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ORIGINAL CONTRIBUTION

Comparative study of hypocholesterolemic and hypolipidemic effects of conjugated linolenic acid isomers against induced biochemical perturbations and aberration in erythrocyte membrane fluidity

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Abstract
Aim of study The purpose of the study was to evaluate hypolipidemic and hypocholesterolemic activities of conjugated linolenic acid (CLnA) isomers, present in bitter gourd and snake gourd seed, in terms of amelioration of plasma lipid profile, lipoprotein oxidation and erythrocyte membrane fluidity after oral administration.
Methods Male albino rats were divided into six groups. Group 1 was control, and others were induced with oxidative stress by oral gavage of sodium arsenite (Sa). Group 2 was kept as treated control, and groups 3–6 were further treated with different oral doses of seed oils to maintaining definite concentration of CLnA isomers (0.5 and 1.0% of total lipid for each CLnA isomer).
Results CLnA isomers normalized cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride contents in plasma and body weight of experimental rats and decreased cholesterol synthesis by reducing hepatic HMG-CoA reductase activity. Administration of Sa caused alteration in erythrocyte membrane fluidity due to increase in cholesterol and decrease in phospholipid content. Tissue cholesterol and lipid contents were also increased by Sa administration. These altered parameters were reversed by experimental oil administration. Protective effect of CLnA isomers on erythrocyte morphology was observed by atomic force microscopy (AFM). Fatty acid composition of erythrocyte membrane showed decrease in polyunsaturated fatty acid (PUFA) and increase in arachidonic acid content after Sa administration, which was normalized with the treatment of these oils. Supplementation of CLnA isomers restored erythrocyte membrane (EM) lipid peroxidation and lipoprotein oxidation.
Conclusion CLnA isomers, present in vegetable oils, showed potent hypolipidemic and hypocholesterolemic activities against biochemical perturbations.

Keywords Conjugated linolenic acid · α-eleostearic acid · Punicic acid · Hypocholesterolemic · Membrane fluidity · Hypolipidemic

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Introduction

Bitter gourd (*Momordica charantia*) and snake gourd (*Trichosanthes anguina*) are the two common vegetables of Asia and widely consumed by the Asian people especially Indians. The seeds obtained from these two vegetables contain appreciable amounts of oils that are rich in α-eleostearic acid and punicic acid, two isomers of conjugated linolenic acid (CLnA), respectively. Bitter gourd seed oil contains more than 50% of α-eleostearic acid (33% *cis* and 66% *trans* molecular composition), and snake gourd seed oil contains more than 40% of punicic acid (66% *cis* and 33% *trans* molecular composition) [1, 2]. Hypocholesterolemic and antioxidant activities of these CLnA isomers have already been extensively studied, and those studies have revealed that CLnA isomers exhibit hypocholesterolemic activity even at very lower doses [1, 3]. Dhar et al. had [3] observed that α-eleostearic acid

significantly reduces plasma lipid peroxidation, lipoprotein peroxidation and erythrocyte membrane lipid peroxidation in both diabetic and non-diabetic blood samples.

Recent findings indicated that some of medicinal herbs or drugs, in addition to their lipid-lowering ability, can also increase the resistance of plasma lipoprotein to oxidation that may contribute to their effectiveness at preventing atherosclerotic disease [4, 5]. It has been known for many years that the type of fat in the diet influences blood lipid levels, and consequently, the risk of the development of atherosclerosis and related cardiovascular diseases [5, 6]. Early studies showed that dietary intake of n-6 polyunsaturated fatty acids, which are abundant in vegetable oils, is inversely related to the incidence of cardiovascular disease. Dietary linoleic acid (C18: 2 n-6) serves as a precursor for the biosynthesis of arachidonic acid (C20: 4 n-6), the substrate for eicosanoid synthesis and thus long been accepted as having hypocholesterolemic effects [6]. Recent studies showed that linolenic acid derivatives, particularly gamma-linolenic acid, were found to be even more potent in reducing blood cholesterol in humans and rats [7]. Those studies revealed that vegetable oil rich in polyunsaturated fatty acids are able to decrease the plasmatic levels of very-low-density lipoprotein (VLDL) and low-density lipoprotein cholesterol (LDL-C) and to increase the plasmatic level of high-density lipoprotein cholesterol (HDL-C) [8, 9].

Erythrocytes, potentially powerful promoters of oxidative processes, are extremely susceptible to oxidative damage as a result of the high polyunsaturated fatty acid content of their membranes and high cellular oxygen and hemoglobin (Hb) concentrations [10]. As is well known, lipid peroxidation (LPO) is the outcome of free radical–mediated chain oxidation reaction of membrane polyunsaturated fatty acids (PUFAs), which disrupts the structural and protective functions of cell membranes, and as a consequence, various pathological events are implicated as a result of this oxidation [11]. Literatures showed that dietary fatty acids may induce extensive modification in the fatty acid composition of erythrocyte membranes [12, 13].

There is an increasing interest in the protective biochemical function of naturally occurring antioxidants in biological systems and on the mechanism of their action. Several plant constituents have been proven to possess considerable physiological beneficial activity. In our previous studies, it has been observed that different isomers of CLnA are able to combat the induced oxidative stress and genotoxicity generated by ingestion of sodium arsenite, at different doses by reducing lipid peroxidation and improving antioxidant enzymes level [14, 15]. In extension to our previous studies, present study has been undertaken to evaluate the hypocholesterolemic and hypolipidemic activities of CLnA isomers in terms of normalization of plasma lipid profile and erythrocyte membrane fluidity on oxidative stress–induced subjects. In some parts of Asia and other developing countries, arsenic contamination and toxicity have become the source of wide range of health hazards starting from cardiovascular disease to dermatological lesions, chromosomal abnormalities so on, and oxidative stress has been identified as an important mechanism of arsenic toxicity and carcinogenicity [16]. Therefore, in this study, we have used sodium arsenite (Sa) as an stress-inducing agent.

Materials and methods

Materials

Sodium arsenite ($NaAsO_2$) was purchased from Sd Fine-Chem limited, Mumbai, India. The dose of Sa (10 mg/kg BW) was chosen on the basis of the previous studies [17–19] done by other workers.

All other chemicals used were of analytical grade and procured from Merck India Ltd., Mumbai, India.

Extraction and quantification of CLnA isomers

Authentic bitter gourd seeds and snake gourd seeds were obtained from the local market of Kolkata, India. Seeds were crushed, and the oil was extracted with solvent petroleum ether. The extracts of the sample were filtered and then concentrated by rotary evaporator. Free fatty acids present in the oil were quantified and then were removed by miscella refining process by adding 10% NaOH solution (20% excess of the required amount) at 40 °C for 30 min [20]. The soap formed was removed by centrifugation, and the organic phase was washed with water. Deacidified oil was recovered by distillation under vacuum and dried. The refined oil was then bleached with tonsil earth (1% w/w) obtained from P.T. Sud-Chemic (Jakarta, Indonesia) and activated carbon (0.2% w/w), supplied by E. Merck India Pvt. Ltd. (Bombay, India), at 60 °C under vacuum for 20 min. After the bleaching operation, the oil was recovered by vacuum filtration and stored at −20 °C under nitrogen.

