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Research Article

In Vitro Antibacterial Activity of Monofloral Bee Pollen from Western Oromia, Ethiopia

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Abstract

Pollen is a natural product collected by bees from flowering plants for brood rearing. It has been used as a medicine and food supplement. However, its biological and nutritional composition primarily depends on floral origin. The aim of the present study was therefore to determine the antioxidant and antibacterial activities of methanolic (99.9%) extract of pollens among the floral origin. The Total Phenolic Compound Content (TPCC) and Total Flavonoid Compound Content (TFCC) were measured following the standard method. *In vitro* antibacterial activity was evaluated using the agar well diffusion method against five bacterial strains. The findings showed that the pollen from *Eucalyptus* plants had the highest TPCC (62.4 ± 0.5 mg GAE) and TFCC (49.6 ± 0.2 mg QE)/100g of pollen), while the pollen from Bidens plants had the lowest TPCC (27.5 ± 0.8 mgGAE) and TFCC (18.8 ± 0.7 mgQE/100g of pollen). Bee pollen exhibited varying levels of antibacterial activity, with *Bidens* spp. showing 6.6 ± 0.6 mm against *Escherichia coli* (ATCC-25922) and *Acinetobacter baumannii* (ATCC-17978) and *Eucalyptus* spp. showing 23.3 ± 0.6 mm against *Staphylococcus aureus* (ATCC-25923). Additionally, it was shown that the antioxidant content and the antibacterial activity were positively correlated. Among all the examined strains, *Eucalyptus* pollen proved to be the most effective, whereas *Bidens* pollen showed the least effectiveness. The results showed the pollen that had more antioxidant content exhibited more inhibition diameter against the tested bacterial strain. It is concluded that pollen could be used as an alternative therapy against diseases caused by bacterial pathogens and free radical compounds while its efficiency influenced by floral origin.

Keywords: Antibacterial activity; Antioxidant activity; Bee pollen; Flavonoid content; Phenolic content

Introduction

Infectious diseases caused by various pathogens are a leading global cause of illness and death [1]. These fast-spreading microbial diseases continue to challenge various health sectors and show resistance to drug treatments [2]. The recent WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) report indicates a rise in antibacterial resistance, particularly in low- and middle-income countries, resulting in significant mortality and morbidity [3]. Around 30% of infants with sepsis perish due to bacterial infections that do not respond to initial antibiotics [4]. Therefore, exploring alternative remedies from natural health products is crucial. A rich supply of protein and minerals, pollen is a highly flexible natural molecule that bees collect for its vast reservoir of bioactive chemicals, which have substantial chemical and medicinal potential [5]. Because of their many bioactive compounds and powerful therapeutic qualities, pollen and bee products have long been regarded as well-liked natural treatments and appreciated for their nutritional content and wide range of medical uses [6]. Bee pollen is an intricate mixture of plant pollens that bees collect. Its elemental makeup varies significantly depending on the type of flower it comes from, where it is located, the type of soil it is made of, and the temperature [7]. Pollen's antibacterial qualities can be ascribed to bioactive substances such flavonoids, phenolic compounds, and other phytochemicals [8].

The antibacterial and antioxidant characteristics of bee pollen are determined by a variety of factors, including plant species, growth circumstances (soil, climate, and location), harvesting time, and extraction technique [9]. For example, rape bee pollen methanol extract was very efficient against *Salmonella enterica*, whereas poppy plant pollen from Slovakia demonstrated great efficacy against *S. aureus* by ethanol extraction [10]. Likewise, *Bacillus subtilis, E. coli, Klebsiella* spp., *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *S. aureus* were shown to be inhibited in growth by bee-pollen extracts obtained from plants belonging to the Papaveraceae, Brassicaceae, and Asteraceae families [11].

Ethiopia leads in honey production in Africa and ranks 10th globally [12]. The country boasts over 10 million bee colonies and more than 800 identified honey-source plants [13,14], showcasing rich plant diversity that yields various biological compounds. Despite this, there is scarce data on the antioxidant contents and antibacterial properties of pollen gathered by *Apis mellifera* L. from Ethiopian flora. Hence, this study aims to assess the total phenol and flavonoid levels and antibacterial effects of bee pollen extracted with methanol from five major bee plants.

