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Stabilization of mitochondrial and microsomal function of fucoidan from *Sargassum plagiophyllum* in diethylnitrosamine induced hepatocarcinogenesis

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ABSTRACT

Crude fucoidan from *Sargassum plagiophyllum* extracted from blade and purified by Q-Sepharose fast flow anion-exchange chromatography and three fucoidan fractions were obtained. Maximum sulphate containing fucoidan fraction was considered as purified fucoidan and purity was checked with agarose gel electrophoresis. The monosaccharides of purified fucoidan analysed by HPLC revealed the presence of the sugars such as fucose as a major sugar were 70.8 mol%. The percentages of other sugars were galactose (13.5%), xylose (2.5%) and mannose (11.2%). GPC was used to analyse molecular weight of purified fucoidan and it was found to be 35 kDa. The levels of ICDH, SDH, MDH, a-KGDH, Phase-I biotransformation enzymes, and Phase-II biotransformation enzymes were decreased in cancer bearing animals which may be due to oxidative stress and mitochondrial damage and fucoidan restored these enzyme activities. The inhibition of carcinogen metabolic activation indicates the anticancer activity of fucoidan in DEN induced liver cancer.

1. Introduction

Brown algal derived fucoidan plays a vital role in human health and nutrition. Furthermore, seaweed processing by-products with bioactive fucoidan can be easily utilized for producing functional ingredients. The possibilities of designing new functional foods and pharmaceuticals to support reducing or regulating the diet related chronic malfunctions are promising. Therefore, it can be suggested that due to valuable biological functions with health beneficial effects, fucoidan has a large potential as an active ingredient for preparation of nutraceutical, cosmeceutical and pharmaceutical products. Until now, most of the biological activities of fucoidan have been observed in vitro or in mouse model systems (Kim & Bae, 2010; Kong, Kim, Yoon, & Kim, 2009; Ordonez, Escrig, & Ruperez, 2010; Shibata, Ishimaru, Kawaguchi, Yoshikawa, & Hama, 2008; Wijesekara, Pangestuti, & Kim, 2011).

The nitrosamines are widely recognized as carcinogenic compounds, but they require metabolic activation to exert their cytotoxic and carcinogenic activities. The International Agency for Research on Cancer (IARC) has recognized that the diethylnitrosamine (DEN) as hepato-carcinogen (IARC, 1978), which on administration to experimental animals, induces cancer in liver and at lower incidences in other organs (Poirier & Beland, 1994; Schuller, 1992). The primary routes of potential human exposure to DEN are ingestion, inhalation, and dermal contact. The general population may possibly be exposed to unknown quantities of DEN, has been found to distribute in tobacco smoke, beverages, herbicides, pesticides and ground water with high level of nitrates. In this context an estimate indicated that air, diet and smoking contribute to potential human exposure at levels of a few μg per day. In addition DEN is present in a variety of foods, including cheeses at concentrations of 0.5–30 μg/kg, soybeans at 0.2 μg/kg, various fish at <1–147 μg/kg, salt-dried fish at 1.2–21 mg/kg, cured meats at up to 40 μg/kg, and alcoholic beverages at 0.1 μg/kg (IARC, 1978). DEN acts as a potent hepato-carcinogen in rats by influencing the initiation stage of carcinogenesis, and during the period of enhanced cell proliferation induces hepatocellular necrosis and forms

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DNA-carcinogen adducts, which results in DNA-strand breaks and in turn leads to hepatocellular carcinomas without cirrhosis through the development of putative preneoplastic focal lesions (Dragan et al., 1991; Tatematsu, Nagamine, & Farber, 1988). It has been suggested that, on metabolic activation, DEN produces the pro-mutagenic products, 06-ethyl deoxy guanosine and 04 and 06-ethyl deoxy thymidine in liver which are responsible for its carcinogenic effects (Nakae et al., 1997; Verna, Whysner, & Williams, 1996). DEN is metabolized to reactive electrophilic reactants, alter the structure of DNA, and form alkyl DNA adducts, inducing chromosomal aberrations and micronuclei in the rat liver (Yoshiji et al., 1991). It is reported that the free radicals participate in DEN-induced hepatocarcinogenesis, which was confirmed by over expression of 8-hydroxyguanine in DEN administered rat liver. In this context, it is suggested that the generation of reactive oxygen species (ROS) by DEN causes carcinogenic effects. ROS are potentially dangerous by-products of cellular metabolism that can produce a direct effect on cell development, growth, and survival. In addition oxidative stress generated by ROS has been reported in membrane lipid peroxidation, DNA damage and mutagenesis associated with various stages of tumour formation process (Cleveland & Kastan, 2000; Parola & Robino, 2001; Sivalokanathan, Ilayaraja, & Balasubramanian, 2005). However, no reports exist concerning the influence of brown seaweed polysaccharide on the mitochondrial and microsomal energy metabolism. The objective of this study is to examine whether fucoidan of *S. plagiophyllum* could be effective against destabilization of mitochondrial and microsomal enzymes of DEN induced Wister albino rat liver.

2. Materials and methods

2.1. Extraction and purification of fucoidan

The blade of *S. plagiophyllum* (fresh weight) was collected along the coast of Mandapam (Lat. 09° 17′N; Long. 79° 08′E), Palk Bay, Tamil Nadu, India in November 2009. After thoroughly washing with the seawater and manual sorting to remove the epiphytes, the fresh biomass was exhaustively washed first with tap water and then with distilled water. The seaweeds were then shade dried and ground to pieces of about 1 mm. One hundred grams of blade algal powder (dry biomass) was soaked in acetone–methanol solvent system (7:3, v:v) for 2 days at shaker at 200 rpm (REMI, Mumbai). The process was repeated twice to ensure the complete decolouration and defatting of dry biomass. This solvent extracted biomass was dried into powder and was dispersed in 1 L of 0.1 M of HCl for 24 h at constant stirring in room temperature. The pellet was re-extracted as above and the supernatants were pooled. The resultant supernatant was kept at 4 °C overnight and precipitated with two vol. of absolute ethanol 1:1 (v:v). The precipitate was collected and again dissolved in water and dialysed against water using the membrane (MW CO 14,000) at 4 °C for 2 days and the dialysate was freeze dried. The crude polysaccharides dissolved in 0.1 M sodium phosphate buffer (pH 7.2) were applied to a column of Q-Sepharose fast flow (4 × 25 cm), followed by stepwise elution with 0.1 M sodium phosphate buffer, 0.2, 0.7, and 1.5 M sodium chloride solutions at a flow rate of 60 mL/h. The eluant (5 mL/tube) was collected and the carbohydrate content was determined by the phenol–sulphuric acid method, using fucose as standard. Finally, three fractions of polysaccharides were obtained, dialysed with water, and lyophilized for further study. Total sugar was determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The sulphate content was measured according to Dodgson and Price (1962). Total uronic acid content was analysed colorimetrically by the m-phenyl phenol method using gluco-uronic acid as standard (Filisetti-Cozzi and Carpita, 1991).

2.2. Monosaccharide identification

To determine the neutral sugar composition of the purified fucoidan, the samples (5 mg) were added with tri-fluoroacetic acid (100% TFA, 4 mL) in round-bottom flask. The mixtures were left overnight at ambient temperature and subsequently refluxed for 2 h. The solutions were then diluted to 80% TFA using deionized water. After refluxed for 30 min, the solutions were diluted to 30% TFA using deionized water and refluxed for 4 h. The TFA was removed using a rotary vacuum evaporator and deionized water was then added to the solids to wash them and this was followed by re-evaporation. This procedure was repeated several times until the hydrolysates obtained were neutral. The dry hydrolysate solids were finally dissolved in deionized water (5 mL) and used in the preparation of the HPLC analysis. Hydrolysed fraction was analysed by Agilante 1100 High Performance Liquid Chromatography (HPLC) (Agilante Technologies, Santa Clara, CA, USA) on a C18 column (ZORBAX Eclipse XDB-C18, 4.6 × 150 mm 3.5 μ).

