The Egyptian Heart Journal (2012) **64**, 147–153

Egyptian Society of Cardiology

The Egyptian Heart Journal

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ORIGINAL ARTICLE

Sulfated polysaccharides of *Turbinaria conoides* dose-dependently mitigate oxidative stress by ameliorating antioxidants in isoproterenol induced myocardial injured rats: Evidence from histopathological study

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Received 14 February 2012; accepted 26 March 2012
Available online 5 July 2012

KEYWORDS

Isoproterenol, Myocardial injury;
Antioxidants;
Oxidative stress;
Turbinaria conoides

Abstract *Objectives:* In recent years, fucoidan is being marketed as a nutraceutical and food supplement because of its various pharmacological activity. Hence, we evaluated the cardioprotective activity of fucoidan extracted from *Turbinaria conoides* in isoproterenol induced myocardial injured rats.

Methods: Wistar rats (180 ± 20 g) were divided into eight groups of six animals each and fucoidan was administered orally in three different doses (50, 100 and 150 mg/kg), for 14 days. At the end of this period, all the rats, except control group and fucoidan alone treated groups, were administered isoproterenol for two consecutive days to induce myocardial injury. After 48 h, rats were anesthetized, sacrificed and the levels of biochemical parameters were determined and histopathological analysis carried out.

Results: Biochemical assessment of myocardial injury was done by measuring the activities of creatine kinase and lactate dehydrogenase, which were significantly elevated in the rats administered

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Peer review under responsibility of Egyptian Society of Cardiology.

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http://dx.doi.org/10.1016/j.ehj.2012.03.003

with isoproterenol. Further, pretreatment of fucoidan significantly normalized both endo and exogenous antioxidant defense system in isoproterenol induced myocardial injured rats. Therefore, fucoidan considerably reduced oxidative stress. In addition, histopathological findings were in line with biochemical findings.

Conclusion: As a consequence, fucoidan possesses cardioprotective and antioxidative effect against isoproterenol induced myocardial injury in rats.

1. Introduction

Cardiovascular diseases are the principal cause of mortality worldwide. Reduction of mortality rate and prevention of myocardial injury (MI) are of utmost importance. MI is the condition of necrosis of the myocardium that occurs as a result of imbalance between coronary blood supply and myocardial demand.[1] Isoproterenol (ISO), a synthetic non-selective β-adrenoceptor agonist, has been exceptionally known to induce MI in rats as a result of disturbed physiological balance between production of free radicals and antioxidative defense system.[2] In addition to these, the reduction of blood pressure that is observed on administration of ISO, by means of peripheral vasodilatation is also suggested to cause MI.[3] Hence, we have chosen the ISO model for this study. Therapeutic intervention by means of suppressing free radical generation and/or augmentation of endogenous antioxidant enzymes may limit the infarct size and attenuate MI.[4]

MI is a devastating disease which badly needs a medical breakthrough. Although modern drugs are effective in treating this condition, they are mostly accompanied with adverse effects. Marine plants, especially seaweeds are a largely unexplored reservoir of bioactive compounds. Brown seaweeds (Phaeophyceae) are known to produce different polysaccharides, namely alginates, laminarans and fucoidans. Fucoidan is a polyanionic sulfated polysaccharide mainly composed of α-1,3 backbones or repeating disaccharide units of α-1,3 and α-1,4 linked fucose residues with branching attached at C2 positions. Depending on the structure of the main chain, fucoidans may be sulfated at C4, C2 or in both positions of the fucose units.[5,6] Besides fucose, fucoidans may also contain minor amounts of other sugars like xylose, galactose, mannose and glucuronic acid.[7] It has been broadly studied in recent years, because it is endorsed with important biological properties such as antioxidant,[8] anticoagulant, antithrombotic,[9] antitumor,[10] and antiviral activities.[11] Additionally, fucoidan administration has no adverse effects on rats.[12]

To the best of our knowledge, efficacy of fucoidan of *Turbinaria conoides* (J. Agardh) in ISO induced MI and oxidative stress related parameters have not been scientifically explored till date. Therefore, the present study was designed to investigate the effects of oral administration of fucoidan on ISO induced MI in rats.

