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Ameliorative Effects of *Acrocarpus fraxinifolius* Leaves Extract Against Liver Damage Induced in Wistar Rats by Acetaminophen.

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ABSTRACT

This study evaluates the possible protective effects of hexane extract of *Acrocarpus fraxinifolius* leaves (*A. fraxinifolius*) against experimental paracetamol (PCM) induced hepatotoxicity in rats. Twenty four male adult Wistar rats were divided into four groups. Group I was the negative control group that received distilled water only, group II that received 500 mg/kg of *A. fraxinifolius extract*, group III was received 600 mg/kg of PCM. Group IV was pre-treated with 500 mg/kg of the hexane leave extract of *A. fraxinifolius* before inducing the liver damage on the 22th day with 600mg/kg of PCM. Forty eight hours after administration, the rats were sacrificed. PCM induced a significant body weight loss with rise in liver relative weight, serum aspartate amino transferase (s-AST), alanine amino transferase (s-ALT), lipid profile, hepatic NOx and MDA with a reduction of serum protein profile, total antioxidant capacity (TAC), hepatic GSH and hepatic SOD activity. The plant extracts showed a remarkable hepato-protective and antioxidant activity against PCM induced hepato-toxicity. Also the extract down regulate the expression of pro- inflammatory cytokines (IL-1β and TNF-α) and up regulate the anti-inflammatory IL-10. *A. fraxinifolius* extract administration ameliorated paracetamol-induced alterations in genes expression of inflammatory cytokines.

Keywords: *Acrocarpus fraxinifolius,* paracetamol, liver, IL-1β, TNF-α.

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INTRODUCTION

Scientists have been paying serious attention to medicinal herbal therapies that have anti-inflammatory and anti-oxidant properties with minimum side effects in the treatment of many diseases. Fabaceae embraces several genera of reported antioxidative and hepatoprotective species (Gamal El-Din *et al.* 2014; Rehman *et al.* 2015;Abd El-Ghffar*et al.,* 2016). *Acrocarpusfraxinifolius*(*A. fraxinifolius*) is a member of the tribe caesalpinieae (family:Fabaceae) (El-nashar *et al.*, 2015). It is a native widespread tree around the world, especially in Africa and Asia (Suirez*set al.,* 2015). Phytochemical studies on this plant revealed the presence of various secondary metabolites including eight flavonoids: quercetin, quercetin 3-O-β-D-glucopyranoside, quercetin3-O-α-L-rhamnopyranoside, myricetin, myricetin 3-O-β-D-galactopranoside, myricetin 3-O-α-L-rhamnopyranoside, desmanthin-1 and naringenin, in addition to four phenolic acids, brevifolin carboxylic acid, ellagic acid, gallic acid and methyl gallate (El-Kashak *et al.,* 2016). *A. fraxinifolius* was reported to have anti-diabetic, anti-proliferative, anti-inflammatory, antioxidant and hepato-protective activities *in-vivo* (AbouZeid *et al.,* 2011; Moustafa*et al.,* 2014;Abd El-Ghffar *et al.,* 2016). Until now, *there* is *not* relevant data on the *hepato-protective effect* of *A. fraxinifolius.* Therefore, the present study was carried out to investigate the hepato-protective activity of hexane extract of *A. fraxinifolius leaf* against PCM induced hepatic damage in male rats.

MATERIAL AND METHODS

Chemicals

Paracetamol (PCM) or acetaminophen (*N*-acetyl-*p*-aminophenol; APAP) was purchased from *Sanofi-aventisegypts.a.e., El Sawah*, El Amiriya, Cairo, *Egypt.*

Preparation of the extract

The plant leaf extract was collected from Giza Zoo Botanical Garden, on January (2015). Leaves of *A. fraxinifolius* were prepared by following the method of Abd El-Ghffar *et al.* (2016). Briefly, 2kg powder of *A. fraxinifolius* was soaked in 80% methanol for 4days and filtered. The filtrate was completely evaporated *in vacuo* at ≈ 55 °C till complete dryness. The dried residue was further successively fractionated with *n*-hexane. The combined *n*-hexane extracts were evaporated *in vacuo* until dryness to give 50 g of a sticky dark greenish material. *N*-hexane extract of *A. fraxinifolius* leaves obtained was preserved in a sterile glass container at 4 °C until further use.