2.3. Agarose gel electrophoresis

Purity of purified fucoidan was checked with Agarose gel electrophoresis according to the method of Björnsson, 1993. The SPs (1 mg/mL dry weight) were electrophoresed in 1.0% by agarose gels using the sample gel procedure and 0.01 M Tris/acetate (pH 8.3) as running buffer. The gel was run at 90 V for 90 min. Staining was done with 0.02% (w/v) toluidine blue O in 3% acetic acid containing 0.5% (v/v) Triton X-100 and the gels were de-stained with 3% acetic acid.

2.4. FT-IR spectral analysis

The Fourier Transmission-Infrared spectrum of 1 mg of purified fucoidan was mixed with KBr and recorded using a spectral range of 450–4500 cm^{-1} (Perkin Elmer MPF 44B, Waltham, MA, USA).

2.5. Molecular weight analysis

Molecular mass of fucoidan was analysed by gel permeation chromatography (GPC). The purified fucoidan (10 mg) was chromatographed on a Sepharose 6B column (Sigma–Aldrich, USA) (90 cm × 1.0 cm) using 100 mM sodium phosphate buffer (pH 7.2) as eluant. The flow rate of the column was 0.6 mL/min, and fractions of 2 mL were collected and checked by the phenol–sulphuric acid reaction (Dubois et al., 1956). The column was calibrated with standard dextrans (500, 70, 40 and 10 kDa).

2.6. Animals and experimental design

Male Wister strain of albino rats weighing ca. 185–220 g was purchased from Small Animal Section, King Institute of Preventive Medicine and Research, Chennai and it was chosen for the in vivo studies. The experiments were designed and conducted according to the ethical norms approved by the Institutional Animal Ethics Committee guidelines, India (IAEC No. 28/09/2011). In the present attempt, effective dosage fixation experiment was conducted with fucoidan on the animals, which revealed that 75 mg/kg body weight significantly ($p < 0.05$) altered the activities of marker enzymes such as acid phosphatase, alkaline phosphatase, aspartate transaminase, alanine transaminase and lactate de-hydrogenase (LDH) in liver tissues. Hence, the dose of 75 mg/kg was chosen for the further study. The experimental animals were divided into four groups, each group comprising of six animals. Group I: normal control rats

Table 1
Yields, compositions and molecular weight of fucoidan obtained by ion exchange chromatography from crude fucoidan.

	Yield (% in mass)	Total sugar (%m/m)	Total sulphate (%m/m)	Total uronic acid (%m/m)	Composition of sugar (mol%)				MW (kDa)
					Fucose	Galactose	Xylose	Mannose	
Purified fucoidan	23.7	63.3	21.9	12.6	70.8	13.5	2.5	11.2	35

(fed with standard diet) for 16 weeks, group II: rats induced with hepatocellular carcinoma by providing 0.01% DEN through drinking water for 16 weeks, group III: rats administrated with 0.01% DEN for a period of 10 weeks followed by treated with fucoidan 75 mg/kg body weight for 5 weeks and group IV: rats treated with fucoidan alone 75 mg/kg body weight for 16 weeks. After the experimental period the rats were anaesthetized, and sacrificed by cervical decapitation and different parameters were recorded.

2.7. Assay of mitochondrial enzymes

1 g of tissue was cut into small pieces and homogenized in 9 mL of 0.25 M sucrose, 10 mM Tris–HCl buffer containing 0.1 mM dithiothreitol. The homogenate was centrifuged at 12,000 rpm, for 30 min, at 4 °C. The pellet formed the mitochondrial fraction and it was washed with the above buffer and used for the estimation of specific activities of enzymes involved in tri-carboxylic acid cycle. Total protein was estimated by the method of Lowry, Rosebrough, Farr, and Randall (1951). The iso-citrate dehydrogenase (ICDH) activity was assayed according to the method of King (1965). The succinate dehydrogenase (SDH) activity was assayed according to the method of Slater and Borner (1952). The malate dehydrogenase (MDH) activity was assayed by the method of Mehler, Kornberg, Grisolia, and Ochoa (1948). The activity of α-ketoglutarate dehydrogenase was assayed by the method of Reed, Jurgensmeier, and Matsuyama (1998).

2.8. Assay of biotransformation enzymes

The liver microsomes were separated according to the method of Boyd and Burka (1978) with slight modification by Kamath and Narayan (1972). 2.5 g of tissue was homogenized with 10 mL of distilled water in Potter-Elvehjem homogenizer fitted with Teflon pestle at 4 °C at uniform speed with 5 return strokes to give a concentration of 25% homogenate. Then the tubes were covered with parafilm and mixed gently by inversion. Then the homogenate was centrifuged for post mitochondrial supernatant at 10,000 rpm for 30 min at 2–4 °C. The floating lipid layer on the top of the supernatant was discarded. Calcium chloride was added to the post mitochondrial supernatant and centrifuged at 15,000 rpm for 10–15 min. The pellet was resuspended in buffer, homogenized and made up to a known volume. The microsomal suspension was used for the analysis of biotransformation enzymes.

2.9. Assay of Phase-I hepatic drug metabolizing enzymes

Cytochrome P450 was estimated by the method of Omura and Sato (1964). The amount of cytochrome b_5 was measured by the method of Omura and Sato (1964). Cytochrome P450 was estimated by the method of Omura and Sato (1964). NADH-cytochrome b_5 reductase activity was assayed by the method of Strittmatter and Verlick (1956).

2.10. Assay of Phase-II biotransformation enzymes

Glutathione S-transferase was assayed by the method of Habig, Pabst, and Jakoby (1974). The UDP-glucuronyl transferase was estimated by the method of Isselbacher, Chrabas, and Quimi (1962) modified by Hollman and Touster (1962).

2.11. Histopathological studies

A portion of the liver was fixed in 10% formalin, processed by routine histology procedures, embedded in paraffin, cut in 5 μm pieces, and mounted on the slide. The samples were stained with haematoxylin and eosin for histopathological examination. Each visual field was magnified at 400×. The average value of at least four different sections from four different rats was counted.

2.12. Statistical analysis

The results were expressed using One-way Analysis of Variance ANOVAand Turkey's Multiple Comparison Test was done to evaluate the significance of difference of means of various treatment groups, using SPSS statistical package (version: 17). The values are presented as mean ± SD and p value less than 0.05 was taken as statistically significant.

3. Results

3.1. Purification and characterization of fucoidan

The resulting crude fucoidan was fractionated by stepwise elution from Q-Sepharose fast flow in aqueous sodium chloride of increasing concentration. The yield and composition of three fractions (F1, F2 and F3) obtained were analysed. Among the three fractions, it was observed that F2 fractions possess high sulphate content. The F2 fraction was considered as purified fucoidan and composition is shown in Table 1. The purity of purified fucoidan checked with agarose gel electrophoresis and single band indicates purity of purified fucoidan that was shown in Fig. 1a. Molecular weight of purified fucoidan analysed using size exclusion chromatography on Sepharose 6B column suggests that the polymer is homogeneous (Fig. 1b). Based on calibration with standard dextrans, the apparent molecular weight of purified fucoidan would be 35 kDa (Fig. 1c). The monosaccharides of purified fucoidan analysed by HPLC revealed the presence of the sugars as fucose, galactose, xylose, and mannose (Table 1). Among these of fucose as a major sugar was 70.8 mol%. The percentages of other sugars were galactose (13.5), xylose (2.5), and xylose (12.2). The FTIR spectrum of purified fucoidan contained intense absorption band at 1240–1270 cm^{-1} (S=O) common to all the sulphate esters and an additional sulphate absorption band recorded at 848 cm^{-1} (C—O—S, secondary axial sulphate) (Fig. 2). Subsequently, purified fucoidan was subjected to further study.

