Studies on Phytochemicals and Biological Properties of Bitter Cumin *Centratherum anthelminticum* (L.) Kuntze

A thesis submitted to the Department of Studies in Biochemistry, University of Mysore in fulfilment of the requirements for the degree of

Doctor of Philosophy in Biochemistry

By

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Certificate

I, Ani. V., hereby certify that, this thesis entitled "**Studies on Phytochemicals and Biological Properties of Bitter Cumin (*Centratherum anthelminticum* (L.) Kuntze)**" is the result of research work done by me under the supervision of **Dr. K. Akhilender Naidu,** at the **Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore 570020, Karnataka, India**. I am submitting this thesis for possible award of Doctor of Philosophy (Ph. D.) degree in **BIOCHEMISTRY** of the University of Mysore.

I further certify that this thesis has not been submitted by me for the award of any other degree or diploma of this or any other University.

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This is to certify that, the thesis entitled **"Studies on Phytochemicals and Biological Properties of Bitter Cumin (*Centratherum anthelminticum* (L.) Kuntze)"** submitted by **Mr. Ani. V.,** for the fulfilment of requirements of the Doctor of Philosophy degree in **Biochemistry** is the result of work carried out by him under my guidance and supervision at the **Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore 570020, Karnataka, India during** the year 2003-2008

Place: Mysore (K. Akhilender Naidu)

Date:

Synopsis of the thesis submitted for the award of Ph.D., degree in Biochemistry of the University of Mysore, India.

Title: Studies on Phytochemicals and Biological Properties of Bitter Cumin *Centratherum anthelminticum* (L.) Kuntze

Background

Human health is inextricably linked with diet and in the last few years research teams have been able to demonstrate the health beneficial effects of food components, spices and medicinal herbs that are a part of the traditional diet. There has been an explosion of research geared towards understanding precisely how diet can affect normal physiology and health of human beings. One of the most remarkable features of medicinal plant research of the last decade is the enormously increasing interest towards the biological activities of phytochemicals such as phenolic compounds including flavonoids. Flavonoids and other phenolics have been suggested to play a preventive role of chronic diseases such as cancer and heart diseases.

Spices have occupied an important place in the lives of people since ancient times. They have been considered indispensable in flavouring, seasoning of foods, flavouring of beverages, in perfumery, cosmetics and medicines. The medicinal activity of spices and spice extracts including hypocholesterolemic, hypoglycemic, antiinflammatory, antimicrobial, antithrombotic, antimutagenic, anticarcinogenic etc., are attributed to their bio-active molecules such as polyphenolic compounds, phenolic glycosides, essential oils, phytosterols etc.,

Cumin is one of the most popular spices all over the world especially Latin America, North Africa, Europe and all over Asia. There are three major varieties of cumin, Normal cumin (*Cuminum cyminum*), Black cumin (*Nigella sativa*) and Bitter cumin (*Centratherum anthelminticum*).

Bitter cumin (*Centratherum anthelminticum* (L.) Kuntze) belongs to the family Asteraceae. It is locally called ‘Kali-ziri’ or ‘Somraj’. It is distributed throughout Indian subcontinent and also cultivated in Sri Lanka. The seeds have a hot, sharp, bitter taste, acrid, astringent to the bowls. The seeds are reported to possess febrifugal, alterative, antihelmenthic, antiulcer, antiphlegmatic, cardiac, diuretic and digestive

properties. In Ayurvedic medicine it is used to treat skin diseases such as leucoderma. In Yunani system of medicine the seeds are used as purgative and for treatment of asthma, hiccough, inflammatory swellings and itching of the eyes. On the Malabar coast an infusion of the seeds is given for the coughs and against flatulency.

There is little scientific data on phytochemicals and their beneficial properties of bitter cumin variety. The main aim of this research programme is to isolate and characterize the bioactive phytochemicals from bitter cumin and study their biological properties in suitable model systems. The main objectives of this study are

1) Isolation and characterization of bioactive phytochemicals from *Centratherum anthelminticum* (L.) Kuntze seeds.

2) To study the biological effects of *Centratherum anthelminticum* (L.) Kuntze phytochemicals on antioxidant, antidiabetic and antimicrobial properties in suitable model systems.

The thesis comprises of five chapters. Chapter I present general introduction about cumin varieties and objectives and scope of the present investigation.

Chapter 2 deals with proximate composition, total phenol content, isolation, characterization and quantification of phenolic compounds from Bitter Cumin (*Centratherum anthelminticum* (L) Kuntze) seeds. The total phenol content of different extracts of bitter cumin was determined in terms of gallic acid and tannic acid equivalents. Aqueous methanol acetone extract of bitter cumin (AMAEBC) showed highest phenolic content among different solvent extracts. The phenolic compounds in AMAEBC was determined and quantified by LC-MS. Interestingly bitter cumin seeds contained a mixture of phenolic compounds such as gallic acid, protocatechuic acid, ellagic acid, caffeic acid, ferulic acid, quercetin and kaempferol.

Chapter 3 describes the antioxidant activity of bitter cumin seed. The antioxidant activity of phenolic compounds of bitter cumin seed was determined by various *in vitro* model systems. The antioxidant assays adopted in this study are phosphomolybdenum reducing assay, potassium ferricyanide reducing method, DPPH radical scavenging, $ABTS^{+}$ radical scavenging, superoxide radical scavenging, soybean lipoxygenase dependent lipid peroxidation, rat liver microsomal lipid

peroxidation, egg lecithin liposomal model system and oxidative DNA damage. AMAEBC showed a wide range of antioxidant activity by scavenging or neutralizing radicals such as DPPH$^{\bullet}$ radical, ABTS$^{\bullet}$ cation radical, hydroxyl ($^{\bullet}$OH), lipid peroxy radical (LOO$^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) and also inhibited oxidative DNA damage. The phenolic acids such as ferulic acid, caffeic acid, gallic acid, ellagic acid, protocatechuic acid and flavonoids such as quercetin and kaempferol found in bitter cumin seeds are reported to have good antioxidant activity. Hence, the phenolic compounds present in bitter cumin could be responsible for its antioxidant activity.

Chapter 4 describes antidiabetic activity of Bitter Cumin. The antidiabetic activity of AMAEBC was tested in *in vitro* and *in vivo* model systems. One of the therapeutic approaches for management of diabetics is decreasing the postprandial hyperglycemia by retarding the absorption of glucose in the small intestine. Hence the modulatory effect of AMAEBC on carbohydrate hydrolyzing enzymes such as intestinal α-glucosidase and human salivary α-amylase has been studied. AMAEBC inhibited salivary α-amylase in a concentration dependent manner with an IC_{50} value of 185.5 ± 4.9 μg. Further, AMAEBC inhibited rat intestinal sucrase, maltase and PNP-glycoside activity at microgram concentrations with IC_{50} values of 34.1 ± 3.8 μg/mL, 62.4 ± 4.5 μg/mL and 500.5 ± 11.9 μg/mL respectively. The inhibitory effect of AMAEBC against sucrase was about 2 and 14 times higher than maltase and PNP-G (p-nitrophenyl glucopyranoside) hydrolysis activity respectively. Further, inhibitory effect of AMAEBC on sucrase and maltase activities is found to 8 and 32-fold higher than DL-catechin, but ≈15 and 624-fold lower than synthetic therapeutic drug acarbose. The higher inhibitory activity of AMAEBC compared to DL-catechin may be because of the additive activity of an array of phenolic compounds present in bitter cumin seeds. The enzyme kinetic studies on α-glucosidase inhibition by AMAEBC showed a non-competitive type of inhibition. The antihyperglycemic effect of AMAEBC was examined on *in vivo* maltose tolerance test in rats. Oral feeding of AMAEBC (50-200 mg/kg body weight) significantly reduced the postprandial plasma glucose levels comparable to that of acarbose. The results demonstrate that AMAEBC may inhibit digestion of carbohydrates in rat intestine leading to a decrease in postprandial plasma glucose level. The fasting blood glucose level of streptozotocin (STZ) induced diabetic rats after AMAEBC treatment for 8 days was reduced by 15.23% when compared to control group. In an another experiment of low STZ and high fructose induced hyperglycemia, AMAEBC caused 13.34% and 19.33% decrease in fasting blood glucose level at 25 mg/kg b.wt and 100 mg/kg b.wt

respectively. These studies clearly show that bitter cumin extract has antihyperglycemic property by modulating the activity of carbohydrate metabolizing enzymes.

Chapter 5 describes the antimicrobial activity of Bitter Cumin. Antibacterial activity of cumin extract was tested against food-borne pathogenic and spoilage bacteria viz., *Bacillus subtilis*, *Bacillus cereus*, *Enterobacter* spp., *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica* by agar diffusion method. Three bacterial species namely *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* were found to be highly sensitive and showed significant inhibition of the growth in presence of AMAEBC. *Enterobacer spp.* and *Listeria monocytogens* were moderately inhibited while *Escherichia coli* and *Yersinia enterocolitica* were not sensitive to bitter cumin extract. Minimum Inhibitory Concentration (MIC) of *B. cereus*, *S. aureus* and *L. monocytogens* were determined and found to be 50 ± 7μg, 260 ± 18 μg and 700 ± 42 μg respectively.

Bitter cumin is a cumin variety which is not used in food preparations due to its bitter taste. However, bitter cumin is used extensively in Ayurvedic and traditional medicine. The present research study demonstrated that the bitter cumin seeds contain an array of bioactive phenolic compounds with a potent antioxidant, antihyperglycemic and antimicrobial activities and thus provide data to supports its usage in ayurveda and traditional medicine. Bitter cumin can be further exploited as a herbal medicine and health related herbal products for the management of different diseases.

Dedicated to

My Village.....

From where I learned the first lessons of natural philosophy.....

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Ani. V

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Abbreviations

µg- Microgram

µL- Microlitre

µM- Micromolar

°C- degree Celsius

ABTS- Azinobis ethyl benzo thiazoline sulfonic acid

AE- Aqueous extract

AGH- α-Glucosidase

AGEs- Advanced glycation end products

AIN- American Institute of nutrition

AMAE- Aqueous methanol-acetone extract

AMAEBC- Aqueous methanol-acetone extract of bitter cumin

AME- Aqueous methanol extract

ANOVA- Analysis of variance

AOAC- Association of Official Analytical Chemists

API- Atmospheric pressure ionization

BC- Bitter cumin

BHA- Butylated hydroxy anisole

BHI- Brain heart infusion

BSA- Bovine serum albumin

c.m- Centimeter

CA- *Centratherum anthelminticum*

CAD- Collision associated dissociation

CE- Collision energy

cfu- colony forming units

cGMP- Cyclic guanosine monophosphate

dL- Deciliter

DMRT- Duncan’s multiple range test

DMSO- Dimethyl sulfoxide

DNA- Deoxy ribonucleic acid

DNS – Dinitro salicylic acid

DP- Declustering potential

DPPH- 1,1- Diphenyl, 2-picryl hydrazyl

EDTA- Ethylene diamine tetra acetic acid

FAME- Fatty acid methyl ester

FC- Folin-Ciocalteu

$FeCl_3$- Ferric chloride

FID- Flame ionization detector

ft- Feet

g- gram

GAE- Gallic acid equivalent

GC- Gas chromatography

H_2SO_4- Sulphuric acid

HCl-Hydrochloric acid

HNE- 4-hydroxy, 2-nonenal

HPLC- High performance liquid chromatography

i.d- internal diameter

KCl- Potassium chloride

kg-Kilogram

L- Litre

LC – Liquid chromatography

LC-MS Liquid chromatography- Mass spectrometry

$LOO^{\bullet}$ - Lipid peroxide radical

LOX- Lipoxygenase

MDA- Malondialdehyde

MeOH- Methanol

mg- Milligram

MIC- Minimum inhibitory concentration

Min- Minutes

mL- Millilitre

mm- Millimetre

mM- Millimolar

Mo- Molybdenum

MS – Mass spectrometry

mU- Milli units

MUFA- Mono unsaturated fatty acids

NADH- Nicotinamide adenine dinucleotide (Reduced)

NBT- Nitroblue tetrazolium

ng- Nanogram

nm – Nanometer

nM- Nanomolar

$O_2^{\bullet -}$- Superoxide anion radical

$OH^{\bullet}$- Hydroxyl radical

PMS- Phenazine methosulfate

PNP-G- Paranitrophenyl gulucopyranoside

PUFA- Poly unsaturated fatty acids

Q- Quenching

RNS- Reactive nitrogen species

ROO•- Peroxy radicals

ROS- Reactive oxygen species

RP-HPLC – Reverse phase high performance liquid chromatography

SEM – Scanning electron microscopy

SFA- Saturated fatty acids

STZ- Streptozotocin

TAE- Tannic acid equivalent

TBARS- Thiobarbituric acid reactive substance

TCA- Trichloro acetic acid

Tris- Tris (hydroxymethyl) aminomethane

UV- Ultraviolet

V- Volts

v- Volume

WHO- World Health Organization

Chapter I

General Introduction

1.1. Food and Health

Human evolution dates back to about 2 million years ago. Food has played a major role in human evolution. Human nutritional requirements and digestive physiology were developed over million years of evolutionary development from anthropoid primates (Milton, 2000). During that time, the micro and macro nutrient requirements were met largely by an herbivorous diet (Milton, 1999; Wood and Brooks, 1999). It was hypothesized that a refinement in food habits including consumption of pre-digested or cooked food and less fibrous food resulted in minimizing dietary bulk which lead to an increase in the development of brain and decrease in the gut size (Aiello and Wheeler, 1995; Milton, 2000). Organized agricultural practices began about 12000 years ago and the green revolution has set a pace for dietary and social changes that outstripped our biological ability to adapt. This has led to a hypothesis that the common 'diseases of civilization' were related to a mismatch between our ancient nutritional diet and the current, contemporary dietary practices (Boyd Eaton *et al*., 1998; Milton, 1999).

Since ancient times, natural products from plants have been used as an important source of prophylactic agents for the prevention and treatment of diseases in humans and animals. About 2500 years ago, *Hippocrates*, the father of modern medicine, conceptualized a relationship between the use of appropriate foods for health and their therapeutic benefits. He quoted that "Let food be thy medicine and medicine be thy food". In recent years, a number of epidemiological studies on the relationship between dietary food habits and disease risk have shown that food has a direct impact on human health. It is generally accepted that plant derived foods such as fruits, nuts, vegetables, grains, legumes, spices, etc., exert many beneficial effects on human health. This fact has encouraged several health organizations around the world to recommend an increase in the intake of plant based foods to improve our health status and to delay the onset of diseases. The capacity of some plant-derived foods to reduce the risk of chronic diseases has been associated, at least in part, to the occurrence of non-nutrient secondary metabolites known as phytochemicals. These phytochemicals have low potency as bioactive

compounds when compared to pharmaceutical drugs, but, since they are ingested regularly and in significant amounts as a part of the diet, they may have noticeable long-term physiological effects in human health.

Functional foods are those that, when consumed regularly, exert a specific health beneficial effect beyond their nutritional properties (i.e., a healthier status or a lower risk of disease) and this effect must be scientifically proven. The new regulation of the European Parliament stipulated that the nutritional and health food products should have scientific support for their health beneficial claims. (Official Journal of the European Union, L 404, volume 49, 30th December, 2006).

There is worldwide concern over side effects and high cost of modern therapeutic methods to cure diseases. Lately, diet is recognized as an important choice for the prevention and cure of such diseases. Nutraceuticals are diet supplements that deliver a concentrated form of a bioactive agent from a food, presented in a non-food matrix and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods (Zeisel, 1999). Nutraceuticals are sold as pills, extracts, tablets etc., similar to drugs. The health benefits of nutraceuticals are mainly because of the presence of phytochemicals. Choice of nutraceuticals is not just going back to nature but also an element of supplementing the body to make it resist the stress and strains of day to day life. Nutraceutical industry is poised for a big growth and scientific research and development can play an important role in its growth. It is estimated that global nutraceutical market is valued at over US $80 billion. USA holds a significant share followed by Japan and European Union. Even though Indian nutraceutical market is in budding stage, it can emerge as fastest growing market as strong economic growth of the country can lead to extensive up gradation and diversification of food and food products. Envisaging a substantial growth in world nutraceutical market and a strong potential in India, many multinational and Indian firms are entering to this arena. In this scenario, extensive research to prove potential health benefits of nutraceuticals will help the healthy growth of nutraceutical industry.

1.2. Spices

Spices are a group of food adjuncts, which have been in use for thousands of years to enhance flavour and sensory quality of food. These spice ingredients impart a characteristic flavour, aroma or piquancy and also colour to the food and some may change the texture of food. The spices are also used in perfumery, cosmetics and toiletries. In addition, several spices have long been recognized to possess medicinal properties such as tonic, carminative, stomachic, antispasmodic and antihelmenthic (Nadkarni and Nadkarni, 1976). Extensive animal studies carried out to evaluate the safety aspect of spices have indicated that red pepper, black pepper and turmeric, even at higher dietary levels (up to 100 times the normal intake) have no adverse effects in experimental animals. In the past three decades, many beneficial physiological effects of spices have been experimentally documented which suggests a definite role of these food adjuncts beyond just taste and flavour (Srinivasan, 2005). Among the health problems that affect mankind, diabetes, cardiovascular disease, inflammatory disorders including arthritis and cancer have received considerable attention. The health beneficial physiological effects of some of the common spices have been summarized Table 1, 2, 3 and 4.

TABLE 1. Spices with antioxidant property

Common name	Botanical name	Family	Reference
Clove	*Syzygium aromaticum*	Myrtaceae	Su-Chen Ho *et al*., 2008
Cinnamon	*Cinnamomum zeylanicum*	Lauraceae	Jayaprakasha *et al*., 2007
Star-anise	*Illicium verum*	Illiciaceae	Padmashree *et al*., 2007
Ginger	*Zingiber officinale*	Zingiberaceae	Stoilova *et al*., 2007
Pepper	*Piper nigrum*	Piperaceae	Agbor *et al*., 2006
Nagkesar	*Mammea longifolia*	Guttiferae	Rathee *et al*., 2006
Coriander	*Coriandrum sativum*	Umbellifereae	Almeida melo *et al*., 2005
Mace	*Myristica fragrans*	Myristicaceae	Chatterjee *et al*.,2007
Saffron	*Crocus sativus*	Iridaceae	Papandreou *et al*., 2006
Curry leaf	*Murraya koenigii*	Rutaceae	Ningappa *et al*., 2008

TABLE 2. Spices with antidiabetic/antihyperglycemic property

Common name	Botanical name	Family	Reference
Curry leaf	*Murraya koenigii*	Rutaceae	Kesari *et al*., 2005
Fenugreek	*Trigonella foenum-graecum*	Fabaceae	Eidi *et al*., 2007
Garlic	*Allium sativum*	Liliaceae	Eidi *et al*., 2006
Rosemary	*Rosmarinus officinalis*	Lamiaceae	Bakirel *et al*., 2007
Turmeric	*Curcuma longa*	Zingiberaceae	Sureshkumar *et al.,* 2005
Lemon grass	*Cymbopogon citratus*	Graminae	Adeneye *et al*., 2007
Jamun	*Syzigium cumini*	Myrtaceae	Prince *et al*., 2004
Onion	*Allium cepa*	Liliaceae	Campos *et al*., 2003
Cinnamon	*Cinnamomum zeylanicum*	Lauraceae	Pham *et al*., 2007
Ginger	*Zingiber officinale*	Zingiberaceae	Al-Amin et al., 2006

TABLE 3. Spices with antimicrobial property

Common name	Botanical name	Family	Reference
Chilli	*Capsicum annum*	Solanaceae	Ribeiro *et al*., 2007
Ginger	*Zingiber officinale*	Zingiberaceae	Perumal Samy, 2005
Cardamon	*Elettaria cardamomum*	Zingiberaceae	El Malti *et al*., 2007
Coriander	*Coriandrum sativum*	Umbelliferae	Delaquis *et al*., 2002
Anise	*Pimpinella anisum*	Umbelliferae	Gulcin *et al*., 2003
Rosemary	*Rosmarinus officinalis*	Lamiaceae	Tsai *et al*., 2007
Clove	*Syzygium aromaticum*	Myrtaceae	Mytle *et al*., 2006
Cinnamon	*Cinnamomum zeylanicum*	Lauraceae	Matan *et al*., 2006
Garlic	*Allium sativum*	Liliaceae	Seydim and Sarikus, 2006
Mint	*Menta piperita*	Lamiaceae	Tassou *et al*., 2000

TABLE 4. Spices with anticancer property

Common name	Botanical name	Family	Reference
Garlic	*Allium sativum*	Liliaceae	Lau *et al*., 1990
Turmeric	*Curcuma longa*	Zingiberaceae	Johnson & Mukhtar, 2007
Cinnamon	*Cinnamomum zeylanicum*	Lauraceae	Schoene *et al*., 2005
Fenugreek	*Trigonella foenum-graecum*	Fabaceae	Amin *et al*., 2005
Rosemary	*Rosmarinus officinalis*	Lamiaceae	Chan *et al*., 1995
Chilli	*Capsicum annum*	Solanaceae	Maoka *et al*., 2001
Ginger	*Zingiber officinale*	Zingiberaceae	Ishiguro *et al*., 2007
Star-anise	*Illicium verum*	Illiaceae	Yadav & Bhatnagar, 2007
Saffron	*Crocus sativus*	Iridaceae	Fernandez, 2006
Onion	*Allium cepa*	Liliaceae	Xiao & Parkin, 2007

1.3. Cumin (*Cuminum cyminum* L.)

Taxonomy

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Apiales
Family	Apiaceae
Genus	*Cuminum*
Species	*Cuminum cyminum*

Cumin is one of the commonly used spices in both vegetarian and non-vegetarian food preparations. Cumin is used in garam masala preparations which is a mixture of a variety of spices. Cumin has been in use since ancient times. Cumin has been mentioned in the Bible in both the Old Testament (Isaiah 28:27) and the New Testament (Matthew 23:23). It was also known in ancient Greece and Rome. Cumin is grown in Iran, Uzbekistan, Tajikistan, Turkey, Morocco, Egypt, India, Syria, Mexico and Chile. The term Cumin is used to represent a number of botanically different species. Of these, four varieties of cumin are very common in India viz., Cumin (*Cuminum cyminum*), Black Cumin (*Nigella sativa*), Kashmiri cumin (*Carum carvi*) and Bitter Cumin (*Centratherum anthelminticum*).

FIGURE 1. Cumin (*Cuminum cyminum*) seeds

TABLE 5. Trivial names of *cumin*

Language	Trivial name
English	Cumin
Sanskrit	Jeeraka, Jeera
Hindi	Jiira
Bengali	Jira
Gujarati	Jiru
Punjabi	Jira
Tamil	Jiiragam
Telugu	Jilakarra
Kannada	Jeerige
Malayalam	Jeerakam

Cumin or *Cuminum cyminum* belongs to the family Apiaceae. It is a small, slender, glabrous and herbaceous annual plant. It usually reaches 25 cm and tends to droop under its own weight. The blue-green linear leaves are finely divided and the white or pink flowers are borne in small compound umbels. Cumin is grown from seed. The seeds come as paired or separate carpels,

and are 3-6mm (1/8-1/4 in) long. They have a striped pattern of nine ridges and oil canals and are hairy, brownish in colour, boat-shaped, tapering at each extremity, with tiny stalks attached.

The word cumin comes from Latin *cuminum*, which was borrowed from Greek *kyminon*. It is cultivated since Biblical times. Cumin is used mainly where highly spiced foods are preferred. It features in Indian, Eastern, Middle Eastern, Mexican, Portuguese and Spanish cookery. It is an ingredient of most curry powders and many savory spice mixtures. It is used in stews, grills - especially lamb and chicken dishes. It gives bite to plain rice, beans and cakes. Small amounts can be used in aubergine and kidney bean dishes. Cumin is essential in spicy Mexican foods such as chile con carne, casseroled pork and enchiladas with chili sauce. In Europe, cumin flavours certain Portuguese sausages and is used to prepare spice cheese. It is burned to smoke cheeses and meats. It is a pickling ingredient for cabbage and Sauerkraut. In the Middle East, it is a familiar spice in fish dishes, grills and stews. It is used to flavour couscous - semolina steamed over meat and vegetables, the national dish of Morocco. Zeera pani is a refreshing and appetizing Indian drink made from cumin and tamarind water. Cumin together with caraway flavours Kummel, the famous German liquor.

In indigenous medicine, cumin seeds have been considered to be stomachic, diuretic, carminative, stimulant, astringent, emmenagogic and antispasmodic. It is valuable in the treatment of dyspepsia, diarrhoea and hoarseness and to relieve flatulence and colic pain. In the West, it is now used mainly in veterinary medicine, as a carminative, but, it is used as a traditional herbal medicine in the East. It is reported to increase lactation and reduce nausea in pregnancy. It has been shown to be effective in treating carpal tunnel syndrome, as well as diarrhoea, indigestion and morning sickness. It is used as a poultice to reduce swelling of the breast or the testicles.

The main constituent of cumin volatile oil is cuminaldehyde (p-isopropyl benzaldehyde, p-cuminaldehyde). Volatile oil of cumin is employed in many types of flavouring compounds. The Indian cumin oil contains significant flavour components, such as cuminaldehyde 18.3% and perillaldehyde 8.17%

and the terpenic hydrocarbons, whereas Egyptian cumin oil contains 39.2% cuminaldehyde (Srinivas, 1986).

Cumin oil, shows antifungal activity, which could be related to the cuminaldehyde present in cumin oil (Lawrence, 1992). The ovicidal activity of cumin essential oil, against insects, has been reported (Tunc *et al*., 2000). Cumin essential oil contains 29.1% α-pinene, 21.5% limonene, 17.9% 1,8-cineole and 10.4% linalool as major compounds with antimicrobial properties (Latif *et al*., 2007). Ishikawa *et al*., (2002) reported isolation and characterization of 12 glucosides from *Cuminum cyminum*. Anti-hyperglycemic effect (Roman-Ramos *et al*., 1995) and hypolipidemic effect of *Cuminum cyminum* on alloxan-induced diabetic rats were also reported (Surya *et al*., 2002). The essential oil present in cumin seeds prevents butter from deterioration and improves its acid value. It has an antihydrolytic effect and is better than conventional synthetic antioxidants (The Wealth of India, 2001).

1.4. Caraway (*Carum carvi* Linn)/kala jira

Taxonomy	
Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Apiales
Family	Apiaceae
Genus	*Carum*
Species	*Carum carvi* L.
Synonym	*Bunium persicum* (Boiss.) B. Fedtsch.

TABLE 6. Trivial names of *Carum carvi*

Language	Trivial Name
English	Caraway
Sanskrit	Karavi, Sushavi
Hindi	Kala jira, Shah jiira
Tamil	Cakacirakam
Bengali	Sa-zira
Marathi	Wilayati zirah
Punjabi	Zira-siah
Kannada	Shime jeerige
Telugu	Sima jirakaia
Malayalam	Shima jeerakam

FIGURE 2. Caraway (*Carum carvi*) seeds

Caraway or *Carum carvi* is a biennial herb and belongs to the family Apiaceae. It is native to North and Central Europe and is also extensively cultivated in Holland, Russia, Poland, Bulgaria, Rumania, Syria, Morocco and to a small extent, in England. It grows wild in India especially in the North

Himalayan regions. It is also cultivated in the plains as a cold season crop and in the hills of Kashmir and Kumaun at an altitude of 2,740 to 3,660 meters, as a summer crop. The plant grows 40-60 cm tall with feathery leaves and thread like divisions. The flowers are pink in colour and grow in umbel. The fruit when ripe, split into narrow elongated carpels of 4-6.5 mm long, curved, pointed at the ends and with longitudinal ridges on the surface. The dried fruit or seed is brown in colour, has a pleasant odour, aromatic flavour, warm and sharp taste.

Caraway seeds on steam distillation yield an aromatic essential oil (4 to 6%), which finds greater use in medicines than the seeds as such. Caraway oil, distilled from fresh seeds, is a colourless or pale yellow oil. Carvone content of oil is 45 to 65%. Caraway grown in Kashmir gives oil conforming to the B.P. standard. Pure carvone is prepared by decomposing the crystalline compound of carvone with hydrogen sulfide. Caraway fruits contain 3-7% essential oil. The aroma of the oil is due to carvone (50 to 85%) and limonene (20 to 30%). Other components of caraway oil are carveol, dihydrocarveol, α- and β-pinene, sabinene and perillyl alcohol which are of minor importance.

Caraway is widely used as a spice for culinary purposes and for flavouring bread, biscuits, cakes and cheese. It is also used in the manufacture of `Kummel` and as an ingredient of sausage seasoning and pickling spice. It is a mild stomachic and carminative, used to control flatulence and colic pain. Its volatile oil is employed more often than the seed itself. Carvone is used as antihelmintic in hookworm infections. Caraway oil is used chiefly for flavouring purposes and in medicine as a carminative. It is also used to correct the nauseating and griping effects of medicines.

Water extract of *Carum carvi* showed strong diuretic action in normal rats (Lahlou *et al*., 2007). The volatile oil of Caraway is reported to have significant larvicidal effect against two mosquito species, *Anopheles dirus* and *Aedes aegypti* (Pitasawat *et al*., 2007). Caraway is a component of STW2 (Iberogast®) mixture which is evaluated for its antioxidant and anti-inflammatory properties (Schempp *et al*., 2006; Germann *et al*., 2006). Caraway is also good in reducing cholesterol and triglycerides in normal and

streptozotocin diabetic rats (Lemhadri *et al*., 2006). d-Carvone or (4*S*)-(+)-carvone the main constituent of caraway oil is used in fragrance and flavour, potato sprouting inhibitor, antimicrobial agent, building block and biochemical environmental indicator (De Carvalho and Fonseca, 2006). Caraway showed antihyperglycaemic effect in diabetic rats (Eddouks *et al*., 2004). Caraway seed extract inhibited cytochrome P450 1A1 (CYP1A1) enzyme activity in hepatoma cells in a dose-dependent manner and reversed the TCDD-dependent induction of cytochrome P450 1A1 activity (Naderi Kalali, 2005).

1.5. Black cumin (*Nigella sativa* L.)

Taxonomy

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Rananculales
Family	Rananculaceae
Genus	*Nigella*
Species	*Nigella sativa* L.

TABLE 7. Trivial names of Black cumin

Language	Trivial name
English	Black cumin
Sanskrit	Krishnajera
Hindi	Kalaunji
Punjabi	Kalonji
Kannada	Kari jirige
Malayalam	Karin jirakam

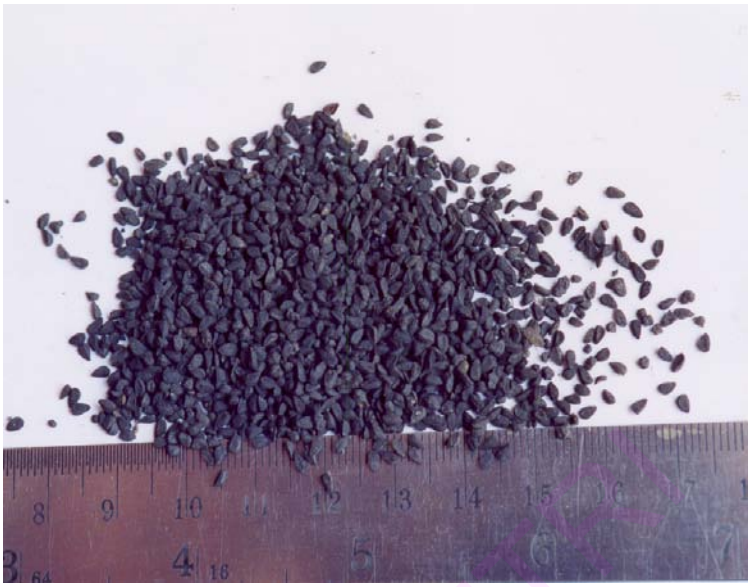


FIGURE 3. Black Cumin (*Nigella sativa*) seeds

Black Cumin (*Nigella sativa*) belongs to the family Rananculaceae. It is an annual plant native to Southwest Asia. In India, it is popularly known as Kalonji. It grows to 20-30 c.m tall, with finely divided, linear (but not thread-like) leaves. The flowers are delicate, and usually coloured pale blue and white, with 5-10 petals. The fruit is a large and inflated capsule composed of 3-7 united follicles, each containing numerous seeds. The seed is used as a *spice*.

Nigella sativa has been used for centuries, both as an herb and pressed into oil, by people in Asia, Middle East, and Africa for medicinal purposes. It has been traditionally used for a variety of conditions and treatments related to respiratory health, stomach and intestinal health, kidney and liver function, circulatory and immune system support, and for general overall well-being. An Arab proverb calls it "the medicine for every disease except death".

Nigella sativa oil contains a number of pharmacologically active constituents. They include thymoquinone, dithymoquinone, thymohydroquinone and thymol

(Ghoshesh *et al*., 1999). Extensive studies were conducted on pharmacological properties of essential oil of Black cumin and thymoquinone. The notable pharmacological properties reported are antitumour (Salomi *et al*., 1992, Islam *et al*., 2004), antioxidant activity (Burits and Bucar, 2000) hepatoprotective (Daba and Abdel, 1998; Nagi *et al*., 1999; Mansour *et al*., 2001), anti-inflammatory (Houghton *et al*., 1995), anti-histaminic (El-Dakhakhny *et al*., 2000), immunomodulatory (Haq *et al*., 1999), antihelminthic (Aboul-Ela, 2002) and antimicrobial activity (Morsi, 2000). Mahgoub (2003) has shown that thymoquinone at 10 mg/kg protected against experimentally induced colitis in rats. Kalus *et al*., (2003) reported a decrease in the IgE and eosinophil levels in patients suffering from allergic rhinitis, bronchial asthma and eczema after oral administration of *Nigella sativa* oil at 40-80 mg/kg/day for 8 weeks. In addition, the volatile oil and its active principle, thymoquinone has shown to inhibit arachidonic acid metabolism and eicosanoid generation in rat peritoneal leukocytes, through inhibition of cyclooxygenase activity (Houghton *et al*., 1995).

1.6. Bitter cumin (*Centratherum anthelminticum* (L.) Kuntze)

1.6.1. Taxonomy

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Asterales
Family	Asteraceae (Compositae)
Genus	Centratherum
Species	*Centratherum anthelminticum*

TABLE 8. Trivial names of Bitter cumin

Language	Trivial name
English	Stone wart
Sanskrit	Somaraji
Hindi	Somraj,bukshi
Gujarathi	Kali jiri
Tamil	Kattu-shiragam
Telugu	Adavi jilakara
Kannada	Kadu-jirage
Malayalam	Kattu-jirakam

FIGURE 4. Bitter Cumin (*Centratherum anthelminticum*, Syn. *Vernonia anthelmintica*) seeds

FIGURE 5. Herbarium of *Centratherum anthelminticum* (Courtesy NISCAIR, New Delhi)

Bitter cumin *(Centratherum anthelminticum* (L.) Kuntze) is a tall robust, annual plant distributed throughout India. It belongs to the family Asteraceae (Compositae). It is commonly known as Somraj and its seeds are known as Kalijiri in Hindi as the seeds are black in colour. It has a bitter taste. It should be noted that *Nigella sativa* (Ranunculaceae), *Bunium persicum* Boiss, (Umbelliferae) and *Centratherum anthelminticum* (L.) Kuntze (Compositae) are all called as kalajiri or black cumin in India. There is a lot of ambiguity in local vernacular of black cumin as the above three species belong to different families but all are called black cumin or kalajiri.

Bitter cumin plant is distributed throughout India up to an altitude of 5500 ft. It is reported to be a medicinally important plant (Dey, 1980). The leaves are 5-9 cm in length and 2.5-3.2 cm in breath. The leaves are lanceolate or elliptic lanceolate in shape, acute tip, serrate margins, more or less pubescent (hairy) on both sides, base tapering into the petiole. Heads (type of inflorescence) 1.2-2 cm diameter, subcorymbose (more or less flat at the top), many (about

40) flowered, with a linear bract (leaf-like structure) near the top of the peduncle (inflorescence axis). Outer involucrar (structures protecting flower or inflorescence bud) bracts are linear, hairy, herbaceous, shorter than those of the inner rows; intermediate bracts with herbaceous hairy tips, linear, acute or subobtuse, often constricted at the base of the herbaceous part, equalling or shorter (rarely longer) than the inner most; innermost bracts usually the longest are linear, subacute, scarious (thin, membranous and dry), often tipped with purple colour. The pappus (a hairy and feather-like modified calyx) are reddish, the exterior row are very short, persistent, the inner ones somewhat flattened, deciduous (easily fall off), much shorter than the glabrous (hairless and smooth) corollas (petals). The achenous (small, dry, indehiscent, one seeded) fruits are 4.5-6 mm long, oblong cylindric, 10 ribbed and pubescent (hairy).

