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HYPOLIPIDAEMIC EFFECT OF *ACANTHOPHORA SPICIFERA* (RED ALGA) AND *CYSTOSEIRA TRINODE* (BROWN ALGA) ON ALBINO RATS*

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Abstract – The polysaccharides of *Acanthophora spicifera* and *Cystoseira trinode* were isolated and their components identified using different chemical and spectral techniques. Their effects were evaluated for the first time on hyperlipidemic rats. Atorvastatin Ca (Lipitor®) was used as a reference drug. Results revealed that the polysaccharides isolated were of sulfate type. *Acanthophora spicifera* lowered the level of total serum lipids, total cholesterol (TC), triglycerides (TG) and low-density lipoproteins (LDL-C) by 48%, 49.6%, 63% and 80.6% respectively. High-density lipoproteins (HDL-C) level was elevated by 1.14 fold. For *Cystoseira trinode* total lipids, TC, TG and LDL-C were decreased by 25.5%, 49%, 51% and 91% respectively. The level of HDL-C was elevated by 1.5 fold in comparison with the hyperlipidemic rats. The histopathological results proved the ameliorated effect after using the isolated polysaccharides of both algae. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities were decreased in hypercholesterolemic groups by values of 63%, 34% and 45%, respectively when treated with *A. spicifera* extract. The treatment with *C. trinode* extract and Atorvastatin exhibited a noticeable amelioration in activity with hypercholesterolemic groups as compared to their corresponding controls.

Keywords – Acanthophora spicifera, Cystoseira trinode, sulfated polysaccharides, hypolipidaemic, histopathology, biochemical parameters

1. INTRODUCTION

Egypt has long seashores represented by the Mediterranean and the Red Sea. These shores contain a national wealth of marine organisms, especially algae. An increasing number of studies have demonstrated that certain compounds produced by marine organisms have potential therapeutic uses. *Acanthophora spicifera* is one of the most abundant red algal species found on reef flates [1]. It contains conjugated eicosapentaenoic acid and conjugated arachidonic acid except glycolipids [2]. The sulfated polysaccharides showed anticoagulant properties [3]. The brown algae *Cystoseira trinode* contains more glucuronic acid than mannuronic acid [4]. Seaweed polysaccharides are highly active natural substances having valuable applications [5, 6]. Various investigations have reported that polysaccharides of many types of seaweed play an important role as a hypocholesterolemic agent [7]. Polysaccharides from red and brown algae have shown hypolipidaemic activity [8]. Some macro-algal polysaccharides and fibers such as alginate, carrageenan, funoran, fucoidan, laminaran, porphyran and ulvan have been noted to produce

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hypocholesterolemic and hypolipidaemic responses. The main objective of this study was to assess, for the first time, the efficacy of the extracted sulfated polysaccharides from the algal species *A. spicifera* and *C. trinode* on various biochemical parameters of hypercholesterolemic rats

2. MATERIALS AND METHODS

I. Phytochemical study

I.1. Algal collection

The red alga *Acanthophora spicifera* (Vahl) Borgesen (Rhodophyta, Rhodophyceae) and the brown alga, *Cystoseira trinode* (Forsskål) Agardh (Heterokontophyta, Phaeophyceae) were collected from the red sea coast, Al-Quoser province, Egypt, in December 2007, at 26° 07' N and 34° 13' E. After collection, the seaweeds were cleaned by washing several times with tap water then with distilled water to remove any epiphytes, salts, sands or any debris. It was then air–dried in shade at room temperature and coarsely powdered to be used for the isolation procedure.

I.2. Determination of carbohydrates content

The dried powdered plants tissue (0.2g) was extracted three times with 80% ethanol at room temperature. After centrifugation of each extract for 15 min. at 4000g, the clear supernatants were combined and decolorized with 2 ml of activated charcoal suspension. The mixture was completed upto a known volume with 80% ethanol and shaken for 5 min and then filtered through Whatman No. 1 filter paper. The clear soluble sugar extract contained reducing and non-reducing sugars.