2. Material and methods

2.1. Animals

All the experiments were carried out with male albino Wistar rats weighing 180–200 g, obtained from The Central Animal House, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India. They were housed in polypropylene cages (47 cm × 34 cm × 20 cm) lined with husk, renewed every 24 h under a 12:12 h light/dark cycle at around 22 °C. The rats had free access to tap water and food. The rats were fed standard pellet diet (Pranav Agro Industries Ltd., Maharashtra, India). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Animal Ethics Committee of Annamalai University (Approval No. 809: 20.04.2011).

2.2. Collection and extraction of fucoidan

Fresh and healthy specimens of *T. conoides*, brown seaweeds, belonging to the Phaeophyceae family were collected along the coast of Tamil Nadu, India, during the year 2011. The species were identified and authenticated by Phycologist Dr. P. Anantharaman, Associate Professor, Centre for Advanced Study in Marine Biology, Annamalai University, Parangipettai, India. One voucher specimen was stored in our aquarium for future reference. The collected alga was initially washed in sea water to remove the macroscopic epiphytes and other extraneous matter and then rinsed in distilled water. The specimen was shade dried and coarsely powdered. Briefly 200 g of the dried seaweed powder was depigmented with acetone for 24 h followed by hot water extraction at 90–95 °C for 3–4 h. The brown colored syrup was then filtered through Whatman no. 4 filter paper, concentrated to 1/4th of the original volume, cooled and precipitated with three volumes of ethanol overnight at 4 °C. The precipitate was collected by centrifugation and dehydrated with diethyl ether to get a dried sulfated polysaccharide, fucoidan. The chemical composition of the extracted fucoidan is carbohydrates 57.62%, uronic acid 7.2%, and sulfate 21.12%. ISO was purchased from Sigma Chemical Company (St. Louis, MO) and all the other chemicals used were of analytical grade.

2.3. Induction of experimental myocardial injury

ISO (100 mg/kg) was dissolved in saline and subcutaneously injected to male albino Wistar rats at an interval of 24 h for 2 days. MI was confirmed by elevated activity of serum creatine kinase (CK) in rats.

2.4. Experimental design

In this experiment, a total of 48 male albino Wistar rats were randomly divided into eight groups of six rats each as follows:

- Group I: Normal control rats.

- Group II–IV: Rats were orally treated with fucoidan (50, 100, and 150 mg/kg, respectively) alone daily for 14 days using an intra gastric tube.
- Group V: Rats were subcutaneously injected with ISO alone (100 mg/kg) at an interval of 24 h for 2 days (on 15th and 16th days).
- Group VI–VIII: Rats were pretreated with fucoidan (50, 100 and 150 mg/kg, respectively) daily for 14 days and then subcutaneously injected with ISO (100 mg/kg) for 2 days (on 15th and 16th days).

At the end of the experimental period, after 12 h of second ISO injection, (i.e. on 16th day) all the rats were anesthetized and then sacrificed by cervical decapitation. Blood was collected and subsequently plasma and serum were separated by centrifugation. The heart tissue was excised immediately from the animals, washed off blood with ice-chilled physiological saline and stored for further biochemical estimations. A known weight of the heart tissue was homogenized in 5 ml of 0.1 M Tris–HCl (pH 7.4) buffer solution. The homogenate was centrifuged at 3000 rpm for 5 min and the supernatant was used for the estimation of various biochemical parameters.

2.5. Assay of cardiac marker enzymes

Activities of CK and lactate dehydrogenase (LDH) were measured in the heart tissue homogenate by standard commercial kits. LDH isoenzymes were separated by agarose gel electrophoresis.[13]

2.6. Assay of antioxidants

The activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GRx) in the heart tissue homogenate were assayed.[14–17] The levels of vitamin C, vitamin E and reduced glutathione (GSH) in the plasma and heart tissue homogenate were also estimated.[18–20] The content of protein in the heart tissue homogenate was also determined.[21]

2.7. Histopathology of myocardium

The heart tissues obtained from all the groups were washed immediately with saline and then fixed in 10% buffered neutral formalin solution. After fixation, the heart tissues were processed and embedded in paraffin. Then, the heart tissues were sectioned and stained with hematoxylin and eosin and examined under a high power microscope (100×), and photomicrographs were taken.

