Original Research Article

Comparative Evaluation of Physical and Phenolic Profiles in Propolis from N. Macedonian Regions

ABSTRACT

Aims/objective:Propolis, a resinous substance collected by bees from plants, is an exceptionally complex mixture whose quality is influenced by the surrounding flora, geography, and climate. It has been used medicinally for centuries and is currently utilized in natural foods, pharmaceuticals, and cosmetics. The aim of this study was to measure the physical parameters and total phenolic content of ethanolic extracts of propolis (EEP). The study also involved a step-by-step approach to ensure maximum extraction efficiency.
Study design: Comparative experimental study.
Place and Duration of Study: Between April and July 2024, suitable ethanolic propolis extracts (4% and 10%) were prepared from raw propolis collected from two different regions (Pelagonia and Polog).
Methodology: Physical parameters such as solubility, conductivity and yield were analyzed in these extracts. Two subsequent extractions, each with two replicates, were performed on the same samples to obtain initial insights into achieving the highest yield from a given amount of propolis. The total phenolic content in the EEP was determined using the Folin-Ciocalteu colorimetric method, with results expressed as mg GAE/g.
Results: Conductivity, yield, and solubility measurements varied across Pelagonia (42 ppm, 17.42%, high solubility) and Polog (33 ppm, 15.38%, lower solubility) propolis extracts, reflecting diverse chemical profiles influenced by geographical factors. Regional analysis of propolis from Pelagonia and Polog regions revealed significant differences in total phenolic content, with Pelagonia extracts showing higher values (189 mg GAE/g) compared to Polog (172 mg GAE/g). Statistical tests confirmed these differences as significant ($p < 0.05$), underscoring the impact of geographic origin on propolis composition.
Conclusions: Variations in bioactive content in propolis are influenced by geographical origin, botanical sources, and extraction methods. Standardized extraction protocols and further research are crucial for optimizing its medicinal potential and utilizing insoluble propolis solids.

Keywords: Propolis extracts, two-stage extraction, physical parameters,phenolic compounds, N. Macedonia.

1. INTRODUCTION

Propolis, known as "bee glue," is collected by honeybees (Apis mellifera) from diverse plant sources and utilized within beehives for structural and protective purposes (Bankova et al., 2000). Recognized for its therapeutic potential, propolis finds applications in pharmaceuticals, food products, and cosmetics due to its varied chemical composition influenced by factors like geographical origin and botanical sources (Bankova et al., 2014; Oršolić et al., 2020). Despite extensive research on its benefits, optimizing extraction methods remains critical to enhance yield and standardize quality for diverse applications (Daugsch et al., 2008; Khalil et al., 2019).

The variability in propolis composition underscores the need for efficient extraction techniques that maximize bioactive compound retention. Physical parameters such as yield, dry matter content, pH, and conductivity are crucial indicators of extract quality, influenced by both extraction methods and propolis' geographic origin (Bankova et al., 2000; Bankova et al., 2014). These factors significantly impact the bioactivity and potential health benefits of propolis extracts, emphasizing the importance of tailored extraction strategies to meet specific application needs (Marcucci, 1995; Khalil et al., 2019).

Total phenolic content, a critical bioactive component of propolis, plays a significant role in its antioxidant and antimicrobial properties (Bankova et al., 2014; Kasiotis et al., 2017). Quantifying total phenolics in propolis extracts provides essential insights into their medicinal potential and suitability for pharmaceutical and natural health applications (Singleton and Rossi, 1965; Kumazawa and Ahn, 2019).

This study addresses the need for comprehensive research on propolis extraction methods, focusing on mountainous regions with diverse flora. It aims to empirically assess physical parameters and total phenolic content, crucial for understanding how geographical origin influences propolis bioactivity. By enhancing our understanding of extraction techniques and their impact on propolis quality, this research seeks to contribute to standardized protocols beneficial to the pharmaceutical and natural health product industries.

2. MATERIAL AND METHODS

2.1. Analysis of raw propolis extraction methods - laboratory tests

Table 1 provides a comprehensive overview of the methodology used for the preparation of propolis extracts over a 24-hour period, including the duration of each step.

Table 1. Methodology for Preparation of Propolis Extracts (24 hours), (Kolovski, 2023)

Order	Procedure Description	Duration (h)
1	Weighing the required quantity and micronization	0.33
2	Blending with ethanol in a blender	0.17
3	Magnetic stirring with heating (multiple cycles)	6.50
4	Cooling and dynamic dissolution (multiple cycles)	15.00
5	Vacuum filtration (using paper macro filters)	1.00
6	Measurement of dry residue and physical parameters	1.00
	Total duration	24.00

2.1.1. Propolis Dissolution Procedure / Dissolution Method

2.1.1.1. Freezing

After weighing 4 g of raw propolis sample, the propolis was frozen at -20 °C to convert crystalline water into ice, inducing expansion and forming micro-cracks within its structure. This method greatly facilitates the subsequent grinding process, resulting in an optimal texture that enhances propolis dissolution in various solvents.

