Research Article
ISSN:2249-1236
Geetha N ***et al. IJRRPAS,(2018),8(1) 001-009***
International Journal of Research and Reviews in Pharmacy and Applied sciences

Hepatoprotective activity of methanolic bark extracts of *Ficus*species

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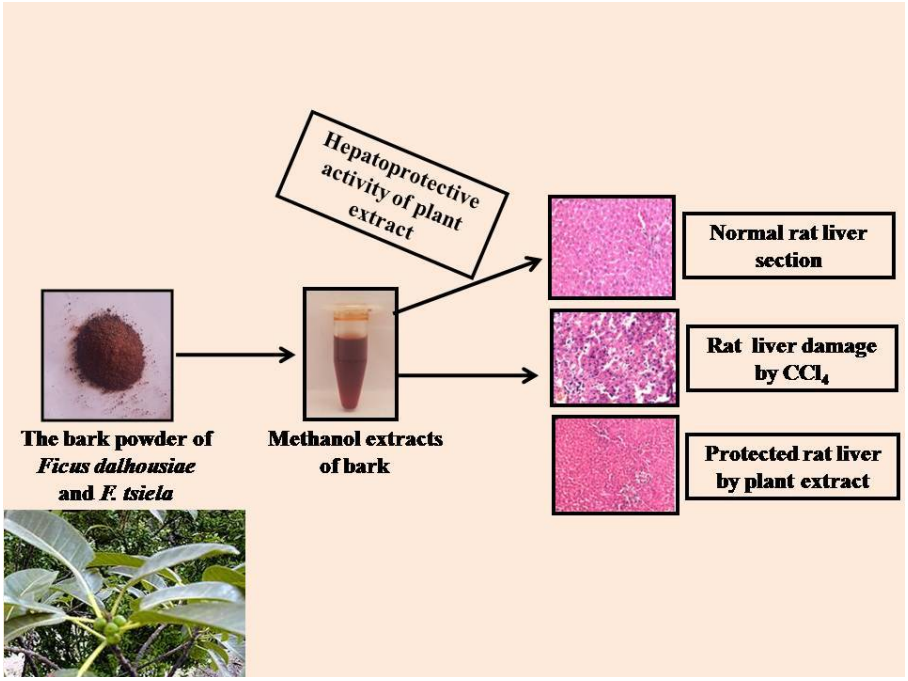

Abstract:
The methanol bark extracts of *F. dalhousiae* (FdBM) and *F. tsiela* (FtBM) extracts were tested for the hepatoprotective activity against Albino Wister rats with liver damage induced by carbon tetrachloride (CCl_4). The bark extracts exhibited a liver protective property by lowering the serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH). The levels of liver cytosolic superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities were raised significantly after oral administration of the FdBM and FtBM extracts at the doses of 150 and 300 mg/kg body weight. The obtained results revealed that the cytosolic enzymes play a crucial role in the cellular defense mechanisms of rats. The hepatoprotective activity was further confirmed by histopathological studies of liver tissue. This study thus has an importance in future development of a novel therapeutic against liver damage.

