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Seasons and bee foraging plant species strongly influence honey antimicrobial activity

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ABSTRACT

Honey has been used in human medicine since ancient times due to its antimicrobial properties. However, honey antimicrobial potential varies due to floral sources, geographical origins, and seasonality. The current study assessed the antimicrobial activity of honey and honeybees' preferred plants namely, *Acacia mellifera*, *Ocimum basilicum*, *Hoslundia opposita*, *Combretum schumannii*, *Grewia bicolor*, *Terminalia brownii*, *Cordia monoica* from Same district in Northern Tanzania, during the short and long rain seasons of 2021/2022. The agar well diffusion method was employed for the antimicrobial assay, and the antimicrobial activity was evaluated by measuring inhibition zones. Significant differences were observed in antimicrobial activities among honey of different seasons ($F = 28.5$, $p = <0.001$) and plant extracts ($F = 15.9$, $p < 0.001$). Honey A and D that were harvested at the end of the short rain season were found with higher antimicrobial activities (10–19 mm inhibition) than that harvested at the end of the long rain season (10–15 mm inhibition), and the most susceptible microorganisms were *Escherichia coli* and *Staphylococcus aureus*. For the tested plant extracts, *T. brownii*, *C. schumannii,* and *H. opposita* showed higher antimicrobial activities (11.3–19 mm inhibition) against pathogenic microorganisms than other tested plants. There was a strong positive correlation in antimicrobial activities ($r = 0.836$, $p = 0.078$, $r = 0.756$, $p = 0.139$, and $r = 0.732$ $p = 0.159$) between honey harvested at the end of the short rain season with some plant extracts from plants blooming during the same season. The study highlighted the variation in antimicrobial activities among honey harvested in different rain seasons and that there is antimicrobial relation between honey and plants that are foraged by honeybees. Thus, the antimicrobial ability of the honey depends much on the plant species foraged by honeybees.

1. Introduction

Honey is among the products that are produced by bees of different species, primarily stinging bees of the genus *Apis* and stingless bees of the genus *Meliponin* [1]. The microbial resistance of pathogenic microorganisms to synthetic antibiotics and antimycotics have increased interest on the use of honey and plants with medicinal potential as an alternative cure [2]. The use of honey in medicine has had historical recognition since ancient times with no reported bacteria resistance [3,4]. Its admiration has grown significantly in medicinal use after discovering its antibacterial properties in 1892 [5].

The plants that are visited by honeybees during foraging contributes to the physical-chemical and biological properties, including the antimicrobial activities of honey [6,7]. For instance, nectar a plant substance foraged by honeybees is used as a primary raw material in honey production; this plant substance is produced by specialized tissue called nectaries, which are found in different plant parts including flowers and leaves [8,9]. Regardless of being produced in different plant parts, nectar is reported to have significant similarities in their composition [10,11]. Thus, the composition of leaf extract and nectar from the same plant have been reported to share notable similarities [11,12].The life of honeybees depends on other plant-delivered resources, such as pollen and resin, in making their food and other hive products [13]. Interestingly, plants with therapeutic value have been reported to account for

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the medicinal potential of honey and other honeybee products in the given region [14]. However, plant's secondary metabolites are responsible for the variation in plant's medicinal potentials, and they are determined and vary with seasonality.

Little has been done regarding the influence of honeybees' preferred forages and seasonality on the antimicrobial activity of honey harvested from different beekeeping potential areas in Tanzania. The current study assessed the antimicrobial activity of honey samples and honeybee's (*Apis mellifera*) preferred plant's leaf extract during different rain seasons and study areas. It can be hypothesized that the antimicrobial activity of honey differ among honey harvested at different beekeeping areas across the rainy seasons, and honey produced from the areas where preferred plants have great potential for medicinal use may have high antimicrobial activity [15].

