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Study on effect of eugenol on anti-metastatic activity and expression of MMPS in TNBC MDA MB: 231 cell line

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Abstract
Breast cancer is the most common cancer affecting women in the world today. Invasiveness or migration through the extracellular matrix (ECM) is a fundamental property of malignant cancer cells. It has been shown that elevated expression of different MMPs is associated with different metastatic stages in the invasion and progression of tumors. MMPs are differentially expressed and their expressions are often associated with a poor prognosis for patients. Most of the chemotherapeutic agents that are currently used to treat this complex disease are highly toxic with long-term side effects. Therefore, novel generation of anti-cancer drugs with higher efficiency and specificity are urgently needed. Eugenol (4-allyl-2-methoxyphenol) is an aromatic phenylpropanoid phenol contained in clove (*Syzygium aromaticum*, *Myrtaceae*), which is traditional medicine as an antiseptic, analgesic or antibacterial, antiviral, antioxidant, antiinflammatory and antiporliferative. In present study, triple-negative breast cancer cell (TNBC) line, MDA-MB-231 was used to investigate the effect of eugenol on mRNA expression of metastatic MMPs. Eugenol showed dose-dependent cytotoxic activity on MDA-MB-231 cell lines with 2.89 mM IC_{50} value after 48 hrs incubation. On the bais of our results eugenol was found nontoxic to RBCs MDA-MB-231 cells cultured and treated with different concentrations of eugenol (1, 5 and 15 uM). Total RNA was extracted from eugenol treated cells and untreated cells to study the effect on mRNA expression levels of MMP 1, 3, 7, 9 and 11 by RT-PCR. The results indicate that mRNA expressions of MMP-1, -3, -7, -9 and -11 were downregulated in eugenol treated cells as compared to untreated cells. Scratch-wound healing assay showed significant inhibition of cell migration at 25 uM and suppressing metastasis of MDA MB-231 cells. Therefore, we suggest that eugenol may be inhibiting of metastasis of human breast cancer by downregulating MMPs.

Keywords: Eugenol, matrix metalloproteinases MMPs, MDA MB-231 cells, breast cancer

Introduction
Breast cancer is the most common leading cause of death among women worldwide due to distant metastasis [1]. Cancer metastasis consists of a complex cascade of events, which ultimately allow for tumor cell escape and seeding of ectopic environments [2]. For breast cancer cells to manifest their malignant potential, they must develop the ability to break through and dissolve extracellular matrix (ECM), particularly the delimiting basement membrane (BM). The degradation of the pericellular BM and ECM is catalyzed by the concerted action of several classes of ECM-degrading enzymes. One important class of ECM-degrading enzymes is the matrix metalloproteinases (MMPs) [3].

Matrix metalloproteinases (MMPs) are a major group of enzymes that regulate the cell-matrix composition [4]. These MMPs are synthesized in most cells, and they are immediately secreted into the extracellular matrix (ECM) [5]. MMPs play important roles as key enzymes in many biological processes such as arthritis, cardiovascular disease, inflammation, angiogenesis, and cancer [6]. MMP is a family of 23 members structurally and functionally related endopeptidases [7]. In the normal mammary gland, constitutive expression of MMPs is low except during times of development, pregnancy, and involution however, in pathologic states such as breast cancer, increased levels of MMPs have been reported in breast tumor cells as well as in the surrounding noncancerous breast tissue. The stage of tumor progression is positively correlated with the expression of MMP family members (MMP-1, 2, 3, 7, 9, 11, and 14) [8]. Several studies have described the presence and role of MMPs in breast cancer. MMP-1, 2, 3, 9 and 14 are implicated as key factors in tumor invasion, metastasis and angiogenesis [9]. MMP-2, -7, -9, -10, -11, -13, -14 and -15 have been involved in the progression of breast cancer [10]. MMP- 1, -2, -7 and -9 have been linked to cancer cell proliferation, whereas MMP-1, -2, -3, -7 and -9 to -18 are involved in breast tumor metastasis [11].

Further, MMP-1 to -4, -9 to -19 have been found to regulate the invasion of breast cancer [12].

