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Antibacterial, Antifungal and Antihelminthic Properties of Ethanolic, Methanolic and Water Extracts of Pollen

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Microorganisms and helminthes can cause serious diseases in humans as well as in animals. The use of antimicrobial and antihelminthic drugs have created selective pressure and caused resistance to antibiotics used against them, thus it necessitates the use of honey bee's derived natural products. One such bee derived product is pollen, collected by worker honey bees from the flowering plants and modify it by adding its salivary secretions. The present study embodies use of pollen as antimicrobial and antihelminthic substance. Among microorganisms 4 Gram (+ve) bacteria; (*Bacillus subtilis, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus pneumoniae*) and 3 Gram (-ve) bacteria; (*Escherichia Coli, Pseudomonas aeruginosa, Salmonella enteric*) and 2 yeasts (*Candida albicans* and *Saccharomyces cerevisiae*) were used and the methodology used disc diffusion assay and broth dilution method. The antihelminthic effect was observed among amphistomes via bioassay method under *in vitro* conditions. For observations three types of pollen extracts (ethanolic, methanolic and water extract) were prepared and positive controls used were; Ampicillin for antibacterial, Amphotericin B for antifungal and Albendazole for anti-helminthes. The antimicrobial activities were determined by measuring the zones of inhibition diameters in millimeters after 24 hours of incubation at optimum temperature for each microbe and also by broth dilution method. Results obtained showed that the water extract of pollen was found to be most effective against bacteria used in the present study where; Gram (+ve) bacteria were

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more susceptible as compared to the Gram (-ve) bacteria. It was also observed that among yeasts; *Saccharomyces cerevisiae* was more susceptible towards ethanolic extract of pollen while *Candida albicans* showed more inhibitions towards water extract of pollen. Results also demonstrated that none of the extracts of pollen was found to be effective against Helminthes (amphistomes) used in the present study.

Keywords: Pollen; antimicrobial; antihelminthic; disc diffusion; bioassay.

1. INTRODUCTION

The problem of bacterial resistance is growing very fast and the use of medicinal plants as antimicrobial and antihelminthic is a common practice. The recent increase in the popularity of alternative medicine and natural products has renewed interests in bee derived products as potential natural remedies. Among them bee pollen is one such valuable product having many pharmacological and medicinal properties through direct action against microorganisms or synergism with antimicrobial drugs. Bee pollen, also known as 'The Life Giving Dust' is collected by worker honey bees from the flowering plants. Worker bees have possessed pollen baskets (corbiculaor) on their hind legs which facilitate pollen transportation to the hive with the aid of salivary secretions. Bee pollen is regarded as functional foods for their high nutritional values as they are rich source of proteins, essential amino acids, sugars, fatty acids, vitamins, macro and microelements and are also rich in polyphenolic compounds [1]. They also contain a wide variety of other health promoting compounds present in functional foods, such as prebiotics, probiotics, fibre, lignans, triterpenes, carotenoids, bioactive peptides and organic acids [1-5]. Therefore it is considered as an excellent substitute of antibiotics [6] and implemented as complementary medicine for a large variety of impaired health conditions [4,7,8]. Bee pollen exhibits antibacterial and antifungal activity [1,3,9-12].

The present study embodies results of investigations undertaken to evaluate the antimicrobial and antihelminthic properties of pollen by using *in vitro* methods. Determination of these activities was done by disc diffusion method and broth dilution method against pathogenic and non pathogenic bacteria and yeasts. In disc diffusion method, the microorganisms were screened for their susceptibility towards pollen (extracted in ethanol, methanol and water), applied on the disc of agar plate at the concentration range of 1.562-300mg/disc and for seeing antihelminthic activity water extract of pollen was used.

2. MATERIALS AND METHODS

2.1 Collection of Pollen

Pollen was collected from the pollen basket of worker honey bees returning to the hive with pollen loads, by installing a pollen trap at the entrance of the beehive in the Dept of Zoology, Punjab University, Chandigarh.

