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Non-peroxide antibacterial activity of honey produced by an Afrotropical stingless bee, *Meliponula* (*Axestotrigona*) *ferruginea*

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Abstract

Honey is a medicinal food that is widely utilized in traditional and complementary medicine. Amidst the contemporary challenge of antibiotic resistance, honey emerges as a promising natural antimicrobial agent. The efficacy of honey in therapy hinges on its mechanisms of antimicrobial activity. Therefore, this study investigated the non-peroxide antibacterial properties of honey sourced from *Meliponula* (*Axestotrigona*) *ferruginea*, a stingless bee species that is commonly managed in the African tropics. The findings reveal that stingless bee honey exhibits remarkable inhibitory effect against both resistant and susceptible bacterial strains. Notably, the studied honey samples retained a substantial portion of their antibacterial potency (89.9 - 98.7%) after the removal of hydrogen peroxide. Interestingly, the antibacterial activity of honey did not correlate with its total phenolic and flavonoid content, suggesting the influence of specific bioactive compounds rather than the overall phytochemical content. Stingless bee honey was most effective against Gram-positive bacterial strains, particularly *Staphylococcus aureus*. These results underscore the therapeutic potential of stingless bee honey for the management of pathogenic bacteria, including resistant strains. Future investigations should focus on elucidating the specific bioactive compounds present in stingless bee honey to bolster its clinical applications.

Key words: antimicrobial resistance, apitherapy, hydrogen peroxide, stingless bee honey, Tanzania

1. Introduction

Bacterial pathogens constantly evolve, acquiring resistance to existing antibiotics. This evolutionary arms race complicates treatment strategies; as once-effective antibiotics lose their efficacy against resistant strains (Jalalifar et al., 2024). The overuse and misuse of antibiotics in both clinical and agricultural settings exacerbate this problem, fostering the emergence and spread of drug-resistant bacteria (Endale et al., 2023). Resistant bacterial strains such as methicillin resistant *Staphylococcus aureus* (MRSA) have become cosmopolitan due to their capacity to spread rapidly (Nishio et al., 2015). Furthermore, the discovery of new antibiotics

has slowed down over the past years due to high costs of drug research, resulting into few effective antimicrobials which are often associated with high costs and multiple side effects for patients (Cardozo et al., 2013; Zainol et al., 2013). For this reason, the search for novel antimicrobial compounds sourced from natural products is paramount.

Honey is a sweet substance produced by bees from the nectar collected from plants. There is a broad array of honey varieties with distinct flavor, color, and odor, originating from various floral sources and bee species. Honey has been used as a food and an integral part of traditional medicine since ancient times (Kuropatnicki et al., 2018). The medicinal uses of honey persisted to the modern era, giving rise to an alternative medicine discipline known as Apitherapy, which utilizes honey and other bee products for treatment (Mandal & Mandal, 2011). The medicinal potential of honey is acknowledged for its antimicrobial, antioxidant, anti-inflammatory, anti-cancer, antidiabetic and immunomodulatory properties (Meo et al., 2017). Due its local availability, affordability, and minimal risks of toxicity and microbial resistance, honey stands out as a valuable alternative for treating bacterial pathogens (Mduda et al., 2023d). Currently, several types of honeys are marketed as medical-grade with standardized levels of antibacterial efficacy. The best known is the Manuka honey which is produced from *Leptospermum* species and is reported to be effective against more than 60 species of bacteria (Mandal & Mandal, 2011; Nolan, 2020).

The diverse components and features of honey collectively contribute to its antimicrobial potency. In an undiluted state, the antimicrobial activity of honey is largely attributed to its high osmolarity and low pH (Zainol et al., 2013). High sugar concentration in honey exerts osmotic pressure on bacterial cells, leading to dehydration and cell shrinkage (Albaridi, 2019). Additionally, the pH of honey (3.2 – 4.5) is far below the optimal pH for the growth of most bacteria which ranges from 6.5 to 7.5 (Almasaudi, 2021). Dilution of honey activates the enzyme glucose oxidase which catalyzes the conversion of glucose to gluconic acid and hydrogen peroxide (Zainol et al., 2013). Hydrogen peroxide is a strong disinfectant which contributes to the antimicrobial efficacy of honey. The maximum level of hydrogen peroxide is achieved when honey is diluted by 30 to 50% (Almasaudi, 2021). However, hydrogen peroxide is susceptible to degradation by catalase enzyme in living tissues making it less effective during therapy (Ewnetu et al., 2013). The antibacterial activity of honey can decrease by up to 100-fold following the removal of hydrogen peroxide (Mandal & Mandal, 2011). Nonetheless, certain varieties of honey can maintain antibacterial potency even after the removal of hydrogen peroxide. The non-peroxide activity results from various elements found in honey, such as phenolic compounds, flavonoids, antibacterial peptides, methylglyoxal, methyl syringate, and other trace components (Zainol et al., 2013). It has also been suggested that synergy between hydrogen peroxide and other bioactive compounds produced the maximum inhibitory effect on bacterial cells (Bucekova et al., 2018; Ng et al., 2020).

