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Research Article

Fraxetin supplementation lowers plasma lipids and enhances antioxidant status in high-fat diet-induced hypercholesterolemic rats

Purushothaman Ayyakkannu[1], Ramalingam Sundaram[1], Meenatchi Packirisamy[1], Sundhararajan Ranganathan[2]

[1]Department of Biochemistry, Mohamed Sathak College of Arts and Science, Affiliated to the University of Madras, Chennai, India
[2]Department of Pharmacy, Mohamed Sathak A. J. College of Pharmacy, Affiliated to the Tamilnadu Dr. MGR Medical University, Sholinganallur, Chennai, India

Abstract

Objectives: Hypercholesterolemia is a serious health concern throughout the world. It is the key risk factor for cardiovascular disease (CVD). The aim of this study was to investigate the antihypercholesterolemic potential of fraxetin on hypercholesterolemic rats given a high-fat diet (HFD).

Methods: A total of 24 male albino Wistar rats weighing 180-200 g were used in this study and were divided into 4 groups: Control (Group 1), hypercholesterolemia-induced (Group 2), hypercholesterolemia-induced and treated with fraxetin (75 mg/kg) (Group 3), and hypercholesterolemia-induced and treated with simvastatin (10 mg/kg) (Group 4). The plasma lipid profile, status of enzymatic and non-enzymatic antioxidants, and the levels of oxidative stress markers of all groups were analyzed.

Results: The plasma level of total cholesterol, triglycerides, very low-density lipoprotein, and low-density lipoprotein cholesterol were significantly increased, and the level of high-density lipoprotein cholesterol was significantly decreased in the hypercholesterolemic rats in comparison with the normal, control rats. Oral administration of fraxetin significantly ($p<0.05$) reversed these altered parameters to near-normal levels. In addition, fraxetin treatment significantly ($p<0.05$) increased the status of antioxidants with a concomitant reduction in oxidative stress markers. Oil red O staining of the thoracic aorta revealed widespread deposition of lipid droplets in the hypercholesterolemic rats (Group 2), whereas the hypercholesterolemic rats treated with fraxetin or simvastatin showed only scattered droplets of fat. The effect of fraxetin on various biochemical parameters was comparable to that of simvastatin.

Conclusion: The results of this study indicated that the lipid-lowering potential of fraxetin at the dosage of 75 mg/kg was comparable to that of the antihypercholesterolemic drug simvastatin. Further studies on the molecular mechanism of action of fraxetin are warranted and in progress in our laboratory at the time of writing.

Keywords: Antioxidants, fraxetin, hypercholesterolemia, oxidative stress

Hypercholesterolemia, the presence of high levels of cholesterol in the blood, is a serious health concern around the world. It is the key risk factor for cardiovascular disease (CVD). Coronary heart disease and stroke are the number one cause of death worldwide, accounting for 17.9 million deaths every year, the equivalent of 31% of all global deaths [1]. To improve the global CVD burden, the World Health Organization has encouraged tobacco control, dietary salt reduction, and strengthening of CVD management in primary health care systems [2]. The major causes of hypercholesterolemia are a sedentary lifestyle, an increased intake of saturated fatty acids, tobacco use, an unhealthy diet, and excessive alcohol consumption. Secondary causes include hypothyroidism, nephrotic syndrome, cholestasis, and certain

Address for correspondence: Purushothaman Ayyakkannu, MD.Department of Biochemistry, Mohamed Sathak College of Arts and Science, Affiliated to the University of Madras, Chennai, India
Phone: +91 44 24501115 **E-mail:** purushothamanbiochem@gmail.com **ORCID:** 0000-0001-5018-5515

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medications, such as cyclosporine, thiazide, and diuretics [3]. Higher levels of female sex hormones, like estrogen, have also been reported to increase cholesterol levels [4]. Hypercholesterolemia results in elevated blood pressure and blood glucose, and is also associated with obesity, all of which are risk factors detrimental to good heart health.

