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Research Paper

Hepatoprotective activity of *Borago officinalis* extract against CCl₄induced hepatotoxicity in rats

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ABSTRACT

The present study aims to investigate the hepatoprotective effect of *Borago officinalis* L. aerial ethanolic extract (BAEE) against CCl₄-induced liver damage in comparison to silymarin, a classical antioxidant liver medicine. The hepatoprotective potential of BAEE in rats was evaluated following oral administration of CCl₄, which enhanced hepatic lipid peroxidation and notably depleted reduced glutathione. Moreover, we found that CCl₄ administration caused over expression of the inflammatory markers TNF- α and NF κ B protein levels, in addition to a significant increase in the release of liver serum biomarker levels. Administration of BAEE showed hepatic protection by significantly reducing elevated levels of serum enzyme levels. Notably, BAEE significantly reduced expression of the TNF- α and NF κ B protein levels comparable to wild type and silymarin. These findings were augmented with the histopathological results in which BAEE was able to show an improvement in the liver condition. The results of the current study indicate that BAEE extract has a potential hepatoprophylactic effect against chronic liver injury.

Key words: Borago officinalis; Carbon tetrachloride (CCl₄); Liver biomarkers.

Abbreviations: BAEE: *Borago officinalis* aerial ethanolic extract, TNF- α : tumor necrosis factor alpha, NF κ B: nuclear factor kappa-B, I κ B: inhibitor of nuclear factor kappa-B, CCl₄: carbon tetrachloride, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, LDH: lactate dehydrogenase, DTNB: 5,5-dithiobis-2-nitrobenzoic acid, EDTA: ethylenediaminetetraacetic acid, ELISA: enzyme-linked immunosorbent assay, GSH: glutathione, H&E: hematoxylin and eosin, 4-HNE: 4-hydroxynonenal, LDH: lactate dehydrogenase, MDA: malondialdehyde, PUFA: polyunsaturated fatty acids, ROS: reactive oxygen species, SDS: sodium dodecyl sulphate, SDS-PAGE: sodium dodecylsulfate polyacrylamide gel, TBA: thiobarbituric acid, TBARS: thiobarbituric acid reactive substance, TCA: trichloroacetic acid, TEMED: tetramethylethylenediamine and TG: triglycerides.

INTRODUCTION

Borago officinalis L. family Boraginaceae is commonly known as borage or starflower. It is used as an ornamental plant and to repel insects (Kaskoos et al., 2012). It was reported previously that *B. officinalis* leaf extract possesses a high antioxidant activity due to their high content of phenolic compounds viz., officinalioside and kaempferol 3-O-β-D-galactopyranoside (Garcia-Herreros et al., 2010; Samy et al., 2015). It was also evidenced that this extract comprises some compounds, which possess a high radical quenching ability (Bandoniene and Murkovic, 2002; Samy et al., 2015). In herbal medicine, infusions of B. officinalis are used to treat urinary infections, bronchitis, colds, rheumatism and skin rashes (Tasset-Cuevas et al., 2013). Its leaves were shown to be diuretic, demulcent, emollient and expectorant (Pieszak et al., 2012). A number of drugs and viral infections have been reported to cause severe hepatic injuries. Hence, identification of an efficient hepatoprotective drug derived from natural sources is an urgent necessity. Therefore, it is important to evaluate plant extracts that can be effective in restoring hepatic functions. Since ancient times, herbs have been used as a remedy for various diseases (Abdel-Hamid et al., 2011). Therefore, the authors of this study investigated the effect of BAEE as a hepatoprotective agent using the CCl₄-induced liver damage as model for study. The hepatoprotective effect of this herb has not been investigated before and this is the first study to address this issue. The reported high antioxidant activity of BAEE due to their high content of phenolic compounds (Garcia-Herreros et al., 2010; Samy et al., 2015) encouraged the authors of this work to screen the hepatoprotective potential against CCl₄-induced hepatotoxicity in rats.

Here, we report for the first time the hepatoprotective effects of BAEE against CCl₄-induced oxidative stress and its role in alleviation of lipid peroxidation and restoration of TNF- α and NF κ B proteins and liver enzymes activities.

