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Effect of geographical origin and process of purification on total phenol content of propolis

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Abstract
This study was conducted to investigate the effect of purification process on total phenol content of ethanolic extracts of propolis with respect to its geographical origin. Propolis samples collected from different geographical regions were extracted with 50% ethanol (at 1:30 w/v). Purification of ethanolic extracts was performed by centrifugation. Significantly higher total phenol was obtained in purified ethanolic extracts of propolis (200.565 mg gallic acid eq/g) than in impure ethanolic extracts (172.989 mg gallic acid eq/g). Moreover, propolis collected from regions of Pantnagar possessed higher total phenol than those collected from Haldwani region. Results of the current study indicated significant effect of interaction between geographical origin of propolis and purification process with respect to its total phenol content. Moreover, the process of purification induced improvement in propolis quality by escalating its total phenol content.

Keywords: Centrifugation; extraction; geographical origin; propolis; purification; spectrophotometer; total phenol content

Introduction
Propolis is a splendid bee product which is gathered from the resins of plants. It has diversified chemical makeup with around 300 chemical compounds have been recognized in propolis (Tang *et al*., 2014) [13]. Its extract is rich in bio-active components *viz*. phenols, flavonoids as well as a large number of amino acids, enzymes, vitamins, polysaccharides and various trace elements. These bioactive constituents impart several pharmaceutical properties to propolis and thus make it a wonder product of honeybee. Environmental factors (diversity of vegetation, climate, and prevailing season) of a particular geographic location where apiary is situated have a considerable influence on amount and quality of propolis (Mendonca *et al*., 2015) [9]. Even the method of extraction, processing, and storage conditions also influences its chemical profile (Kocot *et al*., 2018; Oses *et al*., 2016) [14, 15]. Variations in the chemical composition of propolis eventually influence its biological activities. However, propolis also possesses impurities like wax, resins and balsam. Wax and non-polar compounds may reach from 35 to 40%. As the solvents used in propolis extraction are mostly organic, these contaminants can still be found in propolis extracts (Cottica *et al*., 2011) [3] which allows for easy manipulation of the material in both industrial section and laboratory. This necessitates purification of extracts of propolis in order to remove impurities while retaining its bioactive fraction. Therefore, the aim of the study was to investigate how total phenol content of ethanolic extract of propolis samples is affected by the process of purification along with their geographical origin.

Materials and Methods
Collection of Propolis
Propolis was directly collected from the hives of *Apis mellifera* L. at different geographical regions of Pantnagar *viz.*College of Agriculture, Crop Research Centre (CRC), Honeybee Research and Training Centre (HBRTC) and Haldwani *viz.* Dewalchaur, Tanda in the month of July-September 2021. Raw propolis sample were kept at −20 °C and then crushed into homogeneous powder. The powdered sample was stored at −20 °C till further analysis.

Extraction of Propolis
All samples of raw propolis were extracted with 50% ethanol @ 1:30 (w/v) for 24 hours (Bankova *et al*., 2016) [1].

Method of Purification

It was performed as described by Gregoris and Stevanato (2010) [6], ethanolic extracts of propolis samples were filtered with the help of a strainer to take away insoluble remaining of coarse impurities. After leaving the suspensions for few hours the obtained sediment was subjected to centrifugation for 30 min through Centrifuge.

Estimation of Total Phenol content

10 ml of the Folin-Ciocalteu reagent (Sigma- Aldrich) was dissolved in 90 ml of distilled water for making 10% concentration of the reagent. 7.5% of Na_2CO_3 was prepared by adding 7.5 g Na_2CO_3 in 100 ml distilled water. Then 200 µl of each propolis extract (extracted in 50% ethanol) was mixed with 2.50 ml Folin-Ciocalteu reagent (10%) for 5 min, after that 2.00 ml of 7.5% Na_2CO_3 was added in each sample. Each sample was kept for incubation for 2 hours. Different concentrations of gallic acid was prepared and used as standard. Blank solutions were prepared by adding 2.00 ml of 50% ethanol with 2.50 ml Folin-Ciocalteu reagent (10% concentration) and 2.00 ml of 7.5% Na_2CO_3. Absorbance was measured at 760 nm using Double beam UV-VIS Spectrophotometer Model: 3375, S/N 20200301903. UV-Professional Analysis software was used for processing the spectra and absorbance data.

Statistical analysis

The data obtained was analyzed by two-way ANOVA in OP-Stat software. Each sample was replicated 6 times. Probability values smaller than 0.01 were accepted as statistically significant.

