Author's response to reviews

Title: Alternative preparation of propolis extracts: comparison of their composition and biological activities

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Author's response to reviews: see over
We thank the Reviewers for comments in the microbiological and technological area of our study.

We tried to take into consideration all comments and advice, which are written not only in the points, but also in the additional text.

I. Microbiology

1. An adequate control system is essentially required to demonstrate the genuine antimicrobial activities of the propolis, not PEG 400 or olive oil, especially from diffusion method using well or pitting well as reservoir for tested materials.

2. The details used in diffusion method should be stated and/or the standard methods (i.e. of WHO, FDA, ISO, CLSI etc...) or chapter(s) from text(s) should be cited.

1. Firstly, we have made an additional series of experiments with discs and wells (Fig 1). Results of our experiments with PEG 400 in most cases repeat results obtained in reviewer’s laboratory.

- 1. Ampicillin 10 mcg, 50 µl
- 2. 50 µl PEG400 – disc
- 3. 50 µl PEG400 - well
However, we want to emphasize, that in our experiments for extracts production we have been used solvents containing only 20 % PEG 400.

Carrying out the antimicrobial activity tests, we always check and solvents antimicrobial effect. Our experiments suggests, that solvents do not have anti-microbial effects. As evidence we attached a few pictures (Fig.2).
Fig. 2. Antibacterial effect of differently prepared propolis extracts and solvents against *S. aureus* and *K. pneumoniae*

1 - W₂, 2 - W₁, 3 - A₂, 4 - A₁, 5 – EEP, 6 - W₂ solvent, 7 - A₂ solvent

2. We basically changed description of the microbiological research methodology in the manuscript. We described in detail the microbial cells, medium and Petri dish preparation:

The evaluation of the antimicrobial activity of the investigated solutions was performed using the agar-well diffusion assay. Bacterial cultures were grown for 18 hours at the temperature of 37°C on slant agar (BBL, Cockeysville, USA). The grown cultures were washed off the agar using sterile saline solution and the cell suspensions were adjusted according to McFarland No. 0.5 standard (CLSI, 2007: Clinical and Laboratory Standards Institute: *Performance Standards for Antimicrobial Susceptibility Testing, Seventeenth Informational Supplement M100-S17*. Wayne, PA, USA). The yeast fungus culture grew for 48 hours at the temperature of 25°C on slant Sabouraud Dextrose Agar (BBL, Becton Dickinson and Company). The grown cultures were washed off the agar using sterile saline solution. The cell suspension prepared according to McFarland standard No. 5.
1 ml of the cell suspension was introduced into dissolved and cooled to 45°C agarized medium and thoroughly stirred. The prepared mixture of the suspension of microbial cells and the medium was poured into 9 cm-diameter glass Petri dishes (30 ml of the suspension per each dish). After the medium hardened, 7 wells (8 mm in diameter) were made in it, and 0.12 ml of examined solutions were poured into the wells.

The antimicrobial effect of investigated solutions on bacterial culture was evaluated after 24 hours, and its effect on yeast fungus – after 24-48 hours after cultivation. The evaluation was based on the diameter (in mm) of clear zones formed around the wells. If no clear zones were formed around the wells, we concluded that the investigated solution had no antimicrobial effect on the tested culture. Ampicillin was used as positive control, and A₁, A₂, W₁, W₂ solvents as negative control. Number of experiments – 4 - 6.

3. In the results section we added photos and new research results.

II Technology

1. Abstract, Materials and solvent for chemical analysis and discussion should be shorten. – corrected (see article).
2. In abstract, authors should add the name of method for determination of total phenolic compound. – “Total content of phenolic compounds in extracts was determined using Folin-Ciocalteu method”.
3. Authors have to give more information in Discussion section: Why choose 70°C as extracting temperature? How the author compare the results due to the extraction time of each solvent is not the same. From Table 1, where is the ratio of solvent used come from?

One of the most important indicators determining the quality of propolis solutions is the concentration of phenolic compounds. For this reason, we evaluated the
effectiveness of extraction by quantity of phenolic compounds. The aim of the study was to discover non-ethanolic indifferent solvent capable of dissolving raw propolis, to optimize the sequence of extraction and technological conditions to obtain optimal concentration of phenolic compounds.

Experiments were performed in several stages. We answered the following questions during the experiments:

1. Which solvent is the most efficient for extraction of phenolic compounds?
2. What technological conditions are needed for the highest extraction of phenolic compounds?

Considering diverse technological modifications used in the experiments, we are presenting only the most relevant data.

**Results**

We searched for indifferent solvent allowing better extraction of phenolic compounds. Experiments were made using:

- Different solvents;
- modes of temperature for extraction (room temperature, 70°C and 100°C) to determined influence between thermal mode extraction of phenolic compounds.
- different time (room temperature for 5 hours (mode A) and in 70°C for 15 minutes (mode B).

