



Physicochemical analysis and phytochemical screening of bee propolis from India

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Abstract

Propolis, commonly called as 'bee glue,' is honey bees' most important and fascinating 'chemical weapon.' Resinous exudates from diverse plant parts are collected by the bees and are transferred to their colonies, where they are converted to propolis by enzymes. In this present study propolis was subjected for its physicochemical analysis, extraction using various solvents, phytochemical screening and total phenolic content analysis was also performed. The ash content was 3.46% and moisture content was 4.98%. Solvents such as acetone, chloroform, ethanol, hexane and water were used for the extraction. The presence of secondary metabolites such as phenols, alkaloids, and terpenoids were determined through phytochemical screening of solvent extracts. The total phenolic content was reported to be highest in ethanolic extract which showed 123.50 ± 0.30 mg GAE/g. The values of the acetone, chloroform, hexane, and water extracts were 101.36 ± 0.05 , 94.56 ± 0.16 , 99.54 ± 0.14 , and 73.94 ± 0.35 mg GAE/g of propolis respectively.

Keywords: propolis, solvent extraction, phytochemical screening

Introduction

The products from honey bee i.e. bee propolis possess bioactive compounds, and it is used in pharmaceutical industries in recent years. Honeybees make propolis from a range of plant buds, and exudates, as well as pollen, wax, and hypo-pharyngeal secretions. Propolis is taken from Greek word (pro = defence and polis = city), and it refers to the beehive's defence. It is regarded as the hive's internal immunity, since it protects it from a variety of attackers and environmental conditions (Laaroussi *et al.*, 2021) [12]. Propolis has pharmacological properties that include anti-inflammatory, anti-oxidant, and antibacterial properties. Propolis has been shown to have anti-proliferative and anti-tumour properties *in vivo* and *in vitro* tumour models. Propolis comes in a variety of shapes and sizes, with variable contents according to the place, environment, and season of extraction. More than 300 compounds have already been discovered in various propolis samples. Because propolis contains a huge amount of flavonoids, terpenoids, polyphenolic acids, and other compounds that may reduce Au³⁺ to Au, some types of propolis have been utilised to make gold nanoparticles (Botteon *et al.*, 2021) [7]. Due to the presence of waxes, raw propolis is not overfeeding in food and medical formulations. As a result, propolis should always be used in the form of extracts or tinctures (Kasote *et al.*, 2019) [10]. Geographic location, flora, bee colony strength, climatic zones, and production season all have an impact on the constituents and various properties of propolis, of each country, state, and zone its own uniqueness and diversity (Miguel *et al.*, 2014) [14]. The components of crude propolis vary according to the plant source. It is made up of 50% vegetable balsam and resin,

5% pollen, 10% aromatic and essential oils, 30% wax, and 5% other ingredients, including organic wastes. During the manufacturing of propolis tincture, the wax and organic debris were removed (Nilesh Kumar *et al.*, 2008) [17]. Because India is such a large nation, there are many different types of propolis with different chemical compositions and therapeutic properties such as (Wagh, 2013) [21] antioxidant (Laskar *et al.*, 2010) [13], antimicrobial (Nilesh Kumar *et al.*, 2008) [17], hepatoprotective (Bhadauria & Nirala, 2007) [6], anti-inflammatory (Inui *et al.*, 2021) [8], and antibacterial activities (Airen *et al.*, 2018) [2]. The presence of secondary metabolites in propolis indicates its quality. The lipid content of the propolis is responsible for its water-resistant property (Aliah *et al.*, 2020) [3].

Propolis is in high demand as a nutraceutical, functional food, and food supplement, according to recent statistics, which might be attributed to its key health-promoting bioactive components and biological characteristics (Abdullah *et al.*, 2019; Pant *et al.*, 2021) [1, 19]. The present study aims to carry out the physicochemical analyses of crude propolis and the phytochemical screening of various solvent extracts of propolis. There are no reports available about the phytoconstituents initial screening of Indian propolis extracts which will be very helpful for the further analyses of antimicrobial, anticancer activities, etc., for which the phytoconstituents are majorly contributing for the above activities.

Materials and Methods

Sample collection and sample preparation

The propolis was directly collected from the apiarist. The collection area of the sample was from Kota District, of

Rajasthan. The major plant sources were found to be Sheesham, and Berry. The impurities from propolis were removed manually and the propolis was stored under refrigerated condition in an airtight container. The raw propolis was grated using grater for further analyses. The crude propolis used was depicted in figure-1.



