Bioactivity and bioavailability of lignans from sesame (*Sesamum indicum*. L)

A thesis Submitted to the University of Mysore
in fulfillment of the requirements
for the degree of
Doctor of Philosophy in Biotechnology

by

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DECLARATION

I hereby declare that this thesis entitled "**Bioactivity and bioavailability of lignans from sesame (*Sesamum indicum.* L)**" submitted herewith, for the degree of **Doctor of Philosophy in Biotechnology** of the University of Mysore, Mysore, is the result of work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore, India, under the guidance and supervision of Dr. Sridevi Annapurna Singh, during the period of November, 2003 – September, 2009.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

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CERTIFICATE

I hereby certify that this thesis entitled "Bioactivity and bioavailability of lignans from sesame (*Sesamum indicum.* L)" submitted by Mr. Mahendra Kumar. C to the University of Mysore, Mysore, for the degree of Doctor of Philosophy in Biotechnology, is the result of research work carried out by him in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore, under my guidance and supervision during the period of 2003 – 2009. This work has not been submitted either partially or fully for any other degree or fellowship.

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Bioactivity and bioavailability of lignans from sesame (*Sesamum indicum.* L)

ABSTRACT

The focus of the present investigation is the study of the biological activities of sesame lignans on interaction with proteins possessing physiological and biochemical significance.

Sesame lignans, sesamin and sesamolin were isolated, purified by preparative RP - HPLC and characterized by GC-MS. Sesamin and sesamolin were recovered up to 0.3 g/100 g, and 0.2 g/100 g respectively in the commercially available white sesame seeds. Screening of the different varieties of sesame, for antioxidant contents, with special reference to lignans and γ- tocopherol, was carried out by HPLC. Conditions were standardized for the conversion of sesamolin to sesamol, - a better antioxidant by electrical and infrared roasting. Infrared roasting clearly showed the formation of 1320 mg kg^{-1} at 220°C which is 30% more than the conventional electric roasting.

Antioxidant, free radical scavenging and antibacterial properties of lignans were studied. Sesamol was found to be the best antioxidant and free radical scavenger, amongst the lignan molecules studied. The ORAC-FL value of sesamol was determined to be 4.4 μmol of trolox

equivalents/ml, which is the highest among all the 3 molecules studied lignans. Sesamin and sesamolin had 0.8 and 1.52 μmol of trolox equivalents/ml.

DPPH radical scavenging assay revealed lignans and γ-tocopherol were synergistic in antioxidant action. γ-tocopherol alone has the IC_{50} value of 4.5 μg/ ml. In presence of 10 μg of sesamin, IC_{50} value decreased to 2.74. In the presence of 2 μg sesamol IC_{50} value was found to be 1.6 μg, which is 3 times more potent of γ-tocopherol alone.

The bioavailability and stability to gastrointestinal digestion *in vitro* was evaluated by treating the lignans with gastrointestinal proteases - pepsin and pancreatin. Nearly 45.2, 59.6 and 44.9% of sesamol, sesamin and sesamolin respectively, were available for intestinal absorption.

Sesamol, the phenolic degradation product of sesamolin, inhibited melanin synthesis in mouse melanoma B16F10 cells in a concentration dependant manner, with 63% decrease in cells exposed to 100 μg/ml sesamol.

Sesamol inhibits diphenolase activity and monophenolase activity with midpoint concentrations of 1.9 μM and 3.2 μM, respectively. It is a competitive inhibitor of diphenolase activity with a *K*i of 0.57 μM and a non competitive inhibitor of monophenolase activity with a *K*i of 1.4 μM.

We have studied the role of lignans in mitigating the inflammatory response in the rat model. Rats fed with sesamin had 37% reduced inflammation compared to control group. Sesamol and sesamin were potent inhibitors of 5-lipoxygenase activity, with IC_{50} values of 77 and 192 μM, respectively. Inhibition of lipoxygenase activity could be one of the factors contributing to the anti-inflammatory effects of lignans.

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CONTENTS

ABBREVIATION

ε	Epsilon
µg	Microgram
5- HETE	5-hydroxyeicosatetraenoic acid
5-LOX	5-Lipoxygenase
9S-HETE	9S-hydroxyeicosatetraenoic acid
AA	Arachidonic acid
BHT	Butylated hydroxytoluene
CD	Circular dichroism
DOPA	3,4- Dihydroxy-L- phenylalanine
DPPH	2,2-diphenyl- 1-picrylhydrazyl
DTT	Dithiothreitol
$E^{1\%}$	Absorption coefficient of 1% protein solution
EMS	Ethyl Methane Sulphonate
EDTA	Ethylene diamine tetra acetic acid
FRAP	Ferric reducing antioxidant power
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GIT	Gastrointestinal tract
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HPLC	High performance liquid chromatography

IC_{50}	Midpoint concentration of inhibitor
LDL	Low-density lipoproteins
LOX-1	Lipoxygenase 1
LTB4	Leukotriene B4
kDa	kilo Daltons
K_I	Inhibition constant
K_m	Michaelis constant
MBTH	3- Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate
MIC	Minimum inhibitory concentration
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NaCl	Sodium chloride
$O_2^{\bullet -}$	Superoxide anion
$OH^{\bullet}$	Hydroxyl radical
ORAC	Oxygen Radical Absorbance Capacity
PAGE	Polyacrylamide gel electrophoresis
PDA	Photodiode array
PUFA	Polyunsaturated fatty acids
RP	Reverse phase
ROS	Reactive Oxygen Spices

SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TE	Trolox equivalents
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
TPTZ	2,4,6-tripyridyl-s-triazine
V_{max}	Maximum velocity

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SYNOPSIS OF THE THESIS

BIOACTIVITY AND BIOAVAILABILITY OF LIGNANS FROM SESAME (*Sesamum indicum* L.)

Submitted to the UNIVERSITY OF MYSORE
for the degree of *Doctor of Philosophy* in *Biotechnology*

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BIOACTIVITY AND BIOAVAILABILITY OF LIGNANS FROM SESAME *[Sesamum indicum. L]*

Sesame, an important oilseed, is one of the oldest oilseed known to man and is considered to have not only nutritional value, but also medicinal properties. The oil from this plant, sesame oil is an important ingredient in Ayurvedic remedies in India and is also used in ancient Chinese medicine to increase energy and prevent ageing. Sesame seed contains nearly 50% oil, which is stable against oxidative degradation. The seed has superior nutritional value containing about 20% protein along with various minor nutrients.

Sesame is a source of biologically active phytochemicals. Sesame seeds are rich in lignans, which are methylenedioxyphenyl compounds, such as sesamin, sesamolin, sesaminol and others. The high resistance of sesame oil to oxidative deterioration as compared with other vegetable oils is attributed to the phenolic compound, sesamol, produced from sesamolin. Sesamol is a phenolic compound that is the thermal degradation product of sesamolin and is present in abundance in roasted sesame oils. The quality (flavor, color and oxidative stability) of the roasted sesame oil depends mainly on the roasting conditions. The good flavor generated by roasting sesame seed is also a highly desirable characteristic. The degrees of browning and oxidative stability of the roasted sesame oil are increased by higher roasting temperature.

Many health benefits of sesame are attributed to lignans. In the presence of γ-tocopherols, sesamin lowers the concentrations of total cholesterol, VLDL+LDL and HDL in plasma and liver of rats. Sesamol exhibits powerful inhibitory effects on lipid peroxidation of liposomes induced by Fe^{2+}, on the lipid peroxidation of rat liver microsomes induced by carbon tetrachloride and NADPH and on lipid peroxidation of mitochondria induced by ascorbate/Fe^{3+} ions. It is shown that sesamol, sesamolin, sesamin, γ -tocopherol, browning materials cannot individually account for the high oxidative stability of roasted sesame oil, and a system composed of all these sesame oil components synergistically protect roasted sesame oil against oxidative deterioration. The biological activities of the lignans and their products need careful

characterization. These studies can be carried out *in vitro* or *in vivo*. Some of the key enzymes involved in the metabolic pathways can also be affected by the bioactive molecules resulting in their biological effects. Oxygenases are particularly affected by antioxidants and could be the targets for studying the effects of these compounds. Some of the key oxygenases include tyrosinase, lipoxygenase and cyclooxygenases, which play important roles in melanogenesis and the inflammatory pathways.

The focus of the present investigation is to study the biological activities of sesame lignans with proteins having physiological/biochemical significance using various biophysical approaches and enzyme kinetics. Accordingly, the objectives of the thesis are :

1. To optimize the extraction of major sesame lignans by physical, chemical and enzymatic means. Recovery and isolation of the major sesame lignans – sesamin, sesamolin and sesamol.

2. Bioactivity and bioavailability of sesame lignans *in vitro* and *in vivo* and intracomparison with special reference to antioxidant properties in isolation and combination as well as the synergistic effect.

The major lignans sesamin and sesamolin as well as sesamol the degradation product of sesamolin have been purified, characterized and used for the investigation. Optimization of roasting parameters to obtain sesamol and improve quality of oil is discussed. The biological activities of lignans including antioxidant, free radical scavenging, antiinflammatory and antibacterial activity have been characterized by both *in vitro* and *in vivo* assays. Interaction of sesame lignans with (a) mushroom tyrosinase and melanoma B16F10 cells was studied *in vitro* (b) lipoxygenases (mammalian lipoxygenase and soy lipoxygenase 1) and effect of lignans on λ-carragenaan induced inflammation on rats were studied.

The thesis is organized into four chapters:

Chapter 1 – Introduction: This chapter deals with the general introduction and scope of the present investigation. Literature survey pertaining to the beneficial effects of sesame seeds, roasted sesame oil and the important phytochemical -lignans is presented. Health beneficial effects of natural sesame components in the prevention of various pathological conditions ranging from cancer to cardiovascular diseases are discussed. The importance of oxygenases, their structure and function and their inhibition by natural phytochemicals are reviewed. The role of products of oxygenase reactions in the genesis of various diseases and disorders is also presented. The chapter ends with the aim and scope of the investigation.

Chapter 2 – Materials and Methods: This chapter details the materials and methods used in the investigation, including the experimental approach and data analysis. Isolation, purification and characterization of lignans by preparative HPLC, RP-HPLC and GC-MS to analyze the purity of the molecules are described in detail. Optimization of roasting parameters for the processing of sesame seeds was described. Various antioxidant assays like free radical scavenging, lipid peroxidation assays and antimicrobial assays as well as spectroscopic and kinetic methods used in enzyme inhibition studies are detailed. Experiments involving cell culture procedures of melanoma B16F10 cells studied *in vitro* and animal model experiments are also described.

Chapter 3 – Results and Discussion: Presents the results and discussion of the present investigation under four sections (1 – 4).

Section 1 - Screening of sesame seeds for lignans, preparation of heat resistant sesame seeds, isolation and characterization of selected lignans and optimization of roasting methods for the formation of sesamol

Sesame lignans, sesamin and sesamolin were isolated and purified to homogeneity by chromatographic (preparative HPLC) methods. The homogeneity was established by

checking its purity by analytical high performance liquid chromatography and GC-MS (> 95% purity).

Different sesame varieties, received from Dharwar Agricultural University, were screened for their lignan, tocopherol and polyphenol contents. These varieties were developed from the parent varieties through physical and chemical mutations. Selection of the varieties was based on seed yield, color and seed boldness. The parental variety had sesamin and sesamolin 3.59 and 1.68 g / kg of oil respectively. Sesamin contents of the mutant progeny varied from 1.76 - 10.46 g/kg while sesamolin varied from 0.72 – 3.77 g / kg of the oil. The γ-tocopherol contents were also estimated by normal phase HPLC method. Roasting of sesame seeds degrades sesamolin to sesamol, which increases the oxidative stability of sesame oil synergistically with tocopherols. Different heating methods were investigated for optimizing sesamolin conversion to sesamol. The quality of oils and meal obtained were studied for nutritional, sensory as well as stability.

Section 2 – Bioactivities and bioavailability of sesame lignans: *in vitro* studies

Antioxidant, free radical scavenging and antibacterial properties of lignans were the biological activities studied. Sesamol was the best antioxidant and free radical scavenger amongst the lignan molecules studied. Antioxidant activity in linoleic acid emulsion by sesame lignans was determined by thiocyanate method. All the three lignans, sesamin, sesamolin and sesamol inhibited lipid peroxidation with 96, 86.4 and 98% inhibition, respectively. The ability to scavenge various oxygen free radicals by sesame lignans was studied by Oxygen Radical Absorption Capacity (ORAC) method. At 10 μM concentration, sesamol shows up to 96% scavenging ability compared to 73 % by standard trolox. The DPPH radical scavenging ability of γ-tocopherol is enhanced by three times in presence of sesamol or sesamin synergistically. γ-Tocopherol, alone, has the IC_{50} value of 4.5 μg/ ml and in presence of 10 μg of sesamin, the IC_{50} value decreases to 2.74. In the presence of 2 μg sesamol, IC_{50} value was found to be 1.6 μg which is more than 3 times the potency of γ-tocopherol alone. All 3 molecules shows antimicrobial activity against known food borne pathogenic bacteria.

The bioavailability of these molecules was assessed by *in vitro* digestibility followed by HPLC measurements. After gastric digestion, 81.5, 70.2 and 68.4 % of sesamol, sesamin and sesamolin were recovered. Effectively, all the 3 lignans survived gastric digestion and as well as pancreatic digestion.

Section 3: Inhibition of tyrosinase by sesamol: Effect on melanin and melanin synthesizing melanoma cells

This section deals with the effect of sesame lignans and sesamol on the activity of tyrosinase and the growth and survivability of cultured mouse melanoma B16F10 cells as followed by MTT assay, melanin estimation and apoptotic properties. There is 70 % decrease in the growth of melanoma cells cultured in the medium containing 100 μM sesamol. Cells treated with sesamol and sesamin shows 63% and 5.6% decrease in the melanin content. The results include kinetic and spectroscopic studies of inhibition of both diphenolase and monophenolase activities of mushroom tyrosinase as well as the active site copper. Sesamol is a powerful inhibitor of mushroom tyrosinase with the K_I values of 0.573 μM and 1.4 μM for diphenolase and monophenolase activities, respectively. A mechanism for the inhibition of tyrosinase activity by sesamol is proposed.

Section 4: Inhibition of lipoxygenase by lignans: Evidence of sesame lignans as anti-inflammatory components in animal model.

Lipoxygenase catalyzed products play a major role in the manifestation of inflammatory response in animals. In order to understand the role of physiological role lignans, inhibition of lipoxygenases from plant (soybean lipoxygenase 1) and animal systems (mammalian 5-lipoxygenase) has been followed by enzyme kinetics, spectroscopic and biophysical methods. The role of lignans in mitigating the inflammatory response in the rat model was studied by λ-carrageenan induced post local inflammation on rats. Sesamin lowered inflammation to an extent of 40%, while dietary sesamolin and sesamol reduced the inflammation to an extent of 9 and 15%,

where the paw inflammation at 5 h was 84% of the control. Sesamol and Sesamin were the most effective inhibitor of 5-lipoxygenase with IC_{50} values of 77 and 192 μM respectively. This result suggests that sesame lignans shows antiinflammatory activity by inhibiting the lipoxygenase pathway.

Chapter 4 – A general summary and conclusions of the present investigation is given in this chapter.

Chapter 4 is followed by the **Bibliography** or list of references and list of publications from the present investigation.

Publications:

1. **Mahendra kumar C.,** A. G. Appu Rao and Sridevi Annapurna Singh, (2009) Effect of Infrared Heating on the Formation of Sesamol and Quality of Defatted Flours from *Sesamum indicum* L. *J. Food Sci.* 74(4), H105 – H111.

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Chapter I

Introduction

INTRODUCTION

Sesame seed and its oil are being utilized as important food ingredients since about 6,000 years. The name, sesame, comes from the Arabic word "simsim". The Thebes Medicinal Papyrus (1552 B.C.), an old text found in Egypt, describes the medicinal effect of sesame seed as a source of energy. In traditional Indian medicine, Ayurveda, sesame oil has been used as the basal oil for human body massage since 1100 B.C. (Weiss, 1983; Joshi, 1961). The Japanese traditionally believe that sesame is very good for health (Namiki, 1990; Namiki, 2007).

Bredigian and Harlan (1986) cited India as the origin of sesame, however there is also a belief that the actual origin was Africa, where many wild species are found (Namiki, 1995). A cultivated sesame species, *Sesamum indicum* L., is thought to have originated in the Savannas of Central Africa spreading to Egypt, India, the Middle East, China, and elsewhere.

Sesame has been evaluated as a representative health food and widely used for its good flavor and taste (Namiki, 2007). Recently, scientific researchers have investigated many physiological aspects of sesame seed. Studies on antioxidant, anti-aging, the synergistic effect with tocopherols, serum lipid lowering, blood pressure lowering, and other functions have been reported (Namiki, 2002). Despite such high value being placed on sesame seed, there have been limited scientific studies validating these claims.

Sesame – Cultivation and Production

Sesame belongs to the genus *Sesamum*, one of 16 genera in the family Pedaliaceae. The genus, *Sesamum,* comprises about 35 wild species, besides the only cultivated species, *Sesamum indicum*. Sesame is a broad-leaf summer crop with bell-shaped flowers and opposite leaves. It is an annual plant that can reach 1-1.8 m high. The plant prefers fertile, well drained, and neutral to slightly alkaline soil pH. Sesame is cultivated in tropical areas and needs a growing season of 110-150 frost-free days. The optimum air temperature is 30-35°C and the soil temperature never below 20°C. Flowering starts about 40 days after planting the seeds and continues for almost a further 40 days. A sesame plant with flowers is shown in Figure 1.

Figure 1. A flowering sesame plant

The flowers develop into seed pods (capsules), which contain 70-100 seeds each. The color of seeds varies from white to brown, gold, gray, violet and black. Seeds are tiny and weigh 2-3.5 g/1000 seeds (Figure 2).

Figure 2. Sesame seeds (a) Undehulled sesame seeds (b) Close view of dehulled sesame seeds

Seed yield ranges from 350 to 1700 kg/ha depending on the varieties used and cultivation techniques. The average yields are almost 500 kg/ha. The production of sesame seed in 2008 is reported as 3.69 million tons worldwide, which puts sesame as the 8th highest oil crop (FAOSTAT, www.fao.org). India produces the highest quantity of sesame seeds in the world. Table 1 shows the country-wise cultivation and production of sesame seeds in the year 2007 based on FAO statistics. South Korea is the highest per capita consumer in the world, 6–7 g/day.

Japan is the largest importing country, nearly 150,000 tons a year, 60% of which is processed into oil.

In spite of an increasing demand for this highly valued food, the production of sesame seed has not increased. This is mainly because of the requirement of manual harvesting procedures. Seeds are expelled by pod explosion and hence harvesting has to be carried out at maturity before the pod opens. The small size of the seed is also a hindrance for mechanical harvesting. Following the discovery of a non-dehiscent (non-shattering) mutant by Langham in 1943, plant-breeding efforts have begun toward developing high-yield shatter-resistant varieties. Sesame plants with semi-shattering and non-dehiscent seeds, in which the capsules hold the seeds when they get dry, mechanized harvest is conducive being compared to traditional shattering plants.

Sesame Seed as Food

Sesame seed varies considerably in color, size, and texture of the seed coat. Sesame seed is valued for its high content of oil (about 50%), which is very stable against oxidative degradation. It contains about 20% protein plus various minor nutrients. After cleaning, washing with water and drying, the seeds are usually roasted at 120 - 150ºC for about five minutes. Roasting brings out the characteristic rich flavor. During processing, they are sometimes dehulled mechanically, but they lose some useful nutrients, such as calcium in the process (Namiki, 1995).

Table 1. Country Wise Production of Sesame Seed

Country	Area Harvested (000 acres)	Production (000 tons)
India	4,324	739
Myanmar	3,954	661
China	1,534	615
Sudan	3,781	287
Uganda	692	185
Ethiopia	544	181
Nigeria	484	110
Paraguay	148	58
Bangladesh	198	55
Tanzania	284	51
Thailand	162	47
Central African Republic	99	44
Egypt	77	41

(Ref: FAO, 2007)

Both the seeds and oil are used as food. In China, Korea and other Asian countries, roasted sesame seed is generally used as a topping for many baked foods, such as bread, biscuits and crackers. The roasted seed is also ground into a paste-like product. In Japan, roasted seeds are mixed with common salt and used as a topping on cooked rice. Sesame mash and paste, made by grinding seed in a conical ceramic mortar, are widely utilized as seasoning for salads, cooked rice, boiled meat and other foods. Sesame-tofu (goma-dofu in Japanese) prepared from mashed sesame seed and a starch such as arrowroot starch, is a popular food in Japan (Namiki, 2007). In the Near East and North Africa, sesame paste is generally used. For example, the paste is the base for much of the cooking (called tahina) and for cake (halva). In these uses, a weak roast flavor is preferred. In Africa, the leaves of sesame are eaten. In North America, sesame seed is generally hulled and used for topping on bread and hamburger buns (Namiki, 1998). In India, sesame oil is consumed as hardened oil in margarine. Unlike in East Asia, the oil is not roasted. Other uses in India are for cakes, seasoning and toppings. This has reduced the performance of sesame oil as cooking oil in India. Sesame oil is used as a salad or cooking oil, in shortenings, margarine and to marinate meat and vegetables. Refined sesame oil is mainly used in pharmaceutical and cosmetic industries.

Composition of Sesame Seed

The major constituents of sesame seed are oil, protein and carbohydrates, lignans and the minor constituents such as vitamins and minerals. The compositions of sesame seeds described by Namiki (2007) are summarized in Table 2.

Table 2. Composition of sesame seed (per 100 g)

Energy (calories)	578	Fe (mg)	9.6
Moisture (%)	4.7	P (mg)	540
Fat (g)	51.9	Mg (mg)	370
Protein (g)	19.8	Na (mg)	2
Carbohydrate (g)	18.4	Vitamin C (μg)	12
Fiber (g)	10.8	Vitamin A (IU)	10
Ash (g)	5.2	Carotene (μg)	17
Lignans (g)	1.8	Thiamine (mg)	0.95
K (mg)	400	Riboflavin (mg)	0.25
Ca (mg)	1200	Niacin (mg)	5.1

(Namiki, 2007)

Sesame seed and its defatted form contains protein about 20% and 50% respectively. Protein can be extracted with 10% NaCl and separated into 13*S* (70%), 5*S* (10%), and 3*S* (20%) by centrifugation (Prakash and Nandi

1978). The content of amino acids varies somewhat among species. No significant difference is however, found between the white and black species of sesame. Compared with the standard values recommended by FAO/WHO (1973), the amino acid composition of sesame seed protein is slightly lower in lysine (31 mg/g protein) but greater in other amino acids, especially methionine (36 mg), cysteine (25 mg), arginine (140 mg) and leucine (75 mg). The protein in soybean is rich in lysine (68 mg) but low in methionine (16 mg), so that the simultaneous feeding of both proteins produces good growth in rats (Namiki, 1995). A study on the nutritional quality of the protein fraction, defatted sesame seeds, extracted with isopropanol (DSS-Iso) has shown that rats fed a DSS-Iso based diet exhibit significant decrease in total plasma cholesterol, triglycerides, and VLDL+LDL cholesterol, in comparison with the rats fed a diet containing casein (Sen and Bhattacharyya, 2001). Unroasted seed contained considerable amounts of such taste-enhancing amino acids as glutamic acid, arginine, aspartic acid and alanine, but their amount decreases significantly on roasting at 170°C for 20 min or 200°C for 5 min (Namiki, 1998). The carbohydrate content in sesame seed is about 18–20 wt%. The presence of small amounts of glucose and fructose, and also an oligo-sugar planteose [O-α-D-galactopyranosyl-(1,6)-β-D-fructofuranosyl-α-D-glucopyranoside] is reported (Wankhede and Tharanathan, 1976). However, no starch is present. Most carbohydrates seem to be present as dietary fibers and the content of the dietary fibers

is reported to be 10.8% (STA Japan, 2000). Further, study on the carbohydrates in relation to some functional activities of the water-alcohol extractable fraction of defatted sesame seed is needed.

As shown in Table 2, sesame seed contains a significant amount of vitamin B. However, most of it is present in the hull of the seed and the dehulled seed contains no vitamin B (Brito and Nunez, 1982). In order to utilize vitamin B in sesame seed, it is necessary to use whole sesame seed flour or mashed sesame seed. The presence of vitamin E in sesame seed, among others, is of interest in relation to the effectiveness of sesame seed as a health food. The content of tocopherols in sesame is less than that in soybean and corn oil (Speek et al., 1985). Moreover, the tocopherols in sesame seed are mostly present as the γ-isomer, while the content of *α*-tocopherol is very low. It is known that vitamin E activity of γ-tocopherol is less than 10% that of α-tocopherol (Bieri and Evarts, 1974). Thus, sesame seed appears to contain a little vitamin E activity. Despite this, however, sesame shows clear anti-aging effects in mice.

Very few studies have been done on the nutritive value of various mineral constituents of sesame seed. Among them, calcium and iron, which are often deficient in modern diets, are found in high concentrations (1200 mg/100 g and 9.6 mg/100 g, respectively). As calcium is contained mainly in the hull as an oxalate, nutritionally available calcium may be reduced. Studies show that the bioavailability of calcium in sesame for rats is about 65% (Poneros-schneier and Erdman, 1989), but another

study estimates it to be about one-seventh of the total content, i.e., about 168 mg/100 g due to a large amount of combined calcium (Ishii and Takiyama, 1994). It is also noteworthy that most of the calcium contained in the hull is lost by dehulling and only about 20% of the total calcium remains (Namiki, 1995). The presence of selenium is also unusual, i.e., 36.1 mg/g in isolated sesame protein (Namiki, 1995). As is commonly known, selenium is a constituent of glutathione peroxidase, which is involved in the prevention of physiological peroxidation.

Sesame Oil

The main energy source of sesame seed is the oil. The average content of oil is 55.0% in white-seed strains and that of black-seed strains is 47.8% (Tashiro et al., 1990), although the content can vary considerably depending on the species and cultivation conditions. Fatty acids in the oil are, mainly, oleic (18:1=39.1%) and linoleic (18:2=40.0%) acids, with palmitic (16:0=9.4%) and stearic (18:0=4.76%) acids in smaller amounts and linolenic (18:3=0.46%) acid in trace amounts. Linoleic, linolenic and arachidonic acids are considered to be essential fatty acids for humans. According to recent studies on prostaglandins, n-3 (linolenic acid) and n-6 (linoleic acid) fatty acids have independent roles in the synthesis of prostaglandins. The ratio of n-3 to n-6 fatty acids in the composition of fatty acids is important. From this standpoint, sesame oil, in which the n-3 fatty acid content is low, is inferior to soybean and corn oils (Numa,

1984). Variation in fatty acid composition of different sesame species including a cultivated type and three wild types has been studied (Kamal-Eldin and Appelqvist, 1994a).

Two kinds of sesame seed oil are generally used - raw oil and roasted oil. The former is prepared by expelling steamed seeds and refining through the usual refining process for common vegetable oils (decoloration with acid clay and deodorization by steam distillation *in vacuo*). The product is clear and mild flavored oil, mainly used for frying and as dressing for salad. Roasted oil is produced by expelling the seeds, previously roasted at 180–200°C for 10 to 20 minutes and refining simply by filtration. It has a characteristic roasted flavor and a deep yellow-brown color. It is widely used in China, Korea and Japan as an important seasoning and cooking oil, e.g., for tempura in Japan (Namiki et al., 2002).

Roasting is the most important form of processing of sesame foods and oils; it brings out a characteristic pleasant flavor. The roasted flavor depends greatly on the roasting temperature and the time exposed, from a somewhat grassy, mild, sweet, and desirable aroma to an irritating, scorched smell. This change occurs in the temperature range of 140–230°C. Optimum production conditions are 150–175°C for 15–20 min for roasted seeds. Many chemical studies have been done on the roasted sesame flavor and more than 400 components have been isolated and identified. They have been classified as pyrazines (number of identified compounds: pyridines, pyrroles, furans, thiophenes, thiazoles, carbonyl

compounds, are a few). Among them, alkyl pyrazines are the main components and provide the representative deep-roasted flavor, whereas thiazoles and thiophenes are assumed to contribute to the characteristic roasted sesame flavor (Schieberle, 1995; Shahidi et al., 1997; Namiki, 1998). Roasting of sesame can be carried out by hot air drum roasting that is electrically controlled. Newer methods like infrared roasting present alternatives for better efficiency.

Infrared roasting

Foods and biological materials are heated primarily to extend their shelf life or to enhance taste. Infrared roasting or heating is a relatively new alternative to electrically controlled roasting processes. Energy conservation is one of the key factors determining profitability and success of any unit operation. Heat transfer occurs through one of 3 methods, conduction, convection, and radiation. In conventional heating, which is achieved by combustion of fuels or by an electric resistive heater, heat is generated outside of the object to be heated and is conveyed to the material by convection of hot air or by thermal conduction. By exposing an object to infrared (IR) radiation (wavelength of 1.1 to 1.3 μm), the heat energy generated can be absorbed by food materials. Along with microwave, radiofrequency (RF), and induction, IR radiation transfers thermal energy in the form of electromagnetic (EM) waves and encompasses that portion of the EM spectrum that borders on

visible light and microwaves (Krishnamurthy, et al., 2008). Certain characteristics of IR heating such as efficiency, wavelength, and reflectivity set it apart from and make it more effective for some applications than others. IR heating is also gaining popularity because of its higher thermal efficiency and fast heating rate/response time in comparison to conventional heating. Recently, IR radiation has been widely applied to various thermal processing operations in the food industry such as dehydration, frying, and pasteurization (Sakai and Hanzawa 1994).

IR radiation can be classified into 3 regions, namely, near infrared (NIR), mid-infrared (MIR), and far-infrared (FIR), corresponding to the spectral ranges of 0.75 to 1.4, 1.4 to 3, and 3 to 1000 μm, respectively (Sakai and Hanzawa 1994). The application of infrared radiation to food processing has gained momentum due to its inherent advantages over the conventional heating systems. Infrared heating has been applied in drying, baking, roasting, blanching, pasteurization, and sterilization of food products. Roasted sesame oil, apart from its pleasant flavor, is more resistant to oxidation compared to the raw oil.

Antioxidative Activity of Sesame Oil

Oxygen free radicals or more generally reactive oxygen species (ROS) are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems

(Valko et al., 2006). The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called “redox regulation” (Kovacic and Jacintho, 2001). Excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal function.

A wide range of phenolic compounds such as phenolic acids, flavonoids, anthocyanins, tannins, lignans, catechin, and others are also known to possess antioxidant activities (Kahkonen et al., 1999). These phenolics provide protection against harmful free- radicals and have been known to reduce the risk of certain types of cancer, cardiovascular disease (CVD), coronary heart disease (CHD), stroke, atherosclerosis, and other degenerative diseases associated with oxidative stress (Shahidi and Naczk, 2004).

Antioxidants in food have been found to play an important role in preventing damage due to active oxygen *in vivo*, which lead to various life style diseases such as circulatory disorders, carcinogenesis and aging (Namiki, 1998). The characteristic of sesame oil, which has been proved empirically, is its high resistance to oxidative deterioration. In ancient Egypt, it was used for making mummies. In Japan, it has been evaluated as the best oil for deep frying tempura because of its superior stability against deterioration by heating. As noted earlier, there are two different kinds of sesame oil, roasted and unroasted. The antioxidative activities of these oils were demonstrated in experiments with other common

vegetable oils that were stored in an open dish at 60°C, and autoxidation was determined by increase in weight by peroxidation. Soybean oil, rapeseed oil, and others showed rapid increase in oxidation after about 10 days, whereas both roasted and unroasted sesame oils are very stable. The unroasted oil remains unchanged for 30 days, while no oxidation is observed even after 50 days in the roasted oil (Namiki, 2007). However, no chemical investigations explaining the antioxidative factors contained in these oils were conducted. Two reports exist that sesamol, a degradation product of sesamolin in roasted oil, is the strong antioxidative phenol (Budowsky and Narkley, 1951; Budowsky, 1964). Namiki et al. (2002) conducted investigations on the chemistry and function of antioxidative factors in sesame seed and oil. In their studies, several antioxidative components involving the phenolic lignans sesaminol and sesamolinol have been isolated and identified (Fukuda et al., 1985; Namiki, 1995). Roasted sesame seed oil has a characteristic flavor and red-brown color probably caused by the Maillard-type reaction during roasting.

The antioxidative activity increases mainly in proportion to the roasting temperature along with the brown color, indicating that some products of the roasting reaction contribute to the antioxidative activity. Antioxidation tests on the ether and methanol extracts of the strongly roasted oil indicate that each fraction alone was not so effective. A combination of the extracts shows significant synergistic effect, while

that of three or four fractions exhibit even stronger activity. Thus, the very strong antioxidative activity of the roasted oil might result from the synergistic effect of the combination of such effective factors as sesamol produced from sesamolin, γ-tocopherol, sesamin, and roasted products such as melanoidins (Fukuda et al., 1986a; Koizumi et al., 1996; Fukuda et al., 1996).

Unroasted seed oil is obtained by expelling steamed raw seed and refining the product by decolorization with acid clay and deodorization by steam distillation, *in vacuo*. Its antioxidative activity is much stronger compared to common vegetable oils and it contains no Maillard-type products or sesamol, unlike oil from roasted sesame seeds. Investigation of its antioxidative factors has shown sesamolin in the raw oil is almost completely lost during the decolorization process, whereas a significant amount of a new phenolic lignan named sesaminol, identified to be the principal factor of the antioxidative activity of unroasted oil, could be produced by a novel intermolecular rearrangement of sesamolin, as noted above (Fukuda et al., 1986b; Fukuda et al., 1986c). Figure 3 shows the structure of sesame lignans and the degradation products of sesamolin.

Sesame oil from the whole seed is found to be somewhat more stable than that from the dehulled seed. The oil extracted with a polar solvent (hexane-isopropanol) is apparently better than that extracted with a non-polar solvent (hexane) (Kamal-Eldin and Appelqvist, 1995). Recently, a

new extraction process using supercritical fluid extraction with carbon dioxide (SFE-CO_2) has been introduced. Interestingly, it is found that the lignans are extracted prior to the oil (triglycerides) and therefore, may be obtained easily in a concentrated form at an early stage of the extraction, along with the concentration of antioxidative factors and characteristic roast flavors. Such extraction of lignans has never been accomplished before (Namiki et al. 2002). Detailed investigation into the extraction conditions of sesame oil by SFE-CO_2 also indicated the superiority of the SFE over n-hexane extraction, especially regarding the antioxidative activities of sesame oil (Hu et al., 2004). It has also been shown that when foodstuff, covered with wet material is fried in roasted seed oil, as in the case of Japanese tempura, sesamol is produced by the splitting of sesamolin, resulting in the formation of a strong antioxidative coating on the fried food (Fukuda, et al., 1986a).

Sesame Lignans

Lignans are a group of natural compounds, which are defined as an oxidative coupling product of *β*-hydroxyphenylpropane and widely distributed as a minor component in the plant kingdom, especially in bark of wood. Some lignans are known to have antitumor, antimitotic, and antivirus activities. Interestingly, sesame seed contains significant amounts of characteristic lignans such as sesamin, sesamolin, sesaminol and others. Sesame lignans are being noted as the most important and

characteristic components of sesame seed in view of their various functional activities.

Sesamin and sesamolin have been known to be the only major lignans in sesame seed (Budowsky, 1964) till the identification of sesaminol as another major lignan (Osawa et al., 1985). Sesamin with a typical lignan structure of *β-β ′*(8-8′) linked product of two coniferyl alcohol radicals, and it has been found in other plants such as beech, in small amounts (Figure 3). Sesame seed contains sesamin in large amounts (usually about 0.4% in sesame oil) (Tashiro et al., 1990). Sesamin is highly hydrophobic and easily obtained as a crystalline product from the scum obtained by the vacuum-deodorization process in the purification of unroasted sesame oil production.

A significant positive correlation was observed between the oil content of sesame seed and the sesamin content in the oil, whereas no correlation was found between the oil content and the sesamolin content (Tashiro et al., 1990). Large differences exist in lignan contents among the wild types of sesame. For example, an Indian type has a very low sesamolin content (sesamin: 256.1 mg/100 g; sesamolin: 35.6 mg/100 g) while another type from Borneo, Indonesia, has a remarkably high lignan content (sesamin: 1152.3 mg/100 g; sesamolin: 1360.7 mg/100 g) (Namiki, 1995). Interestingly, differences in the lignan composition and content were observed among four *Sesamum* species, including three wild types.

Sesamin was found in considerable amounts in *S. indicum* cultivars and *S. angustifolium*, in very large amounts (2.40% in oil) in *S. radiatum*, but in very small amounts in *S. alantum*. Sesamolin was detected in considerable amounts in *S. indicum* and *S. angustifolium*, but in very small amounts in *S. alantum* and *S. radiatum*. The major lignan of *S. angustifolium* was found to be sesangolin (3.1 5% in oil) and that of *S. alantum* was 2- epi-sesalatin (1.37% in oil), (Jones et al., 1962; Kamal-Eldin and Appelqvist, 1994b).

Recently, details of the stereo-chemical structure and various thermodynamic properties of the sesamin molecule were elucidated using spectroscopic data and theoretical calculations (Hsieh et al., 2005).

Sesamolin possessing a unique structure involving one acetal oxygen bridge in a sesamin type structure (Figure 3) appears to be a characteristic lignan of sesame seed. Its content is usually about 0.3% in the oil (Tashiro et al., 1990).