As a solvent we chose: aqueous, aqueous with polyethylene glycol 400 (PEG), oil, aqueous with PEG and oil as solvents. For comparison we analyzed ethanolic (70 %) extract of propolis.
This solvent, we have chosen to formulate parenteral drug forms (e.g. eye medicines). In parenteral drug forms - maximal concentration of PEG is 30\% (1). These pharmaceutical form must be sterile (sterile filtration through a 0.22 micrometer pore membrane). Higher PEG and oil concentrations cause significantly more difficult filtration conditions. On the other hand, PEG lower concentration extracted almost similar phenolic compounds as with ethanolic solvent.

Technological conditions: raw propolis 10\%, one of the solvents, extraction performed in room temperature for 5 hours (mode A) and in 70°C for 15 minutes (mode B). Mode A we choose because, the ethanol solutions (extracts, tinctures) in industry are always made at room temperature (2). Propolis ethanol solutions for research are also produced at room temperature (3). To compare the ethanol extract with non-ethanolic, they was made at the same conditions.

(Note. The results of extraction in 100°C are not given because there were no significant differences in concentration of phenolic compounds, compared to 70°C mode).

Solutions were clear, yellow, viscous (with macrogol and oil) liquids. Solutions remain stable when stored, i.e. the colour remains unchanged, no precipitate is observed and they do not turn white.

In the first stage of the experiment we have identified that there is a significant difference in amount of phenolic compounds extracted from raw propolis using different solvents. The concentration of phenolic compounds in obtained solutions ranged from 0.54 mg/ml to 10.74 mg/ml GAE (p<0.05). The highest concentration of phenolic compounds was achieved by extracting propolis with aqueous PEG solution and aqueous PEG, oil solution.
We have also determined a direct influence between thermal mode extraction of phenolic compounds: the amount of phenolic compounds increases significantly (p<0.05) using different thermal modes. This study proves that thermal mode should be used in the production of propolis solutions, since it directly influences the amount of phenolic compounds (table 1).

To determine the optimal time-span for extraction, we evaluated the concentration of phenolic compounds (eg. extract W1: after 2.5 h – 1.49 mg/ml GAE, after 5 h – 1.59 mg/ml GAE, after 24 h – 1.87 mg/ml GAE). The same tendency have been by analyzing thermal made extracts. We have found one noteworthy trend during this experiment: the concentration of phenolic compounds in propolis solutions obtained in thermal mode did not change later during extraction. This can be explained by changes in propolis structure occuring during thermal extraction (rolls are formed) and extraction of phenolic compounds is taking place only during thermal process. To sum up, we have determined that there were no significant changes in amount of phenolic compounds during time spans.

**Conclusion**

1. If raw propolis material is extracted with different solvents, the amount of phenolic compounds changes significantly (p<0.05). The highest concentration of phenolic compounds was achieved by extracting raw propolis material with aqueous PEG solutions and aqueous PEG, oil solution.

2. Thermal extraction mode directly influences the extraction of phenolic compounds: extracting with the same solution at higher temperatures causes the increase of concentration of phenolic compounds (p<0.05).
3. There are no significant changes in concentration of phenolic compounds during time-spans of extraction.

**Table 1.** The influence of technological processes on quantitative and qualitative characteristics of propolis aqueous solutions

<table>
<thead>
<tr>
<th>Solvent</th>
<th>W1</th>
<th>W2</th>
<th>A1</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration of phenolic compounds mg/ml GAE (room temperature for 5 hours) - mode A</strong></td>
<td>1.59±0.35</td>
<td>6.68 ± 0.67</td>
<td>0.54±0.22</td>
<td>6.0 ± 0.54</td>
</tr>
<tr>
<td><strong>Concentration of phenolic compounds mg/ml GAE (70°C for 15 minutes) - mode B</strong></td>
<td>3.10±0.42*</td>
<td>10.74±1.21*</td>
<td>2.60±0.31*</td>
<td>9.53±1.28*</td>
</tr>
</tbody>
</table>

*\(p<0.05\) versus mode A

<table>
<thead>
<tr>
<th>Sample</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>Propolis 10 g; Water ad 100 ml</td>
</tr>
<tr>
<td>W2</td>
<td>Propolis 10 g; PEG 20g; Water ad 100 ml</td>
</tr>
<tr>
<td>A1</td>
<td>Propolis 10 g; Olive oil ad 100 ml;</td>
</tr>
<tr>
<td>A2</td>
<td>Propolis 10 g; PEG 20 g; Olive oil 50 g; Water ad 100 ml</td>
</tr>
</tbody>
</table>
References:

