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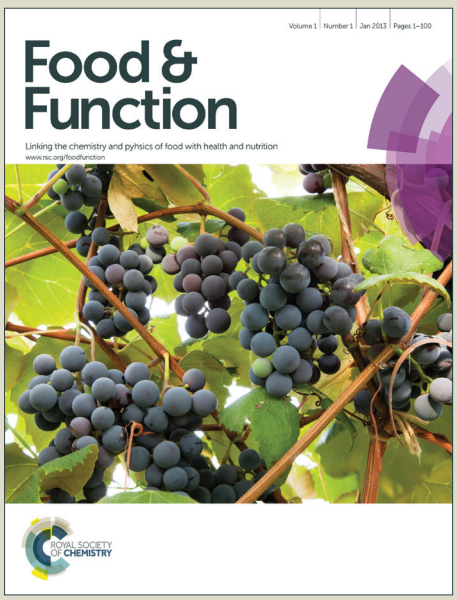

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Antioxidant and hepatoprotective activity of vitex honey against paracetamol in duced liver damage in rats

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Abstract: Fourteen vitex honeys from China were investigated to evaluate its antioxidant and hepatoprotective activity against paracetamol-induced liver damage. All honey samples exhibited high total phenolic content (344-520 mg GAE/kg), total flavonoid content (19-31 mg Rutin/kg), and strong antioxidant activity in DPPH radical scavenging, ferric reducing antioxidant power and Ferrous ion-chelating ability. Nine phenolic acids were detected in vitex honey samples, in which caffeic acid was the main compound. Honey from Heibei Zanhuang (S2) ranked the highest antioxidant activity was orally administered to mice (5 g/kg, 20 g/kg) for 70 days. In high-dose (20 g/kg), vitex honey pretreatment resulting in significant increase in serum oxygen radical absorbance capacity (15.07%) and decrease in Cu^{2+}-mediate lipoprotein oxidation (80.07%), and suppression in alanine aminotransferase (75.79%) and aspartate aminotransferase (74.52%), enhancement in the superoxide dismutase and glutathione peroxidase activities and reduction in malondialdehyde (36.15%) and 8-hydroxy-2'-deoxyguanosine (19.6%) formation compared with paracetamol-intoxicated group. The results demonstrated the hepatoprotection of vitex honey against paracetamol-induced liver damage might attribute to its antioxidant and/or perhaps pro-oxidative property.

Keywords: Vitex honey; Hepatoprotective effects; Paracetamol; Antioxidant

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1. Introduction

Paracetamol, also known as Acetaminophen or N-acetyl-p-aminophenol, is a widely used analgesic and antipyretic drug, which is considered safe if administrated at therapeutic dose. While paracetamol-induced acute liver failure (ALF) largely as a result of unintentional overdoses covers the most of drug-induced ALF reports in the US.[1] Its generally accepted that liver damage occurs when hepatotoxic paracetamol metabolite N-acetyl-p-benzoquinone imine (NAPQI) suppresses the intrahepatic antioxidant defense system by depleting its glutathione pool.[2,3] And it's also confirmed that oxidative stress accounts for the main mechanism of paracetamol-induced liver damage.[4] Therefore, several modern allopathic medicine, representatively, a specific antidote, N-acetyl-cysteine which has the great antioxidation, have been used as a treatment of paracetamol-related liver damage.[5] However, the side effects of these synthetic modern medicine cannot be ignored even in spite of their high cost and limited efficacy.

Honey is a natural product yielded by honey bees using nectar or secretion collected originally from florescent plants. In the long human history, honey has been used as a medicine homological food in virtue of its nutritional and health benefit. These beneficial effects include anti-microbial, anti-inflammatory, antitumour and antineoplastic effects.[6-9] Honey also is a good resource of antioxidants due to its abundant polyphenolic content such as flavonoids and phenolic acids.[10] And accumulating scientific researches demonstrates that the antioxidant activity of honey empowers its therapeutic effect on acting against several chronic disease caused by oxidative damage including cardiovascular, cancer, diabetes and Alzheimer's disease.[11-14] Recently, the protective effects of honey on chemical oxidative liver injury has been validated by increasing researchers, making honey a potential good hepatoprotective product.[15,16,17]

Vitex negundo Linna. Var. *heterophylla* (Franch.) Rehd. is the dominant species and widely distributed in the mountain land of north China, where it represents one of the main resources for unifloral honey production in the late summer. vitex honey is one of the most valuable unifloral honey in the Chinese market, because its flavor

and taste is highly appreciated by the consumer. For recent years, the therapeutic properties of vitex honey have been paid attention to several researchers. Cao et al. reported the high antioxidant capacity of vitex honey, which is conceived in relationship with its high phenolic content.[18] Chai et al. demonstrated the anti-aging effect of vitex honey through drosophila melanogaster experiments.[19] However, very little research (if any research has been done) on the hepatoprotective effects of vitex honey is available.

Therefore, the aim of present research is to evaluate the protective effect of vitex honey on paracetamol-induced liver injury in vitro and in vivo. The total phenolic content (TPC), total flavonoid content (TFC) and antioxidant indexes including DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP), ferrous ion-chelating activity and individual phenolic acid are determined. For investigating the hepatoprotective effects of vitex honey in vivo, the rat experiments were carried out, and the biochemical parameters in serum including the level of ALT, AST, MDA, SOD, GSH-Px, 8-OHdG were also measured. In addition, histopathological examinations also have been conducted.

2. Materials and methods

2.1. Chemicals and reagents

The diagnostic kits for AST, ALT, MDA, SOD, GSH-Px, 8-OHdG and protein were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Folin-Ciocalteu's phenol reagent, DPPH (1,1-diphenyl-2-picrylhydrazyl), ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2, 4-triazine-4`4``-disulfonic acid monosodium salt) were purchased from Sigma-Aldrich (Steinheim, Germany). CCl_4, peanut oil, iron (II) sulfate 7-hydrate ($FeSO_4 \cdot 7H_2O$), sodium carbonate, and ethanol were purchased from Beijing Chemical Co. (Beijing, China). All other chemicals were of analytical grade and were purchased from Xi'an Chemical Co. (Xi'an, China).

2.2. Honey samples

vitex honey samples were collected directly from the honeycomb from three geographical regions of China and were filtered to remove wax and other impurities.

All samples were collected in 2014 and kept at 4 to 5°C until analysis. The data is displayed in Table 1.