2.8. Statistical analysis

Statistical analysis was performed by a one-way analysis of variance followed by Tukey's test using Statistical Package for the Social Science (SPSS) software package version 12.0. Results were expressed as mean ± SD for six rats in each group. P values <0.05 were considered significant.

3. Results

Fig. 1 represents the effect of fucoidan on the activities of heart CK and LDH in normal and ISO induced rats. Rats induced with ISO showed a significant ($P < 0.05$) decrease in the activities of these marker enzymes in heart compared to normal control rats. Prior treatment with fucoidan daily for a period of 14 days significantly ($P < 0.05$) protects the decline of the activities of these enzymes in the heart of ISO induced rats compared with ISO alone induced rats. Agarose gel electrophoretic separation of serum LDH isoenzyme patterns of normal and ISO induced rats is illustrated in Fig. 2. Normal control and fucoidan alone induced rats did not show any increase in the intensities of serum LDH 1 and 2 isoenzyme patterns. ISO induced rats showed increased intensities of LDH 1 and 2 isoenzyme bands compared to normal control rats. Pretreatment with fucoidan (150 mg/kg) normalized the intensities of LDH 1 and 2 isoenzyme bands in ISO induced rats compared with ISO alone induced rats.

In our results, a significant ($P < 0.05$) decrease in activities of SOD, catalase, GPx and GRx in the heart tissue homogenate in ISO induced rats compared to normal control rats was observed. Pretreatment with fucoidan normalized ($P < 0.05$) the activities of enzymic antioxidants in ISO induced rats compared to ISO alone treated rats (Fig. 3). ISO induced rats exhibited significant ($P < 0.05$) decrease in levels of vitamin C, vitamin E and GSH in the plasma and heart tissue homogenate compared to normal control rats. Pretreatment with fucoidan normalized ($P < 0.05$) the levels of vitamin C, vitamin E and GSH in the plasma and heart tissue homogenate in ISO induced rats compared to ISO alone treated rats (Table 1).

Histopathological findings of the normal control rats heart showed normal cardiac muscle fibers (Fig. 4a), and normal rats heart induced with fucoidan showed normal cardiac fibers without any notable damages (Fig. 4b). However, the ISO induced myocardium showed diffuse inflammatory infiltrate in between muscle bundles (Fig. 4c), mononuclear collections (Fig. 4c$_1$) hemorrhage and broken cardiac fibers (Fig. 4c$_2$).

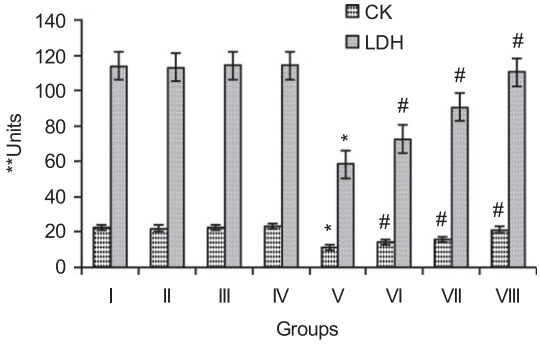

Figure 1 Effect of fucoidan on cardiac markers. Group I: Normal control, Group II: Fucoidan (50 mg/kg) alone, Group III: Fucoidan (100 mg/kg) alone, Group IV: Fucoidan (150 mg/kg) alone, Group V: ISO control (100 mg/kg), Group VI: Fucoidan (50 mg/kg) + ISO (100 mg/kg), Group VII: Fucoidan (100 mg/kg) + ISO (100 mg/kg), Group VIII: Fucoidan (150 mg/kg) + ISO (100 mg/kg). Each column is mean ± S.D. for six rats in each group. Significance was determined by a one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ versus normal control. $^{\#}P < 0.05$ versus ISO control. **Units: CK activity is expressed as μM of phosphorus generated/min/mg protein and LDH activity is expressed as nM of pyruvate liberated/min/mg protein.

