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˙] 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°C) 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).

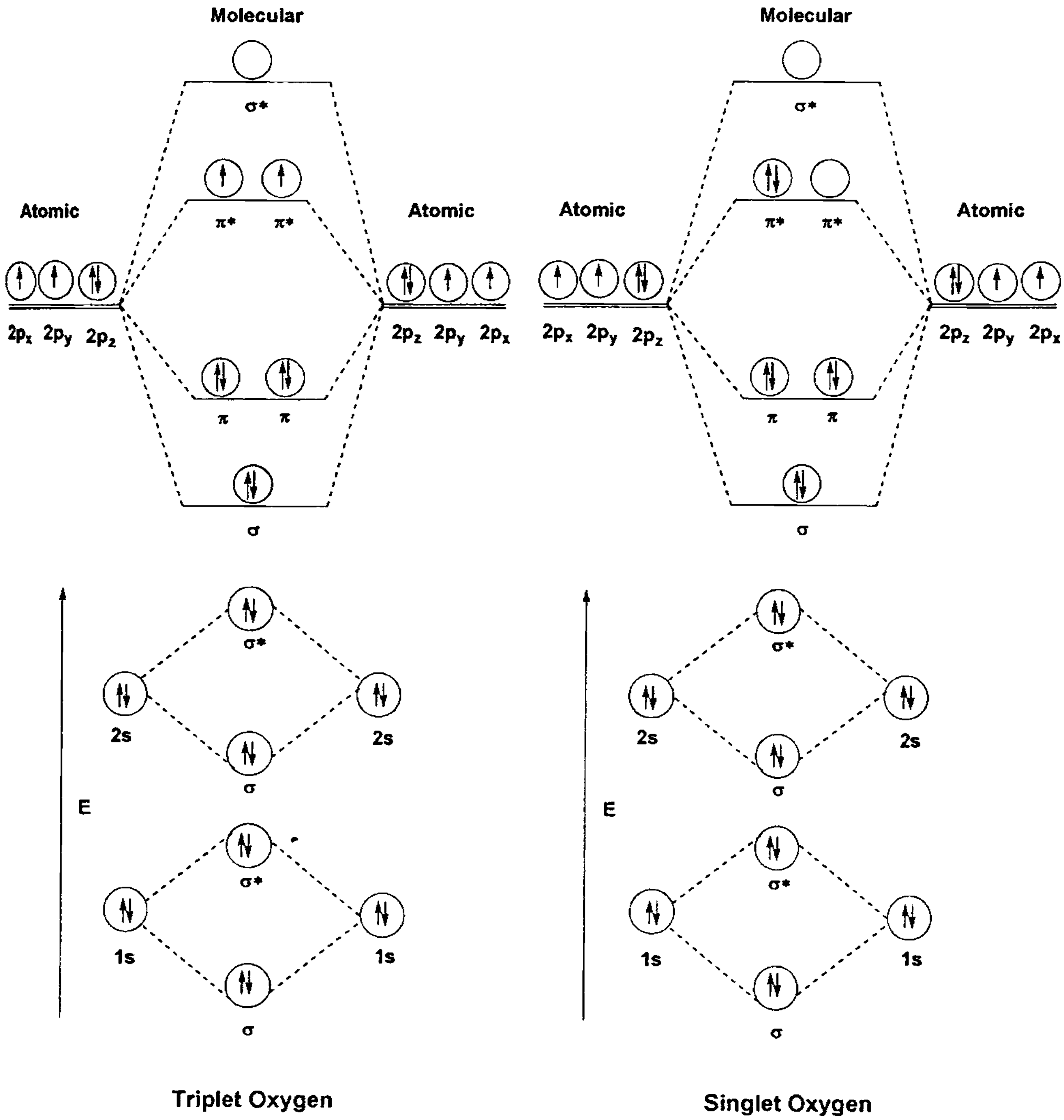

Figure 1.1: Molecular orbitals of Oxygen

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].