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Chemical Analysis and Anti-Microbial Activity of Karnataka Bee Bread of *Apis species*

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Abstract: Bee pollen has been known for its medicinal properties from ancient times are now being used as a natural supplement which is highly packed with protein, vitamins and minerals. The pollen aqueous and pollen ethanolic extracts (PEE 50% and PEE 90%) of bee pollen from Karnataka origin were taken and the anti-oxidative activities were tested and compared with each other. Biochemical tests like Lowry's method of protein estimation and total phenolic contents were carried out. These pollen extracts were also tested for their antagonistic properties against eight different bacterial strains using disc diffusion method. The radio protectiveness and cell repairing property of bee pollen extracts was tested on onion root tips and mitotic indices (MI) were calculated. The TPC (0.99GAE/100gm) and FRAP was highest (4.08mg/ml) in 90% PEE, DPPH with 148.2 g/ml in Pollen aqueous extract and the protein content was 60780mg/ml in 90% PEE. The results showed highest inhibition of *P. aeruginosa*, solely responsible for diseases like skin and soft tissue infections, pneumonia, septic shock, urinary tract infection, blood and gastrointestinal infection. 90% PEE could treat the root cells and helped in repairing the damaged root cells.

Key words: Bee bread · Pollen ethanolic extracts · Anti-oxidative activity and root cells

INTRODUCTION

Bee pollen, which is also known as nature's perfect food is a fine powder-like structure that is collected by honey bees from various flowering plant sources and mixed with nectar and bee secretion and is processed into bee bread. It is considered as a potential source of energy giving food being used for human consumption these days [1]. Bee pollen is especially used for reducing hay fever and allergies and cures other common health problems like flu, cold, ulcers and anemia [2].

Chemical composition of bee pollen varies with varying geographical areas. Bee pollen consists of carbohydrates, amino acids, proteins, vitamins, minerals, lipids and traces of micronutrients necessary for enhancing the human health. Polyphenolic substances, especially flavonoids, are found in pollen [3], which plays a vital role in plant physiology by taking part in plant growth and reproduction processes [1]. They also provide resistance against pathogens due to phytoalexins and protection against plagues [1]. Studies have shown some indications of promoting an early development of the digestive system [4]. Bee pollen consisting of flavonoids quercetin, rutin and chyrisin has shown to increase apoptosis, hence showing a chemo preventive activity [5, 6]. Presence of Phenolic contents in bee bread enhances its health benefits as it reduces oxidative stress, thereby decreasing the chances of degenerative diseases to occur [7, 8].

The present study was carried out to investigate the anti-oxidative, antagonistic potency and radio protectiveness of the pollen extracts collected from Karnataka.

MATERIALS AND METHODS

Sample Collection: The present study was carried out using Pollen obtained from Karnataka in the month of February 2014. The pollen samples were stored in a sterilized container and stored at 4°C. The pollen sample was analysed within a month.

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Preparation of Bee Pollen Extracts: Pollen ethanolic extracts (PEE) were prepared in two concentrations - 50% and 90%. 8g of pollen was weighed and added to 60 ml of 50% and 90% ethanolic solution in Erlenmeyer flasks. For preparation of pollen aqueous extract (PAE), 8g of pollen was added to 60 ml of distilled water. These flasks were kept in a shaker in dark condition for 24 hours at 37°C. Followed by incubation; the mixtures were filtered using a 4-fold sterile muslin cloth followed by filtration with Whatman filter paper. The filtrates were stored in 4°C.

Anti-Oxidative Tests of Pollen Extracts

Ferric Reducing Antioxidant Power (FRAP): The Ferric Reducing Antioxidant Power (FRAP) assay was carried out according the method reported earlier [9]. To 1 ml of each pollen extract, 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferric cyanide (1%) followed by 20 minutes incubation at room temperature. 2.5 ml of Trichloro acetic acid (10%) was added after which centrifugation at 3000 RPM was carried out for 10 minutes. 2.5 ml of supernatant was taken after centrifugation and to it 2.5 ml of distilled water and 0.5 ml of ferric chloride was added and the absorbance was taken at 700 NM. The test was carried out in triplicates for each pollen sample.

