

Eugenol and n-3 Rich Garden Cress Seed Oil as Modulators of Platelet Aggregation and Eicosanoids in Wistar Albino Rats

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Abstract: Eugenol, the active principle of clove oil is a potent antioxidant and a well known anti-inflammatory agent. In an earlier study, we have reported the *in vitro* inhibition of human platelet aggregation and LTC₄ formation in rat PMNL cells. In the present study, an attempt has been made to monitor the *in vivo* and *in vitro* modulation of platelet aggregation and eicosanoids (TXB₂, LTC₄) by eugenol and α -linolenic acid (ALNA) rich garden cress seed oil in adult Wistar rats. Feeding of Garden cress oil (GCO) supplemented diet to rats for 8 weeks moderately inhibited platelet aggregation induced by ADP and arachidonic acid (AA) at 14 and 7% respectively and also TXB₂ levels at 10 and 32% in lung and spleen tissues. Oral feeding of eugenol at 40 mg/kg body weight for one week to GCO fed rats showed 20-30% inhibition of ADP and AA induced platelet aggregation and also TXB₂ levels by ~ 50% in spleen. In an *in vitro* study, eugenol at 15.0 and 45.0 μ M produced 15 and 78% inhibition of LTC₄ levels, 27 and 80% inhibition of TXB₂ in lungs and also 20 and 72% inhibition of LTC₄ levels in the washed platelets respectively. Eugenol and GCO showed synergistic effect against platelet aggregation and TXB₂ levels in spleen and lung tissues. Conclusively, data obtained from the present study provide new insights into modulatory effect of eugenol and α -linolenic acid on platelet aggregation and eicosanoids

Keywords: Eugenol, garden cress oil, n-3 fatty acids, platelet aggregation, TXB₂, LTC₄.

INTRODUCTION

Eugenol (2-methoxy-4-(2-propenyl) phenol) is the active principle of clove (*Eugenia aromaticum*) a commonly used spice in Indian cuisine and a naturally occurring phenolic compound in basil, cinnamon and nutmeg. Eugenol is the major component in clove oil and it is used in dental care, as an antiseptic, analgesic and antibacterial agent against oral bacteria associated with dental caries and periodontal disease [1]. In addition to its antimicrobial activity, eugenol possesses anti-inflammatory, cytotoxic, insect repellent, insecticidal, anticancer and anesthetic properties [2-4]. In an earlier study, we have demonstrated the inhibitory effects of eugenol on soybean lipoxygenase dependent lipid peroxidation, 5-lipoxygenase (5-LOX) enzyme activity and LTC₄ formation which play a vital role in inflammation [5-7]. Further, we have reported the modulatory effect of different spice active principles including on *ex vivo* human platelet aggregation [8].

Fatty acid composition of the oil in our diet plays a vital role in the cardiovascular disease [9,10]. N-3 fatty acids have many beneficial effects in altering the lipoprotein metabolism and decreasing cholesterol and serum triglyceride levels [11]. Important n-3 fatty acids in human nutrition are α -linolenic acid (ALNA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Human body cannot synthesize n-6 and n-3 fatty acids, but can synthesize other long chain n-3 fatty acids from dietary ALNA. EPA and DHA are found mainly in oily cold water fish such as salmon, trout, herring, sardine and mackerel. Flaxseed oil, camelina oil, canola oil, soybean oil are rich sources of ALNA. Mammals cannot interconvert n-6 and n-3 fatty acids due to lack of an enzyme- delta 15-desaturase [12]. Since vegetarians avoid fish and fish oil, there is a need for n-3 fatty acids supplementation from plant sources. Garden cress oil (GCO) is one of the vegetable oils rich in ALNA of 34% [13,14]. GCO is relatively stable oil than flax seed oil [14]. Bioavailability studies with GCO showed significant increase of ALNA levels in serum and conversion to EPA and DHA in tissues in experimental rats [11]. The aim of the study was to determine the modulatory effect of spice principle eugenol and n-3 rich GCO on platelet aggregation, leukotriene and thromboxane levels in *in vivo* and *in vitro* systems.

