Apitherapy products enhance the recovery of CCL4-induced hepatic damages in rats

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Background/aim: Our objective was to identify the antioxidant properties of honeybee products from Turkey, chestnut honey, pollen, propolis, and royal jelly, and their hepatoprotective activity against CCl_4-induced hepatic damage in rats.

Materials and methods: Animals were fed with honeybee products for 7 days following CCl_4 injection. Development of liver damage and oxidative stress were monitored by measuring the activities of the enzymes alanine transaminase, aspartate transaminase, malondialdehyde, superoxide dismutase, and catalase. Antioxidant capacities of the bee products were identified using FRAP and DPPH assays, as well as by measuring total phenolic and flavonoid contents.

Results: The antioxidant activities of the honeybee products were highest in propolis, followed, in order, by pollen, honey, and royal jelly. Despite their different levels of antioxidant capacity, their roles in the prevention of liver damage induced by CCl_4 were very similar, which can be explained through their bioavailability to the treated animals.

Conclusions: Our results suggest that honey, propolis, pollen, and royal jelly significantly enhanced the healing of CCl_4-induced liver damage, partially due to their antioxidant properties and bioavailability.

Key words: Liver damage, honey, pollen, propolis, royal jelly, apitherapy

1. Introduction

The liver is a crucially important organ of the gastrointestinal tract, in which all the metabolic activities and detoxification of xenobiotics take place (1,2). Fatty liver, alcohol, viral and bacterial infections, and several chemical agents such as pesticides, drugs, and heavy metals cause liver damage. Acute and chronic viral hepatitis, alcoholic steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma are some of the major liver diseases, from which millions of patients suffer worldwide (2–4). Although there are some chemical medications for treating liver diseases, clinicians prefer plants or artificially modified versions of natural products, such as silymarin, curcimin, resveratrol, and naringenin, in order to avoid further hepatic complications (2,5,6). Several animal studies have also demonstrated that natural products can effectively inhibit liver damage and treat hepatic diseases (2,5,7).

Hepatic injuries in animal models are induced by various chemicals, such as carbon tetrachloride (CCl_4), ethanol, pesticides, galactosamine, analgesics (acetaminophen), antipyretic drugs, and heavy metal ions (Hg^{2+}, Pb^{2+}) (7,8). CCl_4 is a highly toxic agent metabolized by the cytochrome P450 system, which releases reactive trichloromethyl free radicals and reactive oxygen species, thus initiating lipid peroxidation and cellular necrosis (9,10). According to several reports, CCl_4-induced liver damage affects various organelles of the hepatocyte cells and primarily the mitochondria. Mitochondrial damage in hepatocytes is monitored in a simple way by measuring mitochondrial enzyme activities such as alanine transaminase (ALT), aspartate transaminase (AST), or gamma-glutamyl transferase (GGT) in serum or plasma. Histopathological examination of the liver is also used for the detection of liver injuries (5).

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Apitherapy, which uses bee products, has been employed in traditional medicine for the prevention and treatment of many diseases, such as systemic immune diseases, allergic diseases, viral diseases, and organ-specific inflammatory diseases, since ancient times (11). The main apitherapy products are honey, pollen, propolis, and royal jelly (RJ). These contain compounds with high biological activity such as polyphenols (phenolic acids, flavonoids, anthocyanins), vitamins, essential oils, and minerals (12–14). During the last 2 decades, research into the role of apitherapic products for the prevention and treatment of human diseases has intensified, and their antioxidant, antibacterial, antitumoral, and antiinflammatory potentials have been revealed (11,15,16).

