CHEMICAL AND BIOCHEMICAL STUDIES ON NATURAL ANTIOXIDANTS FROM *SESAMUM* SPECIES

Thesis submitted to
Cochin University of Science and Technology
for the degree of
DOCTOR OF PHILOSOPHY
in
CHEMISTRY

By
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JANUARY 2003

DECLARATION

I, Suja.K.P., hereby declare that, this thesis entitled **'CHEMICAL AND BIOCHEMICAL STUDIES ON NATURAL ANTIOXIDANTS FROM *SESAMUM* SPECIES'** is a bonafide record of the research work done by me and that no part of this thesis has been presented earlier for any degree, diploma of any other University

Thiruvananthapuram
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CERTIFICATE

This is to certify that the thesis entitled ' **CHEMICAL AND BIOCHEMICAL STUDIES ON NATURAL ANTIOXIDANTS FROM *SESAMUM* SPECIES** ' is an authentic record on the research work carried out by Miss.SUJA..K.P under my supervision in partial fulfillment of the requirement for the degree of Doctor of Philosophy of the Cochin University of Science and Technology and further that no part of this thesis has been presented before for any other degree.

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CONTENTS

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SYNOPSIS

Oxidation is an important process in the normal metabolism of animals. But excess oxygen causes a state known as Oxidative stress induced by oxygen radicals. Active oxygen and free radicals jointly described as "Reactive Oxygen Species (ROS)" attack key biomolecules in living systems leading to cancer, inflammation, atherosclerosis and aging. Oxidation of unsaturated lipids initiated by free radicals is a major cause of food quality deterioration and leads to several other chemical reactions that negatively affect food quality. The products of lipid oxidation are known to be important health risk factors. Antioxidants are molecules which, at concentrations much lower than an oxidisable substrate, significantly delay or prevent its oxidation. Synthetic antioxidants such as BHA, BHT and TBHQ have been successfully used to prevent autoxidation. However, there is growing concern about the use of synthetic antioxidants because they are reported to be carcinogenic and toxic. Therefore recent research has focussed on the development and utilization of antioxidants from edible natural sources.

In the present study on natural antioxidants, the focus has been kept mainly on oil seeds, especially sesame and its by-products. Sesame, which has been under cultivation in India for centuries is called the 'Queen of oil seed crops' because of the high yield of oil obtained and the nutritional qualities of the seed, oil, and meal. Though India is the largest producer of sesame in the world, research on the various health benefits of sesame has been carried out by Japanese Sesame has an important place in the foods and traditional medicine of India from time immemorial. Foreseeing the potential of sesame and its byproducts as an important antioxidant source and its availability in bulk, the present study was focussed on Sesamum species. There are not many reports on the wild species of Sesamum in India, especially of the Kerala region. Hence, in the present study we also included antioxidants of Sesamum malabaricum- distributed throughout the coastal region.

The important characteristics of sesame are attributed to the presence of the unique compounds lignans. Lignans are a group of natural products of phenyl

propanoid origin, which are widely distributed in nature. They display important physiological functions in plants, in human nutrition and medicine, given their extensive health promotive and curative properties. Much interest has been focussed on their effectiveness as antineoplastic agents and research in this area has revealed several modes of action by which they can regulate the growth of mammalian cells. Sesame is an important source of furofuran lignans, of which sesamin and the rare oxygenated derivative sesamolin are the most abundant. Others include sesamol and glucosides of lignans. Sesamin and episesamin are reported to have hypocholesterolemic effect, suppressive effect on chemically induced cancer, alleviation of allergy symptoms etc. Sesamol, sesamolin and the lignan glycosides are reported to inhibit lipid peroxidation. Present investigation on sesame and its byproducts have been carried out to explore the possibility of developing a natural antioxidant extract from available resources to be used as a substitute to synthetic ones in vegetable oils and foods. Preliminary analysis showed that sesame cake, a byproduct could still be utilized as a major source of lignans. Sesame cake, which is now used only as a cattlefeed, can be better utilized in the form of a valuable antioxidant source. The present study explains the development of a feasible process for the extraction of antioxidant compounds from sesame cake. The antioxidant extract so prepared from sesame cake has been tested for vegetable oil protection and is found to be effective at low concentration. In addition, studies also include the antioxidant, radical scavenging, anticancer, mosquitocidal and pesticidal activities of extract and individual compounds. The thesis is comprised of four chapters.

CHAPTER 1. INTRODUCTION

Introductory chapter highlights the topic of mechanism of autoxidation, formation of free radicals and subsequent change to the substrate system and how antioxidants intervene and quench free radicals. Chapter1 also covers literature review on natural antioxidants in general and lignans in particular, their structural and functional characteristics and their distribution in nature, especially in an edible oil crop like sesame. It also reveals the importance of choosing sesame as the topic of present study.

CHAPTER 2. MATERIALS AND METHODS

The method of extraction of antioxidants from sesame seed, oil, and cake has been explained. Solvents with varying polarity, such as hexane, isopropanol, ethyl acetate, acetone, ethanol, methanol etc., were tried in cold and hot extraction methods. Quantitation of lignans was carried out by HPLC analysis using reverse phase C_{18} column with standardized methodology. Total phenolic content (TPC) of extracts was measured by AOAC method. Based on TPC values and lignan content values suitable solvents for extraction was selected. Purification of extracts was also tried to improve the lignan content and antioxidant activity. Antioxidant activity was evaluated for crude and purified extracts using various model systems. Preliminary evaluation methods include β-carotene bleaching method, thiocyanate method in the linoleic acid and linoleic acid emulsion systems. β-Carotene bleaching method describes the effect of antioxidants in inhibiting the bleaching rate of β-carotene during co-oxidation of β-carotene and linoleic acid. In the thiocyanate method, the increase in absorbance due to the formation of ferric thiocyanate by the oxidation products of linoleic acid on ferrous chloride and ammonium thiocyanate were measured. Antioxidants hinder the oxidation of linoleic acid and hence colour development will be less. In all these cases different concentrations of extracts were tested and results compared with synthetic antioxidants BHT and TBHQ. Schaal oven test was used for oil storage studies. Further evaluation of oil stability was done by DSC (Differential Scanning Calorimetry) analysis. Radical scavenging effects were studied by[DPPH] assay and xanthine-xanthine oxidase/NBT assay. Anticancer effects pesticidal effect and mosquitocidal effects were also studied using selected methods. The isolation of components present in extract was carried out using chromatographic methods. Column chromatography and preparative HPLC were mainly used. Isolated compounds were identified by IR, UV, NMR and MS techniques. They include sesamol, sesamin, sesamolin, sesaminol triglucoside, sesaminol diglucoside .

CHAPTER 3. RESULTS AND DISCUSSIONS

Chapter 3 consists of results and discussions. For the extraction of antioxidants from sesame, soxhlet extraction using methanol was selected based on lignan content and TPC values. Methanol gives higher (15-20%) yield of lignans compared to other solvents. The crude extract was subjected to a sequence of extraction procedures to get a purified extract with higher lignan content and enhanced antioxidant activity. Separation and quantitation of lignans was done by HPLC analysis. The amount of sesamol, sesamin, sesamolin, sesaminol triglucoside and sesaminol diglucoside were calculated based on calibration with pure compounds. The presence of sesamol in methanolic extract of sesame cake was not so far reported. Crude and purified extracts showed appreciable antioxidant activity. In the peroxidation models, activity was compared with 200ppm BHT. Crude extract at 100 and 200 ppm showed comparable results with that of BHT. Purified extract at lower concentrations showed better activity than that of BHT. The isolated compounds from sesame cake extract included sesamol, sesamin, sesamolin, sesaminol triglucoside and sesaminol diglucoside. Antioxidant activities of each individual compound were evaluated by the β-carotene bleaching method and thiocyanate methods and they showed activity in all model systems. Seed extract of the wild species Sesamum malabaricum showed unusually high amount of lignans compared to the cultivated varieties of Sesamum indicum; the wild species showed high antioxidant activity also.This is the first report on the lignan profile and antioxidant activity of S. malabaricum.

Free radical scavenging capacities of total extracts and individual compounds were evaluated by different methods. DPPH, a highly reactive free radical was used to evaluate the scavenging effect. It had a characteristic absorption at 515 nm. When antioxidants were added, the absorption decreased and reached a steady state.

The effective concentration of antioxidant needed to decrease the DPPH concentration by 50 % (EC_{50}) and hence the antiradical power of extract (ARP) were calculated. The values were treated statistically to explain the kinetic behavior of each antioxidant. At the start of the experiment, [$DPPH^{\cdot}$] was depleted from the medium under pseudo-first-order conditions,($[DPPH^{\cdot}]_0 << [(AH)_n]_0$).Later the reaction follows second order kinetics ie the rate constant is related to the concentration of antioxidant also. Purified extracts showed better activity than crude extracts. Radical scavenging effects and the rate constants of the pure compounds were also studied. Superoxide radical scavenging effects of extract as well as pure compounds were studied by enzymatic methods. One method include xanthine-xanthine oxidase system using cytochrome C. The superoxide radical ($O_2^{-\cdot}$) produced as a by-product during the conversion of xanthine to uric acid, reduces the ferricytochrome C. It was followed by an increase in absorbance at 550nm. Antioxidant activity expressed in terms of decrease in absorbance. Another method for the evaluation of superoxide scavenging was described by NBT method. The superoxide radical reduces NBT to blue colored formazan complex and the reduction was followed by an increase in absorbance at 560nm. In both cases the extract showed antioxidant activity. Activities of isolated compounds were also studied at different concentrations by the NBT method.

Invitro studies were conducted for evaluating sesame extract as an alternate antioxidant source in oil industry. The Schaal oven method of oxidative stability was carried out for this purpose. Three different vegetable oils viz. soybean, sunflower and safflower oils were tested in this invitro experiment. Both crude and purified extracts were tested for antioxidant protection of vegetable oils. The oil samples stored at 50°C for two weeks are analysed for peroxide value, diene value and p-anisidine value. Crude extracts at 100 and 200 ppm showed inhibition effect comparable to BHT at 200 ppm while purified extract at 5,10,15 and 100 ppm showed better effect than BHT. Sesame cake extract was effective in protecting the three different vegetable oils inspite of their different unsaturation levels and vitamin E content. The stability studies also included DSC analysis, making use of the accelerated oxidation of vegetable oil samples at higher temperature (150^0C) in a shorter time. The induction time which is noted as the time instant at which oxidative changes begin, represent the stability of oil. The results of DSC analysis agreed with that of Schaal oven method. Storage stability studies were also

conducted at ambient temperature. Each experiment established the potency of sesame cake extract in protecting vegetable oils.

Biological efficacy of these extracts and pure compounds were also studied. Anticancer effects were studied using cell culture studies in collaboration with Regional Cancer Centre, Trivandrum. The total extract and individual compounds showed anticancer effect and induced apoptosis. Preliminary experiments with extracts and the pure compounds (lignans) showed growth regulatory and ovicidal activities against tested insects. When Aedes mosquitoes were tested with these samples, high larval mortality and Insect growth regulation (IGR) was observed. Seed extract and sesame oil showed mosquitocidal activities.

CHAPTER 4. SUMMARY AND CONCLUSIONS

The conclusion arrived from the present study was summarized in chapterp4. The study establishes the possibility of utilizing sesame cake, a byproduct of oil industry, as an antioxidant source. More over the residue after antioxidant extraction can be still used as cattle feed. Detailed information regarding the antioxidant activity of sesame extract as well as individual compounds present in them were provided.It also establish the presence of sesamol in higher quantities in sesame cake extract The extract can be used as a substitute for synthetic antioxidants in oil industry to protect vegetable oils against oxidation. The extract and pure compounds also showed appreciable anticancer effect. The mosquitocidal and pesticidal activities of extracts and the lignans were also promising. In addition to this the wild species *Sesamum malabaricum* showed high antioxidant, pesticidal and mosquitocidal activities. Thus the thesis work is an earnest attempt to bring out scientific and technological importance of sesame, an important agriculture crop of our country.

CHAPTER 1

INTRODUCTION

1.1.Preamble

Mechanism of oxidation in the context of food processing and storage and consequent quality problems are well documented. Control of oxidation is also extremely vital to maintain functional properties and life of many industrial products. Incorporation of appropriate antioxidants in to food and industrial products, therefore, are accepted industrial practices. Variety of synthetic antioxidants designed to withstand the processing and application conditions are available for the industrial products. However, the lists of synthetic or natural antioxidants permitted in food products are limited primarily due to toxicity on prolonged usage. Apart from the ingestion of oxidized food products with its adverse health effect, endogenous oxidants produced in the biological system through normal process of metabolism have recently been implicated in the genesis of many fatal diseases related to cardiovascular and nervous system and cancer and other debilitating afflictions such as accelerated aging, cataract, memory impairment etc. Strong positive correlation has been brought out by experimental and epidemiological evidences between intake of foods rich in antioxidant and human health. Based on scientific data available now, the options for management of health are prophylactic and therapeutic approaches. The old dictum that " Prevention is better than cure", therefore, is more relevant than ever before with scientific validation. Terminologies like 'nutraceutical', 'phytoceutical', 'cosmoceutical', 'functional foods', 'antioxidants' are coined for prophylactic approach to management of human health and diseases, which is a cheaper alternative to therapeutic option that is not only expensive but also affect quality of life. The resurgence in research on natural products in recent

years, therefore, could be attributed to these scientific evidences. Antioxidants are ubiquitous in nature. However natural product designed for bioactive application, as a commercial product has to undergo several stages including bio prospecting, bioavailability, biopotency evaluation, toxicological scrutiny and techno-commercial evaluation. This limits the viable source of natural products that can be exploited for health care applications. The present investigation on sesame cake based antioxidants is to address primarily the scientific feasibility and techno-commercial to some extent.

1.2. Chemical Oxidation

Oxidation of food components can influence nutritional quality, flavor quality, consumer acceptability and toxicity of food product [1]. The oxidation products of lipid are responsible for the development of rancidity. Oxidation occurs in the presence of molecular oxygen in both the triplet and singlet states [2]. Atmospheric oxygen that is in the triplet state contains two unpaired electrons, while oxygen in the singlet state has no unpaired electrons. The electronic arrangement of triplet oxygen does not allow for a direct reaction with organic compounds, such as unsaturated fats, that exist in the singlet state. Singlet oxygen generated from triplet oxygen by excitation is suggested to be responsible for initiating lipid oxidation of food products due to its ability to directly react with the electron -rich double bonds of unsaturated fats and other singlet-state compounds [3].

Electron structure of molecular oxygen and its excited states can be explained by molecular orbital theory. Molecular oxygen, which consists of two oxygen atoms, has 10 molecular orbitals containing 12 valence electrons. Valence electrons are added to orbitals in order of increasing energy to obtain molecular orbital diagram (Figure1.1).

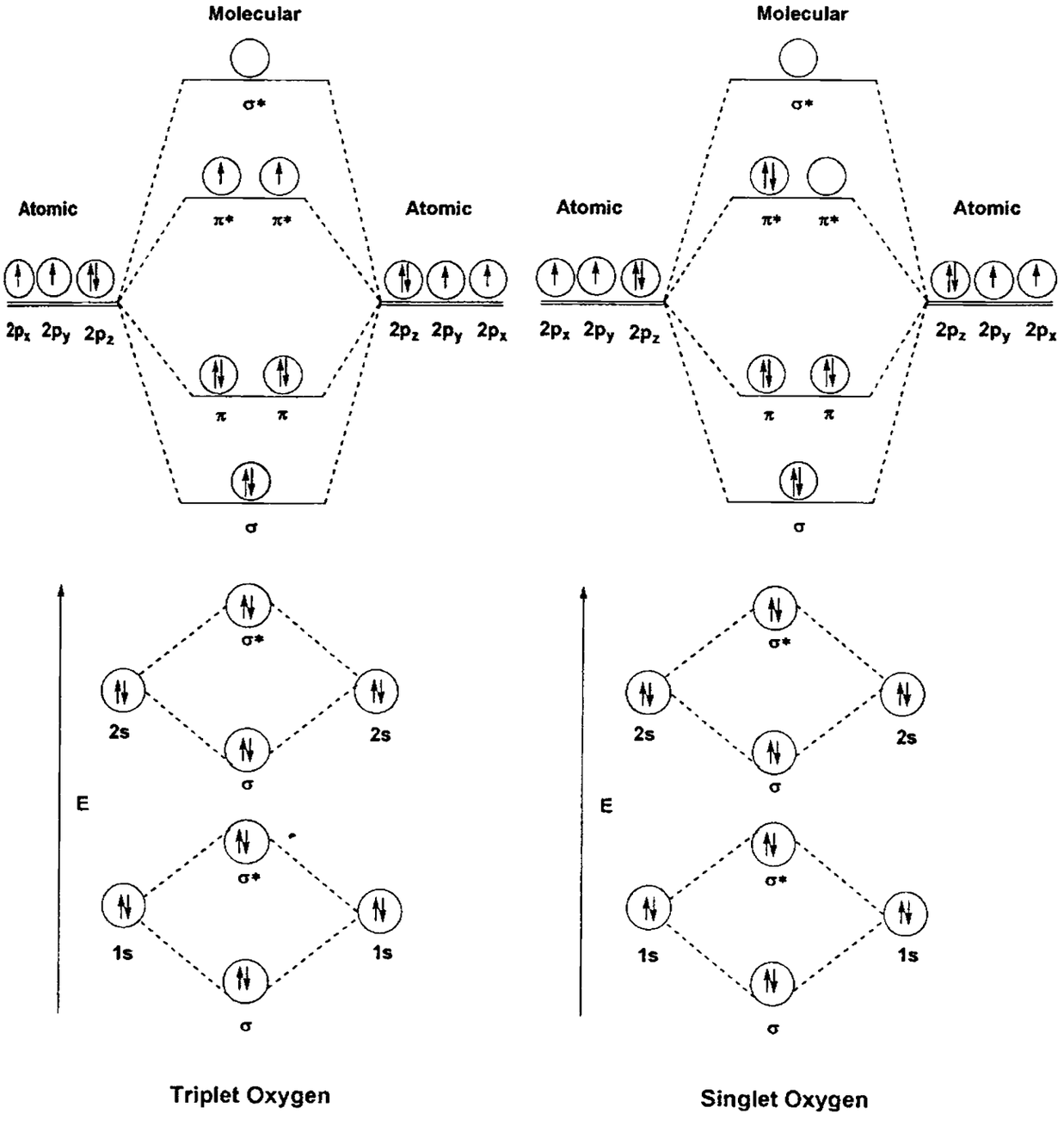


Figure 1.1: Molecular orbitals of Oxygen

According to Paul's exclusion principle only two electrons can occupy each orbital. Hund's rule states that one electron is placed in to each orbital of equal energy one at a time before the addition of second electron. Pauli's exclusion principle also states that electrons in a given orbital must have opposite spins. Accordingly, electrons of opposite spin are assigned to each orbital. The ground state of the molecule is singlet if the resultant spin is zero and hence the multiplicity of the state is 2S+1=1. An excited state is formed by removing one of the electrons from the upper most filled orbital (bonding π) of the ground state to a vacant orbital (antibonding π^*) of higher energy.

Generally, the ground state of most stable molecules containing an even number of electrons is arranged in to pairs with opposite spins, hence the molecule will be diamagnetic. More over, the ground state is singlet until a molecule is excited to the triplet state. Triplet state contains two unpaired elecrons and it will be paramagnetic. Oxygen is an exception to this generality [1]. In the triplet state of oxygen two high energy electrons are present in two degenerate orbitals as unpaired electrons. This shows the diradical nature of oxygen.

Singlet oxygen, whose electrons are paired, is violation of Hund's rule, creating an electronic repulsion that can produce five excited states. Two of the most common excited states include an activated $^1\Sigma$ state that lies 37.5 kcal above the ground state and an activated $^1\Delta$ state occurring 22.4 kcal above the ground state. The $^1\Sigma$ state of oxygen has two electrons with opposite spins in different orbital and is so reactive that it is not able to survive relaxation to the ground state. The less energetic $^1\Delta$ state of molecular oxygen is sufficiently stable long enough to react with other singlet-state molecules. The

$^1\Delta$ state is responsible for most singlet oxygen reactions, therefore singlet oxygen is mostly used to designate as $^1\Delta$ state oxygen [1].

Singlet oxygen is suggested to be responsible for initiating oxidation invitro and invivo, because of its low energy of only 22.4 kcals above the ground state, its relatively long life time, and its highly electrophilic nature, seeking electrons from electron rich compounds to occupy its vacant molecular orbital [4]. Once this active species is formed, it is responsible for initiating singlet oxygen oxidation. Singlet oxygen oxidation that rapidly produces free radicals in turn can initiate a free radical chain reaction. Singlet oxygen can be generated chemically [5], enzymatically [6], and by decomposition of hydro peroxides [7]. Its formation by photochemical methods is most important in food systems in which natural photosensitizers can generate singlet oxygen [8].

1.2.1.Oxidation in food products

Lipid oxidation is primarily responsible for quality problems in food products. There are many lipid or fat components of foods, which spontaneously react with atmospheric oxygen and suffer deterioration in the process of autoxidation. These include fats and oils, mono and diglycerides, and sterols. This autoxidation of food lipid components is a major cause of deterioration in food quality, affecting nutritive value, taste, aroma, color and texture. The process of autoxidation and development of rancidity in foods involves free radical chain reactions.

Autoxidation is a natural process that takes place between molecular oxygen and unsaturated lipids [9]. Original mechanism of autoxidation was proposed by Farmer [10] and Holland and Ten Have [11]. It involves initiation, propagation and termination as cited by S.Cuppet etal.,[12].

Initiation

According to original mechanism, initiation takes place by triplet oxygen.

$$LH + O_2 \longrightarrow L^{\cdot} + {}^{\cdot}OOH$$

Recently other mechanisms have been suggested by Frankel [13]. These include initiation not directly associated with oxygen instead rely on the interaction of unsaturated lipid (LH) with acceptor radicals such as metals, or on interaction with high-energy light that can then transfer the excess energy to a second LH.

In the presence of initiators, unsaturated lipids (LH) lose hydrogen radical ($H^{\cdot}$) to form lipid free radicals ($L^{\cdot}$)

$$LH \longrightarrow L^{\cdot} \quad (1)$$

Propagation

The alkyl radical of unsaturated lipid (L.) contains labile hydrogen that reacts rapidly with molecular oxygen to form peroxyl radicals and alkoxyl radicals.

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \quad (2)$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \quad (3)$$

Termination

The free radicals can react with each other and form non-radical products.

$$LOO^{\cdot} + LOO^{\cdot} \longrightarrow \text{Non-radical products} \quad (4)$$

$$LOO^{\cdot} + L^{\cdot} \longrightarrow \text{non-radical products} \quad (5)$$

$$L^{\cdot} + L^{\cdot} \longrightarrow \text{non-radical products.} \quad (6)$$

A second general mechanism of lipid oxidation involves the presence of light as a means to initiate lipid oxidation. Singlet oxygen which is 1500 times more reactive than triplet oxygen react directly with the LH to form a peroxyl radical ($LOO^{\cdot}$) and ultimately the LOOH. This is called the photo oxidation. It involves a physical excitation of $^3O_2$ by a sensitizer (eg-chlorophyll) in the presence of light to give the active $^1O_2$. $^1O_2$ can then react with the LH to form LOOH.

$$^1S + h\nu \longrightarrow {}^1S^* \longrightarrow {}^3S^*$$

$$^3S^* + {}^3O_2 \longrightarrow {}^1O_2 + {}^1S$$

$$^1O_2 + LH \longrightarrow LOO^{\cdot} + H \longrightarrow LOOH$$

Consumption of oxidized foods is a potential health risk for both humans and animals. The primary product of autoxidation of fat are odorless, tasteless peroxides and hydroperoxides, which are less, absorbed by the intestinal tract. At ambient temperatures, hydroperoxides breakdown and produce a variety of hydrocarbons, aldehydes, ketones, alcohols and organic acids. Many of these oxidized end products that are stable have a carbonyl residue in the molecule such as aldehydes and ketones (Figure 1.2). Due to their strong hydrophilic nature and low molecular weight these components in oxidized fats and oils are easily absorbed and carried to the internal organs in the blood stream and promote lipid oxidation *invivo*[14]. They also tend to react with proteins, amines and DNA. Such processes act as hosts to the degenerative diseases, and thus make a significant contribution to human aging and cancer [15]

1.3.Biological oxidation

Living organisms require continuous supply of free energy for performance of mechanical work, active transport of molecules and ions, and for biosynthesis of macromolecules and other biomolecules from simple precursors. Chemotrophs obtain this free energy by the oxidation of foodstuff whereas phototrophs obtain it by trapping light energy. Part of this energy is used to synthesize the compound, Adenosine triphosphate (ATP) which is used in many energy- driven cellular processes [16].

According to Hans and Krebs there are three stages in the generation of energy from the oxidation of ingested food. In the first stage, macromolecules are broken down into smaller units such as sugars, fatty acids, glycerol and amino acids. In the second stage these free simple units i.e. sugars, fatty acids, glycerol and several amino acids are converted in to acetyl unit of acetyl CoA. The third stage consists of the citric acid cycle

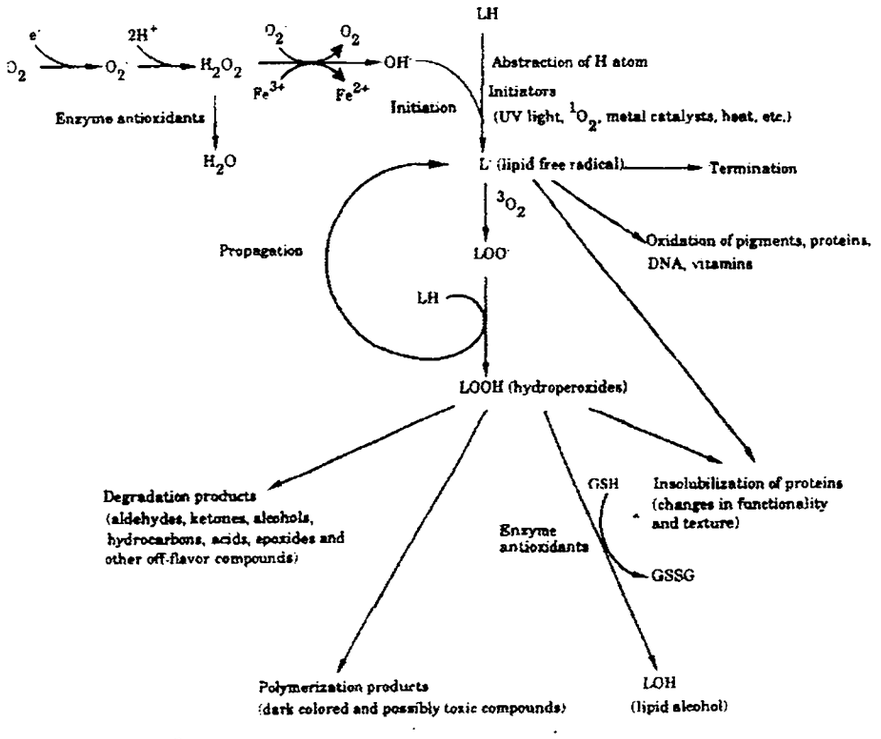


Figure 1.2 : The mechanism and consequences of lipid oxidation in food and in the body and their possible neutralization by enzyme antioxidants

and oxidative phosphorylation, which are the final common pathways in the oxidation of fuel molecules. Acetyl-CoA brings acetyl unit in to this cycle, wherein they are completely oxidized to CO_2. Most of the ATP generated by the degradation of foodstuffs is formed in this final stage. The sub-cellular body or organelle, mitochondrion, carries out these oxidative processes [17]. The oxygen is ultimately converted into water through a relatively safe process. The energy conserved in ATP is essential for numerous metabolic reactions occurring in living cells. Hence oxidation and release of free radicals and active oxygen species is an inevitable part of the metabolic processes.

1.3.1.Free radicals and Reactive oxygen species (ROS) in Biological system

'Free radicals' are any chemical species capable of independent existence that contains one or more unpaired electron [18]. They are in general reactive and attack other molecules. Active oxygen species denote oxygen-containing molecules, which are more active than the triplet oxygen molecule present in air [19]. Some of them have unpaired electrons and are free radicals, but others are not (Table.1.1) and their formation is given in Table1.2.

Table1.1. Reactive Oxygen Species

Radicals	**Non radicals**
Superoxide($O_2^{\cdot -}$)	hydrogen peroxide (H_2O_2)
Hydorxyl radical ($HO^{\cdot}$)	singlet oxygen ($^1O_2$)
Hydroperoxyl radical ($HO_2^{\cdot}$)	lipid peroxide (LOOH)
Lipid radical ($L^{\cdot}$)	iron-oxygen coplex (Fe=O)
Lipid peroxyl radical ($LO_2^{\cdot}$)	hypochlorite (HOCl)
Lipid alkoxyl radical ($LO^{\cdot}$)	
Nitrogen dioxide ($NO_2^{\cdot}$)	
Nitric oxide ($^{\cdot}NO$)	
Thiyl radical ($RS^{\cdot}$)	
Protein radical ($P^{\cdot}$)	

Table1.2: Production of Reactive oxygen species

ROS	Formation
Superoxide($O_2^{\cdot-}$)	Enzymatic and non-enzymatic one electron reduction Of oxygen $O_2 + e \longrightarrow O_2^{\cdot-}$
Hydroxyl radical ($HO^{\cdot}$)	Radiolysis of water,metal catalyzed decomposition of hydrogen peroxide $H_2O_2 + e \longrightarrow OH^- + OH^{\cdot}$
Alkoxyl and peroxyl radicals$LO^{\cdot}$, $LO_2^{\cdot}$	Metal catalysed decomposition of hydroperoxides
Hydrogen peroxide, H_2O_2	Dismutation of superoxide $O_2^{\cdot-} + 2H + e \longrightarrow H_2O_2$
Iron-oxygencomplex	Haemoglobin,myoglobinetc
Singlet oxygen, $^1O_2$	Photosensitized oxidation, bimolecular interactions between peroxyl radicals, reaction of hypochlorite and hydrogen peroxide
Lipid and protein hydroperoxides	Oxidation of lipids and proteins
Nitrogen dioxide	Reaction of peroxyl radical and No, polluted air and smoking
Nitric oxide, $^{\cdot}NO$	Nitric oxide synthase, nitroso thiol, and polluted air
Thiyl radical, $RS^{\cdot}$	hydrogen atom transfer from thiols
Protein radical	Hydrogen atom transfer from protein

Active oxygen and related species are produced to serve important biological functions. For example, Activated phagocytes utilize ROS to destroy some strains of invading bacteria and fungi. Superoxide plays a useful role in the regulation of cell growth and intercellular signaling [19]. Excess production of these free radicals can be very damaging because they are highly reactive and attack almost all molecules including DNA, protein and carbohydrate leading to many disease conditions (Figure.1.3).

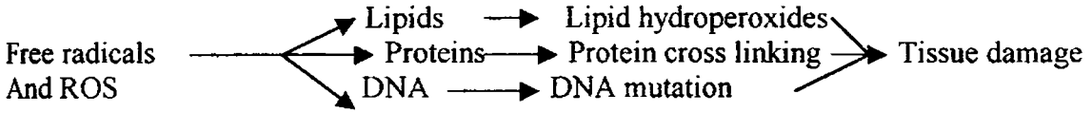


Figure 1.3. Consequence of free radicals and reactive oxygen species (ROS) in diseases and preventive role of antioxidants

1.3.2.Consequences of free radicals in biological system

Lipid peroxidation invivo mainly contributes to the development of cardiovascular diseases, such as atherosclrosis [20]. Membrane lipids became much more susceptible to peroxidation after cell death.The products of lipid peroxidation-particularly malondialdehyde (MDA) and 4-hydroxy nonenal (HNE)- are important because they can cause damage to proteins and to DNA [21, 22].

Oxidative damages to proteins affect function of receptors, enzymes, transport proteins etc and generates new antigens that provoke immune responses [23]. It also

contributed to secondary damage to other biomolecules, such as inactivation of DNA repair enzymes and loss of fidelity of DNA polymerase in replicating DNA. Free radical attack on proteins generates radicals from amino acid residues and electrons can be transferred between different amino acids.

The chemistry of DNA damage by several ROS/RNS has been well-characterized invitro. Nitric oxide ($NO^{\cdot}$) and products derived from it ($NO2^{\cdot}$, HNO_2, $ONOO^{-}$, N_2O_3 etc [24, 25] can cause nitrosation and deamination of amino groups on DNA bases leading to point mutations. Deamination products of purine bases include xanthine (from guanine) and hypoxanthine (from adenine). $O_2^{\cdot-}$ and H_2O_2 do not react with DNA bases, $OH^{\cdot}$ generates many products with DNA. $^1O_2$ attacks upon guanine.

1.4. Antioxidants

'Antioxidants are substances that when present in foods or in the body at low concentration compared to that of an oxidisable substrate, markedly delay or prevent the oxidation of that substrate' [26].

1.4.1.Mechanism of Antioxidant action

The commonly-accepted antioxidant mechanism by which inhibition of lipid oxidation is by radical chain breaking [27].

Antioxidant (AH) breaks radical chains by donating hydrogen atom to the chain carrier peroxyl radicals, $LOO^{\cdot}$, under atmospheric conditions(Reaction1) and to lipid radicals, $L^{\cdot}$, under conditions of limited oxygen availability (Reaction2);

$$AH + LOO^{\cdot} \longrightarrow LOOH + A^{\cdot} \qquad \text{(Reaction 1)}$$

$$AH + L^{\cdot} \longrightarrow LH + A^{\cdot} \qquad \text{(Reaction2)}$$

Under atmospheric conditions, because of the extremely rapid reaction of $L^{\cdot}$ with air oxygen, the termination reaction, Reaction 1 is considered most prevalent in food and biological systems [28].

Hydroperoxides decompose homolytically by producing alkoxy radicals ($LO^{\cdot}$), which undergo β-cleavage (Reaction 3) to form aldehydes and other break down products. These Secondary oxidation products contribute to oxidative and flavor deterioration of food lipids and also cause damage in biological systems.

R2- | - CH($O^{\cdot}$)- | - R1 ⟶ aldehydes+ break down products (Reaction 3)

Antioxidants can inhibit the decomposition reaction (Reaction3) by reacting with the alkoxyl radicals, either by hydrogen donation to form stable hydroxy compounds (Reaction4) or by the termination reaction (Reaction 5) with antioxidant radicals.

$$AH + LO^{\cdot} \longrightarrow LOH + A^{\cdot} \qquad \text{(Reaction 4)}$$

$$A. + LO^{\cdot} \longrightarrow LOA \qquad \text{(Reaction 5)}$$

Usually the activity of antioxidants is measured by their effectiveness to inhibit the formation of hydroperoxides by reaction [1]. However their activity to inhibit aldehyde formation by reaction [4] should also be considered while evaluating their

invitro antioxidant activity. The most commonly observed antioxidant action is through scavenging of lipid peroxyl radicals by donating hydrogen atom [29]. eg. Phenolic compounds. These are called chain breaking antioxidants. Preventive antioxidants are defined either as 'preventing introduction of initiating radicals' [27] or as' reducing the rate at which new chains are started' [30]. Metal chelators are preventive antioxidants by complexing with transition metal ions, thereby hindering metal-catalyzed initiation reactions and decomposition of lipid hydroperoxides [28]. Other antioxidant mechanism includes singlet oxygen quenching, oxygen scavenging, and blocking the prooxidant effects of certain proteins containing catalytic metal sites by binding with them. The oxidation conditions [31,32] and the physical state of the oxidisable substrate [28,33] also affect antioxidant activity.

1.4.2. Endogenous antioxidants

The living organisms have developed powerful and complex antioxidant systems to counteract the damages caused by free radicals and ROS (Figure 1.4). The preventive antioxidants acting in the first defense line suppress the formation of free radicals and active oxygen species. The radical scavenging antioxidants are responsible for the second defense line and inhibit chain initiation and or break the chain propagation. The antioxidant enzymes such as phospholipase, protease, DNA repair enzymes and transferase act as the third line of defense. In addition, the appropriate antioxidant is generated and transferred to the right site at the right time and at the right concentration when the oxidative stress takes place [19].

The toxic effects of normal oxygen metabolism are combated regularly by a number of endogenous antioxidant defense and protective mechanisms in the living cell.

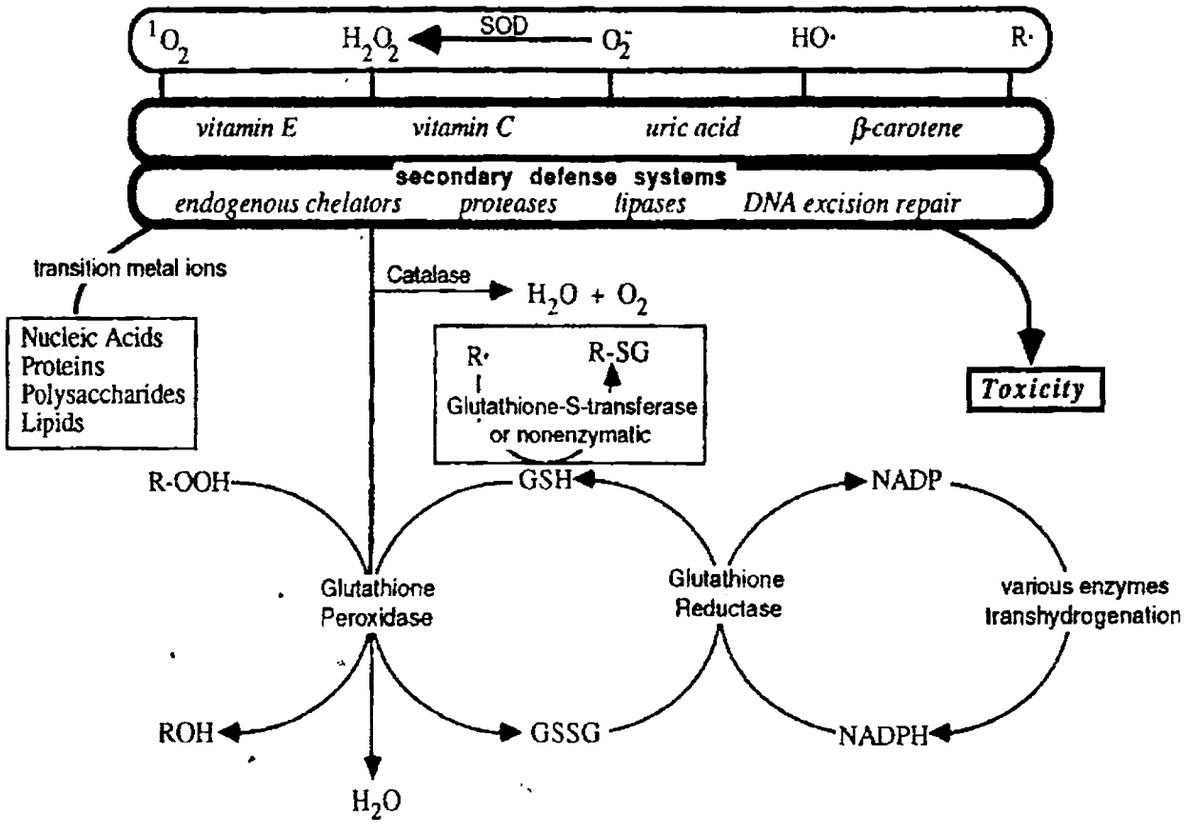


Figure 1.4 : Biological Antioxidant defense systems

The capacity of such protective systems, however, gradually decreases with age leading to a gradual loss of immunity to diseases, malfunctioning of vital organs and eventually to death. Problems associated with aging and age related diseases continue to be a burden on the health care system in many of the developed nations. Hence the need exists to look for new phytochemicals in conventional foods that can offer improved protection against oxygen radical - induced damages. These are usually referred as nutraceutical or phytoceuticals. They require higher dosages to maintain antioxidant status.

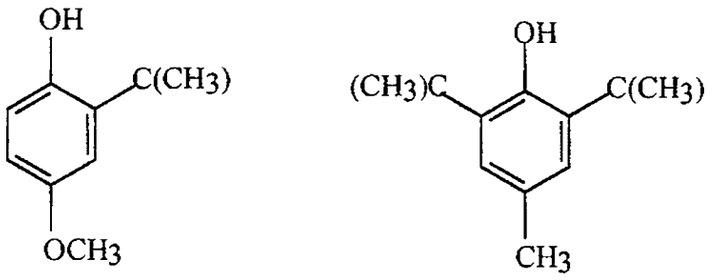
1.4. 3. Nature and applications

Antioxidants are widely used in processed foods and also pharmaceutical, cosmetic, essential oils and plastics for food processing. There are two types of antioxidants, synthetic and natural.

1.4.3.1.Synthetic antioxidants

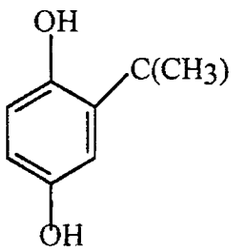
The commonly used synthetic antioxidants in foods to control oxidation and prevent off-flavor developments include Butylated hydroxy anisole(BHA), butylated hydroxy toluene (BHT), Propyl gallate (PG), Tertiary butyl hydroquinone (TBHQ) etc. at a maximum permitted usage level of 200 ppm (Figure 1.5) (9,28,34). BHA and BHT are monohydric phenolic compounds. BHA is more soluble in fats than BHT and are both considered to be more effective in less unsaturated animal fats than polyunsaturated vegetable oils. The trihydric PG is less oil soluble than the monohydric BHA and BHT [9]. Due to their volatile nature, both BHA and BHT are important additives used in packaging materials. TBHQ is regarded as the best antioxidant for protecting frying oils against oxidation [28]. It provides good carry through protection similar to that of BHA and BHT. It is adequately soluble in fats and does not complex with iron and copper,

therefore it does not discolor the treated products unlike PG. Synthetic antioxidants are less expensive than natural antioxidants.

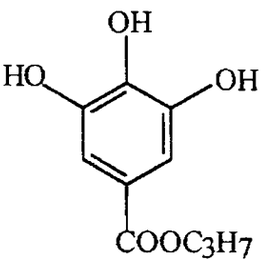


BHT

BHA



TBHQ



Propyl gallate(PG)

Figure 1.5: Structure of Synthetic antioxidants BHT, BHA, TBHQ and Propyl gallate

The role of antioxidants is constantly increasing. However, the consumer is becoming increasingly concerned about the use of traditional synthetic antioxidants due to safety considerations. The possible toxicity of the synthetic chemicals used as antioxidants has been studied for many years. FDA (Food and Drug Adulteration Act)

had expressed concern over the effect of BHT on the conversion of ingested materials in to toxic or carcinogenic substances by the increase of microsomal enzyme [35]. After reports concerning BHA to be carcinogenic in rats. it has been removed from the GRAS (Generally Recognized as Safe) list by the FDA [36]. The most powerful synthetic antioxidant, TBHQ is not allowed for food application in Japan, Canada and Europe and chances are high that other countries may follow the same [37].

1.4.3.2.Antioxidants from Natural sources

There is growing interest in naturally occurring antioxidants because of the adverse effect of synthetic antioxidants and the worldwide trend to avoid or minimize the use of artificial food additives.

