

Full Length Research Paper

***In vitro* and *in vivo* protective action of ethanolic extract of *Triphala* on LDL against glycation-oxidation**

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The modified low-density lipoprotein (LDL; glycated and oxidized) is more atherogenic and are poorly recognized by LDL receptor. In the present study, an attempt has been made to evaluate the effects of *Triphala* on LDL modification in the experimentally treated rats and in human LDL. The effect of ethanolic extract of *Triphala* on LDL oxidation susceptibility in high fat diet fed and STZ induced rats was evaluated. The inhibitory effect of *Triphala* on LDL oxidation and glycation was investigated using copper and glucose induced human LDL, respectively *in vitro*. The co-presence of *Triphala* extract significantly increased lag phase and minimized the conjugated dienes formation in dose dependent manner. Copper mediated LDL oxidation was characterized by elevated indices of thiobarbituric acid reactive substance (TBARS), whereas co-presence of *Triphala* extract significantly minimized the production of TBARS. The results of this investigation shows that *Triphala* probably with their antioxidant properties inhibited LDL glycation and oxidation and provide scientific reasons regarding the possible medical benefits of using *Triphala* to prevent diabetic and cardiovascular complications.

Key words: *Triphala*, low-density lipoprotein (LDL) oxidation, LDL glycation, high fat diet, streptozotocin (STZ).

INTRODUCTION

Atherosclerosis, a cardiovascular disorder posing main cause for the death in developed as well as developing countries (Stachura and Pierzynowski, 2009). Atherosclerosis is characterized by low-density lipoprotein (LDL) deposition in the arterial wall which finally ends in the formation of lesions. Rupture of lesions clinically leads to heart attack and stroke. Atherosclerosis is a complication associated with diabetes. In patients with diabetes, the risk of atherosclerosis is three to five folds greater than in non diabetics. LDL is a major risk factor in this regard.

The LDL consists of 25% apo B-100 protein and 75% of lipid consisting of cholesterol esters and some triglycerides. LDL is the main cholesterol carrying lipoprotein in plasma (Kalyanaraman, 1995). Linoleate is the polyunsaturated fatty acids (PUFAs) present in LDL, which are associated with cholesteryl ester, are very susceptible to oxidative damage. LDL modifications (oxidation and glycation) are strongly related to diabetic complications, atherosclerosis, and other cardiovascular diseases (Lyons, 1993; Hunt, 1991; Li et al., 1996; Picard, 1995). Glycated LDL is more susceptible to oxidation

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than unglycated LDL, and oxidized LDL is more prone to modification by glycation (Picard, 1995; Albertini et al., 2002). Oxidation of LDL is crucial in plaque formation and onset of atherosclerosis (Steinberg, 1997).

Triphala ['three' (tri) 'fruits' (phala)] is a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants, *Terminalia chebula*, *Terminalia bellirica*, and *Phyllanthus emblica*, also known as the 'three myrobalans'. This formulation is a frequently used Ayurvedic medicine to treat many diseases such as anemia, jaundice, constipation, asthma, fever and chronic ulcers. It is an important medicine of the 'rasayana' group and is believed to promote health, immunity and longevity. It corrects constipation, cleanses and tonifies the gastrointestinal tract and also detoxifies the whole body, and improves digestion and assimilation (Nadkarni, 1976). It exhibits anti-viral, anti-bacterial, anti-fungal and anti-allergic properties (Mehta et al., 1999; Singh, 2003). *Triphala* and its constituents act as cardio-tonic, control blood pressure, improve blood circulation and reduce cholesterol levels (Thakur et al., 1988; Tariq et al., 1977). *Triphala* shows immunomodulatory properties and helps in improving the body's defense system (Srikumar et al., 2005). In recent years there are also several reports in the literature which suggest that *Triphala* possesses antimutagenic, and radio protecting activity (Kaur et al., 2005, 2002; Vani et al., 1997; Jagetia et al., 2002, 2004a, b; Arora et al., 2003; Kumar et al., 1996; Naik et al., 2005). *Triphala* has been reported to be a rich source of Vitamin C, ellagic acid, gallic acid, chebulinic acid, bellericanin, β -sitosterol and flavanoids (Jagetia et al., 2002).

