The protective and therapeutic effects of Persian Gulf sea cucumber (*Holothuria leucospilota*) on Carbon tetrachloride - induced hepatotoxicity in Molly Fish (*Poecilia sphenops*)

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Abstract

Carbon tetrachloride (CCl_4) is a well-known toxicant in exposed animals. It is assumed sea cucumber has medical properties. So, the protective and therapeutic effects of ethanol extract of the Persian Gulf sea cucumber (EEPGSC) on CCl_4-induced hepatotoxicity in Molly Fish (*Poecilia sphenops*). A total 90 Molly fish were randomly divided into 9 experimental groups as follows: Group (C_0): had no received injections. Group (C_1): fish injected with olive oil (1 mL/kg, i.m.). Group (C_2): injected with ethanol (100 mg/kg, i.m.) Group (C_3): injected with CCl_4 (1 mL/kg, i.m.) on day 1st. Group (T_1): fish injected with CCl_4 (1 mL/kg, i.m.) and then with EEPGSC (100 mg/kg, i.m.). Group (T_2): injected with CCl_4 (1 mL/kg, i.m.) then with EEPGSC (200 mg/kg, i.m.). Group (C_4): had no received injections for 20 days, then injected with CCl_4 (1 mL/kg, i.m.) on day 21. Group (T_3): injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21. Group (T_4): fish injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21. After the last injection, body length (cm), weight (g), GIS index and liver alkaline phosphatase (ALP), alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were determined. No significant difference detected in body weight and length compared to the control groups (p>0.05). Injection of the different levels of the EEPGSC (100 and 200 mg/kg, i.m.) decreased CCl_4-induced ALP, ALT and AST levels compared to the C_4 groups (p<0.05). These results suggested EEPGSC had protective and therapeutic effect against CCl_4-induced hepatotoxicity in Molly fish.

Keywords: Persian Gulf sea cucumber, Hepatotoxicity, Molly Fish

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Introduction

Liver is the organ which expose to several toxins and infections through hepatic vein in the body. Xenobiotic-metabolizing enzymes (mainly cytochrome P-450) are present in the liver where detoxify and eliminate toxicants. The toxicology of the liver is complicated by the diversity of liver injuries and the dissimilar mechanism through which the injuries are involved (Ujah *et al.,* 2013). Carbon tetrachloride (CCl_4) is an experimental candidate toxicant extensively used to induce lipid peroxidation and toxicity and diseased state in animals (Peng *et al.*, 2010; Abdel Salam *et al.,* 2012 a, b; 2013 a, b). It is reported that CCl_4 promotes hepatocellular carcinoma incidence in rats ( ). There are varieties of enzymes include AST, ALT, ALP which elevated their serum concentrations are a powerful marker for liver damage (Abdel Salam *et al.,* 2012c). Recently, besides physicians, patient's interest increased to the use of botanical medicine and herbal remedies in liver disease management (Fogden and Neuberger, 2003; Karimi *et al.*, 2006; Hayatghaybi and Karimi, 2007; Karimi *et al.,* 2007; Saberivand *et al.*, 2010; Yousofi *et al.,* 2011). On the other hand there is growing interest for therapeutic drugs, products from marine sources (Esmat *et al.,* 2013).

Holothuroidea or sea cucumbers are abundant worm-like and soft-bodied echinoderms found in nearly every marine environment (Haider *et al.*, 2015). Sea cucumbers are the aquatic creatures and have nutritional and therapeutic properties on human health (Mamelona *et al.,* 2007). Sea cucumbers are considered as a culinary delicacy and as a traditional cure for many illnesses in many Asian countries (Liu *et al.,* 2012). Some compounds isolated to date exhibit antimicrobial and anti-inflammatory, antioxidant, anticoagulant, anti-hyperlipidemic and immune modulatory (Hu *et al.,* 2012). It is reported the sea cucumber extract contains physiologically active phenolic compounds with antioxidant activity, which afforded a potential hepatoprotective activity against thioacetamide induced liver injury in a rat model (Esmat *et al.,* 2013). Recently, Dakrory *et al.* (2015) revealed *Holothuria atra* extract exhibits good hepatoprotective, curative and antioxidant potential against DMBA-induced hepato-renal dysfunction in rats. The therapeutic use of sea cucumbers for healing is established, where they used for joint pain, tendonitis and sprains. For instance, sea cucumbers extract decrease serum aminotransferases (ASAT and ALAT) and ALP as well as malondialdehyde (MDA) while increasing the serum albumin, glutathione reduced (GSH) levels in bile duct ligation in rats (Fahmy, 2015). Also, *Holothuria arenicola* extract normalized the antioxidant enzyme, glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) activities (Fahmy, 2015).