MATERIALS AND METHODS

Experimental Animals

Forty male Wistar rats (OUTB-Wistar, IND-cft (2c)) weighing 40-50g were used for the study. The animals were obtained from the stock colony of the Institutes' Animal House Facility. Animals were grouped by randomized design and were housed individually in stainless steel cages, in a room with 12 h light and dark cycles. The temperature was maintained at 25 \pm 2°C with a relative humidity of 60-70%. Animal experiments were carried out based on the ethical guidelines laid down by the committee for the

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purpose of control and supervision of experiments on animals by the Government of India, Ministry of Social Justice and Empowerment.

In Vivo Animal Experiments with GCO and Eugenol

Animals were divided into five groups of eight animals in each group. Group I, II, III were fed with diets containing 10% sunflower oil (SFO), group IV and V were given diets containing 10 % GCO for a period of 8 weeks. At the end of 7th week, group II animals were intubated with 20mg/kg b.wt of eugenol, group III and IV were intubated with 40mg/kg b.wt. of eugenol respectively for one week. Rats were fed with isocaloric semi-synthetic diet *ad libitum*. The diet was prepared every week and stored at 4 °C in air tight containers. Animals were fed with fresh diet every day and had free access to water and diet throughout the experimental period.

Materials

ADP, thromboxane B₂ standard, DMSO were purchased from Sigma Chemical Co. leukotriene C₄ standard from Cayman chemicals, USA. Eugenol was purchased from Loba Chemie, India. Arachidonic acid was procured from Neu-Chek, USA. All other chemicals used for the study were of analytical grade.

Diet

Modified AIN-76A isocaloric semi-synthetic diet was used for animal experiments. The composition of the diet is presented in Table 1. Sunflower oil (SFO) and Garden cress oil (GCO) were supplemented at 10 % level in animal diets.

Table 1. Composition of the Animal Diet

Component (g/kg)	SFO (10%)	GCO (10%)
Casein(fat free)	200	200
Corn starch	500	500
Sucrose	100	100
Cellulose	50	50
Mineral mix ^a	35	35
Methionine	2	2
Choline chloride	3	3
Vitamin mix ^b	10	10
SFO	100	0
GCO	0	100

^a Bernheart Tommerali salt mixture, ^b AIN-76A vitamin mix. SFO-Sun Flower Oil, GCO-Garden cress Oil.

Fatty Acid Analysis

Fatty acid composition of oil used in the diet was analyzed as methyl esters [15] by Gas chromatography (Shimadzu 14B) fitted with Flame ionization detector (FID) and BP21 fused silica capillary column (30m x 0.3mm) was used for separation of fatty acids. GC analysis was carried in isothermal conditions. Column temperature was at

220°C, injector temperature was at 230°C and the detector temperature was at 240°C. Nitrogen was used as the carrier gas with a flow rate of 1ml/min. Individual fatty acids were identified by comparison with retention time of authentic fatty acid standards(Nu-Chek Prep. Inc., MN) and quantified by Clarity integrator.

Ex Vivo Platelet Aggregation Study

After 8 weeks of feeding, rats were fasted overnight and sacrificed under ether anesthesia. Blood was collected by cardiac puncture in 3.8% sodium citrate (8.5:1.5 v/v). Lungs and spleen were excised, weighed and stored at -70°C until analysis. Platelet rich plasma (PRP) was separated by centrifuging the blood at 150g for 20 min at room temperature (25±2°C). Remaining blood was centrifuged at 750g for 20 min to obtain platelet poor plasma (PPP). Platelet count was adjusted to 4.0 x 10⁵ platelets per µl of PRP. Experiments were performed within 2 h of PRP preparation.

Platelet aggregation was monitored in Dual channel Chronolog aggregometer (Model 400; Chronolog Corporation) in a final reaction mixture of 0.5 ml. Platelet aggregation was induced by addition of 50 mM of ADP or 1.0 mM of AA. Threshold aggregating concentration (TAG) of both the agonists was estimated for the prepared PRP. TAG is defined as the minimum concentration of agonist required to induce more than 90% platelet aggregation. The extent of platelet aggregation was measured as the maximal increase in light transmission in 5 min after the addition of the agonist [8,16].

