REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

EVALUATION OF CYTOTOXICITY AND GENOTOXICITY OF EUGENOL IN LYMPHOCYTES

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Pharmaceutical Toxicology Programme
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ABSTRACT

Solmaz, M. Evaluation of cytotoxicity and genotoxicity of eugenol in lymphocytes. Hacettepe University Institute of Health Sciences, MSc. Thesis in Pharmaceutical Toxiclogy Program. Ankara. 2014. Eugenol is a volatile phenolic constituent of clove essential oil obtained from *Eugenia Caryophyllata* buds and leaves. It is a functional ingredient in numerous products. It has been used in the pharmaceutical and cosmetics industry and also in agriculture. Its derivatives have been used in medicine as a local antiseptic and anesthetic. Eugenol is also used in food industry in restricted concentrations. In addition, it is widely used in agricultural applications to protect food from microorganisms during storage. The wide range of pharmaceutical activities of eugenol includes antimicrobial, anti-inflammatory, analgesic, antioxidant and anticancer activities. Although eugenol is considered safe as a food additive but, due to the vast range of different applications and extensive use there has been a great concern about its toxicity in recent years. However, studies about cytotoxicity and genotoxicity of eugenol are very limited.

In the present study, we investigated the *in vitro* cytotoxicity of eugenol on V79 cell line by the Neutral Red Uptake Assay (NRU). Our results demonstrated that eugenol has cytotoxic effect on V79 in a dose dependent manner. But at the concentrations below 340 µM eugenol has been found to have no cytotoxicity in V79 cells.

We also studied the *in vitro* genotoxic effects of eugenol on human peripheral lymphocytes by Single Cell Gel Electrophoresis (Comet) Assay and micronucleus assay. According to the results no significant increase in DNA strand breakage was observed at non cytotoxic concentrations of eugenol when compared with their controls.

Key Words: Eugenol, Cytotoxicity, Genotoxicity, Neutral Red Uptake assay, Single Gel Electrophoresis (Comet) Assay, Micronucleus Assay.

ÖZET

Solmaz, M. Öjenolün lenfositlerde sitotoksik ve genotoksik etkilerinin değerlendirilmesi. Hacettepe Üniversitesi Sağlık Bilimler Enstitüsü, Farmasötik Toksikoloji Programi yüksek lisans tezi, Ankara, 2014. Öjenol, *Eugenia Caryophyllata*'dan elde edilen karanfil uçucu yağının fenolik yapıdaki bir bileşiğidir. Pek çok üründe işlevsel bir bileşen olarak yer alır. İlaç ve kozmetik endüstrisinde ve tarımda kullanılmaktadır. Türevlerin lokal antiseptik ve anestezik olarak tıpta kullanımı bulunmaktadır. Gıda endüstrisinde sınırlı miktarlarda kullanılır. Ayrıca, tarımsal uygulamalarda depolama sırasında besinleri mikroorganizmalardan korumak için yaygın olarak kullanılır. Geniş aralıktaki farmakolojik etkileri arasında antimikrobiyal, antiinflamatuvar, analjezik, antioksidan ve antikanser etkiler yer almaktadır. Öjenol, gıda katkı maddesi olarak genellikle güvenli kabul edilmektedir. Yaygın kullanım alanlarına ve yoğun kullanımına bağlı olarak, toksisitesi son yıllarda ilgi odağı olmuştur. Ancak, öjenol'ün sitotoksisitesi ve genotoksisitesi konusundaki çalışmalar yetersizdir. Bu çalışmada, öjenolün V79 hücre hattında *in vitro* sitotoksisitesi nötral kırmızı alım yöntemiyle incenmiştir. Öjenol doz bağlı bir şekilde V79 hücrelerinde sitotoksik etki göstermiştir. Ancak, 340 µM konsantrasyonun altında öjenolün V79 hücrelerinde sitotoksik etki göstermediği bulunmuştur.

Ayrıca, insan periferal lenfositlerinde öjenolün *in vitro* genotoksisitesi tek hücre jel elektroforez (Comet yöntemi) ve mikroçekirdek yöntemi ile inceledik. Bu sonuçlara göre kontrollerine kıyasla öjenolün sitotoksik olmayan konsantrasyonlarında DNA zincir kırıklarında anlamlı artışa neden olmadığı belirlenmiştir.

Anahtar Kelimeler: Öjenol, Genotoksisite, Sitotoksisite, Nötral Kırmızı Alım Yöntemi, tek hücre jel elektroforez (Comet) yöntemi, mikroçekirdek yöntemi.

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ABBREVIATIONS

ALT	Alkaline Transferase
AST	Apartate Transaminase
B[α] P	Benzo [α] Pyrene
CBPI	Cytokinesis Blocked Proliferation Index
CBMN	Cytokinesis-Blocked Micronucleus
CCl_4	Carbon tetrachloride
CIA	Collagen-induced arthritis
DIC	Disseminated Intravascular Coagulation
DMBA	Dimethyl benzanthracene
DPPH	2, 2-diphenyl-1-picrylhydrazyl
Eug	Eugenic
FDA	The U.S Food and Drug Administration
GLC-MS	Gas-Liquid Chromatography Mass Spectrometry
Glu	Glucose
GRAS	Generally Recognized as Safe
GSH	Reduced Glutathione
HeLa cells	Line of cervical call

IARC	International Agency for Research on Cancer
IFNγ	Interferon gamma
$MAOI_S$	Monoamine oxidase inhibitors
MEG	Methyleugenol
MIC	Minimum Inhibition Concentration
MN	Micronucleus
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMDA	N-methyl-d-aspartate
NR	Neutral Red
NRU	Neutral Red Uptake Assay
OHDA	6-hydroxyl dopamine
ROS	Reactive Oxygen Species
TA	Thioacetamid
TB	Total Bilirubin
TNF	Tumor Necrosis Factor
UDS	Unscheduled DNA synthesis
V79	Chinese hamster lung fibroblast cell line

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INTRODUCTION

The spice known as clove is the dried flower bud of the clove tree, *Eugenia Caryophylata*. Eugenol is derived from the species name *Eugenia Caryophyllata* which contains high level of eugenol (45-90%). Clove has been used in ancient China as spice and fragrance. In Chinese traditional medicine, clove oil, has been used as carminative, antispasmodic, antibacterial and antiparasitic agent, while, the buds were used to treat dyspepsia, acute, chronic gastritis and diarrhea.

Several scientific studies have been carried out on *E.Caryophylata* oil and its main volatile constituent eugenol, revealing pharmacological properties such as anesthetic, analgesic, antimicrobial, antioxidant, antiinflammatory, and anticonvulsant, anticarcinogenic, antimutagenic, repellant and antifumigant activities.

Eugenol and its derivatives have been used in medicine as local antiseptic and anesthetic and in perfumeries and flavorings. Eugenol is also suggested to be a beneficial antioxidant. In dentistry, it is used in combination with zinc oxide for surgical dressing, temporary fillings, and caving liners.

Eugenol is also used in food industry in restricted concentrations. FDA has approved clove oil for use in food as a flavoring agent. Eugenol has been classified as “generally recognized as safe (GRAS)’ by the U.S. Food and Drug Administration. However, in spite of extensive use and availability of clove oil, cytotoxicity and genotoxicity studies of eugenol is lacking.

The aim of this study is to investigate the cytotoxic and genotoxic effects of eugenol. For toxicity V79 cells and Neutral Red Uptake Assay are used as an *in vitro* cytotoxicity test. Single cell Gel Electrophoresis (Comet) assay and Micronucleus assay are used as genotoxicity tests. Genotoxicity studies are carried in lymphocytes.

1 THEORETICAL PRINCIPALS

1.1 General Properties

Cloves; are the aromatic flower buds of a tree (*Syzygium aromaticum*) that belongs to the family of *Myrtaceae* and are commonly used as spice. Cloves are harvested primarily in Indonesia, India, Madagascar, Zanzibar, Pakistan, Sri Lanka and Tanzania (1).

The clove tree is 8-12 meters tall and has large leaves and flower buds which have red color when they are ready for collection. The scientific name of clove is *Syzygium aromaticum*, belonging to the genus *Syzygium*, tribe *Syzygieae,* and subfamily *Myrtoideae* of the family *Myrtaceae.* Oil of cloves, also known as clove oil, is an essential oil from the clove plant, *Syzygium aromaticum* (2).

Clove has been used as herbal medicine, spice and fragrance in China and India for over 2000 years. The medicinal use of clove oil for the treatment of toothache has also been recommended in Europe since 17th century. It has been used as a natural analgesic and antiseptic in dentistry for its main ingredient eugenol. In the United States clove oil and the main active ingredient, eugenol, have been suggested as popular ingredients of consumer products (i.e., soaps, detergents) since the 19th century. Clove oil was a recommended source for use in the synthesis of vanilla during the 20th centuries. Eugenol was isolated from clove oil in 1929 for the first time, and commercial production of eugenol began during the early 1940s in the United States. Clove cigarettes were alternative forms of tobacco which use along with cigars in 1980s. In 1984 and 1985, the US Centers for Disease Control received 11 case reports associated with the development of acute respiratory symptoms because of clove cigarettes (1).

There are three types of clove oil:

- Bud oil is derived from the flower buds of *S. aromaticum.* It consists of 60–90% eugenol, eugenyl acetate, caryophyllene and other minor constituents.
- Leaf oil is derived from the leaves of *S. aromaticum.* It consists of 82-88% eugenol with little or no eugenyl acetate, and other minor constituents.
- Stem oil is derived from the twigs of *S. aromaticum*. It consists of 90–95% eugenol, with other minor constituents (1).

1.1.1 Chemical Structure

Eugenol ($C_{10}H_{12}O_2$or$CH_3C_6H_3$) which is a member of the phenylpropanoid class of chemical compounds is a phenylpropene, an allyl chain-substituted guaiacol. It is a clear to pale yellow oily liquid with the molecular weight of 164.2 g/mol and isextracted from the essential oils especially from clove oil, nutmeg, cinnamon, basil and bay leaf. It has a spicy, clove-like aroma. The name is derived from the scientific name for clove, *Eugenia aromaticum* or *Eugenia caryophyllata*. Eugenol is the main component of the essential oil extracted from cloves, comprising 72–90% of the total and is responsible for the aroma of cloves (1).

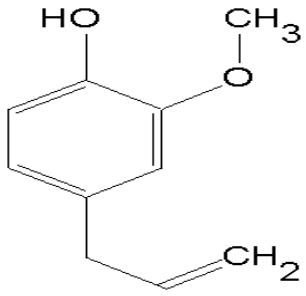


Figure1.1. Chemical structure of eugenol ($C_{10}H_{12}O_2$)

The synonyms of eugenol are given in the below list:

- Phenol, 4-allyl-2-methoxy
- 4-allylcatechol-2-methyl ether
- P-allylguaiacol
- 4-allylguaiacol
- 4-allyl-1-hydroxy-2-methoxybenzene
- Carophyllic acid
- Eugenic acid
- P-eugenol
- 1, 3, 4-eugenol
- 1-hydroxy-2-methoxy-4-allylbenzene
- 4-hydroxy-3-methoxyallylbenzene
- 1-hydroxy-2-methoxy-4-prop-2-enylbenzene
- 2-methoxy-4-allylphenol,
- 2-methoxy-1-hydroxy-4-allylbenzene
- 2-methoxy-4-prop-2-enylphenol
- 2-methoxy-4(2-propenyl) phenol
- Phenol, 2-methoxy-4-(2-propenyl)

1.1.2 Physical Properties

Eugenol is a weakly acidic and volatile phenolic compound which is slightly soluble in water. Eugenol is the major volatile constituent of clove essential oil obtained through hydro distillation of mainly *Eugenia Caryophyllata*

buds and leaves. Eugenol is soluble in organic solvents. It can be mixed with alcohol, chloroform, ether, fixed oils and glacial acetic acid. It is soluble in aqueous sodium hydroxide.

Its molecular weight is 164.2g/mol and specific gravity is 1.067.

The melting and boiling point of eugenol are: -7.5 ^{o}C and 253.2 ^{o}C respectively.

The water solubility of eugenol is 2.46 mg/L 25^{o} C (3).

1.1.3 Occurrence

The spice known as clove (Figure 1.1) is the dried flower bud of the *Eugenia Caryophyllata* tree, with a nail-like appearance. The main constituent of clove oil is eugenol, in addition to acetyleugenol, chavicol, acetyl salicylate and humulenes (4).

Eugenol is widely distributed in the plant kingdom, particularly in cinnamon leaf and bark oil, basil oils (*Ocimum gratissimum*), and sweet basil essential oil *Ocimum basilicum*. Further distillation of clove oil produces a refined product containing almost pure eugenol (i.e., >95%) (5).

Commercial clove oil is a steam distillation product obtained from clove leaves, which contain up to 75-90 % eugenol. Various whiskies aged in oak barrels contain eugenol (i.e., up to about 0.5 mg/L) as a result of the diffusion of the phenolic fraction of an ethanol extract of oak. Clove cigarettes “kreteks” are tobacco products that contain about 60% tobacco and 40% shredded clove buds (1).