The use of honey in traditional medicine is widespread in eastern Africa (Kiprono et al., 2022; Mduda et al., 2023c; Héger et al., 2023). To date, various studies have been conducted to investigate the antimicrobial properties of honeys from this region (Ewnetu et al., 2023; Mokaya et al., 2020; Mduda et al., 2023d; Rikohe et al., 2023; Mduda et al., 2024). Findings from Ethiopia and Tanzania revealed that stingless bee honey was more effective against both Gram-positive and Gram-negative bacteria in comparison to *Apis mellifera* honey (Ewnetu et al., 2013; Mduda et al., 2024). However, little is still known about the mechanisms that underlie the antimicrobial potency of stingless bee honey. The current study investigated for the first time the non-peroxide antibacterial activity of the honey produced by a commonly managed stingless bee species, *Meliponula* (*Axestotrigona*) *ferruginea*. Specifically, honey samples from Siha and Kibiti districts in Tanzania were tested against resistant and susceptible strains of common pathogenic bacteria. The findings of this study will offer valuable insights

into the effectiveness of stingless bee honey as a powerful antimicrobial agent against prevalent pathogenic bacteria, potentially enhancing its use in therapy.

2. Materials and Methods

2.1 Honey samples

Honey samples were collected from colonies of *Meliponula* (*Axestotrigona*) *ferruginea* managed in hives across two districts in mainland Tanzania: Siha and Kibiti (Fig. 1). The Siha district, located in the northern highlands, featured sampling sites at the western foothills of Mount Kilimanjaro, with elevations ranging from 1,618 to 1,880 meters above sea level. This region is characterized by Afromontane vegetation, featuring a diverse, multi-layered, and evergreen flora (Foley et al., 2014). The dominant plant families in this area are Asteraceae and Fabaceae, along with other less prevalent families such as Amaranthaceae, Rubiaceae, Vitaceae, Malvaceae, Celastraceae, and Solanaceae. In contrast, the Kibiti district is situated along the eastern coast, where honey samples were obtained from the Rufiji Delta. This delta is notable for having the largest concentration of mangroves on Africa's eastern coast, encompassing six distinct families: Avicenniaceae, Combretaceae, Meliaceae, Rhizophoraceae, Sonneratiaceae, and Sterculiaceae (Monga et al., 2018). At both sampling locations, the stingless bee colonies are maintained in semi-natural environments, preserving a significant portion of the native vegetation. Sample collection was done in September 2023, with seven hives sampled from each district, resulting in a total of fourteen honey samples. The honey was harvested using the pot-puncture technique that is outlined in Mduda et al. (2023c). Subsequently, all honey samples were filtered using a clean food-grade filter cloth, then transferred into amber plastic containers, and stored at 4°C pending laboratory analyses.

2.2 Test microorganisms

The test microorganisms were obtained from the American Type Culture Collection (ATCC, US). The microbes comprised three Gram-positive bacteria; methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 33592, *Staphylococcus aureus* ATCC 6538P and *Bacillus subtilis* ATCC 6633, and two Gram-negative bacteria; *Escherichia coli* ATCC 11229 and *Salmonella enterica* Typhimurium ATCC 14028.

2.3 Chemicals

The following reagents were sourced from Glentham Life Sciences (UK): Folin–Ciocalteu phenol reagent, gallic acid (99%), quercetin (98%), aluminium chloride, sodium nitrite, sodium chloride, barium chloride, sodium hydroxide, hydrogen peroxide, and methanol. Nutrient Broth (NB) and Mueller-Hinton Agar (MHA) were obtained from Himedia Laboratories Private Limited (India). Additionally, catalase (C100) was purchased from Sigma Aldrich (Germany).

2.4 Instrumentation

A class II biosafety cabinet (BSC-1300IIA2-X, BIOBASE) was used to provide controlled environment for microbial manipulations. Additionally, an incubator (LFZ-TSI-200D, LABFREEZ Instruments) and a UV-Vis spectrophotometer (Cary 60 UV–Vis Spectrophotometer, Agilent Technologies) were used in this study.

