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Suppression of abdominal fat and anti-hyperlipidemic potential of *Emblica officinalis*: Upregulation of PPARs and identification of active moiety

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ABSTRACT

Since ancient time, *Emblica officinalis (E. officinalis)* is being used for the management of various ailments. Phytochemical analysis proves that fruit juice of *E. officinalis* contains high amount gallic acid, which could be responsible for medicinal potentials. Hence in this study, gallic acid and fruit juice of *E. officinalis* were evaluated for anti-hyperlipidemic potential in various experimental animal models. Experimentally, hyperlipidemia was induced through administration of poloxamer-407, tyloxapol and high-fat-diet supplement in rats. Treatment with gallic acid as well as fruit juice of *E. officinalis* decreased plasma cholesterol and reduced oil infiltration in liver and aorta. Mechanistically, *E. officinalis* increased peroxisome proliferator-activated receptors-α (PPARα) expression and increased activity of lipid oxidation through carnitine palmitoyl transferase (CPT) along with decreased activity of hepatic lipogenic enzymes *i.e.* glucose-6-phosphate dehydrogenase (G6PD), fatty acid synthase (FAS) and malic enzyme (ME). Additionally, *E. officinalis* increased cholesterol uptake through increased LDL-receptor expressions on hepatocytes and decreased LDL-receptor degradation due to decreased proprotein convertase subtilisin/kexin type 9 (PCSK9) expression. Simultaneously, *E. officinalis* showed ability to restore glucose homeostasis through increased Glut4 and PPARγ protein expression in adipose tissue. These findings exposed central role of gallic acid in *E. officinalis* arbitrated anti-hyperlipidemic action through upregulation of PPARs, Glut4 and lipogenic enzymes, and decreased expression of PCSK9 and lipogenic enzymes. Findings from this experiment demonstrated that *E. officinalis* is a potential therapy for management of hyperlipidemia and gallic acid could be a potential lead candidate.

1. Introduction

Increased plasma lipid level initiates progression of various pathological changes *viz.* atherosclerosis, myocardial infarction and other cardiovascular disorders. Similarly, increased triglyceride level alters vascular endothelial function and leads to progression of atherosclerosis [1]. Dyslipidemia is one of the common metabolic diseases in developed countries and has becoming a global epidemic at alarming rate. According to WHO report, dyslipidemia is associated with more than 50% global incidence of ischemic heart diseases [2]. Currently, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors (also known as statins) are adopted as first-line therapy for hyperlipidemia and related cardiac disorders despite the serious limitations with the therapy such as; rhabdomyolysis and incidence of elevated AST (Aspartate Aminotransferase) and ALT (Alanine Aminotransferase) levels [3]. Therefore, there is need to identify new moiety with minimal or no adverse event for the management of hyperlipidemia and related cardiac ailments. Certain plant-based phytochemicals like gugulipid, have been utilized for amelioration of dyslipidemia and related disorders but still there is urgent need to identify herbal moiety to provide efficient therapy.

Nature has provided a spectacular toolbox filled with diversified phytochemicals for the management of various ailments. Since ancient era, *Emblica officinalis* (family: Euphorbiaceae), also known as Indian gooseberry or *amla*, has been used for treatment of various maladies including life-style diseases *viz.* diabetes, hyperlipidemia; and life-threatening disorders including metastasis and malignancies [4]. *E. officinalis* contains numerous phytochemicals, to wit: gallic acid, ellagic acid, quercetin, methyl gallate, chebulic acid, corilagin, emblicanins, phyllanemblinins, phyllaemblic acid, punigluconin, pedunculagin, chlorogenic acid and many more [4]. In traditional medicinal system, rather than single isolated compound, whole plant extract or mixture of

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various medicinal plants are more widely used for the management of various maladies. The primary aim of this study was to investigate antihyperlipidemic potential of gallic acid, an active phytoconstituent from fruits of *E. officinalis*, in various animal models of hyperlipidemia and identification of probable mechanism of action. Many clinical and preclinical studies have been reported for antihyperlipidemic potential of *E. officinalis* [5–9]. In our previous studies, we reported anti-hyperlipidemic potential of *E. officinalis* as well as gallic acid in animal models of diabetes-associated hyperlipidemia [10–13]. Recently, Singh et al. also showed antihyperlipidemic potential of *E. officinalis* against arsenic-prompted dyslipidemia through restoring cytokines level [14]. Earlier reports suggest involvement of flavonoids and polyphenols for hypolipidemic potential of *E. officinalis* through inhibition of HMG-CoA reductase, increased Lecithin-cholesterol acyltransferase (LCAT) activity, decreased LDL-oxidation and attenuation of Sterol regulatory element-binding protein 1 (SREBP-1) expression [7,8,15,16]. However, molecular targets of *E. officinalis* need to be elucidated to understand molecular mechanism for its anti-hyperlipidemic potential. This study also explored possible involvement of peroxisome proliferator-activated receptors-α (PPARα), low-density lipoprotein-receptor (LDL-R), pro-protein convertase subtilisin/kexin type 9 (PCSK9), PPARγ, hepatic lipogenic and lipolytic enzymes for *E. officinalis* mediated anti-hyperlipidemic potential.

2. Materials and methods

2.1. Drugs and reagents

Fenofibrate was obtained as a gift sample from Torrent Research Center, Bhatt, Gujarat. Poloxamer-407, tyloxapol (triton WR 1339), Oil-Red-O, CoA, Palmitoyl CoA, acetyl CoA, malonyl CoA, gallic acid, nonfat-dry milk, glut4 and anti-rabbit IgG secondary antibody were purchased from Sigma Aldrich, Co. St. Louis, MO, USA. PPAR-γ and β-actin antibodies were procured from Cell signaling technology, Inc. L-malate, triethanoamine and β-mercaptoethanol were purchased from Spectrochem Pvt. Ltd., Mumbai. NADPH, EDTA, L-carnitine, $MnCl_2$, $NADP^+$, Tris-HCl, $MgCl_2$, glucose 6-phosphate, NAD^+, dithiothreitol (DTT), Bovine Serum Albumin (BSA) and 5,5-dithiodis-2-nitrobenzoic acid (DTNB) were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. All other chemicals and reagents used in study were of analytical grade and procured from either Hi-Media Laboratories Pvt. Ltd., Mumbai, India or S D Fine Chem Ltd., Kolkata, India. Kits for the estimation of serum glucose, total cholesterol, HDL-cholesterol and triglycerides, were obtained from Lab-care Diagnostics Pvt. Ltd. India.