Fig 1: Crude propolis

Solvent extraction

Conventional method of extraction was carried out for the solvent extraction using different solvents. The solvents like hexane, chloroform, ethanol, acetone and water were used for the extraction. Ten grams of propolis sample was treated separately with 100ml of each solvent. At room temperature, the extraction was carried out overnight. Whatman No.1 Filter paper was used to collect the filtrate. Until further analysis, the filtrate was kept in the refrigerator in a dark environment and named as EE (ethanolic extract), AcE (acetone extract), HE (hexane extract), CE (chloroform extract) and AqE (aqueous extract) respectively. The overall methodology of extraction and analysis was represented in fig-2.

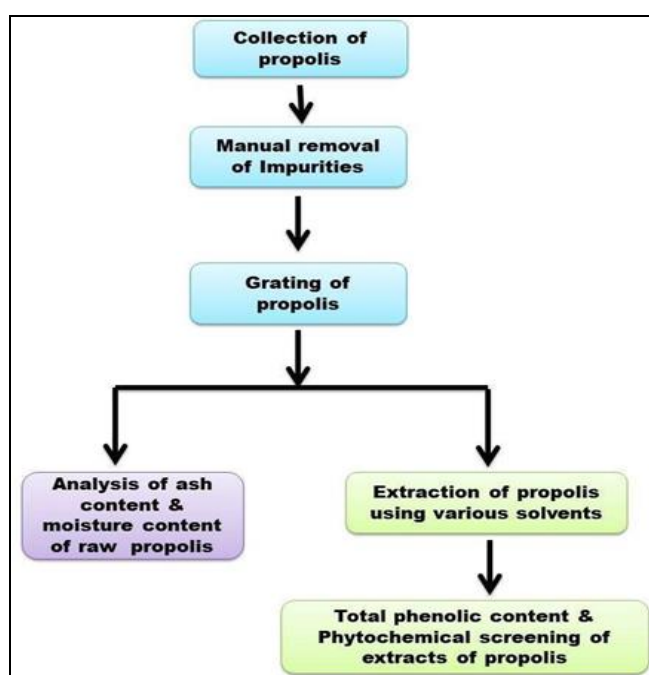


Fig 2: Method of propolis extraction & analysis-summary

Physicochemical parameters

All the analyses were done in triplicates and the mean was determined.

Ash Content

The AOAC technique was used to determine the ash content (Association of Official Analytical Chemists, 2000). About 5 gms of raw propolis sample was transferred into a previously dried and weighed silica crucible. The sample was charred on a hot plate and the crucible was transferred to a muffle furnace which was maintained at 550°C for 5 hrs. Then it was cooled in a desiccator and weighed. It was incinerated to a constant weight and the g% ash was calculated using the below formula.

$$\text{Ash content in g \%} = \frac{W3 - W1}{W2} \times 100 \quad \text{-----} \rightarrow 1$$

W1 = Initial empty weight of the crucible

W2 = Sample weight

W3 = Final weight of the crucible along with ash

Moisture content

The moisture content was calculated using the loss on drying method. About 5 gms of crude propolis sample was taken in the previously dried and weighed container. The sample was kept in the hot air oven which was maintained at 105°C for 3 hrs. The drying was performed in triplicates and the samples were weighed after cooling in a desiccator. The heating, cooling, and weighing process was repeated for 2 hours., until constant weight for achieved. (Ramos & Lima, 2015) [20].

$$\text{Moisture Content (\%)} = \frac{W1 - W2}{W1} \times 100 \quad \text{-----} \rightarrow 2$$

Where, W1= Initial weight of the sample, W2 = Dried weight of the sample.

Phytochemical Screening

By following the standard procedures shown in table-1, different propolis extractions were evaluated for the content of flavonoids, alkaloids, Saponins, carbohydrates, and tannins.

Test for tannins and phenols

Few drops of 0.1 % ferric chloride were mixed to the propolis extract. The presence of tannins and phenols was indicated by a brownish blue or green colour or precipitation. (Khalid *et al.*, 2018) [11] (Iqbal *et al.*, 2015) [9]

Test for alkaloids

A few millilitres of each extract were combined with 1 percent HCl (5 millilitres) and filtered after 5 minutes kept on a water bath. These filtrates were processed for further analysis. (Iqbal *et al.*, 2015) [9]

a. Wagner's test: In 10 ml of distilled water 2g of potassium iodide and 1.27 g of iodine were dissolved, with distilled water, the solution was diluted to 100mL. A dark brown colored precipitate was formed after a few drops of the wagner's solution were added to the filtrate, indicating the presence of alkaloids.

b. Mayer's test: Mayer's reagent (potassium mercuric iodide solution-1ml) was treated with few drops of the filtrate. Cream colored precipitate formation indicates the presence of alkaloids.

c. Dragendorff's test: Few ml of the filtrate was treated with dragendorff's reagent (Potassium bismuth iodide solution-1ml) the formation of orange red precipitate confirms the presence alkaloids.

