ANTIMICROBIAL ACTIVITIES AND PHYTOCHEMICAL CONTENTS OF SUB-TERENEAN NESTING STINGLESS BEE PRODUCTS FROM BARINGO COUNTY

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A RESEARCH THESIS IS SUBMITTED IN PARTIAL FULFILLMENT FOR REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN MEDICAL MICROBIOLOGY OF MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY.

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DEDICATION

This work is dedicated to my parents Reuben Kiget, Irene Kiget and Charles Ng'eno; siblings, husband Jared Rutto and our child Jabari Yegon for supporting and encouraging me throughout my academic journey.

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ABSTRACT

In Baringo County, stingless bee honey is traditionally used to treat various ailments commonly associated with bacterial and fungal infections. However, there is limited experimental data on antimicrobial activities and phytochemical contents of stingless bee products from Baringo County. The objectives of this study were to evaluate the in vitro antimicrobial activities and phytochemical contents of honey and pollen from stingless bees *Meliponula beccarii* and *Plebeina hildebrandti* from Baringo County. Eighteen honey and thirteen pollen samples were conveniently sampled from eighteen wild occurring stingless bee nests in three different ecological zones of Baringo County and used for analysis. The study adopted a factorial experimental design, whereby three increasing concentrations of honey and pollen samples were prepared and tested against *E.coli, H.influenzae, MRSA* and *C. albicans* using agar well diffusion bioassay. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBCs) were measured using broth microdilution assay. Phytochemical screening was done qualitatively using standard methods. Data was analysed using Graphpad Prism software version 7 employing two-way Analysis of Variance and Tukey's post-hoc tests. *M.beccarii* honey samples showed significantly higher mean zones of inhibition against *E. coli* ($p<0.0001$, N=11), *H. influenzae* ($p<0.0001$, N=11), and *MRSA* (p>0.0001, N=11) at 100% v/v compared to distilled water but significantly lower mean zones of inhibition against *H. influenzae (*p<0.0001, N=11*), E. coli (*p<0.0001, N=11) and *MRSA (*8.1±2.8 mm vs *27*±0 mm) in comparison to ciprofloxacin. The mean MICs were 9.38% (v/v), 18.75% (v/v) and 18.75% (v/v), against *E. coli, H. influenzae* and *MRSA* respectively. *M. beccarii* honey was bactericidal only against *MRSA* with a mean MBC of 60.94% (v/v). *P. hildebrandti* honey samples in comparison to distilled water, had significantly higher mean zones of inhibition at 100% v/v against *E. coli* (12.1±2.01 mm vs 0 mm, $p<0.0001$, N=7), *H. influenzae* (10.1±4.8 mm vs 0 mm, $p<0.0001$, N=7), and *MRSA* (14.5±2.4 mm vs 0 mm, p>0.0001, N=7), but significantly lower mean zones of inhibition against *H. influenzae (9.0*±1.05 mm vs 25±2.0 mm, p<0.0001, N=7*), E. coli (10.9*±1.1 mm vs 30.26±0.8 mm, p<0.0001, N=7) and *MRSA (*12.8±1.8 mm vs 26±0.7mm) in comparison to ciprofloxacin. The mean MICs of *13.75* % (v/v), 13.75% (v/v) and 17.71% (v/v), were exhibited against *E. coli, H. influenzae* and *MRSA* respectively and was bactericidal against *E. coli, H. influenzae and MRSA* with mean MBC of 22.5 (v/v), 25% (v/v) and 28% (v/v) respectively. None of the honeys from the two species tested showed activity against *C. albicans* even at 100% v/v. All pollen samples had no antimicrobial activities. Phytochemical analysis revealed the presence of tannins, alkaloids, saponins, triterpenoids, phenols, flavonoids, steroids and glycosides but not terpenoids in all the honey and pollen of *M. beccarii* and only two of *P. hildebrandti*. Five *P.hildebrandti* honey and pollen lacked phenols. Therefore, most but not all honey samples of stingless bees *M. beccarii* and *P. hildebrandti* in Baringo County have antibacterial activities. The samples of pollen and honeys contain a range of valuable phytochemicals. Study confirmed the ethnomedicinal antibacterial uses of *M. beccarii* and *P. hildebrandti* honey and recommends *in vivo* studies and clinical trials.

ABBREVIATIONS AND ACRONYMS

AMR –	Antimicrobial Resistance
ANOVA	Analysis of Variance
CLSI –	Clinical & Laboratory Standards Institute
IREC –	Institutional Review and Ethics Committee
MBC –	Minimum Bactericidal Concentration
MIC –	Minimum Inhibitory Concentration
NACOSTI-	National Commission for Science, Technology and Innovation
SD –	Standard Deviation
SOP –	Standard Operating Procedure
TABDFs –	*Tetragonisca Angustula* Biofilm Destruction Factors
TAM –	Traditional and Alternative Medicine
WHO –	World Health Organisation

OPERATIONAL DEFINITION OF TERMS

Antibacterial activity- is the activity of a chemotherapeutic agent tested in the laboratory that destroy or inhibit the growth of bacteria. In this study, antibacterial activity is the ability of stingless bee honey and pollen to destroy or inhibit the test bacteria.

Antifungal activity- is the activity of a drug that kill or inhibit the growth of fungi. In this study it is ability of honey and pollen to kill or inhibit *C.albicans*.

Antimicrobial activity- is the activity of an antimicrobial agent that inhibit or kill micro-organisms. In this study, it is the ability of stingless bee honey and pollen to inhibit or kill test bacteria and fungi**.**

Potency-is the amount of drug required to produce a defined effect. In this study, it is the expression of the activity of stingless bee honey and pollen in terms of their minimum inhibitory concentration, minimum bactericidal and fungicidal concentrations.

Minimum Inhibitory Concentration - the lowest concentration at which a drug inhibits the multiplication of a microorganism. In this study, MIC is the lowest concentration at which stingless bee honey and pollen can inhibit multiplication of bacteria and fungi.

Minimum Bactericidal Concentration – is the lowest concentration at which an antibiotic kills a bacteria. In this study, MBC is the lowest concentration at which stingless bee honey and pollen can kill bacteria.

Subterranean nesting stingless bees - these are stingless bees that build their nests and live under the ground. In this study, *Plebeina hildebrandti* and *Meliponula beccarii* stingless bees are subterranean-dwelling.

Traditional medicine - are the knowledge, skills and practices based on beliefs, theories and experiences native to different cultures used in prevention and treatment of health aspects. *Plebeina hildebrandti* and *Meliponula beccarii* stingless bee honey and pollen are claimed to be used traditionally as medicine by various communities in tropical and subtropical regions to treat various ailments with topmost being respiratory, stomach disorders and oral thrush.

Phytochemical contents- are naturally occurring bioactive chemical compounds in plants and plant-based products such as honey. In this study, honey and pollen contained phytochemicals including flavonoids and alkaloids.

CHAPTER ONE

INTRODUCTION

This chapter gives the background of the study, the problem statement, research objectives, research questions, justification, significance and scope of study.

1.1 Background information to the study

World Health Organization (WHO) has listed antimicrobial resistance (AMR) among the leading worldwide public health threats (Friedrich, 2019). In 2019, approximately 5 million deaths associated with antimicrobial resistance including 1.27 million deaths directly attributable to bacterial antimicrobial resistance were reported (Murray *et al*., 2022)**.** Kenya had 8500 deaths attributed to and 37300 deaths associated with antimicrobial resistance (Murray *et al*., 2022)**.**

E.coli, S.aureus, K.pneumoniae, A baumannii and *M.tuberculosis* were the top six most AMR bacteria reported in 2019. These pathogens accounted for 752 000 associated deaths and 188 000 attributable deaths in the WHO African region (Sartorius *et al*., 2024)**.** The alarming rate at which AMR is rising indicates that many of the available antimicrobial agents are likely to be ineffective in the near future (Kakkar *et al*., 2021; Shafiq *et al*., 2021)**.**

Accordingly, it is estimated that AMR will cause more than 10 million deaths annually by the year 2050 (Neill, 2014). Therefore, there is urgent need for discovery of novel compounds that can be developed into new effective and safe drugs against infectious pathogens (Khameneh *et al*., 2016) **.**

Plants and animal products have long been used in traditional and alternative medicine (TAM) and have been shown to be rich sources of potential novel antimicrobial agents (Laport *et al*., 2009; Nasir *et al*., 2015; Paul *et al*., 2019). Honey, cerumen, bee bread, cerumen, propolis and pollen (Rozman *et al*., 2022) are the major products that can be harvested from Afro-tropical stingless bees species (Hrncir *et al*., 2016; Roubik, 2023).

Stingless bee honey in particular have been used traditionally as medicine to treat various infections, burns, tumors, kidney diseases and wounds (Zuccato *et al*., 2017) since ancient times by various communities in the tropical and subtropical regions of the world. Recent studies have suggested their potential pharmacological effects (Chanchao, 2009; Lavinas *et al*., 2019; Sanches *et al*., 2017).

The Mayan traditional doctors used stingless bee honey for centuries to cure poisonous stings and treat high fever, pneumonia, wounds, burns, cold and bronchitis (Reyes-Gonzalez *et al.*, 2014; Rosales, 2013). Ethno medicinal uses of honey from various species of stingless bees have also been documented among the local communities in Kakamega and Baringo counties, Kenya (Héger *et al*., 2023; Kiprono *et al*., 2022).

Importantly, several studies have investigated the antimicrobial activities of honey from different species and parts of the world. The honey of *Melipona beecheii species* of Mexico and Central America, for example, have been reported to have in vitro activity against *C. albicans* (Hau-Yama *et al*., 2020) . Honey of *Trigona species* in Ethiopia have been shown to have broad-spectrum antimicrobial activities against *B. subtilis*, *M. luteus, B. megaterium ,B. brevis ,E. coli* and *P. syringae* (Garedew *et al*., 2004). A study on *Trigona carbonaria* honey from Australia has shown its broad-spectrum activities against gram

positive and gram negative bacteria and limited activity against *Candida* (Boorn *et al*., 2010).

A study on honey of *Tetragonula species* of South East Australia against *S. aureus* and *K. pneumoniae* showed high bactericidal effects which were attributed to the high concentration of hydrogen peroxide and phenolic compounds (Massaro *et al*., 2014). Honey of different bee species native to Brazil were also shown to have high antimicrobial activity against *C. albicans , E. coli, K. pneumoniae*, *S. typhimurium* , *S. aureus*, *L. monocytogenes* and *B. cereus* (Ávila *et al*., 2019).

The honey of Costarican *Tetragonisca angustula* has demonstrated inhibition effects on *S. aureus* biofilms with *Tetragonisca angustula* biofilm destruction factors (TABDFs) being responsible for the antibiofilm action (Zamora *et al*., 2017).The potency of honey of *Meliponula species* of Ghana was comparable with that of gentamycin and had activity against *S. aureus* and *P. aeruginosa* with daily conjunctival application of 70 µL twice daily on an animal model (Ilechie *et al*., 2012).

In addition, Malaysian *Trigona* honey was found to have antibacterial and antibiofilm activities against *P. aeruginosa* and *S. pyogenes* in vitro thereby supporting the previous finding that *Trigona* honey can be used as an alternative medicine for various bacterial infections (Al-kafaween *et al.*, 2020). Furthermore, Thailand's *Trigona laeviceps* honey had antimicrobial activity against *E. coli*, *S. aureus*, *A. niger, A. pullulans* and *C. albicans* (Chanchao, 2009). A recent study on honeys from Tanzanian's six stingless bee species including *Dactylurina schmidti* reported their strong antimicrobial activities against *S. aureus, B. subtilis, S. typhi* and *C. albicans* (Mduda *et al*., 2023).

In Kenya, scientific studies have so far reported good antimicrobial activities of *Dactylurina schimidti honey* from Tana River against *P. aeruginosa, S. typhi , E. coli ,S. aureus and B. subtilis* (Muli *et al*., 2008). *Meliponula bocandei* and *Plebenia hylderbrandii* stingless bee honey from Kakamega, Western Kenya have also been shown to exhibit antibacterial activities against drug-sensitive *S. aureus* and *E. coli* (Wavinya *et al*., 2021). However, prior to this study, data on antimicrobial activities of stingless bee products from other parts of Kenya, including Baringo County were still scarce.

The potent antimicrobial activities of stingless bee honey have previously been ascribed to its bioactive components as well as physicochemical properties. Plants and stingless bee products, particularly honey contain common phenolic and flavonoid compounds due to polyphenols including chrysin, apigenin, kaempferol p-coumaric acid, caffeic acid, 4-hydroxybenzoic acid, gallic acid and quercetin-3-O-rutinosoid that are well known to strongly affect their antioxidant activity and thus their antibacterial potency (Cheng *et al*., 2021; Tuksitha *et al*., 2018).

Moreover, phenolic compounds such as vanillin, caffeic acid, rosmarinic acid, mandelic acid, aromadendrin, isoquercitrin, eriodictyol, chlorogenic acid, syringaldehyde, sinapaldehyde, umbelliferone and carnosol were reported in *Meliponinae* bee honey (Biluca *et al*., 2017). The antibacterial effect of honey was also associated with its high osmolarity, low pH and hydrogen peroxide content (Abdullah *et al*., 2019). Studies have also suggested that antifungal activity of stingless bee honey could be attributed to its phenolic composition including coumaric acid, ferulic acid, salicylic acid, and gallic acid (Maringgal *et al*., 2019).

Notably, the chemical content and physicochemical properties of stingless bee products and consequently their pharmacological activities are often greatly dependent on the species and botanical origin (Ávila *et al*., 2019; Biluca *et al*., 2020). However, the phytochemical content of most Kenyan stingless bee products remain largely unexplored, despite their potential as a source of novel anti-microbial agents.

The purpose of this study was to investigate the *in-vitro* antimicrobial activities and phytochemical contents of honey and pollen from two subterranean-dwelling stingless bee species *Meliponula beccarii* and *Plebeina hildebrandti* from various ecological zones of Baringo County, Kenya. The study findings confirmed the *in vitro* antibacterial effects of *Meliponula beccarii* and *Plebeina hildebrandti* honey (Kiprono *et al*., 2022b, Kiprono *et al*., 2022a) and provided evidence of phytochemicals present in the honeys to support their potent antibacterial activities.

1.2 Statement of the problem

The rapid rise in AMR even to the last–resort antibiotics possess a great threat to the global public health (Murray *et al*., 2022). Antimicrobial resistance has been associated with high mortality and morbidity rates, long hospital stays and high cost of care not only in the developing nations but also in the low and middle income countries (Dadgostar, 2019; Shrestha *et al*., 2018).

Out of the 204 countries, Kenya, in 2019, was ranked the 177th highest age-standardized mortality rate per 100,000 population associated with AMR and the 7th highest in the 15 Eastern Sub-Saharan Africa countries with majority of deaths being higher than deaths

from enteric infections, digestive disorders, diabetes, kidney diseases, maternal and neonatal disorders (Murray *et al*., 2022). A review on antimicrobial resistance projected that AMR could cause 10 million deaths per year by 2050 if not addressed (Neill, 2014).

However, despite the current global dramatic emergence and rapid spread of antimicrobial resistance, the development of new safe and effective novel drugs lags behind (Beyer *et al*., 2020). This calls for urgent need for accelerated discovery and development of alternative antimicrobial agents to keep pace with emergence of drug resistance.

More recently, stingless bee products including honey, propolis, cerumen and pollen from different tropical and sub-tropical parts of the world have been shown in several studies to have potent antimicrobial properties which are due to the presence of various regio-specific phytochemicals and unique chemicals produced by the stingless bee species themselves (Ngalimat *et al.*, 2019).

As such stingless bee products from ecological distinct regions and the many stingless bee species represent a rich source of novel compounds, which can potentially be isolated and developed into novel agents against both drug susceptible and resistant microbes. In Baringo County, two subterranean nesting stingless bee species (*Meliponula beccarii and Plebeina hildebrandti*) have been documented.