2.2. Experimental animals

Male albino mice weighing 25–30 g and male wistar rats weighing 200–250 g were used for experiments. Animals were obtained from animal facility of Zydus Research Center, Ahmedabad. They were housed in controlled environment at around 22 ± 2 °C and 50–70% relative humidity (R_H) with 12:12 h light dark cycle and had free access to water and food *ad libitum*. The protocol of animal experiment was approved, *vide* protocol number IP/PCOL/PHD/14-1/013 (approved in 2014) and IP/PCOL/PHD/16/012 (approved in 2015) by the Institutional Animal Care and Use Committee (IACUC) at Institute of Pharmacy, Nirma University, Ahmedabadin accordance to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest & Climate Change, Government of India, New Delhi. All the experimental animals were randomly divided into 5 different groups *i.e.* normal control group, positive control, fenofibrate treated (100 mg/kg/day, *p.o.*), gallic acid treated (100 mg/kg/day, *p.o.*) and fruit juice treated (2 mL/kg/day, *p.o.*), with 6 animals in each group, and treatment regimen was followed accordingly. Animals described as fasted were deprived of food for 16 h or over-night with free access of water *ad libitum*.

2.3. Experimental design and protocol

2.3.1. Poloxamer-407 induced hyperlipidemia

All experimental rats were pretreated with respective drugs for one week prior to poloxamer-407 intoxication. Animals were fasted before starting the final experiment procedures and on ultimate day, hypertriglyceridemia was induced by single injection of poloxamer-407 (100 mg/kg, *i.p.*) in normal saline [17]. Blood was collected from retro-orbital plexus under light ether anesthesia at different time points *i.e.* 0 h, 1 h, 2 h, 4 h, 7 h, 10 h, 12 h, 15 h, 20 h and 24 h after administration of Poloxamer-407 and serum was used for estimation of lipid profile.

2.3.2. Tyloxapol induced hypertriglyceridemia

Hypertriglyceridemia was induced as described by Aziz et al. [18] with slight modification. Briefly, all rats were pre-treated with respective drugs for one week prior to tyloxapol intoxication. Hypertriglyceridemia was induced by single injection of freshly prepared tyloxapol (100 mg/kg, *i.p.*). Blood was collected at 0 h and16 h after tyloxapol administration and used for assessment of lipid profile namely total cholesterol, triglyceride levels and HDL-cholesterol.

2.3.3. Corn oil induced hypertriglyceridemia

All mice were pretreated with respective drugs prior to corn oil administration. After 2 h of drug treatment, corn oil (5 mL/kg, *p.o.*) was administered to all the animals [19] and blood was collected from retro-orbital plexus under light ether anesthesia at different time points *viz.* 0 h, 1 h, 2.5 h and 4 h after oral corn oil load and serum was used for the assessment of total cholesterol and triglyceride levels.

2.3.4. High fat diet (HFD) induced hyperlipidemia

All control rats were fed with normal pelleted diet (NPD) while HFD control, fenofibrate treated, gallic acid treated and fruit juice treated rats were kept on prepared HFD and had free access to water and food *ad libitum*. The regimen was continued for two months and simultaneously treatment was continued until the ultimate day. At the starting of the protocol (Day-0) and after every two weeks, blood was withdrawn from each group for assessment of various biochemical parameters. During treatment period, rats were observed for their body weight, general appearance, behavior, and mortality. At the penultimate day of the treatment régime, OGTT was performed and after 2 days of OGTT test, blood was collected for estimation of various biochemical parameters. Animals were sacrificed; liver, aorta and white adipose tissue were dissected and stored at −80 °C for further analysis.

Liver and white adipose tissue (WAT) was utilized for histoarchitectural study using haematoxylin and eosin staining and liver LDL receptors were immune-stained using anti-LDLR antibody. LDLR expression and adipocytes mean diameter were measured using Image-J software (Version 1.43 u, NIH, USA). Liver steatosis score was documented as described by Hui et al. [20]. Other portion of liver was homogenated and this homogenate was used for estimation of various lipid regulating enzyme activities *viz.* fatty acid synthase (FAS), malic enzyme (ME), glucose 6-phosphate dehydrogenase (G6PD) and carnitine palmitoyl transferase (CPT) activities. WAT and liver were utilized for isolation of total RNA and cellular proteins for polymer chain reaction (PCR) and immunoblotting, respectively. Excised aorta was used for macroscopic *En-face* staining and microscopic HE as well as Oil-Red-O staining with the help of cryo-sectioning technique (detailed procedure is supplied in Supplementary file).

2.4. Statistical analysis

All data are represented as mean ± SEM (Standard Error of Mean) from six animals in each group. Statistical significance between groups are compared using unpaired two-tailed student's *t*-test or one-way

