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Laboratory analysis of propolis obtained from *Apis mellifera* colonies in different districts of Himachal Pradesh, India

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Abstract
Honey bees are one of the most beneficial organisms for human beings. Bee hive products are interesting for their nutritional value and healing power. Honey is the most important product that is obtained from honeybees, however, many other products like wax, propolis, venom, pollen and royal jelly also have immense importance. Among all, propolis is of great medicinal importance, It is a natural composite resin which is collected by *Apis mellifera* from various plants. Propolis is collected by beekeepers however scientific methods of collection are still lacking. The propolis has been tested by various researchers for it's therapeutic properties. It is mainly composed of polyphenol, flavnoids and quercetin substance which makes it medically important. During the present study, some of the medicinal activities were analyzed in Indian propolis obtained from *Apis mellifera* were analyzed. It was found that all the samples collected from different eco-climatic conditions possess therapeutic properties with varying amounts of polyphenols and flavnoids etc.

Keywords: propolis, honeybee, India, polyphenols, alkaloids

Introduction
Honeybees belong to order Hymenoptera, family Apidae and genus *Apis*, distributed all over the globe in all type of climatic conditions. They have become an integral part of agriculture and rural economy both as pollinators and honey producers. Keeping of bees in artificial bee hives is called beekeeping. It has strong connections with horticulture, forest and agriculture industry, which is beyond the ordinary realms of industry. Humans are highly benefited from honeybees as they are providing valuable products as well as contributing in pollination. Plants, including different crops, prosper and the bees flourish sheltered by humans, providing honey and various other products such as beeswax, propolis and royal jelly are main byproducts of beekeeping. Assessment of honey, wax, propolis and other products are very good for the monitoring of the quality of environment (Kumar *et al*., 2022) [17]. Propolis is a product prepared by honeybees from resins obtained from plants. It is used in cosmetics, medicine and food. Since ancient times (300 BC), propolis has been used in traditional medicine and it is still used as a remedy in folk medicine and as a constituent of cosmetics in many parts of the world. Due to various properties of propolis it has been used in various medicines. Bees use propolis as sealing agent (Garcia-Viguera, 1992), to fill in the holes/crevices, and to fix dead invaders (Marcucci, 1995) [19] and to prevent spoilage of small animlas/creatures which are killed by bees invasion of hive by them (Brumfitt *et al*., 1990) [5]. It is believed that propolis hardens the walls as well as contribute in the attainment of an internal sterilized conditions (Ghisalberti, 1979). Propolis is well known for its antioxidative, antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant and antiallergic properties due to presence of more than 150 poyphenolic compounds. Chemical composition of propolis may vary due to different factors like geography, collection time, methods of collection and plant source (Bankova *et al*., 2002) [3] In general, propolis incorporates resin and balsam (40-50%), beewax (25-30%), essential oils (10%), and pollen, minerals and other substances (Ghisalberti *et al*., 1979) [10]. Several investigations have been made on therapeutic properties of propolis such as antibacterial (Bhadauria *et al*., 2007), anticarcinogenic, healing, anaesthetic (Ghisalberti 1979) [10], antiprotozoan and anti-viral (Guler *et al*., 2003). Propolis is a well-known honey bee product having complex chemical composition. Usually, it is made by sticky and balsamic material gathered by honeybees from flower buds, trees, shoots, and other resinous exudates of plants. Honeybees use propolis to protect their hive from microorganisms, seal cracks, sterilize queen bee seats, and wrap them with enemy invaders (Kumar *et al*., 2022) [17]. About 300 years B.C., propolis is treated as a wonderful therapeutic used in many medicines worldwide (Bankova 2005) [1]. Silva-Carvalho 2015 [29] revealed that this product is one of the natural drugs used from ancient times by different cultures. Many products are made from propolis, such as shampoos, antiseptic, chocolates, candies, lotion, and toothpaste, etc., which are being commercialized globally (Ramos and Miranda 2007) [24]. Propolis is mainly used as an immunity booster (Wagh 2013) [31] and in healing infections. Its antibiotic activity blocks out the entry of