2.2 Preparation of the Pollen Extract

Three different extraction solvents such as ethanol, methanol and water were used for the preparation of pollen extract by following the method of Nagai [13] with little modification. For this 3g of fresh bee pollen was taken. It was suspended and extracted by shaking with 10 volumes of solvent (ethanol, methanol and water) at room temperature for one day. The suspension was centrifuged at 10,000g in a refrigerated centrifuge for 20min. Supernatant fraction was collected and filtered. The filtrate was freeze dried. The powder of the extract was dissolved in ice-cold distilled water and filtered through 0.22µ PTFE membrane for sterilization and was stored at cool place for use in experiment.

2.3 Helminthes (Amphistomes)

Gastrothylax crumenifer, were obtained from large intestine of sheep/goat procured from local slaughter house.

2.4 Microorganisms

Microorganisms such as bacteria (*Staphylococcus aureus:* MTCC No- 1144, *Staphylococcus epidermidis:* MTCC No-9040, *Streptococcus pneumoniae:* MTCC No- 2672, *Salmonella enterica:* MTCC No-3231*, E. coli:* MTCC No-2314*, Bacillus subtilis*: MTCC No-2435*, pseudomonas aeruginosa:* MTCC No-3465

and fungi (*Candida albicans* (Yeast): MTCC No-4748, *Saccharomyces cerevisiae* (Yeast): MTCC No- 3090) were procured from IMTECH (Institute of Microbial Technology) Sector-39, Chandigarh, India. The organisms were maintained in suitable/respective media (agar plates at 4^0C). The strains were checked biochemically prior to usage.

2.5 *In vitro* Antimicrobial Activity of Pollen

2.5.1 Disc diffusion method

Antibacterial activity of pollen was evaluated for three types of extracts *viz.* ethanolic, methanolic and water. Organisms initially selected for antimicrobial studies with pollen were nonpathogenic Gram (+ve) and Gram (-ve) bacteria *viz. Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus pneumoniae.* Thereafter putative pathogenic Gram (+ve) bacteria *viz. Staphylococcus aureus, Staphylococcus epidermidis* and Gram (-ve) bacteria *viz*. *Salmonella enterica* which were screened for the inhibitory activity of pollen by disc diffusion method and broth dilution method. The stock solutions of pollen were made at a concentration of 300mg/ml. These were serially diluted to obtain the concentration of 300mg/ml, 200mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml and 1.562 mg/ml. Nutrient agar plates were made and 25-50µl (0.5 x 10^6CFU/ml) of each organism was uniformly spread on the plates. Inoculum was always prepared fresh 24-48hrs. prior to start of the experiment. Then 25 µl of all the above mentioned concentrations were applied on separate agar plates and incubated at their respective growth conditions. After 24-48 hrs. clear zones of inhibitions of culture growth around the discs having pollen were measured.

2.5.2 *Broth dilution method*

The experiments were done in highly sterile conditions under laminar flow. For this test tube containing 2 ml broth media were autoclaved. Different concentrations of pollen (3-60mg/ml) from 300 mg/ml stock solution were added except to the control tube containing only broth. Fresh Inoculum (0.5 x 10^6CFU/ml) of the organism was prepared and 200µl of it was added to each test tube containing broth and bee pollen. Inoculated test tubes were then incubated at the respective growth temperatures of each organism in a shaker incubator till late log phase of growth. Optical density was measured at 600nm for all the test tubes.