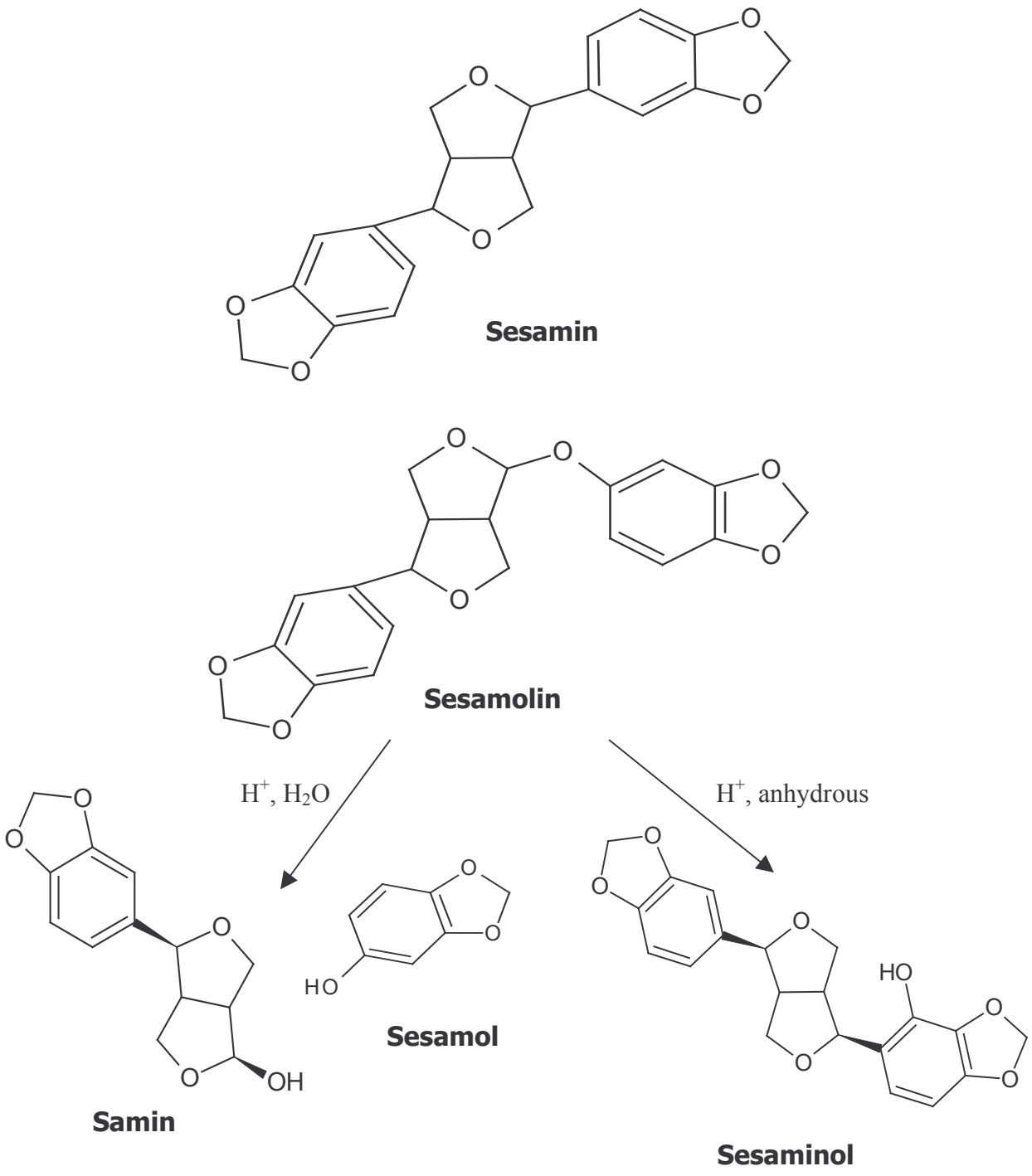


Figure 3. Structure of the major lignans in sesame and the degradation products of sesamolin

Sesaminol is first found to be one of the antioxidative components in sesame seed along with active components including sesamolinol, pinoresinol, and others (Fukuda et al., 1985; Osawa et al., 1985). It has been isolated as the principal antioxidative factor of the refined unroasted sesame oil and verified to be produced as an artifact from sesamolin during the decolorization process of that oil (Fukuda et al., 1986b). This positive change in the antioxidative lignan molecules during decolorization has been demonstrated to occur by a novel intermolecular rearrangement of sesamolin, catalyzed by acid clay. The reaction is initiated by splitting the acetal oxygen bridge of sesamolin with an acidic catalyst (H+) in a nonaqueous system to produce the oxonium samin ion and sesamol. This oxonium ion is immediately linked electrophilically to the ortho-carbon of sesamol to give a new product, sesaminol (see Figure 3). Here, the presence of water, even in trace amounts, led to the hydrolysis of sesamolin to samin and sesamol (Fukuda et al., 1986c). Sesaminol, thus obtained from the scum contains four isomers, but the content of sesaminol in the refined raw sesame oil is about 120–140 mg/100 g, which is very large compared to that is about 1.0 mg/100 g as free form (Nagata et al., 1987). However, considerable amounts of di- or tri-glucosides of sesaminol were isolated and identified from water-alcohol extracts of the defatted meal of sesame seed (Katsuzaki et al., 1992).

It is reported that the deglucosidation of the sesame lignan glucosides is cannot be achieved with the usual *β*-glucosidase alone, but in combination with cellulase. The yield of sesaminol glucosides from defatted sesame meal increases by pretreatment with some microbes (Ohtsuki et al., 2003). In the studies on various functional activities of sesamin and sesaminol, emphasis is laid on the content of these lignans in sesame seeds.

To elucidate the differences in lignan content, the biosynthesis of these antioxidative lignans in sesame seeds has been studied (Kato et al., 1998). The biosynthetic route of sesamolin and sesaminol is not yet clear. Cultivation of sesame is focused on the development of new varieties having high lignan content and functional activities. Shirato and her group have established several new sesame lines with high sesamin and sesamolin content, about twice that of conventional varieties (Shirato et al., 2001).

Bioactivities of lignans

The major sesame lignans, sesamin and sesamolin as well as the degradation product of sesamolin – sesamol, are phenolic and known for their antioxidant, anti-inflammatory, anti-ageing properties and role in the inhibition of enzymes involved in fatty acid metabolism. The health benefits of lignans are empirically known with little scientific evidence. Sesamol (3, 4-methylenedioxy phenol), with profound antioxidant

activity, has a phenolic and benzodioxole group in its structure and is soluble in both aqueous and organic phase. The benzodioxole group is known to scavenge hydroxyl radicals leading to the formation of 1, 2 – hydroxybenzene. Continuous or pulsed UV exposure in aqueous solutions is known to generate radicals of the type sesamolyl, benzoquinone anion, cyclohexadienyl in addition to dimer radicals. Sesamol is converted by UV irradiation to a sesamolyl radical or a dimer radical. The antioxidant activity of sesamol is attributed to the reactivity of stability of the sesamolyl radical (Nakagawa, 1994).

Antioxidant activity is not the only biologically important aspect of phytochemicals as is the understanding of how antioxidants perform within cells. They are likely to influence xenobiotic-metabolizing enzymes, antioxidant enzymes and DNA repair pathways. No single assay can address all of these issues and therefore different approaches are needed. Because the "antioxidant activity" measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay, it is inappropriate and misleading to generalize the data as indicators of "total antioxidant activity" (Huang, et al., 2005). On the basis of the chemical reactions involved, major antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays (Table 3).

The following are some of the most widely used *in-vitro* methods (Mermelstein, 2008)

• **ORAC,** *Oxygen Radical Absorbance Capacity:* When a free-radical generator such as an azo-initiator compound is added to a fluorescent molecule such as beta-phicoerythrin or fluorescein and heated, the azo-initiator produces peroxyl free radicals, which damage the fluorescent molecule, resulting in the loss of fluorescence. Curves of fluorescence intensity vs time are recorded, and the area under the curves with and without addition of an antioxidant is calculated and compared to a standard curve generated using the antioxidant (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, a water-soluble analog of vitamin E -*Trolox*.

• **TRAP** *Total Radical-Trapping Antioxidant Parameter:* This method uses a luminescence spectrometer to measure the fluorescence decay of R-phycoerythrin during a controlled peroxidation reaction. TRAP values are calculated from the length of the lag-phase caused by the antioxidant compared to that of *Trolox*.

• **TEAC** *Trolox Equivalent Antioxidant Capacity:* This method, similar in principle to ORAC, uses a diode-array spectrophotometer to measure the loss of color when an antioxidant is added to the blue-green chromophore ABTS·+, 2,2'-azino-bis(3-ethylbenzthiazoline- 6-sulfonic acid). The antioxidant reduces ABTS·+ to ABTS, decolorizing it. ABTS·+ is a stable radical not found in the human body.

Table 3. List of some of the *In Vitro* Antioxidant Capacity Assays

Assays involving hydrogen atom transfer reactions	ORAC (oxygen radical absorbance capacity) TRAP (total radical trapping antioxidant parameter) Crocin bleaching assay IOU (inhibited oxygen uptake) Inhibition of linoleic acid oxidation Inhibition of LDL oxidation
Assays by electron-transfer reaction	TEAC (Trolox equivalent antioxidant capacity) FRAP (ferric ion reducing antioxidant parameter) DPPH (diphenyl-1-picrylhydrazyl) Copper(II) reduction capacity Total phenols assay by Folin-Ciocalteu reagent
Other assays	TOSC (total oxidant scavenging capacity) Inhibition of Briggs-Rauscher oscillation reaction Chemiluminescence Electrochemiluminescence

• **DPPH** This assay measures by spectrophotometer the ability of antioxidants to reduce 2,2- diphenylpicrylhydrazyl (DPPH), another radical not commonly found in biological systems.

• **TOSC** *Total Oxyradical Scavenging Capacity:* This method is based on the reaction between peroxyl radicals and α-keto-γ-methiolbutyric acid (KMBA), which is oxidized to ethylene. Added antioxidant competes with

KMBA for the peroxyl radicals, reducing the production of ethylene, which is generally measured by gas chromatography. Syft Technologies Ltd. (www.syft.com) has developed a Selected Ion Flow Tube Mass Spectrometric (SIFT-MS) test that is based on TOSC.

• **PSC** *Peroxyl Radical Scavenging :* This method, also similar to ORAC, is based on the degree of inhibition of dichlorofluorescein oxidation by antioxidants that scavenge peroxyl radicals generated from thermal degradation of 2,2'-azobis(amidinopropane).

• **FRAP** *Ferric Reducing/Antioxidant Power:* This method measures the ability of antioxidants to reduce ferric iron. It is based on the reduction of the complex of ferric iron and 2, 3, 5-triphenyl-1, 3, 4- triaza-2-azoniacyclopenta-1, 4-diene chloride (TPTZ) to the ferrous form at low pH. This reduction is monitored by measuring the change in absorption at 593 nm, using a diode-array spectrophotometer.

• **Folin-Ciocalteau Total Phenolic Assay** This assay measures the change in color when metal oxides are reduced by polyphenolic antioxidants such as gallic acid and catechin, resulting in a blue solution with maximal absorption at 765 nm. The standard curve is prepared using gallic acid, and results are reported as gallic acid equivalents.

Cells in culture are not always reliable models as different cell types can differ widely in their redox responses. Comparative information can be obtained by measuring antioxidant activities of test substances (or foods

containing them) *in vitro*. However, this is incomplete without supporting data on their bioavailability and metabolism. If good biomarkers are chosen, experiments with human volunteers allow the evaluation of bioavailability and provide an overview of antioxidant effectiveness.

Although transport and metabolic mechanisms cannot be effectively reproduced, studies can provide a simple predictive instrument to investigate the potential bioavailability of dietary compounds by assessing their stability under conditions mimicking the gastrointestinal tract. Most bioavailability methods are dependent on the diffusion rates of compounds studied and their stability.

Antioxidative Activity of Sesame Lignans

Sesame lignans, which are methylenedoixyphenyl compounds are responsible for the medicinal properties of sesame oil. The presence of 0.5 –1.1 % sesamin and 0.2 – 0.6 % sesamolin and trace amounts of sesamol along with tocopherols are good antioxidants. Various studies have shown that sesamol, in particular, is an inhibitor of several steps in the generation of neoplasia (Soliman and Mazzio, 1998). Sesamin acts as anti-carcinogen (Hirose et al., 1992), helps in lowering blood pressure (Noguchi et al., 2001), reducing serum lipid (Ogawa, et al., 1995) and inhibits absorption and synthesis of cholesterol (Hirose et al., 1991) in rats. There are studies that show defatted sesame extracts and their hulls, which mainly contain lignan glucosides, possess good antioxidant activity both in black and white varieties (Shahidi et. al., 2006).

Sesamin is the main characteristic lignan of sesame seed with a content of about 0.4% in seed oil. It has no free phenol group and showed very weak or no antioxidative effect in conventional *in vitro* tests. However, sesamin exhibits significant physiological activities, assumed to be due to antioxidative activity *in vivo*. To elucidate this discrepancy in the mode of action of sesamin, metabolic changes of sesamin in both *in vitro* and *in vivo* systems have been investigated. In the *in vitro* reaction of sesamin with rat liver homogenate, it is found that sesamin changed into two kinds of metabolites involving the dihydroxyphenyl (catechol type) moiety. These metabolites have also been detected in rat bile after oral administration of sesamin. These products have shown strong radical scavenging activities. Thus, sesamin that is ingested may be incorporated specifically into the liver and metabolized there into materials that possess strong antioxidative activity (Nakai et al., 2003).

Absorption and metabolism of sesame lignans

Recently, the metabolism of sesamin has been investigated, *in vivo*, using four humans and *in vitro*, using human fecal microflora. After ingestion of sesame seed, only small amounts of sesamin, pinoresinol, and other minor lignans contained in sesame seed have been detected in plasma, while after 24 h a rapid increase of large amounts of enterolactone and enterodiol is observed. On the other hand, the fermentation of sesamin with human intestinal microflora has yielded enterolactone and

enterodiol as the main metabolites along with some intermediates named M1 and M2, which are found to be degraded lignans involving a catechol-type moiety. M1 and M2 are identical to those reported by Nakai et al. (2003). These findings suggest that sesamin is not completely absorbed, as such, but is metabolized by the intestinal microflora into a series of demethylenated intermediates, M1 (3-demethylpiperitol) and M2 (3,3′-didemethylpinoresinol), which might be absorbed or transferred, *in situ,* to mammalian lignans such as enterolactone and enterodiol (Penalvo et al., 2005). Production of demethylenated catechol type metabolites is also observed on sesamin and sesaminol triglucoside by culturing with some *Aspergillus* fungi, and these products, especially sesaminol-6 catechol, showed strong antioxidative activity in the DPPH test (Miyake et al., 2005).

Sesamol (3, 4-methylenedioxy phenol), with profound antioxidant activity, has a phenolic and benzodioxole group in its structure and is soluble in both aqueous and organic phase. The benzodioxole group is known to scavenge hydroxyl radicals leading to the formation of 1, 2 – hydroxybenzene. Continuous or pulsed UV exposure in aqueous solutions is known to generate radicals of the type sesamolyl, benzoquinone anion, cyclohexadienyl in addition to dimer radicals. Sesamol is converted by UV irradiation to a sesamolyl radical or a dimer radical. The antioxidant activity of sesamol is attributed to the reactivity of stability of the sesamolyl radical.

Sesamin: Effect on Fatty Acid Oxidation and Synthesis

The effect of sesamin on hepatic fatty acid oxidation and synthesis has been investigated at the genetic level (Ashakumary et al., 1999). Rats were fed diets containing 0, 0.1, 0.2 and 0.5% sesamin (sesamin: episesamin=1:1) for 15 days. Mitochondrial and peroxisomal palmitoyl Co-A oxidation rates increases with dose in sesamin diets. Mitochondrial activity almost double and peroxisomal activity increases more than 10-fold in rats fed 0.5% sesamin diets, compared with rats fed a sesamin-free diet. Dietary sesamin increases the hepatic activities of fatty acid oxidation enzymes as well as the enzymes involved in the auxiliary pathway of β-oxidation of unsaturated fatty acids depending on the dose. The fact that sesamin inhibits the course of fatty acid metabolism is first found in studies conducted by Shimizu et al. (1989) focusing on the production of polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) by fermentation from fatty acids (Shimizu et al., 1989) in microorganisms. Umeda-Sawada et al., 1995, showed that there is remarkably lowering the ratio of n-6/n-3 in rats fed with fed diets containing α-linolenic acid as n-3 fatty acid with sesamin compared to control groups. Sugano et al., 1990, studied the effects of a crude lignan preparation from sesame oil containing mainly episesamin and sesamin on various lipid parameters were studied in rats.

Sesamolin, the second major lignan in sesame seed and with a characteristic structure involving one oxygen bridge, shows no significant antioxidative activity in *in vitro* tests. However, some activities, *in vivo*, such as the suppression of lipid peroxidation and 8-hydroxy-2-deoxyguanosine (8-OhdG) excretion in urine have been observed (Kang et al., 1998). The above results indicate that sesamin and sesamolin have little or no antioxidative activity as such, but act as proantioxidants which become strong antioxidants via *in vivo* metabolic changes in their structure.

Sesaminol: Antioxidant effects

This lignan molecule includes sesamol as a moiety and has far stronger antioxidative activity than sesamol. Sesamol is easily dimerized and its products have lower activity (Fukuda et al., 1986a). The markedly strong and stable antioxidative property may be provided by the presence of a bulky samin group at the ortho position of the phenol group in sesamol similar to the BHT (butylated hydroxytoluene) molecule. The content of sesaminol in the seed is very small in the free form, whereas there are considerable amounts of its di- or tri-glucosides in the seed as well as in the defatted meal or sesame flour. The glucosides found to be weak as active oxygen scavengers in *in vitro* tests, are expected to act as potential antioxidants through deglucosidation by the action of *β*-glucosidase or intestinal microbes (Katsuzaki et al., 1994). Sesaminol suppresses lipid

peroxidation in model systems of *in vivo* peroxidation using the ghost membranes of rabbit erythrocytes and the liver microsome in rat (Osawa et al., 1990). Antioxidative activity of sesaminol is also observed in the lipid peroxidation of low-density lipoprotein induced by 2, 2-azobis (2, 4-dimethyl valeronitrile), where effective radical scavenging activity of sesaminol is demonstrated by isolation and identification of the reaction products of sesaminol with the reagent radical (Kang et al., 1998). In a similar study on the Cu^{2+} induced lipid peroxidation of low density lipoprotein, sesaminol is found to inhibit the peroxidation in a dose dependent manner and show stronger inhibiting activity than α-tocopherol and probcol in the radical scavenger and in the production of lipid peroxidation products such as 4-hydroxy-nonenal and malonaldehyde adduct products (Kang et al., 2000).

Inhibition of oxygenases is indicative of antioxidant activity. Oxygenases need molecular oxygen as a reactant. Tyrosinase and lipoxygenase are key enzymes in the melanin synthesis pathway and the leukotriene pathway, respectively. Lipoxygenase mediated products are implicated in inflammation, while disregulated tyrosinase action is implicated in cancer and pigment disorders.

Melanogenesis

Melanogenesis, a physiological process, results in the synthesis of melanin pigment, which absorbs free radicals generated in the cytoplasm and shields the host from various types of ionizing radiation that may lead to skin carcinogenesis (Figure 4). In humans and other mammals, the biosynthesis of melanin takes place in melanocytes, which contain the enzyme tyrosinase (Robb, 1984). The hydroxylation of L-tyrosine, the initial step in melanin synthesis, is of considerable importance, being the initial step in catecholamine synthesis too.

Tyrosinase, an oxidase, is the rate limiting enzyme for the controlled production of melanin. It is mainly involved in two distinct reactions of melanin synthesis - the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone (Cooksey, et al., 1998) o-Quinone is transformed through a series of non – enzymatic reactions to melanins. Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (Whitaker, 1995; Mayer and Harel, 1998) is a copper containing mixed-function oxidase, widely distributed in microorganisms, animals and plants. Mushroom tyrosinase, a tetramer with a molecular mass of 120 kD, is composed of two H subunits (43 kD) and two L subunits (13 kD) and contains two active sites, each containing two copper atoms as the cofactors (Yong, 1990). Tyrosinase is shown to have a type III site, similar to, though not identical with hemocyanin (Beltramini et al., 1995).

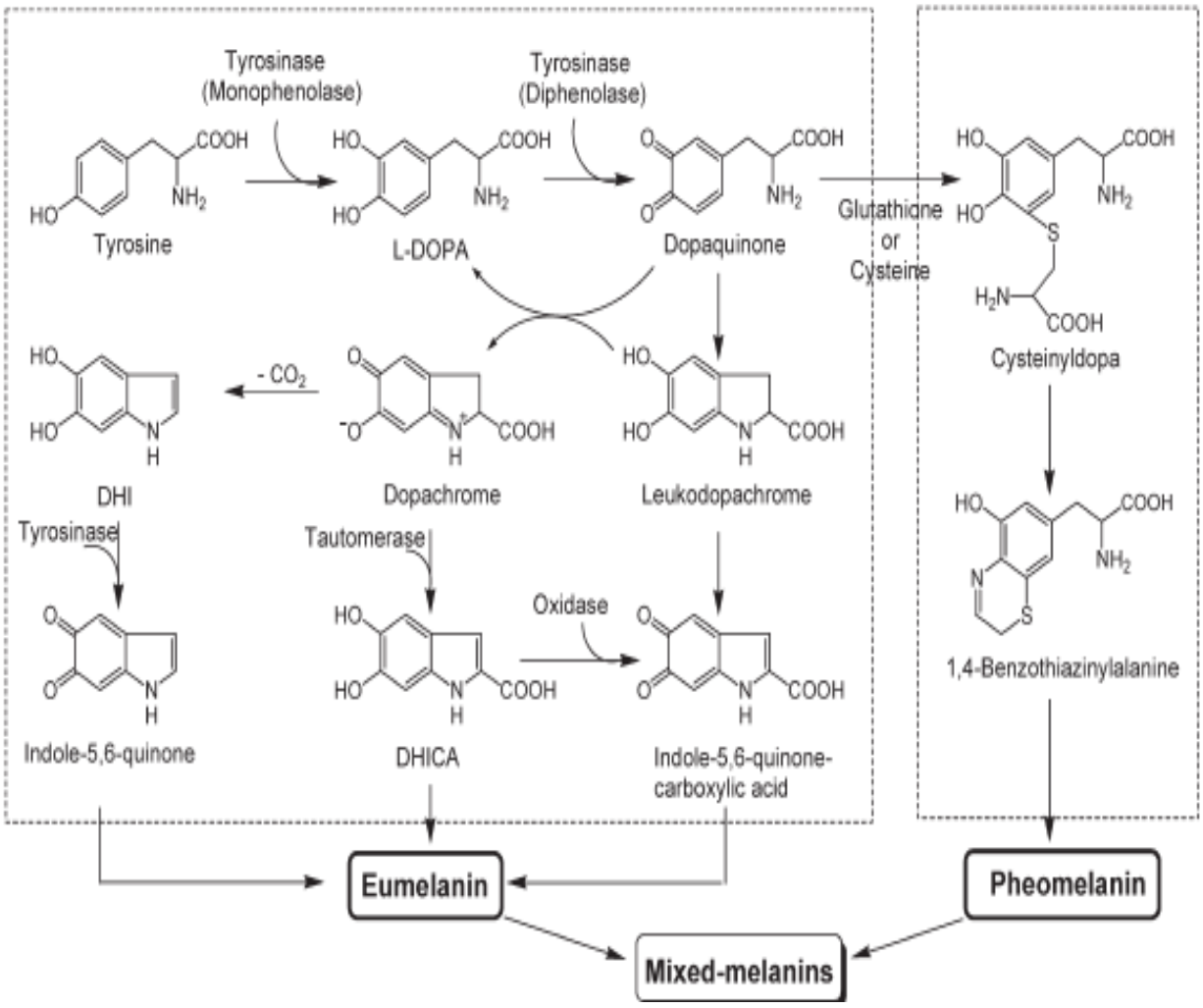


Figure. 4. Biosynthetic pathway of melanin (Adopted from Kim and Uyama, 2005)

Tyrosinase has 3 existing forms: *'met'* (E_m) *'deoxy '*(E_d) *and* 'oxy' (E_o). Both E_m and E_o forms can catalyze diphenolase substrates while the E_o form can also catalyze monophenol substrates. E_d can combine with oxygen (Sánchez- Ferrer et. al., 1990). A structural model has been proposed for the active site of these 3 forms of tyrosinase (Wilcox, et al 1985). Tyrosinase is the rate limiting enzyme in melanin biosynthesis - a physiological process resulting in the synthesis of melanin pigments, which play a crucial protective role against skin photocarcinogenesis. Melanin synthesis and its relationship with betalain formation are given in Figure 5. Alterations in melanin synthesis occur in many disease states, including melanoma.

Disregulation of tyrosinase is encountered in melanoma, where increased melanin synthesis is observed [Solano, 2006]. Several flavonoids, substrate analogues, free radical scavengers and copper chelators have been reported to inhibit tyrosinase (Chen, et al., 1991). In recent times, considerable efforts have been concentrated towards research for the discovery of natural bioactive products (including tyrosinase inhibitors) and their exploitation in medicine and cosmetic formulations for treatment of skin disorders (Kubo and Kinst-Hori, 1999). Table 4 and 5 gives the inhibitors of monophenolase and diphenolase activity of tyrosinase, respectively.

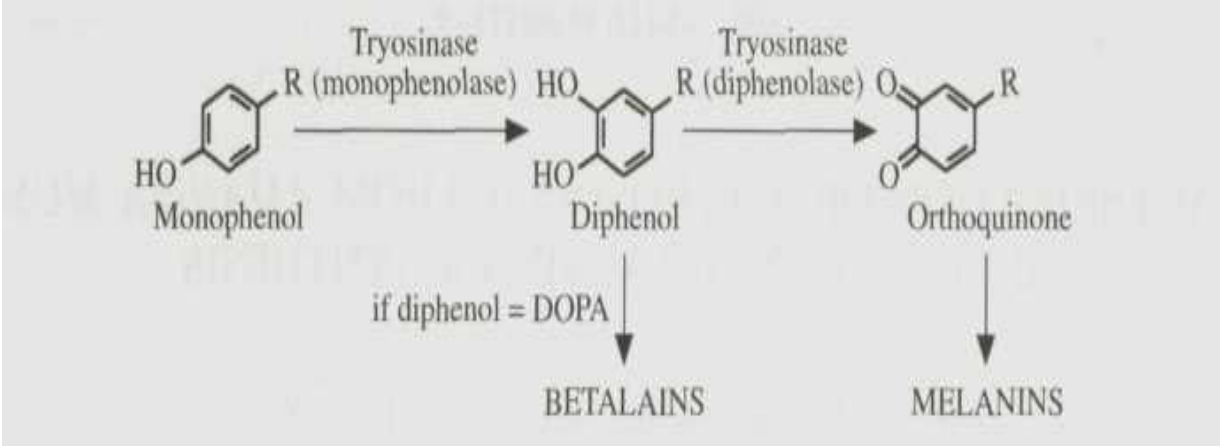


Figure 5. The biosynthetic relationship between betalain and melanin pigments. A monophenolic substrate (tyrosine) is converted into a diphenol by the monophenolase activity of tyrosinase. In the biosynthesis of melanins, the diphenolic intermediate is further oxidized by the diphenolase activity of tyrosinase to yield o-quinones, which polymerize to form melanin-like pigments.

Table 4. Some of the known inhibitors of monophenolase activity of tyrosinase

Compounds	IC_{50} values (μM)	References
4-vinylbenzaldehyde	93	Song et al. (2005)
2-fluorobenzaldehyde	1350	Huang et al. (2006)
Hen egg white lysozyme	0.11	Li et al., (2006)
(-)-Epigallocatechin	35	No et al. (1999)
(-)-Epicatechin gallate	17	No et al. (1999)
4- Hydroxyphenethyl - 3,4,5-trihydroxybenote	4.94	Lee et al. (2007)
4-Vinylbenzoic acid	3010	Song et al. (2005)
Sesamol	3.2	Present study

Table 5. Some of the known inhibitors of diphenolase activity of mushroom tyrosinase

Compounds	IC_{50} values (μM)	References
Quercetin	130	Chen and Kubo (2002)
Cis- 3,5- Dihydroxyxtilbene	405	Song et al.(2006)
Trans-3,5- Dihydroxyxtilbene	705	Song et al. (2006)
Kojic acid	22	Ley, (2001)
3,4-Dihydroxybenzonitrile	45	Khatib et al. (2005)
Triolein	30	Jeon et. al. (2006)
4- hexylresorcinol	5	Dawley, et. al., (1993)
Sesamol	1.92	Present study

Enzymatic browning

Many types of fruits, like apple, bananas, brown rapidly when their tissues are cut or bruised. The brown color is developed due to the enzymatic oxidation of phenols to quinones by polyphenol oxidase (PPO) in the presence of oxygen. Subsequently, these quinones condense and react non-enzymatically with other substances such as phenolic compounds and amino acids to produce complex brown polymers. Enzymatic browning impairs not only the color of fresh fruits but also the flavor and the nutritional quality (Rigal, et al., 2001). Various chemicals are known to be able to reduce enzymatic browning, including PPO inhibitors, reducing agents, acidulants, and chelating agents (Saper and Miller, 1992; Chang, 2009). However, consumers are concerned about the possible dangers of synthetic food additives. This consumer awareness has stimulated the search for natural and safe antibrowning agents.

Lipoxygenase

Lipoxygenases (LOXs)-linoleate oxygen oxidoreductase, (EC 1.13.11.12)- are enzymes ubiquitous in nature. These non-heme, non-sulfur dioxygenases catalyze the dioxygenation of polyunsaturated fatty acids containing a *cis, cis*-1, 4-pentadiene unit to yield *cis, trans* conjugated diene hydroperoxides. following activation, LOX functions through a redox cycle between (a high spin) ferric and ferrous states, fueled by substrate and fatty acid peroxyl radical (Gardner, 1991). Lipoxygenases

are purified and characterized from both plant and animal tissues. The sequences of over 50 lipoxygenases have been reported and many have expressed as active proteins (Kuhn and Thiele, 1999). They range in length from 923 residues (rice LOX-2) to 661 residues (rabbit 15-LOX); the plant sequences are longer than the mammalian sequences. Pair wise sequence identity between plant and mammalian lipoxygenases is 21-27%, while identity among pairs of plant sequences is 43-86%. The sequence similarity between plant and animal lipoxygenases is highest in the region of the catalytic domain near the iron atom (Prigge et. al., 1996). Although the catalytic mechanism of all lipoxygenases may be similar, there are significant differences in the properties of the plant and animal enzymes. Soy LOX is a soluble cytosolic protein, whereas mammalian 5-LOX is membrane bound and requires membrane associated activating protein (FLAP).

The most exhaustively studied plant LOX from soy exists in the form of several isozymes sharing 70% of sequence homology. Soy LOX isozymes LOX1, LOX2 and LOX3 have significantly different properties. LOX1 contains 839 amino acid residues with a molecular weight of 94,262 daltons (Figure. 6 A). The corresponding values for LOX2 and LOX3 are 865 (97,053) and 857 (96,541) respectively (Boyington et. al., 1990). These isozymes further differ with respect to their isoelectric points, showing values of 5.68, 6.25 and 6.15 for LOX1, LOX2 and LOX3 respectively (Siedow, 1991).

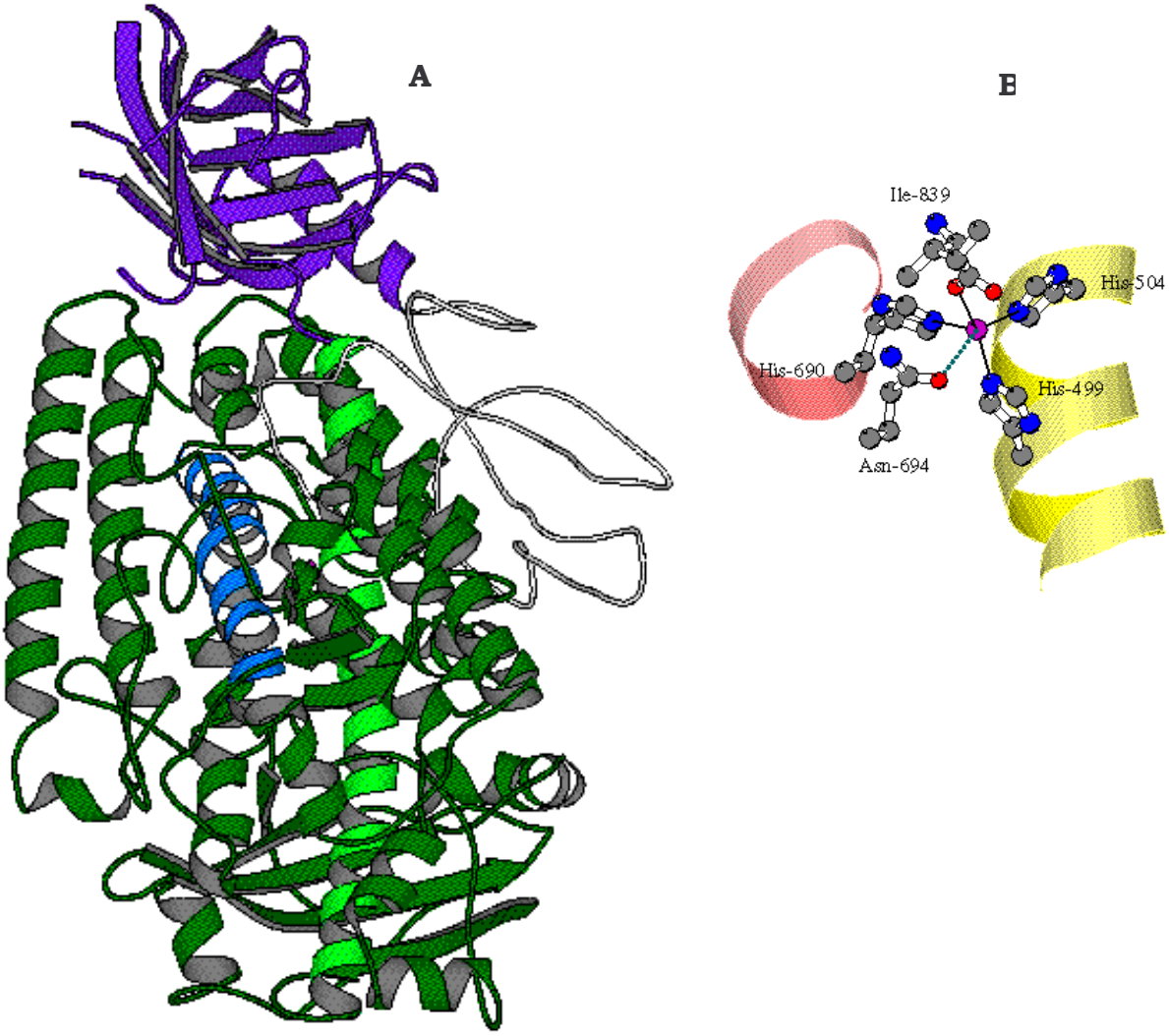


Figure 6. A. Ribbon representation of the soybean lipoxygenase1 (PDB code 2SBL). Colourcodes: Nterminal domain (7-170) = violet; Cterminal domain (255-839) = dark green; helix 9 (474-516) = light green; helix 18 (672-701) = light blue.

B. Active site structure: π helices 494-506 (yellow) and 685-690 (pink) and iron ligands His499, His504, His690, Ile839 and Asn694.

Physiological effects of lipoxygenase and its products

Animal lipoxygenases initiate the arachidonic acid cascade, which is a source of several powerful bioregulators such as prostaglandins, thrombaxanes, leukotrienes, lipoxins and hepoxilins, in a number of cellular responses (Yamamoto, 1997). Hydroperoxides, the products of lipoxygenase mediated pathways, play a major role in the manifestation of chronic inflammatory diseases. The quest for new as well as natural inhibitors of lipoxygenase poses a challenge. Mammalian lipoxygenases are a target for drug design. The inflammatory pathway (schematic) is given as Figure 7.

