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Eucalyptus oil abrogated liver damage, oxidant /antioxidant imbalance, inflammation and apoptosis stimulated by acetaminophen in rats: biochemical, molecular and histological approaches

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ABSTRACT

Acetaminophen is an analgesic antipyretic commonly used. Hepatotoxicity is one of the most common obstacles to acetaminophen therapy. *Eucalyptus* oil is an antioxidant with potent free radical-capturing activities. This research was designed to evaluate the inhibitory impact of *Eucalyptus* oil versus acetaminophen-triggered hepatotoxicity. The composition of *Eucalyptus* was detected utilizing gas chromatography mass spectroscopy. Forty-eight rats were assigned into six groups; control group, acetaminophen group (500mg/kg I.P twice on the 17th and, 20th of the experiment), silymarin group (50mg/kg, I.P once daily for three weeks), *Eucalyptus* oil group (30mg/kg orally once daily for three weeks), acetaminophen +silymarin group and *Eucalyptus* +acetaminophen group. gas chromatography mass spectroscopy discovered four compounds in *Eucalyptus* with eucalyptol representing the main compound. revealed that acetaminophen remarkably elevated serum alanine transaminase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin levels, whereas it declined serum albumin and total protein levels. In addition, hepatic oxidant/antioxidant imbalance was evident in acetaminophen-intoxicated rats by the rising of lipid peroxidation biomarker; malondialdehyde and the downregulation of nuclear factor erythroid 2-related factor 2 and its transcriptional mediators; superoxide dismutase, glutathione peroxidase, and reduced glutathione. Furthermore, following acetaminophen injection, there was a remarkable increase in transforming growth factor-β gene expression, tumor necrosis factor-α and interleukin1-β levels along with a decline in interleukin-10 levels. Immunohistochemical and histopathological examinations were in parallel with the abovementioned results. However, all these abnormalities were significantly abrogated in rats pretreated with *Eucalyptus*. We concluded that prior administration of *Eucalyptus* oil counteracted acetaminophen-mediated hepatotoxicity via powerful antioxidant, anti-inflammatory, and anti-apoptotic impacts.

Introduction

The liver is a crucial organ that regulates essential biochemical and physiological processes (Olorunnisola *et al*., 2011). As a result, it is extremely vulnerable to numerous chemically produced injuries. For instance, acetaminophen (APAP) is an over-the-counter analgesic and antipyretic medication that is usually considered nontoxic when utilized at therapeutic doses (AlWahsh *et al*., 2019). Acute liver failure can result from high APAP dosages in humans and animals (Toyoda *et al*., 2018).

In normal therapeutic doses, the majority of APAP's biotransformation in the liver results in execrable metabolites that are glucuronide and sulphate conjugates (Abed *et al*., 2022). Cytochrome P450 enzymes (CYPs), principally CYP 2E1, biotransform about 5–9% of the residual APAP to the extremely active intermediate metabolite N-acetyl-p-benzoquinone imine (NAPQI) (Lancaster *et al*., 2015). The later compound binds mostly to GSH's sulfhydryl (-SH) group to create an APAP GSH complex, which the kidney excretes as conjugates of cysteine and mercapturic acid (Yoon *et al*., 2016). Additionally, toxic doses of APAP undergo sulfation and glucuronidation pathways to become saturated, which causes GSH depletion and an increase in the concentration of NAPQI with subsequent severe oxidative stress, mitochondrial dysfunction, an inflammatory reaction, and potentially cell death (Neag *et al*., 2020).

Plants are popular for their therapeutic qualities. According to several experimental studies, medicinal plants could effectively shield the liver from hepatotoxicity caused by APAP (Farooqi *et al*., 2022; Shan *et al*., 2023). Silymarin (SLM) is a polyphenolic compound isolated from the milk thistle plant's fruits and seeds, *Silybum marianum* (*Asteraceae* family) (Wagner *et al*., 1974), that is extensively utilized as a reference medication to treat both acute and chronic liver conditions (Abenavoli *et al*., 2010). As a result, SLM has also been used to compare other hepatoprotective medicines in order to assess the effectiveness of these drugs. For example, *Eucalyptus* is the native plant of Australia, which has more than 700 species. Some of them, such as *Eucalyptus globulus* (*E. globulus*), which is a member of the *Myrtaceae* family (Goldbeck *et al*., 2014), were introduced to Europe and North Africa, where they are now thriving along the Mediterranean coasts. Most commonly, *Eucalyptus* is adapted for use in cosmetics, the paper industry, and traditional medicine. The leaf extract or essential oil from the leaves of *E. globulus* has remarkable diverse biological activities, including the cure of liver and gastrointestinal problems, wound healing, chemotherapy, and antifungal, antibacterial, anti-inflammatory, antioxidant, and anthelmintic properties (Dhakad *et al*., 2018). The main ingredient of *Eucalyptus* oil (EUO), 1,8-cineole (eucalyptol), is found in *E. globulus*, also referred to as Tasmanian blue gum (Pino *et al*., 2021), which has significant pharmacological properties, including antioxidant and anti-inflammatory actions. It can scavenge hydroxyl radicals and reduce the symptoms of inflammation (Muchtaridi *et al*., 2010). From there, this study's major goal was to assess EUO's hepatoprotective, anti-inflammatory, and antioxidant properties versus APAP-stimulated hepatic injury in rats.