Keywords:*Ficus*, Hepatoprotective activity, Bark extract, CCl_4

1. Introduction:

Herbal medicines are precious and continuously used in India as 'traditional medicine' from several years for the primary health care. The bioactive compounds used in herbal medicines are secondary metabolites of medicinal plants have several pharmacological properties [1]. The secondary metabolites of plants like phenolic compounds are having positive impact on human health due to their antioxidant properties [2]. The antioxidants have a key role in prevention of several human diseases like cancer, cardiovascular disease, macular degeneration, cataracts and asthma. These phytochemicals have also role in improvement of human immune system [3]. The phenolic compounds investigated from a variety of medicinal plants have anti-microbial, anti-biofilm, analgesic, anti-pyretic, antitumor and hepatoprotective activities [4, 5]. The liver is a vital organ of human being has a key role in maintenance of several physiochemical functions. Therefore, any damage to hepatic cells can lead to its dysfunction. The liver cell damage can occur due to various chemicals and infectious agents. Although a variety of drugs are available in the market which are harmful to liver and also generate free radicals. The excess level of free radicals may cause oxidative damage and chronic liver inflammation [6]. The conventional and synthetic drugs have been used for the treatment of hepatic diseases. These drugs have several adverse effects on human body. Therefore, search of an alternative drug to the conventional drugs is thus of prime importance. The plant resources have been used for the treatment of various diseases due to their no or less side effects on human body [7]. Many plant extracts contain high level of antioxidant compounds having hepatoprotective property [8]. Thus, the development of novel drugs by using plant resources against hepatic diseases could be an alternative drug to traditional and synthetic drugs.

A variety of plants are used in traditional medicine system due to their potential medicinal and therapeutic applications. The *Ficus* is a well known deciduous tree which belongs to *Moraceae* family. They are woody plants or trees have a vast traditional role in indigenous system of medicine like Ayurveda, Siddha, Unani and Homoeopathy etc [9]. The *Ficus* species are being used as important ingredients in many ayurvedic and traditional medicines. The different parts of *Ficus* plants like barks, leaves, fruits and latex are commonly used in Indian traditional medicines. These plants are used for the treatment of several diseases like diabetes, skin diseases, ulcers, dysentery, diarrhea, stomachache and piles etc. They are also used as carminative, astringent, anti-inflammatory, antioxidant, antihepatotoxic and anticancer agents [10]. The phenolic compounds with pharmacological properties are obtained from the fig leaves. They are namely, furanocoumarins (psoralen and bergapten), flavonoids (quercetin 3-O-rutinoside) and phenolic acids (ferulic acid, 3-O-caffeoylquinic acid and 5-O-caffeoylquinic acid) [11].

In the present study, two*Ficus* species such as *F. dalhousiae* and *F. tseila*(Roxb) were used to study their hepatoprotective activity. A fig plant (*F. dalhousiae*Miq) is known an indigenous medicinally important plant scripted in Amarakosa[12]. However, very less research work has been carried out on *Ficus* species with respect to anti-microbial, anti-biofilm and hepatoprotective activities.

2. Experimental:

Chemicals

The chemicals such as glutathione, bovine serum albumin (BSA), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenyl hydrazine (DNPH), trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2; 30%), thiobarbituric acid (TBA), sodium citrate, pyrogallol, and ethylene diamine tetra acetic acid (EDTA) were purchased from Sigma–Aldrich Inc. (Mumbai, India) with highest purity grade and used for experiments as per requirement.

Collection and preparation of the plant extracts:

The barks of different fig plants were collected from KuntiBetta and Kigga, Karnataka, India. The collected plants were identified and validated from the plant taxonomist, Karnataka, India.The shade-dried powered of bark of *F. dalhousiae* and *F. tseila* were prepared. The contents of fine bark powders were successively extracted with methanol (99 %) in a Soxhlet apparatus. The methanol based bark extracts were concentrated by drying on vacuum rotary evaporator. After drying, the obtained bark extracts was stored at 4^{o}C and used for further experiments.

Experiments on rats for biochemical assay:

In these experiments, Albino Wister rats with body weight ranging from 150-200g were used. The protocol used in this study was approved by Ethical Committee of Zoology Department, University of Mysore, Mysuru, Karnataka, India. Initially, the experimental rats were properly acclimatized to laboratory conditions for about 7 days before commencement of the experiments. The FdBM and FtBM extracts were dissolved thoroughly in the sterile distilled water. Two different concentrations of these bark extracts were administered orally. The rats were divided into seven groups and each group consists of six rats (n=6) as shown below:

Group I – (Positive control)

The experimental rats were received only food and water.

Group II – (Negative control)

The experimental rats were received CCl_4 (1 mg/ml); only once on 8^{th} day.

