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RESEARCH ARTICLE

Protective effect of sulphated polysaccharides and aqueous extract of *Ulva lactuca* on N-nitrosodiethylamine and Phenobarbital induced nephrotoxicity in Rats

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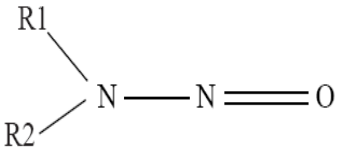
Abstract

The scope of the present study was to investigate the potential effect of sulphated polysaccharides and aqueous extract of *Ulva lactuca* against N-nitrosodiethylamine (NDEA) and phenobarbital (PB) induced renal toxicity. However, nephrotoxicity due to NDEA remains one of the most current investigations challenges. In the present study, an attempt has been made to evaluate the potentiality of *Ulva lactuca* extracts on renal injury markers caused by NDEA. Adult male albino rats were divided into four groups. Three groups (B, C& D) received a single dose of NDEA intraperitoneal (200 mg/kg body weight) for 2, 12 and 24 weeks to provoke nephrotoxicity. Further, the (B, C& D) groups received PB (0.05%) in drinking water after two weeks of NDEA administration, then two (C& D) of which simultaneously received sulphated polysaccharides and aqueous extract of *Ulva lactuca*, respectively by oral gavage (50 mg/kg body weight) along the entire period of study. Saline (0.9%) treated control group (A) was also built-in. The results revealed that NDEA and PB induced an increase in the blood urea nitrogen (BUN), uric acid and creatinine concentrations; diagnosis of renal damage was normalized by simultaneous administration of sulphated polysaccharide better than the aqueous extract of *Ulva lactuca*. Moreover, the extent of kidney damage was appraised by histopathological findings. Simultaneous administration of sulphated polysaccharide treatment significantly attenuated the impairment of renal markers by bringing a decrease in their levels, which might be increase the antioxidant status. These results underline the antioxidant property of sulphated polysaccharides and its nephroprotective action against NDEA-induced nephrotoxicity.

Introduction

Nitrosamines are compounds having the universal structure as shown in, where R1 and R2 are alkyl or aryl groups. Nitroso compounds are one among the common terms of carcinogens extremely present in the food chain and human environment (Preussmann and Stewar, 1984). These compounds are widely reported in numerous foodstuffs, such as meat and milk products, soft drinks, and alcoholic beverages (Tricker et al., 1991; Prasad and Krishnaswamy, 1994; van Maanen et al., 1998; Levallois et al., 2000). Nitroso compounds are immediately formed endogenously in the human body by the reaction of nitrite with amides and amines (Masuda et al., 2000; Ohsawa et al., 2003). Presence of these compounds and their precursors in human surrounding together with the possibility of their endogenous formation in the body has led to suggestions of their potential involvement in various human cancers (Bartsch and Montesano, 1984; Kaplan et al., 2003). N-nitrosodiethylamine (NDEA) promoted by Phenobarbital (PB) has been suggested to cause oxidative stress and cellular injury due to involvement of free radicals (Bansal et al., 2000; Aiub et al., 2003).



Generic N-nitrosamine structure

Nephrotoxicity is a poisonous effect of both toxic chemicals and medicine of the kidneys. There are several sorts of toxicity, nephrotoxicity should not be mixed up with the fact that some medicinal drugs have a predominantly renal excretion and need their dosage adjusted for the decreased renal function. Kellum et al (2011) reported that renal failure is evidenced by abrupt and a reduction in glomerular filtration rate, resulting in accumulation of urea and other chemicals in the blood. It was well known that diagnosis of acute renal injury is based on changes in creatinine, which is believed to be the ultimate marker and might identify patients when it is too late.

Ulva lactuca is widely distributed throughout all levels of the intertidal zone, in calm and protected harbors 10 meters depth. A polysaccharide compound, isolated from *Ulva lactuca*, has been shown to have significant antiviral effects, reducing replication rates of a variety of strains of human and avian influenza viruses (Athukorala et al., 2006). Moreover, the mechanisms of the pharmaceutical effects of biological polysaccharides on diseases have been frequently studied, and several isolated polysaccharides with various curative effects have been tested and recently applied in therapies. The purpose of this study is to investigate the ameliorating effect of sulphated polysaccharides and aqueous extract of *Ulva lactuca* on the NDEA-induced nephrotoxicity.

