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RESEARCH ARTICLE

Exploration of Hepatoprotective Potential and Phytochemicals of *Ziziphus oxyphylla* Edgew

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ABSTRACT

Ziziphus oxyphylla Edgew (ZO) has been traditionally used for the treatment of hepatic diseases in Pakistan. The current study was carried out to investigate the effectiveness and mechanism underlying hepatoprotective effect of ZO. Hepatoprotective potential of root extracts (crude methanolic extract and subsequent fractions) was investigated against carbon tetrachloride (CCl_4) induced hepatotoxicity in BALB/c mice via determination of serum biomarkers, antioxidant enzymes (catalase, superoxide dismutase), lipid peroxidation and histopathology. Membrane stabilizing activity was determined via inhibition of hypotonic solution and heat-induced hemolysis. Crude methanolic extract and chloroform fraction ameliorated CCl_4 induced elevated levels of alanine transaminase (ALT), alkaline phosphatase (ALP), total proteins (TP), total bilirubin (TB) and reversed the antioxidants imbalance in terms of catalase (CAT), superoxide dismutase (SOD) and reduced malondialdehyde (MDA) level. Crude methanol extract inhibited hemolysis induced by hypotonic solution and heat. Histopathological examination of liver specimens of extracts administered mice exhibited recovery from CCl_4 induced pathological changes. Moreover, CCl_4 induced alteration in body and liver weight was countered by the administration of ZO. Compounds betulinic acid (AZO_1), ceanothic acid (AZO_2) and zizybrenalic acid (AZO_3) were isolated from chloroform fraction of the plant. The structures of compounds were deduced using 1D-and 2D-NMR spectroscopy techniques. The results show that *Ziziphus oxyphylla* roots extract possess antihepatotoxic activity mediated by the protection of anti-oxidant defense system and membrane-stabilizing activity, probably due to the presence of pentacyclic triterpenes.

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INTRODUCTION

More than 6000 species of higher plants are reported in Pakistan, 12% of which are used for treatment of various ailments (Ahmad *et al.*, 2014). The increasing use of medicinal plants for various diseases is attributed to synergistic and side effects neutralizing combination of active constituents (Gilani and Atta-ur-Rahman, 2005; Abbas *et al*., 2019). Hepatic disorders are one of the major factors of mortality and morbidity across the globe (Jiang *et al*., 2016). Liver fibrosis, the hallmark of chronic inflammation, is highly prevalent in developing countries affecting millions of people. Moreover, cirrhosis, non-alcoholic steatohepatitis and hepatocellular carcinoma are concerns of all ethnicities worldwide (Torres *et al.*, 2012). The biosynthetic, metabolic, secretory and detoxifying functions of the liver are affected by viruses, obesity, medications, inherited metabolic disorders, immune disorders, cholestatic disturbances, inflammation etc. Oxidative stress, caused by reactive oxygen (ROS) and nitrogen species, is responsible for hepatopathies. ROS accumulation exacerbates the damage in hepatocytes and mitochondria, resulting in pathological degeneration, membrane fragility and enzyme leakage (Siegmund *et al.*, 2005).

Carbon tetrachloride (CCl_4) is one of the widely employed models for hepatotoxicity induction. CCl_4 metabolic activation in liver produces free radicals (trichloromethyl and trichloromethylperoxyl) which results in oxidative stress. It induces hepatotoxicity by inflammation, fatty degeneration, hepatocellular necrosis, fibrosis and carcinogenicity (Zhang *et al.,* 2018).

Some antiviral drugs, used to treat liver disorders, have been known to have potential adverse effects, especially when chronically administrated (Muriel *et al.*, 2008). As allopathic system is inadequate for the treatment of liver disorders, therefore, herbs play a pivotal role in hepatopathies (Chattopadhyay, 2003). Silymarin, a mixture of seven flavonolignans (isosilibinin, silibinin, isosilychristin, silychristin and silydianin) and a flavonoid (taxifolin), obtained from milk thistle, is used as hepatoprotective. However it has low solubility, bioavailability and some side effects such as nausea, dyspepsia, bloating and laxative effect (Federico *et al.,* 2017). Therefore, there is a dire need to search for more effective, safe and economical hepatoprotectives.