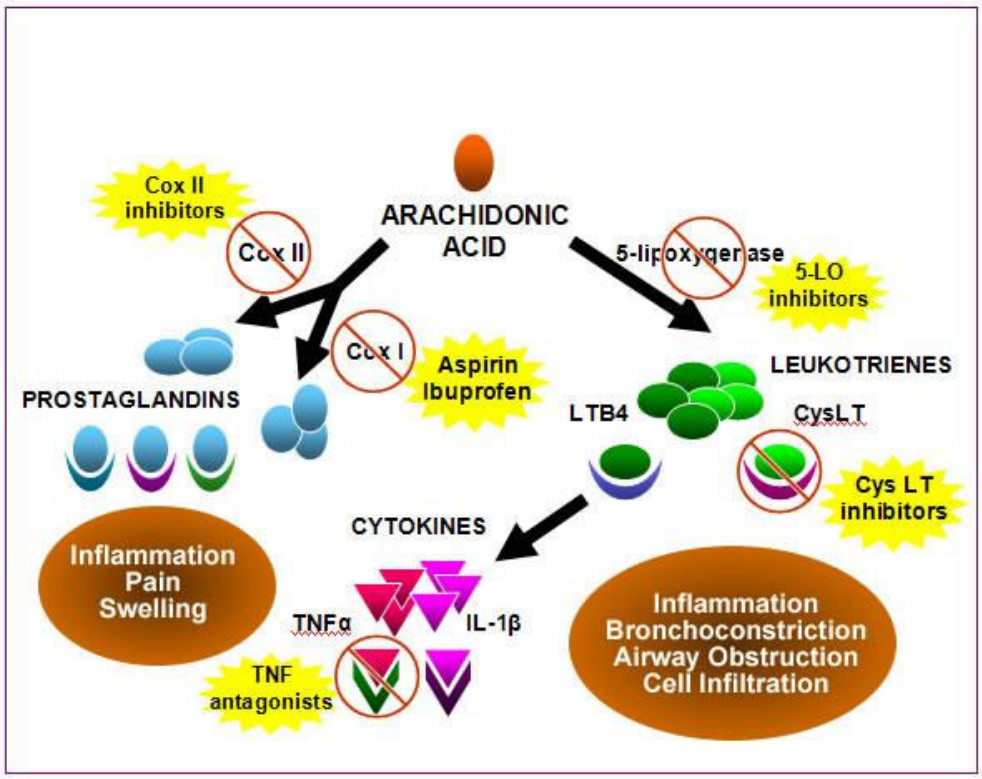


Figure 7. Inflammatory pathway and the effect of enzyme inhibitors on the pathway (www.mydietaryfat.org)

Lipoxygenase products such as dihydroxy fatty acid leukotriene B4 (LTB4) and 9S-hydroxyeicosatetraenoic acid (9S-HETE) are ligands for G-protein coupled receptors and nuclear hormone receptors which regulate pathways involved in inflammation and lipid homeostasis. The activities and products of the LOX enzymes influence atherosclerosis, inflammatory bowel disease, psoriasis, asthma and other immune system disorders, 12-lipoxygenase is over expressed in the pathological lesions of inflammatory bowel disease and psoriasis (Shannon et al.,1993). The ability of 15-LOX (but not other lipoxygenenases) to oxidize low-density lipoproteins (LDL) is suggestive of an important role for this enzyme in the pathogenesis of atherosclerosis. Inhibition of 5-LOX has been shown to have therapeutic value in the treatment of asthma. Mounting evidence suggests that lipoxygenase (LO)-catalyzed products have a profound influence on the development and progression of human cancers. Compared with normal tissues, significantly elevated levels of LO metabolites have been found in lung, prostate, breast, colon, and skin cancer cells, as well as in cells from patients with both acute and chronic leukemias. Agents that block LO-catalyzed activity may be effective in preventing Cancer by interfering with signaling events needed for tumor growth. In fact, in a few studies, LO inhibitors have prevented carcinogen-induced lung adenomas and rat mammary gland cancers (Moody et al., 1998)

Inhibitors of Lipoxygenase

The interest in the inhibitors of lipoxygenase is due to deleterious effects of the products of lipoxygenase reaction on health. Broadly there are three classes of inhibitors (1) substrate analogs, (2) redox inhibitors, those that affect the catalytic iron center and (3) radical trappers, which function by reacting with free radical intermediates of lipoxygenase reaction. The redox inhibitors are very potent but of little pharmaceutical value as they have the tendency to produce toxic side effects. The nonredox inhibitors are more relevant as therapeutic drugs, but their mode of action is difficult to establish, because of the multiple oxidation states of the enzymatic cycle. The biological profile of leukotrienes suggests that inhibitors of the 5-LO pathway may have therapeutic potential in a variety of inflammatory and allergic diseases. 5-LO inhibitors can be classified according to the mechanism of enzyme inhibition. Redox type inhibitors like phenidone reduce the active site iron of the enzyme into the ferrous form (Fe^{2+}) and keep the enzyme in its inactive state. However, redox-active compounds also interact with other biological redox system, which leads to side-effects like methaemoglobin formation (Lau et al., 1992).

Mammalian lipoxygenases are a target for drug design. Since mammalian enzymes are difficult to purify, design of inhibitors rely heavily on structural and mechanistic studies on Soybean lipoxygenase, whose three-dimensional structure and catalytic mechanism have been

explored in detail. Soy LOX-1 is used as *in vitro* biochemical models as it resembles human lipoxygenases, in its substrate specificity and inhibition characteristics (Kingston, 1991). The quest for new as well as natural inhibitors of lipoxygenase poses a challenge. A number of monoenoic fatty acids, which resemble fatty acid substrates but structurally differ in cis, cis, 1-4 pentadiene system are shown to be competitive inhibitors of LOX1. The inhibitors such as 4-nitrocatechol, N-alkylhydroxylamine, disulfiram and isoflavones from soybean are redox inhibitors that inhibit LOX1 activity, by reduction of active yellow form (Fe^{3+}) to its inactive Fe^{2+} form (Clapp, et al., 1985, Mahesha et al., 2007). Inhibitors such as nordihydroguiaretic acid (NDGA), n-propylgallate and butyl hydroxytoluene are antioxidants that function by reacting with free radical intermediates of lipoxygenase catalyzed reaction thereby acting as free radical scavengers. NDGA is shown to inhibit lipoxygenase by reducing the catalytically active ferric enzyme to the catalytically inactive ferrous form thereby acting as an efficient redox inhibitor (Kemal et al. 1987).

In this investigation, the biological effect of sesame lignans and their products have been investigated. Lignans were purified and characterized and their antioxidant properties established by known assay methods. Their effects were studied and mechanism elucidated through their inhibition of two enzyme models – Tyrosinase and lipoxygenase. These studies would help establish the claims of traditional medicine.

SCOPE AND OBJECTIVE OF THE PRESENT INVESTIGATION

Many naturally occurring compounds in plants and animal products have been identified to promote general body development and disease prevention. Food components, that are responsible for changes in health, but do not have the primary role of meeting basic human nutritional needs, are known as bioactives or bioactive ingredients. Bioactive molecules can play roles in human growth and development, and reducing the risks of disease.

Bioactive molecules exhibit a wide range of physiological properties like antioxidant, anticarcinogenic, anti-inflammatory, antimutagenic and so on. These include, among others, flavonoids, anthocyanins, saponins, lectins and lignans. Flaxseed and sesame are very good sources of lignans, which are phenolic in nature.

Sesame seeds are used in traditional Indian medicine and are highly valued for its characteristic flavor. The oil is valued for its mild, pleasant taste and its resistance to oxidative stability. Sesame is reported to have 0.4 – 1.0% sesamin, 0.2 – 0.5% sesamolin, both lignans and traces of sesamol in the oil. Both sesamin and sesamolin are extracted with the oil and together with tocopherols, are responsible for the high stability of oil against oxidative stability. The superior antioxidant quality of roasted oil is attributed to sesamol, which is formed due to the decomposition of sesamolin during roasting.

Sesame lignans are attributed with several medicinal properties like antioxidant activity, anti-ageing and hypocholesterolemic activity. They have been shown to enhance the antioxidative effect of tocopherol and prevent an increase in lipid peroxidation. Both sesamin and sesamolin have been shown to have a cholesterol-lowering effect in humans. They also prevent high blood pressure and increase vitamin E supplies in animals. Sesamin has been reported to protect the liver from oxidative damage. Mammalian lignans such as enterolactone and enterodiol are produced in the colon from sesame lignans and have been suggested as playing a role in cancer-prevention.

While several medicinal properties are associated with sesame lignans and their products, there is not much scientific evidence towards mode of action and synergies, if any.

Accordingly, major objectives of the present investigation were:

- To optimize extraction of major sesame lignans by physical, chemical and enzymatic means. Recovery and isolation of the major sesame lignans – sesamin, sesamolin and sesamol.
- Bioactivity and bioavailability of sesame lignans *in vitro* and *in vivo* and intra comparison with special reference to antioxidant properties in isolation and combination as well as the synergistic effect.

Purification and characterization of sesame lignans and their products was carried out. Roasting conditions with an understanding of the time –

temperature relationship to obtain sesamol were optimized. The antioxidant effects were studied by non – enzymatic and enzymatic models. Enzyme models including oxygenases like tyrosinase and lipoxygenase were purified and the effect of lignans, their inhibition and mode of action determined. This study would help understand the role of these bioactive compounds in maintaining health and provides scientific basis for traditional beliefs.

Chapter II

Materials and methods

Materials and methods

Materials

Sesame (*Sesamum indicum* L.) seeds, commercial variety, were acquired from the local market in Mysore, India. Sesamol, BHT, Trolox (6-hydroxy-2, 5, 8- tetramethylchroman-2- carboxylic acid), Tween 20 (Polyoxyethylene sorbitan monolaurate) emulsifier, 2,2-diphenyl- 1-picrylhydrazyl (DPPH), 2,2′-Azo-bis (2- amidinopropane) dihydrochloride, (AAPH), 2,4,6 – tripyridyl – S- triazin (TPTZ), $FeCl_2$, $FeCl_3$, gallic acid, Mushroom tyrosinase (T-3824), MBTH, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), bovine serum albumin (BSA), tris (hydroxy methyl) amino methane (Trizma base), Acridine orange, Ethidium bromide, $NaIO_4$, 4-tert-butyl-*o*-catachol (*t*BC), L-DOPA and Tyramine hydrochloride used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO. USA) and employed as received. All solvents used were of ACS or HPLC grade, unless otherwise specified.

HPLC columns: Waters Symmetry Shield C_{18} column (4.6 x 150 mm, 5 μm) and Waters Spherisorb® silica column (4.6 x 250 mm, 5μ) were from Waters Corporation MA, USA.

Bacterial cultures

Bacillus cereus (F4810, Public Health Laboratory, London), *Staphylococcus aureus* (FRI 722, Public Health Laboratory, Netherlands)

and *Pseudomonas aeruginosa* (CFR 1734, CFTRI Mysore) were obtained from the Department of Food Microbiology, CFTRI, Mysore. The above cultures were grown in nutrient agar media (HiMedia, Mumbai, India) at 37°C. Pre-culture was prepared by transferring 1 mL of this culture to 9 mL nutrient broth and cultivated for 48 h. The bacterial cells were harvested by centrifugation (1200 g, 5 min), followed by washing with saline, before finally it was suspended it in 9.9 mL of sterilized saline. This was used for plating by spread plate technique.

Methods

Screening of the different varieties of sesame for antioxidant contents

Fifty sesame varieties, obtained after physical or chemical mutation of two parent varieties (DS-1), were received from Dharwar Agricultural University. Several varieties were obtained after chemical or physical mutations using ethyl methane sulphonate (EMS) at 0.5% concentration or gamma rays, at 300 GY, 400 GY and 500 GY dosages. Mutants were isolated from M_3 generation in each treatment from two population *viz.*, lower capsules and upper capsules. Of the several mutants obtained, fifty were selected based on the boldness, color and yield of seeds. The samples received from different mutation methods are given in the Table 5 (in results and discussion section A). Lignan and γ- tocopherol contents of all the samples were analysed by HPLC methods described later under purification.

Dehulling and defatting of sesame seeds

The seeds are precleaned and dehulled using hot lye, were washed and dried at 70°C. Dehulled seeds (roasted or unroasted) are flaked and extracted with food grade hexane for 8 h. The meal is dried and milled before passing through a 44 -mesh screen (Inyang and Nwadimkpa 1992). The meal is used for the experiments. Oil is desolventized before use.

Purification of sesame lignans by Preparative HPLC

The extraction of sesamin and sesamolin was standardized by modification of reported method (Amarowicz et al, 2001). Commercial white sesame seeds were used for recovery of sesamin and sesamolin. Sesame seeds were oven-dried at 60°C for 4 h, cooled to room temperature (27°C) and ground in a commercial coffee mill. The lipophilic constituents were extracted from the seeds with hexane for ~10 h in a Soxhlet apparatus. The extract was desolventized under vacuum at 35°C using a Buchi Rotavapor/Water bath (Models EL 120 and 461, respectively) to recover the oil. The oil was mixed with acetone in a ratio of 1: 10 and left for 8 -12 h at -40°C to precipitate the lipids. The supernatant was recovered, acetone was removed and the yellow oil obtained. The oil was saponified with alcoholic KOH. Unsaponifiable matter was extracted with ether and dissolved in methanol. The unsaponifiable matter contains the lignans and tocopherols.

The lignans in the unsaponifiable matter obtained from sesame oil were further purified by LC- 8A Shimadzu Preparative Liquid Chromatography HPLC system equipped with a C_5 column (250 × 21 mm). The mobile phase consisted of methanol and water with gradient elution of 0-60 % of solvent B (methanol) in 60 min. The flow rate was 5 mL/ min for 60 minutes. Peak fractions, detected at 290 nm, were collected.

Confirmation of purity of sesamin and sesamolin by HPLC and GC-MS Analysis

Purity of sesame lignans was determined from the peak fractions (obtained from preparative HPLC) by an analytical Waters® HPLC system equipped with a C_{18} column (250 x 4.6 mm, 5 μ, Waters®) fitted with a 1-cm guard column (Waters® ODS) and a photodiode array detector (Waters®) as reported earlier (Kikugawa et al. 1983)]. Mobile phase was methanol-water (HPLC grade), 70: 30 v/v (isocratic), at a flow rate 1 mL/ min. Samples (20 μl) were injected and peaks were detected at 290 nm. Sesamol standard was used for detection of sesamol peak.

The chemical structures of purified sesamin and sesamolin standards were confirmed by gas chromatography- mass spectrometry (GC-MS) using a Perkin Elmer Autosystem XL Gas chromatograph coupled to Turbomass Gold mass spectrometer (Perkin Elmer instruments, Norwalk, CT. USA) with an NIST library/ data system. An Elite 1 fused-silica capillary column (30 m × 0.25 mm id, 0.25 μm thickness) was used and analysis were carried out with helium as the carrier gas; helium flow rate was set at 2 mL/ min. The column temperature was set to 150°C initially, for one min, and gradually increased to 290°C at which point it was maintained for 15 minutes. EI mass spectra were recorded at electron energy of 70 eV with the source temperature at 250°C and interphase temperature of 180°C. Purified lignans, prepared in the concentration of 2 mg / mL, were used.

Determination of sesamol and sesamolin in roasted oils

Unsaponifiable fractions obtained from roasted oils (after electrical and infrared roasting of seeds) were analyzed for their sesamol contents by HPLC as described above. The amount of sesamol, in the samples, was calculated from the peak areas (observed at 290 nm) of standards. Sesamolin was isolated by preparative HPLC, characterized by analytical HPLC and GC-MS. The purity was determined to be ~98%. This was used as standard for determining the quantity of sesamolin in the oil, in the absence of commercial standards.

Estimation of γ -tocopherol in sesame oil

Analysis of γ-tocopherol in sesame oil was performed by HPLC (Yen, 1990) using a Waters Spherisorb® silica column (4.6 x 250 mm, 5μ). A mixture of hexane: isopropanol: ethanol (100: 0.3: 0.2) at a flow rate of 1 $mL.min^{-1}$ was used as the mobile phase. Sesame oil, diluted using hexane (HPLC grade), was injected (10 μL) into the column. Detection was at 295 nm. γ-Tocopherol standard (purity >99%, Sigma Chemical co., USA) was used for calculating the γ-tocopherol content in the samples.

Fluorescence measurements

Fluorescence measurements were made using a Shimadzu RF-5000 automatic recording spectrofluorimeter in conjunction with a circulating

water bath fitted with a thermostat. The temperature of the cell was maintained at 27ºC. Lignans and sesamol were taken in 50 μg/mL concentration. Excitation wavelength for sesamol, sesamin and sesamolin was 296, 287 and 288 nm, respectively, and emission spectra were recorded between 300- 450 nm. The emission spectra were recorded with 5 nm bandwidth for both excitation and emission monochromators, respectively.

Electrically heated drum roasting

Roasting of dehulled seeds was carried out in an electrically heated rotating (10 rpm) drum roaster (Indlab grain roaster, Indlab furnaces, Mysore. India) fitted with a thermostat. Roasting was carried out at different temperatures ranging from 160 –240^{o}C (± 5^{o}C), for 30 minutes. The roasted seeds were allowed to cool to ambient temperature before extraction of oil by hexane.

Infrared (IR) roasting

Roasting was carried out in Infrared heating system (Prototype CFTRI, Mysore, India) [Overall dimension: 2.0 m (L) * 0.3 m (W) * 1.2 m (H) IR heating source – quartz infrared (NIR) Wavelength of radiation: 1.1 to 1.3 μm Power input: 6 KW] for 15, 30 and 45 min at temperatures, 180, 200 and 220^{o}C (± 5^{o}C). 1 kg seeds per batch were used in the roasting trials. The seeds were spread in a thin layer evenly on a tray. After

roasting, the seeds were allowed to cool to ambient temperature before extraction of lipids with hexane.

The roasted seeds were flaked and extracted with food grade hexane for 8 h in a Soxhlet apparatus. Oil was obtained after desolventizing using a Büchi rotary evaporator (Model RE 120 Switzerland) at 35°C, under vacuum. Oils were saponified with 5% alcoholic potassium hydroxide, for 2 h. The unsaponifiable fractions were separated and extracted with petroleum ether (60-80°C). Petroleum ether was evaporated and residue dissolved in methanol. This was subjected to lignan analysis by HPLC similar to electrically heated drum roasted seeds.

Preparation of heat resistant sesame seeds

Heat resistant sesame seeds were prepared as described (Singh et al., 2005). Dehulled sesame seeds were immersed in hot water at a temperature of 100°C followed by adding hydrogen peroxide (H_2O_2) at 2% in the hot water (v/v) 1: 5 (seed to water). The seeds were boiled for 1 min and then washed with water. The treated seeds were sprayed with edible solution of gum acacia, calcium salt and food grade color. Finally, the coated seeds were dried in a conventional hot air dryer or a fluidized bed drier at a temperature of 60°C to obtain dry seeds having a moisture content of 3 – 6%. The seeds were baked at 180 – 200°C for 8 – 15 min after sprinkling on proofed buns. The color of the seeds with specific reference to lightness was compared to the untreated seeds.

Color measurement for roasted oil

Samples of sesame seed oil extracted from dehulled seeds (roasted and unroasted) were analyzed for their color characteristics. The defatted flour was also subjected to color measurements by Lab scan XE Hunter lab color measuring system; M/s Hunter Associates Lab inc. Virginia (USA), at 2^{o} angle.

Analysis of defatted roasted flours

Determination of moisture

Moisture content in the roasted flour sample was estimated by using a Sartorius (Switzerland) moisture meter (Model MA 30). Samples, in triplicates, were heated by an infrared element at 110°C to constant weight.

Protein determination

Protein content in defatted flours (roasted as well as unroasted) was determined by the Kjeldahl method. A factor of 6.25 was used for converting nitrogen content to protein content and expressed as g proteins per 100 g sample.

Determination of oxalic acid, phytic acid and tannin contents

Oxalic acid contents in the dehulled and undehulled seeds were determined by a method reported earlier (Wilson et al., 1982). Oxalic acid content is expressed as mg of oxalic acid per 100 g sample. Phytic

acid content was determined as reported (Thompson et al., 1982) and expressed as g phytate phosphorus per 100 g flour. Tannins in sesame seeds as well as roasted flours were estimated colorimetrically (AOAC 1970) and expressed as g tannins per 100 g sesame flour.

Determination of available lysine

Available lysine contents in the flours were estimated by the method reported with some modification (Carpenter et al., 1955) in g per 100 g of protein in the flour.

Determination of methionine and cysteine

Methionine content was determined as reported (Pieniazek et al., 1975) with some modification. Defatted flour (2 g) was partially hydrolyzed by autoclaving in 25 mL of 2N HCl for 1 h. The hydrolysate was neutralized with 10N NaOH to adjust the pH to 6.5 and the volume was made up to 100 mL. The hydrolysates was reacted with sodium nitroprusside and absorbance read at 520 nm. A standard curve was obtained with DL-methionine in the range 1 – 10 mg. The results were expressed in mg methionine/g of sesame protein. Cysteine in defatted sesame flour was estimated in μM free cysteine/g protein. (Beveridge, et al., 1974).

Functional properties of sesame flours

Emulsion capacity and stability were determined as described earlier (Vananuat and Kinsella 1975). Foam capacity and stability, water absorption capacity, fat absorption capacity and protein solubility were determined as described (Okezie and Bello 1988).

Emulsion capacity

One g of sample was mixed with 25 mL water in a blender for 30 s and vegetable oil (refined peanut oil) added at the rate of 10 mL/ min, with continued blending. Blending was continued for an additional 30 s and the sample was transferred to a 50 mL graduated centrifuge tube, incubated at 80°C for 15 min and centrifuged at 3, 000 x g for 40 min. The volume of oil separated from each sample, after centrifugation, was read directly from the tube. Measurements were made in duplicate for each sample and mean values taken. Emulsion capacity was expressed as the amount of oil emulsified and held per g of sample.

Water and fat absorption capacity

One g of sample was taken in a graduated conical centrifuge tube and mixed thoroughly at high speed in a blender with 50 mL of water or oil. The sample was allowed to stand at room temperature for 30 min before centrifuging at 5000 x g for 30 min. The amount of free water or oil was read directly from the graduated centrifuge tube. The amount of oil or

water absorbed (total minus free) was multiplied by its density for conversion to gram. Absorption capacity was expressed as the g of oil or water absorbed (or retained) per g of flour sample. Density of oil (0.92 g/ mL) was determined; water was assumed to have a density of 1 g/ mL. Determinations were made in triplicates and average values reported.

Foam stability

One gram of sample was blended with 100 mL distilled water at high speed, in a blender, for 1 min. The whipped mixture was transferred into a 250 mL graduated cylinder. Distilled water (10 mL) used to rinse the blender jar was added to the graduated cylinder. Foam volume changes in the cylinder were recorded at intervals of 1, 10, 30, 60, 90 and 120 min. For each sample, duplicate measurements were made and the mean values recorded. All the results are expressed mean values ± standard deviation. Means of 3 measurements were compared using a significant level of $P < 0.05$, by one-way analysis of variance.

TBARS assay for oxidative stability of oil

TBARS (2- thoibarbituric acid relative substances) were determined as reported previously (AOCS 1998). TBARS were expressed as mg of malondialdehyde equivalent per kilogram of oil.

Scavenging of DPPH radical

DPPH solution (0.1mM in methanol) was incubated with varying concentrations of test compounds, in three different dilutions (Espin, 2000). The reaction mixture was incubated for 20 min at room temperature in dark and the absorbance of the resulting solution was read at 517 nm against a blank using a spectrophotometer (Shimadzu model 1601, Japan). The scavenging of DPPH radical was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{(\text{Abs}_{\text{control}})} \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH radical + methanol;

$\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical + sesame lignans/standard. BHT was used as standard and all the analysis were carried out in triplicate.

For synergistic antioxidant effects, sesamol (2 µg/ mL) and sesamin (10 µg/ mL) were taken along with γ-tocopherol (0 – 10 µg/ mL) and the DPPH radical scavenging activity assayed as given above.

Antioxidant assay using β-carotene -linoleate model system

β-Carotene 0.2 mg, 20 mg of linoliec acid and 200 mg of tween-20 were mixed in 0.5 mL of chloroform (Hidalgo et. al., 1994). The chloroform

was removed at 40°C under vacuum using a Buchi rotary evaporator. The mixture was immediately diluted with 10 mL of triple distilled water and mixed well to obtain an emulsion. This was further made up to 50 mL with oxygenated water. Aliquots (4 mL) of this emulsion were transferred into different test tubes containing 0.2 mL of sesame lignans (2 mg) in ethanol. Controls contained 0.2 mL ethanol and 4 mL of the above emulsion. BHT, a standard antioxidant was taken in the same concentrations for comparison. Absorbance of all samples were measured at 470 nm at zero time (t=0). The samples were incubated in a water bath at 50°C. Measurements of absorbance were taken at intervals of 15 min till the time when the color of β-carotene disappears in the control reaction tube (t=180) at 15 min intervals. A mixture prepared as above without β-carotene served as blank. All determinations were carried out in triplicates. Antioxidant activity was calculated using the formula :

$$AA= 100[1- (A_0-A_t)/ (Å_0- Å_t],$$

where A_0 and $Å_0$ are the absorbance values measured at zero time of the incubation for test sample and control respectively. A_t and $Å_t$ are the absorbance measured in the test sample and control, respectively, after incubation for 180 min.

Antioxidant activity in linoleic acid emulsion

The total antioxidant activity of sesame lignans was measured using a linoleic acid system (Gow-Chin Yen and Hsieh, 1998). Linoleic acid emulsion was prepared by homogenizing 0.2804 g of linoleic acid, 0.2804 g of Tween-20 and 50 mL of phosphate buffer (0.2 M, pH 7.0). The reaction mixture contained different concentrations of sesame lignans in 0.5 mL of ethanol, 2.5 mL of emulsion and 2 mL of phosphate buffer (pH 7.0, 0.05 M). This was incubated at 37°C in the dark. Aliquots of 0.1 mL were taken at different intervals of time during incubation. The levels of peroxidation were determined by thiocyanate method with sequential addition of ethanol (5 mL, 75%), ammonium thiocyanate (0.1 mL, 30%), sample solution (0.1 mL), and ferrous chloride (0.1 mL, 20 mM in 3.5% HCl). After allowing the above to stand for 3 min, the peroxide value was determined by reading the absorbance at 500 nm Butylated hydroxytoluene (BHT) was used as positive control.

Ferric reducing antioxidant power (FRAP)

The modified FRAP assay was used for determining the ferrous reducing antioxidant power (Loo et al. 2007). FRAP reagent consists of the following:

1. 0.3 M acetate buffer, pH 3.6

2. 10 mM TPTZ 40 mM hydrochloric acid
3. 20 mM ferric chloride in 40 mM hydrochloric acid.

All the above were mixed in the ratio of 10: 1: 1(v/v/v) (acetate buffer: TPTZ: ferric chloride) to obtain FRAP reagent. The reagent was preheated, before analysis, to 38°C and the initial absorbance measured at 593 nm, using acetate buffer blank. Ferric-reducing ability power was measured following the procedure originally described by Benzie and Strain (1996), in which Fe^{3+} to Fe^{2+} ion reduction, at low pH, causes the formation of a colored ferrous–TPTZ complex, resulting in an increase in absorbance at 593 nm. Sesame lignans, in the range 2 – 10 μM, were added to 900 μL of FRAP reagent in test tubes. The reaction mixture was shaken vigorously for 15 s and incubated at 27°C for 90 min. The absorbance was measured at 593 nm at the end of 90 min. Control experiments without lignans or TPTZ were carried out to exclude the effect of the added test compounds. Higher absorbance indicates higher ferric reducing power. The results are expressed as Trolox equivalent reducing power of the compound.

Oxygen Radical Absorbance Capacity

Antioxidant capacity was assessed by the ORAC-fluorescein assay with slight modification of the method reported by Moore et al (2005). Fluorescein was used as the probe. The assay mixture contained 0.067 μM of fluorescein, 60 mM of AAPH, a radical initiator and 300 μL of

sesame lignans or methanol for the reagent blank. Fluorescence of the assay mixture was recorded (λ excitation = 493 nm, λ emission = 512 nm) every minute at 37°C, and the area under the curve of fluorescence vs. time plot was calculated and compared against a standard curve prepared with Trolox. ORAC value is expressed as Trolox equivalents (TE) in micromoles of the test compound. In all the cases, the ORAC- FL was conducted in triplicate.

Inhibition of browning in fruit pulps

Apple (Red delicious), banana (Cavendish variety) and potato were peeled and pulped. Sesamol (0 - 20 μM) was dissolved in water and mixed with the pulp, as described earlier (Eissa et al., 2006). The pulps were incubated at 27°C for 24 h. The samples were centrifuged at 6000 rpm for 10 min and the supernatant absorbance read at 420 nm. Inhibition of browning was calculated as :

$$\text{Inhibition (\%)} = \frac{(A_{420\ nm}\ \text{control} - A_{420\ nm}\ \text{Treated})}{(A_{420\ nm}\ \text{control})} \times 100$$

Growth inhibition assay

Effect of sesame lignans on the growth of food borne pathogenic bacteria was studied by the method of Jayaprakasha et al. (2003). Sesame lignans were filtered through 0.22 μ bacterial filters before use. Appropriate quantities of sesame lignans (dissolved in DMSO) were

transferred into different flasks containing 20 mL of sterile melted nutrient agar to obtain final concentrations of 0.25, 0.5, 0.75, 1 mg/mL. Controls were prepared by transferring an equivalent amount of DMSO to 20 mL of sterile melted nutrient agar. Each culture (100 μL, about 10^4 cfu /mL) was inoculated into the flasks under aseptic conditions. The media was poured into sterile petri-plates (in quadruplet) and incubated at 37°C for 24 h. The colonies were counted and expressed as colony forming units per mL of culture (cfu/ mL). The inhibitory effect was calculated using the following formula,

$$\% \text{ Inhibition} = (1-T/C) \times 100$$

where T = cfu /mL of test sample and C = cfu/mL of control. The minimum inhibitory concentration (MIC) is the lowest concentration of the compound capable of completely inhibiting the growth of the bacterium under the test conditions.

Zone of Inhibition test

The above bacterial cultures were inoculated on nutrient agar media by spread plate method. Small circular wells were dug into agar plates using the back end of a sterile (20 - 200 μL) pipette tip. Lignan samples (2 mg) were suspended in 100 μL of methanol and placed in the wells.

The plates were incubated at 37° C for 24 h. The diameter of the zone of inhibition was measured after the incubation.

Enzymes

Mushroom tyrosinase was obtained from Sigma Chemical Co. (T-3824). The lyophilized powder was redissolved in phosphate buffer, pH 6.8 and dialyzed against the same buffer with three changes. The dialyzed enzyme was used for the experiments.

Soybean LOX-1 was isolated according to the method of Axelrod et al. (1981) with some modifications. The specific activity was 180-200 μmoles/min/mg of protein. The concentration of LOX-1 was calculated by using the value $E_{1\%}$ = 14.0.

5-Lipoxygenase was partially purified by Ficoll-Histopaque density gradient from human peripheral venous blood from healthy individuals. Human peripheral venous blood from healthy individuals, who had not received any medication for at least three months, was collected in tubes containing sodium citrate as anticoagulant.

Polymorph nuclear leukocytes (PMNLs) were isolated from blood by Ficoll-Histopaque density gradient. The cells were resuspended in phosphate buffered saline and sonicated for 20-30 s at 20 kHz to release the cytosolic 5-lipoxygenase into solution. The solution was centrifuged at 10,000 x g for 30 min at 4°C and the supernatant directly

used as a source of enzyme. Protein concentration was estimated by Lowry's method using BSA as standard.

Anti-inflammatory activity of sesame lignans

To examine the post local anti-inflammatory potential of the lignans on animal models, groups of male Wistar rats (100–110 g, 8 week old) were maintained *ad libitum* on semi-synthetic diets. The animal experiments were carried out with approval from the Institutional Animal Ethic Committee. Appropriate measures were taken to minimize pain or discomfort to the experimental animals and all experiments were carried out in accordance with the guidelines laid down by the National Institutes of Health in the USA regarding the care and use of animals for experimental procedures.

Pure sesamin, sesamolin and sesamol were taken in groundnut oil and fed orally in concentration of 5 mg per kg body weight for 15 days. At the end of the feeding period, inflammatory response in the rats was followed by measuring the increase in paw volume after injecting λ-carrageenan. Paw inflammation was induced by injecting λ-carrageenan (2.5 mg kg^{-1} body weight) as a suspension in 200 µL sterile saline into the right hind paw under plantar aponeurosis. The extent of paw inflammation was measured by the mercury displacement method at 1-h intervals up to 5 h.

Thiobarbituric acid reactive substrates (TBARS) formation measurements

Estimation of liver lipid peroxidation

The animals, after measuring inflammatory response in the rats, were sacrificed under deep anesthesia. Blood and liver were used for serum analysis and lipid analysis, respectively.

Liver mitochondria were prepared by tissue homogenization with ice-cold 0.25 M sucrose – 10 mM Tris buffer, pH 7.4, containing 1 mM EDTA and 1% BSA. The homogenate was centrifuged at 800 g for 10 min to remove liver debris and then centrifuged at 3000 g for 10 min to remove cell debris and the nuclear fraction. The resultant supernatant was centrifuged at 10,000 g for 10 min to sediment mitochondria. This pellet was washed twice with 10 mM Tris/HCl buffer containing 175 mM KCl (pH 7.4). The mitochondria, suspended in 10 mM Tris/HCl buffer containing 175 mM KCl (pH 7.4), were stored at -20 °C. The mitochondrial protein was determined by the method of Lowry (1976).

The formation of TBARS was used to monitor lipid peroxidation (Buege, and Aust, 1978). Rat liver mitochondria were incubated at 37°C in 10 mM Tris/HCl buffer containing 175 mM KCl (pH 7.4), and made up to a final protein concentration of 0.7 or 0.35 mg/mL. Peroxidation was initiated by adding Fe^{2+} ($FeSO_4$/ascorbate (Vitamin C). Sesame lignans (1 – 5 mg/mL) were added after dissolving in DMSO to the mitochondria suspension to follow the inhibition of peroxidation, if any. The final

concentration of DMSO in the suspension was 0.2% v/v. There was no appreciable interference to the reaction, at these concentrations, as evidenced by control experiments. The reaction mixture was gently shaken at 37°C and aliquots of the reaction mixture were pipetted out at specific intervals to a TCA– TBA–HCl stock solution (15% w/v trichloroacetic acid; 0.375% w/v TBA; 0.25 N HCl), together with 0.02% w/v BHT. BHT (at this concentration) completely prevents the formation of any nonspecific TBARS, as well as prevents decomposition of ascorbic acid during subsequent boiling. The solution was heated in a boiling water bath for 15 min. After cooling to 27°C, the precipitate was removed by centrifugation. TBARS in the supernatant was determined at 532 nm using the extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Estimation of serum lipid peroxidation

The blood was collected by carotid bleeding and serum separated, after sacrifice of the animals. The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Yagi (1984). Briefly, 0.5 mL of serum supernatant and 0.5 mL of Tris HCl were incubated at 37°C for 2 h. After incubation, 1 mL of 10% trichloroacetic acid was added and centrifuged at 1000 ×g for 10 min. To 1 mL of supernatant, thiobarbituric acid (1 mL, 0.67%) was added and the tubes were kept in

boiling water for 10 min. After cooling 1 mL double distilled water was added and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol of malondialdehyde per mg protein. Serum protein was estimated using the Biuret method and the serum malondialdehyde content expressed as nanomoles of malondialdehyde per milligram of protein.

In vitro bioavailability of sesamin, sesamolin and sesamol

In vitro bioavailability of sesamin, sesamolin and sesamol was determined according to McDougall, et al. (2005). Sesamol at 1 mg/mL concentration in water was used in the study. Sesamin and sesamolin were initially dissolved in DMSO and made up to working concentration of 1 mg/ mL with water. For pepsin-HCl digestion, 1400 units of pepsin were added to the samples (20 mL). The sample pH was adjusted to 2 by addition of concentrated HCl and incubated in a shaking water bath at 37ºC for 2 h. At the end of 2 h incubation, 2 mL was removed and frozen at -20°C. The remaining pepsin digest was transferred to a beaker. A dialysis tubing (made of regenerated cellulose) containing 10 mL water and an amount of $NaHCO_3$ equivalent to the titratable acidity in the sample, was placed in the beaker. The beaker was sealed with parafilm and incubated in a shaking water bath, at 37ºC, until the pH reached about 5 (approximately 30 min). Five milliliter of pancreatin (4

mg/mL) and bile extract (25 mg/mL) mixture was added to the beaker and incubation was continued for an additional 2 h. At the end of the incubation period, the dialysis tubes were removed, rinsed with distilled water and analyzed by HPLC. Accordingly, the solution that entered the dialysis tubing (IN) at the end of incubation represents the material that enters the serum, and the solution outside the dialysis tubing (OUT) represents the material that remains in the gastrointestinal tract and could pass on to the colon. The analyses were carried out in triplicate.

All the results were expressed as the mean ± the standard deviation (SD) of three replicate analyses.

Enzyme Assays

Monophenolase activity

Monophenolase activity of tyrosinase on L- tyramine hydrochloride was determined in the presence of MBTH. The reaction mixture (1 mL), consisted 1 mM tyramine as substrate, 2 mM MBTH and appropriately diluted enzyme. The *o*- quinone formed during the oxidation of tyramine catalyzed by tyrosinase is trapped by MBTH. The reaction was followed at 500 nm (ε= 27200 $M^{-1}cm^{-1}$). The slope of linear zone of the product accumulation curve was used for calculating the activity. One unit of

monophenolase activity of tyrosinase was defined as the amount of enzyme that produces 1 μM of dopachrome per minute.

Diphenolase activity

Diphenolase activity of tyrosinase on L- DOPA was determined by measuring the dopachrome (2- carboxy 2- 3- dihydro – indole-5, 6- Quinone) accumulation at 475 nm. The assay mixture (final volume 3 mL) contained 2.0 mM L-DOPA solution in 0.1 M phosphate buffer (pH 6.8). This was incubated at 25 °C for 5 min. An aqueous solution (30 μL) of mushroom tyrosinase (1.9 μg) was added to the mixture and the initial rate in linear increase of the optical density, at 475 nm, (ε = 3700 $M^{-1}cm^{-1}$) measured for 3 min using a spectrophotometer (Shimadzu UV1601 model, Japan). One unit of enzymatic activity is defined as the amount of enzyme required to increase the absorbance at 475 nm by 0.001 under the test conditions.

Inhibition of tyrosinase activity

Inhibition experiments were carried out for diphenolase and monophenolase activities separately under different conditions of assay. For the inhibition of diphenolase activity, mushroom tyrosinase (1.9 μg/ mL) was taken in 2.3 mL of 0.1 M sodium phosphate buffer (pH 6.8) and 25 μL of ethanol solution containing different concentration of

sesame lignans. The mixture was preincubated at 25°C for 5 min, followed by the addition of 0.6 mL of 20 mM L-DOPA solution. The reaction was monitored at 475 nm for 5 min using a 10 mm path length cuvette.