Animals

The present study was carried out at Zoology Department, Faculty of Science, Ain Shams University, using clinically healthy mature adult male Wistar albino rats. The animals were obtained from the Animal Breeding House of the National Research Centre (NRC), Dokki, Cairo, Egypt, and maintained in clean plastic cages in the laboratory animal house. Their weights ranged from (150-200g) each. On standard pellet diet (Agricultural-Industrial Integration Company, Giza, Egypt), tap water *ad libitum*, and daily dark/light cycle (12/12 hrs.). All animals were humanely treated in accordance with WHO guideline for animal care and the study design was approved by the Ain Shams University Research Ethics Committee. The animals were acclimatized to the laboratory conditions for 2 weeks before being experimented.

Experimental groups

Rats were divided into four groups, each has 6 rats. Control animals (group 1) received an equal volume of distilled water (5 ml/kg b. w) daily for 21 days. The second group of animals received 500 mg/kg hexane leave extract of *A. fraxinifolius* (Abd El-Ghffar*et al.,* 2016) by oral route using a gastric gavage tube, for 21 days. Group three (PCM group) received dist. water daily for 21 days, then the animals received a single oral dose of PCM (600 mg/kg) dissolved in distilled water on day 22. The fourth group (pretreated group) was administered *A. fraxinifolius leaves extract* for 21 days then the animals received a single oral dose of PCM was administered by the same routeon day 22. The rats were sacrificed 48 h after the last administration of PCM or *A. fraxinifolius extract (*on day 24). The blood was collected into clean test-tubes without anticoagulant, centrifuged in a cooling centrifuge for 30 min at 3000 rpm and 4°C to separate the serum and divided into aliquots and preserved at -80^{0}C for further analysis. Liver tissue samples were washed in ice cold physiological

saline and weighted. Portion of liver tissue was homogenized in 5 ml of 50mM potassium phosphate (pH 7.4) per gram tissue. The homogenate was centrifuged at 4000 rpm for 15 min. at 4 °C. The supernatant was divided into aliquots and preserved at −80°C until used.

Biochemical analysis

Determination of s-ALT and s-AST according to Reitman and Frankel, 1957.Determination of total protein, albumin, total lipid, total cholesterol, triglycerides and HDL- cholesterol in serum were carried out as described by Gornal *et al.* (1949), Dumas *et al.* (1971), Knight *et al*. (1972), Richmond (1973), Fassati and Principle (1982) and Burstein *et al.* (1949), respectively. Serum total antioxidant capacity (TAC) was carried out according to Koracevic *et al.,* 2001. Nitric oxide (NO), malondialdehyde (MDA), superoxide dismutase (SOD) and reduced glutathione (GSH) in liver homogenate were estimated by the spectrophotometric method described by Miles *et al.,* 1996, Mihara and Uchiyama, 1978, Nishikimi *et al.,* 1972 and Beutler *et al.,* 1963, respectively. Serum LDL-cholesterol and total globulins level were calculated according to the following equations: LDL-cholesterol = total cholesterol - (TAG/5) - HDL-cholesterol; Total globulins = total protein – albumin.

