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Protective effect of eugenol from *Mesua ferrea* on the oxidative damages caused by 5-fluorouracil in PBMC cells

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5-Fluorouracil (5-FU), a pyrimidine analogue with fluorine at C5 position, is one of the chemotherapy drugs used to treat various cancers. Though 5-FU is a very promising anticancer drug, it can cause side effects such as coronary thrombosis, intestinal mucositis, cognitive impairment, and anaemia through the generation of free radicals, damages the normal cells and also leads to apoptosis. To overcome such toxic effects of 5-FU, in the present work we have explored the cytoprotective potential of eugenol, the antioxidant active compound available in ethanolic extract of an Indian herbal drug *Mesua ferrea*. Aim of the present study was to evaluate the cytoprotective effect of eugenol against 5-FU toxicity in peripheral blood mononuclear cells (PBMC). Based on MTT assay, it is noticed that eugenol significantly prevents the toxicity of 5-FU (73%) in PBMC cells. Eugenol has lowered the generation of intra-cellular ROS (20035.5 AU) when compared to 5-FU treated cells (58918.5 AU). Eugenol exhibited remarkable inhibition of lipid peroxidation (43%) and cell membrane damage (LDH release: 488.02 IU/L). Thus, eugenol prevents apoptosis-induced cell death when compared with 5-FU in PBMC cells and hence it can be used to overcome the cytotoxicity caused by 5-FU and it warrants further study.

Keywords: 5-Fluorouracil, Cytoprotective effect, Eugenol, *Mesua ferrea*, ROS toxicity.

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Introduction

Cancer is a deadly disease and can be treated by radiotherapy and chemotherapy. The use of chemotherapeutic drugs is an established approach for the treatment of various types of cancer. There are several anticancer drugs like 5-fluorouracil (5-FU), cisplatin, cyclophosphamide etc., used for chemotherapy[1]. The cancer drugs are categorized as alkylating agents, anti-metabolites, anticancer antibiotics, topoisomerase inhibitors, and mitotic inhibitors[2]. One of the potent anti-metabolite anticancer drugs is 5-FU, a pyrimidine analogue with fluorine at C5 position and it acts throughout the S phase of cell cycle[3]. It is used for treating cancer types like cervical, gastrointestinal, colon, lung, and skin[4]. It is administered through oral, intravenous and topical routes. When 5-FU reaches the liver, about 80 to 85% is catabolized to inactive metabolites like 5,6-dihydro-5-fluorouracil (DHFU) and α-fluoro-β-alanine (FBAL) by the enzyme dihydropyrimidine dehydrogenase (DPD). These inactive metabolites are finally excreted away. Remaining drug gets converted into three active metabolites i) fluorouridine triphosphate (FUTP) which degrades RNA, ii) fluorodeoxyuridine monophosphate (FdUMP) which inhibits the activity of thymidylate synthase, and iii) fluorodeoxyuridine triphosphate (FdUTP) which damages DNA[5]. Thus, all these active metabolites interfere with both the DNA and RNA synthesis and cause death of cancer cells[6].

Though 5-FU is a very effective anticancer drug, there are few challenges such as short biological half life (5 to 20 min)[7], low selectivity, and having adverse side effects such as intestinal mucositis, inflammation, and ulceration of mucous lining in the gastro-intestinal tract[8,9]. It disturbs the gut microbiota and causes fatigue, loss of appetite and diarrhea. It causes cognitive impairment[10] and damages myelin integrity corresponding to hippocampal neuro-degenerative defects and liver damages[11]. 5-FU can cause cardiac toxicities such as coronary vasospasm, coronary thrombosis and cardiac death[12]. It can also lead to the generation of superoxide in mitochondria[13] and ultimately lead to apoptosis[14].

