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BIOACTIVE COMPOUNDS FROM
OCIMUM SANCTUM LINN. LAMIACEAE

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MARK A. KELM

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BIOACTIVE COMPOUNDS FROM *OCIMUM SANCTUM* LINN. LAMIACEAE

By

Mark Allen Kelm

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ABSTRACT

BIOACTIVE COMPOUNDS FROM *OCIMUM SANCTUM* LINN. LAMIACEAE

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A phytochemical investigation was conducted on various solvent extracts of the herb *Ocimum sanctum* Linn. Lamiaceae resulted in the isolation and characterization of mosquitocidal, anti-oxidant, and anti-inflammatory compounds. Mosquito-bioassay-guided fractionation led to the isolation of two mosquitocidal compounds, eugenol and (*E*)-6-hydroxy-4,6-dimethyl-3-heptene-2-one with minimum inhibitory concentrations at 200 and 6.25 $\mu g \cdot ml^{-1}$, respectively. A novel triglyceride, 1,3-dilinoleneoyl-2-palmitin also was identified. In two other separate studies, anti-oxidant and anti-inflammatory assays yielded eugenol, cirsilineol, cirsimaritin, isothymonin, isothymonin, apigenin, and rosmarinic acid, which were isolated from the acetone extract of fresh leaves and stems of *O. sanctum*. Anti-oxidant activities of many of these compounds namely cirsilineol, isothymonin, and rosmarinic acid were comparable to synthetic anti-oxidants when assayed at 10 μM concentrations. At 1000 μM concentration nearly all of these compounds demonstrated very good anti-inflammatory activity as demonstrated by the inhibition of cyclo-oxygenase-1. In another study, corn earworm-bioassay-guided fractionation led to the characterization of a bioactive porphyrin that exhibited toxicity/anti-feedant activity at 100 ppm concentration.

Structural identification and characterization was facilitated by the use of various spectroscopic experiments (NMR, MS, IR, and UV).

Due to the plant's anti-oxidant activity, it could potentially be used as a cancer preventative agent or even to slow the growth of existing malignant tissues. The anti-inflammatory activity *O. sanctum*, in particular its ability to inhibit cyclo-oxygenase-2 could provide an alternative to currently available drugs. Incorporation of *O. sanctum* either as a ground powder or crude extracts into diet supplements or phytoceuticals could aid in the treatment of the aforementioned maladies. The use of *O. sanctum* has potential in the control of insect pests. Furthermore, because *O. sanctum* is a plant, the acceptance and marketability of it would be enhanced considering the current lean towards natural remedies for human and agriculture pest management.

To Mom, Dad, and my Creator

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LIST OF ABBREVIATIONS

BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
$CHCl_3$	Chloroform
COX	Cyclooxygenase
^{13}C NMR	Carbon nuclear magnetic resonance
DMSO	Dimethyl sulfoxide
DQFCOSY	Double quantum filtered correlated spectroscopy
d	Doublet
dd	Doublet of doublet
DPA-PA	3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid
DEPT	Distortionless enhancement polarization transfer
E	Entgegen
EIMS	Electron impact ionization mass spectroscopy
Et_2O	Diethyl ether
EtOH	Ethanol
EtOAc	Ethyl acetate
FABMS	Fast atom bombardment mass spectroscopy
GC	Gas chromatography
^1H NMR	Proton nuclear magnetic resonance
HPLC	High pressure lipid chromatography
LC	Lethal concentration
m	Mulitplet
MeOH	Methanol
MIC	Minimum inhibitory concentration
MOPS	3-[N-morpholino] propanesulfonic acid
MPLC	Medium pressure lipid chromatography
MS	Mass spectroscopy
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PG	Prostaglandin
PGHS-1	Prostaglandin endoperoxide H synthase-1
PGSH-2	Prostaglandin endoperoxide H synthase-2
PTLC	Preparative thin layer chromatography
s	Singlet
TBA	2-thiobarbituric acid
TBHQ	*tert*-butylhydroquinone
TLC	Thin layer chromatography
UV	Ultraviolet
δ	Chemical shifts
J	Coupling constant
VLC	Vacuum liquid chromatography

INTRODUCTION

Ocimum sanctum Linn. Lamiaceae has been used for generations in Southeast Asian traditional medicine and cuisine. *O. sanctum* (synonym *O. tenuiflorum*) more commonly known by its numerous vernacular names; Holy Basil, Sacred Basil, Tulsi (Unani), Tulasi, Tulssi, Surasa, Krishnamul, Vishnu-priya (Sanskrit), Kala-tulasi (Hindi), Krishna-tulasi (Bengali), and Thulasi (Tamil), has been used to treat a wide variety of ailments. This plant, as many of the common names imply, holds religious significance as well. Sacred Basil is thought to be the transformed nymph, Tulsi, the beloved of Lord Krishna, and thus has been regarded as sacred and as a consequence, it can be found in and around many Hindu temples and homes (Mallick et al., 1989). There are even some Christian sects that consider the herb as holy, since it can be found growing near the tomb of Christ. According to Ayurveda, the traditional Indian system of medicine, teas made from the leaves are prescribed for colds, stomach disorders, and as a source of nourishment for the old and weak (Thakur, 1989). In addition, the roots are alleged to posses aphrodisiac qualities. A decoction made from the roots has been used to treat malarial fever. The leaf juices have been applied to the skin for treatment of ringworms and other cutaneous afflictions (Butani, 1982). According to African folk medicine, *O. sanctum* was used to repel mosquitoes and have a subterfuge and poultice-like effects (Batta and Santhakumari, 1970).

Besides its use in plantings around Hindu temples and homes, *O. sanctum* has little horticultural importance. However, the plant has been considered as an alternative and cheaper source of the highly valued phenyl propanoid, eugenol (Asthana and Gupta, 1984). Eugenol can comprise 30-70% (Asthana and Gupta, 1984) of the essential oil and is used mainly in pharmaceutical formulations, perfumes, toiletries, and as a flavoring agent in foods. Currently eugenol is obtained from the costly spices clove, *Eugenia caryophyllata* L. and cinnamon, *Cinnamon zeylanicum* L.

Much of the impetus for research on *O. sanctum* has been due to its role in traditional folk medicine. Most of these studies on *O. sanctum* were focused primarily on the bioactivity of crude extracts. These studies not only examined pharmacological activities, but also activities pertaining to agriculturally related pest problems such as insects, nematodes, and fungal diseases. Interestingly, a limited number of papers published on *O. sanctum* dealt with the isolation and identification of bioactive compounds and the determination of bioactivity of compounds previously identified in *O. sanctum*.

Many studies pertaining to various biological activities have been completed on crude extracts from *O. sanctum*, as discussed in Chapter II. Also, a number of articles have focused more specifically on the bioactivities of compounds identified from *O. sanctum*, are discussed in Chapter II. It has been my hypothesis that given the body of pre-existing research, as well as preliminary

studies carried out in the Bioactive Natural Products Laboratories (BNPL), *O. sanctum* has the potential to generate bioactive compounds for human and agricultural pest control. Therefore, the primary purpose of the current research was to isolate and identify the presence of biologically active compounds not previously examined. In addition, it was hoped that the present work will add to the body of phytochemcial knowledge that already exists for *O. sanctum*. *O. sanctum* plants were obtained initially from the Hare Krishna of Detroit and grown in the BNPL greenhouses, Department of Horticulture and National Food Safety and Toxicology Research Center, Michigan State University. As previously stated, primarily the crude extracts of *O. sanctum* have been examined for pharmacological as well as agricultural pest related studies. The potential health and pesticidal benefits from *O. sanctum* are therefore apparent given this body of literature and its long use as a traditional medicine. Therefore, I hypothesized that *O. sanctum* contains compound(s) capable of demonstrating antioxidant and anti-inflammatory activities. In addition, *O. sanctum* contains compounds that possess insecticidal and/or antifeedant activity.

This study, extracts and compounds were examined for biological activities. Insect bioassays utilizing mosquito larvae (*Aedes aegyptii* Linn.) and corn earworm (*Heliocoverpa zea* Hubner) were carried out to determine toxicity and antifeedant activities, respectively. Antioxidant and anti-inflammatory assays were conducted on isolated compounds.

The body of this dissertation is comprised of several chapters. Each chapter, excluding the literature review is organized in a scientific journal format, which includes an abstract, introduction, materials and methods, and a results and discussion section.

Chapter 1

LITERATURE REVIEW

This chapter reviews the breadth of literature on the bioactivities of crude extracts and pure compounds from *O. sanctum*. Among the pharmacological activities reported for *O. sanctum* crude extracts were anti-carcinogenic, chemopreventative, anti-oxidant, radioprotective, anti-mutagenic, analgesic, anti-pyretic, anti-inflammatory, anti-spermatogenic, anti-fertility, hypoglycemic, anti-ulcerogenic, immunostimulatory, anti-stress, CNS effects, anti-bacterial, and effect on blood lipid profiles. Bioactivities associated with agricultural pest and other pest problems were; anti-fungal, anti-bacterial, insecticidal, pulicidal, nematicidal, and herbicidal activities. Papers about bioactivities of pure compounds from *O. sanctum* were far fewer, these consisted of antioxidant, hepatoprotective, anti-inflammatory, anti-hyperlipidemia, anti-tumor promotion, and anti-stress. Other miscellaneous articles pertaining to phytochemical studies on *O. sanctum* are presented.

Kelm, M. A. and Nair, M. G. (submitted 1999) Bioactivity and Phytochemical review of *Ocimum sanctum* Linn. *J. Ethnopharmacology*

Pharmacologically related studies

An investigation of the anticarcinogenic effects of the commonly consumed Indian herb, Tulsi revealed that it significantly decreased the occurrence of benzo[a]pyrene-induced neoplasia and 3'methyl-4-dimethylaminoazo benzene induced hepatomas in Swiss mice and Winstar rats, respectively (Aruna and Sivaramakrishnan, 1992). Also, *O. sanctum* exhibited chemopreventative activity against chemical dimethylbenz[a]anthracene (DMBA) induced carcinogenesis. In one study, a topical application of alcoholic *O. sanctum* leaf extract followed by application of DMBA resulted in a significant reduction in skin papillomagenesis of male Swiss albino mice (Prashar et al., 1994). The authors also noted a 25% increase in glutathione-S-transferase activity and a decrease in glutathione content in mouse skin following the topical application of *O. sanctum* leaf extract. In a later study, treatment of mice with the ethanolic extract introduced via gastric intubation and orally were found to be more effective than topical applications in the prevention of DMBA-induced tumor formation (Prashar and Kumar, 1995). Similar results were obtained using Syrian golden hamsters (Karthikeyan et al., 1999). Researchers determined the influence of alcoholic extracts on the activity of certain detoxifying enzymes (Banerjee et al., 1996). Oral doses of *O. sanctum* extract at 400 and 800 $mg \cdot kg^{-1}$ body weight for 15 days significantly elevated the activities of cytochrome p-450, cytochrome b_5, aryl hydrocarbon hydroxylase, and glutathione-S-transferase (Banerjee et al., 1996). These enzymes are

considered to be very important in the detoxification process of carcinogens, mutagens and other poisons.

The anti-thyrodic and anti-oxidant activity of oral doses of aqueous extracts of *O. sanctum* was demonstrated by observing a rise in serum thyroxine concentrations and an increase in hepatic lipid peroxidation, glucose-6-phosphate, and endogenous anti-oxidant enzymes super oxide dismutase and catalase (Panda and Kar, 1998). In another study, it was determined that *O. sanctum* extract possesses free radical scavenging activity *in vitro* (Maulik et al., 1999). It is well known that free radicals, in particular oxygen free radicals, play a major role in the pathology of numerous diseases which include heart disease, arrhythmia, stroke, brain damage, respiratory syndrome, liver damage, cancer, and influenza (Mukhopadhyay et al., 1994 and Das and Maulik 1994). Ursolic acid, a triterpene, isolated from *O. sanctum* demonstrated protective effect against lipid peroxidation (Balanehru and Nagarajan, 1991). In a follow up article, Balanehru and Nagarajan (1992) found that ursolic acid prevented the cardiotoxicity of adriamycin by acting as an antioxidant. Furthermore, a pharmacological review article on ursolic acid discussed its hepatoprotective, anti-inflammatory, anti-tumor, and anti-hyperlipidemia activity (Liu, 1995).

Intraperitoneal injections of 50 $mg \cdot kg^{-1}$ followed by 10 $mg \cdot kg^{-1}$ per day of *O. sanctum* extract for five consecutive days gave the best protection (70% survival) against whole body exposure to ^{60}Co gamma radiation in albino mice

(Devi et al., 1995). In a following report it was found that *O. sanctum* extracts gave *in vivo* protection against ^{60}Co-gamma-radiation induced cytogenic damage (Ganasoundari et al., 1997). The authors speculated that the likely mechanism of the observed radioprotection involved free radical scavenging activity by the *O. sanctum* extracts administered interperitoneally. In a related article, researchers found that the same extracts were beneficial in the protection of bone marrow stem cells in adult Swiss mice against the deleterious effects of ^{60}Co gamma radiation (Ganasoundari et al., 1997). This report also demonstrated the lack of a toxic effect on the part of *O. sanctum* water extracts as compared to the well established radioprotector, WR-2721 (amifostine), an organic thiophosphate. Given this, *O. sanctum* extracts may have an advantage over toxic WR-2721 in clinical uses as a natural and potentially benign substance. In a follow-up report, the same authors had shown the radioprotective effect of bone marrow by the administration of *O. sanctum* water extracts, in addition, the toxicity of WR-2721 was reduced by the presence of *O. sanctum* extracts which also resulted in an increase in the beneficial radioprotective effects synergistically (Ganasoundari et al., 1998). The authors also demonstrated a significant rise in chromosome protection by the use of *O. sanctum* extracts when administered intraperitoneally to Swiss mice. Again, it was suggested that the radioprotection was caused by the free radical scavenging qualities of *O. sanctum* extracts (Ganasoundari et al., 1998).

The anti-mutagenic effect of *O. sanctum* juice were demonstrated in mice that were administered the bone marrow cell mutagens; methylmetnaesulfonate, mitomycin C, and dimethylnitroamine (Lim-Sylianco et al., 1988). *O sanctum* extracts were given orally by gavage, whereas the mutagens were given intraperitoneally.

The analgesic activity of the oil from the seeds of *O. sanctum* was determined in rats and mice (Singh and Majumdar, 1995). Using the tail flick, tail clip, and tail immersion methods, researchers concluded that *O. sanctum* was not effective in raising the pain threshold, indicating that the *O. sanctum* is not centrally active. However, using the acetic-acid-induced writhing method, the oil showed a significantly elevated responses in a dose-dependent manner, which is indicative of peripherally mediated pain. A dose of 3 $ml \cdot kg^{-1}$ of *O. sanctum* oil gave similar results as produced by a 100 $mg \cdot kg^{-1}$ of aspirin. These treatments were given intraperitoneally. In an earlier study, it was observed that rises in hypouricemic and uricosuric effect followed by administration of Tulsi leaves or seeds in rabbits may result in a reduction of uric acid levels (Sarkar et al., 1990). High levels of uric acid are associated with gouty arthritis. These results may, therefore, substantiate one of the traditional uses of Tulsi as a treatment for gouty arthritis or other joint inflammations.

Methanolic and water suspensions of *O. sanctum* both showed analgesic activity in the mouse hot plate method, anti-inflammatory activity in rats, and anti-

pyretic activity in rats (Godwani et al., 1987). 500 $mg \cdot kg^{-1}$ doses of the methanolic extract and water suspensions were found to achieve the same response as seen with 300 $mg \cdot kg^{-1}$ aspirin. However, at the same dose levels, the methanolic extract and the crude water suspension displayed weaker and shorter duration of analgesic activity than that of aspirin.

The fixed oil of *O. sanctum* was 100% effective in the treatment of bovine mastitis after five days alone and after three days when combined with cloxacillin (Singh et al., 1995). Bovine mastitis effects the udder and is characterized by inflammation and bacterial infections largely due to *Staphylococcus aureus*. In another study, the anti-asthmatic and anti-inflammatory of ethanol extracts of both dry and fresh leaves of *O. sanctum* were analyzed. Guinea pigs were used for the study. Interestingly, only the fresh extracts along with the volatile and fixed oils had shown protection against histamine-induced acetyl choline pre-convulsive dyspnea (Singh and Agrawal, 1991). Results from anti-inflammatory studies involving carrageenan-induced hind paw edema indicated inhibition by fresh-leaf volatile and fixed oils. Furthermore, in a later study, anti-inflammatory activity was demonstrated by the oil from *O. sanctum*, since it inhibited arachidonic acid and leukotriene-induced paw edema. Furthermore, the authors postulated that *O. sanctum* may be used as a potential anti-inflammatory agent that blocks both cyclooxygenase and lipoxygenase pathways in arachidonic acid metabolism (Singh et al., 1996).