Based on the literature, scarce information exists on medical properties of the Persian Gulf sea cucumber (*Holothuria leucospilota*). In a sole report, Delghandi Moghadam*et al.* (2016) studied effect of Persian Gulf sea cucumber (*Holothuria leucospilota*) on maturation of mice. So, the protective and therapeutic effects of ethanol extract of EEPGSC on CCl_4-induced hepatotoxicity in Molly Fish (*Poecilia sphenops*) were studied.

Material and methods

Sample preparation

Samples of Persian Gulf sea cucumber *(Holothuria leucospilota)* were collected from Persian Gulf, at the depth of 25-30 meters in 2016. Then samples transported and stored at -20°C in laboratory of Fisheries, Department of Pharmacology, Islamic Azad University, Tehran, Iran.

Persian Gulf sea cucumber extract

The Persian Gulf sea cucumber (EEPGSC) (2000 g wet weight) was cut into small pieces (1 cm), they were put in freeze dried until the tissue was dry(150 g dry weight). Extracts of powder sea cucumber were obtained by using three different solvents including: n-hexane, diethyl ether and methanol. After 24 hours of exposure in n-hexane, the extract was concentrated under low pressure at 30˚C by rotary evaporation. The diethyl ether extract was ready after 48 hours, and then the solvent was removed by rotary evaporation at 35˚C. The methanol extract was ready after 72 hours, then the solvent was removed by rotary evaporation at 40˚C. Ether-methanol was formed by adding Ether in order to separate the methanol-aqueous extract, then the upper phase was separated by separating funnel. The upper phase was combined by n-butanol and aqueous extract was separated by separating funnel. Each extract was shaken bymechanical shaking at room temperature (25°C). All processes were carried out on dark condition. Finally, both crude extracts were kept in freezer (Bligh *et al.*, 1959).

Experimental groups

A total 90 Molly fish (*Poecilia sphenops*) (3±0.2) were randomly divided into 9 experimental groups (n=10). The fish were kept in 2 m^3 tanks with a flow-through circuit, suitable aeration and filtration system and natural photoperiod. The water temperature ranged from 25.1 to 27.8°C. The environmental parameters, mortality and food intake were recorded daily. Before the initiation of the study, fish kept for a week for adaptation to the experimental condition, and then randomly divided into experimental groups as follow in Table 1.

Serum Biomarkers for Liver Functions Tests

Then 24 h after the last injection, fish from each group randomly selected, euthanized and biometric indexes body length (cm), weight (g), and GIS index were determined.

Table 1: the experimental groups.

Group (C_0)	Had no received injections and fish kept as control.
Group (C_1)	Fish injected with olive oil (1 ml/kg, i.m.) for 20 days (given on alternate days).
Group (C_2)	Fish injected with ethanol (100 mg/kg, i.m.) for 20 days (given on alternate days).
Group (C_3)	Fish injected with CCl_4 (1 ml/kg, i.m.) on day 1st.
Group (T_1)	Fish injected with CCl_4 (1 ml/kg, i.m.) and then injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days).
Group (T_2)	Fish injected with CCl_4 (1 ml/kg, i.m.) and then injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days).
Group (C_4)	Had no received injections for 20 days, then injected with CCl_4 (1 ml/kg, i.m.) on day 21.
Group (T_3)	Fish injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 ml/kg, i.m.) on day 21.
Group (T_4)	Fish injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 ml/kg, i.m.) on day 21.