The family *Rhamnaceae* is represented by six genera and twenty-one species in Pakistan. Several triterpene compounds have been isolated from the family *Rhamnaceae*. Triterpenoids are promising hepatoprotective, anticancer, antioxidant and anti-inflammatory agents. They have shown hepatoprotective potential against CCl_4, D-galactosamine, cadmium and acetaminophen-induced toxicity in experimental animals. Pentacyclic triterpenes including asiatic acid, ursolic acid and oleanolic acid have exhibited hepatoprotective effect against CCl_4 and D-galactosamine induced liver toxicity in animal models (Gao *et al.,* 2004; Wang *et al.,* 2017). Betulinic acid, a well known hepatoprotective triterpene has been isolated from *Ziziphus jujuba*. This plant has exhibited anti-hepatotoxic activity against CCl_4 in mice by antioxidant activity and augmentation of the Nrf2 pathway (Liu *et al.,* 2017).

Ziziphus oxyphylla (ZO) Edgew (*Rhamnaceae*) has been investigated for insecticidal, antibacterial, antifungal, cytotoxic, phytotoxic, urease inhibition and analgesic activities. Oxyphylline D, nummularin R, nummularin C (cyclopeptide alkaloids) have been isolated from ZO (Nisar *et al.,* 2010). Similarly, antiglycation and antioxidant compounds (glycosides of kaempferol and quercetin) have been isolated from ZO leaves. Furthermore, antioxidant and acetylcholinestrase inhibition activity of this plant has also been reported (Mazhar *et al.,* 2015).

Keeping in view the folkloric uses and constituents, the current research was carried out to investigate the effectiveness and possible mechanism for hepatoprotection of ZO.

MATERIALS AND METHODS

Plant material, extraction and fractionation: ZO roots were collected from Malakand, Khyber Pakhtunkhwa Pakistan, verified by a Taxonomist from Botany Department, University of Peshawar and the voucher specimen was submitted to Herbarium University of Malakand (specimen number H.UOM.BG. 211). After shade drying it was ground, weighed (5 kg) and soaked in methanol (25 L) for one week followed by filtration. The rotary evaporator was used to concentrate the filtrate at 45°C so as to get the crude methanolic extract that was fractionated into *n*-hexane (NHF), chloroform (CF), ethyl acetate (EAF) and aqueous fractions (AQF).

Isolation of compounds and structure determination: The chloroform fraction was column chromatographed using silica gel for compound isolation. CF (40 g) was loaded to a column, subsequently eluted with pure *n*-hexane and then with ethyl acetate: *n*-hexane in percentage of 5-100. Collected fractions were rotary evaporated at 45°C (de Dieu Tamokou *et al.,* 2012). Fractions having the same R_f value were pooled for repeated chromatography. Isolated compounds structures were determined by EI-MS, ^{1}HMR, and 2D-NMR techniques and comparison of obtained data with literature (Muhit *et al.,* 2010).

Experimental animals: BALB/c mice were obtained from the National Institute of Health (NIH) Islamabad, Pakistan. They were housed in stainless steel cages in the animal house for one week for acclimatization, maintained on a standard diet and 12/12 h dark and light cycle. Approval for experimental protocol was granted by the Research Ethics Committee of the Pharmacy Department, University of Malakand vide letter No. DREC/20160503-15.

Acute toxicity study: Acute toxicity was investigated by oral administration of methanol extract (ZOME) to healthy BALB/c mice according to OECD guidelines 425 on acute oral toxicity (Schlede *et al.,* 2005). The control group was administered vehicle and the test groups (n=6) were orally treated with ZOME at 250-2000 mg/kg. Mice were kept under continuous observation for toxicity and mortality for 1 h and then intermittently for the next 6 h and then at 24 h.

Hepatoprotective activity

Direct method: The experimental animals were randomly grouped into seven having five animals per group. Group A was administered liquid paraffin 0.8 ml/kg body weight/day, i.p. Group B animals were administered CCl_4 in liquid paraffin i.p (30% solution v/v, 0.8 ml/kg body weight/day i.p). Group C mice were treated with CCl_4 (0.8 ml/kg i.p) and silymarin 100 mg/kg p.o. Similarly, groups D-G were treated with ZOME and CF at 200 and 400 mg/kg p.o for one week along with CCl_4 (Navarro *et al.,* 1993).

Biochemical investigation: After experimental protocol, the mice were immediately humanely killed; blood was collected followed by serum separation which was used for determination of alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T.B) and total protein (T.P) as per the manufacturer instructions.

