



## Hepatoprotective activity of phloretin and hydroxychalcones against Acetaminophen Induced hepatotoxicity in mice

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### Abstract

Polyphenolics form a major part of the dietary antioxidant capacity of fruits and vegetables have been identified as chemopreventive or anticancer agents. Hydroxychalcones are polyphenols abundantly distributed throughout the plant kingdom and are compounds with two aromatic rings (benzene or phenol) and an unsaturated side chain. In the present study, effect of phloretin (apple major flavonoid), 4-hydroxychalcone and 4'-hydroxychalcone were investigated against acetaminophen-induced acute liver damage. The study was designed as multiple dose pre- and post-treatments. Mice were administrated acetaminophen (1g/kg and 640 mg/kg for mortality and acute toxicity experiments, respectively). Mortality rate, serum transaminases (SGOT and SGPT) and histological examination were applied. Acetaminophen produced 100% mortality at the dose of 1 g/kg in mice, while pre-treatment and post-treatment (i.p., twice daily for 48 hrs) of animals with phloretin and 4-hydroxychalcone (50 mg/kg) and 4'-hydroxychalcone (25 mg/kg) significantly reduced the mortality rate. Acetaminophen produced acute toxicity at the dose of 640 mg/kg in mice, while pre- and post-treatments of animals with phloretin and hydroxychalcones significantly lowered the rise in SGOT and SGPT. Liver sections collected for histological examination showed cellular changes including centrilobular necrosis, extensive portal inflammation, and micro and macro vesicular structures in the acetaminophen group. These cellular changes were reduced following treatment of mice with Phloretin and hydroxychalcones. Taken collectively, from the results of this study it may be suggested that phloretin and hydroxychalcones have hepatoprotective activity against acetaminophen liver injury in mice.

*Key words:* Phloretin, Hydroxychalcones, Hepatoprotection, Acetaminophen, SGOT, SGPT

### 1. Introduction

Daily diet of human contains approximately 1 g of herbal phenols which can also be found in many traditional medicines. Most of antioxidant capability of fruits and vegetables

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is due to the poly phenols. These compounds include caffeic acid (present in tea and coffee), capsaicin (found in red pepper), resveratrol (found in red grape), gallic acid (found in rhubarb), flavonoids and chalcones [1-3].

Chalcones are secondary metabolite of plants which their chemical structure is similar to curcumine, a known antioxidant. They include 2 benzene rings which relate to each other by an unsaturated -carboxyl group. By now, many therapeutic features have been proposed for chalcones including antifungal, anti-bacterial, anti-malaria, anti-lishemania, anti-mutation and chemopreventive activities. Some of these characteristics have been related to antioxidant capacity and capturing the metallic ions [4-6].

Phloretin (major chalcone of apple, a dihydrochalcone) and isoliquiritigenin (2', 4', 4-trihydroxy-chalcone, a licorice chalcone), are important compounds of this group which have been studied extensively [7]. Until a few years ago, the occurrence of phloretin was considered restricted to apples. The identification and isolation of phloridzin in strawberries, however, extended the knowledge of the natural sources of this polyphenolic compound [8]. It can be expected that phloretin will be found in additional plant species in the future. Despite biological and therapeutical characteristics of chalcones, importance of these compounds has not yet fully understood.

Liver is the largest organ in the vertebrate body and its strategic location between intestinal tract and the rest of the body facilitates its task of maintaining metabolic homeostasis of the body. Therefore, the liver, as opposed to other organs, is the dominant target site of specific toxins. Many medicines such as isoniazid, tetracycline, antidepressants, acetaminophen, halothane, and a number of industrial compounds as well as natural poisons (e.g., Aflatoxin B1) and bacterial infections can cause liver diseases [9-11]. Acetaminophen, an analgesic antipyretic

medicine which is usually sold without prescription (OTC), is mostly metabolized by liver through glucuronidation and sulfation pathways [12]. It is also metabolized to a reactive intermediate namely N-acetyl parabenzoquinoneimine, which can cause liver damage if is not detoxified [13, 14]. The toxic metabolite production will increase following exposure to high levels of the parent compound while alternative detoxification mechanisms are compromised [15]. The compounds presently used in acetaminophen hepatotoxicity have inadequate effects and sometimes accompanied with serious adverse effect. N-acetylcysteine (NAC) is widely used as an antidote to acetaminophen overdose but can cause adverse effects [16]. In the search to find an alternative remedy which can be used more efficiently and safely in liver damages we studied hepatoprotective effect of three members of hydroxy chalcones, namely phloretin, 4-hydroxy chalcone (4-HC) and 4'-hydroxy chalcone (4'-HC) against acetaminophen-induced liver toxicity in mice.

