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Ameliorative effect of *Spatoglossum asperum* and its solvent fractions against acetaminophen-induced liver dysfunction in rats

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Abstract: Acetaminophen (APAP) is a widely consumed drug for pain management and treatment of pyrexia. However, beyond its recommended dose, it becomes harmful for health and may induce acute liver dysfunction. Current work is designed to ameliorate the APAP induced liver toxicity which was induced in rats by giving intra-peritoneal injection of APAP (800mg/kg) dissolved in 40% polyethylene glycol at day 1 and day 14. APAP dosed/intoxicated rats orally administered either with ethanol extract of *Spatoglossum asperum* (200mg/kg) and its fractions including *n-hexane, chloroform* and methanol soluble in a dose of 150mg/kg each daily for 14 days in their respective groups. APAP dosed rats showed remarkable elevation in hepatic biomarkers *viz.,* alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenases, total bilirubin, pro-inflammatory cytokines interleukine-6 and apoptotic protein (caspase-3). In addition, hepatic oxidative stress (lipid per oxidation and indirect nitric oxide) and antioxidant biomarkers (glutathione peroxidase, catalase and reduced glutathione) were also altered. Whereas APAP dosed rats treated with ethanol extract of *S. asperum* and its fractions showed amelioration in concentration of hepatic enzymes, pro-inflammatory cytokines, apoptotic protein and reduction in hepatic oxidative stress by decreasing the lipid peroxidation, indirect nitric oxide and uplifting the activities of antioxidant enzymes and protein.

Keyword: *Spatoglossum asperum,* fractions, acetaminophen, hepatoprotective, cytokines.

INTRODUCTION

Acetaminophen (APAP) is one of the non-steroidal anti-inflammatory drugs (NSAIDS) which is widely used for the treatment of pain, fever and various inflammatory diseases (Meunier and Larrey, 2018). APAP is safer at recommended dosage but if it is overdosed for reducing pyrexia and pain, it becomes problematic for liver function (Caparrotta *et al.,* 2018). It has been reported that APAP generates a toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) by activating cytochrome P450 enzyme system in liver, which induces depletion of reduced glutathione (GSH) and accelerates hepatic necrosis (Xie *et al.,* 2014). Furthermore, NAPQI binds with mitochondrial proteins and causes mitochondrial dysfunction. Mitochondrial impairment leads to the generation of oxidative stress that decreases the activity of antioxidant enzymes glutathione peroxidase and catalase. The oxidative stress also increases the lipid peroxidation and generation of reactive nitrogen species (RNS) which ultimately facilitates the action of pro-inflammatory cytokines and apoptotic proteins (McGill *et al.,* 2012; Win *et al.,* 2018).

Marine macro algae are gaining attention as they possess many bioactive compounds which have promising pharmacological and physiological activities like anticancer, antiviral, antifungal, antibacterial, anticoagulant, anti-inflammatory and antioxidant activities (Dore *et al.,* 2013, Alves *et al.,* 2016, Safavi *et al.*, 2019). Solvent extracts of many species of brown seaweeds including *Spatoglossum* spp., have been investigated against diabetes mellitus (Zhao *et al.,* 2018; Akhtar *et al.,* 2019), neuroprotection (Nho *et al.,* 2020), cardiovascular diseases (Ara *et al.,* 2005), renal dysfunction, acute and chronic hepatotoxicity (Chen *et al.,* 2017; Quintal-Novelo *et al.,* 2018; Hira *et al.,* 2021). Various biological activities of *Spatoglosuum asperum* have been reported earlier (Ara *et al.,* 2005). However, its hepatoprotective activity against drug induced liver dysfunction has not been evaluated. This study was designed to examine the hepatoprotective effect of ethanolic extract of *S. asperum* and its solvent fractions (*n-hexane, chloroform* and methanol) against acetaminophen induced acute hepatotoxicity.

MATERIALS AND METHODS

Seaweed collection

Seaweed (*Spatoglossum asperum*) was collected at low tide from the coastal area of Karachi (Buleji Beach) during the month of November-January. The algal material was identified by an authentic phycologist. Sample was washed in running tap water to remove soil particles and microorganism then air dried under shade. Dried sample was grinded and stored for further use.

