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Hepatoprotective Properties of Starflowers from an Annual Herb, *Borago officinalis* L. (Boraginaceae)

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Abstract:

Traditionally, the decoction of leaves, flowers and stems of *Borago officinalis* was widely used in the treatment of liver diseases. The present work is aimed to assess the hepatoprotective properties of selected ethanolic fractions of *Borago officinalis* flowers in D-galactosamine (GalN)-induced oxidative stress in rats. *In vitro* antioxidant activities were assessed through superoxide radical and ferric ion assays, while hepatoprotective effects were evaluated by GalN-induced oxidative stress in rats. Initially, ethanolic extract of *B. officinalis* flowers was fractionated using column chromatography. The preliminary antioxidant screening of these fractions identified two main bioactive fractions (**BoF2** and **BoF5**), which were found to have significant radical scavenging properties compared to ascorbic acid. Based on the antioxidant profile, **BoF2** and **BoF5** were evaluated for hepatoprotective activity in GalN-induced rats. The Wistar rats were grouped (n = 6) and treated with **BoF2** and **BoF5** (100 and 200 mg/kg), silymarin (25 mg/kg) orally for 7 days, and GalN (200 mg/kg, i.p) was dosed on day 5. this study found that chronic administration of GalN significantly ($P < 0.0001$) altered the liver parameters and oxidative stress markers (MDA, SOD, and CAT). The administration of **BoF5** prominently ameliorated the oxidative stress induced by GalN compared to **BoF2**. Histopathological studies further supported the significant hepatoprotective action of **BoF5**. The present study demonstrates that the *B. officinalis* possess significant antioxidant properties by augmenting the magnitude of the antioxidant enzymes (SOD and CAT), and further reducing MDA levels.

Keywords: Antioxidant; GalN-induced; Hepatoprotective; Oxidative stress; *Borago officinalis*; liver parameters.

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1. Introduction

Annual herb, *Borago officinalis* (synonyms: Borage, starflower; family: Boraginaceae) is widely distributed in Asia, Germany, France, Denmark, and the United Kingdom. In the Kurdish culture, the decoction of leaves, flowers and stems of *B. officinalis* was widely used in the treatment of liver diseases, multiple sclerosis, cough, diabetes, hoarseness, heart problems, asthma, bronchitis, arthritis, abdominal pain and eczema [1,2]. Earlier, few groups established the chemical composition of seed oil of *B. officinalis* and reported it to have a good content of fatty acids such as linolenic acid, oleic acid, stearic acid, palmitic acid, eicosenoic acid and erucic acid [3,4]. Recent pharmacological studies hypothesized various extracts of *B. officinalis* have potential actions to inhibit free-radical and inflammatory enzymes [5-7], and to treat gastrointestinal, respiratory and cardiovascular disorders [8]. So far, no studies established the hepatoprotective potential of *B. officinalis* flower.

The liver plays an essential role in the metabolism of carbohydrates, proteins, and lipids. It is the major site for the detoxification of various toxic substances [9]. The

alteration in the liver's normal functioning leads to abnormal metabolism? and decreased elimination of toxic metabolites that alters the homeostasis[9-10]. The main factors causing liver damage are disorders or diseases, drugs, chronic alcoholism, and various poisonous substances like D-galactosamine (GalN) [11]. The increased production of reactive oxygen free radicals (ROS) by the above-mentioned conditions will ultimately culminate in oxidative stress and tissue inflammation [12].

Oxidative stress plays a dynamic role in the pathogenesis of GalN-associated liver diseases [11]. GalN-induced oxidative stress is generally attributed to the formation of the ROS thereby attenuating antiperoxidative enzymes and damage to the cell membrane. However, supplementation with antioxidants could mobilize the detoxification mechanism and may have a hepatoprotective role in these illnesses [13].

Taken together, the present study aimed to evaluate the antioxidant and hepatoprotective activities of bioactive fractions of ethanol extract of *B. officinalis* flowers against GalN-induced oxidative stress in Wistar albino rats.

