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thesis entitled

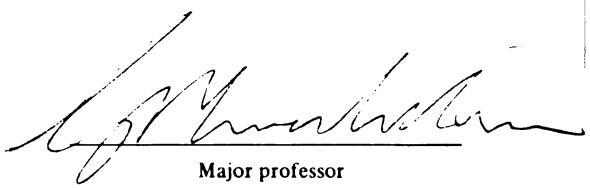
Biologically Active Extracts From *Magnolia* spp. and Compounds From *Magnolia salicifolia*

presented by

Mark Allen Kelm

has been accepted towards fulfillment
of the requirements for

M.S. degree in Horticulture



Major professor

Date January 8, 1996

BIOLOGICALLY ACTIVE EXTRACTS FROM *MAGNOLIA SPP.* AND COMPOUNDS FROM *MAGNOLIA SALICIFOLIA*

By

Mark Allen Kelm

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Horticulture

1996

ABSTRACT

BIOLOGICALLY ACTIVE EXTRACTS FROM *MAGNOLIA SPP.* AND COMPOUNDS FROM *MAGNOLIA SALICIFOLIA*

By

Mark Allen Kelm

Antimicrobial, nematicidal, insecticidal, and anticancer bioassays were done on extracts from *Magnolia salicifolia* Maxim., *Magnolia denudata* Desr., *Magnolia kobus* var. *stellata* Black., and *Magnolia kobus* var. *loebneria* Spong.. Bioassays included mosquito larvae (*Aedes egyptii*), gypsy moth larvae (*Lymantria dispar*), and nematodes (*Panagrellus redivivus* Goody and *Caenorhabditis elegans* Dought.). Anticancer bioassays were performed using mutated yeast, *Saccharomyces cerevisiae* . Most extracts possessed mosquitocidal activity. Extracts from *M. stellata* and *M. denudata* exhibited growth inhibition on *L. dispar* whereas extracts of *M. salicifolia* and *M. stellata* had anticancer activity.

Bioassay-directed work led to the isolation of six and the identification of one mosquitocidal compounds from *M. salicifolia*. Geranial plus neral, *trans*-anethole, methyl eugenol, iso-methyl eugenol, myristicin, and costunolide, gave 100% mortality on 4th

instar larvae at concentrations ranging from 15 to 100 ppm. Parthenolide, identified by HPLC, was not mosquitocidal. Compounds were bioassayed for anticancer and growth inhibitory activity. Costunolide, parthenolide, and myristicin were found to have anti-cancer activity.

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ACKNOWLEDGMENTS

I'd like to thank my major advisor, Muraleedharan Nair, for his advice and encouragement throughout this entire project and my guidance committee members, Drs. John Kelly, Robert Schutzki, and Daniel Herms for the assistance they were able to offer.

A special thanks goes to Dr. Amitabh Chandra for his help in the protocols of the Bioactive Natural Products Laboratory and assistance in spectral interpretation. Dr. James Nitao deserves thanks for his expertise with the insect bioassays. Without these two individuals, the duration of this thesis would have been greatly extended.

I'm also very fortunate to have met so many good people in this department especially the members of my lab, Yu-Chen Chang, Jennifer Miles, Di Zhang, Geoff Roth, Haibo Wang, and Andy Erickson. I'm also grateful for the understanding and help my friend, Sarah Breitkreutz so kindly offered.

I would finally like to thank my parents for believing in me and always being there no matter how tough things got.

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LIST OF ABBREVIATIONS

ACN	.....	Acetonitrile
BNPL	.....	Bioactive Natural Products Laboratory
$CHCl_3$	.....	Chloroform
CD	.....	Circular dichroism
$CDCl_3$	.....	Deuterated chloroform
DEPT	.....	Distortionless Enhancement by Polarization Transfer
DMSO	.....	Dimethyl sulfoxide
EIMS	.....	Electron impact ionization mass spectrometer
EtOAc	.....	Ethyl acetate
ϵ	.....	Molar elipticity
FABMS	.....	Fast atom bombardment mass spectroscopy
HPLC	.....	High performance liquid chromatography
NMR	.....	Nuclear magnetic resonance
PDA	.....	Photodiode array detector
PDA	.....	Potato dextrose agar
TLC	.....	Thin layer chromatography
VLC	.....	Vacuum liquid chromatography
^{1}HNMR	.....	Proton nuclear magnetic resonance
^{13}CNMR	.....	^{13}Carbon nuclear magnetic resonance
δ	.....	Chemical shifts
dd	.....	Doublet of doublet
J	.....	Coupling constant
MeOH	.....	Methanol
m.p.	.....	Melting point
MS	.....	Mass spectroscopy
MW	.....	Molecular weight
m/z	.....	Mass-to-charge ratio
MIC	.....	Minimum Inhibitory Concentration
rel.int.	.....	Relative intensity
sp.	.....	specie
spp.	.....	species
YPDA	.....	Yeast Potato Dextrose Agar

LIST OF APPENDICES

CHAPTER I

Introduction

The genus *Magnolia* has played a multi-dimensional role in the history of man. When one speaks of magnolias, images of beautiful trees with large showy fragrant flowers and attractive vegetation are visualized. For these reasons and the fact that magnolias are generally pest-free, they find their place in many ornamental plantings throughout the world. The campus of Michigan State University contains an abundance of magnolia species. Also, it is known for its occasional use as a source of lumber (Callaway, 1994). Species such as *Magnolia grandiflora* L., *Magnolia virginiana* L., and *Magnolia acuminata* L. have been harvested for lumber in North America. The wood of *Magnolia hypoleuca* Siebold and Zuccarini is prized in Japan for use in making utensils, tools, and toys.

Magnolia spp. were used in traditional medicines in China as far back as 1083 B.C. Interestingly, these traditional medicines are still available and used today in China. There are three entries in the Chinese Pharmacopoeia for *Magnolia spp.* (Tang and Eisenbrand, 1992). "Xinyi", Flos Magnolia is the dried buds of *Magnolia biondii* Pamp., *Magnolia denudata* Desr., or *Magnolia sprengeri* Pamp. Xinyi is used to treat colds and nasal catarrh. "Houpohua", Flos Magnoliae, used as a stomachic, is the dried buds of

Magnolia officinalis Rehd. et. Wils. or *Magnolia officinalis* var. *biloba* Rehd. et. Wils. Finally, the dry stem, branch, and root of *M. officinalis* or *M. officinalis* var. *biloba* known as "Houpo", Cortex *Magnoliae officinalis* is used as a stomachic and antihistamine.

In addition to the three entries, there are a number of other articles in the literature mentioning *Magnolia spp.* as traditional Asian medicines. "Shin-I" (also called "Hsin-I" or "Hsenyi"), a Japanese folk medicine, used to cure rhinitis and nasosinuitis, is from the dried flower buds of *Magnolia salicifolia* Maxim or *Magnolia kobus* DC. Native North Americans as well as Asians have and still use some *Magnolia spp.* for treatment of a variety of ailments (Moerman, 1986). Among the tribes using indigenous *Magnolia spp.* have been the Cherokee, Choctaw, Kosati, Houma, Iroquois and Rappahannock. The bark of *M. acuminata* was used by the Cherokee and Iroquois to treat stomach cramps and tooth aches. The Iroquois also used this species to treat venereal diseases. The Houma used a decoction of leaves and stems of *M. virginiana* as a febrifuge and a cold remedy. Other species had similar uses by different tribes. The Rappahannock were the only tribe to use Magnolia as a hallucinogen. In order to achieve this affect, they reportedly inhaled the leaves or bark of *M. virginiana* contained within cupped hands. It is noteworthy to point out that none of these *Magnolia spp.* used by Native Americans have been analyzed chemically for their purported activities. The bark of *M. acuminata, M. viginiana, and M. tripetala* were first listed as official drugs in the 1787 United States Pharmacopia (USP). They were used to treat malaria, rheumatism, gout, and respiratory ailments (Culbreth, 1927). However, with the advent of more readily available drugs, the

use of magnolia bark declined, and the genus has not appeared in North American pharmacopoeias since 1900.

Much of the driving force on magnolia research has been its long history of use in folk medicine. In addition to the exploration of magnolias for pharmacologically active compounds, there has been some interest in secondary metabolites possessing pesticidal and allelopathic activities.

Many biologically active compounds have been isolated from *Magnolia spp.* as explained in Chapter II. It is our hypothesis that other *Magnolia spp.* also have the potential to generate novel bioactive compounds for human and agricultural pest management. The purpose of the present work is to investigate the presence of novel bioactive compounds in *Magnolia spp.* which were not previously studied. These studies will help lead to a greater understanding of the chemistry of *Magnolia spp.* as well as the potential for new pharmaceutical and pesticidal compounds.

M. salicifolia, *M. denudata*, *M. kobus* var. *stellata* and *M. kobus* var. *loebneria* were selected for phytochemical analyses for this project. Only the pharmacologically active compounds are reported from *M. salicifolia* and to a lesser extent, *M. denudata* and *M. kobus* var. *stellata* (*M. stellata*). *M. kobus* var. *loebneria* has not been investigated for bioactive compounds. In this study, extracts from all species will be examined for activity against fungal and bacterial pathogens, mosquito larvae, gypsy moth larvae, nematodes, and topoisomeraes I and II (anti-cancer assay).

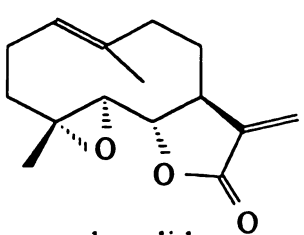
CHAPTER II

Literature Review

This chapter reviews papers dealing with a veritable potpourri of bio-active compounds derived from various *Magnolia spp.* Neolignans, lignans, phenyl propanoids, sesquiterpenes, and alkaloids are among the compounds with reported biological activity. Neolignans, lignans, and phenyl propanoids are derived from the shikimic acid pathway. Sequiterpenes and other terpenes are derived from the mevalonic acid pathway. Alkaloids on the other hand, are hypothesized to come from either biosynthetic pathway depending on whether the alkaloid contains a terpene structure or a phenyl propanoid structure.

Pharmacological studies

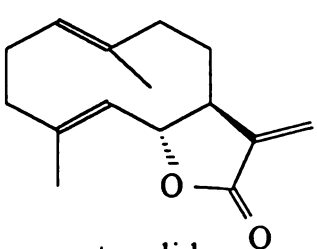
One of the earliest studies conducted on magnolia was part of a search for plants having anti-tumor principles (Wiedhopf et al., 1973). From the petroleum ether leaf extract of *M. grandiflora* L., Wiedhopf and coworkers isolated parthenolide, a sesquiterpene lactone that showed inhibitory activity against human epidermoid carcinoma (skin cancer). The exocyclic double bond adjacent to the carbonyl group of the lactone ring of sesquiterpene lactones, was demonstrated as the most important functionality for



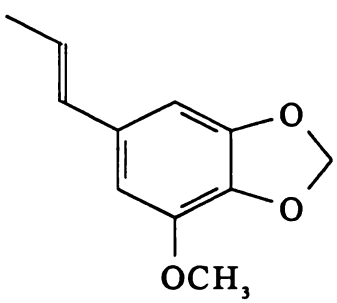
parthenolide

cytotoxicity (Lee et al., 1971).

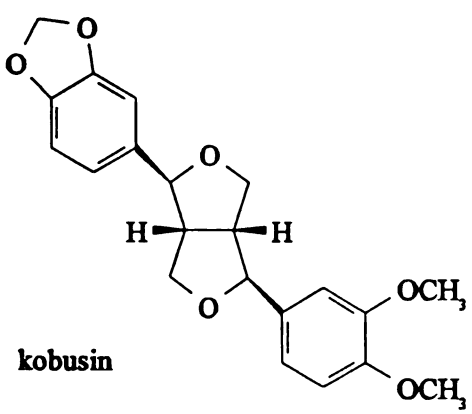
A more recent study conducted on the root bark of *Magnolia denudata* Desr. has yielded compounds cytotoxic to P388 leukemia cells (Funayama et al., 1995). The active constituents , costunolide (1.3 $\mu g \cdot ml^{-1}$) and parthenolide (0.5 $\mu g \cdot ml^{-1}$) were isolated from the $CHCl_3$ extract of the dried root bark. Other less cytotoxic compounds were isolated as well. Among them were a phenylpropanoid, *trans*-isomyristicin and two lignans, sesamin and kobusin. Costunolide had been isolated previously from *M. grandiflora* (El-Feraly, 1979). The lignan kobusin, isolated earlier from *Magnolia campbellii* and *M. mutabilis* (Talapatra et al., 1975), was also later found in *Magnolia stellata* along with sesamin (Iida et al., 1983).



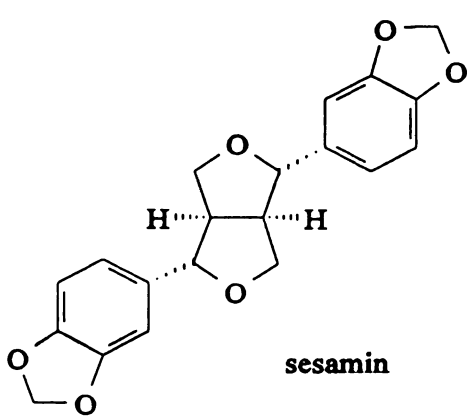
costunolide



trans-isomyristicin

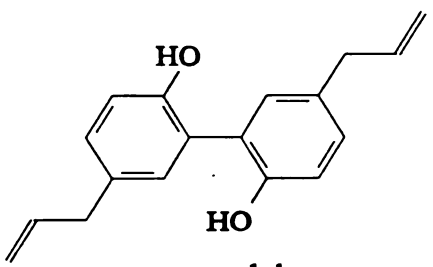


kobusin

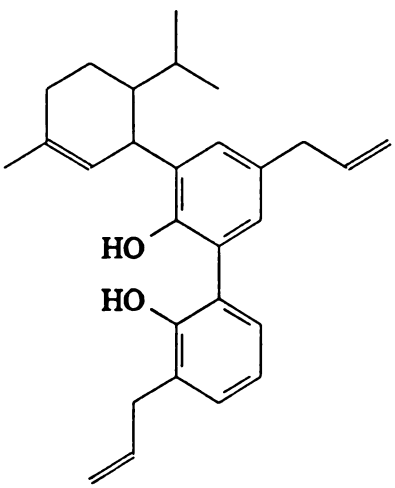


sesamin

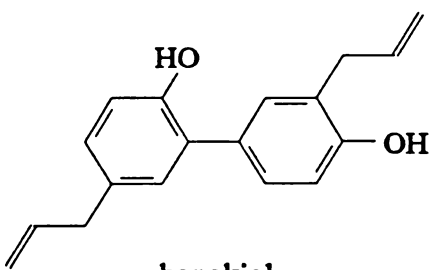
The anti-tumor activities for neolignans of *M. officinalis* also were explored (Konoshima, 1990). Magnolol , honokiol, and monoterpenylmagnolol exhibited inhibitory effects on Epstein-Barr virus (human herpes virus) activation. Among these compounds, magnolol displayed the highest anti-tumor activity. Honokiol was isolated originally from the MeOH extract of the bark of *M. obovata* Thunb. (Fujita et al., 1972).



magnolol

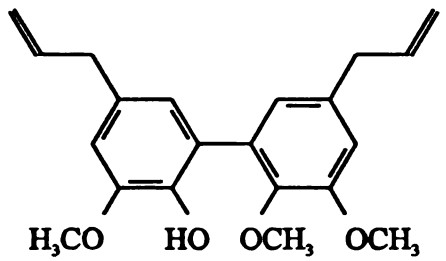


monoterpenylmagnolol



honokiol

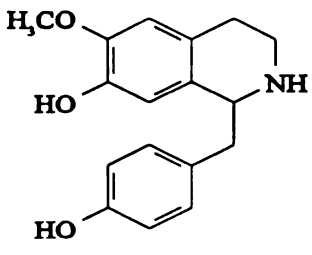
Baek et al. (1992), during the course of screening anti-oxidants from plants, found a neolignan from the bark of *M. officinalis*. 5, 5'-di-2-propenyl-2-hydroxy-3, 2', 3'-trimethoxy-1-1'-biphenyl shown below, was found to have anti-oxidant activity similar to 2, 6-di-*tert*-butyl-4-methylphenol (BHT) or 3-tert-butyl-4-hydroxyanisole (BHA). Anti-oxidants are compounds that inactivate free radicles in the body. Free radicles can promote the growth of cancerous cells by initiating spontaneous mitosis.



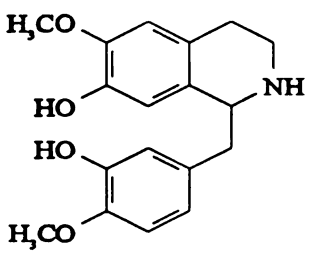
5, 5' -di-2-propenyl-2-hydroxy-3, 2', 3' -trimethoxy-1, 1'-biphenyl

Furthermore, phenolic anti-oxidants in wine have demonstrated the ability to inhibit human low density lipid (LDL) oxidation in vitro (Frankel et al., 1995). Frankel mentions other studies suggesting that oxidation of LDLs may play a major role in atherosclerosis.

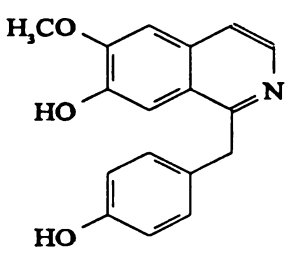
Kimura and coworkers (1983) examined the crude alkaloidal fractions derived from "Shin-I" (a traditional Sino-Japanese medicine) for the presence of neuromuscular blocking activity. From the dried buds of *M. salicifolia*, three alkaloids, d-coclaurine, d-reticulin, and yuzirine were found to reduce acetylcholine-induced twitching of frog skeletal muscle.



d-coclaurine

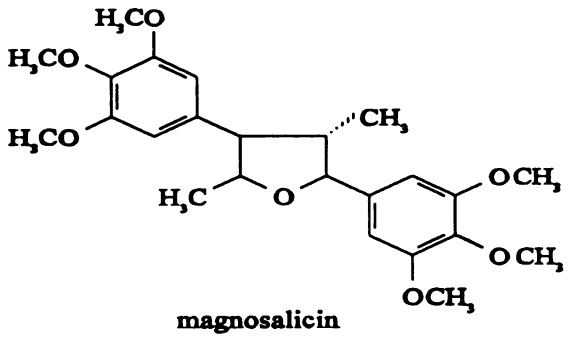


d-reticuline



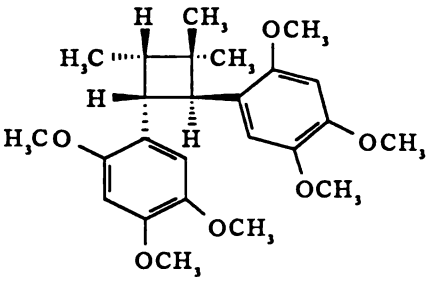
yuzirine

The active component of the purported anti-allergy activity of Shin-I was identified as magnosalicin (Tsurga et al.,1984). Magnosalicin, a neolignan, was found in the $CHCl_3$ extract of *M. salicifolia* buds.

