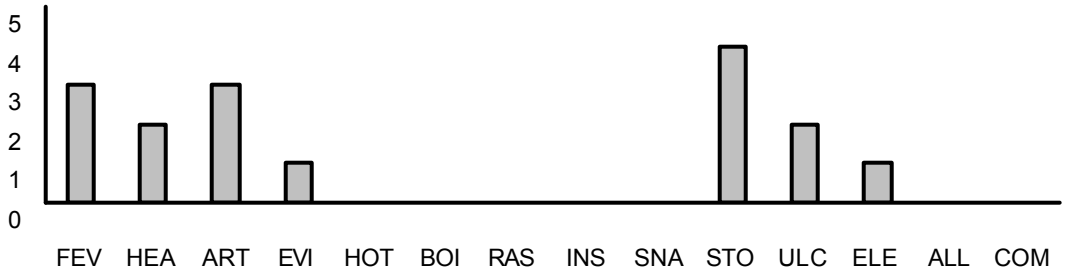
10 µg/mL	100 µg/mL
27.4	21.1

Aritolochia tonduzii O.C. Schmidt

Q'eqchi' names	Translation
Santa Maria kejen	Saint Mary's medicinal plant (Santa Maria = Saint Mary; kejen = medicinal plant)
Sansara kejen	Insence burner medicinal plant (Sansara = clay pot for burning incence; kejen = medicinal plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	1.00	1

Total use reports	Q'eqchi' categories	Cook categories
16	7	5



Preparation

Boil 20 leaves in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

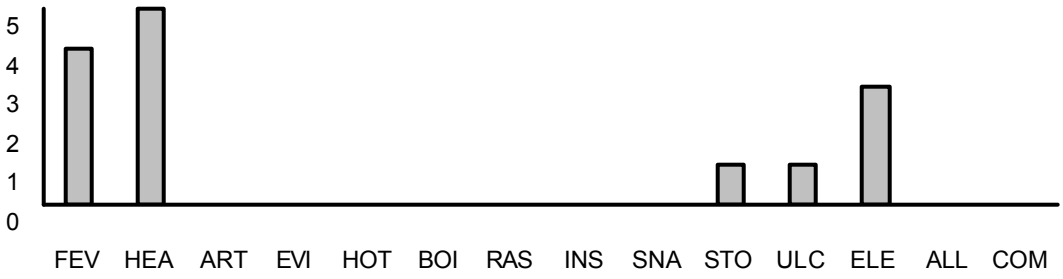
10 µg/mL	100 µg/mL
20.4	-3.5

Bolbitis pergamentacea (Maxon) Ching

Q'eqchi' names	Translation
Re'quaxiru	For crazy person (Re = for; quaxiru = crazy person)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	0.6

Total use reports	Q'eqchi' categories	Cook categories
14	5	4



Preparation

Crush 20 leaves in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
13.3	-1.1

Baccharis trinervis (Lam.) V.M. Badillo

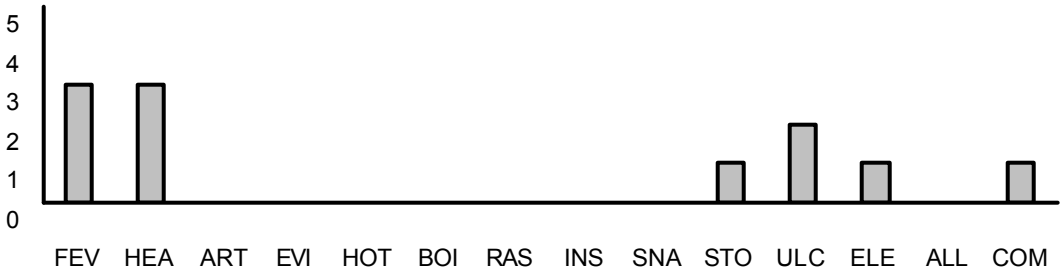


Q'eqchi' names
Cherek sak

Translation
A specific type of grasshopper (Cherek = large square leg grasshopper; sak = grasshopper

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.60	0.8

Total use reports	Q'eqchi' categories	Cook categories
11	6	5



Preparation
Crush 20 tops of plants in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

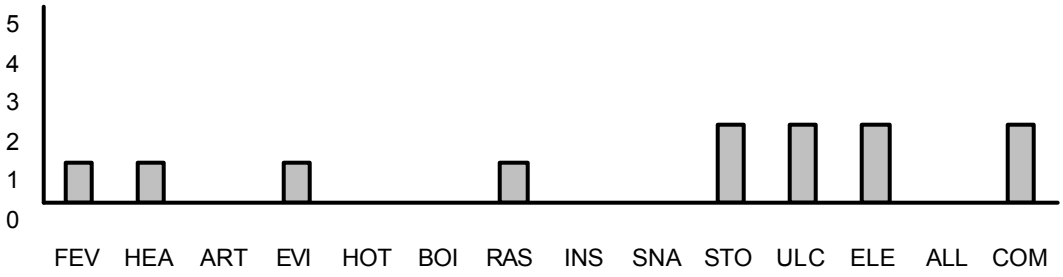
10 µg/mL	100 µg/mL
6.6	37.7

Matricaria recutita L.

Q'eqchi' names	Translation
Menseneya (Creole)	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L, S	0.25	0.8

Total use reports	Q'eqchi' categories	Cook categories
12	8	7



Preparation

Boil leaf and stem of 1 whole plant in 1 gal, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

Mikania leiostachya Benth.



Q'eqchi' names
Juruch aj pak

Translation
Lizard's back (Jurach = back; aj = of; pak = lizard)

Plant part(s) used
L

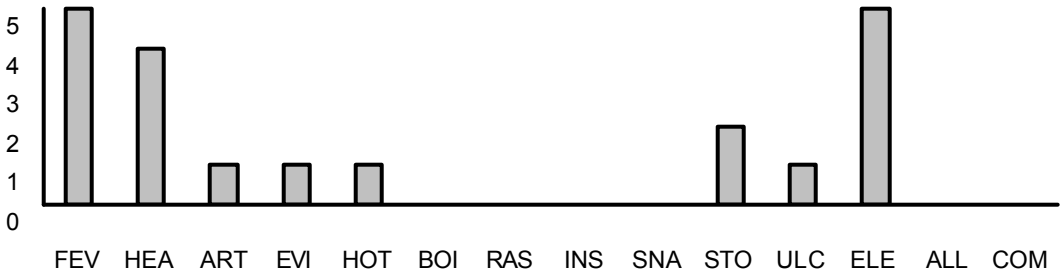
Hot/Cold Score
-0.60

Fuse
1

Total use reports
20

Q'eqchi' categories
8

Cook categories
5



Preparation
Boil or crush 20 leaves in 1 gal cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

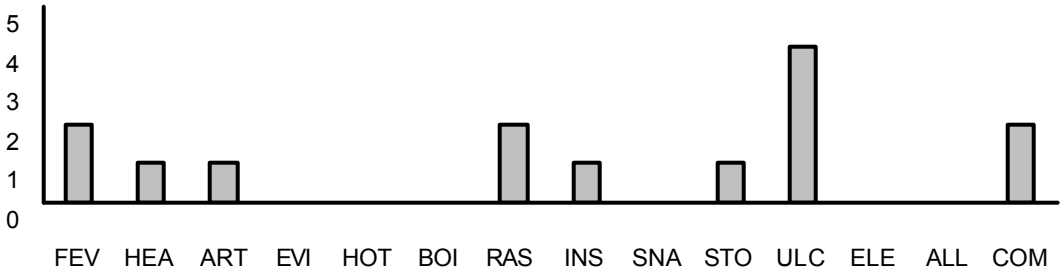
10 µg/mL	100 µg/mL
N.D.	N.D.

Neurolaena lobata (L.) R. Br. ex Cass.

Q'eqchi' names	Translation
K'a mank	Bitter mango (Ka = bitter; mank = mango)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.00	1

Total use reports	Q'eqchi' categories	Cook categories
14	8	7



Preparation

Boil 6 leaves in 1 L of water, drink half in the morning and half in the afternoon.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
2.8	55.4

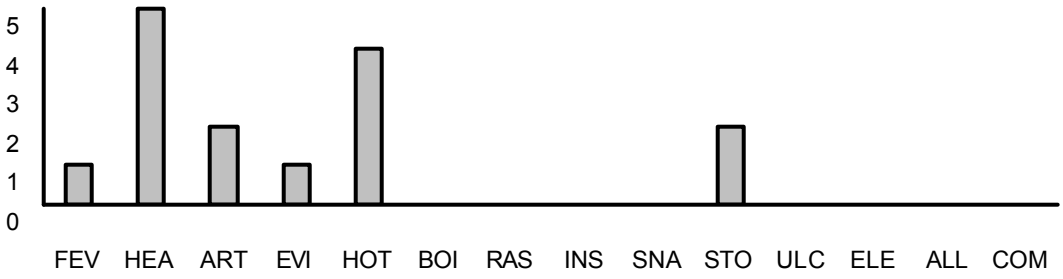
Pluchea carolinensis (Jacq.) G. Don

Q'eqchi' names
Mai pim (#1)

Translation
Pain plant (Mai = pain; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L, R, S	0.60	1

Total use reports	Q'eqchi' categories	Cook categories
15	6	4



Preparation
Boil or crush the leaves of 13 young shoots in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

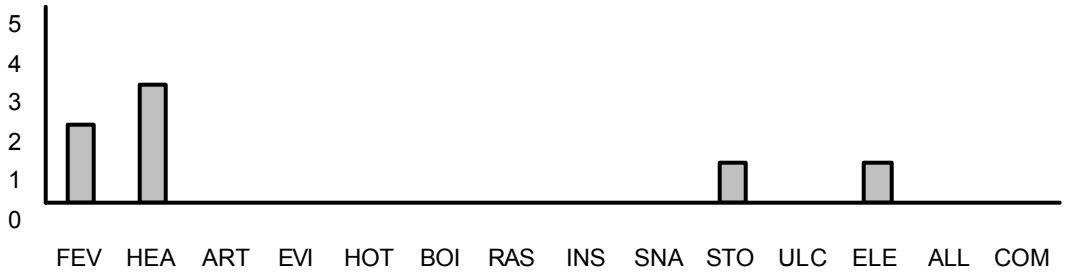
10 µg/mL	100 µg/mL
7.3	7.7

Porophyllum ruderale (Jacq.) Cass.

Q'eqchi' names	Translation
So'sol pim	Vulture plant (So'sol = vulture; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.40	0.8

Total use reports	Q'eqchi' categories	Cook categories
7	4	4



Preparation

Crush 20 tops of plants in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

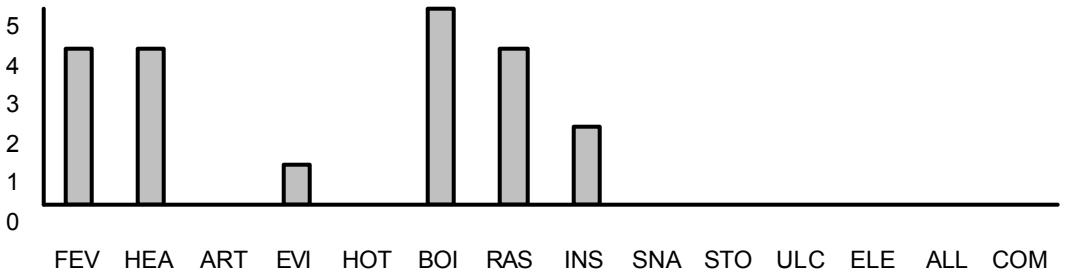
10 µg/mL	100 µg/mL
-5.2	-2.2

Begonia glabra Aubl. Var glabra

Q'eqchi' names	Translation
Pa'ulul	To make a hole through the brain (Pa = to dig/make a hole through; ulul = brain)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
20	6	5



Preparation

Crush 20 leaves in 1 L of cold water, bathe twice daily. Drink a 1/4 cup daily only if necessary.

Anti-inflammatory activity (%)

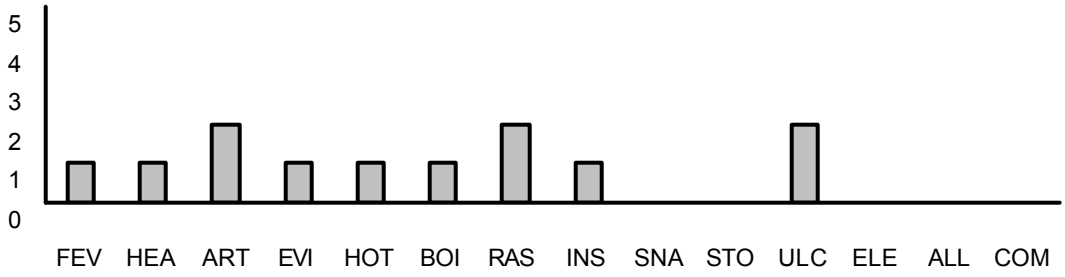
10 µg/mL	100 µg/mL
15.0	6.8

Begonia heracleifolia Schltdl. & Cham.

Q'eqchi' names	Translation
Rutzaj k'opopo'	Frog cane (Rutzaj = cane plant; k'opopo' = frog)
Xak pek (#1)	Plant growing on rock (Xak = leaf; pek = rock)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.40	0.8

Total use reports	Q'eqchi' categories	Cook categories
12	9	6



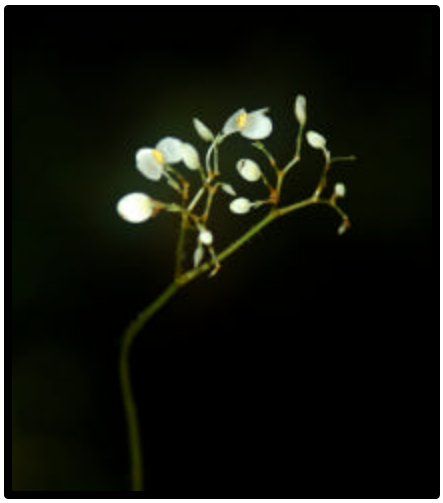
Preparation

Boil or crush 4 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

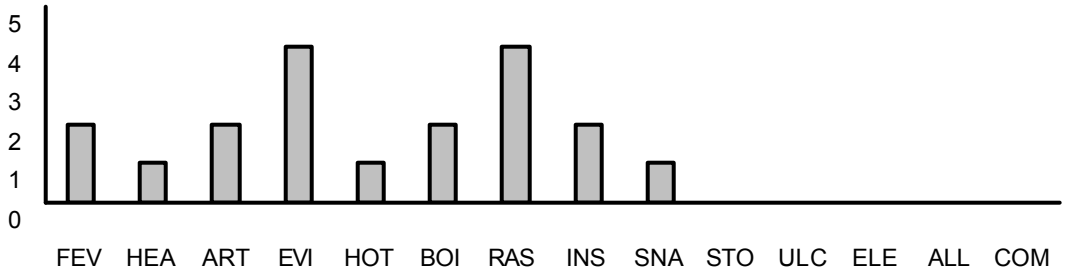
Begonia nelumbiifolia Schltdl. Cham.



Q'eqchi' names	Translation
Xak pek (#2)	Plant growing on rock (Xak = leaf; pek = rock)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.40	1

Total use reports	Q'eqchi' categories	Cook categories
19	9	5



Preparation

Crush 4 leaves in 1 L of cold water, bathe area twice daily. Or, warm 4 leaves, tie warm leaves to the affected area, repeat twice daily.

Anti-inflammatory activity (%)

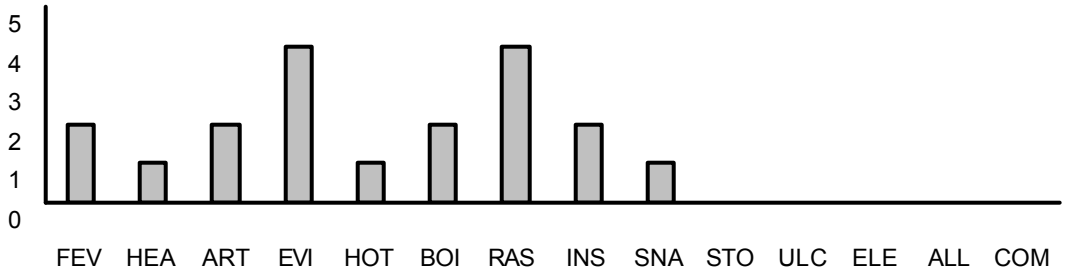
10 µg/mL	100 µg/mL
13.1	9.0

Begonia sericoneura Liebm

Q'eqchi' names	Translation
Xak pek (#3)	Plant growing on rock (Xak = leaf; pek = rock)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.40	1

Total use reports	Q'eqchi' categories	Cook categories
19	9	5



Preparation

Crush 4 leaves in 1 L of cold water, bathe area twice daily. Or, warm 4 leaves, tie warm leaves to the affected area, repeat twice daily.

Anti-inflammatory activity (%)

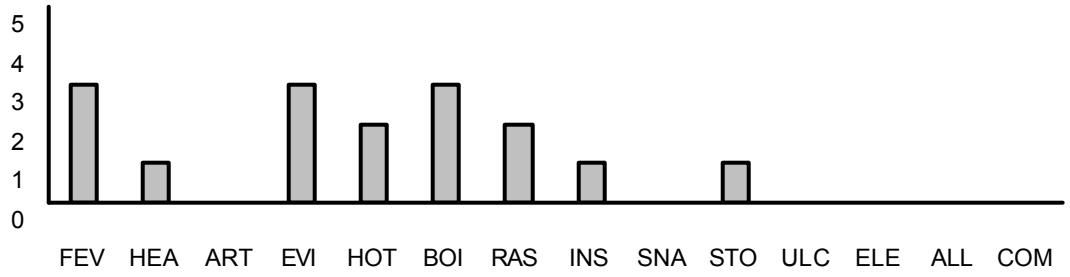
10 µg/mL	100 µg/mL
N.D.	N.D.

Macfadyena unguis-cati (L.) A.H. Gentry

Q'eqchi' names	Translation
Rixij tzunun	Hummingbird toenails (Rixij = toenails or fingernails; tzunun = hummingbird)

Plant part(s) used	Hot/Cold Score	Fuse
L, R	-0.67	0.6

Total use reports	Q'eqchi' categories	Cook categories
16	8	6



Preparation

Crush a combination of root and leaf and apply as a poultice twice daily.

Anti-inflammatory activity (%)

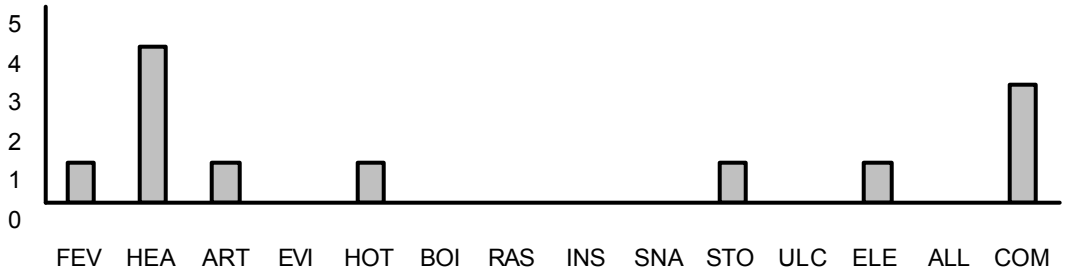
10 µg/mL	100 µg/mL
N.D.	N.D.

Pitcairnia punicea Scheidw.

Q'eqchi' names	Translation
Kis kim i ha	Aromatic water plam (Kis = aromatic; kim = plam-like leaf; i = of; ha = water)
Mes i ha	Water broom (Mes = broom; i = of; ha = water)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.20	0.8

Total use reports	Q'eqchi' categories	Cook categories
12	7	6



Preparation

Boil four bundles of leaves in 1 L of water, drink half in the moring and half in the afternoon.

Anti-inflammatory activity (%)

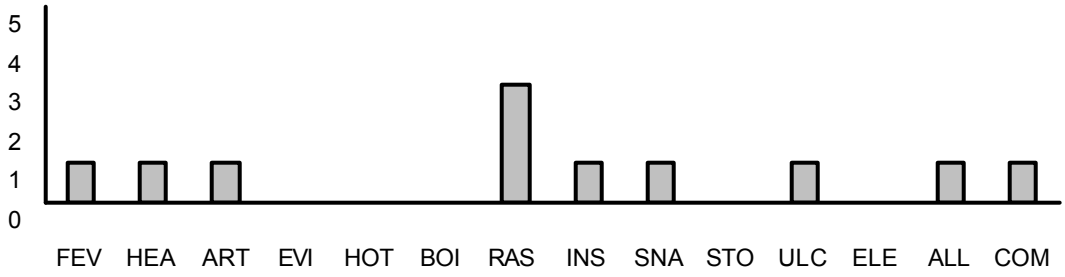
10 µg/mL	100 µg/mL
3.8	5.6

Bursera simaruba (L.) Sarg.

Q'eqchi' names	Translation
Kak kajl	Peeling Red (Kak = red; kajl = peeling)
Gumbo limbo (Creole)	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
B	0.50	0.8

Total use reports	Q'eqchi' categories	Cook categories
11	9	7



Preparation

Boil 4 pieces of bark in 1 L of water, drink 1 cup and bathe twice daily. Or, crush inner bark and rub into the skin for several minutes.

Anti-inflammatory activity (%)

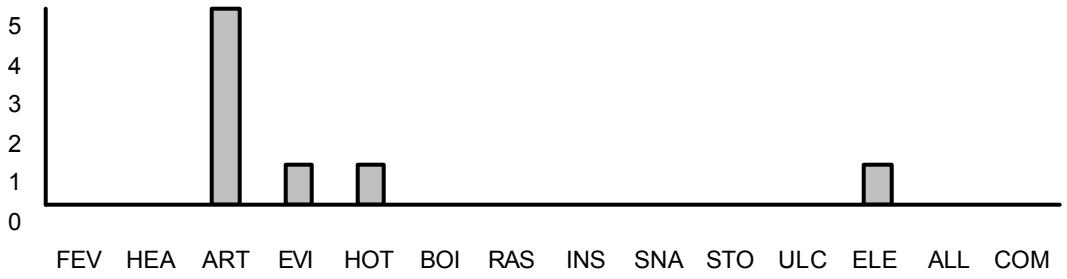
10 µg/mL	100 µg/mL
-13.0	14.1

Epiphyllum crenatum (Lindl.) G. Don

Q'eqchi' names	Translation
Chic'ba'bac (#1)	For joining bones (Chic = to join; ba = of; bac = bone)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.60	1

Total use reports	Q'eqchi' categories	Cook categories
8	4	2



Preparation

Tie a piece to the affected area, replace when dry (approximately every two days).

Anti-inflammatory activity (%)

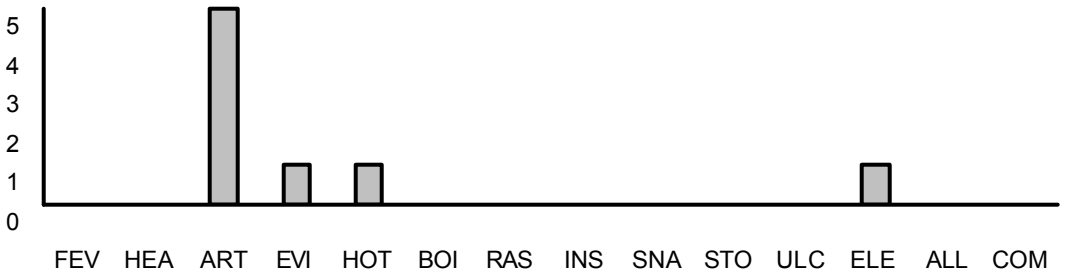
10 µg/mL	100 µg/mL
N.D.	N.D.

Epiphyllum phyllanthus (L.) Haw.

Q'eqchi' names	Translation
Chic'ba'bac (#2)	For joining bones (Chic = to join; ba = of; bac = bone)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.60	1

Total use reports	Q'eqchi' categories	Cook categories
8	4	2



Preparation

Tie a piece to the affected area, replace when dry (approximately every two days).