2.3. Total phenolic content (TPC), total flavonoid content (TFC)

TPC was determined using a modified version of the Folin-Ciocalteu method.[20] Nought point four milliliter of vitex honey solution (2 mg/mL) was added to 1.0 mL of Folin-Ciocalteu reagent and mixture was kept at room temperature for 5 min. Five milliliter of sodium carbonate (1 M) was added to the mixture and the whole mixed gently. The total volume of the mixture was adjusted to 10 mL with distilled water. After the mixture was kept at room temperature for 1 h, the absorbance was read at 760 nm with a 751 UV-visible spectrophotometer (Shanghai Eastsen Analytical Instrument Co., Ltd., China). The standard calibration (0.02-0.12 mg/mL) curve was plotted using gallic acid. The TPC was expressed as the gallic acid equivalents per gram vitex honey (mg GAE/g). TFC was determined according to the method proposed by Jia et al. with a slightly modification.[21] One milliliter of vitex honey solution (2 mg/mL) was placed in a 10 mL volumetric flask and 0.4 mL of 5% sodium nitrite solution was added. Nought point four milliliter of 10% aluminum nitrate was added 6 min later. After 6 min, 4 mL of 4% sodium hydroxide was added and the total was made up to 10 mL with methanol. The solution was mixed well again and the absorbance was measured against a blank at 510 nm 15 min later. Rutin was used as the standard for a calibration curve. The TFC was expressed as the rutin equivalents per gram vitex honey (mg Rutin/g).

2.4. Analysis of antioxidant activities in vitro

2.4.1. DPPH radical scavenging activity

Scavenging activity on DPPH free radical by vitex honey was assessed according to the method reported by Brand-Williams et al. with slight modifications.[22] Briefly, different volumes of the sample (12.5 mg/mL) were placed in a 10 mL volumetric flask with 4.0 mL of 0.1 mM DPPH radical solution added. The total volume of mixture was adjusted to 10 mL with distilled water. Then, the mixture was shaken evenly and allowed to stand at room temperature in the dark for 30 min. Thereafter, the absorbance of the assay mixture was measured at 517 nm against methanol blank

using a spectrophotometer. DPPH radical scavenging capacity was expressed as the percentage inhibition of DPPH radical. The percentage inhibition of DPPH radical by vitex honey was calculated from the absorbance value according to the following equation:

Inhibition of DPPH radical (%) = $(A_0-A_t) / A_0\times100$

where A_0 was the absorbance of control (blank, without sample) and A_t was the absorbance in the presence of sample.

2.4.2. FRAP assay

The ferric reducing antioxidant power (FRAP) assay is based on the reduction at low pH of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine[Fe(III)-TPTZ] to the ferrous complex followed by a spectrophotometric analysis.[23] The reagent was prepared mixing 10 mM TPTZ with 20mM ferric chloride in acetate buffer (pH 3.6). Twenty microliter of diluted honey was dissolved in 2 ml of TPTZ reagent. The absorbance of the assay mixture was measured at 593 nm using a spectrophotometer and the results were expressed as mg Trolox/kg.

2.4.3. Ferrous ion-chelating activity

The ferrous ion-chelating activity of the Vitex hoeny was investigated according to the method of Nandita and Rajini by measuring the absorbance of ferrozine-Fe^{2+} complex at 562 nm.[24] Briefly, the reaction mixture, containing 30 μL of sample (10 mg/mL), iron vitriol (1 mM) 50 μL and ferrozine (1 mM) 20 μL, was adjusted to a total volume of 1 mL with methanol, shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against the blank. The standard calibration (0.05-0.25 mg/mL) curve was plotted using Na_2EDTA. Ferrous ion-chelating activity was expressed as the Na_2EDTA equivalents (mg Na_2EDTA/g honey).

2.5. HPLC analysis

The content of individual phenols in vitex honey was estimated by HPLC-DAD analysis proposed by Liang et al.[25] Agilent 1100 HPLC system (Agilent Technologies Deutschlan, Waldbron,) equipped with a vacuum degasser, a quaternary solvent delivery pump, a manual chromatographic valve, a thermostated column compartment,

and a diode-array detector (Agilent, Palo Alto, CA, USA) was used. The column was a Zorbax SB-C18 column (150 mm×4.6 mm, 5.0 μm). The mobile phase adopted was methanol (A) and 0.15% aqueous acetic acid solution (B) (v/v) using a linear gradient elution of 5-15% A at 0-10 min, 15-35% A at 10-15 min, 35-55% A at 15-20 min, 55-65% A at 20-25 min, and 65-80% A at 25-30 min, 80% A at 30-35 min. The injected volume was 5 μl, and flow rate was 1.0 ml/min. The column was operated at 30ºC. The diode-array detector was performed at 360 nm.

2.6. Animals experiment

2.6.1. Animal studies

Male Kunming mice (weighing 18-22 g), obtained from the Experimental Animal Center of Xi'an Jiaotong University, were used. They were allowed free access to standard dry pellet diet, which was made by Xi'an QinLe Feed Co., Ltd., and water adlibitum. All animals were grouped and housed in polyacrylic cages (29×18×16 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25±2°C, relative humidity 50±10%) with dark and light cycle (14/10 h). The animal ethical approval communication number is SCXK 2012-003. The animal experiments followed the guidelines and regulations of the State Committee of Science and Technology of People's Republic of China. The mice were acclimatized to laboratory condition for 5 days before commencement of experiment. Mice were randomly divided into six groups of 10 animals each. In the control group and APAP intoxicated group, animals were given a single dose of distilled water (0.2 mL/10 g body weight) orally twice daily using a gavage. In the test groups, animals were given 5, and 20 g of vitex honey per kilogram body weight orally twice daily using a gavage. In the honey alone groups, animals were given 20 g of vitex honey per kilogram body weight orally twice daily using a gavage. All administrations were conducted for 10 weeks. On the last day, all mice except those in the control group and honey alone group were given simultaneously an APAP solution (0.4 g/kg body weight) after the last administration 12 h, while the control group and honey alone group received physiological saline alone. Then all the animals were fasted for 20 h and were subsequently tested for the following analysis. The data were shown in

Table 2. All the experiments were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

2.6.2. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay provides a direct measure of hydrophilic chain-breaking antioxidant capacity against preoxyl radical.[26,27] This procedure used an automated plate reader with 96-well plates. Analyses were conducted in phosphate buffer pH 7.4 at 37℃. Peroxyl radical was generated using 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) which was prepared fresh for each run. Flurorescein was used as the substrate. Fluorescence conditions were as follows: excitation at 485 nm and emission at 535 nm. All serum samples suppressed the consumption of fluorescein through neat induction times. The protective effect of serum of administrated vitex honey mice were measured by assessing the area under the fluorescence decay curve (AUC) as compared to that of the blank in which no antioxidant is present.