1. Materials and Methods

1.1. Chemicals

All the reagents and biochemicals were obtained from Sigma Chemicals Co. (St. Louis, MO) (Germany), Diamond Diagnostic Company (Egypt), and Spinreact chemical company (Spain).

1.2. Animals

Male albino rats, initially weighing between 90-100 gm, were used in the experiment and selected from the National Research Center, Dokki, Giza, Egypt. They were housed in a conventional animal facility under the standard laboratory conditions of temperature, pressure and relative humidity (55±5% and a 12 hour photoperiod at 24±1°C). The animals housed in stainless steel cages (6-8 rats per cage) for 2 weeks before the start of the experiment. They were kept under observation for the pre-mentioned period before the start of the experiment to eliminate any intercurrent infections. During the entire period of study, the rats were fed with standard normal pellet diet and water *ad-libitum*. The animal's procedures were maintained under standard conditions, according to the Canadian Committee for Animals Use and Care guideline (Canadian Council on Animal Care, 1993).

1.3. Experimental design (The basic chemical carcinogenesis protocol)

Healthy albino rats were randomly divided into four experimental groups: Group A rats was taken as a normal treated with normal saline, group B rats were the NDEA+PB-treated group that received a single necrogenic, intraperitoneal (i.p.) injection of NDEA (Sigma-Aldrich, St. Louis, MO) (200 mg/kg body weight dissolved in 0.9% saline) at 9 weeks of age. Following 2 weeks of recovery, i.e. after week 11, all the NDEA-initiated rats were given Phenobarbital (PB Sigma-Aldrich) in drinking water (0.05%) 6 days a week and continuing thereafter till the end of the study. Groups C and D-intoxicated rats were administered with oral dosing by means of a stomach tube (50 mg/kg b.wt) sulphated polysaccharides and aqueous extract of *Ulva lactuca*, respectively. Daily food and water intakes were noted and the body weights of the animals from each group were recorded every week. The animals were maintained for 2, 12 and 24 weeks and the rats were sacrificed under light ether anaesthesia at the different

time points, blood was collected from each animal. All the animals were fasted and deprived of water overnight before sacrifice.

1.3.1. Blood sampling and biochemical assays

At the end of each experimental period (2, 12 & 24 weeks), six rats were sacrificed from each group under light diethyl ether anesthesia at overnight fasting state. Trunk blood samples were collected from the cut jugular vein in chilled non-heparinized tubes. Blood samples were collected and allowed to coagulate at room temperature, then centrifuged at 3000rpm for 20 min. The clear, non-haemolysed supernatant sera were quickly removed and stored at -20°C for subsequent biochemical analysis.

1.3.2. Evaluation of renal functions

Determination of blood urea nitrogen (BUN) based on the principle described by (Patton and Crouch, 1977). The uric acid level was estimated according to the methods described by (Barham and Trinder, 1972) on the basis of its oxidation to Allentown which under the influence of POD forming red cinnamon compound which proportionate to the uric acid concentration. The creatinine concentration was determined photometrically at 492nm according to the method of (Henry, 1974).

1.3.3. Histopathological assessment

After sacrification and dissection, kidneys were immediately excised, cleaned, washed, and rinsed in an ice-cold normal saline solution (0.9% NaCl, pH 7.4) until bleached of all the blood and blotted dry on filter paper sheets to remove blood, then kept in 10% neutral buffered formalin (pH 7.4) for at least 24h for histopathological investigation at the histology unit, National Cancer Institute, Cairo, Egypt. Tissue specimens were then dehydrated in a graded ethanol series, cleared in xylene, immersed in Paraplast wax, and sectioned at 10µm thickness. Kidney sections were mounted on positively charged and coated slides (Thermo Scientific, Menzel-Gläser, Braunschweig, Germany). Before staining, tissue sections were consequently deparaffinised and then stained by haematoxylin and eosin for general histological structure.

1.4. Preparation of *Ulva lactuca* aqueous extract and sulphated polysaccharide

Fresh seaweed samples were collected in polythene bags from the intertidal regions of the Red Sea western coastal regions of Egypt, particularly from Marsa Alam (Lat. 25o 04' 0.48" N; Long. 34o 54' 7.2" E) and El-Qusair district (Lat.26o 06' 54" N; Long. 34o 16' 58. 08" E). The air shade dried algae were roughly cut and minced using a mechanical blender. The dried seaweed (100g) was rehydrated with 1:1 of distilled water and heated at 100°C for 1h. After centrifugation, each supernatant was precipitated with EtOH (3volumes) and then freeze dried to give polysaccharide sulphate extracts. The precipitate was washed with distilled water and ethyl alcohol several times to remove salts and minerals and then dried (to remove any traces of alcohol). The aqueous extract dose was prepared by adding 50mg of the powdered algae to 100ml of distilled water and was boiled for 15 minutes, then filtered and was orally given to the rats at doses of 50mg/kg b.wt., daily for 24 weeks. Polysaccharide sulphates were dissolved in boiling q-water and were orally administered at the same dose for the same time course (24 weeks). These doses were used according to previously studied LD50.