2. Materials & Methods

2.1. Collection of herb material

The star-shaped flowers from *Borago officinalis* L. were collected from Nilgiris hills, Nilgiris district, Western Ghats, Tamil Nadu in January 2021. The material was authenticated and deposited in the Herbarium at the Department of Botany, Andhra University, India with voucher number AUDB-2021-12.

2.2. Extraction and fractionation

The shade-dried flowers of *B. officinalis* were ground into a coarse powder using an electrical blender. By maceration technique [14], the powdered material (500 g) was extracted with ethanol for 7 days. The obtained solvent mixture was concentrated under reduced pressure using rotavapor (Buchi R-210 Rotavapor, Marshall Scientific, USA) yielded ethanolic extract of *B. officinalis* (**Bo**, 20 g, 4% w/w) as a dark greenish solid. Using column chromatography (300 mm × 18 mm sintered disc column (Code No.: 6101062), Borosil, India) of mesh size 100-200, **Bo** (30 g) was fractionated using a hexane/ethyl acetate solvent system (100:0 → 0:100), which yielded five fractions (**BoF1-BoF5**).

2.3 In vitro antioxidant activity

2.3.1. Evaluation of superoxide radical scavenging activity

By employing the superoxide radical assay [15] in triplicate, the **BoF1-BoF5** and **Bo** were evaluated for antioxidant activity. To the known concentrations (25-100 µg/ml) of **BoF1-BoF5** and **Bo** added nicotinamide adenine dinucleotide (73 µM) and nitroblue tetrazolium (50 µM), and incubated for 30 min at 37 ˚C. Later, absorbance was measured at 562 nm against the blank. Ascorbic acid was used as a standard drug.

2.3.2. Evaluation of ferric ion reducing power assay

The ferric ion reducing power assay was determined in triplicate by the modified method of Haritha *et al.*[16]. To 2.5 ml of potassium ferricyanide added known concentrations (25-100 µg/ml) of **BoF1-BoF5** and **Bo** and incubated at 50 ºC for 20 min. To it, 0.5 ml of ferric chloride (0.1%) and 2.5 ml of trichloroacetic acid (10%) were added, and the absorbance was noted at 700 nm. Ascorbic acid was used as a standard drug.

2.4. Total phenol and flavonoid contents

The total flavonoid and phenolic content of the extract were evaluated by using aluminium chloride (10%)[17] and Folin- Ciocalteau reagent (0.5 ml) [18], respectively. The total flavonoid and phenolic content of the **BoF2** and **BoF5** were expressed as mg rutin and mg gallic acid equivalent per gram of sample (mg/g), respectively.

2.5. Animals

Adult Wistar albino rats (weighing 190 ± 10 g, age 6-8 weeks) of either sex were used in this study. The animals were given food and water *ad libitum* and were housed in the Animal House under the standard condition with a temperature of 25±2 °C, relative humidity (50±10%), and a 12-h light/12-h dark cycle. This study was approved by the Institutional Animal Ethics Committee (Regd No. 516/PO/c/01/CPCSEA).

2.6. Acute oral toxicity studies

The OECD main test 420 was utilized for acute oral toxicity studies. Rats were randomly divided into four groups (five females) and dosed with 5, 50, 300 and 2000 mg/kg body weight (b.w) of **Bo** suspended in gum acacia and tween-80. The test animals have undergone fasting overnight before administering the **Bo** using oral gavage. The testing was ended until the last three animals survived the upper bound dose, and all of the test animals were observed for up to 14 days and the LD_{50} value was caluculated [19,20].

