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Research Article

Comparative Study of the Phenolic Profile and Antibacterial Activity of Honeybee Propolis from Different Regions of South Punjab, Pakistan

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Authors' Contributions
SI conducted the research. SS supervised the study. EAH co-supervised the study. SS and EAH provided la-boratory facilities and guidance for research work. MA provided laboratory facilities and guidance for HPLC run.

Keywords
Ciprofloxacin, RP-HPLC, propolis, and both Gram-positive and Gram-negative microorganisms

Abstract | There are numerous adverse effects of the COVID-19 pandemic on both public health and the global economy. The population of the third world has been struck particularly severely. The objective of this research is to identify affordable medical resources that can be utilized to treat prevalent bacterial infections that impact the local population. Ten propolis samples from South Punjab were analyzed. Phenolic acids (Sinapic acid, Caffeic acid, and gallic acid) and flavonols (kaempeferol, Quercetin, and myricetin) were quantified by means of UV detection in reverse phase high-performance liquid chromatography (RP-HPLC). The propolis samples exhibited substantial variation ($P < 0.05$) in the total flavonol and phenolic acid contents, which span a range of 52 to 183 mg/kg dried matter respectively. The agar well diffusion method was employed to assess the additional in vitro antibacterial activity of the samples against two Gram positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*) and three Gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium*). The effectiveness of the samples was greater against Gram positive bacteria (MIC = 0.3 mg/mL) compared to Gram negative bacteria (MIC = 0.9 mg/mL). Upon testing the synergy between Ciprofloxacin and one of the samples, it was found to be exceptionally potent.

Novelty Statement | As the use of indigenous materials is of global interest, so we chose propolis- a bio-waste from honeybee hives to investigate its therapeutic potential. The present work is the first report on the phenolic profile and antibacterial activity of Propolis from different regions of South Punjab, Pakistan.

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Introduction

An increasing global concern is the development of antibiotic resistance against pathogenic microorganisms (Davies and Davies, 2010). Moreover, as the adverse effects of contemporary synthetic pharmaceuticals continue to escalate, individuals are reverting to traditional herbal remedies on the grounds that they are more secure (Khan *et al.*, 2018). Asia is a continent whose climate is optimal for supporting its distinct flora and fauna. The vast majority of nations in this area are underdeveloped. Numerous individuals are unable to pay for costly prescriptions due to poverty. As a result, an investigation into the antibacterial medications from

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resources available in these nations is required (Shahbaz *et al.*, 2015). Throughout history, honey bee products have been utilized by humans for medicinal intentions (Bankova *et al.*, 2018). According to the literature (Eteraf-Oskouei *et al.*, 2013; Ghisalberti, 1979; Kuropatnicki *et al.*, 2018), ancient Greeks, Romans, Arabs, Europeans, and even Asians utilized these products to treat a wide range of ailments. A variety of health benefits are associated with propolis, a chemical produced by honey bees (Kuropatnicki *et al.*, 2013). Honey bees (*A. mellifera*) use propolis for protection of their hives (Bankova *et al.*, 2020). Combining bee secretions and beeswax with exudate from tree flowers, fluid flows, or other botanical sources, produces resinous propolis (Simone-Finstrom *et al.*, 2017). This construction protects the hive against dampness, insects, reptiles, birds, and snakes by enclosing any unwanted openings (Figure 1). Ribeiro *et al.* (2020) say propolis' volatile components give it a pleasant sweat smell and boost its bioactivity. Propolis may be red, dark brown, yellow, or green depending on its botanical origin and season (Gur *et al.*, 2020; Santos, 2020).

s: Honeybee's colonies and honeybees making propolis.

Because of its acceptance in folk medicines, it has now become the subject of study. The chemical makeup of propolis has been studied since the early 1900s (Oroian *et al.*, 2020). Over three hundred propolis components have been separated and extracted so far, but new ones are always being discovered. Propolis has a complicated chemical composition and structure (Al-Ghamdi *et al.*, 2017; Thirugnanasampandan *et al.*, 2012). According to Nada a and Alaa (2015), raw propolis contains 10% essential oils, 5% pollen, 50% resin, and 30% wax. Propolis contains flavonoids, phenolic acids, esters, terpenes, caffeic acid phenethyl ester (CAPE), and anthraquinones as its main bioactive components. These active components combine in different proportions to make it an effective antibacterial agent (Rivero-Cruz *et al.*, 2020). According to Pujirahayu *et al.* (2014), flavonoids and phenolic acids are used to evaluate propolis quality. Their concentration depends on the plant source and extraction methods used (Paviani *et al.*, 2013). These chemicals are produced by microbial infections and environmental stress and have several biological properties.