1.6.2. Ethnobotany

Bitter cumin seeds are not commonly used in food preparations due its bitter taste. But it has a wide variety of applications in traditional medicine, especially for the treatment of fever, cough, diarrhoea and also used as a general tonic. The seeds are reported to possess febrifugal, alterative, antihelmenthic, antiulcer, antiphlegmatic, cardiac, diuretic and digestive properties (Kirtikar and Basu, 1984). The seeds have a hot, sharp, bitter taste, acrid, astringent to the bowels. In Ayurvedic medicine it is used to treat skin diseases such as leucoderma. In yunani system of medicine the seeds are used as purgative and for treatment of asthma, hiccough, inflammatory swellings and itching of the eyes. The seeds are also an ingredient of a herbal mixture prescribed for snake-bites (Charaka and Sushruta) and scorpion sting (Charaka, Sushrutha, Vaghbhata, Vrindamadhava, Chakradatta). On the Malabar cost of India, an infusion of the bitter cumin seeds is given to cure coughs and flatulency. In Punjab, it is considered as a febrifuge. The seeds are used instead of quinine for treatment of malaria by the Munds of Chota Nagpur. Powdered seeds are applied externally to treat paralysis of the legs. In Travancore (South Kerala), the seeds ground into a paste along with lime juice, is applied to remove pediculi. The seeds are also reported to have tonic, stomachic and diuretic properties. The seed powder along with castor oil is

consumed to remove round worms. The juice of the leaf is given to cure phlegmatic discharges from the nostrils. In Sri Lanka, the plant is used to cure fever and convulsions. In Assam, powder of bitter cumin seeds are mixed with *Sesamum indicum* and orally taken to cure Leprosy (Saikia *et al*., 2006).

1.6.3. Review of Literature

Bitter cumin seeds are mainly used in traditional medicine and Ayurvedic preparations. They are traditionally used as antifilarial agents against bovine filarial worms (Singhal *et al*., 1992). Bitter cumin seed extracts are reported to have post-coital and anti-implantation activity in rats (Sharma *et al*., 1994). The antimicrobial activity of bitter cumin seed was reported against several human pathogenic bacteria and fungi (Sharma and Mehta, 1991). Bitter principle, novel steroids and antifilarial activity of *C. anthelminticum* seeds have been reported (Asaka *et al*., 1977; Mehta *et al*., 2005; Singhal *et al*.,1992). Methanolic extracts of *Centratherum anthelminticum* at 3.0 mg/mL showed macrofilaricidal activity against adult *Setaria digitata*, the cattle filarial worm (Nisha *et al*., 2007).

1.7. Objectives and scope of the present investigation

Bitter cumin (*Centratherum anthelminticum* (L) Kuntze) or Somraj in Sanskrit or Kalijiri in Hindi is a medicinally important plant used in traditional medicine. Bitter cumin is different from cumin (*Cuminum cyminum*), black cumin (*Nigella sativa*) and Caraway (*Carum carvi*), which are commonly consumed as spices in food preparations. Bitter cumin has a wide variety of applications in traditional medicine, especially for the treatment of fever, cough, diarrhoea and as a general tonic. It has been reported to possess febrifugal, alterative, antifertility, antimicrobial antihelminthic, antiphlegmatic, cardiac, diuretic and digestive properties. However, the bioactive phytochemicals responsible for the medicinal properties of bitter cumin is not reported. The present study was undertaken to investigate the phytochemicals and biological properties of bitter cumin (*Centratherum anthelminticum*) seeds. The major objectives of the present investigation are

1) Isolation and characterization of bioactive phytochemicals from bitter cumin (*Centratherum anthelminticum* (L.) Kuntze) seeds.

2) To study the biological effects of bitter cumin (*Centratherum anthelminticum* (L.) Kuntze) phytochemicals on antioxidant, antidiabetic and antimicrobial properties in suitable model systems.

Chapter II

Isolation and characterization of bioactive phytochemicals from bitter cumin seeds

2.1. Introduction

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. There are more than thousand known phytochemicals. Some of the bioactive phytochemicals are phenolic compounds including phenolic acids and flavonoids. Phenolic compounds are the most ubiquitous group of plant secondary metabolites distributed in various dietary and medicinal plants. Phenolic compounds have many diverse functions in plants viz., antimicrobial function, antifungal function, insect feeding deterrence, screening from damage by solar UV radiation, chelation of toxic heavy metals and antioxidant protection from free radicals generated during the photosynthetic process (Gould and Lister 2006). Epidemiological studies have shown that consumption of foods, rich in phenolic compounds can be correlated with reduced incidence of cardiovascular diseases (Criqui and Ringel, 1994). They retard the progression of arteriosclerosis by acting as antioxidants toward low density lipoproteins-LDL (Frankel *et al*., 1993) and neutralize the free radical mediated oxidative stress (Frankel *et al*., 1995; Meyer *et al*., 1998).

2.1.1. Phenolics

Phenolic compounds embrace a wide range of secondary metabolites that are synthesised from carbohydrates *via* the Shikimate pathway. Phenolics encompass approximately 8000 naturally occurring compounds all of which possess the common structural feature- a phenol.

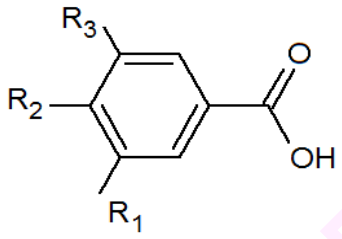
2.1.1.1. Classification of phenolic compounds

Current classification divides the broad category of phenolics into polyphenols and simple phenols based solely on the number of phenol sub units present (Clifford, 1999). Phenolic acids are phenols that possess one carboxylic acid functionality. Polyphenols possessing at least two phenol subunits include the flavonoids and those compounds possessing three or more phenol subunits are known as tannins.

2.1.1.1.1. Phenolic acids

Naturally occurring phenolic acids contain two distinguishing constitutive carbon frame works: hydroxy benzoic and the hydroxy cinnamic structures. Although the basic skeleton remains the same, the number and positions of the hydroxyl groups on the aromatic ring create the variety.

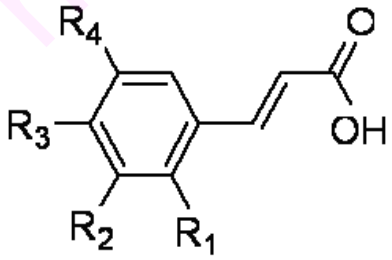
2.1.1.1.1.1. Benzoic acid derivatives



Gallic acid R_1=R_2=R_3=OH, Protocatechuic Acid R_1=H, R_2= R_3=OH

Vanillic acid R_1=H, R_2=OH, R_3=OCH_3, Syringic acid R_2=OH, R_1= R_3=OCH_3

2.1.1.1.1.2. Cinnamic acid derivatives



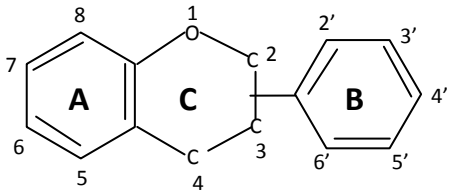
Ferulic acid R_1=R_2=H, R_3=OH, R_4=OCH_3, p-Coumaric acid R_1=R_2=R_4=H, R_3=OH

O-Coumaric acid R_2=R_3=R_4=H, R_1=OH, Caffeic acid R_1=R_2=H, R_3=R_4=OH

Sinapic acid R_1=H, R_3=OH, R_2=R_4=OCH_3

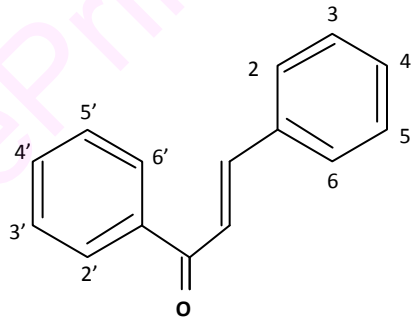
2.1.1.1.2. Flavonoids

Flavonoids are polyphenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain (C_6 -C_3 -C_6 system). Many flavonoids are easily recognized as flower pigments in most flowering plant families.



The chemical structure of flavonoids are based on a C-15 skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3 or 4. Sometimes, the six-member heterocyclic ring C occurs in an isomeric open form or is replaced by a five-member ring. Various subgroups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification. There are 6 major subgroups of flavonoids, they are:

2.1.1.1.2.1. Chalcones



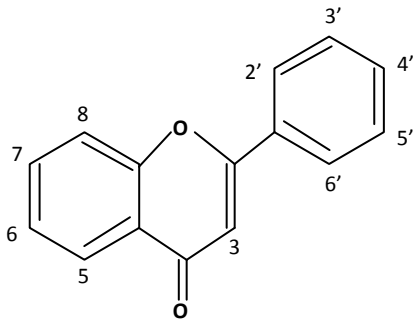
Butein 2'=4'=3=4=OH.

Okanin 2'=3'=4'=3=4=OH,

Licochalcone 4'=3=4=OH, 2=OCH_3

Chalconarigenin 2'=4'=6'=4=OH

2.1.1.1.2.2. Flavone



Apigenin 5=7=4'=OH,

Luteolin 5=7=3'=4'=OH

Diosmetin 5=7=3'=OH, 4'=OCH_3,

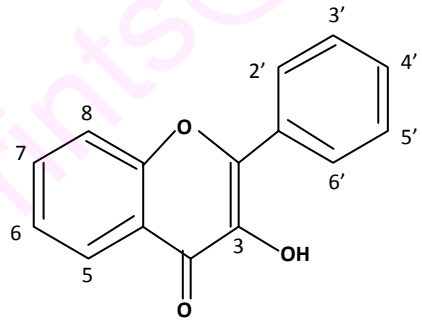
Tricin 5=7=4'=OH, 3'=5'=OCH_3

Sinenstein 5=6=7=3'=4'=OCH_3,

Nobelitin 5=6=7=8=3'=4'=OCH_3

Isovitexin 5=7=4'=OH, 6=Glucose

2.1.1.1.2.3. Flavonol



Fisetin 7=3'=4'=OH,

Kaempferol 5=7=4'=OH

Morin 5=7=2'=4'=OH,

Herbacetin 5=7=8=4'=OH

Quercetin 5=7=3'=4'=OH,

Robenetin 7=3'=4'=5'=OH

Isorhamnetin 5=7=4'=OH, 3'=OCH3,

Myricetin 5=7=3'=4'=5'=OH

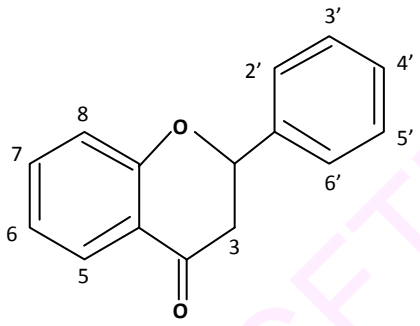
Gossypetin 5=7=8=3'=4'=OH

2.1.1.1.2.4. Flavonol glycosides

Rutin – Quercetin 3-O- rhamnosylglucoside,

Hyperin quercetin 3-O-β-D-galactopyranoside

2.1.1.1.2.5. Flavanone



Naringenin 5=7=4'=OH, Isosakuranetin 5=7=OH, 4'=OCH3

Friodictyol 5=7=3'=4'=OH, Hesperitin5=7=3'=OH, 4'=OCH3

2.1.1.1.2.6. Flavanone glycosides

Prunin – Narigenin 7-glucoside, Naringin – Narigenin 7-neohespiridoside

Narirutin - Narigenin 7- rutinoside, Hesperidin – Hesperitin 7- rutinoside

2.1.1.2. Identification and characterization of phenolic compounds

Although, the number of identified phenolic compounds are increasing exponentially, the phenolic content of most plants constitutes a complex mixture, the chemical nature of which has not yet been completely elucidated for many plant species. Isolation and structural elucidation of these phytochemicals are the initial steps to understand their physiological and biological action.

Most of the phenolic acid derivatives present in the plant matrix are stored in vacuoles. The commonly used solvents for extractions are water, methanol, ethanol, acetone and ethyl acetate. Extraction periods vary from 1 to 6 h using Soxhlet apparatus (Sun *et al*., 2001). Mechanical means are employed to enhance molecular interaction viz., vortexing (Montedoro *et al*., 1992), sonication, mechanical stirring and continuous rotary extraction (Guillen *et al*., 1996).

Hydrolysis of the ester bond to carboxylic acid has been one of the strategies employed to gain a more specific picture of the phenolic acid profile in plants. There are two main procedures to cleave the ester bond, namely acid hydrolysis and alkali hydrolysis or saponification. A third, less prevalent technique is cleavage through the use of enzymes (esterases). Although reaction time and temperature for the acid hydrolysis conditions vary a great deal, the general method involves extraction of plant material in aqueous or alcoholic solvents at reflux or above reflux temperatures for 30 min to over night at room temperature. Saponification involves extraction of the sample with 1-4 M NaOH. Enzymatic reactions have been reported to release phenolic acids (mainly ferulic and p-coumaric acids). Pectinases, cellulases and amylases are usually used for cleaving the carbohydrate linkages. Acid hydrolysis results in the cleavage of acetal or hemiacetal bonds between carbohydrate moieties and the hydroxyl groups of the aromatic ring and not by ester cleavage reactions.

Historically, the most common colorimetric method employed for the quantification of phenolic content was the Folin-Ciocalteu reagent (FCR). FCR involves the reduction of a phosphomolybdic-phosphotungstic acid to a blue coloured complex by phenols in alkaline solution. The blue coloured phosphomolybdic-phosphotungstic phenol complex generated in solution gives detectable absorbance at 760 nm (Singleton and Rossi, 1965). Gallic acid is used as a standard and the total phenol content is expressed as gallic acid equivalents (GAE). Identification of individual phenolic compound is not possible with this method.

For the last two decades, the analytical technique that has dominated the separation and characterization of phenolic compounds is HPLC with reverse phase (RP) column technology coupled with mass spectrum (MS). Many reviews have been published on separation of the flavonoids and phenolic acids (Robards and Antolovich, 1997; Robards *et al*., 1999; Escarpa and Gonzalez, 2001). Considering the diversity of stationary phases available, columns chosen for the determination of phenolic acids are exclusively composed of a C_{18} stationary phase with an internal diameter (i.d) ranging from 2.1 to 5 mm, particle size of 3 or 5 μM and column length of 100 to 300 mm. Investigations using mass spectrometry coupled to liquid chromatography report using columns with smaller i.d of 1.1 mm to 2.1 mm (Cartoni *et al*., 2000).

Although there is a large variation in percent composition of solvent systems, the types of solvents used for separation of phenolic acids remain fairly consistent. Gradient elution systems are more frequently employed than their isocratic counterparts. Linear gradients involving an aqueous phase and an organic phase are commonly used in the separation of phenolic compounds. Typically an acid is added to the aqueous and alcoholic phase (Delage *et al*., 1991). Acetic acid is the most commonly used acidic additive, but, sulphuric acid, perchloric acid, phosphoric acid (Spanos and Wrolstad, 1990), trifluoroacetic acid (Beveridge *et al*., 2000), hydrochloric acid (Delage *et al*., 1991) and formic acids are also being used (Salogoity-Auguste and Bertrand, 1984; Benassi and Cecchi, 1998; Spanos and Wrolstad, 1990). Methanol and acetonitrile are the predominantly used organic solvents with the acidic aqueous medium, but propanol, butanol, tetrahydrofuran and ethyl acetate are also employed in separation of phenolic acids. In certain investigations, the mobile phases (A and B) consist of mixtures of the organic and aqueous solvents. Borzillo *et al*., (2000) report solvent A as methanol-acetic acid-water(10:2:88, v/v/v) and solvent B as containing methanol-acetic acid-water (90:2:8, v/v/v). In a few methods, the mobile phases contain buffers such as H_3PO_4/KH_2PO_4 or acetic acid/ammonium acetate and sodium citrate buffer (pH 5.4). Run times for the methods vary a

great deal, ranging from 30 to 150 min and the column is kept at constant temperature for reproducible separation of phenolic acids.

Detection techniques for HPLC separation of phenolic compounds have been by UV-Visible with photodiode array (PDA) detector, with monitoring wavelengths from 190-380 nm. Phenolic acids with benzoic acid carbon framework have their λ_{max} in the 200-290 nm range. The only exception is gentisic acid, which has an absorbance that extends to 355 nm. The cinnamate derivatives, due to the additional conjugation, show an additional broad absorbance band from 270 to 360 nm. The single most common wavelength routinely used for monitoring phenolic compounds is at 254 nm.

Reverse phase-high performance liquid chromatography (RP-HPLC) represents the most popular and reliable technique for phenolic analysis. Compound elution typical of RP-HPLC is that polar compounds elute first, followed by those of decreased polarity (Nogata *et al*., 1994). Detection is usually based on absorption of UV or less commonly, visible radiation at various wavelengths, characteristic of the class of phenolic compounds (Bengoechea *et al*., 1995). Mass spectrometric (MS) determination of molecular weight of the individual components in the sample enables more precise identification of compounds and provides specific information on both qualitative and quantitative detection of phenolic compounds in high resolution chromatography (Angerosa *et al*., 1995).

Analytical methods for phenolic acids using LC (Liquid chromatography) coupled to MS (Mass spectrum) detection is an emerging field. Diverting a portion of the solvent allows for a small flow rate (11 μL/min) into the electron spray interface (ESI) and MS for ion generation and detection, respectively. The mass detection portion was carried out in the negative ion mode (i.e., [M-H]). High sensitivity with detection limits ranging from 1-6 ng can be detected with LC-MS-ESI technique. Retention time and mass $[M-H]^-$ confirm the identification of phenolic compounds. The interface between the liquid chromatography and the MS detector are carried out with atmospheric pressure ionization (API). After

determining the precursor ion in full scan, negative-ion mode, the product ions are determined using MS/MS.

In this chapter the data on proximate composition and fatty acid composition of bitter cumin seeds, total phenolic compounds, separation and quantification of individual phenolic compounds from bitter cumin seeds by HPLC-MS method are presented.

2.2. Materials and Methods

2.2.1. Chemicals

Standard fatty acid esters and phenolic compounds namely gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid quercetin and kaempferol were purchased from Sigma Chemical Co., MO, USA. Glucose, potassium persulfate, copper sulphate, boric acid and ammonium sulphate were purchased from Hi-media, Mumbai, India. Folin-Ciocalteu reagent was purchased from Sisco research laboratories, Mumbai, India. All other chemicals and solvents used were of analytical grade.

2.2.2. Bitter cumin seeds

Bitter cumin seeds (*Centratherum anthelminticum*) were obtained from local market, identified and authenticated by Dr. H.B. Singh, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India.

2.2.3. Garbling

The dirt and extraneous matter from the seeds were removed by hand.

2.2.4. Grinding

Grinding of the seeds was done in a mixer grinder (Kenstar).

2.2.5. Determination of proximate composition

2.2.5.1. Moisture

Moisture content of bitter cumin was determined according to AOAC method (2000). Sample (2 g) was weighed in an aluminium dish and placed in a hot air oven maintained at 130 ± 1 ºC for 4 h. The sample was cooled to room temperature in a desiccator and the loss in weight in percentage was reported as moisture content of the seeds.

2.2.5.2. Carbohydrate

Carbohydrate content of bitter cumin seeds was estimated by phenol-sulphuric acid method (Dubois *et al*., 1956). 0.5 mL of sample was mixed with 0.3 mL of 5% phenol to which 1.8 mL of concentrated sulphuric acid was added and mixed thoroughly. After 20 min of incubation at ambient temperature the absorbance was measured at 480 nm using a Shimadzu UV-Visible spectrophotometer (Model, 2100). Carbohydrate content was determined by referring the standard graph prepared using D-glucose as standard (0-50 μg/ 0.5 mL).

2.2.5.3. Protein

Protein content of bitter cumin seeds was estimated by micro-Kjeldahl method according to AOAC, 2000. The sample (1 g) was digested with concentrated H_2SO_4 (20 mL) in the presence of catalyst mixture (98 parts of potassium sulphate and 2 parts of copper sulphate) till the solution became clear. The contents of the flask were cooled and the volume was made up to 100 mL with water in a volumetric flask. 5 ml of the digested material was steam distilled in presence of 10 mL of 40% NaOH. The liberated ammonia was absorbed into a container containing 10 ml of 2% boric acid and a few drops of methyl red indicator. This solution was titrated with 0.01 N HCl, till it became bluish green. Simultaneously, a running blank was processed as above, with water in place of sample. Titre value of the blank was deducted from test values. Ammonium sulphate solution was used as the standard to estimate the amount of nitrogen content of the sample. The protein content was calculated in percentage using the factor 6.25.

2.2.5.4. Lipid

Total lipid content of bitter cumin was determined according to AOAC (2000) method. 10 g of the powdered bitter cumin seeds was packed in a thimble and extracted with 200 mL of hexane in a Soxhlet apparatus at 60 ºC for 16 h. The extract was transferred to a previously weighed, dry flat bottom flask and solvent

was evaporated over hot water bath. The flask was dried, cooled and final weight was taken. The fat content was expressed as g/100 g of sample (percentage).

2.2.5.5. Ash

Ash content of bitter cumin was determined by gravimetric method according to the procedure described in AOAC method (2000). 10 g of bitter cumin seeds was weighed in a clean silica crucible and heated in a muffle furnace for 5 h at 550 ºC and the crucible cooled in a desiccator. The weight of the ash was determined and expressed as percentage of original sample.

2.2.6. Determination of fatty acid composition

Fatty acid analysis of bitter cumin oil was carried out according to the method of Morisson and Smith (Morisson and Smith, 1964). 20 μL of bitter cumin oil was transmethylated using boron trifluoride-methanol and 0.5 M methanolic potassium hydroxide and the fatty acid methyl esters (FAME) formed were extracted into hexane. Fatty acid analysis was carried out in Shimadzu gas liquid chromatograph (GC-14B) fitted with 30 m x 0.3 mm fused silica capillary column (BP_{21}) with a flame ionization detector (FID) connected with a Clarity 420 integrator. The analysis was carried out using isothermal conditions. The column temperature was set at 220 ºC, injector temperature 230 ºC and the detector temperature of 240 ºC. Nitrogen was used as carrier gas with a flow rate of 1mL/min. Individual fatty acids were identified by comparison with retention time of authentic fatty acid standards and quantified by Clarity integrator.

2.2.7. Aqueous extract (AE) of bitter cumin seeds

10 g of defatted bitter cumin seed powder was extracted with 100 mL of distilled water by magnetic stirring overnight and the process was repeated thrice. The pooled extract was lyophilized and stored under refrigeration until use.

2.2.8. Aqueous methanol extract (AME) of bitter cumin seeds

100 g of defatted bitter cumin seed powder was soaked in 1 L of methanol: water (8:2, v/v) and kept at ambient temperature for 48 h with occasional shaking. The solvent was filtered with handmade filter paper and the procedure was repeated thrice. The pooled extract was concentrated and treated with 3 volumes of hexane and the aqueous phase was recovered. It was then phase separated with ethyl acetate to remove free phenolics and the aqueous phase was concentrated and dried. The dried extract was refrigerated until use.

2.2.9. Aqueous methanol acetone extract (AMAE) of bitter cumin seeds

10 g of the defatted seed powder was extracted with 100 mL of water, methanol and acetone (7:7:6, v/v/v) with constant stirring on a magnetic stirrer. The extraction was repeated thrice and filtered using filter paper. The pooled extract was centrifuged and concentrated to half volume in a rota vapour at ambient temperature. The concentrated extract was hydrolyzed with 2N HCl and treated with 3 volume of hexane to remove traces of fatty matter. The hydrolyzed phenolics were extracted into ethyl acetate in 1:1 volume and repeated thrice. The ethyl acetate phase was concentrated and dried. The dried extracts were stored under refrigeration until use.

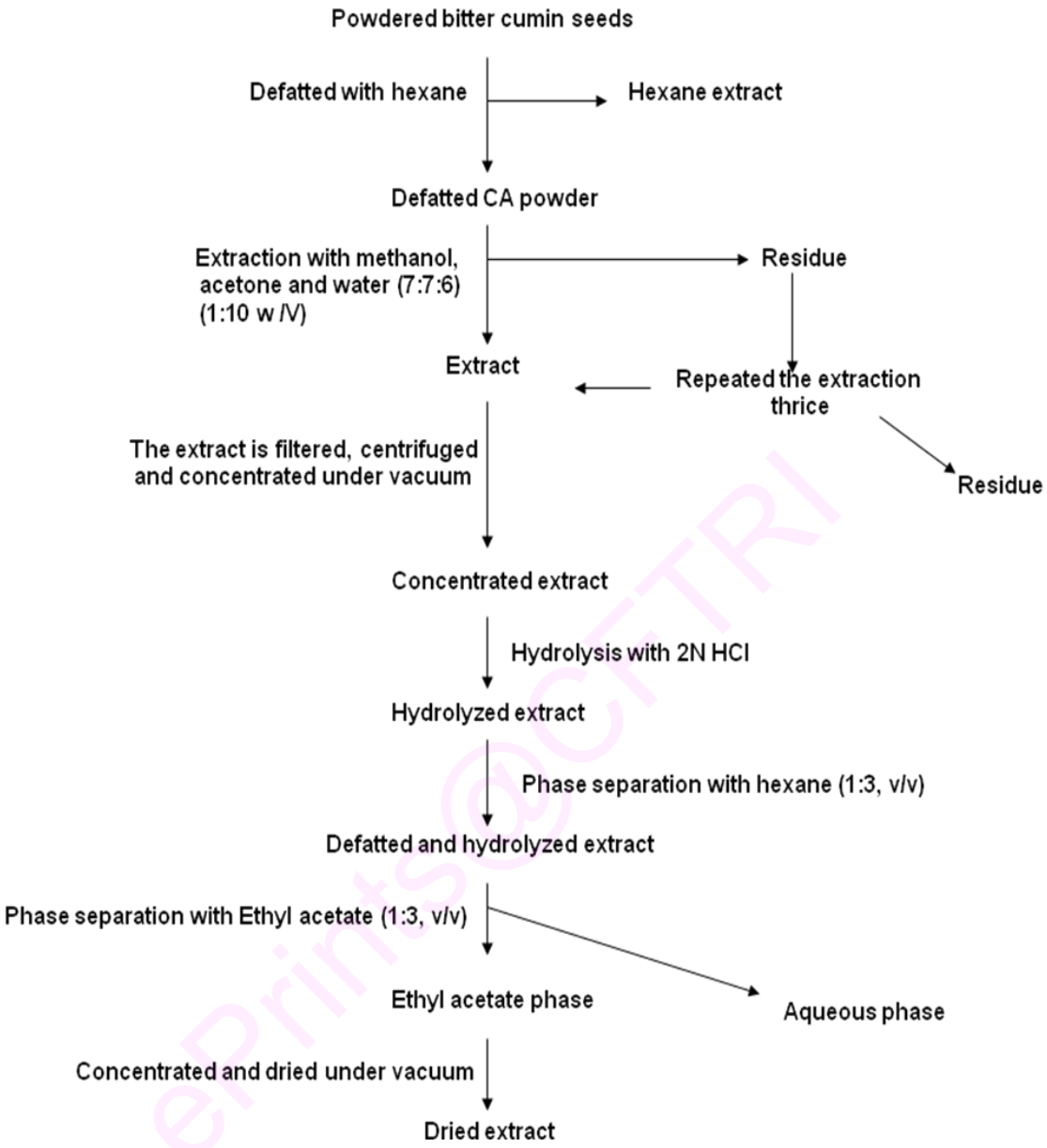


FIGURE 1. Flow chart showing the extraction and hydrolysis of phenolic compounds from bitter cumin seeds.

2.2.10. Estimation of total phenol content

Total phenol content of bitter cumin extracts were measured according to Folin-Dennis method described in Singleton and Rossie (1965). 50 μg of bitter cumin extract was dissolved in 0.5 mL of methanol and the sample was incubated with 2.5 mL of 10% Folin and Ciocalteu's (FC) phenol reagent for 2 min at room temperature. To this 2 mL of 7.5% Na_2CO_3 was added and incubated for 1 h at ambient temperature. The absorbance was measured at 765 nm against a blank developed with 0.5 mL of solvent using a Shimadzu UV-Visible spectrophotometer. Gallic acid and tannic acid were used as standards. Total phenol content in the extract was expressed as gallic acid equivalents (GAE) and tannic acid equivalents (TAE).

2.2.11. Separation and identification of phenolic compounds of bitter cumin extract by HPLC and LC-MS methods

2.2.11.1. HPLC

Phenolic compounds in aqueous methanol acetone extract of bitter cumin (AMAEBC) was dissolved in methanol and subjected to HPLC for qualitative and quantitative analysis. HPLC system (Shimadzu LC-10 A, Japan) equipped with dual pump LC-10AT binary system, UV detector SPD-10A, Phenomenex Luna reverse phase C_{18} column (i.d. 4.6 mm × 250 mm) and data was integrated by Shimadzu Class VP series software. The mobile phase consisted of (A) 1% acetic acid in water and (B) acetonitrile. The gradient programme for HPLC was 18% B at 0 min, 32% at 15.0 min and finally to 50% at 40.0 min. The amount of phenolic compounds (μg/g dry weight of seed powder) was calculated by comparison of peak areas (254 nm) of the individual phenolic compounds with that of standards. Known quantities of phenolic compound standards such as caffeic acid, ellagic acid, ferulic acid, gallic acid, protocatechuic acid, quercetin and kaempferol were used for identification and quantification of phenolic compounds present in the extract of bitter cumin.

2.2.11.2. HPLC-ESI-MS

An API 200 triple quadrupole mass spectrometer (Applied Biosystems) was used for determining the mass of the phenolic compounds. Analysis were performed on a Turbo ions spray source in negative mode by using settings nebuliser gas 16 (N_2) (arbitrary units), focusing potential −400 V, entrance potential −10, declustering potential (DP) 25–60 and collision energy (CE) 15–35. Full scan acquisition was performed by scanning from *m*/*z* 150–700 u at a cycle time of 2 s. MS product ions were produced by collision-associated dissociation (CAD) of the selected precursor ions in collision cell. In all the experiments, both the quadrupoles (Q_1 and Q_3) were operated at unit resolution. Product ion scan of selected molecules were carried out in order to confirm the structure of the compounds.

2.2.12. Statistical analysis

Statistical analysis was done using the software SPSS (Release 7.5.1). The differences in mean values were tested using one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used to determine the significant differences amongst the test materials. Differences were considered to be significant at $P \leq 0.05$.

2.3. Results

Bitter cumin seed powder or defatted bitter cumin seed powder was used for determining the proximate composition and profile of phenolic compounds. The phenolic compounds in AMAEBC were separated and quantified by Reverse phase HPLC coupled with Mass spectrometry.

2.3.1. Proximate composition of bitter cumin seeds

The proximate composition of bitter cumin seeds is presented in Table 1. The carbohydrate content was 14.7%, protein content was 22.5%, lipid content was 21.4%, ash content was 7.2% and fiber content was 29.3%. Bitter cumin seeds are good source of fiber, protein and fat.

TABLE 1. Proximate composition of bitter cumin seeds on dry weight basis (g/100g)

Parameter	Amount (%)
Moisture	4.9 ± 0.10
Carbohydrate	14.7 ± 0.96
Protein	22.5 ± 0.06
Lipid	21.4 ± 2.00
Ash	7.2 ± 0.47
Fiber	29.3 +3.56

Values are mean ± SEM of three estimation

2.3.2. Fatty acid composition of bitter cumin seeds

The fatty acid composition of cumin oil is presented in Table 2. In bitter cumin oil polyunsaturated fatty acids account for 54.3%, saturated fatty acids represent 22.3% and monounsaturated fatty acids account for 14.8% of total fatty acids. The fatty acids in bitter cumin oil was found to be 15.4% of palmitic acid (C16:0), 2.0% of palmitoleic acid (C16:1), 6.9% of stearic acid (C 18:0), 12.8% oleic acid (C 18:1), 49.6% of linoleic acid (C 18:2) and 4.7% linolenic acid (C 18:3). Among the fatty acids linoleic acid was the major fatty acid followed by palmitic acid and oleic acid.

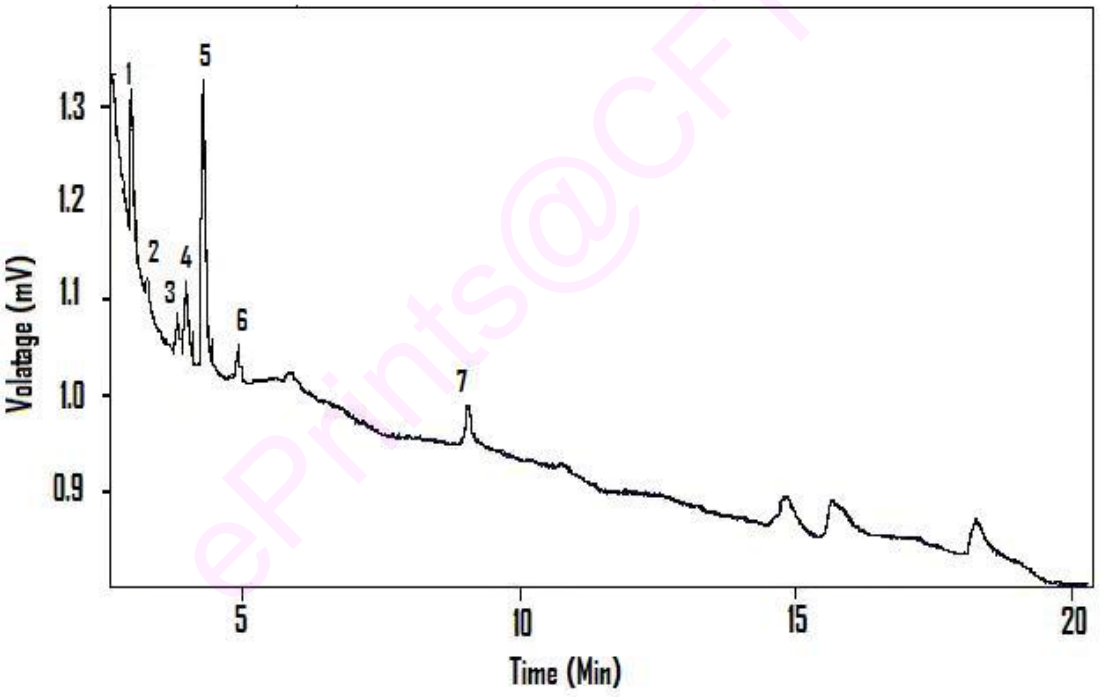


FIGURE 2. Gas chromatogram showing the retention time of fatty acids in bitter cumin seed oil.

(1-Palmitic acid, 2- Palmitoleic acid, 3- Stearic acid, 4- Oleic acid, 5-Linoleic acid, 6- Linolenic acid, 7- Unknown)

TABLE 2. Fatty acid composition of bitter cumin seed oil

Fatty acid		Retention time (min)	Percentage
C16:0	Palmitic acid	2.99	15.4
C16:1	Palmitoleic acid	3.29	2.0
C18:0	Stearic acid	3.80	6.9
C18:1	Oleic acid	3.96	12.8
C18:2	Linoleic acid	4.27	49.6
C18:3	α- Linolenic acid	4.90	4.7
Unknown		9.05	8.6
SFA			22.3
MUFA			14.8
PUFA			54.3

2.3.3. Total phenol content

Bitter cumin seeds were extracted with aqueous (AE), aqueous methanol-acetone (AMAE) and 80% aqueous methanol (AME) separately. The total phenol content in these extracts was determined by Folin-Ciocalteu method and the values were expressed in gallic acid equivalents (GAE) and tannic acid equivalents (TAE) (Figure 3). Significant variation in the phenolic content was observed in different extracts of bitter cumin. Aqueous methanol acetone extract (AMAE) showed highest total phenol content of 551.8 ± 30.8 μg GAE/mg or 840.8 ± 46.9 μg TAE/mg. Aqueous extract (AE) showed lowest total phenolic content of 29.2 ± 1.0 μg GAE/mg or 48.6 ± 1.6 μg TAE/mg. However, 80% aqueous methanol extracted total phenols of 116.87 ± 5.45 μg GAE/mg or 183.52 ± 8.55 μg TAE/mg. AMAE extracted 18.9 fold higher total phenols compared to aqueous extract. The yield of aqueous extract was 8.4 ± 0.65 g/100

g defatted powder or 6.6 ± 0.51 g/100 g seeds. The yield of aqueous methanol extract was 4.1 ± 0.4 g/100g defatted seeds or 3.3 ± 0.3 g/100 g seeds.