Group III

The experimental rats were received the FdBM extract (150 mg/kg b.w.) followed by CCl_4 (1 mg/ml); only once on 8^{th} day.

Group IV

The experimental rats were received the FdBM extract (300 mg/kg b.w.) followed by CCl_4 (1 mg/ml); only once on 8^{th} day.

Group V

The experimental rats were received the FtBM extract (150 mg/kg b.w.) followed by CCl_4 (1 mg/ml); only once on 8^{th} day.

Group VI

The experimental rats were received the FtBM extract (300 mg/kg b.w.) followed by CCl_4 (1 mg/ml); only once on 8^{th} day.

Group VII

The experimental rats were received Silymarin (100 mg/kg b.w.) followed by CCl_4 (1 mg/ml); only once on 8^{th} day.

On the eighth day, the experimental rats of group II to VII were administered with a single oral dose of CCl_4 in olive oil (1:1) at 1 ml of body weight after the last dose of extracts. After 16 h of CCl_4 administration, the experimental rats were sacrificed after anaesthesia. The blood samples were collected through retro-orbital plexus and allowed to clot for 30 min at 28^{o}C. The liver perfused with saline and processed immediately for further biochemical assays.

Measurement of serum biochemical parameters:

The clear serum was obtained by centrifugation of blood sample at 2500 rpm for 10 minutes. The quantity of LDH, ALP, AST and ALT in the serum samples were estimated by using a commercial kit available in market (Agappe Diagnostic limited).

Assessment of glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) activities in the rat liver:

To determine the GSH activity, 0.5 g of liver tissue was homogenized in 5 ml of 1:1 cold buffer (10% TCA, 10 mM EDTA, pH 7.4) at 4°C according to the protocol used in a previous report [13]. The tissue homogenized mixture was further centrifuged at 5000 rpm for 10 min at 4°C. The obtained supernatant was separated and further used for GSH activity. To determine GSH activity, the reaction mixture was prepared by adding 100 µl of supernatant (100 µl) into 3.0 ml of 0.2 M Tris-HCl and 50 µl of Ellman's reagent (0.02% of 5, 5'dithio (bis) nitrobenzoic acid (DTNB). After reaction performed, the developed yellow colour was measured immediately at 412 nm and the enzyme activity was determined. In this experiment,the reaction mixture without DTNP was considered as blank. To determine the CAT and SOD activities, the liver tissue was homogenized (10 % w/v) in 50 mM phosphate buffer of pH 7.4 at ice cold condition. The tissue homogenised mixture was centrifuged at 2500 rpm for 10 minutes at 4^{o}C. After centrifugation, the supernatant was collected and used further to determine the CAT and SOD activities. The CAT activity was carried out by using 3% H_2O_2 (v/v) as the substrate in phosphate buffer and the change in absorbance was measured at 240 nm for two minutes at 30 seconds of intervals [14]. Units of CAT activity were

expressed as the amount of enzyme that decomposes 1 µM of H_2O_2 per minute at 25^oC and the enzyme activity was expressed as units/mg of protein.The SOD activity was determined by using pyrogallol (2mM) autooxidation in Tris buffer according to the previous protocol. The reaction mixture was prepared by adding 0.1ml of Tris Buffer (pH 8.2) and 10% of tissue homogenate. The reaction was started by adding 2 mM of pyrogallol. The absorbance of reaction mixture was measured at 420 nm for two minutes at an interval of 30 s and the SOD activity was determined.

Histopathological studies of rat liver tissues:
The rat liver was transferred to 4% of formalin solution (v/v) for fixation and after that processed for histopathological studies according to the protocol of a previous report [15]. In brief, by using a microtome instrument, several thin sections were prepared, processed and stained with hematoxylin and eosin. The sections were further observed under a Carl-Zeiss microscope attached with camera (Axio Imager 2).

Statistical Analysis:
The obtained data was evaluated by using SPSS 22 software in which multiple groups were analysed by one way analysis of variance (ANOVA). All experiments were performed in triplicates and significance was considered at $P<0.05$.

