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dissertation entitled

PHYTOCEUTICAL AND OTHER BIOACTIVE NATURAL PRODUCTS

FROM APIUM GRAVEOLENS L. SEEDS

presented by

Rafikali A. Momin

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Horticulture

Major professor

Date 10/10/00

PHYTOCEUTICAL AND OTHER BIOACTIVE NATURAL PRODUCTS FROM *APIUM GRAVEOLENS* L. SEEDS

By

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A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Horticulture

2000

ABSTRACT

PHYTOCEUTICAL AND OTHER BIOACTIVE NATURAL PRODUCTS FROM *APIUM GRAVEOLENS* L. SEEDS

By

Rafikali A. Momin

Earlier research on *Apium graveolens* seeds was focused on its phytochemistry and its application in flavor and fragrances industries. Though, celery seeds are well known for its medicinal properties, very little work has been done on the isolation and identification of bioactive compounds. A phytochemical investigation on various solvent extracts of *A. graveolens* seeds resulted in the isolation and characterization of mosquitocidal, nematicidal, antimicrobial, topoisomerase and cyclooxygenase inhibitory and antioxidant compounds. *A. graveolens* seeds were sequentially extracted with hexane and methanol. Bioassay-directed isolation and purification of the hexane extract of *A. graveolens* seeds afforded three compounds, β-selinene (**1**), 3-n-butyl-4,5-dihydrophthalide (**2**) and 5-allyl-2-methoxyphenol (**3**). The triglyceride, 1,3-di[(cis)-9-octadecenoyl]-2-[(cis,cis)-9,12-octadecadienoyl]glycerol (**4**) and compound **3** were isolated for the first time from *A. graveolens* seeds. Hexane and $CHCl_3$ soluble fractions of methanolic extract of *A. graveolens* seeds yielded bioactive compounds, sedanolide (**5**), senkyunolide-N (**6**), senkyunolide-J (**7**) and a novel compound, 3-hydroxymethyl-6-methoxy-2,3-dihydro-1H-indol-2-ol (**8**). Compounds **6** and **7** were isolated for the first time from *A. graveolens.* Antioxidant and cyclooxygenase inhibitory assay directed purification of $CHCl_3$ insoluble fraction of methanolic extract of *A. graveolens* seeds yielded L-tryptophan (**9**)

and 7-[3-(3,4-dihydroxy-4-hydroxymethyl-tetrahydro-furan-2-yloxy)-4,5-dihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy]-5-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chromen-4-one (**10**). The structures of these compounds were established by using various spectroscopic experiments such as 1D- and 2D- NMR, FTIR, and MS.

Compounds **1-3** demonstrated 100 % mortality on fourth-instar *Aedes aegyptii* larvae at 50, 25 and 200 μg mL^{-1}, respectively, in 24 h. The LD_{100} in 24 h for compound **2** was observed at 12.5 and 50 μg mL^{-1}, when tested on nematodes, *Panagrellus redivivus* and *Caenorhabditis elegans*, respectively. Also, it inhibited topoisomerase-I and –II enzyme activities as well as the growth of *Candida albicans* and *Candida kruseii* at 100 μg mL^{-1}. The triglyceride (**4**) was not biologically active in our assays.

The 100 % mortality of the nematode *P. redivivus* was caused by compounds **5-7** at 25, 100 and 100 μg mL^{-1}, respectively. Compound **5** showed 100 % mortality at 50 μg mL^{-1} on nematode, *C. elegans*, and fourth-instar mosquito larvae, *A. aegyptii*. It also inhibited the growth of *C. albicans* and *C. parapsilasis* at 100 μg mL^{-1}. Topoisomerase-I and –II inhibitory activities of compounds **5-7** were recorded at 100, 200 and 200 μg mL^{-1} concentrations, respectively. This is the first report of the mosquitocidal, nematicidal, topoisomerase enzyme inhibitory and antimicrobial activities of compounds **5-7**. Compounds **5-10** were also tested for their cyclooxygenase inhibitory and antioxidant activities. At pH-7 most of the test compounds demonstrated very good cyclooxygenase inhibitory activities as demonstrated by the inhibition of prostaglandin H endoperoxide synthase-I (COX-I) and of prostaglandin H endoperoxide synthase-II (COX-II) at 250 μg mL^{-1} concentration. Compound **8** is here identified as a novel compound that showed the highest COX-I and –II inhibitory activities. Antioxidant activities of compounds **9** and **10**

were comparable to commercially available antioxidants when assayed at 125 and 250 μg mL^{-1} concentrations, respectively.

Humans on a daily basis consume many plants. Natural products isolated from the edible plants with anticarcinogenic and antiinflammatory activities should be readily acceptable as a dietary supplement if the toxicity and side effects are minimal. Both antiinflammatory and antioxidant activities of compounds from *A. graveolens* seeds suggest that consumption of celery seeds may alleviate pain related to gout and arthritis. Also, it has potential in the control of nematodes and other agricultural pests as evidenced by the good nematicidal and mosquitocidal activities of phthalides isolated from celery seeds. Considering the potential phytoceutical and agriculture pest management properties of *A. graveolens* seeds, it may result into novel products which could generate additional income to celery growers.

TO MY PARENTS

ACKNOWLEDGMENTS

I would like to express my sincere gratitude and thanks to my major advisor Prof. Muraleedharan G. Nair for his valuable guidance, constant encouragement, constructive criticism and the final shaping of this dissertation. The timely completion of this dissertation would not have been possible without his support, mentoring and patience.

I am grateful to the members of my advisory committee, Drs. Douglas A. Gage, Randolph M. Beaudry, Gale M. Strasburg and Robert E. Schutzki for their assistance, valuable suggestions and encouragement through out my study. I also gratefully acknowledge Drs. Long Lee and Kermit Johnson for their assistance in conducting various NMR experiments.

I wish to express special thanks to my fellow members and friends, Dr. Seeram, Dr. Ramsewak, Dr. Balasubramanian, Dr. Wang, Dr. Kelm, Dr. Henry, Jennifer Miles, Andrew Erickson, Laura Clifford, Di Wu, Robert Cichewicz and Marvin Weil for their moral support and assistance.

I gratefully acknowledge the financial assistance provided by THE AGA KHAN FOUNDATION, Geneva, Switzerland during the course of this study.

I owe special thanks to my parents, uncle, aunt, brothers and sister for their love, kindness, inspiration, encouragement, moral support and blessings. Finally, I must express deep love and appreciation to my dear wife Almas for her tireless support during long nights of work away from her, and for her understanding, sacrifice, confidence, encouragement, enthusiasm and moral support.

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LIST OF ABBREVIATIONS

BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CD	Circular Dichroism
CFU	Colony forming unit
$CHCl_3$	Chloroform
COX	Cyclooxygenase
^{13}C NMR	Carbon nuclear magnetic resonance
DMSO	Dimethyl sulfoxide
d	Doublet
dd	Doublet of doublet
DPA-PA	3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid
DEPT	Distortionless enhancement polarization transfer
EIMS	Electron impact ionization mass spectroscopy
Et_2O	Diethyl ether
EtOH	Ethanol
EtOAc	Ethyl acetate
FABMS	Fast atom bombardment mass spectroscopy
FTIR	Fourier transfer infrared spectroscopy
GC	Gas chromatography
^1H NMR	Proton nuclear magnetic resonance
HMBC	Heteronuclear multiple bond coherence
HMQC	Heteronuclear correlation through multiple quantum coherence
HPLC	High pressure liquid chromatography
IR	Infrared
J	Coupling constant
LC	Lethal concentration
m	Multiplet
MeOH	Methanol
MIC	Minimum inhibitory concentration
MOPS	3-[N-morpholino] propanesulfonic acid
MPLC	Medium pressure liquid chromatography
MS	Mass spectroscopy
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PG	Prostaglandin
PGHS-1	Prostaglandin endoperoxide H synthase-1
PGHS-2	Prostaglandin endoperoxide H synthase-2
ppm	Parts per million
PTLC	Preparative thin layer chromatography
R_f	Reference value
R_t	Retention time
s	Singlet

TBA	2-thiobarbituric acid
TBHQ	*tert*-butylhydroquinone
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV	Ultraviolet
VLC	Vacuum liquid chromatography
YMG	Yeast maltose glucose
YPDA	Yeast potato dextrose agar
δ	Chemical shifts
$[\theta]$	Molar ellipticity (in Circular dichroism)
λ	wavelength

INTRODUCTION

Apium graveolens L., celery, is a hapaxanthic herb, grown as a biennial and under certain conditions, as an annual. It is a native of Eurasia and grown as a wild plant. It prefers soils containing sodium chloride and, therefore, was grown in mainly coastal regions. Today, celery is widely cultivated in the temperate zones as an important garden crop and the bleached leaf stalks being relished as a popular vegetable. Countries leading in production of celery are France, India, and the USA. Celery, known in Hindi as Ajmud, grows widely in the foot of northwest Himalayas and outlying hills in the Punjab and Uttar pradesh. In India, celery with its various names like Ajamoda, Bastamoda, Kharaashwa, Mayoori, Deepa, Karavee, Lochamostaka is well known for the treatment of a variety of ailments. In the USA, celery was first produced commercially in 1856 in Kalamazoo, Michigan. Celery fruit, commonly known as "celery seed", was largely imported from Europe. Since World War-II, celery has been produced as a domestic crop, especially in Michigan and Wisconsin, and distributed throughout the USA. Celery can be classed both as an ingredient for seasoning and as a vegetable. For seasoning purposes, the seeds and the plant, as well as the essential oil distilled from them, are used (Salzer, 1975). The stalks of the celery plant are eaten raw in salads or cooked as in soups or in stir-fried foods. Celery is also used for its unique texture and appetizing flavor.

The celery seed is ovate, has a dark brown cremocarp and possesses a characteristic aroma with somewhat pungent taste. It is employed as a condiment in the

flavoring of food products. Celery seed oil, celery seed, and celery seed extracts are all extensively used as flavoring ingredients in all major food products, including alcoholic and nonalcoholic beverages, frozen dairy desserts, candy, baked goods, gelatins and puddings, meat and meat products, condiments and relishes, soups, gravies, snack foods, and others. Celery seed oil is reportedly used as diuretic, in dropsy and bladder ailments, antispasmodic, and in rheumatoid arthritis (Styavati and Raina, 1976; Chopra, et al., 1958). In European tradition celery seeds were reportedly used as carminative, stomach ache, diuretic, laxative, for glandular stimulation, gout, kidney stones, rheumatic complaints, loss of appetite and exhaustion. Leaves and petioles of celery were also used for skin problems in addition to the above mentioned uses. In India, celery seeds are used for treating bronchitis, asthma, liver and spleen diseases (Chopra, et al., 1958, Satyavati and Raina, 1976). Polyherbal formulations reputed to have hepatoprotective activity are available in the Indian market, which comprise about more than one hundred Indian medicinal plants including celery seed. Celery is an ingredient of several herbal formulations with reputed liver-protecting activities (Handa et al., 1986).

Earlier research on *A. graveolens* seeds was focused primarily on their phytochemistry and its application in both the flavor and fragrances industries. Celery is also a well known folk medicine with a variety of medicinal properties. For these reasons, celery seed oil has been studied for its compositions (Vernin and Parkanyi, 1994). Most of these studies on *A. graveolens* seed extracts were focused on the bioactivity of crude extracts. Very little work has been done on the isolation and identification of bioactive compounds from *A. graveolens* seeds. Compounds present in celery seeds, which might

contribute to the anticarcinogenic, antiinflammatory, antioxidant and other bioactivities need to be characterized.

Studies on various biological activities of crude extracts, essential oils and pure compounds isolated from *A. graveolens* are discussed in **Chapter-1**. A brief review of the phytochemical constituents of *A. graveolens* seeds is also discussed in **Chapter-1**. The phthalides, naturally occurring in edible umbeliferous plants may be useful chemopreventive against carcinogenesis and tumorigenesis (Zheng et al., 1993). However, additional research must be performed to check the anticarcinogenic effect of the phthalides occurring in celery seeds. Al-Hindawi and co-workers (1989) observed antiinflammatory activity in the ethanol extract of celery plant, but they could not establish any link between known constituents with the observed antiinflammatory activity. The antiinflammatory activities of compounds present in celery seeds need to be investigated. Also, many other constituents present in celery seeds, which might contribute to the anticarcinogenic, antiinflammatory, and antioxidant activities of celery seeds need to be characterized. Therefore, it is my hypothesis that given the body of pre-existing research on celery seeds and preliminary studies carried out in the Bioactive Natural Products Laboratory (BNPL), *A. graveolens* seeds have the potential to yield novel compounds with anticarcinogenic, antiinflammatory, antioxidant, antimicrobial, mosquitocidal and nematicidal activities. This could yield phytoceuticals or agrochemicals and result in additional income to celery growers in Michigan and USA. In order to test this hypothesis, I have conducted a bioassay-directed isolation and characterization of compounds in celery seed extracts by using cyclooxygenase enzymes and Fe^{2+} -induced lipid peroxidation bioassays. These compounds were also evaluated for

antimicrobial, mosquitocidal and nematicidal activities according to published bioassay procedures (Nair, et al., 1989; Roth, et al., 1998). Therefore, the objectives of my research were to conduct bioassay-directed isolation and identification of compounds in celery seeds using chromatographic and spectral methods, and determine the anticarcinogenic, antiinflammatory, antioxidant, antimicrobial, nematicidal and mosquitocidal efficacies of purified compounds. In addition, it is expected that the present work should add to the existing knowledge on the bioactive constituents in *A. graveolens*.

A. graveolens seeds were provided by Asgrow Seed Company, Kalamazoo, Michigan and stored at –20 ^{0}C until extraction. Initially the crude extracts of *A. graveolens* have been examined for insecticidal, nematicidal, antimicrobial and pharmacological studies. Based on the initial bioassay results, bioassay-directed isolation and purification of compounds from *A. graveolens* seeds were performed. I examined crude extracts and pure compounds of celery seeds for their biological activities. Insect bioassay utilizing mosquito larvae (*Aedes aegyptii* Linn.) and nematodes, *Caenorhabditis elegans* and *Panagrellus redivivus*, were carried out to determine such activities for celery compounds. The test organisms *Candida kruseii* (MSU strain), *C. albicans* (MSU strain) and *C. parapsilasis* (MSU strain) were used for antimicrobial bioassays and *Saccharomyces cerevisiae* mutant cell cultures, JN394, JN394t$_{-1}$, and JN394t$_{-2-5}$ were used for the topoisomerase enzyme inhibitory assay on crude extracts and purified compounds. Antiinflammatory and antioxidant assays were conducted on crude extracts and pure compounds using cyclooxygenase enzymes (COX-I and COX-II) inhibitory and Fe^{2+} -induced lipid peroxidation assays, respectively.

This dissertation comprises of five chapters and each chapter except the review of literature is organized as a scientific journal article, which contains an abstract, introduction, material and methods, and results and discussion section.

Chapter 1

LITERATURE REVIEW

The breadth of literature on the bioactivities of crude extracts and pure compounds from *A. graveolens* seeds is reviewed in this chapter. Interesting biological activities related to human health for compounds from *A. graveolens* seeds were anti-carcinogenic, chemopreventative, antiplatelet, anti-inflammatory, diuretic, hepatoprotective, sedative, vasodilatory, antirheumatic, carminative, antispasmodic, effect on blood pressure, tranquillizing, anticonvulsant, antioxidant, and hypotensive activities. Other potential applications in agriculture for *A. graveolens* seeds reported were antifungal and antibacterial activities. Very few papers have been published about bioactivities of pure compounds from *A. graveolens*, which included anti-carcinogenic, chemopreventative, inhibition of platelet aggregation, vasodilatory, and antimicrobial activities. This chapter also covers the phytochemical studies on *A. graveolens*.

Pharmacological Studies

A wide spectrum of secondary products present in dietary supplements has been reported to be associated with protective effects against chemically induced toxicity and carcinogenesis (Hayatsu et al., 1988; Hocman, 1989; Morse and Stonre, 1993). Celery seeds are used in India to treat bronchitis, asthma, liver and spleen diseases (Styavati and Raina, 1976). Polyherbal formulations reputed to have hepatoprotective activity and those are available on the Indian market comprise about one hundred Indian medicinal plants. *A. graveolens* is an ingredient of some of these polyherbal formulations having reputed life-protecting activities (Handa et al., 1986).

Naturally occurring anticarcinogenic components from edible plants have been an important source for obtaining potential cancer chemopreventive agents. *A. graveolens* seed extracts and pure compounds have been shown to possess anticarcinogenic activities (Zheng et al., 1993; Lam and Zheng, 1992; Banerjee et al., 1994). Zheng et al. (1993) studied the chemoprevention of Benzo [α]pyrene-induced fore-stomach cancer in mice by natural phthalides from *A. graveolens* seed oil. Zheng and co-workers (1993) had isolated five natural products, d-limonene, p-mentha-2,8-dien-1-ol, p-mentha-8(9)-en-1,2-diol, 3-n-butyl phthalide and sedanolide from celery seed oil. However, only p-mentha-2,8-dien-1-ol, 3-n-butyl phthalide and sedanolide exhibited appreciable activities by inducing the detoxifying enzyme glutathione S-transferase (GST) in the target tissues of female A/J mice. A total of 3 doses of 20 mg/dose for every two days of 3-n-butyl phthalide, sedanolide, and p-mentha-2,8-dien-1-ol increased the GST activity by 4.5-5.9, 3.2-5.2, and 3.7 times over the controls in mouse liver and small intestinal mucosa,

respectively. These five compounds were also tested for their ability to inhibit benzo[α]pyrene- (BP) induced tumorigenesis in mice. 3-n-butyl phthalide and sedanolide reduced the tumor incidence from 68 to 30 and 11%, respectively (Zheng et al., 1993). These results indicated about 55% of inhibition of tumorigenesis after the treatment of mice with the phthalides. The tumor multiplicity was also significantly reduced as a result of treatment with these phthalides from celery seeds. Sedanolide and 3-n-butyl phthalide reduced more than 83 and 67% of tumor multiplicity in mouse, respectively.

Lam and Zheng (1992) studied the effect of essential oils on glutathione S-transferase (GST) activity in mice including the essential oil from *A. graveolens* seeds. Celery seed oil was one of the most active essential oils studied in this experiment. In this study, the induction of GST enzyme in the cytosols of liver, forestomach, and small intestinal mucosa was examined. The GST enzymes were catalyzed by the conjugation of glutathione with electrophilic species of celery seed oil components to form less toxic, water soluble substances that are readily excreted (Chasseaud, 1979). The enhancement of GST activity suggested an increase in the host's ability to detoxify xenobiotics, including carcinogens. Thus, substances that can elicit increased activity of GST and other detoxifying enzymes are considered to be a potential anticarcinogens and chemoprotecting agents (Lam and Zheng, 1992). Essential oil from celery seed given orally to mice resulted in a 3.29, 3.28 and 1.49 fold increase in GST activities compared to the control in small intestinal mucosa, liver, and forestomach, respectively. Since an increase of GST activity suggests anticarcinogenic potential, the celery seed oil is

expected to contain components that may be useful as chemopreventive agents. Banerjee et al. (1982) investigated the influence of essential oils derived from several edible plants such as celery seed (*Apium graveolens*), cumin seed (*Cuminum cyminum*), cardamom (*Elettaria cardamonum*), coriander (*Coriandrum sativum*), ginger (*Zingiber officinale*), nutmeg (*Myristica fragrans*) and *zanthoxylum* species on the activities of hepatic carcinogen-metabolizing enzymes like cytochrome P_{450}, aryl hydrocarbon hydroxylase and GST and acid-soluble sulfhydryl levels in Swiss albino mice. The celery seed oil caused approximately 2-fold increase in cytosolic GST enzyme activity (Banerjee et al., 1982). Also, GST enzyme activity was elevated in livers of Swiss albino mice by celery seed oil and concluded the enhancement of GST enzyme activity as an indication of its potential role as "blocking agents" in chemopreventive strategies. There were no significant increases in acid-soluble sulfhydryl levels and cytochrome P_{450} enzyme activity in the liver of mice treated with celery seed essential oil. Though the elevated sulfhydryl concentration minimizes the adverse effect of toxic xenobiotic compounds, reduction of the tumorigenic response (Wattenberg, 1985; Shenoy and Choughuley, 1992) and induction of microsomal cytochrome P_{450} and aryl hydrocarbon hydroxylase are suggestive of their participation in activation of lipophilic xenobiotics, including procarcinogens.