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

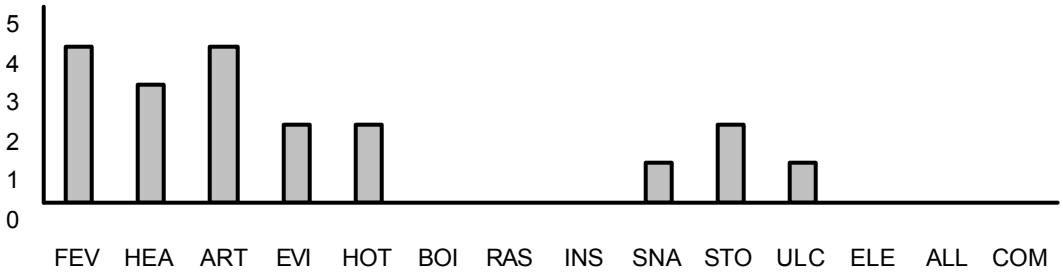
Crossopetalum eucymosum (Loes. & Pittier) Lundell

Q'eqchi' names
Se ruj ajaw chan

Translation
Boa constictor's eye (Se = the; ruj = eye; ajaw chan = boa constrictor)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.50	0.8

Total use reports	Q'eqchi' categories	Cook categories
19	8	5



Preparation
Crush 20 leaves in 1 L of cold water, drink half in the morning and half in the afternoon.

Anti-inflammatory activity (%)

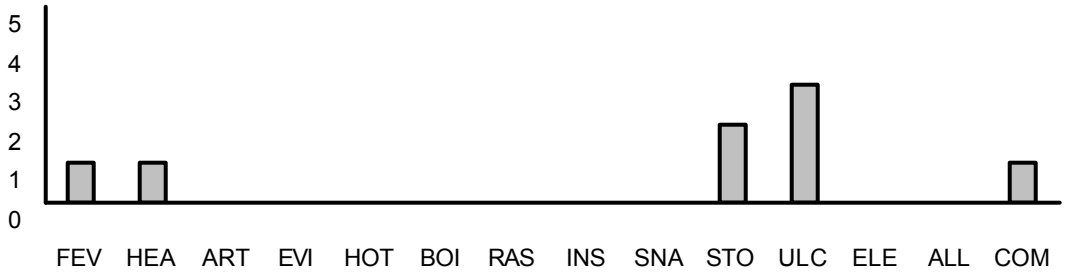
10 µg/mL	100 µg/mL
7.8	27.2

Chenopodium ambrosioides L.

Q'eqchi' names	Translation
Isqij'i'pur	Spice for shells (Isqij = aromatice plant for cooking; i = for; pur = shells)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.25	0.8

Total use reports	Q'eqchi' categories	Cook categories
8	5	4



Preparation

Boil 6 leaves in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

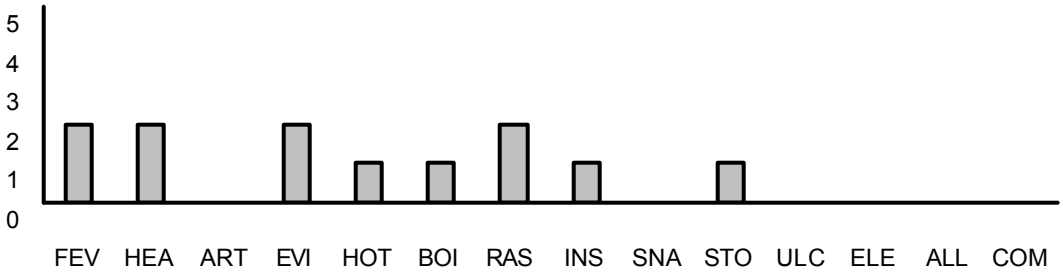
10 µg/mL	100 µg/mL
N.D.	N.D.

Tradescantia spathacea Sw.

Q'eqchi' names	Translation
Ton kit	Bloody bottom (Ton = base/bottom; kit = blood)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	0.6

Total use reports	Q'eqchi' categories	Cook categories
12	8	6



Preparation

Crush 2 leaves and apply as a poultice twice daily.

Anti-inflammatory activity (%)

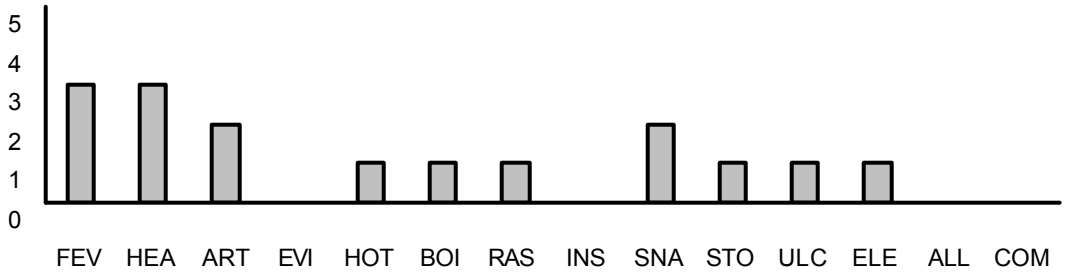
10 µg/mL	100 µg/mL
63.6	98.4

Itzaea sericea (Standl.) Standl. & Steyerm.

Q'eqchi' names	Translation
Iqbolie pim (#1)	Snake plant (Iqbolie = a specific type of snake; pim = plant)
Saki iqbolie pim	White iqbolie pim (Saki = white)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
16	10	7



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

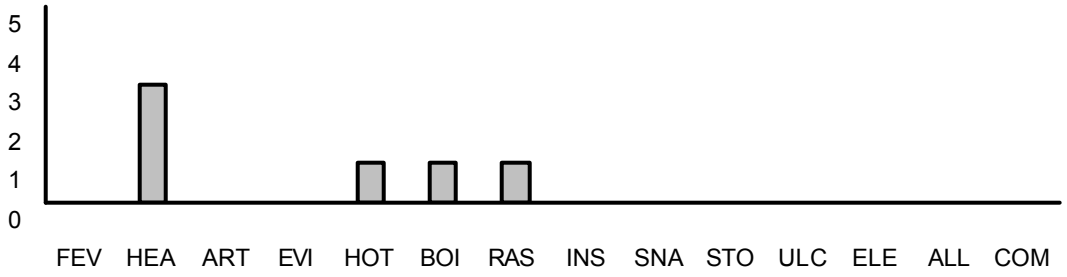
10 µg/mL	100 µg/mL
N.D.	N.D.

Costus pulverulentus C. Presl

Q'eqchi' names	Translation
Kaki chun	Red chun (Kaki = red; chun = this specific plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.60	0.6

Total use reports	Q'eqchi' categories	Cook categories
6	4	3



Preparation

Boil or crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily. Or, crush 20 leaves and apply as a poultice twice daily.

Anti-inflammatory activity (%)

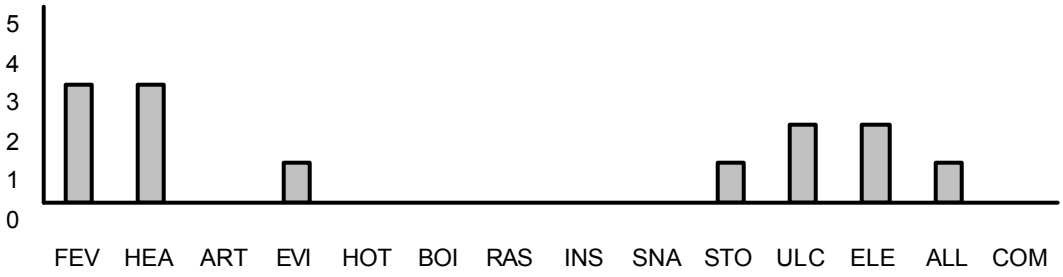
10 µg/mL	100 µg/mL
7.4	45.2

Kalanchoe pinnata (Lam.) Pers.

Q'eqchi' names	Translation
No name	

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	0.6

Total use reports	Q'eqchi' categories	Cook categories
13	7	5



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

Gurania makoyana (Lem.) Cogn.

Q'eqchi' names
Kum pim

Translation
Pumpkin plant (Kum = pumpkin; pim = plant)

Plant part(s) used
L

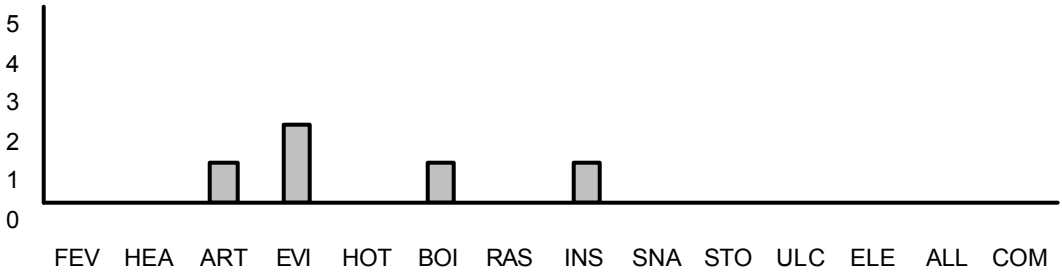
Hot/Cold Score
-1.00

Fuse
0.4

Total use reports
5

Q'eqchi' categories
4

Cook categories
2



Preparation
Crush 4 leaves and apply as a poultice twice daily.

Anti-inflammatory activity (%)

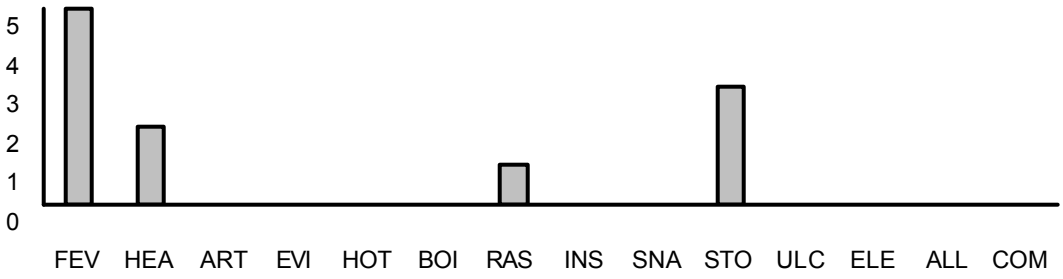
10 µg/mL	100 µg/mL
-1.5	12.9

Momordica charatia L.

Q'eqchi' names	Translation
Sand'ia cho	Rat's watermelon (Sand'ia wantermelon; cho = rat)
Sorosi (Spanish)	Sorosi = specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L, S	0.60	1

Total use reports	Q'eqchi' categories	Cook categories
11	4	4



Preparation

Crush a handful of leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

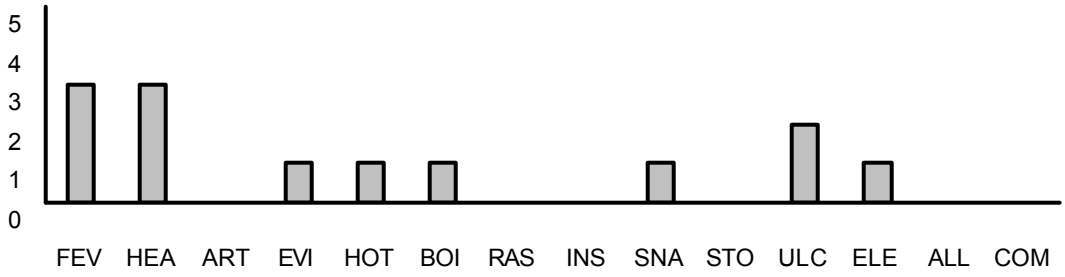
10 µg/mL	100 µg/mL
N.D.	N.D.

Nephrolepis biserrata (Sw.) Schott

Q'eqchi' names	Translation
Ixqu'oq mo'coch	Cohune palm bending down (Ixqu'oq = bending down; mo'coch = cohune palm)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
13	8	7



Preparation

Crush 12 baldes in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

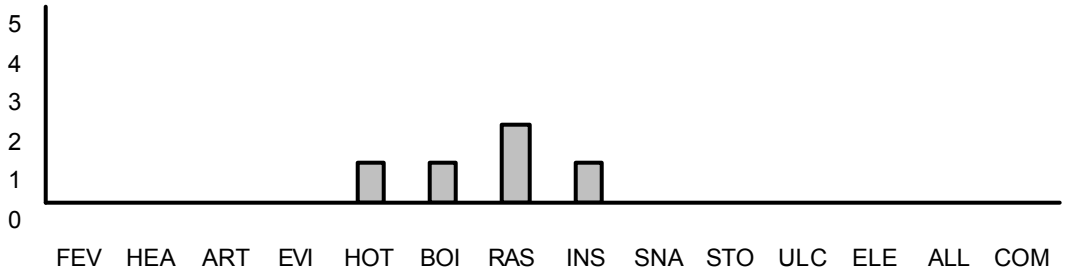
10 µg/mL	100 µg/mL
N.D.	N.D.

Dracaena americana Donn. Sw.

Q'eqchi' names	Translation
Tut	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
B	0.25	0.6

Total use reports	Q'eqchi' categories	Cook categories
5	4	3



Preparation

Crush a handful of whole bark and apply as a poultice twice daily.

Anti-inflammatory activity (%)

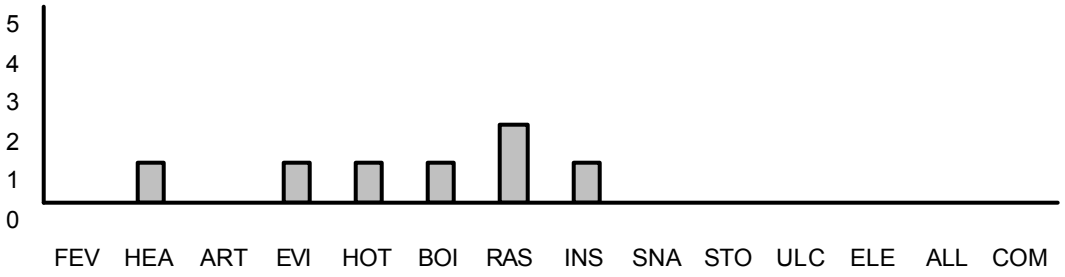
10 µg/mL	100 µg/mL
13.2	-8.9

Croton xalapensis H.B.K.

Q'eqchi' names	Translation
Noq te	Thread tree (Noq = thread; te = tree)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.50	0.8

Total use reports	Q'eqchi' categories	Cook categories
7	6	4



Preparation

Crush 20 leaves in 1 gal of cold water, bathe twice daily. Or, crush 20 leaves and apply as a poultice twice daily. External use only.

Anti-inflammatory activity (%)

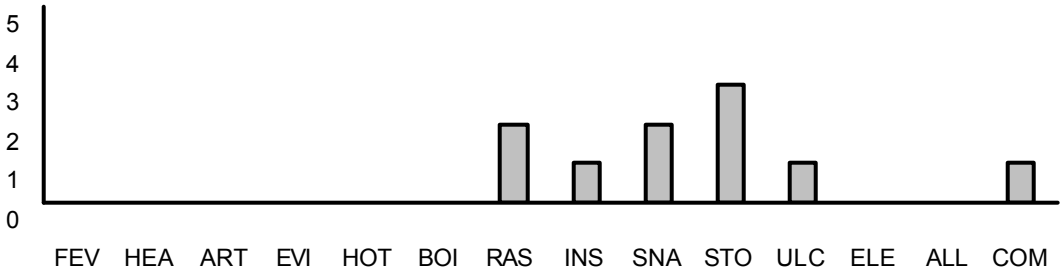
10 µg/mL	100 µg/mL
N.D.	N.D.

Acacia cornigera (L.) Willd.

Q'eqchi' names	Translation
Subin	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L, R, S	0.25	0.8

Total use reports	Q'eqchi' categories	Cook categories
10	6	4



Preparation

Boil 12 leaves and stems in 1 L of water, drink 1 cup and bathe twice daily. Or, boil a 30 cm long piece of root in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

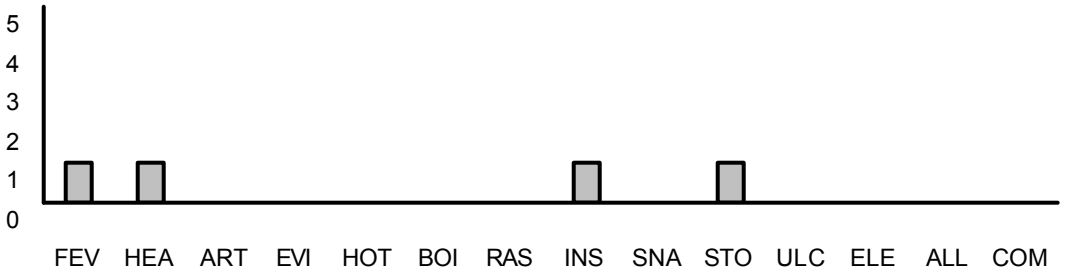
10 µg/mL	100 µg/mL
9.7	33.8

Cojoba graciliflora (S. F. Blake) Britton & Rose

Q'eqchi' names	Translation
Choql ok te	Cloud bean-pod fruiting tree (Choql = cloud; ok = bean-pod fruit of this tree; te = tree)

Plant part(s) used	Hot/Cold Score	Fuse
L, B	0.67	0.4

Total use reports	Q'eqchi' categories	Cook categories
4	4	4



Preparation

Crush a 30 cm long piece of bark and a handful of leaves in 1 gal of cold water, bathe twice daily. External use only.

Anti-inflammatory activity (%)

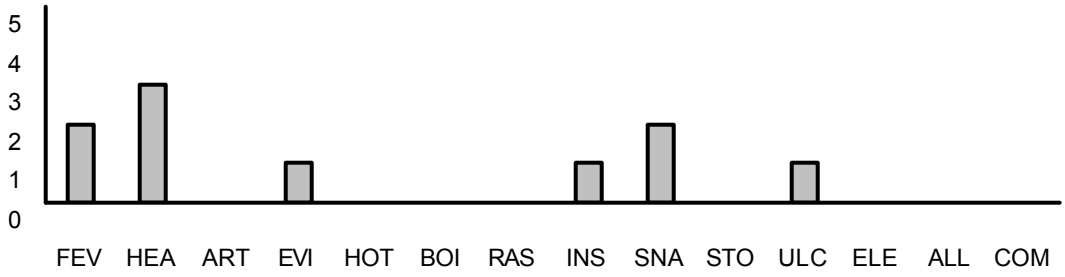
10 µg/mL	100 µg/mL
N.D.	N.D.

Mimosa pudica L.

Q'eqchi' names	Translation
Quare kix	Sleepy kix (Quare = sleepy; kix = this specfic plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.60	0.8

Total use reports	Q'eqchi' categories	Cook categories
10	6	4



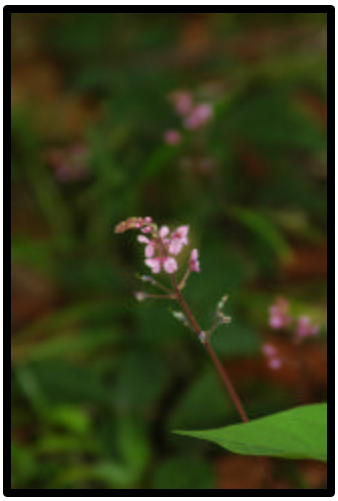
Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

Desmodium adscendens (Sw.) DC.



Q'eqchi' names
Chint pim (#1)

Translation
Chint plant (Chint = this specific plant; pim = plant)

Plant part(s) used
L, S

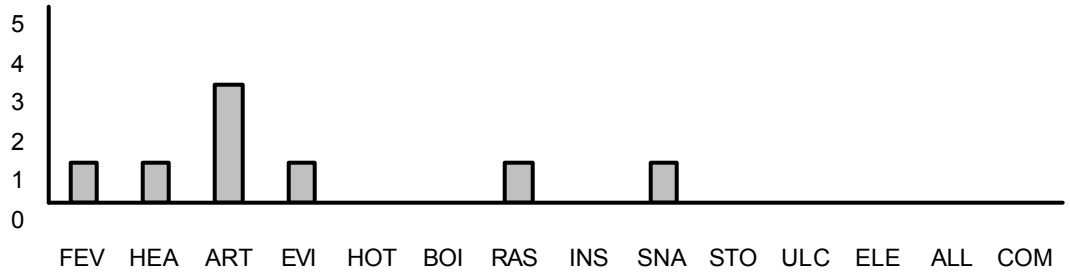
Hot/Cold Score
0.20

Fuse
0.8

Total use reports
8

Q'eqchi' categories
6

Cook categories
4



Preparation
Boil the leaves and stems of 20 whole plants i 1 L of water, drink 1 cup and bathe twice daily. Can use Desmodium adscendens or Desmodium axillare, a combination is preferable.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

Desmodium axillare var. acutifolius Kuntze

Q'eqchi' names
Chint pim (#2)

Translation
Chint plant (Chint = this specific plant; pim = plant)

Plant part(s) used
L, S

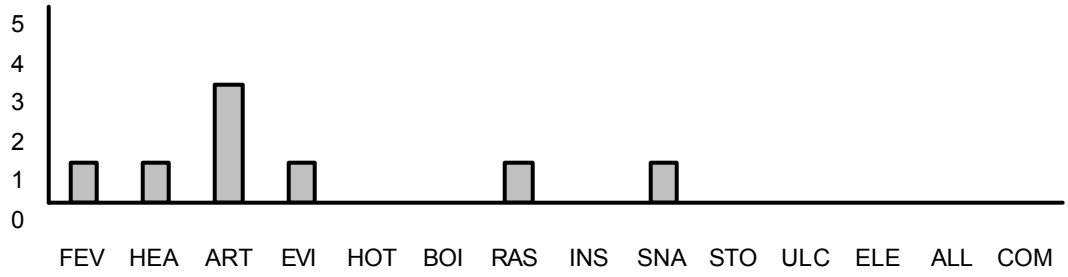
Hot/Cold Score
0.20

Fuse
0.8

Total use reports
8

Q'eqchi' categories
6

Cook categories
4



Preparation
Boil the leaves and stems of 20 whole plants i 1 L of water, drink 1 cup and bathe twice daily. Can use Desmodium adscendens or Desmodium axillare, a combination is preferable.

Anti-inflammatory activity (%)

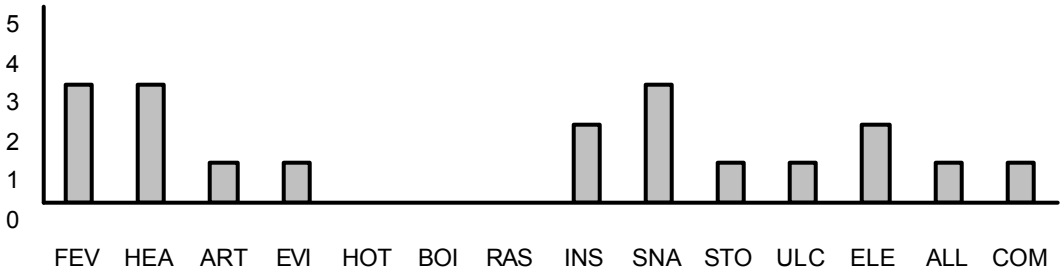
10 µg/mL	100 µg/mL
N.D.	N.D.

Besleria laxiflora Benth.

