This is to certify that the
dissertation entitled

BIOACTIVE CONSTITUENTS IN WASABI (*WASABIA JAPONICA*) AND HORSERADISH (*ARMORACIA RUSTICANA*)

presented by

MARVIN J. WEIL

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in HORTICULTURE

Major Professor's Signature

8/16/03

Date

BIOACTIVE CONSTITUENTS IN WASABI (*WASABIA JAPONICA*) AND HORSERADISH (*ARMORACIA RUSTICANA*)

By

Marvin J. Weil

A DISSERTATION

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

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Department of Horticulture

2003

ABSTRACT

BIOACTIVE CONSTITUENTS IN WASABI (*WASABIA JAPONICA*) AND HORSERADISH (*ARMORACIA RUSTICANA*)

By

Marvin J. Weil

Wasabi (*Wasabia japonica*) and horseradish (*Armoracia rusticana*) are two glucosinolate-rich brassica species cultivated for their rhizomes, which are widely used as a condiment. In this study, a commercial wasabi powder, wasabi and horseradish rhizomes were investigated for the presence of bioactive constituents that may inhibit lipid peroxidation, cyclooxygenase-1 and -2 enzymes activity, and proliferation of human colon (HCT-116), breast (MCF-7), lung (NCI-H460), and CNS (Central Nervous System) (SF-268) cancer cells. The purification of the organic extracts led to the isolation of compounds **1**, **2**, **3**, **5**, **13, 14, 15** and compounds **1**, **3**, **5**, **7**, **9**, **10**, **16**, **17**, **18**, **19** and **20** from the wasabi powder and rhizomes, respectively. Similarly, compounds **3-12** were isolated from the horseradish rhizomes. The identity of these compounds was established as di-(2-ethylhexyl)phthalate (DEHP) (**1**), desulfosinigrin (DSS) (**2**), triglycerides (**3**), plastoquinone-9 (**4**), 3-acylsitosterols (**5**), 6-O-acyl-β-D-glucosyl-β-sitosterols (**6**), 1,2-dilinolenoyl-3-β-galactosylglycerol (**7**), sucrose (**8**), β-sitosterol (**9**), sinigrin (**10**), gluconasturtiin (**11**), phosphatidyl choline (**12**), a mixture of fatty acids (**13**), a mixture of methyl linolenate and methyl oleate (**14**), sitosterol 3-O-glucoside (**15**), α-tocopherol (**16**), ubiquinone-10 (**17**) linolenoyloleoyl-3-β-galactosylglycerol (**18**), L-tryptophan (**19**) and 1,2-dipalmitoyl-3-galactosylglycerol (**20**) by GC-MS, 1H- and 13C-NMR spectral experiments.

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Desulfosinigrin (**2**) promoted the growth of HCT-116 (colon) human cancer cells in a concentration-dependent manner by 27, 42, 69 and 99.5% at 3.72, 7.50, 15 and 60 ppm, respectively. For NCI-H460 human lung cancer cells, DSS at 60 ppm increased the cell number by 20%. Plastoquinone-9 (**4**) showed 28% inhibition of COX-1 enzyme at 60 ppm and a trend towards cancer cell proliferation for all cancer cell lines tested except for SF-268 at 3.75-30 ppm. 6-O-acyl-β-D-glucosyl-β-sitosterols (**6**) inhibited COX-1 by 32%. 1,2-dilinolenoyl-3-galactosylglycerol (**7**) showed 75% inhibition of COX-1 at 250 ppm and inhibited the proliferation of colon cancer (HCT-116) cells by 21.9, 42.9, 51.2 and 68.4% and of NCI-H460 by 30.0, 38.6, 44.2 and 70.5% at 7.5, 15, 30, and 60 ppm, respectively. Sinigrin (**10**) inhibited lipid peroxidation by 71% at 250 ppm. A mixture of fatty acids (**13**) inhibited COX-1 and COX-2 enzymes by 77 and 93%, respectively, at 250 ppm. A mixture of methyl linolenate and methyl oleate (**14**), inhibited COX-1 and COX-2 by 23 and 57%, respectively, at 250 ppm. Both linolenoyloleoyl-3-β-galactosylglycerol (**18**) and 1,2-dipalmitoyl-3-β-galactosylglycerol (**20**) inhibited COX-1 enzyme by 45% at 250 ppm.

These results represent the first report of the tumor cell proliferating activity by a desulfoglucosinolate (**2**). Furthermore, these results represent the first report of the isolation of monogalactosyl diacyl glycerides (MGDGs **7**, **18**, and **20**) with selective COX-1 enzyme inhibitory activity from both wasabi and horseradish rhizomes.

TO MY WIFE AND CHILDREN

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Muraleedharan G. Nair for giving me the opportunity to be a part of his research program, for the financial support in form of a Graduate Assistanship, and also for his kind guidance and friendship.

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I would like to express my thanks to the members of my advisory committee, Dr. Leslie D. Bourquin, Dr. Gale M. Strasburg, and Dr. Robert E. Schutzki for their time, suggestions, and encouragement.

My gratitude goes out to all past and current members of the Bioactive Natural Products and Phytoceuticals Laboratory, who helped me during the realization of my studies, especially to Dr. Yanyung Zhang for the cancer cell experiments.

Special thanks also to Dr. James R. Miller and to Piera Y. Giroux for their invaluable help with the gas chromatographic-mass spectrometric analyses.

For all their love, encouragement, and patience, I thank my wife, Martha, and our children, Melissa and Aldo, to whom I dedicate this dissertation.

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BPDI
CGas
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COX
COX-
CYP-
d
D_2O
dd
DEHE
d-GSI
DMF
DMSO
DNA
DSS
EDTA
GC-M
GSLs
GST
GTP
H_2O
H_2O_2
HAs
HCT-
HPLC
HTCs
J
L
LP
LUVs
m
MeOH
MGDG

KEY TO ABBREVIATIONS

13C-NMR	Carbon Nuclear Magnetic Resonance
1H-NMR	Proton Nuclear Magnetic Resonance
AITC	Allyl glucosinolate
AT	Acetyltransferase
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BITC	Benzyl isothiocyanate
BPDE	(+)-anti-benzo[a]pyrene-diol-epoxide
CGase	Cysteinylglycinase
$CHCl_3$	Chloroform
CNS	entral Nervous System
COX-1	Cyclooxygenase-1 enzyme
COX-2	Cyclooxygenase-2 enzyme
CYP450	Cytochrome P450 enzymes
d	Doublet
D_2O	Deuterium oxide
dd	Doublet of doublet
DEHP	Di-(2-ethylhexyl)phthalate
d-GSLs	Desulfoglucosinolates
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Desulfosinigrin
EDTA	Ethylendiaminetetracetic acid
GC-MS	Gas chromatography-Mass spectroscopy
GSLs	Glucosinolates
GST	Glutathione S-transferase
GTP	Glutamyltranspeptidase
H_2O	Water
H_2O_2	Hydrogen peroxide
HAs	Heterocyclic amines
HCT-116	Human colon carcinoma
HPLC	High-pressure liquid chromatography
ITCs	Isothiocyanates
J	Coupling constant
L	Liter
LP	Lipid peroxidation
LUVs	Large unilamellar vesicles
m	multiplet
MeOH	Methanol
MGDGs	Monogalactosyl diacylglycerides

MNN
MPL
MTI
NADI
NCI
NMR
NNK
PAHs
PAPS
PBIT
PEIT
PGHS
PHIT
PITC
PMI
PPIT
ppm
R.
RNA
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RPM
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TBH
TLC
TXA
UDP

MNNG	N-methyl-N-nitroso-N-nitroguanidine
MPLC	Medium-pressure liquid chromatography
MTT	3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NADPH	Nicotineamide-adenine dinucleotide phosphate, reduced form
NCI	National Cancer Institute
NMR	Nuclear magnetic resonance
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PAHs	Polycyclic aromatic hydrocarbons
PAPS	3`-phosphoadenosine 5`-phosphosulphate
PBITC	4-Phenylbutyl isothiocyanate
PEITC	Phenylethyl isothiocyanate
PGHS-2	Prostaglandin endoperoxide H synthase-2
PHITC	6-Phenylhexyl isothiocyanate
PITC	Phenyl isothiocyanate
PMITC	Phenylmethyl isothiocyanate
PPITC	3-phenylpropyl isothiocyanate
ppm	parts per million
R_f	Reference value
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute-1640 medium
s	Singlet
spp	Species
t	Triplet
TBHQ	tert-Butylhydroquinone
TLC	Thin-layer chromatography
TXA_2	Thromboxane A_2
UDPG	Uridine diphosphate glucose

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INTRODUCTION

In the United States, some 11 million tons of ground roots of horseradish (*Armoracia rusticana*) are used yearly to manufacture 6 million gallons of relishes (HIC, 2003). Since only about 3000 acres are grown in the USA, horseradish must be imported to meet the demand. For the year 2002, US imports of horseradish reached close to 2 million tons (FOT, 2003). Furthermore, horseradish is used as a substitute of wasabi (*Wasabia japonica*) due the scarcity of the latter (OSU, 2000). Consumer preference has raised the demand for the formerly unknown wasabi, once considered an exotic crop in the USA. Exposure of the public to crops such as pak-choi, bamboo shoots, daikon radish and wasabi, and continued immigration from Asian countries have contributed to the high demand for these products (Longbrake et al., 2003).

The State of Michigan ranks second in the USA with regards to agricultural diversity, producing more than 125 commodities on a commercial basis. Agriculture is the second largest industry in the state. Its yearly contribution to Michigan's economy is almost $37 billion (MDA, 2003). In 2001, the State of Michigan announced a grant to develop or enhance specialty crop markets (MDA, 2001) and in 2003, through the Federal-State Marketing Improvement Program, it allocated funds to enhance the state's marketing of agricultural products. One of the main areas of interest of this program was to foster "agricultural diversity through new or niche markets, value-added products, or those that help achieve long-term sustainability of the environment and rural communities." (MDA, 2002). Horseradish and wasabi have been placed on the List of

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Alternative Crops and Enterprises by the Alternative Farming Systems Information Center at the National Agricultural Library (USDA) as an option for diversification, especially for small farmers (AFSIC, 2001).

The use of wasabi in traditional Japanese dishes, its increased consumption in other countries, including the USA, and loss of production areas in Japan have contributed to high market demand and prices for wasabi (Miles and Chadwick, 1996). Both horseradish and wasabi can be consumed either fresh or processed and both have the potential to become an alternative crop for vegetable growers to improve their revenues. Both species are sources of bioactive compounds with many potential uses as a dietary supplement. The determination of these potential uses, especially agronomical, nutritional, and public health applications, becomes a critical part of the process of adding value to any edible plant. To achieve this goal, the elucidation of the bioactive compounds is also critical.

Glucosinolates are probably the only class of bioactive compounds investigated in both wasabi and horseradish. There are several reports that indicate that consumption of glucosinolates from brassica spp. has many health benefits. *A. rusticana* and *W. japonica* are mainly used for their characteristic flavor and a great deal of attention has been devoted to the determination of the properties of the compounds that cause it. The hypothesis of the present study is that both *A. rusticana* and *W. japonica* have the potential to yield value-added bioactive compounds including glucosinolates. Therefore, this study will be focused on the components present in wasabi and horseradish that have potential benefits to human health. For this, lipid peroxidation, cyclooxygenase, and

cancer cell growth inhibitory assays will be used. Thin-layer (TLC), medium-pressure (MPLC) and high-pressure (HPLC) liquid chromatography will be used to purify the bioactive compounds. Gas-chromatography-Mass spectroscopy (GC-MS) and nuclear magnetic resonance (NMR) will be used to elucidate the structures of the pure compounds of interest.

This dissertation comprises five chapters detailing the research results. Chapter One is a literature review in which the botany, chemical constituents, biological activities and uses of wasabi and horseradish are detailed. In Chapter Two, the results of a preliminary evaluation of different extraction protocols on the bioactivity of the extracts are presented. In Chapter Three, the isolation, characterization, and biological activity of two compounds isolated from wasabi rhizomes and powder are discussed. Chapter Four presents all of the compounds isolated from horseradish rhizomes, wasabi rhizomes and wasabi powder, respectively, along with the methods used for their isolation and characterization and the results of the evaluation of their lipid peroxidation, cyclooxygenase, and cancer cell growth and tumor proliferation inhibitory activities. The data presented in Chapters Three and Four has been submitted for publication to peer-reviewed journals and it has been arranged accordingly as manuscripts with sections entitled abstract, introduction, materials and methods, and results and discussion. In Chapter Five a summary and the conclusions are presented.

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CHAPTER ONE

LITERATURE REVIEW

Wasabi: Botany, cultivation and uses

Wasabi (*Wasabia japonica*, Miq. Matsum, syn. *Wasabia pungens*, *Eutrema wasabi* (Sieb.) Maxim., *Cochlearia wasabi* Sieb. *Lunaria japonica* Miq., *Wasabia pungens* Matsum. *Alliaria wasabi*, *Wasabia wasabi* (Sieb) Makino), a perennial herb cultivated as a root crop, belongs to the Brassicaceae and originated in Eastern Asia.

Kingdom	Plantae-Plants
Subkingdom	Tracheobionata-Vascular plants
Superdivision	Spermatophyta-Seed plants
Division	Mangoliophyta-Flowering plants
Class	Manoliopsida-Dicotyledons
Subclass	Dilleniidae
Order	Capparales
Family	Brassicaceae-Mustard family
Genus	Wasabia

Botanical classification of *W. japonica* (USDA, 2002).

The economically important part of the plant is actually not the root, but the rhizome, a thickened stem connected to the much thinner actual roots. The aerial parts of the plant consist of thin, elongated petioles and heart-shaped leaves. To a lesser extent, the aerial parts are also used (Chadwick et al., 1992). In Eastern Asia, especially in Japan, it is cultivated under shadow in inundated soils at temperatures between 8 and 18 ^{o}C, or in mounds or terraces set in shallow waters at temperatures between 11 and 14 ^{o}C.

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The quality of aquatic wasabi is said to be much higher (Sultana et al., 2003). However, its cultivation is costly since the water must have good aeration and a narrow temperature range to maintain an optimum oxygen supply to the roots. (Chadwick et al., 1992).

Figure 1.1: Wasabi plant (Shizuokanet, 2003).

Wasabi has been cultivated in New Zealand and Tasmania, where both the aquatic and terrestrial varieties are grown (Barber and Buntain, 1997; Sultana, 2002; New Zealand Wasabi Limited, 2003). In the USA, it is grown hydroponically (Pacific Farms 2003) and in soil (The Frog Farm, 2003). *W. japonica* is reproduced vegetatively or by seeds. Seeds should not be dried below 30% moisture or frozen for storage in order to keep them viable. Before germination, seeds undergo a period of dormancy for up to eight months (Nakamura and Sathiyamoorthy, 1990). Wasabi plants reach a height of about two feet and can be spread by two feet. The rhizomes are whitish-green both externally and internally, about one inch thick and up to seven inches long. Cultivation of wasabi probably began before the 10^{th} century (Chadwick et al., 1992). Wasabi is

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used in traditional Japanese foods such as sashimi (sliced raw fish and shellfish), sushi (rice ball covered with raw fish or shellfish), and tataki (minced raw fish) (Hasegawa et al., 1999).

Current Japanese production of wasabi is about 5000 tons (fresh weight) per year and about 50% is aquatic (Barber and Buntain, 1997).

Horseradish: botany, cultivation, and uses

Horseradish has been classified as *Armoracia rusticana* P. Gaertn. B. Mey. & Scherb, *Cochlearia armoracia* L., *Cochlearia rusticana*, Lamarck, *Armoracia sativa*, Heller, *Nasturtium armoracia* (L.) Fries, *Radicula armoracia* (L.) B. L. Robinson, *Rorippa armoracia* (L.) A. S. Hitche., and *Armoracia lapathifolia* Gilib (Simon et al., 1984). The commonly used classification is *A. rusticana*. It is a hardy perennial herb cultivated as a root crop. It originated in Europe and Asia and has adapted to North America, where it has become relatively common. Horseradish has been cultivated in Europe since ancient times. It was known to the ancient Greek as a medicinal herb and it is also one of the five bitter herbs used by the Jews during Passover as part of their religious observance. Horseradish was listed as *R. rusticanus* in the Materia Medica of the London Pharmacopeia in the eighteenth century. It has been used as a stimulant of the nervous and digestive systems, as aperient (mildly laxative), rubefacient, diuretic, expectorant, antiseptic, vomitive, vermifuge, and diuretic. It was also used for the treatment of asthma, calculus, scurvy, cystitis, chronic rheumatism, sciatica, gout, joint-

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ache or hard swellings of the spleen and liver and dropsy, and to remove freckles (Grieve, 1931; Crouse et al., 2003).

Kingdom	Plantae-Plants
Subkingdom	Tracheobionata-Vascular plants
Superdivision	Spermatophyta-Seed plants
Division	Mangoliophyta-Flowering plants
Class	Manoliopsida-Dicotyledons
Subclass	Dilleniidae
Order	Capparales
Family	Brassicaceae-Mustard family
Genus	Armoracia

Botanical classification of *A. rusticana* (USDA, 2002).

Temperatures between 5 and 19 °C, deep, moist, and well-drained soils, rich in organic matter and with pH between 5.0 and 7.5 have been reported as ideal conditions for the growth of horseradish plants. Horseradish plants may reach an average height of about two to three feet at the time of flowering. It is propagated from side roots, since it rarely produces seeds. In North America, commercial cultivation of horseradish began in the Midwest around 1850, expanding from Illinois and Wisconsin to Northern California (HIC, 2003). It is generally cultivated as an annual crop and harvest time is usually between October and November (Simon, 1984). Twenty-centimeter-long root pieces are used for propagation, which usually occurs between February and March (Huxley, 1992). The rhizomes are whitish-yellow on the outside, white on the inside, and they reach a thickness of up to two inches at varying lengths. Horseradish rhizomes are a commercially important source of peroxidase, a glycoprotein commonly used as a reagent

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for clinical diagnosis and analytical immunoassays (Mano, 2001). The peroxidase is usually extracted with water and further purified (Yuan and Jiang, 2003).

Figure 1.2: Horseradish rhizomes (Katzer, 2003).

Both wasabi and horseradish are harvested for their rhizomes, which, upon crushing, develop a characteristic pungent odor and taste. Wasabi is mainly used as a condiment, either in fresh form or as a dry powder, for sushi and other seafood dishes in the Japanese cuisine, while horseradish is usually used as a fresh condiment or as an ingredient in relishes. It has been established that the compounds responsible for the pungency of wasabi, horseradish and other members of the genus Brassica (e.g. cabbage, Brussels sprouts, cauliflower, broccoli, mustard, and others) are generated *in situ* from the class of compounds known as glucosinolates by the action of the enzyme myrosinase (Fenwick et al., 1983). In general, products of hydrolysis are pungent, bitter, and possess an acrid smell.

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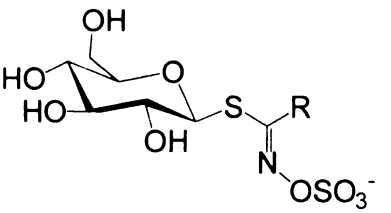
Glucosinolates and isothiocyanates in wasabi and horseradish

Glucosinolates are not exclusively produced by the family Brassicaceae (formerly Cruciferaceae). Glucosinolates have been reported from several hundred species specially in genera belonging to the Capparaceae and the Caricaceae (Rodman, 1981), some of which are cultivated as feed, vegetables, oil seeds and condiments. Table 1.1 lists some common glucosinolate-containing brassica vegetables.

The content of glucosinolates in the brassica vegetables averages between 500 and 2000 μg/g, but the values can largely vary depending on the varieties used and the growing conditions. (Fenwick et al., 1983). Glucosinolates are β-thioglucoside of N-hydroxysulfates whose correct structure was proposed in 1956 (Fahey et al., 2001). Most commonly, glucosinolates are present as the potassium salt.

As a chemical class of compounds, glucosinolates share the structural features shown below:



Glucosinolates possess a sulfur-linked β-D-glucopyranose and an amino acid-derived side chain. Structurally, the N-side chains can be diverse. More than 120 glucosinolates have been identified so far. Among these, straight and branched aliphatic,

ω-methylthioalkyl, aromatic and heterocyclic side chains are the most common. Figure 1.3 shows some of the most common glucosinolates from plants.

Table 1.1: Common brassica vegetables (Fenwick et al., 1983).

Genus	Species	Common name
Armoracia	Rusticana	Horseradish
Brassica	Campestris	Turnip
	Chinensis	Pak choy
	Juncea	Brown mustard
	Napus	Rape, swede, rutabaga
	Nigra	Black mustard
	Oleracea	Cabbage, kale, Brussels sprouts, cauliflower, broccoli, kohlrabi
	Pekinensis	Chinese cabbage
Lepidium	Sativum	Garden cress
Nasturtium	Officinale	Watercress
Raphanus	Sativus	Radish
Sinapis	Alba	Mustard

Nonetheless, most glucosinolates are classified into few groups, such as alkyl/alkenyl, aromatic, and indole glucosinolates. Biosynthetically, only seven glucosinolates are considered as directly derived from one of the protein-building amino acids (tryptophan, valine, alanine, leucine, isoleucine, tyrosine and phenylalanine) (Shapiro et al, 2001).

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(2-hydro
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(3-indol
HO
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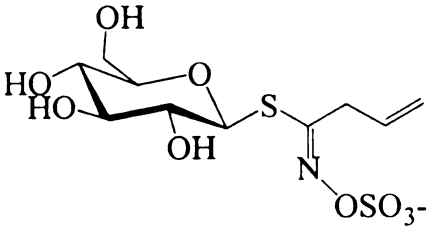
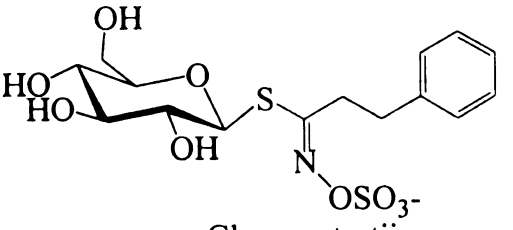
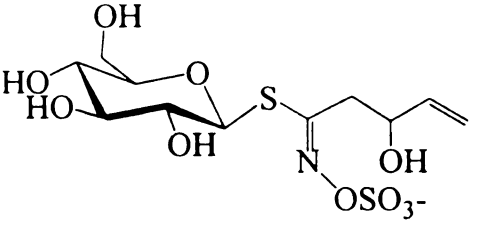
Fig



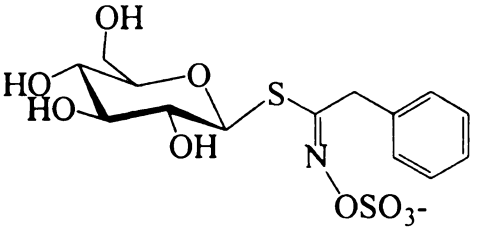
Sinigrin
(2-propenyl glucosinolate)



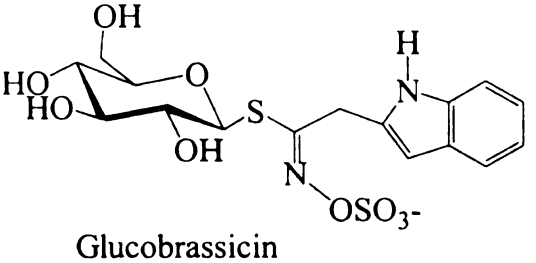
Gluconasturtiin
(2-phenylethyl glucosinolate)



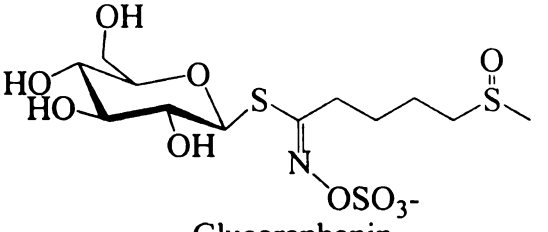
Progoitrin
(2-hydroxy-3-butenyl glucosinolate)



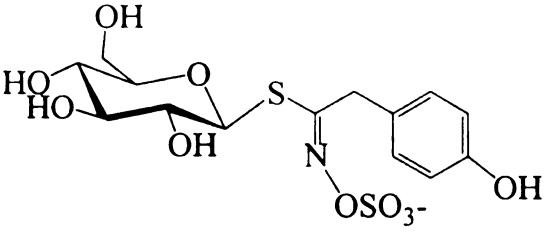
Glucotropaeolin
(phenylmethyl glucosinolate)



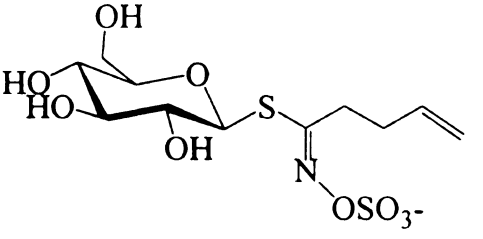
Glucobrassicin
(3-indolylmethyl glucosinolate)



Glucoraphanin
(4-methylsulfinylbutyl glucosinolate)



Glucosinalbin
(phenylmethyl glucosinolate)



Gluconapin
(3-butenyl glucosinolate)

Figure 1.3: Structures of glucosinolates commonly found in brassica vegetables.

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Biosynthesis of glucosinolates

A schematic representation of the biosynthesis of glucosinolates is shown in Figure 1.4. The first stage in the biosynthesis of the glucosinolates is the enzymatic conversion of the corresponding amino acid to an oxime (Du et al., 1995). Cysteine acts as donor of the thiol group to an aci-nitro intermediate to yield an S-alkylthiohydroximate, which is, in turn, cleaved to form a thiohydroximate. The thiohydroximate is then glucosylated by UDPG-thiohydroximate glycosyltransferase to yield a desulfoglucosinolate (Halkier, 1999; Reed, et al., 1993), which is sulphated by desulfoglucosinolate sulfoglucotransferase, a 3'-phosphoadenosine 5'-phosphosulphate (PAPS) (Jain et al., 1990; Dewick, 1998).

Some possible steps leading to structurally different glucosinolates are: 1) chain elongation of amino acids, 2) oxidation of the side chain sulfur to sulfonyl and sulfinyl after glucosinolate biosynthesis, 3) detachment of the ω-sulfinyl group to form a terminal double bond, 4) hydroxylation and 5) methoxylation (Rossiter et al, 1990).

Both wasabi and horseradish produce methylsulfinylalkyl glucosinolates, although only wasabi appears to synthesize ω-methylthioalkyl glucosinolates. The latter have been linked to a characteristic "wasabi flavor" (Ina et al., 1989a; Grob and Matile, 1980). Table 1.2 lists isothiocyanates isolated from ether extracts of wasabi and horseradish (Etoh et al., 1990).

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Figure 1.

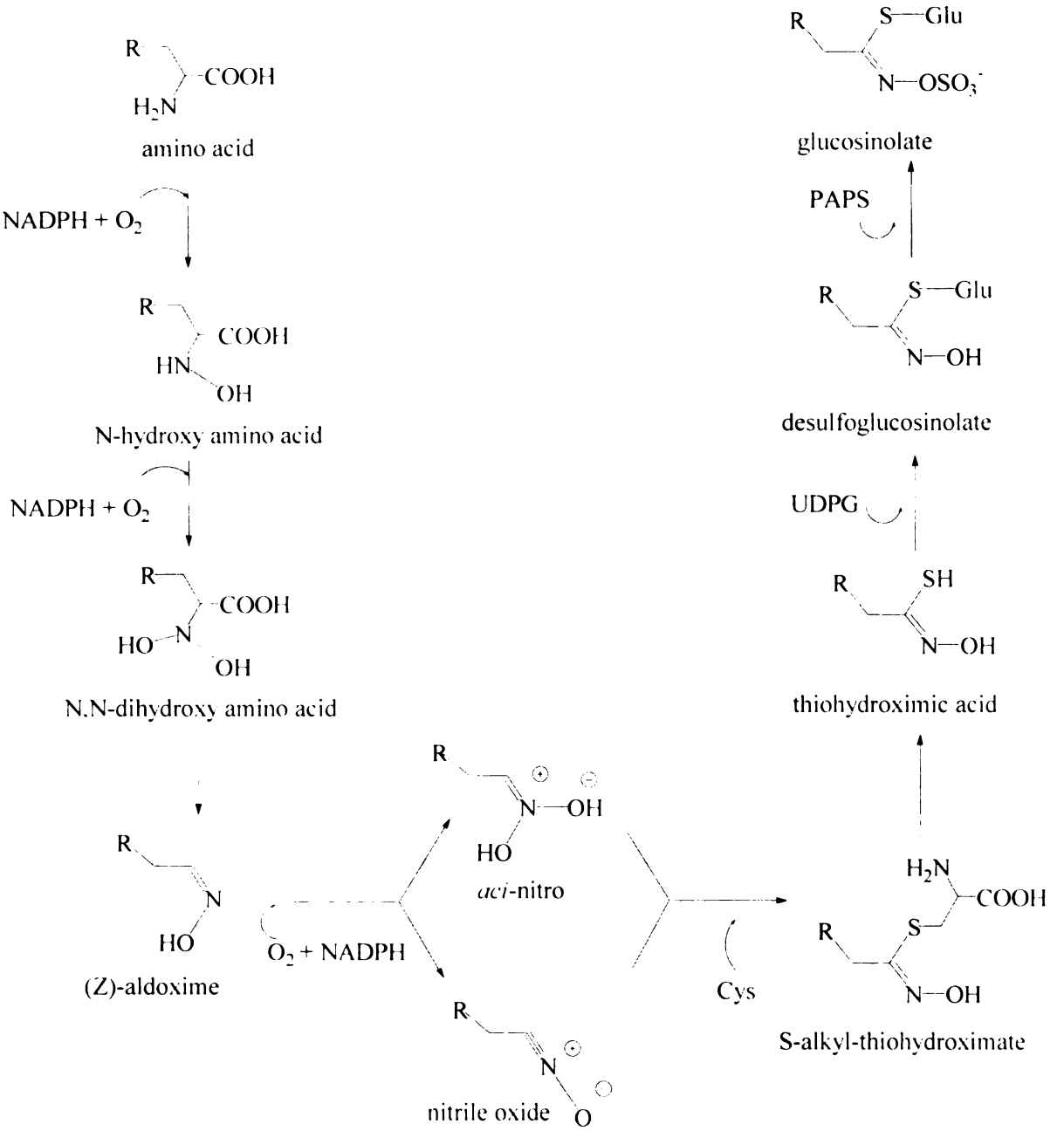

Figure 1.4: Biosynthesis of glucosinolates (adapted from Mikkelsen et al., 2002).