Gene expression analysis

RNA extraction

Total RNA was extracted from liver tissue samples (approximately 100 mg per sample) of experimental rats. Liver samples were flash frozen in liquid nitrogen and subsequently stored at −70°C in 1 ml Qiazol (QIAGEN, Valencia, CA, USA). Frozen samples were homogenized. Then, 0.3 ml chloroform was added to the homogenate. The mixtures were shaken for 30 seconds, followed by centrifugation at 4°C and 16,400 × g for 15 min. The supernatant was transferred to a new set of tubes, and an equal volume of isopropanol was added to the samples, shaken for 15 seconds and centrifuged at 4°C and 16,400 × g for 15 min. The RNA pellets were washed with 70% ethanol, briefly dries up, and then dissolved in diethylpyrocarbonate (DEPC) water. RNA concentration and purity were determined spectrophotometrically at 260 nm.. The ratio of the 260/280 optical density of all RNA samples was 1.7-1.9.

Complementary deoxyribonucleic acid (cDNA) synthesis

For cDNA synthesis, a mixture of 3 µg total RNA and 0.5 ng oligo dT primer (Qiagen Valencia, CA, USA) in a total volume of 11 µl sterilized DEPC water was incubated in the Bio-Rad T100™ Thermal cycle at 65°C for 10 min for denaturation. Then, 2 µl of 10X RT-buffer, 2 µl of 10 mM dNTPs and 100 U Moloney Murine Leukemia Virus (MMuLV) Reverse Transcriptase (SibEnzyme. Ak, Novosibirsk, Russia) were added and the total volume was completed up to 20 µl by DEPC water. The mixture was then re-incubated in BIO-RAD thermal cycler at 37°C for one hour, then at 90°C for 10 min to inactivate the enzyme.

Quantitative PCR analysis

Table 1: Primer sequence and PCR conditions for rat cytokines

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	PCR cycles and conditions
IL-1β (218 bp)	ATGGCAACCGTACCTGAACCCA	GCTCGAAAATGTCCCAGGAA	30 cycles, 61°C 1 min
TNF-α (256 bp)	CCACCACGCTCTTCTGTCTAC	ACCACCAGTTGGTTGTCTTTG	30 cycles, 58°C 1 min
IL-10 (320bp)	GGAGTGAAGACCAAAGG	TCTCCCAGGGAATTCAAATG	30 cycles, 57°C 1 min
GAPDH (309 bp)	AGATCCACAACGGATACATT	TCCCTCAAGATTGTCAGCAA	25 cycles, 52°C 1 min

For quantitative RT-PCR analysis, specific primers for examined genes (Table 1) were used. PCR was conducted in a final volume of 25 µl consisting of 1 µl cDNA, 1 µl of 10 pM of each primer (forward and reverse), and 12.5 µl PCR master mix, the volume was brought up to 25 µl using sterilized, deionized water. PCR was carried out using Bio-Rad T100™ Thermal Cycle machine with the cycle sequence at 94°C for 5 minutes one cycle, followed by variable cycles (Table 1) each of which consists of denaturationat 94°C for one

minute, annealing at the specific temperature corresponding to each primer (Table 1) and extension at 72°C for one minute with an additional final extension at 72°C for 7 minutes. As a reference, expression of glyceraldehyde-3-phosphate dehydrogenase(GPDH) mRNA was examined (Table 1).

Statistical Analysis

Data are presented as mean ±SE. Statistical analysis was performed with ANOVA, and the differences among groups were determined by Tukey's multiple comparison test using Graph Pad Prism version 4.03 for Windows (GraphPad software Inc., San Diego, CA, USA).

RESULTS

Biochemical results

Table (2) and fig (2) revealed that serum hepatic enzymes ALT and AST and lipid profile showed a highly significant increase in PCM group when compared with control group (P<0.05-0.001). On the other hand, HDL-c, total protein, total albumin and TAC were were significantly decreased (P<0.05) when compared with the normal control.

While in pre-treated group with *A. fraxinifolius leaves* extract for 21 days before PCM toxicity the level of s-ALT, s-AST, HDL-c and LDL-c total protein, total albumin and TAC return to the normal level.