MATERIALS AND METHODS

Chemicals: CCl₄, ethylenediaminetetraacetic acid, 5,5-dithiobis-2-nitrobenzoic acid, potassium dihydrogen phosphate, reduced glutathione, trichloroacetic acid, thiobarbituric acid, acrylamide, ammonium persulphate, sodium dodecyl sulphate, tris-HCl, tween 20 and non fat dry milk. All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Plant materials and preparation of BAEE: The aerial parts of BAEE were collected from the Faculty of Agriculture, Minia University in April 2013 and propagated in the experimental station of the Faculty of Pharmacy, Minia University. The plant was identified by Prof. Mahmoud A. H. Abdo, Director of Floriculture Nursery (Aromatic and Medicinal plants), Faculty of Agriculture, Minia University. A voucher specimen number (Mn-Ph-Cog-008) of the plant is deposited in the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Minia University. The aerial parts were air-dried in shade, reduced to fine powder suitable for extraction. The air-dried fine powder (2kg) was macerated in ethanol till exhaustion (4X, 4L, each, with 7 days intervals) and then concentrated under reduced pressure till dryness by using rotary evaporator to yield (65g) ethanolic extract.

Phytochemical screening: Preliminary qualitative phytochemical screening of BAEE was carried out on portions of the residual material, using standard previously reported phytochemical procedures (Harborne, 1998; Trease and Evans, 1985).

Animals and experimental design: Male Sprague-Dawley albino rats $(100g \pm 5g)$ were used. They were purchased from the animal house of Faculty of Agriculture, Minia University. The animals were housed under standardized environmental conditions, fed with standard diet and left to acclimatize to the environment for one week prior to inclusion in the experiment at $22^{\circ}C \pm 2^{\circ}C$ under a 12/12 hrs light/dark cycle. All the animal experiments were conducted in accordance with the guide for the care and use of laboratory animals of the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Animals were divided randomly into 9 groups (10 rats each) as follows: **Group-I**: served as the normal healthy control group: rats received olive oil for 9 weeks. **Groups-II, III and IV:** rats received only BAEE orally (50, 100 and 150mg/kg) without intoxication with CCl₄, respectively. **Group-V:** rats intoxicated with CCl₄ intraperitoneally (0.8ml/kg body weight CCl₄/olive oil; 2 days/week) for 9 weeks to induce chronic liver injury. **Groups-VI, VII and VIII** (BAEE treated): each group consisted of 10 rats receiving CCl₄ intraperitoneally and BAEE orally (50, 100 and 150mg/kg), respectively. **Group-IX** (Silymarin treated): rats received silymarin orally [100mg/kg; dissolved in olive oil (Khan et al., 2012)]. Groups from **VI** to **IX:** rats were given the first dose of the extract and silymarin after 48 hrs of the first dose of CCl₄ injection. At the end of the experiment, the animals were sacrificed to collect blood and livers for experimental analyses. Blood samples were collected on the final day of the experiment after a 12h fast. Blood samples were left for 15 min for *in-vitro* coagulation and then centrifuged at 3000 xg for 15 min for serum collection.

Liver specimen preparation: Each liver specimen was dissected into 2 pieces. One piece was fixed and embedded in paraffin block for histopathological examination, whereas the second piece was stored in liquid nitrogen (when needed), homogenized for total protein extraction. Liver specimens were fixed in 10% neutral buffered formalin for 48h at room temperature. The tissues were then placed in embedding cassettes. To prepare the trimmed liver pieces for embedding in paraffin, specimens were first dehydrated gradually using increasing concentrations of ethanol in order to replace water in liver tissues. The liver tissues were then cleared by infiltrating with xylene. Finally, the specimens were infiltrated with 58-60°C liquid paraffin.