## 2. Materials and Methods

**Chemicals:** 4-Hydroxy chalcone (4-HC) and 4'-hydroxy chalcone (4'-HC) were purchased from Indofine Chemical Company Inc. (NJ, USA). 2', 4', 6', 4-Tetrahydroxydihydrochalcone (phloretin) and methyl cellulose were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). The solvents including ethanol, tween 80 and acetaminophen were supplied by Merck Company (Darmstadt, Germany). All chemicals were of the highest available commercial grade.

**Animals:** All animal experiments were conducted according to the policy of MOH Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes, and the protocol was approved by the Ethics Committee of the Tehran University of Medicine Sciences (TUMS), Tehran, Iran. Male albino mice weighing 25-30 g prepared from Pasteur's

Institute (Tehran, Iran). The animals were kept under 12/12 light/ dark in Animal Care Center, Faculty of Pharmacy, TUMS. The mice were allowed free access to standard laboratory feed and water before experiment. Liver injury was produced in the 12 h fasted rats.

### 2.1. Mortality protection study

Multiple doses pre-treatment: Mice were divided into 11 groups of 6 animals each.

Group 1 (control blank) received vehicle (Normal saline, NS). Group 2 received 4 doses of NS at 12h intervals plus acetaminophen (1 g) orally 1h after the last dose of NS. Groups 3, 6 and 9 treated with 4 doses of phloretin or 4'-HC or 4-HC (25 mg/kg) at 12h intervals and received acetaminophen (1 g) orally 1h after the last dose. Groups 4, 7 and 10 treated with 4 doses of phloretin or 4'-HC or 4-HC (50 mg/kg) at 12h intervals and received acetaminophen (1 g) orally 1h after the last dose. Groups 5, 8 and 11 treated with 4 doses of phloretin or 4'-HC or 4-HC (100 mg/kg) at 12h intervals and received acetaminophen (1 g) orally 1h after the last dose.

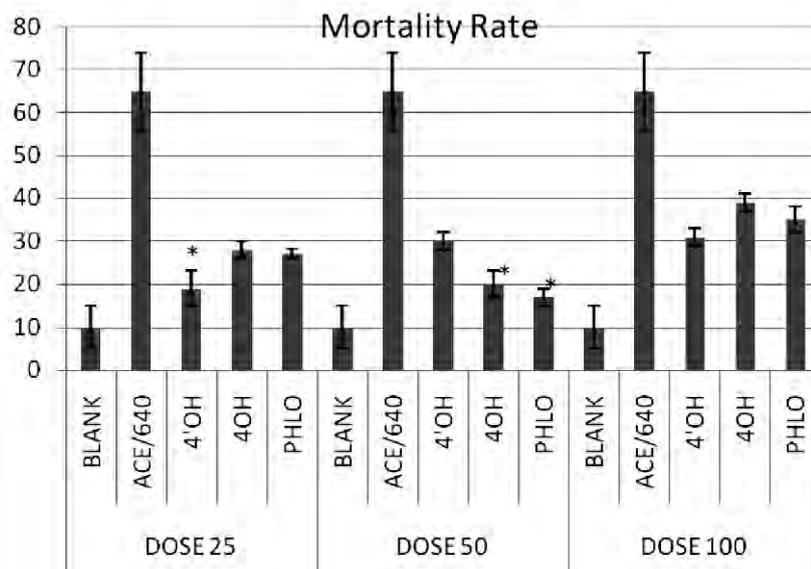
Multiple doses post-treatment: Mice were divided into 11 groups of 6 animals each.

Group 21 (control blank) received vehicle (Normal saline, NS). Group 22 received acetaminophen (1 g) orally at 0h then 4 doses of NS (every 6h until 24 h). Groups 23, 26 and 29 received acetaminophen (1 g) orally at 0h then treated with 4 doses (every 6h) of phloretin or 4'-HC or 4-HC (25 mg/kg). Groups 24, 27 and 30 received acetaminophen (1 g) orally at 0h then treated with 4 doses (every 6h) of phloretin or 4'-HC or 4-HC (50 mg/kg). Groups 25, 28 and 31 received acetaminophen (1 g) orally at 0h then treated with 4 doses (every 6h) of phloretin or 4'-HC or 4-HC (100 mg/kg).