Inhibition of monophenolase activity was followed by incubation of different concentrations of inhibitor with 1.9 μg of enzyme in 1 mL reaction mixture to which MBTH was added for 3 min. This was followed by addition of 1 mM of tyramine and reaction was followed at 500 nm as mentioned earlier. For determination of IC_{50}, the inhibitor was varied at a constant substrate concentration of 1 mM tyramine. K_I was determined by varying the inhibitor and substrate. The data obtained are fitted to a straight line by the method of least squares.

Reversibility of inhibition was studied by the addition of excess substrate (2 mM DOPA) to the assay mixture containing 20 μM sesamol (concentration wherein 100% diphenolase activity is inhibited). Any increase in the absorption at 475 nm was recorded after addition of DOPA.

Data presented are average of at least 3 experiments.

Lipoxygenase-1 assay

Soy LOX-1 activity was determined by following the increase in absorbance at 234 nm due to the formation of hydroperoxide (product, ε = 25000 $M^{-1}cm^{-1}$) at pH 9.0 in 0.2 M borate buffer. The substrate,

linoleic acid, was prepared according the method of Axelrod *et al* (1981). One enzyme unit is defined as the amount of enzyme required for the formation of 1 μM of hydroperoxide per min under the assay conditions.

5-Lipoxygenase-enzyme assay

5-LOX was assayed according to the method of Aharony and Stein (1986) by following the increase in absorbance at 234 nm due to the formation of 5-HETE (ε = 28000 $M^{-1}cm^{-1}$) at 27°C. The substrate, arachidonic acid (AA), was prepared according the method of Axelrod *et al* (1981). The standard reaction mixture contained 100 mM phosphate buffer pH 7.4, 50 μM of DTT, 200 μM of ATP, 300 μM $CaCl_2$, 150 μM of AA and 5 μg of protein. One unit of activity is defined as the amount of enzyme required to form 1 μM of product per min under the conditions of assay.

Inhibition of lipoxygenase activity

For inhibition experiments, lipoxygenase was incubated in presence of inhibitor (0 – 300 μM) for 5 min (in case of soy LOX-1) and 2 min (for 5-LOX) in a 10 mm path length cuvette. The reaction was initiated by the addition of substrate (linoleic acid for soy LOX-1 or arachidonic acid for 5-LOX). For the determination of IC_{50}, the inhibitor was varied at constant substrate concentration of 100 μM linoleic acid and 150 μM arachidonic acid. The % inhibition of LOX activity by lignans was

calculated from the △OD values at 234 nm obtained at the end of 3 min.

Protein estimation

Protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Cell culture experiments

B16F10 mouse melanoma cells were cultured in minimum essential medium (MEM) with 10% fetal bovine serum and penicillin/ streptomycin (100 IU/ 50 mg/ mL) in a humidified atmosphere containing 5% CO_2 in air at 37°C. These cells were used in the study.

Melanin content assay

Melanin content was estimated using a modified method of Hosoi et al. (1985). Cells (1 x 10^5) were cultured in a 6-well plate for 24 h and treated with different concentrations of sesamol or sesamin for 72 h. Cells were harvested by trypsinization followed by washing with phosphate buffered saline. The air- dried cell sample was dissolved in 200 μL of 1 N NaOH containing 10% DMSO, heated at 80°C for 1 h and cooled. The amount of melanin was determined by absorbance at 475 nm after appropriate dilution.

Apoptosis assay

The ability of sesamol/ sesamin to induce apoptosis was determined using ethidium bromide and acridine orange dye method (Powell et al., 2001) followed by observing the cells under the microscope. B16F10 (1 x 10^4 cells/ well) cells were treated with sesamol or sesamin at 0-100 μg/ mL for 72 h^{-1}, 25 μL of cell suspension of both treated and untreated cells were mixed with 1 μL of dye mix and observed under the binocular microscope (Leica, Wetzler GmbH, Germany) at magnification of 40x. The dye was a mixture containing 100 $\mu g.mL^{-1}$ each of acridine orange and ethidium bromide. Viable cell nuclei staining green with acridine orange and apoptotic cell nuclei staining red with ethidium bromide, were counted. The percent apoptosis was compared between control and cells treated with sesamol or sesamin.

Antiproliferative activity

B16F10 cells were grown as a monolayer culture to confluence in a specified medium – DMEM (Dulbecco's modified Eagle's medium) with high glucose (4.5 g/L), 4 mM glutamine, 3.7 g/L sodium bicarbonate and 10% fetal bovine albumin, 25 mM sodium pyruvate, 10 and 100 U/ mL streptomycin and fungizone, respectively, at 37°C in a humidified atmosphere containing 5% CO_2 in air and passaged weekly using 0.25% trypsin.

Sesamol and sesamin were examined for their effect on metastatic mouse melanoma (B16F10) cells, known to produce uncontrolled levels of melanin. Cells (1 x 10^4 cells/ well) were treated with sesamol and sesamin at 0-100 μg/ mL for 72 h. The ability of cells to reduce MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) to a blue formazan product was measured to determine cell survivability (Hansen et al., 1989). Dark blue crystals of formazan solution was measured at 570 nm in a microplate reader (Spectra Max-340, Molecular Devices, Germany) and cell survival rate calculated using the formula :

$$\% \text{ Cell viability} = \frac{(\text{Abs}_{\text{treated group}} - \text{Abs}_{\text{blank}})}{(\text{Abs}_{\text{control group}} - \text{Abs}_{\text{blank}})} \times 100$$

Inhibition of copper chelation

Chelation of copper, a cofactor in tyrosinase, leads to inactivation of enzyme activity. Addition of 10 μM sesamol results in complete inactivation of tyrosinase. In order to study the effect of sesamol on the active site copper, 0.2 – 1 mM copper sulfate was added to the inactivated enzyme (by addition of 10 μM sesamol) and rejuvenation of activity, if any, was followed at 475 nm.

Gel filtration

Gel filtration was carried out by a Waters® HPLC system using a TSK $G2000_{SW}$ column (4.6 x 250 cm, 5μ). Elution was carried out using an

isocratic solvent (0.02 M phosphate buffer pH 6.8) at a flow rate of 1 mL/min. Peaks were detected in the range 210–450 nm using a photodiode array detector.

Circular dichroism (CD) studies

CD measurements were carried out using a Jasco J-810 automatic spectropolarimeter fitted with a xenon lamp. The instrument was calibrated with *d* (+)-10-camphor-sulphonic acid, ammonium salt. The lamp was purged continuously with nitrogen before and during the experiments. All scans were recorded thrice. The far UV CD spectrum was recorded between 190 – 260 nm using a 1 mm path length cell. A protein concentration of 0.22 mg/ mL in 50 mM phosphate buffer, pH 4.5 was used for the experiments. The near UV CD spectrum was recorded in the range 260 – 320 nm using a 1 cm path length cell and a protein concentration of 1 mg/ mL in 50 mM phosphate buffer, pH 6.8.

Statistical analysis

Results were expressed as the mean ± the standard deviation (SD) of three replicate analyses. The difference between the groups was statistically analyzed using one-way ANOVA.

Chapter III

Results and discussion

Results and Discussion

Section 1 - *Screening of sesame seeds for lignans, Isolation and Characterization of selected lignans and optimization of roasting methods for the formation of sesamol*

Screening of the different varieties of sesame for antioxidant contents with reference to lignans and tocopherol.

Among the several varieties of sesame were developed by University of Agricultural Sciences, Dharwad, two are the parents while the rest are the progeny derived from these varieties by physical as well as chemical mutations (Table 6). Selection of the varieties is based on the yield, boldness and whiteness of seeds as well as oil content. All the seeds of selected varieties are white to brown in appearance. Fifty selected sesame varieties developed by University of Agricultural Sciences, Dharwad have been received, screened for lignan and tocopherol contents. Quantification of sesamin and sesamolin in the seeds have been carried out by a Waters® HPLC system equipped with C18 column (Waters SymmetrySheild™ RP_{18}, 5μm, 4.6 x 250 mm). The γ-ocopherol contents have also been estimated by HPLC with a

silica analytical column. (Waters Spherisorb® 5μ, 4.6 x 250 mm). The significant results are given in Table 7.

The parental variety had sesamin and sesamolin 3.59 and 1.68 g / kg of oil, respectively. Sesamin contents of the mutants progeny varied from 1.76 - 10.46 g/kg and sesamolin varied from 0.72 – 3.77 g / kg of the oil. The γ-tocopherol content in the parental variety was 0.6 g/ kg, while the derived progeny had γ-tocopherol content in the range 0.45 – 0.77 g/ kg. The phenolic content of the seeds were estimated and varied in the range 6.3 – 7.4 concentration equivalent of phenol (μg). In the progeny, the phenolic content was in the range 5.5 – 9.00 concentration equivalent of phenol (μg). There was no significant difference seen between the parent and progeny strains in free radical scavenging activity and thoibarbituric acid value.

Table 6. Sesame varieties derived from different mutation methods

Sl. No.	Treatment	Mutants	Total # of mutants
I. Chemical mutagens			
A.	EMS-0.5% (Lower capsule mutants)	699,500,307,211,965,504,40 ,207,21, 946,365,217,822,294,410,10 65, 983, 514,174,23,321,444	22
B.	EMS-0.5% (Upper capsule mutants)	500,51,657	03
II. Physical mutagens			
a.	300 GY (Lower capsule mutants)	144,181,303,191,195,397, 256	07
b.	(Upper capsule mutants)	1041,615	02
a.	400 GY (Lower capsule mutants)	279,119,353,276,224,477,39 1,352, 848	09
b.	(Upper capsule mutants)	383	01
a.	500 GY (Lower capsules mutants)	339	01
b.	(Upper capsule mutants)	949,450,1022	03
	Check (parent)	DS-1	02
		Total	**50**

Table 7. Lignan and γ-tocopherol contents of selected sesame varieties

	Mutant	**Sesamin**	**Sesamolin**	**γ- Tocopherol**	$\bar{X}$
Chemical Mutagens	699 (1)	6.42	2.68	0.53	3.21
EMS 0.5%	500 (6)	5.16	1.86	0.60	2.54
(Lower capsules	307 (7)	4.56	1.99	0.48	2.34
mutants)	211 (12)	6.05	2.25	0.56	2.96
	965 (14)	6.24	1.79	0.53	2.85
	504 (16)	5.26	2.71	0.55	2.84
	40 (19)	5.95	2.15	0.61	2.90
	207 (24)	1.76	1.02	0.52	1.10
	21 (25)	3.08	0.94	0.65	1.56
	946 (26)	6.34	3.13	0.57	3.35
	365 (27)	8.03	2.34	0.54	3.64
	217 (29)	5.64	1.85	0.61	2.70
	822 (30)	4.25	1.86	0.65	2.25
	294 (33)	5.42	1.52	0.48	2.48
	410 (34)	4.48	1.84	0.46	2.26
	1065 (37)	5.90	2.31	0.51	2.91
	983 (39)	8.32	2.98	0.55	3.95
	514 (41)	3.65	1.40	0.54	1.87
	174 (42)	6.46	1.26	0.59	2.77
	23 (43)	9.15	3.77	0.62	4.51
	321 (45)	2.58	0.72	0.70	1.34
	444 (48)	5.42	2.78	0.45	2.89
EMS –0.5%	500 (5)	7.05	1.91	0.62	3.19
(Upper casuals	51 (11)	3.14	1.89	0.53	1.86
mutants)	657 (32)	5.13	1.59	0.60	2.44
Physical Mutagens					
300 GY	144 (21)	5.04	1.89	0.52	2.48
(Lower capsules	181 (23)	4.77	1.36	0.52	2.22
mutants)	303 (31)	4.34	1.11	0.72	2.06
	191 (35)	9.13	2.66	0.77	4.19

	Mutant	Sesamin	Sesamolin	γ- Tocopherol	$\bar{X}$
	195 (38)	4.90	2.01	0.59	2.50
	397 (44)	6.09	2.09	0.65	2.94
	256 (47)	3.00	1.01	0.62	1.54
300 GY (Upper capsules mutant)	1041 (13)	4.57	1.20	0.52	2.10
	615 (17)	5.00	1.56	0.52	2.36
400 GY (Lower capsules mutant)	279 (10)	5.15	2.33	0.57	2.68
	119 (15)	3.21	1.01	0.56	1.59
	353 (18)	5.35	1.31	0.54	2.40
	276 (20)	4.72	2.40	0.72	2.61
	224 (22)	4.31	1.44	0.52	2.09
	477 (28)	4.34	2.25	0.58	2.39
	391 (36)	3.59	1.30	0.45	1.78
	352 (46)	5.95	1.21	0.74	2.64
	848 (40)	5.11	1.61	0.60	2.44
400 GY (Upper capsules mutant)	383 (3)	7.15	2.53	0.72	3.47
500 GY (Lower capsules mutant)	339 (8)	6.35	1.50	0.55	2.80
500 GY (Upper capsules mutant)	949 (2)	7.56	1.90	0.62	3.36
	450 (4)	6.37	2.10	0.59	3.02
	1022 (9)	10.46	3.18	0.59	4.74
Parent 1	DS-1 (49)	3.59	1.68	0.52	1.93
Parent 2	DS-1(50)	3.68	1.70	0.52	1.95

* (Figures in brackets are serial No's. given for analysis. Sesamin, sesamolin and γ-tocopherol were expressed in g /kg of oil)

Extraction and purification of sesame lignans

Sesame lignans– sesamin and sesamolin – from commercial white sesame seeds was carried out employing reported methods with some modification. The unsaponifiable matter of sesame oil, containing the lignans, has been subjected to preparative HPLC using a C5 column. Sesamin and sesamolin obtained have been quantified by analytical RP-HPLC. The purity of the preparations has been ascertained by HPLC as well as GC-MS. Figure 8 shows schematic representation of procedure involved in lignans purification.

Preparative HPLC of the material isolated by saponification exhibited two well resolved peaks originating from sesamin and sesamolin with retention times of 46.03 min and 48.26 min, respectively (Figure. 9). Because of the size of the loop (5 mL), samples were injected a number of times so that a sufficient quantity of the standards could be collected. The mass balance indicated that about 10 mg of sesamin and 5 mg of sesamolin were obtained from 40 mg of injected material.

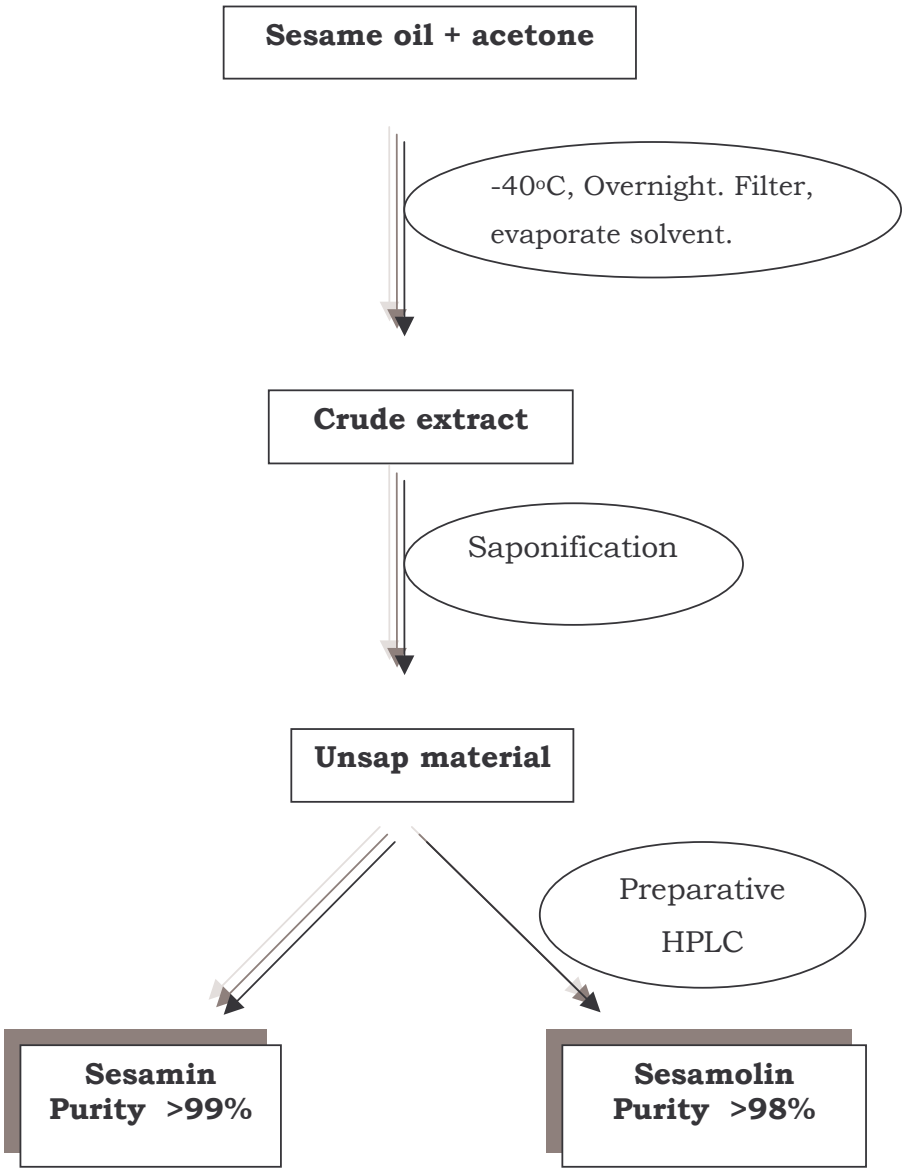


Figure 8. Purification of sesame lignans

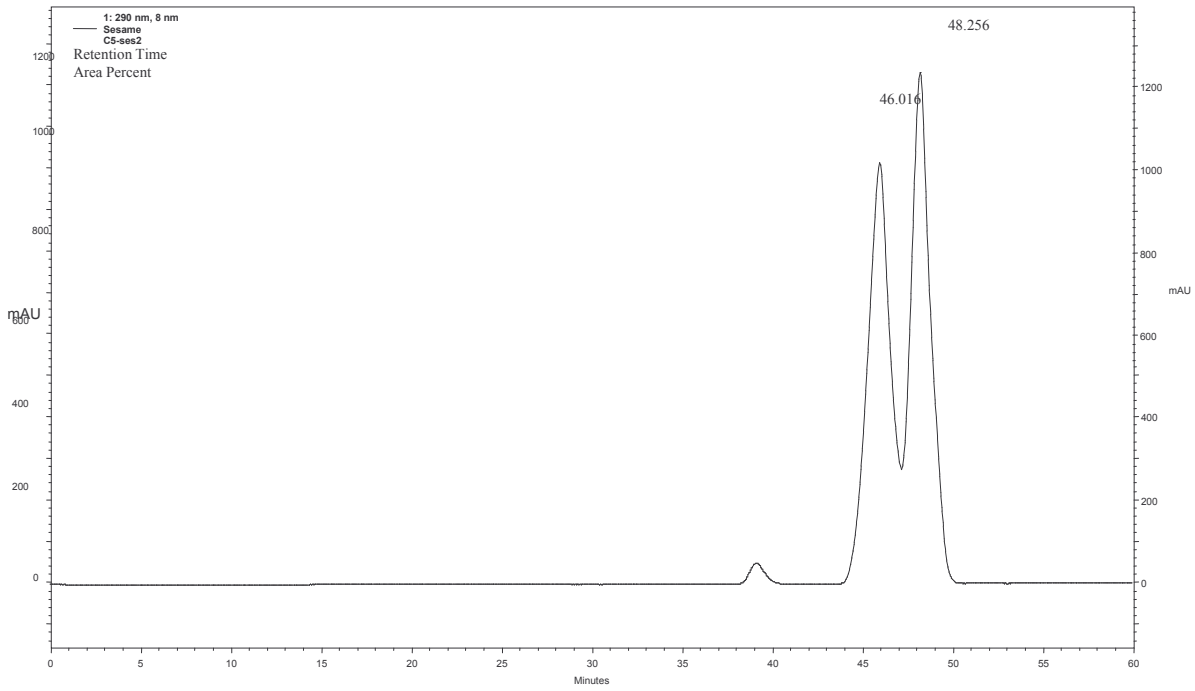


Figure 9. Preparative HPLC profile of Lignans

The lignans in the unsaponifiable matter, obtained from sesame oil, were purified by LC- 8A Shimadzu Preparative Liquid Chromatograph HPLC system equipped with a C_5 column (250 × 21 mm). The mobile phase consisted of methanol and water with gradient elution of 0-60 % of solvent B (methanol) flow rate was 5 mL/ min for 60 minutes. The lignans were detected at 290 nm and the peak fractions collected. Retention time of sesamin is 46.03 min and sesamolin was 48.26 min.

Quantification of lignans by analytical RP-HPLC.

Rechromatography of sesamin and sesamolin, on an analytical HPLC column, confirmed that the purity of the isolated compounds was indeed high (> 98%). The retention times of sesamin and sesamolin was 13.24 and 17.7 min, respectively (Figure. 10), leading to their use as standards for the HPLC analysis of lignans in sesame seeds. Sesamin and sesamolin were recovered up to 0.3%, and 0.2% respectively in the white commercial variety of sesame seeds.

GC- MS analysis of lignans

GC-MS analysis has become necessary to establish the identity of the isolated lignans. The EI mass spectra for sesamin and sesamolin are presented in Figure. 11 while, important mass ion fragments for the two compounds as well as the intensities of the ions observed are summarized in Table 8. The molecular ion radical for sesamin has been detected at a *m/z* of 354 with an intensity of 27 %. The main positive fragment ion generated from electron impact is $ArCO^+$, where Ar represents the 3, 4-(methy1enedioxy)-phenyl group. Based on the chemical makeup and symmetry of sesamin (see Figure 3), $ArCO^+$ being the base peak is not surprising. Compared to that of sesamin, more key fragment ions have been observed in the EI spectrum of sesamolin. The molecular ion was detected at a *m/z* of 370, but only at an intensity of 7 %. Loss of symmetry by the additional oxygen atom in the molecule accounts for the extra relevant MS fragmentation ions observed. Based on radical ion chemistry and

some knowledge of classical fragmentation pathways, a number of the EI assignments in Table 8 indicate which *m/z* signal is that of a radical ion or just a positive ion. The purified lignans have been identified by GC-MS in comparison with the patterns obtained from literature (Amarowicz et al., 1998).

Flouresence spectra of lignans

Fluorescence spectra Sesamin and sesamolin, along with sesamol were taken at 50 μg / ml concentration. Sesamin was excited at 287nm, while sesamolin and sesamol were excited at 288 and 296 nm respectively. Emission pattern of all the 3 molecules were given in Figure 12. Sesamin emission wavelength was at 324 nm, while sesamolin and sesamol showed the emission wavelength of 333 and 345 nm respectively.

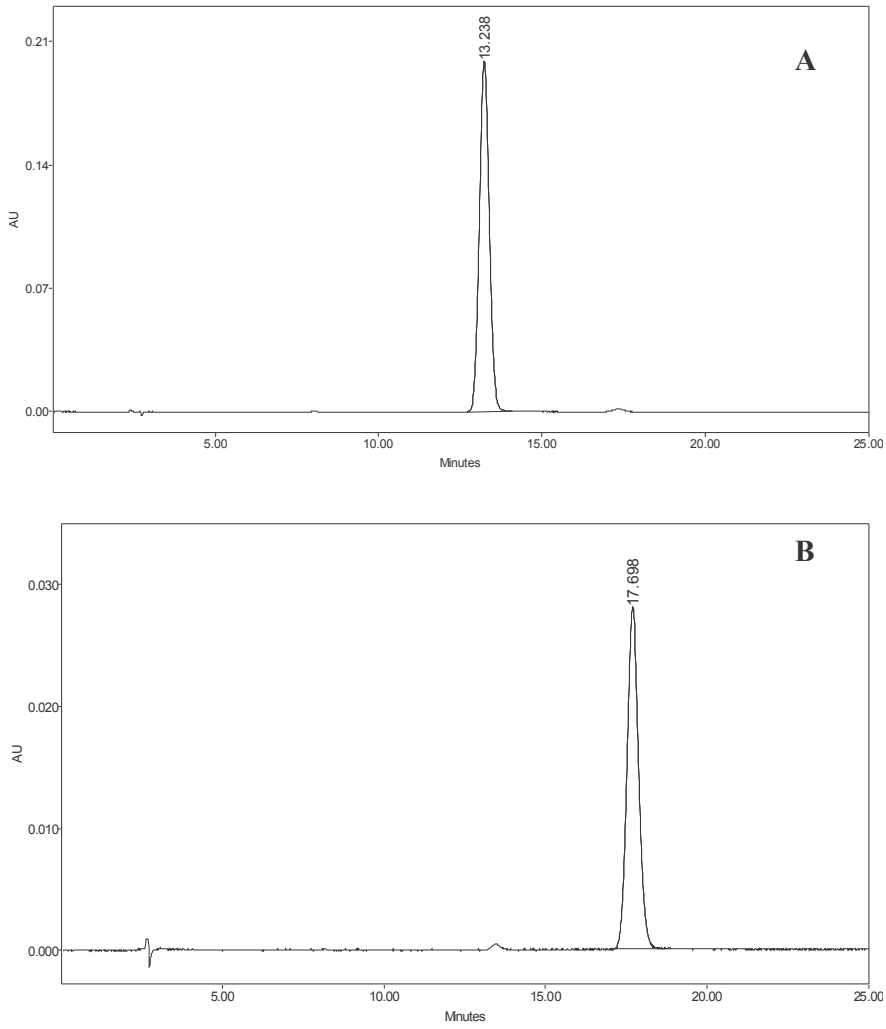


Figure 10. Homogeneity of sesamin (A) and sesamolin (B). Analytical RP-HPLC profiles using a C18 column (25 cm x 4 mm), the purified lignans were detected at 290 nm by isocratic elution in 70% methanol and a flow rate of 1 ml / min.

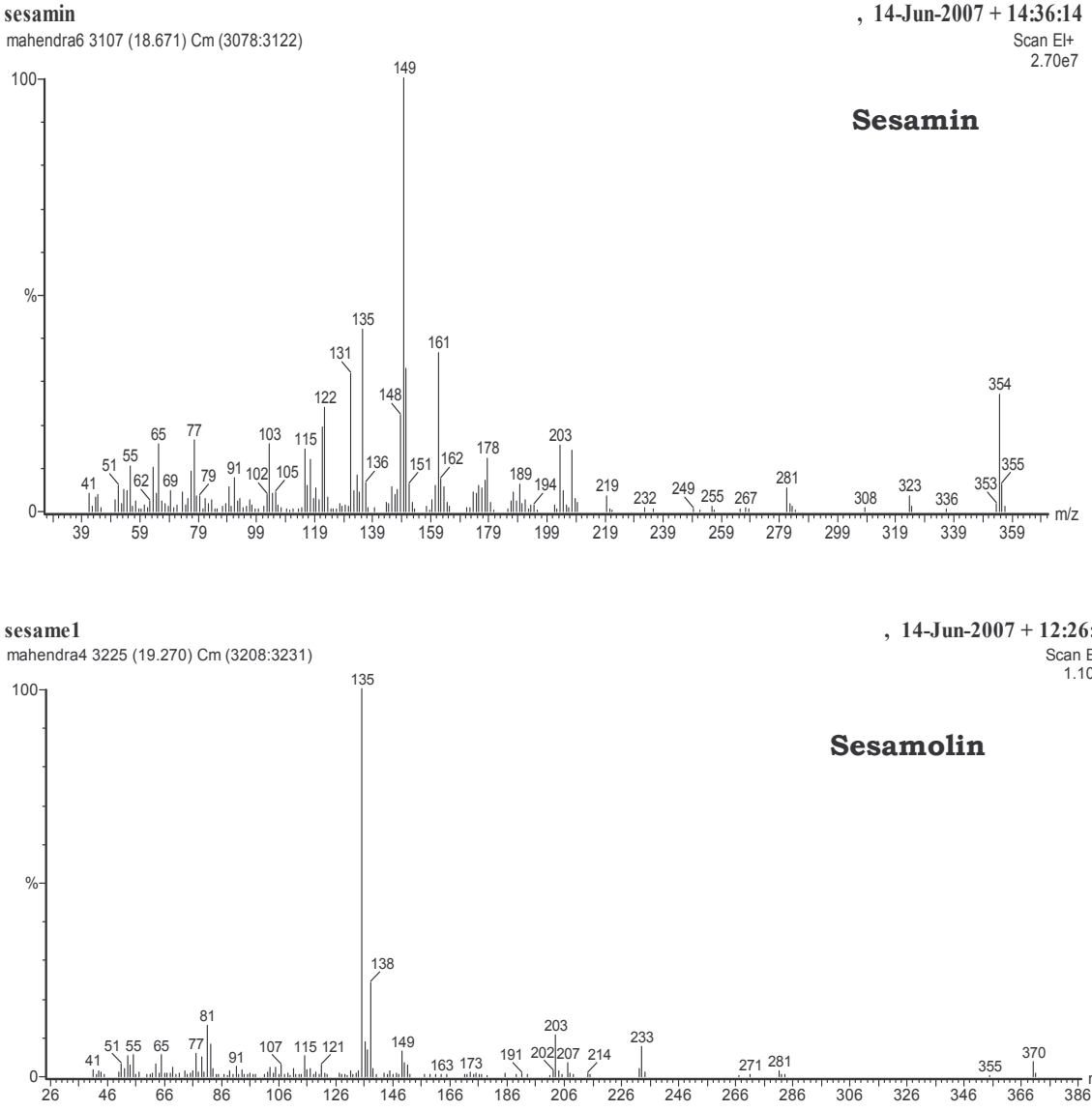


Figure 11. Gas chromatography- mass spectrometry profile of purified lignans using a Parkin Elmer Autosystem XL Gas chromatograph coupled to Turbomass Gold mass spectrometer (Perkin Elmer instruments, Norwalk, CT. USA) with a NIST library/ data system.

Table 8. Important mass ion fragments of the separated lignans (sesamin and sesamolin)*

Sesamin	***m/z***	**Int. (%)**	**Sesamolin**	***m/z***	**Int. (%)**
$M^{+\bullet}$	354	27	$M^{+\bullet}$	370	7
$[M^{+\bullet}- Ar^{\bullet}]^{+}$	233	1	$[M^{+\bullet}- Ar^{\bullet}]^{+}$	249	<1
$M^{+\bullet}- ArCHO^{+\bullet}$	203	4	$[M^{+\bullet}- ArO^{\bullet}]^{+}$	233	12
$[M^{+\bullet}- ArCHO^{+\bullet}- H^{\bullet}]^{+}$	203	16	$[M^{+\bullet}- ArCHO^{+\bullet}- H^{\bullet}]^{+}$	219	1
$[Ar – CH=CH-CH_2]^{+}$	161	37	$M^{+\bullet}- ArOCHO^{+\bullet}$	204	4
$ArCHO^{+\bullet}$	150	33	$[M^{+\bullet}- ArOCHO^{+\bullet}- H^{\bullet}]^{+}$	203	15
$[ArCO]^{+}$	149	100	$[ArOCH_2]^{+}$	151	6
$[ArCH_2]^{+}$	135	43	$ArCHO^{+\bullet}$	150	4
Ar^{+}	121	20	$[ArCO]^{+}$	149	7
			$[ArO]^{+}$	137	22
			$[ArCH_2]^{+}$	135	100
			Ar^{+}	121	6
			Other	173	4

*Where $M^{+\bullet}$ = the molecular ion radical; Int. (%) = the % intensity of the *m/z* signal; Ar = the [3, 4-(methylenedioxy) phenyl] group ($C_7H_5O_2$); and ArO is the [3, 4- (methylenedioxy)-phenoxy] group.

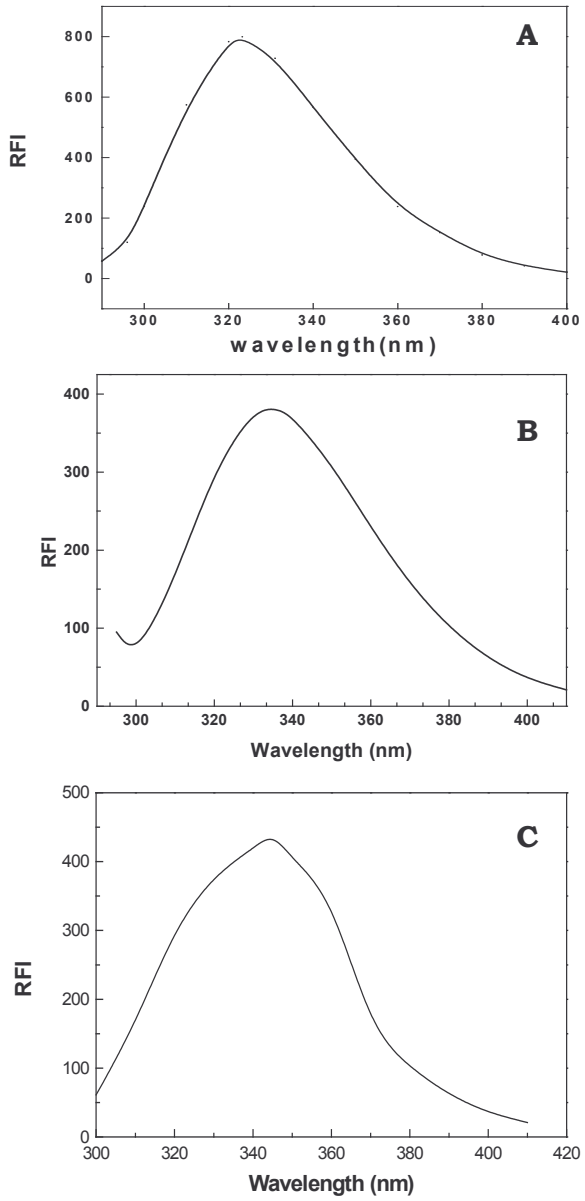


Figure 12. Fluorescence emission spectra of A. sesamin, B. sesamolin and C. sesamol. Excitation and emission slit widths were set at 5 nm and 10 nm, respectively, using a 10 mm path length cuvette containing 50 μg/mL of lignans

Optimization of processing conditions for sesame for maximal production of sesamol the strongest antioxidant in roasted oils

Sesamol is present in negligible quantities in raw seeds. It is formed as the pyrolysis product of sesamolin during refining, acid treatment and roasting. Sesamol is known to have better antioxidant properties compared to sesamolin or sesamin and hence roasted sesame oil is known for its oxidative stability. Conditions have been standardized for the conversion of sesamolin to sesamol using an electric drum roaster as well as an industrial infrared roaster in the temperature 160 - 240°C.

A two factor experimental design, performed in triplicates, has been used to examine the effect of roasting time and temperature and their interaction on the conversion of sesamolin to sesamol. Effect of two factors varied, each at three levels (high, middle and low), has been chosen for this study (Table 9). The crucial factors involved in the studies are sesamol content and roasting temperature. Roasting temperature has been set to 140, 200 and 240 °C for electric drum roasting and 160, 180 and 200°C for infrared roasting. The time of roasting for each temperature is 15, 30 and 45 min. Seeds (1 Kg per batch) have been used for roasting. After roasting, the seeds have been allowed to cool to ambient temperature before lipid extraction.

Table 9. Levels of variables chosen for RSM of sesamol formation

Roasting method	Variables	Levels		
		1	0	-1
Infrared	Roasting temperature (°C) (X_c)	200	180	160
	Roasting time (min) (X_t)	45	30	15
Electric	Roasting temperature (°C) (X_c)	240	200	160
	Roasting time (min) (X_t)	45	30	15

Sesamol contents in roasted oils - Electric drum roasting of sesame seeds

Sesamol was determined from the unsaponifiable fraction of sesame oil roasted by electrical as well as infrared roaster. In the unroasted oil, a negligible amount of sesamol is present (9.2 mg / kg). Roasting in electrical roaster at 200°C for 30 min, increased the sesamol content by ~78- fold to 722 mg/ kg. The formation of sesamol appeared to be dependent on the temperature of roasting (Figure. 13). The content of sesamol in unroasted oil was negligible but increased to 930- 940 mg in oils obtained from seeds roasted at 220 -240°C (Figure 14 and 14 Inset). There was not much increase in the sesamol content after 200°C but the color of the oil was darker and the burnt

flavors more prominent in oils obtained from seeds roasted above 200°C. over half of the sesamolin originally present in the oil (51%) was converted to sesamol when seeds were roasted for 30 min at 200°C.

Infrared roasting of sesame seeds

Infrared (IR) heating offers several advantages over conventional heating in terms of heat transfer efficiency, compactness of equipment and quality of the products. Roasting of sesame seeds degrades the lignan sesamolin to sesamol, which increases the oxidative stability of sesame oil synergistically with tocopherols. IR (near infrared, 1.1 – 1.3 μm, 6 KW power) roasting conditions have been optimized for the conversion of sesamolin to sesamol. The resultant oil has been evaluated for sesamol and tocopherol content as well as oxidative stability.

IR roasting of sesame seeds at 200°C for 30 min increased the efficiency of conversion of sesamolin to sesamol (51% to 82%) compared to conventional heating. There were no significant differences in the tocopherol content and oxidative stability of the oil. IR roasting was better compared to electric drum roasting as the conversion efficiency was better (+30%) (Figure.15). Sesame seeds were roasted at 160 – 240^{0}C for 30 min before the extraction of oil and its saponification. The unsaponifiable fractions were used to estimate lignans by HPLC method. Up to 51 % of the sesamolin originally present in the oil was converted to sesamol when seeds are subjected

to roasting by conventional rotating electric roaster for 30 min at 200°C, as against 82% conversion in infrared roasting for 30 min at 200°C.