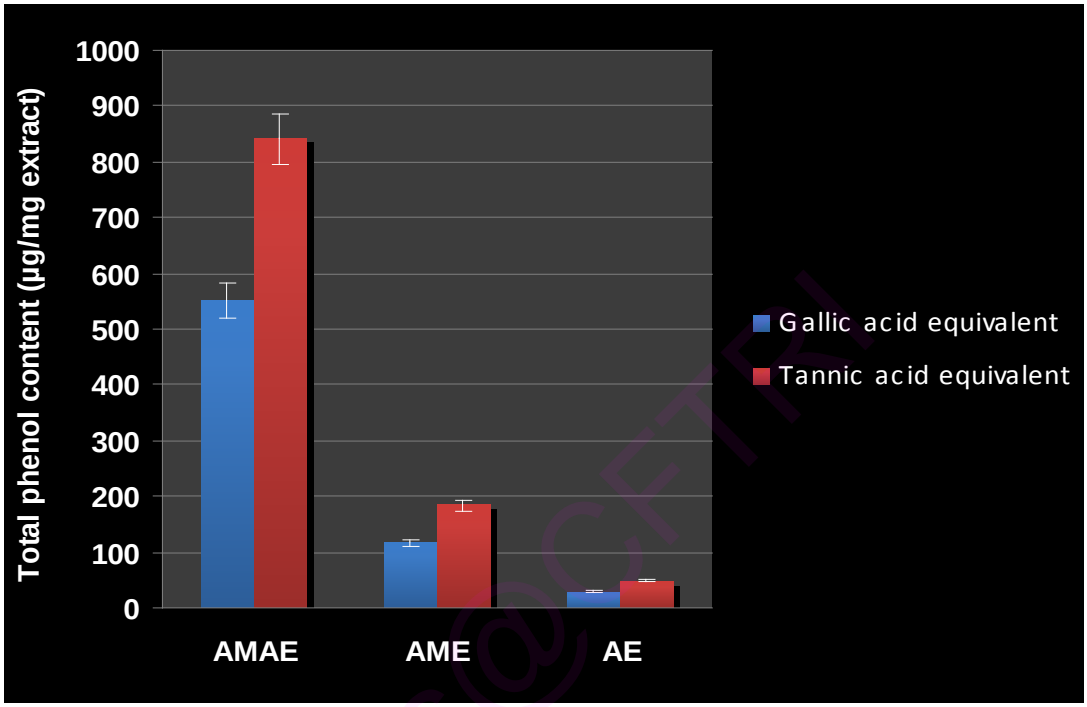


FIGURE 3. Total phenol content of different extracts of bitter cumin.

(AMAE- Aqueous methanol-acetone extract of bitter cumin, AME- Aqueous methanol extract of bitter cumin, AE - Aqueous extract of bitter cumin). Values are mean ± SEM of three estimation

The yield of aqueous methanol acetone extract was 3.4 ± 0.4 g/100 g defatted powder or 2.7 ± 0.35 g/100 g seeds. The yield did not show much difference between aqueous methanol-acetone and aqueous methanol extracts. The recovery of aqueous extract was highest compared to other two extracts. However, the total phenol content was lowest in aqueous extract. Hence aqueous methanol-acetone extract with highest total phenol content was used for further studies such as characterization of phenolic compounds, antioxidant, antihyperglycemic and antimicrobial properties of bitter cumin.

2.3.4. Isolation and identification of phenolic compounds by HPLC and LC-MS

Most phenolic compounds are present normally as glycosides in plants. Hence bitter cumin AMAE was subjected to acid hydrolysis (with 2N HCl) to break the glycoside linkages and the free phenolic compounds in hydrolysate were separated, identified and quantified by LC-MS. HPLC profile of phenolic compounds is shown in Figure 4.

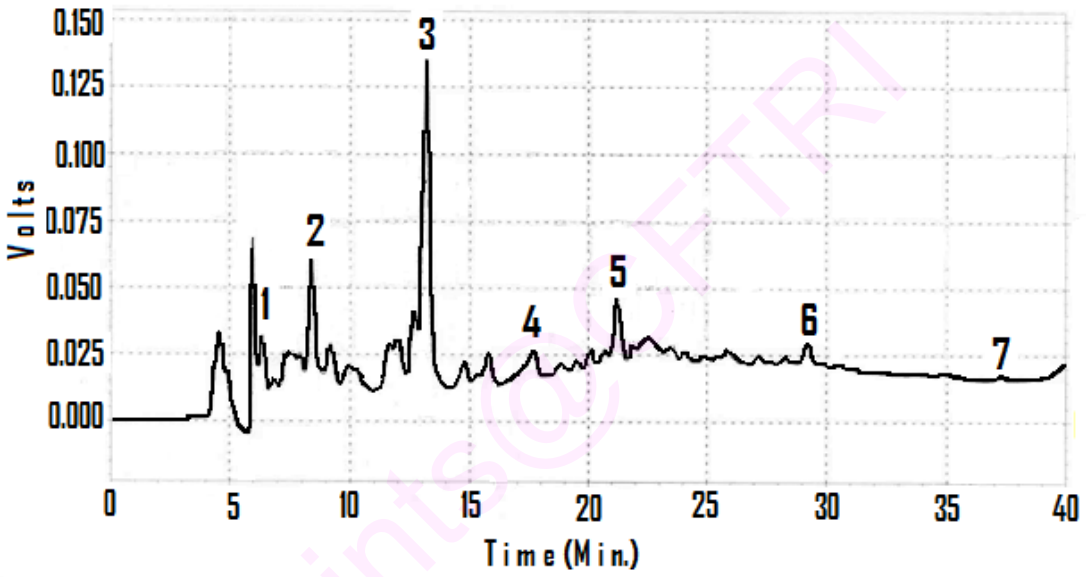


FIGURE 4. HPLC chromatogram of AMAEBC.

The peaks identified are 1) Gallic Acid, 2) Protocatechuic Acid, 3) Caffeic Acid, 4) Ellagic Acid, 5) Ferulic Acid, 6) Quercetin, 7) Kaempferol

The identification and quantification of individual phenolic compound was achieved by comparing retention time and the peak area of AMAE compounds with that of standards. Interestingly, bitter cumin contained a mixture of phenolic acids including gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid and also flavonols such as quercetin and kaempferol (Table 3). The phenolic acids in bitter cumin extract was 500.9 μg/g of caffeic acid, 375.50 μg/g of ferulic

acid, 173.5 μg/g of gallic acid, 150.10 μg/g of ellagic acid and 130.5 μg/g of protocatechuic acid. The flavonoid content was 154.60 μg/g of quercetin and 95.7 μg/g of kaempferol. Caffeic and ferulic acids were the major phenolic acids and quercetin was the major flavonoid among phenolic compounds present in bitter cumin. The concentration of various phenolic compounds identified from AMAE of bitter cumin was in the decreasing order of caffeic acid> ferulic acid> gallic acid > quercetin > ellagic acid > protocatechuic acid > kaempferol.

TABLE 3. Phenolic compounds in bitter cumin seeds

Phenolic Compound	Concentration (μg/g of seed powder)
Caffeic acid	500.90
Ferulic acid	375.50
Gallic Acid	173.50
Ellagic acid	150.10
Protocatechuic acid	130.50
Quercetin	154.60
Kaempferol	94.70

The structure of phenolic compounds were further confirmed by LC-MS. LC-MS characteristics of identified phenolic compounds are given in Table 4 and Fig.6

TABLE 4. HPLC retention time and fragments of phenolic compounds identified from AMAE of bitter cumin through LC-MS

Retention time	$[M-H]^-$	Fragmented ion	Corresponding fragment	Compound
6.37	169	125	$M-COO^-$	Gallic acid
8.41	153	109	$M-COO^-$	Protocatechuic acid
13.47	179	135	$M-COO^-$	Caffeic acid
17.66	300.8	170	M-125	Ellagic acid
		125	Trihydroxy benzene fragment	
21.24	193	178	$M-O^-$	Ferulic Acid
		149	$M-COO^-$	
29.21	301.1	151	M- Free phenol at 2 position and a portion of the benzopyranone ring moiety	Quercetin
37.31	285	133	M-151	Kaempferol
		151	Free phenol at position 2 and a portion of the benzopyranone part	

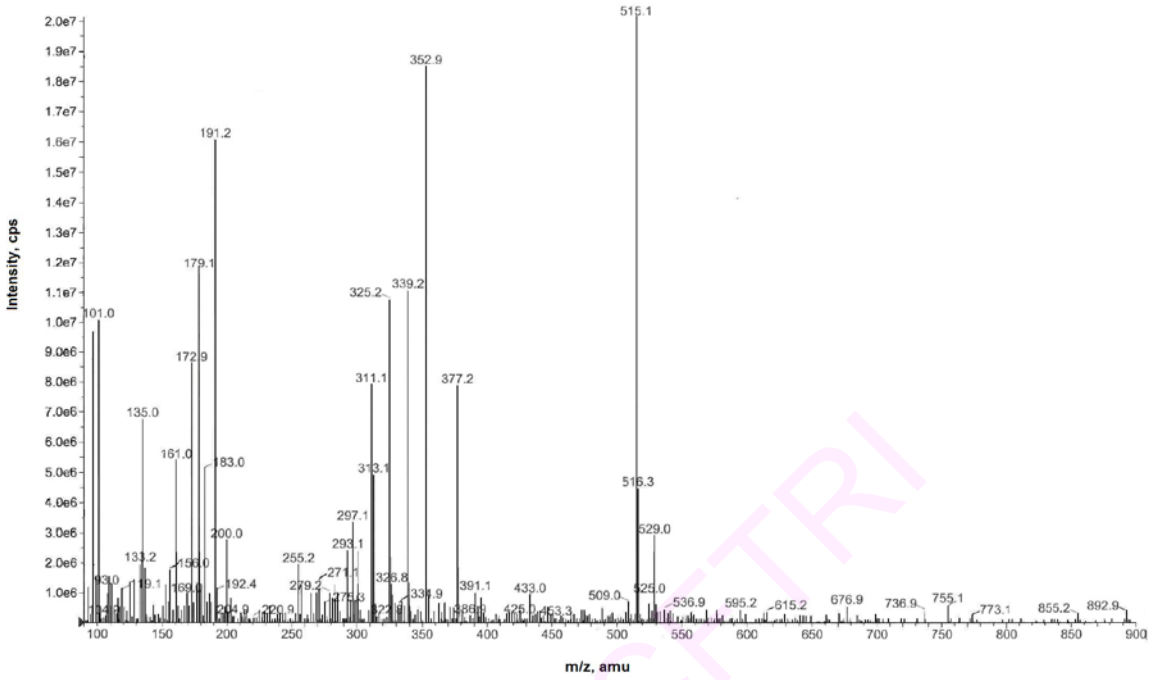


FIGURE 5. Mass spectra of phenolic compounds present in AMAEBC

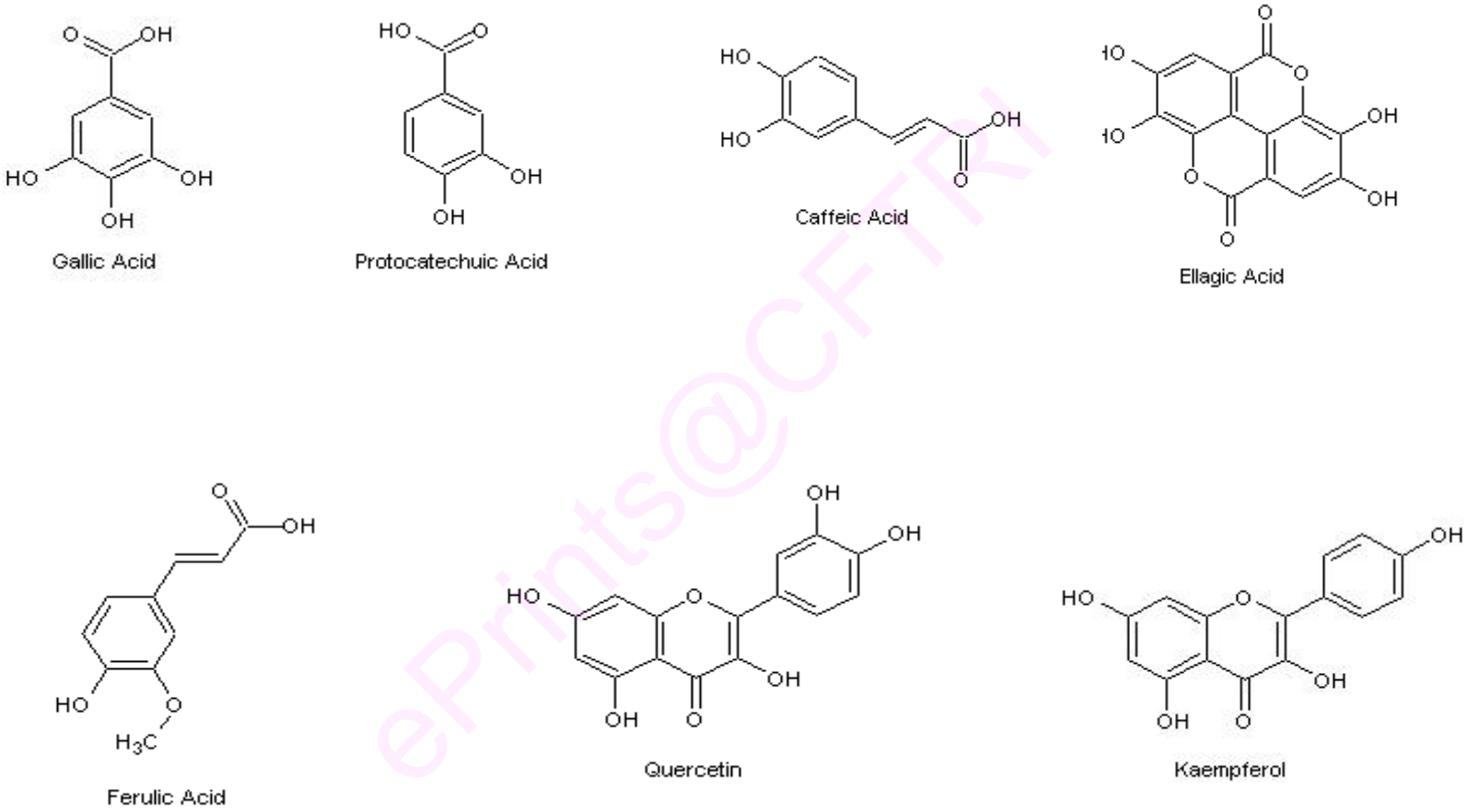


FIGURE 6. Chemical structure of phenolic compounds identified from bitter cumin seeds.

2.4. Discussion

Bitter cumin, which is black in colour, is commonly known as 'Kalijira' in Hindi. The seeds have a bitter taste, acrid, astringent to the bowls, antihelmintic. It is used to cure ulcers, skin diseases, leucoderma and fevers (Ayurveda). The crude extract of the seeds of *Vernonia anthelmintica* Willd is used in traditional Chinese medicine as vermicide and for treatment of vitilago by Uygurs for several centuries (Cheng *et al*., 1987).

Proximate composition, fatty acid composition, total phenols and individual phenolic compounds were estimated to understand phytochemical profile of bitter cumin seeds. Bitter cumin contained 4.9% moisture, 14.7% carbohydrate, 22.5% protein, 21.4% fat, 7.2% ash and 29.3% of fibre. A comparative account of proximate composition of bitter cumin, black cumin and cumin seeds were presented in (Table 5). Bitter cumin seeds were rich in fiber, protein and fat content. Black cumin seeds (*Nigella sativa*) are reported to be rich in carbohydrate, protein and fat content (Takruri and Dameh, 1998). Cumin seeds (*Cuminum cyminum*) have high carbohydrate, protein and fat content.

TABLE 5. A comparative table on proximate composition of cumin varieties (g/100 g)

Parameter	Bitter cumin *(Centratherum anthelminticum)*	Black cumin *(Nigella sativa)*	Cumin *(Cuminum cyminum)*	Caraway (Carum carvi)
Moisture	4.9	11.9	3.8	4.2
Carbohydrate	14.7	36.6	24.1	50.1
Protein	22.5	18.7	23.1	7.6
Lipid	21.4	15.0	36.7	8.7
Ash	7.2	5.8	4.8	4.1
Fiber	29.3	12.0	7.5	25.3

The fatty acid profile of bitter cumin seed oil was estimated by gas chromatography. The major fatty acids found in bitter cumin seed oil were palmitic acid (C16:0) and stearic acid (C18:0) as saturated fatty acids and oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) as the main unsaturated fatty acids. Among the fatty acids linoleic acid was the major fatty acid followed by palmitic acid and oleic acid.

Several flavonoids including 2', 3, 4, 4'-tetrahydroxychalcone, 5, 6, 7, 4'-tetra-hydroxy flavone and butin, were reported from the seeds of bitter cumin *Vernonia anthelmintica* Willd (Tian *et al*., 2004). Bitter cumin seeds were reported to be rich in epoxy acids and contain 60.2% epoxides as epoxyoleic acids (Morris *et al*., 1961). Steroids such as (24α /R)-Stigmasta-7-en-3-one, (24□α /R)-Stigmasta-7,9(11)-dien-3-one, (24α/ S)-Stigmasta-5, 22-dien-3 α-ol and (24□α /S)-Stigmasta-7, 22-dien-3 α-ol were reported from bitter cumin seeds (Mehta *et al*., 2005). A new glycosylated triterpene 3-O-[α-D-glucopyranosyl-(1→3)- α-L-rhamnopyranosyl-(1→2)- α-L-arabinopyranosyl]- 28 -O- [α-D-glucuronopyranosyl-(1→4)- α-L-rhamno pyranosyl -(1→3) - α-D-glucopyranosyl]-hederagenin was also reported from the seeds of bitter cumin (Mehta *et al*., 2004). Yadava and Barsainya (1997) reported a new flavone glycoside, 7-glucosyl (1→4) xyloside from *C. anthelminticum* seeds. Even though a number of steroids and triterpenes have been reported from bitter cumin, the biological activity of these compounds has not been studied in detail. The present study has focused on isolation and characterization of phenolic compounds from bitter cumin and their biological effects in terms of antioxidant, antidiabetic and antimicrobial activity.

Phenols, a major group of antioxidant phytochemicals, have profound importance due to their biological properties. Epidemiological studies have shown that consumption of food, rich in phenolic content can reduce the risk of heart disease by slowing the progression of atherosclerosis by acting as antioxidants towards low-density lipoprotein (LDL) (Kinsella *et al*., 1993). Traditional methods for the determination of the phenolic content relied on colorimetric measurement of total phenols using one of a number of reagents of varying selectivity. Folin-Ciocalteu reagent is the classic reagent

recommended for total phenols (Conde *et al*., 1997; Khokhar and Magnusdottir, 2002). Even though FC reagent is nonspecific to phenolic compounds, the total phenol estimation by FC reagent is convenient and has become a routine assay in studying phenolic antioxidants.

The total phenol content of bitter cumin showed wide variation with the solvents used for extraction. Often a combination of solvents will provide optimum recovery of all phenols or at least a limited range of phenols (Robards, 2003). With dried materials alcoholic solvents presumably rupture cell membranes and enhance the extraction of endocellular materials (Robards, 2003). Extraction method and solvent choice are generally critical and no single solvent will provide optimum recovery of all phenols or even a limited range of phenols. Aqueous mixture of methanol (Conde *et al*., 1997), ethanol (He *et al*., 1997) or acetone (Guyot *et al*., 1998) are often the solvents of choice for recovery of a wide range of phenols from diverse sample types including oats (Peterson *et al*., 2001), fruits and vegetables (Wang and Sporns, 2000; Lister *et al*., 1994) and spices (Areias *et al*., 2000). Hexane is generally employed for extracting non-polar components of a biological sample and it is widely employed in the extraction of lipids. Moreover preliminary cleanup of the sample by a non-polar solvent will give more exact recovery of polar compounds like phenolics. The present study employed water, methanol and acetone as solvents to extract the total phenolics from defatted bitter cumin seeds. Extractions with aqueous methanol and acetone (AMAE) (combination of three solvents) of bitter cumin seeds resulted in highest total phenol content (551.8 ± 30.8 μg GAE/mg) followed by 80% aqueous methanol (combination of two solvents) 116.87 ± 5.45μg GAE/mg, while aqueous medium extracted lowest total phenol content of 29.2 ± 1.0 μg GAE/mg indicating combination of aqueous and organic polar solvents extract more total phenols than aqueous medium alone.

Phenolics are a diverse class of secondary plant metabolites. All plant phenolic compounds share one common feature, namely an aromatic ring with at least one hydroxyl substitute, but may vary greatly in their complexity from simple phenols to the highly polymerized tannins and lignins. They occur

predominantly as conjugates with sugars (mono-, di-, or oligosaccharides), with glucuronic or galacturonic acids, or even with other phenols that are linked to hydroxyl groups or, less frequently, aromatic carbon atoms. In plants, phenolic compounds fulfil essential physiological purposes, such as protection from ultraviolet radiation, pathogens and predators, contribute to their colour and flavour and facilitate growth and reproduction (Bravo, 1998; Harborne and Williams, 2000; Heim *et al*., 2002). Bitter cumin seeds taste bitter and appear to function as deterrent to feeding by animals.

The phenolic compounds from bitter cumin seeds were extracted with aqueous 70% methanol and 70% acetone to facilitate extraction of both low and high molecular weight phenolic compounds. Thus, with the above solvent system the present investigation could extract a number of phenolic acids and flavonols from bitter cumin (Table 3). The total phenol content of this extract was estimated to be 551.8 ± 30.8 μg GAE/mg of extract.

Qualitative and quantitative analysis of individual phenolic compounds is difficult because most phenolic compounds are normally present as glycosides in plants. Hydrolysis of phenolic glycosides to their corresponding aglycones offers a practical method for the quantification of phenolic acids in foods. The rate of acid/base hydrolysis of glycosides depends on the acid/base strength, nature of the sugar moiety and its position in the phenolic acid nucleus. In this study a simple extraction protocol involving 70% methanol and 70% acetone was adopted for extraction of phenolic glycosides followed by a hydrolysis step with acid (2N HCl) as phenolic acids were analyzed as aglycones. Aqueous methanol acetone extract of bitter cumin (AMAEBC) was subjected to acid hydrolysis (2N HCl) for 30 min to break the glycoside linkages. The protocol adopted to extract phenolic compounds from bitter cumin seeds is presented in Figure 1. Further, the phenolic compounds in hydrolysate were separated, identified and quantified by LC-MS.

HPLC-MS is a fast and reliable method for structural analysis of non-volatile phenolic compounds, since better techniques (interfacing systems) have been developed for the removal of the liquid mobile phase before ionization (Careri

et al., 1998). Pietta *et al*., (1994) showed that thermospray liquid chromatography (LC) MS is an excellent technique for the analysis of flavonol glycosides from medicinal plants. Positive ion fast atom bombardment MS and tandem MS have been used to study the glycosidic linkages in diglycosyl flavonoids (Li and Claeys, 1994). HPLC electron spray ionisation (ESI)-MS offers advantages in terms of sensitivity and capacity for the analysis of large, thermally labile and highly polar compounds (Robards and Antolowich, 1997, Careri *et al*., 1998).

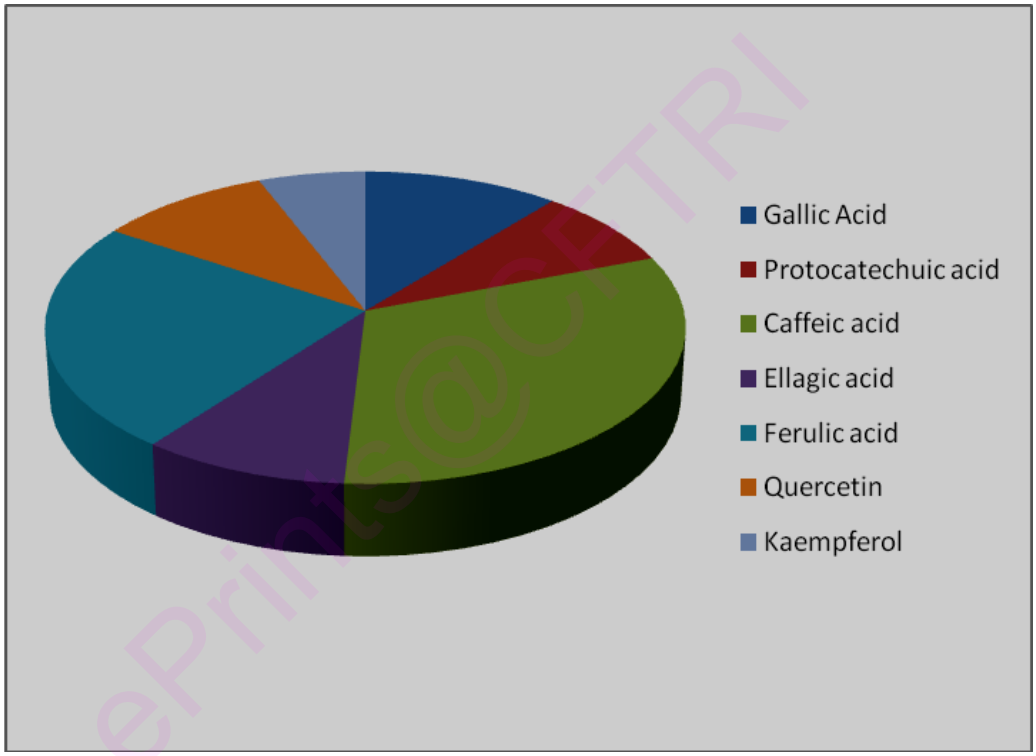


FIGURE 7. Pie Diagram showing different phenolic compounds present in bitter cumin seeds and their relative concentration.

The phenolic compounds in bitter cumin were separated and identified by reverse phase HPLC. The reverse phase C_{18} column combined with a gradient elution system of increasing hydrophobicity was chosen because all compounds of interest were successfully separated. The two solvents were used for gradient elution were 1% acetic acid in water as solvent A and acetonitrile as solvent B. The identification of the individual phenolic compounds was achieved by comparing retention time and the peak area of

cumin extracted compounds with that of reference standards. Interestingly, bitter cumin contained a number of phenolic acids including gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid and also flavonols such as quercetin and kaempferol (Figure 7). Caffeic and ferulic acids were found to be most abundant among phenolic acids present in bitter cumin seeds. Quercetin was found to be the main flavonol in bitter cumin (Figure 7). This is the first report showing the presence of an array of polyphenolic compounds in bitter cumin seeds.

In conclusion, bitter cumin seeds contained a mixture of important phenolic compounds such as gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid and also flavonols such as quercetin and kaempferol. These phenolic compounds are very good antioxidants and also possess antidiabetic and antimicrobial activities. Hence the other biological properties of these phenolic compounds were studied in detail in *in vitro* and *in vivo* model systems and presented in chapter III, IV and V.

Chapter III

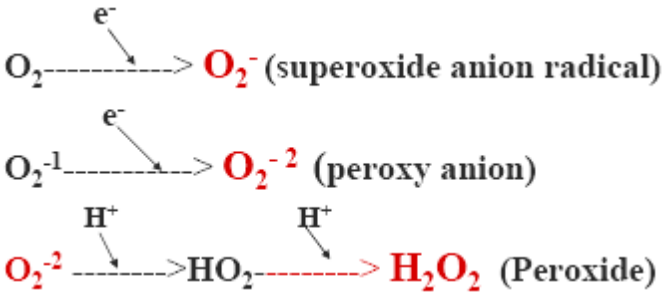
Studies on antioxidant activity of bitter cumin seeds

3.1. Introduction

3.1.1. Reactive oxygen Species (ROS)

Oxygen appeared in the Earths' atmosphere over 2.2 billion years ago, largely due to evolution of photosynthesis by cyanobacteria (Halliwell, 2006). All aerobes including plants, bacteria and human need oxygen for efficient production of energy. But these organisms suffer damage when exposed to oxygen concentrations higher than normal levels. Both plants and animals have evolved antioxidant defence to protect against oxygen toxicity. The toxicity of oxygen was unknown prior to the publication of Gershman's free radical theory of oxygen which states that, the oxygen toxicity is due to partially reduced forms of oxygen (Gerschman *et al*., 1954). The world of free radicals in biological systems was further explored by Denham Harman, who proposed the concept of free radicals' role in the ageing process (Harman, 1956). This work gradually triggered intense research into the field of free radicals in biological systems. A second epoch of the research of free radicals in biological systems was explored in 1969 when McCord and Fridovich discovered the enzyme superoxide dismutase (SOD) and thus provided convincing evidence about the importance of free radicals in living systems (McCord and Fridovich, 1969). A third *era* of free radicals in biological systems began in 1977 when Mittal and Murad (1977) provided evidence that the hydroxyl radical ($OH^{\bullet}$), stimulates activation of guanylate cyclase and formation of the "second messenger" cyclic guanosine monophosphate (cGMP). Since then, a large body of evidence has been accumulated suggesting that, living systems have not only adapted to a coexistence with free radicals, but have developed various mechanisms for the advantageous use of free radicals in various physiological functions.

Oxygen free radicals or reactive oxygen species (ROS), as well as reactive nitrogen species (RNS) are products of normal cellular metabolism. Some of the most relevant ROS are: hydroxyl radical ($OH^{\bullet}$) peroxyl radicals ($ROO^{\bullet}$), nitric oxide radical ($NO^{\bullet}$), superoxide anion radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^{\bullet}$), and hydrogen peroxide (H_2O_2).



There are various sources for specific ROS in human. However, the superoxide radical anion appears to play a central role, since other reactive intermediates are formed in reaction sequences starting with $O_2^{\bullet-}$. It is generated by one-electron reduction of O_2 from xanthine oxidase, NADPH oxidase, or by leakage of the respiratory chain. It has been estimated that about 1-3% of the O_2 we utilize is converted to $O_2^{\bullet-}$ (Fridovich, 1986; Halliwell, 1996).

The most reactive oxygen species, the hydroxyl radical is a very short lived radical of about 10^{-9} seconds as half life period. It is formed *in vivo* by cleavage of water on high-energy irradiation (e.g. X-rays) or from endogenous H_2O_2 in metal-catalysed Fenton reaction. U.V.light can cleave H_2O_2 to yield a molecule of hydroxyl radical. The high reactivity of this radical implies immediate reaction at the place where it is generated (Salem *et al*., 2000; Pastor *et al*. 2000).

The peroxyl radical (ROO') is relatively long lived (seconds) with a considerable diffusion path length in biological systems. It can be generated in the process of lipid peroxidation, which is initiated by the abstraction of an H atom from polyunsaturated fatty acids (PUFA); the hydroxyl radical is capable of starting this reaction sequence (Esterbauer *et al.,* 1992; Reaven and Witzum, 1996).

Singlet oxygen ($^1O_2$) is another non-radical ROS which is suggested to be formed *in vivo* in light exposed tissue. Single oxygen can interact with other molecules either by transferring its excitation energy or by combining chemically. Preferential targets for chemical reactions are double bonds; e.g. in PUFA or guanine in DNA bases (Kanofsky, 1989; Stahl and Sies, 1993; Cadet *et al.,* 1994).

H_2O_2 is a non-radical reactive species and can easily diffuse between living cells. H_2O_2 leads to the depletion of ATP, reduced GSH and NADPH. It induces a rise in cytosolic Ca^{2+} and activates polymerase which leads to cell death. Recent evidence suggests that H_2O_2 is involved in signal transduction regulating the expression of genes through the nuclear factor KB and apoprotein-1 pathways (Schreck and Baeuerle, 1994; Sen and Packer, 1996). It is efficiently converted to water by the enzyme catalase, a process which determines its half-life. However, H_2O_2 can cross cell membranes and react with copper and iron ions to form highly reactive and damaging species such as hydroxyl radicals and peroxynitrite radicals (Roberfroid and Calderon, 1995).

Another important ROS which has attracted attention within the past few years is the nitric oxide radical ($NO^{\cdot}$). It is a signalling compound formed enzymatically from arginine and relaxes smooth muscles in blood-vessel walls resulting in lowered blood pressure. It is also produced by activated macrophages contributing to the primary immune defence. An excess of $NO^{\bullet}$ is cytotoxic. It might react directly with bio molecules or combine with $O_2^{\bullet -}$ to form peroxynitrite ($ONOO^-$). Peroxynitrite radical is capable of inducing lipid peroxidation in lipoproteins and might also interfere with cellular signalling by nitrating tyrosine residues in proteins (Beckman, 1996).

In addition to the above radicals generated *in vivo,* the organisms are also exposed to ROS from external sources. An array of radicals present in cigarette smoke, vehicular smoke and industrial air pollution can also oxidize lipids (Pryor et al. 1995).

3.1.1.1. Implications of Reactive Oxygen Species (ROS)

The development and existence of an organism in the presence of O_2 is associated with the generation of reactive oxygen species (ROS), even under normal physiological conditions. However, over production of ROS/RNS are responsible for the oxidative damage of biological macromolecules such as carbohydrates, proteins and DNA (Halliwell and Gutteridge, 1989; Halliwell, 1996, Sies, 1997). ROS and RNS are well recognized to play a dual role as

both deleterious and beneficial species to living systems (Valko *et al*., 2006). The harmful effect of free radicals, causing potential biological damage is oxidative stress and nitrosative stress (Kovacic and Jacintho, 2001; Ridnour *et al*., 2005; Valko *et al*., 2001). This occurs in biological systems when there is an overproduction of on one side and or a deficiency of enzymatic and non-enzymatic antioxidants on the other. Oxidative stress has been implicated in a number of human diseases as well as in the ageing process.

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko *et al*., 2006). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge, 1999). It is also well known that metal induced hydroxyl radical can also attack other cellular components, involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Siems *et al*., 1995).

The peroxyl radicals (ROO•) generated during lipid peroxidation can be rearranged *via* a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product being malondialdehyde (MDA) (Fedtke *et al*., 1990; Fink *et al*., 1997; Mao *et al*., 1999; Marnett, 1999; Wang *et al*., 1996). The other major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Hydroxynonenal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation.

The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins, are susceptible to oxidation by the action of ROS/RNS (Stadtman, 2004). Advanced glycation end products (AGEs) are the results of a reaction between carbohydrates and free amino group of proteins. The intermediate products are known as Amadori, Schiff base and Maillard products, named after the researchers who first described them (Dalle - Donne *et al*., 2005). Most of the AGEs are very unstable, reactive compounds and the end products are difficult to be completely analysed.

3.1.2. Antioxidants

To counteract the adverse effects of ROS, a number of diverse antioxidant defence systems are operative in biological systems including enzymatic and non-enzymatic antioxidants. Antioxidants are defined as substances, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Halliwell and Gutteridge, 1990; Halliwell, 1995). Endogenous antioxidant defences are both non-enzymatic (e.g. uric acid, glutathione, bilirubin, thiols, albumin, and nutritional factors, including vitamins and phenols) and enzymatic (e.g. superoxide dismutases, glutathione peroxidases and catalase). In normal subjects the endogenous antioxidant defences balance the ROS production. The balance between oxidant and reducing forces is subtle (Abuja, 1998). Trace elements with antioxidant properties such as copper and selenium (Terada *et al*., 1999) may become strongly pro-oxidant both *in vivo* and *in vitro*, as a consequence of their physical properties. Vitamins such as A, C, E (Neuzil *et al*., 1995) may also become pro-oxidant under defined conditions.

3.1.2.1. Dietary and herbal antioxidants

The human diet contains an array of different compounds that possess antioxidant activities or have been suggested to scavenge ROS based on their structural properties. The most prominent representatives of dietary antioxidants are ascorbate (vitamin C), tocopherols (vitamin E), carotenoids and flavonoids. Chemical constituents with antioxidant activity, found in high concentrations in plants, determine their considerable role in the prevention of various degenerative diseases (Challa *et al*., 1997; Diplock *et al*., 1998; Hu and Willett, 2002). Besides fruits and vegetables that are recommended at present as optimal sources of such components, the supplementation of human diet with herbs, containing especially high amounts of compounds capable of deactivating free radicals may have beneficial effects (Madsen and Bertelsen, 1995). The benefits resulting from the use of natural products rich in bioactive substances, has promoted the growing interest of pharmaceutical, food and cosmetic industries, as well as of individual consumers in the quality of herbal produce. The evaluation of antioxidant

properties of the raw material allows the determination of its suitability as high quality food beneficial for human health, and therefore is of considerable importance. One of the aims of present study was to determine the antioxidant activity of bitter cumin (*Centratherum anthelminticum)* seeds in different model systems to evaluate its antioxidant potential.