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Materials and Methods

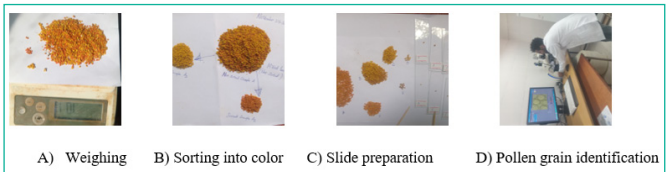
Study Area

The study was carried out at the Haro Sebu Agricultural Research Center on the station, situated 550 kilometers from Addis Ababa in the Western Oromia region, in the Kellem Wollega Zone of the Dale Sedi district. The Illubabor Zone borders it on the south; Dale Wabara borders it on the west; Mirab Welega Zone borders it on the north; and Lalo Kile borders it on the east. Haro Sebu is the district's administrative hub. The location of the pollen collection site was 1,495 meters above sea level. The research region was home to a variety of tropical plants, including cultivated crops, weeds, forest trees, and wild coffee (Coffea arabica).

Bee Pollen Collection and Plant Identification

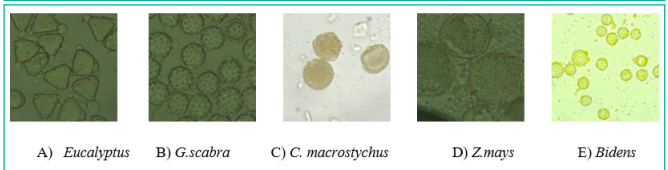
The sample was taken using pollen traps with a 16% pollen catching effectiveness from September 2021 to August 2022. After being taken from the honeybees' rear legs, the pollen samples were scraped off and placed in a tray. After being taken off a tray and put in a fresh paper bag, the pollen pellets were allowed to dry at room temperature for a full day. They were categorized according to color and recognized down to the genus or species level after drying.

Following their collection, weighing, and overnight drying at room temperature, pollen pellets were separated by size and color (Figure 1A-C). Melissopalynological analysis, a routine process, was followed to mount representative pellets of each hue on slides for microscopic inspection. The pellets were then cleaned with ether and coated with glycerin jelly [15]. The slides were inspected using a light microscope with a 400-x magnification after being covered with a cover slip. A light microscope (Zeiss, 2010) connected to a computer program was used to analyze the morphology of pollen, as seen in Figure 1D, and Figure 2 depicted the morphology of each plant's pollen grain. Based on the pollen atlas created specifically for this purpose from Ethiopian bee plants, the observed shape of the pollen grains was confirmed [13].



A) Weighing B) Sorting into color C) Slide preparation D) Pollen grain identification

Figure 1: A) Unsorted (color mixed pollen pellet) and weighing, B) Sorting based on color, C) Slide preparation for each color, D) Microscopically identifying pollen grain morphology.



A) *Eucalyptus* B) *G.scabra* C) *C. macrostychus* D) *Z.mays* E) *Bidens*

Figure 2: Microscopic pollen grain morphology of the selected major bee plants.

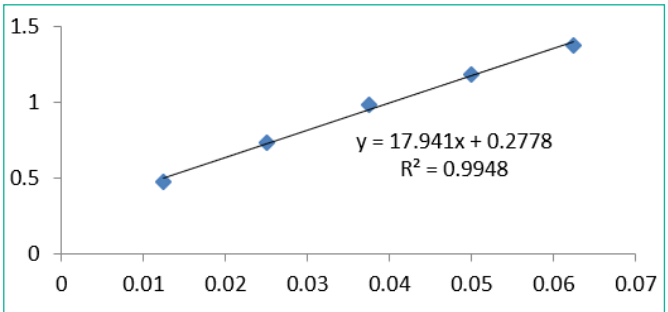


Figure 3: Calibration curve for phenol compound content.

Methanolic Pollen Extraction (MPE)

The pollen sample was extracted using methanol solvent in accordance with Addi et al. [16] methodology. Using a temperature-controlled shaker incubator, two grams of dried pollen powder and 25 mL of methanol were macerated at 25°C for 60 minutes. After filtering the mixture, the leftover residue was extracted twice more using 25 mL of methanol each time. At 40°C, the mixed methanolic extracts evaporated, leaving a dry residue behind. To be used later, this residue was dissolved in 50 mg/mL of methanol and kept at 4°C.