Estimation of antioxidant enzymes: The excised liver (1 g), after homogenization in phosphate buffer (10 ml, pH 7.4, 50 mM), was subjected to determination of catalase (CAT) activity according to previously published protocol (Beers *et al.,* 1952). The reaction mixture (3 ml) was composed of phosphate buffer (1.9 ml, pH 7.0), H_2O_2 (1 ml) and liver homogenate (0.1 ml). The absorbance

change at 240 nm was used to calculate the activity of CAT via UV–visible spectrophotometer. The activity was expressed as units of H_2O_2/mg of tissue.

The activity of superoxide dismutase (SOD) was determined as mentioned previously (Saggu *et al.,* 1989). Sodium carbonate buffer (0.05 Mm, 2.8 ml) was incubated at 30°C for 45 min with 0.1 ml of liver homogenate. The sample was added with the solution of adrenaline (10 µL, 9 mM) and the absorbance was determined at 480 nm. The activity was presented in the form of a unit of SOD /mg of tissue.

Lipid peroxidation: Homogenate of the liver (0.1 ml) was added with trichloroacetic acid (2.0 ml, 20%) followed by centrifugation. The supernatant so obtained (2 ml) was mixed with 2 ml of thiobarbituric acid reagent (0.67%). Incubation of the mixture was done for 20 min at 100°C in water bath and then the absorbance was determined at 532 nm in a double beam spectrophotometer.

In the same way, preparation of 5-25 nmoles of MDA (standard) and the blank was done. Lipid peroxidation was expressed as moles MDA per 100 mg of protein (Sevanian and Ursini, 2000).

Indirect method

Phenobarbital induced sleeping time (PST): PST was determined according to previously reported protocol (Anand *et al.,* 1992). The experiment was carried out as mentioned in the hepatoprotective activity protocol. On day 7, the animals were treated with phenobarbital sodium (40 mg/kg i.p) and observed for PST.

Membrane stabilizing potential

Erythrocyte suspension preparation: Fresh blood (5 ml) was collected from healthy human volunteers and centrifuged for 10 min at 3000 rpm. The supernatant was collected and added with normal saline (1:1). Dissolved red blood pellet volume was measured and reconstituted with the buffered isotonic solution in the form of suspension (40% v/v). The reconstituted erythrocyte suspension was used for the determination of hypotonic solution and heat-induced hemolysis assays.

Hypotonic solution induced hemolysis inhibition: About 50 µL of RBC suspension (40%) was mixed with hypotonic buffer (1.0 ml) and 5 ml of the extracts (100-1000 µg/ml). After incubation at room temperature for 20 min, it was centrifuged (1300 rpm, 3 min). The absorbance of the resultant supernatant was measured at 540 nm. Sodium salicylate, the standard drug (5 ml, 200 µg/ml), was also treated similarly. Percent hemolysis inhibition of RBC by the extracts and standard was determined (Shinde *et al.,* 1999).

Heat induced haemolysis inhibition: Extracts (5 ml, 100-1000 µg/ml) and suspension of RBCs (50 µL) were separately mixed in test tubes and incubated in a water bath for 20 min at 54°C. The other pair was kept in a freezer at -10°C for 20 min. The tubes were centrifuged for 3 min at 1300 rpm and the supernatant was used for estimation of haemoglobin content at 540 nm. The control samples contained RBC suspension (50 µL), 5 ml of 200 µg/ml of sodium salicylate and 5 ml of the vehicle (Shinde *et al.,* 1999). Hemolysis inhibition (as a percentage) by samples/standard was calculated.

Histopathological study: The excised liver was washed with phosphate buffered saline (PBS) followed by fixation in neutral buffered formalin overnight, embedded in paraffin, cut into 4 µ sections with microtome, subsequently stained with H&E dye and observed for histomorphological changes under light microscope (Johnston and Kroening, 1998). Histopathological scoring was carried out according to previously published procedure (Michael, 2008).

Body and liver weight changes: Weight of mice was determined at the start and end of experiment, the percent body and liver weight changes were calculated.

Hepatic Index: Hepatic Index was determined from the ratio of absolute liver weight to the body weight of mice at the end of experimental study (Huang *et al.,* 2012).

Liver index = Liver weight ÷ body weight × 100

Statistical analyses: The results are presented as mean±SEM. Statistical analyses were performed by SPSS version 16 with one-way ANOVA followed by *post hoc* Tukey test. Value of $P<0.05$ was considered significant.

