# ANTIBACTERIAL ACTIVITIES OF *APIS* AND *MELIPONIN* HONEY FROM MARIGAT AGAINST ISOLATED WOUND BACTERIAL STRAINS AMONG PATIENTS ATTENDING NAKURU CTRH

BY

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This thesis is like a celebration and commemoration that marks an account of a great accomplishment in the journey of my procession of academic history. I hereby splendidly send it out as a noble and timeless masterpiece, hoping that many people will find it of much practical utility.

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# ABSTRACT

Wound or burn infections refer to the colonization and invasion of bacteria in human skin or the underlying soft tissues in the body that ignite an immune response and lead to inflammation and injury of tissues. This study sought to establish the efficacy of natural honey produced from Baringo County *Apis* and *Meliponines* bees against some isolated and characterized microorganisms from 34 samples obtained from wound exudate from adult outpatients and surgical wards of The Nakuru County Referral Hospital. Honey samples were purchased from various known beekeepers in Marigat, Baringo County, and their initial qualitative, quantitative and proximate contents were determined using standard procedures. The targeted patients in this experimental study design attended The Nakuru County Referral Hospital with a mean ± SD age of 38.38 ± 16.88, utilizing a self-administered questionnaire to collect data and consent. According to Smith (2013), the value of 'n' sample size was 34 in the sampling technique. The sample size in honey (n=26) was determined according to Okur et al. (2020). The data from this study was analyzed using the IBM SPSS (Statistical Package for Social Sciences) statistics 23 and the variables were analyzed which were found to have a very highly significant difference (P< 0.005) through two-way ANOVA analysis using the Graph pad prism (version 7. The means were then compared using a parametric difference test–the T-test. These honey samples collected showed bacteriostatic and bactericidal effects compared to conventional antibiotics against the identified wound isolates by disc diffusion method. In the present study, *Staphylococcus aureus* was the most frequently isolated bacteria, with 15 (34.8%), followed by *Pseudomonas aeruginosa* – 12 (27.9%), *Klebsiella pneumoniae* – 10 (23.3%), and *Escherichia coli* – 6 (14.0%).

Additional identification tests of the isolates involved the amplification of virulence factors genes encoded by specific primers that were *Staphylococcus aureus* (16Rrna and hla genes), *Pseudomonas aeruginosa* (gyrB and lasI genes), *Klebsiella pneumoniae* (magA and rmpA genes) and *Escherichia coli* (cnf1 and hlyA genes). The stingless bee honey provided mean values of 89.85 ± 5. 07g/100g, 3.86 ± 0.11 and 81.75 ± 10.35mg/g for sugar, pH and moisture, respectively, compared to the honeybee honey, which indicated 90.13 ± 5.76g/100g, 4.07 ± 0.08 and 114.28 ± 26.66 mg/g respectively. The phenolic compounds that acted as antioxidants were in the mean value of total phenolic compounds (92.18 ± 51.20mgGAE/100g), total flavonoids (23.70 ± 5.87 mg RE/100g) and total carotenoids (6.57 ± 0.21 mgβ–carotene/kg) for stingless bee honey compared to honeybee 81 ± 36.25mgGAE/100g, 21.83 ± 6. 16mgRE/100g and 4.41 ± 2.07mgβ –carotene/kg respectively. The isolates showed a differing level of resistance to honey-incorporated discs ($10 \times 10^4$, $20 \times 10^4$, $50 \times 10^4$ and $75 \times 10^4$ µg/ml) with a mean zone of inhibition of 18.23 ± 0.4mm (*Staphylococcus aureus*), 17.49 ± 0.3mm (*Pseudomonas aeruginosa*), 16.05 ± 0.6mm (*Klebsiella pneumoniae*) and 10.19 ± 0.5mm (*Escherichia coli*) with a mean range of 14.54 ± 2.0mm to 17.58 ± 3mm and 10.81 ± 2.5mm to 19.4 ± 4.3mm for both honeybee and stingless bee honey respectively. The mean inhibition diameters of the used antibiotics (Gentamycin, Levofloxacin, Ampicillin, Tazobactum, Meropenem and Chloramphenicol) were 17.6 ± 0.5mm (*Staphylococcus aureus*), 8.9 ± 0.2mm (*Pseudomonas aeruginosa*),

9.3 ± 0.2mm (*Klebsiella pneumoniae)* and 11.5 ± 0.3mm (*Escherichia coli*). Hence, the high prevalence of reduced antibiotic susceptibility amongst most bacterial wound isolates implies that better strategies should be deployed to improve wound treatment and healing.

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# ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| µg | Microgram |
| µl | Microlitre |
| µm | Micrometre |
| $^{0}C$ | Degrees centigrade |
| $^{0}F$ | Degrees Fahrenheit |
| AHL | Acyl-homoserine lactones |
| $ALCl_3$ | Aluminium chloride |
| AMP | Antimicrobial peptide |
| AMR | Antimicrobial resistance |
| AOAC | Association of Analytical Communities |
| CFU | Colony forming unit |
| CoNS | Coagulase negative staphylococcus |
| HIA | Health – care – associated infection |
| KM | Kilometre |
| LOD | Limit of detection |
| MGO | Methylglyoxal |
| MIC | Minimum inhibition coefficient |
| Mmol | Mill moles |
| Mmol/L | Millimoles per Litre |
| $NaNO_3$ | Sodium nitrate |

| | |
|---|---|
| $NaHSO_3$ | Sodium sulphite |
| NaOH | Sodium hydroxide |
| OPrF | Outer membrane protein F |
| pH | Numeric scale specifying the acidity or basicity of an aqueous solution |
| Poise | Unit of dynamic viscosity (p= 0.100kg) |
| ROS | Reactive oxygen species |
| Spp. | Species |
| USDA | U.S. department of agriculture |
| WHO | World Health Organization |

# CHAPTER ONE

# 1.0 INTRODUCTION

## 1.1 Background

Disorders in the configuration and purpose of the skin and the soft tissue architecture are referred to as wounds. Wounds are broadly categorized into acute wounds demonstrating normal physiology and healing anticipated to progress through expected stages, as well as chronic wounds that are physiologically impaired. The wound healing process is well-organized, with potential impediments such as systemic (host) nutrition and local factors like tissue perfusion affecting it. Mainly, open wounds are managed for several days or months until they can be sealed or healed by secondary intent (Al badiri, 2019).

However, microbial contamination leads to delayed wound healing, commonly progressing to colonization, localized sepsis and systemic infections, and multi-organ dysfunction. It may finally lead to life and limb-threatening infections (Gomes et al., 2021). According to Wu et al. (2019), the actual determination of infection in deep wound tissue is the presence of biofilms responsible for stimulating nitric oxide, free radicals and inflammatory cytokines which contribute to keeping the wound in a prolonged inflammation stage. These are formed in a key process known as quorum sensing through specific molecules called auto-inducers ($AI_s$) identified in many species of bacteria depending on the particular species. Hence, to avoid the problem of increased antibacterial resistance to antibiotics, proper wound bed preparations through debridement and antiseptics

are paramount.

Though naturally wounds are known to heal by themselves, the ancient people identified the presence of certain factors and herbal remedies that would speed up or assist the process if undertaken with the necessary hygiene to halt bleeding, leading to the development of wound dressing techniques and surgery (Gwarzo et al., 2022). Among the discovered treatments of wounds by human beings was honey usage, which can be traced back to almost 8000 years past, as faceted by figurines ten thousand years old, as portrayed in the paintings of the Stone Age. Citronella is also mentioned 500 times and more in 900 Egyptian remedies, and it is one of the most common commodities (used in all its medicines is milk and wine) 90% (Cilia et al., 2021).

According to Cebrero et al. (2020), the first antibacterial activity of honey writing was done by Van Ketel in 1892. Sackett then followed in 1919 and found that honey' s antibacterial potency increased with limited dilution. Dold et al. (1937) also contributed to the development of the literature on the antibacterial activity of honey, which led to the scientific name ‘inhibine’ being coined in this area; this term is widely used and applied.

Subsequently, volatile organic compounds (VOCs) of all four Thai kinds of multifloral honey and one Thai multifloral honey collected from different bees were analyzed further and these analyses showed that the concentrations and composition of honey’s volatiles depend on floral source and honey bee species (Tsuruda et al., 2021). The Variety of flower plants the bees feed on, the composition of vegetation and ecological conditions in different areas determine

the chemical structure and the main components of the honey produced (Azonwade et al., 2018).

Another study (Bordoloi et al., 2023) carried out in India on the antiseptic action of methanol, ethanol as well as ethyl acetate mines of both raw and refined honey alongside equally Gram-positive in addition to Gram-negative bacteria indicated both kinds of honey did possess antibacterial properties. However, the methanol extract indicated a more potent activity. At the same time, Gram-negative bacteria showed more susceptibility, a great finding since these bacteria groups seem hard to control due to their cell wall properties (Wadi, 2019).

Also, in another study (Anand et al., 2019) carried out between March 2005 and May 2007, honey samples from natural and exotic plants composed from several Australian provinces stayed in lieu of their antibacterial action using established protocols. A honey activity that may be therapeutic had an incidence of 57%. The antibacterial activity was usually attributed to bee-derived enzyme glucose oxidase hydrogen peroxide. According to Ayurvedic experts (Indian system), honey has also been regarded as valuable in dental health and the treatment of insomnia. It was also used in skin disorders (burns and wounds), cardiac pain, palpation, lung imbalances, anaemia and various eye ailments for improving eyesight and treating cataracts (Naik et al., 2021).

One of the important health problems is respiratory tract infections due to their high incidence and economic costs. Still, the WHO recognizes honey as a potential demulcent management for cough. This is a protective reflex action elicited by

irritation or blockage of the airways. The surplus secretions and foreign bodies are cleared from the lungs by an amalgamation of coughing and the mucociliary escalator (Naveed et al., 2018). It has also been documented to be vigorous against bacteria commonly found in the upper respiratory tract and its sweetness causes reflex salivation, which triggers the assembly of airway mucus, leading to a demulcent consequence on the pharynx and larynx, thus reducing coughs (WHO, 2001).

Among many more studies/documentation, these indicate that honey can be a potential antimicrobial agent. Still, data needs to be generated in a local setup as honey content varies from one geographical location to another and the type of bee involved in making the honey. With the rise in antimicrobial resistance patterns globally, honey could offer alternative Medicare to some pathogens. This is because it is clear that honey has various conditions that it can manage, so the need for scientific validation of its properties is inevitable. Therefore, it's against this background that this study was based as such data is missing locally. However, much more information can be mined from literature based on studies conducted in other world regions.

## 1.2 Statement of the Problem

There is a considerable increase in antibiotic-resistant microorganisms mainly due to antibiotics and misuse, a reduced number of manufacturing companies, and the development of new ones drastically reduced (Mama et al., 2019). Additionally, the drugs in use have been proven to have different side effects, such as toxicities, nausea, vomiting, mucus or bloody stool (Negut et al., 2018). In addition, local

people have been treating their wounds through natural methods such as medicinal plants and honey for a long time which are easily available and affordable. Given the prevalence of current problems due to infections, applying unconventional, non-antibiotic treatments like natural agents, especially honey, in wound healing is of renewed interest. Hence, certain pharmaceutical companies are producing standardized honey-impregnated wound dressings (Parasuraman & Perumal, 2021).

Consequently, scientific agreements must be reached on several strategies for wound management. The concentration level of their antibacterial compounds should be so they can lead to the development of affordable and readily available antibacterial drugs. These polyphenols are a group of plant metabolites that hastens the process of wound healing. These compounds are plant derivatives that increase wound healing without leaving any scars.

### 1.3 Justification

Various microorganisms rescinding on the body's skin, mucous membranes, and the environment are a source of skin and soft tissue infections that enter the body via wounds. They yield very potent virulence factors accountable for sustaining infection and suspending wound healing progression, and they have elevated rates of numerous resistances to the antimicrobial agents frequently used. Various wound types that injure the skin surface include abrasions, avulsion, burns, lacerations and surgical wounds. They are divided into four classes depending on the post-operative risk of a surgical site infection (Okur et al., 2020).

Natural honey contains at least 181 constituents and bioactive substances, which could contribute to its antimicrobial properties (Nguyen et al., 2019). Its quality depends on various factors: ecological conditions and the floristic composition of the plants (Rangel & Fisher II, 2019). However, insufficient scientific reports on honey as an antibacterial agent, as well as comparative physicochemical plus phytochemical possessions of samples collected from diverse bee tribes and regions, has led to a lack of recommendations to justify the routine use of honey in modern clinical practice. Furthermore, although several studies (Albadiri, 2019; Bardoloi et al., 2023 & Girma et al., 2019) have considered honey's antimicrobial properties, very few studies have considered its potential application in wound care. Few of these studies (Bakar et al., 2017; Rao et al., 2016) have considered honey from stingless bees for wound treatment.

Hence, the sole purpose of this study was to assess the effectiveness of natural honey samples formed by both the *Apis* and *Meliponin* bee species from one of Kenya's leading honey production County of Baringo, on selected wound pathogenic bacterial isolates. This antibacterial effectiveness against the isolates was also compared to conventional antibiotics and an analysis of the physicochemical, nutritional and phytochemical properties enhancing its potency was made.

## 1.4 Objectives

### 1.4.1 General Objective

- To determine the effectiveness of stingless (*Meliponines spp.*) and

honeybees (*Apis mallifera)* honey samples from Baringo County against bacterial isolates from infected wounds and their antibiotic susceptibility pattern among adult patients at Nakuru County Referral Hospital

### 1.4.2 Specific Objectives

i. To isolate and characterize selected bacterial isolates from infected wounds among adult patients at Nakuru County Referral Hospital

ii. To establish the antibacterial property of honey samples from stingless bees (*Meliponines*) on bacterial isolates from infected wounds among adult patients at Nakuru County Referral Hospital.

iii. To determine the antibacterial property of honey samples from honey bees (*Apis*) on bacterial isolates from infected wounds among adult patients at Nakuru County Referral Hospital

iv. To compare the antibacterial effectiveness of honey from *Meliponin* and *Apis* bees with other conventional antibiotics against wound bacterial isolates

## 1.5 Null hypotheses

$Ho_1$: *E. coli*, *S. aureus*, *K. pneumoniae* and *P. aeruginosa* cannot be isolated and characterized from the infected wounds among adult patients at Nakuru County Referral Hospital

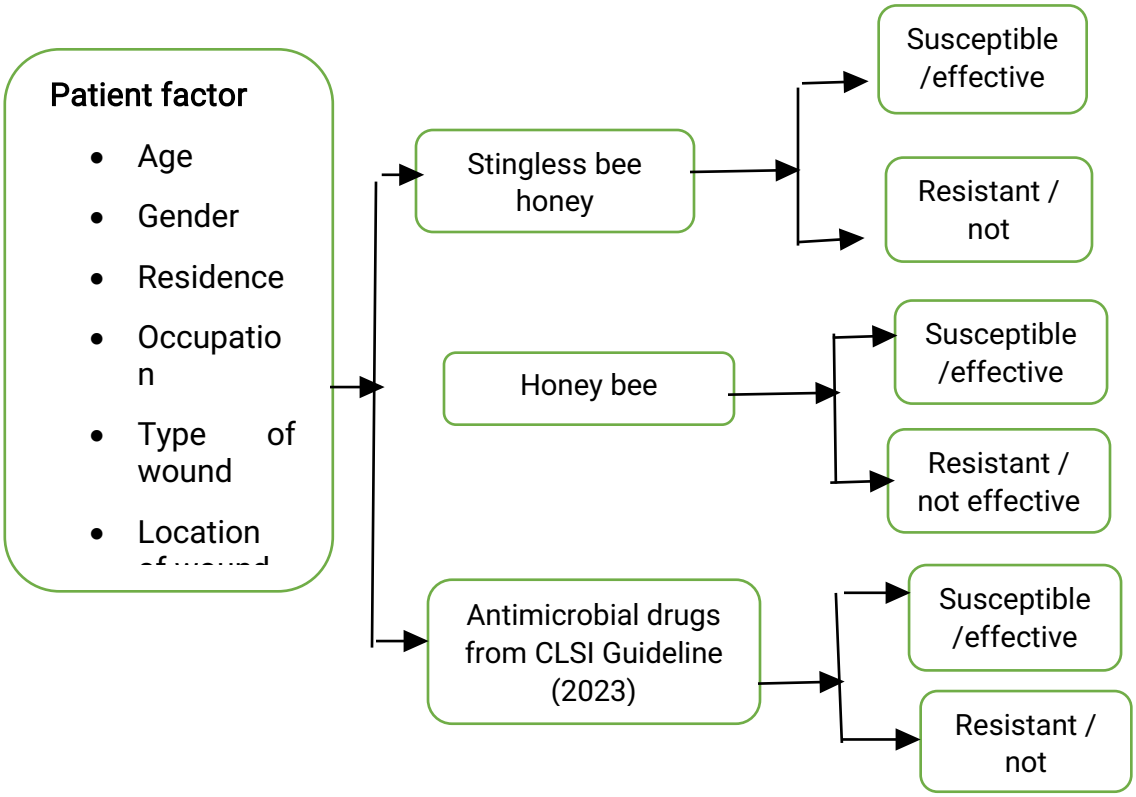
$Ho_2$: Stingless bee honey does not have antibacterial property on bacterial isolates from infected wounds among adult patients at Nakuru County Referral Hospital

$Ho_3$: Honey bee honey does not have antibacterial property on bacterial isolates from infected wounds among adult patients at Nakuru County Referral Hospital

$Ho_4$: Honey from Marigat does not have comparable antibacterial activity with conventional antibiotics

## 1.6 Conceptual framework

Various variables were considered in the conceptualization of this study. The independent variables (Age of the patient, type and location of the wound, occupation, immune status, existing comorbidity and area of residence) influenced the possible outcome of the antibiotic susceptibility pattern.




# CHAPTER TWO

# 2.0 LITERATURE REVIEW

## 2.1 INTRODUCTION

### 2.1.1 Definition of wounds

Dermal wounds are any kind of damage to the skin, mucous membranes or organ texture caused by physical, thermal, chemical or radiologic trauma and resulting in simple wounds (confined to the skin) or complicated wounds (with deeper

involvement of muscles and vessels) (Bowers & Franco, 2020). According to Fuchs et al. (2021), a wound is the interruption of the defensive purpose of the skin or harm of the epithelium continuity of, through or devoid of loss of underlying connective tissue following skin damage instigated by a cut, surgery, a blow, chemicals, blunt force, gunshot, heat/cold, insect bites or due to disease. Wounds may be described by their aetiology, anatomical location, type (acute or chronic), manner of closure, symptoms or appearance of the principal types of tissues in the wound bed (Irfan-Masqsood, 2018). The infected wound is the restricted defect or excavation of the skin or even the fundamental soft tissue whose viable immediate tissues have been invaded by pathogenic organisms.

The common skin and mucosal surface pathogens are the Gram-positive cocci (*Clostridium perfingens Staphylococcus aureus and Enterococcus faecalis,*). Still, Gram-negative aerobes (*Klebsiella* sp., *Proteus* sp. and *Escherichia coli*,) and anaerobes (*Bacteroides* and *Clostridium*) also contaminate the skin at the groin and perennial areas as well as the intrinsic bowel flora (Eyerich et al., 2018). Wound infection causes inflammation and tissue damage by triggering the body's immune response. However, the skin's acid mantle regulates its pH, enabling it to maintain the normal flora when broken. The immune system is compromised, and any microorganisms colonizing it may get hosted into the wound, instigating an infection (Byrd et al., 2018).

Haidari et al. (2023) highlighted the clinical presentation of infected wounds as chills, fever, leukocytosis, malaise and elevated erythrocyte sedimentation rate, tenderness of the wound, increased redness and inflammation, darkening of the

skin at the edges, a soft scab, yellow crust and a foul-smelling pus /cloudy fluid drain from the wound. The lymph nodes draining to the area become large and tender (regional lymphadenopathy) and there is an increase in pain and swelling. If no treatment is established, the infection can blow out to other body parts, causing serious difficulties, including cellulitis, osteomyelitis, sepsis, necrotizing fasciitis and tachycardia (Sen, 2019).

## 2.2 Categories of wounds

Wounds fall into two broad categories: open or closed. Open wounds involve an internal or external break in the skin, mostly by falls or accidents, exposing the internal tissues. Examples include abrasion, laceration, avulsion, puncture and incision (Avila et al., 2018). Closed wounds entail the damage that occurs without exposing the underlying body tissue, but tissue damage and bleeding occur under the skin's surface. Examples include contusions, seroma and hematoma (Bowers & Franco, 2020). According to World Health Organization (WHO) classifications, wounds are classified into four main categories according to the degree of contamination and breaching of the aerodigestive tract epithelium as follows: class I - Clean wounds (uninfected, no inflammation, has 1-5% rate of infection). Class II – Clean- contaminated (8- 11% infection rate, gastrointestinal and respiratory tracts are entered in a controlled fashion). Class III (less than four hours open contaminated fresh accidental wounds). Class IV (dirty traumatic infected wounds containing necrotic tissue, ongoing infection and presence of known organisms) (WHO, 2018).