Anti-Radical Scavenging Activity by DPPH Assay: For depicting the antiradical scavenging activity the protocol followed was as per the method described earlier [10]. A series of test tubes were taken where to each, 200 µL of pollen extracts were added. To each, 600 µL of ethanol and 200 µL of DPPH (394.32 g/Mol) were added. The mixtures were mixed by vortexing vigorously for 2 minutes, followed by the incubation of samples for 30 minutes at room temperature in dark conditions. Absorbance was recorded at 517 nm using a control being an 800 µL ethanol and 200 µL of DPPH. The antiradical scavenging activity of pollen samples was calculated using the following formula:

$$\text{Antiradical Scavenging Activity} = [(A_{control} - A_{test})/A_{control}] \times 100$$

where,
A control = Absorbance of control taken at 517 NM
A test = Absorbance of test sample taken at 517 NM
The test was carried out for each sample in triplicates.

Biochemical Assays of Bee Pollen Extracts

Protein Estimation of Pollen Extract: Protein contents present in bee pollen were estimated using the Lowry's method. Reagents solutions A, B, C and D were freshly prepared. Solution A was prepared by adding 2% Na_2CO_3 in 0.1N NaOH; Solution B by adding 1% sodium potassium tartarate in distilled water; Solution C by adding 0.5% $CuSo_4$.5H2o in distilled water and Solution D (Lowry's buffer) by adding 48 ml of A, 1 ml of B and 1 ml of C. For the preparation of phenol reagent, 1 part Folin-Phenol [2N]: 1 part water was taken. Bovine Serum Albumin, 1 mg/ ml was considered as a protein standard. 0.1 ml of bee product sample with 0.9 ml of distilled water was taken in test tubes where 2 ml of Lowry's buffer was added to each. These were kept for incubation for 10 minutes at room temperature after which 0.2 ml of FC reagent was added to each test tube. After 30 minutes incubation, the observance of each sample was taken at 660 NM. The process was carried out in triplicates.

Total Phenolic Contents (TPC): The total phenolic content of bee pollen extracts were determined by the Folin–Ciocalteu method [11].30 µL of each pollen extract was mixed with 2.37 ml of distilled water in test tubes and 150 µL of 0.2 N FC reagent was added to it. The mixture was mixed by vortex and incubation was done for 2 minutes at room temperature. 450 µL of sodium carbonate solution (0.2 g/ml) was added to the reaction mixture which was followed by incubation for 2 hours at room temperature. Absorbance was taken at 765 mm. The test was carried out for each pollen extract in triplicate.

Antimicrobial Activity of Pollen Extracts

Preparation of Test Organisms: Microorganisms were obtained from the Department of Microbiology, School of Chemistry and Biotechnology, SASTRA University. The pathogens, namely *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Shigella flexneri, Salmonella typhi* and *Proteus mirabilis* were taken as test organisms. The isolates were identified based on standard microbiological techniques and cultured in nutrient agar media at 37°C for 24 hours. Colonies of fresh cultures of the different microorganisms from the overnight grown microorganisms were picked with sterile inoculating loop and suspended it in 3-4 ml nutrient broth contained in sterile test tubes and incubated for 18 hours at 37°C.

Antimicrobial Activity: Antibacterial activity of each bee pollen extract was tested using disc diffusion method against the stated microorganisms. Fresh culture suspension of the test microorganisms (100 µL) was spread on Nutrient agar plates. For screening, 5 mm,

sterile diameter Whatman filter paper discs were impregnated with 10 µL of 50% PEE, 90% PEE and pollen aqueous extract. The standard used was Kanamycin (1.08 µL of 50 µg/µL) and controls were autoclaved distilled water (10 µL) and ethanol (10 µL). The plates were kept for incubation under optimum conditions (37°C) for 24 hours. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. The zone diameters of inhibition (ZDI) were measured in millimeter, including the diameter of the disc. The antimicrobial tests were carried out in triplicates for each pollen extract.