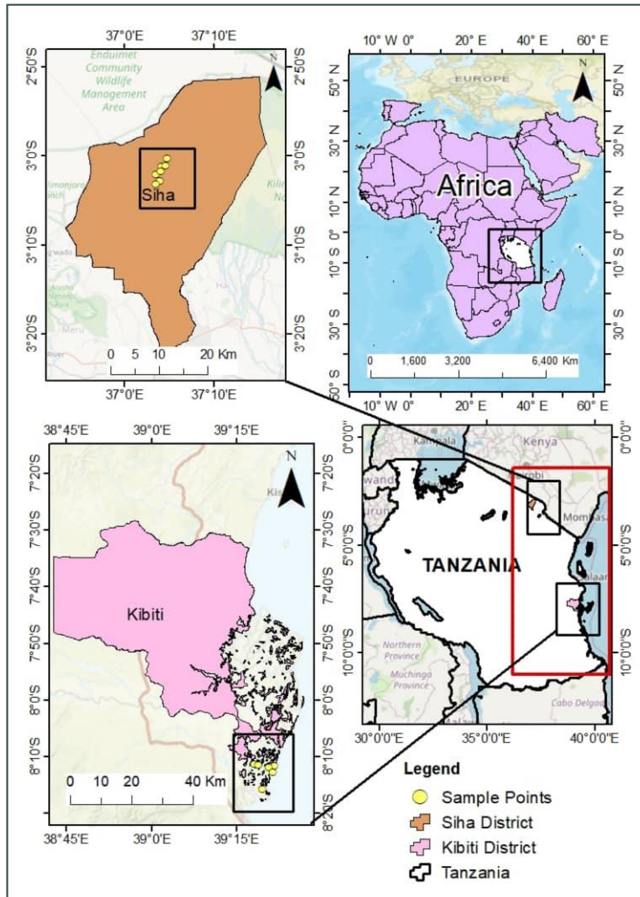

Figure 1. Map of the study area.

2.5 Assessment of the antimicrobial activity of honey

2.5.1 Preparation of inoculum and culture media

Preparation of inoculum and culture media employed the methods outlined in Mduda et al. (2023d). Mueller-Hinton Agar (MHA) medium (38 g of MHA in 1000 mL of distilled water) was prepared and sterilized in an autoclave at 121°C for 15 minutes. The resulting suspension was poured into sterile petri dishes and allowed to solidify at room temperature. Meanwhile, the test microorganisms were inoculated into Nutrient Broth (NB) media (8 g of NB in 1000 mL of distilled water) in test tubes and then incubated at 37°C for 24 hours. A 0.5 McFarland standard solution was prepared by mixing 0.5 mL of 1.175% (w/v) barium chloride with 99.5 mL of 1% v/v sulfuric acid, and then distributed into screw-capped test tubes. Subsequently, 100 microliters of the inoculated microbe sample from the NB medium was added to 5 mL of saline, and the concentration was adjusted to 1.5×10^8 colony-forming units (CFU) per milliliter by comparing the turbidity of the microbial suspension to the prepared McFarland standard.

2.5.2 Agar-well diffusion assay

The agar-well diffusion assay was carried out according to the procedures outlined in Ewnetu et al. (2013). The bacterial strains were inoculated by streaking the surface of an agar plate with a sterile swab until complete coverage of the agar surface was achieved. Wells were created on the agar plates using a sterile cork borer (6 mm). For the determination of total antibacterial activity, 100 μL of 50% (w/v) honey sample in distilled water was added into the agar wells. For non-peroxide activity, 100 μL of 50% (w/v) honey sample in catalase solution (10 mg/mL) was used instead (Zainol et al., 2013). The culture plates were then incubated at 37°C for 24 hours. The diameter of inhibition zone was determined by measuring the clear area surrounding the agar wells. Measurements were done in horizontal and vertical directions using a Vernier caliper and recorded in millimeters (mm). Autoclaved distilled water and Ciprofloxacin (10 μg) were used as negative and positive controls, respectively.

2.5.3 Catalase effectiveness test

Confirmation of the removal of hydrogen peroxide from honey samples was done following the procedure outlined in Zainol et al. (2013) with minor modifications. Two honey samples were selected for the test against *S. aureus* ATCC 6538P. Six tubes of test solutions were prepared and labeled as follows: tube 1 (50% (w/v) honey solution, 45 mmol/L hydrogen peroxide, and 10 mg/mL catalase solution); tube 2 (50% (w/v) honey solution and 10 mg/mL catalase solution); tube 3 (45 mmol/L hydrogen peroxide and 10 mg/mL catalase solution); tube 4 (50% (w/v) honey solution and 45 mmol/L hydrogen peroxide); tube 5 (50% (w/v) honey solution); and tube 6 (45 mmol/L hydrogen peroxide). These solutions were then tested in the same manner as the agar-well diffusion assay.

2.6 Determination of phytochemical content in honey

2.6.1 Total phenolic content

Determination of total phenolic content (TPC) in honey was conducted following the Folin-Ciocalteau method described by Singleton et al. (1999). Initially, three grams of honey sample were mixed with 30 mL of methanol and subjected to sonication for 15 minutes. Subsequently, the mixture was centrifuged at 9000 rpm, after which the supernatant was carefully decanted and stored at 20°C. Following this step, 2.5 mL of diluted Folin-Ciocalteau phenol reagent and 2 mL of 7.5% sodium carbonate solution were added to 0.5 mL of the extracted sample in a separate tube. The mixture was thoroughly mixed and allowed to stand at room temperature for 2 hours. Absorbance was measured at 765 nm against the blank using a UV–Vis spectrophotometer. A standard calibration curve was established using gallic acid concentrations ranging from 0.02 to 0.20 mg/mL. TPC was then calculated and expressed as milligrams of gallic acid equivalent per 100 grams of honey (mg GAE/100 g).