2. Methodology

2.1. Plant materials

The selection of plant species was based on the most reported plants in different studies as honeybees' preferred fodders in Northern Tanzania and elsewhere [16,17], which were as well available and observed in the study areas. Fresh plant leaves of the selected seven plants, *Acacia mellifera*, *Ocimum sinuatum*, *Hoslundia opposita*, *Combretum schumannii*, *Grewia bicolor*, *Terminalia brownii,* and *Cordia monoica* (Fig. 1) were collected directly from plants in the two different study sites (−4.0235862/37.7219419) and (−4.147426 37.9811853) in Same district of Kilimanjaro region in Tanzania. The voucher specimens of the plant species (PS) collected are PS/NM-AIST/001, PS/NM-AIST/002, PS/NM-AIST/003, PS/NM-AIST/004, PS/NM-AIST/005, PS/NM-AIST/006, and PS/NM-AIST/007 have been deposited at the Nelson Mandela African Institution of Science and Technology (NM-AIST). The collected fresh plant leaves were washed with distilled water and left for three weeks at room temperature to dry [18]. The grinder was used to grind dried leaves to fine powder; the powder was stored at room temperature prior to extraction.

2.2. Honey samples collection

Raw honey samples from *Apis mellifera* colonies were harvested from five randomly selected hives in both study areas during the end of the short rain season (January 2022) and the long rain season (May 2022). Honey samples were categorized according to season and area of harvest whereby honey samples A and B were harvested from site I (−4.0235862/37.7219419) and samples C and D from site II (−4.147426 37.9811853). The distance between the study areas (I and II) was 40 km apart. While honey samples A and D were harvested during the end of the short rain season (November to January), honey samples B and C were harvested during the end of the long rain season (February to May) [19]. The collected honey samples per site per season were filtered using double-sieve honey strainer filters and mixed to get one composite sample. Then the samples were stored in 50 mL falcon tubes and kept at a temperature of 20 °C in the University of Dar es salaam food microbiology laboratory prior to antimicrobial assay.

2.3. Microorganisms and sub-culturing

Five pathogenic microorganisms (4 bacteria and 1 fungus) were selected for this study. The selected microorganisms were *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 11775), *Salmonella typhi* (ATCC 14028), and *Candida albicans* (clinical isolate from Muhimbili national hospital). The selected microorganisms were obtained from the food microbiology laboratory at the University of Dar es salaam. The microorganisms were collected purposively to evaluate the antimicrobial potency of honey samples and plant extracts of honeybees' preferred fodders. The sub-culture was conducted where Potato Dextrose Agar (PDA) (HiMedia Laboratories Pvt.Ltd, India) and Muller Hinton Agar (MHA) (HiMedia Laboratories Pvt.Ltd, India) were used for fungi and bacteria growth respectively.

2.4. Plant leaf extraction

Chromatographic method was employed in the extraction of crude extracts [20] with minor modifications. A fine powder of plant leaves of 70 g was dissolved in 700 mL of ethanol (Blulux Laboratories (P) limited,

