In the present study, potential anticancer effect of eugenol on inhibition of cell proliferation and induction of apoptosis in human MCF-7 breast cancer cells was investigated. Induction of cell death by eugenol was evaluated following MTT assay and monitoring lactate dehydrogenase released into the culture medium for cell viability and cytotoxicity, giemsa staining for morphological alterations, fluorescence microscopy analysis of cells using ethidium bromide and acridine orange and quantitation of DNA fragments for induction of apoptosis. Effect of eugenol on intracellular redox status of the human breast cancer cells was assessed by determining the level of glutathione and lipid peroxidation products (TBARS). Eugenol treatment inhibited the growth and proliferation of human MCF-7 breast cancer cells through induction of cell death, which was dose and time dependent. Microscopic examination of eugenol treated cells showed cell shrinkage, membrane blebbing and apoptotic body formation. Further, eugenol treatment also depleted the level of intracellular glutathione and increased the level of lipid peroxidation. The dose dependent increase in the percentage of apoptotic cells and DNA fragments suggested that apoptosis was involved in eugenol induced cell death and apoptosis might have played a role in the chemopreventive action of eugenol.

**Keywords:** Apoptosis, Breast cancer, Eugenol, MCF–7 breast cancer cell

Cancer mortality rate in the developed world have risen throughout in spite of advancement made in cancer diagnosis and therapy during the last several decades and it is the leading cause of worldwide deaths. Breast cancer is one of the most common malignant diseases of women and it is associated with an appropriate morbidity and mortality. Mammary homeostasis is dependent upon establishing and maintaining a balance between cycles of mammary epithelial cell proliferation and death by apoptosis. Risk for spontaneous mammary cancer has been attributed to enhanced proliferation of mammary epithelial cells which leads to the increased accumulation of genetic alterations. It is likely that defective apoptotic pathways may play a role in risk for mammary tumorigenesis. Numerous epidemiological studies on cancer suggest that dietary agents affect the risk of developing cancer either by decreasing or increasing it. In recent years, growing attention has been paid to food components with potential cancer inhibiting effect, with the hope of identifying effective chemopreventive diets or dietary supplements for human use. There are several products of plant origin (phytochemicals) used in traditional medicine which appear to possess significant cytotoxic as well as chemopreventive activity. As therapeutic agents, the phytochemicals promote both growth arrest and apoptosis, leading to cancer remission.

Eugenol (4-allyl-2-methoxy phenol) is a naturally occurring allyl benzene which has been reported to show a number of biological activities. Eugenol is considered to be non-mutagenic and non-carcinogenic and is generally accepted as safe by the Food and Drug Administration. Human exposure to eugenol occurs through its use in dentistry as an analgesic and local anesthetic and its presence in foods and spices. In traditional medicine, eugenol has been used against gastrointestinal diseases and chronic diarrhoea. Several investigations also indicated that eugenol has antioxidant and antimutagenic activities. Eugenol can regulate an array of cellular biochemical processes such as inhibition of lipid peroxidation, cyclooxygenase-2 gene expression and reactive oxygen species. Thus, the reported in vivo and in vitro studies suggest that eugenol can be an effective compound for cancer chemoprevention and chemotherapy. Therefore, the present study was undertaken to determine the cytotoxic activity of
eugenol and its mechanism to induce cell death in human breast cancer cells.

Materials and Methods

Cell culture—MCF-7 (Human breast cancer cell line) was obtained from NFATCC, Pune, India. All cells were grown in MEM supplemented with 10% fetal bovine serum (FBS), insulin (0.1 units/µl), L-glutamine (2 mM), non-essential amino acids (0.1 mM), penicillin (100 units/ml) and streptomycin (100 µg/µl). Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere and 95% air.

Chemicals—Eugenol, MTT, acridine orange, ethidium bromide, dithiobisnitrobenzoic acid (DTNB) and GSH were from Sigma Chemical Co (St. Louis, USA). All other chemicals used were of reagent grade. Stock solutions of eugenol were prepared in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide was less than 0.1%, and it had no effect on MCF-7 cells.

Histopathology study—Cells, after treatment with eugenol, were fixed in 75% methanol and 25% glacial acetic acid for 15 min at room temperature, washed with deionized water and viewed under a phase contrast microscope. For giemsa staining, cells after fixation were treated with dilute giemsa and morphological changes were observed and photographed.

Apoptosis/necrosis in MCF-7 cells—To detect the degree of eugenol induced apoptosis and/or necrosis in MCF-7 cells, the cells (50,000/well) in 24 well culture plates were exposed to various concentrations of eugenol. Cells, after treatment with eugenol, were collected, washed and suspended in phosphate buffer solution (PBS; pH 7.4). After staining with a mixture of acridine orange (100 µg/ml in PBS) and ethidium bromide (100 µg/ml in PBS), cells were examined for morphological features of apoptosis (chromatin condensation, fragmentation and apoptotic body formation) under a fluorescent microscope and photographed. At least 200 cells from randomly selected fields were counted and quantified for each data point. The apoptotic index (percentage of apoptotic cells) was calculated as number of apoptosis cells / total cells counted (× 100).