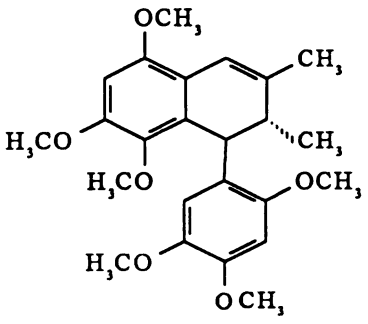


magnosalicin

The anti inflammatory effect of Shin-I was found to be supported by magnosalin's and magnoshinin's ability to inhibit angiogenesis and granuloma formation (Kimura et al., 1990). These lignans were isolated originally from *M. salicifolia* (Kikuchi et al., 1983).



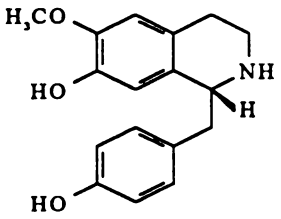
magnosalin



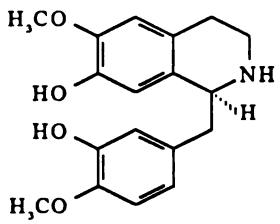
magnoshinin

In a later study, Kimura and workers (1989) demonstrated the negative inotropic effects of (+)-R-coclaurine and (+)-S-reticulin as well as their antagonistic actions to ±higenamine, the cardiotonic principle of aconite root (*Aconitum sp.*). In essence, (+)-R-

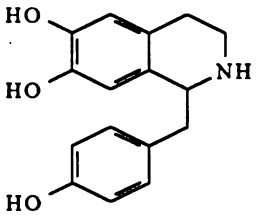
coclaurine and (+)-S-reticulin act as muscle relaxants and to prevent muscle contractions induced by ±higenamine.



(+)-R-coclaurine

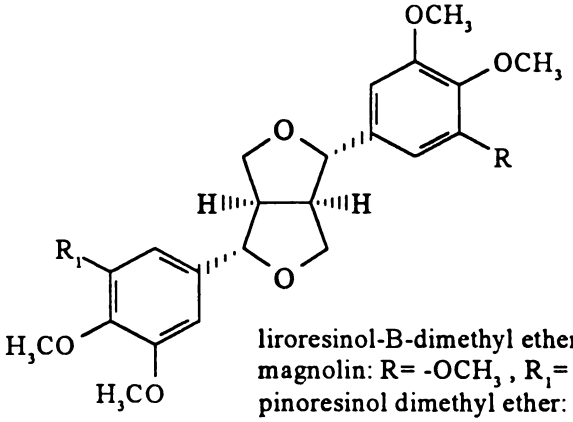


(+)-S-reticulene



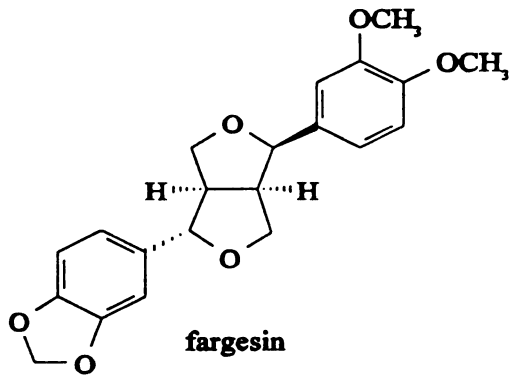
± higenamine

The traditional Chinese drug, Xinyi, was examined for the presence of platelet activating factor (PFA) antagonists or simply, blood anti-coagulants (Pan et al., 1987). Six PFA antagonists were identified from the methylene chloride extract of *M. biondii*. The PFA antagonists were pinoresinol dimethyl ether, magnolin, liroresinol-B-dimethyl ether, fargesin, demethoxyaschantin and aschantin.

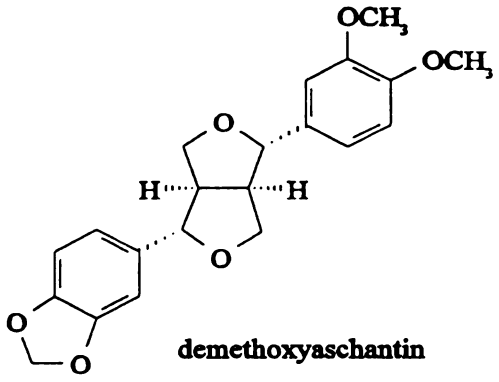


liroresinol-B-dimethyl ether: R= -OCH_3 , R_1= -OCH_3
magnolin: R= -OCH_3 , R_1= -H
pinoresinol dimethyl ether: R= -H, R_1= -H

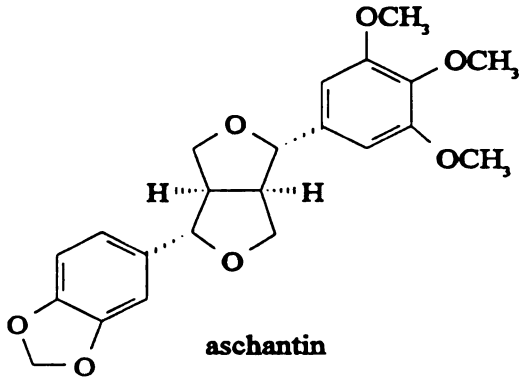
All of these were lignans with similar structures. The first four PFA antagonists were isolated previously from the flower buds of *M. fargesii* (Kakisawa et al., 1972). More recently, pinoresinol dimethyl ether was isolated from *M. saulangiana* (Abdallah, 1993).



fargesin



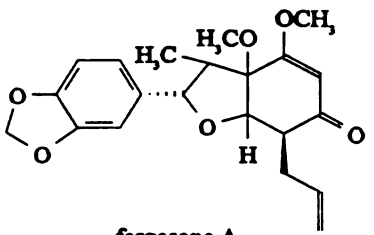
demethoxyaschantin



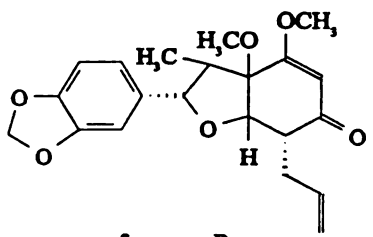
aschantin

Calcium ions are involved in the regulation of muscle contractions. Compounds that interfere with or diminish muscle contractions via Ca^{++} antagonism, may be considered muscle relaxants. Chen et al. (1988) isolated three Ca^{++} antagonistic principles from "Hsin-I". Three neolignans; fargesone A, fargesone B, and denudatin B were purified from the $CHCl_3$ extract of the flower bud from *M. fargesii* Cheng. Denudatin B

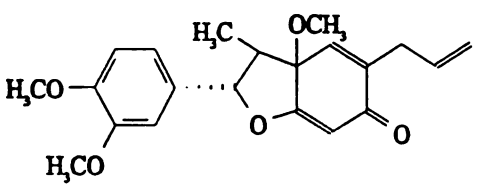
has also been isolated from fresh leaf extract of *M. liliflora* using $CHCl_3$ (Iida and Ito, 1983).



fargesone A

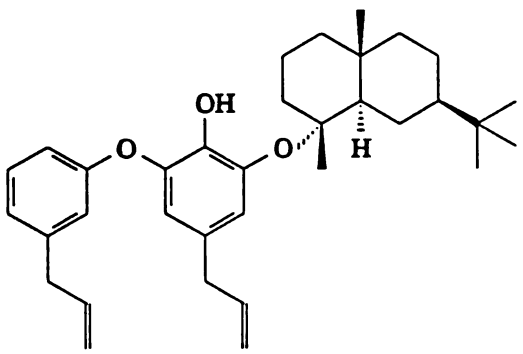


fagesone B

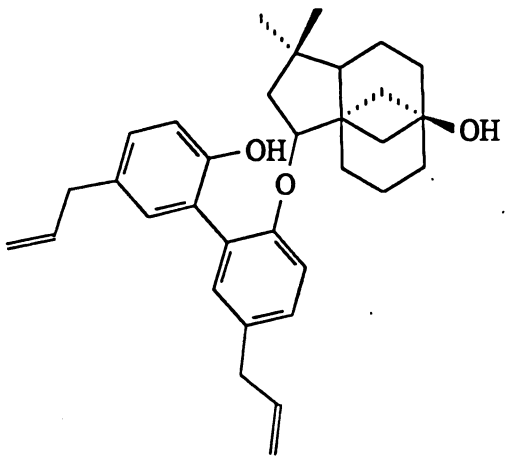


denudatin B

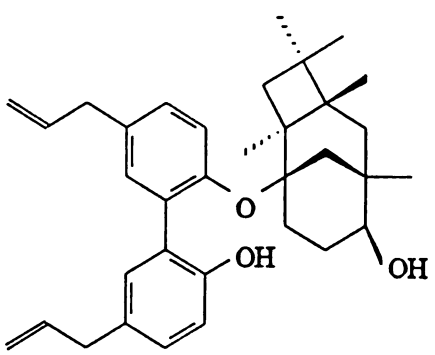
Three compounds possessing neurotrophic activity were characterized from the root bark of *M. obovata* Thunb. (Fukuyama et al., 1989, 1990, 1992). Three novel sesquiterpene neolignans, eudesobovatol A, clovane magnolol, and caryolanemagnolol not only augment neurite sprouting, but also greatly enhance choline acetyl transferase activity. Choline acetyl transferase catalyzes the reaction between acetyl-CoA and choline to yield the neurotransmitter, acetylcholine, which triggers muscle contraction (Voet and Voet, 1990). These results support the alleged use of *Magnolia spp.* as a teatment for neurosis.



eudesbovatol A



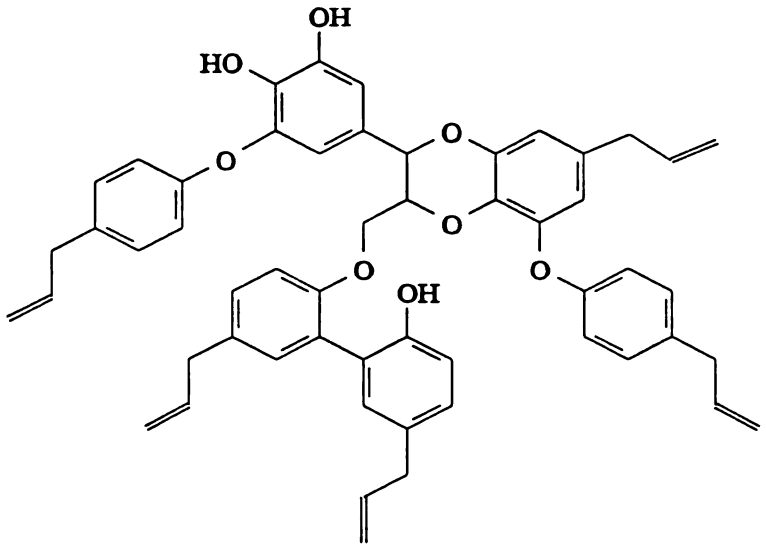
clovanemagnolol



caryolanemagnolol

In another article, Fukuyama et al. (1993) reported a novel trilignan, magnolianin. This unusual molecule was found to possess potent 5-lipoxygenase-inhibitory activity. Lipoxygenase is an enzyme responsible for converting arachidonate into leukotrienes (Voet and Voet, 1990). Leukotrienes are local hormones involved in many of the same functions as prostaglandins. Inflammatory responses, production of pain and fever, regulation of blood pressure, and induction of blood clotting are some of their functions.

The significance of these findings support earlier reports of *Magnolia spp.* being used effectively to treat rhinitis and nasosinuitis.



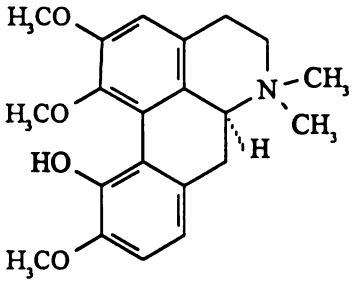
magnolianin

Medicines containing the *M. obovata* bark have been prescribed as stomachic, diuretic, and anti-emetic treatments. Both magnolol and honokiol were determined to be anti-emetic in frogs (Kawai et al., 1993).

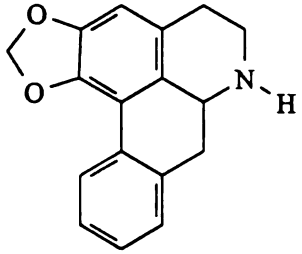
Another lignan, pinoresinol, originally found in the dried fruits of *Forsythia suspensa*, was found to inhibit cyclic adenosine monophosphate (c-AMP) phosphodiesterase (Nikaido et al., 1981). Later, pinoresinol was isolated from *Magnolia spp.* c-AMP phosphodiesterase is responsible for degradation of c-AMP into AMP. c-AMP is a second messenger in the action of many hormones (Stryer, 1988). Inhibitors of c-AMP phosphodiesterase act synergistically with hormones that use c-AMP as a second messenger. For example, the action of the hormone vassopressin (a peptide hormone that

stimulates water resorption) is enhanced when c-AMP phosphodiesterase is inhibited.

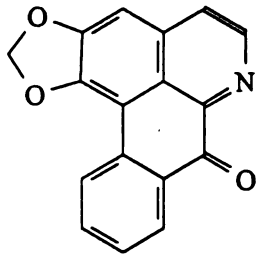
A toxicological study of the methanolic extract of *M. grandiflora* heartwood led to the identification of menisperine (N-methyl isocorydine), a phenolic quaternary alkaloid (Rao and Davis, 1982). Toxicity of this MeOH extract was ultimately due to the neuromuscular blocking action of the alkaloid. Davis (1981) isolated this alkaloid and two antibacterial compounds from *M. grandiflora*. Parthenolide, from the yellowed leaves of *M.gandiflora* exihibited antibiotic activity against *Bacillus subtilis* at an MIC of $3\mu g \cdot ml^{-1}$. Anonaine, demonstrated antibacterial activity at 100 $mg \cdot ml^{-1}$ against *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Candida albicans*. Davis also stated that anonaine can employ a hypotensive effect in mice and rabbits and was shown to be an inhibitor of dopaminergic response. Also, the toxicity of anonaine HCl was demonstrated in mice at 200 $mg \cdot kg^{-1}$. A related compound, liriodenine, had similar antibacterial activity to anonaine.



menisperine

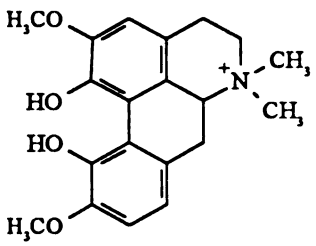


anonaine

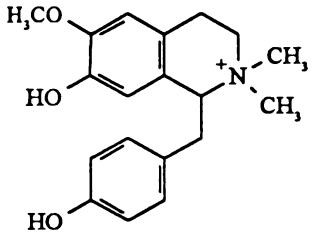


liriodenine

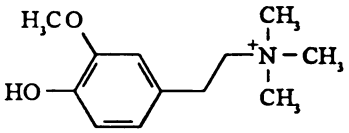
Quaternary alkaloidal compounds associated with ganglionic-blocking activity have been identified in Magnolia extracts (Davis, 1981). Magnocurarine, magnoflorine, salicifoline, and d-tubocurarine have demonstrated this effect in frogs.



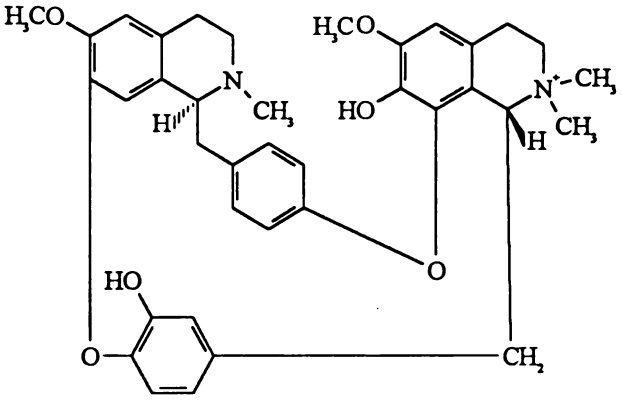
magnoflorine



magnocurarine



salicifoline



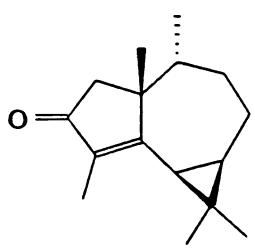
d-tubocurarine

The ether and water extracts of *M. obovata* bark were found respectively to depress activity and induce a quick paralysis of respiration on mice and chicks (Watanabe et al., 1973). In the same study, the ether extract was found also to be anticonvulsant.

Pesticidal, ecological, agricultural, and related studies

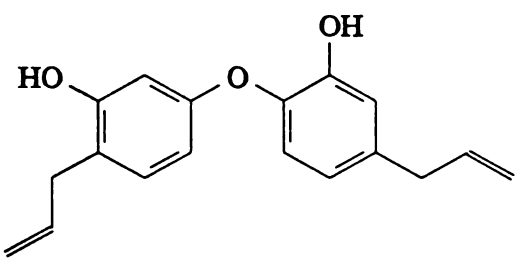
Phenolic constituents of *M. grandiflora* L., namely magnolol, honokiol, and 3, 5'-diallyl-2'-hydroxy-4-methoxy-biphenyl were found to have superior antibacterial activity against *Bacillus subtilis, Staphylococcus aureus*, and *Mycobacterium smegatis* when compared to streptomycin sulfate (Clarke et al., 1981). Additionally, magnolol and honokiol were found to be moderately active against *Candida albicans, Saccharomyces cerevisiae, Aspergillus niger* and strongly active against *Trichophyton mentagrophytes* when compared to amphotericin B. 3, 5'-diallyl-2'-hydroxy-4-methoxy-biphenyl demonstrated milder activity against *S. cerevisae* and strong inhibition of growth against *T. mentagrophytes* when compared to amphotericin B.

Growth inhibitory and antimicrobial activities were demonstrated by cyclocolorenone, a sesquiterpene ketone that was isolated from the leaves of *M. grandiflora* (Jacyno et al., 1991). Cyclocolorenone inhibited the growth of etiolated wheat coleoptiles completely and was comparable to morphactin, juglone, and abscisic acid at 10^{-3} M and 10^{-4} M, respectively. Cyclocolorenone inhibited only 58% whereas morphactin, juglone, and abscisic acid inhibited 38, 100, and 100%, respectively.

