Effect Of *Syzygium aromaticum* Extract And Its Active Component, Eugenol, On The Oxidative Stress-Induced Apoptosis In *Saccharomyces cerevisiae* Cells

Archana, A.K.

(12PBC002)

A Thesis Submitted to Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641 043

In Partial Fulfilment of the Requirements for the Degree of Master of Science in Biochemistry

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S. Mythili

Signature of the Head of the Department

31/3/14

Signature of the Supervisor

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

1. INTRODUCTION

Growth for the sake of growth is the ideology of the cancer cell.

– Edward Abbey

Free radicals are well documented for playing important roles in our body as both beneficial and deleterious species. The excess production of free radicals, coupled with a decrease in antioxidant level, results in oxidative stress. It is a harmful process that can damage cell structures, lipids, proteins, DNA and RNA, leading to a number of diseases (Sen *et al*., 2010).

Reactive oxygen species (ROS) are generated either from the byproducts of biological reaction or from exogenous factors. Excess ROS, if not eliminated by the antioxidant system, result in high levels of free radicals and lipid peroxides, which underlie the pathogenesis of degenerative diseases such as atherosclerosis, carcinogenesis, diabetes, cataract and aging. Plant-derived antioxidants, such as flavonoids and phenolic compounds have multiple biological effects. Plant foods, green leafy vegetables and grains are rich sources of antioxidants (Sreeramulu *et al*., 2013).

Cancer is a group of disease characterized by malignant neoplasm. There are many types and causes of cancer. Oxidative stress is closely related to various diseases including cancer. Oxidative stress leads to gene mutation, which affects the intracellular signal transduction and transcription factors, leading to carcinogenesis (Ahmed *et al*., 2013).

A large number of unspecific influences including radiation, chemical, viruses and inflammation underlie malignant transformation. Six essential alterations in cell physiology in cancer are self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained vascularity and tissue invasion and metastasis. Apoptosis is a co-ordinated process that initiates cell death following a variety of cellular insults. The evasion of apoptosis is a predictable physiological response of tumour cells (Seyfried and Shelton, 2010).

Reactive oxygen species increase proliferative status, supporting the idea that active oxygen may be involved in tumour cells. The effect of ROS on cell fate depends on the level at which ROS are present (Mates *et al*., 2012).

Apoptosis can be triggered by exogenous and endogenous stimulus such as ultraviolet radiation, oxidative stress and genotoxic chemicals. Malfunctioning of apoptosis

may cause human diseases such as cancer, neurodegenerative and autoimmune disorders. Potent apoptosis inducing compounds associated with human health prevent tumour promotion and the occurrence of cellular inflammatory response (Rastogi and Sinha, 2009).

It is logically presumable that any compound that can induce apoptosis or any other form of cell death in cancer cells, possesses anticancer activity. This straight-forward strategy is used by scientists worldwide to characterize anticancer compounds. One of the major side effects associated with anticancer chemicals is that they cause cellular death indiscriminately in both cancerous and non-cancerous (normal) cells in the body. In order to minimize these side effects, the research attention is focussing on identifying agents that can protect non-cancerous cells and kill the cancer cells (Balasubramanian and Padma, 2013). In this pursuit, the present study was undertaken to analyze the extract of clove bud (*Syzygium aromaticum*) and its major phenolic active ingredient, namely eugenol, on *Saccharomyces cerevisiae* cells, which are an excellent model system for non-cancerous eukaryotic cells (Sumathi *et al*., 2011a).

Spices such as clove, oregano, mint, thyme and cinnamon, have been employed for centuries as food preservatives and as medicinal plants mainly due to its antioxidant and antimicrobial activities. Clove received the maximum attention due to the potent antioxidant and antimicrobial activities among the other spices.

The clove tree is frequently cultivated in coastal areas at maximum altitudes of 200m above the sea level. Larger production countries of clove are Indonesia, India, Malaysia, Sri Lanka, Madagascar and Tanzania especially in Zanzibar Island. Clove is an important medicinal plant due to the wide range of pharmacological effects consolidated from the traditional use for centuries. *Syzygium aromaticum* is a rich source of eugenol, eugenol acetate and gallic acid (Francisco *et al*., 2014).

Clove (*Syzygium aromaticum*) is the aromatic dried bud of the tree from the family *Myrtaceae*. It is a commonly used spice to add flavour to food preparations. The extract and the essential oil isolated from clove are widely used for many medicinal purposes such as antifungal, anticarcinogenic, antiallergic and antimutagenic activities. Natural products containing bioactive phytochemicals are potentially important sources of drugs. Eugenol is a phenolic compound representing the most important component of clove (Bachiega *et al*., 2012).

Eugenol has been reported to possess various biological properties like antiviral, antioxidant and anit-inflammatory activities. At low concentrations, it usually acts as an antioxidant and anti-inflammatory agent, whereas at higher concentrations, it acts as a pro-oxidant causing increased generation of tissue damaging free radicals (Jaganathan and Supriyanto, 2012).

Saccharomyces cerevisiae is a unicellular organism, which is an excellent model for apoptotic studies. Apoptosis in yeast has been found to resemble that in higher organisms in both morphological and molecular aspects. When yeast is under stress, typical markers of apoptosis such as DNA fragmentation, phosphatidyl-serine externalization and chromatin condensation can be observed (Cui *et al*., 2012).

The present study focussed on studying the effects of *Syzygium aromaticum* extract and eugenol against oxidative stress induced apoptosis in *Saccharomyces cerevisiae*. The study was formulated with the following objectives:

1. To study the cytotoxic effect of *Syzygium aromaticum* extract and eugenol in *Saccharomyces cerevisiae* cells.
2. To analyze the cellular and nuclear events associated with apoptotic death in *Saccharomyces cerevisiae* cells.

The vast literature pertaining to the study was collected and studied. The following chapter presents a brief review of the same.

\

2. REVIEW OF LITERATURE

Oxidative stress is caused by the imbalance between reactive oxygen species (ROS) and the antioxidant defence system. Reactive oxygen and nitrogen free radicals are produced during immune activity, which triggered by several environmental factors such as pollution, smoke and sunlight. This leads to harmful effects of these reactive species, which include cellular damage, such as RNA, DNA, proteins and lipids (Saleh *et al*., 2010).

Free radicals are produced in normal or pathological cell metabolism. Oxygen is very essential to many living organisms for the production of energy as a form of fuel in biological processes. Uncontrolled production of oxygen radicals in living organisms triggers many diseases such as cancer, rheumatoid arthritis, cirrhosis and arteriosclerosis. All organisms are protected from free radical damage by antioxidants such as superoxide dismutase and catalase (Saleh *et al*., 2010).

It is known that oxidative stress is involved in most of the pathological states and a number of human neurodegenerative disorders, diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders. ROS and other oxidants can cause oxidation of lipids, proteins and DNA. These toxic products of oxidation produce cytostatic effects causing membrane damage and lead into cell death via apoptosis or necrosis. Free radicals arise from various chemical and physical agents. ROS can be produced from endogenous sources, such as from mitochondria, peroxisomes and inflammatory cells activation, as well as exogenous sources, such as environmental agents, pharmaceuticals and industrial chemicals (Klaunig *et al*., 2010).

2.1 FREE RADICALS

Free radicals are highly reactive molecules produced during mitochondrial aerobic metabolism, which can be effectively scavenged by antioxidant molecules (Li *et al*., 2013). Free radicals can be formed from molecules through the hemolytic cleavage of a chemical bond and via redox reactions (Sen *et al*., 2010).

2.2 CELL DEATH

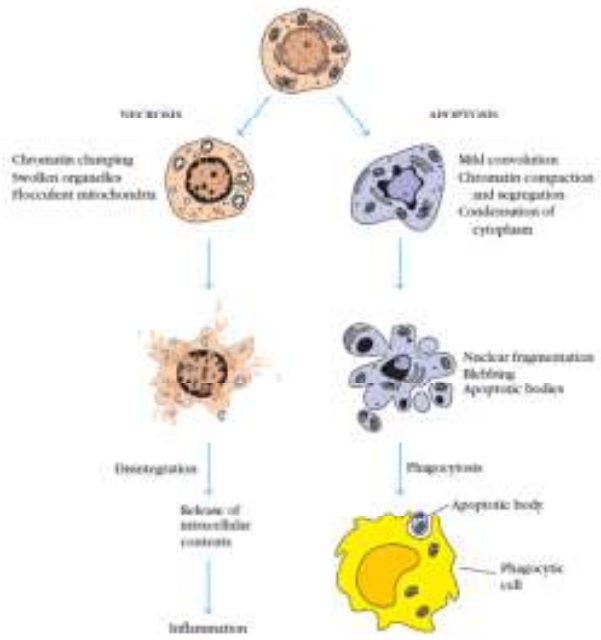
Cell death is the basic cellular process that takes place during the development of an organism in order to maintain homeostasis and structural integrity. There are three commonly observed forms of cell death namely apoptosis, necrosis and autophagy

(Krysko *et al*., 2013). Apoptosis is a programmed cell death occurring in many different kinds of cells, which is characterized by cytoplasm shrinkage, chromatin condensation and fragmentation, apoptotic body formation, caspase activation and maintenance of the plasma membrane (Hanus *et al*., 2013).

2.2.1 APOPTOSIS AND NECROSIS

Apoptotic cell death is important during the development of organism as well as regulation of immune system. The cell death is typically induced within the same tissue. Apoptosis is the major type of programmed cell death. Approximately 10 million cells undergo apoptosis in healthy adult humans each day. The cell undergoes alterations during apoptosis, chromatin condense, cells lose their attachment to surrounding tissue and shrink as the most peculiar property of apoptosis mechanism and the cell membrane starts blebbing (Chaabane *et al*., 2013).

Figure 2.1: Apoptosis and Necrosis



(http://www.symposcium.com/2013/07/qa-what-is-the-difference-between-apoptosis-and-necrosis/)

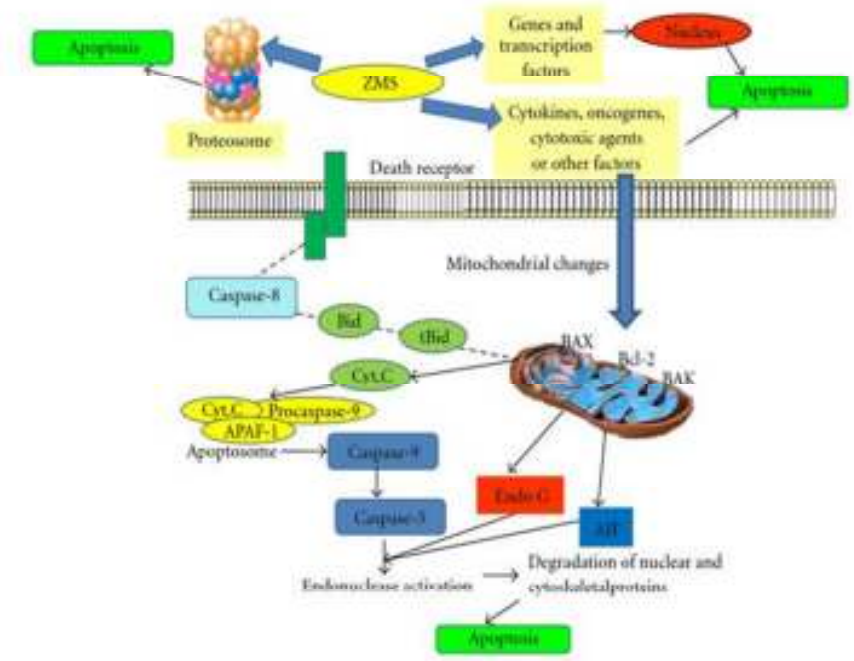
In necrosis, there is an increase in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents. Numerous mediators, organelles and cellular processes occur in necrotic cell death. The inflammatory response is caused by the cytosolic constituents pouring into the intercellular space (Chaabane *et al*., 2013).

The action of necrosis is initiated by Toll-like receptors in the presence of caspase inhibitors. The mitochondrial alteration such as lysosomal changes, nuclear changes, lipid degradation and increase in the cytosolic concentration of calcium leads to over load of mitochondria and activation of non-caspase protease (Galluzzi *et al*., 2009).

2.2.2 MECHANISM OF APOPTOSIS

Apoptosis is a distinct type of cell death, which is one of the most complex signaling pathways and highly conserved process in most eukaryotes. There are two main pathways of apoptosis: extrinsic (death receptor) pathway and intrinsic (mitochondrial) pathway (Figure 2.2). Apoptosis is an evolutionary conserved cell death process that occurs in the variety of physiological situations. Apoptotic stimulus induces an initiation, commitment and degradation phase. Type I extrinsic or death receptor pathway generates an apoptotic aggregation of death ligand. Type II or intrinsic pathway signals through mitochondria (Smith *et al*., 2010).

Figure 2.2: Mechanism of Apoptosis (Intrinsic and extrinsic)



(http://www.hindawi.com/journals/ecam/2011/765029/fig10/)

Apoptosis, derived from the Greek word for a natural process of cells, looks like leaves falling from trees or petals from flowers. It is a distinct form of programmed cell

death. Apoptosis is characterized by a cascade of tightly controlled checks and balances that must be successfully negotiated prior to reaching a point of no-return life-or-death decision that results in the irreversible execution of the cell. This endogenous death program is associated with several biochemical and morphological features including cell shrinkage, membrane blebbing, nuclear and chromatin condensation and DNA fragmentation (Kabbage *et al*., 2013). Apoptosis involves oxidative stress regulation via normal cell death in homeostasis of multicellular organisms (Mates *et al*., 2012).

