Hepatoprotective effects of *Gynura procumbens* against thioacetamide-induced cirrhosis in rats: Targeting inflammatory and oxidative stress signalling pathways

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ABSTRACT

Gynura procumbens is an edible flowering plant that has been utilized as traditional therapy for numerous diseases. The current experiment investigates the hepatoprotective potentials of the ethanol extract of *Gynura procumbens* leaf (EEGPL) against thioacetamide (TAA)-induced liver cirrhosis in rats. Thirty Sprague Dawley rats were randomly divided into 5 clusters: A, rats received orally 10% Tween 80 and intraperitoneal (i.p) inoculation of sterile distal water; B, rats received orally10% Tween 80; C, rats received orally daily 50 mg/kg of silymarin, while groups; D and E, rats received orally daily doses of 200 and 400 mg/kg of EEGPL, respectively. Furthermore, B-E clusters received 200 mg/kg thioacetamide (i.p) three times a week for 60 days.

The liver gross morphology of rats that received only TAA (B) revealed irregular rough surface layers compared to smoother livers of rats that received EEGPL. Histopathology of group B revealed clear hepatic necrosis and fibrous connective tissue, which were significantly reduced in C-E groups. EEGPL treatment caused a significant down-regulation of PCNA and α-SMA protein expressions. Antioxidant (SOD and CAT) enzymes in hepatic homogeneity were meaningfully lower, and MDA levels were significantly higher in TAA controls compared to those of C-E groups. Moreover, EEGPL treatment caused a reduction of TNF-α and IL-6 and increased expression of IL-10 cytokines. Therefore, the hepatoprotective potentials of EEGPL might be contributed to its modulation of detoxification enzymes, anti-inflammatory, and antioxidant activities.

1. Introduction

The liver has been always of biological importance due to its contribution to various metabolic and excretory processes. In recent

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decades, liver dysfunctionality and fibrosis have notably increased, mainly due to environmental carcinogens and lifestyle changes. New data analysis in 2020 has shown that the prevalence of hepatic injury and fibrosis has increased by 13% since 2000, reaching more than 1.5 billion [1]. Statistics have correlated liver cirrhosis with 2.2% of all death and 1.5% of all morbidity cases around the world, making it the 11th reason of death and the 15th causative factor of morbidity in 2016 [2]. Liver cirrhosis can be initiated via various biological reformations of the liver lobules, the build-up of feast fibrosis, and micro-, macro-nodules in parenchymal layers. Such physiological changes are accompanied by portal vein hypertension and cellular proliferation, which are worsened by the accumulation of free radicals and the reduction of endogenous antioxidants (liver defence factors) [3]. Moreover, hepatic injury is considered the outcome of prolonged liver damage due to numerous factors, including chronic disease (diabetes), environmental carcinogens, drugs, alcoholism, and obesity. Liver cirrhosis is considered an end stage of fibrosis, which is changed mainly by 4 major factors: tissue damaged area, the intensity and common paths of pro-fibrogenic factors, and the pro-fibrogenic myofibroblast factors [4].

Another cause of liver cirrhosis is thioacetamide (TAA), a laboratory chemical used by researchers to enhance the formation of hepatocyte injury and cirrhosis in rats, which resembles liver injury detected in humans [5]. The pathophysiology of TAA-induced liver cirrhosis has been linked to its alteration in glutathione (GSH) and endogenous antioxidant enzymes, facilitating lipid peroxidation, free radical formation, oxidative damage, and increased cell necrosis rate [6].

Herbal medicine and its derivatives have a long history as a traditional remedy for liver dysfunctionalities, and in today's world, these products are gaining more interest as alternative medicine due to numerous drawbacks related to synthetic chemicals. In last few decades, numerous research studies have reported the hepatoprotective of traditional herbal medicines including many species of the genus *Gynura* [7,8].

G. procumbens (Merr.) which is known in Malaysia as Sambung nyawa, is broadly dispersed in South-East Asian countries. Various parts of this medicinal herb have been conventionally used for dealing with eruptive fevers, rash, constipation, hypertension, diabetes mellitus, rheumatism, viral diseases of the skin, kidney diseases, migraine, and cancer [9]. The leaves of this plant are not toxic [10] and have been shown to have anti-herpes simplex virus, anti-hyperglycaemic, antihypertensive; anti-ulcerogenic, wound healing; analgesic, and abridged blood hypertension properties (reducing lipids and inflammation) [11].

Inflammatory cytokines are major contributors of the progression or suppression of liver cirrhosis in any stage of this disease. The cytokines tumor necrosis factor (TNF-α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) have been well-known as pro-inflammatory biomarkers that were significantly elevated during hepatocyte damage, which also facilitate the further progression of the disease through their contribution in many biological pathways, including lipid metabolism, protein synthesis (positive and negative acute phase), biliary system obstruction, and fibrosis progression [4]. In contrast, interleukin-10 (IL-10) has been known as an anti-inflammatory cytokine that suppresses cellular pathways involved in hepatic inflammation and further liver damage. The mechanism of action of inflammatory cytokines is set to be monitored by the transcription nuclear factor-kappa B (NF-$_k$B). Moreover, scientists revealed that pro-inflammatory cytokines can induce the pathway actions of NF-$_k$B, thereby creating a continuous auto-controlling cycle that can duplicate the inflammatory process for a prolonged time [12]. Therefore, the evaluation of these inflammatory cytokines becomes one of the main diagnostic tests in healthcare for better managing inflammatory diseases like liver cirrhosis [13].

Quite a lot of studies have revealed that EEGPL displays numerous biological activities due to its phytocontents (flavonoids, terpenoids, saponins, and tannins) [10]. Despite that, until now there is no rigid data on the therapeutic effect of this plant on chemical-mediated liver disease. Therefore, here we try to explore the hepatoprotection efficacy of this plant by evaluating its alteration on the histopathological, immunohistochemical, and antioxidant pathways in TAA-mediated liver cirrhosis in rats.

2. Materials and methods

2.1. Plant preparation

The *G. procumbens* leaves were found at Ethno Resources Sdn Bhd, Malaysia. The plant identification and authentication were done according to criteria recorded at the Herbarium of Rimba Ilmu, Foundation of Natural Science, and the University of Malaya. The GP leaves were desiccated and ground into fine dust. Two hundred grams of the residue were immersed in 1 L of 95% ethanol for 4 days. At that point, the combination was sieved via Whatman paper (#1) and cleansed by condensed pressure in a spinning evaporator. The obtained plant extract was dissolved in 10% Tween 80 and delivered to rats through oral gavage [6].