Estimation of Thromboxane Levels in Rat Tissues

Eight normal rats were sacrificed under ether anesthesia. Lungs and spleen were cut into small pieces and homogenized in a warring blender in 0.1M phosphate buffer pH 7.4. The tissue homogenates were centrifuged at 750g and pH of the supernatant was brought to 3.0 by adding formic acid and loaded on to Sep-Pak C18 column (Waters, Millipore Corp., Milford, MA, USA) [17]. Thromboxane B₂ (TXB₂) was eluted from Sep-Pak column with ethyl acetate and evaporated to dryness under nitrogen. TXB₂ was analyzed by HPLC method using reverse phase C₁₈ column (150 m x 4.6 mm; 5µ diameter) at 200 nm. The mobile phase was water: acetonitrile (60:40 v/v) pH4.0. TXB₂ eluted at 3.4 min was confirmed by authentic standard of TXB₂ from Sigma Chemical Co., USA [17]. Protein was estimated by Lowry's method [18] and the results were expressed as ng of TXB₂ formed per mg of protein.

Estimation of LTC₄ in Rat Lungs

Eight normal rats were anesthetized with ether and sacrificed as described in above section. Lungs were separated, cut into small pieces and homogenized in a warring blender in 0.1M phosphate buffer pH 7.4. Known volume of lung homogenate was incubated with 15.0 and 45.0 µM of eugenol for 5 min. The reaction was initiated by adding 20.0 µM of AA and it was incubated for 30 min at 37°C. Reaction was terminated by reducing the pH to 3.0 with formic acid and reaction mixture was centrifuged at 750g. Supernatant was loaded on to Sep Pak C₁₈ column, eluted with ethyl acetate and evaporated todryness under nitrogen

[19]. LTC₄ was separated by HPLC method using reverse phase C₁₈ column(150 m × 4.6 mm; 5 microns diameter) with a mobile phase of methanol : ammonium acetate (5mM) (70:30 v/v) pH 5.0 at 280 nm. LTC₄ separated at 4.2 min at a flow rate of 0.5 ml/min and was confirmed on the basis of retention time of authentic standard [17].

Estimation of LTC₄ in Platelets Treated with Eugenol

Rats were sacrificed under ether anesthesia. Blood was collected in 3.8% sodium citrate (8.5:1.5 v/v). Platelet Rich Plasma (PRP) was separated as described above. Washed platelets were prepared with slight modification [19]. In brief, PRP obtained was then centrifuged at 1000 g for 15 min at 4°C. Platelets were washed with ice-cold 50mM phosphate buffer pH 7.4 having NaCl (0.15 M) and EDTA (0.2 mM). Platelets (4.0 x 10⁶) were then suspended in the tyrode buffer pH7.5 and incubated with 15 and 45 μM of eugenol for 5 min and the reaction was initiated by adding 20 μM AA. Reaction was stopped by acidifying the mixture with formic acid. LTC₄ formed was estimated by reverse phase HPLC using C₁₈ column after purification of the sample with Sep- Pak C₁₈ column as described in the previous section.

Statistical Analysis

Results were presented as mean ± SD of eight determinations. Statistical differences were calculated by Duncan’s multiple range tests using SPSS statistical software. Level of significance was set at P< 0.05.

RESULTS AND DISCUSSION

Composition of isocaloric semi-synthetic diet is presented in Table 1. Sunflower oil (SFO) supplemented at 10 % level was fed to control groups of animals. GCO oil rich in n-3 fatty acids was supplemented at 10 % in the diet was fed to experimental group of animals for 8 weeks. Table 2 shows the fatty acid composition of the diet

Table 2. Fatty Acid Composition of Diets fed to Experimental Animals

Fatty Acids	SFO (10%)	GCO (10%)
16:0	8.3	9.5
18:0	2.7	3.6
18:1	42.9	24.7
18:2	44.1	12.8
18:3	0.4	28.3
20:0	-	3.6
20:1	-	13.0
22:1	-	4.4
SFA	13.7	16.7
MUFA	42.9	42.1
PUFA	44.5	41.1
n-6/n-3	110.5	0.45

SFA- Saturated fatty acid, MUFA- Monounsaturated fatty acid PUFA- Polyunsaturated fatty Acid.

fed to experimental animals. GCO supplemented diet contained 28% of ALNA and SFO (control) diet contained 0.4% of ALNA. MUFA and PUFA were almost similar in both the diets. n-6 and n-3 ratio of in GCO supplemented diet was 0.45% compared to 110% in SFO diet.