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Preparation of ethanol extract and its fractions

Ethanol extract of *S. asperum* (EEtSA) was prepared by soaking its dried powder (500gm) in 1L of pure ethanol for a week then filtered. The filtrate was further reduced over a rotary vacuum evaporator (Büchi R-200, New Castle, DE) at 40°C until the gummy residue was obtained. 50 g of ethanol extract was first dissolved in *n-hexane* to obtain *n*-hexane soluble fraction (HSF) by using Whatman filter paper. Hexane insoluble portion of extract then dissolved in *chloroform* to obtain chloroform soluble fraction (CSF). Similarly, *chloroform* insoluble portion of extract was finally dissolved in methanol to obtain methanol soluble fraction (MSF). Later, *n*-hexane, *chloroform* and methanol soluble fractions were separately concentrated with the help of rotary vacuum evaporator (Büchi R-200, New Castle, DE) at 35°C and stored at room temperature for further use (Ara *et al.,* 2002).

Experimental animals

Female *Wistar* rats (120-200gm) purchased from Dow University of Health Sciences, Karachi. Rats were housed using polypropylene cages under standard laboratory conditions (23±2°C and 12h light/dark cycle). The cages were bedded with wood shaving and rats were fed with normal pellet diets and tap water. All the experiments were carried out after acclimatization of rats for 7 days, under the guidelines of the Institutional Research Ethics Committee (approval # IBC KU-132/2020).

Effect of EEtSA and its fractions in normal and APAP dosed rat model

Experimental rats were randomly divided into 10 groups (n=6). Rats in group 1 received distilled water in a dose of 1ml/kg body weight, daily for 14 days, Whereas rats in group 2 were orally administered with EEtSA (200mg/kg) and rats of group 3, 4 and 5 respectively with *n-hexane*, *chloroform* and methanol soluble fractions (HSF, CSF and MSF) of ethanol extract in a dose of 150mg/kg daily for the same period. Rats in group 6 received intra-peritoneal injection of APAP (800mg/kg; Sigma Aldrich) dissolved in 40% polyethylene glycol (Merck) at day 1 and 14. Rats in group 7, 8, 9 and 10 respectively administered orally with EEtSA (200mg/kg) and its fractions (HSF, CSF and MSF) in a dose of 150mg/kg along with intra-peritoneal injection of APAP at day 1 and 14. On day 15, rats of all groups were decapitated to obtain serum.

Biochemical assessment of liver, antioxidant and oxidative stress biomarkers

Liver specific biomarkers *viz.,* serum alanine & aspartate aminotransferases (ALT & AST), lactate dehydrogenases (LDH), alkaline phosphatases (ALP) and total-bilirubin were estimated by kit method (Merck, France) using Microlab-300, Merck, France. Serum interleukin-6 (IL-6; pg/ml) and caspase-3 activity (ng/ml) were measured by kit methods (Invitrogen, Thermo Fisher Scientific, Austria and Cloud-Clone Corp, USA respectively) and read at 450 nm using micro-plate reader (iMark-Bio-Rad). The liver was excised and washed with phosphate buffer saline (PBS), the right lobe was cut and used for the evaluation of antioxidants enzymes and protein by standard methods including (GSH) reduced glutathione (Moron *et al.,* 1979), glutathione peroxidase GPx (Flohé and Günzler, 1984), catalase (CAT) activity (Sinha, 1972), lipid peroxidation in term of (MDA) malondialdehyde (Ohkawa *et al.,* 1979) and indirect nitric oxide (NO) (Menaka *et al.,* 2009).

STATISTICAL ANALYSIS

The data were represented as a means ±standard deviation (SD) using SPSS software (version 16). The differences between the means were subjected to one-way ANOVA followed by Tukey's post-hoc test. P-value ≤ 0.05 were considered as a level of significance whereas P-value ≥ 0.05 considered as non-significant.

RESULTS

Effect of ethanol extract of S. asperum* (EEtSA) *and its fractions on liver specific biomarkers

APAP intoxicated rats showed abnormal increase ($p \leq 0.05$) in serum concentrations of ALT, AST, ALP, LDH and total bilirubin when compared with normal control group (table 1). Whereas, intoxicated rats orally administered with EEtSA reduced the hepatic distortion by showing substantial decrease in ALT, AST, ALP, LDH and total bilirubin concentrations (table 1). Similarly, *n-hexane* soluble fraction (HSF) also showed remarkable fall in hepatic enzymes *viz.,* ALT, AST, ALP and LDH concentrations as compared to the APAP intoxicated rats. *Chloroform* and methanol soluble fractions (CSF and MSF) were also found effective in reducing the levels of hepatic enzymes but not found very effective in reducing the level of total bilirubin. Overall, results indicate that ethanol extract of *S.asperum* (EEtSA) and its *n-hexane* fraction (HSF) exhibit more potential in order to reverse APAP induced liver toxicity (table 1).

