Effect of apitherapy products against carbon tetrachloride-induced toxicity in Wistar rats

CĂLIN VASILE ANDRÎTUÎ1–3, VASILE ANDRÎTUÎJ, MAGDALENA CUCIUREANU4, DELIA NICA-BADEA5, NELA BIBIRE6, MARCEL POPA2

1)Apitherapy Medical Center, Balanesti, Gorj, Romania
2)Department of Natural and Synthetic Polymers, “Gheorghe Asachi” Technical University, Iassy, Romania
3)”Vasile Goldiș” Western University, Arad, Romania
4)Department of Morpho-Functional Sciences, “Grigore T. Popa” University of Medicine and Pharmacy, Iassy, Romania
5)Faculty of Health and Behavioral Sciences, “Constantin Brâncuși” University, Targu Jiu, Romania
6)Department of Pharmaceutical Sciences I, “Grigore T. Popa” University of Medicine and Pharmacy, Iassy, Romania

Abstract
The present paper aimed to evaluate the influence of apitherapy diet in Wistar rats with carbon tetrachloride induced hepatotoxicity, by the means of biochemical determinations and histopathological changes of liver, spleen, pancreas and testicular tissue. The experiment was carried out on six groups of male Wistar rats. Hepatic lesions were induced by intraperitoneal injection of carbon tetrachloride (dissolved in paraffin oil, 10% solution), 2 mL per 100 g, every two days, for two weeks. Hepatoprotection was achieved with two-apitherapy diet formulations (containing honey, pollen, propolis, Apilarnil, with/without royal jelly), that have been administered for six up to nine weeks. The biochemical results revealed that the two-apitherapy diet formulations had a positive effect improving the enzymatic, lipid, and protein profiles, coagulation, mineral parameters and also the bilirubin levels, after six weeks of treatment. The histopathological results demonstrated the benefit of the two-apitherapy diet formulations on reducing the toxicity of liver, spleen and pancreas in laboratory animals, after six and nine weeks, respectively. In conclusion, apitherapy products have a hepatoprotective effect in carbon tetrachloride-induced hepatopathy.

Keywords: carbon tetrachloride, hepatotoxicity, apitherapy diet, hepatoprotection.

Introduction
Various pharmaceutical substances and chemicals may induce various levels of hepatopathy, from asymptomatic hepatic lesions to extensive liver necrosis. Carbon tetrachloride (CCl4) is a toxin that was used extensively to induce liver toxicity [1–7]. CCl4 administered to rats induces histologically proven severe hepatopathology, and an increase of serum concentrations of liver enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), that are indicators of liver tissue damage [8].

The biochemical mechanisms involved acquiring CCl4 hepatotoxicity are due to the peroxidation of lipids produced by trichloromethyl radical (CCl3). CCl3 is metabolized by P450 cytochrome to CCl3 radical that induces the peroxidation of membrane lipids and disturbs Ca2+ homeostasis thus inducing liver tissue damage [9–12].

Some studies proved that natural products containing antioxidants protect liver against peroxidation of lipids and depreciation of the antioxidant status induced by CCl4 [13, 14]. Also, the hepatoprotective effect of bee products administered individually was studied [15, 16].

The present experiment follows the same direction of research, by investigating the protective effects of two-apitherapy diet formulations containing a mixture of propolis, pollen, Apilarnil, and honey, with/without royal jelly (RJ), against CCl4-induced toxicity in Wistar rats. The novelty degree is brought by the correlation established between the pathological changes in enzymatic, lipid, protein profiles, the coagulation parameters, minerals, bilirubin, and the histopathological changes of liver, spleen, and pancreas, after six and nine weeks of treatment.

Materials and Methods
Animals and housing
The experiment was unfolded on 60 adult male rats, Wistar strain, having a body weight of 220–250 g. The animals were kept in a light and temperature-controlled room with 12 by 12 hours light–dark cycles, where the temperature (22±0.5°C) and relative humidity (65–70%) were maintained constant. The animals were given free access to standard laboratory diet and water before the experiments.

All the experimental proceedings achieved on laboratory animals (Wistar rats) in this study were in agreement with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and were in compliance with the European Council Directive of 24 November 1986 (No. 86/609/EEC).