Non-polar (benzene, petroleum ether, and ether) and polar extracts (acetone and EtOH) of *O.sanctum* had shown 80-60% and less than 50% anti-fertility, respectively. Anti-fertility activity of *O. sanctum* extracts was determined by the examination of fertile female rats for the presence of spermatozoa in vaginal smears, followed by laparomety to determine the number of implantation sites (Batta and Santhakumari, 1970).

High amounts of *O.sanctum* leaves in the diets of male albino mice decreased in seminal plasma pH, which corresponded to an increase in reducing substances such as alkaline phosphatase, acid phosphatase, mucoproteins, and electrolytes (Kasinathan et al., 1972). Histologically, there was a slight impairment of spermatogenesis. Taken together, the observed sterility was in large part due to these changes. A more recent report compliments these findings (Seth et al., 1981) in which a benzene leaf extract of *O. sanctum* reduced the count and motility of sperm in adult male rats. A reduction in the weight of testes was also observed. Furthermore, over the course of three months of diets including Tulsi leaves, the mating behavior of both male and female rats was severely inhibited (Khanna et al., 1986). This study also showed a decreased sperm count and sperm motility and weight reduction in male sex organs of rats over long-term exposure to Tulsi diets. Contrary to these studies by Khanna et al. (1986) and Kasinathan et al. (1972), researchers found an increase in testes size vs. controls and an increase in cone weight vs. control in

White leghorn male birds (Arneja et al., 1987). These studies, therefore, suggested that antifertility activity is species dependent.

Incorporation of seeds and leaves of *O. sanctum* into artificial diets displayed a progressive and significant hypoglycemic effect during the first week in adult albino rabbits. Both seed- and leaf-containing diets reduced blood sugar levels when compared with the control. However, on a dry weight basis, the leaves were more effective at reducing blood sugar (Sarkar and Pant, 1989). Doses of 100, 150, 200, and 400 $mg \cdot kg^{-1}$ of Tulsi extract were given along with a normal diet to adult male Wistar rats. Significant decreases in sexual behavior were observed by those rats receiving 200 $mg \cdot kg^{-1}$ and 400 $mg \cdot kg^{-1}$ (Kantak and Gogate, 1992). Sexual behavior was observed and scored followed by presenting male rats with a primed ovariectomise female in which 2 μg of estrogen were administered the previous three days followed by progesterone on the fourth day. Reghunandanan et al., (1995) reported a reduction in testicular sperm count and glutamyl transpeptidase activity in Wistar rats after 24 h following intraperitoneal injections of benzene extract of *O. sanctum* leaves (300 $mg \cdot kg^{-1}$).

A definite lowering of blood sugar level in normal, glucose-fed hypoglycemic and streptozotocin-induced diabetic rats was observed following oral administration of alcoholic extracts of *O.sanctum*. Enhancement of the

action of exogenous insulin in normal rats also was observed (Chattopadhyay, 1993).

Researchers concluded that *O.sanctum* extracts have anti-ulcerogenic properties against experimental ulcers, since it can cause a decrease in acid secretion and increased mucous secretion in Wistar albino rats (Mandel et al., 1993). *O.sanctum*-pretreated rat stomach mucosa was able to resist increases in gastric lesions caused by HCl-ethanol treatments. Pretreated rats also displayed a decrease in acidity and an increase in mucosal defense. Both studies (Mandel et al., 1993 and Janardhanan, et al., 1999) seem to justify the use of *O.sanctum* as an anti-ulcerogenic compound.

Steam distillates from fresh *O.sanctum* leaves were used in a study to determine its effects on humoral immune responses *in vitro*. It was found that *O.sanctum* could keep in check or maintain the humoral immune responses by acting on different aspects involved with the immune system such as antibody production, the release of mediators of hypersensitivity reactions, and tissue responses to mediators in target organs (Mediratta et al., 1988). A more recent study reported on the humoral immune response in albino rats by the quantification of agglutinating antibodies and E-rosette formation. Results showned an increase in antibody titre, E-rosette formation and lymphocytosis which are indicative of immunostimulation of humoral immunological responses (Godhwani et al., 1988). Diets containing dry powdered leaf of *O.sanctum* were

fed to birds at 500 mg·day^{-1} orally. Immunomodulating and immunopotentiating activities by dried leaf of *O.sanctum* were shown in naturally IBD (infectious bursal disease) virus-infected poultry (Sadekar et al., 1998). IBD virus is one of the poultry industry's toughest problems.

Connected in part with the immuno-stimulatory properties, researchers also discovered that *O.sanctum* possess anti-stress or adaptogenic properties (Wagner, et al., 1994, Sen et al., 1982, Bahrgava and Singh, 1981,) . *O.sanctum* was found to enhance physical endurance of swimming mice, inhibit stress-induced ulcers in rats, protect from carbon tetrachloride-induced hepatotoxicity in mice and rats, and inhibit milk-induced leucocytosis (Bahrgava and Singh, 1981). Sen and coworkers (1992) analyzed the effects of *O.sanctum* extract and eugenol (major component in *O.sanctum*) on various biochemical stress-induced changes. Both *O.sanctum* and eugenol reduced cholesterol levels caused by restraint stress in male Wistar rats (Sen et al., 1992). Also, *O.sanctum* and eugenol effectively lowered lactate dehydrogenase and alkaline phosphatase levels typically caused by stress (Sen et al., 1992). Red blood cell membrane dynamics also changed due to induction by restraint stress. Increased membrane protein clusterization, increased membrane fluidity and reduced membrane thickness occurred in response to stress; these are lessened or reversed in the presence of either *O.sanctum* or eugenol (Sen et al., 1992).

Action of *O. sanctum* on the central nervous system (CNS) was studied in mouse models (Ahumada et al., 1991 and Sakina et al., 1990). In these studies, it was found that *O. sanctum* extracts acted in synergy with the drug pentobarbital in depressing CNS responses. Sakina et al. (1990) reported a decrease in recovery time and severity due to electroshock and pentylenetetrazole-induced convulsions, in decreased apomorphine-induced fighting time and ability to walk in rodents. This study also indicated a possible interaction of *O. sanctum* extracts with dopaminergic neurons and a synergistic action when combined with bromocryptine, a potent D_2-receptor antagonist. In addition, longer narcosis times were observed in male mice compared to female mice (Ahumada et al., 1991).

Anti-bacterial studies showed ethanolic extracts of *O. sanctum* to be effective against *Staphylococcus aureus* (100 $\mu g \cdot disc^{-1}$), *S. albus* (100 $\mu g \cdot disc^{-1}$), *S. citreus* (250 $\mu g \cdot disc^{-1}$) and *E. coli* NCTC strain (500 $\mu g \cdot disc^{-1}$) (Phadke and Kulkarni, 1989). Water extracts of *O. sanctum* leaves exhibited anti-biotic activity against several strains of tuberculosis bacteria which included: *Mycobacterium tuberculosis* H_{37} Rv TMC102, *M. tuberculosis* SmR_{10}(N)-1, and *M. tuberculosis* 1NHR Rv6 (Reddi et al., 1986). Also, *O. sanctum* juice (30 $\mu l \cdot disc^{-1}$) exhibited inhibitory activity in assays utilizing *Sarcina lutea*. However, activity was not observed on *Bacillus cereus, B. meaterium, B. subtillis, S. epidermis, Proteus sp., Salmonella typhosa,* and *Vibrio cholerae* (Ferdous et al., 1990).

The blood lipid profile of albino rats was significantly lower following the administration of fresh *O. sanctum* leaves (Sarkar et al., 1994). Total serum cholesterol, triglycerides, phospholipids, and LDL cholesterol levels all were reduced significantly. In conjunction, blood HDL cholesterol and total fecal sterol contents were significantly greater. Together, these results suggest that *O. sanctum* has the potential to prevent or treat heart disease as well as other disorders associated with high fat intake.

Agricultural Pest and Insect-Related Studies

In addition to the pharmacologically related studies, agricultural insect control and related studies utilizing *O. sanctum* have been conducted. Much of the driving force behind this research was due to the widespread use of *O. sanctum* as a pest-managing agent. Only crude extracts of *O. sanctum* were studied for pest-managing activity.

Methanol extracts of *O. sanctum* leaves exhibited some fungicidal and bacteriacidal activities on *Trichophyton mertagrophytes* and *Bacillus subtilis* (Ehrenburg) Cohn at concentrations greater than 10^4 ppm but were not active on *Collectotrichum lagenarium* (Passerini) Ellis et Halstead, *Pyricularia oryzae* Cavara, and *Cochliobolus miyabeanus* (Ito et Kuribayashi) Drechsler ex Dastur (Sukari and Takahashi, 1988). However, contradicting results were obtained with ethanolic leaf extracts of *O. sanctum.* These extracts inhibited the growth of

three fungal rice pathogens: rice blast; *P. oryzae*, brown spot; *C. miyabeanus*, and sheath blight; *Rhizoctonia solani* Kühn when applied as a spray (2.5 $g \cdot l^{-1}$) on infected rice plants and in agar plate media at 5.0, 10.0, and 10.0 $g \cdot l^{-1}$, respectively (Tewari and Nayak, 1991). Ethanolic extracts and essential oil of *O. sanctum* showed fungicidal activity against another species of rice blast, *P. grisea* Sac. in both conidial germination and mycelial growth (Tewari, 1995). Ethanolic extracts also showed good performance on par with some synthetic fungicides (ediphenphos and carbendazim) in field studies (Tewar, 1995). Bananas, *Musa spientum* var. Malbhog pretreated with aqueous leaf extracts of *O. sanctum* then infected with the fungi; *Botryodiplodia theobroma*, *Fusarium theobromae*, *F. oxysporum*, *Helminthosporium spiciferum*, *Culvularia lunata*, *Aspergillus flavus*, and *Trichothecium roseum* resulted in significant retardation of fungal pathogen growth (Singh et al., 1993). Aqueous leaf extracts *of O. sanctum* effectively prevented conidial germination and slowed the growth of pre- and post-infected by *H. spiceferum* and *F. scirpi* in sponge gourd, *Luffa cyclindrica* L. (Ahmad and Prasad, 1995). Spore germination, spore growth, and cell wall degrading enzymes of the fruit rot pathogens, *Botryodiplodia theobromae* Pat. and *Rhizopus arrhizus* Fisher were inhibited by *O. sanctum* leaf extracts (Patil, et al., 1992). A reduction of total protein content of the fungi also were observed following treatment with *O. sanctum* leaf extracts.

Soxhletted ethanol extracts of both white and red strains of *O. sanctum* did not show any activity on the fish and shrimp bacterial pathogens, *Acromonas*

hydrophila, *Streptococcus sp.*, and ten strains of *Vibrio* (Direkbusarakom et al., 1998).

Contact toxicity bioassays on cockroach (*Blatella germanica* L.) and red bean weevil (*Callosbrunchus chinesis* L.) gave 100% mortality due to application of 10 mg of *O. sanctum* essential oil (Sukari and Takahashi, 1988). Methanol leaf extracts of *O. sanctum* proved to be effective as antifeedants and repellents but were, not insecticidal against *Anomis sabulifera* Guen., jute semilooper larvae (Mallick and Banerji, 1989). Ethanolic and methanolic extracts of *O. sanctum* were bioassayed against the following aphid species: *Myzus persicae* (Sulzer), *Metopolophium dirhodum* (Walker), *Aphis fabae* (Scopoli), *Sitobion avenae* (Fabricius), and *Acyrthosiphon pisum* (Harris). Mortality of all species was observed following application of the test extract (Stein et al., 1988). The authors concluded that the observed aphidicidal activity cannot be due to eugenol, rather different compounds are more likely causing the observed effect. *O. sanctum* extracts applied at a rate of 10 $kg \cdot ha^{-1}$ on rice TKM9 seedlings resulted in only 5% survival of green leafhopper, *Nephotettix virescens* Dist. (Narasimhan and Mariappan, 1988). Only 33% of transmission rate for rice tungro virus was observed for plants treated with *O. sanctum* compared to the 75% observed in the control. When *O. sanctum* seed are placed in water, they exuded a mucilaginous substance, a polysaccharide of unknown structure. This substance has been found to effectively trap mosquito larvae (*Culex fatigens*) resulting in their death (Hasan and Deo, 1994). Hexane and acetone leaf

extracts also showed excellent to moderate toxicity on *C. fatigans* larvae, respectively (Deshmukh et al., 1982). Arrekul et al., (1988) found that crude extracts of *O. sanctum* a demonstrated repellent effect against the oriental fruit fly, *Daucus doralis*. Paradoxically, Roomi and coworkers (1993) found that *O. sanctum* leaf extracts were acting as attractants in field studies. Methyl eugenol, present in the essential oil, was used as a positive control which attracted *Daucus spp*.

The oviposition deterrent effect of *O. sanctum* leaf extracts on spotted bollworm (*Erias vitella* Fabr.) was demonstrated in a study by Sojitra and Patel (1992). A 5% leaf extract suspension of *O. sanctum* applied to okra plants (*Hibiscus esculentus* L.) was effective at reducing the number of eggs laid. In a later study, researchers (Adiroubane and Letchoumane, 1988) found that 2.5 and 5% water suspensions of *O. sanctum* ground leaf alone and in combination with synthetic pesticides (carbaryl 0.05% and endosulfan 0.035%) were significantly effective in controlling the population of leafhoppers, *Amrasca bigulatta* in the field. Using similar treatments, infestation of spotted bollworm, *Earias spp*. was significantly less than in the nontreated controls.

Hexane and acetone leaf extracts of *O. sanctum* demonstrated pulicidal properties with LC_{50} at 0.05 and 1.45, respectively (Renapurkar and Deshmukh, 1984). Activities were comparable to dichloro-diphenyl-trichloroethane (DDT),

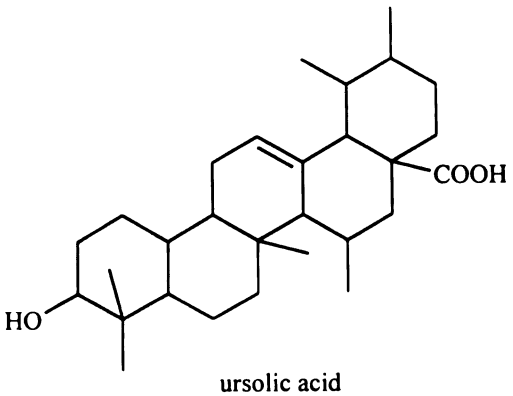
dieldrin, malathion, and fenthion with LC_{50} values at 0.31, 0.39, 0.44, and 0.34, respectively.

Chatterjee et al., (1982) reported nematicidal activity of eugenol and linalool against the nematode, *Meliodogyne incognita*. In another article, eugenol, linalool, cineole, and geraniol (see structures below) were found to be toxic against nematodes; *Anguina tritici*, *Tylenchulus semipenetrans*, *M. javanica*, and *Heterodera cajani* (Sangwan et al., 1990). Okra plants inoculated with *M. incognita* followed by treatments with water decoctions of *O. sanctum* leaves resulted in significant reductions in root protein levels and in the number of root galls (Santi et al., 1984).

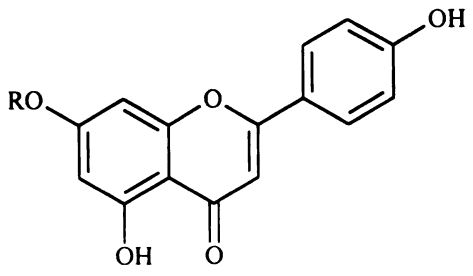
Also, *O. sanctum* extracts were found to inhibit several enzymes completely, namely mitochondrial malate dehydrogenase and malic enzyme in the metabolic pathways of the filarial worm, *Setaria digitata* (Banu et al., 1992).