2.6.2 Total flavonoid content

Determination of total flavonoid content (TFC) in honey followed the methods of Zhishen et al. (1999). One milliliter of the sample solution (5 g of honey in 20 mL of distilled water) was mixed with 4 mL of distilled water and 0.3 mL of 5% sodium nitrite. After a five-minute interval, 0.3 mL of 10% aluminum chloride was introduced into the mixture and allowed to stand for 1 minute. Following this, 2 mL of 1 M sodium hydroxide was added, followed by 2.4 mL of distilled water. The absorbance of the resulting mixture was measured against the blank at 510 nm using a UV/Vis spectrophotometer. A standard calibration curve was created using quercetin concentrations ranging from 0.01 to 0.10 mg/mL. TFC was then reported as milligrams of quercetin equivalent per 100 grams of honey (mg QE/100 g).

2.7 Data analysis

One-way analysis of variance (ANOVA) and a Tukey post-hoc test were employed for the comparison of the total and non-peroxide antibacterial activities of honey samples from the two locations. Non-metric multidimensional scaling (NMDS) ordination plot was created using Euclidean similarity index to highlight the similarities in the total and non-peroxide antibacterial activities. Data were log transformed before generating the NMDS plot to fit them in the same scale. ANOVA was also used to compare susceptibility of the bacterial strains to the studied honey samples. Two sample t-test was conducted to compare the phytochemical content of honey samples from the two locations. Pearson's correlation coefficient was performed to evaluate pairwise relationships between diameters of inhibition zones and phytochemical content of stingless bee honey. Data analysis was done using PAleontological STatistics (PAST) Software V. 4.03 and graphs were plotted using GraphPad Prism V. 9.5.1.

3. Results and Discussion

3.1 Total versus non-peroxide antibacterial activity of stingless bee honey

Results of the antibacterial activity of stingless bee honey from Siha and Kibiti districts are presented in Fig. 2. Diameters of inhibition zones generated by each honey sample against the test microbes are shown in Supplementary Table 1 for untreated honey samples (total activity) and Supplementary Table 2 for catalase-treated honey samples (non-peroxide activity). Results of the catalase effectiveness test (Supplementary Table 3) revealed that the enzyme was effective in removing all hydrogen peroxide molecules from the honey samples.

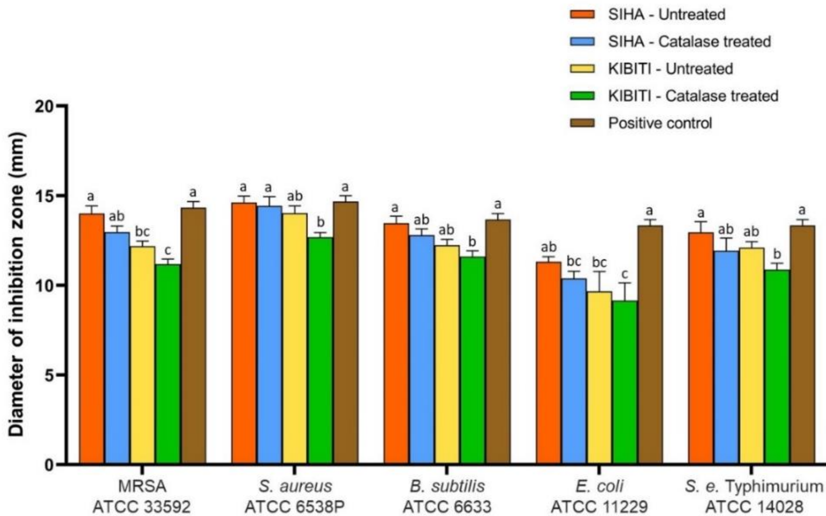

Figure 2. Grouped bar-graphs showing mean diameters of inhibition zones of untreated (total activity) and catalase-treated (non-peroxide activity) honey samples against the bacterial strains. Ciprofloxacin (10 µg) was used as a positive control. Superscripts with different letters within the same group indicate significant differences in diameters of inhibition zones ($p < 0.05$, ANOVA and Tukey post-hoc test).

Stingless bee honey exhibited substantial antibacterial activity against the tested bacterial strains both before and after the removal of hydrogen peroxide. Honey samples from Siha

displayed significantly higher antibacterial activity compared to Kibiti. However, no significant difference was observed between the total and non-peroxide activity for honey samples from the same location (Fig. 2). Stingless bee honey from both locations retained the majority (89.9 – 98.7%) of antibacterial activity after treatment with catalase (Table 1, Supplementary Fig. 1). Similarly, the NMDS plot (Fig. 3) shows minimal differences between the total and non-peroxide antibacterial activity based on the extent to which the convex hulls overlap.

Table 1. Antibacterial activity retained after treatment of honey samples with catalase enzyme.