Total Phenolic Compound Content (TPCC)

To find the TPCC in bee pollen, the Folin-Ciocalteau colorimetric technique [17] was employed. For 2 hours, a combination of 2.0 mL 4% sodium carbonate, 2.5 mL diluted Folin-Ciocalteau reagent (1:10), and 0.5 mL MPE was incubated in the dark at room temperature. Then, at 740 nm, absorbance was measured. Using gallic acid (0-200 mg/mL) as a reference, TPCC was measured in mgs of Gallic acid equivalent (GE) per g of bee pollen dry weight. The calibration formula ($y = 17.941x + 0.2778$; $R^2 = 0.9948$) derived from the calibration curve was used (Figure 3).

Total Flavonoid Compound Content (TFCC)

TFCC was analyzed in accordance with the method outlined by Zou et al. [17]. For this, 4.3 mL of 99.9% methanol, 0.1 mL of 10% Al $(NO_3)_3$, and 0.1 mL of 1 M potassium acetate were combined with 0.5 mL of the MPE (1:10). Using a spectrophotometer (UV-Vis Mini 1240, Shimadzu Co.), the absorbance was measured at 415 nm after 40 minutes at room temperature. A standard of quercetin (0-200 mg/mL) was employed for the calibration curve. Based on the mean of triplicate results, the TFCC was reported as milligrams of Quercetin equivalent (QE) per gram of dry weight bee pollen. The calibration curve (Figure 4) yielded the calibration equation ($y = 9.4688x + 0.2578$; $R2 = 0.9962$).

Test Bacteria Used and Inoculum Preparation

The Ethiopian Public Health Institute (EPHI) provided the standard bacterial strains, which include *S. aureus* (ATCC-25923), *Klebsiella pneumonia* (ATCC 43816), *A. baumannii* (ATCC-17978), *Enterobacter cloacae* (ATCC-13047), *E. coli* (ATCC-25922), and *Pseudomonas aeruginosa* (ATCC-27853).

The preparation of the inoculum was done in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendations [18]. In order to do this, 2-3 colonies were chosen from a culture cultured on their selective media for 24 hours, and they were then put in 5 mL of saline solution (0.85%). After 15 seconds of vortexing, the turbidity of these suspended inoculums was measured by adding saline or colony solution to the microbial stock solution. A visual comparison was made between this and a standardized 0.5 McFarland (10^8 CFU/mL) on white paper with black lines that contrasted [19].

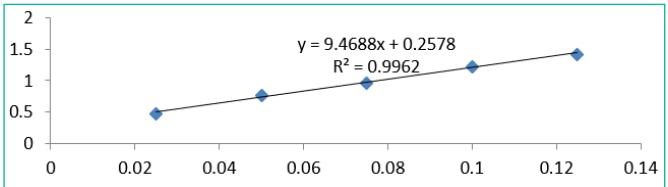


Figure 4: Calibration curve for flavonoid compound content.

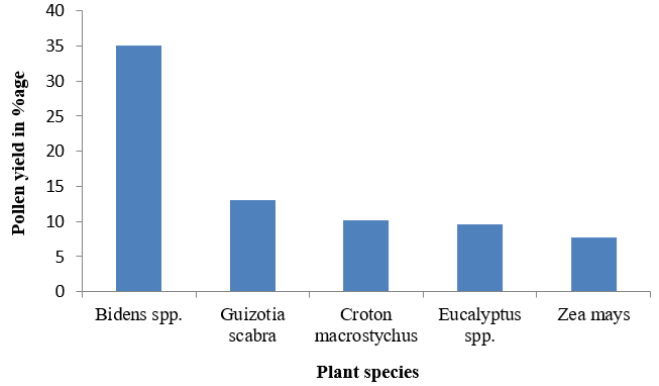


Figure 5: The percentage pollen yield/hive of the tested plants.