Materials and methods

Animals

Forty-eight male Wistar rats (150-200 g) purchased from the Central Animal House at Faculty of Veterinary Medicine, Zagazig University,

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Egypt. They were placed in plastic cages in a temperature-controlled environment with a 12 h light/12 h dark cycle; and fed normal rat pellets and water. This study was conducted in accordance with ethical standards and political considerations approved by the Institutional Animal Care and Use Committee at Zagazig University, Faculty of Veterinary Medicine, (approval no. ZU-IACUC/2/F/419/2022).

Drugs and Chemicals

Acetaminophen and Silymarin (SLM) were bought from EPICO and Sedico Pharmaceutical Co. (Cairo, Egypt), respectively. *Eucalyptus* oil extract (EUO) was bought from Nefertari Body Care Products Manufactory (Heliopolis, Cairo, Egypt). The other chemicals and solvents were of excellent analytical quality.

Gas chromatography-mass spectrometry (GC/MS) analysis of EUO

The EUO essential oil was analyzed utilizing gas chromatography-mass spectrometry (GC/MS). The GC-MS structure consisted of a TG-WAX MS column (30 m x 0.25 mm i.d., 0.25 µm film thickness). The measurements were conducted at the next circumstances: carrier gas, helium at a flow rate of 1.0 ml/min, temperature system: 60°C for 1 min; increasing to 240°C at which the detector and injector were set. A volume of 1µL of diluted samples (1:100 hexane, v/v) was injected. With the aid of electron ionization (EI), mass spectra were determined at 70 eV, and in the m/z 40-450 spectral range. The identification of components was mainly achieved by comparing authentic samples, the Wiley spectral library collection and the NSIT library.

Experimental design

The animals utilized in our investigation were randomly assigned to six groups, with eight rats per group. Group 1; Control: Rats were administered saline (i.p). Group 2: Rats were given an I.P of APAP at a dose 500 mg/kg on the 17th and 20th day of the trial (El-Banna *et al*., 2013). Group 3: Rats were received SLM (50 mg/kg, I.P) once daily for 3 weeks (Pradeep *et al*., 2007). Group 4: Rats were orally given EUO extract (30 mg/kg) once daily for 3 weeks (Shao *et al*., 2020). Group 5: Rats were administered SLM (50 mg/kg, I.P.) once daily for 3 weeks followed by APAP (500 mg/kg) on the 17th and 20th day from the beginning of the experiment. Group 6: Rats were given EUO extract orally (30 mg/kg) once daily for 3 weeks followed by APAP (500 mg/kg) on the 17th and 20th day from the beginning of the experiment.

After 24 h of administration of the last dose, the blood samples were withdrawn from the retro-orbital plexus. Subsequently, we separated the serum by cooling centrifuge (3000 rpm for 15 min, Laboren Zentrifugen, 2K15, Sigma, Germany) and used it to assess serum liver function indicators. The rats were decapitated under ketamine anesthesia. The liver tissues were immediately collected; samples were maintained at -80°C for biochemistry measurements, whereas other samples were stored in 10% buffered formalin for histopathological and immunohistochemical investigations.

Biochemical measurements

Liver function biomarkers

The concentrations of serum alanine amino transaminase (ALT), aspartate amino transaminase (AST), bilirubin and alkaline phosphatase (ALP) were colorimetrically measured (Tietz, 1995). Serum albumin and total protein levels were evaluated (Burtis *et al*., 1999). These biomarkers were assessed using diagnostic tools obtained from Spin React, Gerona, Spain.

Setting up of the liver tissue homogenate

A 10% homogenate was prepared in a 0.05 M phosphate buffer (pH 7) using polytron homogenizer. The prepared homogenate were centrifuged at 10,000 rpm for 20 min, and the obtained supernatant (cytoplasmic extract) was utilized to determine tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and (IL)-10 levels using rat ELISA kits (Cusabio, Houston, TX, USA) and transforming growth factor beta1 (TGF-β1) based on the kit instructions (BOSTER Biological Technology Co., Ltd, China).

Evaluation of liver oxidative stress markers

Following the principle of Ohkawa *et al*., (1979), based on thiobarbituric acid reactive substances, malondialdehyde (MDA), a chief byproduct of lipid peroxidation process, was measured in liver tissue homogenate. Antioxidant action in liver tissues was also evaluated by assessing superoxide dismutase (SOD) activity by its ability to reduce nitro blue tetrazolium (Nishikimi *et al*., 1972), reduced glutathione (GSH) according to Beutler, (1963), and glutathione peroxidase following the principle of Paglia & Valentine, (1967).