Effect of EEtSA and its fractions on GSH, MDA and NO in liver tissues

Lipid peroxidation and generation of reactive nitrogen species (RNS) are the best representatives of oxidative stress in tissues. APAP intoxicated rats showed significant ($p \leq 0.05$) increase in hepatic concentrations of MDA and indirect NO along with remarkable decrease in hepatic concentration of GSH when compared with normal control rats (fig. 1). APAP intoxicated rats treated with EEtSA, and its fractions (HSF, CSF and MSF) showed substantial reduction in adverse effects induced by APAP by promoting the GSH activity and suppressing the MDA level (fig. 1). APAP was responsible to down regulate the activities of CAT and GPx (fig. 2). However, the

intoxicated rats treated with EEtSA and its fractions (HSF, CSF and MSF) showed up-regulation and improvement in the activities of CAT and GPx (fig. 2).

Effect of EEtSA and its fractions on IL-6 and caspase-3 concentration

APAP overdose is responsible for initiating the action of pro-inflammatory cytokines and also inducing the hepatic apoptosis illustrated in fig. 3 and 4. APAP intoxicated rats showed remarkable elevation in serum IL-6 and caspase-3 concentrations when compared with normal control rats. However, APAP dosed rats treated with EEtSA and its *n-hexane, chloroform* and methanol soluble fractions (HSF, CSF and MSF) showed marked reduction in serum IL-6 and caspase-3 concentration. EEtSA and HSF showed more drastic effect in attenuation of inflammatory response and reduction in hepatic apoptosis by decreasing the concentration of IL-6 and caspase-3 levels.

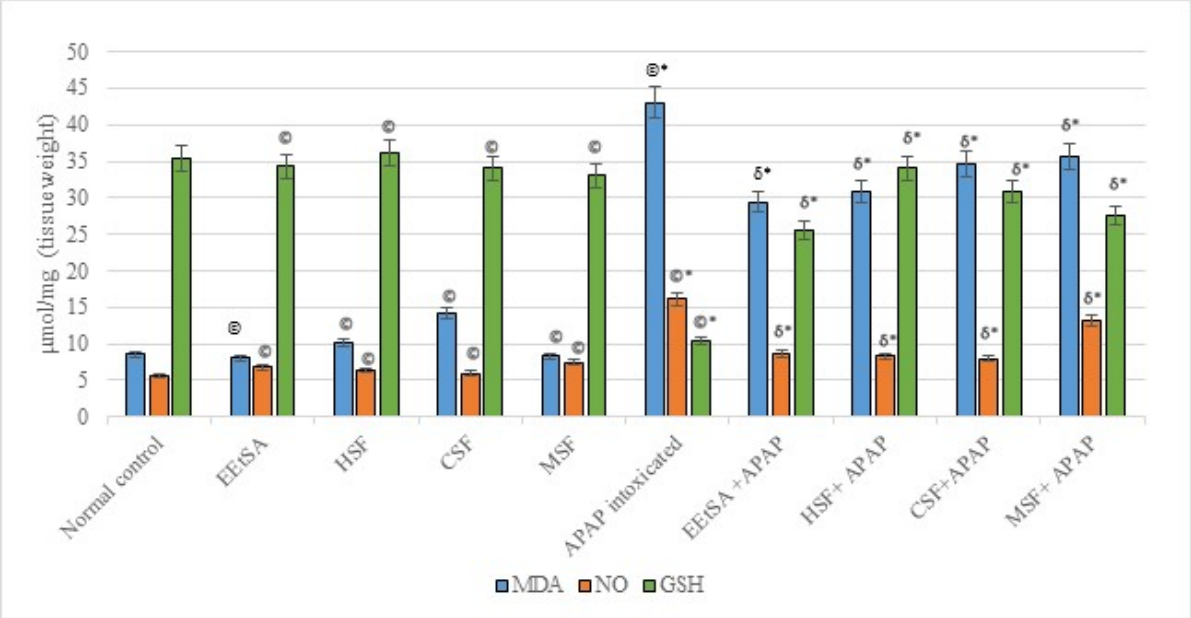
DISCUSSION

Overdose of acetaminophen (APAP) becomes a common cause of drug induced acute liver failure as it activates the hepatic cytochrome P450 system and generates highly reactive metabolites NAPQI (Bernal *et al.,* 2015). Primarily, NAPQI (N-acetyl-p-benzoquinone imine) is detoxified by GSH but at high concentration, it not only depletes GSH level but also bind with cysteine and lysine residues of mitochondrial proteins and persuade mitochondrial dysfunction (McGill *et al.,* 2012; Jaeschke *et al.,* 2019).