Analysis of fatty acid compositions

The fatty acid (FA) compositions of the different dietary oils were determined by gas liquid chromatography (GLC, Agilent Technologies India Pvt. Ltd., Mumbai) techniques after converting the oils (triglycerides) into their corresponding fatty acid methyl esters (FAME) as per Ichihara et al. [21]. The amount of cis-trans isomers present in the oil was determined by dissolving the oil in cyclohexane and measuring the absorbance over wavelength range of

200–300 nm by a UV-Vis Spectrophotometer (Shimadzu corporation, Japan) [22].

Dietary fat blends

Sunflower oil was obtained from Corn Agro Limited (Hyderabad, India). Sunflower oil was mixed with bitter gourd seed oil and snake gourd seed oil to give final oil mixtures with 0.5 and 1.0% by weight of α-eleostearic acid and punicic acid contents, respectively. Table 1 shows the final fatty acid composition of dietary oil mixtures. The dose of CLnA isomers (0.5 and 1.0% of total lipid given) was used according to the previous studies done in the same laboratory [1, 3, 14, 15].

Animal experiment

The work was done under the supervision of the Animal Ethical Committee of the Department of Chemical Technology (University of Calcutta). Male albino rats of Charles Foster strain (selected for the authenticity of the strain) were housed in individual cages. The animals were divided into six groups (average body weight 100–110 g) consisting of six animals in each group. The first group served as normal control, received only vehicles (sunflower oil and deionized water) once per day. Rats in the group 2 were treated with sodium arsenite (Sa; 10 mg/kg BW) along with sunflower oil by oral gavages once per day and served as treated control. Rats in the groups 3 and 4 were treated with α-eleostearic acid (0.5 and 1.0% of total lipid given, respectively) along with sodium arsenite (Sa; 10 mg/kg BW) by oral gavage once per day. Rats in the groups 5 and 6 were treated with punicic acid (0.5 and 1.0% of total lipid given, respectively) along with sodium arsenite (Sa; 10 mg/kg BW) by oral gavage once per day. Weekly body weights of the rats of each group were noted.

The rats were fed experimental diets having the following composition: fat-free casein, 18%; fat, 20%; starch, 55%; salt mixture 4% [composition of salt mixture No. 12 (in g) (Sisco Research Laboratories Pvt. Ltd., India): NaCl, 292.5; KH_2PO_4, 816.6; $MgSO_4$, 120.3; $CaCO_3$, 800.8; $FeSO_4 \times 7H_2O$, 56.6; KCl, 1.66; $MnSO_4 \times 2H_2O$, 9.35; $ZnCl_2$, 0.5452; $CuSO_4 \times 5H_2O$, 0.9988; $CoCl_2 \times 6H_2O$, 0.0476] [23]; cellulose, 3%; and one multivitamin capsule (vitamin A I.P. 10,000 units, thiamine mononitrate I.P. 5 mg, vitamin B I.P. 5 mg, calcium pantothenate USP 5 mg, niacinamide I.P. 50 mg, ascorbic acid I.P. 400 units, cholecalciferol USP 15 units, menadione I.P. 9.1 mg, folic acid I.P. 1 mg and vitamin E USP 0.1 mg) per kg of diet. The diets were adequate in all nutrients.

The animals were killed after 21 days under anesthesia and kept in overnight fasting before killing, and the last dose was administered 18 h before killing. Blood was collected from hepatic vein into heparinized tube, and liver and brain were immediately excised, blotted, weighed and stored frozen (−40 °C) for analysis.

Preparation and oxidative sensitivity of erythrocyte membrane (EM) ghost

After plasma separation, the red blood cells (RBC) were washed 3 times by centrifugation at 3,000×g for 10 min with 3 vol of a cooled isotonic solution containing 0.15 M NaCl and 10^{-5} M EDTA. RBC was hemolyzed using hypotonic solution and centrifuged at 20,000×g for 40 min at 4 °C. The supernatant was removed carefully with a Pasteur pipette. The process was repeated two more times. After the last wash step, the supernatant was removed as much as possible, and the loosely packed milky-looking membrane pellet was resuspended at the bottom of the tube using 0.89% NaCl solution. The concentrated membrane solution was taken into a 2-mL screw-capped vial and stored at –40 °C [24].

A modification of the 2-thiobarbituric acid (TBA) test [25] was used to measure the lipid peroxides. A 0.5-mL aliquot of the red blood cell membrane suspension was mixed with 1.0 mL of 10% TCA and 2.0 mL of 0.67% 2-TBA. The mixture was heated at 95 °C for 15 min,

Table 1 Fatty acid composition of dietary oils and oil blends

Dietary fats	FA composition (% w/w)					
	16:0	18:0	18:1	18:2	18:3 (CLnA, 9*c*, 11*t*, 13*t*-18:3)	18:3 (CLnA, 9*c*, 11*t*, 13c-18:3)
Sunflower oil	6.3	3.5	32.4	57.8	–	–
Bitter gourd oil	1.8	31.6	7.5	6.8	52.3	–
Snake gourd oil	6.2	6.1	26.6	21.0	–	40.1
Sunflower + bitter gourd oil (99:1, w/w)	6.3	3.8	32.2	57.2	0.5	–
Sunflower + bitter gourd oil (98:2, w/w)	6.3	4.0	31.9	56.8	1.0	–
Sunflower + snake gourd oil (98.75:1.25, w/w)	6.3	3.5	32.3	57.4	–	0.5
Sunflower + snake gourd oil (97.5:2.5, w/w)	6.3	3.6	32.3	56.9	–	1.0

cooled and centrifuged. The absorbance of the supernatant was measured at 534 nm in a spectrophotometer (Shimadzu, Tokyo, Japan), and the relative amounts of lipid peroxides were expressed in absorbance units, A_{534} nm [26].

Plasma and EM lipid analysis

The lipid component such as total cholesterol [27] of EM ghost and plasma was analyzed using enzymatic kit supplied by Ranbaxy Diagnostics Ltd. (New Delhi, India). Triglyceride (TG) [28], high-density lipoprotein cholesterol (HDL-C) [29] and low-density lipoprotein cholesterol (LDL-C) [30] of plasma were analyzed using enzymatic kits supplied by Ranbaxy Diagnostics Ltd. (New Delhi, India).

Phospholipid [31] of EM ghost was estimated by standard method and expressed as mg/mg of protein.