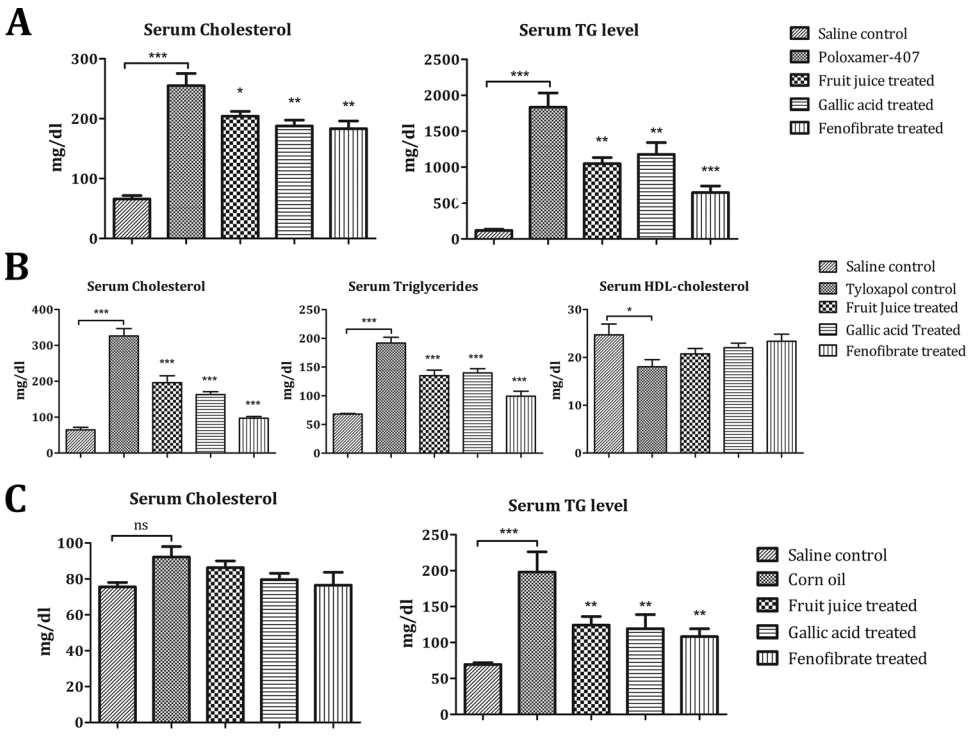

Fig. 1. Gallic acid as well as fruit juice of *E. officinalis* ameliorate dyslipidemia induced by *i.p.* administration of poloxamer-407, *i.p.* injection of tyloxapol and oral administration of corn oil. A) Administration of poloxamer-407 significantly increased serum cholesterol and TG levels which were significantly reduced by treatment with gallic acid as well as *E. officinalis.* B) Tyloxapol is non-ionic surfactant and *i.p.* administration of tyloxapol leads to increase in serum cholesterol and TG levels along with reduced HDL-cholesterol. Oral administration of gallic acid as well as fruit juice of *E. officinalis* protected against tyloxapol-induced hyperlipidemia. C) Oral administration of corn oil leads to significant increase in TG levels ($p < 0.001$) and prior-treatment with gallic acid and *E. officinalis* reduced corn oil prompted increase in TG levels. Values are denoted as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, significantly different from experimental induced dyslipidemia (n = 6).

ANOVA as appropriate using computer based fitting program (Prism v5.00, Graphpad Software Inc., San Diego, CA). When required, the group means were compared by dunnet's *post-hoc* multiple comparisons. Probability values of $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ were used as a measure of statistical significance. p values > 0.05 were considered statistically non-significant.

3. Results

3.1. E. officinalis and gallic acid decreased poloxamer-407 and tyloxapol induced hyperlipidemia

To verify hyperlipidemic potential of gallic acid and fruit juice of *E. officinalis*, acute hyperlipidemia was induced through single intraperitoneal administration of poloxamer-407 and tyloxapol separately in rats. Fenofibrate, an endothelial lipoprotein lipase (LPL) activator, was used as standard positive control. As illustrated in Fig. 1A, administration of poloxamer-407 showed time dependent enhancement in serum lipid profile. Triglyceride levels were increased 2000 times after 8 h and cholesterol level showed constant elevation up to 24 h of poloxamer-407 administration (Fig. S1). Plasma cholesterol and triglycerides were significantly declined in the animals receiving gallic acid as well as fruit juice of *E. officinalis* as compared with those in poloxamer-407 control rats. In all the treatment animals, increased cholesterol started to fall after 8–10 h of poloxamer-407 exposure, but it required more than 24 h to bring cholesterol level at basal level (Fig. S1). In similar manner, after 16 h of tyloxapol administration, serum cholesterol and triglyceride levels were significantly increased ($p < 0.001$) with decreased level of HDL-cholesterol ($p < 0.05$) compared to control animals (Fig. 1B). Treatment with gallic acid as well as fruit juice of *E. officinalis* significantly decreased tyloxapol-provoked hyperlipidemia and hypertriglyceridemia ($p < 0.001$) with non-significant elevation in HDL-cholesterol as compared to control rats.

3.2. E. officinalis and gallic acid diminished corn oil induced triglyceride levels

As depicted in Fig. 1C, after 4 h of corn oil administration, serum triglyceride levels increased approximately 3-fold compared to control animals (198.1 ± 28 *vs* 69.5 ± 2.4, $p < 0.001$). Treatment with gallic acid as well as fruit juice of *E. officinalis* showed remarkable protection against corn oil induced hypercholesterolemia (Fig. S2). Fenofibrate showed maximal fortification against corn oil boosted hypercholesterolemia.

3.3. E. officinalis and gallic acid administration suppresses HFD-induced hyperlipidemia

Chronic administration of high-fat-diet caused metabolic maladies which resulted into 3.2-fold elevation in serum cholesterol level (251.70 ± 25.74) in HFD-control rats as compared to control rats (75.91 ± 6.28, $p < 0.001$). In contrast to this, surprisingly no alteration was observed in plasma triglyceride levels. As shown in Fig. 2A, treatment with gallic acid as well as fruit juice of *E. officinalis* manifested significant decrease in serum cholesterol level which was comparable to the positive control fenofibrate. Treatment control animals (animals on normal pelleted diet with simultaneous treatment) did not display any remarkable alteration in lipid profile (Fig. S3). After eight weeks of dietary manipulation with high fat diet, there was no significant alteration in glucose homeostasis. All animals showed normal blood glucose profile including HFD-control animals and treated animals as well (Fig. 2D). Results from OGTT showed non-significant alteration in $AUC_{glucose}$ level in high-fat-fed rats compared to control rats (Fig. 2E). However, in order to confirm insulin sensitivity, quantitative insulin sensitivity check index (QUICKI) was calculated as a function of insulin sensitivity index and QUICKI results demonstrated no alteration in glucose homeostasis and insulin sensitivity as well (Fig. 2F).

3.4. E. officinalis and gallic acid restored HFD-induced alteration in lipid metabolizing enzymes

As shown in Fig. 3, dietary manipulation with high fat diet significantly altered the level of lipid metabolism regulatory hepatic enzymes. High fat diet supplement demonstrated significantly decreased activity of CPT enzyme ($p < 0.05$) along with significant increase in