Cytotoxicity assay—Growth inhibition of MCF-7 cells by eugenol was determined by using the modified MTT assay as described elsewhere. Briefly, cells were harvested and seeded at a density of 4000 cells/well in flat bottom 96 well plates. The cells were incubated for 24 h at 37°C to allow attachment prior to addition of the drugs. Eugenol, at various concentrations, was added to the cells and incubated at 37°C in a fully humidified atmosphere of 5% CO₂ for 24 h. Each concentration was tested in quadruplicate. After incubation, 25 µl of MTT (2.5 mg/ml) was added per well. The plates were incubated for an additional 4 h and then centrifuged for 5 min at 200 × g. Medium and unconverted MTT was removed by inversion, and 75 µl of DMSO were added to each well. Plates were incubated on a rotator for 10 min and then read at 570 nm by an ELISA reader. The cytotoxic effect was determined by calculating the absorbance of test wells as a % of the control wells.

Measurement of lactate dehydrogenase (LDH) release—Briefly, cells (50,000/well) in 24 well culture plates were treated with various concentrations of eugenol for 24 h. The amount of LDH released into the culture medium, which is a measure of plasma membrane integrity, and cell death was determined by monitoring the oxidation of NADH during the reaction of pyruvate to lactate, following the decrease in absorbance at 340 nm. The % of LDH released was defined as the ratio of LDH released plus the activity measured in the lysate.

Quantitation of DNA fragments—Quantitation of apoptosis induced DNA fragmentation in eugenol treated MCF-7 cells was analyzed using a slight modification of the procedure of Wyllie. Cells after treatment with eugenol were lysed in hypotonic buffer (Tris, 0.1 mM; EDTA, 1 mM; and Triton X-100, 0.2%). Intact and fragmented DNA were separated by centrifugation at 13,000 × g for 10 min and measured by the diphenylamine reaction. Per cent fragmentation was calculated as the ratio of DNA in the 13,000 × g supernatants to the total DNA (supernatant plus pellets).

Measurement of intracellular glutathione—Amount of intracellular glutathione was determined by the reaction of glutathione and 5, 5′-dithiobis-2-nitrobenzene (DTNB) as described. Cells after treatment with eugenol were trypsinized, pelleted and lysed. An aliquot of the cellular homogenate was taken to determine the amount of intracellular glutathione and the results were expressed as nmole GSH reduced/mg protein.

Measurement of lipid peroxidation—TBARS produced by lipid peroxidation was measured at 535 nm according to the TBA method. MCF-7 cells
after treatment with eugenol were suspended in PBS and 1 ml of TBA reagent (0.67% thiobarbituric acid, 20% trichloroacetic acid) was added to the cell suspension. The cell suspension containing TBA reagent was heated for 20 min in a boiling water bath, chilled to room temperature and centrifuged at 1500 × g for 10 min. The absorbance was read at 532 nm.

Protein determination—Amount of protein was determined using bovine serum albumin as a standard.

Statistical analysis—The results were statistically evaluated using Student’s t test. Values are expressed as mean ± SD. The mean difference is significance at the 0.05 level.

Results

Inhibition of growth and proliferation of human breast cancer cells by eugenol—MTT assay was used to assess the cell viability of breast cancer cells to eugenol, in vitro. Breast cancer cells were treated with different concentrations of eugenol (0-4 mM) for different incubation periods. Eugenol inhibited the cell growth and decreased the cell survival through induction of cell death (Fig. 1). Effect of eugenol on MCF-7 was dose and time dependent. In the time course study, cells treated with higher concentrations of eugenol (1, 2 and 4 mM) showed a significant decrease in the number of viable cells. The cell viability of the MCF-7 cells treated with the above concentrations of eugenol decreased further with the extension of incubation time.

Effect of eugenol on LDH release in MCF-7 cells was assessed. Per cent of LDH released into the culture medium after 24 h treatment with 0.125, 0.250 and 0.500 mM concentrations of eugenol were 26, 35, and 45%, respectively (Fig. 2). Cells treated with 1.000, 2.000 and 4.000 mM concentrations of eugenol caused a massive LDH release (75, 79 and 85%, respectively).

Breast cancer cells treated with eugenol were observed under a phase contrast microscope to assess the morphological changes associated with apoptosis. Eugenol treated cells showed morphological characteristics of apoptosis, including cell shrinkage, chromatin condensation, rounding and blebbing (Fig. 3).