Measurement of 3 hydroxy 3-methyl gluteryl CoA (HMG-CoA) reductase activity

HMG-CoA reductase activity was measured by an indirect method [32]. One gram of fresh liver tissue was homogenized in 10 mL saline-arsenate solution, and an equal volume of diluted perchloric acid was added and kept for 5 min. After centrifugation at 2,000 rpm for 10 min, 1 mL of the filtrate was utilized for HMG-CoA analysis with 0.5 mL alkaline hydroxylamine (pH 5.5) and 1.5 mL ferric chloride; 1 mL of the filtrate was utilized for measuring the mevalonate with 0.5 mL acidic hydroxylamine (pH 2.1) and ferric chloride. The absorbance was measured at 540 nm.

Lipoprotein oxidation susceptibility (LOS) test

Non-HDL-cholesterol oxidation was carried out by precipitating apoB-containing lipoprotein (low-density lipoprotein and very-low-density lipoprotein) according to Bachorik and Albers [33]. The LOS test was carried out according to the method described by Phelps and Harris [34].

Analysis of fatty acid composition of EM ghost

The total lipids were extracted from EM ghost with chloroform/methanol mixture [35]. The fatty acid (FA) composition of EM ghost was determined by gas liquid chromatography (GLC, Agilent Technologies India Pvt. Ltd., Mumbai) techniques after converting the lipid into their corresponding fatty acid methyl esters (FAME) according to the method as previously described [21].

Analysis of GSH content in erythrocyte lysate

Two mL of heparinized blood was centrifuged for 10 min at 1,000 × g, and the plasma was removed by suction. The erythrocytes were washed twice with phosphate-buffered saline (PBS), pH 7.4. Distilled water (10 mL) was then added, and the erythrocytes were resuspended by agitation and lysed for 2 h at 4 °C. A mixture of chloroform-ethanol (3:5, v/v, 0.8 mL) and 0.3 mL of water was added to the lysate so as to precipitate the hemoglobin, which was centrifuged at 3,000 g for 10 min at 4 °C [36]. The intracellular GSH content in erythrocyte lysate was quantified spectrophotometrically according to the method of Beutlar et al. [37] and expressed as mg/mg of protein.

Erythrocyte morphology study by atomic force microscopy (AFM)

After plasma separation, red blood cells were washed 3 times with phosphate-buffered saline (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) at 4 °C and finally resuspended in PBS to obtain an RBC suspension at 0.1% (v/v) hematocrit. RBC suspensions (0.1%) were incubated at 37 °C for 3 h under gentle shaking. After 3-h incubation, RBC fixation was done by the addition of glutaraldeyde 0.25% (v/v). For AFM study, 50 μL of each suspension was applied to a cleaned glass coverslip and air-dried. The RBCs were then gently rinsed with deionized water to remove salt crystals and air-dried again before analysis.

All AFM studies were carried out with a Veeco Multimode Iva atomic force microscope (Veeco, Multimode, Nanoscope IIIa) equipped with a j-type scanner. Cell surface studies were carried out in tapping mode by the use of RTESP (Si nitride) tip and silicon cantilevers with a resonant frequency (ω_0) of ~312.14 kHz (Veeco, Multimode, Nanoscope IIIa). Several independently produced samples were analyzed, and several areas were studied on each sample. These images were used to evaluate the roughness of the plasma membrane.

Measurement of protein content

Protein content was measured by the Lowry method [38].

Tissue lipid analysis

The total lipids were extracted from liver and brain with chloroform/methanol mixture and estimated gravimetrically [35]. Total cholesterol [27] of liver and brain was analyzed using enzymatic kit supplied by Ranbaxy Diagnostics Ltd. (New Delhi, India).

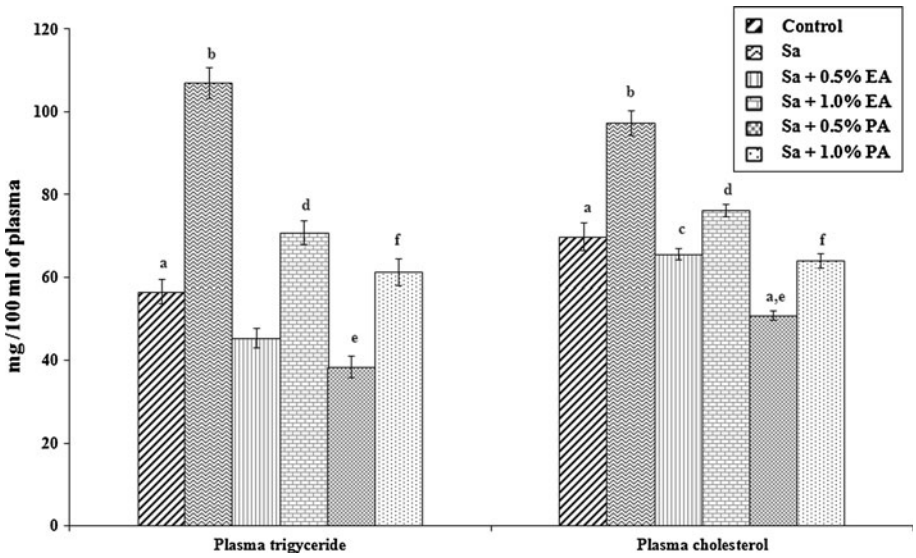

Fig. 1 Effect of CLnA isomers on plasma triglyceride and cholesterol of rats fed blended oils. C, Control; Sa, sodium arsenite (10 mg/kg BW)-treated group; Sa + 0.5% EA, sodium arsenite-treated group fed sunflower oil containing 0.50% α-eleostearic acid; Sa + 1.0% EA, sodium arsenite-treated group fed sunflower oil containing 1.0% α-eleostearic acid; Sa + 0.5% EA, sodium arsenite-treated group fed sunflower oil containing 0.50% punicic acid; Sa + 1.0% EA, sodium arsenite-treated group fed sunflower oil containing 1.0% punicic acid. a = C versus other experimental groups ($P < 0.05$); b = Sa + 0.5% EA versus Sa ($P < 0.05$); c = Sa + 0.5% EA versus Sa + 0.5%PA ($P < 0.05$); d = Sa + 1.0% EA versus Sa ($P < 0.05$); e = Sa + 0.5%PA versus Sa ($P < 0.05$); f = Sa + 1.0%PA versus Sa ($P < 0.05$); $n = 6$

Statistical analysis

The data were expressed as mean ± SEM. A one-way ANOVA was also used for statistical analysis between groups. The F ratio of one-way ANOVA is significant when the P value < 0.05. Tukey's multiple range method [39] was used for comparison.

Results

Effect on plasma lipid profile and body weight

Results showed that administration of Sa increased plasma triglyceride ($P < 0.05$), cholesterol ($P < 0.05$) and LDL-C ($P < 0.05$) contents significantly (Figs. 1, 2) compared to control, but with the supplementation of CLnA isomers, it caused significant reduction in TG, cholesterol, LDL-C contents and restored them almost to control value. Plasma HDL-C content (Fig. 2) decreased significantly ($P < 0.05$) due to the administration of Sa, which was restored completely with the administration of CLnA isomers, and plasma HDL-C content was significantly higher in case of 0.5% α-eleostearic acid-treated group than 0.5% punicic acid-treated group. Maximum reduction in TG and cholesterol occurred in case of 0.5% level of both the isomers. Decrease in plasma cholesterol content was more prominent in case of 0.5% punicic acid-treated group than 0.5% α-eleostearic acid-treated group. Results showed that body weight of Sa-treated animals (Fig. 3) gradually decreased compared to initial body weight before treatment in consecutive 3 weeks though decrease was not significant. In contrast, CLnA-treated animals showed no such change in body weight during experimental period.