Honey, pollen, propolis, and RJ contain a variety of secondary metabolites that are obtained from the plants visited by the honeybee. In general, the term "secondary metabolites of natural products" includes the phenolic compounds, which function in plant defense mechanisms against microorganisms, insects, and herbivores (2,17). Honey is a sweet food collected from the nectar of flower blossoms or the secretions of scale insects. Sugars are the main constituents of honey (95% dry weight), others being organic materials (phenolic substances, organic acids, vitamins, proteins, and minerals) (17). Bee pollen is located at the male gametes of the flower blossom, collected by honeybees in a process of great importance for plant reproduction (18). Bee pollen is a well-balanced food, rich in amino acids, proteins, carbohydrates, fats, minerals, and phenolic substances and used in the honeybees' diet and to feed larvae (19). Several studies have shown that bee pollen extracts protect human cells from oxidative stress (18–20). Bee pollen extracts of *Schisandra chinensis* have been reported to exhibit significant protective effects against acute hepatotoxicity induced by CCl_4 in mice (18). Propolis is a natural resinous product, collected by honeybees from various plant sources such as buds, the barks of some trees, and plants. Its resinous structure is changed and it is stored inside the hive (21). Propolis contains a high percentage of phenolic compounds, such as caffeic acid and caffeic acid phenethyl ester (CAPE), which is its most active component. Many beneficial properties of CAPE have been reported, including antioxidant, antiinflammatory, antibacterial, antitumoral, anticarcinogenic, and immunomodulatory effects (11,22,23). RJ, which represents the major nutrient of the young larvae, is a secretory product from the salivary glands of the worker honeybees. The composition of RJ is approximately 12%–15% proteins, 12%–15% carbohydrates, and 5%–6% lipids, as well as vitamins, minerals, and sterols (11,24,25). RJ has been reported to possess various bioactive components that reduce liver damage and induce proliferation of hepatocytes (15,26).

A previous study of ours showed that chestnut pollen ameliorates hepatic damage induced with CCl_4 in rats and attributed its healing effects to high silibinin levels (27). The aim of this study was to investigate the role of other bee products in preventing liver damage induced with CCl_4. We induced liver damage in rats through injections of CCl_4 and fed them with chestnut honey, pollen, propolis, and RJ to treat the resulting hepatic damage. We also investigated the antioxidant potential of these products by measuring the total phenolic contents and the ferric-reducing antioxidant assay (FRAP) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. Development of liver damage was monitored by measuring the activities of ALT, AST, superoxide dismutase (SOD), and catalase (CAT) enzymes and malondialdehyde (MDA) levels, as well as histopathological examinations. The antioxidant potential of the bee products was correlated with their total phenolic contents. Propolis exhibited the highest potential, followed by pollen, honey, and RJ, in that order. However, these 4 bee products exhibited the same efficiency in the treatment of hepatocyte injury induced by CCl_4.

2. Materials and methods

2.1. Chemicals and samples
The chemicals used were of analytical purity. Methanol, ethanol, thiobarbituric acid (TBA), trichloroacetic acid (TCA), and 1,1,3,3-tetramethoxypropane were procured from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT) was from AppliChem (Darmstadt, Germany) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and quercetin were also procured from Sigma-Aldrich Chemie GmbH, while 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu phenol reagent, and 2,4,6-tri-(2-pyridyl)-s-triazine (TPTZ) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Nitro blue tetrazolium (NBT), xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). AST and ALT diagnostic kits were also purchased from Sigma. Olive oil was obtained from Komili Sızma Company (İzmir, Turkey).

Chestnut honey, pollen, and propolis samples were obtained from experienced beekeepers belonging to the Zonguldak Beekeepers' Association in the Black Sea region of Turkey. Palynological identification showed that *Castanea sativa* L. pollens were the dominant pollens (65%) in the pollen and honey (89%) samples. RJ was obtained from Macahel Apiculture Co. Ltd. (Artvin, Turkey). All samples were from the 2010 season.

2.2. Preparations of the bee samples for antioxidant tests
Approximately 5 g of dried pollen samples was placed in a 100-mL Falcon tube, and 100 mL of methanol was added

and then stirred with a shaker (Heidolph Promax 2020, Schwabach, Germany) for 24 h at room temperature. After shaking, the mixture was sonicated in a sonicator apparatus (Elma Transsonic Digital, Germany) for 3 h. After sonication, the suspension was filtered, and the filtrate was concentrated in a rotary evaporator (IKA-Werke, Staufen, Germany) under reduced pressure at 40 °C. The residue was resolved to a minimal volume in methanol and was kept at 4 °C until use. The same procedure was followed for the honey samples. Raw propolis samples were initially frozen at –20 °C and ground to a fine powder. Next, 5.0 g of powder was placed in a Falcon tube (50 mL) and 30 mL of methanol was added. The suspensions were continuously stirred with a shaker at room temperature for 24 h and sonicated for 3 h. The suspensions were then filtered with a filter paper and concentrated in a rotary evaporator (IKA-Werke) under reduced pressure at 40 °C. The residue was resolved to a minimal volume in methanol and kept at 4 °C until use. Ten grams of raw RJ was dissolved in 50 mL of methanol and stirred at room temperature for 6 h. The suspension was centrifuged, and the supernatant was evaporated in a rotary evaporator (IKA-Werke) at 40 °C. The residue was resolved in minimal ethanol and kept at 4 °C until use.