I.3. Hydrolysis of reducing and non-reducing sugars

One ml of the soluble sugar extract was hydrolysed with 1ml 6N HCl by heating the mixture for 12 min. at 70^{o}C in a water bath. The pH of each sample was neutralized with diluted NaOH using ph.ph. as an indicator, then diluted to a known volume with 80% ethanol, and its total reducing value determined.

The plant residue remained after the extraction of soluble sugars was hydrolyzed with 0.2N H_2SO_4 by heating the mixture in a boiling water bath for one hour [9]. The mixture was centrifuged for 15 min. at 4000g and the clear supernatants were neutralized. The total reducing value of the clear extract was determined.

The reducing value of sugars in each extract was measured by Nelson's test as described by Clark and Switzer [10]. One ml of neutralized sugar extract was mixed with 1 ml freshly prepared Nelson's alkaline copper reagent (Nelson's A:B reagents, 25:1) and heated in a boiling water bath for 20 min, then rapidly cooled in running water. 1 ml of arsenomolybdate reagent was added and shaken several times to dissolve the copper oxide. When effervescence stopped, the mixture was made up to 10 ml with distilled water and its color intensity was measured at 540 nm against water blank treated as the sample. The content of the reducing value was determined using a glucose standard curve, and then calculated as mg sugar g^{-1} dry weight. Subtraction of the reducing sugar contents obtained before and after acid hydrolysis gave the content of non-reducing sugar.

I.4. Extraction of water-soluble polysaccharides

The extraction procedure was carried out according to the method of Pengzhan et al., [11] to yield crude polysaccharides. One hundred gm of dry algae was roughly cut and autoclaved in water at 100^{o}C for 2h. The slurry was centrifuged at 5000 rpm for 20'. The supernatant was dialyzed against tap water for 48

h, and then concentrated at 40°C under reduced pressure and precipitated with 95% ethanol. The mixture was allowed to stand overnight at room temperature. The precipitate was collected and washed twice with absolute ethanol.

I.5. Chromatography for the identification analysis of algal polysaccharides extracts

I.5.1. Acid hydrolysis

A portion (5mg) of each of the crude polysaccharides isolated from *A. spicifera* and *C. trinode* was hydrolyzed with 0.5 M H_2SO_4 at 105°C for 20h. The hydrolysate was neutralized using $BaCO_3$ and centrifuged. The supernatant of each one was concentrated and examined by paper chromatography (PC, descending) using B/A/W (4:1:5 v/v, upper layer) as the solvent system. The hydrolysates were also examined by Thin layer chromatography (TLC) using the system $CHCl_3$/MeOH/H_2O (15:6:2 v/v) [12]. The spots were visualized by spraying with aniline phthalate reagent.

I.5.2. Authentic reference materials for TLC and PC

D-Glucose, D-galactose, D-glucuronic acid, mannuronic acid, mannose, D-xylose and L-fucose were the sugar standards that matched our polysaccharide specimens.

I.6. FT-IR spectral analysis

The dry extracted polysaccharides (about 1mg) were ground with KBr powder (300mg) and pressed into pellets for FT–IR spectra measurements in the frequency range between 100 and 4000 cm^{-1} [13] using an 8201 PC Spectrophotometer.

I.7. HPLC- analysis

The hydrolysates (20µl) were also analyzed by a HPLC, HP1050 model equipped with a UV detector, Hewlett Packard (HPLC laboratory, Agriculture Research center, Cairo) using the following conditions: Column: APS Hypersil column (4.6×200mm i.d.), mobile phase: acetonitrile/water (75:25) v/v., flow rate: 2 ml/min. The compounds were detected by a UV detector set at 192 nm. The sugars were positively identified by matching their retention time data with those of the authentic standards, which were also run under identical analytical conditions. The average retention times being: fucose 1.1, rhamnose 1.4, maltose 1.8, lactose 1.85, sorbose 1.9, Stachyose 2.0, galactose 2.2 , glucose 2.4, ribose 2.6, fructose 2.9, sorbitol 3.2, arabinose 3.6, xylose 3.8, mannitol 3.92, mannose 4.3, glucuronic acid 4.8, galacturonic acid 5.5 and sucrose 6.5.