Anti-Radio Activity of the Pollen Extracts: The protocol followed was as per the methodology described by fresh onion bulbs were taken and dried roots were removed and the root portion of the onion bulb was immersed in a beaker filled with water [12]. The water was changed each day and development of roots took place on the third day. The experiment was carried out from 11 am to 1 pm where the rate of mitotic division is high. The healthy root tips were cut and placed in a beaker containing minimal water so as to prevent roots from drying. The root tips were subjected to UV radiation for 15 minutes. The root tips were placed in 2 ml eppendorf tubes filled with 50% PEE, 90% PEE and pollen aqueous extract for 45 minutes. Distilled water containing irradiated root tips was used as a control. Root squash was prepared with treated root tips on a clean, dry slide and the slides were observed under 45X microscope. The mitotic stages were observed and mitotic indices were calculated by using the formula,

Mitotic Index (MI) = (P+M+A+T)/Total no. of cells in the field X 100

where,
P = Prophase
M = Metaphase
A = Anaphase
T = Telophase

RESULTS

Anti-Oxidative Analysis of Bee Pollen Extracts

Ferric Reducing/Antioxidant Power (FRAP) Assay: The Ferric reducing/antioxidant power (FRAP) assay of the three bee pollen extract had ranged from 3.38 mg/mL to 4.08 mg/mL. Bee pollen extracts that showed higher reducing power had a higher absorbance value at 700nm. This indicates that if the absorbance value is higher then there is more reduction of ferric ions to ferrous ions. 90% PEE (4.08 mg/mL) showed the highest value followed by 50% PEE (4.01 mg/mL). Pollen aqueous extract (3.38 mg/mL) showed the least reducing power (Table 1 and Graph 1).

Anti-Radical Scavenging Activity of Bee Pollen Extracts: The free radical scavenging activity of various bee pollen extracts are done by the DPPH which is stable nitrogen centred radical. According the literature higher the DPPH scavenging activity, higher is the antioxidant capacity of the sample. The DPPH scavenging activity percentage out of the three pollen extracts ranged from 45.69 g/mL to 148.22 g/mL and its percentage was higher in pollen aqueous extract followed by 50% PEE (143.78 g/mL). The DPPH scavenging activity of 90% PEE (45.69 g/mL) having the least antioxidant capacity. Pollen aqueous extract and 50% PEE had antiradical scavenging activity greater than 50% (Table 1 and Graph 1).

Biochemical Analysis of Bee Pollen Extracts

Total Phenolic Content of Bee Pollen Extracts: Seventeen phenolic compounds related to the botanical origins of 13 Anzer pollens from Turkey have been analyzed by RP-HPLC in a study where the mean content of identified total phenolics ranges from 0.5□mg/100□g pollen to 2.6□mg/100□g pollen [13]. The total phenolic content of the 3 bee pollen extracts were in the range of (0.80 mg/L) in pollen aqueous extract to (0.99 mg/L) in 90% PEE. The Gallic acid standard calibration curve was obtained by plotting different concentrations of Gallic acid against the absorbance at 765 NM (Table 2 and Graph 2).