3.2. Mitochondrial TCA cycle enzymes

The experimental data in Fig. 3a–d show the levels of ICDH, SDH, MDH and α-KGDH in liver of control and experimental animals. A highly significant ($p < 0.05$) decrease in the levels of TCA cycle enzymes were observed in group II cancer bearing animals when compared to group I control animals. The fucoidan treatment normalized the levels of ICDH,

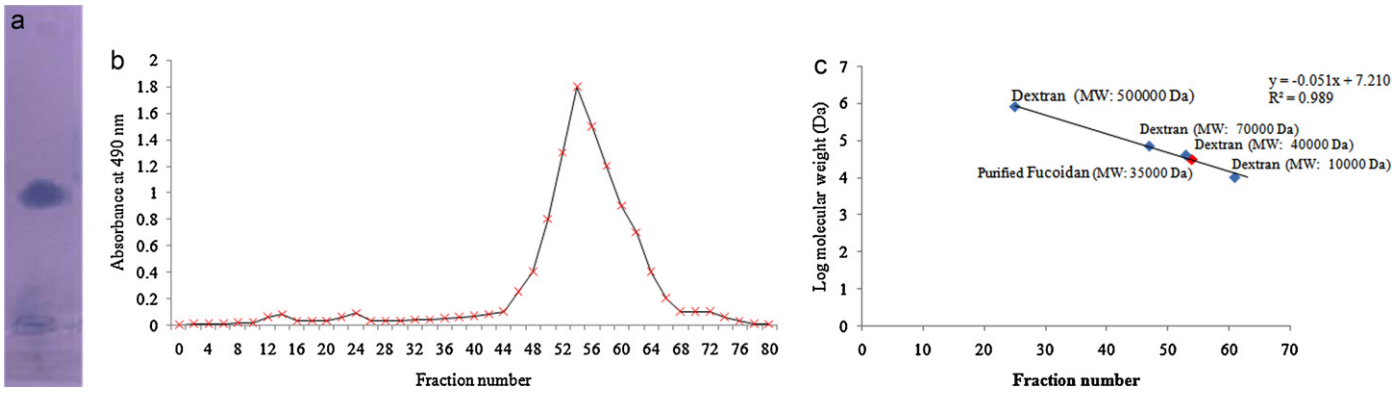

Fig. 1. (a) Purified fucoidan through agarose gel electrophoresis. (b) Gel filtration profile of different fractions on Sepharose 6B column chromatography. (c) Gel permeation chromatogram (GPC) of purified fucoidan calibration curve obtained from dextran standards with molecular weight determination using Sepharose 6B column.

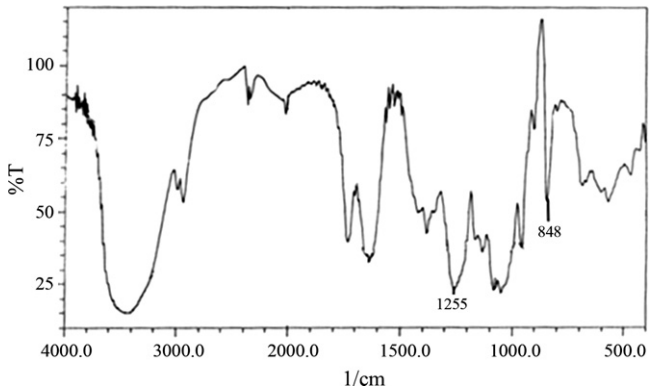

Fig. 2. FT-IR spectrum of purified fucoidan.

SDH, MDH and α-KGDH in group III fucoidan treated animals when compared to group II animals ($p < 0.05$). There was no noticeable change observed in group IV fucoidan alone treated animals.

3.3. Biotransformation enzymes

Data in Fig. 4a–d show the effects of fucoidan on Phase 1 and Phase-II biotransformation enzymes such as cytochrome P450, cytochrome b5, NADPH cytochrome P450 reductase, NADPH cytochrome b5 reductase, GST and UDP-GT of liver microsomes in control and experimental animals. In group II cancer induced animals, the levels of Phase-I biotransformation enzymes were decreased ($p < 0.05$) when compared to the group I control animals. On the other hand, Phase-II biotransformation enzymes were increased in group II cancer bearing animals ($p < 0.05$, Fig. 5). Modulation of these enzymes towards the normal range was observed in purified fucoidan treated group III animals ($p < 0.05$). However, there was no significant change observed in group IV animals when compared to group I control animals.

3.4. Histopathological studies

The morphological changes were presented in Fig. 6. The group I animal showed normal hepatocytes (Fig. 6a), group II

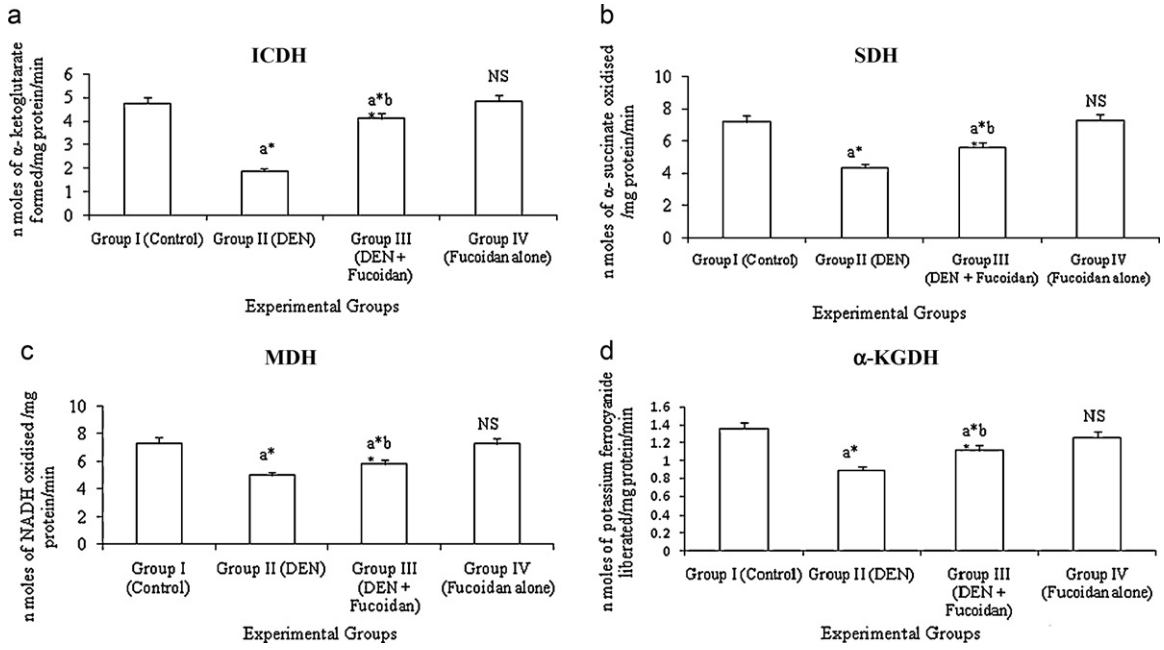

Fig. 3. The level of mitochondrial TCA cycle enzymes in liver of control and experimental animals. (a) The levels of ICDH in groups II, III and IV were compared with group I. (b) The levels of SDH in groups II, III and IV were compared with group I. (c) The levels of MDH in groups II, III and IV were compared with group I. (d) The levels of α-KGDH in groups II, III and IV were compared with group I. $^{*}p < 0.05$, ^{NS}not significant. Each value represents mean ± SD of six animals.