Eugenol has also been identified in several aromatic plants such as *Myristica fragrans Houtt* (nutmeg), *Cinnamomum verum J.Presl* (true cinnamon), *C. loureirii Nees*. (Saigon Cinnamon), *Ocimum gratissimum Forssk* (basil) and *Ocimum Basilicum (*sweet basil). However, commercial eugenol is derived from clove bud/leaf oil; cinnamon leaf oil or basil obtained through steam distillation

which is then further refined (5, 6).

1.1.4 Uses of Eugenol

Clove has been used in medicine since ancient times. In traditional Chinese medicine clove oil has been used as an antimicrobial, antispasmodic and anti-parasitic agent. In the United States, clove oil has been marketed as a dental analgesic and antiseptic, a flavoring agent in food, mouthwashes, and pharmaceutical products, and also as an ingredient in aromatherapy. Eugenol is also used as fragrance and flavoring agent and as an insect repellent (1).

Eugenol and its derivatives have been used in medicine as local antiseptic and anesthetic and in perfumeries and flavorings. They are used in the formulation of insect repellents and UV absorbers, analgesics, biocides, and antiseptics (3).

Eugenol is also used in food industry such as ice cream, baked goods and candy in restricted concentrations. Although the first natural compound which used in the synthesis of vanillin was eugenol, nowadays vanillin is produced from lignin or phenol. Eugenol is also used as an industrial source in the production of isoeugenol and methyleugenol (6).

Eugenol has also been shown to enhance skin penetration of various drugs. This agent is widely used in agricultural applications to protect foods from microorganisms such as *Listeria monocytogenes* and *Lactobacillus* during storage, as a pesticide and fumigant (7).

Eugenol has been used to treat skin infections and digestive disorders. Ingested eugenol is also a beneficial antioxidant. In moderate amounts, some reports suggest that excessive doses of undiluted oil can cause symptoms. In fact, an excessive dose of eugenol was considered as a poison (2,7).

The US Food and Drug Administration (FDA) approved clove oil for use in

food as a flavoring agent, in dentistry as an analgesic and in dental cements, as a fragrance in personal care products and in aromatherapy oils (8).

In the United States, eugenol and clove oil are generally recognized as safe (GRAS) food additive and have been approved for use in foods and dental products. Eugenol is also approved for use in the manufacture of textiles and textile fibers that contact food surfaces. Additionally, eugenol and clove oil are approved for use as fragrance (9, 10).

1.1.4.1 Agricultural Applications

New potential safe strategies for control of postharvest decay in crops are needed due to the problems related to synthetic fungicides. Postharvest diseases cause heavy losses of fruits during storage. Species such as *Phlyctema vagabunda, Penicillium expansum, Monilia fructigena* and *Botrytis cinerea* are reported to damage apples in many regions of the world. The *in vitro* and *in vivo* activities of two eugenol formulations (eugenol-Tween®; eugenol-ethoxylate) against the four apple pathogens revealed growth inhibition of the pathogens incorporated in malt extract agar medium with a minimum inhibition concentration (MIC) value of 2 mg/ml. In addition, the mycelia growth of the four test pathogens was completely inhibited when treated with 150 µl/L of volatile eugenol (11).

Combrinck and et al (12), investigated the effects of eugenol on various pathogens causing postharvest decay of fruits. The lowest concentration required achieving 100% inhibition for *Lasodiplodia Theobromae*, Alternaria citri, *Penicillium Digitatum* and *B. Cinerea* was 500 µM/L. Studies were conducted to determine the ability of eugenol to control spore germination of *Alicyclobacillus acidoterrestris*. The results indicated that spore germination could be inhibited through the use of 80 ppm of eugenol or alternatively through the combination of 40 ppm of eugenol with 20 ppm of cinnamaldehyde.

The effect of eugenol alone and in combination with cinnamaldehyde against the wood decay fungi, white-rot fungus and brown-rot fungus was also evaluated using the MIC method which involved serial dilutions of the compound with sterilized potato dextrose agar. Eugenol exhibited good activity against white-rot fungus. Synergistic interactions were noted when eugenol and cinnamaldehyde were combined in a 1:1 ratio. This synergistic effect was attributed to the interference in fungal cell wall synthesis and cell wall destruction in addition to a radical scavenging effect. The combination of eugenol (0.5 mg/ml) and thymol (0.125 mg/ml) was found to induce a significant increase in the number of damaged cells in comparison to the corresponding single concentration of the two molecules after 4 hours incubation period (13).

Inhibition of the wheat seed germination by clove oil was also investigated and eugenol was found to be responsible for its strong inhibitory activity (14).

In recent years natural insecticides have been developed due to the global concern about air pollution because of the use of synthetic insecticides. Crude essential oils and some of their constituents have been identified as a source of natural pesticides. The repellent effects and fumigant potency of *Ocimum gratissimum* oil (64% of methyleugenol) and eugenol were evaluated against the rice weevil, one of the most severe stored-grain pests worldwide, named *Rust Red Flour Beetle*, and the *Chinese Bean Weevil*. The results showed that fumigant activity and repellency of the oil and eugenol were significantly influenced by concentration and time after treatment (15).

1.1.5 Production of Eugenol

Eugenol can be produced synthetically. The most practical method being the allylation of guaiacol with allylchloride. However, eugenol is first prepared from natural oil sources by mixing the essential oil with an excess of aqueous sodium (3%) or potassium hydroxide solution and shaking, leading to the

formation of a phenolic alkali salt. The insoluble and non-phenolic portion is then extracted with a solvent or via steam distillation. The undissolved portion is removed, and the alkali solution is acidified at low temperatures and the liberated eugenol is purified by fractional distillation (5).

1.1.6 Pharmacokinetical Effects of Eugenol

There are few human studies about the pharmacokinetics of eugenol. Animal studies have suggested that after inhalation of the smoke of clove cigarettes, lungs may absorb minor amounts of eugenol. In some *in vitro* studies suggested that eugenol undergoes biotransformation in hepatocytes. The sulfate, glucuronic acid, and glutathione conjugates have been formed (1).

An investigation in male and female healthy volunteers demonstrated that eugenol was rapidly absorbed and metabolized after oral administration and was almost completely excreted in the urine within 24 hours. Only less than 1% of administrated dose was excreted as non-metabolized in urine. The urine contained conjugates of eugenol and eight metabolites.

The structures of these metabolites, as elucidated by using gas-liquid chromatography-mass spectrometry (GLC-MS) identified as:

- eugenol, 4-hydroxy-3-methoxyphenyl-propane
- cis- and trans-isoeugenol
- 3-(4-hydroxy-3-methoxyphenyl)-propylene-1, 2-oxide
- 3-(4-hydroxy-3-methoxyphenyl)-propane-1, 2-diol
- 3- (4-hydroxy-3-methoxyphenyl)-propionic acid
- 3-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-allylbenzene
- 3-(6-mercapto-4-hydroxy-3-methoxyphenyl)-propane
- 2-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-propionic acid

The amounts of the individual metabolites excreted were determined by GLC. 95% of the dose was recovered in the urine, most of which (greater than 99%) consisted of phenolic conjugates; 50% of the conjugated metabolites were eugenol-glucuronide and eugenol-sulphate. Other observed metabolic routes were the epoxide-diol pathway, synthesis of a thiophenol and of a substituted propionic acid, allylic oxidation, and migration of the double bond (16).

Minor metabolic pathways include oxidation of the side-chain double bond to the epoxide, followed by hydrolysis to the diol and further oxidation. Isomerization to form isoeugenol followed by allylic oxidation and then reduction; conjugation of an oxidation intermediate with glutathione; and reduction of the side chain double bond. All metabolites have an aromatic hydroxyl group that reacts readily with glucuronic acid or sulfate to form the conjugates, which are readily excreted in the urine. Rodent metabolism is similar to that in human (17).

Guenette and coworkers (18) suggested that in rats the half-life ($t_{1/2}$) of eugenol in plasma is about 14 hours and in blood is 18 hours.

Monoamine oxidase inhibitors (MAOIs) are drugs used in the treatment of depression. The antidepressant-like activity of eugenol and three selected eugenol analogues was tested in mice by using an established antidepressant screening test (forced swim test). The results indicated that eugenol exhibited anti-depressant like effects against MAO type A and type B with the concentration required to produce half maximum inhibition Ki of 26 and 211 μM, respectively (19).

1.2 Pharmacological Effects of Eugenol

Eugenol shows pharmacological effects almost in all systems. Several *in vitro* and *in vivo* studies have been conducted to determine the pharmacological properties of eugenol and to elucidate mechanism of action of this agent. The vast range of pharmacological activities of eugenol has been researched and

includes antimicrobial, anti-inflammatory and analgesic, antioxidant and anticancer activities.

1.2.1 Analgesic Effects of Eugenol

Eugenol is widely used for its analgesic properties in dentistry because of its ability to alleviate tooth pain. Eugenol is also shown to be able to alleviate neuropathic pain (5).

The antinociceptive effects of eugenol were examined in ICR mice. The oral administration of eugenol in concentrations of 1-10 mg/kg exhibited an antinociceptive effect in a dose-dependent manner as measured by the number of contractions of the body and this activity could be maintained for at least 30 minutes. Daniel et al, investigated the antinociceptive activity of eugenol in mice Results demonstrated that eugenol at doses of 50, 75, and 100 mg/kg has a significant antinociceptive effect compared with control animals (20).

1.2.2 Anesthetic Effects of Eugenol

Eugenol and clove oil have been used as local anesthetics in dentistry, specifically to reduce the pain associated with dental cavities. Eugenol is an effective, inexpensive and easy to use general anesthetic agent in some fish species. Clove oil is used in these species to reduce stress. Eugenol also has recently been evaluated for anesthetic properties in rats. A reversible, dose dependent anesthesia has been reported after eugenol administration of 5-60 mg/kg i.v. in male rats (21).

1.2.3 Antioxidant Activity of Eugenol

Reactive oxygen species (ROS) are involved in many human diseases such as stroke, cardiovascular diseases, diabetes and cancers. ROS are

continuously generated in aerobic cells by products of oxidative metabolism. Increased production of ROS can damage lipids, proteins and DNA structure which results in cellular injuries. There are so many evidences based on experimental investigations indicating increased rates of lipid peroxidation in diseases and the beneficial effects of antioxidants. There is a growing tendency to replace synthetic antioxidants with natural phenolic compounds. In recent years, many phenolic compounds such as eugenol shown to have antioxidant capacity (5).

Eugenol at low concentrations has shown an antioxidant activity however, at high concentrations it is suggested to act as a pro-oxidant, leading to tissue damage resulting from the enhanced generation of free radicals (22).

The antioxidant activity of eugenol and one of its isomers isoeugenol has been studied by using iron-mediated lipid peroxidation and auto-oxidation of Fe^{2+}. Eugenol had the inhibitory effect on lipid peroxidation (with an IC_{50} value of about 80 μM, which was eight-fold the value of isoeugenol). But this effect was less potent than the effect of isoeugenol. The functional mechanism of these two compounds was evaluated and results suggested that the antioxidant activity of eugenol could be explained by the formation of complexes with reduced metals. The inhibitory effect of isoeugenol on lipid peroxidation may be related to the decreased formation of the perferryl ion or iron-oxygen chelate complex as the initiating factor of lipid peroxidation (23).

In another study by using the hydroxyl radical scavenging and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) tests, results suggested that eugenol exhibited dose-dependently antioxidant capacity. At a volume ranging from 5 to 25 μL, the percentage inhibition ranged from 41 to 93% and from 39 to 62% against the DPPH and hydroxyl radicals, respectively (24).

It was found that eugenol has the inhibitory effects on methaloproteinase (MMP9) via inactivation of extracellular signal-regulated kinases ERK which is a kind of protein molecule. These results suggest that eugenol has the ability as

an excellent agent for prevention of metastasis related to oxidative stress (25).

Masahiro and et al suggest that the inhibition mechanism of eugenol to lipid peroxidation has two steps. It interferes with the chain reactions by trapping the active oxygen. Also eugenol is metabolized to dimer and inhibits lipid peroxidation (26).

An *in vitro* animal study in mice investigated the protective effect of eugenol (1-20 µg/ml) against cellular damage in mice peritoneal macrophages (induced by 10 mM nicotine). The radical generation, lipid, protein, DNA damage and endogenous antioxidant status were analyzed. The results indicated that eugenol could be used as modulator of nicotine-induced cellular damage and immunomodulatory drug against nicotine toxicity (27).

In vitro studies on liver microsomal monooxygenase activities and carbon tetrachloride (CCl_4) induced lipid peroxidation has shown that eugenol has inhibited both. The *in vivo* studies by the same researches also showed a protective effect of eugenol at doses of 5 and 24 mg/kg against CCl4 induced hepatotoxicity (28).

