MEDICAL ETHNOBOTANY AND ANTI-CANCER PROPERTIES OF *VITEX ROTUNDIFOLIA* L. F.

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

BOTANY

DECEMBER 2005

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DEDICATION

This thesis is dedicated to my grandparents, Viola La Rose and Walter Val Hardy. Their eternal support and dedication to my well being has enabled me to pursue my wildest dreams. Thank you. All my love to you both.

ACKNOWLEDGMENTS

I would first like to thank my advisor, Dr. Will McClatchey, without whom this project would not be possible. When he first invited me to join the Marshall Island expedition, I was very excited. I couldn't believe my research dreams were starting to happen. The experience from performing research in the Marshall Islands will stay with me always. I would also like to thank my other committee members Dr. Thomas Hemscheidt and Dr. Mark Merlin. Dr. Thomas Hemscheidt has provided me uncountable hours of his time training me in natural product research and chemical analysis, and for this I am eternally grateful. His patience and perseverance is inspiring. I would also like to thank his entire lab for their constant support. Hillary, Quyen, Guntram, Beca, Phil, and Domos, I will miss working with you. Dr. Merlin has provided great direction in the areas of ethnobotany and biogeography, and his kind ways will always be remembered.

Next, I would like to thank Dr. Bonnie Warn-Cramer, Dr. Patricia Lorenzo, Anne Hernandez and many other wonderful people involved in the Natural Products Research team at the University of Hawai'i Cancer Research Center. Without Dr. Lorenzo and Dr. Cramer this research would not have been possible. Their caring nature and support is unsurpassed, and I am lucky to have worked with them both. I thank Anne for her training me in the MBP assay and providing trouble-shooting advice whenever needed, and her positive loving nature.

My acknowledgments would not be complete without thanking the Marshall Island Research team, including: Dr. Will McClatchey, Dr. Kent Bridges, Dr. Jim Maragos, Daria Siciliano, Dr. Silvia Pinca, and the Marshallese crew from

Rongelap. I would especially like to thank Jim Maragos for being my SCUBA partner during my algal collections on the beautiful and pristine Ailinginae Atoll reefs, as well as Dr. Silvia Pinca for her assistance in algal collections and identification. The research team consisted of an amazing group of professionals and sociable people. I will always remember the songs we sang together, including "The Gambler" and "The Rose." And last, I would like to thank the Rongelap Atoll Government for their collaboration and support of this project, and the Ratik boat crew.

ABSTRACT

Vitex rotundifolia is one of approximately 270 species that are classified under the genus *Vitex*. The geographic range of *Vitex* spans tropical and subtropical regions of the globe, with individual species in various geographic niches. *Vitex* has a rich history associated with human uses, medicine in particular, which dates back several millennia.

It is becoming increasingly more evident in the scientific arena that natural product drug research, combined with ethnobotany and ethnopharmacology, is a highly efficient methodology for pursuing therapeutic resources, whether for complementary and alternative remedies or other biomedical applications. With this increased awareness comes the issue of intellectual property rights. Due to the poor ethics of a few researchers in the past, or simply their ill attempt to clearly state the actual odds of a profitable outcome, researchers interested in ethnopharmacological drug research have an increased number of obstacles to overcome. This project addressed the issue of intellectual property rights with the formation of an agreement with the Rongelap Atoll Government of the Republic of the Marshall Islands. It states that any profitable resources derived from plants collected on the Rongelap and Ailinginae Atolls for chemical analysis would be distributed back to the people of Rongelap Atoll. Thus far, DCM extracts, and fractions thereafter, of *Vitex rotundifolia* have displayed the ability to inhibit phosphorylation activity of MAP kinase. *Vitex rotundifolia* exhibits cancer chemotherapeutic potential, and isolation of the responsible active constituent should be further investigated.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Cancer

There are currently about 22.5 million people in the world living with cancer (WHO 2003). The annual global mortality rate due to cancer is approximately 6 million deaths per year. This is 12% of all deaths worldwide (WHO 2003). Cancer causes more mortality than AIDS, malaria and tuberculosis combined. It is estimated that 1 out of 4 people will be diagnosed with cancer sometime in their life. In the U.S. alone, 1 million people are diagnosed with cancer every year (WHO 2003). There are over 25 different categories of cancer currently defined, including breast, lung, colon, rectum, stomach, oral cavity and pharynx, larynx, pancreas, kidney, cervical, uterine, corpus uterine, ovary, prostrate, testis, bladder, brain, CNS, and skin cancers, as well as numerous forms of Hodgkin's and non-Hodgkins lymphomas and many types of leukemia (Parkin *et al.* 1999). Each different type of tissue prone to cancer usually requires a unique corresponding treatment. This is due to the fact that differentiated cells have unique juxtaposed processes. Further, many cancers are linked with pathogens, such as the bacteria *Helicobacter pylori* and stomach cancer, or the papilloma virus and cervical cancer (Parkin *et al.*1999). Contrary to the popular misconception that there should be one miracle cancer drug, there is a need for many different types of cancer drugs to treat each individual type of cancer. It is for this reason that the search for novel cancer chemotherapeutic

agents is far from over. No potential source of novel medicine should be disregarded.

Plants have always been, and continue to be, a valuable source for therapeutic agents. Some of the most effective drugs of modern medicine are derived from plants. For example, digitoxin, a cardiac glycoside first isolated from *Digitalis purpurea* L. (Scrophulariaceae) used in biomedicine to treat congestive heart failure (Robbers *et al.*1996). More classic examples include salicylic acid from *Salix sp.* L. (Salicaceae), morphine from *Papaver somniferum* L. (Papaveraceae) and most relevant to this study, vincristine, vinblastine from *Catharanthus roseus* (L.) G. Don f. (Apocynaceae) and taxol from *Taxus brevifolia* Nutt. (Taxaceae).

Vincristine and vinblastine are both alkaloids that have become U.S. Food and Drug Administration (FDA) approved cancer chemotherapeutic agents currently used to treat several forms of cancer including breast cancer and Hodgkin's disease, respectively (Johnson *et al.* 1963; Noble *et al.* 1958; Noble 1990). Taxol, a diterpenoid, is also FDA approved, and currently used to treat several forms of cancer including metastatic carcinoma of the ovary and breast cancer (Menzin *et al.* 1994; Wani *et al.* 1971). Vincristine and vinblastine share the same mechanism of action-inhibition of the polymerization of tubulin into microtubules (Duflos *et al.* 2002), and taxol has a unique mechanism in that it actually enhances the polymerization of tubulin, which results in the formation of stable, nonfunctional microtubules (Menzin *et al.*1994). For a list of plants with

anti-cancer properties, see **Table 1.1**, which is based on a compilation of Taylor (2000) and McClatchey and Stevens (2001).

Table 1.1. Plants with anti-cancer activity
(**cancer** = general for cytotoxic or anti-proliferation; **tumor** = solid form of cancer; **leukemia** = cancers of the blood)

Botanical source	Chemical isolate	Activity	Literature
Acronychia baueri Schott.	Acronycine	cancer	Hughes *et al.* 1948; Blasko & Cordell 1998
Ajuga decumbens Thumb.	Cyasterone	tumor	Takasaki *et al.*1999
Annona cherimolia Miller	annocherimolin	tumor	Kim *et al.* 2001
Bazzania novaezelandiae (Mitt.)	Naviculyl caffeate	tumor	Burgess *et al.* 2000
Betula alba L.	Betulinic acid	cancer	Phishe *et al.* 1995
Brucea antidysenterica J.F.Mill	Bruceantin	cancer	Kupchan *et al.* 1973
Camptotheca acuminata Decne	Camptothecin	cancer	Wani & Wall 1969; Wall *et al.* 1986
Casearia arborea (Rich.) Urb.	Casearborins A-E	tumor	Beutler *et al.* 2000
	Cucurbitacin B	tumor	Beutler *et al.* 2000
Catharanthus roseus (L.) G. Don. f.	Vinblastine	tumor, leukemia	Noble 1990
	Vincristine	tumor, leukemia	Noble 1990
Cedronia granatensis Cautrec.	Isobrucein B	melanoma/ colon tumors	Tischler *et al.*1992
	Sergiolide	melanoma/ colon tumors	Tischler *et al.*1992

Table 1.1. (Continued) Plants with anti-cancer activity

Botanical source	Chemical isolate	Activity	Literature
Cephalotaxus harringtonia (Forbes) K. Koch	Harringtonine	leukemia	Powell *et al.* 1970; Blasko & Cordell 1988
	Homoharringtonine	leukemia	Powell *et al.* 1970
Colchicum autumnale L.	Colchicine	tumor	Bullough 1949; Eigsti *et al.* 1949
Combretum erythrophyllum (Burch.) Sond.	Combretastatin	cancer	Hamel & Lin 1983
Crotalaria sessiliflora L.	Monocrotaline	tumor (topical)	Huang *et al.* 1980
Garcinia bracteata C.Y. Wu ex Y.H. Li	Bractatin	cancer	Thoison *et al.* 2000
	Isobractatin	cancer	Thoison *et al.* 2000
Hardwickia binata Roxb.	Harbinatic Acid	cancer (adjunct)	Deng *et al.*1999
Iberis amara L.	Cucurbitacin E	tumors/ melanoma	Lavie 1958; Gilbert & Mathieson 1958; Cardellina *et al.* 1993
Isodon xerophilus (C.Y. Wu & H.W. Li) H. Hara	Xerophilusins A-C	tumor	Hou *et al.* 2000
Picea glehni Mast.	(11E)-14,15-bisbnor-8α-hydroxy-11-labdane-13-one, 9α, 13α-epidioxyabiet-8(14)-en-18-oic acid	tumor (viral induced)	Barrero *et al.* 1991; Kinouchi *et al.* 2000
Podophyllum peltatum L.	Podophyllotoxin	tumor	Pettit *et al.* 1962

Table 1.1. (Continued) Plants with anti-cancer activity

Botanical source	Chemical isolate	Activity	Literature
Portieria hornemannii (Lyngbye) P.C. Silva	Halomon (6(*R*)-bromo-3(*S*)-bromomethyl)-7-methyl-2,3,7-trichloro-1-octene	tumor	Fuller *et al.*1992
Selaginella delicatula (Desv.) Alston	Robustaflavone-4',7-dimethyl ether	tumor	Lin *et al.* 2000
Sophora flavescens Aiton	(2*S*)-2'-methoxykurarinone (-)-kurarinone sophoraflavanone G leachianone A	tumor/ leukemia	Kang *et al.* 2000
Strychnos icaja Baill.	Isosungucine, 18-hydroxysungucine 18-hydroxyisosungucine	cancer	Frédérich *et al.* 2000
Tabebuia cassinoides (Lam.) DC.	Quinones	tumor	Rao & Kingston 1982
Taxus brevifolia Nutt.	Taxol	tumor	Wani *et al.* 1971
Thevetia sp. L.	Neriifolin	lung cancer	Mezey 1950; Cardellina *et al.*1993
Uncaria rhynchophylla (Miq.) Jacks	Uncarinic acids C-E	cancer	Lee *et al.* 2000

The term chemotherapy, which is often thought of as synonymous for cancer treatment, actually refers to drugs used to treat any disease. Chemotherapy therefore, *can* be applied to the chemical treatment of cancer, but to be more specific, it should be referred to as "cancer chemotherapy". Used in lieu of, or complementary to cancer chemotherapy are radiation therapy and/or surgery. The layperson often confuses chemotherapy with radiation therapy.

Cancer chemotherapy is a relatively new development, as it was not developed until the mid 20th century. Since chemotherapy development occurred at the same time as radiation therapy, and the two categories are often used in conjunction, they are easily confused by the layperson.

According to the American Cancer Society (ACS 2005a), the first cancer drug was discovered somewhat serendipitously in the 1940's. During WWI, it was discovered that troops exposed to mustard gas had abnormally low white blood cell counts. This led to experimental cancer treatments during the 1940's, in which mustard gas was given intravenously (rather than by inhalation) to patients with advanced lymphomas. The patients improved remarkably, however temporarily. This breakthrough marked the beginning of cancer chemotherapy research, as we know it today. Chemotherapy is today often the first strategy in cancer treatment, preferred over radiation therapy and surgery. This is often determined, however, by the stage at which the cancer is identified. There are currently over 100 FDA-approved drugs used in cancer chemotherapy, many of which are derived from natural products-several specifically of botanical origin (See **Table 1.2**).

Table 1.2. FDA approved cancer drugs derived from plants

Botanical Source	Drug	Action	Literature
Camptotheca acuminata	Topotecan (Camptothecin analog)	ovarian and small lung cancer	Wall 1998
Camptotheca acuminata	Irinotecan (Camptothecin analog)	metastatic colorectal cancer	Wall 1998; Saltz *et al.* 2000
Catharanthus roseus	Vinblastine	leukemia	Noble 1990; Duflos *et al.* 2002
Catharanthus roseus	Vincristine	Anti-leukemia	Noble 1990
Podophyllum peltatum	Teniposide (semi-synthetic derivative of Podophyllotoxin)	small lung cell and brain cancer	Postmus *et al.* 2000; O'Dwyer 1985
Podophyllum peltatum	Etoposide (semi-synthetic derivative of Podophyllotoxin)	breast cancer	Saphner *et al.* 2000; O'Dwyer 1985
Taxus brevifolia	Taxol/Paclitaxel	ovarian and breast tumors	Wani & Wall 1971; Wall 1998; Menzin *et al.* 1994

The United States National Cancer Institute (NCI) was established in 1937, and by 1955 had developed the Cancer Chemotherapy National Service Center (CCNSC). This development took place not long after the mustard gas breakthrough. For fifty years, the NCI has provided a resource for the pre-clinical screening of compounds and materials submitted by grantees, contractors, pharmaceutical and chemical companies, and other scientists and institutions, public and private, worldwide, and has played a major role in the discovery and development of many of the available commercial and investigational anti-cancer agents (Driscoll 1984). There was a brief period during the early 1980's, when the NCI discontinued their natural products effort due to unsatisfactory findings, however, it was realized that it was not nature that was limiting, it was the

biological assays that were the true bottleneck. The natural product research at NCI resumed and continues to this day.

The term chemoprevention (Sporn 1976) refers to the use of specific natural or synthetic chemical agents to reverse, suppress, or prevent progression to invasive cancer. Chemoprevention studies are based on the hypothesis that interruption of the biological processes involved in carcinogenesis will inhibit it and, in turn, reduce cancer incidence (Kim *et al.* 2002). Specific genes are often the target of such research, mainly *ras* and p53, found in pre-malignant lesions (Kim *et al.* 2002). The *ras* oncogene is suggested to inhibit the tumor suppressor gene p53 (Ries *et al.* 2000).

Although many mechanisms have been studied for identification of new treatments for cancer, great focus is directed toward the study of molecules that interfere with the function of cellular molecules involved in cell proliferation. One of the better-studied targets is Mitogen Activated Protein Kinase (MAPK). MAPK phosphorylates a variety of substrates, including transcription factors critical to cell proliferation and tumor invasion (Sausville *et al.* 2003). Mitogens are proteins that bind to the cell surface receptors and induce cell division (Alberts *et al.* 2002). The discovery of small molecules that inhibit MAPK, or other related kinases, may lead to new cancer chemotherapeutic agents. Protein kinases are a large eukaryotic group of enzymes characterized by their catalytic (kinase) sequence of 250 amino acids (Alberts *et al.* 2002). The main function of kinases is the phosphorylation of substrate molecules. Protein phosphorylation involves the enzyme-catalyzed transfer of the terminal phosphate group of an ATP

molecule to the hydroxyl group on a serine, threonine or tyrosine side chain of a protein molecule. Dephosphorylation occurs via phosphatases. There are hundreds of kinases and their respective phosphatases in eukaryotic cells, each responsible for a different protein or protein group. The kinase of interest for this study is MAPK, more specifically, ERK2 (extracellular-signal regulated kinase). This study examined the ability of crude botanical extracts to inhibit ERK2 from phosphorylating the substrate MBP (myelin basic protein). The objective of this study was to find a novel source of inhibition for this enzymatic reaction. As will be described below, *Vitex rotundifolia* has been revealed through this study to inhibit MAPK.

1.1.2 Vitex rotundifolia taxonomy

Vitex rotundifolia L. is one of approximately 270 species classified in the genus *Vitex*. *V. rotundifolia* has several synonyms, which are provided in **Table 1.3**. As described by Wagner *et al.* (1999), *V. rotundifolia* is a low branching shrub with procumbent stems, often rooting at the nodes and forms mats up to several meters in diameter along coastal areas from China, Japan, Korea and Taiwan, all the way to Mauritius, Sri Lanka, India, Malaysia, and some Pacific Islands such as Hawai'i. *V. rotundifolia* has opposite, simple leaves, rarely compound with 2-3 leaflets that are obovate to suborbicular leaves with a sage-like aromatic scent when crushed. The leaflets are 2-6.5 cm long and 1-4.5 cm wide, with a pale green, densely puberulent abaxial surface and a grayish white densely tomentose adaxial surface. Leaflet margins are entire. *V. rotundifolia*

flowers are perfect, often 3 in cymes aggregated in narrow paniculate inflorescences 3-7 cm long. Individual flowers are bluish-purple, narrowly funnelform, with an upper lip of two lobes about 3.5 mm long, and a lower lip with a lateral lobe about 4 mm long, and a medial lobe about 7.5 mm long, with two white short-pilose markings at the base. *V. rotundifolia* flowers have a superior ovary with two carpels, each 4-celled by false partitioning, with one ovule per cell. Fruit is globose, drupaceous, green turning yellow and red-tinged, becoming bluish black at maturity. *V. rotundifolia* was once classified as a variety of *V. trifolia*, however *V. rotundifolia* differs from *V. trifolia*, in that *V. trifolia* usually takes the form of a small tree whereas *V. rotundifolia* is typically observed as a low lying shrub. Further, *V. trifolia* typically have three leaflets, as reflected in the name. Since it is possible that either of these two *Vitex* species have been mistaken for the other in the literature, they will both be included in the literature review. Most of the papers addressed in this thesis do not make reference to a voucher number, so it is not possible to clearly define which species the authors are actually referring.

Table 1.3. Synonyms of *V. rotundifolia* (Wagner *et al.*1999)

V. trifolia var. *simplicifolia* Cham.
V. trifolia var. *ovata* (Thunb.) Makina
V. trifolia var. *unifoliata* Schauer
V. ovata Thunb.