II. Biological study

II.1. Experimental animals

White male albino rats weighing between 120-140 g were obtained from the animal house colony of the Research Institute of Ophthalmology, Giza, Egypt. They were kept in hygienic conditions, in stainless steel cages, with 12 h light/dark cycles, fed a well balanced diet (ATMID Company, Egypt) and supplied with water *ad libitum*.

II.2. Reference drug

Atorvastatin (Lipitor ®), manufactured by Pfizer Egypt S.A.E. Cairo A.R.E. under authority of Pfizer INC, USA, was used as a reference drug for evaluating the hypocholesterolemic activity.

II.3. Diet

The control group of animals (gp I) was fed a normal diet composed of vitamin mix. (1%), mineral mix. (4%), corn oil (10%), sucrose (20%), casein (10.5%) and starch (54.3%). The other groups were fed a hyperlipidemic diet composed of the same components of a normal diet, but devoid of corn oil and with the addition of cholesterol powder (1%), bile salts (0.25%) and fats (15%) [14] for three weeks.

II.4. Acute toxicity test

Maximum soluble concentration of Sulfated polysaccharide of *A. spicifira* and *C. trinode* (2 g/kg. b w) were administrated orally to the monitored rats to test their toxicity.

II.5. Experimental design

The rats were divided into five groups, each of six animals. The first group (gp I) was kept as the negative control (receiving normal diet). The second group (gp II) is a positive control of hyperlipidemic rats receiving 1ml distilled water orally for four weeks. The third and fourth groups (gp III & gp IV) of hyperlipidemic rats received an oral dose of 200mg/ kg. bw (1/10 max soluble concentration) of sulfated polysaccharides from *A. spicifira* and *C. trinode* respectively for four weeks. The last group (gp V) of hyperlipidemic rats received an oral dose of 0.09 mg/ kg. bwt (=10mg per day) according to Paget and Barnes [15], of the standard drug for four weeks.

II.6. Lipid profile tests

Blood samples from the retro-orbital plexus were drawn after an overnight fasting (more than 12 hr) before treatment (0 week), then after two and four weeks of treatment. Serum total lipids were estimated by using kits (Bio-Diagnostic) [16]. Total cholesterol (Tc), triglycerides (TGs) and high density lipoprotein (HDL)-cholesterol were measured using kits (Spinreact Co., Spain) [17-19]. Low density lipoprotein (LDL)-cholesterol was calculated according to Friedewald *et al.* [20] formula. Serum very low density lipoprotein (vLDL) was determined according to the Norbert [21] formula vLDL - TC = TG / 5.

II.7. Hepatic enzymes determination

The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity was measured by using kits (Quimica Clinica Aplicada S. A.) [22] and the serum alkaline phosphatase (ALP) activity was determined by using kits purchased from Scico Diagnostics [23].

II.8. Histopathological analysis

A portion of the liver tissue, immediately after sacrifice was kept in 10% formalin to fix the tissue. The tissues were washed in running tap water, dehydrated in ethanol and cleared in xylene. The tissues were then embedded in paraffin wax. Sections were cut at 10μm thickness, and stained with haematoxylin and eosin. The sections were then viewed under light microscope for the histopathological changes.

III. Statistical analysis

The values were expressed as mean ± standard deviation (SD, n=5). Differences between groups were assessed by one-way analysis of variance (ANOVA) by using Statistical Package for the Social Sciences (SPSS) software for Windows, version 11; SPSS Inc, Chicago, using the least significance difference test (LSD) and at p – value (≤ 0.05, ≤ 0.01).