viruses, bacteria, and other organisms into the hive. It has been studied that propolis has some antioxidant, liver defending, anti-inflammatory (Daleprane and Abdalla 2013) [8], and anticancer properties (Sawicka 2012) [26]. It has been used in the field of dermatology for the treatment of leg sores, neurodermatitis, and dermatosis. However, propolis has also been used in advanced pulmonary tuberculosis and non-specific bronchitis, especially when the customary therapy has failed. Campos *et al*., 2014 analyzed the antimicrobial, antioxidant and cytotoxic activities of propolis from *Melipona orbignyi*. Propolis obtained from the stingless bees is also well known for its medicinal properties; however, further studies are required to demonstrate its effects in detail. The chemical composition and other medicinal properties were studied in the propolis obtained from the stingless bee *Melipona orbignyi*, in Brazil. The analysis indicated that ethanolic extract of propolis (EEP) have more than 35 aromatic acids, phenolic compounds, alcohols, terpenes and sugars (Campos *et al*., 2014). This extract was found active against 36 bacterium strains. It also exhibited antioxidant property by scavenging free radicals. It also inhibited hemolysis and lipid peroxidation in erythrocytes in human beings. Additionally, it also promoted cytotoxic activity and primarily 39 necrotic deaths in K562 erythroleukemia cells. These results concluded that propolis from *M. orbignyi* has significant therapeutic potential for the treatment and/or prevention of diseases related to microorganism activity and tumor cell proliferation etc.

Methodology

Collection and extraction of propolis

The study was carried out in the laboratory of Zoology Division, Career Point University, Hamirpur, India. The propolis was collected from three different locations i.e. Kangra, Mandi and Hamirpur of Himachal Pradesh. Propolis so collected was crude (Fig.1) and cannot be used as such. Therefore, it was stored in refrigerator until its extraction with suitable solvent to remove the inert materials (Pietta *et al*., 2002) [23]. In the present study, ethanolic (EEP), was prepared by the method of Orsi *et al*. (2015) [22]. Thirty grams of propolis was grinded and 70% ethanol/methanol was added to make the volume upto 100 ml. The constituents were mixed well under at room temperature with reasonable shaking. After about seven days, it was filtered and kept for drying. Then it was stored in refrigerator till further use. The percentage yield of propolis was calculated by the formula:
Percent yield (%): The amount of pure product recovered/Amount of crude material usedx100.
The dilutions of propolis were prepared as per the requirement of study.

Qualitative phytochemical screening

Crude propolis extract was analyzed for detection of different phytochemicals i.e. alkaloids, flavonoids, saponins, and tannins. Color change or formation of precipitate was used as a marker of positive response to these tests (Obadoni and Ochuko, 2001) [21].

Detection of alkaloids

Propolis extract (15mg approx.) was mixed with 2 ml of 5% HCl. It was homogenized and mixed well so that contents gets mixed well properly with the HCl. It was then filtered and three aliquots of this mixture were prepared separately. The drops of reagents i.e. Wagner, Mayer, Bouchardat, and Dragendorff were added to each tube. A red-brown precipitate with Wagner, yellowish-white precipitate with Mayer and brown precipitate with Bouchardat and red-orange precipitate with Dragendorff reagent revealed the presence of each metabolite.

Detection of flavonoids

1. Shinoda's test for the detection of flavonoids: 0.5 mili gram of propolis extract was firstly liquefied in 1ml of isopropyl alcohol. To this, 1 ml of absolute alcohol and three drops of conc. HCl were added. The appearance of red color confirmed the presence of flavones. If red color do not appears, then small amount of magnesium is to be added that produces either orange, red, or magenta color that also confirms the presence of flavones in the propolis extract.

2. Test for flavonoids: Approximately 0.5mg of extract was liquified in 2 ml of isopropyl alcohol. To this, 10% NaOH w/v solution was added drop wise. Appearance of yellow-red, coffee-orange, and purple-red color, confirms the presence of xanthones and/or flavones, flavonols, chalcones, and anthocyanin respectively.