Western blot analysis in liver: To evaluate the effect of BAEE on protein expression levels, western blotting technique was employed. Liver samples were homogenized in ice-cold lysis buffer. 100mg of tissue protein was extracted by homogenization of liver tissues in 50mM Tris buffer pH 7.4 and then protein concentration was determined. Samples were then centrifuged for 15min at 10000 xg at 4°C. Supernatant containing proteins was transferred to a new tube. 15% resolving gel and 5% stacking gel were prepared using acrylamide-bisacrylamide solution. For each sample, 30µg proteins were loaded and electrophoresis was performed at 75 volts followed by 125 volts for approximately 2h. Eelectrophoresed proteins on SDS-PAGE were transferred to a HybondTM nylon membrane (GE Healthcare) and incubated for one hr at room temperature in a blocking solution (5% milk solution). The blotting membrane was incubated overnight at 4°C in antibody solution containing primary antibody (1 in 5000 dilution). The membrane was washed at room temperature for 30-60 min in 5% milk solution. The membrane was then incubated for one hr at room temperature in antibody solution containing appropriate dilution of horse reddish peroxidase (HRP)conjugated secondary antibody (1 in 10000 dilution). The membrane was then washed

for 60 min. The western blotting product was photographed and recorded via DIGI-DOC, UVP, INC England.

Determination of liver enzymes activity: The collected blood was allowed to clot, the serum was separated at 2000 xg and the biochemical markers, including serum ALT, AST (JayaPrada, 2014), ALP (Farah et al., 2012), albumin (Dumas et al., 1997), total bilirubin (Yoshiro et al., 2012), TG (Fossati and Prencipe, 1982) and total cholesterol (Mignarri et al., 2015) were estimated according to previously reported methods. These parameters were determined using available commercial kits (ARE); following the instructions of the manufacturer.

Enzyme-linked immunosorbent assay (ELISA): LDH was measured using ELISA commercially available kits as per manufacturer's regulations (Cameron et al., 2004). *Determination of liver content of TBARS:* A saturated solution was made of thiobarbituric acid in perchloric acid (0.8g % of thiobarbituric acid) as a working solution A. A solution of 20% of trichloroacetic acid was also prepared (working solution B). The final working reagent was made using the prepared previous reagents (A and B) in the ratio (1:3). 0.5ml of 20% liver homogenate was added to 4.5ml of the reagent. The solution was then put for 20 min in boiling water bath and then centrifuged for 10 min in 2000xg. The developed orange color is then measured colorimetrically. Absorbance was recorded at wavelength 532nm against blank. For calculation, the liver content of TBARS as MDA equivalents was determined from the standard curve. MDA content expressed as nMole/g liver tissue (Mahreen et al., 2010).

Determination of hepatic GSH content: GSH content in liver homogenate was determined using Ellman's reagent as previously reported (Davies et al., 1984). The product of the reaction has an intense yellow color and can be determined spectrophotometrically at 412 nm using UV/V is spectrophotometer (CT-2200; SN: RE1103001). A standard calibration curve was constructed using serial dilutions of reduced GSH. The liver content of GSH was determined from the standard curve. GSH content was expressed as μ M/100 mg liver tissue.

Statistical analysis: The statistical analyses of the obtained results were done using GraphPad Prism 5 (Graphpad Software, San Diego California, USA). The results were expressed in terms of means \pm S.E.M. Differences between the mean values for individual groups were assessed by one-way analysis of variance. In all analyses, P<0.05, P<0.01, or P<0.001 was taken to indicate statistical significance. All experiments were repeated at least three times.

RESULTS

Phytochemical screening: Many metabolites were detected in BAEE viz., carbohydrates and/or glycosides, unsaturated sterols and/or triterpenes, flavonoids, saponins, tannins and alkaloids and/or nitrogenous compounds. On the other hand, the extract was found to be free from crystalline sublimate substances, cardenolides and anthraquinones.

Toxicity studies for the BAEE: Three groups of rats were administered 50, 100 and 150 mg of BAEE orally without intoxication with CCl_4 . Liver biomarkers together with creatinine and urea were measured (Table 1). All the results of the former parameters revealed that 150 mg of BAEE is a safe dose comparable to control healthy group.