2.6.3. Cu^{2+} induced oxidative modification of lipoproteins in serum

Using a modified procedure based on Hodgson et al.,[28] we determined the serum lipoprotein. 125 PBS buffer was used to dilute the sera from group I (control group) and group VI (honey group) to 0.5%. We added 12 μmol/l copper ions to obtain a uniform mixture. Oxidation kinetics was determined at 234 nm three times every hour at 37°C to obtain a constant value of absorbance. Serum dilution without Cu^{2+} was used as blank. The area under the oxidation curve (AUC) was determined and percentage inhibition of serum lipoprotein oxidation was calculated according to the following equation:

Inhibition (%) = (AUCcontrol-AUChoney) /AUCcontrol×100,

where AUCcontrol is the under the oxidation curve of control group (group I) and AUChoney is the under the oxidation curve of honey group (group VI).

2.6.4. Liver Index

Liver index was determined as percent of wet liver weight to wet body weight.

2.6.5. Assessment of liver function (ALT and AST levels)

After blood collection, serum was separated by centrifugation at 3000 rpm at room temperature for 10 min. The serum ALT and AST values were measured with commercially available diagnostic kits.

2.6.6. Determination of 8-OHdG levels

The content of 8-OHdG in serum were measured by immunoassay using a corresponding ELISA kit according to the manufacturer's protocols.

2.6.7. Determination of MDA, SOD and GSH-Px activity

Liver was excised immediately after the animals were sacrificed. The liver, except a portion of the left lobe to be used for histopathological sections, was frozen quickly and stored at -80°C. Prior to determinations, thawed tissue samples were homogenized in 9 volumes of ice cold 50 mM phosphate buffer (pH 7.4), centrifuged at 2500 rpm for 20 min at 4°C.

MDA level were measured with the method described by Jain et al. with a few modifications.[29] 100 µl of TCA 10% and 200 µl of TBA 0.6% were added to liposome suspension (100 µl). The mixtures were incubated in a water bath at 100°C for 1 h, cooled under running water and then centrifuged at 3000 g for 10 min. The MDA-TBA was measured at 532 nm using a spectrophotometer. The level of MDA was normalized with protein.

The supernatant was used for determination of SOD, GSH-Px and protein concentration using commercially available diagnostic kits. The levels of SOD and GSH-Px were normalized with protein.

2.6.8. Histopathological examinations

A portion of the left lobe of the liver was preserved in 10% neutral formalin solution for at least 24 h, processed and paraffin embedded as per the standard protocol. Sections of 5 µm in thickness were cut, deparaffinized, dehydrated, and stained with haematoxylin and eosin (H&E) for the estimation of hepatocyte necrosis and vacuolization. Morphological changes were observed including cell gross necrosis, sinusoidal congestion, fatty change, ballooning degeneration, inflammatory infiltration.

2.7. Statistical analysis

All the tests were performed in triplicate. Data analysis as carried out using SAS software, version 8.1 (SAS Institute, Cary, NC, USA). Statistically significant differences between the samples were evaluated by the Tukey's test. Differences at $P < 0.05$ were considered to be significant.

3. Results

3.1. Total phenolic content (TPC), total flavonoid content (TFC) and HPLC analysis

In the present study, we determined the TPC and TFC of vitex honey. As shown in Table 3, a range of 344 to 520 mg GAE/kg of TFC was observed, respectively. It also can be noticed that the TPC and TFC in vitex honey varied significantly between different honey samples from different geographical origins. The individual phenolic acids of vitex honey were determined by HPLC-DAD analysis and the results were shown in Table 4. Nine phenolic acids were detected in vitex honeys, in which caffeic acid was found as the main phenolic acid. Vitex honey sample from Hebei Zanhuang, S2, revealed the highest TPC (520.6 mg GAE/g honey), TFC (31.19 mg Rutin/g honey) and caffeic acid (109.78 mg/kg). Sample from Shaanxi Pingding, S10, had the lowest TPC (344.1 mg GAE/g honey), while from the point of TFC, no significant variation was observed between samples produced in Shaanxi Pingding and Henan Huixian. In general, the TPC of vitex honey investigated in this study, with a mean TPC value of 432.0 mg GAE/kg, is comparable to Portuguse honeys and Italy honeys, which had similar levels of TPC ranging from 226.16 to 727.77 mg GAE/kg and 150 to 980 mg GAE/kg, respectively.[30,31] The average TFC of vitex honey is 24.07 mg Rutin/kg, lower than that of Malaysia honeys (49.04 to 183.43 mg Rutin/kg) but higher than that of Christmas vine honey (10.9 mg/kg), sunflower and rape honey (15-20 mg/kg). [10,30,32,33]

3.2. Antioxidant activities measured by DPPH radical scavenging assay, FRAP assay and iron chelation assay

To evaluate the antioxidant capacity of vitex honey in vitro, the most commonly used antioxidant indexes were investigated and the results were shown in Table 3.

For DPPH radical scavenging activity, the IC_{50} values of vitex honey range from 44.18 (S2) to 55.21 mg/ml (S10)，indicating excellent radical scavenging ability comparing with previous literature data.[30,32,34] Antioxidant capacity of vitex honey was also assessed by FRAP test and Ferrous ion-clelating activity test, giving results of 96.69-132.36 mg Trolox/kg and 31.18-36.36 mg Na_2EDTA/kg respectively. In generally, S2 ranked the highest antioxidant activity in vitro investigations thereby had been applied to the following in vivo studies. Furthermore, we investigated the correlation between TPC as well as TFC and antioxidant activities. The good correlation coefficients were found between TPC/DPPH activity (r^2=0.9366), TPC/FRAP activity (r^2=0.9620), TFC/DPPH activity (r^2=0.7666) and TFC/FRAP activity (r^2=0.8073), DPPH/FRAP (r^2=0.92). Similar results were reported by other researches.[35-38]

3.3. Vitex honey elevated the antioxidant capacity in serum

3.3.1. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay is a well known measurement which gives information on antioxidant or electron donor property of biological samples. The results of ORAC assay were displayed in Fig.1, which showed that all serum samples exhibited increased area under the fluorescence decay curve. For the test group (group VI), administration of vitex honey (20 g/kg) for 10 weeks resulted in more inhibition of serum oxidative damage when compared with control group (group I). The inhibition rate is 15.07%.