On the other hand, Kyaw et al. (2018) stipulated that dermal wounds can be

broadly categorized as the ones that restore themselves or else be able to remain revamped following a systematic plus appropriate manner (acute) and those having halted healing process in the early inflammatory state regardless of the treatment interventions (chronic). Generally, acute wounds result from exterior skin damage, abrasions, minor cuts, lacerations, bites, and burns. They have a hierarchical organization with the possible tissue rebuilding via thrombocytes, microvascular cells, immune surveillance cells, fibroblasts and keratinocytes that perform responsible roles in forming tissue integrity (Byrd et al., 2018). Triggered by tissue injury, acute wounds follow the usual wound-healing processes of four overlapping phases: coagulation, inflammatory, proliferative and granulation, followed by apoptosis, which finally leads to the formation of a cellular scar. The greater risk of infection characterizes them if the wound has debris, bleeding at the wound site, inflammation, healing by primary intervention and may necessitate antiseptic use in case of wound contamination (Raziyeva et al., 2021).

Similarly, Tottoli et al. (2020) also evaluated chronic wounds as those whose healing did not proceed in the expected, orderly and timely manner, especially through the inflammatory phase. Instead, they progress through the repair process without establishing continued anatomical and functional integrity, and the degradation of the fibrin is necessary for healing, hence increasing the inflammation response. These wounds' main signs and symptoms were pain, slow or no healing, exudate, more than four weeks of inflammation, and reactive oxygen species production (Lewis et al., 2018). According to Tottoli et al. (2020), the three types of chronic wounds are infectious, surgical and ulcers classified as different

categories of wounds. Their pathogenesis is attributable to biofilm formation and release of enzymes by infecting bacteria, ischemic conditions that could be due to arterial insufficiency, venous hypertension or pressure injuries. It could also be due to malnutrition, metabolic disorders such as diabetes mellitus, immune suppression or ionizing radiation in cancer treatment.

### 2.3 Prevalence of wound infections causing pathogens

Various studies have acknowledged the occurrence rates of wound infections, thus demonstrating the burden of these conditions. For instance, the findings of Guan et al. (2021) in 195 hospitals across China on identifying the pathogenic bacteria present in infected wounds indicated that the most predominant bacteria isolated was *Staphylococcus aureus* (29.2%). Also, present were *Proteus mirabilis* (8.0%), *Pseudomonas aeruginosa* (11.0%) *Escherichia coli* (11.5%), in addition to *Klebsiella pneumomiae* (5.8%). In India also, a study done on incision and drainage of chronic non-healing ulcers did document the prominence of bacteria isolates [*Staphylococcus aureus* (28.5%), *coagulase-negative Staphylococcus* (23.8%), *Escherichia coli* (11.1%), *Klebsiella* species (22.2%) and *Pseudomonas aeruginosa* (44.4%)] (Kumari et al., 2018). On the other hand, Afroz et al. (2020), who worked with the wound samples within the Department of Microbiology, Sir Salimullah Medical College, Dhaka 2020 mentioned that the most predominant bacteria were *Staphylococcus aureus* (36.9%) more than *Escherichia coli* (35.8%) then *Pseudomonas spp.* (17.3%) and *Proteus spp* (5.8%). Almost comparable findings were documented by Upreti et al. (2018), who specified the prominence of bacterial isolates as *Staphylococcus aureus* (56.9%), *E. coli* (8.6%), *Klebsiella*

*pneumoniae* (5.2%), *Enterobacter* species (5.2%), *Proteus* species (2.5%), *Pseudomonas aeruginosa* (4.3%) and *Citrobacter freundii* (2.3%) in their study.

The prevalence rates in the African continent are not any different, as various studies have documented similar findings. For instance, a study carried out in Tanzania at Kilimanjaro Christian Medical Centre indicated that *Staphylococcus aureus* was the most isolated at 16% from wound swabs, followed by both *coliforms* and *Enterococcus* species at 12.5% each (Abosse et al., 2021). Also, *Enterococcus species* (36.4%) was the most isolated bacteria in diabetical ulcers, while *Staphylococcus aureus* in trauma wounds (40%) and surgical site infection (20.6%). *Streptococcus pneumoniae* was predominantly isolated from cerebral spinal fluid. These outcomes were in tandem with the discoveries of another scholarship in Tanzania by Kumburu et al. (2019).

In Ethiopia, a scholarship conducted at the University of Gondar Referral Hospital indicated that out of all the bacteria isolated, *Staphylococcus aureus* was the most predominant at 38.7%. *Klebsiella* species followed at 17%, then coagulase-negative *Staphylococci (CoNS)* (16%), *Citrobacter* species (5%), *Enterobacter* species (13%), *Pseudomonas aeruginosa* (8%) and *Escherichia coli* (8%) and *Proteus* species at 6% (Misha et al., 2021). These spectra of single bacteria resembled what was reported in the studies conducted in Uganda (Baguma et al., 2020). In the case of Ghana, Janssen et al. (2018) reported that across the sampled species, 72 (38.1%) were clan Enterobacteriaceae, 69 (36.4%) isolates were Gram-positive and 48 (25.4%) were non-fermenters. Out of the 67 samples analyzed, the infection was monomicrobial in 17 samples (25.4%) and

polymicrobial in 50 samples (74.6%), with the study concluding that the most frequently isolated bacterium in infections is both monomicrobial and polymicrobial is *Staphylococcus aureus* and for the polymicrobial infections, the most predominant were *Enterobacteriaceae* and non-fermenters.

In Kenya, the situation is not any different either. For instance, Tuvei et al. (2022) isolated *Proteus* species, *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Staphylococcus aureus* from the wounds of diabetic patients at Jaramogi Oginga Odinga Teaching & Referral Hospital (JOOTRH). This is in tandem with the scholarship of Mutonga et al. (2019) on isolating bacterial species from diabetic foot ulcers in a tertiary hospital. They elucidated *Proteus mirabilis* (11%), *Klebsiella pneumoniae* (7%), *Escherichia coli* (15%), *Staphylococcus aureus* (16%) and *Pseudomonas aeruginosa* (7%). These, amongst many more studies, demonstrate the burden caused by various pathogens in wound manifestations and antibacterial resistance. Therefore, as documented above, proper management is key to reducing such high prevalence rates.

## 2.4 Microbial development in chronic wounds

There are three stages of microbes' existence: free-living, sessile, reversible adhesion, and transient state (Lipsky, 2019). The ongoing competition for space and nutrients between species ultimately determines the adaptation into the niche where those best associate with the local ecological conditions will be the most successful, meaning that bacteria with a lower oxygen demand will have an advantage over those that require more oxygen for survival and this will result in

their domination in the deeper tissues. Isolation of species among the microbial populace of chronic wounds may suggest separated co-isolated pathogens, which appear to be found one after the other at specific locations despite possibly sharing the same milieu that surrounds them even though *Staphylococcus aureus* is one of the main opportunities to grow *Candida albicans* hyphae, where the collaboration is intended to facilitate the invasion of the usually non-motile bacterium into deeper skin tissues (Thaarup et al., 2022).

The possessions of a wound surface will program the microorganism that will ascribe, grow and persist as a component of an initial biofilm. The first colonizing bacteria on every surface (‘pioneering species’) modifies the atmosphere to create a ‘microenvironment’ that inspires their attachment and development. If unchallenged in favorable conditions, a complex community of microorganisms develops, forming a stable ‘climax community’(‘microbial homeostasis’), which can adapt appropriately to outside perturbations (Lanjawani & Channa, 2019). The obligate anaerobes group of microorganisms are present around the wound's surface, especially in devitalized sloughy tissues. They will remain dominant even with frequent attempts to aerate the wound and enjoy a more favorable environment free from the toxic effects of oxygen created by anaerobic bacteria. Most bacteria species have a relatively narrow growth pH spectrum. Still, when hybrids form and occur inside an open wound as biofilms, their pH tolerance increases to ranges inhibiting cells growing in pure cultures. Microbial communities can overcome the forces of the outer macro-environment by promoting their metabolic activities, therefore supporting their existence. Likewise,

the sequential breakdown of several nutrients serves as a way to evolve a simple food chain where the obtained material of one microalgae becomes a food substrate for others. When acquiring microbial contamination, the host presents conditions that are not conducive to disease-causing conditions, and, therefore, organisms involved come together to form synergistic pathologies. To do this effectively, they employ elements of collective defense, such as phagocytosis and intracellular killing, produce growth factors, and inactivate inhibitors of vital species (Bjarnsholt et al., 2022).

As stipulated by Mendoza et al. (2019), microorganisms such as bacteria and fungi, once mature, can attach, forming biofilms, which may be challenging to identify but become more visible, taking the appearance of a viscous shiny film as they grow larger. Through an inflammatory response, the body's natural immune system may help the biofilm by providing nutrients in the form of exudates, making fighting biofilms ineffective while damaging healing tissue, leading to delayed healing of wounds. Recent studies by Johani *et al.* (2018) have indicated that many clinically relevant wound biofilms involve multiple species, i.e., polymicrobials that display impaired wound healing compared with those infected with single-species bacteria. Wounds display increased antimicrobial resistance and tolerance to different ant-biofilm agents in polymicrobial biofilm infections.

### 2.5 Virulence Concepts of Wound Infection Causing Pathogens

According to Wadstrom et al. (2018), virulence is a bacterium's capacity to bind, penetrate and multiply within tissues despite the defensive mechanisms mobilized by the host, thus causing infection. It is enhanced by proteinous factors

synthesized by enzymes and coded for by genes in chromosomal DNA. Their ability to cause disease depends on the infecting bacteria's quantity, entry route to the body, the effects of the host defense mechanisms, and the bacteria's intrinsic features (Liu et al., 2022).

Among the bacteria known to cause wound infection is *Staphylococcus aureus,* a key human pathogen triggering a broad spectrum of diseases due to the passion of various secreted proteins, including beta hemolysin, alpha toxin, enterotoxins, coagulase, lipases as well as cell surface-associated proteins. The virulence genes involved include Alpha-toxin (alpha haemolysis), a major cytotoxic agent that triggers alterations and loss of membrane integrity leading to cell death. The Sea gene was primarily found in the SCCmec III Methycillin. Resistant *Staphylococcus aureus,* whereas the gene was dominant on SCCmecII isolates, seg and sei genes were located within the same isolate, with the majority being agr genes. Others include hla (encodes alpha-toxin), arcA (encodes an enzyme in bacteriocin biosynthesis pathway), luks–pv (encodes a component of PVL- Panton valentine leucocidin), luk E (encodes leucocidin), hlgB (encodes Y-toxin) (Wu *et al.,* 2019). A study by Klutse et al. (2021) did document the presence of pvl- hla – fnbA (28.6%), hla –fnbA (9.6%) and Sea – See, pvc – fnbA (17.9%) genes on *Staphylococcus aureus* isolates. These genes (fibronectin-binding proteins) enable *Staphylococcus aureus* to be more virulent by enhancing one of the mechanisms of its pathogenesis which is adherence to the extracellular matrix of the host, leading both neutrophil and red cell lysis (Sedaghat et al., 2018).

An additional scholarship done by Abdelrahman et al. (2021) on burn patients

indicated the prevalence of *Pseudomonas aeruginosa* virulence genes as plcH (79%), plcN (63.1%), toxA (36.9%), AlgD (70.1%), exoS (21.1%) and lasB (82%). Deinsberger et al. (2022) also documented the presence of similar virulence genes constituted of type II (lasA, ToxA and lasB) as well as type III (pop B) from the *Pseudomonas aeruginosa* isolate obtained in their study. Exotoxin A is an ADP-ribosyl transferase toxin that inhibits protein synthesis. lasA and lasB have an intense elastolytic activity, inactivating an extensive range of biological tissues. PopB can induce necrosis in phagocytes, essential for translocating effector proteins to host cell cytoplasm. The presence of these virulence genes in *Pseudomonas aeruginosa* enhances its virulence since ToxA is a highly toxic protein that inhibits protein synthesis, leading to necrosis at the site of infection. Las A and Las B are involved in proteolysis and elastolysis by creating insertional mutants. They are actively involved in this pathogen's quorum-sensing cascades, which enhances their virulence activities (Wu et al., 2019).

*Escherichia coli* is a pathogen that is commonly associated with wound infections. The Virulence characteristics that enable *E. coli* to colonize and cause disease involve factors such as adhesins (hemagglutinins that assist in adhering in epithelial cells), milking resistance, hemolysis production, cell surface hydrophobicity, the survival rate in phagocytosis, cytotoxic necrotizing factors production, K1 antigen and gelatinase production (Sarowska et al., 2019). Asare et al. (2022) further stipulated the responsibility of toxins in bacterial colonization of host cells mediated through invasion and dissemination (alpha haemolysins and CNF1) that act by causing haemolysis, which decreases the activity of phagocytic

cells and finally leads to direct tissue cytotoxicity. Haemolysin is necessary for the primary incursion of bacteria through the epithelial barrier. At the same time, CNF1 is essential in the spreading and persevering *Escherichia coli* strain through activation of Rho GTPases, which leads to cytoskeletal alterations, thus affecting the cell cycle. The findings of the research done by Moxley (2022) indicated that hlyA and hlyF genes can damage human cells and tissues through complete lysis. The papC and papG genes are the two genes that help the bacterium build a platform for fimbriae growth and then attach to eukaryotic cells for infection.

*Klebsiella pneumoniae,* another wound infection-causing pathogen, is characterized by genes for capsule formation. Among such genes includes the cps (capsule polysaccharide synthesis) gene, which implements the synthesis of capsular polysaccharides; rmpA1, rmpA2 and rmpA (regulator of the mucoid phenotype A1, A2 and A) responsible for regulating the synthesis of the extracellular polysaccharide capsule (Willsey et al., 2018). Shakib (2018) also documented that ycfm, anf, & fimhI genes were responsible for adhesion, enabling the bacterium to adapt to environmental changes for proper multiplication and survival. It also has protein structures with virulence factors like fimbria encoded by fimh genes, enabling biofilm formation in the host cells. Tan et al. (2019) ascertained that mucoviscosity–associated gene A (magA) containing strains produce a significant amount of polysaccharide capsules and are unaffected by serum killing and phagocytosis. The gene plays a vital role in severe infections of *Klebsiella* such as septicaemia, bacteraemia and pneumonia. Therefore, among many genes, these have played a key role in the presence of various traits the

wound infection-causing bacteria exhibited. As such, this could be another plausible area that should be targeted regarding managing such infections, such as dismantling the virulence traits, which means that the pathogen may not be able to induce a given condition. It is also advantageous as targeting virulence traits will not offer selective pressure on the pathogens as targeting the pathogens themselves may lead to rising resistance cases.

## 2.6 Wound infection management principles

### 2.6.1: Introduction

The National Recommendations given for wound infection prevention and management by the World Health Organization (WHO) states that contaminated and infected wounds should not be closed but appropriate care of the surrounding skin (systemic wound toilet) and medical removal of dead or damaged tissue (surgical debridement) should be done. To prevent wound infection, the victim is kept warm and provided with high-energy nutrition and antibiotic prophylaxis, restoring breathing and blood circulation (Lei et al., 2019). Wound management also includes managing the underlying pathology and either permitting the body to heal naturally or using skin grafts to replace the lost tissues. Regenerative therapies in wound treatment have developed a suite of new choices that promise to reprogram the wound's microenvironment from one that promotes inflammation and scar formation to one that speeds up regeneration with minimal scarring (Li et al., 2020).

The objective of wound care involves preventing further infection by translating

those contaminated or infected into clean ones for either surgical closure or second-intention healing, and the wound size, depth, severity and location determine this. Therefore, aseptic techniques should be articulated during wound treatment in the following steps: decontamination (lavage) (removes bacteria and debris), debridement (removal of necrotic and devitalized tissues), application of topical antimicrobials (reduces the number of microorganisms present), and finally wound closure and bandaging (depending on the amount of tissue extension, soft tissue loss and overall patient stability) (Tottoli et al., 2020).

### 2.6.2 Modes of action of commonly used interventions

The range of interactions between the host and the microbial community may reach a point of weakened wound healing progression or resistance to topical agents. The choice of antibacterial agents in wound management to reduce and eradicate the infecting microorganisms must be influenced by their specificity and efficacy, cytotoxicity to human cells, ability to select resistant strains, and allergenicity (Liang et al., 2022).

#### 2.6.2.1 Antibiotics and antiseptics therapy

*i) Antibiotics*

Antibiotics are chemical substances vital in managing wound infection; nevertheless, their haphazard usage has contributed to the development of antibiotic-resilient bacterial strains (Smith et al., 2020). They are grouped based on their biological activity (those acting according to the Gram reaction, broad-spectrum, anti-anaerobic, anti-mycobacterium and beta-lactamase inhibitors),

based according to their action mode (interfering with cell wall synthesis, metabolic pathways inhibition and disrupting the structures of bacterial membrane structures) and finally on their chemical structure (beta-lactams, macrolides, aminoglycosides and quinolones) (Mancuso et al., 2021). It is vital to appropriately recognize the right topical/systemic antibiotics for application on the superficial tissues (skin's subcutaneous layer) and the deeper tissues (deep incisional or organ- space). Although antibiotic therapy is necessary, some decrease the wound's tensile strength, impending ultimate wound closure (Phoon & Hwang, 2020).

According to Bonamonte et al. (2020), topical antimicrobials provide high and sustained concentration at the location of the infection, preventing the overall concentration needed in amalgamation with systemic treatment. Limited potential for systemic absorption and toxicity is also presented, but due to their superficial nature, they cannot be used to treat deep tissue infection. These antimicrobials may cause local allergic reactions or contact dermatitis reactions, altering the normal skin flora and interfering with wound healing. Examples of commonly used antibiotics include Neomycin, which is active against most aerobic Gram-negative rods (except most *Pseudomonas species*) and *Staphylococci,* with resistance and dermatitis developing relatively often. Polymixin with minimal systemic penetration causing scant rashes is effective against some Gram-negative rods (*Pseudomonas species*) and stable to Gram-positive cocci (Liu et al., 2022).

### *ii) Antiseptics*

Antiseptics are broad-spectrum germicides and have persistent biocidal action on unit tissues and some forms of abrasions to kill or inhibit microorganisms since they act on multiple microbial targets but are often cytotoxic to host tissues. As a result, the likelihood of bacteria relating to their mechanism of action and place of action is considerably low, and therefore, they have relatively low levels of bacterial resistance (Barreto et al., 2020).

Several topical antimicrobial agents currently used include Ionic silver (silver sulfadiazine, silver-impregnated dressings), which has a broad spectrum of activity (bactericidal, fungicidal, viricidal and protozoicidal). However, more resistant microorganisms such as spores, cysts and mycobacteria are less eradicated. To be effective, silver ions must reach the target sites at the bacterial cells through a heavy metal ions uptake system switched on and off by the bacteria under particular conditions. Silver has a strong attraction for electron donor groups comprising Sulphur, oxygen and nitrogen, causing inhibition of bacterial enzymes and interfering with respiration at the cell membrane level. It forms irreversible complexes upon reacting with nucleic acid bases and depending on the number of target sites and the extent of damage, it contributes to the overall lethal efficacy (Ray et al., 2019).

Chlorhexidine, being available as diacetate, dihydrochloride and digluconate, exhibits a highly rapid bactericidal activity against a wide range of bacteria through the toxic effect on the outer cell layers and cell membranes and then allows the leakage of the bacterial cytoplasm. Secondly, concentration may cause co-agglutination among intracellular components (Punjataewakupt et al., 2019).

Jiang et al. (2019) have pointed out that iodine, cadexomer-iodine and povidone-iodine have variety of actions against bacteria, mycobacteria, fungi, protozoa and viruses through different cellular effects. It interacts with proteins, nucleotides and fatty acids and alters structure by oxidation of S-H bonds, cysteine, and methionine. Besides, it forms a covalent link with phenolic functional groups of tyrosine. It modifies amino groups of arginine, histidine, and lysine and bases of nucleotides of adenosine, cytosine, and guanine that inhibit hydrogen bonding and alter the molecular structure of the cell membrane by breaking C=C bonds of fatty acids. Iodine shows a lethal effect against microorganisms by quickly traversing the cell wall and subsequently, molecules of protein synthesis will be disrupted. In addition, the function of the respiratory chain enzymes will be affected and both lipid membrane and nucleic acid functions will be impaired. Its function can be altered by formulation, temperature, synergy, and the presence of organic load and dilution (Punjataewakupt et al., 2019).

#### 2.6.2.2 Medicinal plants with wound healing potential

Herbal/traditional medicine has been part of human health for a long time. Their diversity is determined by the local climatic condition and their adaptation capacity in changeable environmental conditions. They contain phytonutrients/phytochemicals, e.g., tannins, flavonoids, saponins and sterols, which interfere with one or more phases of wound healing positively, thus reducing various side effects commonly related to systemic antimicrobials (Mohammed et al., 2018). A study piloted by Ali et al. (2020) on the honey antimicrobial effects and some herbal extracts against multidrug-resistant

bacteria isolated from patients in the local Riyadh Hospital indicated significant antibacterial inhibitions. Plant extracts at low concentrations indicated an inhibition zone diameter range of 11 ± 0.26 to 27 ± 0.21 and the active compounds in the extracts attributed MIC value of 25mg/ml.