Table

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Table 1.2: Isothiocyanates isolated from wasabi and horseradish (mg/100 g fresh weight)

Isothiocyanate	Wasabi			Horseradish root
	Root	Stem	Leaf	
allyl	111	18.6	22.8	96.6
n-butyl	1.74	0.3	0.36	0.42
3-butenyl	1.83	0.06	0.27	0.81
4-pentenyl	3.9	0.66	0.78	0.1
5-hexenyl	1.02	0.3	0.57	0.18
2-phenylethyl				22.5
5-methylthiopentyl	0.48	0.27	0.12	
6-methylthiohexyl	1.89	2.64	1.14	
7-methylthioheptyl	1.44	0.6	0.33	
5-methylsulfinylpentyl	2.17	0.3	0.42	0.81
6-methylsulfinylhexyl	7.8	2.52	5.4	0.9
7-methylsulfinylheptyl	1.41	0.45	1.08	0.78

(Adapted from Etoh et al., 1990)

In spite of the large number of known glucosinolates (Shapiro et al., 2001), allyl isothiocyanate (AITC), derived from allyl glucosinolate (2-propenyl glucosinolate, sinigrin), has been identified as the major pungent compound in wasabi, horseradish, and in mustard (Masuda et al., 1996).

Hydrolysis of glucosinolates

β-thioglucoside glucohydrolases have been shown to be present in plants that produce glucosinolates. These isozymes, known as myrosinases, mediate the hydrolysis

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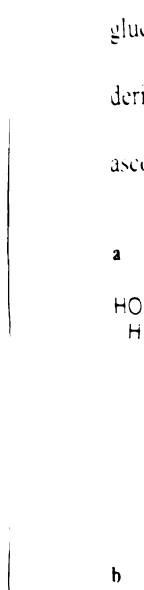
of the glucosinolates (Fenwick et al., 1983). It has been proposed that both the substrate and the enzymes find themselves in separate locations in the intact tissue. It is believed that myrosinase is accumulated in specialized "myrosin cells". According to the "mustard oil bomb hypothesis", glucosinolates would be located in the vacuoles of myrosin cells while myrosinase would be associated with membranes in the cytoplasm of the same cells (Bones and Rossiter, 1996). There is also evidence supporting the notion that the enzyme is located in the interior of the myrosin cell vacuoles (also known as myrosin grains) and that the glucosinolates are located in different cells (Ratzka et al., 2002). The glucosinolate-myrosinase system has probably evolved from the more common system of cyanogenic glycosides and their glucosidases (Rask et al., 2000).

Glucosinolates are chemically stable under physiological conditions in the cell. Only after physical disruption of the cellular integrity by crushing, chewing or cutting will the enzyme and the glucosinolates come together leading to the hydrolysis of the glucosinolates. First, the thioglucosidic bond is hydrolyzed, forming equimolar quantities of sulfate, glucose and aglycone (Figure 1.5). The aglycone formed re-arranges to form products depending on the structure of the side chain and the reaction conditions. Isothiocyanates, thiocyanates and nitriles are the main products. The formation of nitriles is favored by acidic conditions (pH below 3.5) and the presence of iron (II). When iron (II) ions and epithiospecifier protein (a specific cofactor) are present, sulphur is inserted into the terminal double bond of alkenyl aglycones leading to the formation of epithioalkyl compounds like 1-cyano-3,4-epithiobutane (Larsen, 1981; Depree, et al., 1999; Heaney and Fenwick, 1993; Paik et al., 1980). Indole



glucosinolates, like glucobrassicin, form, besides thiocyanate, a series of indole derivatives such as indole-3-carbinol and diindolylmethane (Chevolleau et al., 1997) and ascorbigen (Agerbirk et al., 1998).

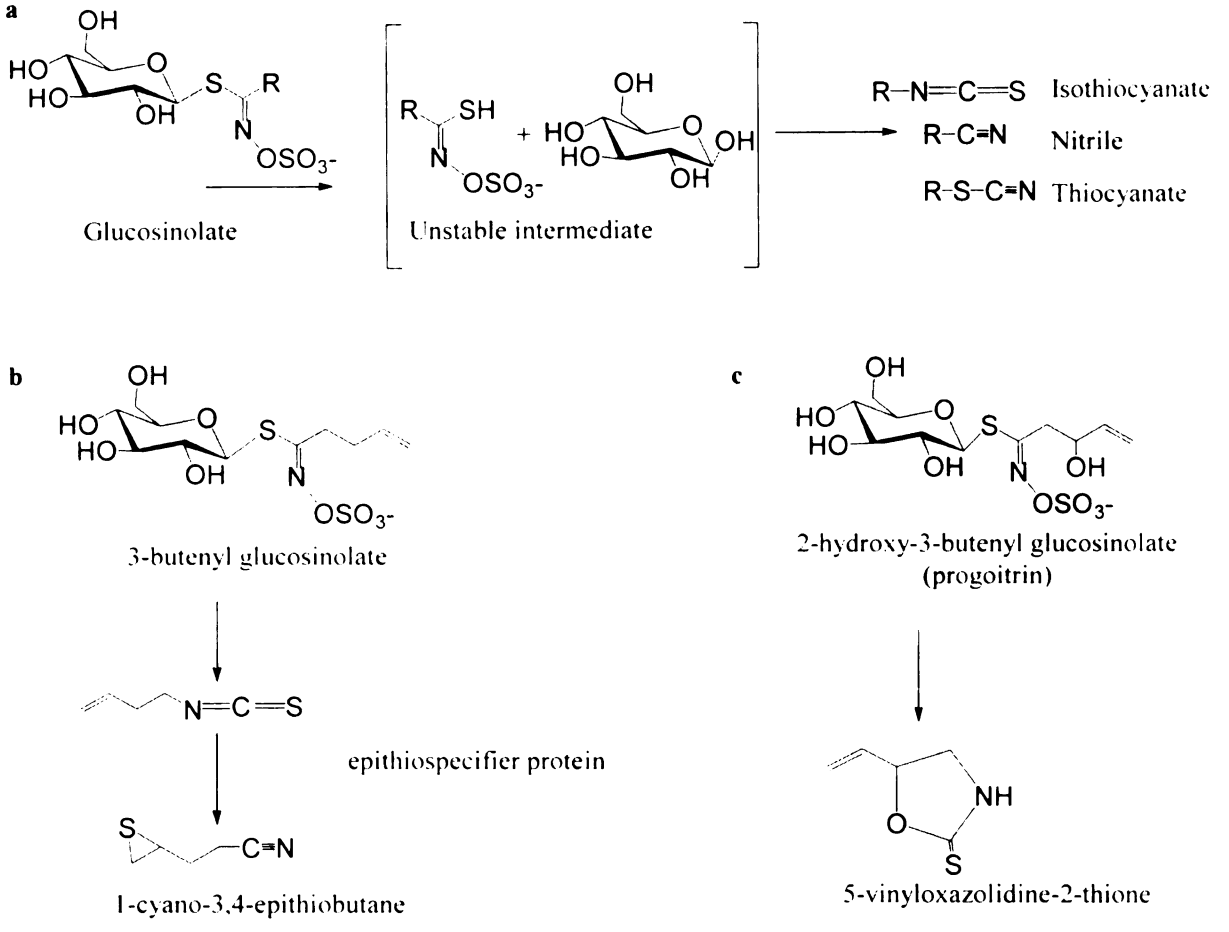


Figure 1.5: Scheme of glucosinolate hydrolysis. (a) Glucosinolates hydrolysis. (b) Rearrangement of isothiocyanates with alkenyl side chain (i.e. from 3-butenyl glucosinolates) to form epithionitriles. (c) Cyclization of isothiocyanates with β-hydroxylated (i.e. from 2-hydroxy-3-butenyl glucosinolate) to form oxazolidine-2-thiones (Mithen, 2001).

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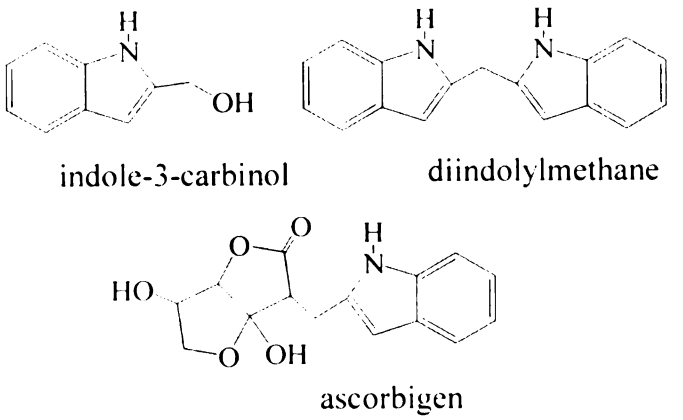

Figure 1.6: Structures of indole-3-carbinol, diindolylmethane, and ascorbigen.

Myrosinase has been shown to be activated by ascorbic acid (Shikita et al., 1999). With a molecular weight of 580 kD, wasabi myrosinase is relatively large, compared to 90-150 kD for other myrosinases (Ohtsuru and Kawatani, 1979). Also compared to other myrosinases, myrosinase from wasabi is somewhat thermally labile with deactivation occurring at 30 °C. The fragility of the wasabi myrosinase makes the preparation of a wasabi powder difficult, since it loses part of its activity. Commonly, myrosinase from other sources or powdered horseradish or mustard have been added to wasabi powder to restore myrosinase activity (Ebisawa et al., 1988).

Biological activity of glucosinolates and their breakdown products

For glucosinolate-producing plants, the release of toxic products resulting from the glucosinolate hydrolysis by myrosinase fulfills important defensive functions against herbivores and pathogens (Rask et al., 2000) and against other plants (Gardiner et al., 1999). However, due to co-evolution of insects and plants, there are insect species that

specialize on glucosinolate-producing plant species. For example, the cabbage pests *Pieris brassicaceae*, *Pieris rapae*, and *Meligethes aeneus* F. (Mewis et al., 2002), the mustard aphid *Liaphis erysimi* (Rask et al., 2000; Muller et al., 2003), the flea beetle *Phyllotetra nemorum* L. (Agerbirk et al., 2001) and the cabbage webworm, *Hellula undalis*, a tropical pest on *brassica spp.* (Mewis et al., 2002) use glucosinolates or their hydrolysis products as positive cues for host plant recognition (Renwick, 2002). Glucosinolates and their hydrolysis products can act not only as feeding stimulants but also as oviposition stimulants (van Loon et al., 1992). They have been shown to induce oviposition in the cabbage root fly, *Delia radicum* L. (Roessing et al., 1997) and in the cabbage webworm, *H. undalis*, with the application of brassica extracts on non-host plants (Mewis et al., 2002). It is unclear how specialist insects perceive glucosinolates since they do not undergo hydrolysis before cell damage, although the presence of glucosinolates in the leaf cuticle as well as additional interactions with other compounds have been proposed (Mewis et al., 2002). It is also unclear how these insects have adapted to the glucosinolate-myrosinase system, but some have been shown to possess sulfatase (Ratzka et al., 2002) or myrosinase enzyme activity which may form hydrolysis products with less toxicity than those produced by the plant myrosinase (Rask et al., 2002). Others have been shown to sequester glucosinolates for defensive purposes (Rask et al., 2000; Muller et al., 2001; Muller et al., 2003).

In general, glucosinolate degradation products have been shown to possess pesticidal (Peterson et al., 1998), fungicidal (Pedras et al., 1998), bactericidal (Ono et al.,

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1998; Ina et al., 1989b) and nematocidal (Lazzeri, et al., 1993; Donkin, et al., 1995; Mitarai et al., 1997) activities.

Allyl isothiocyanate and allyl thiocyanate showed insecticidal activity comparable to or better than chloropicrin, a commercial fumigant, against the common house fly, *Musca domestica*, and the lesser grain borer, *Rhizopertha dominica*. The potency of allyl isothiocyanate was shown to be comparable to that of the commercial nematicide DD, a mixture of 1,2-dichloropropane and 1,3-dichloropropene, in field trials (Mitarai et al., 1997). Intact glucosinolates have been shown to possess no biocidal activity against the second-stage juveniles of the potato cyst nematode, *Globodera rostochiensis*, second-stage juveniles of the sugar beet cyst nematode *Heterodera schachtii* and *Caenorhabditis elegans*. For the myrosinase-induced hydrolysis products, however, mortality was observed (Buskov et al. 2002; Lazzeri, et al., 1993; Donkin, et al., 1995).

The antifungal properties of the hydrolysis products of glucosinolate have long been known (Walker et al., 1937). Intact glucosinolates have been shown to possess no fungitoxic activity. Neither growth nor sporulation of *Fusarium culmorum* were affected by eleven native glucosinolates, but all the glucosinolate-derived isothiocyanates were able to inhibit fungal growth without interfering with sporulation. The hydrolysis products of the glucosinolates glucoiberin, glucotropaeolin and glucoerucin (Figure 1.7) inhibited fungal growth by 50% at 0.1 mg/mL (Manici, et al., 1997).

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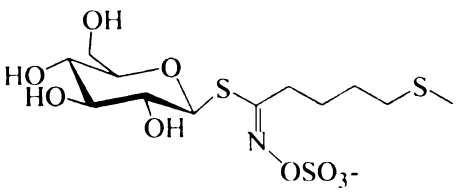
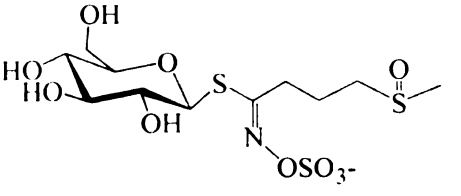


Glucoerucin
(4-methylthiobutyl glucosinolate)



Glucoiberin
(3-methylsulfinylpropyl glucosinolate)

Figure 1.7: Structures of glucoerucin and glucoiberin

ω-methylsulfinylalkyl isothiocyanates of plant origin (including wasabi) have been shown to possess bactericidal activity against *Escherichia coli* and *Staphylococcus aureus*, at 0.1 and 2.0 mg/mL (Ono et al., 1998). 4-methylsulfinylbutyl isothiocyanate isolated from *Arabidopsis thaliana* was tested for activity against a range of fungi and bacteria. For example, it showed 50% growth inhibition at 28 μM against *Pseudomonas syringae* pv tomato (Tierens et al., 2001). Isothiocyanates (mainly AITC) generated *in situ* from brassica spp. vegetables showed fungicidal activity against *Fusarium oxysporum*, *Sclerotium cepivorum*, and *Sclerotinia sclerotiorum* (Smolinska and Horrowicz, 1999). AITC has been shown to suppress the growth of *Gaeumannomyces graminis*, *Bipolaris sorokiniani*, *Phytium irregularie*, *Fusarium graminearum*, and *Rhizoctonia solani* fungi by 50% at concentrations lower than methyl isothiocyanate (0.55-0.79 μmol/L compared to 0.73-0.85 μmol/L), a synthetic fumigant (Sarwar et al., 1998).

The use of glucosinolate-containing plant material for biofumigation, a process by which the glucosinolates are hydrolyzed in field soil liberating the glucosinolate degradation products that exert a suppressive effect on soil-borne plant pathogens, has

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attracted some attention (Angus et al., 1994; Mujtahedi et al., 1991; Mujtahedi et al., 1993; Halbrendt and Jing, 1994; Warton et al., 2001), especially since methyl bromide is ruled to be phased out in the USA after the year 2005 (Borek et al., 1998).

In higher animals, glucosinolate breakdown products have been shown to exert a variety of effects. It was reported that livestock fed with large amounts of *brassica* spp. developed goiter (Stoewsand, 1995). Oxazolidine-2-thiones and thiocyanates exerted a goitrogenic effect in rats and pigs. Goitrogenicity by thiocyanates occurs through a competitive mechanism and the effect can be reduced with higher levels of iodine in the diet, whereas inhibition of thyroxine synthesis is the probable mechanism for the goitrogenic effects by oxazolidine-2-thione.

Growth retardation, liver lesions and necrosis, thyroid hypertropy or hyperplasia, embryonal death, decreased fetal weight, adrenal enlargement (Nishie and Daxenbichler, 1980), renal and pancreatic lesions (Wallig et al., 1988a), lesions of the forestomach (Wallig et al., 1988b), and cancer promotion (Dashwood et al., 1991) were some of the toxicological effects in animals that have been reported for glucosinolates and their hydrolysis products.

Glucosinolates, isothiocyanates, and cancer

Since a reduced incidence of different types of cancer has been linked to an increased consumption of vegetables, especially brassica (Marchand et al., 1989; Cohen, et al., 2000), the glucosinolates and their degradation products have been studied as antimutagens (Nakamura et al., 2001), anticarcinogens (Faulkner et al., 1998; Hecht,

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1999), apoptotic agents (Lund et al., 2001; Gamet-Payrastre et al., 1998; Gamet-Payastre et al., 2000), and as inhibitors of platelet aggregation (Kumagai et al., 1994).

In a prospective cohort study, high consumption of vegetables and fruits was correlated with a decreased colon cancer risk, but not for rectal cancer. In the same study, a lower cancer risk was correlated with high consumption of brassica vegetables in men and women for colon cancer, but the risk was increased in women for rectal cancer (Voorrips et al., 2000). The evaluation of several studies provided no significant evidence that high consumption of brassica vegetables is linked to a reduced prostate cancer risk (Kristal and Lampe, 2002). The epidemiological effects of glucosinolates and their degradation products were confounded as a consequence of genetic polymorphism of biotransformation (Phase I and II) enzymes. In a case-control study, broccoli consumption has been related to lower incidence of colon cancer in subjects with a glutathione S-transferase M1 null (GSTM1 null) genotype (Lin et al., 1998; Lampe and Peterson, 2002). A prospective study among Singapore Chinese (Seow et al., 2002), a population with high dietary isothiocyanate intake via brassica vegetables, demonstrated a significantly lower risk for those individuals who are both GSTM1 and GSTT1 null.

In a case-control study in Japan, a higher risk of colorectal cancer was associated with consumption of radish and cabbage, although no explanation for the observation was attempted (Tajima and Tominaga, 1985). Consumption of glucosinolate-containing vegetables is usually not reported as such in dietary and epidemiological studies in Japan. The expression "yellow-green" vegetables is the preferred term, making it difficult to determine whether brassica vegetables are meant (Nakaji et al., 2001; Kono et al., 1988)

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although some of these studies have categorized broccoli. Consumption of yellow-green vegetables has been correlated with reduced cancer risk in several epidemiological studies in Japan (Wakai et al., 2000; Sauvaget et al., 2003). Consumption of fish may also include isothiocyanates since raw seafood is generally eaten with wasabi. No epidemiological evidence is available correlating the consumption of wasabi or horseradish to cancer protection or cancer risk.

The genotoxic effects of isothiocyanates are ambiguous. They are electrophilic agents capable of directly reacting with proteins, DNA, and RNA. They can cause chromosome aberrations, mutations and cancer (Lee, 1996)(Figure 1.8).

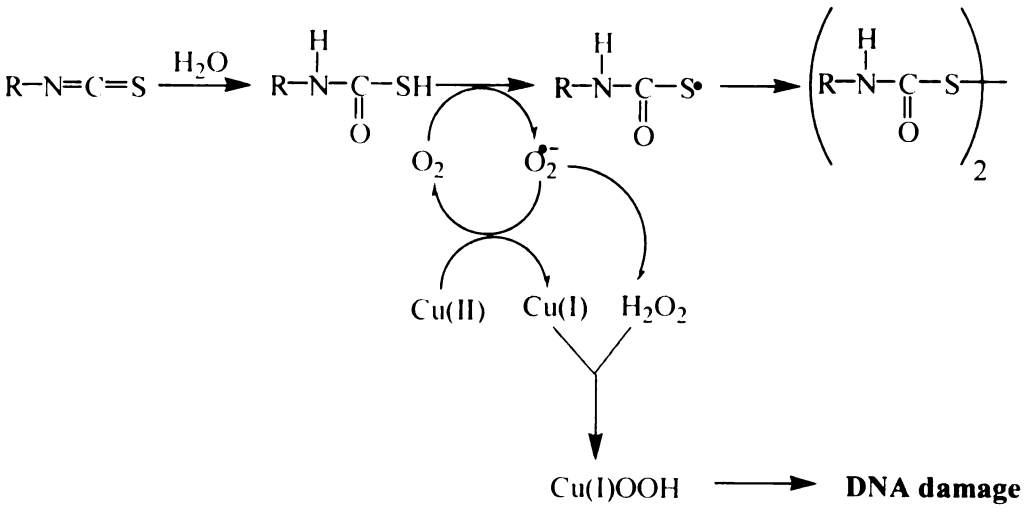

Figure 1.8: Proposed mechanism for oxidative DNA damage by ITCs by isothiocyanates in the presence of Cu(II) (adapted from Murata et al., 2000)

Extracts of brassica vegetables (Brussels sprouts, white cabbage, cauliflower, green cabbage, kohlrabi, broccoli, turnip and black radish) were shown to induce a dose

dependent increase in the number of revertants in *Salmonella* TA98 and TA100, DNA damage in *E. coli* K-12 cells, as well as structural chromosome aberrations in mammalian cells (Chinese hamster ovary cells and SV_{40}-transformed Indian Muntjac cells) (Kassie et al., 2003). Allyl isothiocyanate (AITC) has been reported to be mutagenic towards *Salmonella typhimurium* (Neudecker and Henschler, 1985). Similarly, benzyl isothiocyanate (BITC), phenylethyl isothiocyanate (PEITC), and phenyl isothiocyanate (PITC) were shown to be inducers of chromosome aberrations in an SV_{40}-transformed Indian muntjac cell line (Musk and Johnson, 1993). AITC, BITC, and PEITC have been shown to cause oxidative damage to DNA (Murata et al., 2000) and induce formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG), a marker of oxidative damage, in the presence of copper (II) ions. Strong DNA damaging capacity (even at concentrations as low as 1 ppm) and intracellular generation of reactive oxygen species (ROS) (at 0.01%) by AITC has been demonstrated using *E. coli* deprived of DNA repair capacities and ROS scavenging enzymes (Yonezawa et al., 1999). BITC has been found to be extremely genotoxic in *E. coli* and in human-derived hepatoma cells at concentrations below 2 mg/mL (Kassie et al., 1999).

AITC has been evaluated as carcinogenic to rats by the National Toxicology Program (NTP, 1982). It caused transitional-cell papillomas and epithelial hyperplasia in the urinary bladders of male rats, as well as fibrosarcomas in the subcutaneous tissue in female rats. However, AITC was not carcinogenic for male or female mice (Dunnick et al., 1982).

6-phenylhexyl isothiocyanate (PHITC), used in a single dose at 0.1 μmol, inhibited lung tumorigenesis in A/J mice induced with a single dose of 10 μmol 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Jiao et al., 1994). PEITC has been shown to be an inhibitor of NNK-induced lung adenomas and adenocarcinomas at 3 μmol/g in the diet (Hecht et al., 1996). Wistar WKY male rats, fed with a wasabi powder, were protected against the development of N-methyl-N-nitroso-N-nitroguanidine (MNNG)-induced gastrointestinal tumors compared to the controls (Tanida et al., 1991). 6-methylsulfinylhexyl isothiocyanate has been shown to inhibit the mutation of cancer of skin cells resulting from topical applications of the cancer initiator 9,10-dimethyl-1,2-benzanthracene and the cancer promotor 12-O-tetradecanoylphorbol-13-acetate (Fuke et al., 1997). Also, 6-methylthiohexyl isothiocyanate inhibited 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in mice (Yano et al., 2000).

However, not all isothiocyanates are inhibitors of nitrosamine-induced tumors in animal models. On the contrary, structural analogs have shown both behaviors, strong inhibition and promotion. For example, in rats, dietary PEITC and 3-phenylpropyl isothiocyanate (PPITC) inhibited N-nitrosomethylbenzylamine-induced esophageal tumorigenesis while the longer-chain analog PHITC enhanced esophageal tumorigenesis at 1-2.5 μm/g in the diet (Stoner et al., 1991; Rao et al., 1995; Stoner and Morse, 1996 and 1997). The opposite behavior has been demonstrated for strain A mice with regard to lung tumors at doses of 1 and 5 μmol/day (Stoner and Morse, 1997). BITC and PEITC at 0.1% in the diet have been reported to strongly promote urinary bladder carcinogenesis in rats pretreated with diethylnitrosamine and N-butyl-N-(4-hydroxybutyl)nitrosamine

(Hirose et al., 1998). Even for thisothiocyanates preventing carcinogenesis in the initiation phase, tumor growth promotion in the post-inititation phase cannot be ruled out. At relatively large oral doses in the diet (320 ppm), PEITC caused an increase in non-invasive colon adenocarcinoma in male F344 rats. At twice that concentration, invasive colon adenocarcinoma was also increased. (Rao et al., 1995).

In general, ITCs have been shown to inhibit cancer when administered prior to or during treatment with the carcinogen, but they did not perform as well when administered subsequently to the carcinogen treatment. In addition, the chemoprotective effects of isothiocyanates seemed to be highly specific to both the chemical structure and the cancer model (Hecht, 1999).

Carcinogenesis and mechanisms of cancer chemoprotection by isothiocyanates

Carcinogenesis is considered to be a multifactorial, multistage process that enables certain cells to undergo lasting genetic changes and epigenetic changes. These cells gain a growth advantage and undergo clonal expansion. Mutagens, such as heterocyclic amines (HAs), polycyclic aromatic hydrocarbons (PAHs), and nitrosamines must first undergo metabolic activation to cause DNA damage. Damage left unrepaired can become permanent through DNA replication leading to mutations in critical genes, i.e. oncogenes and tumor suppressor genes, favoring tumor promotion in the carcinogenesis process (Murata et al., 2000). Cells so initiated, in the presence of promoters, become cancerous and metastasize (Verhoeven et al., 1997). A scheme of the evolution and stages of carcinogenesis is shown in Figure 1.9 (Murillo and Mehta, 2001)

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Metabolic activation of carcinogens involves a series of oxidation, reduction and hydrolysis reactions that render them more hydrophilic, hence prone to detoxification. This process is known as Phase 1 metabolism and the enzymes involved, mainly cytochrome P450 enzymes (CYP), are called Phase 1 enzymes. Phase 1 enzymes include monooxygenases, cyclooxygenases, and reductases. Phase 1 metabolites can be activated or deactivated in terms of their reactivity towards DNA. Activated metabolites are electrophiles capable of reacting with nucleophilic sites of DNA and RNA. In Phase 2 metabolism, the corrresponding enzymes conjugate Phase 1 metabolites making them more polar, hence more easily excretable. Glutathione S-transferases, quinone reductases and UDP-glucuronyl transferases are examples of Phase 2 enzymes (Steinkellner et al., 2001; Hecht, 1999).

To rationalize the effects of isothiocyanates with respect to cancer chemoprotection, two sets of putative mechanisms have been identified. One set involves the reduction of metabolic activation by Phase 1 enzymes and, simultaneously, the enhancement of metabolic detoxification by Phase 2 enzymes. The other set involves mechanisms of protection against the development of tumors following initiation. Among these mechanisms enhanced scavenging of free radicals, induction of oxidative stress, up-regulation of apoptosis and cell cycle arrest have been proposed (Fimognari et al., 2002; Loo, 2003; Murillo and Mehta, 2001).

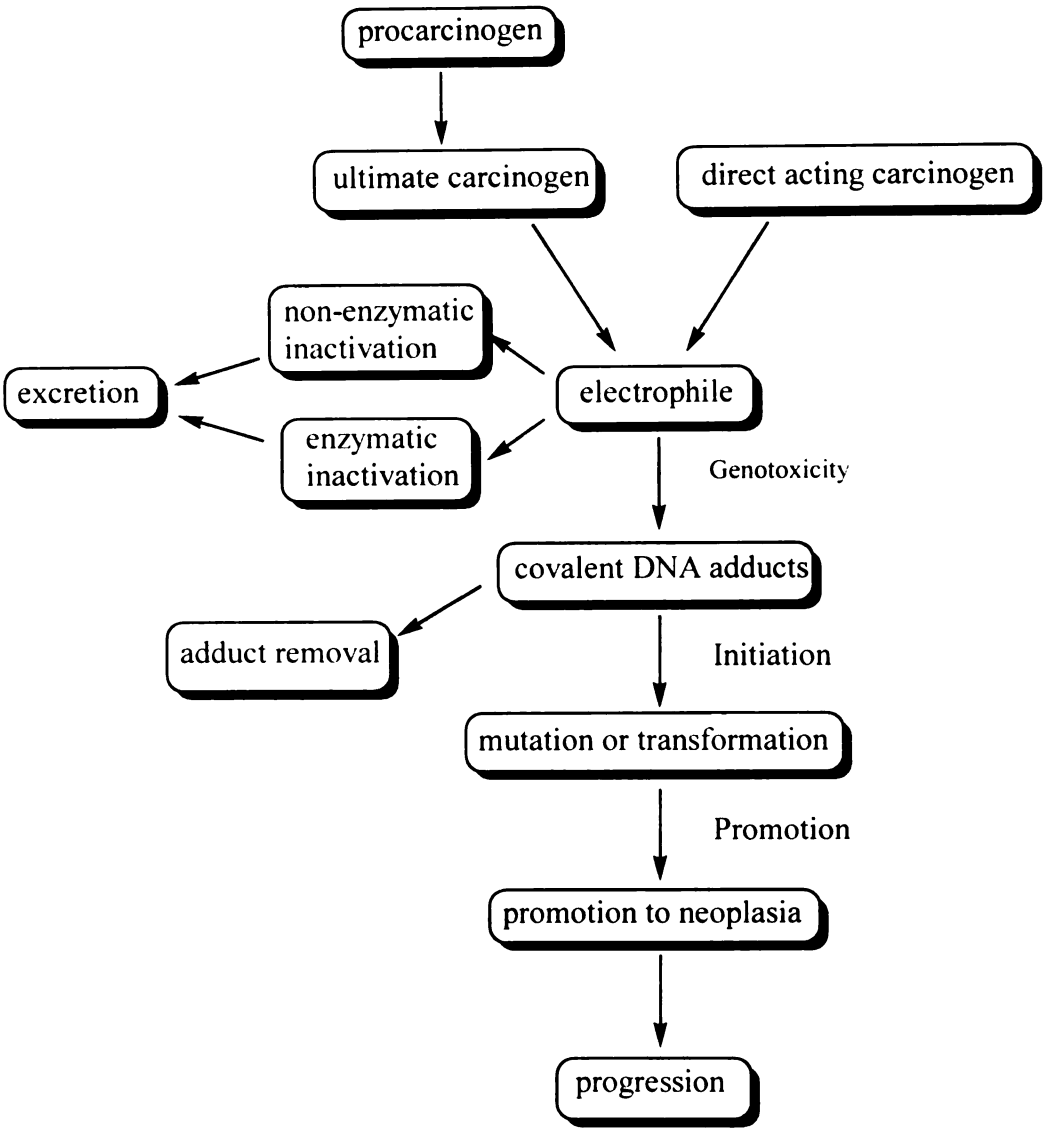


Figure 1.9: Evolution and stages of carcinogenesis (adapted from Murillo and Mehta, 2001).

Regulation of Phase 1 and Phase 2 enzymes

Under the assumption that all Phase 2 enzymes are induced by the same cellular regulatory system, potential Phase 2 inducers can be identified by assaying only one of these enzymes. Usually, induction of quinone reductase or glutathione S-transferase, or

both, is monitored either by in vitro assays with isolated cells or enzyme homogenates or by in vivo studies with rodents. Moreover, inhibition of DNA-damage or the prevention of induced lesions or tumors are used as the endpoints to identify potential Phase 1 inhibitors, Phase 2 inducers or compounds with dual activity (Steinkellner et al., 2001).

Inhibition of Phase 1 enzymes and induction of Phase 2 enzymes, or both, has been demonstrated for a large number of isothiocyanates, supporting the notion that isothiocyanates are effective inhibitors of carcinogenesis (Zhang and Talalay, 1998; Zhang et al., 1992). Glucosinolates are not inducers of cytochrome P450 enzymes. When sinigrin, gluconapin, glucoiberin, glucobrassicin, progoitrin, glucosinalbin, glucotropaeolin and gluconasturtiin were tested, only glucobrassicin breakdown products increased human cytochrome P450 1A1 transcription. The maximum induction was observed at 7.5 μM (Williamson et al., 1996).

Exposure of murine hepatoma (Hepa 1c1c7) cells to 5 μM 4-(methylsulfinyl)butyl isothiocyanate (sulforaphane) selectively raised NAD(P)H:quinone reductase activity by 10-fold and the induction was correlated to the intracellular concentration of sulforaphane (Figure 1.10).

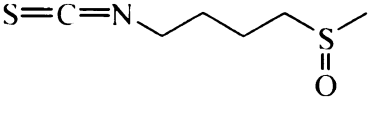


Figure 1.10: Structure of sulforaphane

Sulforaphane has also been shown to increase NAD(P)H:quinone reductase as well as glutathione S-transferase activity in murine liver, forestomach, glandular stomach, proximal small intestine and lung cells. The increase in activity ranged from 17 to 200% and from 37 to 145% for the glutathione S-transferase and the quinone reductase, respectively (Zhang et al., 1992). Treatment of murine hepatoma cells, Hepa 1c1c7, with 2-, 4-, 6- and 8- ω-methylsulfinyl isothiocyanates augmented the induction of quinone reductase in a concentration dependent manner (Fuke et al., 1997).

BITC was shown to induce glutathione S-transferase (GST-S) in the small intestine and liver of female ICR/Ha mice (Sparnins et al., 1982). In animal studies, the tested dose for both the carcinogen and the test compound were usually much higher than the expected exposure to humans. When 3-methylsulfinylpropyl isothiocyanate was tested in rats at doses comparable to those expected in humans, no significant induction of Phase 1 or Phase 2 enzymes was observed (Kore et al., 1991). AITC, when given in the diet to (Ruakura colony of Sprague-Dawley-derived) rats, showed toxicity at >100 μmol/kg/day, causing inflammation, ulceration, and thickening of the stomach wall. At non-toxic doses (5-50 μmol/kg/day), quinone reductase activity was strongly induced in the liver, kidneys, spleen, heart, lungs, glandular stomach, non-glandular stomach (forestomach), duodenum, jejunum, ileum, cecum, colon and urinary bladder (dose dependently). At the non-toxic doses, glutathione S-transferase induction was significant but dose-dependent only in the non-glandular stomach and urinary bladder (Munday and Munday, 2002).

High levels of brassica vegetable consumption induced GST in human blood plasma (Bogaards et al., 1994; Lampe et al., 2000). In a dietary intervention study with Brussels sprouts, broccoli and cooked meat, the level of heterocyclic amines (HA) in the urine of human volunteers was significantly decreased and suggested an increase in the metabolism of HAs as a consequence of the induction of Phase 2 enzymes (Murray et al., 2001).

Cancer chemoprotection afforded by brassica vegetables cannot be exclusively ascribed to the presence of glucosinolates. Treatment of (Hep G2) cells possessing inducible Phase 1 and 2 enzymes with garden cress and water cress juice (0.1-1.25 μL/mL showed that the juices afforded protection against human hepatoma B(a)P-induced DNA damage. However, when the juices were replaced by BITC, DNA damage was observed for BITC (> 2.5 μM) and in synergy (0.6 μM) with B(a)P. PEITC produced no synergistic effect, but it induced DNA damage at 5 μM. BPDE, the genotoxic metabolite of B(a)P, induced significant DNA damage in the presence of the juices. Since BITC and PEITC are the major expected isothiocyanates from garden cress and water cress, respectively, these results indicated that the protective effects of the juices against (B(a)P)-induced DNA damage were not related to the formation of the isothiocyanates nor to the detoxification of BPDE (Kasie et al., 2003).

Cellular oxidative stress

Extracts of brassica vegetables (Brussels sprouts, cabbage, broccoli) have been shown to possess antioxidant but also pro-oxidant activity, depending on the extraction

conditions (Zhu, et al., 2000; Plumb et al., 1996). It has been shown that glucosinolates are not involved in the antioxidant activity of brassica vegetable extracts (Germano et al., 2002). In general, glucosinolates and isothiocyanates did not possess the antioxidant activity that many phenolic phytochemicals exert, although 6-methylthiohexyl isothiocyanate was shown to inhibit NNK-induced formation of the promutagenic adduct O^6-guanine (O^6MG) in mouse lung (Yano et al., 2000). Cancer cells have been shown to constitutively produce high amounts of ROS and H_2O_2 in particular (Loo, 2003). Phenolic phytochemicals generate species capable of scavenging ROS, such as H_2O_2 and $^{\cdot}OH$ (Shahidi and Wanasundara, 1992) thereby inhibiting cancer cell proliferation. The high amounts of constitutive ROS in cancer cells result in what is known as "persistent oxidative stress in cancer" which can lead to additional mutations in the cancer cells. It is believed that H_2O_2 stimulates cancer cell proliferation. Among other events, the levels of glutathione are reduced since it is oxidized by H_2O_2. Reduced levels of glutathione allow for overactivation of protein kinases, which in turn favor cancer cell proliferation. It has been demonstrated that H_2O_2 is a mediator in signal transduction, which leads to the activation of genes that facilitate cell proliferation. For example, COX-2 is expressed at higher levels in cancerous tissues compared to the surrounding normal tissue. However, when H_2O_2 reaches a threshold it can cause cell cycle arrest or apoptosis. Since cancer cells are apparently closer to this threshold than normal cells, the former are more susceptible to undergo cell cycle arrest or apoptosis. Since the difference between proliferation or death of the cancer cell lays in the concentration of H_2O_2 (or ROS in general), a possible mode of action for inhibition of cancer cell proliferation by

phytochemicals and anticancer drugs relies in the modulation of the levels of H_2O_2. Some phytochemicals (including phenolics and isothiocyanates) and anticancer drugs act as prooxidants. They induce oxidative stress, which can, in turn, trigger cancer cell death (Loo, 2003). Isothiocyanates are substrates for GST and are incorporated at a higher ratio in cancer cells than in normal cells (Zhang and Talalay, 1998; Ye and Zhang, 2001), inducing GST production. In addition, cancer cell proliferation is arrested to allow for the detoxification of the isothiocyanates that endanger the viability of the cancer cells (Loo, 2003).

Apoptosis and cell cycle arrest

Apoptosis is a mechanism of cellular self-destruction leading to the elimination of damaged cells in tissue. It is considered to be a defense mechanism against carcinogenesis. It has been shown that inhibition of chemical carcinogenesis in animal models is correlated to the index of apoptosis in treated tumors *in vivo*. Apoptosis has been proposed as a possible mechanism for the inhibition of carcinogenesis by isothiocyanates in the post-initiation phase (Samaha et al., 1997).

Rats supplemented with sinigrin have shown an increase in the number of colon cells undergoing apoptosis as well as a reduction in the number of aberrant crypt foci induced by dimethylhydrazine (DMH) (Smith et al., 1998). Freshly prepared Brussels sprouts juice, as well as fresh, uncooked sprouts, orally administered to male Wistar rats treated with DMH, effectively induced apoptosis in the colon (Smith et al., 2003), as did sulforaphane (SFN) and phenylethyl isothiocyanate (PEITC) in F344 rats (Chung et al.,

2000). SFN, benzyl isothiocyanate (BITC), and PEITC were shown to stimulate apoptosis and also provided protection against DNA damage in human colon cells (Bonnesen et al., 2001). AITC has been shown to be selectively cytotoxic against undifferentiated HT29 cells (Musk et al., 1995a; Smith et al., 1996). Since intestinal epithelia have high rates of mitosis and are constantly exposed to potentially mutagenic agents in foods and to bacterial metabolites, substances that elevate the rate of apoptosis are potential inhibitors of carcinogenesis in the colon (Johnson, 2002).

Furthermore, PEITC, phenylmethyl isothiocyanate (PMITC), 4-phenylbutyl isothiocyanate (PBITC) and 6-phenylhexyl isothiocyanate (PHITC) induced apoptosis in human HeLa cells but PITC did not (Yu et al., 1998). PEITC was found to be pro-carcinogenic toward rat colonic adenocarcinomas (Samaha et al., 1997). 6-methylsulfinylhexyl isothiocyanate from wasabi has been shown to inhibit cell proliferation in human monoblastic leukemia U937 cells through apostosis induction (Watanabe et al., 2003).

Arrest of cell cycle has been proposed as the mechanism for the observed growth inhibition of the carcinoma cell line HT29, although there are contradictory reports about the ability of sulforaphane to inhibit this particular cell line (Lund et al., 2001; Hudson et al., 2001; Figmonari et al., 2002).

Digestion and bioavailabitlity of glucosinolates in humans

Brassica vegetables are usually cut and cooked before consumption. Cutting has been shown to initiate enzymatic hydrolysis resulting in the degradation of the

glucosinolates. Cooking lead to large losses of glucosinolates through thermal degradation, depending on the temperature, the duration and the method of cooking (MacLeod and MacLeod, 1968). Thawing of frozen brassica vegetables without first inactivating myrosinase enzyme had the same consequence (Quinsac et al., 1994).

If ingested in the presence of myrosinase, it can be assumed that the glucosinolates will be degraded to some extent. When myrosinase was deactivated by cooking, a significant portion of the intact glucosinolates reached the large intestine where they were metabolized by the microflora, forming isothiocyanates and other metabolites (Shapiro et al., 1998; Shapiro et al., 2001; Nugon-Baudon et al., 1988; Krul et al., 2002; Combourieu et al., 2001). Hydrolysis of glucosinolates by intestinal bacteria has been demonstrated by incubation of glucosinolates with human faeces (Getahun and Chung, 1999).

The estimation of the average daily intake of glucosinolates and isothiocyanates is difficult because of different patterns of vegetable consumption by individuals from different socioeconomic groups and geographical locations (Stones et al, 1984). Another factor is the influence of growing conditions on the concentration of these compounds in the plant (Fahey et al., 2001). A study calculated the average daily intake of sinigrin from brassica vegetables in the United Kingdom to be close to 11 mg, with a range of 0.4-28 mg. The maximum daily intake was calculated as 170 mg, or 6 μmol/kg/day for a 70-kg individual (Stones et al., 1984). Conversion of glucosinolates to isothiocyanates has been reported during cooking (de Vos and Blijleven, 1988) and passage through the gut (Shapiro et al., 1988; Shapiro et al., 2001; Conaway et al., 2000). The amounts of allyl isothiocyanate

available to humans from brassica vegetables consumption in the United Kingdom have been calculated to be 0.8 μmol/kg/day (Munday and Munday, 2002).

Allyl isothiocyanate has been detected in the contents of the digestive tract of germ-free rats inoculated with a human strain of *Bacteroides thetaiotaomicron* (Elfoul et al., 2001). Nitriles formed in the intestine from glucosinolates have a short residence time since they will be rapidly converted to organic acids and ammonia (Duncan and Milne, 1992).

Partial absorption of intact glucosinolates into the blood stream has been proposed from experiments with poultry (Slominski et al., 1988). Once they enter the epithelial cells of the gastrointestinal mucosa, glucosinolates and isothiocyanates will be metabolized by conjugation with glutathione (mediated by glutathione-S-transferases) followed by hydrolysis and N-acetylation as shown in Figure 1.11 (Shapiro et al., 2001).

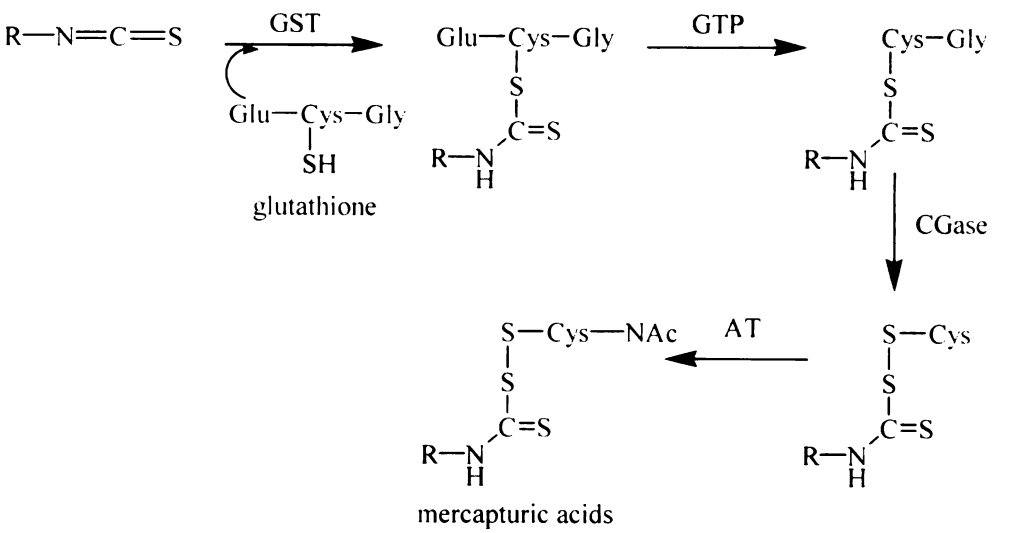


Figure 1.11: Metabolism of isothiocyanates with glutathione S-transferase (GST), γ-glutamyltranspeptidase (GTP), cysteinylglycinase (CGase) and acetyltransferase (AT) (Shapiro et al., 2001).

N-acetylcysteine derivatives (mercapturic acids) were excreted in the urine within 24 h (Chung et al., 1992; Jao et al., 1994). The N-acetylcysteine conjugate of PEITC has been detected in human urine following ingestion of watercress (Chung et al., 1992). Excretion of the mercapturic acids was diminished when myrosinase was absent or inactivated (Shapiro, 1998; Duncan et al., 1997).

Although the extent of the bioavailability and metabolism of glucosinolates are not completely understood, it has been shown that isothiocyanates are rapidly absorbed in the upper gastrointestinal tract (Bollard et al., 1997). Transport of the metabolites to the large intestine following absorption in the small intestine has also been proposed (Smith et al., 2003). Whatever be the absorption mechanism, thiocyanates have been detected in plasma (Murray et al., 2001).

A review of the available literature shows that isothiocyanates derived from the hydrolysis of glucosinolates have a large range of biological activities. They also have been shown to affect humans in both beneficial and detrimental ways. They are potential regulators of the detoxification metabolism by reducing activation of carcinogens and enhancing their excretion. They can also induce high levels of oxidative stress in tumor cells and potentially leading to apoptosis or cell cycle arrest. Even though the relationship between dose and cancer prevention is currently not clear, in the search for compounds with cancer chemoprotective properties they are indeed an interesting class. However, isothiocyanates are also potential tumor promoters and can also be harmful to animals and humans.

The overall objective of the present work will be to investigate fresh horseradish and wasabi rhizomes, as well as a commercial wasabi powder for bioactive compounds with potential lipid peroxidation, cyclooxygenase, cell proliferation and growth inhibitory activities. To achieve this overall objective, this study will be focused on specific objectives, such as the evaluation of different extraction protocols with respect to biological activity, bioasssay-guided fractionation of crude extracts followed by the purification, structure elucidation and efficacy evaluation of the bioactive compounds.

CHAPTER TWO

PRELIMINARY EVALUATION OF THE LIPID PEROXIDATION AND CYCLOOXYGENASE ENZYMES INHIBITORY ACTIVITIES OF WASABI (*WASABIA JAPONICA*) AND HORSERADISH (*ARMORACIA RUSTICANA*) EXTRACTS

Abstract

Horseradish (*Armoracia rusticana*) and wasabi *(Wasabia japonica*) are used as spices mainly due to their pungency. Pungency originates in the enzymatic hydrolysis of glucosinolates. Both species have been reported as having antioxidant and anti-inflammatory activities. Fresh and lyophilized rhizomes of *A. rusticana* and *W. japonica* as well as a commercial wasabi powder were separately extracted using different protocols. The extracts were tested for lipid peroxidation (LP) and cyclooxygenase-1 (COX-1) and -2 (COX-2) inhibitory activities. With 79 and 47 %, respectively, extracts II and III from extraction protocol A inhibited LP significantly stronger than extract VI at 26% from extraction protocol B. This difference in activity was probably due to the hydrolysis of glucosinolates. In COX inhibitory assays no such difference was observed. All extracts of the wasabi powder obtained by protocol C showed very strong LP inhibitory activity (74-98%) when compared to the active extracts of both fresh and lyophilized wasabi rhizomes (35 to 62%). No significant difference was observed in the LP inhibitory activity of the fresh wasabi extracts XI (51%) and XII (53%) from extraction protocol D when compared

to extracts XV (45%) and XVI (62%) of lyophilized wasabi by extraction protocol F. The COX enzymes inhibitory activities of the wasabi powder and rhizomes extracts were unaffected by the extraction protocols used. Nevertheless, extraction with water developed a pungent odor and indicated enzymatic decomposition of the glucosinolates. For a bioassay-guided isolation and characterization of the bioactive compounds, extracts IV-IX and XV-XVII were used (Chapters 3, 4, and 5) to avoid the degradation of glucosinolates.

*Presented as a poster at the 43rd Annual Meeting of the American Society of Pharmacognosy and 3rd Monroe Wall Symposium, July 27-31, 2002, New Brunswick, New Jersey.

Introduction

Both horseradish and wasabi are harvested for their rhizomes, which develop a pungent odor and taste upon crushing. Wasabi is also processed and sold as a powder. Glucosinolates are secondary metabolites that undergo enzymatic hydrolysis once the plant tissue containing them is disrupted. The glucosinolates are hydrolyzed by myrosinase enzyme, a β-thioglucoside glucohydrolyse, producing volatile isothiocyanates. The latter confer characteristic pungent aroma and flavor. For this reason plants with relatively large quantities of glucosinolates are highly valued for culinary purposes. Members of the *Capparidacea*, *Euphorbiaceae*, *Phytolactaceae*, *Resedaceae* and *Tropaeoloaceae* are known for their glucosinolate contents, but generally the highest concentrations are found in *Brassicaceae* (Dewick, 1998). Other possible products of the enzymatic hydrolysis of glucosinolates are thiocyanates, nitriles and cyanoepithioalkanes (Fahey et al., 2001; Watson, 1987). Isothiocyantes (ITCs) from brassica spp., including horseradish and wasabi, have been extensively studied for an array of biological activities ranging from bactericidal, nematocidal, insecticidal, fungicidal and allelopathic to human platelet aggregation and anti-carcinogenic activities (Donkin, et al., 1995; Ono et al., 1998; Velioglu et al., 1998; Morimitsu et al., 2000; Fahey et al., 1997; Verhoeven et al., 1997).

A widely used approach to avoid the enzymatic degradation of glucosinolates is to deactivate the myrosinase enzyme by boiling with MeOH, but the formation of artifacts cannot be precluded. On the other hand, the plant material may be frozen in liquid nitrogen or lyophilized and ground before extraction. The latter approach does not

deactivate the enzyme, but if moisture is excluded no glucosinolate degradation occurs (Heaney and Fenwick, 1993).

The objective of the present study is to determine the effect of different extraction protocols on the lipid peroxidation and COX-1 and COX-2 inhibitory activities of the extracts. The results will be used in the selection of the extraction protocol to be used in a bioassay-guided search for bioactive compounds from wasabi and horseradish.

Materials and methods

Wasabi powder, packaged in one kg bags, was purchased from Oriental Gardens, East Lansing, MI. Fresh wasabi roots were purchased from Pacific Farms (Eugene, OR). All solvents were ACS reagent grade and purchased from Spectrum Chemical Co. (Gardena, CA). *Tert*-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Ibuprofen, Naproxen, dimethyl sulphoxide (DMSO), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Celebrex and Vioxx were physician's professional samples supplied by Dr. S. Gupta. 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), 3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid from Molecular Probes (Eugene, OR). COX-1 and COX-2 enzymes were prepared in the Bioactive Natural Products and Phytoceuticals Laboratory (BNPP) from ram seminal vesicles and prostaglandin endoperoxide H synthase-2 (PGHS-2) cloned insect cell cell lysate, respectively.

Extraction protocols (Figure 2.1)

Extraction protocol A: Fresh horseradish rhizomes (1.025 g, 73% moisture content) were blended with water (1L). The aqueous extract was filtered and the residue was subsequently extracted with methanol (1L x 5) and ethyl acetate (1L x 5). Removal of the solvent under reduced pressure afforded extracts I (134 g,), II (27.9 g), and III (160 mg), respectively.

Extraction protocol B: Lyophilized horseradish rhizomes (345 g) were sequentially extracted in a glass column with hexane (1L x 5), ethyl acetate (1L x5), and methanol (1L x5) and removal of solvents under reduced pressure yielded extracts IV (1.76 g), V (1.31 g), and VI (51 g), respectively.

Extraction protocol C: Wasabi powder (472 g) was sequentially extracted in a glass column with hexane (1L x 5), ethyl acetate (1L x 5) and methanol (1L x 5) and removal of solvents under reduced pressure yielded extracts VII (24.8 g), VIII (5 g), and IX (58 g), respectively.

Extraction protocol D: Wasabi fresh rhizomes (345 g) were sequentially extracted in a glass column with water (1L), methanol (1L x 5), and ethyl acetate (1L x 5) and removal of solvents under reduced pressure yielded extracts X (26.7 g), XI (7.8 g), and XII (200 mg), respectively.

Extraction protocol E: Wasabi fresh rhizomes (643 g) were sequentially extracted with methanol (1L x 5), ethyl acetate (1L x 5), and hexane (1L x 5) and removal of solvents under reduced pressure yielded extracts extracts XIII (35.3 g) and XIV (410 mg) for methanol and ethyl acetate, respectively. No hexane extract was obtained.

Extraction protocol F: Lyophilized wasabi rhizomes (562 g) were sequentially extracted with hexane (1L x 5), ethyl acetate (1L x 5), and methanol (1L x 5) and removal of solvents under reduced pressure yielded extracts XV (2.1 g), XVI (2.2 g), and XVII (22 g), respectively.

Lipid peroxidation inhibitory assay

Inhibition of lipid peroxidation was measured by fluorescence spectroscopy using large unilamellar vesicles (LUVs) of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine containing a fluorescent probe (3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid) and reported after 21 min as relative fluorescence compared to a control. For comparison, tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisolee (BHA) at 1.8, 2.2, and 1.67 ppm, respectively, were used as positive controls. Briefly, the lipid and the probe were dissolved in DMF and evaporated *in vacuo*. The solvent-free mixture was resuspended in 0.15 M NaCl, 0.1 mM EDTA and 0.01 M MOPS (kept over Chelex resin), subjected to ten freeze-thaw cycles using a dry ice-ethanol bath, and extruded through a 100 nm pore size membrane to form the LUVs. The assay buffer used consisted of a mixture of 100 μL HEPES buffer, 200 μL 1 M NaCl, 1.64 mL N_2-sparged water. An aliquot of 20 μL of extracts in DMSO at 2.5% or DMSO (control) and 20 μL of liposome suspension, were mixed with the assay buffer to achieve a concentration of 250 ppm of the extract. Peroxidation was initiated by adding 20 μL 0.5 mM $FeCl_2.4H_2O$. Fluorescence was measured using a Turner model 450 fluorometer (Barnstead Thermolyne, Dubuque, IA) at 384 nm and at 0, 1, 3 and then every three min up to 21 min. The rate of decrease of fluorescence is a measure of the rate of peroxidation.

Relative fluorescence at 21 min (final value divided by initial value) is reported as a measure of lipid peroxidation; higher values correlate with enhanced inhibition (Arora and Strasburg, 1997).

Cyclooxygenase inhibitory assay

Cyclooxygenase enzymes inhibitory activities of extracts were evaluated using COX-1 and COX-2 enzymes. The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction with arachidonic acid as substrate was measured using a Model 5300 biological oxygen monitor (Yellow Spring Instruments, Inc., Yellow Springs, OH). The reaction mixture, consisted of 0.1 M Tris, 1.0 mM phenol, 17 μg hemoglobin, the enzyme, 10 μL extract dissolved in DMSO at 1.5% (DMSO alone as solvent control), was held in a 600 μL micro oxygen chamber (Instech Laboratory, Plymouth Meeting, PA) at 37 ^{o}C. After 3 min of incubation, 10 μL of arachidonic acid (0.25 mg/0.5 mL of Tris buffer) was added to initiate the reaction. Data was recorded using Quicklog for Windows (Strawberry Tree Inc., Sunnyvale, CA). Ibuprofen, Aspirin, Celebrex, Vioxx and Naproxen at 2.06, 180, 1.67, 1.67 and 2.52 ppm, respectively, were used as positive controls (Wang et al., 1999).

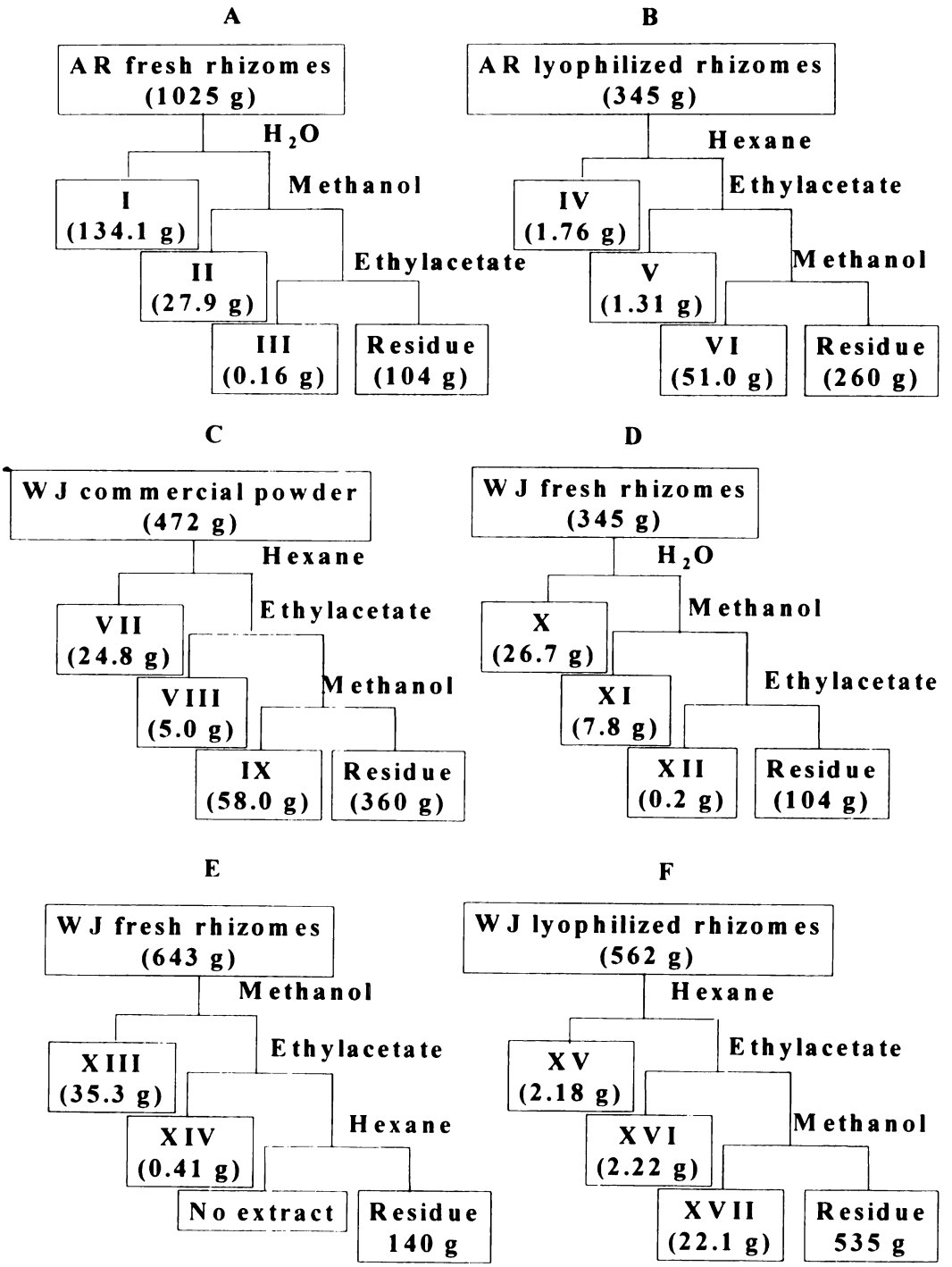


Figure 2.1: Extraction protocols A-F.

Results and discussion

The results of the lipid peroxidation and the cyclooxygenases inhibitory assays for the extracts are shown in figures 2.2 and 2.3, respectively.

A significant difference was observed in the inhibition of lipid peroxidation for the horseradish extracts (I-VI)(Figure 2.2, panel A). No activity was observed for the horseradish aqueous extract (I). The methanol and ethyl acetate extracts of horseradish, II and III, showed 79 and 47% inhibition of lipid peroxidation, respectively, at 250 ppm. In contrast, the methanol extract of the lyophilized horseradish rhizomes (VI) exhibited only 26% lipid peroxidation inhibitory activity while the hexane (IV) and the ethyl acetate (V) extracts were inactive (Figure 2.2, panel B). The difference in lipid peroxidation inhibitory activity between the extracts I-III from extraction protocol A and IV-VI from extraction protocol B was probably due to the hydrolysis of glucosinolates during the extraction with water. Previous reports indicated that glucosinolates are not active in systems assaying for antioxidant activity (Germano et al., 2002). It can be assumed that some of the volatile compounds formed or present in the extract must have been lost since the extracts were evaporated under reduced pressure. There was no significant difference in the results observed for the COX inhibitory assays between extracts I-VI. It is possible that the compounds involved in the inhibitory activity were the same and minor differences observed were attributed to a concentration effect.