Q'eqchi' names	Translation
Jolom masan	Shrimp's head (Jolom = head; masan = shrimp)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	0.6

Total use reports	Q'eqchi' categories	Cook categories
19	11	6



Preparation

Crush 12 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

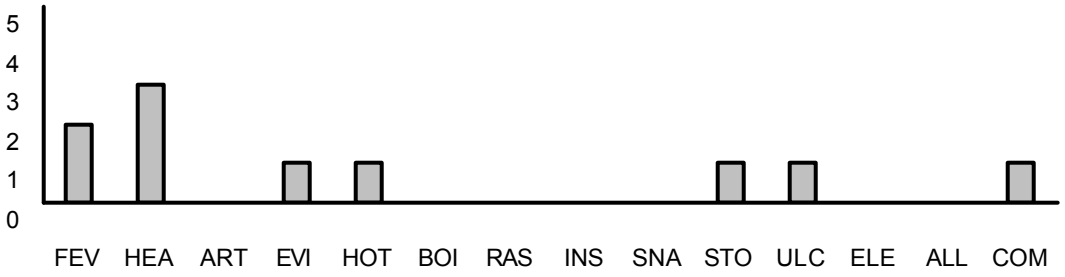
10 µg/mL	100 µg/mL
6.9	-1.5

Codonanthe uleana Fritsch

Q'eqchi' names	Translation
Cacao pim	Cacao plant (Cacao = Theobroma cacao; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	0.6

Total use reports	Q'eqchi' categories	Cook categories
10	7	5



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

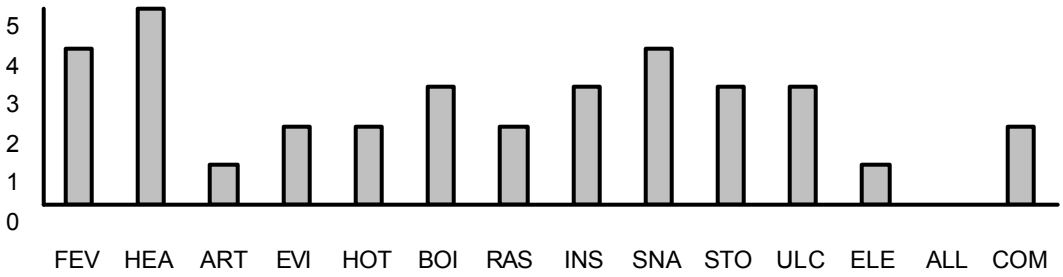
10 µg/mL	100 µg/mL
30.4	19.3

Drymonia serrulata

Q'eqchi' names	Translation
Baknel pim	Snake plant (Baknel = a specific type of snake; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
35	13	8



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
2.6	10.9

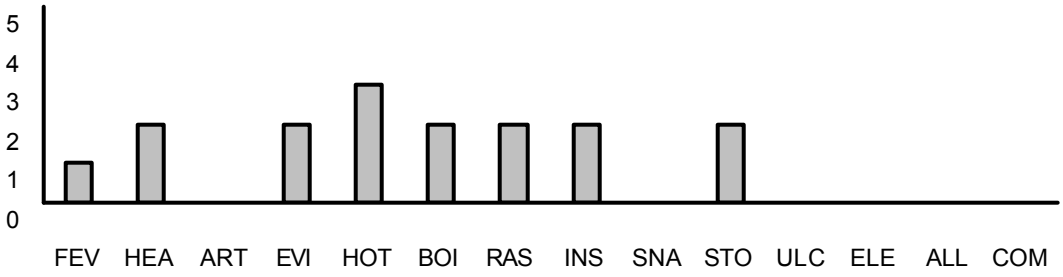
Xiphidium caeruleum Aubl.

Q'eqchi' names
Ixcua'i'kuch

Translation
Hawk's food (Ixcua = food; i = of; kuch = hawk)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	-0.60	1

Total use reports	Q'eqchi' categories	Cook categories
16	8	6



Preparation
Crush 4 leaves and apply as a poultice twice daily. External use only.

Anti-inflammatory activity (%)

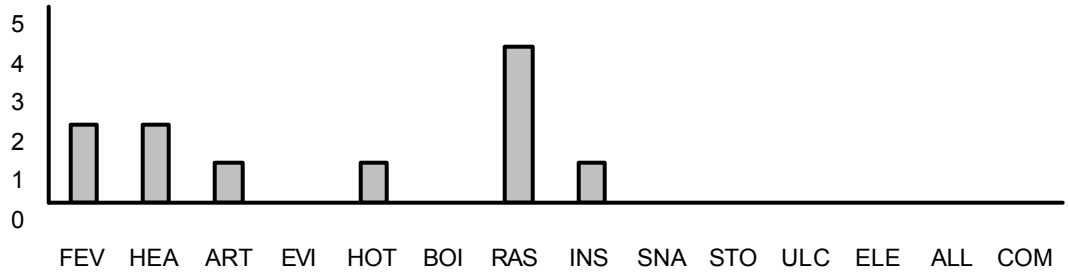
10 µg/mL	100 µg/mL
1.8	11.8

Hyptis capitata Jacq.

Q'eqchi' names	Translation
Se ruj kaway	Horse's eye (Se = the; ruj = eye; kaway = horse)

Plant part(s) used	Hot/Cold Score	Fuse
L	1.00	1

Total use reports	Q'eqchi' categories	Cook categories
11	6	5



Preparation

Boil 20 leaves in 1 gal of water, drink 1/2 cup and bathe twice daily. Do not exceed 1 cup daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
2.0	-42.1

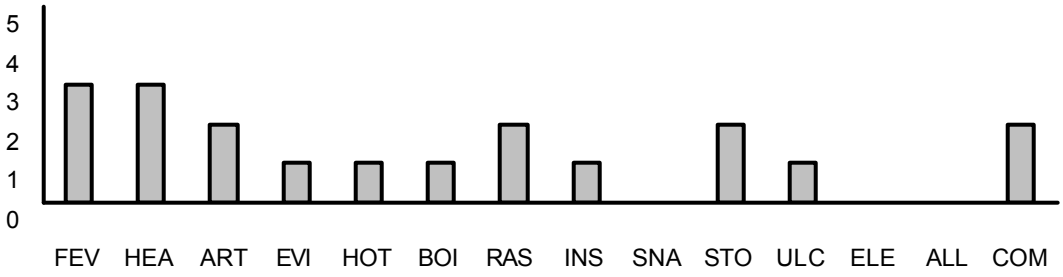
Hyptis verticillata Jacq.

Q'eqchi' names
Chu pim

Translation
Bad smelling plant (Chu = bad smelling; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.60	1

Total use reports	Q'eqchi' categories	Cook categories
19	11	7



Preparation
Boil 20 tops of plants in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
-10.4	66.2

Pectis sp.

Q'eqchi' names
Pericón (Spanish)

Translation
Specific name for this plant

Plant part(s) used
L, S

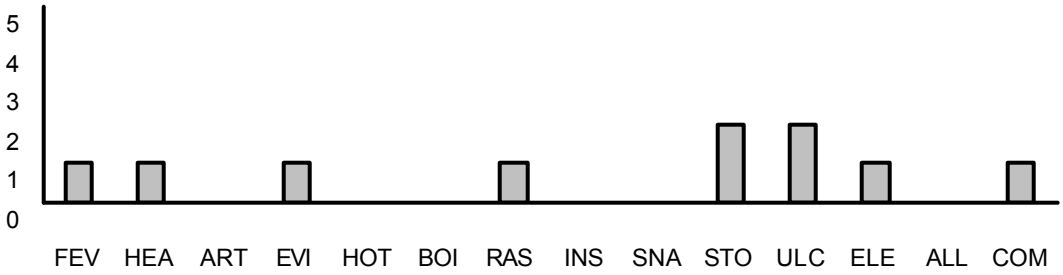
Hot/Cold Score
0.50

Fuse
0.4

Total use reports
10

Q'eqchi' categories
8

Cook categories
6



Preparation
Boil 1 dry bundle of leaves and stem in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

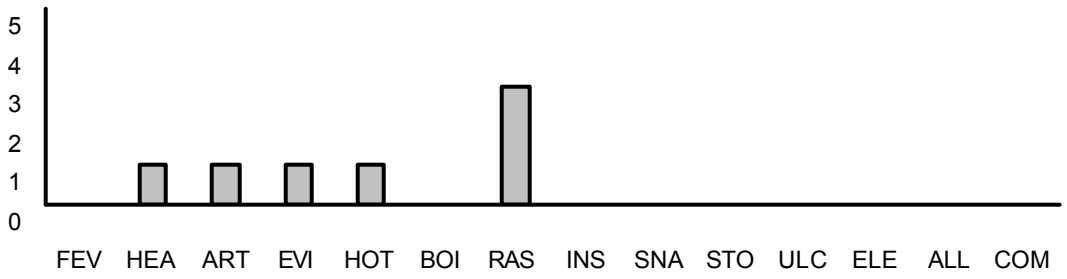
10 µg/mL	100 µg/mL
N.D.	N.D.

Phthirusa pyrifolia (H.B.K.) Eichler

Q'eqchi' names	Translation
Neba pim	Orphan plant (Neba = orphan; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.50	0.8

Total use reports	Q'eqchi' categories	Cook categories
7	5	3



Preparation

Boil 20 leaves in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

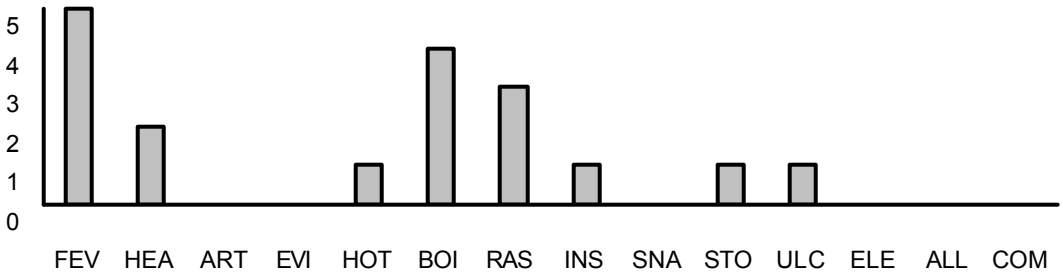
10 µg/mL	100 µg/mL
N.D.	N.D.

Sida rhombifolia L.

Q'eqchi' names	Translation
Mes b'eel	Broom for sweeping (Mes = broom; b'eel = for sweeping)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.60	1

Total use reports	Q'eqchi' categories	Cook categories
18	8	6



Preparation

Crush 12 tops of plants and apply as a poultice, twice daily.

Anti-inflammatory activity (%)

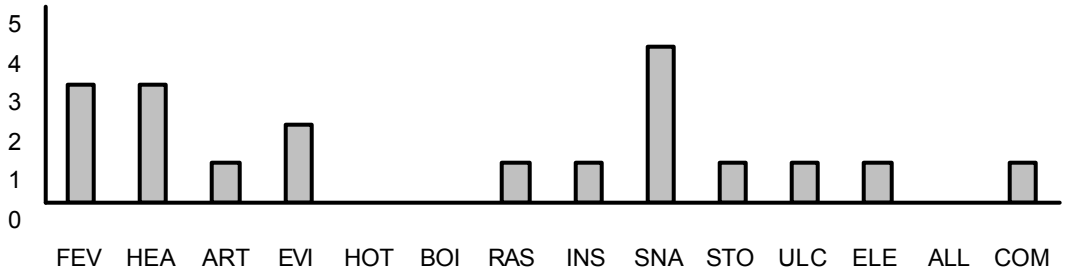
10 µg/mL	100 µg/mL
15.8	23.0

Marcgravia gentlei Lundell

Q'eqchi' names	Translation
Rubelsa'i'xul	Snake's belly (Rubelsa = belly; i =of; xul = a specific snake)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
19	11	7



Preparation

Crush 4 30cm pieces of leaves in1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

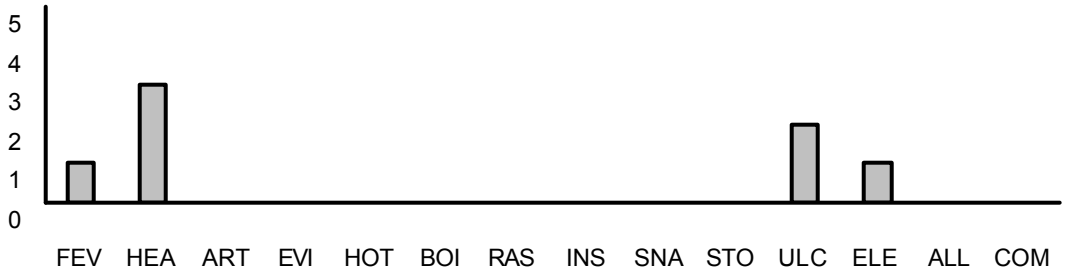
10 µg/mL	100 µg/mL
N.D.	N.D.

Souroubea sympetala Gilg.

Q'eqchi' names	Translation
Hub'ub	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.75	0.8

Total use reports	Q'eqchi' categories	Cook categories
7	4	4



Preparation

Crush 20 leaves in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

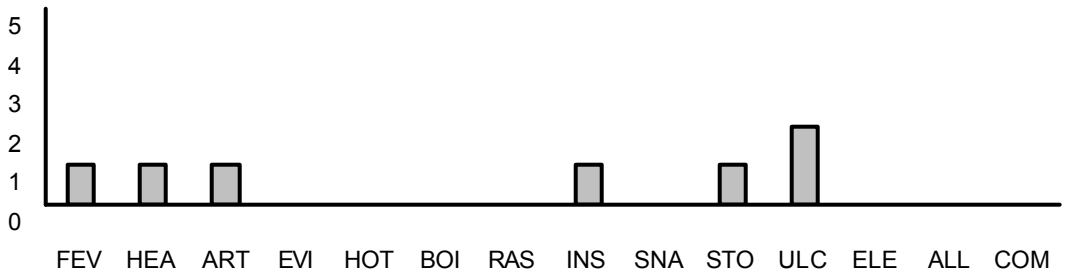
10 µg/mL	100 µg/mL
N.D.	N.D.

Arthrostemma ciliatum Pav. ex D. Don

Q'eqchi' names	Translation
Selek sak	Grasshopper's leg (Selek = leg; sak = grasshopper)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.20	0.4

Total use reports	Q'eqchi' categories	Cook categories
7	6	4



Preparation

Boil 12 tops of plants in 1 L of water, drink half in the morning and half in the afternoon.

Anti-inflammatory activity (%)

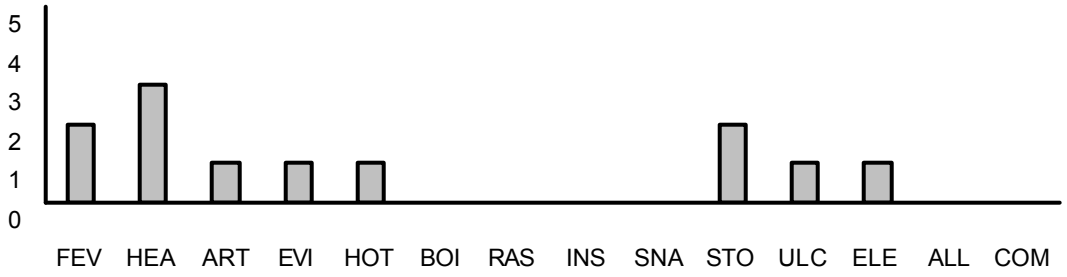
10 µg/mL	100 µg/mL
10.7	3.9

Blackea cuneata Standl.

Q'eqchi' names	Translation
Oxlaho chajom	Thirteen year old teenage boy (Oxlaho = thirteen; chajom = teenage boy)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.33	0.6

Total use reports	Q'eqchi' categories	Cook categories
12	8	5



Preparation

Boil 12 leaves in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

Miconia gracilis Triana

Q'eqchi' names
Roq muqui

Translation
Ground dove's foot (Roq = foot; muqui = ground dove)

Plant part(s) used
L

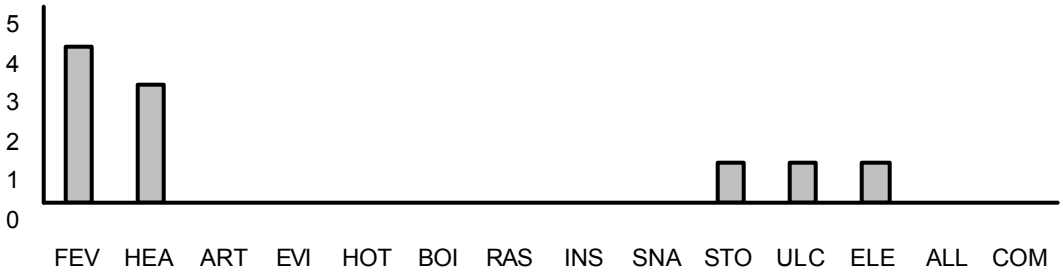
Hot/Cold Score
-0.60

Fuse
0.8

Total use reports
10

Q'eqchi' categories
5

Cook categories
4



Preparation
Crush a handful of leave in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

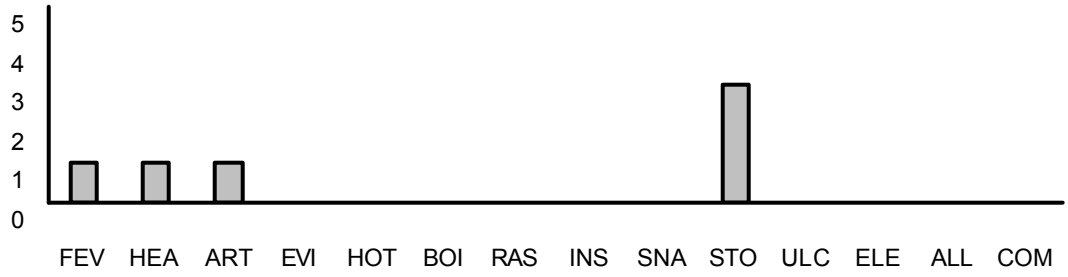
10 µg/mL	100 µg/mL
N.D.	N.D.

Abuta panamensis (Standl.) Krukoff & Barneby

Q'eqchi' names	Translation
Raxi chajom kajam	Green teenage boy vine (Raxi = green or blue; chajom = teenage boy; kajam = vine)

Plant part(s) used	Hot/Cold Score	Fuse
L, B	1.00	0.6

Total use reports	Q'eqchi' categories	Cook categories
6	4	3



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily. Or, boil a handful of bark in 1 L of water, drink 1 cup twice daily.

Anti-inflammatory activity (%)

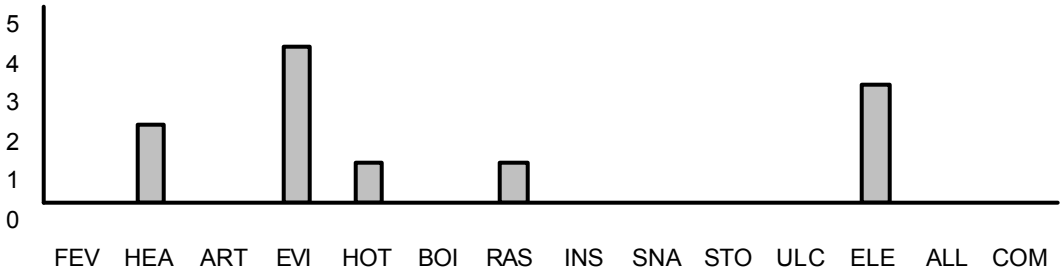
10 µg/mL	100 µg/mL
N.D.	N.D.

Disciphania calocarpa Standl.

Q'eqchi' names	Translation
Roq maus	Devil's foot (Roq = foot; maus = devil)
Xa'ab maus	Devil's shoe (Xa'ab = shoe; maus = devil)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	-0.60	1

Total use reports	Q'eqchi' categories	Cook categories
11	5	4



Preparation

Crush 20 leaves and stems in 1 gal of cold water, drink 1 cup twice daily.

Anti-inflammatory activity (%)

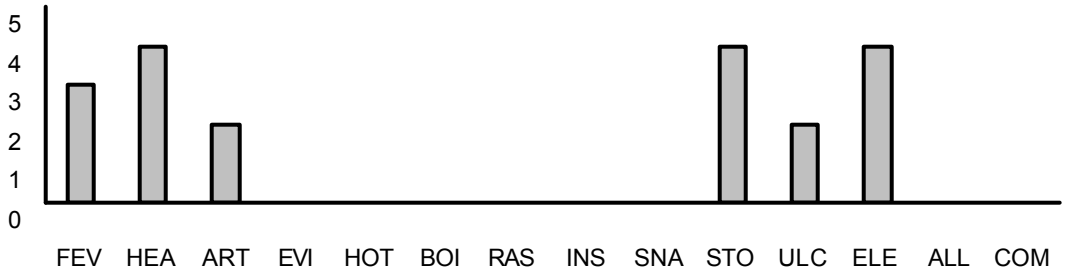
10 µg/mL	100 µg/mL
N.D.	N.D.

Mollinedia guatemalensis Perkins

Q'eqchi' names	Translation
Saki kejen	White medicinal plant (Sake = white; kejen = medicinal plant)
Saki	White plant (Saki = white; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.20	1

Total use reports	Q'eqchi' categories	Cook categories
19	6	4



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

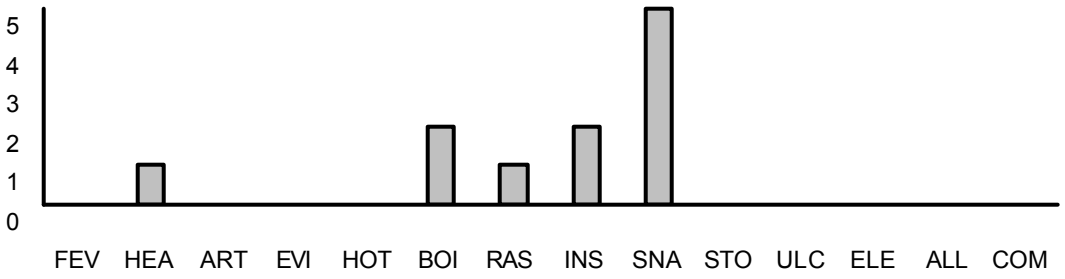
10 µg/mL	100 µg/mL
34.3	89.4

Dorstenia lindeniana Bureau

Q'eqchi' names	Translation
Chacbolie kejen	Tommygoff medicinal plant (Chacbolie = yellow-jaw tommygoff snake; kejen = medicinal plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
11	5	3



Preparation

Boil or crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

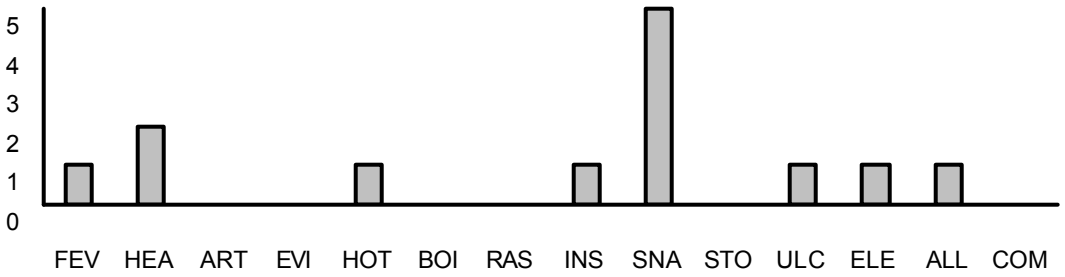
10 µg/mL	100 µg/mL
-0.8	19.1

Oeceoclodes maculata (Lindl.) Lindl.

Q'eqchi' names	Translation
Iqbolie pim (#2)	Snake plant (Iqbolie = a specific type of snake; pim = plant)
Kurarin re kitche	Kurarin re kitche = this specific plant

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
13	8	7



Preparation

Crush 1 leaves and apply as a poultice twice daily.

Anti-inflammatory activity (%)

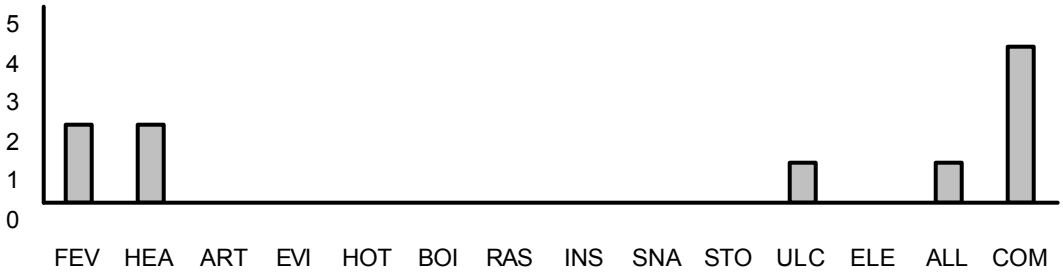
10 µg/mL	100 µg/mL
9.2	12.8

Passiflora oerstedii Hast. var choconiana S. Watson

Q'eqchi' names	Translation
Tu kej kejen	Deer breast medicinal plant (Tu = breast; kej = deer; kejen = medicinal plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.60	1

Total use reports	Q'eqchi' categories	Cook categories
10	5	4



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

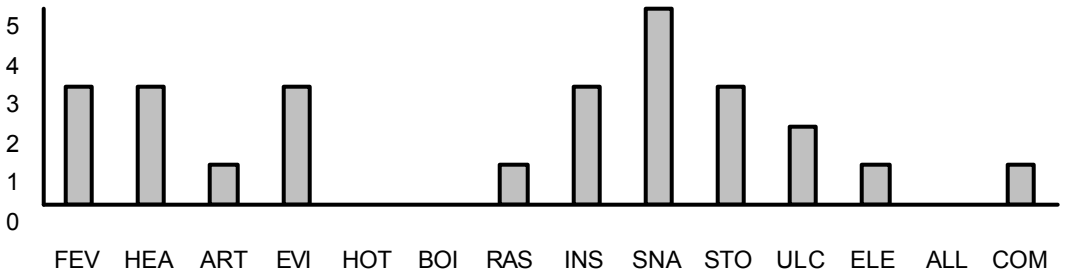
10 µg/mL	100 µg/mL
N.D.	N.D.