1.2 Medical Ethnobotany of *Vitex*

The historical record of *Vitex* reaches back over the past three millennia. Reports on the medical uses of *Vitex* species are common, spanning most continents within tropical and subtropical regions. In Europe, *Vitex* has long been used for gynecological and obstetric applications. Globally, *Vitex* species are used to address ailments ranging from neutralization of snake venom in India to treatment of asthma in Indonesia. However, it is from the long European history of *Vitex* that this genus gained its seemingly most popular name, the Chaste Tree.

Most of the literature on Pacific *Vitex* species has been produced over the past 50 years. Generally it lacks detail about cultural perspectives although varying modes of preparation and administration are discussed. The following sections attempt to summarize the scope of global ethnobotanical reports found in the literature. Traditional uses will first be reported on *Vitex* species in general, other than the published taxa of *Vitex trifolia* L. and *Vitex rotundifolia*, followed by a more detailed description of *V. trifolia* and *V. rotundifolia*. Attention to *V. trifolia* is a result of close association between the two species, as *V. rotundifolia* has been classified as a variety of *V. trifolia*. Also, some confusion seems to exist in the literature, regarding chemistry in particular, between these two *Vitex* species.

In the 20th century, chemists began isolating molecules from various *Vitex* species, including *V. rotundifolia* and *V. trifolia*. Most of these studies cite the traditional medicinal use of *Vitex* as the basis of plant selection. *V. agnus-castus* is at the forefront of these studies because of its traditional and continuing

importance in European health care (Hobbs 2003). As research on the genus *Vitex* grows, more and more species of *Vitex* have been found to display biological activity. Also, a significant number of species are used in traditional pharmacopoeias. The significance of the chemistry and biological activity of *Vitex* will be addressed later.

1.2.1 Europe

The ancient Greek physician Hippocrates completed one of the earliest documentations of Vitex in the 4th century B.C. Hippocrates recommended *Vitex agnus-castus* L. for the treatment of injuries, inflammation, and swelling of the spleen. He also recommended that leaves soaked in wine be used for hemorrhages and the "passing of birth" (Christie and Walker 1997). Hippocrates wrote, "If blood flows from the womb, let the woman drink dark wine in which the leaves of the chaste tree have been steeped" (Hippocrates 400 B.C.). *V. agnus-castus* is also referenced in the works of Dioscorides, Plinius, Paracelsus and Theophrastus (Christie and Walker 1997; Foster 1998). Plinius (Pliny the Elder) in the 1st century A.D. wrote, "The trees furnish medicines that promote urine and menstruation. They encourage abundant rich milk..." (Secundus 1582).

V. agnus-castus is native to the Mediterranean and Central Asia. The name originates from the Latin "castitas" and "agnus" which translate to "chastity" and "lamb," respectively, hence the name Chaste Tree (Stern 2004). Other names applied to *V. agnus-castus* include Abraham's balm, Chaste Lamb Tree, Safe Tree, Indian Pepper and Wild Pepper. *V. agnus-castus* was associated with the ancient Greek festival of Thesmophoria, which was held in honor of

Demeter, the goddess of agriculture, fertility and marriage. During Thesmophoria, women were to remain "chaste" and used the *V. agnus-castus* blossoms for adornment, while the leaves and twigs were used to adorn Demeter's temple (Hobbs 1996). Traditional Greek religion or "mythology" tells of Hera, sister and wife of Zeus, protector of marriage, who was born under a chaste tree. Ancient Rome also utilized *V. agnus-castus* as a symbol of chastity. The vestal virgins carried twigs of the Chaste Tree to represent their vows (Hobbs 1996).

As Christianity began to spread across Europe, the chaste tree was readily incorporated into Christian rituals. Novitiates entering a monastery walked on a path strewn with flowers of the tree, a ritual that is practiced to this day in some regions of Italy (Foster 1998). Monks would use a decoction of the fruit to aid in keeping their vows of celibacy. It is likely that the ancient association of the tree with chastity led to the widely held belief that ingestion of parts of the tree would induce an anti-aphrodisiac effect. The monks would also use the fruit as a source of pepper in addition to suppressing the libido, which led to yet another name of *V. agnus-castus*, "Monk's Pepper".

Through the 19th century A.D., multiple records of *V. agnus-castus* as an anti-aphrodisiac arose. The sixteenth century herbalist Gerard (1597) wrote of *Vitex* for women's health, Duncan (1789) mentions it in his edition of the Edinburgh Dispensatory, and Thornton (1814) in his 1814 Family Herbal. As Europeans crossed the Atlantic and colonized the Americas, they brought *V. agnus-castus* with them. The chaste tree is now naturalized in much of the

Southeastern United States where it continues to be used for medicinal purposes (Wunderlin 1982).

1.2.2 Central and South America

Both bark and fruit from *Vitex polygama* Cham. are used traditionally in Brazil as emmenagogues and diuretics (Correa 1926). In Mexico, several *Vitex* species are used medicinally. *Vitex mollis* Kunth has been reported for treatments including dysentery, scorpion stings, diarrhea and stomachaches, as well as an analgesic and anti-inflammatory medicine (Argueta *et al.* 1994). *Vitex gaumeri* Greenm. is used as an anti-malarial medicine and to treat ulcers (Argueta *et al.* 1994). *Vitex pyramidata* B. L. Rob., *V. pubescens* Vahl., *V. agnus-castus,* and *V. gaumeri* Greenm. are all used in traditional pharmacopoeias for diarrhea and other gastrointestinal problems, not only in Mexico, but also in parts of Malaysia and India (Ahmad and Holdsworth 1995; Argueta *et al.*1994; Bajpai *et al.*1995).

1.2.3 South and Southeast Asia

Traditional pharmacopoeias in India and Malaysia use one or more *Vitex* species. *V. peduncularis* Wall. Ex Schauer in A. DC. is used as a febrifuge (Burkill 1966), and its bark is reportedly used topically on the chest to alleviate chest pains (Kirtikar and Basu 1980). *Vitex negundo* L. is one of the more widely cited *Vitex* species, particularly in Central and Southeast Asia. In India, *V. negundo* is widely used to treat snakebites and inflammatory disorders (Alam and Gomes 2003; Chawla *et al.*1992). *V. negundo* is used in Ayurvedic medicine for a range of ailments many of which overlap with other *Vitex* uses around the

globe (Dey 1980). For example, *V. negundo* is used as an emmenagogue and febrifuge, and to treat asthma, baldness, boils, earaches, rheumatism, tumors, skin diseases and hemorrhage. Further, the leaves are laid over grain to keep insects off and the leaf smoke is used to repel mosquitoes. Several *Vitex* species are reputed to have mosquito deterrent properties or insecticidal activity, as they are used in various agricultural practices. Traditional uses of *Vitex* as mosquito repellent will be discussed later in this paper.

In the Baluchara region of Bangladesh the leaves of *V. trifolia* are used as a topical treatment for rheumatic pains, sprains and inflammations (Kirtikar and Basu 1980). Ghani *et al.* (1998) report claims that the leaves of *V. trifolia* contain insecticidal, anti-tubercular and anti-cancer properties. They also report that the flowers are prescribed to treat fevers accompanied with vomiting. *Vitex* species are reportedly used to treat allergies and related ailments in many traditional societies. For example, Traditional Thai medicine prescribes a decoction from *V. trifolia* flowers, used as a tea, to alleviate asthma (Panthong *et al.* 1986).

On the island of Sumatra in Indonesia, people treat large wounds with a plant mixture containing bark from *V. trifolia*, *Lansium domesticum* Corr. Serr. and *Nephelium lappaceum* L. (Erdelen *et al.*1999). In Madagascar (although geographically near Africa, culturally proximate to other South or Southeast Asian cultures), an infusion of *V. trifolia* stems and leaves is taken before meals to relieve stomach pain (Boiteau and Allorge-Boiteau 2000). In other areas of the Indian Ocean, such as Mauritius and the Seychelles, *V. trifolia* is used to treat hypertension, rheumatism, and even as an antidote against toxic fish (Fakim

1990; Gurib-Fakim *et al.*1994, 1996, and 1997). *V. trifolia* is also used medicinally in Papua New Guinea (Sundarrao *et al.* 1993).

1.2.4 East Asia

Vitex has a long history of reported uses in Traditional Chinese Medicine (TCM), as well as in other East Asian traditional medicines. Traditional Chinese and Japanese medical uses of *V. trifolia* are often intertwined in the literature. For example, Kawazoe *et al.* (2001) report that the fruit of *V. trifolia* is used as folk medicine in both China and Japan for treating colds and inflammation. Kimura and Kimura (1981) report the fruit of *V. trifolia* used in Japan as treatments for headaches, colds, migraines and eye pain.

TCM incorporates *V. trifolia* berries for headaches, catarrh (inflammation of mucous membranes, especially of the nose and throat), watery eyes, and to enhance or initiate beard growth (Shih-Chen *et al.*1973). Most notable to this study is the reported use of *V. trifolia* berries for breast cancer (Shih-Chen *et al.*1973).

But (1996) has reported on the East Asian regional ethnobotany of *V. rotundifolia*. The seeds are reportedly made into a decoction taken orally for colds, headaches, migraine, sore eyes, night blindness, myalgia and neuralgia. The leaves are prescribed either orally or topically for headaches, traumatic injury and rheumatalgia. You *et al.* (1998) report that in Korea, *V. rotundifolia* fruits are sold at local markets for medicinal purposes. Shin *et al.* (2000) report that *V. rotundifolia* is used in Korea to treat headaches in upper respiratory

infections, and for treatment of various allergic diseases through various routes of administration.

1.2.5 Pacific Islands

Other than *V. trifolia* and *V. rotundifolia*, only one *Vitex* species appears to be used medicinally in the Pacific Islands. The Maori of New Zealand use their native tree *Vitex lucens* Kirk. to treat several ailments. Infusions from the boiled leaves are used to bathe sprains and backaches. This same infusion is also used to treat ulcers, particularly under the ear, and as a remedy for sore throats (Brooker *et al.*1987). The infusion was also used to wash the body of the deceased to help with preservation (Dykgraaf 1992). *V. lucens* trees are also associated with funerals and burial sites (Bursial & Sale 1984; Dykgraaf 1992).

Tongan traditional medicine incorporates *V. trifolia* to treat a variety of ailments including inflammation, ulcerated gums and teeth, teething problems, sores on the tongue, stomach, redness around a child's nose, constipation and supernatural ailments (Whistler 1992a; O'Rourke-George 1989). An infusion of the leaves is given to infants to treat mouth infections, and is sometimes taken as a potion to relieve stomachache. The leaves are also used to treat supernatural ailments (Whistler 1992b). The Marshallese, of the Republic of the Marshall Islands, use *V. trifolia* as a mosquito repellent (Merlin *et al.*1994). The Marshallese plant bushes and small trees of *V. trifolia* around their homes to ward off mosquitoes, and in addition, they burn the twigs and leaves, which create a smoke that further deters mosquitoes. Sometimes the flowers are used in ceremonial garlands.

Traditional medicine in the Cook Islands, specifically Rarotanga, includes *V. trifolia* (Holdsworth 1991, Whistler 1985). Whistler (1985) proposed that *V. trifolia* is either native or an aboriginally introduced shrub. *V. trifolia* is casually cultivated on Rarotanga, but rarely found in natural areas. It is used primarily for postpartum complications. Women drink an infusion of the boiled stems in order to ease postpartum pain, and bath in the infusion to remove stale blood. Holdsworth (1991) reports that the leaves are also used for postpartum bathing, and prescribed for a minimum of three nights.

On the island of Rotuma, *V. trifolia* is used to treat headaches (McClatchey 1993). For the treatment of headaches, the leaves of *V. trifolia* are rolled between the hands until well crushed. Then the exudate from the crushed leaves is squeezed under the nose with the head tipped back and a drop of the solution is allowed to enter the nostril. The aromatic properties of *V. trifolia*, similar to sage, are likely responsible for this traditional medical treatment (McClatchey 1993). In Fiji, *V. trifolia* leaves are used to treat stomach pains, gonorrhea, migraine headaches (similar to Rotuma, but also dripped into the ears), hemorrhoids, serious coughs, and serious wounds. Often, the vapor of steamed leaves is used as the main treatment. New shoots are used to remedy colds in children and the stem bark for fractured bones (Weiner 1984). On Futuna (toward the east), *V. trifolia* leaves are chewed with leaves from *Citrus sinensis* (orange tree) and held against a sore tooth to relieve pain (Biggs 1985). Samoan traditional medicine uses *V. trifolia* leaves for internal illness and

inflammation (Cox 1989, 1993). The burned leaves have also been used to repel mosquitoes, as in the Marshall Islands (Whistler 1992).

Traditional Hawaiian healers prescribe the crushed leaves of *V. rotundifolia* as a topical remedy for various skin ailments (Ohai 2004 pers. comm.). The crushed leaves are used as an anti-itch remedy and to heal rashes, as well as any other skin inflammatory condition. Voucher specimens of *V. rotundifolia* from the Bishop Museum Herbarium, Honolulu, Hawaii were examined and it was found that J.F. Rock had collected the earliest voucher in 1908 at Haleiwa Bay, Oahu. M. Neal (BISH voucher number 406030) noted *V. rotundifolia*, collected from Hana, Maui in 1933, was a medicinal plant, however did not mention specifics. A much more recent voucher collected by Evangaline Funk (BISH voucher number 920041) in 1992, mentioned that *V. rotundifolia* leaves are boiled as a solution used to sooth skin irritations, rashes, sunburn and chicken pox.

Conclusion

The ethnomedicinal uses of *Vitex* reported in the literature, in particular *V. trifolia* and *V. rotundifolia*, suggest that this genus has broad medical applications in traditional medicine, as well as for biomedical applications, i.e. cancer chemotherapy. The ethnomedicinal reports of *V. rotundifolia* are less than those of *V. trifolia*, possibly because *V. trifolia* has a much more broad geographic distribution. Often, the widespread use of a particular plant species or genus, such as *Vitex*, suggests either the medicinal claims have strong validity or that the broad geographical distribution, and therefore increased availability are

responsible for the medicinal usage. The broad spatial and temporal use of *Vitex* suggests that there are biologically active components distributed throughout the genus. Also, the fact that *Vitex* has been used by very old and persistent traditional medicines such as TCM, Ayurvedic, and ancient Greek medicine, provides significant support for continued research on the medicinal value of *Vitex*. **Table 1.4** provides a summary of the traditional medicinal uses of *Vitex* discussed in this paper. **Table 1.5** provides a list of common names applied to *Vitex* species, including country and indigenous group (if applicable).

Table 1.4. Summary of cited ethnomedicinal uses of *Vitex* species

Species	Treatment	Plant part(s) used	Source
V. agnus-castus	obstetric, dysmenorrheal, PMS	fruit	Foster 1998
V. gaumeri	malaria, colds, coughing	not specified	Hernandez *et al.*1999
V. lucens	ulcers, sprains, backache, sore throat	not specified	Brooker *et al.*1987
V. mollis	dysentery, scorpion stings, diarrhea, stomachache, analgesic	not specified	Argueta *et al.*1994; Bajpai *et al.*1995
V. negundo	snakebites, inflammation, obstetrics and gynecology, asthma, tumors, mosquito repellent	not specified	Alam and Gomes 2003; Chawla *et al.*1992; Dey 1980
V. peduncularis	febrifuge, chest pains	bark	Kirtikar and Basu 1980
V. polygamma	emmenagogue, diuretic	bark & fruit	Correa 1926
V. pubescens	gastrointestinal	not specified	Ahmad and Holdsworth 1995
V. pyramidata	gastrointestinal	not specified	Argueta *et al.*1994
V. rotundifolia	skin ailments, asthma, eyes, cold, allergies, inflammation, headache	fruit and leaf	Kawazoe *et al.* 2001; But 1996; You *et al.* 1998; Ohai 2004, pers. comm..
V. trifolia	mosquito repellent, eye problems, breast cancer, tuberculosis, fever, inflammation, asthma, postpartum pain	fruit and leaf	Ghani 1998; Shin-Chen *et al.* 1973; Panthong *et al.* 1986; Kimura and Kimura 1981; Whistler 1985

Table 1.5. Examples of common names of *Vitex* species

Vitex species	Country	Common name	Source
V. lucens	Maori New Zealand	*Puriri*	Dykgraaf 1992
V. rotundifolia	Hawaii	*Pohinahina / kolokolo kahakai*	Ohai 2004 Pers. comm.
V. rotundifolia	Japan	*Mankeishi*	Okuyama *et al.*1998a
V. rotundifolia	Korea	*Man Hyung ja*	You *et al.*1998
V. trifolia	Tradition Chinese Medicine	*Man-ching*	Shih-Chen *et al.*1973
V. trifolia	Bangladesh	*Nishcundi*	Hossain *et al.*2001
V. trifolia	Sumatra Indonesia	*Loban*	Erdelen *et al.*1999
V. trifolia	Cook Islands	*Rara*	Whistler 1985; Holdsworth 1991
V. trifolia	Tonga	*Lala tahi*	Whistler 1992a
V. trifolia	Fiji	*Dralakaka*	Weiner 1984
V. trifolia	Marshall Islands	*Utkonamnam*	Merlin *et al.* 1994
V. trifolia	Samoa	*Namulega*	Cox 1989
V. trifolia	Rotuma	*Sa'vao*	McClatchey 1993

The published data regarding the biological activity of *Vitex* is increasing, possibly due to a renaissance of ethnobotany, herbal medicine and botanical based natural products research. *V. agnus-castus* appears to have the most published data regarding biological activity as well as ethnobotany, perhaps followed by *V. negundo*, and then *V. trifolia* and *V. rotundifolia*. The following discussion focuses on the developing data regarding the biological activity of *V. trifolia* and *V. rotundifolia*. In particular, *V. rotundifolia* displays potential in the treatment of multiple ailments, of which most pertinent to this study is the potential as a novel chemotherapeutic agent for cancer.

1.3 Previously reported biological activity of *Vitex trifolia* and *Vitex rotundifolia*

Published data regarding *Vitex trifolia* is highly relevant to a study of *Vitex rotundifolia* because of their shared ethnobotany in areas such as East Asia, and linked taxonomic pasts. There is some overlap regarding the biological activity of *V. trifolia* and *V. rotundifolia*, just as their ethnobotany and botany seem to be intertwined.