Since LDL oxidation and glycation are closely interrelated and both are important factors for the development of atherosclerosis, proper agents with antioxidative and antiglycative properties may provide medical benefits. This formulation is rich in antioxidant and there is no evidence to support *Triphala* has an inhibitory role on LDL oxidation and glycation. Thus, this work was undertaken to investigate the effect of *Triphala* on the modification of LDL both in isolated LDL from human and experimental rat.

MATERIALS AND METHODS

Animals and diet

Male Sprague-Dawley rats (230 \pm 20 g) were used in this study. The animals were housed in large spacious cages, bedded with husk and were given food and water *ad libitum*. The animal room was well ventilated with a 12-h light/dark cycle throughout the experimental period. Animal experimentation was conducted according to the current institutional regulations (IAEC no.: IAEC/39/05/CLBMCP/2013).

Chemicals

Disodium ethylene diamine tetraacetate (Na₂ EDTA), sodium

chloride (NaCl), and disodium hydrogen phosphate (Na₂HPO₄) were purchased from Sigma chemical. *Terminalia chebula*, *Terminalia bellerica*, and *Phyllanthus emblica*, were purchased from the local market.

Extraction

T. chebula, *T. bellerica* and *P. emblica* were bought from the local market and identified by botanist in CSMDRIA, Chennai. The fruits were dried and pulps were coarsely powdered. One kilogram powder was soaked in ethanol for 7 days with intermittent shaking and the solvent was filtered with Whatmann filter paper. The filtrate was evaporated under vacuum drier and the brown mass residue obtained was stored at -4°C for further use(s).

Experimental design

In vitro experiments

To study the effect of *Triphala* on human LDL oxidation and glycation, *in vitro* experiments were conducted on LDL isolated from human plasma.

Blood sampling

Fasting blood samples were collected from normal healthy volunteers. To obtain fresh plasma, blood samples were centrifuged (3000 rpm for 10 min at 4°C) as soon as the samples were collected to avoid auto oxidation.

LDL isolation

Plasma LDL was isolated by precipitation method using heparin-citrate buffer (Ahotupa et al., 1998). The precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 with 5 N HCl, and contained 50,000 IU/L heparin. Plasma sample and precipitation reagents were allowed to equilibrate to room temperature before precipitation of LDL. To 100 μ l of sample, 1 ml of the heparin-citrate buffer was added and mixed using Vortex mixer, and was incubated in room temperature for 10 min; then, was centrifuged at 1000 g for 10 min to remove insoluble lipoproteins. The pellet was resuspended in 1 ml of 0.1 M Na-phosphate buffer, pH 7.4, containing 0.9% of NaCl.

LDL oxidation and glycation

The oxidation of LDL was done by incubating 100 μ g LDL with CuSO₄ 10 mmol/L for 4 h at 37°C (Ghaffari and Ghiasvand, 2006). The ox-LDL was then extensively dialyzed overnight in phosphate buffered saline containing ethylenediaminetetraacetic acid 0.05 mmol/L. The glycation of LDL was induced by incubating with glucose (25 mM) for 7 days at 37°C (Li et al., 1996) with various concentration of *Triphala* extract followed by dialysis and CuSO₄ induced oxidation for 4 h. The degree of oxidation and glycation were measured as the amount of thiobarbituric acid-reactive substances produced.

LDL oxidation kinetics

The total protein was measured after the LDL isolation by Lowry's method. 0.1 ml of native LDL and glycated LDL (100 μ g of protein) was diluted to 0.9 ml with PBS and was incubated with or without

0.1 ml of *Triphala* extract (1, 5 and 10 µg/ml) at 37°C for 30 min. At the end of incubation period, oxidation was initiated by adding 0.01 ml of freshly prepared 10 mM CuSO₄. The LDL oxidation kinetics was measured by continuously monitoring the change in absorbance for 4 h (at 37°C) every 20 min at 234 nm. The lag phase was calculated by drawing a tangent to the slope of propagation phase and extrapolation into the intercept of initial-absorbance axis. The lag phase represents the antioxidant-protected phase during oxidation by *Triphala* extract.