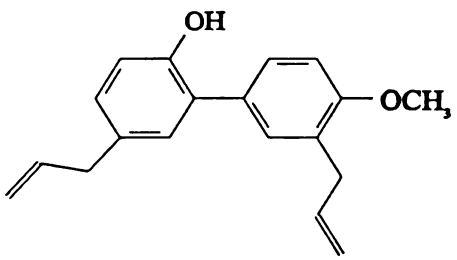


cyclocolorenone

4, 4'-diallyl-2, 3'-dihydroxybiphenyl ether ("biphenyl ether"), 3, 5'-diallyl-2'-hydroxy-4'-methoxybiphenyl, and magnolol from leaves of *M. virginiana* L. displayed antifungal (*C. albicans, Aspergillus flavus, Gloesporum sp.*, and *Rhizoctonia sp.*), antibacterial (*Streptococcus aureus, Staphylococcus epidermis*, and *Escherichia coli*), mosquitocidal (*Aedes egyptii*), and crustaceacidal (*Artemia salina*) activities (Nitao et al., 1991). Additionally, allelochemicals (biphenyl ether and magnolol) of *M. virginiana*



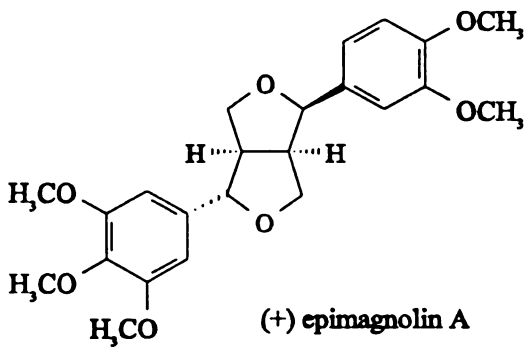
4, 4'-diallyl-2, 3'-dihydroxybiphenyl ether



3, 5'-diallyl-2'-hydroxy-4'-methoxybiphenyl

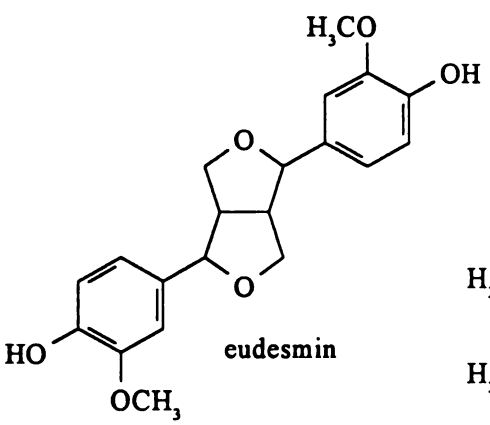
leaves were found to be toxic to *Papilio palamedes* and reduced survival rates of *Papilio troilus* (Nitao et al., 1992). 3, 5'-diallyl-2'-hydroxy-4'-methoxybiphenyl was previously isolated from the seeds of *M. grandiflora* (El-Feraly and Li, 1978).

Two silkworm (*Bombix mori* L.) growth inhibitors were isolated from the leaves of *M. kobus* DC. The structures of the two active compounds were elucidated as lignans, sesamin and kobusin. A novel ligan of the same skeletal form was isoalatd from the buds of *M. fargesii*. (+) epimagnolin A was isolated by bioassay directed fractionation from the CH_2Cl_2 soluble portion of the MeOH fruit extraction and was found to have growth inhibitory activity against larvae of *Drosophila melanogaster* (Miyazawa et al., 1994).

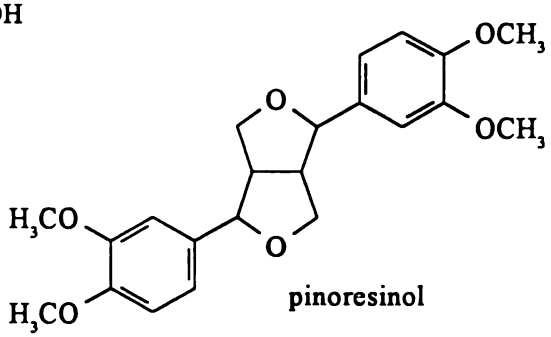


(+) epimagnolin A

The synergistic activity of sesamin, pinoresinol, and eudesamin with pyrethrum insecticides was reported (Haller et al., 1941). However, these compounds were not isolated from *Magnolia spp.* until much later (Fukayama et al., 1995, Kikuchi et al., 1982, Kakisawa et al., 1970, Iida et al., 1983).



eudesmin



pinoresinol

CHAPTER III

Isolation, Purification, Identification, and Quantification of Biologically Active Compounds from *Magnolia salicifolia.*

Abstract

Bioassay-directed isolation and purification of *Magnolia salicifolia* extracts yielded six mosquitocidal compounds. Their structures were determined by ^{1}HNMR, ^{13}CNMR, and MS determinations. The pleasant-smelling monoterpenes, geranial and neral, collectively known as citral, were isolated from hexane extract of the bark. *Trans*-anethole and methyl eugenol, both phenylpropanoids, were isolated from hexane extract of the leaves. Myristicin, a methylenedioxy phenyl compound, was first detected as an additional compound in the ^{1}HNMR spectrum of *trans*-anethole. The positional isomer of methyl eugenol, iso-methyl eugenol, was isolated from the hexane extract of leaves, fruits, and flowers. It also was obtained from the EtOAc extract of fruits. Costunolide, a sesquiterpene lactone was obtained from the hexane and EtOAc extracts of the fruits of *M. salicifolia* for the first time. Iso-methyl eugenol, costunolide, and an additional related sesquiterpene lactone, parthenolide, were detected and quantified in *M. salicifolia* for the first time. Circular dichroisms (CD) of costunolide and parthenolide also are presented for the first time. The CD of costunolide and parthenolide indicated that they are dextro (+)

and levo (-), respectively.

Yield of a geranial and neral mixture was 0.08%. Yield of compounds isolated from leaves were 0.02, 0.25, and 0.43% for *trans*-anethole, methyl eugenol, and iso-methyl eugenol, respectively. Yield of iso-methyl eugenol and costunolide from fruits were 0.92 and 0.60%, respectively. From flowers, 0.24% of iso-methyl eugenol was isolated. Yields were calculated on a dry-weight basis.

Introduction

Previously, compounds isolated from *M. salicifolia* have been those associated primarily with pharmacological activities. In particular, compounds with anti-allergy and anti-inflammatory actions have been identified such as magnosalicin, magnoshinin, and magnosalin (Tsurga et al., 1984, Kimura et al., 1990, Kikuchi et al., 1983). The activity of these compounds supported the purported use of *M. salicifolia* buds as a treatment for rhinitis, sinusitus, and nasosinusitus. The alkaloidal fraction of *M. salicifolia* yielded d-reticuline, d-coclaurine, and yuzirine which were found to possess neuromuscular blocking action (Kimura et al., 1983). Quaternary alkaloidal compounds associated with ganglionic-blocking activity have been identified in Magnolia extracts (Davis, 1981). Magnocurarine, magnoflorine, salicifoline, and d-tubocurarine have demonstrated this effect in frogs.

To a lesser extent, pesticidal compounds isolated from *Magnolia spp.* having been reported. 4, 4'-diallyl-2, 3'-dihydroxybiphenyl ether ("biphenyl ether"), 3, 5'-diallyl-2'-hydroxy-4'-methoxybiphenyl, and magnolol from leaves of *M. virginiana* L. displayed antifungal (*Candida albicans, Aspergillus flavus, Gloesporum sp.*, and *Rhizoctonia sp.*), antibacterial (*Streptococcus aureus, Staphylococcus epidermis*, and *Escherichia coli*), mosquitocidal (*Aedes egyptii*), and crustaceacidal (*Artemia salina*) activities (Nitao et al., 1991).

In chapter IV, hexane extracts from leaves, stems, flowers, and fruits and EtOAc extracts from *M. salicifolia* were found to be 100% lethal against mosquito larvae. There are no reports of mosquitocidal constituents in *M. salicifolia*.

Materials and Methods

General Experimental Procedures

^{1}H and ^{13}CNMR spectra were recorded on Varian VXR 300 and 500 MHz spectrometers and were in $CDCl_3$ solutions. EIMS and FABMS were recorded on JEOL JMS-AX505 and JEOL JMS-HX110 mass spectrometers. The melting point, were recorded on a Thomas Model 40 micro hot-stage apparatus and are not corrected. Purification and subsequent isolation of bioactive constituents from *M. salicifolia* was accomplished by TLC (Analtech 20x20 cm silica gel GF-TLC plates with fluorescent indicator) for stems, leaves, flowers, and fruits. Detection of spots/bands was accomplished by visualizing under a UV lamp at 254 nm and developed by spraying with 10 % H_2SO_4 followed by heating for 5 min at 120°C. Initial purifications were performed by vacuum liquid chromatography (VLC) or column chromatography (CC) over silica gel (Analtech Silica Gel 60Å pore size, 35-75 μm particle size).

HPLC Analyses

Fruit extracts and pure costunolide, parthenolide, and iso-methyl eugenol were analyzed on a capcell pak C-18 (5 μm, 4.6 x 250 mm) column (Dychrome). The mobile phase ACN:H_2O (80:20 v/v) was used under isocratic conditions at a flow rate of 0.5 $ml \cdot min^{-1}$. The samples were filtered through a 0.22 μ PTFE filter (Scientific Resources Inc.), prior to the injection. The injections (10 μl) were performed by the Waters 717 autosampler. A Waters 991 photo diode array detector (PDA), (Millipore Corporation, Milford, Massachusetts) was used to collect data at 217 nm and 222 nm for costunolide and parthenolide, respectively. The calibrations and quantifications were carried out using

Waters Millennium 2010 Chromatography Manager GPC software, version 2.0 (Millipore Corporation, Waters Chromatography division, Milford, Massachusettes).

Circular Dichroism Analyses

Pure costunolide and parthenolide at $1mg \cdot 25ml^{-1}$ in MeOH were analyzed on JASCO J-710 71CD-ORD spectropolarimeter (Jasco Incorporated, Japan). The molar elipticities were spectra were plotted on 7475A Hewlett Packard plotter (Hewlett Packard Corporation, Palo Alto, California). Nitrogen (99.99%) was produced by a nitrogen generator model NG-150 (Peak Scientific, Chicago, Illinois) at a rate of 20 $liters \cdot min^{-1}$.

Geranial (1) and neral (2)

Initial purification of the mosquitocidal hexane extract from the stem (4 g) was carried out by VLC on silica gel using solvent systems 4:1 hexane-ether, $CHCl_3$, 4:1 $CHCl_3$-MeOH, and MeOH, respectively, to yield four fractions, A-D. Fraction B (740 mg) was found to be mosquitocidal. Repeated purification by TLC (25:1, petroleum ether-acetone) gave an oily compound, citral (80.2 mg; R_f 0.19). The presence of geranial (**1**) and neral (**2**) was confirmed in this oil by NMR studies (**Appendices I** and **II** for respective ^{1}HNMR and ^{13}CNMR spectra of citral).

Compound **1**, geranial ($C_{10}H_{16}O$, MW 152); ^{1}HNMR: δ 9.98 (1H, d, J=8.1 Hz, CHO), 5.86 (1H, d, J=8.1 Hz, H-2), 2.56 (2H, t, J=7.5 Hz, H-4), 2.17 (2H, m, H-5), 5.05 (1H, m, H-6), 1.66 (3H, s, H-8), 1.58 (3H, s, H-9), 2.14 (3H, s, H-10); ^{13}CNMR: δ 191.13 (C-1), 127.22 (C-2), 163.70 (C-3), 40.43 (C-4), 25.56 (C-5), 122.39 (C-6), 132.73 (C-7), 17.53 (C-8), 17.40 (C-9), 25.46 (C-10).

Compound **2**, neral ($C_{10}H_{16}O$, MW 152); ^{1}HNMR: δ 9.87 (1H, d, J=8.1 Hz,

CHO

(2H,

190.6

132.7

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0.14) a

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J=8.62

(1H, m

MW 17

(92), 91

J=10.9

CHO), 5.86 (1H, d, J=8.1, H-2), 2.56 (2H, t, J=7.5 Hz, H-4), 2.17 (2H, m, H-5), 5.05 (2H, m, H-6), 1.66 (3H, s, H-8), 1.58 (3H, s, H-9), 2.14 (3H, s, H-10); ^{13}CNMR: δ 190.62 (C-1), 128.47 (C-2), 163.70 (C-3), 32.41 (C-4), 26.86 (C-5), 122.09 (C-6), 132.73 (C-7), 17.53 (C-8), 17.40 (C-9), 24.88 (C-10).

***Trans*-anethole (3), methyl eugenol (4), iso-methyl eugenol (5), and myristicin (6)**

Purification of the mosquitocidal hexane extract from leaves (1.1 g) by medium pressure column chromatography on silica gel gave six fractions, A-E. Solvent systems used were 4:1 and 1:1 hexane-acetone, acetone, and MeOH, respectively. Fractions B (160 mg) and C (478 mg) were found to be mosquitocidal. Purification of fractions B and C by TLC (50:1, hexane-acetone) gave compounds **3** (6.3 mg; R_f 0.42), **4**, (72.2 mg; R_f 0.14) and **5**, (123.7 mg; R_f 0.13). The presence of myristicin (**6**) was confirmed by ^{1}HNMR in a sample of **5**. Myristicin however, was not isolated in the pure form from the leaves of *M. salicifolia*. See **Appendices III, IV, VI,** and **VIII** for ^{1}HNMR spectra of compounds **3**, **4**, **5**, and **6**, respectively. **Appendices V** and **VII** contain ^{13}CNMR spectra for compounds **4** and **5**, repectively.

Compound **3**, *trans*-anethole ($C_{10}H_{12}O$, MW 148); ^{1}HNMR: δ 6.83 (1H, dd, J=8.62 Hz, H-2,6), 7.25 (1H, dd, J=8.6 Hz, H-3,5), 6.34 (1H, d, J=15.8, vinyl, H-7), 6.09 (1H, m, vinyl, H-8), 1.86 (3H, d, J=6.6Hz, H-9), 3.80 (3H, s, OCH_3).

Compound **4**, methyl eugenol; 1,2 dimethoxy-4-(2-propenyl) benzene ($C_{11}H_{14}O_2$, MW 178); EIMS: m/z (%) 178 (100), 163 (100), 147 (100), 135 (60), 115 (43), 103 (92), 91 (90), 77 (44), 65 (30), 51 (23); ^{1}HNMR: δ 6.71 (1H, s, H-3), 6.73 (1H, d, J=10.94, H-6), 6.81 (1H, d, J=7.95, H-5), 3.34 (2H, d, J=6.85, H-7), 5.96 (1H, m, H-8),

5.07 (2H, d, H-9), 3.87 (3H, s, -OCH_3), 3.86 (3H, s, OCH_3); ^{13}CNMR: δ 137.55 (C-4), 132.49 (C-3), 147.22 (C-2), 148.74 (C-1), 115.45 (C-6), 120.34 (C-5), 39.65 (C-7), 111.17 (C-8), 111.10 (C-9), 55.79 (C-10), 55.65 (C-11).

Compound **5**, iso-methyl eugenol; 1,2 dimethoxy-4-(1-propenyl) benzene ($C_{11}H_{14}O_2$, MW 178); EIMS: m/z (%) 178 (100), 163 (100), 147 (24), 135 (30), 115 (15), 107 (61), 91 (53), 79 (18), 65 (13), 51 (9); ^{1}HNMR: δ 6.81 (1H, s, H-3), 6.84 (1H, d, H-6), 6.89 (1H, d, H-5), 6.34 (1H, d, J=15.78, H-7), 6.10 (1H, m, H-8), 1.87 (3H, d, J=6.56, H-9), 3.89 (3H, s, -OCH_3), 3.87 (3H, s, OCH_3); ^{13}CNMR: δ 130.98 (C-4), 130.44 (C-3), 147.98 (C-2), 148.81 (C-1), 118.48 (C-6), 123.64 (C-5), 111.00 (C-7), 108.28 (C-8), 18.227 (C-9), 55.75 (C-10), 55.61 (C-11).

Compound **6**, myristicin; 1-methoxy-2-3-methylenedioxy-5(2-propenyl) benzene ($C_{11}H_{12}O_3$, MW 192.2); ^{1}HNMR: δ 6.38 (1H, s, H-4), 6.35 (1H, s, H-6), 5.93 (2H, s, O-CH_2-O), 5.91 (1H, m, H-8), 5.10 (2H, d, J=7.5, H-9), 3.88 (3H, s, -OCH_3), 3.29 (2H, d, J=6.6, H-7). Compound **5** was isolated also from the fruits and flowers of *M. salicifolia*. Purification of hexane extract of flowers (1.26 g) by VLC over silica gel using 4:1 hexane-acetone, $CHCl_3$, 4:1 $CHCl_3$-MeOH, and MeOH solvent systems, yielded four fractions, A-D, respectively. Fractions A-C (367.8, 587.3, and 58.2 mg, respectively) were mosquitocidal. Repeated purification of fraction B by TLC (15:1 hexane-acetone) yielded compound **5** (100.8 mg).

Purification of the EtOAc extract of fruits (1.26 g) by VLC over silica gel using EtOAc, 4:1 EtOAc-acetone, $CHCl_3$, 3:1 $CHCl_3$-MeOH, and MeOH solvent systems yielded four fractions A-D, respectively. Fraction A (757.8 mg) was mosquitocidal

Purification of fraction A by TLC (25:1 hexane-acetone) gave four bands, I-IV. Further purification of band III by TLC (40:1 hexane-acetone) afforded compound **5** (39.2 mg). ^{1}H NMR confirmed the identity of compound **5**.

Purification of hexane extract of flowers (1.38 g) by VLC gave four fractions A-D. The active fraction, B was purified further by TLC (15:1 hexane-acetone) and yielded compound **5** (108.1 mg). The identity of compound **5** was confirmed by ^{1}H NMR spectra.

Costunolide (7)

Purification of the hexane extract of *M. salicifolia* fruit (1.26 g) by VLC over silica gel using 4:1 hexane-acetone, $CHCl_3$, 4:1 $CHCl_3$-MeOH, and MeOH solvent systems yielded four fractions, A-D, respectively. Fractions A-C (367.8, 587.3, and 58.2 mg, respectively) were equally mosquitocidal. Repeated purification of fraction B by TLC (15:1 hexane-acetone) yielded compound **7**, (63.5 mg; R_f 0.14) as a colorless crystalline solid (**Appendix IX** and **X** for the ^{1}HNMR and ^{13}CNMR spectra of compound **7**, respectively).

Compound **7**, costunolide; 1 (10), 4 (11) (β)-germacratriene-12, 6- olide ($C_{15}H_{20}O_2$, MW 232); m.p. 101-106° C; EIMS: m/z (%) 232 (56), 217 (24), 204 (8), 189 (11), 175 (15), 161 (14), 150 (20), 135 (15), 123 (57), 109 (73), 91 (39), 81 (100), 67 (23), 53 (53); ^{1}HNMR: δ 6.26 (1H, d, J=3.35, H-13'), 5.52 (1H, d, J=3.07, H-13), 4.83 (1H, br dd, J=6.42, 7.81, H-1), 4.73 (1H, br d, J=10.05, H-5), 4.56 (1H, t, J=9.34, 9.36, H-6), 2.55 (1H, t, J=8.23, 7.68, H-7), 2.45 (1H, dd, J=8.51, 7.82, H-3'), 1.97-2.35 (6H, m, H-2', H-3, H-8, H-9/9'), 1.68 (1H, m, H-2), 1.67 (3H, s, H-15), 1.41 (3H, s, H-14). ^{13}CNMR; δ 169.96 (C-12), 140.97 (C-11), 139..57 (C-4), 136.44 (C-10), 126.76 (C-5),

126.54 (C-1), 119.14 (C-13), 81.40 (C-6), 49.90 (C-7), 40.48 (C-3), 38.51 (C-9), 27.53 (C-2), 25.69 (C-8), 16.84 (C-15), 15.60 (C-14).

Purification of the EtOAc extract (1.26 g) by VLC over silica gel using EtOAc, 4:1 EtOAc-acetone, $CHCl_3$, 3:1 $CHCl_3$-MeOH, and MeOH solvent systems yielded four fractions A-D, respectively. Fraction A (757.8 mg) was mosquitocidal. Further purification of fraction A by TLC (25:1 hexane-acetone) gave four bands, I-IV. Repeated purification of band IV by TLC (10:1 hexane-EtOAc) yielded costunolide **7** (31.9 mg). The structure for compound **7** was confirmed by ^{1}HNMR, ^{13}CNMR, DEPT, and MS.

Quantification of Iso-methyl eugenol (5), Costunolide (7), and Parthenolide (8).