The essential oil from *A. graveolens* has a sedative effect upon the central nervous system (Guenther, 1961). The tranquilizing and anticonvulsant activities of the alkaloid fraction obtained from the seeds of *A. graveolens* were studied by Kulshrestha et al. (1970). Potentiation of pentobarbital narcosis, amphetamine group toxicity, conditioned avoidance response (CR), and spontaneous motor activity (SMA) tests were

performed to study the tranquilizing activity of a 2% aqueous sulphuric acid extract of *A. graveolens* in albino mice and rat models. In these tests, the ED_{50} values (in mg per 100 g) were found to be 33.6, 29.8, 24.3, and 68.2 in potentiating pentobarbital narcosis, conditioned avoidance response, spontaneous motor activity, and amphetamine group toxicity tests, respectively. Anticonvulsant activity against strychnine convulsions was tested for celery seed extracts by maximal electroshock seizure, metrazol seizure threshold and strychnine convulsions tests (Swinyard et al., 1952). The *A. graveolens* extract provided protection against only electroshock (ED_{50}- 34.6 mg/100 g). In conclusion, the low toxicity and high protective index in animal experiments suggested the therapeutic use of *A. graveolens* extract in depressant effect on the central nervous system (Kulshrestha et al., 1970). Some anti-depression effects, the tranquillizing and anticonvulsant activities of *A. graveolens* essential oil from seeds, were examined in mice by Kohli et al. (1967). The tranquillizing and anticonvulsant action of an essential oil fraction from celery seed have been demonstrated by using standard methods. The maximum central activity was found out to be in a fraction collected between boiling points of 180 and 265 ^{0}C. These results supported the sedative effect of celery seeds upon the central nervous system (Osol and Farrar, 1955). 3-n-butylphthalide, one of the celery seed constituents was shown to have anticonvulsant effects in experimental chronic epilepsy induced by coriaria lactones in rats (Yu et al., 1988a). The anticonvulsant effects of 3-n-butylphthalide were weaker than those of diazepam, but its ability in counteracting the learning and memory impairment was greater than that of diazepam, causing no damage to brain cells (Yu, et al., 1988a; Yu et al., 1988b). Same authors had reported earlier that 3-n-butylphthalide has low acute and chronic toxicities and no teratogenic activity in experimental animals (Yu, et al., 1988a).

Celery seeds are claimed to have diuretic effects (Houghton and Manby, 1985). The phthalides from celery seeds were shown to have diuretic, antispasmodic and anti convulsion activities (Chevallier, 1998; Boulos, 1983). The volatile oil from *A. graveolens* seeds showed similar activities and was reported to irritate the epithetical tissue of the kidneys, which may cause diuresis. Houghton and Manby (1985) also noted that the flavonoids present in celery seeds may also play some part in diuresis since it is known that they inhibit some of the enzymes in the kidney involved in water reabsorption. Chevallier (1998) has also mentioned that celery seeds have a mildly diuretic and significantly antiseptic action. Flavonoids are an effective treatment for crystitis, helping to disinfect the bladder and urinary tubules. Contrary to these reports, Mahran et al. (1991) found that the volatile oil of *A. graveolens* seeds in doses of 4 and 8 $\mu L\ kg^{-1}$ body weight did not affect urine flow or blood pressure in dogs. Higher doses of volatile oil produced hypotensive response. The ethanolic extract of *A. graveolens* could not be tested pharmacologically for diuretic property because it produced a gelatinous material upon dissolving in saline (Mahran et al., 1991). Also, there was no significant change in urine flow, Na^+, K^+ or Cl^- excretion for more than 2 h in celery seed volatile oil-treated dogs. Boulos (1983) in his book, "Medicinal Plants of North Africa" mentioned the diuretic property and effect of celery fruit on kidneys and bladder. The tincture prepared from matured seeds was used to treat urine retention and other renal disorders (Boulos, 1983).

The ethanol extract of the aerial parts of *A. graveolens* was assessed for its anti-inflammatory activity on intact rats by measuring the suppression of carrageenan-induced paw edema produced by 1/10 of the intraperitoneal LD_{50} doses (Al-Hindawi et al., 1989). Acetylsalicylic acid at doses of 100 and 200 $mg\ kg^{-1}$ body weight was used as a positive

control and the results were in agreement with the known activity of acetylsalicylic acid. The inhibition of carrageenan-induced edema in the rat paw after the intraperitoneal injection of 200 and 358.7 mg g^{-1} of acetylsalicylic acid and *A. graveolens* extract were 66.8 and 53.0%, respectively (Al-Hindawi et al., 1989). However, any link between known constituents in *A. graveolens* with the observed anti-inflammatory activity was not established. Lewis et al. (1985) also studied the anti-inflammatory activity of aqueous extracts of celery stem against two animal models, carrageenan and mouse ear tests. Indomethacin was used as a positive control in both models. Ethanol and aqueous extracts of celery stem were used for this study. Both celery powder (400 mg kg^{-1}) and indomethacin (5 mg kg^{-1}) were anti-inflammatory in the carrageenan model (Lewis et al., 1985). At a lower dose (100 mg kg^{-1}), celery powder showed 30% inhibition of carrageenan-induced inflammation. Celery powder also showed anti-inflammatory activity in the mouse ear test. The ethanol extract was not anti-inflammatory but the celery powder before ethanol extraction inhibited carrageenan-induced inflammation by 71% when measured 5h after the administration of carrageenan. Celery powder residues after ethanol extraction inhibited carrageenan-induced edema by 62%. Two constituents of celery stem, manitol and phytosterols (indosterol), were also studied for their anti-inflammatory activity in these two models (Al-Hindawi et al., 1989). An oral administration of manitol at a dose of 20 mg kg^{-1} did not show antiinflammatory activity. The phytosterols from celery showed antiinflammatory activity when administered orally to rats in an aqueous suspension (Al-Hindawi et al., 1989). Therefore, the observed anti-inflammatory activity in animal models confirmed the basis for the anecdotal claims of celery as a medicinal plant for controlling rheumatic disease (Chopra et al., 1958, Satyavati and Raina, 1976).

Platelets play an important role in the hemostatic process and their abnormal activation will cause arterial thrombosis. Teng et al. (1988) found the inhibitory effect of apigenin, a constituent of *A. graveolens* on platelet aggregation. The effect of apigenin on collagen-induced platelet aggregation and ATP release, aggregation induced by ADP, arachidonic acid, platelet activating factor (PAF), thrombin and ionophore A23187, the thromboxane formation in platelets, and aggregation of whole blood was studied by Teng et al. (1988). In this study, apigenin inhibited the aggregation of washed rabbit platelets induced by collagen, ADP, arachidonic acid and PAF, but not that induced by thrombin or ionophore A23187. It also inhibited the release of ATP from platelets induced by all aggregants used as well as the formation of thromboxane B_2 in platelets challenged by collagen and arachidonic acid. The IC_{50} on collagen-induced platelet aggregation was about 50 μg mL^{-1}. Also, apigenin inhibited the contraction of aortic rings caused by cumulative concentrations of calcium (0.03–3 mM) in high potassium (60 mM) medium, with IC_{50} of about 48 μM (Ko et al., 1991). The pretreatment by apigenin inhibited the norepinephrine (NE, 3 μM)-induced phasic and tonic contraction in a concentration (35-140 μM)-dependent manner with an IC_{50} of 63 μM. The conclusion was that apigenin relaxed rat thoracic aorta mainly by suppressing the Ca^{2+} influx through both voltage- and receptor- operated calcium channels (Ko et al., 1991).

Singh and Handa (1995) studied the antihepatotoxic activity of *A. graveolens* against paracetamol and thioacetamide intoxication in rats. The hepatoprotective effect of methanolic extract of *A. graveolens* seeds on rat liver damage induced by paracetamol (3 g kg^{-1} body weight) or thioacetamide (100 mg kg^{-1} body weight) was monitored by several liver function tests, serum transaminases (SGOT and SGPT), alkaline

phosphatase (SALP), sorbitol dehydrogenase (SSDH), glutamate dehydrogenase (SGLDH) and bilirubin in serum (SBRN) as well as by assaying triglyceride and histopathological alteration in hepatic tissues (Sing and Handa, 1995). A significant reduction observed in the paracetamol and thioacetamide induced increase in the levels of SGOT, SGPT, SALP, SSDH, SGLDH, and SBRN with the pretreatment of rats with 200 mg kg^{-1} body weight of the methanolic extract of *A. graveolens* seeds were 75 and 61.9% overall protection, respectively. Also, the histopathological studies suggested that treatment with methanolic extract of *A. graveolens* reversed the hepatic lesions produced by paracetamol as indicated from the absence of eosinophilia and presence of fewer necrotic zones. They concluded that the observed hepatoprotective activity of the methanolic extract of the seeds of *A. graveolens* against two well known hepatotoxins, paracetamol and thioacetamide in rats differed in their primary mechanism of inducing hepatotoxicity. This study supported the use of *A. graveolens* seeds in several patented Indian herbal preparation for liver ailments (Handa et al., 1986).

Celery juice was also studied for its hypotensive effect (Kiangsu, 1977). Clinically, celery juice is reported to be effective in lowering blood pressure in hypertensive patients. In this study, the celery juice was mixed with equal amounts of honey and 40 mL of the mixture was administered orally three times a day over several days.

Saito et al. (1976) observed antioxidative activity of petroleum ether soluble fraction of celery seed on lard. However, Gazzani (1994) found little or no effect for celery extract at 1-5% for anti- or pro-oxidant activities.

Jain and Jain (1973) studied the effect of some common essential oils on pathogenic fungi. The steam-distilled oils from *A. graveolens* seeds along with some common Indian indigenous herbs were subjected to antifungal evaluations, employing the filter-paper-disk-diffusion-plate method (Vincent and Vincent, 1944). Celery seed oil was tested against *Trichophyton terrestre*, *Trichophyton tonsurans*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Histoplasma capsulatum*, *Candida albicans*, *Monosporium apiospermum*, *Beauveria sp.*, *Philaphora verrucosa*, *Cryptococcus neoformans*, *Microsporum mycetomi*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Sporotrichum schenckii*, *Microsporum cookie*, *Microsporum gypseum*, *Epidermophyton floccosum*, *Nacardia brasiliensis*, *Fusarium oxysporum* and *Curvillaria lanata*. Except, *T. tonsurans*, *T. rubrum*, *T. mentagrophytes*, *P. verrucosa*, *M. mycetomi*, *S. schenckii*, *M. cookie* and *N. brasiliensis*, all the test fungi responded significantly against the celery seed essential oil. Combination of celery seed oil with other essential oils showed synergistic antifungal activity against all of the test fungi (Jain and Jain, 1973). This suggested that celery seed oil can be used for the formulation of suitable topical antifungal formulations.

Phytochemical Studies

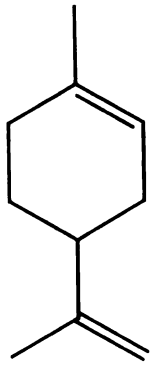
A. graveolens seed contains a wide variety of chemical constituents. The major classes of compounds are monoterpenoids, sesquiterpene, aliphatic alcohol and carbonyl compounds, phenols, aromatic epoxides and phthalide derivatives (Bindler and Laygel, 1986). As a class of bioactive natural products, phthalides occur widely in Umbelliferous plants (Bjeldanes and Kim, 1977; Chulia et al., 1986; Kaouadji and Pouget, 1986a;

Kaouadji et al., 1984; Kaouadji et al., 1986b; Chichy et al., 1984; Banerjee et al., 1982). Phthalides are present in celery seed oil in relatively high amounts (~7%) (Bjeldanes and Kim, 1977) as well as in fresh celery in ppm quantities (MacLeod et al., 1988; Tang et al., 1990).

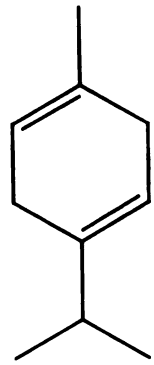
Celery seeds were investigated for their chemical constituents as early as in 1897. Ciamician and Silber (1897) found a sesquiterpene, selinene in celery seed oil, which was later investigated by Schimmel Chemists (1910). The celery seed oil was fractionated into phenols and sesquiterpenes by repeated extraction with a 5% solution of sodium hydroxide and by distilling the separated hydrocarbon over metallic sodium. The presence of selinene in celery seed was also confirmed by Semmler and Risse (1912), who reported that selinene consisted of a mixture of α- and β- isomers, with β-isomer as the predominant form. They also reported that 10 to 15% of selinene was present in celery seed oil.

The Schimmel Chemists (1910) also reported the presence of 2.5 – 3.5% of hydroxylated compounds in celery seed oil but did not characterize the chemistry of these compounds. Ruzicka and Stoll (1923) found that the highest boiling fractions from celery seed oil contained about 1% of bicyclic sesquiterpene alcohol, $C_{15}H_{26}O$. Later they came to the conclusion that selinenol obtained from selinene consisted of a mixture of α-eudesmol and β-eudesmol. In addition, Louveau (1937) reported that the celery seed oil contains 2 – 3% of unidentified terpene alcohol.

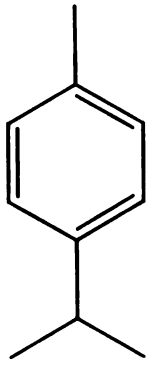
Guenther (1950) noted the presence of sedanoic anhydride in celery seed oil, which was characterized as tetrahydro-n-butylidene phthalide. The same author reported the sedanolide and its corresponding hydroxy acid, sedanolic acid in celery seed oil and concluded that the sedanolide and sedanolic acid anhydride were the two constituents mainly responsible for the characteristic odor of celery seed oil. Limonene was identified by the Schimmel Chemists (1910) in the early part of the twentieth century. They reported that the celery seed oil contains about 60% of d-limonene which was further confirmed by Guenther (1950). The presence of sedanolide in celery seed oil was determined by Barton and De Vries (1963). 3-n-butylphthalide was reported as the main component of celery seed oil (Barton and De Vries, 1963). Phthalides, 3-n-butylphthalide and sedanolide were also identified as the major odor components in celery seed (Bjeldanes and Kim, 1977). They also characterized 3-n-butyl-4,5-dihydrophthalide (sedanenolide) for the first time as a celery-flavor compound and was later confirmed by Uhling et al. (1987). Fehr (1979) also identified 3-n-butylidenephthalide, 3-n-butylidene-4,5-dihydrophthalide (*Z*-ligustilide) and 3-isobutylidene-3a,4-dihydrophthalide in addition to the 27 volatile components in the essential oil from *A. graveolens* seeds. The celery seed oil and extracts were also examined for their phthalide composition by Gijbels (1983) and isolated cnidilide, neocnidilide, butylphthalide, senkyunolide and Z-ligustilide.



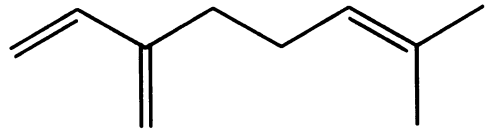
d-limonene



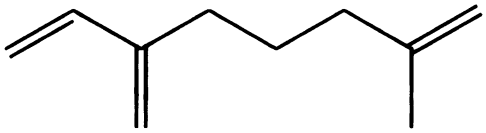
γ-terpinene



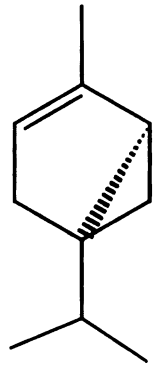
p-cymene



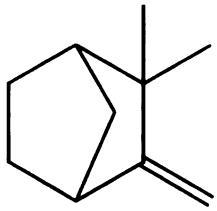
β-myrcene



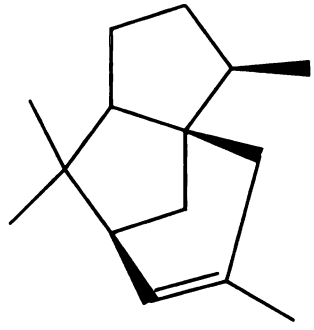
α-myrcene



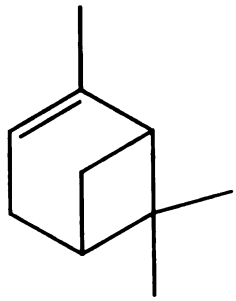
α-thujene



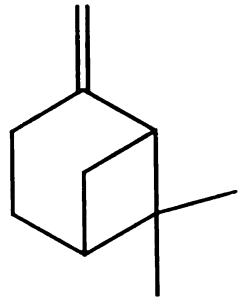
camphene



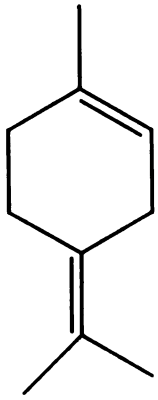
α-cedrene



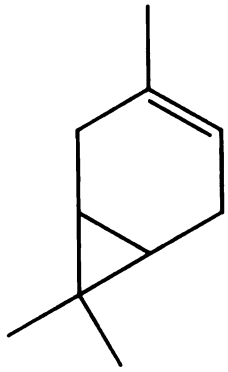
α-pinene



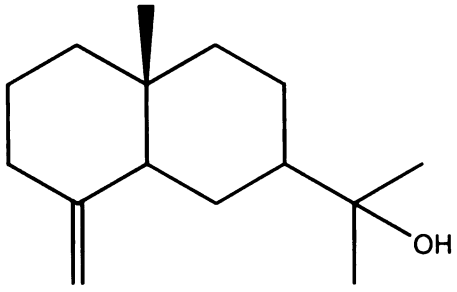
β-pinene



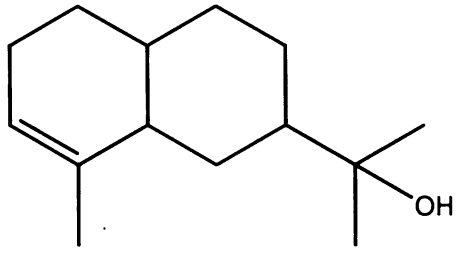
terpinolene



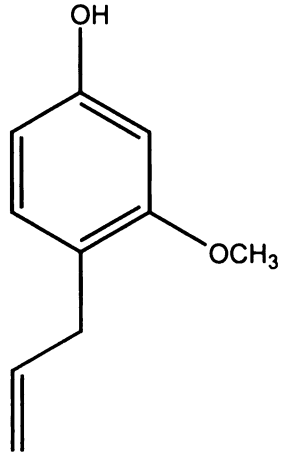
3-carene



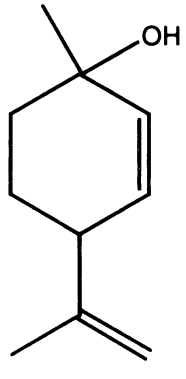
β-eudesmol



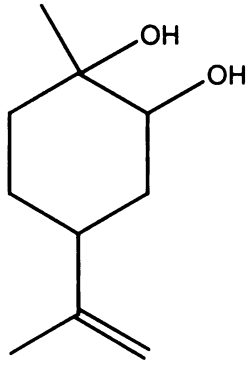
α-eudesmol



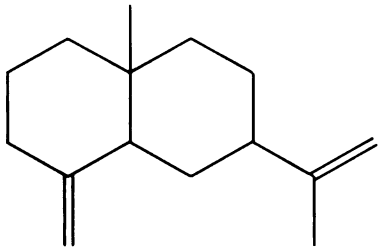
eugenol



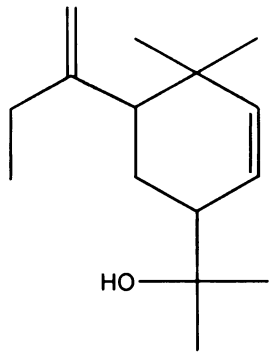
p-mentha-2,8-dien-1-ol



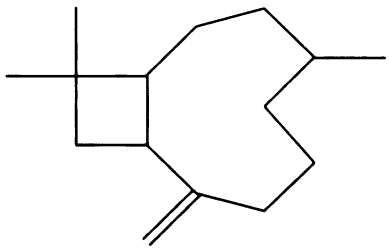
p-mentha-8(9)-en-1,2-diol



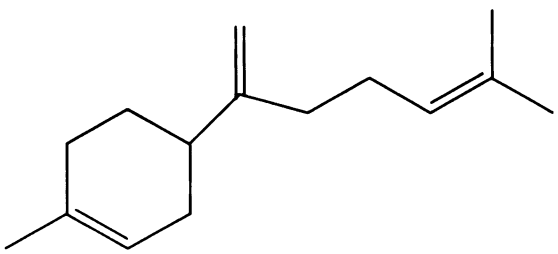
β-selinene



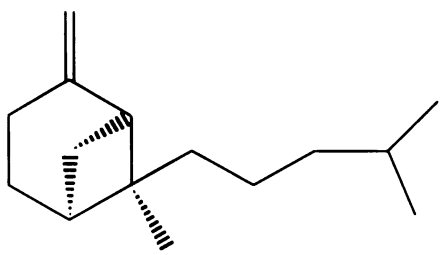
elemol



caryophyllene

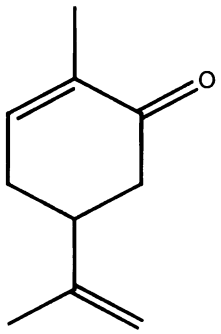


β-bisabolene

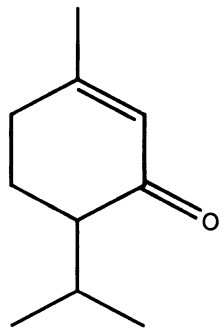


α-bergamotene

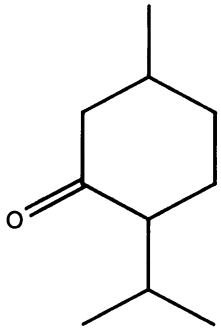
The celery seed oil was analyzed by GC-MS by Masada (1976) and reported the presence of α-pinene, camphene, β-pinene, myrcene, limonene, γ-terpinene, ρ-cymene, octanal, nonanal, octanol, caryophyllene and carvone. This author tentatively identified two terpenoids, selinene and tetradecanal. One year later, Gupta and Baslas (1978) reported the presence of carvone, piperitone, eugenol, α-pinene, terpinolene, δ-3-carene, myrecene, terpinene and menthone in a sample of Indian celery seed oil.