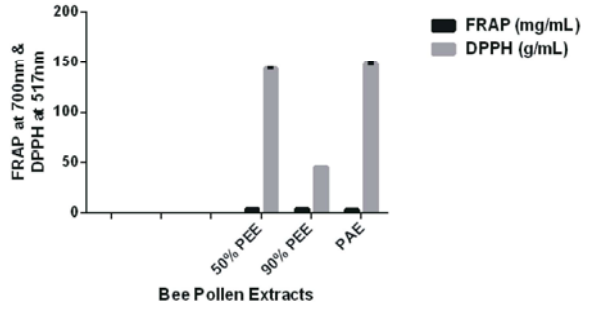
Protein Content of Pollen Extracts: Bee pollen has approximately 40% or 10-40g of protein content in 100 g of pollen bread [14]. It is considered one of nature's most completely nourishing foods. About half of its protein is in the form of free amino acids that are ready to be sued directly by the human body. Lowry's method of protein estimation was performed for three extracts of bee pollen where the total protein content ranged from 33953.33 mg/g to 60780 mg/g, which was determined by using BSA (Bovine Serum Albumin) as standard and it was relatively high in 90% PEE followed by 50% PEE (57036.67 mg/g). The Pollen aqueous extract showed least protein content (Table 2 and Graph 3).

Table 1: Anti-oxidative analysis of bee pollen extracts

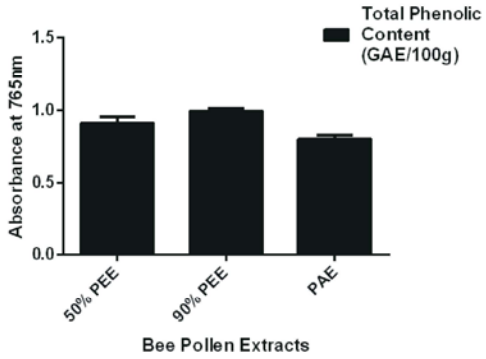
Sl. No.	Bee pollen extract	FRAP (mg/mL)	DPPH (g/mL)
1	50% PEE	4.01±0.18	143.78±0.26
2	90% PEE	4.08±0.15	45.69±0.03
3	Pollen aqueous extract	3.38±0.10	148.22±0.19

Table 2: Biochemical analysis of bee pollen extracts

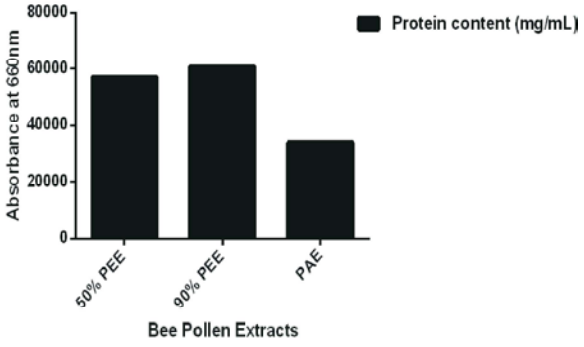
Sl. No.	Bee pollen extract	Protein (mg/mL)	Total phenolic content GAE/100g
1	50% PEE	57036.67±14.83	0.91±0.04
2	90% PEE	60780±21.86	0.99±0.02
3	Pollen aqueous extract	33953.33±4.83	0.80±0.03



Graph 1: Graph showing the ferric reducing/antioxidant power (FRAP) and the anti-radical scavenging activity of bee pollen extracts



Graph 2: Graph showing the Total phenolic content present in bee pollen extracts



Graph 3: Graph showing the protein content in bee pollen extracts

Anti-Microbial Activity of Bee Pollen Extracts: Anti-microbial test was done by agar disc diffusion method where activities of three bee pollen extracts were observed. 50% PEE was highly effective on *Salmonella typhi* (319.01 mm^2) and least on *Klebsiella pneumonia* (61.08 mm^2) but was fairly effective on *Pseudomonas aeruginosa* (214.99 mm^2). 90% PEE had highest anti-microbial activity against *Pseudomonas aeruginosa* (597.14 mm^2) followed by *Salmonella typhi* (493.92 mm^2) and *Proteus mirabilis* (412.26 mm^2) and least effective on *Bacillus subtilis* (111.12 mm^2). Pollen aqueous extract highly inhibited *Pseudomonas aeruginosa* (175.90 mm^2). Collectively the bee pollen extracts successfully inhibit *Pseudomonas aeruginosa, Salmonella typhi* (493.92 mm^2) and *Proteus mirabilis* (412.26 mm^2) and least effective on *Bacillus subtilis* where 90% PEE could show highest among the other extracts (Table 3 and Figure 1).