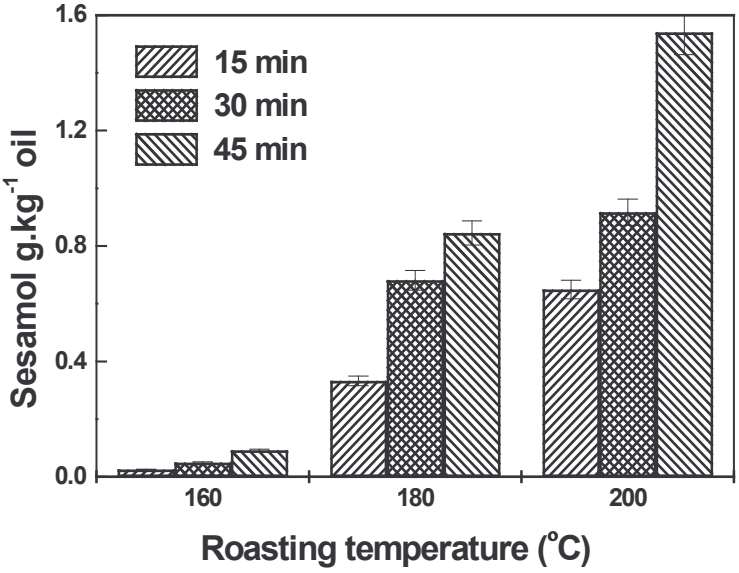


Figure 13. Effect of IR roasting on formation of sesamol in sesame oil.

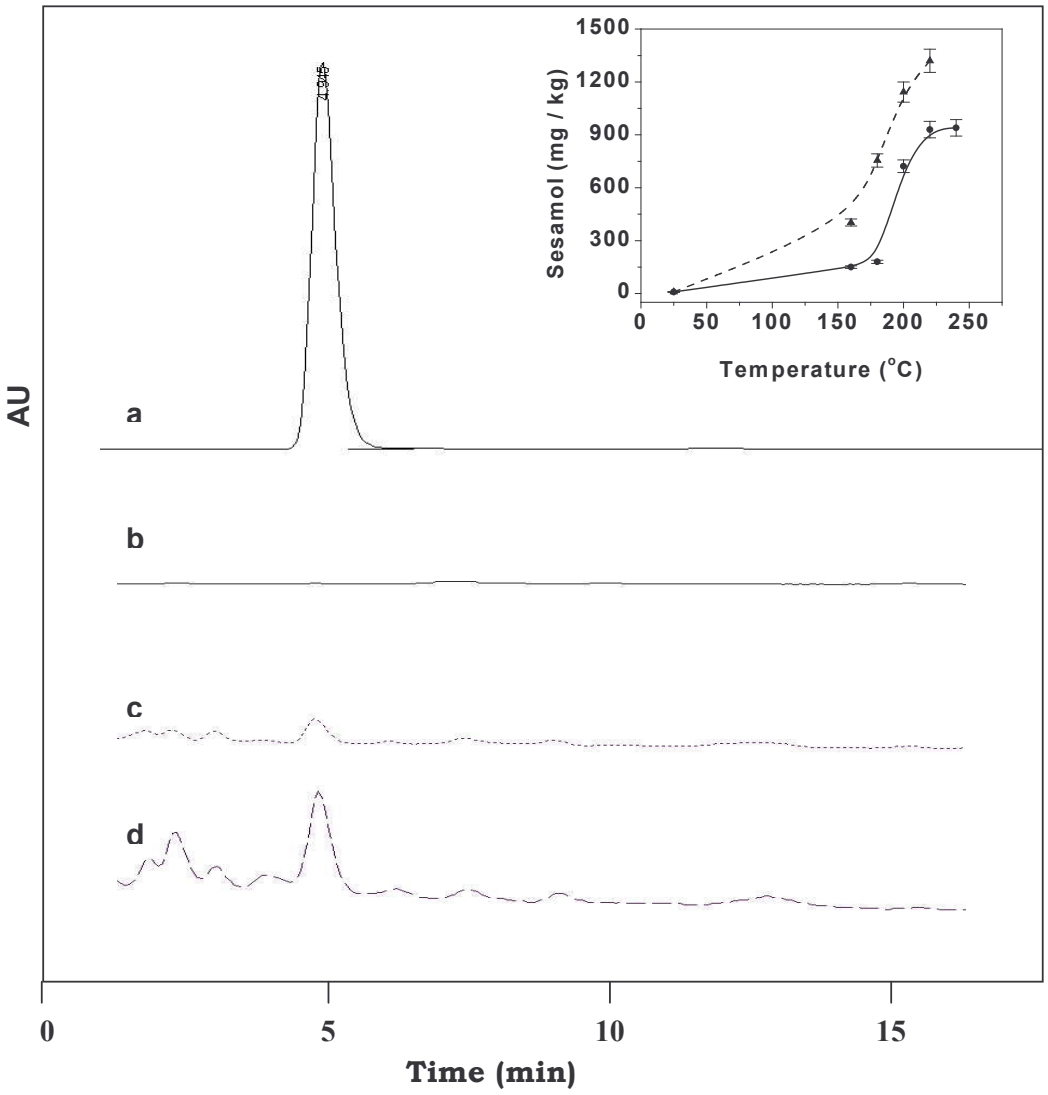


Figure 14. Detection of the formation of sesamol in roasted oils by HPLC. HPLC was performed on a C_{18} column using 70% methanol as the mobile phase with a flow rate of 0.5 ml / min. Standard sesamol (>97% purity) was used to determine the retention time. **a:** Standard sesamol; **b:** unroasted sesame oil; **c:** sesamol from sesame oil obtained from seeds roasted at 180°C and **d:** sesamol from sesame oil obtained from seeds roasted at 200°C.

Inset: Liberation of sesamol by roasting seeds in the temperature range of 160 – 240°C before extraction of oil. 36% of the sesamolin was converted to sesamol under optimized conditions. Electric drum roasting (solid line) and IR roasting (dashed line)

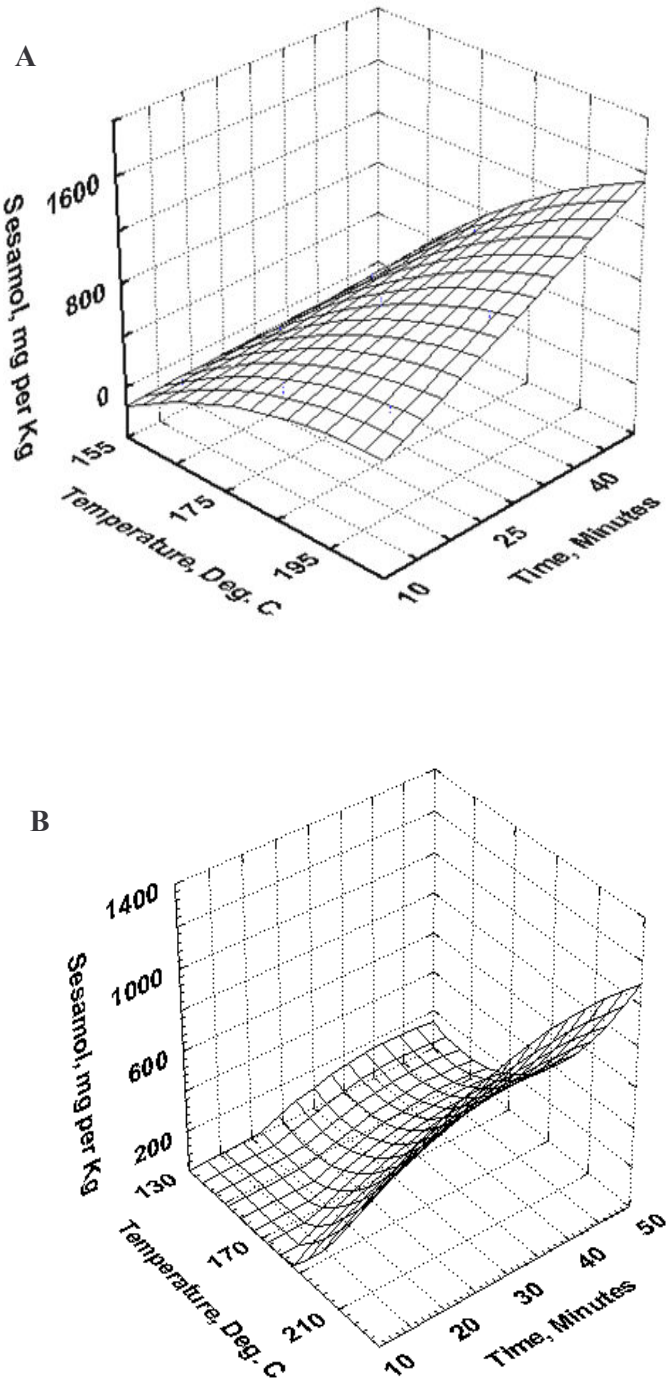


Figure 15. Response surface graph of sesamol formation during (A) infrared roasting and (B) electrical roasting.

Effect of roasting on tocopherol content in sesame oil

Sesame seeds have considerable amounts of tocopherols, of which, γ-tocopherol is the major one, constituting ~97 - 98% of the total (Namiki 1995; Shahidi et al., 1997). The γ-tocopherol content has been estimated to be 395 ± 10 mg. kg^{-1} in the unroasted oil. This is in agreement with literature reports (Namiki 1995; Yen 1990). The tocopherol contents of oils from IR roasted and electrically roasted seeds are summarized in Table 10. The loss in tocopherol contents of IR roasted seeds is marginally lower than drum roasted seeds. Roasting of seeds is found to result in ~20% loss in the γ-tocopherol content of oil up to the temperature of 200°C. The loss is greater at higher temperatures (beyond 200°C).

The oil obtained from seeds electrically roasted at 240°C showed a decrease in γ-tocopherol content of 33%. The effect of combining sesamol with γ-tocopherol antioxidant activity was reported to be a function of temperature and time (Hussain et al., 1986). It was established that the roasting increases the content of sesamol in sesame seeds and sesamol is known to increase the antioxidant activity of γ-tocopherol several folds synergistically (Fukuda et al., 1986).

Table 10. Sesamol and tocopherol contents in the oils obtained from roasted sesame seeds

Roasting temperature	Sesamol (mg.kg^{-1} oil)		γ-Tocopherol (mg.kg^{-1} oil)	
(°C)	**Electrically heated drum roasting**	**Infrared roasting**	**Electrically heated drum roasting**	**Infrared roasting**
Unroasted seeds	6 ± 0.2^{a}	6 ± 0.2^{a}	395 ± 18^{a}	395 ± 18^{a}
140	n.d.	n.d.	374 ± 10^{b}	380 ± 15^{a}
160	150 ± 4^{b}	403 ± 11^{b}	320 ± 11^{c}	334 ± 12^{b}
180	180± 6^{c}	755 ± 24^{c}	318± 14 ^{c}	322 ± 10^{b}
200	722± 13^{d}	1143± 32^{d}	304.4 ± 9^{c}	327 ± 10^{b}
220	930± 21^{e}	1342± 37^{e}	271± 7^{d}	298 ±11^{c}
240	940± 22^{e}	n.d.	265.7 ± 8^{d}	n.d.

Seeds roasted by electrically heated rotary drum roaster and infrared roaster. Time of roasting fixed at 30 min. Each value is a mean of three determinations ± SD and expressed as mg per kg oil. Values in each column with different superscript within the column (a –e) are significantly ($P < 0.05$) different from one another.

n.d. – not determined.

Preparation of heat resistant sesame seeds

The present method provides a process for the preparation of heat resistant seeds that do not brown when used in bakery products.

The heat resistant sesame seeds have whiter and brighter appearance compared to the dehulled seeds (Figure 16), and retain their shape even after exposure to the high baking temperatures (>200°C).

Upon roasting, the sesamol content in the heat resistant seeds is more compared to dehulled sesame seeds. Infrared roasting at 200°C has shown heat resistant seed has 1230 $mg.kg^{-1}$ of sesamol compared to 1143 $mg.kg^{-1}$ in dehulled seeds. Table 11 summarizes the comparison between heat resistant seeds and dehulled seed for the production of sesamol.

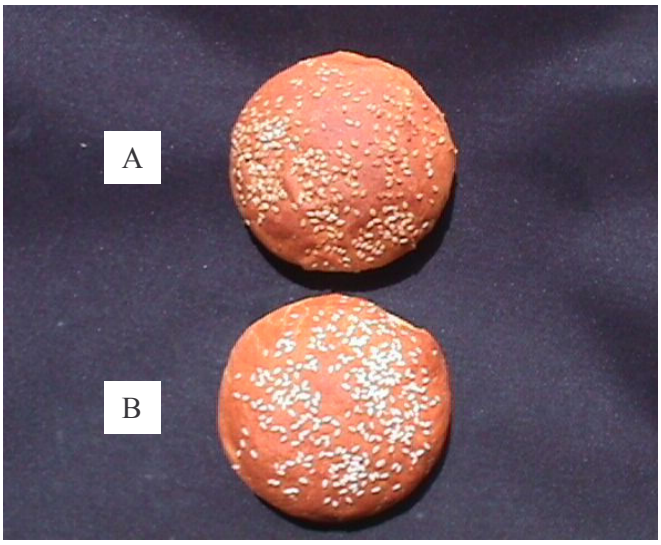


Figure 16. Effect of baking temperature on (A) Dehulled sesame seeds and (B) Heat resistant sesame seeds sprinkled over bread.

Table 11. Lignan content in the heat resistant seeds are more compared to dehulled sesame seeds

Roasting temperature	**Sesamol (mg.kg^{-1} oil)**	
(°C) Infrared heating	**Heat resistant seeds**	**Dehulled seeds**
Unroasted seeds	6 ± 0.2^{a}	6 ± 0.2^{a}
160	485 ±14^{b}	403 ± 11^{b}
180	849± 26^{c}	755 ± 24^{c}
200	1230± 34^{d}	1143± 32^{d}
220	1392± 39^{e}	1342± 37^{e}

Time of roasting fixed at 30 min. Each value is a mean of three determinations ± SD and expressed as mg per kg oil. Values in each column with different superscript within the column (a –e) are significantly ($P < 0.05$) different from one another.

Oxidative stability of roasted sesame oils

Stability of the oils against oxidation was studied by the TBARS assay. Maximum stability was obtained in the oil roasted at 180°C (Figure 17). The oils from roasted seeds in the range 160 -200°C was found to be more stable than unroasted oil. Roasting of seeds at temperatures greater than 200°C resulted in a decrease in the oxidative stability of oil, probably due to the reduction in tocopherol content. The colour formation in sesame oil could be attributed to phospholipid degradation as well as non- enzymatic browning, which occur during heating (Hussain et al., 1986). The non-glyceride fraction of oils had variable composition of tocols and unique components that not only contribute to the oxidative stability of edible vegetable oils but also provide dietary antioxidants (Shahidi and Shukla, 1996).

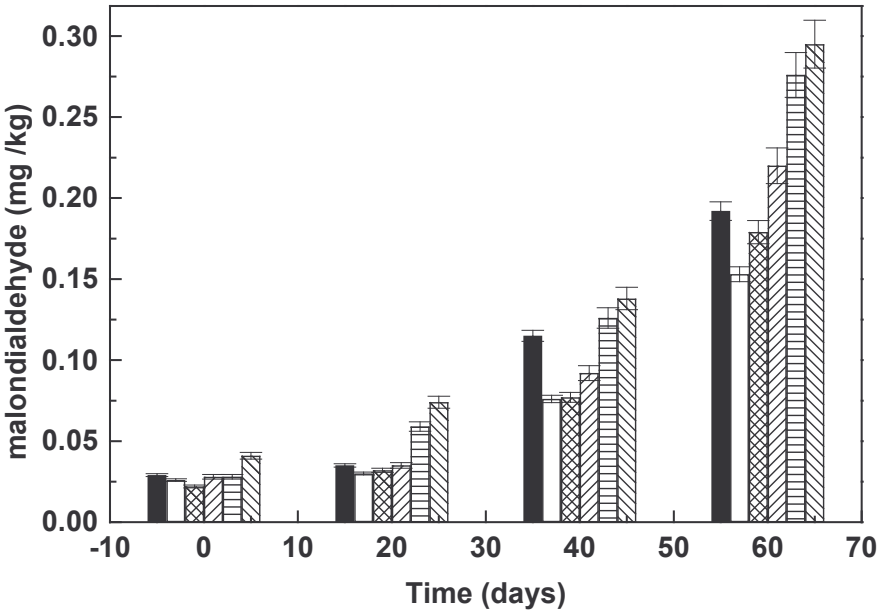


Figure 17. Oxidative stability of sesame oils obtained from seeds after electric roasting. (▬), Unroasted oil; (▭), oil obtained after roasting at 160°C; (▩), roasting temperature 180°C; (▨), roasting temperature 200°C; (▤), roasting temperature 220°C; (▧), roasting temperature 240°C.

Analysis of defatted and roasted sesame flours

Effect of IR and electric roasting on the composition and antinutritional components in the dehulled, defatted and roasted sesame flours is shown in Tables 12 and 13. Oxalic acid has been estimated to be 1.8% in undehulled seeds. Undehulled sesame seeds have higher contents of oxalic acid and dehulling of sesame seeds results in ~50% decrease in oxalic acid content. Oxalic acid is reported to be present as its calcium salt in the hull (Inyang and Ekanem, 1996). No significant decrease in tannin contents of flour on dehulling or roasting is observed (Table 13).

Protein content of the dehulled seeds was 23.0%, which, on defatting, increased to 54.7 %. Roasting did not significantly affect the protein content of flour. The protein content of defatted flour from dehulled seeds was reported to be 59.7% (Inyang and Nwadimkpa, 1992).

Effect of roasting on nutritional indicators: Available lysine and methionine

The method of roasting the seeds did not have any appreciable effect on the loss of available lysine. Roasting of seeds, at 200°C for 30 min, resulted in significant loss (53%) of available lysine, 3.2 to 1.7% (Figure 18). Sesame flour was reported to be rich in sulfur amino acids – methionine and cysteine – in the range of 61 – 78 mg/ g protein (Namiki 1995). In the present study, there was no loss of methionine due to either IR or electric roasting. Methionine contents were determined to be 37 - 43.3 mg/ g protein (Table 12).

Table 12. Effect of infrared roasting of sesame seeds on meal constituents

Parameter (%)	Undehulled seeds	Dehulled seeds	Dehulled unroasted flour (defatted)	Roasted flour (defatted) (Temperature, °C)		
				Infrared roasting of sesame seeds		
				180	200	220
Moisture	4.68 ± 0.2	4.62 ± 0.1	7.06 ± 0.2	7.44 ± 0.2	7.14 ± 0.2	6.2 ± 0.2
Protein	20.3 ± 0.1	23.0 ± 0.1	54.7 ± 0.2	54.8 ± 0.2	55.36 ± 0.1	54.07 ±0.1
Oxalic acid	1.8 ± 0.3	0.85 ± 0.02	0.11 ± 0.01	0.09 ±0.02	0.08±0.001	0.08 ± 0.01
Tannins	0.59±0.02	0.48 ± 0.02	0.36 ± 0.02	0.33 ±0.02	0.33 ± 0.02	0.35 ± 0.02
Available lysine	-	3.2 ± 0.2	2.8 ± 0.4	2.31 ± 0.2	1.83 ± 0.2	1.16 ± 0.3
Methionine	0.037± 0.02	0.038±0.002	0.037±0.001	0.037±0.001	0.043±0.01	0.043±0.001

Each value is a mean of three determinations ± SD.

Table 13. Effect of electrical roasting of sesame seeds on meal constituents

Parameter (%)	Undehulled seeds	Dehulled seeds	Dehulled unroasted flour (defatted)	Roasted flour (defatted) (Temperature, °C)		
Electrically heated drum roasting of sesame seeds						
				160	**200**	**240**
Moisture	4.68 ± 0.2	4.62 ± 0.1	7.06 ± 0.2	8.04 ± 0.2	8.08 ± 0.2	7.47 ± 0.2
Protein	20.3 ± 0.1	23.0 ± 0.1	54.7 ± 0.2	53.8 ± 0.2	53.36 ± 0.1	52.87 ±0.1
Oxalic acid	1.8 ± 0.3	0.85 ± 0.02	0.11 ± 0.01	0.09 ±0.02	0.08 ±0.001	0.07 ± 0.01
Tannins	0.59±0.02	0.48 ± 0.02	0.36 ± 0.02	0.33 ±0.02	0.32 ± 0.02	0.32 ± 0.02
Available lysine	-	3.2 ± 0.2	2.8 ± 0.4	2.71 ± 0.2	1.7 ± 0.2	1.03 ± 0.3
Methionine	0.037± 0.002	.037±0.002	0.037±0.002	0 .037±0.002	0.039± 0.02	0.037± 0.002

Each value is a mean of three determinations ± SD.

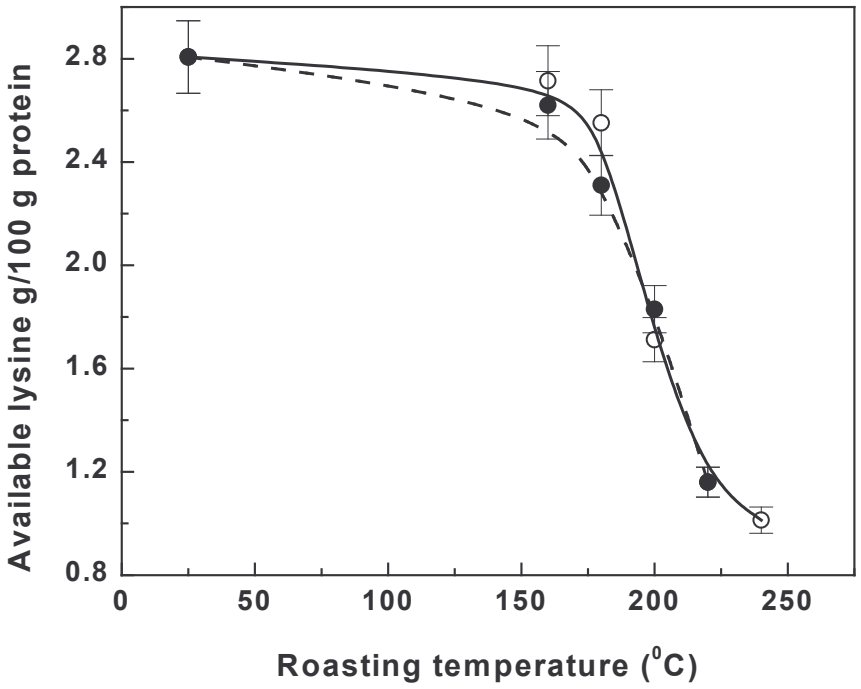


Figure 18. Effect of roasting on the lysine content of defatted sesame flour. Electrically heated drum roasting (solid line) and infrared roasting (dashed line).

Effect of roasting on sensory quality of defatted flours

Flours obtained after oil extraction of roasted seeds were darker in color. The lightness values decreased with increase in roasting temperature, from 84.9 in unroasted flour to 51.4 in roasted flour (200°C). Roasted flours (160 -200°C) had an appealing nutty flavor compared to unroasted flour. Roasted flours above 200°C had a burnt flavor and dark color.

Functional properties of roasted and defatted sesame flours

The effect of roasting on the foaming capacity of defatted sesame flour is given in Table 14. A slight increase in water and fat absorption capacities is observed in roasted flours obtained from roasted seeds. The increase is evident in the temperature range 180 - 200°C. There is no significant change in the foaming capacity when sesame flour is roasted in the range 160 -240°C.

The fat absorption capacity (FAC) of flour reached a maximum in flours obtained from roasted seeds at 200°C (Table 14). There was a slight decrease in the FAC (250 g/ 100 g) in samples obtained from seeds roasted in the temperature range 200 - 240°C, although the value is still higher than in control (225 g/ 100 g flour).

There was a significant increase in the water absorption capacity of flour with increase in the roasting temperature. The flour obtained from seeds roasted at 220°C had the highest water absorption capacity of 240 g/ 100 g flour compared to 177 g/ 100 g flour in the

control. Similar results were obtained with IR roasted flours for fat and water absorption capacities up to 200°C. Beyond 200°C; there was decrease in the water and fat absorption capacity.

The emulsification capacity of unroasted sesame oil was 44 mL/ g flour. The emulsification capacity of flour obtained from roasted seeds at 240°C was 10 (a decrease of 77%).

Table 14. Effect of roasting conditions on functional properties of defatted sesame flour

Parameters	Water absorption capacity (g/ 100g flour)	Fat absorption capacity (g/ 100g flour)	Foaming capacity (mL/ g flour)	Emulsification capacity (mL oil/g flour)
Unroasted flour	177.4 ± 8 [a]	228± 13[a]	46 ± 2 [a]	43.5± 3[a]
Electrically heated drum roasting (Temperature °C)				
160	204.8± 9[b]	243.1± 12[b]	48.5± 3 [b]	32.5± 2[b]
180	224.3± 10[c]	248.7± 13[b]	49± 3 [b]	21± 2[c]
200	236.1± 13[c]	282.3± 13[c]	50.2± 4[b]	17± 2[d]
220	245.6± 12[c]	264± 12[b]	49.5± 4 [b]	16± 2[d]
240	237± 11[c]	258.7± 9[b]	49± 3 [b]	9.5± 1[e]
Infrared roasting (Temperature °C)				
160	212.3± 8[b]	255.1± 10[b]	51± 2[b]	35.4± 2[b]
180	218± 8[c]	260.1± 11[b]	52.4± 2[b]	24± 2[c]
200	221.5± 6[c]	271± 10[c]	49± 3[b]	22 ± 2[c]
220	212.7± 7[b]	254.4± 9[b]	43± 2[b]	13 ± 1[d]

Each value is a mean of three determinations ± SD. Mean values in each column with different superscript (a – e) within are significantly ($P < 0.05$) different from one another.

Discussion

Sesame seed is a rich source of dietary lignans, and is highly valued for its nutritional value in many parts of the world, especially Asian countries. Currently, the consumption of sesame seed products and oils is steadily increasing in Europe and USA (Moazzami et al, 2007). The presence of endogenous antioxidants – lignans that act synergistically with tocopherols in preventing oxidation imparts remarkable stability to the oil (Yoshida 1994). The antioxidant activity of sesame lignans have been adequately demonstrated earlier (Kikugawa et al., 1983; Suja et al., 2004). Sesamin and sesamolin are naturally present lignans in sesame, while sesamol content is negligible. The superior oxidative stability of roasted sesame oil relative to other oils is largely due to sesamol, which is released from sesamolin during thermal processing or storage of the oil (Namiki 1995). Addition of sesamol to sesame oil also leads to increased stability (Fukuda et al., 1986; Kikugawa et al., 1983).

Sesamol is a unique phenolic compound due to its thermal stability and solubility in both aqueous as well as oil phase. The presence of a benzodioxole group, helps to scavenge hydroxyl radical and produce 1,2-dihydroxybenzene (Kumagai et al., 1991). Oils, obtained after roasting seeds by microwaves or electric oven, with higher sesamol contents and antioxidant activity, have been also reported (Yoshida and Kajimoto 1994).

Purification of sesame lignans using C_{18} column has been reported by many researchers previously (Amarowicz et al., 1998; Fukuda et al. 1986). In the preset study lignans have been purified by preparative RP- HPLC using C5 column. This is the first effort to use RP- HPLC column other than C18 column for purification of lignans. With this method we have been able to isolate sesame lignans effectively.

Infrared heating is a rapid as well as cost effective method for processing food for improved sensory and nutritive value compared to conventional electric or natural gas heating (Sakai and Hanzawa 1994). The energy emitted from the infrared heater is converted to heat by interaction with the food molecules and conducted throughout the food. In high temperature resistance ovens, heat is mainly supplied to the surface of the food by forced convection.

The conversion of sesamolin to sesamol by infrared has been compared with electrically heated drum roasting (Table 10). Response surface graph of infrared roasting clearly shows the formation of 1320 mg kg^{-1} at 220°C (Figure.15 A) which is 30% more than the conventional electric roasting (Mahendra, et al., 2009). It can be seen that the formation of sesamol increased linearly as a function of roasting temperature for 15 – 45 min. It is evident that the roasting increases the content of sesamol in sesame seeds and sesamol is known to increase the antioxidant activity of γ-tocopherol several folds synergistically (Fukuda, et al, 1986). Sesamolin and sesamin are

effective in increasing the parameters of hepatic fatty acid oxidation, and are equally effective in decreasing lipogenesis (Lim et al. 2007).

The solubility and functional properties of protein isolate from sesame seed, as influenced by pH and/or salt concentration, is reported (Khalid et al., 2003). All functional properties are greatly affected by pH as well as salt concentrations. The protein isolate being highly soluble in acidic and alkaline pH and hence has better emulsifying as well as foaming properties. The foaming capacity is found to be higher in oils obtained by roasting seeds in the temperature range 160 - 200°C. At higher roasting temperatures, the foaming capacity decreases as a function of temperature.

Infrared roasting is more effective than electric roasting for converting sesamolin to sesamol (82% conversion has been possible against 51% in conventional electrically heated drum roasting of seeds. The contents of both sesamol and γ- tocopherols are critical for the oxidative stability of roasted oils. There is no significant difference in the nutritional or functional qualities of the flours obtained from roasted seeds either with IR or conventional roasting. There is no significant loss in the methionine or cysteine content of the flours due to roasting. IR roasting is a better option for processing of sesame seeds to yield quality oil and meal.

In the next section, the biological activity and the bioavailability of the three molecules i.e. sesamin, sesamolin and sesamol are discussed in detail.

Results and Discussion

Section 2 – Bioactivities and bioavailability of sesame lignans: *in vitro* studies

The antioxidant, free radical scavenging and antibacterial properties of lignans are reported in this chapter. Sesame lignans are unique phenolic antioxidants with many health benefits attributed and also play an important role in plant defense (Namiki, 1995). In the present study we have employed a panel of assays to evaluate the antioxidant potency of pure lignans individually and compare with the known standards. We have also selected FRAP and ORAC-FL methods to evaluate radical quenching ability of sesame lignans along with lipid peroxidation and DPPH radical scavenging assays.

The DPPH radical scavenging assay is commonly employed to evaluate the ability of antioxidants to scavenge free radicals. The use of the DPPH free radical is advantageous in evaluating antioxidant effectiveness because it is more stable than the hydroxyl and superoxide radicals (Liyana-Pathirana, 2006). The DPPH scavenging activities of the sesamin, sesamolin and sesamol at different concentrations are shown in Figure 19. Sesamol scavenged DPPH radicals at the concentration of 20 μg / mL with an IC_{50} value of 5.44 μg / mL. Standard BHT also shows IC_{50} value of 5.81 μg / mL. Both sesamin and sesamolin shows very weak radical scavenging ability with

only 30 and 32% scavenging activity at 250 μg / mL concentration. The high radical scavenging capacity of sesamol is, probably, due to the presence of free hydroxyl group in its structure.

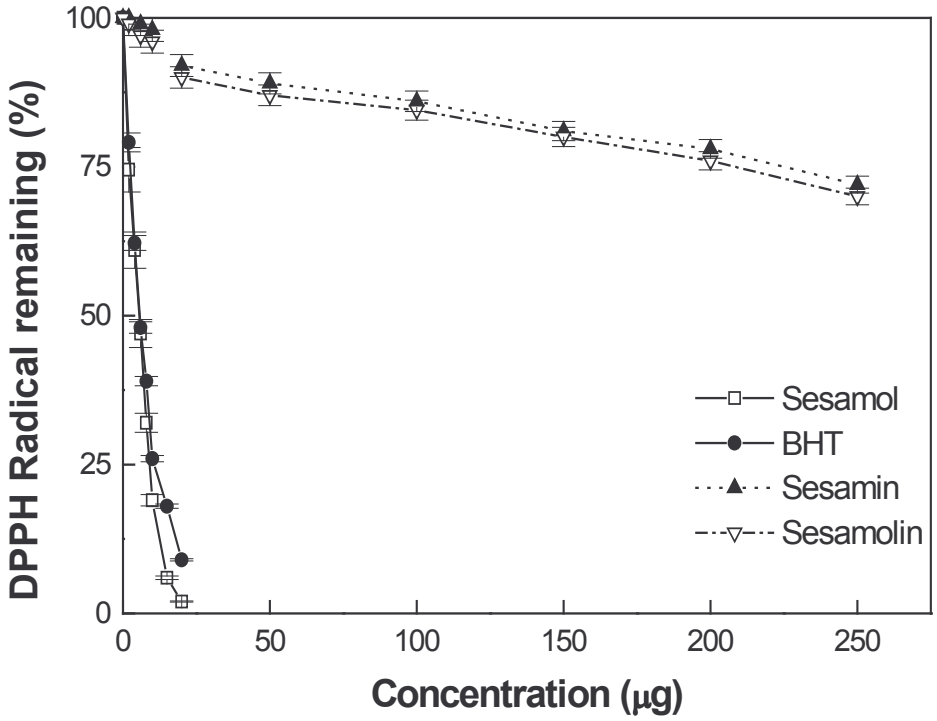


Figure19. Antioxidant activity of sesame lignans by DPPH free radical scavenging method. Scavenging of DPPH free radical was followed at 517 nm spectrophotometrically**.** BHT was used as standard.

Antioxidant Activity by β-carotene - linoleate model system and Linoleic Acid Emulsion

The mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoliec acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β carotene molecules. As β-carotene gets oxidized, it loses its chromophores and characteristic orange color, which can be monitored spectrophotometrically. The presence of sesame lignans can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical in the system. Sesamol, sesamolin and sesamin have antioxidant activity of 78.5, 68 and 62.5 %, respectively (Figure 20) when compared to BHT standard (97%). Sesamol is a unique phenolic compound due to its solubility in the aqueous as well as the oil phase and thermal stability. It has a benzodioxole group, which is known to scavenge hydroxyl radical to produce 1, 2-dihydroxybenzene (Kumagai, 1991). This makes sesamol a better hydroperoxide scavenger compared to sesamin and sesamolin.

The antioxidant effects of sesame lignans on the peroxidation of linoleic acid have been investigated and the results are shown in Figure 21. The oxidative activity of linoleic acid is found to be markedly inhibited by all 3 lignans tested. The antioxidant effects of extracts of sesamin,

sesamolin and sesamol after incubation for 60 hours with linoleic acid are summarized in Table 15. BHT have been included as positive control for phenolic antioxidants. Taken together, the results show that the inhibitory potential follows the order sesamol > sesamin > BHT> sesamolin. Sesamol and sesamin are found to exhibit excellent antioxidant activity with 97.8 and 96 % inhibition of linoleic acid peroxidation respectively (Table 15).

Table 15. Antioxidant activity of sesame lignans linoliec acid emulsion was determined by thiocyanate method

Sample	Absorbance at 500 nm (at 60 hours)	Inhibition % of peroxidation
Control	2.15 ± .025	0.00
Sesamol (200 μg)	0.048 ± .004	97.8 ± 0.02
Sesamin (200 μg)	0.0850 ± 0.019	96.0 ± 0.03
Sesamolin (200 μg)	0.292 ± 0.024	86.4 ± 0.11
Gallic acid (200 μg) (standard)	0.154 ± 0.015	92.8 ± 0.14

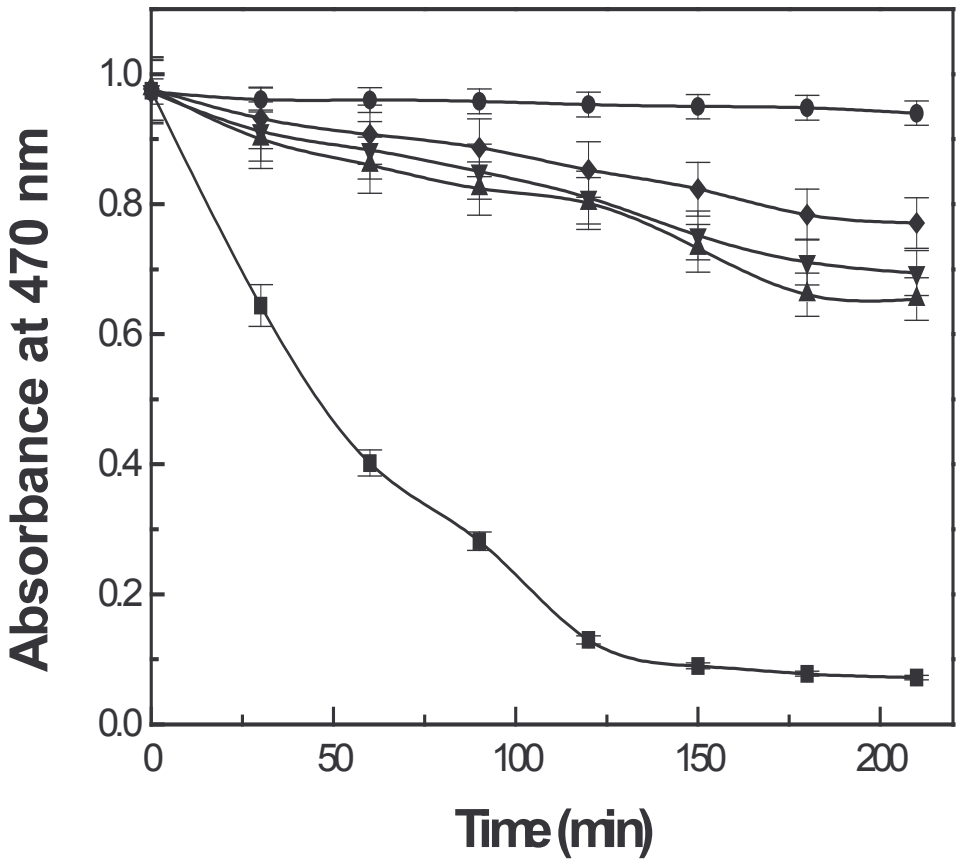


Figure 20. Antioxidant activities of sesame lignans by β-carotene - linoleate model system. lignans and standards were taken at 200 μg ml^{-1} concentration (■) Control, (●) BHT, (◆) Sesamol. (▲) Sesamolin, (▼) Sesamin.