### 2.2. Hepatoprotective study

Multiple doses pre-treatment: Mice were divided into 11 groups of 6 animals each.

Group 41 (control blank) received vehicle (Normal saline, NS). Group 42 received 4 doses of NS at 12h intervals plus acetaminophen (640 mg) orally 1h after the last dose. Groups 43, 46 and 49 treated with 4 doses of phloretin or 4'-HC or 4-HC (25 mg/kg) at 12h intervals and received



**Figure 1.** Mortality rate study, pre-treatment. Effect of pre-treatment with phloretin, 4'-HC and 4-HC (Hydroxychalcone) on mortality rate resulting from acute acetaminophen toxicity (1 g/kg); \* $P < 0.01$ .

acetaminophen (640 g) orally 1h after the last dose. Groups 44, 47 and 50 treated with 4 doses of phloretin or 4'-HC or 4-HC (50 mg/kg) at 12h intervals and received acetaminophen (640 g) orally 1h after the last dose. Groups 45, 48 and 51 treated with 4 doses of phloretin or 4'-HC or 4-HC (100 mg/kg) at 12h intervals and received acetaminophen (640 g) orally 1h after the last dose.

Multiple doses post-treatment: Mice were divided into 11 groups of 6 animals each.

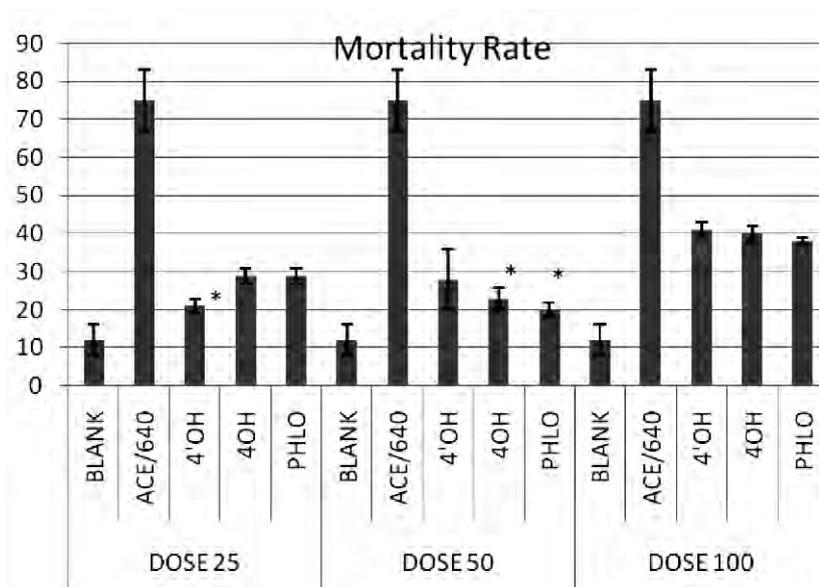
Group 61 (control blank) received vehicle (Normal saline, NS). Group 62 received acetaminophen (640 g) orally at 0h then 4 doses of NS (every 6h until 24 h). Groups 63, 66 and 69 received acetaminophen (640 g) orally at 0h then treated with 4 doses (every 6h) of phloretin or 4'-HC or 4-HC (25 mg/kg). Groups 64, 67 and 70 received acetaminophen (640 g) orally at 0h then treated with 4 doses (every 6h) of phloretin or 4'-HC or 4-HC (50 mg/kg). Groups 65, 68 and 71 received acetaminophen (640 g) orally at 0h then treated with 4 doses (every 6h) of phloretin or 4'-HC or 4-HC (100 mg/kg).

### 2.3. Sample collection & SGOT/SGPT measurement

Mice were anaesthetized with chloroform 24h after the treatment and blood was collected by cardiac puncture using sterile disposable syringes. Serum was separated by centrifugation (5000 g for 5') and serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were estimated on the same day spectrophotometrically using commercial diagnostic kits prepared from Man Laboratory manufacturer in Tehran, Iran.