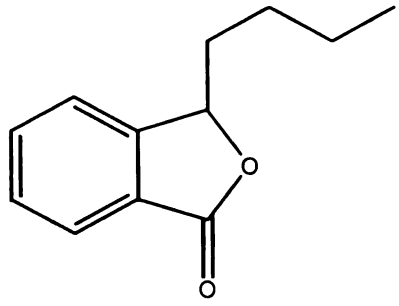
carvone



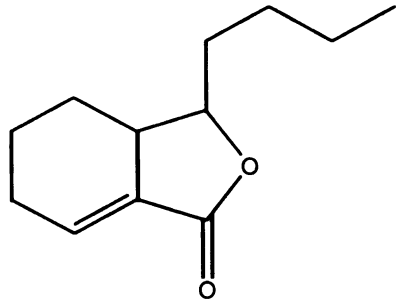
piperiton



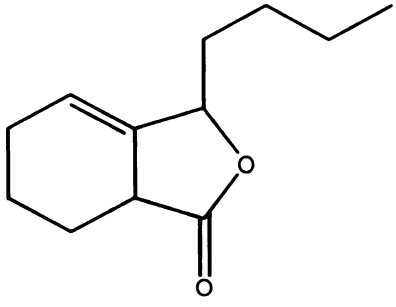
menthone



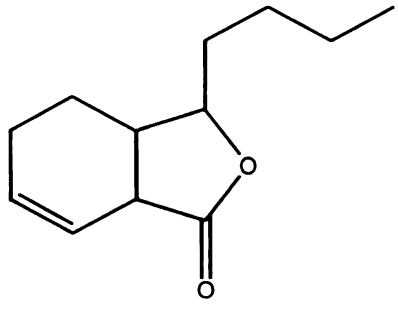
3-n-butylphthalide



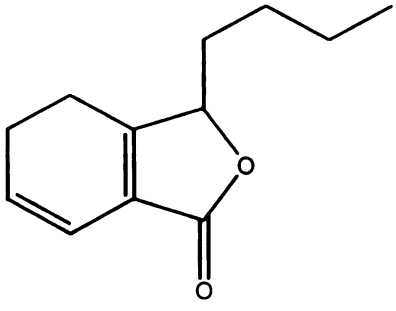
sedanolide



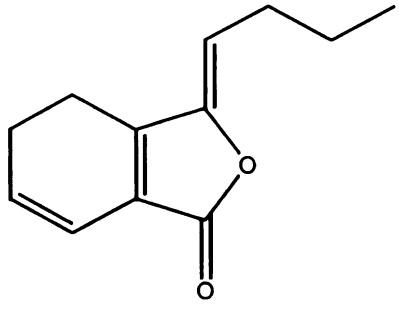
cnidilide



neocnidilide



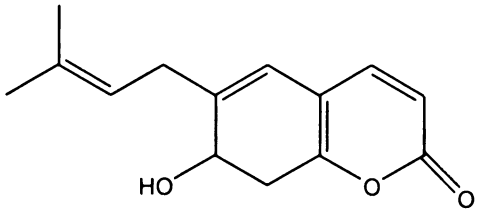
sedananolide



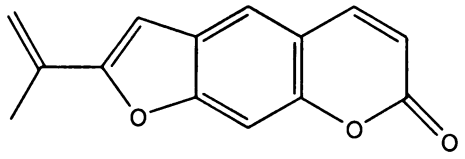
Z-ligustilide

From the cold ether extract of the celery seeds, two dihydrofurocoumarins were isolated using column chromatography and preparative TLC (Garg et al., 1978). These compounds were identified by chemical and spectroscopic methods as rutaretin and its dehydrated derivative apiumetin. In continuation of their work on *A. graveolens* seeds, 3 more compounds were isolated from the Et_2O extract (Garg et al., 1979a). These compounds were identified as 3-methoxy-4,5-methylenedioxybenzoic acid (myristic acid), 8-hydroxy-5-methoxypsoralen and umbelliferone. This was the first report of the natural occurrence of myristic acid in celery seeds. In the later study, Garg et al. (1979b) reported 6 coumarins from the petroleum ether extract of the *A. graveolens* seeds. Three of these coumarins, seselin, isoimperatorin and osthenol were novel and the other two, bergapten and isoimpinellin were known from celery seed (Musajo et al., 1954; Innocenti, 1976). Garg and co-workers (1979c) have also isolated and characterized a new furanocoumarin glucoside, apiumoside from the ethyl acetate extract of the celery seeds. Garg et al. (1980) isolated and identified a new dihydrofuranocoumarin, celereoside and isoquercitrin from the ethyl acetate extract of *A. graveolens* seeds. Other natural coumarins reported were apigravin, apiumetin, apiumoside and bergapten in celery seed (Murray et al., 1982a; Murray et al., 1982b). Ahluwalia et al. (1988) isolated three new furanocoumarin glucosides namely, (+)-2,3-dihydro-9-hydroxy-2[1-(6-sinapinoyl)β-D-glucosyloxy-1-methylethyl]-7H-furo[3,2*g*] [1]-benzopyran-7-one; (-)-2,3,-dihydro-9-O-β-D-glucosyloxy-2-isopropenyl-7H-furo [3,2*g*] [1]-benzopyran-7-one; and 5-methoxy-8-O- β-D-glucosyloxypsoralen besides previously reported celeroside and nodakenin from the seeds of *A. graveolens*.

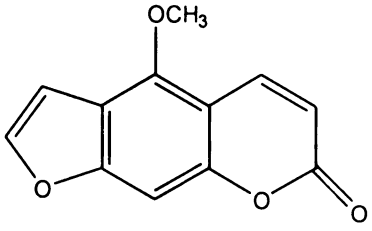
Formacek and Kubeczka (1982) examined an oil of *A. graveolens* using a combination of capillary gas chromatography and ^{13}C-NMR spectroscopy. They found that the oil contained α-piene (0.15%), camphene (0.04%), β-pinene (0.90%), sabinene (0.03%), δ-3-carene (0.03%), myrcene (0.79%), limonene (85.13%), β-phellandrene (0.05%), cis-ocimene (0.01%), trans-ocimene (0.02%, ρ-cymene (0.18%), pentylcyclohexadiene (0.12%), pentyl benzene (1.93%), linalool (0.09%), caryophyllene (0.10%), β-selinene (3.68%), myristicin (0.18%) and 3-n-butylphthalide (1.93%).



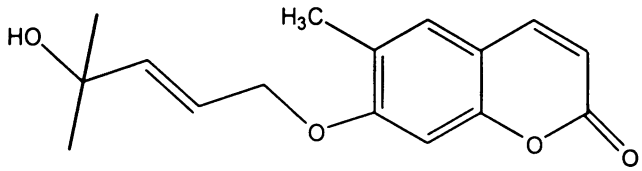
apigravin



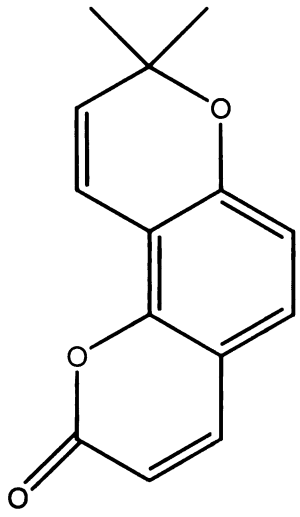
apiumetin



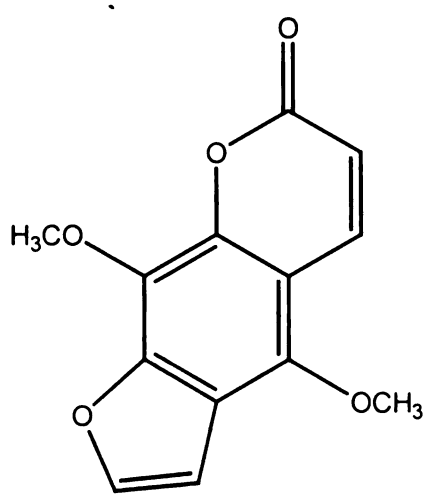
bergapten



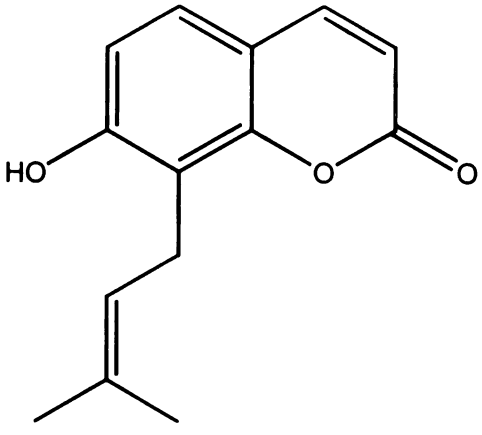
umbelliferone



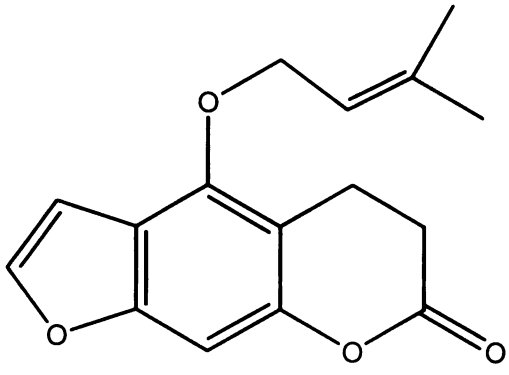
seselin



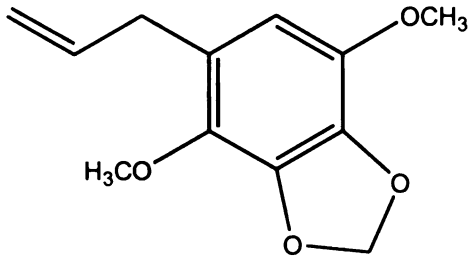
isopimpinellin



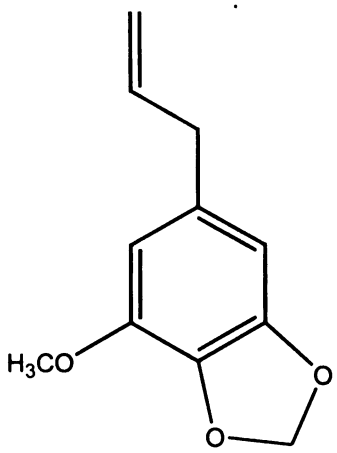
osthenol



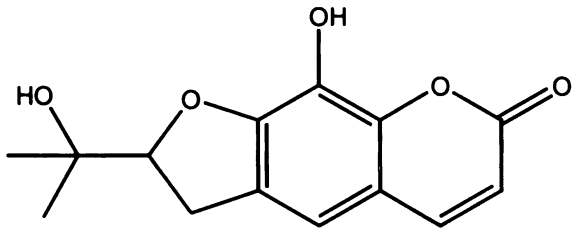
isoimperatorin



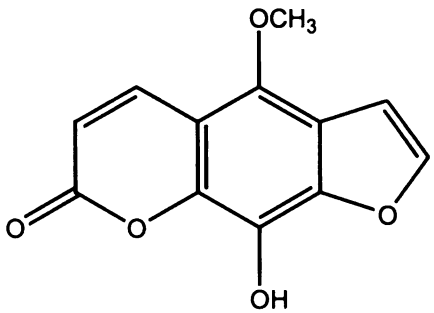
apiol



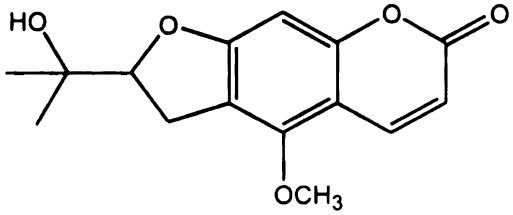
myristicin



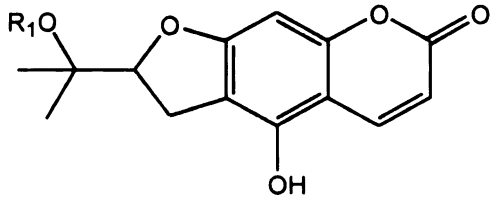
rutaretin



8-hydroxy-5-methoxypsoralen

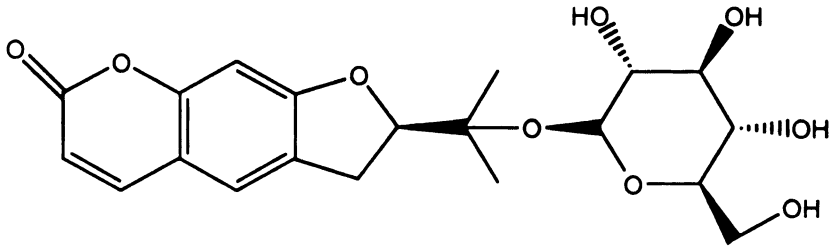


isoquercitin

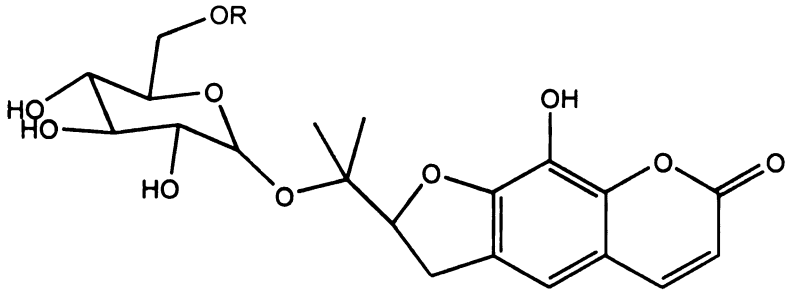


R_1=Glucose

celereoside



nodakenin



R=6'-(p-coumaroyl)-β-D-glucosyl

apiumoside

The volatile components of celery seeds having characteristic spicy aroma and being used as an ingredient of curry powder, were investigated by Kim et al. (1989). Steam distillation followed by extraction with diethyl ether : n-pentane (2:1, v/v) mixture, the volatile components, α-pinene (1.12%), myrcene (0.27%) and limonene (23.44%) were identified by capillary GC and GC/MS. Based upon GC/MS and GC/FTIR analysis of essential oil of celery seed, more that 50 compounds were detected and 27 of which were identified (Cu et al., 1990) (**Table 1.1**). Perineau et al. (1989) studied celery seed essential oil and identified sixteen compounds. In addition, they also reported ligustilide and sedanonic anhydride as components of celery oils.

In 1994, Vernin and Parkanyi analyzed the essential oils from celery seeds by GC/MS. Among the terpene and sesquiterpene derivatives, ten compounds such as α-thujene, (2)- or (3)-methylbutylbenzene, n-hexylcyclohexadiene, 3-pentenylbenzene, α-cedrene, (E)-α-bergamotene, β-bisabolene, δ-selinene, octahydroazulene, Ar-curcumene and elemol were reported as novel from celery seeds. Myrtenal, 1-p-menthen-9-al (two isomers) and 6,10,14-trimethyl-2-pentadecanone were identified as novel compounds (Vernin and Parkanyi, 1994). The t-pinocarveol, myrtenol and verbenol were also detected in the alcohol series (Vernin and Parkanyi, 1994). They have also confirmed the structures of the commonly occurring phthalides present in celery seed essential oil in addition to identifying twenty new compounds.

Table 1.1 The chemical composition of celery seed oil (Cu et al., 1990)

Compound	Methods of Identification	%
α-pinene	GC/MS, GC/FTIR	1.05
camphene	GC/MS	trace
β-pinene	GC/MS	trace
sabinene	GC/MS	0.76
myrcene	GC/MS, GC/FTIR	0.95
δ-3-carene	GC/MS, GC/FTIR	trace
δ-phellandrene	GC/MS	trace
limonene	GC/MS, GC/FTIR	72.16
β-phellandrene	GC/MS	0.02
cis-β-ocimene	GC/MS, GC/FTIR	trace
trans-β-ocimene	GC/MS	trace
p-cymene	GC/MS, GC/FTIR	0.74
pentyl benzene	GC/MS, GC/FTIR	0.02
linalool	GC/FTIR	1.48
isopulegone	GC/FTIR	0.16
caryophyllene	GC/MS, GC/FTIR	0.17
carvone	GC/MS, GC/FTIR	0.09
geranyl acetate	GC/MS, GC/FTIR	0.04
α-ionone	GC/MS	0.05
cinnamic aldehyde	GC/FTIR	0.15
thymol	GC/MS	0.17
β-selinene	GC/MS	12.17
α-selinene	GC/MS	2.05
epoxycaryophyllene	GC/MS	0.55
n-butyl phthalide	GC/MS, GC/FTIR	2.56
eudesmol	GC/MS	0.29
ligustilide	GC/MS	2.41

Schmidt et al. (1995) isolated and detected a new brassinosteroid from celery seed by GC-MS spectral analysis, which they called 2-deoxybrassinolide. Zlatanov and Ivanon (1995) investigated sterol composition of *A. graveolens* oil. Celery seed contains 0.6% of sterol (Zlatanov and Ivanon, 1995), in which sitosterol (27.4%) and stigmasterol have been identified as major components. Gas liquid chromatographic analysis of celery oil also suggested the presence of minor components, cholesterol, brassicasterol, campesterol, Δ^7-campesterol, Δ^5-avenasterol, Δ^7-stigmasterol and Δ^7-avenasterol. The tocopherol and tocotrienol composition of *A. graveolens* seed oil was also investigated using HPLC on silica columns with fluorescence detection (Ivanov and Aitzetüller, 1995).

Enantiomeric distributions of 3-butyl(hexahydro)phthalides in celery seed was investigated by Bartschat et al. (1997) using enantioselective multidimensional gas chromatography with heptakis[2,3-di-O-acetyl-6-O-(tert-butyldimethylsilyl)]-B-cyclodextrin as the chiral stationary phase. They found 3S- configuration of 3-butyl(hexahydro)phthalides as a dominant constituent with trace amount of 3R-configuration of 3-butyl(hexahydro)phthalides in celery seed. Other configurations such as 3S, 3aS, 7aR; 3R, 3aR, 7aS; 3S, 3aR, 7aS; and 3R, 3aS, 7aR were not detectable in celery seed.

It is clear from this review that bulk of the research on celery has focused on the phytochemistry and bioactivities of crude extracts from it. The following chapters report the bioassay-guided fractionation and purification of compounds possessing

mosquitocidal, nematicidal, antimicrobial, topoisomerase inhibitory, antiinflammatory and antioxidant activities.

Chapter 2

Bioactive Compounds and 1,3-di[(cis)-9-octadecenoyl]-2-[(cis,cis)-9,12-octadecadienoyl]glycerol from *Apium graveolens* L. Seeds.*

ABSTRACT- Bioassay-directed isolation and purification of the hexane extract of *Apium graveolens* L. seeds led to the characterization of three compounds, β-selinene **(1)**, 3-*n*-butyl-4,5-dihydrophthalide **(2)** and 5-allyl-2-methoxyphenol **(3)**. The structures of these compounds were established by using ^{1}H- and ^{13}C-NMR spectral methods. Compounds, **1-3** demonstrated 100% mortality on fourth-instar *Aedes aegyptii* larvae at 50, 25 and 200 μg mL^{-1}, respectively in 24 h. Also, compound **2** inhibited the growth of *Candida albicans* and *Candida kruseii* at 100 μg mL^{-1}. It inhibited both topoisomerase-I and -II enzyme activities at 100 μg mL^{-1}. Compound **2** displayed 100% mortality at 12.5 and 50 μg mL^{-1}, respectively, when tested on nematodes, *Panagrellus redivivus* and *Caenorhabditis elegans*. The triglyceride, 1,3-di[(cis)-9-octadecenoyl]-2-[(cis,cis)-9,12-octadecadienoyl]glycerol (**4**) and compound **3** were isolated for the first time from *A. graveolens* seeds, although compound **4** was not biologically active.

*R. A. Momin, R. S. Ramsewak, and M. G. Nair (2000) Bioactive Compounds and 1,3-di[(cis)-9-octadecenoyl]-2-[(cis,cis)-9,12-octadecadienoyl]glycerol from *Apium graveolens* L. Seeds. *J. Ag. Food Chem*, **2000**, 48, 3785-3788.

INTRODUCTION

Apium graveolens L. (Umbelliferae), celery, is a hepaxanthic herb grown as a biennial and under certain conditions, as an annual. Celery is a native of Eurasia and is grown mainly in coastal regions. Celery is widely cultivated in the temperate zones as an important garden crop and the bleached leaf stalks are being relished as a popular vegetable. Celery seeds are used in India to treat bronchitis, asthma, liver and spleen diseases (Satyavati and Raina, 1976). *A. graveolens* is one of the ingredients in eight of the thirty-three Indian polyherbal formulations with reputed life-protecting activity (Handa et al., 1986). Several components from celery seeds were also reported to have anticarcinogenic activity (Zheng et al., 1993).

