



## Alpha-linolenic acid (ALA) rich Garden cress (*Lepidium sativum* L.) seed oil and its vegetable oil blends modulate aggregation of platelets and serum Thromboxane B<sub>2</sub> levels in rats

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### Abstract

Most of the vegetable oils we consume are rich in n-6 PUFAs and deficient in n-3 PUFAs. Blending of oils is necessary to improve n-6/n-3 PUFA ratio in vegetable oils. Garden cress seeds contain 24% of oil in which 32–34% is  $\alpha$ -linolenic Acid (ALA). Garden cress oil (GCO) is more stable due to presence of more balanced MUFA and PUFA ratio and high antioxidants in the oil. In the present study, GCO was blended with edible oils such as sunflower oil (SFO), rice bran oil (RBO), sesame oil (SESO) and flaxseed oil (FLAX), dietary feeding of GCO and its blended oils on platelet aggregation was studied in Wistar rats. Dietary feeding of GCO and its blended oils significantly decreased the adenosine diphosphate (ADP) and collagen induced aggregation of platelets by 25–31% and 22–35% respectively compared to the native oils (SFO, RBO and SESO) fed rats. GCO and its blended oils fed rats showed significantly elevated ALA (5.6 and 2.6–3.1%), eicosapentaenoic acid (3.0 and 2.1–2.5%) content and decreased arachidonic acid (AA) in total fatty acids of platelets compared to native oils fed rat. Further, thromboxane B<sub>2</sub> (TXB<sub>2</sub>) levels were decreased by 25% in SFO+GCO fed rats, 20% in RBO+GCO fed rats, 20% in SESO+GCO fed rats, and 29% in SFO+FLAX fed rats respectively compared to respective native oils fed rats. Thus, dietary feeding of n-3 polyunsaturated fatty acid (PUFA) rich GCO and its blended vegetable oils substituted AA with ALA in the platelet plasma membrane, reduced aggregation of platelets by modulating platelet thromboxane A<sub>2</sub> in rat platelets.

**Keywords:** alpha-Linolenic acid, Blending of vegetable oils, aggregation of platelets, cardiovascular diseases, garden cress oil, Thromboxane B<sub>2</sub>

### Introduction

Digestibility of lipids is affected by their fatty acid profile, unsaturated lipids absorb more compared to saturated lipids [1]. Dietary lipids are known to influence the membrane lipid composition, plasma lipoprotein concentrations, liver metabolism, structure and functions of certain tissues, depending on their constitutive unsaturated or saturated fatty acid contents [2, 3]. Most of the vegetable oils we consume are rich in n-6 PUFA and relatively deficient in n-3 PUFA. N-3 PUFAs namely ALA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential fatty acids with many health beneficial properties [4, 5]. EPA and DHA are the long chain PUFA available in fish and krill oil only. Among plants, Garden cress seeds contain 24% of oil in which 32–34% is ALA. Garden cress seed oil (GCO) is fairly stable oil due to the presence of a high concentration of antioxidants viz., alpha tocopherol, beta carotene, phytosterol and phenolic acids [6, 7]. Dietary feeding of GCO and its blended oils showed significant hypolipidemic effect and significantly enhanced the natural antioxidant status, radical scavenging activity and also antioxidant enzymes in rats [6]. GCO and its blended oil fed rats showed significant accumulation of ALA in all the tissue and it was metabolized to long chain polyunsaturated fatty acid (LCPUFAs) viz., EPA and DHA in serum, liver, heart and brain [8].

Linoleic and  $\alpha$ -linolenic acids are converted to long chain fatty acids through desaturation and elongation process in

liver. The long chain fatty acids build lipid mediators such as eicosanoids that regulate immune and inflammatory responses. Such hormones from 20 carbon atoms long and include prostaglandins, leukotrienes and thromboxanes [9]. The parent fatty acids for eicosanoids are arachidonic acid (AA) from n-6 PUFAs family and eicosapentaenoic acid (EPA) from n-3 PUFAs family. The eicosanoids from n-6 PUFAs cause high risk of asthma, allergic rhinitis, arthritis, psoriasis, colitis and other inflammatory diseases. The functions of the eicosanoids of n-3 PUFAs have the opposite effects [10, 11]. Therefore eating oils containing the proper ratio of n-6 to n-3 PUFAs will supply the body with essential fatty acids ideally for metabolism and will reduce the risk of host diseases including CVD, cancer, diabetes, obesity, arthritis, and asthma [9]. Increased aggregation of platelets is a major risk factor for heart attacks, stroke and thrombosis. LCPUFA, EPA and DHA are reported to modulate aggregation of platelets, co-administration of aspirin with n-3 PUFA inhibited the platelet aggregation and prevented cardiovascular diseases [12, 13]. N-3 fatty acids influence the hemostasis and maintain the bleeding time [14]. Thus, platelets have become key targets to prevent cardiovascular diseases. Collagen, thrombin, thromboxane A<sub>2</sub>, ADP and biological amines like epinephrine, serotonin are the key activators involved in aggregation of platelets. Among them thrombin and collagen are the potent activators of platelets. The antithrombotic therapies that prevent aggregation of platelets is well established. We have earlier