### 2.4. Histopathological studies

For histopathological study, the livers were immediately removed after autopsy and the tissues were fixed in 10% formalin for a period of at least 24 h. The paraffin sections were then prepared (Automatic Tissue Processor, Lipshaw) and cut into 5- $\mu$ m-thick sections in a rotary microtome. The sections were then stained with haema-toxylin-eosin dye (Merck) and mounted with Canada balsam. The histopathological slides were examined and photographs were taken with a Carl Zeiss Jena amplual type photomicro-



**Figure 2.** Mortality rate study, post-treatment. Effect of post-treatment with phloretin, 4'-HC and 4-HC (Hydroxychalcone) on mortality rate resulting from acute acetaminophen toxicity (1 g/kg); \* $P < 0.01$ .

scope ( $3.2 \times 10$  and  $10^2 \times 10$ ).

### 2.5. Statistical analysis

The data obtained were analyzed by one-way analysis of variance (ANOVA) and Student's t-test for the possible significant interrelation between the various groups. The data were analyzed with the help of Instat computer software. Probability levels of less than 0.05 were considered significant.

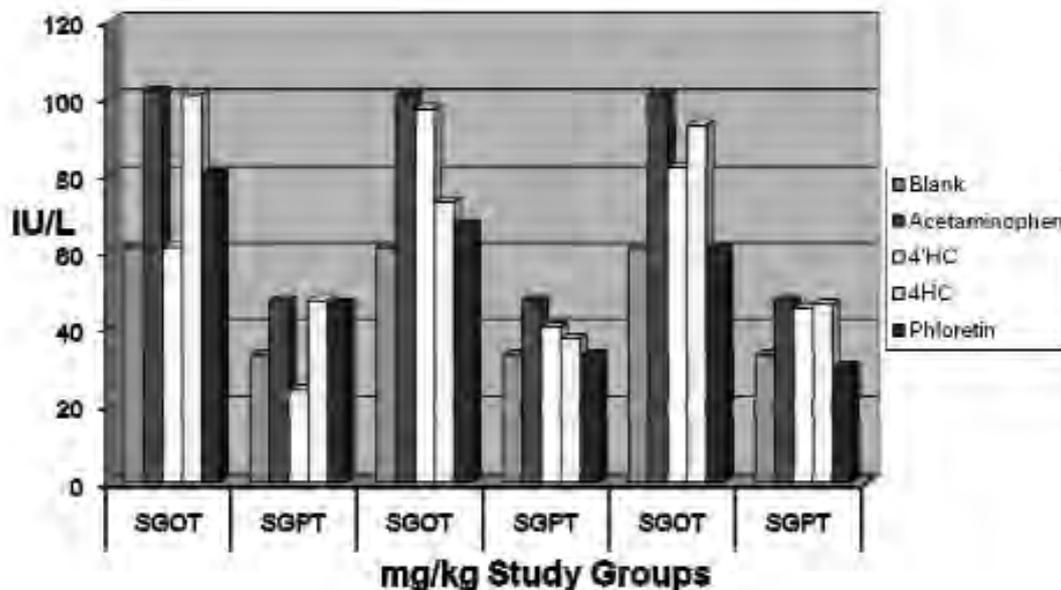
### 3. Results

The preventive effects of phloretin and hydroxychalcones against lethal dose of acetaminophen (1 g/kg) following pre- and post-treatment studies (doses of 25, 50 and 100 mg/kg) were shown in Figure 1. Acetaminophen at the dose of 1 g/kg induced more than 65% lethality in mice (Figures 1&2). Phloretin and hydroxychalcones at the dose of 50 mg/kg remarkably reduced mortality rate of mice resulting in protection against lethal effect of acetaminophen ( $P < 0.01$ ). In this sense, 4'-hydroxychalcone was able to reduce the mortality rate even at the lowest dose of 25 mg/kg.

In the pre-treatment study, 4'HC

significantly ( $P < 0.01$ ) reduced the elevation of SGOT and SGPT at the low dose (50 mg/kg). Phloretin at the all employed doses decreased SGOT values but the SGPT activity was influenced significantly only at 50 mg/kg. 4HC effectively reduced both SGOT and SGPT values at 50 mg/kg and SGOT at 100 mg/kg (Figure 3). The same pattern of protection was demonstrated following post-treatment study but SGPT was not reduced by any of the compounds at the low (50 mg/kg) and high (100 mg/kg) doses. Nevertheless, phloretin and 4HC reduced SGOT at 50 mg/kg which was the best protection observed at the post-treatment study (Figure 4).

Histological analysis of liver sections showed marked pathological changes in liver parenchyma as a result of acetaminophen toxicity as compared to the control. There were severe cell infiltration around the periportal system, vacuolization of the parenchymal cells and focal necrosis. The changes were found to be much less in phloretin and 4HC groups. In the 4'HC group, mild to moderate attenuation in pathological changes were observed (Figure 5).