Although the natives of Baringo county may not really know about AMR, they use the honeys of these stingless bees widely and popularly as a traditional medicinal remedy for various ailments including oral thrush, wounds, respiratory and gastrointestinal disorders

by the community and traditional medical practitioners (Kiprono *et al*., 2022a ; Kiprono *et al*., 2022b).

These ailments treated with stingless bee honey are commonly associated with bacterial and fungal infections, suggesting that the locally harvested stingless bee products might be having potent antimicrobial activities. However, the antimicrobial activities and phytochemical composition of stingless bee pollen and other products from these two species had not been explored.

Therefore, this study explored whether the honey and pollen from the two stingless bees from Baringo county can be sources of novel antimicrobial compounds and if they can be used in conjunction with other remedies against bacterial and fungal-associated infections and showed that the honeys have antibacterial activities suggesting the presence of bioactive compounds that can be determined through a bioassay-guided isolation and purification.

1.3 Study Objectives

1.3.1 Broad objective

To assess the antimicrobial activities and phytochemical contents of honey and pollen samples of *Meliponula beccarii* and *Plebeina hildebrandti* stingless bee species from Baringo County.

1.3.2 Specific objectives

i. To determine the inhibition of bacterial and fungal growth by *Meliponula beccarii* and *Plebeina hildebrandti* honey and pollen samples from Baringo County.

ii. To determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of *Meliponula beccarii* and *Plebeina hildebrandti* honey and pollen samples from Baringo County.

iii. To determine the phytochemical contents of *Meliponula beccarii* and *Plebeina hildebrandti* honey and pollen samples from Baringo County.

1.4 Research Questions

i. Do the honey and pollen samples of *Meliponula beccarii* and *Plebeina hildebrandti* stingless bee species from Baringo County inhibit bacterial and fungal growth in vitro?

ii. What are the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of honey and pollen samples of *Meliponula beccarii* and *Plebeina hildebrandti* stingless bee species from Baringo County?

iii. What are the phytochemical contents of the honey and pollen samples of *Meliponula beccarii* and *Plebeina hildebrandti* stingless bee species from Baringo County?

1.5 Justification

Antimicrobial resistance against all categories of the currently available antibiotics is rising globally at an alarming rate (Murray *et al*., 2022). This calls for urgent and conceited discovery and development of alternative antimicrobial agents including from ancient remedies such as medicinal plant products and honey (Khameneh *et al*., 2016; Paul *et al*., 2019).

The research in this thesis focused on studying stingless bee products from Baringo County as potential sources of novel compounds that could be developed into antimicrobial agents. This was based on the growing evidence that stingless bee products from different other

regions are rich in phytochemicals with potent antimicrobial activities. Unlike individual plants, the honey and pollen consist of phytochemicals collected from different plants, as well as fermentation products. The stingless bee honey and pollen have long also been widely consumed as super food and traditional medicinal remedy. Therefore, the phytochemicals contained in these products are likely to be safe.

Baringo County was selected as the source of study samples because of several reasons. First, recent ethnomedicinal surveys indicated that community members and traditional medical practitioners widely use the costly stingless bee honey as a remedy for various ailments, particularly those associated with bacterial and fungal infections, without any confirmatory laboratory efficacy studies (Kiprono *et al*., 2022a ; Kiprono *et al*., 2022b).

Secondly, Baringo County is unique in terms of plant diversity due to existence of different ecological, climatic zones and highly variable rainfall which can greatly influence the chemical composition of honey and pollen, making them unique in comparison with stingless bee products from other regions and whose antimicrobial activities had been studied.

Thirdly, the diverse topography of Baringo county ranging from flat lands to steep hilly terrains provide suitable habitats for *Meliponula beccarii* and *Plebeina hildebrandti* which are strictly subterranean-nesting unlike the other species studied in other parts of the country which live on trees. This underground environment including the type of soil could influence the contents and pharmacological activity of their products.

E. coli and *H. influenzae* that were used to test the antibacterial activities of honey and pollen samples represent other gram negative bacterial pathogens and are commonly

involved in causing urinary tract, gastrointestinal and respiratory tract infections, respectively (Makvana *et al*, 2015). *MRSA* was used to represent other gram positive bacterial pathogens commonly involved in causing skin disorders and wound infections (Monecke *et al*., 2011). MRSA was also chosen because it is one of the most common multi-drug pathogen of great public health concerns (Ferri *et al*., 2017).

These three, together with *C. albicans* which is the commonly involved in causing of oral thrush, are the most prevalent organisms whose strains have been reported to be resistant to current antibiotics and are the accepted test organisms. This study therefore, tested the stingless bee honey and pollen against these four microorganisms while focusing on the need for new effective antibacterial and antifungals against them.

1.6 Significance of the study

The findings of this study have extended the existing knowledge of antimicrobial activities of stingless bee products from different geographical areas and stingless bee species. The study findings validated the use of stingless bee *Meliponula beccarii* and *Plebeina hildebrandti* honey and provided evidence for their antimicrobial activities. Specifically, the honey showed potent activity against *E. coli, MRSA* and *H. influenzae.* The study findings therefore empower the traditional medical practitioners to continue prescribing the honey to their patients that are suspected to have bacterial infections.

The findings also form the basis for further analysis of the *Meliponula beccarii* and *Plebeina hildebrandti* honey against other common bacterial pathogens. Moreover, the study findings forms a solid basis for isolation and purification of the antibacterial compounds in the honey of *Meliponula beccarii* and *Plebeina hildebrandti* from Baringo. Such compounds may lead to discovery of novel antibacterial compounds. Testing the

honey on animal models of *MRSA* infections and formulating its pure form for use in the treatment of *MRSA*-associated infections have been suggested too.

Most importantly, the findings of this study inform on the necessity for meliponiculture of the two stingless bees for their honeys. The antibacterial potency of the honey of the two stingless bee species showed its possible contribution towards achieving Sustainable Development Goal (SDG) number 3 on good health and well-being through provision of safe and affordable sources of medicine to curb both communicable and non-communicable diseases.

1.7 Scope of the study

1.7.1 Limitations

This study focused on determining the antimicrobial activities and phytochemical contents of honey and pollen of *Meliponula beccarii* and *Plebeina hildebrandti* stingless bees from Baringo County. Convenient sampling technique was applied in determining the stingless bee nests and collecting their honey and pollen in Baringo County. The findings may not be generalizable to other species or same species from other regions.

Hard to reach areas were omitted with the use of this strategy hence the results could be significantly biased since those sampled might not have represented others that were not sampled in the areas that could not be reached easily.Accessing the location of nests was difficult since the two stingless bee species are wild-occurring and live underground in remote areas some with very dense forests and difficult terrain.

1.7.2 Delimitations

This study was delimited to *Meliponulla beccarii* and *Pleibeina hildebrandti* stingless bee honey and pollen from Baringo County and did not cover other stingless bee products such as propolis and royal jelly. The results of this study are applicable only to honey and pollen of *Meliponula becccarii* and *Plebeina hildebrandti* stingless bee species from Baringo County.

This study did not cover antioxidant effects and mechanisms of action of the bioactive contents of the *Meliponula becccarii* and *Plebeina hildebrandti* honey and pollen samples. The study also focused on qualitative phytochemical screening of the honey and pollen samples without isolation and purification of the potentially bioactive compounds.

1.8 Conceptual frame work

Antimicrobial activity of honey and pollen is dependent on species difference, ecological zones and harvesting seasons. The zones of inhibition, MIC, MBC and MFC as well as the phytochemicals are all dependent on the honey and pollen products of the two stingless bee species. The outcome of the assay was establishment of antimicrobial activities, potency levels and phytochemical contents of the honey and pollen of the two stingless bee species. Standard qualitative analytical methods revealed various phytochemicals including phenols and flavonoids which are likely to be responsible for the detected antimicrobial activities.

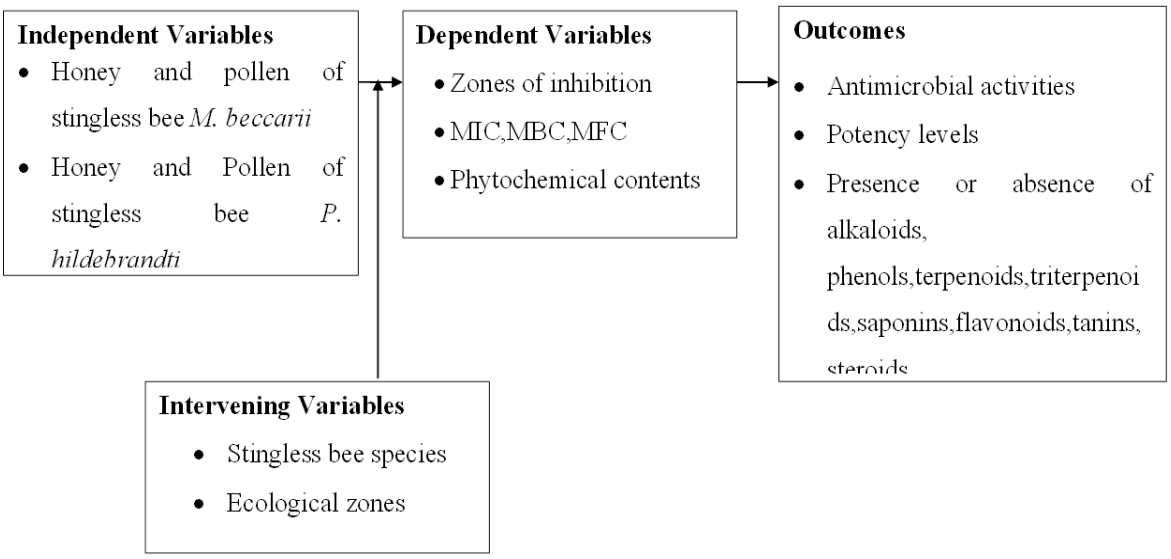


Figure 1.1: Conceptual framework.

The figure shows independent variables, intervening variables, dependent variables and the outcomes.

CHAPTER TWO

LITERATURE REVIEW

This section presents the review of literature guiding the study. The problem of AMR is first reviewed, followed by current state of knowledge on stingless bees, their products and evidence in support of these products as a source of antimicrobial agents. The gaps of knowledge linked to each of the three specific objectives are also identified and reviewed.

2.1 Antimicrobial resistance and need for novel antimicrobial agents

The World Health Organization asserts that antimicrobial resistance constitutes a critical and escalating threat to global public health and economy all over the world and has been classified among the top 10 worldwide public health challenges affecting humans in the 21st century (Friedrich, 2019). In 2010, a study estimated a high mortality rate attributable to six multidrug resistant bacterial pathogens with 43% deaths in patients with hospital acquired infections in Thailand which reflects the situation in other developing nations (Lim *et al*., 2016).

A 2014 review on the state of antimicrobial resistance by then estimated about 700 000 deaths and projected a mortality rate attributed to AMR to reach approximately 10 million deaths per year by 2050 (Neill, 2014). Another study estimated the incidence of bloodstream infections attributed to *E. coli* and *K. pneumoniae* resistant to carbapenem and third-generation cephalosporin globally in 2014 as 0.5 million and 6.4 million, respectively (Matsunaga *et al*, 2018).

In 2015, there were an estimated 671,689 reported cases of infections caused by antibiotic-resistant bacteria in the European Union and European Economic Area (Cassini *et al*., 2019). In 2018, Elizabeth Temkin and her team estimated that around 5 million deaths worldwide were linked to bacterial antimicrobial resistance across 23 pathogens and 88 pathogen-drug combinations, with 1.27 million of those deaths directly attributable to bacterial antimicrobial resistance (Temkin *et al*., 2018).

In 2019, Kenya reported 8,500 deaths directly attributable to antimicrobial resistance and 37,300 deaths associated with it. The bacteria responsible for these deaths included *K. pneumoniae* (6,200 deaths), *E. coli* (5,900 deaths), S. pneumoniae (5,600 deaths), *S. aureus* (5,000 deaths), and *S. typhi* (2,400 deaths) (Murray *et al*., 2022). A study at Kenyatta National hospital revealed increased prevalence of multi-drug resistant *S. typhi* by 28% from 1998 to 2004 (Kariuki *et al*., 2005).

Recently, a study reported resistance in 39 % of isolates from patients hospitalized with infections caused by carbapenem-resistant Enterobacterales and extended-spectrum-lactamase-producing organisms which are also highly prevalent in Kenya (Ita *et al*., 2022). Globally, mortality and morbidity from opportunistic fungal infections, particularly those caused by Candida strains resistant to existing antifungals like azoles and echinocandins, present a significant challenge.

This situation highlights the urgent need for developing new treatments for *Candida species*, particularly through novel compounds derived from natural sources such as honey (Akins, 2005). These evidence of rapidly rising antimicrobial resistance clearly show that the treatment of infections with the existing antimicrobial drugs is may become ineffective in future (Davies *et al* , 2010). This situation underscores the urgent necessity for this study

and similar research to assess the antimicrobial properties of stingless bee honey and other traditional or alternative medicines with demonstrated antimicrobial potential. Such evaluations are crucial for advancing these substances to clinical trials, standardizing them, and recommending them as effective agents to combat antimicrobial resistance.

2.2 Stingless Bees

Stingless bees, which belong to the Meliponinae subfamily within the Apidae family, are social insects that lack a functional sting. They are categorized into two tribes: the Meliponini, which includes only the genus *Melipona* and is predominantly found in the Neotropics, and the Trigonini, which is distributed throughout tropical regions. Over 500 discovered species of stingless bees are confined to tropical and subtropical regions worldwide, including Australasia in Australia, the Paleotropical region in Africa, the Indo-Malayan region in Asia, and the Neotropical region in South America (Roubik, 2023) .

In Africa, they are diverse, including Madagascar. In Kenya, stingless bees can be found in locations such as Kakamega Forest and Mwingi, Arabuko Sokoke along the coast and some parts of the Rift Valley and Nyanza (Macharia *et al*., 2007). Stingless bees nesting sites can be in trees, rocks and trunk cavities, in ant nests, in buildings and undergrounds (Grüter, 2020). The stingless bees' vestigial stings are greatly reduced thus making them harmless to human beings.

These bees are more active in the warm conditions than in the cold. Stingless bees collect and forage on plant-based resources including pollen, nectar, resin, leaves, latex, oils, scents and seeds thereby maintaining the ecosystem (Nunes-Silva *et al*., 2010). Stingless bees collect and store honey which they consume as their source of carbohydrates, propolis,

cerumen and resin which are used to build and maintain the nests and pollen which is their source of proteins (Grüter, 2020).

These bees chemically modify floral nectar and store it as honey in cerumens. Stingless bees are also known for the significant role they play in the tropical and subtropical ecosystems as pollinators (Biluca *et al*., 2016). The abundant vegetation in tropical and subtropical regions where stingless bees are found suggests that stingless bee products may be more promising sources of biologically active compounds compared to those from honey bees (Jalil *et al*., 2017).

2.3 Stingless bee products

2.3.1 Stingless bee honey

Stingless bees produce bee pollen, honey, and propolis (Nordin *et al*., 2018) Stingless bee honey is a floral liquid characterized by a unique aroma, fluid consistency, elevated protein content, increased acidity, higher electrical conductivity, and greater moisture content compared to honey from honey bees. It has a distinctive sweetness with an acidic flavor, lower diastase activity, and tends to crystallize more slowly (Ávila *et al*., 2018; Almeida *et al*., 2013; Nordin *et al*., 2018).

This honey is sourced from floral and plant nectar or from the secretions of insects that feed on plants (Jones, 2013). Stingless bee honey varies in color from light amber to amber brown, with darker shades typically having higher ash content (Nascimento *et al*., 2015) and higher quantity of total phenolics which means higher bioactivity than those with lighter colors (Majid *et al*., 2020). It is also rich in sugars, minerals, flavonoids, proteins and vitamins (Bakar *et al*., 2017).