Examples of some medicinal plants with significant wound healing activity include *Aloe vera* – comprised of many natural bioactive compounds such as pyrocatechol, saponins, and anthraquinones with a stronger antimicrobial capacity counter to Gram-positive than in Gram-negative bacteria (Sharma et al., 2021). The ethanolic extracts of *Morinda gintrifolia* leaves have also been documented to have wound-healing properties Nayak et al. (2019). *Lycopodium serratum* was recorded to contain many alkaloids and its aqueous and methanolic leave extracts indicated were found to have a massive reduction in epithelialization period as well as an increase in the rate of wound shrinkage and reinforcement of tissues at the site of the wound (Wang et al., 2021). Also, *Catharanthus raseus* is a crucial source of monoterpenoid indole, vinblastine, vincristine and alkaloids beneficial in cancer treatment at a dose of 100mg/kg/day of its flower's ethanoic extract (Nayak et al., 2019). *Trigonella foenum – graecum* seeds extracts have also been documented to exhibit substantial wound healing properties by several studies like Al-Dabbagh et al. (2018) and Benabderrahim et al. (2019). Other medicinal plants containing antioxidant, antiulcer, antimicrobial, antidiuretic, and anti-inflammatory properties mainly used in wound management include *Cinnamon, Arctium lappa / Burdock, Eucalyptus / Dinkum oil and Neem* (Farahpour, 2018; Firdous & sautya, 2018; Shedoeva et al., 2019). These, amongst many are a clear indication that

medicinal plants could add value to the process of wound healing.

#### 2.6.2.3 Usage of honey to manage wound infections

The ability of honey to do multiple tasks, including dressing wounds and healing, has been documented. It is a thick and sticky fluid with a tight molecular structure, which creates a barrier between the wound and the external environment while helping to maintain the humidification of the wound. It comprises a high level of sugar and other solutes, creating an osmotic gradient strong enough to move fluids through the sub-dermal cells, plumping up bacteria at the top of the wound, which causes them to rupture (Talebi et al., 2020). Various studies have elucidated that one of the main reasons honey accelerates the healing process of wounds is debridement of the wound bed, accelerated epithelialization and reduced edema, leading to faster wound closure rate and cure. In that span of a few weeks, there will be a slough of necrotic and gangrenous tissues being easily separated from the ulcers such that they can be lifted by forceps without pain and the foul-smelling wounds become odourless (Minden-Birkenmaier & Bowlin, 2018).

Contrary to the case where antibiotics target a specific site in a microbial organism, honey has inhibitory effects on the microbe- causing masses that differ depending on the honey and the microorganisms in question. For example, in that particular study, bacteria class *Staphylococcus aureus* (MRSA -Methicillin - Resistant *Staphylococcus aureus*) cells that were treated with Manuka honey accumulated in the bacteria cell walls wells, which subsequently resulted in the prevention of cell division of Gram-positive due to the bacteria wall component

autolysin (Mama et al., 2019).

Manuka honey also leads to structural alterations in cell wall structures in Pseudomonas aeruginosa, where further tests showed the downregulation of a protein responsible for cell envelope maintenance, leading to cell lysis and death. In the presence of Methicillin-resistant *Staphylococcus aureous*, the stress protein responsible for maintaining the bacterium living in honey-stressed conditions also showed downregulation, translating into knock-on effects on gene expressions controlling virulence, cell-to-cell communication, and biofilm formation. It was noted that the Gram-negative bacteria Manuka honey altered gene expression and showed other dying cell features (Benabderrahim et al., 2019). In addition, 12-72µg/ml of hydrogen peroxide ($H_2O_2$) can be found in honey, and this breaks away one of the oxygen atoms of the surrounding environment and results in free radicals which leads to the impairment of oxidative cell wall and DNA degeneration and this restricts the growth of the bacteria. The existence of the bee defensin 1 exposes the bacteria and hinders their RNA and DNA, eventually making it impossible for their protein production. The glucose oxidase of honey also decreases its pH, killing the bacteria; similarly, the presence of methylglyoxal enhances antibacterial effects because the compound damages the bacterial flagella, making thei,

Also, it was observed that following the association between wound chronicity and biofilms, an effective anti-biofilm agent is required due to the tolerance of biofilms to antibiotics. This involves preventing biofilm formation by interfering with or inhibiting host cell and tissue adherence. Secondly, another approach remains

towards disrupting an established biofilm through establishing biofilm biomass or metallic activity following contact with honey, indicating that a higher concentration of honey is required. Fructose is the dormant sugar in honey which binds to the bacterial lectins, blocking the binding of *Pseudomonas aeruginosa* to erythrocyte receptors. This was investigated in *Streptococcus pyogenes,* where Manuka honey decreased the manifestation of two important surface proteins that act as adhesins that aid in bacterial binding to fibronectin. Microbial species cannot start infection or biofilm formation without connection.

## 2.7 Honey

The honey sacs of bees and related insects are responsible for producing honey, a naturally occurring food product that is golden in color, sweet, and viscous. Enzyme activity, water evaporation, and repeated regurgitation of sugary plant secretions are the mechanisms that yield honey. Due to the amount of water used, it is a silky liquid containing invisible microcrystals of different combinations. The honey is made of 18% nectar and the physiology of the bee manufacturing it with its own physical, chemical, biochemical and sensorial attributes (Almasaudi, 2021). According to Nordqvist (2018), the source of honey contains about 200 different compounds of glucose and fructose, fructose-oligosaccharides, amino acids, vitamins, minerals, and enzymes. It acts as an agent for transporting many of the plant's medicinal properties; hence, it contains antibacterial agents that are active against nearly all common resistant strains of bacteria. Agatz et al. (2019) also, went on to opine that bees must visit 5 million flowers to make one pint of honey through the gathering of nectar, using their "Honey stomach" with the help of their

digestive enzymes (amylase, diastase and gastric acid) to ingest and regurgitate the collected nectar that is finally placed in capped combs sealed with wax for storage.

### 2.7.1 Bee taxonomy

A naturalist, John Ray, introduced the concept of a 'species' in the 17$^{th}$ Century as the basic unit of classification. Harrison et al. (2019) state that all bee species are classified into seven key families (*Andrenidae, Apidae, Colletidae, Halictidae, Megachilidae, Mellittidae and Stenotritidae*). The family *Apidae* includes three subfamilies, namely *Nomadinae, Apinae and Xylocopinae*. The subfamily *Apinae* comprises nineteen species such as Apini for honeybees, *Bombini* for bumble bees and *Meliponini* for stingless bees. The tribes *Meliponini* usually have stingless bees, which are expected mainly in tropical and southern subtropical regions. In contrast, the tribe *Apini* has only one genus, *Apis*, which is the actual social bees with permanent colonies.

#### *2.7.1.1 Stingless Bees*

Stingless bees have highly reduced stingers that cannot be used for defense but use other defensive behaviours and mechanisms, including emitting mandibular secretions such as formic acid that cause unpleasant odours and painful blisters upon crawling on the eyes and ears. These birds prefer to make their nests in hollows or dig burrows in the dust castles of ants' colonies, termite mounds, tree branches or rock crevices, and in wall cavities, which are mostly about one meter deep. They form thimble-sized oval-shaped honey/pollen pots positioned around

the one-cell thick brood area of the combs in a horizontal arrangement in the nests (Appendix I). This differs from perennial, colonial nests from wax constructed by honeybees (Appendix II) (Rueppell & Keneddy, 2019).

According to Azmi et al. (2019), Stingless bees (figure 2.1) are important pollinators of crops because the last pairs of legs have larger surface areas for transporting the pollen back to the hive after collecting food. They are relatively small (2-8mm), silent, never go beyond a radius of 5km when looking for nectar and are less susceptible to diseases, standing out because they produce a particular blend of darker, less viscous and highly acidic honey with superior medical value (Lu et al., 2019).

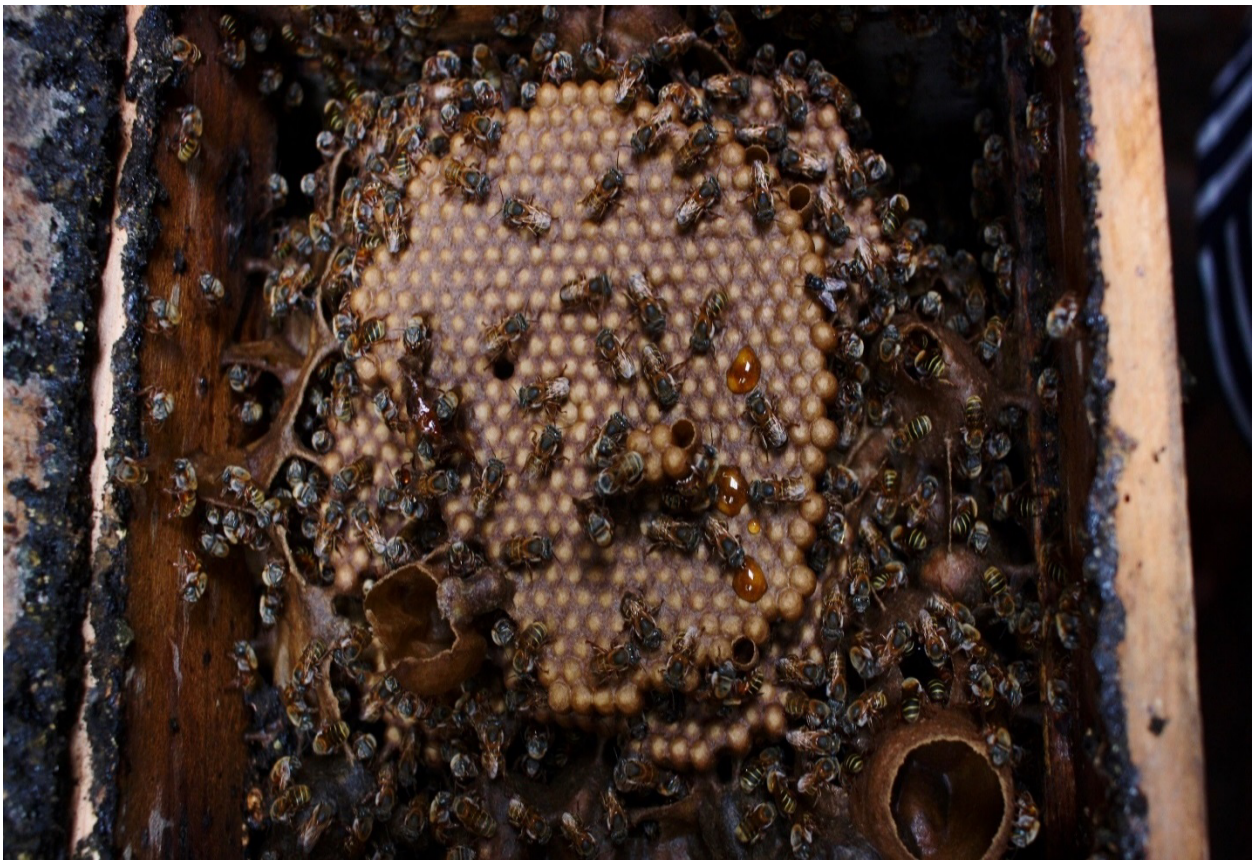


Figure 2.1: Stingless bee image and nest structure (Witkowski, 2018).

### *2.7.1.2 Honeybees*

According to Quigley et al. (2019), honeybees (Apis mellifera) are among the most well-known, popular and economically beneficial insects. They belong to the flying insects of the genus Apis and are known to build annual and large communal structures from wax produced by the workers. They form large colony sizes, as well as surplus production and storage of honey. Beyond the internal coherence of a colony of bees, they also display synchronization with plants since they obligately depend on flowering to obtain pollen and nectar. In contrast, certain plants are obligately reliant on honeybees for pollination.

Rueppell and Kennedy (2019) stipulated that Honey bee (figure 2.2) workers forage for nectar and pollen collected in unique baskets on their hind legs, called corbicula, to feed the colony, they require an ample supply of flowers in their habitat since this is their food source. They have a sophisticated way of communicating; when the hive is threatened, they use chemical signals to help the queen look for mates and direct the foragers to areas where the best foods are found.

Figure 2.2: Honey bees in honeycombs (McAfee, 2020)

### 2.7.2 Ancient Honey uses

According to Zeid et al. (2019), honey has been used by humans for medical purposes since about 8000 years ago. Its use in traditional medicine has continued to this day. Both Islam and Christianity have approved of honey throughout the Bible. This is because it appears in numerous ancient religious writings, including the Quran, Buddhism, the Hebrew Bible, and the New Testament.

According to Testa et al. (2019), honey was used in ancient China since about 2000 BC as medicine because it belongs to the earth element and functions as it

enters the lungs, spleen and large intestine channels. But let's go back to the Eastern Zhou Dynasty (770-256 BC), honey and larvae bees were considered as exotic products and wine for royal families which are mentioned in the Chu Poetry of Qu Yuan, an ancient Chinese patriotic poet.

Arehart (2019) further stipulated that in ancient Greece and Rome, where the medicine was quite advanced, surgeons regularly practiced lobotomies, caesarean sections and amputations as they relied heavily on herbs and the beneficial properties of food. They believed honey is good for mouth sores, tooth pain, pneumonia, pleurisy and snake bites. In India and other Asian countries, it was used as a preservative for fruits and cakes, sweetmeats and foods, and coughs and sore throats. In Egypt, it was used for various purposes, such as as a sweetener, a gift for the gods, and an embalming fluid ingredient. In Ghana, it was used in infected ulcers, while in Mali and Nigeria, it was used in treating gastric ulcers and measles. These, amongst many others, indicate that honey is an old commodity that could have various medicinal properties that benefit humankind.

### 2.7.3 Physico-chemical properties of honey

The physical and chemical characteristics of honey, such as its moisture content, electrical conductivity, ash content, and amount of reducing and non-reducing carbohydrates, have been studied by Azonwade et al. (2018). Honey also contains charcoal. Moreover, the word refers to the amounts of hydroxymethylfurfural (HMF), diastase activity, and free acidity, all of which are impacted by the local climate, environmental factors, and range of native plants. Their study on the

various honey samples collected from various climatic zones of Sudan indicated that the pH, water, total and reducing sugar levels were considerably determined by both season and region. Another study by Imtara et al. (2018) illustrated that the quality characteristics of Palestine's honey samples belonged to diverse botanical origin as categorized by a panel of recognized physicochemical parameters. They stated that the amount of moisture depends on the environmental conditions; ash content determines the mineral richness, which is a biomarker for possible pollution and the subsequent electrical conductivity and is a vital parameter in determining the botanical origin. Many studies have been carried out on different honey types to deduce the significant variations in their physicochemical components and, hence, biological composition (Albadiri, 2019; Lewoyehu & Amare, 2019; Shapla et al., 2018 and Warui et al., 2019).

Honey has been identified to have many attributes that make it antimicrobial. Amongst such properties is the meager availability of water that cannot support microbes' growth due to the high sugar content (fructose and glucose) exerting osmotic pressure on bacterial cells, causing water transport out of the bacterial cells through osmosis, hence dehydration. The other important antibacterial component of honey is its acidic pH (3.2 to 4.5), which is contributed by organic acids and subsequently lower than most bacteria growth pH of 6.5 to 7.5 (Ismail et al., 2021). It also possesses hydrogen peroxide as a vital oxidizing and sanitizing agent produced enzymatically by glucose oxidase which is active at low pH in diluted honey by acting on endogenous glucose (Azonwade et al., 2018). These, amongst many physicochemical properties, are key to the antimicrobial properties

attributed to honey.

### 2.7.4 Phytochemical components of honey

It has also been observed that honey contains phytochemicals; some of which exhibit anti-microbial activity. For instance, honey contains flavonoids, which is a group of phenolic low molecular weight compounds associated with the smell of honey and its antioxidant potential; the polyphenol content or total phenolic content, associated with the taste of honey, is responsible for the natural curative property of honey (Bucekova et al., 2019). This implies that some variations in honey's flavonoid and phenolic content reflect variances in the honey type, bee foraging from floral sources, location, season of collection, honey storage and practices of honey collection. Flavonoids have potential value in clinical settings as treatments for diseases due to inflammation and microbial infections and they are antioxidants. Other phytochemicals that have been documented to be in honey include alkaloids, glycosides, phlorotannins, saponins (steroids and triterpenoids), tannins (polyphenols) and essential oil (terpenoids) (Farooq et al., 2020 and Joshna et al., 2019).

#### 2.7.4.1 Antioxidant properties of honey against reactive oxygen species

Many papers have been written about the quality and identification of honey about its medicinal, antibacterial and antioxidant potentials (Auguskani, 2018; Green et al., 2022; Karadal et al., 2018; Stagos et al., 2018 and Yelin & Kuntadi, 2019). As a result, many previous studies revealed that stingless bee honey exhibits anti-

inflammatory and antimicrobial properties (Abdulla et al., 2020; Biluca et al., 2020; Huanbutta et al., 2020; Mustafa et al., 2020; Wu et al., 2022 and Zeid et al., 2019). It has also been evidenced that it contains antioxidant effects (Al-Hatamleh et al., 2020; Fletcher et al., 2020; Ramadhan et al., 2020) and anti-cancer compounds (Badrulhisham et al., 2020).

Antioxidants are defined as molecules that can protect cells against damage created by several reactive oxygen species such as singlet oxygen, superoxide, and peroxyl as well as hydroxyl radicals both generated by internal stressors and by-products of cellular metabolism and external stressors such as UV light, pollutants, drugs and radiation. Reactive molecules pave the way to the deterioration of lipids, amino acids, and DNA, thus reducing collagen formation and disrupting the proliferation of cells in wound healing. The vasodilation intensification due to iNOS enzyme activation may cause hydrogen peroxide and other ROS to be produced during the damaged tissue healing process, deteriorate the tissues and spread to other organs by inflammation (Fletcher et al., 2020).

There are two classes of antioxidants: non-enzymatic and enzymatic. Non-enzymatic antioxidants include carotenoids, tocopherol, ascorbic acid and phenolic compounds effectively inhibit the formation of free radicals; they can also sometimes stop the destructive chain reaction caused by ROS. On the other hand, Enzymatic antioxidants include ascorbate peroxides, catalase and glutathione S-transferase, which convert free radicals into stable molecules less harmful to the body (Al-Hatamleh et al., 2020).

Based on the research done by Xu et al. (2022) on honey, the findings suggest that most of the physicochemical, antimicrobial, and antioxidant properties of honey and stingless bee honey samples collected from different vegetative growth zones were found to be significant. The antibacterial activities of the honey were assayed using the agar well diffusion method on fourteen bacterial species and two yeast species, all of which, except Streptococcus pyogenes and Candida albicans, were inhibited. The results of the scholarship suggested that samples of stingless bee honey were a better source of antioxidants than honeybees since at a concentration of 2. 5-6. Honey of *Melipona* species at a concentration of 3% (v/v) could inhibit all the tested organisms. At 6.3% and above, it showed inhibition against *Bacillus cereus* 6.3mm, *Cryptococcus neoformans* 6.3mm, and *Cryptococcus tropicalis* 6.3mm. In comparison, 20% (v/v) of the honey samples showed inhibition zones against *Escherichia coli* 12.5mm, *Candida albicans* 12.5mm and at 12.5% (v/v) concentration, it inhibited MDRS *Staphylococcus aureus* 12.5mm and *Pseudomonas aeruginosa* 12.5mm. *HypoTrigona* species honey samples also inhibited all isolates at a concentration range of 10-40% (v/v). The inhibitions obtained included *Bacillus cereus* 12.5mm, MDR *Staphylococcus aureus* 12.5mm, *Pseudomonas aeruginosa* ATCC25783 6.9mm, *Candida albicans* SC5314 25mm and *Cryptococcus neoformans* 12.5mm.

### 2.7.5 Nutritional composition of honey

Honey's composition greatly changes depending on natural and anthropogenic factors that differ depending on the area of its plant source and the additional minerals and heavy metals compounds that significantly impact its quality. (Frey,

2018). According to Brodschneider et al. (2018), the main nutritional composition of honey is the following: carbohydrates 76.4g, 41.8g, Fat 0g, Protein 0.4g, energy 288kcal/1229kj, glucose 34.6g, water 17.5g. It showed that honey comprises vitamins, minerals, trace elements, enzymes and polyphenol compounds. The USDA Nutritional Nutrient database indicates that one tablespoon of honey has 64 calories, 17.3g of sugar, and 0g of fiber, fat and protein. According to this report, the honey's nutritional profile is as follows: Fructose 38.2%, Sucrose 1.3%, Glucose 31.3%, Maltose 7.1%, Water 17.2%, Higher sugars 1.5%, Ash 1.2%, other 3.2%.

Another honey nutritional profile, as documented by Nordqvist (2018) showed that it possessed water 17.10g, Energy 304kcal, Protein 0.30yd, Total lipid (fat) 0.00g, Carbohydrates 82.40g, Sugars (Total) 82.12g and Calcium 6mg. Consequently, an analysis of honey by Hassan et al. (2019) gave the nutritional information for honey (per tablespoon -7gram serving) as Cholesterol 0mg, Sodium 1mg, Potassium11mg, Carbohydrates 17g, Sugars 17g and Protein 0g. The study also identified honey’s micronutrients as Vitamins (Vit A 0IU, Vit C 0mg, Vit E 0mg, Vit B 0mg), Minerals (Calcium 0.5mg, Iron 0mg, Magnesium 0.1mg, Copper 0mg, Phosphorus 0.3mg, Manganese 0mg, Potassium 3.9mg, Zinc 0mg, Sodium 0mg), Total carbohydrates 6.1g, Fat 0g, Calories 24.4cal, Glycemic Index 58GI and Glycemic Load 12 GL.