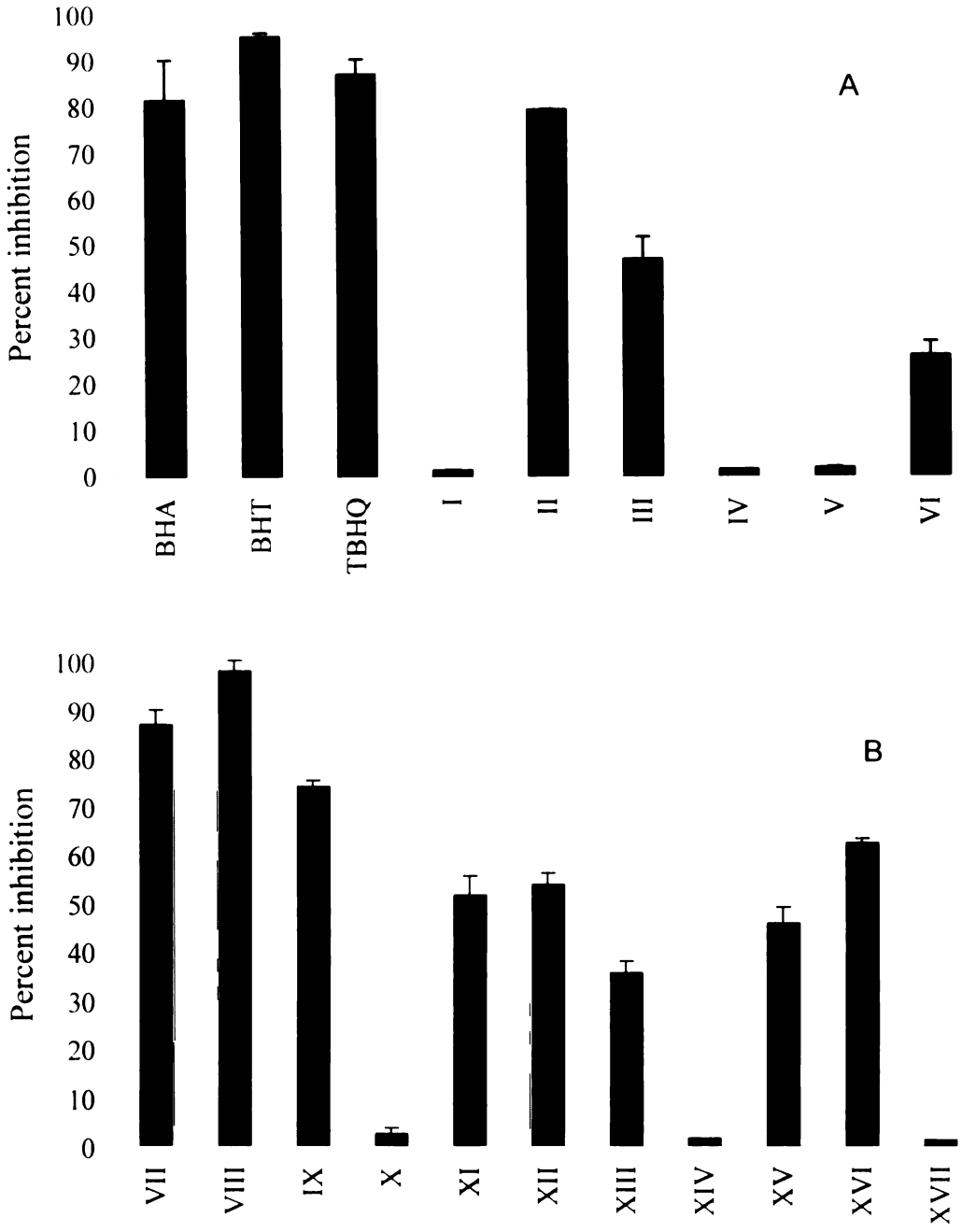


Figure 2.2: Inhibition of lipid peroxidation by horseradish extracts (I-VI, panel A), and wasabi extracts (VII-XVII, panel B) at 250 ppm. Positive controls, BHA, BHT, TBHQ were tested at 1.8, 2.2, and 1.67 ppm, respectively. The experiments were conducted in duplicate and data represent the mean percent inhibition after 21 min. ± one standard deviation.

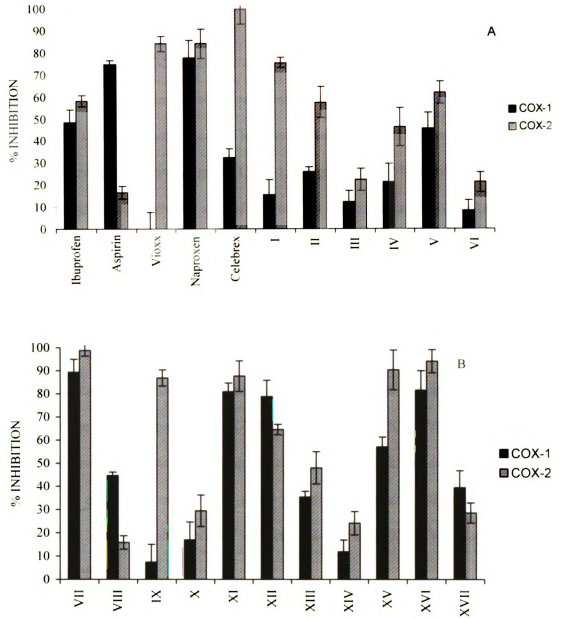


Figure 2.3: Cyclooxygenase enzyme inhibitory activities of horseradish extracts (I-IV, panel A) and wasabi extracts (VII-XVII, panel B) at 250 ppm. Ibuprofen, Aspirin, Celebrex, Vioxx and Naproxen at 2.06, 180, 1.67, 1.67 and 2.52 ppm, respectively, were used as positive controls. Results are expressed as mean value of the percent inhibition of duplicate measurements ± one standard deviation.

All wasabi powder extracts (VII-IX) strongly inhibited lipid peroxidation at 87, 98, and 74%, respectively. Additionally, the hexane extract of the wasabi powder (VII) inhibited COX-1 and COX-2 enzymes at 89 and 99%, respectively, at 250 ppm. The methanol extract (IX) of the wasabi powder inhibited COX-2 enzyme at 87% while it was inactive in the COX-1 enzyme assay. The ethyl acetate extract of the wasabi powder (VIII) showed 45% inhibitory activity against COX-1, but gave only 16% activity against COX-2. The commercial wasabi powder studied did not list the contents on its package and may contain added antioxidants. This was evident from the GC-MS analysis of one of the fractions of extract VII which showed the presence of butylated hydroxytoluene (BHT), a commonly used antioxidant. However, BHT has been previously reported as a natural product (Pala-Paul et al., 2002; Vendramini and Trugo, 2000).

Significant differences in the lipid peroxidation or in the cyclooxygenase inhibitory activities of the extracts from fresh (X-XII) and lyophilized (XV-XVII) wasabi rhizomes were not observed. This was surprising since blending fresh rhizomes with water (extract (X) produced a pungent odor. The lower activity of extracts XIII and XIV in both cycloxygenase assays, compared to extracts XI-XII and XV-XVI may be due to concentration effects.

The results from the lipid peroxidation inhibitory assay of the horseradish extracts (I-VI) indicated that the different extraction protocols yielded extracts with different compounds in addition to compounds formed due to enzymatic hydrolysis of the glucosinolates by myrosinase enzyme. The observed lipid peroxidation and the

cyclooxygenase inhibitory activities of the fresh and lyophilized wasabi extracts were not affected by the extraction protocols. However, it was evident by olfactory detection that extraction with water caused enzymatic hydrolysis of the glucosinolates. The bioassay-guided purification and characterization of bioactive compounds from horseradish (extracts IV-VI), and wasabi (extracts VII-IX) rhizomes as well as from wasabi powder (extracts XV-XVII) will be discussed in Chapters Three and Four.

Partial funding of this project was provided by the MSU Agricultural Experiment Station and the Center for Plant Products and Technologies. Travel funding for the presentation of this work was provided by the Ries Endowed Fund.

CHAPTER THREE

DEHP AND CANCER PROLIFERATING DESULFOSINIGRIN (DSS) IN WASABI (*WASABIA JAPONICA*)

Abstract

A reduced incidence of different types of cancer has been linked to consumption of brassica vegetables and there is evidence that glucosinolates (GSLs) and their hydrolysis products play a role in reducing cancer risk. Wasabi (Wasabia japonica), a Brassica vegetable, is a widely used condiment in the Japanese cuisine and is popular in the USA. Two compounds, di-(2-ethylhexyl)phthalate (DEHP) (**1**) and desulfosinigrin (DSS) (**2**), were isolated from a commercially available wasabi powder and from fresh wasabi roots. DEHP and DSS were evaluated for their inhibitory effects on cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) enzymes, on lipid peroxidation, and on the proliferation of human colon (HCT-116), breast (MCF-7), lung (NCI-H460), and CNS (Central Nervous System) (SF-268) cancer cell lines. DEHP and DSS did not inhibit cyclooxygenase enzymes or lipid peroxidation at 250 ppm. However, DSS promoted the growth of HCT-116 (colon) and NCI-H460 (lung) human cancer cells as determined by the MTT assay in a concentration-dependent manner. At 3.72 ppm, a 27% increase in the number or viable human HCT-116 colon cancer cells was observed; the corresponding increases at 7.50 and 15 ppm were 42 and 69%, respectively. At 60 ppm, DSS doubled the number of HCT-116 cancer cells. For NCI-H460 human lung cancer cells, DSS at 60 ppm increased the cell number by 20%. DEHP did not inhibit the growth of any of the tumor cell lines tested.

However, its presence at 0.5% (w/w) in wasabi powder should be a concern since its toxicological effects are not clear. This is the first report of the tumor cell proliferating activity by a desulfoglucosinolate, the biosynthetic precursor of glucosinolates found in Brassica spp.

Submitted for publication to Nutrition and Cancer.

Introduction

Wasabi (*Wasabia japonica*, Miq. Matsum. syn. *Wasabia pungens*, *Eutrema wasabi*, *Cochlearia wasabi*, *Alliaria wasabi*) belongs to the family Brassicaceae. The plant is harvested for its rhizomes which, upon crushing, developes a characteristic pungent odor and taste. Wasabi is mainly used as a condiment, either in fresh form or as a dry powder, for sushi and other seafood dishes in the Japanese cuisine. Isothiocyanates, the compounds responsible for the pungency of wasabi and other members of the Brassicaceae such as cabbage, brussels sprouts, cauliflower, broccoli, mustard, horseradish are generated *in situ* from glucosinolates by the action of myrosinase enzyme (Fenwick et al., 1983). However, cooking the vegetables deactivates the myrosinase enzyme and the intact glucosinolates undergo hydrolysis by the microflora in the large intestine to yield isothiocyanates (ITCs) and other metabolites (Shapiro et al., 2001). The glucosinolates content reported in the Brassica vegetables ranged from about 1-10 % by dry weight (Fenwick et al., 1983).

Although more than 120 glucosinolates have been identified from different plant families (Fahey et al., 2001), allyl isothiocyanate (AITC), derived from allyl glucosinolate (sinigrin), was identified as the most abundant and pungent compound in wasabi, mustard and horseradish (Masuda et al., 1996). However, a range of other volatile compounds was also formed during the enzymatic degradation of glucosinolates and by rearrangements of the hydrolysed products (Fahey et al., 2001). It has been proposed that the glucosinolate breakdown products protect the plant against pests and predators and act as allelochemicals (Gardiner et al., 1999). The degradation products of glucosinolate were also shown to possess pesticidal, fungicidal, bactericidal and

nematocidal (Fenwick et al., 1983) activities. The glucosinolates in brassica vegetables and their degradation products were studied as inhibitors of platelet aggregation, as antimutagens, anticarcinogens, and apoptotic agents (Kumagai et al., 1994; Conaway et al., 2002). They were also reported to inhibit Phase I enzymes responsible for detoxification and to induce Phase II enzymes, which eliminate electrophilic metabolites formed by Phase 1 enzymes, and reducing their ability to interact with DNA (Zhang and Talalay, 1998).

Several studies indicated that a reduced incidence of cancer was linked to an increased consumption of vegetables such as brassica spp. (Voorrips et al., 2000; Cohen et al., 2000). However, an inverse association was reported for the consumption of brassica vegetables and colon cancer risk in men and women and a positive correlation in women for rectal cancer (Voorrips et al., 2000). The high consumption of brassica vegetables and subsequent reduction of prostate cancer risk was not substantiated (Kristal and Lampe, 2002). The epidemiological effects of glucosinolates and their degradation products were shown to be confounded by genetic polymorphism of Phase I and II enzymes. In a case-control study, broccoli consumption was related to the low incidence of colon cancer in subjects tested positive for a glutathione S-transferase M1 null (GSTM1 null) genotype (Lampe and Peterson, 2002). Also, a study among Singapore Chinese (Seow et al., 2002), a population with high dietary isothiocyanate intake via brassica vegetables, suggested a significantly lowered risk for those individuals who were both GSTM1 and GSTT1 null. Although consumption of glucosinolate-containing vegetables was not reported in dietary and epidemiological studies in Japan, a positive

relationship was observed between radish and cabbage consumption and colorectal cancer (Nakaji et al., 2001; Tajima and Tominaga, 1985).

The use of wasabi in traditional Japanese dishes and its increased consumption in other countries prompted this investigation of the bioactive compounds in wasabi powder and fresh wasabi roots with potential for cell proliferation and growth inhibition. The research objectives also included the comparison of bioactivities of the commercially available wasabi powder and fresh wasabi roots used as a condiment in sushi. In this paper, the potential ill-effects of wasabi powder or fresh root as indicated by in vitro cancer cell assays are reported.

Materials and methods

General Procedures

^{1}H NMR spectra were recorded at 500 MHz on a VRX instrument. ^{13}C NMR spectra were obtained at 125 MHz. Chemical shifts were recorded in $CDCl_3$ or in DMSO-d_6/D_2O and are reported in ppm relative to $CDCl_3$ at 7.24 for ^{1}H NMR and at 77.0 for ^{13}C NMR and relative to DMSO at 2.49 for ^{1}H NMR and at 39.50 for ^{13}C NMR.

A Recycling Preparative Liquid Chromatograph model LC-20, equipped with a model AS-20 fraction collector (both Japan Analytical Industry Co., Tokyo) and a JAIGEL-ODS column (A-343-10, 250 x 20 mm, 10 μm, Dychrom, Santa Clara, CA) was used for the purification of wasabi extracts. Peaks were detected by UV and refractive index detectors attached to a model D-2500 chromato-integrator (Hitachi, Tokyo). Another HPLC system used was equipped with a Waters 600 Controller (Waters Corp.,

Milford, MA), a Waters 717 Autosampler, a Waters 2410 RI detector and a Waters 996 photodiode array detector and an Xterra Prep RP-18 preparative column (10 mm, 19 x 250 mm, Waters Corp.). Data was recorded and processed using Empower Pro (Waters Corp.). Merck Silica gel 60 with a particle size of 35-70 mm and C18 with a particle size of 60 mm (Dychrom, Santa Clara, CA) were used for preparative medium-pressure liquid chromatography (MPLC). For the preparative TLC separation, 250 mm silica gel plates (Analtech, Inc., Newark, DE) were used.

Materials

Wasabi powder, packaged in one kg bags, was purchased from Oriental Gardens, East Lansing, MI. Fresh wasabi roots were purchased from Pacific Farms (Eugene, OR). All solvents were ACS reagent grade and purchased from Spectrum Chemical Co. (Gardena, CA). *Tert*-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Ibuprofen, Naproxen, dimethyl sulphoxide (DMSO), acetic acid and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Celebrexä and Vioxx were physician's professional samples supplied by Dr. S. Gupta. 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), 3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid from Molecular Probes (Eugene, OR). COX-1 and COX-2 enzymes were prepared in the Bioactive Natural Products and Phytoceuticals Laboratory (BNPP) from ram seminal vesicles and prostaglandin endoperoxide H synthase-2 (PGHS-2) cloned insect cell cell lysate, respectively. Fetal bovine serum (FBS) and Roswell Park Memorial Institute-1640

(RPMI-1640) medium were purchased from Gibco BRL (Grand Island, NY). Human tumor cell lines, SF-268 central nervous system (CNS), NCI-H460 (lung), and MCF-7 (breast) were purchased from the National Cancer Institute (NCI, Bethesda, MD) and HCT-116 (colon) from American Type Culture Collection (ATCC, Rockville, MD). All cell lines were maintained at BNPP, Michigan State University.

Extraction of wasabi powder and roots

Wasabi powder (472 g) was sequentially extracted in a glass column with hexane (1L x 5), ethyl acetate (1L x 5) and methanol (1L x 5) and removal of solvent under reduced pressure afforded 24.8, 5, and 58 g of extracts, respectively. Lyophilized wasabi roots (562 g) were extracted in the same manner to yield 2.1, 2.2, and 22 g of extracts, respectively.

Purification of compounds 1 and 2

The ethyl acetate extract (4.3 g) of the wasabi powder was stirred with ethyl acetate and filtered to yield ethyl acetate soluble (4.1 g) and -insoluble (290 mg) fractions. The ethyl acetate soluble fraction (3.6 g) was further purified by silica gel MPLC using chloroform:hexane (200 mL, 1:1, v/v), chloroform (200 mL), chloroform:methanol (500 mL, 1:1, v/v) and methanol (3L) as eluants at 4 mL/min. Fractions were collected in test tubes at 3 min intervals using a fraction collector, combined after TLC analyses to yield nine fractions. Fractions 1 (2.2 g) and 2 (290 mg) showed similar TLC profiles. Fraction 1, a single spot by TLC, compound **1**, was a pale-yellow oil. Fraction 2 (200 mg) was further purified by preparative silica gel TLC (250 μm) using chloroform:hexane (1:1) as the mobile phase to yield compound **1** (145 mg).

The combined yield of compound **1** from wasabi powder was 0.58% by dry weight. The ethyl acetate extract of the wasabi roots (1.9 g) was fractionated by silica gel MPLC using hexane (200 mL), hexane:acetone (150 mL, 10:1, v/v), hexane:acetone (250 mL, 10:2, v/v), hexane:acetone (220 mL, 10:3, v/v), hexane:acetone (220 mL, 10:4, v/v), hexane:acetone (170 mL, 10:5, v/v), hexane:acetone (315 mL 10:7, v/v), hexane:acetone (450 mL, 1:1, v/v), hexane:acetone (280 mL, 7:10, v/v), hexane:acetone (270 mL, 5:10, v/v), hexane:acetone (220 mL, 2:10, v/v), acetone (175mL) and MeOH (300 mL) as eluants at 3.5 mL/min. The fractions were collected in test tubes at 2 min intervals using a fraction collector and combined according to their TLC profiles to yield fifteen fractions. Fraction 2 gave one spot on TLC and gave pure compound **1** (255 mg). Compound **1** was also contained in fraction 1 (440 mg). An aliquot of fraction 1 (100 mg) was fractionated by silica gel PTLC (1000 mm) using $CHCl_3$ as the mobile phase yielded another batch of compound **1** (R_f 0.5, 40 mg). The combined yield of compound **1** from lyophilized wasabi roots was 0.08% by weight.

The methanol extract of the wasabi powder (58 g) was stirred with MeOH, filtered and removal of the solvent yielded MeOH soluble (32.8 g) and insoluble (25.2 g) fractions. The MeOH soluble fraction (10.5 g) was fractionated by C-18 MPLC using MeOH:H_2O (60:40, v/v, 1.2 L) at 4 mL/min followed by MeOH (100%, 2L). The fractions were collected in test tubes at 3 min intervals using a fraction collector and combined according to their TLC profiles to yield nine fractions. An aliquot (101 mg) of fraction 1 (170 mg) was further purified by preparative silica gel TLC (250 mm) using chloroform:methanol:water 5:4:1 (v/v) as the mobile phase. A band collected at R_f =

0.35 (21 mg) was further purified by preparative HPLC (LC-20) with methanol:water (95:5, v/v, 2 mL/min) to yield compound **2** (6 mg) (R_t 41 min). The yield of compound **2** in the wasabi powder was 0.1%. The methanol extract of the wasabi roots (22 g) was processed as in the case of wasabi powder with MeOH to yield MeOH soluble (18 g) and insoluble (4 g) fractions. The MeOH soluble fraction was precipitated with MeOH:H_2O (2:1) and yielded a soluble fraction (16 g) and an insoluble fraction (2 g). An aliquot of the MeOH:H_2O soluble fraction (9.3 g) was further purified by C-18 MPLC. The mobile phases used were MeOH:H_2O (40:60, 480 mL), MeOH:H_2O (60:40,480 mL), MeOH:H_2O (70:30, 180 mL), MeOH:H_2O (80:20 , 240 mL) and MeOH (1 L) at 4 mL/min. The fractions were collected in test tubes at 3 min intervals using a fraction collector and combined according to their TLC profiles to yield five fractions. A portion (7.2 g) of fraction 1 (8.57 g) was purified by C18 MPLC using MeOH:H_2O (1:9, v/v, 500 mL) at 3 mL/min as the mobile phase. The fractions were collected in test tubes at 3 min intervals using a fraction collector and combined according to their TLC profiles to yield five fractions. An aliquot (250 mg) of fraction 3 (300 mg) was further purified by HPLC (Waters) using MeOH:H_2O (5:95, v/v) at 3 mL/min to yield a single peak at 36.1 min, compound **2** (3 mg). The yield of compound **2** in the lyophilized wasabi roots was 0.02%. For bioassays, compound **2**, pure DSS, isolated from the wasabi powder was used.

Compound **1**. ^{1}H-NMR: 7.68 ppm (2H, overlapped doublets, J=5.8 Hz, H-D), 7.50 (2H, overlapped doublets, J=5.8 Hz, H-E), 4.20 (4H, overlapped doublets, J= 6.0 Hz, H-1), 1.65 (2H, m, H-2), 1.23-1.42 (16H, m, H-3,4,5), 0.90 (12H, overlapped triplets,

J=7.4 Hz, H6,8); ^{13}C-NMR: 167.7 ppm (carbon A, quaternary), 132.4 (B, quaternary), 130.9 (E, CH), 128.8 (D, CH), 68.1 (C1, CH_2), 38.9 (C2, CH), 30.3 (C3, CH_2), 28.9 (C4, CH_2), 23.7 (C7, CH_2), 22.9 (C5, CH_2), 14.0 (C6, CH_3), 10.9 (C8, CH_3).

Compound **2**. ^{1}H-NMR: 5.92 ppm (1H, m, H-2'), 5.20 (1H, d, J=17.4 Hz, H3a'), 5.12 (1H, d, J=10.2 Hz, H3b'), 4.73 (1H, d, J=9.7 Hz, H1), 3.66 (1H, dd, J=12.2Hz, 1.5 Hz, H6b), 3.52-2.97 (7H, m, H6a,2,3,4,5,1'); ^{13}C-NMR: 154.6 ppm (C7), 134.0 (C2'), 117.3 (C3'), 81.5 (C5), 81.3 (C1), 78.1 (C3), 72.8 (C2), 69.8 (C4), 60.9 (C6), 36.2 (C1').

Cancer Cell Growth Inhibitory Assay

The tumor cell lines were maintained as adherent cell cultures in RPMI-1640 medium supplemented with 10% FBS, 10 units of penicillin and 10 mg/mL streptomycin at 37 ^{0}C in a humidified incubator at 5% CO_2. Cells were counted using a hemacytometer (Hausser Scientific, Horsham, PA). An aliquot (100 μL) of the supplemented RPMI-1640 medium containing 1000 cancer cells were transferred into 96-well microtiter plates and incubated for 24 h prior to the addition of the test compounds. The test compounds were dissolved in 100 μL of DMSO and diluted with supplemented RPMI-1640 medium to the desired concentration. The test compounds in the appropriate dilution were added to the microtiter wells containing the cells and incubated for 48 h. After the incubation period, 25 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (5 mg MTT per mL phosphate-buffered saline solution) were added to the wells, which were further incubated for 3 h. The medium was aspirated from the wells and the formazan Blue crystals formed were dissolved in 200 μL of DMSO. The optical density of the digest was measured at 570 nm with an

automated microplate reader (Model Elx800, Bio-Tek Instruments, Inc., Winooski, VT). The amount of formazan Blue formed was proportional to the number of viable cells. The change in the number of cells is reported as the mean percentage decrease/increase. Each experiment was carried out in triplicate (Jayaprakasam et al., 2003).

Cyclooxygenase enzymes inhibitory assay

Cyclooxygenase enzymes inhibitory activities of compounds **1** and **2** were evaluated using COX-1 and COX-2. The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction with arachidonic acid as substrate was measured using a Model 5300 biological oxygen monitor (Yellow Spring Instruments, Inc., Yellow Springs, OH). The reaction mixture, consisted of 0.1M Tris, 1.0 mM phenol, 17 mg hemoglobin, the enzyme and 10 μL extract dissolved in DMSO at 1.5% (DMSO alone as solvent control). It was held in a 600 μL micro oxygen chamber (Instech Laboratory, Plymouth Meeting, PA) at 37°C and after 3 min of incubation, 10 μL of arachidonic acid (0.25 mg/0.5 mL of Tris buffer) was added to initiate the reaction. Data was recorded using Quicklog for Windows (Strawberry Tree Inc., Sunnyvale, CA). Ibuprofen, Aspirin, Celebrex, Vioxx and Naproxen at 180, 1.67, 1.67 and 2.52 ppm, respectively, were used as positive controls (Wang et al., 1999).

Lipid peroxidation inhibitory assay

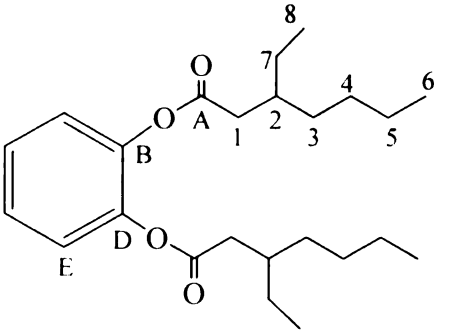
Inhibition of lipid peroxidation was measured by fluorescence spectroscopy using large unilamellar vesicles (LUVs) of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine containing a fluorescent probe (3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid) and reported after 21 min as relative fluorescence compared to a control. For comparison,

tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisolee (BHA) at 1.8, 2.2, and 1.67 ppm, respectively, were used as positive controls. Briefly, the lipid and the probe were dissolved in DMF and evaporated *in vacuo*. The solvent-free mixture was resuspended in 0.15 M NaCl, 0.1 mM EDTA and 0.01 M MOPS (kept over Chelex resin), subjected to ten freeze-thaw cycles using a dry ice-ethanol bath, and extruded through a 100 nm pore size membrane to form the LUVs. The assay buffer used consisted of a mixture of 100 μL HEPES buffer, 200 μL 1 M NaCl, 1.64 mL N_2-sparged water. An aliquot of 20 μL of extracts in DMSO at 2.5% or DMSO (control) and 20 μL of liposome suspension were mixed with the assay buffer to achieve a concentration of 250 ppm of the extract. Peroxidation was initiated by adding 20 μL 0.5 mM $FeCl_2.4H_2O$. Fluorescence was measured using a Turner model 450 fluorometer (Barnstead Thermolyne, Dubuque, IA) at 384 nm and at 0, 1, 3 and then every three min up to 21 min. The rate of decrease of fluorescence is a measure of the rate of peroxidation. Relative fluorescence at 21 min (final value divided by initial value) is reported as a measure of lipid peroxidation; higher values correlate with enhanced inhibition (Arora and Strasburg, 1997).

Results and Discussion

The extraction and purification of wasabi powder and roots resulted in the isolation of compound **1** which was identified as di-(2-ethylhexyl)phthalate (DEHP) (Figure 3.1). The identity of compound **1** was confirmed by spectral experiments and by comparison with published spectral values (Kim and Jonas, 1998). DEHP has been

designated as "reasonable anticipated to be a human carcinogen" (IARC, 2000) based on its ability to increase the incidence of liver tumors in mice and rats (Melnick, 2001), although there is disagreement about its classification as a potential carcinogen (Doull, 1999). DEHP is commonly used in large quantities as a plasticizer (Fromme et al., 2000) but was isolated as a natural product from plants (Lee et al., 2000). The estrogenic activity of DEHP in human and its anti-leukemic and anti-mutagenic (*S. typhimurium*) activities (Lee et al., 2000; Jobling et al., 1995) were reported. Although DEHP did not exhibit any activity in the assays conducted in our laboratory, its presence in substantial quantities in wasabi powder and roots should be a reason for concern.



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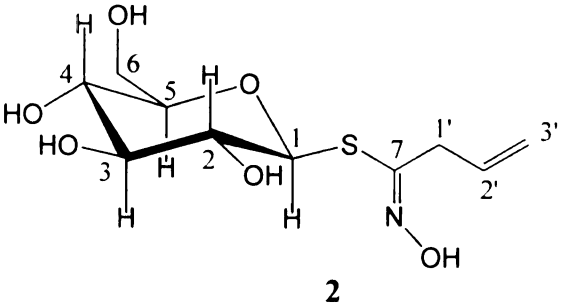


2

Figure 3.1: Structures of compounds **1** and **2**.

The extraction and purification of wasabi powder and roots resulted in the isolation of compound **2**. Its identity was established as desulfosinigrin (DSS) (Figure 3.1) and further confirmed by comparison with previously reported spectroscopic data (Kiddle et al., 2001). In the MTT assays, DSS acted as a growth promoter of HCT-116 (colon) and NCI-H460 (lung) cancer cells (Figure 3.2). The proliferating effect of DSS was strongest for the human HCT-116 colon cancer cells with 27, 42, 69 and 99.5% increase in cell growth at 3.72, 7.50, 15 and 60 ppm, respectively. For lung cancer cells, a 20.6% increase in the number of viable cells was observed at 60 ppm of DSS.