Real time quantitative polymerase chain reaction (RT-qPCR)

The mRNA levels of the CYP2E1 and Nrf2 genes were evaluated by RT-PCR. Liver samples were mixed with Direct-zol RNA Miniprep Plus (Cat# R2072, ZYMO RESEARCH CORP., USA) to obtain the total RNA, and a Beckman dual spectrophotometer (USA) was used to measure the quality and quantity of the RNA. Then, the reverse transcription step was followed by the SuperScript IV One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA, USA). The primers of the CYP2E1 and Nrf2 genes were utilized to amplify the gene products; in addition, the actin gene was employed as the standard gene (Table 1). qRT-PCR was terminated with the DTlite Real-Time PCR Detection System (DNA Technology, Russia) using SYBR Green with low ROX TOPreal™ qPCR 2X PreMIX (enzynomics, Korea) in accordance with the manufacturer's guidelines. By calculating the 2-ΔΔCt for the studied genes, their relative quantification is estimated and normalized according to the housekeeping gene.

Table 1. Primer sequences of target genes for RT-PCR

Target gene	Primers sequences	Reference
β-actin	F: 5′-GGGAAATCGTGCGTGAC-3′ R: 5′-AGGCTGGAAAAGAGCCT-3′	Xu *et al*. (2021)
CYP2E1	F: 5'- TGGGGAAACAGGGTAATGAG-3' R: 5'- CTGGCCTTTGGTCTTTTTGA-3'	Wang *et al*. (2014)
Nrf2	F: 5'-CTCCCAGGTAGCCCCCATTCCC-3′ R: 5'-CTGGGCTCTCGATGTGGCTGG-3′	Zhong *et al*. (2013)

F: Forward; R: Reverse; CYP2E1: Cytochrome P450 2E1; Nrf2: Nuclear factor erythroid 2-related factor 2

Histopathological examination

After successful fixation of collected liver tissue specimens in 10% buffered formalin, the samples were washed, dehydrated, cleared, and embedded in paraffin. Then, the blocks were sectioned at 5 micron thickness, and stained with hematoxylin and eosin (Bancroft and Gamble, 2008) for routine histopathological analysis and were accurately inspected for any lesions under a light microscope (Olympus BX50, Japan).

Histopathological lesion scoring

Histopathological changes were documented and scored as, no alterations (0), mild (1), moderate (2), and severe (3) alterations. The grading of changes was estimated by percentage as follows: <30% (mild), <30% – 50% (moderate), and >50% (severe) (Shamseldean *et al*., 2022).

Immunohistochemistry of liver tissue

Immunohistochemical investigations were done following the principle reported by Hanafi *et al*. (2023). The deparaffinized xylene hepatic tissue sections were dehydrated in grading concentration of alcohol. Antigen retrieval was utilized by citrate buffer (pH 6) pretreatment for 20 min. In a humidified room, we incubated sections for 2 h with rabbit monoclonal anti-Bax antibody [E63] at a ratio of 1:250 (ab32503; Abcam, Cambridge, UK) and rabbit polyclonal anti-Bcl-2 antibody at a ratio of 1:50 (ab59348; Abcam, Cambridge, UK). We incubated the sections with goat anti-rabbit IgG H&L (HRP) (ab205718; Abcam, Cambridge, UK), and the chromogen was 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma). We further stained the slides with hematoxylin and mounted DPX. The primary antibodies on the negative control slides were changed out for PBS.

Evaluation of BAX and Bcl-2 proteins immunostaining

The quantitative immunoreactivity of BAX and Bcl-2 was determined in liver tissue sections of the studied groups (El-Maksoud *et al*., 2020), and five fields were investigated. We assessed immunoreactivity in 10 microscopic fields per section by high-powered microscopic field (x 400). we utilized color deconvolution picture J 1.52 p software (Wayne Rasband, National Institutes of Health (U.S.A.)) to measure the proportion of positively stained cells (%).

Statistical data

The results are represented as mean±SE, and the Shapiro-Wilk test was applied at $p > 0.05$ to verify data normality (Shapiro & Wilk, 1965). To statistically compare the groups, a one-way ANOVA was performed, and Tukey's post hoc test was used to discover remarkable differences between all groups at $p< 0.05$. The Statistical Package for Social Science (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was utilized for all statistical data.

Results

GC-MS analysis of EUO

Four chemical compounds were discovered in EUO utilizing GC-MS analysis technique, as reported in Table 2. The results exhibited that 1,8- Cineole (Eucalyptol) is the main component of EUO with a value of 90.59% of the total identified compounds.

Biochemical studies

Impact of EUO treatment on liver function tests

Intoxication with APAP elicited a marked boost in serum ALT (108.82%), AST (120.74%), ALP (52.43%), and T. bilirubin (1142.86%), levels relative to the normal group. Conversely, pre-administration of EUO remarkably improved liver function as indicated by decreasing serum ALT (23%), AST (39.09%), ALP (16.33%) and T. bilirubin (40.23%) levels compared to APAP-intoxicated group. Furthermore, a remarkable decrease was noticed in serum albumin (84.31%) and T. protein (29.89%) levels following APAP intoxication versus the normal group. A marked improvement in serum albumin (28.46%) and T. protein (16.63%) levels was demonstrated in EUO treated groups, compared to the APAP-intoxicated rats. Moreover, SLM administration to APAP-treated rats succeeded in enhancing these liver function tests. It is worth noting that EUO and SLM treatments showed no liver toxicity indices as demonstrated in Table 3.