Effect on intracellular GSH content of erythrocyte

Intracellular reduced glutathione (GSH) content of erythrocyte (Fig. 4) was decreased significantly ($P < 0.05$) after Sa administration due to oxidative stress, which was reversed after CLnA isomers supplementation. Recovery of erythrocyte GSH content was more efficient in case of 0.5% α-eleostearic acid group than same dose of punicic acid group as GSH content was significantly higher ($P < 0.05$) in case of 0.5% α-eleostearic acid-treated group.

Effect on cholesterol and phospholipid contents of EM ghost

Sa-treated group showed an significant increase ($P < 0.05$) in cholesterol content and decrease $P < 0.05$) in phospholipid content in EM ghost compared to respective control ($P < 0.05$) due to oxidative stress in erythrocyte, and there was complete restoration of EM cholesterol and phospholipid contents after administration of α-eleostearic acid and punicic acid (Table 2). In this respect, there was no difference between the effectiveness of these two isomers.

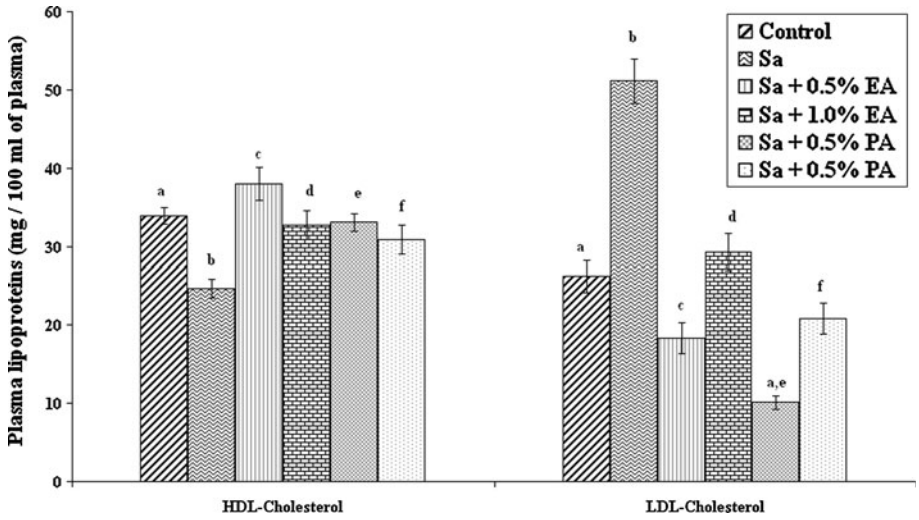

Fig. 2 Effect of CLnA isomers on lipid HDL-cholesterol and LDL-cholesterol of plasma of rats fed blended oils. Other information as in Fig. 1

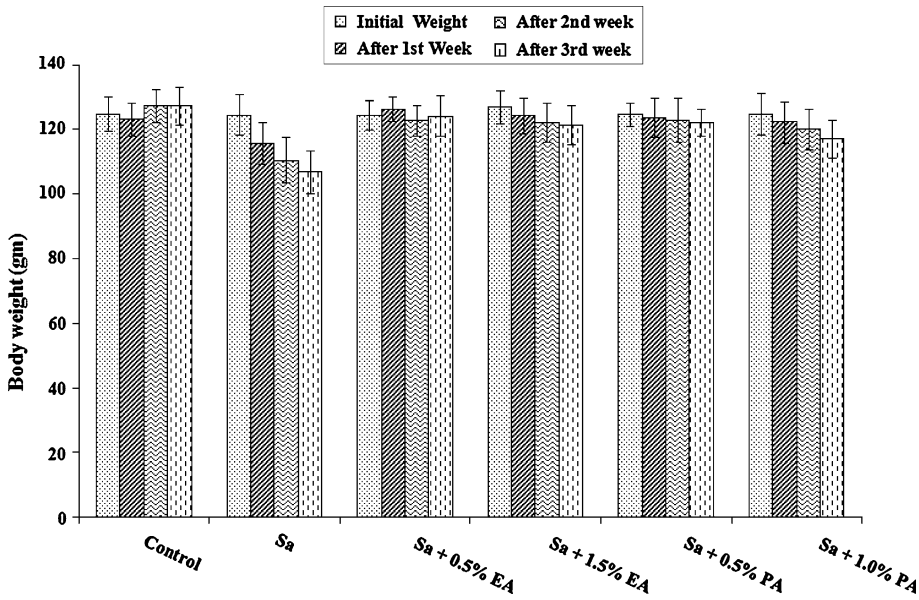

Fig. 3 Effect of CLnA isomers on weekly body weight of experimental groups fed blended products. Other information as in Fig. 1

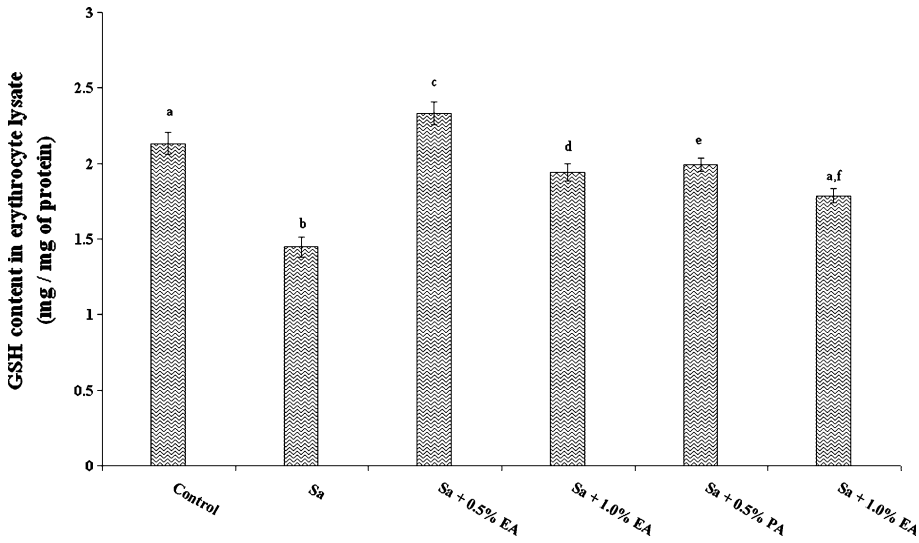

Fig. 4 Effect of CLnA isomers on glutathione (GSH) content of erythrocyte lysate of experimental groups fed blended products. Other information as in Fig. 1

Table 2 Effect of CLnA isomers on total cholesterol and phospholipid content of EM ghost

