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Discrimination of Turkish Propolis from Different Geographical Origins by NMR Spectroscopy

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Abstract In a study utilizing NMR spectroscopy and chemometrics, propolis samples from seven diverse geographic regions across Turkey were analyzed. To identify the optimal method for studying both the antimicrobial properties and compositional variations of propolis from different regions, we investigated metabolite extraction using three solvents: water only, ethanol only, and sequential water-ethanol extraction for residual components. Notably, water-soluble components exhibited significant variation among the samples, which is particularly interesting considering the potability of propolis in water-based solutions. Furthermore, the Muğla sample displayed a distinct water-soluble profile, likely due to its unique coastal location on the Aegean Sea. This specific climate may influence the propolis' chemical composition, resulting in a different mixture of components. Interestingly, the Muğla sample contained pharmaceutically active compounds like cinnamate, ferulate, and verapamil. This research establishes a valuable foundation for further exploration of propolis' antimicrobial potential.

Keywords Turkish propolis, discrimination, statistical analysis, NMR

Introduction

Honeybees (*Apis mellifera*) produce a variety of valuable hive products beyond honey. Propolis, a resinous material, has a long history of medicinal use dating back to 3000 BC in Egypt, where it was employed for wound healing and inflammation reduction.[1] Its antiseptic properties even led to its use in the mummification process.[2] Propolis is a complex mixture of bee secretions, beeswax, and plant resins collected by honeybees from various plant sources surrounding the hive.[3] This resinous material plays a crucial role in maintaining hive health by sealing cracks and providing a barrier against pathogens.[4] The chemical composition of propolis is highly variable and depends on the local flora accessible to the honeybees.[5] This variation in composition is believed to contribute to the diverse biological activities associated with propolis, including effects on cell metabolism, potential anti-cancer properties, anti-inflammatory activity, antioxidant effects, and immune system modulation.[6]

Turkey, situated at the crossroads of Europe and Asia, boasts a remarkable diversity of climates and plant life.[7] This ecological richness translates into a potential diversity of propolis produced by honeybees across the country. As a leading global producer of propolis, Turkey presents a unique opportunity to investigate the influence of regional flora on propolis composition and potential biological activities.[8] It is hypothesized that propolis collected from different

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regions with distinct botanical resources will exhibit variations in its chemical profile, potentially leading to differences in its health benefits.

This study aims to investigate the possibility of discriminating propolis from various Turkish regions based on their metabolic profiles. Nuclear Magnetic Resonance (NMR) spectroscopy, a powerful analytical technique for elucidating the structure and properties of small molecules, will be used to analyze the metabolic fingerprints of propolis samples collected across Turkey. These fingerprints represent the unique set of small molecules present in each propolis sample.[9] By employing NMR spectroscopy, we aim to identify characteristic chemical markers that distinguish propolis from different geographical regions within Turkey. Establishing such a link between propolis origin and its chemical signature would be a significant advancement in the field of propolis research. This approach holds immense potential for not only understanding the influence of regional flora but also for developing reliable methods for the geographical authentication of propolis from various parts of Turkey. Briefly, the most distinct characteristics of each region are as follows: Anzer has a high-altitude continental climate; Artvin, a humid continental climate; Bingöl and Sivas, a severe continental climate; Bursa, a transitional climate blending Mediterranean and continental influences; Hakkari and Ulaşlı, a mountain climate; and Muğla, a Mediterranean climate.

The Muğla region of Turkey was chosen as the primary source of propolis samples due to its unique ecological characteristics. This region boasts a diverse flora, encompassing Mediterranean coastal landscapes, mountainous terrain, and inland valleys.[10] Such ecological variation is known to influence the botanical resources available to honeybees, which in turn can impact the chemical composition of the propolis they produce.[11] By focusing on propolis collected from the Muğla region, the study aimed to explore the potential link between geographical origin and the biological activity of propolis.

Experimental Methods

Propolis extraction - Propolis samples were collected from various regions in Turkey, including Anzer, Artvin, Bingöl, Bursa, Sivas, Muğla, and Ulaşlı (Fig. 1). A separate sample from Bursa-Sivas represented a mixture of propolis from both locations. Two samples were collected from different locations within the Artvin region of Turkey. This study represents an initial exploration of Turkish propolis, with only one sample per region.

Figure 1. Geographical origins of the Turkish propolis used in this study.