3.2. Materials and Methods

3.2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxy anisole (BHA), 2,2' Azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), Thiobarbituiric acid (TBA), Linoleic acid, Soybean type IV Lipoxygenase, Tween 80, Tris base, Calf thymus DNA, Ethidium bromide, Xylene cyanol, Bromophenol blue, Bovine Serum Albumin (BSA) and Agarose were purchased from Sigma Chemical Co., MO, USA. Nicotinamide Adenine Dinucleotide (reduced)-NADH, Copper sulphate, Sodium potassium tartarate and Phenzine Methosulfate (PMS) were purchased from Hi-media, Mumbai, India. Nitro Blue Tetrazolium (NBT) was purchased from Sisco Research Laboratories, Mumbai, India. Plasmid DNA pUC^{18} was purchased from Bangalore Genei, Bangalore, India. All other chemicals and solvents used were of analytical grade.

3.2.2. Bitter cumin extracts

Bitter cumin extracts were prepared as explained in chapter 2

3.2.3. Determination of antioxidant activity

3.2.3.1. Reducing Power

3.2.3.1.1. Phosphomolybdenum reducing power of bitter cumin seed extracts

This assay is based on the reduction of Mo (VI) to Mo (V) by the reductant (antioxidant) in the sample analyte and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH according to the method of Prieto *et al*., (1999). The reagent solution consists of 0.6 M sulphuric acid, 28.0 mM sodium phosphate and 4.0 mM ammonium molybdate. An aliquot of 0.1 mL of bitter cumin extract was combined with 1 mL of reagent solution and incubated in a thermal block at 95°C for 90 min. After the samples were cooled to ambient temperature, the absorbance of the aqueous solution of

each was measured at 695 nm against a blank. The blank solution contained 1mL of reagent solution and the solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. All the experiments were performed in triplicates. The antioxidant capacity was expressed as an increase in absorbance at 695 nm and compared with the synthetic antioxidant BHA. The absorbance was measured by using a Shimadzu UV-Visible spectrophotometer (Model, 2100).

3.2.3.1.2. Potassium ferricyanide reducing method

The reductive potential of the AMAEBC was determined according to the method of Oyaizu *et al*., (1986). Different concentrations of bitter cumin extracts in 0.5 mL of MeOH were mixed with equal volumes of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated for 20 minutes at 50° C. At the end of incubation, an equal volume of 10% trichloroacetic acid was added to the mixture and centrifuged at 3200 xg for 10 minutes. The supernatant was mixed with distilled water and 0.1% ferric chloride at 1:1:0.2 (v/v/v) and the absorbance were measured at 700 nm. An increase in the absorbance of the reaction mixture indicates the potential reducing power of the sample. Ascorbic acid and BHA were used as standards for comparison.

3.2.3.2. Radical Scavenging Activity

3.2.3.2.1. DPPH radical scavenging activity of bitter cumin seed extracts

3.2.3.2.1.1. Static method

1,1-Diphenyl-2-picryl hydrazyl (DPPH) is a stable free radical that accepts an electron or hydrogen atom to become a stable 1,1-Diphenyl-2-picrylhydrazine molecule. The reduction of DPPH radical was determined by decrease in the absorbance at 517 nm. The antioxidant activity of different extracts of bitter cumin seeds and standard synthetic antioxidant BHA was measured in terms of hydrogen donating or radical scavenging ability, using the stable $DPPH^{\bullet}$ method (Brand Williams *et al*., 1995) with some modifications. Briefly 1 mL of

200 μM methanolic solution of DPPH$^{\bullet}$ was incubated with 1mL of bitter cumin seed extracts or BHA and incubated for a period of 20 minutes at ambient temperature. At the end of incubation period the absorbance was measured using a UV-Visible spectrophotometer at 517 nm. The percentage of scavenging or quenching of DPPH radicals (Q) by CA and BHA was calculated using the following formula.

$$Q = 100\,(A_0 - A_c)/A_0$$

Where A_0 is the absorbance of the control tube and A_c is the absorbance of the tube with 'c' concentration of sample. All the experiments were performed in triplicates.

3.2.3.2.1.2. Dynamic Method

To 1 mL of 50 μM DPPH$^{\bullet}$ solution, different concentrations of 50 μl bitter cumin AMAE dissolved in methanol was added. The absorbance of the reaction mixture was monitored continuously for a period of 5 min. A standard curve was prepared by measuring the absorbance at various concentrations of DPPH$^{\bullet}$. The percentage inhibition of absorbance at 517 nm was calculated and plotted as a function of concentration of bitter cumin seed extract.

3.2.3.2.2. ABTS$^{\bullet+}$ radical scavenging by bitter cumin seed extracts

Generation of ABTS radical cation (ABTS$^{\bullet+}$) (Wolfenden and Willson, 1982) forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of various substances. The experiments were carried out using an improved ABTS$^{\bullet+}$ decolourization assay (Re *et al*., 1999).

ABTS radical cation (ABTS$^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark, at ambient temperature for 12–16 h before use. Because ABTS and potassium persulfate react stoichiometrically at a ratio of 1: 0.5, this will result in incomplete oxidation of the ABTS.

The $ABTS^{\bullet+}$ solution was diluted to an absorbance of 0.7 ± 0.05 at 734 nm (Shimadzu UV–VIS Spectrophotometer, Model 2100) with ethanol. To 1 mL of $ABTS^{\bullet+}$ solution, different concentrations of bitter cumin seed extracts/BHA were added. The absorbance was measured every 1.0 min up to 7 min at 734 nm. All the experiments were performed in triplicates.

3.2.3.3. Superoxide anion scavenging of bitter cumin seed extracts

Superoxide anion scavenging activity of bitter cumin seed extract was determined by a modified method of Liu *et al*., (1997). Superoxide radicals were generated in PMS-NADH system by oxidation of NADH, and assayed by the reduction of nitroblue tetrazolium (NBT) (Nishikimi *et al*., 1972). The assay system consists of 100 µl each of 1.0 mM NBT, 3.0 mM NADH and 0.3 mM PMS and the final volume was adjusted to 1mL with 0.1 M phosphate buffer (pH 7.8), at ambient temperature. The reaction mixture (NBT and NADH) was incubated with or without bitter cumin extracts at ambient temperature for 2 minutes and the reaction was started by adding PMS. The absorbance at 560 nm was measured against blank samples for 3 minutes. Decrease in absorbance in the presence of bitter cumin extract indicated superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

Inhibition of superoxide generation (%) $= \boldsymbol{[(A_0-A_1)/A_0] \times 100}$

where, A_0 is the absorbance of the control, and A_1 is the absorbance in presence of bitter cumin extract.

3.2.3.4. Soybean lipoxygenase dependent lipid peroxidation assay

Soybean lipoxygenase dependent enzymatic lipid peroxidation was measured spectrophotometrically, following an increase in the absorbance at 234 nm due to the formation of lipid hydroperoxides, according to method of Narayan *et al*., (1999). Briefly, the reaction mixture in a final volume of 1 mL contained 200 µM linoleic acid and 5 nM soybean lipoxygenase in 50 mM Tris buffer, pH 7.4. The formation of lipid hydroperoxide was measured at 234 nm in a Shimadzu UV-Visible spectrophotometer. Different concentrations of bitter cumin seed extracts were incubated with soybean lipoxygenase for 2 min

prior to initiation of reaction with linoleic acid. The decrease in lipid hydroperoxide formation in the presence of bitter cumin seed extract was calculated using the extinction coefficient (ε = 25000 $M^{-1}cm^{-1}$). The enzyme activity was expressed as μmoles of hydroperoxide formed per minute per 5 nM of enzyme.

3.2.3.5. Rat liver microsomal lipid peroxidation assay

3.2.2.5.1. Preparation of rat liver microsomes

Healthy male Wistar rats weighing 200 ± 10 g were starved overnight and sacrificed after mild anaesthesia with ether. The liver was rapidly removed, washed extensively with ice cold saline and blotted. 2 g of liver was homogenized with 50 mL of 0.25 mM Tris-HCl-Sucrose-EDTA buffer at pH 7.4. The homogenate was centrifuged at 7649 x g for 30 min to remove unbroken cells and cell debris. The supernatant was centrifuged at 100,000 x g in an ultracentrifuge (Beckman L7-65 Ultracentrifuge, USA) for 2 h. The supernatant was discarded and microsomal pellet was suspended in 2 mL of 125 mM KCl. The protein content of the microsomes was estimated. All the procedures were done at 4 °C.

3.2.3.5.2. Estimation of protein

Protein content in liver microsomes was estimated according to Lowry's method (Lowry *et al*., 1951). To 1 mL of protein solution, 5 mL of reagent C containing a mixture of 2% sodium carbonate in 0.1 M sodium hydroxide and 0.5% copper sulphate in 1% sodium potassium tartarate was added. It was allowed to stand for 10 min at ambient temperature. To this, 0.5 mL of 1:1 diluted FC reagent was added, and kept at ambient temperature to develop the colour. The absorbance of the sample was read at 700 nm and the amount of protein present was determined by referring to the standard graph prepared by using Bovine Serum Albumin (BSA).

3.2.3.5.3. Rat liver microsomal lipid peroxidation assay

The liver microsomal lipid peroxidation study was performed according to the method of Miller and Aust (1989). 1 mg protein equivalent of rat liver microsomes was incubated with or without bitter cumin extracts for 10 min at ambient temperature. The peroxidation of microsomal lipids was induced by 0.2 mM $FeSO_4$ and 0.2 mM ascorbic acid. The final volume was made up to 1 mL with phosphate buffer (0.1 M, pH 7.4). The reaction mixture was incubated in a water bath at 37 ºC for 1 h and the reaction was stopped by adding 2 mL of 0.25N HCl containing 15% TCA and 0.375% thiobarbituric acid. Malondialdehyde formed as a result of microsomal lipid oxidation was made to react with thiobarbituric acid. Thiobarbituric acid reactive substances (TBARS) so formed were measured at 535 nm spectrophotometrically (Buege and Aust, 1978). The blank contained all the reagents except microsomes. Malondialdehyde (MDA) formed was calculated by using a molar extinction coefficient of 1.56 $x10^5$ $M^{-1}cm^{-1}$. The results were expressed as nmol of malondialdehyde (MDA) formed per mg protein. Inhibition of lipid peroxidation (%) by AMAE of bitter cumin was calculated by the following equation:

Lipid peroxidation inhibition (%)= $(A_0 - A_i / A_0)100$

where A_0 is the absorbance value of the fully oxidized control and A_i is the absorbance in presence of extract.

The half-inhibition concentration (IC_{50}) value was defined as the amount of bitter cumin extract required for inhibition of 50% of lipid peroxidation. The IC_{50} value was calculated from the graph plotted as the concentration of the bitter cumin extract versus percent inhibition of lipid peroxidation

3.2.3.6. Liposomal model system

3.2.3.6.1. Preparation of egg lecithin

Egg yolk was washed with ice cold acetone and the solvent was discarded repeatedly. The washing was continued till the precipitate became colourless. The precipitate was dried under cold, weighed and dissolved in phosphate buffer.

3.2.3.6.2. Antioxidant assay with egg lecithin

The antioxidant activity of the extracts of bitter cumin seeds and α-tocopherol in a liposome model system was determined according to the method of Pin-Der and Gow-Chin (1997). Egg lecithin (300 mg) was sonicated in 30 mL phosphate buffer (10 mM, pH 7.4) in an ultrasonic sonicator for 30 min to ensure proper liposome formation. Bitter cumin seed extracts and standards were mixed with the liposome preparation (0.5 mL, 10 mg/mL) and incubated for 10 min at ambient temperature. The oxidation of liposomal lipid was started by adding $FeCl_3$ (0.5 mL, 400 mM), and ascorbic acid (0.5 mL, 400 mM). The reaction mixture was incubated in a water bath at 37^0C for 1 h and the reaction was stopped by adding 2 mL of 0.25 N HCl containing 15% TCA and 0.375% thiobarbituric acid. Malondialdehyde formed as a result of microsomal lipid oxidation was made to react with thiobarbituric acid. Thiobarbituric acid reactive substances (TBARS) formed were measured at 535 nm spectrophotometrically (Buege and Aust, 1978). Malondialdehyde (MDA) formed was calculated using a molar extinction coefficient of 1.56 $x10^5$ $M^{-1}cm^{-1}$. The results were expressed as nmol of malondialdehyde (MDA) formed per mg lipid. The antioxidant activity was calculated as

$$\text{Inhibition } (\%) = (Abs_{control} - Abs_{sample})/Abs_{control} \times 100$$

3.2.3.7. Oxidative DNA damage

Oxidative DNA damage study of Calf thymus DNA, prokaryotic bacterial genomic DNA and plasmid DNA was carried out by hydroxyl radical generated by Fenton reaction. 3.0 μg of calf thymus DNA, 2.0 μg of prokaryotic (bacterial) genomic DNA, 1.0 μg of plasmid (pUC18) DNA in phosphate buffered saline were individually incubated with different concentrations of AMAE of bitter cumin for 15 min at ambient temperature. Oxidation of DNA was induced by treating with 1 mM $FeCl_3$ and 10 mM ascorbic acid. Positive control was not treated with AMAE of bitter cumin or by Fenton reaction and negative control was incubated with $FeCl_3$ and ascorbic acid only. The final reaction volume was 9 μl and the reaction mixture was incubated for 1 h at 37 ºC. The reaction was stopped by adding 3 μl loading

buffer (xylene-cyanol, 0.25%; bromophenol blue, 0.25%; and glycerol, 30%) and 9 μl of the reaction mixture was loaded on to agarose gel (1%). Electrophoresis was done in TAE buffer initially for 1 hour at 40 V followed by 2 h run at 60 V. The gel was stained with ethidium bromide (1μg/mL). DNA was visualized and photographed by a digital imaging system (Hero lab, GMBH, Germany).

3.2.4. Statistical analysis

Statistical analysis was done using the software SPSS (Release 7.5.1). The differences in mean values were tested using one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used to determine the significant differences amongst the test materials. Differences were considered to be significant at $P \leq 0.05$.

3.3. Results

3.3.1. Reducing Power

3.3.1.1. Phosphomolybdenum reducing method

The phosphomolybdenum reducing potential of BHA, AMAEBC, AMEBC and AEBC are given in Figure 1. The reducing power of various extracts of bitter cumin and the standard antioxidant BHA increased steadily with increase in concentration. There was a direct relationship between the concentration of bitter cumin extract and reduction of phosphomolybdenum. The correlation coefficient of dose v/s absorbance was 0.9994, 0.9812, 0.9943 and 0.9957 for BHA, AMAEBC, AMEBC and AEBC respectively. The highest reducing potential was shown by BHA followed by AMAEBC, AMEBC and AEBC.

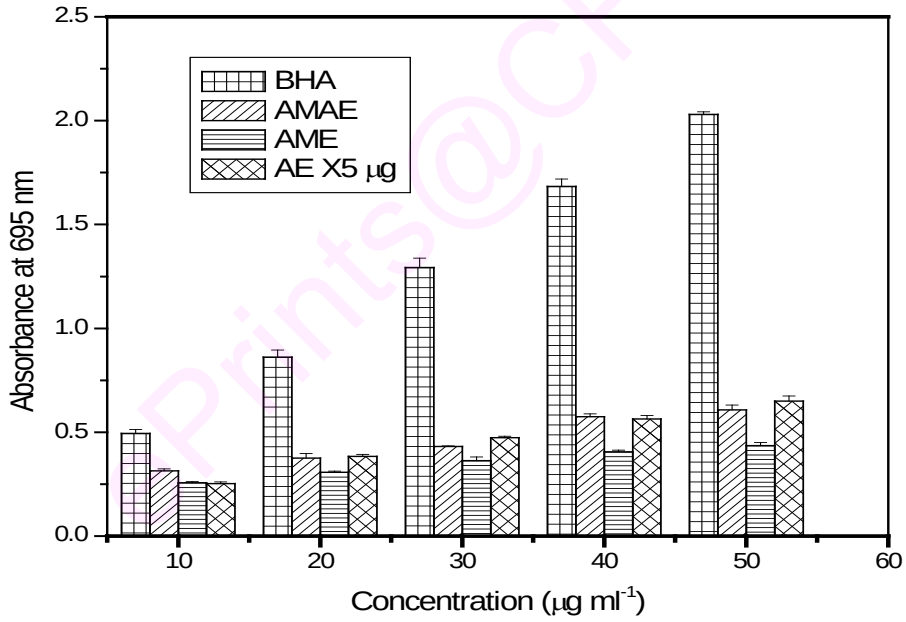


FIGURE 1. Absorbance of different extracts of bitter cumin and standard BHA at various concentrations in phosphomolybdenum reducing power assay.

(BHA- Butylated hydroxy anisole, AMAE- Aqueous methanol-acetone extract of bitter cumin, AME- Aqueous methanol extract of bitter cumin, AE – Aqueous extract of bitter cumin). The values are ± S.E.M of 3 experiments

3.3.1.2. Potassium Ferricyanide reducing power

The reducing power of bitter cumin AMAE, ascorbic acid and BHA are presented in Figure 2. A dose dependent increase in reducing power of AMAEBC was observed. However, the synthetic antioxidants ascorbic acid and BHA showed relatively higher reducing power compared to AMAEBC. The correlation coefficient between dose and absorbance was 0.9937, 0.9988 and 0.9878 respectively for Ascorbic acid, BHA and AMAEBC. The other two extracts of bitter cumin (AME and AE) didn't show appreciable absorbance in this assay.

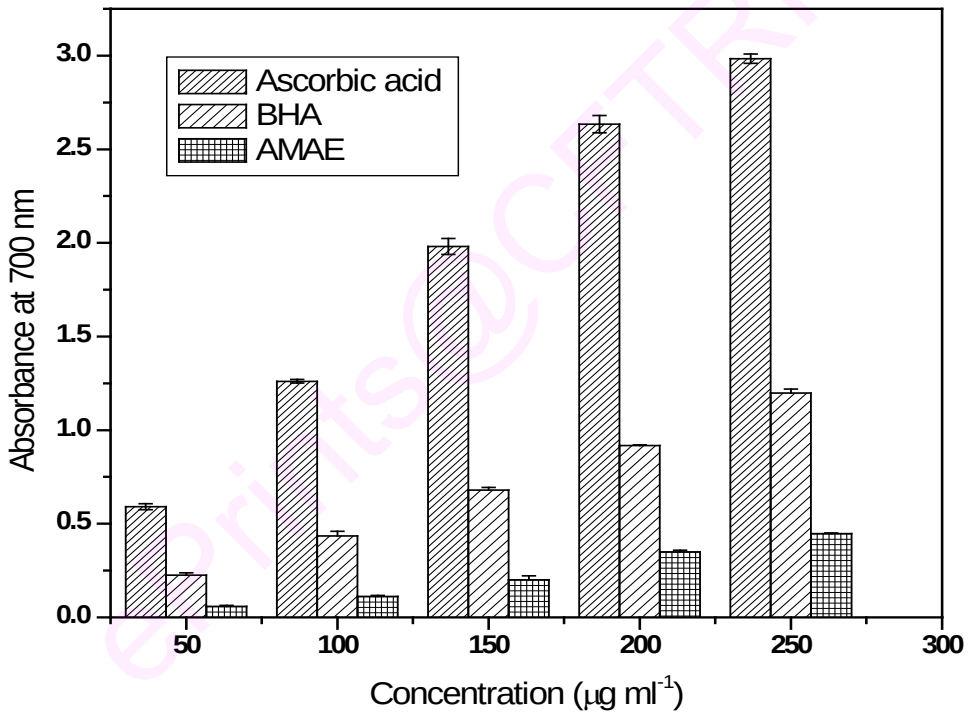


FIGURE 2. Potassium ferricyanide reducing power of AMAEBC, Ascorbic acid and BHA.

(AA- Ascorbic acid, BHA- Butylated hydroxy anisole, AMAE- Aqueous methanol acetone extract of bitter cumin). Values are ± S.E.M of 3 experiments.

3.3.2. Radical scavenging activity of bitter cumin

3.3.2.1. DPPH radical scavenging activity of bitter cumin extracts

3.3.2.1.1. Static Method

The DPPH$^{\bullet}$ scavenging potential of various bitter cumin extracts and BHA are given in Table 1. All the extracts of bitter cumin showed dose dependent scavenging of DPPH radical. As shown in Figure 3, among the extracts tested, AMAEBC showed highest DPPH$^{\bullet}$ scavenging activity with an IC_{50} value of 20.8 ± 0.18 μg followed by AMEBC (IC_{50}= 191.7 ± 9.14 μg) and AEBC (IC_{50}= 639.2 ± 20.91μg).

TABLE 1. Dose dependent DPPH$^{\bullet}$ scavenging activity of BHA and various extracts of bitter cumin.

Amount of inhibitor (μg)	% Inhibition			
	BHA	AMAEBC	AMEBC X10 μg	AEBC X40 μg
02.5	9.32 ± 2.0	08.59 ± 1.7	10.99 ± 1.7	10.58 ± 0.5
05.0	33.05 ± 1.1	14.40 ± 0.4	17.83 ± 2.9	17.10 ± 1.8
10.0	59.91 ± 0.8	26.08 ± 0.1	28.06 ± 1.5	37.44 ± 4.1
15.0	72.71 ±1.5	37.01 ± 1.1	41.08 ± 2.1	47.10 ± 1.7
20.0	81.99 ± 0.9	48.18 ± 0.5	51.55 ± 1.7	59.55 ± 1.8
25.0	87.66 ± 0.2	58.15 ± 1.3	61.20 ± 2.9	68.86 ± 1.5
30.0	89.19 ± 0.5	67.70 ± 1.4	74.03 ± 2.1	77.90 ± 0.8
40.0	91.57 ± 0.2	78.38 ± 2.0	85.26 ± 2.8	86.51 ± 0.1
50.0	92.09 ± 0.1	91.46 ± 0.6	91.58 ± 0.7	86.77 ± 0.2

(BHA- Butylated hydroxy anisole, AMAEBC- Aqueous methanol acetone extract of bitter cumin, AMEBC- Aqueous methanol extract of bitter cumin, AEBC– Aqueous extract of bitter cumin). Values are mean ± S.E.M of 3 experiments.

The correlation coefficient between dose and scavenging activity are 0.8520, 0.9881, 0.9801 and 0.9475 respectively for BHA, AMAEBC, AMEBC and AEBC. BHA, the synthetic antioxidant showed highest DPPH$^{\bullet}$ scavenging activity with an IC_{50} value of 8.2 ± 0.24 μg.

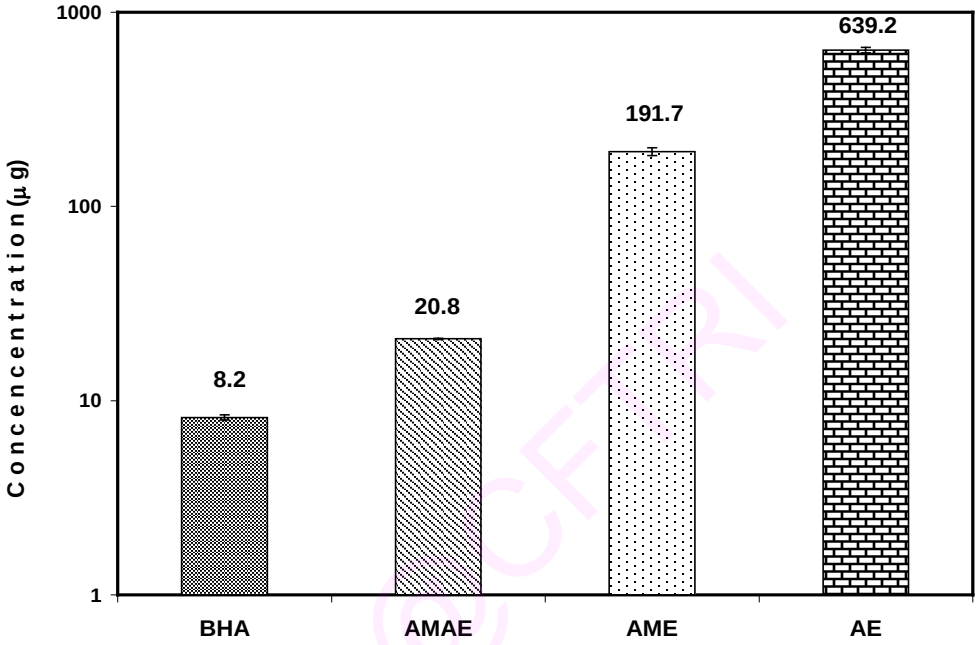


FIGURE 3. IC_{50} value of DPPH$^{\bullet}$ scavenging by BHA and various extracts of bitter cumin

(BHA- Butylated hydroxy anisole, AMAE- Aqueous methanol acetone extract of bitter cumin, AME- Aqueous methanol extract of bitter cumin, AE – Aqueous extract of bitter cumin). Values are mean ± S.E.M of 3 experiments.

3.3.2.1.2. Dynamic or kinetic method

In dynamic or kinetic method the rate of DPPH$^{\bullet}$ decay is immediately followed after addition of the antioxidant. In this method, reactivity is generally characterized by the starting rate of DPPH$^{\bullet}$ decay (Da porto *et al*., 2000). The rate of DPPH$^{\bullet}$ (50 μM) decay over a period of 5 min after addition of different concentrations of AMAEBC is presented Figure 4. It is observed that most of the scavenging of DPPH$^{\bullet}$ occurs during the first 30 seconds of the reaction. The curve was more or less static after the first one minute of the reaction.

The IC_{50} value obtained from the dose response curve was 14.0 ± 0.5 μg and the correlation coefficient between the dose and activity was 0.9375.

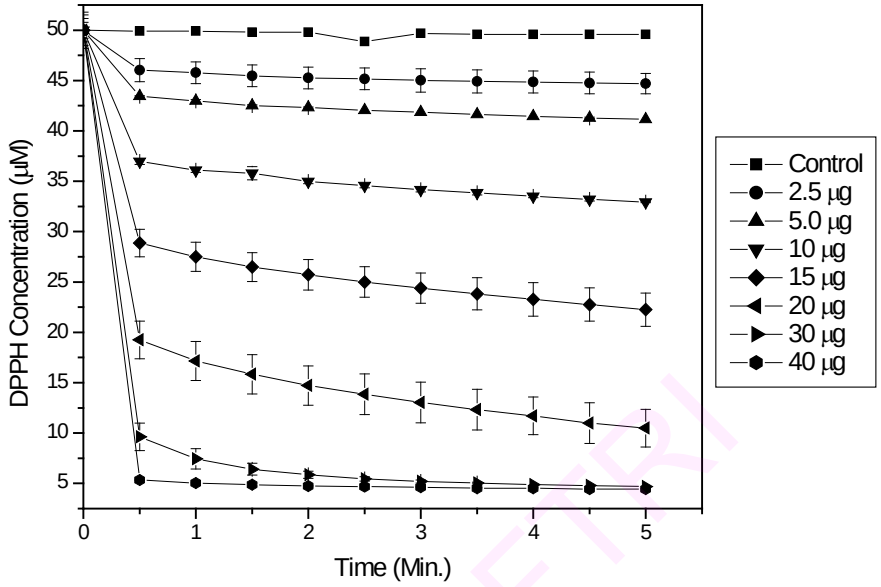


FIGURE 4. Kinetics of DPPH$^{\bullet}$ scavenging by different concentrations of AMAE of bitter cumin

3.3.2.2. ABTS$^{\bullet+}$ scavenging by bitter cumin extracts

Bitter cumin extracts showed significant scavenging of ABTS cation radicals. Figures 5 a-d illustrate the dose dependent scavenging effect of AMAE, AME and AE of bitter cumin and also BHA on ABTS$^{\bullet}$ cation radical. The rate of ABTS$^{\bullet}$ decay after adding different concentrations of AMAE of bitter cumin over a period of 7 min is presented Figure 5 a-c. It is observed that maximum scavenging of ABTS$^{\bullet}$ cation radical occurred during first one minute of the reaction and plateaued till the end of timer period. The IC_{50} value obtained from the dose response curve was presented in Fig.6.

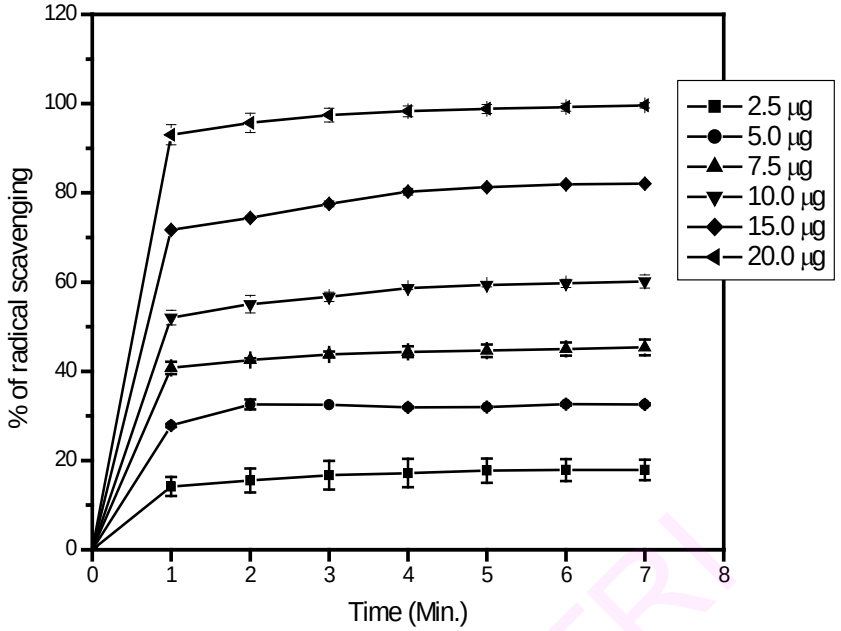


FIGURE 5 a. Kinetics of scavenging of ABTS$^{\bullet+}$ by different concentrations of AMAE of bitter cumin. Values are mean ±SD of 3 experiments.

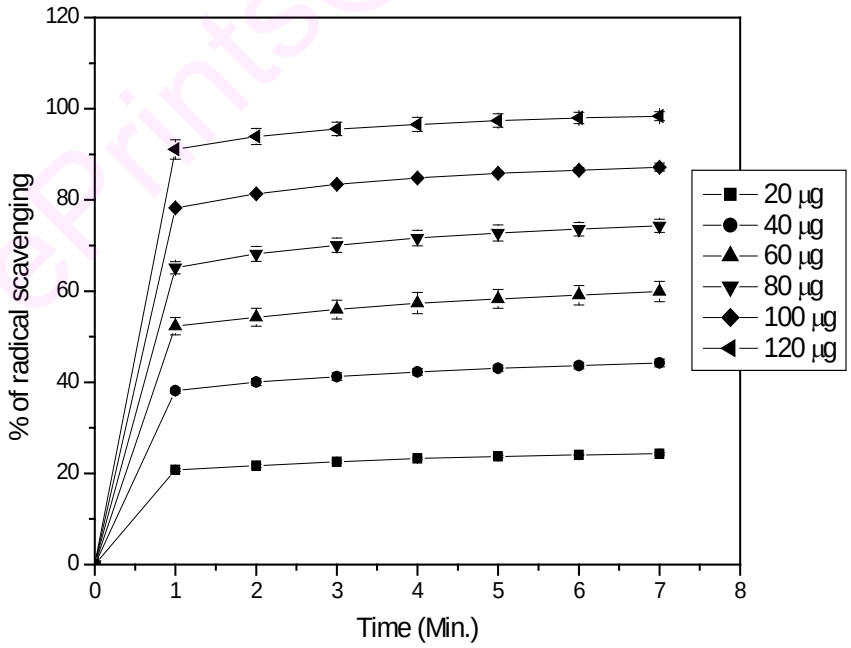


FIGURE 5 b. Kinetics of scavenging of ABTS$^{\bullet+}$ by different concentrations of AME of bitter cumin. Values are mean ± SD of 3 experiments.

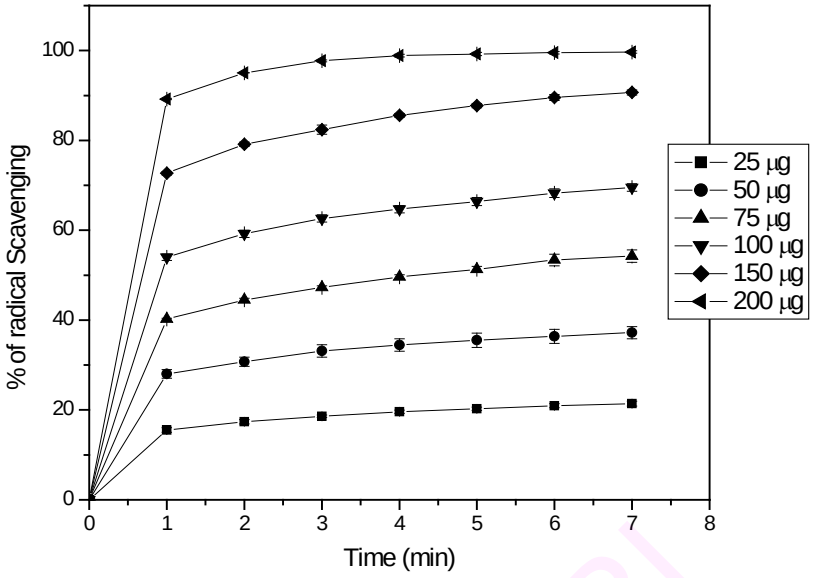


FIGURE 5,c. Kinetics of scavenging of ABTS$^{\bullet+}$ by different concentrations of AE of bitter cumin. Values are mean ±SD of 3 experiments.

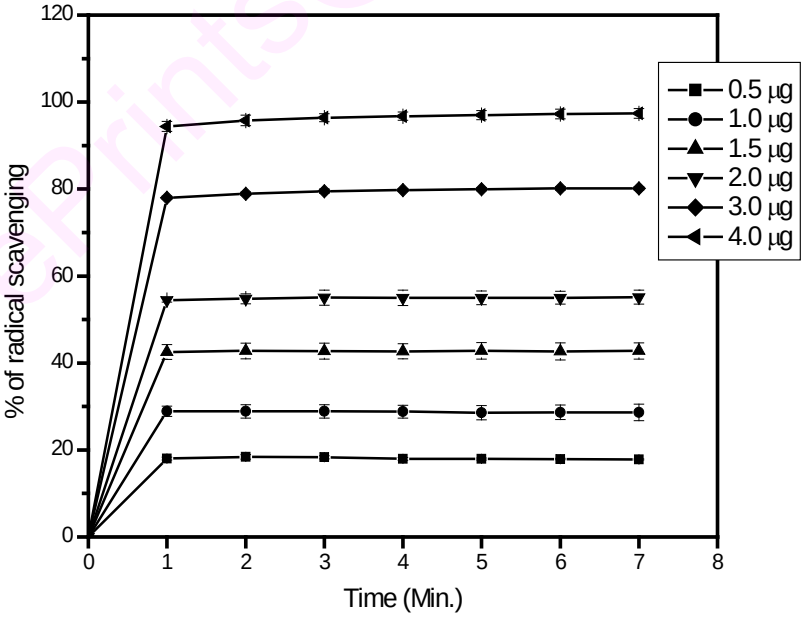


FIGURE 5,d. Kinetics of scavenging of ABTS$^{\bullet+}$ by different concentrations of BHA. Values are mean ± SD of 3 experiments.

All the extracts of bitter cumin demonstrated low scavenging efficiency or higher IC_{50} value of 8.3 ± 0.28, 47.0 ± 1.08 and 68.5 ± 1.6 7 μg for AMAE, AME and AE respectively. However, BHA showed maximum $ABTS^{\bullet+}$ scavenging activity with an IC_{50} value of 1.8 ± 0.06 μg (Figure 6).