2.1.1.2. Grinding / Micronization

A high-powered blender equipped with multiple speeds and a micronization attachment was employed to process and initiate dissolution of the propolis. The blender operated at different blade rotation speeds, ensuring thorough micronization of the propolis particles.

2.1.1.3. Mixing the Micronized Material

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The micronized propolis was blended with ethanol in a high-powered blender, facilitating rapid dissolution of all soluble components and ensuring thorough wetting and separation from the blender body.

For further dissolution of soluble components, two magnetic stirrers with variable rotation speeds and heating capability were employed. The mixing process involved stirring at 500 rpm for 1 hour each at temperatures of 20°C, 25°C, and 30°C. Temperatures were kept below 30°C to prevent degradation of less stable propolis components, aiming to maintain the integrity of biologically active substances for subsequent analysis.

2.1.1.4. Extraction of Raw Propolis in Ethanol

Once the pulverized propolis was immersed in ethanol, it underwent high-speed mixing (>10,000 rpm) for 2 minutes. The resulting mixture was then transferred to a sealed container and subjected to stirring and heating cycles at temperatures of 20°C, 25°C, and 30°C using a magnetic stirrer. Each temperature was maintained for 1 hour, totaling 180 minutes of processing time. Precise equipment was employed to control the duration of each step, alternating between cooling and heating phases with intermittent mixing to ensure thorough dissolution and extraction of propolis components.

Table 2. Measurement of Mass of Tested Ingredients - Propolis and Ethanol (g)

Order	Propolis (g)	Ethanol 70% (g)	Total (g)	Raw Propolis in Mixture (%)
1	4	78.93	82.93	4.82%
2	10	78.93	88.93	11.24%

The table illustrates that Sample 1, comprising 4 g of propolis mixed with 78.93 g of ethanol, achieved a propolis concentration of 4.82%, resulting in a total mass of 82.93 g. Sample 2, with 10 g of propolis in the same volume of ethanol, yielded a higher concentration of 11.24%, with a total mass of 88.93 g. This variation in propolis concentration is critical for evaluating extraction efficiency and extract quality. Comparing the percentages of propolis in the mixtures by volume and mass reveals consistent values, affirming the precision and reliability of the experimental measurements.

2.1.1.5. Filtration

Vacuum filtration, utilizing a Buchner filter, vacuum filtration flask, and a “Jastrebac” laboratory vacuum pump (P=500 W), was employed as the fastest and most efficient method for separating the propolis extract.

2.1.1.6. Dilution to 4% and 10% Solution

After filtration, the ethanol extract was diluted to obtain a 4% and 10% propolis solution.

2.2 Measurement of Physical Parameters

The physical parameters of EEP were measured using the following instruments:

- Solubility(g): Gravimetric Method (Cussler, 2009).
- Conductivity: Xiaomi and TDS & EC conductivity meters (units: µS/cm, ppm; accuracy: d = 0.01)
- Yield: (Pe/Pm*100%)

2.3 Total Phenolic Content Analysis (mg GAE/g)

Total phenolic content in propolis extracts was determined using the Folin-Ciocalteu method (Ayumi et al., 1999) with minor modifications. A 0.2 mL aliquot of each extract was mixed with Folin-Ciocalteu reagent up to 1 mL. After incubation at room temperature, sodium carbonate solution and 95% ethanol were added to a final volume of 3 mL. Samples were incubated in the dark for 50 minutes at room temperature, and absorbance was measured at 725 nm using a UV-VIS spectrophotometer. Gallic acid was used as a standard to generate a calibration curve.(Kolovski, 2023).

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3. RESULTS AND DISCUSSION

Propolis is a complex mixture influenced by local flora, geography, and climate. This study specifically analyzed ethanol extracts from "poplar" type propolis to evaluate their physical, chemical properties, and bioactive components.