To prepare propolis extracts, all crude propolis samples (2 g each) were first subjected to lyophilization for 24 hours to remove any residual moisture. Subsequently, Two grams of lyophilized propolis were divided into two equal portions: one for water extraction and the other for ethanol extraction. For ethanol extraction, the dried sample was mixed with 40 mL of 80% ethanol and incubated at room temperature for two days. On the first day, the mixture was thoroughly vortexed and mixed by hand to ensure complete homogenization. On the second day, the mixture was stirred using a magnetic plate at 150 rpm to facilitate further extraction. Insoluble material was then removed from the extract by centrifugation at 10,000 rpm for 10 minutes at 4 °C, and saved for subsequent water extraction as described below (for water-after-ethanol extraction sample). The resulting supernatant was carefully filtered through a 0.22-micron filter to remove any remaining particulate matter. The filtered extracts were then frozen overnight at -80 °C to ensure complete precipitation. The next day, the frozen extracts were lyophilized until completely dry,

typically taking 1 to 3 days. The dried extracts were finally redissolved in 80% ethanol for further analysis or storage. For water extraction, the sample was mixed with 40 mL of boiling water and autoclaved at 121 °C for 15 minutes. The insoluble material was removed by centrifugation. The resulting supernatant was passed through a centrifugal ultrafiltration device (molecular weight cut-off of 5,000 Da; Merck, Rahway, NJ, USA) followed by the same extraction procedure as used for ethanol.

NMR sample preparation - For total component analysis, 60 mg of extract was dissolved in 600 µL of 100% C_2D_5OD. For water-soluble component analysis, 60 mg of extract was dissolved in 600 µL of 10 mM sodium phosphate buffer (pH 7.0) prepared in 100% D_2O. For ethanol-soluble component analysis, the insoluble fractions remaining after the water-soluble component analysis were dried and then redissolved in 600 µL of 100% C_2D_5OD.

NMR experiments and data processing - All experiments were performed on Bruker Avance II 500 MHz equipped with a TXI probe. A one-dimensional version of NOESY pulse sequence (noesypr1d) was employed. The ^1H spectra were collected with 48 K data points over the spectral width of 12 ppm. The residual water resonance was suppressed by presaturation. The NOESY mixing time was set at 50 ms, and 128 transients were collected per experiment. The raw data were apodized by an exponential window function with a line broadening factor of 0.5 Hz, zero-filled to 64 K, Fourier transformed, and phase adjusted with Mnova NMR (Mestrelab Research, S.L., Santiago de Compostela, Spain). The DSS resonance was used to reference the chemical shift. The statistical analysis was also performed with Mnova NMR.

Results and Discussion

Propolis extraction - To optimize the extraction yield of bioactive compounds, the raw propolis material underwent a two-step size reduction process. Initially, the crude sample was coarsely fragmented using a hammer, followed by a more refined pulverization into a fine powder using a food processor. This meticulous sample preparation facilitated efficient solvent penetration and molecular diffusion, resulting in an approximate extraction yield of 7% (w/w) based on ethanol as the extraction solvent. Notably, the resulting dried extracts displayed a range of physical appearances, varying from crystalline solids to amorphous pastes. This morphological diversity likely reflects intrinsic compositional differences among the original propolis samples or may be attributable to variations introduced during the lyophilization process.

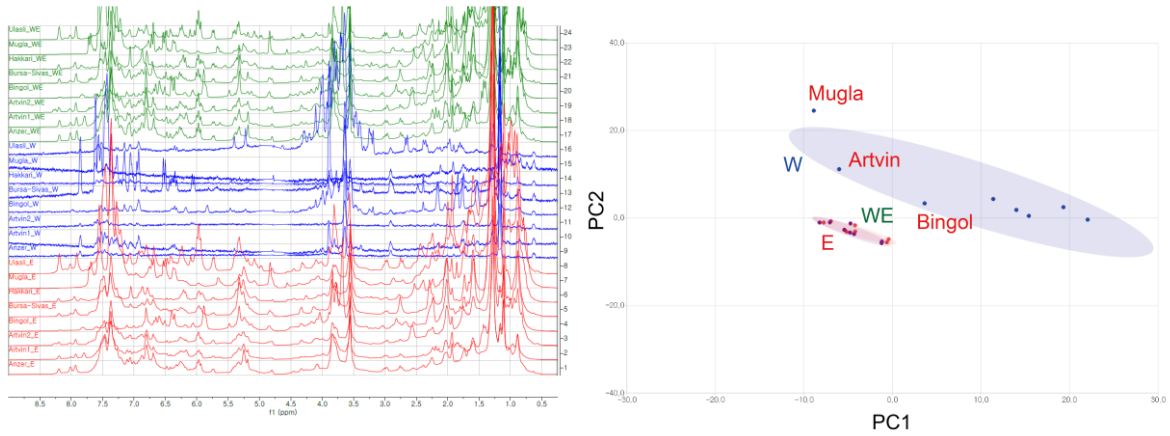

Figure 2. (Left) Comparison of the NMR spectra from three different extractions. Traces were color-coded: red, ethanol; blue, water, and green, ethanol after water. For each extraction method, from bottom to top: Anzer, Artvin, Bingöl, Bursa-Sivas, Muğla, Ulaşlı. (Right) PCA scores plot. E, W, WE represent extraction by ethanol, water, and ethanol after water.