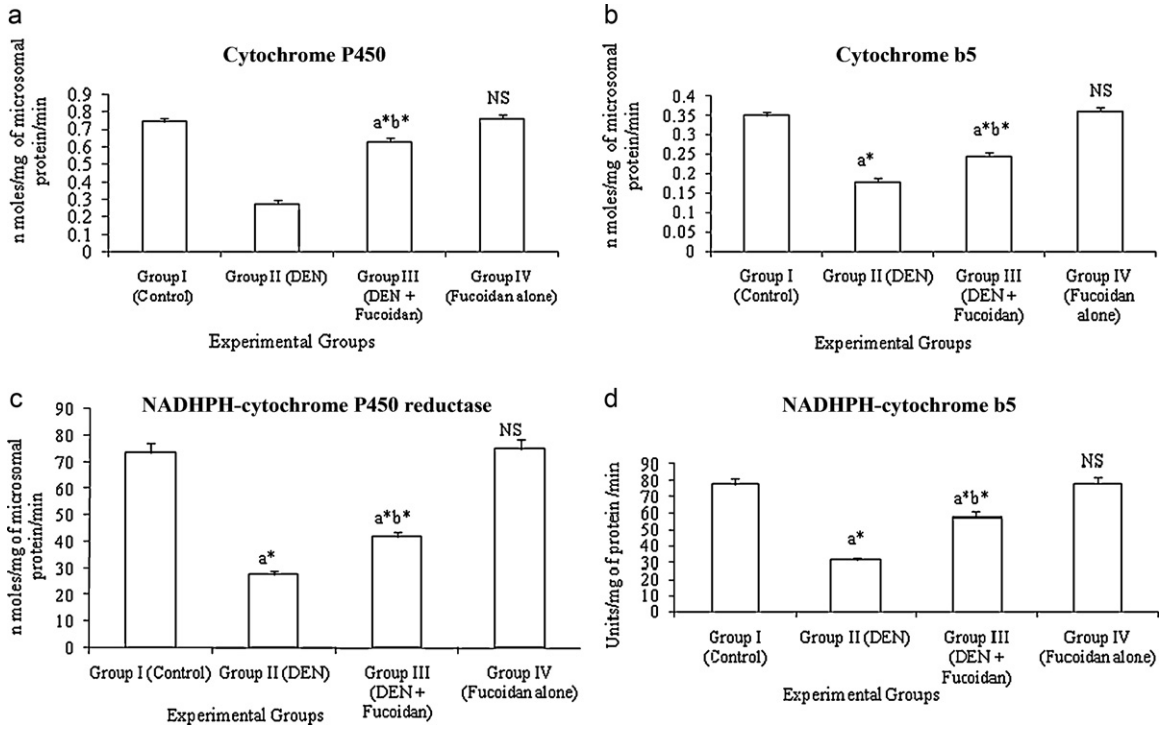

Fig. 4. The level of Phase-I drug metabolizing enzymes in liver of control and experimental animals. (a) The levels of Cytochrome P450 in groups II, III and IV were compared with group I. (b) The levels of Cytochrome b5 in group II, III and IV were compared with group I. (c) The levels of NADHPH-cytochrome P450 reductase in groups II, III and IV were compared with group I. (d) The levels of NADHPH-cytochrome b5 in groups II, III and IV were compared with group I. $^{*}p < 0.05$, ^{NS}not significant. Each value represents mean ± SD of six animals.

animal showed damaged hepatocytic tissue (Fig. 6b), relatively less damaged hepatocytes were found in the fucoidan treated alone group III (Fig. 6c), and group IV animal showed normal hepatocytes (Fig. 6d).

4. Discussion

In recent studies several reports had been indicated that sulphate groups of fucoidan play a major role in the suppression of cancer cell growth by binding with cationic proteins on the cell surface (Koyanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003; Qiu, Amarasekara, & Doctor, 2006; Soeda, Kozako, Iwata, & Shimeno, 2000). This implied that the molecular conformation of fucoidan of brown algae, which might influence the binding properties of sulphate groups, could be another factor affecting the inhibition potency of the cancer cell growth. This is because that if fucoidan was in the compact spherical conformation through the intramolecular interactions, the anionic sulphate groups available to bind proteins on the cell surface may be hidden inside the chains. Consequently, this would cause the reduction of sulphates available to bind the proteins. Hence, in this present study sulphated rich fraction was chosen.

In this present study, the purified fucoidan has low molecular weight and apparent molecular weight 35 kDa. You, Yang, Lee, and Lee (2010) reported that low molecular weight sulphated fucan may have greater molecular mobility and diffusivity than high molecular weight sulphated fucan, which appears to improve the interaction with cancer cell components, and thus induce the enhanced anticancer activity. Haroun-Bouhedja, Ellouali, Sinquin, and Boisson-Vidal (2000) also reported that low molecular weight fucoidans (18.6 kDa) from *Ascophyllum nodosum* at the concentration of 0.1 mg/mL showed more than 70% inhibition activity on CCL39 cell growth. Recent studies also indicated that the sulphated fucan polymers could modulate the cell growth in a different manner depending on their molecular weights. Koyanagi et al. (2003) reported that fucoidan polymers obtained from *Fucus vesiculosus*

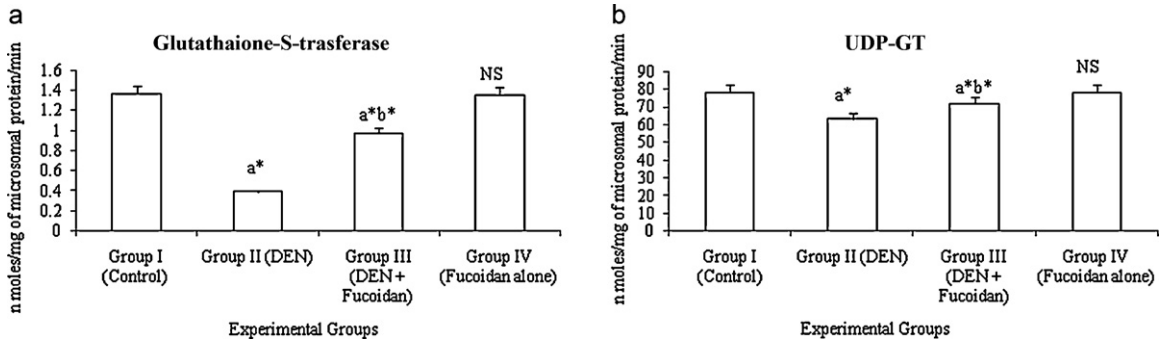

Fig. 5. The level of Phase-II drug metabolizing enzymes in liver of control and experimental animals. (a) The levels of Glutathaione-S-trasferase in groups II, III and IV were compared with group I. (b) The levels of UDP-GT in groups II, III and IV were compared with group II. $^{*}p < 0.05$, ^{NS}not significant. Each value represents mean ± SD of six animals.