Kabuto Et al (29) showed that eugenol has shown a preventive effect on dopamine depression and lipid peroxidation suggesting that eugenol can prevent depression induced by 6-hydroxyl dopamine (OHDA). Eugenol has prevented depression by decreasing lipid peroxidation. Eugenol has stimulated reduced glutation (GSH) and Asc generating systems. Moreover, it was suggested that increased GSH may protect cell death.

1.2.4 Antibacterial Activity of Eugenol

For centuries, natural products have been used to treat microbial infections. Numerous essential plant oil components have demonstrated the ability to inhibit the growth of pathogens.

The effect of eugenol on the growth of some *Gram-positive* bacteria

(*Bacillus cereus; B. subtilis; Staphylococcus aureus*) and *Gram-negative* bacteria (*Escherichia coli; Salmonella typhi; Pseudomonas aeruginosa*) were investigatedby using the agar well diffusion method. At the concentration of (1000 ppm), eugenol inhibited the growth of the bacteria and complete inhibition was observed against *P.aeruginosa* at a high concentration of 2,000 ppm. This inhibition was high in comparison to ampicillin (1 mg/ml) which was used as a positive control. Several other studies also have confirmed the antibacterial activity of eugenol against various pathogens such as *E. coli, B. cereus, Helicobacter pylori, S. aureus, S. epidermidis, Streptococcus pneumoniae* and *S. pyogenes* (30).

The combination of eugenol with a conventional antibiotic has been investıgated to determine the synergistic effect. The combination of eugenol with two antibiotics such as vancomycin and a β-lactam antibiotic resulted in an increase of activity by a factor of 5-1000 with respect to their individual MIC values. This synergistic effect could be explained by the fact that eugenol is able to damage the membrane of *Gram-negative* bacteria. It has been also demonstrated that in combination with eugenol, an increased penetration of vancomycin and β-lactam causes greater antimicrobial effect (31).

The synergistic interactions of eugenol with ampicillin and gentamicin were investigated by using time-kill studies. After 60 min of treatment, the rate of killing in units of bacteria was higher than when eugenol was combined with both antibiotic than were tested alone, suggesting a synergistic interaction between eugenol and other antibiotics (32).

In another study, the antibacterial activity of eugenol, cinnamaldehyde, thymol, carvacrol and the combination of all mentioned agents werecompared against the *Gram-negative* bacteria *E. coli* by using the broth micro-dilution assay. Eugenol hadthe lowest antibacterial activity by the MIC value of 1600 mg/L. Eugenolhad an MIC value of 400 mg/L. However, when eugenol was combined with cinnamaldehyde, thymol and carvacrol, results showed

synergistic interactions in MIC values of 400, 100, 100 mg/L respectively (33).

The effects of eugenol on the bacterial membrane of *Listeria Monocytogenes, Streptococcus pyogenes, Proteus vulgaris* and *E. coli* have been examined by evaluating the mechanism of action of eugenol. Changes in membrane composition and leakage of protein and lipid were observed. The results demonstrated that eugenol induced cell lysis through leakage of protein and lipid contents. Furthermore, both the cell wall and membrane of the treated *Gram-negative* and *Gram-positive* bacteria were significantly damaged and eugenol caused high protein content leakage after about 120 minutes of exposure (34).

The results from tumour necrosis factor (TNF) release and hemolysis assays indicated that S*. aureus* cultured with different concentrations of eugenol (16 to 128µg/ml). Eugenol decreased the release of TNF and also hemolytic activities dose-dependently. It is also found that depending on the concentration of eugenol, *S.aureus* significantly reduced the production of staphylococcal enterotoxin. As a result, it is suggested that eugenol could be applied to food products as an antimicrobial agent to inhibit the growth of bacteria and to suppress the production of exotoxins of *S. aureus* (35).

Eugenol was tested for its effect on total microbial count of bacteria, yeasts and molds in air and was found to reduce the total microbial count of bacteria by 69.4% and yeasts and molds by 58.3% (36).

1.2.5 Antifungal Activity of Eugenol

The antifungal activity of eugenol has been shown in some studies and it was observed that adding a methyl group to eugenol has increased its antifungal activity (37).

In an *in vitro* study, the antifungal activity of eugenol and methyleugenol alone and in combination with fluconazole (an antifungal drug) was evaluated

against 64 fluconazole-sensitive and 34 fluconazole-resistant *C. albicans* strains. All the tested strains were susceptible and the combination of eugenol/ methyleugenol with fluconazole demonstrated a synergistic effect. These results observed that a combination of eugenol/methyleugenol with fluconazole or amphotericin B could be an alternative method to treat *C. albicans* strains which are resistant to fluconazole or other drugs (38).

The effects of eugenol for the prophylaxis and treatment of experimental vaginal candidiasis in immunosuppressed rats has been evaluated. The results demonstrated that after 10 days of prophylactic eugenol treatment, the number of colonies of *C. albicans* in the vaginas of infected rats decreased by 98.9 % (39).

The inhibitory effect of eugenol and other terpenoids have been assessed by using the time-kill method. Eugenol showed highly toxic effect to *C. albicans*ofafter seven minutes of exposure. Results suggested that eugenol can affect membrane integrity and causes cell cycle arrest (40).

It is suggested that eugenol, and other lipophilic agents such as carvacrol and thymol, can easily disperse between the fatty acyl chains making up the bilayers of cell membranes. Eugenol disturbs the cell growth and envelope morphogenesis by modifying the permeability and fluidity of cell membranes. In another study, the antimycotic effects of eugenol were investigated by using the microdilution method. Eugenol exhibited antimycotic activity with MIC values of 1.0, 0.5, 0.25 and 0.13 mg/ml against *Saprolegnia spp*., *A. klebsiana* and *A. piscicida* (33, 41).

Eugenol also has been tested against *T. mentagrophytes* by using the agar dilution method. At the dose of 0.15 mg/ml, eugenol completely inhibited the hyphal growth. It was also suggested that at doses of 0.2 mg/ml of eugenol, the hyphae was collapsed (42).

The activity of eugenol is found to be more potent than some known

synthetic antimicrobials and observed a broad fungi toxic spectrum against *Aspergillus* species, *Alternaria alternata, Botryodiplodia theobromae, Cladosporium herbarum* and *Colletotrichum gloeosporioides* (43).

The use of eugenol (8.205 mg/g) in the grains which were infected with *Aspergillus flavus* demonstrated an antifungal effect with complete inhibition of aflatoxin B1 production (44).

1.2.6 Antiviral Activity of Eugenol

The antiviral activity of eugenol has been tested in an *in vitro* animal study against the herpes simplex: (HSV-1and HSV-2) viruses. The replication of these viruses was inhibited with IC_{50} values of 25.6 µg/ml and 16.2 µg/ml eugenol against HSV-1 and HSV-2 respectively. Additional studies demonstrated synergistic interactions of eugenol with acyclovir, a well-known antiviral drug. Studies have suggested that application of eugenol has delayed the development of keratitis induced by herpes virus in a mouse model (45).

Eugenol also has been evaluated for the anti-HSV properties on standard HSV-1(F), standard HSV-2(G) and ten HSV isolates using the plaque reduction assay. Only HSV-1 isolates were inhibited by eugenol. (46).

1.2.7 AntimalarialActivity of Eugenol

The antimalarial activity of eugenol against the chloroquine-resistant strain *Plasmodium falciparum* (FCR-3) was studied and results demonstrated that eugenol exhibited some activity with an IC_{50} value of 753 µM which was lower than for other essential oils such as nerolidol, linalyl acetae, α-pinene and pulegone (47).

1.2.8 Antiinflammatory Effects of Eugenol

Investigations on antiinflamatory effects of eugenol, have suggested that eugenol is able to suppress the expressions of cyclooxygenase II. Eugenol dimers can inhibit the expression of cytokines in macrophages which are stimulated by polysaccharides. Eugenol also has a prevention effect of NF-Kappa B activation. Eugenol is suggested to be able to reduce the incidence of gastric tumors by suppressing NF-KB activation and is able to modulate the expression of NF-KB target genes which regulate cell proliferation and cell survival. Because of these suggested activities, eugenol has been indicated to have chemopreventive effect (48).

Another investigation based evaluated the effects of eugenol in a murine model suffers from collagen-induced arthritis (CIA). Macroscopic studies and clinical evidence of CIA and treatment with eugenol in a murine model showed that eugenol may have inhibitory effects on mononuclear cell infiltration into the knee joints of arthritic mice. Eugenol has also lowered the levels of cytokines such as tumor necrosis factor (TNF)-α, interferon gamma (IFN-γ) and tumor necrosis factor (TGF-β) within the ankle joints. The *in vitro* cell viability did not affected by eugenol treatment as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Eugenol may cause the treatment of recovery effects on arthritis and can be useful as a beneficial supplement in treating human arthritis (49).

Eugenol was investigated for its antiulcerogenic effects. It was found that gastric ulcer formation, induced by administration of two ulcerogenic agents was significantly reduced through pre-treatment with eugenol (10–100 mg/kg) (50).

In rats with indomethacin-induced ulcers, pretreatment with eugenol (100 mg/kg, orally) 60 min before indomethacin administration reduced gastric mucosal lesions and gastric acid outputs, resulting in a gastroprotective effect. The mechanisms of actions which resulted in antiulcer effect are proposed, were the opening of ATP-sensitive potassium (KATP) channels, free radical

scavenging, decreasing acid-pepsin secretion, increasing mucin production and prevention of the deleterious rise in nitric oxide level (51).

Eugenol has also a myogenic antispasmodic effect on smooth muscle in airways of rats. After EFS in the tracheal muscles, eugenol in concentrations of 1–2,000 µM reduced contractions. This effect was not altered by indomethacin suggesting the effects are not modulated by arachidonic acid derivatives. The mechanisms involved seem to include blockade of voltage- and receptor-operated Ca^{2+}channels, IP 3-induced Ca^{2+}.release from sarcoplasmic reticulum and reduction of the sensitivity of contractile proteins to Ca^{2+} (52).

Eugenol was intragastrically injected in rabbits to evaluate its potential in reducing fever. Results showed that eugenol exhibited pronounced antipyretic activity when given intravenously and intragastrically and may decrease fever through a central action that is similar to that of allopathic antipyretic drugs such as acetaminophen (53).

Eugenol at the concentrations of 0.2 -20 µm is suggested to be able to produce a dose dependent and reversible vasodilator response that are partially dependent on the endothelium (54).

1.2.9 Neuroprotective Effects of Eugenol

The neuroprotective efficacy of eugenol was investigated against neurotoxicity induced by N-methyl-d-aspartate (NMDA), oxygen-glucose deprivation and xanthine/xanthine oxidase in primary murine cortical cultures. The results showed that eugenol at concentrations of 100–300 mM attenuated NMDA induced acute neurotoxicity by 20–60%. Similarly, eugenol at concentration of 300 mM also inhibited NMDA-induced elevation in neuronal Ca2+ uptake by 10–30%. Furthermore, it was observed that addition of eugenol (100–300 mM) prevented acute neuronal swelling and reduced neuronal death by 45–60% in a concentration-dependent manner and oxidative

neuronal injury induced by xanthine/xanthine oxidase was also significantly reduced (75–90%) (55).

Another study has been done about ability of eugenol as a penetration enhancer. The effect of clove oil on the transdermal delivery of ibuprofen was investigated in rabbits using both *in vitro* and *in vivo* experiments. Although the *in vivo* results demonstrated a significant permeation enhancement effect, this enhancement was lower compared to the *in vitro* experiment. GLC-MS results indicated the two major compounds to be eugenol and acetyleugenol (90.93% of the total oil), therefore it was suggested that these constituents contribute to the permeation enhancing ability (56).

1.2.10 Anticancer Effects of Eugenol

Several essential oil constituents, including eugenol and related compounds, have been investigated for their anticancer activity. Eugenol in the concentration of 500 μM was found to be potent to reduce the cell viability of HeLa cells. Eugenol also was tested alone and in combination with a chemotherapeutic drug "gemcitabine" to evaluate its inhibition effect against cancer cells. Eugenol was found to have cytotoxic effect on HeLa cells in comparison to normal cells. However, this effect was dose dependent. Furthermore, eugenol in combination with "gemcitabine", induced growth inhibition and apoptosis at lower concentrations than individual compounds (57).

The anticancer effects of eugenol and its analogues were investigated in two human cancer cell lines: androgen-insensitive prostate cancer cells (DU-145) and oral squamous carcinoma cells (KB) by tetrazolium salt assay. Results demonstrated that analogues of eugenol are more active than eugenol. The obtained results indicated that the nitro and hydroxyl groups play an important role in the activity of these compounds (58).

The anticancer activity of eugenol and methyleugenol was evaluated

on 5 various cell line groups as the HL-60 human promyelocytic leukemia, U-937 human histocytic lymphoma, HepG2 human hepatoma, 3LL Lewis mouse lung carcinoma and SNU-C5 human colon cancer cell lines. Evaluation of the study was based on a colorimetric assay (MTT). Eugenol indicated different degrees of toxicity with IC_{50} values ranging from 23.7 to 129.4 μM. Evaluation of chemical transformation of eugenol to methyleugenol for elucidating of the OH-group in the biological effect of this molecule showed that the activity of methyleugenol was weaker with IC_{50} values ranging from 89.3 to 300 μM (59).