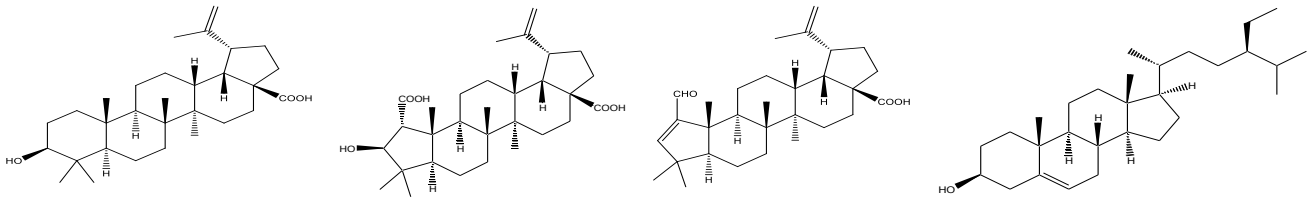
RESULTS

Extraction and compounds isolation: The crude methanolic extract was obtained as semisolid brownish residue with 5.85% yield, while CF was obtained in a percentage yield of 0.7%. It has already been found that crude methanolic extract of ZO contains phenols, flavonoids, tannins, saponins, triterpenes alkaloids and carbohydrates which was in agreement with previous studies (Ahmad *et al.,* 2014). Among various solvent extracts, CF was chosen for the isolation of compounds due to its better hepatoprotective activity. From the NMR and MS data of compounds isolated from CF (spectra appended as supplementary data), the structures were confirmed (Muhit *et al.,* 2010). The isolated compounds were identified as betulinic acid (AZO_1), ceanothic acid (AZO_2), zizybrenalic acid (AZO_3) and beta sitosterol (AZO_4) as mentioned in Fig. 1.

Acute toxicity: ZOME up to 2 g/kg body weight was found safe as shown by lack of writhing, convulsions, respiratory distress, diarrhea, drowsiness and mortality.

Hepatoprotective activity

Biochemical investigation: The effect of CCl_4, standard, ZOME and CF on biochemical parameters is shown in Fig. 2 and Fig. 3. Administration of CCl_4 increased the serum level of ALT, ALP and TB with a significant decrease in the level of TP as compared to the control group. Moreover, concurrent administration of ZOME (200 and 400 mg/kg) and silymarin restored the serum biomarkers exhibiting hepatoprotective potential. CF exhibited more pronounced protection as observed from the reduced serum level of the aforementioned parameters while TP was enhanced at both doses (200 and 400 mg/kg) which is comparable to silymarin.



AZO_1 (betulinic acid) AZO_2 (ceanothic acid) AZO_3 (zizybrenalic acid) AZO_4 (beta sitosterol)

Fig. 1: Structure of phytochemicals.

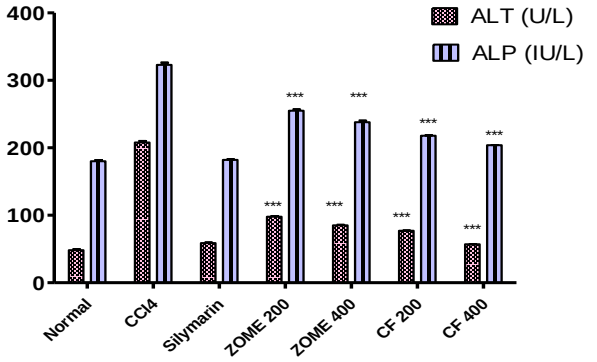


Fig. 2: Effect of ZO on ALT and ALP**.** ***P<0.001 as compare to control. Data was analyzed by one way ANOVA followed by *post hoc* Tukey test.

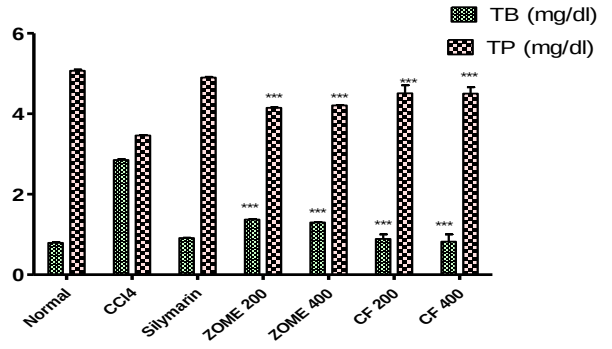


Fig. 3: Effect of ZO on T.B and T.P**.** ***P<0.001 as compare to control. Data was analyzed by one way ANOVA followed by *post hoc* Tukey test.