Figure (2) shown that, PCM administration caused a highly significant increase (*P*<0.001) in hepatic nitric oxide and MDA levels, in contrast, it induced a highly significant decrease (P<0.001) in hepatic SOD activity and GSH level, as compared to control group. Oral administration with *A. fraxinifolius leaves* extract significantly decrease (P<0.05-0.01) hepatic nitric oxide and MDA levels with significant increase in hepatic SOD activity comparing with PCM group. In addition, *A. fraxinifolius leaves* extract treatment 21 days before PCM toxicity succeeded in returning the reduction in GSH level to normal level. Rats consumed *A. fraxinifolius* extract showed significant decrease in hepatic MDA and increase in and GSH levels (P<0.05) compared with the control animals.

Table 2: Effect of the *A. fraxinifolius leaves* extract on serum biochemical parameters in different studied groups

Groups / Parameters	Control	*A. fraxinifolius* (mg/kg)	PCM only(600 mg/kg)	PCM + *A. fraxinifolius* 500
ALT (U/L)	45.88 ± 1.20	42.03 ± 1.08	116.50 ± 1.72 ***	52.72 ± 2.532 †††
AST (U/L)	63.05 ± 2.27	57.59 ± 2.09	146.60 ± 3.65 ***	73.73 ±2.716†††
Total lipid (mg/dl)	618.20 ± 5.35	616.10 ± 3.95	750.10 ± 7.58 ***	644.40 ± 3.89 * †††
Total cholesterol (mg/dl)	119.30 ± 1.70	117.60 ± 2.56	167.00 ± 1.15 ***	135.90 ± 1.57*** †††
TAG (mg/dl)	88.89 ± 1.81	88.57 ± 1.49	176.20 ± 2.01 ***	105.20 ± 1.82 *** †††
HDL-c (mg/dl)	54.21 ± 1.59	56.67 ± 0.98	35.88 ± 1.56***	49.06 ± 1.62†††
LDL-c (mg/dl)	155.80 ± 2.37	156.60 ± 2.21	167.60 ± 2.10*	163.90 ± 2.98
Total protein (g/dl)	6.36 ± 0.34	6.51 ± 0.28	4.87 ± 0.10 **	6.08 ±0.37†
Albumin (g/dl)	3.39 ± 0.12	3.73 ± 0.09	2.27 ± 0.05 ***	3.04± 0.13†††
Globulin (g/dl)	2.97 ± 0.41	2.85 ± 0.21	2.63 ± 0.14	2.35 ± 0.21
TAC (mM/L)	0.79 ± 0.02	0.90 ± 0.03	0.58 ± 0.02***	0.71 ± 0.02†††

Values expressed as mean ± SE., n = 6 rats per group. *A. fraxinifolius*: *Acrocarpusfraxinifolius*, PCM: paracetamol. **P*<0.05, ****P*<0.001: compared with the control group; †††*P*<0.001: compared with the PCM intoxicated group that received vehicle; (One-Way ANOVA with Tukey 's multiple comparison test).