The extrinsic pathway of apoptosis is triggered by the activation of cell surface receptors of tumor necrosis factor (TNF) super family. These TNF super families contributing to the extrinsic pathway of apoptosis are TNFRSF10A, B and TNFRSF6, TNFSF1 R1. Fas receptor is a prototype member of TNF/nerve growth factor receptor superfamily of death receptor. Fas receptor associates with Fas-associated protein death domain (FADD) and pro-caspase, which form a "death inducing signaling complex" (DISC). Deoxyribonuclease, activated by caspase-3 or caspase-8, cleaves BID to cause a change in mitochondrial permeability and release of cytochrome c. Caspase-3-activation is responsible for DNA fragmentation, which exist in the cytosol as inactive ICAD/CAD complex (Darwish, 2010).

The intrinsic pathway is a mitochondria-mediated pathway. Mitochondria play a central role in the integration and circulation of death signals, initiating inside the cells of oxidative stress, and DNA damage in regulating cell death pathway. Some cytotoxic agents are also involved in the formation of pores in the mitochondrial membranes such as nitrogen monoxide, arsenic, streptozotocin and mercury (Sinha *et al*., 2013).

Intrinsic pathway is initiated within the cell. The intrinsic pathway is closely related to the protein of Bcl-2 family. There are two groups of proteins – pro-apoptotic proteins (Bax, Bak, Bad, Bcl-xs) and anti-apoptotic proteins (Bcl-2, Bcl-x, Bcl-w). Anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c. The pro-apoptotic factors induce such release. The release of cytochrome c into the cytosol activates caspase 3 via formation of a complex, termed as apoptosome, which is made up of cytochrome c, Apaf-1 and caspase 9 (Wong, 2011).

2.3 OXIDATIVE STRESS

Oxidative stress is caused by an imbalance between the production of reactive oxygen species and the antioxidant defence system, which leads to oxidative deterioration

of protein, lipids and DNA. ROS has been implicated in the induction of diabetes mellitus, ageing process, cancers, atherosclerosis, acquired immunodeficiency syndrome and neurological disorders (Lobo *et al*., 2010).

2.3.1 DISEASES ASSOCIATED WITH OXIDATIVE STRESS

2.3.1.1 CARDIOVASCULAR DISEASE

Heart disease is the biggest killer that is responsible for about half of all the deaths. In atherosclerosis, progressive lipid disorder and the incidence increases with age. Three most important cell types in the vessel wall are endothelial cells, smooth muscle cell and macrophages, which can release free radical which affects lipid peroxidation (Lobo *et al*., 2010).

2.3.1.2 NEURODEGENERATIVE DISEASE

Parkinson disease is a progressive neurodegenerative movement disorder, which is associated with the loss of dopaminergic neurons in the substantia nigra. The dopaminergic metabolism contributes to the oxidative stress, which leads to intracellular macromolecules. The mitochondrial dysfunction and the consequent increase in reactive oxygen species, trigger a sequence that leads to cell demise. Activated microglia produce nitric oxide and superoxide during neuroinflammatory response, which was aggravated by the damaged dopaminergic neurons (Hwang, 2013).

2.3.1.3 CANCER

Cancer is a multidimensional spectrum of diseases characterized by malignancies. It is a major health disorder occurring in almost every part of the world. Oxidative stress is involved in the pathophysiology of various types of cancers. The human system is constantly exposed to both exogenous and endogenous free radicals. Oxidative stress causes gene mutations, which lead to alterations in the signal transduction pathways, which ultimately leads to the development of cancer (Ahmed *et al*., 2013).

Cancer cells, which exhibit an accelerated metabolism, demand high ROS. In normal cells, low level concentrations of these compounds are required for signal transduction. In cancer cells which exhibit a rapid metabolism, demand for high ROS resistance include the alternative pathways, which can avoid large amount of ROS accumulation compromising the energy demand required by cancer cells. The ROS regulation is highly significant for cancer therapy, as the radio and chemotherapeutic drugs influence tumour outcome through ROS modulation (Sosa *et al*., 2013).

2.3.1.4 AIDS

Oxidative stress plays a major role in the development of various symptoms and processes involved in viral replication, inflammation, decreased immune cell proliferation, loss of immune function and apoptosis (Valle *et al*., 2013). HIV kills $CD4^+$ T lymphocytes which function as regulators and amplifiers of the immune response. The tightly regulated process of apoptosis occurs through receptor mediated signals. Receptor-mediated apoptosis has an essential role in maintaining homeostasis of T lymphocyte number (Wong, 2011).

2.3.1.5 MULTIPLE SCLEROSIS

Multiple sclerosis is a chronic inflammatory disease of the central nervous system, in which the loss of myelin sheath occurs, fatty tissue is deposited around the nerve fibers and allows the conduction of electrical impulses. Oxidative stress plays a major role in the process of development of multiple sclerosis and the supplementation of the dietary antioxidants found major therapy in the treatment of multiple sclerosis (Hadzovic-Dzuvo *et al*., 2011).

In multiple sclerosis, IL-2 deprivation occurs, and Bad is formed, dephosphorylated and translocates to the mitochondrial membrane. This binds to Bcl-2 and Bcl-x and neutralizes their anti-apoptotic capacity. This process leads to loss of mitochondrial membrane potential and leads to apoptosis. Mitochondrial hyperpolarization contributes to apoptosis resistance in SLE upon IL-2 deprivation (Halaby, 2012).

2.4 THE BUDDING YEAST (*Saccharomyces cerevisiae)*

Yeast (*Saccharomyces cerevisiae*) is a unicellular organism that has been used as a model organism for eukaryotic systems. Yeast cell is widely used to identify many cellular mechanisms and cell metabolism, cell division and cell death. It is used as a model for studying programmed cell death (PCD), apoptotic markers including DNA fragmentation and chromatin condensation (Wanichthanarak *et al*., 2013).

2.5 MEDICINAL PLANTS

Plants are good sources of food, which provide essential nutritional values and medicinal properties. Natural products are valuable, especially plants for curing disease. Many bioactive compounds are directly used drugs like digoxin, digitoxin, morphine,

reserpine, taxol, vinblastine and vincristine. Plants give immune response against pathogens and improving body resistance to the disease (Varalakshmi *et al*., 2009).

2.6 *Syzygium aromaticum*

Clove (*Syzygium aromaticum*) are the aromatic dried buds of a tree used as spice in virtually the entire world's cuisine. Cloves have many medicinal uses and have been most famously applied to toothache, mouth and throat inflammation. The scientific classification of clove is as follows:

Kingdom	-	Plantae
Phylum	-	Angiosperms
Unranked	-	Eudicots
Unranked	-	Rosids
Order	-	Myrtales
Family	-	*Myrtaceae*
Genus	-	*Syzygium*
Species	-	*aromaticum*

The clove has been used in India and China for over 2,000 years, as a spice to check both tooth decay and counter halitosis (bad breath). In Persia and China, it is considered to have aphrodisiac properties. Cloves have historically been used in Indian cuisine (both north Indian and south Indian). In the north Indian cuisine, it is used in almost every sauce or side dish made, mostly ground up along with other spices. One of the main constituents of clove oil is eugenol, which exhibits broad antimicrobial activities against Gram-positive, Gram-negative and acid-fast bacteria, as well as fungi. Cloves are well known also for their antiemetic (relieves nausea and vomiting) and carminative properties (Bhowmik *et al*., 2012).

The volatile oil of cloves (about 85-92% eugenol) was highly active against a range of test microorganisms, being classified as bactericidal in nature. Along with the recreational uses of cloves, they are also said to be anthelmintic. In foods and beverages, clove is used as a flavouring agent. In manufacturing, clove is used in toothpaste, soaps, cosmetics and perfumes (Bhowmik *et al*., 2012).

2.6.1 ACTIVE CONSTITUENTS OF *Syzygium aromaticum* (CLOVE) OIL

72-90% of the essential oil extracted from cloves has eugenol. The other essential oil ingredients of clove oil are**,**

1. Acetyl eugenol
2. Beta-caryophyllene and vanillin
3. Crategolic acid, tannins, gallotannic acid and methyl salicylate (pain killer)
4. Flavonoids eugenin, kaempferol, rhamnetin and eugenitin
5. Triterpenoids like oleanolic acid

(Bhowmik *et al*., 2012)

2.6.2 MEDICINAL USES

Clove possesses antibacterial properties, which can be used in various dental creams, tooth pastes, mouth washes and throat sprays for cleanse bacteria. It is also used to relieve pain from sore gums and dental health. Clove effectively cures many digestive problems. Clove has medicinal qualities to cure flatulence, loose motions, indigestion and nausea. Clove and clove oil boost the immune system by purifying the blood and help to fight against various diseases. Cloves can effectively prevent lung cancer as well as skin cancer. Eugenol present in clove helps in minimizing the harmful effects of environmental waste. A very important medicinal use of this clove is controlling the blood glucose levels in diabetic patients. Eugenol is a powerful and effective compound in preventing blood clots. The dried clove bud contains carbohydrates, fixed oil, steam volatile oil, resins, tannins, proteins, cellulose, pentosans and mineral elements (Milind and Deepa 2011).

2.6.3 PHARMACOLOGICAL ACTIVITIES

Clove has anti-viral activity and is a potent antiviral agent. Eugenol isolated from clove shows antiviral activity against Herpes simplex virus. The antioxidant activity of clove bud extract and its major components, eugenol and eugenol acetate, were comparable to that of the natural antioxidant α-tocopherol. Eugenol inhibited 5-lipoxygenase activity and leukotriene C-4 in human PMNL cells. Eugenol also functions as an anti-inflammatory agent (Milind and Deepa 2011).

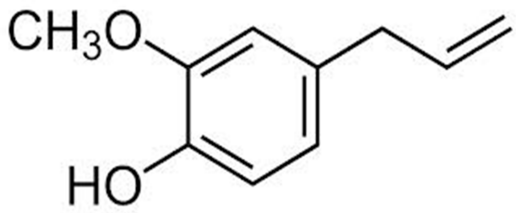
2.7 EUGENOL

Eugenol, an active compound present in *Syzygium aromaticum* mainly exists in clove oil, camphorated oil, cinnamon leaf oil and nutmeg oil. Eugenol is a high volatile compound extracted from clove bud. Eugenol was used in traditional medicine, such as

bactericide, fungicide and anesthetic. Eugenol is a natural phenolic compound (Figure 2.3), present in basils, cinnamon, betel leaves and bay leaves. Eugenol has six natural and synthetic eugenol related compounds, which can inhibit cancer cells. Eugenol possesses antiviral activity *in vitro* and *in vivo* against human herpes virus (Santos *et al*., 2009).

Figure: 2.3

Structure of Eugenol



(https://www.erowid.org/archive/rhodium/chemistry/tcboe/chapter9.html)

2.7.1 ANTIOXIDANT ACTIVITY

Eugenol can avoid functional disorders of endothelial cells resulting from oxidized low-density lipoprotein (LDL) and increase antioxidant activity to inhibit active oxygen generation. Phenols have important antioxidant activity because they can capture the oxygen atom from alkyl radical over the oxidation group by devoting a hydrogen atom of the phenolic hydroxyl group (Kong *et al*., 2014).

2.7.2 ANTIBIOTIC ACTIVITY

Eugenol has antibiotic activity because it can block the proton-motive force, electron stream, active transport and cause coagulation of cell contents. Eugenol has numerous activities such as antibiotic, antiviral, antimycotic and antiparasitic activities. Eugenol has antibacterial efficacy (Kong *et al*., 2014).

2.7.3 ANTICANCER ACTIVITY

The anticancer activity of eugenol has become an important topic in recent years. Eugenol, in combination with gemcitabine, inhibited cancer cell growth. Eugenol can reduce the invasion and cancer angiogenesis by regulating Bcl-2 protein family. It is shown to have good curative effects in skin cancer and melanoma (Kong *et al*., 2014).

Although many reports are available on the biological activities of clove extracts and eugenol, there are no studies that have concentrated on the apoptotic events in yeast cells. The present study focussed on the apoptotic activity of the clove extract and eugenol in *Saccharomyces cerevisiae* cells. The layout of the study and the parameters analysed are presented in the next chapter.

3. EXPERIMENTAL PROCEDURE

Clove is a widely distributed plant in the tropical and subtropical regions which is widely used in toothache and for mouth and throat inflammation. Cloves have historically been used in Indian cuisine (Bhowmik *et al*., 2012). This plant represent one of the richest source of phenolic compounds like eugenol, eugenol acetate and gallic acid and posseses great potential for pharmaceutical, cosmetic, food, and agricultural applications (Francisco *et al*., 2014).

The present study focussed on characterizing the various apoptotic events provoked by the bud extracts of *Syzygium aromaticum* in *Saccharomyces cerevisiae* cells.