2.3. Preparation of the bee samples for animal feedings
Honey, pollen, and RJ samples were diluted with distilled water and administered to the rats orally by gavage. Propolis samples were prepared in 95% ethanol, after which the alcohol degree was reduced to 24% through dilution. The doses administered to the animals were 400 mg/kg honey, pollen, and propolis and 50 mg/kg RJ. CCl_4 was diluted with olive oil (1:1, v/v) and injected intraperitoneally (i.p.).

2.4. Animals and preparation doses
The protocol for this study was approved by the Ethics Committee on Animal Research of Karadeniz Technical University in Turkey (protocol number: 2010/6-03.05.2010). Forty-nine adult female Sprague Dawley rats weighing 250–300 g were obtained from the Surgical Experimental Research Center (Trabzon, Turkey) and housed in a room under controlled temperature (22 ± 2 °C) in a 12-h light/dark cycle. The rats were fed with standard laboratory chow and water during the experiment. They were divided into 7 equal groups (n = 7). The experimental applications were completed in 7 days.

Experimental design:

Group 1: Saline solution/control group: 0.8 mL/kg, i.p.

Group 2: Ethanol/control group: 0.8 mL/kg, i.p.

Group 3: CCl_4 only/untreated group: 0.8 mL/kg, i.p.

Group 4: Honey treatment group: CCl_4 (0.8 mL/kg, i.p.) with 400 mg/kg honey, gavage.

Group 5: Pollen treatment group: CCl_4 (0.8 mL/kg, i.p.) with 400 mg/kg pollen, gavage.

Group 6: Propolis treatment group: CCl_4 (0.8 mL/kg, i.p.) with 400 mg/kg propolis, gavage.

Group 7: RJ treatment group: CCl_4 (0.8 mL/kg, i.p.) with 50 mg/kg RJ, gavage.

Twenty-four hours after the last injection the rats were sacrificed by decapitation. The abdominal cavity was exposed via a midline incision and the liver was quickly removed. Two random specimens from each group were taken for microscopy examination and the remaining livers were divided into 2 pieces and kept in 1.15% KCl solution and 10% formaldehyde for histopathological examination.

2.5. Determination of antioxidant capacity
Total phenolic contents (TPCs) of the samples were determined by the Folin–Ciocalteu method using gallic acid as the standard (28). The amount of total flavonoid was determined using the spectrometric method with aluminum chloride (29) and quercetin as a standard.

The reducing ability of ferric tripyridyltriazine (Fe-III-TPTZ) complex, the FRAP assay, was used for total antioxidant capacity measurement (30). Working FRAP reagent was prepared as required by mixing 25 mL of 300 mM acetate buffer, pH 3.6, with 2.5 mL of 10 mM TPTZ solution in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ solution. Next, 3 mL of freshly prepared FRAP reagent and 100 µL of the samples were mixed and incubated for 4 min at 37 °C, and the absorbance was read at 595 nm against a reagent blank containing distilled water. Trolox was used as a positive control to construct a reference curve (62.5–1000 µM). FRAP values were expressed as µmol $FeSO_4 \cdot 7H_2O$ equivalent/g.

The scavenging of DPPH radicals was assayed using the Molyneux method (31). This method is based on the fact that the DPPH radical has a purple color that decays in the presence of antioxidant agents. The change in absorbance can be monitored at 517 nm in order to detect radical scavenging activity. For each sample, 1.5 mL of the ethanol extract solution was mixed with 1.5 mL of 0.1 mM DPPH (dissolved in methanol), vortexed, and incubated for 50 min in the dark at room temperature. The absorbance was recorded at 517 nm against a blank and a control. The control solution contained DPPH solution without sample. The results were expressed as SC_{50} (mg/mL), which was calculated from the curves by plotting absorbance values, and the SC_{50} values represent the concentration of the extract (mg/mL) required to inhibit 50% of the radicals.

2.6. Biochemical analysis
The SOD activity of plasma and liver tissue was determined by spectrometric assay, using NBT reagents following the method described by Sun et al. (32). The protocol is based on the measurement of absorbance at 560 nm (Beckman-Coulter, DU 530) of the blue-colored formazan product

generated as a result of the reduction of the NBT ion by the superoxide radical. Enzyme activity causing 50% inhibition was regarded as 1 unit using bovine SOD as the standard, and the result was expressed as U/g tissue (32).