2.2. Thioacetamide and standard drug (silymarin)

Thioacetamide was bought from a Swiss company (Sigma-Aldrich)and then it was dissolved in 10% Tween 80 and mixed well till wholly quartzes were liquefied. At that time, 200 mg/kg body mass was given to rats by intraperitoneal injection in 3 dosages weekly for 60 days. The injection of TAA will produce significant tissue damage and biochemical modulations in rats, analogous to liver cirrhosis occurring in humans [6]. The standard silymarin was bought from Sigma Aldrich (Merk, Germany) and was dissolved in sterilized distal water to be orally delivered in a 50 mg/kg dosage [14].

2.3. Acute toxicity experiment

The mature male Sprague Dawley rats, aged 7–8 weeks and weighted 170–180 g, were obtained from the Animal House, Cihan University-Erbil. Rats were kept in separate cages (bottom designed with wide-mesh wire) to prevent coprophagia and ingested with

tap water and standard rat diets (pellets). The experiment began after 7 days of adaptation. For the acute toxicity procedure, rats were divided randomly into three groups; A, normal controls received 10% Tween 80 (5 mL/kg): B, rats received 2 g/kg of EEGPL; C, rats treated with 4 g/kg of EEGPL. Rats were fasting for overnight before treatment delivery. After supplementation, rats were fasting (food and water)foranother 3–4 h. The observation procedure started after supplementation and continued for 48 h (every 8 h) for any possible toxic signs or abnormal changes. After two weeks, rats received an over dose of anaesthesia (ketamine and xylazine) and sacrificed. The intercardial blood puncture were obtained for biochemical analysis and rat organs (liver and kidney) dissected for histological evaluations [15].

2.4. Trial procedure for hepatoprotective effects of EEGPL

The mature rats were aimlessly distributed into 5 clusters (6 rats each):

A, normal control rats had daily oral dose of 10% Tween 80 (5 mL/kg) and weekly three injections (5 mL/kg) of sterile distilled water.

B, cirrhosis rats received daily oral of 10% Tween 80 (5 mL/kg) and weekly three injections of 200 mg/kg of TAA.

C, Reference rats received daily oral dose of silymarin (50 mg/kg) and weekly three injections (200 mg/kg) of TAA.

D and E, EEGPL (ethanol extract of *Gynura procumbens* leaf)-treated rats were given orally 200 and 400 mg/kg, respectively, and weekly three injections (200 mg/kg) of TAA. The experiment continued for 8 weeks, and the body masses of laboratory animals in all groups were taken weekly [5]. Eight weeks later, rats were fasting for overnight and sacrificed after receiving anaesthesia (Ketamine and Xylazine. The blood samples was withdrawn from intracardiac puncture, and placed in yellow cap tubes (containing clot activator gel), spine, and examined for biochemical analysis [6].

2.5. Macroscopic analysis of the liver

The livers were separated, rinsed with normal saline (cold), and placed on the filter papers for the inspection of weight and gross pathological alterations [16].

2.6. Histopathology of the hepatocytes

The sliced liver tissues transferred into10% phosphate buffered formalin for 24 h for the fixation purpose and then transferred into mechanical tissue processing machine (Leica, Germany). Liver tissue slices (3–5 μm thickness) were fixed on slides for histological evaluations by using hematoxylin, eosin, and Masson Trichrome stains [15].

2.7. Immunohistochemistry

The liver fibrosis evaluation was also studied by immunohistochemical technique via estimation of PCNA and α -smooth muscle actin (α-SMA) protein expression. The intensity of stains in liver tissue was determined by calculating values of stained cells divided by 1000 liver cells and mitotic index was found based on detected cellular mitosis [17].

2.8. Endogenous antioxidant determination

Liver tissue samples obtained from both lobes of the liver. Hepatic tissues (1 g) were placed in a flask containing 10 mL (10%) of PBS solution (pH 7.2) before normalization and homogenization procedure by a homogenizer machine at 5000 rpm (15 min at $-4\,^\circ$C). The supernatant part was obtained and stored in a $-80\,^\circ$C freezer. Antioxidant kits were bought from German company (Merk) for the evolution of(catalase (CAT) and superoxide dismutase (SOD)), and malondialdehyde (MDA) contents [18].

2.9. Assessment of inflammatory cytokines

The evaluation of pro-inflammatory cytokines (TNF-α and IL-6) and anti-inflammatory cytokines (IL-10) in serum samples were possible via the application of an easily accessible ELISA kit (Cusabio Biotech Co. China). ELISA inter and intra-assay consistency is calculated via wells obtained from exact plate/assay kit.

Intra-Assay Precision, samples (3) of determined intensity were run three times on one plate. Intra-Assay: CV<8%

Inter-Assay Precision, samples (3) of known concentration were tested in several separate assays. CV (%) = SD/meanX100. Inter-Assay: CV<12%

The procedure began with centrifugation of the blood samples at 3000 g (15 min), and the obtained supernatant (serum) was separated and evaluated for the cytokine contents using a common enzyme-linked immunosorbent assay kit following the producer's guidelines as written in the ELISA kit for rats, TNF-α, IL-6, and IL-10. The concentrations were determined based on the purified reference cytokines [19].

2.10. Biochemistry of liver functions

The blood samples centrifuged and the isolated serum was investigated for the amount of liver enzyme contents including ALT,

alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase. Furthermore, the synthetic and excretory efficiency of the liver were evaluated via detection of the total protein, albumin, and bilirubin levels.