Table 1. Levels of fiver blor	le 1: Levels of liver biomarkers in learny control and unterent doses groups of BALL.						
	Group I	Group II	Group III	Group IV			
Albumin (mg/dl)	4.7 ± 0.034	4.8 ± 0.260	4.9 ± 0.220	4.0± 0.320			
Cholesterol (mg/dl)	72± 3.1	67± 5.9	68±6.4	63±63.6			
Bilirubin (mg/dl)	0.044 ± 0.00058	0.041 ± 0.01000	0.10 ± 0.01400	0.12 ± 0.01400			
TG (mg/dl)	71±4.1	71±4.9	69±4.4	69±5.3			
ALT (U/L)	40± 3.1	34± 2.3	38± 3.5	38± 3.1			
AST (U/L)	100 ± 5.9	97±7.7	88± 8.3	65 ± 6.0			
ALP (U/L)	1.2 ± 0.12	1.9± 0.19	1.7 ± 0.14	1.6 ± 0.067			
LDH (U/L)	570±42	560±44	400±25	550±25			
GSH (µM/100 mg liver)	5.1 ± 0.20	5.1±0.42	5.0 ± 0.18	5.0 ± 0.40			
MDA (nmole/ g liver)	16± 0.37	16± 1.00	15 ± 0.50	18 ± 0.68			
Creatinine (mg/dl)	27± 2.30	25± 0.65	25± 2.10	25 ± 1.40			
Urea (U/L)	5.9 ± 0.50	5.6 ± 0.55	5.8 ± 0.40	5.5 ± 0.37			

Table 1: Levels of liver biomarkers in healthy control and different doses groups of BAEE.

• *Significantly different (P < 0.05). All groups compared with group I (healthy control group).

BAEE treatment restores serum liver enzymes: We first investigated the released markers of liver cell integrity, for example; ALT and AST (in the serum of CCl₄-treated rats). The administration of CCl₄ resulted in an increase in the mean values of ALT levels in comparison to the healthy control (Table 2). Interestingly, treatment with BAEE significantly reduced the elevation in the ALT levels that is caused by CCl₄ in comparison to the healthy control and silymarin/CCl₄. A similar pattern was also observed in case of AST, ALP and LDH enzyme levels.

Table 2	: Levels	of liver	biomarkers	in intoxicated	rats w	ith CCl ₄	compared	with	different	doses
	groups	of BAE	E and silyma	arin.						

	Group V	Group VI	Group VII	Group VIII	Group IX
Albumin (mg/dl)	3.6±0.18	3.2 ± 0.21	3.6±0.21	4.6± 0.11**	4.5±0.17**
Cholesterol (mg/dl)	130± 12.0	130±8.0	120±5.3	64±4.7***	68±3.5***
Bilirubin (mg/dl)	0.390 ± 0.03	0.400 ± 0.08	0.410 ± 0.01	0.130± 0.03*	0.087± 0.03**
TG (mg/dl)	130± 5.9	130±4.6	99±9.1	81±7.4*	75±6.7*
ALT (U/L)	100± 6.9	100 ± 3.4	79± 5.7*	44± 2.2***	42± 2.2***
AST (U/L)	250± 18.0	230 ± 22.0	180± 10.0**	110± 3.5***	110± 4.0***
ALP (U/L)	5.7 ± 0.47	5.1 ± 0.56	4.4 ± 0.44	2.7±0.26***	1.6± 0.16***
LDH (U/L)	1200± 56	1300±91	1200± 82	690± 50***	600± 59***
GSH (µM/100 mg liver)	2.3 ± 0.11	2.4 ± 0.22	2.8 ± 0.24	4.6±0.18***	4.5±0.21***
MDA (nmole/ g liver)	32± 1.80	33±1.20	27± 1.30	17± 0.84***	17± 0.89***

• *Significantly different (P < 0.05). All groups compared with group V (CCl₄)

• No significance (Comparison group VIII (150 mg/kg BAEE) with group IX (100 mg/kg silymarin).

BAEE treatment restores total cholesterol, TG, albumin and bilirubin levels: Table 2 shows the change in the serum levels of total cholesterol, TG, albumin and bilirubin

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compared to the healthy control group. Interestingly, BAEE treatment was significantly able to restore the serum normal levels of total cholesterol, TG, albumin and bilirubin.

BAEE treatment decreases the expression level of TNF- α and NF κ B protein in *CCl₄* intoxicated rats: The expression of the TNF- α and NF κ B proteins was assessed using immunoblot technique in the different experimental groups of rats. CCl₄ treatment resulted in over expression of NF κ B protein when compared to healthy control and silymarin/CCl₄ treated groups. Interestingly, treatment with BAEE resulted in a reduced expression of NF κ B protein when compared to that of CCl₄ treatment (Figure 1).



Figure 1: Effect of BAEE on NFκB and TNF-*α* protein expression levels.