reported the *in vivo* and *in vitro* modulation of aggregation of platelets and eicosanoids (thromboxane B<sub>2</sub>, leukotriene C<sub>4</sub>) by spice active - eugenol and  $\alpha$ -linolenic acid rich GCO seed oil in Wistar rats. Eugenol and GCO showed synergistic effect against aggregation of platelets and thromboxane B<sub>2</sub> levels in spleen and lung tissues of Wistar Rats.<sup>[15]</sup> In this study, we report the modulatory effect of GCO and its blended oils on agonists induced *ex vivo* aggregation of platelets in rats.

## Materials and Methods

### Materials

Garden cress seeds were procured from local commercial suppliers, refined sunflower oil (SFO), Sesame seed oil (SESO), refined rice bran oil (RBO), flax seed oil (FLAX) and starch were procured from local supermarket. Thromboxane B<sub>2</sub> (TXB<sub>2</sub>) was purchased from Cayman chemical co, Ann Arbor, MI, USA. All other chemicals and solvents used in the experiments were of analytical grade.

### Methods

#### Extraction of GCO

Authenticated Garden cress seeds were air dried, flaked in a roller flaker, powdered and extracted using a hydraulic press at the pilot plant facility of the institute to obtain fresh cold pressed oil. The oil was weighed and stored in dark container under nitrogen at -20° C until further use<sup>[7]</sup>.

#### Experimental animals and diet

Male Wistar rats (OUTB—Wistar, IND-cft (2c)) weighing 43 ±1.7 g, were used in this study. The experimental protocol adopted was approved by the Institute's Animal Ethical Committee (IAEC No. 191/11). Rats were divided into eight groups (8 rats/group) by random distribution, housed in individual cages under a 12 h light/dark cycle in animal house facility maintained at 25±2°C and 40–60% relative humidity. Rats were fed AIN-76 diet containing native oils GCO, RBO, SFO, SESO and GCO blended oils (MUFA/FUFA=1) of SFO, RBO, FLAX and SESO [Table 1]. Rats had free access to respective diets and water at all times throughout the study. After 60 days on specified diets, rats were fasted overnight and sacrificed under diethyl ether anesthesia. Blood was drawn by cardiac puncture and immediately used for separation of platelets.

#### Preparation of platelet rich plasma and serum

Blood was drawn by cardiac puncture, collected in plastic tubes containing sodium heparin (1000 units/mL) as anticoagulant at a ratio of 9:1 v/v<sup>[16]</sup>.

#### Measurement of aggregation of platelets

Aggregation of platelets was determined by the turbidometric method using a Chronolog Aggregometer. Aggregation of PRP was performed at 37°C using a computerized dual channel Chronolog Aggregometer (Chrono-Log Corporation, Havertown, PA) as previously described<sup>[16]</sup>. Data were expressed as percent inhibition of aggregation.

#### Estimation of fatty acids in platelets

Platelet rich plasma was obtained as described above. The platelets were separated from plasma by centrifuging for 10

min at 1100 g. The platelets were washed twice in tyrodes buffer pH 7.4. The platelet pellet was suspended in the tyrodes buffer and lipid was extracted as described earlier<sup>[17]</sup>. The extracted lipid were saponified with 0.5 M KOH, methylated with 40% boron trifluoride in methanol<sup>[18]</sup>. The methyl esters of fatty acids (FAMES) were extracted with hexane. Fatty acid analysis was carried out in Shimadzu gas liquid chromatograph (GC-14B) method described by<sup>[8]</sup>. The results are expressed as relative percentage peak area of individual fatty acids.