3. Results and Discussion:
Hepatoprotective activity of FdBM and FtBM extracts:
The FdBM and FtBM extracts were tested for their hepatoprotective activity against the CCl_4 induced hepatotoxicty in a rat model. TheCCl_4 is a well known as liver toxicant and its metabolites such as CCl_3 radicals are responsible for liver damage. The CCl_4 is an extensively used chemical for the investigation of hepatoprotective activity in different experimental models reported earlier[8]. The changes associated with CCl_4-induced liver damage are found to be similar with acute hepatitis infection [16]. The free radicals generated due to stress of CCl_4 are responsible for liver and other tissue damage. A variety of enzymes are present in the cytosol and further they are released into the blood stream. The estimation of these enzymes in the serum is a useful quantitative marker to understand hepatocellular damages[17]. The effects of the FdBM and FtBM extracts on serum marker enzymes are presented in figure 1. The level of serum enzymes like LDH, ALP, ALT and AST were significantly raised in CCl_4 treated rats when compared with the normal control group ($P<0.05$). This observation clearly indicates that the rat liver was significantly damaged. The obtained results are in a good agreement with the previous report [18]. From figure 1, it was seen that the rats treated with the FdBM and FtBM extracts at the dose (150 & 300 mg/kg) decreased the activity of LDH, ALP, ALT and AST in CCl_4 induced liver damage when compared with CCl_4 treated groups ($P<0.05$). Thus, results show that the test samples offer an effective protection to the rat liver against hepatotoxicity of CCl_4 as evidenced by a remarkable reduction in the serum enzymes significantly ($P<0.05$). It is thus depicted that methanolic bark extract of *F. dalhousiae* and *F. tseila* have exhibited a strong hepatoprotective activity. Recently, the hepatoprotective activity was also observed when CCl_4 induced rats were treated with ethyl acetate extracts of *Ficuscarica*[19]. The obtained results are in a good agreement with the standard Silymarin treated rats at the dose of 100 mg/kg body weight. Thus, the oral administration of the FdBA and FtBM extracts have offered a good hepatoprotective activity and preserved the normal physiology of the liver cells which was damaged by CCl_4.

Further, the FdBM and FtBM extracts were used to study the possible effects on enzymatic and non-enzymatic antioxidants of rat liver. The anti-oxidative treatment has been proposed as a potential approach to prevent the toxic liver injury. Hence, in this context, *in vivo* anti-oxidative activity of SOD, CAT and GSH was measured. The SOD and CAT form a mutually supportive team of defense against ROS [13]. The SOD is assumed to be the most effective antioxidant. It is regarded as the first step of defense against superoxide anion and diminishes the toxic effects caused by free radicals. The CAT is another antioxidant enzyme and its highest activity is seen in red cells and liver. The CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals [20]. In CCl_4-induced hepatotoxicity, the balance between ROS production and these antioxidant defenses may have

been lost which results into oxidative stress and dysregulation of cellular function and hepatic necrosis takes place. Glutathione is one of the most abundant tripeptides, non-enzymatic antioxidants found in liver. It plays a major role in detoxification of free radical species like hydrogen peroxide and superoxide radicals and thus, maintain the membrane protein thiols [21]. From table 1, it was observed that the level of liver cytosolic SOD, CAT and GSH activities was significantly depleted in the rat treated with CCl_4.However, the level of liver cytosolic SOD, CAT and GSH activities was raised significantly after oral administration of the FdBM and FtBM extracts at the dose level of 150 and 300 mg/kg body weight (Table 1). Thus, the restoration of SOD, CAT and GSH activities towards the normal values suggested due to the FdBM and FtBM extracts have the protective role in the CCl_4 induced rats.