The chemical composition of plant species varies considerably and this variation is largely the result of selection pressure from environmental and biotic factors. Each plant species has co-evolved with a large number of other plant species, insects, plant pathogens, soil microbes, nematodes, grazing animals, birds and other organism [38]. The response of the plant to evolutionary pressures caused by these interactions is sometimes expressed as the production of specific phytochemicals known as secondary metabolites. They influence the interaction in favor of the plant. Most chemicals that we now value for biological activity are, therefore, likely to be the products of the evolution of chemical defenses against invading organisms. Compounds that are highly toxic to vertebrates and invertebrates have proved to have valuable pharmacological properties and phytochemicals that combat pathogen attacks are often active against human pathogens.

Flavonoids are the flowering pigments in most angiosperm families' [39]. However, their occurrence is not restricted to flowers but include all part of the plant.

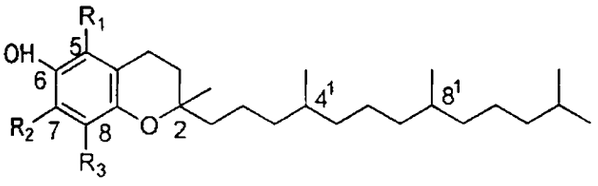
They have tremendous biological functions. Terpenoids, which are constituents of essential oil have been used for many years as preservatives and disinfectants. It follows that the antifungal and antibacterial properties of these compounds must have some role in plant defenses against plant pathogen. The function of Vitamin E and sterols in membranes, Vitamin C as cofactors of enzymes, carotenes as receptors of photones, anthocyanins as pigments for propagation are also known. These all compounds have important properties as phytoceuticals in human health and nutrition

Plant life in its various forms is widely recognized as the most readily available, abundant source of new chemicals to meet the growing needs of the agricultural and pharmaceutical industries [40]. The array of chemicals produced by plant is enormous. Further more, the diversity of biological activities exhibited by different groups of compounds offer countless opportunities for practical applications. Thus plants are an important source of natural antioxidants [41]. They include tocopherols, vitamin C, carotenoids, phenolic compounds, flavonoids, isoflavones, lignans, tannins, coumarins etc.Together, these compounds produce an array of antioxidants, which may act by different mechanisms to confer an effective defense system against free radical attack [43].

Vitamins

Tocopherols (VitaminE compounds) are one of the most important natural antioxidant found in plants especially in oil seeds, legumes and cereals. They occur as different homologues varying in the extent of methylation of the chroman ring (α,β,γ and δ) (Figure 1.6). Tocopherols have a saturated side chain, whereas the tocotrienols have a unsaturated side chain. Tocopherols can interrupt lipid autoxidation by interfering with

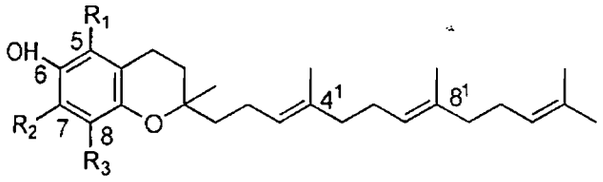
Tocopherols



Tocol

	R_1	R_2	R_3
5,7,8-Trimethyl tocol (α-tocopherol)	CH_3	CH_3	CH_3
7,8-Dimethyl tocol (β-tocopherol)	H	CH_3	CH_3
5,8-Dimethyl tocol (γ-tocopherol)	CH_3	H	CH_3
8-Methyl tocol (δ-tocopherol)	H	H	CH_3

Tocotrienols



Tocol

	R_1	R_2	R_3
5,7,8-Trimethyl tocotrienol (α-tocopherol)	CH_3	CH_3	CH_3
7,8-Dimethyl tocoltrienol (β-tocopherol)	H	CH_3	CH_3
5,8-Dimethyl tocoltrienol (γ-tocopherol)	CH_3	H	CH_3
8-Methyl tocoltrienol (δ-tocopherol)	H	H	CH_3

Figure 1.6: Structure of tocopherols

either through chain propagation or the decomposition process. In addition to its free radical scavenging activity, α-tocopherol is highly reactive toward singlet oxygen and protects food lipids against photosensitized autoxidation (Figure 1.7) [44].

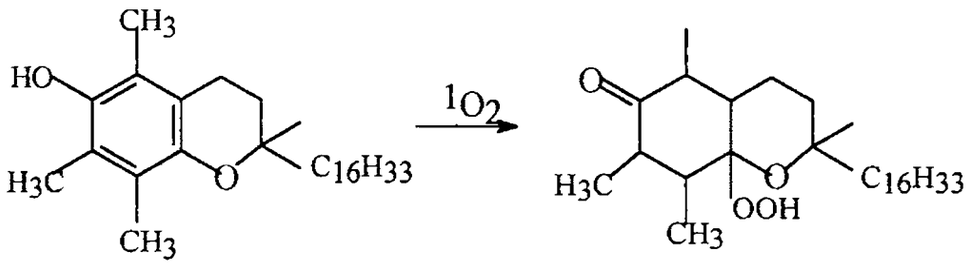


Figure 1.7: Singlet oxidation of α-tocopherol to hydroperoxy dienone

Carotenoids

Carotenes especially βcarotene is an effective $^1O_2$ quencher. The quenching was due to an energy transfer from $^1O_2$ to β-carotene[45]

$$^1O_2 + \beta\text{-car} \text{ --- } ^3O_2 + 3\ \beta\text{-car}$$

The quenching rate depends on the number of conjugated double bonds.

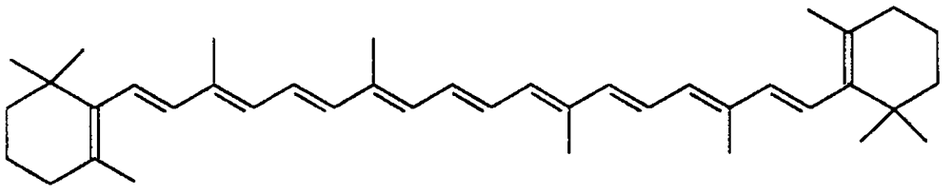


Figure1.8 :Structure of β-carotene

Flavonoids

Flavonoids are known to display a wide array of pharmacological and biochemical action. They are one of the most active natural antioxidant [46]. The potency of flavonoid with polyhydroxylated substitution was affected by the location of the hydroxyl substitution on the B-ring. Hydroxyl substitution in the ortho position in the B ring gave less antioxidant activity, however, hydroxyl substitution in the ortho position accompanied by an additional hydroxyl group in the para position enhanced activity. Strong antioxidant activity was found in compounds having hydroxylation in the para position in the B ring [47].

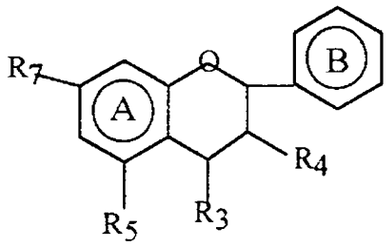


Figure1.9: Structure of Flavonoids

Phenolic acids

Phenolic acids are known to have antioxidant activity. They include caffeic acid, cinnamic acid, ferulic acid sinapic acid etc [48]. They act to inhibit lipid oxidation by trapping the peroxyl radicals.

$$LOO^{\cdot} + ArOH \longrightarrow LOOH + ArO^{\cdot}$$

$$LO^{\cdot} + ArO^{\cdot} \longrightarrow LOOAr$$

Phenolic acids that are hydroxyderivatives of cinnamic acid, such as caffeic , ferulic, sinapic and p-coumaric acids are more active antioxidants than hydroxy derivatives of benzoic acid ie, p-hydroxy benzoic, vanillic, syringic, and 3,4 hydroxy benzoic acid(Figure1.10) [49].

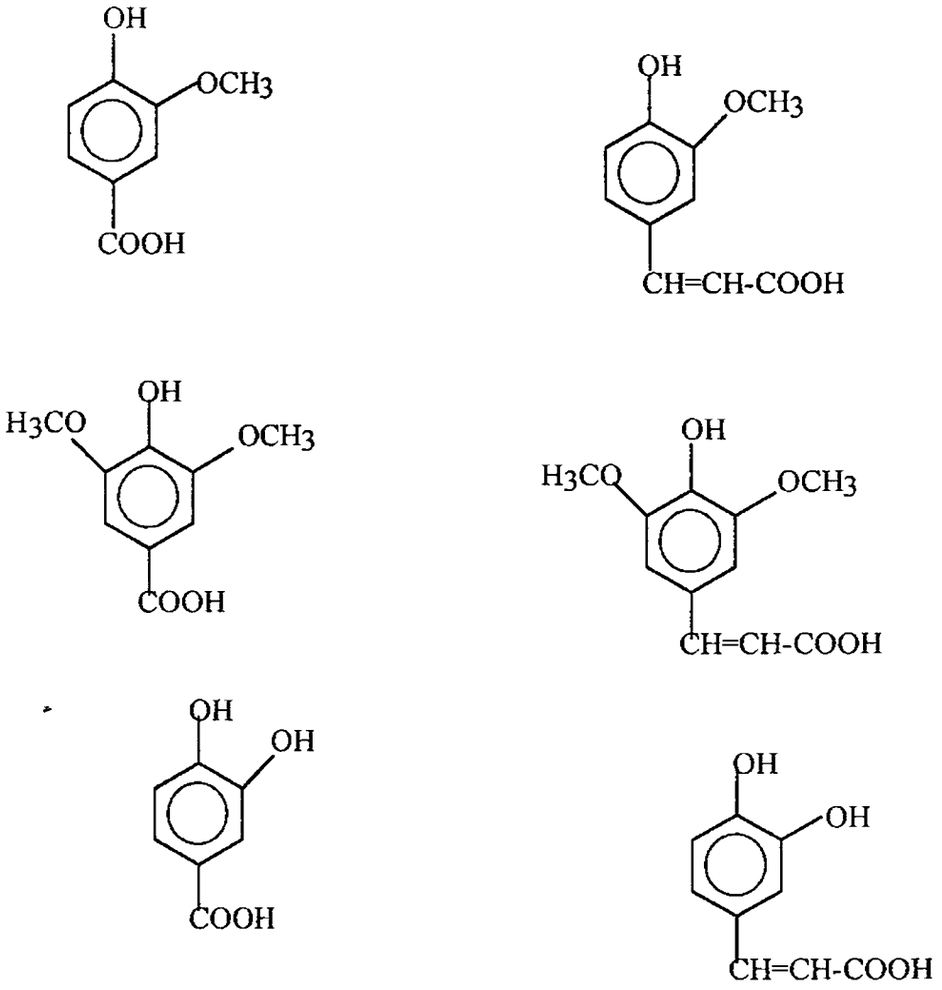


Figure 1:10 : Structure of Phenolic acids

Plant extracts

Herbs and spices and their extracts have shown to extend the shelf life of various foods [35, 50]. Rosemary and tea extracts have been successfully commercialized and have been under use in Japan, Europe and North America. Rosemary extracts provide a major source of natural antioxidants used commercially at present in foods. The antioxidant activity of commercial rosemary extracts is mainly related to its content of phenolic diterpenes carnosic acid and carnosol.

Both epidemiological and clinical studies have provided evidence that phenolic antioxidants present in cereals, fruits, and vegetables are principal contributing factors in accounting for the significantly reduced incidences of chronic and degenerative diseases encountered by populations whose diet is high in the intake of these foods. One of the important studies documented a striking lower incidence of heart diseases and cancer in the Mediterranean countries than in North America and North Europe [51]. The Mediteranean diet, which is rich in fruits and vegetables and olive oil appeared to account for this. Recently, proanthocyanidins have become of interest to medicinal chemistry. Among the many different types of biological activity ascribed to these grape seed components were vascular protecting activity, anti-cancer activity, anti-histamic activity, anti-viral activity, anti-inflammatory activity, antioxidant activity etc. The famous 'French paradox' disclosure was done by St.Leger [52]. In 1972, he made reference to the unusually low level of ischemic heart disease found in the French population that consumed moderate levels of red wine. St.Leger suggested the presence of some active ingredients, which is now known as proanthocyanidins to the protective influence on the cardiovascular system. Isoflavones (genistein and daidzein) are unique because soybeans

are the only significant dietary source of these biologically active compounds. Southeast Asian populations, such as those of Japan, Korea, and Taiwan, who consume 20-80mg of genistein/daidzein have a significantly lower incidence of breast and prostrate cancer [53].

1.4.3.3.Antioxidants from oil seeds

There are several plant constituents that may act as antioxidants to counteract the action of reactive oxygen species. This antioxidative defense mechanism of plant is due to enzymes, carotenoids, peptides etc. Plant seeds are protected from many environmental factors and hence they may contain different antioxidant compounds, which can be made useful [55]. Different types of oil seeds have been in use since antiquity for human consumption.

Olive oil

Olive oil is well known for its antioxidative components. Mediterranean diet is enriched with olive oil and hence its health effects. Considerable amount of phenolic compounds is present in olive [56,57]. Because of its high oleic acid content (53-85%) and antioxidant compounds, olive oil has exceptional stability even during frying.

Soybean

Soybean is cultivated for its seeds, which can be used as human food. The oil is also important. The antioxidants present in soybean include the isoflavones daidzein and genistein [58]. In addition to this, tocopherols and sterols are present in soybean. Sunflower and corn oil are also important dietary sources of Vitamin E and sterols.

Flaxseed

Flaxseed oil has been used mainly for industrial purposes. However it has been used for edible and medicinal purposes in certain cultures around the world. It contains 55-70% n-3 fatty acid, α-linoleic acid [59]. Lignans are an important class of compounds with many biological activities including cancer preventive properties. Flaxseed meal and flour are by far the most significant source of precursors to the mammalian lignans [60].

Rice

Rice is the second largest cereal grain in the world. Ricebran, which is a co-product in the rice milling process, contains 17-23% oil which can be extracted and used for edible purposes. Crude ricebran oil contains a mixture of ferulic acid esters of triterpenoid alcohol which are referred to as γ-oryzanols and exhibit antioxidant activity [61]. It also contains trans ferulic acid and tocopherols [62].

Oil Palm

Oil palm is one of the important sources of edible oil in tropical countries and is the second most widely consumed edible oil in the world after soybean. It contains important antioxidant tocopherols, tocotrienols and carotenoids and sterols [63].

Other important oil seeds include sesame, sunflower, safflower, peanut oil etc. These are also contains variety of antioxidants.

1.5.Sesame

Sesame (*Sesamum indicum* L.,) is probably the most ancient oil seed known and used by humans as a food source [64]. This annual seed crop has been cultivated for centuries, in the developing countries of Asia and Africa, for its high quality oil and protein. Sesame is mainly cultivated for its oil, although whole or dehulled seeds are used

in confectionery foods. Sesame oil is different from all other vegetable oils in many chemical, biological and physiological properties. These properties are due to the presence of endogenous unsaponifiable constituents viz, sesamol,sesamin, and sesamolin [65]. These compounds provide unusual stability to sesame oil. Sesame oil is a natural salad oil, requiring little or no winterization and is one of the few vegetable oils that can be used directly without refining [64]. Sesame is often called the " Queen of oil seed crops" because of the high quality oil and nutritious protein [66]. The meal remaining after oil extraction is rich in methionine, cystine and tryptophan [66,67]. The seed cake is also an excellent protein supplement in the animal feed industry.

1.5.1.World Production and Distribution

Sesame is grown primarily in the tropical and subtropical regions of the world [64]. World oil seed production is approximately 30 million tons of which 10% is sesame seed. Of this, the world sesame trade is nearly 20% of the seed production which is about 0.5 million tons valued over 5 million US$. The major producers and processors of the oil seed consists of India and China (25% each), Myanmar(10-13%), Sudan(10-12%), Japan (approximately 3%) etc. Though Japan is not a producer, is a major importer (25%) of total world imports and consumer followed by European countries, USA, Canada, North America etc. India and China are known for production, use and export of sesame.

1.5.2.History and Origin

Sesame is one of the oldest cultivated plants, and possibly with coconut, perhaps the oldest oil crop used by humans [64,68]. Its center of origin is not certain. Several researchers have described archaeological evidence, which indicated that sesame was a

highly prized oil crop in Babylon and Assyria 4000years ago (Presently Iraq and Syria)[69]. It was used medically and for culinary purposes.

Charred remains of sesame have been found at Harappa excavations and it indicates that sesame was in cultivation during the Indus Valley Civilizations [68]. Sesame seeds are very commonly used in the religious rites of the Hindus and are widely mentioned in Hindu mythology [64,68].

According to some historians, sesame seed was taken from Sudanese Islands to India several thousand years ago. From India, it spread to Egypt, China, Japan, Africa, South America, Central America and Mexico [64,68]. Based on the available archaeological evidence, the Fertile Crescent or the Indian Archipelago can be considered as the primary center of origin of sesame. Genetic variability among cultivated species are seen in India. Bedigian [64] suggested India as the origin of sesame based on many evidences.

1.5.3.Botany

The genus Sesamum is a member of the order Tubiflorae, family Pedaliaceae that comprises 16 genera and some 60 species [64]. There are about 36 species, most of which are wild, distributed mainly in the African Savanna, India, The East Indies and Australia [67]. A cultivated species, Sesamum indicum L; is the major commercial source of sesame seed and is primarily grown in India, China, Mexico and Sudan [64].

Sesamum Indicum L. is the commonly cultivated species of sesame and has 26 somatic chromosomes (2n=26). Other related species include S.alatum, S.lasinatum, S.angolence, S.prostratum, S.radiatum etc. [65].

Sesame is an erect, branched annual herb that grows 100-120 cms high. Two basic sesame types the long season, which has extensive and penetrating root system and the short season sesame, which has less extensive and shallow roots are known. In India the kharif (South west monsoon, May to October) and rabi(North east monsoon, November to April) season are the major sesame types.

Sesame is having simple or branched stems. Sesame types can be distinguished by the extent of hairiness in the stem. The leaves are highly variable in shape and size on the same plant and between varieties. The tubular, two-lipped flower is about 4.5 cm long with a pink or white corolla. One to three attractive flowers, which later develop in to the seedpods (capsules) are borne in each axil. The leaves of sesame are highly variable in shape and size on the same plant and between varieties [64,67].

The sesame fruit is a capsule rectangular in section and deeply grooved with a short, triangular beak. It is usually flat-sided and cylindrical in shape. Each capsule contains up to 50-100 seeds. Sesame seeds are pear-shaped, ovate, small, slightly flattened. The seeds may be smooth or reticulate, and may be white, reddish brown or black [64,67].

Oil, protein and carbohydrate are the major constituent of sesame (Table 1.3). The oil content varies with genetic and environmental factors. It contains 37-63% oil with an average of approximately 50%. Oil content varies considerably among different varieties and growing season. It is also related to color and size of the seed. Fatty acids of the oil mainly consists of oleic acid and linoleic acids, with small amounts of palmitic and stearic acids. The total unsaturated fatty acids amounts to approximately to 80% (Table 1.4) [64,65,66].

Table 1.3: Composition of sesame seeds [64,65,69]

Parameter	Range (%)
Moisture	4-6
Fat	37-63
Protein	19-31
Carbohydrate	14-18
Crude fiber	3-6
Ash	approx.5

Table 1.4: Fatty acid composition of sesame oil

Parameter	Range
Fatty acids(%)	
C< 14	<0.1
C14:0	<0.5
C16:0	7.0-12.0
C16:1	<0.5
C18:0	3.5-6.0
C18:1	35.0-50.0
C18:2	35.0-50.0
C18:3	<1.0
C20:0	<1.0
C20:1	<0.5
C22:0	<0.5

Table 1.5: Characteristics of sesame oil [64, 65, 66]

Characteristics	Range
Iodine value	104-120
Saponification value	187-195
Unsaponifiables(%)	2.0, max
Acid value(%)	
Virgin oil	4.0, max
Nonvirgin oil	0.6, max
Peroxide value (meq/kg)	10.0 max

Table 1.6: Mineral and vitamin contents of sesame seeds [64]

Component	content
Minerals(mg/100g)	
Calcium	1000-1483
Phosphorous	570-732
Iron	10-56
Sodium	60-80
Potassium	725-831
Vitamins	
Vitamin A(IU)	Trace-60
Thiamin(mg/100g)	0.14-1.0
Riboflavin(mg/100g)	0.02-0.34
Niacin(mg/100g)	4.40-8.70
Pantothenic acid (mg/100g)	0.6
Ascorbic acid(mg/100g)	0.5
Folic acid(ug/100g)	51-134
Vitamin E(mg/100g)	
Total(α-tocopherol equv.)	29.4-52.8
αtocopherol	1.0-1.2
β-tocopherol	0.005-0.6
γ-tocopherol	24.4-51.7
δ-tocopherol	0.05-3.2

Sesame seed contains 19-31% protein with an average of 25%. Amino acid composition also varies among the species. Sesame protein is rich in methionine content.

Sesame seed contains 18-20% carbohydrates, which comprised small amounts of glucose, fructose etc. Starch is absent in sesame. Most of the carbohydrate seems to be present as dietary fiber (approximately 11%).

Sesame seed is a good source of minerals especially calcium, Potassium, phosphorous and iron (Table 1.6). The mineral content is approximately 4-6%. Selenium is also present in sesame. Se is a constituent of glutathione peroxidase, which is associated with the prevention of physiological peroxidation, but in excessive amounts, it has a negative effect [70].

Sesame seed is an important source of certain vitamins, especially niacin, folic acid and Vitamin E (Table 1.6). γ-tocopherol is the predominant tocopherol in the sesame. Sesame seed is almost devoid of antinutritional factors and is suitable for consumption as such or after processing. However, it contains oxalate and phytate, which adversely affect mineral availability in human nutrition.

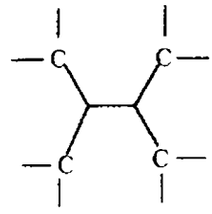
The important properties of sesame are attributed to the presence of unique compound lignans. Lignans are a group of natural products of phenyl propanoid origin, which are widely distributed in nature. They display important physiological functions in plants, particularly in plant defense and are most efficacious in human nutrition and medicine, given their extensive health protective and curative properties. Much interest has been focussed on their effectiveness as antineoplastic agents and research in this area has revealed several modes of action by which they can regulate the growth of mammalian cells [42,71].

The wide spread distribution of lignans, moreover, suggests that they play an important role in plant evolution. They possess antitumor, antimitotic, antiviral,

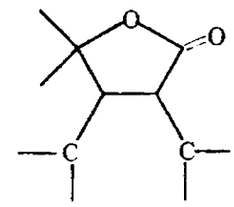
antimicrobial effects etc. Lignans isolated from certain plants have anti cancer effects. They showed activity towards insects and pests [71,72].

Lignans are formed biosynthetically by the Shikimate pathway [71]. They include :

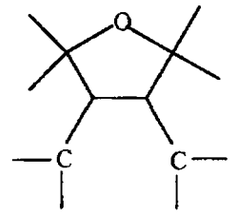
- derivatives of butane (A);
- lignanolide, or derivatives of butanolide (B);
- monoepoxy lignans, or derivatives of tetrahydro furan (C) and
- bis epoxy lignans viz, derivatives of 3,7-dioxabicyclo (3.3.0)-octane (D).
- Tetra hydro naphthalene (E) or naphthalene (F) derivatives are included in cyclolignans. (Figure 1.11)



(A)



(B)



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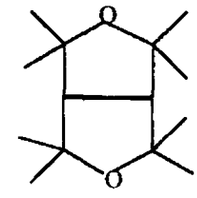

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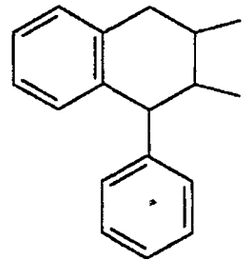

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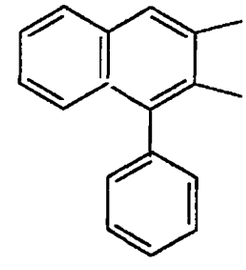


(F)

Figure 1:11 Structure of lignans

1.5.4.Current status on Sesame Phytochemicals

1.5.4.1. Chemistry and antioxidant activity

Sesame seeds are rich in furanofuran lignans, of which sesamin and the oxygenated derivative (sesamolin) are the most abundant. The biosynthetic pathway suggested for their formation is depicted in Figure 1.12 [73]. Pinoresinol seems as the precursor to lignans in sesame seed. Using a combination of radio and isotopically labeled precursor administration experiments, it was found that E-coniferyl alcohol undergoes stereoselective coupling to afford (+) pinoresinol in Sesamum indicum seeds. It is further converted in to (+)piperitol, (+) sesamin and sesamolin.

Sesame seed contains bisepoxy lignans sesamin and sesamolin which are mainly responsible for the important properties of sesame (Figure 1.13). Acetone extract of sesame seed possessed antioxidant activity by thiocyanate method and it contained sesaminol, sesamolinol and trans feulic acid [74,75]. After treating defatted sesame seed flour with β-glucosidase, it was revealed that the phenolic compounds exist as their glycosides, which release their aglcones [76]. Large proportions of the lignans were assumed to be present in the form of lignan glucosides in sesame seed.

Katsuzaki etal., isolated two new lignan glucosides from 80% ethanolic extract of defatted sesame seed [77]. Two pinoresinol diglucosides(kp1 and kp2) that exhibited strong antioxidant activity in a rabbit erythrocyte membrane ghost system have been isolated from sesame seed and identified.

Antioxidative lignan glucosides were isolated from 80% ethanolic extract of defatted sesame seed [78]. The compounds are sesaminol 2'-*o*-β-D-glucopyranoside,

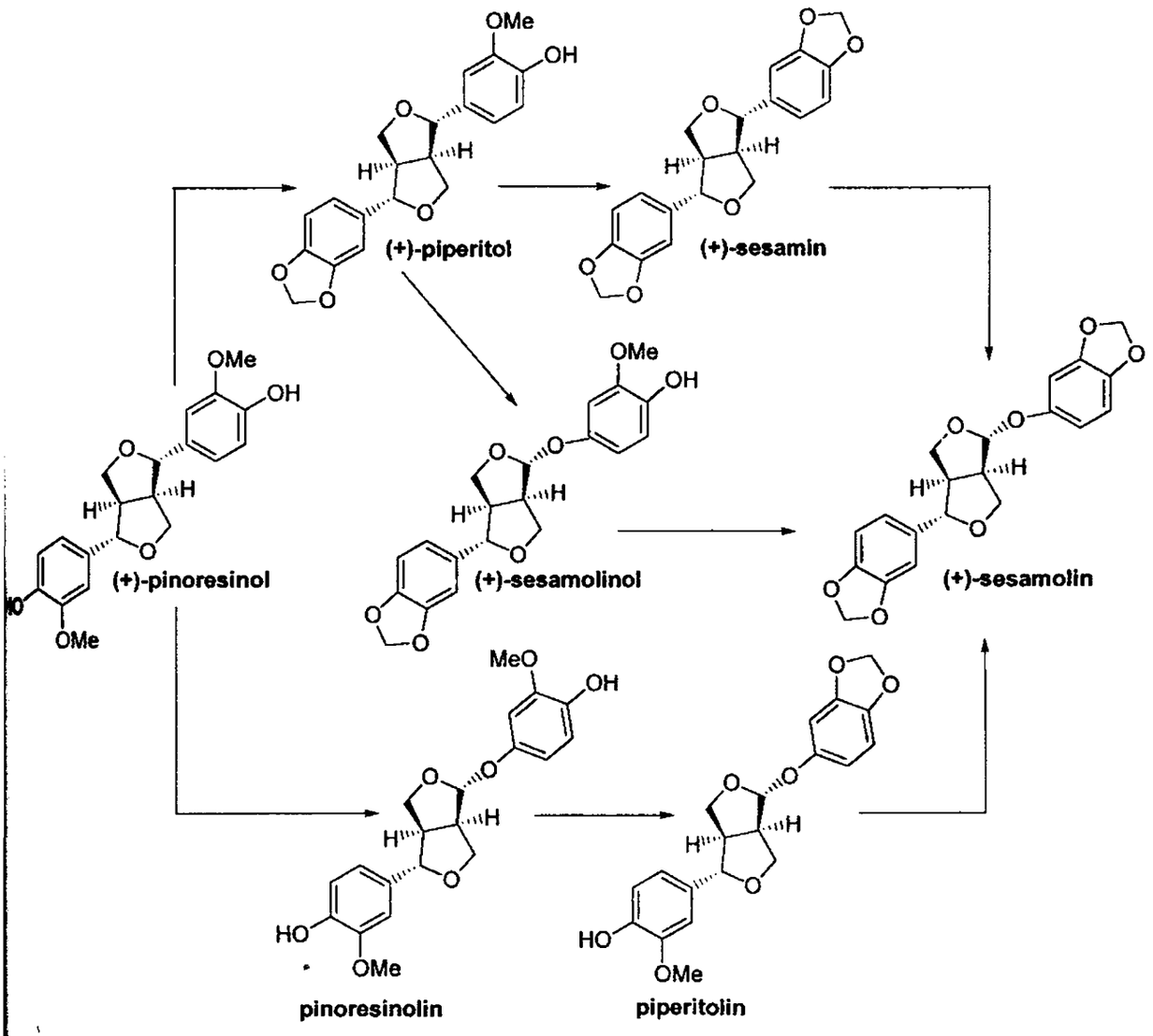
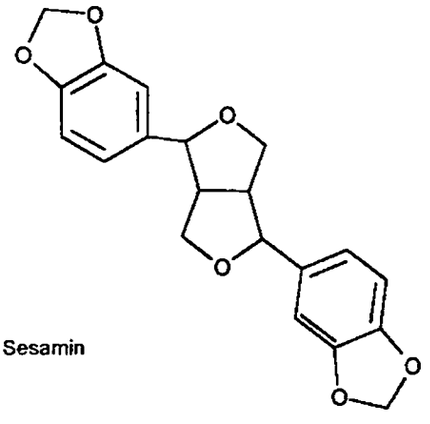
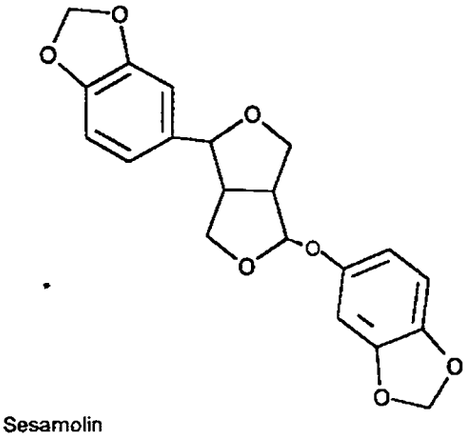


Figure 1.12 Scheme for possible biosynthetic routes to the sesame lignans, (+)-sesamin and (+)-sesamolin from (+)-pinoresinol



Sesamin



Sesamolin

Figure 1:13 : Structure of sesamin and sesamolin

sesaminol 2'-*o*-β-D glucopyranosyl(1-2) -*o*-β-D glucopyranoside and sesaminol 2'-*o*-β-D-glucopyranosyl (1-2)-o-[β-D-glucopyranosyl(1-6)]-β-D-glucopyranoside.

According to Dabrowski and Sosulski [79], 80% ethanolic extract of sesame seed yielded trans caffeic, trans-p-coumaric and trans-ferulic acid in decreasing order of abundance. However, Kozlowska etal, [80] reported that vanillic , syringic sinapic and 0-coumaric acids were also present in sesame seed.

Kamal-Eldin compared various chromatographic methods for separation and quantitation of sesame lignans, tocopherols and sterols in S.indicum L. and three wild species. The extracted oil from the seeds and the unsaponifiable matter of the oil was chromatographed to separate the compounds. They used various chromatographic methods to separate and identify the compounds [81,82].

Sesame oil is obtained from roasted and unroasted seeds [83]. Unroasted sesame seed oil is obtained from expeller pressing and refined by alkaline treatment, bleaching with acid clay and deodorizing. Oil from roasted seed is only filtered to remove contaminants.

Sesaminol is found in high quantities (0.5-1.0 mg/g) in purified unroasted oil, however it is present in minor quantities in sesame seed. This is due to the chemical change occuring during refining process. Fukuda etal, suggested trhat sesaminol was formed from sesamolin under anhydrous conditions in the presence of acid clay catalyst at high temperatures [83,84]. Scission of sesamolin between actal oxygen and carbon produce an oxonium ion and sesamol. Electophilic addition of sesamol at the ortho position to the oxonium ion resulted in the formation of sesaminol (Figure1.14). These changes are taking place during the decolourization step.

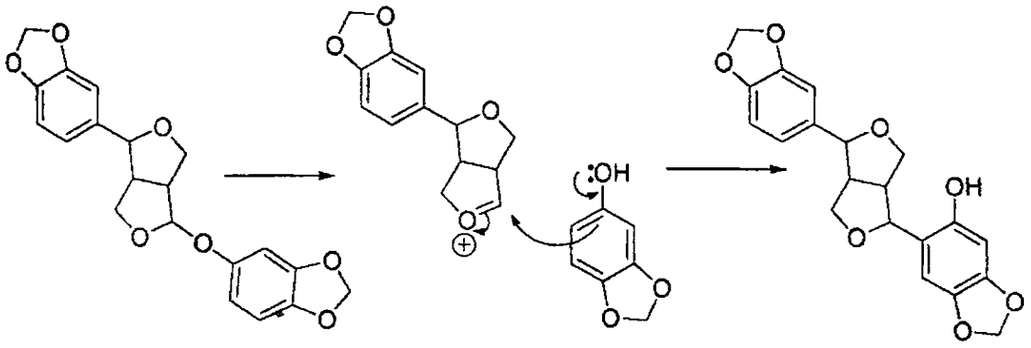


Figure 1.14: Scheme for mechanism of formation of sesaminol from sesamolin

Fresh oils obtained from coated and dehulled sesame seeds of Egyptian and Sudaneese varieties contain sesamin and sesamolin in different ratio (85). Roasting (200^0C, 20 min), steaming (100^0C, 20 min), roasting (200^0C, 15 min) combined with steaming (7min) and microwaving (2450Hz,15 min) of seeds reduced the sesamin content drastically, especially in the oil from dehulled seeds. Storage of these oils under Schall oven test conditions (65^0C) showed a decrease in both sesamin and sesamolin contents. The decrease in sesamolin content was greater during the storage period for all oils. It may probably due to the degradation of sesamolin to sesamol during the thermal treatment. Roasting combined with steaming treatment gave a lesser reduction in sesamin, sesamolin and γ-tocopherol contents.

Sesamolin is the characteristic lignan of sesame seed and serves as the precursor to strong antioxidants. Screening of sesame seed varieties revealed, however, that white sesame seeds contained more sesamolin than the black seed varieties, the ratio of sesamolin to sesamin was higher in black seeds than in white seeds [67].

1.5.4.2.Biological activity

A novel biological function of sesamin, as a specific inhibitor for the Δ5-desaturating enzyme was demonstrated [86].Sesamin and related lignans (sesamolin (+), sesaminol and (+) episesamin) present in sesame seed or its oil are specific inhibitors of Δ5 desaturase in PUFA biosynthesis in both microorganisms and animals.

Mimura et al., produced antioxidative products from methanolic extract of cultured callus cells of sesame [87]. It showed the presence of sesamin and sesamolin. Sesamol, γ-tocopherol or ascorbic acid are absent. However the extract had strong antioixidative activity in the linoleic acid autoxidation system as well as in the peroxide

induced erythrocyte ghost cell membrane system. Antioxidative components in the cells were isolated and identified as new caffeic acid glucosides and they are as active as BHA.

The suppressive activity of sesaminol was observed on lipid peroxidation induced by t-butylhydroperoxide in cultured human diploid fibroblast [88]. Sesaminol was as strongly suppressive as tocopherol in mutagenicity of E.coli induced by peroxidation of membrane lipid of erythrocytes.

In experiments studying the effect of sesame on senescence accelerated mice (SAM), suppressive effect on senescence was observed [89]. The results were obtained also with the addition of sesaminol to a 50% Vit.E deficient diet. Noticeable results concerning the antiaging effect of sesame have been shown in a series of animal experiments conducted by Yamashita and Namiki in 1990. The suppressive effect of sesaminol on invivo lipid peroxidation was also observed in the increase of liver TBARS levels when CCl_4 was administered to rats.

Sesame contains γ-tocopherol with only trace amounts of α-tocopherol [90]. The Vit.E effect of sesame was compared with that of α-tocopherol and γ-tocopherol in rat models. Lipid peroxidation (plasma and liver TBARS values), oxidative hemolysis and plasma pyruvate activity were determined as indices of Vit.E status. The results suggets that the presence of some components in sesame that cause an increase of γ-tocopherol concentrations in plasma and liver, presumably resulting in the prevention of increase in TBARS and other indices caused by a Vit.E free diet. Synergistic effect of sesame lignans may result in enhancement of Vit E.activity of γ-tocopherol and as a result prevent lipid peroxidation invivo [91].

Highly unsaturated fatty acids, such as arachidonic acid, dihomo-γ-linoleic acid, and eicosapentanoic acid (EPA) have important biological functions, in particular as precursors of prostaglandins. To develop large-scale production of these unsaturated fatty acids using a microbiological procedures. Yamada, Shimizu etal., found that fungal mycelia are rich sources of these fatty acids [92]. They also found that incubation with sesame oil increased the dihomo-γ-linoleic acid (20:3, n-6) content and decreased in the arachidonic acid content [93]. This interesting effect of sesame oil was caused by specific inhibiting activity of sesamin and other lignans present in sesame on the Δ5-desaturase in polyunsaturated fatty acid biosynthesis in microorganisms' [94].

Sugano etal., demonstrated that sesamin interferes with Δ5-desaturase even in liver microsomes, which results in considerable modification of the fatty acid profile of liver phospholipids [95]. The specific inhibition of Δ5-desaturase and chain elongation of C_{18} fatty acids by sesamin, especially in n-6 polyunsaturated fatty acid biosynthesis was also observed in rat primary cultured hepatocytes [96].

Sesamin is reported to have hypocholesterolemic activity. Sesamin reduced blood cholesterol levels of rats, which were fed a purified diet or commercial chow irrespective of dietary cholesterol. Sesamin also reduced the concentration of liver cholesterol especially in diet containing cholesterol [97].

The effect of sesamin on 7,12-dimethylbenz-(α)-anthracene- induced rat mammary cancers were studied by Sugano etal., and found that sesamin at the dietary level of 0.2% , considerably reduced the cumulative number and the mean number of mammary cancers ass compared to control [98].

Protective effects of sesamin against liver damage caused by alcohol or carbon tetrachloride were studied in rodents' [95,99]. When a mixture of sesamin and episesamin (51.1:48.2) was given to rats at a dietary level of 0.5% for 13 days, the proportion of dihomo-γ-linoleic acid significantly increased not only in the liver but also in plasma and homocytes suggesting an interference with Δ5-desaturasion by these lignans. Sesamin showed protective effect against accumulation of fat droplets and vacuolar degeneration in the mouse liver. Also, sesamin tended to prevent liver lipid accumulation by carbon tetrachloride in mice. These results suggests that sesamin have the activity to improve liver function [100]

1.6.Relevance of the present investigation

Since an antioxidant is an unavoidable additive, the food, pharmaceutical and cosmetic industries are looking for alternate natural sources. The commonly used tocopherols have weak antioxidant properties. The use of carotenoids is limited due to its high lipid solubility and intense coloration even at low concentrations. Certain antioxidant extracts were also available in market. The disadvantage of these extracts is that they can not be used at high concentrations, especially the odoriferous ones, since they impart undesirable flavour taints to foods and other products. In the case of phenolic extracts, the solubility of such antioxidants in vegetable oils is disputable since they are lipophilic.

Antioxidants has to be developed which will be effective in protecting lipid containing foods as well as emulsion based food systems. To use the antioxidant as a phytoceutical, its solubility in aqueous phase is also essential. Hence, our investigation

has focussed mainly on the above two aspects in developing an antioxidant extract and we concentrated on oil seeds and their industrial byproducts especially sesame.

Sesame is one of the most ancient cultural crops of India. Out of its total production, 80% is used for oil extraction and other for direct edible purposes. Seed and oil are extremely used in culinary purposes. The ancient Indian medical system of Ayurveda mentions the virtues of sesame and sesame oil. In India, sesame oil forms the basis of most of the fragrant or scented oils used in perfumery. Due to high stability it can be used as a carrier for medicines which are administered subcutaneously or intravenously. Though India is known for sesame seed cultivation and use, research works on antioxidant components of sesame was mainly carried out by Japanese.

Sesame oil, which is highly unsaturated and obtained by commercial extraction, is well known for its unusual stability. This is attributable to the antioxidant compounds sesamol, sesamin and sesamolin. Preliminary studies showed the presence of these antioxidants in appreciable amounts in sesame cake also. Hence it appears as a promising source for antioxidants.

Sesame cake is the byproduct of commercial oil milling industry. Presently it is used as a cattle feed. A thorough literature survey showed that this byproduct is not effectively studied to develop it as an antioxidant source. Hence, the current investigation has been carried out on sesame and its byproducts in order to explore the possibility of developing a natural antioxidant source, which can substitute synthetic ones in oil and food industry. The biological effects of the sesame cake extract including radical scavenging effects, anticancer effects etc, were not so far studied. Furthermore, the wild

specis of sesame, *Sesamum malabaricum* found in the coastal regions of Kerala are not studied for their antioxidant and biological effects.

1.7.Structure of presentation

Chapter 1: Introduction-includes objectives and literature survey

Chapter 2: Materials and methods

Chapter3: Results and discussions

Chapter4: Summary and conclusions

References

CHAPTER 2

MATERIALS AND METHODS

2.1.Materials

Commercial samples of sesame seeds (*Sesamum indicum*) were obtained from Agricultural Research Station, Kayamkulam and M/S Idhayam Industries, Thiruchirappally. The three varieties viz. black, red and white seeds were collected. The seeds of wild variety i.e., *Sesamum malabaricum* were collected from the coastal areas of Alappuzha district and were identified with the help of Agricultural Research Station, Kayamkulam where a voucher specimen deposited.

Sesame oil was obtained from 'Idhayam' and 'O.M.S' mills. Commercial samples of sesame cake were obtained from local market as well as from 'Idhayam' industries.

Refined, bleached, deodorized (RBD) Soybean oil without the addition of antioxidants was supplied by Soybean oil Industry (M/S Sakti Soya's, Pollachi, Tamil Nadu, India). Similar RBD samples of Sunflower and Safflower oils without added antioxidants was obtained from M/S Marico Industries, Mumbai, India.

β-carotene, linoleic acid, Tween-20, BHA, BHT, and TBHQ were purchased from E.Merck.

DPPH, NBT, Xanthine, Xanthine oxidase,Cytochrome C, Ferrous chloride, Sesamol were from Sigma Chemical company, USA. All other chemicals were of analytical grade.

2.2.Methods

2.2.1.Preparation of standard sesamin and sesamolin:

Sesamin and sesamolin were purified from sesame oil by the following method [85]. 100 ml of Sesame oil were passed through a packed column of alumina (30 cm x 3.5 cm i.d, filled with 125 g alumina) and eluted with petroleum ether. Fractions were

collected and examined through Badouins' test which involves heating 2 ml of the oil with 1 ml of conc.HCl containing 1% (w/v) sucrose. The portion being eluted immediately below the strong yellow band in the column was removed and continuously extracted with petroleum ether in a soxhlet apparatus for 3 h. Yellow oil was obtained after removal of the solvent; it was saponified with 5 % (w/v) alcoholic KOH for 1 hour. Water (100 ml) was added to the mixture, and the resultant solution was extracted three times with 30 ml portions of diethyl ether. Removal of the solvent afforded approximately 2 g of a yellow resin, which was subsequently dissolved in 10 ml of diethyl ether and left overnight in a refrigerator whereupon 0.5 g of crystalline sesamin was precipitated. Rod- like needles was obtained upon recrystallization of sesamin from ethanol. The residue after removal of the ether was dissolved in 1 ml chlorofom, and petroleum ether was added until the onset of cloudiness. Sesamolin, separated as a white solid, was subsequently recrystallized from ethanol as white plates.