Phytochemical Studies

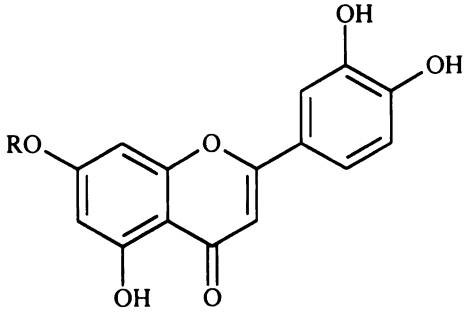
The earliest phytochemical investigations of *O. sanctum* leaves were conducted by Nair et al., (1982). Among the compounds identified were as follows: ursolic acid, apigenin, luteolin, apigenin-7-*O*-glucuronide, luteolin-7-*O*-glucoronide, orientin, and molludistin. Oleanic acid also was reported from *O. sanctum* (Wagner, et al., 1994).



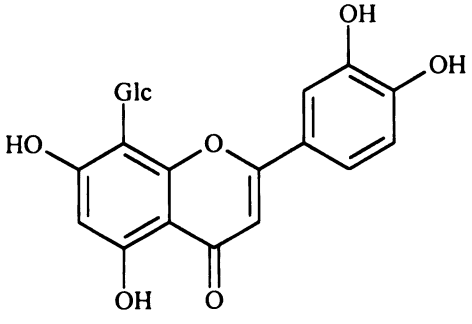
ursolic acid



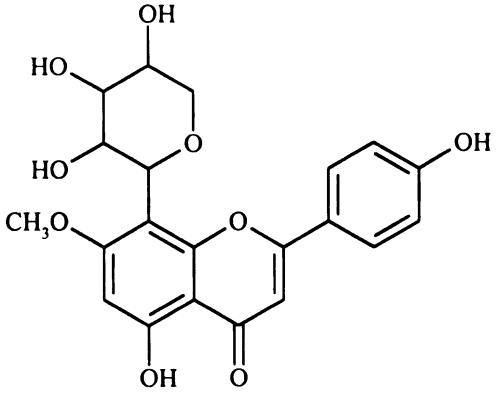
R = H; apigenin
R = glucose; apigenin-7-O-glucoside



R =H; luteolin
R = glucose; luteolin-7-O-glucoside

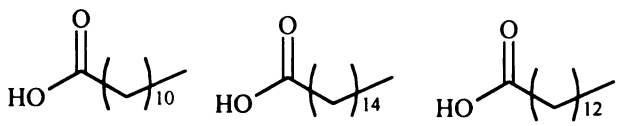


orientin



molludistin

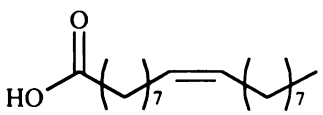
Later the chemical composition of *O. sanctum* seed oil was determined (Malik et al., 1987). Seed oil represented 18.2% by weight of the seed. GC indicated the presence of lauric (2.84%), myristic (1.90%), palmitic (5.24%), stearic (3.12%), oleic (6.0%), linoleic (59.1%), and linolenic (21.7%) acids.



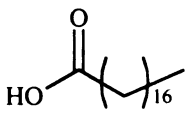
lauric acid

palmitic acid

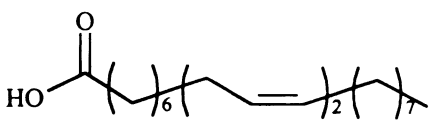
myristic acid



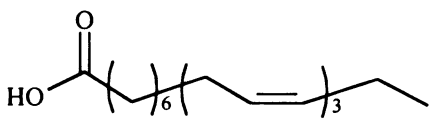
oleic acid



steric acid

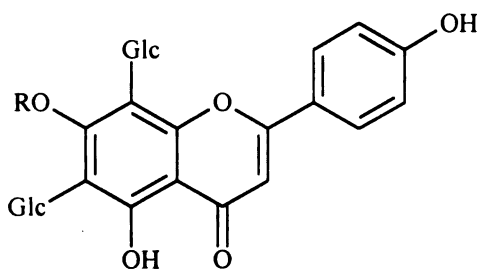


linoleic acid

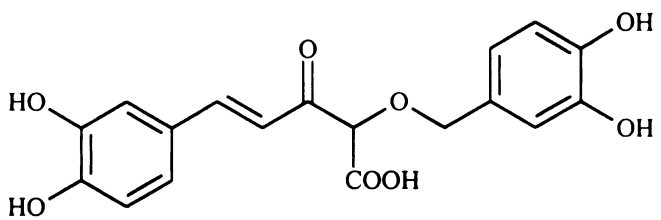


linolenic acid

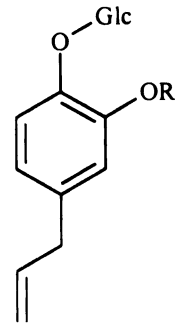
A reinvestigation of *O. sanctum* leaves led researchers to discover the presence of vicenin-2 (apigenin-6, 8-C-diglucoside), rosmarinic acid, galuteolin (luteolin-5-O-glucoside), cirsilineol (5, 4'-dihydrxy-6, 7, 3'-trimethoxyflavone), and



vicenin-2

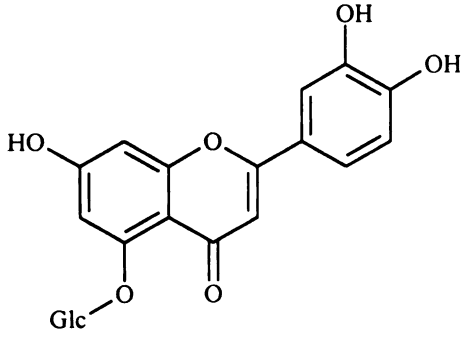


rosmarinic acid

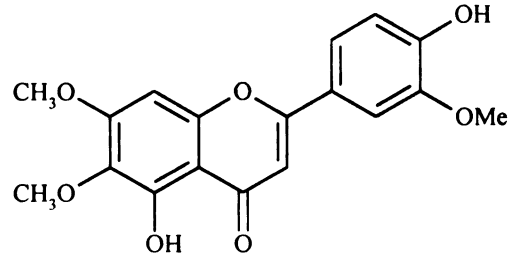


R = H; 4-allyl-1-O-β-Dglucopyranosyl-2-hydroxybenzene

R = CH_3; 4-allyl-1-O-β-D-glucopyranosyl 2-methoxybenzene



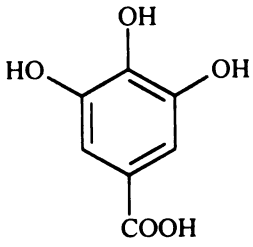
galuteolin



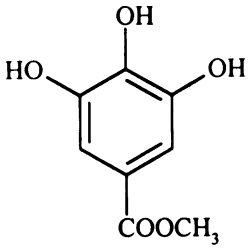
cirsilineol

two phenyl propane glucosides; 4-allyl-1-*O*-β–D-glucopyranosyl-2-hydroxybenzene and -allyl-1-*O*-β–D-glucopyranosyl-2-methoxybenzene (Nörr and Wagner, 1992).

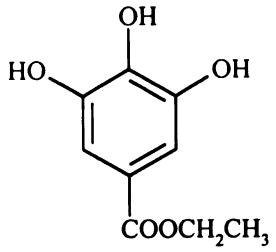
Also, gallic acid, gallic acid methyl ester, gallic acid ethyl ester, protocatechuic acid, vanillic acid, 4-hydroxybenzoic acid, vanillin, hydroxybenzaldehyde, caffeic acid, and chlorogenic acid were identified by UV spectroscopy and HPLC (Nörr and Wagner, 1992).



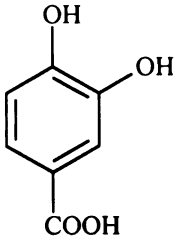
gallic acid



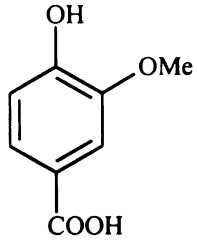
gallic acid methyl ester



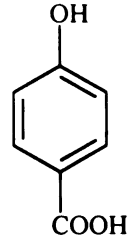
gallic acid ethyl ester



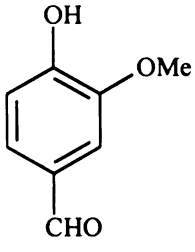
protocatechuic acid



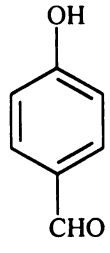
vanillic acid



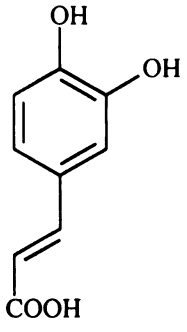
4-hydroxybenzoic acid



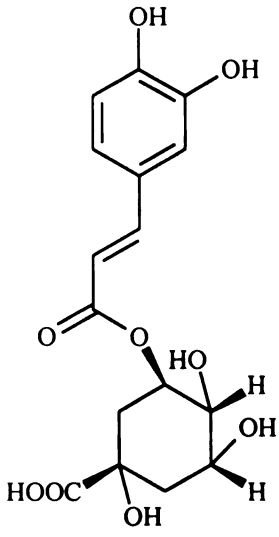
vanillin



4-hydroxybenzaldehyde

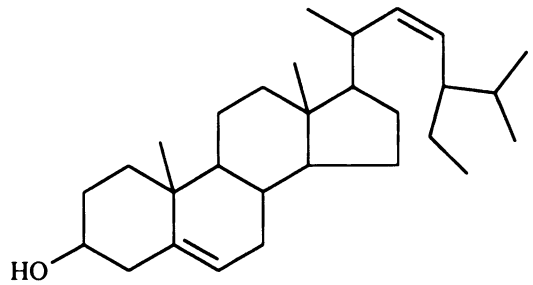


caffeic acid

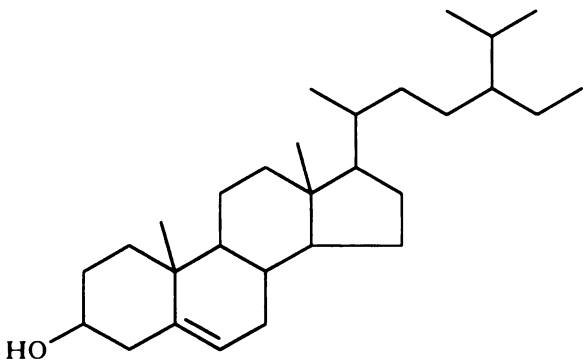


chlorogenic acid

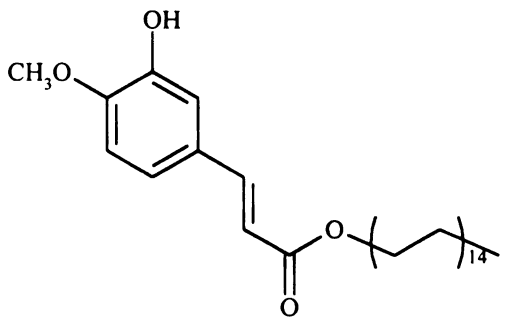
Sukari and coworkers (1995) isolated and identified stigmasterol, β–sitosterol and triacontanol ferulate from the dried bark of *O. sanctum*.



stigmasterol

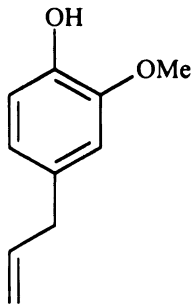


β–sitosterol

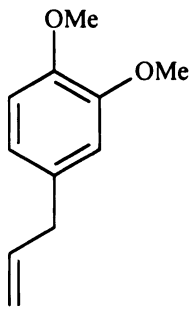


triacontanol ferulate

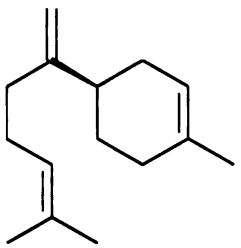
GC-MS and FTIR experiments of the essential oils of *O. sanctum* led to the identification of 20 compounds in one study (Laakso et al., 1990) and 12 compounds in another study (Vasudevan et al., 1997). In the former study eugenol, β–bisabolenes, methyl eugenol, cineole, ocimene, humulene, and β-caryophyllene were the major components derived from the steam and water-distilled oils. Linalool and geraniol were also identified. In the latter study, eugenol, and β-caryophyllene, methyl eugenol, and a number of unidentified compounds the major compounds found in the subcritical fluid extract.



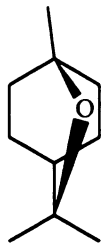
eugenol



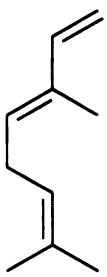
methyl eugenol



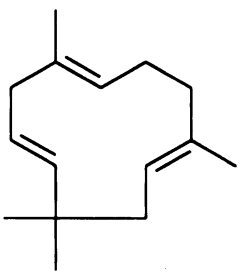
β–bisabolenes



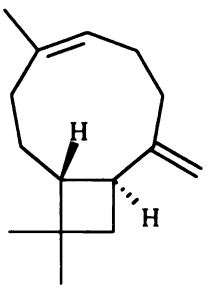
cineole



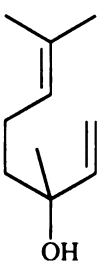
β–ocimene



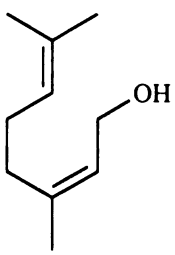
α–humulene



β–caryophyllene



linalool



geraniol

Given the above studies and the lack of studies pertaining to bioactive compounds, this work has shed more light on this deficient area of phytochemical research on *O. sanctum*. Through the process of bioassay-guided fractionation, compounds possessing insecticidal, anti-oxidant, and anti-inflammatory activity were discovered.

Chapter 2

MOSQUITOCIDAL COMPOUNDS AND A TRILGLYCERIDE, 1,3-DILINOLENEOYL-2-PALMITIN FROM *OCIMUM SANCTUM* LINN.*

Abstract - The hexane extract of *Ocimum sanctum* was investigated using mosquito bioassay-guided fractionation and yielded compounds **1** and **2**. The isolation of the triglyceride, 1, 3-dilinoleneoyl-2-palmitin (**3**) from *O. sanctum* leaves and stems is novel. The structures of these compounds were established using ^{1}H- and ^{13}CNMR spectral data. Compounds eugenol (**1**) and E-6-hydroxy-4, 6-dimethyl-3-heptene-2-one (**2**) exhibited mosquitocidal activity at 200 and 6.25 $\mu g \cdot ml^{-1}$ in 24 h, respectively, on fourth instar *Aedes aegyptii* larvae.

* M. A. Kelm and M. G. Nair (1998) Mosquitocidal compounds and a triglyceride, 1, 3-dilinoleneoyl-2-palmitin, from *Ocimum sanctum*. *J. Ag. Food Chem.* **46**, 3092-3094.

Introduction

Ocimum sanctum L. (Lamiaceae) has been used for generations in Southeast Asian medicine and cuisine. *O. sanctum* or "Sacred Basil", as the Ayurvedic herbal drug, has been used to treat a variety of human ailments (Thakur et al., 1989; Butani et al., 1982). Malarial fevers, ringworms, and other cutaneous afflictions also have been treated with this plant (Butani et al., 1982). According to African folk medicine, *O. sanctum* was reported to repel mosquitoes, and have subterfuge and poultice effects (Batta and Santhakumari, 1970). Also, the crude extracts from *O. sanctum* have demonstrated biological activities against certain insects (Risvi, 1981). For example, crude alcoholic extracts exhibited aphidcidal properties (Stein et al., 1988), antifeedant activities on Jute semilooper, *Anomis sabulifera* (Malik and Rafique, 1989), and mosquito repellent and toxic properties (Batta and Santhakumari, 1970; Deshmukh et al., 1982).

Previous phytochemical studies of *O. sanctum* have led to the isolation of flavones and flavone glycosides, including; apigenin (Nair et al., 1982), apigenin-7-*O*-glucoside (Nair et al., 1982), cirsilineol (Nörr and Wagner, 1992), galuteolin (Nörr and Wagner, 1992), luteolin (Nair et al., 1982), luteolin-7-*O*-glucoside (Nair et al., 1982), molludistin (Nair et al., 1982), orientin (Nair et al., 1982), and vicenin-2 (Nörr and Wagner, 1992). Other compounds identified in *O. sanctum* were eugenol (Laakso et al., 1990), rosmarinic acid (Nörr and Wagner, 1992), β-sitosterol (Sukari et al., 1995), stigmasterol (Sukari et al., 1995), triacontanol

ferulate (Sukari et al., 1995), and ursolic acid (Nair et al., 1982). Subsequently, the phenyl propane glycosides 4-allyl-1-*O*-β-D-glucopyranosyl-2-hydroxybenzene and 4-allyl-1-*O*-β-D-glucopyranosyl-2-methoxybenzene were isolated (Nörr and Wagner, 1992). GC-MS and GC-FTIR analyses of the essential oil of *O. sanctum* revealed the presence of 20 compounds (Laakso et al., 1990). From these studies eugenol, methyl chavicol, and β-bisbolenes were found to be the major components in the essential oil.