Chemoprevention by phytochemicals has focused a great attention and considered to be a feasible, readily applicable, acceptable, and accessible approach of cancer control, regression and management. Eugenol is listed by the Food and Drug Administration as "Generally Regarded as Safe" when consumed orally in the unburned form [13]. Eugenol (4-allyl-2-methoxyphenol) (Figure 1) is an aromatic phenylpropanoid phenol contained in clove (Syzygium aromaticum, Myrtaceae), which is well-known for its culinary uses Eugenol is a natural phenolic compound available in honey and the essential oils of cloves, cinnamon, and other aromatic spices. It is added as a therapeutic ingredient in

Various medications to treat digestive disorders [14]. And as an antiseptic, analgesic [15]. Anti-inflammatory, antimicrobial [16] and antioxidant agent [17]. Eugenol has several anticancer properties in colon, liver, prostate, and breast cancer [18]. Eugenol prevents cancer progression by modulating the expression of several genes involved in cell growth, angiogenesis, and apoptosis [13]. Moreover, in a rat model of gastric carcinogenesis, eugenol was observed to induce apoptosis and inhibit invasion and angiogenesis [19]. Studies on MMPs in cancer provide the basis for developing anti-metastatic cancer drugs. In this study we aimed to investigate ant metastatic properties of eugenol and its possible effect on expression of different MMPs in MDA MB-231 breast cancer cell line model.

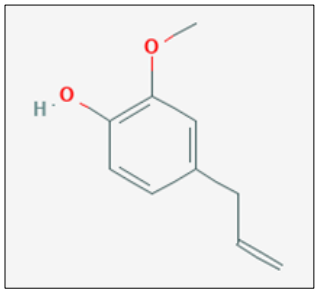

Fig 1: Structure of eugenol

Materials and Methods

Reagents: Following reagents were used for cell culture-Leibovitz-15 media (L-15,Cell clone), Fetal bovine serum (FBS, Cell clone), Penicillin-Streptomycin-Amphotericin B (Cell clone),,Dimethyl Sulphoxides (DMSO, SRL), MTT (AMERCO) was used for cytotoxicity assay, try pan blue solution (SRL), Reverted Reverse Transcriptase (RT) from (Thermo scientific, USA), Dream Taq polymerase from (Thermo Scientific, USA), Revertaid cDNA Kit (Thermo Scientific, USA)

Cell culture and eugenol treatment

Human breast cancer cells MDA-MB-231 were procured from NCCS, Pune, maintained in Leibovitz-15 (L-15,Cell clone) medium containing with 10% fetal calf serum and antibiotics (100 U/ml of penicillin and 100 mg/ml of streptomycin), at 37 °C in a humidified atmosphere. Eugenol was solubilized in ethanol to prepare a stock solution of 100Mm and further dissolved in L-15 medium prepaid working solution.

Cell viability assay using MTT

Viability of triple negative- (MDA-MB-231) breast cancer cells in response to the different concentrations of eugenol was determined by measuring the capacity of cellular oxidoreductase enzymes present in viable cells to convert the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to its insoluble formazan form. In brief, cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 h. The medium was then replaced with fresh medium containing different concentrations of eugenol; untreated cells act as positive control while wells with no cells as negative control. At the end of 48h, 10 µl of MTT reagent was added to each well, and the plates were incubated for 4 h at 37 °C for formazone crystal formation. Next, 100 µl of DMSO was added to each well, and after 1h the amount of formazan was quantified by measuring absorbance at 570 nm using an ELISA reader. Percentage of cell proliferation was calculated relative to control wells designated as 100% viable cells, by using following formula:

% cell proliferation = (OD treated- OD negative control) / (OD positive control – OD negative control) × 100. IC_{50} value was calculated by using Graph pad prism 6.0 software.