Eugenol was also found to show protective effects in skin cancer induced by chemicals. To understand the mechanism of action of essential oil constituents, the *in vitro* antiproliferative effects of eugenol and its isomer isoeugenol on cell cycle progression of human epidermoid carcinoma A431 cells was investigated. The two compounds exhibited an antiproliferative effect by blocking the cells in the G0/G1 phase of the cell cycle. Both compounds favoured the translocation of the aryl hydrocarbon receptor into the nucleus, aryl hydrocarbon receptor target gene expression and aryl hydrocarbon receptor-dependent modulation of cell cycle regulatory proteins (60).

1.3 Toxicity of Eugenol

Eugenol is considered safe as a food additive but due to the wide range of different applications and also the extensive use and availability of clove oil; there is a great concern about its toxicity in recent years.

The estimated acceptable daily oral intake of eugenol is 2.5 mg/kg. Case reports show that the accidental ingestion of very limited amounts of clove oil by infants and young children causes the development of serious toxicity including seizures, coma, metabolic acidosis and hepatic failure and disseminated intravascular coagulation (DIC). But, information about the acute toxicity of

eugenol is limited (1).

A two-year old boy ingested between 5 and 10 ml of clove oil and on arrival at the hospital one hour later presented as normal but slightly drowsy, distressed and crying. Within 3 h his condition deteriorated into deep coma with marked acidosis. Within 8 h, his blood glucose concentration was undetectable and he suffered a generalized seizure, and within 24 h deteriorating liver function as well as disseminated intravascular coagulopathy (DIC) was noted. After intensive symptomatic treatment, the patient regained consciousness on the 6th day after ingestion and eventually made a full recovery. There are several similarities between eugenol and paracetamol poisoning in terms of its hepatotoxic effects (61).

Animal studies have demonstrated that eugenol in the smoke from clove cigarettes, is not highly toxic. In a short-term study, rats were exposed to smoke from tobacco and clove cigarettes for 30 minutes but no significant differences were defected in body weights, food and water intake, respirations, or histological abnormalities between the group that given just tobacco alone and the group given both clove and tobacco. Although in vitro studies suggest that cytochrome P_{450} isoenzymes are able to oxidize eugenol to a reactive quinone methide intermediate, which binds to proteins covalently or conjugates with glutathione. These reactive intermediates can cause necrosis in hepatic cells. In animal studies, depletion of glutathione prior to the administration of eugenol causes substantial liver damage dose dependently (1).

However, intravenous infusion of eugenol in rats at 4 µl and 8 µl (6.52 mol/L) caused acute respiratory distress with hemorrhagic pulmonary edema (62).

In an *in vitro* study using isolated rats hepatocytes, results showed hepatotoxicity with cell death occurring in more than 85% of rat hepatocytes after 5 hours exposure. In the case of an overdose, alternative metabolic pathways are utilized due to saturation, a process supported by acetyl cysteine

administration and cell death in rat hepatocytes was prevented completely by co-administration (63).

Yoqalashmi B and etal (64) investigated the antioxidant, antiinflammatory and DNA protective effects of eugenol on thioacetamide (TA)-induced liver injury in rats. The results demonstrated that eugenol is able to reduce the toxic effects of TA in liver.

A potential mechanism for the possible effects of dietary spices such as cinnamon and clove, on cell functions specially the function of mitochondria was demonstrated in some studies. Because of lipophilic properties, these compounds can permeate the membrane and hence become accessible to various intracellular targets including mitochondria. Recent studies reported the inhibitory effect on the intestinal and kidney Na^+/K^+ ATPase activity and also on alanine transport in rat jejunum. It is possible that mitochondria is another possible target of the actions of spices or toxicity whereby deranging mitochondrial functions cause decrease of ATP level, which then can influence the mechanism of cell growth, viability and aging (65).

Studies on the toxic effects of anesthetic doses of eugenol on African clawed frogs demonstrated damage in kidney and also morphologic changes with renal tubular apoptosis which affect distal tubules in medulla (66).

A 15 days oral dose toxicity study of aspirin eugenol ester in rats suggests that the oral intake of eugenol in different doses during a 15 days period may cause some changes in blood chemistry. Especially causes an increase in Glu, AST, ALT, and total bilirubine (TB) but it seems that such effects are not dose dependent (67).

1.3.1 Cytotoxicity of Eugenol

Studies have investigated to clarify the cytotoxicity mechanisms of eugenol on human submandibular cell line. The cytotoxicity of eugenol, the

reactive oxygen species (ROS) production induced by eugenol, and reduced glutathione (GSH) levels have been evaluated and results suggested that formation of benzyl radicals is the main cause of low GSH levels and high amounts of ROS. In contrast, the cytotoxicity of eugenol is likely to be mediated by ROS -independent mechanisms. Results of the study suggest that the cytotoxic effect of eugenol is less than its isomer; isoeugenol and effects are dose dependent (68).

The cytotoxic effects of eugenol and cytotoxicity mechanism in human osteoblastic cells were investigated with colorimetric method and results indicated that eugenol is potent to decrease the activity of dehydrogenase enzyme in cells in a dose dependent manner (69).

The cytotoxic effects of some of root canal sealer agents based on zinc-oxide eugenol (endofill) and sealer 26 were also studied. Also the production of nitric oxide has been evaluated. Results showed that both agents have cytotoxic effects but the toxicity of sealer 26 agents on macrophages is more than endofill (70).

Anpo M and et al (71) evaluated the cytotoxic effects of eugenol on human pulp cells and also the expression of molecular markers in osteogenic differentiation. Observations suggested that eugenol used for endodontic treatment may have cytotoxic effects to the normal function of stem cells.

Another study evaluated the apoptosis caused by eugenol in human breast cancer cell lines by using the MTT assay. Release of lactate dehydrogenase enzyme and percentage of cell viability and cytotoxicity, morphological alterations, and quantitation of DNA fragments have been recorded. Increase in the percentage of apoptotic cells and DNA fragments is dose dependent and suggested that apoptosis which caused by eugenol can result in cell death and apoptosis might have played a role in the chemopreventive action of eugenol(72).

The cytotoxicity and DNA fragmentation caused by eugenol and related compounds such as (2-methoxy-4-methylphenol, 3,3′-dimethoxy-5,5′-di-2-propenyl- 1,1′-biphenyl-2,2′-diol and 3,3′-dimethoxy-5,5′-dimethyl-1,1′-biphenyl-2,2′-diol) have been studied using leukemia cells (HL-60). The IC_{50} values obtained ranged from 0.18 to 0.38 mM and the strongest DNA fragmentation was induced by 3, 3′-dimethoxy-5, 5′-dimethyl-1, 1′-biphenyl-2, 2′-diol followed by eugenol (59).

The cytotoxic and DNA damaging effects of eugenol and borneol were investigated in malignant HepG2 hepatoma cells, malignant Caco-2 colon cells, and also nonmalignant human VH10 fibroblasts using the trypan-blue exclusion assay. The results suggested that the cytotoxic effect of eugenol against these three cell lines was significantly higher than that of borneol. Moreover, it was demonstrated that borneol is not potent to cause any DNA strand-breaks at the studied concentrations, but eugenol at concentrations lower than 600 μM increased the level of DNA breaks in human VH10 fibroblasts and to a lower degree in Caco-2 colon cells (59, 73).

The effects of eugenol and a chemotherapeutic drug, gemcitabine were investigated. The combination of eugenol (150 μM) and gemcitabine (15 μM) resulted in a decrease in cell viability from 84% (eugenol alone) to 47% combination. Results showed that eugenol alone causes 84% decrease and gemcitabine causes 51% decrease in cell viability. Treatment of colon cancer cells with eugenol resulted in the reduction of intracellular non-protein thiols and also an increase in the earlier lipid layer break. Furthermore, dissipation of mitochondrial membrane potential and generation of reactive oxygen species resulted in eugenol induced apoptosis (74).

In order to determine the mechanism of action of eugenol against HL-60 cell line; HL-60 cells were incubated with eugenol at various concentrations at different time intervals. Results demonstrated a significant increase of fragmented DNA caused by eugenol. Also the ladder pattern of inter

nucleosomal DNA fragmentation was apparent when cells were treated for 4 h with 40 μM of eugenol. Although the DNA fragmentation was totally inhibited by pretreatment with the antioxidant N-acetyl-L-cysteine. Results suggest that eugenol is potent to induce apoptosis in HL-60 cells via ROS. The mechanism is by inducing mitochondrial permeability transition, by reducing anti-apoptotic protein level and cytochrome C release to the cytosol as well as subsequent apoptosis (59).

The inhibition effect of eugenol and its isomer isoeugenol on the proliferation of melanoma cells was investigated in an animal study. Eugenol was able to act as an inhibitor of melanoma cell proliferation. The mechanism caused a significant tumor growth delay, approximately 40% decrease in tumor size, and a 19% increase in the median time to end point. Moreover, about 50% of the animals in the control group died as a result of metastatic growth, whereas in the treatment group none showed any signs of invasion or metastasis. The anti-proliferative mechanism of eugenol was also investigated in the human malignant melanoma cell line (WM1205L) and results showed that eugenol may induce apoptosis by arresting cells in the S phase of the cell cycle (75).

Several studies have demonstrated that in some cases the combination of substances may have a better activity than when just a substance is used and in the diseases such as malaria, HIV and cancer the combination therapy is often more helpful. For instance, the combination of eugenol and 2-methoxyestradiol, an endogenous estrogenic metabolite reported to be an antiproliferative agent in various tumor models resulted an inhibition of the growth of prostate cancer cells and caused apoptosis in a synergistic manner (76).

1.3.2 Carcinogenicity of Eugenol

Recently, animal studies have suggested although eugenol has a short live in the body but may result in hepatic neoplasms by oral intake. However no known health effects and carcinogenicity were reported in human (77).

In an *in vitro* animal investigation, the toxicity and carcinogenic effects of methyleugenol (MEG) were studied in F344/N rats. MEG has been selected because of its widespread use and also because of the similarities of chemical structure between methyleugenol and safrol (a known carcinogen). Results of this two years animal study, demonstrated clear evidence of carcinogenic activity of methyleugenol based on the increased incidences of liver neoplasms and neuroendocrine tumors of the glandular stomach in both male and female rats. The increased incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma and fibroma were also observed (78).

1.3.3 Genotoxicity of Eugenol

Since the twentieth century, attention has been devoted to the genotoxic effects of natural compounds. The data about the genotoxic potential of eugenol is limited and controversial.

In vitro investigation has been done on the genotoxic effects of zinc oxide eugenol and resine based sealers used in dentistry for root canal by using MTT and colorimetric assays and DNA strand break methods. Authors have suggested a moderate to severe toxicity effects of zinc oxide eugenol in V79 cell line and also demonstrated that these effects are dose dependent. They also believe that eugenol has cytotoxic but not genotoxic effects (79).

The *in vitro* investigation on genotoxic effects of eugenol has been done in V79 cell line by using chromosomal aberrations (CAs), with and without rat liver biotransformation (S9). Findings of study indicated that eugenol at the

concentration of 2500 μM significantly induced CAs with significant increases (3.5% aberrant cell). Furthermore, the induction of CAs increased by S9 in a dose-dependent manner to 15% at 2500 μM, with a high frequency of chromatid exchanges. Results confirmed that eugenol is genotoxic and raises the possibility of genotoxicity by inhibition the activity of topoisomerase II. Eugenol induced chromosomal aberrations (CAs) at 2500 μM, and demonstrating cytotoxicity at higher doses (80).

The evaluation of genotoxicity of methyl eugenol in Fischer 344 rats via comet assay resulted that this substance may cause malignancy and induces DNA damage in mouse tissue. The potency of eugenol to reduce the mutagenic and genotoxic effects of benzo(α)pyrene (B(α)P) was evaluated in an *in vivo* study on the k-lac*Z*-transgenic mouse strain 40.6 (MutaT~Mouse). Results indicated no evidence of mutagenicity or genotoxicity of eugenol. However, in comparison with control groups the eugenol intake (0.4% w/w) resulted in apparent growth retardation, although the differences were not statistically significant (81).

On the other hand the chemopreventive effect of eugenol on DNA damage induced by 7, 12 dimethylbenzanthracene (DMBA) has been evaluated in MCF-7 cells. The observations of such study suggested that eugenol was potent to protect against DMBA-induced genotoxicity, presumably through the suppression of the DMBA activation and the induction of its detoxification. These results suggest that eugenol has potential as a chemopreventive substance (82).

The evaluation of antigenotoxicity effects of eugenol in mice with micronucleus test suggests that the antigenotoxic effects of eugenol may be dose related (83).