The loaded proteins were subjected to western blot analysis with the NFκB (1A), TNF-α (1B) and Anti-β tubulin (1C) antibodies. The experimental groups of rats were run as follows: Group-I (lane 1), Group-V (lane 2), Group-IX (lane 3) and Group-IIIV (lane 4).

BAEE treatment increases the reduced GSH level: The hepatic tissue content of GSH was measured in control, CCl_4 , silymarin/ CCl_4 and $BAEE/CCl_4$ -treated rats as shown in (Table 2), one can detect a significant decrease in GSH level in the rat group treated with CCl_4 when compared to control healthy rats. Notably, BAEE treatment effectively prevented the depletion of GSH content of liver as a result of CCl_4 treated group and silymarin/ CCl_4 treated group.

BAEE treatment restores TBARS: A significant increase was detected in the level of MDA in the CCl₄-treated group compared to the healthy control (Table 2). On the other hand, treating rats with BAEE partially prevented the CCl₄-induced over expression of MDA compared to the healthy control and silymarin/CCl₄ treated groups. This observed reduction in MDA equivalent level in BAEE treated/CCl₄treated animals was statistically significant when compared to the CCl₄-treated group. Histopathology of liver sections of rats under experiment: Liver sections from control rats stained with (H&E) revealed normal hepatic architecture. The liver of control rats showed normal hepatocytes, portal tracts and central veins (Figure 2A). The liver of CCl₄-intoxicated rats showed degenerated hepatocytes with microvesicular steatosis, necrosis, distended congested sinusoidal spaces filled with erythrocytes and hepatitis characterized by mild mononuclear cells infiltration mainly lymphocytes and macrophages around central veins and in portal areas (Figures 2B and 2C). The liver of CCl₄-intoxicated rats and treated with silymarin revealed microvesicular steatosis, which was still present in the surrounding liver parenchyma, with noticeable signs of congestion, but reduced in extent and were less frequent compared to the CCl₄ control group (Figure 2B, C). The liver of CCl₄-intoxicated rats and treated with BAEE only exhibited sporadic, markedly small hepatic lesions and microvesicular steatosis in the periportal areas, but without morphological signs of hemodynamic disturbance (Figure 2E).



Figure- 2: The effect of BAEE on liver histology, H&E.
(A): Group-I, (B) and (C): Group-V, (D): Group-IX and (E): Group-IIIV. All X 200 except (A) X 100.

DISCUSSION

 CCl_4 intoxication is the experimental model of choice for liver injury for many scientists all over the world. CCl₄ administration can induce chronic liver injury in rats. It is metabolized by cytochrome P450 system to highly reactive trichloromethyl free radicals and ROS, which initiate liver cell destruction (Jaeschke, 2011). In the current study, we made use of this universal model in order to induce liver injury in rats with the aim of determination of the extent of liver damage. The authors primarily used serum aminotransferases, which are usually referred to as "liver enzymes". Indeed, estimation of these enzyme levels in serum is a useful marker of the extent of hepatocellular damage. Damaged structural integrity of the liver can lead to an increase in the serum level of liver enzymes due to their localization in the cytoplasm. These enzymes are released into the circulation following to cellular breakdown. In the current study, some biochemical parameters including serum transaminases, ALP, triglyceride, total cholesterol levels and serum bilirubin level have been measured to investigate the possible therapeutic effects of BAEE to ameliorate the CCl₄-induced liver injury. In addition to these markers, we determined the level of LDH. It is an oxidoreductase enzyme that is responsible for the reversible reaction in which pyruvate and lactate are interconverted. This enzyme is located in a number of body tissues including the liver. Serum LDH is increased following tissue breakdown such as liver damage. In line with other previous studies (Faddah et al., 2007; Zhao et al., 2013; Al-Sayed et al., 2014; Chae et al., 2014).