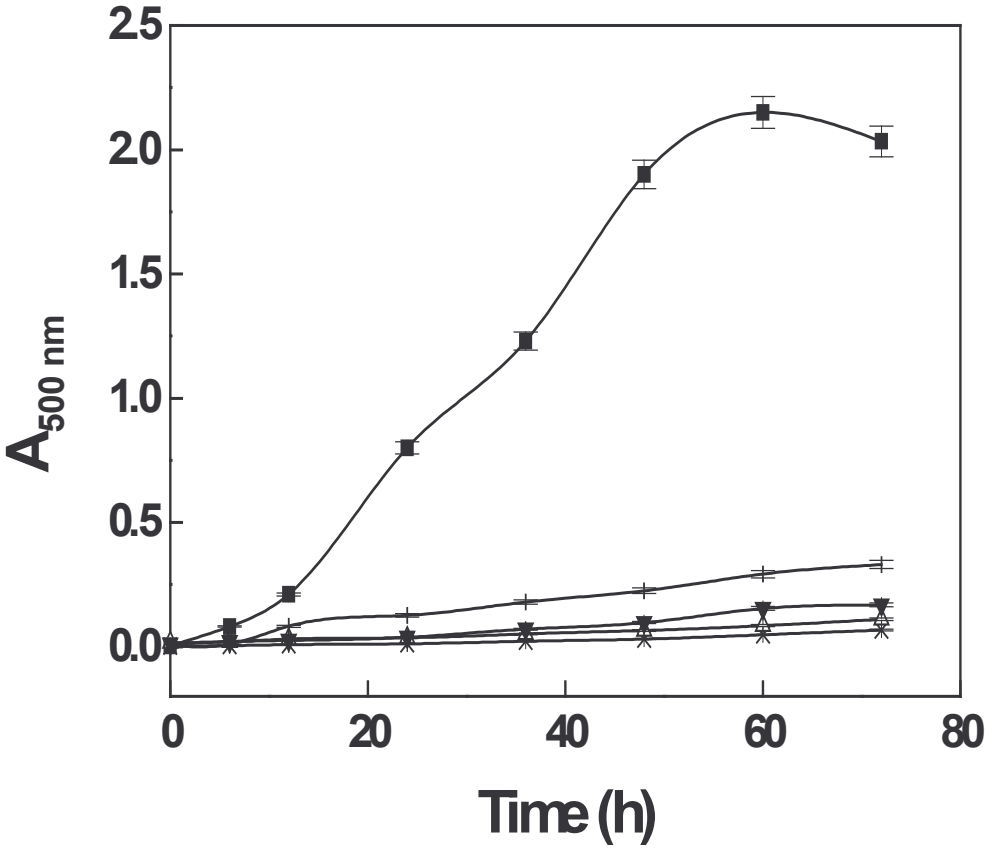


Figure 21. Antioxidant activities of sesame lignans on linoliec acid emulsion by thiocyanate method. All the lignans were taken at 200 μg ml^{-1} concentration. Sesamol (×), sesamin (|), sesamolin (▼), Control (■), Gallic acid (△) at different time intervals.

Ferric Reducing Ability Power and ORAC- FL assays

Of the 3 lignans analyzed (Figure 22), sesamol has shown 1.83 ± 0.08 μM TE (trolox equivalence) reducing power compared to 0.6 ± 0.02 of BHT. Sesamin and sesamolin exhibited very low FRAP values with 0.06±0.01 and 0.12 ± 0.05 TE. This shows that sesamol has potent antioxidant capacity to reduce the Fe^{3+}/ tripyridyl-s-triazine (TPTZ) complex to the ferrous form. Higher absorbance indicates higher ferric reducing power. The results have been expressed as Trolox equivalent reducing power of the compound.

The oxygen radical absorbance capacity assay (ORAC) is based on the chemical damage caused to the fluorescent (Fluorescein, FL) substrate by the peroxyl radicals produced *in situ*, has been quite widely used to assess the free radical absorption activity of pure compounds, fruit and vegetable extracts, wines, and biological fluids (Prior and Cao, 1999). The concentration interval leading to a linear relationship between the net area under the curve (AUC) and the antioxidant concentration have been determined for all lignan samples (Figure 23). Within this interval, any antioxidant concentration is found to give the same oxygen radical absorbance capacity (ORAC-FL) value (Table 16). As expected, the ORAC-FL value of sesamol is 4.4 μmol of trolox equivalents/ml, which is highest among all the 3 lignans. Sesamin and sesamolin show 0.8 and 1.52 μmol of trolox equivalents/ml of ORAC- FL value. Positive

control, BHT, shows a TE value of 2.36 μmol of trolox equivalents, which is lesser compared to sesamol.

Table 16. Relative antioxidant activity by ORAC and FRAP assay of sesame lignans

Serial no.	Compound	ORAC- FL values (μmoles of TE*)	FRAP values (μmoles of TE*)
1	Sesamol	4.4 ± 0.2	1.83 ± 0.08
2	Sesamin	0.8 ± 0.09	0.06 ± 0.002
3	Sesamolin	1.5 ± 0.08	0.102 ± 0.08
4	Trolox	1	1
5	BHT	2.36 ± 0.06	0.6 ± 0.01

*TE: Trolox equivalent value. Values are represented as mean ± SE of three different experiments.

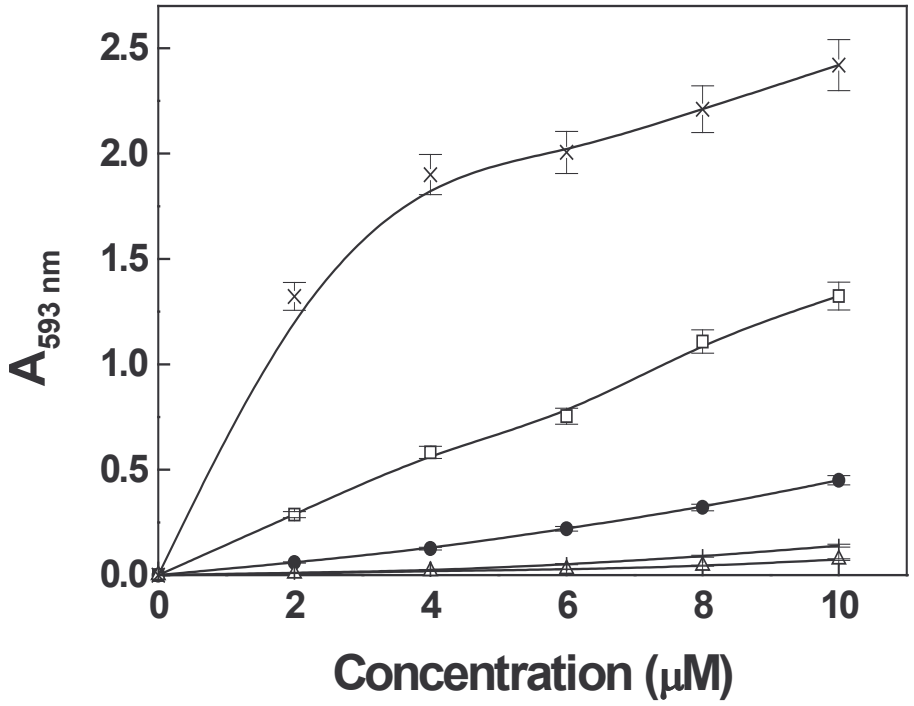


Figure 22. Ferric Reducing Ability Power assay for sesame lignans. All the lignans were taken at 200 μg ml^{-1} concentration. Sesamol (×), sesamin (△), sesamolin (|), trolox (□), BHT (●) at different concentrations.

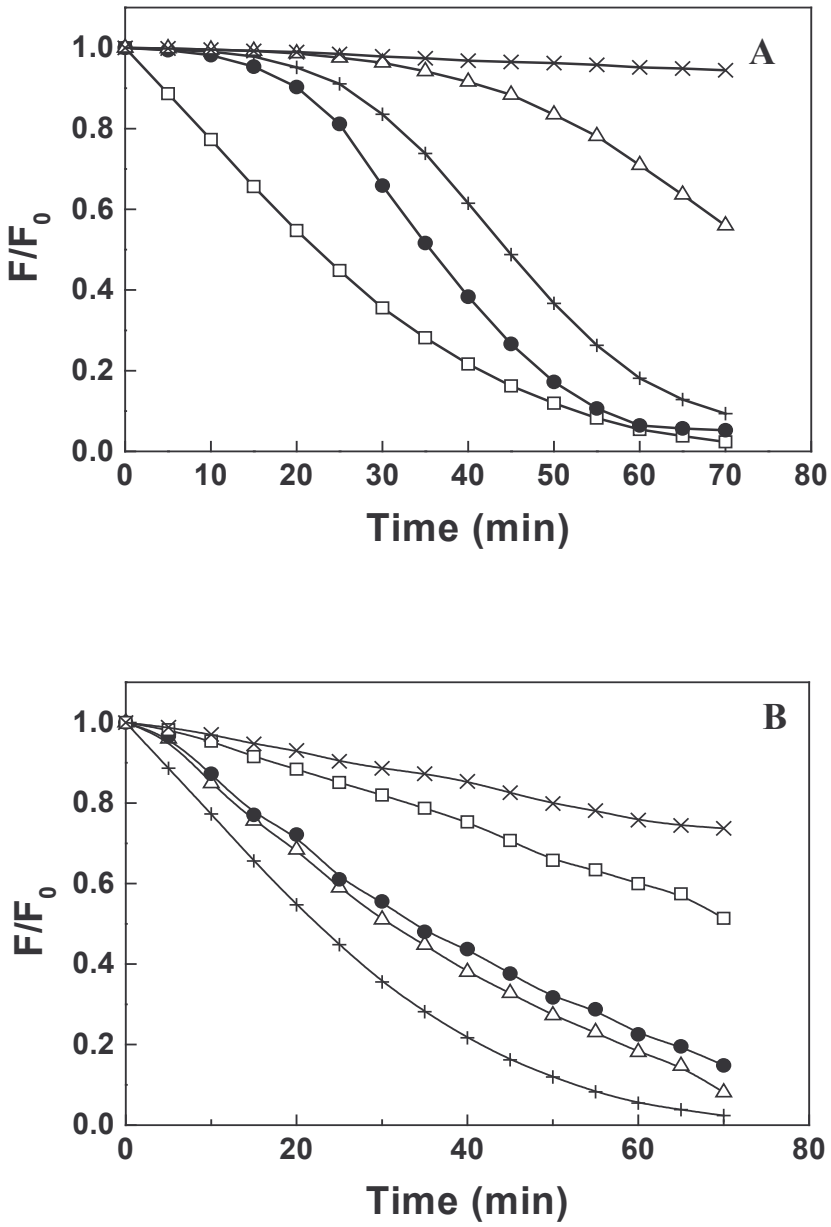


Figure 23. Time-course of the consumption of fluorescein (FL) (70nM) incubated at 37°C in the presence of 10 mM AAPH in absence and presence of (A) Trolox (B) Sesamol. FL fluorescence (493 and 515 nm, for the excitation and emission wavelengths, respectively) decay curve induced by AAPH in the presence of free radical scavenger. F is the relative fluorescence intensity obtained in presence of free radical scavenger; F_0 is the initial fluorescence of fluorescein in the absence of sesamol or trolox. **(A)** (□) Control, (●) 2 µM, (+) 4 µM, (△) 5 µM (×) 10 µM. **(B)** (+) Control, (△) 2 µM (●) 4 µM, (□) 5 µM, (×) 10 µM.

Synergistic antioxidant effects of sesamol and sesamin with γ-tocopherol.

The synergistic effect of γ-tocopherol with sesamol and sesamin were studied by DPPH radical scavenging. The radical scavenging ability of γ-tocopherol is enhanced by three times in presence of sesamol or sesamin. γ-tocopherol alone has the IC_{50} value of 4.5 μg/ ml and in presence of 10 μg of sesamin IC_{50} value decreases to 2.74. In presence of 2 μg sesamol, IC_{50} value is found to be 1.6 μg which is more than 3 times more potent of γ-tocopherol alone (Figure 24). Sesamin has very less antioxidant activity *in vitro* (Figure 19), but it enhances the antioxidant potency of γ-tocopherol.

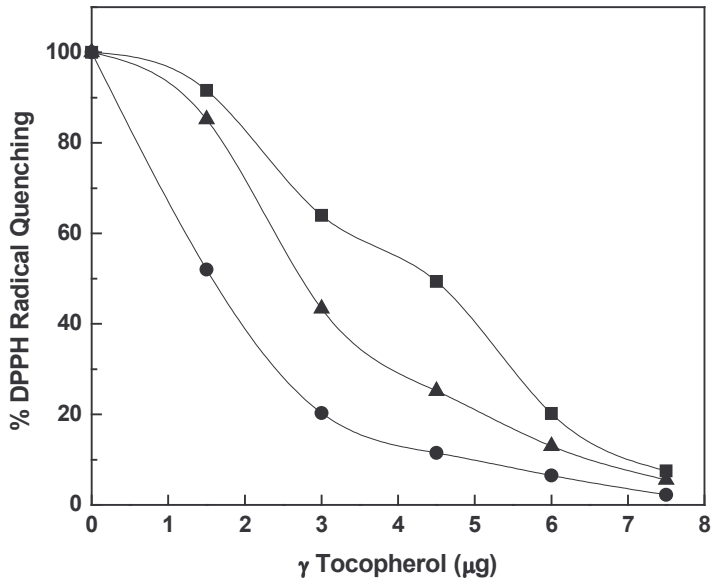


Figure 24. Synergistic antioxidant effects of sesamol and sesamin with γ-tocopherol by DPPH radical scavenging method. (—■—) γ-tocopherol alone; (—●—) γ-tocopherol + 2μg sesamol; (—▲—) γ-tocopherol + 10 μg sesamin.

Inhibition of browning in fruit pulps

Many of the molecules that are antioxidant in nature find application as inhibitors of browning reactions. In the food industry, browning reactions lead to great economic loss due to the deterioration in sensory characteristics. We studied the effect of sesamol in inhibition of browning in fruit pulps. Apple, banana and potato pulps have been used for the study. Sesamol is found to inhibit the browning reactions to the extent of 60 – 65% (Figure 25).

Antibacterial activity

The minimum inhibition concentration (MIC) was calculated by the formula given under materials and methods. Viability test was conducted on three food borne pathogens: *Bacillus cereus, Staphylococcus aureus* and *Pseudomonas aeruginosa.* Figure 26 shows the results of lignans concentration against % inhibition bacterial growth which was carried out to determine MIC value. The results showed that as the concentration of the lignans increases the viability of the organisms decreases gradually in dose dependent manner. The minimum Inhibitory concentration for the sesamol was 2 mg/ml against *Bacillus cereus* and *Staphylococcus aureus*, but it inhibited only 80% of growth of *Pseudomonas aeruginosa* at 2 mg/ml concentrations. Sesamin and sesamolin showed relatively less antimicrobial activity. Sesamin inhibited 69, 69 and 59 % of growth at as high as 2 mg/mL

concentration, 61, 62 and 53 % of growth inhibition as against sesamolin at that same concentration, against *Bacillus cereus, Staphylococcus aureus* and *Pseudomonas aeruginosa,* respectively (Figure 26). Further investigation was carried out to determine antibacterial activity by using agar well diffusion assay. A clear zone of inhibition by the sesame lignans, particularly sesamol at 1 mg/ mL concentration, against *Bacillus cereus* was shown in Figure 27.

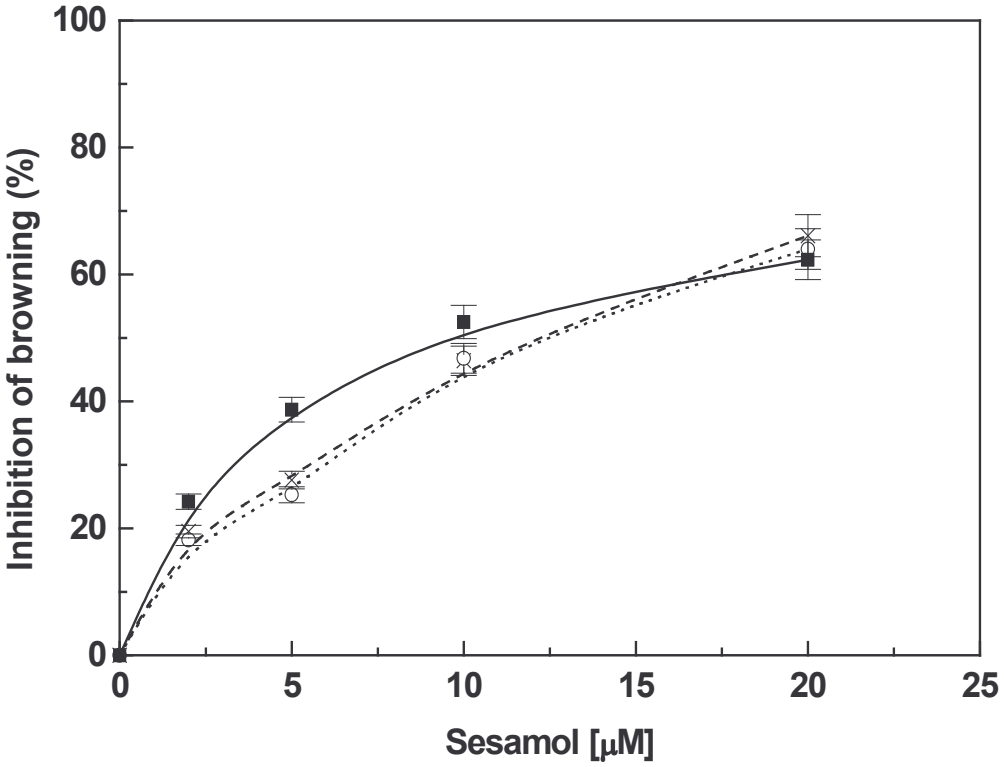


Figure 25. Inhibition of browning by sesamol in fruit pulps. Sesamol (0 – 20 μM) was added to the pulps and the browning studied after a period of 24 h at 27°C. The pulps were stored in a petridish covered by cellulose film. (■) apple, (○) potato, (×) banana

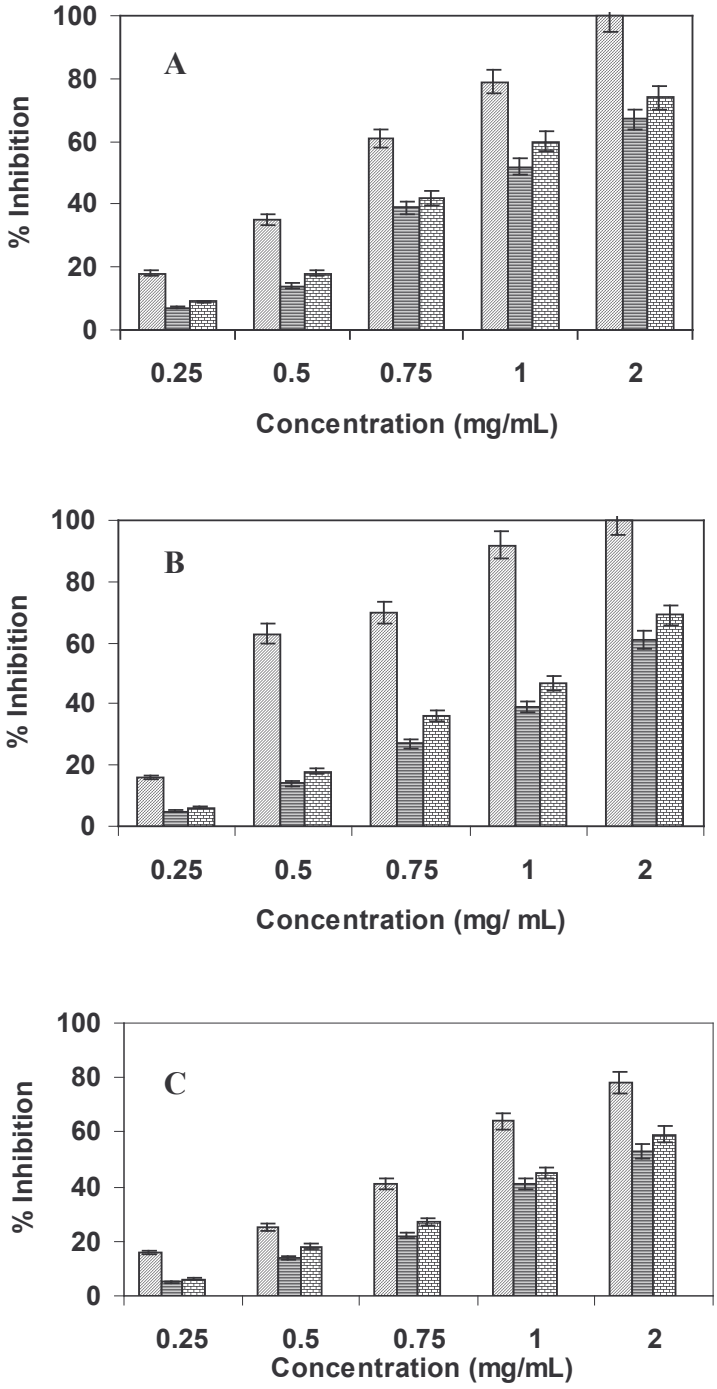


Figure 26. Effect of sesame seed lignans on growth of different bacteria at different concentrations (A) *Bacillus cereus*, (B) *Staphylococcus aureus* and (C) *Pseudomonas aeruginosa*. Sesamin ( ), Sesamolin ( ) Sesamol ( ). Data are presented as means ± SE (n= 4)

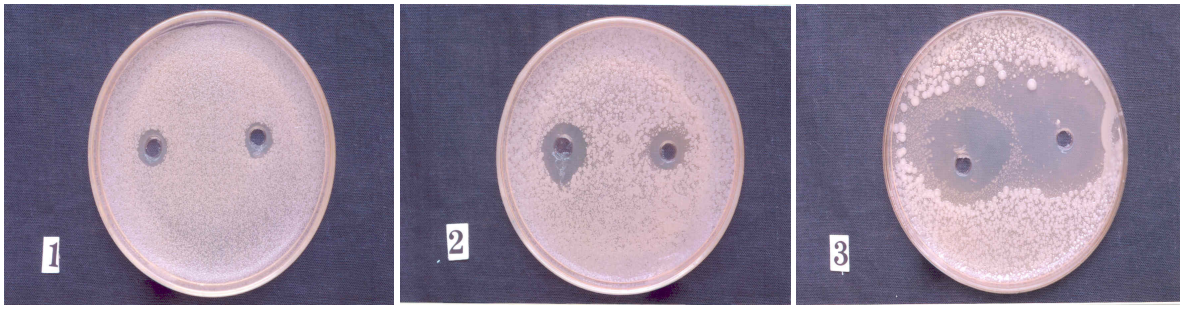


Figure 27. Inhibition of bacterial growth by sesame lignans on *Bacillus cereus*. Plates with Zone of inhibition: (1) sesamolin (2) sesamin and (3) sesamol

In vitro bioavailability of lignans

The bioavailability and stability of sesamol, sesamolin and sesamin to gastrointestinal digestion was evaluated by treating the lignans with gastrointestinal proteases - pepsin and pancreatin. There was little change in the lignan concentration after treatment with pepsin as seen by RP- HPLC profile (Figures 28-30). There was 82, 70.2 and 68.4 % recovery of sesamol, sesamin and sesamolin, respectively, after gastric digestion. Pancreatin treatment, however, showed partial digestion of lignans but the % recovery of lignans in the 'IN' fractions of all three molecules, accounts 45.2, 59.6 and 44.9 for sesamol, sesamin and sesamolin, respectively. Sesamin (>90 % of gastric digest) was absorbed more into the dialysis tube compared to sesamol and sesamolin. However, sesamin was found to be digested to a far greater extent than sesamol. Sesamin recovery in the 'IN' sample was higher, indicating its serum bioavailability to be more than sesamol and sesamolin. Sesamol concentration in the 'OUT' sample was 25.9 %, which is more than sesamin (3.8 %) and sesamolin (9.8%) for the colon bioavailability (Table 17). Total recovery of lignans in 'IN' and 'OUT' sample suggested that sesamol is more bioavailable compared to sesamin and sesamolin.

This methodology has been adapted for determining the potential bioavailability of anthocyanins and other phenolics in different fruit products (McDougall, et. al., *2005*). These studies have found a good

correlation with the bioavailability observed. Nearly 70-82 % of phenols were found to be bioavailable.

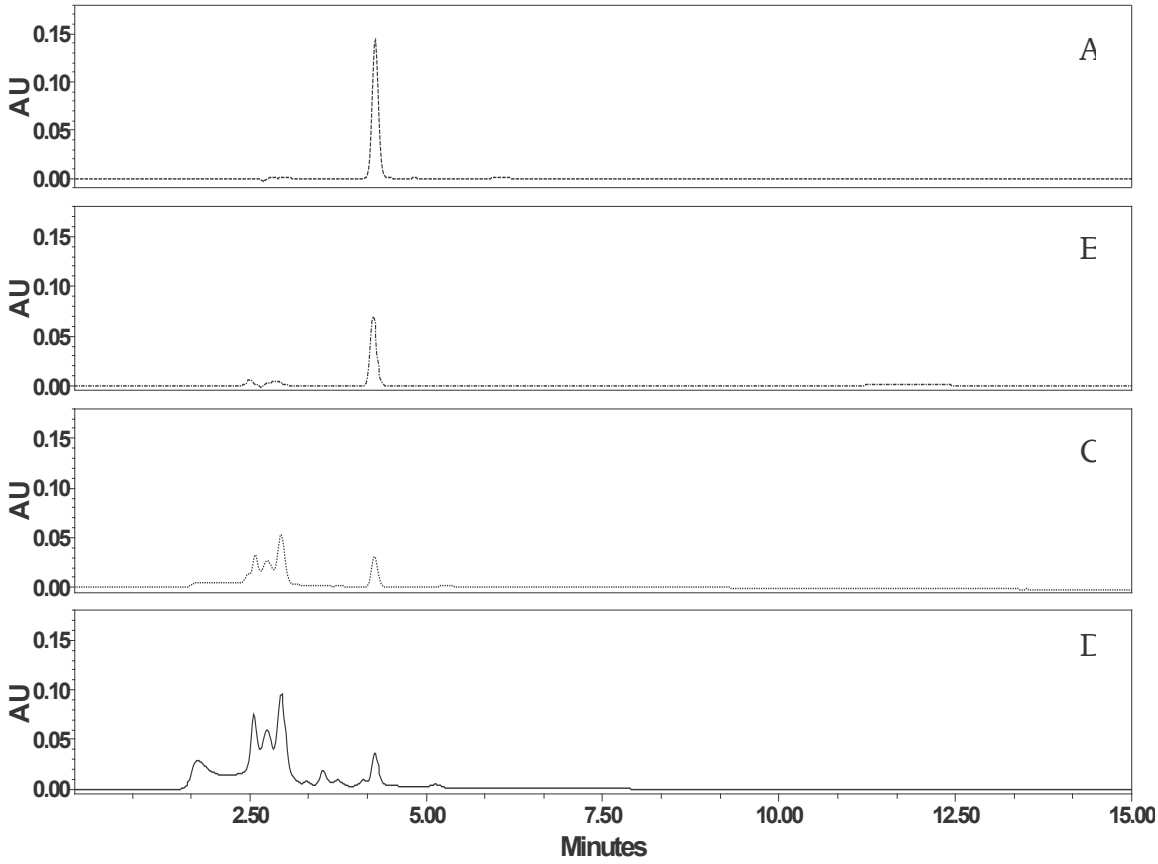


Figure 28. HPLC profile for bioavailability of sesamol. Peak was detected at 4.23 min. The experiment was performed to assess *in vitro* bioavailability and HPLC graphs are as follows sesamol standard (**A,** dashed line); sesamol gastric acid digest (**B,** dash and dotted lines); sesamol –IN- sample (**C,** dotted lines); sesamol –OUT- sample (**D,** solid line). HPLC was carried out with C_{18} column using 70 % methanol with isocratic elution of solvent at 1 mL/ min. peaks were detected at 290 nm.

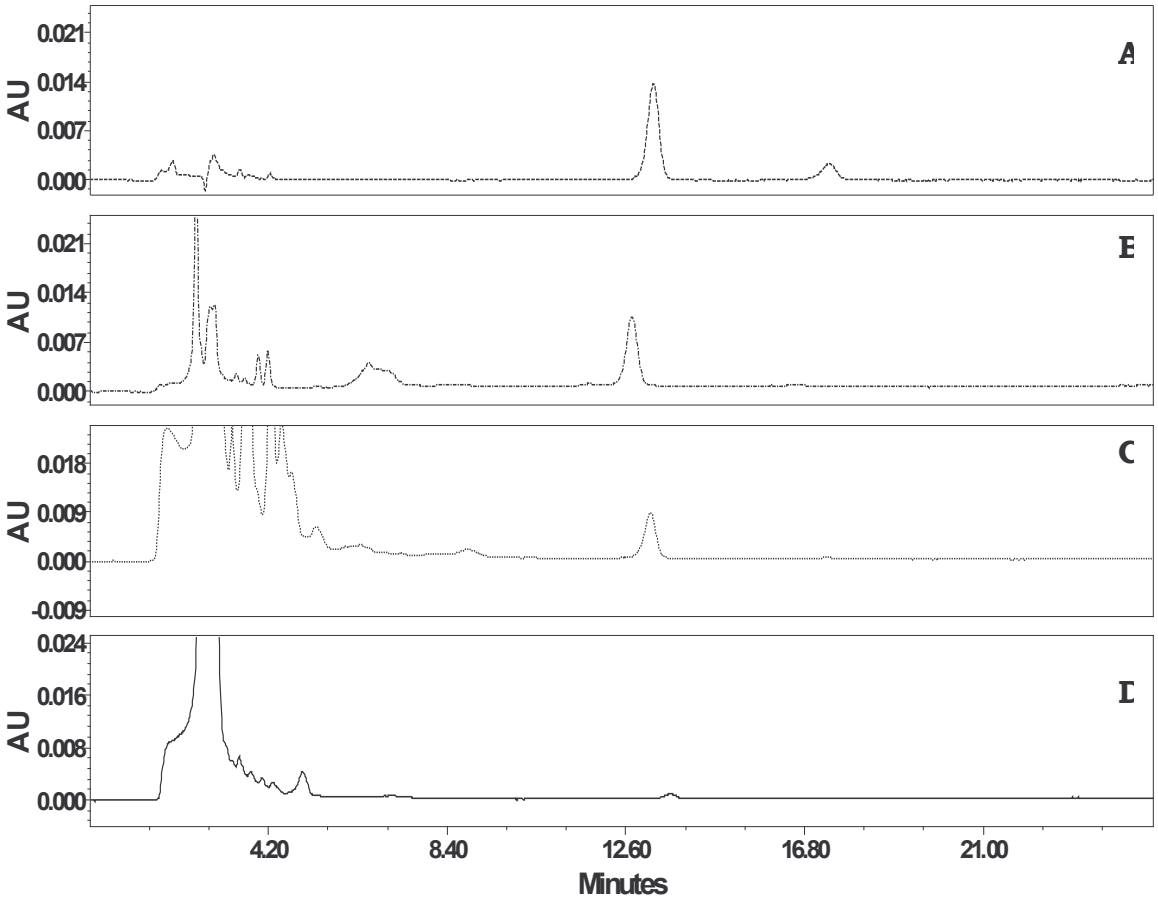


Figure 29. HPLC profile for bioavailability of sesamin. Peak was detected at 13.25 min. The experiment was performed to assess *in vitro* bioavailability and HPLC graphs are as follows sesamin standard (A, dashed line); sesamin gastric acid digest (B, dash and dotted lines); sesamin –IN- sample (C, dotted lines); sesamin –OUT- sample (D, solid line). HPLC was carried out with C_{18} column using 70 % methanol with isocratic elution of solvent at 1 mL/ min. peaks were detected at 290 nm.

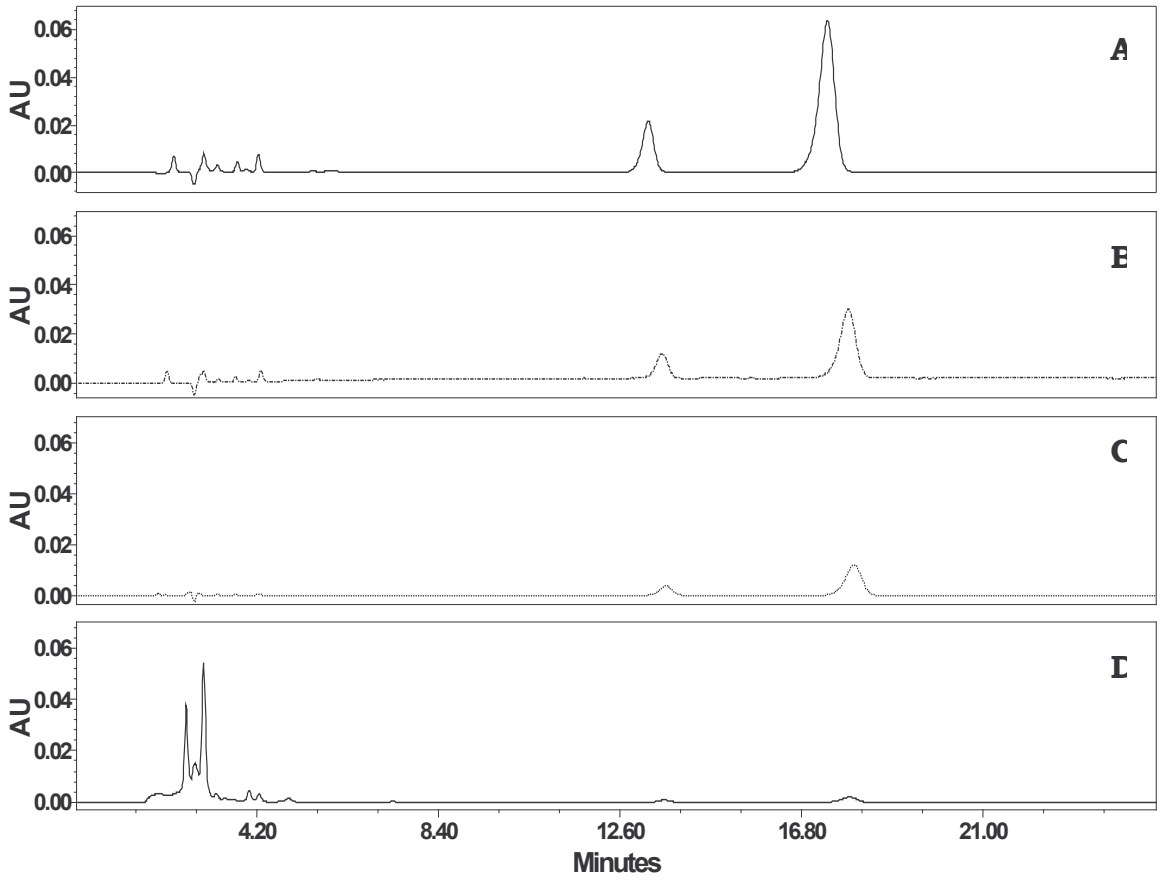


Figure 30. HPLC profile for bioavailability of sesamolin. Peak was detected at 17.39 min. The experiment was performed to assess in vitro bioavailability and HPLC graphs are as follows sesamolin standard (A, dashed line); sesamolin gastric acid digest (B, dash and dotted lines); sesamolin –IN- sample (C, dotted lines); sesamolin –OUT- sample (D, solid line). HPLC was carried out with C_{18} column using 70 % methanol with isocratic elution of solvent at 1 mL/ min. peaks were detected at 290 nm.

Table 17. Recovery of lignans in bioavailability procedure[a]

	Sesamol (µg/ mL)	Sesamin (µg/ mL)	Sesamolin (µg/ mL)
Original (pre digestion)	1000	1000	1000
Gastric Acid digest	815 ± 22	702 ±24	684 ±18
IN	452 ±14	595 ± 15	449 ± 16
OUT	259 ±08	38 ± 04	98 ± 07

[a] Values are represented as mean ± SD of three different experiments.

Discussion

The formation of ROS is prevented by an antioxidant system: antioxidants (ascorbic acid, glutathione, tocopherols), enzymes regenerating the reduced forms of antioxidants, and ROS-interacting enzymes such as SOD, peroxidases and catalases. In plants, many phenolic compounds (in addition to tocopherols) are potential antioxidants: flavonoids, tannins and lignin precursors may work as ROS-scavenging compounds (Blokhina, 2003). Studies have suggested the beneficial effects of antioxidant nutrients such as vitamin E, green tea extract, ginkgo biloba extract, resveratrol and niacin in cerebral ischemia and recirculation brain injury. These results are important in the light of an attenuation of the deleterious consequences of oxidative stress in ischemia and recirculation injury. For instance, silymarin is a polyphenolic flavanoid derived from milk thistle that has anti-oxidant, anti-inflammatory, cytoprotective and anticarcinogenic effects (Dehmlow, 1996). Silymarin significantly reduces the LPS-induced nitrite, iNOS mRNA and protein levels in microglia. It also reduces the LPS induced superoxide generation and nuclear factor kB (NF- kB) activation.