Antioxidant parameters and Lipid peroxidation: Activities of CAT and SOD were reduced and MDA level was increased in CCl_4 administered mice as compared to normal control group indicating marked oxidation of hepatocellular membranes. The concurrent administration of ZOME increased the activities of CAT, SOD and decreased the level of MDA, signifying hepatoprotective effect of ZO as compare to CCl_4 (***P<0.001) (Fig. 4). Administration of CF at 400 mg/kg exhibited more pronounced elevation in CAT and SOD levels and restored them near to normal group and higher than the silymarin group. While the MDA level was higher than the normal control and close to the silymarin group.

Phenobarbital induced sleeping time: The administration of CCl_4 increased the PST in comparison to normal control (P<0.001). On the contrary, the administration of ZOME and CF reduced PST in comparison to CCl_4 (P<0.001) indicating restoration of metabolic function of the liver (Fig. 5).

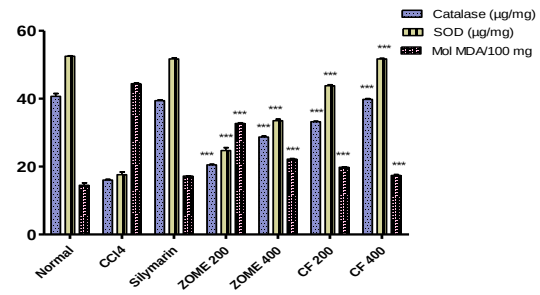


Fig. 4: Effect on antioxidant parameters**.** ***P<0.001 as compare to control. Data was analyzed by one way ANOVA followed by *post hoc* Tukey test.

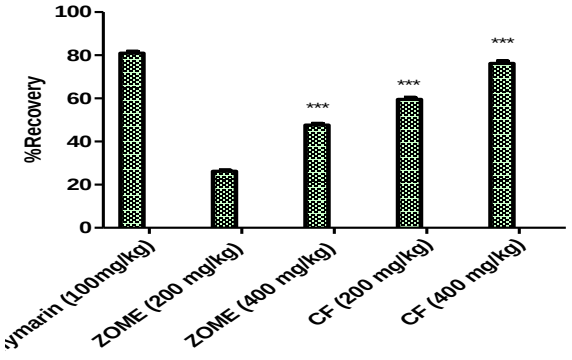


Fig. 5: Effect of ZO on phenobarbital induced sleeping time**.** ***P<0.001 as compare to control. Data was analyzed by one way ANOVA followed by *post hoc* Tukey test.

Membrane stabilizing activity

Hypotonic solution induced hemolysis inhibition: ZOME significantly inhibited hemolysis of erythrocytes as shown in Fig. 6. The inhibition observed was (72.5±2.04% at 1000 µg /ml) as compared to standard (76.43±3.22%).

Heat-induced hemolysis inhibition: ZOME inhibited heat-induced lysis of erythrocytes as shown in Fig. 6. The membrane- stabilizing activity shown by the ZOME was 70.37±1.15% at 1000 µg /ml.

Changes in body and liver weight: Loss of body weight was observed in CCl_4 administered mice that were counteracted by ZO as shown in Table 1. Moreover, CCl_4 administration resulted in marked hepatic intumescence associated with an elevation in liver weight and liver index as compared to the control group (P<0.05). Administration of ZOME and CF resulted in the restoration of the liver and body weight as compared to control (P<0.05).

Table 1: Effect of ZO on body weight, liver weight and liver index

Group	Initial weight	Final weight	% Change	Liver weight (g)	Liver Index %
Normal	22.48±0.787	25.72±0.76	14.41	1.57±0.044	6.104±0.75
CCl4	24.55±0.650	22.49±0.56	-8.39	1.69±0.047	7.514±0.84
Silymarin	24.48±0.346	25.42±0.300	3.84	1.59±0.033	6.254±0.43
ZOME 200	23.63±1.02	22.70±1.33	-3.94	1.61±0.022	7.09±0.12
ZOME 400	23.25±0.93	23.87±0.65	2.66	1.58±0.05	6.62±0.58
CF 200	25.72±0.21	26.05±0.84	1.28	1.59±0.07	6.103±0.26
CF 400	25.21±0.330	25.44±0.55	0.92	1.57±0.04	6.171±0.29