2.11. Statistical analysis

The statistical procedure included one-way analysis of variance (ANOVA) and Graph Pad Prism (version 9.0). Values were presented as MEAN $\pm$ S.E.M., and the significant level was set at $p < 0.05$.
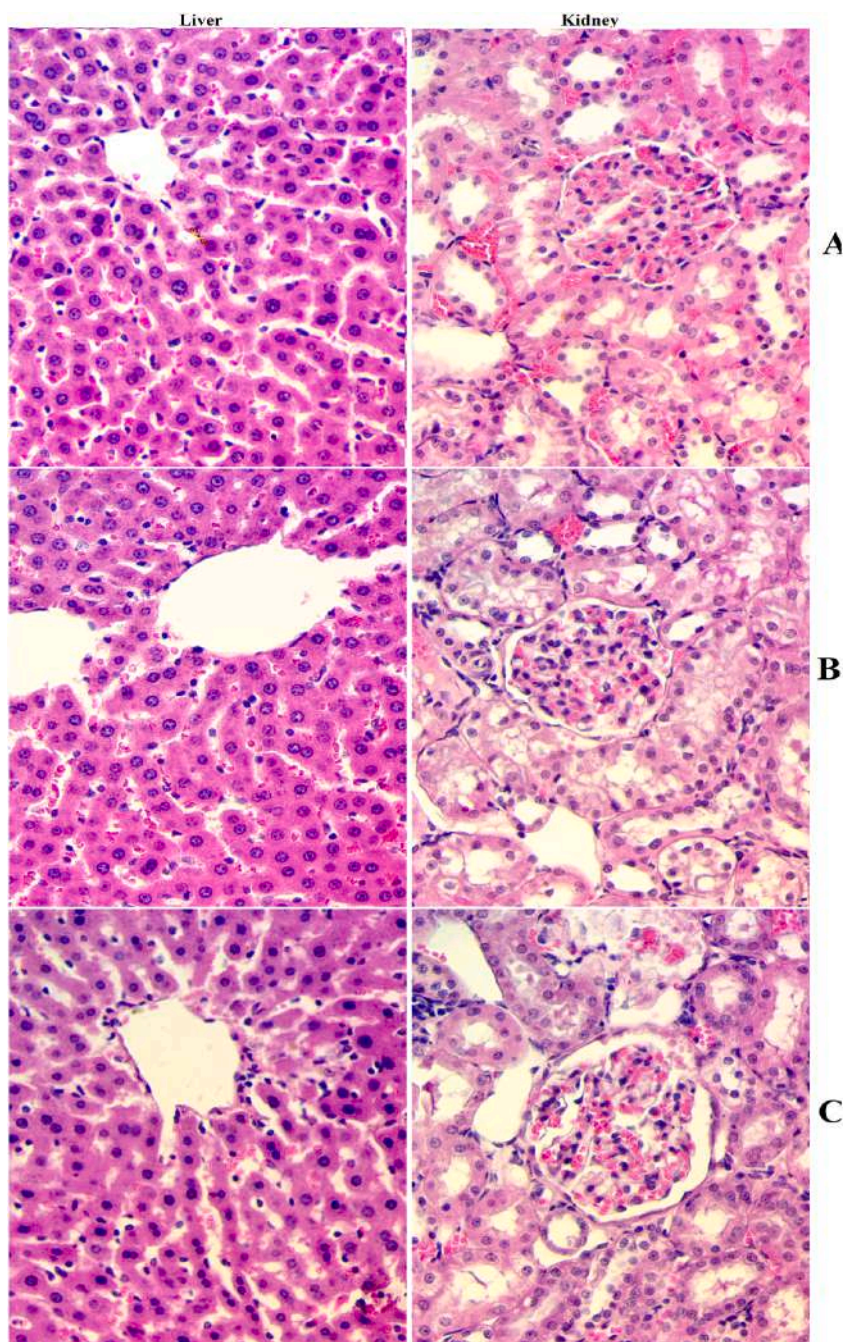

Fig. 1. EEGPL Effects on the tissue structure of the liver and kidney tissues of rats. A, normal control groups; B, rats administered 2 g/kg of EEGPL; C, rats received 4 g/kg of EEGPL. Microscopic view revealed non-statistical variance in the alignment of the kidney and liver tissues layers of rats in the 14 days' toxicity trial (Hematoxylin and Eosin stain, 40x).

3. Results

3.1. Acute toxicity

The present outcomes revealed the absence of any behavioural or physiological changes in rats after two-week administration of 2 g/kg and 4 g/kg of EEGPL. Furthermore, rats consumed equal amounts of food and water without significant differences in their body weight compared to normal controls. The biochemical analysis showed non-significant changes in the serum profiles represented by comparable levels of liver and renal parameters between normal control and supplemented rats. The histopathological investigations of liver and kidneys from EEGPL-treated rats showed the absence of any structure changes, which were very comparable to that of normal control rats. (Table 1A and 1B, and Fig. 1A–C). Indicating that the toxic dosage far EEGPL exceeds the tested dosages.

The present data outcomes for liver (Table 1 A) and kidney (Table 1 B) parameters showed comparable values between plant-treated (2 and 4 g/kg) rats and normal controls, according to the statistics shown below.

The oral supplementation of EEGPL (2 and 4 g/kg) to rats revealed non-significant biochemical changes in the kidney based on the estimated parameters compared to normal controls (Table 1 B and Fig. 1).

3.2. Hepatoprotection of EEGPL

3.2.1. Body and liver masses

The current results have detected significant differentiation between the body weight (BW) of experimental rats compared to those of normal controls. Rats treated with TAA only had statistically lower BW (178.33 ± 2.41 gm) than that 322 ± 5.74, 295.42 ± 2.82, 226.30 ± 3.75, 263.40 ± 4.33 gm of normal control, silymarin, 200 and 400 mg/kg of EEGPL-treated rats, respectively.

The liver weightiness of rats in the TAA control group was significantly higher (13.29 ± 0.06 gm) than that of the normal control, silymarin or EEGPL. Animals nourished with silymarin or EEGPL had positive augmentation of their body mass and liver index (Table 2). The liver index was statistically higher (7.45%) in TAA control compared to 2.63, 3.13, 4.78, 3.93% of normal control, silymarin, EEGPL 200 mg/kg and 400 mg/Kg-treated groups, respectively.

3.2.2. Morphology of liver

The morphological appearance of livers obtained from normal control rats demonstrate smooth surface of hepatic tissues with regular tissue layers. However, the microscopic views of liver tissue dissected form TAA control rats showed numerous micronodules with rough irregularities on the hepatic tissues. Rats treated with standard drug (silymarin) or EEGPL (200 mg/kg and 400 mg/kg) had significantly lower tissue damage (induced by TAA) in their parenchymal tissue, as shown by uniform tissue structure and fewer micronodules (Fig. 2, GA).

3.2.3. Microscopic results

The normal control rats (A) had a normal liver structure in the absence of any notable signs of inflammation or necrosis. The histological results of liver dissection from TAA control rats (B) showed significant tissue damage represented by disrupted endothelium, unclear nuclei, and increased cytoplasmic vacuoles, indicating severe tissue necrosis and inflammation. The results also revealed significant modification of parenchymal cells by fibrous septa that align the collagen linkage in the hepatic triangles, indicating numerous micro-, macro-nodules in the hepatocytes. Such nodules were surrounded by bundles of connective tissues that divide the liver into lobules, which exhibited significant inflammation and tissue necrosis. The liver histology of silymarin-treated rats (C) showed significant recovery from TAA-induced tissue damage as shown by lower infiltrated cells, hepatic micronodules (necrosis), and reduced tissue disruption compared to TAA control. Thus, the structure of the liver was well protected, as shown by normal hepatic lobules and numerous veins spreading throughout connective tissue. The liver tissue analysis of rats receiving 200 mg/kg of EEGPL (D) demonstrated significant retrieval from TAA-induced damage represented by lower scores for fibrotic tissues, lower nucleus damage, and vacuolization, fewer parenchymal cell damage with less tissue fibrosis and micro-nodules than that of TAA control, but not as significant as rats ingested the standard drug silymarin. Finally, the microscopic views of liver tissue showed fewer tissue penetration and parenchymal cell regeneration as expressed by fewer necrotic zones and fewer vacuoles in their endothelial and sub-endothelial layers (Fig. 2, H&E).