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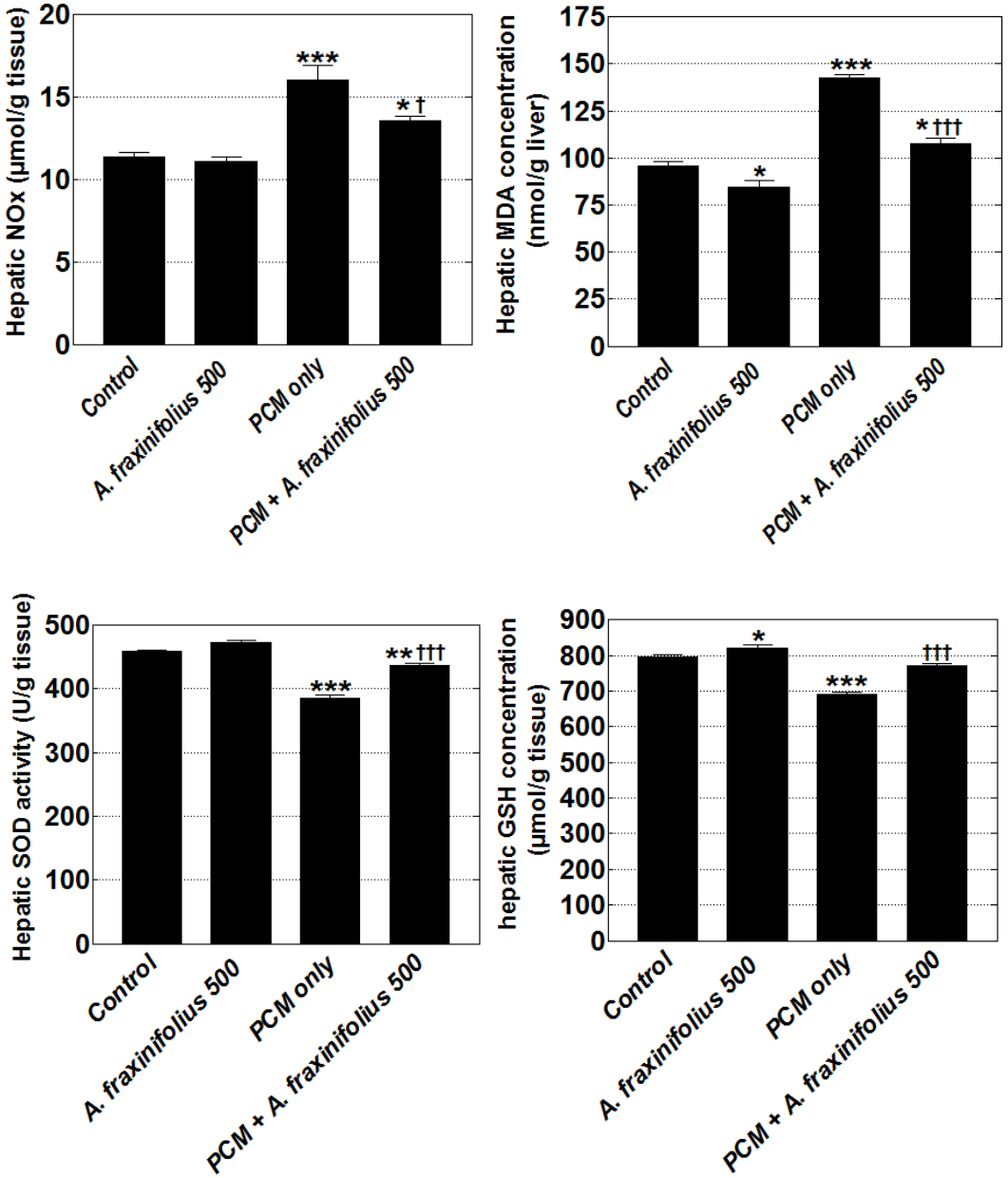

Values are means± SE. *A. fraxinifolius*: *Acrocarpusfraxinifolius*, PCM: paracetamol. **P*<0.05, ***P*<0.01, ****P*<0.001: compared with the control group; †*P*<0.05, †††*P*<0.001: compared with the APAP intoxicated group that received vehicle; (One-Way ANOVA with Tukey 's multiple comparison test).

Figure 2: Effect of the *A. fraxinifolius* extract on hepatic NO, MDA, SOD and GSH of different studied groups.

Results in table (2) and fig (2) cleared that admission of *A. fraxinifolius* leaves extract to the experimental animals do not affect on the measured biochemical parameters when compared to the normal control.

RT-PCR analysis of hepatic cytokine expression

Paracetamol significantly up-regulated ($p < 0.05$) IL-1 β and TNF-α expression compared to control group (table 3). *A. fraxinifolius* leaves extract alone has a minor effect on IL-1β and TNF-α expressions; however, it increased IL-10 expression. *A. fraxinifolius* leaves extract plus paracetamol administration normalized the increase in IL-1β and TNF-α expressions that are observed in paracetamol administered group.