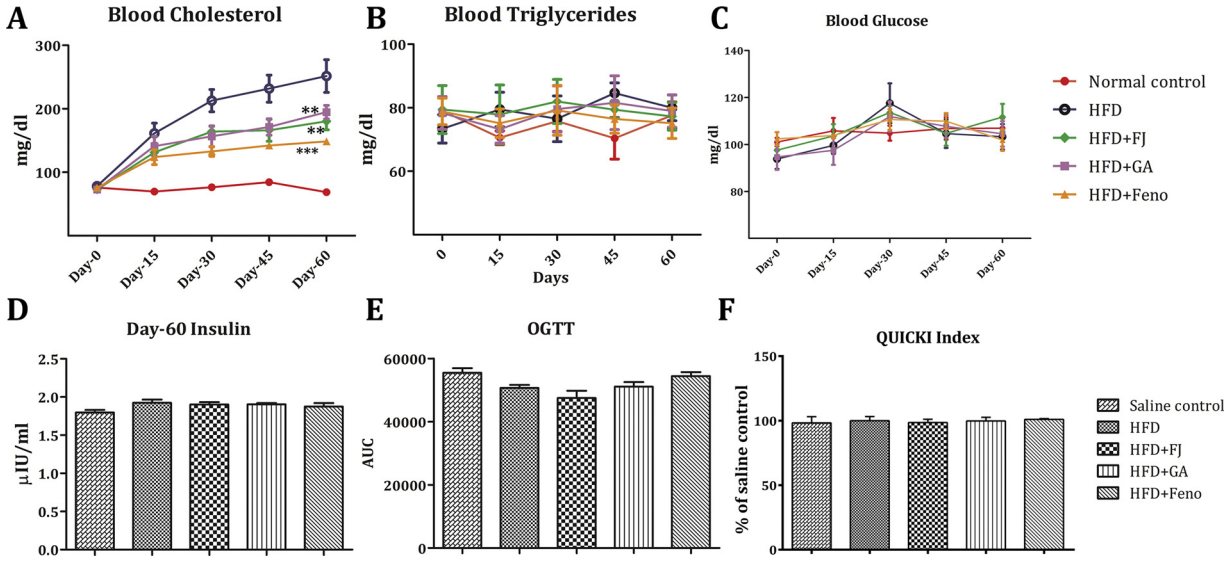

Fig. 2. Gallic acid as well as fruit juice of *E. officinalis* protect against HFD-induced metabolic maladies in rats. A–C) High-fat-fed animals showed significant increase in cholesterol level and administration of gallic acid as well as *E. officinalis* significantly abridged cholesterol level ($p < 0.01$). However, HFD did not show any alteration on triglyceride and blood glucose level. Various glucose homeostasis parameters such as D) blood insulin level, E) AUC in OGTT test and F) QUICKI index were not altered in high-fat-fed animals. OGTT = oral glucose tolerance test, QUICKI = quantitative insulin sensitivity check index, HFD = high fat diet, FJ = fruit juice of *E. officinalis*, GA = gallic acid, Feno = fenofibrate. Values are denoted as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, significantly different from HFD-induced dyslipidemia (n = 6).

G6PD, FAS and ME activity ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively). The animals treated with gallic acid as well as fruit juice of *E. officinalis* showed non-significant elevation of CPT activity in liver tissue along with significant decrease in G6PD, FAS and ME activity.

3.5. *E. officinalis* and gallic acid treatment increased LDL-R expression in lipid along with improved lipid clearance from liver and aortic arc

Liver sections stained with anti-LDL-receptors antibody showed the subordinate level of LDL-receptors in the fat-fed animals as compared to normal control animals (Fig. 4A, B). Animals treated with fruit juice of *E. officinalis* demonstrated significantly higher levels of LDL-R as compared to high-fat-fed animals but surprisingly, gallic acid treated animals showed non-significant protection against high-fat induced drop in LDL-R. In contrast to this, Fenofibrate showed an improvement in LDL-R levels.

Compared to control rat livers, macroscopic observation of HFD-control rat livers was swollen and yellowish in color. As depicted in Fig. 4C, histoarchitectural observations showed clear hepatic lining without oil infiltrations or oil accumulations in control animals while high-fat-fed rats showed high level of oil infiltration along with ballooning hepatocytes, inflammatory cell infiltration and mild necrotic hepatocytes. Treatment with gallic acid as well as fruit juice of *E. officinalis* demonstrated reduced amount of oil infiltration without any alteration in sinusoidal lining with normal hepatic morphology (Fig. 4D). In similar manner, accumulated oil could be seen as vacuoles or empty spaces in the vascular lining of HE stained aortic arc of HFD-control rats (Fig. 4E). Control rats showed clear cellular lining without any fatty infiltration in intimal walls. However, treatment with gallic acid as well as fruit juice of *E. officinalis* ameliorated HFD-induced oil accumulation and pathological changes in the aorta (Fig. 4E). In line with this, ORO *en-face* staining also showed infiltrated lipids as high intensity stained areas in the macroscopic visualization (Fig. S6A). HFD-control animals showed utmost level of ORO stained area compared to control animals while treated animals demonstrated sub-maximal level of stained area than HDF-control animals. However, amongst all treated animals, positive control, fenofibrate, revealed maximum protection against HFD-provoked lipid accumulation in intimal tissues (Fig. S6C).

The size of white adipose tissue cells represents the extent of adipogenesis which could be visualized by analyzing the size of white adipose cells. As portrayed in Fig. 4G, control animals showed high number of adipose cells in unit area with lower cellular size, while HFD-control animals exhibited higher WAT cellular size with lower number of cells in the unit area (Fig. 4F). Nonetheless, treatment with fruit juice of *E. officinalis* and gallic acid showed protective action against HFD-induced increment in adipocytes while fenofibrate treated rats indicated virtually normal adipocyte size in unit area.

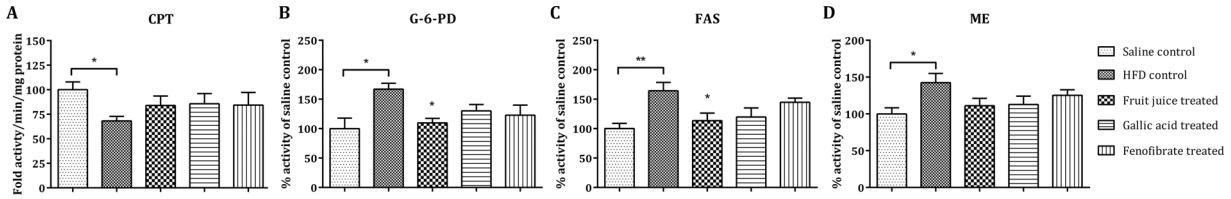

Fig. 3. Effect of gallic acid as well as fruit juice of *E. officinalis* on hepatic lipid metabolizing enzymes. A) High-fat-fed animals showed decreased hepatic lipolytic enzyme *i.e.* CPT activity with increased activity of various lipogenesis enzymes such as B) G-6PD, C) FAS and D) ME. Treatment with gallic acid as well as fruit juice of *E. officinalis* decreased HFD-prompted lipogenic enzymes *i.e.* G-6PD, FAS and ME activity along with reduced CPT activity. HFD = High fat diet, CPT = carnitine palmitoyl-transferase, G-6PD = Glucose-6-phosphate dehydrogenase, FAS = fatty acid synthase, ME = malic enzyme. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, significantly different from HFD-control rats (n = 6).

