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Amrit Research Journal December 2024, Vol. 5, Issue 1, 59-67
ISSN: 2738-9553
RESEARCH ARTICLE

Analysis of Phenolics, Flavonoids Content, Antibacterial, Antioxidant Properties of Honey and Propolis Samples

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(Received: August 12, 2024; Received in Revised form: October 17, 2024; Accepted: October 22, 2024; Available online)

DOI: https://doi.org/10.3126/arj.v5i1.73542

Highlights

- Local and commercial honey and propolis samples were collected and phytochemical analysis, antioxidant and antibacterial activities were evaluated.
- Somewhat higher amounts of phenolics were detected in local honey than commercial honey, flavonoids were absent. Antioxidant activity was observed at higher concentrations, antibacterial activities were not observed for both samples.
- Phenolics and flavonoids were present in propolis extracts. Antioxidant activity was observed at higher concentrations and few extracts showed antibacterial activity against *S. aureus.*
- Quality of honey and propolis are not of superior quality.

Abstract

Honey and propolis are the insect based natural products used to treat various diseases. This study is focused on the analysis of phytochemicals in honey and propolis samples and evaluation of their antioxidant and antibacterial activities. Different extracts of propolis were prepared. Total phenolic and flavonoid content as well as DPPH radical scavenging and antibacterial activities were measured. In screening tests, only phenolics were detected in honey samples while both phenolics and flavonoids were detected in propolis extractsso their content were estimated. In honey samples, total phenolic content varies from 5.802±0.234 to 13.990±0.318 mg GAE/100 g of honey. In propolis samples, total phenolic content varies from 1.59±0.02 (EtOAc extract of PPW) to 38.34±1.04 (MeOH extract of PGW) mg GAE/100 g dry extract. Similarly, total flavonoid content varies from 22.40±2.27 (EtOAc extract of PGS) to 80.61±5.39 (EtOAc extract of PPW) mg QE/100 g dry extract. In antioxidant assay, both honey samples showed DPPH radical scavenging activity at higher concentrations than the propolis samples. Only the ethyl acetate extract of PPW and methanol extracts of PGS and PGW showed activity against S. aureus in antibacterial assay.

Keywords: *Antioxidant, Antibacterial, Honey, Propolis, Phytochemicals*

Introduction

Honey and propolis are natural products derived from honey bees. Honey is one of the most remarkable nature's gifts to mankind as it possesses great nutritional values. It has wide applications in Ayurvedic medicine such as for the treatment of eye diseases, cuts and burns. It is used to treat thirst, vomiting, hiccup, diabeties, diarrhea respiratory disorders such as cough, asthma and phlegm with other herbal preparations. It is also used as a fluid vehicle taken with or after medicine which aids or assists the action of main ingredient [1,2].There are two types of honey available, blossom honey and honeydew honey. Blossom honey,

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also known as floral honey is produced by bees from nectar and it may be monofloral or polyfloral honey. Honeydew honey is obtained from excretions of plant-sucking insects such as aphids. Blended honey is a commercially available honey that is a mixture of two or more honey samples collected from different locations. The composition of honey is directly related to its botanical origin [3]. On the other hand, the quality of honey is generally determined by its sensorial, chemical, physical and microbiological properties [4]. Honey is composed of 80-85 %carbohydrates, such as fructose, glucose, 15-17% water, 0.1-0.4% protein, 0.2% ash, essential and non-essential amino acids. It also contains enzymes like diastase, invertase, glucose oxidase, catalase and acid phosphate, vitamins as well as other substances like phenolic antioxidants. In addition, it also contains phytochemicals like terpenoids, phenolics, alkaloids etc. The chemical compositions and physical properties of natural honey differ according to the botanical origin.This has the great influence on its activity [5].

Propolis or bee glue is a bee product, composed mainly of plant resins and beeswax collected by bees. It is used to seal the cracks, smooth walls, and to keep moisture and temperature stable in the hive all year around. It prevents bee hive from decay and against other flies [6]. Raw propolis is composed of various materials such as plant resins (50%), waxes (30%), essential and aromatic oils (10%), 5% pollens (5%) and other organic substances (5%). Propolis is collected from the resins of some commom trees like poplars, conifers, birch, pine, alder, willow, palm [7]. Like other natural products, propolis is used in traditional as well as modern medicines to prevent diseases like inflammation, heart disease, diabetes, cancer and in food and cosmetics industries [8,9].