3.1 MATERIALS USED AND LAYOUT OF THE STUDY

3.1.1 YEAST STRAIN

Commercially available Baker's yeast was used to study the apoptotic events. The yeast granules were washed several times with sterile PBS and the suspended pellet was used for the experiments.

3.1.2 PREPARATION OF CLOVE EXTRACT

Cloves were purchased from the local market and powered using a blender (Plate 3.1). This powder (5g) was taken in 250ml ethanol and heated at 80°C for 1 hour. The extract was filtered, evaporated and used for each experiment after dissolving in DMSO to a concentration of 1g/ml. Before evaporation, an aliquot of the extract was injected into a reverse phase C18 column of HPLC to check the presence of eugenol, as explained in Appendix I.

Following this, the dose of the extract and eugenol to be used for the quantification of cell death was determined *in vitro* using a spectrophotometric DPPH-scavenging assay, as outlined in Appendix II. This assay was carried out after reconstituting the evaporated residue of the extract in DMSO at a concentration of 1g in 1ml.

3.1.3 TREATMENT GROUPS

The treatment groups set up for the present study were as follows

1. Yeast cells (untreated control)
2. Yeast cells+ H_2O_2 (positive control)

3. Yeast cells + ethanolic extract of *Syzygium aromaticum*
4. Yeast cells + H_2O_2 + ethanolic extract of *Syzygium aromaticum*
5. Yeast cells + eugenol
6. Yeast cells + H_2O_2 + eugenol

The treatment groups were incubated at room temperature for 1 hour. Based on the results of the concentration-dependent study, as presented in the next chapter, 25µl of *Syzygium aromaticum* extract and 5µl of eugenol at a final concentration of 250mM were taken. The oxidant H_2O_2 was used at a final concentration of 200µM.

3.2 PARAMETERS ANALYZED

The viability of the *S. cerevisiae* cells and the events associated with apoptosis were determined in the presence and the absence of the test substances (clove extract and eugenol) and/or the oxidizing agent (H_2O_2).

3.2.1 DETERMINATION OF THE EXTENT OF VIABILITY

The extent of viability in the various treatment groups was determined by the MTT and SRB assays.

3.2.1.1 MTT ASSAY

The viability of the cells in the presence and the absence of H_2O_2, with or without clove extracts and eugenol was estimated by MTT assay as proposed by Igarashi and Miyazawa (2001) and explained in Appendix III.

3.2.1.2 SRB ASSAY

The extent of death in the cells was studied by Sulphorhodamine B assay, as proposed by Skehen *et al*. (1990) as detailed in Appendix IV.

3.2.2 STUDY OF APOPTOTIC EVENTS

The cellular and nuclear events associated with the process of apoptotic death were studied in the various treatment groups using a spectrum of staining methods.

3.2.2.1 MORPHOLOGICAL CHANGES

The morphological characteristics of yeast cells was studied by Giemsa staining as described by Chih *et al*. (2001) and given in Appendix V.

3.2.2.2 NUCLEAR CHANGES

The nuclear changes such as chromatin condensation and fragmentation were investigated in yeast cells by ethidium bromide staining as proposed by Mercille and Massie (1994) with slight modifications, propidium iodide staining as explained by Sarkar *et al*. (2000) and AO/EtBr differential staining (Parks *et al*., 1979), as explained in Appendices VI, VII and VIII respectively. The extent of aopotosis was also quantified using DAPI staining as proposed by Rashmi *et al*. (2003) (Appendix IX).

3.3 STATISTICAL ANALYSIS

The experiments were carried out in triplicates and the results obtained are expressed as mean ± standard deviation. The number of apoptotic cells observed in the various treatment groups were subjected to one-way ANOVA using the SigmaStat statistical package (Version 3.1).

The observations made and the results obtained in the various tests are presented, and discussed in the light of relevant literature, in the next chapter.

PLATE 3.1

The clove used in the present study

4. RESULTS AND DISCUSSION

Oxidative stress is an alteration in the pro-oxidant and antioxidant balance, which causes potential damage, leading to the pathophysiology of many disorders. Oxidative stress arises when the production of reactive oxygen species overwhelms the intrinsic antioxidant defence. This reactive oxygen species play an important role in intracellular signalling aiming at maintaining the cellular homeostasis. But a higher level of oxidation can cause indiscriminate damage to biological molecules, leading to loss of function and cell death (Burton *et al*., 2011).

High level of oxidative stress can cause necrosis, ATP depletion and the induction of controlled apoptotic death. ROS accumulates in aging and plays a crucial role in chronic diseases. Oxidative stress is an important factor in neurodegenerative disease, which damages neurons which could be due to either increased oxidative process or decreased antioxidant content (Shukla *et al*., 2011).

Aromatic plants have been used in folk medicine since ancient times and the essential oils from such plants are known to possess antibacterial, antifungal and antiviral activities (Pinto *et al*., 2009). *Syzygium aromaticum* has been used in the treatment of tooth ache and mouth and throat inflammation (Bhowmik *et al.*, 2012). Almost 72-90% of clove essential oil extract contains eugenol and is reported to possess potential antioxidant activity (Jaganathan *et al.,* 2010).

The present study sought to understand the influence of *Syzygium aromaticum* extract and its major active component, eugenol, on the cell death events induced by imposing oxidative stress in *Saccharomyces cerevisiae* cells. Cloves were purchased from local market, powdered and extracted in ethanol. This extract was tested for the presence of eugenol using HPLC.

4.1 HPLC ANALYSIS OF THE CLOVE EXTRACT

The *Syzygium aromaticum* extract showed a clear peak that corresponded to the eugenol standard. An additional smaller peak was also observed in the extract, indicating the presence of other components. However, it is clear from the HPLC profile that eugenol is the major component in the clove extract, which is depicted in Figures 4.1 and 4.2.

FIGURE 4.1

HPLC Profile of Eugenol

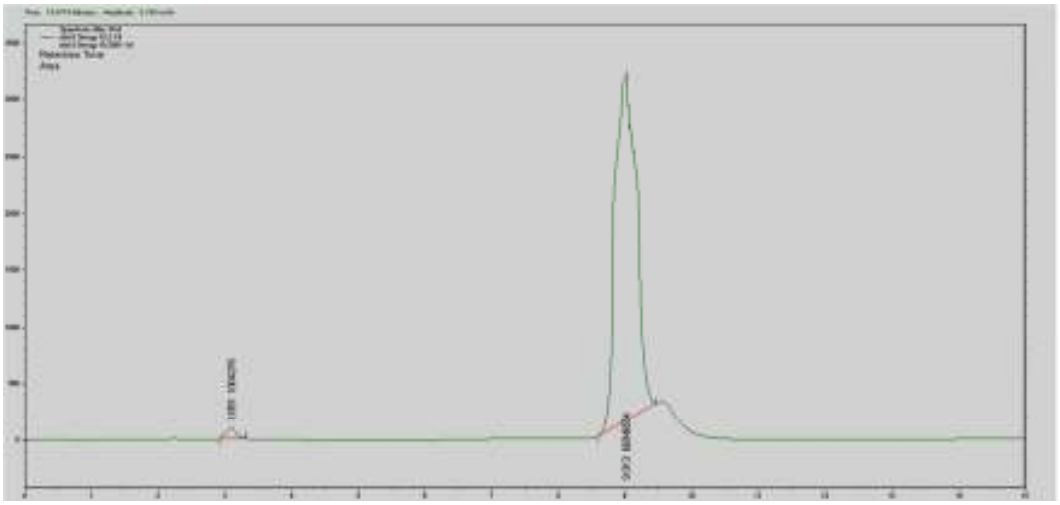
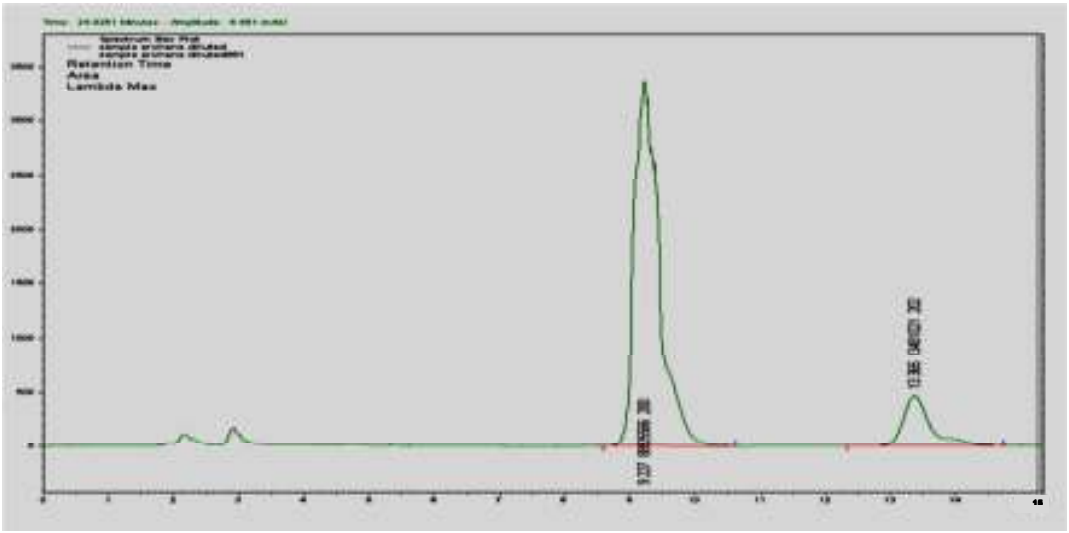


FIGURE 4.2

HPLC Profile of *Syzygium aromaticum* Extract



Several studies have reported the presence of eugenol in many spices. Wan *et al.* (2008) reported that eugenol has been detected in clove bud essential oils. Moreno *et al.* (2013) have also identified the presence of the active compound eugenol in *Acorus calamus*.

Several reports have proved that phenolic compounds present in spices that are used as food adjuncts possess potent antioxidant, anti-inflammatory, antimutagenic and cancer preventive activities. The antioxidative effects of eugenol in clove has been extensively studied (Srinivasan, 2014).

Gundala and Aneja (2014) demonstrated that *Piper betel* leaf constituents induce apoptosis in cancer, due to the presence of eugenol for the chemotherapeutic management of cancer. Venkadeswaran *et al.* (2014) suggested that eugenol from *Piper betel* leaf, possessesd antihypercholesterolemic in hypercholesterolemic Wistar rats.

Having detected the compound of interest in the extract, the extract and the commercially available compound (eugenol) were analyzed for their influence on the oxidatively-stressed *Saccharomyces cerevisiae* cells. In order to optimize the dose level to be used, an antioxidant dose-response effect was carried out using DPPH.

4.2 DPPH RADICAL SCAVENGING EFFECTS OF *Syzygium aromaticum* EXTRACT AND EUGENOL

The extent of DPPH scavenging was determined using varying volumes of *Syzygium aromaticum* extract ranging from 1µl to 50µl. The results obtained are presented in Figure 4.3. It was observed that the clove extract scavenged DPPH radical in a dose-dependent manner. While the extent of scavenging showed an increase in the lower volumes (1-25µl), it plateaued at volumes higher than 25µl. This observation indicated that near-maximum antioxidant activity can be achieved at 25µl dose of the extract, and therefore, this dose was used in subsequent experiments in the present study.

The DPPH scavenging was also assessed for eugenol ranging from 5 to 500µM. The results are presented in Figure 4.4. It was found that eugenol effectively reduced the stable radical DPPH. A similar trend as the clove extract was observed for eugenol, with the extent of scavenging showing a plateau after 125µM concentration. It was, therefore, decided to use 125µM eugenol in the further experiments.

Antioxidant activity has been evaluated using DPPH radical scavenging measurements by several workers. Arteaga *et al.* (2012) reported a remarkable free radical scavenging activity for phenol derivatives, which was attributed to the presence of -R groups located on the phenolic ring, which included eugenol, in clove oil (Farias *et al.,* 2013). The extract of *Eugenia polyantha* bark, which is rich in eugenol has been reported to have strong DPPH scavenging activity, attributable to the presence of rich antioxidative compounds (Lelono and Tachibana, 2013). Adefegha and Oboh (2012) investigated the inhibitory properties of phenolic extracts of clove bud using DPPH and ABTS assays and reported potent antioxidant activities.