Pure costunolide (**7**) and parthenolide (**8**), were used as standards for quantifying these compounds in three replicated *M. salicifolia* fruit extracts. A set of mixed standards of **7** and **8** containing 1mg of each was dissolved in ACN (1 ml), and was used as a the stock solution to generate calibration curves. Serial dilutions of these stock solutions were made to obtain solutions containing 125, 62.5, 31.25, 15.63, 7.81 and 3.91 $\mu g \cdot ml^{-1}$ of ACN. Pure iso-methyl eugenol, compound **5**, was used as a standard for quantifying this compound in *M. salicifolia* fruit extracts. Calibration curves of compound **5**, **7**, and **8**, then were generated from their HPLC data by Millennium 2010 (Millipore Corporation, Milford, Massachusettes) using the following equation: $y = A + Bx$; where y = response calculated by Millennium software for peaks at 217 nm, A = y - intercept of the calibration curve, x = component amount ranging from 125 to 3.91 $\mu g \cdot ml^{-1}$. For compound **5**, y = response was calculated for peaks at 222 nm.

The lyophilized and milled fruits were extracted exhaustively in triplicate, (1.11,

1.08, and 1.06 g, respectively) with MeOH at room temperature to afford three extracts **A-C** (501.6, 502.2, and 462.2 mg, respectively). These extracts were dissolved in ACN (20 $mg \cdot ml^{-1}$). The resultant solutions were filtered through 0.22 μm filters and analyzed (10 μl) by HPLC. All samples were quantified in triplicate. HPLC analyses of fruit extracts showed three major peaks of interest in the PDA chromatogram and were confirmed to be iso-methyl eugenol (**5**), costunolide (**7**), and parthenolide (**8**) at retention times 8.49, 9.49, and 7.38 min, respectively (**Figure 3.1**).

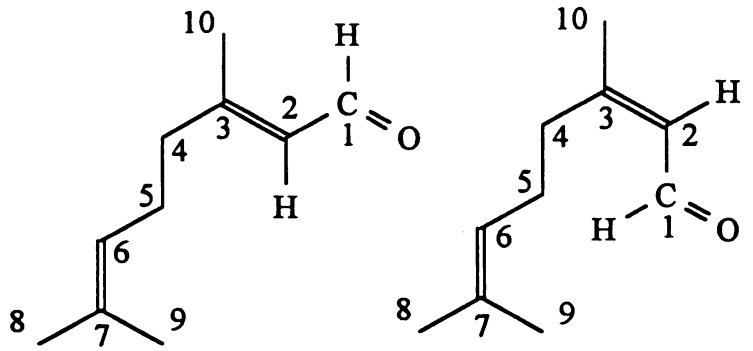
Circular Dichroism (CD) of Costunolide (7) and Parthenolide (8)

Pure costunolide and parthenolide were dissolved in MeOH separately (1 $mg \cdot 25$ ml^{-1}) and determined their CDs under the following conditions: scan mode (wavelength), bandwidth (1.0 nm), sensitivity (100 mdeg), response (2 s), wavelength range (350-200 nm), step resolution (1 nm/data), scan speed (50 $nm \cdot min^{-1}$), and accumulation (2) (**Figure 3.3** and **3.4**).

Results and Discussion

The monoterpene citral, which contains geranial (**1**) and neral (**2**) and the phenylpropanoid *trans*-anethole (**3**) were isolated previously from the leaves of *M. kobus* and *M. salicifolia* respectively (Fujita, 1955). The spectral data of geranial and neral, both geometric isomers of citral, was confirmed from ^{1}H and ^{13}CNMR data. The ratio of geranial to neral was approx. 9:1 in hexane extract of stems. The combined spectra of **1** and **2** gave identical ^{1}H and ^{13}CNMR chemical shift values, with the exception of the aldehydic protons and carbonyl carbons.

The aldehydic proton of **2** is more shielded in the *cis* position than in the *trans*

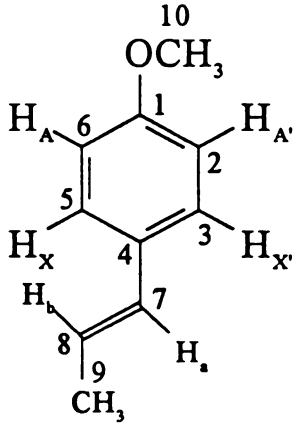


geranial (1) neral (2)

position and therefore, is shifted upfield. The aldehydic proton of **1**, on the other hand, is more deshielded than the corresponding proton in **2** because of its close proximity to the double bond at the C-2 and C-3 positions, thus appearing downfield. The doublet observed at δ 5.86 is characteristic of a vinylic proton whose signal is split due to the adjacent aldehydic proton. The multiplet signal observed at δ 5.05 is also in the characteristic absorption region of olefinic protons. However, it suggested the presence of a neighboring methylene group, and a long-range coupling with another proton at δ 2.56. This is indicative of CH_2 protons adjacent to a double bond. A multiplet at δ 2.17 was assigned the protons on carbon 5. Singlets observed at δ 1.66, 1.58, and 2.14 and integrated for three protons each were assigned to three olefinic groups. Based on the ^{13}CNMR chemical shifts, this molecule contains ten carbons one of which is a carbonyl. Furthermore, according to the ^{1}HNMR spectrum, this carbonyl belongs to an aldehyde functionality. Peaks at δ 127.22, 122.39, and 132.73 were assigned to olefinic carbons. Aldehydic ^{1}H and ^{13}CNMR for **1** and **2** were δ 9.98, 191.13 and 9.87, 190.62, respectively. This confirmed the presence of a mixture of two geometric isomers of this

monoterpene aldehyde. It finally was concluded that **1** and **2** were *trans* and *cis* form, respectively. Therefore, based on these spectral data the structures were elucidated as **1** and **2**.

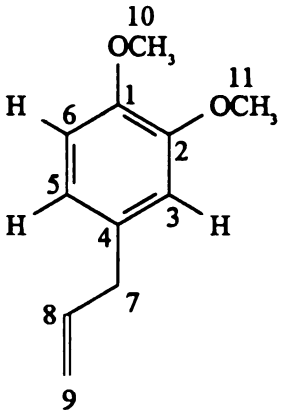
The ^{1}HNMR spectrum of **3** showed three distinctive features. It gave two sets of doublets in the aromatic region at δ 6.83 and 7.25. By Pople notation (Silverstein et al., 1991), this is a classic AA'XX' system or an AB system which is indicative of para disubstituted aromatic ring. The coupling constants, J_{AX} was 8.60 Hz. The sharp singlet at δ 3.80 is typical of an aromatic methoxy group. The presence of benzylic olefinic proton is indicated by a doublet at δ 6.34. The coupling constant of this proton was J_{ab}=15.8 Hz and confirmed a *trans* orientation. The multiplet observed at δ 6.09 was assigned to another olefinic proton on C-8. The doublet at δ1.86 was indicative of a terminal methyl group.



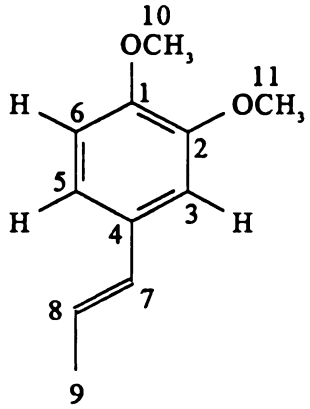
trans-anethole (**3**)

The ^{1}HNMR spectra of **4** was similar to **3** in most respects, since both contained aromatic protons, methoxylated aromatic protons, and an unsaturated side chain. The most striking difference between the two spectra was the presence of an additional

methoxy group. Two intense singlets for these OMe groups appeared at δ3.86 and 3.87. Expansion of the aromatic region revealed the presence of two doublets (much like that of *trans*-anethole) appearing at δ6.81 for C-5 and at δ6.73 for C-6. The proton at C-6 is shielded by the electron-donating ability of an adjacent methoxy group. Coupling constants for aromatic protons at C-5 and C-6 were 7.95 and 10.94Hz, respectively, and indicated that these protons are ortho to one another. A singlet, observed at δ6.71 was assigned to the proton at C-3. A doublet at δ3.34, integrated for two protons, was assigned to the benzylic protons in compound **4**. The olefinic proton, appearing as a multiplet at δ5.96, was assigned to the C-8 proton in compound **4**. The methylene protons were observed as a doublet at δ5.07. ^{13}CNMR data supported aromaticity, two olefinic carbons, one methylene carbon, and two oxygenated carbons. The molecular weight was deduced from the above information and finally confirmed by the molecular



methyl eugenol (4)

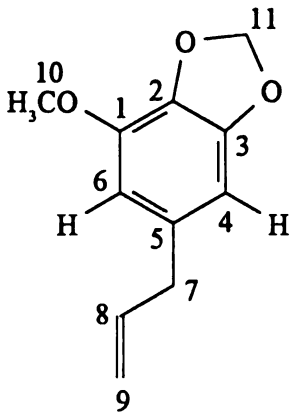


iso-methyl eugenol (5)

ion at m/z 178 observed in the MS spectrum. Spectral data on compound **5** showed

similiarities to compounds **3** and **4**. But **5** was similar in every respect to **4** with only one exception; a vinyl group was positioned in **5** where the allyl group was in **4**.

Compound **6**, the last phenyl propanoid identified, displayed a ^{1}HNMR spectrum most closely resembling that of compound **4**. A key difference was a the singlet at δ5.93 due to the protons of a methylenedioxy phenyl moiety. Another interesting feature observed in the spectrum was the presence of the only singlet at δ7.26 in the aromatic region. This was due to two magnetically equivalent meta protons. Furthermore, only one methoxy group was observed at δ3.88. Based on this data, the methoxy group at C-1, the methylene dioxy moiety at C-2 and C-3, and the allyl functionality at C-5, the structure of the compound was determined to be myristicin (**6**).



myristicin (**6**)

The presence of the phenylpropanoids, methyl eugenol (**4**), iso-methyl eugenol (**5**), and myristicin (**6**) were detected earlier by GC-MS analysis of the $CHCl_3$ extract of *M. salicifolia* buds (Tsurga et al., 1984). *O*-methyl eugenol was isolated from the bark of *M. officinalis* (Baek et al., 1992). Methyl eugenol was detected by GC-MS in the essential oils of *M. liliflora* leaves, branchlets, and flower buds (Fujita, 1989).

The spectral data for compound 7, costunolide, was identical to ^{1}H and $^{13}CNMR$ data published earlier (Ming et al., 1989). Rao and workers (1960) originally isolated costunolide from costus root oil of the costus plant, *Saussurea lappa* Clarke (Asteraceae). Costunolide also has been found in other Magnoliaceae plants, such as the leaves of *M. grandiflora* L. (Castañeda-Acosta et al., 1995), the root bark of *Michelia champaca* (Jacobsson, 1995) and the bark of *Michelia longiflora* Blume (Likhitwitayawuid et al., 1988). This is the first report of costunolide from *M. salicifolia*.

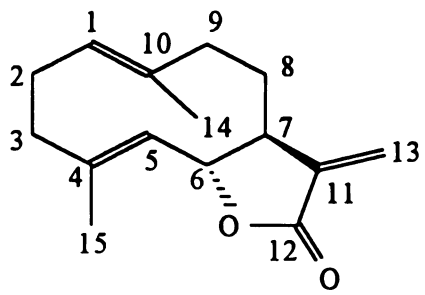
The yields of compounds **1-5** and 7 isolated from respective dried plant parts are shown in **Table 3.1**. The leaves contained the greatest number of active compounds (i.e., **3, 4, and 5**) followed by fruits, flowers, and stems. The fruits however, contained the most active compounds by weight.

The structural features (i.e., a germacrane sesquiterpene lactone) of 7 arose from $^{1}HNMR$, $^{13}CNMR$, DEPT, and mass spectral data. Key components of the $^{1}HNMR$ spectrum of 7 included: two methyl singlets at δ 1.67 and 1.41, two sets of doublets produced by exocyclic methylene protons at δ 6.62 (H-13') and 5.52 (H-13), and a triplet at δ 4.56 (H-6) for lactonic protons. A broad doublet for H-5 was observed at δ 4.73 (J=10 Hz) due to a CH_2 coupling. In the olefinic region, a broad doublet of a doublet was observed at δ 4.83 and assigned to C-1. The $^{13}CNMR$ spectrum of 7 contained 15 carbons with several in the olefinic region, thereby implying the possibility of a sesquiterpene with unsaturations. Also, a peak at δ169.96 (C-12) was indicative of a carbonyl group of an ester or a lactone. Moreover, the presence of a lactone seemed more

Table 3.1 Yield of compounds (**1-5** and **7**) isolated from dried *M. salicifolia* plant parts.

Plant Part	1+2	3	4	5	7
	%	%	%	%	%
stems	0.08	-	-	-	-
leaves	-	0.02	0.25	0.43	-
fruits	-	-	-	0.92	0.60
flowers	-	-	-	0.24	-

plausible, based on the triplet at δ 4.56 in the ^{1}HNMR spectrum. The peak at δ 119.14 (C-13) is representative of a terminal methylene carbon. It was concluded from these data that the partial structure of 7 consisted of a five-membered lactone ring with an exocyclic double bond. Furthermore, based on the remaining spectral data, the lactone was found to be fused to a larger ten-membered ring. Elucidation of the structure of the remainder of the molecule was achieved by DEPT NMR experiments. Two CH carbons at δ 81.40 and 49.90 were assigned to C-6 and C-7, respectively. The C-6 carbon was deshielded, primarily due to the lactone ring and the adjacent olefinic moiety at C-4 and C-5. Peaks appearing at δ 40.48 and 27.53 represented CH_2 carbons at C-3 and C-2, respectively. Another CH and tertiary olefinic carbons were observed at δ 126.54 and 136.44, respectively, for C-1 and C-10. Differences in chemical shifts for these carbons can be explained by the same rationale used for C-4 and C-5 carbons. The peaks at δ 38.51 (C-9) and 25.69 (C-8) were confirmed to be CH_2 carbons. Two peaks observed in DEPT at δ 16.84 (C-15) and 15.60 (C-14) which were assigned to methyl carbons. In the ^{1}HNMR spectrum, H-2', H-3, H-8/8', H-9/9' appeared as multiplets at δ 1.97-2.35.



costunolide (7)

A doublet of a doublet at δ 2.45 (H-3') can be explained by the vicinal coupling. The proton at C-7 appeared as a multiplet at δ 2.55 by coupling with protons on C-6 and C-8. The H-2 proton appeared as a multiplet due to the C-1 and C-3 proton couplings. MS of the compound gave the molecular ion at m/z 232.

HPLC analyses of the fruit extracts of *M. salicifolia* led to the quantification of iso-methyl eugenol, costunolide and its related sesquiterpene lactone, parthenolide, compound **8** (**Figure 3.1** and **3.2**). Parthenolide in *M. salicifolia* extracts was quantified and confirmed by HPLC analyses, using standard parthenolide. The ratio of costunolide to parthenolide was roughly 10:1, as indicated by HPLC quantification. Furthermore, this is the first report of parthenolide in *M. salicifolia.* Parthenolide was isolated previously from *M. grandiflora* L. and *M. denudata* (Wiedhopf et al., 1973 and Funayama et al., 1995).

Circular dichromism (CD) is the differential absorbtion of left and right circularly polarized light (cpl) by a non-racemic sample (Eliel, 1994). Cpl results from the filtering of electromagnetic radiation, so that the tip of its electric vector moves in a helical fashion along whose axis radiation arises. In addition to being non-racemic, the sample must contain two types of chromophores to obtain a CD spectra. Chromophores which are inherently achiral by symmetry such as the carbonyl, carboxyl groups, or carbon-carbon double bonds contain at least one plane of symmetry when considered without substituents. Other chromophore types that need to be considered are those that are inherently chiral.

Costunolide and parthenolide contain both classes of chromophores. The CD for

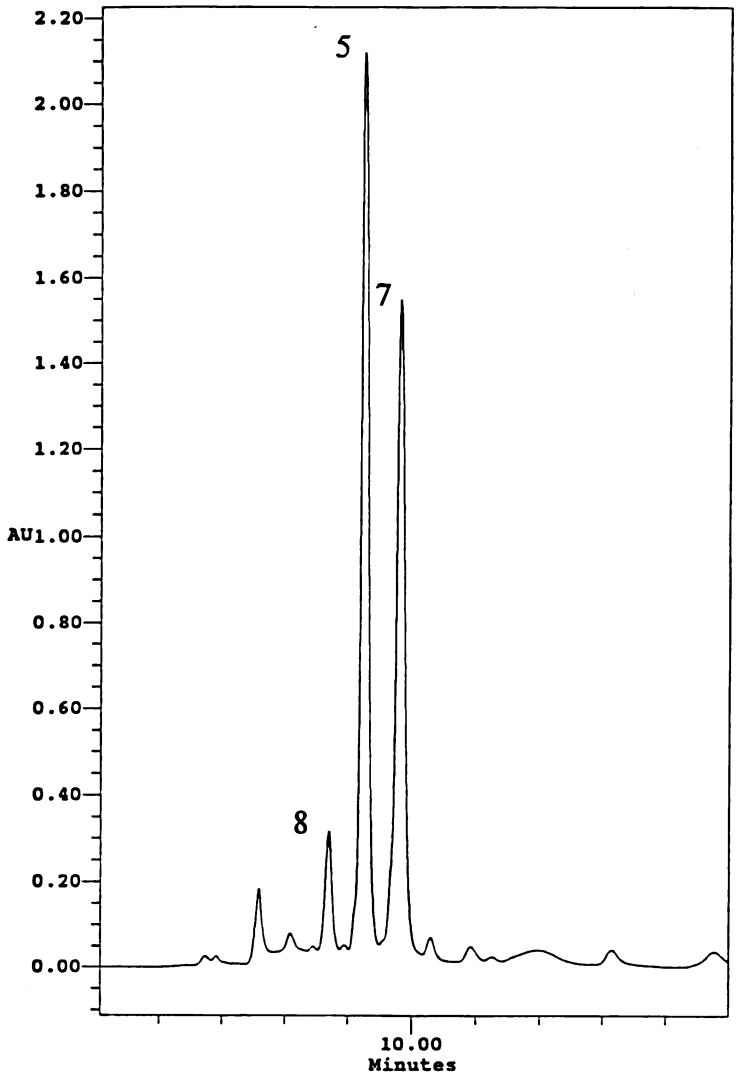


Figure 3.1 A representative HPLC chromatogram of *M. salicifolia* fruit extract showing iso-methyl eugenol (**5**), costunolide (**7**), and parthenolide (**8**) in absorption units, AU. Fruit extracts and pure costunolide, parthenolide, and iso-methyl eugenol were analyzed on a capcell pak C-18 (5 µm, 4.6 x 250 mm) column (Dychrome). The mobile phase ACN:H_2O (80:20 v/v) was used under isocratic conditions at a flow rate of 0.5 ml·min^{-1}. Injection volumes were 10 µl. Data were collected at 217 nm and 222 nm for costunolide and parthenolide, respectively.