Celery seed contains a variety of constituents. As a member of the class of bioactive natural products, phthalides occurs widely in umbelliferous plants (Bjeldanes and Kim, 1977; Chulia et al., 1986; Kaouadji et al., 1984; Kaouadji et al., 1986b, Banerjee et al., 1982). Earlier studies of *A. graveolens* led to the isolation of limonene; p-mentha-2,8-dien-1-ol; p-mentha-8(9)-en-1,2-diol; 3-*n*-butylphthalide, sedanolide (Zheng et al., 1993); selinene (Semmler and Risse, 1912); selinenol (Ruzika and Stoll, 1923) and seselin, isoimperatorin, osthenol, bergapten, isopimpinellin, apigravin, and apiumoside (Grag et al., 1978; Grag et al., 1979a; Grag et al.,1979b).

Previous research on *A. graveolens* was focused on the isolation and structural identification of compounds for perfumery and culinary uses. In this paper, we report,

three biologically active compounds (**1-3**) and a triglyceride (**4**) from the hexane extract of celery seeds.

MATERIALS AND METHODS

General Experimental Procedures.

^{1}H- and ^{13}C NMR, DEPT, Arrayed Decoupling, HMQC, and HMBC spectra were recorded on a Varian INOVA 300 MHz spectrometer. ^{13}C NMR and DEPT spectra were recorded at 75 MHz. Chemical shifts were recorded in $CDCl_3$, and the values are in δ (ppm) based on δ residual of $CHCl_3$ at 7.24 for ^{1}H NMR and $CDCl_3$ at 77 ppm for ^{13}C NMR . Coupling constants, J, are in Hz. The silica gel used for MPLC was Merk Silica gel 60 (30-70 μm particle size). FAB-MS was recorded at 70eV. TLC plates (GF Uniplate, w/binder, 250 μm Analtech, Inc., Newark, DE), and preparative TLC plates (Analtech, Sigel, 20 x 20 cm, 250, 500 μm) after developing, were viewed under UV light (254 and 366 nm). All organic solvents used were ACS reagent grade (Aldrich Chemical Co., Inc., Milwaukee, WI).

Gas chromatography was performed using a HP 6890 gas chromatograph (Algilent Technology, Wilmington, Delaware). Aliquots of 1 μL were injected into a split injector at 200 ^{0}C, and separated on an HP-5 capillary column (30 m x 0.25 mm i.d.) with helium carrier gas at a flow rate of 22 mL min^{-1}. Compounds were detected using flame ionization at 250 ^{0}C. The temperature profile was 150 ^{0}C (2 min) to 200 ^{0}C (1

min) at 10 ^{0}C min^{-1}, then to 250 ^{0}C (2 min) at 2 ^{0}C min^{-1}. Methyl esters of palmitate, linolenate and γ-linolenate were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI.

Plant Material.

Celery seeds were provided by Asgrow Seed Company, Kalamazoo, Michigan and stored at –20 ^{0}C until extraction.

Extraction and Isolation.

The seeds (100 g) were milled using an industrial Warring blender and extracted sequentially with 500 mL of each hexane, EtOAc, and MeOH over a period of 48 h. The hexane extraction afforded 17 g of residue upon removal of solvent. A portion of this hexane extract (5 g) was stirred with acetone to yield acetone soluble (2.3 g) and insoluble (2.6 g) portions. Fractionation of the bioactive acetone soluble portion (1.6 g) was carried out by medium pressure liquid chromatography (MPLC) on silica gel (Sanki Engineering Ltd., Model LBP-V pump operating at 10-15 psi; Chemco MPLC tayperling type glass column, 55 cm in length) at 2 mL min^{-1} flow rates. Fractions collected were I (500 mL) eluted with 100% hexane, II and III with hexane: ether (10:1, 500 mL each), IV (500 mL) with 100% acetone and V (500 mL) with 100% MeOH. The fractions I and IV were mosquitocidal, and fraction IV was antimicrobial, nematicidal, and inhibitory to Top-I and Top-II enzymes.

Fraction I was purified by repeated preparative TLC and yielded fractions A and B. The fraction A was further purified with hexane: ether (10:1) as the mobile phase to yield compound **4** (104 mg). Isolation of compound **1** (30 mg) from fraction B was accomplished by using 10:1 and 50:1 hexane: ether mobile phases in repeated preparative TLC. Fraction IV was purified by preparative TLC using hexane: ether (4:1) and yielded four fractions. The bioactive fraction was separated into hexane soluble and insoluble fractions. The hexane soluble fraction was further purified by preparative TLC (pentane: ether, 4:1) and afforded a pure compound **3** (6 mg). The band at R_f=0.45 was eluted with MeOH on a C_{18}-Sep Pak cartridge and yielded compound **2** (26mg).

Compound 1. ^{1}H NMR ($CDCl_3$) δ 0.71 (s, 3H, H-14), 0.80 (m, 1H, H-7), 1.2-1.6 [m, 10H, H-(1,2,3,6,8) x 2], 1.7 (s, 3H, H-13), 1.9 (m, 2H, H-9), 2.3 (m, 1H, H-5), 4.4-4.7 [bs,4H, H-(12,15)]; ^{13}C NMR ($CDCl_3$) δ 16.3 (C-14); 21.0 (C-13); 23.5, 26.8, 29.5, 36.9, 41.2, 41.9 (C-1,2,3,6,8,9); 36.0 (C-10); 45.9, 49.9 (C-5,7); 105.4, 108.1 (C-12,15); 150.8, 151.0 (C-4,11). ^{1}H NMR data of compound **1** were found to be in agreement with previously published data (Bowden et al., 1978).

Compound 2. ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3H, J=7.2, H-4'), 1.2-1.8 [m, 6H, H-(1',2',3')], 2.45 (m, 4H, H-4,5), 4.9 (m, 1H, H-3), 5.9 (m, 1H, H-6), 6.2 (d, 1H, J=10, H-7); ^{13}C NMR ($CDCl_3$) δ 13.8-22.4 (C-1',2',3',4'); 26.7-31.8 (C-4, 5); 82.5 (C-3); 116.8 (C-7); 128.3 (C-6); 124.5-135 (C-8, 9); 161.4 (C-1). The spectral data confirmed that compound **2** is 3-*n*-butyl-4,5-dihydrophthalide (Bjeldanes and Kim, 1977).

Compound 3. ^{1}H NMR ($CDCl_3$) δ 3.32 (d, 2H, J=7.0, H-7), 3.84 (s, 3H, -OCH_3), 5.08 (m, 2H, H-9), 5.45 (s, 1H, -OH), 5.90 (m, 1H, H-8), 6.66-6.69 (m, 2H, H-3,5), 6.83 (d, 1H, J=8.5, H-6); ^{13}C NMR ($CDCl_3$) δ 39.8 (C-7), 55.8 (-OCH_3), 110.9 (C-9), 114.0 (C-8), 115.4 (C-6), 121.1 (C-5), 131.7 (C-4), 137.8 (C-3), 146.3 (C-2), 146.4 (C-1). The ^{1}H NMR data were found to be in agreement with previously published data for compound 3 (Kurihara and Kikuchi, 1979).

Compound 4. ^{1}H NMR ($CDCl_3$) δ 0.86 [t, 9H, J=6.6, H-(18' x 2), 18"], 1.24 [m, 54H, H-(4'-7',12'-17') x 2 and H-(4"-7", 15"-17")], 1.6 [m, 6H, H-(3' x 2), 3"], 2.02 [m, 12H, H-(8', 11') x 2 and H-(8",14")], 2.3 [t, 6H, J=7.2, H-(2' x 2), 2"], 2.76 [dd, 2H, J=6.1, 5.5, H-11"], 4.12 (dd, 2H, J=18.0, 6.0, H-1a, 3a), 4.34 (dd, 2H, J=16.2, 4.2, H-1b, 3b), 5.3-5.4 [m, 9H, H-(9', 10') x 2, H-(9",10",12",13") and H-2]; ^{13}C NMR ($CDCl_3$) δ 14.0 (C-18"); 14.1 (C-18' x 2); 22.6, 22.7, 24.4, 24.8, 25.6, 26.8, 27.2, 27.3, 29.1-29.7 [C-(4'- 8', 11'-17') x 2, 4"-8", 14"-17"]; 31.5 (C-3"); 31.9 (C-3' x 2); 33.9 (C-2' x 2); 34.0 (C-11"); 34.2 (C-2''); 62.1 (C-1, 3); 68.8 (C-2); 127.9 – 130.5 [C-(9', 10') x2, C-(9",10",12",13")]; 172.8 (C-1"); 173.2 (C-1' x 2). The ^{1}H- and ^{13}C- NMR data for compound **4** were identical to the published finding of Chandra and Nair (1993).

Saponification and Methylation of compound 4 and standards.

Compound **4** (7 mg) was stirred with 5% NaOH in MeOH (1 mL) for 5 min followed by acidification with 6N HCl in MeOH. This solution containing free fatty acids was then dried under a stream of nitrogen. Diazomethane was prepared by reacting N-nitroso-N-

methylurea with concentrated KOH solution in ether (Kelm and Nair, 1998). As the diazomethane product formed, it dissolved into the organic ether phase. This yellow ether solution containing the diazomethane product was collected and used to methylate the free fatty acids obtained in the previous step. Also, the diazomethane solution was used to methylate oleic, stearic and linoleic acids separately. The methylated products were dissolved in hexane and filtered to remove any solids prior to GC analysis.

Mosquitocidal Assay.

A. aegyptii larvae were reared in our laboratory from eggs. Eggs were hatched in 500 mL of distilled, degassed water prepared by sonication (30 min) and larvae were fed with approximately 5 mg of bovine liver powder. Ten to fifteen 4^{th} instar larvae were placed in 980 μL of distilled water in test tubes and 20 μL of DMSO or DMSO solutions containing the appropriate concentration of test compounds were added and left at room temperature. Initially, the crude extracts and pure compounds were tested at 250 and 100 ppm concentration, respectively. The test concentrations for pure compounds were then serially diluted to 0.1 ppm as the final concentration. Pure DMSO was used as solvent control. The assays were conducted in triplicate, and the numbers of dead larvae were recorded at 2,4, 12, and 24 h intervals (Roth et al., 1998; Ramsewak et al., 1999b).

Nematicidal Assay.

The nematode cultures, *C. elegans* and *P. redivivus* were maintained in our laboratory.

C. elegans was grown on NG agar media containing a strain of *Escherichia coli* in disposable petri dishes wet with 2-4 mL of physiological saline solution. *P. redivivus* was maintained in axenic, liquid Basal Heme media (5 mL) in scintillation vials. The cultures were stored at room temperature and subcultured prior to the assay. The assay was conducted in Corning polystyrene 96 well plates. The nematodes were added to 1 mL of physiological saline solution in a scintillation vial. This solution was diluted until the nematodes count were 15-20 in a 48 μL aliquot. Forty eight μL of solution containing nematodes was delivered to each of three wells per treatment. Two μL of DMSO (50%) or DMSO (50%) and test compounds were added to each well. The plate was covered, parafilmed, and kept in a humid chamber. The number of dead nematodes was recorded every at 2, 4, 6, 8, and 24 h by observing under a microscope (Nair et al., 1989).

Antimicrobial and Topoisomerase-I and -II Inhibitory Assays.

The test organisms *Candida kruseii* (MSU strain) and *Candida albicans* (MSU strain) used for antimicrobial bioassays were cultured in Petri dishes containing YMG media (20 mL) and *Saccharomyces cerevisiae* mutant cell cultures, JN394, JN394 t_{-1}, and JN394 $t_{-2\text{-}5}$, used for the topoisomerase assay, were cultured in Petri dishes containing YPDA medium (20 mL). Physiological saline solution (2-3 mL) was added to fully grown plate of each organism and then suspensions were diluted to obtain 5 x 10^6CFU/mL. A 50 μL aliquot of this suspension was then used to inoculate culture tubes containing the corresponding media (930 μL). DMSO or test compounds dissolved in DMSO were

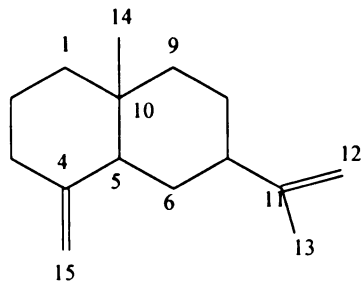
added to the inoculated tubes (20 µL) at concentrations ranging from 100 to 0.1 ppm. The tubes containing cell cultures and compounds were incubated at 27 ^{0}C on a rotary orbital shaker at 120 rpm for 72 h. MIC_{100} values for the test compounds were recorded for each test organism at the end of incubation period (Chang et al., 1995; Roth et al., 1998; Ramsewak et al., 1999b).

RESULTS AND DISCUSSION

Seeds of *A. graveolens* were extracted with hexane, and by successive silica gel MPLC, and preparative TLC gave four compounds, **1-4**. The hexane extract was fractionated into acetone soluble and insoluble fractions. The bioactive acetone soluble fraction was subjected to medium-pressure liquid chromatography. Fraction A eluting with 100% hexane and fraction D eluting with hexane: ether (10:1) was found to be active. Fraction A was purified by repeated preparative TLC to yield 1,3-Di[(cis)-9-octadecenoyl]-2-[(cis,cis)-9,12-octadecadienoyl]glycerol (**4**) and β-selinene (**1**). The repeated preparative TLC of fraction D yielded bioactive compounds 3-*n*-butyl 4,5-dihydrophthalide (**2**) and 5-allyl-2-methoxyphenol (**3**).

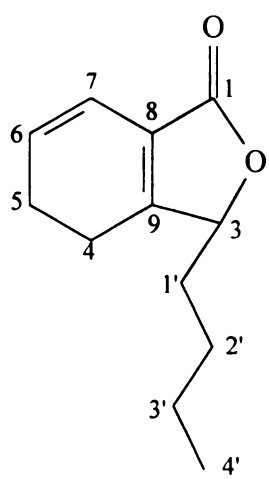
^{1}H- and ^{13}C-NMR and DEPT spectral data were used to determine the structure of compound **1**. Singlets at 1.7 and 0.71 ppm , each integrated for 3 protons, indicated the presence of two methyl groups (C-13, C-14) attached to olefinic and quaternary carbons, respectively. The broad singlet at 4.4-4.7 ppm indicated the presence of vinyl protons at C-12 and C-15 in support to presence of ^{13}C NMR signals at 105.4 and 108.1 ppm. The

DEPT spectrum supported the ^{1}H NMR and ^{13}C NMR data. The ^{1}H NMR spectrum of **1** was identical to the published finding of Bowden et al., (1978).



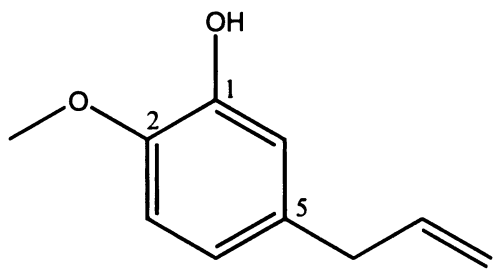
1

The structure of compound **2** was determined by using ^{1}H, ^{13}C NMR, and DEPT spectral data. The two-dimensional HMQC proton-carbon correlations, HMBC proton-carbon long range coupling and arrayed-decoupling experiments facilitated further evidence to support the 1D NMR experiments. The ^{1}H NMR spectrum displays a doublet at 6.2 ppm (1H, J=10 Hz) and a multiplet at 5.9 ppm (1H) for the vinyl protons, H-7 and H-6, respectively; as well as a multiplet at 4.9 ppm for H-3. The ^{1}H NMR spectral data of compound **2** were identical to previously published data (Bjeldanes and Kim, 1977).



2

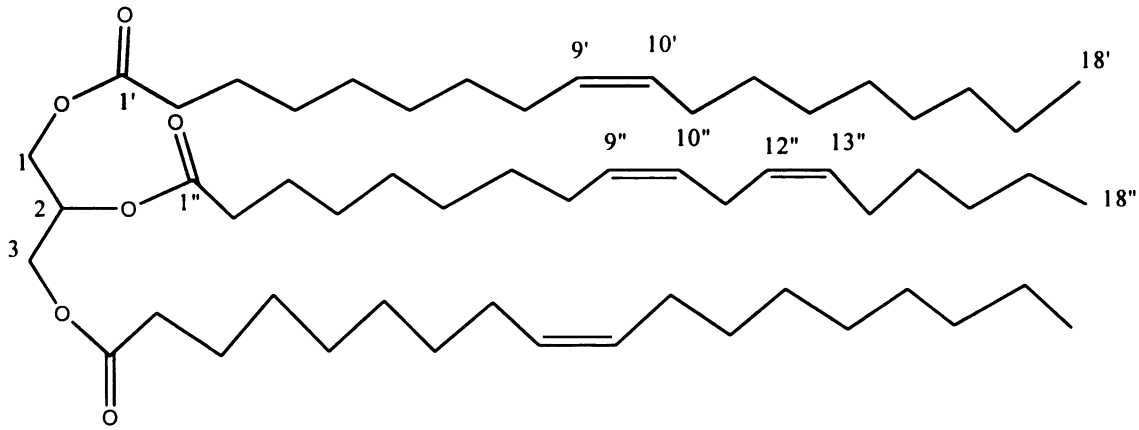
The structure of compound **3** was determined by using ^{1}H, ^{13}C NMR, and DEPT spectral data. A singlet at 3.84 ppm corresponded to methoxy protons. A singlet at 5.45 ppm was determined to be hydroxy group as indicated by the disappearance of this peak following a D_2O shake in the ^{1}H NMR spectrum. Presence of two peaks, at aromatic region (6.66-6.83 ppm), integrated for 3 protons, indicated a trisubstituted aromatic ring. This proton data were found to be in agreement with those previously published (Kurihara and Kikuchi, 1979).



3

The structure of compound **4** was determined by using ^{1}H and ^{13}C NMR, and MS Spectroscopy. The ^{1}H NMR signals at δ 4.12 and 4.34 corresponded to 2 x CH_2-methylene protons of a glycerol backbone. The overlapping multiplets at δ 5.3 for 8 protons correlated with unsaturated carbons at δ 127.9-130.5 indicated the presence of 4 double bonds in this molecule. MS peaks at m/z 883 $[C_{57}H_{102}O_6 + H^+]$, 617 $[C_{57}H_{102}O_6 - C_{18}H_{31}O]^+$, 263 $[C_{18}H_{31}O]^+$, 265 $[C_{18}H_{33}O]^+$ supported the presence of oleic and linoleic acids. The GC profile of methylated hydrolysis product of this molecule confirmed the presence of the methyl esters of oleic and linoleic acids with a ratio of 2:1, respectively. The retention times for both methyl esters were identical to those of authentic samples of

oleic and linoleic acid methyl esters analyzed under the same conditions. The spectral data of compound **4** were identical to the published finding by Chandra and Nair (1993).

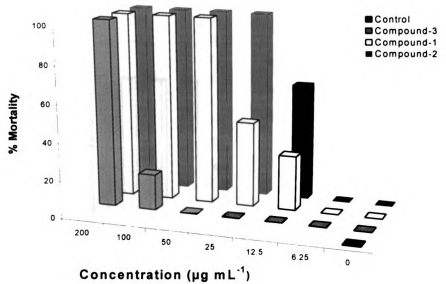


4

Mosquitocidal assays using *Aedes aegyptii* on compounds **1-4** indicated that compounds **1, 2** and **3** were active. Compound **1, 2** and **3** had an LD_{100} (24 h) at 50, 25 and 200 μg mL^{-1}, respectively (**Fig. 2.1**). Compound **2** displayed nematicidal activity on *P. redivivus* and *C. elegans*. LD_{100} values in 24 h were 12.5 and 50 μg $mL^{-1}$for *P. redivivus* and *C. elegans*, respectively (**Fig. 2.2**). Compound **2** showed topoisomerase-I and -II inhibitory activity when tested on *S. cerevisiae* mutant strains. JN394 is hypersensitive to both topoisomerase-I and –II poisons, while JN394 t_{-1} lacks the top-I gene and therefore shows a lack of response to topoisomerase-I poisons. JN394 t_{-2-5} carries the top-II gene, which is resistant to topoisomerase-II poisons but responds to topoisomerase-I poisons. MIC_{100} determination of the compound **3** indicated that it completely inhibited topoisomerase-I and II activity at 100 μg mL^{-1} concentration. The zone of inhibition at 50 μg mL^{-1} concentration of compound **2** on YPDA plate were 1.1,

1.1 and 1.2 cm for JN394, JN394 t_1 and JN394 $t_{2\text{-}5}$, respectively. Antimicrobial assays showed that compound **2** was active when tested on *C. albicans* and *C. kruseii* (MSU strains). The inhibition zone for *C. albicans* and *C. kruseii* was found to be 1.1 and 1.0 cm at the concentration of 50 μg mL^{-1}, respectively. Compound **2** had an MIC_{100} at 100 μg mL^{-1} concentration.