2. Expression of results and statistical significance

Data were analysed with (Graph-Pad Prism V6.01) software packages. One-way analysis of variance (ANOVA) was used to test the significance of differences along the time, between treatments and the control as well. Tukey-Kramer multi-comparison test was also performed to evaluate the changes among different time intervals within the variable using the error calculated from ANOVA. Statistical significance was set at $P<0.05$ for all the values. All the data were presented as mean ± standard error of means (S.E.M.).

3. Results

Table 1. Effect of sulphated polysaccharide and aqueous extract of *Ulva lactuca* on the activities of renal functions during NDEA initiated-renal toxicity.

Parameter	Time (Week)	Group (A)	Group (B)	Group (C)	Group (D)	P_{value}
BUN (mg/dl)	2	32.99±0.71[a] ---- ----	37.55±0.5[a] (13.82)	34.97±0.54[a] (-6.87)	35.99±0.35[a] (-4.15)	<0.0001 --------
	12	34.68±0.73[a] --------	43.18±0.51[b] (24.50)	38.11±0.37[b] (-11.74)	39.21±0.19[b] (-9.19)	<0.0001 --------
	24	33.84±0.44[b] --------	45.54±0.46[c] (34.57)	40.06±0.2[c] (-12.03)	40.34±0.21[c] (-11.41)	<0.0001 --------
Creatinine (mg/dl)	2	0.59±0.01[a] --------	0.89±0.09[a] (50.84)	0.71±0.02[a] (-20.22)	0.74±0.02[a] (-16.85)	0.0001 --------
	12	0.65±0.01[b] --------	1.01±0.02[b] (55.38)	0.80±0.03[b] (-20.79)	0.83±0.02[b] (-17.82)	<0.0001 --------
	24	0.75±0.02[c] --------	1.14±0.03[c] (52)	0.93±0.02[c] (-18.42)	0.96±0.01[c] (-15.78)	<0.0001 --------
Uric acid (mg/dl)	2	2.25±0.04[a] --------	3.38±0.02[a] (50.22)	2.78±0.04[a] (-17.75)	3.00±0.05[a] (-11.24)	<0.0001 --------
	12	2.44±0.03[b] --------	3.67±0.07[b] (50.40)	2.99±0.03[a] (-18.52)	3.02±0.04[a] (-17.71)	<0.0001 --------
	24	2.51±0.02[b] --------	4.12±0.05[c] (64.14)	3.00±0.06[a] (-27.18)	3.09±0.04[a] (-25)	<0.0001 --------

- In the column, mean values with the same superscript letters are non-significant (P>0.01), otherwise are significant (P<0.01).
- Data are expressed as mean±S.E.M. (n=6)
- % changes are calculated by comparing NDEA-intoxicated group with normal and intoxicated treated groups with NDEA-intoxicated group.
- (A) Normal group, (B) NDEA+PB, (C) NDEA+PB+sulphated polysaccharide, (D) NDEA+PB+aqueous extract of algae

Data in table (1) indicated that blood urea nitrogen, uric acid and creatinine activities were significant (P<0.0001) elevated in NDEA-intoxicated rats in comparison to control group. The recorded data were (37.55±0.5mg/dl, 43.18±0.51mg/dl & 45.54±0.46mg/dl) for BUN, (3.38±0.02mg/dl, 3.67±0.07mg/dl & 4.12±0.05mg/dl) for uric acid, and (0.89±0.09mg/dl, 1.01±0.02mg/dl & 1.14±0.03mg/dl) for creatinine along the three time points (2, 12& 24 weeks), respectively. Otherwise, administration of NDEA-intoxicated rats with both sulphated polysaccharides and aqueous extract of *Ulva lactuca* at daily doses of (50 mg/kg b.wt, orally) showed significant (P<0.0001) decrease of these levels as compared with their corresponding intoxicated rats, respectively. The percentages of change were (-6.87%, -11.74% & -12.03%) for BUN, (-17.75%, -18.52% & -27.18%) for uric acid, and (-20.22%, -20.79% & -18.42%) for creatinine by sulphated polysaccharide administration, respectively, while of aqueous extract of *Ulva lactuca* were (-4.15%, -9.19% & -11.41%) for BUN, (-11.24%, -17.71% & -25%) for uric acid, and(-16.85%, -17.82% & -15.78%) for creatinine along the entire time points, respectively.