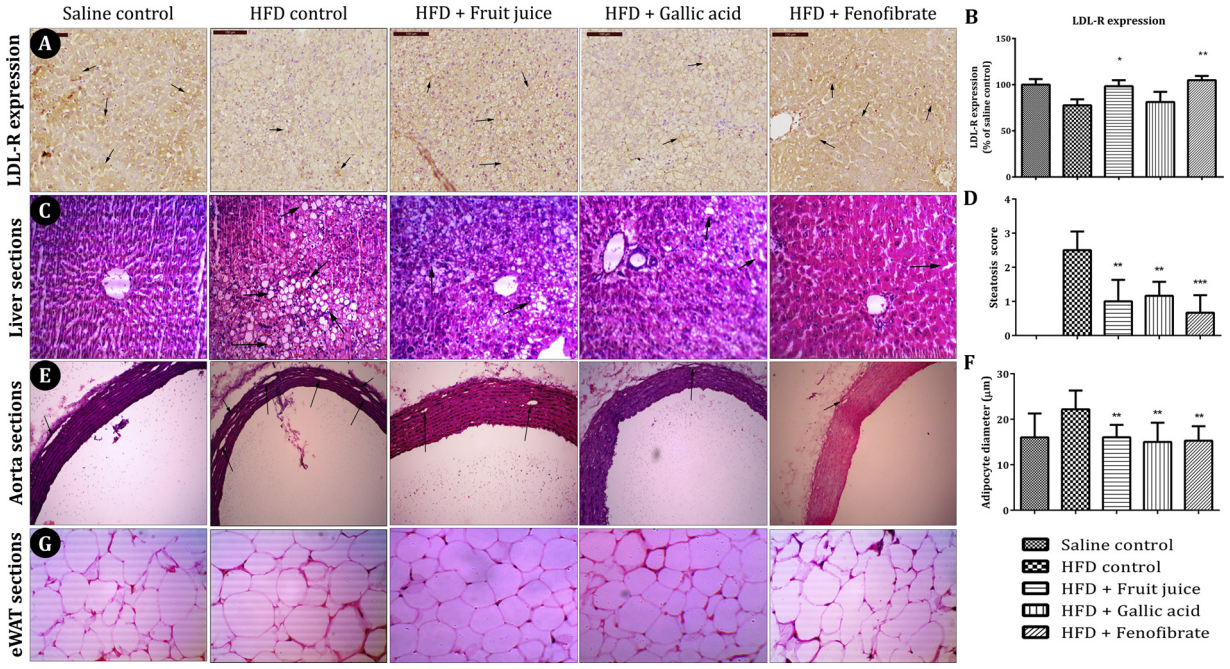

Fig. 4. Gallic acid as well as fruit juice of *E. officinalis* up-regulate LDL-R expression on hepatocytes and protect against HFD-induced fatty infiltration as well as adipogenesis. A, B) HFD decreased hepatic LDL-R expression and treatment with fruit juice of *E. officinalis* showed increase in LDL-R expression but gallic acid failed to restore LDL-R expression. Intensity of brown color represents degree of LDL-R expression (indicated by arrows). C, D) HFD-control rats showed significant increased fatty infiltration *i.e.* balloon formation (indicated by arrows) and steatosis score. Treatment with gallic acid as well as fruit juice of *E. officinalis* significantly decreased HFD-prompted fatty infiltration and steatosis (Steatosis was graded on 0–3 scale; grade-0 indicates absence of steatosis, grade-1 indicates greater than 30% of hepatocytes affected, grade-2 indicates 30–70% of hepatocytes affected and grade-3 indicates more than 70% of hepatocytes are affected). E) There is increased oil accumulation in aortic intimal wall (indicated by arrows) in aorta of HFD-control animals. Gallic acid as well as fruit juice of *E. officinalis* decreased oil accumulation in aortic walls. F–G) Animals manipulated with HFD showed increase in adipocyte mean diameter while gallic acid as well fruit juice of *E. officinalis* treatment showed decrease in adipocyte diameter.HFD = high fat diet. Values are denoted as mean ± SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, significantly different from HFD-induced dyslipidemia (magnification 40×).

3.6. E. officinalis and gallic acid increase the expression of PPARα, LDL-R and adiponectin gene with regulation of PCSK9 and leptin gene

PPAR-α is a key regulator for lipid metabolism which was found to be significantly declined in the animals receiving high fat diet. As shown in Fig. 5, this declined level of PPAR-α expression was significantly restored with the treatment with fruit juice of *E. officinalis*, gallic acid as well as fenofibrate ($p < 0.001$). Simultaneously, the expression of LDL-R was lowered along with elevated PCSK9 levels in HFD-control rats than those in control rats ($p < 0.001$and $p < 0.01$, respectively). Fruit juice of *E. officinalis* and gallic acid supplementation showed increased LDL-R expression with decrease in PCSK9 levels compared to HDF-control animals. Apart from lipid metabolic maladies, adiponectin expression level was significantly decreased along with increased leptin expression in high-fat-fed animals compared to control animals. HFD-boosted decrease in adiponectin and increased leptin mRNA expression levels were significantly restored in gallic acid and *E. officinalis* treated animals as compared to HDF-control animals (Fig. 5).

