Research Article

Hypolipidemic and Anti-atherogenic Effect of Sulphated Polysaccharides from the Green Alga *Ulva fasciata*

Ibrahim H. Borai[1], Magda K. Ezz[1], Maha Z. Rizk[2], M. El-Sherbiny[2], Azza A. Matloub[3], Hanan F. Aly[4], Abd El Razik Farrag[4], Ghadha I. Fouad[2]
[1]Biochemistry Dept., Faculty of Science, Ain Shams University, Cairo, Egypt.
[2]Therapeutic Chemistry Dept., National Research Center, Dokki, Giza, Egypt.
[3]Pharmacognosy Dept., National Research Center, Dokki, Giza, Egypt.
[4]Pathology Dept., National Research Center, Dokki, Giza, Egypt.
*Corresponding author's E-mail: hanan_abduallah@yahoo.com

ABSTRACT

Hypercholesterolemia (HC) is frequently associated with oxidative stress, and release of inflammatory cytokines and results in the formation and accumulation of plaque deposits in the arteries, where it produces numerous functional and structural alterations in the vascular wall that lead to the development of atherosclerosis or coronary heart disease (CHD). Statins have been shown to effectively lower LDL-C levels and reduce both mortality and morbidity associated with coronary heart disease, although there is a significant percentage of individuals for whom statin therapy will not prevent the occurrence of adverse events. There is an obvious need for more efficacious and alternative treatment options. The present study was undertaken to evaluate the atheropreventive activity of sulphated polysaccharides (SPs) of *Ulva fasciata* against induced hypercholesterolemia in rats. Different groups of rats were administered a high cholesterol diet to assess the lipid profile, inflammatory markers (IL-10, MPO) and cell adhesion molecules (ICAM-1 and VCAM-1), oxidative stress marker (NO) before and after treatment with the algal polysaccharides. Oral administration of *Ulva fasciata* crude cold and hot extracts to HC-rats for four consecutive weeks did not exert any side effects, whereas reduced serum lipid profile level and improved the endothelial dysfunction. Treatment with the algal SPs was effectively improved these disorders and diminished the presence of atherogenic plaques in the aorta more than the reference drug fluvastatin. It could thus be concluded that the consumption of UFP (*Ulva fasciata* polysaccharides), may be associated with attenuation of inflammatory markers that in turn lead to control hypercholesterolemia and its related disorders. These results suggest that SPs may be beneficial in ameliorating hypercholesterolemia- associated heart injury probably by attenuation of lipid profile, inflammatory markers, and atherogenic plaque formation in heart, that in turn lead to control hypercholesterolemia and its related disorders; such as obesity, and heart disease.

Keywords: Seaweed, *Ulva fasciata*, Hypercholesterolemia, Atherosclerosis, Hypolipidemic activity, Sulphated polysaccharides (SPs).

INTRODUCTION

Cardiovascular diseases, which include stroke, coronary artery diseases (CAD) and myocardial infarction, are the leading cause of morbidity and mortality in the world. Atherosclerosis, the complex interaction of serum free and esterified cholesterol with the cellular components of the arterial wall, is known to be the primary underlying factor of these cardiovascular events, it is a chronic inflammatory disease started by endothelial injury, followed by sub-intimal focal recruitment of circulating monocytes and T-lymphocytes that heals by fibrosis and calcification and is the leading cause of cardiovascular disease worldwide[1].

Inflammation plays a crucial role in atherogenesis either by local cellular mechanisms or humoral consequences easily measurable in plasma. In most cases inflammation and endothelial dysfunction are triggered by cardiovascular risk factors: hypercholesterolemia, hypertension, smoking or diabetes. Irrespective of its cause systemic inflammation is correlated with cardiovascular events, but currently there are controversial results regarding inflammatory markers and early atherosclerotic process[1]. The selected inflammatory markers are associated with different pathogenic steps in atherogenesis; pro-inflammatory cytokines (MPO); anti-

inflammatory (IL-10), and endothelium activation markers (soluble VCAM-1, ICAM-1).

Hyperlipidemia is known to be the leading risk factor for atherosclerosis. Abnormal increases in serum TC, TG and LDL-C levels are among the indicators of atherosclerosis development, while elevated serum HDL-C is known to be protective against the development of atherosclerosis[2]. Oxidative stress plays a major role in the process of endothelial damage and atherosclerosis. ROS can oxygenate and modify LDL-C to form oxidized LDL-C, leading to the accumulation of cholesterol in phagocytes and to the formation of foam cells, promoting the development of atherosclerosis[3].

Thus, reduction in plasma cholesterol level could improve the endothelial cell function and reduce vascular inflammation[4]. The most commonly used lipid regulators at the present time are statins and fibrates. Although the statins overall safety profile is very good, some concerns have been raised regarding the nature of the side effects, i.e. rhabdomyolysis, essentially because they seem completely linked to the mechanism of action of this class of drugs. In addition, statins do not work for all patients[5]. Therefore there is a need for drugs that would not target an enzymatic pathway, as statins do, or will not induce global changes of gene expression, as fibrates do, but

instead directly target cholesterol present in the blood flow.

In recent years, many polysaccharides with anti-hyperlipidemic activity have been discovered in various food materials, such as *Ulva pertusa*[2], *Ulva lactuca*[6]. Generally, natural polysaccharides from natural materials have antioxidant and anti-hyperlipidemic activities and can be developed as novel potential hypolipidemic agents. Algal SPs have been reported to possess diverse biological activity of potential medicinal value, such as anticoagulant, antitumor, anti-inflammatory, antiviral, antioxidant and anti-hyperlipidemic activity[6,7].

Therefore, this study aimed to evaluate the possible beneficial effect of *Ulva fasciata* polysaccharide (UFP), cold and hot extracts, on serum lipid parameters in hypercholesterolemic (HC) rats fed with cholesterol-rich diet and the histological examination of the aorta, in addition to morphometric measurements of aorta wall thickness were carried out to confirm the biochemical findings and also to develop and utilize effective and natural lipid regulators for more aggressive treatment of hypercholesterolemia.

MATERIALS AND METHODS

Collection of the Algal Sample

Ulva fasciata, belongs to the family *Ulvaceae*, was collected in June 2010 from Abukir, Alexandria. The collected samples of alga were cleaned of epiphytes, barnacle, gastropod and other contaminants at the site. After washing thoroughly in tap water, the samples were air dried at room temperature in the shade, milled coarsely powdered and stored in polyethylene plastic bags in a dry place. Herbarium specimens of the alga were identified by Dr. Shaalan S. A., Professor of Phycology, Faculty of Science, Alexandria University.