# CHAPTER THREE

# 3.0 MATERIALS AND METHODS

## 3.1 Study area

The study was conducted in the Marigat sub-county of Baringo County of Kenya, which has an area of 1663 km2 and a population of about 95,286. This area (Fig 3.1) is located between latitude $00^0$26’ to $00^0$32’N and longitude $36^0$00’ to $36^0$09E, with an average altitude of 900 to 1,200 meters above sea level. It is located within Agro-climatic zones IV and V, according to Koppen and Geiger (Peel et al., 2007). An altitude range of 900-1800 m and an average annual temperature of 24 degrees distinguish it. 60 C and a rainfall that averages 671mm per annum. It is the home of several acacia, low trees and shrubs (*Ezenwa* et al., 2018).

Marigat Sub County was recently formed from the larger Baringo County, which is among the six-county units in Kenya. It is neighbors with Laikipia to the east, East Pokot to the north, Baringo North, Baringo in the west, and Mogotio to the south, covering an area of 1,677 square kilometers. 45 sq. km (Baringo County Government Annual Development Plan 2016/2017)

Livestock keeping is the chief activity in the area, and the main livestock includes East African zebu cattle, goats, sheep, and camels. Other activities include beekeeping and honey production, as shown in Appendix II, as well as hides and skins. Beekeeping and *Aloe vera* plant cultivation are emerging economic activities with local processing at the *Aloe vera* factory in Koriema, which is the only *Aloe vera* processing factory in Kenya. Charcoal burning is another economic activity with the possibility of quick cash rewards, but it is not highly considered since it conflicts with beekeeping interests. The social and economic determinants that aggravate vulnerability to climate risks, such as high poverty and illiteracy levels, subdivided land holdings, inadequate infrastructure, insecurity, poor technology adoption, high input prices and absence of market development, are also present.

(MoALF, 2017).

Figure 3.1: Map of Marigat sub-county, Baringo County, showing the study area (Baringo County Govt. Dev. Plan, 2016/2017).

## 3.2 Study Design

The study design was experimental as the dependent variable was measured once the independent variables were implemented. This study included preliminary phytochemicals determination with potential antibacterial effects, analysis of the nutritional value, and the physico-chemical composition in honey samples collected from honey and stingless bees. It also involved the determination of the honey's antimicrobial sensitivities compared to conventional antibiotics on the bacteria isolated from infected wounds.

### 3.3 Population of the Study

The study entailed the aseptic collection of wound swabs from adult patients attending The Nakuru County Referral Hospital. Some factors of high significance such as site, dislocation, dimension and depth of the actor, were considered. The study considered several types of injuries from different conditions, such as diabetic foot ulcers, venous ulcers and pressure sores, and their duration (chronic and acute).

The study also involved the use of honey samples from targeted beekeepers in the four locations in Marigat Sub County, Baringo County, associated with high honey production and also known to have limited use of herbicides, which included Kibingor, Koriema, Mukutani and Maoi as documented earlier (Henckle et al., 2018) and as shown in Fig. 3.1. The samples targeted included honey from honeybees (*Apis*) collected from beehives as well as from stingless bees (*Miliponines)* collected from clusters of resin egg-shaped pots near the extremities of their nests as shown in Appendix I.

### 3.4 Inclusion and Exclusion Criteria

### *3.4.1 Inclusion criteria*

Wound swabs were aseptically collected from patients' wounds that indicated visible signs of infection, those that had not taken any antibiotics or used any topical wound management therapeutic agent two weeks before the study. Freshly collected honey samples from the beehives of identified beekeepers were used for the study.

### *3.4.2 Exclusion criteria*

Those not to be considered for the study were patients with wounds that did not indicate any sign of infection, those with a history of taking antibiotics, or those who had used any topical wound management therapeutic agent two weeks before the study. Honey samples from regions with known use of many herbicides in farming or previously collected and stored honey were not considered appropriate for the study due to standard and quality discrepancies.

## 3.5 Sample Size Determination and Sampling Techniques

### 3.5.1 Sample Size Determination

Smith's method (2013) determined the sample size for wounds and swabs. This is because the available sample size is fixed, and hence, the formula could be used to generate anticipated precision values. Also, due to the abundance and variety of microorganisms in various wounds, they are influenced by wound category, depth, site, the host immune response and level of tissue perfusion.

$$S= \frac{Z^2X(P)X(1-P)}{C^2}$$

Where, Z – Z value (e.g., 1.96 for 95% confidence level)

P ¬ - Percentage picking a choice, expressed as a decimal.

C - Confidence Interval

$$\text{Hence; } \frac{(1.96)^2x(0.5)x(1-0.5)}{0.25} = 38$$

All patients with wound infections who had not been on any antibiotic treatment and who visited the Nakuru County Referral Hospital during the study period (March – June 2017) were incorporated into the study. Out of the 43 patients who attended the hospital, only 34 were included as study participants who were enrolled through convenient sampling following the laid inclusion/exclusion criteria and supported isolation of all the bacterial species under study.

Okur et al. (2020) employed the method for determining the requisite sample size for honey samples, which is collected to represent the overall population. This meant that the target population was honey samples from bee farmers in the Maoi, Mukutani, Kibingor and Koriema divisions. Hence, the Ministry of Livestock has discovered that there are 1500 bee farmers in 19895 homesteads.

$$N = \frac{Z^2XPXQ}{D^2}$$

N = Sample size

Z = Standard normal value of 1.96 for 5% sig. level

D = Statistical 5% level of significance (0.05)

Q = 1 - P proportion of population without characteristics of interest

P =1500 / 19895 (Average number of bee Farmers in Marigat Sub County / Average number of homesteads) (Mo ALF, 2017).

$$N = \frac{(1.96)^2 X\left(\frac{1500}{19895}\right) X 1\text{-}P)}{(0.05)^2} = \frac{103}{4} = 26$$

### 3.5.2 Sampling Techniques

#### *3.5.2.1 Honey sampling*

The honeybee samples were obtained from beehives using a method that was used before (Williams, 2019). In brief, the smoker was prepared by burning dry branches to give enough smoke which anaesthetise the sense organs of bees so that they cannot produce a chemical signal that has a stinging odour to alert other bees hence reducing their level of aggression. Then the door of the beehive was lifted off. To extract the honey, the thin layers of wax on the frames with the honeycomb were then scraped off with a stainless-steel knife. The exposed honey was then drained through a straining cloth placed over a sieve and into sterile containers. These containers were subsequently labeled with serial numbers, the location, and the date the honey was collected.

These stingless bees can also be controlled by smoke and since it does not sting, the hives were put in a dark room to clear the flies and aphids which may invade the honey nests. Some time was allowed for the swarm to move out of the colony, but the younger bees were moved manually to a safer shelter, then the honey pots

were pricked with a syringe and pressed inside the pot to suck the produce. Not all the honey was removed from the comb, but some were left with the young bees to allow for the continuity of the bee population.

#### *3.5.2.2 Wound swabs*

The wound swabs from surgical wards (outpatients and inpatients) of Nakuru County Referral Hospital were obtained following an ethical clearance from the medical superintendent (Appendix VI) and well-informed patients' consent (Appendix XVI). 34 patients were sampled using well-established SOPs as previously used (Misha et al ., 2021). A patient was considered to have a wound infection if the wound healing was poor, getting bigger or emanating pus or fluid. Those patients on antibiotic treatment two weeks before the scholarship was exempted. Blood samples were collected by a qualified registered clinical officer from both the outpatient diabetic clinic and surgical wards in the Nakuru County Referral Hospital, Kenya, using the standard local departmental protocols.

After performing hand hygiene and putting on gloves, the wound bed was cleansed with normal saline to help confirm that the swab collected denotes the microbiology in the deep wound compartment, thus reducing the likelihood of identifying surface contaminants. A small amount of normal saline was then applied to the head of the sterile swab to facilitate better adhesion of the bacteria to the swab as well as to the surface of the wound. The swab was then rolled in zigzag motions to ensure that the whole head came into contact with the wound's surface while twisting the swab. This was done by starting from the center of the wound to its periphery using constant forceful pressure to squeeze out some form

of liquid from the tissues of the wound (Byrd et al., 2018). One swab per patient was collected to isolate pure colonies from all cultural growth after taking the medication history and receiving informed consent from the patient.

### 3.6 Isolation and Characterization of Microorganisms

The wound swabs were immersed in a Mac Cartney bottle containing Stuart transport medium, which was labeled carefully with the patient's unique serial number and then transported to the laboratory for isolation and proper identification of the probable bacterial species following a set of procedures as stipulated by Oya et al., (2022).

#### 3.6.1 Isolation of Wound Microorganisms

##### *3.6.1.1Streaking on agar plates*

The streaking method was used in the culturing of wound swab samples as used before (Guan et al., 2021). Briefly, after flaming the neck of the bottle and turning the swab against the side of the container to eliminate surplus fluid, the successful dilution of microorganisms in the swab to a low density of recognizable individual colonies was done by dragging a sterile wire loop on the agar plate from the primary inoculum by making four to five zigzag movements hence decreasing the bacterial concentration with each swipe. MacConkey agar, Cystine lactose electrolyte deficient agar (CLED) and Blood agar were used followed by 24-hour incubation at $37^0$ C aerobically with periodic checking for any observable growth. Upon identification of bacterial growth on the surface of the medium, colonial morphology evaluation for characteristics such as form, texture, colour, margin, elevation and the opacity was carried out.

### 3.6.2 Characterization of the selected wound microorganisms

#### *3.6.2.1 Gram staining*

Gram staining technique was used in classifying the isolates obtained from wound swabs as used before (Oya et al., 2022). In brief, a loopful of three colonies of the bacterial isolate was mixed with water to form an emulsion smeared with a clean glass slide and dried by exposure to air and heat fixing, passing it several times through Bunsen flame. The slide was submerged in the crystal violet staining reagent for one minute, and then it was gently washed with tap water for two seconds. This process was repeated an endless number of times. After that, the slide was cleaned again and treated with Gram's iodine, which was left in place for two seconds. After that, the smear was rinsed for ten to fifteen seconds with acetone-alcohol, and then safranin was applied. The smear was then cleaned again and inspected under a microscope using oil immersion. Gram-negative bacteria developed a pinkish-red coloration, while Gram-positive bacteria displayed a blue-purple coloration.

#### *3.6.2.2 Biochemical reactions*

Biochemical characterization and identification of bacterial strain species were done through several biochemical tests under the guidelines of Bergey’s manual (Vakayil et al., 2020). Coagulase, Triple Sugar Iron (TSI), Methylred and oxidase were the principal tests used.

##### *i. Coagulase test*

The coagulase test protocol was used as used previously (Juwita et al., 2022) as it

is commonly used in determining the presence of the various species of the genus *Staphylococcus* that are differentiated into two groups: coagulase-positive *Staphylococcus* and coagulase-negative *Staphylococcus*. Briefly, the frozen rabbit plasma with EDTA was diluted in physiological saline by adding 0.2ml of plasma in 1.8ml physiological saline into a test tube, followed by an emulsion of 3colonies from a fresh culture, incubated at $37^0$ C for one hour and finally, the tubes were observed for clot formation. If the tubes had no observable clot, they were re-examined at 30-minute intervals up to six hours. A positive test demonstrates the agglutination of bacterial cells in plasma.

### *ii. Triple sugar iron agar tests*

The triple sugar iron agar test was also done in isolate identification, as was used in previous studies (Chauhan et al., 2020). This method mainly relies on the dissimilarities in carbohydrate fermentation and the production of hydrogen sulfide gas to classify the organisms. Carbohydrate fermentation is shown by the formation of gas bubbles and by the conversion of the colour of the red litmus paper to yellow. Inoculation in TSI was done using a straight inoculation needle by first stabbing through the TSI medium to the bottom before streaking on the agar surface of the slant. It was then incubated at $35^0$C to $37^0$C at ambient air through loosening of the screw caps for 18 to 24 hours; then the reaction of the medium was recorded.

### iii. *Methyl red (MR) test*

It was performed by inoculating the test microorganism in the glucose phosphate

peptone water contained in a screw-capped bottle followed by incubation at $37^0$ C for 21-48hours 5drops of methyl red and medium colour change to cherry red was considered positive (Islam, 2020).

*iv. Oxidase test*

This method mainly recognizes the ability of bacteria to produce the enzyme cytochrome oxidase through the process of applying a few drops of 1% tetramethyl-p- phenylenediamine dihydrochloride to a filter paper, smearing a small portion of the bacterial isolate to the paper and any development of a purple coloration is considered a positive outcome (Islam, 2020).

### *3.6.3 Molecular characterization of specific bacterial isolates' virulence genes*

*i DNA Extraction*

This was done according to the Fungi / Bacteria DNA premix kit from Zymo, USA, described by Vesty et al. (2020). To summarize, using a growth culture medium on nutrient agar of approximately 50grams, colonies from isolates on an overnight growth culture medium of the microorganisms under investigation which included *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae* were aseptically suspended in 200µl of nuclease-free water. The final homogenate was then transferred to a ZR Bashing Bead lysing tube. The Bashing Bead lysis tube was labelled and 750µl of bashing buffer was pipetted into the tube using the appropriate micropipette and the content in the tube was mixed thoroughly. The tube was then transferred into a 2ml tube holder connected to a bead beater and spun for 3 minutes at 10000xg. A microcentrifuge was used to

spin the ZR Bashing Bead lysis vessel at a rate of 10,000xg for a duration of one minute. The collected supernatant was pipetted onto the zymo-spin III – F filter in a total of 400 microliters. After that, this filter was placed inside a collection tube and centrifuged for one minute at 8000 revolutions per minute. This was accomplished by centrifuging the filtrate in the collection vessel for one minute at 10,000xg. Subsequently, 1200µl of Genomic Lysis Buffer was mixed with 800µl of the mixture and injected into the Zymo-spin II C column. After extracting the liquid from the collecting tube, 200µl of DNA pre-wash Buffer was added to the column. A further round of centrifugation was then carried out for a minute at a speed of 10,000xg. In the following phase, 500 microliters of g-DNA Wash Buffer were added to the Zymo-Spin II C column. The mixture was centrifuged at 10,000 rpm for 10 minutes, and the particle was collected in a sterile 1.5-milliliter microcentrifuge tube. After that, 100 microliters of DNA elution buffer were added to the sample and it was centrifuged for thirty seconds at a speed of 10,000 revolutions per minute.

### *ii Amplification of the Bacterial DNA*

Following the manufacturer's instructions, a total volume of 26µl of PCR master mix reagent was made. Lastly, 0.5µl of forward primer, 0.12µl of reverse primer, 5.5µl of nuclease-free water, and 12.5µl of Taq x 2 were added. After that, the liquid was carefully stirred for a short while using a vortex centrifuge before being

aliquoted. The following preset parameters were followed during the reaction in a machine with an XP thermocycler: One 4-minute cycle at 94$^{o}$Cwas followed by 40 cycles of denaturation (3 minutes at 94$^{o}$C), annealing (thirty seconds at 60$^{o}$C), and extending (2 minutes at 72$^{o}$C). 10 more minutes were spent at 72$^{o}$C. The polymerase chain reaction (PCR) results were examined on a 14-well agarose gel that had been stained with 16µl of ethidium. The ladder, which consisted of 100 base pairs, was introduced using the first well. Next, a mixture of the sample (5µl) and the loading pigment (3µl) was added to the remaining wells using a 20µl micropipette. Electrophoresis was carried out at 50v for 1 hour 15 minutes as used before (Zhang et al., 2020). The amplified bands were visualized under a UV trans-illuminator as their sizes were read using the ladder with specified base pairs (bp). Primers for the DNA amplification were synthesized and adopted from Inqaba Biotech East Africa Ltd (IBE002) (Table 3.1). They were used to determine the virulence genes among the bacterial isolates using well–established procedures (Winand et al., 2019). The master mix reagent for polymerase chain reaction (PCR) was prepared according to the kit instructions, with a total reaction volume of 26 µl. The tube was filled with 12.5 µl of Taq x 2, 0.5 µl of forward primer, 0.12 µl of reverse primer, and 5.5 µl of nuclease-free water. Vortex centrifugation was used to mix the contents of the vessel a few seconds later. An XP Thermocycler machine was used to carry out the reaction. The cycling conditions were as follows: a 4-minute initial denaturation at 94$^{\circ}$C, 40 cycles of denaturation at 94$^{\circ}$C for 3 minutes, annealing at 60$^{\circ}$C for 30 seconds, two cycles of extension at 72$^{\circ}$C, and a 10-minute extension at 72$^{\circ}$C. The PCR products were

visible on a 14-well 1% agarose gel that had been prepared with 16µl of ethidium. The first well had 8µl of the ladder (100 bp), and the ladder was loaded into the other wells. Using a 20µl micropipette, 3µl of loading dye and 5µl of the sample were dispensed. Samples were subjected to electrophoresis for 1 hour 15 minutes at 50v, as was done by Zhang et al. (2020). The amplified bands were illuminated under a UV trans-illuminator, and the sizes of the bands were determined using the ladder containing specified bps. PCR primers for DNA amplification were purchased and obtained from Inqaba Biotech East Africa Ltd (IBE002) (Table 3. 1) and were used to find out the virulence genes among bacterial isolates (Winand et al., 2019).

**Table 3.1:** Primer design for amplifying virulence genes in PCR reactions to characterize the pathogenic bacteria isolated from the collected wound swabs.

| S/NO. | PRIMER | BACTERIA | SEQUENCE | BAR CODE | LENGTH (BP) | REFERENCE |
|---|---|---|---|---|---|---|
| 1 | CNF1 – F | *E. coli* | AGGATGGAGTTTCCTATGCAGGAG | S449B | 24 | El-Shaer et al.,2018 |
| 2 | CNF1 – R | *E. coli* | CATTCAGAGTCCTGCCCTCATTATT | S449C | 25 | El-Shaer etal.,2018 |
| 3 | CNF2 –F | *E. coli* | AATCTAATTAAAGAGAAC | S449D | 18 | El-Shaer etal.,2018 |
| 4 | CNF2 –R | *E. coli* | CATGCTTTGTATATCTA | S449E | 17 | El-Shaer etal.,2018 |
| 5 | HLYA –F | *E. coli* | AACAAGGATAAGCACTGTTCTGGC | S449F | 24 | Khairyetal.,2019 |
| 6 | HLYA –R | *E. coli* | ACCATATAAGCGGTCATTCCCGTC | S44A0 | 24 | Khairyetal.,2019 |
| 7 | HLA –F | *S. aureus* | CTGATTACTATCCAAGAAATTCGATTG | S44A1 | 27 | Elsayed et al.,2015 |
| 8 | HLA –R | *S. aureus* | CTTTCCAGCCTACTTTTTTATCAGT | S44A2 | 25 | Elsayed et al.,2015 |
| 9 | 16SrRNA –F | *S. aureus* | AACTCTGTTATTAGGGAAGAACA | S44A3 | 23 | Ciftci et al.,2009 |
| 10 | 16SrRNA –R | *S. aureus* | CCACCTTCCTCCGGTTTGTCACC | S44A4 | 23 | Ciftci et al.,2009 |
| 11 | KHE –F | *K.. pneumoniae* | GATGAAACGACCTGATTGCATTC | S44A5 | 23 | Whitaker et al.,2018 |
| 12 | KHE –R | *K. pneumoniae* | CCGGGCTGTCGGGATAAG | S44A6 | 18 | Whitaker et al.,2018 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 13 | RMPA –F | *K. pneumoniae* | AGAGTATTGGTTGACTGCAGGATTT | S44A7 | 25 | Hartman etal.,2009 |
| 14 | RMPA –R | *K. pneumoniae* | AAACATCAAGCCATATCCATTGG | S44A8 | 23 | Hartman etal.,2009 |
| 15 | MAGA –F | *K. pneumoniae* | CGAAAGTGAACGAATTGATGCT | S44A9 | 22 | Whitaker et al.,2018 |
| 16 | MAGA –R | *K. pneumoniae* | GTTTCTGCTGCAGATTCGAAGA | S44A | 22 | Whitaker et al.,2018 |
| 17 | LASL –F | *P. aeruginosa* | ATGATCGTACAAATTGGTCGG | S44AB | 21 | Aghamollaei etal.,2015 |
| 18 | LASL –R | *P. aeruginosa* | GTCATGAAACCGCCAGTC | S44AC | 18 | Aghamollaei etal.,2015 |
| 19 | GYRB –F | *P. aeruginosa* | CCTGACCATCCGTCGCCACAAC | S44AD | 22 | Aghamollaei etal.,2015 |
| 20 | GYRB –R | *P. aeruginosa* | CGCAGCAGGATGCCGACGCC | S44AE | 20 | Aghamollaei etal.,2015 |

### *3.6.4 Determination of Honey Microbial Purity*

The adulteration of honey was analyzed using the methods that have been applied in previous research (Klutse et al., 2021). After filtering the raw honey, 25g of honey was aseptically weighed and then dissolved in 100ml of distilled water in the beaker that was autoclaved earlier. The solution was streaked on the blood agar plates using a sterile inoculation loop. The same was done with 50g of honey diluted in 100ml of distilled water to prepare 0.25g/ml and 0.5g/ml, respectively. These plates were then incubated for 72 h at 37$^{o}$C and the samples which did not have bacterial growth on them were selected for analysis. They were kept in the screw-capped bottles with a lid in the dark place.

## 3.7 Analysis of the physico-chemical and nutritive properties of honey samples

from stingless and honeybees

### 3.7.1 The physico-chemical properties of honey samples from stingless and honeybees

All the samples were analyzed in triplicates and in the same period to ensure that the condition was similar and that the results were comparable. This was done based on the standard procedures as highlighted by the International Honey Commission 2009 (Hocine et al., 2018).