Stingless bee honey is not only considered a superfood but also holds significant medicinal value among communities in tropical and subtropical regions as it is used locally to treat digestive, reproductive, ocular and respiratory disorders (Grüter, 2020). Research has highlighted the antimicrobial, antioxidant, natural moisturizing, and anti-inflammatory properties of stingless bee honey, especially its effectiveness in accelerating wound healing. These benefits are attributed to its moisture content, pH level, peroxide and non-peroxide compounds, water activity, phenolic acids, flavonoids, vitamins, and enzymes (Jalil *et al*., 2017). Despite its long history of medicinal and cosmetic use, there is limited scientific evidence supporting the various claimed benefits of stingless bee honey. Additionally, the low production yield of stingless bee honey contributes to its high cost.

2.3.2 Stingless bee pollen

Pollen is a combination of pollen grains collected from shrubs, herbs, trees, flowers and fruits (Vit *et al*., 2018) in different botanical sources and packed into pollen baskets on the stingless bees' hind legs, nectar and salivary enzymes before its fermented in the cerumen pots (Kieliszek *et al*., 2018). Stingless bee pollen is consumed by larvae as an important source of proteins (Grüter, 2020; Michener, 2000).

Stingless bee pollen is known for its benefits in alternative and complementary medicine against infectious diseases, stomach ulcers and prostatitis (Lopes *et al*., 2019). Nevertheless, the daily use of stingless bee pollen is restricted due to its high cost and limited availability (Mohammad *et al*., 2020).

2.4 Medicinal values of stingless bee products traditionally

For many years, stingless bee products, especially honey, have been utilized as traditional medicine in tropical and subtropical regions around the world (Reyes-Gonzalez *et al*., 2014; Rozman *et al*., 2022; Samarghandian *et al*., 2017). In Tanzania, Mduda *et al*., (2023) recently found that honey from *Meliponula beccarii, Meliponula ferruginea, Meliponula togoensis, Plebeina armata, Hypotrigona gribodoi*, and *Dactylurina schmidti* are traditionally used to treat conditions such as asthma, ulcers, fractures, intestinal worms, reproductive issues, burns, and respiratory infections.

Additionally, honey is reported to have protective effects against gastrointestinal problems like stomach disorders and constipation. The Sheka people of Ethiopia have used honey to treat asthma, tuberculosis, and tonsillitis. Honey, renowned for its exceptional natural wound-healing properties, was used by ancient Egyptians, Greeks, Assyrians, and Romans to dress wounds. They applied honey to prevent infections and speed up the healing process (Kassim *et al.*, 2010).

In Brazil, honey has been utilized in traditional medicine to address conditions such as diabetes and snakebites (Mendes *et al.*, 2008). In Uganda, stingless bee honey was used traditionally to alleviate constipation and other stomach disorders (Byarugaba, 2019). In Ghana, stingless bee honey is commonly used as a home remedy for asthma (Karikari, 2007). Respiratory disorders such as coughs, cold, throat infections and bronchitis among the Mayans and natives of Mexico were treated traditionally using stingless bee honey (Reyes-Gonzalez *et al.*, 2014; Rosales, 2013).

In Baringo county, the native community appreciates and widely use stingless bee honey to remedy various diseases top most being respiratory disorders, gastrointestinal disorders, sore throat, infections, and wounds (Kiprono *et al*., 2022a ; Kiprono *et al*., 2022b).

2.5 Antimicrobial activities of stingless bee honey and pollen

A study investigating the antimicrobial properties of honey from Ethiopian Trigona species revealed that it exhibited either bacteriostatic or bactericidal effects against both gram-positive bacteria (such as *M. luteus, B. subtilis , B. megaterium, and B. brevis*) and gram-negative bacteria (including *P. syringae and E. coli*), depending on the concentration of the honey (Garedew *et al*., 2004). Another study found that honey from Trigona species in Malaysia significantly inhibited the growth of *E. coli* cell cultures, suggesting its potential use in treating diseases caused by *E. coli* (Kafaween *et al*., 2019).

In addition, a study on Brazilian honey of *Melipona bicolor, Melipona quadrifasciata* and *Melipona marginata* stingless bees foraging from the same floral region revealed more potent antibacterial activity against gram negative bacteria (*E. coli* and *S. typhimurium*) than gram positive bacteria (Ávila *et al*., 2019). *Trigona honey* of Malaysia and ampicilin combination was reported to have high antibacterial activity and inhibition effect against all *S. aureus* thereby indicating its potential effectiveness in treatment of wounds infected by antibiotic sensitive and resistant strains than when either of them is used alone (Ng *et al*., 2017).

Honey from the *Tetragonisca angustula* species in Costa Rica demonstrated both inhibitory and bactericidal effects against S. aureus biofilms. This activity was attributed to specific biofilm-destroying factors in *Tetragonisca angustula* honey, known as

TABDFs-1 and TABDFs-2, which are believed to enhance the antibacterial effectiveness against S. aureus biofilms (Zamora *et al*., 2017).

Phenolic extracts of honey from *Tetragonula carbonaria* stingless bees species of Australia demonstrated antibacterial activity in vitro against *S. aureus* and *K. pneumoniae* (Massaro *et al*., 2014). Research that was carried out on guinea pigs, reported that administration of one drop of the Ghanian *Meliponula species* stingless bee honey twice daily to *S. aureus* or *P. aeruginosa*–infected conjunctiva reduced infection and convalescence periods. Those results were comparable to that of gentamycin administration in the treatment of conjunctivitis. The authors suggested stingless bee honey as an alternative to treat human conjunctivitis since it is not as expensive as synthetic antibiotics and can be readily available in the rural areas (Ilechie *et al*., 2012).

Another study found that honey from the Australian stingless bee *Trigona carbonaria* exhibited limited antifungal activity against *Candida species* but demonstrated broad-spectrum antibacterial effects against both gram-positive and gram-negative bacteria, including *S. aureus* and *P. aeruginosa*. This suggests that it has potential as a medicinal honey due to its similar properties to other therapeutic honeys (Boorn *et al*., 2010).

Kenyan *Plebeina hildebrandti* stingless bee honey exhibited inhibitory and bactericidal effects against *S. aureus*, as well as bacteriostatic effects against *E. coli*, depending on the concentration. In contrast, *Meliponula bocandei* honey only demonstrated bacteriostatic and bactericidal activity against *S. aureus* and showed no antibacterial activity against *E. coli* at any concentration (Wavinya *et al*., 2021).

Muli *et al.* (2008) reported that while undiluted honey of stingless bee, *Dactylurina schimidti* from Tana River exhibited a notable bacteriostatic effect against *B. subtilis* and *S. typhi* at certain dilutions, it showed neither bacteriostatic nor fungistatic effects against *A. niger, E. coli, P. aeruginosa*, *S. aureus* or *C. albicans.*

While many studies have shown that honey can be effective in treating fungal infections, stingless bee honey is not commonly used for this purpose (Ansari *et al*., 2013; Estevinho *et al*., 2011). A study revealed that honey from the *Melipona beecheii* species in Mexico and Central America exhibits both fungicidal and inhibitory effects against *C. albicans* in vitro. It disrupts the structure and integrity of the fungal cell wall at various concentrations, making it a promising natural source for new antifungal compounds (Hau-Yama *et al*., 2020).

In a separate study, honey from the *Melipona beecheii* species in Cuba was shown to inhibit the growth of *C. albicans* (Alvarez-Suarez *et al*., 2018). Additionally, a study comparing four types of honey from various countries found that honey from *Melipona beecheii* exhibits antifungal activity against *C. albicans* even at very low concentrations (Morroni *et al*., 2018). Among the three Malaysian honey samples examined, stingless bee honey exhibited superior antifungal activity against *C. albicans* and *A. niger* compared to Tualang and Acacia honeys (Hamid *et al*., 2018). All these indicate that studies based on effects of the stingless bee products on *Candida* cells are relevant to establish the antifungal potential of stingless bee products in an attempt to reduce the increasing mortality and morbidity due to resistant strains of *Candida species* (Akins, 2005; Whaley *et al*., 2017).

Although there is limited data on stingless bee pollen, studies have shown its potential as an antimicrobial agent. For example, *Heterotrigona itama* stingless bee pollen was reported

to contain probiotic bacteria whose antimicrobial activity inhibits pathogenic microorganisms in food (Mohammad *et al*., 2020).

2.6 Bioactive compounds of honey and pollen responsible for their antimicrobial effects

P-coumaric acids, naringenin, salicylic acid, caffeic acid, protocatechuic acid, taxifolin, quercetin and aromadendrin are the most commonly discovered phenolic compounds in the stingless bee honeys. For example, *p*-coumarics, quercetin and naringenin were reported to be present in the Malaysian *Heterotrigona itama* honeys (Ismail *et al*., 2016; Majid *et al*., 2020). Quercetin, p-coumarics and naringenin were found in *Tetragonisca angustula* and *Scaptotrigona bipuncatata* honeys from Brazil (Biluca *et al*., 2020).

Similarly, *Melipona marginata* honey of Brazil were found to be rich in quercetin and p-coumarics (Biluca *et al*., 2020) as well as salicylic acid, naringin, caffeic acid, taxifolin, mandelic acid and rosmarinic acid (Biluca *et al*., 2017). Another study on Brazilian *Melipona bicolor* honeys revealed the presence of quercetin (Ávila *et al*., 2019), p-coumarics (Ávila *et al*., 2019; Biluca *et al*., 2017) and naringin (Biluca *et al*., 2017). *Melipona quadriascia*ta honeys have also been reported to have quercetin and p-coumaric acids (Ávila *et al*., 2019; Biluca *et al*., 2017, 2020).

Flavonoids such as quercetin, catechin, gallate and epigallocatechin combined with fluconazole has been reported to act on fluconazole resistant *C. tropicalis* (Silva *et al., 2014)*. Quercetin, catechin and other flavonoids have also been reported in *Melipona* honey (Sierra *et al*., 2016). More studies have shown the antimicrobial contents of stingless bee products including defensin-1 (Leckenby *et al*., 2014) and methylglyoxal (Novi , 2018).

Research conducted on the Kenyan stingless bee honey of *Plebenia hylderbrandii* and *Meliponula bocandei* species unveiled thirty-five organic compounds with the common ones being glycosides, quinolines, amino acids, phenols, flavonoids and peptides which may account for the honeys' bactericidal and bacteriostatic effects against *S. aureus* and *E. coli* (Wavinya *et al*., 2021). A recent preliminary study on stingless bee honeys from Marigat Baringo county, reported the presence of carotenoids, phenols and flavonoids (Mwangi *et al*., 2024)

Stingless bee pollen from *Scaptotrigona affinis postica* has been found to contain high levels of polyphenols, which are responsible for its potent antioxidant and antimicrobial properties (Jorge *et al*., 2020). *Tetragonula biroi* Friese pot pollen of Philippines has also exhibited high content of rutin, hydroxycinnamic acids and quercetin glucoside (Belina-Aldemita *et al*., 2020b). Moreover, *Melipona mandacaia* bee pollen of Brazil was reported to contain phenols, fatty acids and flavonoids and was not contaminated by pathogenic microorganisms thus making it safe for consumption (Bárbara *et al*., 2015).

Melipona compressipes manoasensis stingless bee pollen of Brazil was reported to have active secondary metabolites that are thought to be components for development of antibiotics (Carneiro *et al*., 2019). Prior to the current study there were no study conducted to screen or characterize the phytochemicals found in *Plebeina hildebrandti* and *Meliponula beccarii* stingless bees.

2.7 Factors affecting antimicrobial activity of stingless bee honey and pollen

Stingless bee products particularly honey, have different antimicrobial activities depending on factors including bee species, climatic conditions, floral sources, botanical, geographical origin, harvesting, processing and storage conditions which strongly affect their composition and properties (Ávila *et al*., 2019; Biluca *et al*., 2016; Nordin *et al*., 2018).

According to Mahmood *et al.*, (2021) pollen source influences antimicrobial and antioxidant properties of honey since the type of pollen collected in the study of *Heterotrigona itama* of Indo-Malaya was found to depend on the species of flowering plants within the locality of the nests. A review study on worldwide honey reported that the origin and floral sources of the honey vary depending on geographical distribution (Libonatti *et al*., 2014; Taormina *et al*., 2001).

Different bee species have distinct storage methods that contribute to the varying antibacterial effects observed between honey from honey bees and stingless bees. In Ethiopian honey, stingless bee honey exhibited stronger antibacterial activity compared to honey from honey bees (Yalemwork *et al*., 2013). The botanical origin and processing methods of honey also lead to variations in its antimicrobial activity (Tan *et al*., 2009).

Another study revealed that the concentration of active compounds in the honey which differ according to floral sources determines its antibacterial effects (Taormina *et al*., 2001). The presence of strains of lactic acid bacteria has been stated as a factor contributing to antibacterial and antibacterial features of stingless bee honey (Lani *et al*., 2015).

Certain types of stingless bee species in different geographical zones produce honey that do not inhibit the growth of fungi; no inhibitory activity was detected in Thai's *Tetragonula leaviceps* honey (Suntiparapop *et al*., 2012). For example, no inhibitory activity against *C. albicans* was also detected in Australian *Tetragonula carbonaria* honey even in high concentrations (Boorn *et al*., 2010). However, another study showed that honey from *Melipona beecheii* effectively inhibited the growth of *C. albicans* (Hau-yama *et al*., 2020).

Hydrogen peroxide concentrations in stingless bee honey is influenced by the levels of glucose oxidase and catalase synthesized by the bees and catalase from flower pollens .The hydrogen peroxide levels are known to contribute largely to the honeys' antimicrobial activity (Grecka *et al*., 2018). Deferring physicochemical properties, including high acidity, high osmotic pressure, low pH, and elevated sugar concentration, are also known to contribute to variability in antimicrobial activity of stingless bee honey (Oscier *et al*., 2008).

The chemical composition of pollen collected by stingless bees varies based on factors such as environmental conditions during pollen development, geographical origin, climate zone, bee species, age and nutritional status of the foraging plants, soil fertility, seasonal changes and the method of extraction used (Barth, 2004; Kieliszek *et al*., 2018; Kocot *et al*., 2018).

The variations in the chemical, physicochemical properties and thus the antimicrobial activity of honey and pollen which depends on these factors is the reason as to why stingless bee products from different bee species, different geographical locations and botanical regions should be investigated. This study therefore sought to determine the antimicrobial

activities and phytochemical contents of honey and pollen of the two sub-teranean nesting stingless bee species from the different ecological zones in Baringo.

CHAPTER THREE

MATERIALS AND METHODS

This chapter outlines the methods that were employed in the study in order to collect data and other relevant information. It describes the study area, study design, sampling techniques, sample preparation, experiment assays, data analyses and presentation.

3.1 Study area/site

Stingless bee honey and pollen samples were collected from Baringo County (Appendix III), found in Midwestern part of Kenya (0°40'0" N and 36°0'0" E), covers a locality of 11,015 km^2 and has a population of 666,763 (KNBS, 2019). Baringo County comprises six sub-regions namely; Baringo North, Koibatek, Baringo Central, Marigat, Baringo East and Mogotio, with varying humid, sub-humid, partly humid, arid, semi-arid and extremely arid climatic domains which have varying mean annual rainfall ranging from 450 mm in semi-arid zones to 1100-2700 mm in the humid zones (Ednah *et al*., 2018; Odada *et al*., 2006).

Baringo is divided into highland, midland and lowland zones which have eleven different sub-ecological zones (Ednah *et al*., 2018).The topography of Baringo county have significant variations in elevation, ranging from flat to steep hilly land zones (Ednah *et al*., 2018; Odada *et al*., 2006). The soil types within Baringo County are mainly loam with alluvial deposits mainly derived from tertiary volcanic rock sediments weathered and eroded from uplands.

The county has a tropical climate, with dry seasons from January to mid-March and between July and September and rainy seasons from mid-March to mid-July and between September to December. Bee keeping and livestock farming are the main economic activities that farmers carry out in the lowland parts of the county (MoALF, 2017). The climate, rich plant diversity due to ecological diversity (the highlands, midlands and lowlands) and landscapes in the county provides suitable conditions and habitats for stingless bees including nesting grounds, pollen, nectar and resins (Biluca *et al*., 2017).