2.5.3 *In vitro* anti helminthic activity of bee pollen

Worm motility inhibition assay was employed for the evaluation of anti-helminthic activity of bee pollen under *in vitro* conditions. The *in vitro* anti helminthic activity was conducted at three different concentrations (100, 300, 500 mg/ml) to determine the inhibitory effect of bee pollen extracts on amphistome worms. Mature amphistome worms (*Gastrothylax crumenifer*) were collected from the large intestine of sheep/goat procured from local slaughter house (Fig.1). The worms were washed in phosphate buffered saline (PBS pH 7.2) and then suspended in PBS. Albendazole dissolved in 1% DMSO and diluted in PBS at concentrations of 5, 10 and 15 µg/ml and PBS alone served as positive and negative control respectively. There were three replicates for each treatment concentration. Ten vigorously motile worms were placed in each petri dish containing test solutions and observations were made at 15, 30, 60 and 120 min intervals for cessation of motility by gross visual motility of worms as index for anti-helminthic activity. After exposure to different treatments, the worms were put in lukewarm PBS for 30 min in order to confirm mortality.

2.6 Log Colony Forming Units

The microbial inoculums were prepared by growing their culture in nutrient broth overnight. Bacteria were incubated at 37°C and fungi at 25°C. After incubation, cells were harvested by centrifugation at 8000g for 10 minutes and supernatant was discarded while pellet was washed and suspended in phosphate buffer saline (PBS). Optical density (OD) was then measured at 600nm. Viable count were determined by making serial dilutions and by spread plating on nutrient agar followed by incubation at 37^0 C and counting CFU 24 hours later.

Fig.1. Amphistomes (*Gastrothylax crumenifer*) from stomach of sheep/goat

3. RESULTS AND DISCUSSION

The natural products of both plant and animal origin have wide range of pharmacological activities such as; antimicrobial, anti-inflammatory and anti-modulatory [14-17]. Currently the use of natural products has increased due to reduction in number of effective antibiotics provided by the pharmaceutical industries as well as due to the increasing drug resistance among microorganisms [18-22]. Microbes are evolving various mechanisms of antibiotics resistance and in some cases become multi drug resistant [23-26]. Therefore, a systematic study was carried out to evaluate the antimicrobial potential of pollen against a range of Gram (+ve) and Gram (-ve) bacteria as well as in yeasts by disc diffusion assay and broth dilution method and against amphistome by bioassay method under *in vitro* conditions.

Results obtained are shown in Tables 1-10. The effectiveness of pollen was also compared with standard antibiotic as positive controls, such as; ampicillin (antibacterial), Amphotericin B (antifungal) and Albendazole (anti-helminthes).

3.1 Pollen

3.1.1 *Ethanolic extract*

The values observed for ethanolic extract of pollen for Gram (+ve) bacteria such as *Staphylococcus epidermidis* varied from 15.18±0.78-17.33±0.57mm, the zones of inhibition observed for *Staphylococcus aureus* varied from 13.50±0.89-18.5±1.20mm and for *Bacillus subtilis* from 11.00±0.63-12.55±1.22mm at concentrations ranging from 50-300mg/ml. For *S. epidermidis*, *S. aureus* and *Bacillus subtilis* no inhibition zones observed at concentrations ranging from 1.562-25mg/ml. In case of *Streptococcus pneumoniae* the zones of inhibition ranged from 9.89±0.16-13.46±1.49mm at concentrations from 100-300mg/ml and no inhibition zones were observed from 1.562-50mg/ml of ethanolic extract of pollen as shown in Table 1. The inhibition zones shown by ethanolic extract of pollen against Gram (-ve) bacteria such as *E. coli* varied from 8.00±1.15-10.28±0.33mm at concentrations from 50-300mg/ml. The values observed for *Pseudomonas aeruginosa* varies from 7.5±1.02-8.5±1.07mm at concentrations from 200-300mg/ml, the zones of inhibition observed against *Salmonella enterica* varied from 8.6±1.00-13.0±1.02mm at concentration range of 100-300mg/ml of ethanolic extract of pollen (Table 2). The zone of inhibition obtained against *Candida albicans* was 7.5±1.02mm at 300mg/ml and no inhibitions were observed at concentration lower than this, which showed that for *Candida albicans* the ethanolic extract of pollen was not much effective. The inhibition zones observed against *Saccharomyces cerevisiae* ranged from 13.50±0.89-16.2±1.30mm at concentrations varying from 100-300mg/ml of ethanolic extract of pollen. Here the results obtained showed that *Saccharomyces cerevisiae* was more sensitive as compared to *Candida albicans* to ethanolic extract of pollen used in the present studies (Table 3). The different patterns of sensitivity obtained can be due to variation in phenolic constituents, their solubility in ethanol and impact on the microbial cell wall.