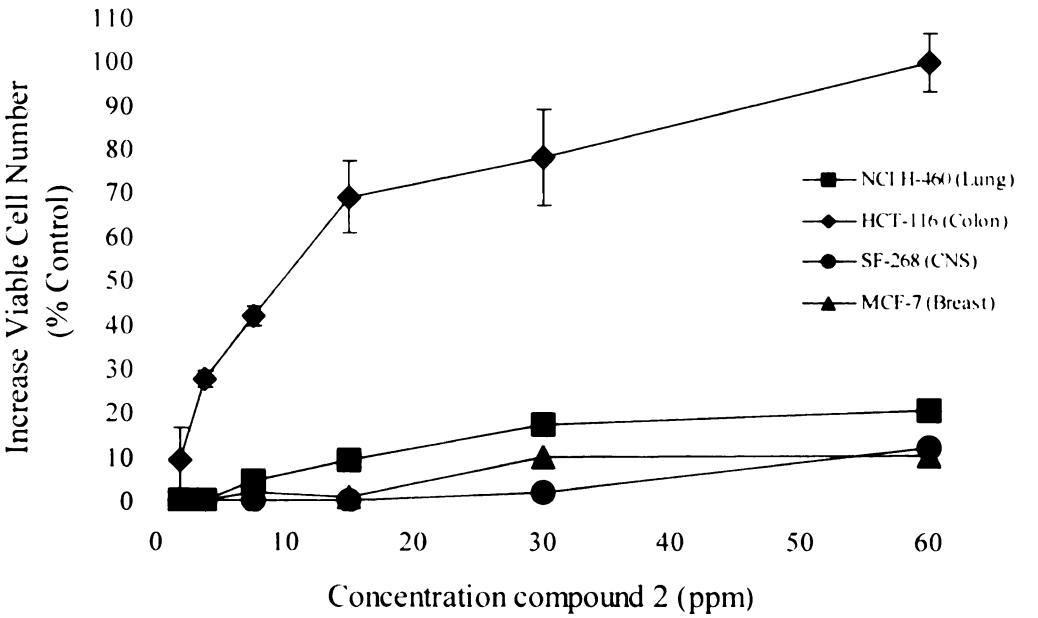


Figure 3.2: Effect of desulfosinigrin (DSS) on the proliferation of human colon (HCT-116), breast (MCF-7), lung (NCI-H460), and CNS (Central Nervous System) (SF-268) cancer cell lines as determined by the MTT assay. The optical density was measured to determine the amount of formazan blue formed by viable cells and compared to the control. The data represents the mean ± SD of the three individual experiments conducted in triplicate.

The cancer cell proliferation showed a strong logarithmic correlation (R^2 >98%. Figure 3.3) with the concentration of DSS for both cancer cell lines. At the concentrations tested, DSS did not show cytotoxicity towards the SF-268 (CNS) and MCF-7 (breast) tumor cells.

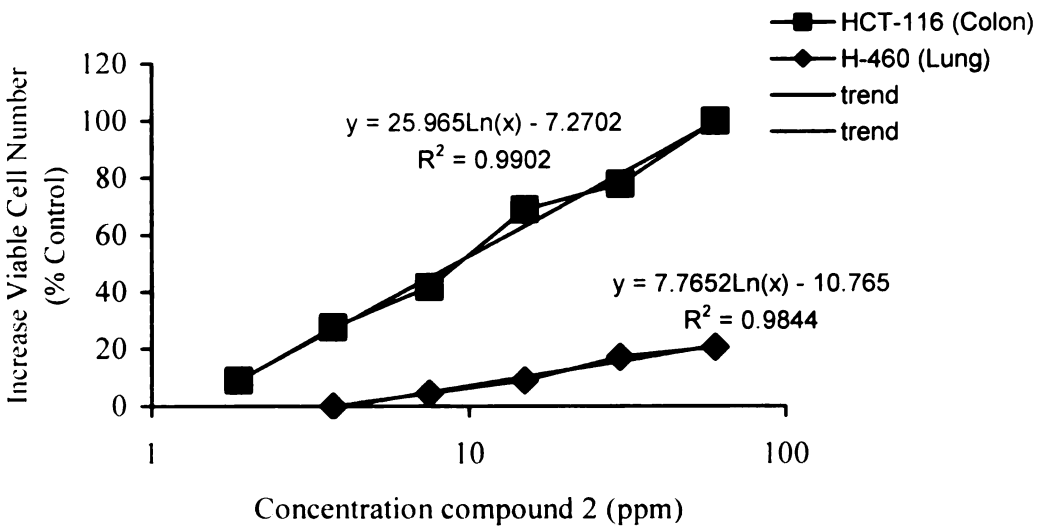


Figure 3.3: Logarithmic correlation between cancer cell proliferation and concentration of DSS for the human colon (HCT-116), and lung (NCI-H460) cancer cell lines.

Rats supplemented with sinigrin have shown an increase in the number of colon cells undergoing apoptosis as well as a reduction in the number of aberrant crypt foci induced by dimethylhydrazine (Smith et al., 1998). Sinigrin has been shown to inhibit hepatocarcinogenesis induced by diethylnitrosamine (Tanaka et al., 1990) and 4-nitroquinoline 1-oxide-induced tongue carcinogenesis (Tanaka et al., 1992) in rats when administered simultaneously with the carcinogens. The reported genotoxic effects of ITCs are ambiguous. They appear to protect rodents against polycyclic aromatic amines.

but they also appear to be potent clastogens in vitro (Musk and Johnson, 1993). On the other hand, AITC, derived from sinigrin, has been shown to cause oxidative damage to DNA, which can lead to inactivation of the p53 tumor suppressor gene, favoring tumor promotion in the carcinogenesis process (Murata et al., 2000). AITC and sinigrin showed cytotoxicity towards a Chinese hamster ovary cell line and induced chromosome aberrations in the same cells (Musk et al., 1995b).

Cyclooxygenase enzymes catalyze the conversion of arachidonic acid to prostaglandins, mediators in the process of inflammation. Two isozymes, COX-1 and COX-2, have been identified. COX-1 is a constitutive enzyme as opposed to COX-2, which is inducible in response to inflammatory stimuli. Inhibition of COX-1 leads to several side effects in humans, including gastric ulceration while differential inhibition of COX-2 can reduce inflammation avoiding these side effects (Laneuville et al., 1994). It has been shown that certain types of precancerous and cancerous cells overexpress COX-2. Epidemiological studies have indicated an association of COX inhibitors with reduced risk of colorectal cancer. Celecoxib, a selective COX-2 inhibitor, was reported to reduce the number of polyps in patients with familial adenomatous polyposis, supporting the notion that COX-2 is a target for cancer prevention and treatment (Subbaramaiah and Dannenberg, 2003). Lipid peroxidation and oxidative stress cause DNA damage via free radicals. This can lead to cell initiation and carcinogenesis. Antioxidants from fruits and vegetables were reported to lower the cancer risk (Kanazawa et al., 2002). Therefore, DSS was tested for COX enzymes and lipid peroxidation inhibitory activities

at 250 ppm, the highest concentration. However, no activity was observed at concentrations tested.

Colorectal cancer is one of the leading causes of cancer death in the United States. In Japan, an upward trend in the age-adjusted mortality rate for colon cancer has been observed over a forty-five year period (Reddy, 2002). The increase in cancer prevalence in Japan has been attributed to the Americanization of the Japanese diet leading to an increased consumption of meat and animal fat and an overall decreased consumption of whole grains and vegetables (Reddy, 2002). It is also possible that proliferation of colon cancer cells may be favored by dietary components found in other sources of food, including vegetables.

Consumption of wasabi, as a condiment with its unique pungent flavor and aroma, is becoming popular in the United States. While the fresh wasabi rhizomes are harder to come by for most people, a wasabi powder is readily available in stores. Wasabi is also served in establishments catering Japanese dishes.

The reports on GSLs, ITCs from brassica spp. and cancer promotion are ambiguous. It is possible that a particular vegetable may contain, concomitantly, chemoprotective and cancer promoting substances. This could be the case for brassica vegetables, including wasabi. The chemopreventive activity of isothiocyanates through the inhibition of Phase I enzymes (Zhang and Talalay, 1998), does not imply that they would not promote tumor growth in the post-initiation phase. Butyl isothiocyanate (BITC) and phenylethyl isothiocyanate (PEITC) were reported to strongly promote urinary bladder carcinogenesis in rats (Hirose et al., 1998).

The reports on the beneficial and adverse effects of GSLs and their enzymatic degradation products refer mainly to GSLs and ITCs and little is known about desulfoglucosinolates (d-GSLs). Since d-GSLs are the biosynthetic precursors of the glucosinolates, it is reasonable to expect that they also contribute to the beneficial and detrimental effects ascribed to the brassica vegetables. To the best of our knowledge, this is the first report of the in vitro cancer cell proliferating effect of a d-GSL. Based on the reports and results from this study, it can be concluded that the relationship between carcinogenesis, tumor promotion, and cancer chemoprotection of GSLs, d-GSLs, and GSL-derived ITCs in brassica spp. is not clear.

Partial funding of this project was provided by the MSU Agricultural Experiment Station and the Center for Plant Products and Technologies.

CHAPTER FOUR

HORSERADISH (*ARMORACIA RUSTICANA*) AND WASABI (*WASABIA JAPONICA*) COMPOUNDS WITH LIPID PEROXIDATION, CYCLOOXYGENASE ENZYME INHIBITORY, AND TUMOR CELL ANTIPROLIFERATIVE ACTIVITIES.

Abstract

Horseradish (*A. rusticana*) and wasabi (*Wasabia japonica*) are glucosinolate-containing brassica vegetables widely used as condiments. Glucosinolates and their enzymatic hydrolysis products have been linked to cancer chemoprotection and tumor growth inhibition. Cyclooxygenase-1 and -2, lipid peroxidation, and human colon (HCT-116), breast (MCF-7), lung (NCI-H460), and CNS (Central Nervous System) (SF-268) cancer cell inhibition assays were used to purify extracts to isolate active compounds from a commercial wasabi powder, horseradish and wasabi rhizomes. Compounds **3-12** were isolated from the horseradish rhizome. Similarly, compounds **3**, **5**, **13, 14, 15** and compounds **3**, **5**, **9**, **16**, **17**, **18**, **19** and **20** were isolated from the wasabi powder and rhizome, respectively. Compounds were identified by GC-MS, ^{1}H- and ^{13}C NMR spectral methods. Compound **3**, a triglyceride, was inactive in all assays. Compound **4**, plastoquinone-9, showed 28% inhibition of COX-1 at 60 ppm while no inhibition for COX-2 was observed. A trend towards cancer cell proliferation was observed for compound **4** for all cancer cell lines except for SF-268 at 3.75-30 ppm. Compounds **5**, 3-acylsitoterols, were not tested due to low solubility. Compounds **6**, 6-O-acyl-β-D-

glucosyl-β-sitosterols, inhibited COX-1 by 32% while no COX-2 inhibition was observed. Compound **7**, 1,2-dilinolenoyl-3-galactosylglycerol, did not inhibit lipid peroxidation. However, it gave 75% inhibition of COX-1 enzyme at 250 ppm. Similarly, compounds **18**, linolenoyloleoyl-3-β-galactosylglycerol, and **20**, 1,2-dipalmitoyl-3-β-galactosylglycerol, inhibited COX-1 enzyme by about 45% at 250 ppm. Furthermore, compound **7** inhibited the proliferation of colon cancer (HCT-116) cells by 21.9, 42.9, 51.2 and 68.4% and of NCI-H460 by 30.0, 38.6, 44.2 and 70.5% at 7.5, 15, 30, and 60 ppm, respectively. Compounds **8**, sucrose, and **9**, β-sitosterol, were not tested. Sinigrin, compound **10**, the major glucosinolate in wasabi and horseradish, inhibited lipid peroxidation by 71% at 250 ppm while it was inactive in the COX and in the cancer cell inhibitory assays. Compounds **11**, gluconasturtiin, and **12**, phosphatidyl choline, were inactive. A mixture of fatty acids, compounds **13**, inhibited COX-1 and COX-2 enzymes by 77 and 93%, respectively, at 250 ppm. Compunds **14**, a mixture of methyl linolenate and methyl oleate, inhibited COX-1 and –2 by 23 and 57%, respectively, at 250 ppm. Compounds **15** (sitosterol 3-O-glucoside), **16** (α-tocopherol), **17** (ubiquinone-10) and **19** (L-tryptophan), respectively, were not tested. These results represent the first report of the isolation of the known inhibitors of cancer cell proliferation MGDGs, such as compounds **7**, **18** and **20** from both wasabi and horseradish. Similarly, it is the first report of their selective COX-1 enzyme inhibitory activity.

Introduction

Horseradish (*Armoracia rusticana*) is a perennial herb of the brassica family. It is cultivated for its rhizomes which are widely used as condiment and as a source of horseradish peroxidase. Horseradish peroxidase is a glycoprotein commonly used as a reagent for clinical diagnosis and analytical immunoassays (Mano, 2001; Yuan and Jiang, 2003). Wasabi (*Wasabia japonica*), also called Japanese horseradish, is another perennial brassica vegetable. Either in fresh form or as a powder, wasabi is commonly used in the Japanese cuisine to garnish traditional dishes, such as sushi and sashimi (Hasegawa et al., 1999).

When the rhizomal tissue of both horseradish and wasabi is mechanically disrupted, for example by mastication, crushing or grating, a characteristic pungent odor and taste develop. Isothiocyanates, generated by the hydrolysis of glucosinolates have been identified as the compounds mainly responsible for the pungency and bitterness of horseradish and wasabi. The hydrolysis of glucosinolates has been shown to be mediated by myrosinase enzyme (Fahey et al., 2001). Since myrosinase and the glucosinolates are stored in different cellular compartments in plants, the enzymatic hydrolysis only occurs after the two have been brought together (Ratzka et al., 2002). The initial step in the formation of isothiocyanates is the actual cleaveage of the thioglucosidic bond to yield glucose and an unstable aglycone. The aglycone then rearranges to form products such as nitriles, epithionitriles and oxazolidine-2-thiones depending on the reaction conditions (Fahey et. al., 2001). However, when the brassica vegetables are cooked before ingestion, myrosinase will be deactivated. It has been shown that glucosinolates can be

hydrolyzed by large intestine microflora to yield isothiocyanates and other compounds (Shapiro et al., 2001).

Sinigrin (2-propenyl glucosinolate, allyl glucosinolate) has been reported as the most abundant glucosinolate in both wasabi and horseradish (Masuda et al., 1996). Gluconasturtiin (2-phenylethyl glucosinolate), the second most abundant glucosinolate in horseradish, is present in trace quantities in wasabi (Masuda, 1996). Both wasabi and horseradish yielded methylsufinylalkyl glucosinolates, although ω-methylthioalkyl glucosinolates, the characteristic wasabi flavor, were found only in wasabi (Ina et al., 1989a; Grob and Matile, 1980). Glucosinolate-derived isothiocyanates were reported to inhibit Phase I enzymes responsible for detoxification. Similarly, they induced Phase II enzymes, which eliminate DNA-interacting electrophilic metabolites formed by Phase I enzymes. The modulation of these enzymes has been shown to play a role in carcinogenesis (Zhang and Talalay, 1998). Fourtehrmore, isothiocyanates have been studied as inhibitors of platelet aggregation, as antimutagens, anticarcinogens, apoptotic agents, and cancer chemoprotectants (Kumagai et al., 1994; Conaway et al., 2002).

Several studies indicated that a reduced incidence of cancer was linked to an increased consumption of vegetables such as brassica spp. (Voorrips et al., 2000; Cohen et al., 2000). In a prospective cohort study, high consumption of vegetables and fruit was correlated with a decreased colon cancer risk, but not for rectal cancer. In the same study, a decreased cancer risk was correlated with high consumption of brassica vegetables in men and women for colon cancer, but the risk was increased in women for rectal cancer (Voorrips et al., 2000). The epidemiological effects of glucosinolates and

their degradation products are not clear. They may be confounded by factors such as genetic polymorphism of Phase I and II enzymes (Lampe and Peterson, 2002). Consumption of yellow-green vegetables has been correlated with reduced cancer risk from epidemiological studies conducted in Japan (Wakai et al., 2000; Sauvaget et al., 2003). It is not clear whether the commonly used term "yellow-green" vegetables includes brassica vegetables (Nakaji et al., 2001;Kono et al., 1988). Consumption of fish may enhance the consumption of isothiocyanates since raw seafood is often consumed with wasabi, but this relationship is no addressed in epidemiological studies. No epidemiological evidence is available to correlate the consumption of wasabi or horseradish to cancer protection or cancer risk except one anecdotal account implicating wasabi in a single case of familial stomach cancer in Japan (Tanida et al., 1991).

Wasabi powder fed to Wistar WKY male rats protected against the development of N-methyl-N-nitroso-N-notroguanidine (MNNG)-induced gastrointestinal tumors (Tanida et al., 1991). Isothiocyanates isolated from wasabi have been shown to prevent the development of cancer in vivo after application of cancer initiators or promoters, such as 9,10-dimethyl-1,2-benzanthracene, 12-O-tetradecanoylphorbol-13-acetate, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Fuke et al., 1997; Yano et al., 2000). In general, ITCs have been shown to inhibit cancer when administered prior to or during dosification with the carcinogen, but they did not perform as well when administered subsequently to the carcinogen treatment. In addition, the chemoprotective effects of isothiocyanates seemed to be highly specific with regards to the type of cancer they are able to prevent (Hecht, 1999). However, tumor promotion by isothiocyanates was also

reported. Benzyl isothiocyanate (BITC) and phenylethyl isothiocyanate (PEITC) at 0.1% in the diet were reported to strongly promote urinary bladder carcinogenesis in rats pretreated with diethylnitrosamine and N-butyl-N-(4)-hydroxybutyl)nitrosamine (Hirose et al., 1998). Although isothiocyanates may prevent carcinogenesis in the initiation phase, they could promote tumor growth in the post-inititation phase.

The assumption by the general public that wasabi and horseradish possess beneficial phytoceuticals prompted this investigation of the bioactive compounds in horseradish and wasabi rhizomes, including a commercial wasabi powder with potential for inhibiting lipid peroxidation, cyclooxygenase enzymes, and cell proliferation.

Materials and Methods

Plant Material

Fresh horseradish roots were purchased from Goodrich's ShopRite Supermarket, East Lansing, MI. The wasabi powder was purchased from Oriental Gardens, East Lansing, MI and fresh wasabi roots were purchased from Pacific Farms (Eugene, OR).

General Procedures

^{1}H NMR spectra were recorded at 300 and 500 MHz on Varian INOVA (for 300) and VRX (for 500) instruments. ^{13}C NMR spectra were obtained at 75 and 125 MHz, respectively. ^{1}H NMR chemical shifts are reported in ppm relative to $CDCl_3$ at 7.24, DMSO-d_6 at 2.49, D_2O at 4.80 and CD_3OD at 3.31 ppm, respectively. ^{13}C NMR, chemical shifts are reported in ppm relative to $CDCl_3$ at 77.0, DMSO at 39.50 and CD_3OD at 49.0, respectively.

A Recycling Preparative Liquid Chromatograph model LC-20, equipped with a model AS-20 fraction collector (both Japan Analytical Industry Co., Tokyo) and a JAIGEL-ODS column (A-343-10, 250 x 20 mm, 10 μm, Dychrom, Santa Clara, CA) was used for the purification of extracts. Peaks were detected by UV and refractive index detectors attached to a model D-2500 chromato-integrator (Hitachi, Tokyo). A second HPLC system (Waters Corp) used was equipped with a Controller, a 717 Autosampler, a 2410 RI detector and a 996 photodiode array detector and an Xterra Prep RP-18 preparative column (10 μm, 19 x 250 mm, Waters Corp.). Data were recorded and processed using Empower Pro (Waters Corp., Milford, MA). Merck Silica gel 60 with a particle size of 35-70 μm and C18 with a particle size of 60 μm (Dychrom, Santa Clara, CA) were used for preparative medium-pressure liquid chromatography (MPLC). For the preparative TLC separation, 1000, 500, and 250 μm silica gel plates (Analtech, Inc., Newark, DE) were used. Gas chromatographic-mass spectrometric (GC-MS) analysis was carried out using a HP 6890 system equipped with an electron capture detector operating at 250 °C, an HP-5MS (30 m x 250 μm x 0.25 μm) column and a 7673 model injector operating at 250 °C in the splitless mode; the injection volume was 1.0 μL. Helium was used as carrier gas at 0.8 mL/min. The temperature was started at 50 °C, held for 2 minutes, elevated to 250 °C at 10 °C/min and held until the end of the analysis. The quadrupole mass filter was set to scan from 40 to 550 m/z units. All samples were dissolved in hexane at a concentration of 1 mg/mL.

Materials

All solvents were ACS reagent grade and purchased from Spectrum Chemical Co. (Gardena, CA). *Tert*-butylhydroquinone (TBHQ), butylated hydroxyanisolee (BHA), butylated hydroxytoluene (BHT), Ibuprofen, Naproxen, dimethyl sulphoxide (DMSO), acetic acid and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Celebrex and Vioxx were physician's professional samples supplied by Dr. S. Gupta. 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), 3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid from Molecular Probes (Eugene, OR). COX-1 and COX-2 enzymes were prepared in the Bioactive Natural Products and Phytoceuticals Laboratory (BNPP) from ram seminal vesicles and prostaglandin endoperoxide H synthase-2 (PGHS-2) cloned insect cell cell lysate, respectively. Fetal bovine serum (FBS) and Roswell Park Memorial Institute-1640 (RPMI-1640) medium were purchased from Gibco BRL (Grand Island, NY). Human tumor cell lines, SF-268 central nervous system (CNS), NCI-H460 (lung), and MCF-7 (breast) were purchased from the National Cancer Institute (NCI, Bethesda, MD) and HCT-116 (colon) from American Type Culture Collection (ATCC, Rockville, MD). All cell lines were maintained at BNPP, Michigan State University.

Cancer Cell Growth Inhibitory Assay

The tumor cell lines were maintained as adherent cell cultures in RPMI-1640 medium supplemented with 10% FBS, 10 units of penicillin and 10 mg/mL streptomycin at 37 ^{0}C in a humidified incubator at 5% CO_2. Cells were counted using a

hemacytometer (Hausser Scientific, Horsham, PA). An aliquot (100 µL) of the supplemented RPMI-1640 medium containing 1000 cancer cells were transferred into 96-well microtiter plates and incubated for 24 h prior to the addition of the test compounds. The test compounds were dissolved in 100 µL of DMSO and diluted with supplemented RPMI-1640 medium to the desired concentration. The test compounds in the appropriate dilution were added to the microtiter wells containing the cells and incubated for 48 h. After the incubation period, 25 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (5 mg MTT per mL phosphate-buffered saline solution) were added to the wells, which were further incubated for 3 h. The medium was aspirated from the wells and the formazan blue crystals formed were dissolved in 200 µL of DMSO. The optical density of the digest was measured at 570 nm with an automated microplate reader (Model ELx800, Bio-Tek Instruments, Inc., Winooski, VT). The amount of formazan blue formed was proportional to the number of viable cells. The change in the number of viable cells is reported as the mean percentage decrease/increase with respect o the DMSO solvent control. Each experiment was carried out in triplicate (Jayaprakasam et al., 2003).

Cyclooxygenase enzymes inhibitory assay

Cyclooxygenase enzymes inhibitory activities were evaluated using COX-1 and COX-2 enzymes. The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction with arachidonic acid as substrate was measured using a Model 5300 biological oxygen monitor (Yellow Spring Instruments, Inc., Yellow Springs, OH). The reaction mixture, consisted of 0.1M Tris, 1.0mM phenol, 17 mg

hemoglobin, the enzyme, 10 μL test sample dissolved in DMSO at 1.5% (DMSO alone as solvent control). It was held in a 600 μL micro oxygen chamber (Instech Laboratory, Plymouth Meeting, PA) at 37°C. After 3 min of incubation, 10 μL of arachidonic acid (0.25 mg/0.5 mL of Tris buffer) was added to initiate the reaction. Data was recorded using Quicklog for Windows (Strawberry Tree Inc., Sunnyvale, CA). Ibuprofen, Aspirin, Celebrex, Vioxx and Naproxen at 2.06, 180, 1.67, 1.67 and 2.52 ppm, respectively, were used as positive controls (Wang et al., 1999).

Lipid peroxidation assay

Inhibition of lipid peroxidation was measured by fluorescence spectroscopy using large unilamellar vesicles (LUVs) of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine containing a fluorescent probe (3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid) and reported after 21 min as relative fluorescence compared to a control. For comparison, tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisolee (BHA) at 1.8, 2.2, and 1.67 ppm, respectively, were used as positive controls. Briefly, the lipid and the probe were dissolved in DMF and evaporated *in vacuo*. The solvent-free mixture was resuspended in 0.15 M NaCl, 0.1 mM EDTA and 0.01 M MOPS (kept over Chelex resin), subjected to ten freeze-thaw cycles using a dry ice-ethanol bath, and extruded through a 100 nm pore size membrane to form the LUVs. The assay buffer used consisted of a mixture of 100 μL HEPES buffer, 200 μL 1 M NaCl, 1.64 mL N_2-sparged water. An aliquot of 20 μL of test sample in DMSO at 2.5% or DMSO (control) and 20 μL of liposome suspension were mixed with the assay buffer to achieve a concentration of 250 ppm of the extract. Peroxidation was initiated by adding 20 μL 0.5

mM $FeCl_2.4H_2O$. Fluorescence was measured using a Turner model 450 fluorometer (Barnstead Thermolyne, Dubuque, IA) at 384 nm and at 0, 1, 3 and then every three min up to 21 min. The rate of decrease of fluorescence is a measure of the rate of peroxidation. Relative fluorescence at 21 min (final value divided by initial value) is reported as a measure of lipid peroxidation; higher values correlate with enhanced inhibition (Arora and Strasburg, 1997).

Derivatization of samples for GC-MS analysis

Diazomethane was prepared as follows: KOH (15 g) was dissolved in water (25 mL) cooled in an ice bath. Diethyl ether (100 mL) and then N-nitroso-N-methylurea (1 g) were slowly added to the KOH solution. Using a separatory funnel, the aqueous layer was discarded. The diazomethane in ether was washed with cold water and the aqueous layer was discarded (Furniss et al., 1978). Samples (2 mg) were hydrolyzed with 5% sodium hydroxide in MeOH (1 mL) for 5 min and then acidified with 6N HCl in MeOH. After evaporation of the solvent, the product was dissolved in anhydrous ether and freshly prepared diazomethane was added until the solution turned yellow. Ether was removed and the residue dissolved in hexane to yield a concentration of 1 mg/mL. Authentic samples of decanoic, undecanoic, nonanoic, octanoic, lauric, docecenoic, myristic, myristoleic, pentadecenoic, pentadecanoic, palmitoleic, heptadecenoic, heptadecanoic, oleic, linoleic, vaccenic, stearic, nonadecenoic, eicosadienoic, eicosenoic, erucic, tricosanoic and lignoceric acids were also methylated using diazomethane. All fatty acid methyl esters, along with commercially available methyl linolenate and methyl palmitate, were used as GC-MS standards during the analysis of the wasabi samples.

Extraction of wasabi powder, wasabi rhizomes, and horseradish rhizomes

Lyophilized horseradish rhizomes (345 g) were sequentially extracted in a glass column with hexane (1 L x 5), ethyl acetate (1 L x 5), and methanol (1 L x 5), and removal of solvents under reduced pressure afforded extracts IV (1.76 g), V (1.31 g), and VI (51 g), respectively (Chapter Two) for extracts I-III and X-XIV). Wasabi powder (472 g) was sequentially extracted in a glass column with hexane (1L x 5), ethyl acetate (1L x 5) and methanol (1L x 5), and removal of the solvents under reduced pressure yielded extracts VII (24.8 g), VIII (5 g), and IX (58 g), respectively. Wasabi fresh rhizomes (643 g) were sequentially extracted with methanol (1L x 5), ethyl acetate (1L x 5) and afforded extracts XIII and XIV, respectively. The numbering of the extracts corresponds to the extraction protocols described in Chapter 2 (Figure 2.1).

Purification and isolation of compounds from horseradish rhizomes

The hexane extract of horseradish (IV, 1.7 g) was partitioned with hexane and MeOH to produce a hexane soluble fraction 1 (1.3 g). Fraction 1 (300 mg) was further purified by preparative silica TLC (1000 μm) with hexane:acetone (25:1, v/v) as the mobile phase. Three bands were collected and yielded compounds **3** (R_f 0.3, 235 mg), **4** (R_f 0.43, 7 mg), and **5** (R_f 0.6, 45 mg), respectively.

The ethyl acetate extract of horseradish (V) was separated into a MeOH soluble fraction 2 (610 mg) and an insoluble residue (30 mg). Fraction 2 (610 mg) was partitioned with hexane and MeOH and yielded a MeOH soluble fraction 3 (570 mg) and the hexane soluble fraction 4 (40 mg). Fraction 3 (325 mg) was further purified by

preparative silica (1000 μm) TLC using hexane:acetone (3:1, v/v) as the mobile phase and yielded fractions 5 (R_f 0.1, 200 mg), and 6 (R_f 0.25, 32 mg). Fraction 6 (15 mg) was submitted to further preparative silica TLC (250 μm) purification with hexane:acetone (3:1, v/v) as the mobile phase to afford compound **6** (R_f 0.125, 10 mg).

Fraction 5 (180 mg) was further purified by preparative silica TLC (250 μm) using MeOH:$CHCl_3$ (1:6, v/v) as the mobile phase and yielded compound **7** (R_f 0.8, 20 mg).

The MeOH extract of horseradish (VI, 46 g) was stirred with $CHCl_3$ to yield a soluble fraction 7 (5.1 g) and an insoluble fraction **8** (41 g). An aliquout of fraction 7 (5 g) was further separated into a $CHCl_3$:MeOH (1:1, v/v) soluble fraction 9 (4.7 g). Fraction 9 (3.5 g) was further purified by preparative silica MPLC using $CHCl_3$ (180 mL), $CHCl_3$:MeOH (10:0.5, v/v, 180 mL), $CHCl_3$:MeOH (10:1, v/v, 180 mL), $CHCl_3$:MeOH (10:2, v/v, 360 mL), $CHCl_3$:MeOH (10:3, v/v, 1 L) and MeOH (540 mL) as the mobile phases at a flow rate of 3 mL/min. Collection of fractions occurred in 2 min intervals using a fraction collector. Fractions were combined according to their TLC profile and yielded fractions 10 (280 mL, 150 mg), 11 (170 mL, 28 mg), 12 (150 mL, 180 mg), 13 (230 mL, 340 mg), 14 (160 mL,350 mg), 15 (460 mL, 815 mg) and 16 (1L, 1.4 g). Compound **8** (220 mg) was isolated as a $CHCl_3$ insoluble portion from fraction 15 (815 mg).

Fraction 11 (25 mg) was further purified by preparative silica (250 mm) TLC using hexane:acetone (5:1, v/v) as the mobile phase and developed twice to yield compound **9** (R_f 0.5, 6.2 mg).

Fraction 16 (1.2 g) was separated into a $CHCl_3$:MeOH (1:1, v/v) soluble fraction 17 (860 mg). Fraction 17 (480 mg) was further purified by preparative silica TLC (1000 μm) with ACN:H_2O (9:1, v/v) as the eluant. Two bands collected gave fractions 18 (R_f 0.280 mg) and 19 (R_f 0.15, 50 mg). Fraction 19 (30 mg) was purified by preparative silica (250 μm) TLC using ACN:H_2O (8.5:1, v/v) as the mobile phase and yielded compounds **10** (8 mg) and **11** (6.3 mg).