The Mason trachoma technique is utilized to evaluate the collagen fibrous deposition in the hepatic tissues. The current results of liver tissue coloured with Masson's trichrome revealed a lack of any collagen deposition in the hepatocyte tissues of normal control rats (Fig. 2, MT (A)). While, TAA-treated rats had severe liver injury indicated by increased collagen fibre deposition with notable concentrated septic fibrous (fibrosis) (Fig. 2, MT (B)). Contrary, rats ingested either silymarin (Fig. 2, C) or EEGPL 200 mg/kg and 400

Table 1A

Effect of EEGPL ingestion on rat's liver functions.

Animals groups	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	T. Bilirubin (μmol/L)	T. Protein (g/L)	Albumin (g/L)
Normal control (10% Tween 80)	81 ± 3.7	37.2 ± 2.44	58.8 ± 1.38	1.30 ± 0.06	73.5 ± 2.65	25.30 ± 2.7
EEGPL (2 g/kg)	72.32 ± 3.20	46.38 ± 3.40	63.00 ± 2.12	1.42 ± 0.07	68.40 ± 2.30	22.384 ± 2.48
EEGPL (4 g/kg)	$77,3 \pm 2.42$	36.4 ± 1.59	60.2 ± 2.45	1.28 ± 0.05	70.39 ± 2.61	28.20 ± 3.20

Data were presented as mean $\pm$ SEM (n = 6 per group).

Table 1B

Effects of EEGPL ingestion on rat's kidney function.

Animals groups	Sodium mmol/l	Potassium mmol/l	Chloride mmol/l	Urea mmol/l	Creatinine μmol/l
Normal control 10% Tween 80	148 ± 2.37	5.1 ± 0.32	107 ± 3.039	4.41 ± 0.21	42.22 ± 3.60
EEGPL 2 g/kg	145.21 ± 2.35	5.32 ± 3.65	136.5 ± 3.82	5.28 ± 0.32	37.23 ± 2.72
EEGPL 4 g/kg	141.22 ± 3.32	5.1 ± 0.30	100.23 ± 2.41	4.72 ± 0.45	40.32 ± 3.20

-Data are shown as mean ± SEM. Results were very much comparable between experimental and normal control groups.

Table 2

Influence of EEGPL on different body parameters of rats exposed to liver cihrrosis.

Groups	Body weight (gm)	Liver weight (gm)	Liver Index LW/BW %
Normal control 10% Tween 80+Distal water	322 ± 5.74[a]	8.48 ± 0.04[a]	2.63 ± 0.01[a]
TAA control 10% Tween 80+ TAA	178.33 ± 2.41[d]	13.29 ± 0.06 [d]	7.45 ± 0.06 [d]
Silymarin (50 mg/kg)+ TAA	295.42 ± 2.82 [b]	9.26 ± 0.03[c]	3.13 ± 0.06 [b]
EEGPL (200 mg/kg) + TAA	226.30 ± 3.75[c]	10.83 ± 0.07 [b]	4.78 ± 0.02[c]
EEGPL (400 mg/kg) + TAA	263.40 ± 4.33 [b]	10.37 ± 0.08 [b]	3.93 ± 0.04 [b]

Values are shown as mean (n = 6) ± S.E.M. Numbers with shared letters within same column indicates non-significant at $p < 0.05$.

mg/kg (Fig. 2, MT (D and E)) had significant liver tissue recovery represented by reduced septic fibrous tissue nodules and a significantly lower intensity of collagen deposition compared to TAA control rats.

3.2.4. Immunohistochemistry by α-SMA and PCNA stains

The current results of the liver fibrosis rate according to the expression of immunohistochemical staining of αSMA protein in the liver tissue significantly differed between all rat groups. Histological evaluation showed the lowest α-SMA intensity in the liver parenchymal tissues of normal control rats (Fig. 3A and F). Contrary, the TAA control rats (Fig. 3B and F) showed the highest α-SMA protein expression compared to all other rat groups, indicating severe tissue injury, fibrosis, and tissue proliferation due to TAA ingestion. The silymarin-treated rats (Fig. 3C and F) had reduced expression of α-SMA stain in their liver tissue, indicating the resistance efficacy of this drug against liver fibrosis and protection of the liver tissues against TAA-induced injury. Rats ingested 200 mg/kg of EEGPL (Fig. 3D and F) presented mild to moderate expression of α-SMA concentration. Moreover, rats ingested 400 mg/kg of EEGPL (Fig. 3E and F) revealed a significant reduction in the hepatocyte cell injury represented by down-regulation of α-SMA stain intensity and decreased mitotic index. Thereby, EEGPL showed significant hepatoprotection through ameliorating fibrosis rate in liver tissue and reduced inflammation rate in the liver parenchymal tissues.

The results of evaluating the hepatocyte proliferation were determined based on the expression of immunohistochemical PCNA stain in the parenchymal cells by utilizing a typical anti-PCNA antibody (Fig. 4).

The normal control rats showed zero expression of PCNA stains, indicating the absence of cell renewal (Fig. 4A). Contrary to this, TAA control rats (Fig. 4B) showed an increased presence of PCNA stains and an up-regulated mitotic index as signs of tissue proliferation and liver cirrhosis induced by TAA. The hepatic tissues obtained from rats treated with silymarin (Fig. 4C), 200 mg/kg EEGPL (Fig. 4D), or 400 mg/kg EEGPL (Fig. 4E) showed significant condensed liver cell renewal as shown by reduced PCNA-stained cells and a significantly lower mitotic index than that of TAA control rats. Thereby, EEGPL has shown a significant inhibitory impact on PCNA expression and mitotic index in a dose-related manner.

3.2.5. Influence of Gynura procumbens on antioxidant enzymes

The current results of antioxidants in liver homogenates significantly varied between the treated and control rats. The normal control rats (A) had the highest concentration of antioxidant activity in their homogenized hepatic tissues, represented by an increased level of SOD (17.87 U/mg), CAD (36.84 U/mg), and reduced lipid peroxidation activity, represented by the lowest MDA level (1.18 U/mg) compared to all other rat groups. In contrast, TAA control rats (B) experienced the lowest antioxidant status expressed by decreased SOD (8.35 U/mg) and CAT (19.95 U/mg) levels and an increased lipid peroxidation status indicated by increased MDA (4.62 U/mg) values (Fig. 5a and b). Contrary to this, rats that ingested silymarin (C) or EEGPL 200 and 400 mg/kg (D and E) had significant improvement in the antioxidant activity and MDA values in the hepatic tissue homogenates. The SOD and CAT of the silymarin group were found to be 12.21 and 28.07 U/mg, which were very comparable to those of the D and E groups, respectively (Fig. 5a and b). Moreover, rats that ingested silymarin had significantly lower MDA (1.54 U/mg) values than those of the TAA control group, but the values were found to be non-significant compared to those (2.16 and 1.78 U/mg) of the D and E groups, respectively (Fig. 5, c).