Effect of GCO and eugenol on platelet aggregation is presented in Figs. (1, 2, 3 and 4). In GCO fed animals, platelet aggregation induced by ADP and AA was inhibited by 14% and 7% respectively. Eugenol at a dose of 20 and 40mg/kg b.wt. fed to SFO group inhibited AA and ADP induced platelet aggregation by 20 and 28 % respectively. Where as in GCO fed animals eugenol at 40 mg/kg b.wt did not show significant inhibition of platelet aggregation induced by AA and ADP compared to SFO group.

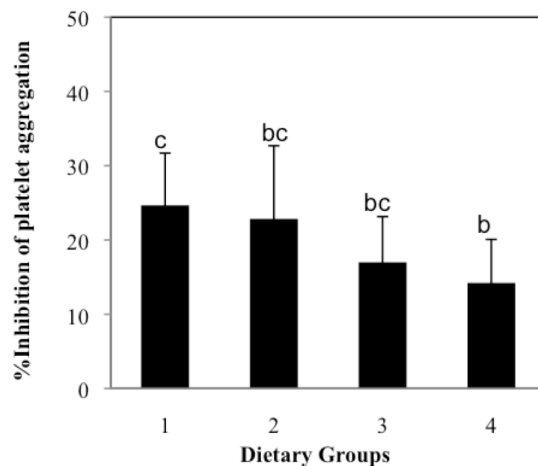


Fig. (1). Effect of eugenol and GCO on ADP induced platelet aggregation in the rat blood. 1. Eugenol (20mg/kg b.wt) + SFO; 2.Eugenol (40mg/kg b.wt) + SFO; 3.Eugenol (40mg/kg b.wt) + GCO; 4.GCO. Values are mean ± SD of eight determinations. Inhibition of platelet aggregation by experimental diets is significantly different from control at P<0.05 levels. b, c indicate the level of significance in the increasing order. (b) Moderately significant. (c) Very significant.

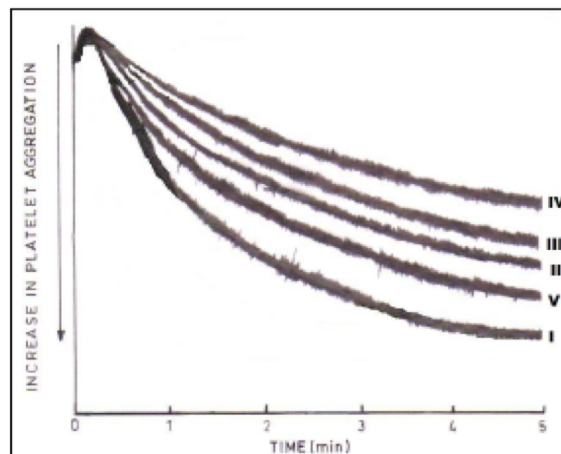


Fig. (2). Effect of eugenol and GCO on ADP induced platelet aggregation in the rat blood. Eugenol and GCO inhibited aggregation of platelets. I. Control (SFO); II. Eugenol (20mg/kg bwt) + SFO; III. Eugenol (40mg/kg b.wt) + SFO; IV. Eugenol (40mg/kg b.wt) + GCO; V. GCO.

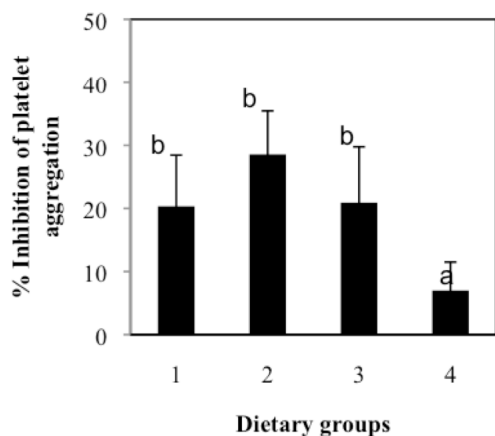


Fig. (3). Effect of eugenol and GCO on AA induced platelet aggregation in the rat blood. 1. SFO + Eugenol (20mg/kg b.wt); 2. SFO + Eugenol (40mg/kg b.wt); 3. GCO + Eugenol (40mg/kg b.wt); 4. GCO. Values are mean \pm SD of eight determinations. Inhibition of platelet aggregation by experimental diets is significantly different from control at $P < 0.05$ levels. a, b indicate the level of significance in the decreasing order. (a) Least significant. (b) Moderately significant.