Foam test for saponins: 20mg propolis extract was liquified in 2 ml of isopropyl alcohol. From this, 2 ml of extract was taken in a clean test tube and shaken properly. Few drops of olive oil were added shaking the mixture continuously. A foamy layer appears that reveals the presence of saponins in the propolis extract.

Detection of glycosides

1. Liebermann's test for detection of glycosides: 0.5mg propolis extract was liquified in 2 ml of acetic acid to which chloroform was added slowly. It was then allowed to cool. After cooling, few drops of conc. H_2SO_4 were added to it. Green color formation reveals the presence of steroidal part of glycosides.

2. Keller-Kaliani test for the detection of glycosides: 10 mg propolis extract was dissolved in 5 ml of distilled H_2O. 4 ml of glacial acetic acid, 2% of $FeCl_3$, 1. 5 ml of conc H_2SO_4 was added to it slowly. A brown ring formation in sandwich like manner confirmed the presence of steroidal glycosides.

3. Salkowski's test for detection of glycoside: 5 mg of propolis extract was dissolved in distilled H_2O. Then 2.5 ml of concentrated H_2SO_4 was added to it. Formation of reddish brown color confirms the presence of steroidal aglycone part of glycoside in propolis extract.

Test for tannins: 10mg propolis extract was taken and dissolved in 1 ml of 70% ethanol. To this, 2 ml of distilled H_2O, 10 drops of 10% ferric chloride aqueous solution (w/v) was added. The appearance of blue or green color reveals the presence of tannins in the propolis extract.

Quantitative assessment of phytochemicals of propolis

Antioxidant Activity: **DPPH free radical scavenging assay**

The free radical scavenging activity in propolis extract was determined by 2,2-diphenyl-1-picryl-hydrazyl (DPPH). The potential of DPPH free radical scavenging activity was calculated by using the below given formula:

DPPH scavenging activity (% of inhibition) = $(A_o\text{-}A_1) \times 100/A_o$

Where,

A_o is the absorbance of the control

A_1 is the absorbance of the sample extract

Assessment of total polyphenolic content

The total polyphenolics in propolis extract were calculated by the Folin-Ciocalteu method of Sidduraju and Becker (2003) [27].

Assessment of alkaloids

The alkaloids in propolis extract were estimated by Obadoni and Ochuko (2001) [21] method with minor modifications.

Determination of flavonoid content

The flavonoid content in propolis extract was assessed by the method of Zhishen *et al*., (1999).

Determination of flavones and flavonol content

Total flavones and flavonols were calculated using aluminum chloride method.

Antibacterial activity

Antibacterial potential of the propolis extract was quantified using turbidity assay.

Antioxidant activity: The antioxidant capacity of all the propolis extract samples was determined using the 2, 2-diphenyl1-picrylhydrazyl (DPPH) free radical scavenging assay as per Moreira *et al*. (2005) with slight modifications. Radical scavenging activity (RSA) of the propolis particles was determined using the following formula:

RSA (%) = (1-As/A0) x 100

Where A0 and AS is the absorbance of mixture without and with the propolis particles, respectively.

Result and Discussion

Determination of Percentage Yield

Results showed a significant difference in the percentage yield. Among solvents tested hydroalcoholic extract showed higher percentage yield 78% in Kangra followed by 64% and 55% in Hamirpur and Mandi respectively (Table 1).

Phytochemical (Qualitative) Screening of Extract

The screening of phytoconstituents in the hydroalcoholic extract of propolis collected from three different districts Kangra, Hamirpur and Mandi showed the presence of alkaloids, steroids, carbohydrates, flavones, tannins, saponins, glycosides, proteins and amino acids and phenols in high, moderate and low concentration as depicted in table 2. It was observed that phytochemicals were present in all the samples obtained from different locations of state when tested with hydroalcoholic and methanolic extract. However, less or no presence was also reported with chloroform and aqueous extracts.

Quantitative Assessment of Phytochemicals

Total Polyphenolic Content

The total polyphenolic content was quantified in the propolis extract collected from Kangra, Hamirpur and Mandi were 60.5, 48.1, and 46.4 mg GA/g (Table 3) respectively.

