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To Implement and Study of Eugenol in Herbal Plants

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Abstract
Eugenol, a phytogenic bioactive component is frequently found in diversified herbal plants possessing well defined functional attributes. Prominent sources of eugenol are clove, cinnamon, tulsi and pepper. Various extraction methods have been practiced globally for the extraction of eugenol and other nutraceutics from plants. The most extensively employed approaches in this regard include solvent extraction, hydrod istillation, microwave-assisted extraction, supercritical carbon dioxide extraction and ultrasound-based extraction. Eugenol has been approved to encompass numerous beneficial aspects against a capacious spectrum of life threatening indispositions including oxidative stress, inflammation, hyperglycemia, elevated cholesterol level, neural disorders and cancer. In addition, eugenol has also shown strong potential as an antimicrobial agent against wide ranges of pathogenic and spoilage causing microorganisms. Predominantly, the principle mechanistic approaches associated with the therapeutic potential of eugenol include its free radical scavenging activity, hindrance of reactive oxygen species' generation, preventing the production of reactive nitrogen species, enhancement of cyto-antioxidant potential and disruption of microbial DNA & proteins. Consequently, this article is an attempt to elucidate the general properties, sources, extraction methods, therapeutic role and associated mechanisms of eugenol.

Keywords: Eugenol, Cloves, tree, soaps, detergents etc.

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.

Theoretical principals
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).

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).

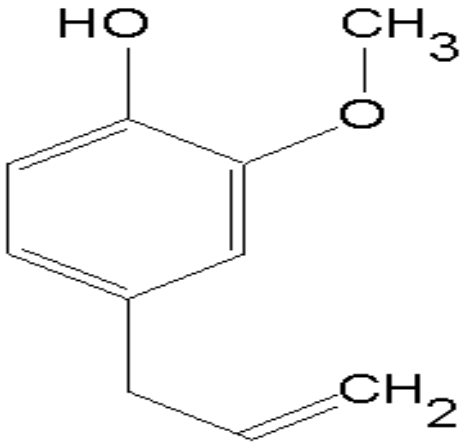

Fig. 1: Chemical structure of eugenol ($C_{10}H_{12}O_2$)

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)

Experimental section

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

Materials and Apparatus

Centrifuge	Heraeus, Hoettich
Cover Slip (24x60mm)	Marienfeld
Deep Freeze (-20oC)	Ariston
Deep Freeze (-800C)	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

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).

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.

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 0C for approximately 18 hours in a humidified atmosphere of 5% CO2 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 0Cwas added to all wells by a multi-channel pipette. The plates were incubated in 37 0C for additional 3 hours in a humidified atmosphere of 5% CO2 in air.
14. At the end of the incubation, the solution was carefully discarded and plate was washed five times with pre-warmed (370C) 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.

Conclusion

This review article explicates the effectiveness of eugenol as a therapeutic tool that can be incorporated to various foods and herbal medicines for contending considerable metabolic disorders. It also contains considerable antimicrobial properties and can be employed to inhibit the growth of microbial populations in many foods. Additionally, derivatives of eugenol have unlocked a new era in the domain of pharmacology, kindling the research interests on this compound. Nevertheless, more studies are required to specify the dosage level of eugenol. 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.

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