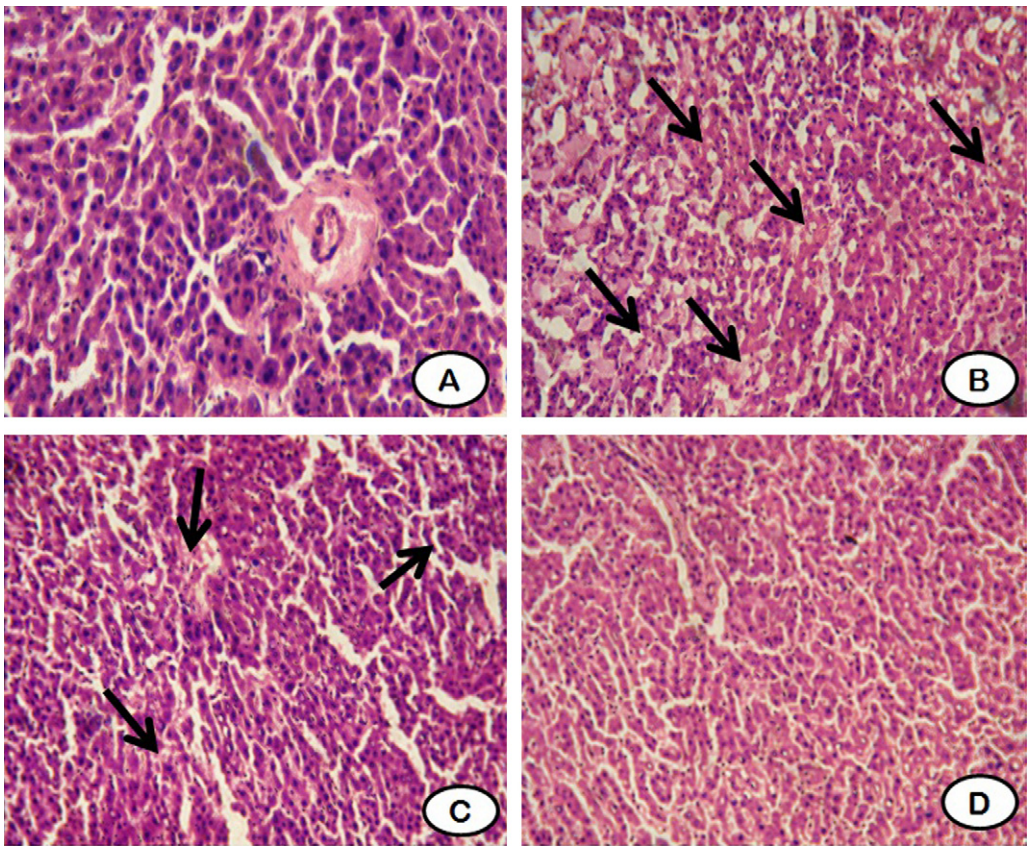

Fig. 6. Histopathological studies by light microscope showing the morphological changes of normal liver and fucoidan treated tissues from Wister albino male rat (a) Control group I. (b) DEN induced rate liver tissue group II. (c) Fucoidan treated on DEN induced rat liver tissue group III. (d) Fucoidan alone treated liver tissue group IV. Tissue sections were stained with haematoxylin and eosin and viewed by light microscopy (400×).

having the Mw of 100–130 kDa effectively suppressed the growth of Sarcoma 180, Lewis lung carcinoma and B16 melanoma cells by inhibiting the neovascularization, called angiogenesis, due to their anti-angiogenic potency. On the other hand, low molecular weight fucoidans (16 kDa and 4 kDa) obtained from *A. nodosum* enhanced the neovascularization of human umbilical vein endothelial cells (HUVEC) and endothelial progenitor cells (EPC) in the presence of fibroblast growth factor-2, which resulted in the increase of cell proliferation (Matou, Helley, Chabut, Bros, & Fischer, 2002; Zemani et al., 2005).

4.1. Mitochondrial TCA cycle enzymes

Mitochondria are important for cell viability due to the nature of their functions. They play a central role in the regulation of intracellular Golgi apparatus especially during toxic insult or stress (Carafoli, 1987) and mitochondrial oxidative phosphorylation provides approximately 95% of cellular energy needs (Erecinska & Wilson, 1982). Numerous toxic compounds that targeted mitochondria and mitochondrial injury have been investigated as a potential initiating factor in various organ toxicities caused by chemicals (Wong, Card, & Racz, 2000). Since mitochondrial oxidative phosphorylation plays a central role in the maintenance of cellular energy supply (Burcham & Harman, 1991), the deleterious effect of xenobiotics on mitochondrial respiration may have serious consequences for the cell viability. Donnelly, Walker, and Racz (1994) suggested that mitochondrial respiratory function acts as a possible initiator for an early inhibition of drug induced hepato cellular carcinoma (HCC). Since mitochondrial integrity is important for cell viability, the protective effect of the fucoidan was assessed against DEN induced HCC in Wister rats.

DEN administration resulted in the significant decrease in the levels of liver mitochondrial enzymes such as SDH, ICDH, α-KGDH and MDH. Among these enzymes succinate dehydrogenase (SDH) is an important enzyme of TCA cycle and it is also associated with the electron transport chain due to its ability to transfer electrons to respiratory chain (Singh, Agarwal, & Shankar, 1990). SDH is known to contain a number of cysteine rich sulphur clusters and it can be inhibited by a number of agents that modify sulphhydryl groups. The N-acetyl para benzo quinone imine (NAPQI) directly interacts with sulphhydryl groups on SDH, causing the loss of its activity (Burcham & Harman, 1991). The NAPQI also oxidizes other essential protein sulphhydryl groups in the mitochondrial respiratory chain, thereby limiting the ability of the mitochondria to meet the energy demands of the cell and disrupting cellular energy homeostasis (Streeter, Dahlin, Nelson, & Baillie, 1984).

The increased superoxide production results in oxidative damage to mitochondria, thus comprising their ability to meet cellular energy demands (Sohal & Sohal, 1991), which could have reduced the activities of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. The accumulation of pyruvate and α-ketoglutarate results in the lowering of activities of iso-citrate dehydrogenase and succinate dehydrogenase (Zhang, Redman, Churchill, & Churchill, 1990). Ironically treatment with fucoidan prevented the decrease in the levels of liver and kidney SDH and also the levels of ICDH, α-KGDH and MDH probably by promoting the elimination of NAPQI by mitochondrial GSH, thereby protecting the critical nucleophilic sites on the enzymes against the toxic electrophilic metabolite of NAPQI (Burcham & Harman, 1991). This may be due to the antioxidant nature of the fucoidan, which nullified the mitochondrial damage due to DEN administration.

4.2. Biotransformation enzymes

4.2.1. Phase-I hepatic drug metabolizing enzymes

In the present study, there was an increase in the Phase-I enzymes such as cytochrome P450, cytochrome b5, NADPH cytochrome c (P450) reductase and NADPH cytochrome b5 enzymes in DEN induced HCC in Wister rats. This may be due to induction of Phase-I enzymes by DEN (Nagata et al., 1985). It is widely accepted that the metabolic activation of nitrosamines by Phase-I enzymes to reactive electrophiles is required for their cytotoxic, mutagenic and carcinogenic activities. The expression of the Phase-I enzymes is preferentially localized to the most common sites where tumour arises (Godoy et al., 2002).