Also, the liver samples obtained and the tissue alkaline phosphatase (ALP), alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were determined using commercial detecting kits (Reitman and Frankel, 1957; Belfield and Goldberg, 1971).

Statistical analyses

Effect of EEPGSC was analyzed by repeated measure two-way analysis of variance (ANOVA) using SPSS 21.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data is presented as mean±SEM. For treatment showing a main effect by ANOVA, means compared by Tukey-Kramer test. $p<0.05$ was considered as significant differences between treatments.

Results

The protective and therapeutic effects of EEPGSC on CCl_4 -induced hepatotoxicity in Molly Fish (*Poecilia sphenops*) is presented in Figs. 1-6. According to the results despite there was difference between the experimental groups, no significant difference obseved in body weight gain compared to the control groups ($p>0.05$) (Fig. 1).

The protective and therapeutic effects of EEPGSC on body length (cm) of Molly fish was presented in Fig. 2. According to the results, no significant difference detected on body length (cm) compared to the control group ($p>0.05$), however, the man body length was higher in fish injected with olive oil (1 mL/kg, i.m.) for 20 days (given on alternate days).

As seen in Fig. 3, no significant difference detected in GIS index in fish injected with EEPGSC, olive oil, ethanol and CCl_4 compared to the control group ($p>0.05$).

As seen in Fig. 4, no significant difference detected in tissue ALP levels in fish injected with olive oil (1 mL/kg, i.m.) for 20 days (given on alternate days) and fish injected with CCl_4 (1 mL/kg, i.m.) on day 1st compared to the control group ($p>0.05$). Furthermore, tissue ALP level was significantly higher in fish injected with CCl_4 compared to the compared to the control group ($p<0.05$).

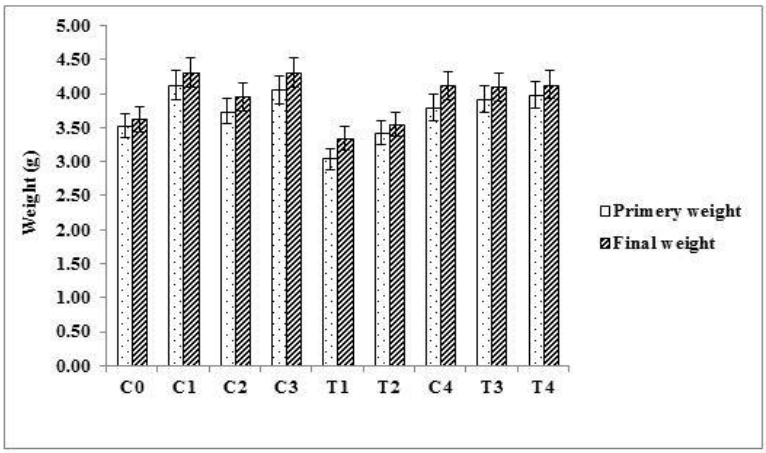

Figure 1: The protective and therapeutic effects of EEPGSC on body weight (g) of Molly fish (*Poecilia sphenops*). EEPGSC: ethanol extract of the Persian Gulf sea cucumber; C_0: had no received injections and fish kept as control; C_1: fish injected with olive oil (1 mL/kg, i.m.) for 20 days (given on alternate days); C_2: fish injected with ethanol (100 mg/kg, i.m.) for 20 days (given on alternate days); C_3: fish injected with CCl_4 (1 mL/kg, i.m.) on day 1st; T_1: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days); T_2: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days); C_4: had no received injections for 20 days, then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_3: fish injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_4: fish injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21.