2.2.2.Preparation of sesamol dimer:

To a solution of 1.0 g of sesamol in 100 ml of water, 4.0 ml of 31 % H_2O_2 and 20 mg of HRPO were added [101]. The solution immediately turned violet and afforded a precipitate. The mixture was kept at room temperature for 5 minutes, then diluted with 500 ml of water and extracted with 300 ml of chloroform three times. The combined extract was subjected to TLC. The extracts were evaporated to dryness in vacuum to obtain a gum. The gum was applied to a column of silica gel (60 g) and eluted with $CHCl_3$ to afford the product sesamol dimer II and a small amount of III. II was rechromatographed on a column of silica gel to obtain pure sesamol dimer.

2.2.3.HPLC analysis

High Performance Liquid Chromatographic analysis was carried out in a Shimadzu make binary system with LC-10AD model pump, a Rheodyne injector fitted with a 20 µl sample loop, a SPD-10A UV- Visible detector, with a C-R7Ae plus integrator for data acquisition, analysis and display.

Column : Reverse phase column, Waters µ-bondapak C_{18} column (4.6 mmX 25cm) with a guard column of C_{18}(Supelco)

Mobile phase : Methanol : water (70:30)

Flow rate : 1 ml/minute

Wave length of detection : 290 nm

The UV-Visible detector was set at 290 nm, with a detector sensitivity of 0.005 AUFS.

The extracts were filtered through PTFE membrane before injection into HPLC. Standard sesamol was dissolved in methanol. Concentration of the standard was determined spectrophotometrically using Beer-Lambert's law. Various concentration of standard in the range of 0.2-1µg/20µl in methanol was prepared and injected to HPLC to check the linearity between concentration and peak areas, and response factor was calculated. Standard solution of sesamin and sesamolin was also prepared by dissolving the required amount in methanol and suitable concentration was injected to know the retention time. Lignan glucosides were calculated based on the response factor for standard sesamol.

Preparative HPLC was done with Shimazdu system with ODS column. The solvent system was Methanol:water(70:30)

Spectrophotometric readings were taken in a Shimadzu 160-UV specrophotometer.

IR spectra were recorded in a Nicolet IR spectrophotometer.

NMR spectre was taken in a Bruker AVANCE DPX_{300} series. Chemical shifts are expressed in ppm units with respect to TMS.

MS were taken in a GCMS-QP5050 Shimadzu spectrometer with EI mode.

2.2.4.Total phenolic Content (TPC)

TPC of the extract was measured by method (9.110) of the Association of Official Analytical Chemists AOAC(1984) [102]. Briefly, sesame extract (0.1ml) was accurately transferred to a 100 ml volumetric flask containing 75 ml distilled water. 5 ml Folin-Ciocalteu reagent and 10 ml of saturated sodium carbonate solution were added to the flask and diluted to 100 ml with distilled water. The mixture was then shaken for 1 minute and allowed to stand at room temperature for 30 minutes. Absorbance of the solution was measured in a Shimazdu make (model UV-160A) UV-Visible spectrometer at 760 nm. The experiment was repeated thrice and the mean value taken.

2.3.Composition analysis of seed/cake

2.3.1.Moisture

This was determined by air oven method as outlined in AOAC [102].

2.3.2.Fat Determination

Fat was extracted from 10g of samples using solvent hexane in a soxhlet apparatus according to AOAC procedure [102].

2.3.3.Total sugars

Preparation of standard glucose solution

0.25 g of glucose was weighed out accurately, transferred to a 100 ml standard flask and made up to the mark with distilled water. 1 ml of this solution was pippetted out in to a 10 ml standard flask and made up to the mark with distilled water.

Preparation of reagent

2.5 g thiourea and 0.125 g of anthrone were weighed out in 250 ml of 66% H_2SO_4 (165 ml H_2SO_4 + 85 ml H_2O) heated at 80-90^{0}C and cooled.

Sample preparation

The dry methanolic extract was redissolved in methanol and made up to 100 ml. From it 5 ml was taken and made upto 50 ml.

Procedure

0.2, 0.4, 0.6, 0.8, 1 ml of glucose solution and 0.2. 0.4, 0.6, 0.8 ml sample solution were pippetted in to stoppered tubes, made up to 1 ml with water. 10 ml of anthrone reagent was added, warmed in a boiling water bath for 15 minutes, cooled in tap water, kept in dark for 30 minutes. Absorbance was measured at 620 nm.

2.3.4.Determination of Protein- Kjeldahl's method

Reagents

(1) Digestion mixture - Mixture of 2.5 g SeO_2, 100 g K_2SO_4 & 20 g $CuSO_4$.

(2) Boric acid solution (2%) - 2 g boric acid in 100 ml H_2O

(3) Mixed indicator - 0.1 % bromocresol green in ethanol & 0.1 % methyl red in CH_3OH (10:2)

(4) 40% NaOH - 40 g NaOH in 100 ml Distilled water.

Procedure

1 g of sample and 0.5 g digestion mixture was taken in a kjeldhal's flask. 10 ml of concentrated H_2SO_4 was added to it. The flask was heated on a hot plate in slanting position until the color of the solution changes to blue green. This clear solution was made up to 50 ml in a standard flask (Cold condition).

RB flask, half filled with water was connected to the kjeldahl's apparatus and heated on a mantle. 20 ml of boric acid and 1 ml indicator was taken in a small conical flask and was placed under the condenser. 5 ml sample with 20 ml 40% NaOH and a few ml H_2O were added to the distillation tube through the funnel. Water inside the RB boils and steam passes in to the distillation tube. NH_3 evolved from the distillation tube was trapped in boric acid and when maximum ammonia was evolved the color of the boric acid solution changes to blue. Then keep it for 2 more minutes. It was then titrated against 0.01N HCl.

Lowry's method

Reagents

(1) 2% Na_2CO_3 in 0.1 N NaOH (Reagent A)

(2) 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate (Reagent B)

Alkaline Cu reagent: Mix 50 ml A + 1 ml B

(3) Folins-ciocalteu reagent

(4) Protein solution (stock standard)- 50 mg BSA dissolved in 0.1 N NaOH and made up to 50 ml in a standard flask.

Procedure

Sample preparation

Dry methanolic extract was redissolved in methanol and made up to 100 ml.

0.2, 0.4, 0.6, 0.8, 1 ml of standard and 0.1, 0.2, 0.4 ml of sample were taken, made up to 1 ml with 0.1 N NaOH , kept for 10 minutes. Then add 5 ml of alkaline Cu reagent, kept for 10 minutes. Shake well. Finally 0.5 ml of Folin's reagent added kept for 30' in dark and OD read at 660 nm.

2.3.5.Peroxide value [103]

Reagents

(1) 0.01 $Na_2S_2O_3$ solution [0.1Nsolution = 2.48 g $Na_2S_2O_3.5H_2O$ in 100 ml distilled water. 25 ml of this solution is diluted to 250 ml to get 0.01Nsolution, eq.wt of $Na_2S_2O_3.5H_2O$ = 248]

(2) 10% KI solution (2g in 20 ml)

(3) Concentrated HCl

(4) Starch

(5) 0.01N $K_2Cr_2O_7$[0.1Nsolution-0.49g in 100ml]

(6) 3:2 $CHCl_3$-CH_3COOH

(7) Saturated KI solution

Procedure

1. Standardisation of $Na_2S_2O_3$ solution

To 10 ml $K_2Cr_2O_7$ solution, 6 ml 10% KI solution and 1 ml conc.HCl were added and titrated against 0.1N $Na_2S_2O_3$ solution until a green color was formed.

2.Determination of peroxide value

To accurately weighed 5 g of sample, 30 ml 3:2 $CHCl_3$-CH_3COOH was added followed by 0.5 ml saturated KI solution is added and kept in dark for 1 minute. It was then titrated against $Na_2S_2O_3$ solution, added 30 ml H_2O. When a pale yellow colored solution was formed starch was added and titrated until the blue color disappears.

$$PV = \frac{V\ Na_2S_2O_3 \times N\ Na_2S_2O_3 \times 1000}{W\ sample}$$

2.3.6.Para anisidine value [104]

Reagents

(1) Trichloroacetic acid - (1.5 g TCA in 100 ml CH_3OH)

(2) P-anisidine - [0.25g in 100 ml CH_3OH]

(3) CH_3OH

(4) $CHCl_3$

Procedure

0.5 g oil in 25 ml $CHCl_3$ was prepared. Solutions in the proportions as shown in the table were taken in stoppered test tubes. These solutions were then heated at 60^0C for 60' and the absorbance at 400 nm of the colored solution was taken

$$P.A.V. = \frac{[E - E_0 - E_1] \times 10}{n}$$

E = Absorbance of sample solution

E_0 = Absorbance of common blank

E_1 = Absorbance of sample blank

n = weight of fat in 2 ml fat solution

2.3.7.Diene value [105]

Oil (0.02-0.04 g) was weighed in to a 25 ml volumetric flask, dissolved in isooctane and made up to the mark with the same solvent. The solution was thoroughly mixed and the absorbance was read at 234 nm. Pure isooctane was used as the reagent blank. The conjugated diene was calculated.

$$\text{Conjugated Diene value} = \frac{\text{Absorbance at 234 nm}}{\text{Conc.of oil in g/100ml x length of the cell in cm}}$$

$$\text{Conjugated triene value} = \frac{\text{Absorbance at 269 nm}}{\text{Conc.of oil in g/lx length of cell in cm}}$$

2.4.Extaction of antioxidants from Sesame seed/oil/cake

Sesame seed: (a) Dried and ground sesame seeds were extracted with Methanol for 16 hrs.in a soxhlet extractor. The extract was filtered, and the filtrate concentrated to get residue. It was redissolved in methanol and kept under refrigeration.

(b) Dried and ground sesame seeds were defatted with hexane for 12 hrs. After removal of oil the residue was dried and further extracted with methanol for 16 hrs in a Soxhlet extractor. The extract was filtered and the filtrate concentrated in a vacuum rotary evaporator at 50^0C. The residue dissolved in methanol and kept under refrigeration.

Sesame cake

(a) Sesame cake was dried and ground. The powdered sample was extracted with hexane, isopropanol, ethyl acetate, acetone, ethanol and methanol for 16 hrs in a soxhlet extractor. Each time the filtrate was concentrated and residue redissolved in methanol and kept under refrigeration.

Dried and ground sesame cake was successively extracted with Petroleum ether, chloroform, ethanol and methanol each time in 16 hrs in a soxhlet extractor. Each time filtrate collected. Filtrate was concentrated in a rotary evaporator at 50^0C and the residue redissolved in methanol.

Purification of extract

Sesame cake was dried and ground well. 100g was defatted with 1500 ml of hexane (3 to 4 times with 500ml hexane) under room temperature. The residue was dried. It was then washed with water (3 to 4 times with 500 ml of distilled water). Water washed residue was dried at 60-70^0C. Then it was again extracted with methanol (1500 ml) in a soxhlet extractor. The extract concentrated and redissolved in methanol.

2.5.Isolation and Characterisation

Methanolic extract (1g) was applied on to silicagel column(50g). The column was then eluted with hexane followed by a gradient of hexane:ethyl acetate(100% hexane through 100% ethyl acetate). 20 ml fractions were collected. Altogether 45fractions were obtained and they were combined according to their TLC profiles to yield 10 primary fractions. Antioxidative components were eluted in 5,6, 8,9,10 fractions. The glycosidic portions and sesamol were obtained by prep.HPLC of the extract. Altogether 5 compounds were isolated. Pure compounds were subjected to UV, IR, NMR and MS.

2.6.Antioxidant assays

2.6.1. β-carotene bleaching method

The antioxidant activity of extracts was evaluated by the β-carotene -linoleate model system [106, 107]. 0.2 mg of β-carotene, 20 mg of linoleic acid and 200 mg Tween-20 were mixed in 0.5 ml chloroform and the solvent evaporated under vacuum. The resulting

mixture was diluted with 50 ml oxygenated water. To 4 ml of this emulsion, 0.2 ml of test samples in ethanol was added. BHT was used for comparative purposes. A solution with 0.2 ml of ethanol and 4 ml of the above emulsion was used as control. The tubes were covered with aluminum foil and were placed at 50^0C in a water bath. Absorbance was taken at zero time (t=0) and after every 15 minutes. Measurement of absorbance was continued until the color of βcarotene disappeared in the control reaction (t=120 minutes). The antioxidant activity of extracts was based upon three different parameters Antioxidant activity (A_A), the oxidation rate ratio (R_{OR}), and the antioxidant activity coefficient (C_{AA}).

Antioxidant activity (A_A) was determined as percent inhibition relative to control sample

$$A_A = [(R_{control}-R_{sample})] \times 100$$

Where $R_{control}$ and R_{sample} represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively. Degradation rates (R_D) were calculated according to the first order kinetics.:

$$R_D = \ln(A_t/A_x)x1/t$$

Where ln is the natural log, A_t is the initial A_{470} (t=0) and Ax is the A470 at t=10,20,30 minute.

The oxidation rate ratio (R_{OR}) was calculated by:

$$R_{OR}=R_{sample}/R_{control}$$

Where R_{sample} and $R_{control}$ were described earlier.

The antioxidant activity coefficient (C_{AA}) was calculated using:

$$C_{AA}=[(A_{S(120)} - A_{C(120)} / A_{C(0)} - A_{C(120)})]x1000$$

Where $A_{S(120)}$ is the absorbance of the sample containing antioxidant at t=120min, $A_{C(120)}$ is the absorbance of the control at t=120 min, and $A_{C(0)}$ is the absorbance of the control.

2.6.2.Thiocyanate method-Linoleic acid emulsion system

Antioxidant activity of sesame cake extract was evaluated using the thiocyanate method [108,109]. The reaction mixture consists of 0.28g of linoleic acid, 0.28 g of Tween-20 and 50 ml of phosphate buffer (0.2M,pH7.0). To 2.5 ml of above emulsion, 0.5 ml of test sample and 2.5 ml of phosphate buffer (0.2M,pH7.0) were added and incubated at 37^0C for 120 hrs. The mixture prepared, as above without test sample is the control. The readings were taken after each 24 hrs. 0.1 ml of the mixture was taken and mixed with 5.0 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 M ferrous chloride in 3.5 % HCl and allowed to stand at room temperature. Precisely 3 minutes after the addition of ferrous chloride to the reaction mixture, the absorbance at 500 nm was measured.

2.6.3.Thiocyanate method-Linoleic acid system

Autoxidation of linoleic acid was carried out by the following method [110]. Different amounts of samples dissolved in 0.1 ml of ethanol (100,200ug/ml) were added to a reaction mixture in a screw cap vial. Each reaction mixture consisted of 2.5 ml of 0.02 M linoleic acid in ethanol and 2.0 ml of 0.2 M phosphate buffer (pH7.0). The vial was incubated in an oven at 40^0C. at regular intervals during incubation, 0.1 ml aliquot of the mixture was diluted with 4.0 ml of 75% ethanol, which was followed by the addition of 20mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the

absorbance at 500 nm was measured. The solution without added samples were used as blank. The test were run twice and averaged.

2.7.Oil Storage studies

2.7.1.Schaal oven test method for the oxidative stability of oil

Schaal oven test was conducted to evaluate the effect of antioxidants against oxidation during the accelerated storage of oils [111]. The storage tests were carried out in three different vegetable oils like soybean, sunflower and safflower oils.

Refined, bleached, deodorized soybean oil without any added synthetic antioxidants such as TBHQ supplied by soybean oil manufacturer was used for storage studies. Oil samples stored in uniform glass containers at 60^0C for a definite period in an incubator. The following sets of samples were included in the study. Levels of antioxidant extracts tested at 60^0C included 5, 10,50 and 100 ppm by weight of the extract. Experiments were also carried out with synthetic antioxidants such as TBHQ and BHT at 200 ppm level and a control set without added antioxidants. Samples were analyzed after 3,6, 9,12 and 15 days for peroxide value, diene value and p-anisidine value to follow the oxidative changes. The percentage inhibition of oil oxidation is given by,

100-[(PV increase of sample/PV increase of control)x100]

All experiments were conducted in duplicate and analyses of samples were run in duplicate and averaged.

The above experiments were repeated with sunflower and safflower oils.

2.7.2.DSC analysis

DSC analysis was carried out in a Mettler Toledo instrument. This technique is used for studying various heat-related phenomena in materials by monitoring associated

changes in enthalpy [112,113]. Oxidation is an exothermic process and the heat of reaction involved makes it possible to employ DSC for the evaluation of oxidative stability of oils. The measurement was carried out on a, macroscopic quantity of sample. The oil without additives was first studied under dynamic heating regime from 90^0C to 200^0C and the temperature of onset of oxidative changes was noticed from the DSC curve as the point of inflection. The samples were then analyzed isothermally at a temperature 10 degrees below the above said temperature. Oxidative stability of vegetable oils was evaluated under isothermal oxidation at 150^0C under a stream of oxygen at 40 ml/min. Heat of reaction was measured. An aliquot (7-10mg) of sample was kept in the aluminum sample cell and another pan without sample was kept as reference. The flow of nitrogen was 200 ml/min. The vegetable oil sample containing synthetic antioxidants and sesame cake extracts were analyzed isothermally at the temperature. The time at which the onset of oxidation occurred was noted and this induction period was taken as indicative of the oxidative stability of oil.

2.8.Radical scavenging studies

2.8.1.Free radical scavenging method by DPPH

The method of Brand-Williams etal., [114] modified by Sanchez-Moreno [115] was followed. 0.025g/L DPPH solution was prepared in methanol. Various concentrations of standard compounds were also prepared. To 3.9 ml of DPPH. solution, 0.1 ml of appropriately diluted standard solutions were added and absorbance at 515 nm were measured at different time intervals in a 160A Shimazdu UV-Visible spectrophotometer for 30 minutes.

The DPPH· concentration in the reaction medium was calculated using the following equation obtained by linear regression.

$A_{515nm}=2936.68[DPPH]_T-2.18x10^{-3}$

The percentage of remaining DPPH· was calculated as

$\%DPPH_{REM} = [DPPH]_T/[DPPH]_{T=0}$

The percentage of remaining DPPH against the standard concentration was plotted to obtain the EC_{50} concentration (the amount of antioxidant required to decrease the initial DPPH concentration by 50%).

Kinetic analysis

Graphs and fittings of the experimental data were carried out in Microsoft Origin 6 programme [116]. The data points of spectrophotometer readings of the disappearance of DPPH. in the presence of various antioxidant compounds were taken. Second order rate constants(k_2) were calculated to determine the radical scavenging capacity (RSC) of the different compounds.

2.8.2.Superoxide oxide radical scavenging

2.8.2.1.Xanthine-Xanthine oxidase/ NBT method [117]

Methodology

Reagents

Carbonate buffer 50mM, pH 10.2 containing EDTA

Xanthine (8.7 mg/ml)

Xanthine oxidase(10mg/ml)

NBT (6 mg/ml)

Experimental procedure

The assay mixture consists of 1ml of sodium carbonate-EDTA buffer (carbonate buffer 50mM, pH 10.2 containing EDTA), 50 ml xanthine (8.7 mg/ml), 20µl xanthine oxidase(10mg/ml) (18). The time course for the reduction of NBT into formazan complex was followed for about 12 minutes at 560 nm. The percentage of NBT reduction was calculated as follows

% of NBT reduction == [Rate of change of absorbance of sample/Rate of change of absorbance of control] x100

Radical scavenging ability == 100-% of NBT reduction

2.8.2.2.Xanthine-xanthine oxidase/ Ferricytochrome C [118]

Methodology

Reagents

Potassium dihydrogen phosphate

Disodium hydrogen phosphate

EDTA

Xanthine

Xanthine oxidase

Ferricytochrome C

Solution A

0.76 mg (50µM) xanthine in 10 ml of 0.001N sodium hydroxide and 24.8 mg (20µM) Cytochrome C are admixed with 100 ml of 50 mM phosphate buffer, pH 7.8 containing 0.1 mM EDTA.

Solution B

Freshly prepared solution of xanthine oxidase in 0.1 mM EDTA= 0.2 units/ml

Experimental

The assay was performed in a 3.0 ml cuvette. The assay mixture consisted of 2.9 ml Solution A (containing 50 μM xanthine and 20μM cytochrome C) and 0.05 ml sample solution. A solution of 0.05 ml xanthine oxidase (0.2 unit/ml) was added to start the reaction and the rate of reduction of Ferricytochrome C was determined by using UV-Visible spectrophotometer at 550nm.Here methanol is used as carrier solvent for standard compounds and samples.

2.9. Biological studies

2.9.1. Anticancer studies

Cell culture studies using sesame cake extract and isolated compounds were carried out at Regional Cancer Centre (R.C.C), Thiruvananthapuram using their standardized methodology. Antioxidant activity was studied by TBARS method [119]. Apoptosis was studied by Tunel assay, Annexin-V-Biotin assay [120] and MTT assay [121]

2.9.2. Insecticidal activity

Prelimenary studies on insecticidal activity of extract and individual compounds were studied [122] in the Entomology section of Natural Products Division of RRL.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Extraction kinetics of lignans

This section deals with compositional aspects and extraction kinetics of sesame lignans. Sesame cake is by-product of sesame oil industry. As already detailed in Chapter 1, the antioxidant potential of sesame cake has not been investigated and exploited for commercial use. Hence the current investigation was undertaken to explore the feasibility of preparing an antioxidant extract from sesame cake. It is known that lignans especially sesamol,sesamin and sesamolin in sesame oil imparts its extraordinary oxidative stability. Presuming that substantial quantity of these compounds could remain in the deoiled cake, a low value product, the present investigation was carried out. The focus of the present investigation therefore has been on the abundantly available cake as a potential source for natural antioxidant that could substitute synthetic antioxidants, in the near future.

3.1.1. Proximate composition

Sesamum Indicum is the predominantly cultivated and commercially available sesame seed. Hence for Proximate composition analysis *S.indicum* seeds were used. Proximate composition of sesame seed (*Sesamum indicum*) is given in Table 3.1. Seed contained 47.5% oil, 21% protein, 19% sugar and 11.5% crude fiber. Sesame cake is the byproduct of oil milling industry and commercial cake was taken for analysis. Commercial cake is available normally as a mixture of locally cultivated varieties and therefore wide variations are expected. Sesame cake (raw, commercial variety) contained 11% oil, 32.5% protein, 7.5% sugar, 9.5% ash and 9.5% crude fiber. Sesame cake was defatted followed by water washing to enrich lignan. The cake treated thus (partially purified) contained 0.5% oil, 30.5% protein, 0.9% sugar and 20.5% ash. The minerals

present in the ash content included calcium, iron and selenium. The ash content increased in the partially purified extract whereas the fat and sugar content decreased.

Sesame is a rich source of oil and protein and the seed has been used as food since ancient time. Sesame has long been regarded as a health food, which reported to increase energy and prevents aging [67]. Sesame seeds differ considerably depending on varieties, size, color and coat thickness. The major and minor components also vary considerably [64]. Oil, protein and carbohydrates are the major constituents of sesame [66]. Minor components included lignans, triterpenes, steroids, vitamins etc [64]. Sesame seed has been subject of studies as an oil seed crop of commercial significance. Marked compositional variations have been observed due to agroclimatic conditions, varieties and season [64, 67]. The oil content of the seed reported here falls within the range reported by other researchers [123, 124]. White seeds contain more oil than the black seed. Fatty acids of oil consist mainly of oleic and linoleic acids with small amounts of palmitic and stearic acids. The total unsaturated fatty acid amounts to approximately 80%. The sugar contents included low amounts of glucose and fructose. The sugar and protein content of seed also agreed with the reported values [64,66, 67].

Table 3.1: Proximate composition (%) of commercial sesame seed (*Sesamum indicum*) and cake (commercial)

Sample	Sesame seed	Sesame cake, raw	Sesame cake, purified
Moisture	8.0	6.5	6.5
Fat	47.5	11.0	0.5
Protein	21.0	32.5	30.5
Sugar	19.0	7.5	0.9
Crude fibre	11.5	9.5	10.5
Ash	7.5	9.5	20.5

• Values are expressed as means of triplicate experiments

3.1.2. Extraction kinetics

Objectives of kinetic studies were to obtain maximum lignans in a suitable solvent and a concentrate. Kinetic studies were designed to select appropriate solvent and optimize extraction time, temperature etc. Standardization of HPLC method to separate and quantify lignans was also carried out. Commercial sesame cake procured from local market was used to study the extraction kinetics primarily aiming at future commercial prospects for natural antioxidants from this abundantly available source.

Selection of Solvents: Antioxidants were extracted from sesame cake with various solvents of differing polarity to establish the solvent power of each solvent in terms of extractability of lignans under identical conditions. They included hexane, ethyl acetate,

isopropanol, acetone, ethanol and methanol (Table 3.2). All extracts were tested for Total phenolic content (TPC) and lignan content and average of three independent extractions were recorded. In hexane extract the total lignans were 259ppm with no sesamol. Ethyl acetate extracted more lignans than isopropanol. The lignan content of acetone, ethanol, and methanol extracts were 1071, 1370, 1560 ppm respectively. It is clear from the table that polar solvent especially alcoholic solvents extracted antioxidants more effectively than less polar solvents like hexane, isopropanol.

The total phenolic content (TPC) was also estimated for the extracts. Quantitatively TPC was far less than that of total lignans in the respective solvent extracts. This may be due to the different response of the phenolic compounds to different reagent [125]. Hence TPC and lignans cannot be directly correlated here.

Sesame cake was successively extracted with petroleum ether, chloroform, acetone and methanol to obtain maximum recoverable lignans. The results are presented in Table 3.3. Sesamol could not be extracted with Petroleum ether and chloroform, but its extractability progressively increased with increased polarity of the solvent, with methanol showing the highest. Similar trend was observed for sesamolin. Sesamin was extracted in all solvents. The total lignan, extracted in all solvents amounts 2064 ppm while using single solvent extraction with methanol alone extracted 1560ppm lignans. During successive extraction, considerable amounts of lignans were separated at each stage of extraction. Exhaustive extraction like this is not practical and hence for subsequent studies only methanol was used that extracted 2/3 of the lignans in single step. More over successive extraction was time consuming and therefore not economical. Besides, chlorinated solvents are not allowed for edible use. Based on the above results

methanol was selected as solvent considering its higher extraction efficiency of lignans besides being a permitted solvent in food industry.

Table 3.2: Extraction of antioxidant compounds from sesame cake using various solvents*

	Lignans and TPC in ppm in cake				
Extracting Solvent	Sesamol	sesamin	sesamolin	Total lignan	TPC
Hexane	-	157	102	259	50
Ethyl acetate	86	720	166	972	167
Isopropanol	68	394	22	484	88
Acetone	218	833	20	1071	173
Ethanol	380	865	125	1370	201
Methanol	477	895	189	1560	224

*10g sesame cake was soxhlet extracted with 150 ml solvent, for 16hrs.

TPC-Total phenolic content; All values are expressed as means of three independent experiments

Table 3.3: Successive extraction of sesame cake for antioxidant compounds with various solvents*

	Lignans in ppm in cake			
Solvent	Sesamol	sesamin	sesamolin	Total lignan
Pet.ether	-	324	18	342
↓				
Chloroform	-	163	37	200
↓				
Acetone	53	237	160	450
↓				
Methanol	294	588	190	1072
Total	347	1312	405	2064

*10g sesame cake was soxhlet extracted with 150ml solvent for 16hrs.

Values are expressed as means of three independent experiments

Optimization of Temperature: Cold extraction (extraction at room temperature) and soxhlet extraction (60-70^{0}C) were tried for sesame cake. It was found that sesamolin was not extracted during cold extraction. Hence Soxhlet extraction was preferred.

Optimization of Extraction Time: The extraction of antioxidants from sesame cake at various times is given in Figure 3.1. Sesame cake samples were subjected to methanol extraction (10g in 150ml methanol, soxhlet extraction, 60-70^{0}C) separately and the extracts were analyzed for lignans by HPLC. About 80% of total lignans were extracted in 8hrs and solubility was linear during this time period. The remaining 20% were obtained in the next 8hrs. However the individual lignans showed a different pattern. Sesamin that accounted for more than 80% of the total lignans followed the trend of total lignan whereas sesamolin and sesamol were extracted in 2hrs. Sesamin being most dominant one and about 90% of it obtained in 16hrs, this extraction time was fixed for subsequent extractions. Material to solvent ratio was fixed at 1:15 based on the results with 1:10, 1:15 and 1:20. The extractability of lignans from sesame cake in various solvents was not so far studied.

Standardization of HPLC method for sesame lignans

Reverse phase HPLC using μ-bondapak C_{18} column was used for the analysis of lignans. Due to the polar nature of compounds, the separation was more effective in reverse phase column. The solvent system, flow rate and the wavelength of detection was selected based on many trials. During HPLC analysis, the total lignan content was calculated based on the amount of sesamol, sesamin, and sesamolin. Total lignan glucosides included sesaminol diglucoside and sesaminol triglucoside. Linearity was

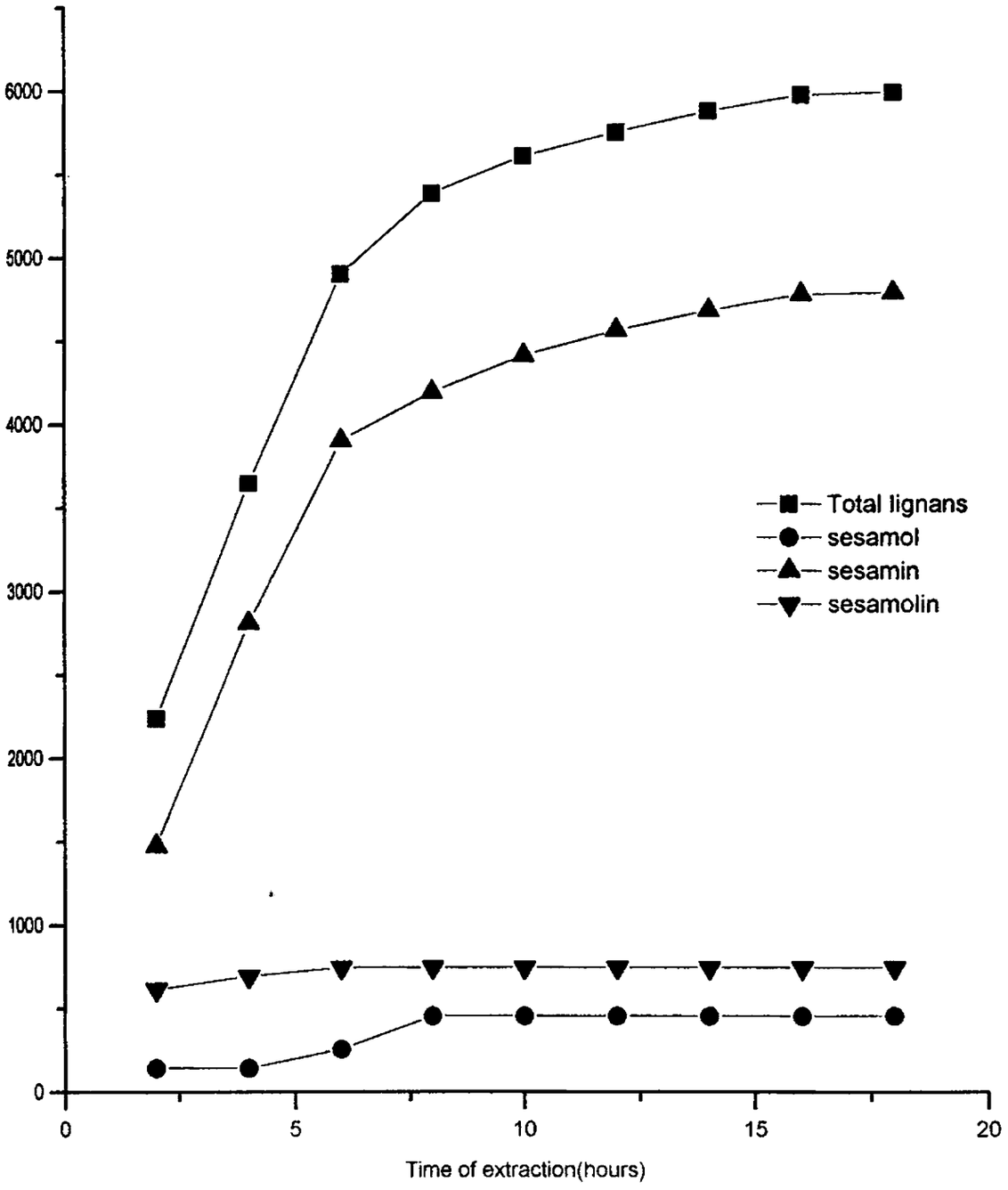


Figure3.1: Extraction kinetics for sesame antioxidants in cake with respect to time [Soxhlet extraction with methanol]

assessed for standard sesamol. Detector response was found to be linear with a correlation coefficient, γ =0.9988.

Sesamol and sesamin were quantitated based upon the areas relative to standard calibration curves obtained with corresponding standards. All other compounds were quantitated using the calibration factor for standard sesamol. Other standard compounds were injected to obtain the retention time of each compound to enable identification of lignans in samples. The HPLC profile of standard sesamol, sesamin, cake extract, and seed extract are given in Figures 3.2, 3.3, 3.4 and 3.5.

Fukuda etal., [84] used HPLC using ODS column for the analysis of lignans present in sesame oil. Sesame meal extracts were analysed by Shyu etal., [126] and they quantitated sesamin, sesamolin, and the lignan glycosides from 80% alcoholic extract of defatted sesame meal. The analysis time was higher, i.e., more than 60 minutes. In the present investigation, the lignans were analyzed in less than 25 minutes in single run. Sesamol was not reported in any of the seed or cake extract. In oil, sesamol was reported in trace amounts [64]. Sesamol was not extracted if aqueous alcohol was used for extraction. It was observed in the present investigation that sesamol could be extracted in 100% methanol and therefore this may be the first report regarding the presence of sesamol in higher amounts in sesame cake extract as well as seed extract.

The optimized extraction conditions for sesame cake was applied to three sesame seed varieties i.e. white, red and black to understand their variability in terms of lignan content. The results are presented in Table 3.4. The whole seed from the three varieties were defatted with hexane to separate the oil. The defatted meal and oil were extracted with methanol as described before. The lignan content of oil and meal were analyzed by

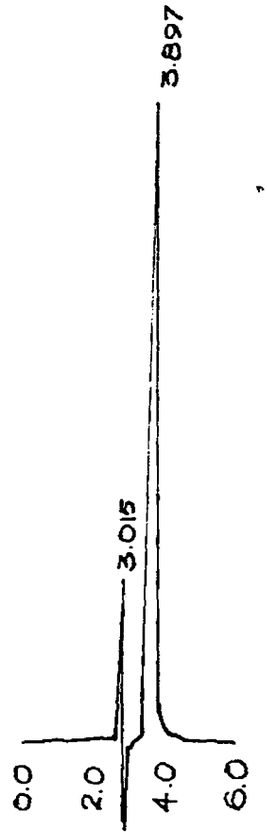


Figure 3.2: HPLC Profile of standard sesamol (μ-bondapak column; Methanol : water (70:30); λ=290nm) 1.sesamol

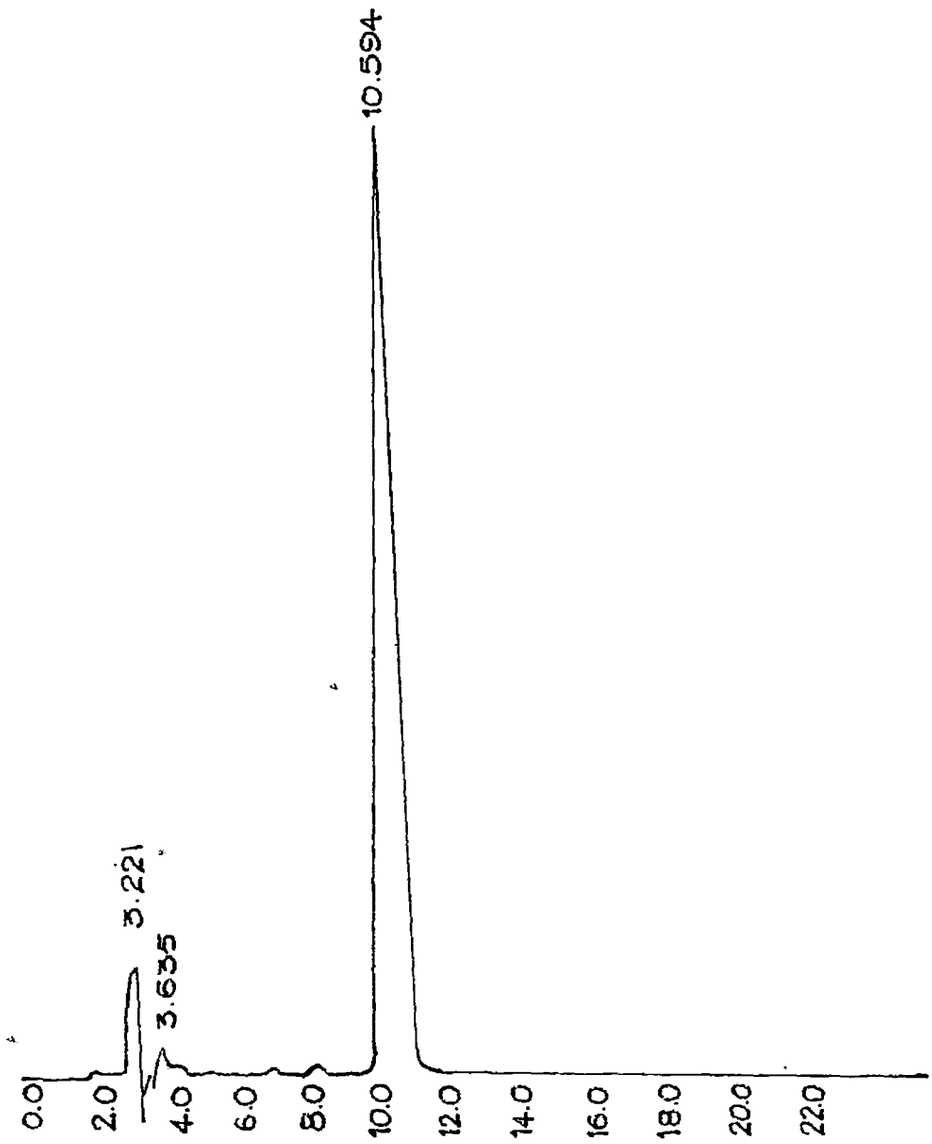


Figure 3.3: HPLC Profile of standard sesamin (μ-bondapak column; Methanol : water (70:30); λ=290nm) 1.sesamin

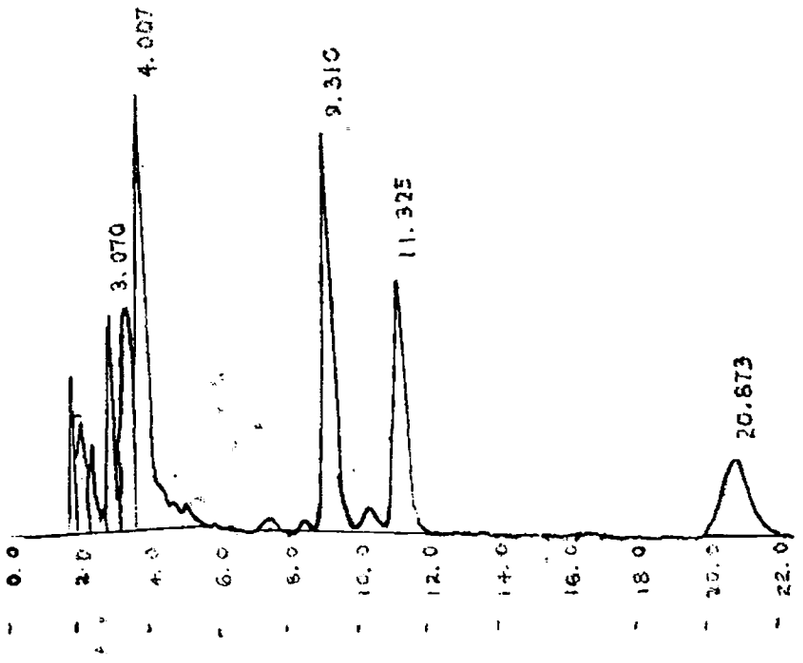


Figure 3.4: HPLC Profile of methanolic extract of sesame seed (μ-bondapak column; Methanol : water (70:30); λ=290nm) 1.sesamin 2.sesamin 3.sesamolin

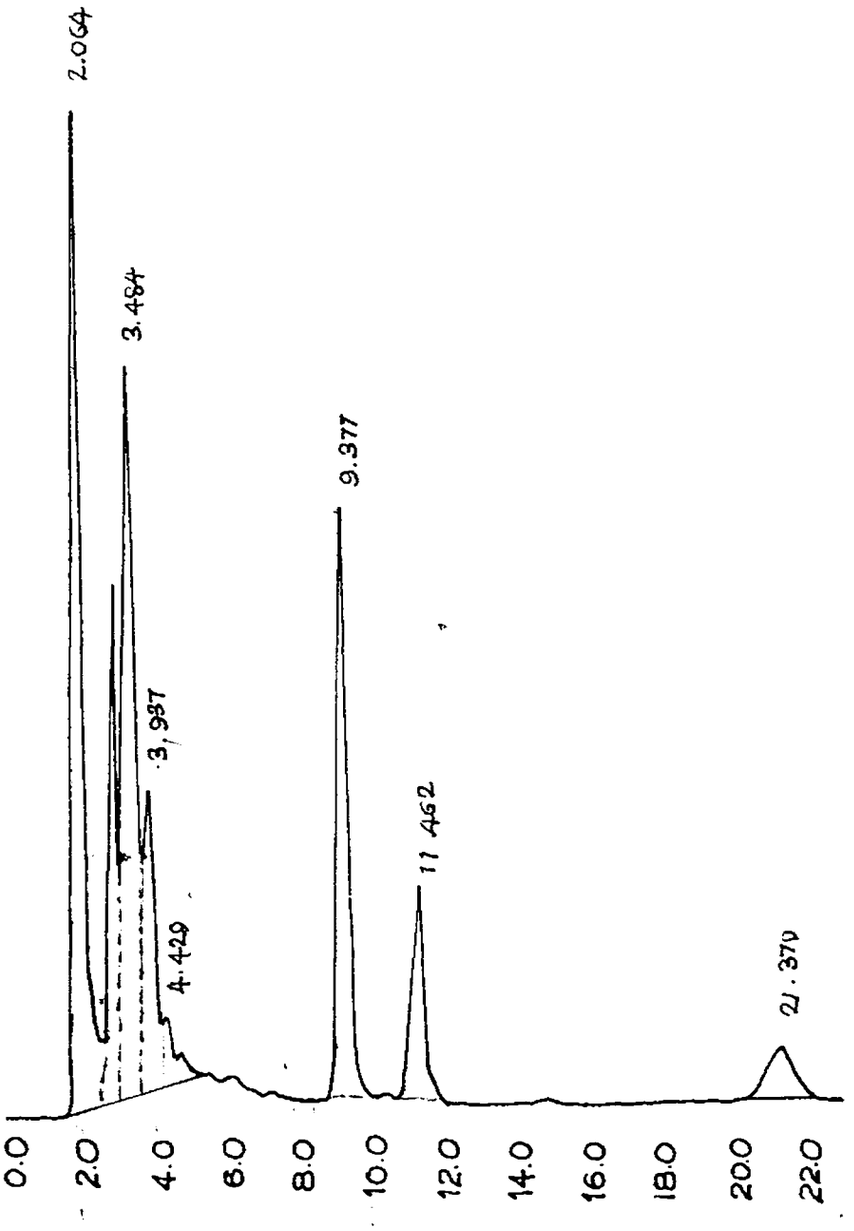


Figure 3.5: HPLC Profile of methanolic extract of sesame cake (μ-bondapak column; Methanol : water (70:30); λ=290nm) 1.sesamin 2.sesamin 3.sesamolin

HPLC. Commercial cake sample was directly extracted with methanol. The total lignan content in the seed was computed from values obtained for oil and meal.