3.2.6. Inflammatory cytokines

The results of inflammatory and anti-inflammatory biomarkers were significantly different between all rat groups due to differentiation in the liver damage grades. The normal rats (A) showed the lowest TNF-α and IL-6 and the highest IL-10 cytokines compared
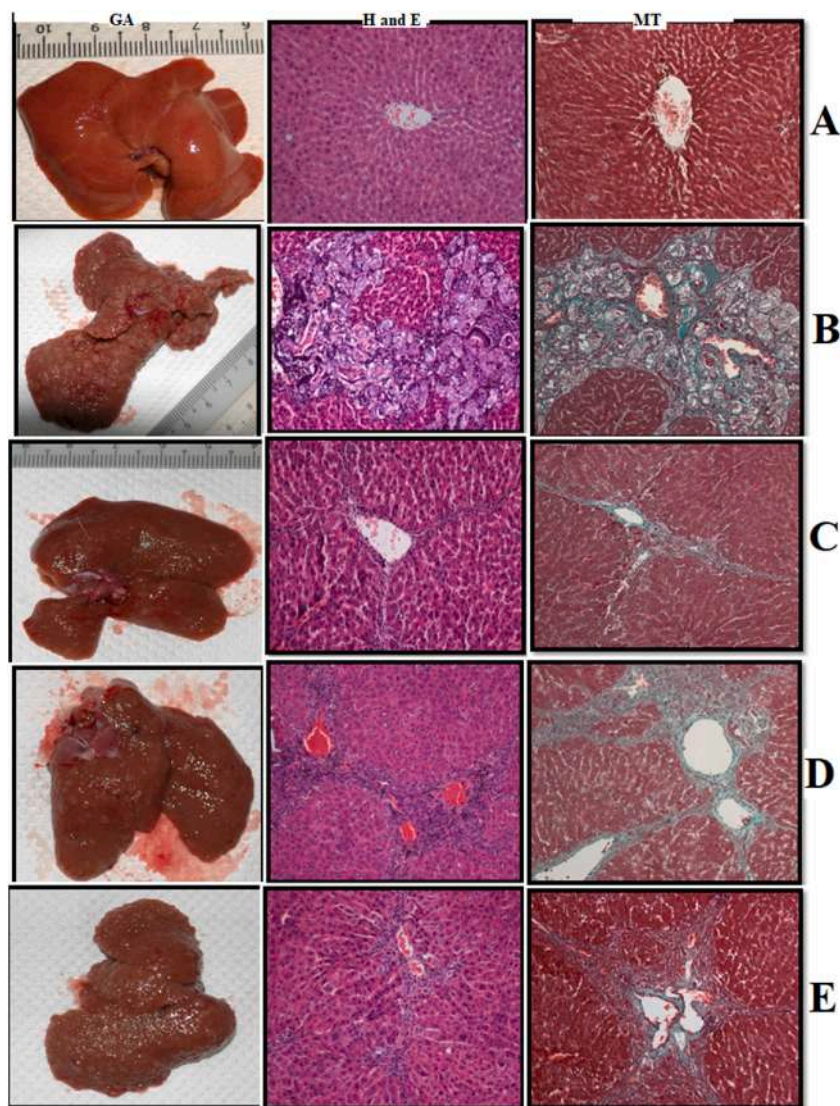
Fig. 2. The liver histopathology by using different stains H&E, Hematoxylin and Eosin; MT, Mason Trichrome stains; GA, gross appearance. A, TAA control rats had normal liver tissue architecture; B, rats treated with TAA only had an elongated bile duct, enlarged fibrous septum, numerous nodules, and severe collagen deposition. C, rats ingested TAA + silymarin had fewer liver injuries, as demonstrated by less fibrous septa and reduced collagen deposition. D, rats treated with TAA+200 mg/kg EEGPL had less liver injury than TAA controls, as expressed by an almost normal amount of fibrous septum and less tissue nodules. E, rats ingested TAA+400 mg/kg EEGPL showed comparable liver tissue structure to that of silymarin-ingestedrats, as their livers had fewer fibrous septa, lower amount of nodules, and reduced collagen deposition. The coloured hepatic tissues were observed by a Nikon microscope (Y-THS, Japan). 20X magnification.

to all other rat groups. While rats receiving only TAA had significant up-regulation of TNF-α and IL-6 values, and down-regulation of IL-10 cytokines, indicating severe inflammation initiated by TAA. In contrast, rats treated with silymarin (C) or EEGPL (D and E) had a significant reduction in the TNF-α and IL-6 values and a significant increase in the IL-10 in the serum samples compared to those of TAA control rats (Fig. 6a-c).