It has been hypothesized that DEN undergoes reductive oxidation at cytochrome P450 of liver microsomes to produce reactive alkylating agents, which in turn produce alkylated DNA adducts. During biotransformation of DEN the microsomal mixed function oxidase, superoxide ions are released within the system. Aruoma, Halliwell, Laughton, Quinlan, and Gutteridge (1989) has suggested that the Phase-I enzymes are involved in lipid peroxidation (LPO) and at least partially resemble the drug-hydroxylating system. In addition approximate parallelism was observed between the formation of malondialdehyde and the loss of microsomal enzymes and cytochrome P450 (Reiner, Athanassopoulos, Hellmer, Murray, & Uehleke, 1972). In the present study, the observed decrease in Phase-I enzymes of cancer bearing animals may either be due to result of damage to membrane lipids by free radicals or to complex formation between alkylating agents and DNA (Sarkar, Bishayee, & Chattejee, 1995).

Cytochromes P450 catalyse the oxidation reactions and also are inducible, particularly by xenobiotics that bind to specific intracellular receptors and consequently modulate the expression. These enzymes are expressed in many tissues, but the highest levels are found in liver (Vrzal, Ulrichova, & Dvorak, 2004). In the present study, decreased level of this enzyme was observed in cancer bearing animals, which may be due to increased utilization of this enzyme to excrete the carcinogen. Cytochrome b5 is involved in the cytochrome P450 mediated biotransformation through electron donation by NADH via cytochrome b5 reductase (Timbrell, 1991). In the present investigation, a decreased level of cytochrome b5 was observed in DEN-induced HCC Wister rats. This inhibition may be due to the alterations in metabolism of DEN. In DEN induced biotransformation the NADPH are transferred through cytochrome P450 reductase to cytochrome P450 via cytochrome b5. DEN may attack and inactivate the reductase and thus, electron transport from NADPH to cytochrome P450 is interrupted. The rate of drug metabolism is more closely linked to NADPH-cytochrome P450 reductase than to the amount of cytochrome P450 present (Testa & Jenner, 1976). The inhibition of this enzyme resulted from binding of the toxin, either to the reductase or to cytochrome P450 (Koster & Slee, 1980). Feuer (1988) has reported a 40% reduction in the enzyme activity in HCC. NADH is unable to replace NADPH efficiently in many microsomal mixed function oxidase reactions but in the presence of NADPH, a synergistic effect of NADH on metabolic process is apparent (Testa & Jenner, 1976). The decreased activity of NADPH-cytochrome P450 reductase in cancer bearing animals may lead to the inhibition of NADH-cytochrome b5 reductase.

In fucoidan treated group III animals, the Phase-I enzymes levels were reverted back to near normal, when compared to group II animals, and this may be due to the anticancer activity of the fucoidan.

4.2.2. Phase-II hepatic drug metabolizing enzymes

In the present investigation, there was a decrease in the Phase-II enzymes such as GST and UDP-GT in DEN induced HCC in Wister rats. Reduction in carcinogenic effect is mainly carried out by GST, which catalyses the nucleophilic substitution of the nitro group of DEN with thiols. Greater thiol loss under toxic and hypoxic conditions suggested oxidation by superoxide, peroxide or hydroxyl radical formed in the course of DEN reductions (Varnes & Biaglow, 1979). Oxidative stress produced by DEN reduction depletes thiol and inactivates GST and UDP-GT, which cause mutation and HCC.

Similarly, GST is a soluble protein located in the cytosol and plays an important role in detoxification and excretion of xenobiotics (Bansal, Bansal, Soni, & Bhatnagar, 2005; Sreepriya & Bali, 2005; Sivaramakrishnan & Devaraj, 2009). GST catalyses the conjugation of the thiol functional groups of GSH to electrophilic xenobiotics and results in increasing solubility. The xenobiotic-GSH conjugate is then either eliminated or converted to mercapturic acid (Rao, Rao, Pushpangadan, & Amiie, 2006). Induction of xenobiotic detoxifying enzymes is an additional mechanism by which antioxidant rich compound may act as anti-carcinogens as they compete with steps in xenobiotic activation and metabolize toxic compounds to non-toxic ones (Bergmeyer and Bernt, 1974). As the activity of GST increased in fucoidan treated group III rats, it appears that the drug induces greater coupling of electrophilic intermediates with GSH. It was found that liver activities increased in the fucoidan treated rats, suggesting that fucoidan administration contributes to the liver protection against the oxidative induced injury. In this regard, it has been demonstrated that different natural compounds and terpenes are reported to induce GST as one of the principal anti-carcinogenic mechanisms (Kuo, Chou, Young, Chang, & Wang, 2005). In addition some report pointed out that the induction of GST as a mechanism to protect against chemically induced cancer and oxidative stress by increasing the metabolism of electrophilic intermediates and ROS (Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005; Sehrawat & Sultana, 2006). Uridine 5′-diphospho-glucuronyl transferases (UDP-GT) catalyse the transfer of glucuronic acid from UDP-glucoronic acid to phenols, hydroxylamines, carboxylic acid, etc. UDP-GT is a family of integral proteins of the endoplasmic reticulum membranes and the nuclear envelope which are present in many tissues of vertebrates. In the present investigation, increased level of UDP-GT was observed in cancer bearing animals. It was suggested that the synthesis of glucuronides by microsomal UDP-glucuronyl transferase is the major pathway for the inactivation and subsequent excretion of both endogenous and xenobiotic organic compounds (George, 1994; Lech & Vodicinik, 1985; Mulder, Coughtrie, & Burchell, 1990).

In this study, a salient increase in the activities of UDP-GT and GST is observed. Hepatic microsomal UDP-GT and GST are known to be important pre-neoplastic and neoplastic markers to evaluate the extent of free radical damage caused by exposure to various carcinogens. In addition to marginal changes in the activities of all the bio-transforming enzymes tested in the fucoidan treated group III rats, there is a significant reduction in the activities of GST and UDP-GT compared to carcinogen induced group II rats in liver tissue. The probable mechanism of this is that fucoidan arrests the formation of free radicals and oxidative threat to the animals generated by exposure to DEN. UDP-GT is constrained to phospholipids of the microsomal membrane (Erickson & Zakim, 1978) and hence the observed decrease in fucoidan treated animals may be due to peroxidation damage to the microsomal lipids in cancer conditions. Hence, the present investigation suggested that purified fucoidan has been shown to prevent chemically induced cancer presumably by the up-regulation of Phase-I and down regulation of Phase-II metabolizing enzymes.

The histopathological studies of the liver tissues supported the biochemical alterations well and thus inevitably confirm the anti-neoplastic activity of fucoidan against experimental liver cancer. Further studies are in progress to elucidate the detailed mechanism of action of fucoidan against DEN induced hepatocellular carcinoma.

5. Conclusion

In the present study results revealed that, the levels of ICDH, SDH, MDH and α-KGDH were decreased in cancer bearing animals which may be due to oxidative stress and mitochondrial damage and fucoidan restored these enzyme activities. Hepatic microsomal drug metabolizing enzymes play a vital role in DEN induced carcinogenesis, because of their involvement in activation and detoxification of DEN. The Phase-I biotransformation enzymes namely cytochrome P450, cytochrome b5 NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase and Phase-II enzymes GST, and UDP-GT were significantly decreased in HCC bearing animals. On the other hand the Phase-II enzymes GST, and UDP-GT were increased in cancer bearing animals, and fucoidan acts as a functional inducer and hence activities of all these enzymes were reverted back to near normal in fucoidan treated animals. The inhibition of carcinogen metabolic activation indicates the anticancer activity of fucoidan in DEN induced liver cancer Wister rats.

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References

Aruoma, O. I., Halliwell, B., Laughton, M. J., Quinlan, G. J., & Gutteridge, J. M. (1989). The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron(II)–iron(III) complex. *Biochemical Journal*, *258*(2), 617–620.