Table 1. Antimicrobial activity of ethanolic extract of pollen against Gram (+ve) bacteria

Gram (+ve) Bacteria					
Ethanolic extract of pollen (EEP)		***Bacillus subtilis***	***Staphylococcus epidermidis***	***Staphylococcus aureus***	***Streptococcus pneumoniae***
S. No	**(mg/ml)**	**Zones of inhibitions (mm)**			
1.	1.562-25	NI	NI	NI	NI
2.	50	11.00±0.63*	15.18±0.78	13.50±0.89	NI
3.	100	10.85±1.16	15.40±0.98	14.2±0.30	9.89±0.16
4.	200	11.05±1.57	17.35±1.00	16.2±1.30	11.99±0.29
5.	300	12.55±1.22	17.33±0.57	18.5±1.20	13.46±1.49

All the values are expressed as mean ± S.D (n=5). NI-no inhibition. ZOI-zone of inhibition. mm-milimeter

Table 2. Antimicrobial activity of ethanolic extract of pollen against Gram (-ve) bacteria

Gram (-ve) Bacteria				
Ethanolic extract of pollen		***Escherichia Coli***	***Pseudomonas aeruginosa***	***Salmonella enterica***
S. No	**(mg/ml)**	**Zones of inhibitions (mm)**		
1.	1.562-25	NI	NI	NI
2.	50	8.00±1.15*	NI	NI
3.	100	8.10±0.86	NI	8.6±1.00
4.	200	9.20±0.71	7.5±1.02	9.4±2.01
5.	300	10.28±0.33	8.5±1.07	13.0±1.02

All the values are expressed as mean± S.D (n=5). NI-no inhibition. ZOI-zone of inhibition. mm-milimeter

Table 3. Antimicrobial activity of ethanolic, methanolic and water extracts of pollen against yeasts

Pollen extracts		***Candida albicans***			***Saccharomyces cerevisiae***		
		WEP	**EEP**	**MEP**	**WEP**	**EEP**	**MEP**
S. No	**mg/ml**	**Zones of inhibitions (mm)**					
1.	1.562-6.25	NI	NI	NI	NI	NI	NI
2.	12.5	NI	NI	NI	8.70±0.34*	NI	NI
3.	25	NI	NI	NI	10.43±0.33	NI	NI
4.	50	NI	NI	NI	11.50±0.08	NI	NI
5.	100	NI	NI	NI	12.65±0.24	13.50±0.89	8.6±0.96
6.	200	9.8±0.06	NI	NI	14.43±0.43	14.2±0.30	8.22±1.02
7.	300	11.8±0.32	7.5±1.02	NI	14.05±1.54	16.2±1.30	10.54±2.01

All the values are expressed as mean± S.D (n=5). NI-no inhibition. WEP-water extract of pollen. EEP- ethanolic extract of pollen. MEP- methanolic extract of pollen