Preparation of *U. fasciata* polysaccharides (UFP) crude extracts

Chemical Extraction

Air-dried alga were soaked in 30% volume (w/v) of distilled water and kept overnight at 4 to 5°C. Then the material was stirred well and allowed it to return to room temperature. The cold water extract was first filtered through muslin cloth and then with filter paper. The process was repeated till complete exhausted of polysaccharide (negative molish test). Extract was concentrated to 1/10 of its original volume under reduced pressure at 40°C using rotary evaporator with vacuum (BÜCHI Rota vapor R 200), and precipitated by the addition of 4-fold volume of 95% (v/v) ethanol, centrifuge at 3000 rpm for ten min. The algal residue of cold water was soaked in sufficient distilled water and heated at 100°C for 3h and hot water extract was obtained following the same procedure used for the cold water extract. The precipitate was washed twice with absolute ethanol, the dried by freeze dryer to obtained a crude polysaccharide cold and hot then keep in

refrigerator for chemical and biological[8]. The yields of polysaccharides of *Ulva fasciata* were calculated on the basis of the dry weight of algal sample (w/w).

Chemicals

All chemicals and reagents were purchased from **Biodiagnostic Company** for diagnostic and research reagents, Egypt. Reference drug (Fluvastatin) was purchased from **NOVARTIS** Pharmaceuticals. ELISA kits were provided by **Uscn** (U.S.A.) for MPO and **Invitrogen** (U.S.A.) for IL-10, **Eiaab** (U.S.A.) for both VCAM-1and ICAM.

Induction of Hypercholesterolemia

Hypercholesterolemia was induced in rats according to the method of **Adaramoye**[9], by feeding rats high-fat diet (cholesterol), cholesterol was orally administrated at a dose of (30mg/0.3ml olive oil /1 kg animal) five times a week for twelve consecutive weeks, lard fat was mixed with normal diet (One kilogram of animal lard was added to 5Kgs of normal diet), the occurrence of hypercholesterolemia was determined by measuring the lipid profile (TC, LDL-C, HDL-C, TG), the hypercholesterolemic rats were only used.

Doses and Routs of Administration

- Negative cold extract: Normal rats given *U.fasciata* cold extract at a dosage of 175 mg/kg body weight dissolved in distilled water for 4 weeks, this dose was calculated from the therapeutic dose for rats[8].

- Negative hot extract: Normal rats given *U.fasciata* hot extract at a dosage of 175 mg/kg body weight dissolved in distilled water for 4 weeks.

- Hypercholesterolemic (HC) rats and negative control rats received an oral dose of cold UFP extract; 175 mg/kg body weight dissolved in distilled water daily for 4 weeks.

- Hypercholesterolemic (HC) rats and negative control rats received an oral dose of hot UFP extract at a dose of 175 mg/kg body weight dissolved in distilled water daily for 4 weeks.

- Hypercholesterolemic (HC) rats received an oral dose of 2 mg/kg body weight dissolved in distilled water of the anti-hypercholesterolemic reference drug; fluvastatin, dissolved in distilled water orally by gastric intubation for four weeks.

Experimental Design

Rats

A total of 105 Male Wister rats weighing 120 ± 10gm, were provided from the animal house of the National Research Center (NRC) and housed in a temperature-controlled environment (26-29°C), in steel mesh cages on wood-chip bedding, with a fixed light/dark cycle for two weeks as an acclimatization period with free access to water and food *ad libitum*. The present study was

approved by the Ethical Committee of the National Research Center (NRC), Egypt, which provided that the animals will not suffer at any stage of the experiment.

Animals were randomly divided into seven equally sized groups of 15 rats each [n=15] as follows:

Group 1

Normal controls (NC), given normal diet and distilled water.

Group 2

Negative cold extract controls: Normal rats given cold UFP extract.

Group 3

Negative hot extract controls: Normal rats given hot UFP extract.

Group 4

Hypercholesterolemic (HC) positive control rats.

Group 5

HC-rats received an oral dose of cold UFP extract.

Group 6

HC-rats received an oral dose of hot UFP extract.

Group7

HC-rats received an oral dose of the anti-hypercholesterolemic reference drug; Fluvastatin.

Normal groups were continued to be provided with the common commercial rat chow. By the end of the experiment (4 weeks), the animals were fasted for 12h, weighted then sacrificed using diethyl ether anesthesia.

Blood Collection & Tissue Sampling

By the end of the experiment (4 weeks), the animals of different groups were fasted for 12h, weighted then blood samples were collected from the sublingual vein, then left to coagulate at room temperature and centrifuged at 3000 rpm for 15 min. The clear, non hemolyzed, supernatant sera were quickly removed and kept at -80°C till used for biochemical investigations of lipid profile, liver function, kidney function parameters and inflammatory markers (IL-10, MPO) and cell adhesion molecules (VCAM-1, ICAM-1). Then animals sacrificed using diethyl ether anesthesia and liver tissue was rapidly excised and accurately weighed; 0.5 g from each liver was homogenized in 5 ml phosphate buffer using (pH 7.4) electrical homogenizer. The clear homogenate was used for estimation of non-enzymatic antioxidant defense system; nitric oxide (NO).

Biochemical Examination

Lipid Profile

Serum total lipids concentration was determined according to the method of **Zollner and Kirsch**[10], serum triglycerides (TGs) concentration was determined according to the method of **Fassati and Prencipe**[11], serum cholesterol concentration was estimated according to the method of **Allain**[12] and serum HDL-C concentration was measured according to the method of **Lopez-Virella**[13]. Serum LDL-C concentration was determined according to the equation of **Friedewald**[14]. Serum very low density lipoprotein (VLDL-C) was determined according to **Norbert**[15]. The Atherogenic index (AI) was calculated according to the method of **Harnafi**[16].

Endothelial Dysfunction Markers

1. Nitric oxide

Liver nitric oxide (NO) was determined according to the method of **Montgomery and Dymock**[17].

2. Cell adhesion molecules

Rat soluble Intracellular Adhesion Molecule-1 (s-ICAM-1) concentrations and rat soluble Vascular Cell Adhesion Molecule-1 (s-VCAM-1), were determined using Enzyme linked ImmunoSorbent assay (ELISA).