Result

Purification of propolis is an essential procedure in order to remove wax and other undesirable ingredients while retaining the polyphenolic fraction which contains most of the bioactive components. In the current study, Total phenol content in ethanolic extract of propolis was significantly affected by the geographical origin of propolis. Propolis sample from CRC, Pantnagar was recorded for higher total phenol content (196.839 mg gallic acid eq/g) whereas sample from Tanda, Haldwani contained lowest total phenol (176.481 mg gallic acid eq/g). Similar effect was also reported by Gorecka *et al.* (2022) [4], Srinivas *et al.* (2021) [12], Ozkok *et al.* (2021) [11] and Keskin *et al.* (2019) [8] in other experiments. The effect of purification process on total phenol content was also found significant. It was found maximum in purified ethanolic extract of propolis samples (200.565 mg gallic acid eq/g) and minimum in the ethanolic extract of propolis samples which were not subjected to purification process (172.989 mg gallic acid eq/g) (Table 1). In general, Polyphenols in propolis and plant extracts have been found to be rather thermal-stable (Gonzalez, Gomez, Tereschuk, & Molina, 2009; Volf, Ignat, Neamtu, & Popa, 2014) [16, 17]. That is why longer centrifugation at a relatively high temperature as applied in the method of purification performed in the current study, may lead to more effective wax and organic waste removal without any negative effect on the total phenols. Kalogeropoulos *et al.* (2009) [7] also reported the importance of purification process even for the extracts of propolis.

Interaction between geographical origin of propolis and purification process was significant and data pertaining to interaction is shown in Table 2 and Fig 1. Amongst all samples of different geographical locations significantly highest total phenol content was recorded for purified ethanolic extract of proplis collected at CRC, Pantnagar (212.70 mg gallic acid eq/g). Lowest total phenol was obtained in the impure ethanolic extract of propolis sample collected at Tanda, Haldwani (162.25 mg gallic acid eq/g). The results of the current study are in accordance with the findings of Graikini *et al.* (2019) [5].

Table 1: Mean of total phenol content in ethanolic extract of propolis as influenced by different treatments.

Treatments	Total Phenol Content in Ethanolic Extract of Propolis (mg gallic acid eq/g)
Geographical Origin of Propolis	
College of Agriculture, Pantnagar	195.085
CRC, Pantnagar	196.839
HBRTC, Pantnagar	186.082
Dewalchaur Haldwani	179.399
Tanda Haldwani	176.481
SEm ±	1.638
CD at 1%	4.666
Purification Process	
Without Purification Process	172.989
After Purification Process	200.565
SEm ±	1.036
CD at 1%	2.951

Table 2: Effect of interaction between geographical origin of propolis and purification process for total phenol content of ethanolic extracts of propolis.

Total Phenol Content in Ethanolic Extract of Propolis (mg gallic acid eq/g)		
Factors (A/B)	**Without Purification Process**	**After Purification Process**
CRC, Pantnagar	177.469	212.700
College of Agriculture, Pantnagar	187.895	205.783
HBRTC, Pantnagar	172.015	200.148
Dewalchaur Haldwani	165.317	193.482
Tanda Haldwani	162.25	190.712
Mean	172.989	200.565
SEm ±	2.316	
CD at 1%	6.599	

Factor A- Geographic Origin of Propolis; Factor B-Purification Process

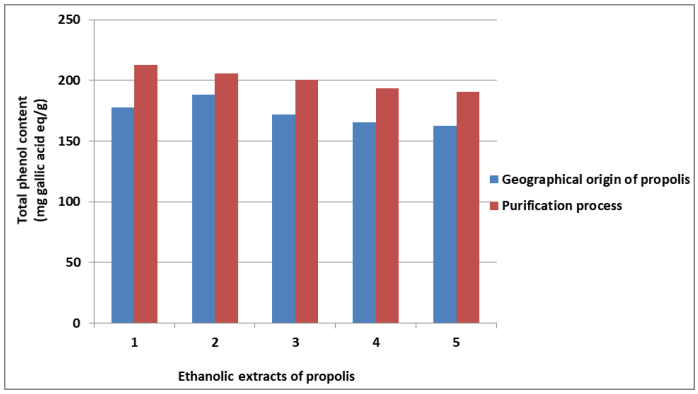

Fig 1: Effect of geographical origin and process of purification on total phenol content of propolis.

Conclusion

Results of the current study indicated significant effect of interaction between geographical origin of propolis and

purification process with respect to its total phenol content. It is evident that the process of purification induces improvement in propolis quality by escalating its total phenol content and consequently increasing its worth as a food with therapeutic properties. Thus it can be concluded that centrifugation may be the most effective process for purifying propolis to raise its grade and can be considered as a crucial step that should be used for both experimental and commercial applications.

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