Experimental group	Parameters	
	EM cholesterol content (mg/mg of protein)	EM phospholipid content (mg/mg of protein)
Control	0.083 ± 0.005[a]	3.47 ± 0.17[a]
Sa	0.149 ± 0.009[b]	2.21 ± 0.17[b]
Sa + 0.5% α-eleostearic acid	0.069 ± 0.007[c]	3.31 ± 0.21[c]
Sa + 1.0% α-eleostearic acid	0.082 ± 0.008[d]	3.16 ± 0.22[d]
Sa + 0.5% punicic acid	0.072 ± 0.005[e]	3.23 ± 0.20[e]
Sa + 1.0% punicic acid	0.084 ± 0.008[f]	3.12 ± 0.10[f]

Sa, Sodium arsenite (10 mg/kg BW)-treated group; Sa + 0.5% EA, sodium arsenite-treated group fed sunflower oil containing 0.50% α-eleostearic acid; Sa + 1.0% EA, sodium arsenite-treated group fed sunflower oil containing 1.0% α-eleostearic acid; Sa + 0.5% EA, sodium arsenite-treated group fed sunflower oil containing 0.50% punicic acid; Sa + 1.0% EA, sodium arsenite-treated group fed sunflower oil containing 1.0% punicic acid

The superscript letters represent statistical significance at $P < 0.05$

All values are Mean ± SEM, $n = 6$

[a] Control versus other experimental groups

[b] Sa + 0.5% EA versus Sa

[c] Sa + 0.5% EA versus Sa + 0.5% PA

[d] Sa + 1.0% EA versus Sa

[e] Sa + 0.5% PA versus Sa

[f] Sa + 1.0% PA versus Sa

Effect on hepatic HMG-CoA reductase activity

Hepatic HMG-CoA reductase activity was measured indirectly by determining the HMG-CoA/mevalonate ratio. Sa-treated rats showed a little increase in HMG-CoA reductase activity as the HMG-CoA/mevalonate ratio was slightly lower than control rats (Table 4), but the difference was not significant. After the administration of vegetable oils containing CLnA isomers, HMG-CoA activity decreased significantly than Sa-treated and control rats. Results showed that HMG-CoA/mevalonate ratio was much higher in CLnA-treated rats than Sa-treated and control rats, which imply that enzyme activity was significantly decreased in CLnA-treated groups than non-CLnA-treated groups. Punicic acid was more efficient in reducing hepatic HMG-CoA reductase activity than α-eleostearic acid.

Effect on oxidation susceptibility of lipoprotein (LOS) and EM lipid peroxidation

Oxidation of lipoprotein as observed from LOS test ($P < 0.05$) and EM lipid peroxidation ($P < 0.05$) increased significantly compared to respective control due to Sa administration. But with the CLnA administration, both the oxidation parameters were restored and the effectiveness of α-eleostearic acid at 0.5% dose with respect to oxidation susceptibility of lipoprotein and EM lipid peroxidation was more efficient than punicic acid at the same dose (Fig. 5).

Effect on fatty acid composition of EM ghost

Fatty acid composition of EM lipid showed that most of the polyunsaturated fatty acids such as eicosapentaenoic acid (EPA; $C_{20:5}$) and docosahexaenoic acid (DHA; $C_{22:6}$) decreased due to oxidative stress induced by arsenite. Administration both the CLnA isomers caused complete restoration of the PUFA levels (Table 3). Although α-eleostearic acid was more efficient in restoring EPA level, 0.5% level of both the isomers showed maximum protective effect in terms of restoration of PUFA. Fatty acid composition of EM lipid of arsenite-treated rats showed increase in arachidonic acid (ARA; $C_{20:4}$) level and decrease in alpha linolenic acid (ALA; $C_{18:3}$) content, which was an indicator of inflammation. Results showed that administration of oils containing CLnA isomers caused significant decrease in ARA content and ALA content compared to Sa-treated rats at different doses, and there was no difference between the effectiveness of these isomers in reducing ARA content though α-eleostearic acid was slightly more effective than punicic acid in terms of restoring ALA content.

Effect on erythrocyte morphology

Atomic force microscopy study of erythrocyte morphology showed changes in the biconcave disk-shaped morphology after Sa treatment compared to control group (Fig. 6).

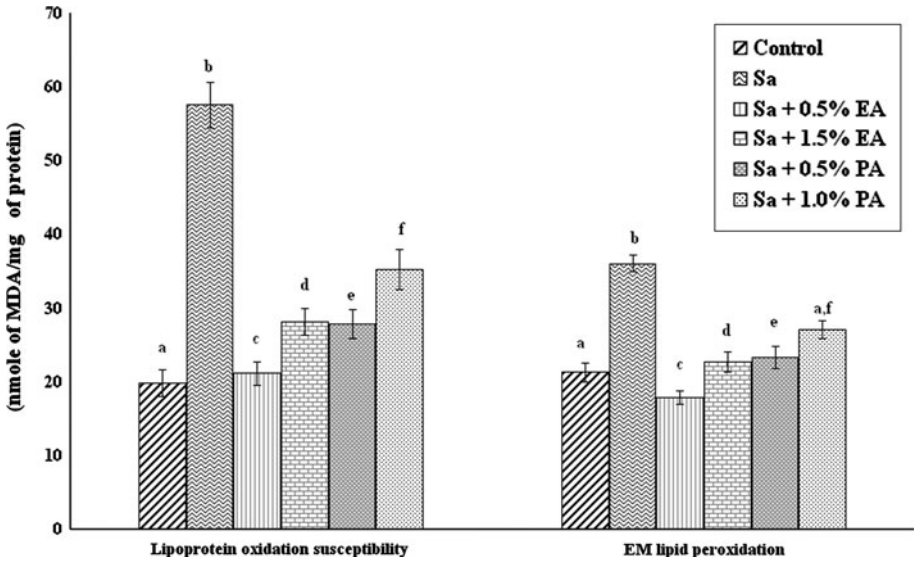

Fig. 5 Effect of CLnA isomers on lipoprotein oxidation susceptibility and EM lipid peroxidation of experimental groups fed blended products. Other information as in Fig. 1

Table 3 Effect of CLnA isomers on fatty acid composition of EM lipid of experimental groups