Two main subterranean nesting stingless bee species; *Meliponula beccarii and Plebeina hildebrandti* have been recently documented to inhabit forests, farmlands and riparian lands in Koibatek, Baringo central and Baringo North Sub-Counties where the nesting sites were sampled from in this study.

Specifically, for *Meliponula beccarii* six honey and six pollen samples were harvested from Koibatek, four in Lembus and two in Sabatia forest. In Baringo North, one sample was obtained from Katimok forest. In Baringo Central, four samples were derived, three from Morop-Tambaras and one from Kipngochoch (Appendix III).

For *Plebeina hildebrandti* honey, one and three honey samples were collected from Solian and Lepolos forests, in Koibatek. One honey was obtained from Sacho-Kibereke found in Baringo Central. One *Plebeina hildebrandti* pollen sample was derived from Solian forest within Koibatek and one other pollen sample from Sacho-Kibereke in Baringo Central (Appendix III).

3.2 Study design

The antimicrobial activities of *Meliponula beccarii* and *Plebeina hildebrandti* species honey and pollen against *H. influenzae*, *MRSA*, *E. coli* and *C. albicans* was determined using a (3 (1) × 3 (1) ×18 (13)) factorial experimental study design arranged in a completely randomized design at Mount Kenya University Research Laboratory whereby the factors were 3 increasing concentrations (50% v/v, 75% v/v and 100% v/v) for *Meliponula beccarii* honey and 1 concentration of pollen (10% w/v) and 3 concentrations for *Plebeina hildebrandti* honey and 1 concentration of pollen (10% w/v); 3 bacterial strains (*E. coli, H. influenzae* and *MRSA*) and 1 fungi (*C. albicans*); 18 non-pooled honey samples 11 (*Meliponula beccarii)* and 7 (*Plebeina hildebrandti)* , 13 pollen samples ;11 (*Meliponula beccarii)* and 2 (*Plebeina hildebrandti*).

3.3 Sample size

A total of 11 wild occurring stingless bee nests were located from which 11 honey and 11 pollen samples from *Meliponula beccarii* stingless bees were obtained. For *Plebeina hildebrandti* stingless bee honey a total of 7 wild occurring stingless bee nests were located from which 7 honey samples were obtained and 2 pollen samples from *Plebeina hildebrandti* stingless bees were obtained and used for analysis.

3.4 Sampling technique

The 18 stingless bee nests and samples were sampled conveniently in Koibatek, Baringo Central and Baringo North Sub-Counties. The location of the nests in the forests was difficult hence the experienced wild honey gatherers located the underground stingless bee nests by chance and honey and pollen were collected only from the nests that were searched and found within the fieldwork timeline.

3.5 Sample collection

Wild occurring stingless bee nests were located with the aid of experienced local honey gatherers. Once located, the nests were carefully excavated to expose the storage areas. The honey pots were then identified and 50 ul sterile syringes were used to suck the honey, which were then transferred into sterile 50 ml well labeled bottle/tube for each nest (with identification of the species, sub counties and date of collection).

Pollen pots were then identified and the pollen which was not mixed with honey was collected using a pair of forceps and placed in sterile polyethene ziplock bags, which were well pre-labelled. The samples were then transported to Masinde Muliro University of Science and Technology public health laboratory, and stored in the fridge at 4 ^{0}C until needed for experimental analysis.

The 11 *Meliponula beccarii* and the first two *Plebeina hildebrandti* honey and pollen samples were collected between January and April 2022 and the other 5 *Plebeina hildebrandti* honey samples were collected in November 2023, which were the dry and harvesting seasons in Baringo County.

3.6 Preparation of test honey dilutions

Pure honey (100 % v/v) collected were diluted with sterile distilled water to obtain other two lower concentrations of 75 % (v/v) and 50 % (v/v). This was done by measuring 0.75 mls, and 0.5 mls of pure honey and toping up using sterile distilled water to exactly 1 ml to eventually obtain 75% (v/v) and 50% (v/v) concentrations respectively after conducting a pilot test on all the samples. All the working honey concentrations were prepared in triplicates (Mama *et al*., 2019).

3.7 Preparation of pollen solutions

The stingless bee pollen was prepared as follows; the pollen was carefully separated from the pot.100 mg of the pollen sample was then weighed using an electronic analytical weighing balance (ATY 224, Shimadzu) and put in a 2 ml cryovial tube well labelled with sample laboratory identification number.1 ml of sterile distilled water was added into the cryovial tube containing the pollen sample and mixed thoroughly till the pollen dissolved to make a homogenous solution of 10% (w/v) concentration for each of the 13 pollen samples, which were then kept in a refrigerator at 4-8 ^{0}C until needed for experiments.

3.8 Study microorganisms

A total of four microorganisms including three bacterial strains; *E. coli* (ATCC 25922) and *H. influenzae (*ATCC 49766*)* which are gram negative and *MRSA* (ATCC 12393) which is gram positive and one fungus; *C. albicans* (ATCC 10231) were used to determine the microbial activities of both honey and pollen samples. All the microorganisms were pure cultures with referenced ATCC numbers and were obtained from Mount Kenya University Research Centre Microbiology section.

Prior to their use, they were sub-cultured on nutrient agar for bacteria and sabouroud dextrose agar for the fungi for 24 hours and 48 hours respectively to check their viability and to obtain fresh colonies. This was done by aseptically picking a loopful culture from the respective original stock and streak-plating to freshly prepare on the respective media.

3.9 Culture media and inoculum Preparation

3.9.1 Culture media preparation

Nutrient agar (HiMedia), Nutrient Broth (HiMedia) and Sabouraud Dextrose Agar (HiMedia) were used as culture media for bacteria and fungi, respectively. The preparation

of these media was done as per the manufacturer's instructions. Briefly, Nutrient Agar (28 g), Nutrient Broth (13g) and Sabouraud Dextrose Agar (65 g) were weighed using electronic balance (ATY224; Shimadzu) then suspended in 1000 mls of distilled water and heated to boiling point to ensure complete dissolution of the culture media powders. The well dissolved media was then sterilized by autoclaving at 121 ^{0}C and 15 psi for 15 minutes using vertical pressure steam sterilizer (Jibimed LS-50 LD). Approximately, 20 mls of the sterile media was then dispensed aseptically into sterile disposable petri dishes (90×15 mm).

3.9.2 Preparation of the culture inoculums

The bacteria and fungi inoculums were prepared as described by Matuschek *et al*., (2014) with some modifications. The 24 hour old bacteria and fungi colonies were suspended in sterile distilled water and the resultant turbidity compared to 0.5 McFarland standard. The turbidity of the inoculum was adjusted either by addition of more colonies or sterile distilled water until the turbidity of the inoculums matched the McFarland standards for the respective test bacteria and fungi.

3.10 Antimicrobial Activity Assays

3.10.1 Agar well diffusion for antibacterial activities

The agar well diffusion technique was used to test for the antibacterial activity of honey and pollen using the technique previously described for natural products by Valgas *et a*l., (2007) and Carneiro *et al*., (2019) for pollen. Briefly, 1 ml of the inoculum of each test bacterial strain was pipetted and added into each petri dish.20 mls of the already prepared nutrient agar was measured using a sterile measuring cylinder and added into each petri

dish. The content in each petri dish was mixed well and entirely spread uniformly using a sterile cotton wool swab and allowed to solidify.

Five 6 mm wells were then made on the solidified nutrient agar in each petri dish using a sterile cork borer 6 mm in diameter. 30 µl of each of the three increasing concentrations per honey sample (50% v/v, 75% v/v and 100% v/v) were loaded onto three different wells, respectively.15 µl of Ciprofloxacin (30 µg/ml) standard (Thermo Fisher Scientific) and 15 µl of sterile distilled water was loaded to the remaining two wells in each petri dish as positive and negative controls, respectively, using a 5-50 µl micropipette (DragonLab).

For each of the pollen sample, 30 µl of 10% w/v pollen solution was loaded in triplicates per petri dish, with 15 µl of Ciprofloxacin (30 µg/ml) standard and 15 µl of sterile distilled water (negative control).The inoculated petri dishes were then left to set for 15 minutes at room temperature to allow for diffusion of both honey and pollen extracts into the media prior to incubation at 37 °C for 18 hours in an incubator (D-91126 Schwabach FRG; Memmert GmbH). The zones of inhibition diameters were then measured in mm using a ruler and recorded. All samples were tested in triplicates.

3.10.2 Agar well diffusion for antifungal activities

The agar well diffusion technique was used to test for the antifungal activity of honey and pollen using the technique previously described for natural products by Valgas *et a*l., (2007) and Carneiro *et al*., (2019) for pollen. Briefly, 1 ml of *C. albicans* inoculum was pipetted and added into each of the sterile petri dishes. Thereafter, 20 mls of Sabouraud Dextrose Agar was added and mixed well before spreading it uniformly using a sterile cotton wool swab and allowed to solidify.

Five wells measuring 6 mm in diameter were then punched on the solidified Sabouraud Dextrose Agar using a sterile cork borer measuring 6 mm in diameter. 30 µl of each of the three increasing concentrations per honey sample (50% v/v, 75% v/v and 100% v/v) were loaded onto three different wells, respectively. 15 µl of nystatin standard (0.4 mg/ml) (Thermo Fisher Scientific) and 15 µl of sterile distilled water was loaded into the remaining two wells in each petri dish as positive and negative controls, respectively using a micropipette (DragonLab).

For each of the pollen sample, 30 µl of 10% w/v pollen solution was loaded in triplicates per petri dish, with 15 µl of Nystatin (0.4 mg/ml) standard and 15 µl of sterile distilled water (negative control). All the inoculated petri dishes were left to set for 15 minutes at room temperature to allow for diffusion of both honey and pollen extracts into the media prior to incubation at 35 °C for 24 hours in an incubator (D-91126 Schwabach FRG; Memmert GmbH). The diameters of the inhibition zones were measured in mm using a ruler and recorded. The experiments were done in duplicates.

3.11 Determination of Honey and Pollen Potency

3.11.1 Minimum Inhibitory concentration (MIC) for antibacterial assay

The broth microdilution technique was used to measure the minimum inhibitory concentration of honey as previously described for natural products by Valgas *et a*l., (2007) and for honey by Mama *et al*., (2019). MIC was determined only for honey samples that had zones of inhibition >13 mm in the agar well diffusion assay. For *Meliponula beccarii* honey against *E. coli and MRSA*, the selected one and four samples, respectively exhibited antimicrobial activity at concentration of 75% v/v.

Therefore, 100 µl of honey sample (75% v/v) was subjected to four stepwise two-fold serial dilutions in 96-well microtitre plate (Thermo Scientific-nunc # 167008) and the MIC determined. For *H. influenzae* two *of* selected samples exhibited antimicrobial activity at concentration of 50% v/v and one at 75% v/v. Therefore, 100 µl of honey samples (50% and 75% v/v) were subjected to four stepwise two-fold serial dilutions in 96-well microtitre plates (Thermo Scientific-nunc # 167008).

For *Plebeina hildebrandti* honey against *E.coli* and *H. influenzae*, the five samples selected exhibited antimicrobial activity at concentration of 25% v/v. Therefore, 100 µl of the honey samples (25% v/v) were subjected to four stepwise two-fold serial dilutions in 96-well microtitre plate (Thermo Scientific-nunc # 167008). For MRSA, five of the selected honey samples exhibited antimicrobial activities at concentration of 25% v/v and one at 75% v/v. Therefore, 100 µl of honey samples (25% and 75% v/v) were subjected to four stepwise two-fold serial dilutions in 96-well microtitre plates (Thermo Scientific-nunc # 167008).

All the microtiter plates were incubated at 37°C for 18 hours in an incubator (D-91126 Schwabach FRG; Memmert GmbH) after which, freshly prepared aqueous solution of resazurin indicator was added and incubated for 30 minutes. The first well in which pink color was observed was then located and the concentration at the well that preceded it was taken as the minimum inhibitory concentration. The MIC was determined as the lowest concentration of honey sample that inhibited the growth of the respective test microorganisms. This was done in triplicates for each honey sample.

3.11.2 Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration of the honey and pollen extracts was determined according to criteria described by National Committee for Clinical Laboratory Standards, (2011). The contents of the nutrient broth used for MIC tests were picked from the respective wells on the microtitre plates using a sterile wireloop and individually sub-cultured by streak-plating on freshly prepared sterile nutrient agar plates and incubated at 37 °C for 18 hours in an incubator (D-91126 Schwabach FRG; Memmert GmbH). The inoculated plates were then observed for bacterial growth such that the least concentration of honey that did not have the growth of the test bacteria was recorded as the MBC of the respective honey samples.

3.12 Phytochemical contents screening using qualitative methods

The phytochemical contents for each of the eighteen honey and thirteen pollen samples were screened preliminarily using standard qualitative test procedures referring to Harborne, (1998),with slight modifications. Presence or absence was denoted by (+) and (-), respectively.

3.12.1 Detection of alkaloids

1 ml of honey (100% v/v) and pollen (1g) was put into respective labelled test tubes. 10% H_2SO_4 was added into each of the test tubes and both test tubes were warmed in a water bath for 5 minutes and left to cool. 2 mls of the content from each of the test tubes was pipetted out and put into two separate test tubes for Dragendorrf's test whereby a blue litmus paper was put into the content in each of the test tubes and left to set until it changed color to pink.

Ammonia solution was then added into each of the test tubes and left to set until the litmus paper changed color to blue. Chloroform was then added into separating funnels labelled with each sample's identity. The content from each of the test tubes was then added into the respective separating funnels containing the chloroform and rocked gently and slowly in order to fractionate the content in the separating funnels. The resulting fractions were then separated by slowly releasing each of the extracts into the respective evaporating dishes and discarding the debris.

The extracts were left to evaporate on an oven.1-2 drops of 10% H_2SO_4 was added into each of the evaporating dish.1-2 drops of Dragendorrf's reagent was added into the evaporating dishes. The formation of orange-red precipitate on the evaporating dishes indicated the presence of alkaloids (Harborne, 1998).

3.12.2 Detection of flavonoids

NaOH test was used to detect the presence of flavonoids in honey samples. Briefly, honey (1 ml) was put in each of the three test tubes and added 1-2 drops of aqueous NaOH .1-2 drops of HCl was then added into each of the test tubes. The formation of yellow-orange color indicated the presence of flavonoids (Harborne, 1998)

Ammonia test was used to detect the presence of flavonoids in pollen samples as follows; pollen (1 g) was put in three test tubes.70% EtOH was added into each of the test tubes and warmed in a hot water bath for 5 minutes. A strip was dipped into each of the test tubes' content and exposed to ammonia vapour in ammonium chamber. The formation of a yellow color in 3 minutes on the strip indicated the presence of flavonoids (Harborne, 1998).

3.12.3 Detection of triterpenoids

Honey (1ml) and pollen (1g) samples were dissolved in 2ml chloroform in test tubes. Acetic anhydride (2 drops) was added followed by concentrated H_2SO_4 (2 drops).The appearance of a reddish-violet color indicated the presence of triterpenoids (Harborne, 1998).

3.12.4 Detection of Phenols

Honey (1 ml) and pollen (1g) were put in their respective test tubes .70% ethanol was then added into each of the test tubes and all the test tubes were warmed in a hot water bath for 5 minutes.5% Ferric chloride was then added into the content in each test tube and all the test tubes were shaken. The change of color to green indicated the presence of phenols (Harborne, 1998).

3.12.5 Detection of Glycosides

Modified Borntrager test was used to detect the presence of glycosides in the honey and pollen samples as follows; honey (1ml) and pollen (1g) were put in labelled test tubes and 2 mls of Sulphuric acid was added into each of the test tubes followed by addition of 5% Ferric chloride (2 drops). All the test tubes were placed in a hot water bath for 5 minutes after which the content in each test tube was decanted into other test tubes to remove the debris.