MDA levels were measured with a colorimetric test with TBA, which is used to assess endogenous lipids (33). Fresh tissue samples obtained from the treated rats were kept at –80 °C until analysis. Liver tissues were weighed and homogenized in ice-cold 1.15% KCl. The homogenate was centrifuged at $2000 \times g$ for 10 min. The breakdown product of 1,1,3,3-tetramethoxypropane was used as the standard and tissue MDA levels were calculated as nmol/mL plasma/g tissue.

CAT activity was determined using the method described by Aebi (34). Decomposition of H_2O_2 was monitored at the absorbance of 240 nm.

2.7. Histopathological analysis

For histopathological analysis, the liver tissue samples were immediately fixed in 10% formaldehyde solution, dehydrated with ethanol series, cleared with xylene, embedded in paraffin, and sectioned. Next, 5-μm tissue sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus BX-51; Olympus Optical Co., Ltd., Tokyo, Japan). All liver tissue slides were examined under high magnification, and images were recorded by a blinded histologist. Liver sections from each study group were evaluated for structural changes. Liver damage severity was assessed semiquantitatively using the following criteria: hepatocyte degeneration, vascular congestion, sinusoidal dilatation, congestion in enlarged sinusoids, and fatty degeneration. Each specimen was scored on a scale of 0 to 3 (0: none, 1: mild, 2: moderate, 3: severe). A mean histological score was calculated for each group.

2.8. Statistical analysis

Statistical calculations were performed with SPSS 15.0 for Windows and Microsoft Excel for Windows XP. The results are presented as mean ± standard deviation (SD). One-way analysis of variance was used for evaluation of values. The mean values found to be statistically different from each other were compared using Duncan's multiple comparison test. P < 0.05 was regarded as statistically significant.

3. Results

3.1. Antioxidant potential of the honeybee products

The antioxidant values of bee products used in the study are summarized in Table 1. The TPCs of the honeybee samples were measured using the commonly used Folin assay for the methanolic extracts. We found significant differences in the amounts of TPC among the honeybee products tested, ranging between 0.072 mg GAE/g (RJ) and 183 mg GAE/g (propolis) in raw samples (P < 0.01). In descending order, based on TPC, it was propolis > pollen > honey > RJ. Propolis exhibited the highest TPC and RJ the lowest. Similar to total phenolic substances, propolis samples exhibited the highest total flavonoid contents (TFCs) and RJ samples the lowest.

The reducing ability of the Fe-III-TPTZ complex reflects the total antioxidant capacity of honeybee products. In this method, higher FRAP values indicate higher antioxidant activity. The calculated FRAP values of the samples are given in Table 1, and they ranged from 1.02 to 1416 μmol $FeSO_4.7H_2O$/g sample. The ranking was similar to that recorded for TPC and TFC, i.e. propolis, pollen, honey, and RJ.

The antioxidant potential of the bee product samples can also be measured through their DPPH radical

Table 1. Total phenolic and flavonoid contents (TPCs and TFCs) of the studied honeybee products representing their antioxidant potential (FRAP and DPPH).

Honeybee product	TPC (mg GAE/g)	TFC (mg quercetin/g)	FRAP (μmol $FeSO_4.7H_2O$/g)	DPPH (mg/mL)
Honey Sig.	0.95 ± 0.07[a] 0.833	0.56 ± 0.03[a] 0.291	1.02 ± 0.02[a] 0.143	19.64 ± 1.45[c] 1.000
Pollen Sig.	13.78 ± 0.34[c] 1.000	1.64 ± 0.11[b] 0.121	48.75 ± 2.60[b] 0.118	0.49 ± 0.01[b] 1.000
Propolis Sig.	183.86 ± 6.35[d] 1.000	106.61 ± 2.36[c] 0.097	1416.20 ± 0.07[c] 1.000	0.02 ± 0.00[a] 1.000
Royal jelly Sig.	0.072 ± 0.05[b] 1.000	0.02 ± 0.00[d] 1.000	0.95 ± 0.06[a] 0.143	38.72 ± 3.20[d] 1.000
F	158.331	155.359	143.493	110.942

a, b, c, d: The values with different letters are significantly different from each other (P < 0.01).
FRAP: Ferric-reducing antioxidant assay.
DPPH: 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity.

scavenging ability. The results were expressed as SC_{50} (mg/mL) values and the lower SC_{50} values represent higher radical scavenging activity. All the bee products scavenged the DPPH radicals, but there were considerable differences among the studied samples. The propolis and pollen samples exhibited higher radical scavenging activity than the honey and RJ samples (Table 1).

