Apitherapy products enhance the recovery of $\text{CCL}_4$-induced hepatic damages in rats

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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, curcumin, 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 ($\text{CCL}_4$), ethanol, pesticides, galactosamine, analgesics (acetaminophen), antipyretic drugs, and heavy metal ions ($\text{Hg}^{2+}, \text{Pb}^{2+}$) (7,8). $\text{CCL}_4$ 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, $\text{CCL}_4$-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 the activities of the enzymes 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).
A previous study of ours showed that chestnut pollen ameliorates hepatic damage induced with CCl₄ 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 CCl₄. We induced liver damage in rats through injections of CCl₄ 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 CCl₄.

2. Materials and methods

2.1. Chemicals and samples
The chemicals used were of analytical purity. Methanol, ethanol, thio BARBITURIC 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-Aldrich Chemie GmbH. Olive oil was obtained from Komili Sızm 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 powdered 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) under reduced pressure 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 water and administered to the rats orally by gavage. Propolis 

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: CCl₄ only/un-treated group: 0.8 mL/kg, i.p.
Group 4: Honey treatment group: CCl₄ (0.8 mL/kg, i.p.) with 400 mg/kg honey, gavage.
Group 5: Pollen treatment group: CCl₄ (0.8 mL/kg, i.p.) with 400 mg/kg pollen, gavage.
Group 6: Propolis treatment group: CCl₄ (0.8 mL/kg, i.p.) with 400 mg/kg propolis, gavage.
Group 7: RJ treatment group: CCl₄ (0.8 mL/kg, i.p.) with 50 mg/kg RJ, gavage.

Twenty-four hours after the last injection the rats were sacrificed by decapsulation. 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 FeCl₃·6H₂O 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 FeSO₄·7H₂O 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 SC₅₀ (mg/mL), which was calculated from the curves by plotting absorbance values, and the SC₅₀ 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 × 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 H2O2 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.

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, and 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 FeSO4·7H2O/g. 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).

<table>
<thead>
<tr>
<th>Honeybee product</th>
<th>TPC (mg GAE/g)</th>
<th>TFC (mg quercetin/g)</th>
<th>FRAP (µmol FeSO4·7H2O/g)</th>
<th>DPPH (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey Sig.</td>
<td>0.95 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.56 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.02 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.64 ± 1.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.833</td>
<td>0.291</td>
<td>0.143</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Pollen Sig.</td>
<td>13.78 ± 0.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.64 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.75 ± 2.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.000</td>
<td>0.121</td>
<td>0.118</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Propolis Sig.</td>
<td>183.86 ± 6.35&lt;sup&gt;d&lt;/sup&gt;</td>
<td>106.61 ± 2.36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1416.20 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.02 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.000</td>
<td>0.097</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Royal jelly Sig.</td>
<td>0.072 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02 ± 0.00&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.95 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.72 ± 3.20&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.000</td>
<td>1.000</td>
<td>0.143</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>158.331</td>
<td>155.359</td>
<td>143.493</td>
<td>110.942</td>
</tr>
</tbody>
</table>

<sup>a, b, c, d</sup>: 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\textsubscript{50} (mg/mL) values and the lower SC\textsubscript{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\textsubscript{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\textsubscript{4} attenuated the liver damage in the CCl\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 ethanol 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\textsubscript{4}-induced liver damage.