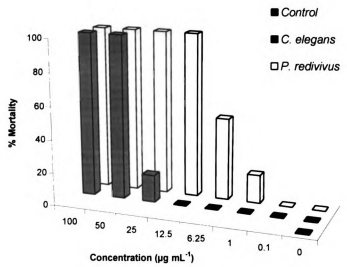
Figure 2.1. Percent mortality of 4th instar *A. aegyptii* larvae for compounds **1-3** at 24 h. Statistical analysis was done using ANOVA ($p \leq 0.01$) and the means were compared by calculating least significant difference (LSD).



The flavor of celery seed oil is mainly due to β-selinene and phthalides (Lund, 1978). Terpenes, terpenoids and phthalides have been implicated as being natural

insecticides, insect pheromones, mammalian sex hormones and they have been used since antiquity as ingredients of flavors, preservatives, perfumes, medicines, narcotics and pigments. The present study supports that the observed biological activity of celery seeds is part due to the presence of terpenes and phthalides. To the best of our knowledge, this is the first report of compounds **3** and **4** from *A. graveolens* seeds, although compound **4** is not biologically active. Till now, there are no reports on the biological activity of compounds **1-3** although compound **2** was used as one of the component of perfume.

Figure 2.2. Percent mortality of *C. elegans* and *P. redivivus* nematodes for compound **2** at 24 h. Statistical analysis was done using ANOVA ($p \leq 0.01$) and the means were compared by calculating least significant difference (LSD).



Chapter 3

Mosquitocidal, Nematicidal and Antimicrobial compounds from *Apium graveolens* L. seeds*

ABSTRACT- The methanolic extract of *Apium graveolens* seeds was investigated for bioactive compounds. Mosquitocidal, nematicidal and antimicrobial compounds, sedanolide (**5**), senkyunolide-N (**6**) and senkyunolide-J (**7**) were isolated and characterized from *A. graveolens* seeds. Their structures were determined by ^{1}H and ^{13}C NMR spectral methods. Compounds **5-7** gave 100% mortality at 25, 100 and 100 μg mL^{-1}, respectively, on the nematode, *Panagrellus redivivus*. Compound **5** showed 100% mortality at 50 μg mL^{-1} on nematode, *Caenorhabditis elegans*, and fourth-instar mosquito larvae, *Aedes aegyptii*. Also, it inhibited the growth of *Candida albicans* and *Candida parapsilasis* at 100 μg mL^{-1}. Compounds **6** and **7** were isolated for the first time from *A. graveolens*. This is the first report of the mosquitocidal, nematicidal and antimicrobial activities of compounds **5-7**.

*R. A. Momin and M. G. Nair (2000) Mosquitocidal, nematicidal and antimicrobial compounds from *Apium graveolens* seeds. *J. Ag. Food Chem.*, Submitted, August, 2000.

INTRODUCTION

Apium graveolens L., celery, is widely grown in the temperate zone as a garden crop and its leaf stalks are used as a popular vegetable. In the USA, celery fruit, commonly known as "celery seed", was largely imported from Europe but since World War-II, it has been supplied from domestic growers, especially in Michigan and Wisconsin. Celery seeds possess a characteristic aroma and pungent taste and are used as a condiment in the flavoring of food products. Celery can be classed both as a seasoning and as a vegetable. For seasoning purpose, the seeds and the plant, as well as the essential oil distilled from the seeds are used (Salzer, 1975). Celery seed extracts are extensively used as flavoring ingredients in many food products, including alcoholic and nonalcoholic beverages, frozen dairy desserts, candy, baked goods, gelatins, puddings, meat products, condiments and relishes, soups, gravies, snack food, and others. Celery seeds are used for treating bronchitis, asthma, liver and spleen diseases (Chopra et al., 1958; Satyavati and Raina, 1976).

Sedanolide, considered to be one of the celery flavor compounds (Bjeldanes and Kim, 1977; Tang et al., 1990) was isolated and identified from celery seed oil in 1950 (Guenther, 1950). Since the isolation of sedanolide, several other phthalides such as sedanenolide, 3-n-butylphthalide, cnidilide, neocnidilide, ligustilide and 3-isobutylidene-3a,4-dihydrophthalide were reported from celery seed oil (Bjeldanes and Kim, 1977; Fehr, 1974; Uhling et al., 1987). In this paper we report for the first time the mosquitocidal, nematicidal and antimicrobial activities of compounds **5-7**.

MATERIALS AND METHODS

General Experimental Procedures.

All NMR spectra (^{1}H and ^{13}C) were recorded on a Varian INOVA 300 MHz spectrometer. ^{13}C NMR spectra were recorded at 75 MHz. Chemical shifts were recorded in $CDCl_3$, and the values are in δ (ppm) based on δ residual of $CHCl_3$ at 7.24 for ^{1}H NMR and $CDCl_3$ at 77 ppm for ^{13}C NMR. Coupling constants, J, are in Hz. The silica gel used for VLC and MPLC was Merk Silica gel 60 (30-70 μm particle size). For preparative HPLC (LC-20, Japan Analytical Industry Co., Tokyo) purification, two JAIGEL-ODS, A-343-10 (20 mm x 250 mm, 10 μm, Dychrom, Santa Clara, CA) columns were used in tandem. Peaks were detected using a UV detector equipped with model D-2500 Chromato-integrator (Hitachi, Tokyo). The CD analysis of compounds **6** and **7** were performed on JASCO J-710 CD-ORD spectropolarimeter. Nitrogen was generated by a nitrogen generator model NG-150 at a rate of 40 L min^{-1}. Compounds **6** and **7** were dissolved in methanol separately, and the CD was determined at 185-400 nm. All solvents were ACS reagent grade and were purchased from Aldrich Chemical Co., Inc.

Plant material

Celery seeds were donated by Asgrow Seed Company, Kalamazoo, Michigan and stored at –20 ^{0}C until extraction.

Extraction and isolation

The seeds (905 g) were milled using an industrial Warring blender and extracted with hexane (4 x 1.5 L, 48 h) to yield the hexane extract (85 g). The residue was then extracted with MeOH (3 x 1.5 L, 48 h) to yield MeOH extract (20 g). The solvents were evaporated under reduced pressure at 40 ^{0}C to yield crude extracts. The MeOH extract (19 g) was stirred with hexane (2 x 400 mL, 15 min) to yield hexane soluble (7.4 g) and insoluble (11.5 g) fractions. The hexane soluble fraction (6 g) was further separated by VLC followed by MPLC on silica gel (Sanki Engineering Ltd., Model LBP-V pump operating at 10-15 psi; Chemco MPLC tayperling type glass column, 55 cm in length) using hexane with increasing amount of acetone and finally with MeOH as the eluting solvents. The fractions collected were: A (404 mg, hexane-acetone, 4:1, 250 mL); B (142 mg, hexane-acetone, 2:1, 80 mL); C (483 mg, hexane acetone, 2:1, 100 mL); D (79 mg, hexane-acetone, 2:1, 120 mL); E (61 mg, hexane-acetone, 1:1, 100 mL); F (89 mg, hexane acetone, 1:1, 200 mL); G (35 mg, hexane-acetone, 1:1, 100 mL); H (14 mg, 100% acetone, 20 mL); I (33 mg, 100% acetone, 130 mL), and J (8 mg, 100% MeOH, 250 mL). Mosquitocidal, nematicidal and antimicrobial bioassays on these fractions revealed that fraction B was active. The bioactive fraction B (135 mg) was purified by preparative TLC (hexane-acetone, 20:1 x 3) to yield three major bands: I (48 mg, R_f 0.18); II (65 mg, R_f 0.35) and III (16 mg, R_f 0.50). Band II was biologically active and was further purified by repeated preparative TLCs (hexane-ether 8:1 x 4 and 2:1 x 1) to yield a pure compound **5** (22 mg).

The hexane insoluble fraction (11 g) of MeOH extract was separated into chloroform soluble (2.1 g) and insoluble (8.2 g) fractions by stirring it with chloroform (2 x 50 mL, 15 min). The chloroform soluble fraction (2.1 g) was further separated into hexane soluble (607 mg) and insoluble (1.5 g) fractions. The hexane insoluble fraction was further separated into methanol-water, 75:25 soluble and insoluble fractions and precipitates were removed by centrifugation. The soluble fraction (684 mg) was separated into 8 fractions by preparative HPLC using methanol-water, 75:25 as a mobile phase at flow rate of 3 mL min^{-1}. Fraction II (R_t = 48 min, 75 mg) was further purified by HPLC using methanol-water, 60:40 as a mobile phase at flow rate of 2 mL min^{-1} to yield 1:1 mixture of compounds **6** and **7** (R_t = 65 min, 12 mg). This mixture was separated in to pure compounds **6** and **7** by HPLC using H_2O-THF, 90:10 as a mobile phase at the flow rate of 4 mL min^{-1}.

Compound 5. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.83 (3H, t, J=7.1 Hz, H-11), 1.00-2.20 (10H, m, H-4,5,8,9,10), 2.22 (1H, m, H-3a), 2.41 (2H, m, H-6), 3.87 (1H, m, H-3), 6.66 (1H, dd, J=6.3, 3.0 Hz, H-7); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 13.87 (C-11), 20.74 (C-5), 22.50 (C-10), 24.96 (C-4), 25.34 (C-3a), 27.49 (C-8), 34.30 (C-9), 43.04 (C-6), 85.35 (C-3), 131.11 (C-7a), 135.20 (C-7), 170.24 (C-1). Compound **5** was identified as sedanolide. The spectral data of compound **5** were identical to the published values for sedanolide (Zheng et al., 1993).

Compound 6. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, t, J=7.2 Hz, H-11), 1.30-1.45 (4H, m, H-9,10), 1.50-2.21 (4H, m, H-5,8), 2.35-2.40 (2H, m, H-4), 3.91 (1H, ddd, J=9.8, 6.1, 3.3 Hz, H-6), 4.41 (1H, dddd, J=6.1, 2.5, 2.0, 1.9 Hz, H-7), 4.86 (1H, ddd, J=8.1, 3.6,

2.4 Hz, H-3); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 13.80 (C-11), 21.3 (C-4), 22.4 (C-10), 26.5 (C-5), 26.8 (C-9), 31.9 (C-8), 67.4 (C-7), 71.5 (C-6), 82.9 (C-3), 126.3 (C-7a), 166.7 (C-3a), 173.0 (C-1). Compound **6** was identified as senkyunolide-N by comparison of its ^{1}H- and ^{13}C- NMR spectral data with published values (Naito et al., 1992).

Compound 7. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, t, J=7.2 Hz, H-11), 1.30-1.45 (4H, m, H-9,10), 1.50-2.21 (4H, m, H-5,8), 2.35-2.40 (2H, m, H-4), 3.91 (1H, ddd, J=9.8, 6.1, 3.3 Hz, H-6), 4.41 (1H, dddd, J=6.1, 2.5, 2.0, 1.9 Hz, H-7), 4.86 (1H, ddd, J=8.1, 3.6, 2.4 Hz, H-3); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 13.80 (C-11), 21.1 (C-4), 22.3 (C-10), 26.3 (C-5), 26.6 (C-9), 31.8 (C-8), 67.3 (C-7), 71.3 (C-6), 82.8 (C-3), 126.1 (C-7a), 166.5 (C-3a), 172.9 (C-1). Compound **7** was identified as senkyunolide-J by comparing its ^{1}H- and ^{13}C- NMR spectral data with published values (Naito et al., 1992).

Mosquitocidal Assay.

Fourth instar mosquito larvae, *A. aegyptii*, were reared from neonates in our laboratory. Ten to 15 larvae were placed in 980 μL of degassed distilled water and 20 μL of DMSO or DMSO solution containing test extracts or pure compounds were added. Extracts were tested at 250 μg mL^{-1} and pure compounds were tested at a 1-200 μg mL^{-1} concentrations. There were three replicates per treatment. The numbers of dead larvae were recorded at 2-, 4-, 6- and 24- h intervals. The control was prepared with 980 μL of degassed distilled water and 20 μL of DMSO solution to which larvae were added (Roth et al., 1998).

Nematicidal Assay.

The nematode cultures, *P. redivivus* and *C. elegans* were maintained in our laboratory. *P. redivivus* was grown in axenic, liquid Basal heme media (5 mL) in scintillation vials. *C. elegans* was maintained on NG agar media containing a strains of *Escherichia coli* in disposable petri dishes wet with 2-4 mL of physiological saline solution. The cultures were stored at room temperature and sub-cultured prior to the assay. The assay was conducted in Corning polystyrene 96-well plates. The nematodes were added to 1 mL of physiological saline solution in a scintillation vial. This solution was diluted until the nematodes count were 15-20 in a 48 μL aliquot. This solution (48 μL) containing nematodes was delivered to each of three wells per treatment. Two μL of DMSO (50%) and test compounds were added to each well. The plate was covered, parafilmed, and kept in a humid chamber. The number of dead nematodes recorded every at 2-, 4-, 6-, 8- and 24- h by observing under a microscope (Nair et al., 1989).

Antimicrobial Assay.

Compounds **5-7** were evaluated for antimicrobial activities according to the reported procedure (Chang et al., 1995). The test organisms, *C. albicans* (MSU strain) and *C. parapsilasis* (MSU strain) used for antimicrobial bioassays were cultured in petri dishes containing YMG media (20 mL). Two to three mL of physiological saline solution was added to fully grown plate of each organism and then suspensions were diluted to obtain 5×10^6 CFU mL^{-1}. Bioassays were conducted by spreading 50 μL of the desired cell suspension on petri dishes of the YMG media. DMSO or test compound dissolved in

DMSO (20 μL) were spotted carefully on the bioassay plates at varying concentrations. The plates were allowed to dry in a laminar flow hood and then incubated at 27 ^{0}C for 72 h. The zone of inhibition was measured in mm (Chang et al., 1995, Roth et al., 1998). Minimum inhibitory concentration (MIC_{100}) was determined for compounds **5-7** according to the published procedure (Nair et al., 1989).

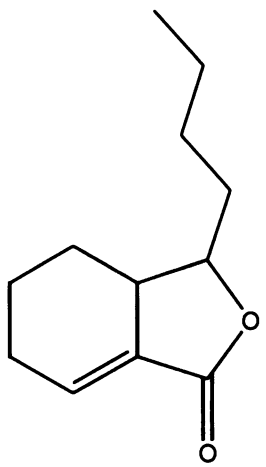
RESULTS AND DISCUSSION

Celery seeds were milled and extracted sequentially with hexane and MeOH. MeOH extract was partitioned into hexane soluble and insoluble fractions. Preliminary bioassays indicated that hexane soluble fraction was active on *C. albicans*, *C. parapsilasis*, mosquito larvae, *A. aegyptii*, and nematodes, *C. elegans* and *P. redivivus*, where as hexane insoluble fraction was active on nematode, *P. redivivus*. The hexane soluble fraction was purified by VLC and MPLC on silica gel using hexane with increasing amount of acetone and finally with MeOH as the eluting solvents. Ten fractions, A-J, were collected and tested for nematicidal activity. The active fraction B, eluting with hexane-acetone was further purified by repeated preparative TLC to yield compound **5**.

The hexane insoluble fraction of the MeOH extract of celery seed was further partitioned to yield chloroform soluble and insoluble fractions. The chloroform soluble fraction was active against *P. redivivus*. This fraction was further fractionated in to hexane soluble and insoluble fractions. The hexane insoluble fraction was dissolved in MeOH and precipitated with water and centrifuged. Purification of the MeOH-H_2O

soluble fraction was carried out by HPLC on two JAIGEL-ODS columns using MeOH-H_2O. The fraction II (R_t = 48 min) was active on *P. redivivus* and was further purified by HPLC using MeOH-H_2O as the mobile phase to yield a mixture of compounds **6** and **7** (R_t = 65 min, 12.5 mg). This mixture was separated into compounds **6** and **7** by HPLC using H_2O-THF as the mobile phase.

The structure of compound **5** was confirmed by ^1H- and ^{13}C- NMR spectral data and was identical to the published spectral data for sedanolide (Zheng et al., 1993).



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Compounds **6** and **7** were identified as senkyunolide-N and senkyunolide-J, respectively, by comparison of their ^1H- and ^{13}C- NMR spectral data to that of the published data (Naito et al., 1992). The CD spectrum (**Fig. 3.1**) of compound **6** showed a positive Cotton effect at 194 nm indicating that compound **6** has the 6S and 7S configuration, while compound **7** showed a negative Cotton effect at 194 nm indicating that **7** has 6R and 7R configuration (Naito et al., 1992). Thus, the structural identities and stereochemistry of Compounds **6** and **7** were confirmed as senkyunolide-N and senkyunolide-J (Naito et al., 1992). Compounds **6** and **7** were previously reported from

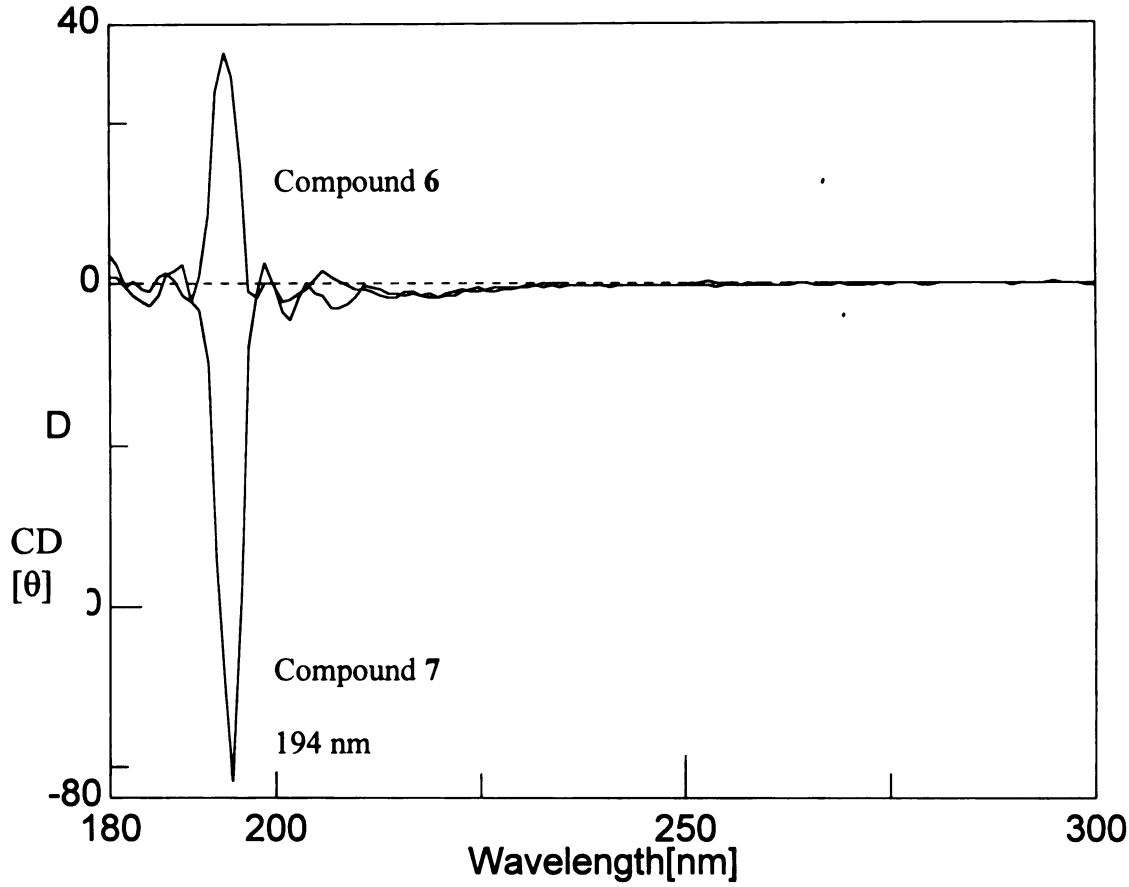
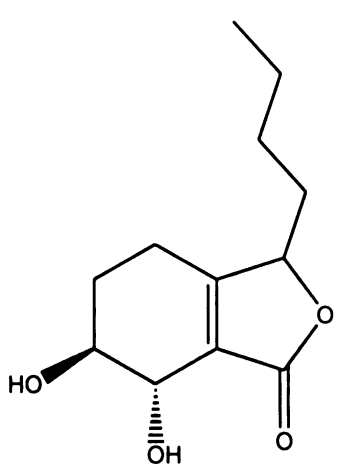


Figure 3.1. CD Spectra of compounds **6** and **7** in MeOH



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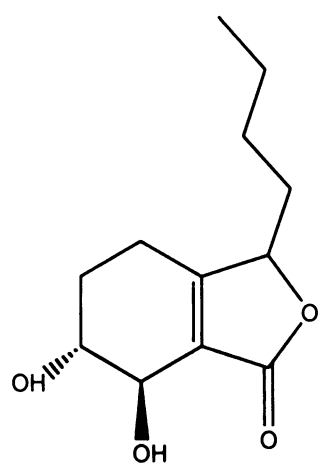

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the dried rhizomes of *Ligusticum chuangxions* (Naito et al., 1992). This is the first report of the isolation of compounds **6** and **7** from *A. graveolens*.