Table 2: Histomorphological effects of ZO on liver

Observation	Control	CCl_4	Silymarin	ZOME 200 mg	ZOME 400 mg	CF 200 mg	CF 400 mg
Fatty Changes	-	+++	+	++	+	+	-
Piecemeal necrosis	-	+++	+	++	+	+	-
Centrilobular necrosis	-	+++	-	+	-	-	-
Congestion in sinusoids	-	+++	+	+	-	-	-
Lymphocyte infiltration	-	+++	+	+	++	+	+
Central vein degeneration	-	++	-	-	-	-	-

-, absent; +, minimal, mild; ++, moderate; +++, severe, ++++, very severe.

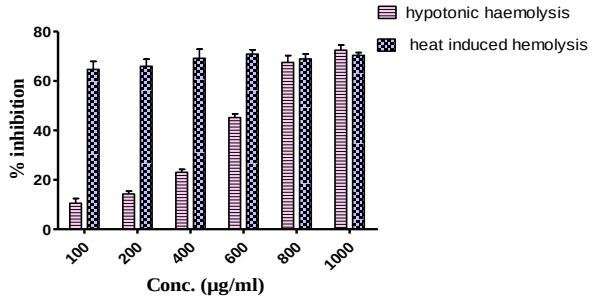


Fig. 6: Percent inhibition of hemolysis by ZOME.

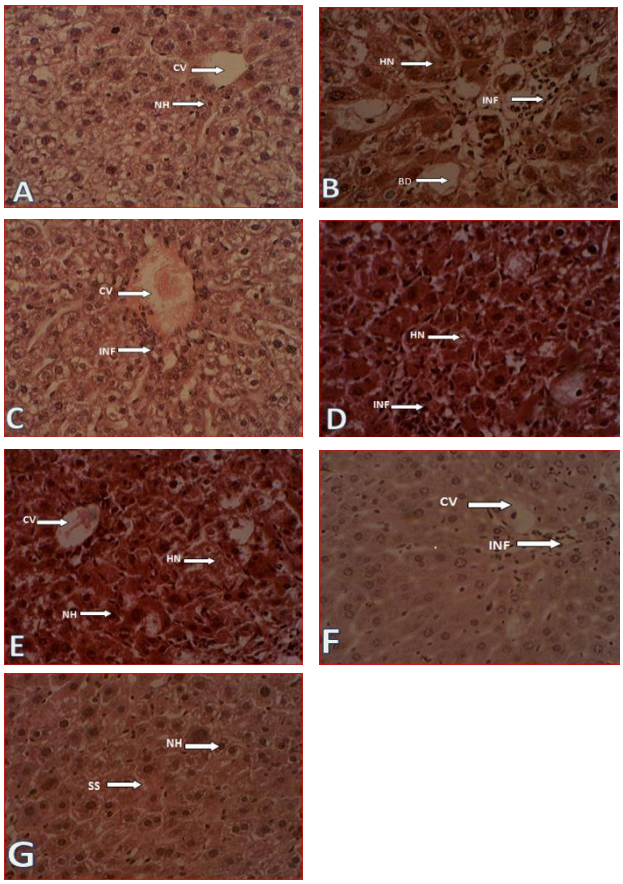


Fig. 7: Light micrograph of liver of ZO treated mice (A) normal control mice (B) CCl_4 intoxicated mice (C) silymarin administered mice (D) ZOME (200 mg/kg) administered mice (E) ZOME (400 mg/kg) administered mice (F) CF (200 mg/kg) administered mice (G) CF (400 mg/kg) administered mice. CV=Central venule, NH=Normal hepatocyte, INF=Cellular infiltration, BD=Ballooning degeneration, HN=Hepatocyte necrosis, SS=Sinusoids.

Histopathological study: Animals treated with CCl_4 exhibited extensive hepatocellular necrosis, severe sinusoidal congestion, fatty changes and lymphocytic infiltration as shown in Table 2 and Fig. 7A, 7B. The mice treated with ZOME (200 mg/kg) exhibited moderate necrosis, fatty changes, mild infiltration and sinusoidal congestion (Fig. 7C, 7D). While ZOME at 400 mg/kg resulted in mild fatty changes, hepatocyte necrosis and moderate infiltration of lymphocytes as compared to standard (Fig. 7E). Moreover, the administration of CF had a more pronounced restoration of CCl_4 induced histological damage (Fig. 7F, 7G).