<table>
<thead>
<tr>
<th>Treatment groups (n = 7)</th>
<th>Weight change (%)</th>
<th>AST (U/L)</th>
<th>ALT plasma (U/L)</th>
<th>MDA (liver)</th>
<th>SOD (U)</th>
<th>CAT (kU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>Liver</td>
<td></td>
<td>Plasma</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(U/mL)</td>
<td>(U/g tissue)</td>
<td>(U/mL)</td>
<td>(U/mL)</td>
<td>(U/g tissue)</td>
</tr>
<tr>
<td>G1 Sig. Control 0.9 %SF</td>
<td>+4.21 ± 0.90\textsuperscript{a} 0.101</td>
<td>220 ± 46\textsuperscript{c} 0.419</td>
<td>61 ± 10\textsuperscript{b} 0.818</td>
<td>9.28 ± 1.00\textsuperscript{d} 1.000</td>
<td>0.16 ± 0.14\textsuperscript{c} 0.235</td>
<td>1.39 ± 0.30\textsuperscript{c} 1.000</td>
</tr>
<tr>
<td>G2 Sig. Ethanol</td>
<td>−0.93 ± 0.03\textsuperscript{a} 0.059</td>
<td>179 ± 31\textsuperscript{d} 0.419</td>
<td>54 ± 6.0\textsuperscript{d} 0.818</td>
<td>11.24 ± 0.40\textsuperscript{b} 0.399</td>
<td>0.04 ± 0.06\textsuperscript{b} 0.235</td>
<td>1.75 ± 0.34\textsuperscript{b} 1.000</td>
</tr>
<tr>
<td>G3 Sig. CCl\textsubscript{4} (0.8 mL/kg)</td>
<td>−3.65 ± 0.80\textsuperscript{a} 1.000</td>
<td>1303 ± 225\textsuperscript{d} 1.000</td>
<td>1080 ± 20\textsuperscript{d} 1.000</td>
<td>21.23 ± 1.19\textsuperscript{d} 1.000</td>
<td>0.02 ± 0.04\textsuperscript{b} 0.235</td>
<td>6.40 ±1.09\textsuperscript{a} 1.000</td>
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<tr>
<td>G4 Sig. Honey (400 mg/kg)</td>
<td>+3.42 ± 0.68\textsuperscript{a} 0.101</td>
<td>424 ± 110\textsuperscript{c} 0.520</td>
<td>199 ± 56\textsuperscript{a} 0.514</td>
<td>14.03 ± 0.42\textsuperscript{b} 1.000</td>
<td>0.09 ± 0.05\textsuperscript{a} 0.235</td>
<td>4.41 ± 2.22\textsuperscript{a} 0.377</td>
</tr>
<tr>
<td>G5 Sig. Pollen (400 mg/kg)</td>
<td>−2.29 ± 0.52\textsuperscript{a} 1.000</td>
<td>445 ± 140\textsuperscript{c} 0.520</td>
<td>222 ± 120\textsuperscript{a} 1.000</td>
<td>12.50 ± 0.50\textsuperscript{b} 1.000</td>
<td>0.11 ± 0.07\textsuperscript{a} 0.235</td>
<td>4.33 ± 1.23\textsuperscript{a} 0.377</td>
</tr>
<tr>
<td>G6 Sig. Propolis (400 mg/kg)</td>
<td>−2.78 ± 0.70\textsuperscript{a} 1.000</td>
<td>409 ± 68\textsuperscript{c} 0.520</td>
<td>205 ± 42\textsuperscript{a} 0.514</td>
<td>10.71 ± 0.70\textsuperscript{a} 0.058</td>
<td>0.12 ± 0.06\textsuperscript{a} 0.235</td>
<td>3.52 ± 0.35\textsuperscript{a} 1.000</td>
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<tr>
<td>G7 Sig. Royal Jelly (50 mg/kg)</td>
<td>+3.17 ± 0.64\textsuperscript{a} 0.101</td>
<td>410 ± 112\textsuperscript{a} 0.520</td>
<td>204 ± 73\textsuperscript{a} 0.514</td>
<td>16.65 ± 0.65\textsuperscript{a} 1.000</td>
<td>0.07 ± 0.05\textsuperscript{a} 0.235</td>
<td>3.85 ± 0.67\textsuperscript{a} 1.000</td>
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<tr>
<td>F</td>
<td>5.363</td>
<td>31.079</td>
<td>37.617</td>
<td>171.750</td>
<td>4.345</td>
<td>2.376</td>
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</table>

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 CCl₄-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 CCl4-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 FeSO4/7H2O/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).

Chesnut 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 CCl4-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 apitherapeutic 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 CCl4 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 CCl4-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 CCl4, 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 CCl4-only group (Group 3) revealed serious liver necrosis (Figure 1C). Many studies have reported that CCl4 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 CCl4- 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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**References**

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