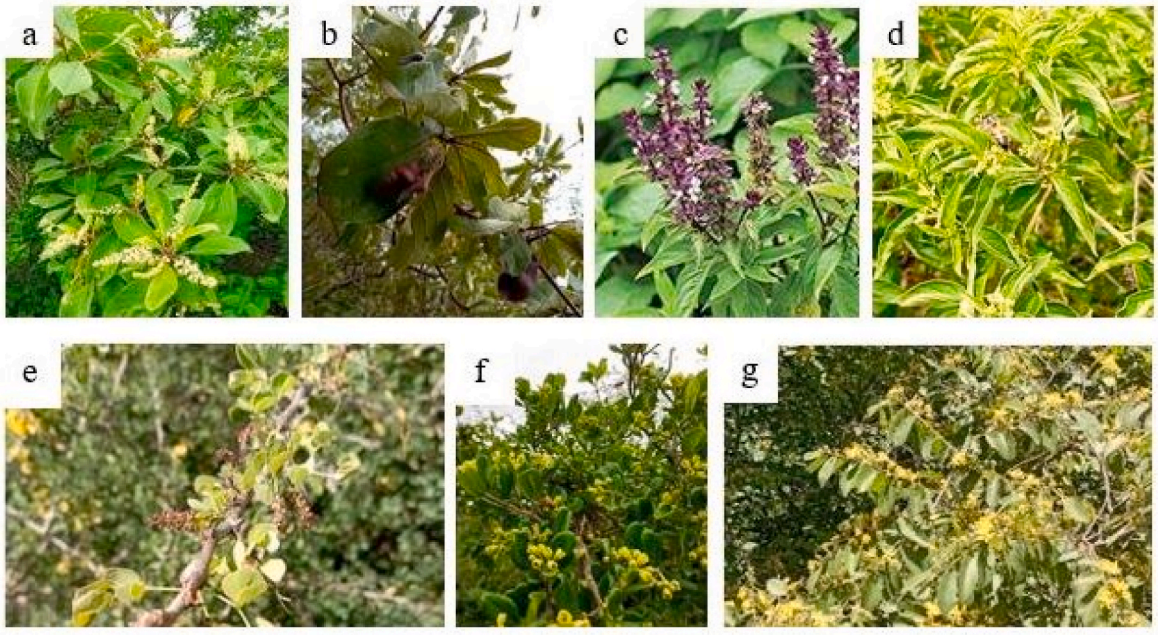


Fig. 1. Honeybees' fodders that were selected for antimicrobial assay, where (a) *T. brownii* (b) *C. schumannii* (c) *O. basilicum* (d) *H. oposita* (e) *A. mellifera* (f) *C. monoica* (g) *G. bicolor*.

India), the mixture was then shaken slightly and left for 48 h at room temperature, followed by filtration using a Whatman filter paper of 125 mm diameter. The filtrates were then subjected to a rotary evaporator (Jinan Biobase Biotech Co., Ltd, China) at the temperature of 40 °C and a speed of 100 RPM to obtain crude extracts. The extracts were left at room temperature to allow evaporation of the remaining ethanol. The crude extracts were then stored at a temperature of 4 °C prior to antimicrobial assay.

2.5. Preparation of crude extracts and honey samples for antimicrobial assay

During the preparation of the samples for antimicrobial assay, 100 mg of plant crude extracts were dissolved in 1 mL of Dimethyl sulfoxide (DMSO) (Loba Chemie Pvt Ltd, India) to make a stock of 100 mg/mL. To obtain a 9:1 honey sample, each sample of honey was mixed in a 9:1 (honey: pure water respectively). The vortex mixer was used to mix separately each of the crude extracts and honey samples, ensuring they dissolved completely in solvents. The resulting mixtures were used for antimicrobial assay accordingly.

2.6. Preparation of inoculum

An overnight Nutrient agar/Potato dextrose agar culture (HiMedia Laboratories Pvt.Ltd, India) of the test microorganisms were used to prepare the inocula. A loopful of cells from the stock cultures were transferred to test tubes containing Sabouraud dextrose broth (HiMedia Laboratories Pvt.Ltd, India) for fungi and Nutrient broth (HiMedia Laboratories Pvt.Ltd, India) for bacteria. The two were then incubated for 24 h at 37 °C and 25 °C, respectively, without agitation to create the active cultures for the assays [21]. 0.2 mL of the culture was added to 5 mL of Sabouraud dextrose broth and nutrient broth, and it was then incubated until it attained the required turbidity of 0.5 McFarland solution at 600 nm and absorbance of 0.08–0.1, or $1.5x10^8$ CFU/mL.

2.7. Antimicrobial susceptibility test assay

In the antimicrobial assay, the agar well diffusion method was employed to test the antimicrobial activity of both extract and honey. Fluconazole and Chloramphenicol were used as a positive control during experiments for fungi and bacteria, respectively.

2.7.1. Agar well diffusion

The agar well diffusion method was used as described by Bello and co-workers (2022) with minor modifications [22]. Growth media: Muller Hinton agar for bacteria and potato dextrose agar for fungi were prepared as per manufacturer instruction. The media were autoclaved at the temperature of 121 °C and pressure of 15 pounds per square inch (psi) for 15 min and then allowed to cool in a sterilized fume hood chamber (Jinan Biobase Biotech Co., Ltd, China). 20 mL of freshly sterilized prepared nutrient agars, Muller Hinton Agar, and Potato Dextrose Agar were added in 9 cm diameter disposable petri dishes and left for 5 min to solidify at room temperature. After the solidification of nutrient agar, inoculum prepared from 0.5 standard McFarland for each microorganism was spread into the disposable petri dishes using sterilized cotton swabs. During the preparation of the wells, stainless steel borer was used to punch the wells of 6 mm diameter. A 50 μL for both plant extracts and honey samples was added to prepared wells, while 30 μL of fluconazole and chloramphenicol were added in separate wells in each plate as a positive control. The plant extracts, honey, fluconazole and chloramphenicol were endorsed to defuse, followed by incubation for 24 h at 37 °C for bacterial strains and 48 h at 27 °C for fungi [23]. This treatment of honey, plant extracts as well as fluconazole and chloramphenicol were done in triplicate. A transparent ruler calibrated in millimetres was used to measure the zone of inhibition's diameters.