Fraction 18 (200 mg) was further purified by preparative silica (1000 μm) TLC with $CHCl_3$:MeOH:H_2O (5:3:0.5, v/v) as the mobile phase. A single band was isolated, fraction 20 (R_f 0.6, 55 mg) and further purified (30 mg) by preparative silica TLC (250 μm) using $CHCl_3$:MeOH:H_2O (5:3:0.5, v/v) as the mobile phase to afford compound **12** (R_f 0.5, 9.6 mg).

Compound **3** (pale yellow oil): ^{1}H-NMR ($CDCl_3$): 5.35 (10 H), 5.25 (1H), 4.28 (2H), 4.13 (2H), 2.79 (6H), 2.30 (6H), 2.05 (8H), 1.60 (6H), 1.22-1.37 (40H), 0.96 (3H), 0.87(6H); ^{13}C-NMR ($CDCl_3$): 173.12, 173.09, 172.68 , 131.86, 130.13, 130.11, 128.23, 128.17, 127.72, 127.71, 127.07, 68.87, 62.02, 34.12, 33.98, 33.95, 31.87, 31.72, 31.46, 28.92-29.70, 25.57, 25.43, 24.81, 24.77, 22.62, 66.59, 20.49, 14.19, 14.03, 13.98.

Compound **4** (pale yellow oil): ^{1}H-NMR ($CDCl_3$): 6.44 (1H, t., J = 1.8 Hz, H6), 5.07-5.16 (9H, b. t., J = 7.2 Hz, H2', 6', 10', 14', 18', 22', 26', 30', 34'), 3.10 (2H, d., J = 7.2 Hz, H1'), 2.04 (3H, s, H3'), 2.01 (3H, s, H2'), 2.06 and 1.96 (32H, m, 4', 5', 8', 9', 12', 13', 16', 17', 20', 21', 24', 25', 28', 29', 32', 33'), 1.66 (3H, d, J = 1.0, Hz, H36'), 1.60 (3H, d, J = 1.0 Hz, H3"), 1.58 (24H, b. s. H7", 11", 15", 19", 23", 27", 31", 35"); ^{13}C-NMR ($CDCl_3$): 187.79 (C1), 187.65 (C4), 147.99 (C5), 140.96 (C3), 140.57 (C2), 139.68,

135.42 (C3'), 134.88-134.93 (C7', 11', 15', 19', 23', 27', 31'), 132.04 (C6), 131.22 (C35'), 124.2-124.4 (C10', 14', 18', 22', 26', 30', 34'), 123.82 (C6'), 118.18 (C2'), 39.69-39.75 (C5', 9', 13', 17', 21', 25', 29', 33'), 27.41 (C1'), 26.69-26.78 (C4', 8', 12', 16', 20', 24', 28'), 26.51 (C32'), 25.67 (C36'), 17.66 (35"), 16.14 (C3"), 15.90-16.06 (C7", 11", 15", 19", 23", 27", 31"), 12.35 (C3"'), 12.01 (C2"').

Compound **5** (colorless oil): ^{1}H-NMR ($CDCl_3$): 5.25-5.45 (6H, m, 9', 10', 12', 13', 15', 16'), 5.10 (1H, m, H6), 4.60 (1H, m, H3), 2.78 (4H, b. t., J = 5.6 Hz, H11', 14'), 2.20-2.35 (4H, overlapping t. and d., J = 7.6 Hz, H2', 4), 0.90-2.10 (H1, 2, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 24, 25, 28, 3', 4', 5', 6', 7', 8', 17'), 1.00 (3H, s, H19), 0.95 (3H, t., J = 7.5 Hz, H18), 0.90 (3H, d., J = 7.6, H21), 0.82 (3H, d., J = 7.5 Hz, H29), 0.80 (3H, d., J = 7.5 Hz, H27), 0.78 (3H, d., J = 7.5 Hz, H26), 0.60 (3H, s, H18); ^{13}C-NMR ($CDCl_3$):173.20 (C1'), 139.72 (C5), 131.93 (C16'), 130.26 (C9'), 128.27 (C12'), 128.26 (C13'), 127.72 (C10'), 127.13 (C15'), 122.56 (C6), 73.67 (C3), 56.71 (C14), 56.07 (C17), 50.06 (C9), 45.87 (C24), 42.32 (C13), 39.74 (C4), 38.18 (C12), 37.02 (C1), 36.61 (C10), 36.15 (C20), 34.69 (C2'), 33.97 (C22), 32.20 (C7), 31.91 (C8), 31.86 (C2), 29.56 (C7'), 29.0-29.4,(C4', 5', 6') 28.23 (C16), 27.83, 27.15 (C8'), 25.61 (C11'), 25.53 (C14'), 25.03 (C3'), 24.29 (C15), 23.10 (C28), 21.04, 21.03 (C11) 20.54, 19.79 (C19), 19.30 (C27), 19.05 (C26), 18.78 (C21), 14.25 (C18'), 11.97 (C18), 11.84 (C29).

Compoound **6** (colorless solid): ^{1}H-NMR ($CDCl_3$): 5.25-5.40 (7H, m, H6, 9", 10", 12", 13", 15", 16"), 4.43 (1H, dd, J = 12.0, 4.0, H6a), 4.38 (1H, d, J = 7.6 Hz, H1'), 4.25 (1H, dd, J = 12.0, 2.0, H6'b), 3.50-3.60 (2H, m, H5', 3'), 3.44 (1H, m, H3), 3.30-3.40 (2H, m, H2', 4'), 2.80 (4H, b. t., H11", 14"), 2.35 (4H, m, H2", 4), 2.00 (4H, m, H8", 17"),

0.80-1.90, 0.98 (3H, s, H19"), 0.95 (3H, t., J = 7.5 Hz, H18"), 0.90 (3H, d., J = 7.6, H21"), 0.82 (3H, d., J = 7.5 Hz, H29"), 0.80 (3H, d., J = 7.5 Hz, H27"), 0.78 (3H, d., J = 7.5 Hz, H26"), 0.60 (3H, s, H18"); ^{13}C-NMR ($CDCl_3$): 174.41 (C1"), 140.41 (C5), 130.25 (C16"), 130.03 (C9"), 129.96 (C13"), 129.86 (C12"), 128.37 (10"), 128.30 (15"), 122.14 (C6), 101.27 (C1'), 79.61 (C3), 76.22 (C3'), 74.05 (C5'), 73.74 (C2'), 70.38 (C4'), 63.32 (C6'), 56.88 (C14), 56.25 (C17), 50.33 (C9), 46.02 (C24), 42.43 (C13), 39.88 (C12), 39.00 (C4), 37.36 (C1), 36.81 (C10), 36.18 (C20), 34.28 (C2"), 34.09 (C22), 31.99 (C2), 31.93 (C7, 8), 29.17-29.90 (C28, 3", 4", 5" 6"), 28.24 (C16), 27.26 (C8"), 26.39 (C23), 25.68 (C11", 14"), 24.33 (C15), 23.21 (C28), 22.69 (C17"), 21.15 (C11), 19.79 (C19), 19.36 (C27), 19.11 (C26), 18.23 (C21), 14.05 (C18"), 12.00 (C18), 11.89 (C29).

Compound **7** (colorless oil): ^{1}H-NMR (CD_3OD): 5.25-5.45 (13H, m, H2, 9', 10', 12', 13', 15', 16', 9", 10", 12", 13", 15", 16"), 4.43 (1H, dd, J = 12.1, 3.0 Hz, H1a), 4.23 (1H, d, J = 7.5, H1"'), 4.20 (1H, dd, J = 12.1, 6.7 Hz, H1b), 3.82 (1H, dd, J = 11.0, 5.5 Hz, H3b), 3.69-3.80 (3H, overlapping m, H3"'a, 6"'a, 6"'b), 3.45-3.55 (3H, overlapping m, 5"', 3"', 2"'), 2.78 (8H, b. t., H11', 14', 11", 14"), 2.30 (4H, overlapping t., H2', 2"), 1.98-2.10 (8H, m, H17', 17", 8', 8"), 1.60 (4H, m, H3', 3"), 1.20-1.40 (16H, H4', 4", 5', 5", 6', 6"), 0.95 (6H, t, J = 7.6 Hz, 18', 18"); ^{13}C-NMR ($CDCl_3$): 173.67 (C1'), 173.34 (C1"), 131.93 (C16', C16"), 130.17 (C9', 9"), 128.31 (C12', 12"), 128.22 (C13', 13"), 127.99 (C10', 10"), 127.12 (C15', C15"), 104.03 (C1"'), 74.62 (C5"'), 73.51 (C3"'), 71.46 (C2"'), 70.23 (C2), 69.29 (C4"'), 68.22 (C3), 62.78 (C1), 62.25 (C6"'), 34.26 (C2"), 34.11 (C2'), 29.58 (C7', 7"), 29.04-29.29 (C4', 4", 5', 5", 6', 6"), 27.18 (C8', 8"), 25.61 (C11', 11"), 25.52 (C14', 14"), 24.85 (C3', 3"), 20.50 (C17', 17"), 14.17 (C18', 18").

Compound **8** (white solid): ^{1}H-NMR (DMSO): 5.16 (2H, overlapping d, OH, H1'), 5.01 (1H, d, OH), 4.74 (3H, m, 3OHs), 4.46 (1H, d, OH), 4.36 (2H, overlapping d, OHs), 3.87 (1H, t, J = 8.1 Hz, H4), 3.76 (1H, dd, H5'), 3.64 (1H, overlapping ddd, H5), 3.52-3.60 (4H, m, CH_2-6, CH_2-6'), 3.47 (2H, m, 3', OH), 3.39 (2H, d, OH), 3.34 (2H, s, H1), 3.17 (2H, overlapping d, OH, H3), 3.11 (1H, dd, H4'); ^{13}C-NMR (DMSO): 104.05 (C2), 91.75 (C1'), 82.59 (C5), 77.11 (C3), 74.34 (C4), 72.90 (C5'), 72.82 (C3'), 71.65 (C2'), 69.90 (C4'), 62.13 (C1, C6), 60.54 (C6').

Compound **9** (white solid): ^{1}H-NMR ($CDCl_3$): 5.31 (1H, d, J = 3.5, H6), 3.45 (1H, m, H3), 2.24 (2H, overlapping t. and d., J = 7.6 Hz, H 4), 0.90-2.10 (27H, m, H1, 2, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 24, 25, 28), 1.00 (3H, s, H19), 0.95 (3H, t., J = 7.5 Hz, H18), 0.90 (3H, d., J = 7.6, H21), 0.82 (3H, d., J = 7.5 Hz, H29), 0.78 (3H, d., J = 7.5 Hz, H27), 0.78 (3H, d., J = 7.5 Hz, H26), 0.60 (3H, s, H18); ^{13}C-NMR ($CDCl_3$): 140.21 (C5), 130.25 (C16"), 121.62 (C6), 71.77 (C3), 56.83 (C14), 56.23 (C17), 50.24 (C9), 46.95 (C24), 42.37 (C13), 39.85 (C12), 38.90 (C4), 37.32 (C1), 36.54 (C10), 36.19 (C20), 34.04 (C22), 31.98 (C2), 31.94 (C7), 31.70 (C8), 29.31 (25), 28.22 (C16), 26.31 (C23), 24.31 (C15), 23.17 (C28), 21.12 (C11), 19.78 (C19), 19.37 (C27), 19.09 (C26), 18.27 (C21), 11.99 (C18), 11.86 (C29).

Compound **10** (white solid): ^{1}H-NMR (DMSO): 5.93 (1H, m, H2'), 5.49 (b. s, OH), 5.21 (1H, dd, J = 1.8, 17.0 Hz, H3'a), 5.12 (1H, J= 1.6, 9.8 Hz, H3'b), 4.74 (1H, d, J = 9.8 Hz, H1), 3.68 (1H, J = 11.8 Hz, H6b), 3.45 (1H, dd, J = 17.0, 7.2 Hz, H6a), 3.0-3.4 (6H, H2, 3, 4, 5, CH_2-1').

Compound **11** (white solid): ^{1}H-NMR (DMSO): 7.28 (4H overlapping dd, J = 3.8 Hz, H2', 3', 5', 6'), 7.18 (1H, overlapping dd, H4') 5.53 (b. s, OH), 5.35 (b. s, OH), 5.16 (b. s, OH), 4.76 (1H, d, J = 9.8 Hz, H1), 3.61 (1H, b. d, J = 11.8 Hz, H6b), 3.30-3.40 (H6a, CH_2-8), 3.23 (t, J =8.6 Hz), 3.0-3.16 (3H, m, H2, 3, 4), 2.78-2.92 (3H, m, H5, CH_2-8); ^{13}C-NMR (DMSO): 154 (C7), 140.94 (C1'), 128.22 (C2', 3', 5', 6'), 125.86 (C4'), 81.92 (C1), 81.22 (C5), 78.05 (3), 72.86 (C2), 68.75 (C4), 60.79 (C6), 33.38 (C8), 32.88 (C9).

Compound **12**: ^{1}H-NMR (DMSO): 5.15-5.40 (8H, m, H9', 10', 12', 13', 15', 16', 9", 10"), 5.05 (1H, m, H2), 4.28 (1H, dd, J = 12.0, 2.8, H1a), 4.07 (1H, dd, J = 12.0, 7.2, H1b), 4.00 (2H, b. m., H1"'), 3.70 (2H, dd, J = 11.2, 6.6, H3), 3.50 (2H, b. t., J = 4.9 Hz, H2"'), 3.12 (9H, s, H3"'), 2.75 (4H, overlapping t., J = 5.9 Hz, H11', 14'), 2.25 (4H, overlapping t., H2', 2"), 2.00 (6H, m, H8', 17', 8", 10"), 1.50 (4H, m, H3', 3"), 1.15-1.35 (28H, b. s., H4', 5', 6', 7', 4", 5", 6", 7", 12", 13", 14", 15", 16", 17"), 0.92 (3H, t., J = 7.5 Hz, H18'), 0.84 (3H, b. t., H18"); ^{13}C-NMR (DMSO): 172.34 (C1"), 172.07 (C1'), 131.33-126.79 (C9', 10', 12', 13', 15', 16', 9", 10", 12", 13", 15", 16"), 70.43 (C2), 65.50 (C2"'), 62.32 (C1, 3), 58.11 (C1"'), 53.07 (C3"'), 33.44 (C2'), 33.25 (C2"), 30.91 (C16"), 28.06-28-63 (C4', 5', 6', 7', 8'), 24.27 (C3'), 24.21 (C3"), 21.85, 21.72, 19.84, 13.84(C18'), 13.64 (C18").

Purification and isolation of compounds from wasabi powder

The hexane extract of the wasabi powder (VII) (15 g) was fractionated by preparative silica MPLC using hexane (200 mL), hexane:acetone (4:1, v/v, 250 mL),

hexane:acetone (3:1, v/v, 300 mL), hexane:acetone (2:1, v/v, 300 mL), hexane:acetone (1:1, v/v, 300 mL) and acetone (500 mL) at 3 mL/min as the mobile phases. Collection of fractions in 2 min intervals using a fraction collector started 20 min after elution began. Fractions were combined according to their TLC profile. Fractions 21 (18 mg), 22 (22 mg), 23 (125 mg), 24 (7.9 g), 25 (230 mg), 26 (1.27g) and 27 (2.78 g) were collected. Fraction 24 (330 mg) was further purified by preparative silica TLC (500 μm) with hexane acetone (20:1, v/v) as the mobile phase. A single band was collected (R_f 0.8, 11 mg) and found to be by NMR spectral analysis identical to compound **5**.

Fraction 24 (900 mg) was submitted to preparative silica (500 μm) TLC with hexane:acetone (20:1, v/v) as the mobile phase. Fraction 27 (R_f = 0.5; 786 mg) was collected as a single band and purified (33 mg) by silica (250 μm) TLC with hexane:acetone (10:1, v/v) as the eluant to yield a single band (R_f 0.6, 20 mg) which was identical to compound **3**, as confirmed by its spectroscopic data.

An aliquot of fraction 24 (503 mg) was further purified by silica MPLC with $CHCl_3$:acetone 10:0.5 at 3 mL/min. Collection of at 2 min intervals yielded fraction 28 (40 mL, 410 mg) and compound **13** (150 mL, 62 mg).

The methanol extract of the wasabi powder (IX, 58 g) was stirred with MeOH, filtered and removal of the solvent yielded a MeOH soluble fraction 30 (32.8 g). It was fractionated (10.5 g) by C-18 MPLC using MeOH:H_2O (60:40, v/v, 1.2 L) at 4 mL/min followed by MeOH (100%, 2L). The fractions were collected in test tubes at 3 min intervals using a fraction collector. No collection occurred during the first 20 min. Fractions were combined according to their TLC profiles to yield fractions 31(170 mg),

32 (1.6 g), 33 (2.9 g), 34 (1.4 g), 35 (180 mg), 36 (230 mg), 37 (230 mg), 38 (200 mg) and 39 (25 mg). Fraction 34 (973 mg) was separated into a $CHCl_3$ soluble fraction 41 (573 mg) and a residue. Fraction 41 (200 mg) was further purified by by preparative silica (1000 μg) TLC with hexane:acetone 4:1.as the mobile phase. The band isolated at R_f 0.6 (7.3 mg) was identical to compound **13**, as confirmed by NMR experiments.

Extract VIII (4.3 g) from wasabi powder was stirred with ethyl acetate and filtered to yield the soluble fraction 42 (4.1 g) and the insoluble fraction 43 (290 mg). Fraction 42 (3.6 g) was further purified by silica gel MPLC using $CHCl_3$:hexane (200 mL, 1:1, v/v), chloroform (200 mL), $CHCl_3$:methanol (500 mL, 1:1, v/v) and methanol (3L) as eluants at 4 mL/min. Fractions were collected in test tubes at 3 min intervals using a fraction collector, combined after TLC analyses to yield fractions 44 (264 mL, 2.2 g, see compound **1**, Chapter 3), 45 (192 mL, 290 mg), 46 (132 mL, 730mg), 47 (432 mL, 120 mg), 48 (180 mL, 110 mg), 49 (240 mL, 30 mg), 50 (240 mL, 20 mg), 51 (2 L, 80 mg), 52 (50 mL, 20 mg). Fraction 51 (35 mg) was further purified by preparative silica (250 μm) TLC with hexane:acetone 2:1 as the mobile phase to yield compound **14** (Rf 0.75, 20.6 mg).

Fraction 43 (200mg) was further separated into the $CHCl_3$ soluble fraction 53 (185 mg) and a residue. Fraction 53 (180 mg) was separated into a MeOH soluble fraction 54 (117 mg) a residue. Fraction 54 (110 mg) was further purified by preparative silica (250 μm) TLC with MeOH: $CHCl_3$ 1:4 as the mobile phase and afforded compound **15** (R_f 0.7, 6.0 mg).

Compound **13** (pale yellow oil): ^{1}H-NMR ($CDCl_3$): 5.35 (2 H), 2.79 (2H), 2.30 (2H), 2.05 (4H), 1.60 (2H), 1.22-1.40 (20H), 0.87(3H).

Compound **14** (colorless oil): ^{1}H-NMR ($CDCl_3$): 5.35 (3H), 3.64 (3H), 2.75 (1H), 2.30 (2H), 2.05 (3H), 1.60 (2H), 1.22-1.40 (18H), 0.87(3H); ^{13}C-NMR ($CDCl_3$): 174.3, 130.20, 130.0, 129.74, 128.02, 127.88, 51.4, 34.08, 31.90, 31.51, 29.08-29.75, 27.18, 24.93, 22.67, 22.57, 14.11, 14.07.

Compound **15** (colorless solid): ^{1}H-NMR (DMSO): 5.31 (1H, d., J = 3.4 Hz, H6), 4.90 (3H, b. s, 2'-OH, 3'-OH, 4-'OH), 4.60 (1H, m, H3), 4.45 (1H, b. s, 6'-OH), 4.20 (1H, d., J = 7.7 Hz, H1'), 3.64 (1H, d., J = 11.2 Hz, H6'a), 2.95-3.15 (3H, m, H3', 4', 5'), 2.87 (1H, dd, J = 8.0, 4.5 Hz, H2'), 2.35 (1H, dd, J = 13.0, 3.4, H4a), 2.12 (1H, dd, J = 13.0, H4b), 0.80-2.0 (27H, m, H1, 2, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 24, 25, 28), 0.94 (3H, s, H19), 0.89 (3H, d, J = 6.5, H21), 0.81 (3H, d, J = 7.0, H29), 0.80 (3H, d, J = 7.0, H27), 0.78 (3H, d, J = 7.0, H26), 0.64 (3H, s, H18); ^{13}C-NMR (DMSO): 140.44 (C5), 121.24 (C6), 100.77 (C1'), 76.87 (C3), 76.70 (C3'), 76.68 (C5'), 73.46 (C2'), 70.07 (C4'), 61.07 (C6'), 56.17 (C14), 55.40 (C17), 49.60 (C9), 45.12 (C24), 41.86 (C13), 38.29 (C4), 36.83 (C1), 36.22 (C10), 35.50 (C20), 33.33 (C22), 31.42 (C8), 31.40 (C7), 29.28 (C2), 28.68 (C25), 27.83 (C16), 25.38 (C23), 23.88 (C15), 22.59 (C28), 20.60 (C11), 19.74 (C27), 19.12 (C19), 18.62 (C21, C26), 11.80 (C29), 11.70 (C18).

Purification of extracts and isolation of compounds from wasabi rhizomes

The hexane extract of the wasabi roots (XV, 1.80 g) was separated into the $CHCl_3$ soluble fraction 55 (1.4 g) and a residue. Fraction 55 (1.3 g) was subsequently

fractionated by preparative silica MPLC with hexane (150 mL), hexane:acetone (10:0.5, v/v, 150 mL), hexane:acetone (10:1, v/v, 150 mL), hexane:acetone (10:2.5, v/v, 240 mL), hexane:acetone (10:4, v/v, 180 mL), hexane:acetone (1:1, v/v, 330 mL), acetone (30 mL) and MeOH (240 mL) at 3 mL/min as the mobile phases. Collection of fractions in 2 min intervals using a fraction collector started 20 min after elution began. Fractions were combined according to their TLC profile. Fractions 56 (36 mL, 80 mg) and 57 (84 mL, 85 mg), compound **5** (150 mL, 30 mg), fractions 58 (9 mL, 40 mg), 59 (54 mL, 660 mg), 60 (84 mL, 400 mg), 61 (54 mL, 20 mg), 62 (96 mL, 3 mg), 63 (60 mL, 7 mg), 64 (84 mL, 10 mg), 65 (126 mL, 14 mg), 66 (72 mL, 6 mg), 67 (132 mL, 5 mg), 68 (156 mL, 5 mg), 69 (60 mL, 13 mg) and 70 (250 mL, 40 mg) were obtained.

Compound **9** (140 mg) was isolated as the MeOH insoluble portion of fraction 60 (245 mg).

Fraction 59 (650 mg) was separated into the MeOH soluble fraction 71 (100 mg) and the insoluble fraction 72 (550 mg). Fraction 71 (90 mg) was further purified by preparative silica (500 μm) TLC using $CHCl_3$ as the mobile phase. A single band obtained (R_f 0.65, 24 mg) yielded fraction 73 which was further purified (20 mg) by preparative silica (250 μm) TLC with hexane:acetone (10:1.5) as the mobile phase and yielded compound **16** (R_f 0.56, 3.3 mg).

Fraction 72 (420 mg) was fractionated by preparative silica (500 μm) TLC with hexane:acetone (10:0.8, v/v) as the eluant. Two bands were isolated and yielded fractions 74 (R_f 0.56, 45 mg) and 75 (R_f 0.78, 250 mg), respectively. Fraction 74 (20 mg) was

further purified by preparative silica (250 μm, binder-free) using hexane:acetone (10:1.2, v/v) as the mobile twice. A single band isolated was compound **17** (R_f 0.6, 3.8 mg).

Fraction 75 (150 mg) was further purified by preparative silica (500 μm) using hexane:acetone (10:1.5, v/v) as the mobile phase. Compound **3** (R_f 0.65, 111 mg) was isolated as a single band.

The ethyl acetate extract (XVI, 1.9 g) of wasabi roots was fractionated by preparative silica MPLC with hexane (140 mL), hexane:acetone (10:1, v/v, 150 mL), hexane:acetone (10:2, v/v, 250 mL), hexane:acetone (10:3, v/v, 220 mL), hexane:acetone (10:4, v/v, 220 mL), hexane:acetone (10:5, v/v, 170 mL), hexane:acetone (10:7, v/v, 315 mL), hexane:acetone (1:1, v/v, 450 mL), hexane:acetone (7:10, v/v, 280 mL), hexane:acetone (5:10, v/v, 270 mL), hexane:acetone (2:10, v/v, 220 mL), acetone (175 mL), acetone:MeOH (1:1), v/v, 200 mL) and MeOH (200 mL) as mobile phases at 7 mL/min. No collection occurred during the first 20 min of elution. Fractions were collected with a fraction collector in 2 min intervals and yielded fraction 76 (105 mL, 440 mg), compound **1** (Chapter 3, 77 mL, 255 mg), fractions 77 (180 mL, 270 mg), 78 (336 mL, 160 mg), 79 (133 mL, 8 mg), 80 (70 mL, 30 mg), 81 (315 mL, 230 mg), 82 (270 mL, 23 mg), 83 (770 mL, 300 mg) and 84 (455 mL, 230 mg). Fraction 83 (290 mg) was separated into a acetone:MeOH (1:1, v/v) soluble fraction 86 (230 mg) and a residue. Fraction 86 (200 mg) was further purified by preparative silica (250 μm) TLC with $CHCl_3$:MeOH:H_2O (4:1:0.1, v/v) to afford fraction 87 (R_f 0.7, 70 mg). Fraction 87 (60 mg) was further purified by preparative silica (250 μm) TLC with $CHCl_3$:MeOH:H_2O (3.5:1:0.1, v/v) as the mobile phase to afford compound **18** (R_f 0.7, 15 mg).

The methanol extract of the lyophilized wasabi (XVII, 21.2 g) was separated into a MeOH soluble fraction 88 (17 g) and a residue. Fraction 88 (16.9 g) was further purified with MeOH:H_2O (5:1, v/v) into a soluble fraction 89 (16 g) and an insoluble fraction 90 (900 mg). Fraction 89 (9.5 g) was subsequently fractionated by preparative C18 MPLC using MeOH:H_2O (2:3, v/v, 480 mL), MeOH:H_2O (3:2, v/v, 480 mL), MeOH:H_2O (7:3, v/v, 180 mL), MeOH:H_2O (4:1, v/v, 240 mL) and MeOH (1L) as eluants at 4 mL/min. Fractions were collected after the first 20 min using a fraction collector at 2 min intervals. Fractions were combined based on their TLC profiles and yielded fractions 91 (200 mL, 8.57 g), 92 (120 mL, 176 mg), 93 (488 mL, 60 mg), 94 (320 mL, 20mg) and 95 (700 mL, 540 mg). Fraction 92 (140 mg) was futher fractionated by preparative silica (250 μm) TLC with MeOH:$CHCl_3$ (1:1, v/v) as the mobile phase to afford fractions 96 (R_f 0.1, 38 mg), and 97 (R_f 0.5, 36 mg). Fraction 96 (30 mg) was further purified by preparative C-18 HPLC (LC-20, MeOH:H_2O, 20:80, v/v, 4 mL/min) to yield compound **19** (6 mg). Fraction 97 (30 mg) was further purified by preparative silica (250 μm) PTLC with $CHCl_3$ as the mobile phase. Compound **9** was isolated as a single band (R_f 0.4, 20 mg).

Fraction 90 (880 mg) was fractionated by preparative silica MPLC with $CHCl_3$ (135 mL), $CHCl_3$:MeOH (95:5, v/v, 270 mL), $CHCl_3$:MeOH (80:20, v/v, 325 mL) and MeOH (1L) at 3mL/min as mobile phases. Fractions were collected at 3 min intervals using a fraction collector and combined according to their TLC profiles. Fractions 98 (120 mL, 60 mg), 99 (225 mL, 80 mg), 100 (297 mL, 300 mg), 101 (225 mL, 390 mg) and 102 (135mL, 40 mg) were collected. Fraction 101 (340 mg) was separated into a

MeOH soluble fraction 103 (300 mg), and a residue. Fraction 103 (220 mg) was further purified by preparative silica MPLC with $CHCl_3$ (120 mL) $CHCl_3$:MeOH (10:0.5, v/v, 150 mL), $CHCl_3$:MeOH (4:1, v/v, 185 mL), and MeOH (1L) at 2 mL/min as mobile phases. Fractions were collected in 3 min intervals using a fraction collector. Fractions were combined according to their TLC profiles. Fractions 104 (70 mg) and 105 (140 mg) were collected. Fraction 105 (120 mg) was further purified by preparative C18 MPLC using MeOH:H_2O (2:8, v/v, 80 mL), MeOH:H_2O (3:7, v/v, 80 mL), MeOH:H_2O (4:6, v/v, 80 mL), MeOH:H_2O (5:5, v/v, 80 mL), MeOH:H_2O (6:4, v/v, 80 mL), MeOH:H_2O (7:3, v/v, 80 mL), MeOH:H_2O (8:2, v/v, 80 mL), MeOH:H_2O (9:1, v/v, 80 mL) and MeOH (900 mL) at 4mL/min as eluants. Fractions were collected in 2 min intervals and combined according to their TLC profiles to yield fractions 106 (400 mL, 25 mg), 107 (480 mL, 50 mg), and 108 (400 mL, 25 mg).

Fraction 107 (30 mg) was further purified by preparative silica (250 μm) TLC with MeOH:$CHCl_3$ (6:1, v/v) as the mobile phase and yielded compound **20** (R_f 0.2, 16 mg) .

Compound **16** (yellow oil): ^{1}H-NMR ($CDCl_3$): 4.13 (1H, b. s, C6-OH), 2.58 (2H, t., J = 6.9 Hz), 2.14 (3H, s., C7-CH_3), 2.11 (6H, s., C8-CH_3, C5-CH_3), 1.76 (2H, m, H3), 1.10-1.58 (21H, H1', 2', 3', 4', 5', 6', 7', 8', 9', 10', 11', 12'), 1.21 (3H, s., C2-CH_3), 0.86 (3H, overlapping d., J = 6.6 Hz, H12''), 0.84 (3H, overlapping d., J = 6.6 Hz, H13'), 0.83 (3H, overlapping d., J = 6.6 Hz, H4'), 0.82 (3H, overlapping d., J = 6.6 Hz, H8''); ^{13}C-NMR ($CDCl_3$): 145.57 (C-9), 144.54 (C-9), 122.62 (C-8), 120.99 (C-7), 118.44 (C-5), 117.36 (C-10), 75.53 (C-2), 39.84 (C-1'), 39.38 (C-11'), 37.49 (C-3'), 37.46 (C-9'), 37.43

(C-5'), 37.29 (C-7'), 32.80 (C'-8'), 32.71 (C-4'), 31.58 (C-3), 27.97 (C-12'), 24.79 (C-10'), 24.44 (C-6'), 23.79 (C-2-), 22.70 (C12"`), 22.61 (C13'), 21.06 (C-2'), 20.76 (C-4), 19.74 (C-4"), 19.65 (C-8"), 12.18 (C-7), 11.76 (C-8), 11.25 (C-5).