DISCUSSION

ALT is mainly located in hepatocytes cytosol and its elevated serum level suggest leakage due to liver cells injury. The rise in serum ALP level indicates hepatocytes damage and bile flow obstruction (Dong *et al*., 2014). In the current study, a significant rise in ALT, T.B, ALP, and fall in the level of T.P was observed due to the administration of CCl_4. The level of these biomarkers was restored by ZOME and CF extracts shows their effectiveness as a hepatoprotective agent.

Oxidative stress, occurs when the antioxidant system is overwhelmed, is implicated in the progression of hepatotoxicity that ultimately damage the macromolecules including lipids, DNA and protein (Habib *et al.*, 2015). Parameters of oxidative stress such as SOD, CAT and MDA levels were determined in this study because many medicinal plants exert their hepatoprotective effect due to antioxidant and membrane-stabilizing activity (Abdullah *et al.*, 2017). Administration of ZO extracts decreased MDA level that signifies decreased ROS generation. While the levels of CAT and SOD were raised by ZO extracts due to restoration of the balance of the antioxidant enzyme system. Reduction in the level of MDA and improvement of endogenous antioxidants is probably caused by triterpenes which is in corroboration with previous studies.

One of the possible interventions to treat hepatotoxicity is inhibition of Cytochrome p450 enzyme system as it is involved in the formation of more potent hepatotoxic metabolites. Previous studies have shown cytochrome p450 inhibitory potential of triterpenes (Kim *et al.*, 2004). Cytochrome p450 enzymes of the liver metabolizes phenobarbital. Any drug which can inhibit this enzyme system will increase the duration of

phenobarbital induced sleeping time and vice versa. In the case of hepatotoxicity induced by CCl_4, prolongation of righting reflex occurs due to the destruction of the cytochrome P450. In the current study, the shortening of PST observed after administration of ZOME and CF shows the ability of these extracts to inhibit this enzyme.

The lysis of the erythrocyte membrane occurs due to heat or hypotonic solution, with consequent hemolysis and oxidation of haemoglobin. During hepatotoxicity and liver inflammation, there is lysis of lysosomes causing biomarkers release. NSAIDs exert their antiphlogistic effect by stabilizing the membrane of lysosomes or inhibition of lysosomal enzymes. Inhibition of hemolysis has been considered as a measure of hepatocyte membrane stabilization and thus inhibition of release of liver biomarkers due to the similarity between RBC and lysosomal membranes (Shinde *et al.,* 1999). Therefore, an extract that stabilizes the membrane of lysosomes can inhibit the release of enzymes from lysosomes to the surrounding tissue. ZOME successfully inhibited hypotonic solution and heat-induced hemolysis indicating its potential as a cell membrane stabilizing agent.

Histopathological investigation of mice liver treated with CCl_4 exhibited hepatocellular necrosis, infiltration of inflammatory cells (Fig. 7B). However, these pathological alterations were not observed in ZO extracts administered animals (Fig. 7D, 7E) reflecting potential hepatoprotective activity. The liver specimens of CF treated mice exhibited more efficient protection of hepatic architecture except for cellular infiltration (Fig. 7F, 7G).

Changes in the weight of the body and liver caused by chemicals are indicators of tissue injury and recovery. It has been considered that progressive loss of body weight is associated with reduced food intake and absorption after the administration of CCl_4 (Chao *et al*., 2019). ZOME and CF improved the loss in body weight caused by CCl_4 intoxication. Moreover, CCl_4 treatment resulted in the increase of liver weight that was attenuated by the plant extracts (Table 1).

Conclusions: It can be concluded from the current study that solvent extracts of ZO show hepatoprotective activity. Moreover, CF offered good hepatoprotection against CCl_4 due to the presence of triterpenes especially betulinic acid. The possible mechanism for the overall observed hepatoprotective activity of the plant is due to the protection of endogenous antioxidant defense system and membrane stabilization activity that help to ameliorate the biochemical and morphological alterations in liver of mice. Moreover, the activity of isolated compounds is in progress in our lab.

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Authors contribution: Abdullah carried out experimental work under the supervision of MAK and AA while WA, AK and MK helped in the refining of the manuscript. MI and MR helped in the interpretation of data.

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