2.7.2. Statistical analysis

The Shapiro-Wilk test was employed to assess whether the data were normally distributed or not. For normally distributed data an Analysis of variance (ANOVA) was used to see if there were significant differences in the antibacterial activity of samples of honey and plant extracts. Pearson's correlation relation and Principal component analysis (PCA) were employed to determine the relationship in antimicrobial activity between honey samples and plant species in different rain seasons. The statistical analysis software used was JAMOVI version 2.3.18 (2022), with significance set at 5% level of significance ($p < 0.05$).

3. Results

3.1. The antimicrobial activities of honey

Honey samples A and D exhibited higher antimicrobial activity against the tested microorganism than other samples (Fig. 2), with zones of inhibition ranging from 11.0 to 17.3 mm and 11.7–19 mm (Table 1). The least honey sample in inhibiting microorganisms' growth was sample B, with the lowest zone of inhibition for all tested microorganisms ranging from 10 to 11 mm (Table 1). The highest susceptible test microorganisms were *E. coli* and *S. aureus,* while the fungi *C.albicans* was the least inhibited microorganism by all tested honey samples. Moreover, there was a significant difference in the antimicrobial activity of honey samples harvested in different rain seasons to pathogenic organisms ($F = 28.5$ $p < 0.001$).

3.2. The antimicrobial activity of plant extracts

The *C. schumannii*, *H. opposita,* and *T. brownii* showed higher antimicrobial activity against test microorganisms than other plant extracts (Fig. 3), with zones of inhibition ranging from 11.3 to 17.7 mm, 14–16.7 mm and 11.7–19 mm, respectively (Table 2). While *A. mellifera* was the most diminutive plant in inhibiting microorganisms' growth, its zone of inhibitions ranged from 10 to 12 mm (Table 2). Further, *B. subtilis* and *S. aureus* were the most susceptible microorganisms to test plant extracts, followed by *E. coli*. Moreover, *C. albicans* was the least inhibited microorganism with the lowest recorded zones of inhibitions (Table 2, Fig. 3). There was a significant difference among plant extracts in antimicrobial activity against the test microorganisms ($F = 15.9$, $p < 0.001$).

3.3. Antimicrobial activities of honey and plant extracts against test microorganisms

The Principal Component Analysis (PCA) explained 75% of the variance in antimicrobial activities of honey and plant extracts against test microorganisms (Fig. 4). Inhibitions of *S. typhi* and *B. subtilis* correlated toward positive x-axis and negative y-axis, associated with *C. schumannii, H. opposita* extracts and honey sample C while inhibitions of *S. aureus* and *E. coli* correlated toward positive x-axis and y-axis associated with *T. brownii, G. bicolor* extracts and honey sample A (Fig. 4). On the other hand, the inhibitory activity of the honey sample D, C, some values of sample A and *T. brownii*, *H. opposita* and *C. schumannii* extracts correlated toward positive x-axis values (Fig. 4).

3.4. Comparison between honey samples and plant extracts antimicrobial activities per rain seasons

The honey samples A and D that were harvested at the end of the short rain season were compared with individual plants that flowered during the same rain season; *C. schumannii*, *G. bicolor*, *C. monoica,* and *T. brownie.* Similarly, samples B and C that were harvested at the end of the long rain season were compared with *O. basilicum*, *A. mellifera,* and *H. opposita* that flowered during the same rain season. There was a strong positive correlation in antimicrobial activity between honey