**Figure 3.** Effect of pre-treatment on serum enzymes. Effect of pre-treatment with phloretin, 4'-HC and 4-HC (Hydroxychalcone) on SGOT and SGPT Values.

#### 4. Discussion

The study provides evidence that phloretin and the employed hydroxychalcones (50 mg/kg) remarkably reduced mortality rate of mice resulting from protection against lethal effect of acetaminophen (Figures 1&2,  $P < 0.01$ ). In this sense, 4'-hydroxychalcone was able to reduce the mortality rate even at the lower dose of 25 mg/kg. Phloretin and its glucoside phloridzin are abundantly present in apples, particularly in the peel, and in strawberries [8, 17, 18]. Recent experimental research has suggested that phloridzin could be potentially used to treat various disease states such as diabetes, obesity, and hyperglycemia. Inhibition of glucose cotransporter 1, however, has been proposed as the main biological action of phloretin described in the literature [19, 20]. Nevertheless, phloretin possesses antioxidative properties and other studies have established the pharmacophore responsible for the antioxidative activity of phloretin [21, 22]. Recently a study demonstrated that some of the biological actions of phloretin may contribute to the observed protective cardiovascular effects of

diets rich in flavonoids [23].

The present study in agreement with other studies showed that overdose of acetaminophen can cause liver damage which is pronounced clinically as jaundice, steatosis and fibrosis [24]. Acetaminophen induced hepatotoxicity is a commonly model used to examine liver damage and to screen the hepatoprotective activity of compounds [25]. Its reactive metabolite N-acetyl-p-benzo-quinoneimine (NAPQI) causes free radical production as peroxynitrite [26]. Structural damage and the function integrity of hepatocytes can be studied using a series of cellular enzymes including AST, ALT and LDH [27 – 30]. The increased serum level of these enzymes is a valuable indicator of liver membrane damage and necrosis; since they are cytoplasmic enzymes in location and their release into the systemic circulation is attributed to the cellular damage [28]. Our findings demonstrated that; high dose administration of acetaminophen (640 mg/kg) in animals causes severe hepatocellular injury as indicated by the significant elevation of aminotransferase enzyme activities which account as indicator of the cellular damage

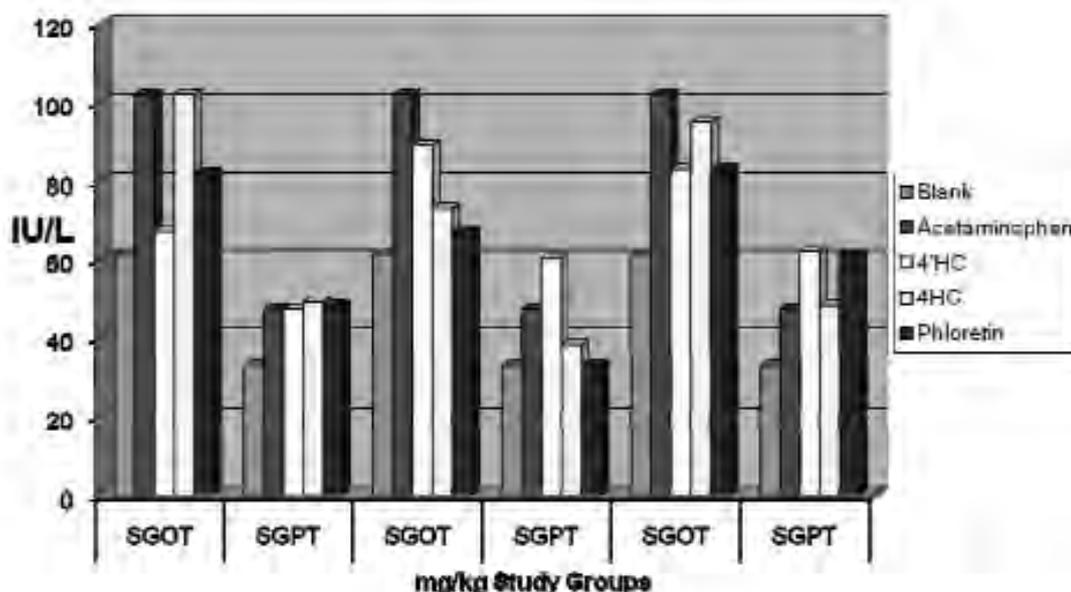


Figure 4. Effect of post-treatment on serum enzymes. Effect of post-treatment with phloretin, 4'-HC and 4-HC (Hydroxychalcone) on SGOT and SGPT Values.