Radio Protective Potential of Bee Pollen Extracts: The radio protectiveness and cell repair mechanism was measured by calculating the mitotic indices of the UV irradiated onion root cells which were treated with respective extracts for a given period of time (45 min). The slides were prepared and observed under a binocular microscope. In each field the number of mitotic stages (prophase, metaphase, anaphase and telophase) was calculated. Mitotic index for control was zero as no dividing cells were present. 50% PEE and aqueous pollen extract treated root cells could not show any improvements in the damaged cells. 90% PEE could treat the root cells where all the four mitotic stages were observed that is, it helped in repairing the damaged onion root cells.

Hence it is concluded that bee pollen not only has the anti-oxidative and antimicrobial capacity but also has the property to repair irradiated damaged cells. It showed higher protein content compared to that of honey and propolis. It showed high values of FRAP and DPPH than that of honey and propolis (Table 4 and Figure 2).

Table 3: Anti microbial activity of the Bee pollen extracts by disc diffusion method

Sl. No.	Sample	*Escherichia coli* Area of inhibition (mm^2)	*Staphylococcus aureus* Area of inhibition (mm^2)	*Pseudomonas aeruginosa* Area of inhibition (mm^2)	*Bacillus subtilis* Area of inhibition (mm^2)
1	50% PEE	139.58±2.31	122.50±1.73	214.99±3.51	90.38±2.31
2	90% PEE	256.84±1.53	139.58±1.53	597.14±2.52	111.12±3.05
3	PAE	148.41±2.12	157.42±2.52	175.90±1	75.38±1
4	Kan (Std)	566.75±3.79	452.30± 1	127.10±3.21	301.54±5.29
5	Control	68.45±2.08	90.38±1.15	214.99±0.58	33.78±1.15
Sl. No.	Sample	*Proteus mirabilis* Area of inhibition (mm^2)	*Salmonella typhi* Area of inhibition (mm^2)	*Shigella flexneri* Area of inhibition (mm^2)	*Klebsiella pneumonia* Area of inhibition (mm^2)
1	50% PEE	106.11±2.08	319.01±6.18	122.45±1	61.08±0.58
2	90% PEE	412.26±2.12	493.92±6.95	139.58±2.31	139.58±0.58
3	PAE	106.11±1.53	110.13±1.5	122.50±1.73	106.10±1.15
4	Kan (Std)	692.50±1.15	432.08±2.5	508.17±3.21	725.57±4
5	Control	122.50±1.73	190.23±0.96	157.42±0.58	278.85±5.03

Table 4: Radio protective potential of the Bee pollen extracts by microscopic observation

Sl. No.	Microscopic field no.	Total no. of cells in field (under 45X)	No. of mitotic stages	No. of dividing cells	Mitotic Index (M.I.) (in %)
1.	1	101	28 P, 2 M, 1 A	31	30.6931%
2.	2	134	12 P, 6 M, 5 A, 1 T	24	17.9104%
3.	3	254	24 P, 3 M, 6 A, 2 T	35	13.7795%