Bansal, A. K., Bansal, M., Soni, G., & Bhatnagar, D. (2005). Protective role of vitamin E pretreatment on N-nitrosodiethylamine induced oxidative stress in rat liver. *Chemico-Biological Interactions*, *156*, 101–111.

Bergmeyer, H. U., & Bernt, E. (1974). Amino-transferases and related enzymes. In H. U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis 2* (2nd ed., pp. 735–763). New York: Academic Press.

Boyd, M. R., & Burka, L. T. (1978). In vivo studies on the relationship between target organ alkylation and the pulmonary toxicity of a chemically reactive metabolite of 4-ipomeanol. *Journal of Pharmacological Experimental Therapy*, *207*(3), 687–697.

Burcham, P. C., & Harman, A. W. (1991). Acetaminophen toxicity results in site specific mitochondrial damage in isolated hepatocytes. *Journal Biological Chemistry*, *226*, 5049–5054.

Carafoli, E. (1987). Intracellular calcium homeostasis. *Annual Review of Biochemistry*, *56*, 395–433.

Cleveland, J. L., & Kastan, M. B. (2000). A Radical approach to treatment. *Nature*, *407*, 309–311.

Dodgson, K. S., & Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, *84*, 106–110.

Donnelly, P. J., Walker, R. M., & Racz, W. J. (1994). Inhibition of mitochondrial respiration *in vivo* is an early event in acetaminophen-induced hepatotoxicity. *Archives of Toxicology*, *68*(2), 110–118.

Dragan, U. P., Rizvi, T., Xu, Y. H., Hully, J. R., Bawa, N., Campbell, H. A., et al. (1991). An initiation-promotion assay in rat liver as a potential complement to the 2-year carcinogenesis bioassay. *Fundamentals of Applied Toxicology*, *16*, 525–547.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Erecinska, M., & Wilson, B. F. (1982). Regulation of cellular energy metabolism. *Membrane Biology*, *70*, 1–14.

Erickson, R. H., & Zakim, D. A. (1978). Preparation and properties of a phospholipid-free form of microsomal UDP glucuronyl transferase. *Biochemistry*, *17*, 3706–3711.

Feuer, G. (1988). Hepatic metabolism and carcinogenesis. Its role in hepatoma and adenocarcinoma. *Annals of the New York Academy of Sciences*, *534*, 541–551.

Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*, 157–162.

George, S. G. (1994). Enzymology and molecular biology of Phase-II xenobiotic-conjugating enzymes in the fish. In D. C. Malins, & G. K. Ostrander (Eds.), *Aquatic toxicology: Molecular, biochemical and cellular perspectives* (pp. 37–85). Boca Raton, USA: Lewis Publishers/CRC Press.

Godoy, R. M., Albano, E. G., Moraes, P. R., Pinho, R. A., Nunes, E. H., Saito, C., et al. (2002). CYP2A6/2A7 and CYP2E1 expression in human oesophageal mucosa: Regional and interindividual variation in expression and relevance to nitrosamine metabolism. *Carcinogenesis*, *23*(4), 611–616.

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione-S-transferase. The first enzymatic step in mercapturic acid formation. *Journal Biological Chemistry*, *249*, 7130–7139.

Haroun-Bouhedja, F., Ellouali, M., Sinquin, C., & Boisson-Vidal, C. (2000). Relationship between sulphate groups and biological activities of fucans. *Thrombosis Research*, *100*(5), 453–459.

Hollman, S., & Touster, O. (1962). Alterations in tissue levels of UDP-glucose dehydrogenase, UDP-glucuronic acid pyrophosphatase and glucuronyl transferase induced by substances influencing the production of ascorbic acid. *Biochimica et Biophysica Acta*, *62*, 338–352.

IARC. (1978). *Some A-nitroso compounds. IARC monographs on the evaluation of carcinogenic risk of chemicals to humans* Lyon, France: International Agency for Research on Cancer., pp. 365.

Isselbacher, K. J., Chrabas, M. F., & Quimi, R. C. (1962). The solubilization and partial purification of a glucuronyl transferase from rabbit liver microsomes. *Journal of Biological Chemistry*, *237*, 3033–3036.

Kamath, S. A., & Narayan, K. A. (1972). Interaction of Ca^{2+} with endoplasmic reticulum of rat liver: A standardized procedure for the isolation of rat liver microsomes. *Analytical Biochemistry*, *48*(1), 53–61.

Kim, T. H., & Bae, J. S. (2010). Ecklonia cava extracts inhibit lipopsaccharide induced inflammatory responses in human endothelial cells. *Food and Chemical Toxicology*, *48*, 1682–1687.

King, J. (1965). Practical clinical enzymology. In D. Van (Ed.), *The dehydrogenases or oxidoreductase lactate dehydrogenase* (pp. 83–93). London: Nostrand Company Limited.

Kong, C. S., Kim, J. A., Yoon, N. Y., & Kim, S. K. (2009). Induction of apoptosis by phloroglucinol derivative from *Ecklonia cava* in MCF-7 human breast cancer cells. *Food and Chemical Toxicology*, *47*, 1653–1658.

Koster, J. F., & Slee, R. G. (1980). Lipid peroxidation of rat liver microsomes. *Biochimica et Biophysica Acta*, *620*(3), 489–499.

Koyanagi, S., Tanigawa, N., Nakagawa, H., Soeda, S., & Shimeno, H. (2003). Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. *Biochemical Pharmacology*, *65*, 173–179.

Kuo, W. H., Chou, F. P., Young, S. C., Chang, Y. C., & Wang, C. J. (2005). Geniposide activates GSH S-transferase by the induction of GST M1 and GST M2 subunits involving the transcription and phosphorylation of MEK-1 signaling in rat hepatocytes. *Toxicology and Applied Pharmacology*, *208*(2), 155–162.

Lech, J. J., & Vodicinik, M. J. (1985). Biotransformation. In G. M. Rand, & S. R. Petrocelli (Eds.), *Fundamentals of aquatic toxicology: Methods and applications* (pp. 526–557). New York, USA: Hemisphere Publishing Corporation.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Masella, R., Di Benedetto, R., Vari, R., Filesi, C., & Giovannini, C. (2005). Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione-related enzymes. *Journal of Nutritional Biochemistry*, *16*, 577–586.

Matou, S., Helley, D., Chabut, D., Bros, A., & Fischer, A. M. (2002). Effect of fucoidan on fibroblast growth factor-2-induced angiogenesis *in vitro*. *Thrombosis Research*, *106*(4–5), 213–221.

Mehler, A. H., Kornberg, A., Grisolia, S., & Ochoa, S. (1948). The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate. *Journal Biological Chemistry*, *174*, 961–977.

Mulder, G. J., Coughtrie, M. M. H., & Burchell, B. (1990). Glucuronidation. In G. J. Mulder (Ed.), *Conjugation reactions in drug metabolism* (pp. 51–105). London: Taylor and Francis.

Nagata, K., Buppodom, P., Matsunaga, T., Ishimatsu, M., Yamato, H., Yoshihara, S., et al. (1985). Purification and characterization of seven distinct forms of liver microsomal cytochrome P-450 from untreated and inducer-treated male Wistar rats. *Journal of Biochemistry*, *97*(6), 1755–1766.

Nakae, D., Kobayashi, Y., Akai, H., Andoh, N., Satoh, H., & Ohashi, K. (1997). Involvement of 8-hydroxyguanine formation in the initiation of rat liver carcinogenesis by low dose levels of N-nitrosodiethylamine. *Cancer Research*, *57*, 1281–1287.