Atherogenic Markers

In vivo quantitative measurements of MPO, and IL-10, were performed by ELISA; a sandwich enzyme Immunoassay.

Fixed heart specimens were processed to form paraffin cubes, then thin sections of 5µm thickness were stained with Hematoxylin & Eosin (H&E) to assess the cellular changes induced in the myocardium in all treatment modalities.

Histopathological Analysis

Parts of aorta were kept in neutral buffered neutral formalin for 8 hours for fixation then processed in automatic processors and paraffin blocks were obtained. Sections of 3-5µ thickness were stained using Hematoxylin and Eosin (H&E) stain to assess the cellular changes induced in the aorta in all treatment modalities; like the presence of foam cells in subintima as well as media, interruption of elastic lamina and presence or absence of fibrosis. The slides were examined and photographed under a light microscope at a magnification power of x150.

Statistical Analysis

Data were analyzed by comparing values for different treatment groups with the values for individual control. All data were expressed as mean ± S.D. of 15 rats in each group. Significant differences between the groups were statistically analyzed using SPSS computer program; one-way analysis of variance (ANOVA) combined with Student T-test, the slight significant level at P value ≤ 0.05, significant at P ≤ 0.001 and highly significant at P ≤ 0.001.

RESULTS

The current study was designed to examine the anti-hypercholesterolemic, antioxidative and anti-

inflammatory, anti-atherosclerotic and activities of green alga *U. fasciata.*

Antihyperlipidemic Activity in Rats

Body Weight

Negative control rats received both UFP extracts showed insignificant change as compared to normal control untreated rats.

As compared to negative control rats, high-fat fed rats showed significant increase in body weight. Treatment of HC rats with cold, hot extracts of *U. fasciata* and fluvastatin showed significant increase in body weight (initial and final) and BWG (Body weight gain), (Table 1).

Lipid Profile

Insignificant change was detected in lipid profile in healthy negative control rats treated with both algal extracts as compared to normal control rats.

As compared to negative control rats, feeding rats with cholesterol-enriched diet for 12-weeks gave rise to a significant elevation in serum total cholesterol (+ 81.38%), total lipids (63.74%) and triacylglycerol (176.30%). Oral supplementation of cold and hot SP algal extract resulted in insignificant change in the levels of serum total lipids by 7.02, and 14.03% respectively, TC by 3.72, and 2.84%, respectively and TG by 4.34 and -14.42%, respectively, the same for fluvastatin-treated HC rats which showed insignificant change by 8.77% and 13.36% respectively for serum total lipids and TC, while for TG it showed significant decrease reached to 85.75% (Table 2).

It was obvious that, serum HDL-C level was significantly decreased in the HC-rats (85.74%), whereas serum LDL-C, VLDL-C levels and AI of HC- rats were significantly augmented, as compared to normal control group with percentages amounting to 323.86, 175.21 and 2671.1%, respectively (Table 3). Treatment of HC-rats with cold, hot extracts and fluvastatin showed significant decrease in the serum LDL-C, VLDL-C and the AI with percentages decrease reached to 62.56, 3.99 and 43.22%, respectively for cold UFP extract. Treatment of HC-rats with hot extract recorded insignificant change of LDL-C (19.63), VLDL-C (14.71), while recorded 25.83% for AI (significant at P≤ 0.001). Fluvastatin treatment showed percentages decrease of 23, 85.50 and 39.39%, respectively for LDL-C, VLDL-C and AI, as compared to normal control rats. However, HDL-C level showed insignificant change (P ≤ 0.05) amounting to 12.69, 9.55 (significant at P ≤ 0.001) and 3.20%, respectively for cold, hot SP extracts and fluvastatin.

As compared to atherogenic HC-group; treatment of HC-rats with cold, hot UFP extracts and fluvastatin showed, marked reduction in TC, TG and total lipids levels, with percentages decrease of 42.81, 62.24, and 34.64 %, respectively for cold extract and by 46.43, 69.03, and 30.36%, respectively for hot extract, while fluvastatin showed mild percentages decrease reached to 37.50,

32.77 and 33.57%, respectively. Concerning lipoproteins, treatment of atherogenic rats showed significant decrease in LDL-C, VLDL-C levels and the AI with percentages decrease of 61.65, 62.2, and 94.83%, respectively for cold UFP, and 81.04, 69.01 and 97.32% (significant at P ≤ 0.001), respectively for hot UFP. Fluvastatin treatment showed percentages significant decrease reached to 70.98, 32.60, and 94.97%, respectively and in contrast, HDL-C level was significantly increased (P ≤ 0.05) in reached to 512.36 and 578.88%, respectively for cold SP extract and fluvastatin respectively, and 668.31 for hot UFP (significant at P≤ 0.001).

Effect of UFP on Endothelial Dysfunction

Nitric Oxide (NO)

Insignificant change was detected in nitric oxide (NO) levels in healthy negative control rats treated with both algal extracts as compared to normal control rats.

The HC-rats of the present study exhibit high significant elevation of hepatic NO concentration, by a percentage increase of +300.29% (significant at P ≤ 0.001), as compared to normal control rats. HC-rats treated with cold and hot algal extracts of UFP showed insignificant change in the NO concentration as compared to normal control (Table 3).

Comparing to atherogenic HC-rats, UFP treatments showed marked increase in NO level achieved significant decrease; 38.95, 69.46 and 58.19 %, respectively for cold, hot extracts as well as fluvastatin.

Soluble Adhesion Molecules

Insignificant change was observed in healthy negative control rats treated with both algal extracts as compared to normal control rats.

The HC-rats showed significantly increased in serum ICAM-1 (9.67%) and VCAM-1(36.09%) as compared to the negative control group (P ≤ 0.05), (Table 3).

As compared to the negative control group (P ≤ 0.05) ICAM-1 was insignificantly changed by 3.72% for cold UFP, 3.19% for hot UFP and 1.83 for the reference drug, the same was noticed for VCAM-1 by 26.15% for cold UFP, 23.34% for hot UFP and 28.83 for fluvastatin.

In comparison with diseased HC-rats, their treatment with cold, hot UFP extracts as well as fluvastatin, both CAMs were significantly reduced by 5.43 and 7.31%, respectively for cold extract, while hot extract recorded 5.91 and 9.37%, respectively, whereas fluvastatin showed percentages decrease reached to 7.15 and 5.34%, respectively.

Anti-inflammatory Effect of UFP

Insignificant change was detected in both (IL-10) and (MPO) levels in healthy negative control rats treated with both algal extracts as compared to normal control rats.