The dose dependent morphological changes induced by eugenol were confirmed by giemsa staining. Control MCF-7 cells exhibited polygonal morphology (Fig. 4a). Cells treated with lower concentrations of eugenol (0.250 and 0.500 mM) appeared round and exhibited cell shrinkage, chromatin condensation, fragmented nuclei and cell membrane blebbing (Fig. 4b, c). Cells treated with higher concentrations of eugenol (above 0.500 mM) showed flattened appearance, less dense cytoplasm and vacuole formation (Fig. 4d-f). A decrease in the viable cell count was observed with increase in the incubation period.

Induction of apoptosis in human breast cancer cells by eugenol—Exposure of human breast cancer cells to various concentrations of eugenol was found to induce cell death, which was dose and time dependent. To detect eugenol induced apoptosis, cells after treatment with eugenol were stained with acridine orange/ethidium bromide and observed under a fluorescent microscope. Cells exhibiting features of apoptosis were counted and apoptotic index was calculated. The

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![Image](image.png)

Fig. 1—Effect of eugenol on viability of MCF-7 cells as measured by MTT assay: Dose response and time course study. [Values are mean ± SD; significant at ***P < 0.001 Vs control]

Fig. 2—Effect of eugenol on LDH release in MCF-7 cells: Dose dependent study [Values are mean ± SD (n=4); significant at ***P < 0.001 Vs control]
same microscopic fields were examined at various time points. Eugenol treatment significantly increased the amount of cells undergoing apoptosis (Table 1). The percentage of apoptosis was found to increase with increasing concentrations of eugenol.

DNA fragmentation, one of the hallmarks of apoptosis was evaluated in eugenol treated MCF-7 cells in a dose dependent manner. The percentage of DNA fragmentation was significantly increased with increasing eugenol concentration (Fig. 5).

The dose-dependent changes in the levels of intracellular glutathione and lipid peroxidation products induced by eugenol treatment were determined to assess the effect of eugenol on the

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**Fig. 3**—Microphotography of (a)-Control MCF-7 cells; and MCF-7 cells treated with (b)-0.250; (c)-0.500; (d)-1.000; (e)-2.000; and (f)-4.000 mM concentration of eugenol for 24 h showing cell shrinkage and chromatin condensation (magnification 200 x).
redox status of the breast cancer cells. Exposure of breast cancer cells to eugenol depleted the intracellular glutathione concentration which was associated with an increase in the level of lipid peroxidation products (Table 2).

**Discussion**

Apoptosis has been reported as one of the most fundamental biological processes in eukaryotes that plays a key role in both physiological and pathophysiological conditions. It has also been

Fig. 4—Microphotography of MCF-7 cells stained with giemsa. (a)-Control MCF-7 cells; and MCF-7 cells treated with (b)-0.250; (c)-0.500; (d)-1.000; (f)-2.000; and (e)-4.000 mM concentration of eugenol for 24 h, showing cell shrinkage, vacuole formation, membrane blebbing, chromatin condensation and apoptotic body formation (magnification 200×).
Table 1—Percentage of apoptosis induced by eugenol treatment for 12 and 24 h in MCF-7 cells: A dose and time response study.  
[Values are mean± SD (n=4)]

<table>
<thead>
<tr>
<th>Eugenol concentration (µM)</th>
<th>Treatment time</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.3 ± 1.0</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>0.125</td>
<td></td>
<td>40.3 ± 4.3***</td>
<td>50.5 ± 3.3***</td>
</tr>
<tr>
<td>0.250</td>
<td></td>
<td>45.6 ± 5.4***</td>
<td>57.4 ± 4.6***</td>
</tr>
<tr>
<td>0.500</td>
<td></td>
<td>53.3 ± 4.2***</td>
<td>64.7 ± 5.5***</td>
</tr>
<tr>
<td>1.000</td>
<td></td>
<td>60.5 ± 3.7***</td>
<td>72.6 ± 5.6***</td>
</tr>
<tr>
<td>2.000</td>
<td></td>
<td>67.6 ± 4.7***</td>
<td>85.4 ± 6.4***</td>
</tr>
<tr>
<td>4.000</td>
<td></td>
<td>75.4 ± 4.3***</td>
<td>93.5 ± 6.3***</td>
</tr>
</tbody>
</table>

***P < 0.001 vs control

Table 2—Effect of eugenol on the level of intracellular GSH concentration and TBARS formation in MCF-7 cells.  
A dose response study  
[Values are mean± SD (n=4)]