Location		MRSA ATCC 33592	*S. aureus* ATCC 6538P	*B. subtilis* ATCC 6633	*E. coli* ATCC 11229	*S. enterica* Typhimurium ATCC 14028
Siha	Mean total activity (mm)	14.0 ± 1.1	14.6 ± 1.0	13.5 ± 1.0	11.3 ± 0.8	13.0 ± 1.6
	Mean non-peroxide activity (mm)	13.0 ± 0.9	14.4 ± 1.4	12.8 ± 0.9	10.4 ± 1.1	11.9 ± 1.9
	Retained activity	92.6%	98.7%	95.1%	91.8%	92.1%
Kibiti	Mean total activity (mm)	12.2 ± 0.78	14.0 ± 1.1	12.2 ± 0.9	9.7 ± 3.0	12.1 ± 0.9
	Mean non-peroxide activity (mm)	11.2 ± 0.8	12.7 ± 0.7	11.6 ± 0.9	9.1 ± 2.6	10.9 ± 1.0
	Retained activity	91.8%	90.4%	94.8%	94.7%	89.9%

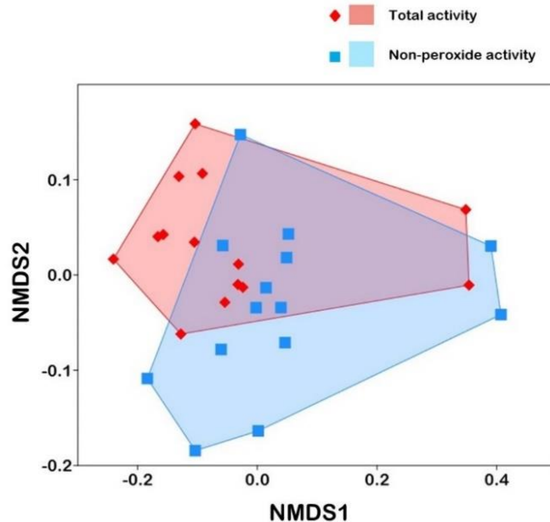

Figure 3. Non-metric multidimensional scaling (NMDS) ordination plot showing honey treatments exhibiting total and non-peroxide antibacterial activity. Overlapping of convex hulls indicate the degree of similarity between sample treatments. Stress value of the NMDS plot is 0.159.

Honey exhibits potent antimicrobial activity due to various attributes such as its low pH, high osmolarity and the presence of hydrogen peroxide and non-peroxide components

(Mandal & Mandal, 2011). Previous studies have reported the pH of *M. ferruginea* honey to be in a range between 3.8 and 4.9 (Mokaya et al., 2022; Mduda et al., 2023a) which is low enough to be inhibitory to bacteria. Honey acidity is influenced by the presence of organic acids, particularly gluconic acid which is the dominant acid in honey (Dardón et al., 2013). However, pH is raised when honey is diluted making it less effective as an antimicrobial factor. Additionally, *M. ferruginea* honey has lower sugar content (70.3 – 73.9 °Brix) and higher water content (26.1 - 28.8%) compared to *A. mellifera* honey (Mokaya et al., 2022; Mduda et al., 2023a), resulting into low osmolarity.

Honey produces hydrogen peroxide through the enzyme glucose oxidase, which converts glucose into gluconic acid and hydrogen peroxide (Mandal & Mandal, 2011). The antimicrobial potency due to hydrogen peroxide is the most common in many types of honey, with maximum activity when honey is diluted (Almasaudi, 2021). The downside of the peroxide activity is that hydrogen peroxide can be easily destroyed by heat or in the presence of catalase enzyme (Ewnetu et al., 2013). In that regard, the effectiveness of hydrogen peroxide as an antimicrobial agent is limited when honey is mixed with bodily fluids (Almasaudi, 2021; Mduda et al., 2024).

Previous studies have highlighted the prevalence of non-peroxide antibacterial activity in stingless bee honey (Temaru et al., 2007; Zainol et al., 2013; Jibril et al., 2020). Honey samples from 14 stingless bee species displayed remarkable non-peroxide activity against Gram-positive and Gram-negative bacterial strains (Temaru et al., 2007). Further, Jibril et al. (2020) reported that stingless bee honey retained 98.9% of the antibacterial activity after treatment with catalase (Jibril et al., 2020). Contrary, non-peroxide activity is uncommon in *Apis mellifera* honey except for special honey types such as Manuka honey (Johnston et al., 2018). For example, honey samples from various origins in UK and Denmark had no detectable non-peroxide activity despite exhibiting broad-spectrum total antibacterial activity (Sulaiman et al., 2012; Matzen et al., 2018). Additionally, the removal of hydrogen peroxide by catalase resulted in substantial decrease in the antibacterial activity of *A. mellifera* honeys from Western Australia (Roshan et al., 2017).

3.2 Phytochemical content of stingless bee honey

Honey comprise a diverse array of phytochemicals including polyphenols which are derived from the nectar of flowers. Phenolic acids and flavonoids can exhibit antibacterial potency by interfering with the bacterial cell functioning, disrupting cell growth and effecting cell lysis (Shehu et al., 2016; Kumar Singh et al., 2019). These compounds can play a critical role in the non-peroxide antibacterial activity of honey (Tuksitha et al., 2018). Previous research findings have indicated a strong correlation between the antimicrobial activity of honey and both total phenolic content (TPC) and total flavonoid content (TFC) (Sousa et al., 2016; Mduda et al., 2023d).