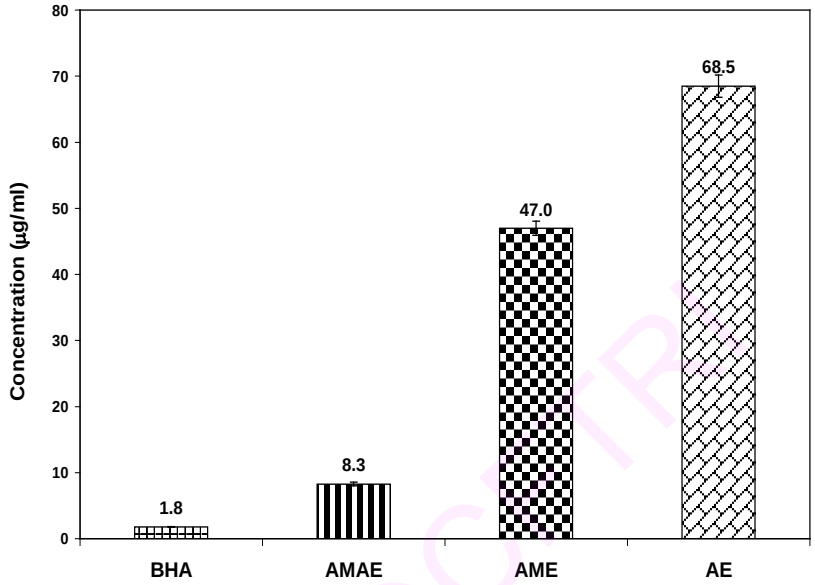


FIGURE 6. IC_{50} value of $ABTS^{\bullet+}$ scavenging by BHA and different extracts of bitter cumin.

(BHA- Butylated hydroxy anisole, AMAE- Aqueous methanol acetone extract of bitter cumin, AME- Aqueous methanol extract of bitter cumin, AE – Aqueous extract of bitter cumin). Values are mean ± SD of 3 experiments.

3.3.3. Superoxide anion ($O_2^{\bullet-}$) scavenging by bitter cumin extracts

The effect of bitter cumin extract (AMAE) and gallic acid on scavenging of superoxide anions is illustrated in Figure 7. AMAE of bitter cumin and gallic acid significantly scavenged superoxide anions in a dose dependent manner. The other two extracts of bitter cumin viz., AME and AE were not able to produce effective scavenging of superoxide anion radicals. The IC_{50} values were 125.4 ± 8.7μg/mL and 11.4 ± 0.79 μg/mL respectively for AMAEBC and gallic acid. The correlation coefficient between dose and scavenging activity

was 0.9458 for AMAEBC and 0.8583 for gallic acid. However, gallic acid was much more potent in scavenging superoxide anions than AMAEBC.

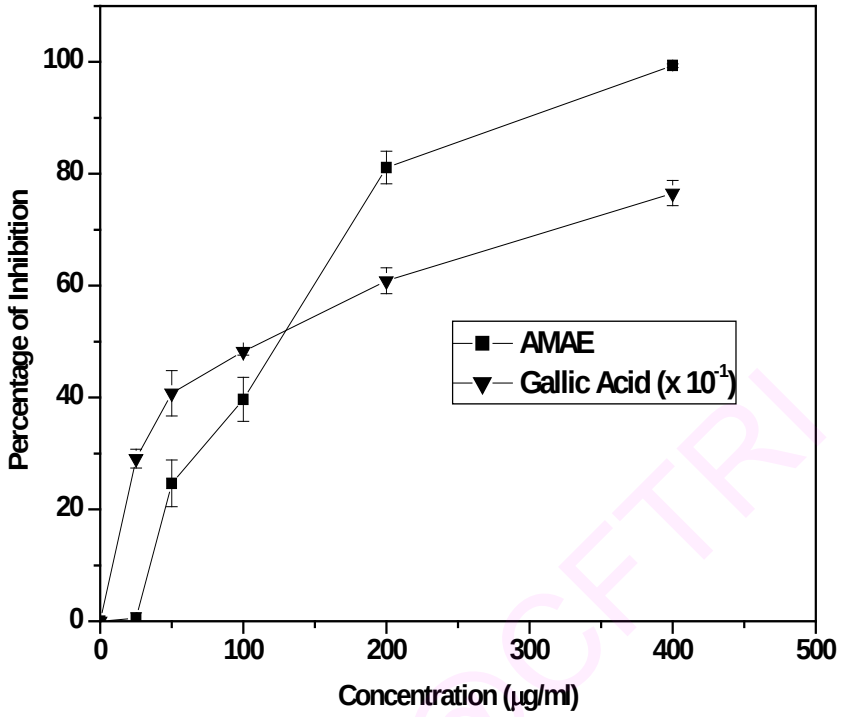


FIGURE 7. Superoxide scavenging activity of AMAEBC and gallic acid. Values are mean ±SD of 3 experiments.

3.3.4. Inhibition of soybean lipoxygenase dependent lipid peroxidation by bitter cumin

Soybean lipoxygenase-dependent lipid peroxidation is an enzymatic lipid peroxidation assay, used to determine the antioxidant activity of test compounds. AMAEBC and BHA are found to inhibit LOX activity and the inhibitory effect was found to be dose dependent (Figure 8). The inhibition of lipid peroxidation was nearly complete as shown in linearity curve. IC_{50} concentration was found to be 28.0 ± 3.0 μg for AMAEBC, 61.8 ± 6.9 for AME, 173.3 ± 9.9 AE and 14.0 ± 0.25 μg for BHA.

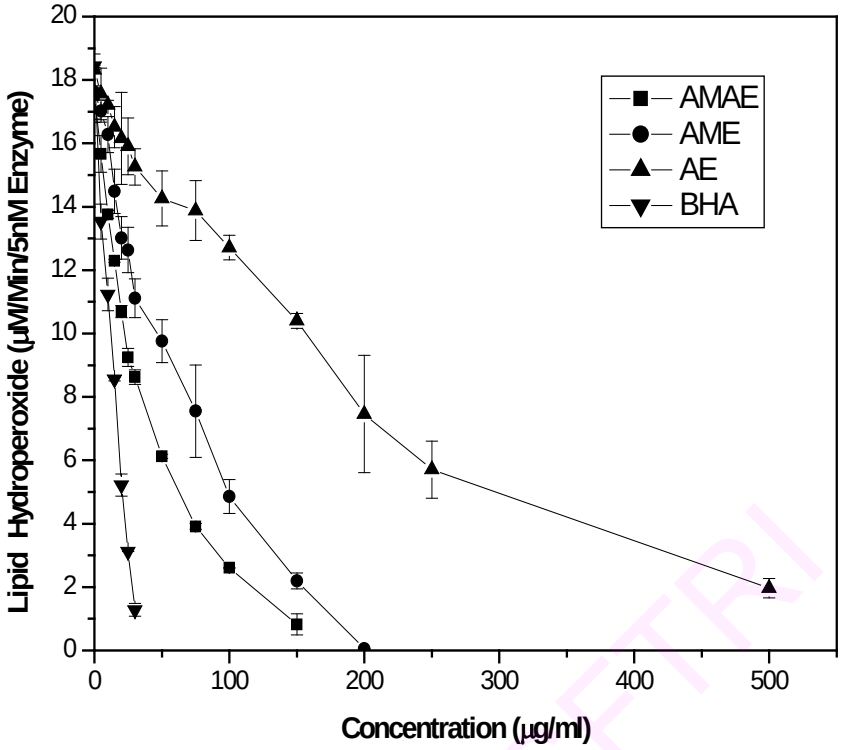


FIGURE 8. Inhibition of soybean lipoxygenase dependent lipid peroxidation by AMAEBC and BHA.

(BHA- Butylated hydroxy anisole, AMAE- Aqueous methanol acetone extract of bitter cumin, AME- Aqueous methanol extract of bitter cumin, AE – Aqueous extract of bitter cumin). Values are mean ± SEM of three individual experiments.

3.3.5. Inhibition of rat liver microsomal lipid peroxidation by bitter cumin

AMAEBC significantly inhibited hydroxyl radical induced lipid peroxidation in rat liver microsomes. Figure 9 shows the inhibition of rat liver microsomal lipid peroxidation by AMEBC and BHA. The other two extracts of bitter cumin were not much effective in inhibiting lipid peroxidation. IC_{50} concentration for inhibition of microsomal lipid peroxidation was found to be 69.97 ± 2.4 µg for BHA and 110 ± 14 µg for AMAEBC respectively. BHA showed better inhibitory effect compared to AMAEBC on microsomal lipid peroxidation.

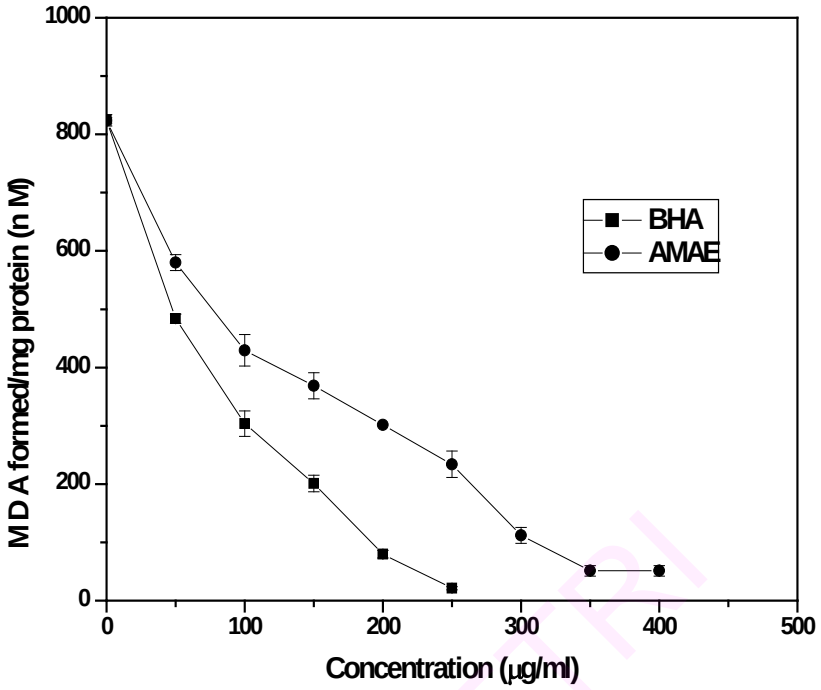


FIGURE 9. Inhibition of rat liver microsomal lipid peroxidation by AMAEBC and BHA. Values are mean ± SEM of three individual experiments.

3.3.6. Inhibition of lipid peroxidation of egg lecithin liposomes by bitter cumin

Bitter cumin extracts showed concentration dependent inhibition of egg liposomal lipid peroxidation as shown in Figure 10. AMAEBC showed highest inhibitory activity and AEBC showed lowest activity in inhibiting phospholipid peroxidation induced by Fenton reaction. α-tocopherol, the standard antioxidant showed relatively lower inhibitory effect compared to bitter cumin extracts. The IC_{50} values of inhibition of liposomal lipid peroxidation by bitter cumin extracts and α-tocopherol are given in Table 2. AMAEBC, AMEBC and AEBC were found to be 41, 24 and 3 times more potent than α-tocopherol in inhibiting liposomal lipid peroxidation. Thus, the inhibitory efficiency of bitter cumin extracts was in the order of AMAEBC> AMEBC> AEBC> α-tocopherol. The correlation between bitter cumin extracts and

antioxidant activity was found to be 0.9041 for AEBC, 0.8288 for AMAEBC, 0.7560 for AMEBC and 0.7364 for α-tocopherol.

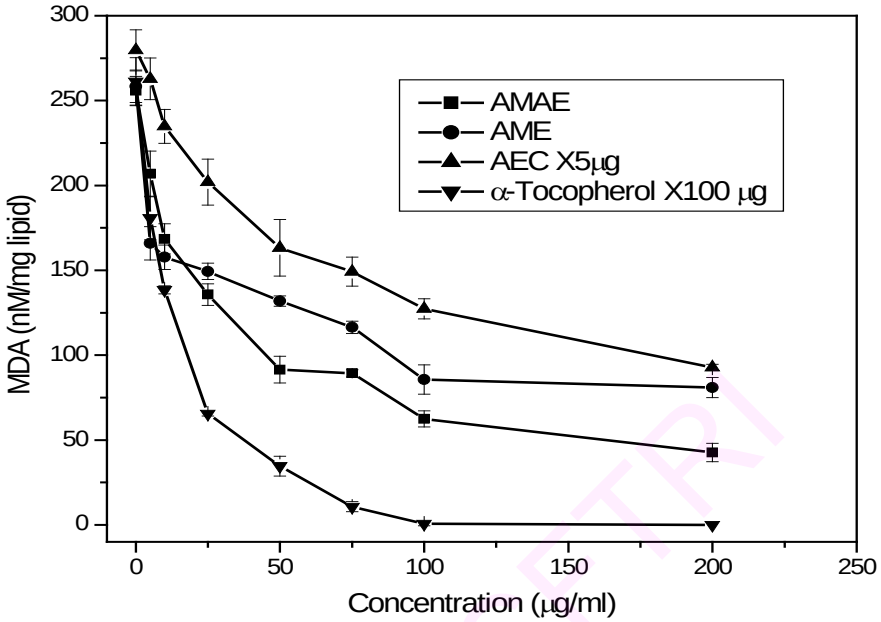


FIGURE 10. The inhibition of liposomal lipid peroxidation by different extracts of bitter cumin and α-tocopherol.

(BHA- Butylated hydroxy anisole, AMAE- Aqueous methanol acetone extract of bitter cumin, AME- Aqueous methanol extract of bitter cumin, AE – Aqueous extract of bitter cumin). Values are ± S.E.M of 3 experiments.

TABLE 2. IC_{50} values of bitter cumin extracts and α-tocopherol in inhibition of liposomal lipid peroxidation.

Bitter cumin extracts	IC_{50} Value (μg/ mL)
AMAEBC	014.3 ± 01.9
AMEBC	024.0 ± 05.9
AEBC	210.9 ± 20.9
α-Tocopherol	580.0 ± 89.5

Values are ± S.E.M of 3 experiments.

3.3.7. Inhibition of oxidative DNA damage

DNA is susceptible to oxidative damage and hydroxyl radicals oxidise guanosine or thymine to 8-hydroxyl-2- deoxyguanosine and thymine glycol which change DNA and lead to mutagenesis and carcinogenesis (Ames *et al*., 1993). In this study, hydroxyl radicals generated by Fenton reaction were found to induce DNA strand breaks in calf thymus DNA, prokaryotic genomic DNA and uncoiling of supercoiled DNA.

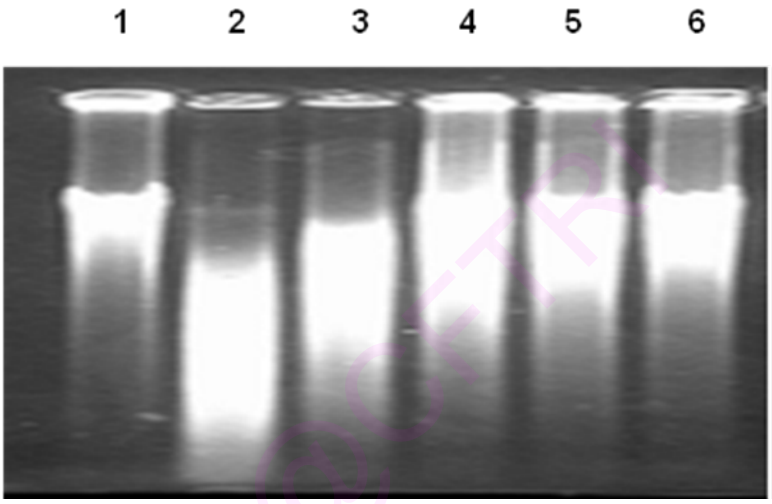


FIGURE 11. Protective effect of AMAEBC on hydroxyl radical induced damage to calf thymus DNA. A significant protection of DNA damage can be observed at 0.5-2.0 μg of AMAEBC

Lane 1 – Native calf Thymus DNA

Lane 2 – DNA + 1.0 mM $FeCl_3$+10.0 mM AA

Lane 3 – DNA + 1.0 mM $FeCl_3$ +10.0 mM AA + 0.5μg AMAEBC

Lane 4 – DNA + 1.0 mM $FeCl_3$ +10.0 mM AA + 1.0 μg AMAEBC

Lane 5 – DNA + 1.0 mM $FeCl_3$ +10.0 mM AA + 1.5 μg AMAEBC

Lane 6 – DNA + 1.0 mM $FeCl_3$ +10.0 mM AA + 2.0 μg AMAEBC

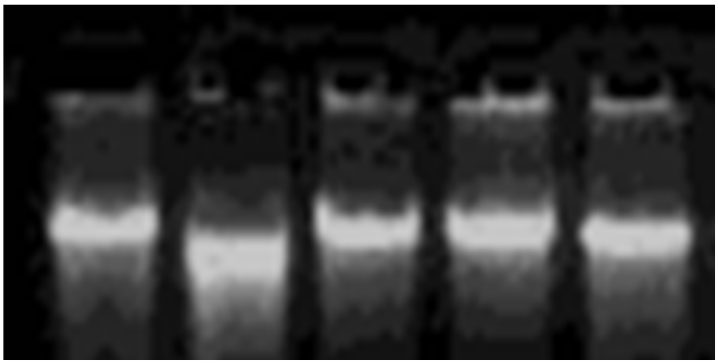


FIGURE 12. Protective effect of AMAEBC on hydroxyl radical induced oxidative damage on prokaryotic genomic DNA. A significant protection of genomic DNA damage can be observed at 1.0 and 2.5 μg of AMAEBC. The protection offered by AMAEBC is comparable to that of BHA

Lane 1 – Native genomic DNA of *Bacillus*.

Lane 2 – DNA + 1.0 mM $FeCl_3$ +10.0 mM AA

Lane 3 – DNA + 1.0 mM $FeCl_3$ +10.0 mM AA + 1.0μg BHA

Lane 4 – DNA + 1.0 mM $FeCl_3$ +10.0 mM AA + 1.0 μg AMAEBC

Lane 5 – DNA + 1.0 mM $FeCl_3$ +10.0 mM AA + 2.5 μg AMAEBC

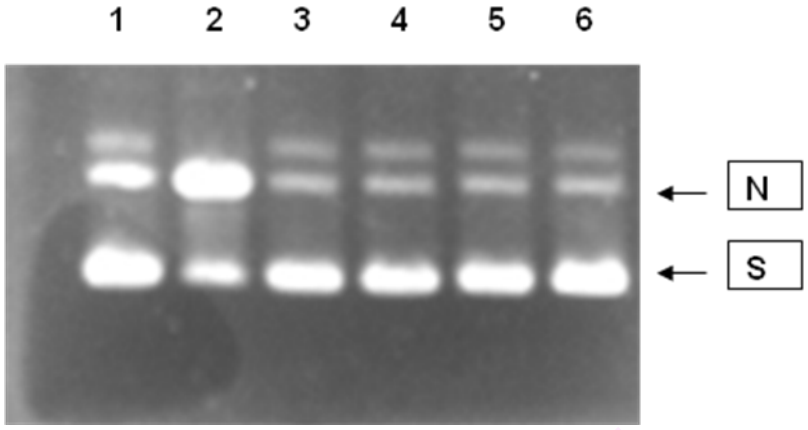


FIGURE 13. Protective effect of AMAEBC on hydroxyl radical induced oxidative damage on PUC^{18} DNA. Exposure of supercoiled DNA to hydroxyl radicals resulted in nicking of DNA. 0.5, 1.0 and 2.5 μg of AMAEBC offered protection against nicking of supercoiled DNA by hydroxyl radicals.

S- Supercoiled DNA, N- Nicked DNA.

Lane 1- pUC18 DNA.

Lane 2- pUC18 DNA + 10.0 μM $FeSO_4$ + 60.0 μM AA + 6.0 mM H_2O_2.

Lane 3- pUC18 DNA + 10.0 μM $FeSO_4$ + 60.0 μM AA + 6.0 mM H_2O_2 + 0.5 μg AMAEBC

Lane 4- pUC18 DNA + 10.0 μM $FeSO_4$ + 60.0 μM AA + 6.0 mM H_2O_2 + 1.0 μg AMAEBC

Lane 5- pUC18 DNA + 10.0 μM $FeSO_4$ + 60.0 μM AA + 6.0 mM H_2O_2 + 2.5 μg AMAEBC

Lane 6- pUC18 DNA + 10.0 μM $FeSO_4$ + 60.0 μM AA + 6.0 mM H_2O_2 + 2.5 μg BHA

3.4. Discussion

Oxygen free radicals or reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well known to play a dual role as both deleterious and beneficial species as they can be either beneficial or harmful to living systems (Valko *et al*., 2006). Beneficial effects of ROS occur at low/moderate concentrations and involve in cellular responses to noxia, the induction of a mitogenic response and in defence against infectious agents and in a number of cellular signalling systems. The harmful effect of free radicals include oxidative stress and nitrosative stress (Kovacic and Jacintho, 2001; Ridnour *et al*., 2005; Valko *et al*., 2001). The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called "redox regulation". The harmful effects of ROS in biological systems manifest when there is an overproduction of ROS/RNS and or a deficiency of neutralizing enzymatic and non-enzymatic antioxidants. The oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant/ antioxidant reactions in living organisms. The excess ROS can damage cellular lipids, proteins, or DNA and thus compromise their normal physiological function. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the ageing process. The process of "redox regulation" protects living organisms from various oxidative stresses and maintains "redox homeostasis" by controlling the redox status *in vivo* (Dore, 2002).

Phenolic compounds present in plants have many health beneficial effects, even though they are considered as non-nutritive compounds. A considerable amount of research has been directed towards their activity as antioxidants as well as their antimicrobial, antimutagenic and anti-carcinogenic properties. The antioxidative properties of phenolic acids play an important role in the stability of food products, as well as in the antioxidative defence mechanisms of biological systems (Macheix and Fleuriet, 1998).

An antioxidant is a substance that, when present at a low concentration compared to that of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1995). Even though plant phenols are not always treated as real antioxidants in the literature, many *in vitro* studies have demonstrated the antioxidant potential of phenols as direct aqueous phase radical scavengers and as agents capable of enhancing the resistance to oxidation of low density lipoproteins implicated in the pathogenesis of coronary heart disease (Rice-Evans *et al*., 1995). It is demonstrated that, a part of the antioxidant capacity of many fruits and berries is derived from flavonoids (Wang *et al*., 1996, Heinonen *et al*., 1998) and, in fact, all the major phenolic constituents of food show greater efficacy in these systems as antioxidants on a molar basis than the antioxidant nutrients vitamin C, vitamin E, and β-carotene (Vinson et al., 1995). Differences between the antioxidant potential of selected compounds can be measured using many different techniques. Because most phytochemicals are multifunctional, a reliable antioxidant protocol requires the measurement of more than one property relevant to either foods or biological systems (Frankel and Meyer 2000). In this chapter, the effect of bitter cumin extracts on different antioxidant systems such as reducing power, antiradical activity, enzymatic and non-enzymatic lipid peroxidation activity, superoxide anion scavenging activity and protection against oxidative DNA are presented.

The reducing power of bitter cumin extracts were tested on phosphomolybdenum reduction and potassium ferricyanide reduction. The reduction of Mo(VI) to Mo(V) by reducing agent is being employed to test the antioxidant activity of the sample. The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex with a maximal absorption at 695 nm. Bitter cumin extracts (AMAEBC) significantly reduced phosphomolybdenum and there was a direct relationship between the concentration of bitter cumin extract and reduction of phosphomolybdenum.

The reducing power of a compound is related to its electron donation ability and therefore serves as an indicator of antioxidant activity. In potassium ferricyanide reducing assay, the reductants (antioxidants) in the samples would reduce Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} can be monitored by measuring the complex formation with Perl's Prussian blue at 700 nm (Chung *et al*., 2002). Bitter cumin extract (AMAEBC) showed reducing power activity and the antioxidative power of bitter cumin extracts seems to be due to the electron donating ability of the phenolic compounds present in them.

The model of scavenging the stable $DPPH^{\bullet}$ is a widely used method to evaluate the free radical scavenging ability of various samples (Lee *et al*., 2003; Nagai *et al*., 2003). Use of DPPH• provides an easy and rapid way to evaluate the antiradical activities of antioxidants. This method is useful to assess both polar and non-polar samples. In the DPPH• free radical method antioxidant efficiency is measured at ambient temperature and thus eliminates the risk of thermal degradation of the molecules tested. The $DPPH^{\bullet}$ which is a nitrogen centered radical has deep purple colour and characteristic absorption maxima at 517 nm, whereas reduction product, $DPPH_2$ is yellowish colour and does not have absorption at 517nm. The degree of dissociation indicates the scavenging potential of antioxidant.

DPPH• + AH --------- DPPHH + A•

DPPHH is the reaction product between DPPH• and antioxidant. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability. But, on the basis of the kinetic analysis of the reaction between phenols and $DPPH^{\bullet}$, it was suggested that the reaction, in fact, behaves like an electron transfer reaction (Foti *et al*., 2004). However, the reaction mechanism between the antioxidant and $DPPH^{\bullet}$ depends on the structural conformation of the antioxidant. In this study, bitter cumin extracts (AMAEBC) showed significant $DPPH^{\bullet}$ scavenging activity in both static and kinetic methods. In static method, the amount of $DPPH^{\bullet}$ scavenged by bitter cumin extract was tested which determines the stoichiometry for the reaction of $DPPH^{\bullet}$ with H-donor and quantity of active OH- group in complex mixture.

In kinetic method, the scavenging of the DPPH$^{\bullet}$ by various concentrations of AMAEBC was studied at different time intervals. Figure 4 presents the curves obtained during the scavenging of DPPH$^{\bullet}$ by AMAEBC and it was evident that DPPH scavenging could be separated in two phases. In the first phase a rapid scavenging of DPPH$^{\bullet}$ was observed in first 30 s, and in the second phase the radical was being scavenged at a very slow rate for 5 min. In the first phase the decrease of DPPH concentration was 50 to 90 times rapid in 30 s. The stoichiometries of the rapid and slow kinetic reaction are basic elements for the explanation of the antiradical activity, because it reveals the contribution of the different functional groups to scavenging reactions. The antiradical effect could be due to hydrogen-donating ability of mixture of phenolic compounds present in bitter cumin extracts. Thus bitter cumin extracts could serve as a potent free radical inhibitor or scavenger and act as primary antioxidant.

Generation of the ABTS [2,2'-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] radical cation (Wolfenden and Willson, 1982) forms the basis of one of the spectrophotometric methods that have been applied for the measurement of the total antioxidant activity of solutions of pure substances (Rice-Evans *et al*., 1996, Miller *et al*., 1996), aqueous mixtures and beverages (Salah *et al*., 1995; Rice-Evans and Miller, 1995). The production of blue/green ABTS$^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate has an absorption maxima at 734 nm. Antioxidants reduces the ABTS radical cation (ABTS$^{\bullet+}$), which results in decolourization of ABTS$^{\bullet+}$ chromatophore and the degree of decolourization indicates the scavenging potential of the antioxidant. The method is useful to study both polar and non-polar samples. In this study, the antioxidant potential of bitter cumin extracts was assessed to quench ABTS$^{\bullet+}$ cation radical. AMAEBC, AMBC and AEBC showed good scavenging efficiency with IC_{50} values of 8.3 ± 0.28, 47.0 ± 1.08 and 68.5 ± 1.6 7 µg respectively. The ABTS cation radical scavenging activity can also be correlated with the total phenolic content of bitter cumin extracts. AMAEBC with highest phenolic content showed highest antiradical scavenging activity and AEBC with lowest phenolic content showed least antioxidant activity.

Superoxide anion is a reduced form of molecular oxygen and plays an important role in the formation of other types of ROS such as hydrogen peroxide, $OH^{\bullet}$ and singlet oxygen (Lee *et al*., 2004). Superoxide has also been observed to initiate lipid peroxidation (Wickens, 2001). Despite involvement in many pathological processes, the superoxide radicals by themselves are not as reactive as the hydroxyl radicals. But, they can give rise to highly reactive hydroxyl radicals which can damage bio-macromolecules either directly or indirectly (Cotelle *et al*., 1992). The superoxide radicals have been implicated to play important roles in ischemia-reperfusion injury (Radi, *et al*., 1991). Superoxide radicals are produced by a number of enzyme systems in autooxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complexes such as cytochrome *c*. Thus, scavenging of these radicals would be a promising remedy to alleviate free radical mediated oxidative stress. Bitter cumin extracts showed significant scavenging ability of superoxide anion with an IC_{50} of 125.4 ± 8.7μg/mL of AMAEBC.

Lipoxygenase (LOX) (EC1.13.11.12) is an enzyme that is found in many plants and animals, which catalyses the oxygenation of polyunsaturated fatty acids (PUFA) to form fatty acid hydroperoxides. Lipoxygenase has negative implications for colour, off-flavour and antioxidant status of plant based foods (Casey *et al*., 1996). In mammals lipoxygenase plays an important role in generation of leukotrienes which are implicated in pathophysiology of chronic inflammatory diseases such as asthma, allergic rhinitis, psoriasis and rheumatoid arthritis. Lipoxygenases are, therefore, considered as one of the potential targets for herbal inhibitors for the treatment of a variety of disorders and autoimmune diseases. In this study the modulatory effect of bitter cumin extracts on soybean lipoxygenase mediated lipid peroxidation was studied. During lipid peroxidation reaction, the native resting lipoxygenase enzyme in the ferrous state ($E\text{-}Fe^{2+}$) is activated to an active ferric state ($E\text{-}Fe^{3+}$) by low levels of hydroperoxide present as a contaminant in substrate and initiates the lipid peroxidation reaction (De Groot *et al*., 1975). The lipid peroxy radicals generated by oxidation of linoleic acid by soybean lipoxygenase are highly reactive radicals and further initiate oxidation of lipid and propagate lipid

peroxidation. Plant phenols and flavonoids are known to inhibit lipid peroxidation by quenching lipid peroxy radicals and or reduce or chelate iron in lipoxygenase enzyme and thus prevent initiation of lipid peroxidation reaction (Takahama, 1985; Kubo *et al*., 2006; Sadik *et al*., 2003). Similarly, the phenolic compounds such as quercetin, caffeic acid, ferulic acid and ellagic acid present in AMAEBC being free radical scavengers could react with peroxy radical before the fatty acid react with peroxy radicals and thus inhibit lipid oxidation. The data obtained from the present study demonstrated the ability of AMAE, AME, and AE of bitter cumin to significantly inhibit lipoxygenase dependent lipid peroxidation. Thus the phenolic compounds present in bitter cumin may prevent the oxidation of lipids and formation of oxidized lipids in *in vivo* systems.

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. Hydroxyl radicals have the highest 1-electron reduction potential (2310 mV) and can react with everything in living organisms at the second-order rate constants of 109–1010 mol/s. The hydroxyl radical has a high reactivity, making it a very dangerous radical with a very short *in vivo* half-life of approx. 10^{-9} s (Pastor *et al*., 2000). Hydroxyl radicals react with lipid, polypeptides, proteins, and DNA especially thiamine and guanosine. In biological systems, lipid peroxidation (oxidative degradation of polyunsaturated fatty acids in the cell membrane) generates a large number of degradation products such as malondialdehyde (MDA), which are found to be an important cause for cell membrane destruction and cell damage (Kubow,1992). Microsomes, especially smooth surfaced endoplasmic reticulum, are particularly susceptible to oxidative stress because of the high content of polyunsaturated fatty acids, hence have been widely used as a model for oxidative stress and antioxidant studies (Cos *et al*., 2001). Iron (Fe^{2+}) plus a reducing reagent is an extensively used system for generating hydroxyl radicals to induce lipid peroxidation. MDA, one of the major products of lipid peroxidation, has been extensively used as an index for lipid peroxidation and as a marker for oxidative stress. The reaction of MDA with TBA has been widely adopted as a sensitive assay method for lipid

peroxidation (Ohkawa *et al*., 1979). The present study has used hydroxyl radical generated by Fenton reaction to induce peroxidation of rat liver microsomes to investigate the antioxidant effect of bitter cumin seed extract. AMAEBC showed good inhibition of hydroxyl radical mediated rat liver microsomal lipid peroxidation with an IC_{50} value of 110 ± 14 μg. The antioxidant activity of AMAEBC could be accounted for the direct interaction or scavenging of hydroxyl radicals or due to a metal chelating activity by phenolic compounds of bitter cumin (Carbonaro *et al*., 1996).

Cellular membranes, which contain abundant phospholipids, such as phosphatidylcholine (lecithin), are major targets of free radicals which induce lipid peroxidation and thereby cause malfunctioning of membranes by altering membrane fluidity and membrane bound enzyme and receptor functions (Jana *et al*., 1990). Further, several harmful by products of the lipid peroxidation can damage other biomolecules including DNA, away from the site of their generation (Box and Maccubbin, 1997; Esterbauer, 1996). Liposomes are spherical, self closed vesicles of colloidal dimensions, in which (phospho) lipid bilayer sequesters part of the solvent, in which they freely float (Bangham and Home, 1964). The use of liposomes is suggested to be the most promising method of assessing antioxidant properties relevant to human nutrition, since these systems allow investigations on the protection of an antioxidant in a model biological membrane or lipoprotein. Phospholipid liposomes undergo rapid peroxidation when incubated with $FeCl_3$ and ascorbic acid at pH 7.4. Ascorbic acid at low concentration accelerates lipid peroxidation by reducing iron to the active ferrous state (Magdalena *et al*., 2001). Al-Ismail and Aburjai (2004) reported that water and alcohol extracts of the seeds of anise (*Pimpinella anisum* L) and dill (*Anethum graveolens* L) inhibited liposomal peroxidation and the activity was directly related to total phenolic content. In addition, Chen *et al*., (2007) shown that the antioxidant activities of herbs in a liposome system correlated significantly with the total phenolic compounds ($r = 0.968$, $P < 0.05$). In this study, AMAEBC with high phenolic content showed highest antioxidant activity and AEBC with lowest phenolic content showed least antioxidant activity against liposomal oxidation. However, it has been reported that tomato, broccoli, turmeric, lotus stem and

Brussels sprout, which have low phenolic contents, showed high antioxidant activities (Charanjit and Harish, 2002). Thus the high antioxidant activity of bitter cumin seeds can be related to the content of mixture of phenolic compounds which may have additive antioxidant activity against liposomal peroxidation.

DNA damage results in mutations and altered cell functions. A large proportion of these mutagenic steps precede carcinogenic events. In this study, hydroxyl radical mediated DNA damage was induced using Fenton reaction (Ferric chloride and ascorbic acid). AMAEBC was tested for its potential to inhibit the DNA damage induced by hydroxyl radical in calf thymus DNA, prokaryotic genomic DNA and plasmid DNA (puc^{18}). Figure 11 shows the calf thymus DNA damage by hydroxyl radicals and the dose dependent protection by AMAEBC. The relative mobility of oxidized DNA and AMAEBC treated DNA were comparable to that of native undamaged DNA indicating the protection offered by bitter cumin extract. Similarly the hydroxyl radical mediated oxidative damage to bacterial DNA was inhibited by AMAEBC and BHA (Figure 12). However, there were no marked differences in the protective effect with increased concentration of AMAEBC. The pUC^{18} is a 2.69 kb plasmid with an ampicillin resistant marker on it. In this study hydroxyl radicals induced breakage of super coiled DNA to nicked DNA (Figure 13). Different concentrations of AMAEBC protected plasmid DNA from non specific DNA breakage as shown in Figure 13 in lanes 3 to 5. Aqueous extracts of chilli, black pepper and turmeric are reported to inhibit radiation induced oxidative damage to DNA (Sharma *et al*., 2000). The protective effect of AMAEBC against hydroxyl induced damage to DNA and supercoiled DNA can be related to the scavenging of hydroxyl radical by polyphenolic compounds present in AMAEBC.

Free radicals (reactive oxygen species (ROS) like superoxide, hydroperoxide, peroxyl and hydroxyl radicals and reactive nitrogen species (RNS) like nitric oxide) are constantly produced in the body during physiological processes, such as aerobic respiration in mitochondria, xenobiotic metabolism or disposal of infected cells by phagocytes (Ames *et al*., 1993). Once formed, these

radicals rapidly react with macromolecules viz., lipids, lipoproteins, proteins, carbohydrates, RNA and DNA and thus starting self-propagating radicals which alter or destroy the structure and function of important cell components. Alternatively, the ROS may react with other free radicals to form stable products or be scavenged by antioxidants, or transformed into non-radical species, while the antioxidants become 'antioxidant-radicals', which are much less reactive and do not efficiently attack adjacent macromolecules (Halliwell, 1996). The excess formation of free radicals caused by an imbalance of oxidative and antioxidative processes, leads to oxidative stress (Sies, 1997), which is believed to be the basis of many degenerative diseases, such as atherosclerosis, cardiovascular disease (CVD), stroke, cancer, arthritis, and Alzheimer's disease (Davies, 1995).