3.1.2 Methanolic extract

The values observed for methanolic extract of pollen for Gram (+ve) bacteria such as *Staphylococcus epidermidis* varied from 9.55±0.47-11.25±0.53mm at range of concentrations from 100-300mg/ml. The zones of inhibition observed for *Staphylococcus aureus* varied from 8.6±1.00-12.4±.098mm at range of concentrations from 50-300mg/ml of methanolic extract of pollen; for *Bacillus subtilis* it was 8.43±1.39-9.72±1.09 mm at range of concentrations from 100-300mg/ml. The values for *Streptococcus pneumoniae* ranged from10.29±0.39-11.26±1.19mm at concentrations from 200-300mg/ml and no inhibition zones observed from 1.562-100mg/ml of methanolic extract of pollen as shown in Table 4. The present studies were in agreement with Sramkova [27] where the antimicrobial effect of pollen samples was tested by using the agar well diffusion method and the results showed that the most sensitive bacteria to ethanolic extract of poppy pollen was *Staphylococcus aureus.* The zones of inhibition observed for methanolic extract of pollen against Gram (-ve) bacteria such as *E. coli* varied from 7.025±0.83-8.925±0.44mm at range of concentrations from 100-300mg/ml. No inhibition zones were observed from 1.562-50mg/ml of methanolic

extract of pollen. Moreover *Salmonella enterica* was found to be insensitive against all the concentrations 1.562-300mg/ml used for evaluating the antibacterial activity of pollen methanolic extract. The value observed against *Pseudomonas aeruginosa* was 8.56±0.98mm at 300mg/ml of methanolic extract of pollen and no inhibitions was observed at lower concentrations as shown in Table 5. From the results obtained it was concluded that highest inhibition was found against *E. coli* and least against *Salmonella enterica* with all the extracts. Abouda [9] also obtained similar results by analyzing samples of bee bread and bee pollen from different aromatic and medicinal plants and observing them for their antimicrobial activities. They observed that Gram (+ve) bacteria were more sensitive to bee bread and bee pollen than Gram (-ve) bacteria. Further results obtained for yeasts revealed that there were no zones of inhibition found against *Candida albicans* by using the entire range of concentrations (1.562-300mg/ml) of the methanolic extract of pollen, while *Saccharomyces cerevisiae* showed inhibitions ranging from 8.6±0.96-10.54±2.01mm at concentrations varying from 100-300mg/ml of methanolic extract of pollen thus concluding that *Saccharomyces cerevisiae* was more sensitive to methanolic extract of pollen as compared to *Candida albicans* as shown in Table 3.

3.1.3 Water extract

The zones of inhibition observed for water extract of pollen against Gram (+ve) bacteria such as *Staphylococcus epidermidis* was observed to be in the range of 9.80±0.28-16.00±0.32mm at concentrations ranging from 12.5-300 mg/ml. No inhibition zones were observed at lower concentrations. The values observed for *Staphylococcus aureus* were from 8.70±0.34-15.49±1.51mm at concentration range of 6.25-300mg/ml and there was no inhibition at lower concentrations. The range of inhibition observed for *Streptococcus pneumoniae* was from 8.90±0.27-11.45±1.09mm at concentrations ranging from 25-300mg/ml, no inhibitions were observed at concentrations less than 25mg/ml of water extract of pollen. The range of inhibitions observed for *Bacillus subtilis* was 8.90±0.47-11.25±0.53mm at concentrations from 25-300mg/ml water extract of pollen. No inhibitions against *Bacillus subtilis* were observed at concentrations of 1.562-12.5mg/ml water extract of pollen (Table 6). The antimicrobial activity observed by using water extract of pollen against Gram (-ve) bacteria showed no inhibition zones at concentrations ranging from 1.562-6.25mg/ml. The most sensitive Gram (-ve) bacteria was found to be *E. coli* and the values observed varied from 7.98±0.41-11.25±0.44mm at concentration ranging from 12.50-300mg/ml. The zones of inhibition observed for *Salmonella enterica* ranged from 6.0±1.47-10.8±0.77mm at concentration varying from 12.50-300mg/ml. The values observed for *Pseudomonas aeruginosa* were from 9.8±0.06-15.0±0.08mm at 100-300mg/ml for water extract of pollen as shown in Table 7. The results obtained against *Candida albicans* varied from 9.8±0.06-11.8±0.32mm at concentrations ranging from 200-300mg/ml and no inhibitory effect of water extract of pollen was observed at lower concentrations. For *Saccharomyces cerevisiae* results obtained showed zones of inhibition ranging from 8.70±0.34-14.05±1.54mm at concentrations 12.5-300mg/ml. No inhibition zones were observed at concentrations less than 12.5mg/ml of water extract of pollen. Data obtained suggested that *Saccharomyces cerevisiae* was more sensitive as compared to *Candida albicans* to the water extract of pollen used in the present studies (Table 3). The present studies have shown that ethanolic, methanolic and water extracts of pollen possess variation in antimicrobial activities which might be due to solubility of phytoconstituents and hence solvent dependent. From the tested extracts of pollen, water extract was found to be most inhibiting and that too on Gram (+ve) microorganisms which authenticated/validated the results obtained in previous studies of ethanolic, methanolic and water extract of pollen [28]. So due to great biodiversity of bee pollen sources, more research is required for a better understanding of the functional properties of bee products along with bee pollen.