Eugenol was investigated *in vitro* in order to evaluate its antigenotoxic effects in unscheduled DNA synthesis (UDS) assay with established mutagens assay and the *Salmonella typhimurium* mutagenicity assays. In addition, the

effect of *in vitro* treatment with eugenol on benzo[α]pyrene (B[α] P)-induced genotoxicity in the human hepatoma cell line Hep G2 was evaluated by comet assay. The mutagenicity of B[α]P in the *S. typhimurium* mutagenicity assay was lower in liver S-9 fractions prepared from rats treated orally with eugenol (1,000 mg/kg body weight) than in liver S-9 fractions from control rats, while in the unscheduled DNA synthesis assay, eugenol showed no effect. *In vitro* treatment of cultured cells with eugenol resulted in an increase in genotoxicity of benzo[α]pyrene. The findings of this study suggested that there is very limited support for the antigenotoxicity potential of eugenol *in vivo* (84).

Another study investigated the oxidative mutagenesis induced by tert-butylhydroperoxide (TBH) in *E. coli* and found that when eugenol was added to TBH, at 300–400 µg/plate, a significant decrease of (50%) in the oxidative mutagenesis by TBH was recorded (85).

In the mouse bone marrow micronucleus test, pretreatment with trans anethole and eugenol resulted insignificant antigenotoxic effects against cyclophosphamide procarbazine,N-methyl-N′-nitro-N-nitrosoguanidine and urethane (86).

Another study evaluated the effect of eugenol on tobacco-induced mutagenesis using the Ames Salmonella/ microsome assay. The results indicated that eugenol inhibited tobacco-induced mutagenicity at concentrations of 0.5 and 1 mg/plate. However, a study on humans showed contrasting results. No significant differences on the cytogenetic parameters were noted after ten healthy non-smoking males ingested 150 mg of eugenol per day suggesting that eugenol has no genotoxic potential in humans (87).

1.3.4 Effects of Eugenol on Immune System

Several adverse effects related to the use of dental products containing eugenol have been observed and include localized irritation of the skin, ulcer

formation, allergic contact dermatitis, tissue necrosis, reduced healing and in rare cases even anaphylactic-like shock (88).

Eugenol is found in soaps, antiseptic solutions and emollient creams used by healthcare workers who frequently wash their hands, dentists are exposed to eugenol in mouthwashes, impression materials and periodontal packings, eugenol is present in 27% of household products, it is commonly included in cutting fluids used by metalworkers and massage therapists are frequently exposed due to the use of essential oils in their trade. A study on fragrance as an occupational allergen was conducted on a total of 24,046 patients at 2002. For eugenol they found that (25.5%) health care workers, (16.5%) non-health care workers, (39.39%) metal workers and (16.3%) people in other occupations were allergic to eugenol (89).

Eugenol is a primary irritant and sensitizer and can cause contact dermatitis as well as irritation of the skin, eyes and respiratory tract. This compound is among the most frequently reported and well recognized consumer allergens in the European Union. But, eugenol was found to induce allergic contact dermatitis in guinea pigs (90).

The potential of eugenol and clove leaf oil to induce delayed skin hypersensitivity in human or to elicit reactions due to pre-existing skin sensitization was evaluated. But analysis of patch-test data demonstrated that eugenol alone or clove oil has a very low potential to cause these effects (91).

Recently another study evaluated the potential of eugenol to cause allergic contact dermatitis in a repeat open application test (ROAT) for a leave-on product. Five volunteers previously sensitized to eugenol were included in the ROAT study where the maximum allowed concentration of eugenol was applied for 4 weeks. Four of the five volunteers reacted during this time, confirming the ability of eugenol to cause contact dermatitis (92).

An 8 years old boy reported type I immediate hypersensitive reaction to

the eugenol after root canal was medicated with sodium hypochlorite and sealed with zinc oxide eugenol. About 1 min after the zinc oxide eugenol placement the patient was anxious and excited, with evident erythema on the face, neck, torso, upper limbs, lower limbs and itchiness and redness extending behind the ear. Cutaneous examination revealed extensive weals of various sizes and shapes. There was no angioedema or mucosal involvement. Owing to the fact that the erythema was noticed after the placement of zinc oxide eugenol, by underwent skin prick test for zinc oxide eugenol the patient showed positive response for eugenol (10%) and negative responses for zinc oxide (10%), formaldehyde (1% aq) and sodium hypochlorite(93).

Intravenous injection of 100 mg of hydrocortisone hemi succinate was administrated immediately and zinc oxide eugenol temporary dressing was replaced with non-eugenol containing material. Forty-five minutes later the patient presented reduced erythema on the face, neck, hands (93).

According to the studies, and three reaction types may be promoted by eugenol, direct tissue damage due to the nature of the medication, contact dermatitis and true allergic reaction. Although eugenol allergy is uncommon, eugenol causes allergic contact dermatitis, possibly because it can react directly with proteins to form conjugates and reactive haptens (93).

1.3.5 Mutagenicity of Eugenol

In vivo study on antimutagenicity effect of eugenol against mutagenicity of cyclophosphamide (CP), mitomycin C(MMC), ethylmethanesulfonate (EMS) and benzo (α) pyrene (B(α)P in the rodent bone marrow cells done by using micronucleus assay. After oral administration of eugenole (0.4 % in the diet) for two weeks, the frequency of micronucleated erythrocytes was decreased significantly. The results provided that eugenol has the capacity of mutagenicity

in male mouse and causes mutation particularly in the anaphase of polychromatic erythrocytes (94).

An investigation has been done on the genotoxic capacity of various phenolic compounds such as eugenol, isoeugenol and safrole in the wing spot test of *Drosophila melanogaster* (common fruit-fly) by using the Drosophila wing somatic mutation and recombination tests. Results of this study demonstrated that isoeugenol was clearly non-genotoxic at the same milimolar concentrations as used for eugenol. Observed results also demonstrated that eugenol and safrole produced a positive recombinagenic response that was related to a high CYP P450 dependent activation capacity. Final results suggested that the reactive metabolites of eugenol and recombinagenic compounds were responsible for the genotoxicity of eugenol (95).

1.4 Cytotoxicity Assays

1.4.1 Neutral Red Uptake Assay

The neutral red uptake assay is one of the most used tests in cytotoxic tests with many biomedical and environmental applications such as toxicity of chemical agents, viral cytopathogenicity and treatment effectiveness, estimation of cytotoxic T-lymphocyte populations, phototoxicity, tumoral cell growth and chemotherapy effectiveness and determination of toxins in biological and environmental samples or biotechnological products. It is also used in ranking chemicals according to their toxic potencies. This test is based on the ability of viable cells to incorporate and bind to the neutral red dye. Neutral red (toluene red, $C_{15}H_{17}ClN_4$) is a weakly cationic dye which penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes. Neutral red dye binds to the phosphate groups or anionic groups of lysosomes by electrostatic hydrophobic bonds. Then by using an acidified ethanol solution, neutral red dye is extracted from the viable cells and the absorbance of the solubilized dye is quantified by spectrophotometric method (96).

The uptake of dye is depends on the production of ATP. Cells which are able to produce ATP have the capacity to maintain pH gradients. At physiological pH, the net charge of dye is close to zero, and dye is enabling to penetrate the membranes of the cells. There is also a proton gradient in the lysosomes which enable them to maintain pH lower than pH of the cytoplasm. Thus, the dye is charged and retained inside the lysosomes. But when the cells die or the pH gradient is reduced, the dye is not charged and extracted from the cells (96).

The absorbance of the solubilized dye is quantified by using spectrophotometry at 540 nm wavelengths. An increase or decrease in the number of cells results in a concomitant change in the amount of dye incorporated by the cells in the culture. This indicates the degree of cytotoxicity

caused by the test material (97).

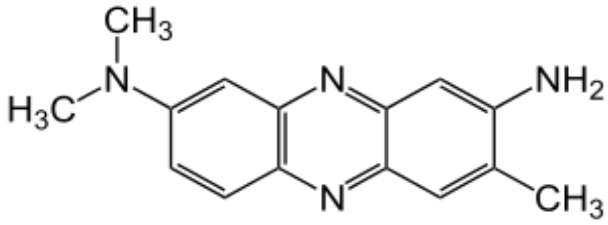


Figure1.2. The chemical structure of Neutral Red

1.5 Genotoxicity Assays

1.5.1 Single Cell Gel Electrophoresis Assay

The Single Cell Gel Electrophoresis assay (known as the comet assay) is a simple, economical and extremely sensitive method for measuring DNA strand breaks and DNA damage induced by chemicals at the individual eukaryotic cells. This is a molecular method that measures deoxyribonucleic acid strand breaks. The term "comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet (98).

The most common form of the assay is the alkaline comet assay. In this technique, cells are embedded in a low-melting-point agarose suspension on a microscope slide. The agarose forms a matrix of carbohydrate fibers that encapsulate the cells and anchoring them on slide. The agarose is considered to be osmotic- neutral, therefore solutions can penetrate the gel and affect the cells. Cells are lysed with a solution which consists of a highly concentrated aqueous salt and a detergent (such as Triton X-100) to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Then the pH of the lysis solution can be adjusted (usually between the neutral and alkaline pH) (98).

The aqueous salt disrupts proteins and their bonding patterns within the cell as well as disrupting the RNA content of the cell. The detergent dissolves the cellular membranes. The cells are destroyed through the action of the lysis solution. All proteins, RNA, membranes and cytoplasmic and nucleoplasmic constituents are disrupted and diffuse into the agarose matrix. The DNA of the cell remains, and unravels to fill the cavity in the agarose that the whole cell formerly filled. This structure is called nucleoid (a general term for a structure in which DNA is concentrated) (99)

After lysis of the cells (typically 1 to 2 hours at 4°C) the slides are washed in distilled water to remove all salts and immersed in an electrophoresis solution. In alkaline conditions the DNA double helix is denatured and the nucleoid becomes single stranded (99).

DNA damage is detected after electrophoresis of single cells embedded in agarose where, in alkaline pH (pH>13).The broken DNA strands move towards the anode and results in comet shapes, observed by fluorescence microscopy. Indeed, the electric current pulls the charged DNA from the nucleus and broken DNA fragments migrate. The intensity of the comet tail relative to the head shows the number of DNA breaks (98,99).

In this method, undamaged DNA retains a highly organized association with matrix proteins in the nucleus. In damaged DNA, this organization is disrupted. The individual strands of DNA lose their compact structure and relax, expanding out of the cavity into the agarose. When the electric field is applied the DNA, which has an overall negative charge, is drawn towards the positively charged anode. Undamaged DNA strands are too large and do not leave the cavity, whereas the damaged fragments, move in a given period of time. Therefore, the amount of DNA that leaves the cavity is a measure of the amount of DNA damage in the cell (98).

The image analysis measures the intensity of the fluorescence for the whole nucleoid and the fluorescence of the migrated DNA and compares the two

signals. The more damaged DNA presents stronger signal. The overall structure resembles a comet shape with a circular head corresponding to the undamaged DNA that remains in the cavity and a tail of damaged DNA.The brighter and longer the tail, the higher the level of damage (99).

1.5.2 The Micronucleus (MN) Assay

The cytokinesis-block micronucleus assay is successfully recognized as one of the most successful and reliable assays for screening various genotoxic compounds in both *in vivo* and *in vitro* cytogenetic studies especially in chromosome damages, and also in various research fields such as nutrigenomics and pharmacogenomics (100).

A micronucleus (MN) is the third nucleus that is formed during the metaphase/ anaphase transition of mitosis or meiosis (cell division). Micronuclei (Howell-Jolly bodies) are cytoplasmic bodies which are expressed in dividing cells. Some divided cells contain chromosome breaks lacking centromeres (acentric fragments) or whole chromosomes that are unable to travel to the spindle poles during the anaphase of mitosis. MN may arise from a whole lagging chromosome (an eugenol event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei (101).

The formation of MN results in the daughter cell lacking a part or all of a chromosome. These chromosome fragments or whole chromosomes normally develop nuclear membranes and form as micronuclei. After cytokinesis, one daughter cell ends up with one nucleus and the other ends up with one large and one small nucleus, i.e., micronuclei. There is a chance of more than one micronucleus forming when more genetic damage has happened (101).

DNA damage events are scored specifically in once-divided binucleated (BN) cells and include:

1. Micronuclei (MNi), a biomarker of chromosome breakage or whole chromosome loss.
2. Nucleoplasm bridges (NPBs), a biomarker of DNA mis repair or telomere end-fusions,
3. Nuclear buds (NBUDs), a biomarker of elimination of amplified DNA or DNA repair complexes.

Scoring of micronuclei can be performed easily on different human cell lines such as: lymphocytes, fibroblasts and exfoliated epithelial cells. An *in vitro* analysis of lymphocytes in the presence of cytochalasin-B (Cyt-B), allows distinguishing easily between mononucleated cells which did not divide and binucleated cells which completed nuclear division. Cyt-B is an inhibitor of actin microfilament. These microfilaments are required for the completion of cytokinesis (100).