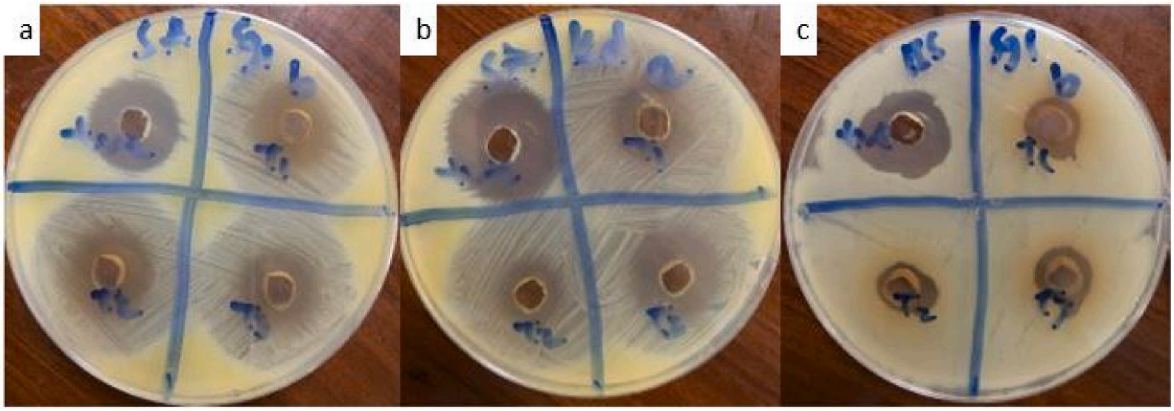


Fig. 2. Zones of inhibition (ZI) of some honey samples against tested microorganisms, where (a) sample D against *S. aureus* (b) sample A against *S. aureus*, (c) sample D against *B. subtilis*.

Table 1
Antimicrobial activity of honey samples on different test pathogenic microorganisms.

Honey Sample	Inhibition zone for different microbe (mm) Mean ± SD.				
	B. subtilis	C. albicans	E. coli	S. aureus	S. typhi
A	12.0 ± 0.0^{c}	11.0 ± 0.0^{b}	11.7 ± 1.2^{c}	17.3 ± 1.2^{b}	12.7 ± 1.2^{bc}
B	10.3 ± 0.6^{c}	10.0 ± 0.0^{b}	10.7 ± 0.6^{c}	11.0 ± 1.0^{d}	10.0 ± 0.0^{d}
C	14.7 ± 1.2^{b}	10.3 ± 0.6^{b}	14.3 ± 0.6^{b}	15.0 ± 0.0^{c}	12.3 ± 0.6^{c}
D	14.3 ± 0.6^{b}	11.7 ± 0.6^{b}	15.7 ± 0.6^{b}	19.0 ± 0.0^{b}	13.0 ± 0.0^{b}
Chloramphenicol/ Fluconazole	18.3 ± 0.6^{a}	21.3 ± 1.2^{a}	20.0 ± 0.0^{a}	22.0 ± 0.0^{a}	16.3 ± 1.2^{a}

***SD**- Standard deviation, * Superscripts of different letters in the same column shows values that significantly differ from each other.

sample A with *T. brownii* (r = 0.756, p = 0.139) and *C. monoica* (r = 0.732, p = 0.159), while honey sample D strongly correlated with *T. brownii* (r = 0.836, p = 0.078), and *C. monoica* (r = 0.732, p = 0.159) (Table 3). In addition, Table 4 shows the correlation relation of sample C with *H. opposita* (r = 0.660, p = 0.226).

4. Discussion

The results from this study indicated that all assayed honey samples have potential bactericidal and fungicidal activities against the selected pathogenic microorganisms. These findings were similar to other studies conducted elsewhere that reported the antimicrobial activities of honey against ranges of pathogenic drug-resistant microorganisms in both *in vitro* and clinical trials [7,24]. However, the antimicrobial activity of honey varies in different samples against the test microorganisms [25,26]; honey samples A and D were found to have a higher antimicrobial activity against the test pathogenic microorganisms than honey samples B and C. This variation in antimicrobial activities among honey could be due to the differences in their chemical and biological properties that are highly determined by plant sources from which honeybees collect resources, which are used as primary raw materials during honey production [27,28] and the season of collection [29]. Regarding the honeybees-plant interaction, the medicinal properties of foraged plants as were observed with *C. monoica* and *T. brownie* (during the short rain season) and *H. opposita* and *A. mellifera* (during the long rain season) have an influence and may relate to that of produced honey [30–32], which could be due to their synthesized secondary metabolites, as reported in other studies [33,34]. The correlation in microorganisms' inhibition between honey samples and plant extracts that were harvested and bloomed respectively during the same season as were explained through PCA, further explains the role of plant resources from which honeybees collects resources and season of collection in determining the antimicrobial activity of the honey. Our results on variations in antimicrobial activities among plant species are in agreement with the findings from other studies [35,36].