Total Alkaloids Content

The total alkaloid content was quantified in the hydroalcoholic leaf extract of propolis. The total alkaloid content in the propolis extract collected from Kangra, Hamirpur and Mandi were 70.9, 55.9 and 47.9 mg/g (Table 3).

Total Flavonoids Content

The hydroalcoholic extract of propolis extract collected from Kangra, Hamirpur and Mandi were evaluated for total flavonoid content. The total flavonoid content in the propolis extract collected from Kangra, Hamirpur and Mandi were measured about 30.32, 24.2, and 18.1 mg QE/g (Table 3).

Total Flavones and Flavonols

The hydroalcoholic extract of propolis extract collected from Kangra, Hamirpur and Mandi were evaluated for total flavones and flavonols content. The total flavones and flavonols content in the propolis extract collected measured about 20.75, 8.75, and 4.54 mg RE/g (Table 3).

Antioxidant Activity: DPPH Free Radical Scavenging Assay

DPPH free radical scavenging activity of propolis extract collected was observed as shown in figure 1, 2 & 3. The propolis extract at different concentrations ranged from 1 to 100μg/ml was estimated with vitamin C (ascorbic acid) as a standard. The results revealed % inhibition by propolis in district Kangra as 32.01 ± 0.82, 36.78 ± 0.97, 38.88 ± 0.94, 49.99 ± 1.01, 57.88 ± 0.91, 59.91± 0.99, 62.98 ± 0.93, 65.93 ± 1.51, 79.98 ± 0.95, 83.02 ± 0.99, 86.04 ± 0.96 and 92.88 ± 0.97 at 1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80 and 100 µm/ml concentration. In Hamirpur, the % inhibition by propolis was recorded to be 27.01 ± 0.92, 32.78 ± 0.97, 34.88 ± 0.94, 44.99 ± 1.01, 50.89 ± 0.91, 51.94± 0.99, 56.98 ± 0.93, 58.91 ± 1.51, 77.99 ± 0.95, 80.02 ± 0.99, 82.04 ± 0.96 and 88.86 ± 0.97. In Mandi district, the values obtained for % inhibition by propolis were 25.01 ± 0.89, 29.88 ± 0.99, 32.83 ± 0.90, 40.94 ± 1.05, 50.79 ± 0.89, 52.94± 0.97, 57.87 ± 0.94, 63.91 ± 1.56, 68.89 ± 0.99, 77.04 ± 0.95, 79.04 ± 0.98 and 85.78 ± 0.99 at 1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80 and 100 µm/ml concentration.

The propolis samples were found to have flavonoids, flavones, polyphenols and alkaloids. The free radical scavenging activity was also reported in all the samples. The results were in accordance with Bankova *et al*., 2000 [3], Kujumgiev *et al*., 1999 [15], Silva *et al*., 2012 [28], Kocot *et al*., 2018 [14], Grecka *et al*., 2019 [11], Kharsany *et al*., 2019 [13], Touzani *et al*., 2019 [30], Dezmirean *et al*., 2021 [9], Rivera-Yanez *et al*., 2021, Kumar *et al*., 2022 [17], who reported the presence of plant secondary metabolites in propolis which are responsible for therapeutic properties of propolis.

Table 1: Percentage (%) yield screening of propolis collected from various districts of Himachal Pradesh

Sr No.	Locations	% Yield of Extract
1	Kangra	78%
2	Hamirpur	64%
3	Mandi	55%

Table 2: Qualitative phytochemical screening of propolis collected from various districts of Himachal Pradesh