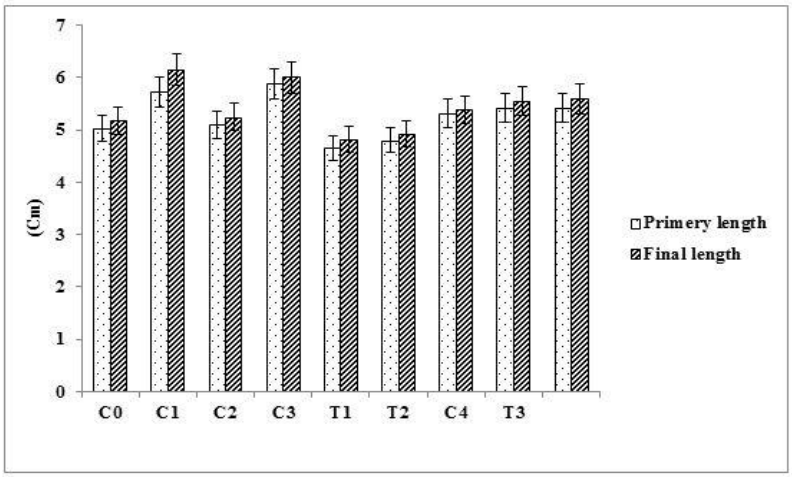

Figure 2: The protective and therapeutic effects of EEPGSC on body length (cm) of Molly fish (*Poecilia sphenops*). EEPGSC: ethanol extract of the Persian Gulf sea cucumber; C_0: had no received injections and fish kept as control; C_1: fish injected with olive oil (1 mL/kg, i.m.) for 20 days (given on alternate days); C_2: fish injected with ethanol (100 mg/kg, i.m.) for 20 days (given on alternate days); C_3: fish injected with CCl_4 (1 mL/kg, i.m.) on day 1st; T_1: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days); T_2: fish injected with CCl_4 (1 mL /kg, i.m.) and then injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days); C_4: had no received injections for 20 days, then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_3: fish injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_4: fish injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21.

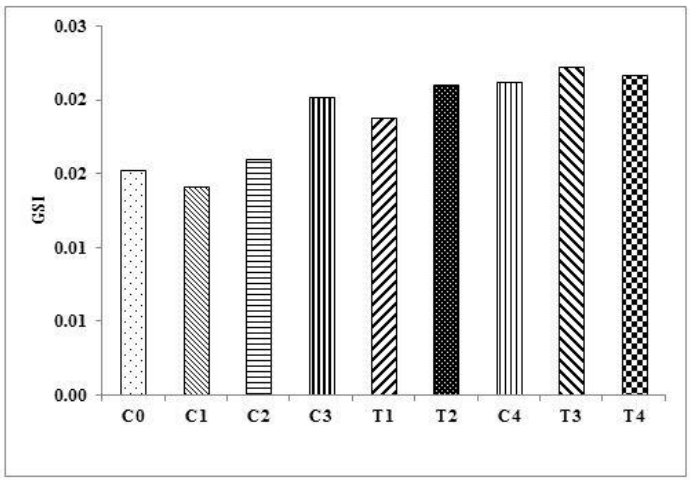

Figure 3: The protective and therapeutic effects of EEPGSC on GSI index in Molly fish (*Poecilia sphenops*). EEPGSC: ethanol extract of the Persian Gulf sea cucumber; C_0: had no received injections and fish kept as control; C_1: fish injected with olive oil (1 mL/kg, i.m.) for 20 days (given on alternate days); C_2: fish injected with ethanol (100 mg/kg, i.m.) for 20 days (given on alternate days); C_3: fish injected with CCl_4 (1 mL/kg, i.m.) on day 1st; T_1: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days); T_2: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days); C_4: had no received injections for 20 days, then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_3: fish injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_4: fish injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21.