Table 1: Effect of ethanol extract of *S. asperum* (EEtSA) and its fractions on hepatic biomarkers

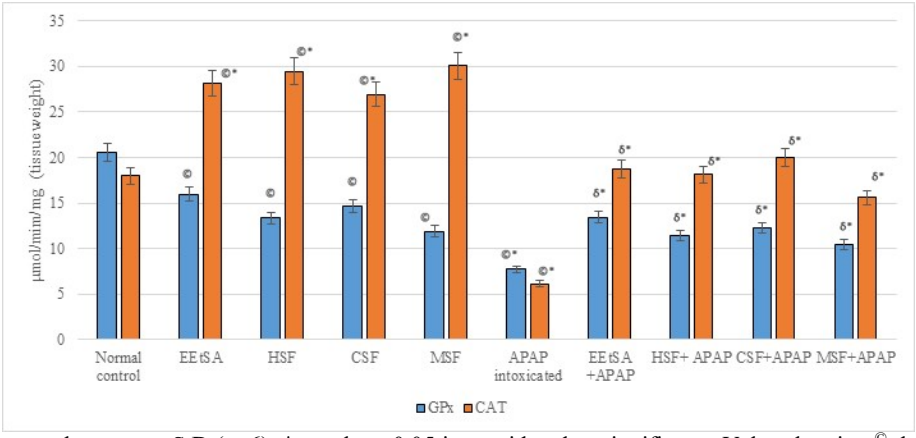
Groups	ALT (μL^{-})	AST (μL^{-})	ALP (μL^{-})	LDH (μL^{-})	Total bilirubin ($mgdL^{-}$)
Normal control	31.5±4.5	51±1.0	105.3±1.5	138.0±3.0	0.25±0.05
EEtSA	46±1.7$^{©*}$	132±2.6$^{©*}$	163.6 ± 4.6$^{©*}$	180.0±2.0$^{©*}$	0.166±0.05$^{©NS}$
HSF	47.6±1.4$^{©*}$	91.5±1.2$^{©*}$	142.6±2.3$^{©*}$	146.3±0.5$^{©NS}$	0.21±0.01$^{©NS}$
CSF	51.3±0.5$^{©*}$	116.0±8.7$^{©*}$	150.0±8.0$^{©*}$	159.0±3.4$^{©*}$	0.23±0.05$^{©NS}$
MSF	56.6±2.3$^{©*}$	102.5±0.5$^{©*}$	128.3±1.1$^{©*}$	192.0±6.9$^{©*}$	0.11±0.02$^{©*}$
Acetaminophen (APAP) intoxicated group	142±5.1$^{©*}$	211.2±6.1$^{©*}$	447.3±18.47$^{©*}$	321.0±7.0$^{©*}$	1.2±0.1$^{©**}$
EEtSA + APAP	76.1±11.5^{δ*}	142.3±0.5^{δ*}	160.3 ± 0.5^{δ*}	200.16±0.2^{δ*}	0.25±0.05^{δ*}
HSF + APAP	38.3±1.1^{δ*}	124.6±0.5^{δ*}	134.6±2.08^{δ*}	217.6±1.1^{δ*}	0.45±0.05^{δ*}
CSF + APAP	105.3±3.5^{δ*}	126.5±0.5^{δ*}	164.0±3.0^{δ*}	122±1.0^{δ*}	0.18±0.02^{δ*}
MSF +APAP	70.3±3.5^{δ*}	162±14.1^{δ*}	201.1±1.7^{δ*}	235.3±1.5^{δ*}	0.35±0.05^{δ*}

Each value is expressed as mean ± S.D (n=6); *p- value ≤0.05 is considered as significant. Values bearing © show the comparison with normal control. Whereas values bearing δ show the comparison with APAP intoxicated group. NS = non-significant. HSF= Hexane soluble fraction, CSF=Chloroform soluble fraction, MSF= Methanol soluble fraction