Kangra						
Sr. No.	**Phytochemicals**	**Test**	**Hydroalcoholic Extract**	**Methanolic Extract**	**Chloroform Extract**	**Aqueous Extract**
1.	Alkaloids	1. Mayer's Test	++++	++	+	-
		2. Dragondroff's Test	++++	+	-	++
2.	Steroids	1. Salkowski's Test	+++	++	-	+++
		2. Liebermann's Burchard's Test	+++	++	+	-
3.	Carbohydrates	Molisch Test	+	++	++	+
4.	Flavones	1. Aqueous Test	++++	++	+	+
		2. H_2So_4 Test	++++	++	+	+
5.	Tannins	Ferric chloride Test	+++	++	+	+++
6.	Saponins	Aqueous Test	+++	++	+	++
7.	Glycosides	1. H_2So_4 Test	+++	+++	-	+
		2. Kellar Kilani Test	+++	++	++	+
8.	Proteins and Amino Acids	Millon's Test	+++	+	-	
9.	Phenols	Phenol Test	+++	++	-	+
Hamirpur						

1.	Alkaloids	1. Mayer's Test	+++	+++	+	-
		2. Dragondroff's Test	++	+	-	++
2.	Steroids	1. Salkowski's Test	++	++	-	+++
		2. Liebermann's Burchard's Test	++	++	+++	-
3.	Carbohydrates	Molisch Test	++	+++	++	+
4.	Flavones	1. Aqueous Test	++	++	+	+
		2. H_2So_4 Test	+++	+++	+	+
5.	Tannins	Ferric chloride Test	+++	++	+	+++
6.	Saponins	Aqueous Test	+++	+++	+	++
7.	Glycosides	1. H_2So_4 Test	+++	+++	-	+
		2. Kellar Kilani Test	++	++	++	+
8.	Proteins and Amino Acids	Millon's Test	+	-	-	+
9.	Phenols	Phenol Test	+++	++	-	+
Mandi						
1.	Alkaloids	1. Mayer's Test	++	+++	+	-
		2. Dragondroff's Test	+++	+	-	++
2.	Steroids	1. Salkowski's Test	+++	++	-	+++
		2. Liebermann's Burchard's Test	++	++	+++	-
3.	Carbohydrates	Molisch Test	-	+++	++	+
4.	Flavones	1. Aqueous Test	++	++	+	+
		2. H_2So_4 Test	++	++	+	+
5.	Tannins	Ferric chloride Test	++	++	+	+++
6.	Saponins	Aqueous Test	++	++	+	++
7.	Glycosides	1. H_2So_4 Test	++	+++	-	+
		2. Kellar Kilani Test	+++	++	++	+
8.	Proteins and Amino Acids	Millon's Test	+	+	-	+
9.	Phenols	Phenol Test	+++	++	-	+

Symbols: High conc. (+++); moderate conc. (++); low conc. (+); absence (-).

Table 3: Quantitative phytochemical screening of propolis collected from various districts of Himachal Pradesh

Phytochemicals (per gram of extract)	Concentration (mg/g)
Kangra	
Polyphenols	60.5
Flavonoids	30.32
Flavones and flavonols	20.75
Alkaloids	70.9
Hamirpur	
Polyphenols	48.1
Flavonoids	24.2
Flavones and flavonols	8.75
Alkaloids	55.9
Mandi	
Polyphenols	46.4
Flavonoids	18.1
Flavones and flavonols	4.54
Alkaloids	47.9