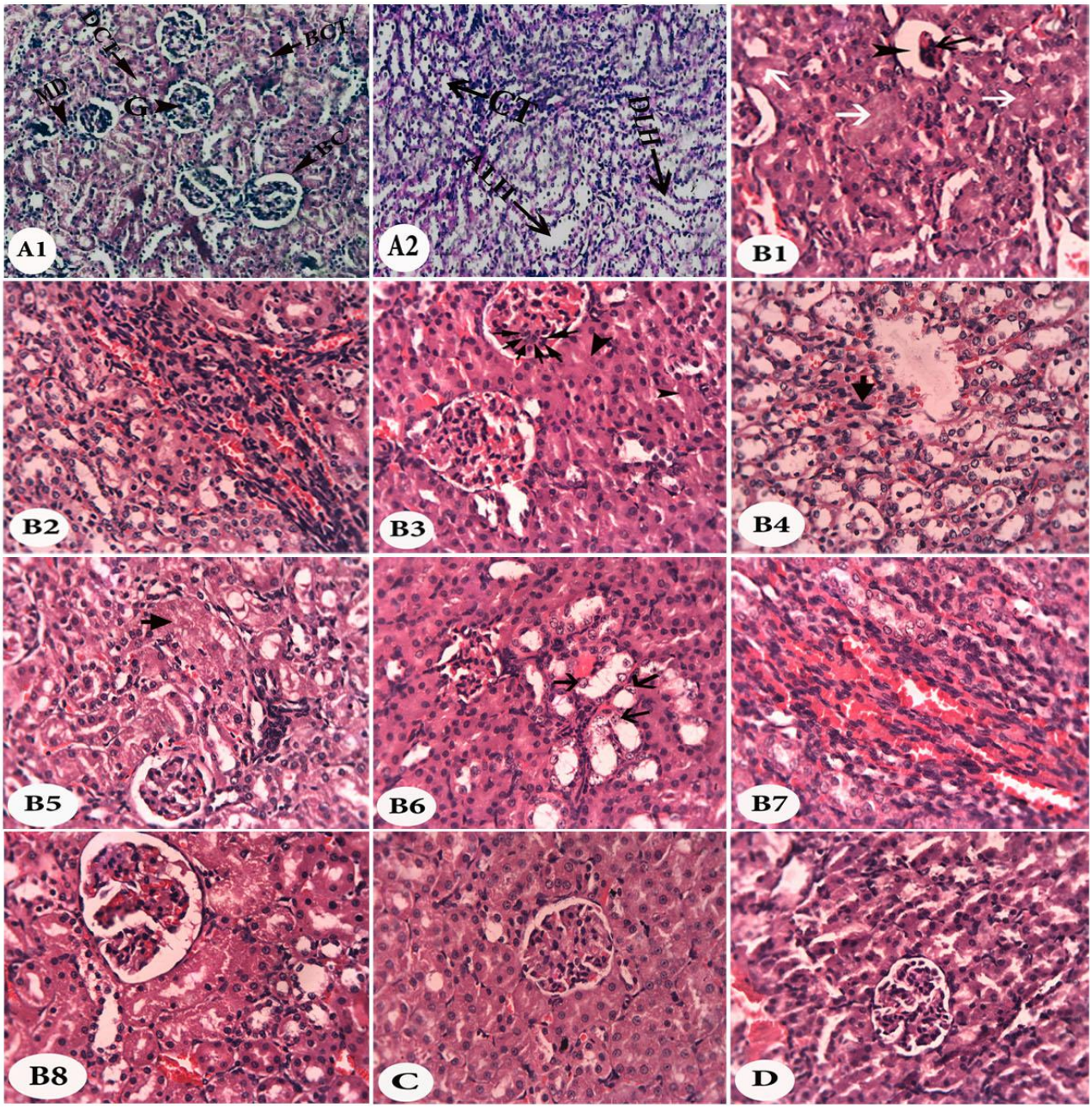


Fig. 1: Photomicrographs of kidney specimens stained with H& E. (A1-2): kidney from control rat (saline-treated) showing normal renal tubular histology with normal glomerular capillary lumen, intact basement membrane, and small mesangial space. Moreover, normal Bowman's space, proximal and distal tubular system (PCT& DCT), appropriate diameter of loop of Henle segments ascending and descending (ALH& DLH) and normal macula densa are illustrated (x, 20); B: kidney from rat treated with NDEA+PB showing focal necrosis (white arrows) accompany abnormal glomeruli (with enlarged Bowman's space (headed arrow) and shrunken glomerular tufts (black solid arrow) in the outer renal cortex (B1, x 40), multifocal neutrophilic infiltrates in tubular lumens and mononuclear cells within the interstitium (B2, x 40), nephritic syndrome; glomerulus is crowded with cells and filling of glomerular loops, hypercellularity due to cell proliferation and the solid arrows point to areas with increase of cellularity (mild proliferation in this case) besides necrotic cells (headed arrow) (B3, x 40), acute tubular necrosis and degeneration (ATN) and

pyknotic nuclei (arrow) (B4, x 40), acute tubulointerstitial nephritis (ATIN); the interstitium has mononuclear inflammatory cell infiltrates accompanied by diffuse necrosis (arrow) (B5, x 40), tubular necrosis and degeneration accompany glomerulonephritis (B6, x 40), massive alveolar haemorrhage infiltrate the medullary region (Wegener's granulomatosis) (B7, x 40), multifocal necrosis accompany chronic tubulointerstitial nephritis (CTIN) (B8, x 40); C: kidney from treated rats with NDEA+sulphated polysaccharides, showing normal architectural and arranged cortical cells with normal glomerular loop and exact Bowman's space (C, x 40); D: kidney from treated rats with NDEA+aqueous extract of *Ulva lactuca* showing not improvements of renal tissues with a focus of neutrophil infiltrate associated with abnormal architectural cells (x 40).

Histopathological investigation

Kidneys showed normal architectural cells under saline treated conditions, but NDEA administration caused severe granular degeneration and coagulative necrosis in the kidneys.