Bee products
Honey, propolis, Apilarnil, and pollen were provided by Stupina LLC, Bâlănești, Gorj, Romania, while RJ was...
commercially acquired from the local market (lyophilized RJ produced by ICDA, Bucharest, Romania).

Study design

The experimental model of hepatic lesion was induced by intraperitoneal (i.p.) injection of CCl₄ (dissolved in paraffin oil, 10% solution), 2 mL per 100 g, every two days, for two weeks.

Treatment was achieved with two formulations of apitherapy diet:

- formulation I, consisted of honey, propolis, Apilarnil, and pollen;
- formulation II consisted of all ingredients from formulation I plus RJ.

The formulations were prepared daily. Formulation I was administered to groups II and V, in doses of 3.535 g/100 g bw/day, while formulation II was administered to groups III and VI, in doses of 3.635 g/100 g bw (Table 1).

**Table 1 – The daily intake of the two apitherapy diet formulations calculated for 100 g bw**

<table>
<thead>
<tr>
<th>No.</th>
<th>Bee products</th>
<th>Formulation I</th>
<th>Formulation II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Honey</td>
<td>2.5 g</td>
<td>2.5 g</td>
</tr>
<tr>
<td>2.</td>
<td>Propolis</td>
<td>0.01 g</td>
<td>0.01 g</td>
</tr>
<tr>
<td>3.</td>
<td>Apilarnil</td>
<td>0.025 g</td>
<td>0.025 g</td>
</tr>
<tr>
<td>4.</td>
<td>Pollen</td>
<td>1 g</td>
<td>1 g</td>
</tr>
<tr>
<td>5.</td>
<td>RJ</td>
<td>–</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Total amount</td>
<td>3.535 g</td>
<td>3.635 g</td>
<td></td>
</tr>
</tbody>
</table>

The Wistar rats were randomly divided into six groups of 10 animals each, as follows:

- Group I (control group on standard food) – served as control, and was fed with standard food;
- Group II (control group apitherapy diet) – fed with apitherapy diet (formulation I – 3.535 g/100 g bw/day, for six weeks);
- Group III (control group apitherapy diet + RJ) – fed with apitherapy diet and RJ (formulation II, 3.635 g/100 g bw/day, for six weeks);
- Group IV (CCl₄ group) – i.p. administration of 2 mL of 10% paraffin oil solution of CCl₄ per 100 g, every two days, for two weeks, and fed with standard food;
- Group V (group CCl₄ + apitherapy diet) – i.p. administration of 2 mL of 10% paraffin oil solution of CCl₄ per 100 g, once at two days, for two weeks, and fed with apitherapy diet (formulation I – 3.535 g/100 g bw/day, for six weeks);
- Group VI (group CCl₄ + apitherapy diet + RJ) – i.p. administration of 2 mL of 10% paraffin oil solution of CCl₄ per 100 g, once at two days, for two weeks, and fed with apitherapy diet and RJ (formulation II – 3.635 g/100 g bw/day, for six weeks).

The calculated amounts for each rat of the two-apitherapy diet formulations were added to a mixture of cereals (oat, barley, rye and wheat). The feeding was done individually for each rat (housed in separate individual cages) and took place twice a day: in the morning and at noon.

In the end, after nine weeks of apitherapy treatment, the animals were sacrificed.

Biochemical analysis

Anesthesia was achieved with Thiopental (dose of 1 mL/100 g using a 0.01% Thiopental solution), and blood samples were collected by cord puncture using a Vacuette® system and submitted to biochemical analysis.

The investigated parameters were:

- enzymatic parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT);
- lipid profile: total cholesterol (TC), triglycerides (TG), very low density lipoproteins (VLDL), high-density lipoproteins (HDL);
- protein profile: total proteins (TP), albumin (ALB), globulins (GLO), α₁-globulins (ALPHA-1), α₂-globulins (ALPHA-2), β-globulins (BETA), γ-globulins (GAMMA), and albumin/globulin ratio (A/B);
- coagulation parameters: Quick’s time (PT), thrombin time (TT), fibrinogen (F), International Normalized Ratio (INR);
- minerals: iron (Fe), potassium (K), serum and ionized calcium (sCa and iCa);
- bilirubin: total bilirubin (TB), direct bilirubin (DB), indirect bilirubin (IB).