3.1.Results of measurements of the physical parameters of ethanol extracts of propolis with various concentrations

Table 3. Solubility Dependency on Quantity of Solids vs. Solvent Concentration

Solvent Concentration	Pelagonia EEP Insoluble Solids (g)	Pelagonia EEP Solubilized Solids (g)	Polog EEP Insoluble Solids (g)	Polog EEP Solubilized Solids (g)	Initial Quantity of Propolis (g)
4.00%	0.12	0.85	0.24	0.79	1
10.00%	0.92	1.58	1.27	1.23	2.5

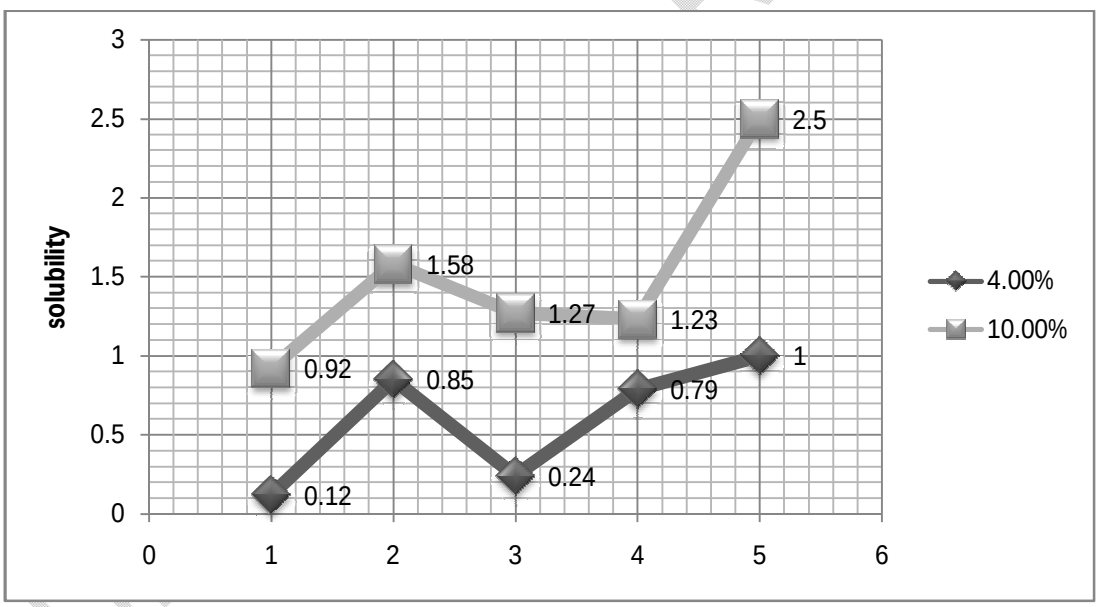

Fig.1. **Relationship between the quantity of solids and solvent concentration at EEP**

Table 3 and Figure 1 illustrate the relationship between solids quantity and solvent concentration in terms of solubility for Pelagonia and Polog propolis extracts. The data reveals that at lower solvent concentrations (4%), a higher proportion of solids can be solubilized. For example, Pelagonia EEP solubilizes 0.85 g of solids from 1 g of propolis at 4% solvent concentration, while Polog EEP solubilizes 0.79 g from the same initial quantity. However, as solvent concentration decreases to 10%, solubility of solids significantly diminishes. At 10% solvent concentration, Pelagonia EEP solubilizes 1.58 g of solids from 2.5 g of propolis, and Polog EEP solubilizes 1.23 g from the same initial amount. This trend underscores that increasing solids quantity and reducing solvent relative to solids content

reduces solubility. Consequently, solvent saturation increases notably from 4% solids (96% solvent) to 10% solids (90% solvent) (Ibishi et al., 2023).
Therefore, for enhanced efficiency in dissolving solids, starting with a larger amount of solvent and concentrating the solution is preferable over attempting to dissolve a high quantity of solids in a smaller solvent volume. This method ensures solubility limits of the solvent are not exceeded, thereby improving dissolution efficiency (Bankova et al., 2021; Kolovski, 2023).