Note: P- Prophase; M- Metaphase; A- Anaphase and T- Telophase

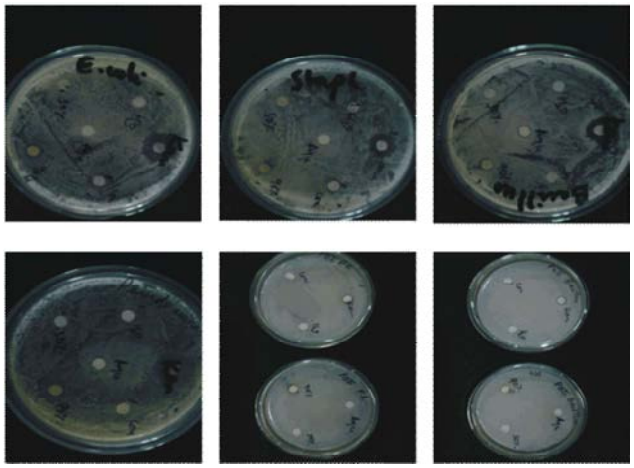


Fig. 1: Antimicrobial activity of bee pollen extracts by diffusion method

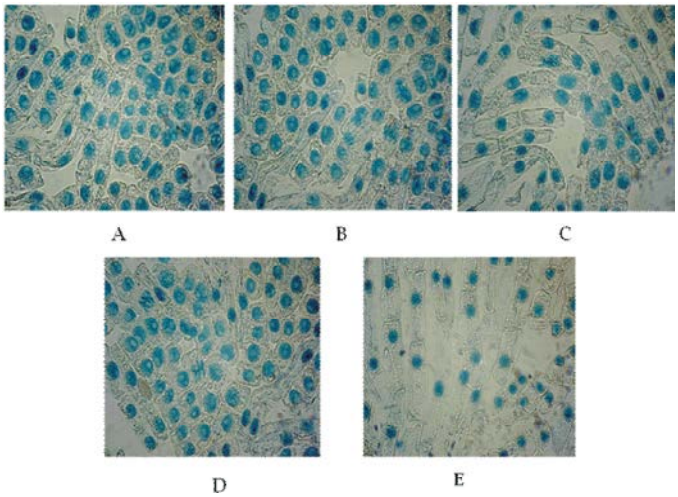


Fig. 2: A to D showing Mitotic stages observed in the 90° % PEE slider and E showing th eControl

DISCUSSION

Anti-Oxidative Activity of Bee Pollen: High anti-oxidant values of bee pollen have been reported which seems to be due to the presence of phenolic compounds [15] and mostly flavonoids, their glycosides and cinnamic acid derivatives are present in floral pollen [16]. Reports have indicated that antioxidant property of bee pollen is species specific [17, 18, 19 and 20]. It was believed that high concentrations of phenolic compounds go hand in hand with high anti-oxidative property of pollen. The property is independent of its geographical origins [17]. Several studies have indicated a close relationship of antioxidative property with levels of phenolic compounds [18, 19, 21 and 22]. It was also shown that pollens with high levels of flavonoids and phenolics did not show good antiradical activity [15]. Its property decrease with increased storage period [22]. The present studies indicated high FRAP and DPPH values of bee pollen extracts. The bee pollen also had high protein content which showed that bee pollen is an excellent source of protein.

Anti-Microbial Activity of Bee Pollen: There has not been an in-depth study done on antimicrobial activity of bee pollen until where they tested antagonistic activity of bee pollen extract and the strongest effect was observed against *E. coli, S. typhi* and *Enterobacteriaceae* [23]. The antimicrobial activity of bee pollen extracts which showed its effects on the selected strains of pathogenic bacteria, fungi and yeasts [24]. The anti-microbial property of monofloral bee pollen extract where it acted against *E. coli* CCM 3988 and *Salmonella enterica* CCM 4420 were studied [25]. Bee pollens of *Ranunculus sardous* and *Ulex Europeans* had an antimicrobial activity against *P. aeruginosa* [14]. Quercetin derivatives of pollens of *Eucalyptus globulus* did not show any antibacterial activity [26]. Antifungal activity was significantly shown by bee pollen [27, 28].

The present study showed highest inhibition of *P. aeruginosa* that are solely responsible for diseases like skin and soft tissue infections, pneumonia, septic shock, urinary tract infection, blood and gastrointestinal infection. They are also known to cause infections in burns and wounds that could be now treated with certain bee pollen extracts after conducting further studies.

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