Table 1: Phytochemical screening test procedures used for the propolis extract

Sl.No	Phytoconstituents	Test Procedure	Observation
1.	Test for Alkaloids: a. Mayer's Test	Few ml of extract + 5ml of 1% HCl (Heat for 5 mins & filter). Filtrate + 1 ml of Mayer's reagent (Potassium mercuric Iodide Solution)	Formation of cream colour precipitate.
	b. Wagner's Test	Wagner's Reagent (2g of Potassium iodide, 1.27g of Iodine dissolved in 100ml of water). 1ml of filtrate + few drops of wagner's reagent.	Formation of brown colour precipitate.
	c. Dragendorff's Test	Few ml of filtrate + 1ml of dragendorff's reagent (Potassium bismuth iodide solution)	Orange red precipitate formation.
2.	Test for Tannins & Phenols:	Filtrate + few drops of 0.1% ferric chloride	Brownish blue (or) Green colour formation
3.	Test for Flavonoids: Alkaline Reagent Test:	Filtrate+ 2-3 drops of sodium hydroxide solution	Formation of yellow colour. (When adding some drops of H2SO4 solution changes to colourless).
4.	Test for Carbohydrates: Molisch's test:	Filtrate + two drops of α -naphthol Solution+ few drops of Con. H2SO4 along the sides of the tube.	Formation of violet colour.
5.	Test for Steroids & Terpenoids: a) Salkowski test:	Filtrate + chloroform + Con. H2SO4 along sides of the tube.	Reddish brown coloration.
	b) Liebermann – Burchard Test:	Filtrate + chloroform + few drops of acetic anhydride (boiled in water bath & cooled) + Con. H2SO4 along sides of the tube.	Steroids-brown ring formation at the junction & upper layer changing to green colour. Terpenoids-formation of deep red colour.

Test for flavonoids (Alkaline reagent test)

About 2-3 drops of sodium hydroxide solution was added to the extract. The presence of flavonoids was indicated by the yellow colour development, and the solution in the test tube became colorless once a few drops of sulphuric acid were added. (Khalid *et al.*, 2018) [11].

Test for carbohydrates (Molisch's test)

Two drops of alcoholic α -naphthol solution were added to the filter. Then, along the tube's wall, a few drops of con. Sulphuric acid were carefully added. The presence of carbohydrates is indicated by the production of violet colour at the junction or interface of two liquids (Khalid *et al.*, 2018) [11].

Tests for steroids and terpenoids

a. Salkowski test: The propolis extract was shaken separately with chloroform, then con. sulphuric acid was added along the side of the test tube, resulting in a reddish brown colour at the interface, indicating the presence of terpenoids. (Iqbal *et al.*, 2015) [9].

b. Liebermann-Burchard test: In a test tube, each extract was shaken with chloroform, then added 2 ml of acetic anhydride, heated in a water bath, and quickly cooled in ice water. Then, to the sides of the tube, con. sulphuric acid was introduced. The presence of steroids is shown by the production of a brown ring at the junction of two layers, with the top layer turning to green colour, whereas the presence of terpenoids is indicated by the formation of a deep red color (Iqbal *et al.*, 2015) [9].

Biochemical properties

Total phenolic content

With slight modifications, the total phenolic content of propolis extracts was measured using Folin-Ciocalteu's method (Belmehtdi *et al.*, 2021) [5]. Gallic acid (50-1000 g/ml) was used to create a standard curve. 1.0 ml of folin

ciocalteu's reagent and one ml of 10% sodium carbonate was added to 0.1 ml of propolis extracts (EE, AcE, HE, AqE, CE). With distilled water, the final volume in all of the test tubes was increased to 12ml. The absorbance was measured at 760nm after 2 hours of incubation at Room Temperature (RT). The results were given in mgs of Gallic acid equivalents (GAE) per g of propolis extract.