FIGURE 4.3

DPPH Radical Scavenging Activity of *Syzygium aromaticum* Extract

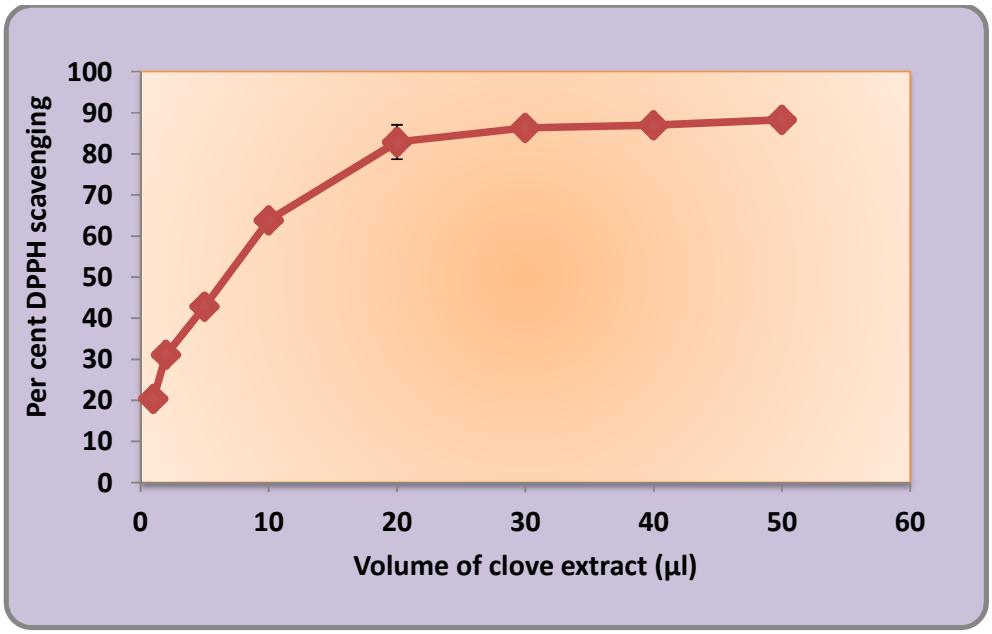
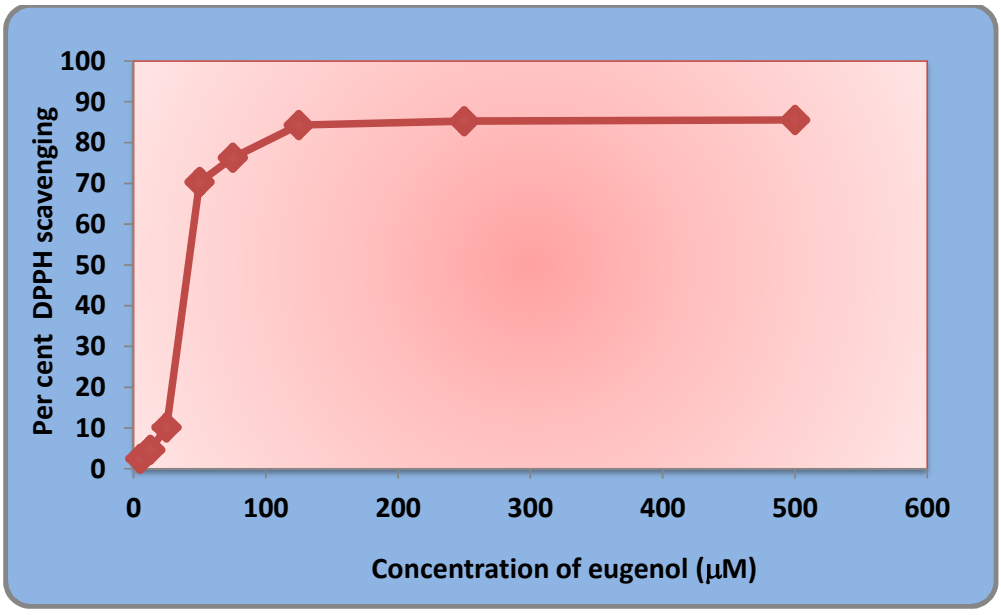


FIGURE 4.4

DPPH Radical Scavenging Activity of Eugenol



Nam and Kim (2013) also showed the presence of eugenol as a major compound in the clove and *Magnoliae flos,* which exhibited remarkable free radical scavenging effects. Hamed *et al.* (2012) reported that the microemulsion of eugenol showed potent antioxidant and antimicrobial activities than crude clove bud essential oil.

In the present study, the results showed that the *Syzygium aromaticum* extract and eugenol exhibit potent DPPH scavenging ability. This assay was done *in vitro*. However, the effect of any compound or extract needs to be confirmed in the intact cell, because the conditions in the living cell/tissue are complex and results from an interplay of several factors and conditions, including the permeability of the compounds into the cells, the effect of various cellular components like enzymes on the compounds, as well as the possible toxic effect of the compound to the cells themselves.

Therefore, the protective effects of clove extract and eugenol were also proved in intact, live cells (*Saccharomyces cerevisiae)*. These cells have been reported to be an excellent model for representing the eukaryotic organisms as a whole (Rego *et al.,* 2013). It has also been shown that oxidative stress can result in various events culminating in apoptotic death in the yeast cells (Cui *et al.,* 2012). Thus, the present study was designed to follow the extent of cell death in *Saccharomyces cerevisiae* cells exposed to oxidative stress in the presence and the absence of the test substances (clove extract or eugenol), and to understand their effects on the cellular and nuclear features associated with apoptotic death. The results of these tests are presented below.

4.3 EFFECTS OF *Syzygium aromaticum* EXTRACT AND EUGENOL ON THE APOPTOTIC EVENTS IN *Saccharomyces cerevisiae*

Yeast cell is an easily manipulated model system that is used to determine the preliminary cytotoxicity of compounds (Limberger *et al.,* 2011). The apoptotic events induced by oxidative stress imposed by H_2O_2 in *S. cerevisiae* cells were studied by determining the extent of viability (MTT, SRB) and by various staining methods (Giemsa, PI, EtBr, DAPI and AO/EtBr). The results obtained are presented and discussed below.

4.3.1 VIABILITY ASSAYS

The viability assays were carried out in order to study the effect of *Syzygium aromaticum* bud extract and eugenol in *Saccharomyces cerevisiae* cells, in the presence and the absence of H_2O_2.

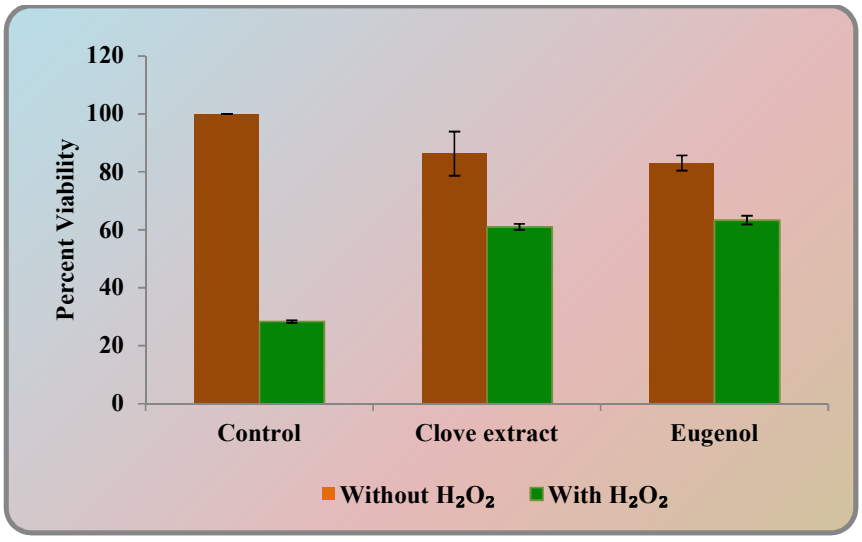
4.3.1.1 MTT ASSAY

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase in actively growing cells to produce a purple formazan product. Hence, it is widely used to measure cell proliferation and for screening of anticancer drugs (Zaidi *et al.,* 2012).

MTT assay was performed to analyse the ability of *Syzygium aromaticum* bud extract and eugenol in the presence or the absence of H_2O_2. The oxidant treated cells showed a steep fall in the viability of *S. cerevisiae* cells. This cytotoxic effect was significantly decreased by the presence of both clove extract and eugenol, indicating their protective effects. The results are depicted in Figure 4.5.

FIGURE 4.5

Effect of *Syzygium aromaticum* extract and eugenol on *Saccharomyces cerevisiae* Cells subjected to Oxidative Stress (MTT Assay)



The values are mean ± S.D of triplicates

Several studies are available in the literature, which support our findings. Palaniswamy and Padma (2011) reported that the methanolic extract of *Majorana hortensis* leaves increased *Saccharomyces cerevisiae* cell viability under the oxidative stress conditions, which showed that the cytotoxicity of H_2O_2 was counteracted by the administration of *M. hortensis* leaf extract. Similarly, Lakshmi (2012) showed that

artemisinin and dihydroartemisinin prevented the cytotoxicity induced by H_2O_2 in *Saccharomyces cerevisiae.*

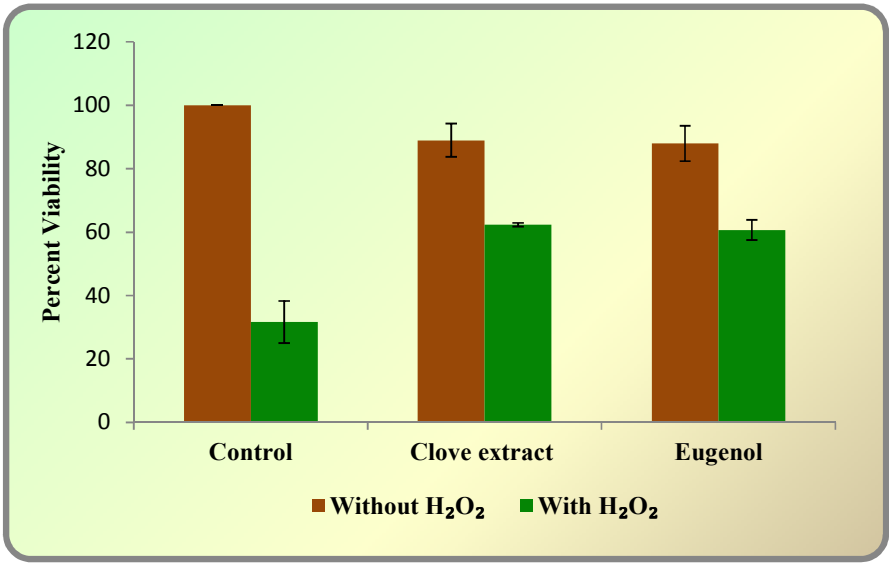
The *Curcuma amada Roxb* rhizome and leaf extract rendered protection to yeast cells exposed to oxidative stress and MTT assay revealed that the extract by itself did not cause any cellular damage (Sumathi *et al.,* 2013). Farias *et al*. (2013) proved that eugenol derivatives had no cytotoxic effect in the liver cells. Similarly Jeong *et al.* (2014) indicated that HS-1793, a resveratrol analogue, significantly increased the cell viability of irradiated Chinese hamster ovary (CHO)-K1 cells. All the above studied render support for the results obtained in our study.

4.3.1.2. SRB ASSAY

Sulphorhodamine B (SRB) assay was used to evaluate the viability of cells, which directly determines the metabolic activity of the cells (Populo *et al.,* 2013). SRB assay was performed to confirm the viability in *Saccharomyces cerevisiae* cells treated with clove extract or eugenol in the presence or the absence of H_2O_2. The oxidant caused a steep decrease in cell survival. The viability of the cells increased markedly in the presence of clove extract and eugenol when co-treated with H_2O_2. The results also indicated that both the treated groups performed well in preventing the cytotoxicity, as shown in Figure 4.6.

FIGURE 4.6

Effect of *Syzygium aromaticum* extract and eugenol on *Saccharomyces cerevisiae* Cells subjected to Oxidative Stress (SRB Assay)



The values are mean ± S.D of triplicates

The methanolic extract of *Nyctanthes arbor-tristis* leaves improved the percent viability of yeast cells exposed to oxidant treatment as estimated by SRB assay (Jayapratha, 2010). Similarly, earlier studies in our laboratory in *S. cerevisiae* exposed to oxidative stress *in vitro* by SRB showed that the methanolic extract of *Euphorbia antiquorum* is protective to the yeast cells (Sumathi *et al*., 2011a).

A report by Yamuna (2014) showed that the flower extracts of *C. pulcherrima* improved the viability of the yeast cells in the presence of oxidant as analysed by SRB assay. Sivaprabha (2014) has reported that the *Curcuma amada Roxb.* leaf and rhizome extracts alone did not induce any harmful effects to yeast cells. Abdallah *et al.* (2011) also demonstrated that caffeic acid and quercetin prevented the toxic effects of Lambda-cyhalothrin treated rat erythrocytes.

Similar trend as the MTT assay was also followed in the results of SRB assay, which proved that the clove extract and eugenol decreased the cell death in the presence and absence of H_2O_2. In order to confirm this, the apoptotic cells were observed and counted by the various staining methods. The results are given below.

4.3.2 CELLULAR AND NUCLEAR EVENTS ASSOCIATED WITH APOPTOSIS

The cells undergoing morphological and nuclear changes such as membrane blebbing, chromatin condensation, cell shrinkage and nuclear fragmentation during apoptosis were observed and counted in the presence and the absence of extract, compound and/or H_2O_2. The numbers of apoptotic cells and normal cells were counted under phase contract microscope with various dyes such as Giemsa, ethidium bromide, propidium iodide, DAPI and AO/EtBr.

4.3.2.1 MORPHOLOGICAL CHANGES BY GIEMSA STAINING

The number of apoptotic cells present in the various treatment groups is presented in Table 4.1. The apoptotic ratio was calculated from this number and the values obtained are represented in Figure 4.7. Plate 4.1 shows the photographic record of the cells showing apoptosis. The number of apoptotic cells increased in the oxidant treated group, which decreased significantly on co-treatment with clove extract and eugenol.