3.2. Physiological findings

We detected no major physical disorders or weight loss in the control and treatment groups, except for weight loss (3.65%) in the CCl_4-only treated rats (Group 3). The honey- and RJ-treated groups (Group 5 and Group 6, respectively) remained at the same weight, while the pollen- and propolis-treated groups (Group 4 and Group 7, respectively) both gained weight by the end of the treatment. The relevant results are given in Table 2.

We measured ALT and AST enzyme activity in plasma in order to determine whether CCl_4 attenuated the liver damage in the CCl_4-treated rats (Table 2). AST and ALT enzyme activities of Group 3 were significantly higher than in the control groups (Groups 1 and 2), approximately 6 and 17 times higher, respectively. AST and ALT enzyme activities decreased significantly in the rats fed with the bee products (pollen, propolis, honey, and RJ; Groups 4–7) following CCl_4 administration. However, none of these treatment groups differed significantly from one another in terms of lowering AST and ALT enzyme activity.

We measured MDA levels and SOD and CAT enzyme activity using liver and plasma samples in order to determine changes in antioxidant activity at the cellular level (Table 2). The liver MDA levels increased significantly in rats treated with CCl_4 only (Group 3); however, MDA levels remained close to control group levels in animals fed with the bee products (Groups 4–7). Among the treatment groups, the group receiving propolis (Group 6) had the lowest MDA levels, followed by the pollen and honey groups (Groups 5 and 4), in that order. Nonetheless, RJ treatment partially reversed oxidative stress induced by CCl_4 treatment. We also measured MDA levels in the rat plasma samples. However, those findings were below the detection limits and the results were omitted from Table 2. Similar to MDA measurements, liver SOD activity increased only in CCl_4-treated rats (Group 3) and decreased to close to control levels following honeybee product treatments (Groups 4–7), although the changes in plasma did not achieve any significance. CAT activities increased in the groups receiving CCl_4 treatment, representing higher liver damage, and increased slightly in the honeybee product-treated groups compared to the control group (Group 6).

Since propolis is not water-soluble, we used ethanolic propolis extracts. In order to identify the effect of ethanol on liver markers, antioxidant enzymes, and lipid peroxidation, we established an ethanol group (Group 2)

Table 2. The enzyme analysis of the animal samples treated with the honeybee products following CCl_4-induced liver damage.