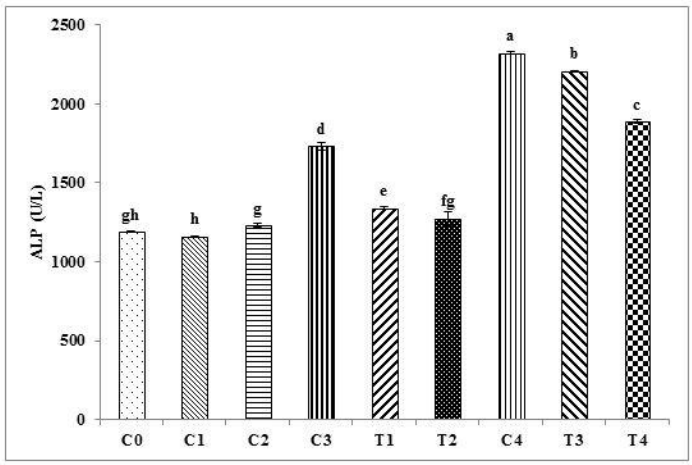

Figure 4: The protective and therapeutic effects of EEPGSC on liver ALP levels in Molly fish (*Poecilia sphenops*). EEPGSC: ethanol extract of the Persian Gulf sea cucumber; ALP: alkaline phosphatase; C_0: had no received injections and fish kept as control; C_1: fish injected with olive oil (1 mL/kg, i.m.) for 20 days (given on alternate days); C_2: fish injected with ethanol (100 mg/kg, i.m.) for 20 days (given on alternate days); C_3: fish injected with CCl_4 (1 mL/kg, i.m.) on day 1st; T_1: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days); T_2: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days); C_4: had no received injections for 20 days, then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_3: fish injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_4: fish injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21. There are significant differences between groups with different superscripts in a column (p<0.05).

Injection of the different levels of the EEPGSC (100 and 200 mg/kg, i.m.) significantly decreased CCl_4-induced ALP levels compared to the C_4 groups (p<0.05). These results suggest EEPGSC has protective and therapeutic effect against CCl_4-induced hepatotoxicity in Molly fish.

No significant difference observed in liver ALT levels in Molly fish injected with olive oil (1 mL/kg, i.m.) for 20 days and ethanol (100 mg/kg, i.m.) for 20 days compared to control group (p>0.05) but injectin of the CCl_4 (1mL/kg) on day 1st significantly increased liver ALT levels in comparison to control group (p<0.05). Additionally, liver ALT levels significantly increased in group C_4 (no received injections for 20 days, then injected with 1 mL/kg of CCl_4 on day 21) compared to control group) while injection of the EEPGSC (100 and 200 mg/kg, i.m.) for 20 days significantly decreased CCl_4-induced ALT levels (p<0.05) Fig. 5). These results suggest EEPGSC had protective and therapeutic effect CCl_4-induced hepatotoxicity in Molly fish.