The renal cortex of the control rats contained glomeruli, vessels, tubules and interstitium (Fig. 4. A1). When evaluating these renal specimens by light microscopy on an H-E-stained section, the following glomerular features were inspected: the overall cellularity of the glomerulus, the symmetry of the glomerulus and the thickness of the capillary walls. Renal tubules (the long and winding neck) formed as the proximal tubule, the loop of Henle and the distal tubule (Fig. 4. A2). Light microscopic findings of kidney sections from NDEA-treated rats are summarized as follows: Focal necrosis accompany shrunken glomerular tufts with enlarged Bowman's space, multifocal neutrophilic infiltrates in tubular lumens and mononuclear cells within the interstitium, nephritic syndrome due to hypercellularity, acute tubular necrosis and degeneration (ATN) with pyknotic nuclei, acute tubulointerstitial nephritis (ATIN) indicated by mononuclear inflammatory cell infiltrates within interstitium accompanied by diffuse necrosis, glomerulonephritis accompanied by tubular necrosis and degeneration, massive haemorrhage infiltrate the medullary region (Wegener's granulomatosis) and multifocal necrosis accompany chronic tubulointerstitial nephritis (CTIN) (Fig. 4. B1-8). Otherwise, the kidneys from treated rats with sulphated polysaccharides, showing normal architectural and arranged cortical cells with normal glomerular loop and exact Bowman's space (Fig. 4C). On the other hand, kidneys of treated rats with aqueous extract of *Ulva lactuca* showing little bit improvements of renal tissues and focus of neutrophilic infiltrates associated with abnormal architectural cells were seen (Fig. 4D).

4. Discussion

Nephrotoxicity of the N-nitrosodiethylamine is well documented (Sharma and Janmeda, 2013). The kidney is a highly energetic organ and therefore relies heavily on aerobic metabolism for the ATP production by oxidative phosphorylation and their functions will suffer from the generation of these reactive species. In the present study, sulphated polysaccharides and aqueous extract of *Ulva lactuca* ameliorated NDEA-induced nephrotoxicity as it reduced serum urea and creatinine as well as uric acid levels and restored the histological patterns. In accordance with these results, Josephine et al (2006, 2007) reported that isolated sulphated polysaccharide reduced blood urea nitrogen and serum creatinine levels and attenuated renal tubular damage in renal glomerular injury in rats.