Assay for conjugated dienes and products of lipid peroxidation

At the end of 4 h incubation, the formation of conjugated dienes was calculated as conjugated dienes equivalent content (nmol/mg-protein) at 240 min. The conjugated dienes concentration was calculated by using the extinction coefficient for diene conjugates at 234 nm (29500 L/mol.cm).

After 4 h incubation, samples were incubated with 0.5 ml of 20% trichloroacetic acid and 1 ml of 0.67% aqueous solution of thiobarbituric acid (Scoccia et al., 2001). The reaction mixtures were heated at 100°C for 20 min and then centrifuged at 2000 g for 5 min. The absorbance of the supernatant fractions was estimated at 532 nm. The concentration of TBARS was calculated using the extinction coefficient of 165,000 mol/cm and expressed as nmol of malondialdehyde equivalents per mg LDL protein

In vivo study

The study was divided into two phases: the atherosclerotic progression and regression studies. The models used were the male Sprague-Dawley rats. The animals were randomly divided into 8 groups of six animals each. The total duration of the experiment is 8 weeks.

Group 1: Control (0.5% dimethyl sulfoxide [DMSO]); Group 2: Control + extract (200 mg/kg body weight in 0.5% DMSO); Group 3: high fat diet (HFD) + STZ; Group 4: HFD + extract (100 mg/kg body weight in 0.5% DMSO) + STZ; Group 5: HFD + extract (200 mg/kg body weight in 0.5% DMSO) + STZ; Group 6: HFD + STZ + extract (100mg/kg body weight in 0.5% DMSO); Group 7: HFD+ STZ + extract (200 mg/kg body weight in 0.5% DMSO); Group 8: HFD + STZ + 2 mg/kg body weight atorvastatin in 0.5% DMSO.

Progression study

For the progression study, 12 standard deviation (SD) rats were randomly divided into two groups (4 and 5), fed with high fat diet and received *Triphala* 100 and 200mg/kg of body weight orally along with HFD. After 4 weeks, the animals were subjected to an overnight fast and injected with STZ (35 mg/kg body weight in 0.1 M citrate buffer, pH 4.5). Animals had free access to food and water after the STZ injection. The animals continued on their HFD diet for the duration of the study.

Regression study

Twelve SD rats were randomly divided into two groups (6 and 7), fed with HFD. After 4 weeks, the animals were subjected to an overnight fast and injected with STZ (35 mg/kg body weight in 0.1 M citrate buffer, pH 4.5) and received *Triphala* 100 and 200 mg/kg of body weight orally. The animals continued on their HFD diet for the duration of the study.

At the end of the first and second phases of the experimental period, the rats were fasted overnight, anesthetized with phenobarbital

(25 mg/kg intravenously) and sacrificed. The tissues were removed quickly, washed with cold saline, and stored at 20°C until analyzed. Various biochemical analysis and histopathologic analysis of the aorta were performed.

LDL isolation and effect of *Triphala* on LDL oxidation

Plasma LDL was isolated by precipitation method using heparin-citrate buffer (Ahotupa et al., 1998) as described earlier. The total protein was measured after the LDL isolation by Lowry's method. 0.1 ml of LDL (100 µg of protein) was diluted to 0.9 ml with phosphate buffer saline (PBS) and was incubated with or without 0.1 ml of *Triphala* extract (1, 5 and 10 µg/ml) at 37°C for 30 min. At the end of incubation period, oxidation was initiated by adding 0.01 ml of freshly prepared 10 mM CuSO₄.

Assay for conjugated dienes and products of lipid peroxidation

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Statistical analysis

Each data value was presented the mean ± (SD) of three experiments performed in duplicate. Results were expressed as mean ± SD. Statistical evaluation of the data was done by one way ANOVA followed by Duncan's multiple range test (DMRT) test. Differences were considered significant at P<0.05.