These studies were in agreement with Abouda [9] who reported antimicrobial activities of bee bread and bee pollen against bacterial strains isolated from pathological conditions in man. They revealed that Gram (+ve) bacteria are more sensitive to bee bread and bee pollen than Gram (-ve) bacteria. This was also supported from the studies of Pascoal [29], where antimicrobial activity of bee pollen against Gram (+ve) was being more sensitive as compared to Gram (-ve) bacteria. In his studies *Staphylococcus aureus* was the most sensitive and *Candida glabrata* was found to be most resistant of the microorganisms studied. Sramkova [27] determined antioxidant and antibacterial activity

of monofloral bee pollen samples against pathogenic bacteria. The antimicrobial effect of pollen samples were tested by using the agar well diffusion method. The most sensitive bacteria to the poppy pollen ethanolic extract was *Staphylococcus aureus* (70%) The most sensitive bacteria to rape bee pollen methanolic extract (70%) and sunflower ethanolic extract (70%) was *Salmonella enterica*.

3.2 Broth Dilution Method

For determination of inhibitory concentrations of the honey bee product pollen against the organisms listed previously and to study the effect of a range of concentrations of different extracts on the growth of an organism, experiments were done with broth dilution method (nutrient broth). Organisms were grown in presence of pollen at concentrations ranging from 3mg/ml-60mg/ml. Growth of Gram (+ve) and Gram (-ve) non-pathogenic bacteria *viz. Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus pneumoniae* was measured at late log phase and viable counts were determined ($0.5x10^6$CFU/ml). Then pathogenic Gram (+ve) bacteria *viz. Staphylococcus aureus, Staphylococcus epidermidis* and Gram (-ve) bacteria *viz*. *Salmonella enterica* were screened separately for the inhibitory activity of bee pollen by broth dilution assay. Growth of each organism was measured at late log phase by taking O.D. at 600nm (Table 8). Determination of antimicrobial activity by broth dilution method for bee pollen revealed that there is concentration dependent decline in growth of organisms under study. Therefore this concludes the antimicrobial properties of pollen. The antimicrobial properties observed for bee pollen could be due to cell wall lyses and plama membrane degradation, which leads to a loss of potassium ions and the damage, caused provoking cell autolysis [30].

3.3 *In vitro* Anti Helminthic Activity of Bee Pollen

Parasitic infections have always been a major concern to the medical field and among them amphistomes are the prominent causal agents of diseases in humans as well as in animals especially sheep and goat, which ultimately cause considerably economic losses to livestock industry and hence to the economic development of a country. Over the past few years, medical science has led many milestones in parasitological research but still efficient products to control helminthes are yet to discover. Moreover the drugs used for this purpose has caused resistance considerable toxicity to humans beings through foods derived from livestock causing serious health hazards [31]. This makes the necessity of use of natural products as antihelminthic drugs. Among them use of medicinal plants continues to be the most fruitful approach towards antihelminthic drugs [32]. The present study was undertaken to evaluate anti helminthes activity of pollen by Petri dish method [33], in comparison with a standard drug Albendazole, against amphistome (*Gastrothylax crumenifer)* parasitizing the large intestine of sheep/goat through *in vitro* studies by the worm motility inhibition assay.