Compound **5** exhibited 100% mortality (LD_{100}) at 25 and 50 μg mL^{-1} when tested on *P. redivivus* and *C. elegans*, respectively, (**Fig. 3.2 and 3.3**). At 100 μg mL^{-1}, compounds **6** and **7** showed 100% mortality when tested on *P. redivivus* (**Fig. 3.2**). Also, compound **5** gave 100% mortality at 50 μg mL^{-1} when tested on fourth instar *A. aegyptii* larvae. In the preliminary bioassay, compound **5** gave a zone of inhibition of 11 mm each for *C. albicans* and *C. parapsilasis* at 100 μg mL^{-1} on YMG culture plates. Also, it completely inhibited the growth of *C. albicans* and *C. parapsilasis* at 100 μg mL^{-1}. Compounds **5-7** were showed no activity when tested against bacteria, *Escherichia coli*, *Staphylococcus epidermidis* and *Streptococcus aureus* (Chang et al., 1995).

Phthalides are bioactive natural products and occur widely in umbelliferous plants. Besides its role in the characteristic odor of celery, sedanolide was previously reported as tumor inhibitor in forestomach of female A/J mice by inducing glutathione S-transferase enzyme activity (Zheng et al. 1993). The Chinese traditional plant, *L. chuangxiong* has been used to treat headache, anemia, feeling of cold and irregularity of menstruation. Senkyunolide-N and –J were reported from the rhizomes of *L. chuangxiong* (Naito et al., 1992). These results support the notion that phthalides are biologically active. The five-member lactone ring along with butyl side chain in phthalides may be important for observed biological activities. Zheng et al. (1993) reported that the five member lactone ring in phthalides is important for the high glutathione S-transferase activity. Celery seed extracts and oil which are used in

perfumery, for flavoring, seasoning and pharmaceutical preparation have the potential to be used as dietary supplements, if toxicity and side effects are minimal. The nematicidal and mosquitocidal activity of compounds **5-7** suggest that further examination is required for their potential toxicity and side effect.

Figure 3.2. Percent nematicidal activity against *P. redivivus* for compounds **5-7** at 24 h. Statistical analysis was done using ANOVA ($p \leq 0.01$) and the means were compared by calculating least significant difference (LSD).

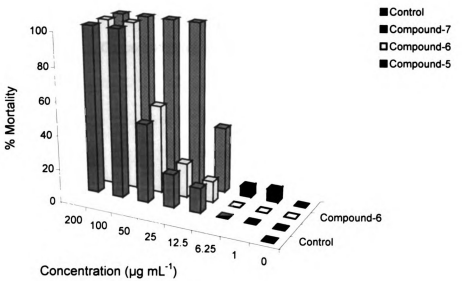
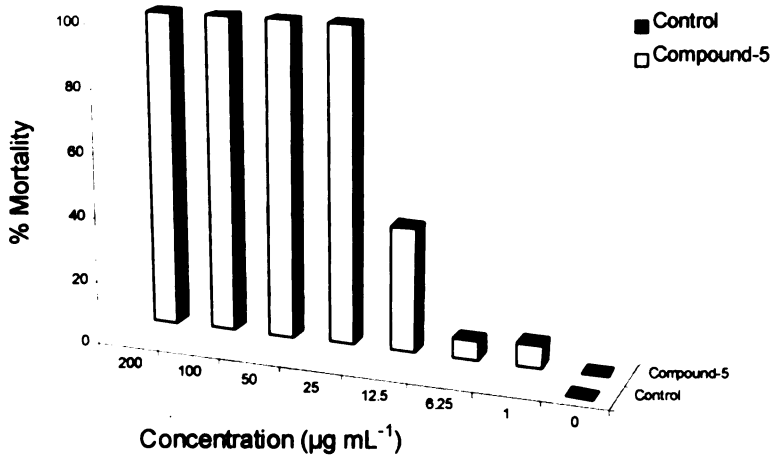


Figure 3.3. Percent nematicidal activity against *C. elegans* for compound **5** at 24 h. Statistical analysis was done using ANOVA ($p \leq 0.01$) and the means were compared by calculating least significant difference (LSD).



Chapter 4

Antioxidant, Cyclooxygenase and Topoisomerase Inhibitory compounds from *Apium graveolens* Linn. Seeds.*

ABSTRACT– Cyclooxygenase inhibitory and antioxidant bioassay-directed extraction and purification of the celery seeds yielded compounds; sedanolide [**5**], senkyunolide-N [**6**], senkyunolide-J [**7**], 3-hydroxymethyl-6-methoxy-2,3-dihydro-1H-indol-2-ol [**8**], L-tryptophan [**9**], and 7-[3-(3,4-dihydroxy-4-hydroxymethyl-tetrahydro-furan-2-yloxy)-4,5-dihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy]-5-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chromen-4-one [**10**]. Compounds **5-7** were isolated and characterized according to procedures described in **Chapter 3**. The structures of compounds **5-10** were established using spectroscopic methods. Isolation and identification of compound **8** from celery seeds is for the first time and compound **8** is a novel natural product. At 250 μg mL^{-1}, compounds **5-10** displayed 24, 32, 36, 52, 31 and 29 % of prostaglandin H endoperoxide synthase-I (COX-I) and 24, 29,25, 40, 32 and 21 % of prostaglandin H endoperoxide synthase-II (COX-II) inhibitory activities at pH 7, respectively. The acetylated product (**11**) of compound **8** also inhibited the COX-I and COX-II enzymes at 31 and 28 %, respectively, when tested at 250 μg mL^{-1} concentration. The COX-I and COX-II enzyme inhibitory activities of compounds **5-10** were also studied at different concentrations. Compounds **9** and **10** exhibited good antioxidant activity at 125 and 250 μg mL^{-1} concentrations. Compounds **5-7** exhibited topoisomerase-I and –II enzyme inhibitory activity at 100, 200 and 200 μg mL^{-1} concentrations, respectively.

*R. A. Momin and M. G. Nair (2000) Antioxidant, Cyclooxygenase and Topoisomerase Inhibitory compounds from *Apium graveolens* Linn. Seeds. Will be submitted to *J. Nat. Prod.*

INTRODUCTION

A review of the literature indicates that celery has been cultivated for the last 3000 years, notably in pharaonic Egypt, and it was known in China in the 5th century BC (Chevallier, 1998). Throughout history, celery has been used as a food, and at various times both the whole plant and the seeds have been taken medicinally.

The characteristic odor of celery essential oil is due to a series of phthalide derivatives (Bjeldanes and Kim, 1977). Sedanolide, sedanonic anhydride, 3-n-butyl phthalide, and other minor phthalides are reported to be the major constituent of celery seed oil (Lund, 1978). Celery seed or celery seed extracts are used as flavoring for preparing herbal combinations sold as dietary supplements and also in antirheumatic formulations. Celery seeds are also used specifically for arthritic pain relief (Chevallier, 1998). The seeds also have a reputation as a carminative with a mild tranquillizing effect (Chevallier, 1998). Today, celery seeds are used for treating rheumatic conditions and gout. They help the kidneys dispose of urates and other unwanted waste products, as well as working to reduce acidity in the body as a whole (Chevallier, 1998; Boulos, 1983). The seeds are useful in detoxifying the body and improving the circulation of blood to the muscles and joints (Houghton and Manby, 1985). Celery seeds have diuretic and antiseptic action (Boulos, 1983; Houghton and Manby, 1985; Chevallier, 1998). They are an effective treatment for cystitis, and to disinfect the bladder and urinary tubules. Celery seeds are beneficial for chest problems such as asthma, bronchitis and when used in combination with other herbs, help to reduce blood pressure (Chevallier, 1998; Styavati and Raina, 1976; Kiangsu, 1977). Some nitrogenous and non-nitrogenous portions of the

celery seed essential oil were separated and tested for their effects on central nervous system (CNS) (Kulshrestha et al., 1967; Kulshrestha et al., 1970; Satyavati and Raina, 1976). Only the nitrogenous part was found to have CNS activity. It provided protection against supramaximal seizure threshold test and has tranquillizing activity (Kulshrestha et al., 1967). Although celery seeds are often used in preparations for treating rheumatism, arthritis and as a sedative and spasmolytic, the German Federal Health Office's Commission E monograph does not recommend its use because of insignificant scientific proof of efficacy (Houghton and Manby, 1985). *A. graveolens* was assessed for its antiinflammatory activity on intact rats by measuring the suppression of carrgeenan-induced paw edema (Al-Hindawi et al., 1989). However, any link between known constituents in celery with the observed antiinflammatory activity was not established.

Much of the impetus for research on *A. graveolens* has been due to its role in traditional folk medicine. Most of these studies on *A. graveolens* seeds were focused primarily on the bioactivity of crude extracts. Therefore, the primary purpose of the current research was to isolate and identify the presence of biologically active compounds not previously examined. In this chapter, we report the cyclooxygenase and topoisomerase inhibitory and antioxidant activities of compounds **5-10** from *A. graveolens* seeds.

MATERIALS AND METHODS

General Experimental procedures

^{1}H- and ^{13}C NMR, DEPT, HMQC and HMBC spectra were recorded on Varian INOVA 300 and 500 MHz spectrometers. ^{13}C NMR and DEPT spectra were recorded at 75 and 126 MHz. Chemical shift were recorded in $CDCl_3$, CD_3OD and DMSO-d_6, and the values are reported in δ (ppm) based on δ residual of 7.24, 3.3 and 2.29 for ^{1}H NMR and 77, 49 and 39.5 for ^{13}C NMR for $CDCl_3$, CD_3OD and DMSO-d_6, respectively. Coupling constants J, are in Hz. EIMS were recorded at 70 eV. The silica gel used for VLC and MPLC was Merk Silica gel 60 (30-70 μm particle size). For preparative HPLC (LC-20, Japan Analytical Industry Co., Tokyo) purification, two JAIGEL-ODS, A-343-10 (20 mm x 250 mm, 10 μm, Dychrom, Santa Clara, CA) columns were used in tandem. Peaks were detected using a UV detector equipped with model D-2500 Chromato-integrator (Hitachi, Tokyo). The CD analysis of compounds **6** and **7** were performed on JASCO J-710 CD-ORD spectropolarimeter. Compounds **6** and **7** were dissolved in methanol separately, and CD was determined at 185-400 nm. All solvents were ACS reagent grade and were purchased from Aldrich Chemical Co., Inc.

Plant Material

Celery seeds were donated by Asgrow Seed Company, Kalamazoo, Michigan and stored at –20 ^{0}C until extraction.

Extraction and Isolation

The ground seeds (905 g) were extracted with hexane followed by MeOH to yield 20 g of MeOH extract. The MeOH extract (19 g) was stirred with hexane (2 x 400 mL, 15 min) to yield hexane soluble (7.4 g) and insoluble (11.5 g) fractions. The hexane soluble fraction (6 g) was further fractionated by VLC followed by MPLC on silica gel using hexane with increasing amount of acetone and finally with MeOH as eluting solvents. The fractions collected were: A (404 mg, hexane-acetone, 4:1, 250 mL); B (142 mg, hexane-acetone, 2:1, 80 mL); C (483 mg, hexane-acetone, 2:1, 100 mL); D (79 mg, hexane-acetone, 2:1, 120 mL); E (61 mg, hexane-acetone, 1:1, 100 mL); F (89 mg, hexane-acetone, 1:1, 200 mL); G (35 mg, hexane-acetone, 1:1, 100 mL); H (14 mg, 100% acetone, 20 mL); I (33 mg, 100% acetone, 130 mL), and J (8 mg, 100% MeOH, 250 mL). Topoisomerase and cyclooxygenase bioassays on fractions A-J revealed that fraction B was active. The bioactive fraction B (135 mg) was purified by preparative TLC (hexane-acetone, 20:1 x 3) to yield three major bands: I (48 mg, R_f 0.18); II (65 mg, R_f 0.35) and III (16 mg, R_f 0.50). Band II was biologically active and was further purified by repeated preparative TLC (hexane-ether 8:1 x 4 and 2:1 x 1) to yield a pure compound **5** (22 mg).

Hexane insoluble fraction (11 g) of the MeOH extract was separated into $CHCl_3$ soluble (2.1 g) and insoluble (8.2 g) fractions by stirring it with $CHCl_3$ (2 x 50 mL, 15 min). The $CHCl_3$ soluble fraction (2.1 g) was further separated into hexane soluble (607 mg) and insoluble (1.5 g) fractions. The hexane insoluble fraction was further separated into methanol-water (75:25 v/v) soluble and insoluble fractions by removing the

precipitate by centrifugation. The aqueous MeOH portion (684 mg) was purified by preparative HPLC using methanol-water (75:25 v/v) as the mobile phase at a flow rate of 3 $mLmin^{-1}$. Among the 8 fractions collected, the active fraction B (R_t = 48 min, 75 mg) was further purified by HPLC using methanol-water (60:40 v/v) as the mobile phase at a flow rate of 2 mL min^{-1}. This yielded a mixture of compounds **6** and **7** (R_t = 65 min, 12 mg) and a pure compound **8** (R_t = min, 8.5 mg). The mixture of compounds **6** and **7** was separated into pure compounds **6** and **7** by HPLC using H_2O-THF (90:10 v/v) as the mobile phase at a flow rate of 4 mL min^{-1}. The acetylation of **8** was performed according to the published procedure (Ramsewak et al., 1999a) to yield compound **11**.

The $CHCl_3$ insoluble fraction of MeOH extract of *A. graveolens* seeds (10.2 g) was partitioned into MeOH-water (75:25 v/v) soluble (8 g) and insoluble fractions (1.6 g) by removing the precipitate by centrifugation. The aqueous MeOH portion (7.5 g) was further fractionated by MPLC on C_{18} silica column. The fractions collected were: A (5.25 g, MeOH:H_2O, 50:50, 100 mL); B (1.59 g, MeOH:H_2O, 50:50, 130 mL); C (126.6 mg, MeOH:H_2O, 70:30, 160 mL); D (47.1 mg, MeOH:H_2O, 90:10, 60 mL); E (139.2 mg, MeOH:H_2O, 90:10, 120 mL); F (75.9 mg, MeOH:H_2O, 90:10, 100 mL) and G (161.7 mg, 100% MeOH, 225 mL). The bioactive fraction E was purified by preparative HPLC (LC-20, Japan Analytical Industry Co., Tokyo) using methanol-water (60:40 v/v) as the mobile phase at the flow rate of 3 mL min^{-1}. This yielded bioactive compound **10** (R_t = 54 min, 4.5 g). The MPLC fraction B (1.5 g) was separated into MeOH soluble (1.2 g) and insoluble (200 mg) fractions by centrifugation. The MeOH soluble fraction (1.2 g) was further fractionated into four fractions by MPLC on C_{18} silica column with MeOH-

H_2O (60:40, 540 mL) at 2 mL min^{-1} flow rate. Bioactive fraction four (125 mg) was purified by preparative HPLC using MeOH-H_2O (30:70 v/v) as the mobile phase at the flow rate of 3 mL min^{-1} to yield pure compound **9** (R_t = 84.8 min, 5.5 mg).

Compound 8. pale yellow oil; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.08 (m, 1H, H-3), 3.86 (dd, 1H, J=6, 2.1 Hz, H-8a), 3.88 (s, 3H, H-9), 4.23 (dd, 1H, J=9, 3 Hz, H-8b), 4.72 (d, 1H, J=4 Hz, H-2), 5.60 (b, 1H, NH, exchanged with D_2O), 6.80 (m, 1H, H-7), 6.85 (m, 1H, H-5), 6.88 (m, 1H, H-4); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 54.09 (C-3), 55.92 (C-9), 71.62 (C-8), 85.82 (C-2), 108.54 (C-4), 114.21 (C-5), 118.92 (C-7), 132.82 (C-3a), 145.16 (C-7a), 146.64 (C-6).

Acetate of 8 (11). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.04 (s, 3H, -OAc), 2.28 (s, 3H, -OAc), 3.08 (m, 1H, H-3), 3.84 (s, 3H, H-9), 3.92 (dd, 1H, J=9, 3 Hz, H-8a), , 4.26 (dd, 1H, J=9, 3 Hz, H-8b), 4.78 (d, 1H, J=4 Hz, H-2), 6.81 (m, 1H, H-7), 6.86 (m, 1H, H-5), 6.88 (m, 1H, H-4).

Compound 9. ^{1}H NMR (CD_3OD, 500 MHz): δ 7.69 (d, 1H, J=8 Hz, H-4), 7.34 (d, 1H, J=8 Hz, H-7), 7.18 (s, 1H, H-2), 7.10 (m, 1H, H-6), 7.04 (m, 1H, H-5), 3.85 (dd, 1H, J=9.5, 3.5 Hz, H-9), 3.52 (dd, 1H, J=15.5, 4.5 Hz, H- 8a), 3.15 (dd, 1H, J=15.5, 9.5 Hz, H-8b); ^{13}C NMR (CD_3OD, 126 MHz): δ 174.4 (C-10), 138.42 (C-7a), 128.51 (C-3a), 125.13 (C-2), 122.76 (C-6), 120.12 (C-5), 119.34 (C-4), 112.43 (C-7), 109.62 (C-3), 56.77 (C-9), 28.45 (C-8).

Compound 10. Yellow amorphous solid; ^{1}H NMR (CD_3OD, 300 MHz): δ 7.84 (1H, d, J=9 Hz, H-5'), 7.48 (1H, s, H-3), 6.84 (1H, d, J=3 Hz, H-8), 6.80 (1H, dd, J=9, 2.4 Hz, H-6'), 6.62 (1H, d, J=3 Hz, H-6), 6.53 (1H, d, J=2.4 Hz, H-2'), 5.45 (1H, d, J=3 Hz, H-1'''), 5.15 (1H, d, J=6 Hz, H-1''), 3.5-4.1 (11H, m, H-2", H-3", H-4", H-5", H-6", H-2''', H-4''', H-5'''), 3.34 (3H, s, H-7'); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 181.83 (C-4), 165.48 (C-4'), 164.35 (C-2), 162.58 (C-5), 161.08 (C-7), 156.85 (C-9), 148.42 (C-3'), 128.57 (C-1'), 116.62 (C-2'), 116.04 (C-5'), 110.21 (C-6'), 108.68 (C-1'''), 105.30 (C-10), 102. 73 (C-3), 99.26 (C-8), 98.19 (C-1"), 94.84 (C-6), 79.23 (C-3'''), 76.99 (C-3"), 76.76 (C-2'''), 76.02 (C-5"), 75.70 (C-2"), 73.94 (C-5'''), 69.90 (C-4"), 64.18 (C-4'''), 60.49 (C-6"), 55.90 (C-7').

Cyclooxygenase Inhibitory Assay

Pure compounds **5-11** were assessed for their cyclooxygenase inhibitory activity at 37 ^{0}C by monitoring the initial rate of O_2 uptake using an O_2 electrode (Instech Laboratories, Inc., 5209 Militia Road, Plymouth Meeting PA 19462-1216) (Laneuville et al., 1994; Meade et al., 1993). Cyclooxygenase-I (COX-I) activities were measured by using prostaglandin H synthase isozymes-I (PGHS-I) from ram seminal vesicles. Cyclooxygenase-II (COX-II) activities were measured by using human PGHS-II enzyme obtained from cloned insect cell lysate. Each assay mixture contained 600 µL of 0.1 M Tris buffer (pH 7), 1mM phenol, 100 µM arachidonic acid and 17 µg hemoglobin. Cyclooxygenase activities were initiated by the addition of 10 µL of COX-I or 20 µL of COX-II enzymes. Instantaneous inhibition was determined by measuring the cyclooxygenase activity initiated by adding aliquots of microsomal suspensions of

PGHS-I (10 μM O_2 min^{-1} cyclooxygenase activity/aliquot) to assay mixtures containing 10 μM arachidonate and 1-500 μg mL^{-1} concentrations of the test compounds. Ibuprofen and naproxen were assayed at their IC_{50} values, 2.52 μg mL^{-1} and 2.06 μg mL^{-1} concentrations, respectively.

Antioxidant Assay

Antioxidant bioassays were performed on crude extracts and pure compounds by analysis of liposome oxidation using fluorescence spectroscopy (Arora et al., 1998; Arora et al., 1997). A mixture containing 5 μM of 1-steroyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar lipids, Inc., Alabaster, AL) and 5 μM of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (molecular Probes, Inc., Eugene, OR) was dried in a foil-covered round-bottom flask under vacuum using a rotary evaporator at room temperature. MBSE buffer (500 μL), consisting of 0.15 M NaCl, 0.1 mM EDTA (Ethylenediaminetetraacetic acid di-sodium salt) and 10 mM MOPS [3-(N-morpholine propane sulfonic acid), adjusted to pH 7] was used to suspend this lipid film. Buffer solutions were treated with Chelex 100 chelating resin to remove trace metal ions. The lipid-buffer suspension was subjected to 10 freeze-thaw cycles using a dry ice-EtOH bath. After the last thaw, the lipid-buffer suspension was then extruded 29 times through a Liposo Fast extruder (Avestin, Inc., Ottawa, Canada) containing a polycarbonate membrane with a pore size of 100 nm to produce large unilamellar liposomes (LUVs). A 20 μL aliquot of this liposome suspension was diluted to a final volume of 2 mL in Chelex 100 treated HEPES buffer (100 μL, pH 7), 1M NaCl (200 μL), N_2-sparged water and DMSO solution containing the test compound (20 μL), vortexed, and placed in the

cuvette holder of the spectrophotometer. Peroxidation then was initiated by the addition of 20 μL of 0.5 mM $FeCl_2$ stock solution to achieve a final concentration of 5μM of Fe^{+2} in the presence of test compounds or crude extract dissolved in DMSO. The control sample did not contain either Fe^{+2} or the test compounds. The positive controls BHA, BHT, TBHQ, and α-tocopherol (Vitamin E) were all tested at a final concentration of 1.8, 2.2, 1.66 and 4.31 μg mL^{-1}, respectively. Fluorescence intensities of these liposome solutions were measured at an excitation wavelength of 384 nm for every 3 min over a period of 21 min using a Turner model 450 digital fluorometer (Barnstead Thermolyne, Dubuque, IA). The decrease of relative fluorescence intensity with time indicated the rate of peroxidation. Relative fluorescence was calculated by dividing the fluorescence value at a given time point (F_t) by that at t = 0 min (F_0).