Rats of high fat diet showed significant increase in pro-inflammatory cytokines, MPO (+10.94%), and in contrast significant decreases in significant decrease in anti-atherogenic cytokine IL-10 (35.62%), was recorded as compared to normal control rats (p ≤ 0.05). Treatment of HC-rats showed significant increase in IL-10 level amounting to 16.26% for cold UFP, 23.75% for hot UFP and 13.65% for fluvastatin drug.

On the other hand, atherogenic marker MPO was significantly augmented by 8.15, 3.63 and 4.95%, for cold UFP, hot UFP and fluvastatin, respectively, (p ≤ 0.05) as shown in (Table 4).

As compared to atherogenic HC-rats, treatment of HC-rats with cold, hot extracts and fluvastatin showed insignificant change in atherogenic (inflammatory) marker MPO with percentages decrease of 17.21, 13.13 and 14.32 % for cold extract, hot extract and fluvastatin respectively.

While, significant increase (P ≤ 0.05) in anti-atherogenic marker IL-10 levels reached to 30.08, 18.45 and 34.13, respectively for cold, hot SP extracts and fluvastatin respectively.

Pathological Examination

Morphometric Analysis

Insignificant change was detected in morphometric analysis in healthy negative control rats treated with both algal extracts as compared to normal control rats.

Morphometric measurements indicated a slight increase of medial cross-sectional area accompanied by a decrease in total smooth muscle layer, with hypercholesterolemia. Hypercholesterolaemia was found to be associated with intimal thickening, this effect was statistically significant marked increase (P ≤ 0.05) as in HC-rats recorded percentage of +95.67 % (Table 5).

Morphometric measurements of atherosclerotic lesion extent and composition were altered by treatment; both cold and hot extract UFP induced more or less significant similar reduction percentages in medial cross-sectional area (19.50 and 26.46%, respectively) and 34.53 % for fluvastatin.

As compared to HC-rats, treatment of HC-rats with cold, hot extracts and fluvastatin showed marked reduction in morphometric parameters with percentages decrease of 38.93, 35.37, and 31.25% for cold extract, hot extract, and fluvastatin respectively.

Histopathological Investigations of Aorta Histology

Aorta of normal control rats showed normal architecture and normal aorta thickness. The normal rat aorta was arranged with a smooth intima, consisting of endothelial cells, with normal contour and normal endothelial corrugation of the intima (Fig. 1). There was no evidence of inflammatory cell infiltration into the intimal and sub-intimal layers. Additionally, there were no adipocytes in the adventitia. The mid-layer comprised an abundant amount of elastic fibers, between which were a large number of smooth muscle cells with a diamond nucleus and small amounts of fibrous components. Normal amount of collagen fibers and connective tissues are exhibited in the tunica adventitia of the aortic tissue of normal rats. Most of the medial smooth muscle cells of the tunica media oriented horizontally to the aortic canal. Similar results were achieved for normal control rats received cold and hot extract of alga (Figs. 2, 3).

In contrast, the aorta of HC-rats (Fig. 4) demonstrated, multifocal degeneration, necrosis, disorientation of smooth muscle cells and irregularity of the wall of the aorta with increased of wall thickness, loss of normal corrugation and discontinuity of endothelium and desquamation in the lining endothelium and hemorrhage in perivascular tissue with vacuolation in the cells of the tunica media, and minor increase in the thickness of aorta wall.

Treatments of HC rats with both cold and hot algal extracts of *U. fasciata* showed no oblivious lesions, the intimae were thin and lacked swelling; the endothelial cells were basically intact and did not desquamate. Furthermore, there was no migration of smooth muscle cells to the under-intimae, no proliferation of smooth muscle cells was observed and the smooth muscle cells were arranged in a regular pattern (Figs. 5, 6).

Normal amount of collagen fibers and connective tissues are exhibited in the tunica adventitia of the aortic tissue of normal rats (Fig. 1). Most of the medial smooth muscle cells of the tunica media oriented horizontally to the aortic canal. In contrast, multifocal degeneration, necrosis, and disorientation of smooth muscle cells were shown in the aortic tissue of HC rats (Fig. 4). No remarkable lesions were shown in aortic tissue of HC rats treated with SP extracts (Figs. 5, 6). Fluvastatin showed the lowest observed restoration on the histopathological levels, with irregular thickening of the wall of the aorta that contains small areas of vacuolation (Fig. 8).

Table 1: Body weight, organs weight of negative controls, hypercholesterolemic control and treated rats.

Parameters Groups		Initial wt.	Final wt.	BWG	Liver wt.	Liver wt.*100/b-wt	Abd. fat	Abd. factor
Negative control	Mean ± S.D.	114.25 ± 4.349	136.500 ± 5.0662	22.25 ± 3.5940	4.7250 ± 0.4113	3.4575 ± 0.1941	2.5750 ± 0.2500	1.8850 ± 0.1406
Negative Cold extract	Mean ± S.D.	117.00 ± 2.5820	139 ± 3.9158	22 ± 2.4495	3.9375 ± 0.2213	2.9800 ± 0.3741	2.6175 ± 0.2845	1.8775 ± 0.08
	% Change to control	2.41	1.83	-1.12	16.91	13.87	1.16	4.23
Negative	Mean ± S.D.	117.50 ± 6.455	138.250 ± 2.3629	20.75 ± 6.9940	3.3500 ± 0.3109	2.4225 ± 0.2419	2.5550 ± 0.04	1.8475 ± 0.02

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Hot extract	% Change to control	2.84	1.28	6.74	29.18	29.19	0.78	3.70
HC-rats	Mean ± S.D.	209 ± 13.291*	236 ± 8.7560*	27 ± 4.546*	6.9425 ± 1.0417*	2.9550 ± 0.5288	3.7025 ± 0.2344*	1.5675 ± 0.04*
	% Change to control	82.93	72.89	21.35	46.72	14.45	43.41	16.93
HC-Cold extract	Mean ± S.D.	202 ± 7.2572*,**	207.50 ± 6.137*,**	5.500 ± 1.914*,**	4.3500 ± 0.2646**	2.055 ± 0.09	2.6075 ± 0.1559**	1.4050 ± 0.2711*
	% Change to control	76.80	52.01	75.28	8.03	40.58	1.16	25.40
HC-Hot extract	Mean ± S.D.	150.2500 ± 8.139*,**	167 ± 21.556*,**	16.75 ± 13.744*,**	3.8875 ± 1.4174**	2.282 ± 0.6067	2.6175 ± 0.2845**	1.6000 ± 0.3633*
	% Change to control	31.51	22.34	24.72	17.76	34.10	1.55	15.34
HC-Fluvastatin	Mean ± S.D.	175.00 ± 9.96*,**	181.750 ± 12.34*,**	6.7500 ± 2.753*,**	5.0500 ± 0.5447**	2.990 ± 0.1257	3.2825 ± 0.3520 **	1.8075 ± 0.1396**
	% Change to control	53.17	33.15	69.66	6.77	1.01	27.13	4.23

(BWG) Body weight gain, Data presented as mean ± SD, n=15 for each treatment group, (*) is significant to control normal rats, (**) is significant to HC positive control.