Impact of EUO treatment on hepatic oxidative stress biomarkers

As depicted in Table 4, a remarkable elevation in hepatic MDA (143.67%) level with reduction in GSH (71.18%), SOD (46.91%), and GPX (74.37%) contents was noticed in APAP –administered group, relative to the normal rats. On the contrary, pretreatment with either EUO or SLM enhanced GSH, SOD, and GPX levels, as well as decreased MDA content compared to the APAP-injected rats. Also, no marked changes have been demonstrated in these oxidant and antioxidant biomarker levels following SLM or EUO per se administration, relative to the normal rats.

Impact of EUO treatment on hepatic inflammatory biomarkers

The incidence of hepatic inflammation following APAP administration to rats has been confirmed by marked boosting in TNF-α (47.40%) and IL-1β (141.61%) contents, as well as a decrease in IL-10 (48.42%) levels, in comparison with the control group. Conversely, pretreatment with EUO followed by APAP administration succeeded in controlling the hepatic inflammatory process via inhibiting TNF-α and IL-1β levels, and rising IL-10 level, compared to APAP-intoxicated groups (Fig. 1A, B &C). Moreover,

Table 2. Results of the GC-MS analysis of EUO.

Peak	RT (min)	Compound name	Molecular formula	Area (%)
1	3.14	alpha-Pinene	$C_{10}H_{16}$	3.66
2	6.17	1,8-Cineole	$C_{10}H_{18}O$	90.59
3	9.94	Camphor	$C_{10}H_{16}O$	4
4	10.43	Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl) -	$C_{10}H_{18}O$	1.57

RT: retention time

Table 3. Influence of EUO administration on serum liver function markers in APAP-caused hepatotoxicity in rats.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	Albumin (g/dL)	T. protein g/dL)	T. bilirubin (mg/dL)
Control	34.00±3.06 $^{a, c}$	90.00±1.73 ^{a}	164.67±3.28 ^{a}	4.27±0.22 ^{a}	7.46±0.24 ^{a}	0.14±0.01 ^{a}
APAP	71.00±1.15^{b}	198.67±4.33 ^{b}	251.00±5.19 ^{b}	2.67±0.15 ^{b}	5.23±0.09 ^{b}	1.74±0.04 ^{b}
SLM	30.67±0.88^{a}	80.33±0.88 ^{a}	159.34±1.86 ^{a}	3.86±0.09 $^{a, c}$	7.20±0.12 ^{a}	0.07±0.01 ^{a}
EUO	30.65±3.84^{a}	82.66±2.18 ^{a}	167.31±2.60 ^{a}	3.93±0.14 $^{a, c}$	7.23±0.09 ^{a}	0.14±0.01 ^{a}
SLM + APAP	44.00±2.65$^{c, d}$	101.33±1.85 ^{c}	187.33±2.18 ^{c}	3.76±0.07 $^{a, c}$	6.90±0.06 ^{a}	0.92±0.04 ^{c}
EUO + APAP	54.67±2.18^{d}	121.00±1.53 ^{d}	210.00±1.52 ^{d}	3.43±0.06 ^{c}	6.10±0.11 ^{c}	1.07±0.09 ^{c}
F ratio	40.99	310.44	135.53	17.18	41.95	236.03
p <	0.05	0.05	0.05	0.05	0.05	0.05

Values are expressed as mean±SE (n=6). Results which have dissimilar letters are remarkably different at $p<0.05$ (Tukey's test).

Table 4. Influence of EUO treatment on hepatic oxidant/anti-oxidant status biomarkers in APAP-intoxicated rats.

Groups	MDA (nmol/mg)	GSH (mU/mg)	SOD (U/mg)	GPX (mU/mg)
Control	3.87±0.14[a]	152.66±2.03[a]	123.67±0.88[a]	5.97±0.15[a]
APAP	9.43±0.38[b]	44.00±2.51[b]	65.66±2.40[b]	1.53±0.23[b]
SLM	3.63±0.09[a]	155.67±7.22[a]	121.33±4.09[a]	6.04±0.18[a]
EUO	3.67±0.28[a]	156.33±8.99[a]	133.67±2.40[a]	6.44±0.40[a]
SLM + APAP	5.82±0.19[c]	113.34±4.90[c]	101.33±4.70[c]	4.30±0.31[c]
EUO + APAP	7.23±0.52[c]	91.00±4.62[c]	88.67±0.33[c]	3.23±0.24[c]
F ratio	98.99	65.55	76.24	53.3
p <	0.05	0.05	0.05	0.05

Values are expressed as mean±SE (n=6). Results which have dissimilar letters are remarkably different at p<0.05 (Tukey's test).

Table 5. histopathological lesion scoring of the liver.