Past research on *O. sanctum* focused on the biological activity of crude extract. Typically, phytochemical investigations carried out on *O. sanctum* extracts did not lend to the isolation and structural identification of biologically active compounds. The work in our laboratory, in part, involves the preliminary screening of many plant and microbial extracts in order to determine the presence of any biologically active compound. In this paper, we report, for the first time, the isolation and structure determination of a novel triglyceride (**3**) and two mosquitocidal compounds (**1**, **2**) from the leaf and stem hexane extract of *O. sanctum*.

Materials and Methods

General Experimental. ^{1}HNMR, DQFCOSY, NOESY, and HMQC spectra were recorded at 300 and 500MHz. ^{13}CNMR and DEPT spectra were recorded at 126 MHz. Chemical shifts were recorded in $CDCl_3$, and the values are in δ

(ppm) based on residual of $CHCl_3$ 7.24 and $CDCl_3$ 77.0. Coupling constants, J, are in Hz. EIMS were recorded at 70 eV. Particle size of silica gel used in VLC and MPLC was 35-70 μm. All PLTC purifications were carried out on 250- and or 500-μm silica gel plates. Spots and bands were visualized under UV light (366 and 254 nm).

Gas chromatographic analyses were performed utilizing a flame ionization detector which was set at 260°C. The capillary column used for the analysis was a DB-5 (30 m x 0.25 mm ID). The temperature for the analysis was programmed from 150° (4 min) to 250°C (5 min) at $4°C \cdot min^{-1}$ with a helium carrier gas at a linear velocity of 34 $cm \cdot sec^{-1}$ and with split injections.

Plant Material. A voucher plant specimen (MSC 360851) was filed with the Beal-Darlington herbarium, Department of Botany and Plant Pathology, Michigan State University. Leaves and stems of *O. sanctum* were harvested from plants maintained in the Pesticide Research Center greenhouses at Michigan State University. Plant materials were then freeze-dried, milled, and stored at -20°C until extraction.

Mosquitocidal Bioassay. Fourth instar mosquito larvae, *Aedes aegyptii* L. were reared in our laboratory from eggs (courtesy of Drs. Alexander Raikal and Alan Hays, Department of Entomology, Michigan State University). Eggs were hatched in 500 mL of distilled, degassed water prepared by sonication (30 min).

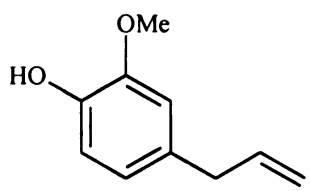
Approximately 5 mg of bovine liver powder was added to the water to provide a food source. After four days, the fourth instar mosquito larvae were ready for bioassay. At least 10 larvae were placed in 980 µL of degassed, distilled H_2O. To this, 20 µL of DMSO containing the appropriate concentration of test extract or purified compounds was added and left at room temperature. Extracts were tested at 250 ppm. Pure compounds were tested initially at 100-250 ppm then were diluted serially and subsequently bioassayed to determine LC_{100}. 4 mL test tubes were used for the bioassay. There were three replicates per treatment. The number of dead larvae were recorded at 2-, 4- and 24-h intervals. The control tube containing at least 10 larvae received 20 µL of DMSO alone, and mortality was recorded as in the case of test compounds. These bioassays were conducted according to previously published works (Roth et al., 1998; Nitao et al., 1991; Nair et al., 1989).

Saponification and Methylation of Compound **3**. With stirring, 6.6 mg of **3** was treated with 5% NaOH in MeOH (1 mL) for 5 min. Methanolic 6N HCl then was added to acidify this solution. This material was dried under a stream of nitrogen. Diazomethane was prepared by reacting N-nitotroso-N-methyl urea with concentrated KOH solution under ether. As the diazomethane product formed, it dissolved into the organic ether phase. This yellow-colored ether solution containing the diazomethane product then was collected and used to methylate the free fatty acids obtained in the previous step. The methylated product was filtered to remove any solids prior to GC analyses.

Extraction and Isolation of Compounds **1**, **2**, *and* **3**. The freeze-dried *O. sanctum* leaves and stems (440 g) were extracted sequentially with hexane, EtOAc, and MeOH. 750 mL of each solvent was used for the first 12 hours. Thereafter, a second 750 mL of fresh solvent was used to extract the plant material for an additional 12 hours. The hexane extraction afforded 13.1 g residue upon removal of solvent. A portion of this residue (10.4 g) was fractionated on a silica gel VLC (200 g) using: 4:1 pentane-Et_2O as the mobile phase. A 600 mL sintered funnel was used for the VLC. Dimensions of the MP column were 250 mm x 25 mm. Fraction collection was based on the color of bands observed through the VLC sintered funnel as well as the MP column. Flow rates for MPLC experiments were approximately 1-2 mL per min. Fractions collected in the VLC experiment were 1 (375 mL), 2 (200 mL), and 3 (300 mL). Next, elution with 2:1 pentane-Et_2O yielded fractions 4 (650 mL) and 5 (250 mL). This was followed by elution with 1:1 pentane-Et_2O, which gave fractions 6 (250 mL), 7 (500 mL), and 8 (300 mL), and then elution with Et_2O which gave fractions 9 (325 mL) and 10 (200 mL). Finally, elution with $CHCl_3$ and MeOH afforded fractions 11 (500 mL) and 12 (875 mL), respectively. All fractions were bioassayed, and only fraction 2 was found to be mosquitocidal. Fraction 2 was purified further with silica gel MPLC using hexane-Me_2CO solvent systems to yield 7 fractions. The seven solvent systems used and the volume of each fraction collected were as follows: 1 (hexane; 110 ml), 2 (40:1; hexane:acetone; 90 mL), 3 (25:1; hexane:acetone; 90 mL), 4 (10:1; hexane:acetone; 90 mL), 5 (4:1; hexane:acetone; 190 mL), 6 (acetone; 95 mL), and 7 (acetone; 250 mL).

The seventh fraction was found to be mosquitocidal. The MeOH-soluble portion of this fraction was finally purified by repeated preparative TLC to give compounds **1** (31.9 mg), **2** (10.1 mg) and **3** (8.8 mg). Initially, compound **1** was purified using 100% EtOAc as the mobile phase. 8:1 hexane-EtOAc followed by 10:1 hexane-acetone mobile phase ultimately led to the isolation of compound **1**. Like compound **1**, compounds **2** and **3** were purified initially with 100% EtOAc as the mobile phase. Isolation of compounds **2** and **3** was accomplished using 2:1 and 1:1 EtOAc-hexane mobile phases, respectively.

Compound **1**, a pale brown oil: ^{1}HNMR ($CDCl_3$) δ: 3.30 (d, 2H, J=6.6 Hz, H-7), 3.86 (s, 3H, -OCH_3), 5.04 (dd, 1H, J=9.0 Hz, J=1.5 Hz, H-9 *cis*), 5.05 (dd, 1H, J=18.0 Hz, J=2.4 Hz, H-9 *trans*), 5.47 (s, 1H, -OH), 5.93 (m, 1H, H-8), 6.67 (d, 1H, J=7.8 Hz, H-6), 6.67 (s, 1H, H-3), 6.83 (dd, 1H, J=8.7 Hz, J=1.5 Hz, H-5); ^{13}CNMR ($CDCl_3$) δ: 39.9 (C-7), 55.8 (-OCH_3), 111.0 (C-9), 114.1 (C-8), 115.5 (C-6), 121.1 (C-5), 131.9 (C-3), 137.8 (C-4), 143.7 (C-2), 146.3 (C-1). The spectral data of this compound were identical to an authentic sample purchased from Aldrich.



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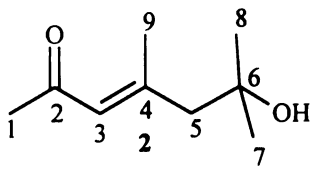
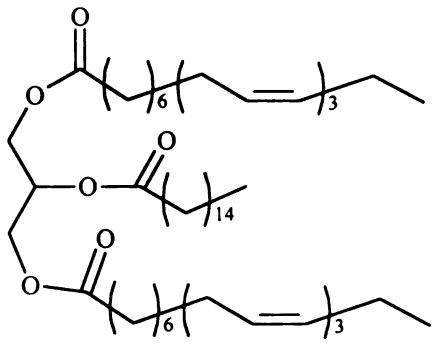
Compound **2**, a colorless oil: ^{1}HNMR ($CDCl_3$) δ: 1.23 (s, 6H, H-7, 9), 1.88 (s, 3H, H-8), 2.14 (s, 3H, H-1), 2.56 (s, 2H, H-5), 4.25 (s, 1H, -OH), 6.01 (s, 1H, H-3); ^{13}CNMR ($CDCl_3$) δ: 21.0 (C-8), 27.8 (C-1), 29.3 (C-7, 9), 53.8 (C-5), 69.8

(C-6). 124.5 (C-3), 157.4 (C-4), 202.3 (C-2). Therefore, it is identified as *E*-6-Hydroxy-4, 6-dimethyl-3-heptene-2-one. ^{1}HNMR data were found to be in agreement with previously published data (Kimura et al., 1982).



Compound **3**, a pale yellow oil: EI-MS *m/z* (rel. int.): 611 $(M^{+}-C_{16}H_{31}O]^{+}$ (9), 262 $[C_{18}H_{29}O+H, 85]^{+}$ (85), 261 $[C_{18}H_{29}O]^{+}$ (42), 239 (15), 230 (15), 108 (69), 95 (100), 81 (96), 53 (93); ^{1}HNMR ($CDCl_3$) δ: 0.86 (bt, 3H, H-16"), 0.95 (t, 6H, J=7.5 Hz, H-18'), 1.25 [m, 40H, H-(4'-7')×2 and H-(4"-15")], 1.58 [m, 6H, H-(3'×2), 3"], 2.01 [m, 8H, H-(8', 17')×2], 2.29 (t, 4H, J=7.5 Hz, H-2'×2), 2.28 (t, 2H, J=7.5 Hz, H-2"), 2.76 (m, 8H, H-11', 14'×2), 4.12 (dd, 2H, J=18.0, 6.0 Hz, H-1a, 3a), 4.36 (dd, 2H, J=16.2, 4.2 Hz, H-1b, 3b), 5.24 (m, 1H, H-2), 5.34 (m, 12H, H-9', 10' 12', 13', 15', 16'×2); ^{13}CNMR ($CDCl_3$) δ: 14.1 (C-16"), 14.3 (C-18'×2), 27.2 (C-8', 17'×2), 22.6, 22.7, 24.8, 24.9, 29.0-29.7 (C-4'-7'×2, 4"-15"), 25.5, 25.6 (C-11', 14'×2), 31.5 (C-3'×2), 31.9 (C-3"), 34.0 (C-2'×2), 34.2 (C-2"), 62.1 (C-1, 3), 68.9 (C-2), 127.1-131.9 (C-9', 10', 12', 13', 15', 16'×2), 172.3 (C-1'×2), 173.3 (C-1"). The spectral data confirmed that this compound is 1, 3-dilinoleneoyl-2-palmitin.



3

Results and Discussion

Three compounds, **1-3**, were isolated from the leaf and stem hexane extract of *O. sanctum* by successive silica gel VLC, MPLC, and preparative TLC. The ^{1}HNMR spectrum of compound **2** contained only five singlets. The peak furthest upfield at δ 1.23, integrated for 6 protons, indicated the presence of two deshielded magnetically equivalent methyl groups, C-7 and C-8. Deshielding of these methyl groups occurred as a result of a hydroxy group at C-6. Methyl protons at C-1 and C-9 at δ 2.14 and 1.88, respectively, were indicative of methyls attached to a carbonyl carbon and an olefinic carbon. From the ^{13}CNMR spectrum, it was concluded that compound **2** contained an α, β unsaturated ketone moiety (C-3, C-4, and C-2, respectively) with an additional oxygenated carbon (C-6). This oxygenated carbon was determined to be a tertiary alcohol as indicated by the disappearance of a singlet at δ2.56 following a D_2O shake in the ^{1}HNMR spectrum. The DEPT spectrum supported the ^{1}HNMR and ^{13}CNMR data as well as indicating the presence of three non-protonated carbons at 202.3, 157.4, and 69.8 ppm for one carbonyl, one olefinic, and one oxygenated carbon, respectively. Finally, the stereochemistry of **2** was determined to be *E* by

NOESY since no correlations were observed for the olefinic proton on C-3 and the methyl protons on C-4. Therefore, compound **2**, was confirmed to be *E*-6-hydroxy-4, 6-dimethyl-3-heptene-2-one. The ^{1}HNMR spectrum of **2** was identical to previously published findings (Kimura et al., 1982). The structure of compound **3** was determined using ^{1}H- and ^{13}CNMR, DEPT, and DQFCOSY spectral data. Also, two-dimensional HMQC proton-carbon correlations facilitated the assigning of saturated carbons in the fatty acid side chains and provided further evidence to 1D-NMR experiments. The ^{1}HNMR signals at δ 4.12, 4.36 and 5.24 and ^{13}CNMR signals at δ 62.09 and 68.87 along with MS fragments at m/z 611 $[M^{+}-C_{16}H_{31}O]^{+}$, 262 $[C_{18}H_{29}O+H]^{+}$, and 261 $[C_{18}H_{29}O]^{+}$ confirmed that compound **3** is a triglyceride with C_{16} and C_{18} fatty acid esters. The overlapping multiplets at δ 5.34 for twelve protons correlated with unsaturated carbons at δ 127.09 to 131.94 in the HMQC spectrum indicated the presence of six double bonds in this molecule. Support for the chemical nature of the side chains came from the GC analyses of the methyl esters of fatty acids obtained from the hydrolyzed compound **3**. The GC profile confirmed the presence of the methyl esters of linolenic and palmitic acids with a ratio of 2:1, respectively. Also, retention times for both methyl esters were identical to those of authentic samples of linolenic and palmitic acid methyl esters analyzed under the same conditions.

The novel triglyceride, compound **3**, was not mosquitocidal. LD_{100} were 200 and 6.25 $\mu g \cdot mL^{-1}$ in 24 h for compounds **1** and **2**, respectively, when tested

against fourth instar *Aedes aegyptii* larvae. There was no mortality for control larvae.

In a previous report the phenylpropanoid, eugenol was found to act as an attractant to the beetle, *Maladera matrida* (Ben-Yakir et al., 1995). Eugenol, in previous studies, was found to comprise 30-70% of the essential oil in *O. sanctum* (Asthana et al., 1984). Compound **2** originally was isolated from green and red bell peppers, *Capsicum annum* L. (Kimura et al., 1982) and later identified in the culture broth $CHCl_3$ extract of *Streptomyces olivaceus* (Grote et al., 1990). The synthesis of **2** has been reported by Duperrier et al. (1975). To the best of our knowledge, compound **3** has not been reported previously as a natural product.

The isolation and identification of mosquitocidal compounds in *O. sanctum* supports earlier findings that had shown insecticidal activity in crude extracts (Batta and Santhakumari, 1970; Risvi, 1981; Stein et al., 1988; Malik and Rafique, 1989; Deshmukh et al., 1982). Additional insecticidal compounds will be isolated from extractions of *O. sanctum* leaves and stems using more polar solvents such as acetone. **Chapters 3**, **4** and **5** describe other biologically active compounds in the acetone extracts of *O. sanctum*.

Chapter 3

ANTIOXIDANT COMPOUNDS FROM *OCIMUM SANCTUM* LINN.*

Abstract –Antioxidant bioassay-directed extraction of the fresh leaves and stems of *Ocimum sanctum* and purification of the extract yielded compounds, cirsilineol (**1**), cirsimaritin (**2**), isothymusin (**3**), isothymonin (**4**), apigenin (**5**) and rosmarinic acid (**6**). Appreciable quantities of eugenol (70-80%) also were isolated along with these compounds. The structures of compounds **1**-**6** were established by spectroscopic methods. Compounds **1**, **5**, and **6** were isolated previously from *O. sanctum*, whereas compounds **2**, **3**, and **4** are identified for the first time in *O. sanctum*. Eugenol and compounds **1**, **3**, **4**, and **6** exhibited good antioxidant activity at 10 μM concentration.