3. RESULTS

I. Phytochemical study

Carbohydrates content of the algae species

It was clear that the total carbohydrates content of *Cystoseira trinode* was higher than *Acanthophora spicifera.* The soluble and insoluble sugars content of *C. trinode* equaled 1.2 and 2.6 fold, respectively when compared to *A. spicifera* (Table 1).

Table 1. Sugar concentration of *Acanthophora spicifera* and *Cystoseira trinode* tissues

Alga extract	Soluble sugar conc. (mg g^{-1} dry wt)			Insoluble Sugar (mg g^{-1} dry wt)	Total Carbohydrate (mg g^{-1} dry wt)
	Reducing	Non -Reducing	Total		
A. spicifera	4.29±0.67	67.48±6.83	71.76±6.71	92.97±1.38	164.74±6.62
C. trinode	6.94±1.00	75.34±6.00	82.08±6.04	347.9±6.35	429.97±8.76

Wt., weight Values are means of 5 replicates ± SD

Algal polysaccharide extracts identification

The absorbance of sulfate functional groups of both *A. spicifera* and *C. trinode* extracts were detected in the FT-IR spectra at bands of wave numbers 1262.2 & 1257.4 cm^{-1} (S=O) and 842 & 835 (C-O-S) [24], respectively, (Fig. 1). The stretching of C = O of uronic acids were assigned at bands 1652.4 and 1653.2 cm^{-1}, corresponding respectively to *A. spicifera* and *C. trinode*, and the vibration of the C-O-C bridge of glucosides were recorded at wave numbers 1072.5 and 1044.8 cm^{-1}, respectively .