Lesions	Control	SLM	EUO	APAP	SLM+APAP	EUO+APAP
Degeneration of hepatocytes	0	0	0	3	1	2
Necrosis of hepatocytes	0	0	0	2	1	1
Mononuclear inflammatory cell infiltration in portal areas	0	0	0	2	0	0
Edema of the portal area	0	0	0	2	0	0

The score system was designed as follows: score 0 = absence of the lesion in all rats of the group (n= 5), score 1= (<30%), score 2= (<30% – 50%), score 3= (>50%).

oral administration of APAP to rats resulted in boosting of hepatic TGF-β (233.51%) content in comparison with the normal rats. While, a marked decrease in the hepatic TGF-β levels have been observed in EUO-treated rats compared to APAP-administered animals (Fig. 1D). On the other side, the results of the SLM-treated groups are recorded in Fig. 1.

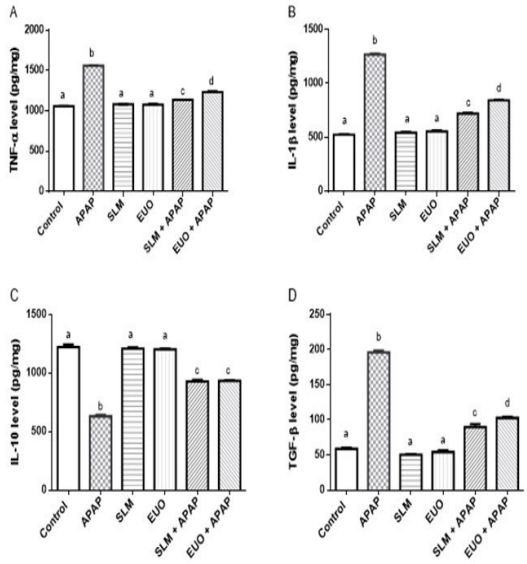

Fig. 1. The influence of EUO treatment on hepatic inflammatory biomarkers; TNF-α (A), IL-1β (B), and IL-10 (C) levels, and hepatic TGF-β (D) in APAP-injected rats. Data are expressed as mean±SE (n=6). Columns which have dissimilar letters are markedly different at p<0.05 (Tukey's test).

Impact of EUO treatment on the hepatic Nrf2 and CYP2E1 genes

Figure 2A represents the significant downregulation in hepatic Nrf2 gene expression following injection of APAP compared with the normal animals. In contrast, pretreatment of APAP-intoxicated rats with EUO significantly up-regulated the level of hepatic Nrf2 gene expression, compared with APAP-treated rats. A remarkable upregulation in the expression of the hepatic CYP2E1 gene has been noticed in APAP-administered rats, relative to the control rats (Fig. 2B). On the contrary, a marked decrease in the hepatic CYP2E1 gene level was observed in the EUO-treated group, compared with APAP-intoxicated rats. The results of EUO were statistically compared with those of SLM, as documented in Fig. 2.

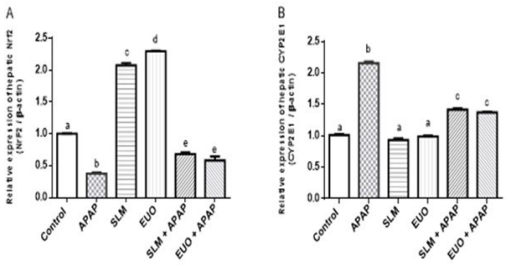

Figure 2. Influence of EUO treatment on relative expression of hepatic Nrf2 (A), and CYP2E1 (B) genes in APAP-injected rats. Results are expressed as mean±SE (n=6). Columns which have dissimilar letters are remarkably different at p<0.05 (Tukey's test).

Histopathological findings

Histopathological studies of livers of the control, SLM, and EUO groups revealed normal histological structures of hepatocytes (figs. 3 a, b, and c), the APAP treated group exhibited severe vacuolar degeneration of a considerable number of hepatic cells (Fig. 3 d), coagulative necrosis of hepatic cells was present (Fig. 3e), mononuclear inflammatory cell infiltration and edema dispersing portal area connective tissue of portal area of this group also was evident (Fig. 3f), SLM+APAP and EUO+ APAP treated groups showed mild degeneration of hepatocytes and coagulative necrosis of few hepatocytes (Figs. 3 g & h).

Histopathological lesion score

Recorded lesions in the liver were scored in accordance with their severity (Table 5).

Immunohistochemical findings of BAX and Bcl-2 protein expression

Immunoexpression of BAX and Bcl-2 proteins percentage area in the liver of experimental groups is illustrated in figs. 4 and 5 g, respectively. Expression of BAX protein in the liver showed very weak or no immunoexpression in the control, SLM, and EUO groups (FIgs.4 a, b, and c). BAX protein expression in the APAP group showed strong immune expression (Fig. 4 d). SLM+APAP and EUO+APAP groups showed weak immune expression of BAX (Figs. 4 e, and f). Bcl-2 expression in control, SLM, EUO, and APAP groups showed weak immuno- expression (Figs. 4 a, b, c, and d), while SLM+APAP and EUO+APAP groups showed strong immuno-expression in Bcl-2 (Figs. 4 e, and f).