3.3.2. Cu^{2+} induced oxidative modification of lipoproteins in serum

Estimation of inhibitory effect of vitex honey against Cu^{2+} induced lipid peroxide gives information of its antioxidant capacity against oxidative stress. Therefore, we determined the serum oxidative curve by plotting the absorbance values at 234 nm against time. Fig.2 shown that, pretreatment with vitex honey (20 g/kg) for 10 weeks inhibited the Cu^{2+} induced oxidative modification of lipoproteins in serum by 80.07% compared to the control mice.

3.4. Hepatoprotective effect of vitex honey against paracetamol-induced liver damage

3.4.1. Vitex honey decreased liver index

Fig.3 showed the liver index of all experimental groups. It was observed from the model group (group II) that administration of paracetamol leaded to the significant increase of liver index by 51.79%, indicating the existence of paracetamol-related liver damage. However, the liver index of the rats treated with silymarin (0.4 g/kg per day) and different doses of vitex honey (5 g/kg, 20 g/kg twice per day) reduced by 23.95%, 14.70% and 19.78%, respectively.

3.4.2. Vitex honey inhibit the ALT and AST activities in serum

The protective effect of vitex honey on paracetamol-induced liver damage was investigated by estimation of serum ALT and AST activities. As shown in Fig.4, dramatically elevated serum ALT and AST levels from model group (group II) were observed when compared with control group (group I). The levels of serum ALT and AST in model group were 173.41 U/L and 666.63 U/L, which were 6.22 and 16.43 times higher than that of control group, respectively. Compared with model group, administration of vitex honey (5 g/kg, 20 g/kg twice per day) could inhibit the elevation of the levels of serum ALT and AST effectively in a dose dependent manner. The inhibition rates of serum ALT and AST in low-dosed group (5 g/kg) were 66.92% and 66.36%, and that in high-dosed group (20 g/kg) were 75.79% and 74.52%, respectively, which was almost equipotent with the reference drug, silymarin. Pretreatment with silymarin (0.4 g/kg) could even reduce the serum ALT and AST levels by 80.35% and 84.97% only.

3.4.3. Vitex honey decreased hepatic MDA, SOD and GSH-Px activities

To evaluate the oxidative damage of liver, we determined the indicators of oxidative stress (MDA, SOD and GSH-Px). From Fig.5, signs of oxidative liver pathology arising from paracetamol-intoxication were detected by significant increase in hepatic MDA level (63.47%) and decrease in the activities of antioxidant enzymes such as SOD (10.35%) and GSH-Px (23.50%). In the present investigation, pretreatment with vitex honey (5 g/kg, 20 g/kg) for 10 weeks prior to paracetamol-intoxication resulted in suppression of MDA formation (13.44%, 36.15%) as well as restoration of SOD (4.89%, 9.15%) and GSH-Px activities

(8.46%, 25.65%). And it has to be noticed that the administration of vitex honey at a dose of 20 g/kg exhibited remarkable hepatoprotective effect, of which the MDA reduction rate as well SOD and GSH-Px improvement were 1.08, 0.97 and 2.27 higher than that of positive group which treated with silymarin at a dose of 0.4 g/kg body weight per day.

3.4.4. Vitex honey decreased the level of 8-OHdG in serum

The level of 8-OHdG in serum reflects the extent of oxidative DNA damage.[39] Therefore, we determined the serum 8-OHdG content and displayed the results in Fig.5 (D). Administration of silymarin (0.4 g/kg) and vitex honey (5 g/kg, 20 g/kg) could decrease the serum 8-OHdG concentration by 4.1% and 19.6%, respectively.

3.5. Histopathological observations

The hepatoprotective effect of vitex honey on paracetamol-induced liver damage was further evaluated by histological examinations. As shown in Fig.6A, rats in control group showed regular hepatic microarchitecture. While, remarkable liver damage characterized by congested central vein, dilated sinusoidal spaces, ballooning degeneration and inflammatory cell infiltration was observed in paracetamol-intoxication group (Fig.6B). However, pretreatment with vitex honey effectively inhibit the loss of hepatic microacrchitecture to some extent. The livers of rats in low-dosed group (5 g/kg) showed moderate congestion of centrol vein, mild fatty changes and ballooning degeneration of hepatocytes (Fig.6D). The high-dose group (20 g/kg) displayed marked amelioration in liver damage with a pattern of very less percentage of hepatocellular necrosis, less extent of hepatocelluar hypertrophy and ballooning degeneration around the central vein (Fig.6E), indicating excellent protective effect against hepatic lesions which was comparable to silymarin-treated group (Fig.6C). This was in line with the above serum analysis.

4. Discussion

Liver, taking charge of homostasis in human body, is a versatile organ that masters plenty of functions, among which the biotransformation and detoxification functions are most noteworthy. But it is for that very reason that initiates the

paracetamol-related toxicity which subsequently leads to liver damage. Paracetamol is an over-the-counter (OTC) drug, primarily used as antipyretic or analgesic agent. However, paracetamol-overdose accounts for the most of drug-induced hepatic failure in the U.S due to its easy accessibility.[40] The hepatotoxicity of paracetamol begins with its cytotoxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which combines with glutathione (GSH) to form non-toxic GSH-adduct in the body. While after exhausts the cellular GSH pool, excessive NAPQI primarily caused by paracetamol-overdose covalently bind to macromolecules in cell including proteins of mitochondria, resulting in the mitochondrial dysfunction and ultimately the loss of cell function or cell death.[41-46]

The treatment of paracetamol-induced liver damage is basically based on the use of a N-acetyl-cysteine (NAC). As a precursor of GSH, NAC attenuates the toxicity of NAPQI by elevating GSH level,[47] competing against NAPQI-induced oxidative stress through scavenging reactive oxygen radicals(González et al., 2009) and increasing interleukin-6 production.[48,49] However, the use of NAC usually associated with frequent adverse effects related to concentration and the standard regimen is complex thereby not easily established.[5] As a result, it's imperative to look for new effective treatments with minimal side effects.