2.7. Experimental protocol of hepatoprotective activity of BoF2 and BoF5

At the beginning of the experiment, rats were randomly divided into seven groups (six rats in each group). In group 1 (normal control), rats were administered orally

with only 0.2 ml of the vehicle for 7 days. In group 2 (toxic control), rats were dosed intraperitoneally with only 0.2 ml of the GalN (200 mg/kg) for 7 days. In group 3 (standard), rats received 25 mg/kg b.w of silymarin orally for 7 days. Rats in groups 4 and 5 received 100 mg/kg b.w (as a low dose) and 200 mg/kg b.w (as a high dose) of **BoF2**, respectively, orally for 7 days. Rats in groups 6 and 7 received 100 mg/kg b.w (as a low dose) and 200 mg/kg b.w (as a high dose) of **BoF5** respectively, orally for 7 days. On day 5, GalN (200 mg/kg, i.p) was administered in all rats, except the normal control group [11]. After 48 h of GalN administration, animals were anesthetized for obtaining the blood and sacrificed to collect the liver organs. In the present study, 1/20 and 1/10 of the LD_{50} value (i.e., 2000 mg/kg b.w) were chosen as low and high dosages of test samples.

2.8. Collection of serum samples

The blood samples were obtained from the portal vein and 0.5 ml of blood samples were transferred into laboratory tubes containing pre-autoclaved nutrient broth medium (Sigma-Aldrich, Germany) and put in an incubator at 37°C. The remaining blood samples were decanted gently into collection plastic tubes and centrifuged at 4000 rpm for 5 min. Then serum was obtained, aliquoted into microtubes, and stored at -80 °C for biochemical analysis [21].

2.9 Tissue homogenization

At the end of the study, by cervical dislocation, all the rats were sacrificed. The livers were removed, weighed up, and washed thoroughly. Some portion of tissue was stored immediately in buffered formalin (10%) for histopathological studies, and the remaining tissue was processed. In 0.05M of ice-cold phosphate buffer saline (pH 7), the tissue was minced into small pieces and homogenized with a Homogenizer (Remi Homogenizer, Mumbai, India) to obtain 10% whole homogenate. To the homogenate, an equal volume of trichloroacetic acid (10%) was mixed and centrifuged (Sigma-3-30 KS, USA) for 10 min at 5000 rpm [22].

2.10 Assessment of oxidative stress parameters

The above-obtained supernatant was subjected to estimate the oxidative stress parameters, namely malondialdehyde (MDA) levels, superoxide dismutase (SOD) levels, and catalase (CAT) activity using the established procedure [23].

2.11 Assessment of serum biochemical parameters

The serum biochemical parameters such as alkaline phosphate (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) were estimated as per the standard method described in the EXCEL kit leaflet (EXCEL Pvt Ltd, India).

2.12 Histopathological studies

The thin sections of formalin-fixed liver tissues were made using paraffin blocks and stained by hematoxylin and eosin stain. These stained sections were inspected under a light microscope.

3. Results and Discussion

Since ancient times, plants and their parts are being used to treat hepatotoxicity both in Ayurveda and Unani systems [24, 25]. Based on this evidence, the search for new lead molecules for liver disorders from natural sources is a crucial point of interest [26]. As a result, many plants and their phytochemicals were tested for antioxidant and hepatoprotective potentialities [27,28]. Therefore, in the present study, *B. officinalis* flowers were extracted with ethanol (**Bo**) and fractionated (**BoF1-BoF5**).

The antioxidant activity of **BoF1-BoF5** and **Bo** was evaluated by superoxide-free radical scavenging activity of ferric ion reducing power assays. All the tested samples exhibited a considerable antioxidant activity compared with ascorbic acid (Table 1).

The concentration of **Bo, BoF2** and **BoF5** needed for 50% inhibition of superoxide radicals were found to be 38.51±2.11 µg/ml, 55.02±2.44 µg/ml, and 52.20±1.53 µg/ml, while ascorbic acid was 26.00±1.98 µg/ml (Table 1). Alternatively, the IC_{50} values of **Bo, BoF2** and **BoF5** on ferric ions were found to be 44.10±2.02 µg/ml, 79.22±4.32 µg/ml, and 55.21±1.86 µg/ml whereas ascorbic acid value was 35.10±1.98 µg/ml (Table 1).