#### Estimation serum thromboxane B<sub>2</sub>

Collected serum was used for estimation of TXB<sub>2</sub> by HPLC method described by<sup>[16]</sup>. TXB<sub>2</sub> levels were quantified by comparing with authentic standard. Data are expressed as pg/mL of serum.

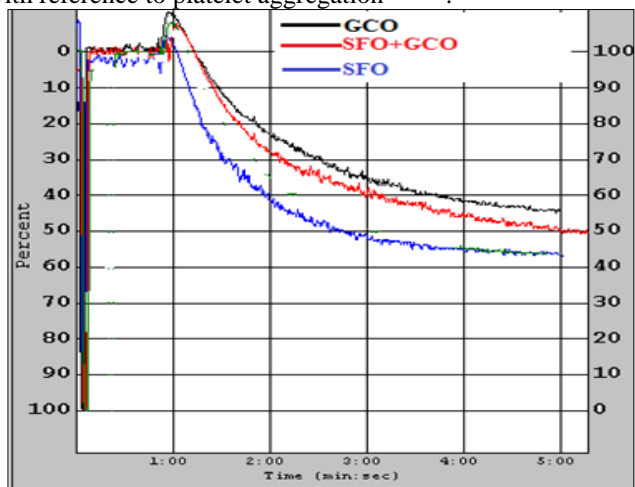
#### Statistical analysis

Data are expressed as mean ± SD and analyzed by one way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons test for significance at P< 0.05 using Graphpad statistical software (Graphpadinstat).

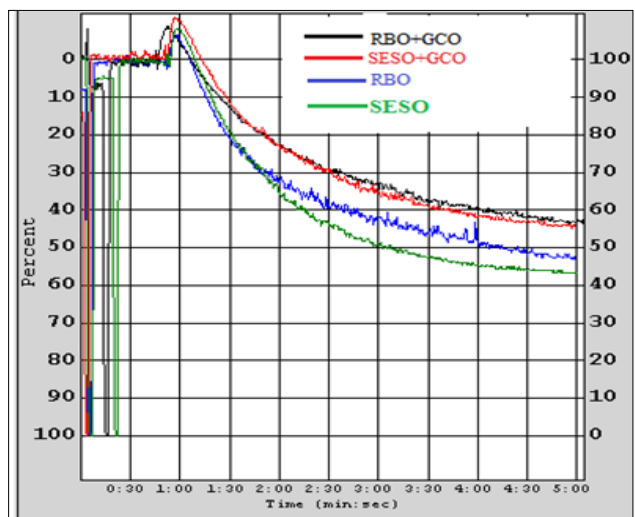
## Results and Discussion

The fish-derived omega-3 fatty acids namely EPA and docosahexaenoic acid (DHA) have demonstrated strong cardio protective effects.<sup>[19,20,21,22,23]</sup> EPA and DHA supplementation has been shown to reduce platelet aggregation, and blood pressure.<sup>[22]</sup> Dietary feeding of GCO decreased ADP and collagen induced aggregation of platelets by 31% and 35.6% respectively, compared to SFO fed rats (Table 2). In blended oils fed rats, ADP induced aggregation of platelets was reduced by 19.6% in SFO+GCO group (Fig-1), 14.2% in RBO+GCO group and 16.8% in SESO+GCO group compared to SFO, RBO and SESO groups (Fig-2). Similarly, collagen induced aggregation of platelets was significantly decreased by 19-28% in GCO blended oil fed groups compared to the respective native oils fed rats. (Table 2). AA is the major substrate for proinflammatory eicosanoids like prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and TXA<sub>2</sub> which aggravate the inflammation in thrombotic stroke. Replacement of AA with ALA in the platelets, the metabolites of ALA namely EPA and DHA in platelets thus reduces the inflammation associated with thrombotic stroke. Recent studies have revealed cardiovascular disease (CVD) preventive effect of ALA vs EPA+DHA, and opined that an increase in dietary ALA decreases the CVD risk<sup>[24, 25]</sup>. However, randomized controlled clinical trials are necessary to confirm the health benefits of ALA. It is unclear, whether the effect is due to the action of ALA itself or ALA being a precursor for EPA+DHA. In a critical review of human studies, the conversion of ALA to EPA was estimated to be a modest of 8%; while the extent of conversion to DHA was much smaller at 0.05%.<sup>[26]</sup> The consensus is that conversion of ALA to EPA+DHA in humans is limited with an average overall conversion rate of about 4-5%.<sup>[27, 25]</sup> Most of the studies on ALA was other than platelet aggregatory risk factors like low density Lipoproteins (LDL), total cholesterol levels and inflammatory mediators etc. As per our knowledge, very few studies have been conducted on the role ALA on CVD risk