Statins (hydroxymethylglutaryl-CoA reductase inhibitors) have become the most extensively prescribed rhabdomyolysis drug worldwide since their introduction in the 1980s [5, 6]. However, these drugs can have notable side effects, such as hyperuricemia (gout), diarrhea, flushing, nausea, myositis, gastric irritation, dyspepsia and gallstones, abnormal liver function, and rhabdomyolysis, which may lead to renal failure [7]. Hence, there is still a need to discover anti-hypercholesterolemic drugs with better efficacy and fewer potentially adverse effects. In recent years, chemical principles from plant sources have become a focal point of research to develop novel hypocholeterolemic drugs. Phytotherapy could be a suitable alternative or complementary therapy for hyperlipidemia [8].

Coumarin (1,2-benzopyrone) is a natural phenolic compound found in many plant species, including green tea [9]. Coumarin and some coumarin derivatives *viz*. esculetin, scoparone, and 4-methylumbelliferone, have been reported to have lipid-lowering effects [10]. Fraxetin (7, 8-dihydroxy-6-methoxycoumarin), a coumarin derivative widely present in citrus fruits, tomatoes, vegetables, green tea, and many natural food products, has attracted research interest as an antioxidant, antidiabetic, anti-inflammatory, antiviral, antitumor, and neuroprotective agent [11-13]. A literature survey yielded no reports on a lipid-lowering effect of fraxetin. Therefore, this study was designed to investigate the hypolipidemic and antioxidant potential of fraxetin on high-fat diet (HFD)-induced hypercholesterolemic rats.

Materials and Methods

Source of chemicals and HFD components

Cholesterol used in the study was procured from Sisco Research Laboratories Pvt. Ltd., of Mumbai, Maharashtra, India. Bile salt was purchased from Central Drug House Pvt. Ltd., of New Delhi, India. Egg yolk power was obtained from SKM Egg Products Export (India) Ltd., Erode, Tamil Nadu, India and lard from a local market in Chennai, Tamil Nadu, India. The standard reference drug simvastatin was purchased from Micro Labs Ltd., Pondicherry, Puducherry, India. Oil red O staining solution was purchased from HiMedia Laboratories Pvt. Ltd. of Mumbai, Maharashtra, India. All other chemicals and reagents used were of analytical grade.

Experimental design

A total of 24 male albino Wistar rats of 8 week old, weighing 180-200 g were procured from the Central Animal House Facility, Mohamed Sathak A. J. College of Pharmacy (affiliated with The Tamil Nadu Dr. MGR Medical University), Chennai, Tamil Nadu, India. The subjects were maintained at an ambient temperature of 25±2°C and a 12/12-hour light/dark cycle. The animals were given standard commercial rat chow and water *ad libitum*. The experiments were conducted according to the ethical norms approved by the Government of India Ministry of Social Justice and Empowerment Institutional Animal Ethics Committee Guidelines. The subjects were divided into 4 groups of 6 animals: control (Group 1), hypercholesterolemia-induced (Group 2), hypercholesterolemia-induced and treated with fraxetin (75 mg/kg) [14] (Group 3), and hypercholesterolemia-induced and treated with simvastatin (10 mg/kg) (Group 4).

Induction of hypercholesterolemia

The HFD was prepared according to the method described by Xie et al. [15]. It comprised normal rat chow (84.3%), lard (5%), egg yolk powder (10%), cholesterol (0.2%) and bile salt (0.5%), and was fed to the rats orally for a period of 8 weeks. To confirm the induction of hypercholesterolemia, blood samples were collected in a heparin-coated tube from the retro-orbital sinus, and the plasma obtained after centrifugation was used to estimate the level of cholesterol. Rats with a fasting plasma cholesterol of >250 mg/dL were used for the study. Once hypercholesterolemia was confirmed, the HFD was maintained and treatment with fraxetin (75 mg/kg dissolved in 0.5 mL of saline administered intragastrically) or simvastatin (10 mg/kg) dissolved in 0.5 mL of saline and delivered intragastrically via gavage was initiated on the next day, which was considered day 1 of treatment and continued for 30 days. At the end of treatment (56 days of induction + 30 days of fraxetin or simvastatin treatment), the animals were anesthetized with sodium thiopentone (40 mg/kg) and euthanized. Blood was collected and cardiac tissue was collected for analysis.