Chloroform was then added to the extracts in the separating funnels whereby the extracts were fractionated and released into other separate test tubes. Dilute ammonia was then

added to test tubes and observed for the formation of a rose-pink to red color in the ammoniacal layer which indicated the presence of glycosides (Harborne, 1998).

3.12.6 Detection of saponins

Honey (1 ml) and pollen (1 g) samples were put in labelled test tubes and dissolved with 5 mls of distilled water, boiled for 2-3 minutes and cooled for 15 minutes, shaken vigorously for 15 seconds and allowed to stand for 10 minutes. Formation of a stable persistent foam indicated the presence of saponins (Harborne, 1998).

3.12.7 Detection of Tanins

Ferric Chloride test was done whereby honey (1ml) and pollen (1g) was dissolved with 5 mls of distilled water. The content in the test tubes were warmed in a hot water bath inorder to extract. The extracts were filtered and 3 drops of 5% Ferric chloride solution was added into each of filtrates in the test tubes, which were then shaken. The formation of greenish black color indicated the presence of tanins (Harborne, 1998).

3.12.8 Detection of Terpenoids and steroids

Salkowski's test was used to detect the presence of terpenoids and steroids in the honey and pollen samples as follows; honey (1 ml) and pollen (1g) were put in respective testtubes.10 mls of Anhydrous Chloroform was carefully added to the honey and pollen in each of the test tubes and filtered into separate test tubes.0.5 mls of acetic acid and acetic anhydride were added. Concentrated H_2SO_4 was then added carefully and slowly into the content in each of the test tubes and observed for the formation of a blue-green color which indicates the presence of steroids or a reddish brown color at the interface which indicates the presence of terpenoids. (Harborne, 1998).

3.13 Data Quality Control

To ensure quality data, a prepared standard operating procedure (SOP) was adhered to in all stages of the experiment. All the culture media that were used were prepared strictly adhering to the manufacturer's instructions, and the sterility was checked such that only sterile media was used in the experiments. The old colonies of test microorganisms were subcultured to ensure they were viable and to obtain fresh 24- hour colonies for use in the experiments.

Pre-testing was done to check the viability and quality of pure cultures before they were used for the actual experiment. The incubators used were always cleaned and sterilized before use to prevent any contaminations during incubation. All the experiments were conducted in a biosafety chamber. The antibacterial assay experiments and phytochemical screening were all done in triplicates while antifungal tests were done in duplicates. All steps of every test were also recorded in detail and any issue that affected the processes was documented as described by National Committee for Clinical Laboratory Standards, (2011).

3.14 Data analysis

Data that included zones of inhibition, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration and Minimum Fungicidal Concentration were imported into the Microsoft excel 365 spreadsheet. Descriptive statistics was done and data expressed as mean values with standard deviation (± SD). Data analysis was conducted using Graphpad prism software (version 7). Two-way analysis of variance (ANOVA) with Benferroni's multiple comparison test was used to compare treatment means, with statistical

significance set at $P \leq 0.05$ and Tukey's post hoc tests for multiple comparison. The results are presented in forms of tables and graphs.

3.15 Ethical considerations

Ethical approval and permission to conduct the research was obtained from Masinde Muliro University of Science and Technology Review and Ethics Committee (MMUST/IREC/117/2023, Appendix I). Research permit was also obtained from The National Commission for Science Technology (NACOSTI) (NACOSTI/P/23/24316, Appendix I I).

CHAPTER FOUR

RESULTS

This chapter presents the study findings. It shows graphical representation of antibacterial activities of *Meliponula beccarii* and *Plebeina hildebrandti* honey samples. Furthermore, tables were used to represent MIC, MBC and qualitative phytochemical contents.

4.1 *In vitro* inhibition of bacterial growth by *Meliponula beccarii* honey samples

The antibacterial activity of *Meliponula beccarii* honey was determined by agar well diffusion and results expressed as mean diameter of growth-inhibition zones. *Meliponula beccarii* honey samples had significantly higher mean zones of inhibition against *E. coli* at 75% v/v ($p<0.0001$, N=11) and 100% v/v ($p<0.0001$, N=11), but not at 50% v/v ($p>0.05$ Figure 4.1 A) in comparison to distilled water (negative control) but a significantly lower mean zones of inhibition at all concentrations ($p<0.0001$, N=11, Figure 4.1A) compared to ciprofloxacin standard (30 ug/ml).

However, out of the 11 honey samples tested, only 9 had activity against *E. coli* at a concentration of 100% v/v with inhibition zones ranging from 8.67±0.58 mm to 14.33±0.58 mm. At 75% v/v, 7 honey samples exhibited antibacterial activity against *E. coli,* albeit with smaller zones of inhibition compared to 100% v/v. Only 3 honey samples had mild activity against *E. coli* at 50% v/v (Table 4.1).

When tested against *H. influenzae,* the honey samples exhibited significantly higher mean zones of inhibition compared to distilled water (negative control) at 50% v/v ($p<0.001$, N=11), 75% v/v ($p<0.0001$, N=11) and 100% v/v ($p<0.0001$, N=11) (Figure 4.1 B), but which was lower than ciprofloxacin standard ($p<0.0001$, N=11), Figure 4.1 B).

Importantly, the individual honey samples exhibited varying activity at the different concentrations. At concentration of 100% v/v, *H. influenzae* was susceptible to 10 out of the 11 honey samples. At a concentration of 75% v/v 9 honey samples exerted activity on *H. influenzae,* albeit with smaller zones of inhibition compared to 100% v/v and 7 honey samples had mild activity against *H. influenzae* at 50% v/v (Table 4.1).

Overall, *Meliponula beccarii* honey samples also showed antibacterial activity against *MRSA.* As shown in Figure 4.1 C the mean zone of inhibition of honey samples was significantly higher compared to distilled water (negative control) at the concentrations of 75% v/v ($p<0.001$, N=11) and 100% v/v ($p<0.0001$, N=11), but not at concentration of 50% v/v (5.2± 5.3 mm vs 0 mm). Similarly to *E.coli and H. influenzae*, the zone of inhibition of ciprofloxacin against *MRSA* was significantly higher than those of all the three honey sample concentrations (27mm vs 8.1±2.8 mm, Figure 4.1 C*)*.

Notably, individual honey samples exhibited varying zones of inhibition with 2 honey samples showing no activity against *MRSA* at concentration of 100 v/v (Table 4.1). At concentrations of 50% v/v and 75% v/v, 5 honey samples and 3 honey samples, respectively, also did not show any activity against *MRSA* (Table 4.1).

Table 4.1: Mean diameter of inhibition zones (mm) of individual 11 *Meliponula beccarii* honey samples evaluated by agar well diffusion method

		Zones of inhibition (mm) of test microorganisms±SD										
		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
E.coli	100%	14.33 (±0.58)	10.33(± 0.58)	10.67 (±0.58)	11.33 (±0.58)	9	11.33 (±0.58)	0	11	12	0	8.67(±0.58)
	75%	11	9	10	9.67(±0.58)	0	9.67 (±0.58)	0	8.67 (±0.58)	9.33 (±0.58)	0	0
	50%	9	0	0	7.33 (±0.58)	0	7.33 (±0.58)	0	0	0	0	0
	iprofloxacin 30 ug/m	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)	31.67 (±1.53)
MRSA	100%	16.33 (±0.58)	14.67 (± 2.89)	0	10.67 (± 0.58)	11.67 (± 0.58)	13.67(± 1.16)	0	11.67 (±0.58)	18.33(±5.77)	11.67(± 0.58)	9.67 (± 0.58)
	75%	19.00 (± 1.00)	11.33 (± 0.58)	0	8.67 (± 0.58)	9.33 (± 0.58)	11.00(±1.0 0)	0	-	15.67(± 5.51)	9.33(± 0.58)	7.67 (± 0.58)
	50%	11.67 (± 0.58)	9	0	0	7	9.33 (± 0.58)	0	-	13.67)±5.5 1)	7	0
	iprofloxacin 30 ug/m	27	27	27	27	27	27	27	27	27	27	27
HI	100%	20.333 (±0.58)	16.33(±0.57)	13.33 (±1.16)	11.67(±0.5 8)	10.67(±0.5 8)	10.67(± 0.58)	0	11.33(±0.5 8)	10.33(±0.5 8)	8.67 (± 0.58)	9.33 (± 0.58)
	75%	18.33 (±0.58)	14.67(±0.58)	11.333 (±1.16)	10.33(±0.5 8)	8.67(±0.58)	9.67(±0.58)	0	10	9	7.67(±0.58)	0
	50%	15.00 (±1.00)	13.00(±1.00)	0	9	7.33(±0.58)	8.67(±0.58)	0	9	7.33(±0.58)	0	0
	iprofloxacin 30 ug/m	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	24.67 (±0.58)

Mean zones of inhibition (mm) includes the well (6 mm), N=3; (0) = no clear zone of inhibition. Samples 1 and 2 were from Chemusu forest; samples 3 and 4 from Sabatia forest, sample 5 was from Narasha forest, sample 6 from Sigoro, all in Koibatek sub-county. Samples 7, 8, 9, 10 and 11 were collected from Katimok, Kipngochoch, Kapkomoi, Sacho Kaplel and Kituro respectively.

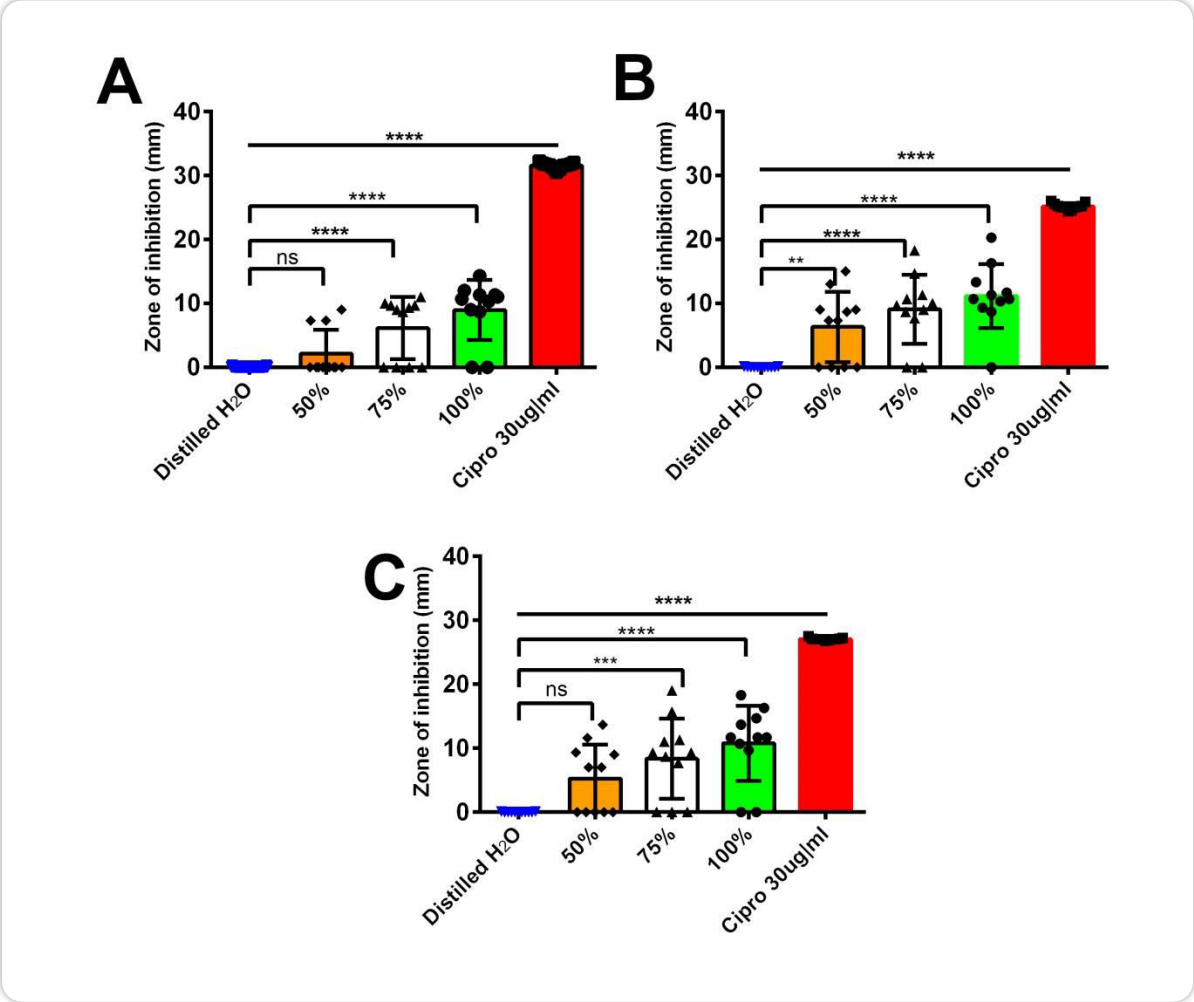


Figure 4.1: Antibacterial activities by increasing concentrations of *Meliponula beccarii* honey in agar well diffusion assay A) Shows concentration-dependent increase in the mean zone of inhibition of honey against *E.coli* **B)** Shows concentration-dependent increase in the mean zone of inhibition of honey against *H. influenzae*, **C)** Shows concentration-dependent in the mean zone of inhibition of honey against *MRSA* in comparison with ciprofloxacin positive control (30 ug/ml). Data was analysed by two-way ANOVA followed by Tukeys post hoc test and all data presented as mean±SD. Abbreviation, ns; not significant, cipro; ciprofloxacin, ug; microgram, ml; millilitres, **; $p<0.01$, ***; $P<0.001$, ****; $p<0.0001$.

4.2 *In vitro* inhibition of bacterial growth by *Plebeina hildebrandti* honey

The antibacterial activity of *Plebeina hildebrandti* honey was determined by agar well diffusion and results expressed as mean diameter of growth-inhibition zones. Compared to distilled water (negative control), *Plebeina hildebrandti* honey samples exhibited significantly higher mean zones of inhibition against *E. coli* at 50 % v/v (10±0.8 mm vs 0 mm, $p<0.0001$, N=7) and 75 % v/v (10.5±1.51 mm vs 0 mm, $p<0.0001$, N=7) and at 100% v/v (12.1±2.01 mm vs 0 mm, $p<0.0001$, N=7) Figure 4.2A).

Compared to ciprofloxacin standard (30 ug/ml) the honey samples exhibited significantly lower mean zones of inhibition at all concentrations (10.9±1.1 mm vs 30.26±0.8 mm, $p<0.0001$, N=7, Figure 4.2A). All the 7 honey samples tested, had activity against *E. coli* at a concentration of 100% v/v with inhibition zones ranging from 9.33±0.58 mm to 15 mm. At 75% v/v, all the 7 honey samples exhibited antibacterial activity against *E. coli,* albeit with smaller zones of inhibition compared to 100% v/v ranging from 9.00±1.0 mm to 13 mm.

Only 5 out of 7 honey samples had mild activity against *E. coli* at 50% v/v with inhibition zones ranging from 9.67±0.58mm to 11 mm (Table 4.2). When tested against *H. influenzae,* the honey samples exhibited significantly higher mean zones of inhibition compared to distilled water (negative control) at 50% v/v (8.1± 3.8 mm vs 0 mm, $p<0.0001$,N=7), 75% v/v (8.6±4.0 mm vs 0 mm, $p<0.0001$, N=7) and 100% v/v (10.1±4.8 mm vs 0 mm, $p<0.0001$, N=7) (Figure 4.2B), but which was lower than ciprofloxacin standard (9.0±1.05 mm vs 25.0±2.0 mm, $p<0.0001$, N=7), Figure 4.2B).Notably, *H. influenzae* was susceptible to 6 out of the 7 honey samples tested individually at all the concentrations (Table 4.2).