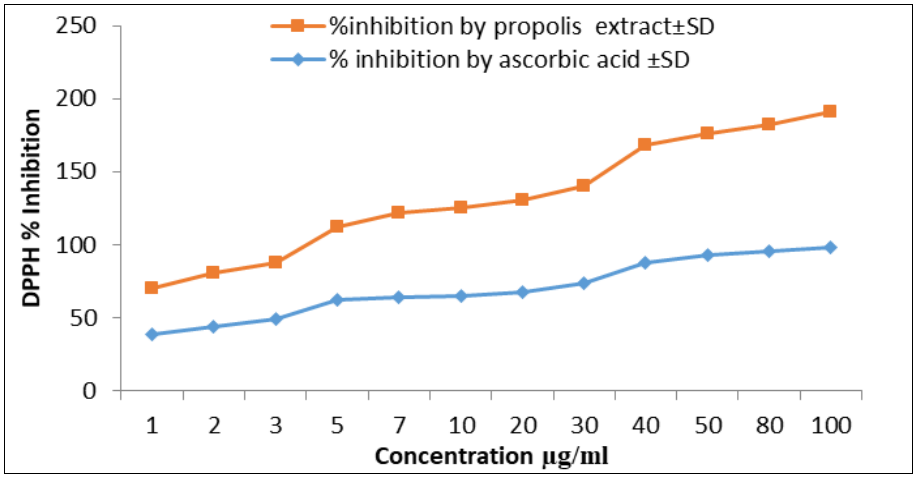

Fig 1: % inhibition by propolis in district Kangra samples

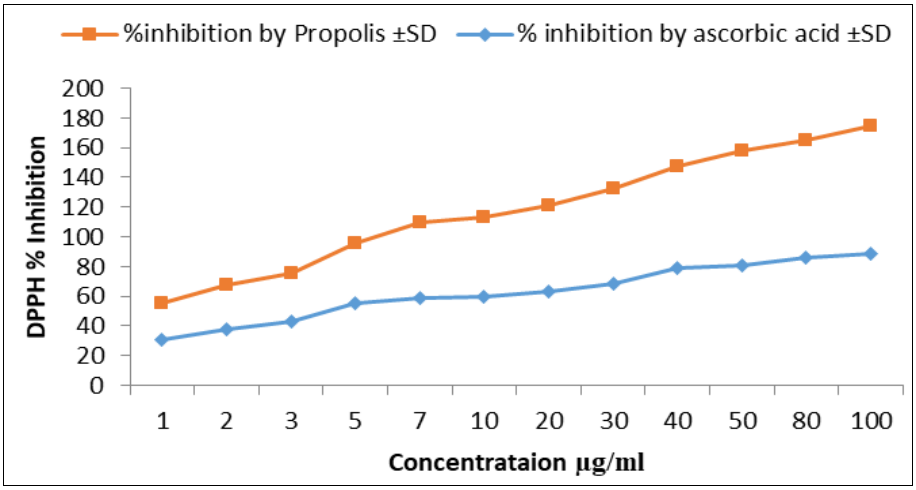

Fig 2: % inhibition by propolis in district Hamirpur samples

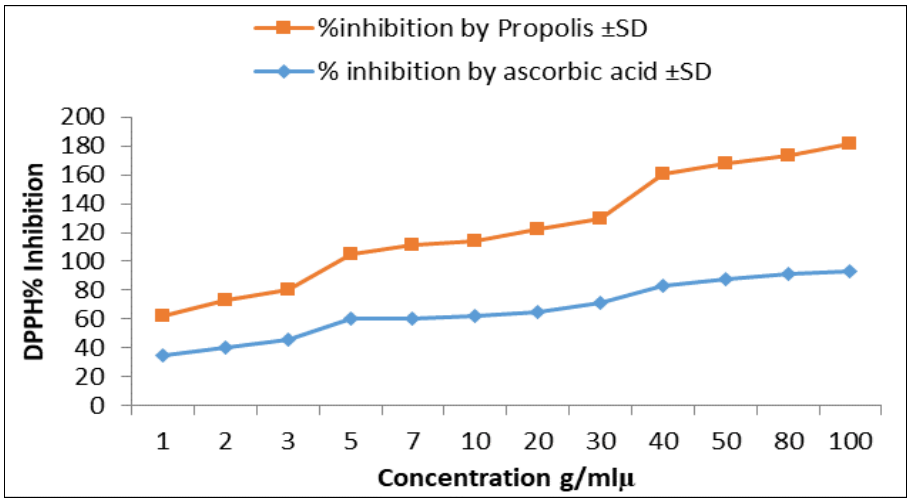

Fig 3: % inhibition by propolis in district Mandi samples

Conclusion

It may be concluded at the end of study that emphasis ay be given to extract valuable products from beekeeping other than honey. These products are of great medicinal importance and may be used in modern therapeutic system. Propolis being having strong therapeutic properties may be explored scientifically so that is can be utilized to the fullest and beekeeping economics may also be improved.

Conflict of Interest

It is declared that there is no conflict of interest among the authors.

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