To overcome the cytotoxicity of 5-FU on normal cells through free radicals-mediated oxidative damage, antioxidant phytochemicals could be used. It is

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reported that quercetin and rutin are able to protect normal hepatic cells in rats from 5-FU cytotoxicity[15]. It is also reported that ascorbic acid attenuates the 5-FU induced gastrointestinal toxicity in rats[16]. A mucoadhesive formulation containing curcuminoids were used against the 5-FU induced cellular toxicity in oral mucositis cells[17]. In addition, *Kochia indica* extract, *Muricid* extract, *Artichoke* extract, and Diadzein were also reported against 5-FU toxicity[18-21]. In this context, we have identified antioxidant phytochemical eugenol as a major constituent of the Indian Siddha herbal drug *Mesua ferrea* (Fig. 1). It belongs to the family Calophyllaceae and widely distributed in tropical countries like India. The dried flower bud of *M. ferrea* has been used in Indian Siddha system of medicine to treat various ailments. A recent study in our lab evidenced the antioxidant potential of ethanolic extract of *M. ferrea* containing eugenol as a major active constituent[22]. Eugenol is a major phenolic compound available in various plants like *Eugenia aromatica*, *Cinnamomum* and *Ocimum* spp. with strong antioxidant properties[23-26]. It is already reported that cisplatin ototoxicity has been prevented by eugenol[27]. But till date, there is no study indicating ameliorating effect of eugenol against 5-FU toxicity. Hence, the present study was conducted with the aim to study the effect of eugenol, a major antioxidant constituent of *M. ferrea*, on the oxidative damages caused by 5-Fluorouracil in peripheral blood mononuclear cells (PBMC).

Materials and Methods

Materials

For the experimental purpose, we purchased 5-FU (Sigma), eugenol (Merck), Histopaque (Sigma), PBS (Phosphate buffered saline, Merck), RPMI (Roswell Park Memorial Institute-1640 media (Himedia), MTT (Himedia), acidic isopropanol (Merck), LDH kit (Biosystems, Cat. No.11580), TBA (Sigma aldrich), TCA (Merck), DNPH (Sigma aldrich), AO/EB dual stain (Cat. No. E607308) and $DCFH_2$-DA (Sigma). Flower buds of *Mesua ferrea* were purchased from local herbal market in April-2018 and authenticated at the Center for Advanced Studies in Indian System of Medicine (CARISM), SASTRA Deemed University using pharmacognostic tools[22]. Ethanol extract of *Mesua ferrea* dried flower bud was prepared, dried and re-dissolved in water at 0.1 mg/mL concentration for further experiments.

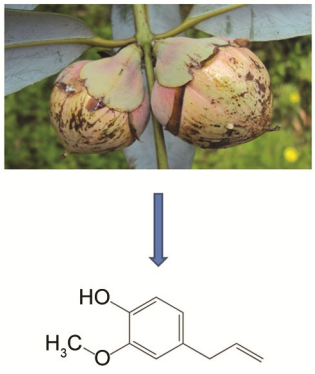

Fig. 1 — Flower bud of *Mesua ferrea* (An Indian herbal drug used in Siddha system of medicine) and its major antioxidant constituent eugenol[22].

Isolation of PBMC cells

The isolation of PBMC was performed based on Prasad *et al.*[28] protocol. Blood (5 mL) was collected from healthy volunteers (Ref. No. SASTRA/IBSC/8/2018 dt. 27-04-2018) in a 15 mL falcon tube containing ethylenediamine tetraacetic acid (EDTA). Histopaque (5 mL) was added with equal volume of blood along the walls and centrifuged for 40 min at 1800 rpm. Based on different densities, the blood components were separated into four layers: the uppermost layer containing plasma, a buffy layer containing the PBMC cells, histopaque layer and final down layer containing red blood cells. The upper layer was removed and buffy coat layer was collected in another tube, washed with PBS buffer and centrifuged at 2500 rpm for 10 min. The pellet was suspended in RPMI-1640 medium supplemented with 10% FBS solution and used for all the experiments.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay was used to check the viability of PBMC cells upon treatment with 5-FU and eugenol[29]. To the PBMC cells (150 µL) in a 96 well plate, various concentrations of 50 µL of 5-FU (625, 312.5, 156.25, 78.13, 39.05, 19.59, 9.86, 4.87, 2.44 and 1.22 µg/mL) was added to check the cytotoxicity. Cytoprotective effect of eugenol (25 µL) of different concentrations (1250, 625, 312.5, 156.25, 78.13, 39.06, 19.53, 9.77, 4.88, 2.44 µg/mL) was studied against 5-FU (625 µg/mL) toxicity. Finally, cytoprotective effect of *M. ferrea* extract (25 µL) of different concentrations (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2 µg/mL) was studied against 5-FU (625 µg/mL, 25 µL). After the addition of 5-FU/extract/eugenol to the cells, it was incubated for 2 h at 37 °C and then 20 µL MTT (5 mg/mL) was added in each well and further incubated for 3 h at 37 °C. Finally, 20 µL of acidic isopropanol (70%) was added and incubated for 20 min