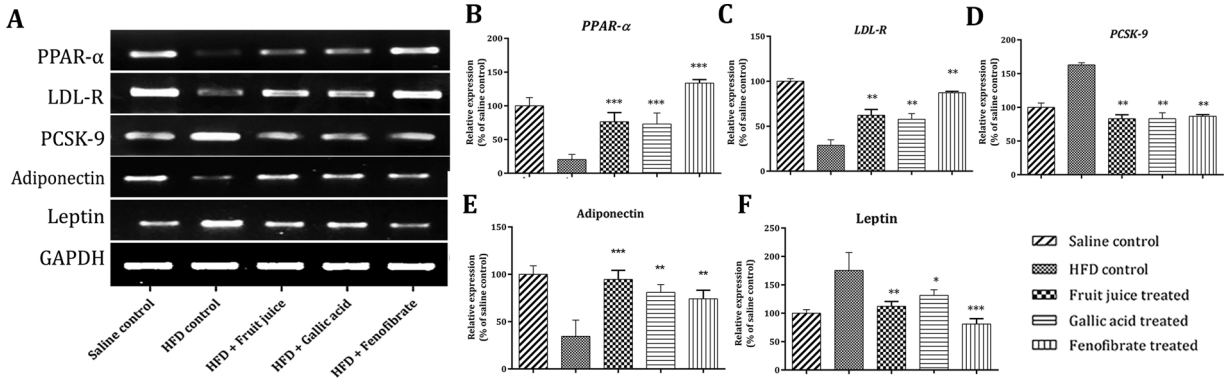

Fig. 5. Effect of gallic acid as well as fruit juice of *E. officinalis* on high-fat-fed animal. A) Representative PCR data from HFD- and treated animals. High-fat-fed animals showed significant reduction in B) PPAR-α and C) LDL-R expression with increased D) PCSK-9 expression. HFD-control animals also demonstrated diminished level of E) adiponectin expression along with elevated F) leptin expression. HFD = High fat diet, PPAR-α = peroxisome proliferator-activated receptor-α, LDL-R = low-density lipoprotein-receptor, PCSK-9 = proprotein convertase subtilisin/kexin type 9, GAPDH = glyceraldehyde 3-phosphate dehydrogenase. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, significantly different from HFD-control rats.

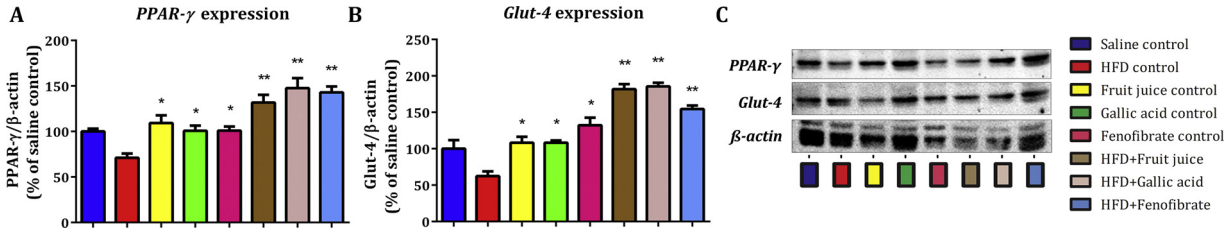

Fig. 6. Effect of gallic acid as well as fruit juice of *E. officinalis* treatment on PPAR-γ and glut-4 protein expression. High fat diet control animals showed reduced level of A) PPAR-γ and B) Glut-4 protein expression in eWAT. Simultaneous treatment with gallic acid as well as fruit juice of *E. officinalis* showed up-regulation of PPAR-γ and Glut-4 protein expression. C) Representative immunoblot of eWAT from HFD- and treated animals. Western blots were carried out on lysates from epididymal white adipose tissue. HFD = High fat diet, PPAR-γ = peroxisome proliferator-activated receptor-γ, eWAT = epididymal white adipose tissue. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, significantly different from HFD-control rats.

3.7. Treatment with E. officinalis and gallic acid improved Glut-4 and PPARγ expression in eWAT

Immunoblotting revealed non-significant reduction in Glut-4 protein expression in white adipose tissue of HFD-control rats (0.712 ± 0.074) compared to control rats (1.141 ± 0.134) (Fig. 6). The decreased level of Glut-4 was significantly restored in the gallic acid and *E. officinalis* treated animals when compared with HFD-control rats. As illustrated in Fig. 6, PPAR-γ expression was also found to be decreased with chronic exposure to high-fat-diet and treatment with fruit juice of *E. officinalis* and gallic acid restored the level of total PPAR-γ in the high-fat-fed animals.

4. Discussion

Cholesterol plays vital role in maintenance of cell membrane fluidity and permeability, but high lipid level is recognized as a major contributor for health problems worldwide and leads to cardiovascular events. Higher plasma cholesterol leads to lipid deposition in arterial walls and starts forming plaque and ultimately develops atherosclerosis [21]. For the treatment of atherosclerosis and related lipid disorders, numerous traditional medicines were employed along with the current treatment [22]. In present investigation, the protective effect of gallic acid, one of the chief phytochemical present in the *E. officinalis*, on the various animal models of dyslipidemia was evaluated. Results from current study suggested the protective role of gallic acid against experimental hyperlipidemia; as evidenced through increased gene expression of PPARα and LDL-R, increased hepatic oil clearance and activating hepatic lipid metabolizing enzymes.

Poloxamer-407 is a non-ionic surfactant and systemic administration of poloxamer-407 elevates plasma lipid levels by direct inhibition of endothelial lipoprotein lipase and indirect activation of rate limiting enzyme in *de-novo* cholesterol biosynthesis, HMG-CoA reductase [23]. Also, poloxamer-407 administration induces atherogenic response by decreasing LDL-R expression on hepatocytes and lipid oxidation [23,24]. In similar manner, administration of tyloxapol-407 also produces hyperlipidemia by displacing cholesterol from liver to serum compartment and increasing sterol synthesis [25]. In agreement with this observation, current data also showed increase in plasma cholesterol and triglyceride levels upon injection of poloxamer-407 and tyloxapol in rats. Treatment with gallic acid as well as fruit juice of *E. officinalis* showed significant reduction in elevated triglycerides and cholesterol levels. These data support our previous lipid lowering observations of fruit juice of *E. officinalis* [10,11].