Peperomia hirta C.CD.

Q'eqchi' names	Translation
Ixcua'i'xul (#1)	Snake's food (Ixcua = food; i =of; xul = a specific snake)

Plant part(s) used	Hot/Cold Score	Fuse
L, R, S	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
26	11	7



Preparation

Crush 13 leaves in 1 cup of cold water, add 1 cup of boiling water, drink the mixture and repeat every 1-2 hours.

Anti-inflammatory activity (%)

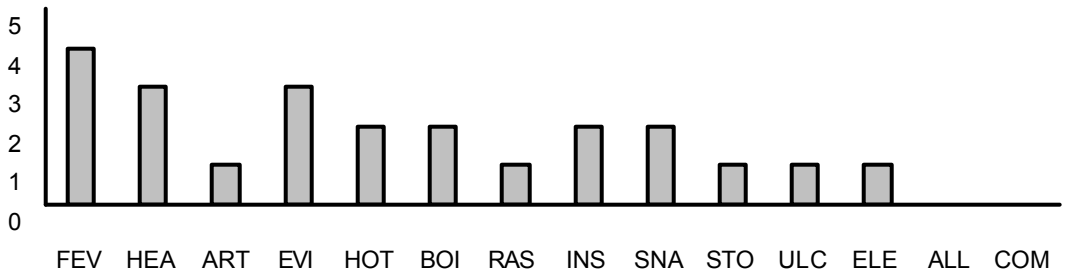
10 µg/mL	100 µg/mL
11.2	11.0

Peperomia macrostachya (Vahl) A. Dietr.

Q'eqchi' names	Translation
Mai pim (#2)	Pain plant (Mai = pain; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L, R, S	-1.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
23	12	7



Preparation

Crush 40 leaves in 1 gal of cold water, drink 1 cup and bathe twice daily. Use an equal mixture of Peperomia macrostachya and Peperomia obtusifolia.

Anti-inflammatory activity (%)

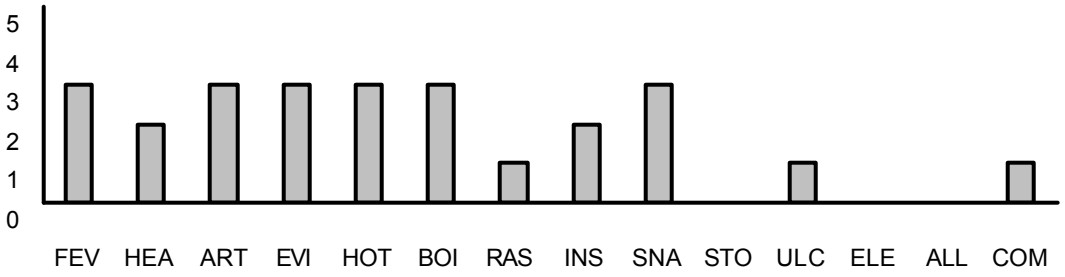
10 µg/mL	100 µg/mL
-0.8	11.4

Peperomia obtusifolia (L.) A. Dietr.

Q'eqchi' names	Translation
Ixcua ajaw chan	Boa constictor's food (Ixcua = food; ajaw chan = boa constrictor)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
25	11	7



Preparation

Crush 12 leaves in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

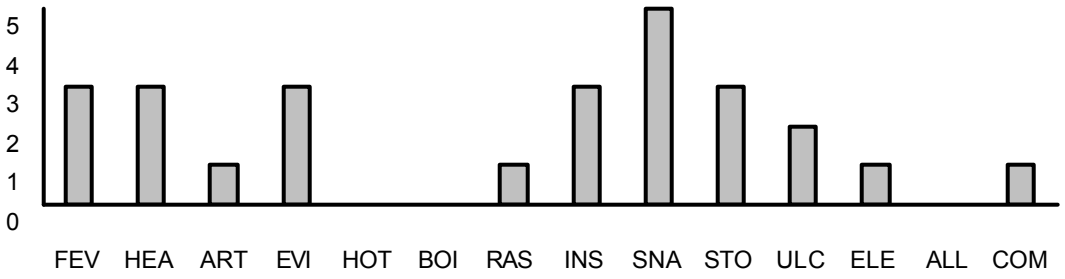
10 µg/mL	100 µg/mL
8.3	22.2

Peperomia urocarpa Fisch. & C.A. Mey

Q'eqchi' names	Translation
Ixcua'i'xul (#2)	Snake's food (Ixcua = food; i =of; xul = a specific snake)

Plant part(s) used	Hot/Cold Score	Fuse
L, R, S	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
26	11	7



Preparation

Crush 13 leaves in 1 cup of cold water, add 1 cup of boiling water, drink the mixture and repeat every 1-2 hours.

Anti-inflammatory activity (%)

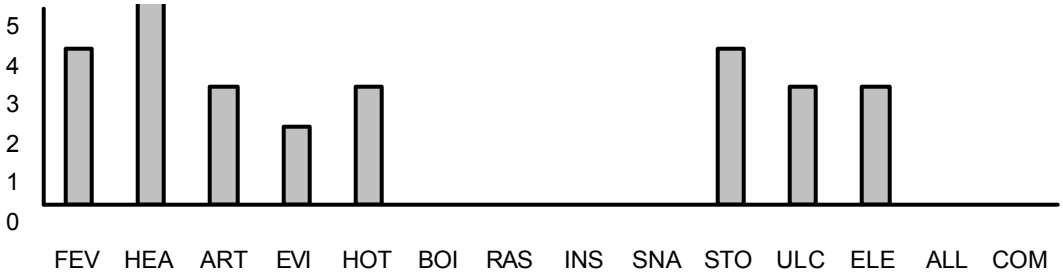
10 µg/mL	100 µg/mL
17.1	30.4

Piper aequale Vahl

Q'eqchi' names	Translation
Kan pom	Yellow insence (Kan = yellow; pom = insence)
Pu'chuch remuch kej	Piper plant for cramps (Pu'chuch = piper plant; re= for; much kej = cramps)
Pu'chuch re'tzu'ul	Piper plant for mountain (tzu'ul = mountain)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	0.40	1

Total use reports	Q'eqchi' categories	Cook categories
28	8	5



Preparation

Boil or crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

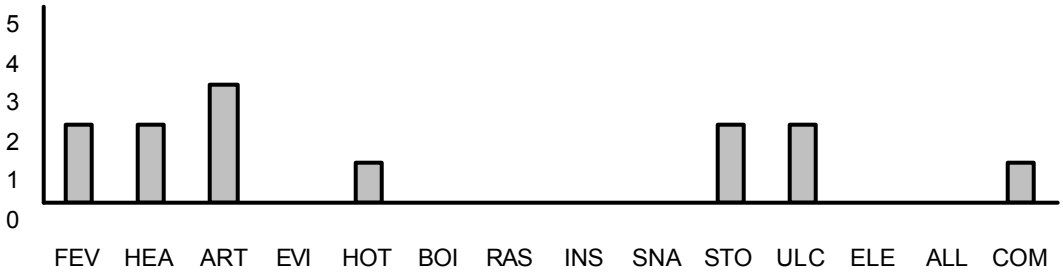
10 µg/mL	100 µg/mL
10.9	41.2

Piper amalogo L.

Q'eqchi' names	Translation
Tziritok	Small and fragile

Plant part(s) used	Hot/Cold Score	Fuse
L	1.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
13	7	5



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
N.D.	N.D.

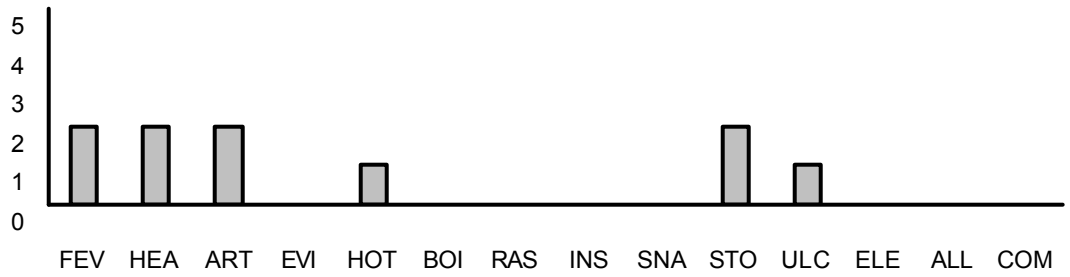
Piper arboreum Aublet

No photo available

Q'eqchi' names	Translation
Tyut'it pu'chuch (#1)	Tied node piper (Tyut = tied; it = node; pu'chuch = piper plant)

Plant part(s) used	Hot/Cold Score	Fuse
L, R, S	1.00	0.6

Total use reports	Q'eqchi' categories	Cook categories
10	6	4



Preparation

Boil the leaves, roots, and stems of 4 young plants in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

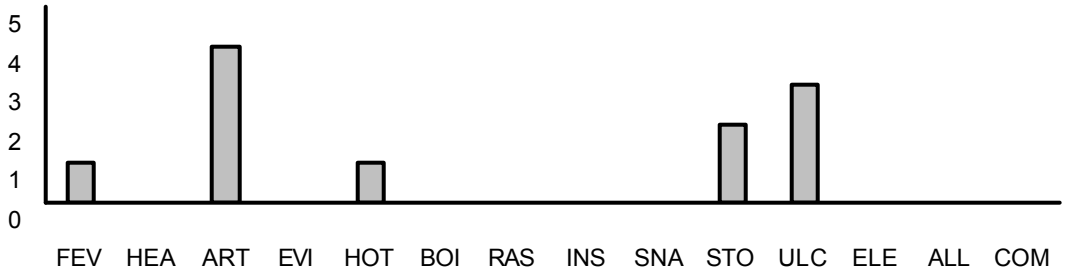
10 µg/mL	100 µg/mL
N.D.	N.D.

Piper auritum Kunth

Q'eqchi' names	Translation
Ubel	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L, R	1.00	1

Total use reports	Q'eqchi' categories	Cook categories
11	5	3



Preparation

Boil a handful of leaves and roots in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
7.8	16.3

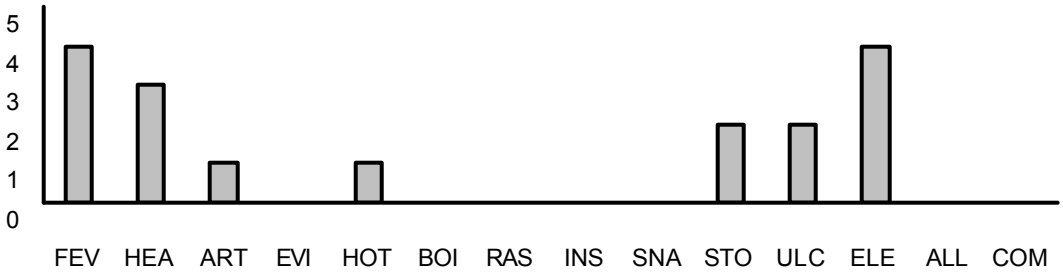
Piper glabrescens (Miq.) C.DC.

Q'eqchi' names
Pu'chuch rekanil

Translation
Piper plant for fear (Pu'chuch = piper plant; re= for; kanil = fear)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	0.60	1

Total use reports	Q'eqchi' categories	Cook categories
17	7	5



Preparation
Boil 20 leaves in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

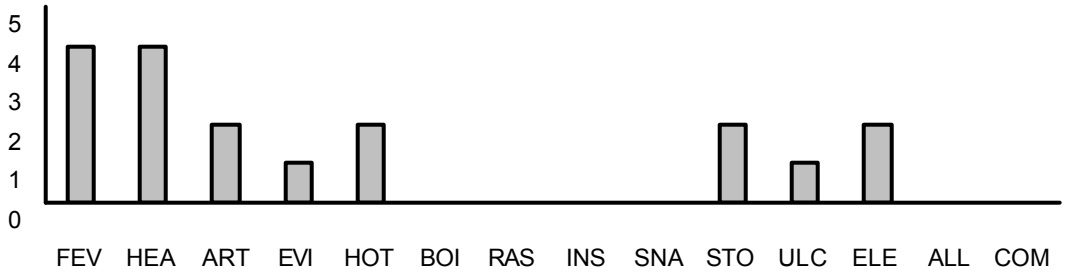
10 µg/mL	100 µg/mL
N.D.	N.D.

Piper hispidum Sw.

Q'eqchi' names	Translation
Rax pu'chuch	Green piper (Rax = Green or blue; pu'chuch = piper plant)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	1.00	1

Total use reports	Q'eqchi' categories	Cook categories
18	8	5



Preparation

Crush 13 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

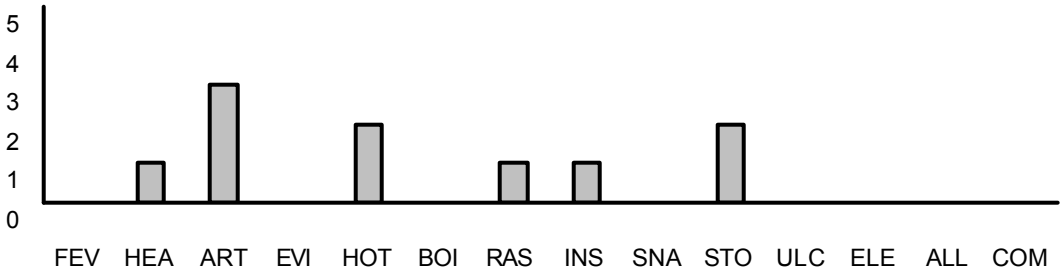
10 µg/mL	100 µg/mL
24.5	17.4

Piper peltatum L.

Q'eqchi' names	Translation
Saki tyut it	White tied node (Saki = white; tyut = tied; it = node)
Tyut it	Tied node (Tyut = tied; it = node)

Plant part(s) used	Hot/Cold Score	Fuse
L, R	-0.40	0.6

Total use reports	Q'eqchi' categories	Cook categories
10	6	5



Preparation

Boil 4 leaves and 4 roots in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
4.7	3.3

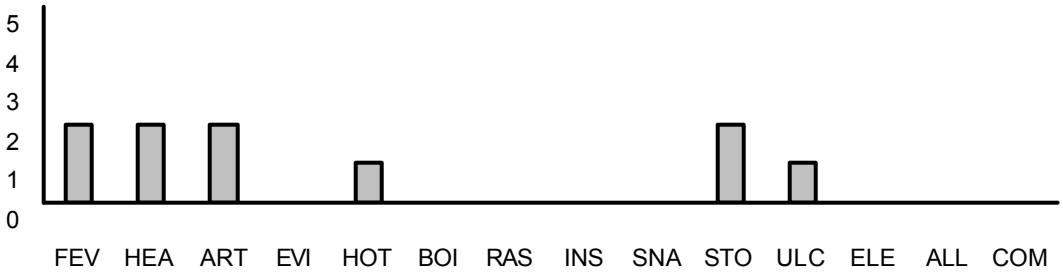
Piper sanctum (Mig.) Schltl. ex C.CD.

Q'eqchi' names
Tyut'it pu'chuch (#2)

Translation
Tied node piper (Tyut = tied; it = node; pu'chuch = piper plant)

Plant part(s) used	Hot/Cold Score	Fuse
L, R, S	1.00	0.6

Total use reports	Q'eqchi' categories	Cook categories
10	6	4



Preparation
Boil the leaves, roots, and stems of 4 young plants in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

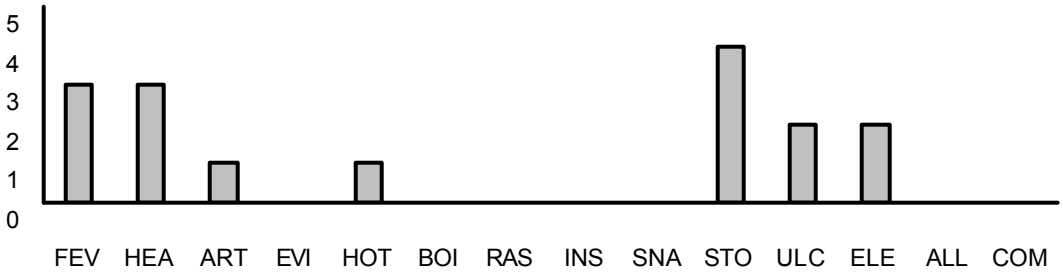
10 µg/mL	100 µg/mL
7.0	14.8

Piper tuerckheimii C. CD. Ex Donn. Sm.

Q'eqchi' names	Translation
Cux sawi	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
W	0.20	1

Total use reports	Q'eqchi' categories	Cook categories
16	7	5



Preparation

Boil or crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

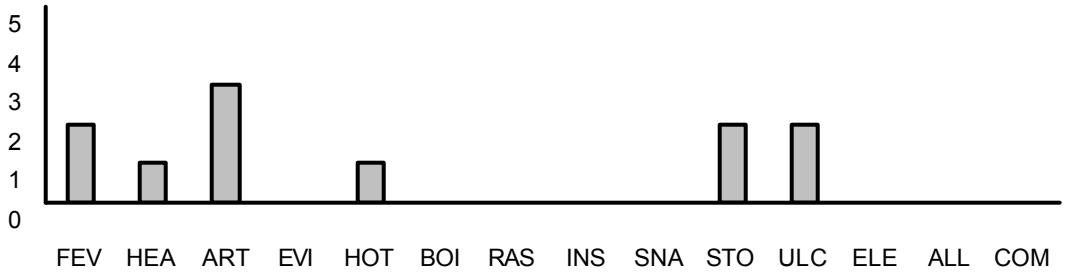
10 µg/mL	100 µg/mL
30.0	91.8

Piper yucatanense C.CD.

Q'eqchi' names	Translation
Che pu'chuch	Piper tree (Pu'chuch = piper plant; che = tree)
Pu'chuch rebakel	Piper plant for bones (Pu'chuch = piper plant; re= for; bakel = bones)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	1.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
11	6	4



Preparation

Boil 20 leaves and stems in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

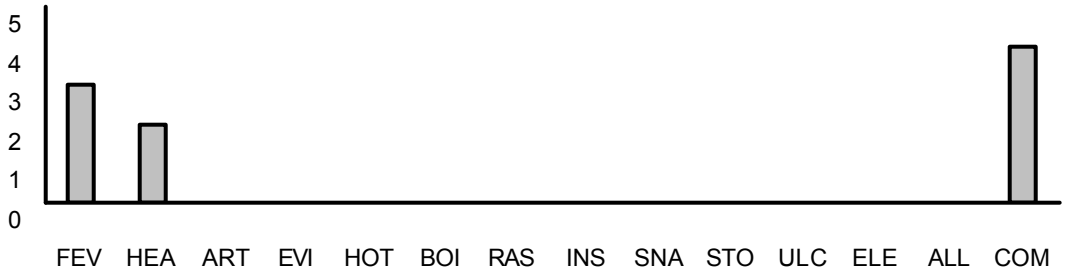
10 µg/mL	100 µg/mL
9.2	14.4

Cymbopogon citratus (DC.) Stapf

Q'eqchi' names	Translation
Kis kim	Aromatic plam (Kis = aromatic; kim = plam-like leaf)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.00	1

Total use reports	Q'eqchi' categories	Cook categories
9	3	3



Preparation

Boil 20 leaves in 1 L of water, drink half in the morning and half in the afternoon.

Anti-inflammatory activity (%)

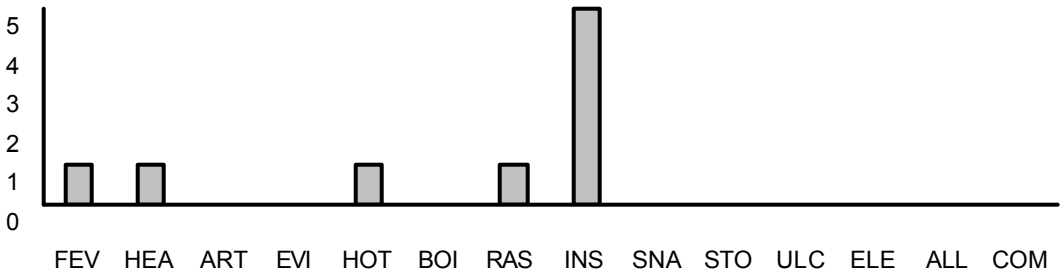
10 µg/mL	100 µg/mL
N.D.	N.D.

Gouania polygama (Jacq.) Urban

Q'eqchi' names	Translation
Kek xeb	Black wax (Kek = black; xeb = wax)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
9	5	5



Preparation

Crush 5 leaves and apply as a poultice twice daily.

Anti-inflammatory activity (%)

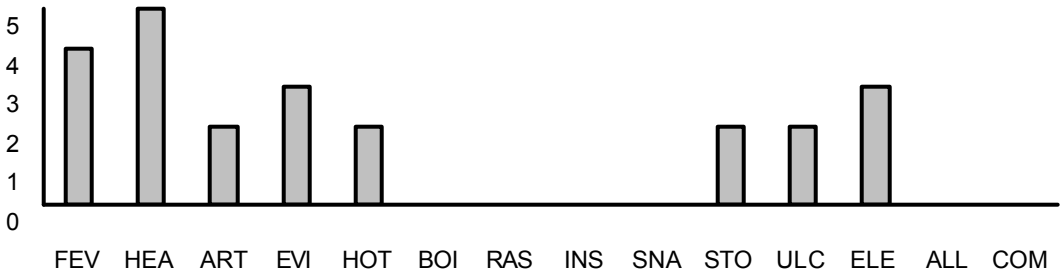
10 µg/mL	100 µg/mL
29.0	7.7

Gonzalagunia panamensis (Cav.) K.Schum.

Q'eqchi' names	Translation
Tzu'ul che	Mountain tree (Tzu'ul = mountain; che = tree)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	-0.20	1

Total use reports	Q'eqchi' categories	Cook categories
23	8	5



Preparation

Boil or crush 20 leaves in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

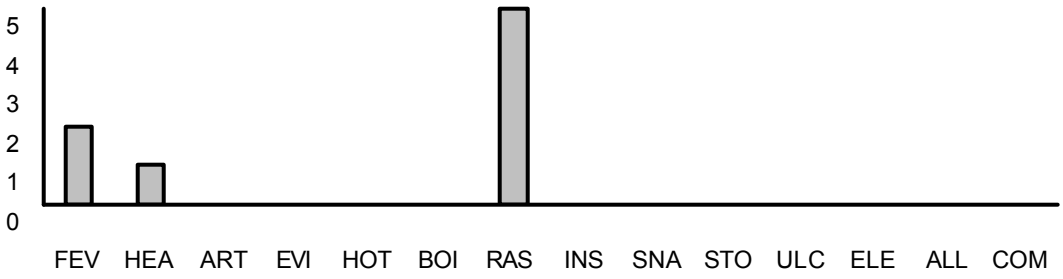
10 µg/mL	100 µg/mL
N.D.	N.D.

Hamelia patens Jacq.