In this study, stingless bee honey exhibited remarkable levels of TPC (197.0 – 263.1 mg GAE/100 g) and TFC (118.5 – 156.7 mg QE/100 g) (Fig. 4). However, neither TPC nor TFC showed significant correlation with the diameters of inhibition zones against any of the bacterial strains (Fig. 5). When comparing the two locations, honey samples from Kibiti produced smaller mean diameters of inhibition zones (Fig. 2), despite having significantly higher levels of TPC and TFC. Tuksitha et al. (2018) also observed that honey samples with the highest TPC failed to inhibit the growth of Gram-negative bacteria. In addition, honey samples from Brazil and Scotland showed no correlation between TPC and antimicrobial activity against a variety of bacterial strains including *Shigella dysentery*, *Salmonella enterica* Typhimurium, *Staphylococcus aureus* and *Bacillus cereus* (Bueno-Costa et al., 2016; Fyfe et al., 2017). These findings indicate that the antimicrobial activity displayed by the studied

honey samples may be attributed to specific polyphenolic compounds or other bioactive components present in honey.

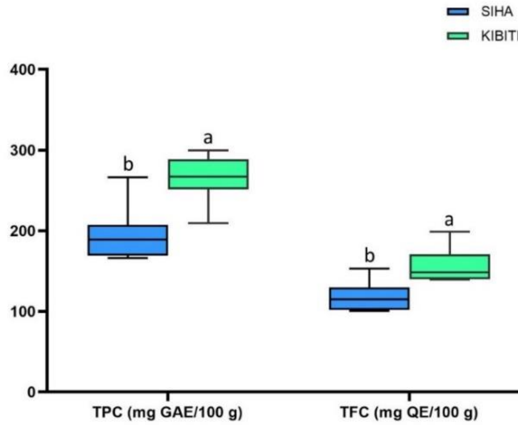

Figure 4. Grouped bar-graph showing total phenolic and flavonoid content of stingless bee honey from the studied locations. Superscripts with different letters within the same group indicate significant differences in diameters of inhibition zones ($p < 0.05$, ANOVA and Tukey post-hoc test).

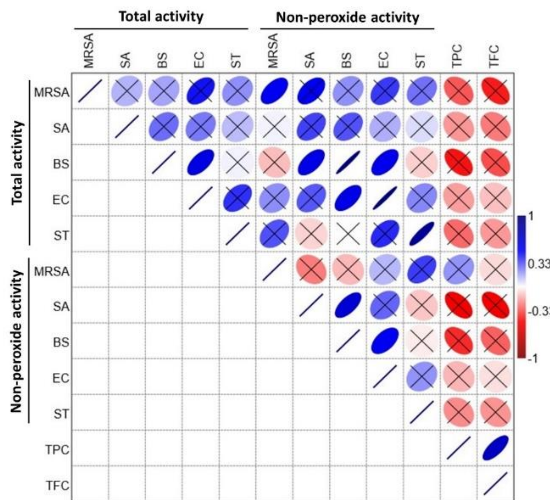

Figure 5. Correlation matrix showing pairwise Pearson's coefficients among variables. Crossed boxes (×) indicate correlations which are not statistically significant ($p > 0.05$). MRSA = methicillin resistant *S. aureus* ATCC 33592, SA = *S. aureus* ATCC 6538P, BS = *B. subtilis* ATCC 6633, EC = *E. coli* ATCC 11229, ST = *S. enterica* Typhimurium ATCC 14028, TPC total phenolic content, TFC = total flavonoid content.

The amount and types of phenolic compounds present in honey vary depending on the botanical source, geographical location as well as the bee species origin (Mduda et al., 2023b). Specific phenolic acids such as syringic acid, chlorogenic acid, caffeic acid and

hydroxycinnamic acids, and flavonoids such as naringenin, apigenin, quercetin and myricetin, have been identified to exhibit broad spectrum antibacterial activity (Jibril et al., 2019). Additionally, other bioactive compounds have been reported to contribute to the non-peroxide antibacterial activity of honey. Notably, methylglyoxal and methyl syringate, which are predominantly found in Manuka honey, have been extensively studied (Johnston et al., 2018; El-Senduny et al., 2021; Hossain et al., 2023). Popova et al. (2021) also highlighted a range of organic acids in *M. ferruginea* honey that may possess antimicrobial effects. Therefore, future research should aim to identify the specific bioactive compounds responsible for the antimicrobial potency observed in stingless bee honey sourced from the African tropics.