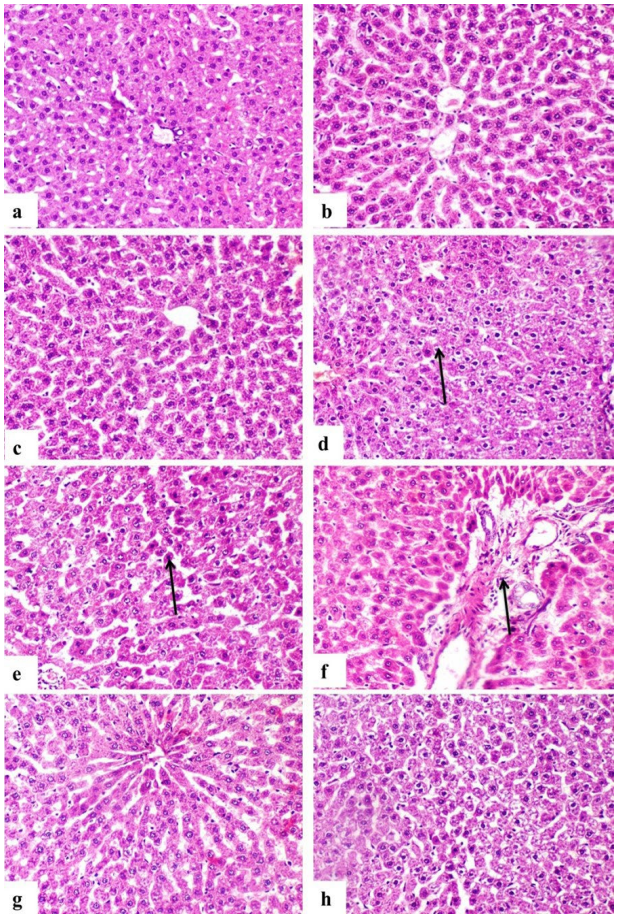

Figure 3. photomicrograph, rat liver, (a), (b), and (c) control, SLM, and EUO groups showing normal histological structure of hepatocytes. (d) PMC group showing severe diffuse vacuolar degeneration of hepatocytes (arrow). (e) PMC group showing necrosis of hepatocytes (arrow). (f) PMC group revealing mononuclear inflammatory cell infiltration in the portal area (arrow). (g) SLM+PMC group revealing mild vacuolar degeneration of hepatic cells. (h) EUO+PCM group showing mildly degenerated hepatocytes. (H&EX, 200)

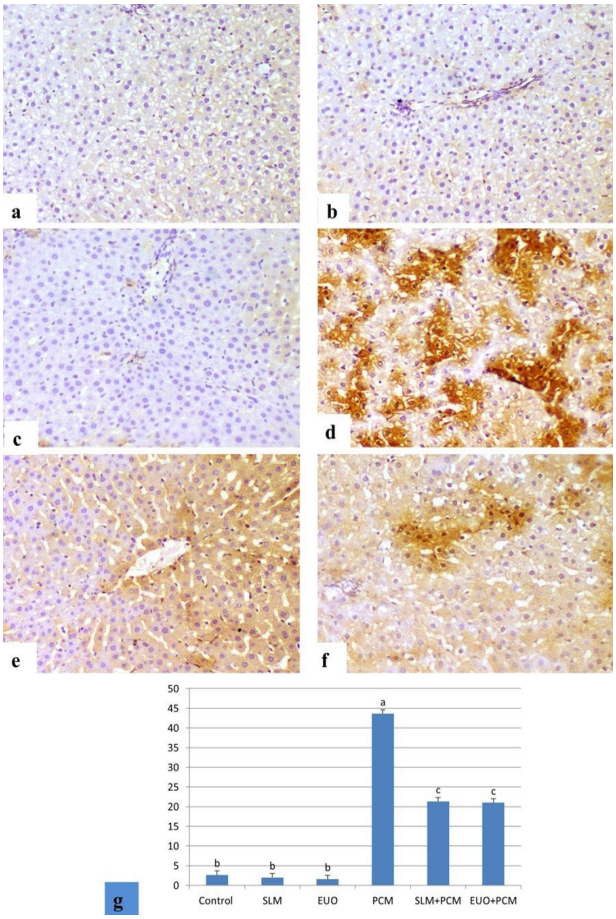

Figure 4. Immunostaining of BAX in the liver, (a), (b), and (c) control, SLM, and EUO groups, respectively, showing weak or no immuno- expression of BAX. (d) PMC group showing strong BAX expression. (e) and (f) SLM+PCM and EUO+PCM groups, respectively, exhibiting weak immune expression of BAX (BAX, X200). (g) area % of BAX expression in administered groups (Value was presented as mean±SE, Dissimilar letters denoting marked differences at $p < 0.05$).

Discussion

It is well recognized that APAP is a major contributor to drug-induced liver damage on a global scale and that its toxicity is dose-dependent (Bao *et al*., 2020). It is responsible for 44% of adult self-poisoning cases in the emergency rooms of hospitals (Wong & Graudins, 2017). So, our study evaluated the prospective influence of natural oil (EUO) as a protective agent versus APAP-induced liver toxicity.