Q'eqchi' names	Translation
Chaj max	Pine tree spider monkey (Chaj = pine tree; max = spider monkey)
Jolom'i'posp	Matchstick head (Jolom = head, i = of, posp = matchstick)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.40	1

Total use reports	Q'eqchi' categories	Cook categories
8	3	3



Preparation

Boil 20 leaves in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

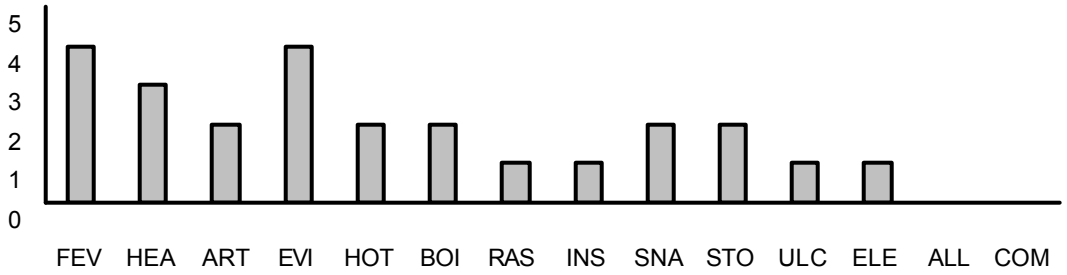
10 µg/mL	100 µg/mL
3.5	1.7

Hoffmania ghiesbreghtii (Lem.) Hemsl.

Q'eqchi' names	Translation
Mai pim (#3)	Pain plant (Mai = pain; pim = plant)
Kaki mai pim	Red mai pim (Kaki = red)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.60	1

Total use reports	Q'eqchi' categories	Cook categories
25	12	7



Preparation

Crush 4 leaves in 1 L of cold water, drink 1 cup and bathe area once daily.

Anti-inflammatory activity (%)

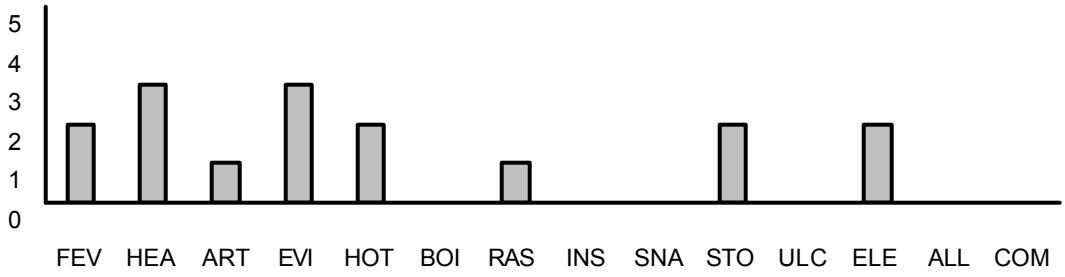
10 µg/mL	100 µg/mL
11.0	20.4

Psychotria pleuropoda Donn. Sm.

Q'eqchi' names	Translation
Kolaras	Beaded Maya necklace

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.33	0.6

Total use reports	Q'eqchi' categories	Cook categories
16	8	6



Preparation

Crush 20 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
1.1	6.9

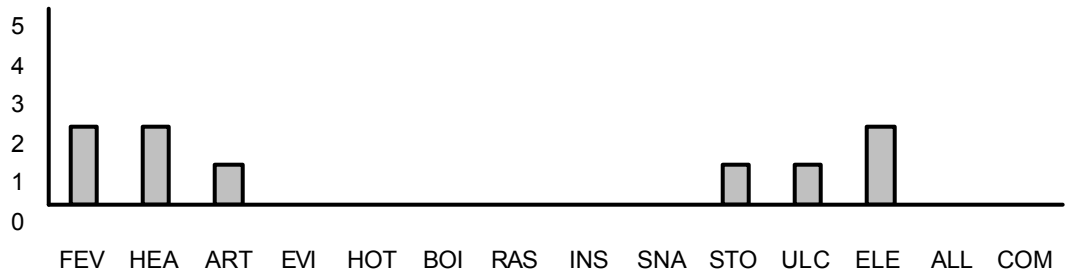
Ruta graveolens L.

No photo available

Q'eqchi' names	Translation
Ruda (Spanish)	Rue

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	0.4

Total use reports	Q'eqchi' categories	Cook categories
9	6	5



Preparation

Crush 1 bundle of leaves in 1 gal of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

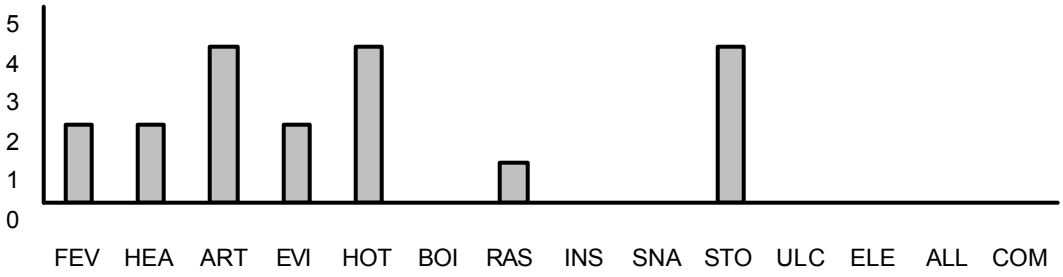
10 µg/mL	100 µg/mL
N.D.	N.D.

Paullinia costata Schltdl. & Cham.

Q'eqchi' names	Translation
Korona kix	Crown of thorns (Korona = crown; kix = thorns)

Plant part(s) used	Hot/Cold Score	Fuse
W	0.20	1

Total use reports	Q'eqchi' categories	Cook categories
19	7	5



Preparation

Boil 1 handful of leaves in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

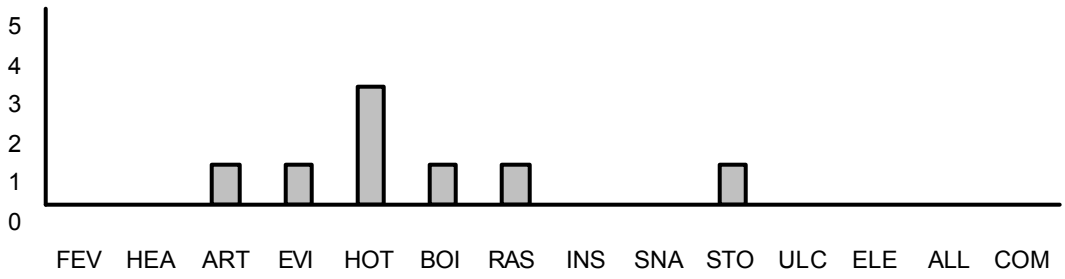
10 µg/mL	100 µg/mL
N.D.	N.D.

Serjania mexicana (L.) Willd.

Q'eqchi' names	Translation
Bolon Yok	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L	1.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
8	6	3



Preparation

Crush 1 handful of leaves in 1 gal of cold water, bathe whole body twice daily. External use only.

Anti-inflammatory activity (%)

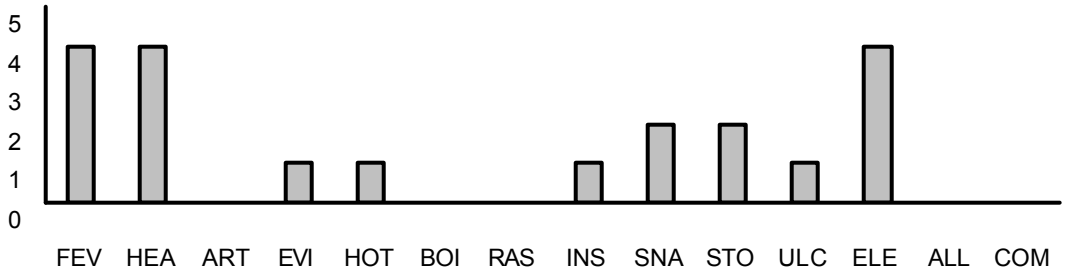
10 µg/mL	100 µg/mL
N.D.	N.D.

Lygodium heterodoxum Kunze

Q'eqchi' names	Translation
Ruxb'i'kaak (#1)	Thunder vine (Ruxb = vine; i = of; kaak = thunder)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
20	9	6



Preparation

Crush 26 leaves in 1 gal of cold water, drink 1 cup and bather twice daily. Use an equal mixture of Lygodium heterodoxum and Lygodium venustum.

Anti-inflammatory activity (%)

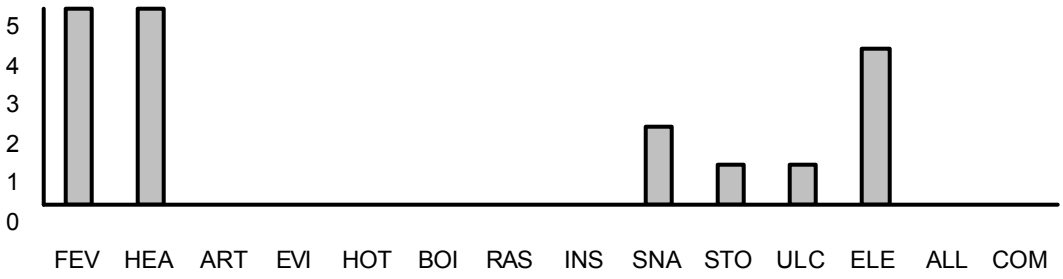
10 µg/mL	100 µg/mL
N.D.	N.D.

Lygodium venustum Sw.

Q'eqchi' names	Translation
Ruxb'i'kaak (#2)	Thunder vine (Ruxb = vine; i = of; kaak = thunder)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
18	6	5



Preparation

Crush 26 leaves in 1 gal of cold water, drink 1 cup and bather twice daily. Use an equal mixture of Lygodium heterodoxum and Lygodium venustum.

Anti-inflammatory activity (%)

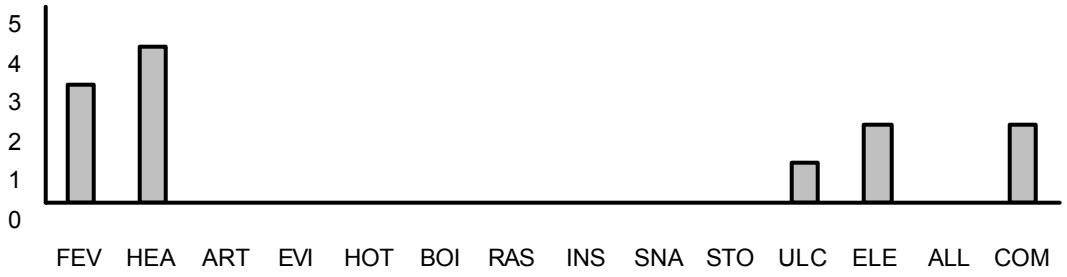
10 µg/mL	100 µg/mL
N.D.	N.D.

Selaginella sp.

Q'eqchi' names	Translation
Xquq'i'pek	Hangs off of rock (Xquq = hangs off; i = of; pek = rock)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
12	5	5



Preparation

Crush 13 leaves in 1 L of cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

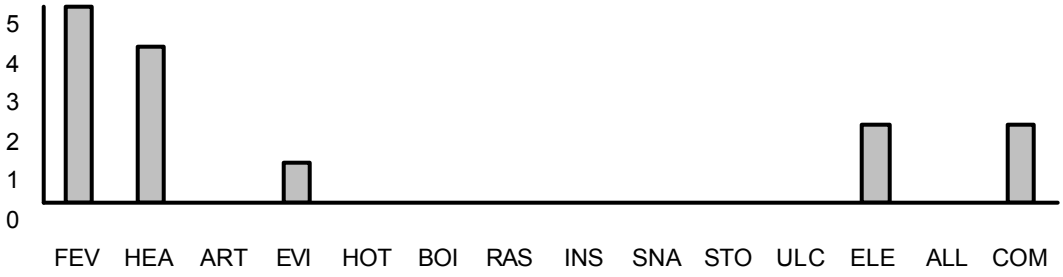
10 µg/mL	100 µg/mL
N.D.	N.D.

Selaginella umbrosa Lem. ex Wieron

Q'eqchi' names	Translation
Choql pim	Clound plant (Choql = cloud; pim = plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	-1.00	1

Total use reports	Q'eqchi' categories	Cook categories
14	5	5



Preparation

Crush 20 leaves in 1 L of cold water, drink half in the morning and half in the afternoon.

Anti-inflammatory activity (%)

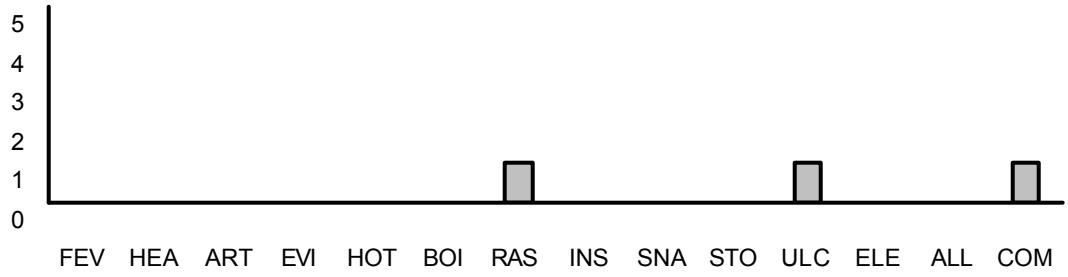
10 µg/mL	100 µg/mL
N.D.	N.D.

Smilax sp.

Q'eqchi' names	Translation
Ruchire ak	Pacarry teeth (Ruchire = teeth; ak = pecarry)
Sarsafaria (Spanish)	Sarsafaria = specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L	0.00	0.4

Total use reports	Q'eqchi' categories	Cook categories
3	3	3



Preparation

Boil 20 leaves in 1 L of water, bathe twice daily. External use only.

Anti-inflammatory activity (%)

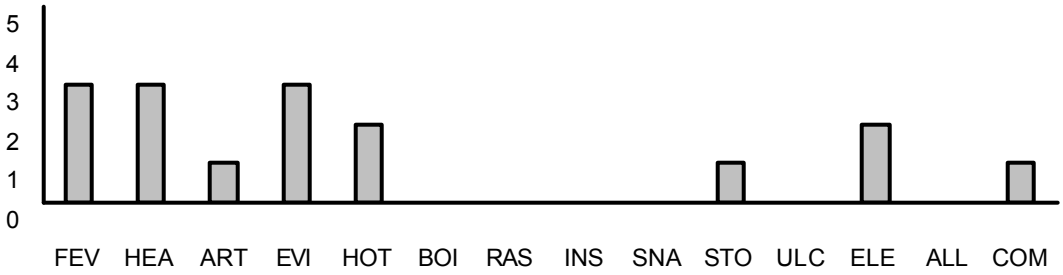
10 µg/mL	100 µg/mL
N.D.	N.D.

Cestrum megalophyllum Dunal

Q'eqchi' names	Translation
Ik che	Pepper tree (Ik = pepper; che = tree)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.40	1

Total use reports	Q'eqchi' categories	Cook categories
16	8	6



Preparation

Boil or crush 20 leaves in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

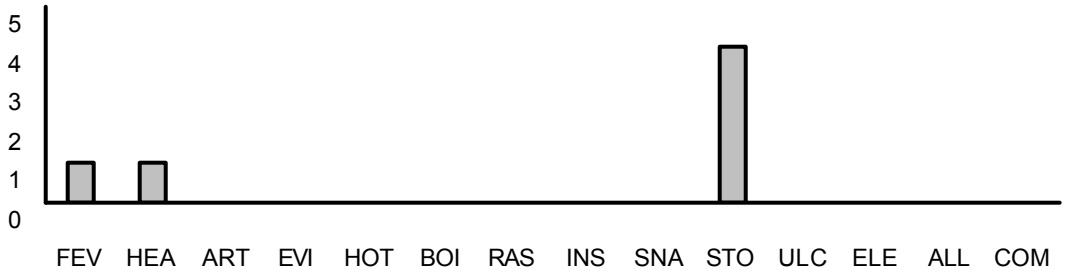
10 µg/mL	100 µg/mL
N.D.	N.D.

Solanum nudum Dunal

Q'eqchi' names	Translation
Na'i'pajl	Mother of pajl (Na = mother, i = of; pajl = a specific plant)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.40	0.8

Total use reports	Q'eqchi' categories	Cook categories
6	3	3



Preparation

Boil 20 leaves in 1 L of water, drink half in the morning and half in the afternoon.

Anti-inflammatory activity (%)

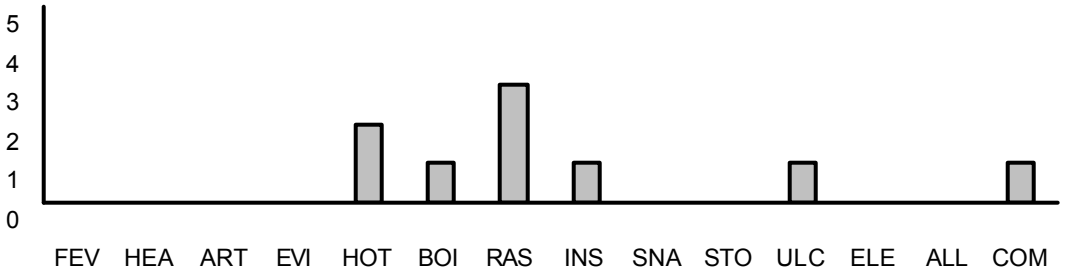
10 µg/mL	100 µg/mL
5.1	15.1

Solanum torvum Swartz.

Q'eqchi' names	Translation
Pajl	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L	0.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
9	6	5



Preparation

Boil 20 leaves in 1 L of water, drink half in the morning and half in the afternoon.

Anti-inflammatory activity (%)

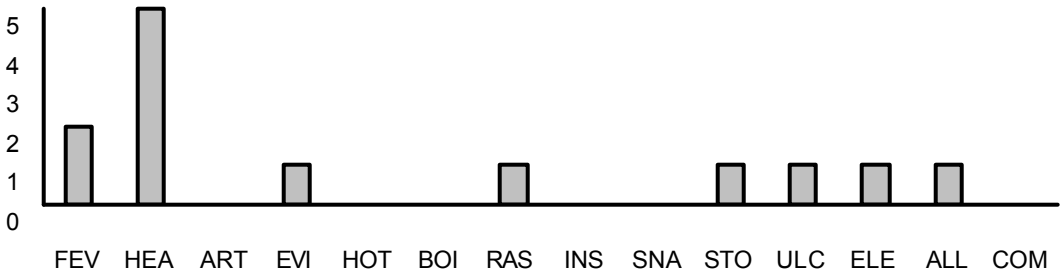
10 µg/mL	100 µg/mL
N.D.	N.D.

Cornutia grandifolia (Schltdl. & Cham.) Schauer

Q'eqchi' names	Translation
Roq xa'an	Old lady's foot (Roq = foot; xa'an = old lady)

Plant part(s) used	Hot/Cold Score	Fuse
L	-0.20	1

Total use reports	Q'eqchi' categories	Cook categories
13	8	7



Preparation

Boil or crush 13 leaves in 1 L of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

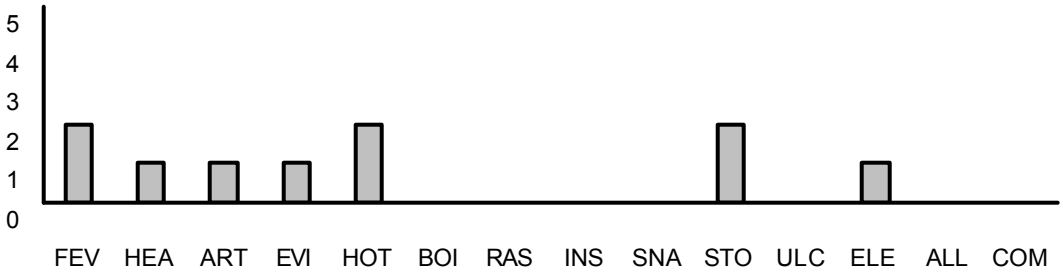
10 µg/mL	100 µg/mL
N.D.	N.D.

Cornutia pyramidata L.

Q'eqchi' names	Translation
Hob'lob'te	Hollow tree (Hob'lob = hollow; te = tree)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.50	0.8

Total use reports	Q'eqchi' categories	Cook categories
10	7	5



Preparation

Boil 20 leaves in 1 gal of water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

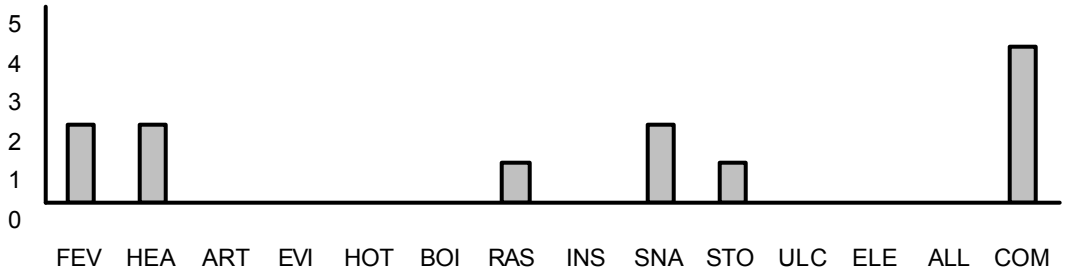
10 µg/mL	100 µg/mL
N.D.	N.D.

Lantana trifolia L.

Q'eqchi' names	Translation
Tulush	Dragonfly (Tulush = dragonfly)

Plant part(s) used	Hot/Cold Score	Fuse
L	0.60	1

Total use reports	Q'eqchi' categories	Cook categories
12	6	6



Preparation

Boil a handful of leaves in 1 L of water, drink half in the morning and half in the afternoon.

Anti-inflammatory activity (%)

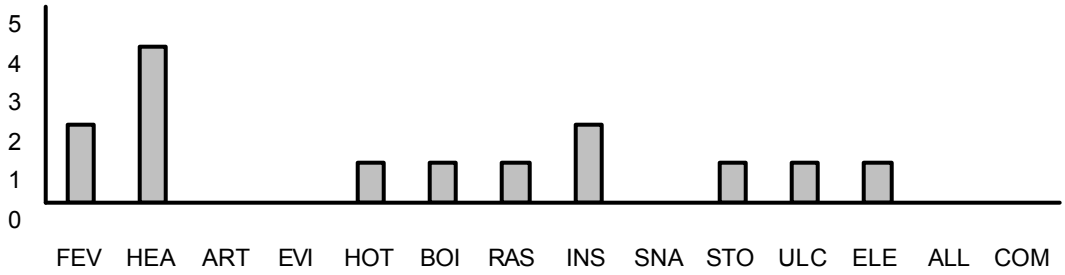
10 µg/mL	100 µg/mL
23.6	93.0

Stachytarpheta frantzii Pol.

Q'eqchi' names	Translation
Xtye aj pak	Lizard's tail (Xtye = tail; aj = of; pak = lizard)

Plant part(s) used	Hot/Cold Score	Fuse
L, S	-1.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
14	9	7



Preparation

Crush the leaves and stems of 12 young shoots in 1 L cold water, drink 1 cup and bathe twice daily.

Anti-inflammatory activity (%)

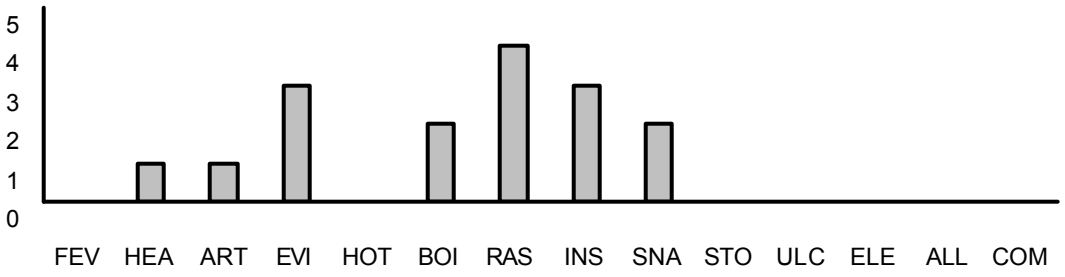
10 µg/mL	100 µg/mL
N.D.	N.D.

Cissus microcarpa Vahl.

Q'eqchi' names	Translation
Roq ab	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L, S	-0.60	1

Total use reports	Q'eqchi' categories	Cook categories
16	7	4



Preparation

Crush 20 leaves and stems and rub into the skin for several minutes, repeat four times daily. Or, crush 20 leaves and apply as a poultice, twice daily.