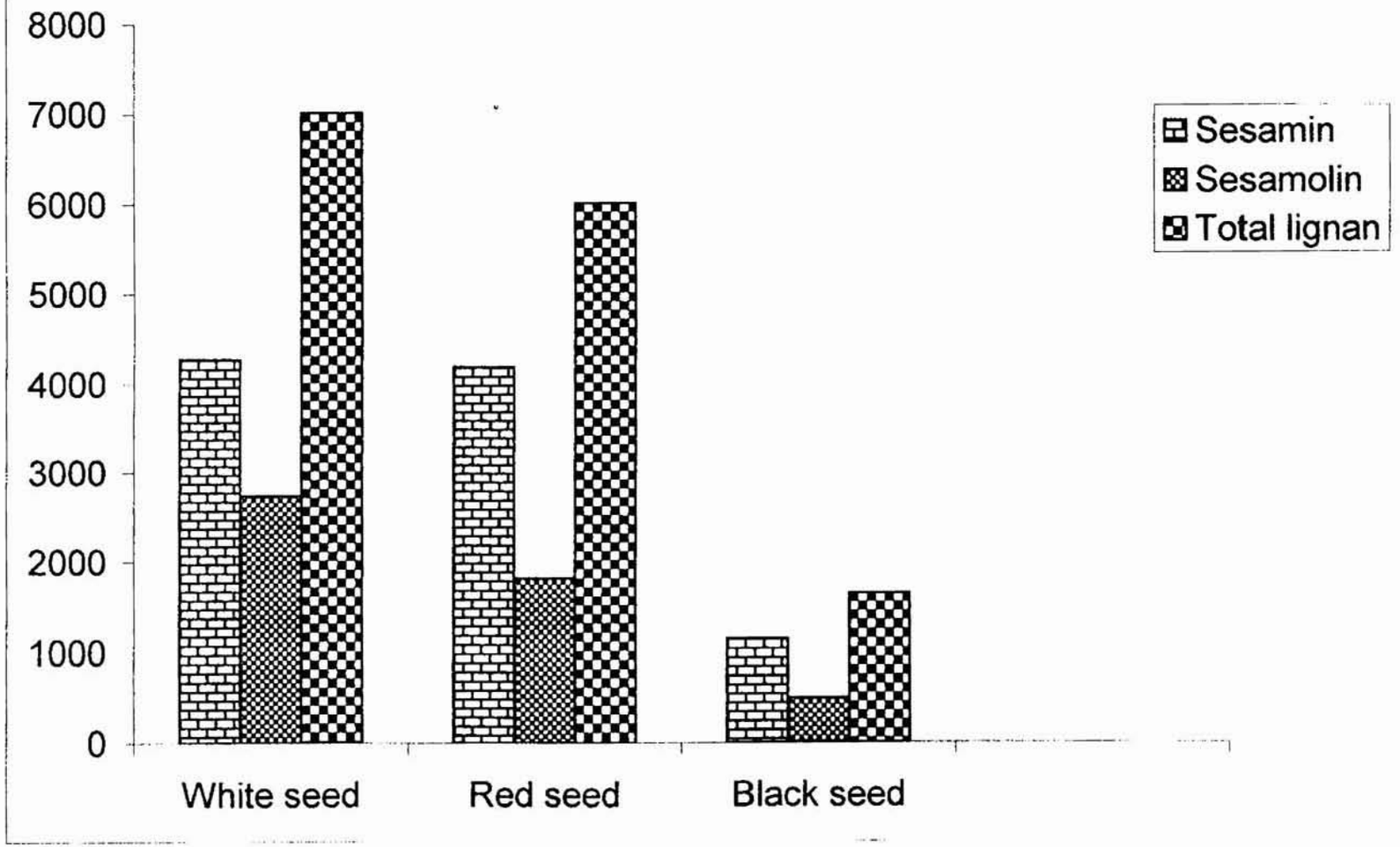
Oil from white, red and black sesame seeds contained 7018, 6014 and 1653ppm lignans respectively. The lignan in oil were comprised of sesamin and sesamolin and only trace amounts of sesamol were present in oil. Oil from white, red and black seeds contained 4278, 4193 and 1154 ppm sesamin and 2740, 1821 and 502 ppm sesamolin respectively. The amount of sesamin, sesamolin and total lignans were higher for oil from white seed and lower for black seed varieties. The variations in the lignans in oil from different seeds are represented in Figure 3.6.

Commercial oil samples contained high lignan content, i.e. 19161 and 17434 ppm and there was considerable increase in sesamin and sesamolin content than the other seed oils studied. They contained 6638, 3060 ppm sesamin and 12523, 14374 ppm sesamolin. Sesamolin content was found to be higher compared to sesamin content.

Previously, Bailey [64] and Namiki [67] correlated the color types with oil content and its lignan content. Oil content and sesamin content is high for white seeded strains. Black seed strains are having lower oil content and sesamin content. In this study, the oil and sesamin contents were positively correlated in both the white and black seeded strains and the lignan content of oil. The high lignan content of commercial oil samples may be due to the mixing of different varieties of sesame seeds and also differences in the various processing stages [84, 85, 127, 128].

Methanolic extract of defatted seeds from white, red and black varieties contained 3420, 3237 and 2038 ppm lignans. The total lignans in defatted seed extract included sesamol, sesamin and sesamolin. The content of sesamol in the extract of white,

Figure 3.6: Variation in lignan content of oil among various seeds



red and black seeds were 755, 1334 and 401 ppm. They also contained 2351, 1679, 1129 ppm sesamin and 314, 223, 508 ppm sesamolin. Sesamin content was higher compared to sesamol and sesamolin content in the extract. The amount of sesamol was lower in black variety and higher in red variety and sesamolin content was higher in black variety. The variations in lignans in different seeds are represented in Figure 3.7. The total lignan content of white seed variety was comparatively higher than that of others.

Commercial cake samples I and II contained 1300 and 3300ppm lignans. Total lignan content included sesamol, sesamin and sesamolin. Extracts of I and II contained 277 and 461ppm sesamol, 607 and 2104ppm sesamin and 309 and 928ppm sesamolin. In this case also, sesamin content was higher than that of other lignans. The wide range in lignan content is due to different sources of sesame cake. Seasonal variation also affects the lignan content.

Total lignan content (lignan content in seed) was calculated based on lignan content in oil and meal. It was based on assumption that lignans from seed were distributed in oil and meal. In the present case, 10g seed gave 4g oil and 6g meal. It can be deduced from the results of Table 3.4 that the cake retained nearly 50% of lignans even after oil extraction using solvent. The common practice employed in the industry is deoiling of sesame seed by mechanical pressing that could leave more lignans in the cake.

Figure 3.7: Variation in the lignan content of meal extract among different seeds

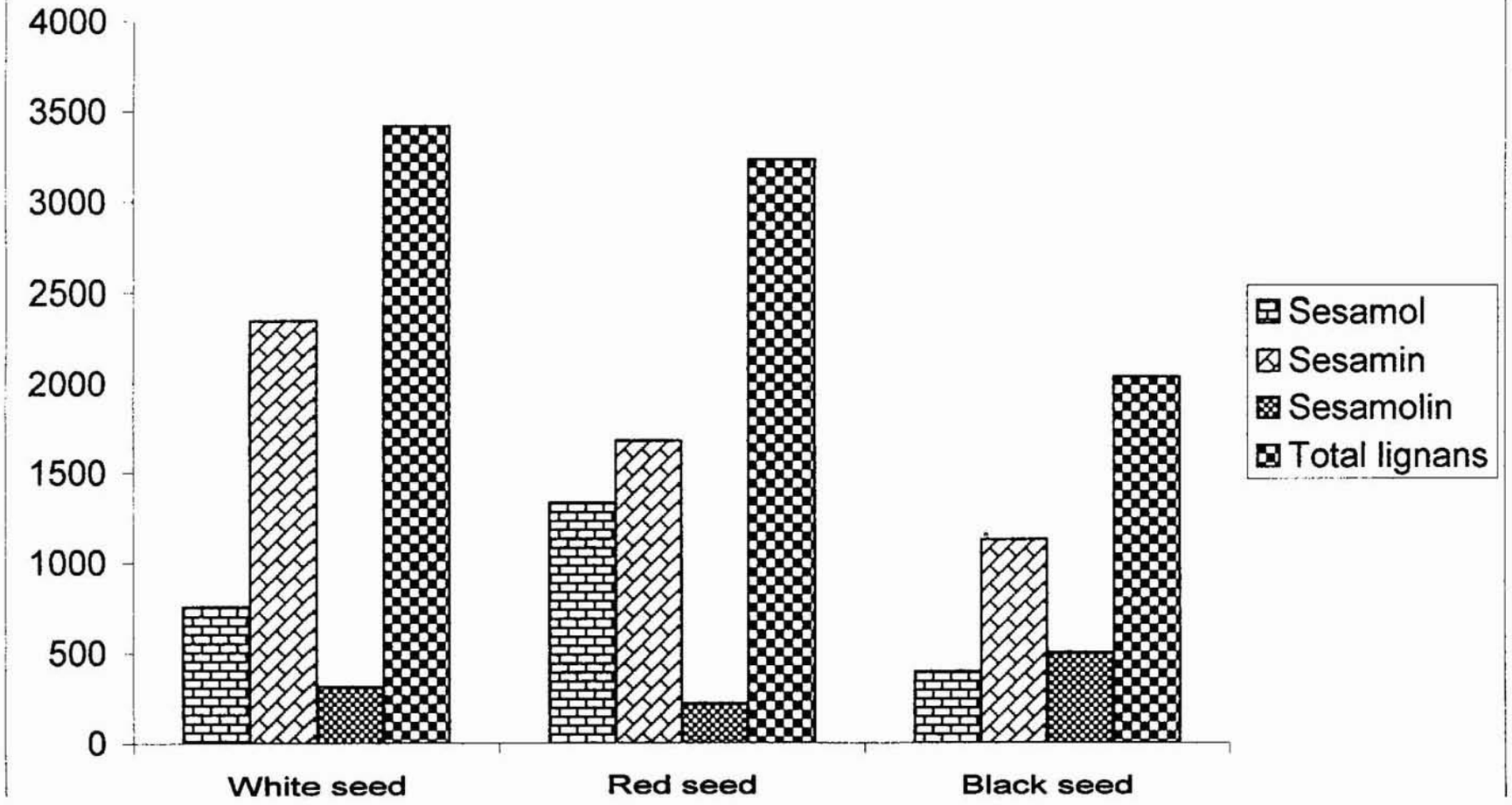


Table 3.4: Antioxidant compounds in oil and meal obtained from different varieties of sesame seeds*

	Lignans in ppm in sample			
Sesame variety	sesamol	sesamin	sesamolin	Total lignans
White seed				
Oil	trace	4278	2740	7018
Meal	755	2351	314	3420
Total(oil+meal)	453	3122	1284	4859
Red seed				
Oil	trace	4193	1821	6014
Meal	1334	1679	223	3237
Total(oil+meal)	800	2685	862	4347
Black seed				
Oil	trace	1154	502	1653
Meal	401	1129	508	2038
Total(oil+meal)	241	1139	506	1886
Commercial cake 1	461	607	309	1377
Commercial cake 2	277	2104	928	3309
Commercial oil 1	trace	6638	12523	19161
Commercial oil 2	trace	3060	14374	17434

* oil-oil extracted from seed with hexane, and lignans extracted with methanol; meal-defatted seed extracted with methanol

3.1.3.Preparation of partially purified extract

The following experiments were designed to enrich the lignan content and antioxidant activity of the extract through elimination of interfering constituents like fat and sugar. As a first step the dried raw material, sesame cake, was defatted with hexane, at room temperature (28 ± 2^0C). Prolonged soxhlet extraction at high temperature was avoided to minimize the loss of lignans in hexane. Instead, 100g of powdered sesame cake was washed 3 to 4 times with 500 ml of hexane for 12 hours. Hexane extract was analyzed by HPLC and found to contain only negligible amount of lignans i.e. approximately 0.03%. It was further observed that sesamol and sesamolin were not extracted and therefore only small amounts of lignans were going with hexane. The fat content decreased from 11 to 0.2% after hexane wash. The methanolic extract of crude sesame cake and defatted sesame cake found to contain 7729 and 23659ppm lignans.

The defatted material was dried and then washed 3 to 4 times with distilled water (500ml). The loss of lignans during water washing was approximately 0.5%. Water washing was done to remove maximum amount of sugar. The sugar content decreased to 0.82% from 7.5. The water washed residue was dried at 50-60^0C and finally the residue was extracted with methanol in a soxhlet extractor for 16 hours. The extract contained 0.5g residue. The methanolic extract of water washed cake contained 141074ppm lignans. (The details of the process are covered under a patent proposal submitted, US Patent Application No.60/404.004).

The lignan content and yield at various stages of purification is given in Table 3.5. 3.1 fold purification was attained after defatting which further increased to 18.2 fold by water washing. The total lignan content of the material was increased by 2.3 and 3.6

times by the above purification steps i.e. defatting and water washing. The individual lignan content of the crude and partially purified extracts is represented in Table 3.6. The ratio of sesamol : Sesamin : sesamolin were 2.5 : 4.7 : 1.0 and 1.8 : 8.5 : 1.0 respectively. In the purified extract the individual lignans sesamol, sesamin and sesamolin increased in the ratio of 10 :24 : 13 compared to crude extract. There was significant increase in the amount of all compounds by purification, especially sesamin. The increase in lignans is due to the increased extractability attained by removal of interfering constituents like fat and sugar. As the fat was removed, polar solvent could more effectively percolate through the raw material and thus the extraction was more effective and hence the higher extraction efficiency.

It was found that extractability of lignans was significantly increased by these simple steps, which were more economical. More over, the extract contained lignan compounds, which are reported to possess many additional antioxidant and biological effects. This will promise the use of this extract in place of synthetic antioxidants, which will provide additional health benefits.

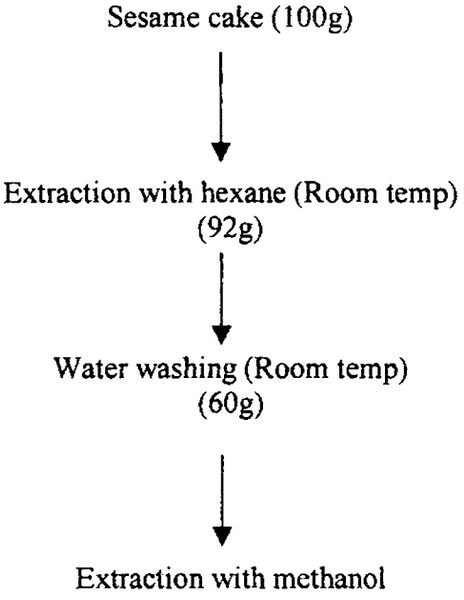


Figure 3.8: Schematic diagram for purification of sesame cake

Table 3.5: Partial purification of sesame cake and the lignan concentration (ppm) at various stages of purification for enhancing antioxidant activity

Sample	Quantity (g)	Weight of extract(g)	lignans/g extract	Total lignans	Fold purification
Sesame Cake	100	15	7729	115940	-
↓					
Defatted Cake	92	12.5	23659	261195	3.1
↓					
Defatted, water washed Cake	60	5.0	141074	412800	18.2

* Sesame cake samples i.e. crude, defatted, defatted and water washed were extracted with methanol.

Table 3.6: Antioxidants extracted from sesame cake with methanol

	Lignans in ppm in extract	
Sample	Crude extract	Purified extract
Sesamol	2359	22677
Sesamin	4431	105893
Sesamolin	939	12504
Total lignans	7729	141074
Sesaminol triglucoside	5061	6792
Sesaminol diglucoside	1756	6506
Total glucoside	6817	13298
TPC	1709	5438

3.2.Antioxidant activity studies using extracts from sesame cake

As explained in Chapter 1, the behavior of antioxidant compounds towards various assay methods mainly depends upon their mode of action. Hence, more than one model system was used for evaluation of antioxidant activity. One of the important properties of antioxidant is inhibition of lipid peroxidation. In the following assay methods the inhibition of lipid peroxidation is taken as the index of activity under different assay conditions. The activities of crude and purified extracts were studied by the following invitro methods.

3.2.1. β-carotene bleaching method

There are reports on the antioxidative effects of sesame seed [84]. However, little is known about the qualitative and quantitative aspects of antioxidants present in sesame cake and their activities. The antioxidant activity of sesame cake extract was evaluated by the β-carotene bleaching method where the oxidation of β-carotene is induced by linoleic acid peroxides. It is a free radical mediated phenomenon whereby hydroperoxides formed from linoleic acid oxidises the β-carotene molecule with consequent loss of its chromophore [107]. As a result, the orange colour of β-carotene decreases and the extent of reduction in color can be followed spectrophotometrically at 470 nm. Presence of added antioxidants in the assay mixture inhibits the β-carotene oxidation by neutralizing the free radicals generated by linoleic acid. The extent of inhibition of β-carotene oxidation by antioxidants is taken as antioxidant activity index. The bleaching rate of β-carotene with and without antioxidants is represented as oxidation rate ratio. The results are expressed as means of three independent determinations.

Figure 3.9. shows the antioxidant effect of sesame cake extracts in comparison with BHT. Crude and purified extracts were tested at 100 and 200 ppm levels and BHT at 200ppm. Since the maximum permissible level allowed for synthetic antioxidants for food use is 200 ppm, this concentration was used as reference. It can be seen that both crude and purified extracts showed appreciable antioxidant activity. Crude extract showed 42% inhibition at 100ppm and 47% inhibition at 200ppm concentration. BHT at 200ppm showed 45.6% inhibition. The activity of crude extract was comparable to BHT at 200 ppm.

Purified extract was far more active than that of either crude extract or BHT at comparable concentration. At 200ppm concentration, the antioxidant activity index for purified extract, crude extract and BHT were 61.1, 46.6 and 45.6% respectively. The results for purified and crude extracts in terms of dose-response relationship are represented in Table 3.7. The activity index for purified extract at 50 ppm was 81.6% which was almost double than that obtained for BHT at 200 ppm (46.6%) and crude extracct at 200ppm(45.6%). Activity index follows the order 50ppm > 100ppm > 10ppm > 5ppm > 200ppm > 200ppm (crude extract) > BHT > 100ppm (crude extract). Purified extract showed better activity at lower concentrations compared to 200ppm.

The data's for oxidation rate ratio and activity coefficient support the antioxidant activity index. Oxidation rate ratio bears inverse relationship with antioxidant activity index while activity coefficient has direct relationship. The oxidation rate ratio for purified extracts were lower than that for crude extracts and BHT. Activity coefficient increases directly with the increase in value of antioxidant activity index. C_{AA} value was

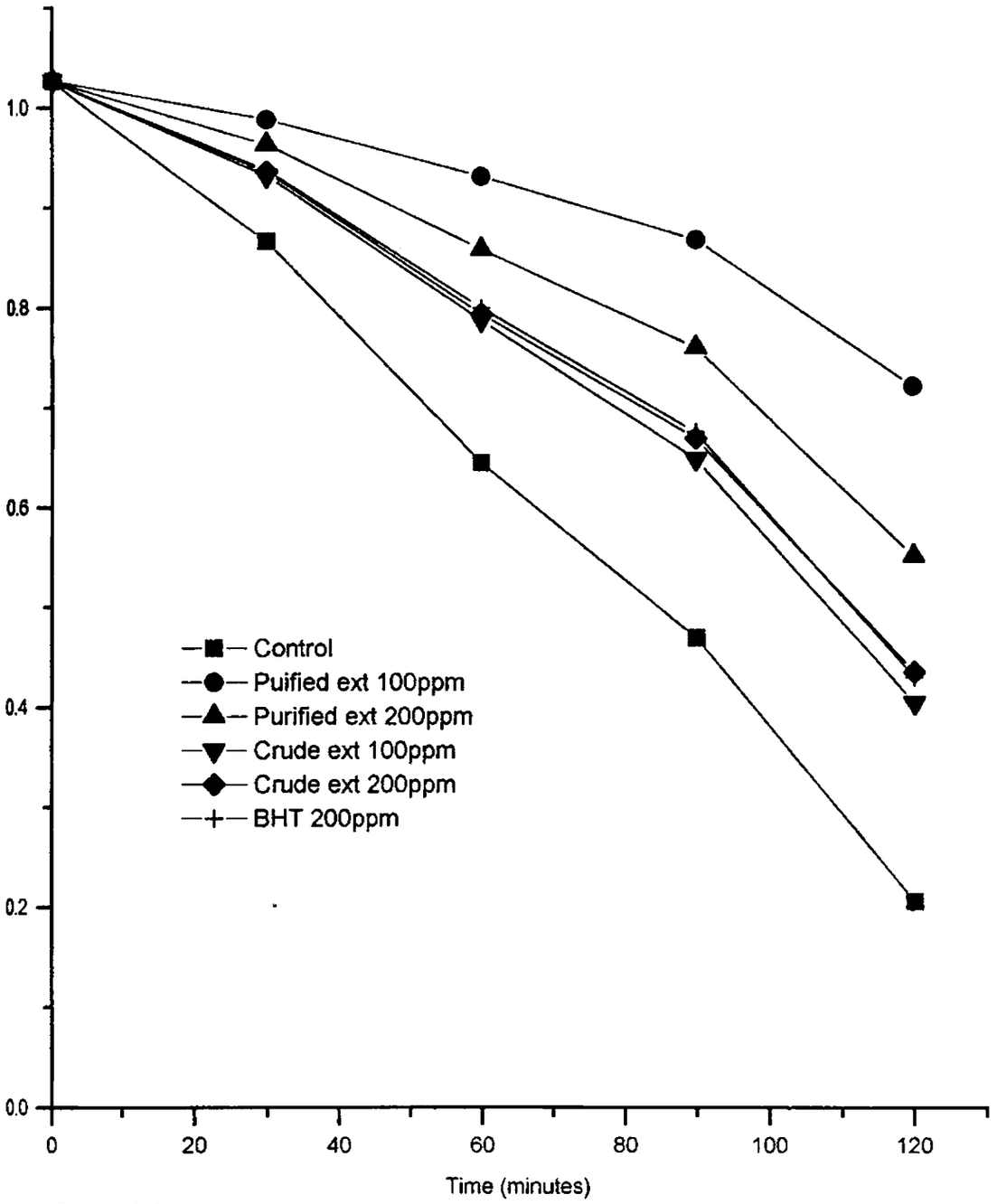


Figure 3.9:Antioxidant activity of sesame extracts and BHT by the β-carotene bleaching method

highest for purified extract at 50ppm (904.9) and lowest for crude extract at 100ppm (242.4)

Table 3.7: Concentration dependent antioxidant activity of sesame cake extracts by β-carotene bleaching method

Concentration (ppm)	A_A	R_{OR}	C_{AA}
Purified extract			
5	63.0	0.37	601.9
10	67.1	0.33	662.1
50	81.6	0.18	904.9
100	77.9	0.22	628.5
200	61.1	0.39	421.4
Crude extract			
100	41.7	0.58	242.4
200	46.6	0.54	280.2
BHT 200	45.6	0.54	274.1

* The values are expressed as mean of three independent determinations.

A_A-Antioxidant activity index
R_{OR} - Oxidation rate ratio
C_{AA} - Activity coefficient

3.2.2. Thiocyanate method

This method is primarily employed to measure lipid oxidation through linoleic acid. Oxidation of linoleic acid generates hydroperoxides, which further decomposes in to secondary oxidation products. The oxidized products react with ferrous sulphate to form ferric sulphate, which further reacts with ammonium thiocyanate to form ferric thiocyanate, which is red in color. As a result of this oxidation, the absorbance increases. After a certain time, the formation of peroxides will be decreased due to non-availability of linoleic acid and hence the oxidation of ferrous sulphate will be reduced with consequent decrease in absorbance [107]. Presence of antioxidants delays the oxidation of linoleic acid, which in turn delays color formation. Thus efficacy of antioxidants can be measured from the extent of inhibition of linoleic acid oxidation, by this method.

Linoleic acid system and linoleic acid emulsion systems were used for evaluation in the present investigation. Foods generally consist of multiphase in which lipid and water co exists with some additional emulsifiers. Hence, instead of single phase system for eg oil, heterogeneous system such as oil-in-water emulsion is also required in the assay to simulate conditions exists in foods. Autoxidation of linoleic acid in ethanol-buffer system is one of the simple conditions of oxidation in heterogeneous system for evaluation of antioxidant [129]. Linoleic acid system can be correlated with homogenous system or bulk oil phase system. Linoleic acid emulsion system can be correlated with the biological system or with food.

3.2.2.1.Linoleic acid emulsion system

Sesame cake extracts (crude and purified) showed activity in linoleic acid emulsion system (Figure 3.10). The percentage inhibition for crude extract at 100,

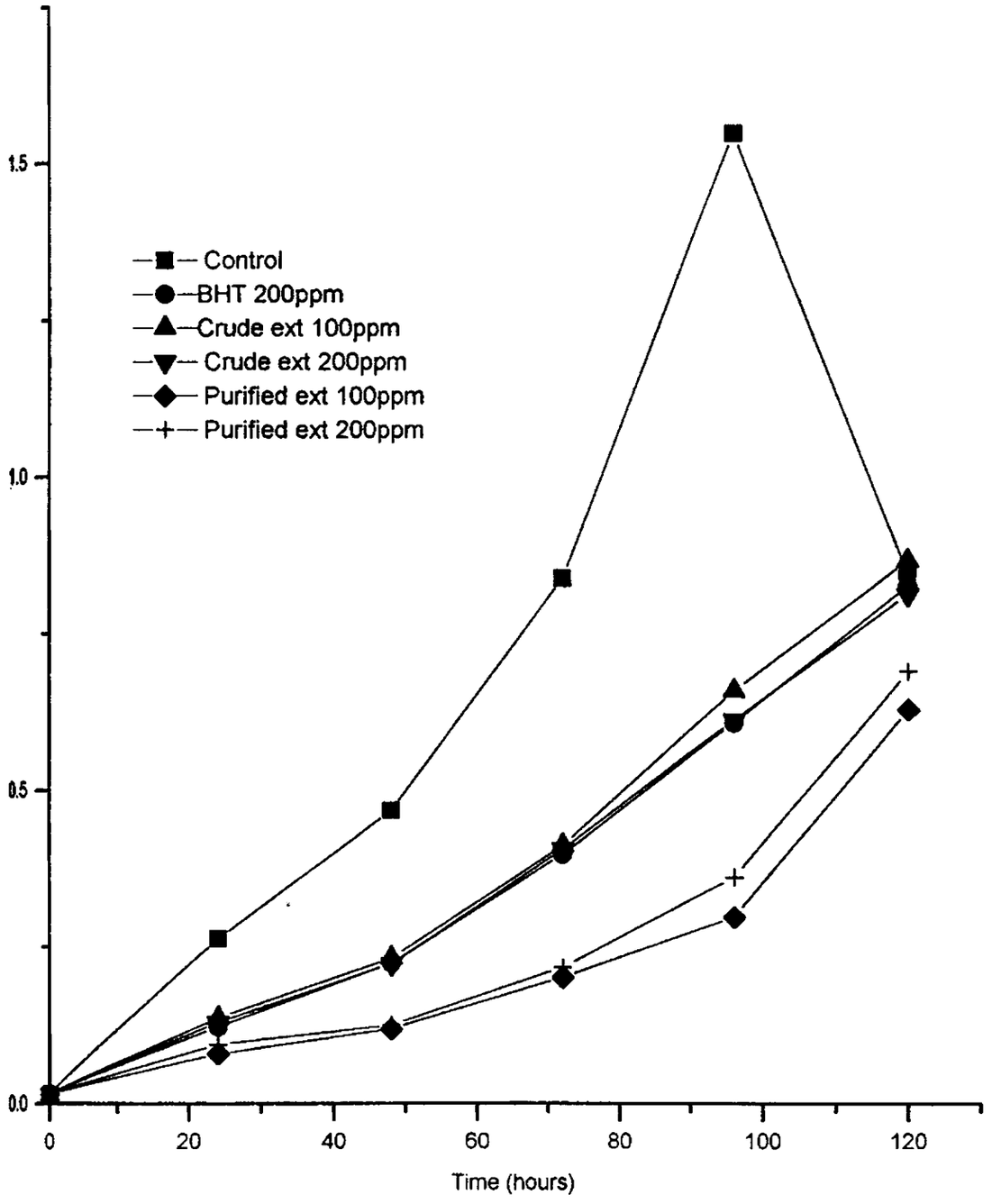


Figure 3.10: Antioxidant activity of sesame extracts and BHT by the Thiocyanate method-linoleic acid emulsion system

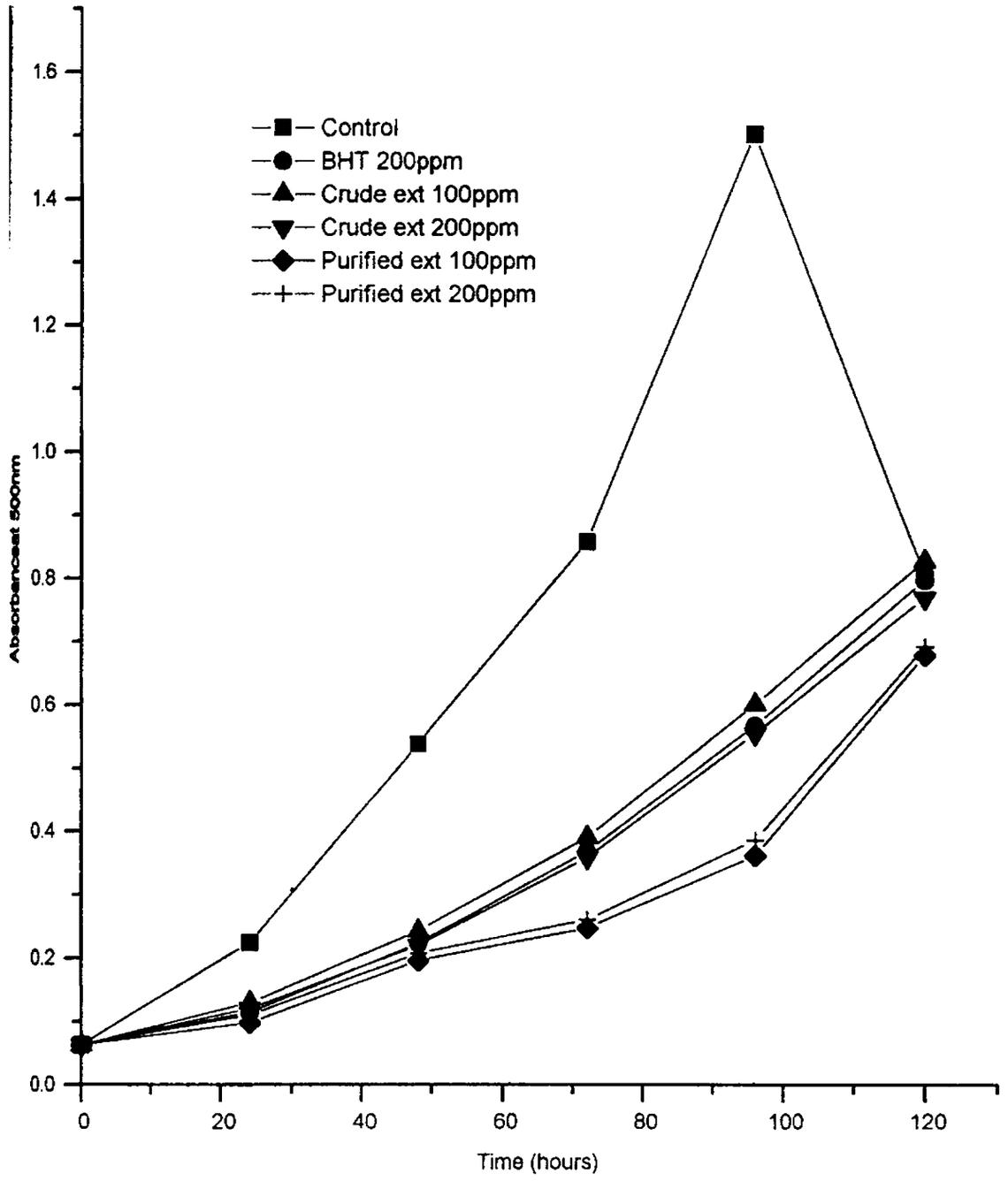


Figure 3.11: Antioxidant activity of sesame extracts and BHT by the Thiocyanate method-Linoleic acid system

200ppm, purified extract at 100, 200ppm, BHT at 200ppm were 57, 61, 81, 77 and 61% respectively. The antioxidant efficacy of purified extract was markedly higher than that of crude extract and BHT. Activity of crude extract was comparable to that of BHT at comparable concentrations. The result obtained by this method is by and large are similar to those of β-carotene method in terms of inhibition of lipid oxidation.

3.2.2.2.Linoleic acid system

Antioxidant activity profile of sesame cake extract evaluated by linoleic acid system is shown in Figure 3.11. Crude extract at 100 and 200ppm showed 62 and 63% inhibition respectively, while purified extract at 100 and 200ppm and BHT at 200ppm showed 76 and 74and 62% inhibition respectively by this assay. The activity of purified extract was higher than that of crude extract and BHT while the activity of crude extract and BHT were comparable. The percentage inhibition of extracts showed similar trend in both model systems. The results from linoleic acid system and β-carotene assay were mutually supportive in terms of the ability of sesame cake extracts to inhibit peroxidation.

3.2.3.Antioxidant efficacy of sesame lignans in vegetable oils

Autoxidation of vegetable oils/lipids and consequent quality deterioration in the bulk oil or in food systems is a major quality problem with huge economic implications. Currently several synthetic antioxidants are in use in food industry to control lipid oxidation. There are several standard methods to assess oxidation status of vegetable oils and food system. In the present investigation, Schaal oven test method and Differential scanning calorimetry (DSC) analysis were employed to assess the oxidation of vegetable oils and cake extracts were used to assess their ability to inhibit oxidation in comparison with commonly used synthetic antioxidants such as BHT, TBHQ etc.

3.2.3.1. Schaal oven test method

The oxidative stability studies were carried out at 60^0C in an incubator. Determination of peroxide value (PV) of oils oxidized at 100^0C is unreliable because hydroperoxides decompose at elevated temperature [130]. Therefore the antioxidant efficacy of sesame cake extracts in soybean, sunflower and safflower oils were carried out at 60^0C.

The antioxidant efficacy was initially studied in soybean oil with crude extract. BHT and TBHQ at 200 ppm were used for comparison. PV, Diene value and p-anisidine values were measured periodically (after each week) for 4 weeks. Sesame cake extracts were tested at 50, 100 and 200 ppm level. The peroxide values for soybean oil with different concentrations of methanolic extracts from sesame cake are shown in Figure 3.12. There were significant differences in the peroxide values among the control oil and the oil with added sesame extracts and BHT. The TBHQ containing oil had significantly lower peroxide value and the percentage inhibition was highest (61%) compared to all other treatments. The oil with sesame cake extract at 200 ppm, 100ppm and BHT at 200 ppm showed almost similar PV after four weeks of storage (92.8, 93.0, and 92.7) and these values were lower than that of control (105). Up to three weeks of storage, sesame extracts at 200, 100 and 50 ppm and BHT showed similar PV developments. There was significant protection offered by crude sesame cake extracts to vegetable oils and the efficacy was comparable to BHT.

Purified extract was used for all the subsequent experiments with different vegetable oils (soybean, sunflower and safflower) employing Schaal oven method.

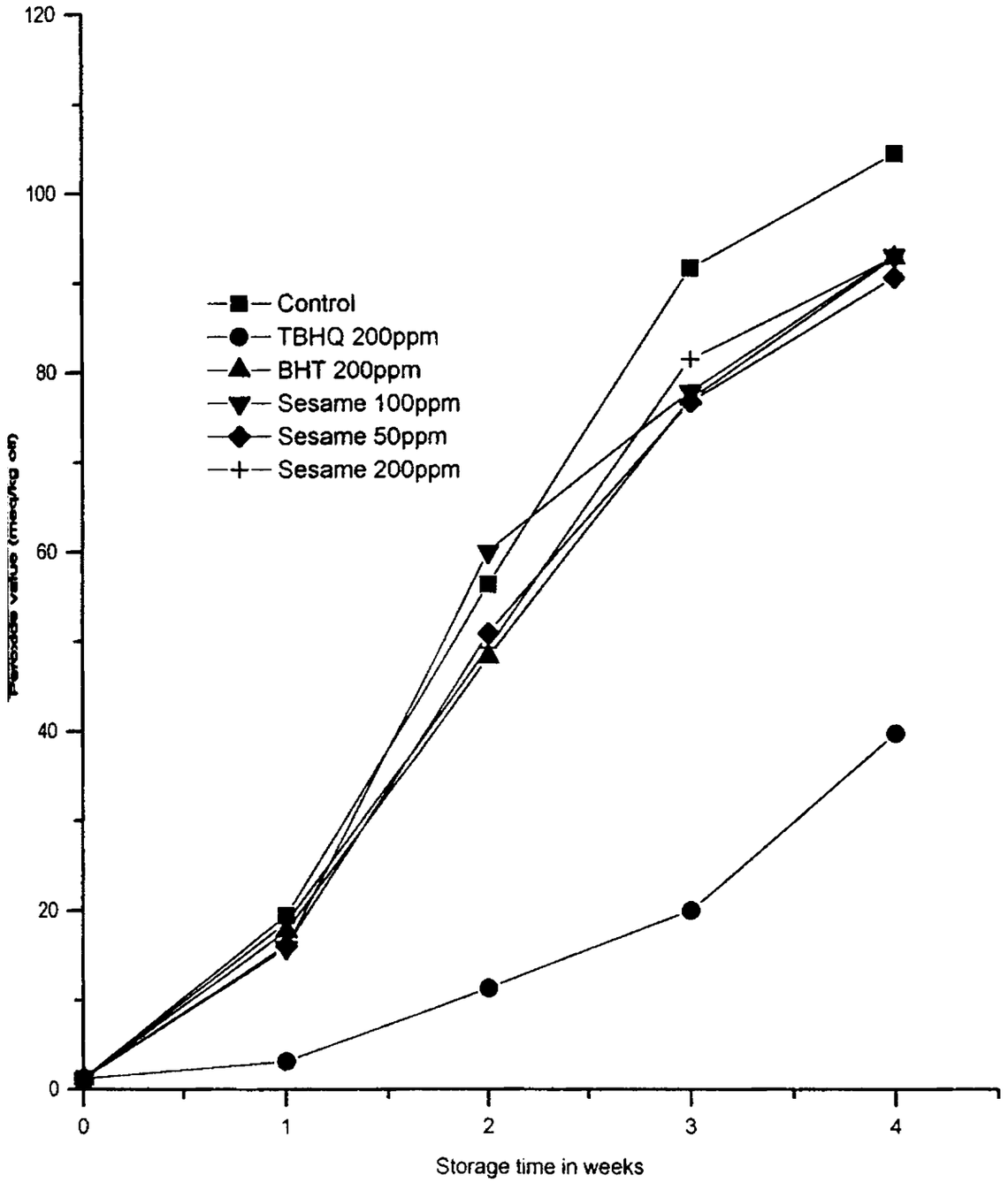


Figure 3.12 Antioxidant activity of sesame cake extract (crude) in terms of peroxide value inhibition in soybean oil by Schaal oven method at 60^0C

Soybean oil (SBO): Oil storage studies were further carried out with purified sesame cake extract.Figure.3.13.shows the peroxide developments during the storage of SBO at 60°C for 15 days with various concentrations of sesame cake extracts (SCE). Samples were analyzed at 3, 6, 9, 12, and 15 days. Other treatments in the study included TBHQ and BHT at 200 ppm and a control without no additives. SBO without the antioxidant (control) reached a maximum PV of 89.2 meq/kg after 15 days of storage. A significant difference ($P<0.05$) in PV was observed between the control and SBO containing sesame cake extract (SCE), BHT and TBHQ. The PV of SBO with 5,10,50 and 100 ppm of SCE, 200 ppm BHT and 200 ppm TBHQ were 74.2, 65.7, 72.9, 69.4, 80 and 41.4 respectively. The corresponding inhibition rates were 16.7, 26.3, 18.2, 19.8, 10.3 and 53.6% after 15 days of storage as compared with the control. Eventhough the effect due to four different SCE concentrations were varying during the incubation periods, the total effect is more or less equal. These results indicated that SCE inhibited soybean oil oxidation effectively. Further, the antioxidant effect of SCE at 5,10,50 and 100 ppm was far better than BHT at 200 ppm. During incubation period, TBHQ maintained a significantly lower PV than all other treatments. During initial trials, the protective effect offered at 200 ppm was comparable with that at 100 ppm concentration. Hence for subsequent experiments 200 ppm was eliminated.