On the other hand, the total flavonoid value of **BoF2** and **BoF5** was equivalent to 4.68 ± 0.10 and 8.22±0.12 mg rutin/g dried extract, respectively, while the total phenolic value was equal to 110.20 ± 1.70 and 144.55 ± 2.40 mg gallic acid/g dried extract, respectively (Table 2).

Earlier, total phenolic content and antioxidant evaluation of various (methanol, acetone, and water) extracts of Borage flowers was performed and among all, methanol extract was found to have better phenolic content, as well as, activity against DPPH, β-carotene/linoleic acid, and ferric ions [29]. However, our present total phenolic content and antioxidant results were consistent with this study. Thus, the two obtained fractions (**BoF2** and **BoF5**) exhibited significant superoxide-free radical scavenging activity and ferric ion reducing power capabilities attributed to the substantial quantity of phenol and flavonoid compounds [30, 31] (Table 1).

Table 1. Primary screening of antioxidant activity of Bo and its fractions.

Samples	Percentage inhibition at different concentrations (%)				IC_{50} values (µg/ml)
	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	
DPPH free radicals					
Bo	43.33±5.02[a]	65.51±2.43	78.30±5.40[a]	96.14±1.35	38.51±2.11[c]
BoF1	9.89±0.91[c]	13.09±0.50[c]	17.65±0.96[c]	19.88±1.66[c]	>100
BoF2	29.29±4.28[c]	46.11±2.95[b]	66.62±3.60[b]	75.96±4.97[c]	55.02±2.44[c]
BoF3	9.18±0.25[c]	18.18±1.45[c]	22.79±2.85[c]	30.62±2.63[c]	>100
BoF4	7.01±0.14[c]	15.06±1.54[c]	21.83±1.51[c]	27.96±1.71[c]	>100
BoF5	35.51±6.28[b]	47.20±1.34[b]	77.21±1.65[a]	86.73±2.77[a]	52.20±1.53[c]
Ascorbic acid	48.63±1.20	68.58±1.82	81.71±3.10	97.56±2.07	26.00±1.98
Ferric ions					
Bo	27.95±1.92[b]	56.69±2.46[b]	66.23±2.40[b]	78.27±2.49[c]	44.10±2.02[b]
BoF1	2.32±0.20[c]	4.96±0.49[c]	10.28±0.93[c]	12.01±0.49[c]	>100
BoF2	28.85±2.81[b]	34.21±4.18[c]	47.66±4.12[c]	67.59±2.35[c]	79.22±4.23[c]
BoF3	6.00±0.40[c]	10.34±0.45[c]	14.35±1.36[c]	21.91±1.56[c]	>100
BoF4	4.56±0.85[c]	7.20±0.73[c]	11.20±1.16[c]	17.98±1.45[c]	>100
BoF5	20.69±1.91[c]	46.31±1.55[c]	65.04±2.30[b]	76.93±2.42[c]	55.21±1.86[c]
Ascorbic acid	38.50±1.36	67.21±2.52	78.74±4.06	95.23±4.99	35.10±1.98

Values are represented as mean ± SD (n = 3). Where, [a]P < 0.05, [b]P < 0.001, [c]P < 0.0001 as compared with ascorbic acid was considered as statistically significant using one-way ANOVA with Dunnett's multiple comparison test.

Table 2. Total flavonoid and phenol contents of BoF2 and BoF5.

Sample	Total flavonoid content (mg rutin /g dried extract)*	Total phenol content (mg gallic acid /g dried extract)*
BoF2	3.34 ± 0.90	124.14 ± 3.74
BoF5	10.11 ± 0.74	166.47 ± 3.54

*mean ± SD (n = 3)

An acute toxicology study of **Bo** was carried out with five female albino mice at 5, 50, 300 and 2000 mg/kg b.w. No mortality, abnormal signs in locomotor activity, or diarrhea was observed over the course of two weeks. Therefore, the LD_{50} value of **Bo** was noted as above 2000 mg/kg b.w, and the low (1/20) and high (1/10) dosage was fixed as 100 and 200 mg/kg b.w, respectively.