There are twenty-seven reports specifically focusing on the biological activity of *Vitex rotundifolia*. These papers are reviewed in this discussion. Previous to this study, research on *V. rotundifolia* was mainly carried out in East Asia from research groups in Japan and Korea. This is likely due to the fact that *V. rotundifolia* is widely used as a medicinal plant in this region.

Most biological activity reports on *V. rotundifolia* involve some type of flavonoid. The term "flavonoid" defines a large group of polyphenolic compounds that occur ubiquitously in foods of plant origin. Flavonoids are among the most widely distributed natural products in plants with over 4000 compounds currently described; these occur in a free state or as glycosides (Hollman and Katan 1999; Robbers *et al.*1996). Flavonoids, like all phenylpropanoids, are derived from the shikimic acid pathway.

The second most commonly reported category of bioactive molecules from *V. rotundifolia* is that of diterpenes. The term "diterpenoids" describes a large group of C_{20} compounds derived from geranylgeranyl pyrophosphate

(Robbers *et al.*1996). There are over 20,000 terpenoids described, and these are by far the largest group of natural compounds (Robbers *et al.*1996).

Shinozaki (1921) published the earliest report on the chemical constituents of *Vitex rotundifolia*. Further reports do not appear until almost 50 years later (Kimura *et al.* 1967; Asaka *et al.* 1973; Hirotsu and Shimada 1973). The first chemical moieties isolated from *V. rotundifolia* include the flavone vitexicarpin (=casticin), and the diterpenes rotundifuran and prerotundifuran. The significance of these compounds will be elaborated in the following discussion.

1.3.1 Anti-cancer activity

Hernandez *et al.* (1999) performed a series of biological activity tests on extracts from *Vitex trifolia*. They produced three extracts of both *V. trifolia* stems and leaves with hexane, dichloromethane (DCM) and methanol. The first of their assays focused on the cytotoxic properties of *V. trifolia* on four human tumor cell lines: cervix carcinoma (SQC-1 UISO), ovarian cancer (OVCAR-5), colon cancer (HCT-15 COLADCAR), and nasopharyngeal carcinoma (KB). The hexane and DCM leaf extracts displayed the most interesting activity. The DCM leaf extract was the most active, demonstrated with an ED_{50} (effective dose, i.e., dose that causes 50% effect) less than 1 μg/ml toward the colon cancer cell line, which proved to be the most sensitive cell line tested in their study. Extracts with an ED_{50} of 20 μg/ml or less were considered active. Hernandez *et al.* (1999) suggest that the positive activity displayed by the DCM extract could be attributed to compounds in the most non-polar fraction. The same compounds could also be present in the hexane extract, which also displayed high cytotoxic properties.

Sundarrao *et al.* (1993) reported that *V. trifolia* extracts exhibited anti-tumor properties. The methanolic leaf extract of *V. trifolia* displayed significant anti-tumor activity against sarcoma cells (soft tissue). Kobayakawa *et al.* (2004) reported that several flavonoids isolated from methanolic extracts of *V. rotundifolia* fruits initiated G2-M arrest and anti-mitotic activity in KB cells (human epidermoid carcinoma cells) but not in 3T3 Swiss Albino or TIG-103 cells (normal mouse embryo and human fibroid cells). Several compounds were isolated and found to explain part of the activity. Casticin was found to exhibit the most activity (IC_{50} = 0.23 μM), compared to artemetin, quercetagetin and 3'5-dehydroxy-4',6,7-trimethoxyflavone (IC_{50} = 15.3 –18.6 μM).

Polymethoxyflavonoids isolated from methanolic extracts of *V. rotundifolia* fruit have reportedly inhibited growth of human myeloid leukemia HL-60 cells by induction of apoptosis (Ko *et al.* 2000). They isolated vitexicarpin (= casticin), artemetin and 2',3',5-trihydroxy-3,6,7-trimethoxyflavone. All three moieties inhibited the HL-60 cells in a dose-dependant manner. Ko *et al.* (2001) found that rotundifuran, a labdane-type diterpene, isolated from the fruit of *V. rotundifolia,* induced apoptosis in human myeloid leukemia HL-60 cells. The mechanism of action of rotundifuran remains to be elucidated.

Ko *et al.* (2002) later found that the flavonoid luteolin, also isolated from the fruit of *V. rotundifolia*, inhibited proliferation in human myeloid leukemia HL-60 cells by means of inducing apoptosis. They suggested that luteolin has strong potential applications as both a chemopreventive and chemotherapeutic agent.

However, the exact mechanism of action of how luteolin induced apoptosis was not reported.

You *et al.* (1998) found that vitexicarpin, isolated from the fruit, inhibits mouse lymphocyte proliferation. Using cells lines EL-4 (lymphoma, ATCC TIB 39), P815.9 (mastocytoma, ATCC TIB 64) and L929 (fibroblast, ATCC CCL 1), they found that vitexicarpin displayed inhibition at > 0.1 μM against concanavaline A (Con A) and lipopolysaccharide (LPS)-induced lymphocyte proliferation. This study did not report a mechanism of action, however, it proposed that the anti-proliferative activity of flavonoids in general, is dependent on a C-2, 3 double bond, and that the potency of inhibition is exclusively dependent on the number and position of hydroxylation(s). Further, they suggest that the inhibitory activity exhibited by vitexicarpin is likely a downstream mechanism of lymphocyte proliferation. When vitexicarpin was added at a later phase of lymphocyte growth, inhibition still occurred, unlike other similarly active flavonoids, which have only inhibited growth during the early phase of lymphocyte proliferation. This is significant pharmacologically with respect to autoimmune diseases, such as rheumatoid arthritis, because lymphocytes are already activated and proliferating by the time symptoms arrive and treatments are prescribed (You *et al.*1998). Vitexicarpin also appears to be selectively cytotoxic against specific lymphocyte cell lines, including those used in this study, but not towards other types of cell lines such as human keratinocytes. Such selectivity suggests that vitexicarpin is a promising candidate for novel treatment of lymphomas, autoimmune diseases and other similar ailments.

Sato (1989) found that extracts of *V. rotundifolia* inhibited the human cancer cell line JTC 26. Because this article is in Japanese, the methodology cannot be discussed in this paper. For details, see Sato (1989). *V. rotundifolia* has also displayed weak activity toward P388 leukemia cells (Suffness *et al.*1988).

1.3.2 Anti-microbial activity

The second and third assays carried out by Hernandez *et al.* (1999) focused on anti-microbial properties of *V. trifolia*, specifically, anti-fungal and anti-bacterial. Hexane extracts of dried leaves were prepared, followed by residual extractions using dichloromethane and then methanol. Five different fungi were employed for the anti-fungal assay: *Penicillium sp.*, *Aspergillus flavus*, *A. parasiticus*, *Tricoderma sp.*, and *Fusarium sp.* 100% growth inhibition of *Fusarium sp.* was observed with hexane leaf extracts, followed by 54% growth inhibition by the DCM leaf extract. No other significant anti-fungal activity was observed. The anti-bacterial assay employed six bacteria: *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Proteus mirabilis*, *Shigella sonei*, and *Salmonella typhi*. At 10 mg/ml each leaf extract (hexane, DCM and methanol) exhibited 100% growth inhibition against all bacterial cell lines, except against *S. typhi*, which only exhibited 50% growth inhibition. At 5 mg/ml, the DCM extract displayed the greatest anti-bacterial activity inhibiting all cell lines by 100%, except *S. typhi* by 50%, as just mentioned above. As extract concentrations decreased, anti-bacterial activity became more parallel between the three extracts. At 2.5 mg/ml all three extracts displayed 100% inhibition on

only two bacteria, *S. aureus* and *S. faecalis*, with the exception of the methanolic extract on *Streptococcus*, which only displayed 50% inhibition.

In Bangladesh, Hossain *et al.* (2001) investigated the anti-microbial properties of *V. trifolia* leaves. Two extractions were prepared: 1) a petroleum ether extract, and 2) an ethanol extract. They then used a disc diffusion technique to test activity against five Gram-positive and fourteen Gram-negative bacteria. They reported that both extracts were moderately active against the majority of both the Gram positive and negative bacteria. They suggested that the antibacterial activity of *V. trifolia* leaves could provide support to a variety of traditional uses.

In Papua New Guinea, Sundarrao *et al.* (1993) investigated *V. trifolia* along with 23 other native medicinal plants for anti-bacterial properties. All plants were extracted with methanol. A methanolic leaf extract of *V. trifolia* was tested against several bacteria, including both Gram positive and Gram negative. Significant growth inhibition was observed in the Gram-positive bacteria, *Staphylococcus albus* and *Bacillus subtilus*.

Bae *et al.* (1998) tested a large number of herbal medicines obtained from a medicinal herb store in Seoul, Korea against *Helicobacter pylori*. *V. rotundifolia* was among the traditional herbs tested. A water extract of *V. rotundifolia* was found to inhibit *H. pylori* growth with a minimum inhibitory concentration (MIC) of 1-2 mg/ml. No mechanism of action was reported in this study. *V. rotundifolia* was also found to be active against *Streptococcus mutans*, the pathogen responsible for dental caries (Chen *et al.*1989). Similar to *V. trifolia*, *V.*

rotundifolia has demonstrated significant anti-bacterial activity towards *Staphylococcus aureus* (Kawazoe *et al.* 2001). Kawazoe *et al.* (2001) report the first biological activity observed from the roots of *V. rotundifolia*. Methanolic extracts of the roots inhibited growth of methicillin resistant *Staphylococcus aureus* (MRSA). Several novel phenylnaphthalene compounds were reported in this study, and were all found to inhibited MRSA growth.

1.3.3 Anti-asthma activity

Ikawati *et al.* (2001) identified *V. trifolia* as displaying promising activity as an anti-asthmatic remedy. They found that both n-hexane and residual ethanol extracts of *V. trifolia* leaves inhibited mast-cell degranulation by over 80%. They also found that *V. trifolia* had both bronchospasmolytic activity and an inhibitory effect on histamine release. With this dual action, *V. trifolia* leaves may prove to be a highly significant asthma therapy.

In attempt to determine the responsible constituents for *V. trifolia*'s anti-asthmatic activity, Alam *et al.* (2002) performed liquid-liquid partitioning of a n-hexane extract of *V. trifolia* leaves and then tested the resulting fractions in two separate guinea pig trachea *in vitro* assays. The first assay tested the fractions for their ability to inhibit histamine induced spontaneous contractions in isolated guinea pig trachea. The second assay tested the fractions for their ability to inhibit contractions in ovalbumin sensitized isolated guinea pig trachea. They found the responsible constituents to be viteosin-A and vitexicarpin. Both were active in the spontaneous contraction assay, however, only vitexicarpin was active in the ovalbumin-sensitized assay. They also reported that both viteosin-A

and vitexicarpin inhibited the tracheal contractions in a dose dependant manner, and that their mechanism of action is a non-competitive antagonism to histamine. This mechanism is different from diphenhydramine and other common commercial anti-histamines, which are competitive antagonists to histamine.

1.3.4 Anti-allergy activity

Using rat basophilic leukemia (RBL-2H3) cells, Kataoka and Takagaki (1995) found that aqueous extracts of *V. rotundifolia* fruit inhibited β-hexosaminidase release from RBL-H3 cells. β-hexosaminidase is a chemical mediator released from cells by the biotinyl IgE-avidin complex. This report suggests that *V. rotundifolia* has an anti-allergy property.

Bae *et al.* (2000) tested 54 Korean herbal medicines for induction of IgA in primary Peyer's patches cells (lymphoid follicles containing white blood cells) in search for novel treatments of food allergies. They found that extracts of *V. rotundifolia* displayed moderate activity in this assay. Several other reports on the biological activity of *V. rotundifolia* include anti-inflammatory activity (i.e., Han *et al.*1972), which is noteworthy due to the widespread traditional use of *Vitex* species for the treatment of inflammation related ailments (See **Table 1.4**).

1.3.5 Smooth muscle contraction inhibition

A broad screening of the Samoan ethnopharmacopoeia by Cox *et al.* (1989) included *V. trifolia*. Ethanolic crude extracts of *V. trifolia* leaves displayed inhibitory activity in an *in vitro* assay measuring the ability of extracts to induce or inhibit electrically induced guinea pig ileum contractions. No mechanisms were reported in this study.

1.3.6 Vascular activity

Okuyama *et al.* (1998a) observed that the methanolic extracts of the fruit from *V. rotundifolia* produced a vascular relaxant effect in rat aortic strips. In a follow up study, Okuyama *et al.* (1998b) found that the responsible constituents include vitexfolin A, 10-O-vanilloylaucubin, vanilloy-β-D-(2'-O-*p*-hydroxybenzoyl) glucoside together with agnuside, and dihydrodehydrodiconiferylalcohol-β-D-(2'-O-*p*-hydroxybenzoyl) glucoside. No mechanism was reported in this study.

1.3.7 Anti-viral activity

Kim *et al.* (2000) claims that a traditional Korean herbal preparation made from the fruit of *V. rotundifolia* can mildly inhibit the rotavirus. The rotavirus is responsible for over 50% of infant and childhood diarrhea around the globe, and is one of the leading causes of infant and child death, worldwide. In this study, 50µl of 10^{-3} diluted WA virus (a wild type of human rotavirus, $1x10^3$ pfu) was used to infect 100µl of MA104 cells (Macaccus Rhesus monkey kidney cells, $3x10^5$ cells/ml) prior to addition of 50µl *V. rotundifolia* herbal preparation. This study used a $TCID_{50}$ (50% tissue culture infectious dose) of $1.27x10^6$ $TCID_{50}$/ml and plaque forming unit (pfu) of WA virus of $8.8x10^5$ pfu/ml. The fruit of *V. rotundifolia* was found to inhibit rotavirus infection by 25%. This study is extremely vague and focuses much more on several other plants, which were more active than *V. rotundifolia*.

Zheng (1988) found that aqueous extracts of *V. rotundifolia* moderately inhibited Herpes Simplex Virus 1 (HSV-1) infection in muscle-skin monolayer cells of human embryo. Cell cultures were exposed to HSV-1 one hour before

addition of the *V. rotundifolia* extract. Assay concentration of the extract appears to be 10 mg/ml, however this value seems high. The LVI (logarithm of virus inhibition) against HSV-1 was between 3 and 4, which was considered moderately effective against HSV-1, compared to a LVI between 2 and 3 as low effectiveness, and a LVI above 4 as highly effective. In a follow up study, Minshi (1989) found that *V. rotundifolia* was also effective at inhibiting HSV-2, but with a LVI between 2 and 3, which was evaluated using the same scale as Zheng et al. (1988).

1.3.8 Inhibition of cataract formation

Cataracts are a significant cause of blindness in diabetic and elderly people and there are limited treatments for this physical ailment. The sorbitol pathway, an accessory pathway to glucose metabolism, is known to play a fundamental role in cataract formation. The sorbitol pathway is primarily composed of two enzymes: aldose reductase (which reduces glucose to sorbitol), and polyol dehydrogenase (which oxidizes sorbitol to fructose). When the lens is exposed to high concentrations of glucose, it penetrates the lens and is partly converted to sorbitol, which is partly impermeable. As the impermeable sorbitol accumulates, it raises the cytoplasmic osmolarity, which draws in water causing the lens to swell, which then ultimately opacifies. Chiou *et al.* (1992) found that the TCM tablet ZYM (Zhang-Yan-Ming), which contains *V. rotundifolia*, among eleven other herbs, significantly inhibits sorbitol formation in rabbit lenses. Chiou *et al.* (1992) suggest that ZYM can become a safe and effective popular treatment for cataracts, if administered as eye drops.

1.3.9 Mosquito deterrent activity

Watanabe *et al.* (1995) isolated rotundial, a cyclopentene dialdehyde, from an extract of fresh *V. rotundifolia* leaves. It was found to function as a potent mosquito repellent, particularly against *Aedes aegypti.* This is particularly noteworthy due to the fact that multiple ethnobotanical reports include the use of *Vitex* leaves as insect and mosquito repellent, including reports from Tonga and the Marshall Islands (See section 1.2).

1.3.10 Anti-mutagenic activity

Miyazawa *et al.* (1995) observed anti-mutagenic activity from (+)-polyalthic acid, isolated from a methanolic extract of commercially available *V. rotundifolia* dry powder.

1.3.11 Anti-inflammatory activity

V. rotundifolia has also been shown to be generally anti-inflammatory (Han *et al.*1972) and more specifically to be an inhibitor of cyclooxygenase (Min *et al.* 1996). Anti-inflammatory actions could account for some of the traditional uses and assist in the treatment of cancer.

Conclusion

The list of biologically active properties of *V. rotundifolia* provides support for its medicinal potential, particularly for the treatment of cancer. Traditional medicinal uses inspired most of the biological activity studies reported in this paper. The collective reports display the potential for traditional medicines. Some noticeable reoccurring traditional treatments with corresponding biological activities include cancer, asthma, inflammation, insect deterrent, and treatment of

female reproductive ailments. A summary of the biological activities of *V. rotundifolia* discussed in section 1.3 is provided below in **Table 1.6**. **Table 1.7** provides data published regarding the lack of biological activities exhibited by extracts from *V. rotundifolia*. **Table 1.8** provides some examples of various *Vitex* species and their reported biological activities.