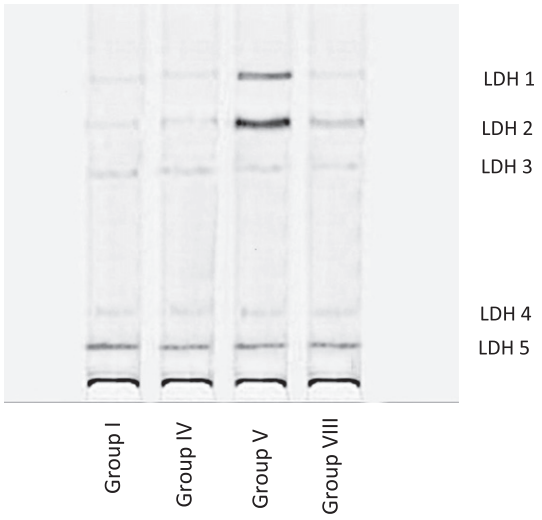

Figure 2 Effect of fucoidan on serum lactate dehydrogenase isoenzyme pattern. Group I: Normal control, Group IV: Fucoidan (150 mg/kg) alone, Group V: ISO control (100 mg/kg), Group VIII: Fucoidan (150 mg/kg) + ISO (100 mg/kg).

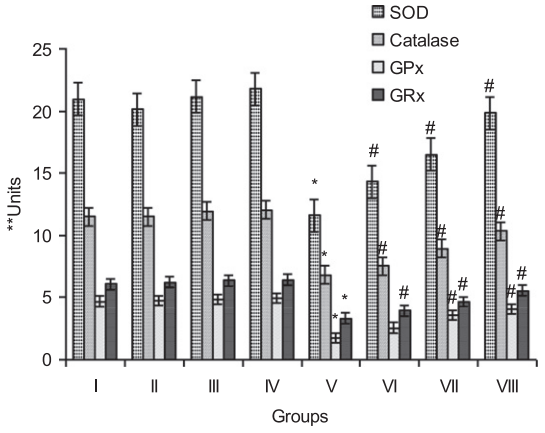

Figure 3 Effect of fucoidan on enzymatic antioxidants. Group I: Normal control, Group II: Fucoidan (50 mg/kg) alone, Group III: Fucoidan (100 mg/kg) alone, Group IV: Fucoidan (150 mg/kg) alone, Group V: ISO control (100 mg/kg), Group VI: Fucoidan (50 mg/kg) + ISO (100 mg/kg), Group VII: Fucoidan (100 mg/kg) + ISO (100 mg/kg), Group VIII: Fucoidan (150 mg/kg) + ISO (100 mg/kg). Significance was determined by a one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ versus normal control. $^{\#}P < 0.05$ versus ISO control. **Units: SOD: U/mg protein. SOD: one unit is defined as the enzyme concentration required inhibiting the OD at 560 nm of chromogen production by 50% in 1 min, Catalase: μM of H_2O_2 consumed/min/mg protein, GPx: μg of GSH consumed/min/mg protein and GRx: nM of NADPH oxidized/min/100 mg protein.

Fig. 4d showed normal cardiac fibers without notable damages in rat's heart pretreated with fucoidan in ISO induced rats. Table 2 illustrates the effects of fucoidan on the degree of histological changes in the heart of normal and ISO induced myocardial injured rats. Normal control rats and normal rats treated with fucoidan showed normal cardiac muscle fibers without any notable changes (−). ISO induced rats showed diffuse inflammatory infiltrate (+ + +), mononuclear collections (+ + +), broken cardiac fibers (+ + +) and hemorrhage (+ + +). Pretreatment with fucoidan in ISO induced rats showed normal cardiac fibers without hemorrhage (−), broken cardiac fibers (−) mononuclear collections (−) and diffuse inflammatory infiltrate (−). Fucoidan pretreatment (150 mg/kg) to ISO induced rats showed a significant effect on all the biochemical parameters studied. There was no considerable change in the normal control rats treated with fucoidan (150 mg/kg) daily for a period of 14 days.