Plasma lipid profile

The levels of total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-c) were estimated using biochemical kits (Agappe Diagnostics Ltd., Ernakulam, Kerala, India). To determine the level of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol, Friedewald's formula [16] was used:

VLDL cholesterol = Triglyceride/5 and

LDL cholesterol = Total cholesterol-(VLDL+HDL cholesterol)

Status of antioxidants and estimation of oxidative stress markers

The enzymatic antioxidant superoxide dismutase (SOD) was assayed according to the method described by Marklund and Marklund [17]. Catalase (CAT) activity was assayed using the method of Sinha [18], and glutathione peroxidase (GPx) was determined by the method reported by Rotruck et al. [19]. Non-enzymatic antioxidants were also analyzed. Vitamin C was

measured using the method of Omaye et al. [20], vitamin E was estimated with the method of described by Desai [21], and reduced glutathione (GSH) was assayed according to the method of Moron et al. [22]. The Okhawa et al. [23] method was used to measure the level of lipid peroxides as thiobarbituric acid-reactive substances (TBARS). Lipid hydroperoxides (LOOH) were measured with the method of ferrous oxidation in xylenol orange as described by Jiang et al. [24] and Wolff [25].

Oil red O staining of the aorta

Thoracic aorta tissue was quickly dissected from the rats and stored at -80°C until use. The frozen tissue was processed with a cryomicrotome using sections of 5 µm in thickness and stained with oil red O stain. The stained aortic tissue samples were examined and photographed under a light microscope to assess the presence of lipid droplets/atherosclerotic plaque lesions. Digital images were obtained using an Olympus BX51 microscope equipped with a digital camera (Olympus Corp., Tokyo, Japan). All of the images were recorded at 40x magnification.

Statistical analysis

The results were statistically evaluated with Student's t-test using SPSS for Windows, Version 16.0 (SPSS Inc., Chicago, IL, USA) software and analysis of variance. The differences between the groups were considered significant at $p<0.05$. All of the results were expressed as mean±SD. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD).

Results

The plasma level of total cholesterol, triglycerides, VLDL and LDL cholesterol were significantly increased and the HDL cholesterol was significantly decreased in the hypercholesterolemic rats in comparison with the control rats. Oral administration of fraxetin significantly reversed all of these altered parameters to near-normal levels (Table 1).

The effect of fraxetin on the status of antioxidants was examined by assessing the enzymatic and nonenzymatic antioxidants in the serum and cardiac tissue of the control and experimental animals. Table 2 illustrates the activity of enzymatic antioxidants in the serum and cardiac tissue of the control and experimental groups. A significant decrease ($p<0.05$) in the activity of SOD, CAT, GPx was seen in the HFD-induced hypercholesterolemic rats (Group 2) when compared with the controls (Group 1). Similarly, the levels of non-enzymatic antioxidants of vitamin C and vitamin E, and reduced GSH (Table 2) were significantly decreased ($p<0.05$) in the HFD-induced hypercholesterolemic rats (Group 2) when compared with the controls (Group 1). These changes were modulated and brought back to near-normal levels with fraxetin or simvastatin treatment.

Table 2 depicts the effect of fraxetin on the level of TBARS and LOOH in the cardiac tissue of control and experimental subjects. The extent of lipid oxidation was significantly higher in the HFD-induced hypercholesterolemic rats (Group 2) when compared with the control rats. Fraxetin treatment resulted in free radical scavenging and thereby significantly ($p<0.05$) decreased the extent of lipid peroxidation (LPO), thus restoring the antioxidant activity to near-normal levels.

Figure 1a-d are photomicrographs of oil red O-stained aortic specimens. The histology of the aorta is normal in the control group (1a). In the HFD-only group, widespread deposition of lipid droplets and inflammatory cells is seen (1b). The aorta of hypercholesterolemic rats treated with fraxetin significantly reversed the HFD- induced histological changes and showed only scattered fat droplets (1c, 1d). The effect is comparable to that of simvastatin.