3.2.7. Impact of EEGPL on biochemical parameters

The serum biochemical results revealed significant up-regulation in the TAA control rats (B) compared to the normal control rats (A), indicating clear enzyme leakage due to severe hepatic tissue penetration induced by TAA. Rats who had oral ingestion of silymarin (C) or EEGPL (D and E) supplementation showed lower liver enzymes and bilirubin concentrations in their serum compared to those of TAA control rats (Table 3). The liver synthetic function of TAA control rats was significantly reduced, as represented by reduced detection of total protein and albumin in their serum, indicating severe liver tissue injury. The TAA-mediated hepatotoxicity was less affecting the protein synthesis in the silymarin or EEGPL-treated rats represented by increased total protein and albumin content than
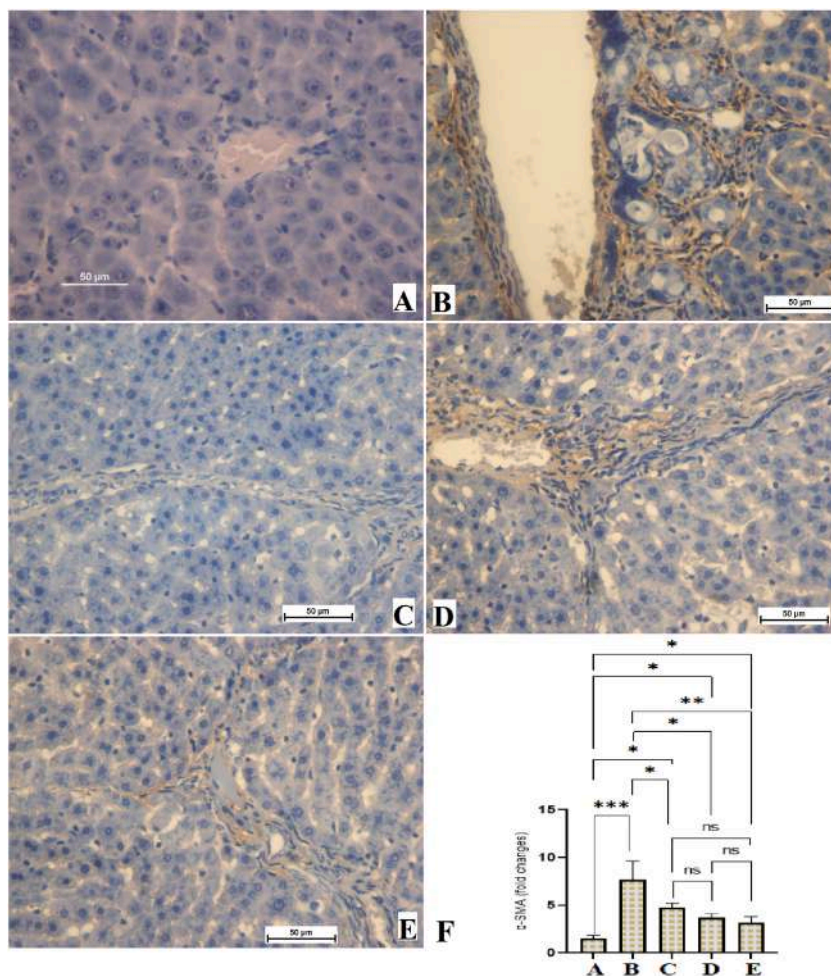
Fig. 3. EEGPL effects on α-SMA protein appearance in the liver of rats exposed to TAA hepatotoxicity. A, normal control; B, TAA control rats; C, Silymarin-treated rats; D and E were from rats that ingested 200 and 400 mg/kg EEGPL, respectively. The stained hepatocytes were viewed by a Nikon microscope (Y-THS, Japan). 100X magnification.

that of the B group, evidencing toxic resistance to EEGPL and normalization of liver synthetic functions (Table 3).

4. Discussion

The present EEGPL supplementation (2 and 4 g/kg) to rats in a 14-day toxicity trial did not cause any abnormalities in the serum biochemical (liver and kidney) parameters or their organtissue structure, with a zero mortality rate even after the experimental period. Suggesting that the toxic dose for this plant exceeds the 4 g/kg dosage. Similar results were reported in the absence of any physiological change or mortality among rats administered 5 g/kg of EEGPL for 2 weeks [20].

The present work revealed that rats treated only with TAA (B) had significantly lower body weight and increased liver weight compared to the normal control. Similar results have been reported by numerous researchers during their investigations of different liver cirrhosis trials [5,21,22]. However, rats that received silymarin or EEGPL had almost normal body weight and liver weight. This can be correlated with the lower inflammation rats in those groups compared to the TAA control. Comparable results were reported previously [23,24].

The current study showed that TAA control rats experienced significant hepatomegaly and augmented the weightiness/body mass ratio, which could be correlated with the accumulation of fat and deterioration in the hepatocytes (alcoholic steatosis). This effect was in line with a previous statement on the improved liver heaviness/body heaviness relation in hepatotoxic rats [14,22]. The drop in liver/body weightiness seen in rats ingested EEGPL might be due to its anti-lipidemia [25], anti-inflammatory [26], and anti-radical properties [27], as previously explained.

In the current study, the TAA injection caused severe liver cirrhosis in rats, which could be due to its effects on liver enzymes, as results indicated significant up-regulation of ALP, ALT, and AST enzymes in TAA control rats. Studies have linked the TAA efficacy in the elevation of liver enzyme production with its reaction with the nucleic acids (DNA and RNA), thereby causing extracellular liver
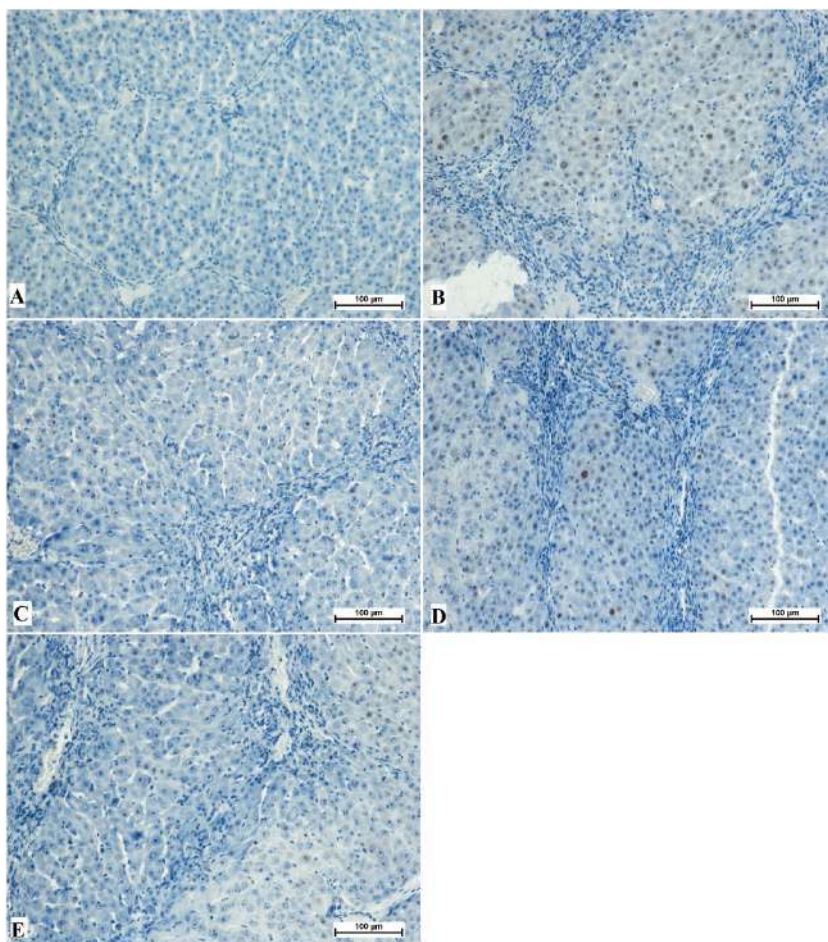
Fig. 4. Effect of EEGPL on the expression of PCNA stains in liver tissue. A, normal control rats; B, cirrhosis control rats; C, silymarin-treated rats; D and E, rats ingested orally with 200 and 400 mg/kg EEGPL, respectively. Hepatic tissues were viewed by a Nikon microscope (Y-THS, Japan). 20X magnification.

damage that accelerates the hepatic enzyme formation and their leakage into the extracellular fluid [28]. In contrast, the EEGPL treatment caused significant retention in the enzymatic function of the liver, which could be correlated with its efficacy in reducing liver tissue damage and enzymatic leakage. Similarly, numerous studies have shown the protection of EEGPL against CCl4-induced inflammation and correlated it with its antioxidant and anti-lipidemic potentials. Such biological activities were mainly correlated with its phytochemical contents, flavonoids, and phenolic compounds [29]. Moreover, researchers have reported the hepatoprotective effects of ethanol extracts of GP stem in ethanol-induced liver steatosis and they have linked this action with its TAA regulation of lipid metabolism processes including MAPK/SREBP-1c-dependent and -independent mechanisms [30].

