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CLINICAL RESEARCH

A Study of Antimicrobial Activities of Aqueous and Ethanolic Extract of Bee Pollen against *Escherichia Coli*, *Shigella Boydii*, *Staphylococcus Aureus* and *Bacillus Subtilis*

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ABSTRACT

BACKGROUND

Ethiopia is one of plants species-rich countries in the world and center of origin of many medicinal plants. Studying antimicrobial activities of pollen is vivacious to investigate plants resources for medicinal values and the study was conducted to evaluate antimicrobial properties of bee pollen against mentioned bacteria.

METHODS

Completely randomised design was used for laboratory work. After adjusting turbidity, consistent growth of bacterial culture was made using sterilized cotton swap. 20 grams of bee pollen was added to 200 ml of distilled water as well as ethanol and finally the extract was filtered by Whatman filter by paper, dried and weighted and stock solutions was made as follows, 3.6 gram. was added to 12 ml of distilled water to prepare stock solutions as follows 3.6:12 = 0.3 × 10^6 = 3 × 10^5 ppm stock solution and antimicrobial activities of pollen was tested against mentioned bacteria. Data were imported to R software version 3.44. Multilevel analysis was used to see interaction between bacteria species and each concentration of pollen and Anova was used to see significance of these concentrations on bacteria species. A p-value of <0.05 was considered as statistically significant.

RESULTS

Results indicated that bacteria were more inhibited at 72 hours than 48 hours and 24 hours and the results shown ethanolic extract of bee pollen had antimicrobial activities against both Gram negative and Gram-positive bacteria mentioned above. Time has significant effects on tested bacteria ($p = 0.000$) and treatments have significant effects on tested organisms ($p = 0.000$). Ethanolic extract inhibited the growth of more Gram-negative bacteria:

Escherichia coli and *Shigella boydii*. *Bacillus subtilis* was mostly inhibited by aqueous extract of bee pollen than others.

CONCLUSION

Ethanolic extract of pollen had antibacterial activities against all tested bacterial strains even though it is concentration and time based. Ethanolic extract inhibited more Gram-negative bacteria relatively while aqueous extract inhibited more Gram-positive relatively. Negative controls (sterilized water) didn't show any antimicrobial properties, while positive control (Chloramphenicol) had antimicrobial activities. Further isolation and characterization of bioactive compound from pollen is useful to develop novel botanical formulation for further applications from pollen of medicinal plants.

KEYWORDS

Antimicrobial activities; Aqueous extract; Bee pollen; Ethanol extract

INTRODUCTION

Ethiopia is one of plants species-rich countries in the world and center of origin of many medicinal plants. Many naturally occurring compounds present in plants, herbs, and spices have been shown to possess antimicrobial effect against foodborne pathogens and the production of bee pollen might help to increase economic profits and thus help beekeepers to rectify their financial difficulties [1]. Bee pollen is promoted as a healthy food with a wide range of nutritional and therapeutic properties [2]. Human societies have been in close contact with their environments since the beginning of their formation and used the ingredients of the environment to obtain food and medicine [3]. An interest in substances of natural origin has been a subject that is increasing constantly-both those known for many years and recently discovered are of great interest to the researchers and this interest also applies to bee products because of their extensive nutritional and therapeutic properties; these products are known and used for several thousand years, but only recently, they became the subject of sparse documented scientific research and Greeks believed that honey and pollen are the food of kings, giving the youth and life [4]. Pollen, another bee product, is recognized as an important part of traditional medicine in several countries; because of its nutritional and therapeutic properties, pollen is considered as a functional food in the food industry because of phenolic compounds such as gallic, caffeic and trans-cinnamic acid are the components of pollen that are responsible for antibacterial and antioxidative effects [5]. Bee-collected pollen is a well-known functional food, Honeybees process the collected pollen and store it in the hive, inside the comb cells and the processed pollen is called bee- bread or ambrosia and it is the main source of proteins, lipids, vitamins, macro-and micro-elements in honeybee nutrition and nowadays there is an increasing interest regarding honeybee products, their bioactivity and implementation in alternative medicine and apitherapy [6]. The use of natural products is becoming an ever more popular approach in both medical treatments and the preservation of foods [7]. Bee pollen is consumed for api-therapeutical, nutritional and medicinal properties and its actions are attributed to its chemical composition and mostly phenolics, carotenoids, fatty acids and vitamins [8]. Among the bee products, bee pollen resulting from the agglutination of flower pollen and nectar with salivary substances, emerge as food for worker-bees, with therapeutic properties given its composition [9]. Bee pollens are rich source of essential amino acids and are often considered as complete food for human beings and Apitherapy has become popular as an alternative treatment in recent years and pharmaceutical properties of bee products depend on biological activities such as antioxidant and