Treatment groups (n = 7)		Weight change (%)	AST (U/L)	ALT plasma (U/L)	MDA (liver)	SOD (U) Plasma (U/mL)	SOD (U) Liver U/g tissue	CAT (kU/L) Plasma (U/mL)	CAT (kU/L) Liver (U/g tissue)
G1 Sig.	0.9 %SF Control	+4.21 ± 0.90[a] 0.101	220 ± 46[a] 0.419	61 ± 10[a] 0.818	9.28 ± 1.00[a] 1.000	0.16 ± 0.14[a] 0.235	1.39 ± 0.30[a] 1.000	1.28 ± 1.09[a] 0.187	22.78 ± 2.20[a] 0.127
G2 Sig.	Ethanol	−0.93 ± 0.03[b] 0.059	179 ± 31[a] 0.419	54 ± 6.0[a] 0.818	11.24 ± 0.40[b] 0.399	0.04 ± 0.06[a] 0.235	1.75 ± 0.34[b] 1.000	2.20 ± 0.56[a] 0.187	24.18 ± 2.65[a] 0.127
G3 Sig.	CCl_4 (0,8 mL/kg)	−3.65 ± 0.80[c] 1.000	1303 ± 225[c] 1.000	1080 ± 20[c] 1.000	21.23 ± 1.19[f] 1.000	0.02 ± 0.04[a] 0.235	6.40 ±1.09[f] 1.000	2.55 ± 0.08[a] 0.187	34.05 ± 4.40[b] 0.610
G4 Sig.	Honey (400 mg/kg)	+3.42 ± 0.68[a] 0.101	424 ± 110[b] 0.520	199 ± 56[b] 0.514	14.03 ± 0.42[e] 1.000	0.09 ± 0.05[a] 0.235	4.41 ± 2.22[e] 0.377	1.98 ± 0.50[a] 0.187	25.20 ± 3.21[a] 0.127
G5 Sig.	Pollen (400 mg/kg)	−2.29 ± 0.52[c] 1.000	445 ± 140[b] 0.520	222 ± 120[b] 0.514	12.50 ± 0.50[e] 1.000	0.11 ± 0.07[a] 0.235	4.33 ± 1.23[e] 0.377	1.65 ± 0.56[a] 0.187	27.46 ± 2.80[a] 0.127
G6 Sig.	Propolis (400 mg/kg)	−2.78 ± 0.70[c] 1.000	409 ± 68[b] 0.520	205 ± 42[b] 0.514	10.71 ± 0.70[c] 0.058	0.12 ± 0.06[a] 0.235	3.52 ± 0.35[c] 1.000	1.16 ± 0.92[a] 0.187	24.50 ± 3.60[a] 0.127
G7 Sig.	Royal Jelly (50 mg/kg)	+3.17 ± 0.64[a] 0.101	410 ± 112[b] 0.520	204 ± 73[b] 0.514	16.65 ± 0.65[d] 1.000	0.07 ± 0,05[a] 0.235	3.85 ± 0.67[d] 1.000	1.89 ± 0.23[a] 0.187	28.01 ± 3.42[a] 0.127
F			5.363	31.079	37.617	171.750	4.345	2.376	0.943	2.484

a, b, c, d: The values with different letters are significantly different from each other ($P < 0.05$). G = Group.

(0.8 mL kg^{-1} day^{-1}) for control purposes. Ethanol treatment did not change AST and ALT enzyme activity in plasma, but it significantly altered the MDA level and SOD and CAT enzyme activities in the liver compared to the control group (Group 1).

3.3. Histopathological findings
We analyzed the liver tissue sections from the study groups using light microscopy (Figure 1). Liver tissue was histologically normal in the control group (Figure 1A). In the ethanol and CCl$_4$-treated groups (Groups 2 and 3), extensive intracellular fatty degeneration and sinusoidal dilatation were observed (Figures 1B and 1C). In the honey-treated group (Group 4), a decrease in intracellular fatty degeneration was observed, especially around the portal area (Figure 1D). In the pollen and the propolis groups (Groups 5 and 6), common intracellular fatty degeneration was observed around the central veins, but normal hepatocytes were present around the portal area (Figures 2A and 2B). In the RJ-treated group (Group 7), minimal intracellular fatty degeneration was observed around the central veins, although extensive abnormal hepatocytes were present in the same regions (Figure 2C).
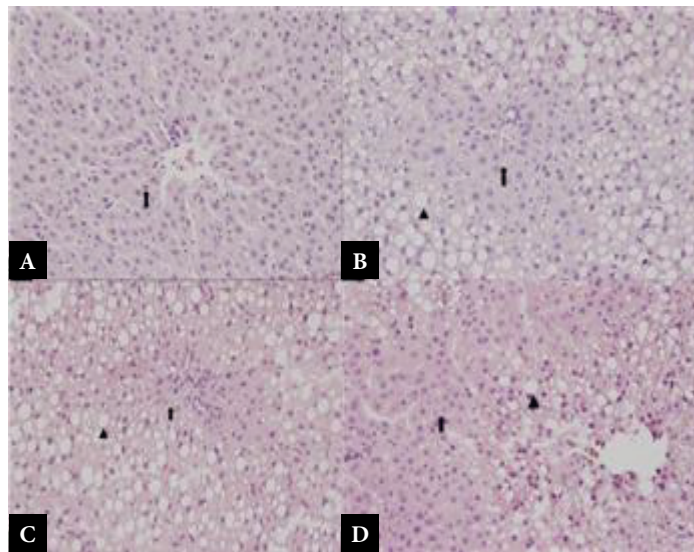