Histological studies:

The histopathological examination of liver sections ofcontrol rat group and the test rats was carried out. The normal cellular architecturewith distinct hepatic cells, sinusoidal spaces andcentral vein were seen in the control group [I] as shown in figure 2. The cellular architecture of CCl_4-treated liver tissue of group [II] revealed the marked loss of hepatic architecture with centrilobular necrosis, loss of cellular boundaries, enlarged nuclei, and scattered masses of necrotic tissue as shown in figure 2.The liver sections of the rats treated with the FdBM and FtBM extracts and silymarin followed by CCl_4 intoxication showed a sign of protection as it was evident by the absence of centrilobular necrosis, enlarged nuclei and scattered masses of necrotic tissue as shown in figure 2. Thus, the bark extracts of *Ficus* species showed a potent hepatoprotective activity. Such property of plant extracts might be due their anti-oxidants or inhibition of cytochrome P450s effect whichimpair the bio-activation of CCl_4 into their corresponding reactive species [22]. Overall, experimental findings revealed that the bark methanolic extracts of *F. dalhousiae* and *F. tsiela* are found to be an effective plant extracts shows hepatoprotective property.

Conclusions:

From the obtained results, it is concluded that *F. dalhousiae* and *F. tsiela* bark methanolic extracts showed the hepatoprotective activity in the CCl_4 induced rat liver damage due the anti-oxidant property of bioactive compounds present in bark extracts of *Ficus* species. Thus, this study revealed the importance of bark extracts of *Ficus* species for the future development of 'therapeutic agent' against the liver damage.

Conflict of interests:

All authors have declared that there is no conflict of interests.

Ethics approval:

Ethical approval was taken as per rule from the ethical committee of zoology department, university of Mysore, Mysuru, Karnataka, India.

Acknowledgements:

Authors would like to acknowledge to Department of Biotechnology (DBT, New Delhi, India) for providing financial assistance in form of DBT-JRF Fellowship.

All authors are thankful to University of Mysore, Mysuru, Karnataka under the IOE (Institution of Excellence) programme from MHRD & UGC, New Delhi, India and Garware Research Centre, SavitribaiPhule Pune University, Pune, Maharashtra, India for providing all the instrumental facilities.

Table 1. Effects and FdBM, FtBM extracts at the dose level of 150 and 300mg/kg/b.w. and silymarin at dose of 100 mg/kg/b.w. on GSH, CAT and SOD enzymes in CCl_4 induced liver toxicity.

Sr. No.	Groups	GSH (µg/mg protein)	CAT (µmoles H_2O_2/min/mg protein)	SOD (Unit/mg protein)
1.	Control (I)	2.72 ± 0.35^{c}	10.95 ± 0.43^{d}	2.28 ± 0.16^{d}
2.	CCl_4 (II)	0.90 ± 0.064^{a}	3.92 ± 0.07^{a}	0.46 ± 0.02^{a}
3.	$FdMB_{(150mg)} + CCl_4$ (III)	1.37 ± 0.08^{b}	7.12 ± 0.45^{b}	$1.7 \pm 0.07^{b,c}$
4.	$FdMB_{(300mg)} + CCl_4$ (IV)	$2.27 \pm 0.28^{b,c}$	$8.25 \pm 0.89^{b,c}$	1.35 ± 0.12^{b}
5.	$FtMB_{(150mg)} + CCl_4$ (V)	$2.48 \pm 0.50^{b,c}$	$9.73 \pm 0.72^{c,d}$	$1.68 \pm 0.10^{b,c}$
6.	$FtMB_{(300mg)} + CCl_4$ (VI)	$1.71 \pm 0.13^{b,c}$	$9.63 \pm 0.70^{b,c,d}$	1.38 ± 0.16^{b}
7.	$Silymarin_{(100mg)} + CCl_4$ (VII)	2.89 ± 0.184^{c}	$10.52 \pm 0.11^{c,d}$	$2.05 \pm 0.08^{c,d}$

Each value in the table is represented as mean ± SE (n = 6). Superscript letters with different letters in the same column, indicate significant difference ($P < 0.05$) analysed by Duncan's multiple range test.