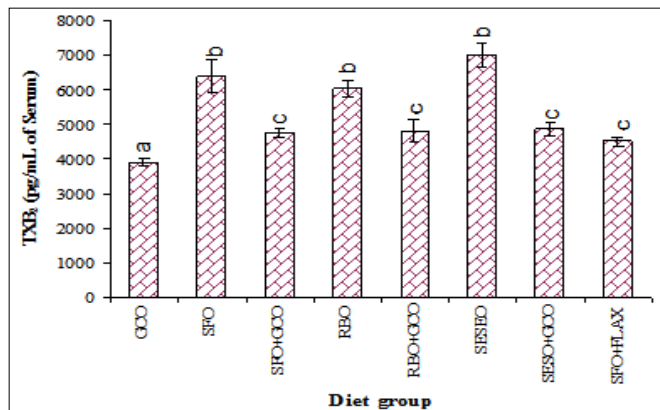
with reference to platelet aggregation [13, 15].



**Fig 1:** Effect of feeding of GCO, SFO and SFO+GCO on ADP induced platelet aggregation



**Fig 2:** Effect of feeding of RBO, SESO, SESO+GCO and SESO+GCO on ADP induced platelet aggregation



Values are mean  $\pm$  SD of 4 rats). Values sharing the same type of alphabet on bar are not statically significant at ( $p < 0.05$ ). Garden cress oil; SFO: Sunflower oil; RBO: Rice bran oil; SESO: Sesame oil; FLAX: Flax seed oil.

**Fig 3:** Serum TXB<sub>2</sub> levels of rat fed with GCO and its blended oils

### Serum TXB<sub>2</sub> levels of rat fed with GCO and its blended oils

Serum TXB<sub>2</sub> levels were altered by feeding GCO and its blends in rat platelets. Serum TXB<sub>2</sub> level was significantly decreased by 37% in rats GCO fed rats. In blended oil groups the TXB<sub>2</sub> level was reduced by 25% in SFO+GCO group, 20% in RBO+GCO group 31% in SESO+GCO group and 29% in SFO+FLAX group compared to respective n-6 rich native oils fed rats. (Fig. 1). In our study the TXB<sub>2</sub> levels in serum was decreased by 37% in GCO group than SFO group. Dietary feeding of flax seed oil supplemented in spray dried milk powder inhibited platelet aggregation by lowering AA levels by 44 % in platelets and serum TXA<sub>2</sub> by 35% of total fatty acids with respect to controls in rats. The results of the present study are consistent with previous findings but at much lower concentrations of ALA in blended oils compared to ALA rich flax seed oil. [13] AA (n-6 PUFA) is an important fatty acid in platelet phospholipid, and it is a substrate for cyclooxygenase for the production of potent platelet proaggregator eicosanoid TXA<sub>2</sub>.

### Effect of GCO and its blended oils on fatty acid composition of platelets

Fatty acid composition of platelet of rats fed with GCO and its blended oils is presented in Table 3. The platelets of GCO and blended oils fed rats showed highest ALA content of 5.9% and 2.6% and 3.1% respectively of total fatty acids, compared to native oils. The EPA levels in GCO (3.0%) and in blended oils SFO+GCO (2.1%), RBO+GCO (2.5%) and SESO+GCO (2.4%) was significantly higher compared to native oils. Linoleic acid (18:2) content was significantly decreased in GCO and blended oils fed rats. AA content in GCO fed rats was decreased by 54% and in blended oils it was also decreased by 11 % and 25% of total fatty acids compared to respective native oils fed rats. The DHA content was detected in traces in platelets of GCO and all blended oils fed rats. In our study the platelet AA levels were decreased by 54% in GCO fed group compared to SFO fed control group. In GCO blended oils fed group the Platelet AA levels were decreased by 11-25 % of total fatty acids compared to their respective control. Thus our results show a better absorption and metabolism of ALA in GCO oil compared to flax seed oil where the platelet AA levels were decreased by 45% [13]. N-3 PUFA,  $\alpha$ -linolenic acid (ALA), is present in plant seeds including flaxseed, chia, camelina, garden cress and walnuts. However, the beneficial effect of ALA intake on CHD risk is less well-established [28]. Comprehensive studies on health beneficial effects of ALA are urgently needed, because of the low cost and greater potential for global supply of ALA from plant source as opposed to EPA and DHA from fish oil which is declining due to over exploitation of fishing and contamination of toxic heavy metals.