In the present study, it has been demonstrated in the preliminary experiments that all the 14 samples of vitex honey has excellent DPPH free radical scavenging activities, ferric reducing antioxidant power (FRAP) and ferrous ion-chelating capacity, while S2 ranked the highest antioxidant activity, and then was applied to mice in the following experiments. As expected, it had been evinced that vitex honey has potential ability for increasing antioxidant capacity in vivo which endows the living organism with resistance to oxidative stress, as evidenced by the dramatically inhibition of fluorescein consumption in ORAC assay and conjugated diene formation in Cu^{2+}-mediated lipoprotein peroxidation in the honey-pretreated group. It hints that pretreatment with vitex honey might be a reasonable therapeutic strategy to cope with paracetamol-induced liver damage. For this reason, successive investigations have been conducted.

The determination of serum ALT and AST is a golden standard in measurement of hepatic disease. When hepatic injury occurs, the two intracellular aminotransferase enzymes are leaked into plasma due to the disturbance of hepatocellular transport functions.[50] In this study, obvious increases in the levels of serum ALT and AST were observed of the rats in model group after paracetamol delivery (0.4 g/kg body weight) compared to the control group, indicating the deterioration of the hepatic functions as a result of cell injury to some extent which is attributed to the development of hepatotoxicity. Similar results were obtained by other researchers.[3,51,52] Silymarin, a high antioxidant flavonoid, also is a hepatoprotective drug that is reported to have effects of scavenging free radicals, stabilizing hepatocyte membrane and protecting intracellular enzyme system.[53] Therefore, we chose it as a positive reference. As seen in table 4, caffeic acid and vanillic acid, which have been certified to own the hepatoprotection against oxidative stress,[54,55] are detected in vitex honey as the main phenolic acids. Therefore, pretreatment with vitex honey (4 g/kg, 20 g/kg) does some help in enhancement in hepatic biological functions as observed by significantly ($P<0.01$) inhibition of the increase in the serum ALT and AST concentration in a dose-dependent manner. It suggested that vitex honey may be capable of stabilizing the hepatocellular membranes or stimulating the regeneration of hepatocytes thereby appears to be a protective agent in alleviating the injury caused by paracetamol. In the positive reference, silymarin (0.4 g/kg), as investigated in other studies, showed a super protection on liver injury.[56]

Oxidative stress, characterized by excessive production of reactive oxygen radicals (ROS) combined with weakened endogenous antioxidant defense system, underlies the disruption of homostasis. In the organism, the endogenous enzymatic and non-enzymatic antioxidants cooperate to form the antioxidant defense system that holds the key to counterbalance the deleterious ROS. However, once the antioxidant defense system fails, irreversible cellular oxidative damage will arise. The harmful effects of oxidative stress were reported by several researchers who pointed out that oxidative stress initiates or promotes the pathological progresses of

diverse human diseases. And there is evidence supporting oxidative stress as a detrimental pathway that intensifies the hepatocytes injury caused by paracetamol.[4] Superoxide dismutase (SOD), an enzymatic antioxidant, catalyses the dismutation of superoxide radicals to hydrogen peroxide which is still cytotoxic and further detoxified to water by catalase (CAT) and glutathione peroxidase (GSH-Px). In the paracetamol-intoxication group, paracetamol metabolism resulted in the gravely compromised antioxidant capacity of the mice as observed in the significantly decreased SOD and GSH-Px levels. While, vitex honey pretreatment prevented the reduction in the SOD and GSH-Px activity which in turn moderated the oxidative stress, a critical role in accentuation the hepatocyte injury. Therefore, potential hepatoprotective effect of vitex honey may be readily relevant to its role in maintain or restoration endogenous antioxidant enzyme levels. Parallel findings have been previously reported by James, who demonstrated that administration of encapsulated SOD decrease the paracetamol-induced toxicity.[57] In our primarily study, DPPH radical scavenging activity, ferrous ion-chelating had been confirmed. Moreover, pretreatment with vitex honey for 10 weeks significantly increases oxygen radical absorbance capacity (ORAC) and anti-lipid peroxidative activities of serum in mice. Thus, there is a hypothesis that radical-scavenging as well as ion-chelating activities of vitex honey contributed partly to its effect of amelioration in paracetamol-induced oxidative stress by restoration or maintain enzymatic antioxidant status. Furthermore, malondialdehyde (MDA) and 8-OHdG, as a product of lipid peroxidation and DNA oxidative damage respectively, were observed to have significantly elevation after paracetamol intoxication, indicating the development of lipid oxidative modification and DNA oxidative damage. Whereas the rats in honey-pretreated groups (4 g/kg, 20 g/kg) reflected limited increase in MDA and 8-OHdG levels, which reinforced the hepatoprotective potential of vitex honey.

Histopathological observations of the liver further supported the biochemical studies and the serum assays. Paracetamol intoxication caused remarkable liver damage which characterized by congested central vein, dilated sinusoidal spaces, fatty changes and loss of acrchitecture. While pretreatment with vitex honey

exhibited a more normal acrchitecture , less extent of hepatocellular hypertrophy and very less percentage of cell necrosis, which basically confirmed the hepatoprotective effect of vitex honey.

Polyphenols, which can act as a free radical-scavenger, metal ion-chelator and/or singlet oxygen-quencher, are considered as the key contributor to the antioxidant capacity of honey that seems to mainly responsible for the hepatoprotective property of vitex honey. This is in agreement with the broadly accepted concept that phenolic compounds from honey are bioactive which could enter the serum during metabolism thereby increasing the activity of intracorporal antioxidant defense system. Recently, it has been reported that phenolic compounds exhibit pro-oxidative action through promoting the production of hydrogen peroxide that induces oxidative stress. While the mild oxidative stress does not indicate toxicity entirely but confers cells with resistance to successive oxidative damage and the coordination of cell functions.[58,59,60] Thus, it seems logical to make an assumption that the hepatoprotective effect of vitex honey may be due to the strengthened oxidative resistance of rats which results from pro-oxidative property of vitex honey. In our investigation, vitex honey displayed excellent protective effect against paracetamol-induced liver damage as well as, if not better than, the reference drug silymarin, notwithstanding, the phenolic content of it is not very high. More interesting, the ferrous ion-clelating activity of vitex honey revealed a weak link with either TPC or TFC, those which stand for the most part of antioxidant capacity of honey. The most plausible explain is that antioxidants other than phenolic compounds that exist in vitex honey play an un-neglected role in its ion-chelating plus antioxidant activities. These antioxidants include ascorbic acid, amino acids, proteins, products of Maillard reaction and several enzymes.[61] Therefore, it's necessary to unveil the possible synergistic effect of those antioxidants.