Honey and propolis are complex mixture of primary and secondary metabolites and the medicinal properties are due to presence of different phytochemicals.The composition, aroma and color vary from hive of different locations and season of collection. On the other hand, the composition is determined by bee species, botanical source and geographical conditions from where they were collected by bees. The main phyto-constituents that have been isolated and identified from the propolis are polyphenolics, chalcone, terpenes, aromatic acids and their esters [10-12]. Literature search revealed that there were reports available related to the polyphenol content, antioxidant and antibacterial activities of honey and propolis [13-16], chemical constituents of propolis and their biological activities[17-21] and physico-chemical characteristics of honey samples [22] from different regions of Nepal.

The consumers in these days are more conscious about the quality and nutritional values of foodstuffs. The processing method also determines the quality of the honey. Honey can be easily adulterated. In this study, we have reported the quality of honey and propolis in terms of polyphenol content, antioxidant and antibacterial activities. As the quantity of polyphenol depends on the floral diversity, geographical origin and climatic condition of the collection sites, it is always interesting to quantify the polyphenol content. Depending on the quantity of polyphenols, the quality of honey can be access in terms of antioxidant and antibacterial activities.

Samples collection

The local honey samples (A and B) and propolis from winter (PPW) were collected from the traditional hives from beekeepers of Palpa districtin March 2020. Commercial samples (C and D) were brought from the apiary maintained at Pashupati bee products, Palpa. Fresh propolis samples from winter (PGW) and summer (PGS) were collected from beekeepers Gulmiof district in March and September 2020.

Extraction

Freshly collected propolis samples (50 g each) were extracted successively with ethyl acetate (100 mlx4), methanol (100 ml x4) and 50% aqueous methanol (100 mlx2) using cold percolation method. The extracts were filtered and concentrated separately using rotatory evaporator. The dried extracts were stored in a refrigerator.

Screening for Phytochemicals

The presence of phytochemicals in honey and propolis samples were detected by reacting with specific reagents according to the standard method [23].

Determination of total phenolic content (TPC)

The total phenolic content was measured using Folin-Ciocalteureagent [24]. Gallic acid was used for the construction of calibration curve. In a 96 well plate, 20 µlgallic acid of different concentrations (10, 20, 30, 40, 50, 60, 70, and 80 µg/ml) and 20 µl of honey/propolis extracts (50 mg/ml) were added in triplicate. Then 100 µl of FC reagent (10%) and 80 µl of Na_2CO_3(7%)

solution were added. The plate was kept away from light source for 15 minutes. Absorbance was taken at 765 nm using a microplate reader. The total phenolic content in the sample was calculated using the formula C=cV/m where C the total phenolic content in mg GAE/g dry extract. Concentration of gallic acid obtained from calibration curve in mg/ml is indicated by c. Volume of extract in ml is represented by V, mass of extract in gram is indicated by m.

Determination of total flavonoid content (TFC)

The total flavonoid content of the samples was determined by aluminum chloride method [25]. In a 96 well plate, 130 µl of different concentrations of standard quercetin solutions (10, 20, 30, 40, 50, 60, 70, and 80 µg/ml) were added in triplicate. 20 µl of honey/propolis extract (50 mg/ml) and additional 110 µl of distilled water was added in triplicate in each well-containing test samples to maintaining the final volume of 130 µl. Then 60 µl ethanol, 5 µl $AlCl_3$, and 5 µl potassium acetate buffer were added separately in each well-containing quercetin standard and test samples. It was placed away from light source for 30 minutes. Absorbance was taken at 415 nm using a microplate reader. The total flavonoid content in the samples was calculated as in the case of phenolics and expressed as milligram of quercetin equivalent per 100 gram of dry weight (mg QE/100g).

Determination of Antioxidant activity using DPPH free radical

DPPH free radical was used for the determination of antioxidant activity with slight modification [26]. 100 µl of propolis samples of different concentrations (100, 200, 400, 800, 1000 and 2000 µg/ml)and 100 µlquercetin of different concentrations(5, 10, 20, 40, 80 and 100 µg/ml) were added to 96 well plate in triplicate. Control well was prepared in triplicate by adding 100 µl of methanol instead of samples. Then, 100 µl of DPPH solution (0.1 mM) was added to each well of 96 well plate. The plate was incubated for 30 minutes away from the light source. Absorbance was taken at 517 nm using a microplate reader. The percentage DPPH radical scavenging was calculated by using the equation (2)

$$\% \text{ inhibition} = \frac{(\text{Acontrol} - \text{Asample})}{\text{Acontrol}} * 100\% \quad \text{.....(2)}$$

Antibacterial assay

The antibacterial activities of honey samples and propolis extracts were determined against four bacteria,*Staphylococcus aureus* (ATCC 6538)*, Escherichia coli* (ATCC8739)*, Klebsiella pneumonia* (ATCC700603)*, Salmonella typhi* (Clinical sample) using agar well diffusion method [27]. The samples (50 µl) of each concentrations (100 mg/ml prepared in 50% DMSO) was introduced into each well (6 mm diameter) seeded with respective microorganism. The amount of extract in each well was 5 mg. 50 µl of 50% DMSO was used for negative control. Standard antibiotic, ciprofloxacin (1mg/ml) was used for positive control. Clear inhibition zones of bacterial growth around the wells were observed at the end of incubation period which were measured.