The biochemical evaluation also revealed that TAA injection caused a significant reduction in the levels of albumin and total proteins in TAA control rats. This has been linked with the effect of this chemical on lowering the transcriptional processes (mRNA) and significantly with the RNA program from the nucleus to the cytoplasm, initiating severe injury in the cell membranes and leading to significant enzyme leakages. Prolonged liver leakage consequently leads to diminished proteins in cells and extracellular fluids [31]. Similar results were detected in our investigation, in which the level of liver proteins was significantly dropped, but they were significantly retrieved almost to a normal state in rats ingested silymarin or EEGPL. Accordingly, scientists have shown the efficiency of many herbal products in normalizing the liver proteins in hepatotoxic-induced rats [17,32,33].

The current gross study showed increased production of micro-, macro-nodules on the parenchymal tissues of the liver obtained from TAA control rats. Similarly, numerous studies have shown the efficacy of TAA in the reformation of the liver surface tissues (irregular surface), indicating proliferative cells (tumors) and presence of severe hepatocyte injury. In contrast, silymarin or EEGPL-treated rats had livers with a more regular surface smooth area, indicating significant protection against TAA-induced liver damage. Similar outcomes were reported previously [34,35].

The histological examination of liver tissue stained with H and E stains revealed significant liver injury in TAA control rats who received only TAA (TAA controls). Moreover, livers obtained from the TAA control group showed significant collagen deposition (using Masson's trichrome stain), indicating severe alteration of the membrane permeability of hepatocytes. In contrast, rats
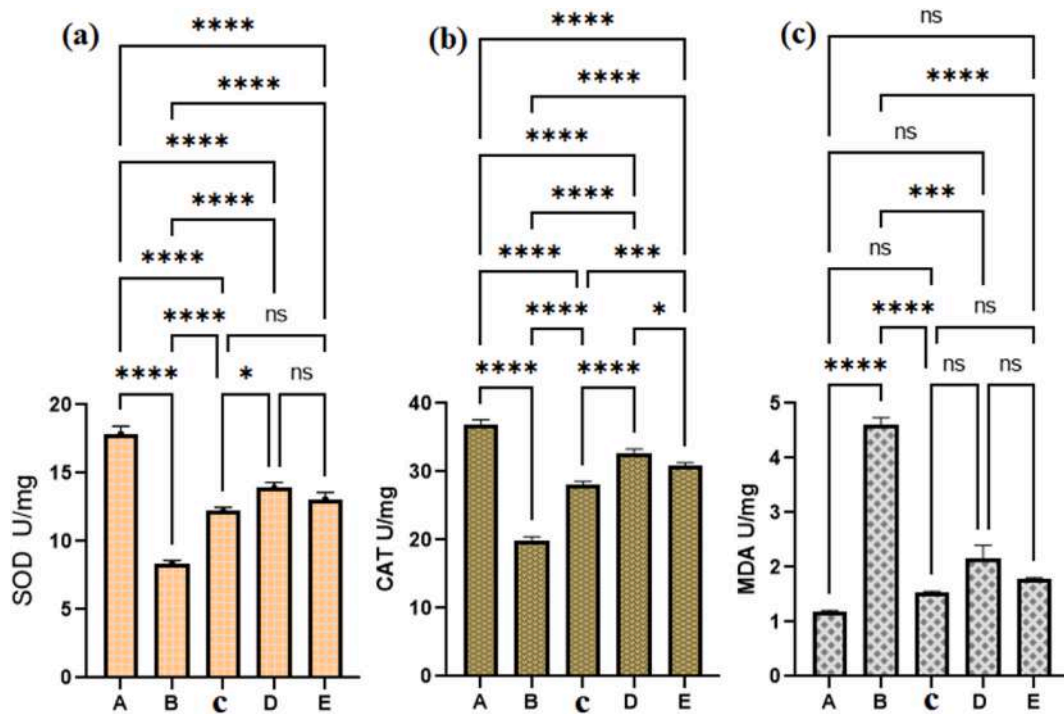
Fig. 5. EEGPL effects on the liver antioxidants (SOD, CAT and MDA) in the liver homogenates obtained from TAA-produced cirrhosis in rats. Values were demonstrated as mean (n = 6) ± S.E.M. ns, non-significant; *, $p < 0.05$; ***, $p < 0.001$, and ****, $p < 0.0001$.
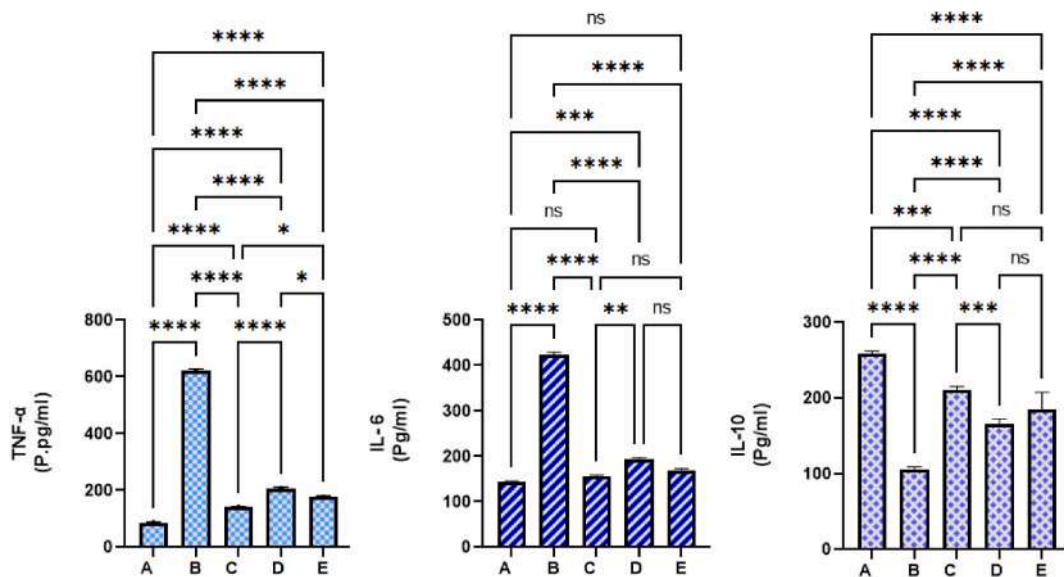

Fig. 6. EEGPL effects on inflammatory indicators in rats exposed to hepatotoxicity. A, control group; B, TAA group; C, silymarin group; and D and E were rats receiving 200 and 400 mg/kg of EEGPL, respectively. ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, and ****, $p < 0.0001$.

administered silymarin or EEGPL showed lower colour intensity in their liver tissue, meaning less collagen deposition occurred. Accordingly, numerous scientists have reported the efficacy of TAA in releasing collagens from liver cells and the potential of herbal medicines in turning over this action [24,36].