Table 2: Effects of UFP (cold and hot extracts) and fluvastatin supplementations on body serum lipid profile serum lipoproteins, and atherogenic index (AI) in different therapeutic groups.

Groups Parameters		TC (µg/dl)	TG (µg/dl)	Total lipids (mg/dl)	LDL-C (µg/dl)	HDL-C (mg/dl)	VLDL-C	AI
Negative control	Mean ± S.D.	55.63 ± 10.85	23.72 ± 8.96	1000 ± 52.6	19.66 ± 9.24	31.21 ± 1.48	4.76 ± 1.79	0.782 ± 0.27
	% Change to HC	44.87	63.80	38.93	76.41	601.35	63.66	96.39
Negative Cold extract	Mean ± S.D.	54.03 ± 5.45	24.21 ± 5.49	1105.26 ± 52.65	22.07 ± 2.69	27.25 ± 4.77	4.86 ± 1.07	0.983 ± 0.19
	% Change to control	2.88	2.07	10.53	12.26	12.69	2.10	25.70
	% Change to HC	46.45	63.06	38.93	73.51	512.36	62.90	95.55
Negative Hot extract	Mean ± S.D.	57.65 ± 8.28	24.84 ± 5.19	1105.26 ± 105.25	25.1 ± 5.10	27.74 ± 3.09	4.96 ± 1.02	1.08 ± 0.14
	% Change to control	3.63	4.72	10.53	27.67	11.12	4.20	38.11
	% Change to HC	42.86	62.09	32.50	69.88	517.30	62.14	95.02
HC-rats	Mean ± S.D.	100.9 ± 11.24*	65.54 ± 7.05*	1637.43 ± 96.64*	83.33 ± 8.68***	4.45 ± 1.48*	13.10 ± 1.40*	21.67 ± 6.23***
	% Change to control	81.38	176.30	63.74	323.56	85.74	175.21	2671.1
HC-Cold extract	Mean ± S.D.	57.7 ± 6.23**	24.75 ± 1.90**	1070.2 ± 60.79**	31.96 ± 12.05**,*	27.25 ± 2.27**	4.95 ± 0.38**,*	1.12 ± 0.08**,*
	% Change to control	3.72	4.34	7.02	62.56	12.69	3.99	43.22
	% Change to HC	42.81	62.24	34.64	61.65	512.36	62.21	94.83
HC-Hot extract	Mean ± S.D.	54.05 ± 5.43**	20.3 ± 3.4**	1140.33 ± 80.40**	15.80 ± 3.30**	34.19 ± 1.50***	4.06 ± 0.65**	0.58 ± 0.09***
	% Change to control	2.84	14.42	14.03	19.63	9.55	14.71	25.83
	% Change to HC	46.43	69.03	30.36	81.04	668.31	69.01	97.32
HC-Fluvastatin	Mean ± S.D.	63.06 ± 8.30**	44.06 ± 8.95**,*	1087.73 ± 80.40**	24.18 ± 7.99**,*	30.21 ± 2.27**	8.83 ± 1.79**,*	1.09 ± 0.13**,*
	% Change to control	13.36	85.75	8.77	23	3.20	85.50	39.39
	% Change to HC	37.50	32.77	33.57	70.98	578.88	32.60	94.97

(TG): Triglycerides and (TC): Total cholesterol, (LDL-C): low density lipoprotein cholesterol; (VLDL-C): very low density lipoprotein cholesterol; (HDL-C): high-density lipoprotein cholesterol; (AI): Atherogenic Index. Data presented as mean ± SD, n=15 for each treatment group, (*) is significant to control normal rats, (**) is significant to HC positive control, (***) is significant at P ≤ 0.001.

Table 3: The antioxidant effect of UFP (hot and cold) extracts and fluvastatin supplementation on endothelial dysfunction in hypercholesterolemic rats and different therapeutic groups.

Groups Parameters		NO mg/g tissue	I-CAM (ng/ml)	V-CAM (ng/ml)
Negative control	Mean ± S.D.	13.63 ± 4.54	251.40 ± 0.21	12110.63 ± 2.67
	% Change to HC	75.02	5.39	26.52
Negative Cold extract	Mean ± S.D.	17.26 ± 4.54	244.73 ± 0.32	13473.51 ± 46.46
	% Change to control	26.63	2.65	11.25
	% Change to HC	68.37	7.90	18.25
Negative Hot extract	Mean ± S.D.	15.72 ± 4.54	249.21 ± 0.17	13237.07 ± 33.06
	% Change to control	15.33	0.87	9.30
	% Change to HC	71.19	6.21	19.69
HC-rats	Mean ± S.D.	54.56 ± 9.12***	265.71 ± 0.21 *	16482.26 ± 85.32*
	% Change to control	300.29	5.69	36.09
HC-Cold extract	Mean ± S.D.	18.17 ± 4.54**	255.99 ± 0.32 **	15276.97 ± 84.07 **

	% Change to control	33.31	1.83	26.15
	% Change to HC	38.95	-3.66	7.31
HC-Hot extract	Mean ± S.D.	16.66 ± 2.62**	259.41 ± 0.26 **	14437.60 ± 55.31 **
	% Change to control	22.23	3.19	19.21
	% Change to HC	69.46	2.37	12.40
HC-Fluvastatin	Mean ± S.D.	22.81 ± 4.54*,**	261.75 ± 0.21 **	15302.6 ± 74.63**
	% Change to control	67.35	4.12	28.83
	% Change to HC	58.19	1.49	5.34

(NO): Nitric oxide, (VCAM-1): Vascular cellular adhesion molecule-1, (ICAM-1): Intracellular adhesion molecule-1, Data presented as mean ± SD, n=15 for each treatment group, (*) is significant to control normal rats, (**) is significant to HC positive control, (***) is significant at P ≤ 0.001.