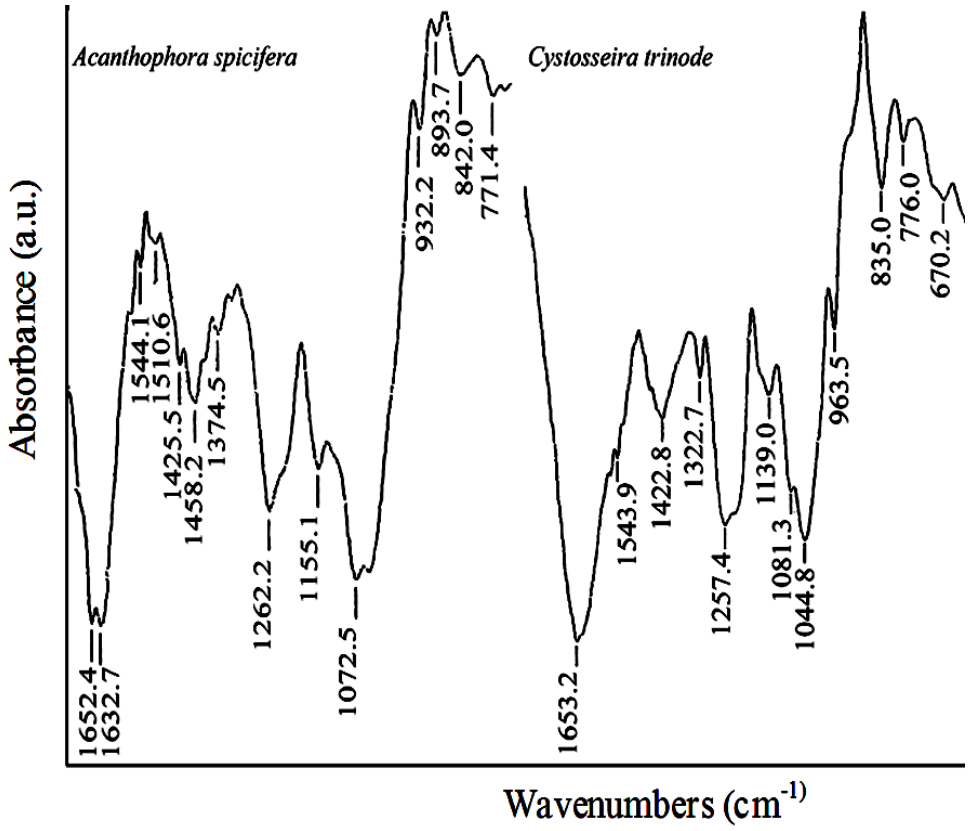

Fig. 1. The FT-IR spectra of the algal extracts

The monosugars content of both *A. spicifera* and *C. trinode*, obtained after hydrolysis and subjected to HPLC analysis, proved the presence of glucose, fucose, galactose, xylose and glucuronic acid at retention time 2.4, 1.1, 2.2, 3.8 and 4.8, respectively.

Identification of the hydrolysates of polysaccharides

Results of qualitative PC and TLC of the hydrolysates in the water–soluble fraction of *A. spicifera* and *C. trinode* revealed the presence of glucose, fucose, galactose, xylose, mannuronic and glucuronic acid.

II. Biological study

a) Antihypercholesterolemic activity

From the results displayed in Table 2, it was obvious that oral treatment for four weeks with sulfated polysaccharides from *A. spicifera* and *C. trinode* exhibited a significant decrease in total lipids, TC, TG, LDL-C and VLDL-C together with a significant increase in HDL-C in comparison with the hypercholesterolemic group. On the other hand, in comparison with the normal group, the treatment with the sulfated polysaccharides from *A. spicifera* exhibited a decrease in TC, while TG, LDL-C and VLDL-C returned to normal value and the HDL-C was lower than the normal value. On treatment with sulfated polysaccharides of *C. trinode*, there was a decrease in TC and LDL-C below the normal value, while the value of total lipids, TG, VLDL-C was higher than the normal and the HDL-C returned to the normal value.

Table 2. Effect of Sulphated polysaccharides of *Acanthophora spicifera* and *Cystoseira trinode* on lipid profile of normal and hypercholesterolemic rats

Groups	Total lipids mg/dl	TC mg/dl	TG mg/dl	HDL mg/dl	LDL-C mg/dl	VLDL-C mg/dl	TC/ HDL	LDL/ HDL
gp I	112.5±4.7	44.1±3.04	43.35±2.92	26.8±1.47	8.67±1.58	8.67±0.59	1.65±0.06	0.32±0.05
gp II	228.4±1.35***	79.9±3.63^{+++}	120.77±2.07^{+++}	10.5±0.61^{+++}	45.25±3.46***	24.15±0.41***	7.63±0.53***	4.32±0.43***
gp III	118.8±5.6^{+++}	40.3±2.17^{+++}	44.7±2.3^{+++}	22.55±0.82^{+++}	8.77±1.85^{+++}	8.94±0.46^{+++}	1.79±0.09^{+++}	0.39±0.08^{+++}
gp IV	170.3±4.76^{+++}	41.7±0.87^{+++}	59.08±1.5^{+++}	26.61±1.23^{+++}	4.07±0.68^{+++}	11.82±0.3^{+++}	1.57±0.06^{+++}	0.12±0.05^{+++}
gp V	154.13±4.01^{+++}	39.3±1.5^{+++}	45.2±3.09^{+++}	27.2±1.52^{+++}	3.83±0.64^{+++}	9.04±0.92^{+++}	1.44±0.04^{+++}	0.11±0.05^{+++}
LSD at 0.05	2.5	1.39	1.37	0.72	1.03	0.27	0.13	0.11

gp I, negative control; gp II, positive control; gp III, sulfated polysaccharides from *A. spicifera* receiving orally a dose of 200mg/kg b.wt.; gp IV, Sulfated polysaccharides from *C. trinode* receiving orally a dose of 200mg/kg b.wt.; TC, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoproteins; LDL-C, low density lipoproteins; VLDL-C, very low density lipoproteins LSD, least significance difference test; Values significantly different compared to normal and hypercholesterolemic $^{+++}$ $P \leq 0.001$, Values are means of 5 replicates ± SD.