In the present studies sesamin, sesamolin and sesamol have shown a wide range of antioxidant activities from prevention of lipid peroxidation to free radical scavenging. They also exhibit free antimicrobial activities especially with food borne pathogenic bacteria.

Sesamin and sesamolin, with two methylenedioxy bridges, have potentially 4 functional OH groups and scavenge ROS in cell-free condition better than sesamol, with 2 OH groups. This suggests that the higher inhibition of LPS-induced NO production by sesamin and sesamolin may be due to the difference in numbers of functional OH group (Hou, 2003). A water soluble form of sesamol, 1, 2, 4-benzenetriol, has a similar potency in inhibiting LPS-induced NO production. The Methylenedioxy bridge of sesamin and sesamolin is formed by a redox reaction of cytochrome P450 in the presence of O_2 and NADPH. Sesamin is metabolized in the liver and converted to an antioxidative form that inhibits superoxide production in aortic endothelium (Nakano, 2002).

Sesame lignans are known to inhibit the course of fatty acid metabolism *in vivo* in animal models and also in microorganisms by specifically inhibiting the rate limiting enzyme Δ5 desaturase (Fujiyama-Fujiwara et al 1992). In the present study, all the 3 lignans have exhibited inhibition of lipid peroxidation *in vitro,* resulting in prevention of β-carotene bleaching and also formation of linoleate peroxyl radical. Ghafoorunissa et al (2004) showed the synergistic effect of lignans on lipid peroxidation in rat liver and mitochondrial microsomes in presence of tocopherols. The order of inhibitory effects of different lignans in combination with *α*-tocotrienol is as follows: sesamol > sesamin > sesamolin (Ghafoorunissa et al 2004).

Sesamin is known to act as insecticide and used in combination of pyrethrin insecticides (Nascimento 2004; Beroza M 1954). Wynn et al. (1997) have reported that sesamol is a potent inhibitor of *Mucor circinelloides* inhibits the microbial growth upon assimilation. These lignan molecules, once assimilated, will be metabolized in to catachol-like compounds, which could inhibit the growth of bacterial colonies.

The *in vitro* digestion procedure provides a simple, rapid, and upgradeable screening method to assess the potential stability of phytochemicals from fresh, extracted as well as processed foods. In the present study, sesame lignans and sesamol show considerabl stablility under *in vitro* conditions that mimic those of the upper gastrointestinal tract. Sesamol and sesamin being the most stable lignans withstand up to 80 and 70% of the gastric digestion. Sesamin's most absorption to the IN sample suggests that its absorption in the intestinal tract may explain the rapid but transient increase in serum sesamin noted in animal and human studies

On the basis of the results of this study, it is clearly indicated that sesame lignans have significant antioxidant activity against various lipid peroxidation systems *in vitro* and also synergistically enhance biological activity of other molecules, such as tocopherols. Their bioavailability *in vitro* is also makes them as important nutraceutical molecules.

The various antioxidant and antimicrobial activities of sesamol sesamin and sesamolin are summarized in Table 18.

The next section deals with the effect of sesame lignans and sesamol on the activity of tyrosinase and the growth and survivability of cultured mouse melanoma B16F10 cells. A mechanism for the inhibition of tyrosinase activity by sesamol is proposed.

Table 18. Summary of the non enzymatic bioactivities studied for sesame lignans*

Bioactivity	Sesamin	Sesamolin	Sesamol
Non enzymatic assays			
DPPH radical scavenging	–	+	+++
Ferric Reducing Ability Power	++	+	+++
Antioxidant assay by thiocyanate method	+++	+++	+++
Antioxidant assay by β-carotene linoleate model	+++	+++	+++
ORAC	+	+	+++
Anti bacterial activity	+	+	+++

* +++ = Highly active
++ = Moderately active
+ = Less active
– = No activity

Results and Discussion

Section 3 – Inhibition of tyrosinase by sesamol: Effect on melanin and melanin synthesizing melanoma cells

In the present study, we have looked into the nature and mechanism of inhibition of tyrosinase activity by sesamol, a phenolic compound formed in roasted sesame oil. The effect of sesamol on tyrosinase activity from mushroom has been used as a model to establish its mode of action in comparison to mammalian tyrosinase. This is probably, one of the early reports of the mechanism of tyrosinase inhibition caused by lignans which may help in the use of these naturally occurring lignans.

Tyrosinase, an oxidase, is the rate limiting enzyme for the controlled production of melanin. It is mainly involved in two distinct reactions of melanin synthesis - the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone. *o*-Quinone is transformed through a series of non – enzymatic reactions to melanins.

Effect of sesame lignans on mouse melanoma B16F10 cells

The lignans – sesamin and sesamolin as well as the phenolic degradation product of sesamolin - sesamol from sesame seed, known for their health promoting properties, were studied for their effect on

melanin synthesis using mouse melanoma B16F10 cells. Cultured B16F10 (1×10^4 cells/well) cells were treated with sesamol, sesamolin and sesamin, at 25, 50 and 100 μg mL^{-1}, for 72 h. The ability of the cells to reduce MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) to a blue formazan product was measured to determine cell survivability. Cells treated with sesamolin were not affected. However, sesamol inhibited 50% cells at 100 μg/ mL concentration. The effect appeared to be mediated through inhibition of tyrosinase activity, since loss in viability was proportional to the reduction in melanin pigment produced $cell^{-1}$ (Table 19). Sesamol inhibited the synthesis of melanin by 63%, while sesamin and sesamolin showed negligible effects (Table 19). Only sesamol and sesamin were used for dose depending studies on melanin synthesis. Reduction in melanin content of the cells was associated with the concomitant loss of viability and proliferation indicating a role of melanin in B16F10 cell survival (Table 20). Factors that reduce melanin were, therefore, believed to inhibit melanoma cell growth.

Table 19. Total melanin content in B16F10 melanoma cells following treatment with sesame lignans

	Melanin content (%)	No. of Viable cells	(%) Viable cells
Control	100	92	100
Sesamol	37	57	61.5
Sesamin	95	81	88
Sesamolin	99	90	97.8

Treatment with sesamol sesamin and sesamolin at 100 μg ml^{-1} for 72 h; data are expressed as percentage of melanin content per well observed with the control and each column represents the mean ± S.D. of 4 determinations.

Table 20. Relationship between melanin decreases with concentration of sesame lignans.

	Melanin content decreased (%)	
Concentration (μg ml^{-1})	Sesamin	sesamol
0	0	0
25	3	18.1
50	5.8	29.4
75	8.4	42.7
100	12.1	63.4

Treatment with sesamol and sesamin for 72 h; data are expressed as percentage of melanin content per well observed with the control and each column represents the mean ± S.D. of 4 determinations.

Effect of sesame lignans on cell apoptosis

Sesame lignans, sesamin and sesamol, were studied for their apoptotic properties on metastatic mouse melanoma B16F10 cells. Viable cell nuclei, staining green with acridine orange and apoptotic cell nuclei, staining red with ethidium bromide, were counted. Cells treated with sesamin showed no symptoms of death, as they were well differentiated and remained adhering to the cover slip similar to those of untreated cells (Figure 31). However, cells treated with sesamol showed visible changes, such as spherical shape and lifting of cells, indicative of cell death. Nearly 40% of apoptotic cells were observed with distinct morphological changes as evident from nuclear/ chromatin structures, increase in cell volume and membrane disruption, cell membrane blebbing and intracellular bridges, oozing of cytoplasmic contents and formation of apoptotic bodies (Figure 31). These observations were substantiated by the appearance of 40 to 50% apoptotic cells as evaluated by acridine orange and ethidium bromide staining methods

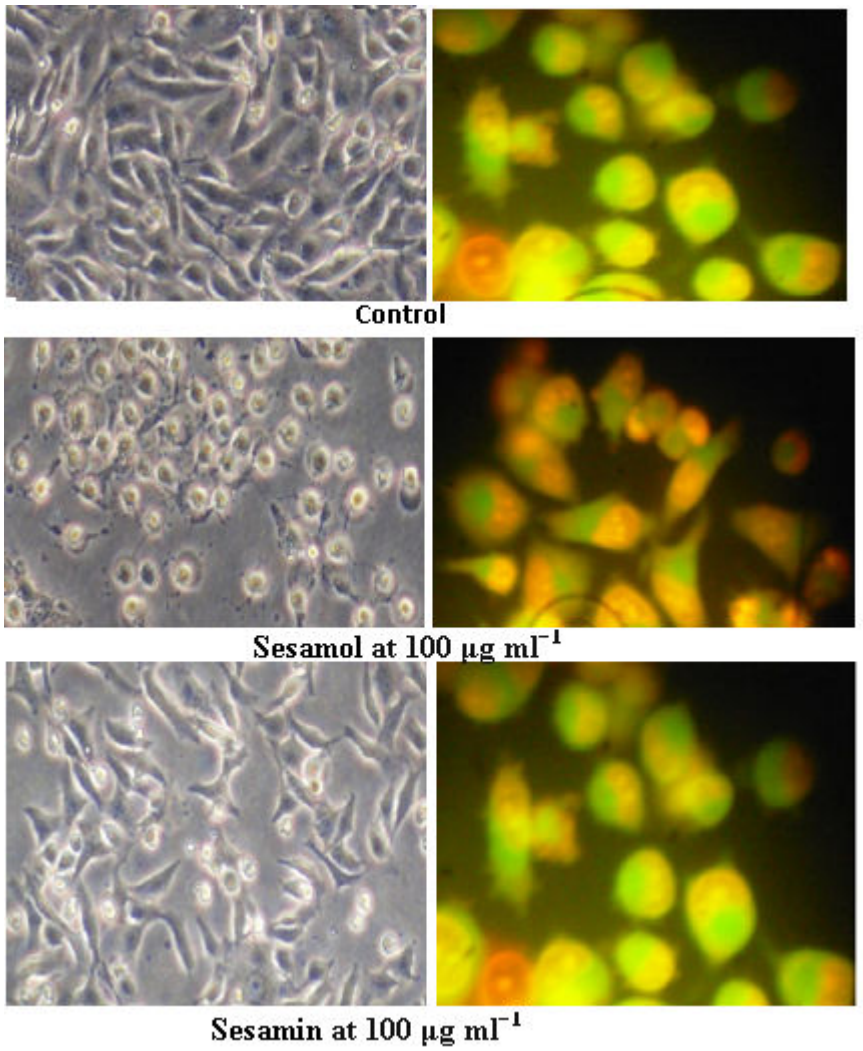


Figure 31. Microscopic observation of cell culture suspension of melanoma cells which were mixed with 1 µL of dye mix containing 100 µg mL^{-1} each acridine orange and ethidium bromide and observed under the microscope at 40×. Viable cell nuclei stained green with acridine orange and apoptotic cell nuclei stained red with ethidium bromide were counted. **A**. Control; **B**. Sesamol treated cells; **C**. Sesamin treated cells.

Effect of sesame lignans on mushroom tyrosinase

In order to understand the mechanism of inhibition of tyrosinase, mushroom tyrosinase has been used as model. There are two activities associated with tyrosinase – monophenolase and diphenolase activity; either of which or both activities could be inhibited. The scavenging of oxygen or chelation of copper, a cofactor of tyrosinase, can also result in the inactivation of tyrosinase. Investigations on the inhibition of both diphenolase and monophenolase activities of mushroom tyrosinase indicated that sesamol was a powerful inhibitor of diphenolase and monophenolase activities, in a competitive and non-competitive manner, respectively.

Inhibition of monophenolase and diphenolase activity

Mushroom tyrosinase used for the study had a monophenolase activity of 142 U/ mg protein and a diphenolase specific activity of 122 U/ mg protein. The effect of sesame lignans – sesamolin, sesamin and sesamol – on the monophenolase and diphenolase activities of mushroom tyrosinase indicated only sesamol is very effective in inhibiting both monophenolase and diphenolase activities.

Sesamol is a powerful inhibitor of mushroom monophenolase activity with IC_{50} of 3.2 μM (Figure 32 A & B) and a corresponding K_I value of

1.4 μM (Figure 32D). Tyramine hydrochloride with addition of MBTH showed a better catalytic constant compared to tyrosine. The presence of MBTH decreases the lag phase during tyramine hydroxylation. Addition of sesamol increased the initial lag phase with a concomitant decrease in the reaction velocity (Figure 32A). The Lineweaver Burk plot indicates (Figure 32C) that sesamol is a non-competitive inhibitor of monophenolase activity. The V_{max} value decreases at higher concentrations of sesamol without any change in the K_m.

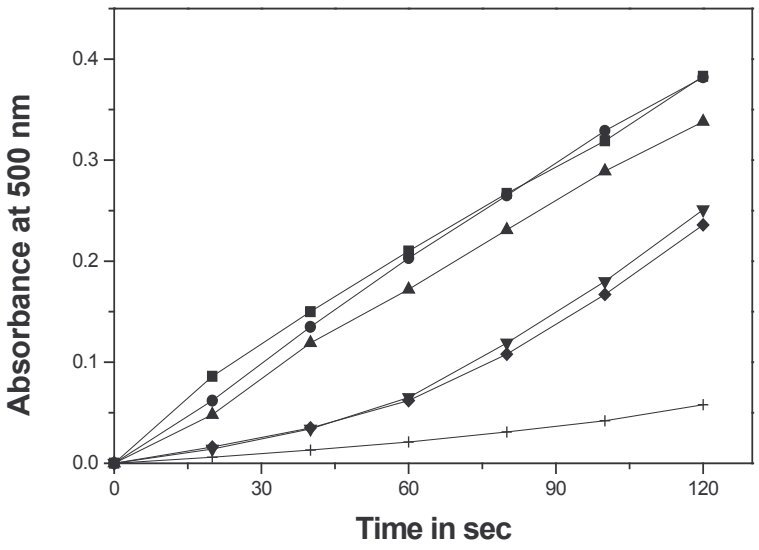


Figure 32 A. Progressive curves of the inhibition of monophenolase activity of mushroom tyrosinase by sesamol. Sesamol was incubated with tyrosinase for 3 min in 0.1 M phosphate buffer (pH 6.8) before assaying for monophenolase activity with 1 mM L - Tyramine hydrochloride as substrate. The reaction medium contained 2 mM MBTH as a neutrophile. Formation of products with MBTH adduct was followed at 500 nm. The concentration of sesamol were 0 (–■–), 2 (-•-), 4 (-▲-), 6 (-▼-), 8 (-♦-) and 10 μM (-+-).

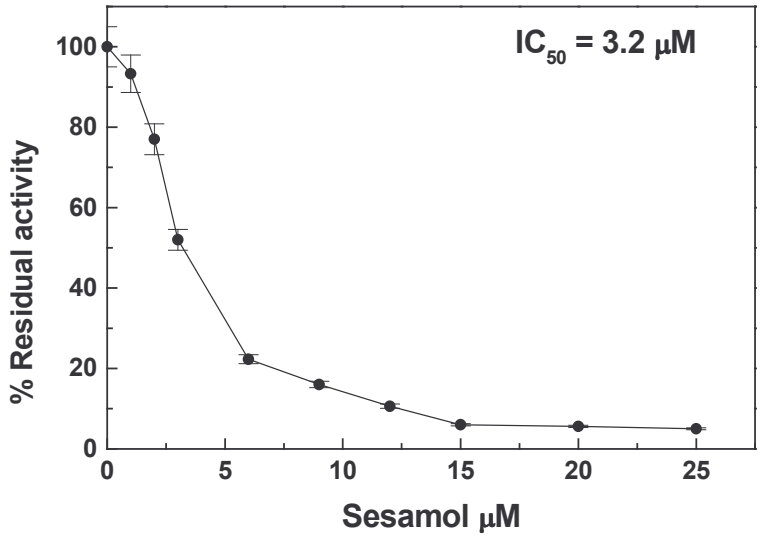


Figure 32 B. Effect of sesamol concentrations on the monophenolase activity of mushroom tyrosinase for the oxidation of tyramine as a substrate. The reaction media contained 1 mM of substrate. 2 mM MBTH was used as a neutrophile in a final concentration of 0.1 M phosphate buffer, pH 6.8.

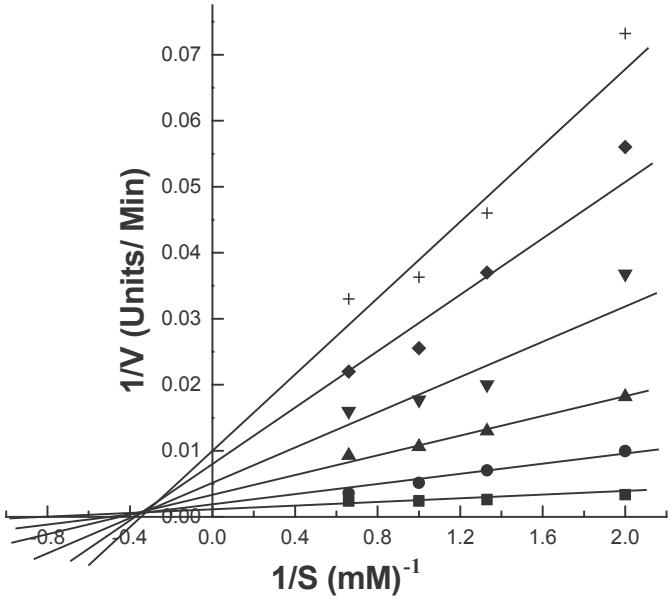


Figure 32 C. Linweaver- Burk plots for inhibition of sesamol on mushroom tyrosinase for the catalysis of tyramine hydrochloride (substrate concentration was 0.5- 1.5 mM) at 25° C and pH 6.8. Concentrations of sesamol were 0 - 10 μM. The enzyme concentration was 1.9 μg. Data were obtained as mean values of 1/V of 3 independent tests.

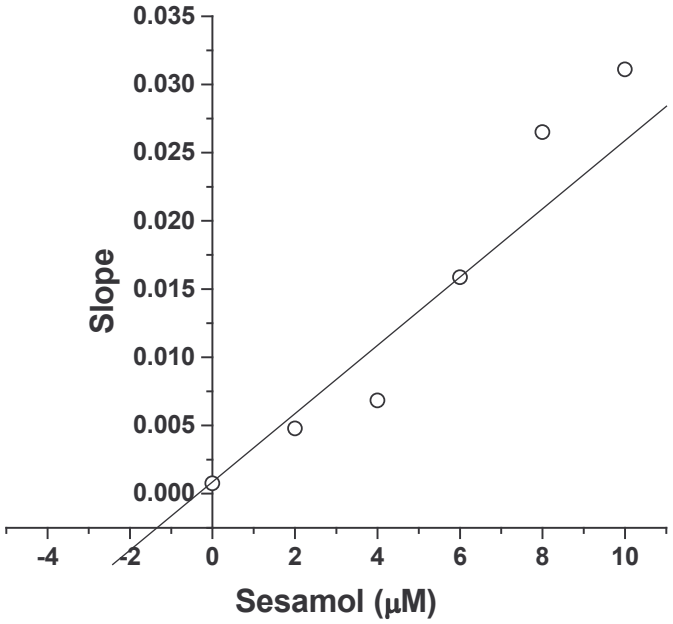


Figure 32 D. Determination of K_I of sesamol on monophenolase activity. The slope (K_m / V_{max}) of the lines described by the double reciprocal plot is plotted against the sesamol concentration in order to determine K_I values of sesamol. All values are the average of three experiments.

A progressive curve of the inhibition of diphenolase activity of mushroom tyrosinase by sesamol shown in Figure 33 A. Sesamol is a competitive inhibitor of diphenolase activity (Figure. 33 C). The IC_{50} for inhibition was 1.92 μM (figure 33 B) with a corresponding K_I of 0.57 μM (Figure. 33 D). In comparison to known inhibitors of tyrosinase activity, this is one of the most potent inhibitor of tyrosinase activity reported. Only cupferron, which binds to the copper containing active site, was 4-fold better than sesamol (Chang, 2009).

Corresponding K_I values were 1.4 μM and 0.57 μM for monophenolase and diphenolase activities, respectively, comparable to the known standard, quercetin and kojic acid, respectively, which inhibited with an IC_{50} value of 130 and 22 μM, respectively. Sesamol also affected the state of copper in tyrosinase as evidenced by spectroscopic studies.

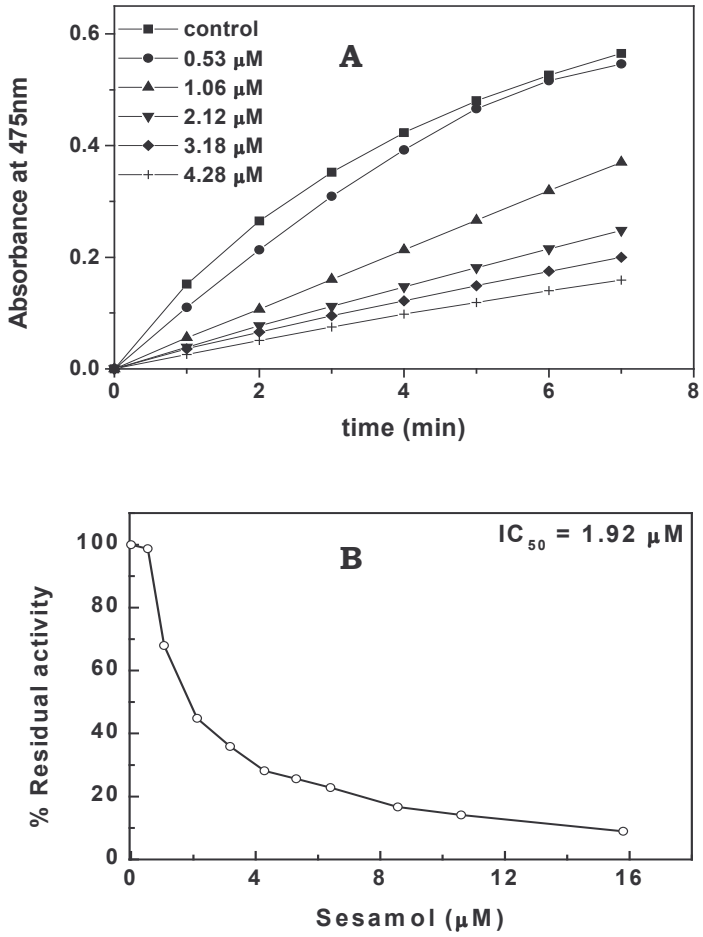


Figure 33 A. Progressive curves of the inhibition of diphenolase activity of mushroom tyrosinase by sesamol. Sesamol was incubated with tyrosinase for 3 min in 0.1 M phosphate buffer (pH 6.8) before assaying for diphenolase activity with 2 mM L- DOPA as substrate.

B. Effect of sesamol concentration on the activity of mushroom tyrosinase for the oxidation of L- DOPA.

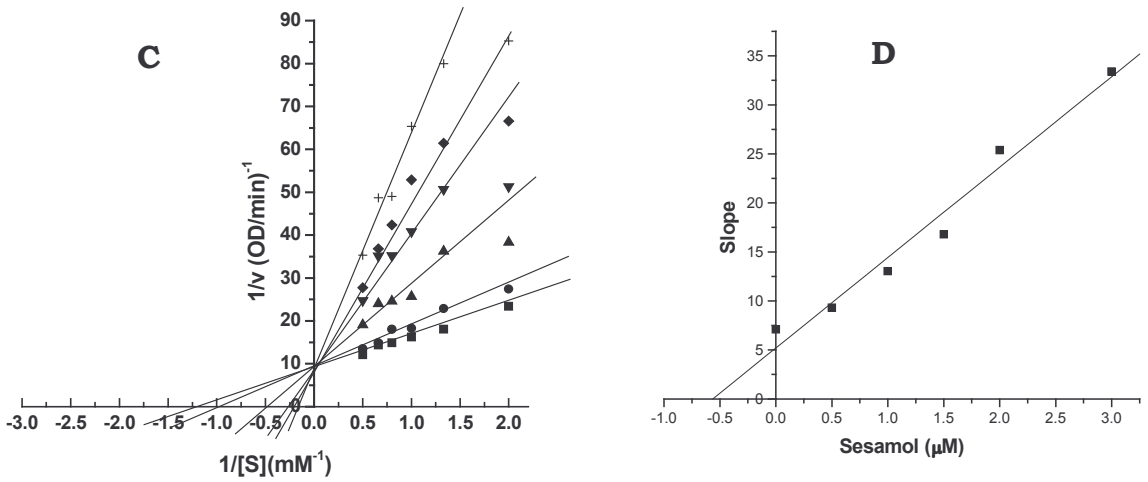


Figure 33 C. Linweaver- Burk plots for inhibition of sesamol on mushroom tyrosinase for the catalysis of L- DOPA at 25° C in sodium phosphate buffer pH 6.8. Concentrations of sesamol were 0 - 3.0 μM. The enzyme concentration was 1.44 μM. Data was obtained as mean values of 3 independent tests. 1/V is defined as the inverse of the increase of absorbance at the wavelength of 475 nm per min.

D. Determination of K_I of sesamol for the diphenolase activity of the tyrosinase. The slope (K_m / V_{max}) of the lines described by the double reciprocal plot is plotted against the sesamol concentration in order to determine K_I values of sesamol. All values are the average of three experiments.

Mechanism of tyrosinase inhibition: Spectroscopic studies

Formation of a tyrosinase – sesamol complex

Tyrosinase, in phosphate buffer, absorbs at 282 nm, while sesamol in the same buffer absorbs at 296 nm. The absorption spectrum of tyrosinase, in the absence and presence of sesamol, is given in (Fig. 34). In presence of sesamol, an increase in the intensity at 291 nm is observed. There is a red shift in the peak at lower concentrations of sesamol (<200 μM), while two distinct peaks are seen at higher concentrations of sesamol (>200 μM) – one at 265 nm and the second at 320 nm. With time, a third peak at 400 nm is observed with development of yellow color. The peak intensity at 291 and 400 nm increases with time indicating a change in the tyrosinase structure and the formation of a quinone.

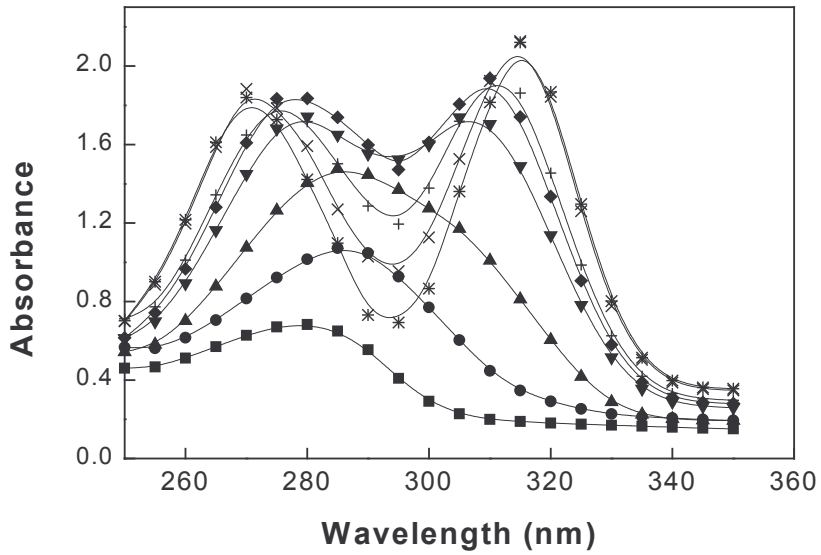


Figure 34. Absorption spectra of mushroom tyrosinase in the presence of different concentrations of sesamol. The curves represented are tyrosinase alone (–■–), tyrosinase with different sesamol concentrations, 100 μM (–•–), 200 μM (–▲–), 300μM (–▼–), 400 μM (–♦–), 500 μM (–+–) and 600 μM (–×–).

Sesamol binding to tyrosinase: effect on active site copper

In order to investigate the effect of sesamol on copper present in the active site of tyrosinase, the enzyme assay was carried out in the presence of copper sulfate (1 mM) to the sesamol-inactivated enzyme. No regain in activity was observed indicating the irreversibility in the activation of the enzyme (Figure 35).

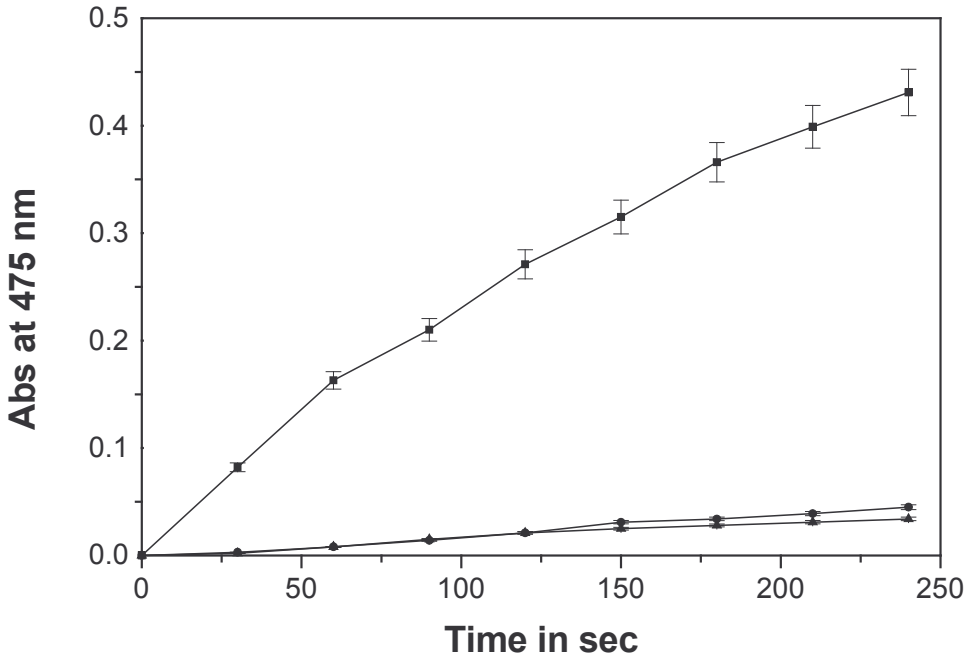


Figure 35. Effect of copper sulfate on reversibility of tyrosinase inhibition. native tyrosinase (—■—); tyrosinase activity in presence of 10 μM sesamol (—▲—) and 1 mM copper sulfate (—●—).

CD spectrum of tyrosinase in the visible region showed minima at 290 and 350 nm (Figure 36). Addition of sesamol to tyrosinase resulted in a decrease in the molar ellipticity at 350 nm, in a dose and time dependent (after 30 h of incubation) manner, with the molar ellipticity reaching zero at 290 nm and 350 nm, indicating a change in the environment around the aromatic residues and copper ligand in tyrosinase, in presence of sesamol.

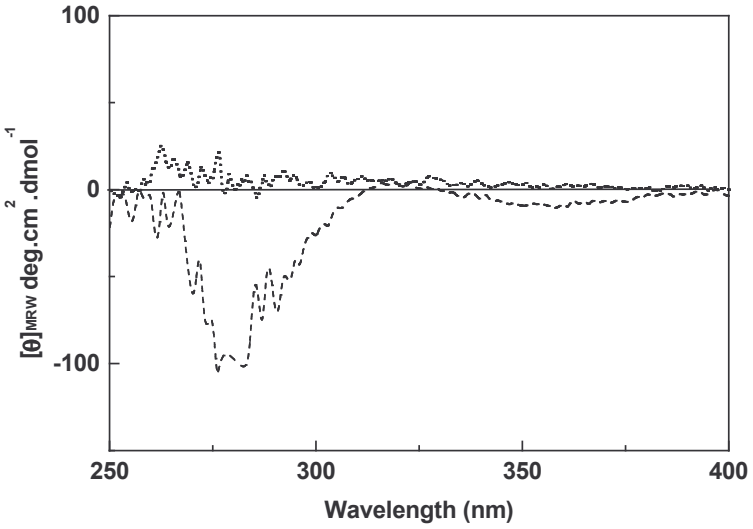


Figure 36. CD spectrum of tyrosinase in the visible region in presence (dotted and dashed lines) and absence (dotted lines) of sesamol.

Gel filtration by HPLC

The formation of a complex between sesamol and tyrosinase, if any, was followed by gel filtration on HPLC and observation for any change in the retention time. The peak at ~19.3 min shifted to 17.2 min after nearly 24 h. There was a third peak at ~27 min that is concomitant with the appearance of yellow color indicating the presence of quinone (figure not shown).

Interaction of sesamol with o-quinones

To determine whether sesamol interacts with *o*-quinones through oxido-reduction with regeneration of the corresponding *o*-diphenol or there is a formation of a new conjugate, the *o*-diphenol *t*BC has been oxidized by $NaIO_4$ in the absence and presence of sesamol and the spectra recorded (Figure 37). Sesamol alone has a peak at 295 – 300 nm and another peak at 232 nm (curve a). The oxidation of *t*BC (with λ_{max} at 279 nm, curve b) by $NaIO_4$ has given rise to the corresponding 4-tert-butyl-*o*-benzoquinone (*t*BQ) with λ_{max} at 400 nm (curve c). When sesamol is added to the *o*-quinone, the maximum shift is found from 400 nm to 470 nm (curve d). The color of the solution turns red from yellow, indicating the formation of a new conjugate (Figure. 37 inset). Increase in the absorption at 300 nm has also been observed, which is very close to the absorption of sesamol. The absorption increases with time before saturating at 2 min.

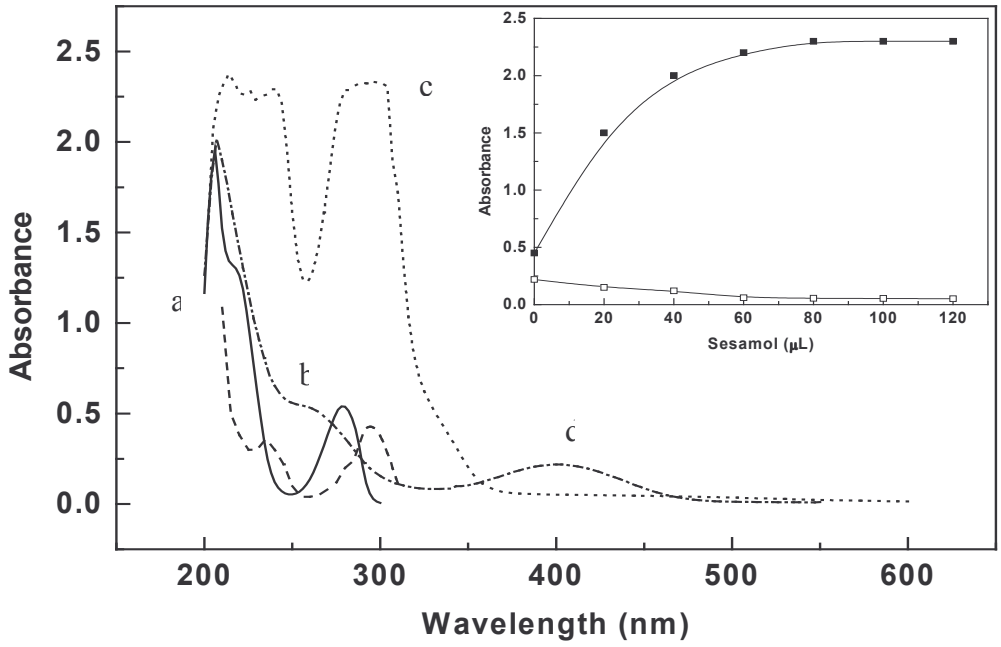


Figure 37. Interaction of sesamol with *o*-quinones. The absorption spectra of $NaIO_4$ in the absence and presence of sesamol. a, Sesamol alone in phosphate buffer pH 6.8; b, tyrosinase alone in phosphate buffer pH 6.8; c, Tyrosinase and sesamol; d, tyrosinase, sesamol and $NaIO_4$.

Discussion

The various biological properties of sesame lignans are thought to be attributable to the presence of 3, 4 - methylenedioxyphenyl group (-O-CH_2-O-) in the molecular structure (Figure. 3). Some of these biological effects result from the activation or inhibition of key enzymes involved in the different metabolic pathways. Sesamin is known to have hypocholesterolic and anti-inflammatory activity, which specifically inhibit the Δ5-desaturase activity in liver microsomes that regulates the production of prostaglandins (Shimizu, et al. 1989). Sesamol, a better antioxidant compared to sesamin or sesamolin, is an excellent free radical scavenger. The increased antioxidant activity of sesamol, compared to sesamin or sesamolin, may be due to the presence of free hydroxyl group, which can easily participate in redox reactions.

Tyrosinase is the key enzyme in the melanin synthesis pathway and its inhibitors can be expected to inhibit melanin production also. The effect of sesame lignans on B16F10 melanoma cell lines has been examined to determine whether melanin synthesis alone or cell growth too is affected in addition to alterations in catalytic activity of mushroom tyrosinase. Among the three lignans studied, only sesamol is found to inhibit melanin production, concomitant with decreased cell viability of B16F10 mouse melanoma cells. The increased melanoma B16F10 cell death is observed only in presence of sesamol that exhibited higher antioxidant activity, and not with sesamolin or sesamin (which are

having less antioxidant activity *in vitro*) suggesting that antioxidative property is responsible for melanoma cell death. Interestingly, cell death is also thought to be associated with reduction in melanin production that is essential to maintain the survivability of melanoma cells. It is intriguing to note that melanoma pigments are forced to produce in higher levels in melanoma cells, to overcome the cellular stress induced on cells, particularly on exposure to UV and cosmic radiations. The protective effect of melanin against UV induced skin damage probably gets hindered due to the presence of prooxidant short chain melanin polymers. Apoptotic effect of these lignans on mouse melanoma cells has also yielded similar results. Sesamol brings about apoptosis in mouse melanoma cells in a dose dependent manner.