antibacterial activities [10]. Bee-collected pollen and beebread are appreciated mainly for their high nutritional value. Both products are rich in proteins, essential amino acids, sugars, fatty acids (including ω-3 and ω-6 fatty acids), vitamins, macro and microelements [6]. Pollen is a product harvested by bees and the pollen transferred to the hive in the form of pollen loads is called the "bee pollen" it is the product that contains valuable substances such as essential amino acids, phenolic compounds, vitamins, pigments [11]. Pollen, another bee product, is recognized as an important part of traditional medicine in several countries. Because of its nutritional and therapeutic properties, pollen is considered as a functional food in the food industry [5]. Bee pollen is a food supplement widely used in the world due to the benefits promoted by the bioactive compounds present in it [12]. Biologically active substances of natural origin always focus a great interest. This also applies to bee products because of their powerful healing properties and Bees' life is still the subject of scientific interest and a source of inspiration for artists, and the bees remain the symbol of diligence and thrift [4]. Natural products can be utilized in the discovery of new antimicrobial drugs and in the treatment of infectious diseases [7].

MATERIAL AND METHODS

Sample Size and Design of the Experiment

The experimental design was Completely Randomised Design for laboratory (CRD). Three working concentrations were used for the experiment and replicated thrice for these bacteria species at exposure experimental periods 24 hours, 48 hours and 72 hours.

Description of the Study Area and Location of the Study Area

The study was conducted at Hawassa University Biology laboratory which is found in Hawassa is a city in Ethiopia, which is found on the shores of Lake Hawassa in the Valley. It is located 273 km south of Addis Ababa. It has latitude and longitude of 7°3′N 38°28′E; 7°3′N 38°28′E and an elevation of 1708 meters above sea level. Hawassa has a tropical savanna climate though it borders on a subtropical highland climate www.hu.edu.et Background of Hawassa University October 2, 2013.

Solvent Extractions of Pollen Samples and Preparation of Working Concentrations

Different amounts of bee pollen were milled, homogenized and individually extracted 20 grams. of bee collected pollen with aqueous and ethanol was added to 250 ml of beakers that contains 200 ml of distilled water and shacked for 24 hours and filtered by Whatman filter paper and then dried in water bath. The extracts of pollen were measured by sensitive balance and then stock solutions and working concentrations were prepared the resulting solutions were stored at 4°C until antibacterial activity determination [8]. Twenty gram of bee pollen was added to 200 ml of ethanol (70%), shacked for twenty-four hours and filtered by Whatman filter paper and dried in water bath 78°C stayed in a water bath at 70°C, for 30 minutes [5]. The extracts of pollen were measured by sensitive balance and then stock solutions and working concentrations were prepared as follows 3.6 grams of extracts was added to 12 ml of distilled water and calculated as 3.6:12 = 0.3 × 10^6 = 3 × 10^5 ppm stock solution and then working concentrations of 100%, 70% and 30% were prepared from stock solution prepared. The resulting solutions were stored at 4°C until antibacterial activity determination [10].

Inoculum Preparation and Inoculation of Bacteria Strains in Laboratory

Four species of bacteria were selected; two Gram positive: *Staphylococcus aureus* and *Bacillus subtilis*. two-Gram negative: *Escherichia coli* and *Shigella boydii* and they were cultured. With the help of a sterile wire loop, the test bacteria were transferred into test tubes having a sterile nutrient broth and incubated at 37°C for 24 hours until the noticeable turbidity and density was equal to that of 0.5 McFarland standards by adding a 0.5 ml of $BaCl_2$ solution in to 99.5 ml of H_2SO_4. After adjusting turbidity, a sterile cotton swab was dipped into suspension and streaked over the whole surface of the plate to make sure the consistent growth of bacterial culture [13].