Plebeina hildebrandti honey samples also showed antibacterial activity against *MRSA.* As shown in Figure 4.2C the mean zone of inhibition of honey samples was significantly higher compared to distilled water (negative control) at the concentrations of 50% v/v (11± 2.6 mm vs 0 mm, $p<0.01$, N=7), 75% v/v (12.8±2.6 mm vs 0 mm, $p<0.001$, N=7) and 100% v/v (14.5±2.4 mm vs 0 mm, $p<0.0001$, N=7).

Similarly to *E.coli and H. influenzae*, the zone of inhibition of ciprofloxacin against *MRSA* was significantly higher than those of all the three honey sample concentrations (12.8±1.8 mm vs 26.0±0.7 mm, Figure 4.2C). Notably, individual honey samples exhibited varying zones of inhibition with all the 7 honey samples showing activity against *MRSA* at all the concentrations tested (Figure 4.2C and Table 4.2).

Table 4.2: Mean diameter of inhibition zones (mm) of individual seven (7) *Plebeina hildebrandti* honey samples evaluated by agar well diffusion method

		Zones of inhibition (mm)						
	Concentrations	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
	100%	11	15	13	12.3	14	9.3	10
	75%	10	13	11	11	11	9	9
	50%	9.7	11	10	10	11	0	0
E.coli	Ciprofloxacin 30 ug/ml	30	30	30.3	30	31	31.7	31.7
	100%	15	12	16	14	17	10.3	16.5
	75%	13	10	15	12.7	16	9.3	15.7
	50%	10	7	13	12	15	2.7	11.7
MRSA	Ciprofloxacin 30 ug/ml	25.7	25	25.7	25.7	25.7	27	27
	100%	11	13	12	10	15	9.6	0
	75%	10	12	10	10	10	8.7	0
	50%	9	11	10	10	10	7.3	0
H.influenzae	Ciprofloxacin 30 ug/ml	25.3	25	25	25	25	24.7	24.7

Mean zones of inhibition (mm) includes the well (6mm), N=3; (0) = no clear zone of inhibition. Samples 1, 2, 3, 4 and 5 were collected from Lepolos in Koibatek sub-county. Sample 6 was from Solian in Koibatek sub-county and sample 7 was from Sacho Kibereke in Baringo Central.

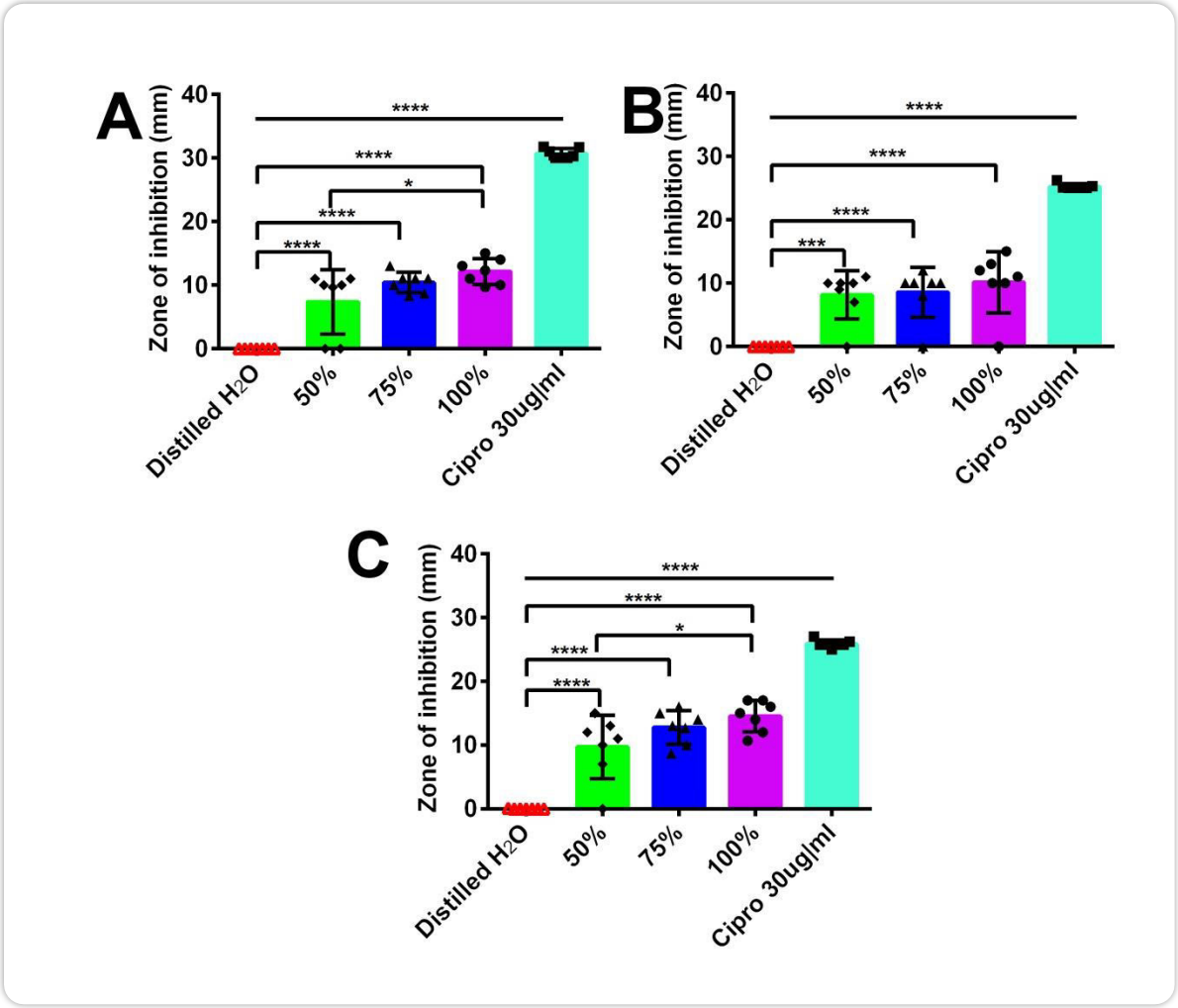


Figure 4.2: **Antibacterial activities by increasing concentrations of *Plebeina hildebrandti* honey in agar well diffusion assay A)** Shows concentration-dependent increase in the mean zone of inhibition of honey against *E.coli* **B)** Shows concentration-dependent increase in the mean zone of inhibition of honey against *H. influenzae*, **C)** Shows concentration-dependent increase in the mean zone of inhibition of honey against *MRSA* in comparison with ciprofloxacin positive control (30 ug/ml). Data was analysed by two -way ANOVA followed by Tukeys post hoc test and all data presented as mean±SD. Abbreviation, ns; not significant, cipro; ciprofloxacin, ug; microgram, ml; millilitres, *; $p<0.05$, ***; $P<0.001$, ****; $p<0.0001$.

4.3 Comparative in vitro inhibition of bacterial activity by *Meliponula beccarri* and *Plebeina hildebrandti* honey

The comparison of the mean zones of inhibition recorded against the three studied bacteria between the honey samples from the *Meliponula beccarri* and *Plebeina hildebrandti* at 50% v/v, 75% v/v and 100% v/v is presented in the Figures 4.3. At 50% v/v concentration, honey samples from the *Plebeina hildebrandti* recorded significantly larger mean zones of inhibition as compared to the honey samples from the *Meliponula beccarii* against *E.coli* and *MRSA* (10±0.8 mm vs 2.1 ±3.7 mm, $p<0.01$,) and (11±2.6 mm vs 2.1 ±3.7 mm $p<0.01$,) respectively (Figure 4.3 A).

However, at similar 50% v/v concentration the mean zones of inhibition recorded by the honey samples from both the *Meliponula beccarii* and *Plebeina hildebrandti* were not significantly different from each other against *H. influenzae* (Figure 4.3 A).At concentration levels of 75% v/v and 100 % v/v, the honey sample from both the *Meliponula beccarii* and *Plebeina hildebrandti* species recorded no significant difference in the mean zones of inhibition recorded against the three bacteria; *E.coli, H. influenzae* and *MRSA* respectively (Figure 4.3 B, and Figure 4.3 C).

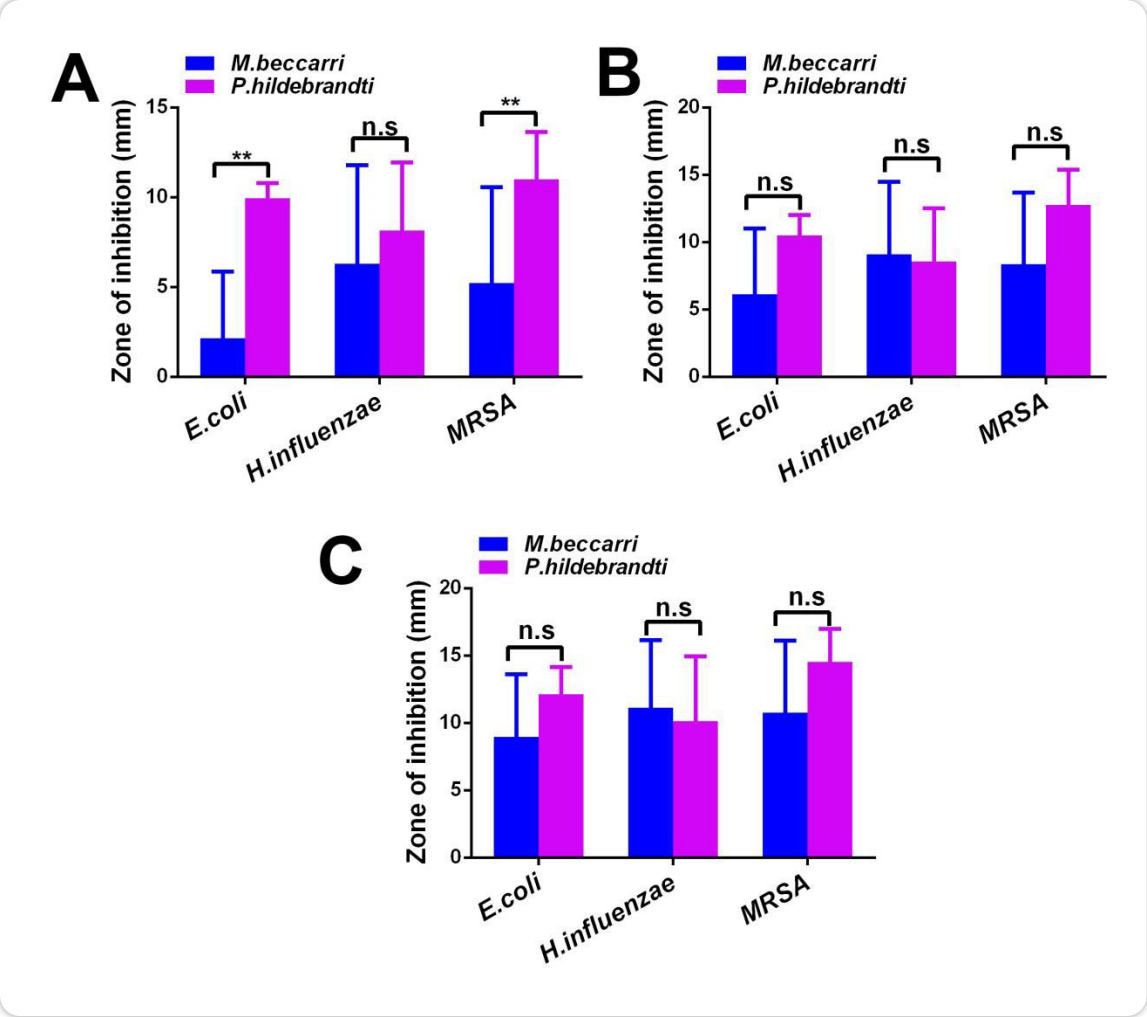


Figure 4.3: **Graphical representation for comparative zones of inhibition between *Meliponula beccarii* and *Plebeina hildebrandti* honey on different bacteria at different concentrations A)** Shows the mean zones of inhibition of honey against *E.coli, H. influenzae* and *MRSA* at concentration of 50 % v/v, **B)** Shows the mean zones of inhibition of honey against *E.coli, H. influenzae* and *MRSA* at concentration of 75 % v/v, **C)** Shows the mean zones of inhibition of honey against *E.coli, H. influenzae* and *MRSA* at concentration of 100 % v/v. Data was analysed by two -way ANOVA with Bonferroni's multiple comparisons and all data presented as mean±SD. Alpha set at 0.05. N=15. Abbreviation, ns; not significant, **; $P<0.01$.

4.4 *Meliponula beccarii* and *Plebeina hildebrandti* pollen exhibit no in vitro inhibition of bacterial growth

The antibacterial activities of *Meliponula beccarii and Plebeina hildebrandti* pollen (10% w/v) were determined by agar well diffusion and results expressed as mean diameter of growth-inhibition zones. The result shows that none of the 11 *Meliponula beccarii* pollen samples tested exhibited significant activity against *E. coli* (Figure 4.4A), *H.influenzae* (Figure 4.4B) and *MRSA* (Figure 4.4C). Similarly, all the 2 *Plebeina hildebrandti* pollen samples tested did not exhibit activity against *E. coli* (Figure 4.4D), *H .influenzae* (Figure 4.4E) and *MRSA* (Figure 4.4F).

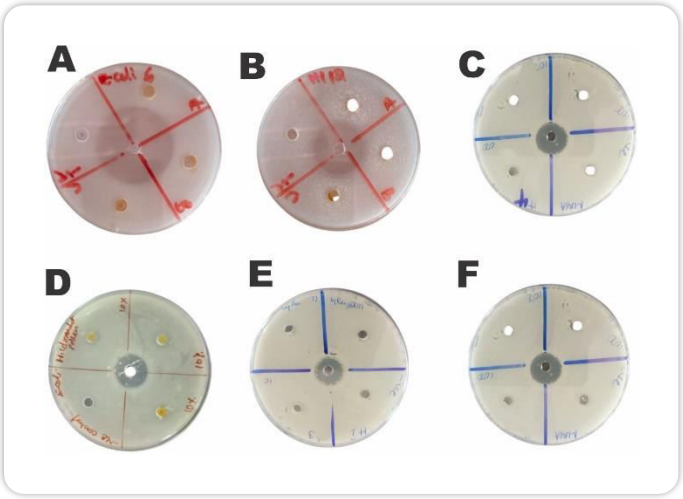


Figure 4.4: **Pollen of *Meliponula beccarii* exhibit no antibacterial activity in agar well diffusion assay.** **A)** Shows *Meliponula beccarii* pollen against *E. coli,* **B)** Shows *Meliponula beccarii* pollen against *H. influenzae*, **C)** Shows *Meliponula beccarii* pollen against *Methicillin resistant staphylococcus aureus* (*MRSA*).**D)** *Plebeina hildebrandti* pollen against *E. coli,* **E)** Shows *Plebeina hildebrandti* pollen against *H. influenzae*, **F)** Shows *Plebeina hildebrandti* pollen against *Methicillin resistant staphylococcus aureus* (*MRSA*) (–ve)-negative control. The clear zones of inhibition in the middle of the plates are for the positive controls. Letters A, B and C mean the same concentration of pollen in triplicates

4.5 *Meliponula beccarii* and *Plebeina hildebrandti* honey and pollen exhibit no activity against *C. albicans*

The antifungal activities of *Meliponula beccarii and Plebeina hildebrandti* honey (50%, 75% and 100%) and pollen (10% w/v) were determined by agar well diffusion and compared with nystatin (0.4mg/ml) and results expressed as mean diameter of growth-inhibition zones. Notably, the result shows that *C. albicans* was not susceptible to both *Meliponula beccarii* (Figure 4.5 A) and *Plebeina hildebrandti* honey (Figure 4.5 C) at all concentrations tested. Similarly, none of the *Meliponula beccarii* pollen samples (Figure 4.5B) and *Plebeina hildebrandti* pollen (Figure 4.5D) exhibited any activity against *C.albicans* at the concentrations tested.