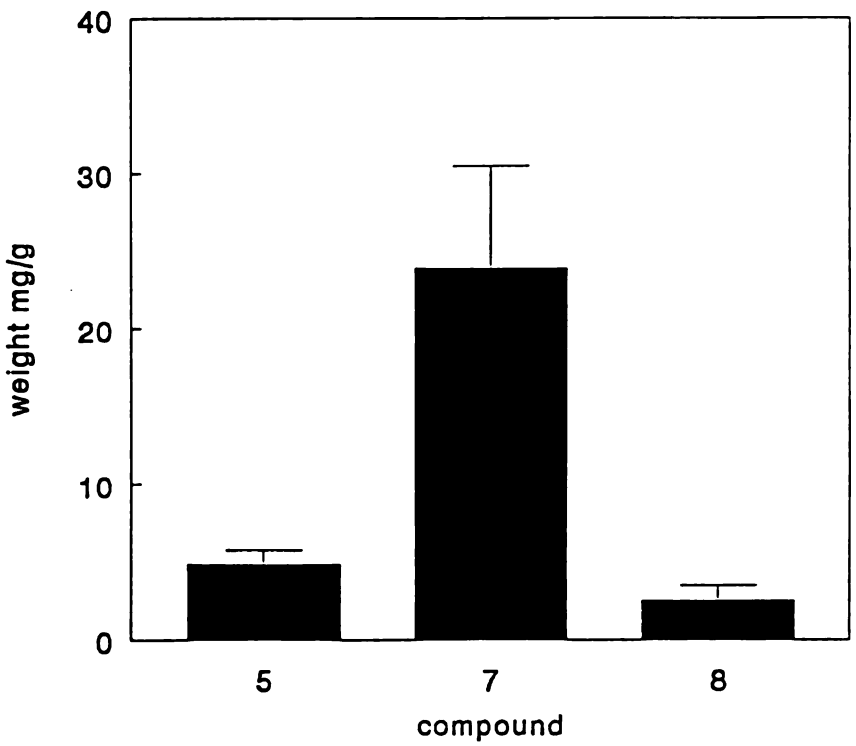


Figure 3.2 Yield ($mg \cdot g^{-1}$) of iso-methyl eugenol (**5**), costunolide (**7**), and parthenolide (**8**) from *M. salicifolia* fruit extracts.

costunolide and parthenolide indicate + and - absorptions for their respective structures (**Figure 3.3** and **3.4**, respectively). The source of these absorptions are the result of cumulative electronic or vibrational transitions associated with the chirotopic chromophores in parthenolide and costunolide, which causes right and left cpl to be absorbed differentially. Therefore, the absorbances $A_L \neq A_R$ and $\Delta A = A_L - A_R$ are measures of CD. Furthermore, since the molar concentrations are known, $\Delta A = \Delta \epsilon c l$ where c is the molar concentration (mol·liter^{-1}) and l is the path length (cm), therefore, $\epsilon_L - \epsilon_R = \Delta\epsilon$. ϵ_L and ϵ_R are the molar absorption coeffecients for left and right cpl, respectively. Subsequently, $\Delta\epsilon$ defines the sign of the CD. It can be concluded by the above relationships that costunolide, giving a predominately + CD spectrum, absorbs more left cpl, thus giving largely a positive molar elipticity as well. The molar elipticities of costunolide were 1.155E+02 mdeg (220 nm) and -7.815E+00 mdeg (262 nm) for the maximum and extremum, respectively. Parthenolide, giving a predominately - CD spectrum, absorbs more right cpl, and therefore, has mainly a negative molar elipticity. The molar elipticity of parthenolide was at the extremum of -2.942E+01 mdeg (208 nm). A literature search revealed that CD data for costunolide and parthenolide are not reported. This is the first report of the CD for costunolide and parthenolide.

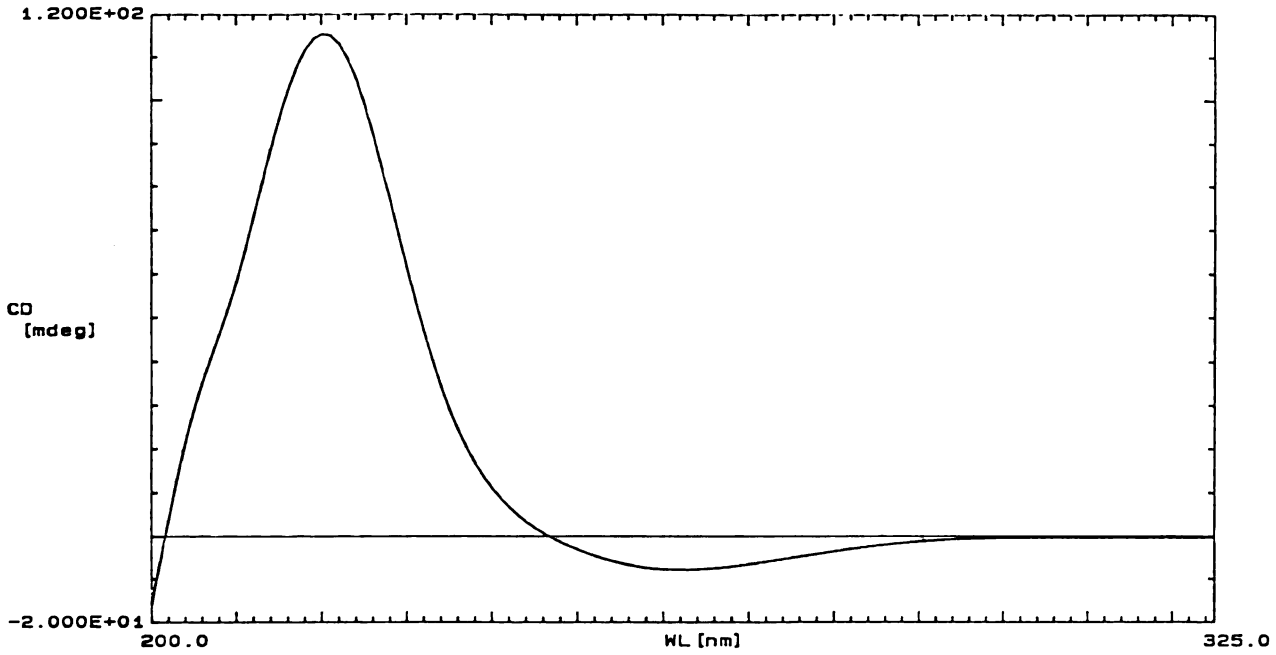


Figure 3.3 CD spectrum of costunolide, 7 (1 mg·ml^{-1}) at 220 nm shows a molar elipticity, ϵ at a maximum 1.155E+02 mdeg. At 262 nm an extremum at 7.005E+00 mdeg was observed for costunolide.

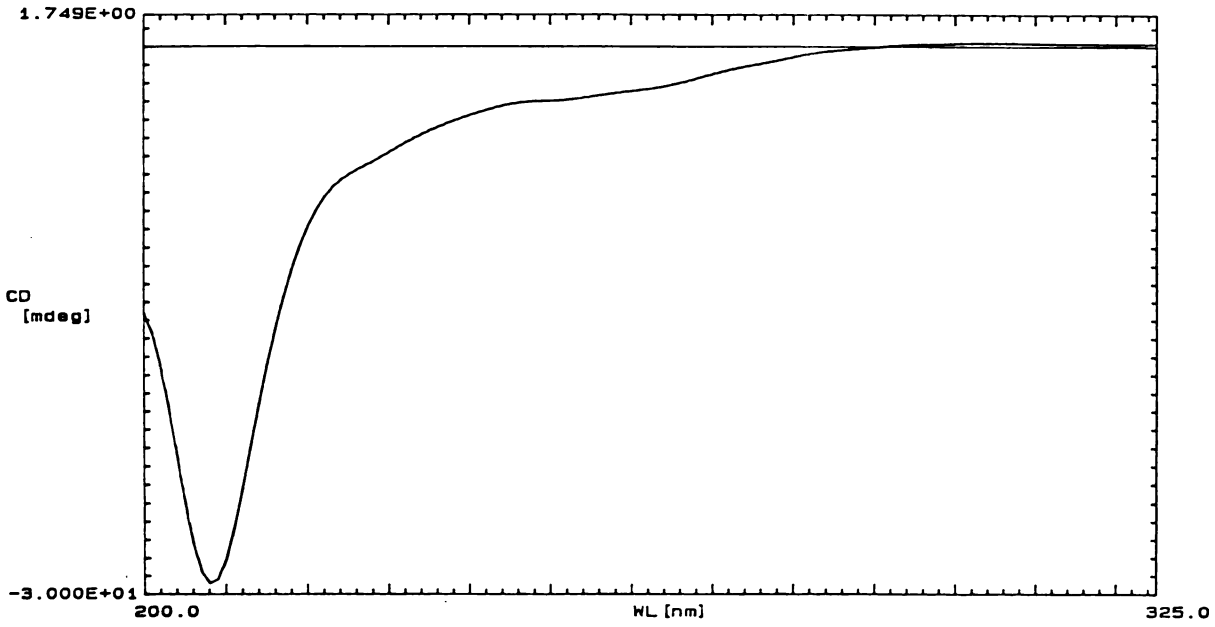


Figure 3.4 CD spectrum of parthenolide, **8** (1 mg·ml^{-1}) at 208 nm shows a molar elipticity, ϵ at a extremum at 2.942E+01 mdeg.

CHAPTER IV

Biologically Active Extracts from *Magnolia spp.* and Compounds from *Magnolia salicifolia*

Abstract

Preliminary antimicrobial, nematicidal, mosquitocidal and anti-cancer bioassays were carried out for leaf, stem, flower, and fruit extracts of ornamental *Magnolia salicifolia, Magnolia denudata* 'Yulan', *Magnolia kobus* var. *stellata* 'Star', and *Magnolia kobus* var. *loebneria* 'Merrill'. Crude extracts were bioassayed against microbial plant and human pathogens. Additional bioassays included mosquito larvae, *Aedes aegyptii;* gypsy moth larvae, *Lymantria dispar;* forest tent moth larvae, *Malacosoma distria;* and nematodes, *Panagrellus redivivus* and *Caenorhabditis elegans*. Anti-cancer bioassays were conducted on mutant *Saccharomyces cerevisae* strains to determine the presence of topoisomerase I or II poisons. Antimicrobial or nematicidal activities were absent for crude extracts at 250μg per spot on solid agar plates and 250 ppm in solution assays, respectively. Many extracts possessed larvacidal activity at 250 ppm against mosquito, *A. egyptii*. Extracts of *M. kobus* var. *stellata* and *M. denudata* demonstrated significant growth inhibition on gypsy moth larvae, *L. dispar.*

Minimum inhibitory concentrations (MIC) for mosquitocidal compounds from *M.*

salicifolia were determined. Geranial and neral (**1** and **2**), isolated from the bark resulted in 100% mortality at 100 ppm in 24 h. *Trans*-anethole (**3**), isolated from the leaves exhibited 100% mortality at 20 ppm in 24 h. 1, 2-dimethoxy-4-(2-propenyl) benzene (**4**) isolated from leaves and 1, 2-dimethoxy-4-(1-propenyl) benzene (**5**) isolated from leaves, fruits, and flowers, resulted in 100% mortality at 60 and 80 ppm, respectively, in 24 h. Costunolide (7), isolated from fruits, killed all *A. aegyptii* larvae at 15 ppm. Parthenolide (**8**), was not mosquitocidal when tested at 50 ppm. However, costunolide and parthenolide were found to be effective topoisomerase I poisons at 15 μg spot on solid agar plates lawned with test organism. Also, some of the extracts were active as top I and or top II poisons.

Introduction

As mentioned earlier, many phytochemical studies carried out on *Magnolia spp.* examined predominately pharmacological activities. A wide variety of compounds and activities associated with them have been identified. The anti-cancer activity of the sesquiterpenes, costunolide and parthenolide has been well documented (Funayama et al., 1995 and Wiedhopf et al., 1973). The alkaloids, (+)-R-coclaurine and (+)-S-reticulin, were found to possess negative inotropic effects (Kimura et al., 1989). Six lignans, derived from the buds of *M. biondii* were identified as blood anticoagulents (Pan et al., 1987). Other notable compounds possessing neurotrophic activity, were characterized from the root bark of *M. obovata* Thunb. (Fukuyama et al., 1989, 1990, 1992). The three novel sesquiterpene neolignans, eudesobovatol A, clovane magnolol, and caryolanemagnolol were resposible for the neurotrophic activity.

The phytochemical literature of *Magnolia spp.* relative to pesticidal activities, as mentioned before, are minimal. Yet, some studies have been done in this area. Phenolic constituents of *M. grandiflora* L., namely magnolol, honokiol, and 3, 5'-diallyl-2'-hydroxy-4-methoxy-biphenyl were found to have superior antibacterial activity against *Bacillus subtilis, Staphylococcus aureus,* and *Mycobacterium smegatis* when compared to streptomycin sulfate (Clarke et al., 1981). Additionally, magnolol and honokiol were found to be moderately active against *Candida albicans, Saccharomyces cerevisiae, Aspergillus niger* and strongly active against *Trichophyton mentagrophytes* when compared to amphotericin B. 3, 5'-diallyl-2'-hydroxy-4-methoxy-biphenyl demonstrated milder activity against *S. cerevisae* and strong inhibition of growth against *T.*

mentagrophytes when compared to amphotericin B.

Materials and Methods

Plant Materials

Stems of *M. salicifolia* were collected from trees growing on the campus of Michigan State University (East Lansing, Michigan) in February 1994. Stems, leaves, flowers and fruits of *M. salicifolia, M. denudata, M. kobus* var. *stellata,* and *M. kobus* var. *loebneria* were collected during the summer of 1994. Fresh weights were recorded for each of the plant parts. Large stems and fruits were cut into smaller pieces in order to facilitate efficient freeze drying. All plant parts were lyophilized at 5°C under vacuum for at least 24 h or until the plant material was dried. Following the dry weight determination, the dried plant parts were milled and stored at -20°C until extraction. **Table 4.1** reports percent dry matter for respective plant and plant parts.

Extraction

Separate extractions were conducted for each plant part. Dried ground plant materials were placed in an extraction column. All plant parts were extracted sequentially with hexane, EtOAc, and MeOH. The plant materials were soaked in solvent for at least 24 h with one exchange of fresh solvent after 12 h. Solvents were removed *in vacuo* and stored at -20°C until bioassay and purification. **Table 4.2** indicates the yield of extract generated from respective *Magnolia spp.* plant parts.

Bioassays

Microbial bioassays. The microorganisms assayed were: Hyphales; *Botrytis spp.*, *Aspergillus flavus* (MSU strain), *Fusarium oxysporum* (MSU-SM-1322), *F. moniliforme*

(MSU-SM-1323), Melanconiales; *Gloesporum spp.*, Agonomycetales; *Rhizoctonia spp.* (MSU strain), *Candida albicans* (MSU strain), Bacteria; *Staphylococcus epidermidis* (ATCC 25923), *Streptococcus aureus* (MSU strain), *Escherichia coli* (ATCC 25922) and nematodes; *Panagrellus redivivus* Goody and *Caenorhabditis elegans.* Bacteria, yeast, and fungi, respectively, were grown on Emmons, YMG, PDA agar media and maintained in the Bioactive Natural Products Laboratory (BNPL). These assays were conducted according to published methods (Nair et al., 1989). Samples were prepared by dissolving a known amount of the extract into an appropriate volume of DMSO such that the final concentration was $250\mu g \cdot 20\mu l^{-1}$. The microbial organisms were lawned onto petri dishes containing the appropriate agar media. Aliquots of 20 µl of test samples were spotted, respectively, onto lawned plates. Plates were allowed to incubate at 26°C for approximately 5-7 days. Thereafter, plates were analyzed for the presence of inhibition zones. None of the *Magnolia spp.* tested extracts possessed anti-microbial activity.

Mosquitocidal bioassays. Fourth instar mosquito larvae, *Aedes aegypti* L. (Culicidae), were reared in the BNPL from eggs. Eggs were obtained from Dr. Alexander Raikel, Department of Entomology, Michigan State University. At least 10 larvae were placed in 980 µl of degassed distilled H_2O and 20 µl of DMSO containing test extracts or purified compounds. Resulting concentrations were 250 ppm for crude extracts. Initial concentrations for purified compounds were 100 ppm which then were diluted serially to determine MIC. The tube containing the control larvae received 20 µl of DMSO. Treatments and controls were left at room temperature. There were three replications per treatment. The number of dead larvae were recorded at 2, 4, 6, and 24 h intervals. The

tube containing the control larvae received 20 µl of DMSO alone, and the number of dead larvae were recorded as in the case of the test compounds (Nair et al., 1989 and Nitao et al., 1992). Many of the extracts had mosquitocidal activity at 250 ppm. MICs for compounds **1+2**, **3**, **4**, **5**, and **6**, were 100, 20, 60, 80, and 15 ppm, respectively. Compound **7** was not mosquitocidal when tested at 50 ppm.

Nematicidal bioassays. These assays were performed on free-living nematodes *Panagrellus redivivus* and *Caenorhabditis elegans* from cultures maintained in the BNPL. Test samples were prepared by dissolving a known weight of extract into DMSO such that the concentration was $12.5\mu g \cdot 2\mu l^{-1}$. An aliquot of 48µl of media containing 30-50 nematodes at various developmental stages, were transferred aseptically from stock solutions into each sterile well (0.7 cm diameter × 1.0 cm deep) of a 96-well Corning flat-bottomed tissue culture plate. 2µl of the test solution was added to the 48µl thereby bringing the final concentration to $12.5\mu g \cdot 50\mu l^{-1}$ or 250 ppm. Plates were covered and placed in a sealed container at high humidity. Plates were monitored for 2, 24, and 48 h intervals.

Gypsy moth caterpillar bioassay. Gypsy caterpillar eggs were obtained from The Forest Pest Management Institute, Sault Ste. Marie, Ontario Canada via Dr. Daniel A. Herms of the Department of Entomology, Michigan State University. Eggs were stored under refrigeration until needed. Eggs were hatched in a growth chamber at ambient temperature with 15 h photoperiod. After 2-3 days, larvae were moving freely. Crude extracts and purified compounds were dissolved in DMSO to give a concentration of 1250 $\mu g \cdot 25\mu l^{-1}$ and 500 $\mu g \cdot 25\mu l^{-1}$, respectively. 25 µl of stock test solutions were mixed

completely with 845 mg of dry diet mix (see **Appendix XI** for recipe). Agar held at 50°C, then was added until total diet weighed 5 g. The final concentration of test extracts and compounds were 250 and 100 ppm, respectively. The warm agar was mixed thoroughly with the dry portion of diet and then poured into 15 disposable polystyrene conicle cups (3.5 ml capacity, Sarstedt). The freshly poured diets were allowed to set 30 min in order for excess moisture to evaporate. Thereafter, larvae were added to the vials. For the gypsy moth assay, each vial received 1 larva. There were a total of 48 extracts with 15 replications per treatment. After six days, the larvae were weighed, these weights were averaged and then compared with the control weight average. Data were analyzed using Dunnet's test, where all means are compared with a control.

Anti-cancer bioassays. Saccharomyces cerevisae mutant cell cultures of JN394, $JN394t_1$, and $JN394t_{2\text{-}5}$ were supplied by Dr. John Nitiss of St. Jude Children's Hospital, Memphis, Tennessee and maintained in our laboratory. JN394 is hypersensitive to topoisomerase I poisons, while $JN394t_1$ is isogenic to JN394 except for the deletion of the top I gene, therefore showing a lack of response to topoisomerase I poisons. $JN394t_{2\text{-}5}$ carries top II gene which is resistant to topoisomerase II poisons, but responds to topoisomerase I poisons. These organisms were cultured in petri dishes containing 20 ml YPDA media. Plates were spotted with crude extracts, pure mosquitocides (**1-6**), parthenolide (**7**), and camptothecin and etoposide as positive controls. Plates were incubated at 26°C for one week. Camptothecin, a top I poison, is inhibitory to JN394 and $JN394t_{2\text{-}5}$. Etoposide, a top II poison, is active against JN349 and $JN394t_1$. MICs were determined for pure compounds by assaying a range of serial dilutions beginning at 250

$\mu g \cdot 20\ \mu l^{-1}$.