and then the absorbance was measured at 600 nm using spectrophotometer. The cytotoxicity was calculated using the formula (% Cytotoxicity = ([Control OD - Test OD] / Control OD) * 100) and graph was plotted with cytotoxicity Vs. concentrations.

LDH assay

The cell membrane damage induced by 5-FU was detected by lactose dehydrogenase (LDH) release into the medium from the damaged cells[30]. PBMC cells in RPMI medium were taken in an eppendorf tube and exposed to 500 µL of 5-FU (625 µg/mL) and eugenol (1250 µg/mL) or *M. ferrea* extract (100 µg/mL) and then centrifuged at 3000 rpm for 5 min. To the supernatant (100 µL), 1 mL mixture of reagent A (100 mM Tris, 2.75 mM Pyruvate, 225 mM NaCl, pH 7.2) and reagent B (1.55 mM NADH and 9.5 g/L sodium azide) was added from LDH kit (Biosystems, Cat. No.11580). After 2 min of incubation, the absorbance was measured at 340 nm in an UV-Vis spectrophotometer and the LDH activity was calculated.

AO/EB assay

The AO/EB assay was performed as explained by Antonova *et al.*[31] to study the apoptosis induced by 5-FU in PBMC cells. The cells in RPMI medium was treated with drug 5-FU (625 µg/mL), eugenol (1250 µg/mL) and *M. ferrea* extract (100 µg/mL). The cells were incubated for 2 h at 37 °C and then centrifuged at 2500 rpm for 8 min. The pellet was collected, washed twice with PBS buffer, added with AO/EB stain (2 µL, 100 µM) and incubated for 30 min at 37 °C in dark. Then the cells were mounted on a glass slide and images were recorded in green and red filters under fluorescence microscope.

ROS assay

The ROS assay was performed by following the protocol explained by Figueroa *et al.*[32] to measure the intracellular free radical generation in PBMC in response to 5-FU treatment. The PBMC cells in RPMI medium were taken in eppendorf tubes and treated with 5-FU (75 µL, 625 µg/mL), eugenol (75 µL, 1250 µg/mL) or *M. ferrea* extract (75 µL, 100 µg/mL) separately. All the tubes were incubated for 3 h and centrifuged at 3500 rpm for 8 min. The pellet was collected and 100 µL $DCFH_2$-DA (2 µg/mL) was added and further incubated for 30 min at dark. The cells were harvested, mounted on a glass slide and images were captured using fluorescent microscopy and the absorbance was recorded at excitation (480 nm) and emission (530 nm) and also the quantification results were obtained from spectro-fluorometer.

TBARS assay

The level of lipid peroxidation in PBMC cells due to 5-FU toxicity was measured using TBARS assay as explained by Dawn-Linsley *et al.*[33] PBMC cells in RPMI medium (500 µL) was taken in an eppendorf tube and treated with 5-FU (75 µL, 625 µg/mL), eugenol (75 µL, 1250 µg/mL) or *M. ferrea* extract (75 µL, 100 µg/mL). The tubes were incubated for 3 h and centrifuged at 2500 rpm for 3 min. The pellet was re-suspended in 1 mL of PBS and homogenized for 1 min. Cell homogenate (500 µL) was added with 1 mL of TBA (Thiobarbituric acid, 0.375%, w/v) and 1 mL of TCA (Trichloro acetic acid, 15%, w/v). The tubes were placed in a water bath at 90 °C for 20 min, cooled, contents were centrifuged at 2000 rpm for 5 min and the absorbance was measured at 540 nm.