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Table 3: Effect of *A. fraxinifolius* extract on mRNA levels of some cytokines

Groups / Gene	Control	*A. fraxinifolius* (mg/kg)	PCM (600 mg/kg)	PCM + *A. fraxinifolius* (500mg/kg)
IL-1β	1.0 ± 0.35	0.9 ± 0.24	2.1 ± 0.61**	1.4 ± 0.06*
TNF-α	1.0 ± 0.64	1.1 ± 058	1.9P ± 0.27 **	1.2 ± 0.14
IL-10	1.0 ± 0.26	1.06 ± 0.03	1.4 ± 0.58*	2.3 ± 0.71**

The data represent the mean ±S.E values;(*) Significant different at P <0.05 and (**) highly significant at P <0.001

DISCUSSION

The present study aims to evaluate the possibility of using a natural extract as a protective for liver injury. Herbal plants and plant-derived medicines which possess flavinoides have been widely used in the treatment of hepatic diseases for a long time. Where the flavonoids compounds have a potential hepato-protective effect due to its antioxidant properties (Kumar *et al*., 2011 and El-Gendy, 2012; Azab *et al.,* 2013).

In the current study PCM administration lead to a highly significant increase in s-AST, s-ALT, serum cholesterol and triglyceride levels. These elevated levels of serum hepatic enzymes are indicative of hepatic cellular leakage and loss of functional integrity of cell membranes (Larrey, 2003). On the other hand, a significant decrease in serum total protein and albumin levels are related to the function of the hepatic cells revealing the functional status of the hepatic cell (Basu *et al.,* 2012).

The presence of these flavinoid compounds in *A. fraxinifolius* extract may be responsible for the protective effect on PCM induced liver damage in rats. Pre-treatment with *A. fraxinifolius* hexane extract offered protection by preserving the structural integrity of the hepatic membrane against PCM toxicity.

In the present study, *A. fraxinifolius* leaves extract decreased the elevated serum lipid profile with the elevation of total protein and albumin concentration in the PCM treated rats. The extract also ameliorated the harmful effect PCM on liver of male rats. These results are in agreement with Abd El-Ghffar*et al.* (2016) who observed a significant decreases in serum hepatic markers and serum lipid profile by *A. fraxinifolius* hexane extract in male rats intoxicated with PCM. In the present investigation, reported that PCM oral administration in male rats decreased serum TCA as evidenced by decreased activity of the antioxidant defense mechanisms to prevent overproduction of reactive oxygen species (ROS). Lipid peroxidation(LPO), expressed as MDA, in hepatic tissue increased significantly in in male rats intoxicated with PCM. While, the activities of protective enzymes such as hepatic SOD activity and GSH content were lowered after PCM oral administration. Moreover, enhanced NOx, LPO and reduced activities of SOD, as well as GSH content, in liver tissue are indications of generation of free radical stress as a mark of hepatic tissue injury due to PCM toxicity. The link between oxidative stress and the excess consumption of fat is well known (Jang et al., 2012). The current investigation showed that *A. fraxinifolius*hexane extract pre-treatment prevented the reduction in the antioxidant enzyme activities and consequent oxidative damage to the liver. It is known that TAC, an indicator of enzymatic and non-enzymatic antioxidants, reflects the total antioxidant capabilities (Burton and Traber, 1990 and Azab et al., 2013). The maintenance of adequate antioxidant levels is essential to scavenge the free radical that cause the oxidative stress , so TAC could be a reliable diagnostic biomarker. The administration of *A. fraxinifolius* hexane extract completely improved TAC of rats intoxicated with PCM.