Statistical Analyses of Gypsy Caterpillar Data

The statistical analyses of the data concerning the gypsy moth larvae bioassay involved analysis of variance (ANOVA) for a completely randomized design (CRD) followed by Dunnett's test for each experiment. The ANOVA were done to get a preliminary feel if the treatments had any effect on the test organism. Dunnett's method was chosen because we are interested in determining whether the mean of the control group is significantly different than each of the means of the treatments. Due to the time involved in this bioassay, treatments were split into six separate experiments. Experimental treatments consisted of crude extracts and/or pure compounds. Geraniol and nerol (Aldrich Chemical, Milwaukee, Wisconsin) the respective corresponding alcohols of compounds **1** and **2** also were included in the study.

Results and Discussion

Leaves, stems, flowers and fruits of *M. salicifolia, M. kobus* var. *stellata* 'Star', *M. denudata* 'Yulan', *and M.kobus* var. *loebneria* were extracted sequentially with organic solvents. Dry weights for the various *Magnolia spp.* plant parts are shown in **Table 4.1**. Generally, the dry weights for the same plant part for different species were nearly equivalent. Extract weights for all plant parts from each *Magnolia spp.* are shown in **Table 4.2**. The yields of MeOH extracts in all instances were larger than hexane and EtOAc extracts combined.

Preliminary bioassays were performed on these extracts for their antifungal, antibacterial, mosquitocidal, and nematicidal activities. Other preliminary bioassays were

Table 4.1 Dry matter content for *Magnolia spp.* plant parts.

Plant species	% dry matter
M. salicifolia	
leaves	32. 9
stems	44.5
flowers	10.3
fruits	19.8
M. kobus* var. *stellata	
leaves	41.8
stems	46.6
fruits	8.9
flowers	18.5
M. denudata	
leaves	7.9
stems	38.9
fruits	9.9
flowers	21.0
M. kobus* var. *loebneria	
leaves	28.7
stems	48.9
fruits	11.6
flowers	20.0

Table 4.2 The weight of extracts from various *Magnolia spp.* plant parts.

Plant species	Dry weight of plant material	Hexane extract	EtOAc extract	MeOH extract
	g	g	g	g
M. salicifolia				
leaves	100.27	3.77	2.51	11.88
stems	100.00	4.07	0.57	11.80
flowers	50.15	1.55	0.50	15.42
fruits	120.27	12.66	3.16	23.68
M. kobus var. *stellata*				
leaves	5.82	0.23	0.09	0.70
stems	100.26	2.98	1.76	5.67
flowers	20.00	1.96	0.18	4.59
fruits	2.83	0.07	0.04	0.27
M. denudata				
leaves	10.49	0.58	0.27	0.94
stems	100.15	1.48	0.87	5.34
flowers	20.00	0.32	0.38	4.53
fruits	2.51	0.09	0.04	0.21
M. kobus var. *loebneria*				
leaves	10.76	0.50	0.30	1.14
stems	100.00	3.34	0.89	8.16
flowers	6.34	0.22	0.18	2.35
fruits	4.33	0.25	0.30	0.64

conducted to determine the presence of growth inhibitors for gypsy moth larvae and the topoisomerase I and II poisons. Hexane (**Table 4.3**) and EtOAc extracts (**Table 4.4**) from all *Magnolia spp.* were found to be lethal to the larvae of *A. egyptii* at 250 ppm. The MeOH extracts displayed only minor mosquitocidal activity. All extracts of *M. kobus* var. *stellata* and fruit extracts of *M. denudata* demonstrated growth inhibition against *L. dispar* (**Table 4.8**). No other extracts had shown any significant reduction in weight (**Tables 4.5-4.7** and **4.9**). Preliminary anti-cancer bioassays indicated the presence of active components in stem, leaf, and fruit hexane extracts of *M. salicifolia.* Leaf EtOAc extracts of *M. salicifolia* and hexane and EtOAc extracts of *M. stellata* also were found to exhibit anticancer activity. It was concluded that compounds contained in these extracts were topoisomerase I poisons, since activity was observed for JN394 and $JN394_{t-1}$. None of the extracts showed any antimicrobial or nematicidal activities.

Mosquitocidal activities were evaluated for compounds **1-5, 7** and **8** (**Table 4.10**). Compound **6**, myristicin, was reported earlier to be mosquitocidal at 25 ppm and fungicidal against *Cladosporium cucumerinum* at 20 μg (Marston et al., 1995). Myristicin also has exhibited toxicity towards fruit flies, *D. melanogastar* at an LD_{50} of 0.34 mg and against mice at an LD_{50} 200 $mg \cdot kg^{-1}$ (Lichtenstein et al., 1974). In the same study, myristicin was found to act synergistically at 2% w/v with 0.1% w/v pyrethrins against house flies, *Musca domestica.* Synergisms also were observed when a mixture of myristicin and parathin, a carbaryl, were tested against fruit flies. Berenbaum and Neal (1985) explained that the mode of action of methylenedioxyphenyl compounds like myristicin, is through inhibition of the activity of mixed function oxidases (MFO), the

Table 4.3 Mortality of *A. egyptii* for hexane extracts of Magnolia plant parts at 250 ppm after 24 h.

	Percent Mortality*			
Magnolia spp.	**leaves %**	**stems %**	**flowers %**	**fruits %**
M. salicifolia	100	100	100	100
M. kobus var. stellata	100	56.8±13.0	NA	100
M. denudata	100	85.6±10.6	100	100
M. kobus var. loebneria	100	16.7±17.0	100	100

NA=not active at 250 ppm
***DMSO controls had 100% survival**

Table 4.4 Mortality of *A. egyptii* for EtOAc extracts of Magnolia plant parts at 250 ppm after 24 h.

	Percent Mortality*			
Magnolia spp.	leaves %	stems %	flowers %	fruits %
M. salicifolia	40 ±21.6	NA	NA	100
M. kobus var. stellata	100	22.1 ±5.6	NA	100
M. denudata	87.9 ±4.3	93.6 ±4.5	96.7 ±4.7	97.0 ±4.3
M. kobus var. loebneria	51.7± 13.2	23.3 ±4.7	100	56.7 ±12.5

NA=not active at 250 ppm
*DMSO controls had 100% survival

Table 4.5 Average weights of *L. dispar* larvae after six days. Treatments for pure compounds were bioassayed at 100 ppm. DMSO was used for the control.

treatment	weight (mg)
trans-anethole (3)	9.9, -
citral (1 & 2)	11.8, -
geraniol	11.3, -
nerol	11.2, -
control	9

*Data was analyzed using Dunnett's Test, $P \leq 0.01$
+ Significant reduction in weight
- No significant reduction in weight

Table 4.6 Average weights of *L. dispar* larvae after six days. Treatments for crude *Magnolia spp*. extracts and pure compounds were bioassayed at 250 ppm and 100 ppm, respectively.

treatment	weight (mg)
M. salicifolia	
stem hexane extract	9, -
stem EtOAc extract	11, -
stem MeOH extract	11.3, -
leaf hexane extract	10.4, -
leaf EtOAc extract	8.3, -
leaf MeOH extract	9, -
flower hexane extract	15.4, -
flower EtOAc extract	10, -
flower MeOH extract	10.2, -
fruit hexane extract	11.2, -
fruit EtOAc extract	8.3, -
fruit MeOH extract	11.3, -
M. kobus var. *loebneria*	
stem hexane extract	11.2, -
stem EtOAc extract	12.1, -
stem MeOH extract	9.3, -
Compounds	
costunolide (7)	10.8, -
iso-methyleugenol (5)	10.8, -
methyleugenol (4)	9.1, -
control	9.5

*Data was analyzed using Dunnett's Test, $P \leq 0.01$

\+ Significant reduction in weight

\- No significant reduction in weight

Table 4.7 Average weights of *L. dispar* larvae after six days. Treatments for crude *Magnolia spp*. extracts and pure compounds were bioassayed at 250 ppm and 100 ppm, respectively.

treatment	weight (mg)
M. denudata	
flower hexane extract	9.2, -
flower EtOAc extract	8.2, -
flower MeOH extract	8, -
stem hexane extract	7, -
stem EtOAc extract	8.2, -
stem MeOH extract	9.1, -
leaf hexane extract	10.2, -
leaf EtOAc extract	6.1, -
leaf MeOH extract	9.2, -
M. kobus var. *loebneria*	
fruit hexane extract	11.1, -
fruit EtOAc extract	8.4, -
fruit MeOH extract	9, -
flower hexane extract	10.5, -
flower EtOAc extract	9.2, -
flower MeOH extract	10.4, -
leaf hexane extract	9.6, -
leaf EtOAc extract	8.3, -
leaf MeOH extract	9.1, -
control	9.6

*Data was analyzed using Dunnett's Test, $P \leq 0.01$

\+ Significant reduction in weight

\- No significant reduction in weight

Table 4.8 Average weights of *L. dispar* larvae after six days. Treatments for crude *Magnolia spp*. extracts were bioassayed at 250 ppm.

treatments	weight (mg)
M. denudata	
fruit hexane extract	9.9, +
fruit EtOAc extract	9.3, +
fruit MeOH extract	10.1, +
M. kobus var. *stellata*	
leaf hexane extract	9.2, +
leaf EtOAc extract	6.8, +
leaf MeOH extract	7.3, +
stem hexane extract	10.2, +
stem EtOAc extract	8.2, +
stem MeOH extract	7.9, +
flower hexane extract	9.8, +
flower EtOAc extract	10.3, +
flower MeOH extract	9.6, +
fruit hexane extract	9.3, +
fruit EtOAc extract	14.1, +
fruit MeOH extract	17.8, -
control	20.1

*Data was analyzed using Dunnett's Test, $P \leq 0.01$

+ Significant reduction in weight

- No significant reduction in weight

Table 4.9 Average weights of *L. dispar* larvae after six days. Treatments for pure compounds were bioassayed at 100 ppm.

treatments	weight (mg)
myristicin (6)	14, -
parthenolide (8)	15.7, -
control	15.6

*Data was analyzed using Dunnett's Test, $P < 0.01$
+ Significant reduction in weight
- No significant reduction in weight

enzymes that metabolize many lipophilic xenobiotics in both vertebrates and invertebrates. MFOs are the primary means by which insects detoxify naturally occurring plant toxins. They found in their study that at as little as 0.1% myristicin in an artificial diet increased the toxicity of xanthotoxin to corn earworm, *Heliothis zea*. Mode of action, of the other compounds (**1-5**, **7**, **8**) of the this study have not been determined. However, observations regarding lethality of compounds relative to structural differences may be inferred.

Phenylpropanoids, **3**, **4**, **5**, and **6** had activities ranging from 20 to 80 ppm concentrations. Among them, compound **3** having a methoxy group para to the benzylic double bound, was the most potent mosquitocide. Addition of another methoxy group in compound **5** gave reduced mosquitocidal activity. However, compound **4** was more active than **5**, due to the presence of an allyl group instead of the benzylic double bond. Compound **6**, more active than **4** or **5**, may derive its toxicity for the reasons mentioned above. When applied in 20 mg quantities, compounds **1** and **2** collectively, were reported to have antifungal activity against *E. coli*, *B. subtilis*, and *Staphylococcus aureus* (Onawani et al., 1984). Citral also has demonstrated phototoxicity to cabbage loopers, *Trichoplusia ni* at concentrations of 300 ppm in an artificial diet (Green and Berenbaum, 1995).

Table 4.10 Mosquitocidal activities of pure compounds from *M. salicifolia* on 4th instar *A. egyptii* after 24 h.

Compounds	LD_{100} (ppm)†
1 + 2	100
3	20
4	60
5	80
6	25*
7	15
8	NA

*Reported earlier by Marston et al., 1995
NA=not active at 50 ppm
†DMSO controls had 100% survival.

The introduction of allyl groups onto aromatic rings can increase antibacterial activity of some phenolics (Bae et al., 1986). However, extrapolation of this observation with that of the present data, may be spurious. Compound 7, the most active of all the compounds isolated, gave an MIC of 15 ppm. The exocyclic double bond and carbonyl group were demonstrated to be the most important factors responsible for the cytotoxicity among sesquiterpene lactones (Lee et al., 1971). Interestingly, parthenolide (**8**) did not show any mosquitocidal activity when assayed at 50 ppm. These data suggest that the presence of a double bond rather than an epoxide at carbons 4 and 5 in costunolide is required for mosquitocidal activity. However, information regarding the structural activity relationships with respect to insecticidal activity for these compounds is lacking.

The F tests for the ANOVA of first and third gypsy moth bioassay indicated no overall significance among the mean weights. Furthermore, according to Dunnett's method, none of the treatment mean weights differed significantly from the control mean weights (**Tables 4.5** and **4.7**). The F test for the ANOVA of second gypsy moth bioassay demonstrated an overall significance at $P \leq 0.01$ among the mean weights. However, Dunnett's test had shown that none of the treatment mean weights were significantly different than that of the control mean weights (**Table 4.6**). The F test for the ANOVA of the fourth gypsy moth bioassay indicated an overall significance at $P \leq 0.01$ among the mean weights. Additional support came from Dunnett's test where it was shown that 14 out of 16 treatment mean weights were significantly lower than the weight of the control mean weights (**Table 4.8**). The last gypsy moth bioassay showed no significant reduction in weight after six days when parthenolide and myristicin were tested at 100 ppm (**Table**

4.9). In conclusion, all treatment extracts of *M. kobus* var. *stellata* and fruit extracts of *M. demudata* significantly reduced the weights of the larvae when tested at 250 ppm. Compounds with mosquitocidal activities had little or no effect on gypsy moth larvae weight when at 100 ppm.

Only compounds **6, 7** and **8** showed activity against JN394 and $JN394_{t2\text{-}5}$ indicating inhibitory activity against topoisomerase I (**Table 4.11**). Compounds **7** and **8** were equally active at 15 μg per spot, whereas **6** was only active at 250 μg per spot . Results for **7** and **8** support earlier findings expressed by Lee and coworkers (1971). Topoisomerase I and II are enzymes that change the DNA linkers by catalyzing a three-step process. This involves the cleavage of one (topoisomerase I) or both (topoisomerase II) strands of DNA, movement of a segment of DNA through this break, and then resealing the DNA break (Stryer, 1988). The lack of antibacterial or antifungal activities of these extracts suggests that the phytochemistry of northern North American *Magnolia spp.* are quite different than those grown in the southern United States.

Table 4.11 Anti-cancer activity of citral (1 and 2), trans-anethole (3), methyl eugenol (4), isomethyl eugenol (5), myristicin (6), costunolide (7), parthenolide (8), camptothecin and etoposide.

Compounds	µg/spot	*S. cerevisae*		
		JN394	JN394 t_1	JN394 $t_{2.5}$
1 + 2	250	-	-	-
3	250	-	-	-
4	250	-	-	-
5	250	-	-	-
6	250	+, 1.7 cm	-	+, 1.7 cm
7	15	+, 1.1±0.1 cm	-	+, 1.1±0.1 cm
8	15	+, 1.1±0.1 cm	-	+, 1.1±0.1 cm
*camptothecin	25	+, 2.6±0.2 cm	-	+, 2.6±0.2 cm
*etoposide	25	+, 2.5±0.1 cm	+, 2.5 cm	-

+ active, zone of inhibition diameter
- not active
*standards purchased from Sigma Chemical Company

CHAPTER V

Summary and Conclusion

Leaves, stems, flowers, and fruits of four *Magnolia spp.* were extracted and preliminary bioassays were performed on these extracts at 250 ppm concentrations to test for the presence of antifungal, antibacterial, mosquitocidal, and nematicidal activities. Bioassays also were conducted on all extracts on gypsy moth larvae, *L. dispar* to test for growth inhibition. Anti-cancer bioassays were evaluated for all crude extracts which utilized mutant *S. cerevisae* strains. All crude extracts were found to be mosquitocidal on the larvae of *A. egyptii*. Extracts of *M. kobus* var. *stellata* and *M. denudata* were found to significantly reduce the growth of *L. dispar* larvae. However, crude solvent extracts from these *Magnolia spp.* did not show any antimicrobial or nematicidal activities when tested on Hyphales; *Botrytis spp., Aspergillus spp., Fusarium oxysporum, F. moniliforme,* Melanconiales; *Gloesporum spp.,* Agonomycetales; *Rhizoctonia spp., Candida albicans,* Bacteria; *Staphylococcus spp., Streptococcus spp., Escherichia coli* and nematodes; *Panagrellus redivivus* Goody and *Caenorhabditis elegans* at 250 ppm concentrations. However, some crude extracts exhibited moderate anti-cancer activity.

Through the process of bioassay directed fractionation six mosquitocidal compounds were isolated from the various plant parts of *M. salicifolia*; citral (**1** and **2**), *trans*-anethole (**3**), methyl eugenol (**4**), iso-methyl eugenol (**5**), and costunolide (**7**).

Myristicin (**6**) was detected by ^{1}HNMR along with anethole in leaf extracts of *M. salicifolia*. HPLC analysis of the fruits of *M. salicifolia* led to the identification and quantification of the sesquiterpene lactone, parthenolide. Costunolide and iso-methyl eugenol also were quantified in an HPLC analysis of the fruit extracts. Structures of compounds **1-7** were confirmed by ^{1}HNMR, ^{13}CNMR, and MS experiments (**Chapter III**). Mosquitocidal and anticancer activities were evaluated for compounds **1-8** (**Chapter IV**). The most potent mosquitocide was found to be the sesquiterpene lactone, costunolide. The MIC for this compound was 15 ppm. Parthenolide was not active against mosquito larvae when tested at 50 ppm. Citral, trans-anethole, methyl eugenol, iso-methyl eugenol, and myristicin had mosquitocidal activities at 100, 20, 60, 80, and 25 ppm, respectively. Costunolide and parthenolide displayed anti-cancer activity and their MICs were 15 μg. Myristicin also demonstrated anticancer activity at 250 μg.

Mode of action of these compounds for their insecticidal activity has not been determined. However, observations regarding lethality of compounds relative to structural differences may be inferred. Phenylpropanoids, **3**, **4**, **5**, and **6** had activity concentrations ranging from 20 to 80 ppm. Among them, compound **3**, having a methoxy group para to the benzylic double bound, was the most potent mosquitocide. Addition of another methoxy group in compound **5** gave reduced mosquitocidal activity. However, compound **4** was more active than **5**, due to the presence of an allyl group instead of the benzylic double bond. Compound **6** with a methylenedioxy functionality, was the second most potent mosquitocide. Its activity was quite similar to that of **3** and may be due to its allyl functionality.

The introduction of allyl groups onto aromatic rings increased antibacterial activity of some phenols and biphenols (Bae et al., 1986). However, extrapolation of this observation with that of the present data, requires additional research. Compound 7, the most active of all compounds isolated, had an MIC of 15 ppm. Exocyclic double bonds and carbonyl groups were the important factors responsible for cytotoxicity among sesquiterpene lactones (Lee et al., 1971). Parthenolide showed no mosquitocidal activity when bioassayed at 50 ppm. This data suggests that the presence of a double bond rather than an epoxide at carbons 4 and 5 in costunolide is required for mosquitocidal activity. However, information regarding the structure activity relationships with respect to insecticidal activity for these compounds is lacking.

The work contained herein has yielded known compounds with new activities. These compounds were reported previously. Additionally, compound 7, parthenolide, has been reported for the first time in *M. salicifolia*. Similarly, this is the first report of spectra for CD for parthenolide and costunolide.

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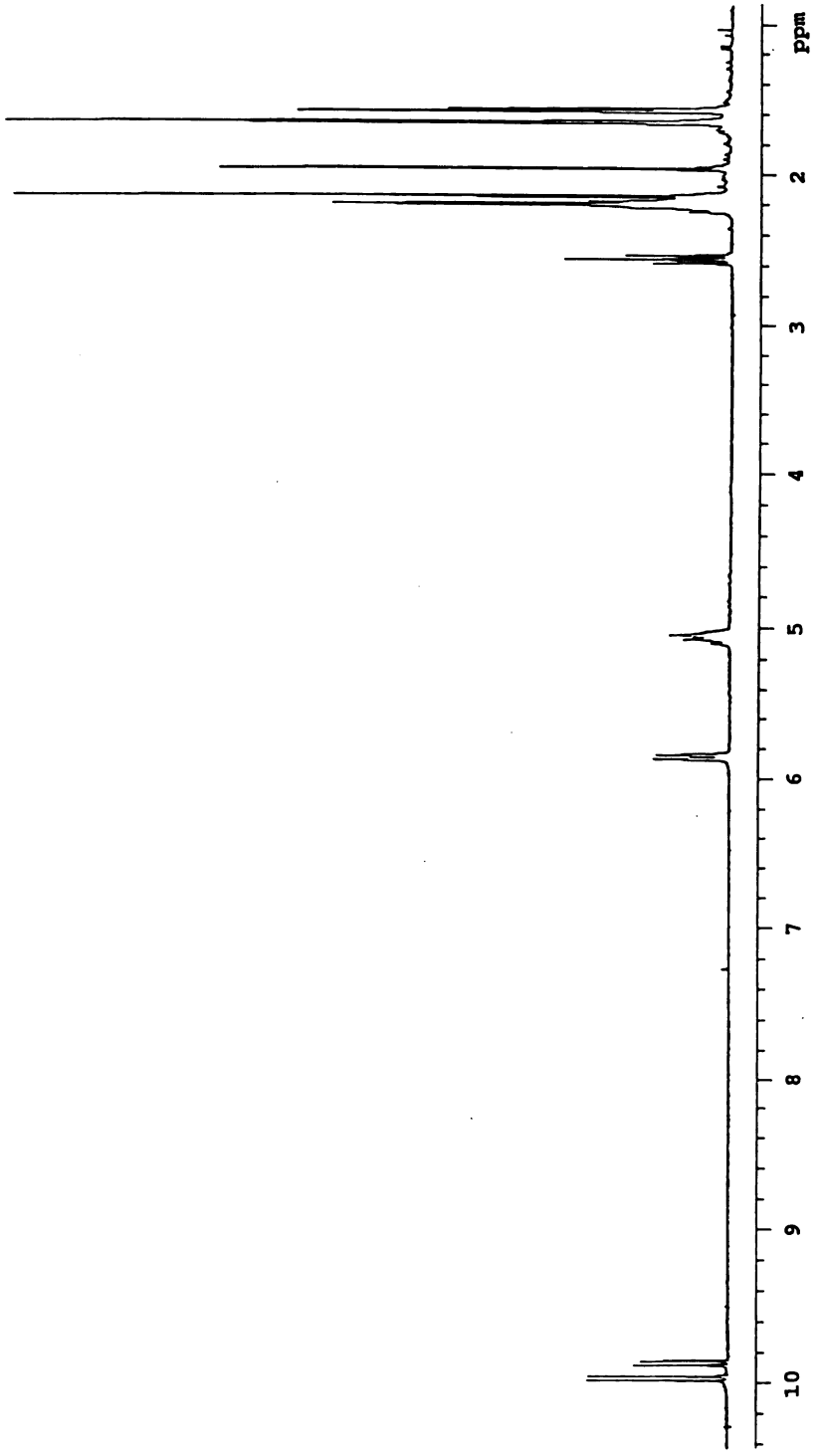
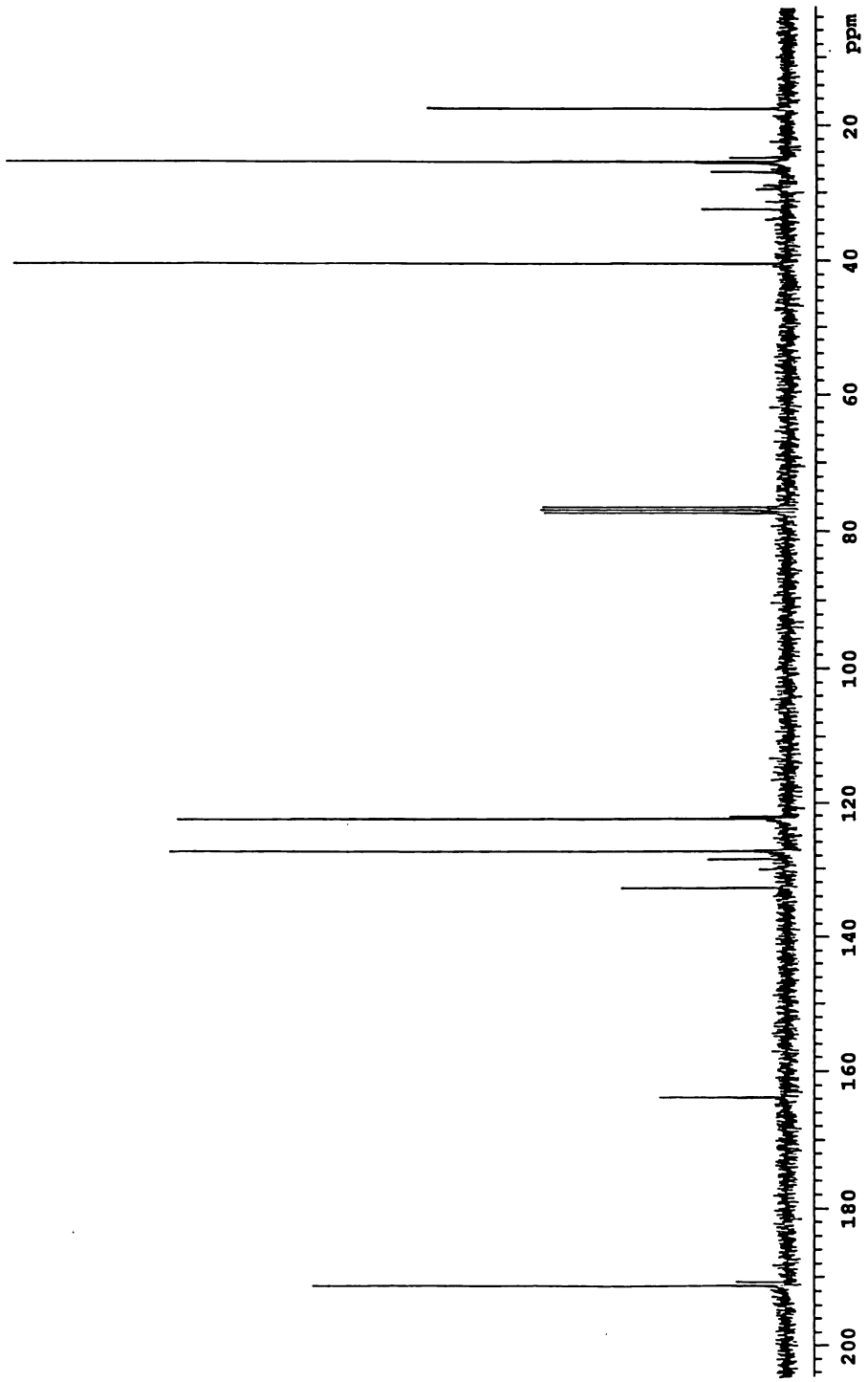
APPENDICES

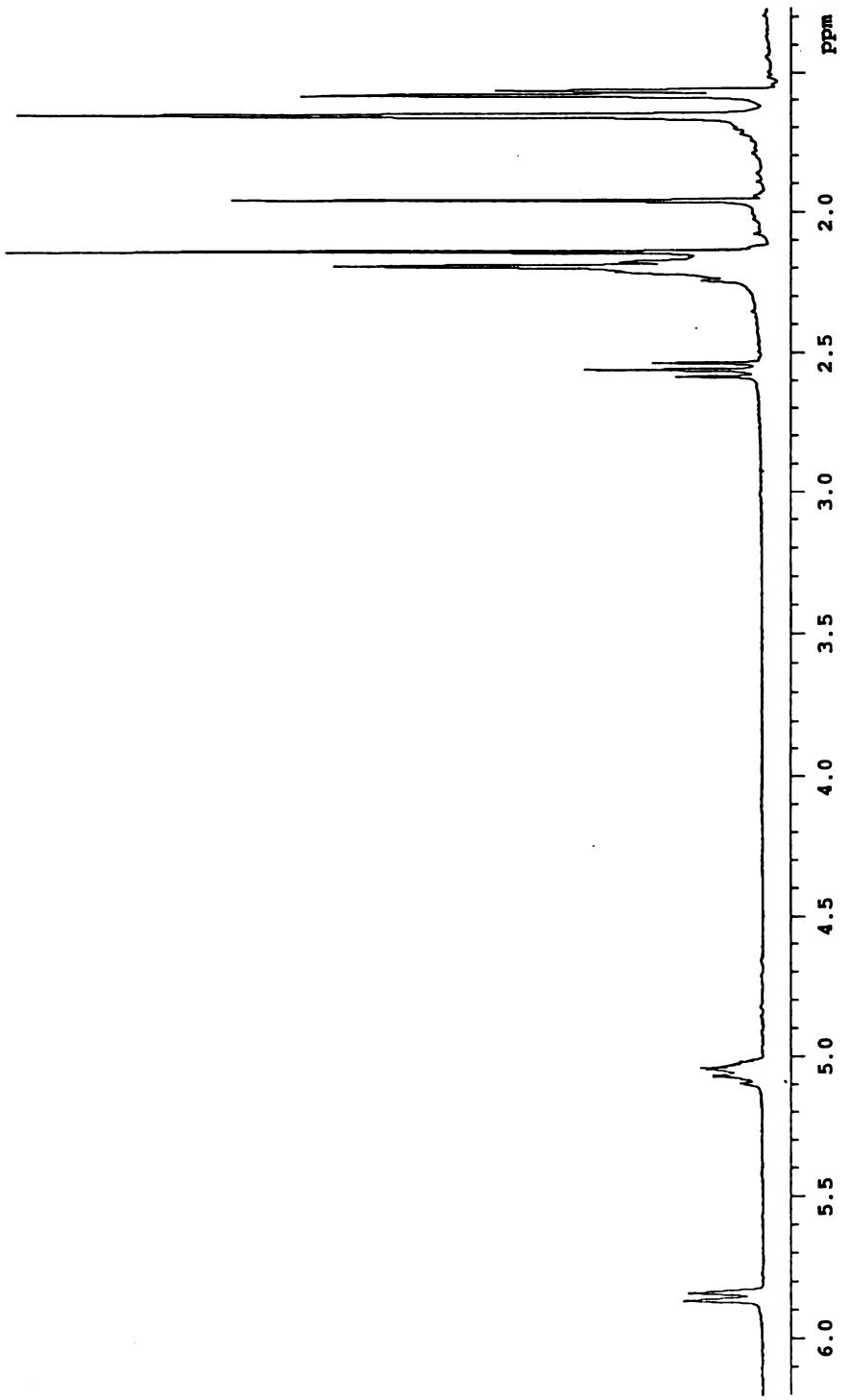
APPENDIX I
^{1}HNMR - citral (1 and 2)



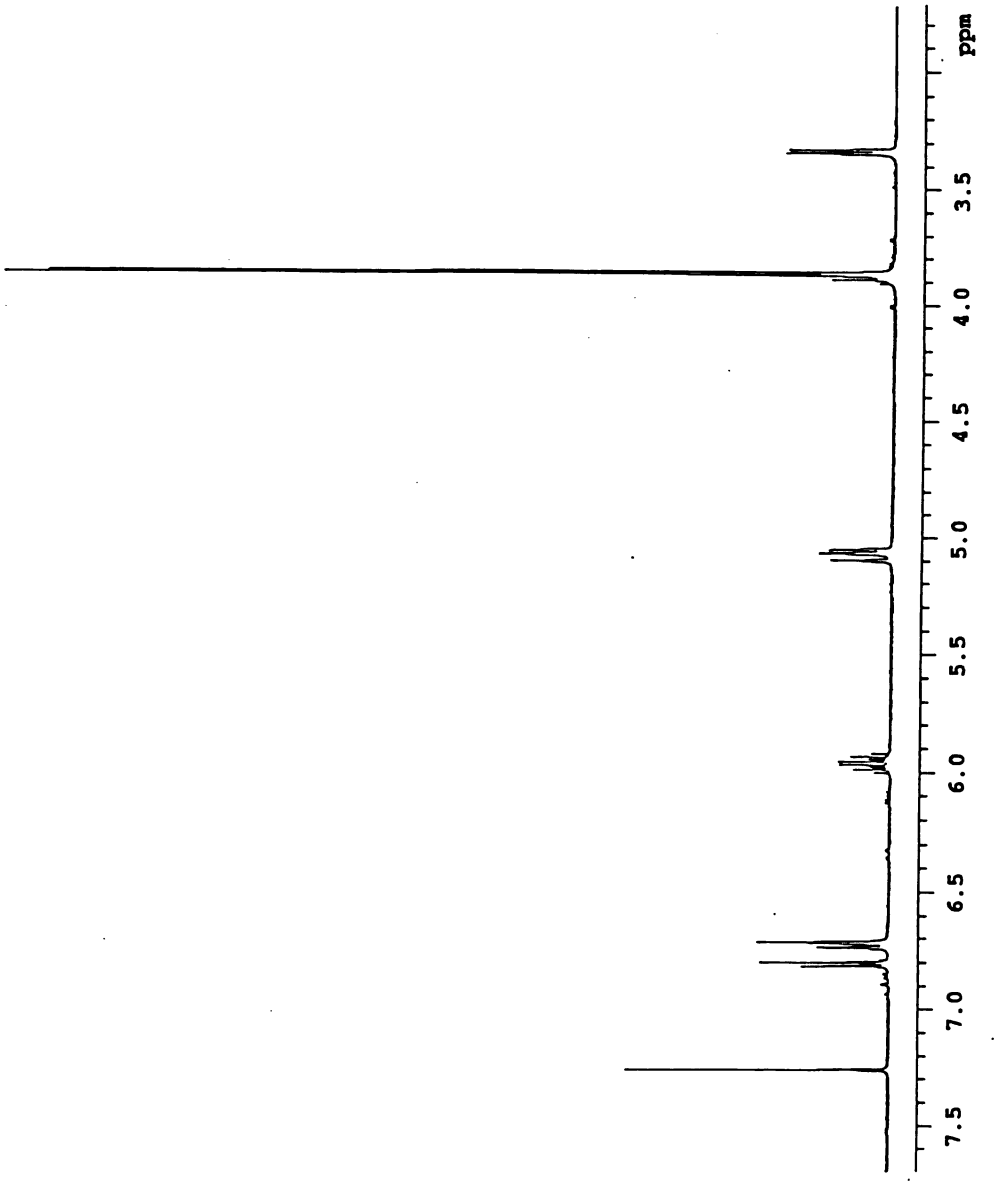
APPENDIX II
^{13}CNMR - citral (1 and 2)



APPENDIX III
^{1}HNMR - *trans*-anethole (3)

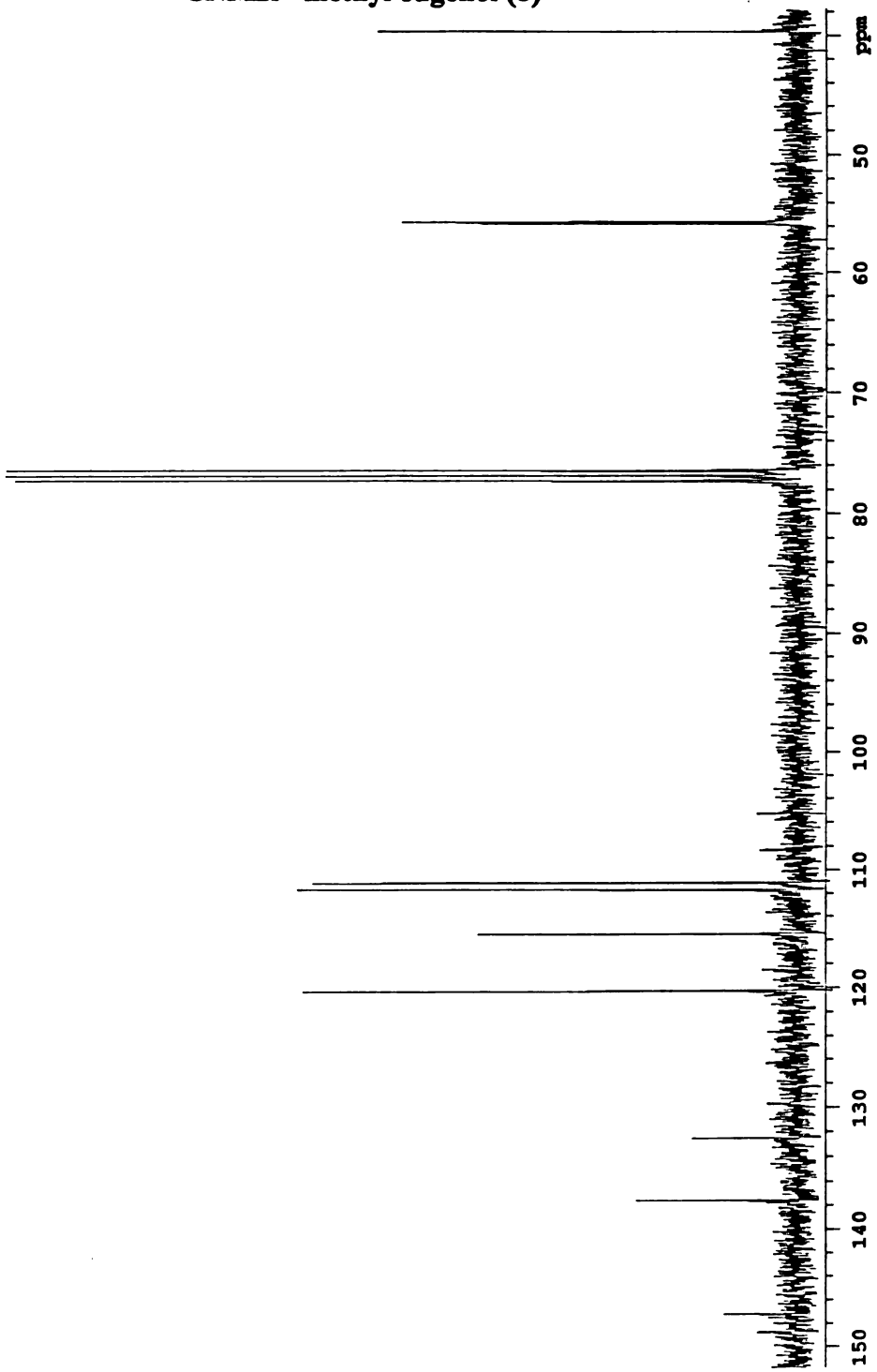


APPENDIX IV
^{1}HNMR - methyl eugenol (4)