Table 1.6. Reports on the biological inactivity of *V. rotundifolia*
(retrieved from NAPRALERT database, January, 31 2005, University of Illinois, Chicago)

Activity	Plant part(s)	Source
Aldose reductase inhibition	fruit	Shin *et al.*1994
Analgesic	fruit	Okuyama *et al.*1998a
Inhibition of cataract formation	?	Chiou *et al.*1992
Anti-*Helicobacter pylori*	?	Bae *et al.*1998
Anti-HSV-1/HSV-2	?	Zheng 1988; Minshi 1989
Anti-leukemia (P388)	?	Suffness *et al.*1988
Anti-microbial	?	Chen *et al.*1987
Anti-mutagenic	dried powder (?)	Miyazawa *et al.*1995
Anti-oxidant	?	Kim *et al.*1994
Anti-rotavirus	?	Kim *et al.*2000
Anti-*Staphylococcus*	root	Kawazoe *et al.* 2001
Anti-*Streptococcus*	?	Chen *et al.*1989
Anti-tumor	?	Sato 1989
Apoptosis in human leukemia cells	fruit	Ko *et al.* 2000; Ko *et al.* 2001
ß-hexosaminidase inhibition	fruit	Kataoka and Takagaki 1995
G2-M arrest anti-mitotic	fruit	Kobayakawa *et al.* 2004
Induction of IgA	?	Bae *et al.* 2000
Inhibition of cyclooxygenase	?	Min *et al.* 1996
Inhibition of histamine release	?	Shin *et al.*2000
Inhibition of ileum contraction	?	Itokawa *et al.*1983
Inhibition of lymphocyte proliferation	fruit	You *et al.*1998
Inhibition of proliferation of leukemia cells	fruit	Ko *et al.* 2002
Mosquito repellent	leaf	Watanabe *et al.*1995
Oxytocic and anticholinergic	?	Lee and Lee 1991
Vascular relaxant	fruit	Okuyama *et al.*1998a

Table 1.7. Reports on the null biological activity of *V. rotundifolia*
(NAPRALERT database, January 31, 2005)

Acetylcholinesterase inhibition	Lee *et al.*1997
Analgesic effects	Chow *et al.*1976
Anti-*Bordetella*, -*E. coli*, -*Pseudomonas*, -*Salmonella*	Chen *et al.*1987
Anti-neoplastic activity	Park *et al.*1993
Anti-tumor	Suffness *et al.*1988
Anti-tumor activity	Itokawa *et al.*1982
Anti-mutagenic	Ishii *et al.*1984
Cell-mediated immunity activity	Kuo *et al.*1995
Hepatoprotective activity	Lee *et al.*1992
Inhibition of cyclooxygenase/lipooxygenase	You *et al.*1999
Inhibition of dopa oxidase activity of tyrosinase	Shin *et al.*1997
Inhibition of glutamate-pyruvate-transaminase	Lee *et al.*1992
Inhibition of hepatitis B virus DNA replication	Nam *et al.*1996
Promotion of hair growth	Kubo *et al.*1988
Promotion of hair growth	Tanaka *et al.*1980
Protein kinase C receptor binding	An *et al.*1997
Urease inhibition	Bae *et al.*1998

Table 1.8. ISI data on the biological activity of the genus *Vitex* (except *V. rotundifolia*)

Vitex species	Biological activity	Plant part	Source
Vitex agnus-castus	estrogen receptor, β selective		Jarry *et al.* 2003, Liu *et al.* 2004
	cytotoxic/apoptotic	fruit	Ohyama *et al.* 2003
	PMS	fruit	Schellenberg 2001
	cyclical mastalgia		Halasaki 1999
	luteal-phase defects		Milewicz *et al.* 1993
	inhibit prolactin secretion		Sliutz *et al.* 1993
	anti-microbial		Kustrak *et al.* 1987
Vitex doniana	anti-hepatotoxic	bark	Ledeji *et al.* 1996b
	blood pressure	bark	Ledeji *et al.* 1996a
	termite deterrent		Epila *et al.*1988
Vitex negundo	mosquito repellent	leaves	Hebbalkar *et al.* 1992
	biocidal	leaves	Kaushik *et al.* 2003
	male reproductive (fertility enhancement)	seeds	Das *et al.* 2004
	analgesic/anti-inflammatory	leaves	Dharmasiri *et al.* 2003
	cytotoxic	leaves	Diaz *et al.* 2003
	anti-venom	root	Alam & Gomes 2003
	anti-inflammatory		Gaidhani *et al.* 2002
	anti-androgenic	seeds	Bhargava 1989
Vitex peduncularis	anti-inflammatory		Suksamrarn *et al.* 2002
Vitex polygama	anti-viral		Goncalves *et al.* 2001
Vitex trifolia	anti-bacteria		Hossain *et al.* 2001

1.4 Chemical moieties previously isolated from *V. rotundifolia*

Several types of compounds have previously been isolated from *V. rotundifolia*, mainly flavonoids, various mono- and diterpenes, glucosides, phenylpropanoids and lignans. The flavonoids casticin and luteolin are the most significant to the study, as they both demonstrate potent anti-proliferative properties in multiple cell lines, as discussed in section 1.3. **Table 1.9** is an attempt at a comprehensive list of all known chemical constituents thus far isolated from *V. rotundifolia*. The fruit is, by far, the most studied anatomical part of *V. rotundifolia*, followed by the leaves. A few studies have reported isolates from the seed and root (Asaka *et al.* 1973; Kawazoe *et al.* 1999; Kondo *et al.* 1986). **Figure 1.1** through **Figure 1.9** provide the corresponding structures to the isolates listed in **Table 1.9**.

Table 1.9. Compounds previously isolated from *V. rotundifolia*

Compound Isolated	Plant Part	Class	Source
(rel 5*S*,6*R*,8*R*,9*R*,10*S*)-6-Acetoxy-9-hydroxy-13(14)-labdane-16,15-olide	fruit	diterpene	Ono *et al.* 2001
(rel 5*S*,6*R*,8*R*,9*R*,10*S*)-9-Acetoxy-9-hydroxy-15-methoxy-13-(14)-labden-16,15-olide	fruit	diterpene	Ono *et al.* 2001
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*R*)-6-Acetoxy-9,13-epoxy-15-methoxy-labdan-15,16-olide	fruit	diterpene	Ono *et al.* 2001
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*R*,15*R*)-6-Acetoxy-9,13;15,16-diepoxy-15-methoxylabdane	fruit	diterpene	Ono *et al.* 1999
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*R*,15*S*)-6-Acetoxy-9,13;15,16-diepoxy-15-methoxylabdane	fruit	diterpene	Ono *et al.* 1999
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*R*,16*S*)-6-Acetoxy-9,13-epoxy-16-methoxy-labdan-15,16-olide	fruit	diterpene	Ono *et al.* 2001
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*)-6-Acetoxy-9,13-epoxy-15-methoxy-labdan-15,16-olide	fruit	diterpene	Ono *et al.* 2001
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*R*)-6-Acetoxy-9,13;15,16-diepoxy-15-methoxylabdane	fruit	diterpene	Ono *et al.* 1999
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*R*, 16*R*)-6-Acetoxy-9,13;15,16-diepoxy-15,16-dimethoxylabdane	fruit	diterpene	Ono *et al.* 1999
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*S*)-6-Acetoxy-9,13;15,16-diepoxy-15-methoxylabdane	fruit	diterpene	Ono *et al.* 1999
(rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,16*S*)-6-Acetoxy-9,13-epoxy-16-methoxy-labdan-15,16-olide	fruit	diterpene	Ono *et al.* 2001
(rel 5*S*,6*S*,8*R*,9*R*,10*S*)-6-Acetoxy-9-hydroxy-13(14)-labdane-16,15-olide	fruit	diterpene	Ono *et al.* 2001
(rel 5*S*,8*R*,9*R*,10*S*,13*S*,15*R*,16*R*)-9,13;15,16-Diepoxy-15,16-dimethoxy-labdane	fruit	diterpene	Ono *et al.* 2001

Table 1.9. (Continued) Compounds previously isolated from *V. rotundifolia*

Compound Isolated	Plant Part	Class	Source
(rel 5*S*,8*R*,9*R*,10*S*,13*S*,15*R*,16*S*)-9,13;15,16-Diepoxy-15,16-dimethoxy-labdane	fruit	diterpene	Ono *et al.* 2001
(rel 5*S*,8*R*,9*R*,10*S*,13*S*,15*S*,16*R*)-9,13;15,16-Diepoxy-15,16-dimethoxy-labdane	fruit	diterpene	Ono *et al.* 2001
(rel5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*R*,16*S*) -6-Acetoxy-9,13;15,16-diepoxy-15,16-dimethoxylabdane	fruit	diterpene	Ono *et al.* 1999
(rel5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*S*,16*R*) -6-Acetoxy-9,13;15,16-diepoxy-15,16-dimethoxylabdane	fruit	diterpene	Ono *et al.* 1999
(rel5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*S*,16*S*) -6-Acetoxy-9,13;15,16-diepoxy-15,16-dimethoxylabdane	fruit	diterpene	Ono *et al.* 1999
5,3'-dihydroxy-6,7,4'-trimethoxyflavanone	fruit	flavonoid	Kobayakawa *et al.* 2004
Abieta-9(11)-12(13)-di-alpha-epoxide	fruit	diterpene	Sakurai *et al.* 1999
Abieta-9(11)-12-diene	fruit	diterpene	Sakurai *et al.* 1999
Abietatrien-3-β-ol	fruit	diterpene	Ono *et al.* 1999
Agnuside	fruit, leaf	iridoid	Koundo *et al.* 1988
Artemetin	seed, fruit	flavonoid	Kobayakawa *et al.* 2004
Casticin (vitexicarpin)	fruit, leaf	flavonoid	Kobayakawa *et al.* 2004
Dihydrodehydrodiconiferylalcohol-β-D-(2'-O-*p* hydroxybenzoyl) glucoside	fruit	glucoside	Okuyama *et al.* 1998
Erythroguaiacylglycerol	fruit	phenyl-propanoid	Okuyama *et al.* 1998
Eucommiol	fruit	iridoid	Ono *et al.* 1997
1-oxo-eucommiol	fruit	iridoid	Ono *et al.* 1997
Eurostoside	leaf	iridoid	Koundo *et al.* 1988
Eurostoside, *cis*	leaf	iridoid	Koundo *et al.* 1988

Table 1.9. (Continued) Compounds previously isolated from *V. rotundifolia*

Compound Isolated	Plant Part	Class	Source
Ferruginol	fruit	diterpene	Ono *et al.* 1999
p-Hydroxybenzioc acid	fruit	phenolic acid	Kondo *et al.* 1986
Iridolactone	fruit	iridoid	Ono *et al.* 1997
Iso-ambreinolide	fruit	diterpene	Ono *et al.* 2002
Luteolin	fruit	flavonoid	Ko *et al.* 2002
Pedicularis-lactone	fruit	iridoid	Ono *et al.* 1997
Penduletin		flavonoid	Okuyama *et al.* 1998b
(+)-Polyalthic acid	entire plant	diterpene	Miyazawa *et al.* 1995
Prerotundifuran	leaf	diterpene	Asaka *et al.* 1973
Previtexilactone	fruit	diterpene	Kondo *et al.* 1986
Quercetagetin	Fruit	flavonoid	Kobayakawa *et al.* 2004
Rotundial	leaf	cyclopentene dialdehyde	Watanabe *et al.* 1995
Rotundifuran	leaf and seed	diterpene	Asaka *et al.* 1973
Threoguaiacylglycerol	fruit	phenyl-propanoid	Okuyama *et al.* 1998
Trisnor-γ-lactone	fruit	diterpene	Ono *et al.* 2002
Vanillic acid	fruit	phenolic acid	Kondo *et al.* 1986
10-O-vanilloylaucubin	fruit	iridoid	Ono *et al.* 1997
Vanilloyl-β-D-(2'-O-*p*-hydroxybenzoyl) glucoside	fruit	glucoside	Okuyama *et al.* 1998
Viteoid I	fruit	iridoid	Ono *et al.* 1997
Viteoid II	fruit	iridoid	Ono *et al.* 1997
Viteoside A	fruit	diterpene	Ono *et al.* 1998
Vitetrifolin D	fruit	diterpene	Ono *et al.* 2002
Vitex lignan 7	root	lignan	Kawazoe *et al.* 2001

Table 1.9. (Continued) Compounds previously isolated from *V. rotundifolia*

Compound Isolated	Plant Part	Class	Source
Vitex lignan 8	root	lignan	Kawazoe *et al.* 2001
Vitexfolin A	fruit	phenolic β-D-glucoside	Okuyama *et al.* 1998
Vitexfolin B	fruit	phenolic β-D-glucoside	Okuyama *et al.* 1998
Vitexfolin C	fruit	phenolic β-D-glucoside	Okuyama *et al.* 1998
Vitexifolin A	fruit	diterpene	Ono *et al.* 2002
Vitexifolin B	fruit	diterpene	Ono *et al.* 2002
Vitexifolin C	fruit	diterpene	Ono *et al.* 2002
Vitexifolin D	fruit	diterpene	Ono *et al.* 2002
Vitexifolin E	fruit	diterpene	Ono *et al.* 2002
Vitexilactone	fruit	diterpene	Kondo *et al.* 1986
Vitrofolal A	root	lignan	Kawazoe *et al.* 1999
Vitrofolal B	root	lignan	Kawazoe *et al.* 1999
Vitrofolal C	root	lignan	Kawazoe *et al.* 1999
Vitrofolal D	root	lignan	Kawazoe *et al.* 2001
Vitrofolal E	root	lignan	Kawazoe *et al.* 2001
Vitrofolal F	root	lignan	Kawazoe *et al.* 2001

1.4.1 Flavonoids

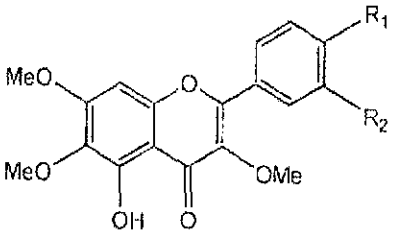
Both vitexicarpin and luteolin, isolated from *V. rotundifolia* (compounds **1** and **5**, respectively, in **Figure 1.1**), have demonstrated anti-proliferative properties. Vitexicarpin (casticin) has proven effective against multiple cell lines, including human epidermoid carcinoma lines KB and A431, human myeloid leukemia HL-60 cells, T-lymphocytes, B-lymphocytes, EI-4 lymphoma cells, and P815.9 mastocytoma cells (Ko *et al.* 2000; You *et al.* 1998). Luteolin and artemetin have both inhibited growth of human myeloid leukemia HL-60 cells, while luteolin also inhibits growth of human melanoma HMB-2 cells (Ko *et al.* 2002; Horvathova *et al.* 2005). Quercetin, not isolated from *V. rotundifolia*, but very similar to luteolin, (compound **6**, Figure 1.1) also inhibits human melanoma HMB-2 cells (Horvathova *et al.* 2005).

Both traditional claims and biological activity studies report *V. rotundifolia* has anti-allergy and anti-asthma properties. Thus, it is noteworthy that luteolin is very similar in structure to quercetin, which is proven to be effective in the treatment of allergies and asthma (Miller 2002; White and Pearce 1982).

1.4.2 Glucosides

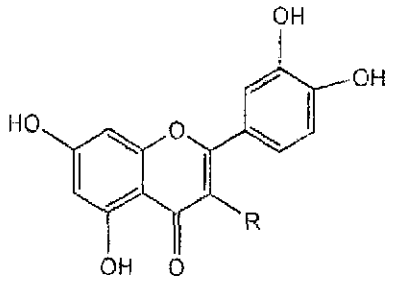
Several glucosides have been isolated from *V. rotundifolia* (**Figure 1.2**). Both vitexifolin A (compound **2**) and dihydrodehydrodiconiferylalcohol-β-D-(2'-O-p-hydroxybenzoyl) glucoside (compound **1**) exhibit analgesic effects (Okuyama *et al.* 1998b). Thus far, the glucosides have not exhibited significant biological activity according to the literature reviewed in this study. This could, however, be due to lack of investigation.

Figure 1.1. Flavonoids previously isolated from *V. rotundifolia*



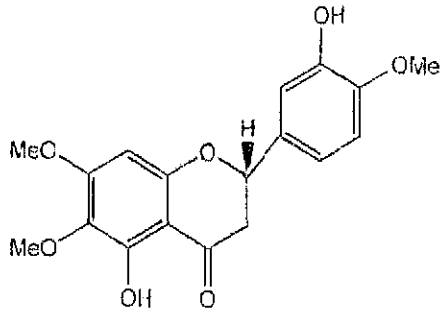
	R_1	R_2
1 vitexicarpin (casticin)	OMe	OH
2 artemetin	OMe	OMe
3 quercetagetin	OH	OH
4 penduletin	OH	H

(Kobayakawa *et al.* 2004)



	R_1
5 luteolin	H
6 quercetin	OH

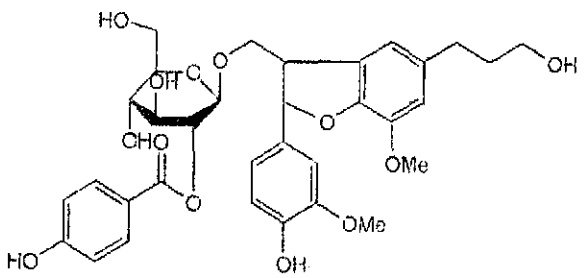
(Ko *et al.* 2002)



7 3',5-dihydroxy-4',6,7-trimethoxyflavanone

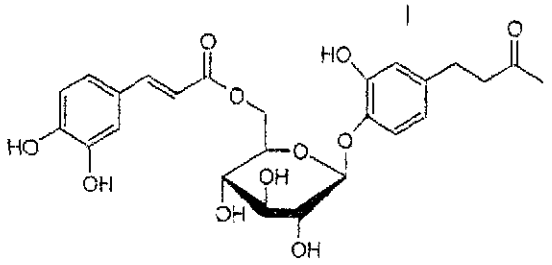
(Okuyama *et al.* 1998)

Figure 1.2. Glucosides previously isolated from *V. rotundifolia*



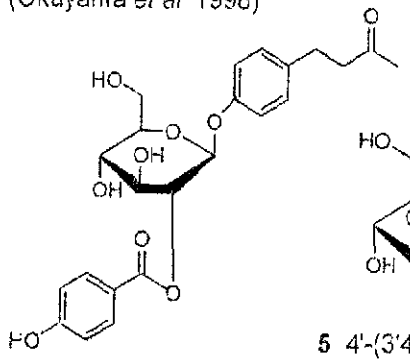
1 dihydrodehydrodiconiferylalcohol-β-D-(2'-O-p-hydrobenzoyl)glucoside

(Okuyama *et al* 1998)



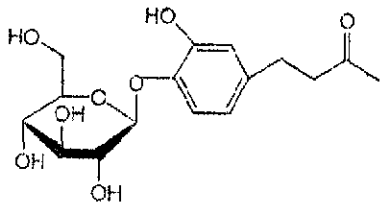
4 Vitexfolin C

(Okuyama *et al.* 1998)



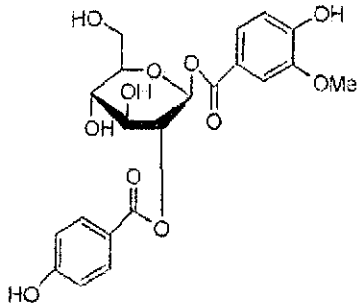
2 Vitexfolin A (7'',8''-trans)
3 Vitexfolin B (7'',8''-cis)

(Okuyama *et al.* 1998)



5 4'-(3'4'-dihydroxyphenol)-butan-one-4'-O-β-D-glucoside

(Koundc *et al.* 1988)



6 Vaniloyl-β-D-(2'-O-p-hydroxybenzoy)-glucoside

(Okuyama *et al.* 1998)

1.4.3 Lignins

Kawazoe *et al.* (1999, 2001) isolated several aryl- and phenylnaphthalene lignins from *V. rotundifolia* (above in **Figure 1.3**). Kawazoe *et al.* 2001 found that lignins **3**, **4**, and **9** exhibited anti-bacterial activity against 8 out of 18 methicillin-resistant *Staphylococcus aureus* (MRSA) strains, a problematic pathogen, particularly in nosocomial infections.