Fatty acids (%w/w)	Control	Sa	Sa + 0.5% α-eleostearic acid	Sa + 1.0% α-eleostearic acid	Sa + 0.5% Punicic Acid	Sa + 1.0% Punicic Acid
$C_{8:0}$	1.28 ± 0.18	1.98 ± 0.06	1.48 ± 0.22	0.35 ± 0.04	1.27 ± 0.24	1.92 ± 0.17
$C_{10:0}$	5.04 ± 0.71	5.9 ± 0.55	4.81 ± 0.75	4.64 ± 0.74	4.35 ± 0.97	3.98 ± 0.99
$C_{12:0}$	7.19 ± 0.52	8.4 ± 0.31	6.44 ± 0.61	6.07 ± 0.63	5.80 ± 0.91	3.94 ± 1.01
$C_{14:0}$	4.75 ± 0.67	7.22 ± 0.92	4.12 ± 0.61	3.85 ± 0.29	4.14 ± 0.90	3.84 ± 0.62
$C_{16:0}$	14.24 ± 1.12	12.87 ± 0.45	12.91 ± 1.65	14.84 ± 0.42	13.67 ± 0.93	15.40 ± 2.97
$C_{16:1}$	5.75 ± 0.52	5.62 ± 0.13	5.39 ± 0.86	5.44 ± 0.11	4.27 ± 0.33	3.75 ± 0.12
$C_{16:2}$	12.58 ± 0.95	9.48 ± 1.11	12.06 ± 1.30	12.74 ± 1.22	11.46 ± 1.01	9.95 ± 1.02
$C_{18:0}$	5.88 ± 0.42	6.51 ± 0.58	5.77 ± 0.77	6.82 ± 0.83	6.20 ± 0.05	7.40 ± 0.97
$C_{18:1}$	8.95 ± 0.92[a]	5.74 ± 0.28[b]	8.83 ± 0.10[c]	7.77 ± 0.23[d]	8.88 ± 0.89[e]	8.01 ± 0.17[f]
$C_{18:2}$	10.8 ± 1.04[a]	7.84 ± 0.78[b]	11.36 ± 1.10[c]	9.37 ± 0.54[d]	11.36 ± 1.11[e]	10.70 ± 1.02[f]
$C_{18:3\ (ALA)}$	1.81 ± 0.06[a]	0.76 ± 0.06[b]	2.01 ± 0.31[c]	1.56 ± 0.04[d]	1.86 ± 0.14[e]	1.62 ± 0.07[f]
$C_{20:4}$	1.12 ± 0.09[a]	4.84 ± 0.57[b]	1.21 ± 0.14[c]	1.76 ± 0.10[d]	1.11 ± 0.07[e]	1.47 ± 0.19[f]
$C_{20:5}$	4.67 ± 0.78[a]	2.30 ± 0.24[b]	4.90 ± 0.99[c]	4.15 ± 0.31[d]	4.49 ± 0.54[e]	4.08 ± 0.82[f]
$C_{22:1}$	2.48 ± 0.24	3.05 ± 0.19	3.47 ± 0.01	3.23 ± 034	3.43 ± 0.58	3.37 ± 0.52
$C_{22:6}$	1.56 ± 0.11[a]	0.33 ± 0.02[b]	1.47 ± 0.04[c]	1.25 ± 0.12[d]	1.46 ± 0.54[e]	1.39 ± 0.11[f]
$C_{24:1}$	4.53 ± 0.56	1.49 ± 0.15	1.83 ± 0.20	3.02 ± 0.10	2.12 ± 0.27	1.84 ± 0.13
Others	7.38 ± 0.50	15.69 ± 1.95	11.97 ± 1.71	13.16 ± 1.94	14.15 ± 2.25	17.38 ± 2.43

For other abbreviations, see Table 2

The superscript letters represent statistical significance at $P < 0.05$

All values are mean ± SEM, $n = 6$

[a] Control versus other experimental groups

[b] Sa + 0.5% EA versus Sa

[c] Sa + 0.5% EA versus Sa + 0.5% PA

[d] Sa + 1.0% EA versus Sa

[e] Sa + 0.5% PA versus Sa

[f] Sa + 1.0% PA versus Sa

Untreated erythrocytes appeared as typical discocytes, while exposure to Sa resulted in a significant change in the cell shape and distinct echinocyte formation. Upon treatment of cells with Sa, formation of multiple blebs on cell membrane was observed. After CLnA administration, any morphological alterations in the cells were not found.

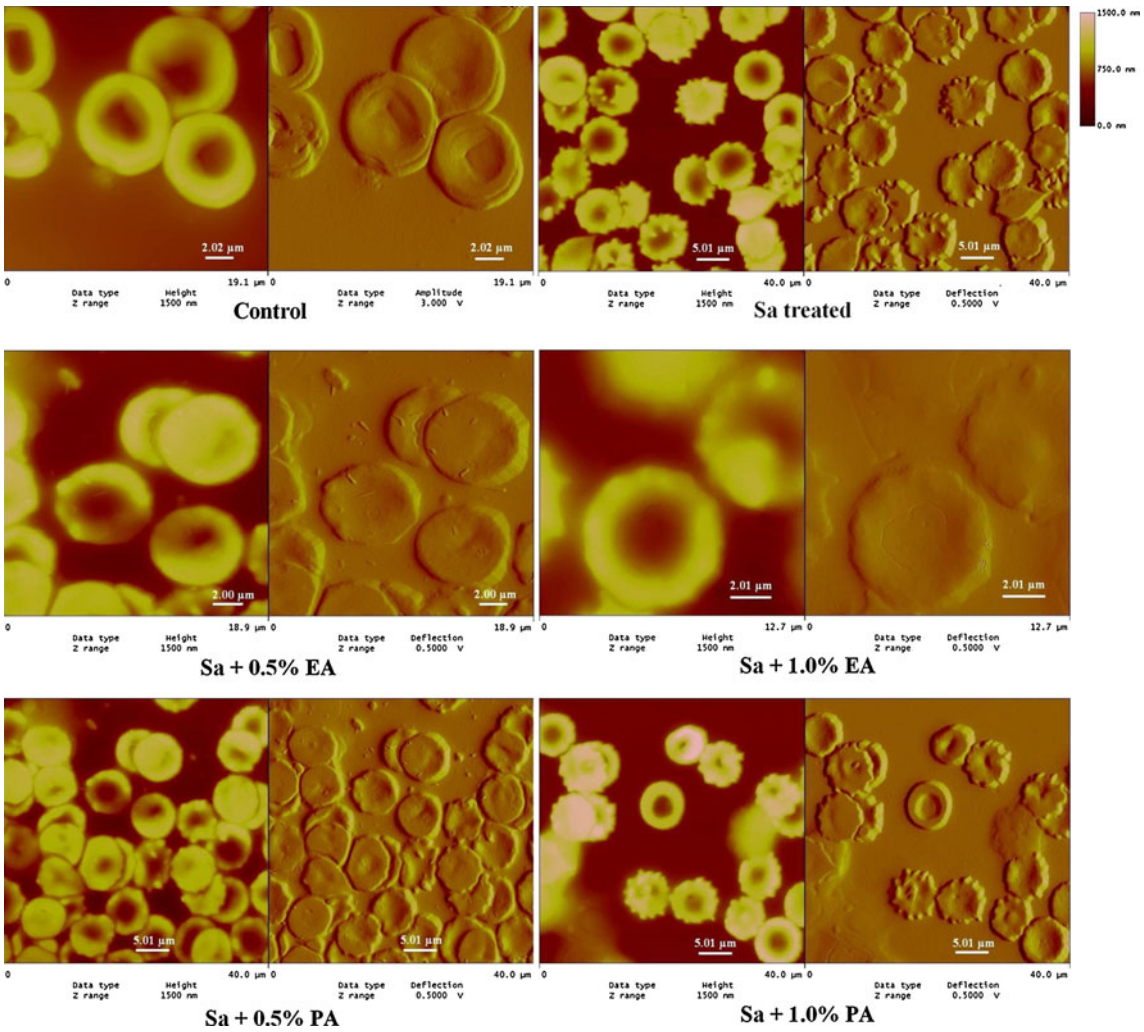

Fig. 6 Effect of CLnA isomers on erythrocyte morphology of experimental groups fed blended products observed by atomic force microscopy. Other information as in Fig. 1

However, under the experimental conditions, CLnA significantly inhibited the Sa-induced morphological alterations.