3.3 Microbial susceptibility to stingless bee honey

The bacterial strains displayed different levels of susceptibility to both untreated and catalase-treated honey samples (Fig. 6). Stingless bee honey was more effective in inhibiting the growth of Gram-positive bacteria compared to Gram-negative bacteria. The largest and smallest diameters of inhibition zones were observed against *S. aureus* ATCC 6538P and *E. coli* ATCC 11229, respectively. Previous studies have also reported stingless bee honey to be more effective against Gram-positive bacteria. Malaysian stingless bee honey exhibited remarkably high antibacterial activity against *S. aureus*, surpassing that of the standard medicinal Manuka honey (Zainol et al., 2023). Similarly, honey samples from two *Scaptotrigona* species effectively inhibited the growth of two MRSA strains, while being least effective against strains of *E. coli* (Nishio et al., 2016). In contrast, Ng et al., (2020) reported Malaysian stingless bee honey to be highly effective against *E. coli*. Variation in microbial susceptibility to honey may result from differences in growth rate and cell-wall permeability to antimicrobial components (Dżugan et al., 2020). Additionally, honey samples from different origins may contain bioactive compounds with different effects on bacterial cells.

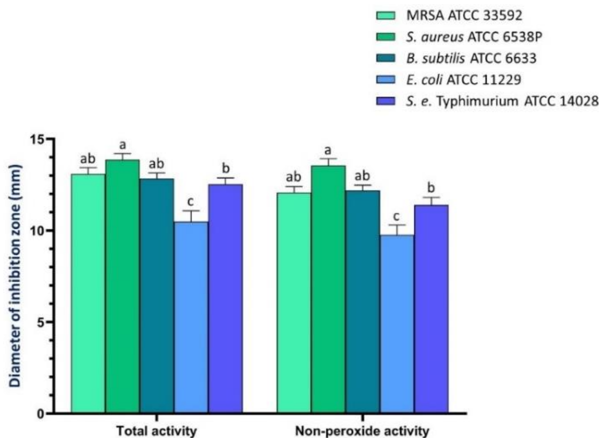

Figure 6. Grouped bar-graph showing differences in microbial susceptibility to the untreated (total activity) and catalase-treated (non-peroxide activity) honey samples. Superscripts with different letters within the same group indicate significant differences in diameters of inhibition zones ($p < 0.05$, ANOVA and Tukey post-hoc test).

5. Conclusions

The studied honey samples demonstrated broad-spectrum antibacterial activity against common pathogens. The antibacterial effect of stingless bee honey was primarily attributed to its non-peroxide components, indicating substantial therapeutic value. Its notable effectiveness against methicillin resistant *S. aureus* (MRSA) suggests it could serve as a potent natural remedy for treating wound infections and drug-resistant pathogens. However, further research is needed to unravel the underlying mechanisms and bioactive compounds responsible for the observed antibacterial activity. Such investigations will help determine the clinical applicability of stingless bee honey in treating bacterial infections, including resistant strains.

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Conflict of interests

The authors declare that they have no competing interests.

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Supplementary Table 1. Diameters of inhibition zones (mm) produced by honey samples before treatment with catalase against five bacterial strains.

Test microbes	MRSA ATCC 33592	*S. aureus* ATCC 6538P	*B. subtilis* ATCC 6633	*E. coli* ATCC 11229	*S. enterica* Typhimurium ATCC 14028
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
SH01	13.0 ± 1.0	16.0 ± 0.0	14.9 ± 0.3	11.7 ± 0.6	11.3 ± 1.2
SH02	15.0 ± 0.5	15.7 ± 0.6	12.5 ± 1.1	11.0 ± 1.0	13.3 ± 1.2
SH03	16.0 ± 0.5	14.3 ± 0.6	12.3 ± 0.6	10.5 ± 0.3	12.0 ± 0.0
SH04	13.0 ± 1.0	13.7 ± 1.2	14.0 ± 0.3	10.5 ± 0.5	11.7 ± 0.6
SH05	14.0 ± 1.0	14.3 ± 0.6	13.8 ± 0.3	12.5 ± 0.5	13.0 ± 0.0
SH06	13.5 ± 0.5	13.5 ± 1.2	12.5 ± 0.5	11.0 ± 0.6	16.0 ± 1.5
SH07	13.5 ± 0.3	14.8 ± 0.3	14.4 ± 0.6	12.0 ± 0.0	13.3 ± 1.5
KB01	12.0 ± 0.0	13.3 ± 1.5	13.0 ± 1.1	10.0 ± 0.0	11.7 ± 0.6
KB02	11.0 ± 0.0	14.2 ± 0.6	11.2 ± 0.3	*6.0 ± 0.0	12.3 ± 0.6
KB03	12.5 ± 1.5	13.3 ± 0.6	11.0 ± -.3	*6.0 ± 0.0	10.5 ± 0.5
KB04	13.5 ± 0.5	13.7 ± 1.2	12.5 ± 0.6	14.5 ± 0.5	12.8 ± 0.3
KB05	11.8 ± 0.3	16.3 ± 0.6	12.3 ± 0.6	11.0 ± 0.0	13.3 ± 0.6
KB06	12.0 ± 0.0	13.7 ± 0.6	13.5 ± 0.3	10.0 ± 0.0	12.0 ± 1.0
KB07	12.5 ± 0.5	13.7 ± 0.6	12.3 ± 0.6	10.2 ± 0.8	12.0 ± 0.0

Samples with codes SH and KB originated from Siha and Kibiti districts, respectively. Size of the agar well is 6.00 mm. Values with * sign indicate no microbial inhibition.