* M. A. Kelm, G. M. Strasburg, and M. G. Nair Antioxidant compounds from *Ocimum sanctum* Linn. *Phytomedicine.*

Introduction

Flavonoids represent one of the most ubiquitous classes of polyphenolic secondary compounds found in higher plants. Many common fruits, vegetables, herbs, and plant products such as wine, juices, dried fruits are rich sources of flavonoids. More importantly, many of these compounds have demonstrated rather potent anti-oxidant activity by being able to block and or scavenge free radicals (Saija et al., 1995). The formation of oxygen free radicals is a normal phenomenon carried out in aerobic cells. The interaction of these free radicals with lipids produces hydroperoxides and peroxides, which in turn may act adversely with biological systems, resulting in cancer. By way of the free radical scavenging mechanism, flavonoids effectively negate the deleterious effects of hydroperoxides and peroxides. Free radical formation also can result from the presence of transition metal ions such as Fe^{+2}, which can act as free radical initiators. Alternatively, flavonoids can prevent the formation of free radicals by chelation or complexation with the transition metal free radical initiator.

Concerning structural activity relationships (SAR) of flavanoids, hydroxyl groups at the C_5 and C_7 and the C_2 - C_3 double bond were shown to be necessary for high inhibitory activity on xanthine oxidase (Cos et al., 1998). Xanthine oxidase forms superoxide radicals and hydrogen peroxide and is involved in the oxidation of hypoxanthine to xanthine to uric acid. Researchers also have found that a hydroxyl group at C_3' and at C_3 were essential for high

superoxide scavenging activity (Cos et al., 1998). In addition, substitution patterns on the B ring were important to antioxidant activity of flavonoids, with hydroxyl groups increasing activity by preventing lipid peroxidation (Arora et al., 1998 and Arora et al., 1997).

Many of the previous studies on *Ocimum sanctum* Linn. Lamiaceae have focused largely on the biological activity of crude extracts. A triterpene, ursolic acid, isolated from *O. sanctum* was shown to be effective in protecting against lipid peroxidation (Balenehru and Nagarajan, 1991). We have reported mosquitocidal compounds eugenol and (*E*)-6-hydroxy-4, 6-dimethyl-3-heptene-2-one from *O. sanctum* (Kelm and Nair, 1998). In the present study, we report for the first time the extraction, purification, and structural identification of anti-oxidant compounds from the fresh leaves and stems of *O. sanctum*. Also, the importance of the A ring substitution relative to anti-oxidant/free-radical scavenging activity are discussed.

Materials and Methods

General Experimental. ^{1}HNMR spectra were recorded at 300 and 500MHz. ^{13}CNMR and DEPT spectra were recorded at 126 MHz. Chemical shifts were recorded in DMSO-d_6, CD_3OD, and $CDCl_3$. The values are in δ (ppm) based on residual of DMSO 2.29; DMSO-d_6 39.7 and $CHCl_3$ 7.24; $CDCl_3$ 77.0. Coupling constants, J, are in Hz. EIMS were recorded at 70 eV. UV

experiments were carried out on a Shimadzu UV-260 spectrophotometer. Shift reagents were prepared and used according to Markham (1982). UV samples were prepared at 12.5-50 ppm concentrations. Particle size of silica gel used in VLC was 35-70 μm. PLTC purifications were carried out on 250, 500, 1000, and 2000 μm silica gel plates and 200 μm KC18 silica gel plates (60 Å). Spots and bands were visualized under UV light (366 and 254 nm).

Anti-oxidant assays. Anti-oxidant assays were conducted on crude extracts and pure compounds by analysis of model liposome oxidation by fluorescence spectroscopy peroxidation (Arora et al., 1998 and Arora et al., 1997). The procedure for the anti-oxidant assay is as follows. A mixture containing 5 μmol of 1-steroyl-2-linoleneoyl-*sn*-glycerol-3-phosphocholine and 5 μmol of fluorescence probe 3-(p-6-phenyl)-1, 3, 5-hexatrienyl) phenyl propionic acid was dried in a foil-covered round-bottom flask (to prevent degradation of probe) on a rotary evaporator. The resulting lipid film was suspended in 500 μL of MBSE buffer. The MBSE buffer contained 0.15 M NaCl, 0.1 mM EDTA (ethylenediamine tetra acetic acid di-sodium salt), 10 mM MOPS [3-(N-morpholine propane sulfonic acid), adjusted to pH 7.0] buffer and then treated with Chelex 100 chelating resin to remove trace-metal ions. The lipid-buffer mixture was subjected to ten-freeze thaw cycles using a dry ice/ethanol bath. Following the last thaw, the lipid-buffer suspension was extruded 29 times through a Liposofast extruder containing a polycarbonate membrane with a 100 nm pore size to produce unilamellar liposome. A 20 μL aliquot of this liposome

suspension was diluted to 2 mL with 200 μL of HEPES buffer (adjusted to pH 7.0), 100 μL of 1M NaCl (treated with Chelex 100), and 1.68 mL nanopure H_2O then incubated for 5 min at room temperature, followed by incubation at 23°C in a spectrophotometric cuvette. Peroxidation then was initiated by the addition of 20 μL of 0.5 mM $FeCl_2$ stock solution to achieve a final concentration of 5 μm of Fe^{+2} in the presence of test compounds or crude extracts (dissolved in DMSO). Two controls were used in which one contained test solvent (DMSO), liposome, and buffer, whereas the second one contained Fe^{+2}. Fluorescence intensity of these liposome solutions were measured at an excitation wavelength of 384 nm for every 3 min over a period of 21 min. The decrease in relative fluorescence intensity with time indicated the rate of peroxidation. TBHQ, BHT, and Vitamin E were used as positive controls for this study.

Plant Material. A voucher plant specimen (MSC 360851) of *O. sanctum* was filed with the Beal-Darlington herbarium, Department of Botany and Plant Pathology, Michigan State University. Leaves and stems of *O. sanctum* were harvested from plants maintained in the Center for Integrated Plant Systems greenhouses at Michigan State University. *O. sanctum* plants were grown in 11-inch plastic pots, using a mixture of 50% loam and 50% Bacto mix. Plants were watered on a daily basis and fertilized once a week with 20-20-20 Peters brand fertilizer. Leaves and stems were harvested periodically from 4 to 6 month-old plants.

Generalized Extraction of Plant Material. Three separate extractions (I-III) were carried out for the isolation of eugenol and compounds **1**-**6**. The following is a representative protocol used for the extraction of the plant material. 2.58 kg of fresh *O. sanctum* leaves and stems were chopped, blended with acetone (6.5 L), and allowed to set for 24 h. The acetone was removed *in vacuo* to yield an aqueous green suspension. Solids were collected in a Büchner funnel lined with a No. 4 Whatman filter paper to give extract A (18.33 g). The aqueous filtrate was sequentially extracted with $CHCl_3$ (5 x 250 mL) followed by EtOAc (5 x 250 mL) to provide extracts B (4.58 g) and C (12.12 g), respectively. The remaining aqueous phase was dried *in vacuo* to yield extract D (56.40 g).

Extraction I. 763.9 g of fresh leaves and stems were chopped into pieces approximately 10-15 mm long. These pieces were blended with acetone (2 L) the allowed to soak for 24 h. Thereafter, the mixture was filtered through a Büchner funnel lined with a No. 4 Whatman filter paper. The plant material was washed with several aliquots of acetone (3x50 mL). The filtrate was distilled to remove acetone, resulting in the production of a green precipitate suspended in an aqueous solution. The solids (Extract A; 10.5 g) were recovered by centrifuging at 10,000 rpm for 10 min at 4 °C. The remaining water was extracted with $CHCl_3$ (3 x 50 mL) to give upon drying *in vacuo*, Extract B (0.786 g). Extract C (17.9 g) was obtained simply by drying the water-soluble phase *in vacuo*.

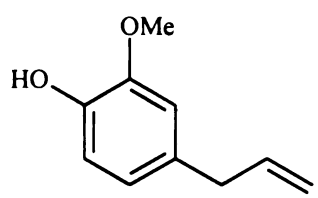
Extraction II. The second extraction carried out was essentially the same as the previous with the exception of several modifications as described below. 450 g of fresh *O. sanctum* was chopped, blended with acetone (1.2 L), and allowed to set for 24 h. The acetone was removed *in vacuo* to yield a green precipitate suspended in an aqueous solution. Solids were collected in a Büchner funnel lined with a No. 4 Whatman filter paper to give Extract A (4.53 g). The aqueous filtrate was sequentially extracted with $CHCl_3$ (3 x 50 mL) followed by EtOAc (3 x 50 mL) to provide Extract B (0.49 g) and Extract C (1.16 g), respectively. The remaining aqueous phase was dried *in vacuo* to yield Extract D (7.1 g).

Extraction III. The third extraction utilized 1.37 kg of plant material. The same procedure was followed as for the second extraction; however, larger volumes of solvents were used. Acetone (3.3 L), $CHCl_3$ (5 x 200 mL), and EtOAc (5 x 200 mL) were used to obtain Extracts A, B, and C, respectively. Weights of Extracts A, B, C, and D were 3.3, 3.3, 9.1, and 33.3 g, respectively.

Isolation of eugenol and compound **1.** Preparative TLC (silica gel; 3 x 2000 μm) of Extract B (477.3 mg) using $CHCl_3$:MeOH (15:1) as the mobile phase, afforded fractions, i-v. Fraction i (342 mg) and ii (14.4 mg) were determined to be pure by TLC. Fraction iii was purified further (silica gel; 500 μm) using $CHCl_3$:MeOH (25:1) which gave fractions 1-4. Here, fraction 3 (9.7 mg) was determined by TLC to be identical to fraction ii. A second preparative

TLC (silica gel; 4 x 2000 μm) of Extract B (290 mg) using $CHCl_3$:MeOH (15:1) resulted in fractions i-v. In this purification fractions i (195 mg) and ii (6.6 mg) determined to be pure, by TLC, were identical to fractions obtained from the first purification of Extract B. Fractions i from the first and final purification were combined and subsequently determined by NMR to be eugenol.

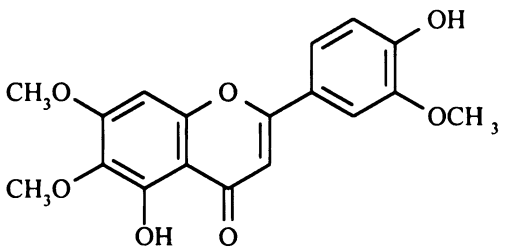
Eugenol, a pale brown oil: ^{1}HNMR ($CDCl_3$) δ: 3.30 (d, 2H, J=6.6 Hz, H-7), 3.86 (s, 3H, -OCH_3), 5.04 (dd, 1H, J=9.0 Hz, J=1.5 Hz, H-9 *cis*), 5.05 (dd, 1H, J=18.0 Hz, J=2.4 Hz, H-9 *trans*), 5.47 (s, 1H, -OH), 5.93 (m, 1H, H-8), 6.67 (d, 1H, J=7.8 Hz, H-6), 6.67 (s, 1H, H-3), 6.83 (dd, 1H, J=8.7 Hz, J=1.5 Hz, H-5); ^{13}CNMR ($CDCl_3$) δ: 39.9 (C-7), 55.8 (-OCH_3), 111.0 (C-9), 114.1 (C-8), 115.5 (C-6), 121.1 (C-5), 131.9 (C-3), 137.8 (C-4), 143.7 (C-2), 146.3 (C-1). The spectral data of this compound was identical to an authentic sample purchased from Aldrich Chemical Company, Inc. Milwaukee, WI 53233.



eugenol

Fractions ii, iii, and ii from the first, second, and third purifications, respectively were combined and subsequently determined to be compound **1** by NMR. **Cirsilineol; 5, 4'-dihydroxy-6, 7, 3'-trimethoxyflavone (1).** UV λ_{max} (MeOH) 341 nm, 275 nm; (5% $AlCl_3$/MeOH) 378 nm, 284 nm, 262 nm; ($AlCl_3$/HCl) 366 nm, 287 nm, 260 nm. ^{1}HNMR (DMSO-d_6) δ: 12.98 (bs, 1H, 5-OH), 7.67 (d, 1H, J=9 Hz, H-6'), 7.54 (s, 1H, H-2'), 6.92 (s, 1H, H-3), 6.90 (d, 1H,

J=8.0 Hz, H-5'), 6.89 (s, 1H, H-8), 3.91 (s, 3H, -OCH_3), 3.87 (s, 3H, -OCH_3), 3.72 (s, 3H, -OCH_3). ^{13}CNMR (DMSO-d_6) δ: 56.56 (-OCH_3), 57.09 (-OCH_3), 60.70 (–OCH_3), 92.28 (C-8), 103.25 (C-3), 105.65 (C-10), 116.57 (C-5'), 120.95 (C-6'), 121.35 (C-1'), 132.44 (C-6), 148.92 (C-5), 152.45 (C-9), 152.82 (C-3'), 153.31 (C-4'), 159.13 (C-7'), 164.78 (C-2), 182.68 (C-4).



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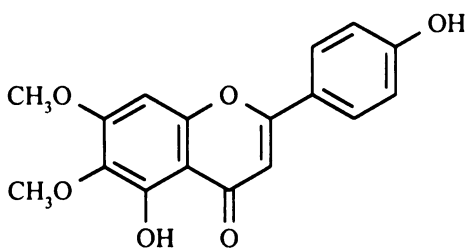
Isolation of compound **2**. Extract B from Extraction I was fractionated twice as previously mentioned. The third fraction (iii) from each of the purifications, 4.2 and 22.5 mg, respectively were found to be identical by TLC. Extract B (490.4 mg) from Extraction II was loaded onto preparative silica gel TLC plates (3x2000 μm) and developed with $CHCl_3$: MeOH (15:1) as the mobile phase. Bands i-v were isolated. Band i (318 mg) was determined to be compound **1** by comparing its TLC with a previously identified sample obtained. Band ii (10 mg) was found to be identical to both fractions iii above and these subsequently were combined. The combined fractions were subjected to further purification by preparative TLC (2x250 μm) with $CHCl_3$:MeOH (20:1) as the mobile phase. Bands i-iv were obtained. Fraction iii (3.5 mg) was found to be suitable for NMR and afterwards was determined to be compound **2**.

Cirsimaritin; 5, 4'-dihydroxy-6, 7-dimethoxyflavone (2). UV λ_{max} (MeOH) 329

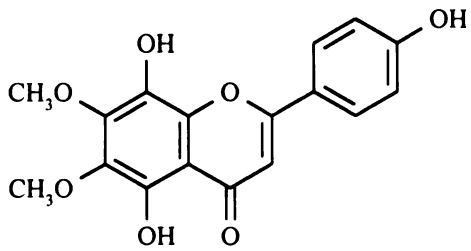
nm, 276 nm; (5% $AlCl_3$/MeOH) 362 nm, 284 nm; ($AlCl_3$/HCl) 356 nm, 288 nm. ^{1}HNMR (DMSO-d_6) δ: 7.94 (d, 2H, J=9.0 Hz, H-2', 6'), 6.92 (s, 1H, H-3), 6.90 (d, 2H, J= 9Hz, H-3', 5'), 6.82 (s, 1H, H-8), 3.91 (s, 3H, -OCH_3-6), 3.72 (s, 3H, -OCH_3-7). ^{13}CNMR (DMSO-d_6) δ: 56.51 (-OCH_3-7), 60.10 (-OCH_3-6), 91.55 (C-8), 102.30 (C-3), 105.04 (C-10), 116.20 (C-3', 5'), 120.30 (C-1'), 128.48 (C-2', 6'), 131.83 (C-6), 152.09 (C-5), 152.61 (C-9), 158.55 (C-7), 162.35 (C-4'), 164.23 (C-2), 182.16 (C-4).