Statistical analysis

Data was analyzed by using Graphpad Prism 5.0 statistical program. The results were expressed as mean±standard deviation. ANOVA was applied to analyze the significant differences among the values (*P <0.05, ** P <0.01, and *** P <0.0001).

Results and Discussion

Cytoprotective effect of eugenol

In the present project, Histopaque was used to separate PBMC cells based on the gradient density principle. Histopaque is a sterile, endotoxin tested solution of ficoll, adjusted to a density of 1.077 g/mL. The blood cells are separated as plasma, PBMC layer, histopaque and red blood cells based on their different densities[34]. The PBMC cells were obtained as a second layer from the top below the plasma layer. The yield of PBMC cells was about 15 x 10^6 per 5 mL blood.

Even though 5-FU is frequently used in the clinical treatment of various cancer types, it is reported to cause toxicity to normal cells via free radical-mediated damage. In the present work, the cytotoxicity of 5-FU in PBMC cell model was investigated and from Fig. 2a it could be inferred that the cytotoxicity (%) increases as the concentration of the 5-FU increases. At high concentration (625 µg/mL), 5-FU exhibited maximum cytotoxicity of 73% in PBMC. Similarly, the 5-FU cytotoxicity of 95-110% was observed in HCT116 and HT-29 cells[35]. Treatment of 5-FU leads to ROS production and

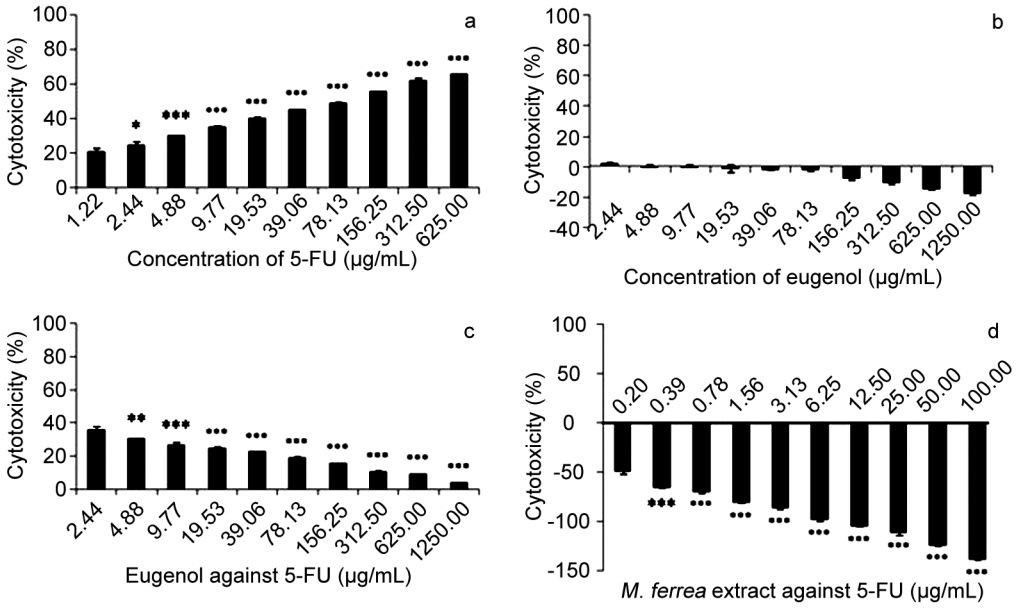

Fig. 2 — Cytotoxicity, a) 5-FU, b) Eugenol, c) Cytoprotective effect of eugenol, d) *M. ferrea* extract against 5-FU toxicity assessed in PBMC cells. Bars with * indicates P <0.05, ** indicates P <0.001 and *** indicates P <0.0001 level of significant difference of each value when compared to the control (lowest concentration) according to ANOVA with Dunnett's multiple comparison test.

oxidative damage to various biomolecules of cell membrane and organelles, and hence it exhibits remarkable cytotoxicity to the normal cells. So, usage of 5-FU is effective in killing cancer cells during clinical therapy, but it also affects normal cells like PBMC, particularly when it is administered intravenously.