#### *3.7.1.1 Determination of sugar content*

It was estimated using a spectrophotometer following the method of Fox and Robyt (2018). The weighed honey sample (2.0g) was then measured using a 150ml beaker and transferred into a clean and dry weigh boat before gradually adding 10ml of dimethyl sulfoxide (DMSO) (25% v/v) while stirring until a homogeneous solution formed. This was done before incubation for 20 minutes in a water bath maintained at 100 $^{\circ}$C. This was accomplished by diluting 0.5 ml of the obtained combination in a 100 ml test tube, and then adding the resultant mixture to 9.5 ml of distilled water. A 0.5 ml solution of 5% phenol was pipetted into the mixture that had been diluted in a test tube. After giving the test tube a good shake, 2 ml of sulfuric acid ($H_2SO_4$), which had a 75% concentration, was introduced gradually down the side. After that, the mixture was put into a cuvette, and its absorbance at 492 nm was calculated by comparing it to a standard glucose solution. Three separate versions of the studies were conducted.

#### *3.7.1.2 Determination of moisture in honey samples*

The method described by Azemi et al. (2021) was utilized to determine the amount of moisture that was present. Following a precise weighing process, a hot oven was used to heat the 2.0-gram honey samples to a temperature of 70 °C. The samples were taken out of the oven once they had reached a uniform mass, indicating that all of the moisture had been eliminated. Weighing the desiccated samples again after they had cooled allowed us to determine their oven-dry mass. The moisture content was then defined as;

$$Mc\ \% = \frac{\text{Initial wt. – Oven- dry weight}}{\text{Oven – dry wt.}} \times 100\%$$

All the tests were performed in three replicates unrelated to one another.

### *3.7.1.3 Determination of pH in honey samples*

According to Yakubu et al. (2021), a digital pH meter with pH calibration scales of 4 and 10 was used to determine the pH of the honey. 75 milliliters of distilled water were used to dissolve 10 grams of honey to create the honey solution. Following the preparation of the honey solution, an electrode and stir bar were standardized. By analyzing the figure on the meter, the pH of the solution was ascertained. The trials were carried out three times using three different setups to guarantee the validity of the findings.

### *3.7.1.4 Determination of titratable acidity in honey samples*

Nemo and Bacha (2020) provided an account of the standard operating methods used to determine the titratable acidity of honey. A 250-ml container containing honey was filled with 75 ml of distilled water. The mixture was then shaken some

more after the pH/reference electrode and stir bar were dipped into the honey solution. The pH was brought to 8.5 by adding 0.05M sodium hydroxide, and the discharge rate was set at 5 milliliters per minute. After that, the reaction was allowed to happen, and the outcomes were recorded. During the experiment, 0.50 ml of hydrochloric acid was added to the solution to bring its pH up to 8.3. A 0.05M NaOH solution in 10 ml was used for the titration. The first step was creating a blank determination procedure using seventy-five milliliters of deionized water. The pH was then brought down to 8.5.

For computation, free acidity is the amount of total acidity that can be neutralized by sodium hydroxide up until it reaches the same level as the neutralizing agent.

$$\text{F.A. (meq/kg)} = \frac{\text{ml of 0.05M NaOH used x NaOH Molarity blank x1000}}{\text{Weight of sample (g)}}$$

### *3.7.1.5 Determination of Hydroxy- methyl furfural (HMF)*

Using the spectrophotometric approach, the UV absorbance of honey solutions prepared both with and without the addition of sodium metabisulfite was measured (Yakubu et al., 2021). Determining the HMF concentration required these information. Five grams of honey are dissolved in twenty-five milliliters of distilled water to form a control sample. Next, the resultant mixture is poured into a volumetric vial with a capacity of 50 milliliters. Subsequently, 0.15 ml of Carrez solution I-15 ml (15 grams of potassium ferrocyanide in 100 ml of distilled water) and 0.5 ml of Carrez solution II – Zinc acetate solution (30 grams per one hundred milliliters of distilled water from Carrez) were added to precipitate the proteins. Filtration was performed with filter paper, and the first ten milliliters of the filtrate were discarded. The ingredients were increased to a total volume of fifty milliliters using distilled water. For this experiment, aliquots of five milliliters each were

moved to two different test containers. One test tube was filled with the sample solution after it had been diluted with five milliliters of distilled water. The other test tube was filled with a fresh sodium metabisulfite solution that contained 0.2% sodium. The absorbance of these solutions was then measured with a UV-visible small ± 1240 Shimadzu spectrophotometer at two different wavelengths: 284 nm and 336 nm. Every experiment was carried out three times to guarantee that the outcomes could be precisely repeated.

Calculation: (Abs 284 – Abs 336) x 149.7 x 5 x D / w

#### *3.7.1.6 Determination of hydrogen peroxide in honey*

The technique described by Makhloufi et al. (2020) was used to accurately determine the hydrogen peroxide concentration in honey samples. Three grams of honey were dissolved and then weighed out to create a solution with a thirty percent (w/v) weight-to-volume ratio and a pH of eight. 1 ml of distilled water was then added. After that, a pH meter was used to measure and correct the solution's pH to the proper value. Subsequently, the mixture was heated to 37°C in a water bath for thirty minutes, or sixty minutes. The detection of hydrogen peroxide was achieved using test strips. After submerging the segments in the solution for a single second, the solution was taken out. The concentration of hydrogen peroxide in the honey sample was then determined by comparing the color code with the color that had grown on the strip.

All the samples were analyzed in triplicate to help detect any technical variability within the experiment and for accuracy purposes to obtain good data. The results were then recorded for analysis.

### 3.7.2 The nutritive properties of honey samples from stingless and Honeybees

### *3.7.2.1 Determination of sugar content*

Determining the sugar concentration in the honey samples was done as described by Fox and Robyt (2018). Add 2.0g of honey sample into a 150 ml beaker and mix with 10 ml of 25% (v/v) dimethyl sulfoxide solvent. The mixture was then stirred for further 30 minutes until the colour became that of a clear, homogenous solution. The mixture was stirred until all the contents of the flask were evenly distributed. These solutions were allowed to stand in a hot water bath at 100$^{o}$C for 20 minutes. After incubation, 0.5 ml of the solution was then added to 9.5 ml of distilled water in a 100 ml test tube. To this diluted mixture, 0.5 ml of phenol was added to the solution. Then, slowly, 2 ml of 75% sulfuric acid ($H_2SO_4$) was added down the side of the test tube. The sample mixture was then taken in a burette and the absorbance of the solution was then determined at 492nm regarding a standard glucose solution. All the tests were performed three times.

### *3.7.2.2 Determination of Honey Protein Concentration*

In the previous literature, the Kjeldahl method was used to determine the crude protein content in honey, as was done in this study (Brown et al., 2020). 0.5g of honey was measured into Kjeldahl digestion tubes and 2.5g of a catalytic mixture consisting of 10 grams of sodium sulfate and 1 gram of copper sulfate pentahydrate were added then the solution was shaken. This was followed by adding 7 ml of sulfuric acid to each tube, which was then mixed. The labeled tubes were then placed in a block digester and the temperature of the block digester was raised from 50$^{o}$C to 400$^{o}$C and it was again kept at the temperature for 5 hours.

These samples were then digested and dissolved in 10ml distilled water. In another set of 125 ml Erlenmeyer flasks, 15 ml of 5% boric acid solution was added and then 5 drops of methyl red/bromocresol green mixed indicator was added. The digestion tubes were filled up to 20ml 50% Sodium hydroxide until the sample turned dark blue. The samples were then titrated against 0.01M hydrochloric acid in a burette until the endpoint when the solution turned pink. This titration data was used to calculate the protein content.

$$\% \text{ protein} = \frac{\text{V x M x FC x 0.004 x 100 x 6.25}}{\text{M}}$$

Where V = Volume of HCl used in titration

M = Molarity of HCL

FC = Correlation factor of HCL

6.25 = Correlation factor for proteins

M = Sample weight

### *3.7.2.3 Determination of Vitamin C Content*

The amount of vitamin C in honey samples was determined recently by using several standard methods as mentioned in the previous papers (Sawicki et al., 2020). To the honey samples, 100 ml of each honey sample was accurately weighed into respective flasks, and 10 ml of 1% metaphosphoric acid was added

to the honey samples. Then the contents were stirred for approximately half an hour at room temperature to form the solution to be homogeneous. It was then filtered using Whatman filter paper No. 4. 1 ml of the filtrate was mixed with 9 ml of 0.005% 2,6-dichlorophenolindophenol (DCPIP) solution. The absorbance of the colored complex at 515nm wavelength was then measured after 30 minutes of reaction from the spectrophotometer.

#### *3.7.2.4 Determination of Water-Soluble Vitamins in Honey ($B_2$, $B_3$, $B_5$ and $B_9$)*

To estimate the concentration of vitamins in the honey samples, the amount of vitamins B2, B3, B5 and B9 was determined using the technique described earlier (Alvarez-Suarez et al., 2018). 10g of each honey sample was weighed and dissolved in 10 ml of distilled water by stirring. To this solution, 1 ml of 2M sodium hydroxide was added and stirred well until the honey dissolved completely. Next, 12.5 ml of 1 M phosphate buffer with the required pH of 5.5 was added and dissolved into the solution properly. The solution was then diluted up to 50ml mark with distilled water in a volumetric flask. The prepared sample solutions were injected through a filter and the absorbance was measured at selected wavelengths specific to each vitamin: 210 nm for both vitamin B2 and B5, 254 nm for vitamin B3 and the same wavelength of 210 nm but for vitamin B9.

#### *3.7.2.5 Determination of Calcium, Magnesium, Iron and Zinc in Honey*

As in the past, the elemental composition of the minerals was ascertained using an Atomic Absorption Spectrophotometer (AAS) (Tutun et al., 2019). Ten grams of each honey sample were heated under an infrared light source until they weighed

five hundred grams to create the foam. This was done to manipulate the foam. The obtained ash samples were broken down using 10 ml each of 60 % $KMnO_4$ and 65% $HNO_3$. Using Whatman filter paper No. 4, the solutions were filtered until they were clear. Using the ash solutions with an air acetylene flame and a hollow cathode lamp, a qualitative evaluation of the individual minerals was carried out. The apparatus was, at the very least, calibrated once a week using criteria that were previously established for every minerality. A wide range of wavelengths were applied to the metals under examination: zinc at 213 nm, copper at 324 nm, iron at 248 nm, and magnesium at 285 nm.

#### *3.7.2.6 Determination of Sodium and Potassium in Honey Samples*

According to Lanjwani and Channa (2019), this was carried out in compliance with the procedures that are generally followed when looking into situations of this kind. An accurate weight measurement of 0.5 grams was made before the material was transferred to a 15-milliliter polypropylene vial. The tube was then gently shaken after 0.4 milliliters of a caesium chloride solution with a weight-to-volume ratio of 5% were added. The solution was then added to till it was 10 milliliters in volume by adding N-propanol. After that, the mixture was constantly stirred until it turned transparent. First, the internal standards were made by diluting ethanol and yttrium at a ratio of 1:10 (m/v). Next, the absorbance at 371 nm was measured. The sample solutions were obtained by pipetting, and 100µl of each sample solution was atomized into the flame using a micro funnel that was attached to a pneumatic nebulizer. The analysis was done in Flame Emission Mode at a specific wavelength for the minerals under test: potassium at 766 nm and Sodium at 589

nm.

### *3.7.2.7 Determination of Phosphorous in Honey Samples*

Lanjwani and Channa (2019) determined phosphorus in honey samples: Around 10g was placed in a porcelain crucible from each honey sample while 5 ml of 1N nitric acid was added. These solutions were then aliquoted into the respectively labeled tubes and subjected to heat for 3 minutes in the water bath to dissolve the solute as much as possible and make the solutions as straightforward as possible. Each honey sample was dissolved in distilled water and distilled water was added to complete the volume of 100ml. After that, the honey samples were filtered through the Whatman filter paper. 5ml of each of the prepared test solutions was then pipetted into a 100-millilitre volumetric flask. To each flask, 10ml of 0.1mg/ml of this solution was then added with 10ml of 6 N nitric acid and the mixture was stirred. This was followed by adding 10ml of 0. A 2% ammonium vanadate solution and 10ml of a 5% ammonium molybdate solution. The samples were then diluted to 100ml with distilled water and agitated and left to sediment for 15 minutes before color development. Last of all, the absorbance of the respective solution was measured at 400 nm wavelength in comparison with the reagent blank and the concentration of phosphorus was determined from a standard formula.

$$\frac{Pmg}{100g} = \frac{Abs\ am \times 1\ Vo \times 10}{Ab_{std} \times Vp \times W}$$

Where: $Ab_{sam}$ - Absorbance sample.

$Ab_{std}$ - Absorbance Standard (1mg/ml).

Vo. – Total volume (ml).

Vp. – Volume of a diluted sample (ml).

W – Sample weight (g).

## 3.8 Determination of the preliminary phytochemical Properties of Honey of the honey from stingless and honeybees

### 3.8.1 Total Content of Phenolic Compounds (TCPC)

Identification and determination of phenolic compounds in honey samples followed a method described by Avila et al. (2019) based on liquid chromatography of the phenolic extracts obtained from honey samples. 5 g of each honey sample was mixed with fifty milliliters of distilled water and the resultant mixture was stirred to ensure homogeneity for one hour using a magnetic stirrer and then filtered using Whatman filter paper. To the filtrate, 2.5ml of 0.2N Folin-Ciocalteu reagent was added, and the contents were mixed and allowed to stand at room temperature for 5 minutes. Then, 2ml of 75g/l sodium carbonate ($Na_2CO_3$) solution was pipetted to the mixture and stirred before the mixture was allowed to stand in the dark at room temperature for 2 hours. The absorbance of the reaction mixture was read at 760 nm against the methanol blank on the spectrophotometer. Gallic acid was used as a reference compound, and the concentration of the individuals was expressed as milligrams per equivalent gallic acid (mgGAE). The samples of all the homey ingredients were collected thrice through triplicate measurements, the observations were then

recorded and ultimately the mean was determined. The total phenolic content in the honey samples was analyzed using the method described by Avila et al. (2019). In preparing the sample solution, 5 grams of every honey sample procured was dissolved in 50 milliliters of distilled water after which the mixture was stirred for one hour to ensure homogeneity before filtering through the Whatman filter paper. To the filtrate, 2.5ml of 0.2N Folin-Ciocalteu reagent was introduced to the tube then the contents were mixed and left at room temperature for 5 minutes. After that, 2ml of 75g/l sodium carbonate ($Na_2CO_3$) solution was added to the mixture and then shaken, the samples were incubated at room temperature in the dark for approximately 2 hours. The absorbance of the reaction mixture at 760nm as the reference was calibrated using a methanol blank in a spectrophotometer. Gallic acid was used as the reference compound and the contents were expressed as milligrams of gallic acid equivalents (mgGAE). For all the homey samples, measurements were taken in triplicate, the result was recorded, and the mean value was determined.'

### 3.8.2 Total Content of Flavonoid (TCF)

The quantity of flavonoids in the honey samples was determined using a method developed by Bagheri et al. (2021). 0.5 grams of the honey was weighed into a beaker, and 5 ml of fifty percent methanol was added and mixed to form a sample solution. The above solution was then filtered through Whatman No. 1 filter paper to remove any solid that may not dissolve. 5ml of the filtered honey solution was then reacted with 5ml of 2% aluminum chloride ($AlCl_3$) by diluting both solutions and stirring the two solutions together. The mixture was then left for half an hour

at room temperature to form the aluminum–flavonoid complex. After incubation, the absorbance of this complex was determined using a UV-visible spectrophotometer at 420nm. Rutin at a concentration of 0-100mg/L was used as standard. The TFC of the honey samples was chosen as the mean value of triplicate determinations in milligrams of rutin equivalent per gram of honey.

### 3.8.3 Total Content of Carotenoids (TCC)

The total carotenoid content was estimated using various methods, as Sawicki et al. (2020) mentioned. 1 gram of each honey sample was mixed with 10 ml of n-hexane. An acetone mixture was added to the slurry and homogenized for 10 minutes. The above-prepared mixture was, therefore, stirred and left in the darkness at laboratory temperature for around 30 minutes. The assimilate of each test sample was prepared as follows: After incubation, the solution was poured into Whatman No. 1 filter paper and absorbance of the filtrate was measured at 450nm with the help of a spectrophotometer. The total carotenoids were quantified and reported in milligrams of β-carotene equivalents (mg β-carotene/kg honey). These findings were measured as the mean of the absorbency triplicate assays.

### 3.8.4 Determination of Vitamin C (ascorbic acid) Content

The analysis of honey samples for their ascorbic acid (vitamin C) content, the procedure described by Sawicki et al. (2020) was used. For each honey sample, 100g was taken into a beaker and this 10ml of 1% metaphosphoric acid was added and then stirred until homogeneous at room temperature. The obtained

solution was then filtered using the Whatman filter paper brand. To 1 ml filtrate, 9 ml of 0.005% 2,6-dichlorophenolindophenol (DCPIP) solution. The absorbance of this final mixture was then taken within the next 30 minutes at 515 nm. These were determined in triplicate and presented in milligrams of ascorbic acid per kilogram of honey.

### 3.9 Quality Assurance

Standardization procedures and additional checks were also applied to all the laboratory assessments, which aimed to maintain the false-positive and false-negative rates and provide quality control for the study results. Controlling the power of equipment, reagents, and procedures of each procedure was appropriate and done aseptically. A chemically produced honey, produced by dissolving 67g of glucose, 80g fructose, 3g sucrose and 15g maltose with distilled water in 34ml, was used as the negative control. Bacterial strains used for this study were obtained from the American Type Culture Collection (ATCC). They included *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 27736), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC). The bacterial strains were obtained from Nakuru Veterinary Laboratory and were used according to the Clinical and Laboratory Standards Institute (CLSI, 2016).

### 3.10 Comparison of the antibacterial effectiveness of honey from stingless and honeybees with other conventional antibiotics against the isolated pathogens from cutaneous wounds

#### 3.10.1 Preparation of honey discs

Honey samples from honey and stingless bees were used to prepare different concentrations by weighing 10g of the honey into a 100ml volumetric flask, 2ml distilled water was added and the solution was mixed well, then topped to 100ml mark with distilled water. The same was repeated with 20g, 50g and 75g sterile Whatman No. 1 filter paper discs (6mm) were impregnated with each concentration of every honey type ($10x10^4$, $20x10^4$, $50x10^4$ and $75x10^4$ µg/ml) and allowed to dry under sterile conditions.

### 3.10.2 Disc diffusion method

The disc diffusion (Kirby-Bauer) technique was earlier used in the study of (Khan et al., 2019). Mueller-Hinton agar plates were prepared according to the manufacturer's instructions provided in the manual. For each bacterial isolate, an inoculum was prepared by suspending, in distilled water, two colonies from a bacterial colony, and the turbidity was standardized to that equivalent to 1. McFarland turbidity standards are five times one hundred and eight colony-forming units per milliliter.

The disc diffusion (Kirby-Bauer) method was used as described in the previous study (Li et al., 2020; Mama et al., 2019). The cauterized cotton-tipped scalpel was soaked in the bacterial suspension prepared at $1.5x10^8$ CFU/ml per McFarland standards and the swab was rolled across the side of the tube to eliminate excess fluid.

A swab was spread uniformly across the MHA plates for inoculation. The plates were left for approximately 15 minutes to dry after which discs were impregnated

with the honey samples in different concentrations of ($10 \times 10^4$, $20 \times 10^4$, $50 \times 10^4$ and $75 \times 10^4$ µg/ml), reference standard samples (positive control) and discs containing only artificial honey (negative control) were inoculated on the Petri dishes using sterile forceps and gently pressed until the discs made direct contact Care was taken to ensure that all the bacterial inocula were 15mm away from the plate edges to avoid interference between zones of inhibition.

The honey samples were spread on the inoculated plates and kept at 4°C for 2 hrs and at 37°C for 24 hrs. Subsequently, the diameters of the inhibition zones should be measured on all the Petri dishes. The following antibiotic cartridges were employed: ampicillin (10µg), Levofloxacin (5µg), meropenem (10µg), tazobactam (110µg), chloramphenicol (30µg) and gentamycin (10µg). A control was made using a weak reaction on the coded, autoclaved and sterile plain filter paper discs. The zone diameters were then used as measure of the isolate's resistance to the drug activity or the rate of drug diffusion to the agar media as highlighted by the CLSI-2018. Each inhibition zone diameter was measured to the nearest millimeter on an aspect perpendicular to the petri dish using a ruler placed perpendicularly on the petri dish. These were selected because they are readily available, not expensive, effective on Gram-positive and Gram-negative bacteria and because ampicillin and penicillin derivatives are often used to manage wound infections.

### 3.10.3 Evaluation of the bacteriostatic and bactericidal activity of honey

The effectiveness of the collected honey samples against bacterial isolates was determined using the microdilution technique (Hasan et al., 2020). This study sought to determine and compare stingless bee honey's bacteriostatic and

bactericidal properties on four bacterial isolates obtained from wounds. The microdilution method on the stingless bee honey samples determined the MIC or the concentration below, for which any test microorganisms had no visible growth.