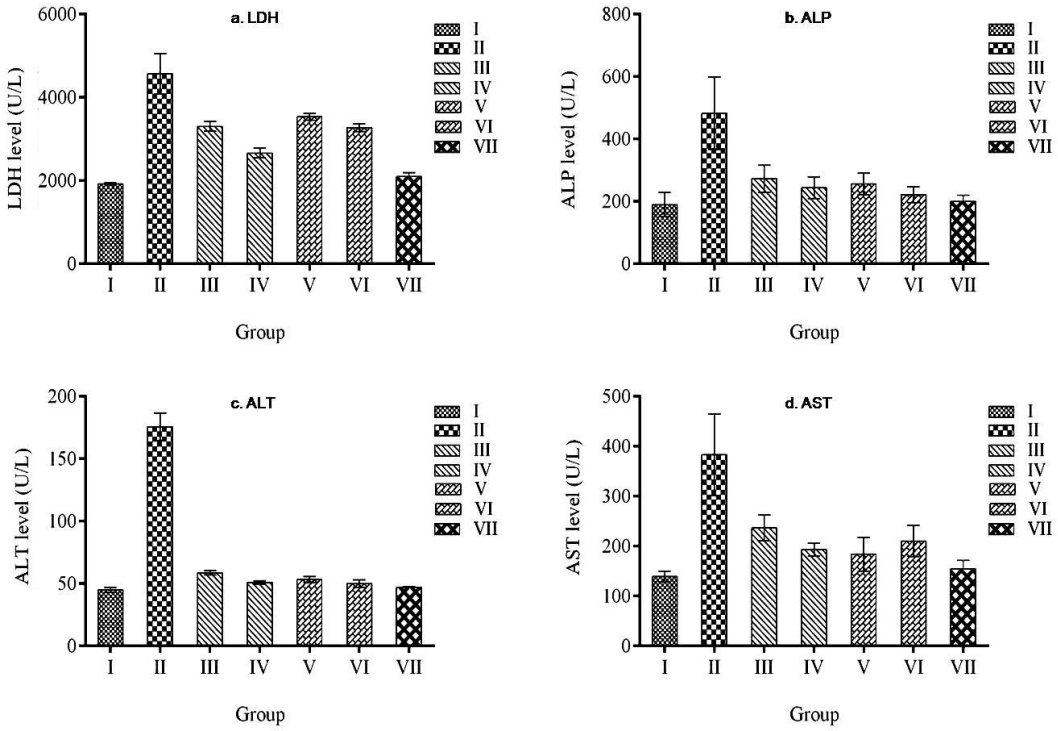

Figure 1. Protective effect of methanolic extract of the bark of FdBM and FtBM on CCl_4 hepatotoxicity: Serum enzyme Group I – control; Group II – CCl_4; Group III– FdBM 150 mg/kg b.w. + CCl_4; Group IV– FdBM 300 mg/kg b.w. + CCl_4; Group I – FtBM 150 mg/kg b.w. + CCl_4; Group VI– FtBM 300mg/kg b.w. + CCl_4 and Group VII – Silymarin 100 mg/kg b.w. + CCl_4. Each bar represents the mean ±SE, n=6, Where, $p< 0.05$ when compared with control.