Diene value is the measure of conjugated double donds of hydroperoxides and therefore expected to follow the patteren of PV. Figure 3.14 represents the diene value of the SBO samples at different storage periods. The diene value of the control reached 11.9 from an initial value of 2.9 after 15 days of storage. The values for cake extracts at 5, 10, 50,100 ppm, 200 ppm BHT and 200 ppm TBHQ were 10.2, 9.9, 10.1,9.8, 10.0 and 7.1

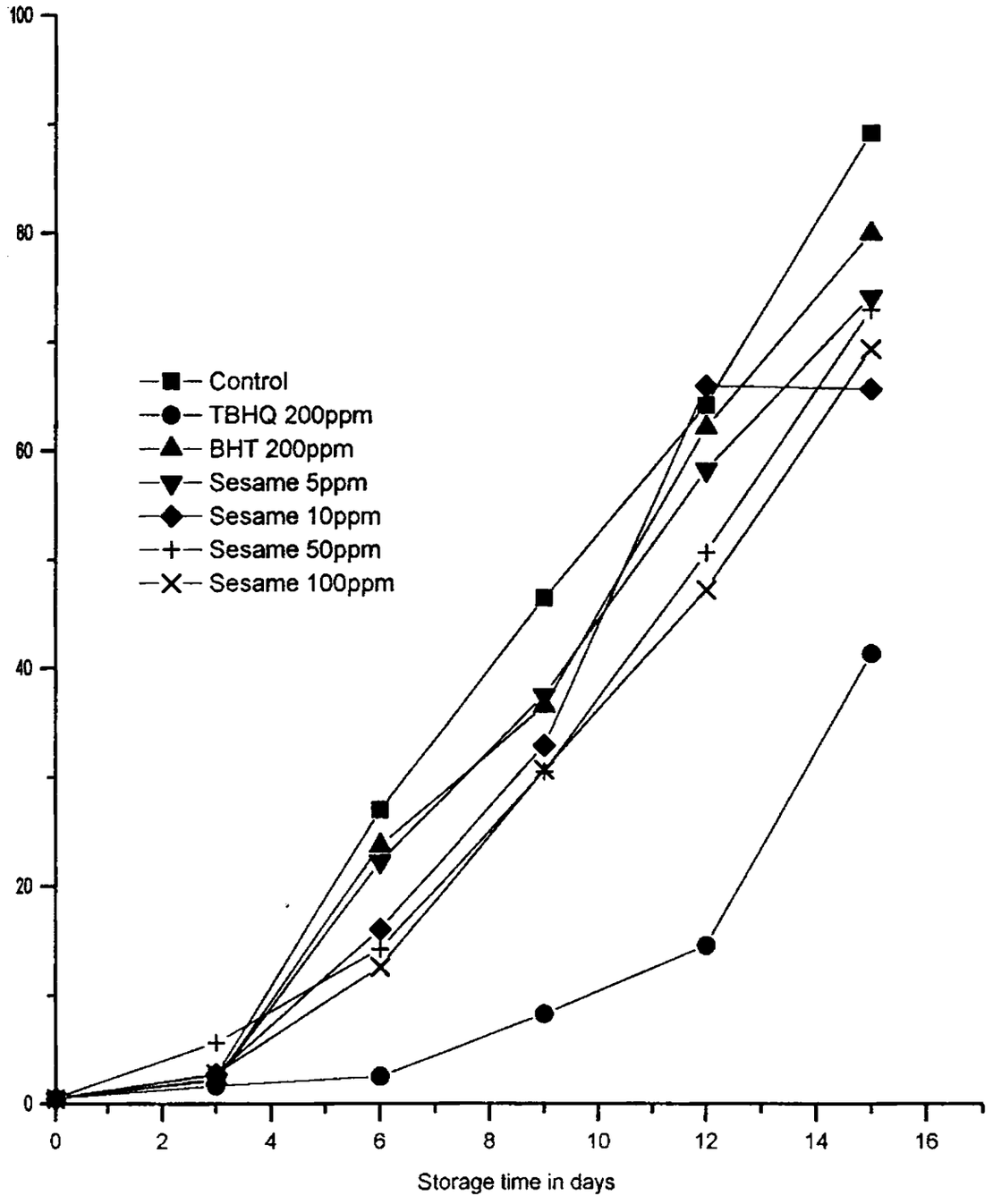


Figure3.13: Antioxidant activity of sesame cake extract(purified) in terms of peroxide value inhibition in soybean oil by Schaal oven method at 60^0C

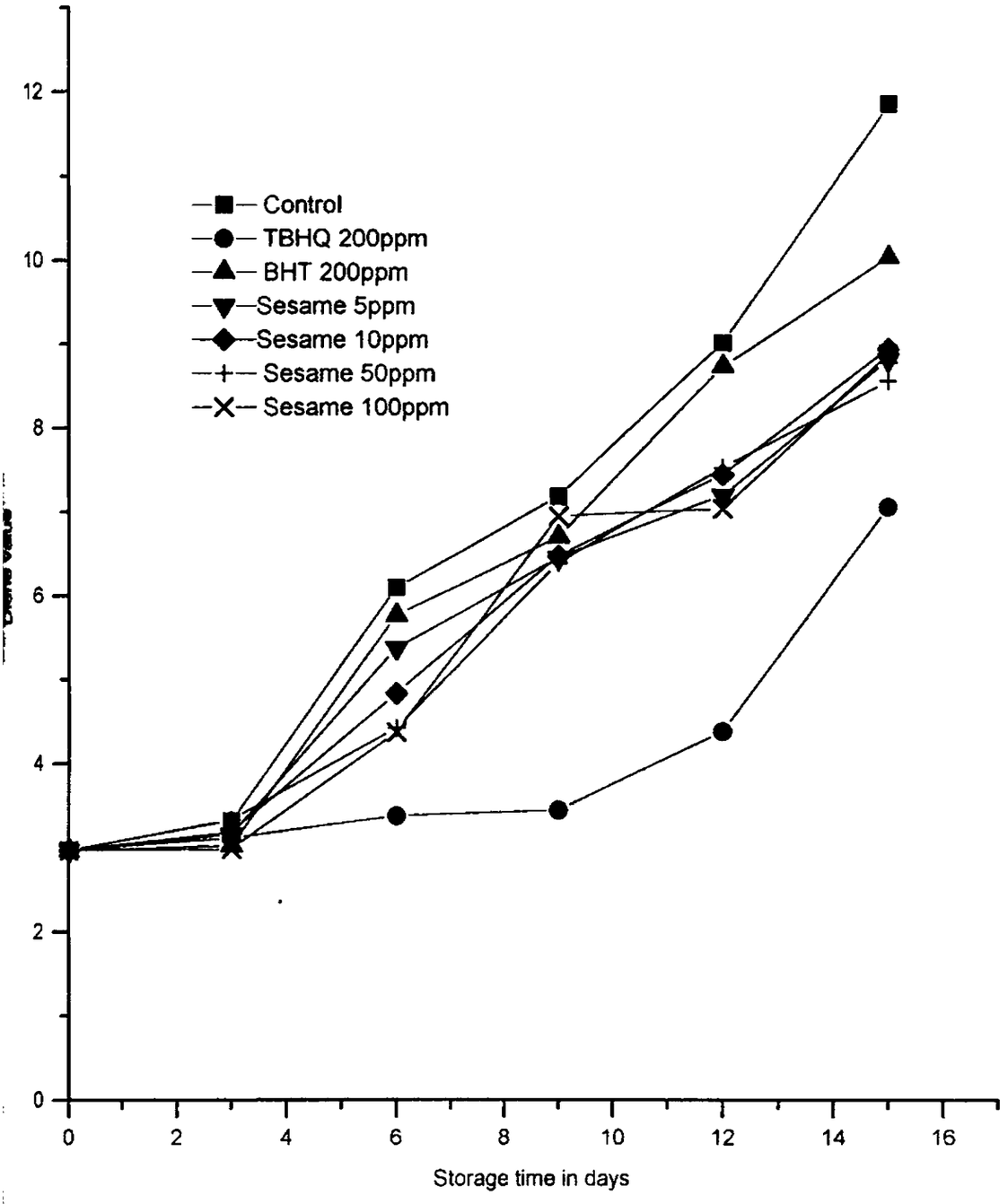


Figure3.14:Antioxidant activity of sesame cake extract (purified) in terms of diene value in soybean oil by Schaal oven method at 60^0C

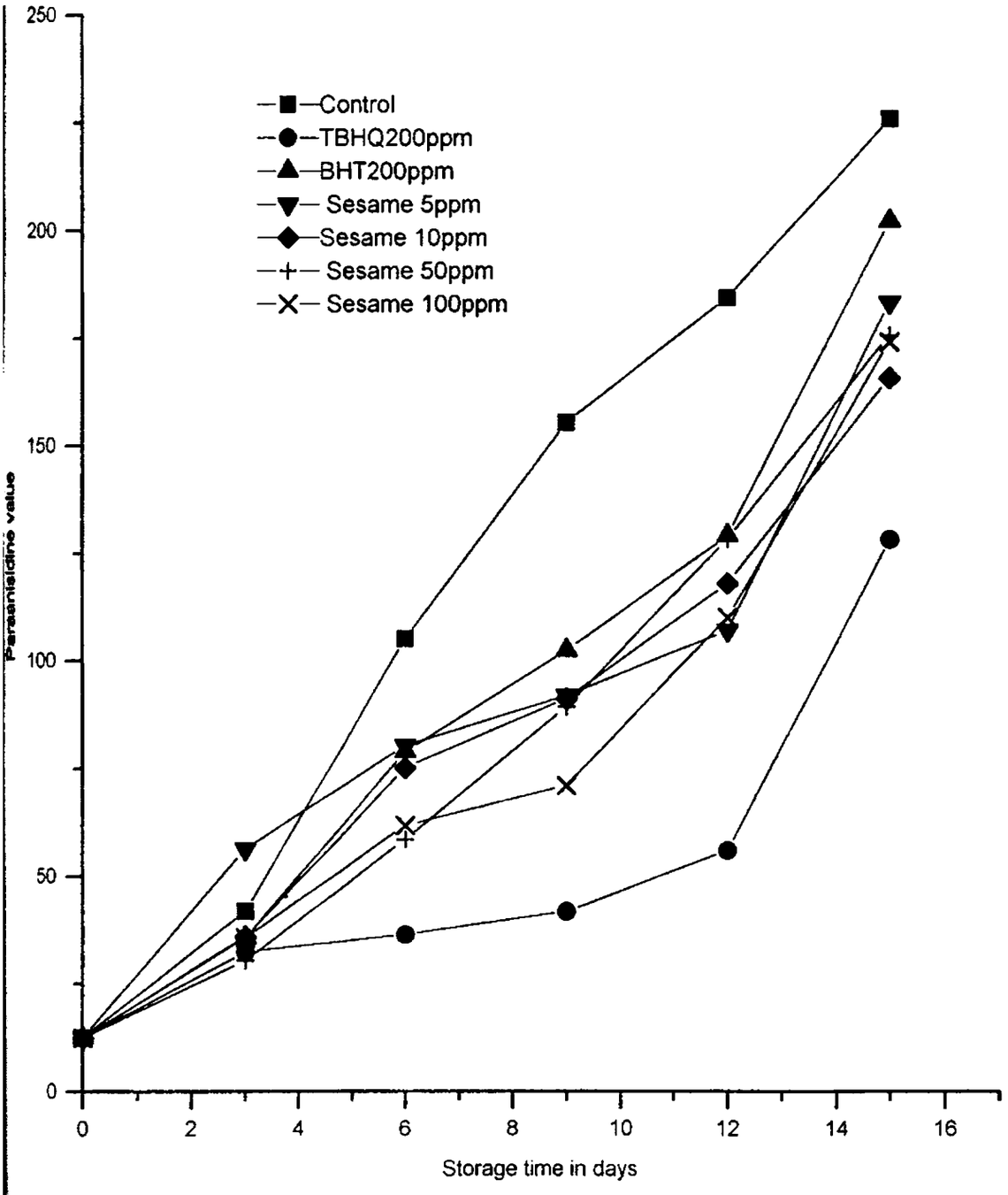


Figure3.15:Antioxidant activity of sesame cake extract (purified) in terms of p-anisidine value in soybean oil by Schaal oven method at 60^0C

respectively. The diene values of all treatments were significantly lower than that of control and exhibited similar pattern observed for PV.

Changes in p-anisidine value, which represent the secondary oxidation products produced during the oxidative degradation of oil, are shown in Figure 3.15. The formation of secondary oxidation products also increased during storage. The p- anisidine value of control reached a maximum of 226.0 from an initial value of 12.4 after 15 days of storage. The values for SCE at 5,10,50 and 100ppm, BHT at 200 ppm and TBHQ at 200 ppm were 183.2, 165.8,175.6, 174.1, 202.2 and 128.2 respectively. A sudden increase in p-anisidine value was noticed after 12 days of storage. A significant difference was noticed between the values for control and experimental samples.

Antioxidants are mainly used in lipids to delay the accumulation of primary oxidation products and thus to improve the oxidative stability. The primary products of lipid peroxidation are hydroperoxides, which are generally referred to as peroxides. Therefore the results of peroxide value estimation is an indication of lipid autoxidation. For further confirmation of these results, other oxidation parameters such as diene value and p-anisidine value were also measured. Thus PV, diene value and p- anisidine value of soybean oil that contained the extract were significantly lower than that of control, which are farmore effective than BHT but lesser than TBHQ.

Sunflower oil (SUFO): The PV developments of SUFO with sesame cake extract at 5,10,50,100 ppm, BHT 200 ppm, TBHQ 200 ppm and of the control sample are represented in Figure 3.16. PV of control increased from an initial value of 1.5 to 84.9. The values for SCE at 5, 10, 50 and 100ppm, BHT 200 ppm and TBHQ 200 ppm were 56.4, 57.4, 61.4, 57.9, 79.0 and 15.8 respectively. The percentage inhibition for TBHQ

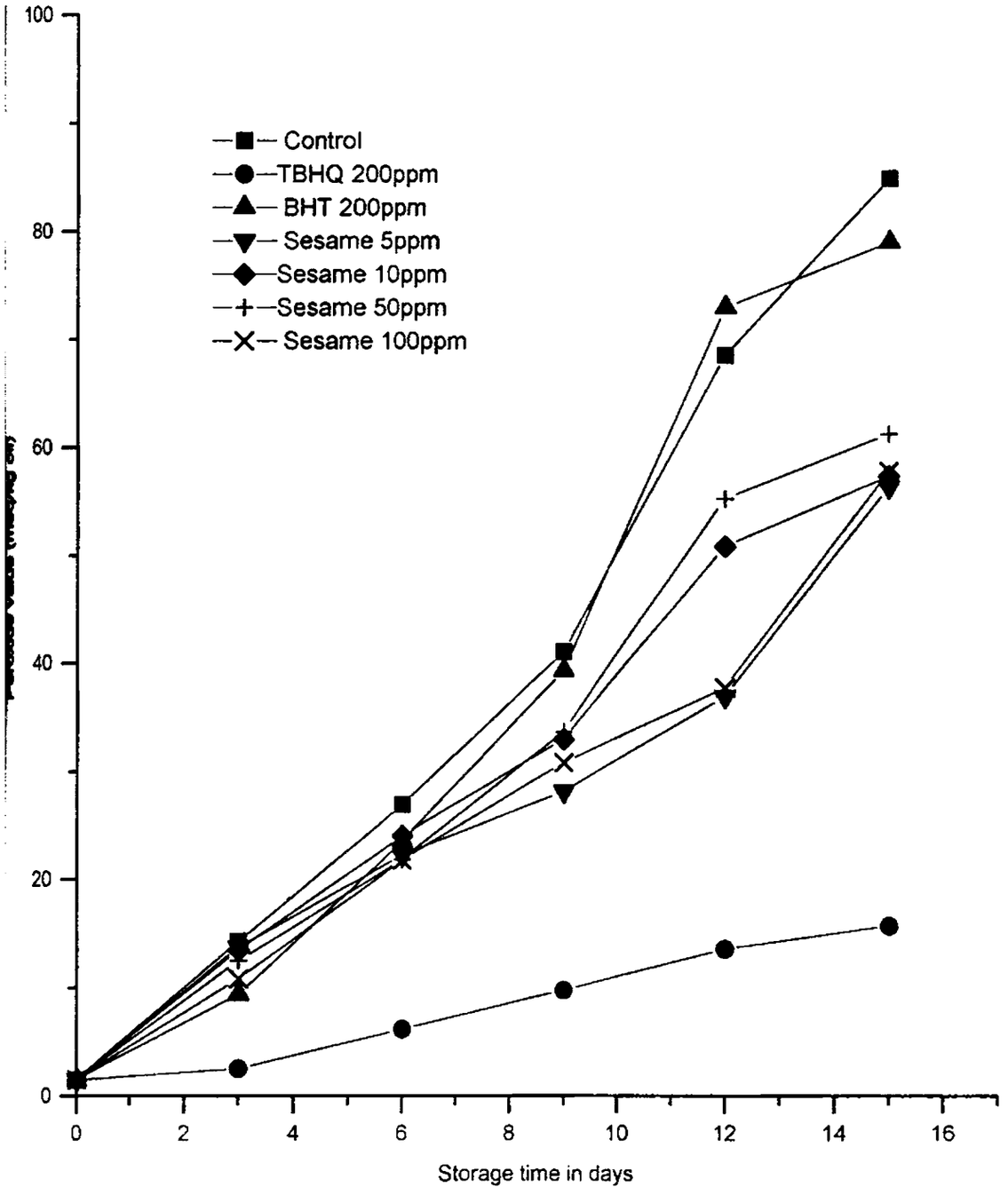


Figure3.16:Antioxidant activity of sesame cake extract (purified) in terms of peroxide value inhibition in Sunflower oil by Schaal oven method at 60^0C

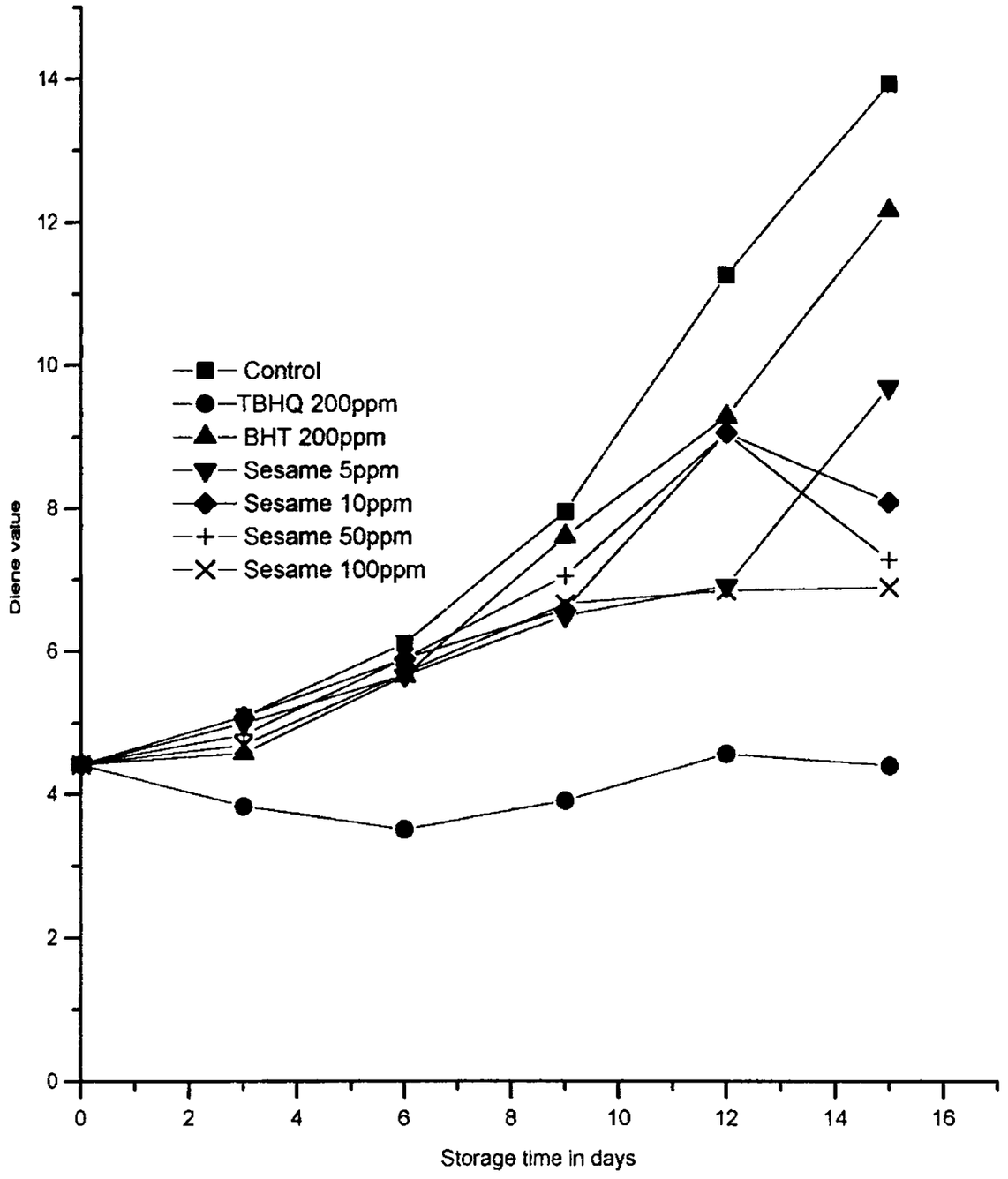


Figure3.17: Antioxidant activity of sesame cake extract (purified) in terms of diene value in Sunflower oil by Schaal oven method at 60^0C

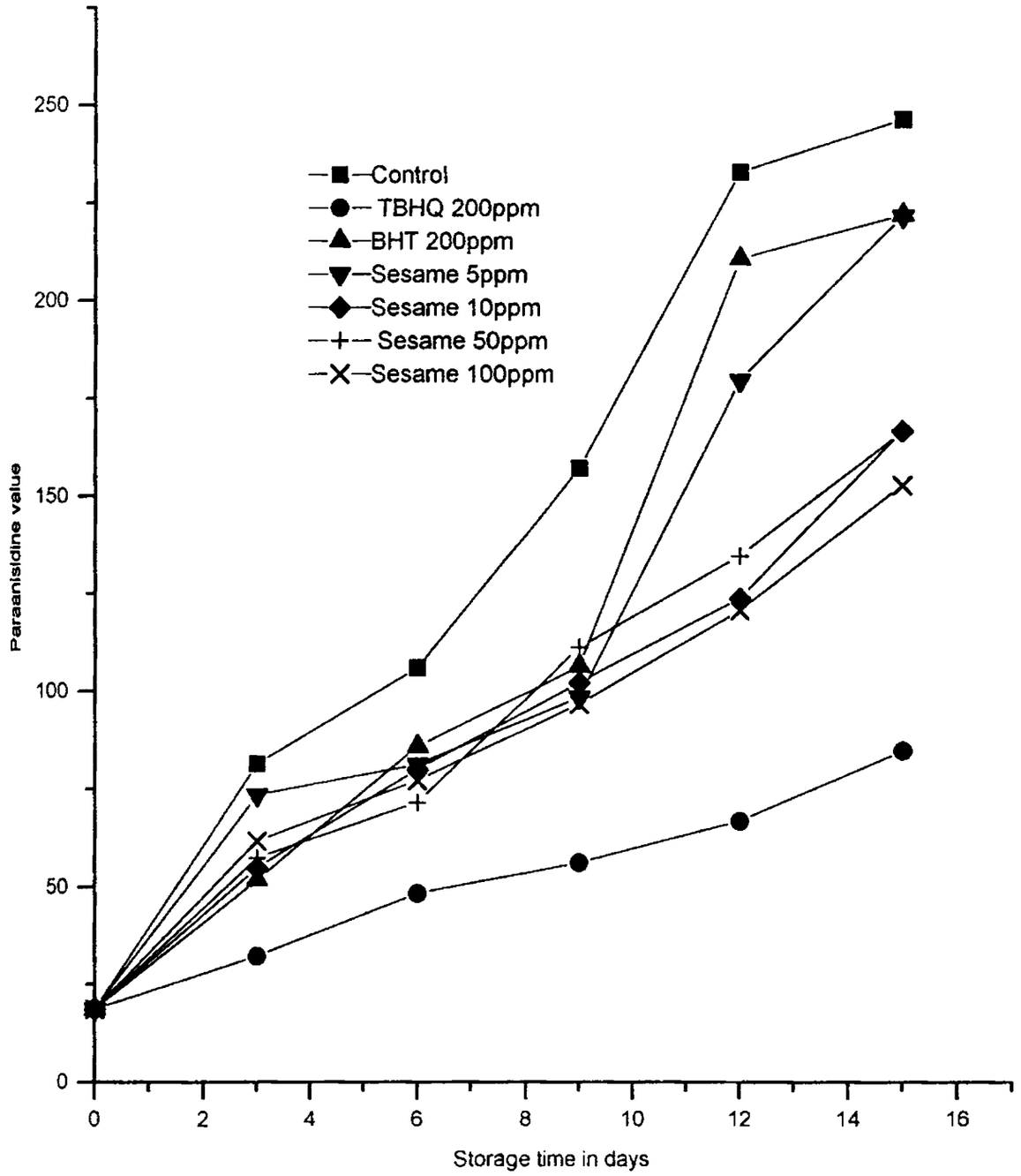


Figure3.18:Antioxidant activity of sesame cake extract(purified) in terms of p-anisidine value in sunflower oil by Schaal oven method at 60^0C

was very high. The antioxidant activities were calculated for BHT 200, SCE 5, 10, 50 and 100 as 6.9, 33.5, 32.4, 27.8 and 31.8% respectively and significant differences were found between these values, indicating that SCE exhibited an inhibitory effect on sunflower oil oxidation. Moreover, SCE at 50 and 100ppm levels were far superior to that of BHT at 200 ppm.

The diene value of control SUFO, increased to 13.9 from 4.4 after 15 days of storage (Figure 3.17). The diene value of SUFO with 5, 10, 50 and 100 ppm SCE, 200 ppm BHT and 200 ppm TBHQ were 11.2, 10.2, 10.0, 6.9, 12.5 and 4.5 respectively. These values are significantly lower than that of control stored for 15 days.

The p-anisidine value of SUFO (Figure.3.18) without added antioxidant (control) increased from an initial value of 18.8 to 246.7 after 15 days. The p-anisidine value of SUFO with 5, 10, 50, 100 ppm SCE, 200ppm BHT and 200 ppm TBHQ were 221.9, 167.1, 166.8, 153.1, 222.2 and 84.9 respectively. These results confirmed the protective action of sesame extracts on the oxidative deterioration of sunflower oil.

Safflower oil : The peroxide value developments for safflower oil with and without added antioxidants are shown in Figure 3.19. The peroxide formation for control was 85.7 (initial value=1.5) after 15 days. The PV for SCE at 5, 10, 50, 100, BHT at 200 ppm and TBHQ 200 ppm levels were 72.1, 74.7, 74.7, 56.9, 78.2and 19.2 meq of oxygen/kg respectively. There is significant difference between the control and other treatments. SCE 5, 10 and 50 ppm showed more or less equal antioxidant activity while SCE 100 ppm showed higher activity.

The diene value and p-anisidine value for the experimental samples are shown in Figures 3.20 and 3.21. The diene value of the control reached 12.7 from an initial value

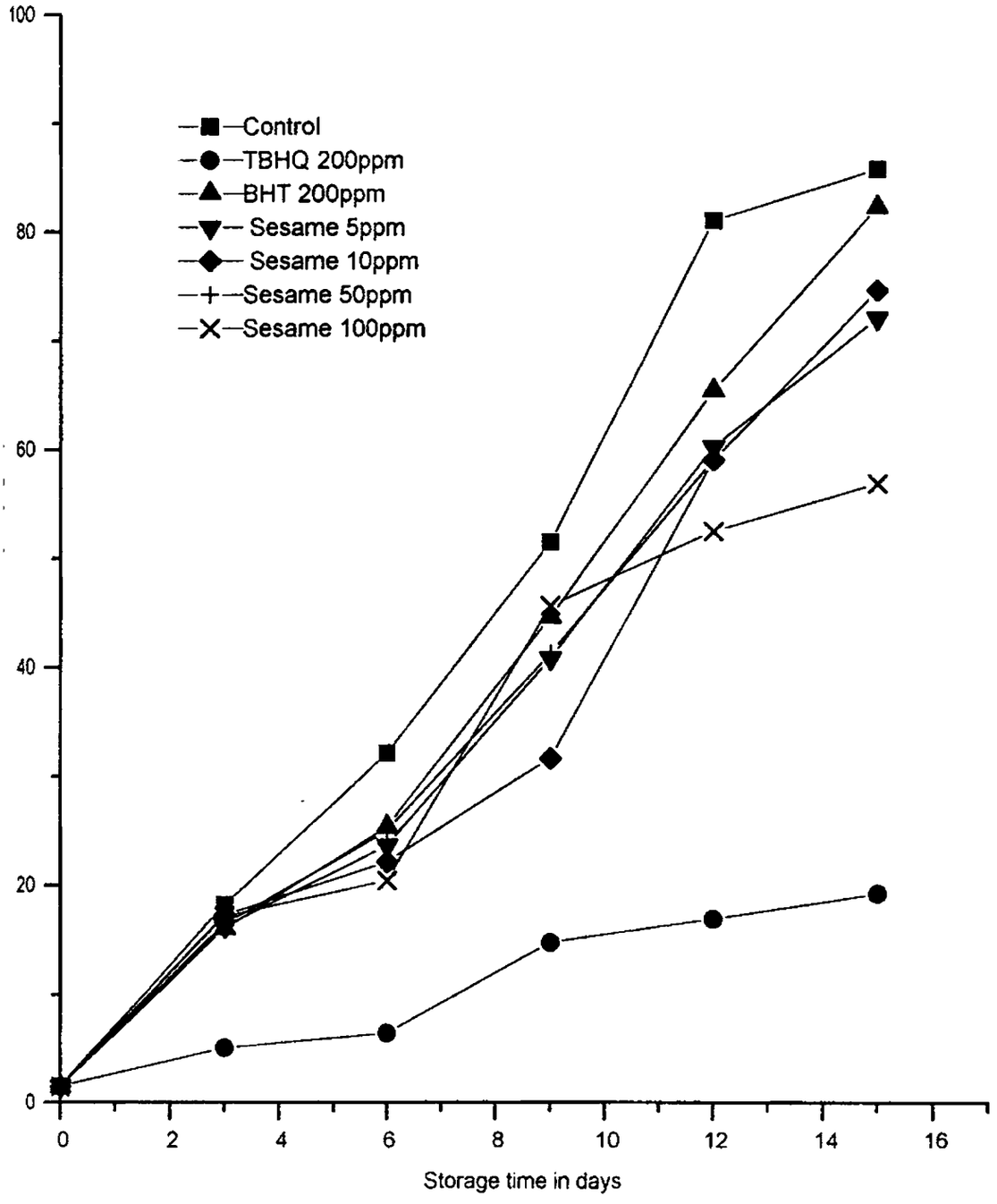


Figure3.19: Antioxidant activity of sesame cake extract (purified) in terms of peroxide value inhibition in Safflower oil by Schaal oven method at 60^0C

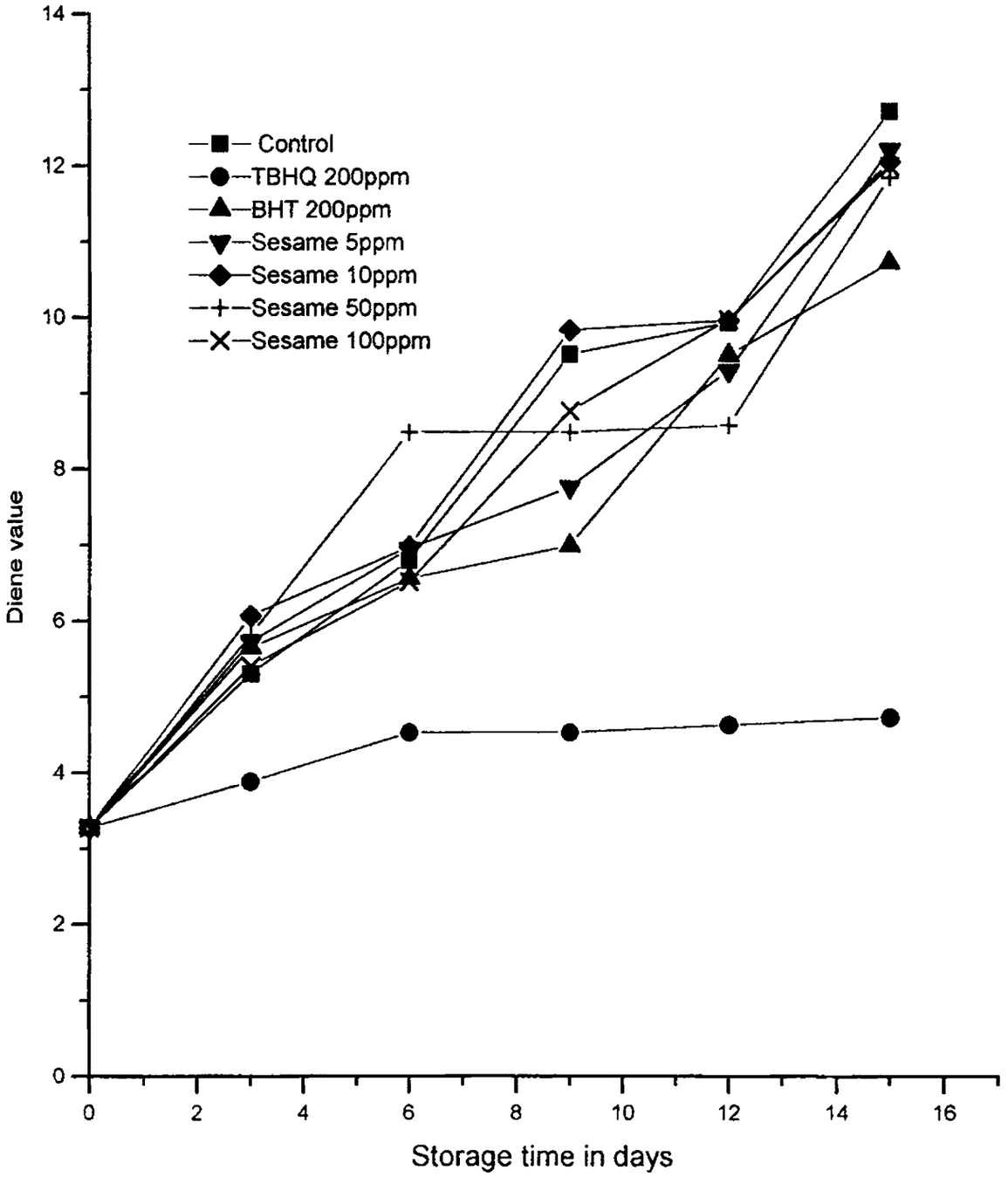


Figure3.20: Antioxidant activity of sesame cake extract (purified) in terms of diene value in Safflower oil by Schaal oven method at 60^0C

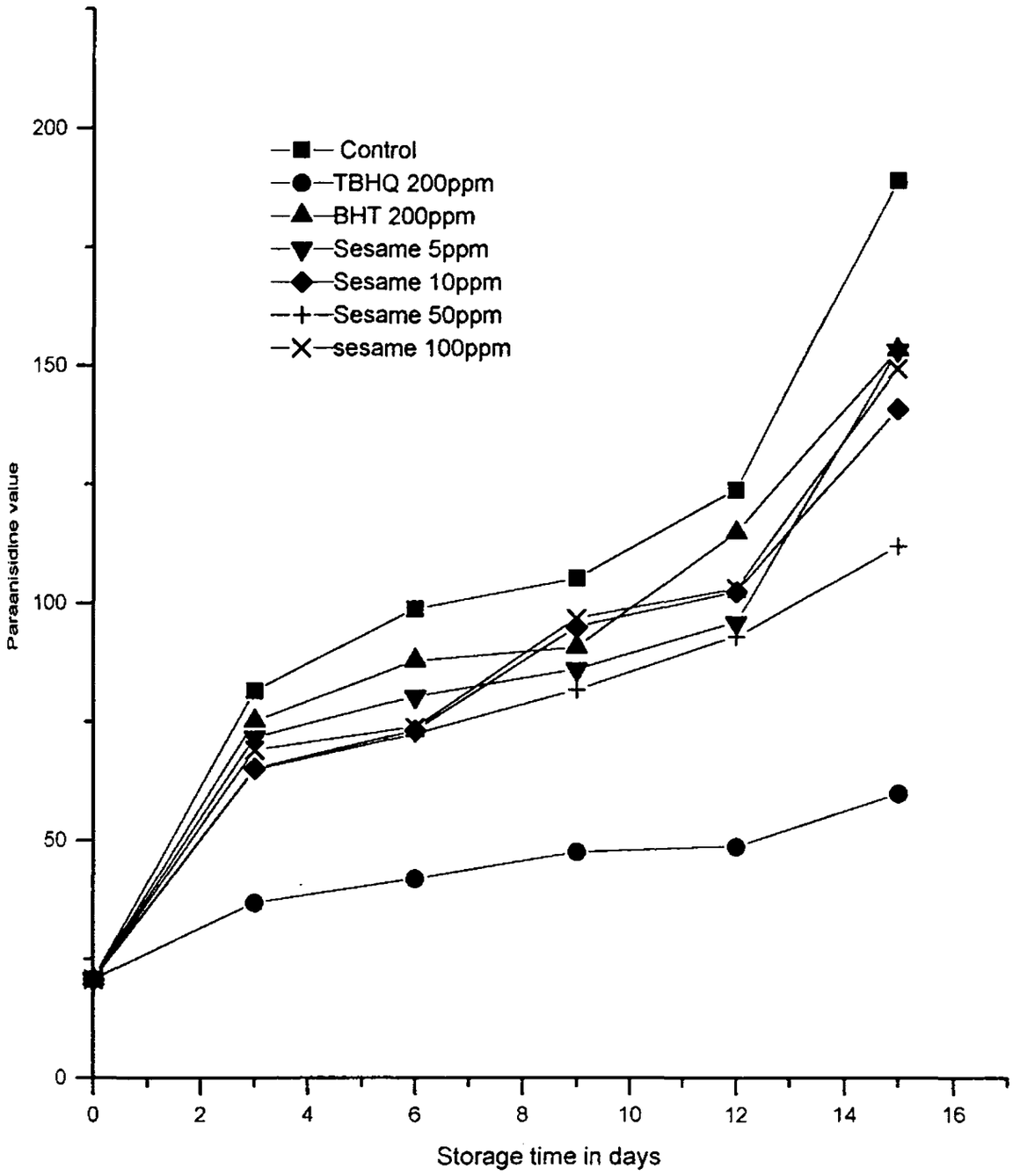


Figure3.21:Antioxidant activity of sesame cake extract (purified) in terms of p-anisidine value in Safflower oil by Schaal oven method at 60^0C

of 3.2 while that of SCE at 5, 10, 50, 100, BHT 200 and TBHQ 200 ppm levels were 10.2, 10.1, 9.8, 10.0, 10.7 and 4.7 respectively after 15 days. The p-anisidine value of control was 189.0 (initial value=20.7). The SCE at 5, 10, 50, 100, BHT 200, and TBHQ 200 ppm showed p-anisidine values as 153.3, 141.0, 112.0, 149.5, 153.4 and 59.9. There was significant difference in the diene and p-anisidine values of control and experimental samples. The antioxidative effects of SCE were better than that of BHT in safflower oil.

The oxidative stability studies were also conducted during storage at ambient (28+ 2^0C) temperature according to Schaal oven test method. Oil was filled in containers with or without headspace. The peroxide value developments are shown in Figures 3.22 and 3.23.The PV of soybean oil stored at ambient temperature, without headspace, were increased from 0.55 to 89.07 after 12 weeks of storage. The peroxide development upto 4 weeks was very slow and after that it showed rapid and progressive increase. The increase in peroxide value for oils treated with sesame extract at 10, 50 and 100ppm were 44.8, 35.08 and 42.2 after 12 weeks. Purified sesame cake extract at 50ppm showed 65% inhibition while at 10 and 100ppm had 50 and 52% inhibition respectively. TBHQ provided maximum protection of 79%. The inhibitory effect of sesame extract at 10 and 100ppm were similar to that of BHT while sesame extract at 50ppm showed higher protective effect. The oils stored with headspace showed slightly higher PV developments. It may be due to the higher surface to volume ratio. The inhibition effects when stored, with headspace, for SCE 10, 50 and 100 were 45, 61 and 48% respectively.