We found that honey samples harvested during the end of the short rain season showed higher antimicrobial activity than those harvested at the end of the long rain season. Our findings align with other studies that evaluated honey antimicrobial activities across the seasons [27,29]. This

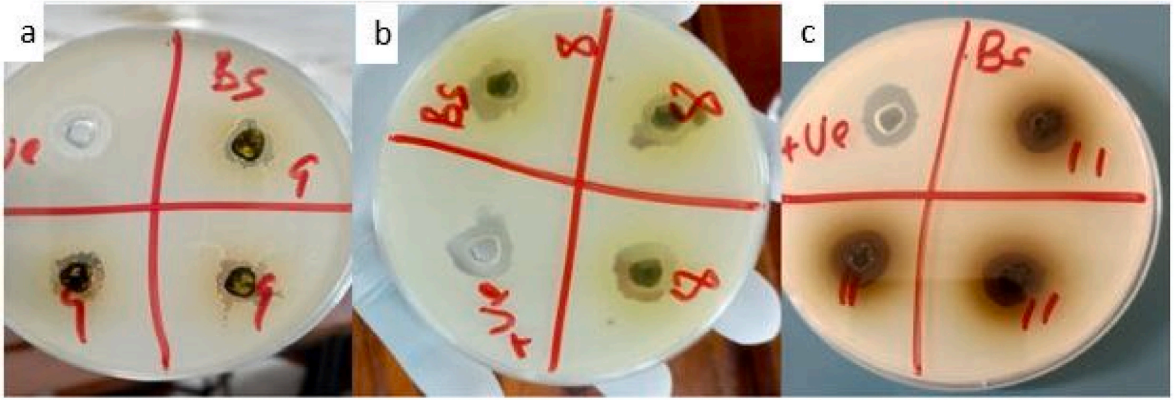
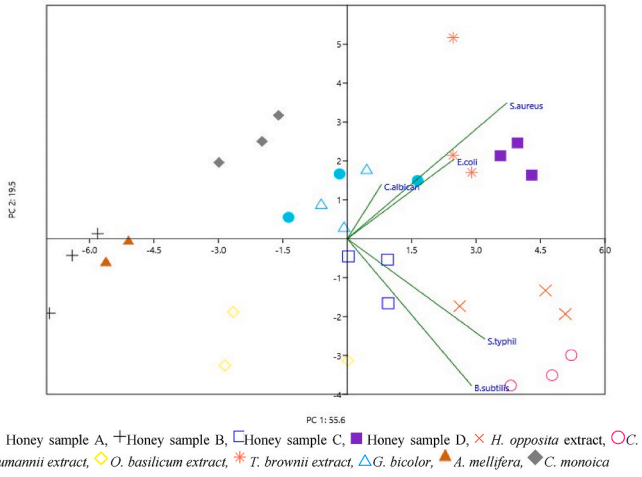


Fig. 3. Zones of inhibition (ZI) of some plant extracts against the test microorganisms, where (a) *T. brownii* against *B. subtilis* (b) *C. schumannii* against *B. subtilis*, (c) *H. opposita* against *B. subtilis*.

Table 2
Zone of inhibitions of plant extracts against tested pathogenic microorganisms.

Plant extracts	Zones of Inhibitions (mm) of plant extracts against microorganisms, Mean ± SD.				
	B. subtilis	C. albicans	E. coli	S. aureus	S. typhi
H. opposita	15.3 ± 0.6^{bc}	12.0 ± 1.7^{b}	14.0 ± 0.0^{bc}	16.7 ± 0.6^{bc}	16.3 ± 1.2^{a}
C. schumannii	17.7 ± 0.6^{ab}	11.3 ± 1.2^{b}	14.7 ± 0.6^{bc}	15.7 ± 0.6^{cd}	16.0 ± 1.0^{a}
O. basilicum	16.0 ± 1.0^{abc}	11.7 ± 1.5^{b}	10.7 ± 0.6^{d}	13.3 ± 0.6^{de}	10.7 ± 1.2^{b}
T. brownii	13.7 ± 1.5^{c}	13.7 ± 0.6^{b}	13.7 ± 1.2^{c}	19.0 ± 1.0^{b}	11.7 ± 1.2^{b}
G. bicolor	14.3 ± 0.6^{c}	12.3 ± 0.6^{b}	16.7 ± 0.6^{b}	14.0 ± 1.0^{cde}	10.0 ± 0.0^{b}
A. mellifera	11.0 ± 0.0^{d}	11.3 ± 0.6^{b}	10.3 ± 0.6^{d}	12.0 ± 0.0^{e}	10.0 ± 0.0^{b}
C. monoica	11.0 ± 0.0^{d}	14.7 ± 0.6^{b}	14.3 ± 1.2^{bc}	12.0 ± 1.7^{e}	10.0 ± 0.0^{b}
Chloramphenicol/ Fluconazole	18.3 ± 0.6^{a}	21.3 ± 1.2^{a}	20.0 ± 0.0^{a}	22.0 ± 0.0^{a}	16.3 ± 1.2^{a}