Measurement of minimal hemolytic concentration (MHC) of eugenol

The MHC was determined following the protocol of (Singh *et al.,* 2012) with modification [20]. Canine venous blood was collected in heparinized vial from Veterinary Poly Clinic, IVRI and was centrifuged at 400g for 10mins with his opaque to remove serum and buffy coat. Collected RBCs were washed once with 1X PBS and suspended in 1X PBS. The process was repeated twice followed by seeding onto 96well cell culture plate (100µl/well). Then RBCs were treated with the above mentioned drugs at concentrations of 10µM to 2000µM of eugenol with PBS and 0.1%Triton-X-100 as control. Then the plates were incubated for 1h at 37 ºC and centrifuged at 200g for 5min. the supernatant was transferred to another plate and reading was taken at 550nm. The percentage of hemolysis was calculated by plotting concentration of phytocompounds vs absorbance.

Scratch/Wound-healing assay

To determine cell motility determination, MDA MB-231 cells (1×105cells/ ml) were seeded in 6-well tissue culture plate and grown to 100% confluence. After aspirating the medium, the center of the cell monolayers was scraped with a sterile micropipette tip to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, and the cells were exposed to various concentrations of eugenol (0, 1, 10, 25 µM). The wound closure was monitored and photographed at 0, 24 and 48 h with a Nikon eclipse TiS inverted microscope and camera. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation. Wound area was measured by using Images software in 3 random fields from each triplicate treatment.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Cells were treated with increasing doses of eugenol (Sigma, USA) 1µM, 10µM, 25µM and were incubated for 48h. Cells without any treatment were taken as control. Following exposure to the drugs the cells were continuously observed under microscope for morphological changes and subsequently the total RNA was isolated for subsequent PCR

reactions. Total RNA was extracted from MDA MB 231 cells using the Trizol reagent as per manufacture guidance. Total RNA (2 µg) was transcribed to 20 µl cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific) as per the manufacturer's protocol. CDNA synthesized after the treatment with drugs, was used for amplification of the MMP1, MMP3, MMP 7, MMP 9, MMP11 and GAPDH using specific set of primers in Step One™ System. PCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific, USA) under the following conditions: 40 cycles of 95 °C for 20 seconds, 56 °C for 15 seconds, 72 °C for 30 seconds preceded by one step of 10 min at 95 °C to yield the amplification of an endogenous control gene (GAPDH) and the specific target genes of interest. Following amplification, melting curve analysis was performed to verify the correct product according to its specific melting temperature (Tm). Experiments were performed in triplicate. The following appropriate primers were used for polymerase chain reaction (PCR) amplifications.

Table 1: Oligonucleotide sequences of primers used in this study

Gene	Forward Primer sequence	Reverse Primer sequence
MMP1	5'-AGGTCTCTGAGGGTCAAGCA-3'	5'- CTGGTTGAAAAGCATGAGCA-3'
MMP3	5'- GAAGAGAAATTCCATGGAGCCAGG-3'	5'-AGAAATAAAAGAACCCAAATTCTTCAAAAACA-3'
MMP7	5'- AGATGTGCAGTGCCAGATGTT -3'	5'- TTGCCCCACATGTTTAAAGCC -3'
MMP9	5'- GATGCGTGGAGAGTCGAAAT -3'	5'- CACCAAACTGGATGACGATG -3'
MMP11	5'- AGAGGTTCGTGCTTTCTGG -3'	5'- TCACATCGCTCCATACCTTTAG -3'
GAPDH	5'-TTGAGGTCAATGAAGGGGTC -3'	5'- GAAGGTGAAGGTCGGAGTCA -3'

Comparison of the relative change in mRNA level of the target gene in the treated cells with the untreated cells were determined as fold change after normalization with GAPDH internal control. Method described by Pfaffl (2001) was used for calculation of relative fold changes in gene expression[21].

Statistical Analysis

All experiments were performed in triplicate and analyzed by one way ANOVA using SPSS software for significant differences. P values of <0.05 were considered statistically significant. Where appropriate, the data are presented as the mean±SD.

Results

Cell cytotoxicity assay: The optimal concentration of eugenol to inhibit breast cancer cell, was determined. Eugenol significantly inhibited the growth of cell lines in a dose-dependent manner. The IC_{50} values were 2.89 ± 0.23mM. Figure 2 showed that eugenol at concentrations up to 12.5 mM did not significantly affect cell viability. However, concentrations of 0.78, 1.56, and 3.12 mM significantly decreased the viability of MDA-MB-231 cells by 78, 59, and 39%, respectively, at 48-h incubation period, whereas, On the other hand, high eugenol concentrations (12.5 and 26 mM) markedly inhibit cell proliferation by <5% compared to untreated cells.