TABLE 3. Antioxidant activity of AMAEBC in different antioxidant assays

Antioxidant assay	Radical	IC_{50} values/absorbance	
		AMAEBC	BHA
Phosphomolybdenum reducing power	-	0.31 ± 0.010	0.49 ± 0.019
Ferricyanide reducing Power	-	0.20 ± 0.020	0.68 ± 0.014
ABTS radical assay	$ABTS^{\bullet+}$	8.3 ± 0.28 μg	1.8 ± 0.06 μg
DPPH radical assay	$DPPH^{\bullet}$	20.8 ± 0.18 μg	8.2 ± 0.24 μg
Liposomal lipid peroxidation	$OH^{\bullet}$	14.3 ± 1.9 μg	580.0 ± 0.09 μg (α-Tocopherol)
Soybean lipoxygenase lipid peroxidation	$LOO^{\bullet}$	28.0 ± 3.0 μg	14.0 ± 0.25 μg
Rat liver microsomal lipid peroxidation	$OH^{\bullet}$	110.0 ± 14 μg	70.0 ± 2.4 μg
Superoxide radical	$O_2^{\bullet-}$	125.4 ± 8.7μg	11.4 ± 0.79 μg

Values are ± S.E.M of 3 experiments.

The body defends itself against oxidative damage through an antioxidant network such as vitamin C, vitamin E, glutathione etc. In addition to the endogenous antioxidants, the phenolic compounds from medicinal plants are suggested to offer protection against oxidative stress by neutralizing the ROS. In this context, the present study has evaluated the antioxidant potential of bitter cumin extract containing a mixture of phenolic compounds. Aqueous methanol acetone extract of bitter cumin (AMAEBC) demonstrated significant antioxidant activity at microgram concentration including reducing power, radical scavenging ability and protection against DNA damage. However, aqueous methanol extract (AMEBC) and aqueous extract (AEBC) of bitter cumin showed relatively less antioxidant activity due to less total phenol content compared to AMAEBC. Thus antioxidant activity of bitter cumin is correlated with total phenol content of the extracts. AMAEBC showed a wide range of antioxidant activity by scavenging or neutralizing radicals such as DPPH $^{\bullet}$ anion radical, ABTS$^{\bullet}$ cation radical, hydroxyl ($^{\bullet}$OH), lipid peroxy radical (LOO$^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) and also inhibited oxidative DNA damage (Table 3 and Figures 11, 12 and 13). The phenolic acids such as ferulic acid, caffeic acid, gallic acid, ellagic acid, protocatechuic acid and flavonoids such as quercetin and kaempferol found in bitter cumin seeds are reported to have good antioxidant activity. Hence, the phenolic compounds present in bitter cumin could be responsible for its antioxidant activity.

Chapter IV

Studies on anti-diabetic activity of bitter cumin seeds

4.1 INTRODUCTION

Diabetes is a major health problem associated with excess morbidity and mortality. One of the major public health challenges of the 21st century is increasing diabetes in the world. Today, diabetes causes 9% of the total world's mortality. It is the 5th leading cause of death worldwide, the leading cause of blindness in working-aged adults and accounts for 50% of non traumatic lower-limb amputations and 35% of new case of end-stage kidney disease. Diabetes causes a 2-4 fold increase in the risk of heart diseases.

4.1.1. Prevalence of Diabetes

WHO estimates that, by 2025, about 200-300 million people worldwide may suffer from this disease. The prevalence of diabetes is steadily increasing more in Asian countries like India and China. In the year 1995, there were 135.5 million diabetics in the world, of whom 19.4 million were in India. In 2000, the figures were 151 million and 31.7 million, respectively. By 2025, there will be over 57.2 million diabetics in India and for this reason, India is being considered as the *"World's diabetes capital".* A study was undertaken under a programme of Prevalence of Diabetes in India (PODIS), to determine the prevalence of diabetes and impaired glucose tolerance (prediabetic stage) in subjects aged 25 years and above in India. The study was carried out in 77 centres (40 urban and 37 rural areas) and 40000 subjects were studied. The standardized prevalence rate for diabetes in the Indian urban semi urban and rural populations was 4.3, 5.9 and 2.7% respectively. The corresponding pre diabetes rates in the three populations were 5.2, 6.3 and 3.7% respectively. The urban prevalence of diabetes and impaired glucose tolerance was significantly greater than in the rural population.

National Urban Diabetes Study (NUDS), a study undertaken to assess the prevalence of diabetes and prediabetes in six major cities, covering all the regions of the country was published in 2001. This study showed that age standardized prevalence of diabetes and prediabetes were 12.1% and 14.0% respectively, with no gender difference.

The reason for increasing incidence of diabetes is prolonged survival rate that is, increasing pool of elderly population, increased weight, urbanization and sedentary physical activity. Urbanization is associated with sedentary life style, availability of food in plenty, affluence and stress of urban culture and life. There is external migration to western countries and internal migration from villages to cities, not to mention urbanization of villages with the availability of comforts in villages. Thus villages are slowly converting to towns and towns are becoming cities. Few studies have shown that increasing TV viewing is associated with increasing obesity in children and adolescents. Participation of children and youth in sports activities is decreasing leading to obesity, over eating while watching TV and thus contributing to increasing prevalence of diabetes in the country.

4.1.2. Role of dietary and medicinal plants in the treatment of diabetes

Despite the great strides that have been made in understanding and management of diabetes, the disease and disease-related complications are increasing unabated. The therapeutic approach to manage type I and some type II diabetics include, use of insulin and other agents like amylin analogues; and for type II include alpha glucosidase inhibitors like acarbose, miglitol, voglibose, sulphonylureas and biguanides. However, most of the antidiabetic drugs have certain adverse effects like at higher doses, liver problems, lactic acidosis and diarrhoea. Thus, the management of diabetes without any side effects is still a formidable challenge. In addition to the currently available therapeutic options, many herbal medicines have been recommended for the treatment of diabetes. Traditional plant based medicines are used throughout the world for a range of diabetic presentations. Herbal drugs are prescribed widely because of their effectiveness, less side effects and relatively low cost (Venkatesh *et al*., 2003). Therefore, investigation on bioactive phytochemicals from traditional medicinal plants has become an important area of research in India (Suba *et al*., 2004). India has a rich history of using various potent herbs and herbal components for treating diabetes since ancient times. Many Indian plants have been investigated for their beneficial use in different types of diabetes.

α-amylase (EC 3.2.1.1.) is the most abundant enzyme in human saliva and it catalyzes initial break down of starch to oligosaccharides in mouth during chewing of food. α-glucosidase (AG) (EC 3.2.1.20) is a complex group of carbohydrases that catalyzes the liberation of glucose from disaccharides in the final step of digestive process of carbohydrates. Thus inhibition of specific α-amylase and α-glucosidases can retard the dietary uptake of carbohydrates and suppress postprandial hyperglycemia. A number of plant extracts have shown to have inhibitory effect on both α-glucosidase and α- amylase activity and some of them have clinical importance (Yoshikawa *et al*., 1998).

This chapter deals with the modulatory effect of bitter cumin seed phenolic extract on α-amylase, α-glucosidase, postprandial hyperglycaemia in maltose over loaded rats, blood plasma glucose levels in STZ diabetic rats and high fructose fed, low STZ injected diabetic rats.

4.2. Materials and Methods

4.2.1. Chemicals

Human salivary α-amylase, Dinitro salicylic acid (DNS), Streptozotocin, Thiobarbituric acid and Tris base were purchased from Sigma Chemicals (St.Louis, MO, USA). p-nitrophenyl glucopyranoside (PNP-G), glucose and fructose were purchased from E.Merck (Darmstadt, Germany). DL-Catechin was purchased from ICN biomedicals Inc. (Irvine, CA). Starch, maltose, sucrose, Sodium dodecyl sulphate (SDS), and heparin were purchased from Sisco Research Laboratories (Mumbai, India). Acarbose (Glucobay, 50 mg/tablet) was obtained from Bayer medical company (Leverkusen, Germany). Glucose estimation kit was purchased from Monozyme India Pvt. Ltd. (Secunderabad, India). D-fructose was purchased from Ace laboratories Bangalore, India. All other chemicals and solvents used were of analytical grade.

4.2.2. Bitter cumin extract

Bitter cumin extract (AMAEBC) was prepared as explained in the chapter II

4.2.3. Effect of bitter cumin seed extract on α-glucosidase activity

4.2.3.1. Isolation of α-glucosidase crude enzyme from rat intestine

Male CFT Wistar rats weighing around 150 g were used for this experiment. The rats, after overnight fasting, were dissected under mild anaesthesia with ether. The small intestine was dissected open and washed with ice cold saline. The epithelial membrane of the intestine was carefully scrapped out and homogenized with phosphate buffered saline for 1 min. The homogenate was centrifuged at 11952xg for 20 min at 4°C. The supernatant was used as crude α-glucosidase enzyme. All the preparations were carried out at 4 °C. The protein content of the enzyme preparation was estimated by Lowry's method (Lowry *et al*., 1951).

4.2.3.2. Inhibition of rat intestinal α-glucosidases by bitter cumin seed extract

Rat intestinal α-glucosidase activity was studied according to the method of Matsui *et al*., (2001). The rat intestinal maltase, sucrase and p-nitrophenol glucopyranoside (PNP-glucoside) hydrolase activity were studied using the substrates sucrose, maltose and PNP-glucoside.

4.2.3.2.1. PNP-glucoside hydrolase activity

0.5 mg protein of crude α-glucosidase enzyme was incubated with different concentrations of AMAE of bitter cumin for 5 min at ambient temperature. The reaction was started by adding 0.7 mM PNP-glucoside and final volume of the reaction mixture was made up to 1 mL with 100 mM phosphate buffer pH, 6.8. The reaction mixture was incubated for 20 min at 37 °C and the reaction was stopped by adding 1 mL of 0.5 M Tris base. α-glucosidase activity was determined by monitoring the amount of p-nitrophenol liberated from PNP-glucoside at 400 nm using a Shimadzu UV-Visible spectrophotometer (Model, 2100). The inhibitory effect of bitter cumin on α-glucosidase will cause a decrease in the release of p-nitrophenol from PNP-glucoside.

4.2.3.2.2. Maltase activity

0.5 mg protein equivalent of crude α-glucosidase preparation was incubated with different concentrations of AMAE of bitter cumin for 5 min at ambient temperature. The reaction was started by adding 6.0 mM maltose and final volume of the reaction mixture was made up to 1 mL with 100 mM phosphate buffer pH, 6.8. The reaction mixture was incubated for 30 min at 37 °C and the reaction was stopped by adding 1 mL of 0.5 M Tris base. Maltase activity was determined by monitoring the amount of glucose liberated from maltose using glucose oxidase method (Trinder, 1969). The inhibitory effect of bitter cumin on maltase activity will cause a decrease in the release of glucose from maltose.

4.2.3.2.3. Sucrase activity

0.5 mg protein equivalent of crude α-glucosidase preparation was incubated with different concentrations of AMAEBC for 5 min at ambient temperature. The reaction was started by adding 45.0 mM sucrose and final volume of the reaction mixture was made up to 1 mL with phosphate buffer (0.1 M, pH, 6.8). The reaction mixture was incubated for 45 min at 37°C and the reaction was stopped by adding 1 mL of 0.5 M Tris base. Sucrase activity was determined by monitoring the amount of glucose liberated from sucrose using glucose oxidase peroxidase method (Trinder, 1969). The inhibitory effect of bitter cumin on sucrase activity will cause a decrease in the release of glucose from sucrose.

4.2.3.3. Enzyme kinetic study on α-glucosidase inhibitory effect of bitter cumin seed extract

The enzyme kinetics on inhibition of α-glucosidase activity was studied by using different concentrations of substrates sucrose, maltose and PNP-glucoside versus IC_{20}, IC_{40} and IC_{60} concentrations of sucrase, maltase and PNP-glucoside hydrolase activity of AMAEBC as described above. Double reciprocal plots of enzyme kinetics were constructed according to Lineweaver and Burk method (Lineweaver and Burk, 1934). K_m, V_{max} and K_i values were calculated from Lineweaver and Burk plots.

4.2.4. Inhibitory effect of bitter cumin seed extract on human salivary α-amylase activity

α- amylase activity was estimated as per the method of Kim *et al*., (2005) with some modifications. Human salivary α-amylase enzyme (60 mU) was incubated with or without AMAEBC for 5 min at ambient temperature in 50 mM phosphate buffer pH 7.4 in a final volume of 1 mL. The reaction was initiated by adding 1.0 mL of 1% starch solution. The reaction mixture was incubated for 10 min at 37°C and the reaction was terminated with 1.0 mL of 3, 5-dintrosalicyclic acid (DNS) reagent and the tubes were boiled for 10 min in a boiling water bath. The absorbance of the solution was measured at 540 nm in Shimadzu model UV-VIS spectrophotometer. The blank was made of all the constituents except the enzyme.

4.2.5. *In vivo* studies on antihyperglycemic activity of bitter cumin seed extract

4.2.5.1. Animals

Male Wistar rats [OUTB-Wistar, Ind-cft (2c)] were obtained from Animal House facility of Central Food Technological Research Institute, Mysore. They were housed in a room, maintained at 25 ± 2°C with a relative humidity of 60–70% and exposed to a light and dark cycle of 12 h duration and were provide with standard food and water *ad libitum*. Protocols adopted for animal experiment were based on the animal ethical committee guidelines on control and supervision of experiments on animals by the Government of India, Ministry of Social Justice and Empowerment. Animals were kept individually in stainless steel cages.

4.2.5.2. Effect of bitter cumin extract on *in vivo* oral maltose tolerance test

This experiment is based on measuring the postprandial plasma glucose levels at different time intervals following oral maltose ingestion. The animals were allowed to acclimatize for one week with standard pellet diet. All the animals were randomly divided into five groups. The animals were starved overnight and the individual groups were fed orally with the following combinations.

Group 1	Control	
Group 2	Acarbose	(10 mg/kg b.wt)
Group 3	AMAEBC	(50 mg/kg b.wt)
Group 4	AMAEBC	(100 mg/kg b.wt)
Group 5	AMAEBC	(200 mg/kg b.wt)

The control group received normal saline (0.9 % NaCl); experimental groups were orally fed by gastric intubation with 10 mg/kg b.wt of acarbose and 50, 100 and 200 mg/kg b.wt of AMAE of bitter cumin. 15 min after the above treatment, all the rats were orally fed with 2.0 g/kg b.wt of maltose. Blood samples were collected immediately and at 30, 60, 90,120 min after maltose ingestion under mild anaesthesia from retro orbital plexus. Plasma was separated and glucose was estimated by glucose oxidase method (Trinder, 1969).

4.2.5.3. Effect of bitter cumin extract on streptozotocin induced diabetic model

Induction of diabetes in rats with streptozotocin was followed according to the method of Hatch *et al*., (1995). After one week of acclimatization, the rats were subjected to 12 h fast. Diabetes was induced by a single intraperitoneal injection of 55 mg/kg b.wt of STZ dissolved in 0.01 M citrate buffer pH 4.5. The rats were given 5% glucose solution in drinking water for two days and normal water thereafter. On the 8th day after the STZ injection, the animals were subjected to overnight fast. Blood samples from retro orbital plexus were collected under mild anaesthesia into heparinized tubes. The blood plasma was separated within 30 min after blood collection and plasma glucose was estimated by glucose oxidase method (Trinder, 1969). The rats with blood glucose of >300 mg/dL were considered to be diabetic and used for the experiment. The rats were randomly divided into two groups with six animals in each group.

Group 1 Diabetic control

Group 2 AMAEBC (50 mg/kg b.wt)

STZ diabetic control group received by oral intubation normal saline (0.9 % NaCl), the diabetic experimental group were given 50 mg/kg b.wt of AMAEBC for a period of 8 days. The food intake and body weight gain were periodically measured. Rats were subjected to overnight fast and killed under mild anaesthesia. Blood samples were collected in heparanized tubes (20 U/mL of

blood) and plasma was separated. The plasma glucose was estimated by glucose oxidase method (Trinder, 1969). Liver, kidney, brain, testes and intestine were excised, washed with cold saline and stored at -80°C till further analysis.

The lipid peroxidation product malondialdehyde (MDA) levels in different tissues were measured as TBARS according to the method of Ohkawa *et al*., (1979). To 1 mL of 10% tissue homogenate 0.2 mL of SDS (8.1 %) was added and the solution was precipitated with 1.5 mL of acetic acid, pH 3.0. To this solution, 1.5 mL of 0.8% freshly prepared thiobarbituric acid (TBA 10 %) was added and vortexed well. The tubes were incubated for 1 h in a boiling water bath. The colour was extracted into equal volume of butanol and measured at 535 nm. The results were expressed as nmoles of malondialdehyde (MDA) formed per mg protein of the tissue using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

4.2.5.4. Effect of bitter cumin extract on high fructose fed low streptozotocin injected diabetic model

High fructose and low streptozotocin diabetic model was adopted according to the method of Luo *et al*., 1998. Male Wistar rats after one week of acclimatization with basal diet were randomly divided into six groups. The first group received semi synthetic diet containing normal starch as source of carbohydrate, second group in addition to the diet mentioned for the first group received 100 mg/kg b.wt of AMAEBC through oral intubation. The third group received fructose (60%) diet and hyperglycemia was induced in fourth, fifth and sixth group of rats by intra peritoneal injection of STZ at a dosage of 25 mg/kg b.wt after 2 weeks of fructose feeding and were continued with diet containing fructose (60%) as the sole source of carbohydrate. STZ treated rats were given 5% glucose solution for a day. On the 8^{th} day after STZ injection, the animals were subjected to overnight fast. Blood samples from retro orbital plexus were collected under mild anaesthesia into heparinized tubes. The blood plasma was separated within 30 min after blood collection and plasma glucose was estimated by glucose oxidase method (Trinder,

1969). The rats of groups 4, 5 and 6 with blood glucose of >150 mg/dL were considered to be hyperglycemic and used for the experiment. The composition of starch and fructose based diets are given in Table1 and 2. Fifth and sixth group of rats were orally intubated with 25 and 100 mg/kg b.wt of AMAE BC for a period of four weeks.

Group 1 Starch diet (SD)

Group 2 Starch diet and AMAEBC (100 mg/kg b.wt)

Group 3 Fructose diet (FD)

Group 4 STZ diabetic rats on fructose diet

Group 5 STZ diabetic rats on fructose diet and AMAEBC 25 mg/kg b.wt

Group 6 STZ diabetic rats on fructose diet and AMAEBC 100 mg/kg b.wt

TABLE 1. The diet composition of starch and fructose based diets

Composition of diet	g/ Kg diet	
	Starch fed group	Fructose fed group
Starch	600	-
Fructose	-	600
Casein	200	200
Groundnut oil	50	50
Cellulose	96	96
Salt mixture	35	35
AIN-76 Vitamin mixture	10	10
Methionine	7	7
Choline	2	2

TABLE 2. Composition of AIN-76 Vitamin mixture (g/kg diet)

Vitamins	Amount (g/Kg)
Thiamine hydrochloride	0.6
Riboflavin	0.6
Pyridoxine hydrochloride	0.7
Nicotinic acid	3.0
D-Calcium pantothenate	1.6
Folic acid	0.2
D-Biotin	0.02
Cyanacobalamin (Vit. B12)	0.001
Retinyl acetate	0.4 (4,00,000 IU)
DL-α-tocopherol acetate	7.7 (5000 IU)
Cholecalciferol	0.0025 (1,00,000 IU)
Menadione	0.005

The food intake and body weight gain were periodically measured. Rats were subjected to overnight fasting and killed under mild anaesthesia. Blood samples were collected in heparanized tubes (20 U/ mL of blood) and plasma was separated within 30 min after blood collection. The plasma glucose was estimated by glucose oxidase method (Trinder, 1969). Liver, kidney, brain, and testes were excised and weighed.

4.2.6. Statistical analysis

All the *in vitro* experiments were done at least in triplicates and the results were expressed as ± SEM. An *in vivo* experimental value represents ± SEM of at least 5 readings. Statistical significance between the groups were calculated by one way ANOVA using the software SPSS (Release 7.5.1) and the individual comparison were obtained by Duncan's Multiple Range Test. A difference in the mean values of $P \leq 0.05$ was considered to be statistically significant.

4.3. Results

4.3.1. Inhibitory effect of bitter cumin extract on α-glucosidase

The inhibitory activity of AMAEBC on maltase, sucrase and PNP- glucoside hydrolase is shown in Figure1 and IC_{50} values of inhibition are shown in Table 3. Phenolic components of AMAEBC inhibited rat intestinal sucrase, maltase and PNP- glycoside hydrolase in a dose dependent manner as shown in Figure1. AMAEBC at 50-100 µg concentration significantly inhibited maltase and sucrase activity compared to synthetic substrate PNP-glucoside.

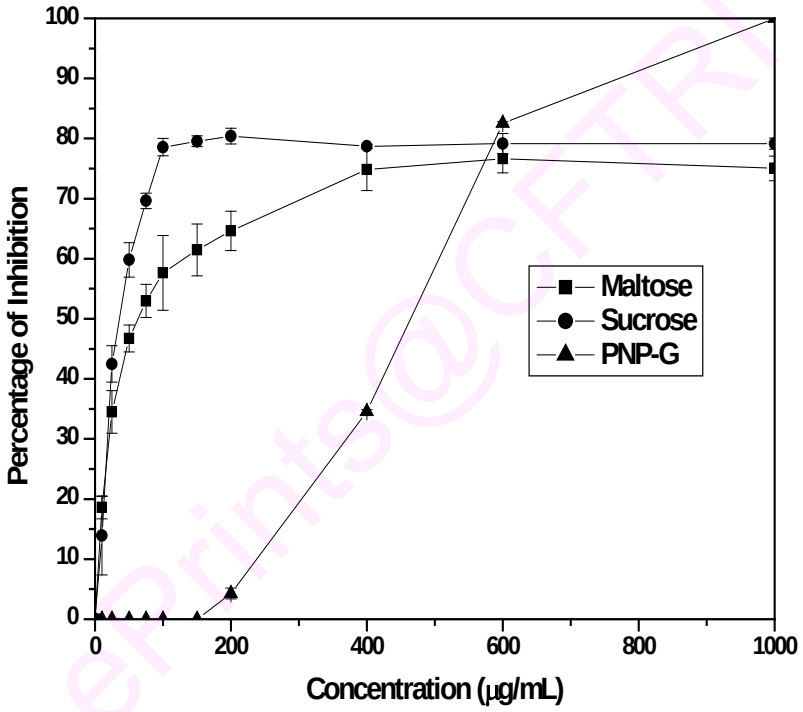


Figure 1. Dose dependent inhibition of rat intestinal maltase, sucrase and PNP-glucoside hydrolase activity by AMAEBC. The values are ± SEM of 3 independent experiments.

The IC_{50} values obtained from the dose response curves are presented in Table 3 for maltase, sucrase and PNP- glucoside hydrolase activities. The IC_{50} values for sucrase, maltase, and PNP-glucoside hydrolase are 34.1 ± 3.8, 62.4 ± 4.5 and 500.5 ± 11.9 µg of AMAEBC respectively. The inhibitory effect of CA against sucrase was about 2 and 14 times higher than maltase and PNP-G hydrolase activity respectively. The inhibitory activity of AMAEBC

on sucrase, maltase and PNP-glucoside hydrolase was relatively less efficient than synthetic AGH inhibitor acarbose (Table 3). However, the inhibitory effect of AMAEBC on sucrase and maltase activities was found to be 8.6 and 29.4 fold higher than DL-catechin, a reported phenolic α-glucosidase inhibitor, but, ≈15 and 624 fold lower than synthetic and therapeutic drug acarbose.

TABLE 3. IC_{50} values of AMAEBC, acarbose and DL-catechin on rat intestinal sucrase, maltase and PNP-glucoside hydrolase inhibition.

Substrates	IC_{50} (µg)		
	AMAEBC	Acarbose	DL-Catechin
Sucrose	34.1 ± 3.8^{b}	2.30 ± 0.2^{c}	1003 ± 80^{a}
Maltose	62.4 ± 4.5^{b}	0.10 ± 0.001^{c}	537.5 ± 7.9^{a}
PNP-G	500.5 ± 11.9^{a}	54.70 ± 3.2^{b}	NI

Values are ± SEM of 3 experiments. Means of the same row followed by different letters differ significantly at $P \leq 0.05$. NI: No inhibition

4.3.1.2. Enzyme kinetic studies on α-glucosidase inhibition by bitter cumin seed extract

Substrate dependent enzyme kinetic studies were carried to understand the nature of inhibition of disaccharidases and PNP-glucoside hydrolase by AMAEBC. Double reciprocal plots of inhibition of maltase, sucrase and PNP-glucoside hydrolase are shown in Figures. 2, 3 and 4. Table 4 summarizes the apparent Michaelis constants (K_m) values V_{max} and K_i values calculated by Lineweaver-Burk plots in Figures 2, 3, and 4. The apparent K_m value varied with the substrate tested. PNP-glucoside showed low Km value of 0.59 mM followed by maltose of 3.3 mM and sucrose of 23.8 mM. This data clearly indicated that the affinity of PNP- glucoside is 5.6 times greater than maltose and 40.3 times greater than sucrose. Since maltose is specific for maltase, sucrose for sucrase, and the use of PNP-glucoside would be valid for

evaluating the overall inhibitory effect of AMAEBC against rat glucosidases composed of a variety of carbohydrases on the surface of small intestines.

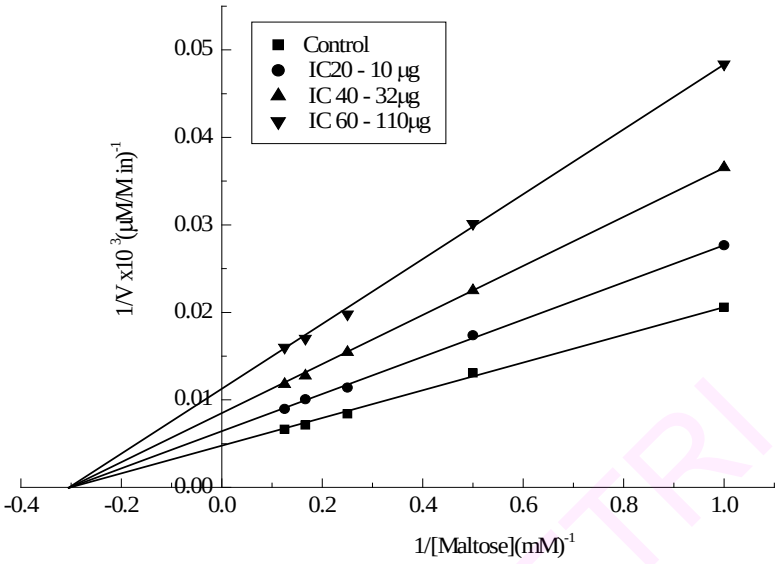


FIGURE 2. A double reciprocal plot of substrate dependent enzyme kinetics on inhibition of rat intestinal maltase activity by phenolic compounds mixture of AMAEBC.

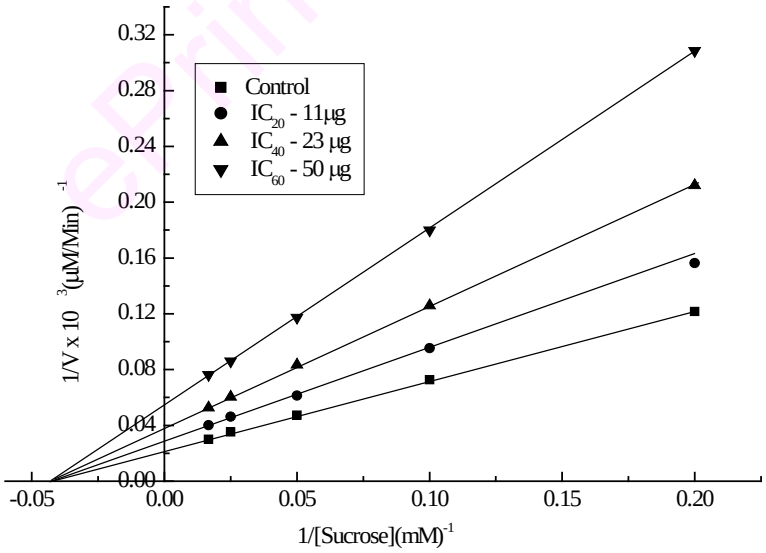


FIGURE 3. A double reciprocal plot of substrate dependent enzyme kinetics on inhibition of rat intestinal sucrase activity by phenolic compounds mixture of AMAEBC.

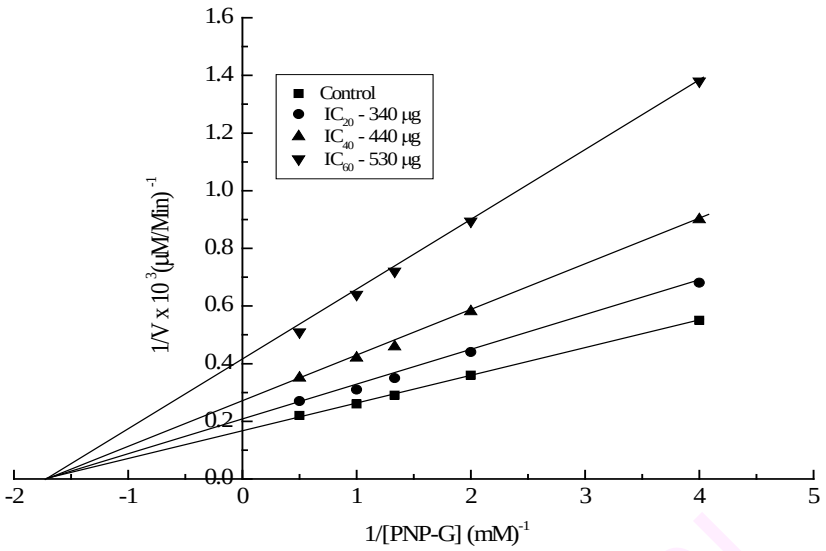


FIGURE 4. A double reciprocal plot of substrate dependent enzyme kinetics on inhibition of PNP- glucoside hydrolysis by AMAEBC

Table 4. Summary of the kinetic behaviour of AMAEBC on inhibition of α-glucosidase

Substrate	Treatment	K_m (mM)	1/ V_{max} 10^3 (µM/min)$^{-1}$	K_i (µg)
	Control	3.52	0.0047	
Maltose	AMAEBC 10 µg	3.39	0.0064	
	AMAEBC 32 µg	3.30	0.0085	76.7
	AMAEBC 110 µg	3.12	0.0112	
	Control	23.51	0.0207	
Sucrose	AMAEBC 11 µg	22.93	0.0291	30.2
	AMAEBC 23 µg	24.35	0.0387	
	AMAEBC 50 µg	24.46	0.0555	
	Control	0.5732	0.1693	
PNP- glucoside	AMAEBC 340 µg	0.5859	0.2088	341.6
	AMAEBC 440 µg	0.6131	0.2695	

4.3.2. Inhibitory effect of bitter cumin extract on α-amylase activity

The inhibitory effect of AMAEBC on human salivary α-amylase is presented in Figure 5. A dose dependent inhibition of α-amylase by AMAEBC was observed with an IC_{50} value of 185.5 ± 4.9 µg and the correlation coefficient between dose and inhibition percent was 0.9864. Inhibitory effect of acarbose was 10 fold higher than AMAEBC with an IC_{50} value of 17.4 ± 0.9 µg.

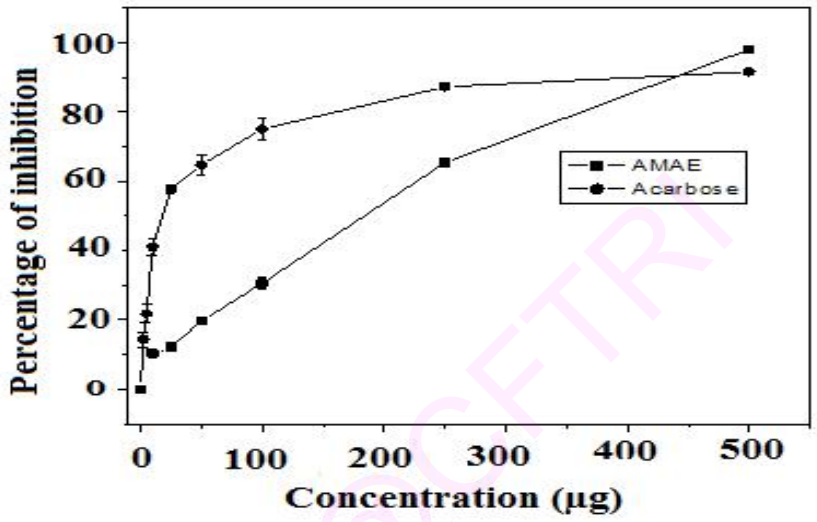


FIGURE 5. Inhibitory effect of AMAEBC and acarbose on human salivary α-amylase activity. Values are ±SEM of three experiments

4.3.3. Effect of bitter cumin seed extract on oral maltose tolerance test

The effect of different concentrations of AMAEBC (50, 100 and 200 mg/kg b.wt) and acarbose (10 mg/kg b.wt) on blood plasma glucose levels after maltose (2.0 g/ kg b.wt) administration is shown in Table 5. The blood plasma glucose level was increased from 64.57 ± 1.65 to 197.33 ± 5.27 mg/dL after 30 min of maltose feeding in control group. A dose dependent decrease in postprandial blood plasma glucose level was observed at 30 and 60 min after oral feeding of 50–200 mg/kg b.wt of AMAEBC when compared with the control and acarbose treated group (Table 5). The decrease in postprandial glucose levels in AMAEBC pre treated rats (50-200 mg/kg b.wt) was significantly more when compared with that of synthetic drug acarbose, but, at a higher dosage.

TABLE 5. Effect of AMAEBC on postprandial plasma glucose levels in oral maltose tolerance test in normal rats

Time (min)	Blood plasma glucose level (mg/dL)				
			AMAEBC (mg/kg b.wt)		
	Control	Acarbose (10 mg/kg b.wt)	50	100	200
0	64.57 ± 1.65[a]	67.46 ± 2.80[a]	59.82 ± 4.04[a]	56.39 ± 4.20[a]	59.50 ± 3.94[a]
30	197.33 ± 5.27[a]	174.83 ± 5.67[b]	165.89 ± 9.22[c]	165.40 ± 5.69[c]	151.46 ± 7.32[d]
60	180.50 ± 3.83[a]	155.52 ± 5.94[b]	144.62 ± 6.49[c]	141.23 ± 5.13[c]	139.55 ± 2.88[c]
90	144.18 ± 3.14[a]	130.67 ± 2.97[b]	130.41 ± 2.34[b]	126.75 ± 4.90[b]	127.61 ± 1.19[b]
120	113.22 ± 2.29[a]	114.58 ± 3.81[a]	107.62 ± 3.78[b]	118.40 ± 2.66[a]	117.44 ± 2.12[a]

Values are mean ± SEM of 5 rats in each group.

Means of same row followed by different letters differ significantly according to Duncan's post hoc test at $P \leq 0.05$.

4.3.4. Anti-hyperglycaemic effect of bitter cumin extract on streptozotocin induced diabetic rats

The body weight gain of STZ diabetic control rats during AMAEBC treatment for a period of 8 days is 0.5 ± 11.5 g. However, STZ diabetic rats treated with AMAEBC (50 mg/kg b.wt) showed a body weight gain of 7.3 ± 9.9 after 8 days. The increase in body weight gain in treated groups was found to be significantly higher than STZ-diabetic control group. Thus oral administration of AMAEBC has a positive effect in maintaining the body weight gain in diabetic rats.

Fasting blood plasma levels of STZ diabetic rats and STZ diabetic rats treated with AMAEBC at 50 mg/kg b.wt for 8 days is shown in Figure 6. The fasting blood plasma glucose level of STZ induced diabetic group was 506.4 ± 20.8 mg/dL. AMAEBC treatment significantly reduced fasting blood glucose level by 15.23% compared to untreated control diabetic group.