Table 1. Effect of fraxetin on the plasma lipid profile in control and experimental rats

Parameters	Group 1 control	Group 2 hypercholesterolemia	Group 3 hypercholesterolemia + fraxetin	Group 4 hypercholesterolemia + simvastatin	p
Cholesterol	122.6±9.07	351±12.60[a*]	180.5±11.30[b*]	171.6±7.42[cNS]	<0.05
HDL-c (mg/dL)	57.16±3.54	28.5±3.01[a*]	50.16±3.18[b*]	52.66±3.26[cNS]	<0.05
LDL-c (mg/dL)	45.73±11.6	238.2±9.79[a*]	88.4±14.5[b*]	79.26±8.68[cNS]	<0.05
VLDL-c (mg/dL)	19.7±1.6	84.3±2.10[a*]	41.99±1.6[b*]	39.73±1.84[cNS]	<0.05
TG (mg/dL)	98.8±8.40	421.5±10.65[a*]	209.66±8.16[b*]	198.6±9.20[cNS]	<0.05

Data expressed as mean±SD (n=6). Statistically significant at *$p<0.05$ with post hoc least significant difference test for [a]control versus hypercholesterolemic rats, [b]hypercholesterolemic rats versus fraxetin-treated hypercholesterolemic rats and [c]fraxetin-treated hypercholesterolemic rats versus simvastatin-treated hypercholesterolemic rats. HDL-c: High-density lipoprotein cholesterol, LDL-c: Low-density lipoprotein cholesterol, VLDL-c: Very low-density lipoprotein cholesterol, NS: Non-significant, TG: Triglyceride