b) Serum hepatic enzymes

A. spicifera had no significant change on (ALT) activity, whereas *C. trinode* and Atorvastatin induced significant increase and decrease actions, respectively, as compared to the normal rats group. The hypercholesterolemic group exhibited a significant increase ($P \leq 0.001$) of ALT activity as compared to the normal group. Meanwhile, the treatment of hypercholesterolemic rats recorded great improvement (Fig. 2). *A. spicifera*, *C. trinode* extracts and Atorvastatin significantly decreased AST concentration by values 34, 24 and 28.6%, respectively, as compared to their corresponding control ones. Although in the case of the hypercholesterolemic rats, AST was activated significantly by a value 2.7 fold in comparison with the normal rats (Fig. 2). The oral administration with *A. spicifera* extract exhibited a significant increase (2%) in ALP activity, while at the same time, *C. trinode* extract and Atorvastatin showed significantly

decreased ALP activity by values of 3 and 52.6%, respectively, as compared to the control rats (Fig. 3). On the other hand, treatments with the studied materials exhibited a highly significant decrease ($P \leq 0.001$) in ALP activity of the hypercholesterolemic rats.

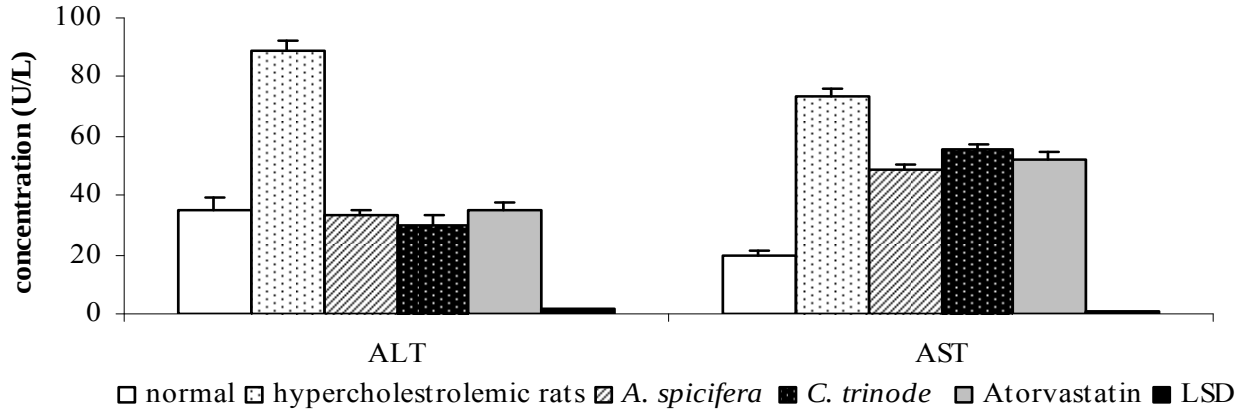

Fig. 2. Effect of *Acanthophora spicifera*, *Cystoseira trinode* and atorvastatin on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes activities of hypercholesterolemic rats