The determination of the values of the investigated parameters was achieved with an automated analyzer (Aeroset, Abbott) and commercial kits (Abbott, USA).

Histopathological examination

When the absence of vital signs (respiratory rate, heart rate, reflexes) was ascertained, the animals were dissected in order to collect the samples of liver, spleen, pancreas and testicular tissue for evaluating the histopathological modifications.

The collected samples were fixed in 10% buffered formalin for at least 24 hours, progressively dehydrated in solutions containing an increasing percentage of ethanol (60, 80, 90, and 98%, v/v), clarified with Amylic Alcohol, embedded in paraffin under vacuum, sectioned at 5 μm thickness, deparaffinized, and stained with Hematoxylin–Eosin (HE).

The samples for pathological anatomy were collected after six and nine weeks.

Statistical analysis

The statistical interpretation of the results was performed with One-Way ANOVA test and Tukey’s post-hoc test. The results were given as mean ± standard deviation. The value of \( p < 0.05 \) was considered significant.

**Results**

Biochemical analysis

The results presented below in the tables contain the significance from the statistical point of view, defined by one of the letters (*a*), (*b*), (*c*), (*d*) or (*e*), meaning:

* \( p < 0.05 \) vs. group I – the value is statistically significant in comparison with group I (control group standard food);
* \( p < 0.0001 \) vs. group II – the value is statistically significant in comparison with group II (control group apitherapy diet);
* \( p < 0.0001 \) vs. group III – the value is statistically significant in comparison with group III.
Effect of apitherapy products against carbon tetrachloride-induced toxicity in Wistar rats

(control group apitherapy diet + RJ); *d, p<0.0001 vs. group IV – the value is statistically significant in comparison with group IV (CCl4 group); *e, p<0.0001 vs. group V – the value is statistically significant in comparison with group V (group CCl4 + apitherapy diet).

Table 2 – Results regarding the enzymatic profile

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>153.85±31.33</td>
<td>67.54±27.25</td>
<td>0.6±0.05</td>
<td>108±21.25</td>
</tr>
<tr>
<td>II</td>
<td>68.71±29.62</td>
<td>42.17±6.73</td>
<td>0.52±0.07</td>
<td>101.7±15.81</td>
</tr>
<tr>
<td>III</td>
<td>51.71±8.48</td>
<td>35.07±4.85</td>
<td>0.47±0.04</td>
<td>98.65±16.47</td>
</tr>
<tr>
<td>IV</td>
<td>318.3±48.19</td>
<td>100.9±32.1</td>
<td>1.22±0.23</td>
<td>156.1±24.9</td>
</tr>
<tr>
<td>V</td>
<td>44.7±7.65</td>
<td>43.03±7.65</td>
<td>0.43±0.17</td>
<td>107.6±12.03</td>
</tr>
<tr>
<td>VI</td>
<td>39.7±3.43</td>
<td>36.2±5.4</td>
<td>0.16±0.1</td>
<td>100.1±13.69</td>
</tr>
</tbody>
</table>

Table 3 – Results regarding the lipid profile

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC [mg/dL]</th>
<th>VLDL [mg/dL]</th>
<th>TG [mg/dL]</th>
<th>HDL [mg/dL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>72.2±14.44</td>
<td>14.2±6.94</td>
<td>47±20.72</td>
<td>50.0±7.82</td>
</tr>
<tr>
<td>II</td>
<td>62.5±7.45</td>
<td>18.8±7.64</td>
<td>82.5±16.64</td>
<td>52.5±7.61</td>
</tr>
<tr>
<td>III</td>
<td>55.4±9.16</td>
<td>23.8±3.07</td>
<td>114±20.35</td>
<td>53.4±6.97</td>
</tr>
<tr>
<td>IV</td>
<td>125.9±27</td>
<td>69.2±12.64</td>
<td>336.5±55.68</td>
<td>40.5±8.54</td>
</tr>
<tr>
<td>V</td>
<td>58.8±9.1</td>
<td>12.9±4.05</td>
<td>64.7±19.69</td>
<td>53.6±7.78</td>
</tr>
<tr>
<td>VI</td>
<td>60.5±4.9</td>
<td>22.7±5.1</td>
<td>111±24.66</td>
<td>63.9±3.96</td>
</tr>
</tbody>
</table>