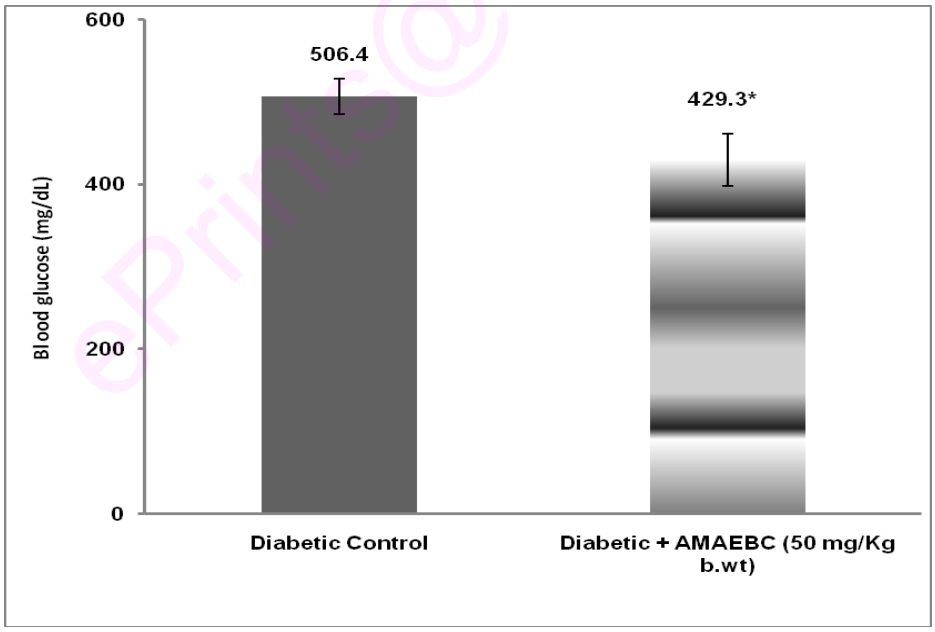


FIGURE 6. Effect of acarbose and AMAEBC on fasting blood plasma glucose levels of STZ-diabetic albino rats. Values are mean ± SEM of 6 animals. *Significantly different from the diabetic group at P ≤ 0.05

Effect of AMAEBC on lipid peroxidation was studied in STZ diabetic rats by estimating MDA levels in plasma and vital organs (Table 6). In STZ diabetic rats, AMAEBC treatment significantly lowered MDA levels in plasma, liver, kidney, brain and testis with respect to control diabetic animals.

TABLE 6. Effect of AMAEBC on MDA levels in different tissues of STZ diabetic rats

Tissues	Control (diabetic)	AMAEBC (50 mg/kg b.wt)
Plasma	0.41 ± 0.04^{a}	0.32 ± 0.05^{b}
Liver	6.40 ± 0.62^{a}	5.72 ± 0.49^{b}
Kidney	7.62 ± 0.88^{a}	5.64 ± 0.64^{b}
Brain	3.19 ± 0.21^{a}	2.60 ± 0.26^{b}
Testes	5.00 ± 0.42^{a}	2.69 ± 0.35^{b}

Values are mean ± SEM of 6 rats in each group. Means of same row followed by different letters differ significantly according to Duncan's post hoc test at $P \leq 0.05$.

4.3.5. Anti-hyperglycaemic effect of bitter cumin extract on high fructose fed and low streptozotocin induced diabetic rats

Food intake and body weight gain of different groups of animals are shown in Table 7. Compared to starch fed group a small, but, significant decrease in food intake was observed in AMAEBC fed starch group, fructose fed group, fructose + low STZ group and fructose + low STZ and AMAEBC (25 mg/Kg b.wt) fed rats. Rats in the high fructose fed group weighed significantly less (161.67 ± 23.3 g) compared to other groups. The apparent decrease in body weight in fructose group may be attributed to less food intake by the same group (Table 7).

TABLE 7. Effect of AMAEBC on food intake and body weight gain of high fructose and low STZ induced diabetic rats.

Group	Food intake	Body weight
Control	13.24 ± 3.1^{a}	175.00 ± 21.5^{ab}
Control + AMAEBC (100 mg/kg b.wt)	11.94 ± 4.2^{cd}	171.41 ± 30.1^{bc}
Fructose	11.38 ± 3.1^{d}	161.67 ± 23.3^{c}
Fructose + STZ	12.20 ± 4.2^{bc}	169.10 ± 21.3^{bc}
Fructose + STZ+ AMAEBC (25 mg/kg b.wt)	11.50 ± 3.2^{cd}	171.61 ± 23.8^{bc}
Fructose + STZ+ AMAEBC (100 mg/kg b.wt)	12.93 ± 4.2^{ab}	177.08 ± 27.9^{ab}

Values are mean $\pm$ SEM of five rats. Means of same column followed by different letters differ significantly according to Duncan's post hoc test at $P \leq 0.05$.

The absolute organ weights of control, fructose, low STZ and high fructose rats fed with AMAEBC is presented in Table 8. No significant variation in liver, kidney and testes tissues was noticed between different groups, but, weight of the brain was significantly less in fructose + STZ group.

TABLE 8. Effect of AMAEBC on absolute organ weights of high fructose and low STZ induced diabetic rats.

Groups	Liver	Kidney	Testis	Brain
Control	6.73 ± 1.50[a]	1.90 ± 0.40[a]	2.80 ± 0.62[a]	1.63 ± 0.06[a]
Control +AMAEBC (100 mg/kg)	6.60 ± 0.42[a]	1.95 ± 0.35[a]	2.75 ± 0.35[a]	1.60 ±0.14[a]
Fructose	6.77 ± 0.32[a]	2.13 ± 0.42[a]	2.57 ± 0.42[a]	1.50 ± 0.06[ab]
Fructose + STZ	6.56 ± 1.04[a]	2.04 ± 0.34[a]	2.52 ± 0.31[a]	1.40 ± 0.13[b]
Fructose+ STZ+ AMAEBC (25 mg/kg)	7.27 ± 0.58[a]	2.21 ± 0.49[a]	2.61 ± 0.20[a]	1.53 ± 0.10[ab]
Fructose+ STZ+AMAEBC (100 mg/kg)	7.19 ± 0.86[a]	2.19 ± 0.25[a]	2.71 ± 0.22[a]	1.61 ± 0.09[a]

Values are mean ± SEM of five animals per group. Means of same column followed by different letters differ significantly according to Duncan's post hoc test at $P \leq 0.05$.

The fasting blood plasma glucose levels of starch fed group, high fructose fed group, high fructose and STZ treated group and AMAEBC treated groups is presented in Table 9. There was no significant difference in fasting blood plasma glucose levels in high fructose fed group compared to starch fed control group. However, fasting blood plasma glucose concentrations were significantly elevated in high fructose and low STZ group (36.9 %) compared to fructose fed and starch fed group of rats.

TABLE 9. Effect of AMAEBC on blood plasma glucose levels in high fructose low STZ induced diabetic rats

Group	Glucose (mg/dL)
Control	116.91 ± 9.70[c]
Control + AMAEBC (100 mg/kg b.wt)	106.82 ± 9.60[c]
Fructose	120.10 ± 5.60[c]
Fructose + STZ	160.74 ± 1.40[a]
Fructose + STZ + AMAEBC (25 mg/Kg b.wt)	139.33 ± 16.1[b]
Fructose + STZ+ AMAEB (100 mg/Kg b.wt)	129.67 ± 13.8[b]

Values are mean ± SEM of five rats. Means of same column followed by different letters differ significantly according to Duncan's post hoc test at $P \leq 0.05$.

AMAEBC at 100 mg/kg b.wt did not induce significant decrease in fasting plasma glucose levels in starch fed animals. There was an increase in fasting blood glucose of the rats fed with high fructose and injected with low dose of STZ. But, feeding of AMAE of bitter cumin at 25 and 100 mg/Kg b.wt to high fructose fed low STZ injected rats decreased fasting blood glucose to 13.3 and 19.3% respectively compared to high fructose fed low STZ injected group.

4.4. Discussion

Carbohydrates are one of the best sources of energy and give about 4 calories of energy/g. Much of the carbohydrate in food is converted by the digestive enzymes to monosaccharide- glucose, the principal source of energy. Insulin released from pancreatic beta cells in response to rise in the level of blood glucose facilitates the storage of glucose in liver as glycogen. However, in diabetic condition, excess blood glucose is not easily converted into glycogen due to insufficient insulin or insulin insensitivity or resistance and leads to hyperglycaemic condition. Type 1 diabetes or insulin dependent diabetes (IDDM) is caused by insulin deficiency due to the dysfunction of insulin secreting pancreatic langerhans β-cells. The principal treatment of type 1 diabetes is replacement by exogenous insulin. Type 2 diabetes or Non-insulin dependent diabetes mellitus (NIDDM) is manifested due to a combination of defective insulin secretion from pancreatic langerhans β-cells and, or reduced insulin sensitivity due to excess glucose absorption. Hyperglycaemia in type 2 diabetes can be reversed by improving insulin sensitivity or reducing glucose production by the liver. Globally, the percentage of non-insulin dependent diabetes is more than 90% (Zimmet *et al*., 2001). At present, the direct clinical therapy and prophylaxis in NIDDM is to optimize or control the postprandial blood glucose level (Stanik and Marcus, 1980). One of the therapeutic approaches for decreasing postprandial hyperglycaemia, is to retard the absorption of glucose by the inhibition of carbohydrate hydrolyzing enzymes namely, α-glucosidase and α-amylase in the digestive organs (Toeller, 1994; Saito *et al*., 1998, Holman *et al*., 1999).

α- glucosidase (AGH) is a membrane bound enzyme located at the epithelium of small intestine and catalyzes the cleavage of glucose from disaccharides (Hauri *et al*., 1982). Thus, the modulation of the action of α-glucosidase by inhibitors is one of the most effective approaches to control NIDDM (Toeller, 1994). Synthetic α-glucosidase inhibitors such as acarbose, miglitol and voglibose are being prescribed for the management of Type 2 diabetes mellitus. These drugs are helpful to keep blood glucose levels within safe limit

by slowing the rate of intestinal absorption of glucose from food. But these drugs have certain adverse effects such as hypoglycaemia at higher doses, liver problems, lactic acidosis and diarrhoea. Traditional medicinal plants are used throughout the world as natural anti-diabetic agents for a range of diabetic presentations. Herbal drugs are perceived to have limited side effects and are cost effective compared to prescription drugs even though their biologically active compounds and efficacy are still under investigation (Li *et al*., 2004). Thus, plant derived compounds continue to provide valuable therapeutic agents, in both modern and traditional medicinal system. To date, there are many studies on functional herbs and food materials to control the blood glucose level for the prophylaxis of hyperglycaemic condition (Morimoto *et al*., 1999; Suresh babu *et al*., 2004; Li *et al*., 2005).

Yeast α-glucosidase has been frequently used to identify the inhibitory potency of herbal and medicinal plants (Rao *et al*., 2003; Matsui *et al*., 1996). However, α-glucosidase activity from rat intestinal epithelium closely mimics the mammalian system (Ohta *et al*., 2002) and therefore, may be a better model to identify, design and develop antihyperglycemic agents particularly for the management of postprandial hyperglycaemia in diabetes. Therefore the present study has investigated the antihyperglycemic effect of aqueous methanol-acetone extract of bitter cumin (AMAEBC) on some carbohydrate hydrolyzing enzymes namely rat intestinal α-glucosidase, and human salivary α-amylases in *in vitro* model systems.

Preliminary studies with different extracts of bitter cumin on AGH inhibition showed that aqueous methanol acetone extract of bitter cumin (AMAEBC) is the most potent inhibitor; hence further studies were undertaken with this extract. AMAEBC inhibited rat intestinal sucrase, maltase and PNP-glucoside hydrolase activity at microgram concentrations with IC_{50} values of 34.1 ± 3.8 µg/mL, 62.4 ± 4.5 µg/mL and 500.5 ± 11.9 µg/mL respectively. The inhibitory effect of AMAEBC against sucrase was about 2 and 14 times higher than maltase and PNP-glucoside hydrolysis activity (Table 3). Further, inhibitory effect of AMAEBC on sucrase and maltase activities is found to be 8 and 32-fold higher than DL-catechin (Table 3), but 15 and 624 -fold lower than synthetic therapeutic drug acarbose. The higher inhibitory activity of AMAEBC

compared to DL-catechin may be because of the additive activity of an array of phenolic compounds present in AMAEBC (Ani *et al*., 2006)

Screening of antihyperglycemic natural compounds with α-glucosidase inhibitory activity is an important area of research to develop alternative antidiabetic medications (Ali *et al*., 2002). Phenolic compounds are ubiquitous molecules in plants and are capable of inhibiting intestinal α-glucosidase (Honda and Hara, 1993; Matsui *et al*., 2001). The present study showed that, AMAE of bitter cumin is a strong inhibitor of rat intestinal α-glucosidase. There was a direct dose dependency in the percentage of inhibition by the extract. LC-MS studies have shown that AMAEBC is rich in various phenolic compounds such as gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol. Caffeic acid is the major phenolic compound present in bitter cumin extract which is a dihydroxy derivative of cinnamic acid. Caffeic acid and its derivatives like 6-0-caffeoylsophorose are known inhibitors of α-glucosidase (Matsui *et al*., 2004). Wansi *et al*., (2007) showed that out of 13 compounds isolated from *Terminalia superba* extracts, only gallic acid and methyl gallate showed significant α-glucosidase inhibition activity. Inhibition of α-glucosidase and α-amylase by 6 groups of flavonoids is reported (Tadera *et al*., 2006). In their experiment it is showed that quercetin is more active in inhibiting α-glucosidase than kaempferol. In another interesting experiment Yoshida *et al*., (2008) showed that substitution of sugar moiety of quercetin or kaempferol with that of a phenolic acid, in particular, caffeic acid, could enhance their inhibitory activity. Ellagic acid derivatives with α-glucosidase inhibitory activity have been purified from *Terminalia superba* (Tabopda *et al*., 2008). α-glucosidase inhibitory activity of two ferulic acid derivatives, N-p-coumaroyl-N'-feruloyl putresciene and N, N'-difuroylputersciene purified from corn gluten meal were studied by Niwa *et al*., (2003). In short, most of the phenolic compounds reported from bitter cumin extract or their derivatives are reported to be potent inhibitors of α-glucosidase inhibitors in various studies. As a result, it is quite obvious that, bitter cumin with a combination of these phenolic compounds exerts a very strong α-glucosidase inhibition either due to additive activity and or due to hetero-polymerisation.

Another important feature of AMAEBC compared to acarbose and DL-catechin is that it is a more efficient inhibitor of sucrase activity compared to maltase activity. Caffeic acid, the major phenolic component of bitter cumin extract also showed a similar activity of high inhibition against sucrase than maltase (Matsui *et al*., 2004). Sucrose being the major sweetening agent in human diet, inhibitors of sucrose hydrolysis will be much effective in controlling hyperglycaemia after sweet servings.

Enzyme kinetic studies were conducted to understand the nature of inhibition of sucrase, maltase and PNP-glucoside hydrolase by AMAEBC. Rat intestinal α-glucosidase preparation showed maximum affinity towards PNP-glucoside with a *K*m of 0.59 mM, followed by maltose (K_m = 3.3 mM), and sucrose (K_m = 23.81 mM).The V_{max} of an enzyme is a measure of the speed of the reaction it catalyzes once the enzyme substrate complex is formed and therefore, provides the information on inhibitory nature and type of enzyme inhibition. Table 4 presents data on K_m and $1/V_{max}$ data in presence of different concentrations of substrate and AMAEBC. AMAEBC decreased the maximum velocity of enzyme activity or V_{max} without much change in the *K*m values. Hence, the mechanism of inhibition of sucrase, maltase and PNP-glucoside by AMAEBC was of reversible, non-competitive nature. The K_i values represent the efficacy of inhibitory molecules to bind to the enzyme and cause inhibition. The K_i values calculated from Lineweaver-Burk plots were found to be 30.24, 76.67 and 341.6 µg of AMAEBC respectively, for sucrase, maltase and hydrolysis of PNP- glucoside.

α-amylase catalyzes the hydrolysis of the (1-4) - α-D-glycosidic linkages of starch, viz., amylose, amylopectin; glycogen and maltodextrins. Two types of α-amylases are produced by many mammals viz., salivary α-amylase from parotid gland and pancreatic α-amylase from the pancreas. Thus digestion of starch starts with salivary α-amylase in mouth and further digestion takes place in small intestine by pancreatic α-amylase. Recent studies have shown that phenolic compounds inhibit amylase and therefore have a great potential for the management of type 2 diabetes (McCue and Shetty, 2004). Tea polyphenols were reported as inhibitors of α-amylase both *in vivo* and *in vitro* systems (Matsumoto *et al*., 1993; He *et al*., 2006). Further, α-amylase

inhibitory effect of phenolic extracts of pears, cocoa and lentils support the inhibitory effect of polyphenols on α-amylase activity (Quesada *et al*., 1995).

The inhibitory effect of AMAEBC (IC_{50} 185.5 ± 4.9 µg) on α-amylase was found to be low when compared with the synthetic and therapeutic drug acarbose (IC_{50}17.4 ± 0.9 µg). However, the present investigation support the finding that the natural α-amylase and α-glucosidase inhibitors from plants have lower inhibitory effect against α-amylase and a stronger inhibitory activity against α-glucosidase (Kwon *et al*., 2006). An effective strategy for the type 2 diabetes management was suggested to be a mild inhibition of α-amylase and strong inhibition of intestinal α-glucosidase activity (Krentz and Bailey, 2005). Therefore, AMAEBC has the potential to be used as a natural herbal drug capable of modulating both α-amylase and α-glucosidase and thus aid in suppression of the postprandial hyperglycaemia.

In traditional medicine a variety of dietary and medicinal plants are used in the prevention and treatment of diabetes. Although some of these plants have been reported to inhibit α-glucosidase activity, most of the experiments have been limited to *in vitro* or to normal or chemically induced diabetic models (Kim *et al*., 1999; Yoshikawa *et al*., 2003). AMAE of bitter cumin significantly reduced plasma glucose levels, in maltose loaded overnight fasted normal rats. The results suggest that AMAE interferes with the transit, digestion or absorption of sugars in the small intestine. The combined findings of *in vitro* inhibition of α-glucosidase and *in vivo* inhibition of hyperglycaemia in maltose overloaded normal rats suggest that AMAEBC inhibit carbohydrate digestion by inhibiting α-glucosidase activity. Thus the results demonstrate that AMAEBC is an effective α-glucosidase inhibitor.

Phenolic extracts of plants were found to be effective inhibitors of intestinal α-glucosidase (Matsui *et al*., 2001) and α-amylase (He *et al*., 2006) and are being used to control Type 2 diabetes mellitus (Toeller, 1994; Bischoff, 1994). Phenolic acids such as caffeic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, 3-hydroxy-4-methoxy cinnamic acid, 4- methoxy-*trans*-cinammic acid, eufenlin, casuarictin and ellagitannin were reported to be good inhibitors of α-glucosidase (Matsui *et al*., 2001; Toda *et al*., 2000; Adisakwttana, *et al*.,

2004). In this study, AMAEBC, the phenolic extract of bitter cumin seeds containing a mixture of phenolic compounds viz., gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol showed significant inhibition of rat intestinal α-glucosidase (maltase and sucrase) activity, human salivary α-amylase and also reduced postprandial hyperglycaemia in rats. Thus phenolic compounds present in bitter cumin could be responsible for inhibition of α-glucosidase and α-amylase activity thus indicating a possible mechanism in antihyperglycemic effect of bitter cumin seeds.

The present study was focussed on antihyperglycaemic activity of AMAEBC in STZ induced diabetic rats. Streptozotocin is an antibiotic derived from *Streptomyces achromogenes* and structurally is a glucosamine derivative of nitrosourea. Rakieten *et al*., (1963) first demonstrated the diabetogenic property of STZ in dogs and rats. STZ causes hyperglycaemia mainly by its direct cytotoxic action on the pancreatic beta cells and consequent insulin deficiency (Junod *et al*., 1969; Szkudelski, 2001). STZ is a preferred agent to induce experimental diabetes in animals. STZ diabetic animals are most widely used for screening the compounds including natural products for their insulinomimetic, insulinotropic and other antihyperglycemic activities (Jones *et al*., 1997; Pele Tounian *et al*., 1998; Bates *et al*., 2000). Intraperitoneal administration of STZ (55 mg/kg b.wt) effectively induced diabetes in normal rats, as reflected by hyperglycaemia and bodyweight loss compared with normal rats. The present study observed that AMAEBC treatment reduced the STZ induced hyperglycaemia in diabetic rats and also in high fructose and low STZ diabetic rats. Flavonoids, sterols/triterpenoids, alkaloids and phenolics are known to have antidiabetic activity (Oliver-Bever, 1986; Ivorra *et al*., 1989; Rahman and Zaman, 1989; Rao *et al*., 1997). Flavonoids are known to regenerate the damaged beta cells in the alloxan diabetic rats (Chakravarthy *et al*., 1980). Polyphenolic compounds from plants are also known to cause insulin like effects in glucose utilization in mammals (Manickam, 1997; Toda *et al*., 2000). Thus the phenolic compounds present in bitter cumin may have stimulatory effect on the remnant beta cells to

secrete insulin and could be responsible for antihyperglycemic action in diabetic rats.

There exists a couple of animal models (spontaneous as well as induced) for the study of type 2 diabetes, however, the pattern of disease initiation and development in most of these animal models do not appear to be analogous to the clinical situation in humans. There are certain genetic models namely Zucker diabetic fatty (ZDF) rat and db/db mouse which develop diabetes spontaneously resembling human type 2 diabetes. However, the development of diabetes in them is predominantly genetically determined unlike in humans (Luo *et al*., 1998; Shafrir, 2003). Moreover, the observations derived from these highly inbred genetic strains may not always be satisfactorily extended to the human population as a whole because of the large heterogeneity in the latter. In addition, these animals are expensive and are not easily available for the investigative purposes as well as regular screening experiments. Further, in induced diabetic models, most of the animals (adult or neonates) require relatively high dose of streptozotocin (STZ > 50 mg kg−1) (Shafrir, 2003). The development of hyperglycaemia in these rats following STZ injection is primarily due to the direct pancreatic beta cell destruction resulting in insulin deficiency rather than the consequence of insulin resistance (Shafrir, 2003; Rerup, 1970). Thus, they depict symptoms and characteristics more typical of human type 1 than type 2 diabetes and further are not very responsive to the effects of drugs like insulinotropic (e.g. glipizide, tolbutamide) and insulin-sensitizing compounds (e.g. pioglitazone, rosiglitazone) (Weir *et al*., 1981; Hofmann *et al*., 1991; Portha *et al*., 1994).

Fructose has been suggested as an important dietary source of carbohydrate for diabetic patients because it elicits lower blood glucose and insulin secretary response than other simple carbohydrates (Bantle *et al*. 1986; Gerrits and Tsalikian, 1993). However, the high levels of dietary fructose and severe hyperglycaemia is reported to have interactive effects which may contribute to the development of complications associated with diabetes. Dietary fructose does not alleviate glycaemia, circulating insulin concentrations or growth in the diabetic rats (Bell *et al*., 1996). In human subjects and rats, high fructose intake significantly increases the level of

serum cholesterol and triglyceride level in hyperinsulinemic and nonhyperinsulinemic human subjects (Gerrits and Tsalikian, 1993; Hollenbeck, 1993; Bell et al. 1996; Reiser *et al*., 1989; Kannappan *et al*., 2006). Thus, although fructose supplementation has been advocated for patients with diabetes mellitus, the lack of positive effects on glycemic control coupled with adverse side-effects suggests that high fructose intake is not recommended for diabetic populations whose glucose concentrations are uncontrolled (Bell *et al*., 1996).

High fructose and low STZ diabetic model is an important rat model to study type- 2 diabetes. High dosage of fructose in the diet has been documented to induce insulin resistance accompanied by deleterious metabolic consequences including hyperinsulinemia, hyperglycaemia, glucose intolerance, hypertriglyceridemia and hypertension in rodents (Hwang *et al*, 1987; Thorburn *et al*., 1989). Fructose-fed rodents become insulin-resistant and hyperinsulinemic but were able to maintain normal glucose homeostasis (Zavaroni *et al*., 1980), a condition similar to the prediabetic state in humans (Warram *et al*., 1990; Lillioja *et al*., 1988). In the present investigation, high fructose and low STZ fed rats showed significantly elevated fasting blood plasma glucose concentrations (36.9 %) compared to fructose fed and starch fed group of rats.

AMAEBC at 25 and 100 mg/kg b.wt significantly decreased the fasting blood plasma glucose levels by 13.3 and 19.3 % respectively compared to high fructose fed and low STZ injected group. The antihyperglycemic effect of AMAEBC in high fructose fed and STZ injected rats can be related to possible stimulatory effect on the remnant pancreatic beta cells.

In summary, the present study clearly demonstrated the antidiabetic effect of bitter cumin seed extract (AMAEBC) by modulating the activity of human salivary α-amylase activity and rat intestinal sucrase, maltase and PNP-glucoside hydrolase activities. AMAEBC showed non-competitive and reversible inhibitory effect on above enzymes. Further, AMAEBC is found to be inhibitor of α-amylase but a strong inhibitor of intestinal α-glucosidase activity. In maltose tolerance test AMAEBC showed significant suppression of

postprandial blood glucose level. Thus AMAEBC inhibits the enzymes involved in hydrolysis of dietary carbohydrates especially sucrose and maltose in small intestine and thus reduce blood glucose levels. The inhibitory effect of AMAEBC can be related to an array of phenolic compounds present in it which are reported to be good inhibitors of amylase and glucosidase activities. *In vivo* STZ induced diabetic model and high fructose and low STZ diabetic model AMAEBC at 25-50 mg/kg b.wt significantly decreased fasting blood glucose level, thus demonstrating that AMAEBC may stimulate insulin secretion in these diabetic models. In conclusion, the present study has demonstrated that bitter cumin seeds are effective in controlling the hyperglycaemia and thus may have a potential beneficial effect in controlling diabetes.

Chapter V

Studies on antimicrobial activity of bitter cumin seeds

5.1. Introduction

Human beings owe their existence to a great extent to plant life. Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and continue to provide mankind with new remedies. Herbs and spices are known for their antimicrobial and antioxidative properties. Food safety is a fundamental concern both for consumers and food industries, since there are a number of reported cases of food associated epidemics. Food preservation, however, is the basis of most of the food industries worldwide. Since food processors and consumers have expressed a concern regarding the use of synthetic chemicals in food preservation, interest on herbs and spices as source of natural alternative antimicrobial has been explored. The significance of phenolics in human diet and antimicrobial activity has been established (Nychas *et al*., 2003; Rauha *et al*., 2000). The antioxidant properties of these compounds are often claimed for the protective effects of plant-based beverages against cardiovascular disease, certain forms of cancer and photosensitivity reactions. It was also found that they inhibit human immunodeficiency viral replication (HIV), human simplex virus (HSV), glucosyl transferases of *Streptococcus mutans* (dental carries), ascorbate auto-oxidation (green tea), cytotoxic effects, tumour promotion and xanthine, monoamine oxidases (Havsteen, 2002; Mattila *et al*., 2000; Middleton *et al*., 2000).

In recent years, drug resistance in human pathogenic microorganisms is increasing due to indiscriminate use of antibiotics in the treatment of infectious diseases. Of the 2 million people who acquire bacterial infections in US hospitals each year, 70% of cases now involve strains that are resistant to at least one drug (IDSA, 2004). A major cause for concern in the UK is methicillin- resistant *Staphylococcus aureus*, which was at low levels a decade ago, but, now accounts for approximately 50% of all *S. aureus* isolates (Adcock, 2002). Substantial investment and research in the field of antimicrobials are warranted to overcome drug resistance of infective pathogens. One of the effective means to tackle the drug resistance problem is the structural modification of the drug to which resistance has developed.

This method has been successfully applied in antifungal agents such as azoles (Jeu *et al*., 2003), antiviral agents such as non-nucleotide reverse transcriptase inhibitors (De Clercq, 2001) and various antibacterial agents including β-lactams and quinolones (Poole, 2001). Therefore, it is very important to develop new class of antimicrobial agents that acts on different target site than those in current use (Anonymous, 2000; Kimberlin and Whiteley, 1996). Rational drug design may not always yield effective antimicrobial agents. In the past, potent enzyme inhibitors have been successfully designed and synthesized but they had only modest antibacterial activity, probably owing to the complexity of drug uptake by cells. Broad empirical screening of chemical entities for antimicrobial activity represents an alternative strategy for the development of novel drugs. Natural products have been a particularly rich source of anti-microbial agents, for example, the penicillin in 1940, the tetracyclines in 1948 and the glycopeptides in 1955 (Silver and Bostian, 1990).

Scientists are in search of new antimicrobial substances from plants which are good sources of novel antimicrobial chemotherapeutic agents. The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents (Salvat *et al*., 2001; Arias *et al*., 2004). Thus, studies have been focused on the antimicrobial agents and on the antimicrobial properties of plant-derived active principles such as spices and essential oils, which have been used for a long time in traditional medicine to overcome microbial infections (Cowan, 1999). The use of crude extracts instead of isolated constituents may be more appropriate, since a specific compound could be bioactive, but, the presence of other compounds in the extracts could have a synergistic/additive inhibitory property.

Since ancient times, spices have been used as natural, alternative remedy to heal many infectious diseases (Parekh *et al*., 2005). Spices are the common dietary adjuncts that contribute to the taste and flavour of foods. In addition, spices are also used to prevent the spoilage of foods and food products due to microbial contamination (Kizil and Sogut, 2003). Several scientific reports

have described the inhibitory effect of spices on a variety of microorganisms, although considerable variation exists for resistance of different microorganisms to a given spice and of the same microorganisms to different spices (Arora and Kaur, 1999). Spices are rich source of biologically active antimicrobial compounds. The Gram positive bacterial strains are more sensitive to the antimicrobial compound of spices than Gram negative bacterial strains (Lai and Roy, 2004; Russell, 1991). Arora and Kaur (1999) tested different spices and found that only garlic and clove exhibited antimicrobial activity. Indeed, some bacteria, which showed resistance to certain antibiotics were sensitive to extracts of both garlic and clove.

Black cumin *(Nigella sativa)* seeds extract (diethyl ether) at 25–400 µg caused concentration dependent inhibition of Gram positive bacteria represented by *Staphylococcus aureus*, Gram negative bacteria represented by *Pseudomonas aeruginosa* and *Escherichia coli* and a pathogenic yeast *Candida albicans* (Hanafy and Hatem, 1991). The aqueous decoction of cumin (*Cuminum cyminum* L., Umbelliferae) exhibited significant inhibitory activity against *Micrococcus roseus*, *Plesiomonas shigelloides* , *Alcaligenes spp., Citrobacter* spp., *Klebsiella pneumoniae*, *Aeromonas hydrophila, Klebsiella ozaenae, Pseudomonas aeruginosa, Enterobacter aerogenes* and *Staphylococcus aureus* (Chaudhry and Tariq, 2008). Similarly, the aqueous decoction of black cumin (*Nigella sativa* L., Ranunculaceae family) revealed a significant antibacterial activity against *Staphylococcus aureus*, *Streptococcus mutans, Micrococcus roseus, Streptococcus morbillorium*, *Streptococcus sanguis*, *Streptococcus intermedius*, *Klebsiella ozaenae*, *Aeromonas hydrophila*, and *Streptococcus salivarius* (Chaudhry and Tariq, 2008). Essential oil of *Cuminum cyminum* showed antimicrobial activity against the mold *Aspergillus niger*, the Gram positive bacteria *Bacillus subtilis* and *Staphylococcus epidermidis* as well as the yeast *Saccharomyces cerevisiae* and *Candida albicans* (Jirovetz *et al*., 2005). This chapter deals with the antimicrobial activity of bitter cumin extract (AMAEBC) on different food spoilage and pathogenic bacteria.

5.2. Materials and Methods

5.2.1. Preparation of bitter cumin extract

Aqueous methanol acetone extract of bitter cumin (AMAEBC) was prepared as described in Chapter 2.

5.2.2. Microorganisms and culture media

5.2.2.1. Microorganisms

Gram positive and negative bacterial strains such as *Bacillus cereus* (F-4810, Public Health laboratory, London, U.K.), *Bacillus subtilis* (CFR-1604), *Staphylococcus aureus* (FRI-722, Public Health Laboratory, The Netherlands), *Listeria monocytogenes* (Scott A), *Escherichia coli* (MTCC-108, Microbial type culture collection, Institute of Microbial Technology, Chandigarh), *Enterobacter spp.*, *Yersinia enterocolitica* (MTCC-859, Microbial type culture collection, Institute of Microbial Technology, Chandigarh) were used in this study. Stock cultures of these strains were obtained from the Food Microbiology Department of Central Food Technological Research Institute. Each bacterial strain was grown separately in Brain Heart Infusion (BHI) broth at 37 ºC for 12-24 h. Each bacterial suspension was subsequently streaked on plate count agar plates and incubated at 37 ºC for 48 h. A single colony was transferred to 10 mL of BHI broth and incubated at 37 ºC for 16-24 h. This culture was used for the antimicrobial assay.

5.2.2.2. Culture Media

All types of media and gamma irradiated disposable plates were procured from Hi Media, Mumbai, India. The composition of BHI broth, BHI agar and plate count agar are given below.

5.2.2.2.1 Brain heart infusion broth (gL^{-1})

calf brain, infusion from	200
beef heart, infusion from	250
proteose peptone	10
dextrose	2
sodium chloride	5
disodium phosphate	2.5
pH	7.4 ± 0.2

5.2.2.2.2. Brain heart infusion agar (gL^{-1})

calf brain, infusion from	200
beef heart, infusion from	250
proteose peptone	10
dextrose	2
sodium chloride	5
disodium phosphate	2.5
agar	15.0
pH	7.4 ± 0.2

5.2.2.2.3. Plate count agar (gL^{-1})

casein enzymic hydrolysate	5.00
yeast extract	2.50
dextrose	1.00
agar	15.00
pH	7.4 ± 0.2

Requisite quantity of the above media was dissolved in known volume of distilled water, dispensed into suitable glass containers and sterilized by autoclaving at 120 lbs for 20 min.

5.2.3. Antibacterial activity of bitter cumin

5.2.3.1. Agar well diffusion method

The agar well diffusion method of Brantner *et al*., (1994) was employed for the preliminary screening of antimicrobial activity of AMAEBC. Antibacterial activity was tested against food-borne pathogenic and spoilage bacteria viz., *Bacillus subtilis*, *Bacillus cereus*, *Enterobacter* spp., *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica*. The test cultures were grown in BHI broth and pour plated individually in PCA medium and allowed to set. Five equidistant wells of 5.0 mm diameter each were made on the solidified agar medium using sterile stainless steel cork borer. Different concentrations of 100 µL of cumin aqueous methanol–acetone extract (AMAEBC) in DMSO were added to the wells and the control well received the same volume of the vehicle DMSO. Initially, the plates were kept at 6 ºC for 3 h and then incubated for 22 h at 37 ºC. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the wells. The growth inhibition diameter was an average of three measurements, taken at four different directions. All tests were performed in triplicate.

5.2.3. 2. Determination of Minimum Inhibitory Concentration (MIC)

Bacillus cereus, Staphylococcus aureus and *Listeria monocytogenes* were inoculated individually in BHI broth and incubated for 24 h at 37 ºC. It was subsequently streaked on a plate count agar plate and incubated at 37 ºC for 24 h. A single colony was transferred to 10 mL of BHI broth and incubated at 37 ºC for 6 h. The suspension was centrifuged at 15 ºC for 20 min at 7012 x g and cell pellet was recovered, washed and re-suspended in 10 mL of 100µM PBS (pH 7.0). The cell suspension was serially diluted to achieve a

concentration of 10^5 cfu/mL. 1 mL of this cell suspension was transferred into empty sterile tubes.

Effect of AMAEBC was tested on *B. cereus, S. aureus* and *L. monocytogenes* to determine MIC. Various concentrations of AMAEBC was added to 1 mL of the diluted bacterial suspension and incubated for 18 h at 37 ºC in a shaker incubator. The control tube carried equal volume of vehicle DMSO and was incubated under the same conditions. After the incubation period, the whole suspension was pour plated with plate count agar and the plates were incubated overnight at 37 ºC. The number of colony forming units formed in each concentration of AMAEBC was recorded and MIC was determined as the lowest concentration of AMAEBC at which no colony was observed. All the experiments were performed in triplicates.