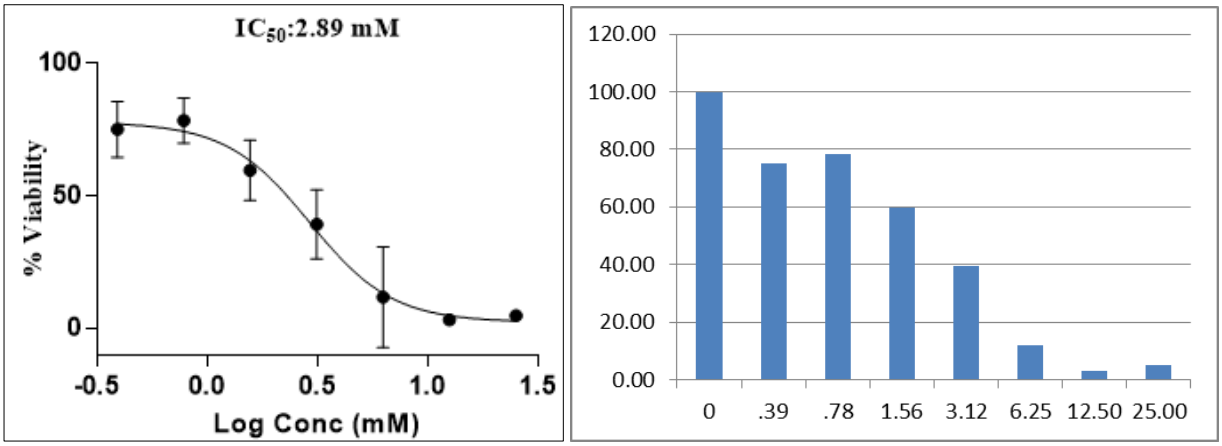

Fig 2: Survival of human breast cancer MDA MB-231 cells following treatment with eugenol. Cells were seeded in 96-well tissue culture plates and 24 h later eugenol was added. Cells were incubated for 48 h with different concentrations of apigenin (0 to 10 mM) and their viability was determined with a modified colorimetric MTT assay. The results are shown as mean values ± SD. *statistically significant compared with the corresponding control (P<0.05) post hoc test. *p<0.05

Measurement of hemolytic concentration (MHC) of eugenol using dog RBC

The minimal hemolytic concentration (MHC) was determined by treating the RBCs with both the drugs at concentrations ranging from 10µM to 2000µM for eugenol in PBS while 0.1% triton-100 was used as control. Percentage of hemolysis in canine RBCs with different concentrations of both drugs with respect to the controls shown in Table 2. 5% hemolysis of canine RBCs was observed around 1000µM for eugenol (Fig 3).