Mašek *et al.* (2018) and Ghisalberti *et al.* (1977) identified bactericidal effects in propolis. By preventing bacterial cell division, it may harm the cytoplasm or cell wall and limit protein synthesis (Al-Fahdawi, 2015; Rufatto *et al.*, 2011). Propolis boosts the body immune system and give us a natural defence against harmful pathogens (Morsy *et al.*, 2021). The chemical composition and biological activity of propolis depend on its botanical and geographical origin (Cunha *et al.*, 2004; Markham *et al.*, 1996), therefore discovering novel bioactive compounds in unknown propolis variations is vital. Numerous researches showed its antibacterial, hepatoprotective, antiviral, anti-inflammatory, and antifungal effects. Presently the antimicrobial potential of propolis is a subject of interest both in animal and plant research (Ali *et al.*, 2017). Previous investigations on animal and human models have shown that propolis is non-toxic and has pharmacological capabilities (Jalali *et al.*, 2020; Zampini *et al.*, 2021). Punjab, being an agricultural land has several plant species and propolis as gifts.

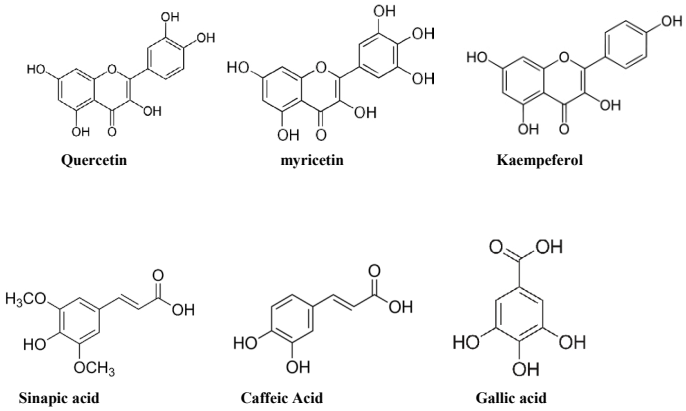


Figure 2: Detected flavonoids and phenolic acids.

Limited research has been undertaken thus far regarding this significant bee product originating from this area (Khan *et al.*, 2018). The purpose of this study is to determine whether ten propolis samples contain flavonols (kaempeferol, quercetin and myricetin) and phenolic acids (sinapic acid, caffeic acid, and gallic acid) (Figure 2). Additionally, two Gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and three gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium)* were subjected to in vitro assessments of their antibacterial activity (Cibanal *et al.*, 2020; Hochheim *et al.*, 2020; Surek *et al.*, 2020). The selection of these bacteria was based on the frequency with which they caused food poisoning, diarrhea, cutaneous infections, urinary tract infections, and respiratory infections among the local population. There have been reports of fever and vomiting due to these microbes (Miryan *et al.*, 2021; Ghoshal *et al.*, 2021; Lind *et al.*, 2013). Ciprofloxacin, a fluoroquinolone antibiotic with a broad spectrum of activity (Hamdan *et al.*, 2021), was also examined for potential synergy with propolis in one of the

samples, given that propolis has been shown to synergize with a variety of antibiotics in the literature (Fernandes Jr *et al*., 2005). It halts bacterial cell division by eliminating essential enzymes required for bacterial DNA separation, including topoisomerase II and topoisomerase IV (also known as DNA gyrase) (Khondker *et al*., 2021).

Materials and Methods

Sample collection

Ten propolis samples were collected from beekeepers during 2018 to 2020, from South Punjab (Dera Ghazi Khan (Latitude 30.0489 ^{0}N, Longitude 70.3301 ^{0}E), Bahawalnagar (Latitude 30.0025 ^{0}N, Longitude 73.2412 ^{0}E), Layyah (Latitude 30.9693 ^{0}N, Longitude 70.9428 ^{0}E), Vehari (Latitude 29.9719 ^{0}N, Longitude 72.4258 ^{0}E), and Multan (Latitude 30.1575 ^{0}N, Longitude 71.5249 ^{0}E) in summer as well as in winter (P1 to P10). Samples were carefully transported, and stored in refrigerator at -20˚C until further use.

Chemicals

Standards of phenolic acids (sinapic acid, caffeic acid, gallic acid) and flavonoids (myricetin, quercetin, kaempeferol) were provided by Sigma Chemicals Co. (St. Louis, MO, USA). The additional compounds employed in this investigation, namely hydrochloric acid, ethanol, acetonitrile, and methanol, were supplied by Merck (Darmstadt, Germany). Stock solutions of flavonoids and phenolic acids were prepared in methanol at respective concentrations of 100 µg/ml and 10 µg/ml. Working solutions and stock were stored in the dark within a refrigerator at 4 ˚C. Calibration curves were constructed by comparing peak area to concentration.