Compound **17** (yellow oil): ^{1}H-NMR ($CDCl_3$): 5.10 (8H, b. t., 6.9 Hz, H10", 14", 18", 22", 26", 30" 34", 38"), 5.06 (1H, t., 6.9 Hz, H6"), 4.95 (1H, t., J=7.1 Hz, H2"), 3.99 (3H, s., H6'), 3.98 (3H, s., H5'), 3.19 (2H, d., J = 7.1 Hz, H1"), 2.01 (3H, s., H3'), 1.95-2.10 (36H, b., H4", 5", 8", 9", 12", 13", 16", 17", 20", 21", 24", 25", 28", 29", 32", 33", 36", 37"), 1.74 (3H, s., H3"`), 1.67 (3H, s., 39"`), 1.60 (24H, b. s., H7"`, 11"`, 15"`, 19"`, 23"`, 27"`, 31"`, 35"`, 39"`), 1.57 (3H, s., 39"`); ^{13}C-NMR ($CDCl_3$): 184.78 (C4), 183.92 (C1), 144.29 (C6), 144.14 (C5), 141.65 (C2), 138.86 (C3), 137.63 (C3"), 135.25 (C7"), 134.88-135.00 (C11", 15", 19", 23", 27", 31", 35", 39"`), 131.27 (C39"), 124.10-124.36 (10", 14", 18", 22", 26", 30", 34", 38"), 123.8 (C6"), 118.78 (C2"), 61.15 (C5', 6'), 39.7 (5", 9", 13", 17", 21", 25", 29", 33", 37"), 26.6-26.7 (C8", 12", 16", 20", 24", 28", 32", 36"), 26.48 (C4"), 25.71 (C40", $CH_3$), 25.28 (C1"), 17.68 (C39"), 16.33 (C3"`), 16.01 (C7"`, 11"`, 15"`, 19"`, 23"`, 27"`, 31"`, 35"), 11.96 (C3').

Compound **18** (colorless oil): ^{1}H-NMR (CD_3OD): 5.20 (1H, m, H2), 4.43 ((1H, , H1a), 4.23 (1H, H1"`), 4.20 (1H, H1b), 3.82 (1H, H3b), 3.69-3.80 (3H, overlapping m, H3"`a, 6"`a, 6"`b), 3.45-3.55 (3H, overlapping m, 5"`, 3"`, 2"`), 2.30 (4H, overlapping t., H2', 2"), 1.60 (4H, m, H3', 3"), 1.20-1.40 (48H, H4'-15', H4"-15"), 0.95 (3H, H18'), 0.88 (3H, H18"); ^{13}C-NMR ($CDCl_3$): 173.67 (C1'), 173.34 (C1"), 131.93 (C16', C16"), 130.17 (C9', 9"), 128.31 (C12', 12"), 128.22 (C13', 13"), 127.99 (C10', 10"), 127.12 (C15', C15"), 104.03 (C1"`), 74.62 (C5"`), 73.51 (C3"`), 71.46 (C2"`), 70.23 (C2), 69.29

(c4"`), 68.22 (C3), 62.78 (C1), 62.25 (C6"`), 34.26 (C2"), 34.11 (C2'), 29.58 (C7', 7"), 29.04-29.29 (C4', 4", 5', 5", 6', 6"), 27.18 (C8', 8"), 25.61 (C11', 11"), 25.52 (C14', 14"), 24.85 (C3', 3"), 20.50 (C17', 17"), 14.17 (C18', 18").

Compound **19** (yellow oil): ^{1}H-NMR (DMSO): 10.92 (1H, s, H1), 7.54 (1H, d, J = 7.7 Hz, H7'), 7.33 (1H, d, J = 8.0 Hz) H4'), 7.19 (1H, d, J = 1.7, H2'), 7.05 (1H, t, J = 7.3 Hz, H6'), 6.96 (1H, t, J = 7.3 Hz, H5'), 3.40 (1H, br. s., H1'), 3.28 (1H, dd, J = 15.1, 3.4 Hz, H3a), 2.93 (1H, dd, J = 15.1, 8.9 Hz, H3b); ^{13}C-NMR (DMSO):174.75 (C1), 136.33 (C9'), 127.28 (C8'), 124.00 (C2'), 120.89 (C6'), 118.39 (C4'), 118.26 (C5'), 111.32 (C97), 109.78 (C3'), 54.83 (C2), 27.81 (C3).

Compound **20** (colorless oil): ^{1}H-NMR ($CDCl_3$): 5.30 (1H, m, H2), 4.30 (1H, dd, H1a), 4.20 (2H, H1", H1b), 3.80 (1H, dd, J = 11.0, 5.5 Hz, H3b), 3.69-3.80 (3H, overlapping m, H3"`a, 6"`a, 6"`b), 3.20-3.40 (3H, overlapping m, 5"`, 3"`, 2"`), 2.30 (4H, overlapping t., H2', 2"), 1.60 (4H, m, H3', 3"), 1.20-1.40 (24H), 0.88 (6H, C16', C16``).

Results and Discussion

Rhizomes of wasabi and horseradish and a commercial wasabi powder were separately extracted. Extractions were performed successively with hexane, ethyl acetate, and methanol. The bioassay-guided fractionation and purification of the organic extracts resulted in the isolation of compounds **3-18** using a series of chromatographic procedures which included preparative thin-layer (TLC), medium pressure (MPLC) and high pressure (HPLC) liquid chromatography.

Compounds **3-12** were isolated from the horseradish rhizome, compounds **3**, **5**, and **13-15** from the wasabi powder and compounds **3**, **5**, **7**, **9**, **10** and **16-18** from the wasabi rhizome. Nuclear magnetic resonance (NMR) and gas-chromatography-Mass spectroscopy (GC-MS) were used to elucidate the identities of these compounds. The structures of compounds **3-18** are presented in Figures 4.1 through 4.16.

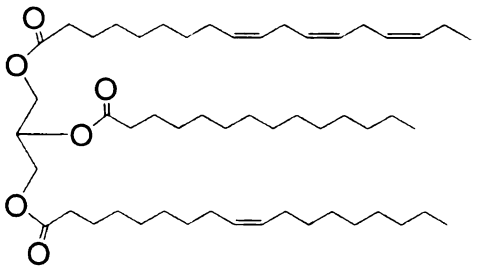


Figure 4.1: Compound **3**.

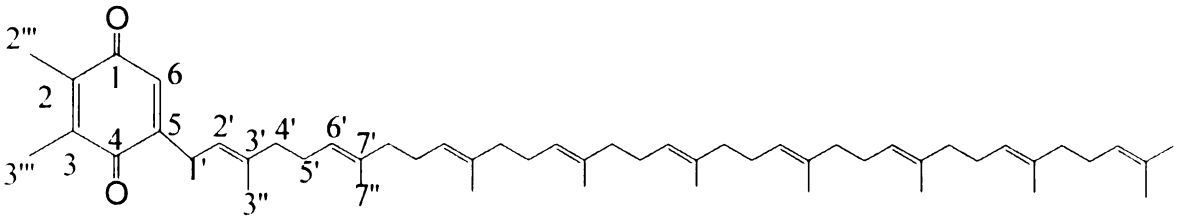


Figure 4.2: Compound **4**.

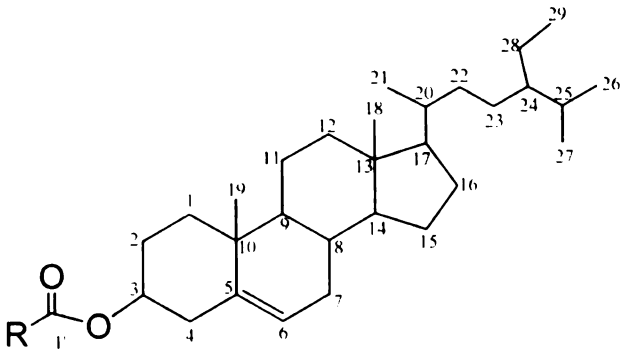


Figure 4.3: Compound **5**.

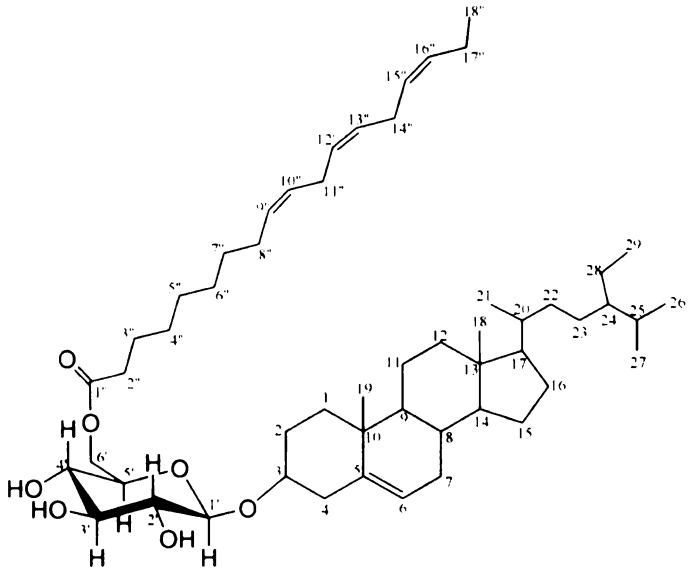


Figure 4. 4: Compound **6**.

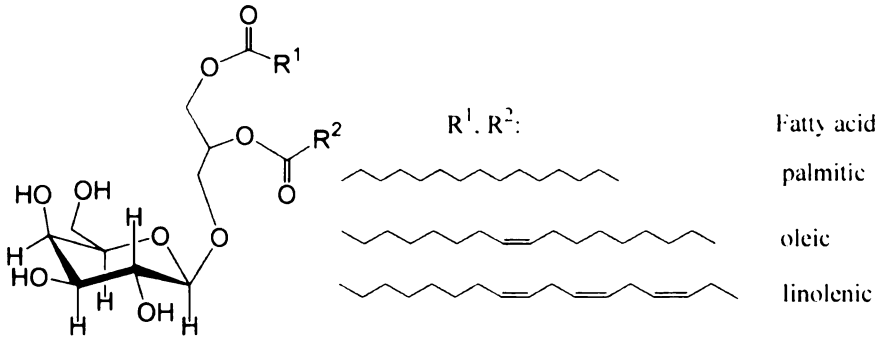


Figure 4.5: Compounds **7** (R1=R2= linolenic), **18** (linolenic and oleic) and **20** (R1=R2= palmitic).

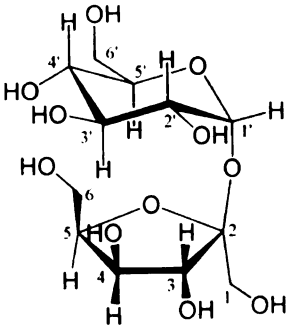


Figure 4.6: Compound **8**.

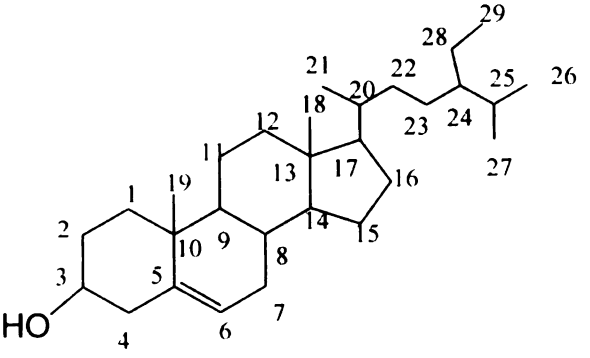


Figure 4.7: Compound **9**.

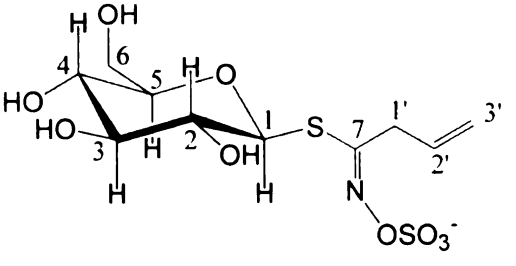


Figure 4.8: Compound **10**.

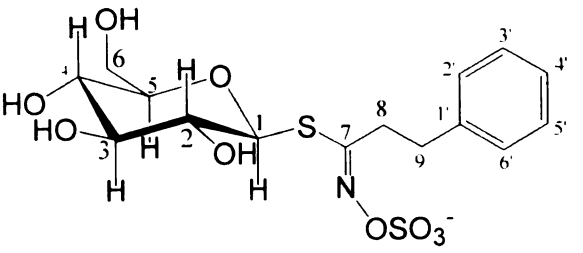


Figure 4.9: Compound **11**.

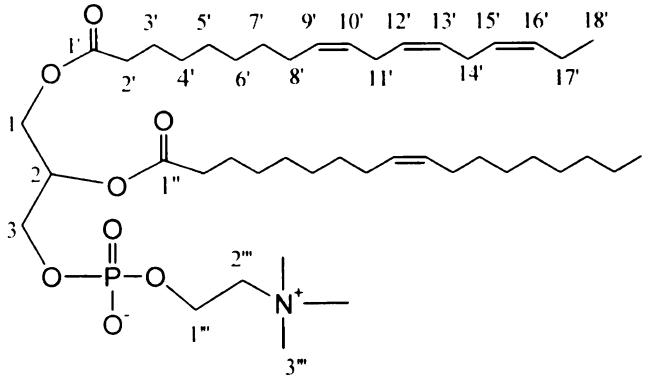


Figure 4.10: Compound **12**.

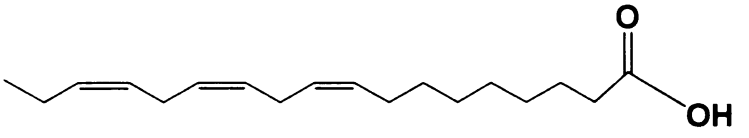


Figure 4.11: Compound **13** (linolenic acid shown)

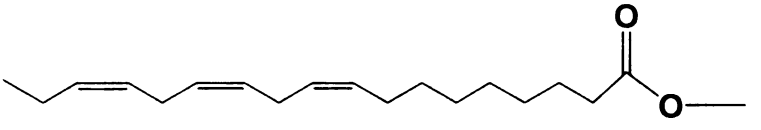


Figure 4.12: Compound **14** (methyl linolenate shown).

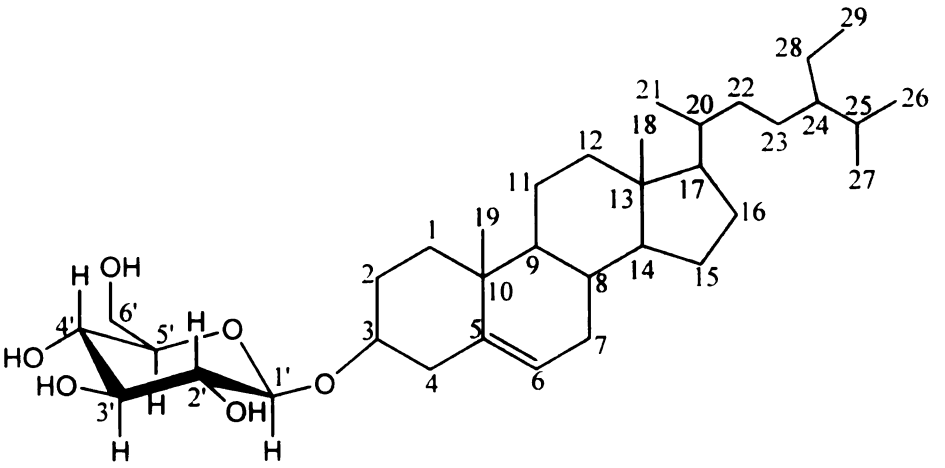


Figure 4.13: Compound **15**

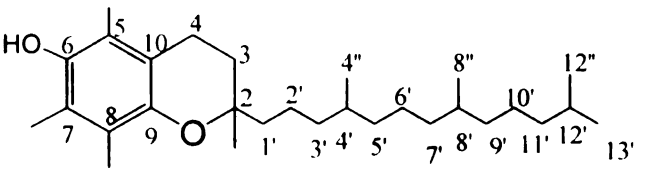


Figure 4.14: Compound **16**

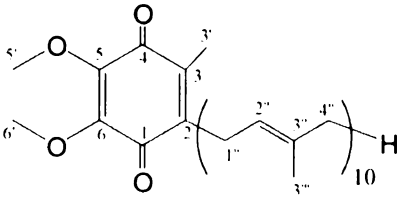
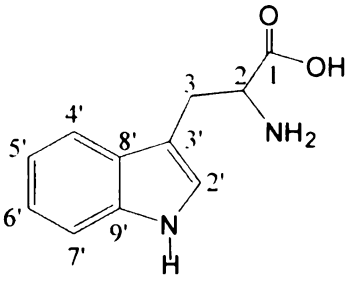


Figure 4.15: Compound **17**.



Compound **3** (Figure 4.1) was isolated from all three plant materials evaluated. Evaluation of its NMR spectral data indicated the presence of a triglyceride. Compound **3** was hydrolyzed and the products were methylated and analyzed by GC-MS. For compound **3** isolated from horseradish rhizomes, tha fatty acid composition was established as linolenic, linoleic, oleic and palmitic acids in a ratio of 5:2:1:1. Similarly, for compound **3** isolated from wasabi rhizomes gave only palmitic, oleic, and linolenic acids in a ratio 1:1:1 were detected. Compound **3** isolated from wasabi powder contained linolenic, eisosenoic, oleic and erucic acid in a ratio 1:1:2:2. No lipid peroxidation inhibitory activity was observed for compound **3**. It inhibited COX-1 and COX-2 enzymes by 35 and 24%, respectively, at 250 ppm (Figure 4.17). It is possible that compound **3** may have contained free fatty acids. Fatty acids have been shown to possess both lipid peroxidation and COX enzymes inhibitory activities (Henry et al., 2002). However, no lipid peroxidation inhibition was observed and the presence of fatty acids in compound **3** was ruled out. The COX inhibitory assays were conducted at pH 7.4 and therefore a partial hydrolysis of the triglyceride was a distinct possibility.

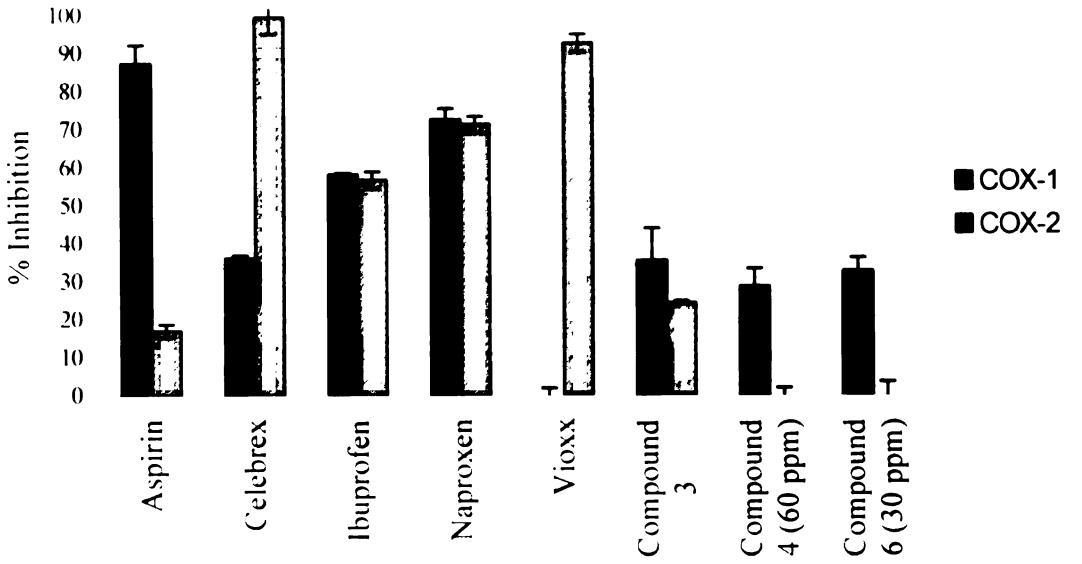


Figure 4.17: Cyclooxygenase enzyme inhibitory activities of compounds **3**, **4** and **6** at 250 ppm. Ibuprofen, Aspirin, Celebrex, Vioxx and Naproxen at 2.06, 180, 1.67, 1.67 and 2.52 ppm, respectively, were used as positive controls. Results are expressed as mean value of the percent inhibition of duplicate measurements ± one standard deviation.

Compound **4** was isolated from horseradish rhizomes. Its identity was established as plastoquinone-9, also known as 2,3-dimethyl-5-solanesyl-benzoquinone, 2,3-dimethyl-5-[(2E,6E,10E,14E,18E,22E,26E,30E)-3,7,11,15,19,23,27,31,35-nonamethyl-2,6,10,14,18,22,26,30,34-hexatriacontanonaenyl]-2,5-cyclohexadiene-1,4-dione. The structure was further confirmed by comparison with previously reported data (Boers et al., 2002a). In the COX inhibitory assays (Figure 4.17), it inhibited COX-1 by 28% at 60 ppm (Figure 4.8).

In the MTT assay (Figure 4.18), compound **4** did not inhibit the cell growth of the cancer cells at concentrations between 3.75 - 30 ppm. However, a concentration dependent trend towards cell proliferation was observed for colon and lung cancer cells.

For the lung cancer cells (NCI-H460), the number of viable cancer cells was 20% higher at 30 ppm, when compared with the DMSO control.

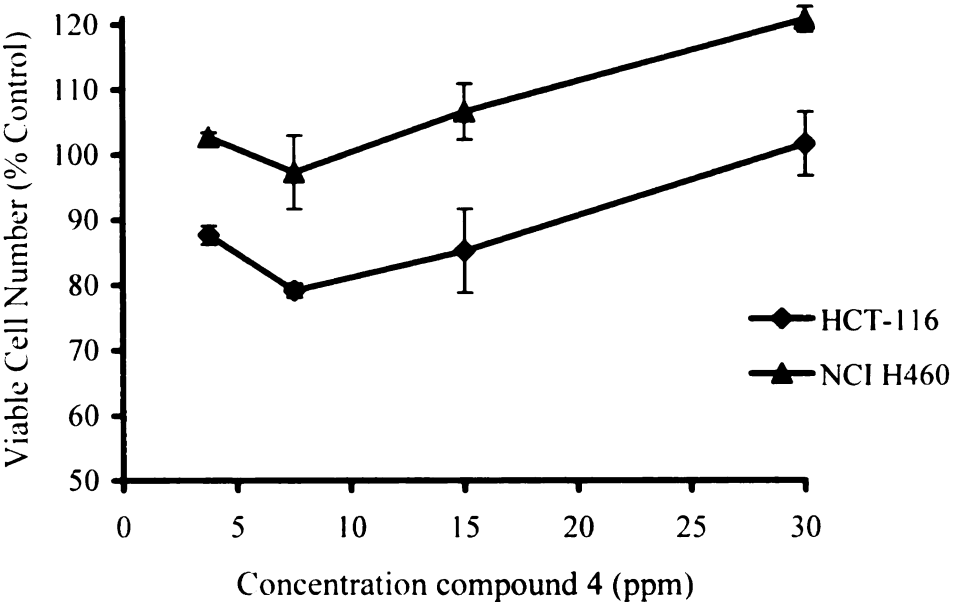


Figure 4.18: Effect of compound **4** on the proliferation of human colon (HCT-116) and lung (NCI-H460) cancer cell lines as determined by the MTT assay. No activity was observed breast (MCF-7) and CNS (Central Nervous System) (SF-268) cancer cells. The optical density was measured to determine the amount of formazan blue formed by viable cells and compared to the control. The data represents the mean ± SD of the one experiment conducted in triplicate.

In plants, plastoquinone is located in the thylakoid membranes of chloroplasts. In its reduced form, plastoquinone functions as an antioxidant in chloroplasts (Hundal et al., 1995). No lipid peroxidation inhibitory activity was observed for plastoquinone in the assay used at 250 ppm.

The evaluation of the NMR spectral data of compound **5** suggested the presence of a 3-O-acyl sitosterol with a long chain fatty acid moiety (Figure 4.3). Compound **5** isolated from horseradish rhizomes, wasabi rhizomes and wasabi powder was

hydrolyzed, the hydrolysis products were methylated and analyzed by GC-MS. Compound **5** isolated from horseradish gave a 1:1 ratio of methyl palmitate and methyl linolenate. Similarly, compound **5** from wasabi rhizomes and powder gave a 1:1 ratio of methyl linolenate and methyl palmitate and a ratio of 1:1:1.5 of palmitic, oleic and stearic acid methyl esters, respectvely. Bioassays were not conducted for compound **5** due to its low solubility, but esterified phytosterols have been shown to lower total cholesterol levels in human serum (Wester, 2000)

Compound **6** was isolated from the horseradish rhizomes. Its NMR data suggested the presence of a 6-O-acyl-β-D-glucosyl-β-sitosterol (Figure 4.4). Compound **6** was hydrolyzed, the hydrolysis products were methylated and analyzed by GC-MS. A ratio of 1:1:1 of methyl palmitate, oleate and linolenate was determined. Compound **6** was identified as an inseparable mixture of 6-O-acyl-β-D-glucosyl-β-sitosterols and its identity was confirmed by comparison with previously reported NMR data (Guevara et al., 1989; Dehmlow et al., 2000).

The evaluation of the biological activity of compound **6** was complicated by its low solubility in DMSO and other suitable solvents. The highest concentration used was 30 ppm. In the lipid peroxidation inhibitory assay, compound **6** gave 11% inhibition after 21 min. Also, it gave selective COX-1 enzyme inhibitory activity at 32% (Figure 4.17). It did not inhibit COX-2 enzyme.

In the MTT assay, no growth inhibitory or cell proliferative activities were observed for compound **6** except for a slight trend towards the inhibition of breast cancer

cells (Figure 4.19). However, with only 16% inhibition at 30 ppm, the inhibitory activity is considered as marginal.

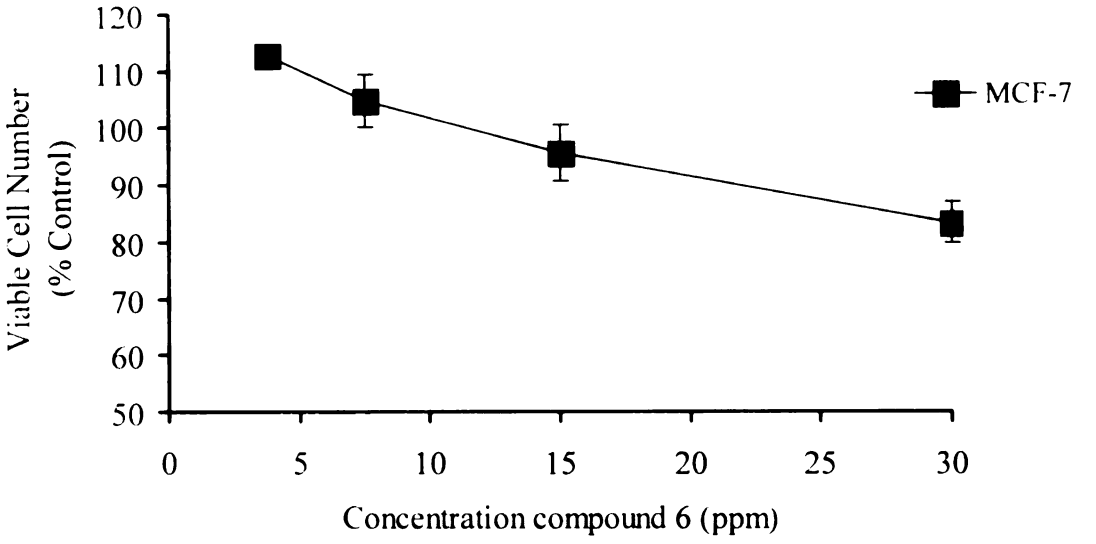


Figure 4.19: Effect of compound **6** on the proliferation of human breast (MCF-7) cancer cell lines as determined by the MTT assay. No activity was observed for colon (HCT-116), lung (NCI-H460), and CNS (Central Nervous System) (SF-268) cancer cells. The optical density was measured to determine the amount of formazan blue formed by viable cells and compared to the control. The data represents the mean ± SD of one experiment conducted in triplicate.

Mixtures of 6-O-acyl-β-D-glucosyl-β-sitosterols have been previously isolated from the flowers of the yellow paperbush, *Edgeworthia chrysantha* and showed piscicidal activity against killie fish, *Oryzia latipes* at 0.1 ppm within 3 h (Hashimoto et al., 1991). A mixture of sitosterol derivatives containing linoleic and palmitoleic acid moeities, inhibited tumor growth in mouse myeloma cells at 50 ppm (Kiriakidis et al., 1997). Furthermore, a mixture of 6-O-acyl-β-D-glucosyl-β-sitosterols containing mainly palmitoyl and linoleyl moeities, isolated from fig (*Ficus carica*) latex, was reported as cytotoxic against severval lines of cancer cells, including the breast cancer cells MCF-7

(57% at 25 ppm) (Rubnov et al., 2001). 6-O-palmitoyl-β-D-glucosyl-β-stigmasta-5,25-(27)-diene was isolated from *Momordica charantia*. It also showed antimutagenic activity by reducing the number of micronucleated polychromatic erythrocytes induced by mitomycin C in mice (Guevara et al., 1990).

Compound **7**, isolated from horseradish rhizome, and compounds **18** and **20**, isolated from wasabi rhizome showed similar spectral data. The NMR spectral data suggested that these compounds belong to the class of compounds known as monogalactosyl diacylglycerides (MGDGs). Compound **7** was hydrolyzed, the hydrolysis products were methylated and analyzed by GC-MS. The identity of compound **7** was established as 2,3-bis[[(9Z,12Z,15Z)-1-oxo-9,12,15-octadecatrienyl]oxy]propyl β-D-galactopyranoside (1,2-dilinolenoyl-3-β-galactosylglycerol, 3-(galactosyloxy)linolenin) and further confirmed by comparison with published data (Wang et al., 2002; Jung et al., 1996). MGDGs with linoleic and oleic acids were detected as minor components. Similarly, compounds **18** and **20** were hydrolyzed, the hydrolysis products were methylated, and analyzed by GC-MS. Methyl oleate and methyl linolenate in a ratio 1:1 were detected in the derivatized product of compound **18**. Since the distribution of the fatty acid substituents at the glycerol backbone was not established, it is possible that compound **18** consisted of a mixture of positional isomers. Minor quantities of other fatty acid methyl esters were also detected for **18**. Analysis of compound **20** gave methyl palmitate as the only fatty acid moiety in the molecule. Its identity was established as 1,2-dipalmitoyl-3-β-galactosylglycerol.