Topoisomerase-I and –II Inhibitory Assay

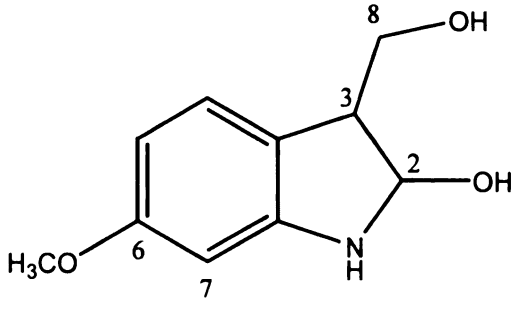
Compounds **5-7** were evaluated for topoisomerase-I and –II inhibitory activities according to the reported procedure (Chang et al., 1995). The *S. cerevisiae* mutant cell cultures, JN394, JN394t_{-1} and JN394t_{-2-5} used for the topoisomerase inhibitory assays were cultured in petri dishes containing YPDA medium (20 mL). Two to three mL of physiological saline solution was added to fully grown plate of each organism and then suspensions were diluted to obtain 5 x 10^6 CFU mL^{-1}. Bioassays were conducted by spreading 50 μL of the desired cell suspension to petri dishes of the YPDA media. DMSO or test compound dissolved in DMSO (20 μL) were spotted carefully on the bioassay plates at varying concentrations. The plates were allowed to dry in a laminar flow hood and then incubated at 27 ^{0}C for 72 h. The zone of inhibition was measured in

mm. Minimum inhibitory concentrations (MIC_{100}) for compounds **5-7** were determined according to the published procedure (Nair et al., 1989).

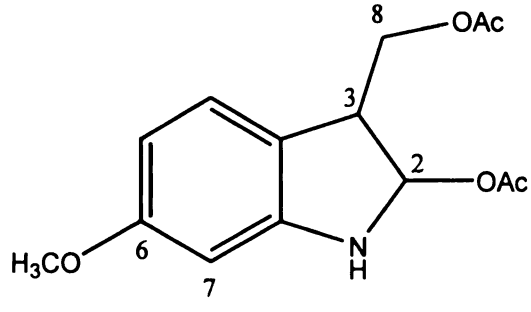
RESULTS AND DISCUSSION

Compounds **5-7** were isolated and characterized as described in **Chapter 3, Results and Discussion** section.

The structure of compound **8** was deduced by using ^{1}H and ^{13}C NMR, DEPT, HMQC, HMBC, IR and GCMS spectral techniques. ^{1}H NMR spectrum of compound **8** gave a singlet, integrated for three protons at 3.88 ppm, was corresponded to a methoxy group. A broad peak at 5.6 ppm was determined to be an –NH group, as indicated by the disappearance of this peak with D_2O shake in its ^{1}H NMR spectrum. The ^{13}C NMR spectrum of **8** revealed ten peaks, which were assigned to three aromatic carbons (δ 108.5, 114.2, 118.9), one methoxy carbon (δ 55.92), three quaternary carbons (δ 132.8, 145.2, 146.6), two CH (δ 85.8, 54.09) and one CH_2 (δ 71.6) as indicated by the DEPT spectrum of **8**.



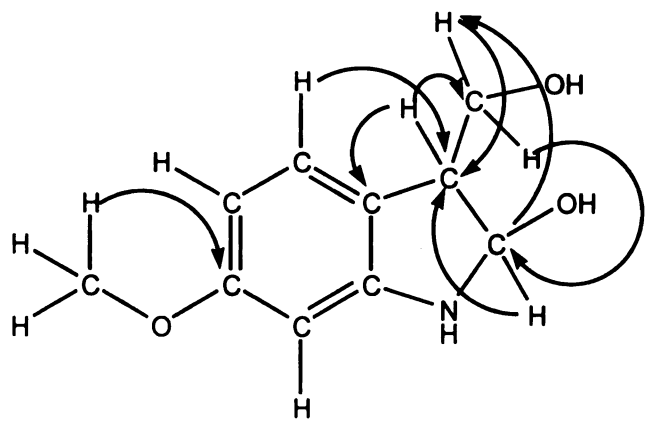
8



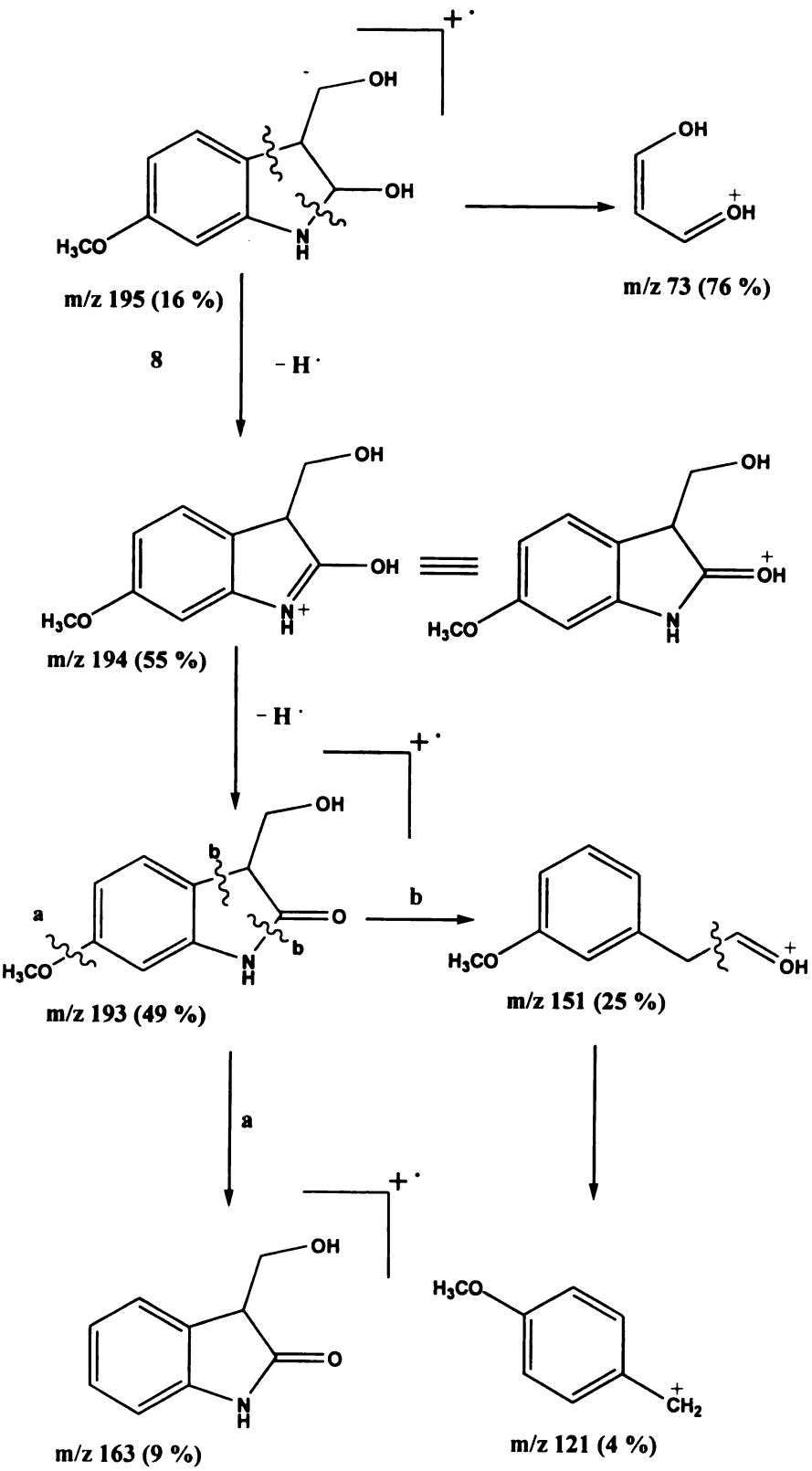
11

Both HMQC and HMBC spectral data provided confirmation of the structure proposed for compound **8**. The important connectivities in HMBC spectrum of **8** were those observed for C-3a and C-8 (**Fig. 4.1**). The signal for C-3a correlated to H-3 and H-2 signals while C-8 displayed cross signals to H-2. Also, the correlation of C-2 to H-8a and H-8b as well as the correlation of C-3 to H-8a, H-8b and H-2 supported the proposed structure (**Fig. 4.1**). Three methoxy protons showed correlation to C-6. The presence of the OH groups in **8** were suggested by a broad peak at 3372 cm^{-1} in its IR spectrum and confirmed by the formation of a di-acetate of **8**, compound **11**. The MS analysis of **8** gave a weak M^+ ion peak at m/z 195 (9 %). The fragmentation pattern of **8** under EIMS condition is shown in **Scheme-1**. The EIMS fragmentation pattern with ions at m/z 73, 151, 163, 193 and 194 was suggestive of the proposed structure for compound **8** (**Scheme-1**). The NMR analysis of the acetate of **8**, compound **11**, showed the presence of two acetoxy groups. The MS of **11** gave peaks at m/z 279 and 280 confirming the acetylation of two OH groups in **8**. Based on all spectral data of the natural product and its acetate, compound **8** was deduced to be 3-hydroxymethyl-6-methoxy-2,3-dihydro-1H-indol-2-ol. Compound **8** is a novel natural product.

Figure 4.1. HMBC connectivities in compound **8**

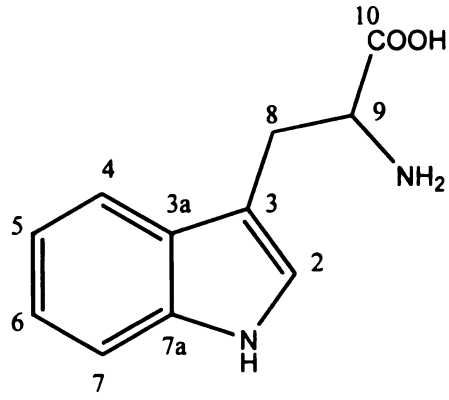


Scheme 1



The $CHCl_3$ insoluble fraction of MeOH extract of *A. graveolens* seeds was partitioned into MeOH-water (75:25, v/v) soluble and insoluble fractions by removing the precipitate by centrifugation. The aqueous MeOH portion was fractionated by MPLC in C_{18} silica column and seven fractions, A-G, were collected. The bioactive fraction E was further purified by preparative HPLC using MeOH-water, which yielded a bioactive compound **10**. The active MPLC fraction B was partitioned into MeOH soluble and insoluble fractions by centrifugation. The MeOH soluble fraction was further fractionated into four fractions by MPLC on C_{18} silica column with MeOH: water (60:40, v/v, 540 mL). Bioactive fraction IV was further purified by preparative HPLC using water-MeOH (70:30, v/v) as the mobile phase to yield a pure compound **9**.

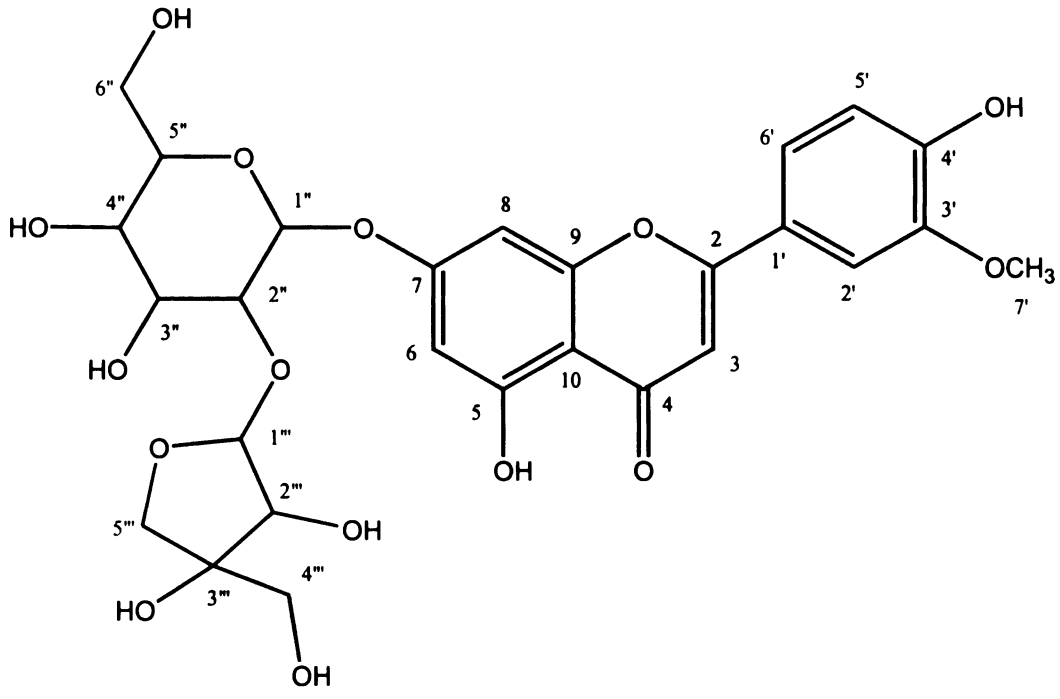
The structure of compound **9** was deduced by detailed analysis of the ^{1}H- and ^{13}C-NMR spectra. The ^{1}H NMR spectrum of **9** gave two doublets at δ 7.69 and 7.34 and two multiplets at δ 7.04 and 7.1 suggested a disubstituted aromatic ring in the molecule. A singlet at δ 7.18 correlated to a carbon peak at δ 125.13 in HMQC and the chemical shift of C-7a (δ 138.42) suggested the presence of heteroatom in a five-membered ring attached to an aromatic ring. A peak in the ^{1}H NMR spectrum of **9** at δ 10.82 in DMSO-d_6 which corresponded to a carbon peak at δ 174.4 in its ^{13}C NMR spectrum suggested the presence of a carboxylic acid group in the molecule. The presence of one CH_2 and one CH group next to each other was also confirmed by their splitting pattern in ^{1}H NMR spectrum, HMQC and HMBC correlation spectra. From the spectral data, compound **9** was characterized as L-tryptophan. ^{1}H- and ^{13}C- NMR spectra of compound **9** are in agreement with the reported values of L-tryptophan (Lee and Phillips, 1992; Goodnow et al., 1991; Morales-Rios et al., 1987).



9

Detailed analyses of the ^{1}H- and ^{13}C- NMR spectra helped to characterize the structure of **10**. In the ^{1}H NMR spectrum of **10**, a doublet at δ 7.84 and a doublet of doublet at δ 6.80 suggested that the B ring in **10** was substituted at C-3' and C-4'. The signal at δ 7.48, a singlet integrated for one proton, was assigned to H-3. Another three aromatic protons (δ 6.84, 6.62 and 6.53), all doublets with coupling constants of 3, 3 and 2.4 Hz, respectively, indicated a meta coupling and thus assigned to H-8, H-6 and H-2', respectively. Two doublets at 5.45 and 5.15 ppm were assigned to anomeric protons of fructose and glucose, respectively. The sugar identities were further confirmed by ^{13}C NMR experiments. ^{13}C NMR spectral data of compound **10** are in agreement with the published data for apiin (Forgacs et al., 1978), a related structure of **10**. The difference between apiin and compound **10** is the presence of a methoxy group at C-3'. This methoxy group appeared as a singlet at δ 3.34 in ^{1}H NMR spectrum corresponded to a peak at δ 55.9 in ^{13}C NMR spectrum. However, in the published report of apiin by Forgacs et al. (1978), the carbon assignments for C-7, C-8, C-9 and C-10 are incorrect. This is evident from the detailed published ^{13}C NMR values for other related phenolic glycosides (Ramsewak et al., 1999a). The compound **10** was identified as 7-[3-(3,4-dihydroxy-4-hydroxymethyl-tetrahydro-furan-2-yloxy)-4,5-dihydroxy-6-hydroxymethyl-

tetrahydro-pyran-2-yloxy]-5-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chromen-4-one. Compound **10** was reported earlier by Seshadri and Vydeeswaran (1971) and Farooq et al., (1953) from celery seeds. This is the first report of ^{1}H- and ^{13}C- NMR spectral data for 3'-methoxy apiin (**10**).



10

Compounds **5-11** were tested for their cyclooxygenase inhibitory activities using COX-I and –II enzymes at 500-50 mg/mL (**Fig. 4.2-4.8**). All test compounds demonstrated good COX-I and COX-II inhibitory activities at 500 mg mL^{-1}. There was no significant difference in COX-I and COX-II inhibition at 250, 100 and 50 mg mL^{-1} for compound **5**. Compounds **6** and **7** showed similar inhibitory activities on COX-I and –II enzymes at their respective concentrations. COX-I inhibition decreased with a decrease in concentration for both compounds, **6** and **7**. Compounds **8** and its acetate (**11**) demonstrated 57.1 and 31.6 % of COX-I inhibition and 54.2 and 31.8 % of COX-II

inhibition, respectively, when assayed at 500 mg mL^{-1}. The positive controls ibuprofen (2.06 μg mL^{-1}) and naproxen (2.52 μg mL^{-1}) showed 54.3 and 47.5 % of COX-I inhibition whereas 39.8 and 41.3 % of COX-II inhibition, respectively (**Fig. 4.2-4.8**). Also, compounds **9** and **10** showed 31 and 29 % COX-I inhibition as well as 32 and 21 % COX-II inhibition at 250 μg mL^{-1}, respectively.

Figure. 4.2. *In vitro* dose response of COX-I and COX-II inhibitory activities of compound **5**. Both ibuprofen and naproxen were tested at 2.06 and 2.52 μg mL^{-1}, respectively.

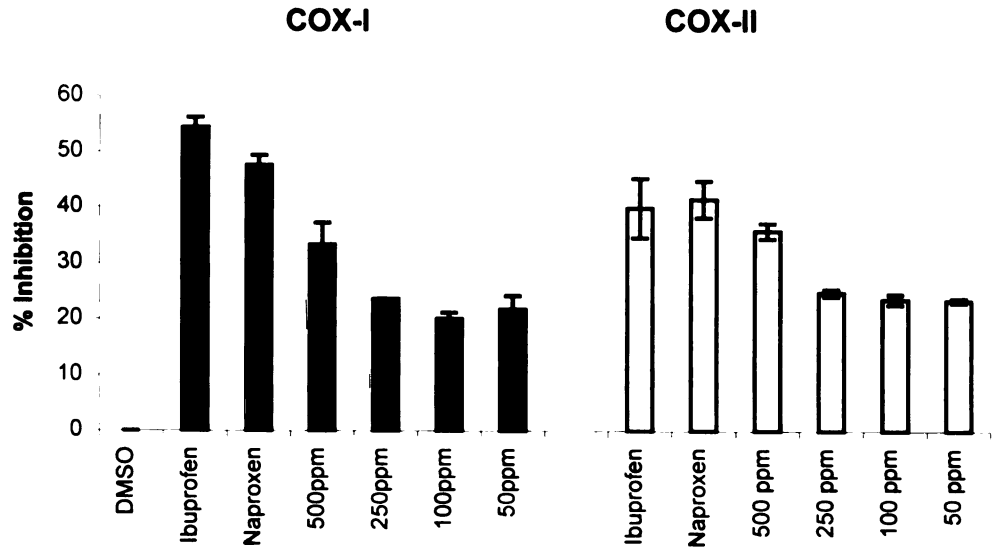


Figure. 4.3. *In vitro* dose response of COX-I and COX-II inhibitory activities of compound **6**. Both ibuprofen and naproxen were tested at 2.06 and 2.52 μg mL^{-1}, respectively.

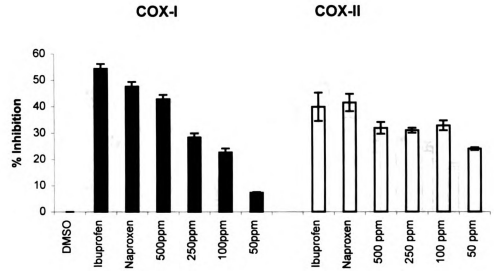


Figure. 4.4. *In vitro* dose response of COX-I and COX-II inhibitory activities of compound **7**. Both ibuprofen and naproxen were tested at 2.06 and 2.52 μg mL^{-1}, respectively.