The Antibacterial Efficacy of these Products against Pathogenic Gram- Positive Bacteria. (B. Subtilis, S. Aureus) and Gram-Negative Bacteria (S. boydii and E. coli) by Ditch Diffusion Method

Four species of bacteria were selected for experiment; two Gram positive: *Staphylococcus aureus* and *Bacillus subtilis* [14]. Two-Gram negative: *Escherichia coli* and *Shigella boydii* and they were cultured. As well, nectar was used directly in all concentrations since no need of extraction nectar. Antimicrobial activities of pollen were tested against mentioned bacteria after they incubated. Disks were mixed into working concentrations of 100%, 70% and 30% and then added to labelled bacteria species inoculated into Petri dish respectively and stayed for 10 minutes - 15 minutes until introduced with media, inverted, and brought to incubator incubated at 37°C. The inhibition zone of bacteria was checked continuously for 24 hours, 48 hours and 72 hours and measured at 48 hours and 72 hours. The antibiotic was placed aseptically in the middle of the plate and was incubated at 37°C for 24 hours. The inhibitory zone diameter was measured in mm [10].

Data Analysis

Pollen was collected and dried at the laboratory and then grinded and tested against mentioned bacteria and data were inserted to inserted to computer Microsoft excel 2010 and imported to R software version 3.44. Multilevel analysis was used to see interaction between bacteria species and each concentration and Anova was used to see significance of these concentrations on bacteria species. A p-value of <0.05 was considered as statistically.

RESULTS

In the study, it is observed that, when the same concentration of the two solutions of poly pollen, against four bacteria a different mean of inhibition zone is observed. Among two solution of poly pollen water extract solution with 100% concentration with the mean inhibition zone of 13.05 ± 3.42, 11.84 ± 4.24, 11.14 ± 3.53, 11.69 ± 4.18 against *E. coli*, *S. boydii*, *S. aureus* and *B. subtilis* respectively. Time has highly significant effects on all species of bacteria on both Gram-negative and Gram-positive bacteria (Table1). Which means inhibitions zones of bacteria depends on exposure periods that at 24 hours it might be less inhibition, but at 48 hours and 72 hours there may be more inhibition. In this study, the inhibition zones were measured against the test bacteria at 48 hours and 72-hours' time interval. Almost all the solution with three different concentrations (30%, 70%, and 100%) produce the higher inhibition zone after 72 hours exposure than 48 hours exposure. The 30% concentration poly pollen ethanol extract also showed the lower (1.83 ± 0.68) result at the time of 72 hours than 48 hours (2.05 ± 0.08) against *S. aureus*. In the study the relatively higher inhibition zone is observed against Gram-negative bacteria and Gram-positive bacteria at the time of 72 hours, this might be due to reaction of bioactive compounds found in the solution of poly pollen.

Tested products at t1	*E. coli*	*S. boydii*	*S. aureus*	*B. subtilis*
Drug (Positive) Control	8.46 ± 0.51	6.91 ± 0.22	17.63 ± 0.49	20.49 ± 0.49
Pp30%w	1.72 ± 0.28	1.63 ± 0.39	2.13 ± 0.93	1.47 ± 1.53
Pp70%w	9.27 ± 3.13	10.31 ± 3.72	9.13 ± 1.91	9.22 ± 3.03
Pp100%w	13.05 ± 3.42	11.84 ± 4.24	11.14 ± 3.53	11.69 ± 4.18
Pp30%E	1.57 ± 0.47	1.65 ± 0.47	2.05 ± 0.08	1.87 ± 0.89
Pp70%E	10.43 ± 2.96	9.54 ± 1.73	9.09 ± 2.84	10.5 ± 3.71
Pp100%E	13.61 ± 3.34	13.18 ± 3.09	11.66 ± 3.86	11.75 ± 4.77
Sterilized Water. Water (Negative Control)	0	0	0	0

Values are mean ± standard deviation of three replications of these concentrations of the extract

Table 1: Mean ± standard deviation of inhibitions zone of bacteria by tested products after 48 hours.