Effect on total tissue lipid and cholesterol content

Total lipid (Table 4) and total cholesterol (Fig. 7) contents of liver and brain tissue increased significantly ($P < 0.05$) after arsenite treatment, which was completely restored by CLnA, and maximum restoration was at 0.5% level in case of both the isomers. Punicic acid was more effective in reducing cholesterol in liver and brain that α-eleostearic acid at 0.5% level.

Discussion

The hypocholesterolemic effect of dietary CLnA has been observed in rats [1] and of dietary conjugated linoleic acid (CLA) has been observed in hamsters [40]. Reduction in serum low-density lipoprotein cholesterol and triacylglycerol in cholesterol-enriched diet–fed rabbits after supplementation of 0.5 g/d CLA has been reported by Lee et al. [41]. Dietary linoleic acid serves as a precursor for the biosynthesis of arachidonic acid, the substance for eicosanoid synthesis through activity of the enzyme cyclooxygenase and 5-lipoxygenase. CLA has long been accepted as having hypocholesterolemic effects [6]. Our observation of hypocholesterolemic activity of CLnA isomers has been confirmed by another study with some vegetable oils [8]. They have shown that substitution of the intake of saturated fatty acids by unsaturated fat induces a decrease in total cholesterol concentration, VLDL, LDL-C and increase in HDL-C. During cholesterol biosynthesis, HMG-CoA is converted into mevalonate by HMG-CoA reductase, a rate-limiting enzyme of cholesterol biosynthesis. In this study, dietary CLnA significantly decreases HMG-CoA reductase activity and thus reduces biosynthesis of cholesterol in the

Table 4 Effect of CLnA isomers on total lipid content of liver and brain and hepatic HMG-CoA/mevalonate ratio of experimental groups

Experimental group	Parameters		
	Liver total lipid content (mg/g of tissue)	Brain total lipid content (mg/g of tissue)	Hepatic HMG-CoA/ mevalonate ratio
Control	116.28 ± 4.15[a]	98.06 ± 2.36[a]	3.11 ± 0.22
Sa	169.29 ± 4.59[b]	146.17 ± 1.56[b]	2.38 ± 0.25[b]
Sa + 0.5% α-eleostearic acid	105.70 ± 5.39[c]	106.40 ± 2.38[c]	7.37 ± 0.34[a]
Sa + 1.0% α-eleostearic acid	122.92 ± 5.47[d]	114.79 ± 1.71[d]	6.68 ± 0.26[a,d]
Sa + 0.5% punicic acid	114.49 ± 4.58[e]	99.93 ± 2.01[e]	8.64 ± 0.40[a,e]
Sa + 1.0% punicic acid	129.31 ± 5.31[f]	113.79 ± 1.17[f]	7.70 ± 0.52[a,f]

For other abbreviations, see Table 2

The superscript letters represent statistical significance at $P < 0.05$

All values are mean ± SEM, $n = 6$

[a] Control versus other experimental groups

[b] Sa + 0.5% EA versus Sa

[c] Sa + 0.5% EA versus Sa + 0.5% PA

[d] Sa + 1.0% EA versus Sa

[e] Sa + 0.5% PA versus Sa

[f] Sa + 1.0% PA versus Sa

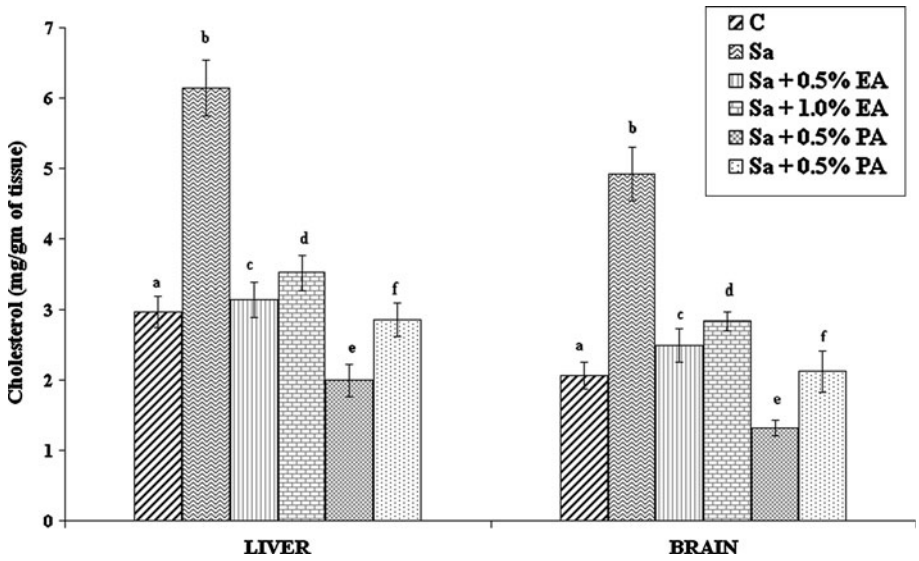

Fig. 7 Effect of CLnA isomers on cholesterol content of liver and brain of experimental groups fed blended products. Other information as in Fig. 1

liver and also decreases hepatic cholesterol content. In addition, dietary CLnA may exert the hypocholesterolemic effect at least in part through a decreased hepatic secretion of very-low-density lipoprotein-cholesterol and thus reduces plasma LDL-C, TG and increases HDL-C. Apart from these, CLnA could inhibit uptake of cholesterol in the gastrointestinal tract or decrease intestinal absorption of cholesterol. A little and insignificant increase in HMG-CoA reductase activity in Sa-treated rats than control is due to increased lipid peroxidation after Sa administration. Due to suppressive activity of CLnA on HMG-CoA reductase, cholesterol biosynthesis in brain cells has also been inhibited, and as a result, concentration of cholesterol in brain is decreased significantly after CLnA administration. Gradual decrease in body weight of treated animal is due to toxic effect of arsenic, which has been circumvented by the administration of dietary CLnA.