Test microorganisms

Staphylococcus aureus (ATCC 25923), *Bacillus cereus* (ATCC 14579), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 700931).

Propolis extraction

Each individual sample weighed 30g. The material was subsequently filtered through Wattman filter paper after being submerged in a 1:10 solution of 95% ethanol and agitated for 24 hours at 30 degrees Celsius in an Irmeco swaying incubator (Germany). The aforementioned procedure was iterated triple. The filtrates were combined and desiccated at 70-75 degrees Celsius in a Heilhoff Laborta 4000 effective rotary evaporator. Table 1 presents the % yield of all extracts (P 1-10) calculated as.

$$\%Yield = \frac{weight\ of\ extract}{weight\ of\ raw\ sample}$$

HPLC analysis

Chromatographic analysis was performed utilizing an Agilent 1100 series HPLC system equipped with the following components: A quaternary pump (G1311A Quat pump), a DAD detector (G1315B DAD), an auto-sampler/autoinjector (G1313A ALS), and a column compartment (G1316A Colcom). At a flow rate of 1.0 mL/min, the mobile phase was utilized; for flavonoids, it comprised 50% tri-fluoroacetic acid (0.3%), 30% acetonitrile, and 20% methanol; for phenolic acids, it was 40% tri-fluoroacetic acid (0.3%), 40% acetonitrile, and 20% methanol. Before elution, the mobile phase underwent degassing and filtration via a 0.45 m Nylon membrane filter. The isolation and detection of flavonoids and phenolic acids were accomplished by employing isocratic elution and detection at 360 nm and 280 nm, respectively. Through a comparison of their retention periods with those of authentic standards (Table 1), the compounds were identified. The software

Table 1: % Yield and HPLC analysis of propolis samples from South Punjab.

Sample code	% yield	Flavonoids (µg/mg)			Phenolic acids (µg/mg)		
		Myricetin	Quercetin	Kaempeferol	Sinapic acid	Caffeic acid	Gallic acid
P 1	12.27	0.16(16)±0.030	0.31(31)±0.022	0.05(5) ±0.004	0.25(25)±0.012	0.05(5)±0.021	1.10(110)±0.010
P 2	8.05	0.26(26)±0.006	ND*	ND*	0.20(20) ±0.001	0.08(8)±0.011	0.36(36) ±0.001
P 3	13.33	0.23(23)±0.020	0.08(8) ±0.020	0.1(10) ±0.004	0.30(30) ±0.001	0.32(32)±0.001	1.21(121)±0.011
P 4	9.34	0.20(20)±0.040	ND*	0.05(5) ±0.004	0.35(35) ±0.001	0.05(5) ±0.001	0.85(85) ±0.011
P 5	13.34	0.15(15)±0.004	ND*	0.27(27)±0.002	0.28(28) ±0.001	0.19(19)±0.001	1.10(110)±0.013
P 6	9.02	0.05(5) ±0.004	0.12(12)±0.001	0.20(20)±0.001	ND*	0.20(20)±0.001	0.50(50) ±0.023
P 7	13.86	0.27(27)±0.010	0.08(8) ±0.003	0.02(2) ±0.001	0.42(42) ±0.011	0.05(5) ±0.001	0.33(33) ±0.002
P 8	9.34	0.10(10)±0.009	0.05(5) ±0.001	ND*	0.05(5) ±0.001	ND*	1.10(110)±0.022
P 9	10.22	0.17(17)±0.012	0.01(1) ±0.032	0.20(20)±0.001	ND*	0.21(21)±0.001	0.60(60) ±0.111
P 10	7.02	0.07(7) ±0.008	ND*	0.05(5) ±0.001	ND*	ND*	0.87(87) ±0.002

*ND, not detected.

Agilent Chem Station was utilized to analyze the chromatographic outcomes.

Antibacterial activity

Antibacterial activity was assessed utilizing the Agar well diffusion method (Perez, 1990). A fresh 100 µl bacterial culture containing 108 CFU/ml was added to each Petri plate along with 25 ml of sterile selective medium and allowed to solidify at room temperature. Wells were created and three known concentrations (5 mg/mL, 10 mg/mL, and 20mg/ mL) of each propolis extract were added. Plates were incubated at 37°C for an entire day and zone of inhibition was measured in millimeters. Macro broth dilution method was used to measure the minimum inhibitory concentration (MIC) for each bacterial strain using 48-well plates (Wiegand *et al.*, 2008). Because propolis extracts were ineffective against *Salmonella typhimurium*, the minimum inhibitory concentration (MIC) could not be determined.