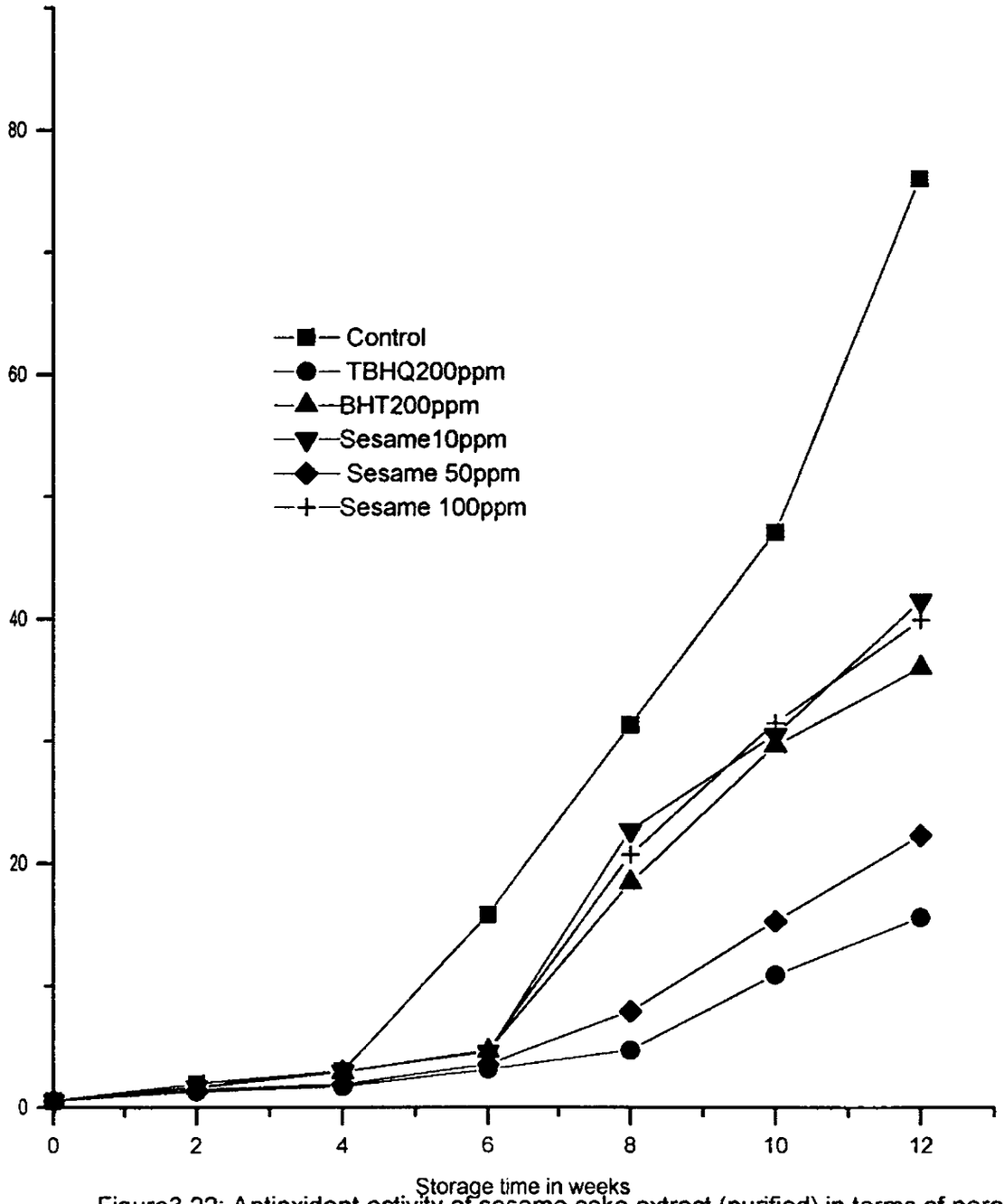


Figure3.22: Antioxidant activity of sesame cake extract (purified) in terms of peroxide value inhibition in Soybean oil stored at ambient temperature (with head space)

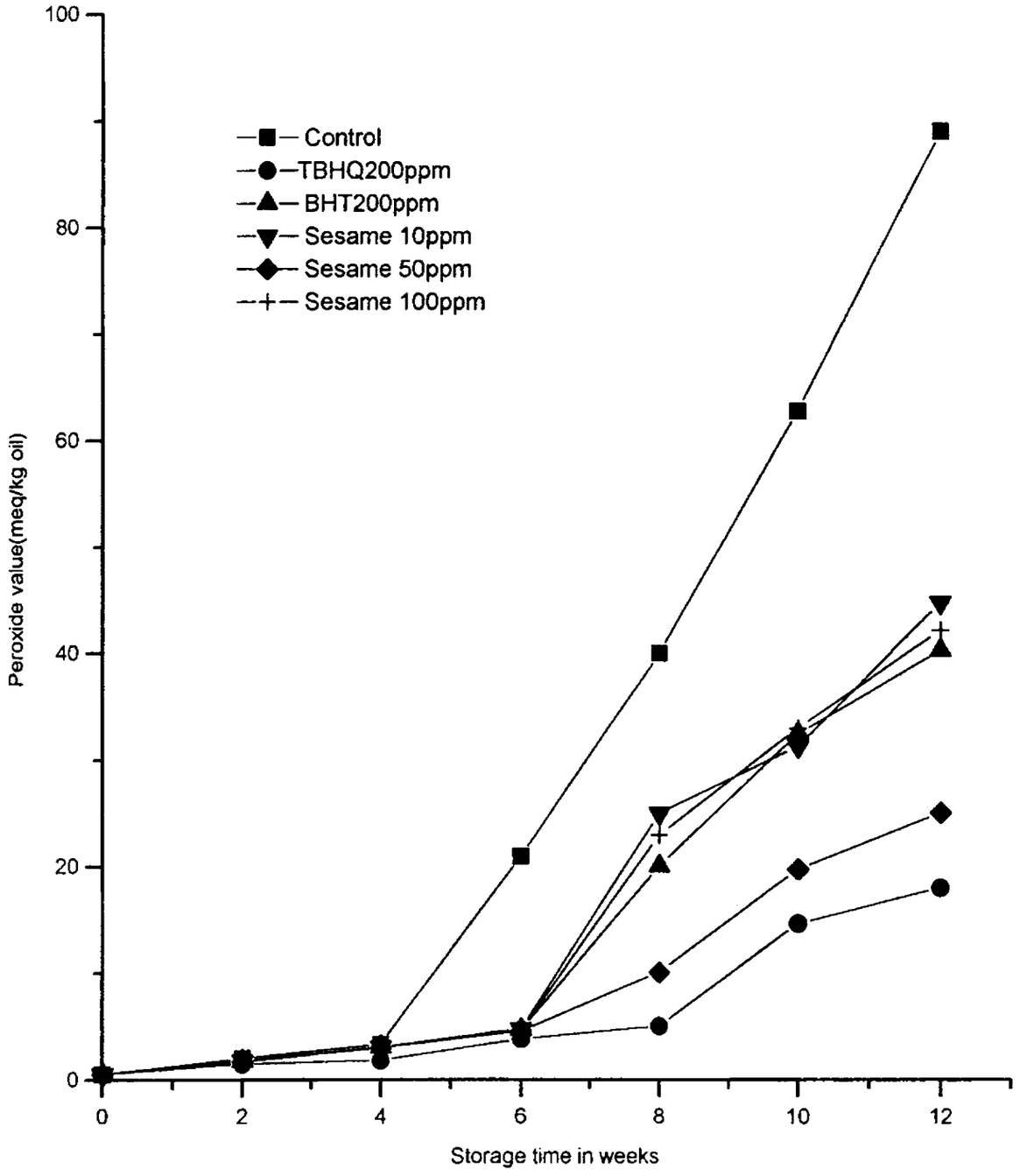
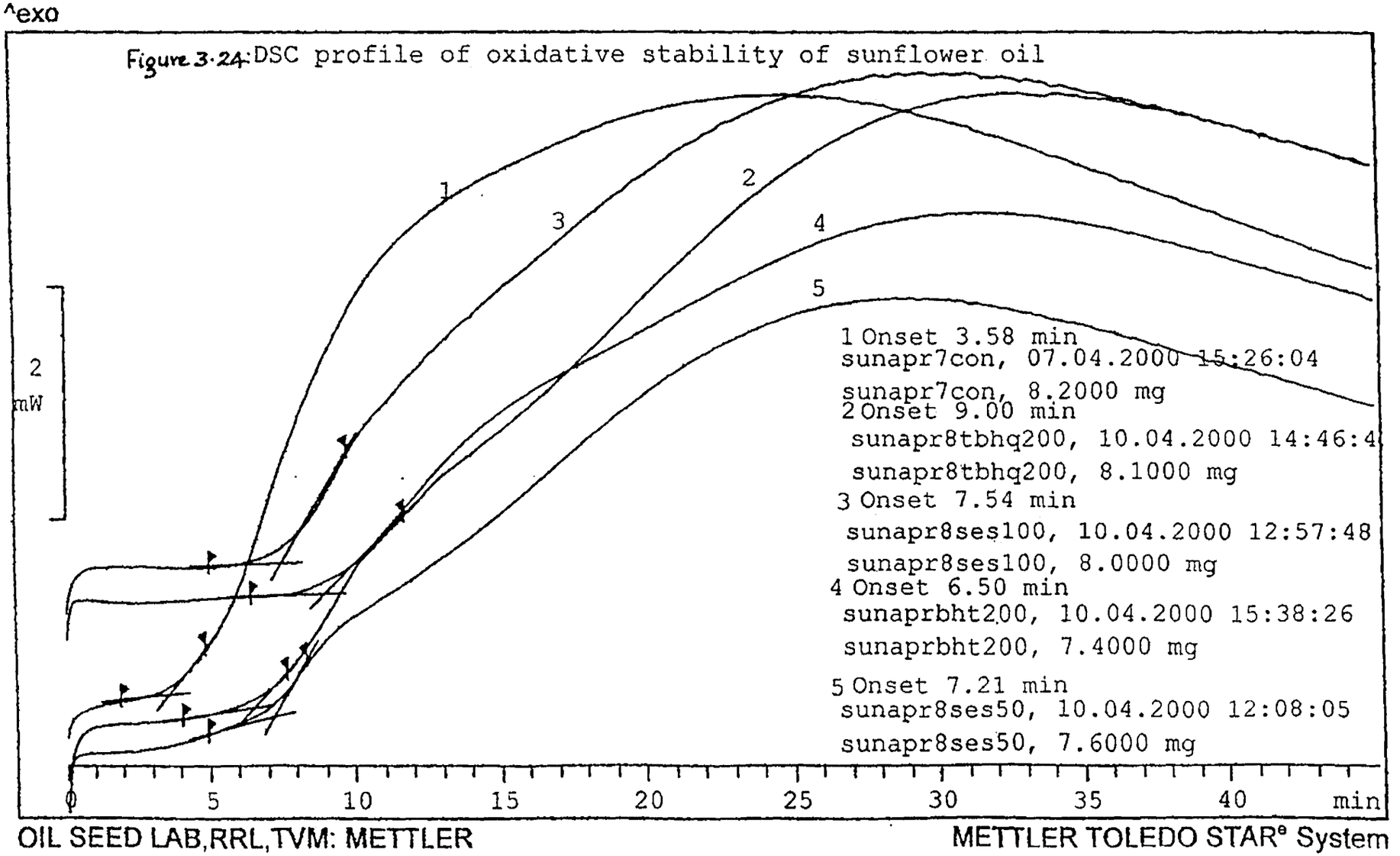


Figure3.23:Antioxidant activity of sesame cake extract (purified) in terms of peroxide value inhibition in Soybean oil stored at ambient temperature(without head space)

3.2.2.2. Differential Scanning Calorimetric (DSC) analysis for antioxidant efficacy of sesame cake extract

DSC provides unique energy profile information, which significantly measures the temperature and heat flows associated with material transitions as a function of time and temperature. The DSC profile of sunflower oil is shown in Figure 3.24. When heated at 150^0C for 45 minutes, the onset of oxidation of control was at 3.58 minutes. However in the case of oil containing SCE at 100 ppm, 50 ppm and BHT 200ppm the induction period was 6.93, 6.98 and 6.34 minutes respectively. Samples with TBHQ showed the induction period at 8.71 minutes. These results indicate that even at higher temperatures, sesame extract is capable of protecting the oil to the same extent as BHT at 200 ppm levels. Moreover, in soybean and safflower oils, sesame extract was more effective in protecting vegetable oils at lower concentration levels than BHT. DSC analysis can be adopted as a fast and reliable method for evaluation of oil stability. Oil samples, which require 15 days using Schaal oven method, could be evaluated for their oxidative stability in less than one hour by DSC method as demonstrated here.

The antioxidant activity of sesame cake extracts was evaluated by the widely used β-carotene bleaching assay, since β-carotene is extremely susceptible to free-radical mediated oxidation because of its eleven pairs of double bonds [131]. Moreover β-carotene, is a physiologically significant compound involved in photosynthesis in plants, precursor of Vitamin A and antioxidant in human system [132]. Furthermore, when β-carotene is used as a food color, its discoloration due to oxidation would affect quality adversely [133].


^exo
Figure 3·24: DSC profile of oxidative stability of sunflower oil
1
2
3
4
5
2
mW
1 Onset 3.58 min
sunapr7con, 07.04.2000 15:26:04
sunapr7con, 8.2000 mg
2 Onset 9.00 min
sunapr8tbhq200, 10.04.2000 14:46:4
sunapr8tbhq200, 8.1000 mg
3 Onset 7.54 min
sunapr8ses100, 10.04.2000 12:57:48
sunapr8ses100, 8.0000 mg
4 Onset 6.50 min
sunaprbht200, 10.04.2000 15:38:26
sunaprbht200, 7.4000 mg
5 Onset 7.21 min
sunapr8ses50, 10.04.2000 12:08:05
sunapr8ses50, 7.6000 mg
0
5
10
15
20
25
30
35
40
min
OIL SEED LAB,RRL,TVM: METTLER
METTLER TOLEDO STAR® System

The significant difference in antioxidant index (A_A) values found between crude extract and purified extract as reported here are probably due to the higher lignan content and also less interfering compounds like fat and sugar in the purified extract. Also, the A_A index values provide a useful and easily determinable index for antioxidant effect of the extract. Moreover, the antioxidant activity obtained for purified extract in the present study is comparable with other natural extracts reported. For instance evening Primrose seed extracts reported to have comparatively less activity at 250ppm than purified sesame cake extract at 50ppm as shown here [134]. Grape seed extracts also shown to have lower activity at higher concentrations of 100, 150 and 200ppm as compared to those of sesame extracts.. The advantage of sesame cake extract is its higher antioxidant power at lower concentration compared to other natural extracts and synthetic antioxidants except TBHQ. Moreover the extract might be useful to protect β-carotene.

Owing to the major role played by lipids in various biological and food processes as source of free radicals, antioxidants are used primarily to inhibit lipid peroxidation. In the present case, lipid peroxidation was studied using two model systems representing homogeneous (single phase) and heterogeneous phase (food system) by linoleic acid and linoleic acid emulsion system respectively. The antioxidant activity in heterogeneous system might be affected by several parameters, including concentration of antioxidants, partitioning and interaction with other compounds such as transition metals [13].

In the present experiments, both crude and purified sesame cake extracts showed potent inhibition of lipid peroxidation in the linoleic acid and linoleic acid emulsion system. These results would imply that sesame cake extracts works well at both interfaces and therefore has both lipophilic and hydrophilic properties. The theoretical basis for

these results is supported by similar studies wherein interaction between individual antioxidants in the extract and the lipid substrate are explained [32]

Previously acetone extract of sesame seed was studied for antioxidant activity by thiocyanate method and the active compounds isolated found to be aglycone of simplexoside, ferulic acid and sesamolinol [76]. Aqueous alcoholic extract of sesame seed has also been reported to be active by thiocyanate method [77]. However, antioxidant activity of sesame cake extracts so far has not been reported in two different systems. Moreover, there are very few natural extracts, which are active in both systems. The positive results obtained for sesame cake extracts here therefore has potential to be used in different types of lipid based food systems.

Antioxidant activity of natural extracts in protecting vegetable oils during storage are reported. Tian and White conducted a series of experiments to evaluate the antioxidant activity of oat extract in soybean and cottonseed oil [135]. The added antioxidant concentration was based on total phenolic content and they used higher concentration at 200 and 300 ppm and the extent of inhibition offered at this concentrations are comparable with that of SCE at 50ppm in the present study indicate that SCE far more potent than oat extracts..

Duh etal., examined the antioxidant activity of mung bean hulls in soybean oil oxidation [136]. They compared the activity with that of BHA and tocopherol and the activity was less than that of BHA and tocopherol. Antioxidant efficacy of peanut hull extracts was evaluated in soybean and peanut oil by Pin-Der Duh etal., [137]. They used higher concentration of extract and obtained comparable result with that of BHA.

The effectiveness of sesamol and tocopherol and their mixtures at different concentrations on the oxidative stability of tocopherol stripped oils (soyabean, rapeseed and safflower) was studied under microwave heating conditions by Yoshida etal.[138]. A combination of tocopherol (400ppm) and sesamol (200-400ppm) was found to be useful in protecting these oils. The unusual stability of sesame oil is also known [139]. However, an antioxidant extract obtained from sesame cake which can be utilized at a very low concentration (10-100ppm) compared to that of other natural extracts, tocopherol and sesamol has not been investigated so far. The SCE protect different vegetable oils, with different levels of unsaturation and tocopherol levels as demonstrated through Schaal oven test method in this study.

Some of the commercially available antioxidant extracts such as Rosemary are considered high potency natural antioxidants. However they are used at 0.02, 0.05 and 0.1% levels [31], whereas sesame cake extracts are effective at a concentration of 0.01% and below (10-100ppm) for comparable protection levels at comparatively lesser cost (US Patent Application No 60/404.004). Thus sesame antioxidant extract will be highly competitive and efficient, especially when produced from a byproduct like sesame cake. Since it is derived from an oil seed , it will be more compatible with vegetable oils. The fact that the extracts impart no color and odor and has the benefits of antioxidants such as sesamol, sesamin, sesamolin that are known to possess many biological properties are added advantages. The protection offered by the sesame cake extract in vegetable oils is far greater than that of widely used synthetic antioxidant like BHT at very low concentration as demonstrated through this study make SCE an ideal natural product for vegetable oils and processed foods.

3.2.3.Antioxidant activity of the wild species, Sesamum malabaricum

Sesamum malabaricum is the wild species growing in the coastal areas of Southern Kerala which shows sturdy growth. There are no research reports regarding the antioxidant content, activity and biological effects of phytochemicals of this wild species. The oil content was very low for this wild variety. The oil content of *S.indicum* and *S.malabaricum* were 48% and 17% respectively. The methanolic extracts and oil samples were analyzed for lignan content by reverse phase HPLC using μ-bondapak C_{18} column. .Quantitation included sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside. The total lignan content for the oil samples were 5152 and 11095 ppm for *S.indicum* and *S.malabaricum* respectively. The extract contained 15036 ppm lignans for *S.indicum* and 98802 ppm for *S.malabaricum.* Total phenolic contents of the extracts were analyzed by the AOAC method [3] and the values are presented in Table.3.8.

Antioxidant activity was evaluated for the extracts using β-carotene bleaching method . It was found that BHT, *S.indicum* and *S.malabaricum* extract showed 33.3, 44.7 and 82.2% inhibition respectively.Free radical scavenging power of extract was evaluated by [DPPH·] method . At 200 ppm level, the radical scavenging effect of S.indicum extract, S.malabaricum extract and BHT were 55.4, 92.8 and 49.6% respectively.

Preliminary analysis of seed extracts showed growth regulatory and ovicidal activities against tested insects. When mosquitoes were tested with these samples, high larval mortality and Insect growth regulation (IGR) were observed.

Above results strongly indicate the higher antioxidant, pesticidal and mosquitocidal properties of the wild species of sesame. Secondary metabolites are known to protect the plants, directly or indirectly from pests. Here also the natural occurrence of

higher lignan content may be considered as part of the protective mechanism of the wild species for its survival.

Table 3.8.Comparison of Biochemical Characteristics of the two varieties of seeds

S.No	Parameters	S.indicum	S.malabaricum
1.	Fat content (%)	48	17.1
2.	Total phenolic content of extract(%)	1.80	11.14
3.	Lignan content of oil(ppm)	6966	11095
4.	Lignan content of extract(ppm)	15036	98801
5.	Lignan glycoside content of extract (ppm)	10803	42215

3.3.Radical scavenging studies with sesame cake extracts

Currently various methods are available to evaluate the antioxidants under different conditions, to establish their ability to function be it food system or biological system. It is general practice, therefore to apply more than one condition of oxidation to evaluate antioxidant [140]. Systematic studies under different environments simulate actual conditions are, therefore rendered before antioxidants are put in to practical use. One of the well accepted mechanisms is to measure ability of antioxidants to scavenge free radicals. In the present investigation the radical scavenging effects of sesame cake extracts therefore, were studied in detail.

3.3.1. Free radical scavenging effects by DPPH

Lipid oxidation is a free radical mediated reaction. The antioxidant activity can be expressed in terms of radical scavenging activity during reaction with a specific radical such as [DPPH$^{\cdot}$] or [LOO$^{\cdot}$]. [DPPH$^{\cdot}$] study is more widely used, because of its simplicity and reliability. It measures the hydrogen donating ability of antioxidants in a relatively short time as compared to other methods and spectrophotometric characterization is also possible. In the case of [LOO$^{\cdot}$] kinetics, the peroxide value is determined by Active Oxygen Method (AOM) in oil or linoleic acid model system which is time consuming [141]. The reaction kinetics or the rate at which the antioxidant reacts with radicals can also be determined on time scale.

Crude and purified extracts of sesame cake were studied for DPPH scavenging activity. The scavenging reaction between [DPPH$^{\cdot}$] and antioxidant can be expressed in following equations,

$$[DPPH^{\cdot}] + (AH) \longrightarrow DPPH\text{-}H + (A^{\cdot})$$

The new radical formed can undergo radical-radical interaction to render stable molecules [142, 143].

$$DPPH^{\cdot} + A^{\cdot} \longrightarrow DPPH\text{-}A$$

$$A^{\cdot} + A^{\cdot} \longrightarrow A\text{-}A$$

Absorbance decreases as the radical is scavenged by antioxidants through donation of hydrogen to give rise to the reduced form [DPPH-H] with the result the color changes from purple to yellow. The more rapidly the absorbance decreases the more potent is the antioxidant compound in terms of its hydrogen donating ability. In the presence of antioxidants, decrease in the absorbance at 515 nm was measured until a steady state is observed.

Various concentrations of the extracts were allowed to react with DPPH. At each concentration, a graph was plotted with Time Vs % $DPPH_{REM}$(DPPH remaining) (Figure 3.25, 3.26, 3.27 and 3.28). This graphic presentation showed the behavior of each antioxidant compound. From the graph the % $[DPPH]_{REM}$ at steady state were noted. The percentage of remaining DPPH against the standard concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% that is expressed as EC_{50} values of the antioxidant [115]. The EC50 values for crude extract and purified extract were 154153 and 5632 respectively. From the figures it could be deduced that there was a sharp decrease in absorbance values for the first one minute, thereafter the rate of decrease was steady. It can persumed that in the initial stages, the scavenging power depended only on the concentration of [DPPH] and thereafter it depends both on concentration of antioxidant and [DPPH]. Hence the behavior of compounds were explained on the basis of kinetics.

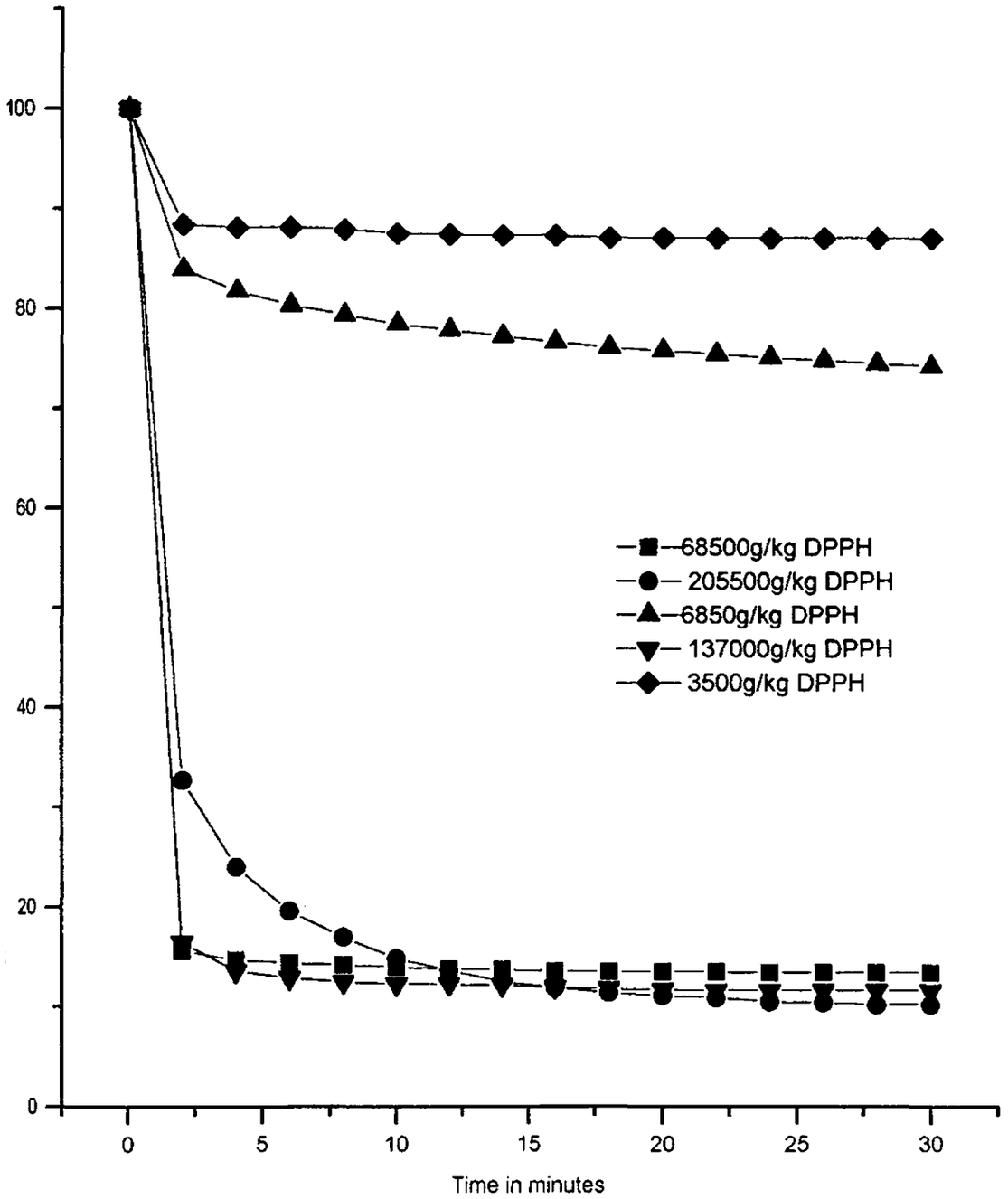


Figure 3.25 : DPPH scavenging effect of crude sesame cake extract

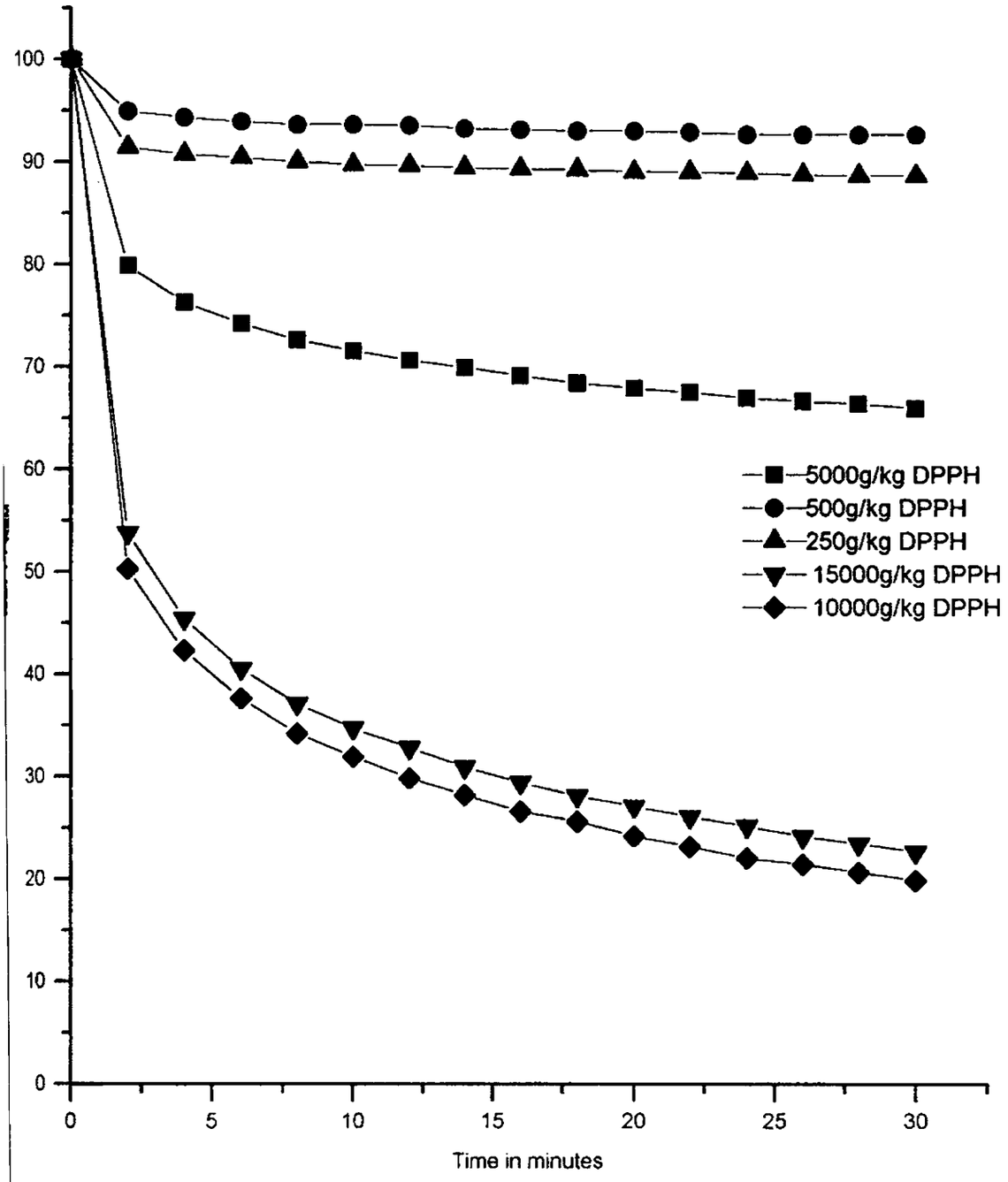


Figure 3.26: DPPH scavenging effect of purified sesame cake extract

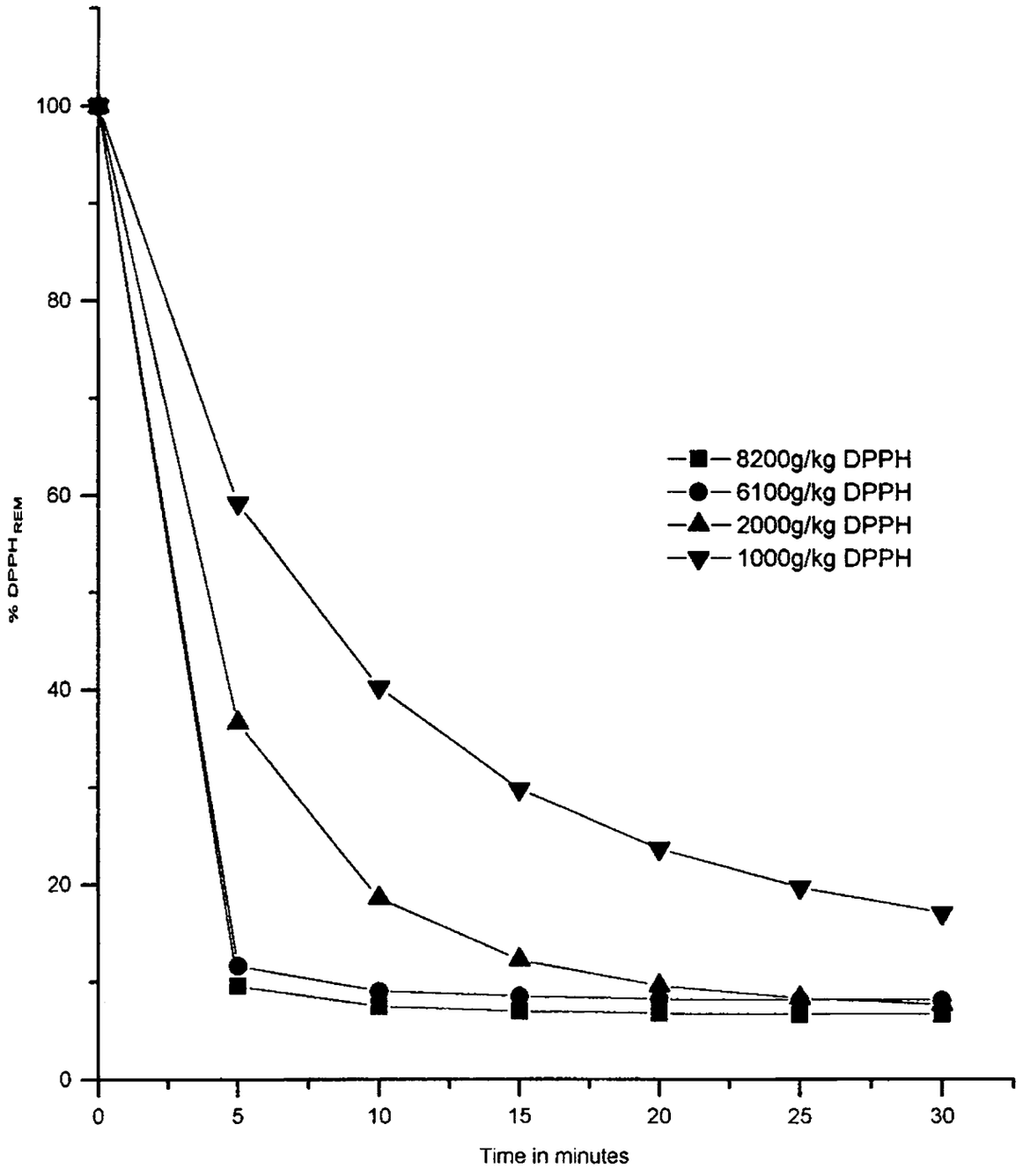


Figure 3.27: DPPH scavenging effect of BHT

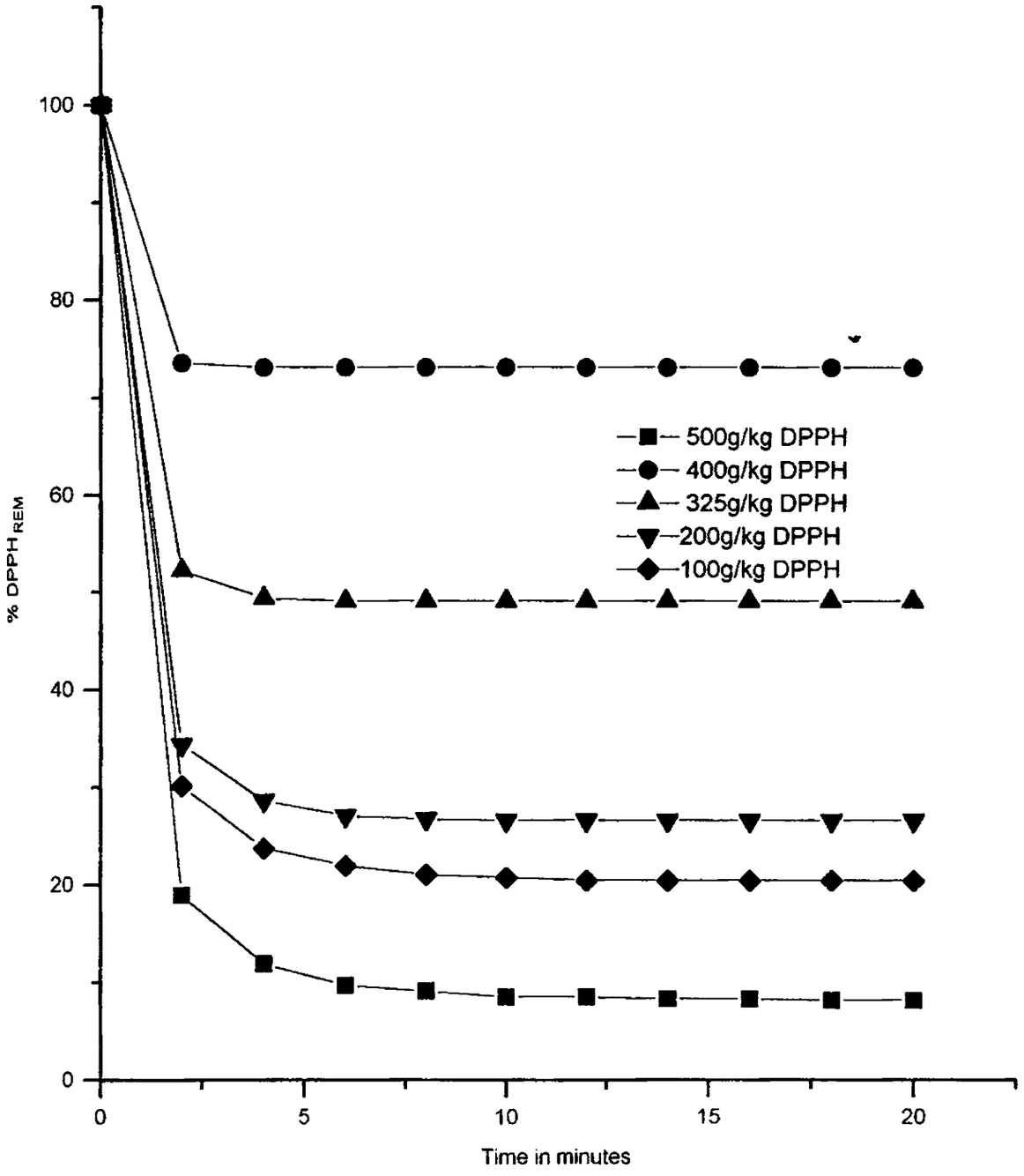


Figure 3.28: DPPH scavenging effect of α-tocopherol

The reaction followed pseudo-first-order kinetics according to the equation,

$$[DPPH^{\cdot}]_t = [DPPH^{\cdot}]_0 \, e^{-k_{obsd}^{t}} \longrightarrow (1)$$

where $[DPPH^{\cdot}]_t$ is the radical concentration at any time t, $[DPPH^{\cdot}]_0$ is the radical concentration at time zero and k_{obsd} the pseudo-first-order rate constants. This constant (k_{obsd}) was linearly dependent on the concentration of antioxidants, and from the slope of this plot, the second order rate (k_2) was determined [116, 143, 144].

The mean of three determinations of k_2 is shown in Table (3.9). The initial estimations of k_{obsd} was obtained from the slope on the origin of the straight line resulting from the fit by linear regression of the experimental data of $[DPPH^{\cdot}]_t$ and t to the equation,

$$\ln [DPPH^{\cdot}]_t = \ln [DPPH^{\cdot}]_0 - k_{obsd} \, t \longrightarrow (2)$$

Equation (2) becomes,

$$\frac{-d[DPPH^{\cdot}]_t}{dt} = k_{obsd}[DPPH^{\cdot}]_t = k_2[(AH)]\,[DPPH^{\cdot}]_t \longrightarrow (3)$$

The pseudo-first-order rate constant, k_{obsd} when plotted against antioxidant concentration, a linear graph was obtained. From these plots, the second order- rates constant $k_{2\ were}$ calculated for the scavenging reaction of different antioxidants. This rate constant is related to the Radical scavenging capacity (RSC) of the antioxidants and it is considered as a measure of the rate of disappearance of DPPH. The RSC of the antioxidants from sesame were compared with that of α-tocopherol and BHT.

Table 3.9: Second-Order Rate Constants (k_2) and EC_{50} values for the Reactions between DPPH$^{\cdot}$ and Sesame extract

No	Antioxidant compound	k_2,(g /kg) $^{-1}$ s^{-1}	EC_{50}
1.	BHT	$0.18x10^{-5}$	700
2.	α-tocopherol	$1.33x10^{-5}$	250
3.	Crude extract	$0.04x10^{-5}$	154153
4.	Purified extract	$0.24x10^{-5}$	5632

3.3.2.Superoxide radical scavenging ability of sesame extracts

Xanthine oxidase (XOD) is one of the main enzymatic sources of Reactive oxygen species (ROS) invivo. XOD is present in normal tissues as a dehydrogenase enzyme and it transfers electrons to NAD^+ [145]. When it oxidizes xanthine or hypoxanthine to uric acid, under certain stress conditions the dehydrogenase is converted into an oxidase enzyme by oxidation of essential thiol groups, or by limited proteolysis. Upon this conversion, the enzyme reacts with the same electron donors, reducing oxygen instead of NAD^+, thus producing superoxide anion and H_2O_2 [146]. Superoxide is a biologically important radical, which can be decomposed to form stronger oxidative

species such as singlet oxygen and hydroxyl radicals [147]. The highly reactive OH radicals can cause oxidative damage to DNA, lipids and proteins. Xanthine oxidase therefore is considered to be an important biological source of superoxide radicals' [148]. Under normal physiological conditions, the superoxide scavengeres in the system neutralize such free radicals and protect tissues. This invivo reaction is simulated invitro so as to use it an analytical tool to evaluate antioxidant properties.

Superoxide scavenging ability of sesame extracts using Xanthine-xanthine oxidase/NBT method : Superoxide radical produced in the xanthine-xanthine oxidase system reduces Nitroblue tetrazolium to form formazan complex. It is followed by an increase in absorbance at 560nm. In the presence of antioxidants, this reaction is interrupted hence the absorbance is reduced and the percentage reduction or inhibition is a measure of scavenging power. So far there has been no report on the superoxide radical scavenging activities of sesame antioxidants.

The superoxide radical scavenging effect of sesame cake extract (purified extract) was studied by the NBT assay using xanthine-xanthine oxidase. Various concentrations of each antioxidant compounds were tried. The results indicated the concentration dependent effect of extract towards superoxide radical (Table 3.10). Extract showed 38 and 44% efficacy at 100 and 200ppm concentrations. This was more than that of 35% efficacy exhibited by Trolox at 200ppm. Extract had appreciable superoxide radical scavenging effect at lower concentration of 5,10 and 25 ppm corresponding to 34.5, 34.5, 38.9 and 25.2% respectively whereas Trolox at 5, 10, 25, 40 and 500ppm concentration corresponded to 23, 22.6, 19.4, 29.2 and 25.9% respectively.

Superoxide radical scavenging ability of sesame cake extracts using Xanthine-xanthine oxidase/Cytochrome C method : The superoxide radical produced as byproduct during the conversion of xanthine to uric acid, reduces the ferricytochrome C with consequent increase in absorbance at 550nm. The ability of antioxidants to retard this reaction is measure of radical scavenging efficacy expressed in terms of percentage reduction in absorbance. The radical scavenging effects of sesame cake extract, sesamol and tocopherol were shown in figure 3.29 and 3.30. The extract at 100ppm and 450ppm concentration showed 61 and 65% inhibition. Standard sesamol showed 44% efficacy at 20ppm and 30% efficacy at 40ppm. The efficacy of α-tocopherol at 200ppm was 46%.

The percentage inhibition or radical scavenging ability of sesame cake extract obtained by NBT method was comparatively lower than that obtained by Cytochrome C method, but the order of efficacy was same. This may be due to the difference in response of the reagents in the model system. In NBT method α-tocopherol was used for comparison while in cytochrome C Trolox was used. In both cases sesame cake extract showed superoxide radical scavenging ability, significantly higher than the standard compounds.