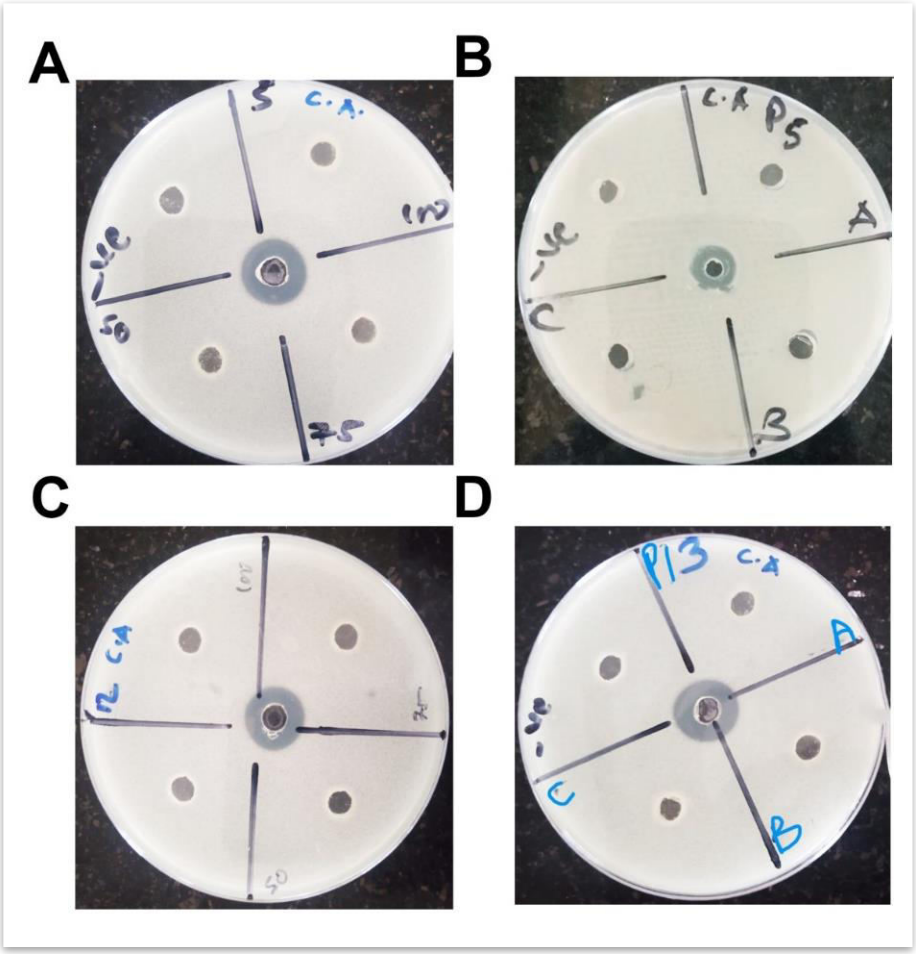


Figure 4.5: **Honey and pollen of both *Meliponula beccarii* and *Plebeina hildebrandti* exhibit no activity against *C. albicans* in agar well diffusion assay. A)** Shows zones of inhibition of *Meliponula beccarii* honey against *C. albicans* at increasing concentrations of 50%, 75% and 100% v/v**, B)** Shows zones of inhibition of *Meliponula beccarii* pollen against *C. albicans* at 10% w/v, **C)** Shows zones of inhibition of *Plebeina hildebrandti* honey against *C. albicans* at increasing concentrations of 50%,75% and 100% v/v, **D)** Shows zones of inhibition of *Plebeina hildebrandti* pollen against *C. albicans* at 10% w/v .(–ve)-negative control. The clear zones of inhibition in the middle of the plates are for the positive controls. Letters A, B and C mean the same concentration of pollen in triplicates

4.6 Minimum Inhibitory Concentration (MIC) of *Meliponula beccarii* and *Plebeina hildebrandti* honey

The MIC for the selected *Meliponula beccarii* honey against the three test bacteria was evaluated using broth microdilution technique in comparison with ciprofloxacin (0.6 ug/ml) as the standard and results expressed as means as shown in Table 4.3. The result revealed that while both *H influenzae* and *MRSA* had the highest MIC value of 18.75% v/v. *E.coli* had the lowest MIC value of 9.38 % v/v. The mean MIC of ciprofloxacin (positive control) was 0.075 µg /ml against *H. influenzae*. However, the tested concentrations of the positive control against *E.coli* and *MRSA* did not result in detectable MIC.

The MIC for the selected *Plebeina hildebrandti* honey against the three test bacteria was evaluated using broth microdilution technique in comparison with ciprofloxacin (15 µg /ml) as the standard and results expressed as means as shown in Table 4.4. While *MRSA* had the highest MIC value of 17.71% v/v, both *E.coli* and *H. influenzae* had the lowest MIC value of 13.75 % v/v. The mean MIC of ciprofloxacin (positive control) was 1.875 µg /ml against *MRSA* and *3.*75 µg /ml *against E.coli*. However, the tested concentrations of the positive control against *H. influenzae* did not result in detectable MIC.

Table 4.3 Mean MIC of *Meliponula beccarii* honey and ciprofloxacin (positive control)

Micro-organism	***Meliponula beccarii* honey (% v/v)**	**Ciprofloxacin (µg /ml)**
E.coli	9.38	ND
H. influenzae	18.75	0.075 µg /ml
MRSA	18.75	ND

N/D: bactericidal activity not detected

Table 4.4 Mean MIC of *Plebeina hildebrandti* honey and ciprofloxacin (positive control)

Micro-organism	***Plebeina hildebrandti* honey (% v/v)**	**Ciprofloxacin (µg /ml)**
E.coli	13.75	3.75
H. influenzae	13.75	N/D
MRSA	17.71	1.875

N/D: bactericidal activity not detected

4.7 Minimum Bactericidal Concentration (MBC) of *Meliponula beccarii* and *Plebeina hildebrandti* honey

Mean MBC for *Meliponula beccarii* honey against the three *E.coli, H.influenzae* and *MRSA* were as shown in Table 4.5. The result shows MBC of 60.94% (v/v) for the honey against *MRSA*. However, bactericidal effect on *E.coli and H. influenzae* at the specified tested concentrations was not detected. Ciprofloxacin standard showed MBC value of 0.3 µg /ml against *H. influenzae,* but bactericidal effect on *E.coli* and *MRSA* was not detected at the tested concentrations. Altogether, these findings show the bactericidal effect of *Meliponula beccarii* honey against *MRSA* only, which was not observed on against *E.coli and H. influenza*e.

On the other hand, the MBC of *Plebeina hildebrandti* honey against the test organisms are shown in Table 4.6. The results revealed that the mean MBC of *Plebeina hildebrandti* honey were 28 % v/v, 25 % v/v and 22.5 % v/v against *MRSA, H. influenzae* and *E. coli* respectively. However, out of the 5 honey samples tested against *H. influenzae*, one honey sample did not result in detectable bactericidal effect. Furthermore, 5 out of the 6 honey samples tested against *MRSA* resulted in detectable bactericidal effect. At the tested concentrations, ciprofloxacin (positive control) exhibited mean MBC values of 7.5 µg /ml for E.coli and *H. influenzae,* but no detectable bactericidal effect for *MRSA.* These results indicate that *Plebeina hildebrandti* honey is bactericidal against *E.coli, H. influenza*e and *MRSA*.

Table 4.5 Mean MBC of *Meliponula beccarii* honey and ciprofloxacin (positive control)

Micro-organism	***Meliponula beccarii* honey (% v/v)**	**Ciprofloxacin (µg /ml)**
E.coli	N/D	N/D
H. influenzae	N/D	0.3 µg /ml
MRSA	60.94	N/D

N/D: bactericidal activity not detected

Table 4.6 Mean MBC of *Plebeina hildebrandti* honey and ciprofloxacin (positive control)

Micro-organism	***Plebeina hildebrandti* honey (% v/v)**	**Ciprofloxacin (µg /ml)**
E.coli	22.5	7.5
H. influenzae	25	7.5
MRSA	28	N/D

N/D: bactericidal activity not detected

4.8 Phytochemical screening of *Meliponula beccarii* honey and pollen

Preliminary qualitative phytochemical screening of *Meliponula beccarii* honey samples revealed the presence of phenols, tanins, saponins, alkaloids, glycosides, steroids, flavonoids and triterpenoids. Notably, terpenoids were absent in all the analysed 11 honey samples (Table 4.7).

Table 4.7 Phytochemicals in honey samples from *Meliponula beccarii*

Phytochemical	Test	Honey samples and Inferences										
		1	2	3	4	5	6	7	8	9	10	11
Phenols	Ferric chloride	+	+	+	+	+	+	+	+	+	+	+
Tanins	Ferric chloride	+	+	+	+	+	+	+	+	+	+	+
Saponins	Foaming	+	+	+	+	+	+	+	+	+	+	+
Alkaloids	Dragendoff's	+	+	+	+	+	+	+	+	+	+	+
Glycosides	Modified Borntrager	+	+	+	+	+	+	+	+	+	+	+
Steroids	Salkowski's	+	+	+	+	+	+	+	+	+	+	+
Flavanoids	NaOH test	+	+	+	+	+	+	+	+	+	+	+
Triterpenoids	Liebermann-burchard	+	+	+	+	+	+	+	+	+	+	+
Terpenoids	Salkowski's	-	-	-	-	-	-	-	-	-	-	-

(+) presence; (-) absence; 1-11 represent individual honey samples. Samples 1 and 2 were from Chemususu forest; samples 3 and 4 from Sabatia forest, sample 5 was from Narasha forest, sample 6 from Sigoro, all in Koibatek sub-county. Samples 7, 8, 9, 10 and 11 were collected from Katimok, Kipngochoch, Kapkomoi, Sacho Kaplel and Kituro, respectively.

Similarly, to the honey samples, the screening of the *Meliponula beccarii* pollen samples showed the presence of phenols, tanins, saponins, alkaloids, glycosides, steroids, flavonoids and triterpenoids (Table 4.8). Notably, terpenoids were absent in all the analysed 11 pollen samples (Table 4.8).

Table 4.8 Phytochemicals in pollen samples from *Meliponula beccarii*

Phytochemical	Test	Pollen samples and Inferences										
		1	2	3	4	5	6	7	8	9	10	11
Phenols	Ferric chloride	+	+	+	+	+	+	+	+	+	+	+
Tanins	Ferric chloride	+	+	+	+	+	+	+	+	+	+	+
Saponins	Foaming	+	+	+	+	+	+	+	+	+	+	+
Alkaloids	Dragendoff's	+	+	+	+	+	+	+	+	+	+	+
Glycosides	Modified Borntrager	+	+	+	+	+	+	+	+	+	+	+
Steroids	Salkowski's	+	+	+	+	+	+	+	+	+	+	+
Flavanoids	Ammonia test	+	+	+	+	+	+	+	+	+	+	+
Triterpenoids	Liebermann-burchard	+	+	+	+	+	+	+	+	+	+	+
Terpenoids	Salkowski's	-	-	-	-	-	-	-	-	-	-	-

(+) presence; (-) absence; 1-11 represent individual honey samples. Samples 1 and 2 were from Chemususu forest; samples 3 and 4 from Sabatia forest, sample 5 was from Narasha forest, sample 6 from Sigoro, Samples 7, 8, 9, 10 and 11 were collected from Katimok, Kipngochoch, Kapkomoi, Sacho Kaplel and Kituro, respectively.

4.9 Phytochemical screening of *Plebeina hildebrandti* honey and pollen

Phytochemical screening of the *Plebeina hildebrandti* honey samples showed the presence of tanins, saponins, alkaloids, glycosides, steroids, flavonoids and triterpenoids. Notably, phenols were absent in all the first 5 honey samples analysed and terpenoids were absent in honey sample 6 and 7 (Table 4.9).

Table 4.9 Phytochemicals in honey samples from *Plebeina hildebrandti*

Phytochemical	Test	Honey samples and Inferences						
		1	2	3	4	5	6	7
Phenols	Ferric chloride	-	-	-	-	-	+	+
Tanins	Ferric chloride	+	+	+	+	+	+	+
Saponins	Foaming	+	+	+	+	+	+	+
Alkaloids	Dragendoff's	+	+	+	+	+	+	+
Glycosides	Modified Borntrager	+	+	+	+	+	+	+
Steroids	Salkowski's	+	+	+	+	+	+	+
Flavanoids	NaOH test	+	+	+	+	+	+	+
Triterpenoids	Liebermann-burchard	+	+	+	+	+	+	+
Terpenoids	Salkowski	+	+	+	+	+	-	-

(+) presence; (-) absence; 1-7 represent individual honey samples. Samples 1,2,3,4,5 were from Lepolos in Koibatek sub-county, sample 6 was from Solian in Koibatek and sample 7 was from Sacho Kibereke in Baringo Central.

On the other hand, preliminary screening of two *Plebeina hildebrandti* pollen samples showed the presence of phenols, tanins, saponins, alkaloids, glycosides, steroids, flavonoids and triterpenoids. Terpenoids were absent in both pollen samples (Table 4.10).

Table 4.10 Phytochemicals in *Plebeina hildebrandti* pollen

Phytochemical	Test	Pollen samples and Inferences	
		6	7
Phenols	Ferric chloride	+	+
Tanins	Ferric chloride	+	+
Saponins	Foaming	+	+
Alkaloids	Dragendoff's	+	+
Glycosides	Modified borntrager	+	+
Steroids	Salkowski's	+	+
Flavanoids	Ammonia	+	+
Triterpenoids	Liebermann burchard	+	+
Terpenoids	Salkowski	-	-

(+) presence; (-) absence; 6-7 represent individual honey samples. Sample 6 and 7 were from Solian in Koibatek and Sacho Kibereke in Baringo Central respectively.

CHAPTER FIVE

DISCUSSION OF THE RESULTS

The results of the in vitro inhibition of microbial growth, minimum inhibitory concentration, minimum bactericidal concentration and phytochemical composition of *Meliponula beccarii* and *Plebeina hildebrandti* stingless bee honey and pollen are interpreted and discussed in this chapter in comparison with previous studies.

5.1 *In vitro* inhibition of bacterial and fungal growth by *Meliponula beccarii* and *Plebeina hildebrandti* honey and pollen samples from Baringo County

Stingless bees *Meliponula beccarii* and *Plebeina hildebrandti* stingless bees of Baringo , Kenya, produce honey which is valued for its ethnomedicinal uses in the treatment of various health conditions associated with bacterial and fungal infections (Kiprono *et al*., 2022a; Kiprono *et al*., 2022b).The subterranean-nesting stingless bees also produce pollen whose antimicrobial activities have not been investigated. This study explored the *in vitro* antibacterial, antifungal properties and screened for phytochemical composition of *Meliponula beccarii* and *Plebeina hildebrandti* nest products.

All of the *Meliponula beccarii* honey tested except two, at concentration of 100% v/v exhibited significant activity against *MRSA*, *E.coli* and *H. influenzae* as demonstrated by zone of inhibition with observed ranges from 8.67±0.58 mm to 20.33±0.58 mm (Figure 4.1 A-C). Similar to *Meliponula beccarii* honey, all the *Plebeina hildebrandti* honey samples also had inhibitory effects against *MRSA* and *E.coli* at 100 % volume / volume except one sample whose activity against *H. influenzae* was not significant (Figure 4.2 A-C).

These results agree with Wavinya *et al*., (2021) who demonstrated activity of *Plebenia hylderbrandii* and *Meliponula bocandei* honey of Western Kenya against *E. coli* and *S. aureus*. In Malaysia, honey produced by *Heterotrigona itama* species (Hasali *et al*., 2018) and *Geniotrigona thoracica* species (Nuratiqah *et al*., 2020; Tuksitha *et al*., 2018) have been shown to have antimicrobial effect. In addition, the antimicrobial properties of *Tetragonula laeviceps* honey from Thai have been demonstrated (Khongkwanmueang *et al*., 2020).

Previous studies attributed the antibacterial activities of honey to phenol and flavonoid composition (Tuksitha *et al*., 2018). Therefore, the antibacterial activity of *Meliponula beccarii* and *Plebeina hildebrandti* honeys may be linked to their phenol and flavonoid contents (Table 4.7 and Table 4.9).