Dhar et al. [3] has reported the antioxidant effects of α-eleostearic acid in bitter gourd oil on lipid peroxidation. They have demonstrated that in compounds with higher number of conjugated double bonds, conjugation increased the rate of oxidation, and as a result, in an in vivo

condition, CLnA isomers are also quickly oxidized by picking up free radicals, thereby eliminating or reducing the formation of hydroperoxides. Noguchi et al. [42] demonstrate that bitter gourd oil has potent in vivo hypocholesterolemic action on rat. EM lipid peroxidation and lipoprotein oxidation have been increased significantly due to Sa treatment as observed from LOS test. Reactive oxygen species (ROS) are known to be the initiators of lipid peroxidation [43]. Malondialdehyde (MDA), the well-characterized product of the LPO of erythrocytes, is a highly reactive and bifunctional molecule, which is shown to cross-link erythrocyte phospholipids and proteins to impair a variety of the membrane-related functions, which ultimately lead to diminished erythrocyte survival [44, 45]. Protective effect of CLnA isomers against EM lipid peroxidation and lipoprotein oxidation is in agreement with the previous findings of Ip et al. [46] who reported that the antioxidant activity of CLA serves as an in vivo defense mechanism against membrane attack by free radicals. Recent studies showed that some of medicinal herbs or drugs, in addition to their lipid-lowering ability, can also reduce the production of ROS and increase the resistance of plasma lipoprotein to oxidation that may contribute to their effectiveness at preventing diseases [4, 5]. Decrease in phospholipid is due to increase in lipid peroxidation in arsenite-treated rats compared to their respective control. Due to the decrease in lipid peroxidation in EM and MDA, phospholipid content restored to normal value after CLnA supplementation.

Toxic effect of arsenic was accompanied by significant reduction in intracellular GSH level, implicating a diminution in the effectiveness of antioxidant defense system. Exhaustion of GSH levels played a leading role in allowing oxidative stress–induced loss of functional integrity in erythrocytes making cell death inevitable during arsenic toxicity. As a result of oxidative stress after arsenic treatment, extensive damage of erythrocyte occurs as shown in AFM study [47]. Thus, reduced cellular deformability resulting from the altered shape of red cells during arsenic toxicity may share mechanisms associated with proportional enhancement of cholesterol. Erythrocyte deformability is a basic rheological property of the cells and refers to the ability of the red cells to change shape during their flow in the microcirculation [47]. Changes in the cellular deformability are due to increased membrane rigidity. The fluidity of the erythrocyte membrane depends on various factors among which cholesterol content and fatty acid composition have significant influences [48]. Dietary CLnA improves membrane fluidity owing to its hypocholesterolemic and antioxidant effect. Cholesterol homeostasis between cell membrane and lipoprotein surface is driven by HDL-C, and in this study, CLnA supplementation causes increase in plasma HDL-C content, which helps in the removal of cholesterol from membrane. Fatty acid composition of EM lipid showed that due to increase in lipid peroxidation in Sa-treated rats, most of the polyunsaturated fatty acids such as EPA and DHA have been decreased significantly. Increase in ARA and ALA contents in EM lipid is an indication of mild inflammation in arsenite-treated rats, and occurrence of inflammation due to arsenic exposure is in agreement with the previous findings of Chen et al. [49]. During the conversion of dienes to arachidonic acid, CLnA can be incorporated into the pathway due to biohydrogenation. Result shows that in CLnA-treated groups, ALA content is higher than the arsenite-treated group, which indicates less conversion of dienes to ARA, and it is a combating mechanism of action of CLnA against inflammation. Therefore, these two CLnA isomers have protective effect on erythrocyte membrane fluidity and morphology owing to its hypocholesterolemic and antioxidant activities.

Increase in cholesterol content in tissues after Sa treatment is in agreement with the findings of Mann et al. [50] who suggested that the antagonism between arsenic and liver X receptor (LXR) leads to hypercholesterolemia (i.e., decrease in LXR signaling by arsenic could increase cholesterol levels). In this study, CLnA exhibits its hypocholesterolemic activity at the tissue level and reduces cholesterol content of liver and brain. Result shows that punicic acid is slightly more efficient in reducing total cholesterol content. This can be attributed that punicic acid has 66% *cis* configuration, so it might acted like essential fatty acids (EFA) such as linoleic or n-6 linolenic acid, which normally reduce the cholesterol level in plasma and other tissues. Increase in total lipid content is due to the accumulation of triglyceride and cholesterol in the liver and brain; this is in agreement with the previous findings of Santra et al. [51], and they suggested that increased oxidative stress can lead to mitochondrial damage within the hepatocytes and, in turn, decreased mitochondrial oxidation of fatty acids. Thus, fatty acids are shunted toward esterification pathways resulting in the accumulation of triglycerides within the hepatocytes. Total lipid content of brain also increased significantly in case of arsenite-treated rats due to the accumulation of triglyceride and cholesterol. In this study, CLnA significantly reduces total lipid content in liver and brain. According to Park et al., dietary CLA reduces fat accumulation in adipose tissues [52]. They have suggested that this is due to increased oxidation of fatty acids as reflected by enhanced activity of carnitine palmitoyl transferase in fat pad and skeletal muscle. According to the study of Kim et al., suppression of fatty acid synthase plays a significant role in the hypolipidemic effects observed in rats fed ALA-rich perilla oil, and these effects were associated with the increase in hepatic microsomal EPA and DHA contents [53]. From above-mentioned

observations, it is clear that CLnA isomers have better effectivity at lower concentration (0.5% of total lipid), which is in agreement with the previous findings of Dhar et al. [3]. They have suggested that at higher concentrations, CLnA acts as prooxidant rather then antioxidant. Although in this study, protective effect of CLnA isomers have also been observed at 1.0% level, effectivity has been decreased in comparison with 0.5% treated groups. Therefore, our observations regarding hypocholesterolemic and hypolipidemic activities of CLnA isomers have been well supported by aforesaid studies.

Conclusion

Present study all along supports the hypocholesterolemic and hypolipidemic activities of CLnA isomers. All the biochemical parameters strongly suggest that though there are some differences in the effectiveness of α-eleostearic acid and punicic acid due to the difference in their structural *cis-trans* configuration, both can effectively act as hypocholesterolemic and hypolipidemic agents. Results showed that punicic acid is slightly more efficient in reducing total cholesterol content due to its high *cis* content (66%) (*cis* -9, *trans*-11, *cis*-13-octadecatrienoic acid), and so it might have acted like essential fatty acids (EFA) such as linoleic or n-6 linolenic acid. Overall, CLnA isomers have showed better effectivity at lower dose (0.5% of total lipid), and effectiveness of α-eleostearic acid seems to be more prominent due to its higher oxidative stability and *trans* content (66%) (*cis*-9, *trans*-11, *trans*-13-octadecatrienoic acid). In terms of hypocholesterolemic activity on EM and antioxidant effect on fatty acid composition of EM lipid (effect on DHA, EPA, ARA and ALA contents), CLnA isomers have protective role in amelioration of EM fluidity and morphology. CLnA isomers also have some anti-inflammatory action on EM as it reduces ARA content. Hypocholesterolemic and hypolipidemic activities of different vegetable oils containing CLnA isomers have been established by this study.

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