To understand the role of lignans in the inhibition of melanin synthesis, the effect of sesamol on mushroom tyrosinase, a copper containing oxygenase with mono and diphenolase activities has been studied. Tyrosinase has 2 distinct catalytic functions: the hydroxylation of monophenol and the oxidation of *o*-diphenols. The inactive monophenolase is in the '*met*' state. This gets reduced by enzyme reaction with a catecholic substrate that is oxidized simulataneously to its corresponding quinone, thereby converting the enzyme to its '*deoxy*' state. In this state, the enzyme is able to bind molecular oxygen and oxidize the molecular phenol substrate. The active site of tyrosinase has two copper atoms. While the E_m form can catalyze diphenol substrates

alone, the E_o forms can catalyze both monophenol substrates and diphenol substrates; E_d can combine with oxygen (Epsin, 2001). A structural model has been proposed for the active site of these 3 forms of tyrosinase.

Sesamol is found to inhibit tyrosinase activity effectively. Melanin reduction is believed to be associated with the inhibition of tyrosinase activity. The low IC_{50} value of sesamol reveals that the compound is more potent than kojic acid (IC_{50} = 22.72 μM) that is generally considered to be the benchmark inhibitor (Ley, 2001). Neither sesamin nor sesamolin is found to inhibit tyrosinase activity.

Monophenolase activity is non-competitively inhibited by addition of sesamol. The inhibition is seen through an increase in the lag phase of the reaction. The copper environment is also affected (CD data), indicating a decrease in the conversion of the enzyme from the inactive state to its active state. The monophenolase reaction requires oxygen as one of the reactants and removal of oxygen would affect this phase. The antioxidant nature of sesamol, by its ability to quench oxygen radicals may be one of the mechanisms of inhibition. Several indirect techniques have shown that sesamol scavenges free radicals. However, the reaction is not well characterized. The presence of a methylene dioxy group is reported to be mainly responsible for the various biological activities of lignans (Joshi, 2005). The stereochemistry of the furan-phenyl bond also contributes to the activity. In case of sesamol and sesamol dimer,

the presence of a hydroxyl group with a methylene dioxy group may be responsible for the higher activity, compared to the other lignans.

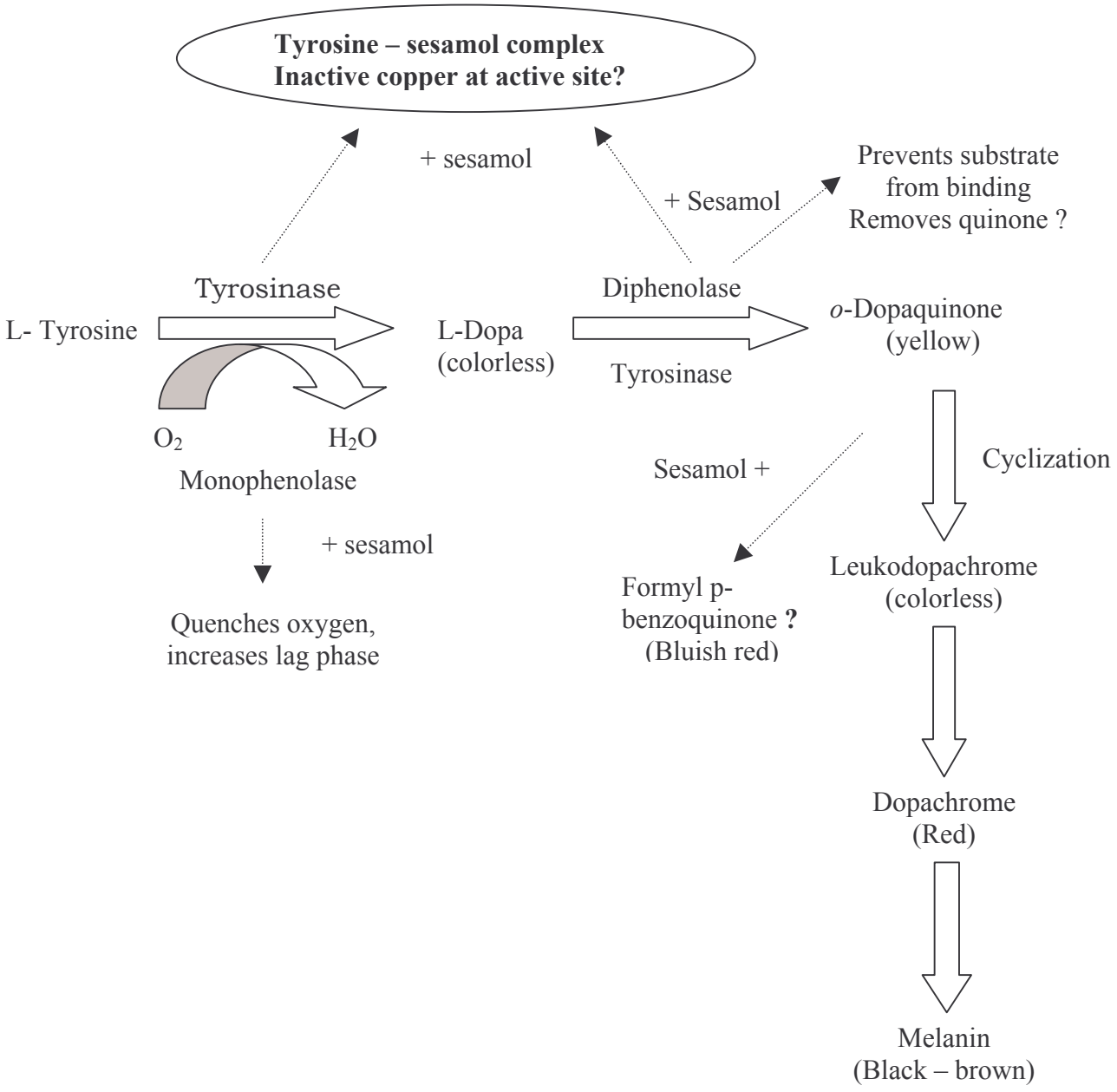
Sesamol competitively inhibits diphenolase activity also, suggesting that it combines with free enzyme molecule converting the E_m and E_o forms to E_mI and E_oI, respectively. Formation of E_mI and E_oI complex is a slow and reversible reaction (Solomon 1996). Sesamol is a potent inhibitor of diphenolase activity of mushroom tyrosinase with a K_I value of 0.57 μM. Sesamol has a hydroxyl group, attached to benzene ring in its 3, 4-methylenedioxy phenol moiety (Figure. 3). The hydroxyl group attached to the benzene ring of flavonoids like oxyresveratrol structure reportedly plays an important role in inhibiting tyrosinase activity (Chen, et al., 1991).

The active site Cu^{2+} is affected (Figure 35). Tyrosinases (type 3 copper proteins) are composed of two H subunits of 43 kDa and L subunits of 13 kDa each. Two binuclear coppers (coordinated to two histidines) are present in its active sites per tetramer. Type 3 copper proteins share a similar active site, though they differ in structure and sequence. Tyrosinase is a type 3 copper protein whose active site entrance is covered by an aromatic amino acid (tyrosine or phenylalanine) that is removed for activating the enzyme. This amino acid is highly conserved and considered to be a place holder for the substrate, tyrosine. It is in close vicinity to the highly active oxygen species. It has been postulated that an inhibitor with a phenylalanine or tyrosine like structure can

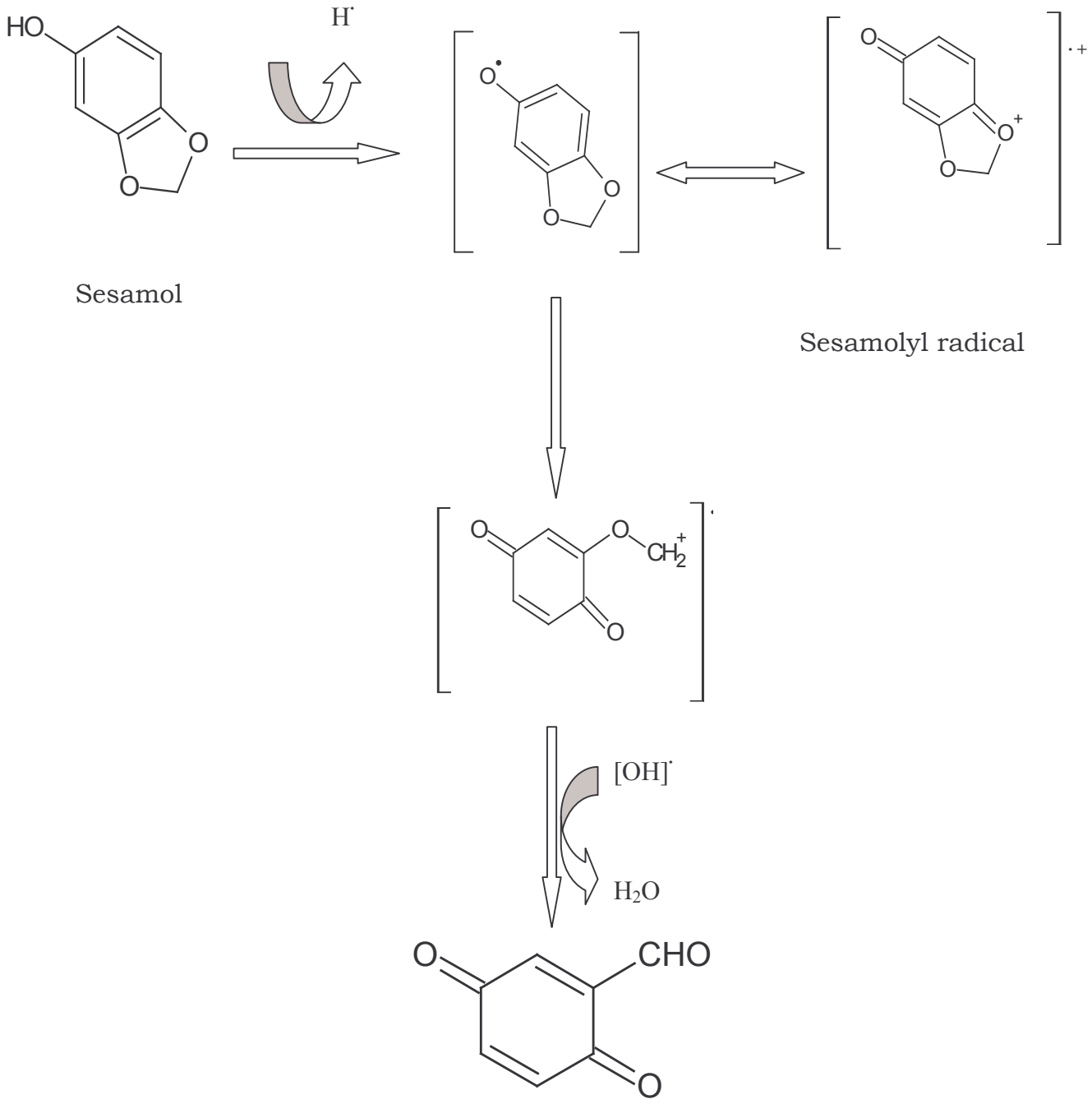
bring about inhibition through blocking the entrance of the active site. The potential of sesamol to inhibit diphenolase activity is higher when compared to monophenolase activity. The mechanism of inhibition of tyrosinase by sesamol is given in scheme 1. The conversion of sesamol to formyl-p-benzoquinone (?) (deep bluish red) may also contribute to its more potent inhibition of this activity (scheme 2). Sesamol is reported to form the benzoquinone anion radical if the H• attacks the 3-position of sesamol benzene ring.

Sesamol is, therefore, one of the few molecules reported so far that is capable of inhibiting the monophenolase as well as diphenolase activities together with quenching of free radicals.

In summary, the kinetic study has revealed that sesamol is the most potent inhibitor of both diphenolase and monophenolase activities of mushroom tyrosinase. Our results suggest that sesamol has a high potential for being developed into an effective antibrowning and depigmenting agent.



Scheme 1: Mechanism of tyrosinase inactivation by sesamol



Formyl p- benzoquinone
(formed more easily than *o*-quinone)
(deep bluish red color)

Scheme 2: Probable mechanism of O_2 radical scavenging by sesamol

Section 4 – Inhibition of lipoxygenase by lignans: Evidence of sesame lignans as anti-inflammatory components in animal model

The study of the chemistry and biology of natural products in relation to the mode of action of bioactive components may lead to applications of enzymes as drug targets. Several enzymes are now recognized as being potentially able to produce ROS and perhaps the most important among these is lipoxygenase. The interest in inhibition studies of LOX have been spurred by the role played by the products of LOX reaction with unsaturated fatty acids, in inflammation and immediate hypersensitivity. In recent years, a number of compounds isolated from plants, have been shown to inhibit LOXs (Mahesha et al., 2007)

Anti-inflammatory activity of sesame lignans

In the present study, a state of local inflammation was produced by injection 1% w/v λ- carrageenan (0.1 ml/ paw) subcutaneously in the planter surface of the left hind paw of the rat and right paw (saline treated) served as control. A comparison of the extent of carrageenan-induced paw inflammation at 3.5 h in various lignan molecules-fed animals is shown in Figure 38. Dietary sesamin lowered inflammation to an extent of 38 %, while dietary sesamol and sesamolin reduced the inflammation to an extent of 9% and 8% respectively (Figure 39). Thus sesamin had effective anti-inflammatory action compared to sesamol or sesamolin.

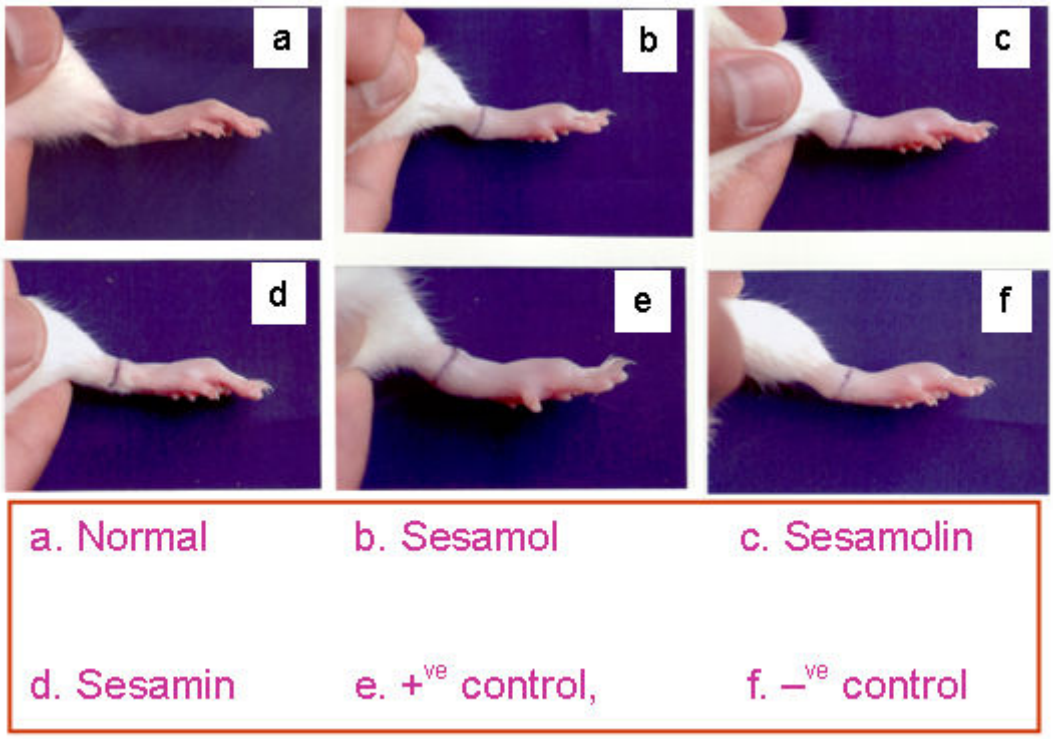


Figure 38. Reduction in carrageenan induced paw inflammation by sesame lignans at 3.5 hr after injecting carrageenan. $+^{ve}$ and $-^{ve}$ controls are the fed with groundnut oil and coconut out without any lignans respectively.

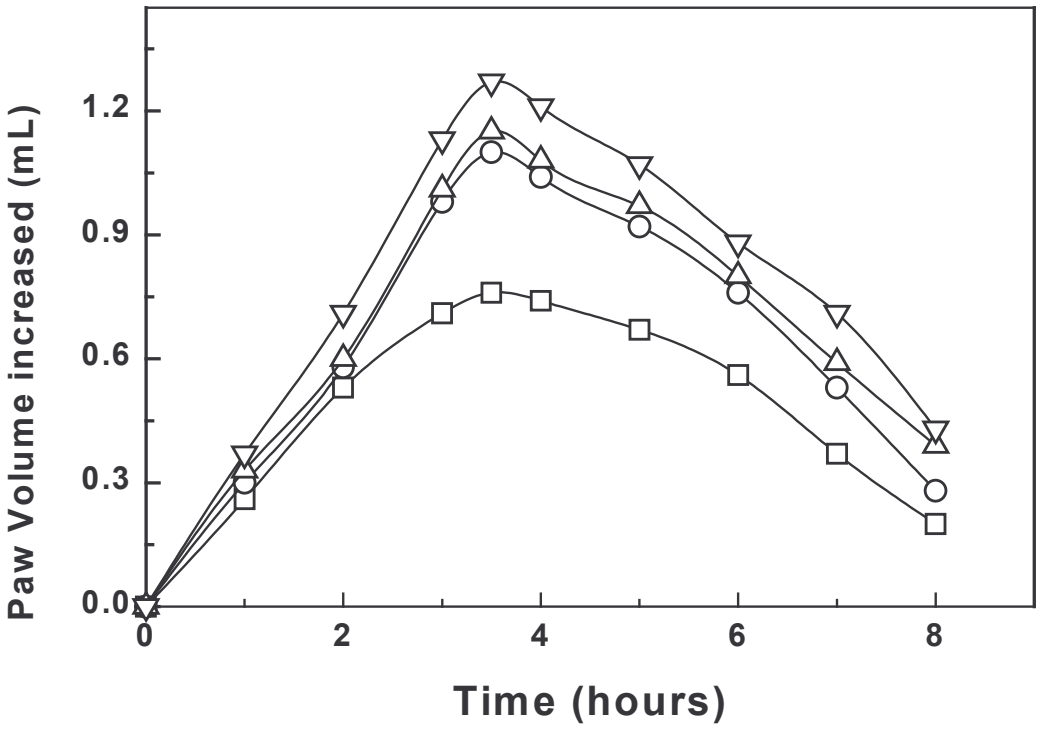


Figure 39. Measurement of reduction in paw inflammation by sesame lignan. Sesamin (–□–); sesamolin (– ○–); sesamol (–△–); Control (–▽–)

Inhibition of 5- lipoxygenase from leucocytes:

From the animal study, we found that sesame lignans show anti-inflammatory activity. The anti-inflammatory effect may be due to inhibition of either cyclooxygenase or lipoxygenase pathway. 5-Lipoxygenase (5- Lox) was partially purified by Ficoll-Histopaque density gradient and lysis of erythrocytes peripheral venous blood from healthy individuals. For inhibition of 5-Lox, sesamol was incubated for 2 min, and assayed with Arachidonic acid substrate. Sesamin and sesamol inhibited the 5- Lox activity dose dependently. Sesamol and Sesamin were the most effective inhibitor of 5-lipoxygenase with IC_{50} values of 77.15 and 192 μM, respectively (Figure 40). The result indicated that sesamol is a more powerful inhibitor of 5-Lox compared to sesamin. Sesamin brings about its anti-inflammatory effect not only by inhibiting Lox activity but also through other yet undiscovered mechanisms.

Since mammalian enzymes are difficult to purify, design of inhibitors based on structural and mechanistic studies of soybean lipoxygenase, whose three-dimensional structure and catalytic mechanism have been explored in detail. We have followed the enzyme inhibition kinetics using soy Lox -1 as the model.

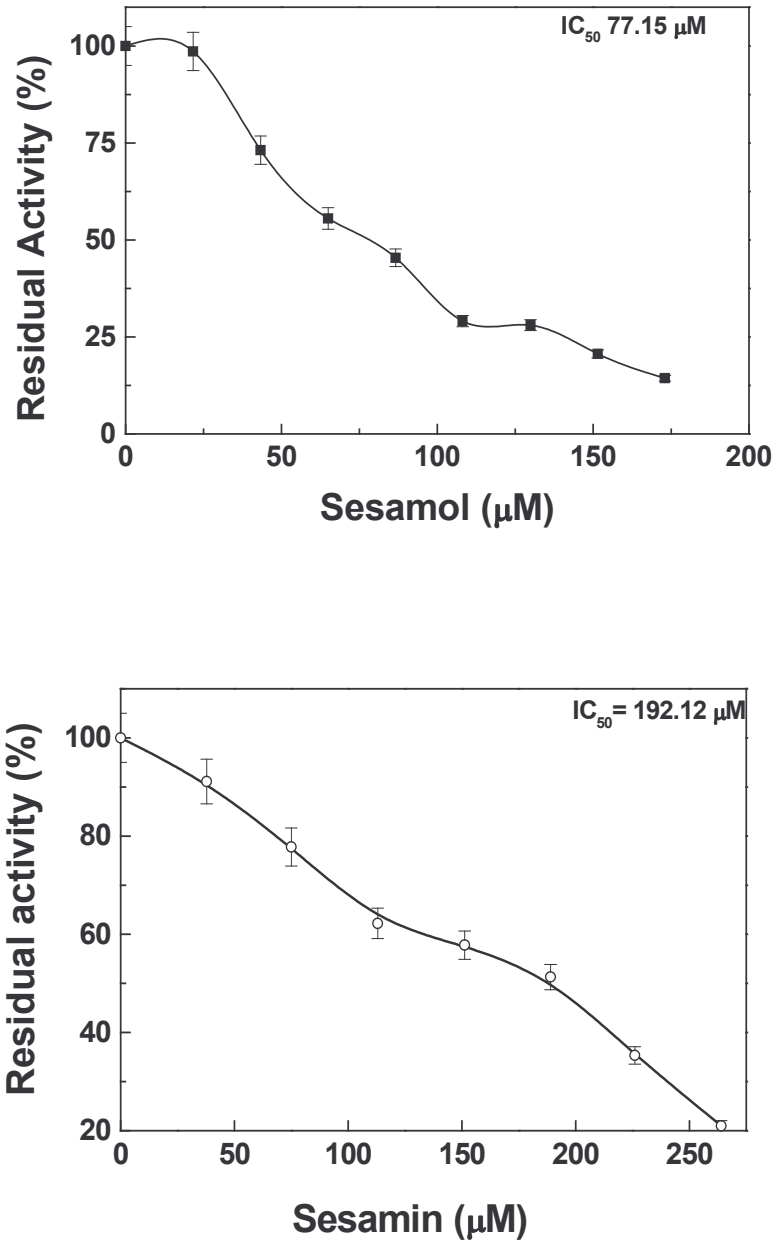


Figure 40. Inhibition of human PMNL 5- Lipoxygenase by sesamin and sesamol.

Studies on inhibition of soybean lipoxygenase 1 by sesame lignans

Lipoxygenase 1 from soybean was purified by reported methods (Axelrod, 1981). The SDS PAGE gel pattern is shown in Figure 41. Sesamol, sesamin and sesamolin inhibit the activity of Lox-1 with sesamol having maximum inhibitory effect. (Figure 42).

Figure 41. SDS PAGE of purified LOX 1. A 5 % stacking gel and 12 % resolving gel was used and electrophoresis was run according to Laemmelli (1970). The lanes are two different concentrations of the enzyme.

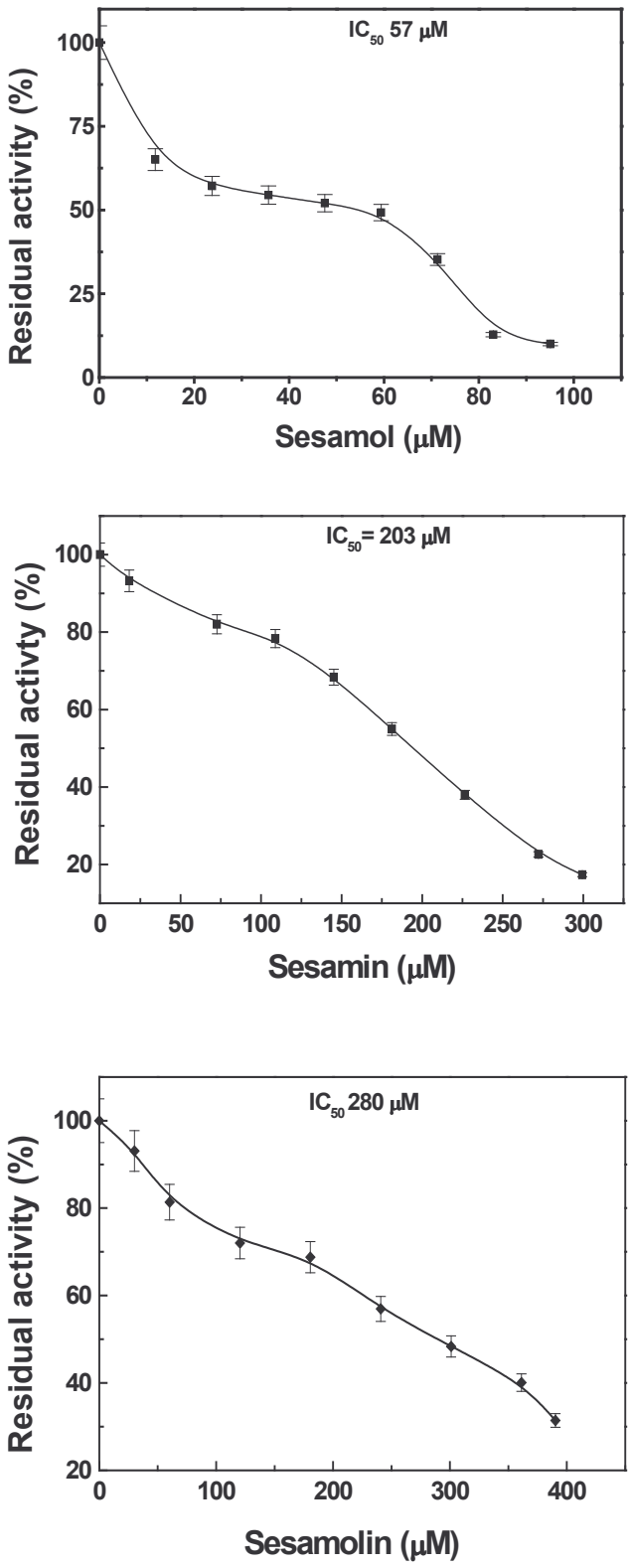


Figure 42. Inhibition of soy lipoxygenase 1 by sesame lignans

Effects of dietary sesamin, sesamolin and sesamol on iron-induced lipid peroxidation in rat serum and liver are presented in Table 21. The results of the present study demonstrate that excess iron introduced by adding $FeSO_4$ and ascorbic acid induce oxidative stress by increasing lipid peroxide levels in liver as well as serum samples. The addition of iron significantly elevated the hepatic lipid peroxides (418% increases in control group) and the levels of TBARS in liver were lower in animals fed sesamin, sesamolin and sesamol. The decreases were 38, 24 and 12% in the respective diet groups. Dietary sesamin, sesamolin and sesamol significantly reduce the severity of iron-induced lipid peroxidation in liver. Induction of peroxidation by iron (II) also results in higher lipid peroxides in serum (Table 21). The increase in serum TBARS value in control rats as a result of iron (II) addition is 76%. Dietary sesamin, sesamolin and sesamol lower serum lipid peroxide levels by 45, 10 and 9%, respectively. Our data demonstrates that the lignans derived from sesame have antioxidant properties and effectively inhibit non enzymatic lipid peroxides in animal tissues.

Table 21. Effect of lignans on serum and liver lipid peroxidation in rats

		Serum lipid peroxidation		Liver lipid peroxidation	
Group [a]	Dose (mg/ Kg body weight)	nmoles of MDA/dL	% Inhibition	nmoles of MDA/g liver	% Inhibition
Control	-	258.6 ±6	-	7.26 ± 0.6	-
Sesamin	5	141 ± 8.5	45.3	4.48± 0.1	38.3
Sesamolin	5	235 ± 5	10.0	5.47± 0.2	24.4
Sesamol	5	230 ± 6.5	9.8	6.33± 0.09	12.1

a Values show the mean ± SD (*n=5* rats)

Discussion

Animal lipoxygenases initiate the arachidonic acid cascade, which is a source of several powerful bioregulators such as prostaglandins, thrombaxanes, leukotrienes, lipoxins and hepoxilins, in a number of cellular responses (Prigge, et al. 1996). Hydroperoxides, the products of lipoxygenase mediated pathways, play a major role in the manifestation of chronic inflammatory diseases (Mulshine, 1998). The quest for new as well as natural inhibitors of lipoxygenase poses a challenge. Mammalian lipoxygenases are a target for drug design. Inhibition studies have been performed on lipoxygenases with a view to understand the key mechanistic aspects of its inactivation. Soy LOX-1 contains a non-heme ferrous ion that is oxidized by hydroperoxides to yield the catalytically active ferric enzyme (Papatheofanis, 1985). Soy isoflavone genistein scavenges lipid peroxyl radicals derived from the enzyme reaction. It also converts the active form of enzyme to the resting state. Mahesha et al, (2007) have shown that an electron donated by isoflavones is accepted by the ferric form (Fe^{3+}) of LOX, which is reduced to resting ferrous form (Fe^{2+}), thus inhibiting LOX. Similarly sesame lignans which inhibit the peroxidation of lipids both enzymatically and non enzymatically could inhibit the activity of both types of lipoxygenases.

The primary mechanism for peroxidation of liver and serum lipid is believed to be the iron-catalyzed decomposition of lipid peroxides. The role of iron in *in vivo* and *in vitro* lipid peroxidation has been well studied (Ryan and Aust 1992). Dietary curcumin and capsaicin, individually are protective to LDL oxidation both in vivo and in vitro, to iron-induced hepatotoxicity and to carrageenan-induced inflammation. These beneficial effects generally appeared to be higher when the two compounds are fed in combination (Manjunatha and Srinivasan, 2006). In our study the individual lignans are found to effectively inhibit the peroxidation, especially sesamin which inhibited the liver as well as serum lipid peroxidation. Sesamin which is known to reduce absorption and synthesis of cholesterol (Hirose et al., 1991) is a good fat reducing molecule in the diet.

The products of the lipoxygenase pathway of arachidonic acid metabolism are known to stimulate cellular proliferation, both directly and as intermediaries in the growth factor-mediated mitogenic signaling Pathway (Rioux et al., 1998). Although the exact mechanism is as yet unclear, modulation of this pathway is a target for the drug therapy of lung cancer, asthma. Indeed, recent studies have examined the use of lipoxygenase inhibitor compounds as potential chemopreventive agents (Hussey et al. 1996). The biological profile of leukotrienes suggests that inhibitors of the 5-LO pathway may have therapeutic potential in a variety of inflammatory and allergic diseases. Our findings using animal

model as well as enzyme kinetics, suggest that inhibition of lipoxygenase activity could be one of the factors contributing to the anti-inflammatory effects of lignans.

Chapter IV
Summary and Conclusion

Summary and Conclusions

Many health promoting effects of sesame are attributed to the presence of lignans. The biological activities of sesame lignans and their products were characterized in this study. The salient findings of this investigation are as follows:

1. Differences in lignan contents were investigated in fifty varieties of white sesame. The varieties were screened for lignan and tocopherol contents. These were selected from several progeny obtained after physical and chemical mutations of two parent strains of DS-1 variety. Sesamin contents of the mutants progeny varied from 1.76 - 10.46 g/kg and sesamolin varied from 0.72 – 3.77 g / kg of the oil. The γ-tocopherol content in the parental variety was 0.6 g/ kg, while the derived progeny had γ-tocopherol content in the range 0.45 – 0.77 g/ kg.
2. Infrared roasting was more effective than electric roasting for converting sesamolin to sesamol (82% conversion was possible against 51% in conventional electrically heated drum roasting of seeds).
3. There was no significant difference in the nutritional or functional qualities of the flours obtained from roasted seeds either with IR or conventional roasting. There was no significant

loss in the methionine or cysteine content of the flours due to roasting. However the color of the roasted flour was darker.

4. A simple RP- HPLC method was developed to purify the sesame lignans sesamin and sesamolin from sesame seed oil using RP C-5 column. Sesamin and sesamolin were obtained in purity of > 98 %.
5. The biological activities of these lignans were studied by comparing their antioxidant properties as well as antimicrobial activity. Radical scavenging, reducing power ability as well as antimicrobial activity of the 3 compounds was in the order of sesamol > sesamin > sesamolin.
6. The marked antioxidant activity of sesamol was probably due to presence of free hydroxyl group in its structure which readily donates electrons and reacts with free radicals to convert them to more stable products there by terminating free radical chain reaction.
7. Sesamol was the most potent antioxidant amongst the molecules studied.
    a. Sesamol and sesamin exhibited excellent antioxidant activity with 97.8 and 96 % inhibition of linoleic acid peroxidation respectively.
    b. Sesamol scavenged DPPH radicals efficiently at 20 μg / mL with an IC_{50} value of 5.44 μg / mL.

c. The ORAC-FL value of sesamol was 4.4 μ mol of trolox equivalents/ml, which was highest among all the 3 lignans. Sesamin and sesamolin shows 0.8 and 1.52 μ mol of trolox equivalents/ml of ORAC- FL value. The oxygen radical absorbance capacity assay (ORAC) is based on the chemical damage caused to the fluorescent (Fluorescein, FL) substrate by the peroxyl radicals. This assay was used to assess the free radical absorption activity of pure compounds.

d. Sesamol showed 1.83 ± 0.08 μM TE (trolox equivalence) ferric reducing ability power compared to 0.6 ± 0.02 of BHT standard. Sesamin and sesamolin exhibited less FRAP values with 0.06 ± 0.01 and 0.12 ± 0.05 TE.

e. The radical scavenging ability of γ-tocopherol was synergistically enhanced by three times in presence of sesamol or sesamin. γ-Tocopherol, alone, had the IC_{50} value of 4.5 μg/ ml and in presence of 10 μg of sesamin, IC_{50} value decreases to 2.74. In presence of 2 μg sesamol, IC_{50} value was found to be 1.6 μg which is more than 3 times more potent than γ-tocopherol alone.

8. Sesame lignans showed antibacterial activities against food borne pathogens. The minimum inhibitory concentration for

sesamol was 2 mg/ml against *Bacillus cereus* and *Staphylococcus aureus*, but it inhibited only 80% of growth of *Pseudomonas aeruginosa* at 2 mg/ml concentration. Sesamin and sesamolin show relatively less antimicrobial activity.

9. The bioavailability and stability to gastrointestinal digestion was evaluated by treating the lignans with gastrointestinal proteases - pepsin and pancreatin. The percent recovery in the 'IN' fractions of all three molecules, accounted for 45.2, 59.6 and 44.9 of sesamol, sesamin and sesamolin, respectively suggesting these molecules were fairly stable to gastric digestion.
10. Serum and liver lipid peroxidation was prevented by sesame lignans in the order of sesamin > sesamol > sesamolin in the rats fed with lignans for 15 days.
11. Sesamol, the phenolic degradation product of sesamolin, inhibited melanin synthesis in mouse melanoma B16F10 cells in a concentration dependant manner with 63% decrease in cells exposed to 100 µg/ml sesamol.
    a. Sesamol has found to inhibit melanin production, concomitant with decreased cell viability of B16F10 mouse melanoma cells.
    b. Apoptosis was induced by sesamol there limiting proliferation.

12. Inhibition of melanogenesis by sesamol was found to be through inhibition of the key regulatory enzyme of melanin synthesis pathway - tyrosinase. Tyrosinase exhibit monophenolase and diphenolase activity.
    a. Sesamol inhibited monophenolase activity with midpoint concentration of 3.2 μM. It is a non competitive inhibitor of monophenolase activity with a K_I of 1.4 μM.
    b. Sesamol inhibited diphenolase activity with midpoint concentrations of 1.9 μM. It is a competitive inhibitor of diphenolase activity with a K_I of 0.57 μM.
    c. The state of copper in the active site during the oxidation and reduction cycle was arrested by sesamol.
    d. Based on the kinetic and spectral data, a mechanism for inhibition of tyrosinase by sesamol is proposed.
13. Anti-inflammatory activity of lignans is postulated to be through the inhibition of Δ5-desaturase activity leading to decreased production of the pro-inflammatory leukotrienes. We have studied the role of lignans in mitigating the inflammatory response in the rat model.
    a. Rats fed with sesamin had 37% reduced inflammation compared to control.

b. Sesamol and sesamin were potent inhibitors of 5-lipoxygenase activity with IC_{50} values of 77 and 192 μM, respectively.

c. Soybean lipoxygenase-1 was inhibited by sesamol, sesamin and sesamolin with IC_{50} values of 57, 203 and 280 μM, respectively.

d. Inhibition of lipoxygenase activity could be one of the factors contributing to the anti-inflammatory effects of lignans.

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Appendices