Table 4 – Results regarding the protein profile

<table>
<thead>
<tr>
<th>Groups</th>
<th>TP [g/dL]</th>
<th>ALB [%]</th>
<th>GLO [%]</th>
<th>ALPHA-1 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5.98±0.45</td>
<td>29.6±0.79</td>
<td>70.4±0.79</td>
<td>28.9±0.55</td>
</tr>
<tr>
<td>II</td>
<td>7.16±0.5</td>
<td>40.8±2.4</td>
<td>59.2±2.15</td>
<td>24.5±1.91</td>
</tr>
<tr>
<td>III</td>
<td>7.47±0.62</td>
<td>39.9±1.42</td>
<td>60.1±1.21</td>
<td>25.8±1.31</td>
</tr>
<tr>
<td>IV</td>
<td>4.7±0.32</td>
<td>1.6±0.11</td>
<td>98.3±0.8</td>
<td>29.4±0.53</td>
</tr>
<tr>
<td>V</td>
<td>7.32±0.47</td>
<td>41.1±1.55</td>
<td>58.8±1.55</td>
<td>25.8±9.59</td>
</tr>
<tr>
<td>VI</td>
<td>7.2±0.3</td>
<td>39.2±1.61</td>
<td>60.7±1.63</td>
<td>25.36±1.88</td>
</tr>
</tbody>
</table>

Table 5 – Results regarding the coagulation parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>PT [s]</th>
<th>TT [s]</th>
<th>F [mg/dL]</th>
<th>INR</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>18.2±1.02</td>
<td>21.78±0.65</td>
<td>322.2±8.59</td>
<td>1.57±0.09</td>
</tr>
<tr>
<td>II</td>
<td>16.8±0.38</td>
<td>18.67±1.34</td>
<td>214±8.83</td>
<td>1.37±0.02</td>
</tr>
<tr>
<td>III</td>
<td>15.6±0.39</td>
<td>19.9±1.2</td>
<td>208.1±5.13</td>
<td>1.29±0.03</td>
</tr>
<tr>
<td>IV</td>
<td>27.4±1.75</td>
<td>30.9±3.82</td>
<td>408.6±42.9</td>
<td>2.21±0.24</td>
</tr>
<tr>
<td>V</td>
<td>17.1±0.88</td>
<td>19.41±1.51</td>
<td>233.9±37.62</td>
<td>1.42±0.09</td>
</tr>
<tr>
<td>VI</td>
<td>16.0±0.5</td>
<td>18.8±1.47</td>
<td>213.1±8.45</td>
<td>1.32±0.04</td>
</tr>
</tbody>
</table>

Table 6 – Results regarding the minerals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fe [µg/dL]</th>
<th>K [mmol/L]</th>
<th>iCa [mg/dL]</th>
<th>sCa [mg/dL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>56.4±5.34</td>
<td>5.18±0.26</td>
<td>4.18±0.13</td>
<td>8.79±0.9</td>
</tr>
<tr>
<td>II</td>
<td>69.5±9.1</td>
<td>5.3±0.3</td>
<td>4.4±0.2</td>
<td>9.8±0.5</td>
</tr>
<tr>
<td>III</td>
<td>93.8±7.73</td>
<td>5.3±0.21</td>
<td>4.5±0.16</td>
<td>10.5±0.39</td>
</tr>
<tr>
<td>IV</td>
<td>269.6±79.62</td>
<td>6.27±0.29</td>
<td>3.38±0.49</td>
<td>7.27±0.4</td>
</tr>
<tr>
<td>V</td>
<td>131.4±0.56</td>
<td>5.49±0.48</td>
<td>4.4±0.21</td>
<td>9.9±0.67</td>
</tr>
<tr>
<td>VI</td>
<td>128.5±30.73</td>
<td>5.41±0.21</td>
<td>4.54±0.17</td>
<td>10.4±0.51</td>
</tr>
</tbody>
</table>
Histopathological examination