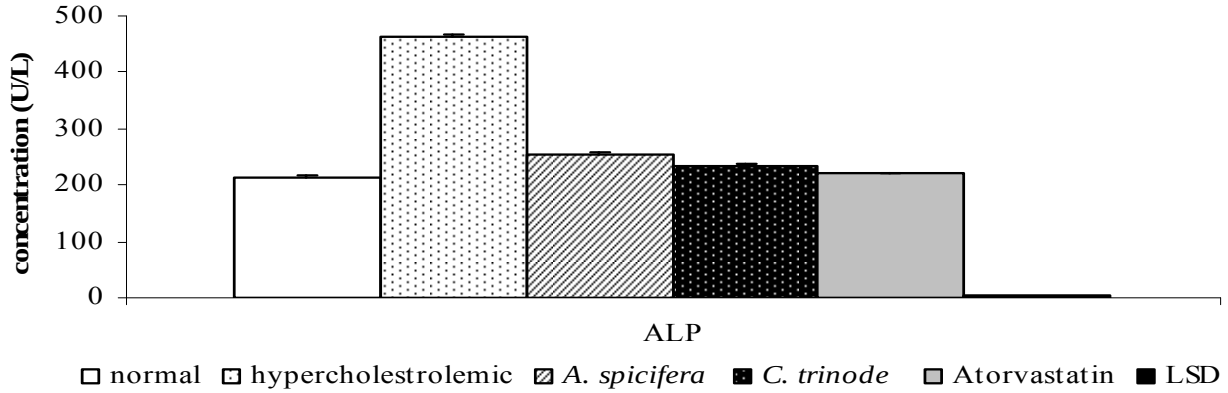

Fig. 3. Effect of *Acanthophora spicifera*, *Cystoseira trinode* extracts and atorvastatin on serum alkaline phosphatase (ALP) enzyme of hypercholesterolemic rats

c) Histopathological changes

The Histopathological findings in the liver sections (H & E, 100x) from four experimental groups are presented in Fig. 4. The control group showed normal liver structure (Fig. 4a). Liver sections of the hypercholesterolemic group produced marked changes like inflammation around portal triad with patchy microvesicular fatty degeneration (Fig. 4b). Group III and IV, treated with sulfated polysaccharides of *A. spicifera* and *C. trinode* (Fig. 4c & 4d.), showed considerable reduction in the pathological changes and exhibited an almost normal Figure as the control.