NMR Experiments and Data Interpretation - To comprehensively characterize the chemical constituents of propolis samples from diverse geographical regions, we implemented a differential partitioning approach utilizing water and ethanol. This methodology involved the initial addition of 100% D_2O to the dried ethanol extract to selectively isolate water-soluble compounds. Subsequently, the undissolved residue was dissolved in 100% C_2D_5OD for independent analysis. A control sample was prepared by directly dissolving the original dried ethanol extract in 100% C_2D_5OD to establish a reference point for comparative analysis. The initial spectral analysis of the three different extracts reveals a significantly lower number of peaks, indicating a reduced component profile in the water extraction samples compared to the other fractions. This suggests that propolis-based beverages (water extracts) might potentially offer lower efficacy as health supplements when compared to the powdered form, which contains a broader spectrum of bioactive compounds.

By employing this straightforward partitioning technique, the distinct chemical profile of the Bursa-Sivas sample became apparent (4th blue trace in Fig. 2 (left)). This sample exhibited a unique composition, characterized by a high abundance of water-soluble aromatic compounds (6 to 8 ppm). In contrast, the Muğla, Anzer, and Artvin samples displayed significantly lower sugar levels (3 to 5 ppm) compared to those from Ulaşlı and Bingöl. While the water-soluble fractions revealed pronounced variations across samples, the ethanol-soluble fractions (green and red traces in Fig. 2 (left)) demonstrated remarkable similarity. The spectral complexity of the ethanol fractions suggests a significantly richer composition in terms of ethanol-soluble components compared to the water-soluble counterparts.

Statistical Analysis of the NMR Data. To elucidate the underlying chemical diversity and potential therapeutic constituents within propolis samples, we employed Principal Component Analysis (PCA) on NMR spectroscopic data. The resultant scores plot (Fig. 2 (right)) revealed a striking contrast in sample variability between the ethanol and ethanol-water extract groups. While the former exhibited minimal compositional differences across diverse propolis sources, the water-soluble fractions demonstrated significantly greater chemical heterogeneity, suggesting a more pronounced influence of geographical origin on the water-soluble constituents of propolis.

The pronounced separation of the Muğla sample within the PCA scores plot prompted a deeper investigation into its unique metabolic profile. Through the application of the Chenomx database, we successfully identified a suite of metabolites, including cinnamic acid, ferulate, ethanol, ribose, acetate, methylsuccinate, 2-hydroxybutyrate, verapamil, and valine. Comparative analysis with propolis samples from other regions revealed a marked enrichment of verapamil in the Muğla sample.