An experimental protocol of hepatoprotective activity of **BoF2** and **BoF5** was established using five animals in each group. In the toxic control group, the chronic administration of GalN ($P < 0.0001$) significantly elevated the serum AST, ALT and ALP levels compared with the normal control group (Figure 1). The animal group administered with **BoF2** did not show significant variation in ALT levels at a 100 mg/kg dose. In contrast, the rat group administered with **BoF5** offered significant dose-dependent protection and significantly reduced the elevated levels of liver parameters at both low and high doses (Figure 1). Moreover, administration of 200 mg/kg dose of **BoF5** ($P < 0.0001$) offered almost equivalent protection in the liver parameters compared with silymarin (25 mg/kg) (Figure 1).

In general, acetaldehyde accumulation in the liver inhibits the transport pumps present on the hepatocytes' membrane, thereby outflowing the liver enzymes such as AST, ALT and ALP into serum resulting in hepatotoxicity [11,32]. In the current investigation, similar observations were noticed in the toxic control group. Administration of **BoF2** and **BoF5** significantly reduced the elevated levels of AST and ALP (Figure 1). Notably, administration of a higher dose of **BoF5** displayed equivalent protection in the liver parameters compared with silymarin (Figure 1).

Oxidative stress is the pathogenesis of numerous chronic diseases that affects the imbalance between the antioxidants and reactive oxygen species [33-36]. The endogenous antioxidant enzymes like SOD and CAT play a vital role in catalyzing the H_2O_2 into the water, the major precursor of ROS. The depletion of SOD and CAT results in oxidative stress in surrounding tissues and

causes tissue and organ necrosis [37, 38]. Moreover, the excess generation of ROS plays a vital role in hepatotoxicity's etiology. The chronic administration of GalN activates the kuffer cells, thereby generating ROS and pro-inflammatory substances [39, 40]. These excess ROS covalently binds to the hepatocytes' membrane and results in the oxidation of polyunsaturated lipids that markedly elevate the MDA levels. Further, excess accumulation of both ROS and MDA levels together causes oxidative stress in hepatocytes [41-43].

The GalN-treated group ($P < 0.0001$) significantly reduced the endogenous antioxidant enzymes, namely SOD and CAT, compared with the normal control group. The animal group administered with both the fractions (**BoF2** and **BoF5**) offered significant protection against the GalN-toxicity in level (Figure 2).

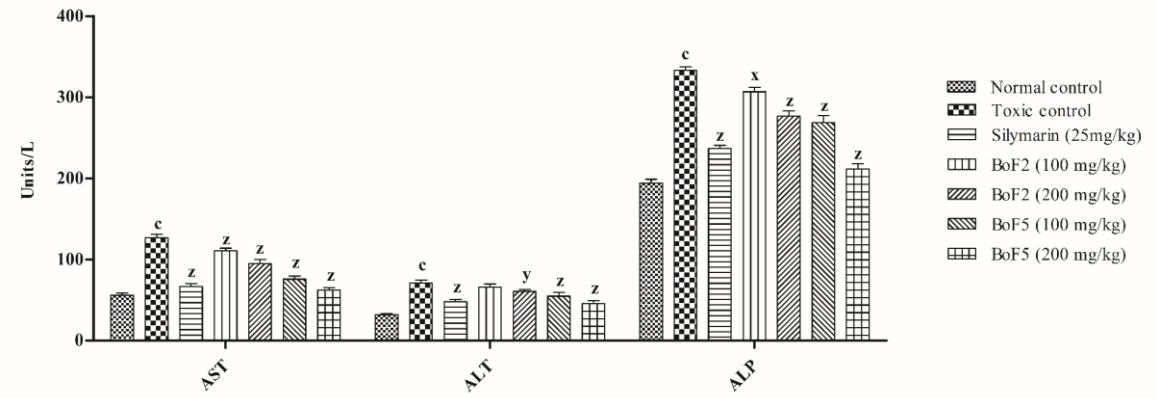

Figure 1. Effect of BoF2 and BoF5 on liver function tests. All the values were expressed as mean ± SEM (n = 6). Where, $^{c}P < 0.0001$ as compared with normal control, and $^{x}P < 0.05$, $^{y}P < 0.001$, $^{z}P < 0.0001$ as compared with toxic control was considered as statistically significant using one-way ANOVA with Dunnett's multiple comparison test.