Results and Discussion

Physicochemical characterization

The ash content and moisture content of the crude propolis sample was 4.98 and 3.46 respectively and are summarized in the Table-2. The results were in accordance with the previous research work with little differences. These differences might be due to the collection method, flora and the collection period (Pant *et al.*, 2021) [19]. Excessive water might cause hydrolysis of active compounds, favouring microbial growth, hence high moisture content is critical for the preservation of propolis samples (Ramos & Lima, 2015) [20].

Table 2: Ash content & Moisture content values of the crude propolis

Sample	Ash (%)	Moisture (%)
Raw Propolis	3.46±0.05	4.98±0.01

Values are means \pm SD of triplicate analysis.

Phytochemical Screening

Secondary metabolites such as tannins, phenols, flavonoids were analysed in phytochemical analyses of several propolis extracts, as shown in table 3. The phytochemical compounds are known to be responsible for the bioactive activities. For example, terpenoids are the volatile components of propolis that are known to produce odour or aroma. It's also useful for the biological properties, like anti-inflammatory and antimicrobial properties of propolis (Nur *et al.*, 2021) [18]. Flavonoids and phenolic chemicals, which have been shown to be capable of scavenging free radicals,

are the main pharmacologically active components of propolis (Mohammadzadeh *et al.*, 2007)^[16]. The presence of alkaloids indicates the chance for the presence of antinutrient compounds in propolis extracts.

Table 3: Results for the phytochemical analyses.

Sl.No	Phytoconstituents	Observation (Presence / Absence)				
		EE	ChE	HE	AcE	AqE
1.	Test for Alkaloids: a. Mayer's Test	+++	++	+++	+++	++
	b. Wagner's Test	+++	+++	++	++	++
	c. Dragendorff's Test	+++	++	++	+++	++
2.	Test for Tannins & Phenols:	+++	+	++	+++	+
3.	Test for Flavonoids: Alkaline Reagent Test:	+++	++	+++	++	++
4.	Test for Carbohydrates: Molisch's test:	+++	+++	-	-	++
5.	Test for Steroids & Terpenoids: a. Salkowski test:	+++	++	-	-	+
	b. Liebermann-Burchard Test: 1. Steroids 2. Terpenoids	+++	+	+	-	-
		+++	++	+++	-	-

+++ = Presence of phytoconstituents: ++ = presence of phytoconstituents within 3 mins: + = presence of phytoconstituents within 5 mins: - = absence of phytoconstituents:

Total phenolic content

Using spectrophotometric methods, the total phenolic content of various solvent extracts of propolis was determined. The findings were represented in μg GAE/g (Gallic acid equivalent) of propolis and depicted in figure 3. The phenolic content of propolis ranged from 123.50 ± 0.30 to 73.94 ± 0.35 μg GAE/g of propolis. The phenolic concentration of the ethanolic extract was found to be the greatest. Whereas the aqueous extract was the lowest. The descending order of the phenolic content of propolis extracts were as follows: EE > AcE > HE > ChE > AqE. The results of the total phenolic content of the propolis extracts are in accordance with the previous research work in which the four moroccan propolis samples collected from different regions analysed for their phenolic, flavonoid and antioxidant activities (Belmehdi *et al.*, 2021).

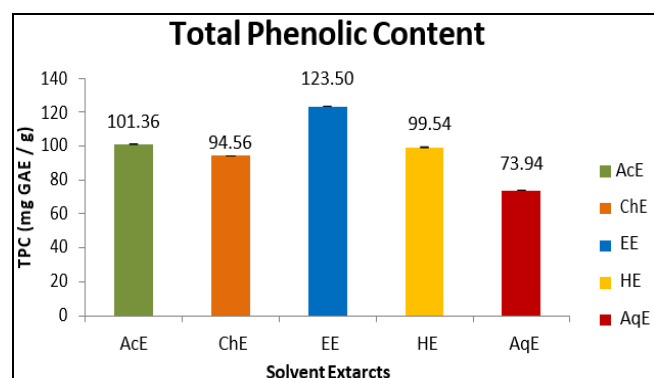


Fig 3: Total phenolic content of solvent extracts of propolis

Conclusion

The present study concluded that the secondary metabolites like flavonoids, phenols, carbohydrates, terpenoids, and alkaloids are present in the various solvent extracts of propolis. The total phenolic content noticed to be high in ethanolic propolis extract (123.50 ± 0.30 μg GAE/g). These phytoconstituents render the therapeutic properties of propolis collected from Indian origin. Furthermore,

biological studies and antinutrients quantification are required related to our research to analysis the therapeutic properties and its responsible secondary metabolite component.

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