In conclusion, the results of the present study manifested the hepatopretective effect of vitex honey against paracetamol-induced liver damage. The protective effect may be, at least in part, due to its antioxidative property and/or perhaps pro-oxidative action. Further studies are required to identify the active constituents

that responsible for the pro-/anti-oxidative and hepatoprotective properties of vitex honey. For the purpose of ascertaining whether the hepatoprotective effect is specific for paracetamol, the detailed mechanisms that involved in the protect effect is also need to be studied.

Acknowledgements

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Table 1. Characterization of the analyzed vitex honey samples.

Sample no.	Type of honey	Geographical origin	Vitex agnus-castue L. (Verbenaceae) pollen frequency (%)
S1	Monofloral	Hebei Zanhuang	74±4.6
S2	Monofloral	Hebei Zanhuang	87±5.1
S3	Monofloral	Hebei Zanhuang	72±4.2
S4	Monofloral	Hebei Zanhuang	83±3.7
S5	Monofloral	Hebei Zanhuang	77±7.3
S6	Monofloral	Hebei Zanhuang	73±8.6
S7	Monofloral	Shanxi Pingding	88±2.8
S8	Monofloral	Shanxi Pingding	69±4.9
S9	Monofloral	Shanxi Pingding	59±4.1
S10	Monofloral	Shanxi Pingding	71±5.2
S11	Monofloral	Shanxi Pingding	67±6.4
S12	Monofloral	Henan Huixian	75±3.8
S13	Monofloral	Henan Huixian	57±4.5
S14	Monofloral	Henan Huixian	55±6.1

Results presented in the table are expressed as mean ± standard deviation (SD) for 3 replications.

Table 2. Groups of all tested animals.

Groups	Treatment	Dose of APAP	Tested Sample
Group I	blank control	0	none
Group II	APAP alone	0.4g/kg body weight	none
Group III	APAP＋silymarin	0.4g/kg body weight	silymarin (0.04g/kg twice daily)
Group IV	APAP＋low honey	0.4g/kg body weight	honey (5g/kg twice daily)
Group V	APAP＋high honey	0.4g/kg body weight	honey (20g/kg twice daily)
Group VI	high honey alone	0	honey (20g/kg twice daily)

Table 3. TPC (mg GAE/g), TFC (mg Rutin/g) and antioxidant activity in vitro of vitex honey.

Sample	TPC (mg GAE/kg)	TFC (mg Rutin/kg)	DPPH (IC_{50} mg/ml)	FRAP (mg Trolox/kg)	Ferrous ion-chelating activity (mg EDTA-Na_2/kg)
S1	518.7±18.2 ^{a}	30.14±4.13 ^{a}	45.628±2.448 ^{c}	132.36±9.28 ^{a}	35.58±3.45 ^{a}
S2	520.6±20.4 ^{a}	31.19±3.29 ^{a}	44.187±2.038 ^{c}	129.54±7.35 ^{a}	35.69±4.23 ^{a}
S3	498.1±17.3 ^{a}	29.32±2.94 ^{a}	47.159±2.247 ^{c}	124.04±7.29 ^{ab}	34.89±2.63 ^{a}
S4	473.2±15.2 ^{a}	26.63±3.01 ^{a}	49.061±2.603 ^{bc}	122.39±8.12 ^{ab}	34.37±4.13 ^{ab}
S5	486.1±25.2 ^{a}	27.75±3.28 ^{a}	47.894±3.196 ^{bc}	126.67±6.19 ^{a}	32.25±5.11 ^{b}
S6	465.4±22.1 ^{a}	26.67±2.74 ^{a}	49.406±2.048 ^{bc}	119.25±8.53 ^{ab}	31.56±3.68 ^{b}
S7	378.1±17.7 ^{c}	21.18±3.56 ^{b}	53.492±3.621 ^{ab}	107.26±5.32 ^{b}	31.18±4.29 ^{b}
S8	394.5±19.5 ^{c}	22.06±2.49 ^{b}	53.090±3.524 ^{ab}	108.28±5.92 ^{b}	33.79±3.28 ^{b}
S9	367.2±14.8 ^{c}	20.69±2.19 ^{bc}	53.929±2.963 ^{ab}	104.58±7.23 ^{b}	35.17±2.18 ^{a}
S10	344.1±13.2 ^{c}	19.85±3.03 ^{c}	55.154±3.288 ^{a}	96.69±6.22 ^{c}	36.14±5.29 ^{a}
S11	359.8±15.1 ^{c}	20.03±4.28 ^{bc}	55.212±2.677 ^{a}	100.17±7.81 ^{bc}	34.04±4.77 ^{ab}
S12	407.9±18.3 ^{b}	19.83±3.29 ^{c}	49.786±2.178 ^{b}	110.32±8.29 ^{b}	36.36±3.75 ^{a}
S13	419.6±20.6 ^{b}	21.06±4.26 ^{b}	50.315±2.040 ^{b}	117.37±5.91 ^{ab}	33.43±4.18 ^{b}
S14	414.7±23.7 ^{b}	20.58±4.82 ^{bc}	51.375±1.872^{b}	111.29±4.29 ^{b}	35.75±2.86 ^{a}

Results presented in the table were expressed as the mean values ± standard (SD) for 3 replications.

Different lower case letters correspond to significant differences at $p < 0.05$.

Table 4. Individual phenolic acids of vitex honey (mg/kg honey).