Table 4: The anti-inflammatory effect of UFP (hot and cold extracts) and fluvastatin supplementation on serum MPO and IL-10 levels in normal and hypercholesterolemic rats.

Groups Parameters		MPO (pg/ml)	IL-10 (pg/ml)
Negative control	Mean ± S.D.	125.63 ± 0.02	66.36 ± 0.27
	% Change to HC	9.86	55.34
Negative Cold extract	Mean ± S.D.	123.63 ± 0.00	64.40 ± 0.25
	% Change to control	1.59	2.95
	% Change to HC	11.29	50.75
Negative Hot extract	Mean ± S.D.	127.60 ± 0.01	65.27 ± 0.18
	% Change to control	1.57	1.64
	% Change to HC	8.46	52.78
HC-rats	Mean ± S.D.	139.37 ± 0.60 *	42.72 ± 0.24**
	% Change to control	10.94	35.62
HC-Cold extract	Mean ± S.D.	115.39 ± 0.01*	55.57 ± 0.47*,**
	% Change to control	8.15	16.26
	% Change to HC	17.21	30.08
HC-Hot extract	Mean ± S.D.	121.07 ± 0.01*	50.6 ± 0.41*,**
	% Change to control	3.63	23.75
	% Change to HC	13.13	18.45
HC-Fluvastatin	Mean ± S.D.	119.41 ± 0.32 *	57.3 ± 0.22*,**
	% Change to control	4.95	13.65
	% Change to HC	14.32	34.13

(MPO): Myeloperoxidase. Data presented as mean ± SD, n=15 for each treatment group, (*) is significant to control normal rats, (**) is significant to HC positive control.

Table 5: Morphometric investigations on the effect of UFP (hot, cold extracts) and fluvastatin supplementation in of hypercholesterolemic rats and different therapeutic groups.

Groups Parameters		Wall thickness of aorta (mm)
Negative control	Mean ± S.D.	25.43 ± 4.93
	% Change to HC	48.89
Negative Cold extract	Mean ± S.D.	26.63 ± 10.2
	% Change to control	4.72
	% Change to HC	46.48
Negative Hot extract	Mean ± S.D.	27.56 ± 3.75
	% Change to control	8.36
	% Change to HC	44.61
HC-rats	Mean ± S.D.	49.76 ± 12.75**
	% Change to control	95.67
HC-Cold extract	Mean ± S.D.	30.39 ± 5.95*,**
	% Change to control	19.50
	% Change to HC	38.93
HC-Hot extract	Mean ± S.D.	32.16 ± 11.79*,**
	% Change to control	26.46
	% Change to HC	35.37
HC-Fluvastatin	Mean ± S.D.	34.21 ± 4.89*,**
	% Change to control	34.53
	% Change to HC	31.25

Data presented as mean ± SD, n=15 for each treatment group, (*) is significant to control normal rats, (**) is significant to HC positive control.
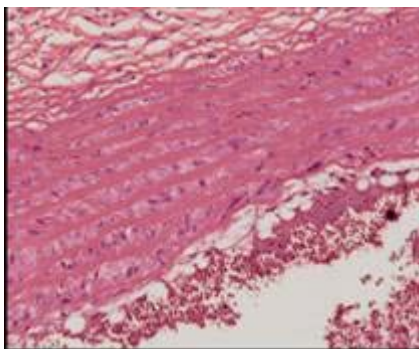
7
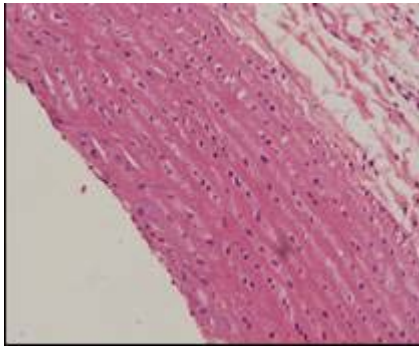
Figure 1: Micrograph of transverse of aorta of negative control rats showing a normal histological structure (H & E X 150).
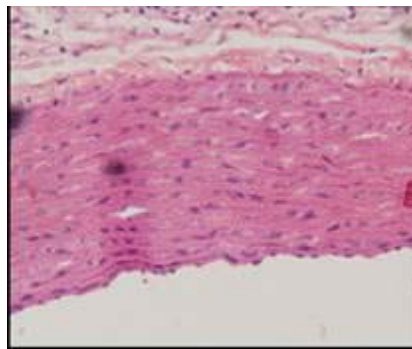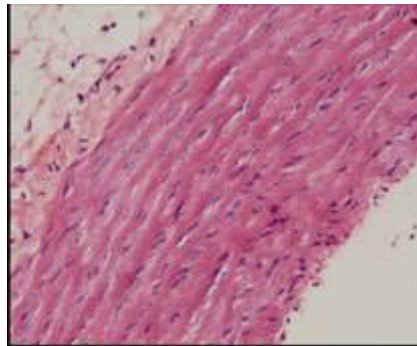

Figure 2: Micrograph of transverse of aorta of negative control rats treated with cold extract of the green alga (*Ulva fasciata*) showing the normal histological structure of the tunica intima, tunica media and tunica adventitia (H & E X 150).
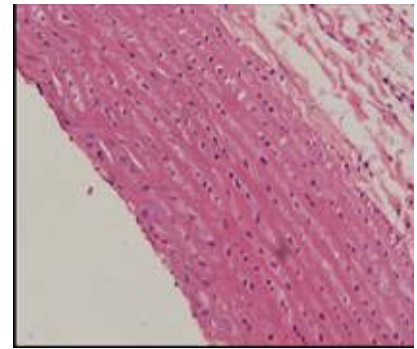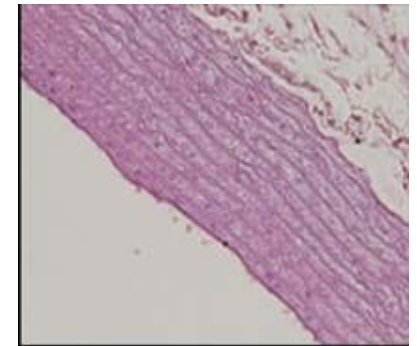

Figure 3: Micrograph of transverse of aorta of negative control rats treated with hot extract of the green alga (*Ulva fasciata*) showing a normal histological structure (H & E X 150).
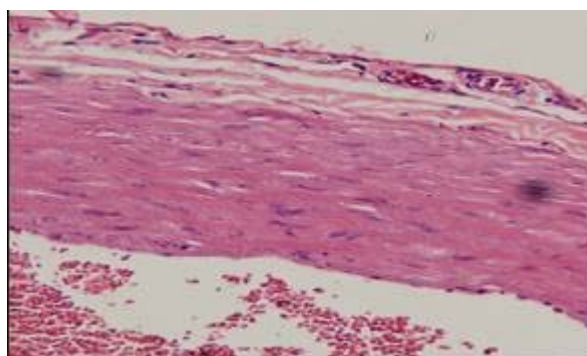

Figure 4: Micrograph of transverse of aorta of hypercholesterolemic rats showing a significant atherosclerosis, vacuolation in the cells of the tunica media and marked luminal narrowing (H & E X 150).