2

*Isolation of compounds **3** and **4**.* Fraction iii from Extract B of Extraction I and fraction iv from Extract B of Extraction II were combined (17.6 mg). Preparative TLC silica gel (2x250 μm) using $CHCl_3$:MeOH (10:1) as the mobile phase was carried out on the mixture. Bands 1-4 were obtained, and fraction 4 was determined to be pure by TLC and subsequently was determined to be compound **3** (6.7 mg) by NMR studies. **Isothymusin; 5, 8, 4'-trihydroxy-6, 7-dimethoxyflavone (3).** UV λ_{max} (MeOH) 328 nm, 306 nm; (5% $AlCl_3$/MeOH) 360 nm, 322 nm, 289 nm, 234 nm; ($AlCl_3$/HCl) 352 nm, 322 nm, 288 nm. ^{1}HNMR (CD_3OD) δ: 7.93 (d, 2H, J= 9 Hz, H-2', 6'), 6.92 (d, 2H, J=9.3 Hz, H-3', 5'), 6.63 (s, 1H, H-3), 4.02 (s, 3H, -OCH_3), 3.91 (s, 3H, -OCH_3). ^{13}CNMR (CD_3OD)

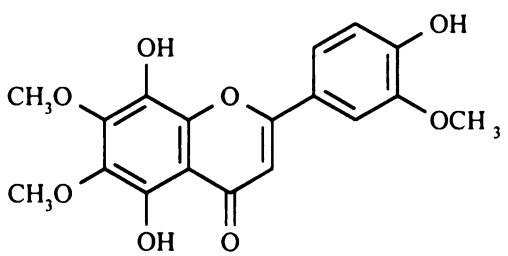
δ: 61.35 (–OCH_3), 61.99 (–OCH_3), 104.46 (C-3), 107.94 (C-10), 116.99 (C-3', 5'), 123.30 (C-2', 6'), 132.25 (C-8), 137.81 (C-6), 142.86 (C-7), 146.42 (C-5), 149.20 (C-9), 162.92 (C-4'), 166.74 (C-2), 184.84 (C-4).



3

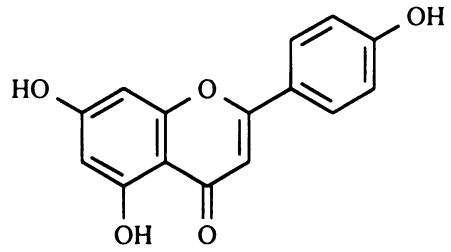
Band 4 from both purifications of Extract B, Extraction I were combined (5.2 mg) and purified using silica gel preparative TLC (1x250 μm) and $CHCl_3$:MeOH (15:1) as the mobile phase. Bands 1-3 were isolated. The NMR analysis of fraction I resulted in the identification of compound **4** (1 mg).

Isothymonin; 5, 8, 4'-trihydroxy-6, 7, 3'-trimethoxyflavone (4). UV λ_{max} (MeOH) 340 nm, 288 nm; (5% $AlCl_3$/MeOH) 450 nm, 371 nm, 327 nm, 292 nm, 263 nm; ($AlCl_3$/HCl) 450 nm, 364 nm, 331 nm, 293 nm, 259 nm. ^{1}HNMR (CD_3OD) δ: 7.61 (dd, J= 2.1, 9.0 Hz, 1H, H-6'), 7.60 (d, J=1.8 Hz, 1H, H-2'), 6.93 (d, J=8.7 Hz, 1H, H-5'), 4.03 (s, 3H, -OCH_3-6), 3.96 (s, 3H, -OCH_3-7), 3.91 (s, 3H, -OCH_3-3').



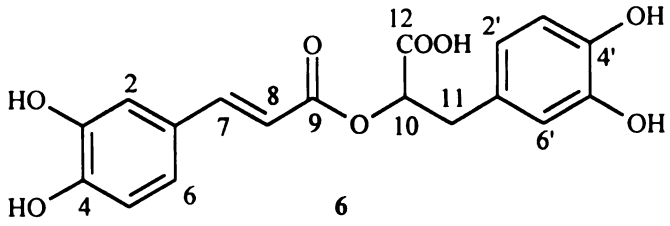
4

Isolation of compound ***5***. Fractionation of Extract B from Extraction III was carried out using VLC. Extract B (3 g) was loaded onto a bed of silica gel (75 g) preconditioned in hexane. Fractions were collected in aliquots of 125 (1), 150 (2), 100 (3), 120 (4), 160 (5), 160 (6), 150 (7), 210 (8), and 1,200 (9) mL using hexane, hexane:acetone (1:1), hexane:acetone (1:1), hexane:acetone (1:1), acetone, $CHCl_3$, $CHCl_3$:MeOH (1:1), $CHCl_3$:MeOH (1:1), and MeOH, respectively. The acetone-soluble portion of fraction 3 (190 mg) was dissolved in MeOH and concentrated. This was allowed to stand overnight at room temperature, resulting in the precipitation of solids. The mother liquor was recovered and dried *in vacuo* to yield a brown gum (160 mg). A portion of this was subjected to repeated silica gel PTLCs using $CHCl_3$:MeOH (30:1, 10:1), to afford compound **5**, apigenin (1.6 mg). **Apigenin; 5, 7, 4'-trihydroxyflavone (5).** UV λ_{max} (MeOH); (5% $AlCl_3$/MeOH); ($AlCl_3$/HCl). ^{1}HNMR (DMSO-d_6) δ: 12.86 (bs, 1H, 5-OH), 7.73 (d, 1H, J=8.7 Hz, H-6'), 7.70 (d, 1H, J=8.7 Hz, H-2'), 6.79 (d, 1H, J=8.7 Hz, H-5'), 6.76 (d, 1H, J=8.7 Hz, H-3'), 6.34 (s, 1H, H-3), 5.80 (s, 1H, H-6), 5.57 (s, 1H, H-8), 3.44 (bm, 2H, 7, 4'-OH).



5

Isolation of Compound ***6***. Prior to fractionation, the EtOAc extract C (1.16 g) was first dissolved in MeOH (1 mL), stirred with $CHCl_3$ (16 mL) and refrigerated overnight. The precipitate (630 mg) was recovered and dried *in vacuo*. This precipitate (630 mg) was purified by medium pressure silica gel column chromatography. Fractions were collected in aliquots of 750 (1), 1000 (2), 1000 (3), 1000 (4), 1500 (5), 200 (6), 900 (7), and 850 (8) mL using $CHCl_3$:MeOH (3:1), MeOH:$CHCl_3$ (2:1, 4:1, 4:1), 100% MeOH, 2 x 1% HCOOH (in MeOH), 100% MeOH, respectively. The active fraction 2 (233 mg) was dissolved in MeOH (5 mL) and precipitated with $CHCl_3$. The pale yellow solids were recovered and dried *in vacuo* to yield compound **6** (62.4 mg). **Rosmarinic acid (6)**. UV λ_{max} (MeOH) 349 nm, 338 nm, 223nm. IR ν_{max} cm^{-1} 3350, 3250,1689, 1600, 1524. ^{1}H-NMR (DMSO-d_6) δ: 7.37 (d, 1H, J=15.9 Hz, H-7), 7.04 (d, 1H, J=1.8 Hz, H-2), 6.92 (dd, 1H, J=9.0, 2.1, 2.1 Hz, H-6), 6.75 (d, 1H, J=2.1 Hz, H-5), 6.67 (d, 1H, J=2.1 Hz, H-2'), 6.60 (d, 1H, J=7.8 Hz, H-5'), 6.49 (dd, 1H, J=9.0, 2.1, 2.1 Hz, H-6'), 6.18 (d, 1H, J=16.2 Hz, H-8), 4.89 (1H, m H-10), 3.04 (1H_a, m, H-11), 2.77 (1H_b, m, H-11). ^{13}C-NMR (DMSO-d_6) δ: 173.23 (C-12), 166.46 (C-9), 148.38 (C-4), 145.73 (C-3), 144.82 (C-1), 144.50 (C-4'), 143.49 (C-3'), 130.01 (C-1'), 125.76 (C-8), 121.24 (C-7), 119.93 (C-2'), 116.70 (C-2), 116.03 (C-5'), 115.47 (C-5), 114.99 (C-6'), 114.87 (C-6), 75.98 (C-10), 30.76 (C-11).



6

Results and Discussion.

Seven compounds, eugenol and **1-6**, were isolated from the fresh leaf and stem extracts of *O. sanctum*. Structure determination was facilitated by ^{1}H and $^{13}CNMR$ experiments. Final position assignments of flavone hydroxyl groups were achieved by the use of different shift reagents in UV fluorescence experiments. (Markham, 1982). According to Markham's *Interpretation of UV spectra*, compound **1** in MeOH indicated the presence of a flavone with strong absorption at 341 and 275 nm. In fact, the UV spectra of compounds **2-5** indicated the presence of flavone. Following the addition of $AlCl_3$ stock solution, a +37 nm shift in Band I in the spectrum of compound **1** was observed. This shift was indicative of a hydroxyl substitution at the C-5 position. The presence of the 5-OH hydrogen bonded to the carbonyl oxygen at C-4 was confirmed by the chemical shift of the –OH signal at δ 12.98. As deduced from the $AlCl_3$/HCl spectrum, the presence of A or B ring *o*-diOH substitutions was not observed. Two doublets at δ 7.67 (J = 9.0 Hz) and at 6.90 (J = 8.0 Hz) corresponded to protons H-6' and H-5', respectively. Singlets at δ 6.92 and δ 6.89 represented

protons at H-3 and H-8, respectively. Intense, strong three proton singlets at δ 3.91, 3.87, and 3.72 were assigned to methoxy protons. These proton data were found to be in agreement with those previously published (Martínez, et al., 1987).

Compound **2**, cirsimaritin was isolated previously from the methanolic extract of aerial parts of *Baccharis trimera* (Asteraceae) Less. (Grayer and Veitch, 1998), the diethyl ether extract of freeze-dried leaves of *Becium grandiflorum* (Lamiaceae) Pic. Serm. (Yousseff and Frahm, 1995), the ethanolic extract of the aerial parts of *Centaurea scoparia* (Asteraceae) Sieb. (Zhu et al., 1996), the ethanolic (70%) extract of the air-dried root bark of *Clerodendrum mandarinorum* (Verbenaceae) Diels. (Cuvelier et al., 1996), and was identified by HPLC in two genera of the Lamiaceae family, *Salvia officinalis* L. and *Rosmarinus officinalis* L. (Barberán et al., 1996).

The 5-OH and the 4'-OH for compound **2** was observed at δ 12.93 and 10.34, respectively. Two three-proton singlets at δ 3.91 and 3.72 were assigned to methoxy groups at C-7 and C-6, respectively. Two proton doublets δ 7.94 (J=9.0 Hz) and 6.90 (J=9.0 Hz) indicated the presence of a *para* substituted aromatic ring. These data were found to be in agreement with previously published data (Zhu et al., 1996). A +25 nm shift in the UV spectrum following the addition of $AlCl_3$/HCl further indicated the presence of the 5-OH substitution. With $AlCl_3$ alone, shifts in band I were not large enough to indicate the presence of any *o*-diOH substitution, in particular the A ring of the molecule.

Isothymusin, compound **3**, was isolated from a natural source, *B. grandiflorum* for the first time (Yousseff and Frahm, 1995). Isothymusin (6, 7-dimethoxy-5, 8, 4'-trihydroxyflavone) was previously known as a conversion product via a Wessely-Moser rearrangement of the natural product, isothymusin (7, 8-dimethoxy-5, 6, 4'-trihydroxyflavone). A protocol for this conversion was reported (Horie et al., 1995).

In the present study, the identification of compound **3** relied largely on UV experiments, since ^{1}HNMR data for thymusin and isothymusin are interchangeable. A comparison of $AlCl_3$ and $AlCl_3$/HCl spectra of compound **3** revealed only a small change, 8 nm in Band I, which indicated the lack of *o*-diOH substitution pattern on the A ring. Our results are in agreement with those previously published by Barberán et al. (1985). Two 2H proton doublets at δ 7.93 (J = 9.0 Hz) and 6.92 (J = 9.3 Hz) were indicative of a *para* disubstituted aromatic ring. The above chemical shifts were assigned to H-2', 6' and H-3', 5', respectively. Two intense three-proton singlets at δ 4.02 and 3.91 were assigned to methoxy groups on the B ring at C-6 and C-7, respectively. ^{1}HNMR data is consistent with previously published work (Horie et al., 1995). Isothymusin can be formed from its isomer by a Wessely-Moser rearrangement (Ferreres et al., 1985).

Compound **4**, isothymonin, is reported herein for the first time as a natural product. Isothymonin, like isothymusin is obtained by means of acidic treatment

and Wessely-Moser rearrangement. The UV spectral interpretation for compound **4** was much like that carried out for compound **3**. ^{1}HNMR spectral data were very similar to data obtained for isothymusin with the addition of a three-proton singlet at δ 3.91 which was assigned to a methoxy group. The UV and ^{1}HNMR data for compound **4** were found to match closely previously published data (Barberán et al., 1985 and Horie et al., 1995).

Apigenin is probably one of the most common of the flavones. Apigenin, compound **5**, was previously identified in *O. sanctum* leaves (Nörr and Wagner, 1992), *O. basilicum* (Grayer et al., 1996), *B. trimera* (Youseff and Frahm, 1995), and in the extracts of *S. officinalis* and *R. offincinalis* (Cuvelier et al., 1996). The antioxidant activity of apigenin was reported previously (Cholbi et al., 1991). However, in another study (Chen et al., 1996), researchers found that apigenin was unable to prevent the oxidation of lipids in canola oil.

The isolation and identification of compound **6**, rosmarinic acid was reported from *O. sanctum* (Nörr and Wagner, 1992) and *S. officinalis* (Wang et al., 1998). Portions of the ^{1}HNMR spectrum, namely chemical shifts at δ 7.37 (J=15.9 Hz), 7.04 (J=1.8 Hz), 6.92 (J = 9.0, 2.1, 1.8 Hz), 6.75 (J = 8.4 Hz), and 6.18 (J=16.2 Hz) matched closely with those of caffeic acid. Furthermore, the acidic hydrolysis of **6** yielded caffeic acid, as indicated by comparative TLC. The chemical shifts at δ 7.37 (J = 15.9 Hz) and 6.18 (J = 16.2 Hz) were for doublets that integrated for one proton each. Coupling constants for both of these olefinic

protons (H-7, 8) indicated that they existed in a *trans* geometry. Protons at positions 2, 5, and 6 gave identical splitting patterns with 2', 5', and 6', respectively. Chemical shifts for H-2 and 2' were δ 7.04 (J = 1.8 Hz) and 6.67 (J = 2.1 Hz), respectively. Their J values were indicative of *meta* coupling protons. *Ortho* coupling was observed for H-5 and 5' doublets. A 1H doublet of doublet was assigned to H-6 and 6' at δ 6.92 and 6.49, respectively. Both *ortho* and *meta* coupling was observed for H-6 and 6' where J values were 9.0, 2.1, 1.8 Hz and 9.0, 2.1, 2.1 Hz, respectively. ^{13}CNMR experiments indicated the presence of two carbons at δ 173.23 and 166.46 which corresponded to the carboxylic acid and ester functional groups, respectively. Both ^{1}HNMR and ^{13}CNMR data agreed with published data (Wang et al., 1998).

Anti-oxidant assays were conducted according to previously published procedures (Arora et al, 1998 and Arora et al., 1997). Results for anti-oxidant bioassay are presented in Figure 1 and 2. All flavones with the exception of compound **2** demonstrated excellent anti-oxidant activity. Eugenol and rosmarinic acid (**6**) also displayed good anti-oxidant activity. The most notable compound in regards to anti-oxidant activity was compound **3**, isothymusin. These results suggest that hydroxy substitution on the A ring at C-8 enhances anti-oxidant activity, whereas the addition of a methoxy group at C-3' position inhibits the activity, as in the case of compounds **3** and **4**. However, in the case of compounds **1** and **2**, when the hydroxy at C-8 is lacking, the more active compound contains a methoxy at the C-3' position. Isothymusin, compound **3**,

was the best performer in regards to its anti-oxidant activity. Compound **3** was about 50% more active than the synthetic antioxidants TBHQ and BHT at the same 10 μM concentration. Compound **4** performed as well as compound **1** and better than BHT and TBHQ at 10 μM concentration. Compound **1** performed equally well if not better than TBHQ and BHT in our anti-oxidant assays (Figure 1, 2). Surprisingly, due to structural similarities, compound **2** displayed poor anti-oxidant activity in our anti-oxidant assays (Figure 1, 2).