Sample	galic acid	protocatechuic acid	p-hydroxybenzoic acid	chlorogenic acid	vanillic acid	caffeic acid	sinapic acid	ellagic acid	rosmarinci acid
S1	7.08±0.09	36.51±2.68	40.16±3.26	2.19±1.83	22.97±2.34	86.05±5.68	-	-	0.64±0.04
S2	9.42±0.31	35.36±2.54	42.11±4.11	6.60±0.72	74.49±5.43	109.78±8.26	-	-	-
S3	5.71±0.27	29.87±3.13	40.76±2.73	1.58±0.15	-	95.30±7.75	-	18.65±1.01	0.39±0.02
S4	7.49±0.43	28.81±2.75	29.16±2.14	1.12±0.09	55.97±4.86	49.48±3.62	0.19±0.01	13.41±0.99	0.35±0.02
S5	11.85±0.89	22.89±2.18	35.51±3.85	0.66±0.08	-	69.56±5.13	0.11±0.00	47.37±3.63	0.89±0.06
S6	8.08±0.94	23.62±1.96	45.96±5.88	1.54±0.21	23.55±2.03	77.84±6.51	-	-	0.86±0.05
S7	4.88±0.39	26.95±2.01	26.41±2.17	1.81±0.17	55.18±4.83	38.52±0.27	-	-	-
S8	9.58±0.65	29.18±1.85	21.69±1.89	1.46±0.09	42.43±4.41	55.34±5.43	0.31±0.02	-	-
S9	8.83±0.81	14.43±0.92	19.14±1.06	1.66±0.13	29.47±2.43	75.39±3.66	0.33±0.02	-	0.03±0.00
S10	6.41±0.53	27.92±3.04	40.38±3.44	2.39±0.35	11.22±1.83	54.78±4.36	-	-	0.34±0.03
S11	8.76±0.77	12.30±2.53	17.83±1.52	-	32.72±2.93	56.87±4.07	0.35±0.01	-	0.49±0.04
S12	7.84±0.53	14.37±0.96	22.61±1.99	1.38±0.08	32.64±2.85	56.65±6.38	0.48±0.04	-	0.49±0.05
S13	7.36±0.84	34.69±4.04	37.80±2.74	1.84±0.16	18.56±1.36	63.63±3.77	-	-	0.53±0.04
S14	4.32±0.59	21.38±1.87	38.46±3.53	2.62±0.31	-	95.49±6.33	-	-	-

Results presented in the table were expressed as the mean values ± standard (SD) for 3 replications.

Figure captions:

Fig.1 Effects of vitex honey on ORAC assay. Different lower case letters correspond to significant differences at $p < 0.05$. Group I was control group. Group VI was given 20 g/kg twice per day of vitex honey only.

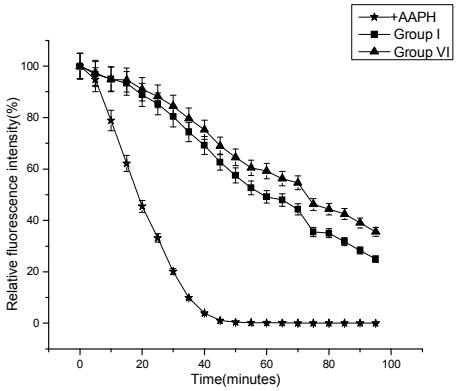
Fig.2 Effects of vitex honey on Cu^{2+} induced oxidative modification of lipoproteins in serum. Group I was control group. Group VI was honey alone group served as 20 g/kg twice per day of the vitex honey.

Fig.3 Effects of vitex honey on liver index. Different lower case letters correspond to significant differences at $p < 0.05$. Group I was control group. Group II was given only APAP. Group III was APAP plus 0.4 g/kg/d of silymarin. Group IV was APAP plus 5 g/kg twice per day of vitex honey. Group V was APAP plus 20 g/kg twice per day of vitex honey.

Fig.4 Effects of vitex honey on serum ALT and AST activity. Different lower case letters correspond to significant differences at $p < 0.05$. Group I was control group. Group II was given only APAP. Group III was APAP plus 0.4 g/kg/d of silymarin. Group IV was APAP plus 5 g/kg twice per day of vitex honey. Group V was APAP plus 20 g/kg twice per day of vitex honey.

Fig.5 Effects of vitex honey on serum MDA content (A), SOD activity (B), GSH-Px activity (C) and 8-OHdG content (D). Group I was control group. Group II was given only APAP. Group III was APAP plus 0.4 g/kg/d of silymarin. Group IV was APAP plus 5 g/kg twice per day of vitex honey. Group V was APAP plus 20 g/kg twice per day of vitex honey.

Fig.6 Effects of vitex honey on the hepatic morphological analysis (×400 H&E): control group (A), APAP model group (B), APAP plus 0.4 g/kg/d of silymarin (C), APAP plus 5g/kg twice per day of vitex honey (D), APAP plus 20 g/kg twice per day of vitex honey (E)



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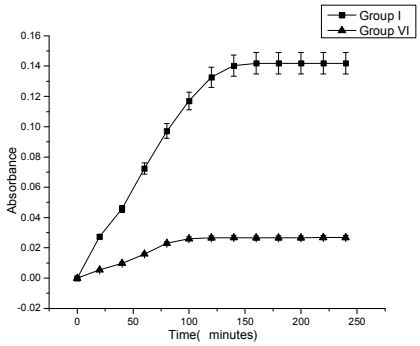

Fig.2. Effects of vitex honey on Cu^{2+} induced oxidative modification of lipoproteins in serum. Group I was control group. Group VI was honey alone group served as 20 g/kg twice per day of the vitex honey.

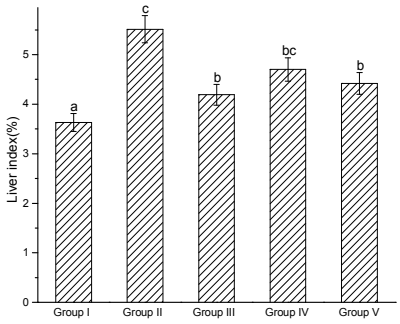

Fig.3. Effects of vitex honey on liver index. Different lower case letters correspond to significant differences at $p < 0.05$. Group I was control group. Group II was given only APAP. Group III was APAP plus 0.4 g/kg/d of silymarin. Group IV was APAP plus 5 g/kg twice per day of vitex honey. Group V was APAP plus 20 g/kg twice per day of vitex honey.