Agar well Diffusion Assay

The agar well diffusion test was performed [20]. Mueller Hinton Agar was poured on the petridishes and using a sterile cork borer, wells of 6 mm in diameter and 4 mm in depth were made in the agar medium. Then, the solidified medium was uniformly seeded on using a sterile cotton swab from an inoculated saline solution containing bacteria strains. After that, the plates were placed on the bench to absorb extra liquids. Afterwards, a micropipette was used to add 60 µL of MEP samples at a 75% concentration (3g of MEP combined with 1 mL of dimethyl sulfoxamide) to the plate wells. Additionally, dimethylsulfoxamide; a negative control, and ciprofloxacin (10 µg/60 µL); a positive control, were added to the wells. For twenty-four hours, the plates were incubated at 37°C. The diameters of the inhibition zones around well were measured in millimeters using a ruler, and the results were recorded and replicated three times.

Data Analysis

Means ± standard deviations of the recorded data were calculated using SAS Software. Determination of the significant differences between MEP was done using one-way ANOVA. Inhibition zones of the tested MEP were used for mean separation. Multiple pairwise comparison between the mean values was done using the Least Significant Difference (LSD).

Results and Discussions

Plant Identification

Figure 5 illustrates the different percentages of the bee pollen plant species that contribute to the pollen output. Five key bee plants namely: *Bidens* spp., *G. scabra*, *C. macrostychus*, *Eucalyptus* spp., and *Z. mays* were selected based on their notable production and sample availability for examination. *Z. mays* had the lowest pollen output at 7.7%, whereas *Bidens* spp. had the greatest at 35%.

Numerous scholars have identified these plants as important Ethiopian bee plants [16,21]. After June to August, which is the major wet season, herbaceous plants such as *Guzotia* and *Bidens* blossomed abundantly, offering nectar and pollen to bees. Single-flower honey is produced by *G. scabra*, which grows well in a variety of environments including open grasslands, woodland borders, and farmed areas [22].

Total Phenolic Compound Content (TPCC) and Total Flavonoid Compound Content (TFCC)

Figure 6 and 7 showed the TPCC and TFCC for various pollen kinds, respectively. The results showed that, of the pollen types tested, *Eucalyptus* had the highest TPCC (62.4 ± 0.5 mg GAE) and TFCC (49.6 ± 0.2 mg QE)/100g of pollen), while the pollen from *Bidens* had the lowest TPCC (27.5 ± 0.8 mg GAE) and TFCC (18.8 ± 0.7 mg QE/100g of pollen). The present study's TPCC is lower than the ranges reported in Eastern Croatia (7.08 to 15.27 mg GAE/g) [24], Poland (12.93 to 82.43 mg GAE/g) [25], and Italy (5.78 to 20.15 mg GAE/g) [23]. The TPC in pollen grains of Castanea sativa varied from 64.02 ± 0.26 to 103.8 ± 6.72 mg GAE/g, according to another research [26].

The TFCC in this research (18.8 ± 0.7 to 49.6 ± 0.2 mg QE/100g of pollen) was lower than that from Northwest Algeria (TFC, 8.92 mg QE/g) [27], the Baltic region (TF, 6.1 to 11.6 mg QE/g) [28], Thailand (7.53± 0.30 to 56.40± 4.85 mg QE/g) [29]. In line with prior research [30], variations in the botanical and geographic origins of bee pollen samples may account for the diversity in phenolic compounds and flavonoids. Furthermore, as noted by previous research [31], the variations in phytochemical composition found in various bee pollen samples, especially those originating from the same plant, may be due to a variety of factors, including climate and beekeeping techniques, in addition to their botanical provenance.

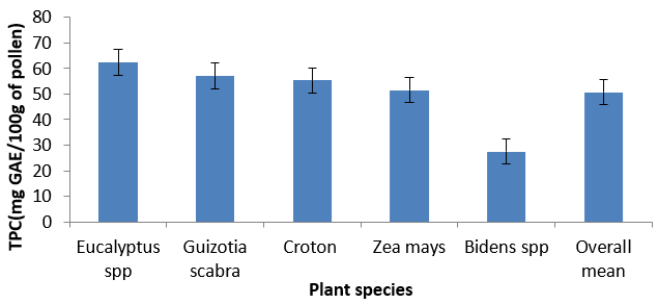


Figure 6: Average total phenolic compound content among plant species.