5.2.3.3. Scanning Electron Microscopic (SEM) studies

The effect of AMAEBC on *Bacillus cereus* and *Staphylococcus aureus* was studied by using scanning electron microscopy. 1 mL of the bacterial suspension of *B. cereus* and *S. aureus* was incubated with AMAEBC at 50 μg/mL and 260 μg/mL respectively for 18 h at 37 ºC in a shaker incubator as describe above. The control tube contained equal volume of DMSO instead of AMAEBC. After incubation, the bacterial cells were collected by centrifugation, washed with phosphate buffered saline (PBS) and fixed with 2% gluteraldehyde for 24 h. The fixed cells were serially dehydrated with ethanol (10, 20, 40, 60, 80 and 96%). The dried samples were spread on a double sided conducting adhesive tape pasted on a metallic stub coated with gold (100 μ) in a sputter coating unit for 5 min and observed under scanning electron microscope (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, U.K) at 20 KV.

5.3. Results

5.3.2. Agar well diffusion method

The effect of AMAEBC on growth of bacterial strains is presented in Table 1. Three bacterial species namely *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* showed maximum inhibition in presence of AMAEBC. *Enterobacter spp*. and *Listeria monocytogenes* showed moderate growth inhibition in presence of AMAEBC. *Escherichia coli* and *Yersinia enterocolitica* showed little or no inhibition of growth.

Table 1. Antimicrobial activity of bitter cumin extract by agar well diffusion method

Bacterial strain	Inhibition	Inhibition zone (mm)
Bacillus subtilis	++	26.5 ± 3.5
Bacillus cereus	++	31.0 ± 1.4
Enterobacter spp.	+	14.5 ± 2.1
Escherichia coli	–	5.5 ± 0.7
Listeria monocytogenes	+	17.0 ± 2.8
Staphylococcus aureus	++	24.5 ± 2.1
Yersinia enterocolitica	–	6.0 ± 1.4

Values are ± SEM of 3 experiments. - Low/No inhibition (< 10 mm); + Moderate inhibition (10-20 mm); ++ High inhibition (> 20 mm)

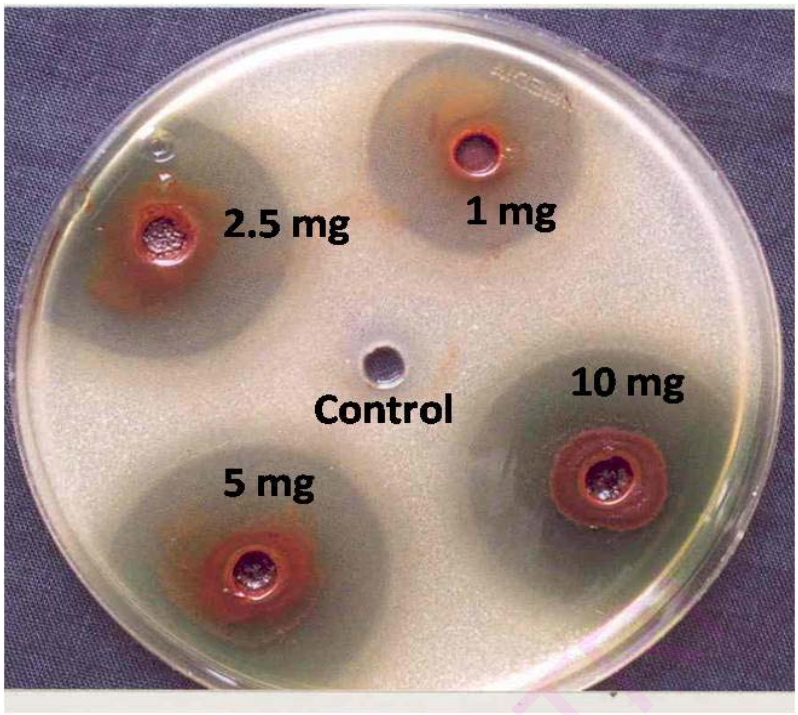


Figure 1. Inhibition of *B. cereus* by AMAEBC as determined by agar well diffusion method

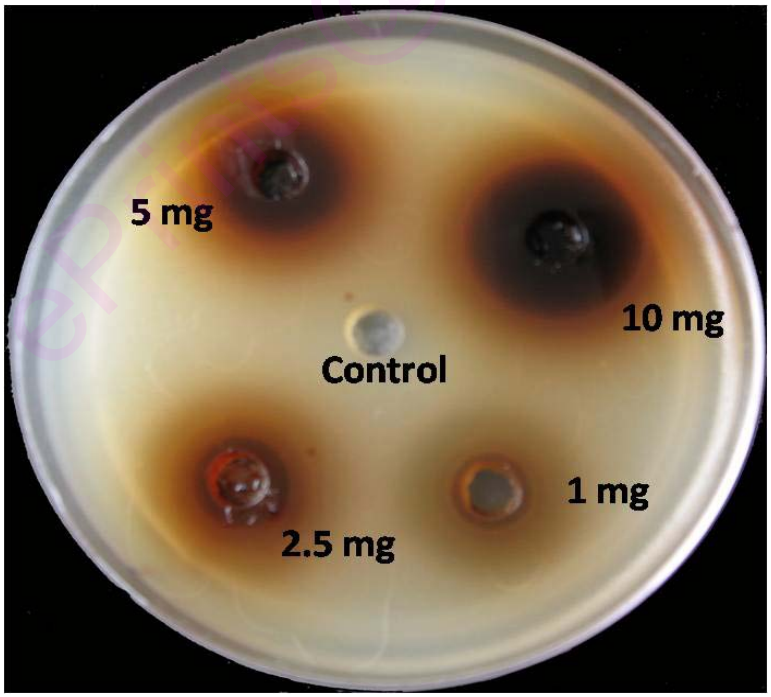


Figure 2. Inhibition of *S. aureus* by AMAEBC as determined by agar well diffusion method

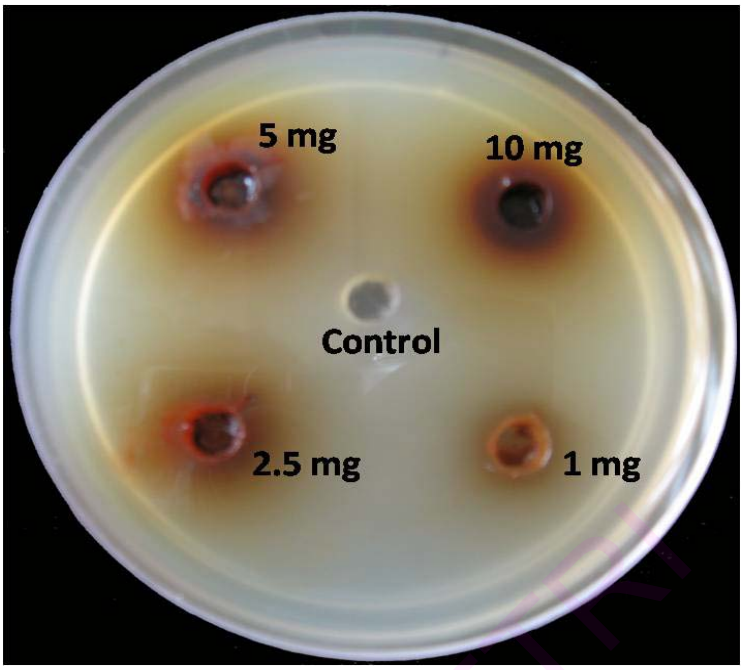


Figure 3. Inhibition of *B. cereus* by AMAEBC as determined by agar well diffusion method

5.3.3. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of cumin extract was studied on *B. cereus, S. aureus* and *L. monocytogenes*. MIC of these organisms was represented graphically in Figure 4. *B. cereus* showed the lowest MIC of 50 ± 7 μg/mL while *S. aureus* and *L. monocytogenes* showed an MIC of 260 ±18 and 700 ± 42 μg/mL respectively.

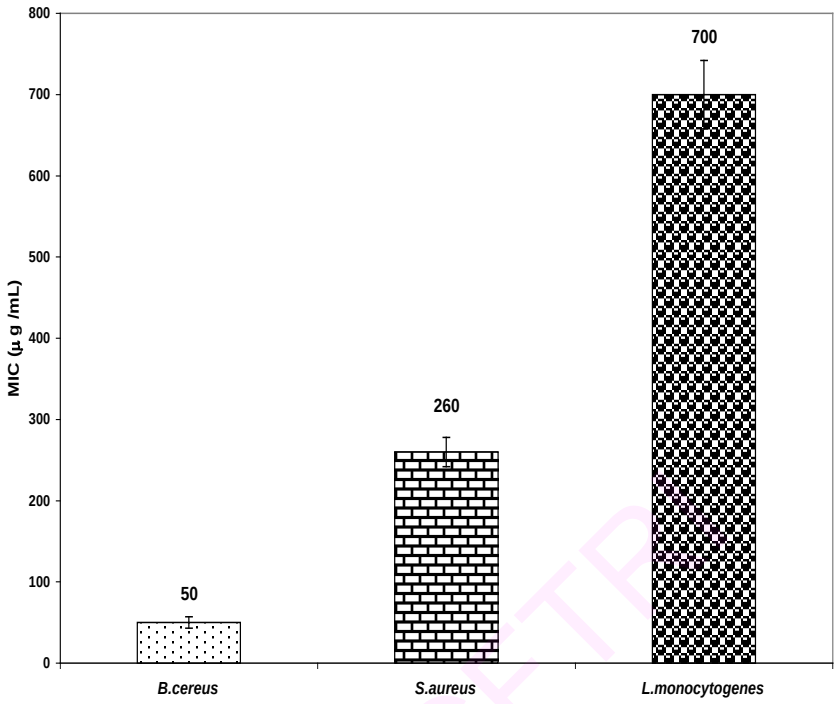
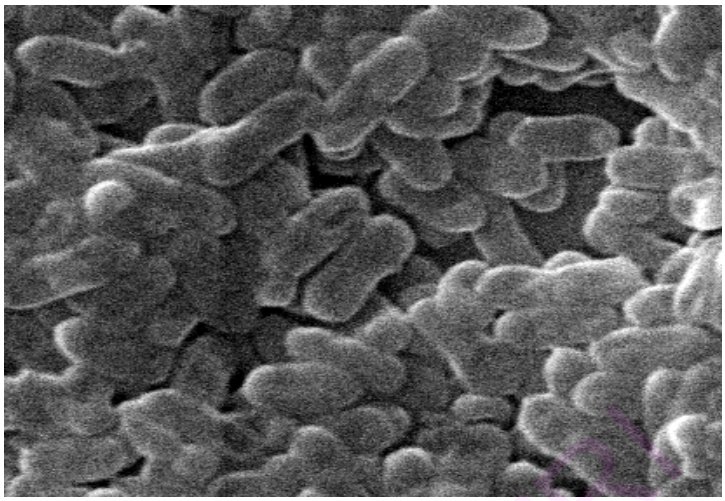


Figure 4. MIC of AMAEBC against *B. cereus*, *S. aureus* and *L. monocytogenes*.

5.3.4. SEM studies

Scanning electron micrographic studies were carried out to understand the possible mechanism of action of bitter cumin extract on bacteria. As shown in Figure 5 (A and B) bitter cumin extract (AMEBC) treated *Bacillus cereus* showed alteration in membrane integrity with cell membranes being disrupted and damaged resulting in cell lysis (Figures 5 A and B). Similarly AMAEBC treated *Staphylococcus aureus* showed small pits in the bacterial membrane surface (Fig 6, A and B).

A



B

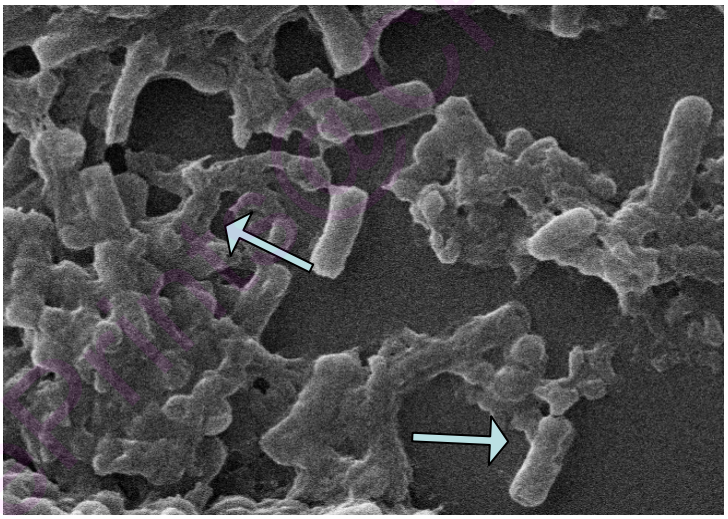
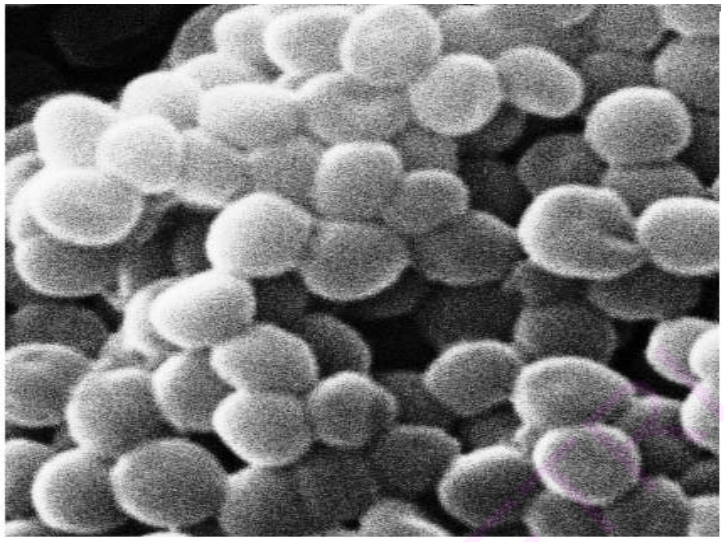


Figure 5. Scanning electron micrograph of *Bacillus cereus*. A) Control cells, B) Cells treated with bitter cumin extract. The arrows indicate the lysed cells in presence of AMAEBC.

A



B

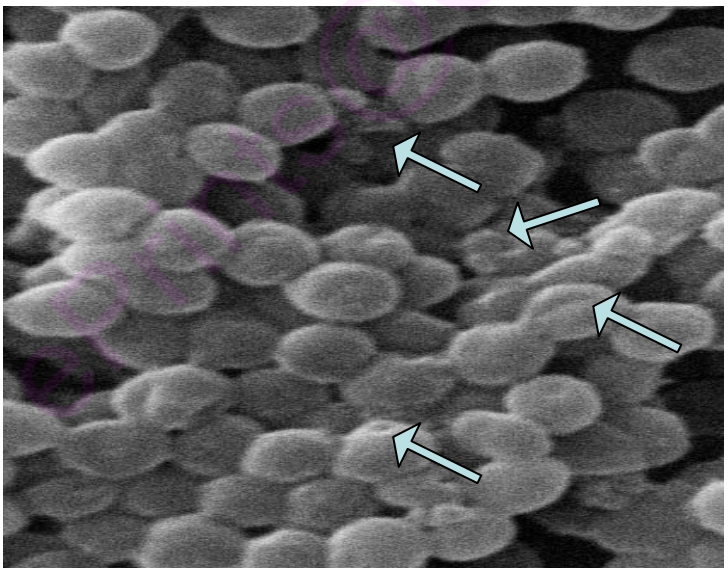


Figure 6. Scanning electron micrograph of *Staphylococcus aureus*. A) Control cells B) Cells treated with bitter cumin extract. The arrows indicate the presence of disruptions on cell membrane in presence of AMAEBC.

5.4. Discussion

To a great extent, human life depends upon plant life. Well before the discovery of microbes man found that certain plants had curing potential against infectious diseases. Man learnt use of plants against diseases from his instinct or from the observation of animals and birds. At present, the development of multiple drug resistance by human pathogenic bacteria and search for natural antimicrobial alternatives in food processing has forced scientists to search for new antimicrobial substances from natural sources.

Bitter cumin (*Centratherum anthelminticum*), a member of the botanical family Asteraceae is widely used as a traditional remedy for helmintic parasites. A preliminary study on *in vitro* antimicrobial activity of different extracts of *C. anthelminticum* was done by Sharma and Mehta (Sharma and Mehta, 1991).

The present study has determined the antibacterial activity of cumin extract – AMAEBC- by agar well diffusion method, determined MIC and carried out SEM studies against selected food spoilage and pathogenic bacteria. Agar well diffusion is one of the extensively used methods to investigate the antibacterial activity of natural plant extracts. This assay is based on the use of discs or holes as reservoir containing the solutions of test substance to be examined (Brantner *et al*., 1994). The present study has tested the antibacterial effect of cumin extract (AMAEBC), which contained an array of phenolic compounds, against selected Gram positive and Gram negative bacteria. DMSO which was used as vehicle to dissolve AMAEBC showed no inhibitory effect on bacterial growth. The growth inhibition pattern shown by different organisms in agar well diffusion method is given in Table 1. Among the bacteria tested, Gram positive bacteria were most susceptible to AMAEBC. The present investigation results are in corroboration with earlier reports on bitter cumin and other plant extracts (Jirawan *et al*., 2006; Burt, 2004; Maillard, 2002). Bacterial strains such as *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* were the most sensitive to AMAEBC. *Enterobacter spp.* and *Listeria monocytogenes* were moderately sensitive and *Escherichia coli* and *Yersinia enterocolitica* were resistant to AMAEBC. LC-

MS studies on AMAEBC showed that it contained an array of phenolic compounds such as gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol. Thus antibacterial activity can be related to the phenolic compounds present in AMAEBC.

The minimum inhibitory concentration (MIC) of AMAEBC required to inhibit the growth of *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes* is given in Figure 4. AMAEBC showed strongest inhibitory effect against *Bacillus cereus* followed by *Staphylococcus aureus* and *Listeria monocytogenes*. A comparison of the MIC values of aqueous methanol acetone extract of bitter cumin, aqueous and methanol extracts of spices such as ginger, onion and other herbs against above bacterial strains is presented in Table 2, 3 and 4. Bitter cumin extract was found to strongest inhibitor of above bacterial strains compared to ginger and onion.

Table 2. MIC of AMAEBC and other plant extracts on *Bacillus cereus*

Extract/ Compound	MIC (µg/mL)	Reference
Centratherum anthelminticum - AMAE	50 ± 7	Present study
Pimpinella anisum - MeOH extract	125	Al-Bayati, 2008
Corylus avellana -aqueous extract	100	Oliveira *et al*., 2007
Zingiber officinale- MeOH extract	660	Alzoreky & Nakahara, 2003
Chloramphenicol	0.5	Rabanal *et al*., 2002

Table 3. MIC of AMAEBC and other plant extracts on *Staphylococcus aureus*

Extract/Compound	MIC (µg/mL)	Reference
Centratherum anthelminticum- AMAE	260 ± 18	Present study
Pimpinella anisum -MeOH extract	62.5	Al-Bayati, 2008
Metasequoia glyptostroboides -MeOH extract	250	Bajpai *et al*., 2007
Zingiber officinale MeOH extract	2640	Alzoreky and Nakahara, 2003
Maximpime	7.8	Al-Bayati, 2008

Table 4. MIC of AMAEBC and other plant extracts on *Listeria monocytogenes*

Extract/Compound	MIC (µg/mL)	Reference
Centratherum anthelminticum AMAE	700 ± 42	Present study
Hippophae rhamnoides aqueous extract	750	Chauhan *et al*., 2007
Zingiber officinale MeOH extract	2640	Alzoreky and Nakahara, 2003
Allium sativum- aqueous extract	5000	Singh *et al*., 2001
Streptomycin	64	Margolles *et al*., 2001.

SEM studies have shown that bitter cumin extract caused changes in cell wall features of micro-organisms studied. These changes were more pronounced in *B. cereus* but minimal in *S. aureus*. *Bacillus cereus* treated with bitter cumin extract showed disruption and disintegration of cell wall resulting in the

release of cell materials in cytoplasm. There was slighter disruption in the cell wall integrity of *S. aureus* upon treatment with bitter cumin extract in comparison to control. These disruptions may lead to the leakage of cytoplasmic materials, loss of cell integrity and eventual death of organisms.

Antifungal and antibacterial potential of spice essential oils and oleoresin has been demonstrated (Singh *et al*., 2007). Thymol, carvacrol, cinnamaldehyde and eugenol from cloves, cinnamon showed inhibitory effect on oral infectious bacteria (Didry *et al*., 1994). Essential oils such as carvacrol, thymol, eugenol, perillaldehyde, and cinnamaldehyde showed antibacterial activity at 0.2 to 10 μL against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Shigella dysenteria*, *Bacillus cereus* and *Staphylococcus aureus* (Burt, 2004).

Phenolic compounds are known to be synthesized by plants in response to microbial infection. It is therefore logical that they have effective antimicrobial substances against a wide group of microorganisms (Cowan, 1999). Phenolic compounds either synthetic or purified from various plants and their derivatives are reported to possess antimicrobial activity and inhibit the growth of different classes of microbes (Anchana *et al*., 2007). Phenolic compounds such as gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid, quercetin, kaempferol etc., are reported to have antibacterial activity (Penna *et al*., 2001; Wen *et al*., 2003; Theim and Goslinska, 2004; Kwon *et al*., 1997; Rauha *et al*., 2000; Lim *et al*., 2007). Flavonoids such as quercetin and kaempferol are reported to inhibit the synthesis of DNA, RNA and related macromolecules (Ferrell *et al*., 1979; Beretz *et al*., 1978; Meltz and MacGregor, 1981; Ishitsuka *et al*., 1982; Cody *et al*., 1986; Havsteen, 1983). Interaction and mispairing activity towards the nucleic acid bases have been proposed because of the similar planar structures of flavonoids and the bases of DNA and RNA. The B-ring of flavonoids may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases, which is reflected in the inhibitory action of DNA or RNA synthesis (Akihisa *et al*., 1987). Thus AMAEBC containing a mixture phenolic acids and flavonoids may interfere with synthesis of DNA or RNA and inhibit the growth of bacterial strains.

Phenolic compounds are reported to have different modes of action against microorganisms. Phenolic acids, are weak acids and can dissociate at cell membranes at a biological pH of 6.8 to 7.2 (Choi and Gu, 2001). This dissociation will create a proton gradient across the cell membrane which can alter the function of many proton pumps on the cell membrane. The dissociation of a proton from the carboxyl or hydroxyl group of phenolic compounds may result in hyper-acidification at the plasma membrane interface of microorganisms (Shetty and Labbe, 1998; Shetty and Wahlqvist, 2004). The hyper-acidification can lead to change in resting potential of the membrane, since it causes an increased positive charge outside of the membrane. This disruption in proton and electrostatic gradient across the membrane can have several implications. The ion pump channels such as Na^{+}/K^{+} or Ca^{2+} pumps are critical for regulating many cellular functions such as motility and cell division. Thus alterations of these cellular functions by phenolic compounds can lead to the death of microorganisms.

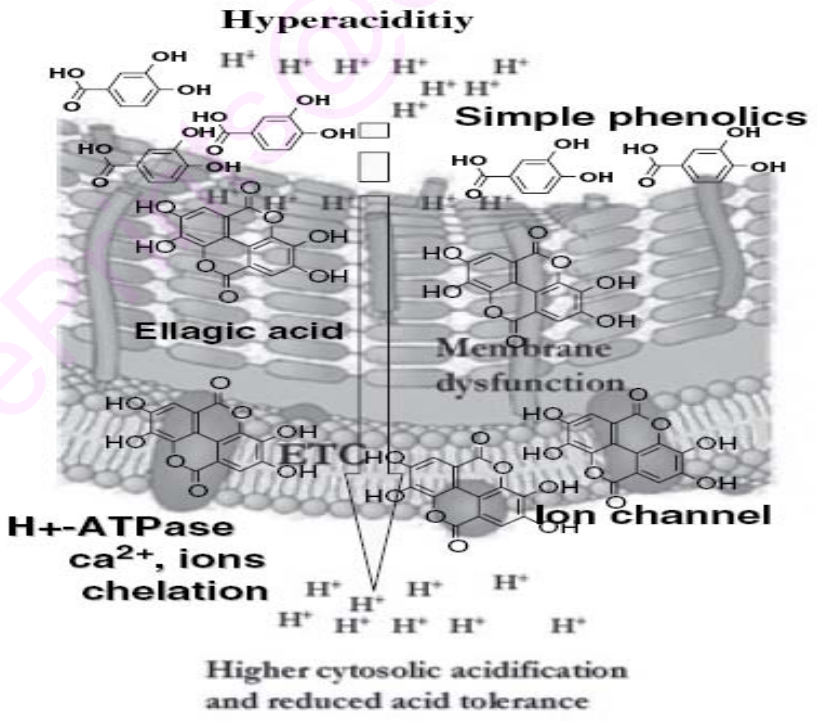


Figure 7. Proposed mechanism of antibacterial action by phenolic compounds on Gram positive bacteria (adopted from Vattem and Shetty, 2005)

The cell membrane is a site of electron transport and of ATP generation in prokaryotes. Phenolic compounds which are very good antioxidants have an ability to quench electrons from free radicals and to delocalize them within the phenolic ring (Rice-Evans *et al*., 1997). Consequently, they can easily quench electrons from the electron transport chain along the bacterial membrane. This could disrupt the flow of the electrons at the level of cytochromes and inhibit the growth of bacteria by disrupting oxidative phosphorylation (Shetty and Labbe, 1998; Shetty and Wahlqvist, 2004). Localized protonation and reduced plasticity of the membrane can diffuse the protein gradient that is essential for the functioning of H^+-ATPase required for ATP synthesis. The diffusion in the proton gradient results in the lowered efficiency of the H^+-ATPase, and therefore, the organism will synthesize less ATP or no ATP. Because of the disruption in the membrane transport induced by phenolic compounds, the reduced intake of nutrients could increase the demand for ATP, and the disruption of ATPase activity could prove fatal to the survival of the microorganism.

Many key proteins and receptors on the membrane are responsible for the receptor mediated transport of nutrients and co-factors. These proteins and receptors are sensitive to pH and ionic strength. Thus phenolic compounds induced disruption of hydrogen ion concentration or electrochemical gradient across the membrane could cause the inactivation of the receptors involved in the uptake of nutrients and thus will cause the death of microorganisms. Some phenolic compounds can inhibit the growth of microorganisms by sequestering metal ions that are critical for the growth and metabolism of microbes (McDonald *et al*., 1998). Thus the array of phenolic compounds and flavonoids present in bitter cumin seed extract might alter or disrupt proton pump and ion channels, disrupt oxidative phosphorylation and ATP synthesis, disrupt DNA and RNA synthesis, and might alter receptor mediated transport of nutrients in bacterial cell membrane which can lead to inhibition of growth and death of bacteria.

In conclusion the present study demonstrated that bitter cumin possess antibacterial activity especially against Gram positive bacteria. Thus bitter cumin, with an array of phenolic compounds, can be exploited as a natural antibacterial agent to control food borne pathogens and spoilage microorganisms.

Summary and Conclusions

The major objectives of this study were to investigate the phytochemicals from bitter cumin seeds (*Centratherum anthelminticum* (L.) Kuntze and their biological properties in terms of antioxidant, antidiabetic and antimicrobial activities. The major findings of this study can be summarized as follows:

1. Bitter cumin (*Centratherum anthelminticum* (L.) Kuntze or Kalijeera in vernacular name belongs to family Asteraceae. The seeds have a sharp bitter taste.

2. Bitter cumin is used in traditional medicine for the treatment of fever, cough, and diarrhoea and also used as herbal tonic and antidiabetic agent. The seeds are reported to possess acrid, febrifugal, alterative, astringent, antihelmenthic, antiulcer, antiphlegmatic, cardiac, diuretic and digestive properties.

3. Proximate analysis showed that bitter cumin seeds contained 14.7% carbohydrates, 21.4% fat, 22.5% protein, 29.3% fiber, 7.2% ash and 4.9% moisture.

4. Bitter cumin oil contained 50% linoleic acid which is one of the essential fatty acids. The other fatty acids present in bitter cumin oil are palmitic acid, palmitoleic acid, stearic acid, oleic acid and linolenic acid.

5. The total phenols of bitter cumin seeds were extracted with water, aqueous methanol and aqueous methanol acetone. Among these extracts, aqueous methanol acetone extract of bitter cumin (AMAEBC) showed highest phenolic content.

6. The AMAEBC contained a mixture of phenolic compounds such as gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid and flavonoids viz., quercetin and kaempferol as determined by LC-MS.

7. The major phenolic compound present in AMAEBC was caffeic acid. The various phenolic compounds present in bitter cumin extract was in the decreasing order of caffeic acid> ferulic acid> gallic acid> quercetin> ellagic acid> protocatechuic acid> kaempferol.

8. The biological properties of bitter cumin extract (AMAEBC) were studied on antioxidant, antidiabetic and antimicrobial systems.

9. The effect of bitter cumin extract was studied on different antioxidant systems such as DPPH, ABTS and superoxide anion radical scavenging, phosphomolybdenum and potassium ferricyanide reducing power, soybean lipoxygenase dependent lipid peroxidation, rat liver microsomal lipid peroxidation, liposomal oxidation and oxidative DNA damage.

10. Bitter cumin showed a potent antioxidant activity in the above assay systems. Among various extracts of bitter cumin, aqueous methanol-acetone extract showed highest antioxidant potential. There was a very strong correlation between total phenol content and antioxidant activity. The polyphenolic compounds present in AMAEBC were established antioxidant molecules. Therefore antioxidant activity can be attributed to the polyphenolic compounds present in bitter cumin seeds.

11. Bitter cumin extract (AMAEBC) showed significant antioxidant activity by scavenging DPPH radical, ABTS radical, superoxide anion radical, reduced phosphomolybdenum and potassium ferricyanide, inhibited lipoxygenase dependent lipid peroxidation, Fe^{2+} and ascorbate induced rat liver microsomal lipid peroxidation and protected against hydroxyl radical mediated oxidative damage to DNA. Thus AMAEBC was able to scavenge or neutralize $DPPH^{\bullet}$, $ABTS^{\bullet+}$, superoxide anion radical, lipid peroxy radical, hydroxyl radical and reduce phosphomolybdenum and ferricyanide molecules.

12. The antidiabetic potential of AMAEBC was studied both in *in vitro* and *in vivo* model systems. In *in vitro* model, the effect of AMAEBC was studied on carbohydrate hydrolysing enzymes such α-amylase, sucrase, maltase and PNP-glucoside hydrolase. *In vivo* model studies were conducted on normal rats, streptozotocin induced diabetic rats and high fructose and low streptozotocin diabetic rats.

13. AMAEBC dose dependently inhibited rat intestinal sucrase, maltase and PNP-glucoside hydrolase. The inhibitory potential of bitter cumin was less than the therapeutic α-glucosidase inhibitor acarbose, but relatively higher than DL-catechin, a reported α-glucosidase inhibitor from tea polyphenols.

14. Sucrase inhibitory activity of bitter cumin was found to be higher than maltase inhibitory activity in contrast to most of the reported natural α-glucosidase inhibitors. This property may be due to the presence of high concentration of caffeic acid in bitter cumin which is reported to possess higher inhibitory effect on sucrase activity than maltase activity.

15. Enzyme kinetic studies on α-glucosidase inhibition showed that the K_m values remain unchanged with different concentrations of AMAEBC, but the $1/V_{max}$ values increased with an increase in concentration of AMAEBC. This pattern of inhibition indicates the bitter cumin inhibited α-glucosidase activity in a non-competitive manner.

16. Studies on human salivary α-amylase showed that bitter cumin can inhibit α-amylase activity in a dose dependent manner. The efficacy of bitter cumin in inhibiting α-amylase was less when compared to acarbose. But bitter cumin extract with strong inhibitory effect on α-glucosidase and weak α-amylase inhibitory activity indicates it is more ideal for the control of type 2 diabetes.

17. Bitter cumin significantly reduced postprandial hyperglycemia in overnight fasted maltose loaded normal rats confirming its potential antihyperglycemia activity. The antihyperglycemic activity was found to be better than acarbose but at a higher concentration of bitter cumin extract.

18. In streptozotocin induced diabetic rats an oral feeding of bitter cumin extract for a period of one week reduced the fasting blood glucose level compared to control streptozotocin induced diabetic rats. Bitter cumin extract with very rich phenolic content may be having insulin like activity

or as a strong antioxidant it may be involved in the rejuvenation of β-cells, or may increase the output of insulin from the existing β-cells.

19. High fructose and low streptozotocin type 2 diabetic animal model for oral feeding of bitter cumin extract (AMAEBC) for a period of 4 weeks reduced fasting blood glucose level in a dose dependent manner.

20. Antimicrobial study of bitter cumin extract (AMAEBC) was studied on food borne pathogenic and spoilage bacteria.

21. The preliminary screening of antibacterial activity of bitter cumin extract by agar diffusion method showed that it is more effective on Gram positive bacteria than on Gram negative bacteria.

22. Bitter cumin showed marked growth inhibition of *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* and moderate inhibition of *Listeria monocytogenes* and *Enterobacter spp*. The species like *Escherichi coli* and *Yersinia enterocolitica* are insensitive to bitter cumin extract.

23. Minimum inhibitory concentration (MIC) of AMAEBC against *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes* was 50 ± 7, 260 ±18 and 700 ± 42 µg/mL respectively.

24. Scanning electron microscopy on *B. cereus* and *S. aureus* showed that bitter cumin seed extract caused changes in cell wall configurations leading to cell wall lyses and to cell death.

Future Research

This study has demonstrated that bitter cumin seeds are a rich source of an array of dietary phenolic compounds and flavonoids. The bitter cumin seeds extract showed potent antioxidant, antihyperglycemic and antibacterial activity. The mechanism of antioxidant, antihyperglycemic and antibacterial activities of bitter cumin seeds has been elucidated to certain extent.

Future areas of potential study include-

i) Further clinical investigations are required to confirm antihyperglycemic effect of bitter cumin in treatment of Type 2 diabetes.

ii) The mechanism of antifilarial activity of bitter cumin remains to be established.

iii) Studies on human cancer cells could be useful to examine whether bitter cumin seeds have anticancer property.

iv) Bitter cumin seed powder can be used to control filarial worm infections and also post prandial hyperglycemia. However toxicological and safety profiles of bitter cumin seeds need to be established.

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List of Publications

Original Papers

1. **Ani, V**., Varadaraj, M.C., Akhilender Naidu, K., 2006. Antioxidant and antibacterial activities of polyphenolic compounds from bitter cumin (*Cuminum nigrum*). *Eur Food Res Technol*., **224**:109-115.

2. **Ani, V**., Akhilender Naidu, K., 2008. Antihyperglycaemic activity of polyphenolic components of black/bitter cumin *Centratherum anthelminticum* (L.) Kuntze seeds. *Eur Food Res Technol*., **226**:897-903.

3. **Ani, V**., Akhilender Naidu, K., 2008. Antioxidant activity of bitter cumin (*Centratherum anthelminticum* (L.) Kuntze) in various *in vitro* model systems (to be communicated).

4. **Ani, V**., Akhilender Naidu, K. *In vivo* antidiabetic activity of bitter cumin (*Centratherum anthelminticum* (L.) Kuntze) seeds (to be communicated).

5. **Ani, V**., Varadaraj, M.C., Akhilender Naidu, K. Antimicrobial activity of bitter cumin (*Centratherum anthelminticum* (L.) Kuntze) seeds (to be communicated). .

Posters Presented in symposia

1. **Ani, V**., Akhilender Naidu, K., 2004. Antioxidant and antimicrobial activity of bitter cumin (*Cuminum nigrum*). 16th Indian Convention of Food Scientists and Technologists (ICFOST), Mysore, India.

2. **Ani, V**., Akhilender Naidu, K., 2005. α-Glucosidase inhibitory activity of *Cuminum nigrum* seeds. 74th annual meeting of Society of Biological Chemists (India), Lucknow, India.

3. **Ani, V**., Akhilender Naidu, K., 2006. Antihyperglycaemic activity of *Cuminum nigrum* L. seeds in *in vitro* and *in vivo* model systems. International conference on ethnopharmacology and alternative medicine, Thrissur, India.

4. **Ani, V**., Akhilender Naidu, K., 2007. Modulation of hyperglycaemia and oxidative stress in streptozotocin diabetic rats by phenolic extract of bitter cumin *Centratherum anthelminticum* (L.) Kuntze seeds. International conference on emerging trends in free radical and antioxidant research, Lonavala, India.