Figure 5: Micrograph of transverse of aorta of hypercholesterolemic rats treated with cold extract of the green alga (*Ulva fasciata*) showing normal histological structure of the tunica intima, tunica media and tunica adventitia (H & E X150).

Figure 6: Micrograph of transverse of aorta of HC-rats treated with hot extract of the green alga (*Ulva fasciata*) showing the normal histological structure of the tunica intima, tunica media and tunica adventitia (H & E X 150).

Figure 7: Micrograph of transverse of aorta of HC rats treated with Fluvastatin showing a perivascular hemorrhage and edema in the adventitia (H & E X 150).

DISCUSSION

The fat enriched diet is regarded as an important factor in the development of cardiac diseases since it leads to the development of hyperlipidemia, atherosclerosis and abnormal lipid metabolism[18]; thus there is keen interest in unraveling the underlying biological mechanisms that account for these differences. In the current study, the hypolipidemic and anti-atherogenic effects of hot and cold extracts of UFP in HC-rats were investigated. It was reported that the rats fed with high cholesterol diet showed significant increase in body weight which leads to secondary complications clinically[19]. In this study, body

weights in HC-rats were decreased significantly upon treatment with UFP and fluvastatin.

In the present study the cholesterol-enriched diet for 12-weeks resulted in a dramatic surge in serum total cholesterol (81.83%), total lipids (63.74%), and triacylglycerols (176.30%). Concerning lipoproteins and AI, it was oblivious that, circulating serum HDL-C level (the good cholesterol) was significantly diminished (85.74%) in the HC- rats, whereas atherogenic lipoproteins; LDL-C (the bad cholesterol) and VLDL-C levels in addition to AI, were significantly raised as compared to normal control group with percentages amounting to 323.86%, 175.21%, and 2671.1%, respectively, thus providing a model for dietary hyperlipidemia. The increase of lipid parameters had been shown to be a strong risk factor for coronary heart diseases in many populations[20]. These results run in parallel with Jang.[21]. The high level of LDL-C found in hypercholesterolemic rats may be attributed to a down regulation in LDL receptors by cholesterol and saturated fatty acids included in the diet[22,23].

As compared to atherogenic group; treatment of HC-rats with cold, hot UFP extracts and fluvastatin showed, marked reduction in TC, TG and total lipids levels, with percentages decrease of 42.81, 62.24, and 34.64 %, respectively for cold extract and by 46.43, 69.03, and 30.36%, respectively for hot extract, while fluvastatin showed mild percentages decrease reached to 37.50, 32.77 and 33.57%, respectively. Concerning lipoproteins, treatment of atherogenic rats showed significant decrease in LDL-C, VLDL-C levels and the AI with percentages decrease of 61.65, 62.2, and 94.83%, respectively for cold UFP, and 81.04, 69.01% and 97.32% (significant at P≤ 0.001), respectively for hot UFP. Fluvastatin treatment showed percentages significant decrease reached to 70.98, 32.60, and 94.97%, respectively and in contrast, HDL-C level was significantly increased (P ≤ 0.05) in reached to 512.36 and 578.88%, respectively for cold SP extract and fluvastatin respectively, and 668.31 for hot UFP (significant at P≤ 0.001). Thus there was a marked reduction in the TC, TG and total lipids, mainly by both algal extracts than the reference drug.

Atherogenic indices are powerful indicators of the risk of heart disease: the higher the value, the higher the risk of developing cardiovascular disease and *vice versa*[24]. In this study, the positive control HC-rats exhibited a profound increase in atherogenic index (AI) as compared to normal ones that was markedly reduced by algal treatment; thus this treatment provides us with a good indicator of reducing atherogenicity, that leads in consequence to a decreased possibility of CVD occurrence.

Therefore, treatment of hypercholesterolemic rats with UFP (*U. fasciata* cold and hot sulphated polysaccharide extracts) induced marked significant decrease of serum total lipids, total cholesterol, triacylglycerols and LDL-C concentrations as compared to the positive control rats.

Rats treated with both cold and hot algal extracts showed enhanced HDL-C level; which may be due to the ability of the extract to hasten the decomposition of free radical species generated during cholesterol administration[25]. Treatment of HC-rats with both UFP extracts was shown to significantly reduce atherogenic index. Low atherogenic indices and high HDL-C levels are protective and could contribute to its anti-atherogenic properties, including its capacity to inhibit LDL-C oxidation and protect endothelial cells from the cytotoxic effects of oxidized LDL-C[26].

Hypercholesterolemia-induced oxidative stress produced an elevation of 300.29% (significant at P≤ 0.001) in liver nitric oxide (NO) concentration, as compared to normal controls (Table 3). Our findings are in accord with the experiment previously performed by Antoniades[27] and Tall[28], where it had been shown that ox-LDL-C can injure endothelial cells and lead to a decrease in nitric oxide synthase activity, thus inhibiting the production of NO, and also that, LDL-C can also significantly inhibit the production of NO from vascular endothelial cells, whereas HDL-C can enhance endothelial NO release. In the present study, high fat diet significantly increased the hepatic NO level in hyperlipidemic rats, algal treatment caused decreased NO production, a result that may be attributed to antioxidative effect of ulvan sulphated polysaccharide and to the increased HDL-C level and decreased LDL-C level.

HC-rats treated with cold and hot algal extracts of UFP showed insignificant change in the NO concentration as compared to normal control, this proves the antioxidative effect exerted by sulphated polysaccharides of this green alga.