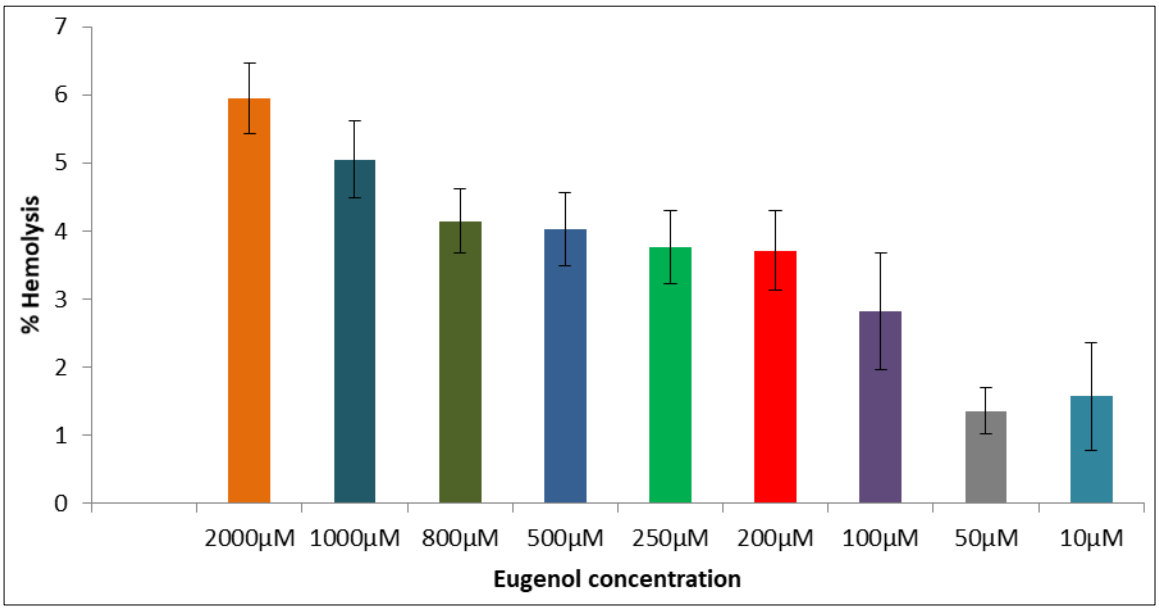

Fig 3: The percent Hemolysis at different concentration of eugenol (0.1% tritonx-100 was used as positive control)

Table 2: Minimal hemolytic concentration of Eugenol using canine RBCs

Eugenol Concentration µM	% Haemolysis
10	1.49
50	1.29
100	2.32
200	3.38
250	3.52
500	3.77
800	4.11
1000	5.29
2000	6.24
Triton X-100(0.1%)	100

Wound scratch assay: The scratch wound healing assay was conducted to determined effect of eugenol on migration MDA MB-231 cells. As exhibited in figure 4, cell migration was significantly decreased at concentrations of 25µM.

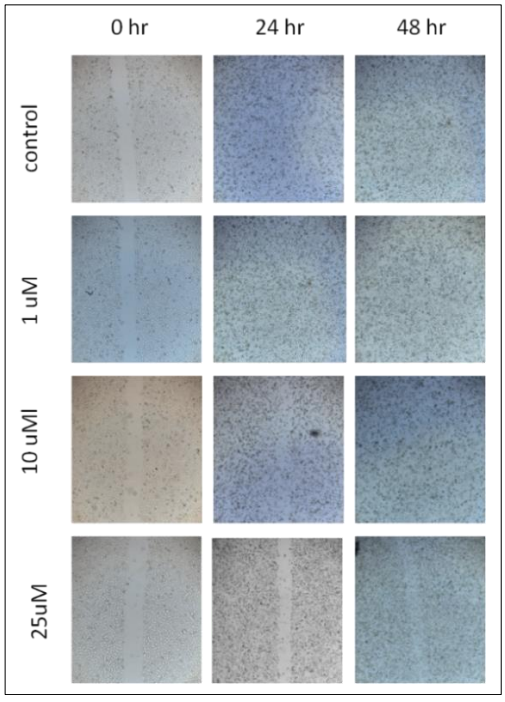

Fig 4: Effect of eugenol on cell migration of MDA-MB-231: Eugenol delayed the wound- healing time in human MDA-MB-231 cells. The inhibitory effects of eugenol on the cells were determined using a wound healing assay. The distance of the scratch was measured in the control and eugenol groups using Image J software.

The expression of MMP 1, 3, 7, 9 and 11 gene in eugenol treated and untreated Breast cancer MDA MB-231 cells

In cancer, invasion, and metastasis, the primary cause of death, is mediated by a group of genes (e.g., MMPs) via degradation of the extracellular matrix with complicated steps Therefore, suppression of MMPs (expression or secretion) may be an effective strategy in preventing cell migration and Invasion. Earlier trials with MMPs inhibitors in breast cancer revealed serious dose-limiting toxicity or failure to reach therapeutic plasma levels, which may be due in part, to their inability to target specific MMPs. To verify the effect of eugenol on the MMP-1, 3, 7,9, and 11 mRNA expressions, MDA MB-231 cells with the 1 µM, 10 µM and 25µM concentrations of eugenol for 48h. (P<0.05, Figure 5).