Indeed, in these conditions the frequencies of mononucleated cells provide an indication of the background level of chromosome/genome mutations accumulated *in vivo* and the frequencies of binucleated cells with MN a measure of the damage accumulated before cultivation plus mutations expressed during the first *in vitro* mitosis. Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells. Further information regarding mechanisms leading to MNi, NPBs and NBUDs formation is obtained using centromere and telomere probes (101).

2. EXPERIMENTAL SECTION

2.1 Chemicals

Cytochalasin B (Cyt-B)	Sigma
Dimethyl Sulfoxide (DMSO)	Sigma
Ethanol	Sigma
Ethidium Bromide (EtBR)	Sigma
Ethylene Diamin Tetra Acetic Acid Disodium (EDTA)	Sigma
Eugenol	Sigma
Fetal Calf Serum (FCS),	Biological Industries
Giemsa	Merck
Glacial Acetic Acid	Merck
Heparin (Sodium Salt)	Nevparine®
Histopaque-1077	Sigma
Hydrochloric Acid (37%)	Merck
Hydrogen Peroxide (35%)	Merck
L-Glutamine,	Biological Industries
Low Melting Point Agarose (LMA)	Sigma
Methanol	Sigma
Minimum Essential Medium Eagle (MEM)	Sigma
Neutral Red (NR) Dye	Sigma
Nitric Acid	Sigma
N-Lauryl Sarcosinate sodium Salt	Sigma

Normal Point Melting Agarose (NMA)	Sigma
Penicillin–Streptomycin,	Biological Industries
Phosphate Buffered Saline (PBS)	Sigma
Phytohaemaglutinin-M (PHA-M)	Biological Industries
Potassium Chloride (KCl),	Sigma
Potassium Dihydrogen Phosphate	Sigma
RPMI 1640	Biological Industries
Sodium Chloride (NaCl)	Merck
Sodium Hydrogen Phosphate Dihydrate	Sigma
Sodium Hydroxide (NaOH)	Merck
Tris	Sigma
Triton X-100	Sigma
Trypan Blue	Sigma
Trypsin–EDTA	Biological Industries

2.2 Materials and Apparatus

Centrifuge	Heraeus, Hoettich
Cover Slip (24x60mm)	Marienfeld
Deep Freeze (-20^{o}C)	Ariston
Deep Freeze (-80^{0}C)	Revco
Electrophorese	Biometra Analitik
Electrophorese Power Supply	Power Pack P 25
Etuve	Dedeoğlu

Fluorescent Microscope	Leica
Incubator	Heraeus Instruments
Inverted Microscope	Leica
Laminar Flow	Heraeus
Magnetic Mixer	Stuart Scientific, 7801Dottingen,M-21
Micro Centrifuge	Heraeus
Micropipettes (1-10 µl, 0, 5-40 µl, 40-200 µl, 200-1000 µl, 1-5ml)	Finnpipette,Gilson, Biohit
Neubauer Slide	Marienfeld
Comet Analysis Software,version 3.0	Perceptives Kinetic Imaging
PH meter	Cyberscan
PH meter Electrode	Sensorel
Scale	Schimadzu Libror
Slides (26x76mm)	Marienfeld
Spectrophotemeter	Schimadzu Libror
Ultrasonic Bath	Transsonic 460/H
Vortex	Heidolph 2000

2.3 Cytotoxicity Assays

The cytotoxicity of eugenol was performed in V79 cells (purchased from Ankara University, Faculty of Pharmacy) by Neutral Red Uptake (NRU) assay following the protocols described by Virgilio et al. (102) and Saquib et al (103).

2.3.1 Solutions of Neutral Red Uptake Assay

1. **Eugenol Stock Solution**

Eugenol solution is prepared at the concentration of 2 µM. Eugenol is dissolved in distilled water containing 1 % DMSO. Before use, the chemical solution is filtered using Millipore filter.

2. **Neutral Red Stock Solution**

20 mg of NR powder is dissolved in 5 ml distilled water. The solution must be kept in darkness at 4^0C temperature. The NR Stock Solution can be stored in the dark at 4 ^{0}C for up to one month.

3. **Neutral Red Standard Solution**

625 µL from stock NR is mixed with 50 ml modified Eagle's medium (MEM). The NR standard solution must be prepared 18 hours before the experiment and must be kept in incubator at 37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air.

4. **Neutral Red Fixation Solution**

A 100 ml of ethanol and 2 ml of acetic acid are mixed with 98 ml distilled water.

2.3.2 Procedure of Neutral Red Uptake Assay

All the procedure must be carried out in the laminar flow safety cabinet. The safety cabinet has been thoroughly cleaned and all equipment have been wiped down with 70% ethanol before use.

1. V79 Cells were seeded in MEM supplemented with 10% fetal calf serum and 1% penicillin streptomycin solution. Cells were cultured in 25 ml cell culture flasks.
2. Cells were incubated at 37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air for 24 hours.
3. After 24 hours medium were aspirated with the aspirator pump. 5 ml of 37^0C warm trypsin-EDTA (10X) was added to the flask to wash the cells, and then the trypsin was aspirated. This procedure was repeated once, after trypsinization the cells were incubated at 37 ^{0}C.
4. After incubation, cells were detached from the flask and checked under the microscope to ensure cells being detached.
5. 10 ml of 37 ^{0}C medium with fetal calf serum was added to the flasks to stop the reaction.
6. Cells were centrifuged for 5 min at 1200 rpm. The supernatant was discarded by aspiration. Cells were suspended in 2 ml of culture medium.
7. 90 µL of cells were mixed with 10 µL of trypan blue and the cells were counted using Neubauer slide.
8. 10000 cells/ well in 200 µL medium were seeded in a 96-well plate. Each plate was controlled under a phase contrast microscope.
9. Plates were incubated at 37 ^{0}C for 24hours in a humidified atmosphere of 5% CO_2 in air.
10. After 24 hours, the viability and also the contamination of cells cultured in plates are controlled microscopically. Typical signs of contamination are changes in color or clouding of cell medium and changes in cell shape.

11. Medium was discarded from the plate. The cells were cultured with different concentrations of eugenol for an additional 24 hours.
12. Plates were incubated at 37 ^{0}C for approximately 18 hours in a humidified atmosphere of 5% CO_2 in air.
13. At the end of the incubation, the solution was discarded from the plates and 200 µl of NR standard solution at 37 ^{0}Cwas added to all wells by a multi-channel pipette. The plates were incubated in 37 ^{0}C for additional 3 hours in a humidified atmosphere of 5% CO_2 in air.
14. At the end of the incubation, the solution was carefully discarded and plate was washed five times with pre-warmed (37^{0}C) sterile PBS under the safety cabinet. For washing procedure, 200 µL of the PBS was added to each well by a multichannel pipette and then discarded. Each plate firmly was tapped on absorbent paper cloth to remove any remaining liquid from the wells.
15. 200µl of NR fixative solution was added to each well by a multichannel pipette.
16. Plates were placed on shaker for 20 minutes at 600 rpm. Plates were wrapped in foil to be kept in the dark.
17. Plate lids were removed just before placing each plate on the plate reader.
18. The absorbance of the samples was recorded at 540 nm wavelength spectrofotometrically.

The data were expressed as average values obtained from 8 wells for each concentration. The results were expressed as the mean percentage of cell growth inhibition. Cell viability was plotted as percent of control (assuming data obtained from the absence of eugenol as 100%).

The cytotoxicity experiment was repeated three times.

2.4 Genotoxicity Assays

2.4.1 Assessment of DNA Damage in the Peripheral Lymphocytes by Alkaline Single Cell Gel Electrophoresis

The basic alkaline technique of Singh and et al. as further described by H. Anderson was followed. (104,105)

2.4.1.1 Solutions of Comet Assay

1. Electrophoresis Buffer

1750 ml cold distilled water, 52.8 ml NaOH 10 N, 8.8 ml 200mM EDTA are mixed properly. It should be freshly prepared on the day of the experiment.

2. EDTA 200mM

14.89 g EDTA salt is dissolved in 200 ml distilled water. The pH is adjusted to 10 and the solution is kept at room temperature.

3. Ethidium Bromide Staining Solution

To prepare the stock staining solution, 10 mg ethidium bromide is dissolved in 50 ml distilled water. Then 1 ml of this solution is taken and the volume is adjusted to 10 ml with distilled water. Both solutions are kept at room temperature.

4. Low Melting Agarose (LMA) 0.5% Solution

125 mg LMA is dissolved in 25 ml PBS in microwave avoiding the boiling of agarose. After the agarose is dissolved, the solution has been divided into small amounts and kept in refrigerator.

5. Stock Lysing Solution

146.1 g NaCl, 37.2 g EDTA, 1.2 g Tris are dissolved in 500 ml of distilled water. After adjusting pH to 10 by 10 g of NaOH, 10 g of N-Lauryl Sarcosinate sodium salt is added. The contents should be properly mixed, and then the volume is adjusted to 1000 ml with distilled water. The stock lysing is kept at room temperature.

6. Lysing Solution

178 ml of stock lysing solution is mixed with 20 ml of DMSO and 2 ml of Triton-X100. It should be freshly prepared 1 hour before the experiment and should be kept at 4^0C.

7. Neutralization Buffer

48.5 mg Tris is dissolved in 750 ml distilled water and the pH is set to 7.5. The volume is adjusted to 1000 ml distilled water and the buffer is stored at the room temperature.

8. Normal Melting Agarose (NMA) 0.5% Solution

125 mg NMA is dissolved in 25 ml PBS in hot water avoiding the agarose to boil. The solution is kept in small volumes in refrigerator.

9. Sodium Hydroxide (NaOH) 10 Normal

200 Ml NaOH is dissolved in 500 ml distilled water and the solution is kept at room temperature. The shelf life of the 10 N NaOH stock solutions is limited to 2 weeks.

10. Phosphate Buffered Saline (PBS) Buffer

One tablet of PBS is dissolved in 200 ml distilled water and then the buffer is sterilized and stored at 4 ^{0}C.

2.4.1.2 Blood sampling and Lymphocyte Preparations

1. A 2-ml heparinized (50 units/mol sodium heparin) whole peripheral blood sample was taken by venipuncture from the individual (with no smoking and alcohol drinking habit).
2. The whole blood which was diluted with 6 ml PBS was gently layered on 2 ml Ficoll (lymphorep) by a Pasteur pipette in a centrifuge tube.
3. The mixture was centrifuged for 20 minutes at 1400 rpm and the lymphocytes were isolated by Ficoll-Hypaque density gradient.
4. The cells on to the Ficoll layer in the interphase were carefully taken by a Pasteur pipette after centrifugation.
5. PBS was added to the lymphocytes and mixed.
6. The cells were washed by centrifugation at 1800 rpm for 10 minutes.
7. The supernatant was poured off and the lymphocytes were washed with PBS again in the same manner.
8. The 1 x 10^4 cells in 50 µl were treated with the increasing concentrations of eugenol (50, 100, 150, 200, and 250 µM) for 30 min at 37°C for the assessment of DNA damage.
9. After the pretreatment of eugenol for 30 min, oxidative damage was induced by replacing the medium with PBS containing 50 µM H_2O_2 and then incubating for 5 min on ice to assess the antigenotoxicity of eugenol.
10. Then the lymphocytes were centrifuged and washed with PBS for removing the H_2O_2 solution. A negative control sample (PBS) and positive control 50 µM H_2O_2 were also included in the experiments.
11. After centrifugation at 3000 rpm, supernatant was discarded and the cells were suspended in 75 µl of 0.5% LMA.

2.4.1.3 Slide Preparation

1. The suspensions were embedded on the slides pre-coated with a layer of 1% NMA using a coverslip.
2. The slides were allowed to solidify on ice for 5 min.
3. After removal of the coverslip, the slides were immersed in cold freshly made lysing solution for a minimum for 1 hour at 4 ^{0}C.

2.4.1.4 Electrophoresis

1. The slides were removed from the lysing solution, drained and were left in the electrophoresis solution for 20 min at 4 ^{0}C to allow unwinding of the DNA and expression of alkali-labile damage.
2. Electrophoresis was conducted also at a low temperature (4 ^{0}C) for 20 min using 25 V and adjusting the current to 300 mA by raising or lowering the buffer level.
3. The slides were neutralized by washing 3 times in 0.4 M Tris-HCl (pH 7.5) for 5 min at room temperature.
4. After neutralization, the slides were incubated in 50%, 75%, and 99% of alcohol for 5 min, respectively.
5. The dried microscope slides were stained with ethidium bromide (EtBr 20 µg/ml in distilled water, 35 µl / slide), covered with a coverslip prior to analysis.

2.4.1.5 Slide Scoring

For visualization of the DNA damage, the slides were examined with a Leica fluorescence microscope under green light.

The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel.

In order to visualize DNA damage, slides were examined at 60 x. One hundred cells from two replicate slides were assayed for each experiment. Results were expressed as tail length, tail intensity, and tail moment.

The experiment was repeated three times.