Omura, T., & Sato, R. (1964). The carbon monoxide binding pigment of liver microsomes. *Journal of Biological Chemistry*, *239*, 2370–2378.

Ordonez, E. G., Escrig, A. J., & Ruperez, P. (2010). Dietary fibre and physicochemical properties of several edible seaweeds from northwestern Spanish coast. *Food Research International*, *43*, 2289–2294.

Parola, M., & Robino, G. (2001). Oxidative stress-related molecules and liver fibrosis. *Journal of Hepatology*, *35*, 297–306.

Poirier, M. C., & Beland, F. A. (1994). DNA adduct measurements and tumor incidence during chronic carcinogen exposure in rodents. *Environmental Health Perspectives*, *102*, 161–165.

Qiu, X. D., Amarasekara, A., & Doctor, V. (2006). Effect of oversulfation on the chemical and biological properties of fucoidan. *Carbohydrate Polymers*, *63*, 224–228.

Rao, G. M. M., Rao, C. V., Pushpangadan, P., & Amiie, S. (2006). Hepatoprotective effects of rubiadin, a major constituent of *Rubia cordifolia* Linn. *Journal of Ethnopharmacology*, *103*, 484–490.

Reed, J. C., Jurgensmeier, J. M., & Matsuyama, S. (1998). Bcl-2 family proteins and mitochondria. *Biochimica et Biophysica Acta*, *1366*(1–2), 127–137.

Reiner, O., Athanassopoulos, S., Hellmer, K. H., Murray, R. E., & Uehleke, H. (1972). Formation of chloroform from carbon tetrachloride in liver microsomes, lipid peroxidation and destruction of cytochrome P-450. *Archives of Toxicology*, *29*(3), 219–233.

Sarkar, A., Bishayee, A., & Chattejee, M. (1995). Beta-carotene prevents lipid peroxidation and red blood cell membrane protein damage in experimental hepatocarcinogenesis. *Cancer Biochemistry and Biophysics*, *15*, 111–125.

Schuller, H. M. (1992). Nitrosamine-induced lung carcinogenesis and Ca^{2+}/calmodulin antagonists. *Cancer Research*, *52*, 2723–2726.
Sehrawat, A., & Sultana, S. (2006). Tamarix gallica ameliorates thioacetamide-induced hepatic oxidative stress and hyperproliferative response in Wistar rats. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *21*(2), 215–223.
Shibata, T., Ishimaru, K., Kawaguchi, S., Yoshikawa, H., & Hama, Y. (2008). Antioxidant activities of phlorotannins isolated from *Japanese Laminariacea*. *Journal of Applied Phycology*, *20*, 705–711.
Singh, O. K., Agarwal, K. N., & Shankar, R. (1990). Effect of under nutrition on succinate dehydrogenase and acetyl choline esterase in developing rat brain. *Indian Journal Experimental Biology*, *28*, 868–870.
Sivalokanathan, S., Ilayaraja, M., & Balasubramanian, M. P. (2005). Efficacy of *Terminalia arjuna* (Roxb.) on N-nitrosodiethylamine induced hepatocellular carcinoma in rats. *Indian Journal Experimental Biology*, *43*(3), 264–267.
Sivaramakrishnan, V., & Devaraj, S. N. (2009). Morin regulates the expression of NF-KB-p65, COX-2 and matrix metalloproteinases in diethylnitrosamine induced rat hepatocellular carcinoma. *Chemico-Biological Interactions*, *180*(3), 353–359.
Slater, E. C., & Borner, W. D. (1952). The effect of flurise on the Succinic oxidase system. *Biochemical Journal*, *52*(2), 185–196.
Soeda, S., Kozako, T., Iwata, K., & Shimeno, H. (2000). Oversulphated fucoidan inhibits the basic fibroblast growth factor-induced tube formation by human umbilical vein endothelial cells: Its possible mechanism of action. *Biochimica et Biophysica Acta: Molecular Cell Research*, *1497*(1), 127–134.
Sohal, R. S., & Sohal, B. H. (1991). Hydrogen peroxide release by mitochondria increases during aging. *Mechanisms of Aging and Development*, *57*, 187–202.
Sreepriya, M., & Bali, G. (2005). Chemopreventive effects of embelin and curcumin against N-nitrosodiethylamine/phenobarbital-induced hepatocarcinogenesis in Wistar rats. *Fitoterapia*, *76*, 549–555.
Streeter, A. J., Dahlin, D. S., Nelson, S. D., & Baillie, T. A. (1984). The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation *in vitro*. *Chemico-Biological Interactions*, *48*, 349–366.
Strittmatter, P., & Verlick, S. F. (1956). A microsomal cytochrome reductase specific for diphosphopyridine nucleotide. *Journal Biological Chemistry*, *221*, 277–286.
Tatematsu, M., Nagamine, Y., & Farber, E. (1988). Stable phenotypic expression of glutathione-S-transferase placental type and unstable phenotypic expression of y-glutamyl transpeptidase in rat liver preneoplastic and neoplastic lesions. *Carcinogenesis*, *9*(2), 215–220.
Testa, B., & Jenner, B. (1976). The concept of regioselectivity in drug metabolism. *Pharmacy and Pharmacology*, *28*(10), 731–744.
Timbrell. (1991). *Principles of biochemical toxicology* (2nd ed.). London: Taylor and Francis.
Varnes, M. E., & Biaglow, J. E. (1979). Interactions of the carcinogen 4-nitroquinoline 1-oxide with the non-protein thiols of mammalian cells. *Cancer Research*, *39*(8), 2960–2965.
Verna, L., Whysner, J., & Williams, G. M. (1996). N-nitrosodiethylamine mechanistic data and risk assessment: Bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. *Pharmacological Therapy*, *71*(l–2), 57–81.
Vrzal, R., Ulrichova, J., & Dvorak, Z. (2004). Aromatic hydrocarbon receptor status in the metabolism of xenobiotics under normal and pathophysiological conditions. *Biomedical Papers of the Medical Faculty of the University Palacky, Olomouc, Czech Republic*, *148*(1), 3–10.
Wijesekara, I., Pangestuti, R., & Kim, S. K. (2011). Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. *Carbohydrate Polymers*, *84*, 14–21.
Wong, S. G. W., Card, J. W., & Racz, W. J. (2000). The role of mitochondrial injury in bromobenzene and furosemide induced hepatotoxicity. *Toxicological Letters*, *116*, 171–181.
Yoshiji, J. H., Nakae, D., Kinugasa, T., Matsuzaki, M., Denda, A., & Tsujii, T. (1991). Inhibitory effect of dietary iron deficiency on the induction of putative preneoplastic foci in rat liver initiated with DEN and promoted by phenobarbital. *British Journal of Cancer*, *64*, 839–842.
You, S. D., Yang, C., Lee, H. Y., & Lee, B. Y. (2010). Molecular characteristics of partially hydrolyzed fucoidans from sporophyll of *Undaria Pinnatifida* and their *in vitro* anticancer activity. *Food Chemistry*, *119*, 554–559.
Zemani, F., Benisvy, D., Galy-Fauroux, I., Lokajczyk, A., Colliec-Jouault, S., & Uzan, G. (2005). Low-molecular-weight fucoidan enhances the proangiogenic phenotype of endothelial progenitor cells. *Biochemical Pharmacology*, *70*, 1167–1175.
Zhang, W. W., Redman, K., Churchill, S., & Churchill, P. (1990). Comparison of D-beta-hydroxybutyrate dehydrogenase from rat liver and brain mitochondria. *Cell Biology*, *68*, 1225–1230.