The presence of eugenol in *O. sanctum* is well known. In fact, eugenol can comprise 38% of the water distilled essential oil of *O. sanctum* (Laakso et al., 1990) and up to 86% in the steam distillate (Sukari et al., 1988). In the same study, the essential oil of *O. sanctum* showed some inhibitory activity on *Trichophyton mertagrophytes* (Robin) Blanchard and *Bacillus subtilis* (Ehrenberg) Cohn at 10^4 ppm and 100% mortality on *Blattella germanica* L. and *Callosobrunchus chinensis* L. in the presence of 10 mg of the neat oil. In a later study, it was found that eugenol could demonstrate anti-stress activity by effecting stress-induced changes brought about by various biochemical parameters and cell membrane dynamics utilizing red blood cells (Sen et al., 1992). Eugenol's anti-oxidant activity prior to this paper was reported by Priyadarsini et al., 1998. In their study, inhibition of lipid peroxidation of rat brain homogenates induced by ferric ion, ferrous ion, and cumene hydroperoxide was demonstrated by eugenol at IC_{50} 74.2, 11.3, 136.8 $\mu mol \cdot dm^{-1}$, respectively. Eugenol's anti-oxidant activity was comparable to compound **6** (Figure 1, 2). The

presence of eugenol's 4-hydroxy group could be involved in the observed activity by formation of a less reactive phenoxyl radical.

Compound **1**, cirsilineol was previously identified in *O. sanctum* (Nörr and Wagner, 1992), *O. basilicum* (Grayer et al., 1996), and *Artemisia assoana* Willk. (Martínez et al., 1987). Cirsilineol was found to have potent 3', 5'-cyclic adenosine monophosphate phosphodiesterase (cAMP-PDE) inhibitory activity (Nagasugi et al., 1998). In this study the authors were able to correlate high cAMP-PDE-inhibitory activity with averaged ^{13}CNMR chemical shifts.

Cirsimaritin showed a 77% antimutagenic activity at 25 μM per plate in a modified Ames test. Apigenin was 85.2% effective at the same concentration in the same study (Grayer and Veitch, 1998). *S. officinalis* and *R. officinalis* extracts that contained cirsimaritin and apigenin demonstrated anti-oxidant activity as measured by accelerated auto-oxidation of methyl linoleneate (Barberán et al., 1996).

Compound **6** compared to other compounds in the study demonstrated high anti-oxidant activity. Compound **6** certainly was more active than Vitamin E, and compounds **2** and **5** (Figure 1, 2). The anti-oxidant activity of rosmarinic acid was reported previously from sage, *Salvia officinalis* where it was found to scavenge DPPH (2, 2-diphenyl picryhydrazyl) free radical-induced oxidation (Wang et al., 1998).

Figure 1. Anti-oxidant activity of synthetic and isolated compounds assayed at 10μM.

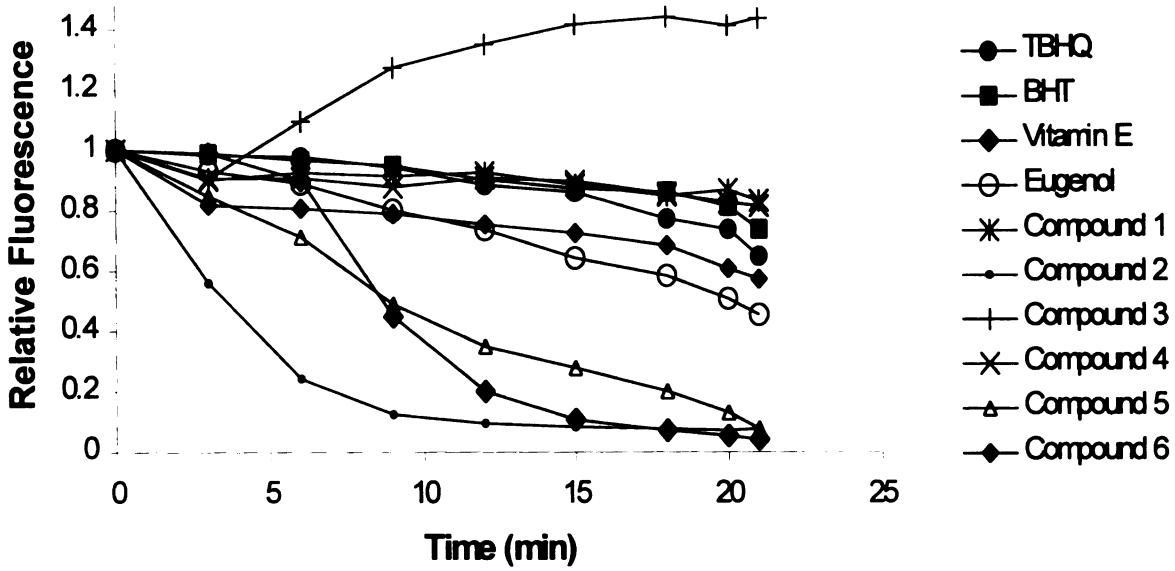
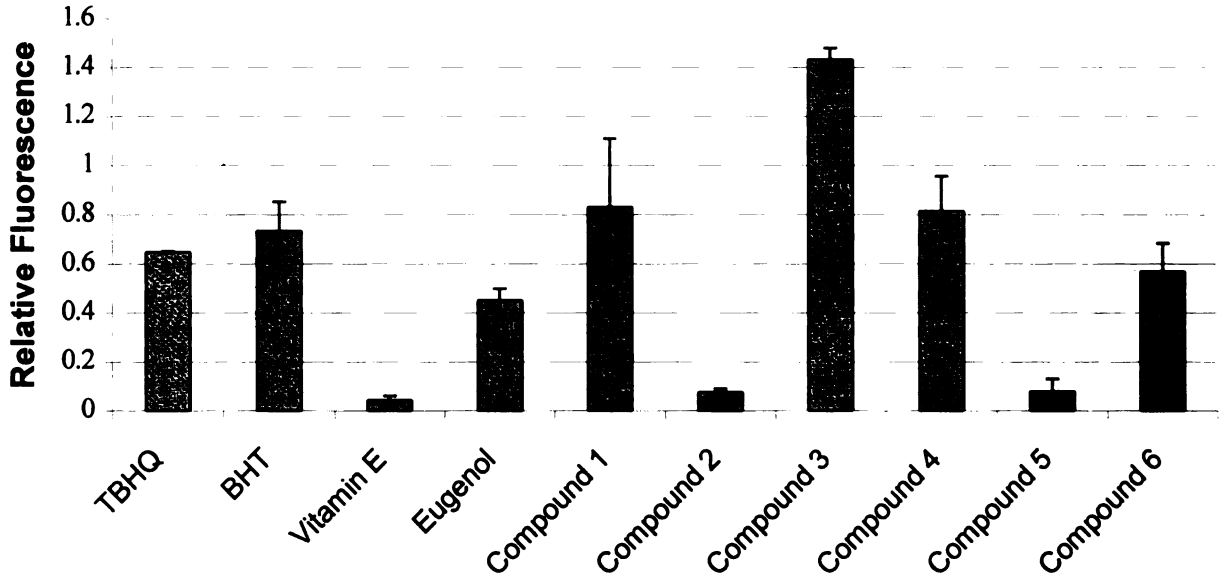


Figure 2. Anti-oxidant activity at 21 minutes for synthetic and isolated compounds at 10 μM.



Chapter 4

ANTI-INFLAMMATORY COMPOUNDS FROM *OCIMUM SANCTUM* LINN.

Abstract – Eugenol and compounds **1-6** described in the previous chapter were subjected to anti-inflammatory assays. Eugenol demonstrated 97% COX-1 inhibitory activity when assayed at 1000 μM concentrations. Compounds **1**, **2**, and **4-6** displayed 37, 50, 37, 65, and 58 % COX-1 inhibitory activity, respectively, when assayed at 1000 μM. Eugenol and compounds **1**, **2**, **5**, and **6** demonstrated COX-2 inhibitory activity at slightly higher levels when assayed at 1000 μM. The activities of compounds **1-6** were comparable to ibuprofen, naproxen, and aspirin tested at 10, 10, and 100 μM, respectively.

Introduction

The formation of prostaglandins from arachidonic acid by prostaglandin synthase is a well studied process. The formation and subsequent effect of prostaglandins can result in the stimulation of inflammation and associated pain (Stryer, 1988). Prostaglandin synthase contains both cyclo-oxygenase and hydroperoxidase components; however, it is well understood that two distinct isoforms of cyclo-oxygenase (COX) exist, namely COX-1 and COX-2 which are both involved in the conversion of arachidonic acid to prostaglandins (Lipsky et al., 1998). COX-1 is found throughout the body, particularly in the gastrointestinal tract, kidneys and platelets, whereas COX-2 is found predominantly in inflamed tissues. Both COX-1 and COX-2 are inhibited by traditional non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, ibuprofen, naproxen, sulindac, diclofenac, etc. Many of these drugs are taken to releive inflammation pain such as musculoskeletal pain including arthritis and tendonitis, as well as other general aches and pains. Since traditional NSAIDS are not specific in the inhibition of both COX forms, the inhibition of COX-1 seems to be associated with gastrointestal damage, renal dysfunction, and platelet abnormalities (Simon et al., 1998). Therefore, much of the research in the 1990s has been directed toward the discovery of NSAIDS with specific inhibition towards the enzyme COX-2.

As stated in the previous chapter, flavonoids are widespread components in the plant portion of the human diet. And, many of these have low toxicity in

mammals. Their action as anti-inflammatory agents and their low toxicity make them prime candidates in plant drug research. The anti-inflammatory activity of flavonoids is believed to be controlled, at least in part, by the addition of cyclo-oxygenase (Kim et al., 1998). Flavonoids such as 3-hydroxyflavone, galangin, quercetin, and kaempferol demonstrated good cyclo-oxygenase inhibitory activity in assays utilizing rat mixed peritoneal leukocytes (Hoult et al., 1994). Other researchers have reported similar findings (Kim et al., 1998, Tordera et al., 1994, Middleton and Kandaswani, 1992). In a study looking at structural activity relationships of flavonoids, it was determined that flavonoids lacking 3', 4'-dihydroxy substitution as well as fewer overall hydroxy groups had greater cyclo-oxygenase inhibitory activity (Moroney et al., 1988). More specifically, it was demonstrated that B-ring hydroxyl substitution decreased a flavonoid's ability to inhibit cyclo-oxygenase. Baumann et al., (1979) indicated that ortho dihydroxy-substituted phenols can act as initiators of cyclo-oxgenase by acting as cofactors in prostaglandin generation, specifically in cell-free assays.

The compounds isolated from *O. sanctum* are described in **Chapter 3** were eugenol, cirsilineol (**1**), cirsimaritin (**2**), isothymusin (**3**), isothymonin (**4**), apigenin (**5**) and rosmarinic acid (**6**). These compounds were subjected to anti-inflammatory assays in order to determine COX-1 and COX–2 inhibitory activities. Of the compounds tested, eugenol (Saeed et al., 1995), apigenin (**5**) (Todera et al., 1994 and Kim et al., 1998), and rosmarinic acid (**6**) have demonstrated anti-inflammatory activity. The COX-1 inhibitory activity of flavones, **1**, **2**, and **4** are described here for the first time. Eugenol, compounds

1, **2**, **5**, and **6** demonstrated better COX-2 inhibitory activity compared to COX-1 inhibitory activity. This would indicate specific enzyme inhibition towards COX-2.

Materials and Methods

Refer to **Chapter 3**, **Materials and Methods** section for *General Experimental, Plant Materials*, *Extraction*, and *Fractionation* procedures.

Anti-inflammatory Assay. Human prostaglandin H synthase isozymes (hPGHS-1) were expressed in *cos*-1 cells as described previously (Laneuville et al., 1994; Meade et al., 1993). Cyclo-oxygenase activity (COX) was measured by utilizing microsomal membranes (*ca.* 5 mg protein/ml in 0.1 M TrisHCL, pH 7.4) from sham-transfected *cos*-1 cells or from *cos*-1 cells transfected with the plasmid pOSML - PGHS - 1. Cyclo-oxygenase assays were performed at 37 °C by monitoring the initial rate of O_2 uptake using an O_2 electrode (Instech Laboratories, Inc., 5209 Militia Hill Road, Plymouth Meeting PA 19462-1216). Each assay mixture contained 3 mL of 0.1 M TrisHCl, pH 8.0, 1 mmol phenol, 85 µg hemoglobin and 100 µmol arachidonic acid. Reactions were initiated by adding 5 to 25 µg of microsomal protein in a volume of 10-20 µL.

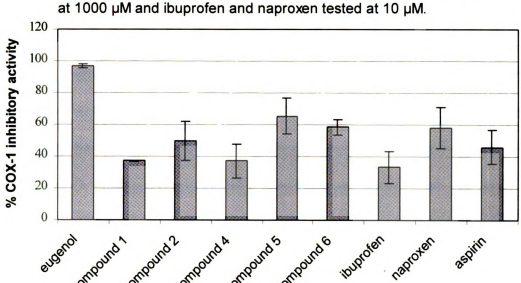
Instantaneous inhibition was determined by measuring the cyclooxygenase activity initiated by adding aliquots of microsomal suspensions of hPGHS-1 (10 µmol O_2/min cyclooxygenase activity/aliquot) to assay mixtures containing 10 µmol arachidonate and 1000 µM concentrations of the test

hPGHS-1 (10 μmol O_2/min cyclooxygenase activity/aliquot) to assay mixtures containing 10 μmol arachidonate and 1000 μM concentrations of the test compounds (Figure 3, 4). Ibuprofen and naproxen were assayed at 10 μM and aspirin at 1000 μM.

Results and Discussion

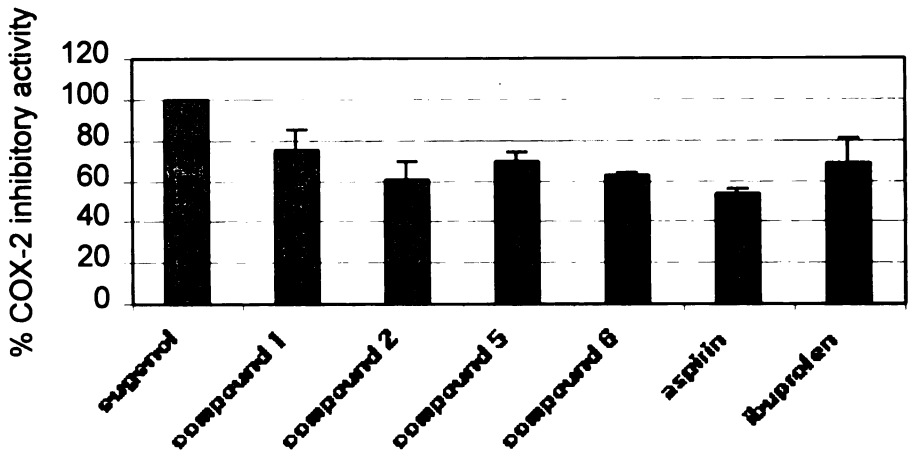
The in vitro anti-inflammatory activity of eugenol, compounds **1**, **2** and **4-6** all demonstrated inhibitory activity against COX-1 as expressed in Figure 3.

Figure 3. In vitro anti-inflammatory assay of eugenol, compounds 1-6 and synthetics. Eugenol, compounds 1-6, and aspirin tested at 1000 μM and ibuprofen and naproxen tested at 10 μM.



rosmarinic acid demonstrated 58% COX-1 inhibitory activity in comparison to the other compounds. The activities of eugenol, apigenin (**5**), and rosmarinic acid (**6**) were reported by Saeed et al., 1995, Todera et al., 1994, and Kim et al., 1998. The COX-1 inhibitory activities of compounds **1**, **2**, and **4** are reported here for the first time. At 1000 μM, compounds **1-6** demonstrated COX-1 inhibitory activity comparable to ibuprofen, naproxen, and aspirin at 10, 10, and 100 μM concentrations, respectively. Ibuprofen, naproxen, and aspirin demonstrated 33, 58, and 46% COX-1 inhibitory activity, respectively. COX-2 inhibitory activities were demonstrated by eugenol, compounds **1**, **2**, **5**, and **6** when assayed at 1000 μM (Figure 4). Compound **4** was unavailable for testing COX-2 inhibitory activity. Compound **3** did not inhibit COX-1 or COX-2 activity. This present study supports earlier findings and suggests possible phytochemicals relative to research on the anti-inflammatory activity of *O. sanctum*: Early studies had shown that methanolic and aqueous suspensions of *O. sanctum* showed anti-inflammatory activity in rats (Godwani, 1987); however, anti-inflammatory activity was found to be less active than aspirin. Singh et al., 1996, found that the volatile oil *of O. sanctum* could inhibit arachidonic and leukotriene-induced inflammation. They concluded that inhibition of cyclo-oxygenase and lipo-

Figure 4. In vitro anti-inflammatory activity of eugenol, compounds 1, 2, 5, 6, and aspirin tested at 1000 μM and ibuprofen tested at 10 μM.



oxygenase pathways in arachidonic acid metabolism might be occurring. The present findings tend to support these earlier findings; however, inhibition of enzymatic pathways might be only part of the overall activity. Tulsi has been shown to decrease levels of uric acid in rabbits (Sarkar et al., 1990). Elevated levels of uric acid are associated with gouty arthritis and other joint inflammation.