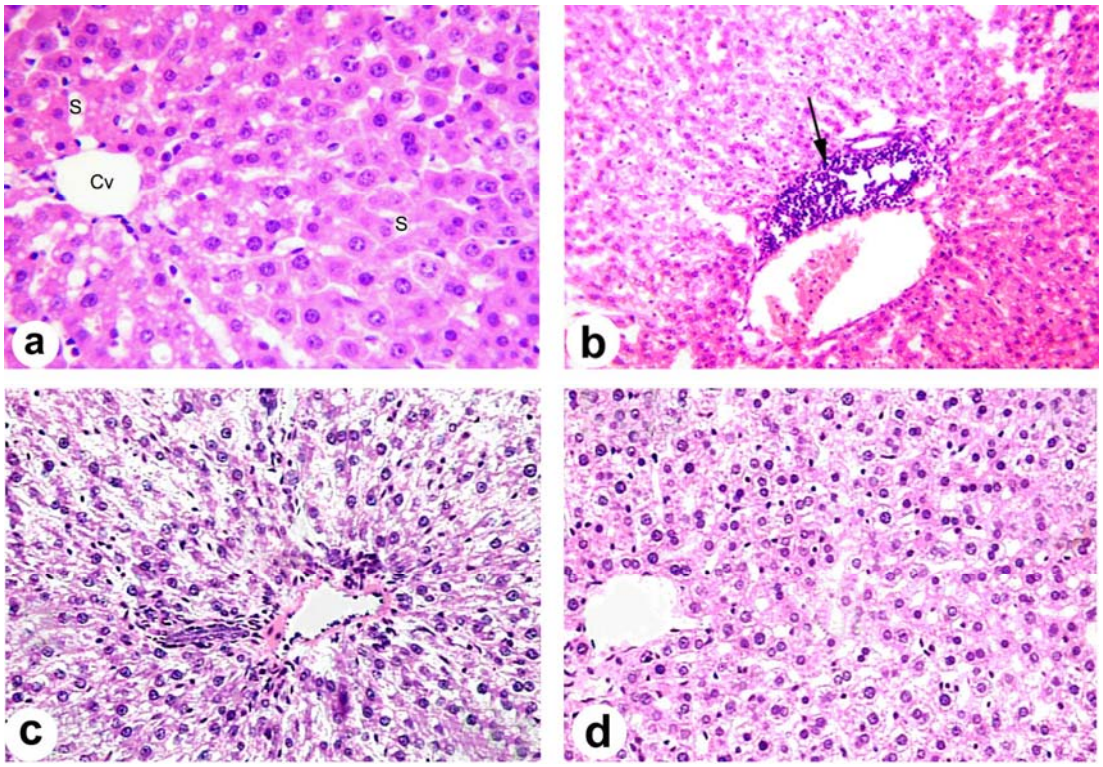

Fig. 4. Histopathological light micrographs of a cross section in liver (100x) showing the hepatic cord, inflammation around portal triads (arrow), normal portal triads (arrow) and normal architecture findings in liver sections from four experimental groups: a) control, b) hypercholesterolemic, c) treated with sulfated polysaccharides from *Acanthophora spicifera* and d) treated with sulfated polysaccharides from *Cystoseira trinode*, respectively. Central vein (Cv), sinusoid (s)

4. DISCUSSION

Sulfated polysaccharides are associated with the surfaces of animal cells and are involved in biological activities such as cell recognition, cell adhesion or regulation of receptor functions, which are of interest in medicine [25]. The signal at 1230 cm^{-1} and two shoulder bands at 835 and 788 cm^{-1} were indicative of the presence of sulfate ester substitutions in the FT-IR spectra [26]. It was known that seaweeds contain numerous bioactive substances that have been shown to lower cholesterol, reduce blood pressure, promote healthy digestion and antioxidant activity [27, 28]. The present study is the first reported study of the protective role of sulfated polysacchrides on liver and the prevention of acute increase in triglycerides and total cholesterol of hypercholesterolemic treated rat. The sulphur compounds in the extracts are capable of reducing the excessive accumulation of intracellular triglycerides [29]. The reduction in serum total cholesterol levels and dietary cholesterol absorption in the treated groups (fed with the algal extracts and drug) relative to the hypercholesterolemic control group was a likely explanation for the observed reduction in LDL-C levels [30]. The elevated level of LDL-C was significantly reduced in the algal treated rats, this may be due to the antioxidant property of the extracts, which were capable of inhibiting the LDL-C peroxidation [31]. The HDL-C is a free radical scavenger and prevents peroxidation of beta lipoproteins [32]. Rats Treated with algal extracts showed improved high levels of HDL-C, which may be due to the ability of the extract to hasten the decomposition of free radical species generated during cholesterol administration [27]. In contrast, Wong et al., [33] reported that some seaweeds such as *Ecklonia cava*, *Colpomenia sinuosa* and *Sargassum hemiphyllum* did not reduce but rather elevated serum cholesterol levels due to the increase of endogenous synthesis of cholesterol in the liver. The liver enzymes are normally found in circulation in small amounts because of hepatic growth and repair. ALT, AST and ALP

activities were elevated in hypercholesterolemic groups, meanwhile, the treated groups showed an obvious enhancement [34, 35]. The antihypocholesterolemic activity was due to reduced cholesterol absorption in the gut [36, 37]. This is often coupled with an increase in the faecal cholesterol content and a hypoglycemic response [38]. Others have reported lower levels of total cholesterol, free cholesterol and triglycerides [39]. The co–treatment of rats with sulfated polysaccharides reduced the increased accumulation of total cholesterol and favourably modulated the lipid profiles.

5. CONCLUSION

In conclusion, the sulfated polysaccharide of both investigated algal species showed high anti-hyperlipidemic activity in rats, but the mechanism by which they ameliorated TC, TG and LDL-C blood levels in hyperlipidemic rats is still uncertain.

Author's Statements

a) Competing Interests

The authors declare no conflict of interest.

b) Animal Rights

The institutional and international guide for the care and use of laboratory animals was followed. See the experimental part for details.

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