The UFP-treatment resulted in insignificant change in both ICAM-1 and VCAM-1 levels, as compared to negative control rats, whereas in comparison with diseased HC-rats, their treatment with cold, hot UFP extracts as well as fluvastatin, both CAMs were significantly reduced. On the basis of the presented data, both SP algal extracts of were observed to inhibit the expression of VCAM-1 and ICAM-1 as they known to be protective against the progression of atherosclerosis. These effects of UFPs may be due to antioxidative effects that reduced the oxidation of LDL-C to ox-LDL-C. Furthermore, VCAM-1 can also mediate the adhesion and migration of monocytes. These cells, located under the endothelium, become activated and differentiated into macrophages. Finally, these monocytes become foam cells *via* the aggregation of lipids. Additionally, the vascular smooth muscle cells gradually proliferate and migrate from the media to intima, promoting further development of atherosclerotic lesions. Ox-LDL-C can also stimulate endothelial cells to produce adhesion molecules, increasing the atherogenicity[29].

The cells involved in the atherogenesis secretion are activated by soluble factors; the cytokines. The immune-inflammatory response in atherosclerosis is modulated by

regulatory pathways, in which the balance between anti-inflammatory and pro-inflammatory cytokines plays a crucial role as a major determinant of plaque stability[30]. Thus, in HC-rats, we observed strong activation of several inflammation markers such as; myeloperoxidase MPO, as they are implicated in pathophysiological alterations. By algal treatment, the results clearly showed that the mean levels of detected IL-10 were significantly increased. Han[31] suggested that IL-10 induced scavenger receptor activity and uptake of pro-inflammatory modified LDL-C by macrophages, that may be efficient for removal of the harmful modified lipoproteins from the artery wall and disposal of cytotoxic free cholesterol, thereby decreasing inflammation and apoptosis in the lesion thereby, retarding early atherosclerotic lesion development. In addition, improvement in endothelial function and attenuation of endothelial activation, may be attributed to reduction in pro-inflammatory markers of endothelial function, this may lead to a reduction in the progression of atherosclerosis and local production of the cytokines by inflammatory accumulated cells.

Morphometric measurements of atherosclerotic lesion extent and composition were altered by treatment; both cold and hot extract induced more or less similar reduction percentages in medial cross-sectional area (19.50 % and 26.46 %, respectively), this was proved by the histological findings that revealed the diminution of the atherosclerotic plaques by the algal treatment of the atherogenic rats which showed a thick layer of fats recorded a percentage of 95.67% as compared to the normal healthy controls.

Concerning histopathological findings, aorta of normal control rats showed normal architecture and normal aorta thickness. There was no evidence of inflammatory cell infiltration into the intimal and sub-intimal layers. In contrast, the aorta of HC-rats demonstrated, multifocal degeneration, necrosis, disorientation of smooth muscle cells and irregularity of the wall of the aorta with increased of wall thickness, loss of normal corrugation and discontinuity of endothelium and desquamation in the lining endothelium and hemorrhage in perivascular tissue with vacuolation in the cells of the tunica media, and minor increase in the thickness of aorta wall[32]. Additionally, the smooth muscle cells proliferated, became disordered and migrated to the underintima, causing histologic changes. Oedema and foam cell infiltration were also observed under the intima. Proliferation of smooth muscle cells of tunica media associated with thickening were observed in aorta of rats from positive control group fed high fat, cholesterol-diet[33], (Fig. 4). These findings agreed with Lecanu[34], who found that 13 weeks of a cholesterol-enriched diet induced the formation of atheroma and lipid deposition in guinea pig aortas as compared to animals fed with a standard diet. The present histopathological findings indicated that hyperlipidemia causes mild structural abnormalities manifested as thickened media-intima layer in comparison to control group; this effect might be

due to accumulation of fatty vacuoles cells in the tunica media which resulted into narrowing of the aorta diameter. In accordance with the present results, Rioufol and Finet[35] indicated that hyperlipidemia causes accumulation of fatty plaque deposits in the arteries and aggravate narrowing of the arterial diameter, which restricts blood flow to vital organs. Consistently, several studies indicated that, inflammation causes abrasion of the overlying endothelium of the blood vessels through the exposure to the immune cells monocytes/macrophages and deposition of LDL-C leading to arteries stenosis even in normal lipid profile individuals[36]. Histopathology of aorta of cholesterol fed rat showed increased number of myointimal cells, thickening of underlying media and loss of normal arrangement of elastic lamellae of the media whereas in aorta of UFP supplemented rat showed no significant changes (Fig. 4).

Treatments of HC rats with both cold and hot algal extracts of *U. fasciata* removed the formed atheromas and prevented the formation of new atheroma deposits and it showed no oblivious lesions, the intimae were thin and lacked swelling; the endothelial cells were basically intact and did not desquamate. The aortas were similar to the animals fed with a standard diet and no lipid-containing elements were visible. The morphology, structure and cytological features of the aortas from treated animals also did not differ from the control group. Furthermore, there was no migration of smooth muscle cells to the under-intimae, no proliferation of smooth muscle cells was observed and the smooth muscle cells were arranged in a regular pattern (Figs. 7 and 8).

The present study was conducted to evaluate the relationship between biochemical, and histological investigations before and after the course of induction and treatment of hypercholesterolemia. The biochemical findings were confirmed by histological observations. The changes mostly include atherosclerosis, fatty accumulation, inflammatory cells infiltration and other histological manifestations which were also consistent with the findings of above-mentioned authors. The dramatically increase in TC, TG, total lipids, LDL-C, VLDL-C and AI was supported with the histological studies, and this proves the correlation between cholesterol level and heart disease.

CONCLUSION

Thus, it could be concluded that there is a correlation between cholesterol level and heart disease, however; that does not prove causation. Moreover, inflammation emerges to be independent risk factor for the development of atherosclerosis even in normal cholesterol level. In absence of the inflammatory reactions and oxidative stress, hyperlipidemia alone is not the principal risk factor for atherosclerosis. Supplementation of *U. fasciata* cold and hot extracts to HC-rats showed an effect of attenuation of serum lipid profile by reducing the plasma total cholesterol

triglyceride, LDL-C, and VLDL-C and oxidative stress and provided the cardiac protection from hypercholesterolemia.

In conclusion, UFP cold and hot extracts caused a protective effect against induced hypercholesterolemia and improved the biochemical parameters. As, these sulphated polysaccharides has a cardioprotective effect against injury in the aorta of HC-treated rats.

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