All the concentrations of eugenol were noted as safe (Fig. 2b). In agreement to our results, eugenol showed 100% cell viability in PBMC[36]. Cytotoxicity of PBMC in response to 5-FU, eugenol, and *M. ferrea* extract treatments was evaluated using MTT assay, which is a simple and rapid spectrometric method used to check the cell viability. The mitochondrial enzymes such as NADPH dependent oxidoreductase can react with MTT and forms purple formazan, if the cells are viable[37]. Then acidic isopropanol was added to the cells to dissolve the formazan crystals and the absorbance of formazan is directly proportional to cell viability.

Fig. 2c indicates that the cytotoxicity of 5-FU (625 µg/mL) was ameliorated with increasing concentrations of eugenol. This shows that eugenol can protect the 5-FU toxicity even at a low concentration of 2.44 µg/mL. In agreement to our results, Gelen *et al.*[15] also reported phytochemicals like quercetin and rutin protected liver cells from 5-FU toxicity. Different concentrations of *M. ferrea* extract also offered complete protection to PBMC against 5-FU toxicity (Fig. 2d). An earlier report of the authors indicated strong antioxidant capacity of *M. ferrea* extract[22] and hence, it protected the PBMC cells by scavenging intracellular ROS generated by 5-FU. Hence, either *M. ferrea* extract in crude form or its major active compound eugenol could be used as a promising cytoprotective agent to prevent 5-FU toxicity in normal cells like PBMC. As the *M. ferrea* extract was dissolved in 0.1% DMSO, the toxicity of vehicle control was also analyzed and found that it does not have toxicity in PBMC (Supplementary Fig. 1).

Cell wall damage

LDH is a cytosolic, oxidoreductase enzyme that catalyses the inter-conversion of pyruvate to lactate[38]. If the cell membrane gets damaged, LDH will be released into the medium and thus measurement of this enzyme in the cell culture medium indicates the cell wall damage. From Fig. 3, it is inferred that 5-FU can cause release of higher amount of LDH (1321.745 µg/mL) when compared to control (155.39 µg/mL) due to cell membrane damage. When the cells are treated with eugenol, it significantly prevented the LDH release (488.02 µg/mL) as it controlled the cell membrane damage caused by 5-FU. Thus, experimental results suggest that eugenol can prevent cell membrane damage and protects normal cells against 5-FU toxicity. In agreement to the present results, chrysin inhibited LDH release (242 nanomole NADH oxidized/min) in renal tissue against 5-FU treatment (374 nanomole NADH oxidized / min)[39]. Similarly, *M. ferrea* extract also exhibited low LDH

release (389.36 µg/mL) and thus offered cytoprotective effect against cell wall damage caused by 5-FU in PBMC.

Cellular apoptosis

AO/EB assay is used to detect the apoptosis of the cells, in which acridine orange is capable of diffusing into both live and dead cells and gives green fluorescence, whereas the ethidium bromide is permeable only into the apoptotic cells and exhibits red fluorescence[40]. Apoptosis induced in large number of PBMC cells by 5-FU was visualized from Fig. 4. The PBMC cells that were treated with 5-FU experience oxidative stress due to over-production of ROS and it leads to membrane/organelle damage and ultimately results in cellular apoptosis. The intracellular generation of free radicals by 5-FU treatment might damages the membrane of mitochondria and activate apoptotic pathway. The PBMC cells treated with eugenol showed more number of viable cells and few apoptotic cells and similar trend was noticed in *M. ferrea* extract treatment. It is understood that treatment with eugenol-rich *M. ferrea* extract or eugenol might prevents the oxidative stress caused by 5-FU in normal cells and hence they prevents apoptosis in PBMC. In agreement to our results, quercetin also reported to control apoptosis caused by 5-FU in HCT15 cells[41].