In the COX enzyme inhibitory assays (Figure 4.20), compound **7** selectively inhibited COX-1 (75% at 250 ppm) with marginal COX-2 inhibition (12%). COX-1 inhibition for compounds **18** and **20** was about 45% (Figure 4.21). This value remained unchanged irrespective of the fatty acid composition of the MGDG.

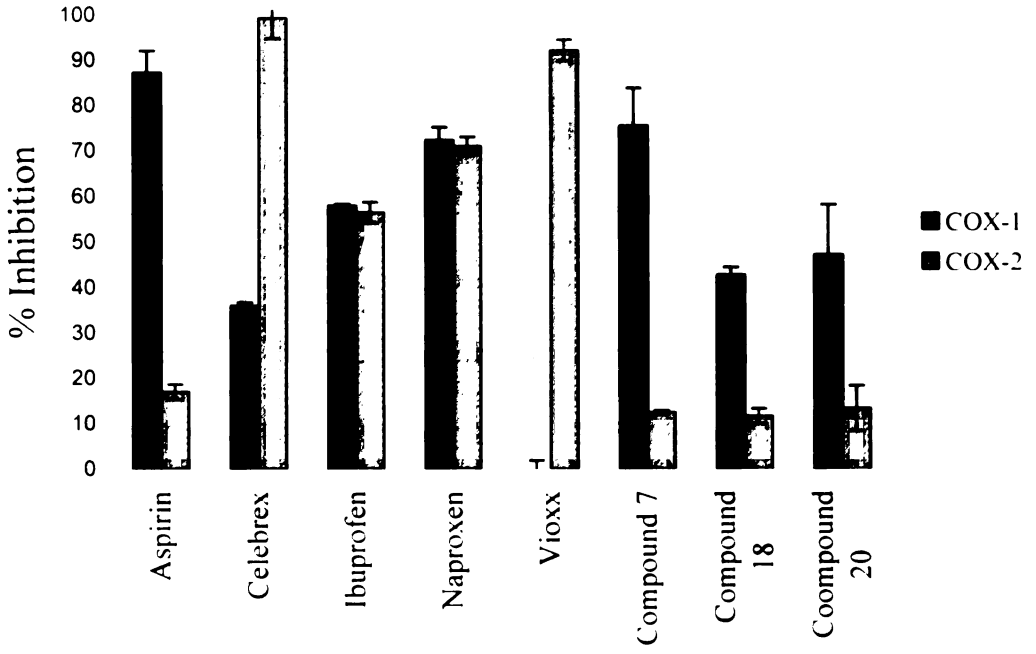


Figure 4.20: Cyclooxygenase enzyme inhibitory activities of compounds **7**, **18** and **20** at 250 ppm. Ibuprofen, Aspirin, Celebrex, Vioxx and Naproxen at 2.06, 180, 1.67, 1.67 and 2.52 ppm, respectively, were used as positive controls. Results are expressed as mean value of the percent inhibition of duplicate measurements ± one standard deviation.

This is the first report of the selective COX-1 activity of MGDGs. Cyclooxygenase (COX) enzymes mediate the rate-limiting step in the conversion of arachidonic acid to prostanoids such as prostaglandins, thromboxanes, and prostacyclin. Prostaglandins are involved in physiologic processes such as cytoprotection of the gastric

mucosa, gestation, and parturition. Prostaglandins also participate in inflammation and cancer (Sakamoto, 1998)

Two cyclooxygenases have been identified, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues while COX-2 is induced in response to inflammatory stimuli (Laneuville, et al., 1994). COX inhibitors are used for the long-term treatment of inflammatory conditions (Bing and Lomnicka, 2002). Non-steroidal anti-inflammatory drugs that inhibit both isozymes, COX-1 and COX-2, can cause side effects such as gastric ulceration as a consequence of COX-1 inhibition. Coxibs, a class of selective COX-2 inhibitors can reduce inflammation, fever, and pain without causing these side effects (Bing and Lomnicka, 2002). Only COX-1 is expressed in platelets, where it mediates the formation of thromboxane A_2 (TXA_2). TXA_2 plays a role in platelet aggregation, vasoconstriction and atherogenesis. Aspirin, a non-selective COX inhibitor, is routinely used to prevent ischemic heart disease since it inhibits platelet TXA_2 formation by irreversibly blocking COX-1 (FitzGerald, 2002).

Compound **7** was tested in the lipid peroxidation inhibitory assay and did not inhibit peroxidation. Therefore, compounds **18** and **20**, the MGDGs isolated from wasabi, were not tested.

In view of the results observed for compound **7** in the lipid peroxidation and for compounds **7**, **18** and **20** in the COX inhibitory assays, compound **7** was selected as a representative MGDG and tested in the cancer cell assays. In the MTT assay (Figure 4.21), compound **7** inhibited the growth of colon (HCT-116) and lung cancer (NCI-H460) cells in a concentration dependent manner between 7.5 and 60 ppm (9.7 - 77.5 μM). It

inhibited the growth of colon cancer cells by 21.9, 42.9, 51.2 and 68.4% and lung cancer cells by 30.0, 38.6, 44.2 and 70.5% at 7.5, 15, 30, and 60 ppm, respectively. Compound **7** showed a similar inhibitory effect for both cell lines.

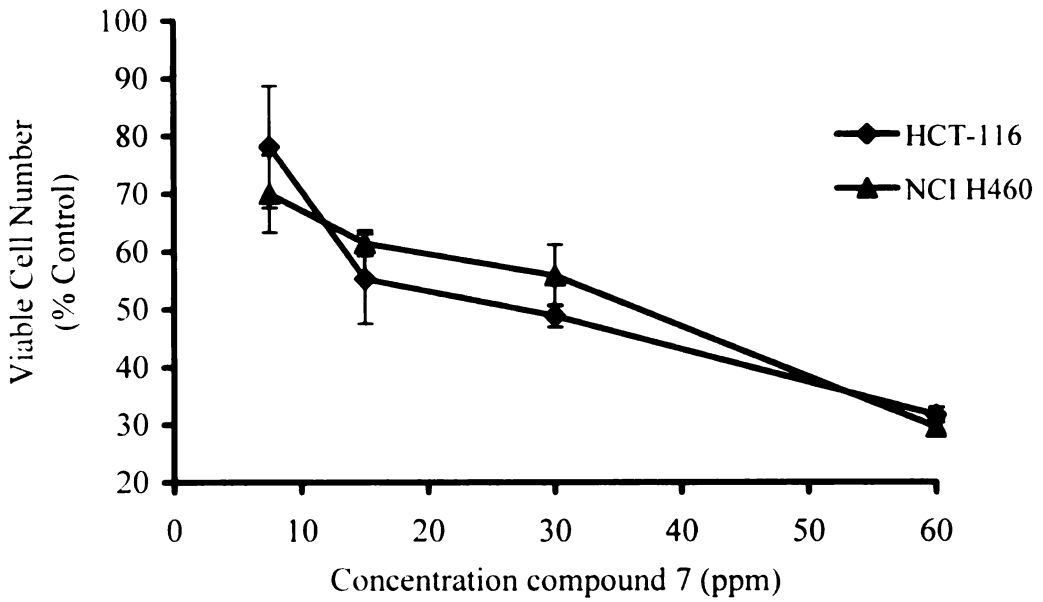


Figure 4.21: Effect of compound **7** on the proliferation of human colon (HCT-116) and lung (NCI-H460) cancer cell lines as determined by the MTT assay. No activity was observed for breast (MCF-7) and CNS (Central Nervous System) (SF-268) cancer cells. The optical density was measured to determine the amount of formazan blue formed by viable cells and compared to the control. The data represents the mean ± SD of one experiment conducted in triplicate.

MGDGs are a common class of compounds in plants. Together with other glycosyl diacylglycerides, they belong to the major lipids present in the chloroplasts and in membranes. MGDGs have been reported as inhibitors of 12-O-tetradecanoylphorbol-13 acetate (TPA)-induced tumor promotion in vitro and in mouse models (Wang et al., 2002; Colombo et al., 2002; Colombo et al., 2000; Morimoto et al., 1995; Cateni et al.,

2001; Murakami et al., 1995). They have been shown to be inducers of apoptosis and selective inhibitors of mammalian polymerases (Murakami et al., 2003).

1,2-dilinolenoyl-3-β-galactosylglycerol, isolated from *Euphorbiacyparissias* L., was reported to exert topical antiinflammatory activity in a mouse oedema model (Cateni et al., 2001). However, this is the first report of MGDGs isolated from horseradish and wasabi rhizomes with specific COX-1 enzyme inhibitory activity.

Upon ingestion, it is expected that MGDGs will be enzymatically cleaved at position 1 by pancreatic lipase. This may affect their ability to inhibit cancer cell proliferation. In a recent study, MGDGs were hydrolyzed with pancreatic lipase and the resulting monogalactosyl monoacylglycerides (MGMGs) were compared to their MGDG parents in cancer cell inhibitory assays. Cancer cell growth was inhibited by both classes of compounds and the LD_{50} values were approximately 40 μg/mL (as measured in the MTT asssay)(Murakami et al., 2003).

Diets rich in plant foods have been related to a reduced risk of cardiovascular disease and cancer (Wahrburg et al., 2002). Many different classes of compounds have been implicated in this protection, including unsaturated fatty acids, vitamins, and antioxidants (Simopoulos, 2001). Given their abundance in plant foods, MGDGs may play a role in reducing cancer risk and the occurrence of cardiovascular disease in vegetable and fruit-rich diets.

Compound **8** was identified as sucrose (Figure 4.6) after evaluation of its NMR spectroscopic data. Its identity was confirmed by comparison with previously reported

data (Jarrel et al., 1979; Forsgren et al., 1985). It was isolated from both horseradish and wasabi rhizomes. Sucrose was not tested in any of the assays.

Compound **9**, identified as β-sitosterol (Figure 4.7), was isolated from horseradish and wasabi rhizomes. Its identity was confirmed by comparison with previously published data (Della Grecca et al., 1990). Compound **9** was not tested for biological activity, but consumption of phytosterols has been shown to reduce cholesterol absorption and to lower its levels in human serum (Wester, 2000).

Compound **10** was identified as sinigrin, 2-propenyl glucosinolate (Figure 4.8). Its identity was confirmed by comparison with previously published literature data (Prestera et al., 1996; Kiddle et al., 2001). Sinigrin inhibited lipid peroxidation in the liposome model by 71% at 250 ppm (Figure 4.22). Although it has been proposed that glucosinolates in general have no participation in the antioxidant activity of juices from vegetables (Germano et al., 2002), sinigrin was identified as one of the components of Brussels sprouts extracts that afforded protection against DNA oxidative damage (Zhu et. al., 2000). The COX-1 enzyme inhibitory activity of sinigrin at 250 ppm was about 20% and no COX-2 enzyme inhibitory activity was observed (Figure 4.23).

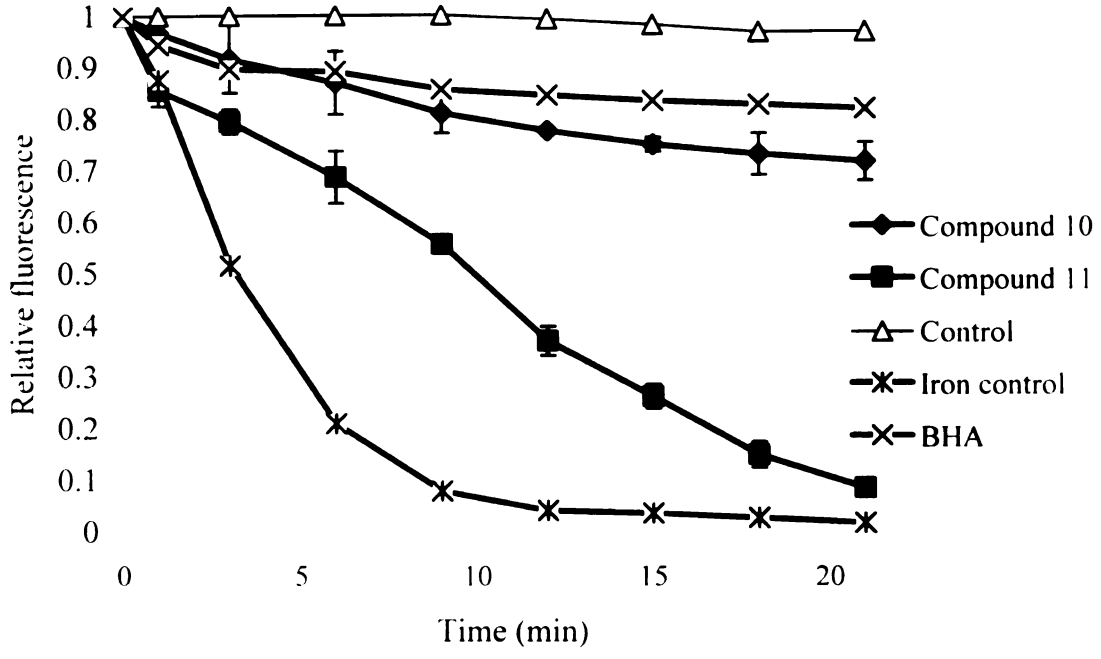


Figure 4.22: Inhibition of lipid peroxidation by compounds **10** and **11** at 250 ppm. BHA was tested at 1.67 ppm. The experiments were conducted in duplicate and data represents the relative fluorescence from 0-21 min. ± one standard deviation.

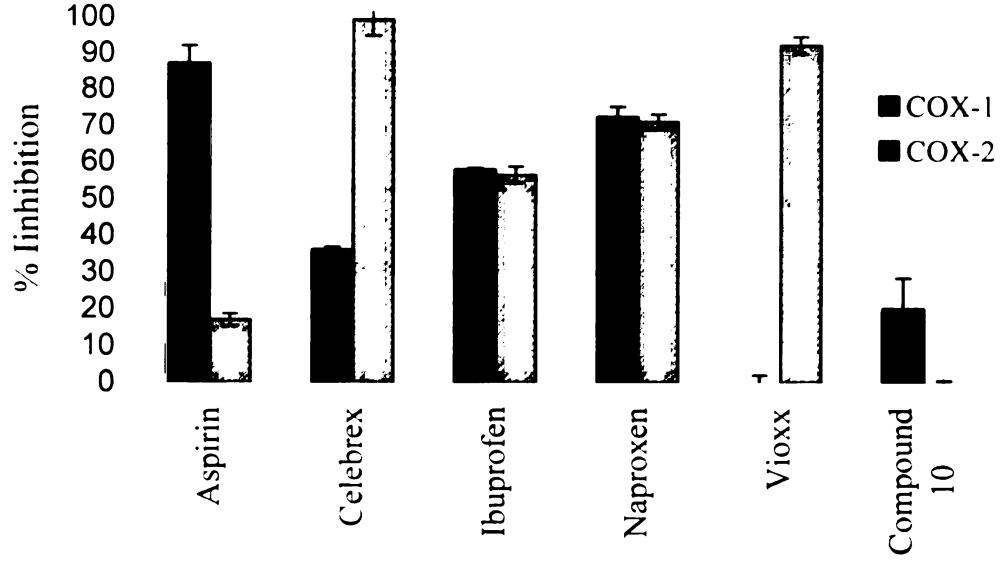


Figure 4. 23: Cyclooxygenase enzyme inhibitory activities of compound **10** at 250 ppm. Ibuprofen, Aspirin, Celebrex, Vioxx and Naproxen at 2.06, 180, 1.67, 1.67 and 2.52 ppm, respectively, were used as positive controls. Results are expressed as mean value of the percent inhibition of duplicate measurements ± one standard deviation.

In the MTT assay (Figure 4. 24) sinigrin was not able to significantly inhibit cancer cell growth. It inhibited the growth of the breast cancer cells by 20% at 60 ppm. The results observed for sinigrin are in sharp contrast to those observed for desulfosinigrin (DSS, compound **2**, Chapter Two). DSS, the biosynthetic precursor of sinigrin was inactive in the lipid peroxidation inhibitory assay, while it promoted the growth of two cancer cell lines in the MTT assay (Chapter Three). No data is avaliable in the literature comparing the metabolism of sinigrin and desulfosinogrin or their ROS-scavenging or metal chelating abilities.

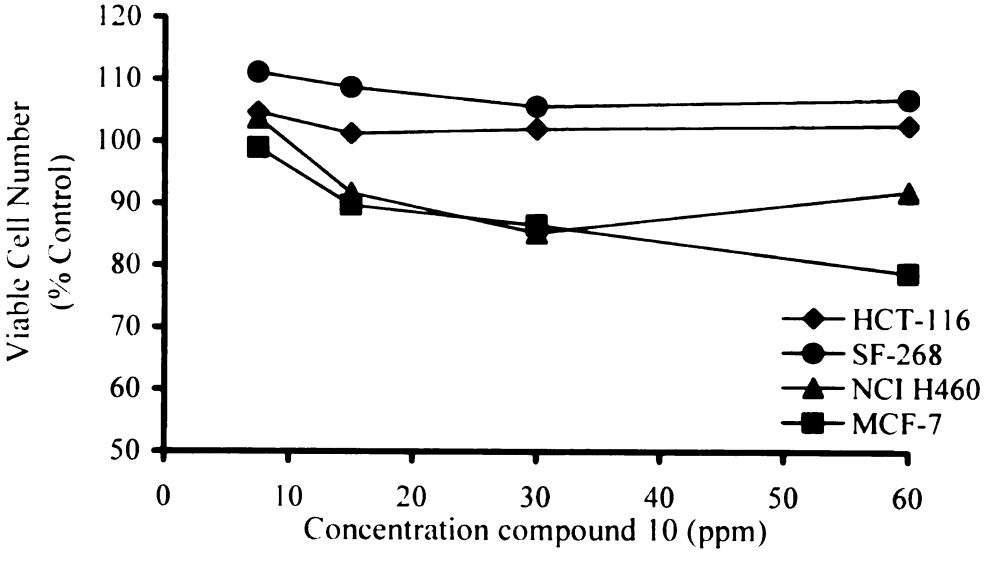


Figure 4.24: Effect of compound **10** on the proliferation of human colon (HCT-116), breast (MCF-7), lung (NCI-H460), and CNS (Central Nervous System) (SF-268) cancer cell lines as determined by the MTT assay. The optical density was measured to determine the amount of formazan blue formed by viable cells and compared to the control. The data represents the mean of one experiment conducted in triplicate.

Sinigrin was not isolated in pure form from wasabi or from wasabi powder, but its presence was established by analytical TLC and by comparison with sinigrin isolated from horseradish rhizomes

Compound **11** was identified as 2-phenylethyl glucosinolate (gluconasturtiin) (Figure 4.9). Its identity was confirmed by comparison with previously published literature data (Kiddle et al., 2001). 2-phenylethyl glucosinolate differed from sinigrin in its ability to inhibit lipid peroxidation (Figure 4.22). Although no significant inhibition remained after 21 min, the rate of peroxidation was slower than that of the Fe(II) control (Figure 4.14). No significant growth inhibition was observed in the MTT assay for 2-phenylethyl glucosinolate. It was also inactive in both COX enzyme inhibitory assays.

The NMR spectral data of compound **12** suggested the presence of a phosphatidylcholine (Figure 4.10). The estructure of compound **12** was confirmed by comparison with previously reported data (Basti and LaPlance, 1990; Gaede and Stark, 2001; Han et al., 1991). GC-MS of the methylated products from the hydrolysis of compound **12** revealed the presence of palmitic, oleic, linoleic and linolenic acid methyl esters at a ratio of 1:2:2:4. The identity of **12** was established as a mixture of phosphatidylcholines with four major fatty acid moeties. Phosphatidyl cholines are a major component in cell membranes. In the lipid peroxidation inhibitory assay, compound **12** was not active.

Compound **13** was isolated from wasabi powder. The TLC profile and the NMR spectral data of compound **13** (Figure 4.11) indicated the presence of a fatty acid. Compound **13** was methylated and analyzed by GC-MS. The GC-MS results showed that

it contained 28, 21, 20, 12, 12, and 4% linoleic, erucic, linolenic, eicosenoic and palmitic acids, respectively. Compound **13** was active in the lipid peroxidation (Figure 4.25) and cyclooxygenase (Figure 4.26) inhibitory assays at 250 ppm.

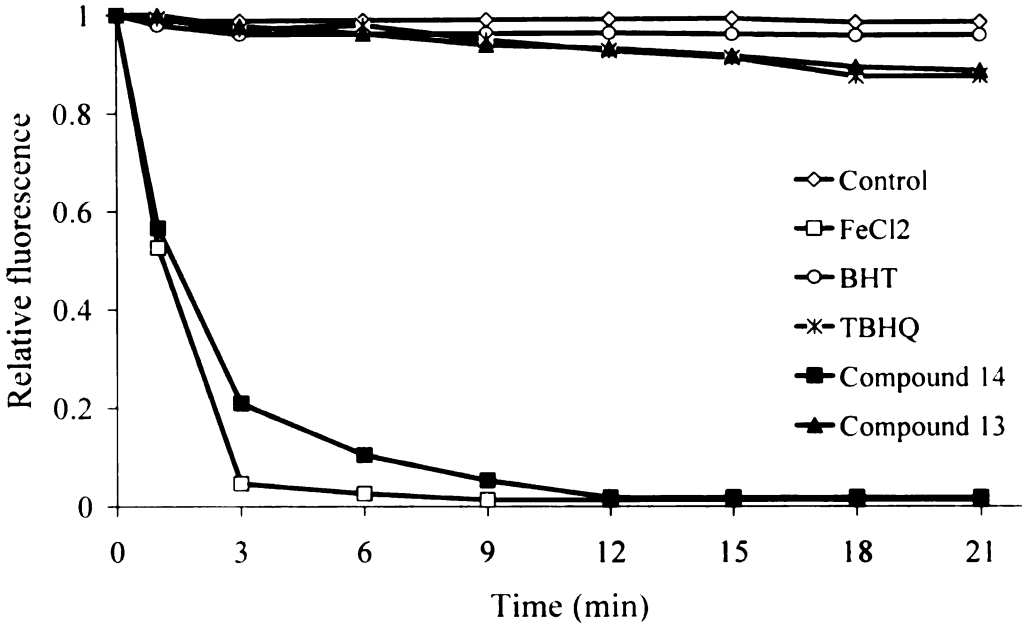


Figure 4.25: Inhibition of lipid peroxidation by compounds **13** and **14** at 250 ppm. BHT, and TBHQ were tested at 2.2 and 1.8 ppm, respectively,. The experiments were conducted in duplicate and data represents the mean relative fluorescence from 0-21 min ± one standard deviation.

It has been previously shown that fatty acids are good inhibitors of lipid peroxidation in the liposome model at much lower concentrations (Henry et al., 2002). At 250 ppm, compound **13** inhibited COX-1 and COX-2 by 77 and 93%, respectively. Erucic acid, oleic acid, and eicosenoic acid have been reported as inactive in the COX-1 and COX-2 inhibitory assays, whereas linoleic and linolenic acids showed very intense activity (Ringborn et al., 2001; Henry et al., 2002)(Figure 4.27). The content of this fatty

acid fraction in the wasabi powder was calculated at about 0.25%. At high concentrations, erucic acid has been related to the development of myocarditis and to the accumulation of fatty tissue in and around the heart of animals but not in humans (Guil et al., 1997).

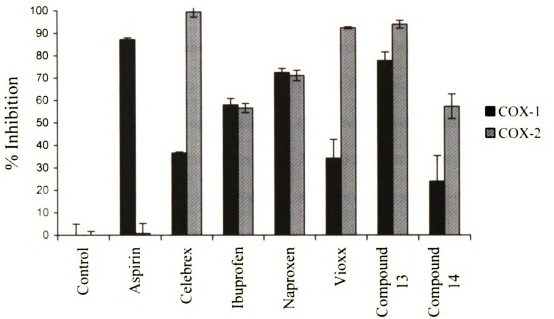


Figure 4.26: Cyclooxygenase enzyme inhibitory activities of compounds **13** and **14** at 250 ppm. Ibuprofen, Aspirin, Celebrex, Vioxx and Naproxen at 2.06, 180, 1.67, 1.67 and 2.52 ppm, respectively, were used as positive controls. Results are expressed as mean value of the percent inhibition of duplicate measurements ± one standard deviation.

Several active fractions containing fatty acids were obtained from wasabi and horseradish rhizomes. They were not characterized, but they contributed to the lipid peroxidation and COX inhibitory activity of the crude extracts.

The NMR and the GC-MS data of compound **14** allowed the identification of two components, methyl linoleate (47.2%) and methyl oleate (52.8%). No inhibitory activity was detected in the liposome peroxidation assay, but selective inhibitory COX-2 activity was measured (Figure 4.27). Under the assay conditions (pH 7.4) hydrolysis of the fatty acid methyl esters is probable so that the observed activity may be explained by the presence of linoleic acid in the reaction microchamber.

The identities of compounds **15**, **16**, **17** and **19** were established as stigma-5-en-3-O-β-glucoside (Figure 4.13), α-tocopherol (Figure 4.14), ubiquinone-10 (Coenzyme Q10)(Figure 4.15) and L-tryptophan (Figure 4.25), respectively, and confirmed by comparison with the literature data (Faizi et al., 2001; Baker and Myers, 1991; Boers et al., 2002b; Morales-Rios et al., 1987; Saito et al., 1984). Sitosterol 3-O-glucoside, α-tocopherol, ubiquinone and L-tryptophan were not tested in the assays.

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

Wasabi and horseradish rhizomes are widely used as condiments mainly for their characteristic flavor and aroma. Wasabi is also commercially available as a powder. It has long been known that glucosinolates are hydrolyzed by myrosinase enzyme to form isothiocyanates, the compounds responsible for the pungency of both wasabi and horseradish. In Chapter One, an account of the present knowledge with respect to glucosinolates and their hydrolysis products, including their biological activity was presented. Based on this account, it was concluded that wasabi and horseradish had the potential to yield bioactive compounds with lipid peroxidation, cyclooxygenase, cell proliferation and growth inhibitory activities. For the cancer proliferation assays, human colon (HCT-116), breast (MCF-7), lung (NCI-H460), and CNS (Central Nervous System) (SF-268) cancer cell lines were used.

Wasabi and horseradish rhizomes and a commercial wasabi powder were subjected to a series of different extraction protocols in order to determine their effect on the lipid peroxidation and cyclooxygenase (COX) inhibitory activities of the extracts. These results are presented in Chapter Two. A significant difference in the lipid peroxidation inhibitory activity was observed for horseradish extracts from different extraction protocols, but not for wasabi. The results of the COX inhibitory assays did not reveal any difference between extraction protocols for horseradish and wasabi. However, it was evident that extraction with water resulted in the hydrolysis of glucosinolates.

Therefore, the wasabi powder, and wasabi and horseradish rhizomes were subjected to extraction with organic solvents.

The organic extracts of horseradish, wasabi, and wasabi powder were subjected to a series of fractionation and purification procedures and led to the isolation of di-(2-ethylhexyl)phthalate (DEHP) (**1**), desulfosinigrin (DSS) (**2**), a triglyceride (**3**) plastoquinone-9 (**4**), 3-acylsitoterols (**5**), 6-O-acyl-β-D-glucosyl-β-sitosterols (**6**), 1,2-dilinolenoyl-3-β-galactosylglycerol (**7**), sucrose (**8**), β-sitosterol (**9**), sinigrin (**10**), gluconasturtiin (**11**), phosphatidyl choline (**12**), a mixture of fatty acids (**13**), a mixture of methyl linolenate and methyl oleate (**14**), sitosterol 3-O-glucoside (**15**), α-tocopherol (**16**), ubiquinone-10 (**17**) linolenoyloleoyl-3-β-galactosylglycerol (**18**), L-tryptophan (**19**) and 1,2-dipalmitoyl-3-galactosylglycerol (**20**).

Purification and biological activites of DEHP (**1**) and desulfosinigrin (DSS, **2**) obtained from wasabi powder and wasabi rhizomes are presented in Chapter Three. While DEHP was inactive in the bioassays, its presence should be cause of concern since its toxicological effects are unclear. DSS showed a strong proliferating effect on colon cancer cells and a moderate proliferating effect towards lung cancer cells. The isolation and characterization of compounds **3-20** are presented in Chapter Four. Furthermore, the bioactivity of some of these compounds is also presented in Chapter Four. Plastoquinone-9 (**4**), a mixture of 6-O-acyl-β-D-glucosyl-β-sitosterols (**6**), 1,2-dilinolenoyl-3-β-galactosylglycerol (**7**), linolenoyloleoylgalactosylglycerol (**18**) and 1,2-dipalmitoyl-3-β-galactosylglycerol (**20**) exhibited moderate COX-1 enzyme inhibitory activity. While compound **4** showed a trend towards proliferation in all cancer cells

tested except for SF-268, compound **7** was a moderate inhibitor of colon and lung cancer cells. While sinigrin (**10**) was active in the lipid peroxidation inhibitory assay, its lack of activity in the cancer cell assays was perhaps more impressive when compared to the strong proliferating activity of DSS (**2**), its biological precursor. During the course of this study, it became clear that the lipid peroxidation and COX inhibitory activities of the extracts of both wasabi and horseradish were due to fatty acids (**13**).

The present study is a contribution to the current knowledge of the phytochemical compositions in wasabi and horseradish and their bioactivities. Also, the present study reports for the first time, the isolation of the monogalactosyl diacylglycerides (MGDGs) **7**, **18**, and **20** from wasabi and horseradish. These compounds inhibited the growth of human tumor cell lines. The topical anti-inflammatory activity of MGDGs has been previously reported, but their selective COX-1 enzyme inhibitory activity is reported here for the first time. Although there is no epidemiological evidence linking MGDGs with cancer chemoprotection, the cytotoxicity of these ubiquitous compounds may help explain the lower cancer incidence in societies with high intakes of fruits and vegetables. More importantly, the cancer cell proliferating activity of the desulfosinigrin (**2**) is reported for the first time. Wasabi is widely consumed in several Asian countries, especially in Japan, as part of the traditional cuisine. Although wasabi and horseradish contain beneficial phytochemicals, the presence of cancer cell proliferating DSS and other desulfoglucosinolates merits further study to establish wheter it may help explain the high incidence of certain cancers -including colon- in Japan.

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