Figure 1. Photomicrograph of liver sections. A) Normal histological appearance of hepatocytes (↑) in control group (Group 1). B) Significant dilatation in sinusoids (↑) and increased intracellular fatty degeneration (▲) in ethanol-treated group (Group 2). C) Significant dilatation in sinusoids (↑) and common increased intracellular fatty degeneration (▲) in CCl4-only group (Group 3). D) Normal hepatocytes (↑) and intracellular fatty degeneration around the central veins (▲) in honey-treated group (Group 4). H&E, 200×.
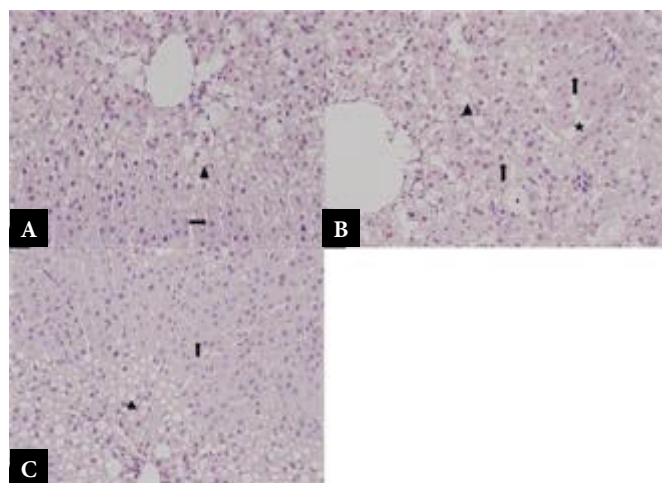

Figure 2. Photomicrograph of liver sections. A) Normal hepatocytes (↑) and intracellular fatty degeneration around the central veins (▲) in pollen-treated group (Group 5). B) Normal hepatocytes (↑), increased fatty degeneration (▲), and sinusoidal dilatation (★) in propolis-treated group (Group 6). C) Extensive normal hepatocytes (↑) and rare intracellular fatty degeneration around the central veins (▲) in RJ-treated group (Group 7). H&E, 200×.

4. Discussion

Liver diseases are one of the most common illnesses in the world, from which hundreds of millions of people suffer and die each year. Liver damage is primarily caused by viral infections, such as hepatitis B, hepatitis C, and the human immunodeficiency virus, as well as bacterial infections, chemical agents, antibiotics, and pesticides (1,5,7,8). Since the liver is a major organ that processes food and most medications, natural medicines, such as milk thistle (silymarin) and dandelion, are preferred over chemical drugs for treating liver damage (6,14). Several clinical studies have used natural medicines, which are rich in secondary metabolites such as phenolic agents, to treat liver disorders. In recent decades, apitherapy, the use of bee products for healing, has also been used to treat liver disorders (7,14,15). In our study, we tested and compared the potentials of various bee products in different samples in the treatment of CCl_4-induced liver damage in rats.

Before experimental investigation of their hepatoprotective roles in rats, we evaluated the antioxidant properties of the honeybee products. Most honeybee products exhibit biological properties such as antioxidant, antibacterial, antitumoral, and antiinflammatory activities, which are mostly associated with phenolic acids, flavonoids, anthocyanins, and several aromatic acids and esters within them (35). The phenolic contents of natural medicines have been identified as the major agents involved in counteracting reactive oxygen species in the healing of the damaged liver (17). We initially analyzed TPC and various antioxidant properties of the honeybee products in order to compare relations between structure and liver healing. Propolis contained the highest TPC (183.86 mg GAE/g) and RJ the lowest (0.072 mg GAE/g). In our previous study, the TPC levels were between 115 and 210 mg GAE/g in Turkish propolis (21). TPC in all bee products, as well as other antioxidant substances, largely depends on the geographical location and biodiversity involved (36,37). Ulusoy and Kolayli (38) reported TPC levels between 44 and 124 mg GAE/g in Anzer pollen samples from Turkey. In another study, TPC levels in Sonoran Desert pollen were reported as being between 5.91 and 34.85 mg GAE/g (19). In parallel to the TPC levels, TFC levels were highest in propolis, followed by pollen, honey, and RJ, in that order. In association with its phenolic structures, propolis exhibited the highest antioxidant capacity, as well as the highest FRAP value (1416.2 µmol $FeSO_4.7H_2O/g$) and the lowest radical scavenging activity (0.02 mg/mL), followed by pollen, honey, and RJ, in that order. Our findings suggest a positive correlation between TPC/TFC and antioxidant potentials of honeybee products, in agreement with earlier studies (17,39,40).