TABLE 4.1

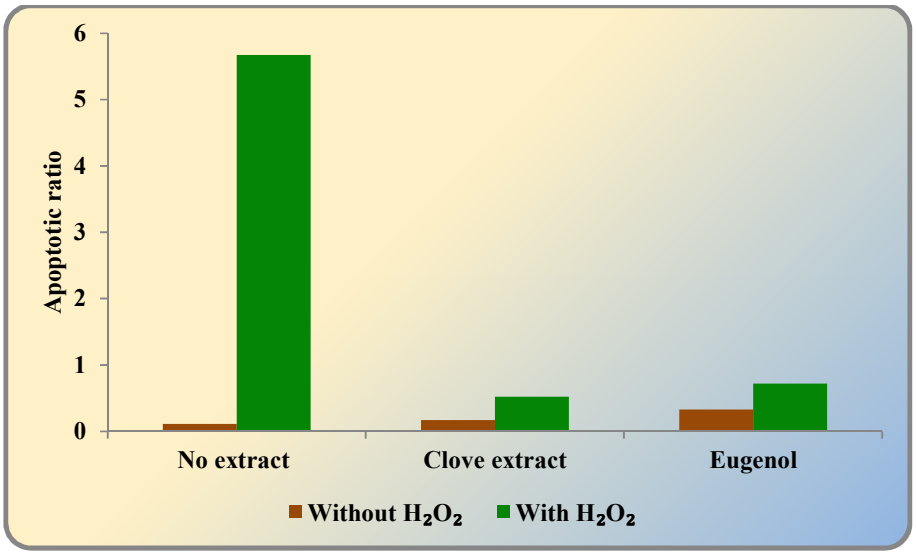
Effect of *Syzygium aromaticum* Extract and Eugenol on the Morphological Changes in *Saccharomyces cerevisiae* Subjected to Oxidative Stress (Giemsa staining)

Treatment groups	No. of Apoptotic cells/100 cells	
	Without H_2O_2	With H_2O_2
No extract	10 ± 2	85 ± 1 [a]
Clove extract	15 ± 2 [a]	34 ± 3 [a,b,c]
Eugenol	25 ± 1 [a]	42 ± 6 [a,b,c]

The values are mean ± SD of triplicates
a - Statistically significant ($p<0.001$) compared to untreated control group
b - Statistically significant ($p<0.001$) compared to H_2O_2 treated group
c - Statistically significant ($p<0.001$) compared to the respective group treated with the extract/compound alone

FIGURE 4.7

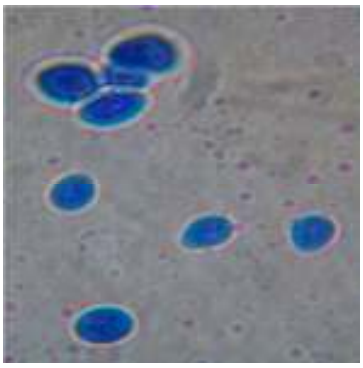
Effect of *Syzygium aromaticum* Extract and Eugenol on the Morphological Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative stress (Giemsa staining)



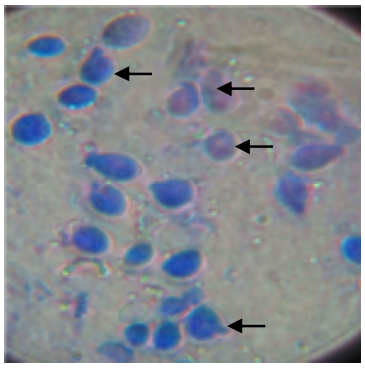
The values are mean ± S.D of triplicates

PLATE 4.1

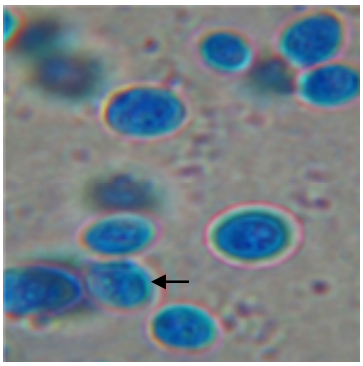
***Saccharomyces cerevisiae* Cells stained with Giemsa**



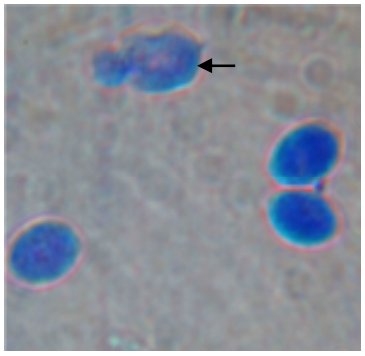
Untreated control



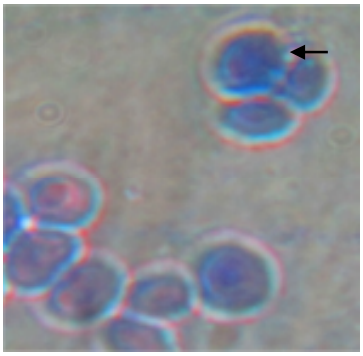
H_2O_2



***S. aromaticum* extract**



***S. aromaticum* extract + H_2O_2**



Eugenol

Eugenol + H_2O_2

Giemsa is a stain which is commonly used to differentiate the apoptotic and normal cell based on the morphological changes. A significant decrease in apoptotic cells was observed in *S. cerevisiae* cells following the treatment with *Piper betle* leaf extract (Sulekha, 2011). The effect of *Rhinacanthus nasutus* on oxidative stress induced apoptosis was also studied in yeast cells by the Giemsa staining to differentiate the apoptotic and normal cells (Nirmaladevi, 2008).

Morphological features of apoptotic cells include shrinkage, condensation of chromatin and cytoplasm (Palaniswamy and Padma, 2011). Muthukumari *et al.* (2013) reported the apoptosis by Giemsa staining, wherein anethole and etoposide combinatorial drug treatment group showed more blebbing and cell shrinkage when compared with untreated groups. *Clitoria ternatea* showed a strong protection against oxidative damage in CCl_4 induced hepatotoxicity in rats (Jayachitra *et al.,* 2012).

A report of Chavasco *et al.* (2014) demonstrated the extracts of stem and flower of *Heliconia rostrata* and the leaf and stem of *Plinia cauliflora* did not show any cytotoxicity in C6-36 larvae cells of the *Aedes albopictus* mosquito. Our findings, in relation to these reports, proved the protective effects of clove extract and eugenol in the oxidant treated yeast cells.

4.3.2.2 ETHIDIUM BROMIDE STAINING

EtBr staining was implemented to observe the nuclear changes of the apoptotic process, which is depicted in the Table 4.2, Figure 4.8 and Plate 4.2. The results of the present study revealed that the *Syzygium aromaticum* extract and eugenol can provide a significant protective effect against H_2O_2 in yeast cells.

Ethidium bromide stains nucleus in cells and emits red fluorescence under fluorescence microscope (Choudhari *et al*., 2013). To demonstrate that apoptotic events have been reduced by *A. vulgaris m*ethanolic plant extract, yeast cells were analysed by EtBr staining (Sharmila, 2014).

In another study, the nuclear changes were observed and quantified in the normal chick embryo fibroblasts exposed to etoposide in the presence and the absence of methanolic extract of latex of *Euphorbia antiquorum*. The results revealed that latex did not induce apoptosis in normal cells but it modulated the apoptotic effects produced by etoposide (Sumathi *et al*., 2011b).

TABLE 4.2

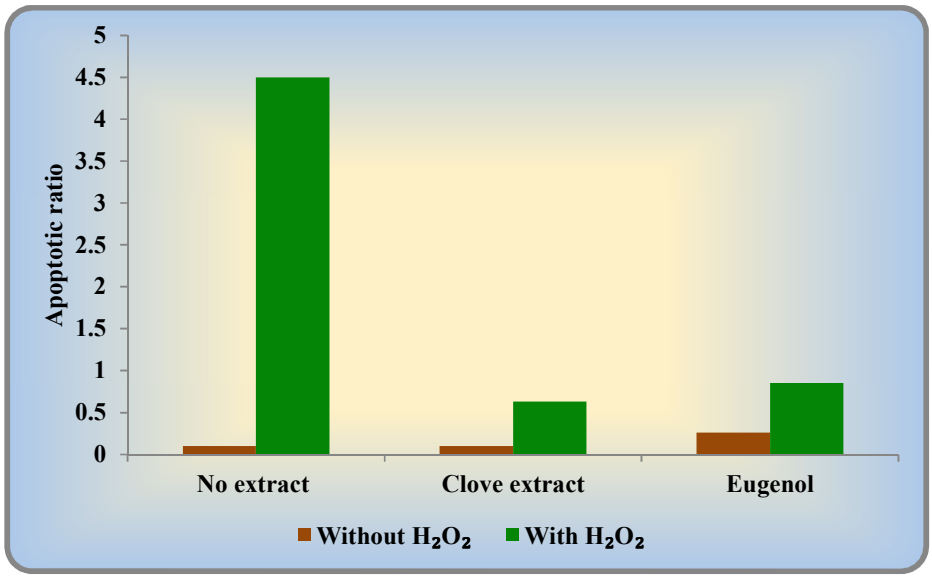
Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (Ethidium bromide staining)

Treatment groups	No. of Apoptotic cells/100 cells	
	Without H_2O_2	With H_2O_2
No extract	10 ± 2	82 ± 3[a]
Clove extract	11 ± 2	39 ± 1[a,b,c]
Eugenol	21 ± 2[a]	46 ± 1[a,b,c]

The values are mean ± SD of triplicates
a - Statistically significant ($p<0.001$) compared to untreated control group
b - Statistically significant ($p<0.001$) compared to H_2O_2 treated group
c - Statistically significant ($p<0.001$) compared to the respective group treated with the extract/compound alone

FIGURE 4.8

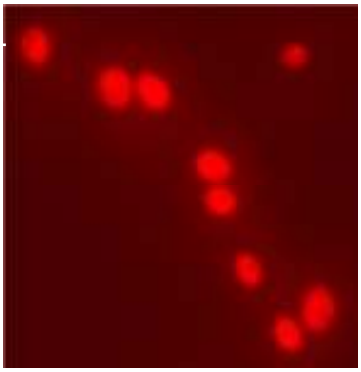
Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (Ethidium bromide staining)



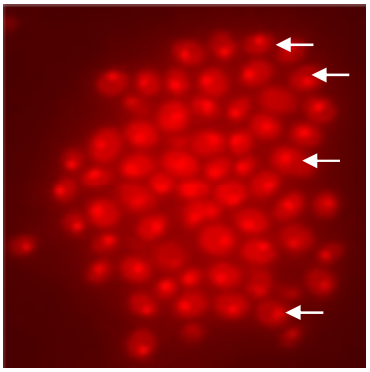
The values are mean ± S.D of triplicates

PLATE 4.2

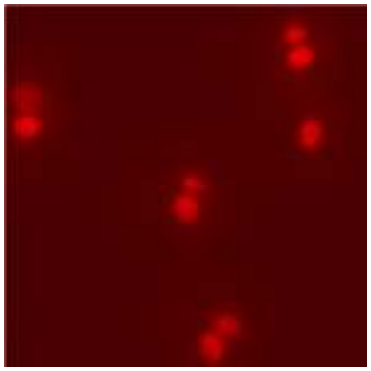
Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (Ethidium bromide staining)



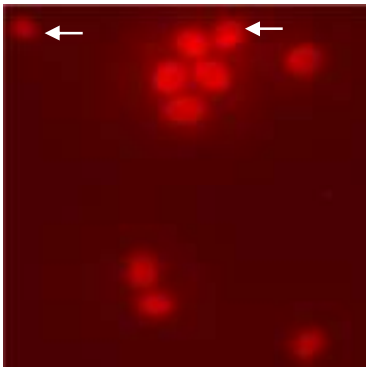
Untreated control



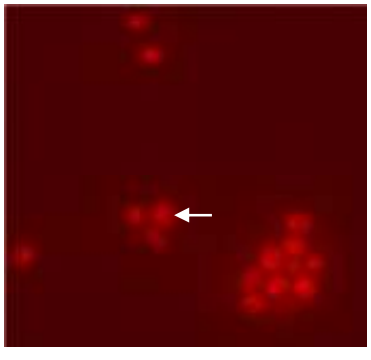
H_2O_2



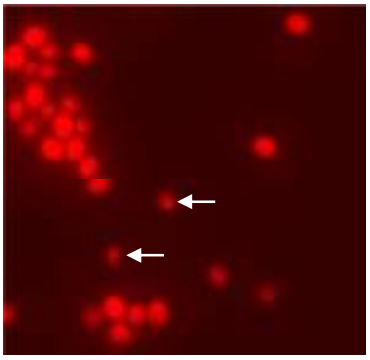
***S. aromaticum* extract**



***S. aromaticum* extract + H_2O_2**



Eugenol



Eugenol + H_2O_2

Solanum nigrum leaves with black and red berries showed the prevented the cellular damage in oxidative stress imposed goat liver slices *in vitro* (Padma and Kalaivani, 2013). The results of Lassiter *et al.* (2010) showed the prevention of oxidative stress in pyuravate treated chicken embryo fibroblast. In tune with these reports, the present study also showed the protective effect of clove extract and eugenol, reiterating the interference that clove extract and eugenol render protection to the normal cells against oxidative death.