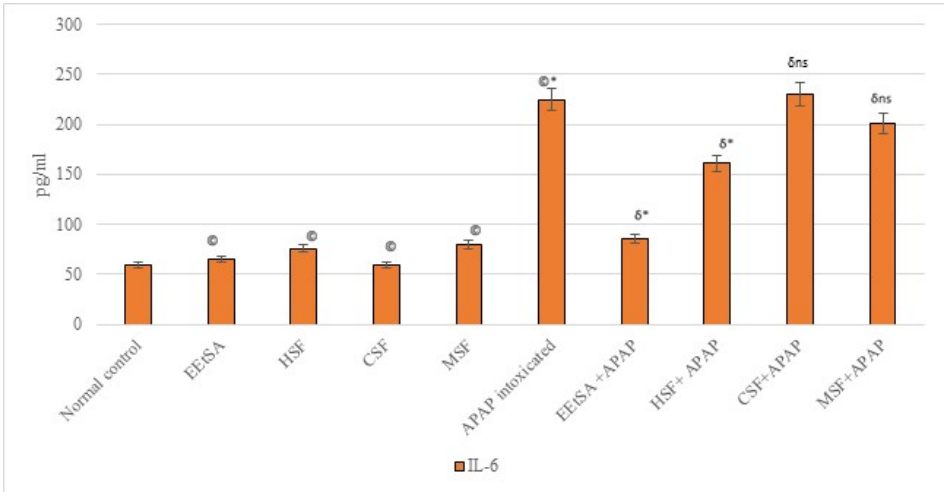
Each column represents the mean ± S.D (n=6), *p- value ≤ 0.05 is considered as significant. Values bearing © show the comparison with normal control, whereas values bearing δ show the comparison with APAP intoxicated group. HSF= Hexane soluble fraction, CSF=Chloroform soluble fraction, MSF= Methanol soluble fraction.

Fig. 1: Effect of ethanol extract of *S. asperum* (EEtSA) and its fractions on oxidative stress biomarkers



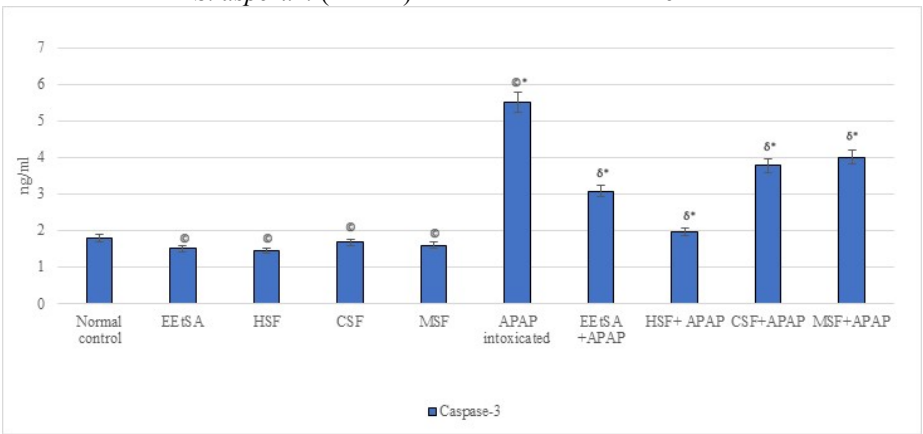
Each column represents the mean ± S.D (n=6), *p- value ≤0.05 is considered as significant. Values bearing © show the comparison with normal control, whereas values bearing δ show the comparison with APAP intoxicated group. HSF= Hexane soluble fraction, CSF=Chloroform soluble fraction, MSF= Methanol soluble fraction.

Fig. 2: Effect of ethanol extract of *S. asperum* (EEtSA) and its fractions on GPx and CAT activities



Each column represents the mean ± S.D (n=6), *p- value ≤0.05 is considered as significant. Values bearing © show the comparison with normal control. Whereas values bearing δ show the comparison with APAP intoxicated group. ns = non-significant. HSF= Hexane soluble fraction, CSF= Chloroform soluble fraction, MSF= Methanol soluble fraction.

Fig. 3: Effect of ethanol extract of *S. asperum* (EEtSA) and its fractions on IL-6 concentration



Each column represents the mean ± S.D (n=6), *p-value ≤0.05 is considered as significant. Values bearing ©show the comparison with normal control, whereas values bearing δ show the comparison with APAP intoxicated group. HSF= Hexane soluble fraction, CSF=Chloroform soluble fraction, MSF= Methanol soluble fraction.

Fig. 4: Effect of ethanol extract of *S. asperum* (EEtSA) and its fraction on serum caspase-3 concentration.