<table>
<thead>
<tr>
<th>Eugenol concentration (µM)</th>
<th>GSH (nmole glutathione reduced/mg protein)</th>
<th>TBARS (nmol malondialdehyde/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.2 ± 1.53</td>
<td>0.194 ± 0.010</td>
</tr>
<tr>
<td>0.125</td>
<td>10.3 ± 1.10***</td>
<td>0.283 ± 0.011 ***</td>
</tr>
<tr>
<td>0.250</td>
<td>9.2 ± 1.83***</td>
<td>0.332 ± 0.020***</td>
</tr>
<tr>
<td>0.500</td>
<td>8.3 ± 1.02***</td>
<td>0.372 ± 0.021 ***</td>
</tr>
<tr>
<td>1.000</td>
<td>7.9 ± 0.93***</td>
<td>0.404 ± 0.043***</td>
</tr>
<tr>
<td>2.000</td>
<td>6.2 ± 0.89***</td>
<td>0.492 ± 0.072***</td>
</tr>
<tr>
<td>4.000</td>
<td>5.0 ± 1.01***</td>
<td>0.523 ± 0.051 ***</td>
</tr>
</tbody>
</table>

***P < 0.001 vs control

demonstrated that the efficacy of anticancer treatment may be associated with the ability of cancer cells to respond by apoptosis\(^{22}\). The possibility of modulating cancer cell responsiveness by apoptosis, therefore, opens new anticancer strategies.

Growth inhibition effect of eugenol has been observed in human hepatoma cell line Hep G2\(^{23}\) and human promyelocytic leukemia cell HL-60. In leukemia cells, eugenol treatment induced cytotoxicity and internucleosomal DNA fragmentation\(^{13}\). Eugenol transduces the apoptotic signal via reactive oxygen species generation, thereby inducing mitochondrial permeability transition, reducing anti-apoptotic protein bcl-2 level, inducing cytochrome C release to the cytosol and subsequent apoptotic cell death\(^{24}\). Eugenol inhibited the proliferation of HT-29 cells and the mRNA expression of COX-2\(^{25}\).

Growth inhibition of cancer cells correlated significantly with the degree of treatment induced cell death and induction of apoptosis. Eugenol blocks cells in the replication phase to repair DNA damage and the cells either re-enter the cell cycle or activate apoptosis in case of massive DNA damage. Melanoma cells treated with eugenol remain blocked in the S phase and undergo apoptosis\(^{26}\).

Cytotoxic effect of eugenol is mainly concerned with its possible metabolic activation via a peroxidative reaction to form an intermediate namely quinone methide, which is an electrophile\(^{27,28}\). The highly reactive intermediate, quinone methide, binds to cellular proteins and intracellular glutathione\(^{23,29}\).

Involvement of intracellular redox status as a key mediator of apoptosis has been well established in many cancer cell lines. Reduced glutathione (GSH), the most abundant cellular thiol and the major determinant of cellular redox equilibrium has been shown to be an important factor in apoptosis. Glutathione contributes to an extraordinary range of metabolic processes including the maintenance of the intracellular redox status and plays a role in detoxification of intracellular H\(_2\)O\(_2\), lipid peroxides and also in the metabolism of exogenously derived compounds, including therapeutic drugs, mutagens and carcinogens\(^{30}\). Depletion of intracellular glutathione renders the cancer cells more sensitive to apoptotic agents.

As intracellular glutathione plays an important role in maintaining the cellular redox equilibrium that is pivotal for cell growth and function, it is speculated that the reaction of eugenol with glutathione may
change the intracellular redox status and thus trigger apoptosis and inhibit cell proliferation.

In MCF-7 cells, the intracellular glutathione levels were depleted following exposure to eugenol and the effect was dose-dependent. Cell proliferation is recognized to be associated with diminished lipid peroxidation\(^\text{31}\). The extent of lipid peroxidation was low in proliferating cancer cells when compared to control cells. This may be related to increased GSH redox cycle. GSH in conjunction with GPx, GST and GR plays a regulatory role in cell proliferation\(^\text{32}\). This depleted level of glutathione in eugenol treated MCF-7 cells may lead to the accumulation of intracellular \(\text{H}_2\text{O}_2\) and lipid peroxidation.

Eugenol treated MCF-7 cells also showed a dose-dependent increase in lipid peroxidation products at the concentrations used in the present study. The products of lipid peroxidation are known to be toxic and may promote internucleosomal DNA fragmentation\(^\text{33}\). Increased membrane lipid peroxidation has also been implicated as being associated with apoptosis\(^\text{34}\). Thus, the observed increase in the level of lipid peroxidation could be due to the depletion of intracellular protein thiol and glutathione by eugenol metabolite, quinone methide, as conjugates.

In conclusion, the present study indicated that eugenol treatment inhibited the growth and proliferation of human breast cancer cells and induced apoptosis, which was dose and time dependent. Eugenol treatment altered the intra cellular redox status of the breast cancer cells by depleting the level of glutathione through conjugation and increasing the level of lipid peroxidation products. These results supported a role for eugenol as a chemopreventive agent.

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