The concentration shows the amount needed to inhibit bacterial growth, revealing that the stingless honey sample inhibited all the isolated bacteria, which was 625µg/ml. In comparison, the MBC was 2500µg/ml. However, in this study, the bacteriostatic activity was found to be highest for honey samples at 1250µg/ml as depicted in Table 3.6 isolated below with Escherichia coli being the most vulnerable isolate as it exhibits the highest MIC activity at a concentration of 1250µg/ml and was eradicated at higher concentration of 2500µg/ml. Consequently, it is possible to conclude that honey possesses bacteriostatic and bactericide effects like an antibiotic.

### 3.11: Analysis and Presentation

The raw data was tabulated and then processed to reduce the complexity of programming and analysis in the study using IBM SPSS Statistics 23 to analyze variance and frequency. Two–way ANOVA test using Graph Pad Prism (Version 7. 0) statistical software to get the P – value < 0. 05 to compare significant variations in the experiments and drawing of graphs were also done. Variables were deemed significant by and highly significant when P < 0. 01(*), P < 0. 001 (***), P< 0. 0001(****). All the experiments were performed in triplicate independently and the data was represented as Mean ± SD.

### 3.12 Ethical Consideration

Research and ethics clearance for the research proposal was acquired from the

research ethics committee of the University of Eastern Africa, Baraton and the National Commission for Science & Technology (NACOSTI) (Appendix V & VI, respectively). The medical superintendent granted wound swab collection authority- to County Referral Hospital – Nakuru (Appendix VII), while the Chief Medical Officer authorized the use of the bacteriology laboratory through the Chief Medical Laboratory Technologist – at Egerton University (Appendix VIII). Individual informed consent (Appendix IX) was also sought from every patient before taking the wound swabs.

# CHAPTER FOUR

# 4.0 RESULTS

## 4.1 Introduction

### 4.1.1 General bacteriology

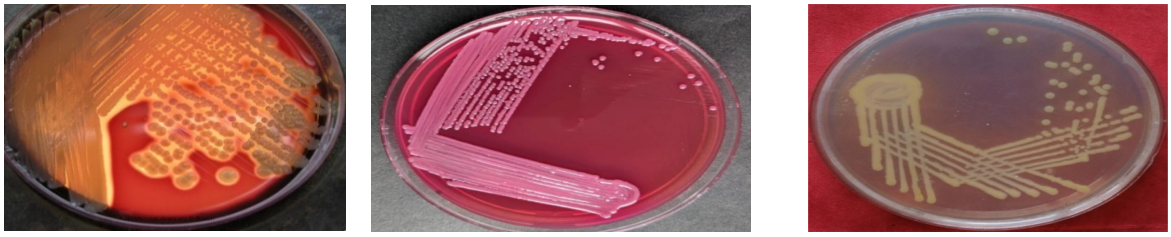
The bacterial isolates from burns and cutaneous wounds were identified depending on the colonial morphologies and Gram staining methods and the biochemical results. The bacterial isolates were then further characterized based on the presence of virulence genes of the respective bacterial species relating to the pathogenesis of wound infection. The honey samples obtained from honey and stingless bee species were then diluted in different concentrations and their bacteriostatic and bactericidal efficacy were determined as shown below. Thus, the following characteristics of honey and stingless bee honey samples exposing the bacterial isolates were emphasized in this study.

## 4.2 Isolation of bacterial isolates from cutaneous wounds

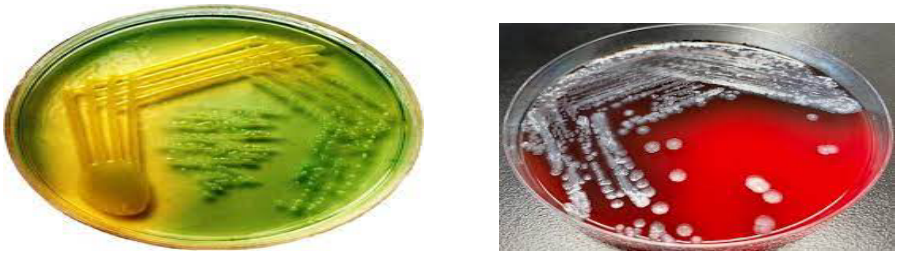
### 4.2.2 Cultural characteristics

Therefore, the following morphological colony variants were identified from the

data collected in this cross-sectional study on the macroscopic examination of bacterial colonies from wound swab samples. Colonies formed a halo of clear zones of beta hemolysis on blood agar as demonstrated in Fig 4.1a, pointing more to the presence of *Staphylococcus aureus*. Floaters, big, circular, red/pink donut-shaped colonies formed on MacConkey agar with a zone of precipitated bile salts around it, as depicted in Figure 4.1b. The biochemical test indicated that the patient had *Escherichia coli* as some of this bacteria's characteristics were seen: Fig 1c, thick, grey-whitish moist colonies on nutrient agar as shown in Fig 4.1c. Yellow to whitish blue colony, very mucoid on CLED agar (which is not recommended for supporting all the circulating strains of the organism) as seen in *Klebsiella pneumoniae* shown in figure 4.1d. Different large, white to pink, flat, irregular colonies with alligator skin-like surfaces and with the typical grape-like odour, as described in Fig 4.1e. It was inferred from 1e that the isolate was *Pseudomonas aeruginosa*.



(a) (b) (c)



(d) (e)

**Figure 4.1**: Colonial morphology of bacterial isolates

**Colonial morphology of bacterial isolates as shown on the plates; Colonies surrounded by clear zones of beta haemolysis on blood agar, Uniform re/pink donut-shaped colonies surrounded by precipitated bile salts in MacCConkey agar, Opaque, yellow lactose fermenting colonies on MacConkey agar, Thick greyish white, moist, smooth opaque colonies on Nutrient agar, Mucoid lactose fermenting on CLED agar and Pink, flat, irregular colonies with alligator skin-like surface on MacConkey agar.**

Of the total 4 burn and 24 cutaneous wound swabs, 28 samples (82.35%) showed good bacterial growth associated with wound infection. In comparison, 6 samples out of 1 burn and 5 cutaneous wound swabs (17.6%) showed low bacterial growth, as depicted in Table 4.1. Among the study patients, 18 (52%) were farmers with surgical wounds, while 9 (27%) were motorbike riders with abrasion and burn wounds. Pure bacterial cultures were derived from a mixed culture by transferring single colonies onto nutrient agar and spreading it across the surface to separate individual cells. Of the total, 15 (44.1%) of the wound swabs were found to produce 1 isolate, 11 (32.4%) produced 2 isolates each, and two (5.9%) produced 3 isolates each, thus giving a total of 43 pure isolates.

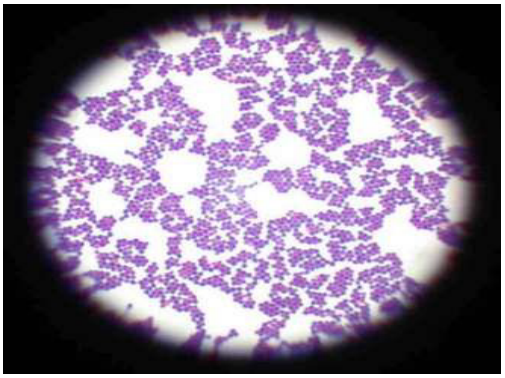
**Table 4.1:** Cultural proportion of pure bacterial isolates from infected cutaneous wounds

| Type of swab | No. collected | No. infected | No. of pure isolates | Frequency (%) |
|---|---|---|---|---|
| Burn | 6 | 4 | 6 | 14.7 |
| Wound | 28 | 24 | 37 | 82.4 |

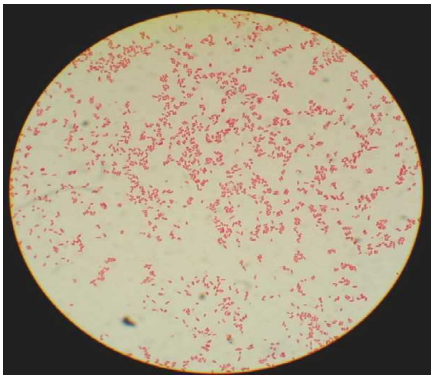
## 4.3 Characterization and identification of bacteria

### 4.3.1 Gram stain

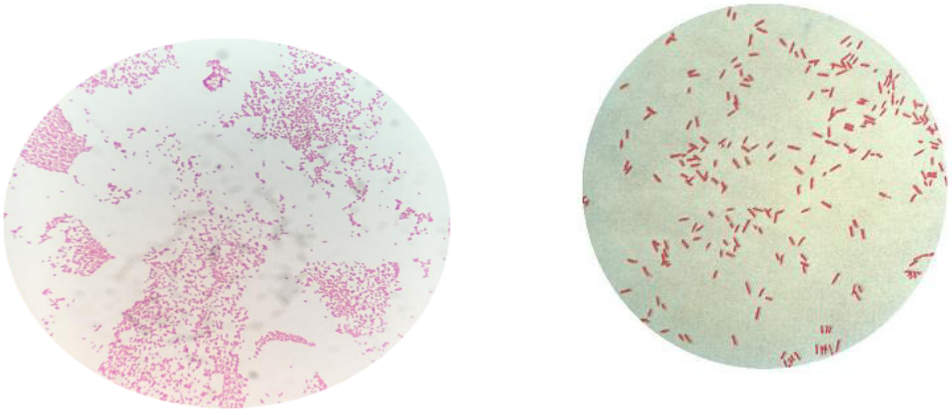
The below Gram reactions were noted in Gram staining as follows: Gram-positive (purple) cocci-shaped bacteria in clusters 'grape-like' formations suggesting the presence of Staphylococcus aureus bacteria as depicted in figure 4.2a below. Single and pairs of tiny Gram-negative pink-red rod-shaped bacteria indicating the presence of *Escherichia coli* are represented in Figure 4.2b below. Pink-red rod-shaped bacteria predominantly present as isolated rods, paired and in short chains with the morphological configuration of appearing capsulated, resembling *Klebsiella pneumoniae,* as demonstrated in Figure 4.2c below. Finally, Gram-negative encapsulated rod-shaped bacilli that appeared to be slightly slender and paler than those of *Enterobacteriaceae*, suggestive of *Pseudomonas aeruginosa,* are depicted in Figure 4.2d.



(a)



(b)



(c) (d)

**Figure 4.2:** Gram stain reaction of bacterial isolates

**Gram stain reaction of bacterial isolates: Gram-Positive grape-like cocci, Gram-Negative rods arranged singly and in pairs, Plump rod-shaped Gram-Negative bacilli and Slimmer pale-staining Gram-Negative rod-shaped bacilli**

The proportion of wound swabs that contained the varying Gram reaction microorganisms was also indicated as tabulated in Table 4.2.

**Table 4.2:** Proportion of Gram staining reactions of bacterial isolates from the collected wound swabs

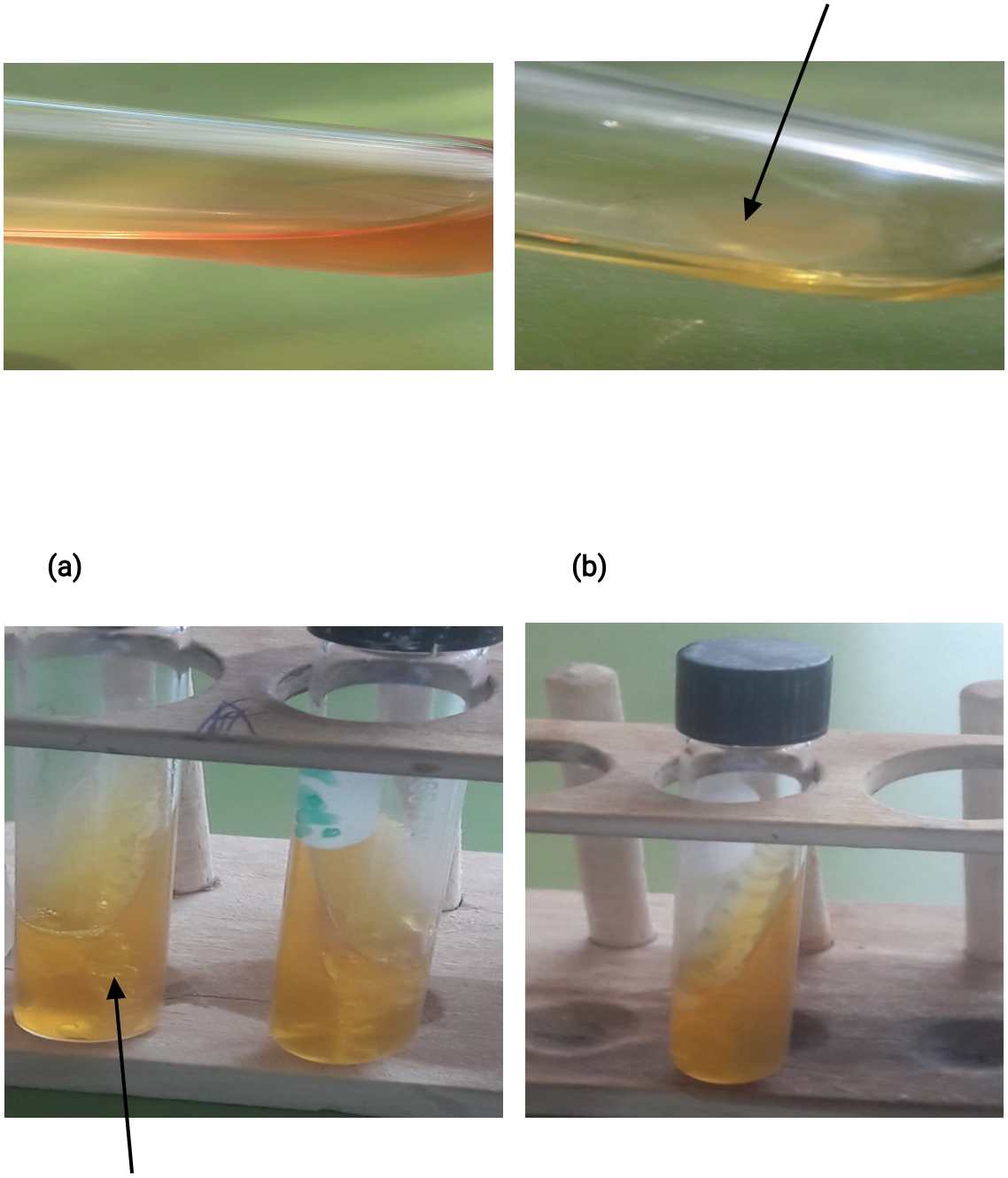
| Bacterial isolates | Gram reaction | Morphology | No. of swabs | Frequency (%) |
|---|---|---|---|---|
| Gram stain 1 | Gram-positive Gram | Cocci | 10 | 29.4 |
| Gram stain 2 | Negative | Rods (single/pair) | 12 | 35.3 |
| Gram stain 3 | Gram | Rods (plump/short | 4 | 11.8 |

| | | | | |
|---|---|---|---|---|
| | Negative Gram | chains) | | |
| Gram stain 4 | Negative | Rods (slimmer) | 3 | 8 |
| Gram stain 5 | Nil ( no growth) | | 5 | 14.7 |

### 4.3.2 Biochemical reactions

The coagulase test, triple sugar iron agar (TSI), methyl red test, and oxidase test will be employed to test for the target bacteria. Out of the samples isolated in this study, 35% reacted positive for coagulase since the bacteria belong to *Staphylococcus aureus* as depicted in Figure 4.3(a) and Table 4.3. The study showed that when the positive, were sweet in Triple Sugar Iron Agar (TSI) 37.2% of the samples that tested positive and the isolate had an acidic slant (yellow) and an acidic butt (yellow). This indicates that the isolate was a glucose, lactose, or sucrose fermenter, as depicted in the figures 4.3 c & d. Carbon dioxide production as a product of fermentation was also confirmed using bubbles in the tube as illustrated in figure 4 below. These are presented in Table 3 (c) and Table 4.3 below, indicating that the isolate could be *Escherichia coli*.

Fibrin clot



(a) (b)

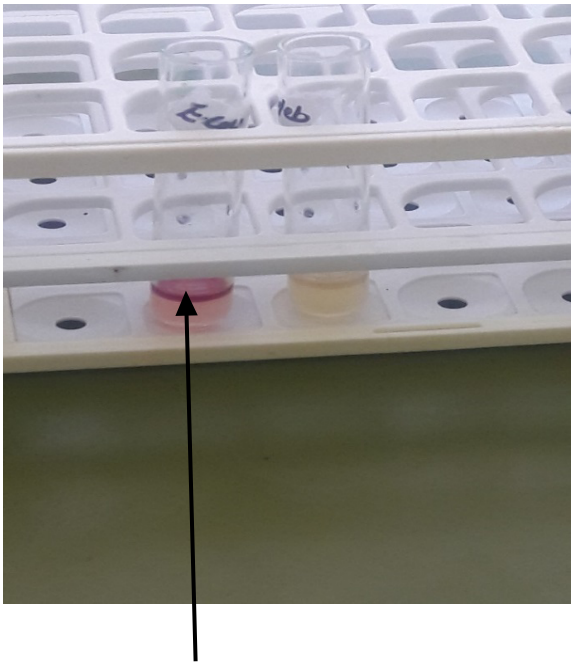
(c) Gas bubbles (d)

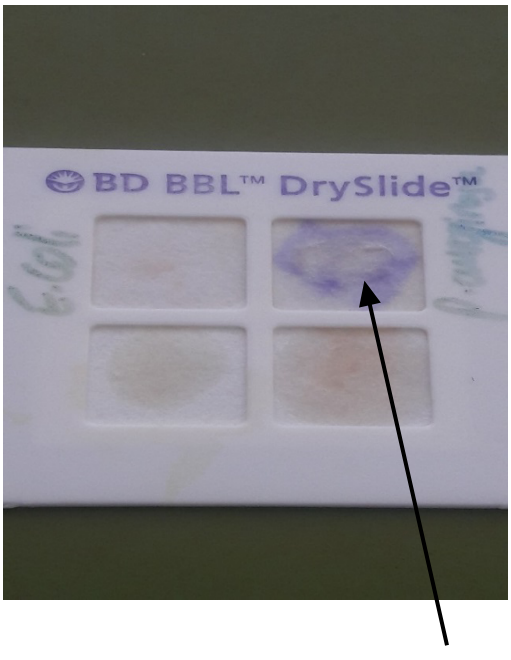
**Figure 4.3**: Biochemical reactions of the bacterial isolates

**Biochemical reactions of the bacterial isolates: Coagulase positive *Staphylococcus aureus*, arrow shows clumping of cells due to fibrinogen coming**

**into contact with the bacterial cell wall. Triple sugar iron agar (TSI) yellow butt and slant with gas bubbles– as shown by the arrows indicating *Escherichia coli***

Methyl red was also used to confirm the presence of other organisms that failed to undergo the fermentation process of glucose. Based on the information and data, 13.95% formed a red ring and hence the sample was methyl red positive, thus being *Escherichia coli*. Regarding the negative sample for this test, it was 23.26 % which focuses on *Klebsiella pneumonia* as shown in Figure 4.4(a) and Table 4.3 below. Finally, an Oxidase test was conducted on the isolated bacteria to differentiate between *Pseudomonas aeruginosa* from Other similar bacteria like *Escherichia coli*. One of the most popular identification tests to identify an organism with cytochrome oxidase/indophenol oxidase; is an enzyme that oxidizes the colorless reagent form to form a dark blue or purple compound. Finally, the samples showing 28% positivity that pointed to Pseudomonas aeruginosa are depicted in Figure 4.4(b) and Table 4.3 below.





(a) Methylred ring (b) Oxidase positive

**Figure 4.4:** Biochemical reaction of bacterial isolates

**Biochemical reaction of bacterial isolates: Methyl red (MR) test positive (see the red ring as shown by the arrow) and Oxidase test positive (purple colour as shown by the arrow on the slide)**

**Table 4.3: Biochemical tests**

| | | Gram reaction | | | |
|---|---|---|---|---|---|
| | | Gram-negative n=3 (75%) | | | Gram -positive |
| **Biochemical test** | | **n=1(25%)** | | | |
| Coagulase | | | | + (35%) | - 0%) |
| TSI | Butt/slope | Acid/Acid | Acid/ Acid | Alkaline/Alkaline | Alkaline/ Alkaline |
| | | + (37.2%) | + | - (62.8%) | - |
| | Gas | + (37.2%) | + | - (62.8%) | - |
| | $H_2S$ | - | - | - | - |
| MethylRed | | + (13.95%) | - (23.26%) | | |
| Oxidase | | - | - | + (27.91%) | - (13.95%) |
| Indole | | | | | |
| Possible pathogen | | *E. coli* (13.95%) | *K. pneumoniae* (23.26%) | *P. aeruginosa* (27.91%) | *S. aureus* (35%) |

### 4.3.3 Molecular identification of bacteria through amplification of virulence genes

In this study, molecular techniques (Polymerase Chain Reaction - PCR assays and gel electrophoresis) were used to reveal the prevalence of 10 virulence genes including 16srRNA (756bp), hla (229bp), cnf1(426bp), cnf2 (543bp), hlyA (1011bp), rmpA (461bp), LasL (600bp), gyrB (411bp), khe (77bp) and magA (128bp) using appropriate primer sequences (Table 3.1 above). 36 out of 43 isolates (84%) were genotypically characterized, as shown in Figure 4.5 below. It was observed that all 15 (100%) *Staphylococcus aureus* bacterial isolates were carrying 16RrRNA – 756bp and hla – 209bp genes, as shown in lane 1 and 2, respectively, in Figure 4.5 and Table 4.4 below, confirming the presence of its associated genes. One out of the 6 *Escherichia coli* isolates (16.7%) indicated the presence of cnf1- 498bp, 2 of the isolates (33.3%) indicated the presence of hly – 1177bp (67%) and no amplicons were indicated for cnf2 as shown in lanes 4, 5 and 6 of figure 4.5 below confirming the presence of genes commonly associated with *Escherichia coli*.