Statistical analysis

The results obtained from each sample were analyzed in triplicate, and the average (n= 3 SD) is presented. A variance analysis (ANOVA) was conducted utilizing Minitab 2000 Version 13.2 (Minitab Inc., PA, USA), a statistical programme. The threshold for a statistically significant difference was set at $p < 0.05$.

Results and Discussion

Propolis samples obtained from various regions of South Punjab were purified via maceration (Oroian *et al.*, 2020). Diverse plant origins and collection locations might account for the fluctuating yield percentages observed in the samples (Table 1) (de Lima *et al.*, 2016). Propolis's diverse bioactive properties are attributed to its polyphenolics, which consist of phenolic acids and flavonoids (Bankova *et al.*, 2020; Hindi, 2015). Gargouri and Fernández-Muiño (2019) reported a new type of propolis rich in flavonoids that have a good antibacterial activity. Nevertheless, variations in the concentration of polyphenolics in propolis can be attributed to geographical dissimilarities and botanical provenance (Bankova *et al.*, 2020). The investigation assessed the concentrations of total phenolics and flavonols, including Sinapic acid, Caffeic acid, Gallic acid, kaempeferol, Myricetin, and Quercetin, in the various propolis samples (Table 1). Not with standing the fact that Gallic acid was detected in the maximum concentration in all ten propolis samples, Quercetin was only detectable in six of them. Similar inconsistencies were observed in the quantity and composition of total phenolics and flavonols examined in this research as were observed in China, Brazil, and Taiwan. The antibacterial effectiveness of each extract was found to be promising in comparison to ciprofloxacin against a variety of bacterial isolates (Table 2). A reference medication, ciprofloxacin has been selected on account of its broad-spectrum efficacy against Gram positive and Gram negative microorganisms. At a concentration of 5 mg/ml, the extracts exhibited no or very little antibacterial activity against *Pseudomonas aeruginosa* (Pa), *Escherichia coli* (Ec) and *Salmonella typhimurium* (St) while activity enhanced by increasing the concentration to 20 mg/mL.

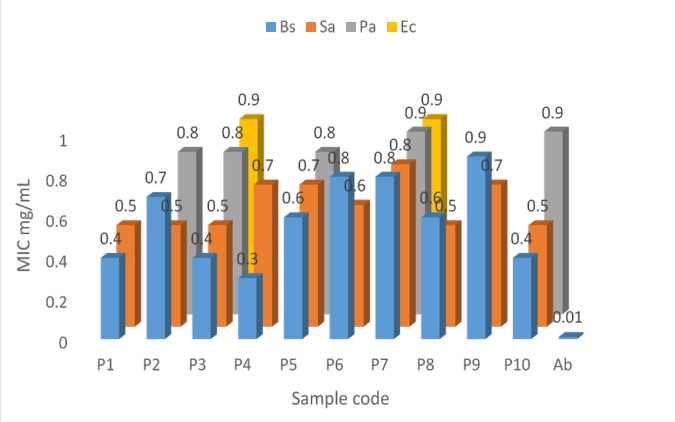
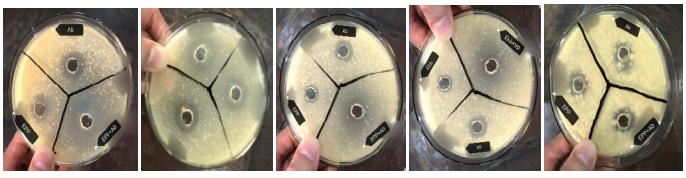


Figure 3: MIC of EPPs against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, respectively.

The MIC for P4's antibacterial activity against *Staphylococcus aureus* is 0.3 mg/mL, which is lowest among all the tested samples while values increases for rest of the strains. A comparable pattern was observed in other samples of propolis (Figure 3). Augmenting an ethanolic propolis extract in conjunction with antibiotics may reduce the risk of drug resistance, treatment doses, and adverse effects associated with pharmaceuticals, according to a prior study. When compared to all bacterial isolates, P4 in combination with Ciprofloxacin demonstrated a greater zone of inhibition against G+ve bacteria but no discernible alteration against G-ve bacteria (Figure 4). Consequently, propolis might have enhanced the effectiveness of the medication (Noori, 2012; Wojtyczka *et al.*, 2013). Further in vivo studies are required to see the effectiveness of present research.