Liver tissue

In six weeks of treating the lab rats with CCl4 to induce hepatopathy, it was noticed: portal zone with dilated vessels, slight sclerosis, slight chronic inflammatory infiltrate, biliary neocanaliculi, centrolobular stasis, hyperplasia or Kupffer cells (Figure 1). In nine weeks, there was severe lipid dystrophy, microvesicular and macrovesicular, inflammatory infiltrate into portal zones with intralobular intrusions and chronic inflammatory infiltrate into portal zones with slight sclerosis and incipient intralobular intrusions, congestion, isolate lysis of the liver cells, polypoid nuclei (Figure 2) that indicates the advancement of liver destruction, accentuated in time, and tissue architecture destruction, after CCl4 intoxication. Instead, when treating the lab animals with CCl4 concomitantly with apidiet, there was normal aspect (Figure 5), but after nine weeks, there was vascular congestion and after nine weeks, there was vascular congestion, isolated lipid infiltration and beginning of cells lysis in the pancreatic acinus (Figure 6).

Renal tissue

In the present study, the renal tissue showed hyaline cylinders, interstitial infiltrate, medullar and glomerular congestion in lab animals with CCl4-induced hepatopathy for six weeks (Figure 7). In nine weeks, there was interstitial and glomerular congestion, granular dystrophy of contort tubes, peritubular congestion with granular dystrophy, peritubular congestion with damage to the brush border, granulovacuolar dystrophy of contort tubes, inflammatory infiltrate (Figure 8). However, upon treating the lab animals with apidiet with/without RJ there were minor renal lesions after six or nine weeks (Figures 7 and 8).

Testicular tissue

During this experiment, there were no testicular changes noticed at the animals with CCl4-induced toxic damage (Figure 9). Upon treating the animals with apidiet after six weeks, there was normal testis (Figure 9), and after nine weeks, there was noticed normal spermatogenesis, normal seminiferous tubes, and normal architecture (Figure 10). Instead, upon treating the animals with CCl4 concomitantly with apidiet, there was normal testis after six weeks (Figure 9), but after nine weeks, interrupted germinal line was noticed (Figure 10).

Table 7 – Results regarding the values of bilirubin

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB [mg/dL]</td>
</tr>
<tr>
<td>I</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>II</td>
<td>0.12±0.005</td>
</tr>
<tr>
<td>III</td>
<td>0.085±0.005*</td>
</tr>
<tr>
<td>IV</td>
<td>0.158±0.01***</td>
</tr>
<tr>
<td>V</td>
<td>0.11±0.01**</td>
</tr>
<tr>
<td>VI</td>
<td>0.09±0.008**</td>
</tr>
</tbody>
</table>

Figure 1 – Histopathological examination of liver tissue after six weeks of treatment (HE staining). CCl4 group: (A) Portal zone with dilated vessels (×400); (B) Stasis, slight sclerosis (×100); (C) Slight chronic infiltrate, detail (×200).
Effect of apitherapy products against carbon tetrachloride-induced toxicity in Wistar rats

Figure 1 (continued) – Histopathological examination of liver tissue after six weeks of treatment (HE staining). CCl₄ group: (D) Biliary neocanaliculi (×400); (E) Middle lobular stasis (×200); (F) Kupffer hyperplasia (×400). Api group: (G) Microvesicular steatosis (×400); (H) Portal zone with inflammatory infiltrate (×400); (I) Centrolobular stasis (×400). Api + RJ group: (J) Infiltrate, necrosis, apoptotic cells (×200); (K) Slight microvacuolar dystrophy (×400); (L) Centrolobular steatosis (×400).

Figure 2 – Histological examination of liver tissue after nine weeks of treatment (HE staining). CCl₄ group: (A) Lipid micro- and macrovacuolar dystrophy (×400); (B) Inflammatory infiltrate (×400); (C) Portal zone fibrosis, pseudolobule (×100); (D) Neocanaliculi (×400); (E) Hepatocyte lysis (×100); (F) Dystrophy and polyploidy (×400); (G) Congestion (×100); (H) Lipid dystrophy, apoptosis (×200); (I) Nodule (×200).
Figure 2 (continued) – Histological examination of liver tissue after nine weeks of treatment (HE staining). Api group: (J) Portal zone with inflammatory infiltrate (×200); (K) Inflammatory infiltrate, slight sclerosis (×100); (L) Kupffer hyperplasia (×40); (M) Portal zone infiltrate. Kupffer hyperplasia (×400); (N) Normal hepatocytes (×400); (O) Normal hepatocytes (×400). Api + RJ group: (P) Portal zone infiltrate (×400); (Q) Congestion (×400); (R) Dystrophy, congestion, infiltrate (×400).