BAEE decreased the serum levels of ALT, AST, LDH and other liver biomarkers. The return of elevated levels of serum enzymes to near normal values is an indication of the stabilization of the plasma membrane and the repair of hepatic tissue damage caused by CCl₄. The reversal of increased serum enzymes in CCl₄induced liver damage by BAEE may have occurred due to prevention of leakage of intracellular enzymes. We suggest that the reason behind this is the antioxidant activity of BAEE, which blocked at least partly, the effect of released free radicals by CCl₄ which leads to lipid peroxidation and hence membrane destabilization and eventually liver cell injury. Our findings are inline with previous reports emphasizing

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the high antioxidant activity of BAEE due to their high content of phenolic compounds (Garcia-Herreros et al., 2010; Samy et al., 2015). This antioxidant activity was attributed to the presence of some compounds, which possess a high radical quenching ability (Bandoniene and Murkovic, 2002; Samy et al., 2015).

To investigate our hypothesis, we proceeded with examining TBARS serum level. CCl₄ hepatotoxicity is attributed to the release of free radicals, which leads to liver injury via combination with PUFAs of hepatic cellular membranes, causing elevation of TBARS; a major reactive aldehyde resulting from the peroxidation of PUFAs (Zhang et al., 2013). Lipid peroxidation is believed to be an important cause of destruction and damage to cell membranes. In the present study, TBARS level was notably increased in CCl₄-treated rats liver as compared with those of the control, indicating that CCl₄ exposure induced oxidative stress. Interestingly, BAEE treatment decreased TBARS productions in the CCl₄-treated rat liver homogenates, which may be due to its powerful antioxidant and free radical scavenging activities (Shinomol and Muralidhara, 2007). Results suggest that BAEE could at least partly attenuate oxidative stress by decreasing levels of ROS and lipid peroxide in CCl₄-treated rats. The significant reduction in the level of liver enzyme into the serum also confirmed the inhibitory effect of BAEE against lipid peroxidation. In line with previous reports, we suggest that the reversal of elevated serum liver enzymes following hepatocellular damage by BAEE might have been occurred secondary to prevention of intracellular enzyme leakage due to membrane stabilization and antioxidant activity of BAEE (Khan et al., 2012; Al-Sayed et al., 2014; Samy et al., 2015).

Furthermore, GSH has a central role in protecting cells from damage that may result from release of ROS resulting from CCl_4 intoxication. GSH plays this role by covalently binding to trichloromethylperoxyl free radicals released from CCl_4 , this leads to a chain reaction, which results in lipid peroxidation of cellular membranes and eventually leads to cell membrane disruption (Brattin et al., 1985; Srivastava and Shivanandappa, 2010). It has been reported previously that CCl_4 -induced hepatic damage in rats causes a significant reduction in hepatic GSH content (Wills and Asha, 2012; Khan et al., 2012). Inline with these reports, we show that CCl_4 injection produced a significant decrease in hepatic GSH content. Treatment of rats with BAEE resulted in significant increase in the content of liver GSH compared with the control group, which again suggests an antioxidant effect for this extract.

All the former results were supported and confirmed with the histopathological investigations in which BAEE was able to show an improvement in the liver condition. Screening the literature we found that many antioxidant compounds usually possess anti-inflammatory effects as well. Inflammatory cytokines play a crucial role in the pathogenesis of liver disease (Wang et al., 1999). Indeed, TNF- α has been reported before as a key mediator in many experimental liver injury models (Bradham et al., 1998; Schümann and Tiegs, 1999). It has been shown before that patients with inflammatory diseases have high serum TNF- α levels compared to healthy people (Gratacós et al., 1994; Zhao et al., 2013) and hence TNF- α was considered to be a biomarker for inflammatory status (Reyes-Gordillo et al., 2007). Moreover, NF κ B is an inducible transcription factor that regulates the expression of genes involved in the inflammation process. It is mainly present in the cytoplasm. NF κ B is retained in an inactive form by I κ B (inhibitor of NF κ B). Following induction, NF κ B is unbound from I κ B and then translocated to the nucleus, where it binds to

DNA and activates transcription. In agreement with previous studies (Zhao et al., 2013), the authors of this paper showed that BAEE has an anti-inflammatory effect through inhibiting the synthesis of TNF- α and NF κ B proteins, where BAEE treated rats showed a lower expression level of TNF- α and NF κ B compared to its expression level in the rats of CCl₄ treated group.

CONCLUSION

Finally, we conclude that BAEE has potential hepatoprotective effects against chronic liver injury, which we propose to be due to the antioxidant and anti-inflammatory effects that inhibit CCl₄ free radical derivative formation and prevent cellular damage.

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