2.4.2 Cytokinesis-Blocked Micronucleus Assay

The presence of MN in a binuclated cell using the protocol of Fenech (100) was determined with minor modifications. Human peripheral blood cultures were used for the CBMN test.

2.4.2.1 Solutions of Micronucleus Assay

1. Culture Medium

100 ml Fetal Calf Serum 12.5 ml PHA-M 10 ml L-Glutamine and 10 ml penicillin-streptomycin are dissolved in 500 ml RPMI 1640. This culture medium should be kept in -20^{0}C.

2. Hypotonic Solution

1.397 g KCl is dissolved in 250 ml dissolved water. This solution should be stored at -20 ^{0}C.

3. Fixation Solution

70 ml acetic acid is dissolved in 210 ml methanol. (3:1 is the ratio of methanol: acetic acid.)This solution should be stored at -20^{0}C.

4. Nitric Acid 65%

68.75 ml nitric acid is dissolved in 1000 ml distilled water. This solution is stored at dark.

5. Cytochalasin-B (Cyt-B)

1 mg Cyt-B is dissolved in 1.475 ml stilled DMSO. This solution should be stored at -20 ^{0}C.

6. Buffer A

11.34 g KH_2PO_4 is dissolved in 250 ml distilled water. pH is adjusted to 4.8.

7. Buffer B

7.37 g $Na_2HPO_4.2H_2O$ is dissolved in 250 ml distilled water. pH is adjusted to 9.3.

8. Giemsa Dye

15 ml buffer A and 15 ml buffer B and 15 ml Giemsa dye mixed with255 ml distilled water.

2.4.2.2 Blood Sampling

1. A 2-ml heparinized (0.2 ml heparin per 5 ml whole blood) peripheral blood sample was taken by venipuncture from the individual (with no smoking and no alcohol drinking habits).
2. 0.5 ml blood was added to the tubes containing 5 ml cultured medium. Medium should be warmed to 37^{0}C.
3. Tubes were incubated at 37^{0}C in a humidified atmosphere containing 5% CO_2.
4. After 24 hours (one cell cycle) different concentrations of eugenol (50, 100, 150, 200, 250 µM) were added to the tubes alone and also in combination with 100 µM H_2O_2. In all sets of experiments, an untreated negative control as well as a positive control (100 µM H_2O_2) was also run simultaneously. All tubes were incubated at 37^{0}Cand 5 % CO_2.

5. After another 20 hours (total 44 hours), 50 µL Cyt-B (an inhibitor of the mitotic spindle) was added to all tubes and were incubated at 37 ^{0}C and 5 % CO_2.
6. 28 hours after adding Cyt-B, the tubes were centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and 0.5-0.7 ml of ice-cold hypotonic KCl solution was added to tubes very slowly. Tubes were kept at 4^{0}C for 5 minutes.
7. Tubes were centrifuged at 1000 rpm for another 10 minutes. The supernatant was discarded and 5 ml of fixation solution was added to the tubes very slowly. Tubes were kept at 4^{0}C for 15 minutes. This step should be repeated twice.
8. After the last centrifuge, the supernatant was discarded and the 0.5-0.7 ml of cell suspension was retained in the tubes and was homogenized by Pasteur pipette.
9. The slides were allowed to air dry and stained in 5% Giemsa for 10 min and coded for different concentrations and were stored at -20 ^{0}C in 70 % ethanol.
10. The fixed cells were dropped onto slides previously cleaned with nitric acid.

2.4.2.3 Examination of slides and assessment of MN

After staining, the slides were air-dried and binucleated cells were counted. Slides were examined at 100 x objectives using a light microscope. A score should be obtained for slides from each duplicate culture. The number of cells scored should be determined depending on the level of change in the MN index that the experiment is intended to detect and the expected standard deviation of the estimate. For each slide the following information should be obtained: The number of micronuclei (MNi) in at least 1000 binucleate (BN) cells should be scored .The criteria for scoring MNi in BN cells were detailed below.

The distribution of BN cells with zero, one or more MNi; the number of MNi in a single binucleated cell normally ranges from 0 to 3 in lymphocytes of healthy individuals but can be greater than 3 on occasion depending on chemical exposure and age.

The frequency of micronucleated BN cells in at least 1000 BN cells and the proportion of mononucleated, binucleated, tri-nucleated and tetra-nucleated cells per 500 cells were scored.

The Nuclear Division Index can be derived as below:

[1 x N1] + [2 x N2] + [3 x (N3+N4)] / N, where N1-N4 represent the number of cells with 1-4 nuclei, respectively, and N is the total number of cells scored.(100)

The cytokinesis-blocked cells that may be scored for MN frequency should have the following characteristic:

a) The cells should be binucleated.

b) The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.

2.5 Statistical Analysis

For alkaline comet assay, statistical analysis was performed by SPSS for windows 22.0 computer program. The results were expressed as the mean ± standard deviation. Difference between the means of data are compared by the one way variance analysis (ANOVA) test and the post hoc analysis of group differences by least significant difference (LSD) test. *P* value of less than 0.05 was considered as statistically significant. For statistical analysis of CBMN assay results, the z-test was applied for the percentage of MN and CBPI. The results were given as the mean ± standard error.

3. RESULTS

3.1 Determination of the Cytotoxicity of Eugenol by Neutral Red Uptake Assay

The cytotoxicity of eugenol was assayed in V79 cell line by NRU assay. The V79 cell line was chosen because of its high sensitivity to various chemicals, high cloning efficiency, and excellent properties in colony formation. The cytotoxic effect of the different concentrations of eugenol as measured by the NRU assay has been shown in Figure 3.1.

According to the results, compared to the same concentrations of untreated cells (control group); eugenol was found to have cytotoxic effects in concentrations more than 500 µM. A concentration dependent toxicity was observed in V79 cells after 18 hours exposure to eugenol.

The concentrations up to 250 µM had no effect on V79 cell viability during 18 hours exposure but at concentrations higher than 500 µM, the cell viability decreased below 50 %. IC_{50} value of eugenol in V79 cell line was found to be 341.5 µM.

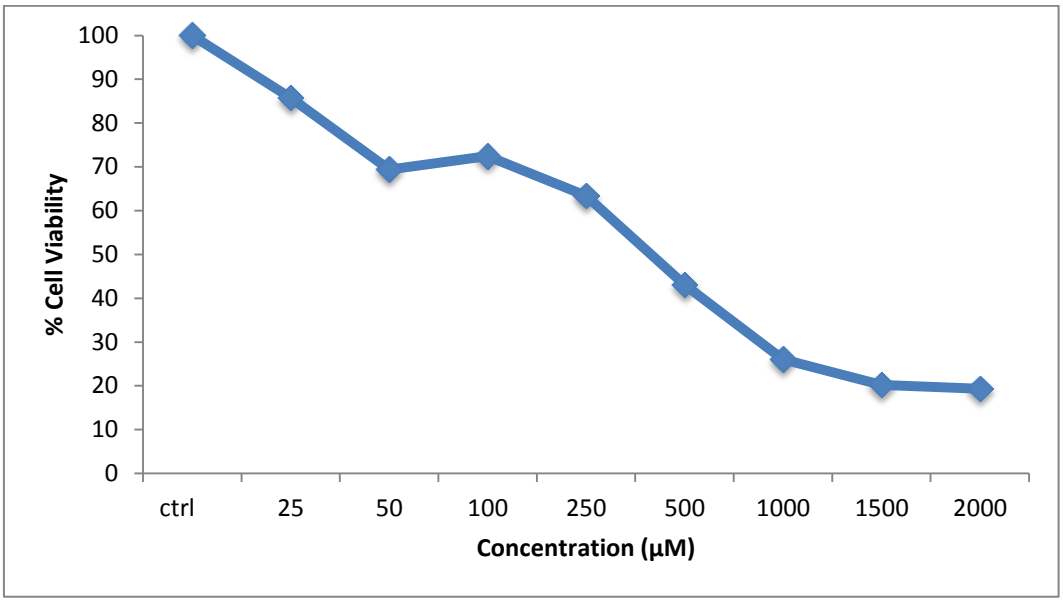


Figure 3.1. Effects of Eugenol on cell viability in V79 cells

Results of cytotoxicity assay (NRU assay) were expressed as the mean percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as percent of control.

(Assuming data obtained from absence of eugenol as 100%)

3.2 Assessment the Genotoxicity of Eugenol by Comet Assay

The results for the assessment of the genotoxicity and antigenotoxicity of eugenol by alkaline comet assay were shown in Figure 3.2. According to the three independent experiments, no significant increase in DNA strand breakage as expressed as DNA tail length, DNA tail intensity and DNA tail moment was observed at different concentrations of eugenol (50, 100, 150, 200, and 250 µM) when compared with their controls (Table 3.1).

At the concentration of 150µM, eugenol seemed to decrease the H_2O_2 induced DNA damage expressed as decreases as in the DNA tail length, tail moment and tail intensity. ($p< 0.05$). Although at the higher concentrations of 200 and 250 µM of eugenol decreased the tail intensity induced by H_2O_2, such effect was not confirmed by the evaluation of tail moment and tail length.

3.3 Assessment the Genotoxicity of Eugenol by Micronucleus Assay

The results of the experiments for the clastogenicity and anticlastogenicity testing by MN formation were shown in Table 3.2. The lymphocytes treated with different concentrations of eugenol (50-250 µM) caused no genotoxic effects alone at mentioned concentrations as compared with the control group. The treatment of cells with eugenol and H_2O_2 resulted in some decrease in the cytokinesis blocked proliferation index; however the differences were not significant. The treatment of eugenol with H_2O_2 revealed a reduction in the frequency micronuclei at all concentrations in a dose dependent manner when compared to the samples treated with H_2O_2 only (Table 3.2).

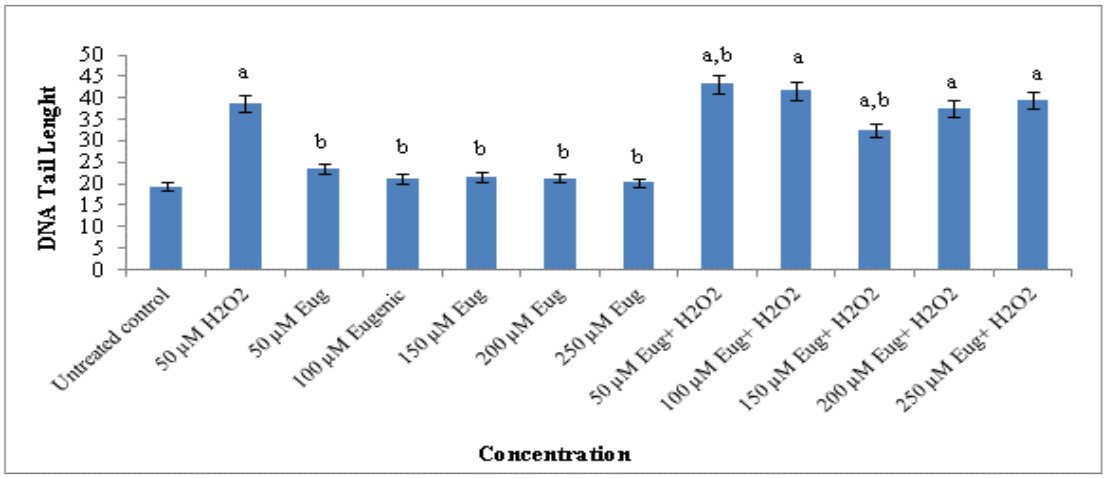
Table 3.1. Genotoxicity Findings of Eugenol by Comet Assay*