Table 3.10: Superoxide radical scavenging activity of sesame cake extract by Xanthine-xanthine oxidase/NBT method

Sl.No	Sample	Concentration of antioxidant (ppm)	Efficacy (%)
1.	cake extract	100	37.8
		200	44.1
		50	25.2
		25	38.9
		10	34.5
		5	34.5
2.	Trolox	200	35.0
		500	25.9
		40	29.2
		25	19.4
		10	22.6
		5	23.0

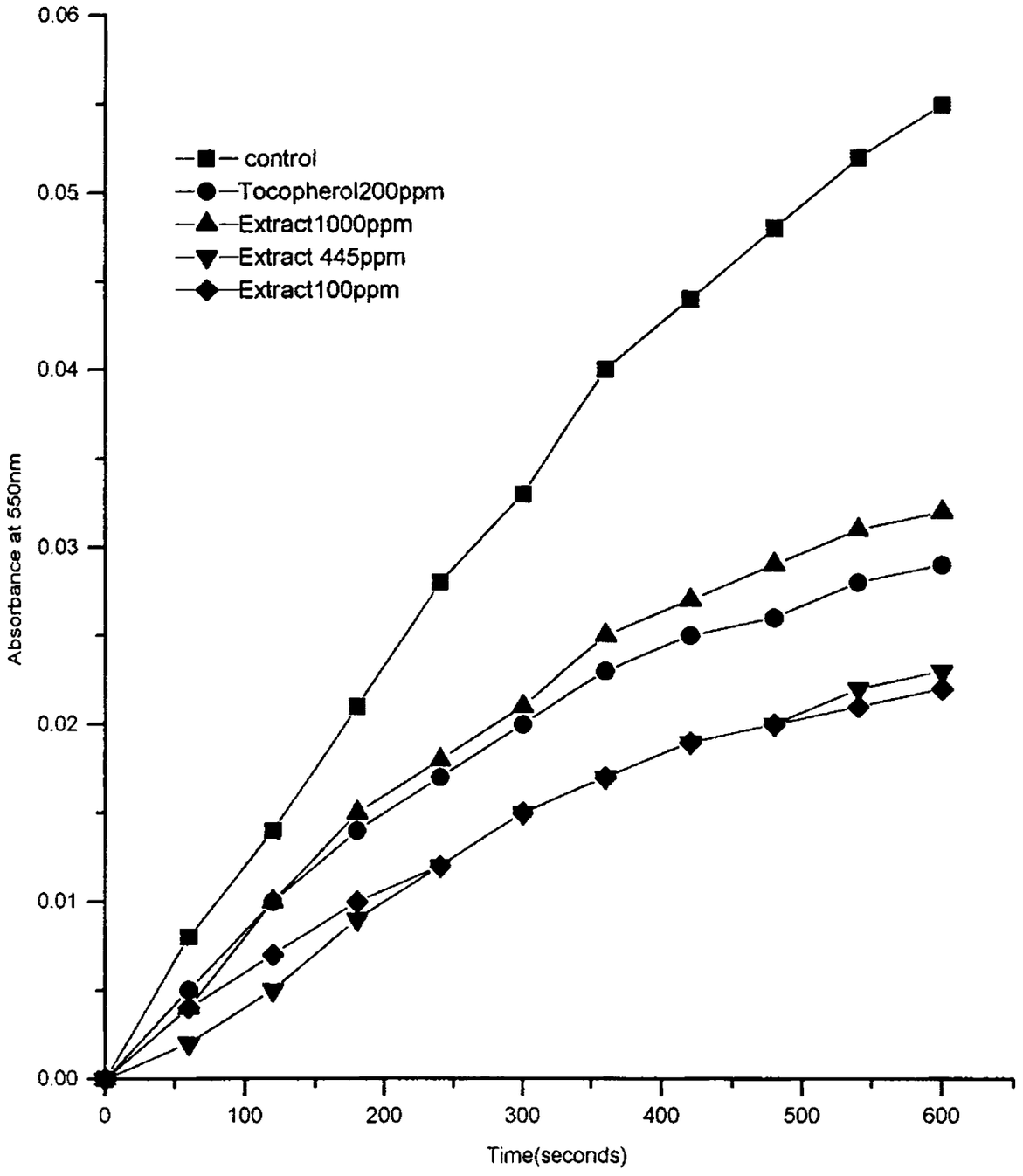


Figure 3.29:Superoxide radical scavenging effect of sesame cake extract by Xanthine-xanthine oxidase/CytochromeC method

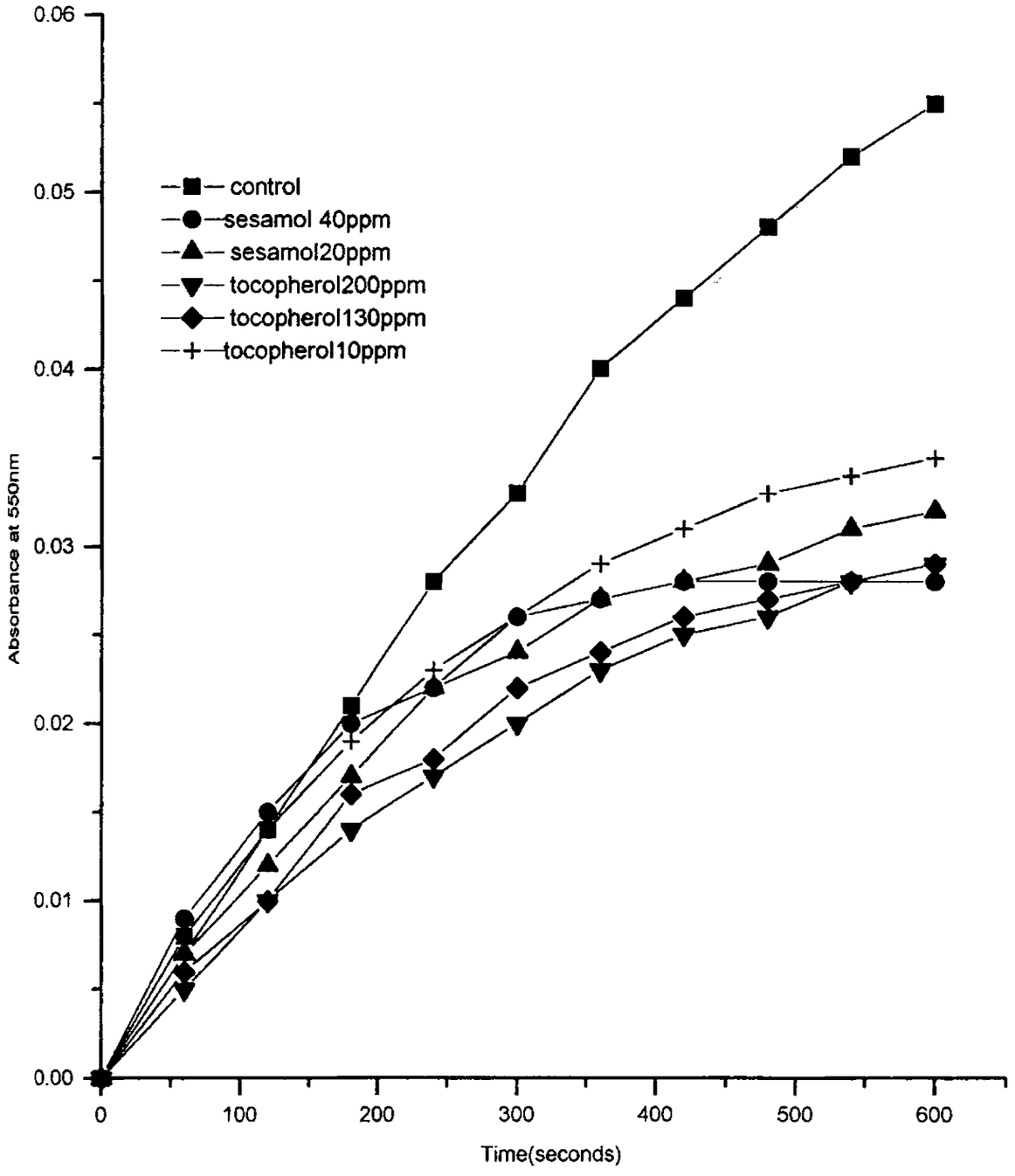


Figure 3.30:Superoxide radical scavenging effect of sesamol and α-tocopherol by xanthine-xanthine oxidase/CytochromeC method

The model system of scavenging DPPH free radicals is a simple method to evaluate the antioxidant activity of compounds and it measures one aspect of antioxidant activity, that is hydrogen donating ability of antioxidants [114,149]. There are many reports about the radical scavenging activity of extracts and compounds by DPPH assay [150, 151, 152]. In all cases, they measured the percentage scavenging effect of compounds in a particular time interval. Brand Williams [114] measured the EC_{50} values and calculated the Antiradical power($ARP=1/EC_{50}$) for the compounds. Sanchez Moreno [115] described a method to measure the antioxidant efficacy (AE) based on EC_{50} and TEC_{50}. Later, Espin etal, calculated second order rate constants to evaluate the RSC values of anthocyanins [116].

Radical scavenging activity of sesame antioxidants based on kinetic behavior has not been so far investigated. From the behavior of sesame extracts towards DPPH (Figure3.25 and 3.26) it was deduced that the scavenging activity can not be successfully explained on the basis of EC_{50} and TEC_{50}. Hence a kinetic approach was selected which explained the behaviour in terms of second order rate constant. The RSC of sesame cake extracts obtained based on kinetics were higher than that of other natural extracts especially berry extracts [116]. Comparing with individual compounds, purified extract possesses higher activity than that of BHT. Only tocopherol had more RSC values than that of purified extract. The higher scavenging ability of purified extracts may be due to the high lignan content.

Inhibition of superoxide anion is an effective therapeutic approach for many disease conditions [153, 154]. In view of the crucial role of superoxide anion ($O_2^{\cdot-}$) in the

development of inflammation and age-related disease, and the suggested role of superoxide generation inhibition as a contributing factor in the process [155], we evaluated the effects of the extracts on superoxide scavenging invitro. The sesame cake extract when assayed for ($O_2^{\cdot-}$) radical scavenging effect, by cytochrome C method and NBT method showed positive radical scavenging effects. This shows that the antioxidant compounds do not inhibit xanthine-oxidase leading to superoxide anion production, which is a natural biological process. The inhibitory effect of antioxidants could be attributed to scavenging of the superoxide anion rather than XOD inhibition.

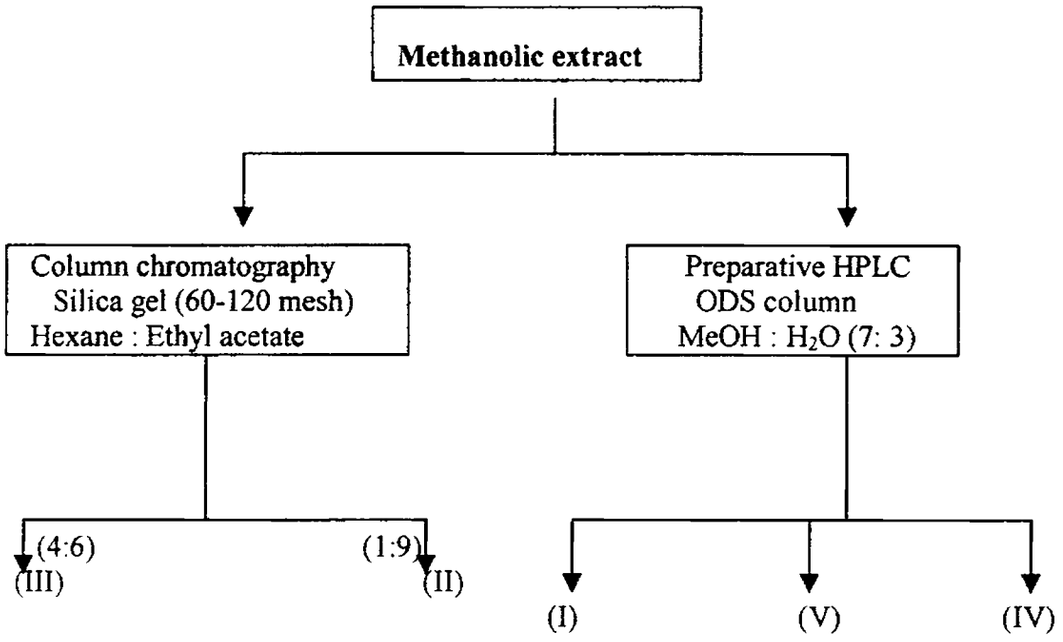
3.4.Antioxidant effects of pure compounds

The antioxidant efficacy of sesame cake extract was studied by various methods and results are presented in the previous sections (3.2 and 3.3). Studies with crude and partially purified extract could not yield results related to the active compounds and their relative efficacy. Therefore experiments were designed to isolate, characterize and to evaluate individual compounds in the sesame cake extract. This section deals with the results obtained from the above studies.

3.4.1. Isolation and Characterization

Methanolic extract obtained as described elsewhere was subjected to silica gel column chromatography with gradient elution using hexane: ethyl acetate. At solvent ratio 4:6and 1:9 (hexane:ethyl acetate) active compounds were eluted. These were termed as fractions II and III. Fractions II and III found to contain pure compounds on TLC and HPLC analysis. The compounds were purified and recrystallized from ethanol to get sesamin and sesamolin. Since the isolation of other compounds was not successful with column chromatography, Preparative HPLC of the methanolic extract was carried out. The fraction corresponding to each peak was collected. They were lyophilized and further purified. The main fractions were termed as I, IV and V and found to contain pure compounds by TLC. Fraction I, IV and V were analyzed and found to contain sesamol, sesaminol diglucoside and sesaminol triglucoside. The scheme for isolation is presented in Figure.3.31.The identity of the compounds was confirmed by HPLC, IR, NMR and MS techniques. The presence of sesamol was not reported so far in any of the seed extract and cake extract. The presence of sesamol was ensured in this study by the IR,

NMR and MS spectra obtained for the corresponding fraction from preparative HPLC (Figures 3.32, 3.33, 3.34 and 3.35). The structure of the compounds isolated from sesame cake extract was shown in Figure 3.36.



I-sesamol; II- sesamin; III-sesamolin; IV-sesaminol diglucoside; V-sesaminol triglucoside

Figure 3.31: Scheme for isolation of components from sesame cake extract

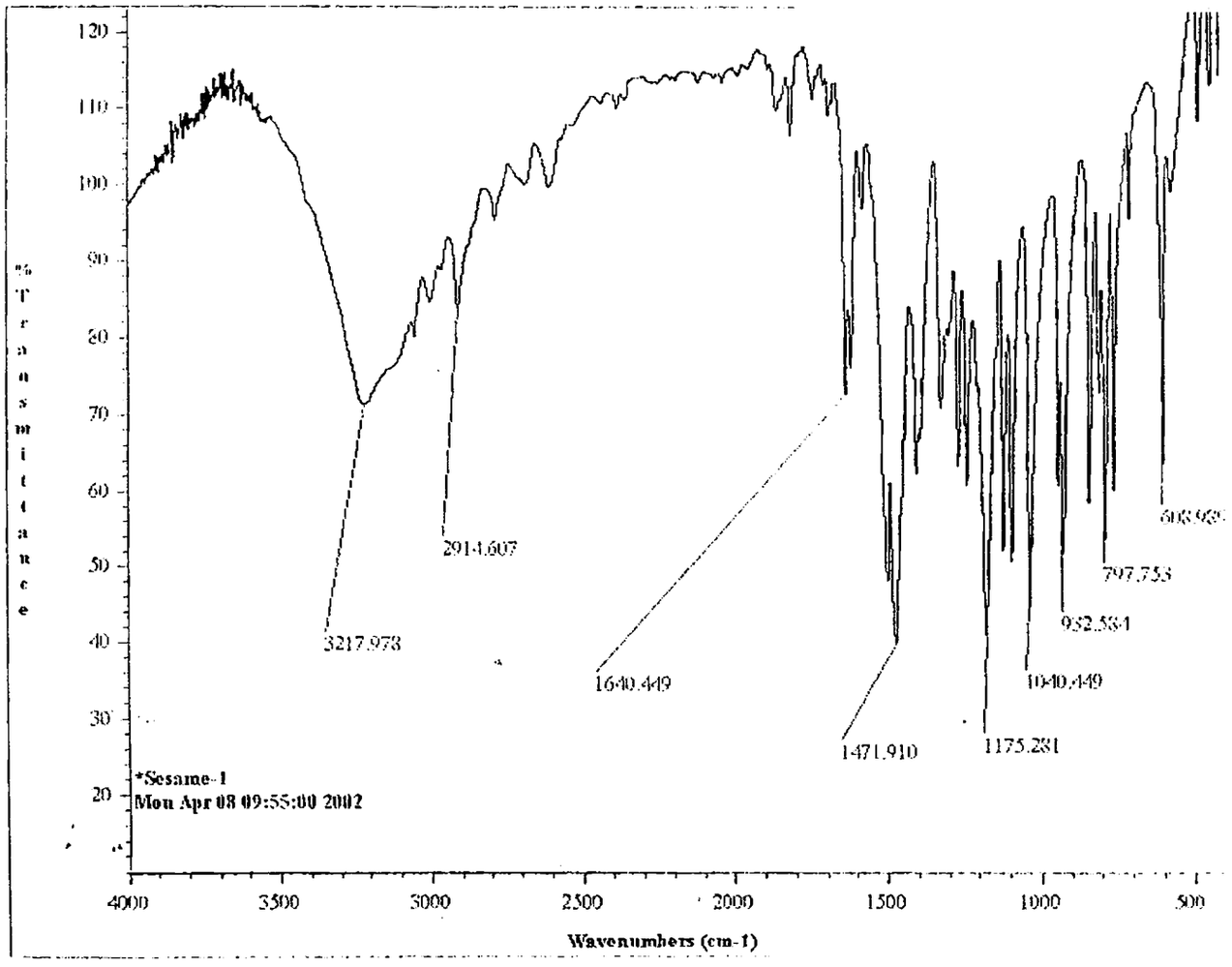


Figure 3.32: IR Spectrum of sesamol

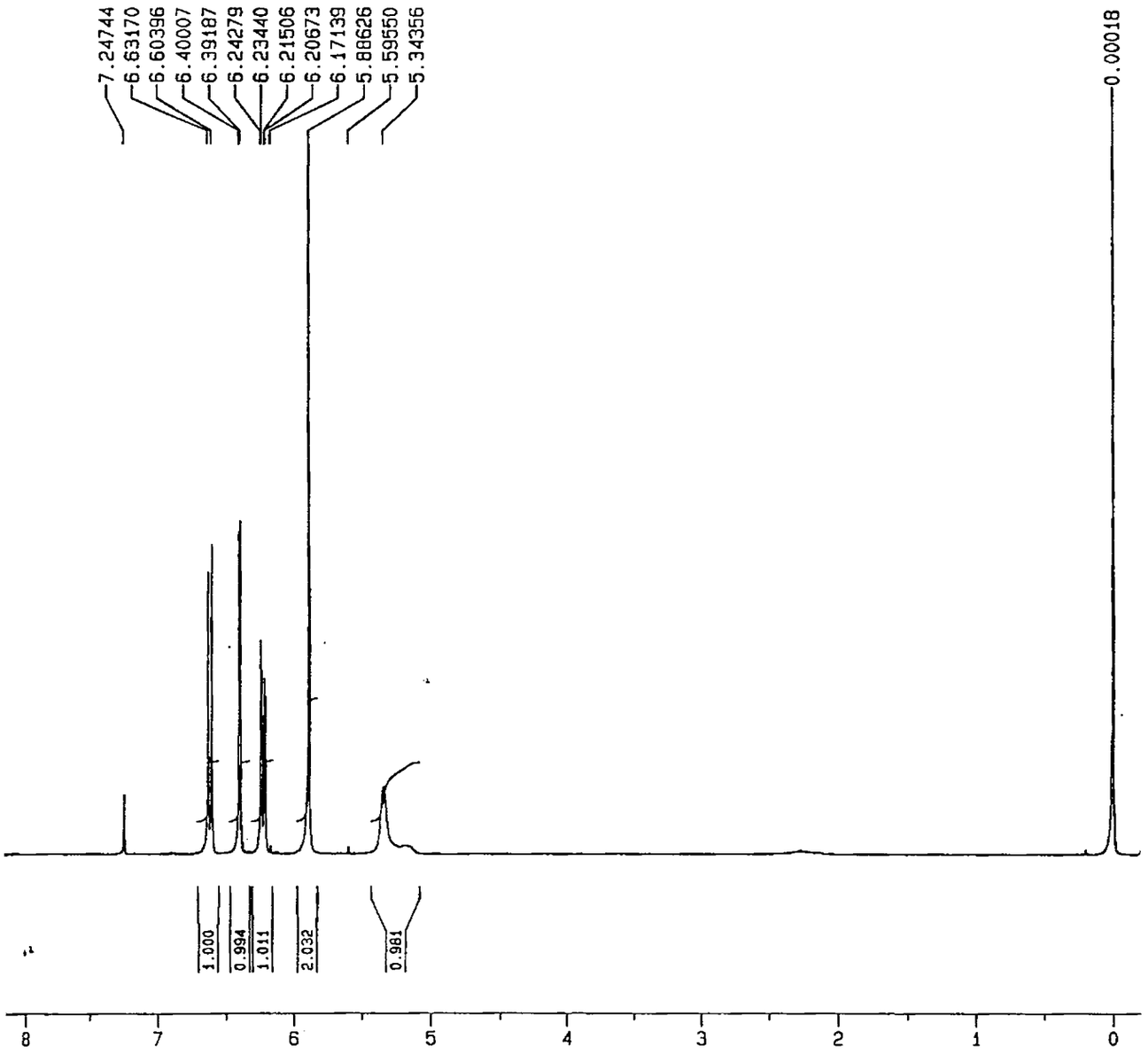


Figure 3.33: ^{1}H NMR spectrum of sesamol

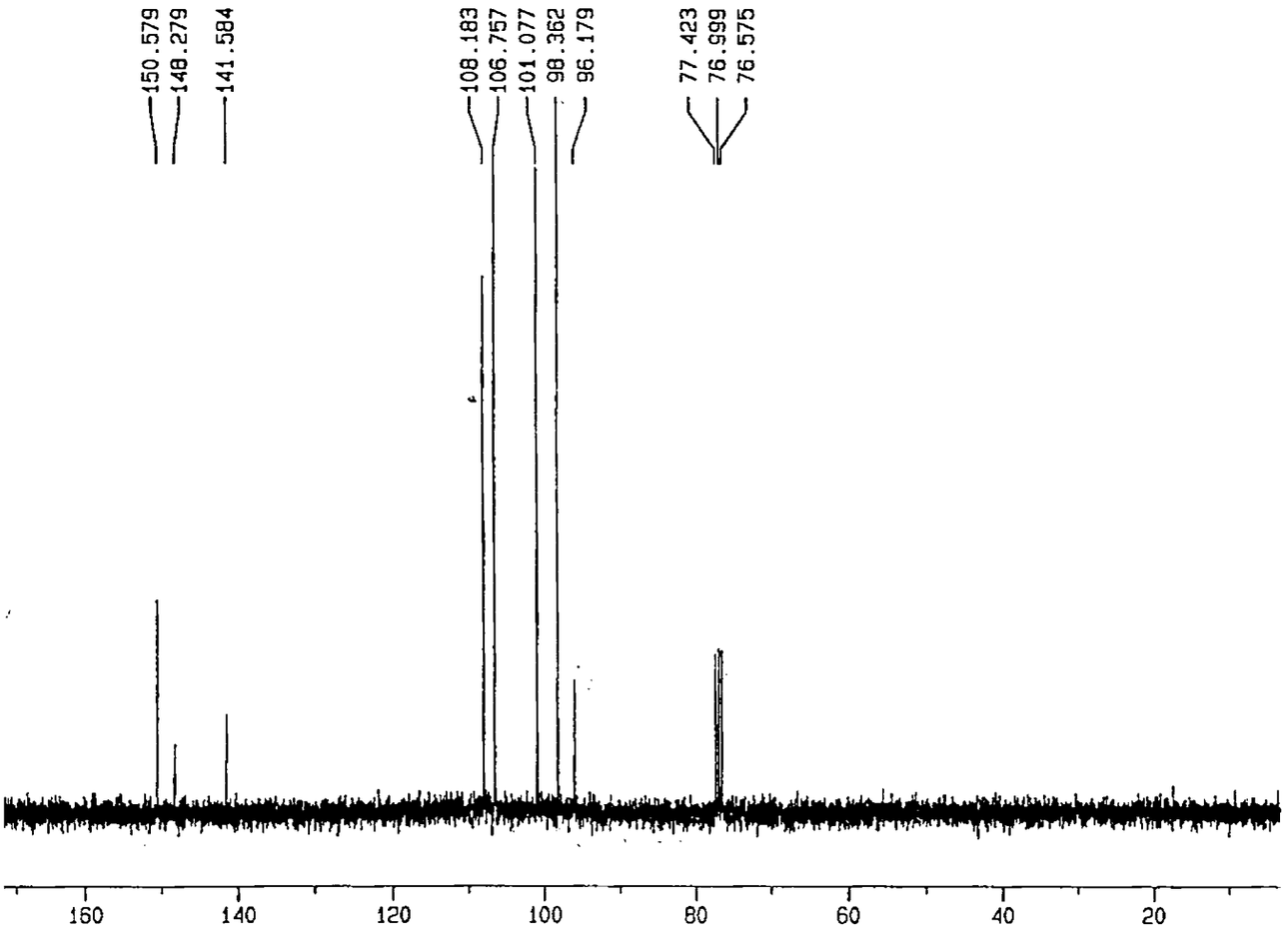


Figure 3.34: ^{13}C NMR spectrum of sesamol

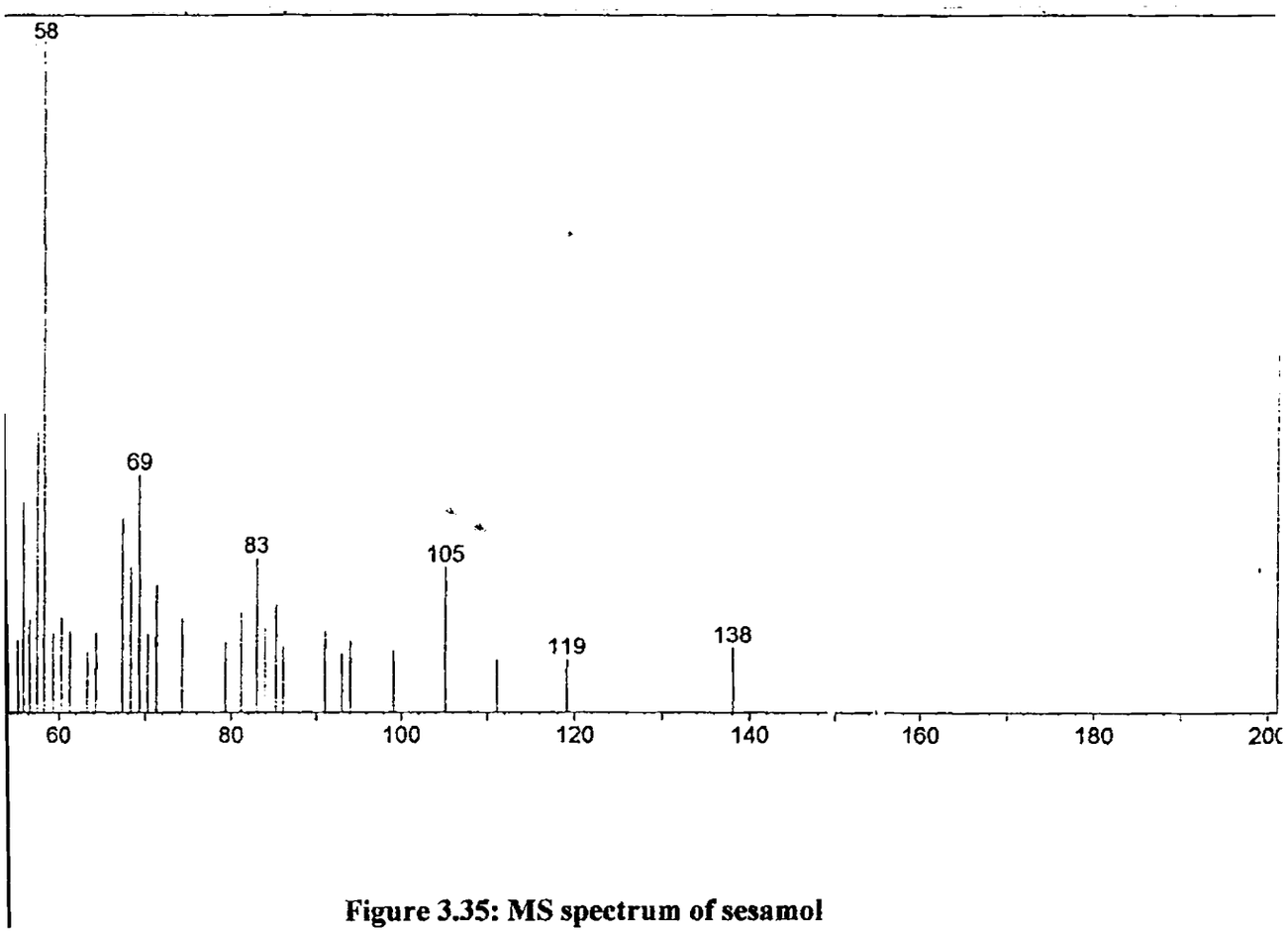


Figure 3.35: MS spectrum of sesamol

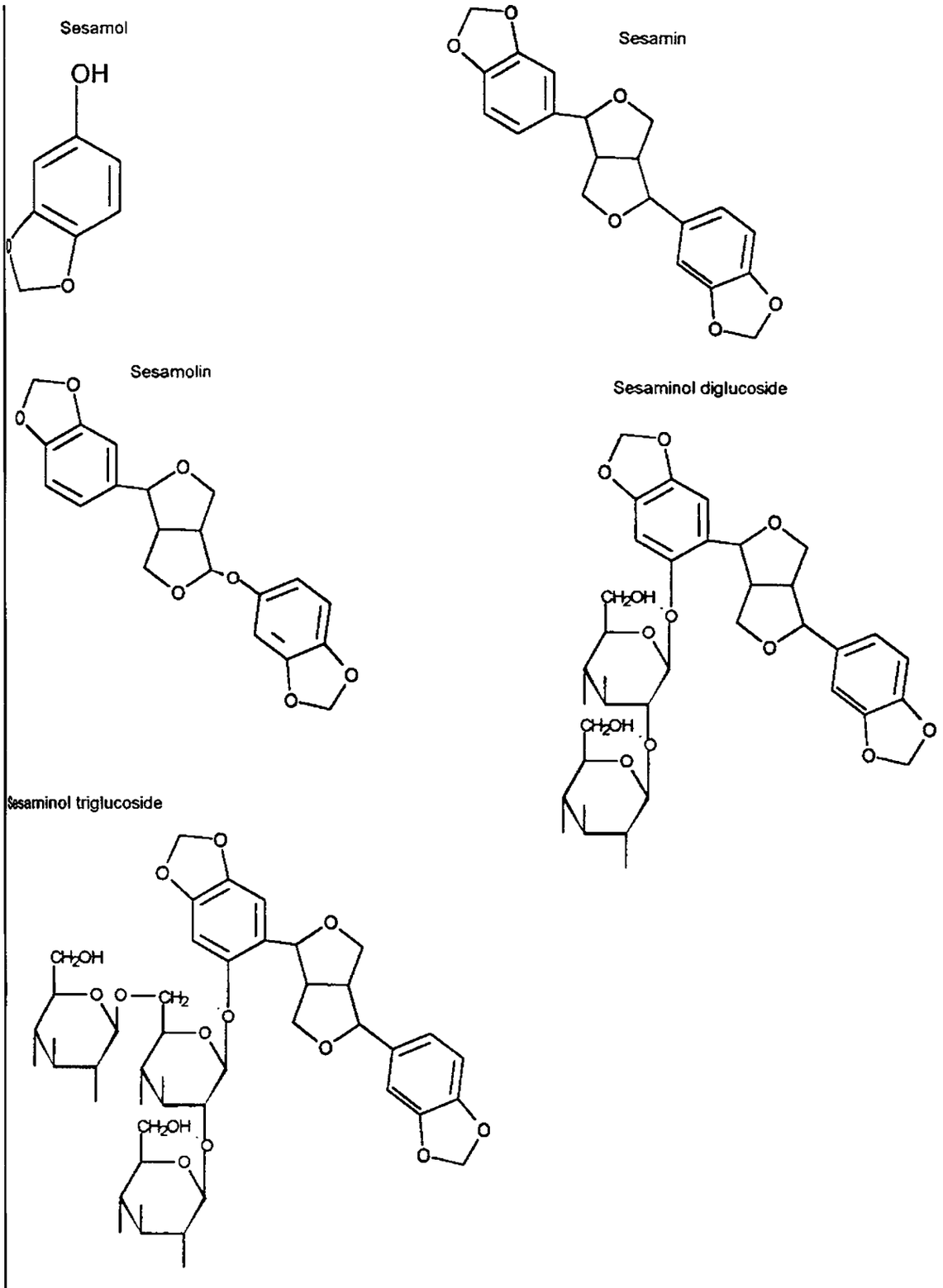


Figure 3.36: Structure of compounds isolated from sesame cake extract

Structural identification of I, II, III, IV, and V.

1.Sesamol:

IR $\nu_{max}^{CHCl_3}$: 3218,2914,1640,1471,1175,1040,933,798,609

MS Pattern : 137 (55), 136 (30), 52(100)

^{1}H NMR($CDCl_3$, TMS):δ ppm $\nu_{max}^{CHCl_3}$

6.24(1H,m,H2); 6.63(1H,d,H3); 6.40(1H,d,H6); 5.88(-O-CH_2-O-)

^{13}C NMR($CDCl_3$)

150.57(C-OH), 148.27(C-3), 141.58(C-4), 108.18, 106.75, 101.07(C-2, C-5, C-6), 98.36(CH_2)

2.Sesamin (Results compared with reported value)

IR $\nu_{max}^{CHCl_3}$: 2921, 2860, 1734, 1485, 1451, 1256,1047,932

MS Pattern: 356(10), 163(50), 149(100), 177(50), 136(20)

^{1}H NMR: ($CDCl_3$,TMS) δppm

3.03(2H,m,H1,H5); 4.70(2H,d,J=4.0)

3.87(2H,d,d,J=3.39, H-4a/8a); 4.24(2H,d,d,J=6.83 and 7.55, H-4e/8e)

-O-CH_2-O- 5.94(4H,s); 6.82(6H,m)

Sesamolin

IR $\nu_{max}^{CHCl_3}$: 2928,2853, 1714, 1492, 1451, 1256, 1195, 1053,979,932

MS pattern: 370(5), 303(10), 279(10), 253 (20), 226(40), 206(72), 191(80), 175(35), 169(100), 126(50), 112(70)

^{1}H NMR: ($CDCl_3$,TMS) δppm

3.49, 2.77(H1,H5);3.66(H-8a); 4.30(H-8e);4.15(H-4a);4.26(H-4e), 4.28(H-6);5.96 (-O-CH_2-O-)

Sesaminol diglucoside (Results compared with reported value)

IR $\nu_{max}^{CHCl_3}$:3390, 2928,2860,1593,1269,1026,764,683

MS pattern: 663(10), 649 (20),536(20),399(60),317(40), 231(25), 138(100)

^{1}H NMR(CD_3OD)

4.87, 4.60,(Anomeric protons, G1, G1^1,); 5.93,5.66(-O-CH2-O-); 6.79, 6.91,6.93, 7.10(Ar-H, 3^1, 6^1, 2^{11}, 5^{11}); 2.81, 5.06, 4.10, 2.89(furan protons)

Sesaminol triglucoside (Results compared with reported value)

IR $\nu_{max}^{CHCl_3}$:3420, 2921, 2860,1734, 1593, 1444, 1276, 1027,811

MS Pattern: 854(65), 835 (100),750(45),670(80)

^{1}H NMR (CD_3OD)

4.87, 4.87, 4.38(Anomeric protons, G1, G1^1, G1^{11}); 5.99, 5.93(-O-CH_2-O-);6.81, 6.87, 6.75, 6.74(Ar-H, 3^1,6^1, 2^{11}, 5^{11},6^{11}); 2.91,2.88, 4.51,5.16, 3.76, 4.12, (furan protons, H1, H2, H4, H5, H6, H8)

3.4.2.Antioxidant studies using isolated compounds from sesame cake

The isolated compounds from sesame cake extract include sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside. Each compound was analyzed by β-carotene bleaching method, thiocyanate method and radical scavenging assays for their individual antioxidant activity, as described before.

3.4.2.1.β-carotene bleaching method

The activity of individual compounds isolated from sesame cake extract was studied by the β-carotene bleaching method. All compounds were tested at concentrations, 100 and 200 ppm, and BHT was used for comparison. The individual compounds isolated and evaluated by this method were sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside. The antioxidant activity in the decreasing order was, sesamol> sesamolin > sesamin > BHT > triglucoside > Diglucoside at 200 ppm levels and the corresponding activity indices were 76, 65, 59, 48, 30 and 22% respectively (Figure 3.37). The activity indices at 100 and 200 ppm for theses compounds are given in Table 3.11. BHT showed better activity than that of glucosides. The activity index at 100ppm for sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside were 61, 50, 47, 26 and 17% respectively. All compounds showed activity by this method. Sesamol showed 76% activity at 200ppm while sesame extract had 61% inhibition at 200ppm. However the activity of the extract was 81% at 50ppm. Glucosides showed less activity at 100 and 200ppm by this method. Sesamol, sesamin and sesamolin had more activity than BHT. The activity of sesamin was slightly higher than that of sesamolin at 100 ppm and lower at 200 ppm.

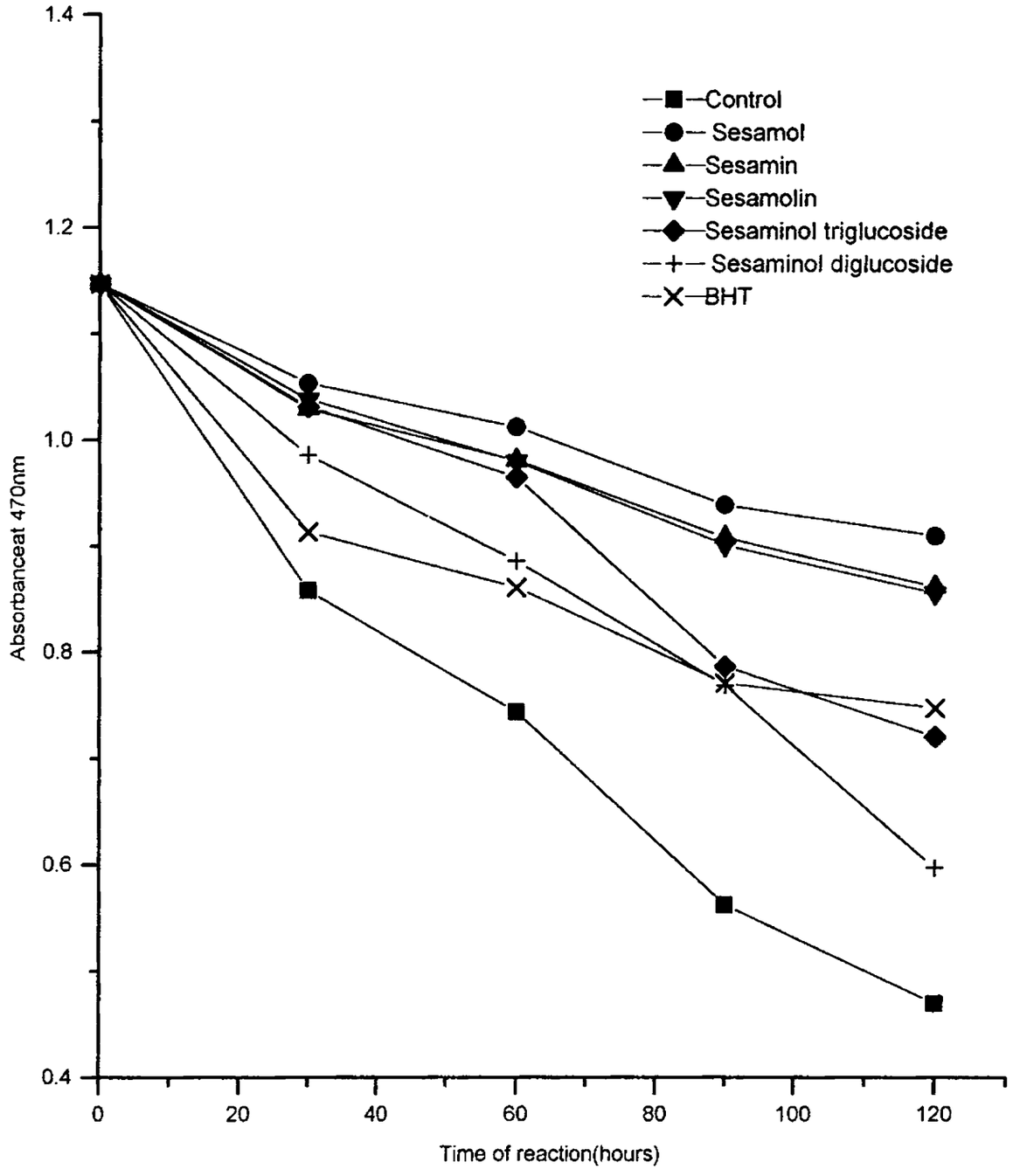


Figure 3.37: Antioxidant activity of sesame antioxidants and BHT (200ppm) by β-carotene bleaching method

Table 3.11: Concentration dependent antioxidant activity (%) for different compounds isolated from sesame cake extract by β-carotene bleaching method

Sample	100ppm	200ppm
Sesamol	61	76
Sesamin	50	59
Sesamolin	47	65
Sesaminol triglucoside	26	30
Sesaminol diglucoside	17	22
BHT	-	48

3.4.2.2.Thiocyanate method

Individual compounds isolated from sesame cake extract were also studied by linoleic acid emulsion system (Figure 3.38) as described before.. All compounds were evaluated at 200ppm level. The percentage inhibition for sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside and BHT were 77, 60, 69, 25, 32 and 49 respectively. The activity indices of these compounds in the decreasing order was: sesamol > sesamolin > sesamin > BHT > sesaminol triglucoside > sesaminol diglucoside. Sesamol (77%), sesamin (60%) and sesamolin (69%) showed more activity than BHT (49%). The activities of glucosides were less than that of BHT. Sesamolin showed slightly higher activity than that of sesamin.