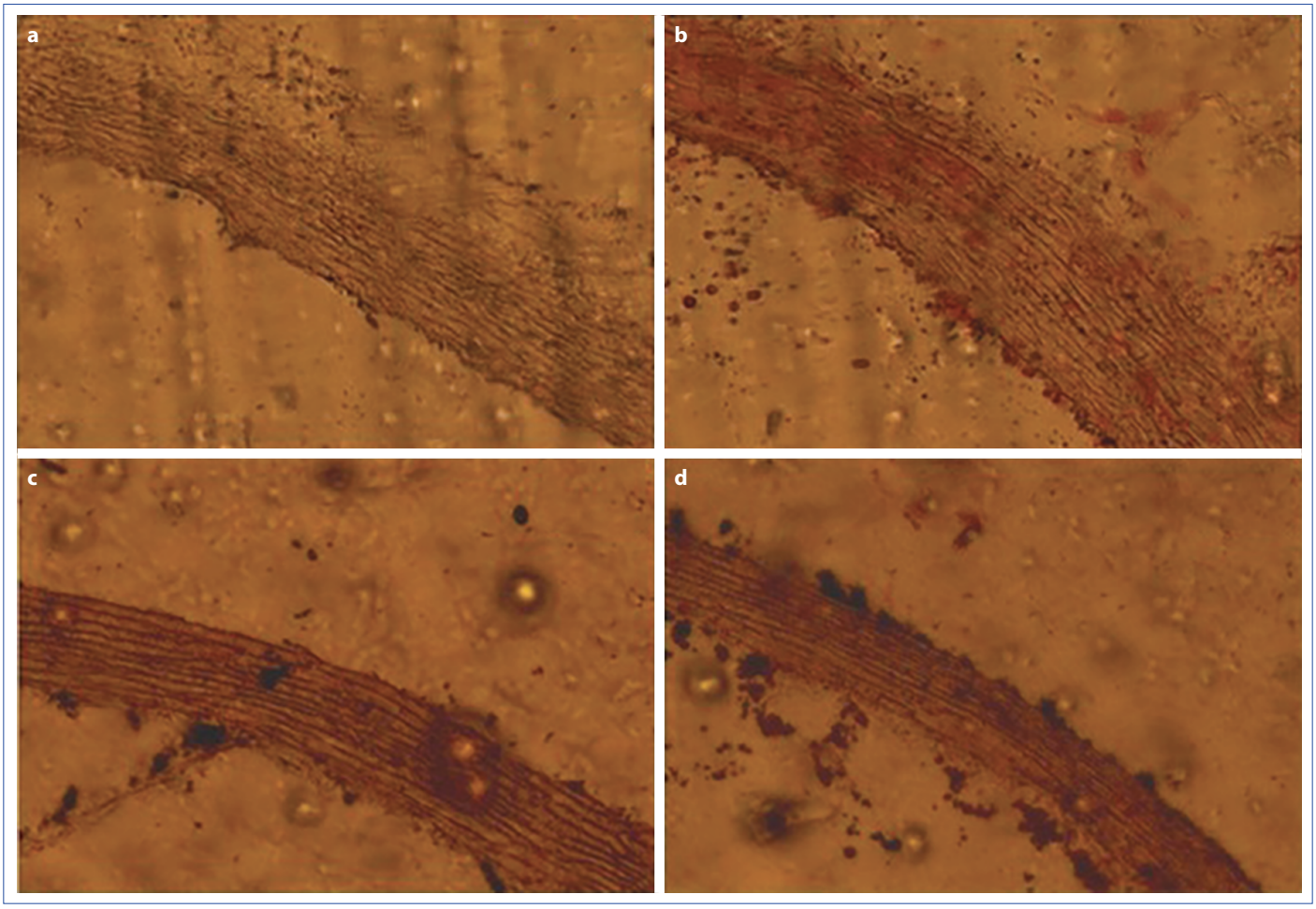

Figure 1. Photomicrographs showing the results of oil red O staining of aorta tissue samples from the control and experimental groups of rats (40x). (a) control, (b) hypercholesterolemia-induced, (c) hypercholesterolemia + fraxetin (75 mg/kg), and (d) hypercholesterolemia + simvastatin (10 mg/kg).

Discussion

Hypercholesterolemia is characterized by elevated levels of blood cholesterol and serum triglycerides. It is the major risk factor for the development of CHD [26, 27]. In this study, the therapeutic efficacy of fraxetin against HFD-induced hypercholesterolemia in rats was examined. Oral administration of fraxetin or simvastatin significantly reversed all of the plasma lipid profile parameters. The level of HDL cholesterol reflects the rate of removal of excess peripheral cholesterol. An elevated plasma level of LDL cholesterol is a major risk factor for coronary heart disease [28]. Hypercholesterolemic rats treated with fraxetin (75 mg/kg) demonstrated an increase in HDL cholesterol with a concomitant decrease in LDL cholesterol, which indicates a reduced risk of atherosclerosis and CVD. Fraxetin appears to have lipid-lowering potential.

Earlier studies have reported that an imbalance of oxidant/antioxidant status, i.e., increased oxidative stress, and weakened antioxidants could be part of the pathogenesis of CHD and ischemic stroke [29-33]. To study the antioxidant potential of fraxetin, we analyzed the status of the enzymatic antioxidants of SOD, CAT, and GPx. The level of the non-enzymic antioxidants vitamin C, vitamin E, and GSH were also analyzed in the serum and cardiac tissue of the control and experimental groups. Our results indicated that both the enzymatic and non-enzymatic antioxidants were significantly decreased ($p<0.05$) in the HFD-induced hypercholesterolemic rats. Endogenous antioxidant enzymes such as SOD, CAT, and GPx play an active role in the cellular defense mechanism against reactive oxygen species (ROS) [34]. Superoxide anion (O_2^-), a highly reactive molecule, can have a deleterious effect on cellular macromolecules by generating hydroxyl radicals, hydrogen peroxide, and hypochloric acid, leading to oxidative stress and tissue damage [35]. SOD is the first antioxidant enzyme to contend with oxyradicals by accelerating the dismutation of superoxide to hydrogen peroxide [36]. CAT is involved in the removal of hydrogen peroxide formed during the reaction catalyzed by SOD. Thus, SOD and CAT are reciprocally supporting antioxidative enzymes and provide a protective defense against ROS. In the present study, the SOD and CAT levels were significantly compromised in the untreated hypercholesterolemic rats (Group 2), which may be due to the oxidant/

Table 2. Effect of fraxetin on the level of enzymatic, non-enzymatic antioxidants, and oxidative stress markers in control and experimental rats