Anti-inflammatory activity (%)

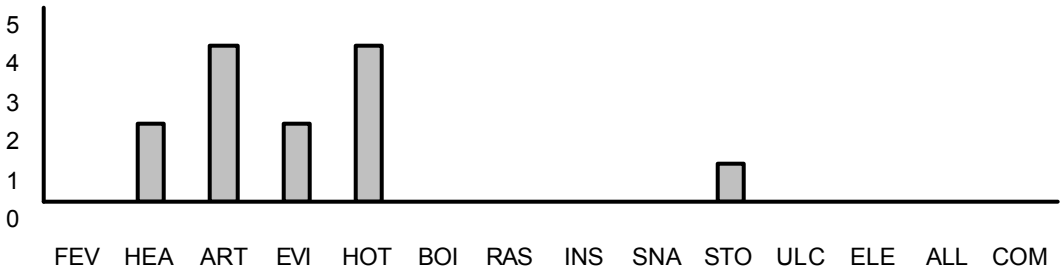
10 µg/mL	100 µg/mL
N.D.	N.D.

Renealmia aromatica (Aubl.) Griseb.

Q'eqchi' names	Translation
Cux tzi	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
L	0.80	1

Total use reports	Q'eqchi' categories	Cook categories
13	5	3



Preparation

Warm 4 leaves, tie warm leaves to the affected area, repeat twice daily.

Anti-inflammatory activity (%)

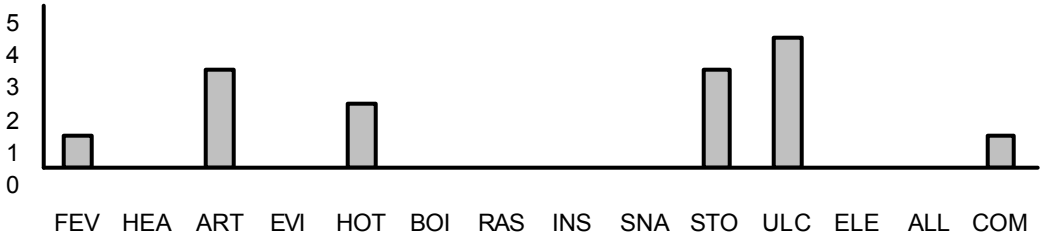
10 µg/mL	100 µg/mL
N.D.	N.D.

Zingiber officinale

Q'eqchi' names	Translation
Xan xir	Specific name for this plant

Plant part(s) used	Hot/Cold Score	Fuse
R	1.00	0.8

Total use reports	Q'eqchi' categories	Cook categories
14	6	4



Preparation

Boil 1-2 roots in 1 L of water, drink 1 cup twice daily.

Anti-inflammatory activity (%)

10 µg/mL	100 µg/mL
28.2	13.1

Appendix IIa – Residual values from plant families regression

Plant family	Total number of species in checklist (+ new)	Total number of species used by the QMHA	Residual
Piperaceae	46	14	11.23
Asteraceae	154	6	2.50
Araceae	47	6	3.21
Adiantaceae	40	5	2.31
Fabaceae	295	5	1.11
Begoniaceae	4	4	2.70
Verbenaceae	40	4	1.31
Rubiaceae	142	4	0.55
Gesneriaceae	13	3	0.99
Acanthaceae	47	3	0.21
Solanaceae	57	3	0.10
Lamiaceae	27	2	-0.45
Melastomataceae	97	3	-0.22
Marcgraviaceae	7	2	0.36
Menispermaceae	8	2	0.28
Cactaceae	10	2	0.14
Schizaeaceae	13	2	-0.01
Selaginellaceae	13	2	-0.01
Cucurbitaceae	25	2	-0.41
Zingiberaceae	4	1	-0.30
Sapindaceae	37	2	-0.64
Crassulaceae	1	1	0.53
Haemodoraceae	1	1	0.53
Chenopodiaceae	2	1	0.11
Monimiaceae	2	1	0.11
Dracaenaceae	3	1	-0.13
Costaceae	4	1	-0.30
Araliaceae	5	1	-0.44
Davalliaceae	5	1	-0.44
Rhamnaceae	6	1	-0.55
Burseraceae	7	1	-0.64
Vitaceae	7	1	-0.64
Smilacaceae	8	1	-0.72
Loranthaceae	9	1	-0.79
Aristolochiaceae	10	1	-0.86
Celastraceae	11	1	-0.91
Commelinaceae	19	1	-1.24
Passifloraceae	22	1	-1.33
Amaranthaceae	24	1	-1.38
Moraceae	36	1	-1.63
Malvaceae	44	1	-1.75
Bignoniaceae	45	1	-1.76
Bromeliaceae	50	1	-1.83
Convulvulaceae	50	1	-1.83
Aspleniaceae	58	1	-1.91
Euphorbiaceae	104	1	-2.27
Orchidaceae	279	1	-2.86

Appendix IIb – Residual values from plant class and subclass regression

Plant class and subclass	Total number of species in checklist (+ new)	Total number of species used by the QMHA	Residual
Magnoliopsida subclass asteridae	757	26	11.37
Magnoliopsida subclass rosidae	836	19	3.95
Magnoliopsida subclass magnoliidae	129	17	9.87
Polypodiopsida	230	9	-0.58
Magnoliopsida subclass dilleniidae	327	8	-3.07
Liliopsida subclass ariflorae	48	4	1.06
Magnoliopsida subclass caryophyllidae	79	4	-1.05
Liliopsida subclass liliiflorae	330	3	-8.11
Liliopsida subclass zingiberidae	32	2	0.78
Liliopsida subclass bromeliiflorae	55	2	-1.51
Lycopodiopsida	21	2	2.57
Liliopsida subclass commeliniflorae	435	1	-11.28
Magnoliopsida subclass hamamelidae	78	1	-4.00

Appendix III

Preliminary results from the bioassay guided isolation of anti-inflammatory principles from *Monstera acuminata*

Materials and Methods

Plant material

This project received ethical approval from the University of Ottawa Research Ethics Board (file H 03-07-01). Plant collecting and export permits were obtained from the Belize Forest Department (Ref. No. CD/60/3/08(33)) and authenticated voucher specimens deposited at the University of Ottawa herbarium, the Juvenal Valerio Rodriguez herbarium of the Universidad Nacional de Costa Rica, and the herbarium of the Missouri Botanical Garden. Fresh plant material was collected on field excursions with members of the QMHA and immediately preserved in alcohol. Wet and shredded plant material was extracted twice with EtOH (80% in H2O) at a ratio of 1 g plant material to 10 ml 80% EtOH over 24h at room temperature. The combined extracts were evaporated in vacuo, lyphophillized, and homogenized using a mortar and pestle.

Anti-inflammatory assays

The anti-inflammatory activity of plant extract was assessed by measuring TNF-α reduction in an LPS stimulated THP-1 monocyte assay (Zhao et al, 2005). THP-1 cells (human monocyte culture TIB-202, ATCC, Manassas, VA, USA), were cultured in RPMI 1640 media (ATCC, Manassas, VA, USA) supplemented

with 1% 0.05 mM beta-mercaptoethanol, 1% penstrep (Invitrogen, Mississauga, ON, Canada) and 10% fetal bovine serum (Invitrogen, Mississauga, ON, Canada), in a 37°C humidified environment with 5% CO_2. Cells were transferred ($3x10^4$ cells/well) to the wells of a 96-well plate, followed by the addition of plant extract dissolved in 80% EtOH for a final volume of 300 µl/well and a final EtOH concentration 0.5%. Plant extract was assayed at 10 and 100 µg/mL, parthenolide (Sigma-Aldrich, St-Louis, MO, USA) was used as a positive control at 1 and 10 µg/mL, and 0.5% EtOH was used as a vehicle control. Extract and controls were assayed in quadruplicate. Following the addition of extracts and controls, cells were incubated for 2 hours, and then stimulated with 1 µg/mL LPS purified from E. coli (Sigma-Aldrich, St-Louis, MO, USA) and allowed to incubate for 20 hours. An unstimulated control containing 0.5% EtOH but no LPS was also assayed. After incubation, cells were centrifuged at 1200 RPM for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. DuoSet® ELISA development kits (R & D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol to measure TNF-α levels in cell culture supernatants. Raw TNF-α values were transformed to a % activity of the parthenolide 10 µg/mL control.

Solvent series fractionation

A four-step solvent series extraction of the crude 80% extract was undertaken to generate primary fractions. This fractionation involved extraction with solvents that ranged from non-polar to polar in the following order: hexanes,

ethyl acetate, methanol and 80% ethanol. Each solvent extraction was performed until exhaustion, and each fraction was dried and re-dissolved in 80% EtOH.

Thin Layer Chromatography (TLC)

Silica TLC plates were used to profile primary solvent-series fractions using a range of non-polar to polar solvents in a coplin jar.

Analytical scale high performance liquid chromatography (HPLC)

In addition to profiling fractions using TLC plates as described in *2.5*, analytical scale HPLC was used to generate more detailed chromatograms. The HPLC-diode array detection (DAD) analyses were performed on an Agilent 1100 Series LC system. The HPLC system consisted of an auto-sampler with a 100µL built in loop, a quaternary pump with a maximum pressure of 400 bars, a column thermostat and a diode array detector set to scan from 200-400nm. For HPLC-DAD analysis the solvents were sonicated at 5 min/L prior to analysis and the column (Gemini C18(2), 250 × 4.6 mm, particle size 5 micron, Phenomenex, Torrance, CA, USA) was equilibrated for 15 min with the starting conditions prior to analysis. The mobile phase consisted of A = water and B = acetonitrile. The optimal elution conditions were a linear gradient of 5–35% B in 7 min followed by increasing B up to 100% in 3 min and maintaining isocratic conditions for 5 min. The column was brought back to initial conditions in 5 min and equilibrated for 3 min prior to subsequent injections. The column operated at a flow rate of 2 mL/min and column temperature was maintained at 55°C. Samples were filtered

though 0.22 µm PTFE membrane and 2 µL of each extract was injected into the HPLC system through the auto-sampler.

Preparative scale high performance liquid chromatography (Prep-HPLC)

Secondary fractionation was performed using and Agilent 1200 Series Preparative-LC-DAD system. The HPLC system consisted of an auto-sampler with a 5000µL built in loop, two single channel preparative pumps, a diode array detector set to scan from 200-400nm, and an automated fraction collector. For Prep-LC fractionation the solvents were sonicated at 5 min/L prior to analysis and the column (hand packed Sephadex in a glass column, 45 x 2.5cm, 250ml volume) was equilibrated for 60 min with the starting conditions prior to fractionation. The mobile phase consisted of 50% MeOH in water held at isocratic conditions over 130 minutes and 30 x 35ml fractions were collected. Following complete elution, the column was flushed with 100% MeOH over 90 minutes and 21 x 35ml fractions were collected. The column was brought back to initial conditions and equilibrated for 30 min prior to subsequent injections. The column operated at a flow rate of 8 mL/min and column temperature was maintained at room temperature. Samples were filtered through 0.4 µm PTFE membrane and 5000 µL of sample was injected into the Prep-LC system though the auto-sampler.

Results

Bulk extraction yield

A bulk 80% ethanol extract of *Monstera acuminata* was prepared from 4108.9g of raw material (leaves) preserved in alcohol. Following an exhaustive extraction procedure, the final extract yield was 116.8g (2.84% raw).

TLC results

Silica gel chromatography was originally going to be used for the generation of primary fractions, but silica gel TLC profiling of the *Monstera acuminata* plant extract produced poor separation and revealed the presence of many polar compounds not suitable for fractionation using silica gel. As such, it was decided that primary fractionation would be carried out using a solvent-series fractionation.

Primary solvent-series fractionation

A solvent-series extraction was performed on the crude bulk extract as depicted in rows 1 and 2 of the fractionation scheme (Figure 1). Both the aliquot crude extract used for initial screening and the bulk crude extract used for fractionation were assayed in order to ensure that the activity of the aliquot was conserved following bulk extraction (Figure 2). The activity of both the aliquot and bulk crude extracts were comparable at the highest concentration tested (95.0% activity for the aliquot extract and 92.3% activity for the bulk extract). However, at the lowest concentration tested there was an important loss of activity as the

aliquot extract displayed 91.9% activity and the bulk extract only displayed 40.2% activity. Chromatographic profiling of both aliquot and bulk crude extracts reveals that the composition of both extracts is fairly similar (Figure 3a and 3b), however there are some differences in the composition and concentration of phytochemicals in the more polar region of the chromatogram (Figure 3c). It is possible that these differences are responsible for the observed loss of activity at the lower concentration tested.

Primary solvent-series fractions were assayed at 10 and 100 µg/mL, and all fractions displayed some anti-inflammatory activity in a dose-dependent manner (Figure 2). The hexane and ethyl acetate fractions were less active than the methanol and 80% ethanol fractions, and this difference was most notable at the lowest concentration tested. Methanol and 80% ethanol fractions displayed 61.0% and 84.7% of 10 µg/mL parthenolide activity, respectively, at the highest concentration tested.

None of the primary fractions demonstrated anti-inflammatory activity comparable to the bulk crude extract at the highest concentrations tested. This result was unexpected, as we would anticipate the compounds responsible for the activity of the crude extract to be concentrated in one of the primary fractions and therefore demonstrate greater activity than the crude extract. As this was not the case, the fractions that were collected after the solvent-series extraction were then combined in a factorial design and compared to the crude bulk extract activity to test for synergistic effects and to determine whether the activity of the crude bulk extract could be reproduced with a re-constituted combination of all

four fractions. Consequently, the noted loss of activity could not be attributed to synergy because no significant differences in activity were observed when the extracts were tested in various combinations. More specifically, the combined fractions did not display greater activity than their individual parts (Figure 2). Methanol and 80% ethanol fractions showed the highest activity both individually, as aforementioned, and together (85.1% of 10 µg/mL parthenolide), and had similar phytochemical profiles (Figure 4a and 4b). For these reasons, the methanol and 80% ethanol fractions were selected as the most active fractions and combined for secondary fractionation as shown in row 2 of Figure 1.

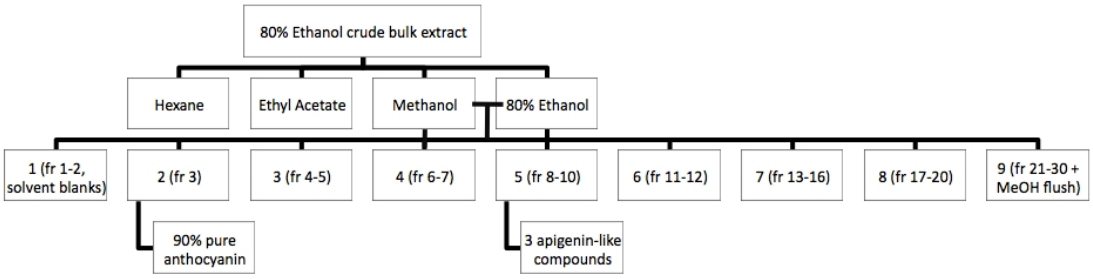


Figure 1. Bioassay-guided fractionation scheme. Row 1 shows starting material; row 2 depicts solvent-series primary fractionation; row 3 displays pooled secondary fractions from Preparative-HPLC; row 4 shows identified phytochemicals.

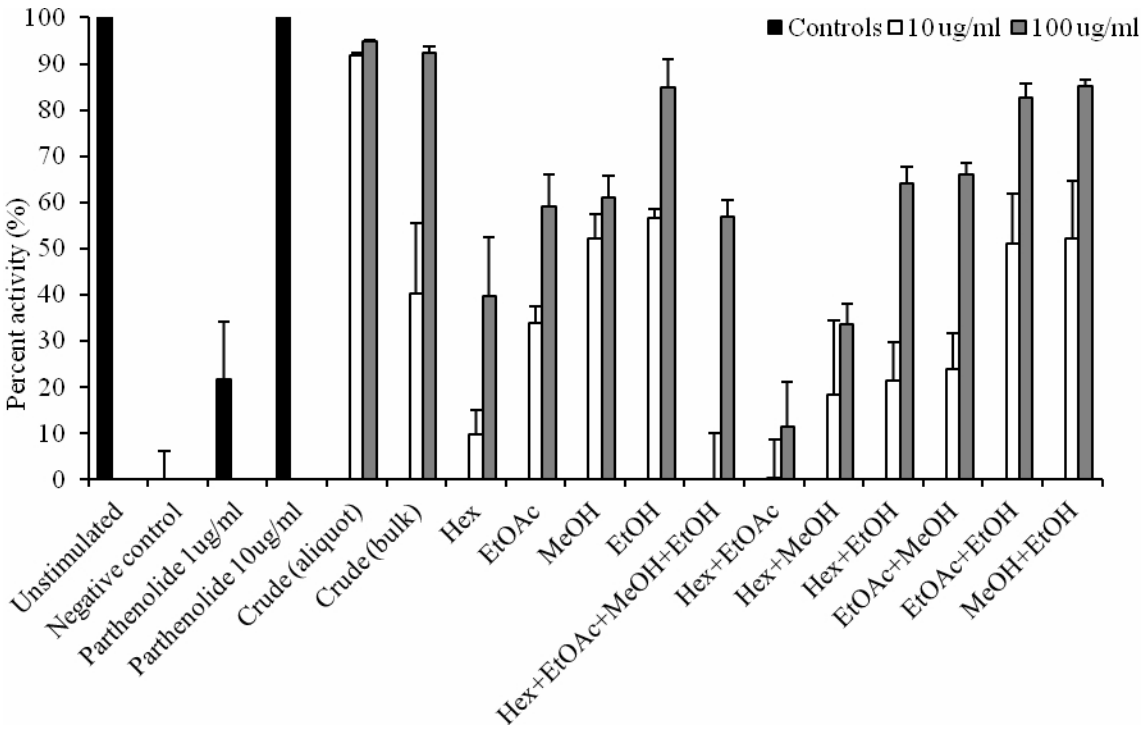


Figure 2. Percent activity of individual and combined fractions from a solvent-series extraction at varied concentrations of *Monstera acuminate* relative to the 10µg/mL parthenolide control in LPS-stimulated THP-1 monocytes.

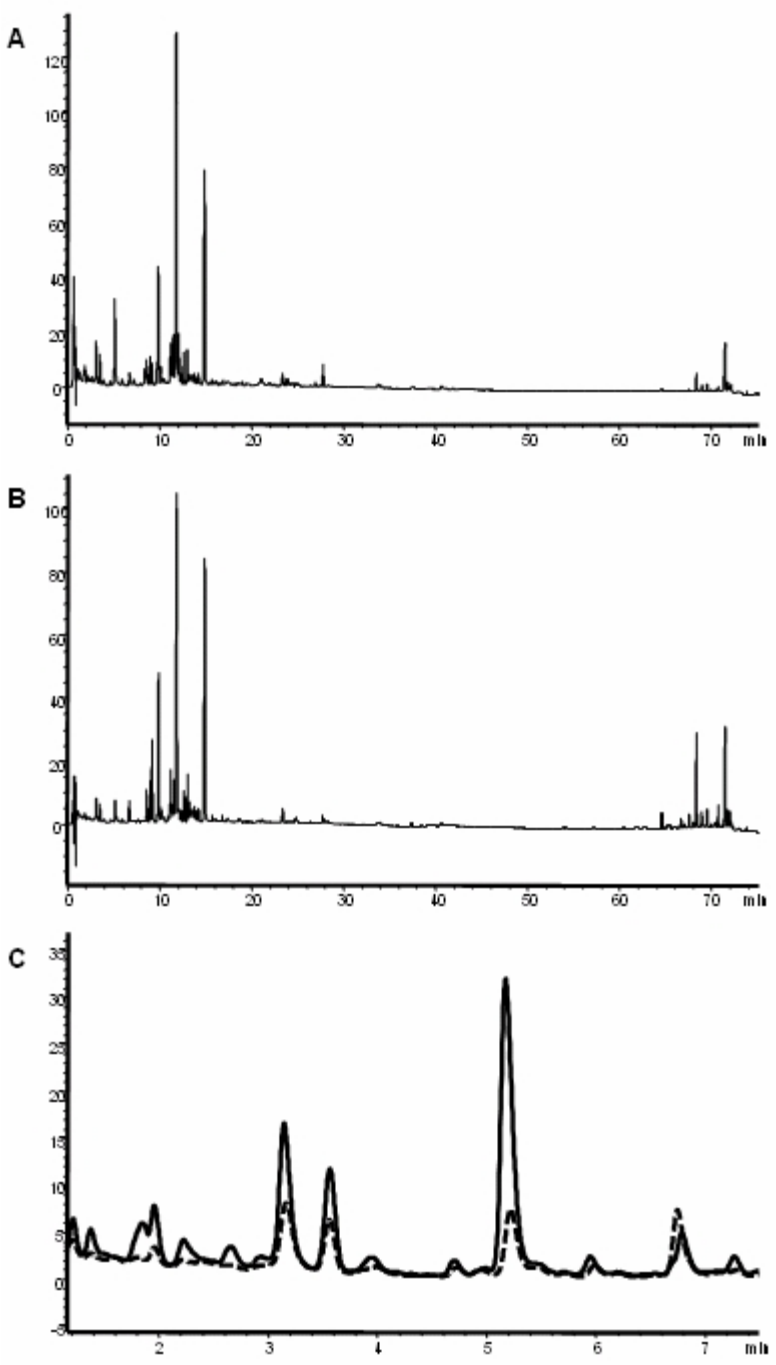


Figure 3. Chromatographic profiles of crude *Monstera acuminata* extracts at 330nm. Panel A is the aliquot crude extract, and panel B is the bulk crude extract. Panel C is a magnified view of the most polar region of the chromatograms (Rt = 1-8 min) shown in A and B with the solid line representing the aliquot extract and the dotted line representing the bulk extract.

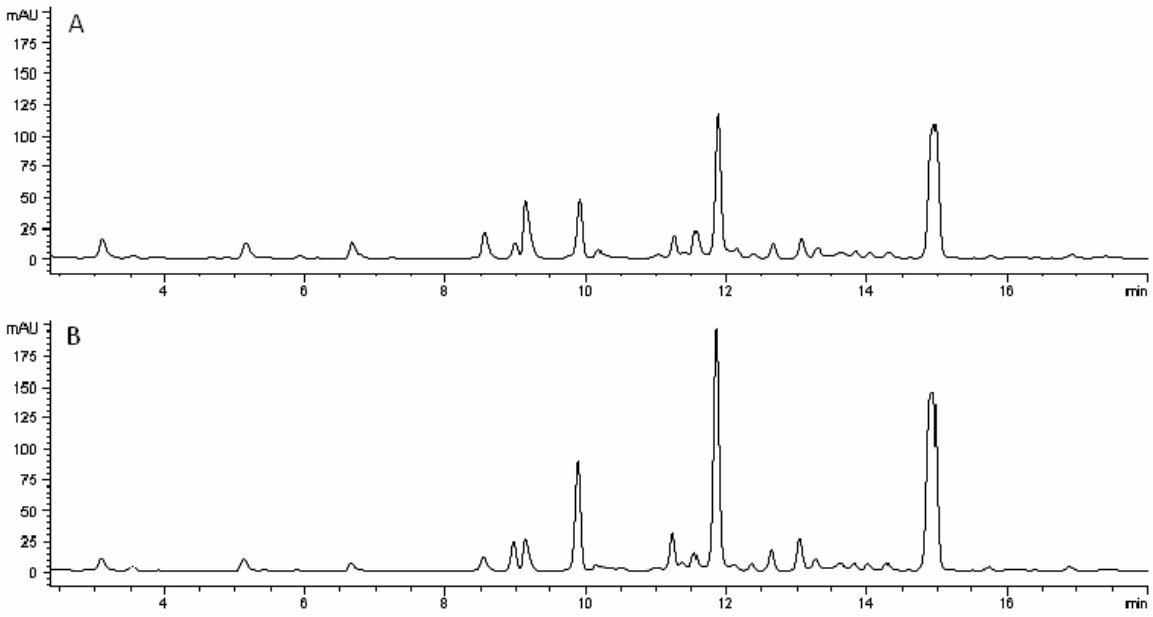


Figure 4. Chromatographic profiles of two primary solvent-series fractions of *Monstera acuminata* at 330nm. Panel A is the MeOH primary fraction, and panel B is the 80% EtOH primary fraction. These fractions were combined because they were highly similar, then subjected to secondary fractionation.