Development of overweight can be directly correlated with high energy intake and energy intake is often high when high-fat-diet is consumed in high amount. Animals will develop obesity upon HFD consumption and subsequently develop metabolic syndrome like condition *i.e.* hyperlipidemia, hepatic steatosis, insulin resistance, hyperglycemia and hypertension due to alteration in cholesterol and triglyceride levels in plasma and liver [26–28]. In present study, significant increase in plasma total cholesterol level was observed in rats supplemented with high-fat-diet. However, in contrast to previous data, there was no obvious increase in triglyceride levels or bodyweight gain. As hypothesized, gallic acid as well as *E. officinalis* decreased HFD-elevated total cholesterol level, suggesting that treatment might have an important role in regulation of hepatic lipid metabolizing enzymes. It is well documented that high-fat-diet supplement increases oxidative stress in liver and adipose tissue and this leads to weight-gain, insulin resistance and hyperlipidemia through upregulation of genes involved in sterol biosynthesis [29,30]. In line with these results, recent publication from Muthu et al. has also shown antioxidant property of extract of *E. officinalis* against HFD-induced oxidative imbalance in liver and kidney [31]. Thus, antioxidant potential of gallic acid might be one of the mechanism for protection against HFD-prompted oxidative maladies [32]. Activity of hepatic lipogenic enzymes like G6PD, FAS and ME initiates *de-novo* synthesis of cholesterol and fatty acid biosynthesis while reduction in lipogenic enzymes decrease fatty acid availability required for synthesis of triglycerides [33]. Berndt et al. also demonstrated linear correlation between increased FAS gene expression and body fat accumulation [34] and Zhao et al. showed increased adipose fat accumulation with increased activity of FAS, ME and G6PD [35]. In accordance with this data, current study also showed significant reduction in hepatic lipogenic enzymes in the gallic acid and *E. officinalis* supplemented rats. Concurrently, treatment with gallic acid as well as fruit juice of *E. officinalis* also increased activity of fatty acid oxidation rate-limiting enzyme *i.e.* CPT in hepatic tissue. Former report showed increased intracellular lipid accumulation with inhibition of CPT enzyme [36]. In accordance to this finding, Stefanovic-Racic et al. also proved substantial decrease in hepatic triglyceride level with moderate increase in CPT activity [37]. Hence, hypolipidemic potential of gallic acid and fruit juice of *E. officinalis* might be credited to suppression of lipogenesis through reduction in lipogenic enzyme activity and increased fatty acid oxidation via CPT activity.

Plasma cholesterol level is maintained through net balance between cholesterol biosynthesis and degradation or uptake of cholesterol through hepatic LDL-R [38]. Liver is the vital organ for regulation of lipid level through expression of LDL-R and HMG-CoA reductase enzyme. In general, elevation in plasma LDL-cholesterol level is strongly associated with incidence of cardiac and vascular disorders. Nammi et al. demonstrated that long-term consumption of HFD alters body lipid homeostasis which leads to increased cholesterol biosynthesis along with reduced hepatic LDL-R protein in rat liver [39]. In agreement with this data, present study also demonstrated significant decrease in LDL-R expression on hepatocytes from HFD-control rats and treatment with *E. officinalis* showed increase in LDL-R expression while gallic acid showed non-significant increase in LDL-R expression. Fruit juice of *E. officinalis* contains abundant amount of phytochemicals including ellagic acid, quercetin, flavone glycosides, alkaloids and sesquiterpenes [4], and *E. officinalis* mediated significant increase in LDL-R expression could be attributed to presence of other phytochemicals. Lee et al. has reported that treatment with ellagic acid can reduce oxidized

LDL in human endothelial cells [40]. Results from Pal et al. and Koshi et al. also support increase in LDL-R expression upon treatment with polyphenols and *E. officinalis* [41].

High-fat-diet supplementation alters lipid homeostasis through up-regulating lipogenesis and down-regulation of lipolysis which results into hepatic fatty infiltration and steatosis [35,37,42]. Similarly, in present study high-fat-diet increases incidence of fatty liver and infiltration of leucocytes in liver tissue due to activation of proinflammatory cytokines like TNFα and IL-6 which activate resident macrophages in liver [43]. Gallic acid and *E. officinalis* ameliorated steatosis through dramatic decrease in lipid accumulation and decrease in hepatic tissue inflammatory response. In atherosclerosis, chronic inflammation leads to accumulation of lesions on vessel walls which activates macrophage mediated inflammatory response and leads to oil accumulation in intimal wall [44]. In present experiment, higher oil accumulation was observed in intimal wall of aortic arc of HFD-control rats while animals treated with gallic acid as well as fruit juice of *E. officinalis* showed decreased oil accumulation in aortic intima. This study exposed multiple mechanisms for gallic acid mediated hypolipidemic activity and amalgamation of these hypolipidemic activities potentially play an important role in the decreased oil accumulation in base of aortic arc. Recently, many reports showed hypolipidemic potential of *E. officinalis* through activation of various molecular mechanisms including antioxidant potential [31,45,46].

Chronic inflammation has clinical correlation with progression of obesity and related metabolic disorders. Chronic administration of high-fat-diet activates inflammatory response in adipose tissue and results into release of various inflammatory cytokines and chemokines like IL-1α, IL-1β, IL-6, IL-10, TNFα, GM-CSF, MIP1α and MIP1β [47]. These inflammatory cytokines increase adipocyte differentiation which leads to increase in adipocyte size and increase in weight [47,48]. In line with this fact, there was increase in adipocyte size in epididymal adipose tissue from high-fat-diet rats. However, there was no weight-gain in HFD animals but treatment with gallic acid and *E. officinalis* showed decrease in adipocyte size. Recently, Huang et al. also reported that administration of *E. officinalis* decreases hypertriglyceridemia and adipose tissue weight in high-fructose-fed animals through activation of protein kinase C-zeta (PKC-ζ) [49].

PPARα is a member of nuclear receptor family which regulates lipid homeostasis through genetic expression of fatty acid transport and acyl-CoA synthase. Activation of PPARα increases fatty acid ß-oxidation and this leads to increase in HDL level [50]. Previous studies conducted on PPARα$^{-/-}$ mice showed essential role of PPARα in regulation of lipid homeostasis in high-fat-diet models [51,52]. In accordance with this data, current study also revealed increase in PPARα gene expression in rats treated with gallic acid as well as fruit juice of *E. officinalis*. PCSK9 and LDL-R expression share a common regulatory pathway and expression of PCSK9 was found to be positively correlated to the plasma lipid levels [53]. PCSK9 binds with LDL-R and targets it for lysosomal degradation in cells. In this study, PCSK9 gene expression was decreased in gallic acid treated animals and increased LDL-R on hepatocytes might be attributed to decreased expression of PCSK9.

Leptin is adipocytes-derived hormone which regulates energy metabolism in adipose tissue and food intake. Adiponectin regulates glucose homeostasis and maintains insulin sensitivity [48,54]. In accordance with earlier reported data, decreased adiponectin and increased leptin levels were observed in rats receiving high-fat-diet and gallic acid restored HFD-induced alteration in adiponectin and leptin expression.

PPARγ is a nuclear receptor which acts as transcription factor and regulates fat storage and glucose homeostasis in adipose tissue. PPARγ agonists have been reported for various biological potentials *viz.* antioxidant, antifibrotic, antiapoptotic and anti-inflammatory [55]. In protein expression study, gallic acid treatment demonstrated increase in expression of PPARγ and Glut4 protein in adipose tissue. Bak et al. also demonstrated gallic acid induced improved glucose tolerance in obese mice through increased PPARγ expression [56]. However, in contrast to this data, Sato et al. demonstrated anti-obesity effect of *E. officinalis* through inhibition of PPARγ [57], while Huang et al. supported induction of Glut4 and PPARγ expression in animals treated with gallic acid isolated from *Punica granatum* [58].