The immunohistochemical outcomes have shown that TAA controls experienced severe liver cirrhosis, fibrosis, and cellular proliferation, as shown by increased concentrations of α-SMA and PCNA proteins in their liver tissue. In contrast, silymarin or EEGPL-treated rats revealed significantly lower expression of these two proteins, indicating less liver tissue damage. Similar results were

Table 3
Effects of EEGPL ingestion on liver synthetic functions in rats exposed to hepatoxicity.

Groups	ALP (IU/L)	ALT(IU/L)	AST(IU/L)	Total bilirubin uM	Protein (g/L)	Albumin (g/L)
Normal control	95.34 ± 3.21[a]	61 ± 3.2[a]	168 ± 4.55[a]	2.5 ± 0.3[a]	65 ± 2.8[a]	12.8 ± 0.43[a]
TAA control	231 ± 4.30[c]	204 ± 4.82[c]	309 ± 5.43[c]	8 ± 0.22[c]	53 ± 2.5[c]	7 ± 0.65[c]
Silymarin (50 mg/kg)+ TAA	127 ± 3.22[b]	74 ± 5.4[b]	184 ± 4.25[b]	5 ± 0.36[b]	65 ± 3.7[b]	12 ± 0.44[b]
EEGPL **(200 mg/kg)+ TAA**	172 ± 3.47[b]	86 ± 3.53[b]	210 ± 4.25[b]	6 ± 0.74[b]	58 ± 2.3[b]	10 ± 0.23[b]
EEGPL **(400 mg/kg)+ TAA**	144 ± 3.24[b]	77 ± 4.2[b]	194 ± 3.67[b]	5 ± 0.49[b]	62 ± 2.4[b]	11 ± 0.53[b]

Values are shown as mean (n = 6) ± S.E.M. Numbers with shared letters within same column indicates non-significant at $P < 0.05$.

reported on the up-regulation of these proteins by TAA and their down-regulation by medicinal plants or their natural products [21, 37]. Previously, scientists have also demonstrated the efficacy of EEGP stems in reducing the expression of immunohistochemical stains in nanochemical toxicant-induced liver injury in mice, and they have correlated this bioactivity with the plant's phytoconstituents, namely caffeoylquinic and non-caffeoylquinic acids [38].

The evaluation of antioxidants (SOD and CAT) in the liver homogenates has become one of the main indicators of oxidative stress-causing liver injury [17]. The current data analysis showed significant oxidative stress in TAA control rats, presented by reduced SOD and CAT levels with up-regulation of MDA levels (lipid peroxidation) in their hepatic tissue homogenates, which play a crucial role in the progression of liver cirrhosis, as scientists explained [39]. TAA has been linked with the induction of free radical formation in liver tissues, initiating oxidative and consequently leading to hepatic necrosis and liver cell apoptosis [40]. Contrary to cirrhosis control rats, rats fed with EEGPL had shown higher free radical quenching enzymes and lower lipid peroxidation.This anti-radical action of EEGPL can be explained through its flavonoids and phenolic contents (caffeoylquinic acid, kaempferol, quercetin, coumaroyl-*O*-hexoside, coumaroylquinic acid, and caffeoyl-*O*-hexoside) as previously reported in details [10,41].

The immune defence changes are another physiological alteration caused by TAA in hepatotoxic rats. Immunity modulation mainly includes increased production of pro-inflammatory cytokines (TNF-α and IL-6) and decreased anti-inflammatory cytokines (IL-10), thereby causing more ROS formation and oxidative tension [40]. Studies have shown that chemical-induced liver injury will significantly increase levels of pro-inflammatory cytokines and decrease anti-inflammatory cytokines in the hepatocytes [42]. Similar results were detected in TAA control rats, which experienced increased TNF-α and IL-6 levels and significantly reduced IL-10 values than that of control rats. But such inflammatory changes were curbed in EEGPL-treated rats, which showed lower TNF-α and IL-6 levels and higher IL-10 levels than those of TAA control rats, thereby showing EEGPL as an effective anti-inflammatory product in cirrhotic livers. Similar outcomes were reported by the previous researchers regarding the anti-inflammatory potentials of EEG stems and leaves in different in-vitro and in-vivo experiments, and they have correlated this bioactivity with its phytochemical contents (phenolic and flavonoids) [23,29,38,43]. The above data could be considered as suffecient scientific explanation for the hepatorpotective roles of *G. procumbens*. The current outcome scientifically backup the therapeutic efficacy of this plant that could serve as new medicinal source for better management of patients with liver cirrhosis.

5. Conclusions

The ethanolic leave extracts of *Gynura procumbens* have shown significant hepatoprotective potentials based on the histological, immunohistochemical and biochemical evaluations. The molecular mechanism behind these action can be explained through its positive augmentation on the inflammatory cytokines, antioxidant enzymes, immunohistochemical proteins (α-SMA and PCNA), altogether resulted in decreased liver cell proliferation, and lower mitotic index. Hepatoprotection of EEGPL can be also correlated with its inducing actions on the SOD and CAT enzymes and its inhibitory potentials on MDA levels. The current study faced many obstacles including, a small sample size, unavailability and expensiveness of laboratory reagents, and inability to extract specific active ingredients of this plant due to poor facility and lack of specialized instruments.

Ethics approval

The study protocol approved by ethics committee in Cihan University-Erbil (No. 278, October 20, 2022). The procedure was according to the standards set by national and international organization for animal use in the laboratory [44].

Author contribution statement

Ahmed A.j. Jabbar: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Zaenah Zuhair Alamri, Nur Ain Salehen, Zakia Salim Amur Al Sinawi, Soliman Mohammed Alfaif: Contributed reagents, materials, analysis tools or data.

Mahmood Ameen Abdulla: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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