Chestnut honey (*Castanea sativa*) is a dark amber-colored product with a high fructose/glucose ratio (>1.52) and is used as a medicinal honey in clinics worldwide due to its high TPC and antioxidant capacity (20,41–43). We used chestnut honey obtained from the Zonguldak region of Turkey, which has already been shown to have higher TPC levels (98.0 mg GAE/100 g honey) than some blossom honeys in Turkey (43), in order to evaluate its hepatoprotective effect following CCl_4-induced liver damage. Honeybees use a highly pure composition of RJ to feed their larvae and young bees owing to its high nutritional and bioactivity properties. As researchers reviewed its therapeutic potential, RJ became one of the most popular natural products in apitherapic applications (24,39). Several studies have shown that RJ protects cells against oxidative stress (25,44). Its bioactivity properties are mainly attributed to high concentrations of fatty acids, proteins, and phenolic compounds (24). However, TPC levels among the bee products we analyzed were lowest in RJ (0.072 mg GAE/g, 13 times less than in honey), which may suggest that its bioactivity depends not only on phenolic acids, but also on other constituents (fatty acids, proteins) within its structure.

The purpose of this study was to evaluate the effects of bee products on hepatoprotective activities in experimental rat groups. The experimental animals were exposed to CCl_4 toxicity in order to induce hepatic damage. Seven different groups were studied. Significant weight loss was determined in the rats treated with ethanol (Group 2), the CCl_4-exposed group (Group 3), and the pollen (Group 5) and propolis (Group 6) groups. Since the propolis was dissolved in ethanol for injection, the weight loss in the propolis-treated animals may be due to the ethanol solvent rather than to the propolis. We observed no weight loss in the honey- or RJ-treated groups (Groups 4 and 7), which may suggest that the rich levels of carbohydrates and other nutrients in honey and RJ compensated for the weight loss caused by the liver injury.

Administration of CCl_4 at a dose of 0.8 mL kg^{-1} day^{-1} leads to severe acute necrosis in the liver, since AST and ALT activities of plasma enzymes were significantly elevated in the rats. In addition, microscopic examination of liver tissues in the CCl_4-only group (Group 3) revealed serious liver necrosis (Figure 1C). Many studies have reported that CCl_4 is a hepatotoxic agent that can induce lipid peroxidation and cellular damage (8–10,27). Fatty degeneration in hepatocytes was detected in liver sections from the CCl_4- and ethanol-treated groups. A significant improvement in these degenerations was evident in the groups treated with the honeybee products (Groups 4–7). Kanbur et al. (44) investigated hepatoprotective effects in paracetamol-induced liver damage and reported a marked protective effect on liver damage in mice. RJ has been reported to exhibit hepatoprotective effects against fumonisin-induced liver damage in rats (25). Cheng et

al. (18) reported that pollen extracts from *Schisandra chinensis* reduced CCl$_4$-induced liver damage in mice. Ethanolic propolis extract has also been reported to protect against AlCl$_3$-induced hepatic injury in a mouse model (40). Chestnut propolis has been reported to exhibit a protective effect against alcohol-induced liver damage (16). Dietary honey consumption has been shown to reduce hepatotoxicity in CCl$_4$-induced liver damage (45). These studies, and our own results, suggest that honeybee products have substantial potential applications for the healing of liver damage to various extents depending on their antioxidant capacity.

Although our honeybee products exhibited different levels of antioxidant characteristics, their healing potentials in liver damage did not differ significantly from one another. This may be due to their bioavailability and their absorption by the rats' gastrointestinal tracts. It has been reported that honey, propolis, pollen, and RJ possess different bioavailability properties. Honey exhibited the highest absorption rate followed by pollen, RJ, and propolis, in that order (46).

In conclusion, chestnut honey, pollen, propolis, and RJ are rich in natural antioxidant products. Propolis exhibited the highest phenolic and flavonoid contents and thus exhibited more pronounced antioxidant activity in the FRAP and DPPH assays. Weight loss resulting from CCl$_4$-induced liver damage was successfully compensated for by the honey and the RJ, but not by the propolis or the pollen. Overall, we conclude that honey, propolis, pollen, and RJ enhance recovery from CCl$_4$-induced liver damage in a manner partially dependent on their antioxidant properties and bioavailability, which has been reported in previous studies. These honeybee products can therefore be used for the prevention and treatment of various liver diseases. The mechanism of hepatoprotective activity on hepatocytes of these bee products requires further in vitro analyses in future studies.

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