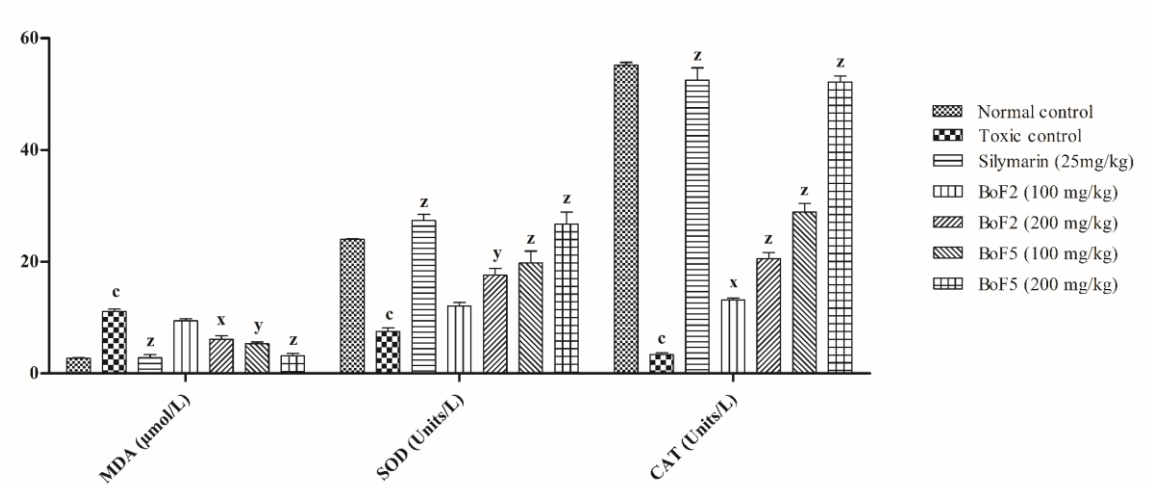

Figure 2: Effect of BoF2 and BoF5 on tissue oxidative stress markers. All the values were expressed as mean ± SEM (n = 6). Where, $^{c}P < 0.0001$ as compared with normal control, and $^{x}P < 0.05$, $^{y}P < 0.001$, $^{z}P < 0.0001$ as compared with toxic control was considered as statistically significant using one-way ANOVA with Dunnett's multiple comparison test.

The high dose of **BoF2** significantly elevated the SOD and CAT levels compared with its low dose and toxic control group (Figure 2). On the other hand, administered **BoF5** offered dose-dependent protection and the high dose of **BoF5** ($P < 0.0001$) showed a potent elevation of SOD and CAT levels similar to that of silymarin (Figure 2).

Further, the toxic control group ($P < 0.0001$) significantly elevated the serum levels of MDA compared with the normal control group (Figure 2). The administration of **BoF2** and **BoF5** significantly depleted the elevated MDA levels compared with the GalN-induced group (Figure 2). A 200 mg/kg dose of **BoF2** ($P < 0.05$) significantly decreased the MDA levels, while 100 mg/kg of **BoF2** was non-significant in controlling MDA levels. **BoF5** markedly diminished the MDA levels in a dose-dependent manner, and the effects at a higher dose (200 mg/kg) were comparable with silymarin (Figure 2).