From the total 10 *Klebsiella pneumoniae* isolates, the following genes were indicated: magA - 121bp by 2 (20%) isolates, rmpA 106bp by 4 (40%) isolates, while khe did not indicate any amplicons as shown in lanes 8, 9, and 10 of figure 4.5 below confirming for the presence of genes commonly associated with *Klebsiella pneumoniae*. Further to the study, out of all 12 isolates analyzed, 10 (83.3%) indicated the presence of both gyrB 222bp and lasL 600bp genes, as shown in lanes 12 and 13 of Figure 4.5 confirming the presence of genes commonly associated with *Pseudomonas aeruginosa.* The control organisms for *staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* namely ATCC 25923, ATCC 25922, ATCC 27736 and

ATCC 27853, respectively, were also tested as confirmation for the presence of the virulence genes in the isolated bacteria.

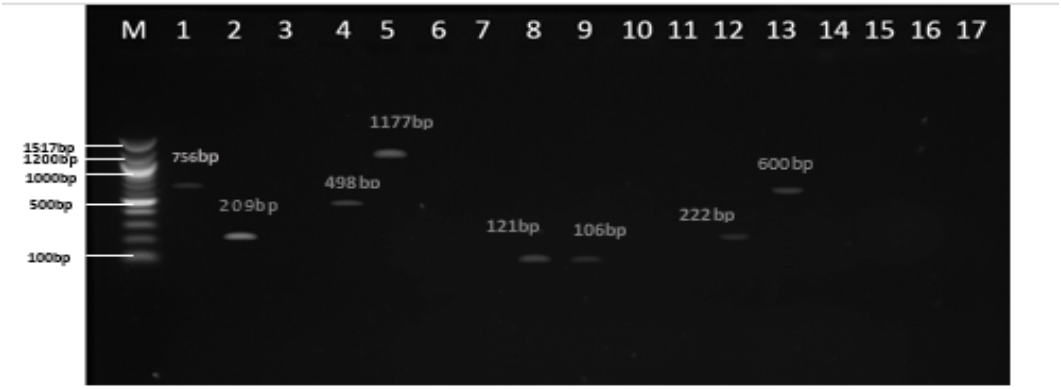



Figure 4.5: Agarose gel (1%) electrophoresis showing the typical amplicons of the virulence genes products of the bacterial isolates *Staphylococcus aureus* (Lane 1 and 2), *Escherichia coli* (lane 4 and 5), *Klebsiella pneumoniae* (Lane 8 and 9) and *Pseudomonas aeruginosa* (Lane 12 and 13). Lanes 6 and 10 were negative for the genes and hence did not indicate any amplicons, while lanes 3, 7 and 11 were set as the negative control. M is a ladder (standard) containing a mixture of DNA fragments of predetermined sizes (10 fragments with unique restriction sites ranging from 100bp to 1000bp) that can be compared against the unknown DNA samples.

The prevalence of the virulence genes among the selected isolates obtained from the study findings are summarized in Table 4.4. The outcomes demonstrate that all the Staphylococcus aureus isolates possessed 16SrRNA (756) and hla (209) virulence genes. In contrast, cnf2 genes for *Escherichia coli* and khe genes for *Klebsiella pneumoniae* were not found to be present on the pathogens isolated from wounds.

Table 4.4: virulence genes for molecular identification

A summary of the virulence genes amplified in the molecular identification of bacterial isolates

| Bacterial isolate | Virulence gene (BP) | Number positive/Freq. | Number negative/Freq. | PC (CONTROL MICROORGANISMS) |
|---|---|---|---|---|
| *Staphylococcus aureus* | 16RrRNA (756) | 15 (100%) | 0(0%) | *S. aureus* (ATCC25923) |
| *Staphylococcus aureus* | Hla (209) | 15(100%) | 0(0%) | *S. aureus* (ATCC25923) |
| *Escherichia coli* | Cnf1 (498) | 1 (16.7%) | 5(83.3%) | *E. coli* (ATCC25922) |
| *Escherichia coli* | Hly (1177) | 2 (33.3%) | 4(66.7%) | *E. coli* (ATCC25922) |
| *Escherichia coli* | Cnf2 (543) | 0 (0%) | 6(100%) | *E. coli* (ATCC25922) |
| *Klebsiella pneumoniae* | MagA (121) | 2(20%) | 8(80%) | *K. pneumoniae* (ATCC27736) |
| *Klebsiella pneumoniae* | rmpA (106) | 4(40%) | 6(60%) | *K. pneumoniae* (ATCC27736) |
| *Klebsiella pneumoniae* | Khe (77) | 0(0%) | 10(100%) | *K. pneumoniae* (ATCC27736) |
| *Pseudomonas aeruginosa* | lasL (600) | 10(83.3%) | 2(16.7%) | *P. aeruginosa* (ATCC27853) |
| *Pseudomonas aeruginosa* | gyrB (222) | 10(83.3%) | 2(16.7%) | *P. aeruginosa* (ATCC27853) |

## 4.4 Physico-chemical and nutritive properties of honey samples from stingless and honeybees

Analysis of the stingless and honeybee honey samples indicated pH, moisture content, free acidity, water-soluble vitamins, sugar content, vitamin C, crude protein and minerals. The statistical data were described as mean ranges and /or mean averages ± standard deviations (SD). The results for the analyzed honey

samples were compared with the Codex Alimentarius Commission standard set in 2001. The Codex Alimentarius is an international collection of food standards and codes of practice that are used as the main source of honey standards, with the commission urging all nations to adopt them. The criteria in the Codex Alimentarius were used to compare analyzed honey samples with the standards.

### 4.4.1 Physicochemical properties of honey samples from stingless and honeybees

#### *4.4.1.2 Hydromethylfurfural (HMF)*

For the Hydromethylfurfural (HMF) analysis, it was deduced that all the honey samples analyzed recorded a mean range HMF level of 9.1 ± 0.1mg/kg to 42.3 ± 0.38 mg/kg (average mean of 19.21 ± 13.91mg/kg). The stingless bee honey indicated a higher HMF mean concentration (22.77 ± 13.6mg/kg) compared to the honeybee samples (15.65 ± 15.2 mg/kg) and notably compared well with the reference sample which indicated a mean concentration of 16.35 ± 2.5 mg/kg. The text implies that the honey samples used in the study complied with the quality standards set by the International Regulations (Codex Alimentarius Commission, 2001) on honey from the tropical zone. Hydromethylfurfural (HMF) is used to gauge honey freshness and purity and should range from 5mg/kg to 80mg/kg. The fact that HMF content in the samples was low points at the fact that honey had not been stored for a long time after its collection, storage conditions were appropriate, and no high temperature was applied during honey processing. In addition, analyzing the obtained results, statistically significant differences ($P<0.6$).

A significant variation (P<0. 05) of the examined honey samples was observed regarding HMF level, as presented in Figure 4.6.

#### *4.4.1.3 Sugar concentration*

The text contains information regarding glucose concentration in the assessed honey samples. The mean glucose content varied between 80. 83 ± 1. 60 g/100g to 95. 2 ± 5. 07g/100g, with an overall average of 90. 00 ± 5.u= 45 g/100g. Honey samples collected from honeybees had a slightly higher mean glucose level of 90. 13 ± 5. 8g/100g) was recorded higher than those of stingless bees (89. 85 ± 6. 0 g/100g). In particular, Maoi and Mukutani who were from arid-semiarid areas, had slightly higher mean sugar levels of 94.0 ± 1.26 g/100g) higher than those from medium altitude (Koriema and Kibingor) (86.0 ± 5.02 g/100g). Compared to the control sample, all the analyzed samples had similar mean concentrations of 89.7 ± 0.8g/100g and range between 60g/100 to 800g/100 on average, as stated by Codex Alimentarius Commission (2001). This explained the readiness of the honey at the time of harvesting as it contained high glucose that acted as a macronutrient and facilitated other health benefits such as moisture retention, low crystallization and anti-microbial properties. These results indicate significant differences (P<0.05) among the samples, as depicted in Figure 4. 6.

#### *4.4.1.4 Moisture*

The text provides information about the moisture content in honey samples from honeybees and stingless bees. The mean moisture content ranged from 71.7 ± 2.52 to 147.3 ± 2.08 mg/g, with an overall average of 98.08 ± 25.53 mg/g.

Stingless bee honey samples had a lower mean moisture content of 81. 75 ± 10. 4 mg/g than honeybee samples, which contained a relatively higher moisture content 114. 28 ± 26. 6 mg/g. The mean moisture content for honeybee samples was also higher than the control honey sample, with a mean value of 76. 87 ± 3. 1 mg/g. According to the obtained results, the mean moisture content of the analyzed honey samples was 6.5 mg/g to 210 mg/g per gram, as recommended by the Codex Alimentarius Commission (2001). This moisture content range shows how well the honey resists granulation and fermentation by osmophilic yeasts; because honey is hygroscopic, few bacteria can live in it. Further, variance analysis indicated a highly significant difference (P<0. 05) in the moisture content among honey samples from the different sampled regions as depicted in Figure 4. 6.

#### *4.4.1.5 pH and Free acidity*

According to the analysis, the text demonstrates the values of pH and free acidity of honey samples under discussion. On average, the pH range was 3.70 ± 0.10 to 4.17 ± 0.47, with an average of 3.97 ± 0.14. The average pH of the stingless bee honey samples is slightly lower than that of the honeybee honey i.e., stingless bee honey (3.86 ± 0.11) and honeybee honey (4.06 ± 0.08). All the samples had a pH level within the recommended range of 3.2 to 4.5 as recommended by the Codex Alimentarius Commission (2001). The low pH levels observed in this study might have been a sign that the honey samples' freshness impacted the honey's ability to prevent the growth of certain pathogenic bacteria. Regarding free acidity, the mean range was 0.03 ± 0.0 to 0.15 ± 0.03 meq/kg, with an average of 0.06 ± 0.04

meq/kg. Both stingless bee and honeybee samples contained considerable levels of free acidity, 0.063 ± 0.0 and 0.064 ± 0.005 meq/kg, respectively, which were within the recommended levels of less than 50 meq/kg by the Codex Alimentarius Commission (2001). The mean free acidity values in all the samples were slightly lower than the control mean value of 0.03 ± 0.01 meq/kg and there was no sign of fermentation which pointed toward low moisture content. The free acidity and pH of the samples investigated in this case were not significantly different (P > 0.05) as shown in Figure 4. 6.

#### *4.4.1.6 Hydrogen peroxide*

The text details the hydrogen peroxide concentration in the analyzed honey samples. The mean range of hydrogen peroxide concentration was 0.5 ± 0.0 mM to 1.0 ± 0.0 mM, with an average of 0.81 ± 0.37 mM. Honey types of stingless bees had a higher ratio of 1 ± 0. 41 mM compared to honeybee 0.62 ± 0.25 mM. All the honey samples produced in the Mukutani region showed the highest mean concentration (1.25 ± 0.35 mM). All the samples were not significantly different from the control mean value of (0.69 ± 0.3 mM) and within the required limit of 0.5 to 2.5 mM. Recommended by the Codex Alimentarius Commission (2001).

However, as stated before, low concentrations of hydrogen peroxide as a by-product of oxidative metabolism are essential for DNA degradation in bacteria; honey inhibits bacterial growth and kills them with the help of other components, even if hydrogen peroxide is present only in small amounts. From the results of this study, it was noted that there was no significant difference in the levels of hydrogen peroxide in honey samples obtained from various regions as shown in Figure 4.6.

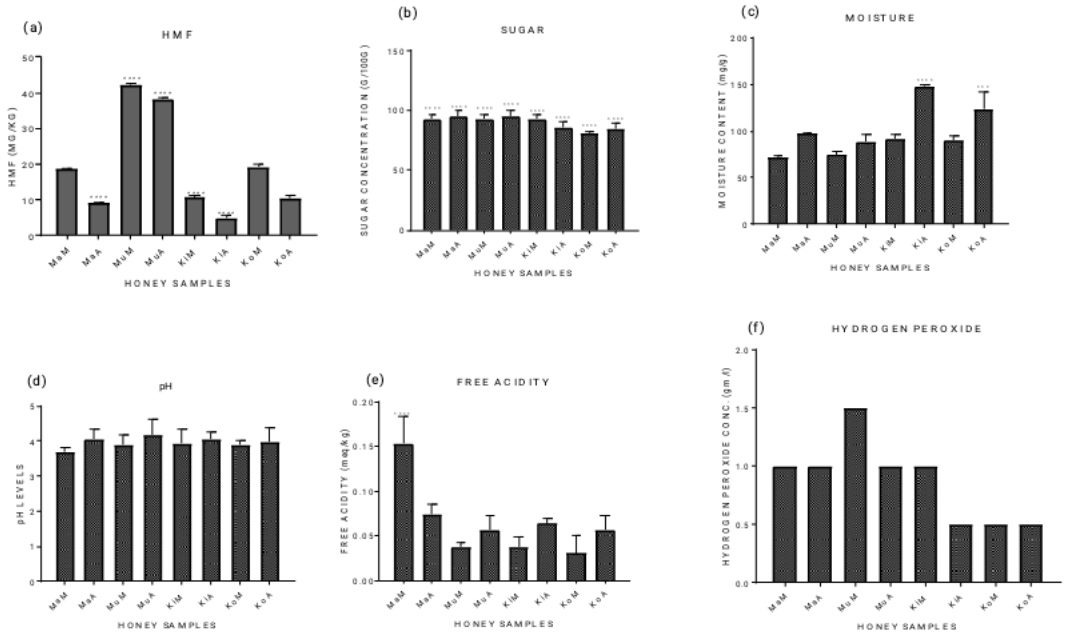



**Figure 4.6: Physico-chemical properties: HMF, Sugar, Moisture, pH, Free acidity and Hydrogen peroxide. The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05, **P<0.01, ***P<0.001 and **** P<0.0001).**

### 4.4.2 Nutritive properties of honey samples from stingless and honey bees

#### *4.4.2.1 Water - Soluble vitamins*

The investigation's findings show that honey has a range of water-soluble vitamins, with the following mean concentrations: Vitamin B1, B2, B3, B5, and B9 concentrations varied from 0.21 ± 0.03 to 0.64 ± 0.02 mg/l (mean average 0.40 ± 0.16 mg/l), 0.72 ± 0.03 to 2.39 ± 0.07 mg/l (mean average 1.32 ± 0.62 mg/l), 0.65 ± 0.5 to 4.19 ± 0.02 mg/l (mean average 2.65 ± 1.07), 0.01 ± 0.0 to 1.22 ± 0.03 mg/L (average concentration of 0.50 ± 0.52 mg/l), and 0.11 ± 0.00 to 1.64 ± 0.10 mg/l (mean average 1.16 ± 0.52 mg/l). The samples of honey and stingless bee honey had mean vitamin concentrations of 1.14 ± 0.86 mg/l and 1.32 ± 0.92 mg/l, respectively. The most common vitamin combinations were B3 > B2 > B9 > B5 > B1. This finding is in line with the experimental results.

In the positive control sample, the amounts of Vitamin B1, B2, B3, B5, and B9 were 0.39 ± 0.07 mg/l, 1.06 ± 0.21 mg/l, 3.48 ± 0.36 mg/l, 0.09 ± 0.09 mg/l, and 1.07 ± 0.06 mg/l, in that order. Throughout the inquiry, these values were determined. According to the inquiry, Maoi honey was included in all of the honey samples that were evaluated for their ability to identify vitamins. Vitamins B1 and B3 were present in comparatively high concentrations in the Maoi honey samples. On the other hand, Figure 4.7 below shows that the Kibingor honey samples included a significant amount of vitamins B1, B2, and B9. Honey is a nutrient-dense food that can be eaten to replace the water-soluble vitamins that the body constantly excretes in urine. The findings fell between the 1.10 mg/kg and 1.75 mg/kg permitted Range of Detection (LOD) by the International Union of Pure and Applied Chemistry. However, honey is not able to achieve this. Vitamins B1 and B2 were likewise found to not exhibit statistical significance ($P>0.05$) when honey and

stingless bees were examined. However, it was shown that vitamins B2, B3, and B9 have statistical significance ($P<0.05$). According to the study's findings, water-soluble vitamins such vitamins B1, B2, B3, B5, and B9 are present in honey at the mean range concentrations listed below: The following vitamin levels are reported: Vitamin B1: 0.21 ± 0.03 to 0.64 ± 0.02 mg/l (mean average 0.40 ± 0.16 mg/l), Vitamin B2: 0.72 ± 0.03 to 2.39 ± 0.07 mg/l (mean average 1.32 ± 0.62 mg/l), Vitamin B3: 0.65 ± 0.5 to 4.19 ± 0.02 mg/l (mean average 2.65 ± 1.07 mg/l), Vitamin B5: 0.01 ± 0.0 to 1.22 ± 0.03 mg/l (mean average 0.50 ± 0.52 mg/l), and Vitamin B9: 0.11 ± 0.00 to 1.64 ± 0.10 mg/l (mean average 1.16 ± 0.52 mg/l).

Vitamin B3 was the vitamin at which the concentration peaked, then Vitamin B2, Vitamin B9, and Vitamin B5, and Vitamin B1 brought the concentration down to its lowest point. As a result, the mean vitamin concentration of the honey and stingless bee honey samples used in this study varied; the former had a value of 1.14 ± 0.86 mg/l while the latter had a value of 1.32 ± 0.92 mg/l.

All of the honey samples had vitamin levels above the detection limit, and the findings shown below agreed with the values of the positive control sample. As shown in Figure 4. 7, the Maoi sample had higher amounts of vitamins B1 and B3, whereas the Kibingor sample had higher levels of vitamins B1, B2, and B9.

The International Union of Pure and Applied Chemistry states that these findings were between the 1.10 mg/kg and 1.75 mg/kg acceptable range. Since most vitamins are water soluble, it is possible to replace the vitamins lost through urine even though honey does not have a high concentration of vitamins.

The vitamin B1 and B2 samples, honey, and stingless bee samples did not exhibit a statistically significant difference (P>0.05) in the results. Nonetheless, there was a significant difference (P<0.05) between the vitamin B2, B3, and B9 samples.

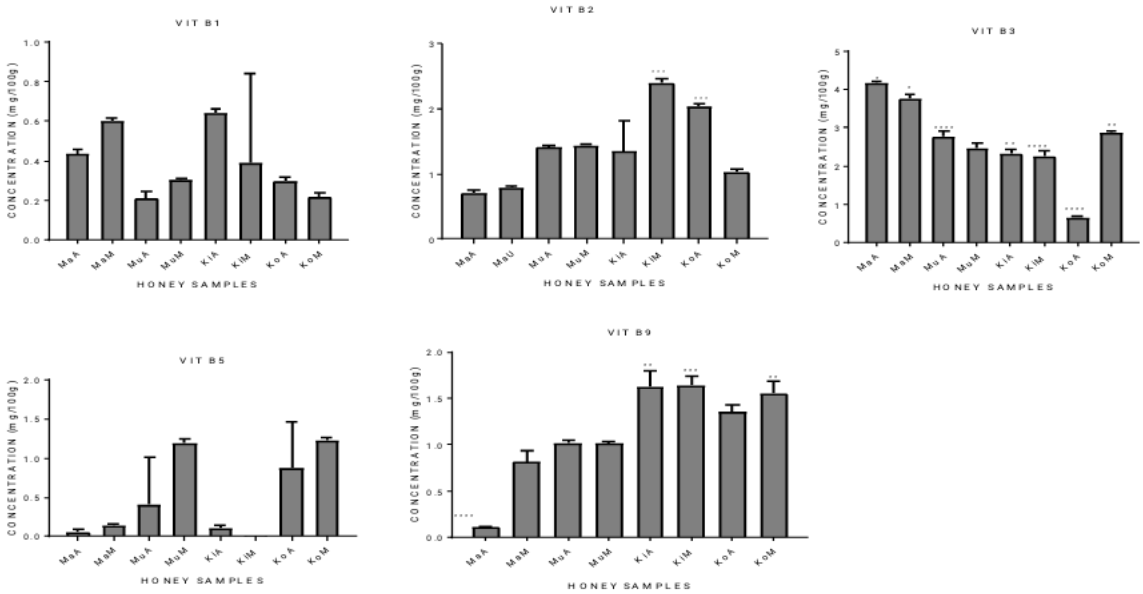



**Figure 4.7: Water soluble vitamins: Vitamin $B_1$, Vitamin $B_2$, Vitamin $B_3$, Vitamin $B_5$ and Vitamin $B_9$. The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05,**P<0.01, ***P<0.001 and **** P<0.0001).**

### *4.4.2.2 Minerals*

The study's findings show that different mineral elements, such as sodium, iron, zinc, magnesium, calcium, and phosphorus, are present in honey in different amounts. But potassium was the mineral most commonly found in honey, and this

was found everywhere. With a mean value of 17.78 ± 1.29 mg/l, the potassium content ranged from 16.17 ± 2.6 to 20.37 ± 1.7 mg/l.