RESULTS

Oxidation kinetics of both glycated and oxidized LDL was measured by change in absorbance at 234 nm for every 20 min as shown in Figures 1A and 2A. It clearly shows copper increased LDL oxidation. The conjugated dienes formation, marker of the LDL oxidation, was decreased by the incubation with *Triphala* extract. Lag phase was also calculated as shown in Figures 1B and 2B. Four hours after copper addition, the extent of LDL oxidation was estimated from TBARS and conjugated dienes measurements as shown in Figures 1C, 1D, 2C and 2D. TBARS production was decreased in the presence of 1 µg, 5 µg and 10 µg of *Triphala* concentrations. As shown in Figures 1C, 2C and 3, this reduction was dependent on *Triphala* concentrations.

The TBARS and conjugated dienes formation in experimental animals was also studied and measurements were given in graph 3A and B. *In vitro* LDL oxidation studies revealed that HFD+STZ treated rats showed

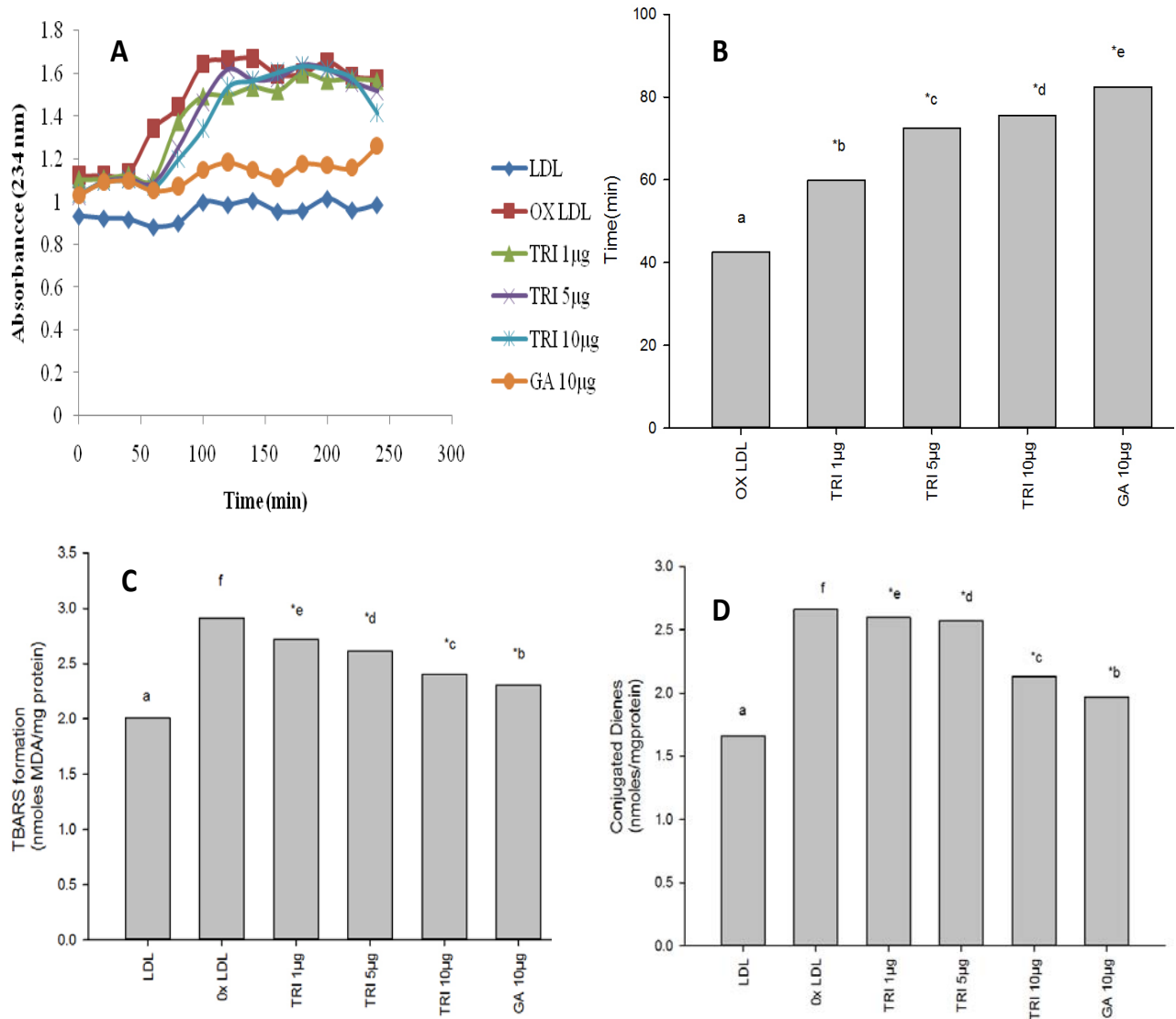


Figure 1. (A) The effect of *Triphala* extract on the LDL oxidation kinetics. Each point represents the mean of three experiments. (B) The effect of *Triphala* extract on the lag time (The lag phase represented the length of the antioxidant - protected phase during LDL oxidation by extract). Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Ox-LDL. (C) The effect of *Triphala* extract on the formation of TBARS. Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Ox-LDL. (D) The effect of *Triphala* extract on the formation of conjugated dienes. Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Ox-LDL.

significantly lowered lag phase time, increased CD concentration and significantly elevated levels of TBARS, whereas no significant change was observed in lag phase of *Triphala* alone treated controls rats as compared to untreated control rats (group 1). However, supplementing *Triphala* simultaneously along with HFD (groups 4 and 5) significantly increased the lag phase, lowered the CD formation and TBARS level as compared to the rats supplemented with *Triphala* after four weeks treatment with HFD and induction of diabetes with STZ (groups 6 and 7).

DISCUSSION

Natural antioxidants and lipid lowering interventions such as food supplements, spices, and herbs find extensive application in prevention of atherosclerosis, because of their ability to prevent *in vitro* LDL oxidation (Chang et al., 2006; Chu et al., 2009) and plaque formation (Ho et al., 2010).

LDL oxidation is mediated by free radical and the process is initiated by the removal of hydrogen atom from a methylene (CH₂) group of PUFA moiety of LDL. The