***Bs*:** ***Sa*:** ***Pa*:** ***Ec*:** ***St*:**

Figure 4: Synergism of P4 + Ciprofloxacin (Ab) against 5 bacterial strains (*Bs*: *Bacillus cereus*, *Sa*: *Staphylococcus aureus*, *Pa*: *Pseudomonas aeruginosa*, *Ec*: *Escherichia coli*, *St*: *Salmonella typhimurium*).

Table 2: Zone of inhibition (mm) of propolis samples from south Punjab.

EEP/antibiotic	Conc. (mg/ml)	Microbes and their zone of inhibition (mm)				
		Bs	Sa	Pa	Ec	St
Ciprofloxacin (Ab)	20	26.0±0.4	24.0±0.5	22.0±0.4	24.0±0.6	20.0±0.4
P 1	20	18.0±0.4	17.0±0.5	9.0±0.4	9.5±0.6	NA*
	10	17.0±0.4	15.0±0.7	8.5±0.6	8.5±0.4	NA*
	5	15.5±0.3	13.0±0.6	NA*	NA*	NA*
P 2	20	16.0±0.6	18.0±0.4	14.0±0.3	10.0±0.3	9.3±0.3
	10	15.0±0.4	17.5±0.3	12.0±0.4	8.5±0.3	NA*
	5	13.0±0.3	16.0±0.2	11.0±0.6	NA*	NA*
P 3	20	20.0±0.6	18.0±0.5	12.0±0.5	11.5±0.6	11.0±0.9
	10	18.0±0.4	16.0±0.3	9.5±0.4	10.0±0.6	8.5±0.3
	5	14.0±0.3	12.0±0.4	8.5.0±0.4	8.0±0.3	NA*
P 4	20	20.0±0.5	15.5±0.4	10.0±0.2	10.0±0.6	9.00±0.3
	10	18.5±0.3	13.5±0.4	8.5±0.3	8.5±0.2	NA*
	5	16±0.2	11.0±0.3	NA*	NA*	NA*
P 5	20	16.0±0.5	15.0±1.4	10.5±0.5	10.0±1.3	NA*
	10	14.0±0.7	12.5±0.3	9.00±0.4	8.5±0.4	NA*
	5	12.0±0.3	12.0±1.0	8.5±0.3	NA*	NA*
P 6	20	14.0±0.3	17.0±0.3	10.0±0.4	NA*	NA*
	10	10.0±0.4	15.0±0.5	8.5±0.4	NA*	NA*
	5	8.0±0.8	10.0±0.3	NA*	NA*	NA*
P 7	20	15.0±1.4	15.0±0.3	10.0±0.4	10.0±0.4	8.5±0.6
	10	13.5±0.4	12.0±0.3	8.5±0.3	8.7±0.3	NA*
	5	12.0±0.3	11.0±0.5	NA*	NA*	NA*
P 8	20	17.5±0.5	18.0±0.3	11.0±0.3	12.0±0.4	11.0±0.7
	10	16.0±0.4	16.0±0.2	9.5±0.5	10.0±0.6	9.0±0.3
	5	14.5±0.3	14.5±0.2	NA*	8.5.0±0.7	NA*
P 9	20	14.5±0.4	16.0±0.1	NA*	8.5±0.9	NA*
	10	10.0±0.7	10.0±1.1	NA*	NA*	NA*
	5	8.5±0.5	8.5±0.4	NA*	NA*	NA*
P 10	20	20.0±0.7	17.0±0.5	12.0±0.6	9.0±0.5	NA*
	10	16.5±0.4	15.5±0.7	9.5±0.6	NA*	NA*
	5	14.0±0.5	14.0±0.5	8.5±0.4	NA*	NA*

Ab, Antibiotic; *Bs*, *Bacillus cereus; Sa*, *Staphylococcus aureus; Pa*, *Pseudomonas aeruginosa; Ec*, *Escherichia coli; St*, *Salmonella typhimurium;* *NA, not active.

Conclusions and Recommendations

In conclusion, this research has established that propolis samples procured from South Punjab, Pakistan, comprise phenolics, which consist of flavonoids and phenolic acids. The concentration of propolis samples influences their antimicrobial activity. *Salmonella typhimurium* exhibited the highest resistance, while *Bacillus cereus* and *Staphylococcus aureus* were the most susceptible microorganism. In addition, propolis also synergistically enhanced the efficacy of antibiotic, ciprofloxacin. The possibility exists that this synergistic effect could inspire the development of novel pharmaceutical combinations intended to combat bacterial infections.

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Conflict of interest

The authors have declared no conflict of interest.

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