Table 4.Measurements of electrical conductivity (EC) of EEP

Concentration	Pelagonia EEP (ppm)	Polog EEP (ppm)
4.00%	24.5	25.8
10.00%	42	33

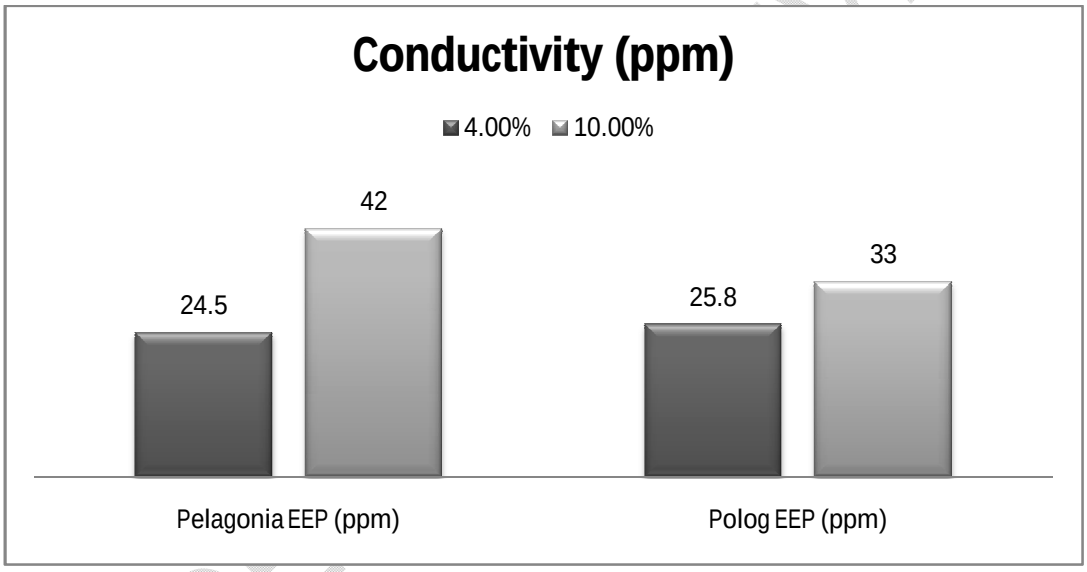

Fig.2. Electrical conductivity (EC) of propolis extract in (ppm).

Table 4 and Figure 2 illustrate the electrical conductivity (EC) measurements of Pelagonia and Polog propolis ethanol extracts at 4% and 10% concentrations. At 4% concentration, Pelagonia EEP exhibits an EC of 24.5 ppm, while Polog EEP measures 25.8 ppm. Upon increasing the concentration to 10%, Pelagonia EEP shows a significant rise in EC to 42 ppm, whereas Polog EEP displays a smaller increase to 33 ppm. These findings indicate that the electrical conductivity of propolis extracts increases with higher concentrations, suggesting a greater presence of ionizable compounds in the solution. The higher EC observed in Pelagonia EEP compared to Polog EEP at both concentrations may reflect variations in extract composition due to geographical and botanical differences, as discussed by Tosi et al. (2007), who highlighted regional variations in propolis chemistry and bioactivity. This trend aligns with the results reported by Ozdemir and Karagoz (2024), where they observed a positive correlation between propolis extract concentration and electrical conductivity, indicating that higher concentrations enhance the presence of ionizable components in solution. However, the smaller increase in EC for Polog EEP at 10%

concentration suggests a potential saturation point, where further increases in solids may not significantly elevate the concentration of ionizable species in the solution.

Table 5. Yield of EEP from different regions

Region	Concentration	(Pe) Raw propolis (g)	(Pm) net EEP(g)	Yield (%)
Pelagonia	4.00%	1	14.88	6.72
	10.00%	2.5	14.35	17.42
Polog	4.00%	1	16.90	5.92
	10.00%	2.5	16.25	15.38

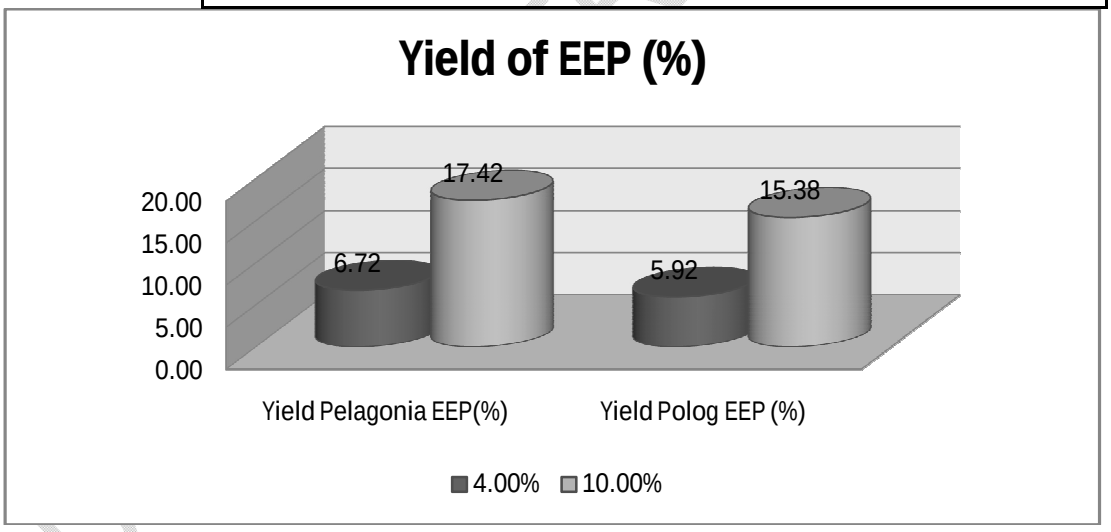

Fig. 3. Yield of propolis extracts from Pelagonia and Polog

The provided data concerns the filtration of raw propolis using different ethanol concentrations and filtration materials. The objective is to calculate the yield of the net propolis solution after filtration using the formula: $\boldsymbol{Yield = (P_e / P_m) \times 100}$