4.3.2.3 PROPIDIUM IODIDE STAINING

The apoptotic events in *Saccharomyces cerevisiae* cells, as seen by nuclear changes, were assessed using PI staining method. These results are shown in Table 4.3, which prove that the *Syzygium aromaticum* extract and eugenol showed significant apoptotic inhibitory effect against oxidant induced stress in yeast cells. The photographic view of *S. cerevisiae* showing apoptosis is presented in Plate 4.3, and the calculated apoptotic ratios are depicted in Figure 4.9.

TABLE 4.3

Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (Propidium iodide staining)

Treatment groups	No. of Apoptotic cells/100 cells	
	Without H_2O_2	With H_2O_2
No extract	11 ± 1	82 ± 2[a]
Clove extract	18 ± 2 [a]	30 ± 3 [a,b,c]
Eugenol	31 ± 4 [a]	40 ± 3 [a,b,c]

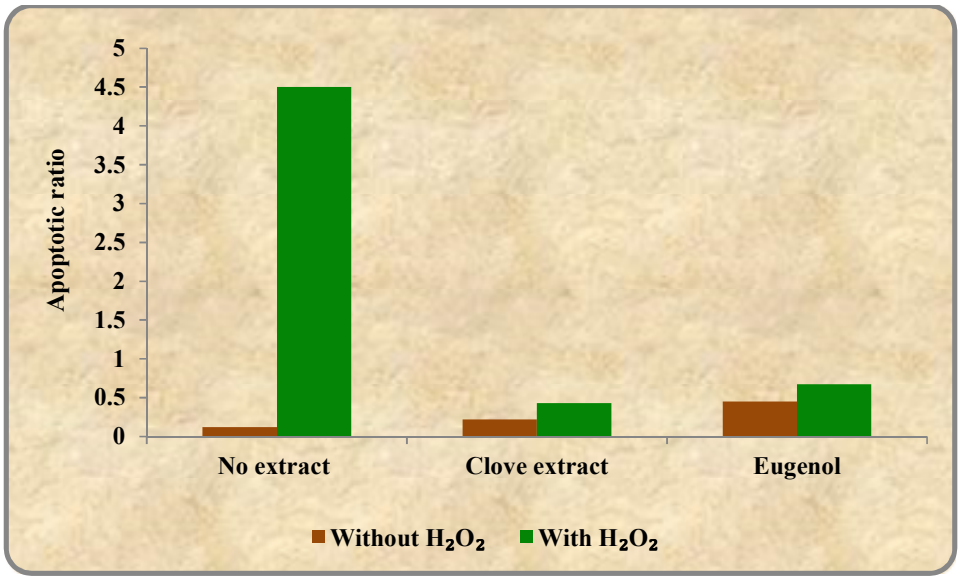
The values are mean ± SD of triplicates
a - Statistically significant ($p<0.001$) compared to untreated control group
b - Statistically significant ($p<0.001$) compared to H_2O_2 treated group
c - Statistically significant ($p<0.001$) compared to the respective group treated with the extract/compound alone

Philip *et al.* (2012) reported that the red fluorescing dye propidium iodide (PI) is only permeable to dead cells and cannot enter the intact plasma membrane of living cells.

Radha (2010) proved that the *Bacopa monnieri* leaf extract protected the yeast cells from oxidative stress induced cell death. Quercetin was shown to inhibit doxorubicin-induced cytotoxicity in murine spleen cells (Du *et al.,* 2010). The findings of Spincemaille *et al.* (2014) indicated the potential of *Arabidopsis thaliana* - derived decapeptide to prevent Cu-induced apoptosis in yeast cells.

FIGURE 4.9

Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (Propidium iodide staining)

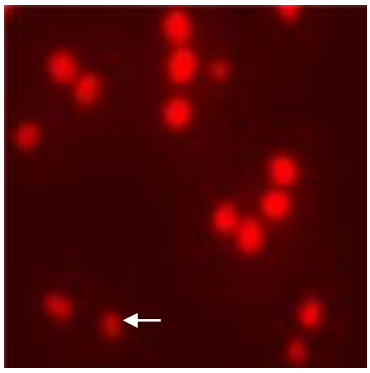


The values are mean ± S.D of triplicates

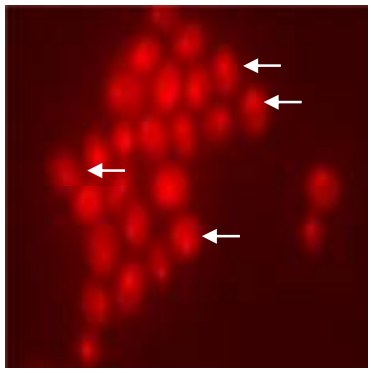
The results of Haniya and Padma (2013) showed that the leaf extracts of *Artemisa vulgaris* have strong ability to improve the antioxidant status in the oxidatively stressed liver slices. Similar results was also proved by Yamuna and Padma (2013) using yellow, pink and orange flowers extract of *Caesalpinia pulcherrima*, *Swartz* and Sivaprabha *et al.* (2013) using *Curcuma amada* in the precision-cut goat liver slices exposed to oxidative stress.

PLATE 4.3

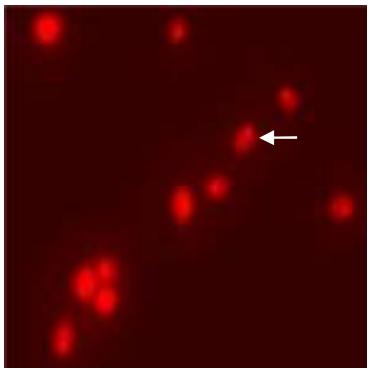
Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (Propidium iodide staining)



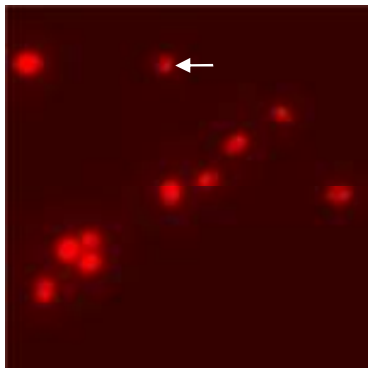
Untreated control



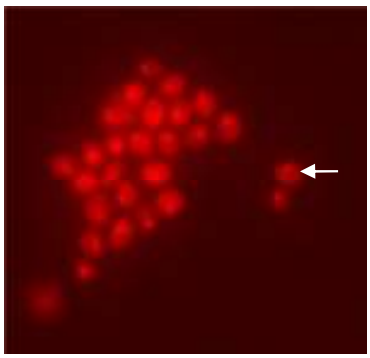
H_2O_2



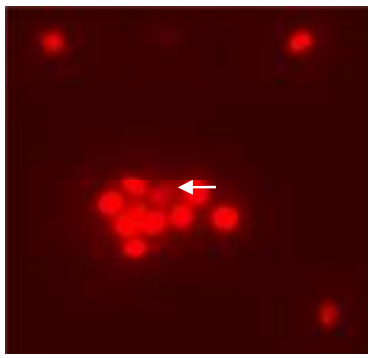
***S. aromaticum* extract**



***S. aromaticum* extract + H_2O_2**



Eugenol



Eugenol + H_2O_2

Sharmila (2014) reported that the human peripheral blood lymphocytes with nuclear changes were found to be increased in etoposide treated group and remarkable decrease when treated with the leaf extract *Artemisa vulgaris*. The results of our study reveals that the clove and eugenol posses the antioxidative property, which strongly support the earlier findings of our research group.

4.3.2.4 ACRIDINE ORANGE/ETHIDIUM BROMIDE STAINING

In order to study the difference between apoptotic and normal cells under the fluorescent microscope AO/EtBr staining was used. In AO/EtBr staining, apoptotic cells are observed as orange fluorescence and necrotic cells are observed as red fluorescence due to their loss of membrane integrity (Vivek *et al.,* 2013). In the present study, the induction of apoptosis in *Saccharomyces cerevisiae* was decreased on treatment with *Syzygium aromaticum* extract and eugenol. The results are depicted in Table 4.4, Figure 4.10 and Plate 4.4.

TABLE 4.4

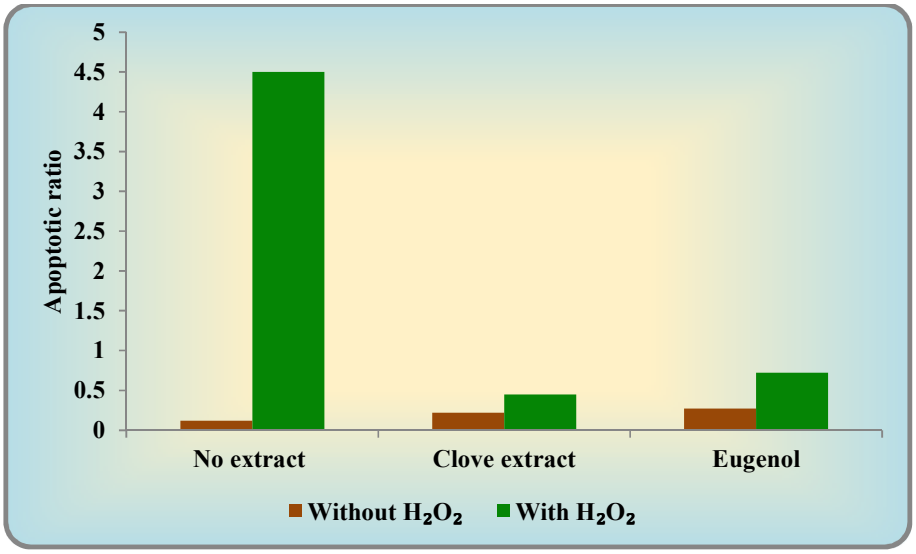
Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (AO/EtBr staining)

Treatment groups	No. of Apoptotic cells/100 cells	
	Without H_2O_2	With H_2O_2
No extract	11 ± 1	80 ± 2[a]
Clove extract	18 ± 2 [a]	30 ± 3 [a,b,c]
Eugenol	31 ± 4 [a]	40 ± 3 [a,b,c]

The values are mean ± SD of triplicates
a - Statistically significant ($p<0.001$) compared to untreated control group
b - Statistically significant ($p<0.001$) compared to H_2O_2 treated group
c - Statistically significant ($p<0.001$) compared to the respective group treated with the extract/compound alone

FIGURE 4.10

Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (AO/EtBr staining)



The values are mean ± S.D of triplicates

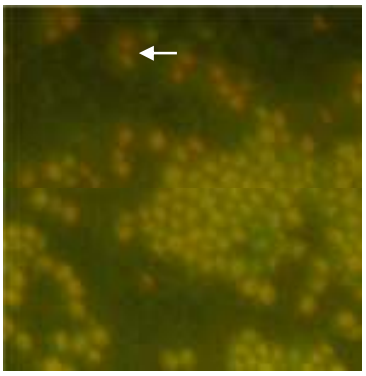
In AO/EtBr staining, the control cells fluoresced uniformly green and had normal features. The cells in the H_2O_2-treated group fluoresced red, indicating apoptosis. The proportion of cells stained red markedly decreased in the grapes treated with clove extract/eugenol along with H_2O_2, clearly demonstrating the protective effect render by both the extract and the pure compound.

The induction of apoptosis in yeast cells was analyzed by AO/EtBr and the results showed that apoptotic nuclei were found more frequently in H_2O_2 treated cells. This was reverted significantly by the *Majarona hortensis* leaf extract (Palaniswamy and Padma, 2011).

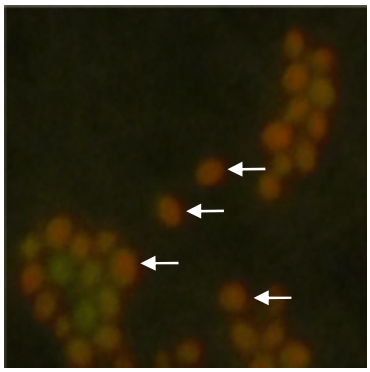
Sreelatha and Padma (2010) evaluated the efficacy of *Moringa oleifera* leaves against carbon tetrachloride (CCl_4)-treated liver slices *in vitro*, wherein the leaf extracts effectively suppressed CCl_4-induced oxidative stress. Sumathi *et al.* (2011b) also proved that the treatment with hydrogen peroxide decreased the antioxidant status of goat liver slices and co-treatment with the *Nyctanthes arbor-tristis* extract improved the antioxidant status of liver slices.

PLATE 4.4

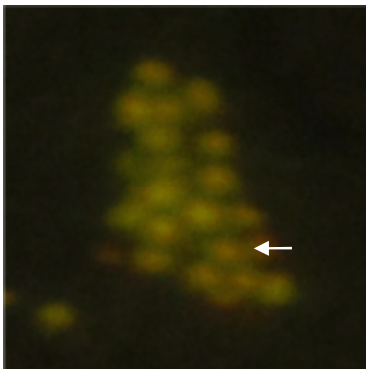
Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (AO/EtBr staining)



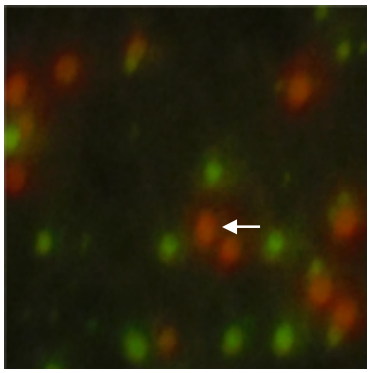
Untreated control



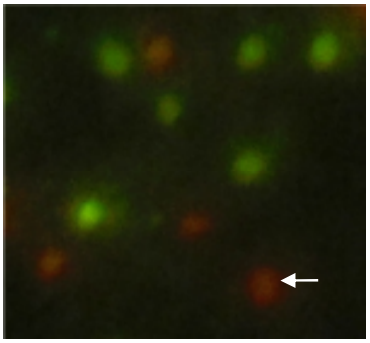
H_2O_2



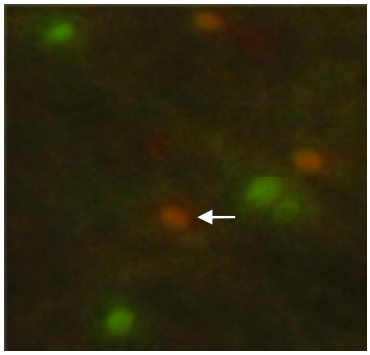
***S. aromaticum* extract**



***S. aromaticum* extract + H_2O_2**



Eugenol



Eugenol + H_2O_2

Balasubramanian and Padma (2012) also showed the protection of precision-cut goat liver slices by *Zea mays* leaf extracts from hydrogen peroxide induced oxidative stress. The clove extract and eugenol proved to be the anti-apoptotic in oxidative agent treated cells.

4.3.2.5 DAPI STAINING

The nuclear changes like cornering was observed using DAPI stain. The exposure of *Saccharomyces cerevisiae* cells to H_2O_2 caused a very significant proportion of cells to commit to apoptosis. The co-exposure to clove extract and eugenol caused a statistically significant reduction in the proportion of dying cells. However, the extent of cell survival did not reach basal (untreated control) values. The results are shown in Table 4.5, Figure 4.11 and Plate 4.5.

TABLE 4.5

Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (DAPI staining)

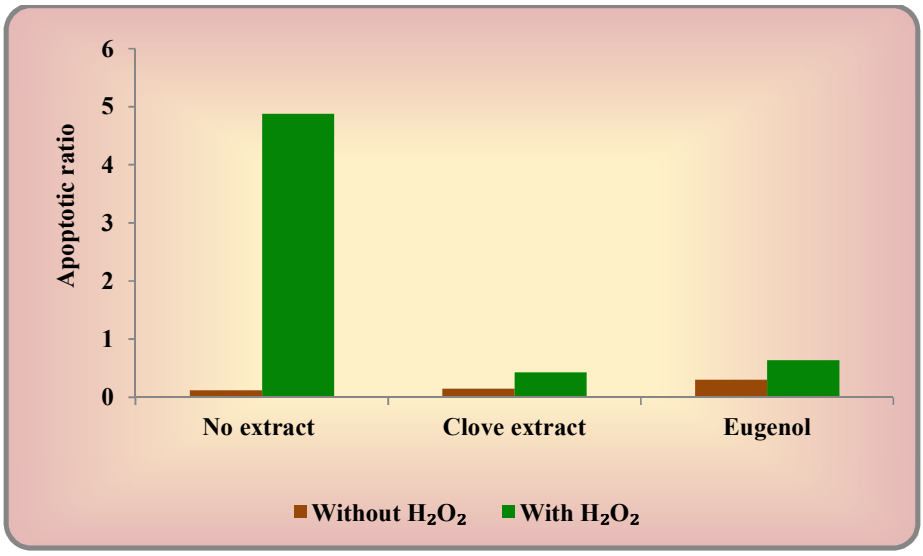
Treatment groups	No. of Apoptotic cells/100 cells	
	Without H_2O_2	With H_2O_2
No extract	11 ± 1	83 ± 1 [a]
Clove extract	13 ± 2	30 ± 4 [a,b,c]
Eugenol	23 ± 2 [a]	39 ± 3 [a,b,c]

The values are mean ± SD of triplicates
a - Statistically significant ($p<0.001$) compared to untreated control group
b - Statistically significant ($p<0.001$) compared to H_2O_2 treated group
c - Statistically significant ($p<0.001$) compared to the respective group treated with the extract/compound alone

DAPI staining can be adopted for apoptotic studies to evaluate the nuclear changes of apoptosis (Morrison *et al*., 2010). Oorschot *et al.* (1997) studied the long-term expression of apoptin in normal human fibroblasts and revealed that apoptin has no toxic or transforming activity in these cells. The maximum inhibition of H_2O_2-induced apoptosis was exhibited by the methanolic extract of *Zea mays* leaf in chick embryo fibroblast cells (Kiruthika *et al.,* 2013).

FIGURE 4.11

Effect of *Syzygium aromaticum* Extract and Eugenol on the Nuclear Changes in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (DAPI staining)



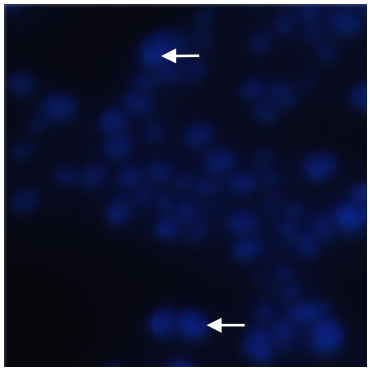
The values are mean ± S.D of triplicates

Artemisia vulgaris leaf extract significantly reduced the apoptotic events in yeast and primary chick embryo cells, as evidenced by DAPI staining (Haniya, 2010). DAPI staining was used to prove the protective effect of *C. pulcherrima* against oxidant induced apoptosis in *S.cerevisiae* (Yamuna, 2014). The cell death induced by methanolic extract of *Prosopis cineraria* leaf extract was studied in HBL100 normal breast cells, and the results showed that the extract did not exhibit any harmful effects to the cells (Sumathi *et al.,* 2013).

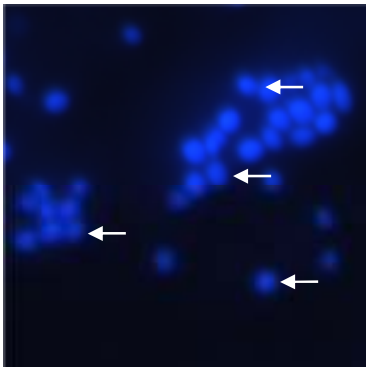
The results of present study show that *Syzygium aromaticum* extract and eugenol act as strong protective agents in *Saccharomyces cerevisiae* cells against oxidative stress-induced cell death. The results also confirmed the use of *Saccharomyces cerevisiae* as a model organism for study the oxidative stress by applying various staining methods.

PLATE 4.5

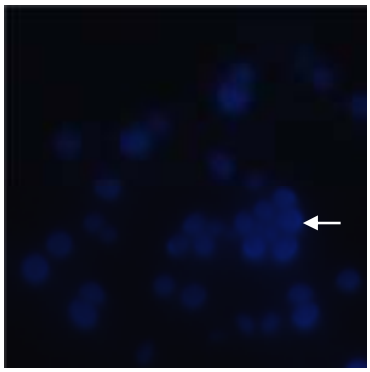
Effect of *Syzygium aromaticum* Extract and Eugenol in *Saccharomyces cerevisiae* Cells Subjected to Oxidative Stress (DAPI staining)



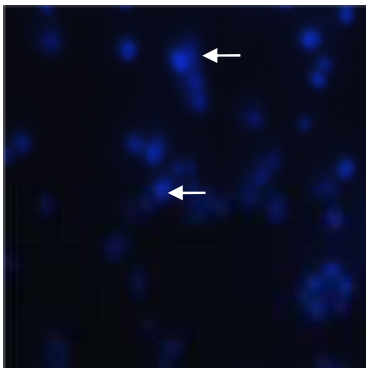
Untreated control



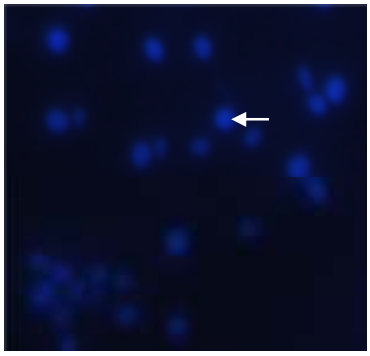
H_2O_2



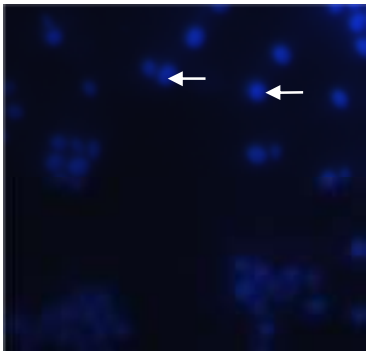
***S. aromaticum* extract**



***S. aromaticum* extract + H_2O_2**



Eugenol



Eugenol + H_2O_2

It can be concluded from the present study that clove extract and eugenol can effectively revert the damage caused by oxidizing agents by rescuing the cells from death. This is a significant observation, as several chemotherapeutic agents (like etoposide) are known to cause oxidative apoptosis in the untransformed eukaryotic cells, the results of the present study throws light on the fact that clove extract and eugenol can protect normal (non-cancerous) cells against such toxicity. These results are summarized in the next chapter, along with the conclusions and inference that can be drawn.

5. SUMMARY AND CONCLUSION

The traditional Indian cuisine is rich with a variety of health-promoting substances, offering a wide range of components that can directly influence the metabolism and well being. Adoption of the western style of life, especially in the food habits, which include the fad of fast foods that is fast-becoming the preference of the younger generation, is resulting in the slow loss of such balanced supply of the components of the traditional diets. It becomes imperative to understand the importance of the components of such preparations and reiterate the values of the age-old traditions by unearthing the scientific basis of the action of the components.

One such major component of the diet constitutes the spices. The spices in the Indian diet fall into a large class of characteristic agents, which include flavour-enhancing, texture-enhancing, taste-influencing and other such condiments. One such component is the clove, which is predominantly used as a flavour-enhancing component in the Indian kitchens.

Clove is known to possess several medicinal benefits, as documented in the traditional medicinal systems. The consumption of clove is associated with curative effects for digestive disorders, bacterial and fungal infections, oral hygiene, sinusitis, skin problems, hair loss, diabetes, cardiovascular disorders and a variety of other conditions. The traditional medical implications of the consumption of clove also include anti-septic, local anaesthetic, anti-inflammatory, rubefacient (warming and soothing), carminative and anti-flatulent properties, and it is considered to be a good stress reliever. The part of the plant that is used as a dietary ingredient is the bud of clove.

The clove bud is a source of the clove oil, which is used as an essential aromatic oil. The major component of this oil, and hence of the clove bud, is recognized to be eugenol, an aromatic phenolic phytochemical with several health influencing properties. The presence of eugenol has been identified in several other plants like the betel leaves, tulsi leaves, basil, celery, cinnamon and nutmeg. It is recognised to be responsible for the spicy aroma of clove.

Several studies have probed the health beneficiary effects of the clove bud (*Syzygium aromaticum*). They have been recognized to have strong analgesic, antimicrobial and antioxidant properties. Some studies have also shown the anticancer properties of

clove. However, there are no systematic studies reported on the influence of the clove bud and its components on the non-cancerous or untransformed cells under the influence of oxidative stress.

The present study was undertaken with the objective of analyzing and characterizing the effects of clove extract and its major component, eugenol, on the extent of cell death induced in *Saccharomyces cerevisiae* (yeast) cells, imposed by oxidative stress. Oxidative damage is well established to be the root cause for several, outwardly unrelated, diseases and disorders, including diabetes, cancer, cardiovascular diseases, autoimmune and neuro-degenerative disorders. Thus, it is presumable that any agent that possesses strong antioxidant property and is able to counteract or decrease the damage caused by oxidizing agents, can also render good protection against the multitude of diseases resulting from oxidative damage.

In the present study, *S. cerevisiae* cells were chosen as a model system to study the effects of clove extract and eugenol on oxidative stress-induced cell death, because the model offers several advantages. The yeast cells have a short doubling time, and therefore, grow rapidly. They constitute a very reliable test system due to the genomic and proteomic homology with higher eukaryotes in general and the human system in particular. Thus, any effects observed in the budding yeast system can be reliably extrapolated to the human system.

For the present study, the clove buds were purchased from the local market in Coimbatore. They were powdered using a blender and extracted with hot ethanol to maximize the yield of eugenol in the extract. This extract was first tested for the presence of eugenol, using HPLC. The HPLC profiles showed a clear peak that corresponded to the eugenol standard, confirming that eugenol was the major component in the extract. Therefore, the extract was tested alongside the commercially available eugenol pure compound, for their influence on the oxidative stress-induced death process in *Saccharomyces cerevisiae*.

Before initiating the assays in *S. cerevisiae* cells, the dose to be used was optimized in an *in vitro* DPPH-scavenging assay, both for clove extract and eugenol. Different volumes of the clove extract (ranging from 1µl to 50µl) and varying concentrations (5µM to 500µM) were tested in the DPPH assay. It was found that both clove extract and eugenol scavenged

DPPH in a dose-dependent manner. From this assay, the optimum doses of the extract and eugenol were found to be 25µl and 125µM respectively. Therefore, these doses were used in the subsequent assays.

Oxidative stress was imposed in *Saccharomyces cerevisiae* cells using H_2O_2 at 200µM final concentration. The cells were incubated with or without the test compounds (clove extract or eugenol) in the presence and the absence of the oxidizing agent, at 37°C for one hour with mild shaking. The cells were pelletted down after incubation, and were subjected to quantification of the viability (by MTT and SRB assays) and apoptotic events (by various staining methods like Giemsa, EtBr, PI, AO/EtBr and DAPI).

MTT and SRB assays complement each other in the quantification of the extent of cytotoxicity. The results showed that the exposure to H_2O_2 imposed a severe oxidative stress in the yeast cells, as reflected by the steep increase in the extent of cell death. When clove extract or eugenol were co-administered to the cells, there was a significant reduction in the proportion of dead cells, with the levels reaching values closer to the untreated control group. These observations proved that the test substances, namely clove extract and eugenol, were very effective in protecting the yeast cells against oxidative stress.

Among the staining procedures used, Giemsa helps in the visualization of the cellular morphology. Apoptosis is characterized by cell shrinkage and plasma membrane disturbances resulting in blebbing. The morphological changes induced in the various treatment groups also proved that H_2O_2 caused a significant increase in the number of apoptotic cells. The cells were rescued from this cytotoxic effect by the co-treatment with the clove extract as well as eugenol.

The nuclear changes in apoptosing cells, which include chromatin condensation, DNA fragmentation, nuclear cornering and apoptotic body formation, were visualized using various fluorescent stains (EtBr, PI, AO/EtBr and DAPI). All the nuclear staining protocols reiterated the findings of the cytotoxic assays and the morphological observations, proving without doubt, the protective effect rendered by the extract and eugenol against oxidative stress-induced cell death in *S. cerevisiae*.

The outcome of the present study clearly demonstrate that both clove extract and eugenol can effectively neutralize oxidative stress *in vitro* (against DPPH) as well as in

intact cells (*S. cerevisiae*). Intense research over a period of a few decades has shown that oxidative damage to various biomolecules acts as the underlying causative step in the onset of myriad diseases, including cancer, diabetes, CVD, neurological disorders and infectious diseases. Thus, the antioxidant potential exhibited by clove extract and its active component, eugenol, gains significance, with the implication that they can protect the body against the oxidative damage that lead to several disease conditions. This inference implies that clove can be used as a health supplement in addition to the culinary uses.

The cells used in the present study are established as a model system representing higher eukaryotic organisms, including humans. Thus, it is inferable that clove extract and eugenol can render protection to untransformed (normal) cells in the body against oxidative stress-induced death. This property will be very useful in planning treatment strategies directed towards the cancer cells, wherein several chemotherapeutic agents cause death in the cells via an oxidative process. When used as a combination therapy, clove and its component eugenol, have the potential to rescue the normal cells from the toxicity associated with anticancer chemicals. Thus, the results of this study scientifically validates the medicinal property of clove in acting as a supportive therapy during cancer management.

SUGGESTIONS FOR FUTURE RESEARCH

1. Clove oil can be tested in the yeast cells to understand if the protective effects are caused to the same extent.

2. The effects of the clove extract and eugenol can be studied in different cancer cell types.

3. Other spice components in the Indian cuisine can be tried for the apoptosis-inducing properties in various cell types.

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APPENDIX I

HPLC ANALYSIS OF THE PRESENCE OF EUGENOL IN CLOVE EXTRACT

Principle

HPLC is a form of liquid chromatography used to separate compounds that are dissolved in solution. The HPLC instrument consists of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a sample mixture into the column, which is a narrow-bore stainless steel column, which is usually packed with very small spherical particles of silica coated or stationary phase bonded to a porous polymer, where the liquid mobile phase is forced through under considerable pressure. The mobile phase is a miscible solvent mixture, which either remains constant or may be changed continuously in its proportions, by including a mixing chamber into the set-up.

The different components in the mixture pass through the column at different rates due to differences in their partition behavior between the mobile phase and the stationary phase. Those components are monitored as they elute off the column by means of detector (Photo Diode Array detector, Fluorescence detector, Differential Refractive Index detector or Fluorescence detector). A comparison is made between the chromatograms obtained for the extract and standards to identify the compounds present.

ANALYSIS OF CLOVE EXTRACT BY HPLC

All the mobile phases and samples were filtered through a 0.45μm nylon membrane filter and the mobile phase was degassed in an ultrasonic bath prior to use. The residue of plant extracts was dissolved in an appropriate volume of HPLC grade methanol and 20μL of the sample (clove extract or eugenol) was injected into the reverse phase C18 column of the HPLC (Shimadzu, 100A, 250mm × 4.60mm particle size). Detection was carried out using a diode array detector (Prominence SPD-M20A). Chromatographic data were obtained and processed with software LC-WorkStation VP^{TM} 6.14. The peaks were identified by comparing their retention time and UV-Visible spectra with the standard compound, eugenol. The mobile phase used was water and methanol in the ratio of 40:60 and the flow rate was 1ml/minute at 30ºC. The chromatograms were monitored at a broad spectrum window of 210-600 nm.

APPENDIX-II

DPPH SCAVENGING ASSAY

(Mensor *et al*., 2000)

The ability of the *Syzygium aromaticum* extract to scavenge the DPPH radical was quantified using a spectrophotometric assay.

Principle

DPPH (2,2-diphenyl-2-picryl hydrazyl) radical reacts with an antioxidant compound that can donate hydrogen and gets reduced. DPPH, when acted upon by an antioxidant is converted into diphenyl-picryl hydrazine. This can be identified by the conversion of purple to light yellow colour.

Reagents

1. DPPH (0.3mM in methanol)
2. Methanol

Procedure

The *Syzygium aromaticum* extract (50µl to 250µl) was added to a methanolic solution of DPPH and the mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH solution without extract as the positive control. After 30 minutes of incubation, the discoloration of the purple colour was measured at 517nm in spectrophotometer (Genesys 10S, USA). The radical scavenging activity was calculated as follows,

$$\% \text{ Scavenging} = \frac{\text{A(Control)} - \text{A(Sample)}}{\text{A(Control)}}$$

APPENDIX III

CELL VIABILITY ASSAY USING MTT

(Igarashi and Miyazawa, 2001)

The method of Igarashi and Miyazawa (2001) was employed for the assessment of cytotoxicity in the oxidant-induced cells both in the presence and the absence of the extract or eugenol.

Principle

MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2,4-tetrazolium salt) is converted into its formazan derivative by living cells. The amount of formazan produced is directly proportional to the number of surviving cells. After solubilization of the formazan in a suitable solvent, the cell viability can be measured in a microtitre plate reader.

Reagents

1. Phosphate buffer saline (PBS)
2. MTT - 3mg/ml in PBS
3. Acid-propanol (Isopropanol containing 0.4% of 0.04N HCl)

Procedure

The H_2O_2 treated cells, after centrifugation were incubated with 50µl of MTT at 37°C for 3 hours. After incubation, 200µl of acid-propanol was added to all the samples. The liquid was then carefully aspirated. Then 200µl of acid propanol was added and left overnight in the dark. The absorbance was read at 650nm in a microtitre plate reader (Bio-rad, USA). The viability of the control cells were fixed as 100% viability and the percent viability of cells in the other treatment groups were calculated relative to the control group.

APPENDIX IV

CYTOTOXICITY ASSAY USING SRB

(Skehen *et al*., 1990)

The cell viability in the H_2O_2 treated yeast cells in the presence and the absence of the clove extract or eugenol was studied using SRB.

Principle

Sulphorhodamine B (SRB) is a bright pink aminoxanthine dye with two sulphonic groups. Under mildly acidic conditions, SRB binds to basic amino acids in proteins in TCA fixed cells to provide a sensitive index of cellular protein content, which is directly proportional to cell viability.

Reagents

1. TCA (40%)
2. PBS
3. Sulphorhodamine B - 0.4% in 1% trichloroacetic acid (TCA)
4. Acetic acid (1%)
5. Tris (10mM, pH 10.5)

Procedure

After centrifugation, 350µl of ice cold 40% TCA was layered on top of the treated cells and incubated at 4°C for one hour. The cells were then washed 5 times with 200µl of cold PBS. The buffer was removed, 350µl of SRB stain was added to each well and left in contact with the cells for 30 minutes at room temperature. The unbound dye was removed by washing 4 times with 350µl portions of 1% acetic acid. Then 10mM tris (350µl) was added to each well to solubilize the protein-bound dye and the plate was shaken gently for 20 minutes. Then the tris layer in each well was transferred to a new 96-well plate and the absorbance was read in a microtitre plate (Bio-rad, USA) at 492nm. The cell survival was calculated as percent absorbance compared to the control cells.

APPENDIX V

MORPHOLOGICAL CHANGES BY GIEMSA STAINING

(Chih *et al.*, 2001)

The morphological changes of the cells were observed in the presence and the absence of the clove extract or eugenol and/or H_2O_2. The coverslip with cells were stained with Giemsa (diluted to 1:2 ratio with saline) for 10 minutes and viewed under a phase contrast microscope (Nikon, Japan).

Reagents

1. Normal saline
2. Liquid Giemsa stain (1:2 dilution in saline)

Procedure

The cells were collected by centrifugation after treatment and resuspended in saline, and spread on microscopic slides with 10µl of diluted Giemsa stain. The slides were then observed by phase contrast microscope at 400X magnification for morphological changes. The apoptotic ratio was calculated using the formula.

$$Apoptotic\ ratio = \frac{Number\ of\ apoptotic\ cells}{Number\ of\ normal\ cells}$$

APPENDIX VI

NUCLEAR CHANGES BY ETHIDIUM BROMIDE STAINING

(Mercille and Massie, 1994)

Ethidium bromide is an intercalating agent and when exposed to UV light, it fluoresces with a red-orange colour, intensifying almost 20 fold after binding to DNA.

Reagents

1. PBS
2. Ethidium Bromide – 50µg/ml in PBS

Procedure

The treated cells were incubated for 5 minutes with 10µl of ethidium bromide and spread by placing a cover slip over the microscopic slides. The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei in fluorescent microscope (Nikon, Japan) using UV – 2A filter at 400X magnification. The apoptotic ratio was calculated as given earlier.

APPENDIX VII

PROPIDIUM IODIDE STAINING

(Sarkar *et al*., 2000)

Propidium iodide is an intercalating agent and a fluorescent molecule. It can be used to stain DNA and to differentiate necrotic, apoptotic and normal cells.

Reagents

1. Acetone : methanol (1:1)
2. Saline (0.9% NaCl)
3. Propidium iodide - (5µg/ml in PBS)

Procedure

After treatment with H_2O_2 and clove extract/eugenol, the cells were collected by centrifugation, washed with saline and spread on a glass slide. They were then permeabilized with 50µl of acetone: methanol at -20°C for 10 minutes. Then, 10µl of PI was added, spread with a coverslip and incubated for 30 minutes in the dark. The apoptotic cells were detected using the green filter of fluorescence microscope (Nikon, Japan) at 400X magnification. The apoptotic ratio was calculated by the formula as mentioned earlier.

APPENDIX VIII

AO/EtBr STAINING

(Parks *et al.*, 1979)

Acridine orange is a nucleic acid-selective fluorescent cationic dye useful for cell cycle determination. Ethidium bromide is a DNA intercalator, inserting itself between the base pairs in the double helix. Acridine orange and ethidium bromide can be used in combination as a dual stain for evaluating the proportion of apoptotic cells.

Reagents

1. PBS (Phosphate buffered saline)
2. Stock solution (100X) – AO (15mg) and EtBr (15mg) were dissolved in 1ml of 95% ethanol and frozen as 1ml aliquots.
3. Working solution – 1ml aliquot of the 100X stock solution was thawed and diluted 100 times in PBS. The solution was mixed well and stored at 4ºC up to one month.

Procedure

The treated cells were diluted with equal volumes of AO/EtBr staining solution and immediately viewed under fluorescent microscope using B2A filter (Nikon, Japan). Cells that stained green represented the viable cells, yellow represented early apoptotic cells and reddish or orange represented the late apoptotic cells. The apoptotic ratio was calculated as mentioned earlier.

APPENDIX IX

NUCLEAR CHANGES BY DAPI STAINING

(Rashmi *et al*., 2003)

Diamino-2-phenylindole (DAPI) forms fluorescent complexes with double stranded DNA. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) can be scored in the dying cells by DAPI staining. The presence of nuclear apoptotic bodies and chromatin margination can also be observed after DAPI staining.

Reagents

1. PBS
2. Paraformaldehyde (3%) in PBS
3. Triton X-100 (0.2%) in PBS
4. DAPI – 1µg/ml in PBS

Procedure

The treated cells were fixed with Paraformaldehyde (50µl) for 10 minutes at room temperature. The fixed cells were then permeabilized with 0.2% triton X-100 (50µl) for 10 minutes at room temperature and incubated for 3 minutes with 10 µl DAPI after placing a cover slip over the cells.

The apoptotic cells were determined by counting the cells with condensed chromatin and fragmented nuclei under inverted fluorescent microscope (Moticam, Hong Kong) using DAPI filter at 400X magnification. The apoptotic ratio was calculated as given earlier.