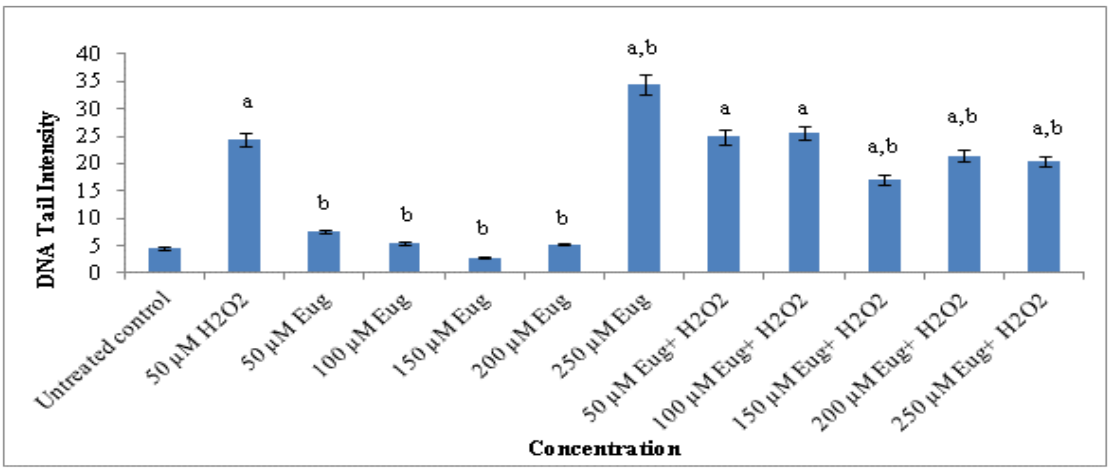
DNA Tail Length		**Mean**	**Standard Deviation**
1	Negative Control	19.398	2.63
2	50 µM H_2O_2	38.887 [a]	26.12
3	50 µM Eug	23.497 [b]	8.38
4	100 µM Eug	21.544 [b]	6.39
5	150 µM Eug	21.013 [b]	3.64
6	200 µM Eug	21.382 [b]	4.32
7	250 µM Eug	20.255 [b]	2.63
8	50 µM Eug+ H_2O_2	43.294 [a,b]	25.98
9	100 µM Eug+ H_2O_2	41.725 [a]	25.54
10	150 µM Eug+ H_2O_2	32.413 [a,b]	20.63
11	200 µM Eug+ H_2O_2	37.504 [a]	24.9
12	250 µM Eug+ H_2O_2	39.479 [a]	22.27
DNA Tail Intensity			
1	Negative Control	4.62	5.81
2	50 µM H_2O_2	24.34 [a]	26.01
3	50 µM Eug	7.619 [b]	11.01
4	100 µM Eug	5.997 [b]	8.13
5	150 µM Eug	4.596 [b]	6.44
6	200 µM Eug	5.288 [b]	8.41
7	250 µM Eug	34.496 [a,b]	42.56
8	50 µM Eug+ H_2O_2	24.868 [a]	27.07
9	100 µM Eug+ H_2O_2	25.590 [a]	27.52
10	150 µM Eug+ H_2O_2	17.098 [a,b]	22.67
11	200 µM Eug+ H_2O_2	21.360 [a,b]	25.17
12	250 µM Eug+ H_2O_2	20.422 [a,b]	24.83
DNA Tail Moment			
1	Negative Control	0.651	0.92
2	50 µM H_2O_2	6.872 [a]	9.93
3	50 µM Eug	1.329 [b]	2.74
4	100 µM Eug	0.914 [b]	1.52
5	150 µM Eug	0.667 [b]	1.13
6	200 µM Eug	0.810 [b]	1.55
7	250 µM Eug	2.455 [b]	4.57
8	50 µM Eug+ H_2O_2	8.219 [a,b]	12.00
9	100 µM Eug+ H_2O_2	8.410 [a,b]	11.96
10	150 µM Eug+ H_2O_2	4.972 [a,b]	8.22
11	200 µM Eug+ H_2O_2	6.656 [a]	10.24
12	250 µM Eug+ H_2O_2	6.050 [a]	8.90

*****DNA damage expressed as DNA tail length, DNA tail intensity and DNA tail moment treated with eugenol and eugenol with H_2O_2. Values are given as mean ± standard deviation. n=3.

[a] P<0.05, significantly different from negative control.

[b] P<0.05, statistically different from positive control (50 Mµ H_2O_2).





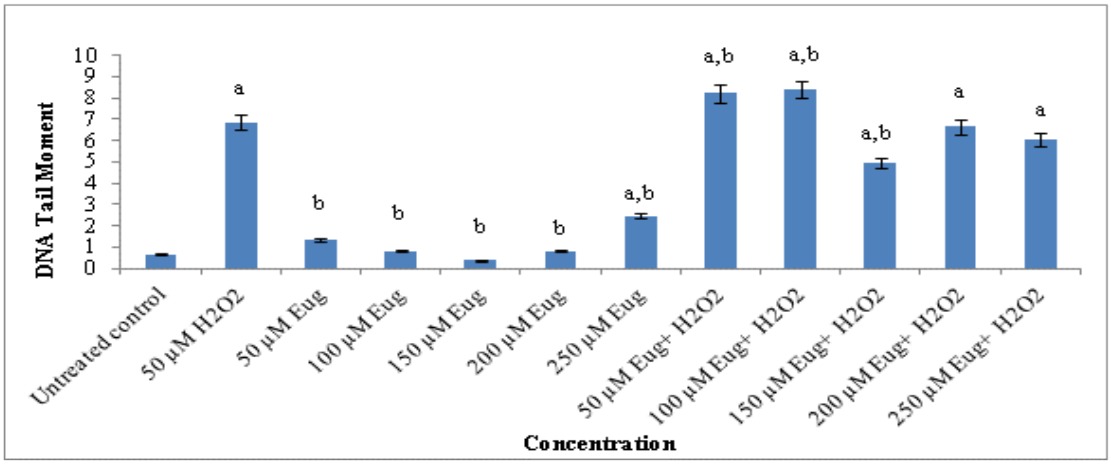


Figure 3.2.DNA damage expressed as DNA tail length, DNA tail intensity, and DNA tail moment. Values are given as mean ± standard deviation, *n*=3.

[a]*p*<0.05, statistically different from negative control.

[b]*p*<0.05, statistically different from positive control (50µM H_2O_2).

Table 3.2. Genotoxicity Findings of Eugenol by Micronucleus Assay*

Treatment group	BN cells scored	Number of BN cells according to donors			MN /10^3 cells	MN% ± SE	CBPI ± SE
		1st	2nd	3rd			
Untreated control	3000	1	2	1	1.33	0.13± 0.07	1.356 ± 0.3
50 µM H_2O_2	3000	10	11	10	10.33	1.03± 0.18 *	1.407±0.30
50 µM eugenol	3000	3	4	1	2.67	0.27 ± 0.09	1.35±0.30
100 µM Eugenol	3000	5	2	4	3.67	0.37± 0.11	1.306±0.30
150 µM Eugenol	3000	2	4	2	2.67	0.27± 0.09	1.368±0.30
200 µM Eugenol	3000	1	3	1	1.66	0.17± 0.08	1.288±0.29
250 µM Eugenol	3000	7	1	3	3.67	0.37± 0.11 #	1.251±0.29
50 µM Eugenol+ H_2O_2	3000	4	3	4	3.67	0.37± 0.11 #	1.368±0.30
100 µM Eugenol+H_2O_2	3000	7	2	3	4	0.4 ± 0.12 #	1.419±0.31
150 µM Eugenol + H_2O_2	3000	2	2	2	2.33	0.20 ± 0.08 #	1.35±0.30
200 µM Eugenol + H_2O_2	3000	2	3	2	2.33	0.23 ± 0.09 #	1.236±0.29
250 µM Eugenol + H_2O_2	3000	2	5	4	3.66	0.37± 0.11 #	1.27±0.29

*Micronucleus frequencies and the cytokinesis block proliferation index in human lymphocytes for the genotoxicity and antigenotoxicity of eugenol.

BN=binucleated ; **MN**=micronucleus; **SE**=standard error; **CBPI**=cytokinesis blocked proliferation index; Negative control=untreated cells; Positive control= 50 µM H_2O_2 treated cells.

*p< 0.05 significantly different from negative control (z-test)

#p<0.05 significantly different from positive control (z-test)

4. DISCUSSION

Eugenol, a major volatile constituent of clove essential oil, is derived from the *Eugenia Caryophyllata*. Its pharmacological properties which include antimicrobial, anti-inflammatory, analgesic, antioxidant and anticancer activities have been the subjects of many studies. Since the twentieth century, attention has been devoted to the antimutagenic and genotoxic effects of natural compounds and in recent years there has been a great concern about the activity and the toxicity of eugenol due to its wide range of usage, as well as its antioxidant activity due to the presence of its phenolic group. Eugenol has been classified as generally recognized as safe (GRAS) by the U.S Food and Drug Administration. However, its cytotoxicity and genotoxicity studies are very limited and contradictory.

The cytotoxic properties of eugenol have been investigated in different cell lines. The cytotoxicity of eugenol has been studied in three different human derived cell lines i.e. malignant Hep G2 hepatoma cells, malignant Caco-2 colon cells and nonmalignant human VH10 fibroblasts. Eugenol showed cytotoxic effect in all cell lines and acted as a genotoxicant in human VH10 fibroblasts and Caco-2 colon cells but not in Hep G2 hepatoma cells. Eugenol at concentrations below 600 µM significantly increased the level of DNA breaks in human VH10 fibroblast cells and to a lower degree in Caco-2 colon cells. The DNA damaging effect was not observed in Hep G2 cells (73).

The cytotoxic effect of eugenol was also investigated in human osteoblastic cell line. Eugenol showed a cytotoxic effect in human osteoblastic cell line in a dose-dependent manner. The IC_{50} of eugenol in this study was approximately 0.75 mmol/L. Eugenol also inhibited cell proliferation during a 4-day culture period. At the concentrations higher than 0.01 mmol/L eugenol seemed to have significant toxicity potential (69).

Yoo CB and et al (59) examined the cytotoxicity of eugenol by using MTT assay in HL-60 cancer cells. Eugenol showed different degrees of cytotoxicity in

these cells and it inhibited 50% cell growth in HL- 60 cells at the concentration of 23.7 µM.

Another study investigated the effects of eugenol on the growth of human colon cancer cells. HT-29 cells were treated with various concentrations of eugenol (0-250 µM). Eugenol inhibited the growth of cells in a dose and time dependent manner. After 24h exposure, the growth of cells was reduced below 50% at the 250 µM concentrations of eugenol (74).

Martins et al (106) examined the genotoxic and apoptotic activities of eugenol in AA8 and EM9 cells. Dose dependent decreases in viability were observed. For a 24h exposure, the cell viability was reduced below 50 % when cells were treated with concentrations higher than 500 µM for AA8 cells and 1000 µM for EM9 cells. The ability of eugenol to induce DNA damage was assessed with alkaline comet assay. In AA8 cells, DNA damage was induced by eugenol, but with no statistical significance. In EM9 cells, eugenol did not induce DNA damage.

Studies demonstrated that all the zinc-oxide eugenol based root canal sealers have moderate to severe cytotoxic effects in V79 cultured cells but such effects are different due to the dose and duration of exposure. However, the results did not indicate the genotoxic effects of these dental products (79).

In this study we investigated the *in vitro* cytotoxicity of eugenol by the NRU test in V79 cell line which is widely used healthy cell line in many *in vitro* assays. Our results demonstrated that the concentrations of eugenol up to 250 µM did not affect the viability of V79 cells during 18 hours exposure, but above this concentration the cytotoxicity of eugenol was observed and the cell viability decreased below 50% at the concentration of approximately 341.5 µM.

The data of our study is consistent with the data of Martins et al (106) that indicated cytotoxicity of eugenol at high concentrations, although the IC_{50} value determined in this study is lower than our finding. IC50 values of eugenol have

been found to be different according to the cell-line, duration of incubation and the method used in different studies. In these studies, generally cancer cell-lines were used however V79 were used in our study.

The genotoxicity of eugenol in V79 cells was investigated *in vitro*. Eugenol was found to increase chromosomal aberrations with significant increases (3.5% aberrant cells) at 2500 µM, demonstrating cytotoxicity at higher doses (80).

A dose and time dependent study in rats investigated the genotoxicity of methyleugenol (MEG) by using comet assay. Results demonstrated no significant differences in DNA damage after 24 hours exposure with doses that produce tumors in rodents (107).

In our study, the genotoxicity potential of eugenol was investigated by the alkaline comet assay, a commonly used assay, and the cytokinesis-blocked micronucleus assay (CBMN), at non-cytotoxic concentrations (50-250 µM).

Furthermore, the MN and comet assays were performed to investigate whether eugenol provided protection against H_2O_2 induced DNA damage in human peripheral lymphocytes. H_2O_2 is a highly reactive oxygen species and is able to induce damage to cell membranes, proteins, nucleic acids. It is known to cause oxidative DNA damage primarily through the hydroxyl radical which results from the Fenton reaction. H2O2 has been reported to cause DNA damage in the form of chromosomal aberrations, single and double strand breaks (108).

The genotoxic effect of eugenol was investigated by Comet assay in the range of 50-250 µM concentrations.

However, only 250 µM eugenol indicated genotoxic effect based on DNA tail intensity data. The same effect was not observed with tail moment and tail length. At the concentrations of 50, 100 µM eugenol, no decrease in the H_2O_2-induced DNA damage was seen. When eugenol used in combination with H_2O_2,

eugenol appeared to prevent H_2O_2-induced DNA damage only at 150 µM concentration according to DNA tail length, tail intensity and tail moment data. Eugenol alone, in all study concentrations did not induce any increase in MN. On the other hand, eugenol, in all concentrations, decreased H_2O_2-induced DNA damage. According to this data, it seems that eugenol prevents H_2O_2-induced DNA damage in all study concentrations.

In conclusion, the results of this study suggest that eugenol might have cytotoxic effects in a dose dependent manner. However, eugenol in the concentrations used below the IC_{50} values showed no significant genotoxic effects. Our results of MN assay also showed that eugenol might protect against H_2O_2-induced genotoxicity. As our study is composed only an *in vitro* experiments, further *in vivo* animal studies are required to understand the genotoxic and antigenotoxic properties of eugenol in detail.

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