**SD- Standard deviation, * Superscripts of different letters in the same column shows values that significantly differ from each other.*



*● Honey sample A, ┼Honey sample B, □Honey sample C, ■ Honey sample D, × *H. opposita* extract, ○*C. schumannii extract,* ◇*O. basilicum extract,* ✳*T. brownii extract,* △*G. bicolor,* ▲*A. mellifera,* ◆*C. monoica*

Fig. 4. Principal component analysis (PCA) indicating the relationship between antimicrobial activities of honey and plant extracts against test microorganisms.

Table 3
Pearson correlation in antimicrobial activity between plant species and honey sample.

Plant species (extract)	Honey sample A		Honey sample D	
	Pearson's r	*p-value*	Pearson's r	*p-value*
C. schumannii	+0.506	0.384	+0.487	0.405
G. bicolor	+0.586	0.299	+0.541	0.349
T. brownii	+0.756	0.139	+0.836	0.078
C. monoica	+0.732	0.159	+0.732	0.159

Table 4
Pearson Correlation relation in antimicrobial activity between plant species and honey sample.

Plant species (extract)	Honey sample B		Honey sample C	
	Pearson's r	*p*-value	Pearson's r	*p*-value
O. basilicum	+0.203	0.744	+0.477	0.416
H. opposita	+0.424	0.477	+0.660	0.226
A. mellifera	+0.497	0.394	+0.170	0.785

could be due to physiological responses by plants toward environmental stress during this period [37]. The short rain season in our study areas was characterized by little rainfall due to semi-arid nature of the sites [19], which could have triggered competition for resources amongst plants and that led to increased production of phyto-compounds by plants as a competition avoidance strategy [38]. The higher formulated phyto-compounds by plants during this period could have contributed to the observed higher antimicrobial activity of both plant extracts and honey as honeybees may have collected nectar composed of these compounds and used it for honey production [39]. On the other hand, the low antimicrobial activity observed in honey samples harvested at the end of the long rain season and plant extracts could be due to the dilution of nectar and the small content of phyto-compound contained in plants [40] as any alteration in nectar contents have been reported to affect honey's chemical and biological properties [27]. Likewise, it has been reported that seasonality influences the variation in different plant secondary metabolites [41]. For instance Ref. [42], reported that during the rain shortage period, the diversity, concentration level, and complexity of the secondary metabolites produced by some plants are higher compared to the long rain period. Additionally, regardless of available plants with flowers in the long rainy season, regular and consistent rainfall during these days negatively affects the honeybees' foraging paradigm and flower visitation [43], which in turn hinders honeybees from exploiting, and benefiting from the available flower resources compared to the short rain season [44].

We further found variation in susceptibility amongst test microorganisms to honey and plant extracts, in which some microbes were more susceptible to almost all the samples than others. These results are similar to findings from other studies [45,46]. The discrepancies among test microorganisms' responses to honey and plant extract samples might be attributed to the fact that microbes differ in their cellular organization [26]. Besides, the resistance of some microorganisms, especially the *Candida* species towards the antimicrobial substances could be due to their molecular mechanisms [47] and the antifungal resistance phenotypes found in different species of fungi [48]. Our study's findings highlights on the influence and contribution of seasonality and floral sources to the antimicrobial activity variation amongst honey samples in the study area and elsewhere where beekeeping activities are conducted.

5. Conclusion

This study has revealed that honey differs in its antimicrobial activities depending on the harvesting season and floral sources. There is a significant correlation in antimicrobial activity between honey samples and plant species that are available in the areas and in a given season. Honey that can be harvested at the end of the short rainy season offers a higher antimicrobial potential against microbial infections than honey harvested at the end of the long rainy season. This information is of high importance in the beekeeping industry as it can be used to inform effective use of honey as a medicinal product. However, we recommend further studies on the variation of honey antimicrobial activities in relation to available plant species especially during the short rain season.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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