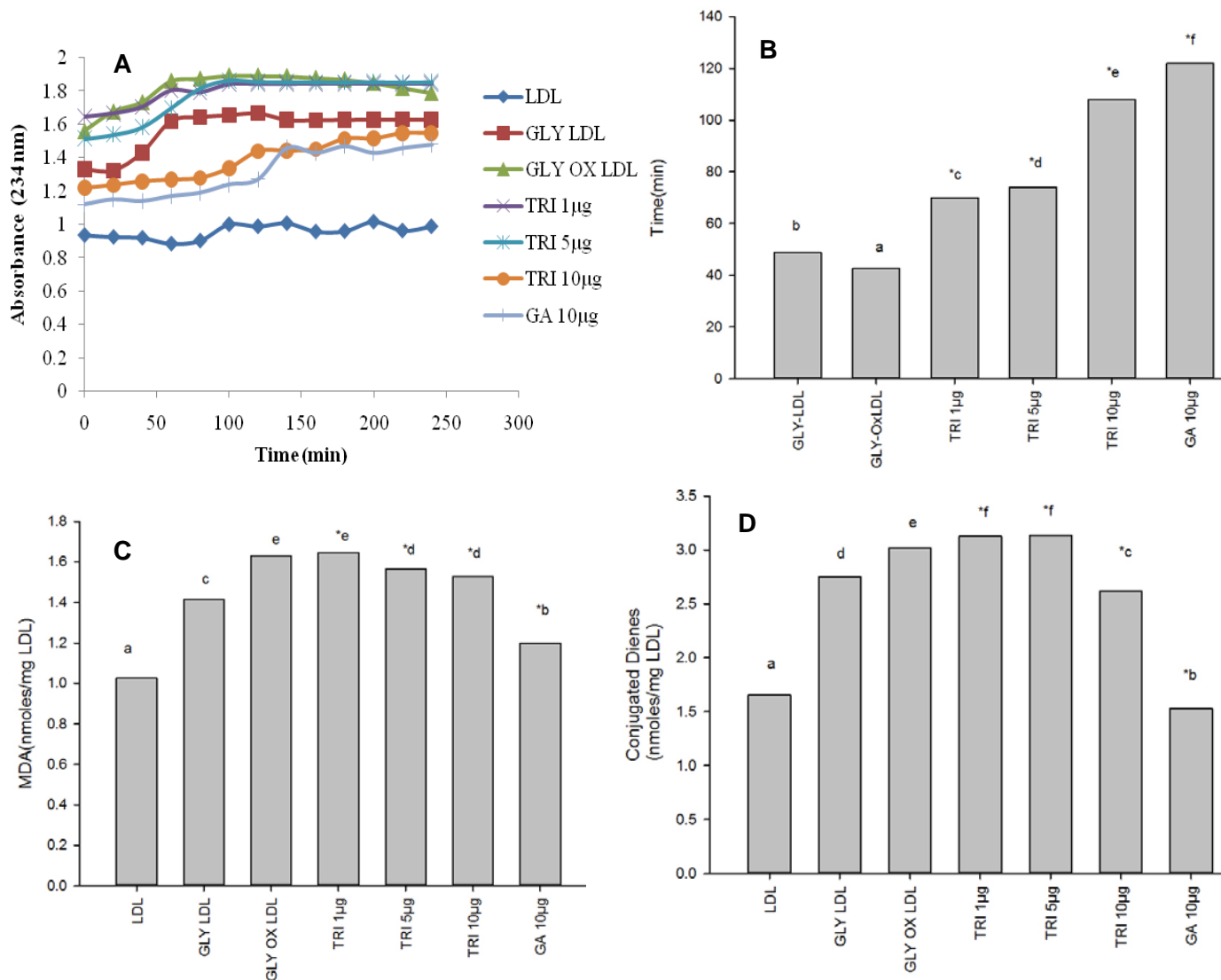


Figure 2. (A) The effect of *Triphala* extract on oxidation kinetics of glycated- oxidized LDL. Each point represents the mean of three experiments. (B) The effect of *Triphala* extract on the lag time on Gly-Ox-LDL (The lag phase represented the length of the antioxidant-protected phase during LDL oxidation by extract). Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Ox-LDL. (C) The effect of *Triphala* extract on the formation of TBARS in glycated-oxidized LDL. Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Gly-Ox-LDL. (D) The effect of *Triphala* extract on the formation of conjugated dienes in glycated-oxidized LDL. Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Gly-Ox-LDL.

resultant unstable carbon atom undergoes rearrangement to form stable conjugated diene. Further, conjugated diene will react with oxygen to form peroxy radical that further abstracts hydrogen atom from adjacent PUFA and cholesterol to form lipid hydrogen peroxide and oxysterols, respectively. The lipid hydrogen peroxide is cleaved to form short aldehyde chain, that is, malondialdehyde (MDA) and 4-hydroxynonenal. Later, these aldehydes will form covalent adduct to lysine residue in Apo-B in LDL. This derivatization introduces a negative charge to the LDL molecule and it is recognized

by the scavenger receptor of macrophages, resulting in increased LDL uptake by macrophages and their transformation into foam cells (Young and McEneny, 2001). This is a hallmark event to onset of atherosclerosis. Foam cell formation is thought to be mediated by modified LDL. The formation of foam cells *in vitro* can be induced by OxLDL and acetylated LDL (AcLDL). OxLDL can induce proliferation of peripheral macrophages *in vitro*. OxLDL induces PPAR- γ expression.