The present study's findings indicate that GalN administration markedly elevated the MDA levels by depleting the endogenous antioxidants (SOD and CAT) (Figure 2). In contrast, the administration of fractions significantly antagonized the oxidative stress induced by GalN by enhancing the SOD and CAT levels and simultaneously depleting the MDA levels in a dose-dependent manner when compared with toxic control (Figure 2). The administration of 200 mg/kg of **BoF5** exhibited marked protection of tissue oxidative stress markers similar to silymarin. Moreover, our outcomes are identical to the previous study models of hepatotoxicity [11,39,41].

The liver's architecture is clear, and the hepatocytes were arranged clear in normal control (Figure 3A). In contrast, the GalN administration results in disruption of the architecture, infiltration of inflammatory cells, vacuolated hepatocytes deposition of lipids, and degenerated nuclei (Figure 3B). At a high dose, administration of **BoF5** remarkably reversed the alterations induced by GalN than **BoF2**, supporting the research findings of biochemical (Figure 3D-E). These histopathological results support the hepatoprotective potentiality of *B. officinalis* against GalN-induced rats.

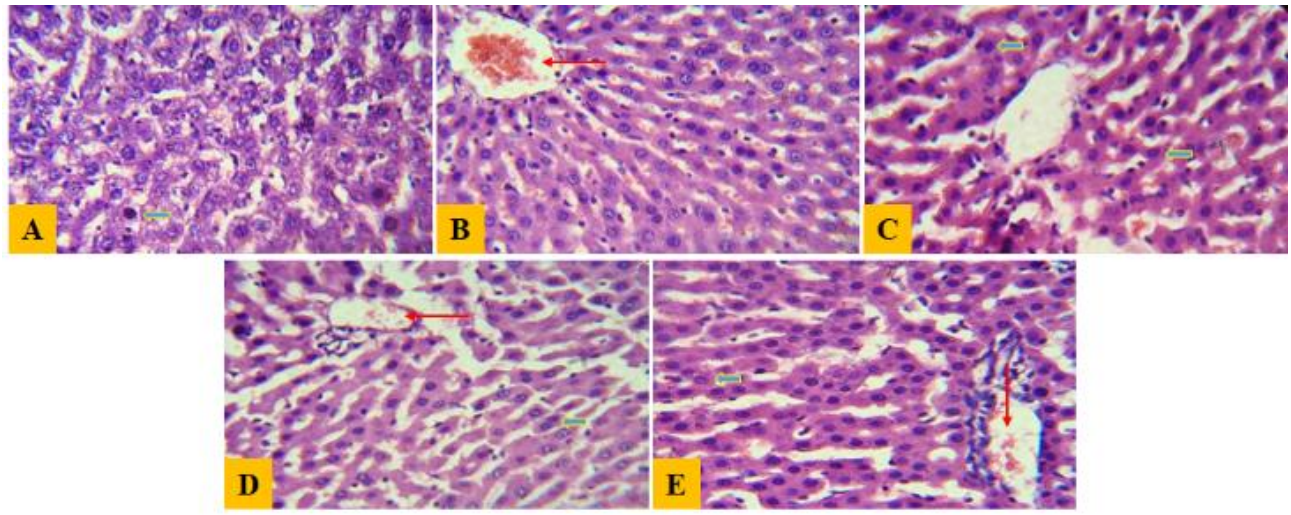

Figure 3. Histopathological studies of liver tissues of BoF2- and BoF5-treated groups against GalN-induced oxidative stress. (A) Normal control; (B) Toxic control; (C) standard (silymarin, 25 mg/kg); (D) BoF2 (200 mg/kg); (E) BoF5 (200 mg/kg). Green arrow indicates hepatocytes; Red arrow indicates infiltration of inflammatory cells.

4. Conclusion

To conclude, our findings demonstrated that *B. officinalis* possesses hepatoprotective and antioxidant activity, which is evidenced by lowered serum hepatic marker enzyme activities in GalN-induced Wistar albino rats. Among the two dosages tested, 200 mg/kg body weight showed promising hepatoprotective activity, and is comparable to the standard drug silymarin. Hence, *B. officinalis* could be used in the treatment of different liver ailments.

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