Table 4. Antimicrobial activity of methanolic extract of pollen against Gram (+ve) bacteria

		Gram (+ve) Bacteria			
Methanolic extract of pollen (MEP)		***Bacillus subtilis***	***Staphylococcus epidermidis***	***Staphylococcus aureus***	***Streptococcus pneumoniae***
S. No	**(mg/ml)**	**Zones of inhibitions (mm)**			
1.	1.562-25	NI	NI	NI	NI
2.	50	NI	NI	8.6±1.00*	NI
3.	100	8.43±1.39	9.55±0.47	8.22±1.02	NI
4.	200	8.78±1.00	10.20±0.43	10.54±2.01	10.29±0.39
5.	300	9.72±1.09	11.25±0.53	12.4±.098	11.26±1.19

All the values are expressed as mean ± S.D (n=5). NI-no inhibition. ZOI-zone of inhibition. mm-milimeter'

Table 5. Antimicrobial activity of methanolic extract of pollen against Gram (-ve) bacteria

	Gram (-ve) Bacteria			
Methanolic extract of pollen (MEP)		***Escherichia Coli***	***Pseudomonas aeruginosa***	***Salmonella enterica***
1.	1.562-50	NI		
2.	100	7.025±0.83*	NI	NI
3.	200	8.175±0.59	NI	NI
4.	300	8.925±0.44	8.56±0.98	NI

All the values are expressed as mean± S.D (n=5). NI-no inhibition

Table 6. Antimicrobial activity of water extract of pollen against Gram (+ve) bacteria

		Gram (+ve) Bacteria			
Water extract of pollen (WEP)		***Bacillus subtilis***	***Staphylococcus epidermidis***	***Staphylococcus aureus***	***Streptococcus pneumoniae***
S. No	**(mg/ml)**	**Zones of inhibitions (mm)**			
1.	1.562-3.125	NI	NI	NI	NI
2.	6.25	NI	NI	8.70±0.34*	NI
3.	12.5	NI	9.80±0.28	10.43±0.33	NI
4.	25	8.90±0.47	10.23±1.08	11.50±0.08	8.90±0.27
5.	50	10.13±0.34	11.35±0.44	12.65±0.24	9.63±0.74
6.	100	10.55±0.66	13.18±0.60	14.43±0.43	10.85±0.56
7.	200	11.13±0.73	14.58±0.21	14.05±1.54	10.19±0.99
8.	300	11.25±0.53	16.00±0.32	15.49±1.51	11.45±1.09

***.**

All the values are expressed as mean ± S.D (n=5). NI-no inhibition. ZOI-zone of inhibition. mm-milimeter

Table 7. Antimicrobial activity of water extract of pollen against Gram (-ve) bacteria

		Gram (-ve) Bacteria		
Water extract of pollen (WEP)		***Escherichia Coli***	***Pseudomonas aeruginosa***	***Salmonella enterica***
S. No	**(mg/ml)**	**Zones of inhibitions (mm)**		
1.	1.562-6.25	NI	NI	NI
2.	12.5	7.98±0.41*	NI	6.0±1.47
3.	25	8.65±0.37	NI	6.5±0.56
4.	50	9.88±0.57	NI	8.30±0.55
5.	100	10.0±0.49	9.8±0.06	9.2±1.29
6.	200	10.05±0.31	11.8±0.32	10.0±0.76
7.	300	11.25±0.44	15.0±0.08	10.8±0.77