Further, GCO and its blended oil contain other bioactive such as  $\alpha$ -tocopherols, carotenoids and phenolic acids [6] which may contribute to the antiplatelet activity of GCO. However, further studies are needed to confirm the effect of minor components present in GCO on platelet aggregation.

**Table 1:** Composition of diet supplemented with GCO and its blended oils

Diet composition g/kg	Diet groups								
	GCO	SFO	SFO + GCO	RBO	RBO + GCO	SESO	SESO + GCO	SFO + FLAX	
Casein	200	200	200	200	200	200	200	200	
Corn starch	500	500	500	500	500	500	500	500	
Sucrose	100	100	100	100	100	100	100	100	
Cellulose	50	50	50	50	50	50	50	50	
Mineral mix <sup>a</sup>	35	35	35	35	35	35	35	35	
Methionine	2	2	2	2	2	2	2	2	
Choline chloride	3	3	3	3	3	3	3	3	
Vitamin mix <sup>b</sup>	10	10	10	10	10	10	10	10	
Oil	GCO	100	-	50	-	40	-	40	-
	SFO	-	100	50	-	-	-	-	65
	RBO	-	-	-	10	60	-	-	-
	SESO	-	-	-	-	-	100	60	-
	FLAX	-	-	-	-	-	-	-	35

**Table 2:** ADP and collagen induced aggregation of platelets in GCO and its blended oils fed rats

Diet group	% of Aggregation		Rate of aggregation	
	ADP	Collagen	ADP	Collagen
GCO	42± 2 <sup>a</sup>	34.5±1.5 <sup>a</sup>	8.4±0.4 <sup>a</sup>	6.9±0.3 <sup>a</sup>
SFO	61±3 <sup>b</sup>	53.5±2 <sup>b</sup>	12.2±0.6 <sup>b</sup>	10.7±0.4 <sup>b</sup>
SFO+GCO	49±1.5 <sup>c</sup>	40.5±1 <sup>c</sup>	9.8±0.3 <sup>c</sup>	8.1±0.2 <sup>c</sup>
RBO	56±3 <sup>d</sup>	48.0±2.0 <sup>b</sup>	11.2±0.6 <sup>d</sup>	9.6±0.4 <sup>b</sup>
RBO+GCO	48±1 <sup>c</sup>	38.5±1 <sup>c</sup>	9.6±0.2 <sup>c</sup>	7.7±0.2 <sup>c</sup>
SESO	60±2.5 <sup>b</sup>	49.5±1.5 <sup>b</sup>	12.0±0.5 <sup>b</sup>	9.9±0.3 <sup>b</sup>
SESO+GCO	50±1 <sup>c</sup>	35.5±2 <sup>a</sup>	10.0±0.2 <sup>c</sup>	7.1±0.4 <sup>a</sup>
SFO+FLAX	52±1.5 <sup>cd</sup>	37.5±1.5 <sup>ac</sup>	10.4±0.3 <sup>cd</sup>	7.5±0.3 <sup>ac</sup>

Values are mean ± S.D of 4 rats. Values in the same row with different superscript are significantly different at P<0.01, (Rate of aggregation was calculated dividing % aggregation for 5 min. Garden cress oil; SFO: Sunflower oil; RBO: Rice bran oil; SESO: Sesame oil; FLAX: Flax seed oil.