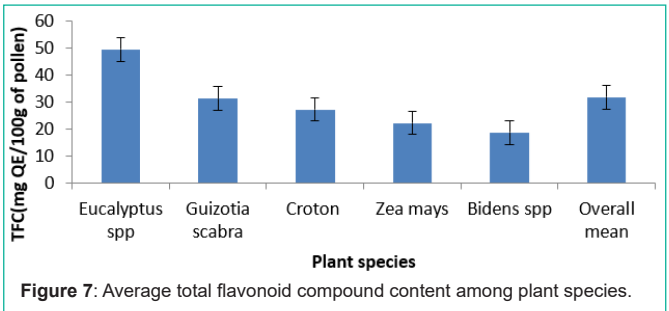


Figure 7: Average total flavonoid compound content among plant species.

Table 1: Inhibition zone (mean ± standard deviation) among pollen types against the strains.

Bacterial strain	Pollen types (mean ± standard deviation of the inhibition diameter zone; mm)										
	Eucalyptus spp.	*Guizotia* spp.	*Croton macrostychus*	*Zea mays*	*Bidens* spp.	Ciprofloxacin	DMSM	LSD	p-value	CV	R^2
SA (ATCC-25923)	23.3± 0.6b	22.0± 1b	16.6± 1.5c	14.0± 1.0d	12.3± 0.6e	33.0± 1.0a	NIZ	1.56	<.0001	4.84	.99
KN (ATCC-43816)	18.3± 1.2b	17.0± 1b	15.0± 1c	12.0± 1.0d	9.0± 1.0e	27.6± 0.6a	NIZ	1.56	<.0001	5.87	0.98
AB (ATCC-17978)	16.6± 0.6b	15.3± 0.6c	13.3± 0.6d	9.6± 0.6e	6.6± 0.6f	25.3± 0.6a	NIZ	0.86	<.0001	3.67	0.99
ECL(ATCC-13047)	17.3± 0.6b±	16.0± 1.0b±	13.6± 1.5c	9.3± 1.2d	7.3± 0.6e	26.3± 0.6a	NIZ	1.66	<.0001	6.81	0.98
PA (ATCC-27853)	14.3± 0.6b	13.0± 1c	10.3± 0.6d	9.3± 0.6d	8.0± 1.0e	26.6± 0.6a	NIZ	1.28	<.0001	5.7	.99
EC (ATCC-25922)	14.6± 1.1b	13.3± 0.6b	11.3± 0.6c	8.0± 1.0d	6.6± 0.6de	23.0± 1.0a	NIZ	1.43	<.0001	6.80	0.98

Values in the same row followed by the same letter are not significantly different by Tukey's multiple range tests ($p < 0.05$). SA (*S. aureus*), KN (*K. pneumonia*), AB (*A. baumannii*), ECL (*E. cloacae*), EC (*E. coli*), PA (*P. Aeruginosa*), DMSM (Dimethylsulfoxamide), LSD (Least Significant Difference), CV (Coefficient of Variation), ATCC (American Type Culture Collection) and NIZ (No Inhibition Zone)

Table 2: Pearson correlation coefficient.

	Phenol	Flavonoid
Phenol	**1**	
Flavonoid	0.73407**	1
SA (ATCC-25923)	0.78501***	0.87375***
KN (ATCC-43816)	0.88581***	0.84034***
AB (ATCC-17978)	0.90266***	0.85298***
ECL(ATCC-13047)	0.84354***	0.84411***
PA-27853	0.79420***	0.88958***
ECOLI25922	0.82483***	0.87037***

Antibacterial Activity Among Pollen Types

Table 1 displays the inhibitory diameters of the methanol (99.9%) extracts of monofloral bee pollens that were evaluated as natural antibacterial agents against common human bacterial strains in the current investigation. Using the well diffusion technique, the antibacterial activity of bee pollen against *S. aureus* varied from 23.3 ± 0.6 mm for pollen from *Eucalyptus* spp. to 6.6 ± 0.6 mm for pollen from *Bidens* spp. against *E. coli* and *A. baumannii*. In comparison to other pollen kinds, bee pollen from *Eucalyptus* and *Guizotia* displayed statistically equivalent inhibitory diameters ($p>0.05$) and shown better efficacy against *S. aureus* and *K. pneumonia*. Furthermore, against every pathogenic strain examined, *Eucalyptus* pollen proved to be much more efficient ($p<0.0001$) than *Bidens* pollen. When compared to the positive control (an antibiotic; Ciprofloxacin), all pollen types shown statistically significant differences ($p<0.0001$) and were less effective; in contrast, the negative control, dimethylsulfoxamide, exhibited no inhibition against the tested strains. The test organism that was shown to be most vulnerable to all forms of pollen was *S. aureus*.