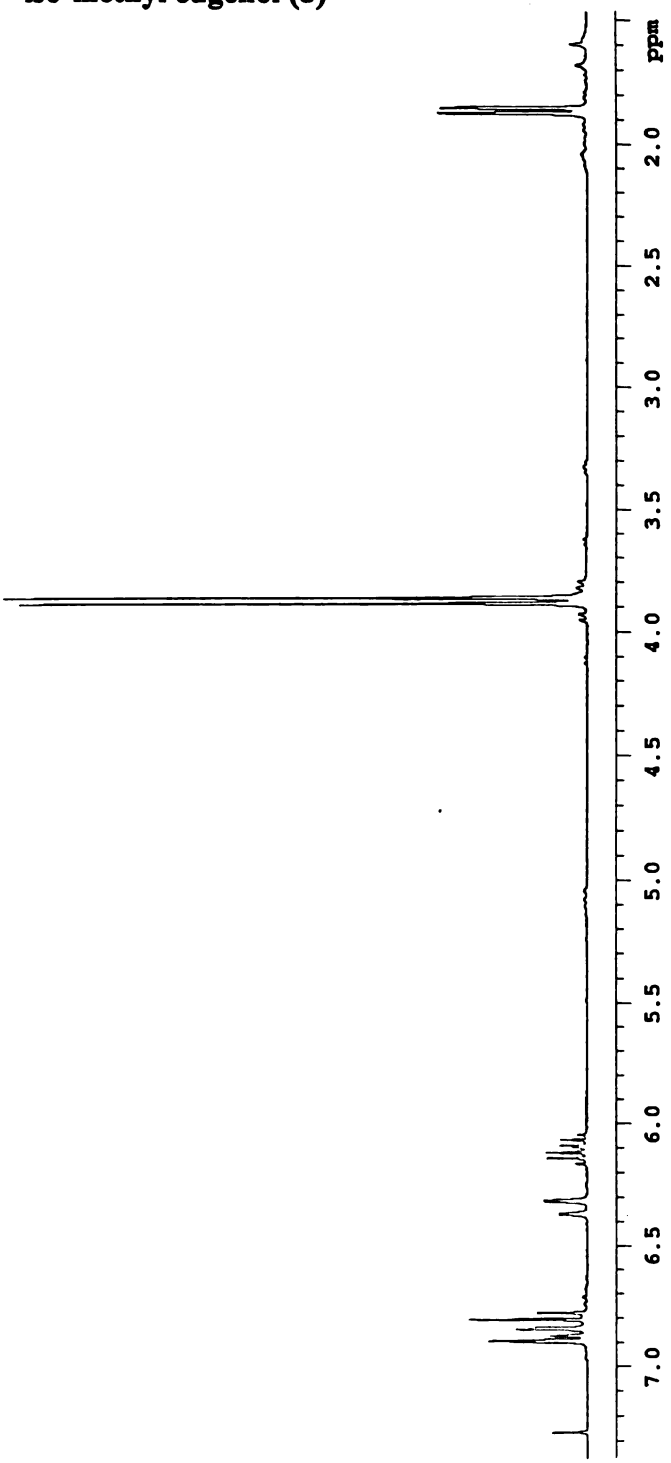


APPENDIX V

^{13}CNMR - methyl eugenol (5)

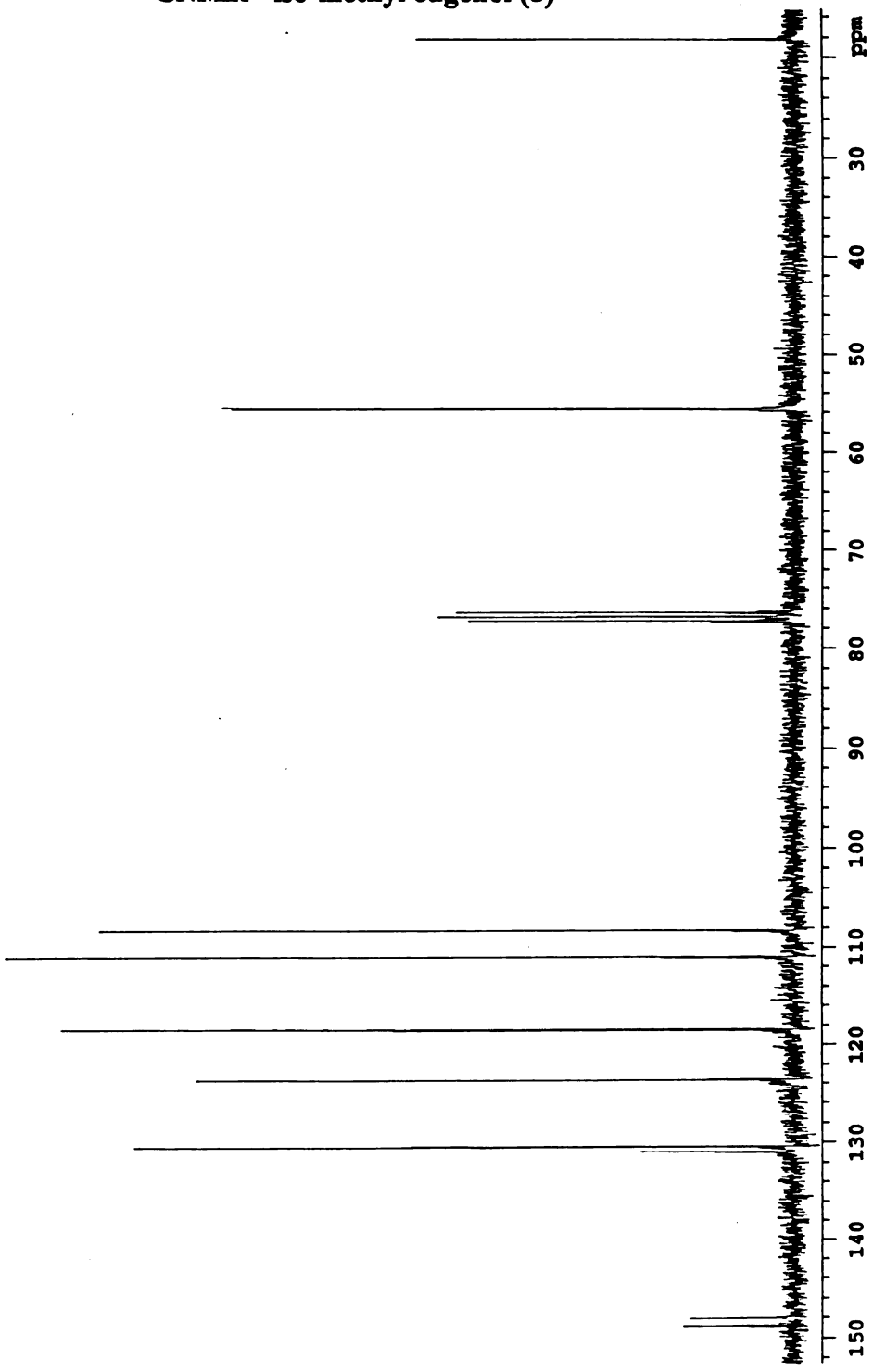


APPENDIX VI
^{1}HNMR - iso-methyl eugenol (5)

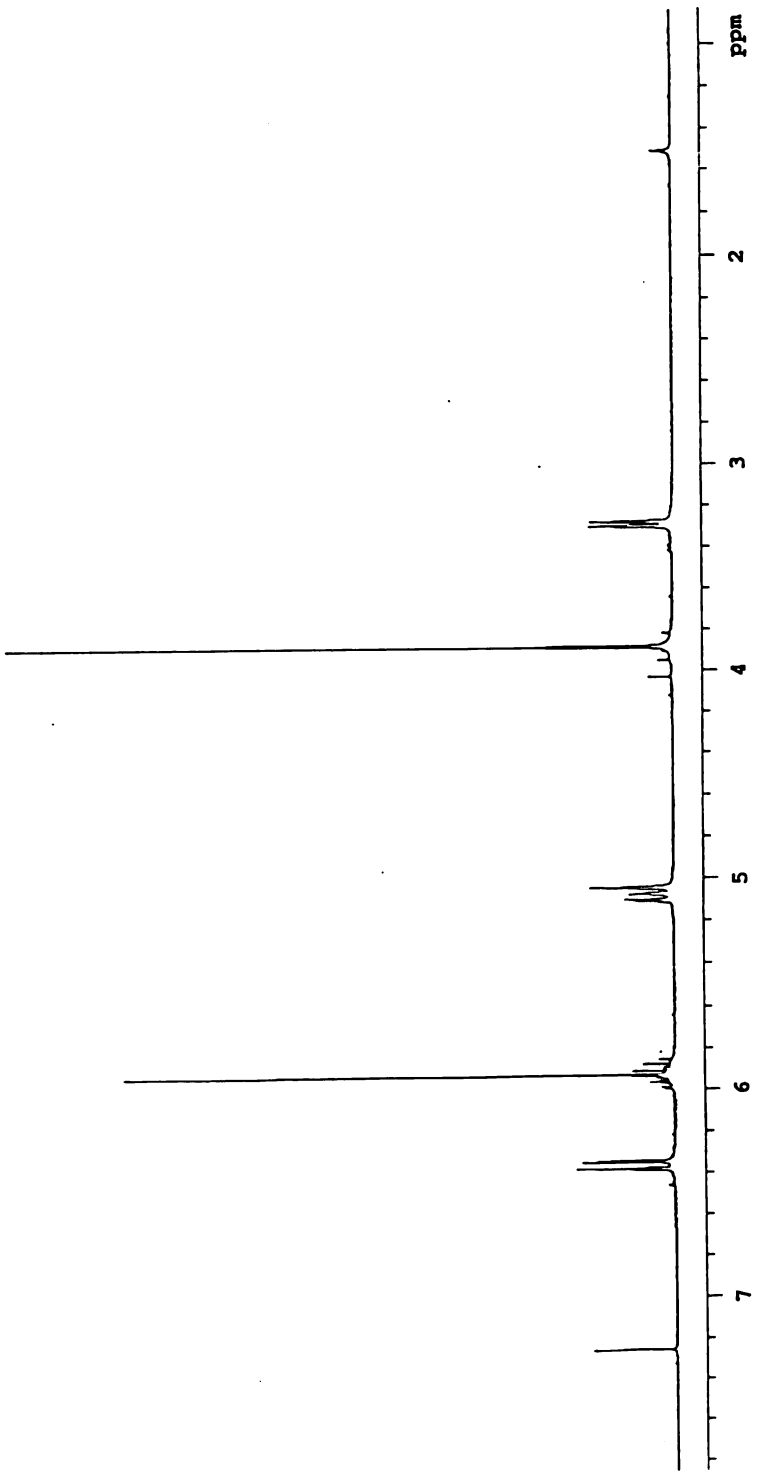


APPENDIX VII

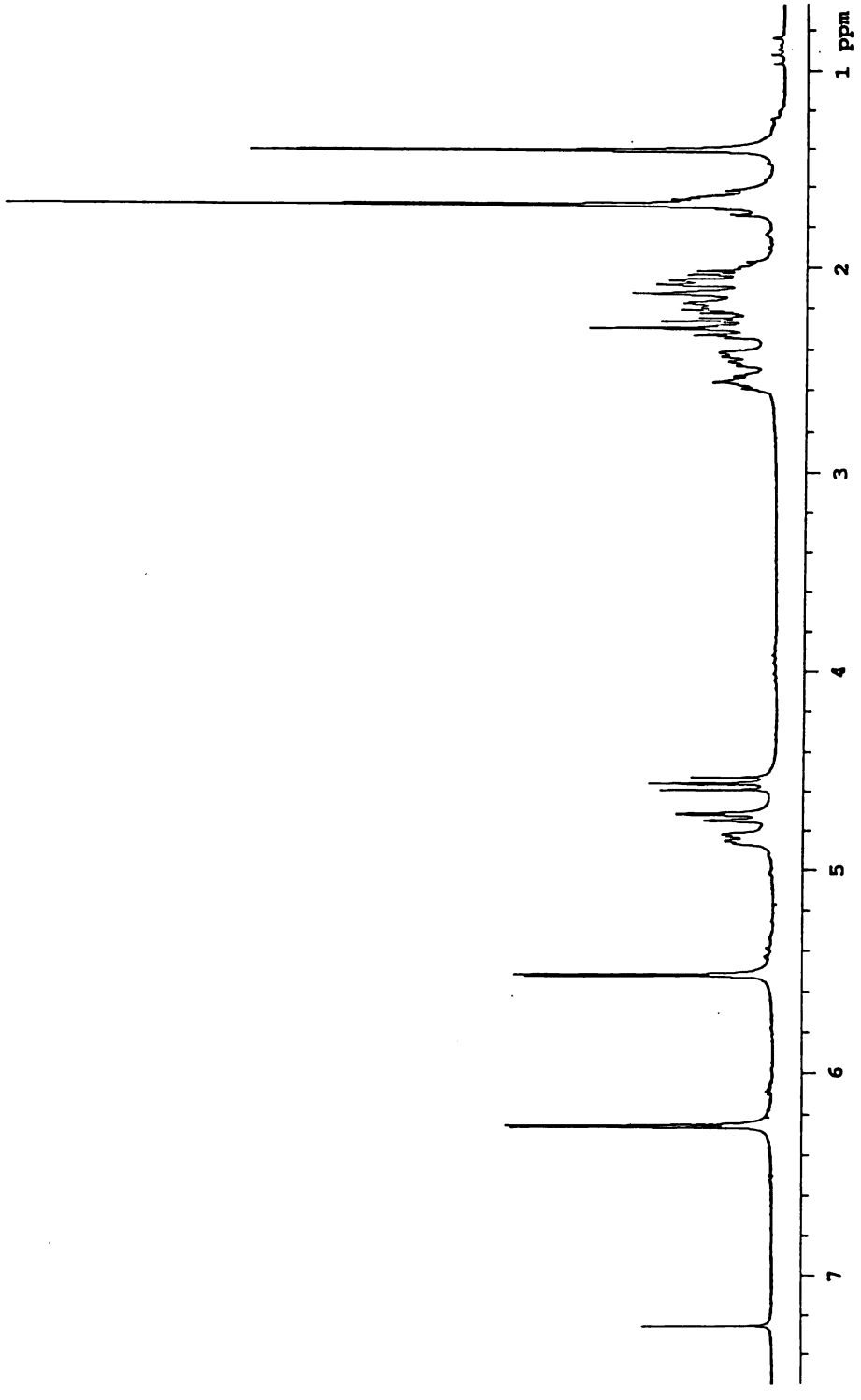
^{13}CNMR - iso-methyl eugenol (5)



APPENDIX VIII
^{1}HNMR - myristicin (6)

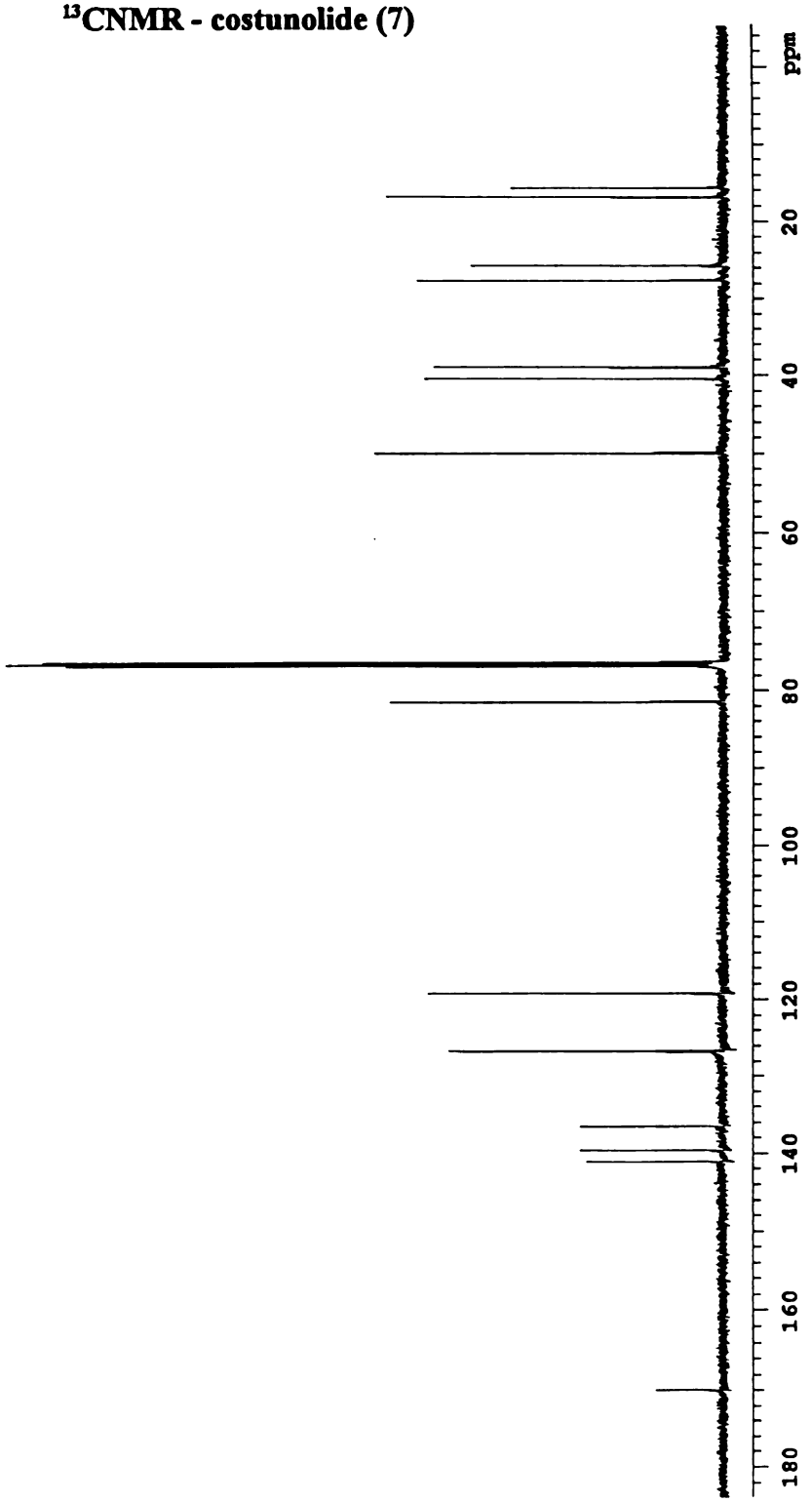


APPENDIX VIIII
^{1}HNMR - costunolide (7)



APPENDIX X

^{13}CNMR - costunolide (7)



APPENDIX XI

Gypsy moth catapillar diet

Gypsy Moth, *Lymantria dispar* dry diet ingredients:

wheat germ	36 g
casein	7.5 g
wesson salts	2.4 g
sorbic acid	0.6 g
methyl paraben	0.3 g
vitamin mix (Hoffman-LaRoche)	3.0 g