Results and discussion

Extractive values of propolis

The propolis samples (50 g each) from Palpa collected in winter (PPW) and Gulmi collected in summer (PGS) and winter (PGW) were extracted with ethyl acetate, methanol and 50% aqueous methanol at room temperature. The yields of crude extracts obtained with different solvents are presented in Table 1.For all three samples,the highest yield was obtained with methanol. Both samples from Gulmi provided relatively high amounts of extracts with 50% aqueous methanol in comparison to Palpa sample. Low amounts of extracts were obtained with ethyl acetate for all three samples. This indicated that all propolis samples contain greater amounts of highly polar phytoconstituents. The results are presented in Table 1.

Table 1. Percentage yield of different Propolis samples

Extracts	Percentage yield of Propolis samples			Organoleptic properties
	PPW	PGW	PGS	
Ethyl acetate	2.52	1.38	1.15	Orange
Methanol	76.14	52.90	39.43	Dark brown
50% aq. methanol	1.21	12.32	10.55	Light brown

Screening for phytochemicals in honey and propolis samples

In chemical screening all honey samples, alkaloids, coumarins and flavonoids were found to be absent. Polyphenols, terpenoids, quinones, tannins and saponins were present in all samples. The results are presented in Table 2. This qualitative screening indicated that there was no difference between local (A and B) and commercial (C and D) honey samples in terms of phytochemicals. The main phenolic compounds reported in honey are vanillic, caffeic, syringic, p-coumaric, ferulic, rosmarinic, ellagic, benzoic, 3-hydroxybenzoic, 4-hydroxybenzoic, chlorogenic acids, quercetin, kaempferol, myricitine, pinocembrin, pinobanksin, chrysin, galangin, hesperetin. However the main functional components of honey are flavonoids rather than phenolic acids[28, 29].

Table 2.Phytochemicalsof honey samples

Phytochemicals	A	B	C	D
Alkaloids	-	-	-	-
Polyphenols	+	+	+	+
Flavonoids	-	-	-	-
Terpenoids	+	+	+	+
Coumarins	-	-	-	-
Glycosides	+	+	+	+
Quinones	+	+	+	+
Reducing sugars	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+

In the chemical screening of propolis samples, most of the phytochemicals were present in all three samples with few exceptions. Alkaloids and saponins were found to be absent in all samples. Terpenoids were absent in the methanol extract of PPW, quinones and tannins were absent in the ethyl acetate extract of PPW. Similarly, coumarins were absent in methanol extract of PGW and reducing sugars were absent in ethyl acetate extracts of PGS.The results of phytochemical screening of propolis samples are presented in Table 3.This qualitative screening study indicated that the propolis samples collected from Palpa and Gulmi districts were also nearly similar. It is well known that the propolis is the sources of flavonoid compounds,more than 300 compounds have been reported so far from propolis and many bioactive flavonoids have been reported from Nepalese propolis [30-33].

Table 3. Phytochemicals of propolis extracts

Phytochemicals	PPW			PGW			PGS		
	E	M	MW	E	M	MW	E	M	MW
Alkaloids	-	-	-	-	-	-	-	-	-
Polyphenols	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+
Terpenoids	+	-	+	+	+	+	+	+	+
Coumarins	+	+	+	+	-	+	+	-	+
Glycosides	+	+	+	+	+	+	+	+	+
Quinones	-	+	+	+	+	+	+	+	+
Reducing sugars	+	+	+	-	+	+	-	+	+
Saponins	-	-	-	-	-	-	-	-	-
Tannins	-	+	+	-	+	+	-	+	+

E-Ethyl acetate, M-Methanol, MW-50% aq. methanol

Phenolics and flavonoids content of honey and propolis samples

The total phenolic content in different honey samples and propolis extracts was calculated from the calibration curve using regression equation y= 0.039x +0.019, R^2 =0.996 and expressed as mg GAE/100 g of extract in dry weight (mg/100g). The results are given in Table 4. Among honey samples, in comparison to the localsamples (A:13.99±0.318 mg GAE/100 g and B:

12.89±0.813 mg GAE/100 g), relatively low amounts on phenolics were detected in commercial samples (C:6.67±0.318 mg GAE/100 g and D:5.80±0.243 mg GAE/100 g).The polyphenol content in honey samples collected from different altitudes of Nepal has been reported,the content varies from 41.90±3.42 to 154.87±3.41 mg GAE/100g [13]. The polyphenol content in the monofloral honey samples from four different floral sources has been reported. The highest polyphenol content was observed in *Fagopyrum esculentum* honey (59.34 ± 2.77 mg GAE/100 g) while the lowest was observed in *Diploknema butyracea* honey (17.82 ± 1.61 mg GAE/100 g) [15]. In comparisonto the reported date, our samples were found to contain relatively low amounts of phenolic compounds. Honey production and quality is influenced by the vegetations/floral sourcesaround the bee hives. On the other hand, bees are forced to feed on commercial sweetners or commercial honey is adulterated with sugar syrups and other adulterants. This could be the reasons for the low content of phytochemicals in honey samples.

Among three propolis samples, PPW, PGW and PGS, the highest amounts of phenolics were obtained in PGW with three different extraction solvents, ethyl acetate, methanol and 50% aq. methanol. When solvent polarity for extraction was taken into account,the highest amounts of phenolics were detected in the methanol extract of propolis collected from Gulmi (PGW) (38.34 ±1.04 mg GAE/100 g extract) and the lowest amounts were detected in the ethyl acetate extract of propolis collected from Palpa (PPW) (1.591±0.029 mg GAE/100 g extract).For all three propolis samples, low amounts of phenolics were detected in ethyl acetate extracts and high amounts of phenolics were detected in methanol extracts. Thus, the phenolics content were in the order, methanol> 50% aq. methanol>ethyl acetate extracts for all propolis samples.The total content of phenolics in propolis samples collected from different regions of Nepal has been reported and it ranged between 127.36±5.50 mg GAE/gto 242.70±4.50 mg GAE/g. The reported values are very high than our findings. This chemical parameter varies depending on the location of hive. Similarly, geographical origin of the honey as well as season of collection and plant biodiversity where honeybee feed determine these chemical parameters [16].

The total flavonoid content in honey samples and different propolis extracts was calculated from the calibration curve using regression equation y=0.013x+ 0.010, R^2 =0.993 followed by the formula C=cV/m and expressed as mg QE/100 g of extract in dry weight. In our analysis, all the honey samples were devoid of flavonoids. The total flavonoid contents in different extracts of propolis are given in Table 4. Among three propolis samples, PPW, PGW and PGS, higher amounts of flavonoids were detected in PPW with two extraction solvents, ethyl acetate and 50% aq. methanol. However, in PGW and PGS, higher amounts of flavonoids were detected with methanol. When solvent polarity for extraction was taken into account, higher amounts of flavonoids were detected in the ethyl acetate extract of propolis collected from Palpa in winter (PGW) (80.61±5.39mg CE/100 g extract) and lower amounts were also detected in the ethyl acetate extract of propolis collected from Gulmi in summer (22.40 ± 2.27 mg CE/100 g extract). Similarly, total flavonoid content in propolis samples collected from different regions of Nepal has been reported and it ranged from 1.31±0.02 QE mg/ gto 5.39±0.02 QE mg/g [16].These values are very high than our findings.

Table 4. Total phenolic and flavonoid content in different honey samples and propolisextracts

Sample	Extracts	TPC (mg GAE/100g)dry extract (Mean ± S.D) (n=3)	TFC (mg CE/100g)dry extract (Mean ±S.D) (n=3)	IC_{50}(mg/ml) in DPPH assay(Mean ±S.D) (n=2)
Honey A	Aqueous solution	13.99±0.31	-	>5.00
Honey B	Aqueous solution	12.89±0.81	-	>5.00
Honey C	Aqueous solution	6.67±0.31	-	>5.00
Honey D	Aqueous solution	5.80±0.24	-	>5.00
Propolis	Ethyl acetate	1.59±0.02	80.61±5.39	1.48± 0.24
PPW	Methanol	27.63±0.03	23.26±1.38	3.28±0.18
	50% aq. methanol	22.64±0.04	40.32±3.46	2.69±0.21
Propolis	Ethyl acetate	6.33±0.87	25.60 ±3.76	4.30±0.34