and liver functions ( $P < 0.01$ , Figures 3&4). In the pre-treatment study, phloretin, 4'HC and 4HC markedly ( $p < 0.01$ ) reduced the elevation of ALT and AST at 50 mg/kg (Figure 3). This indicates that 2 days administration of the chalcones can protect the animals against acetaminophen induced liver damage. The same pattern of protection was demonstrated following post-treatment study but SGPT was not reduced by any of the compounds at the low (50 mg/kg) and high (100 mg/kg) doses. Nevertheless, phloretin and 4HC reduced SGOT at 50 mg/kg which was the best protection observed at the post-treatment study (Figure 4).

Histopathological data of the acetaminophen intoxicated animals showed liver toxicity including severe centrilobular necrosis, fatty infiltration and lymphocytes infiltration. The findings were significantly decreased in the hydroxychalcones treated groups (Figures 4 and 5). The findings further support the biochemical data.

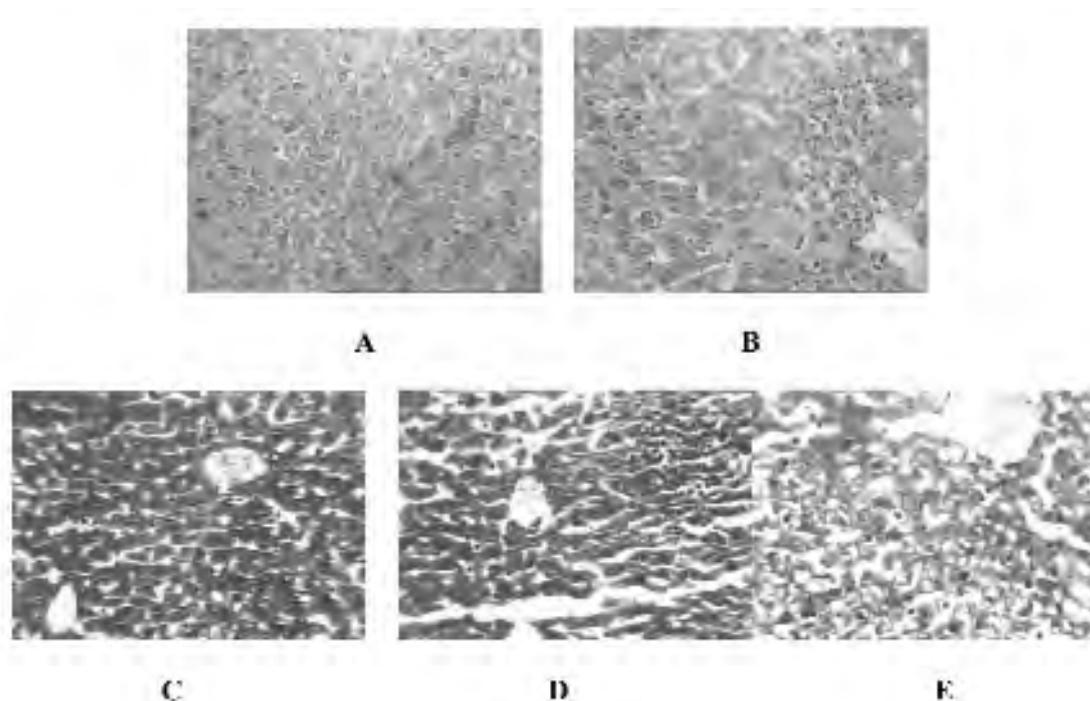
Taken collectively, from the results of this study it may be suggested that phloretin and the two employed hydroxychalcones have hepatoprotective activity against acetaminophen liver injury in mice. Thus, the present study provides a scientific rationale for the application and commercial uses of natural products in management of liver diseases.

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#### Declaration of interest:

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.



**Figure 5.** Histological studies. Histological images of mice livers displaying normal lobular architecture with central veins and radiating hepatic cords in saline control mice (A). Slide (B) depicted the significant cell infiltration around the periportal system, vacuolization of parenchymal cells, focal necrosis and central vein dilation in acetaminophen control mice. Pretreatment with Phloretin (C), 4'-HC (D) and 4-HC (E) (50 mg/kg) significantly reduced degree of liver damage and degeneration (H&E, 40X).

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