The increased concentration of serum toxicity markers like urea, creatinine and uric acid have long been thought of investigating chemicals induced nephrotoxicity in man and animals (Bennett et a., 1982). Normally, the serum urea is well known in the liver protein that derived from the diet or tissues and in eliminated in the urine. On the other side, creatinine is generally derived from endogenous sources by the tissue creatinine breakdown (Venkatesan et al., 2000). Thus the BUN concentration is frequently reckoned a more reliable renal function predictor than serum creatinine. Nephropathy well known expressed as a decrease in glomerular filtration rate (GFR), which resulting in reduced renal clearance of urea, uric acid and creatinine and subsequently elevated concentration of these substances in the blood. The marked elevation in the levels of serum constituents was significantly prevented by sulphated polysaccharides co-administration. This suggests the nephroprotective role of sulphated polysaccharides isolated from *Ulva lactuca*.

Currently, the elevated blood urea nitrogen, uric acid and creatinine levels along the three time points 2, 12 and 24 weeks of the NDEA and PB co-treatment are considered good indicators for renal disorders. Urea is the end product of protein catabolism, and presence of some toxic compounds might increase blood urea and decrease

plasma protein (Varely et al., 1987). Enhanced catabolic proteins and accelerated amino acid deamination for gluconeogenesis is possibly an acceptable postulate to interpret the elevated urea levels (Bishop et al., 2005). Uric acid is the end product of nucleic acid catabolism in tissues, and the increment in its concentration might be due to degradation of purines or by either overproduction or inability of excretion. Elevated serum creatinine is indicative of renal injury and associated with abnormal renal function, particularly as it relates to glomerular function (Bennett, 1996; Bishop et al., 2005). Upon treatment of intoxicated rats with sulphated polysaccharides and aqueous extract of *Ulva lactuca* improve and normalize the levels of urea, creatinine and uric acid. These results indicated that these extracts may protect against NDEA-induced renal toxicity. There is evidence that fucoidan, a sulphated polysaccharide from marine brown algae retards renal injuries by increasing renal blood flow (Bojakowski et al., 2001).

The antioxidant activity of seaweeds has previously been documented (Cornish and Garbary, 2010) the restorative effect of *S. dentifolium* extract could be attributed to its ability to decline the NDEA metabolism into more toxic metabolites. Moreover, co-treatment with sulphated polysaccharide and aqueous extract of *Ulva lactuca* significantly abrogated the enhanced level of these markers. It was noticed that sulphated polysaccharides exhibited greater efficiency than the aqueous extract and thereby rendering the protein catabolism and amino acid deamination and this may relate to its unique chemical composition like sulphated polyaldobiuronan, rhamnosylated saccharides and a potential source of iduronic acid which required in the synthesis of heparin analogs with antithrombotic activities (Bokang et al., 2012; Tabarsa et al., 2012). Besides, the ulvan and its oligosaccharides have antitumor and modulate immune system (Ketterer, D.J. Meyer, 1989; Vasquez-Garzon et al., 2009).

Nephrotoxicity induced by NDEA and PB is evident from the abnormal histological findings. Our histopathological inspections also confirm the significant tubular vascular transformation, tubular necrosis and tubular degeneration in this nephropathy model. Histologic alterations provide a clear evidence of nephrotoxicity following NDEA administration, including proximal tubular transformation, interstitial mononuclear cell infiltrates, hemorrhage, and tubular degeneration following NDEA treatment have also been described in this exemplar. Acute and chronic tubulointerstitial nephritis were demonstrated in this study and was also described earlier (Baker and Pusey, 2004; Clarkson et al., 2004; Appel and Bhat, 2006) and considered the most relevant histopathological change. On the other hand, treatment with sulphated polysaccharides had shown better improvement in the architecture of the tubular cells and normal glomeruli than the aqueous extract of *Ulva lactuca*.

Conclusion

In conclusion *Ulva lactuca* extracts offer significant nephroprotection against NDEA-induced nephropathies using sulphated polysaccharides in particular by inhibiting inflammation and improving renal microcirculation and renal functions. The activity elicited by the sulphated polysaccharides was better than the aqueous extract and might be due to its ability to enhance the antioxidant enzymatic system. Therefore, further trials by using higher doses of these polysaccharides in combination with other nephroprotective agents should be tested as prophylactic agents to combat NDEA-induced nephropathy.

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Disclosure statement

Authors have declared that no competing interests exist.

Ethical Standard

The present study does not contain clinical studies or patient data.

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