**Table 3:** Fatty acid composition of platelets of GCO and its blended oils fed rats

Fatty acid	Platelet fatty acid composition (%)							
	GCO	SFO	SFO+GCO	RBO	RBO+GCO	SESO	SESO+GCO	SFO+FLAX
14:0	1.2±0.1 <sup>a</sup>	0.7±0.2 <sup>b</sup>	0.9±0.1 <sup>b</sup>	0.5±0.4 <sup>b</sup>	0.6±0.1 <sup>b</sup>	0.2±0.1 <sup>c</sup>	0.4±0.3 <sup>b</sup>	0.3±0.1 <sup>bc</sup>
16:0	23.5±0.9 <sup>a</sup>	21.6±0.8 <sup>a</sup>	25.7±0.8	24.6±1.1	25.4±1.2	23.8±1.3	24.3±0.8	26.3±1.4
16:1	2.7±0.2 <sup>a</sup>	2.3±0.2 <sup>a</sup>	2.3±0.1 <sup>a</sup>	2.4±0.2 <sup>a</sup>	2.1±0.1 <sup>a</sup>	1.9±0.1 <sup>a</sup>	2.7±0.1 <sup>a</sup>	1.5±0.1 <sup>b</sup>
18:0	23.7±0.8 <sup>a</sup>	25.6±1.2 <sup>a</sup>	22.0±1.2 <sup>a</sup>	26.8±1.5 <sup>a</sup>	24.1±0.9 <sup>a</sup>	25.8±1.2 <sup>a</sup>	22.5±0.9 <sup>a</sup>	23.7±0.6 <sup>a</sup>
18:1	28.2±0.6 <sup>a</sup>	29.3±1.6 <sup>a</sup>	29.4±1.0 <sup>a</sup>	28.5±1.7 <sup>a</sup>	28.0±1.4 <sup>a</sup>	30.9±0.9 <sup>a</sup>	30.2±1.6 <sup>a</sup>	29.5±1.1 <sup>a</sup>
18:2	5.7±0.3 <sup>a</sup>	10.8±0.6 <sup>b</sup>	7.1±0.5 <sup>c</sup>	9.6±0.6 <sup>b</sup>	6.8±0.5 <sup>a</sup>	8.1±0.6 <sup>c</sup>	6.6±0.1 <sup>a</sup>	7.2±0.5 <sup>ac</sup>
18:3	5.9±0.2 <sup>a</sup>	Nd <sup>b</sup>	2.3±0.2 <sup>c</sup>	Nd	3.0±0.2 <sup>c</sup>	Nd <sup>b</sup>	3.4±0.1 <sup>c</sup>	3.4±0.1 <sup>c</sup>
20:4	4.6±0.1 <sup>a</sup>	9.7±0.3 <sup>b</sup>	7.3±0.2 <sup>c</sup>	7.6±0.3 <sup>c</sup>	6.8±0.2 <sup>d</sup>	9.3±0.4 <sup>b</sup>	7.0±0.3 <sup>c</sup>	6.2±0.2 <sup>d</sup>
20:1	1.5±0.3 <sup>a</sup>	Nd <sup>b</sup>	0.5±0.1 <sup>c</sup>	Nd <sup>b</sup>	0.6±0.1 <sup>c</sup>	Nd <sup>b</sup>	0.8±0.2 <sup>c</sup>	Nd <sup>b</sup>
20:5	3.0±0.2 <sup>a</sup>	Nd <sup>b</sup>	2.1±0.2 <sup>c</sup>	Nd <sup>b</sup>	2.5±0.1 <sup>c</sup>	Nd <sup>b</sup>	2.4±0.4 <sup>c</sup>	1.8±0.2 <sup>c</sup>
22:6	0.6±0.1 <sup>a</sup>	Nd <sup>b</sup>	0.4±0.1 <sup>a</sup>	Nd <sup>b</sup>	0.2±0.1 <sup>c</sup>	Nd <sup>b</sup>	0.3±0.1 <sup>c</sup>	0.2±0.1 <sup>c</sup>
ΣSFA	48.4	47.9	49.1	51.9	50.1	49.8	47.2	50.3
ΣMUFA	33.6	31.6	32.2	30.9	31.3	32.8	33.8	31.2
ΣPUFA	19.2	20.5	19.2	17.2	19.3	17.4	19.7	18.8
n-6/n-3	9.7	20.5	14.4	17.2	13.6	17.4	13.6	13.4

Values are means ± SD (n=4). Means not sharing a common superscript letter in a row are significantly different at p < 0.05. Nd -Not detected. Garden cress oil; SFO: Sunflower oil; RBO: Rice bran oil; SESO: Sesame oil; FLAX: Flax seed oil.

### Conclusion

This is the first report that the edible oil blends with ALA from GCO decreases the aggregation of platelets by decreasing AA and TXA<sub>2</sub> levels in rats. Thus ALA blended oils have health beneficial effects of decreasing platelet aggregation by modulating fatty acid profile and proaggregatory eicosanoids in platelets and prevent atherothrombotic disease.

### Acknowledgments

The authors thank University Grant Commission (UGC) New Delhi, for providing Senior Research Fellowship, KAN

acknowledges the financial support in the form of a Project (SR/SO/HS-0005/2010) awarded by the Department of Science and Technology (DST), New Delhi, India

### Conflict of interest

The authors declare no conflict of interest in this publication.

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