Administration of rats with APAP revealed liver impairment as detected by marked rises in serum ALT, AST, ALP, and total bilirubin. Besides, APAP caused a decline in serum albumin and total protein compared to the normal rats. Our findings go hand in hand with (Geresu *et al*., 2022). Also, (Islam *et al*., 2021) claimed that overabundance of APAP produced reactive metabolite (NAPQI), which disrupted hepatic cellular permeability by inducing lipid peroxidation, causing greater levels of ALT and AST to leak into serum. Hepatic toxicity conduces to biliary congestion, which impairs the body's ability to excrete ALP from the body, resulting in an elevation in serum, as seen in the APAP group. The liver's function includes the creation of serum proteins. Rats given APAP were shown to have hypoproteinemia, an established indication of liver damage (Islam *et al*., 2021). Bilirubin cannot conjugate appropriately after a liver injury, leading to an elevation in the serum total bilirubin (Ozougwu, 2017). In contrast, administration of EUO before APAP partially restored liver functions to normal, as evidenced by a decrease in serum liver enzymes, ALP and total bilirubin levels, whereas serum albumin and total protein levels increased. These findings go in agreement with Mousa *et al*. (2020) who demonstrated that *E. globulus* reduced the alteration in liver function biomarkers caused by diclofenac sodium.

Additionally, acetaminophen encouraged an imbalance between antioxidants and oxidants, which was followed by cellular damage and necrosis (Kaplowitz *et al*., 2015). Our study reported that APAP-intoxicated rats exhibited a remarked elevation in the hepatic lipid peroxidation biomarker MDA and a decline in the protective antioxidants SOD, GPx and GSH, which are the same as those shown by Henneh *et al*. (2022) and Islam *et al*. (2021). Over administration of APAP induces the oxidation of unsaturated fatty acids within the cell membrane, leading to a significant amount of lipid peroxidation (Zoubair *et al*., 2013). APAP toxicity causes excessive NAPQI production and a decline in cellular GSH and SOD (Jaeschke *et al*., 2012). GSH is considered one of the most essential endogenous antioxidants that has a great role in hepatoprotection, synthesized by the liver, and performs as a substrate for glutathione peroxidase. Furthermore, it eliminates reactive oxygen species as superoxide, hydrogen peroxide, and alkoxy while maintaining membrane protein stability (Alabi *et al*., 2017). Nrf2 which is the major transcription factor of the antioxidant response element, showed a notable downregulation in the expression of its gene in rats administered APAP. It is now a crucial cellular target for the development of possible treatments and preventative measures for numerous illnesses (Abed *et al*., 2015). Eraky & El-Magd, (2020) reported that NrF2/BACH1/HO-1 have a great role in liver injury induced by APAP-overdose. On the other hand, pretreatment with EUO showed antioxidant influence by a remarkable decline in hepatic MDA, a rise in hepatic GSH, GPX, and SOD levels and an upregulation in the gene expression of Nrf2. Our outcomes are consistent with those of Zhao *et al*., (2021) who discovered that EUO may mitigate LPS-stimulated oxidative damage in mice by substantially reducing free radical levels, postponing SOD and GSH-Px activities, and lowering MDA levels. Furthermore, it has been demonstrated that *E. globulus*'s essential oil possesses anti-inflammatory and antioxidant qualities (Chandorkar *et al*., 2021).

Our research revealed that APAP inflammatory reactions are demonstrated by a remarkable rise in hepatic IL1β and TNF-α levels and, in addition, a decline in the anti-inflammatory IL10. Our findings concur with those of Ullah *et al*. (2022). The liver damage and inflammation caused by APAP are significantly influenced by pro-inflammatory cytokines. An imbalance between antioxidants and oxidants triggers the production of cytokines, which then use a positive feedback system to regulate inflammation in the liver tissue (Khan *et al*., 2020). Moreover, TGF-β is a cytokine that is essential for cell division, proliferation, and death (Crosas-Molist & Fabregat, 2015) and is synthesized by hepatic endothelial and Kupffer cells. Upregulation of TGF-β expression was found to be highly linked to the development of fibrotic disease, particularly in the liver, through the deposition of collagen and excessive matrix material (Roderburg *et al*., 2011). In accordance with other studies (Kaya *et al*., 2018; McMillin *et al*., 2019), which supported our findings, TGFβ1 signaling influences necrotic cell death, inflammatory reactions, oxidant/antioxidant imbalance, and

hepatocyte regeneration, all of which are important aspects of APAP-induced liver damage. On the other side, pretreatment with EUO followed by APAP administration succeeded in controlling the hepatic inflammatory process by increasing IL-10 and inhibiting TNF-α and IL-1β levels relative to APAP-intoxicated groups. This supports the outcomes of Arooj *et al.* (2023) and Zhao *et al.* (2021) who found strong anti-inflammatory properties of EUO. Furthermore, (Dhakad *et al.*, 2018) stated that 1,8-cineole is mostly responsible for the anti-inflammatory properties of EUO.