Figure 3 – Histological evaluation of spleen tissue after six weeks of treatment (HE staining). CCl₄ group: (A) Dilated capillaries and red pulp (×100); (B) Normal, but dilated capillaries (×100). Api group: (C) Red and white pulp. Normal aspect (×100); (D) White pulp. Normal aspect (×200); (E) Normal aspect (×100). Api + RJ group: (F) Stasis in red pulp (×100); (G) Stasis in red pulp (×200).
Figure 4 – Histological evaluation of spleen tissue after nine weeks of treatment (HE staining). CCl₄ group: (A) Red pulp hypertrophy, hemosiderin deposits (×100); (B) Red pulp stasis (×200); (C) Red pulp stasis (×400). Api group: (D) Congestion (×200); (E) Perivascular pigment (×400); (F) Pigment (×400). Api + RJ group: (G) Normal aspect (×100); (H) Normal aspect (×100); (I) Congestion (×200); (J) Hyperplasia of Malpighi corpuscles (×100); (K) Pigment in macrophages (×100); (L) Pigment in macrophages (×400).

Figure 5 – Histological evaluation of pancreas tissue after six weeks of treatment (HE staining). CCl₄ group: (A) Insular hypertrophy (×100); (B) Insular hypertrophy, detail (×200); (C) Detail (×400). Api group: (D) Normal aspect (×100); (E) Normal aspect (×200); (F) Islets of Langerhans (×400).
Figure 5 (continued) – Histological evaluation of pancreas tissue after six weeks of treatment (HE staining). Api + RJ group: (G) Normal aspect (×100); (H) Normal aspect (×200).

Figure 6 – Histological evaluation of pancreatic tissue after nine weeks of treatment (HE staining). CCl4 group: (A) Slight congestion, detail (×400); (B) Lipid infiltrate (×200); (C) Necrosis (×200). Api group: (D) Vascular congestion (×200); (E) Normal aspect (×200); (F) Normal aspect (×400). Api + RJ group: (G) Vascular congestion (×100); (H) Isolated lipid infiltration (×100); (I) Beginning of acinar lysis, detail (×1000).

Figure 7 – Histological evaluation of renal tissue after six weeks of treatment (HE staining). CCl4 group: (A) Hyaline cylinders, detail (×400); (B) Perivascular inflammatory infiltrate (×200); (C) Interstitial inflammatory infiltrate (×200); (D) Glomerular congestion, inflammatory infiltrate (×400); (E) Glomerular congestion, detail (×400); (F) Congestion and inflammatory infiltrate (×100).
Figure 7 (continued) – Histological evaluation of renal tissue after six weeks of treatment (HE staining). Api group: (G) Glomerular congestion (×100); (H) Glomerular congestion, detail (×400); (I) Glomerular congestion, detail (×100). Api + RJ group: (J) Glomerular congestion (×100); (K) Glomerular congestion (×400).

Figure 8 – Histological evaluation of renal tissue after nine weeks of treatment (HE staining). CCl₄ group: (A) Glomerular congestion (×100); (B) Interstitial congestion (×100); (C) Vascular congestion (×100); (D) Glomerular congestion (×200); (E) Peritubular congestion (×1000); (F) Glomerular dystrophy (×1000). Api group: (G) Renal congestion (×400); (H) Glomerular congestion and mild vascular dystrophy (×400). Api + RJ group: (I) Vascular and glomerular congestion (×400); (J) Tubular dystrophy (×400); (K) Glomeruli inflammation (×400).


Discussion

Enzymatic profile

It was noticed that treating rats with CCl₄ produced significant histological changes that indicated liver damage such as: necrosis [17–19], fibrosis [17, 20–22], mononuclear cell infiltration [22, 23], steatosis and foamy degeneration of hepatocytes, increased mitotic activity [8] and liver cirrhosis [23, 24]. Also, it had been reported that CCl₄ causes liver apoptosis [25–27].