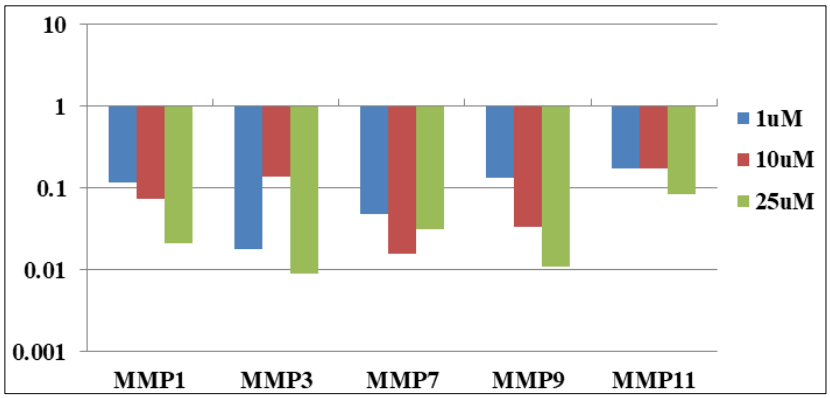

Fig. 5: The effects of eugenol on MMPs mRNA expression in MDA MB-231 cells: The cells were treated with eugenol at the 1, 10 and 25 uM concentrations. The levels of MMP-1, 3, 7, 9 and 11 mRNA were analyzed by real-time PCR. The results are representative of three independent experiments. The values shown are the means SEM. *P<0.05 versus control.

After 48-hr incubation, there was significant difference observed in the MMP1, MMP9 and MMP11 gene expression at the concentration of 1 µM, 10 µM and 25µM compared to the untreated cells. MMP1 was significantly decreased by 0.117, 0.075 and 0.021 folds respectively as compared to untreated cells. There was no significant decrease observed in the MMP3 gene expression with 0.018, 0.137 and 0.009 fold change at 1 µM, 10 µM and 25 µM concentration of eugenol compared to the untreated cells. The expression of MMP7 decreased with increased in concentration of eugenol. The MMP7 gene expression was decreased in response to eugenol treatment with 1 µM, 10 µM and 25 µM by 0.048, 0.031 and 0.016 folds respectively. MMP9 gene expression was decreased significantly in response to eugenol treatment with 1 µM, 10 µM and 25 µM by 0.136, 0.034 and 0.001 folds respectively. There was significant decrease observed in the MMP11 gene expression with 0.176, 0.173 and 0.084 fold change at 1 µM, 10 µM amd 25 µM concentration of eugenol compared to the untreated cells.

Discussion

Eugenol had a concentration dependent effect on MDA-MB-231 cell proliferation, suggesting the potential anti-proliferative and ant metastatic effects of eugenol in MDA-MB-231 cells. Eugenol was found to possess anticancer properties by inhibiting the proliferation of MDA-MB231 cells with an IC_{50} of 2.89 mM. Some studies reported that eugenol has no cytotoxic activity or cytotoxicity only when present in the mM range [22]. Previous studies showed the IC_{50} value of eugenol in MDA MB-231 cells were 1.7 µM, 15.1 µM and ~1600 µM [23, 24, 25]. In studies with HSG (human submandibular gland adenocarcinoma) and HSC-2 (human oral squamous cell carcinoma) cells, eugenol caused cytotoxicity when in the mM range. Yoo and coworkers studied the anticancer mechanism of eugenol against human leukemia cells (HL-60). They initially estimated the IC_{50} values of eugenol against various cancer cell lines like U-937, HL-60, HepG2, 3LL Lewis, SNU-C5 which were found to be 39.4, and 23.7, 118.6, 89.6 and 129.4 µM, respectively. So with comparision to the other cell lines, inhibition of MDA MB 231 cells need high concentration of eugenol to cause 50% inhibition. The observed variation in the cytotoxic effect of eugenol across the literature could be due to the variation in used concentrations, purity of eugenol, and the source or types of cell lines. However, its cytotoxic mechanism remains unknown. Junior *et al.* demonstrated that mechanisms of action eugenol involves cytoplasm membranes disruption, decreasing in mitochondrial membrane potential (MMP) accompanied by a burst in ROS production and cell-cycle deregulation even in chemo-resistant cells line such as MDA-MB-231[24]. A recent study reported that eugenol promotes cytotoxicity against breast cancer cells (TNBC) and animal model and synergistic chemotherapeutic effects with cisplatin by the inhibition of the NF-κB signaling pathway, which resulted in the inhibition of the p50 and p65 subunits phosphorylation, and its consequence migration to the cellular nucleus, reducing IL- 6 and IL-8 levels [26]. Eugenol in µM concentrations causes cytotoxicity to MCF-7, T47-D (human breast carcinoma) and MDA-MB-231 (human breast adenocarcinoma) cells through down-regulation of E2F1 and its downstream anti-apoptosis target, surviving independently of the status of p53 and ER [23].