4. Discussion

There is a huge global demand for safe and effective natural nutraceutical ingredients that can provide preventative health benefits. In recent years, extensive pharmaceutical researches have been done on fucoidan, as a result, it is now being marketed as a nutraceutical and food supplement.[22] In our experiment, we found that fucoidan of *T. conoides* protected the myocardium from ISO induced myocardial functional and structural injury via favorably maintained biochemical, electrophoretic and histopathological parameters suggesting its cardioprotective action.

In our study, we observed decreased activities of cardiac marker enzymes such as CK and LDH in the myocardium. Myocardial cells containing these enzymes are damaged or destroyed due to insufficient oxygen and glucose supply and the cell membrane becomes permeable or may rupture, which results in the leakage of these enzymes. This action may be due to the damage caused to the sarcolemma by ISO rendering it leaky.[23] This accounts for the decreased activities of these enzymes in the heart of ISO induced MI in rats. Pretreatment with fucoidan normalized the activities of these enzymes by their antioxidant activity,[8] which may explain their ability to protect the myocardium from damage by preventing the leakage of these enzymes from the heart. LDH isoenzymes exist in five different isoforms (LDH 1–LDH 5). In cardiac tissue, LDH 1 and LDH 2 are predominant, so the detection of elevated concentrations of this enzyme in serum has become a definitive diagnostic and prognostic criterion for various diseases and studies of its isoenzymes have found importance in the location of tissue damage.[24] It can be differentiated from other types of tissue damage, because LDH isoenzymes begins to rise in 12–24 h following MI and peaks in 2–3 days then gradually dissipating in 5–14 days.[25] The increased abundance of serum LDH 1 and LDH 2 bands in ISO induced rats observed in this study could be due to ISO induced cardiac injury, which is supported by previous report.[26] Normalization of the intensities of LDH 1 and LDH 2 bands in ISO induced rats by pretreatment with fucoidan (150 mg/kg) might probably be due to its free radical scavenging potential, thereby suggesting good antioxidant activity.[8]

We further studied the underlying mechanisms of fucoidan in ISO induced rats. Pharmacological augmentation of endogenous myocardial antioxidants such as SOD, catalase, GPx, and GRx has been identified as a promising therapeutic approach in diseases associated with increased oxidative stress, i.e., the first line of cellular defense against oxidative stress. The observed decreased activities of SOD and catalase in ISO induced cardiac tissue might be due to $O_2^{\cdot-}$ generated at

Table 1 Effect of fucoidan on the levels of non-enzymatic antioxidants in normal and ISO induced myocardial injured rats.