In the study groups, it was observed that the animals with CCl₄-induced hepatopathy showed a severe increase in liver enzyme profile for AST, GGT, ALT and FA that was to be expected; the increase of hepatocytolysis enzymes reflected precisely the liver lesions, a fact proven by various previous studies [8, 15, 19, 27–33]. However, using apidiet and apidiet plus RJ, on lab animals with CCl₄-induced hepatopathy determined a decrease of the liver enzymes to values that were comparable to the reference ones. Besides, it had been mentioned that RJ feeding alone stopped notably the CCl₄-induced liver damage, fact proved by the decrease of the activity of AST and ALT in serum [29]. The treatment with propolis or caffeic acid phenethyl ester (CAPE), a compound of propolis, reduced significantly the increased levels of AST, ALT, FA, total and conjugated bilirubin. Furthermore, it was established that the histopathology changes observed after CCl₄ treatment had disappeared [34, 35]. Histopathology evaluations showed that CCl₄-induced hepatopathy was diminished after RJ treatment [29].

Lipid profile

According to a previous study, lipid peroxidation was deemed the most important mechanism in CCl₄-induced liver damage pathogenicity [36–38]. Significant increase of lipid profile indicated a severe lipid peroxidation and...
physiological damage to organs after administering CCl₄ [39].

Regarding the studied groups, of the present study, an increase of the parameters of the lipid profile was observed in the animals with CCl₄-induced hepatopathy, concerning total cholesterol, triglycerides, VLDL and also lowering HDL; administering apidiet with or without RJ determined a regulation of these parameters. Increased HDL was noticed at lab animals with CCl₄-induced hepatopathy on apidiet and on apidiet + RJ. The highest HDL was observed in group VI that was on apidiet + RJ, that was significantly higher than in any other study group. That led to the fact that RJ intervened in the regulation of that lipid fraction, by increasing it up to the point it became a cardiac protector, which is important in liver and cardiovascular diseases, obesity and diabetes. Besides, it had been previously proven that hepatic lipid peroxidation induced by CCl₄ was prevented by CAPE [34]. Furthermore, antioxidant activity and/or inhibition of free radicals production were important in regards to the protection of liver against CCl₄-induced damage [38, 40]. It had been proven that CAPE had strong protective effects against liver damage induced by CCl₄ in rats [19].

Protein profile

It was noticed total protein decrease, albumin decrease, albumin/globulin ratio decrease and globulin increase in rats with CCl₄-induced hepatopathy that lead to severe liver damage correlated with tissue histarchitecture. By treating with apidiet and apidiet + RJ the lab animals with CCl₄-induced hepatopathy and comparing against the group with CCl₄-induced hepatopathy on standard diet it was noticed total protein and albumin increase, globulin decrease, that proved the recovery of liver synthesis function, because it is well known that albumin increase represented a crucial factor in restoring liver function. Besides, one of the main targets of the treatment of liver damage is the rise of albumin levels (that are generally low once the damage of liver tissue progresses).

That led to the fact that RJ intervened in the regulation of that lipid fraction, by increasing it up to the point it became a cardiac protector, which is important in liver and cardiovascular diseases, obesity and diabetes. Besides, it had been previously proven that hepatic lipid peroxidation induced by CCl₄ was prevented by CAPE [34]. Furthermore, antioxidant activity and/or inhibition of free radicals production were important in regards to the protection of liver against CCl₄-induced damage [38, 40]. It had been proven that CAPE had strong protective effects against liver damage induced by CCl₄ in rats [19].

Protein profile

Coagulation

The co-administration of CCl₄ with apidiet and apidiet + RJ determined the return to normal levels of the parameters F, TQ and TT up to value comparable to those of the animals without hepatopathy.

Minerals

Treating the lab animals with CCl₄-induced hepatopathy with apidiet and apidiet + RJ determined: low Fe concentrations, high levels of ionic and serum calcium. It was supposed that propolis aided the absorption and use of various minerals through its various organic acids derivatives that improved physiologic functions by regulating enzyme dependent ionic activities. The beneficial effects of pollen and/or propolis on the metabolism of iron, calcium, phosphorus and magnesium had been already been proven [41, 42]. Numerous plasmatic proteins, including albumin, alpha and β-globulins, coagulation factors and transport proteins, are synthesized by liver.