All the values are expressed as mean± S.D (n=5). NI-no inhibition. ZOI-zone of inhibition. mm-millimeter

Table 8. Optical density observed against Gram (+ve) and Gram (-ve) bacteria with water extract of pollen

Pollen		**(O.D) for Gram (+ve) Bacteria**			**(O.D) for Gram (-ve) Bacteria**		
Conc. (mg/ml)	***Bacillus subtilis***	***Staphylococcus epidermidis***	***Staphylococcus aureus***	***Streptococcus pneumoniae***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Salmonella enterica***
Control	0.99	1.55	1.45	1.65	1.68	1.47	1.42
3mg/ml	0.850	1.42	1.36	1.53	1.60	1.20	1.34
7.5mg/ml	0.81	1.31	1.21	1.46	1.43	1.16	1.21
15mg/ml	0.79	1.22	1.16	1.31	1.35	1.09	1.15
30mg/ml	0.65	0.99	0.98	1.20	1.22	0.96	1.08
60mg/ml	0.50	0.82	0.88	1.11	1.15	0.88	0.78

Table 9. Optical density observed against yeasts with water extract of pollen

Bee product	O.D for *Candida albicans*						O.D for *Saccharomyces cerevisiae*					
	Control	3mg/ml	7.5mg/ml	15mg/ml	30mg/ml	60mg/ml	Control	3mg/ml	7.5mg/ml	15mg/ml	30mg/ml	60mg/ml
Pollen	2.25	1.85	1.79	1.72	1.65	1.44	2.65	2.57	2.32	2.10	1.90	1.78

O.D-optical density

Table 10. Antihelminthic activities of bee pollen, positive control (Albendazole) and negative control (Normal saline)

Bee products Extracts	Concentrations	15min	30min	60min	120min
O.D	optical density	O.D	optical density	O.D	optical density
O.D	optical density	O.D	optical density	O.D	optical density
O.D	optical density	O.D	optical density	O.D	optical density
O.D	optical density	O.D	optical density	O.D	optical density
O.D	optical density	O.D	optical density	O.D	optical density
O.D	optical density	O.D	optical density	O.D	optical density
O.D	optical density	O.D	optical density	O.D	optical density

Water extracts of pollen was used for this study as this was observed to be the most effective for microorganisms tested during the *in vitro* study. Mortality was observed after every 15, 30, 60 and 120 min in the entire test group. The data obtained is presented in Table 10. Perusal of the results obtained revealed that pollen at the highest concentration tested (500mg/ml) after completion of 120min of the experiment did not give any more mortality than the negative control (3 and 4 live amphistome respectively) and was therefore not effective in controlling the parasite. The positive control using Albendazole, however at much lower concentration (5, 10, 15µg/ml) was able to arrest the parasite almost cent percent at the end of the experiment. Results therefore suggested that bee pollen was not potent anti helminthic agents and is not suitable for application against amphistome; *Gastrothylax crumenifer*.

4. CONCLUSION

4.1 *In vitro* Antimicrobial Activity

Zone of inhibition measurement done for the organisms studied showed that *Staphylococcus aureus* was most susceptible to ethanolic and methanolic extracts of pollen, followed by *S. epidermidis* and *Streptococcus pneumonia*. *Staphylococcus epidermidis* was also inhibited by water extract of pollen. In yeast, higher inhibition was observed for *Saccharomyces cerevisiae* than *Candida albicans* with ethanolic extract of pollen while higher inhibition was observed with water extract of pollen against *Candida albicans.* The results obtained also showed that negative controls (ethanol and methanol) did not show any inhibitory effect on tested microorganisms, while positive control (ampicillin) showed the highest antimicrobial activity.

4.2 *In Vitro* Antihelminthic Activity

Bee pollen was found to be not effective against amphistomes (*Gastrothylax crumenifer)* used in the present studies. While the positive control using Albendazole was very effective even at much lower concentrations.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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