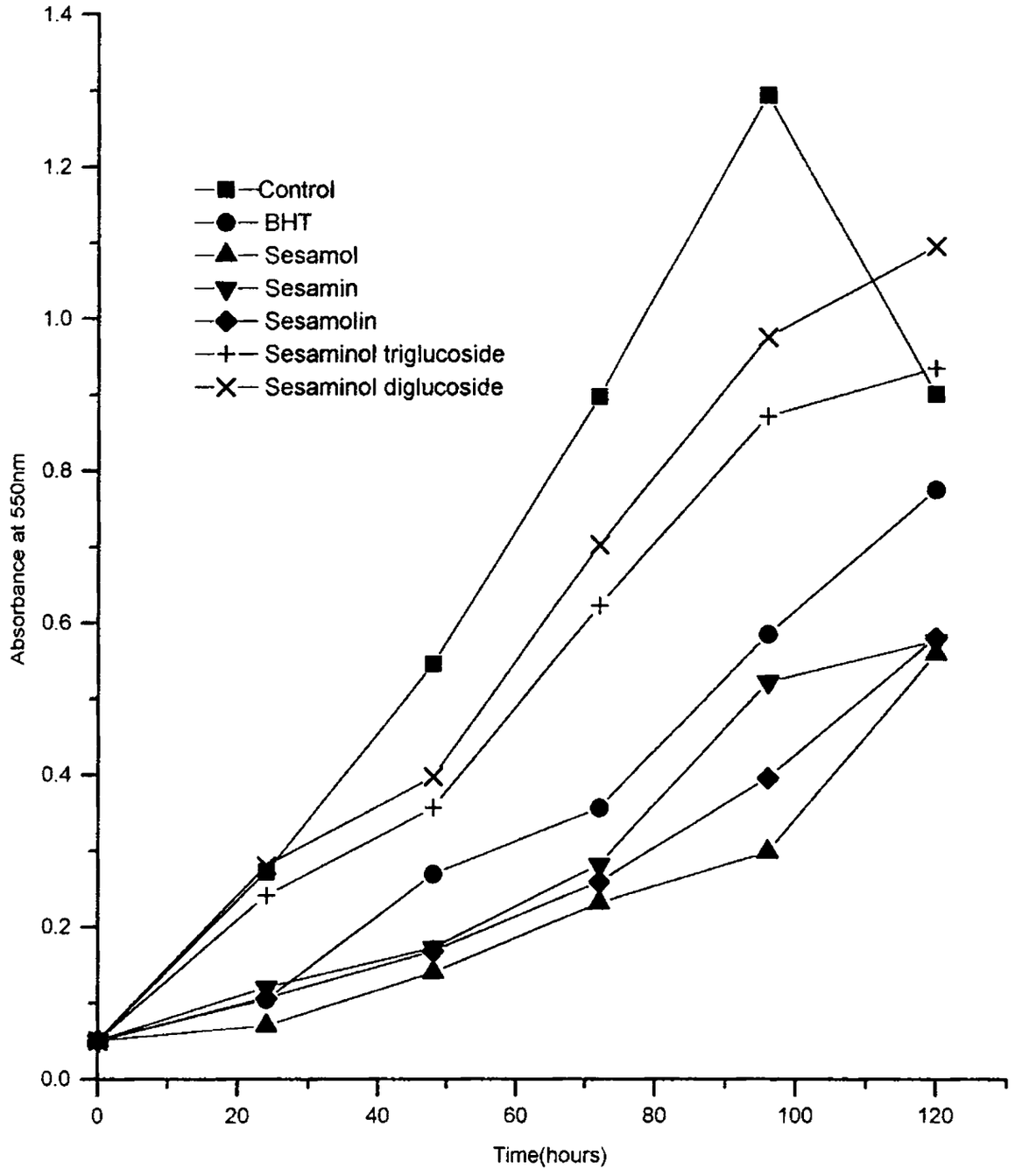


Figure 3.38: Antioxidant activity of sesame antioxidants and BHT (200ppm)by the Thiocyanate method-Linoleic acid emulsion system

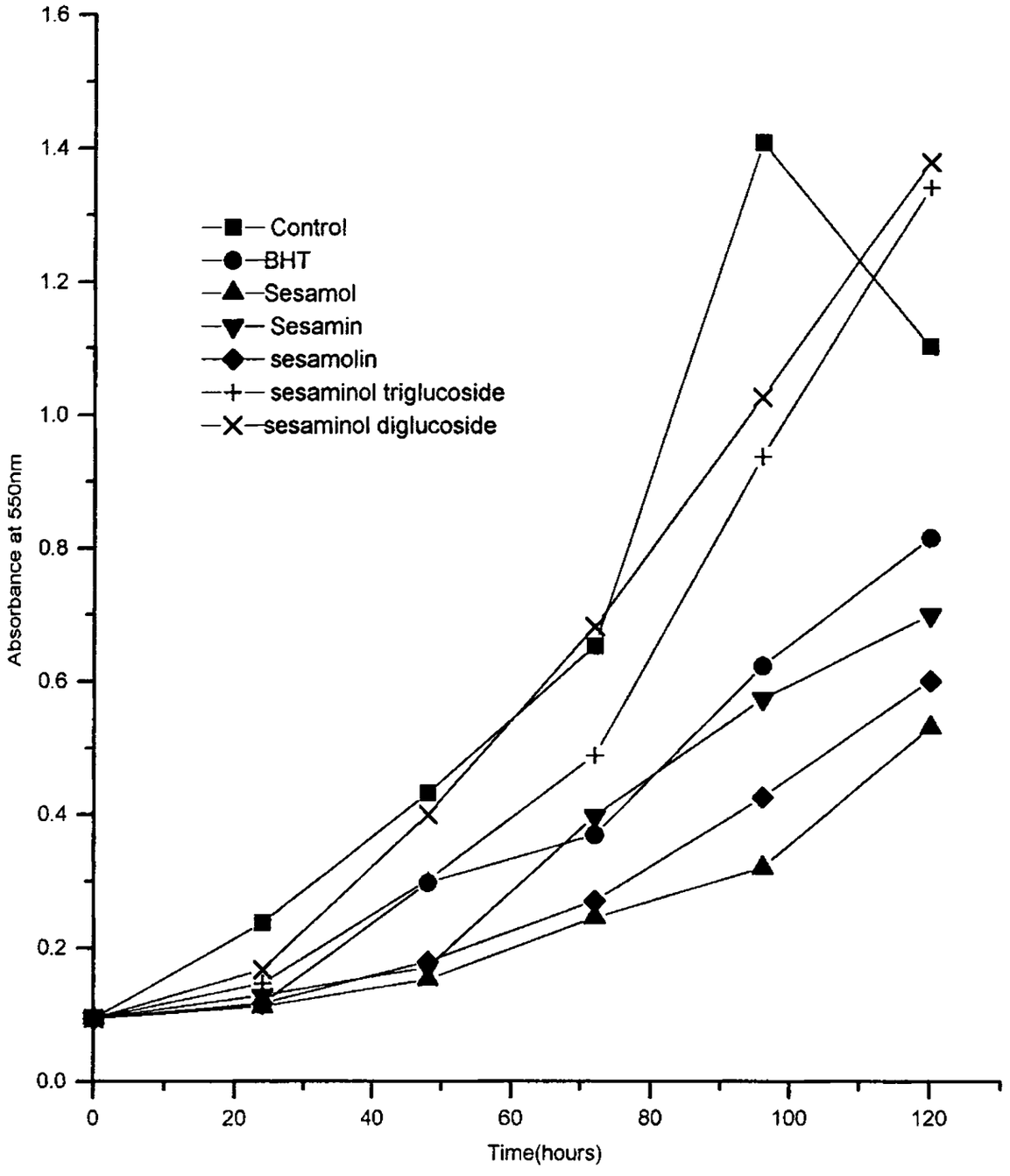


Figure 3.39:Antioxidant activity of sesame antioxidants and BHT (200ppm) by Thiocyanate method-Linoleic acid system

Individual compounds isolated from sesame cake extract were studied by linoleic acid system (figure3.39). Sesamol, sesamin, sesamolin, sesaminol diglucoside, sesaminol triglucoside and BHT showed 78, 59, 70, 27, 33 and 56% inhibition on oxidation in the above system. The activity index followed the order: sesamol > sesamolin > sesamin > BHT > sesaminol triglucoside > sesaminol diglucoside. Activities of lignan glucosides were less than that of BHT and the activities of sesamol, sesamin and sesamolin were more than that of BHT. Sesamolin showed higher activity than sesamin.

3.4.2.3.Free radical scavenging effect by DPPH

Pure compounds were isolated from defatted sesame cake extract by preparative HPLC and their identification was confirmed by IR, NMR and MS techniques as described before. The compounds, at various concentrations, were allowed to react with $DPPH^{\cdot}$ .Sesamol dimer, which was detected in a few sesame extracts, was also isolated and studied by the above method.

In this study, the antiradical effectiveness of lignans and lignan glycosides isolated from sesame cake extract were calculated according to second order rate values. Sesamol was having the highest rate constant followed by α-tocopherol, BHT, sesamol dimer, sesamin, sesaminol triglucoside, sesamolin and sesaminol diglucoside in the decreasing order. The k_2 values for sesamol, sesamol dimer, sesamin, sesamolin, sesaminol triglucoside and sesaminol diglucoside were $2.66x10^{-5}$, $0.166x10^{-5}$, $0.11x10^{-5}$, $0.04x10^{-5}$, $0.10x10^{-5}$, $0.01x10^{-5}$ $(g/kg)^{-1}s^{-1}$respectively (Table 3.12). The Radical scavenging capacity (RSC) of sesamol dimer, sesamin, and sesaminol triglucoside were almost comparable to BHT. This is the first report on the kinetic behavior of lignans and

lignan glucosides isolated from sesame (Figure 3.40, 3.41, 3.42 and 3.43). The EC_{50} values are also given Table 3.11. According to the assay results, all these compounds possess radical scavenging activity towards [DPPH·], but to different degrees.

Table 3.12: Second-Order Rate Constants (k_2) for the Reactions between DPPH· and Sesame Antioxidant compounds

No	Antioxidant compound	k_2,(g /kg) $^{-1}$ s^{-1}	EC_{50}
1.	sesamol	$2.66x10^{-5}$	75
2.	sesamol dimer	$0.16x10^{-5}$	1450
3.	sesamin	$0.11x10^{-5}$	4400
4.	sesamolin	$0.04x10^{-5}$	5000
5.	sesaminol triglucoside	$0.10x10^{-5}$	2400
6.	sesaminol diglucoside	$0.01x10^{-5}$	7000
7.	BHT	$0.18x10^{-5}$	700
8.	α-tocopherol	$1.33x10^{-5}$	250

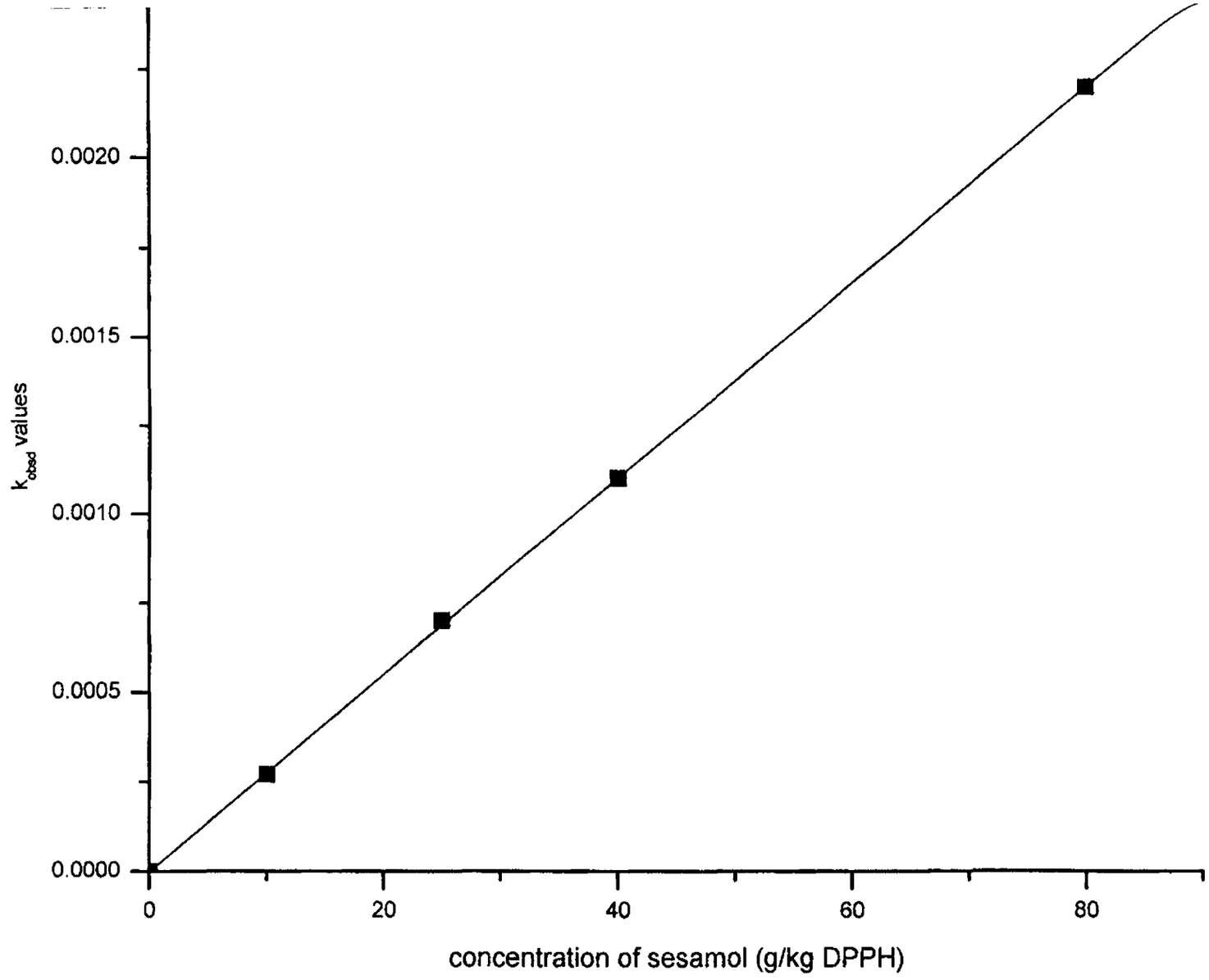


Figure 3.40: Dependence of pseudo-first-order rate constants on the concentration of sesamol

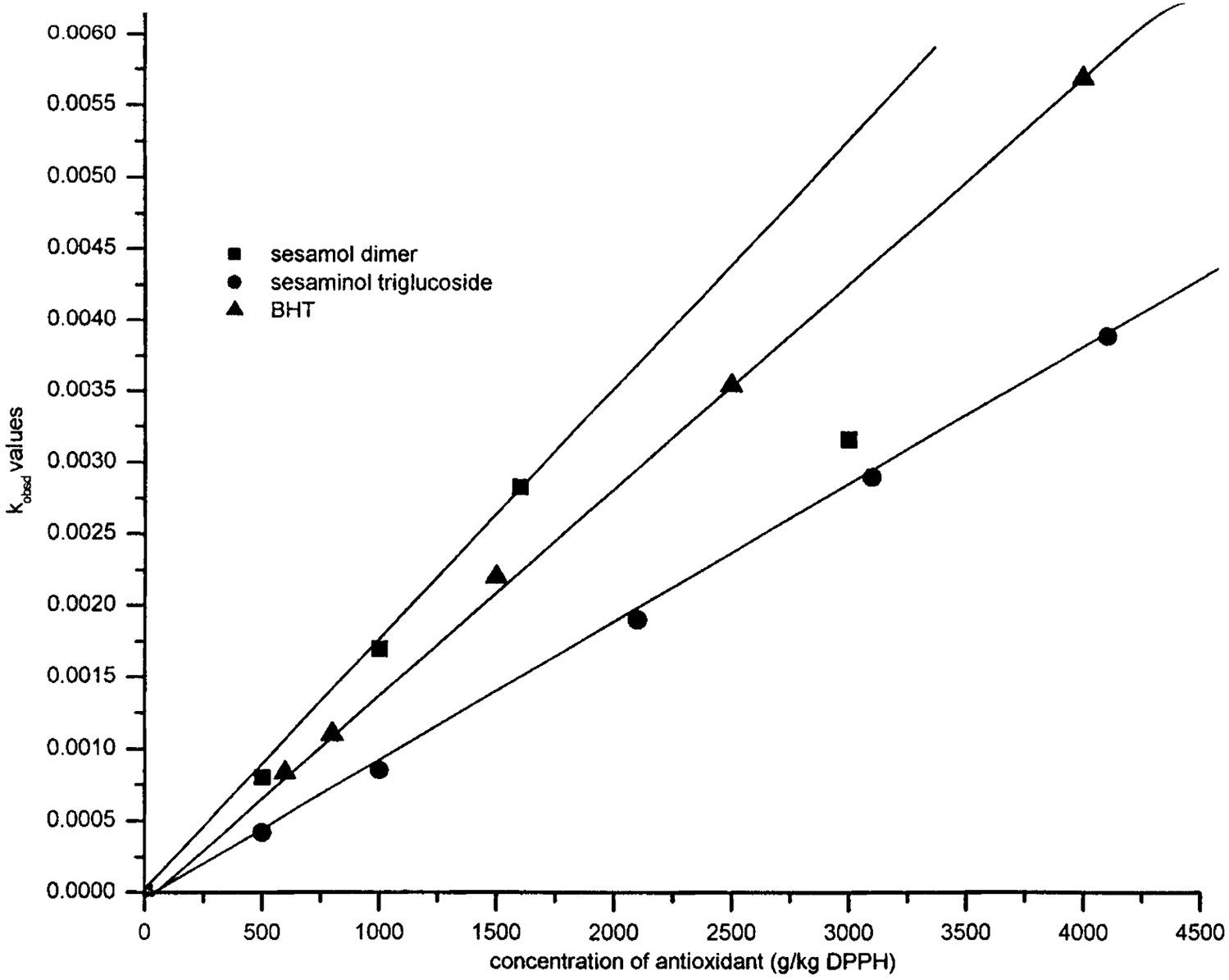


Figure 3.41: Dependence of pseudo-first-order rate constants on the concentrations of sesame antioxidants

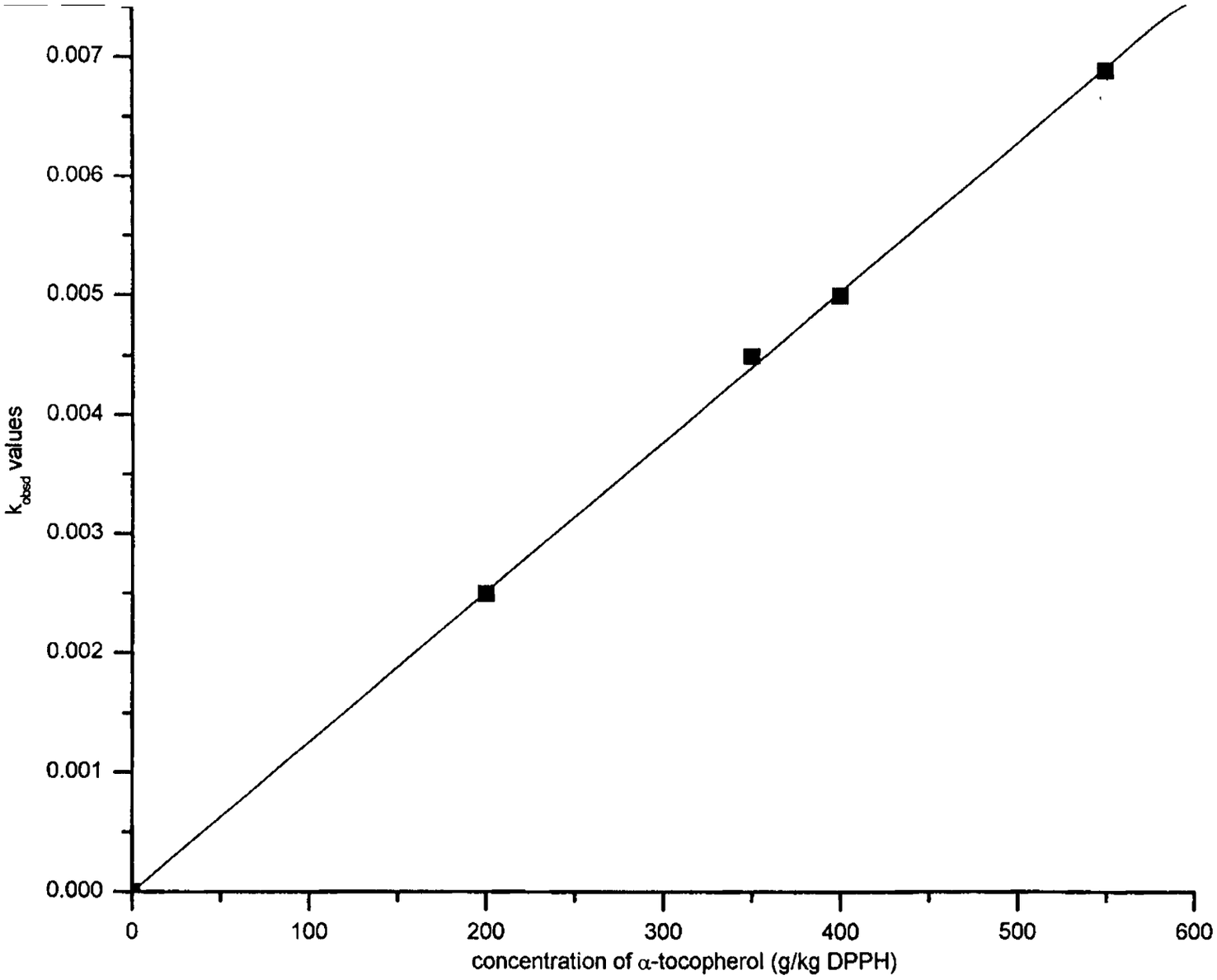


Figure 3.42: Dependence of pseudo-first-order rate constants on the concentration of α-tocopherol

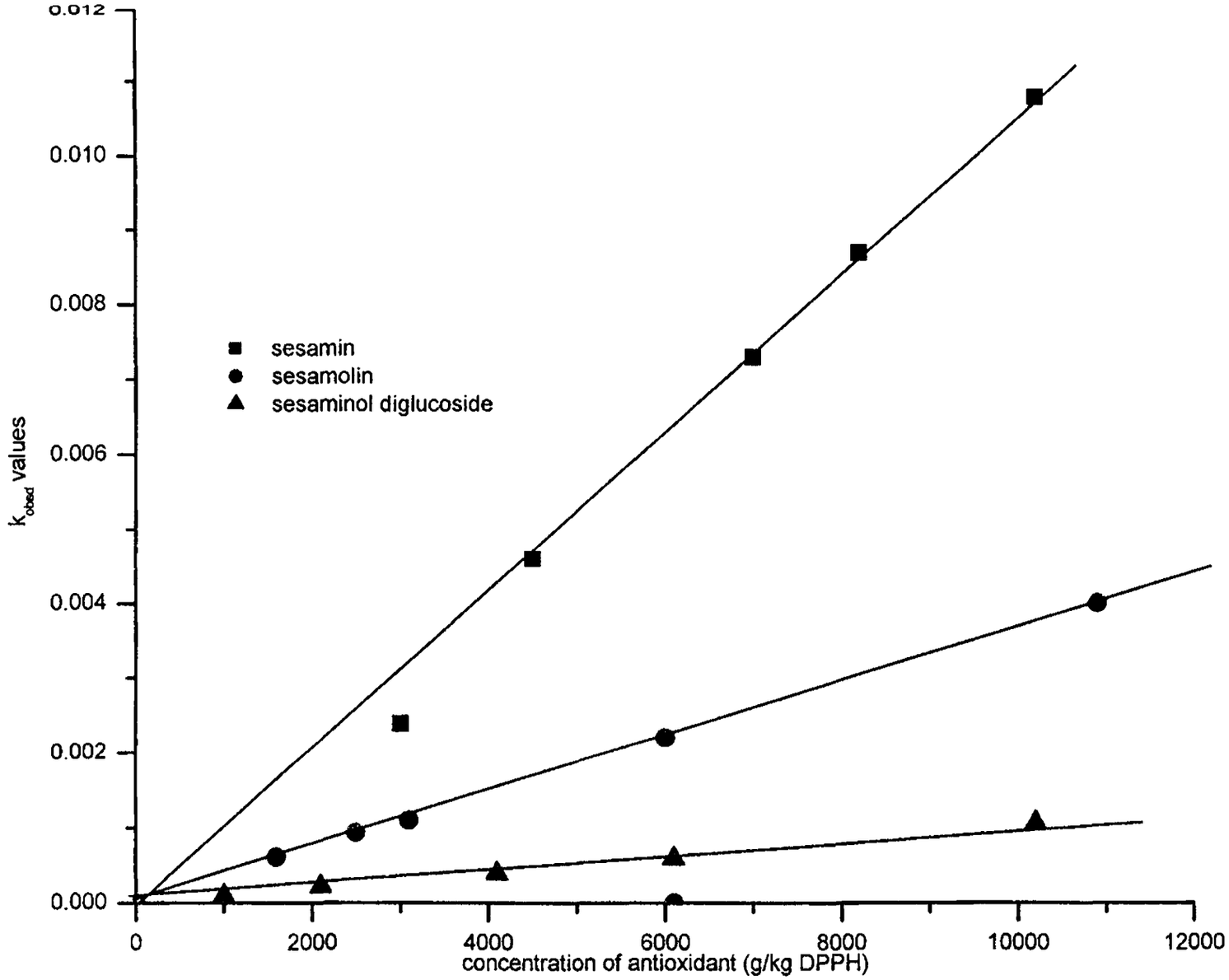


Figure 3.43: Dependence of pseudo-first-order rate constants on the concentrations of sesame antioxidants

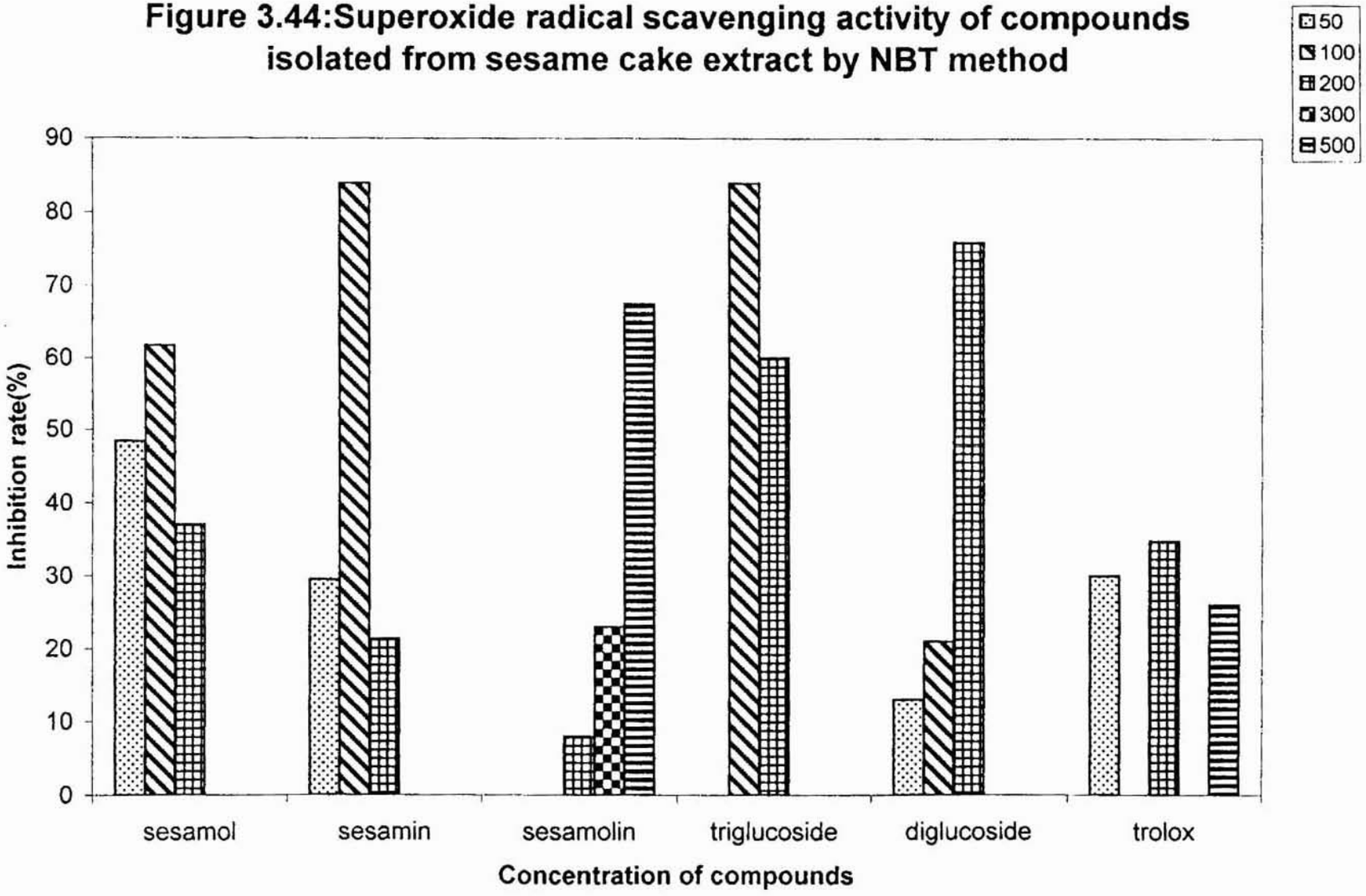
3.4.2.4.Superoxide radical scavenging effects

The superoxide radical scavenging effects of individual compounds isolated from sesame cake extract were studied by the NBT assay using xanthine-xanthine oxidase/NBT assay. Various concentrations of each antioxidant compounds were tried. It showed the concentration dependent effect of lignans towards superoxide radical (Table 3.13). The superoxide radical scavenging effects of lignans and lignan glucosides were compared with Trolox. Sesamol showed more efficacies at 100ppm. The efficacy of sesamol at 50, 100 and 200ppm were 48.4, 61.7 and 34% respectively. The efficacy of sesamin at 100ppm was more than that of sesamol at 100ppm, however at 50 and 200ppm it showed fewer efficacies. Sesamolin showed negative effect at 50 and 100 ppm levels. At higher concentration of 300 and 500ppm, the efficacy was 23 and 67%. Sesaminol diglucoside showed less effect at 50ppm and the effect was more at 200ppm. Sesaminol triglucoside had negative effect at 50ppm. At 100ppm it showed efficacy in the order of 84%. All compounds showed higher activity than Trolox at some concentration. All compounds except sesamolin had appreciable effects at 50, 100 and 200 ppm. The results were expressed as a bar diagram (Figure 3.44). The antioxidants do not inhibit xanthine-xanthine oxidase leading to superoxide anion production. They scavenge the deleterious superoxide radicals [148]. This antioxidant effect is more useful in biological systems.

Table 3.13: Superoxide radical scavenging activity of sesame antioxidants by Xanthine-xanthine oxidase/NBT method

Sl.No	Sample	Concentration of antioxidant (ppm)	Efficacy(%)
1.	Sesamol	50	48.4
		100	61.7
		200	34.0
2.	sesamin	50	29.4
		100	84.0
		200	21.4
3.	sesamolin	200	7.9
		300	22.9
		500	67.4
4.	sesaminol	50	13.0
	diglucoside	100	21.2
		200	76.5
5.	sesaminol	50	-ve
	triglucoside	100	83.9
		200	60.1
6.	Trolox	200	35.0
		500	25.9
		40	29.2
		10	22.6

Figure 3.44:Superoxide radical scavenging activity of compounds isolated from sesame cake extract by NBT method



The high stability of sesame oil against oxidative deterioration has been known for a long time to be due to the antioxidant property of sesamol and tocopherol present in it [71, 156]. Sesamol exhibits antimutagenic activity against oxygen species mediated mutagenicity in rats [157]. It also exhibits powerful inhibitory effects on lipid peroxidation of liposomes induced by Fe^{2+}, on the lipid peroxidation of rat liver microsomes induced by CCl_4 and NADPH and on lipid peroxidation of mitochondria induced by ascorbate/Fe^{3+} ion [158]. Sesamin possess various physiological actions. It possess antihypertensive effects in rats [159, 160] and alleviation of hepatic injury caused by alcohol or CCl_4 in mice [100]. Sesamin also affects lipid metabolism, inhibits cholesterol absorption from the intestine, reduces 3-hydroxy-3-methyl-glutaryl CoA reductase activity in liver microsomes [86, 88]. Some lignan glucosides inhibited the formation of TBA reacting substances [161]. Glucosides are also reported to possess skin lightening properties.

These studies on lignans are mainly on the biological activities. The present study investigates the invitro activities of lignans and lignan glucosides of sesame cake extract inorder to understand their utility in food and biological systems. Inhibition of lipid peroxidation is shown by preventive antioxidants [162]. Lignans and lignan glucosides were effective in inhibiting β-carotene bleaching and linoleic acid oxidation. Sesamol, sesamin and sesamolin showed more activity than the glucosides. This may be due to the lipophilic nature of lignans and hydrophilic nature of glucosides.

Significantly high radical scavenging activity was shown by sesamol which was higher than that of tocopherol. Presence of methylene dioxy group is reported to be mainly responsible for the various biological activities of lignans. Steriochemistry of

furan-phenyl bond also contribute to activity [71]. In the case of sesamol and sesamol dimer the presence of hydroxyl group along with methylene dioxy group may be responsible for the higher activity.

The compounds which scavenge superoxide anion generated by XOD, can be grouped as chain-breaking antioxidants [162]. Lignans and lignan glucosides showed appreciable superoxide radical scavenging effects, but to varying degrees and also the activity is concentration dependent. Glucosides are more effective as superoxide scavengers than lipid oxidation inhibitors. This again may be due to their hydrophilic nature. From the above studies it is clear that the compounds from sesame cake extract possess antioxidant activity in different model systems and this may contribute to the higher activity of sesame cake extract. Moreover, the above properties of isolated compounds can also be used in food and biological system.

3.5. Biochemical studies

3.5.1. Ex-vivo studies on Anticancer effects of sesame antioxidants

Oxidants by-products of normal metabolism cause extensive damage to DNA, protein and lipids. Healthy organisms maintain a delicate balance between pro-oxidants and antioxidants. However, a shift in this balance, in favor of pro-oxidants, can accelerate cell damage and may lead to malignant cell transformation. This damage appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular diseases etc. Epidemiological studies were proved to suggest the role of dietary antioxidants in prevention of such diseases. Invivo antioxidant effect of sesame seed which has been used since ancient times, is already reported. However, studies relating to their efficacy in tumors, which are known to express a higher amount of oxidation end products, are not clear. In this context, preliminary level cell culture studies were carried out in collaboration with Regional Cancer Centre, Thiruvananthapuram to evaluate the anti cancer effects of sesame extracts in cancer cells using their standardized methodology. Antioxidant assay by TBARS method was carried out in DLA (Dalton's Lymphoma Ascites) cells. The extract containing 1.075 mg/ml of lignans showed approximately 60% inhibition of lipid peroxidation shown by reduction in TBARS values compared to that of standard sesamol at a concentration of 1.075 mg/ml. Both sesamol and extract showed antioxidant activity in a culture of DLA cells. Apoptosis is programmed cell death and is a normal physiological process, which occurs during embryonic development as well as maintaince of tissue hemostasis. It is characterized by certain morphological features. This include changes in the plasma membrane, condensation of cytoplasm and nucleus and internucleosomal cleavage of DNA. In the

final stage, the dying cells become fragmented in to apoptotic bodies which are eliminated by phagocytic cells. SiHa cells (cervical carcinoma) were used for screening of apoptotic activity. 50µl of sesame extract (1.075 mg/ml) were used. In Tunel assay and Annexin-V-Biotin assay, apoptosis was observed with sesame extract and sesamol. Based on the above results further cell culture studies were conducted with pure compounds isolated from sesame extract.

MTT-Cell Proliferation assay was conducted in ME-180 and PAI cells. Compounds from sesame extract inhibited the proliferation of human cervical cancer cells and ovarian cancer cells (ME 180 & PAI) in a dose dependent manner. However, the extent of inhibition varied between the cell lines. Moreover, it is documented that PAI cell were more sensitive to these compounds. Out of the five compounds tested, sesamolin had solubility problem in DMSO and hence was not considered for the experimentation. Moreover, the compound 5, i.e., the triglucoside, did not exhibit a dose response curve in the present experiment.

Out of the three compounds showing a dose response curve, sesamol showed more activity than sesamin in both the cell lines tested (Figure 3.45). The phenotypic characteristics of treated cells were evaluated for nuclear condensation and apoptosis. Treatment of 100µM sesamol for 24h resulted in the formation of nuclear condensation. When cells were treated with 200µM sesamol for 48 h, cells formed apoptotic bodies. In contrast, cells treated with control medium were well spread, with flattened morphology. The cells treated with diglucoside were more resistant to cell death when compared to the other two compounds. One reason may be that activity of the compound may be only there when the glucoside moiety was removed. Thus in conclusion these preliminary

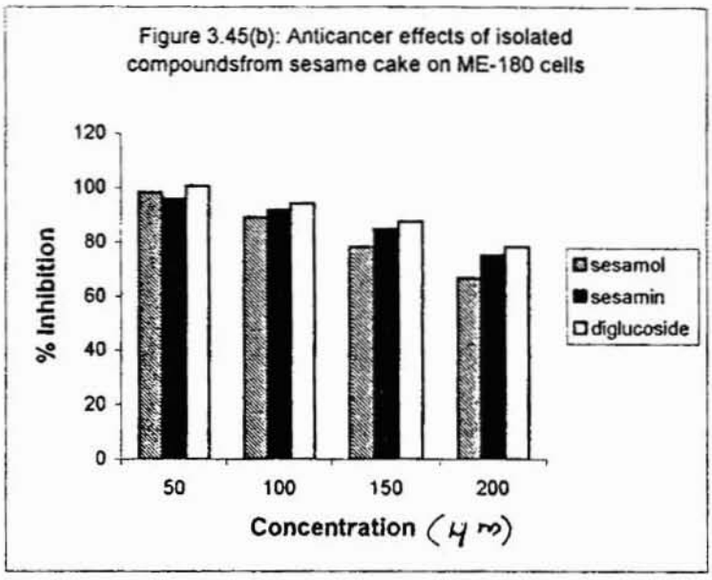


Figure 3.45(b): Anticancer effects of isolated compoundsfrom sesame cake on ME-180 cells
120
100
80
60
40
20
0
% Inhibition
50
100
150
200
Concentration (μm)
sesamol
sesamin
diglucoside

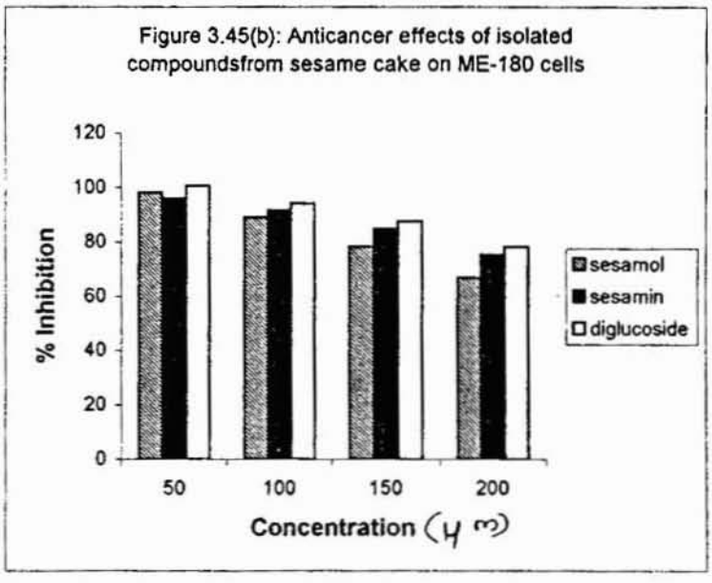


Figure 3.45(b): Anticancer effects of isolated compoundsfrom sesame cake on ME-180 cells
120
100
80
60
40
20
0
% Inhibition
50
100
150
200
Concentration (μm)
sesamol
sesamin
diglucoside

studies suggest the sesame extract and isolated compounds to have significant antioxidant properties, which has potential for chemopreventive strategies. The ability of the extract to induce apoptosis of tumor cells reflects possible anti tumor activity of the compound.

3.5.2. Biological effects

The search for ecologically acceptable methods for crop protection led to the development of alternate strategies for insect control [163]. Recent research in chemical ecology have established that some of the secondary metabolites of plants are not toxic themselves but influence the morphogenetic cycles of insects [164] thereby disrupting the normal life cycle. Some of the natural products are known to have insect growth regulatory effects [165, 166]. The following studies were conducted at the preliminary level for development of economically viable pest management strategies. Sesamin present in sesame was reported to have insect growth regulatory effects in milkweed bug [71].

Orthopterans are generally considered as serious pests and causes heavy damage to the crops worldwide. In the present study, sesamin and sesamolin isolated from sesame cake extract were tested for their growth regulatory properties against an Orthopteran, Teleogryllus mitratus, which feeds on leafy vegetables. The compounds were applied topically on the last instar nymphs (0-24hrs old) on the dorsal side of the body. Both the treated and the control nymphs were kept undisturbed and observed at regular intervals till the adult emerged.

On treatment with 15μg sesamin , the adult emerged showed deformed appendages, wings, abdomen and survived only for few hours after emergence. With

12µg sesamolin, the treatment restricted insect moulting. The treated nymphs were unable to perform ecdysis and half moults were observed that were unable to survive.

The present investigation showed that the compound interferes with the morphogenetic cycle and ecdysis. This may be due to a possible interference of these compounds on ecdysteroid titres of the insects, which normally initiates the release of the moulting hormone for normal ecdysis.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Natural Products, have been in use since antiquity, be it for preservation and fortification of foods or health care and disease management. Recent resurgence in natural products emanate from wide spread and prolonged use of synthetic chemicals in foods and pharmaceuticals and consequent adverse effects on environment and health. A group of compounds namely antioxidants gained attention of late primarily due to their association with genesis of various diseases and disabilities brought out in recent time through epidemiological and experimental evidences. Naturally occurring antioxidants, therefore, received attention in this context in pursuit of identifying and exploiting them for substituting for synthetic antioxidants. Oil seeds and their co-products are repository of various phytochemicals that are bio-active including antioxidation properties. Deoiled sesame cake was identified for the present investigation, since it is an abundantly available co-product of sesame oil industry and incidentally India is the largest producer of sesame in the world (5 to 7 lakh tonnes/year). The objective of this study was two fold viz (i) chemical characterization of antioxidant in sesame cake in quantitative and qualitative terms and (ii) standardization of protocol for extraction, enrichment and scientific validation of antioxidants from sesame cake The results obtained through this investigation and conclusion drawn there from are summarized below:

Sesame seeds, from major cultivars and the deoiled meals were characterized for their lignans by HPLC and compared with commercial deoiled cake. Deoiled meals from white, red and black seeds contained 3420, 3237 and 2038ppm lignans. Commercial cake contained 1300-3000ppm lignans. It could be stated from the result that more than 50%

lignans are left in the deoiled cake. Seamol, an important compound with known bio-active properties retained in the cake. This has not been identified and exploited till now.

4.2 Kinetic studies were conducted in order to standardize protocol to extract lignans using solvents. Based on the results methanol was selected as the most efficient solvent that could extract at <80^{0}C in 16hours. Lignan content of crude methanol extract was 1560ppm.

4.3 Enrichment of lignan in crude methanol extract was achieved by partial purification and enrichment factor or fold purification obtained was 18, with final purified extract, containing as high as 15% lignan as compared to 0.7% in the crude extract.

4.4 A series of experiments were conducted to evaluate the antioxidant efficacy of the extract (crude and purified) using the invitro methods such as schaal oven test, β-carotene bleaching, linoleic acid oxidation, radical scavenging (DPPH), xanthine oxidase assay (NBT & cytochrome C) Synthetic antioxidants like BHT, TBHQ, Trolox and tocopherol standards were used for comparison. The results showed that purified extract at very low concentration range (5 to 200 ppm) inhibited peroxidation (30 to 80 %) significantly higher than comparable concentration of pure compounds such as BHT, Trolox, tocopherol etc. The results obtained from stability studies using vegetable oils (Soybean, sunflower, safflower) indicated that purified extract could be used as a substitute for synthetic BHT. Radical scavenging studies showed the hydrogen donating ability and superoxide scavenging properties of the extract, which had biological significance.

4.5 Detailed studies on separation of lignans and lignan glucosides were carried out. They were isolated, identified and antioxidation kinetics were studied for each compounds. Sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside showed antioxidant efficacy in the decreasing order, sesamol >sesamolin > sesamin > sesaminol triglucoside> sesaminol diglucoside in peroxidation model system. The radical scavenging kinetics followed the order sesamol > sesamol dimer > sesamin > sesaminol triglucoside > sesamolin > sesaminol diglucoside. No studies so far have been reported on the radical scavenging effects of these compounds.

4.6 Preliminary studies suggest that sesame extract, sesamol, sesamin and sesaminol diglucoside to have significant antioxidant properties, which has potential for chemopreventive strategies. The ability of the extract and pure compounds to induce apoptosis of tumor cells reflects possible antitumor activity of the compounds. Preliminary level studies showed pesticidal and mosquitocidal effects for sesame cake extract and isolated compounds.

4.7 Detailed investigation on the lignans and their derivatives from deoiled sesame cake , hitherto, not reported has been conducted and scientifically validated their antioxidant efficacies as extract and individual compounds vis-à-vis reference compounds and established the commercial potential of sesame lignans towards substituting synthetic antioxidants . The economics being in favor , scale up studies and commercial applications in edible oil as first step with industrial collaboration are further works to be

undertaken before commercialization. A patent has been filed for the process and application based on the present studies.

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ACHIEVEMENTS

Patents filed

1.Process for extraction of Antioxidants from Sesame seed/cake : US Patent Application No.60/404.004

Papers

1.Antioxidant activity of sesame cake extract (communicated to Food Chemistry)

2.Antioxidant efficacy of sesame cake extract in vegetable oil protection (communicated to Food Chemistry)

3.Free radical scavenging behavior of antioxidant compounds from sesame (*Sesamum indicum*) in DPPH system (communicated to Journal of Agricultural and food chemistry)

4.Invitro studies on antioxidant activity of lignans isolated from sesame cake extract (communicated to Journal of Science and Food Agriculture)

5.Antioxidant and biological activities of wild species of sesame, *Sesamum malabaricum* (Communicated to Journal of American Oil Chemical Society as letter to editor)