The results emphasize the potential of pollen to inhibit human bacterial growth and highlight the influence of floral origin on its biological activities. Ethiopia has not carried out any study of this kind to compare with the results of the current finding but compared with research done at somewhere. Additionally, bee flora is not always available in all nations. For instance, *Citrus aurantium* pollen extracted with 50% ethanol demonstrated a less effective inhibition diameter (6 ± 0.01) against *A. baumannii, E. cloacae, E. coli, K. pneumonia*, and *P. aeruginosa* when compared to different plant types, whereas *Ruta graveolens* pollen showed a higher effectiveness (22.33 ± 1.20) against *S. aureus*. A pollen extract from Morocco was less effective against Gram-negative while Gram-positive (*S. aureus*) was susceptible [32]. Pollen extracts from *Quercus ilex* and *Punica granatum* demonstrated substantial antibacterial activity against *S. aureus*, with inhibition zones of 19.33 ± 0.33 mm and 22.33 ± 1.20 mm, respectively [33]. Nonetheless, *E. coli* (14 ± 0.57 mm), *A. baumannii* (13 ± 0.88 mm), and *S. aureus* (14 ± 0.57 mm) were all successfully combated by *Ruta graveolens* pollen extract.

Phenolic and flavonoid chemicals found in bee pollen have been linked to a range of medical benefits, including antibacterial, anti-inflammatory, antioxidant, and antitumor effects [34]. The antibacterial qualities of bee pollen are attributed to its bioactive constituents, which include flavonoids, plant phenolics, glucose oxidase, and secondary metabolites [35]. It has been proposed that polyphenols provide a novel approach to counteract bacterial resistance [36]. It has been shown that pollen grains from various plant species and geographical regions vary in their antibacterial effectiveness [37,38]. The content and properties of pollen are influenced by a variety of factors, including the kind of plant, growth circumstances such as soil and climate, harvesting season, extraction solvent, and laboratory techniques [39]. Differences in the composition of bacterial walls may account for the differing susceptibilities of gram-positive and gram-negative bacteria to certain kinds of pollen [40]. Compared to gram-negative bacteria, gram-positive bacteria have a cytoplasmic membrane that is richer in anionic phospholipids, which may be the reason why certain gram-negative bacteria are naturally resistant to antibiotics [41]. For instance, daptomycin, a lipopeptide antibiotic, works well against gram-positive bacteria but not gram-negative ones because it cannot cross the outer membrane, which is an essential step in its antibacterial activity [42].

Pearson Correlation Coefficient

One useful method for determining correlations between different research factors is the correlation test. The relationships between antioxidant levels and antibacterial activity are shown in Table 2. Due to the high concentration of bioactive components in the pollens, phenolics and flavonoid contents were shown to significantly positively correlate. A significant positive correlation was discovered between the antioxidant content and the antibacterial activities against different strains of bacteria, as well as between the flavonoid content and the antibacterial activities displayed by the methanol extract of various pollens against all strains of bacteria. This finding is consistent with other studies showing an antibacterial activity-antioxidant content relationship [43,44].

Conclusion

This is the first study to look at how five different botanical sources of bee pollen affect different strains of human bacteria. The findings show that these bee pollen extracts have potent antibacterial qualities

as well as a high concentration of natural antioxidants. In particular, the greatest concentrations of antioxidant chemicals and antibacterial activity were found in pollen derived from *Eucalyptus* and *G.scabra* flowers. *S. aureus* were more vulnerable than gram-negative bacteria. In the methanol extracts of different pollen types against all bacterial strains, a significant association was found between the flavonoid content and antibacterial activities, the phenol content and antibacterial activities, and the flavonoid content and phenol content. A thorough examination of the biological and chemical components of the pollen samples was not carried out due to a lack of resources. Hiwever, this research will further our knowledge of the antimicrobial and antioxidant characteristics of pollen derived from various plant sources.

Author Statements

Author Contributions

Ofijan Tesfaye: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing – original draft and Writing – review & editing.

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