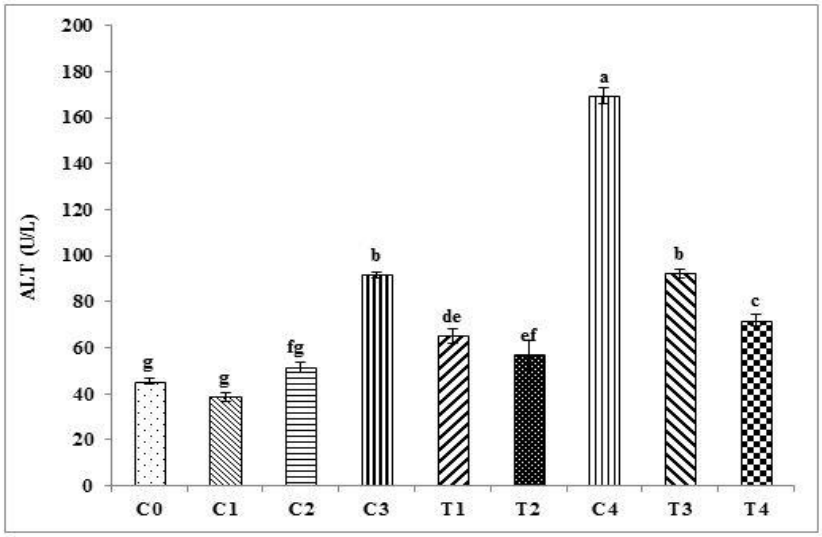

Figure 5: The protective and therapeutic effects of EEPGSC on liver ALT levels in Molly fish (*Poecilia sphenops*). EEPGSC: ethanol extract of the Persian Gulf sea cucumber; ALT: alanine aminotransferase; C_0: had no received injections and fish kept as control; C_1: fish injected with olive oil (1 mL/kg, i.m.) for 20 days (given on alternate days); C_2: fish injected with ethanol (100 mg/kg, i.m.) for 20 days (given on alternate days); C_3: fish injected with CCl_4 (1 mL/kg, i.m.) on day 1st; T_1: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days); T_2: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days); C_4: had no received injections for 20 days, then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_3: fish injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 ml/kg, i.m.) on day 21; T_4: fish injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21. There are significant differences between groups with different superscripts in a column (p<0.05).

As seen in Fig. 6, CCl_4 (1 mL/kg, i.m.) on day 1st significantly elevated tissue AST level compared to the control group (p<0.05). Also, tissue AST levels

significantly decreased in fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) (p<0.05). Injection of the different levels of the EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21 significantly decreased CCl_4-induced AST compared to the C_4 group (p<0.05). These results suggest EEPGSC was able to minimize CCl_4-induced hepatotoxicity in Molly fish.

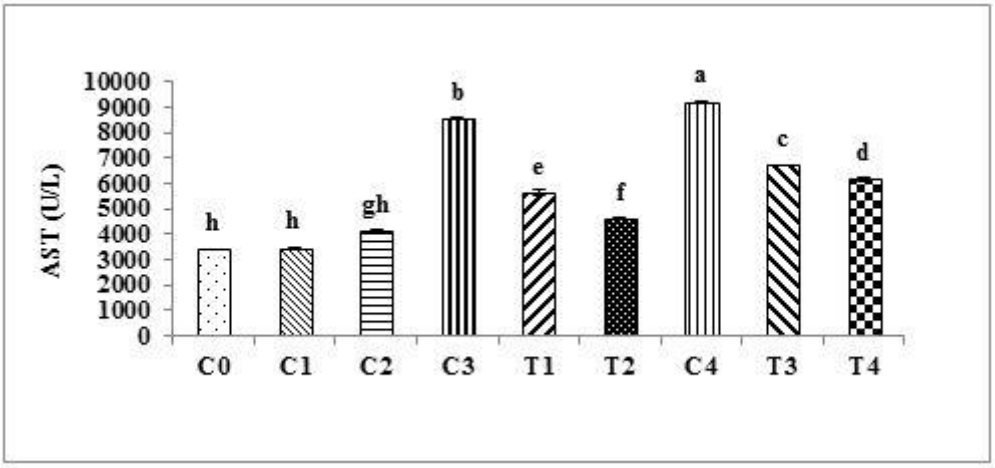

Figure 6: The protective and therapeutic effects of EEPGSC on liver AST levels in Molly fish (*Poecilia sphenops*). EEPGSC: ethanol extract of the Persian Gulf sea cucumber; AST: Aspartate aminotransferase; C_0: had no received injections and fish kept as control; C_1: fish injected with olive oil (1 mL/kg, i.m.) for 20 days (given on alternate days); C_2: fish injected with ethanol (100 mg/kg, i.m.) for 20 days (given on alternate days); C_3: fish injected with CCl_4 (1 mL/kg, i.m.) on day 1st; T_1: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days); T_2: fish injected with CCl_4 (1 mL/kg, i.m.) and then injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days); C_4: had no received injections for 20 days, then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_3: fish injected with EEPGSC (100 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21; T_4: fish injected with EEPGSC (200 mg/kg, i.m.) for 20 days (given on alternate days) then injected with CCl_4 (1 mL/kg, i.m.) on day 21. There are significant differences between groups with different superscripts in a column (p<0.05).

Discussion

To the best of this study is first report on the protective and therapeutic effects of Persian Gulf sea cucumber (*Holothuria leucospilota*) on Carbon tetrachloride -induced hepatotoxicity in Molly fish. As detected, no significant difference detected in body weight gain and body length but different levels of the EEPGSC (100 and 200 mg/kg, i.m.) significantly decreased CCl_4-induced ALP, ALT and AST levels.