It is well known that flavonoids and phenolic acids are potent antioxidant agents (Olech *et al.,* 2012). Recently, El-Kashak *et al.,* in 2016 reported that aqueous methanolic extract have very strong antioxidant effect due to presence of quercetin-3-O-β-D-glucopyranoside, quercetin-3-O- α-L-rhamnopyranoside, myricetin-3-O-β-Dgalactopyranoside and myricetin-3-O-α-L-rhamnopyranoside which exhibited strong antioxidant activity. Also, the other, flavonoids quercetin 3-O-α-L-rhamnopyranoside, myricetin 3-O- α-L-rhamnopyranoside and myricetin 3-O-β-D-galactopyranoside, exhibited significant antioxidant activity. Therefore, the presence of these phytochemicals in extract of *A. fraxinifolius* rules out any possibility of degenerative changes in liver tissue, in the present study.

In the present investigation, *A. fraxinifolius* extract also showed a potent anti-inflammatory effect evidenced by decreased hepatic NOx production. This might be due to hydroxyl radicals scavenging activities of the *A. fraxinifolius* hexane leaves extract. Higher activity in hexane extract may be due to presence of α-tocopherol, which has a powerful antioxidant activity in scavenging the free radicals, stabilization of the cell membrane and structure restoration (Abd El-Ghffar et al. 2016). The co-effectiveness of *A. fraxinifolius* extract on LPO and oxidative stress indicates a strong point of this medicinal plant. This implies the reduction in free radical production and subsequent decrease the damage oh hepatocellular membranes (Metwally *et al.,* 2009).

The mechanism of hepato-protection by *A. fraxinifolius* extract against PCM toxicity might be due to restoration of the GSH level indicating decreased free NAPQI level in the blood. These results are in agreement with Abd El-Ghffar *et al.* (2016) who reported that pre-treatment with *A. fraxinifolius*hexane extract for one week decreased LPO, as well as increased TAC and glutathione system, and exerted a hepato-protective effect against PCM-induced liver damage in male rats.

To confirm the anti-inflammatory activity of *A. fraxinifolius* hexane extract during hepatic toxicity, the expression of acute phase cytokines were examined. The. *A. fraxinifolius* hexane extract administration down-regulated the increase of IL-1β and TNF-α expressions in paracetamol administered group (AbouZeid *et al.,* 2011) IL-1β and TNF-α are the major inducers of acute phase response (Ganjali *et al.,* 2014). They act as hepatotrophic factors as evidenced by circulating levels of TNF-α and IL- 1β that are increased in rats with liver damage (Yang *et al.,* 1998). *A. fraxinifolius* hexane extract modulates the inflammatory response by down-regulating the activity of TNF-α and IL-1β. The results confirmed that *A. fraxinifolius* hexane extract decreased mRNA expression of IL-1β and TNF-α that are increased in liver of paracetamol administered group.

Moreover, *A. fraxinifolius* hexane extract co-administration with paracetamol increased expression of IL-10, which is a known regenerative cytokine (Fiorentino *et al.,* 1991). IL-10 is produced mainly by monocytes with pleiotropic actions (Pestka *et al.,* 2004). IL-10 down regulates T helper 1 cytokines expression, inhibits IL-1β and IL-6 production (Fiorentino *et al.,* 1991), configures the development of the immune response and decrease pro-inflammatory cytokine expression (Moore *et al.,* 2001). Therefore, the increase in IL-10 expression is a mean to control the degree of toxicity induced by paracetamol and to counteract the increase in expression of IL-1β and TNF-α.

CONCLUSION

In the present study, *A. fraxinifolius* hexane extract offered potential hepato-protection against PCM-induced hepatic damage, normalizing biochemical parameters in male rats plausibly by augmenting endogenous antioxidant defense mechanisms. Finally, *A. fraxinifolius* hexane extract possessed superior hepato-protective activity which may be due to its free radical-scavenging and antioxidant activity, resulting from the presence of some flavonoids constituents. Also the extract has the ability to up- regulate the anti-inflammatory cytokines and down regulate the inflammatory cytokines in the experimental animal model.

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