The results show increased zones of inhibition with increasing concentrations of *Meliponula beccarii* honey (Figure 4.1 A-C and Table 4.1) and *Plebeina hildebrandti* honey (Figure 4.2 A-C and Table 4.2). In this regard, it is possible that the antibacterial activities of the honey samples is agent specific or dependent on the concentration of the bioactive agent.

Some honey samples from both species did not show antibacterial activity even at concentration of 100% v/v (Table 4.1 and Table 4.2) with up to two *Meliponula beccarii* honey against *E.coli* and *MRSA* and one *Plebeina hildebrandti* honey against *H.influenzae*. It is hypothesized that the active antibacterial phytochemicals in such agents are absent or are available in lesser amounts than the observed minimum inhibitory concentrations.

These findings disagree with those reported in a previous study on *Dactylurina schimidti* species from the Kenya coastal region which exhibited bacteriostatic effect on both *S. aureus* and *E. coli* (Muli *et al*., 2008). The difference can be ascribed to difference in stingless bee species and floral sources of nectar in different geographical regions and ecological zones which majorly determine honey's bioactive molecules (Ávila *et al*., 2019; Biluca *et al*., 2016).

Furthermore, this study shows no antifungal activity against *C. albicans* by honey from *Meliponula beccarii* (Figure 4.5 A and B) and *Plebeina hildebrandti* (Figure 4.5 C and D) stingless bee species. These findings are contrary to the report of ethno medicinal study in Baringo County which revealed that honey from the subterranean nesting stingless bees is widely utilized by the community for managing oral thrush (Kiprono *et al*., 2022a; Kiprono *et al*., 2022b). Several other previous studies on different stingless bee honeys from various regions have reported antifungal activities against *C. albicans* contrary to the findings of this study. For example, antifungal activities of *Melipona beechei* (Hau-Yama *et al*., 2020) and *Kelelut* of *Trigona* (Hamid *et al*., 2018) against *C.albicans* have been reported.

Moreover, various stingless bee species of Tanzania have demonstrated antifungal effect on *C.albicans* (Mduda *et al*., 2023). In addition,various Brazillian stingless bee species produce honey which have shown activity on *C.albicans* (Ávila *et al*., 2019). This difference could be due to variation in species and geographical regions which determine the phytochemistry honey *(Ávila et al*., 2019; Nordin *et al*., 2018).

Stingless bee pollen is a valuable therapeutic product, normally made from plant pollen, nectar and bee secretions. Previous studies have correlated antibacterial effects of pollen with their phenol contents (Sulbarán *et al*., 2018). However, despite having similar

phytochemical profile as the honey samples, none of the *Meliponula beccarii* and *Plebeina hildebrandti* pollen investigated herein showed no activity against *E.coli, H. influenzae* and *MRSA* (Figure 4.4 A-F) or activity against *C. albicans* (Figure 4.5 B and D).

These findings concur with those of a previous study on Brazillian *Melipona compressipes Manaosensis* pollen which was shown to lack activity against *S.aureus* and *E.coli* (Carneiro *et al*., 2019). Findings of another previous study on *Frieseomelitta, Tetragonisca* and *Melipona* stingless bee species pollen extracts reported that it lacked antibacterial activity against *E.coli* (Sulbarán-Mora *et al*., 2018).

Several other research work done contradicted the findings in this study. For example, *Melipona compressipes Manaosensis* bee pollen of Brazille was shown to be antibacterial against *P.aeruginosa* and *M.smegmatis* as well as *C.albicans* (Carneiro *et al*., 2019). Moreover, *Tetragonula carbonaria, Tetragonula hockingsi* and *Austroplebeia australi,* of Australia extracted using ethanol and methanol worked effectively on *E.cloacae, E.coli, S.aureus, P.aeuruginosa* and *B.subtilis* (Pérez-Pérez *et al*., 2018).

Venezuelan *Frieseomelitta, Tetragonisca* and *Melipona* stingless bee species glycolic pollen extracts demonstrated activity against *B.subtilis, E.cloacae, S.aureus* and *P.aeruginosa* (Sulbarán-Mora *et al*., 2018). In addition, *S.aureus* and *B.cereus* were susceptible to *Heterotrigona itama* pollen (Akhir *et al*., 2017).

This study suggests the possibility that the bioactivity of honey and pollen might be affected as they undergo differential processing and storage after their collection (Ávila *et al*., 2019). Furthermore, the antimicrobial activity might be dependent on specific bioactive

compound and its concentration such that lack of them in pollen or their presence in very low concentrations compared to honey cannot be effective against the test microorganisms.

5.2 Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) of *Meliponula Beccarii* and *Plebeina hildebrandti* honey

Meliponula beccarii honey samples showed the lowest MIC of 9.38% v/v against *E.coli* compared to *H.influenzae* and *MRSA*, which exhibited almost twice higher MIC of 18.75% v/v, (Table 4.3). Similarly, *Plebeina hildebrandti* honey exhibited inhibition against *E.coli* and *H.influenzae* at the minimum concentration of 13.75 % v/v compared to 17.71% v/v for *MRSA* (Table 4.4). These findings indicate that gram negative bacteria are more susceptible to honey from both species than gram positives.

These findings oppose several previous studies that have shown higher sensitivity of stingless bee honey on gram positive bacteria than gram negative bacteria. Higher sensitivity of Gram-positive bacteria than Gram-negative bacteria to *M. scutellaris Latrelle* and *M. subnitida Ducke* honey have also been demonstrated (Sousa *et al*., 2016). Several studies have reported *S. aureus* as the most susceptible bacteria to stingless bee honey. For example, Costa Rican *Tetragonisca angustula* and *Melipona beechei* honey have been found to inhibit *S. aureus* (Zamora *et al*., 2014). Moreover, another study on *Kelelut* honey of Malaysia showed that it inhibited *S. aureus* (Zainol *et al*., 2013).

In addition, *Plebeina hildebrandti* honey from Western Kenya inhibited *S. aureus* and *E. coli* whereas *Meliponula bocandei* honey effectively inhibited *S. aureus*, but not *E. coli* (Wavinya *et al*., 2021). *MRSA* exhibited higher MIC value of 18.75 % v/v with *Meliponula beccarii* honey compared to 17.71% v/v with *Plebeina hildebrandti* honey (Table 4.3 and 4.4).

Among the gram negative bacteria, *Meliponula beccarii* honey had MIC values ranging from 9.38% v/v to 18.75 % v/v while *Plebeina hildebrandti* honey had MIC values ranging from 13.75% v/v to 17.71% v/v (Table 4.3 and 4.4). Comparatively, a study on individual and *Scaptotrigona postica* and *Scaptotrigona bipunctata* honeys revealed higher MIC values (2.5-10%) with the gram negative bacteria than among the gram positive bacteria whose MIC was between 0.63-10% (Nishio *et al*., 2016).

Meliponula beccarii honey exhibited bactericidal effect against *MRSA* at 60.94% v/v which is comparatively higher but no cidal effect on and *H. influenzae* and *E. coli* (Table 4.5). *Plebeina hildebrandti* honey was bactericidal on *E.coli, H.influenzae* and *MRSA* at much lower concentrations that is; 22.5% v/v, 25% v/v and 28% v/v respectively (Table 4.6). Generally, these findings indicate that honey from both *Meliponula beccarii* and *Plebeina hildebrandti* from Baringo have a greater bactericidal effect against *MRSA* compared to *E. coli* and *H. influenzae*.

Wavinya *et al*., (2021) similarly demonstrated a lower bactericidal activity of honey produced by *Plebenia hylderbrandii* and *Meliponula bocandei* on *E. coli* compared to *S. aureus*. These findings suggest the potential of *Meliponula beccarii* and *Plebeina hildebrandti* honey to become an antibacterial agent against *MRSA* which is known as a threat to public health development in recent times. Moreover, these results show that *Plebeina hildebrandti* honey could contain novel bactericidal compounds against *E.coli* and *H.influenzae*. All these suggest further investigations to isolate and test the potential bioactive compounds in these honey samples individually on various antibiotic resistant pathogens.

5.3 Phytochemical contents of *Meliponula beccarii* and *Plebeina hildebrandti* honey and pollen

The biological activities of honey from stingless bees can be justified by its phytochemical composition. This study conducted the first qualitative phytochemical screening of *Plebeina hildebrandti* as well as *Meliponula beccarii* products in Kenya. The findings reveal that *Meliponula beccarii* honey and pollen are both rich in alkaloids, steroids, phenols, flavonoids, triterpenoids, tannins, saponins and glycosides but not terpenoids.

5 out of 7 *Plebeina hildebrandti* honey and pollen contained alkaloids, steroids, terpenoids, flavonoids, triterpenoids, tannins, saponins and glycosides but lacked phenols while 2 samples from Solian in Koibatek subcounty and Sacho Kibereke in Baringo Central had alkaloids, steroids, phenols, flavonoids, triterpenoids, tannins, saponins and glycosides but not terpenoids. These findings indicate that the honey and pollen from the two species are important natural sources of bioactive compounds. The composition of honey is dependent on several factors including geographical variations, floral sources around the bees, different bee species and climatic differences (Ávila *et al*., 2018).

The presence of phenols and flavonoids in honey of *Meliponula beccarii* and *Plebeina hildebrandti* contain flavonoids and phenols which concur with the findings of several studies done on honey from stingless bee species from different geographical regions of the world. For example, several Indonesian stingless bees in East and Northern Kalimantan were reported to produce honey containing tannins, alkaloids, flavonoids, triterpenoids and saponins but not steroids (Syafrizal *et al*., 2020).

The Brazillian *Melipona bicolor, Melipona quadrifasciata, Melipona marginata,* and *Scaptotrigona bipunctata* stingless bee honeys were also reported to have flavonoids,

tannins and phenolics (Ávila *et al*., 2019). Furthermore, honey from *Melipona marginata, Scaptotrigona bipunctata, Tetragonisca angustula, Tetragona clavipes, Melipona quadrifasciata* and *Trigona hypogea* stingless bees were found to be rich in phenols and flavonoids (Biluca *et al*., 2020). Phenols and flavonoids were reported in Malaysian *Geniotrigona thoracica* and *Heterotrigona itama* stingless bees (Bakar *et al*., 2017).

Peru's stingless bees *Melipona crinita, Melipona grandis, Nannotrigona melanocera, Melipona eburnea, Partamona epiphytophila, Melipona illota, Ptilotrigona lurida, Tetragonisca angustula Scaura latitarsis,* and *Scaptotrigona postica* were reported to contain phenolics and flavonoids (Rodríguez *et al*., 2009). Saponins, phenols and flavonoids were found in *Trigona itama* bees rubber honey but not alkaloids, tannins, triterpenoids, and steroids (Adalina *et al.*, 2020).

These results agree with Indonesian's *Tetragonula laeviceps* stingless bee honey which was found to have alkaloids, steroids, triterpenoids, phenolics, saponins, tannins (Hariani *et al*., 2022). Flavonoids, tannins and glycosides in *Meliponula bocandei* and *Plebenia hylderbrandii* honey of Kakamega forest (Wavinya *et al*.,2021).

The findings of qualitative phytochemistry of *Meliponula beccarii* and *Plebeina hildebrandti* stingless bee pollen are consistent with those of 8 Philippine *Tetragonula biro Friese* stingless bee pollen which were reported to contain phenolics, flavonoids and phytosterols (Belina-Aldemita *et al*., 2020a). Bárbara *et al*., (2015) also demonstrated that Brazil's *Melipona mandacaia* pollen is rich in flavonoid and phenol contents. Furthermore, Carneiro *et al*., (2019) found polyphenols, tanins, saponins and flavonoids in methanol extracts of Brazillian stingless bee pollen.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

i. *Meliponula beccarii* and *Plebeina hildebrandti* honey from Baringo have *in vitro* activity against *H.influenzae*, *MRSA* and *E.coli* but no activity was observed against *C.albicans*.

ii. The inhibitory effect of *Meliponula beccarii* honey is apparent at much lower concentration of 9.38% v/v on *E.coli* and at much higher concentrations of 18.75% v/v on *MRSA* and *H.influenzae*. The MIC of *Plebeina hildebrandti* honey on *E.coli* and *H. influenzae* is lower (13.75% v/v) compared to *MRSA* (17.71% v/v)

Meliponula beccarii honey exerts bactericidal effect on *MRSA* only at 60.94% v/v. *Plebeina hildebrandti* honey is bactericidal against *H.influenzae, E.coli,* and *MRSA* at 25% v/v, 22.5% v/v and 28% v/v respectively.

iii. *Meliponula beccarii and Plebeina hildebrandti* products particularly pollen and honey contain various classes of phytochemicals, which can be further investigated for the discovery of natural antibacterial agents especially against *MRSA, E.coli* and *H.influenzae*

6.2 RECOMMENDATIONS

6.2.1 Recommendations for action

The honey of *Meliponula beccarii* and *Plebeina hildebrandti* from Baringo may be utilized as alternative drug for treatment of diseases associated with bacterial infections and especially *E.coli, H.influenzae* and *MRSA* infections as claimed by the natives, traditional medicine practitioners and confirmed by this study findings.

The honey and pollen of *Meliponula beccarii* and *Plebeina hildebrandti* from Baringo can be sources of valuable phytochemicals.

6.2.2 Recommendation for further research

Meliponula beccarii and *Plebeina hildebrandti* honey should be tested in animal models with *MRSA* infections and be formulated directly for use in *MRSA* infections.

In addition, potential novel antibacterial compounds in the investigated *Meliponula beccarii* and *Plebeina hildebrandti* honey samples should be isolated, characterized and tested individually on various microorganisms.

Investigation of the mechanism of action of active compounds in *Meliponula beccarii* and *Plebeina hildebrandti* honey responsible for the observed activity against MRSA in future.

Testing the effect of the honey on more pathogenic fungal strains should be considered.

Physicochemical properties including pH and osmolality should be investigated in future studies.

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APPENDICES

Appendix I: MMUST ISERC Approval letter

MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY
Tel: 056-31375
Fax: 056-30153
E-mail: ierc@mmust.ac.ke
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P. O. Box 190,
50100.
Kakamega,
KENYA

Institutional Scientific and Ethics Review Committee (ISERC)

REF: MMU/COR: 403012 Vol 6 (01) Date: February 17th, 2023

To: Christine Chepkemoi

Dear Ms.,

RE: ASSESSMENT OF ANTIMICROBIAL EFFECTS AND PHYTOCHEMICAL CONTENT OF SUB-TERENEAN-NESTING STINGLESS BEE PRODUCTS FROM BARINGO COUNTY.

This is to inform you that the ***Masinde Muliro University of Science and Technology Institutional Scientific and Ethics Review Committee (MMUST-ISERC)*** has reviewed and approved your above research proposal. Your application approval number is **MMUST/IERC/117/2023.** The approval covers for the period ***February 17th, 2023 to February 17th, 2024.***

This approval is subject to compliance with the following requirements;

i. Only approved documents including informed consents, study instruments, MTA will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by ***MMUST-ISERC.***
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to ***MMUST-ISERC*** within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to ***MMUST-ISERC*** within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to ***MMUST-ISERC.***

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://research-portal.nacosti.go.ke and also obtain other clearances needed

Yours Sincerely,

Prof. Gordon Nguka (PhD)
Chairperson, Institutional Scientific and Ethics Review Committee

Copy to:
- The Secretary, National Bio-Ethics Committee
- Vice Chancellor
- DVC (PR&I)

Appendix II: Approval letter from NACOSTI

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 474241

Date of Issue: 04/April/2023

RESEARCH LICENSE



This is to Certify that Miss.. Chepkemoi christine Christine of Masinde Muliro University of Science and Technology, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Baringo on the topic: Assessment of Antimicrobial Effects and Phytochemical Content of Sub-tereneau-nesting Stingless Bee Products From Baringo County for the period ending : 04/April/2024.

License No: NACOSTI/P/23/24316

474241

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

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See overleaf for conditions

Appendix III: Map of study area