CCl_4 as an applicable candidate for other halogenated hydrocarbons has been one of the most broadly studied hepatotoxicant to date (Weber *et al.*, 2003). Acute poisoning with CCl_4 becomes manifested as a multi-organ disorder (Gitlin, 1996). It seems that liver is very vulnerable to this messy

hepatotoxin and a single exposure can quickly lead to severe necrosis and steatosis. Organ function tests are useful to evaluate amount or severity of CCl_4-induced toxicity. In this line, assaying activities of several enzyme like LDH, AST, ALT, CPK, ALP, Glutathione-S-transferase (GST) and Glucuronyltransferase, catalase, superoxide dismutase, glutathione peroxidase has been frequently used to determine CCl_4-induced toxicity (Peng *et al.*, 2010; Weerachayaphorn *et al.*, 2010; Abdel Salam *et al.*, 2012 a, b; 2013 a, b). Other studies that have a mechanistic view regarding CCl_4-induced toxicity usually measure biomarker of oxidative stress like reduced glutathione (GSH) and malondialdehyde (MDA) (Wills *et al.*, 2006).

It is reported *Holothuria atra* extract decrease serum AST, ALT and ALP as well as MDA levels in bile duct ligation in rats (Fahmy, 2015). Also, the sea cucumber extracts increase GSH and normalized GST, SOD, CAT levels (Fahmy, 2015). So, it seems sea cucumber extracts have potential role against hepatotoxicity in rat model. In the current study, because of the limitations we were not able to determine effects of the EEPGSC on reactive oxidation species (ROS) like MDA, SOD and CAT. The modern medicinal system relies heavily on synthetic chemicals being used as drugs, but these unnatural synthetic drugs often pose serious side effects. Therefore, the development of novel chemotherapeutic agents would play a key role in the treatment of many refractory diseases.

The Persian Gulf sea cucumber (*Holothuria leucospilota*) is a marine invertebrate of the phylum Echinoderm and the class *Holothuroidea* found on the sea floor worldwide (Farjami *et al.*, 2014). Dry sea cucumber contains approximately 20 mg/g glucosylceramide. It is reported dietary sea cucumber contacting glucosylceramide decreased liver cholesterol and triglyceride compared to glucosylceramide free sea cucumber in mice but not affect body weight (Hossain *et al.*, 2011). Polysaccharides can act as stimulators of bile acid synthesis and circulation and increasing the fecal excretion of bile acids so that fewer bile acids return to the liver (Hossain *et al.*, 2011). The present study explores the protective and curative roles of the EEPGSC on ALP, ALT and AST which the findings were similar to Fahmy (2015). As observed in the current study, injection of CCl_4 elevated ALT, AST and ALP in Molly fish. Increase in ALP levels confirmed the damage produced by the bile duct ligation in rats (Baldo *et al.*, 2011). This increase may be attributable to the retention of bile salts that damaged the membrane and consequently leads to the passing of the ALP enzyme into circulation (Baldo *et al.*, 2012). In this regard, Esmat *et al.* (2013), reported administration of the *Holothuria leucospilota* extract attenuated the hepatic enzymes as evident by 77, 70

and 47% improvement in the activities of serum AST, ALT and ALP respectively in the rats. In the current study, our hypothesis for using fish as animal model firstly was because of anatomical and comparative physiological difference among animal (marine vs. mammalian) and secondly for determining possible effects EEPGSC in fish as sea food source for human. These results suggested sea cucumber not only could use as medical substance but also, it can be used as food additive in fishery industry to improve quality of the fish meat and its safety for human consumption (Farjami *et al.*, 2014).

In conclusion these results suggested EEPGSC has protective and therapeutic effect against CCl_4-induced hepatotoxicity in Molly fish. To date, several bioactive components and medical properties of the sea cucumber have been identified. The obtained results of the current study could count as new medical properties of the Persian Gulf sea cucumber in medicine, pharmacology and even veterinary. However, further researches needed to determine accuracy of the results and the possible molecular and cellular mechanisms for effect of EEPGSC for clinical trials.

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