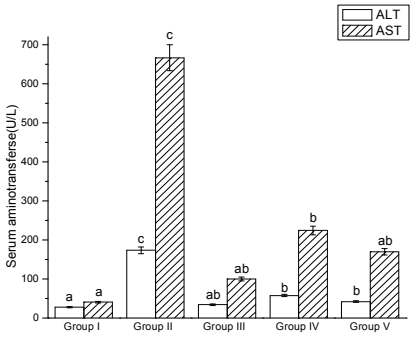

Fig.4. Effects of vitex honey on serum ALT and AST activity. Different lower case letters correspond to significant differences at $p < 0.05$. Group I was control group. Group II was given only APAP. Group III was APAP plus 0.4 g/kg/d of silymarin. Group IV was APAP plus 5 g/kg twice per day of vitex honey. Group V was APAP plus 20 g/kg twice per day of vitex honey.

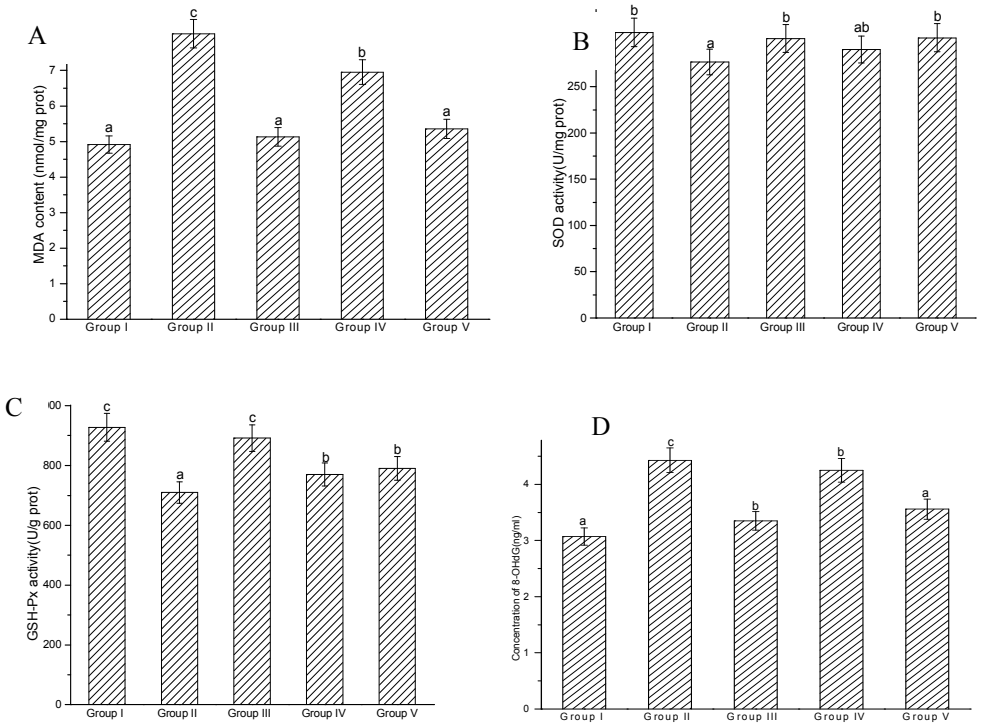

Fig.5. Effects of vitex honey on serum MDA content (A), SOD activity (B), GSH-Px activity (C) and 8-OHdG content (D). Group I was control group. Group II was given only APAP. Group III was APAP plus 0.4 g/kg/d of silymarin. Group IV was APAP plus 5 g/kg twice per day of vitex honey. Group V was APAP plus 20 g/kg twice per day of vitex honey.

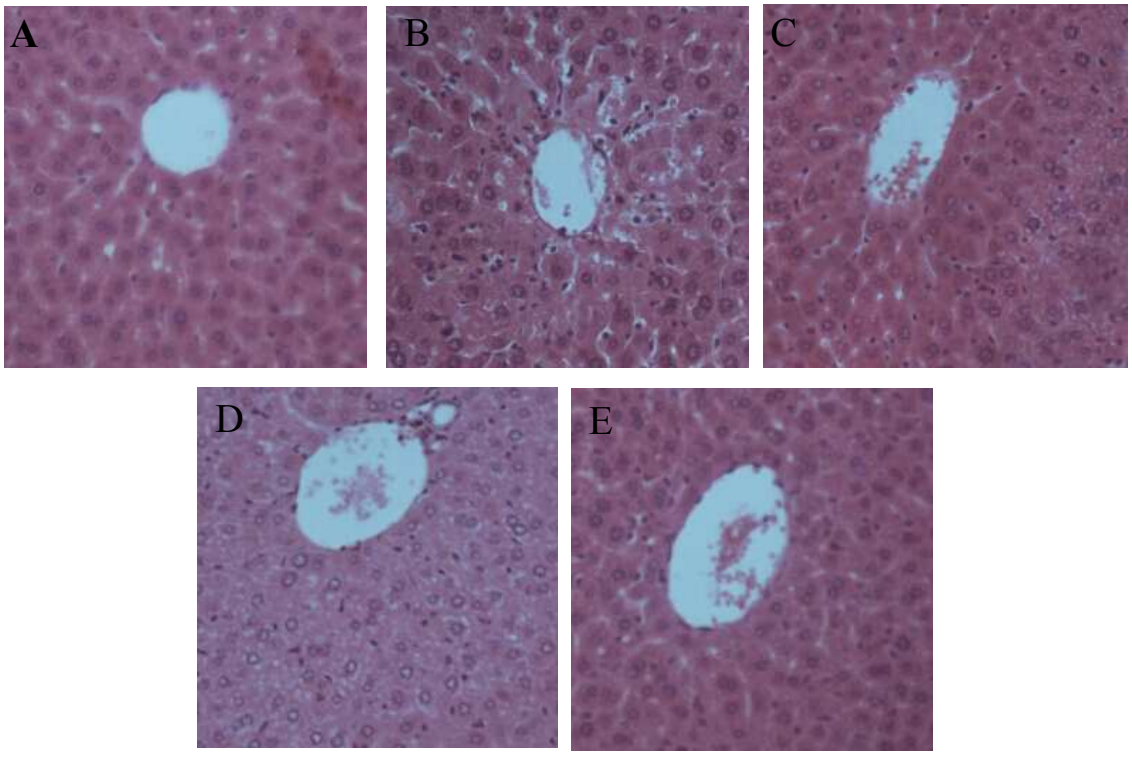

Fig.6. Effects of vitex honey on the hepatic morphological analysis (×400 H&E): control group (A), APAP model group (B), APAP plus 0.4 g/kg/d of silymarin (C), APAP plus 5g/kg twice per day of vitex honey (D), APAP plus 20 g/kg twice per day of vitex honey (E)