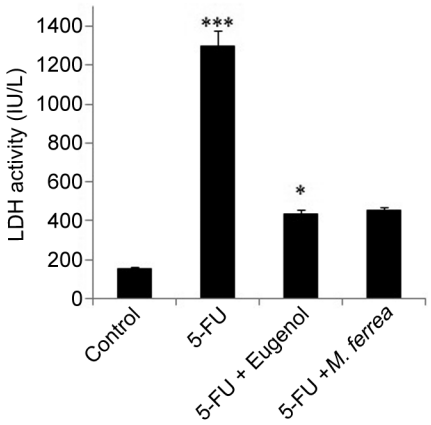

Fig. 3 — LDH release from PBMC cells treated with 5-FU and eugenol. Bars with * indicates P <0.05 and *** indicates P <0.001 level of significant difference of each value when compared to the control according to ANOVA with Dunnett's multiple comparison test.

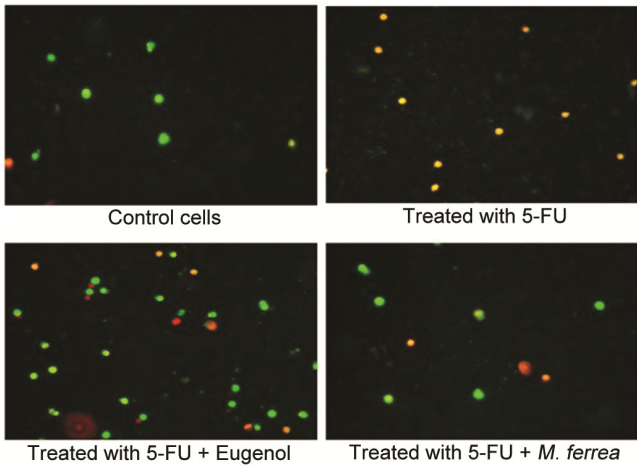

Fig. 4 — Images of AO/EB assay indicating apoptosis in PBMC cells treated with 5-FU and eugenol.

Intracellular ROS generation

In this assay, a cell permeable reagent 2,7-dichlorofluoresin diacetate ($DCFH_2$-DA) was used to measure the ROS level in PBMC cells. When the $DCFH_2$-DA is diffused into the cells, $DCFH_2$-DA is deacetylated to a non-fluorescent compound, 2,7-dichlorofluoresin ($DCFH_2$) by cellular esterases. Once it reacts with ROS, $DCFH_2$ is oxidized to a fluorescent compound DCF, whose fluorescent intensity is directly proportional to ROS level[32]. From Fig. 5, it can be seen that the cells exposed to 5-FU

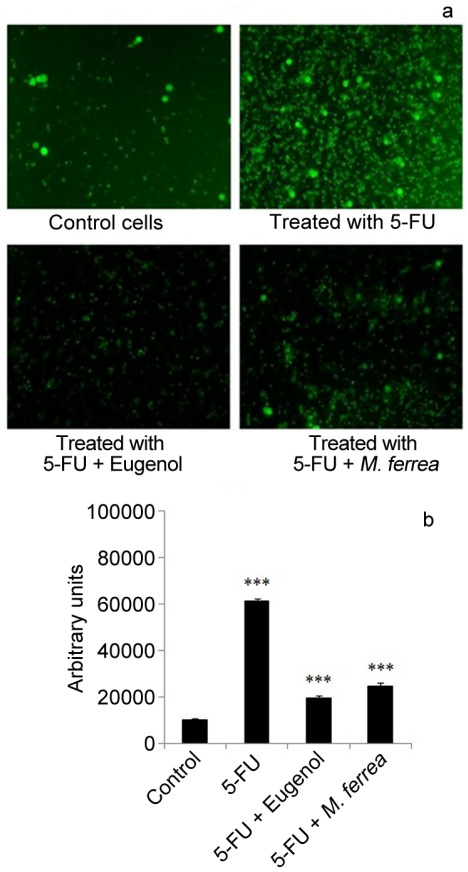

Fig. 5 — The images of ROS generation, a) PBMC cells and, b) their quantification results. Bars with *** indicate P <0.001 level of significant difference of each value when compared to the control according to ANOVA with Dunnett's multiple comparison test.