Structural activity relationships (SAR) of flavonoids **1**-**5**, relative to COX-1 inhibitory activity seemed dependent on number and position of hydroxy and methoxy groups on their A and B rings. Similar conclusions regarding SAR can be said of compounds **1**, **2**, **5**, and **6** regarding COX-2 inhibitory activity. Due to the small number of compounds being compared, unequivocal deductions

regarding SAR for these compounds cannot be made. Regardless, we can discuss to some extent, the results obtained. The 5, 7, 4'-trihydroxyflavone, apigenin (**6**) had the greatest COX-1 inhibitory relative to the other flavones. Methoxy groups at 6, 7, and 3' positions resulted in a decrease in activity. Compound **2**, slightly more active that **1**, had an additional methoxy group at the 3' position. One notable exception to this is the activity of compounds **3** and **4**. Compound **4** showed modest COX-1 enzyme-inhibitory activity, whereas compound **3** totally lacked COX-1 enzyme inhibitory activity. The only difference between these two compounds was a methoxy group at the 3' position.

These findings present some compounds that lend support to earlier observed anti-inflammatory activities of crude extracts from *O. sanctum*. Furthermore, these results support the traditional use of Tulsi as a remedy for inflammation and pain.

Chapter 5

A PORPHYRIN COMPOUND FROM *OCIMUM SANCTUM* LINN. WITH CORN EARWORM ANTI-FEEDANT/TOXICITY ACTIVITY

Abstract- A porphyrin compound characterized as phylloerythrin like compound was isolated from the crude acetone extract of *O. sanctum* by bioassay-guided fractionation. This compound was anti-feedant to corn earworm, *Helicoverpa zea* at 100 ppm.

Introduction

Insect toxicity (Narasimhan and Marriappan; 1988, Stein et al., 1988; Sukari and Takahashi, 1988), anti-feedant, repellant (Mallick and Banerji, 1989, Arrekul, et al., 1988) and oviposition deterrent activities (Sojitra and Patel, 1992) were reported for extracts of *O. sanctum*. In their investigations, only crude extracts were analyzed for bioactivity against insects. Biologically active components of these crude extracts, remain unknown.

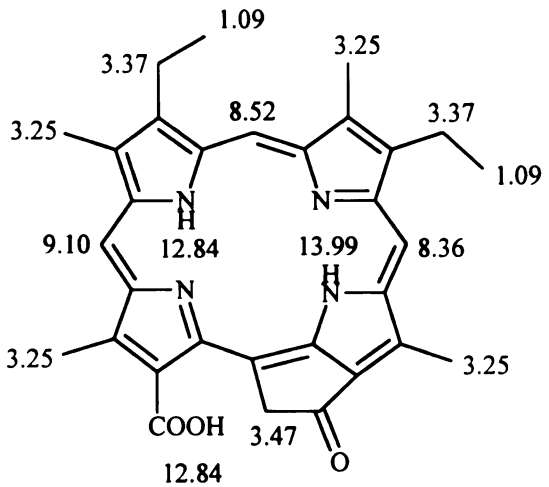
Preliminary bioassays indicated that the crude acetone extract from *O. sanctum* contained a compound or compounds that were demonstrating anti-feedant activity against *Helicoverpa zea*, corn earworm. This study undertook the bioassay-guided fractionation approach to determine the presence of insect anti-feedants or toxins utilizing *H. zea*, corn earworm bioassays. My initial hypothesis of finding compounds responsible for corn earworm anti-feedant activity evolved into a search for a porphyrin type compound as the fractionation progressed. A red fluorescing compound always seemed to be associated with the active fractions.

Materials and Methods

Refer to **Chapter 3,** Materials and Methods section for *General Experimental, Plant Materials*, and *Extraction* procedures.

Fractionation of crude acetone extract. A quantity of crude acetone extract (12.5 g) was suspended in acetone (125 mL) and then kept in a freezer at -20°C overnight to cause precipitation. After 24 h, the suspension was filtered through a bed of celite. The bioactive filtrate (3.3 g) subsequently was fractionated using reverse phase (Supelco bonded phase silica C-18) medium pressure column chromatography (MPLC). Solvents used and corresponding fractions collected were as follows: A (70:30; MeOH:H_2O), B (90:10; MeOH:H_2O), C-I (100% MeOH). Two fractions, H and I were found to be active and identical according to TLC and subsequently were combined. The combined fractions (173.2 mg) were subjected to preparative TLC (6x200 μm, KCF18 silica gel plates, 60 Å) using 80:20 MeOH:H_2O as the mobile phase which resulted in five fractions (A-E). The bioactive fraction, fraction D (6 mg) was subjected to a final preparative TLC experiment (1 x 200 μm, KCF18 silica gel plates, 60 Å) that resulted in two bands, A (1.6 mg) and B (1.9 mg). Band A was pure enough to conduct UV experiments. The UV absorption spectrum obtained was found to be identical to the published data for the porphyrin, phylloerythrin (Perrin, 1958); however, this band may contain other related porphyrin compounds as impurities with similar UV absorption spectra profiles. These other porphyrins may have similar biological activities. Similarities between ^{1}H-NMR data of porphyrins provided by Kenner et al. (1973) and the isolated compound were found. The tentative structure for the active compound was elucidated using UV and NMR studies. **Band A**; **Porphyrin**: UV λ_{max} (MeOH) 659 nm, 584 nm, 557 nm, 512 nm, 420 nm. ^{1}H-NMR (DMSO-d_6) δ: 12.84 (1H, s, N-H), 13.99 (1H, s, N-H),

12.84 (1H, s, COOH), 9.10, 8.52, 8.36 (3H, s, olefinic), 3.47 (2H, s, $-CH_2CO-$), 3.25 (4H, s, $-CH_3$), 3.37 (4H, m, CH_2CH_3), 1.09 (6H, t, CH_2CH_3).



Corn earworm Anti-feedant Assay. *Helicoverpa zea* Boddie Noctuidae eggs were hatched in an incubator at 27°C. Dry diet (940 mg) obtained from North Carolina Insectory was placed into glass vials. Crude extracts were dissolved in DMSO to give a concentration of 1250 mg·20μL^{-1}. Subsequent fractions were assayed at progressively lower concentrations. DMSO test solutions (20 μL) were mixed thoroughly with the dry diet (940 mg). DMSO (20μL) plus dry diet (940 mg) was used as the control. Agar solution/suspension (1.4%) was autoclaved 5 min at 15 psi at 125°C to encourage melting of the agar. The temperature of the agar was dropped to 45°C by the use of a water bath set. The agar solution then was pipetted into individual vials containing the test mixtures until a weight of 5 g was obtained. The vials were mixed thoroughly and poured into 3.5 mL polystyrene vials. One neonate larvae was place in each

vial. Vials were capped and placed in a growth chamber with a photoperiod of 16-h days and 8-h nights. Day and night temperatures averaged 27°C. There were 15 replications per treatment. Larvae were weighed after six days. Bioassay protocols were adapted from previously published work (Bell, R. A., and Joachin, F. G., 1976 and Joyner, K. and Gould, F., 1985). An analysis of variance (ANOVA) was conducted on the data using a completely randomized design (CRD).

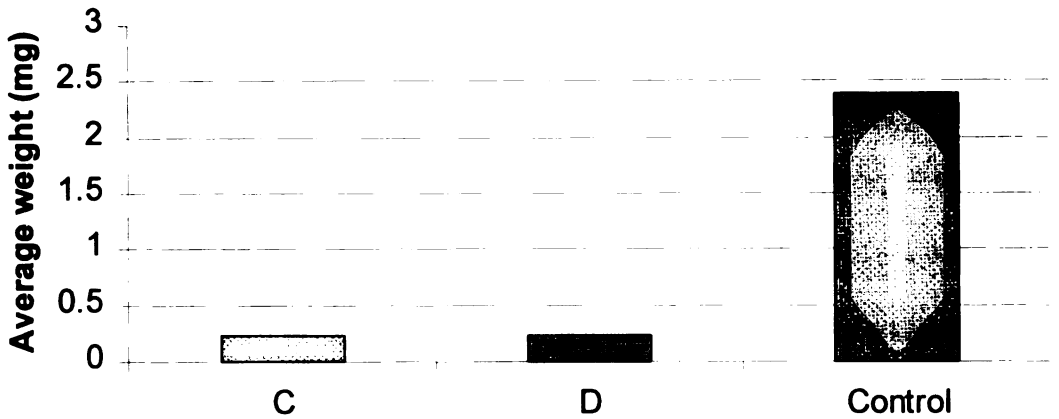
Results and Discussion

The porphyrin-containing fraction, responsible for the corn earworm anti-feedant activity, has a UV absorption spectrum identical to previously published data (Perrin, 1958); however, several other compounds including phylloerythrin methyl ester, have similar UV absorption spectra profiles (Wolf and Scheer, 1973). ^{1}HNMR data indicated a mixture of compounds similar to previously published data (Kenner et al., 1973) namely, peaks for the ethyl, methyl, and unsaturated protons.

The results of the corn earworm bioassay indicated that the treatments assayed at 100 ppm had a highly significant ($p < 0.005$) effect on the caterpillar weight (Figure 5). It is important to point out that the fractions assayed (C and D) contained the isolated compound in addition to smaller amounts of other

porphyrin-type compounds. Therefore, in actuality, biological activity might be due to other porphyrin type compounds working in concert or independently.

Figure 5. Corn earworm anti-feedant/toxic activity of two fractions containing isolated porphyrin. Significant at P < 0.005.



Porphyrin-based compounds, such as phylloerythrin, result from the normal rumen microbial breakdown of chlorophyll from ingested forage plants in livestock animals. The phylloerythrin is absorbed and transported to the liver. Healthy livers transfer the phylloerythrin to the bile for excretion. Livers damaged by the fungal toxin, sporidesmin, cannot properly metabolize phylloerythrin, which then accumulates in peripheral blood. Therefore, circulating phylloerythrin in the blood causes the photosensitization reaction in nonpigmented skin (Cheeke, 1997). Photosensitization manifests itself often as facial eczema. The observed eczema is an example of "secondary photosensitization," in which the skin

lesions are really the secondary result of liver damage, rather than the direct result of a plant toxin (Hansen et al., 1994). Extrapolation of the above information to our corn earworm bioassays and the activity that was observed, may not be necessarily relative to photoxicity to *H. zea* caterpillars. However, it is possible that phototoxicity resulting from abnormal chlorophyll metabolism might be occurring in the *H. zea* gut and resulting in mortality and weight loss. As of 1995, phototoxin-mediated effects and phototoxicity in insects resulting from abnormal chlorophyll metabolism is not know to exist (Berenbaum, 1995); however, akin to this phenomenon, involved the administering of synthetic compounds which interfere with heme biosynthesis from protoporphyrin IX (Rebeiz et al., 1990 and 1988). Lethal accumulation of protoporphyrin IX in *Trichoplusia ni* Hubner and *H. zea* larvae was found to result following the administration of 2, 2'-dipyridyl alone or in combination with δ-aminolevulenic acid (Rebeiz et al., 1988). Lethal accumulation of protoporphyrin IX in the insect was associated with regurgitation, convulsions, and loss of body fluids. Larval death occurred in the dark and light, however, the treatment appeared to be photodynamic in nature. Also, exogenous applications of protoporphyrin and Mg-protoporphyrin also exhibited photodynamic damage. The compound, 1, 10-phenanthroline exhibited similar effects in *T. ni* (Rebeiz et al., 1990).

The preliminary evidence in this study indicates that a porphyrin like compound is responsible for the observed mortality of corn earworm larvae. Whether or not a phototoxic or related effect is occurring, remains unknown.

Whatever the mode of action, this work may provide a basis for further research on the insecticidal activity of porphyrins.

Chapter 6

SUMMARY

The body of information about *O. sanctum* prior to this work was presented in **Chapter 1** and also formed much of the impetus for the current work. Much of the literature summarized in **Chapter 1** focused primarily on the biological activity of crude extracts of *O. sanctum*. This early work stimulated the formulation of a multidimensional hypothesis namely that, *O. sanctum* contains compounds capable of exhibiting anti-oxidant and anti-inflammatory as well as insecticidal and anti-feedant activities. Compounds possessing these activities were isolated and identified as discussed in **Chapters 2-5**. Identification of isolated compounds was determined by the use of various spectral techniques.

Chapters 2 and **5** focused on mosquitocidal and corn earworm anti-feedant activities, respectively. Isolated as oils, eugenol and *E*-6-hydroxy-4, 6-dimethyl-3-heptene-2-one demonstrated mosquitocidal activity at 200 and 6.25 $\mu g \cdot mL^{-1}$ in 24 h, respectively on fourth instar *A. aegyptii* larvae. Both compounds with the addition of a novel triglyceride, 1, 3-dilinoleneoyl-2-palmitin were isolated from the leaf and stem hexane extract of *O. sanctum*. Bioassay directed fractionation experiments conducted in **Chapter 5** resulted in the identification of a porphyrin compound that displayed toxic activity against corn earworm larvae. Porphyrins are known for their photochemical activity. Therefore, the porphyrin isolated may possess a phototoxic or photosensitizing effect that results in the

death and lower weigh of the test insect by either interfering with heme biosynthesis or interaction with other chemical aspects of the insect. Given these preliminary data, further research into the insect toxicology of porphyrins is warranted.

Preliminary anti-oxidant assays on crude extracts from *O. sanctum* had shown excellent anti-oxidant activity. With antioxidant activity in mind, the isolation and identification of eugenol, cirsilineol, cirsimaritin, isothymusin, isothymonin, apigenin, and rosmarinic acid from acetone extracts of fresh *O. sanctum* leaves and stems was carried out in **Chapter 3**. Eugenol, cirsilineol, isothymusin and rosmarinic acid demonstrated good antioxidant activity when assayed at 10 μM. Cirsimaritin, isothymusin, and isothymonin were identified for the first time in *O. sanctum*. Isothymonin was also reported as a natural product for the first time.

The antioxidant compounds isolated and identified in **Chapter 3** were subjected to COX-1 inhibitory assays (**Chapter 4**). Eugenol was by far the most active, showing 97% COX-1 inhibitory activity when assayed at 1000 μM. The activity of eugenol exceeded the activity of aspirin by 51% at the same molar concentration. Apigenin and rosmarinic acid also performed well, demonstrating 65 and 58% COX-1 inhibitory activity, respectively. The other compounds demonstrated comparable activity with ibuprofen and aspirin at 10 μM and 1000

μM, respectively. Some of the same compounds demonstrated slightly higher levels of COX-2 inhibition at the 1000 μM thereby suggesting specificity.

Natural mosquito control could conceivably be accomplished by utilizing conventional aerosol sprays or novel chemical dispersion technologies in the deployment of essential oils from *O. sanctum*. Human health concerns as well as an impact on the environment would be alleviated by the use of *O. sanctum* as a natural form of insect pest control.

The anti-feedant and/or toxic nature of the porphyrin compound against corn earworm is new and therefore warrants further investigation. Determination of mode of action, phototoxicity, and its possible applications would be worthwhile endeavors for additional research.

These research results could provide support for the use of *O. sanctum* in food supplements or nutraceuticals in the prevention of cancer and in the reduction of inflammation. The acceptance of *O. sanctum* as an alternative to conventional treatments would be well received by the public considering the current trend towards natural alternatives to drug treatments.

This research has added to the body of literature dealing with *O. sanctum*. More specifically, the current work has led to the first identification of biologically active compounds from *O. sanctum*. Additional studies should be undertaken to

determine the presence of other biologically active compounds contained in *O. sanctum*. These compounds may hold potential for pharmaceutical and or agricultural applications in addition to being a worthwhile research endeavors.

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