Time has highly significant effects on all species of bacteria on both Gram-negative and Gram-positive bacteria (Table 2). Which means inhibitions zones of bacteria depends on exposure periods that at 24 hours it might be less inhibition, but at 48 hours and 72 hours there may be more inhibition. In this study, the inhibition zones were measured against the test bacteria at 48 hours and 72-hours' time interval since no more inhibitions zone was seen at 24 hours. Almost all the three solutions with three different concentrations (30%, 70%, and 100%) produce the higher inhibition zone after 72 hours exposure than 48 hours exposure. The 30% concentration bee collected pollen ethanolic extract showed the lower (1.83 ± 0.68) result at the time of 72 hours than 48 hours (2.05 ± 0.08) against *S. aureus*. In the study the relatively higher inhibition zone is observed against Gram-negative bacteria and Gram-positive bacteria at the time of 72 hours, this might be due to reaction of bioactive compounds found in the solution of bee collected pollen.

Tested products at t2	Bacterial Isolates			
	E. coli	*S. boydii*	*S. aureus*	*B. subtilis*
Drug	13.41±0.81	10.5±2.73	22.35±0.58	25.82±0.57
Pp30%w	2.71±0.67	2.64±1.12	2.26±0.87	2.16±0.65
Pp70%w	13.89±1.25	12.34± 2.67	12.15±2.21	14.27± 1.43
Pp100%w	17.36±2.04	15.57±3.02	15.07±2.17	17.43± 1.16
Pp30%E	2.19±0.59	2.63± 1.20	1.83± 0.68	1.97±0.86
Pp70%E	14.91±0.31	13.07±2.79	12.53±1.84	14.52±0.49
Pp100%E	18.16± 0.10	15.6±3.48	15.41± 2.03	17.29±0.23
S. water	0	0	0	0

Values are mean ± standard deviation of three replications of these concentrations of the extract

Table 2: Mean ± standard deviation of inhibitions zone of bacteria by tested products after 72 hours exposure periods.

DISCUSSION

The present study summarizes bee collected pollen had high antimicrobial effects against both Gram-negative and Gram-positive bacteria; however, highly inhibition differs from species to species, working concentrations and exposure periods. *E. coli* and *S. boydii* were more inhibited by ethanolic extract than aqueous extract and similar results was reported that different extracts exhibited different antibacterial activities [10]. Ethanolic extract of pollen showed highest inhibitions zone against both Gram negative and Gram positive bacteria and it was reported that ethanolic extract of Pollen had the highest activity against all the bacteria's used [14].This study was limited to study antimicrobial activities of aqueous and ethanolic extracts against these mentioned bacteria and studying

many indigenous plants' pollen antimicrobial activities at against more test organisms by different concentration of different solvent is important to find alternative solutions for resistant developing bacteria against prescribed drugs. The inhibition zones were different, according to the extraction solvent used and also pollen concentration and this result is analogs to reports of [8]. Based on the solvents, used for extraction variable antimicrobial activity may be seen even against the same bacterial strains similar results was reported by [6]. The results showed that bee pollen extract has an inhibitory effect against all Gram negative and Gram-positive bacteria and similarly is reported that Turkish bee pollen extract had an inhibitory effect against all pathogens [15,16]. The Result Obtained from this research showed that pollen, had antibacterial activity against both the Gram-Positive and Gram-Negative bacteria (Table 1 and Table 2) this is in relation with the work conducted by [14].

CONCLUSION

In the present research work, we concluded that the Ethanolic extract had more inhibitory effects against Gram-negative bacteria than aqueous extract. Higher concentration of aqueous and ethanolic extracts had higher inhibitions zone than lower concentrations. Pollens might be an alternative natural food resource due to the anticipatory properties as indicated by this result. Time has significant effects against bacteria species with this p-value of 1.72e-08 *** and treatments have significant effects with this p-value (3.80e-14 ***). From the above table we can conclude that ethanolic extract has significant effects than aqueous extract even though its significance effects differ based on bacteria species and exposure periods. An upcoming study is compulsory to pinpoint the functional components in a wide variety of honeybee forages' pollen and test their biological goings-on.

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ETHICS APPROVAL AND CONSENT PARTICIPATE

The purpose of the study was explained to Dr. Zufan Bedewi the head of department of natural and computational science at Hawassa University, Ethiopia and she agreed to test antimicrobial activities of aqueous extract and ethanolic extract of bee collected pollen against mentioned Gram negative and Gram-positive bacteria.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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