These factors influence homeostasis (for example by binding proteins they modulate the total concentration of circulating Ca²⁺ and Mg²⁺ and that of numerous drugs), white serum albumin concentration regulates the colloid-osmotic pressure of plasma thus influencing the dynamic of fluids in between blood and tissues [43]. Honey is well-known for stimulating calcium absorption in vitro and in vivo for lab rats. There had been reported a positive effect of honey on patients with hepatopathy [44].

Bilirubin

Increased bilirubin synthesis or its defective hepatic metabolism (because of defective uptake, conjugation and excretion in the bile) determines hyperbilirubinemia [45, 46], that is frequent in liver damage. This was found to be also true in this study for the group treated with CCl₄. However, treating the lab animals with CCl₄-induced hepatopathy with apidiet with/without RJ tends to revert total, direct and indirect bilirubin to normal levels. Bilirubin, hydrophobic and potentially toxic substance, circulates in plasma bound to albumin, this is why it cannot be filtrated and eliminated renal. For its elimination, it is necessary to convert it to hydro-soluble conjugates in bile [47]. That led to the conclusion that apitherapy intervened into that exact mechanism.

Histopathological aspect

The favorable results with respect to the liver tissue shown in our study, could be possible due to the accelerate regeneration of parenchymatous cells because of various bioactive compound, such as flavonoids and their esters that are present in propolis and that help prevent membrane fragility, later on decreasing the release of marker enzymes into circulation. Biochemical results combined to the histopathological ones showed an improvement of liver histarchitecture regarding treated groups of lab rats, fact confirmed by previous studies for propolis [15]. Furthermore, there had been studies to demonstrate the involvement of retinoic acid present in bee products in the evolution of liver fibrosis induced by CCl₄ in rats [48]. Retinoic acid reduced the proliferation of stellate liver cells and collagen synthesis in experiments on cell cultures [49, 50]. Retinoic acid proved to regulate collagen production by decreasing it. It reduces the synthesis of collagen I in stellate liver cells activated by suppressing α2(1)collagen [51, 52]. This suppression is mediated by specific nuclear receptors RARβ and RXRα, and by their interactions with their coregulators [52, 53]. Besides, retinoic acid had been shown to have a protective effect against damage induced by oxidative stress on cellular level [54, 55].

The alleviation of the histopathological aspect was also observed in spleen, renal and pancreatic tissue, results that are in agreement with literature data [56]. CAPE is a major component of propolis, claimed to be responsible of protecting renal tubes against damage and increased urea in blood [57, 58].

The examination of the testicular tissue had been frequently neglected by previous studies when studying the toxic effects of drugs or other chemicals. Even though there were no testicular changes noticed at the animals from group IV, our results indicate that long-term intake of
RJ affected the testicular function by inhibiting spermatogenesis, that is a minor thing regarding the use of this product for male patients.

Conclusions
Apitherapy products interfered positively upon the enzymatic, lipid and protein profiles, improved the levels of coagulation parameters, minerals, bilirubin, and also had a benefic effect on the hooarchitectue of liver, spleen, pancreas after six and nine weeks of treatment. Apitherapy products had a hepatoprotective effect in CCl₄-induced hepatoapathy.

Acknowledgments
The authors are grateful to Professor Gioconda Dobrescu, MD, PhD from “Grigore T. Popa” University of Medicine and Pharmacy, Iassy, Romania, for interpreting the micrographs.

References

Borek C, Antioxidant health effects of aged garlic extract, J Nutr, 2011, 131(3s):1015S–1017S.


Corresponding author
Nela Bibire, Associate Professor, PhD, Discipline of Analytical Chemistry, Department of Pharmaceutical Sciences I, Faculty of Pharmacy, “Grigore T. Popa” University of Medicine and Pharmacy, 16 Universității Street, 700115 Iassy, Romania; Phone +40740–236 507, e-mail: nelabibire@yahoo.com

Received: March 3, 2014
Accepted: August 27, 2014