PPW	Methanol	27.63±0.03	23.26±1.38	3.28±0.18
	50% aq. methanol	22.64±0.04	40.32±3.46	2.69±0.21
Propolis	Ethyl acetate	6.33±0.87	25.60 ±3.76	4.30±0.34
PGW	Methanol	38.34 ±1.04	51.34±3.09	1.06±0.17
	50% aq. methanol	18.12±0.78	27.34± 2.48	2.36±0.29
Propolis	Ethyl acetate	2.24±0.79	22.40 ±2.27	3.02±0.11
PGS	Methanol	25.37±0.81	42.48 ±5.39	1.47±0.14
	50% aq. methanol	14.34±1.76	25.18±0.49	2.55±0.35
Quercetin	-	-	-	0.018±0.002

Antioxidant activity

In DPPH assay, all the honey samples and propolis extracts showed very weak free radicals scavenging capacities. All the tested honey samples showed IC_{50} values greater than 5mg/ml. In the case of propolis, the extracts having higher amounts of flavonoids showed lower IC_{50} values. For instance, the ethyl acetate extract of PPW (IC_{50}1.48±mg/ml), methanol extract of PGW (IC_{50}1.06±mg/ml) and methanol extract of PGS (IC_{50}1.47± mg/ml) showed lower IC_{50} values than other extracts. The results are presented in Table 4. It was reported that the IC_{50} values of honey samples ranged from 56-72 mg/ml [13] and propolis samples showed strong antioxidant capacity which was greater than the standard antioxidant [16]. The components such as phenolic acids, flavonoids, vitamins, and enzymes, as well as a small amount of mineral areresponsible for the antioxidant properties of honey. However, the mechanism is still not clear and synergistic effects of all these components play a key role [34].

Antibacterial Assay

In antibacterial assay, all the tested honey samples were found to be inactive against all the tested bacteria, *S.aureus, K.pneumoni, S.typhi, A. baumanni*and *S. sonni.* In the case of propolis extracts, the ethyl acetate extract of PPW, methanol extracts of PGW and PGS showed activity against gram positive bacteria, *S. aureus*. It could be due to the presence of higher amounts of flavonoids in these extracts than other extracts (Table 4).The inhibition zone ranged from 8-18 mm.The antibacterial activity of honey could vary depending on the countries of originas well as floral diversity [35].On the other hand, hydrogen peroxide and polyphenols, also play role in antibacterial action [36]. Again, antimicrobial activity is determined by various physicochemical properties. Among them, the high content of reducing sugars, high viscosity, high osmotic pressure, low pH, low water activity, low protein content and the presence of hydrogen peroxide [37].

Table 5: Antibacterial activity of propolis samples

Sample	Extracts	Inhibition zone in mm				
		S.aureus	*K.pneumoni*	*S.typhi*	*A.baumanni*	*S. sonni*
PPW	Ethyl acetate	18	-	-	-	-
	Methanol	-	-	-	-	-
	50% aq. methanol	-	-	-	-	-
PGW	Ethyl acetate	-	-	-	-	-
	Methanol	11	-	-	-	-
	50% aq. methanol	-	-	-	-	-
PGS	Ethyl acetate	-	-	-	-	-
	Methanol	8	-	-	-	-
	50% aq. methanol	-	-	-	-	-
Neomycin		20	19	16	18	16

Conclusions

The quality of honey and propolis sample were investigated in terms of polyphenol content, antioxidant and antibacterial activities. The local and commercial honey samples as well as propolis samples collected from Palpa and Gulmi districts were included. In local honey, somewhat greater amounts of polyphenols were observed than commercial honey, but flavonoids were not detected in all honey samples. In propolis, methanol extracts contained greater amounts of phenolics followed by 50% aq. methanol and ethyl acetate extracts. Flavonoids content in propolis samples from Palpa and Gulmi districts were found to be different. In Gulmi samples, methanol extracts contained greater amounts of flavonoids followed by 50% aq. methanol and ethyl acetate extracts. In Palpa samples, ethyl acetate extract contained greater amounts of flavonoid followed by50% aq. methanol and methanol extracts. DPPH radical scavenging activity was observed for both honey and propolis samples at high concentrations only. Only the ethylacetate extract of propolis PPW, methanol extracts of PGW and PGS showed antibacterial activity against *S. aureus*. Both honey and propolis sample contained low amounts of phenolics and flavonoids so they did not show significant antioxidant and antibacterial activities. Thus, thequality of honey and propolis samples collected from Palpa and Gulmi districts are not of superior quality. However, it is necessary to analyze other parameters as well to get concrete results.

Acknowledgements

We Acknowledge to UGC, Nepal (Grant Number FRG76/77) for financial support.

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