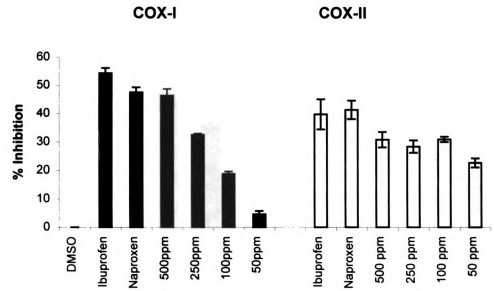


Figure. 4.5. *In vitro* dose response of COX-I and COX-II inhibitory activities of compound **8**. Both ibuprofen and naproxen were tested at 2.06 and 2.52 μg mL^{-1}, respectively. .

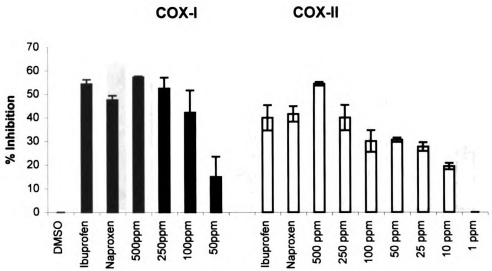


Figure. 4.6. *In vitro* dose response of COX-I and COX-II inhibitory activities of compound **9**. Both ibuprofen and naproxen were tested at 2.06 and 2.52 μg mL^{-1}, respectively.

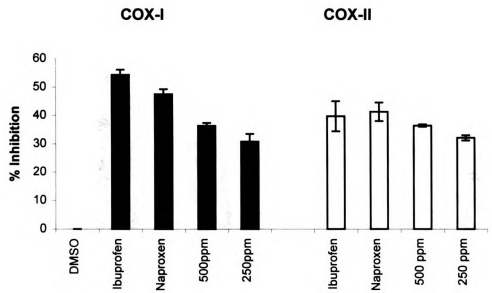


Figure. 4.7. *In vitro* dose response of COX-I and COX-II inhibitory activities of compound **10**. Both ibuprofen and naproxen were tested at 2.06 and 2.52 μg mL^{-1}, respectively.

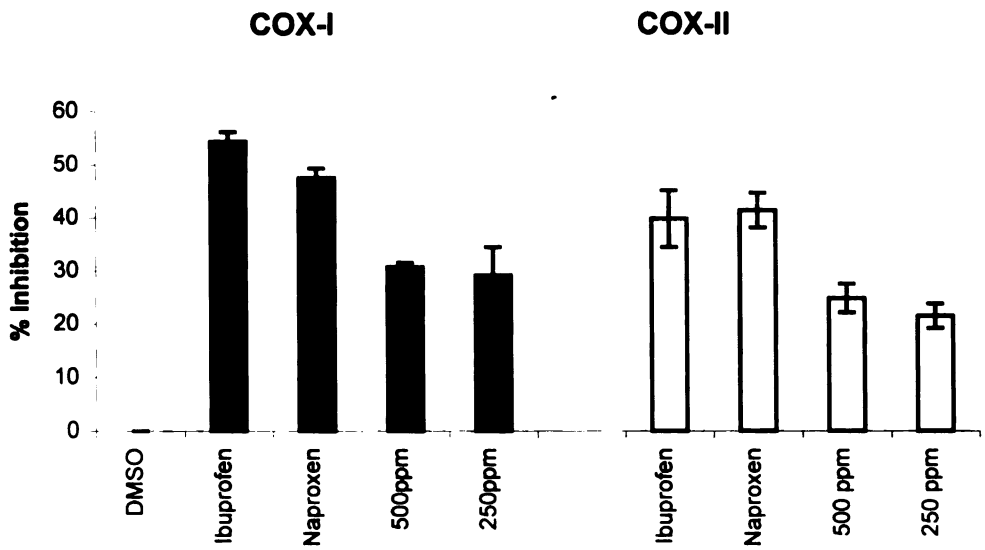
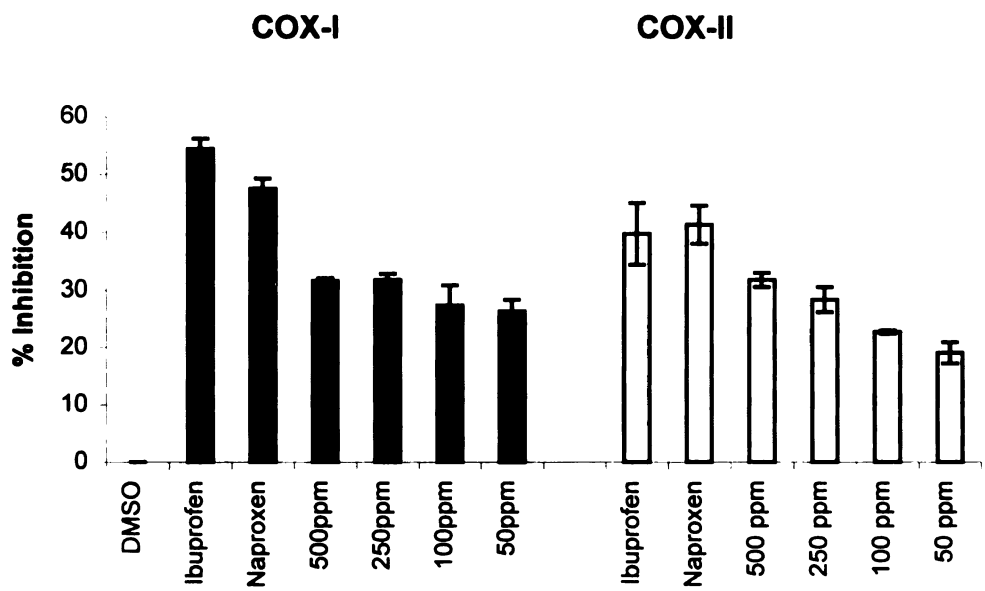


Figure. 4.8. *In vitro* dose response of COX-I and COX-II inhibitory activities of compound **11**. Both ibuprofen and naproxen were tested at 2.06 and 2.52 μg mL^{-1}, respectively.



Compounds **5-7** demonstrated topoisomerase-I and –II inhibitory activities when tested on *S. cerevisiae* mutant strains. *S. cerevisiae* mutant strains, JN394 is hypersensitive to topoisomerase-I poisons, while strain $JN394_{t-1}$ lacks the top-I gene and therefore shows a lack of response to topoisomerase-I poisons. The strain $JN394t_{-2-5}$ carries the top-II gene, which is resistant to topoisomerase-II poisons but responds to topoisomerase-I poisons. The MIC_{100} determination of compounds **5-7**, indicated that compound **5** completely inhibited topoisomerase-I and –II activities at 100 μg mL^{-1}, while **6** and **7** inhibited only topoisomerase-II enzyme at 200 μg mL^{-1}.

Compound **9**, L-tryptophan, exhibited very good antioxidant activity at 250, 125 and 50 μg mL^{-1}, respectively (**Fig. 4.11, 4.12**). Compound **10** also inhibited lipid peroxidation induced by Fe^{2+} at 250 and 125 μg mL^{-1} concentrations. The rest of the compounds isolated from *A. graveolens* seeds did not show significant antioxidant activities (**Fig. 4.11, 4.12**).

Earlier reports showed that the inhibitory effect of flavonoids and flavonoids glycosides on Fe^{2+} lipid peroxidation was attributed due to their ability to chelate Fe^{2+} with the formation of inert complexes that are unable to initiate peroxidation (Afanas'ev et al., 1989). Also, the Fe^{2+} complexes of flavonoids are considered to retain their free radical scavenging activities and therefore, can scavenge the free radical intermediate in lipid peroxidation (Wang et al., 1999). Therefore, the observed antioxidant activity of compound **10** suggests that it may function as an Fe^{2+} chelator as well.

Figure 4.9. Antioxidant activities of some commercial antioxidants. Data represent the mean of duplicate experiments.

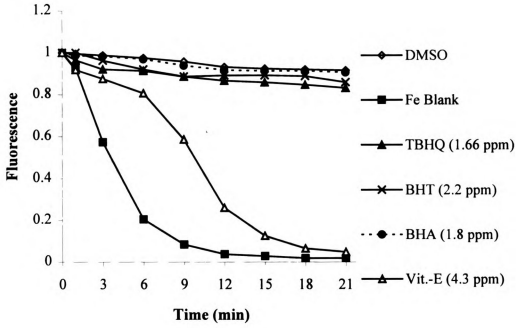


Figure 4.10. Antioxidant activity at 21 min for some commercial antioxidants. Data presented are the mean of duplicate experiments.

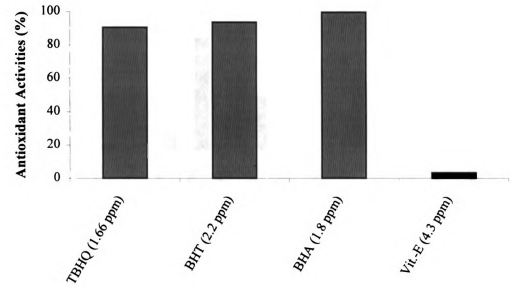


Figure 4.11. Antioxidant activities of compounds **5-11**. Data represent the mean of duplicate experiments.

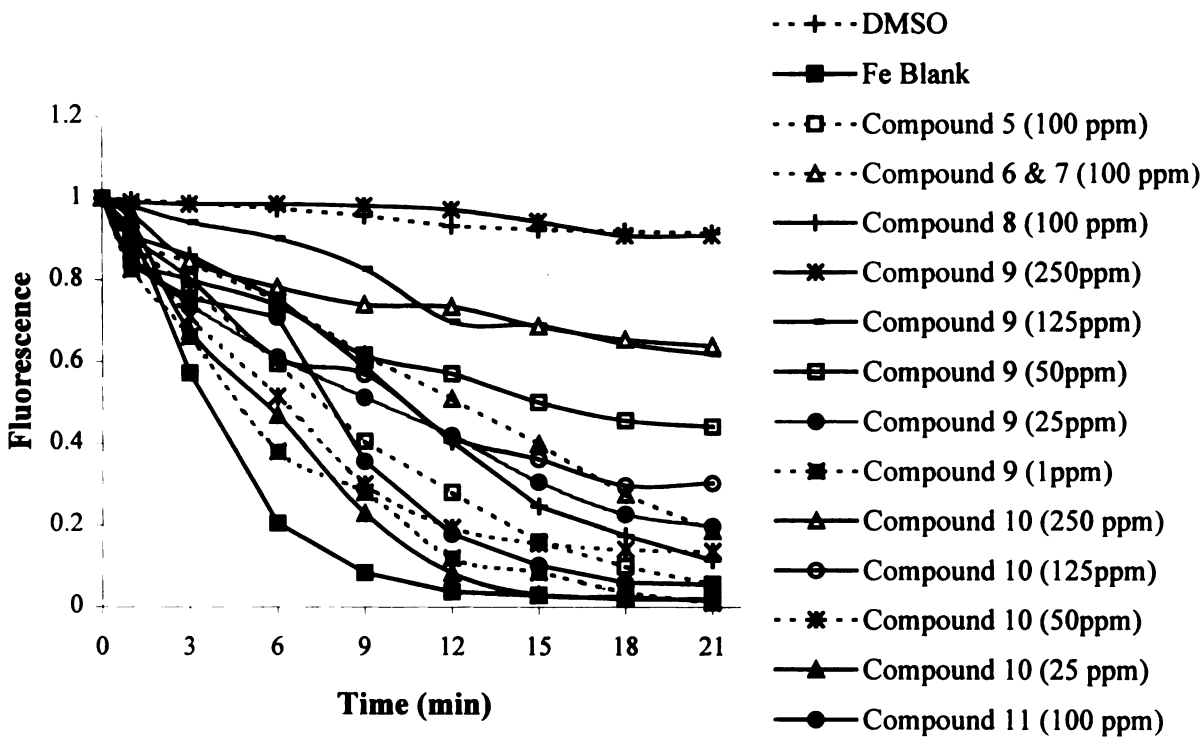
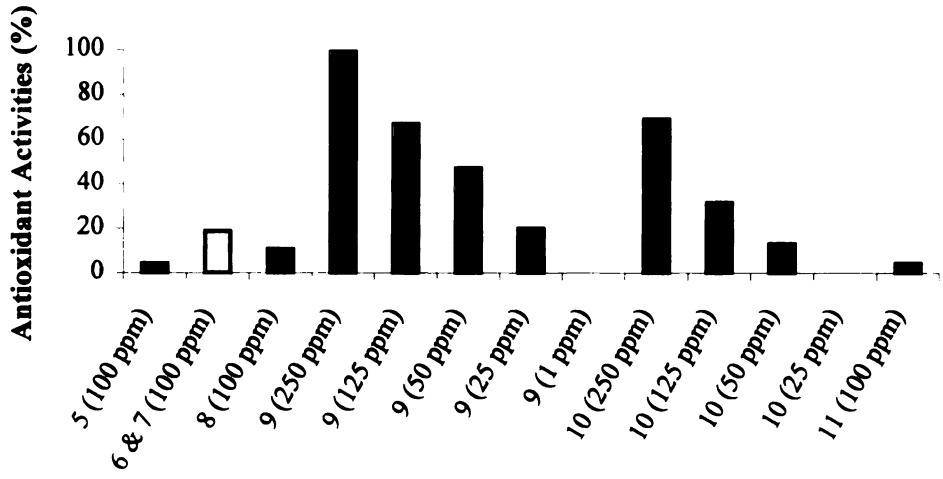


Figure 4.12. Antioxidant activity at 21 min for compounds **5-11**. Data presented are the mean of duplicate experiments.



Indole like compounds and indole alkaloids found in the plant kingdom have many constituents whose pharmacological properties are significant to mankind (Hutchinson, 1980). Several indole alkaloids were reported as antitumor agents (Wall et al., 1966; Yates et al., 1975). Tryptophan, a precursor for indole biosynthesis is well documented for its effect on central nervous system and for its therapeutic use in depression, mania and aggression (Young et al., 1984; Curzon, 1980). Seltzer et al. (1984) reported that the dietary supplementation of tryptophan combined with a high carbohydrate and reduced protein and fat diet resulted in a significant reduction in the pain intensity experienced by chronic pain patients. The observed biological activities of compounds **8** and **9** supported these findings. The present study also supports the medicinal use of celery seeds to alleviate gout and arthritic related pain.

Chapter 5

Summary and Conclusion

The celery seed oil is one of the important components used by flavor and fragrance industries. It is also known for its medicinal uses by the folklore in India and many parts of the world. For these reasons, the celery seed oil has been widely studied for its chemical composition. The research prior to our present study pertaining to phytochemistry and bioactivities of crude extracts and pure compounds from *A. graveolens* seeds was presented in **Chapter 1**. The Literature review summarized in **Chapter 1** is focused primarily on the biological activity of crude extracts of *A. graveolens* seeds. It was our hypothesis that *A. graveolens* seeds contain compounds with an array of biological activities. Our research therefore, has yielded a series of compounds with antioxidant, topoisomerase and cyclooxygenase inhibitory and other agricultural related bioactive compounds from *A. graveolens* seeds.

Bioassay directed isolation and purification of compounds from *A. graveolens* seeds are discussed in **Chapters 2-4**. The structures of purified celery seed compounds were determined by various spectral techniques such as ^{1}H- and ^{13}C- NMR, DEPT, HMQC, HMBC, FTIR and MS techniques. *A. graveolens* seeds were sequentially extracted with hexane and methanol. The separation and purification of hexane extract of *A. graveolens* seeds by various chromatographic techniques yielded three biologically active compounds, β-selinene (**1**), 3-*n*-butyl-4,5-dihydrophthalide (**2**), 5-allyl-2-methoxyphenol (**3**) and a triglyceride, 1,3-di[(cis)-9-octadecenoyl]-2-[(cis,cis)-9,12-octadecadienoyl]glycerol (**4**) (**Chapter 2**). Compounds **3** and **4** were isolated for the first

time from *A. graveolens* seeds. However, compound **4** was not active in our bioassays. Bioassay-directed isolation and purification of the methanolic extract of *A. graveolens* seeds afforded sedanolide (**5**), senkyunolide-N (**6**) and senkyunolide-J (**7**) (**Chapter 3**). Both of these compounds, **6** and **7**, were isolated for the first time from *A. graveolens* seeds. Their structures were elucidated by ^{1}H and ^{13}C- NMR spectral data and further confirmed by their CD spectra. A novel natural product, 3-hydroxymethyl-6-methoxy-2,3-dihydro-1H-indol-2-ol (**8**) and two additional compounds, L-tryptophan (**9**) and 3'-methoxy apiin (**10**), were also isolated and characterized from the methanolic extract of *A. graveolens* seeds (**Chapter 4**). Isolation and purification of these compounds were performed by MPLC and repeated preparative HPLC on C_{18} columns. The structure of compound **8** was deduced by using ^{1}H- and ^{13}C- NMR, DEPT, HMQC, HMBC, FTIR, and GCMS spectral techniques.

Mosquitocidal, nematicidal, antimicrobial, antioxidant, cyclooxygenase and topoisomerase inhibitory activities of these pure compounds were also discussed in **Chapters 2-4**. β-selinene (**1**), 3-*n*-butyl-4,5-dihydrophthalide (**2**) and 5-allyl-2-methoxyphenol (**3**) isolated from *A. graveolens* seeds, as oily compounds, demonstrated 100 % mortality on fourth-instar *A. aegyptii* mosquito larvae at 50, 25 and 200 μg mL^{-1}, respectively, in 24 h. Compound **2** inhibited the growth of *C. albicans*, *C. kruseii* and topoisomerase-I and –II enzyme activities at 100 μg mL^{-1}. Also, compound **2** displayed 100 % mortality on nematodes, *P. redivivus* and *C. elegans* at 12.5 and 50 μg mL^{-1}, respectively (**Chapter 2**).

Biologically active compounds, sedanolide (**5**), senkyunolide-N (**6**) and senkyunolide-J (**7**) were isolated and identified from methanolic extract of *A. graveolens* seeds as discussed in **Chapter 3**. Compounds **5-7** gave 100 % mortality at 25, 100 and 100 μg mL^{-1}, respectively, on the nematode, *P. redivivus*. Sedanolide (**5**) showed 100 % mortality at 50 μg mL^{-1} on nematode, *C. elegans*, and fourth-instar mosquito larvae, *A. aegyptii*. Also, it inhibited the growth of *C. albicans* and *C. parapsilasis* at 100 μg mL^{-1}.

3-hydroxymethyl-6-methoxy-2,3-dihydro-1H-indol-2-ol (**8**), L-tryptophan (**9**) and 3'-methoxy apiin (**10**) were identified from methanolic extract of *A. graveolens* seeds and evaluated for biological activities (**Chapter 4**). Cyclooxygenase and topoisomerase inhibitory and antioxidant activities for compounds **5-10** were discussed in **Chapter 4** as well. Compounds **5-10** displayed 24, 32, 36, 52, 31 and 29 % of COX-I inhibitory activity when assayed at 250 μg mL^{-1} at pH-7, respectively. Also, compounds **5-10** demonstrated 24, 29, 25, 40, 32 and 21 % of COX-II inhibition at pH-7 when assayed at 250 μg mL^{-1}, respectively. The acetylated product (**11**) of compound **8** also inhibited the COX-I and COX-II enzymes at 31 and 28 %, respectively, when tested at 250 μg mL^{-1} concentration. The activities of these compounds were compared with ibuprofen and naproxen at 2.06 and 2.52 μg mL^{-1}, respectively. A dose response study of **5-11** were carried out for the COX-I and COX-II inhibitory activities. Compounds **6-8** showed a dose response in COX-I inhibitory bioassay whereas compound **8** and its acetate (**11**) showed a dose response in COX-II assay.

All compounds isolated from methnol extract of *A. graveolens* seeds in my research were evaluated for topoisomerase-I and –II enzyme inhibitory activities.

Compound **5** exhibited topoisomerase-I and –II inhibitory activities at 100 μg mL^{-1} where as compounds **6** and **7** exhibited only topoisomerase-II enzyme inhibitory activities at 200 μg mL^{-1} when tested on *S. cerevisiae* mutant cell cultures. Compounds **5-11** were also evaluated for antioxidant activity. Only compounds **9** and **10** exhibited appreciable antioxidant activity at 125 and 250 μg mL^{-1} concentrations, respectively.

Topoisomerase inhibitory and antioxidant activities of crude extracts and pure compounds isolated from *A. graveolens* seeds supported the anecdotal claims such as the anticarcinogenic and chemopreventative properties of celery seeds. Compounds isolated from *A. graveolens* seeds showed comparable cyclooxygenase inhibitory activities with the commercially available drugs. Our *in vitro* results support the notion of the use of celery seeds in folk medicine to control gout related pain and in the reduction of inflammation. The use of *A. graveolens* seeds as food supplements or nutraceuticals would be desirable by the public, considering the current trend towards natural alternative medicines.

Mosquitocidal, nematicidal and antimicrobial affects of phthalides and a variety of natural products resulted from my research on *A. graveolens* seeds are new and therefore warrants further investigation. The crude extract or purified compounds from *A. graveolens* seeds have potential for pharmaceutical applications since they exhibited anticarcinogenic, antiinflammatory and antioxidant activities. Although, these compounds have potential as nematode managing agents, further examination is required for their efficacies under field conditions. Determination of mode of action and

appropriate formulations of celery seed extracts or seed powder would be worthwhile endeavors for additional research.

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