Groups	Plasma (mg/dl)			Heart (mM/mg protein)		Heart GSH (mM/g wet tissue)
	Vitamin C	Vitamin E	GSH	Vitamin C	Vitamin E	
Normal control	3.54 ± 0.3	2.06 ± 0.11	30.13 ± 2.4	1.81 ± 0.1	0.7 ± 0.05	8.49 ± 0.7
Fucoidan (50 mg/kg)	3.51 ± 0.3	1.85 ± 0.12	29.56 ± 2.4	1.79 ± 0.1	0.69 ± 0.05	8.41 ± 0.7
Fucoidan (100 mg/kg)	3.55 ± 0.3	1.96 ± 0.12	30.25 ± 2.4	1.82 ± 0.1	0.72 ± 0.05	8.59 ± 0.7
Fucoidan (150 mg/kg)	3.61 ± 0.3	1.99 ± 0.12	30.82 ± 2.5	1.85 ± 0.1	0.74 ± 0.05	8.67 ± 0.7
ISO (100 mg/kg)	1.02 ± 0.09[a]	0.96 ± 0.06[a]	14.96 ± 1.2[a]	1.05 ± 0.1[a]	0.33 ± 0.04[a]	4.52 ± 0.4[a]
Fucoidan (50 mg/kg) + ISO (100 mg/kg)	1.85 ± 0.1[b]	1.25 ± 0.12[b]	19.56 ± 1.2[b]	1.25 ± 0.1[b]	0.39 ± 0.04[b]	5.25 ± 0.4[b]
Fucoidan (100 mg/kg) + ISO (100 mg/kg)	2.65 ± 0.1[b]	1.56 ± 0.12[b]	24.35 ± 1.7[b]	1.65 ± 0.1[b]	0.53 ± 0.05[b]	6.96 ± 0.7[b]
Fucoidan (150 mg/kg) + ISO (100 mg/kg)	3.3 ± 0.2[b]	1.85 ± 0.12[b]	29.27 ± 1.8[b]	1.77 ± 0.1[b]	0.65 ± 0.05[b]	8.32 ± 0.7[b]

Each column is mean ± S.D. for six rats in each group. Significance was determined by a one-way ANOVA followed by Tukey's test.
[a] $P < 0.05$ versus normal control.
[b] $P < 0.05$ versus ISO control.