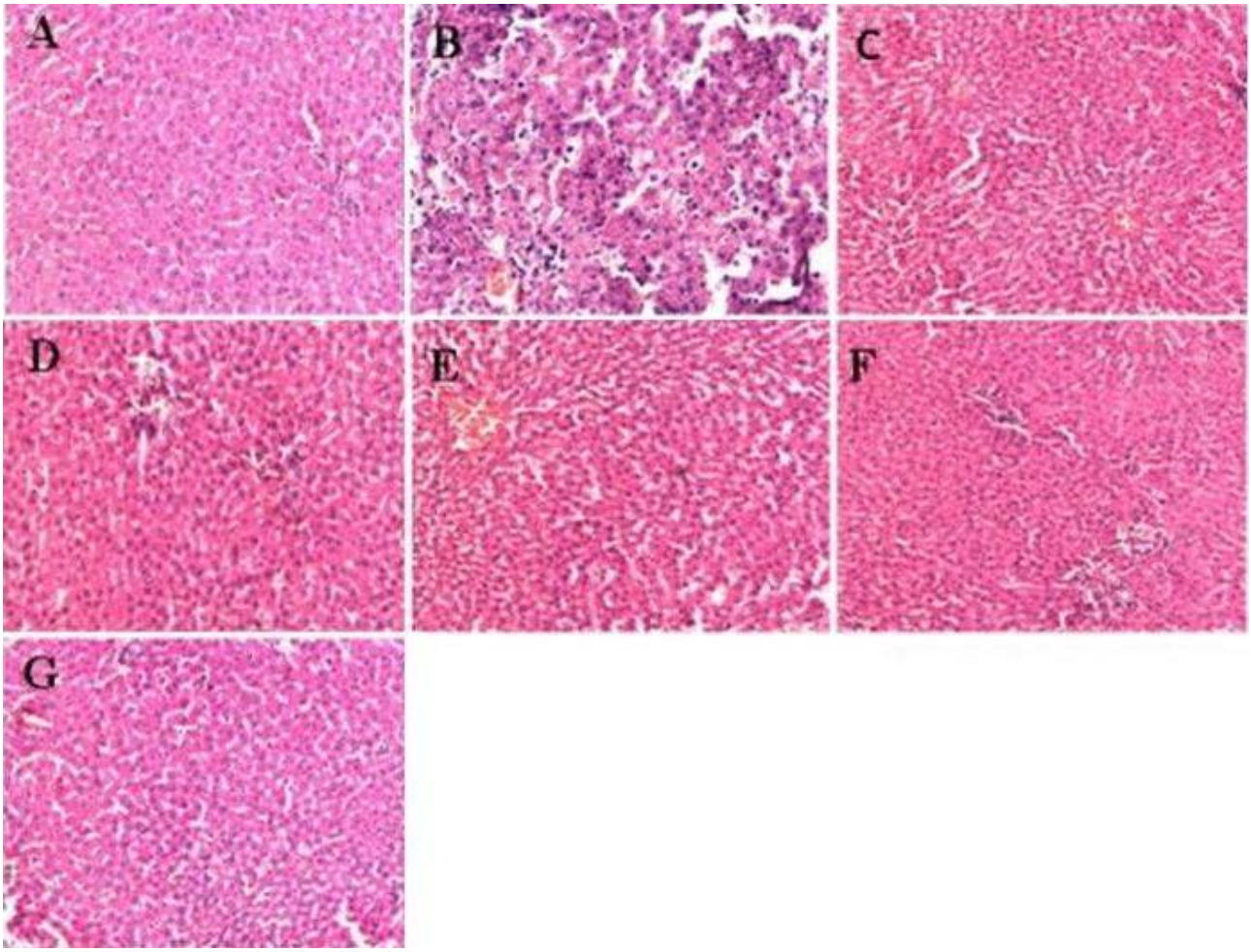

Figure 2. Effect of *F. dalhousiae* and *F. tsiela*on CCl_4-induced hepatotoxicity in rats (histopathology). A: Normal control, histology of the liver sections of normal control animals showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus and well brought out central vein; B: Disease control (CCl_4-treated), the liver sections of CCl_4-treated animals showed hepatic cells with severe toxicity characterized by centrilobular; C: FdBM 150 mg/kg b.w. + CCl_4; D: FdBM 300 mg/kg b.w. + CCl_4; E: FtBM 150mg/kg b.w. + CCl_4; F: FtBM 300 mg/kg b.w. + CCl_4 and G: Silymarin (100 mg/kg) + CCl_4-reversed CCl_4-toxicity.

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