Parameters	Sample	Group 1 Control	Group 2 Hypercholesterolemia	Group 3 Hypercholesterolemia + fraxetin	Group 4 Hypercholesterolemia + simvastatin	p
SOD	Serum	9.38±1.26	2.75±0.76[a*]	7.82±0.84[b*]	9.27±0.86[cNS]	<0.05
	Cardiac tissue	11.35±0.98	3.21±0.30[a*]	9.08±0.78[b*]	9.67±0.83[cNS]	<0.05
CAT	Serum	68.56±2.35	44.36±1.47[a*]	53.36±1.54[b*]	63.61±2.78[cNS]	<0.05
	Cardiac tissue	57.12±8.58	36.40±3.67[*]	52.18±6.92[b*]	54.02±6.70[cNS]	<0.05
GPx	Serum	9.74±0.64	6.34±0.53[a*]	8.26±0.64[b*]	8.68±0.73[cNS]	<0.05
	Cardiac tissue	11.93±0.72	6.89±0.37[a*]	9.96±0.57[b*]	10.6±0.69[cNS]	<0.05
Vitamin C	Serum	2.65±0.32	1.48±0.19[a*]	2.24±0.14[b*]	2.46±0.18[cNS]	<0.05
	Cardiac tissue	3.88±0.24	192±0.16[a*]	3.38±0.22[b*]	3.52±0.26[cNS]	<0.05
Vitamin E	Serum	1.86±0.13	1.27±0.08[a*]	1.64±0.15[b*]	1.87±0.17[cNS]	<0.05
	Cardiac tissue	2.46±0.24	1.15±0.13[a*]	2.23±0.21[b*]	2.56±0.27[cNS]	<0.05
Reduced glutathione	Serum	9.87±0.86	6.85±0.57[a*]	7.79±0.58[b*]	9.62±0.79[cNS]	<0.05
	Cardiac tissue	12.82±0.87	7.36±0.69[a*]	8.64±0.72[b*]	12.63±0.96[cNS]	<0.05
TBARS (mmol/g tissue)	Cardiac tissue	0.71±0.06	1.72±0.10[a*]	1.20±0.17[b*]	1.16 ± 0.10[cNS]	<0.05
Hydroperoxide (mmol/g tissue)	Cardiac tissue	60.40±5.20	122.30±8.12[a*]	75.52±7.56[b*]	72.10±0.11[cNS]	<0.05

Data expressed as mean±SD (n=6). Statistically significant at *p<0.05 with post hoc least significant difference test for [a]control versus hypercholesterolemic rats, [b]hypercholesterolemic rats versus fraxetin-treated hypercholesterolemic rats, and [c]fraxetin-treated hypercholesterolemic rats versus simvastatin-treated hypercholesterolemic rats. Units: Superoxide dismutase is expressed as unit/mg protein, Catalase is expressed as µmoles hydrogen peroxide utilized/min/mg protein, Glutathione peroxidase is expressed as µmoles of reduced glutathione consumed/min/mg protein, Vitamin C, vitamin E, and reduced glutathione are expressed as µg/mg protein. CAT: Catalase, GPx: Glutathione peroxidase, GSH: Reduced glutathione, NS: Non-significant, SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reactive substances

antioxidant imbalance. Furthermore, GPx has been reported to reduce lethality and malondialdehyde levels in ischemic rats treated immediately after ischemia, signifying an anti-ischemic potential [37, 38]. Hence, the reduced levels of GPx and other antioxidant enzymes observed in the present study denote that antioxidant capacity declined in the HFD-induced hypercholesterolemic rats.

The non-enzymatic antioxidant systems are the subsequent line of defense against free radical damage. Vitamins C and E protect cellular membranes, thereby preventing degenerative changes [39]. Glutathione is an important intrinsic non-enzymatic antioxidant and it acts directly as a free radical scavenger by donating a hydrogen atom to a hydroxyl radical [40]. It maintains the cells in a normal redox state and counteracts the harmful effects of oxidative stress [41]. Fraxetin treatment led to a significant increase in these parameters in our study, which may have been due to consistent improvement in antioxidant status, thereby averting a favorable cellular environment for oxidative damage.

Lipid peroxidation (LPO) involves the direct reaction of oxygen and lipid to form radical intermediates and semi-stable peroxidases, which in turn damage the enzymes, nucleic acids, membranes, and proteins [42]. Our results are in agreement with previous findings: We observed an increase in LPO in the HFD-induced hypercholesterolemic rats (Group 2). Administration of fraxetin or simvastatin to HFD rats significantly decreased the oxidative stress markers to near-normal levels, indicating that fraxetin may have the potential to improve hypercholesterolemia-induced oxidative damage.

Conclusion

Based on the results of the biochemical parameter analysis and oil red O staining observations, it can be concluded that fraxetin has a potentially antihyperlipidemic effect. It may have possible use as a hypolipidemic drug; however, additional studies are warranted to further explore the molecular mechanism of action of fraxetin.

Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this article.

Ethics Committee Approval: This experimental study was approved by the Institutional Animal Ethical Committee (IAEC), Central Animal Facility, Mohamed Sathak A. J. College of Pharmacy, Sholinganallur, Chennai-600119, India.

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