have high fluorescence, which indicates that the formation of more number of ROS in PBMC. In treatment with eugenol, the ROS production was lower (20035.5 AU) when compared to 5-FU treated PBMC cells (58918.5 AU). In agreement to the present results results, gamma-tocotrienol also controlled the ROS generation by 60% when compared to 5-FU treated oral epithelial cells[42]. As the phytochemical (eugenol) is a strong antioxidant, it could effectively scavenge the ROS produced by 5-FU and thus showed less fluorescence. Similarly, *M. ferrea* extract also inhibited/scavenged the ROS in PBMC cells generated due to 5-FU and hence showed low fluorescence (25416 AU). ROS levels controlled by eugenol or *M. ferrea* extract in PBMC cells corresponds to their cytoprotective effect, prevention of cell wall damage, and induction of apoptosis.

Lipid peroxidation

Lipid oxidation caused by free radicals in cell system leads to the formation of end product such as malondialdehyde (MDA), which can be measured by TBARS assay[43]. TBARS value or MDA level could be used as biomarker to assess the oxidative stress of cells in terms of lipid peroxidation. The principle behind this assay is that the MDA reacts with TBA (Thiobarbituric acid) in an acidic medium and forms a pink colour chromogen, whose absorbance is directly correlated with the level of lipid peroxidation. Treatment of cells with 5-FU caused lipid peroxidation and production of higher MDA level (0.28 µg of MDA/mL) when compared to untreated control (Fig. 6). When the PBMC cells are administered with eugenol or *M. ferrea* extract, it showed the formation of low level of MDA (0.1-0.12 µg of MDA/mL). Hence, it could be realized that the antioxidant compound eugenol or eugenol-rich *M. ferrea* might prevent lipid peroxidation by ROS scavenging and thus offered cytoprotective effect against 5-FU in PBMC cells. Similarly, ascorbic acid was reported to reduce the MDA level from 430 to 350 µM in intestinal tissues of 5-FU administered SW rats[16].

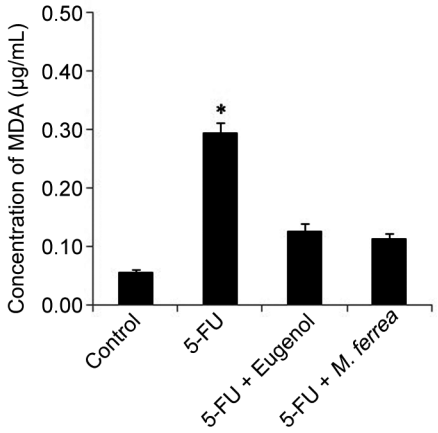

Fig. 6 — Lipid peroxidation (TBARS) levels in PBMC cells treated with 5-FU and eugenol. Bar with * indicates P <0.05 level of significant difference when compared to the control according to ANOVA with Dunnett's multiple comparison test.

Conclusion

From the results of MTT, ROS, LDH, AO/EB, and TBARS assays, we could conclude that 5-FU can cause cytotoxicity to the PBMC cells through ROS generation and oxidative stress-induced apoptosis. The antioxidant compound eugenol or eugenol-rich *M. ferrea* extract could prevent the cytotoxicity of 5-FU in PBMC cells. Eugenol offered cytoprotection in PBMC cells against 5-FU by inhibiting intra-cellular ROS production, inhibiting the cell membrane damage, and also by lowering the lipid peroxidation. So, antioxidants like eugenol could be co-administered to prevent the toxic effects of 5-FU drug on normal cells during clinical cancer therapy. In depth *in vivo* experiments and clinical studies are essential for future application of eugenol along with the anticancer drug 5-FU for better and safer medication.

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Conflict of interest

The authors declare that they have no conflict of interest.

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