Secondary fractionation, phytochemical isolation and identification

The Prep-HPLC Sephadex fractionation of the pooled MeOH and 80% EtOH primary solvent-series fractions generated 30 secondary fractions, and a MeOH column flush was also collected. Following HPLC-DAD chromatographic profiling, these fractions were pooled to 9 fractions based on phytochemical similarity (Row 3 of Figure 1) and assayed for their anti-inflammatory activity (Figure 5). Fractions 2 to 6 all displayed important levels of dose-dependent anti-inflammatory activity, with fraction 2 being the most active at 85% the activity of the 10 µg/ml parthenolide positive control. Fraction 1, the solvent blank, displayed no anti-inflammatory activity, and fractions 7 to 9 became progressively less active.

The HPLC chromatographic profile of the pooled MeOH and 80% EtOH primary solvent-series fractions used for secondary fractionation is composed of two distinct regions (Figure 6a). Retention time, Rt = 1-2 min comprised very polar compounds with similar UV spectra, and Rt = 3.5-5.0 min consisted of compounds with medium polarity that share similar UV spectra. The pooled secondary fraction 2 (Figure 6b) and the pooled secondary fraction 5 (Figure 6c) demonstrated a good separation of several representative peaks from these two regions. Due to the relatively simple nature of pooled secondary fractions 2 and 5 when compared with the other pooled secondary fractions (chromatograms not shown), and their potent anti-inflammatory activity, these two fractions were subjected to a more detailed phytochemical analysis. The pooled secondary fraction 2 is composed of approximately 90% of peak 1, as approximated by its proportion of the total area under the curve for the entire chromatogram. The UV spectrum for this peak showed a peak absorbance at 240nm (Figure 7a), which is characteristic of anthocyanins (glucosides of anthocyanidins – chemical skeleton shown in Figure 7b). The UV spectra for peaks 2-4 (Figures 7c-7e) are very similar to the UV spectrum of apiginen (UV spectrum in Figure 7f, chemical structure in Figure 7g). Peaks 2, 3, and 4 have percent matches of 89.7%, 91.3% and 91.4% with the UV spectrum of apigenin, respectively.

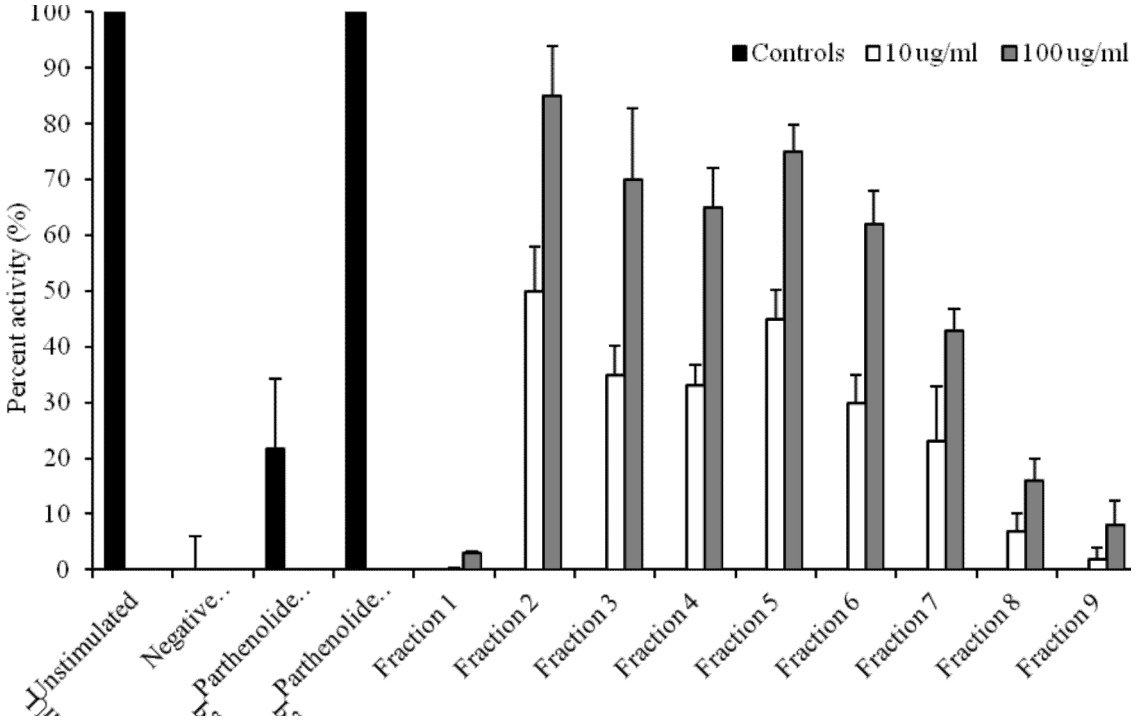


Figure 5. Percent activity of secondary *Monstera acuminata* fractions at varied concentrations relative to the 10 µg/mL parthenolide control in LPS-stimulated THP-1 monocytes.

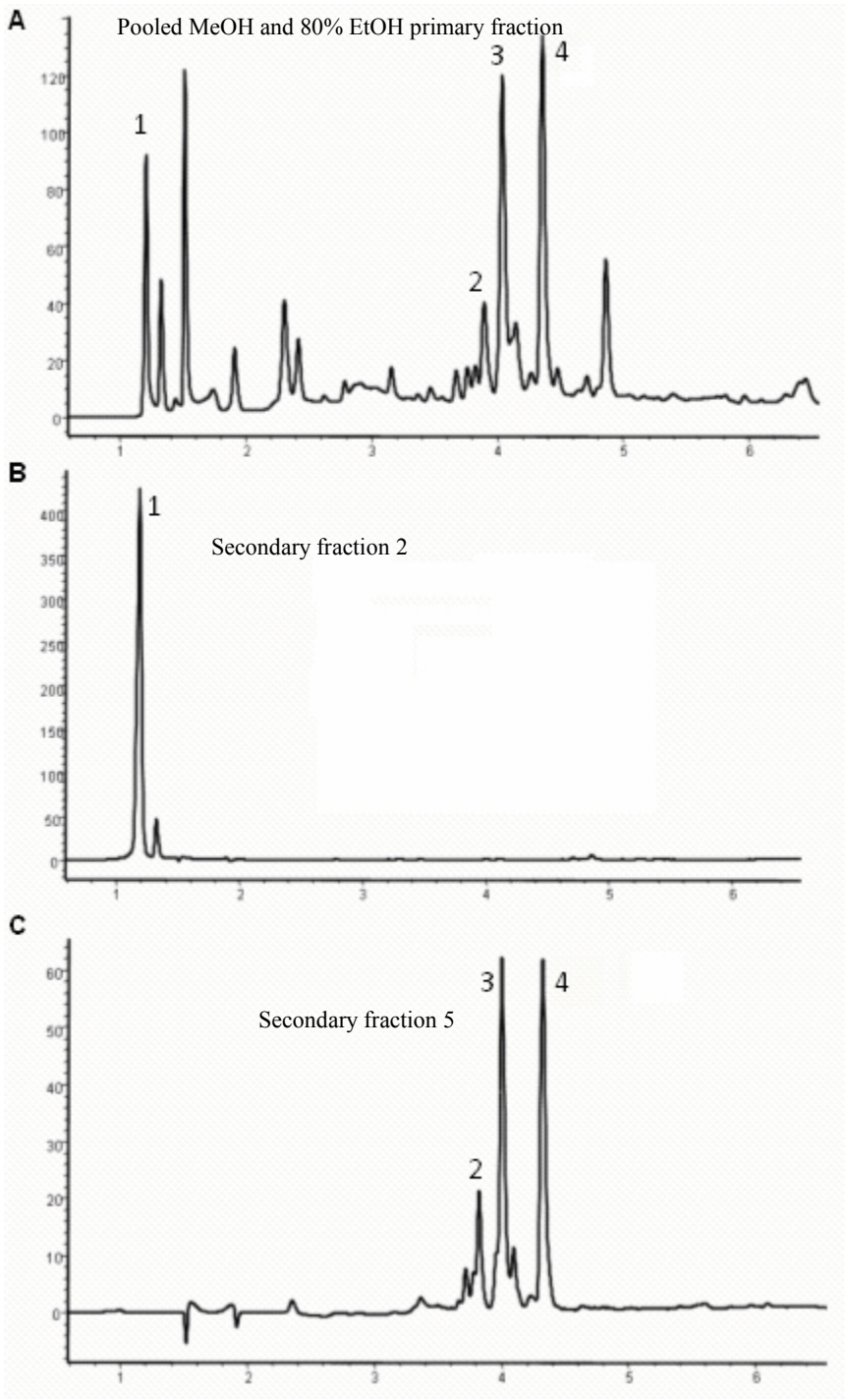


Figure 6. Chromatographic profiles of pooled primary and pooled secondary fractions of *Philodendron* sp. at 254nm (band width = 4). Panel A is pooled MeOH and 80% EtOH primary fractions subjected to secondary fractionation; panel B is the pooled secondary fraction 2; panel C is the pooled secondary fraction 5. Peaks of interest are labeled 1-4.

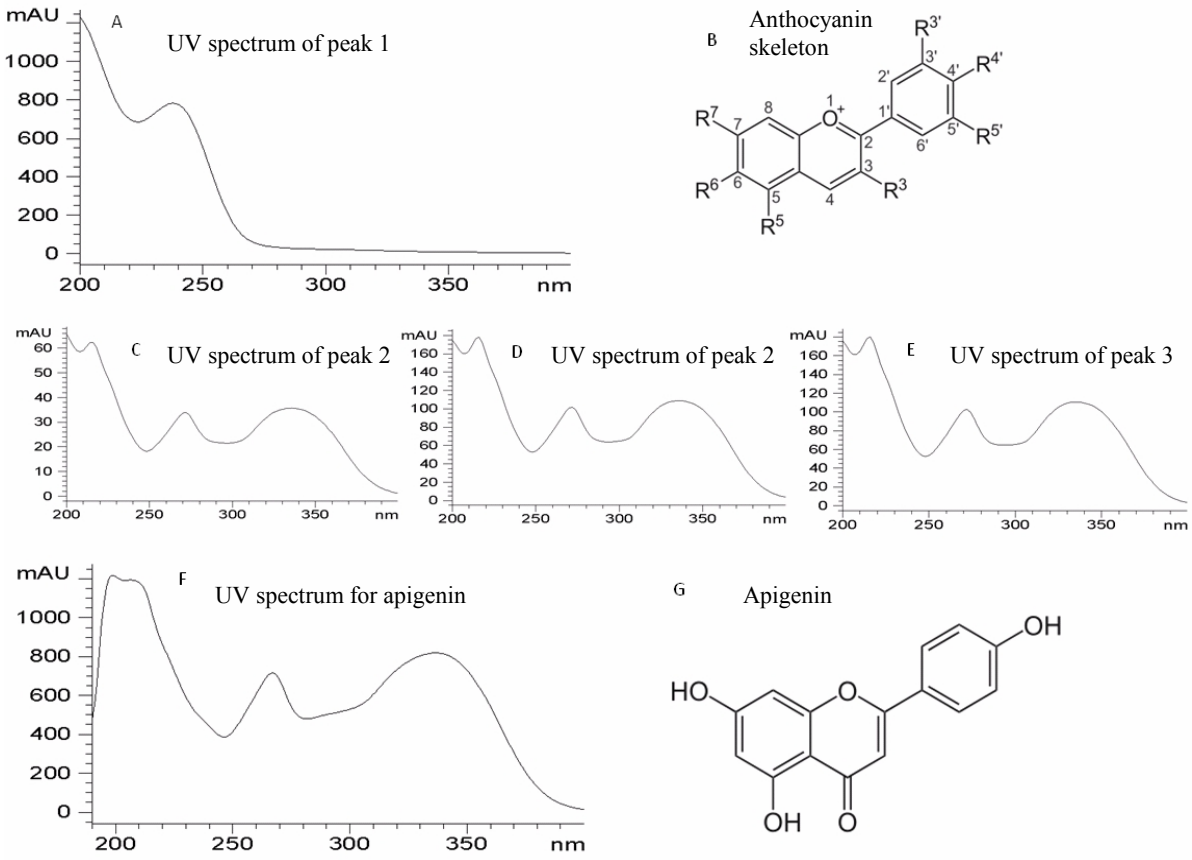


Figure 7. UV spectra and tentatively identified compounds from pooled secondary fractions 2 and 5. Panel A is the UV spectrum of peak 1; panel B is the anthocyanin chemical skeleton, R groups represent potential glycoside arrangements; panel C is the UV spectrum for peak 2; panel D is the UV spectrum for peak 3; panel E is the UV spectrum for peak 4; panel F is the UV spectrum for apigenin; panel G is the structure of apigenin.

Appendix IV

Preliminary results from the bioassay guided isolation of anti-inflammatory principles from *Tradescantia spathacea*

Materials and Methods

Plant material

This project received ethical approval from the University of Ottawa Research Ethics Board (file H 03-07-01). Plant collecting and export permits were obtained from the Belize Forest Department (Ref. No. CD/60/3/08(33)) and authenticated voucher specimens deposited at the University of Ottawa herbarium, the Juvenal Valerio Rodriguez herbarium of the Universidad Nacional de Costa Rica, and the herbarium of the Missouri Botanical Garden. Fresh plant material was collected on field excursions with members of the QMHA and immediately preserved in alcohol. Wet and shredded plant material was extracted twice with EtOH (80% in H2O) at a ratio of 1 g plant material to 10 ml 80% EtOH over 24h at room temperature. The combined extracts were evaporated in vacuo, lyphophillized, and homogenized using a mortar and pestle.

Anti-inflammatory assays

The anti-inflammatory activity of plant extract was assessed by measuring TNF-α reduction in an LPS stimulated THP-1 monocyte assay (Zhao et al, 2005). THP-1 cells (human monocyte culture TIB-202, ATCC, Manassas, VA, USA), were cultured in RPMI 1640 media (ATCC, Manassas, VA, USA) supplemented

with 1% 0.05 mM beta-mercaptoethanol, 1% penstrep (Invitrogen, Mississauga, ON, Canada) and 10% fetal bovine serum (Invitrogen, Mississauga, ON, Canada), in a 37°C humidified environment with 5% CO_2. Cells were transferred ($3x10^4$ cells/well) to the wells of a 96-well plate, followed by the addition of plant extract dissolved in 80% EtOH for a final volume of 300 µl/well and a final EtOH concentration 0.5%. Plant extract was assayed at 10 and 100 µg/mL, parthenolide (Sigma-Aldrich, St-Louis, MO, USA) was used as a positive control at 1 and 10 µg/mL, and 0.5% EtOH was used as a vehicle control. Extract and controls were assayed in quadruplicate. Following the addition of extracts and controls, cells were incubated for 2 hours, and then stimulated with 1 µg/mL LPS purified from E. coli (Sigma-Aldrich, St-Louis, MO, USA) and allowed to incubate for 20 hours. An unstimulated control containing 0.5% EtOH but no LPS was also assayed. After incubation, cells were centrifuged at 1200 RPM for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. DuoSet® ELISA development kits (R & D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol to measure TNF-α levels in cell culture supernatants. Raw TNF-α values were transformed to a % activity of the parthenolide 10 µg/mL control.

Silica Gel Column Fractionation and Pooling using TLC

A 3L chromatography column with a glass and wool plug inserted just above the Teflon stop switch was assembled. The column was loaded with silica

(SiO_2) and a 3 cm sand layer was places at the top. 100g of silica gel was weighed into a glass container and 10ml of deionized water was added to deactivate the silica. *Tradescantia spathacea* crude extract (56g) was mixed with the 100g of SiO_2 and was then subjected to normal phase column chromatography, eluted with a solvent gradient ranging from nonpolar to polar; Hex:EtOAc (1:1, 4:6 3:7, 2:8, 1:9), EtOAc, EtOAc:MeOH (95:5, 9:1, 8:2. 7:3, 6:4, 1:1, 25:75). MeOH and MeOH:water (9:1) at 1L x 3 for each solvent concentration. Fractions were collected (200ml initially and 500ml after 1:1 EtOAc:MeOH) from the varying solvent gradients. Fractions were dried and homogenized by mortar and pestle and then subjected to thin layer chromatography (TLC) and HPLC-DAD analysis in order to pool fractions which exhibited similar phytochemical profiles. Thin Layer Chromatography was performed on every second fraction, and where possible, pooled based on similar phytochemical profiles to form a smaller number of primary pooled fractions. Silica TLC plates and a coplin jar were used for TLCs using a range of non-polar to polar solvents. HPLC was also used for fractions with thin layer chromatograms that did not provide adequate pooling information.

HPLC-DAD Analysis

In addition to profiling fractions using TLCs, analytical scale HPLC- diode array detection (HPLC-DAD) was used to generate more detailed chromatograms. The HPLC-DAD analyses were performed on an Agilent 1100

Series system consisting of an autosampler with a 100µL built in loop, a quaternary pump with a maximum pressure of 400 bar, a column thermostat and a diode array detector. All solvents were HPLC grade quality (Fisher Scientific, CA, USA). For HPLC-DAD analysis the solvents were sonicated at 5 min/L prior to analysis and the column (Luna C18(2). 250 X 4.6 X 5µm, SN:398501-1 (Phenomenex)) was equilibrated for 15 min with the starting conditions prior to analysis. The mobile phase consisted of A = water and B = acetonitrile. The optimal conditions for *Tradescantia spathacea* extract and its fractions were isocratic conditions of 90:10 A: B for 4 min followed by a 100% flush of B for 6 min. The column was brought back to initial conditions and equilibrated for 8 minutes prior to subsequent injections (total run time 18 min). The column operated at a flow rate of 3 ml/min and column temperature was maintained at 55 °C with maximum pressure limit of 400 bar. One ml of *Tradescantia spathacea* extract and its fractions were filtered through 0.22 µm PTFE membranes and 1 µL of each extract were injected into the HPLC system though the autosampler. Monitoring wavelengths were set at 210nm, 256nm, 280nm, 330nm and 410nm.

HPLC-DAD preparative scale fractionation

Secondary fractionation of Fr12, the most active pooled primary fraction, was also done using the same HPLC-DAD method described above, with the following modifications. *Tradescantia spathacea* fraction Fr12 was injected (100µl) at a concentration of 6.2mg/ml, and 5 secondary fractions were manually collected at 0.6 min, 1.1min, 1.9min, 2.2min, 2.6 min. Secondary fractions (Fr12-

1 to Fr12-5) were pooled following multiple rounds of collection, and re-injected to verify peak purity. The identification of peaks in secondary fractions of Fr12 was done using a phenolics spectra library (Saleem, 2009). The matching criterion was based on a multiple search choices option and spectral matching conditions were set so that the threshold for each peak was based on individual signal to noise ratio. Peak purity was assessed using ChemStation software (Agilent 1100 Series system) with a threshold of 99% spectral purity set to qualify a peak as pure. Fraction purity was a measure of the percent area under the curve over total area under the curve for the entire integrated chromatogram at 280nm.

UPLC-QTOF analysis

UPLC conditions: Acquity BEH C18 1.7um 2.1 x 100mm column connected with a VanGuard Pre-column 2.1 x 5mm. Mobile phase, A, water+0.1% formic acid, B-acetonitrile+0.1% formic acid (Fisher Optima LC-MS). Flow rate 0.5 mL/min. Column temperature, 50 °C, sample temperature 10 °C. Mobile phase composition, 0-1 min 5% A isocratic, 1-6 min linear gradient 5-50% B, 6-8 min 50-95%B, 8.01-10 min 5% A isocratic (total run time 10 min). Sample injection conditions: 1uL injection followed by a strong wash 200uL (90% acetonitrile+10% water) and weak wash 600uL (10% acetonitrile+90% water). QTOF analysis conditions: MassLynx software, MSe ESI+ mode, lock mass Leucine Enkephalin 12C 556.2615, source temperature 120 °C, desolvation

temperature 400 °C, Cone gas (N2) flow 50 L/hr, desolvation gas (N2) flow 1195 L/hr. MSe conditions, mass range 100-1500 Da, F1 CE, 6V, F2 CER 10-30V, Cone voltage 20V, Scan time 1 sec. Calibration, 50-1000 Da sodium formate. Statistical analysis conditions: Principal component analysis (PCA) and discriminate analysis (OPLS-DA) were performed by MarkerLynx (version 8.03).

Results

Silica Gel Column Chormatography and Pooling Results

A total of 191 fractions were attained from silica gel column chromatography, using a solvent gradient from non-polar to polar: Hexanes, EtOAc, MeOH, flush using H_2O. Thin layer chromatography was performed on every second fraction, and where it was evident, fractions were pooled based on similar phytochemical profiles to form a smaller number of primary pooled fractions. HPLC was also used for fractions with thin layer chromatograms that did not provide adequate pooling information. Based on TLC and HPLC phytochemical profiles, 21 primary fractions were pooled from the 191 fractions.

During the pooling of fractions, precipitate was observed in some of the fractions. A light brown precipitate was found in fractions 161-175, and HPLC analysis revealed it to be the same compound. Another fraction (pooled F11) contained crystallized rods which were collected separately. The pure compounds were tested individually for their anti-inflammatory activity.

Anti-Inflammatory Activity

All 21 pooled primary fractions displayed some anti-inflammatory activity in a dose dependant manner, although there were clear peaks of activity (Figure 1). Fractions 1-21 and the two precipitates ranged in activity from 2.06% to 47.0% relative to the parthenolide control when tested at 10µg/ml and from 4.71% -81.5% at when tested at 100µg/ml. Precipitates from primary fractions F11 and F169 showed the least amount of anti-inflammatory activity. Fractions 1-7, F13-14, F17-F21 and both precipitates displayed little activity ranging from 2.07% to 15.9% activity at 10µg/ml and 4.07% to 47.9% at 100µg/ml. There were 2 clear peaks of activity F10-12 and F15-16, all of which had percent activity ranging between 56.1-81.4% at 100µg/ml. F12 was chosen for further isolation because it had the highest percent activity at 100µg/ml, 81.5%, and its HPLC-DAD chromatogram was relatively simple (Figure 2) in comparison to other fractions and most suitable for compound isolation.