1.4.4 Diterpenes

Diterpenes are by far the most abundant reported compounds isolated from *V. rotundifolia*. Ono *et al.* (1998, 1999, 2001, 2002) have reported the majority of diterpenes thus far isolated from *V. rotundifolia*. Two of these diterpenes have displayed anti-cancer properties. Rotundifuran, compound **4** in **Figure 1.7**, induces apoptosis in human leukemia HL-60 cells (Ko *et al.* 2001), and trisnor-γ-lactone, compound **7** in **Figure 1.6**, has exhibited anti-cancer properties, using human lung cancer cells PC-12 and human colon cancer cells HCT116 in a MTT assay (Ono *et al.* 2002). Ono *et al.* (1999) report that several of the diterpenes, mainly ferruginol (compound **9**), abietatrien-3-β-ol (compound **10**), and vitetrifolin D (compound **6**) from **Figure 1.6** have exhibited strong antioxidant properties.

Diterpenes numbered 1 through 8 in **Figure 1.4** correspond to the following molecules listed in **Table 1.9**: **1)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*R*,15*R*)-6-Acetoxy-9,13;15,16-diepoxy-15-methoxylabdane,
2) (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*R*,15*S*)-6-Acetoxy-9,13;15,16-diepoxy-15-methoxylabdane, **3)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*S*)-6-Acetoxy-9,13;15,16-

diepoxy-15-methoxylabdane, **4)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*R*)-6-Acetoxy-9,13;15,16-diepoxy-15-methoxylabdane, **5)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*S*,16*R*)-6-Acetoxy-9,13;15,16-diepoxy-15,16-dimethoxylabdane,

6) (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*R*,16*R*)-6-Acetoxy-9,13;15,16-diepoxy-15,16-dimethoxylabdane, **7)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*S*,16*S*)-6-Acetoxy-9,13;15,16-diepoxy-15,16-dimethoxylabdane,

8) (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*R*,16*S*)-6-Acetoxy-9,13;15,16-diepoxy-15,16-dimethoxylabdane. Diterpenes numbered 1 through 10 in **Figure 1.5** correspond to the following molecules: **1)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*)-6-Acetoxy-9-hydroxy-13(14)-labdane-16,15-olide, **2)** (rel 5*S*,6*S*,8*R*,9*R*,10*S*)-6-Acetoxy-9-hydroxy-13(14)-labdane-16,15-olide, **3)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*)-9-Acetoxy-9-hydroxy-15-methoxy-13-(14)-labdane-16,15-olide,

4) (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,16*S*)-6-Acetoxy-9,13-epoxy-16-methoxy-labdane-15,16-olide, **5)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*R*,16*S*)-6-Acetoxy-9,13-epoxy-16-methoxy-labdan-15,16-olide, **6)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*)-6-Acetoxy-9,13-epoxy-15-methoxy-labdan-15,16-olide, **7)** (rel 5*S*,6*R*,8*R*,9*R*,10*S*,13*R*)-6-Acetoxy-9,13-epoxy-15-methoxy-labdan-15,16-olide, **8)** (rel 5*S*,8*R*,9*R*,10*S*,13*S*,15*S*,16*R*)-9,13;15,16-Diepoxy-15,16-dimethoxy-labdane,

9) (rel 5*S*,8*R*,9*R*,10*S*,13*S*,15*R*,16*S*)-9,13;15,16-Diepoxy-15,16-dimethoxy-labdane, **10)** (rel 5*S*,8*R*,9*R*,10*S*,13*S*,15*R*,16*R*)-9,13;15,16-Diepoxy-15,16-dimethoxy-labdane. The diterpenes numbered 1 through 8 in **Figure 1.6** correspond to the following molecules listed in Table 1.9: **1)** vitexifolin A,

2) vitexifolin B, **3)** vitexifolin C, **4)** vitexifolin D, **5)** vitexifolin E, **6)** vitetrifolin D, **7)** trisnor-γ-lactone, **8)** iso-ambreinolide, **9)** ferruginol, **10)** abietatrien-3β-ol.

Figure 1.3. Lignins previously isolated from *V. rotundifolia*

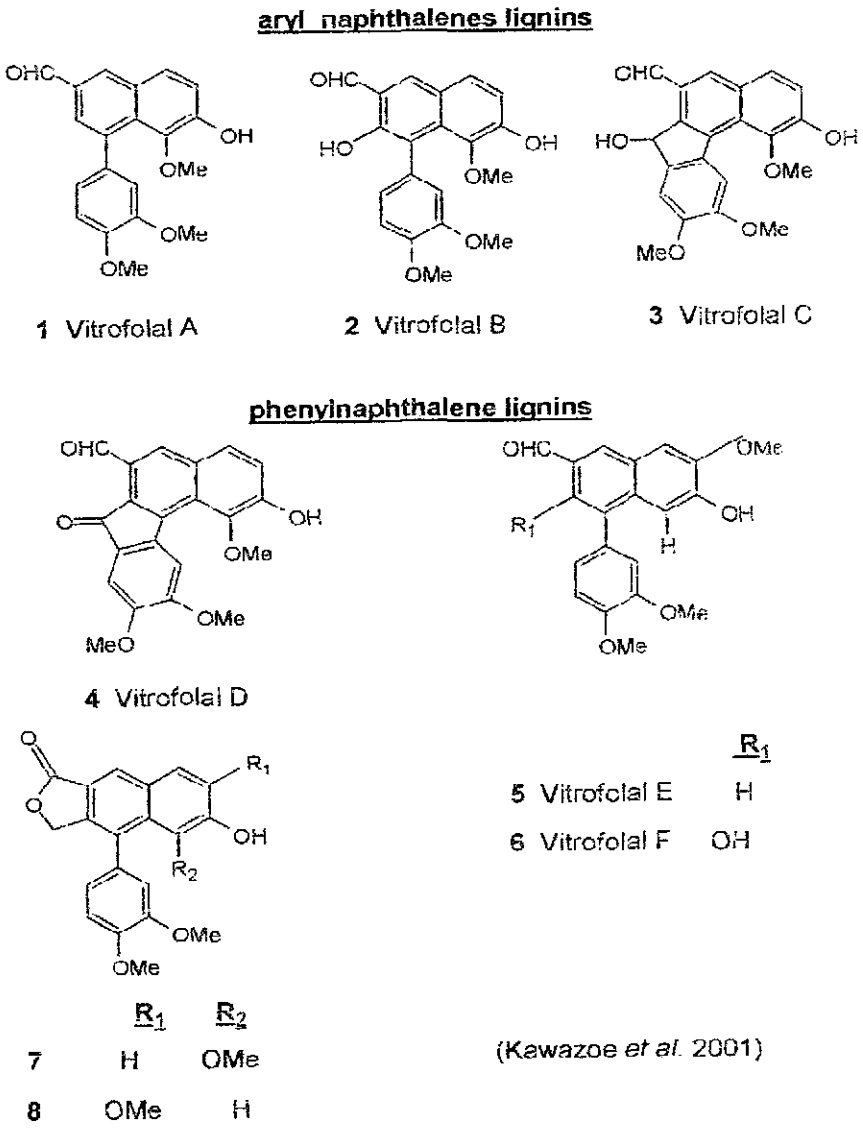
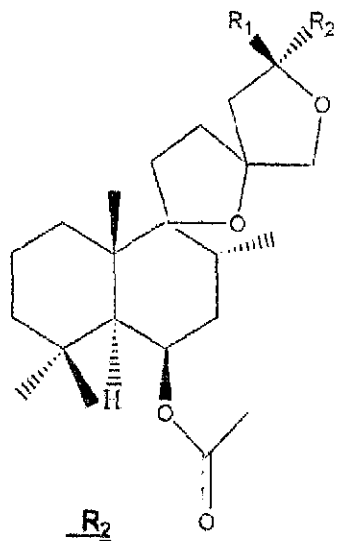
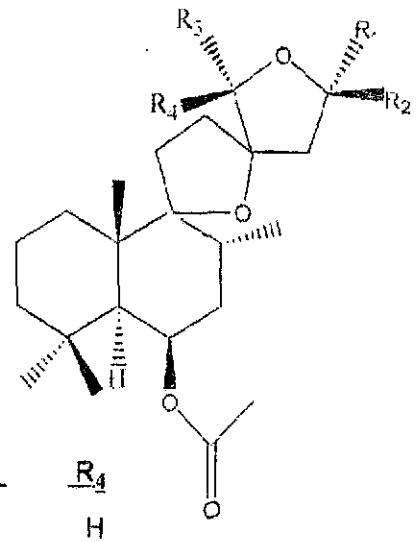


Figure 1.4. Diterpenes I: Previously isolated from *V. rotundifolia*



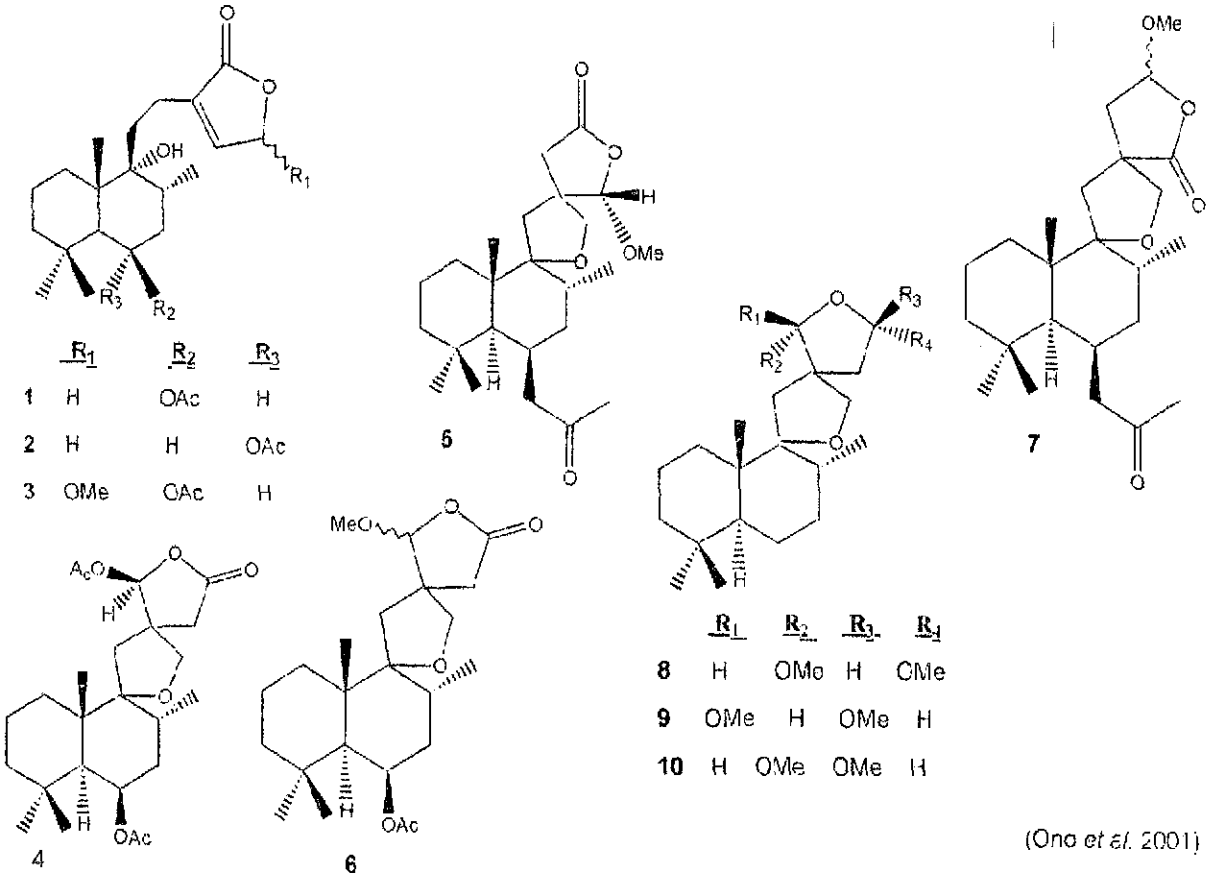
	R_1	R_2
1	OCH_3	H
2	H	OCH_3



	R_1	R_2	R_3	R_4
3	OCH_3	H	H	H
4	H	OCH_3	H	H
5	OCH_3	H	OCH_3	H
6	H	OCH_3	OCH_3	H
7	OCH_3	H	H	OCH_3
8	H	OCH_3	H	OCH_3

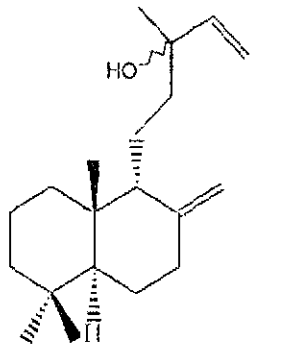
(Ono *et al.* 1999)

Figure 1.5. Diterpenes II: Previously isolated from *V. rotundifolia*

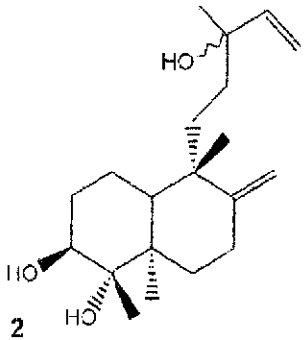


(Ono *et al.* 2001)

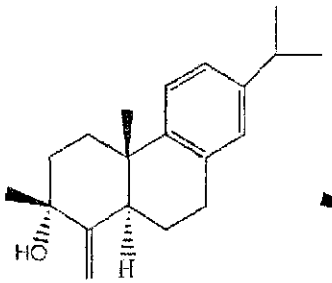
Figure 1.6. Diterpenes III: Previously isolated from *V. rotundifolia*



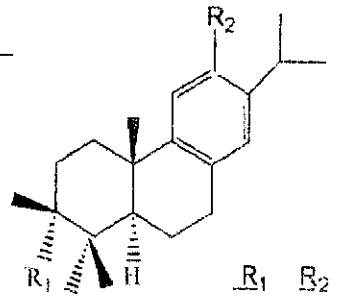
1



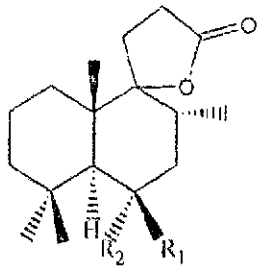
2



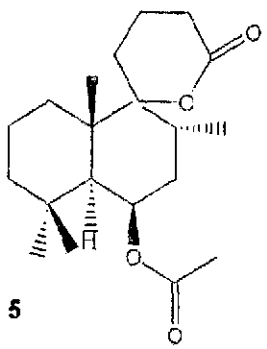
3



		R_1	R_2
9	ferruginol	H	OH
10	abietatrien-3β-ol	OH	H

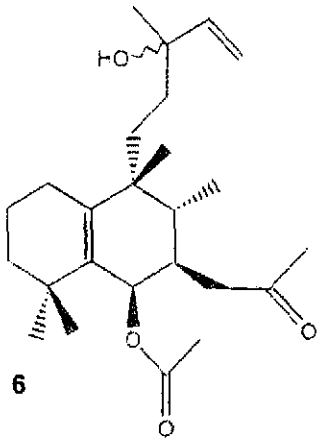


	R_1	R_2
4	H	OAc
7	OAc	H
8	H	H



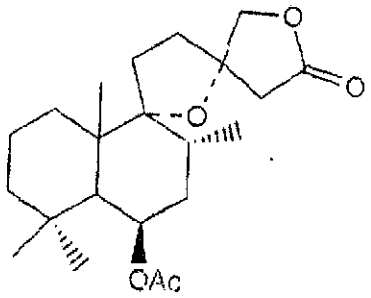
5

(Ono *et al.* 2002)

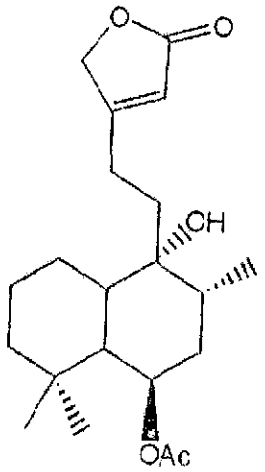


6

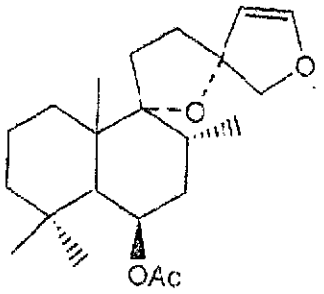
Figure 1.7. Diterpenes IV: Previously isolated from *V. rotundifolia*



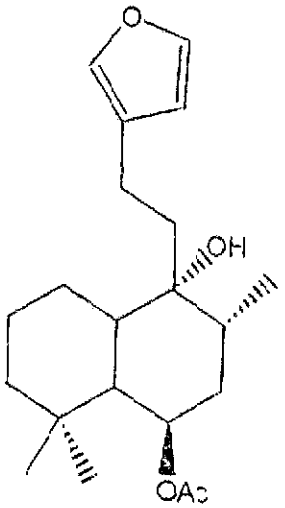
1 previtexilactone
(Kondo *et al.* 1986)



2 vitexilactone
(Kondo *et al.* 1986)



3 prerotundifuran
(Asaka *et al.* 1973)



4 rotundifuran
(Asaka *et al.* 1973)

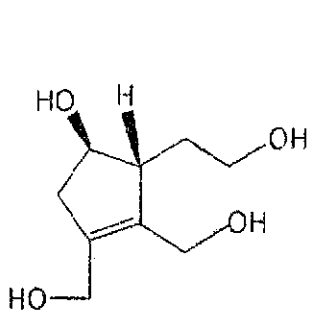
1.4.5 Iridoids

Biological activity reports regarding iridoids (**Figure 1.8**) previously isolated from *V. rotundifolia* are scarce, but several examples of other plant iridoids might suggest their medicinal potential. Agnuside, compound **7** in **Figure 1.8**, is found in several *Vitex* species. Suksamrarn *et al.* (2002) found that agnuside exhibits anti-inflammatory activity. They isolated agnuside from the stem bark of *V. peduncularis*. Konoshima *et al.* (2000) found that the iridoid glycoside 8-acetylharpagide isolated from *Ajuga decumbens* exhibits inhibitory effects on two-stage carcinogenesis on mouse skin tumors and on mouse hepatic tumors. Not drawn in Figure 1.8 are the *trans*- and *cis* eurostoside, which is very similar to agnuside, but the *p*-hydroxybenzoyl is replaced with either a *trans* or *cis* *p*-hydroxycinnamoyl (Koundo *et al.* 1988).