where P_e is the amount of raw propolis (in grams) and P_m is the amount of the net solution obtained (in grams). The yield calculation, expressed as a percentage, indicates the efficiency of the filtration and the quantity of propolis solution obtained.
At a 4% concentration, Pelagonia EEP shows a yield of 6.72%, while Polog EEP exhibits a slightly lower yield of 5.92%. Increasing the concentration to 10% results in Pelagonia EEP achieving a yield of 17.42%, and Polog EEP showing a yield of 15.38%. These findings demonstrate that raising the propolis concentration from 4% to 10% significantly boosts the extract yield for both regions. However, consistently higher yields in Pelagonia compared to Polog suggest that Pelagonia propolis may contain more extractable compounds or possess more efficient extraction properties (Kolovski, 2023; Ibishi et al., 2023).
The variation in yields between regions can be attributed to differences in propolis botanical sources and regional environmental conditions, which influence propolis chemical composition and solubility. This observation is supported by previous research, such as Popova et al. (2007), which highlighted significant regional variations in propolis bioactive properties due to flora and climate differences. Furthermore, the higher yields observed at 10% concentration for both regions align with research principles from Banskota et al. (2001), indicating enhanced extraction efficiency with increased raw material concentrations.

Table 6. Total Phenolic Content at EEP from different regions (t-test)

Region	Concentration	Yield (%)	Conductivity (ppm)	Total Phenolic Content (mg GAE/g)
Pelagonia	**10.00%**	**17.42**	**42**	**189 ***
Polog	**10.00%**	**15.38**	**33**	**172***

*Significant difference ($p < .05$)

Table 6 presents the laboratory analysis results for Pelagonia and Polog propolis extracts, highlighting their physical parameters, yield, conductivity, and total phenolic content.
At a concentration of 10.00%, Pelagonia EEP exhibited a yield of 17.42% and a conductivity of 42 ppm. The total phenolic content was measured at 189 mg GAE/g, indicating a high concentration of phenolic compounds essential for bioactivity. These results underscore the efficiency of the extraction process for Pelagonia propolis, producing a potent extract rich in phenolics.
In contrast, Polog propolis extract at the same concentration showed a yield of 15.38% and a conductivity of 33 ppm, with a total phenolic content of 172 mg GAE/g. Despite slightly lower values compared to Pelagonia EEP, the Polog extract still demonstrates significant phenolic content, reflecting effective extraction methods.
The differences in yield and phenolic content between Pelagonia and Polog extracts suggest variations in propolis plant sources and extraction conditions, influencing the bioactive composition of the extracts.
The higher conductivity values observed in the propolis extracts indicate a greater presence of ionic substances, which correlates with the concentration of phenolic compounds in the extracts. This observation aligns with previous studies that have shown higher yields and conductivity measurements to be associated with increased phenolic content. For example, Bankova et al. (2000) demonstrated significant variations in the chemical composition and antibacterial activity of propolis based on its geographic origin.

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Comparison of Pelagonia and Polog propolis extracts reveals notable differences in their mean total phenolic content, as determined by laboratory analyses. Pelagonia propolis exhibited a higher mean total phenolic content of 189 mg GAE/g, whereas Polog propolis showed 172 mg GAE/g. This difference was statistically significant ($t = 5.38$, $df = 18$, $p < 0.05$), indicating that the variations observed in phenolic compounds between these two regions are unlikely due to random chance.

These findings are consistent with the research objectives, emphasizing regional variations in propolis composition influenced by geographic and environmental factors. Understanding such differences is crucial for assessing the potential health benefits and applications of propolis sourced from different regions.

4. CONCLUSION

The study highlights significant variations in propolis extracts from the Pelagonia and Polog regions, impacting their physical parameters and bioactive content. Geographical origin influences propolis composition, affecting solubility, conductivity, and total phenolic content. Standardizing extraction protocols is essential to ensure consistent quality and maximize the medicinal potential of propolis. Further research should focus on identifying specific botanical sources and environmental factors contributing to regional variability. This understanding will enhance the strategic use of propolis in pharmaceuticals, cosmetics, and functional foods.

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