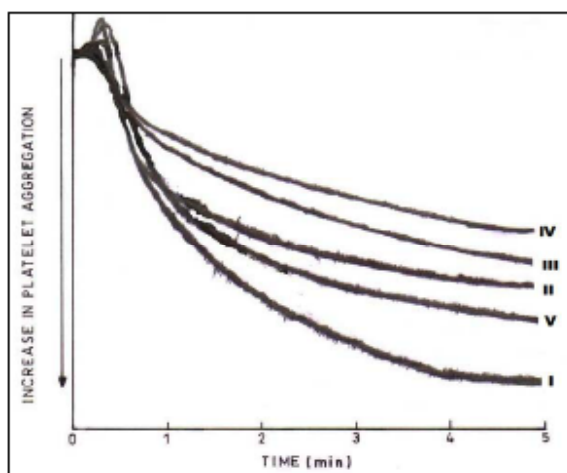


Fig. (4). Effect of eugenol and GCO on AA induced platelet aggregation in the rat blood. Eugenol and GCO inhibited platelet aggregation. I. Control (SFO); II. Eugenol (20mg/kg b.wt) + SFO; III. Eugenol (40mg/kg b.wt) + SFO; IV. Eugenol (40mg/kg b.wt) + GCO; V. GCO.

Effect of GCO and eugenol on TXB₂ level in the spleen and lungs is presented in Figs. (5 and 6). Eugenol treatment inhibited TXB₂ by 66.6 and 75 % respectively in spleen. GCO alone inhibited TXB₂ level by 48 % compared to SFO group and significant inhibition was observed in combination with eugenol in spleen. Eugenol treatment produced 26.8 % inhibition of TXB₂ in lungs. GCO alone produced marginal inhibition of about 12 % compared to SFO group. However, in GCO group, eugenol treatment produced significantly higher inhibition of TXB₂ (53.6 %) in lungs.

In vitro effect of eugenol on rat lungs TXB₂ and LTC₄ is presented in Figs (7, 8 and 9). TXB₂ level was inhibited

by 28% to 80% and LTC₄ level by 15% and 78% respectively by eugenol.

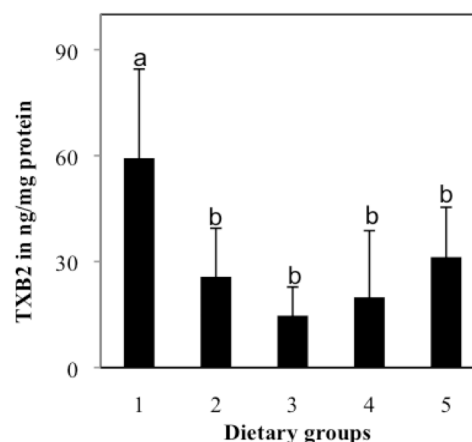


Fig. (5). *In vivo* effect of SFO, GCO and eugenol treatment on levels of TXB₂ in rat spleen. 1. Control-SFO; 2. SFO + Eugenol (20mg/kg b.wt); 3. SFO + Eugenol (40mg/kg b.wt); 4. GCO + Eugenol (40mg/kg b.wt); 5. GCO. Values are mean \pm SD of eight determinations. Inhibition of TXB₂ level is significantly different from control at $P < 0.05$ levels. a, b indicate the level of significance. (a) Least significant. (b) Moderately significant.

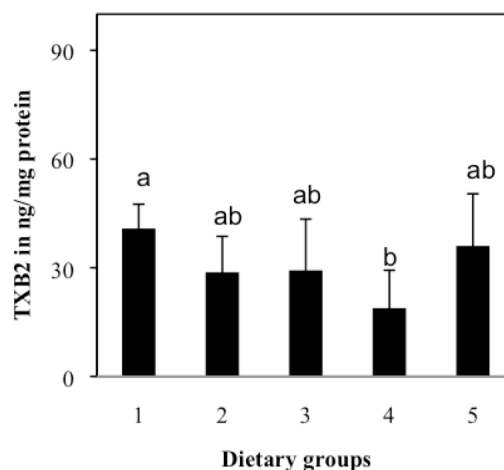


Fig. (6). *In vivo* effect of SFO, GCO and eugenol treatment on levels of TXB₂ in rat lungs. 1. Control-SFO; 2. SFO + Eugenol (20mg/kg b.wt); 3. SFO + Eugenol (40mg/kg b.wt); 4. GCO + Eugenol (40mg/kg b.wt); 5. GCO. Values are mean \pm SD of eight determinations. Inhibition of TXB₂ level is significantly different from control at $P < 0.05$ levels. b indicate the level of significance. (a) Least significant. (b) Moderately significant.

Eicosanoids are highly reactive lipid mediators synthesized from AA and EPA by the action of cyclooxygenase and lipoxygenase enzymes. Cyclooxygenase catalyzes conversion AA and EPA to prostacyclins of 2 and 3 series and prostaglandins and thromboxane of 3 and 4 series from respectively. 5-lipoxygenase is responsible for the synthesis of 4 and 5 series of leukotrienes from AA and EPA respectively [12,20]. TXA₂ is a potent vasoconstrictor and platelet aggregator, while TXA₃ is a weaker platelet aggregator. Similarly, LTB₄ is a powerful chemotactic agent, LTC₄, LTB₄ and LTE₄ are vasoconstrictors and increase vascular

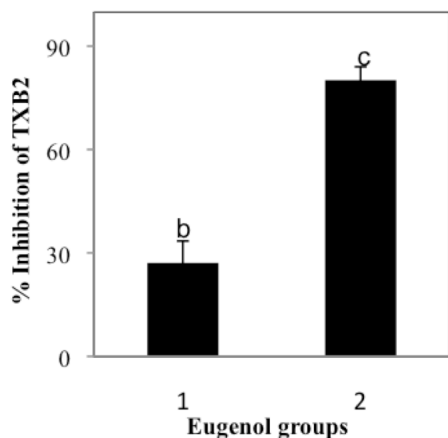


Fig. (7). *In vitro* effect of eugenol on AA induced TXB₂ in the rat lungs. 1. Eugenol (15 μM); 2. Eugenol (45 μM). Values are mean ± SD of eight determinations. Inhibition of TXB₂ in the rat lungs by eugenol is significantly different from control at P<0.05 levels. b, c indicate the level of significance. (b) Moderately significant. (c) Very significant.

permeability. Oppositely, the corresponding 5 series of leukotrienes derived from EPA, are very weak chemotactic and vasoconstrictor agents [21, 22]. Since n-3 PUFA antagonizes the production of inflammatory eicosanoid mediators from AA, there is a potential benefit of n-3 fatty acids in many chronic inflammatory diseases. In addition, n-3 PUFA regulate the production of some inflammatory cytokines and down regulate the expression of a number of genes involved in leukocyte adhesion, blood coagulation and fibrinolysis. Many studies have reported beneficial effects of ALNA rich diet in reducing the platelet aggregation, total cholesterol and further reduction in severity of cardiovascular disease (CVD) [9, 23].

Since ages, spices and their active principles are known for rabbit platelets by acting as TXA₂ receptor antagonist [25]. Earlier we have reported significant inhibitory effect of different spice active principles on platelet aggregation [26]. The results presented here demonstrate that eugenol, the major ingredient and active principle in clove and

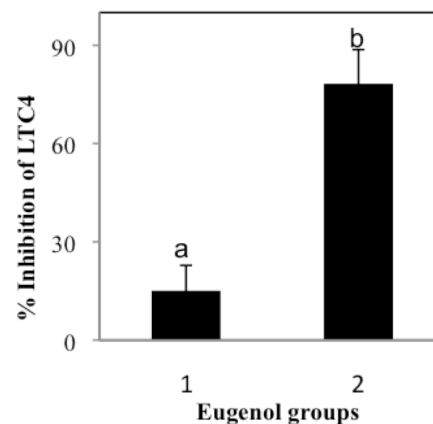


Fig. (9). *In vitro* effect of eugenol on AA induced LTC₄ in the rat platelets. 1. Eugenol (15 μM); 2. Eugenol (45 μM). Values are mean ± SD of eight determinations. Inhibition of LTC₄ in the rat platelet by eugenol is significantly different from control at P<0.05 levels. a, b indicate the level of significance in the decreasing order. (a) Least significant. (b) Moderately significant.

clove oil inhibits platelet aggregation, TXB₂ levels in rat platelets, lung and spleen. TXB₂ is a stable end product of TXA₂ which plays a major role in platelet aggregation and is a very potent vasoconstrictor. TXA₂ are autocrine or paracrine in their actions and exert their effects by binding to G_q receptors on platelet surface and activate inositol pathway which leads to increase in cytosolic Ca²⁺ levels. In our earlier studies, we have shown that eugenol was 30 times more potent than that of aspirin in inhibiting TXB₂ in human platelets [8]. Preferential inhibition of AA induced platelet aggregation by eugenol could be due to decreased formation of TXA₂ in platelets. Further, eugenol was also shown to inhibit the ADP induced platelet aggregation by modulating the Ca²⁺ influx in rat platelets [8,27,28]. Thus it can be suggested that, anti-platelet their beneficial effects on human health [24]. Extracts of *Piper longum* L. were shown to inhibit platelet aggregation in activity of eugenol could be due to modulation of thromboxane and Ca²⁺ levels in platelets.

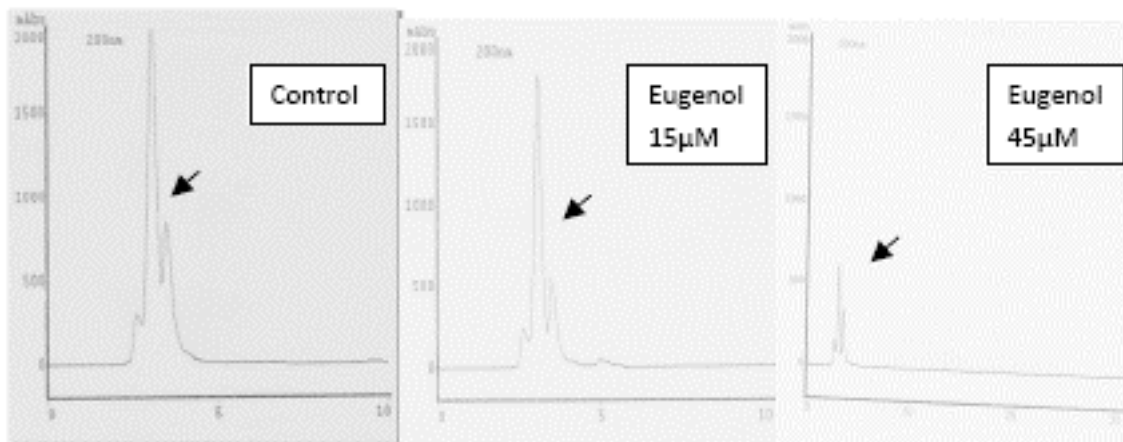


Fig. (8). *In vitro* effect of eugenol on AA induced TXB₂ synthesis in the rat lung. TXB₂ was analyzed by reverse phase HPLC using C18 column (150x 4.6) at 200 nm by mobile phase of water : acetonitrile (60:40 v/v) pH4.0 with elution at 3.4 min.

Spleen is a major secondary lymphoid organ which plays a major role in immune system and where as lungs are affected by inflammation in asthma. Eugenol at the dose of 40mg/kg b.wt. produced more than 50% reduction in TXB₂ in rat spleen (Fig. 5). But, eugenol in combination with GCO did not show additive effect in reducing the TXB₂ levels. In case of lungs, both eugenol and GCO combination showed nearly 50% reductions in TXB₂ level. Further, in an *in vitro* study eugenol significantly inhibited LTC₄ levels in rat lungs indicating the possible inhibition of transcellular metabolism and 5-lipoxygenase activity in these tissues [7, 29].

Studies in our laboratory and others showed the modulatory effects of ALNA on the fatty acid profile and lipid metabolism in rats [11, 30]. Supplementation of GCO inhibited ADP induced platelet aggregation (Figs 1, 2, 3 and 4). GCO is very rich in ALNA (Table 2) a precursor for long chain fatty acids viz., EPA and DHA [9, 31]. Rats fed with GCO showed a significant increase in ALNA levels in serum and tissues and also a significant decrease in LA and its metabolite AA in serum and liver compared to SFO fed rats [11]. AA in the platelet membrane plays a major role in production of TXA₂. Thus low levels of AA in GCO fed animals could be responsible for reduced platelet aggregation activity. In Conclusion, the results clearly demonstrated that eugenol and ALNA modulates platelet aggregation and formation of eicosanoids such as LTC₄ and TXB₂ in lungs, spleen and platelets. Thus, eugenol can be considered as a nutraceutical adjuvant from clove in the treatment of diseases related to platelet aggregation, such as thrombosis, transient ischemia, headache and other inflammatory disorders.

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