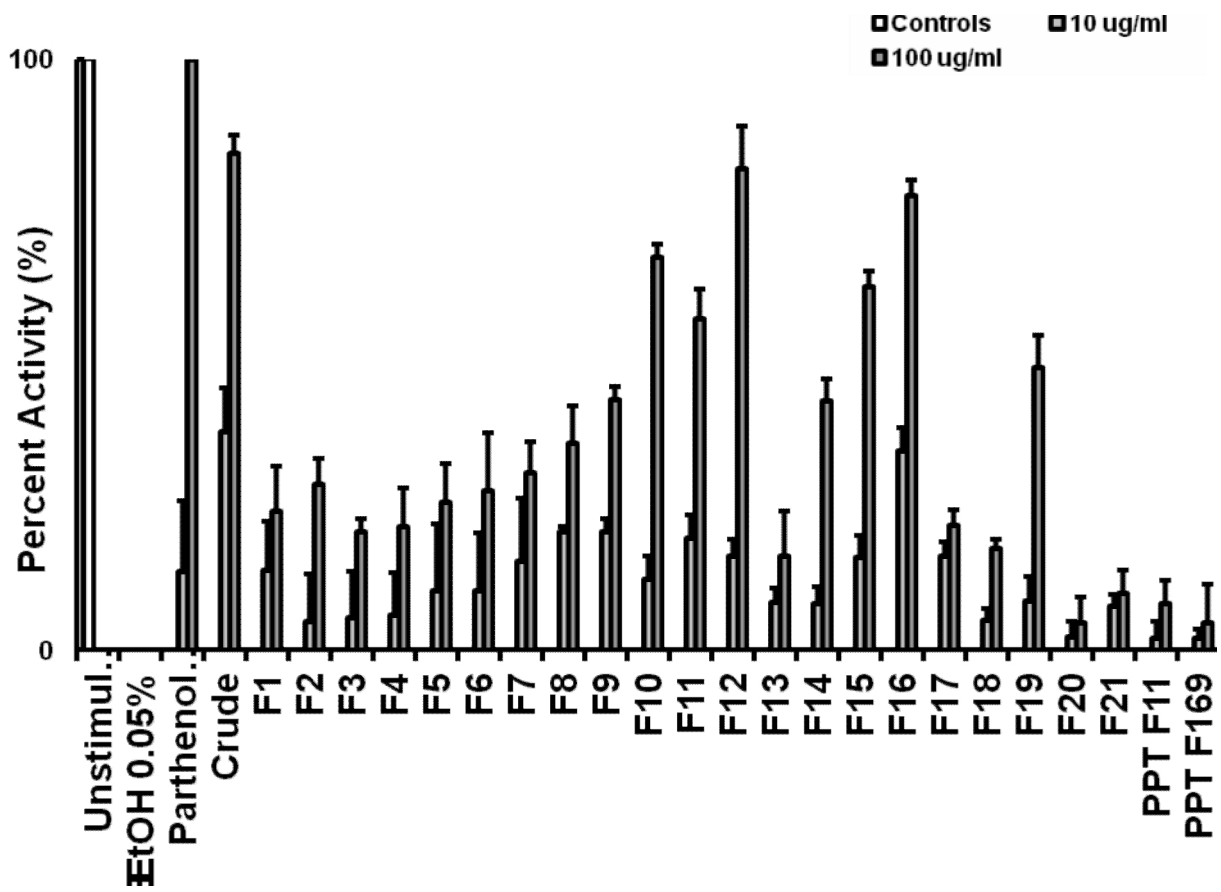


Figure 1: Percent activity relative to the 10µg/ml parthenolide positive control of *Tradescantia spathacea* pooled primary fractions (10µg/ml and 100µg/ml) as well as two precipitates (1µg/ml and 10µg/ml) in LPS-stimulated THP-1 monocytes

HPLC-DAD analysis of fraction Fr12 resulted in five distinct areas (Fr12-1 to Fr12-5) as indicated in Figure 2. Upon HPLC-DAD assisted fractionation, Fr12-3 was isolated at a retention time 1.985minutes (peak purity >99%) and a fraction purity of 62.4% (Figure 3). A tentative identification of Fr12-3 was done using the phenolics spectra library (Saleem, 2009) where a 96% match to catechol was found, indicating that Fr12-3 is a compound closely related to catechol (Figure 4) Fr12-4 was isolated at a retention time of 2.436 minutes (peak purity >99%) and a fraction purity of 84.1% (Figure 5). A tentative identification of Fr12-4 was done

using the phenolics spectra library where a 96% match to chlorogenic acid was found indicating that Fr12-4 is a compound closely related to chlorogenic acid (Figure 6). Curiously, Fr12-1 was found to contain primarily a mixture of the major peaks from Fr12-3 and Fr12-4, indicating the potential for the lack of chromatographic retention due to a highly concentrated injection, or due to carryover between multiple injections. This large peak was not seen in smaller injections of Fr12, and was only an artifact following larger volume injections. Fr12-2 and Fr12-5 were found to contain a mixture of compounds in relatively small amounts.

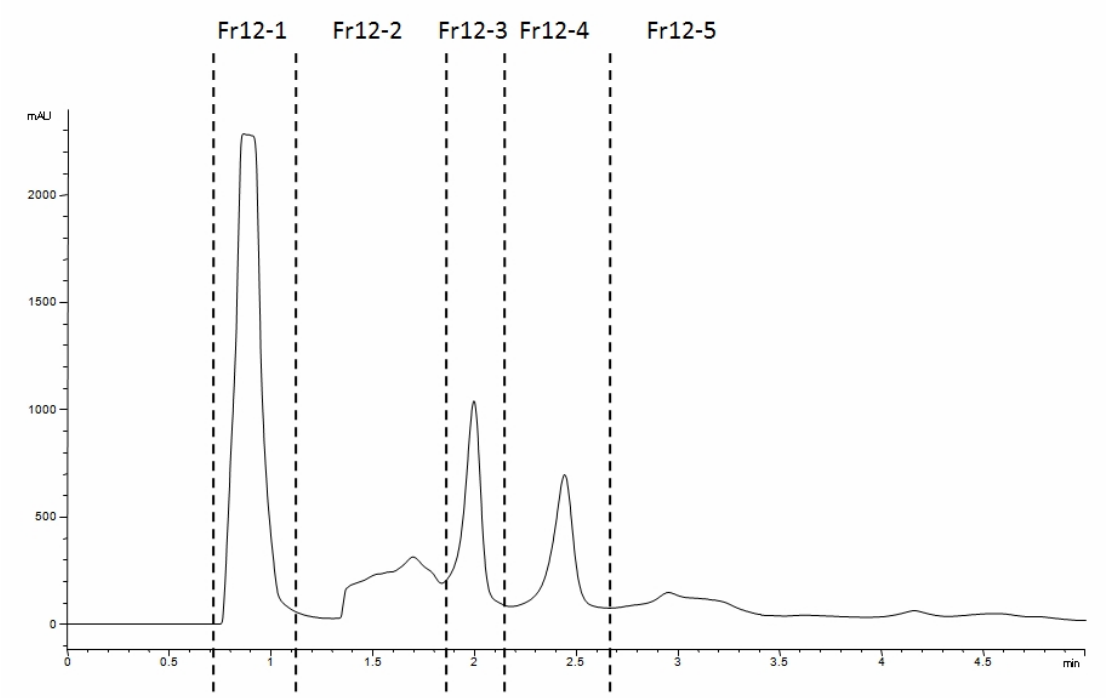


Figure 2: Chromatographic profile of F12 fraction of *Tradescantia spathacea* at 280nm with an injection volume of 100ul . Secondary fractionation indicated as Fr 12-1 through to Fr12-5. Fr12-3 and Fr12-4 are peaks of interest.

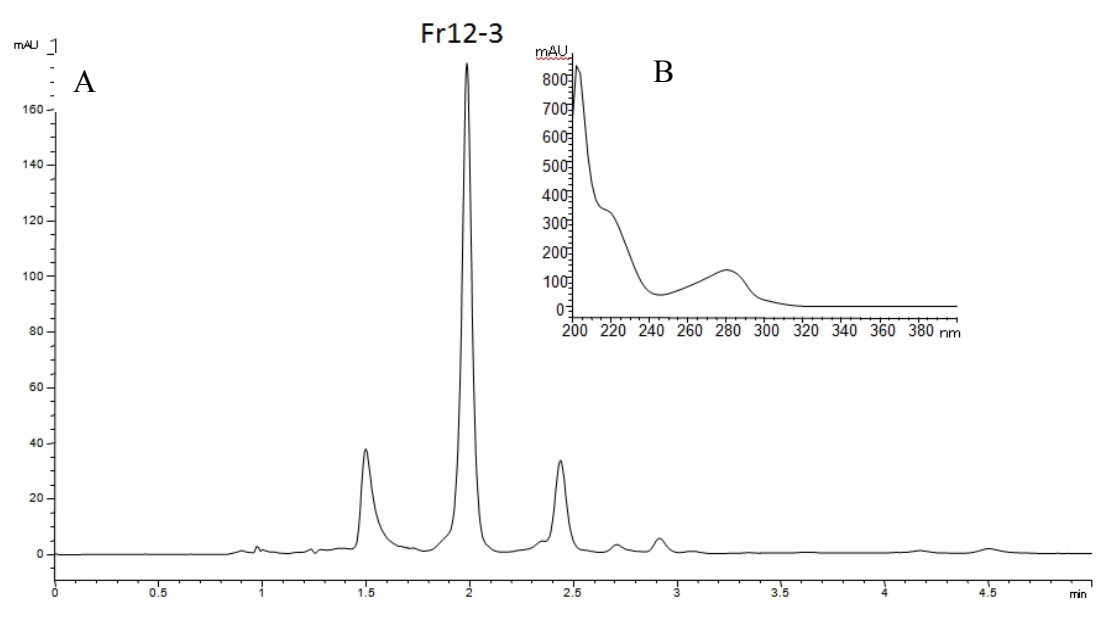


Figure 3-A: Chromatographic profile of F12-3 of *Tradescantia spathacea* at 280nm at a retention time of 1.985 minutes (peak purity >99%) and a fraction purity of 62.4%. 3-B: UV spectrum of Fr12-3.

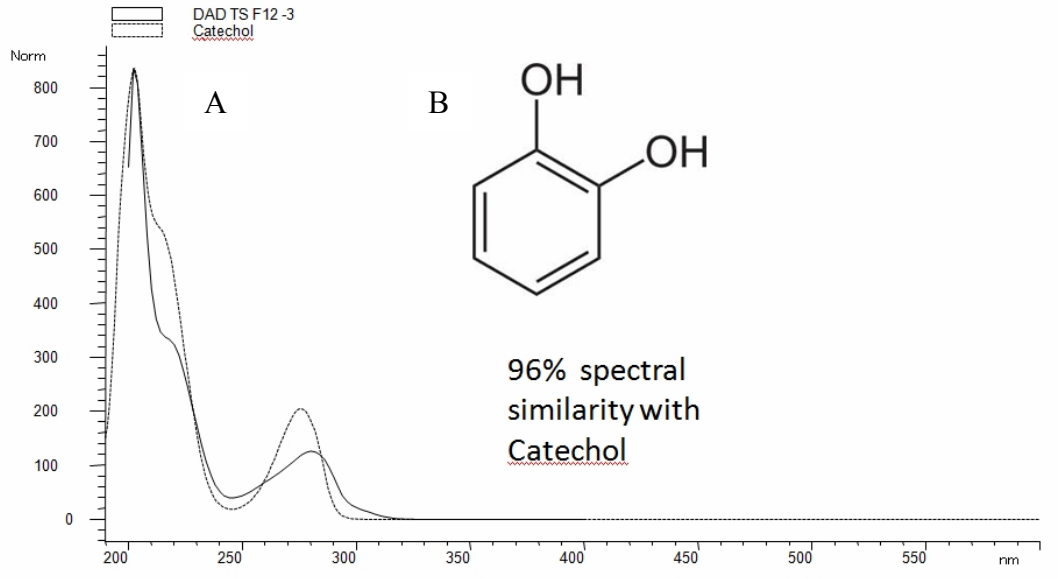


Figure 4 A: UV spectrum of F12-3 and overlayed with a UV spectrum of catechol. Fr12-4 has a 96% spectral similarity to catechol using phenolics spectra library (Saleem, 2010). 4-B: Structure of catechol.

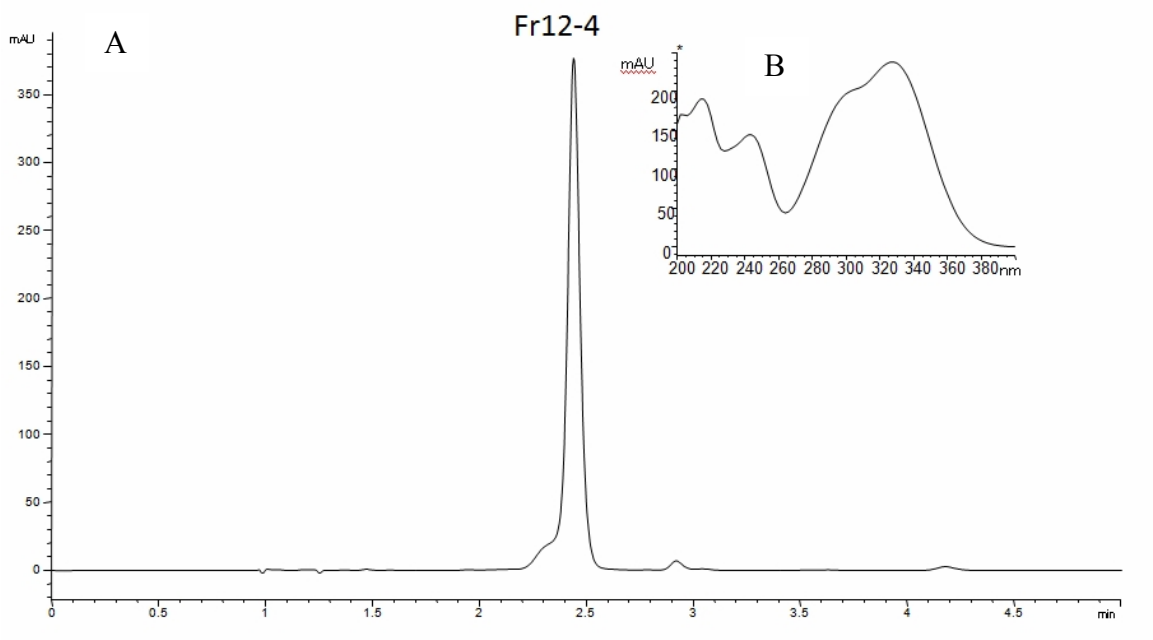


Figure 5-A: Chromatographic profile of F12-4 of *Tradescantia spathacea* at 330nm at a retention time of 2.436 minutes (peak purity >99%) and a fraction purity of 84.1%. 5-B: UV spectrum of Fr12-4.

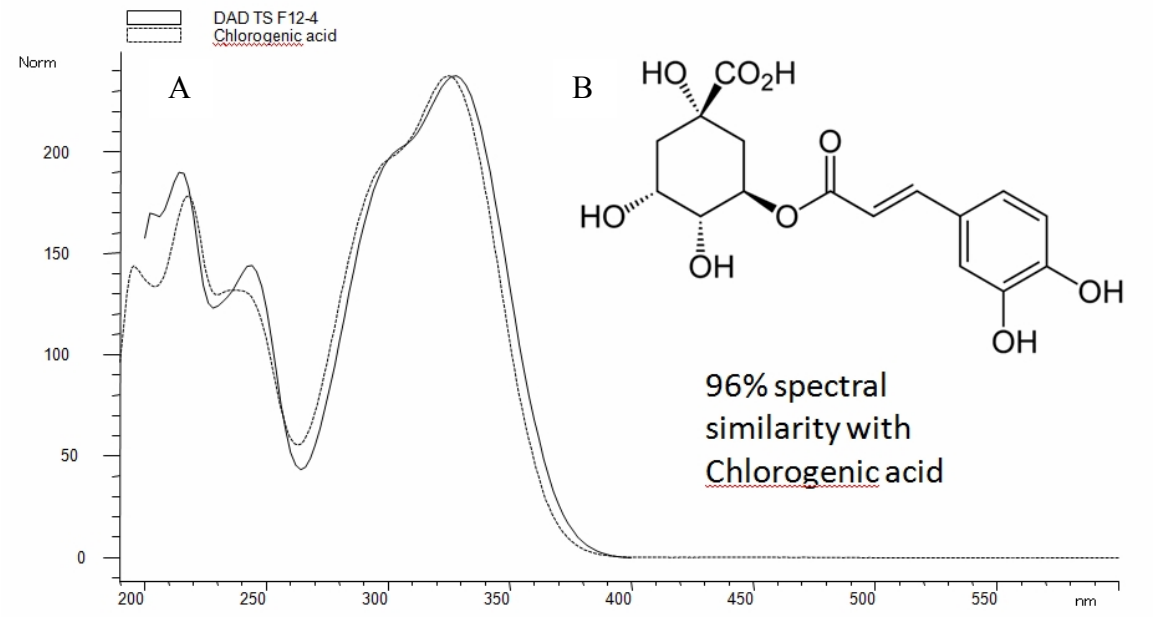


Figure 6-A: UV spectrum of F12-4 and UV spectrum of chlorogenic acid. Fr12-4 has a 96% spectral similarity to cholorgenic acid using phenolics spectra library (Saleem, 2010). 6-B: Structure of chlorogenic acid.

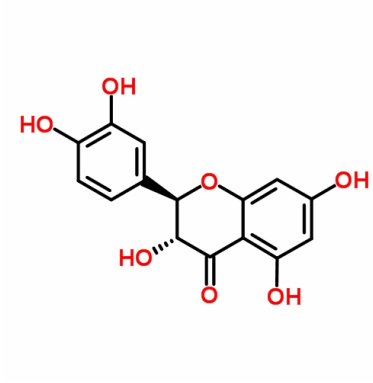
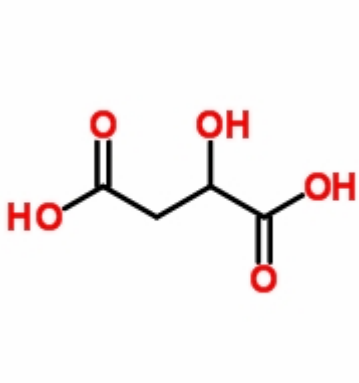
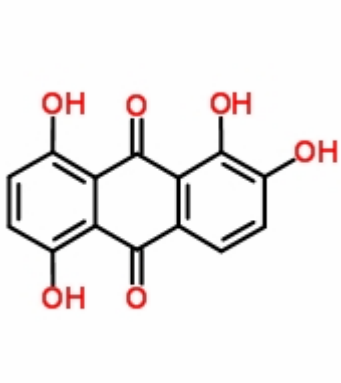
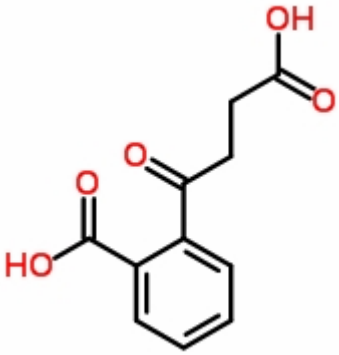
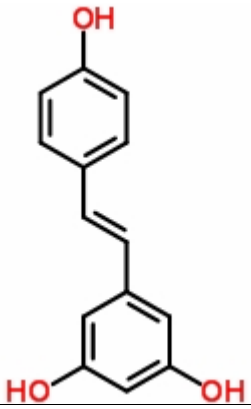
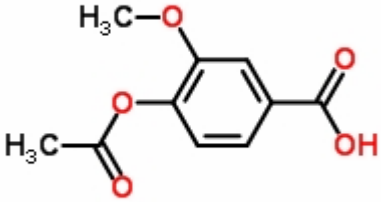
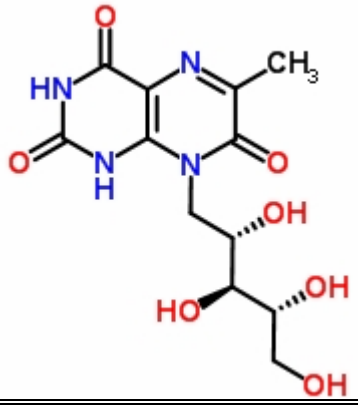
UPLC-QTOF Results

Seven phytochemical identifications were achieved by UPLC-QTOF using electrospray ionization in positive mode with high mass accuracy (mass error less than 3 ppm). Among the compounds identified, taxifolin, resveratrol, malic acid and vanillic acid have reported anti-inflammatory properties (Table 1, Table 2). Their retention times were 2.369min, 6.427 min, 0.936 min, and 6.428 min respectively. Quinalizarin (2.274 min), 2-succinylbenzoate (2.568 min) and 7-Hydroxy-6-methyl-8-ribityl lumazine (6.400 min) were also phytochemicals identified in *Tradescantia spathacea* extract.

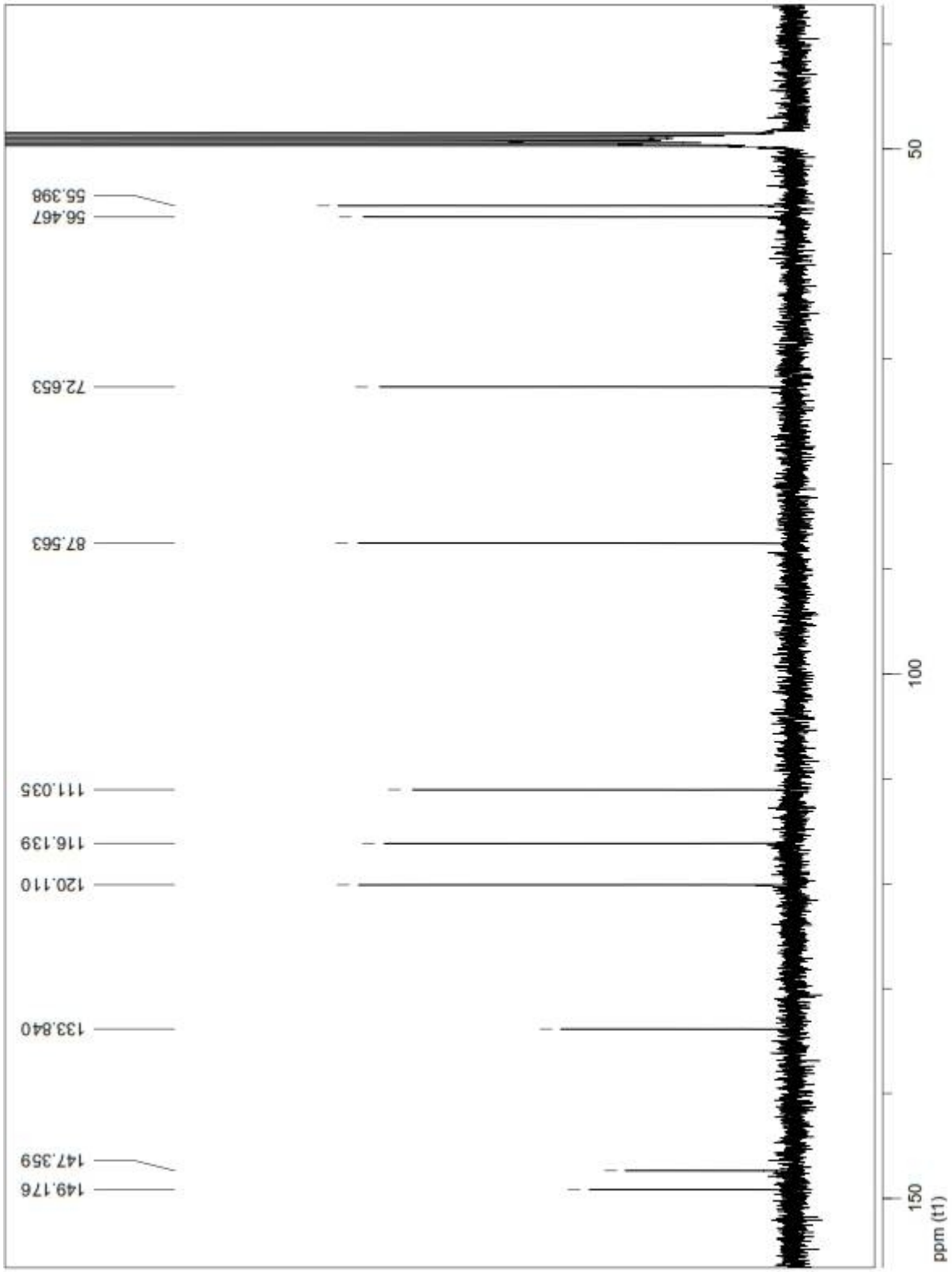
Table 1: Phytochemical characterization of anti-inflamatory metabolites from *Tradescantia spathacea* by UPLC-Q-TOF by electrospray ionization in positive mode.

#	Name	Ret. Time	$[M+H]^+$	Formula	Type	Anti-Inflammatory Reference
1	Taxifolin	2.369	305.0168	C15H12O7	Flavanoid	Choi *et al, Phytotherapy Research* (2011)
2	Malic acid	0.936	134.98	C4H6O5	Small molecule	Obertreis *et al, Arzneimittelforschung* (1996)
3	Quinalizarin	2.274	272.9901	C14H8O6	Anthraquinone	
4	2-succinylbenzoate	2.568	223.0218	C11H10O5	DHNA precursor	
5	Resveratrol	6.427	229.0638	C14H12O3	Phenolic	Qureshi *et al, Lipids in Health and Disease* (2012)
6	O-Acetylvanillic acid	6.428	211.0588	C10H10O5	Phenolic	Min-Cheol *et al, Immunopharmacology and Immunotoxicology* (2011)
7	7-Hydroxy-6-methyl-8-ribityl lumazine	6.400	329.1438	C12H16N4O7	Riboflavin	

Table 2: Structures of phytochemicals listed in Table 1.

1. Taxifolin 	2. Malic Acid 	3. Quinalizarin 
4. 2-succinylbenzoate 	5. Resveratrol 	6. O-Acetylvanillic acid 
7. 7-Hydroxy-6-methyl-8-ribityl lumazine 		

Appendix Va – ^{13}C NRM of (+)-pinoresinol



Appendix Vb – ^{13}C NRM of (+)-lariciresinol-9'-*p*-coumarate