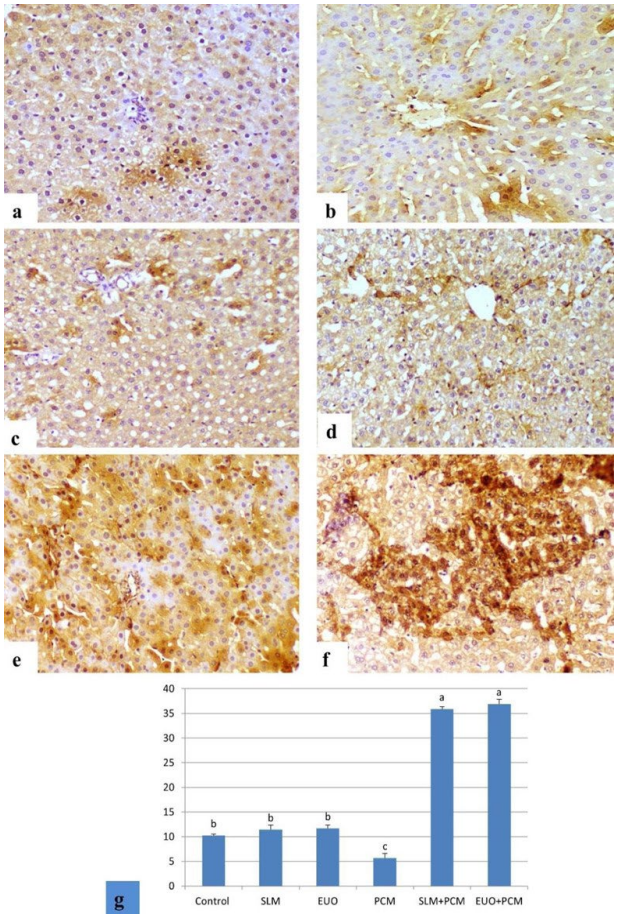

Figure 5. Immunostaining of BCL-2 in the liver, (a), (b), (c), and (d) control, SLM, EUO, and PMC groups showing weak immuno- expression of BCL-2. (e) and (f) SLM+PCM and EUO+PCM groups showing strong immune expression of BCL-2 (BCL-2, X200). (g) area % of BCL-2 expression in dissimilar groups (Value was expressed as mean±SE, dissimilar letters denoting marked differences at $p < 0.05$).

Acetaminophen-injected rats showed upregulation in the mRNA expression of CYP2E1 in comparison with the normal group. These findings are in harmony with those of (Yao *et al.*, 2019). The primary cause of APAP-induced liver impairment is the liver's creation of a toxic metabolite of NAPQI during the CYP2E1, 1A2, and 3A4 metabolism of APAP (Yoon *et al.*, 2016). Conversely, treatment with EUO followed by APAP administration downregulated the expression of CYP2E1 compared to APAP-intoxicated rats, which could be linked to its high eucalyptol content. According to the study by Jiang *et al.* (2019), administration of eucalyptol, which lowers the plasma concentration of APAP into NAPQI, decreased the significantly increased expression of CYP2E1 caused by APAP. Consequently, eucalyptol reduces liver damage.

Bcl-2/BAX/caspase-3 apoptosis signaling is a major controller of both apoptosis and cell survival, and inhibiting this cascade is thought to be a key approach for reducing cell death. (Gao *et al.*, 2008). Strong expression of BAX and weak expression of antiapoptotic Bcl-2 in APAP-administered rats relative to the control group indicated apoptosis. Subsequently, there was a notable increase in the quantities of proteins linked to apoptosis, such as caspase-3 and BAX, indicating a crucial role for apoptosis in hepatic injury caused by paracetamol (El-Gendy *et al.*, 2021). On the other side, administration of EUO followed by APAP injection revealed weak expression of BAX and strong expression of the anti-apoptotic Bcl-2 protein. Also, Ghareeb *et al.* (2019) are in harmony with us and verified that the anti-apoptotic impact of EUO might be because of its actions against oxidation and inflammation.

The histopathological results of PCM-intoxicated rats matched the changes observed in the molecular and biochemical studies, such as degenerative and necrotic hepatic cells, inflammatory cell infiltration and edema in portal areas which is in consistent with (Eraky & El-Magd, 2020). EUO succeeded in ameliorating all of these harmful impacts. The cause could be due to its activity against oxidation, inflammation, and apoptosis, which are linked with stimulation of the Nrf2 signaling pathway (Ghareeb *et al.*, 2019).

Conclusion

The current research proved the protective influence of EUO (30 mg/kg) versus APAP-mediated hepatic injury as defined by enhanced liver function parameters and corrected pro-oxidant and antioxidant imbalances in the hepatic tissue. Furthermore, EUO suppressed the inflammatory and apoptotic responses and corrected the histopathological alterations induced by APAP injection. Therefore, EUO can be utilized as a protective agent to improve APAP-stimulated hepatotoxicity due to its powerful antioxidant, anti-inflammatory, and anti-apoptotic characteristics. Further research is encouraged to figure out the additional mechanism of action of EUO as a therapy for liver disease before application.

Conflict of interest

The authors declare that they have no conflict of interest.

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