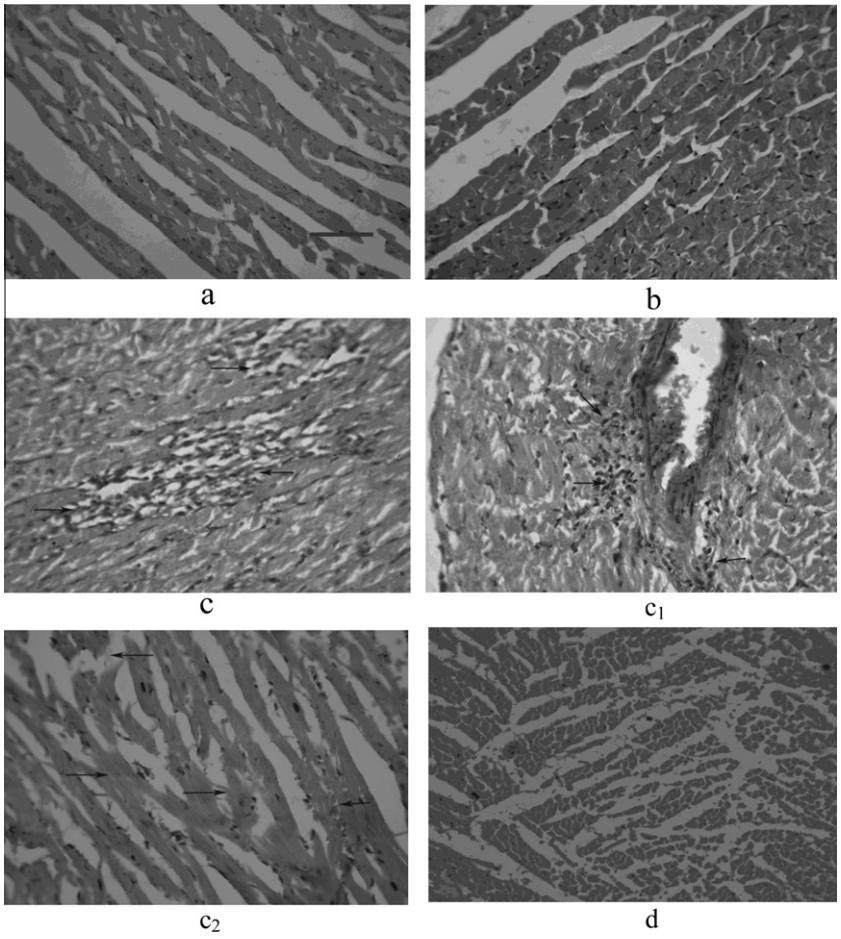

Figure 4 Histopathological changes of myocardium. *Scale bar* in a = 100 μm in all (heamatoxylin and eosin (HE) staining 100×). (a) Normal untreated rats showing normal cardiac muscle fibers (Group I). (b) Fucoidan (150 mg/kg) alone treated rats showing the normal cardiac fibers without any notable damages (Group IV). (c) Isoproterenol induced myocardium showing diffuse inflammatory infiltrate in between muscle bundles (Group V). (c_1) Isoproterenol induced myocardium showing mononuclear collections (Group V). (c_2) Isoproterenol induced myocardium showing hemorrhage and broken cardiac fibers (Group V). (d) Fucoidan (150 mg/kg) pretreated + isoproterenol (100 mg/kg) induced rats showing the normal cardiac fibers without any notable damages (Group VIII).

the site of damage, which modulates SOD and catalase resulting in decreased activities and leads to the accumulation of the $O_2^{\cdot-}$, which damages the myocardium.[27] Pretreatment with fucoidan normalized the activities of these enzymes in ISO induced rats. Accordingly, fucoidan scavenges $O_2^{\cdot-}$ and reduces myocardial injury caused by free radicals in the myocardium due to ISO treatment. The glutathione dependent enzymes GPx and GRx are lowered in ISO induced cardiac tissue.

Table 2 Effects of fucoidan on the degree of histopathological changes in normal and ISO induced myocardial injured rats.

Groups	Diffuse inflammatory infiltrate	Mononuclear collections	Hemorrhage	Broken cardiac fibres
Normal control	−	−	−	−
Fucoidan (150 mg/kg)	−	−	−	−
ISO (100 mg/kg)	+ + +	+ + +	+ + +	+ + +
Fucoidan (150 mg/kg) + ISO (100 mg/kg)	−	−	−	−

Photomicrographs were used to evaluate the degree of damage in the heart tissues: (−) no changes. (+ + +) marked changes.

The decreased activity of these enzymes in ISO induced rats is due to the decreased concentration of their substrate, GSH.[28] The enhanced protective mechanism toward oxidative stress in MI may consume GSH and depress GSH levels. The observed decrease in GSH might also be due to increased utilization in protecting thiol containing proteins from lipid peroxides and from other reactive oxygen species. Prior treatment with fucoidan normalized the activities of GSH dependent enzymes and the levels of GSH in ISO induced rats. Cellular injury can be prevented by increasing intracellular GSH. The enhanced concentration of GSH observed in fucoidan pretreated ISO induced rats resulting in the enhanced activities of GSH related enzymes and thus prevents cellular injury in myocardium. The antioxidant effect of fucoidan is clearly revealed by this effect.

The second line of cellular defense against oxidative stress consists of the non-enzymatic scavengers namely, vitamin C, vitamin E, ceruloplasmin and sulfhydryl containing compounds, which scavenge the residual free radicals escaping from decomposition by the antioxidant enzymes. An inverse correlation exists between plasma levels of vitamin E and mortality from MI.[29] The lowered concentrations of vitamins C and E observed in ISO induced rats might be due to neutralizing increased production of free radicals. Prior treatment with fucoidan normalized the levels of these vitamins in ISO induced rats. The retained concentration of these vitamins may protect the heart against ISO mediated free radicals in the fucoidan pretreated ISO induced group.

Histopathological findings of the myocardial injured rat's heart pretreated with fucoidan (150 mg/kg) showed a well preserved normal morphology of cardiac muscle without notable changes compared to ISO induced rat's heart. Thus, fucoidan protected the myocardium against injury caused by ISO and inhibited lipid peroxidation and thus protected the near normal architecture of the myocardium.

5. Conclusion

Oral administration of fucoidan (150 mg/kg) extracted from brown seaweeds, *T. conoides* significantly protects the myocardium by maintaining the biochemical, electrophoretic and histopathological parameters against ISO induced myocardial injury. These effects could be due to its antioxidant and membrane stabilizing properties. The results of this present investigation may trigger a renewed interest in the use of *T. conoides* fucoidan for MI.

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