5. Conclusion

In summary, these findings have confirmed anti-hyperlipidemic potential of *E. officinalis* through multiple mechanisms including increased PPARs expression, increased expression LDL-R on hepatocytes, decreased PCSK9 levels, increased activity of hepatic lipolytic and decreased lipogenic enzymes. *E. officinalis* restored HFD-induced alteration in lipid metabolism and decreased hepatic oil infiltration along with decreased oil accumulation in intimal wall of aorta. Gallic acid, a chief phytoconstituent present in the *E. officinalis*, found to be bio active constituent responsible for anti-hyperlipidemic potential of *E. officinalis*. These data support anti-hyperlipidemic along with cardio- and hepato-protective potential of *E. officinalis.*

Conflict of interest

The authors declare that there are no conflict of interest pertaining to this manuscript.

Author contributions

BV, AB and YC designed, performed, analyzed and interpreted studies data and wrote the manuscript. SP and JH designed, analyzed and interpreted experimental data, contributed to writing the manuscript and contributed to study supervision.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.09.158.

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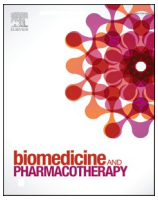

Corrigendum to "Suppression of abdominal fat and anti-hyperlipidemic potential of *Emblica officinalis*: Upregulation of PPARs and identification of active moiety" Biomed. Pharmacother. 108 (2018) 1274–1281

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The authors regret that in the published article, there was an error in Fig. 4G as published. These errors occurred in preparation of composite figures from individual images, which were inadvertently placed. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The authors would like to apologise for any inconvenience caused. The corrected Fig. 4G appear below.

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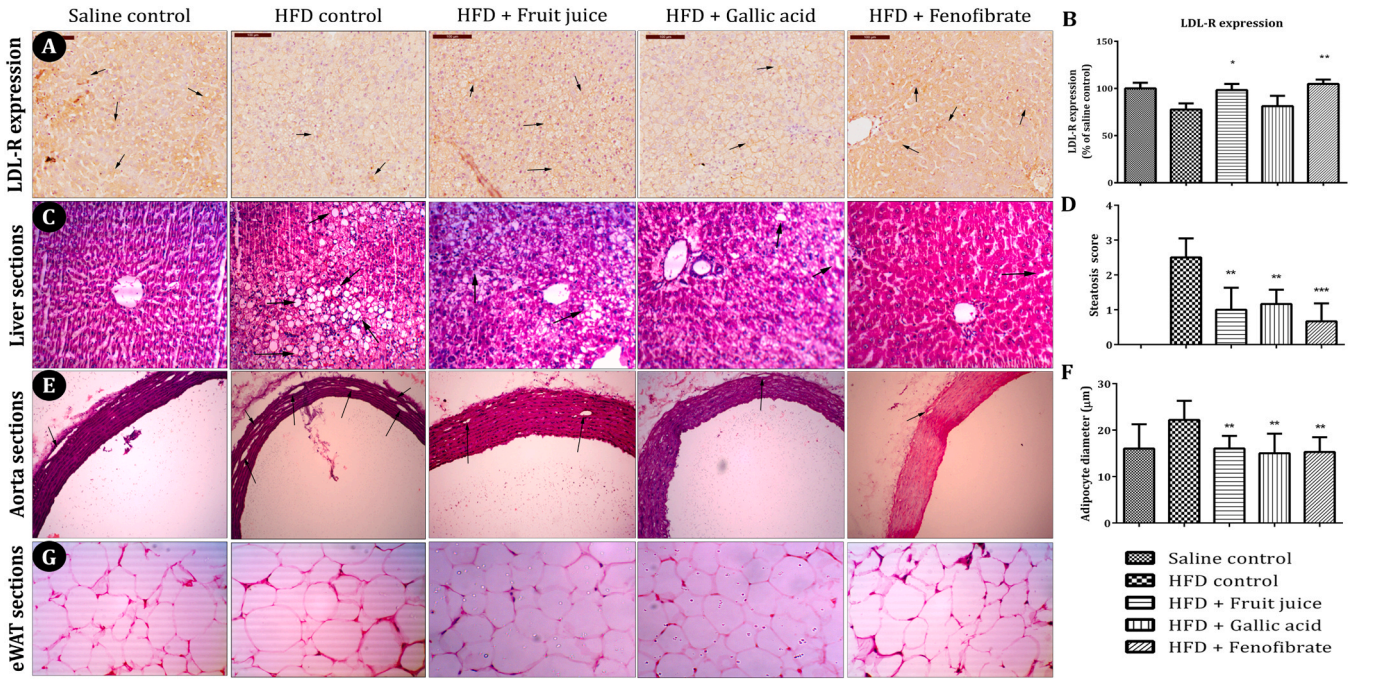

Fig. 4. Gallic acid as well as fruit juice of E. officinalis up-regulate LDL-R expression on hepatocytes and protect against HFD-induced fatty infiltration as well as adipogenesis. A, B) HFD decreased hepatic LDL-R expression and treatment with fruit juice of E. officinalis showed increase in LDL-R expression but gallic acid failed to restore LDL-R expression. Intensity of brown color represents degree of LDL-R expression (indicated by arrows). C, D) HFD-control rats showed significant increased fatty infiltration i.e. balloon formation (indicated by arrows) and steatosis score. Treatment with gallic acid as well as fruit juice of E. officinalis significantly decreased HFD-prompted fatty infiltration and steatosis (Steatosis was graded on 0–3 scale; grade-0 indicates absence of steatosis, grade-1 indicates greater than 30 % of hepatocytes affected, grade-2 indicates 30–70 % of hepatocytes affected and grade-3 indicates more than 70 % of hepatocytes are affected). E) There is increased oil accumulation in aortic intimal wall (indicated by arrows) in aorta of HFD-control animals. Gallic acid as well as fruit juice of E. officinalis decreased oil accumulation in aortic walls. F–G) Animals manipulated with HFD showed increase in adipocyte mean diameter while gallic acid as well fruit juice of E. officinalis treatment showed decrease in adipocyte diameter.HFD=high fat diet. Values are denoted as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, significantly different from HFD-induced dyslipidemia (magnification 40×).