Eugenol showed greater than 5% hemolysis beyond 1000µM so it can be concluded than it is not hemolytic at its effective concentrations used in present study. So any drug to be exploited for its pharmacological action should not cause hemolysis beyond 5% at its pharmacologically effective concentration, so eugenol and piperine were assessed for their hemolytic ability.

Breast cancer cells treated with eugenol were observed under bright field microscope to assess the morphological changes associated with apoptosis, including granulated cells, cell shrinkage, chromatin condensation, rounding and blebbing (Fig 4). Previous studies reported eugenol induces alteration on cellular architecture probably by perturb cytoskeleton dynamics leading to a cytoplasmic retraction, shrinkage, pyknosis and cell detachment culminating in anoikis, especially in MCF-7, SiHA and SK-MEL-28 cells. Alterations in cytoplasm membrane were identified on A2058 and MDA-MB-231; however these cells maintained their

adherence. Besides, blebs formation was noticed in both MDA-MB-231 and MCF-7 cells [24].

MMPs function in the remodeling of the extracellular matrix that is integral for many normal and pathological processes. MMPs are up regulated and often associated with a poor prognosis for patients [27, 28, 29]. The stage of tumor progression is positively correlated with the expression of MMP family members (MMP-1, 2, 3, 7, 9, 11, and 14) [8]. The overproduction of MMPs in cancer has long been correlated with tumour progression and metastasis. Eugenol treatment with increasing concentration decreased in MMP1, MMP3, MMP7, MMP9 and MMP11 genes expression levels. Multiple signaling pathways regulate the expression of MMPs [30, 31]. Thus, targeting genes in these signaling pathways may suppress or decrease metastasis and consequently reduce cancer mortality. MMP9 was reduced significantly in response to eugenol treatment after 48 hr incubations [32]. A study found that 100 μM eugenol has an antioxidant effect on human fibro sarcoma cells, and can inhibit MMP-9 activity and expression [35]. A correlation between high expression levels of MMP-2 and MMP-9 and lymph node metastasis and tumor staging in breast cancer patients was reported [33].

Previous studies observed that eugenol inhibited MMP activities in PMA-stimulated HT1080 cells and exerts inhibitory effects on MMP via inactivation of ERK. Therefore, these results suggest that eugenol could be available as an excellent agent for prevention of metastasis related to oxidative stress [30, 34]. Al-Shairf *et al.*, reported that eugenol could suppress breast cancer cell migration by decreasing the MMP-9 and Paxilin gene expression Moreover, eugenol up-regulated the versatile cyclin dependent kinase inhibitor p21 WAF1 protein, and inhibited the proliferation of breast cancer cells in a p53-independent manner [35]. Eugenol in the mM range also inhibits the growth of human breast carcinoma MCF-7 cells accompanied by cell shrinkage and an increase in the percentage of apoptotic cells and DNA fragments. Eugenol impaired cell migration, at the 25uM dosage, Similar findings were reported by Fangjun and Zhijia in lung cancer cells [36]. Abdullah *et al.* in MDA-MB-231 and SK-BR-3 breast cancer cells [37]. Baharara *et al.* in MCF-7 breast cancer cells [38]. The present study demonstrates that eugenol exhibits an antiproliferative, antimetastatic, anti-breast cancer effect via targeting the matrix metalloproteinase and may be considered as a potential therapeutic agent for triple negative breast cancer.

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