In the present study, APAP has been administered at a dose of 800mg/kg, it exerts excessive pressure on hepatocytes as a result of which marked elevation was found in serum aminotransferases (ALT and AST), phosphatase (ALP) and dehydrogenase (LDH) concentrations which clearly showed that overdosing of APAP enhanced the destruction of hepatocytes and induced hypoxia. Abnormal increased in total bilirubin concentration was also found in APAP dosed rats, which was again an evidence of liver dysfunction. Studies showed that consumption of APAP at high concentration is responsible for inducing excessive stress to liver that results in abnormal release of hepatic enzymes and metabolites in serum that proves liver dysfunction (Zhao *et al.,* 2018). However, APAP dosed rats administered with ethanol extract of *S. asperum* (EEtSA) significantly decreased the serum concentration of ALT, AST, ALP, LDH and total bilirubin in test rats. *n*- Hexane and *chloroform* fractions (HSF and CSF) of EEtSA significantly reduced the hepatic enzymes and metabolites levels demonstrated that they have potential to diminish the APAP toxicity. Previously, brown algae have shown tremendous effect against hepatotoxicity induced by different toxicants as they are rich source of terpenes, phenols and other various antioxidants (Quintal-Novelo *et al.,* 2018).

In the current study, APAP dosed rats showed notable low concentration of GSH and decreased activities of GPx and CAT. These findings validate the previous studies that describe APAP at a dose of 800mg/kg stimulates liver cytochrome P450 system and converts itself into highly reactive metabolites NAPQI which ultimately causes depletion in activities of antioxidants enzymes and their concentrations. Similarly, APAP dosed/intoxicated rats showed increased in lipid peroxidation in terms of elevated level of hepatic MDA. Furthermore, APAP intoxicated rats demonstrated marked increase in indirect NO concentration which indicated that APAP overdose also motivates the generation of RNS which contribute in destruction of hepatocytes. Previous reports suggested that ethanol extract of brown seaweeds are rich with terpenes, fucoxanthin, flavonoids and polyunsaturated fatty acids (PUFAs) which showed hepatic preventive activities against toxicants (Dore *et al.,* 2013; Hira *et al.,* 2021). APAP dosed rats treated with EEtSA and its solvent fractions (HSF, CSF and MSF) showed improvement in antioxidant status of liver by increasing the concentration of GSH and activities of antioxidant enzymes (GPx, CAT). This aspect is more strengthen by observing significant reduction in hepatic MDA and indirect NO concentrations. Among them, EEtSA and its HSF possess more capability to revert the hepatotoxic effect of APAP overdose.

Cytokines are small proteins which have a huge role in detection of toxicity. These are usually secreted in response to toxicity and promote the inflammatory response. As APAP overdose promotes the generation of cytotoxic metabolites NAPQI which is responsible for activating the inflammatory mechanism of liver and ultimately causing the over expression of pro-inflammatory cytokines IL-6 (Wang *et al.,* 2018). Similarly, in the present study, APAP dosed rats showed significant ($p<0.05$) elevation in serum concentration of IL-6 which represented the liver inflammation. Previously, it has been seen that APAP intoxication provokes the apoptotic pathways to control further liver damage such as caspase-3 protein (Xu, 2018). In this study, APAP dosed rats also displayed increase in apoptotic protein caspase-3, secreted by destructed hepatocytes. Thereby, these data suggest that, usage of APAP beyond its prescribed dose is responsible for significant destruction of hepatocytes and become a major cause of acute liver dysfunction. Rats administered with EEtSA and its solvent fractions remarkably reduced the serum concentration of IL-6 and caspase-3 protein, these findings suggested that ethanol extract of *S. asperum* and its solvent fractions have tendency to delay the inflammatory response and cell death which were accelerated in response of APAP overdose. Conclusively, ethanol extract and *n-hexane* fraction of *S. asperum* showed more significant results against APAP overdose. Previous data showed that, ethanol extracts of brown seaweeds are rich with alkaloids, flavonoids tannins and PUFAs which contribute central role in alleviating the generation of reactive oxygen (ROS) and improving the activity of membrane bound enzymes, antioxidant enzymes and the shuttles mechanism of liver (Motshakeri *et al.,* 2014).

CONCLUSION

APAP overdose is the major cause of acute liver toxicity. In the present work, ethanol extract of *S. asperum* and its fractions (*n-hexane*, *chloroform* and methanol) minimized the toxic effect of APAP overdose in rats by reducing the action of inflammatory cytokines, apoptotic protein and oxidative stress indices by accelerating the efficacy of antioxidant enzymes and protein and by normalizing the levels of liver biomarkers.

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