In the present study, an attention has been paid to the

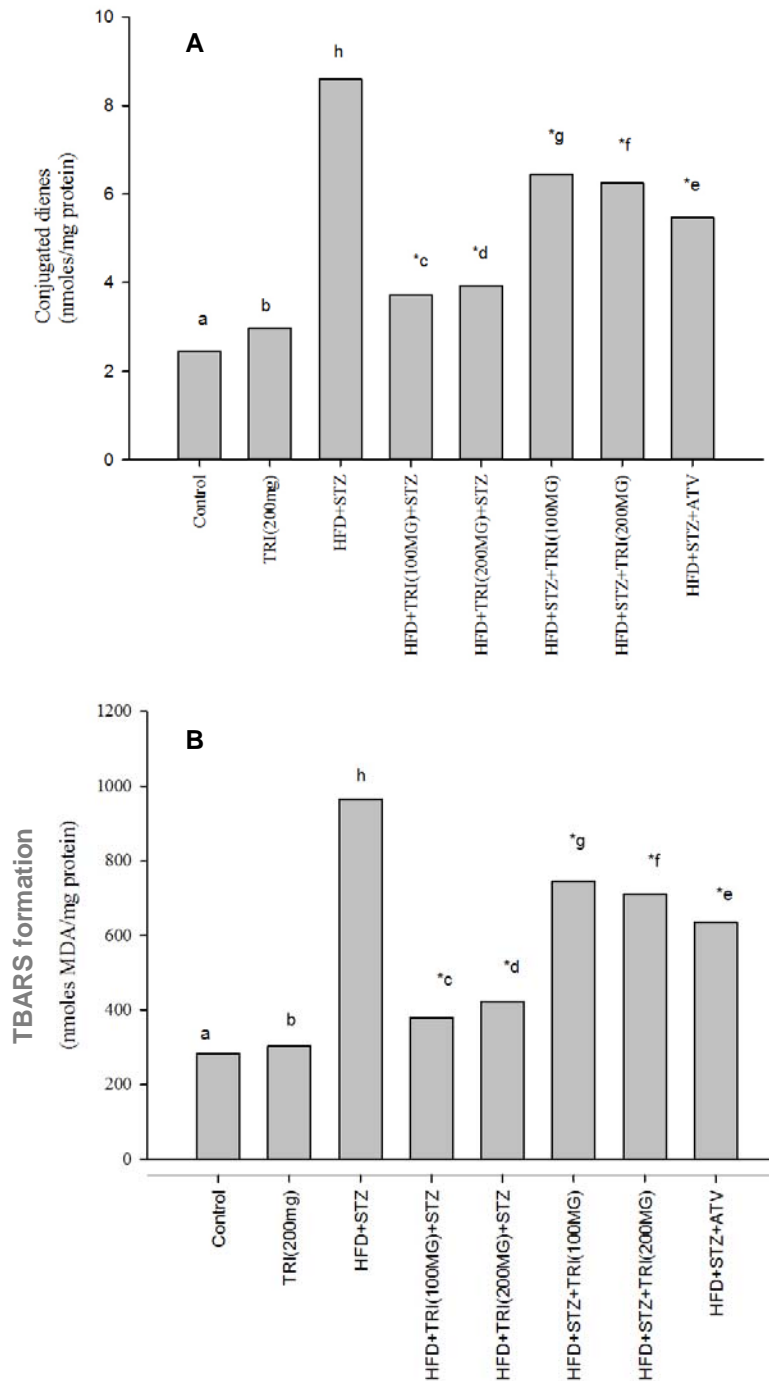


Figure 3. (A) The effect of *Triphala* extract on the formation of conjugated dienes in experimentally treated rats with HFD and STZ (n=6). (*) is the symbol for significant difference compared to HFD+STZ treated rats. (B). The effect of *Triphala* extract on the formation of TBARS in experimentally treated rats with HFD and STZ (n=6). (*) is the symbol for significant difference compared to HFD+STZ treated rats.

antioxidant effect of *Triphala* towards the oxidation and glycation of LDL, which is thought to be a key process in diabetes and atherosclerosis. Our result show *Triphala* extract showed prolonged lag phase in copper mediated

native LDL and glycated LDL oxidation kinetics. It also reduced the conjugated dienes formation, and also, significant reduction in the formation of various LDL oxidation intermediary products (MDA). This indicates the

role of *Triphala* extract as a chain breaking antioxidant.

Also, this study demonstrated that *Triphala* at a dose of 100 and 200 mg/kg body weight per day modulated the conjugated dienes (CDs) and TBARS levels in rats fed with HFD and treated with STZ in progression phase more significantly than in regression phase of the study. These results can be attributed to the established antioxidant and free radical scavenging property of *Triphala* extract (Deepa et al., 2013). Previous study from our laboratory has established that *Triphala* extract is rich in polyphenols (Deepa et al., 2013). The present study shows that *Triphala* is a potent antioxidant in protecting plasma LDL against oxidation and glycation.

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