Supplementary Table 2. Diameters of inhibition zones (mm) produced by catalase-treated honey samples against five bacterial strains.

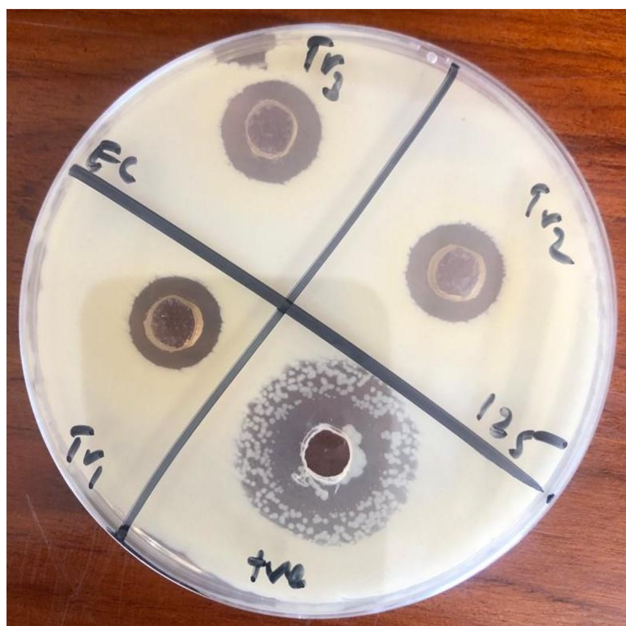
Test microbes	MRSA ATCC 33592	*S. aureus* ATCC 6538P	*B. subtilis* ATCC 6633	*E. coli* ATCC 11229	*S. enterica* Typhimurium ATCC 14028
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
SH01	12.3 ± 0.3	16.0 ± 0.0	14.2 ± 0.3	10.3 ± 0.6	9.8 ± 0.3
SH02	13.8 ± 0.8	15.0 ± 0.5	12.3 ± 0.6	10.3 ± 1.2	12.5 ± 0.5
SH03	14.5 ± 0.3	14.0 ± 1.0	11.7 ± 1.1	8.7 ± 0.6	11.8 ± 0.3
SH04	12.0 ± 0.0	13.0 ± 1.0	13.3 ± 0.6	9.7 ± 0.6	11.0 ± 0.0
SH05	13.0 ± 0.5	16.0 ± 0.0	13.3 ± 0.6	12.0 ± 1.0	12.5 ± 0.5
SH06	12.8 ± 0.8	12.5 ± 0.5	11.8 ± 1.1	10.3 ± 0.6	15.5 ± 0.5
SH07	12.5 ± 0.5	14.5 ± 0.5	13.0 ± 0.6	11.3 ± 0.6	10.5 ± 0.8
KB01	10.5 ± 0.5	12.5 ± 0.5	12.3 ± 0.6	9.7 ± 0.6	10.5 ± 0.5
KB02	10.0 ± 0.0	13.0 ± 1.0	10.7 ± 1.1	*6.0 ± 0.0	11.0 ± 1.0
KB03	11.0 ± 0.5	12.8 ± 0.3	10.3 ± 0.6	*6.0 ± 0.0	9.3 ± 0.6
KB04	11.8 ± 0.3	13.0 ± 1.0	11.7 ± 0.6	13.7 ± 0.6	10.5 ± 0.5
KB05	11.5 ± 0.5	11.5 ± 0.5	11.7 ± 0.3	9.7 ± 0.6	12.5 ± 0.5
KB06	11.3 ± 0.3	13.8 ± 0.3	12.8 ± 0.3	9.0 ± 1.0	11.3 ± 0.3
KB07	12.3 ± 0.8	12.3 ± 0.8	11.7 ± 0.6	10.0 ± 0.0	11.0 ± 0.0

Samples with codes SH and KB originated from Siha and Kibiti districts, respectively. Size of the agar well is 6.00 mm. Values with * sign indicate no microbial inhibition.

Supplementary Table 3. Results of the catalase effective test showing diameter of inhibition zones (mm) of treatments against *Staphylococcus aureus* ATCC 6538P.

Treatments	Without a catalase solution		With a catalase solution (10 mg/mL)	
	SH01	KB02	SH01	KB02
Honey solution (50% w/v) + Hydrogen peroxide (45 nmol/L)	26.5 ± 0.5	25.8 ± 0.3	16.0 ± 0.5	13.5 ± 0.3
Honey solution (50% w/v)	16.3 ± 0.3	14.3 ± 0.3	15.8 ± 0.8	13.3 ± 0.3
Hydrogen peroxide (45 nmol/L)	29.3 ± 3		*6.0 ± 0.0	

Values are recorded in mean ± SD. Size of the agar well is 6.00 mm. Values with * sign indicate no microbial inhibition.



Supplementary Figure 1. A representative agar plate showing zones of inhibition produced by stingless bee honey. Wells in Tr1 and Tr2 received catalase-treated honey (Non-peroxide activity) while Tr3 received untreated honey (Total activity).