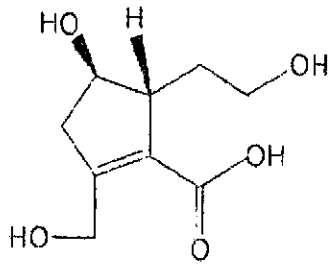
1.4.6 Miscellaneous Molecules

Several molecules isolated from *V. rotundifolia* did not fit into one of the large groups designated in **Figures 1.1** through **1.8**, so they were assigned to **Figure 1.9**. The polyalthic acid, molecule **3** in **Figure 1.9** has been found to exhibit strong anti-mutagenic activity (Miyazawa *et al.* 1995).

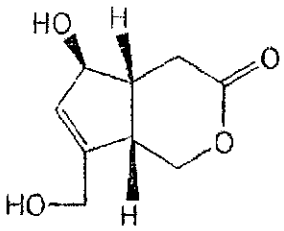
Figure 1.8. Iridoids previously isolated from *V. rotundifolia*



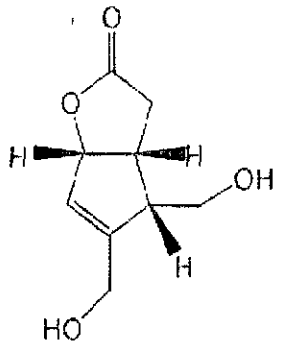
1 eucommiol



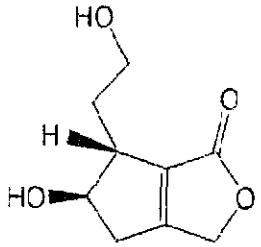
2 1-oxo-eucommiol



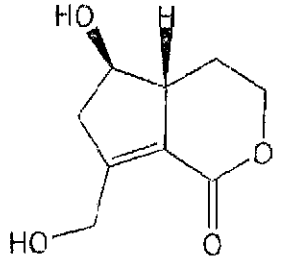
3 iridolactone



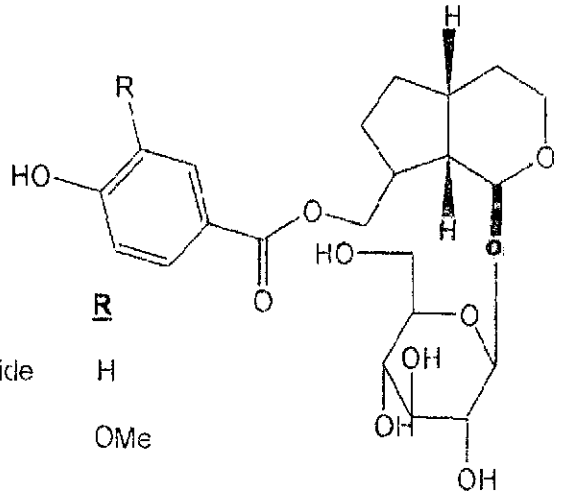
4 pedicularis-lactone



5 viteoside I



6 viteoside II

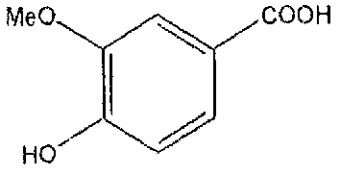


7 agnuside H

8 VR-I OMe

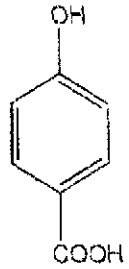
(Ono *et al.* 1997)

Figure 1.9. Miscellaneous compounds isolated from *V. rotundifolia*



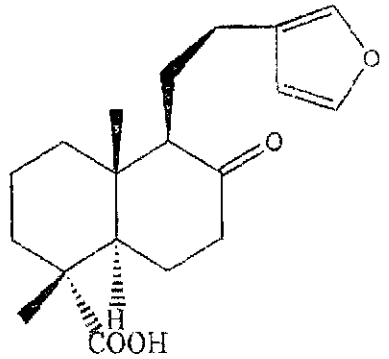
1 vanillic acid

(Kondo *et al.* 1986)



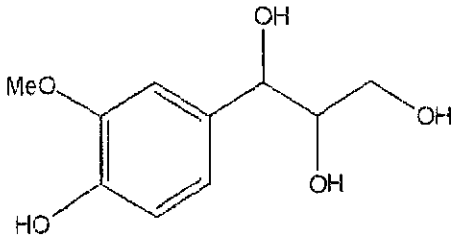
2 *p*-hydroxybenzoic acid

(Kondo *et al.* 1986)



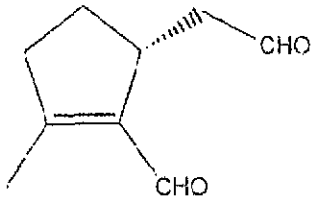
3 (+)-polyalthic acid

(Miyazawa *et al.* 1995)



4 erythro- and threoguaiacylglycerol

(Okuyama *et al.* 1993)



5 rotundial

(Watanabe 1995)

CHAPTER 2

VITEX ROTUNDIFOLIA L.F.: A NOVEL SOURCE OF INHIBITION FOR MAP KINASE (ERK2)

Abstract

V. rotundifolia is a tropical shrub that grows throughout Central and East Asia, and Hawai'i. The genus *Vitex* has a long ethnomedical history with origins in both Old Europe and Traditional Chinese Medicine. *V. rotundifolia* is used to treat ailments such as inflammation, childbirth complications and eye problems, and in Bangladesh, the leaves are used to treat illnesses claimed to be cancer. Methylene chloride (DCM) extracts of *V. rotundifolia* leaves have been found to inhibit mitogen activated protein kinase (MAPK) phosphorylation. MAPK inhibition is suggestive of anti-cancer properties due to the fact that the MAPK cascade is a central component to cell proliferation. *V. rotundifolia* also displayed anti-proliferative activity of human embryonic kidney (HEK) 293 cells. HEK 293 cells were exposed to the *V. rotundifolia* DCM leaf extract and cell viability was detected by the mitochondrial enzyme reaction of tetrazolium MTS to MTS Formazan. The results of this research suggest *V. rotundifolia* is a potential source of new chemotherapy agents and that the biochemistry and pharmacology of *V. rotundifolia* should be further investigated.

Key words: *Vitex rotundifolia*, MAP kinase, ERK2, phosphorylation, inhibition, ethnopharmacology, ethnomedicine

2.1 Introduction

The Marshall Islands consist of 29 low-lying coral atolls and 5 table reefs (flat coral islands with no lagoons) in the Central Pacific, within the larger area called Micronesia (Mueller-Dombois and Fosberg 1998). Plants have been a very important resource for people in the Marshall Islands, ever since the early Polynesians arrived in the Marshall Islands around *circa* 2000 years ago on their voyaging canoes (Nandwani and Dasilva 2003). With the exception of introduced weeds, almost every species of plant growing in the Marshall Islands has a specific use. Approximately 60 plants growing in the Marshall Islands are known to have medicinal value (Nandwani and Dasilva 2003). Most medicinal plant species are native such as *Tournefortia argentea* and *Scaevola sericea.* Several species are anthropogenic, brought along on the voyaging canoes thousands of years ago, such as *Tacca leontopetaloides* and *Colocasia esculenta*. A few more recent introductions, such as *Cocos nucifera*, have also found their place among Traditional Marshallese medicine. The water from *C. nucifera* is often used as a medicinal solvent or as a medicine alone (Nandwani and Dasilva 2003). It is not clear whether *V. trifolia* is native to the Marshall Islands or arrived by anthropogenic sources, but the leaves are used to repel mosquitoes (Merlin *et al.* 1993). According to Nandwani and Dasilva (2003), much of the Marshallese medicinal practice focuses on mothers and their babies, and most remedies are a mixture of plants rather than a single plant species.

This study was designed around the hypothesis that plants growing in the Marshall Islands would display biological activity, in particular, anti-cancer

properties. In order to test this hypothesis, bioassay guided fractionation was employed centered on MAPK activity. The goal was to investigate whether or not any of the extracts could interfere with the MAPK signaling pathway, which is critical to cell proliferation. Investigation of kinase inhibitors, and promoters, is common in cancer chemotherapy research because cancer is a signaling disease (Sausville *et al.* 2003). The discovery of agents that inhibit cell growth can potentially inhibit cancer cell growth. The key is to finding a chemotherapeutic that can inhibit cancer cell growth and not normal cell growth. Often, with increased tissue-specificity comes an increase in efficacy and safety.

2.2 Materials and Methods

2.2.1 Botanical Collections

V. rotundifolia was collected on Rongelap atoll, in the Republic of the Marshall Islands (RMI). *V. rotundifolia* was one of many plants collected for anti-cancer research during the month of June 2002, as part of a larger expedition aimed at establishing Rongelap's neighbor, Ailinginae atoll, as a world heritage site. Plants for anti-cancer screening were collected on both Ailinginae and Rongelap atolls, which lie in the northeastern Ralik (sunset) Chain, at a latitude of 11° 8'-11° 11' North and a longitude of 166° 17'-166° 2' East, and 11° 9'-11° 29' North and 166° 38'-167° 4' East, respectively. An attempt was made to collect every species of terrestrial plant growing on these atolls except for palms and grasses to provide a diverse range of taxa for anti-cancer studies. Plants were collected systematically along transects identified for other purposes

(Bridges and McClatchey 2005) and opportunistically as they were seen. In total, 90 terrestrial plant species comprising 197 samples, and 39 marine algal species comprising 39 samples were collected for chemical analysis (see **Table 2.1**). The additional samples extracted and assayed were samples provided by the laboratory of Will McClatchey at University of Hawai'i at Manoa. Overall, 129 species were extracted and assayed. For plants with a woody habit, 2-4 plant samples were collected (i.e., root, bark, flower or fruit, leaf), and for plants with herbaceous habit, 1-3 plant samples were collected (i.e., leaf, flower, root). Marine algal samples were collected as entire thalli. 236 lipophilic and 236 aqueous extracts were prepared from both the terrestrial and algal species collected in the Marshall Islands, as well as some miscellaneous samples in the lab waiting to be extracted (MN and a few WCM samples). All species were collected carefully without exposing the plant to possible chemical contaminants (i.e., insect repellent). Immediately upon collection, plant samples were placed in 250 ml Nalgene polyethylene plastic bottles containing 70% isopropyl alcohol. These were then kept refrigerated in the survey ship until shipped cold to Hawaii.

A voucher was prepared for each species collected. Vouchers were prepared in triplicate, except for the algal species, from which only one voucher was prepared. A minimum of one voucher from each species was submitted to the newly developed herbarium of the Rongelap Atoll. Duplicate voucher specimens were submitted to BISH and HAW.

2.2.2 Extraction Methodology

In the field, plant samples were preserved in 70% isopropyl alcohol and kept as cool as possible until they arrived at the University of Hawai'i at Manoa, Hawai'i, where they were placed in a -20°C freezer. Plant samples were individually placed in a blender with 250ml 70% ethyl alcohol and ground into a fine pulp. The extract was then filtered through cheesecloth and a cellulose qualitative standard filter paper (Whatman No. 1001-110) over a Buchner funnel attached to an Erlennmeyer flask to collect the extract. This method yielded a series of semi-aqueous extracts. The plant pulp was then further extracted with 250ml of distilled methylene chloride (DCM), allowed to steep for 15-30 minutes before filtration by the same methodology just described, thereby yielding a series of lipophilic extracts. The alcohol extracts were concentrated using a rotavap, and stored as liquid at –80°C. The DCM extracts were evaporated, re-suspended with ether, and stored as a liquid at -80°C.

To observe whether or not the MAPK inhibitory activity was exclusive to the Marshall Islands *V. rotundifolia* leaf extract, a secondary sample of *V. rotundifolia* leaves was collected on the island of Oahu, extracted following the same protocol, and screened for MAPK activity. Once both *V. rotundifolia* specimens demonstrated positive inhibitory activity of MAPK, a third, scale-up extraction of *V. rotundifolia* leaves, also collected on Oahu, was performed with a wet leaf mass of 335g. The scale-up extraction followed a similar protocol to that of the original extracts, except that the DCM extracts were not re-suspended with ether, and all extracts were stored evaporated, free of solvent, at -20°C. Also,

the scale up extraction allowed the leaves to steep in the DCM for 24-48 hours before filtration.

2.2.3 Biological Activity

Except for *V. rotundifolia*, only lipophilic extracts have thus far been screened for biological activity. All 235 lipophilic plant extracts (terrestrial plants and marine algae from the RMI) were tested in the enzyme based MBP assay, which focuses on the ability of MAP kinase (ERK2), an extracellular signal-regulated kinase, to phosphorylate the substrate myelin basic protein (MBP). Extracts that at least partially inhibited MAPK phosphorylation were sequentially tested in a cell based cytotoxic/antiproliferative MTS assay using HEK 293 cells. In addition, extracts that caused 25% or more stimulation of MAPK phosphorylation compared to the solvent control, were singled out for the cell-based, MAP kinase stimulation assay. *V. rotundifolia* was also tested in this assay, but rather to observe if the inhibitory action of MAPK phosphorylation could be implemented in cell culture.

2.2.3.1 MBP assay:

The MBP assay refers to the enzyme based MAPK assay, which focuses on the ability of an extract to inhibit ERK2 mediated MBP (Sigma-Aldrich) phosphorylation by detecting the incorporation of radiolabeled phosporus from ATP (Redivue^{TM} [γ-^{32}P] ATP, Amersham Biosciences). In order to activate the ERK2, MEKR4F (MAP kinase kinase) was allowed to react with ERK2 for two hours at 30°C in a kinase buffer (10mM Tris pH 8, 1mM dithiothreitol, 1mM benzamidine, 10mM magnesium chloride) and 50μM ATP (both purified

recombinant proteins, ERK2 and MEKR4F, generously provided by the Bonnie Warn-Cramer lab). Extracts and controls were diluted and added to reaction tubes along with kinase buffer, 9µg MBP and 1µCi [γ-^{32}P] ATP, with a final activated ERK2 concentration of 5.6 µg/ml.

5-iodotubercidin (Calbiochem), a known potent and competitive inhibitor of MAP kinase phosphorylation (Massillon *et al.* 1994) was used for the positive control. Two concentrations of 5-iodotubercidin (2µM and 0.5µM) were run parallel to the samples. A negative vehicle control, DMSO (dimethylsulfoxide, Sigma-Aldrich) for all original extracts and DMF (N,N-dimethyl-formamide, Sigma-Aldrich) for all extract fractions were run parallel to the samples. Controls containing no ERK2 were also assayed as reagent blanks. All samples, including the controls, were run in duplicate.

The negative control reaction tubes consisted of: 5µl activated ERK2 mixture, 15µl [γ-^{32}P] ATP mixture, and 10µl DMSO/DMF. The positive control reaction tubes consisted of: 5µl activated ERK2 mixture, 15µl [γ-^{32}P] ATP mixture, and 6µl 5-iodotubercidin (2mM control) and 4µl DMSO/DMF to the high positive control, and 1.5µl 5-iodotubercidin (0.5mM control) and 8.5µl DMSO/DMF to the low positive control. The ERK2 blank reaction tubes were prepared by adding: 15µl [γ-^{32}P] ATP mixture, 10µl DMSO/DMF and 5µl milli-Q water in lieu of extract and the ERK2 mixture, respectively. The extract sample tubes consisted of a final crude extract assay concentration of 33µg/ml, 5µl activated ERK2 mixture, and 15µl [γ-^{32}P] ATP mixture.

The assay reaction tubes were incubated at 30°C for 10 minutes. The reaction was stopped with 1.5µl glacial acetic acid. Samples were spotted onto P81 cellulose phosphate paper circles (Whatman) and allowed to dry briefly. Papers were washed with 0.85% phosphoric acid in water four times, followed by one wash with acetone. Papers were placed in vials and γ-^{32}P incorporation was quantified in a Packard Tri-Carb 2900TR liquid scintillation counter. Extracts that fell within the range of the 5-iodotubercidin positive controls (or above) were considered active. For a list of the extracts tested see **Table 2.1**.

2.2.3.2 MTS cytotoxic/proliferation assay

Due to time constraints, only 20 extracts other than *V. rotundifolia* were screened in the MTS assay. Promega CellTiter 96 one solution cell proliferation assay, which detects cell viability based on the mitochondrial enzyme reaction of tetrazolium MTS → MTS formazan, was used to test the ability of the extracts to inhibit cell proliferation. MTS formazan is detected spectrophotometrically at 490nm. In brief, human embryonic kidney HEK 293 cells grown to 80% confluency (5,000 cells plated per well), were exposed to plant extracts at 37°C for 48 hours, where upon 20 µl tetrazolium MTS was added to each well, followed by a second incubation at 37°C for 2 hours. After the 2-hour incubation, levels of MTS formazan were recorded spectrophotometrically at 490 nm. Extracts with a low absorbance reading were considered positive, conversely, a high absorbance, similar to the media control, was considered negative. A list of the plant extracts tested in the MTS assay is provided in **Figure 2.4**.