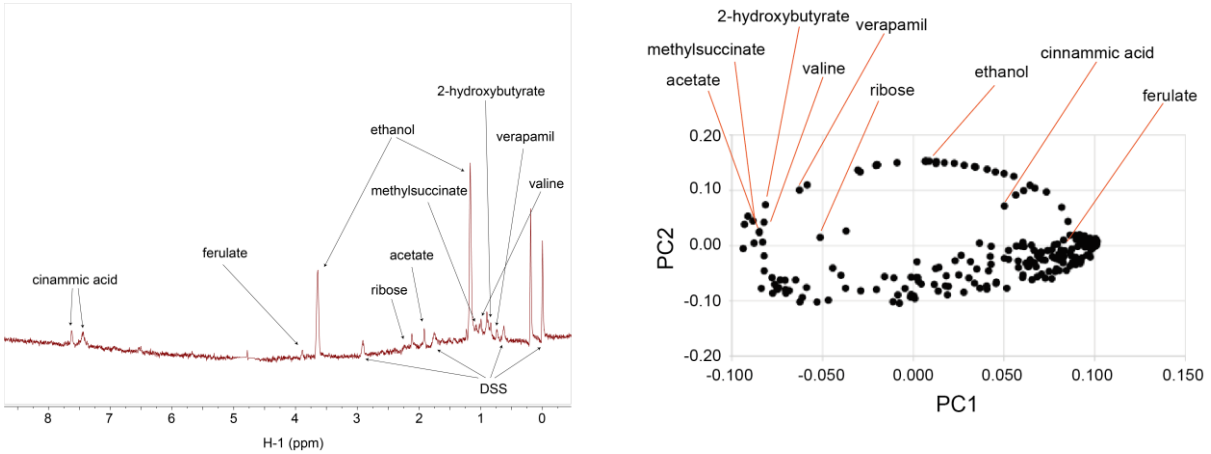

Figure 3. (Left) ^{1}H spectrum of Muğla sample. Identified metabolites are indicated. (Right) PCA loadings plot. Corresponding metabolites are indicated.

Notably, verapamil is a clinically established calcium channel blocker employed in the management of hypertension and angina pectoris.[12] Surprisingly, the Muğla sample exhibited significantly lower concentrations of cinnamic acid, renowned for its anti-inflammatory properties, and ferulate, a potent antioxidant widely distributed in plants such as ginseng and various herbal teas. This unexpected finding underscores the complex and region-specific phytochemical composition of propolis.[13, 14] These marked compositional variations across different propolis samples strongly suggest that the observed disparities in therapeutic efficacy for specific health conditions may be attributable to the unique phytochemical profiles characteristic of each region. Further investigations are warranted to elucidate the precise mechanisms underlying these region-specific effects and to identify potential biomarkers for predicting propolis efficacy.

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References

1. G. Papa, R. Maier, A. Durazzo, M. Lucarini, I. K. Karabagias, M. Plutino, E. Bianchetto, R. Aromolo, G. Pignatti, A. Ambrogio; et al., *Biology*. **11** (2), 233(2022)
2. M. El-Sakhawy, A. Salama, H. S. Tohamy, *Arch Dermatol Res*. **316** (1), 61(2023)
3. S. I. Anjum, A. Ullah, K. A. Khan, M. Attaullah, H. Khan, H. Ali, M. A. Bashir, M. Tahir, M. J. Ansari, H. A. Ghramh; et al., *Saudi J Biol Sci*. **26** (7), 1695-1703(2019)
4. A. E. Pérez Matos, G. Bacci, L. Borruso, M. Landolfi, D. Petrocchi, S. Renzi, B. Perito, *Microorganisms*. **11** (5), 1130(2023)
5. V. C. Toreti, H. H. Sato, G. M. Pastore, Y. K. Park, *Evid Based Complement Alternat Med*. **2013**, 697390(2013)
6. P. Elumalai, N. Muninathan, S. T. Megalatha, A. Suresh, K. S. Kumar, N. Jhansi, K. Kalaivani, G. Krishnamoorthy, *Evidence-Based Complementary and Alternative Medicine*. **2022** (1), 5901191(2022)
7. J. Noroozi, G. Zare, M. Sherafati, M. Mahmoodi, D. Moser, Z. Asgarpour, G. M. Schneeweiss, *Frontiers in Ecology and Evolution*. **7**, (2019)
8. R. Aliyazıcıoglu, H. Sahin, O. Erturk, E. Ulusoy, S. Kolayli, *International Journal of Food Properties*. **16** (2), 277-287(2013)
9. E. S. Elgin, S. Çatav Ş, A. Babayeva, H. Kim, E. Dibek, B. Çöl, Y. K. Chae, İ. Kıvrak, *J Appl Microbiol*. **134** (1), (2023)
10. N. Böke Sarıkahya, A. C. Gören, G. Sümer Okkalı, F. O. Çöven, B. Orman, D. Kırcı, B. Yücel, D. Kışla, B. Demirci, M. Altun; et al., *Phytochemistry Letters*. **44**, 129-136(2021)
11. P. P. Wieczorek, N. Hudz, O. Yezerska, V. Horčinová-Sedláčková, M. Shanaida, O. Korytniuk, I. Jasicka-Misiak, *Molecules*. **27** (5), (2022)
12. C. D. Baggott, B. Aagaard-Kienitz, *Neurosurgery Clinics of North America*. **25** (3), 497-528(2014)
13. J. S. Kim, *Prev Nutr Food Sci*. **21** (3), 263-270(2016)
14. Z. Rezaei, S. Momtaz, P. Gharazi, M. Rahimifard, M. Baeeri, A. R. Abdollahi, M. Abdollahi, A. Niknejad, D. Khayatan, M. H. Farzaei; et al., *Antiinflamm Antiallergy Agents Med Chem*. **23** (1), 21-30(2024)