2.2.3.3 MAP kinase activation assay

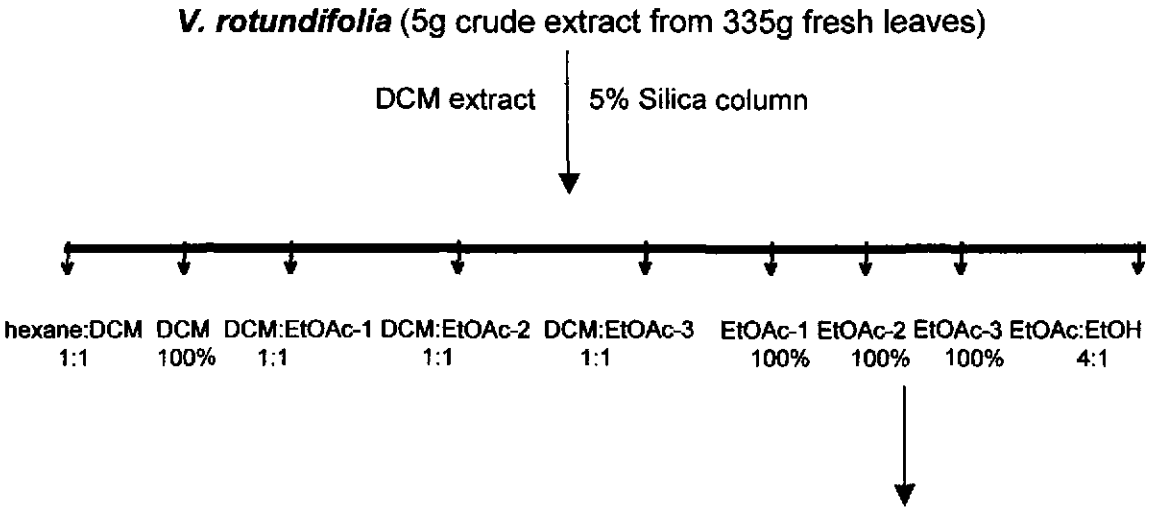
Extracts that stimulated, rather than inhibited, MAPK kinase phosphorylation in the MBP assay were tested for their ability to affect MAP kinase activation in cell culture. HEK 293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37°C for 48 hours to achieve 80% confluence. Cells were serum-starved overnight and then treated for 15 minutes with 30 µg/ml of crude extracts, or positive and negative controls. For the positive control, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) was used, at 1µM final concentration. TPA is a well-known activator of protein kinase C (PKC) (Ron and Kazanietz 1999), which is upstream from MAPK, and therefore activates the MAP kinase pathway. The negative control consisted of vehicle (DMSO) alone. After the 15-minute incubation at 37°C, cells were harvested on ice in lysis buffer (50mM Tris-HCl buffer, pH 7.4, containing 150mM NaCl, 1% IGEPAL, 1mM $MgCl_2$, 1mM DTT, 1 mM EDTA, 1mM NaF, 1mM sodium orthovanadate, and 1 tablet/10ml protease buffer of Mini-Complete protease inhibitors (Roche)). Proteins in the total cell lysates were resolved by 10% SDS-PAGE followed by Western blotting using a phospho-44/42-ERK2 antibody (Cell Signaling).

2.2.4 Fractionation

Normal phase flash chromatography on silica gel was performed on both the original Marshall Island *V. rotundifolia* DCM leaf extract and the first Hawaiian *V. rotundifolia* DCM leaf extract. A step gradient was used: cyclohexane: DCM (1:1), DCM (100%), DCM:ethyl acetate (EtOAc) (1:1), EtOAc (100%), and

EtOAc:ethyl alcohol (EtOH) (4:1). 100mg of sample extract was loaded, equal to 5% of the bed volume (2g silica). Solvent volumes equaled 3x the bed volume. Five fractions were collected. Fractions were rotavapped and placed on a vacuum pump for 24 hours to ensure all solvent was evaporated. The mass was determined for each fraction, which in total were close to the mass of the loaded sample, suggesting good recovery of plant extract. For the first scale up, 5g crude extract was yielded from 335g leaf material. A larger silica column was performed using 100g silica. The same normal phase column chromatographic technique was used as with the initial columns, however, 9 fractions were collected: one cyclohexane:DCM, one DCM, three DCM:EtOAc, three EtOAc, and one EtOH fraction. Aliquots of each fraction were then tested in the MBP assay. See **Figure 2.1** for a schematic drawing of fractionation methodology.

Figure 2.1. Flow chart of MBP assay guided fractionation of *Vitex rotundifolia* L. f.



2.3 Results

Table 2.1. List of extracts prepared from Marshall Islands plants (and a few opportunistic samples) and cumulative MBP assay results.
(++ indicates active, + indicates active within 10% below the 5-IT control and – indicates inactive) (WCM = Will C. McClatchey, MN = Mark Nickim, CLH = Carrie Lynn Harrington)

Plant Species	Voucher No.	Plant Part	MBP Results
Abutilon abutilon (L.) Rusby. Malvaceae	MN 149C		-
	MN 149		-
Acer saccharinum L. Aceraceae	MN 38A		-
Alliaria alliaria (L.) Britton. Brassicaceae	MN 79		-
Artocarpus altilis (Park.) Fosberg Moraceae	WCM 2852	leaf	-
	WCM 2852	root	-
	WCM 2852	stem	-
Bidens pilosa L. Asteraceae	WCM 2849	stem	+
	WCM 2849	leaf	+
	WCM 2849	flower	+
	WCM 2849	root	+
Boerhavia diffusa L. Nyctaginaceae	WCM 2815	leaf	-
	WCM 2815	stem	-
	WCM 2815	root	+
Boerhavia tetrandra L. Nyctaginaceae	JJS 982	whole plant	-
Bruguiera argentea L.f. Rhizophoraceae	WCM 2837	fruit	-
	WCM 2837	flower	-
	WCM 2837	stem	-
	WCM 2837	root core	-
	WCM 2837	root	-
	WCM 2837	leaf	-
Calophyllum inophyllum L. Guttiferae	WCM 2729	stem	-
	WCM 2729	leaf	-
Canavalia microcarpa (DC.) Piper Fabaceae	WCM 2857	root	-
	WCM 2857	stem	-
	WCM 2857	flower	-
	WCM 2857	petiole/leaf	-

Table 2.1. (Continued) List of extracts and cumulative MBP assay results.

Plant Species	Voucher No.	Plant Part	MBP Results
Cassytha filiformis L. Lauraceae	JJS 983	whole plant	-
Casuarina equisetifolia L. Casuarinaceae	WCM 2864	leaf	-
		stem	-
		fruit	-
		flower	-
Caulerpa sp. Caulerpaceae	CLH 0034	algae	-
Caulerpa sp. Caulerpaceae	CLH 0035	algae	-
Caulerpa sp. Caulerpaceae	CLH 0036	algae	-
Caulerpa sp. Caulerpaceae	CLH 0044	algae	-
Caulerpa sp. Caulerpaceae	CLH 0056	algae	-
Caulerpa sp. Caulerpaceae	CLH 0058	algae	-
Caulerpa sp. Caulerpaceae	CLH 0059	algae	-
Caulerpa sp. Caulerpaceae	CLH 0062	algae	-
Caulerpa sp. Caulerpaceae	CLH 0067	algae	-
Caulerpa sp. Caulerpaceae	CLH 0072	algae	-
Celtis occidentalis L. Ulmaceae	MN 73B		-
cf. *Bignonia* Bignoniaceae	WCM 2863	stem	-
	WCM 2863	leaf	+
Chamaesyce prostrata Gray Euphorbiaceae	WCM 2848	root	-
	WCM 2848	leaf	-
	WCM 2848	seed	-
	WCM 2848	stem	-
	WCM 2848	leaf	-
	WCM 2848	stem/flower	-
Cichorum intybus L. Asteraceae	MN 114		+
Clerodendron inerme (L.) Gaertner Verbenaceae	WCM 2855	stem	-
		flower	+
		root	-
		leaf	-
Cocos nucifera L. Araceae	WCM 2869	root	-

Table 2.1. (Continued) List of extracts and cumulative MBP assay results.

Plant Species	Voucher No.	Plant Part	MBP Results
Conyza canadensis (L.) Cronq. Asteraceae	WCM 2850	leaf	-
		root	-
		stem	-
		flower	-
Cordia subcordata Lam. Boraginaceae	WCM 2862	flower	+
		leaf	+
		fruit	-
		stem	-
Cornus stolonifera Michx. Cornaceae	MN 85C		-
Crataegus sp. Rosaceae	MN 47	flower	-
	MN 75B		-
Crinum asiaticum L. Amaryllidaceae	WCM 2854	root	-
		leaf	-
		stem	-
Fleurya ruderalis (Forst.f.) Gaudichaud	WCM 2701	leaf	-
		entire plant	+
Gaura coccinea (Nutt.) Pursh Onagraceae	WCM 2001	stem	-
Gomphrena globosa L. Amaranthaceae	WCM 2700	stem/leaf	-
		flower	-
Guettarda speciosa L. Rubiaceae	JJS 985	stem	-
		fruit	-
		leaf	-
Halimeda sp. Halimedaceae	CLH 0039	algae	-
Halimeda sp. Halimedaceae	CLH 0040	algae	-
Halimeda sp. Halimedaceae	CLH 0043	algae	+
Halimeda sp. Halimedaceae	CLH 0047	algae	-
Halimeda sp. Halimedaceae	CLH 0048	algae	+
Halimeda sp. Halimedaceae	CLH 0049	algae	-
Halimeda sp. Halimedaceae	CLH 0051	algae	+
Halimeda sp. Halimedaceae	CLH 0052	algae	-
Halimeda sp. Halimedaceae	CLH 0053	algae	-
Halimeda sp. Halimedaceae	CLH 0054	algae	-
Halimeda sp. Halimedaceae	CLH 0055	algae	-

Table 2.1. (Continued) List of extracts and cumulative MBP assay results.

Plant Species	Voucher No.	Plant Part	MBP Results
Halimeda sp. Halimedaceae	CLH 0060	algae	-
Halimeda sp. Halimedaceae	CLH 0061	algae	-
Halimeda sp. Halimedaceae	CLH 0063	algae	-
Halimeda sp. Halimedaceae	CLH 0064	algae	-
Halimeda sp. Halimedaceae	CLH 0065	algae	-
Halimeda sp. Halimedaceae	CLH 0066	algae	-
Halimeda sp. Halimedaceae	CLH 0068	algae	+
Halimeda sp. Halimedaceae	CLH 0069	algae	-
Halimeda sp. Halimedaceae	CLH 0073	algae	-
Heliotropium ovalifolium (Cham.) Merr Boraginaceae	WCM 2866	leaf	-
		root	-
		flower	-
Heliotropium ovalifolium (Cham.) Merr Boraginaceae	WCM 2867	bark	-
		flower	-
		mature leaf	+
		young leaf	+
identification in progress	MN 53B		-
identification in progress	MN 65C		-
identification in progress	MN 66		-
	MN 66B		-
identification in progress	MN 92		-
identification in progress	MN 93		-
identification in progress	MN 100		-
	MN 101C		-
identification in progress	MN 107		+
identification in progress	MN 108		+
identification in progress	MN 115		-
identification in progress	MN 120D		-
identification in progress	MN 122		-
	MN 122C		-
identification in progress	MN 126		+
	MN 126C		+
identification in progress	MN 132B		-

Table 2.1. (Continued) List of extracts and cumulative MBP assay results.

Plant Species	Voucher No.	Plant Part	MBP Results
identification in progress	MN 134B		-
identification in progress	MN 140		-
identification in progress	MN 142		-
identification in progress	MN 144		-
identification in progress	MN 146		-
Ipomea alba L. Convulvulaceae	WCM 2842	stem	-
Ipomea pes-carprae (L.) R.Br. Convulvulaceae	WCM 2870	leaf	-
		stem	-
		root	-
Juglans nigra L. Juglandaceae	MN 63B		-
Juniperus occidentalis Hook. Cupressaceae	WCM 1840	leaf	-
Juniperus sp. L. Cupressaceae	MN 103		-
	MN 103B		+
Lepidium campestre (L.) R.Br. Brassicaceae	MN 89		-
Lepidium virginicum L. Brassicaceae	MN 90		-
Lilium tigrinum Andr. Liliaceae	MN 151		+
Medicago lupulina L. Fabaceae	MN 97		-
Meliotus officinalis (L.) Lam. Fabaceae	MN 141B		+
	MN 141		-
Microdictyon sp. Chlorocystidaceae	CLH 0041	algae	-
Microdictyon sp. Chlorocystidaceae	CLH 0042	algae	-
Microdictyon sp. Chlorocystidaceae	CLH 0046	algae	-
Microdictyon sp. Chlorocystidaceae	CLH 0057	algae	-
Microsorum scolopendria Burm. f. Polypodiaceae	WCM 2851	leaf	-
		petiole	-
		rhizome	-
Microsorum scolopendria Burm. f. Polypodiaceae	JJS980	stem	-
		leaf	-
		petiole	-
Mirabilis linearis (Pursh) Heimerl. Nyctaginaceae	WCM 2092	leaf/stem/flower	-

Table 2.1. (Continued) List of extracts and cumulative MBP assay results.

Plant Species	Voucher No.	Plant Part	MBP Results
Morinda citrifolia L. Rubiaceae	WCM 2841	root	-
		leaf	-
		stem	-
		fruit	-
Ochrosia parviflora (Forster) Henslow Oleaceae	WCM 2858	fruit	-
		Leaf	-
Ochrosia parviflora (Forster) Henslow Oleaceae	WCM 2820	leaf	-
		stem	-
		petiole	-
Paeonia hybrid Paeoniaceae	MN 36D		-
Pemphis acidula Forster Lythraceae	WCM 2816	stem	-
Pemphis acidula Forster Lythraceae	JJS 986	leaf	-
		stem	-
Phyllanthus amarus Schum. & Thonning Euphorbiaceae	WCM 2865	flower	-
		stem	+
		Root	-
Physalis angulata L. Solanaceae	WCM 2847	stem	-
		root	+
		leaf	+
		fruit	+
Phytolacca americana L. Phytolaccaceae	MN 111		+
	MN 111C		+
	MN 111D		-
Piper auritum Kunth Piperaceae	WCM 2879	root	-
		leaf blade	+
		stem node	-
		fruit spike	-
		internode	-
		petiole	+
Piper auritum Kunth Piperaceae	WCM 2880	storage root	-
Pisonia grandis R.Br. Nyctaginaceae	WCM 2838	leaf	-
		bark	-

Table 2.1. (Continued) List of extracts and cumulative MBP assay results.

Plant Species	Voucher No.	Plant Part	MBP Results
Portulacca lutea Sol. Portulacaceae	WCM 2709	stem	-
		root	-
		leaf	-
Portulacca sp. Portulacaceae	WCM 2817	leaf	-
Premna serratifolia L. Verbenaceae	WCM 2853	stem	+
		leaf	+
Scaevola frutescens (Mill.) Krause Goodeniaceae	WCM 2840	stem	-
		leaf	-
		flower	-
Sida fallax Walpers Mavaceae	WCM 2860	leaf	-
		root	-
		stem	-
		flower	+
Solanum dulcamara L. Solananceae	MN 41		-
Soulamea amara Lam. Simaroubaceae	WCM 2861	leaf	-
		fruit	-
		stem	-
Suriana maritime L. Surianaceae	JJS 984	leaf	-
		stem	-
Syringa vulgaris L. Oleaceae	MN 113		-
Tacca leontopetaloides (L.) Merr. Taccaceae	WCM 2856	flower	-
		leaf	+
		petiole	+
		root	-
Terminalia samoensis L. Combretaceae	JJS 981	fruit	-
		stem	-
		leave	+
Tournefortia argentea L.f. Boraginaceae	WCM 2843	flower	-
Toxylon pomiferum Raf. Moraceae	MN 102		-
Tradescantia ohiensis Raf. Commelinaceae	MN 117		+

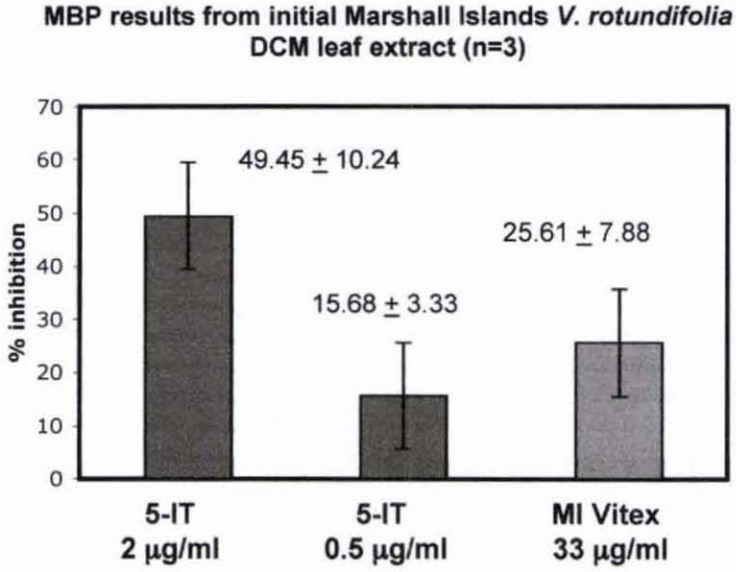
Table 2.1. (Continued) List of extracts and cumulative MBP assay results.

Plant Species	Voucher No.	Plant Part	MBP Results
Triumfetta procumbens Forster f. Tiliaceae	WCM 2726	stem	-
		leaf	-
Triumfetta procumbens Forster f. Tiliaceae	WCM 2859	fruit	-
		stem	-
		leaf	-
		root	-
unknown algae	CLH 0038	algae	-
unknown algae	CLH 0045	algae	-
unknown algae	CLH 0070	algae	-
unknown algae	CLH 0071	algae	+
Verbascum thapsus L. Scrophulariaceae	MN 148C		-
Vitex rotundifolia L. fil. Lamiaceae	WCM 2868	leaf	++
		fruit	-
		stem	-
Wollastonia biflora (DC.) L. Asteraceae	WCM 2715	stem	-
		leaf	-
		root	-
Xanthium strumarium L. Asteraceae	WCM 2036	root	-
Xanthium strumarium L. Asteraceae	MN 188B		-

Figure 2.2. MBP assay results from the primary Marshall Islands (MI) *Vitex rotundifolia* L. f. DCM leaf extract, and from the primary Oahu (HI) *Vitex rotundifolia* L. f. DCM leaf extract.

(A) *V. rotundifolia* data from the initial MI series of *in vitro* MBP assay. The positive control, 5-IT, is run parallel in a concentration gradient. **(B)** The original *V. rotundifolia* (MI) tested parallel with the Hawaii-collected *V. rotundifolia*. Both DCM extracts display low, but positive activity.

(A)



(B)

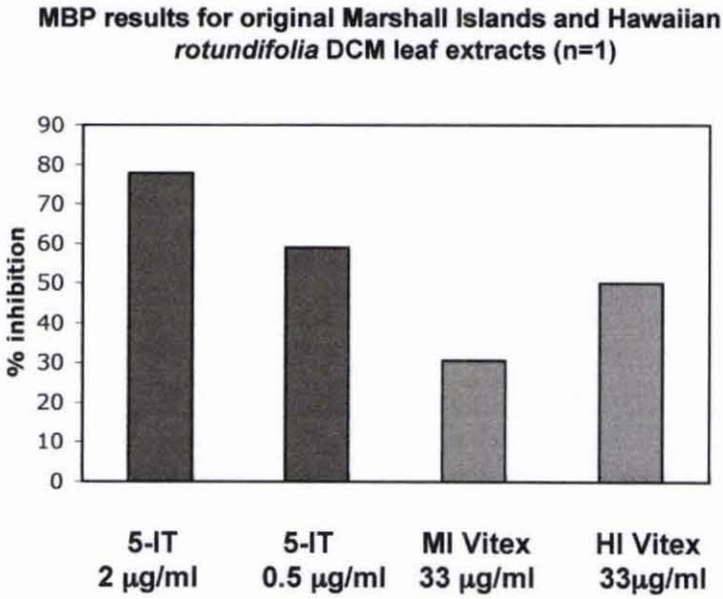
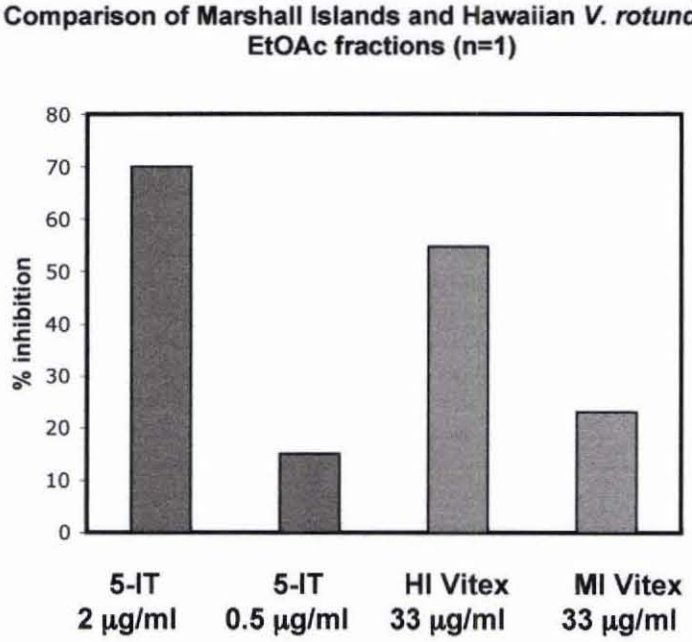


Figure 2.3. MBP assay results from fractionation of both MI and Oahu (HI) *Vitex rotundifolia* L. f.

(A) Primary fractionation via flash chromatography on silica with the solvent system: cyclohexane:DCM (1:1), DCM, DCM:EtOAc (1:1), EtOAc, EtOAc:EtOH (4:1) yielded active EtOAc fractions in both HI and MI samples. **(B)** Following the same chromatographic methods as the initial fractionation on a scale up DCM leaf extract, except with three EtOAc fractions collected rather than one, displayed an activity gradient with the peak centered on the second EtOAc fraction. The EtOH and DCM whole extracts were run in parallel, which also displayed activity.

(A)



(B)

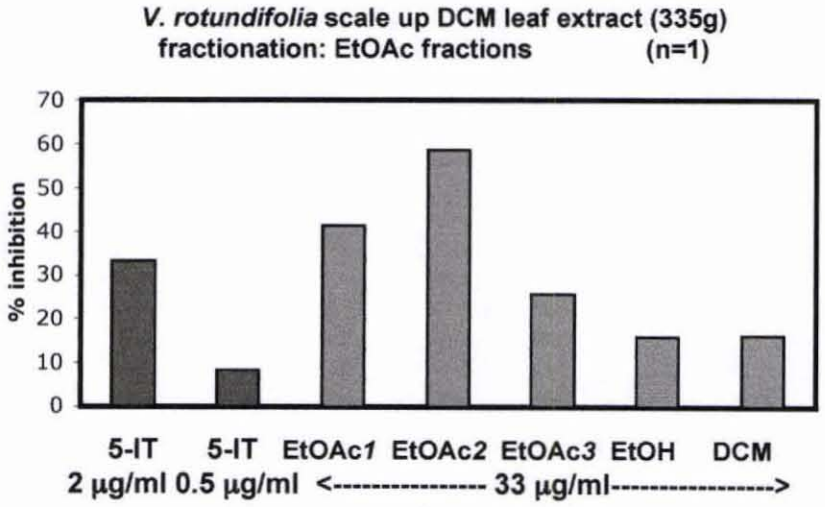


Figure 2.4. MTS proliferation assay results on Marshall Islands DCM extracts, including *Vitex rotundifolia* L. f. (WCM 2868 leaf).
Columns 1-2 and 24-25 are the negative solvent controls (DMSO). Column 4 displays that HEK 293 cell proliferation is inhibited by the DCM leaf extract *V. rotundifolia* as well as several other DCM plant extracts (see **Table 2.2**).

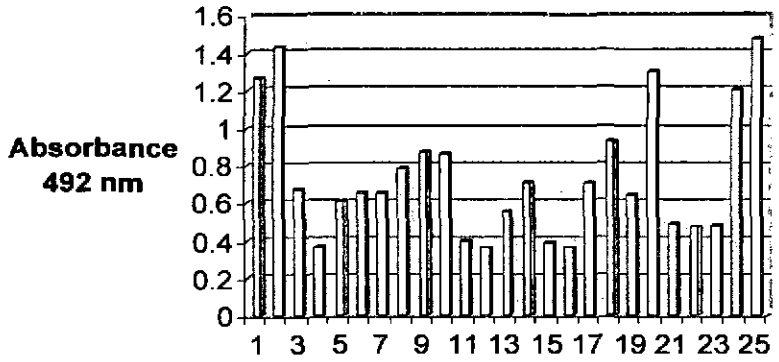


Table 2.2 Plants tested in the MTS assay (Figure 2.4)

Column number	Plant Species	Plant part	Voucher No.
1		DMSO control	
2		media control	
3	*Triumfetta procumbens*	stem	WCM 2859
4	*Vitex rotundifolia*	leaf	WCM 2868
5	*Bidens pilosa*	leaf	WCM 2849
6	*Halimeda sp.*	algae	CLH 0043
7	*Halimeda sp.*	algae	CLH 0068
8	*Phyllanthus amarus*	stem	WCM 2865
9	*Medicago lupulina*		MN 97
10	Identification in progress		MN 100
11	*Juniperus sp.*		MN 103
12	*Juniperus sp.*		MN 103B
13	*Soulamea amara*	stem	WCM 2861
14	*Heliotropium ovalifolium*	mature leaf	WCM 2867
15	*Heliotropium ovalifolium*	young leaf	WCM 2867
16	*Halimeda sp.*	algae	CLH 0049
17	*Halimeda sp.*	algae	CLH 0051
18	*Guettarda speciosa*	leaf	JJS 985
19	*Pemphis acidula*	leaf	JJS 986
20	*Pemphis acidula*	stem	JJS 986
21	Identification in progress		MN 107
22	Identification in progress		MN 108
23	*Phytolacca lutea*		MN 111
24		DMSO control	
25		media control	

Table 2.3. Results from the MAP kinase activation assay
(extracts tested were considered stimulatory in the MBP assay)

Plant sample	MAPK activation
WCM 2843 leaf	-
WCM2851 petiole	-
WCM2851 rhizome	-
WCM 2866 root	-
WCM 2868 fruit	-
WCM2868 stem	-
WCM2820 stem	-
WCM2820 petiole	-
JJS985 stem	-
CLH0034 algae	-
CLH0035 algae	-
CLH0039 algae	-
CLH0041 algae	-
CLH0063 algae	-
MN41	-
MN47	-
MN53B	-
MN63B	-

Discussion

Several of the culturally significant medicinal plant species (Nandwani and Dasilva 2003) collected in the Marshall Islands displayed a minimal degree of biological activity in the MBP assay. Plants tested include the stems of *Triumfetta procumbens* (WCM2859), the flowers from *Clerodendron inerme* (WCM2855), the root of *Boerhavia diffusa* (WCM2815), both flowers and leaves from *Cordia subcordata* (WCM2862), flowers from *Sida fallax* (WCM2860), stems from *Phyllanthus amarus* (WCM2865), petioles from *Tacca leontopetaloides* (WCM 2856), and both the stems and leaves from *Premna serratifolia* (WCM2853). The cumulative results from the MBP screening can be observed in

Table 2.1. *V. rotundifolia* is the only sample out of the 236 lipophilic plant extracts that exhibited significant inhibition of MAP kinase phosphorylation of MBP, as displayed in **Figure 2.1.A**. Significant activity was defined as a percent inhibition within or above the 5-IT positive control concentration gradient.

V. rotundifolia, *Heliotropium ovalifolium*, and *Phytolacca americanum* inhibited proliferation of HEK 293 cells, observed by the inability of mitochondrial enzymes to convert tetrazolium MTS to MTS formazan (**Table 2.2**). Inhibition by 50% or more compared to the negative controls was considered significant. One *Halimeda* algal species, extract CLH0049 displayed significant inhibition of HEK 293 cell proliferation. The only species that displayed strong activity in both the MBP and MTS assay was *Vitex rotundifolia*. *V. rotundifolia* displayed strong inhibitory effects on MAPK phosphorylation, as well as cellular proliferation of HEK 293 cells.

Although *V. rotundifolia* is not noted as a significant medicinal plant in the Marshall Islands, it has been employed as a medicine throughout Eastern and Southern Asia for centuries. The Marshallese, however, do use *V. trifolia* to repel mosquitoes. And as noted earlier, *V. rotundifolia* was once thought to be the same species as *V. trifolia*. The two species share many similar traits, including morphology, traditional uses and biological activity. Mosquito repellent is a common traditional use of many *Vitex* species in tropical and subtropical regions, and plants employed by a society for food, protection or medicine is often suggestive of biological activity (Etkin 1993). It is not clear whether or not *V. rotundifolia* is native or naturalized in the Marshall Islands, similar to its close

relative *V. trifolia*, but *V. rotundifolia* was collected on Rongelap Atoll. The flora of Rongelap Atoll is much more disturbed than that of Ailinginae, since Ailinginae has just recently had human contact after 50 years of isolation as a result of United States atomic bomb testing on nearby Bikini Atoll in the 1950's and the resultant outfall of radioactive material. A paucity of data exists regarding the native flora and fauna of the Northern Marshall Islands, with the exception of work by Taylor (1950), Thomas *et al.* (1989), and Mueller-Dombois and Fosberg (1998). The absence of *V. rotundifolia* in the above references does not necessarily mean that *V. rotundifolia* is not native to the Northern Marshall Islands, for *V. rotundifolia* could be native, just low in abundance and therefore often overlooked.

After *V. rotundifolia* displayed activity in three replicates of the MBP assay, thereby confirming the positive activity, the DCM leaf extract, along with lesser positive extracts, were tested for anti-cell proliferation properties in the MTS assay. Results from this assay are provided in **Figure 2.4**. *V. rotundifolia* (column number four), demonstrated potent anti-proliferative properties in comparison to the DMSO vehicle- and media control, as well as to the other extracts tested. Samples MN103 and MN103B (*Juniperus* sp.), WCM2867 (*Heliotropium ovalifolium*) and CLH0049 (*Halimeda* sp.) also exhibited significant inhibition of cell proliferation. Though not necessarily directly related, the fact that *V. rotundifolia* displayed anti-proliferative properties of HEK 293 cells, and inhibited ERK2 phosphorylation activity, cumulatively suggests that *V. rotundifolia* has chemotherapeutic potential.

The MAP kinase cell-based assay employed in this study (MAPK stimulatory assay) yielded all negative results. None of the plant extracts tested promoted phosphorylation of PKC, and thereby the activation of MAP kinase. The MAP kinase cell-based assay was relevant to this study because ERK2 is stimulated most strongly by growth factors and tumor-promoting phorbol esters (Davies *et al.* 2000). Phorbol esters, such as the TPA used in the MAP kinase cell-based assay, are small molecules derived from members of the Euphorbiaceae family and have been found to promote certain types of tumors. Investigation into the mechanisms behind MAPK pathway disruptors, inhibitors or promoters, can provide valuable information about the mechanisms that cause cancer.

Once the MAPK inhibitory activity of the Marshall Islands *V. rotundifolia* DCM leaf extract was confirmed in the primary screening, as well as anti-proliferative properties in the MTS assay, a second extract was prepared from a Hawaiian *V. rotundifolia* specimen to confirm if the biological activity was common among all *V. rotundifolia* specimens, or if activity was specific to the Marshallese *V. rotundifolia*. The Hawai'i collected *V. rotundifolia* proved just as potent as the original Marshall Island collected *V. rotundifolia* in the MBP assay, see **Figure 2.1.B**. After finding that both the Marshallese and Hawaiian *V. rotundifolia* extracts exhibited strong activity in the MBP assay, they were fractionated via normal phase flash chromatography on silica and screened in the MBP assay as described in materials and methods. Both the Marshallese and

Hawaiian *V. rotundifolia* DCM leaf extracts yielded EtOAc fractions equal to, or more potent than the positive control, 5-iodotubercidin, see **Figure 2.3.A**.

The first scale up extraction was prepared from locally growing *V. rotundifolia* (at the University of Hawai'i at Manoa). 335 g of fresh leaf material was collection, which yielded ~5g of crude dried extract. Normal phase chromatography was performed using the same methodology as the original fractionation, however nine fractions were collected rather than six. Because the MAPK inhibitory activity from the first fractionation was observed in the EtOAc fraction, three separate fractions were collected for both DCM:EtOAc and EtOAc. The three EtOAc fractions displayed a biological activity gradient, with the peak at EtOAc fraction number two, see **Figure 2.3.B**. EtOAc fraction number two (EtOAc-2) displayed MAPK inhibition activity in the same range as the high 5-IT positive control. This is significant because 5-IT used in this assay is a purified compound and known potent inhibitor of MAPK, where as the EtOAc-2 is still in crude form.

Fractionation of EtOAc-2 was performed via solvent-solvent partitioning using hexanes, toluene, and ether. These fractions were tested in the MBP assay, resulting in an active toluene fraction. The toluene fraction was then further fractionated with a Sephadex LH-20 column, however activity was lost at this stage. Also, material became limited at this time.

Another problem with this study is the general sensitivity of the MBP assay (MAPK inhibition). The MAPK enzyme based assay implemented in this study is much more sensitive than most of the existing MAPK inhibition assays, however,

even with this increased sensitivity, a recurring problem of overlap among the low positive 5-IT control and the negative DMSO or DMF control could not be avoided in the results. Because of the problem of overlapping results, different and/or improved assays should be considered. The overlap became a noticeable problem after the partitioning of the active EtOAc-2. In attempt to confirm activity multiple assays needed to be performed, many more assays than the normal parameters set in reliable and reproducible assays. Further, upon repeat assays of the partition fractions, the activity began to shift between the fractions derived from EtOAc-2 and the activity could not be pinpointed. There is no question that DCM extracts of *V. rotundifolia leaves*, and the subsequent EtOAc fraction(s), inhibit MAPK in the enzyme based assay used in this study, however, further fractionation of the EtOAc fraction triggers unreliable data. It is recommended that further analysis be focused on the EtOAc-2.

One recognized internal component of the MBP assay that began to cause problems during the testing of the fractions, was the concentration of the MAPK substrate, MBP. It is critical to ensure that the substrate concentration is high enough to provide significant differentiation among the samples regarding the amount of detectable radiolabeled γ-^{32}P incorporated into the substrate. If the substrate concentration is not high enough, the low positive 5-IT control can become very close in percent inhibition in comparison to the negative DMSO or DMF control.

Conclusion

Vitex rotundifolia leaves sampled from specimens collected in the Marshall Islands and Hawai'i inhibit MAPK phosphorylation of the substrate MBP, suggesting that *V. rotundifolia* should be further investigated for chemotherapeutic properties. The MAP kinase inhibitory activity of *Vitex rotundifolia* addresses the hypothesis of this project. Inhibition of MAPK (ERK2) is suggestive of anti-cancer properties because MAPK activity is critical for cell growth. Upon activation, MAPK enters the nucleus where it plays a role in the regulation of gene transcription. Transformed cells (cancerous cells) are often the results of constitutive activation of components in signaling pathways involved in cell growth and proliferation. Most frequently the component(s) involves the MAPK pathway (Davis 1993). It is for this reason, that novel agents that inhibit MAPK are considered potential cancer chemotherapeutics.

Further research is necessary to determine the active component(s) in *V. rotundifolia*. Follow up research should include further fractionation, such as a Sephadex LH-20 column and HPLC (high pressure liquid chromatography). In order to elucidate the structure of the chemical moiety responsible for the inhibition of MAPK, and ^{1}H NMR should be implemented. All fractions should be screened in the MAPK enzyme assay.

There are several secondary metabolites that seem to be distributed throughout the genus *Vitex* which have been reported to exhibit anti-cancer properties (See section 1.3). Examples of these compounds include vitexicarpin (casticin), agnuside, luteolin and a variety of terpenes. Since no mechanism of

action has been reported in any of the previous reports, it is possible that vitexicarpin, or one of the above mentioned compounds, is responsible for the mechanism of action studied here, inhibition of MAPK phosphorylation. Some of the previously isolated compounds could be tested in the MBP assay.

The ethnobotanical data provides support for the medicinal potential of *V. rotundifolia*, particularly for cancer, as it is used in both Traditional Chinese Medicine (TCM) and traditional Bangladesh medicine to treat breast cancer. The fact that *V. rotundifolia* is used frequently in traditional medicine, such as TCM, in conjunction with the fact that *V. rotundifolia* has demonstrated anti-cancer properties in the laboratory, provides strong support for further research on the medicinal value of *V. rotundifolia*.

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