The second most common mineral in the honey samples was found to be calcium. The average calcium value was 1.86 ± 0.33 mg/l, with a range of 1.33 ± 0.02 to 2.32 ± 0.08 mg/l. Sodium, iron, zinc, magnesium, and phosphorus followed, with the corresponding mean concentrations for each shown below: Sodium concentration ranges from 0.37 ± 0.23 to 2.47 ± 1.02 mg/l (mean average 1.43 ± 0.96 mg/l), 0.36 ± 0.11 to 2.16 ± 0.40 mg/l (mean average 1.33 ± 0.67 mg/l), 0.27 ± 0.06 to 0.44 ± 0.07 mg/l (mean average 0.41 ± 0.06 mg/l), Magnesium from 0.09 ± 0.02 to 0.74 ± 1.36 mg/l (mean average 0.34 ± 0.19 mg/l), and phosphorus from 0.056 ± 0.02 to 0.21 ± 0.02. The mean value is 0.12 ± 0.05 mg/l.

The results were like the control honey samples with mean values of 1.56 ± 0.46 mg/l for calcium, 0.52 ± 0.08 mg/l for magnesium, 1.06 ± 0.13 mg/l for iron, 0.31 ± 0. 0.05 mg/l for zinc and 1.64 ± 0.29 mg/l for sodium, 14.39 ± 0.62 mg/l for potassium and 0.16 ± 0.13 mg/l for phosphorous.

In addition, the honey obtained from stingless bees was found to contain high mean levels of calcium, magnesium, iron, sodium and potassium as 2.11 ± 0.20 mg/l, 0.60 ± 0.47 mg/l, 0.76 ± 1.84 mg/l, 1.24 ± 0.95mg/l and 17.94 ± 1.7 mg/l while the honeybee mean levels detected were 1.67 ± 0.35 mg/l, 0.45 ± 0.15 mg/l, 0.65mg/l, 0.93 mg/l and 17.63 mg/l respectively. From the honey samples, it could be deduced that they were not rich in minerals but were lower than the limit set by Codex Alimentarius Commission, 2001 of 3.23 to 236.8 mg/l, 4.85 to 218.0 mg/l,

2.18 to 563.72 mg/l, 16.66 – 1249.34 mg/l, 0.41 – 224.0 mg/l and 0.153 – 7.22 mg/l, for sodium, calcium, magnesium, potassium, iron, and phosphorus respectively, which could be due to the botanical differences it came with clear indications on the safety of honey for human consumption. Comparing the results shown in Figure 4.8, Potassium, Iron, Sodium and Phosphorus differentiated not significantly ($P<0.05$).

#### *4.4.2.3: Crude proteins*

From the information given above, honey and stingless bee honey samples were found to contain proteins. Most of the proteins found in honey are released through the salivary and hypopharyngeal glands of bees, and these proteins may differ from one bee species to another and from one floral source of nectar to another.

The average protein content of the honey samples was 0.72 ± 0.23 g/100g, while the stingless bee honey samples had a higher average protein content of 1.33 ± 0.89 g/100g. These concentrations were slightly higher compared to the norm of well water standard at 0. 9 to 0. 5 g/100g as the Codex Alimentos Commission had suggested in 1999.

The results showed a highly significant difference ($P < 0.05$) in the protein content of honey from honeybees and stingless bees, as depicted in Figure 4.8.

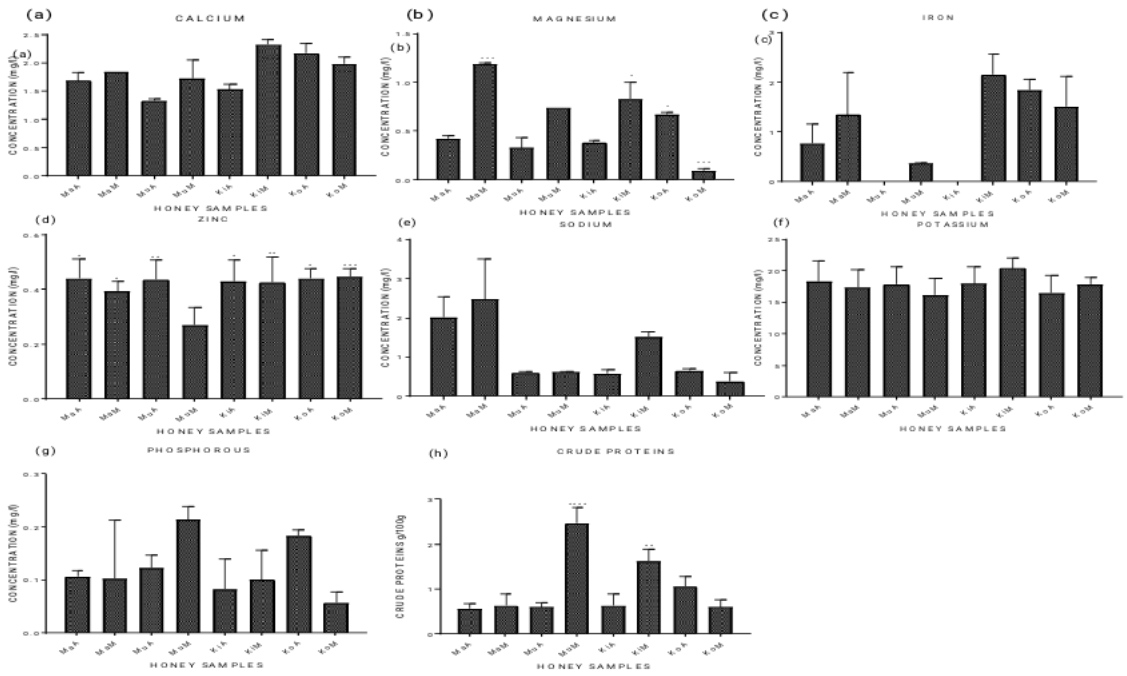



**Figure 4.8: Minerals and crude proteins; Calcium – ca, Magnesium – mg, Iron- fe, Zinc –zn, Sodium – Na, Potassium - K, Phosphorous - P and Crude proteins. The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05,**P<0.01, ***P<0.001 and **** P<0.0001).**

#### 4.4.2.4 Correlation of physicochemical and nutritive properties of honey from stingless and honeybees

They showed a significant positive correlation between vitamins and proteins; sugars and moisture; and a negative correlation between vitamins and sugars as indicated in Table 4.5. This correlation indicates that high sugar molecules in honey cause a strong interaction with water molecules, leading to a high vitamin osmolarity responsible for antibacterial activity. Proteins, the primary components of most cells, support their growth and development, supporting the development of wound strength through collagen formation. Unlike vitamins, sugars stimulate fibroblast growth and the production of collagen, which decreases epithelialization, collagen synthesis and the development of tissue granulation.

**Table 4.5:** Correlation matrix (Pearson Correlation Coefficient) of the nutritive components between honey and stingless bee honey samples.

| | | PROTEINS | VIT | SUGARS | MOISTURE |
|---|---|---|---|---|---|
| PROTEINS | Pearson Correlation | 1 | 1.000** | -1.000** | -1.000** |
| | Sig. (2-tailed) | | | | |
| | N | 2 | 2 | 2 | 2 |
| VIT | Pearson Correlation | 1.000** | 1 | -1.000** | -1.000** |
| | Sig. (2-tailed) | | | | |
| | N | 2 | 2 | 2 | 2 |
| SUGARS | Pearson Correlation | -1.000** | -1.000** | 1 | 1.000** |
| | Sig. (2-tailed) | | | | |
| | N | 2 | 2 | 2 | 2 |
| MOISTURE | Pearson Correlation | -1.000** | -1.000** | 1.000*** | 1 |
| | Sig. (2-tailed) | | | | |
| | N | 2 | 2 | 2 | 2 |

KEY: ** Correlation is significant at the 0.01 level (2-tailed).

## 4.5 Phytochemical properties of honey from stingless and honeybees

The phytochemical composition of honey determined were Total flavonoid content, Total phenolic content, Vitamin C and Total carotenoid content are presented in mean range ± SD (mean average ± SD) about Codex Alimentarius Commission 2001 as detailed in Figure 4.9.

### 4.5.1 Total phenolic content of honey samples from stingless and honeybees

All the honey samples analyzed had a mean range of Total Phenolic Content of 40.87 ± 1.95 mgGAE/100g to 155.83±7.06 mgGAE/100g (mean average 86.49 ± 41.50 mgGAE/100g). This was lower than the mean value of the positive control

sample of 86.20 ± 41.50 mgGAE/100g. Higher mean concentrations were recorded from stingless bees' honey samples compared to those from honeybees of 92.18 ± 51.20 mgGAE/100g and 80.81 ± 36.25 mgGAE/100g, respectively. According to the above figures, it was clear that the mean phytochemical contents of the analyzed samples of honey were within the allowable limits of total phenolic content as recommended by the Codex Alimentarius Commission, 2001 for honey between 161 mgGAE/100g and 186 mgGAE/100g as depicted in Figure 4.9.

### 4.5.2 Total flavonoid of honey samples from stingless and honeybees

All the honey samples analyzed had a mean range Total Flavonoid Content of 17.57 ± 0.76 mgRE/100g to 31.63 ± 1.27 mgRE/100g (mean average 22.75 ± 5.66 mgRE/100g), lower mean concentrations of 22.75 ± 5.66 mgRE/100g were recorded in the positive control honey sample but higher mean concentrations in stingless bees compared to the honey bee samples at 23.7 ± 5.87 mgRE/100g and 21.83 ± 6.16 mgRE/100g respectively. The mean values that were obtained were as follows, the mean phytochemical components of the analyzed honey samples were within the recommended range required by the (Codex Alimentarius Commission, 2001) for total flavonoid content with a range of from 15.6mgRE/100g to 54.23mgRE/100g as indicated in Figure 4.9.

### 4.5.3 Total carotenoid content of honey samples from stingless and honeybees

The total Carotenoid Content mean range in all the analyzed honey samples was 1.6 ± 0.7 mg βcarotene to 6.73 ± 0.11 mg βcarotene (mean average 5.33 ± 1.87mg βcarotene), which was lower than the positive honey control samples of 5.33 ±

1.87 mg βcarotene. Mean carotenoid content shows that stingless bee honey samples have a mean of 6.57 ± 0.21 while honeybee was found to have 4.41 ± 2.07 mgβcarotene. The mean levels derived meant that the mean phytochemical constituents of the studied honey samples were within the permissible regulatory guide for Total carotenoid for honey, which is 1.29 mgβ-carotene to 11.6 mgβ-carotene as depicted in Figure 4 9.

### 4.5.4 Total Vitamin C content of honey samples from stingless and honeybees

The two honey samples had an average vitamin C concentration of 1.52 ± 0.63 mg/100g, ranging from 0.73 ± 0.06 mg/100g to 2.27 ± 0.17 mg/100g. Significant amounts were found in the honey samples from stingless bees and honeybees, 1.51 ± 0.7 mg/100g and 1.53 ± 0.64 mg/100g, respectively. These readings were compared to the mean value of 1.79 ± 0.06 mg/100g for a control sample of honey. The average results found suggested that the average content of total Vitamin C in the phytochemical components of the honey samples tested was within the Codex Alimentarius Commission's (2001) recommended limit. The figure below, Figure 4.9, illustrates this.

Total carotenoid, total flavonoid, and vitamin C contents varied from 161 mgGAE/100g to 186 mgGAE/100g, along with the phenolic content. The study's findings suggest that the high quantity of phenolic acids, flavonoids, vitamin C, and carotenoids present in the Marigat honey under investigation contribute to its potent antioxidant action. Furthermore, Figure 4.9 will show how these chemicals'

concentrations vary significantly between different places.

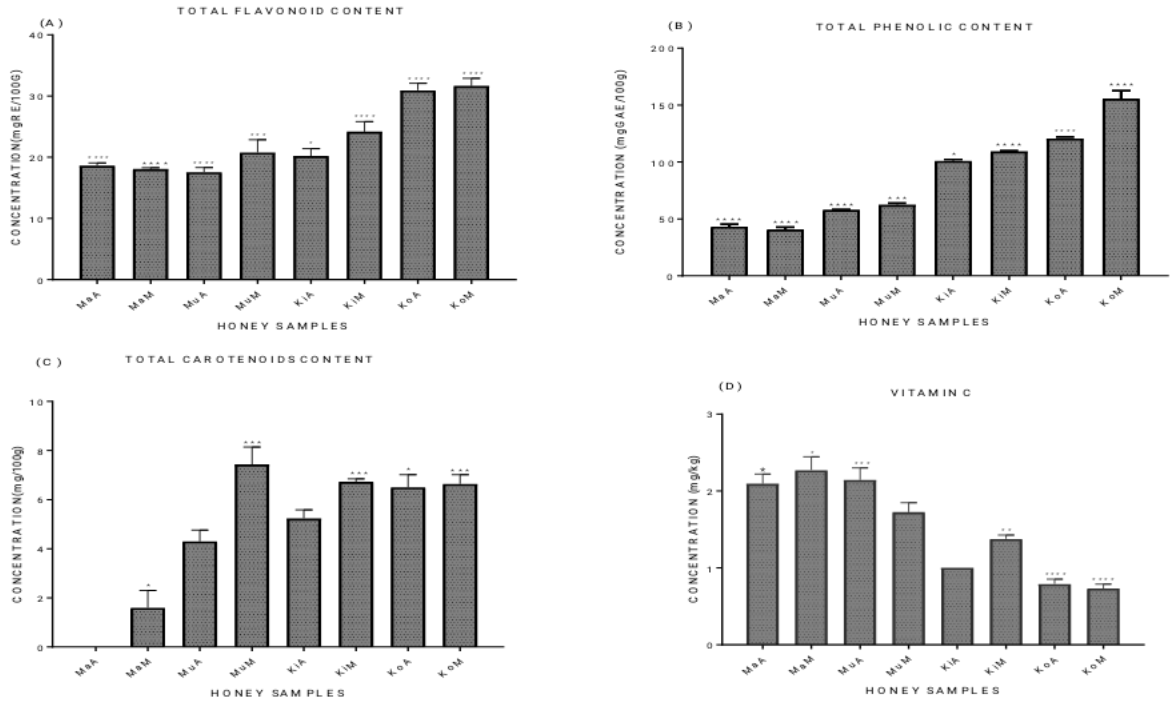



Figure 4.9: Total flavonoid content – TFC, Total phenolic content – TPC, Carotenoids and Vitamin. The values are represented in mean ± SD as error bars

represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05,**P<0.01, ***P<0.001 and **** P<0.0001).

### 4.5.5 Correlation between the phytochemical components of honey samples from honeybees and stingless bees

The correlation findings designate a positive correlation between the phenolics, as shown in Table 4.6. This interaction indicates that flavonoid, phenolic and carotenoid content are all involved in the regulation of cytokine release, including the tumor necrosis factors, a protein that reduces inflammation in tissues and activates white blood cells. These compounds enhance the formation of new collagen, increasing the tissue tensile strength, which increases wound contraction and reduces the period of epithelization.

**Table 4.6:** Correlation matrix (Pearson Correlation Coefficient) between the phytochemical component of honey and stingless bee honey samples

| | | TPC | TFC | TCC |
|---|---|---|---|---|
| TPC | Pearson Correlation | 1 | .900* | .768* |
| | Sig. (1-tail) | | 0.001 | 0.013 |
| | N | 8 | 8 | 8 |
| TFC | Pearson Correlation | .900* | 1 | .661* |
| | Sig. (1-tail) | 0.001 | | 0.037 |
| | N | 8 | 8 | 8 |
| TCC | Pearson Correlation | .768* | .661* | 1 |
| | Sig. (1-tailed) | 0.013 | 0.037 | |
| | N | 8 | 8 | 8 |

KEY: ** Correlation is significant at 0.90 level (1tailed), TPC - Total Phenolic Concentration TFC - Total Flavonoid Concentration and TCC - Total Carotenoids Concentration

## 4.6 Antibacterial activity of honey samples and selected antibiotics against isolates obtained from wounds and burns

### 4.6.1: Introduction

The antibacterial effect of locally produced honey samples from honeybees and stingless bees was also compared to clinically used antibiotics here as well. Sterile discs with honey samples in different concentrations: To evaluate the antibacterial activity the following concentrations of extracts were tested: $10x10^4$, $20x10^4$, $50x10^4$ and $75x10^4$ µg/ml per disc along with the standard antibiotic discs of Ampicillin (10µg), Levofloxacin (5µg), Meropenem (10µg), Tazobactam (110µg), Chloramphenicol (30 µg) and Gentamicin (10µg) and the mean inhibition zones obtained were recorded.

### 4.6.2: Disc diffusion

All eight honey samples in this study showed variable inhibitory activities against the target bacterial isolates from burn and cutaneous wound swab samples by disc diffusion method and this was significantly ($p < 0.005$) affected by the honeybee tribe as well as the region of honey sample collection. The mean zones of inhibitions obtained compared well with those of the bacterial control species of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 27736) as well as *Pseudomonas aeruginosa* (ATCC 27853).

These findings of clear zones suggest that honey possess strong antibacterial properties at concentrations of 50 $x10^4$ µg/ml and 75 $x10^4$ µg/ml of honey per disc but low inhibition or none at the concentrations of 10 $x10^4$ µg/ml and 20 $x10^4$ µg/ml of honey per disc. This could be due to unstable putative agents that would have been inactivated during the dilution of honey.

This study revealed that all eight honey samples had variable inhibitory activity against the target bacterial isolates from burn and cutaneous wound swab samples by disc diffusion. This activity was significantly influenced ($p < 0.005$) by the honeybee tribe and region of sample collection. Mean zones of inhibitions that were obtained agreed with the zones of bacterial control species of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 27736).

Inhibition of the antibacterial activity was observed in the concentrations of honey 50 x $10^4$ µg/ml and 75 x $10^4$ µg/ml per disc with no inhibition or minimal inhibition in the concentrations of honey 10 x $10^4$ µg/ml and 20 x $10^4$ µg/ml per disc. This gap could be attributed to unstable putative agents that are neutralized during the honey dilution process.

***Staphylococcus aureus**:* According to this study, it was able to indicate the highest susceptibility to the honey sample with a mean inhibition of 6.0 ± 0.0 mm and 6.13 ± 0.19 mm for honey and stingless bee honey at 10 x $10^4$ µg/ml, 6.0 ± 0.0 mm and 6.15 ± 0.19 mm at 20 x $10^4$ µg/ml, 12.8 ± 2.17mm to 21.73 ± 0.37 mm at 50 x $10^4$ µg/ml and 15.29 ± 0.31 mm to 25.22 ± 0.49 mm for 75 x $10^4$ µg/ml. The samples

indicated a significant inhibition difference between the sample collection region and the bee species, as shown in Figure 4.10.

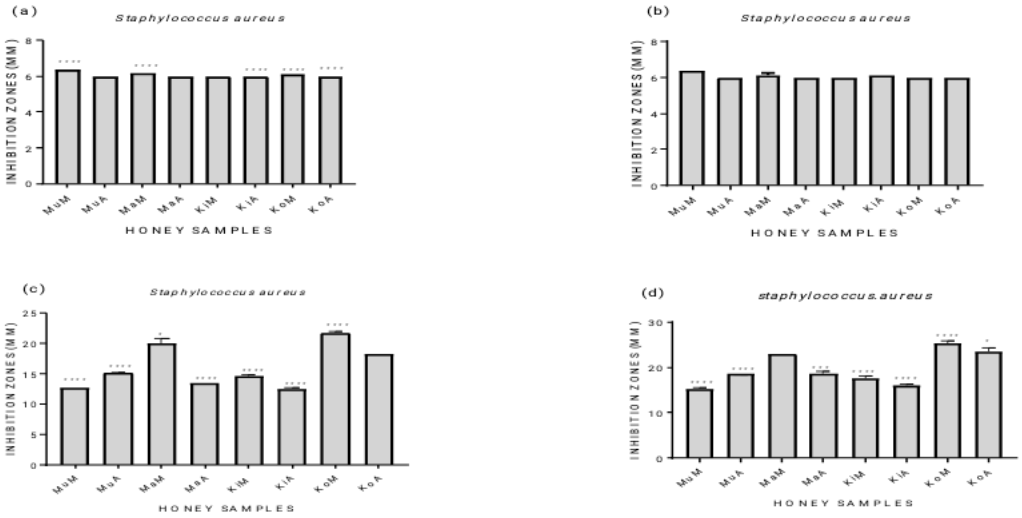



Figure 4.10: Mean zones of inhibition for both honey and stingless bee honey samples against *Staphylococcus aureus* at $10x10^4$µg/ml, $20x10^4$µg/ml, $50x10^4$µg/ml and $75x10^4$µg/ml. The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05,**P<0.01, ***P<0.001 and **** P<0.0001) (KEY: MaM – Maoi Meliponin, MaA – Maoi Apis, MuM – Mukutani Meliponin, MuA - Mukutani Apis, KiM – Kibigor Meliponin, KiA- Kibingor Apis, KoM- Koriema Meliponin, KoA - Koriema Apis.

*Pseudomonas aeruginosa*: According to the study findings, it showed a mean inhibition of 6.03 ± 0.05 to 6.05 ± 0.11mm at $10x10^4$µg/ml, 6.04 ± 0.05 to 6.14 ±

0.22mm at 20x10$^4$µg/ml, 11.80 ± 2.18 to 19.42 ± 0.07mm at 50x10$^4$ µg/ml and 14.67 ± 0.83 to 21.63 ± 0.0mm at 75x10$^4$ µg/ml as shown in figure 4.11.

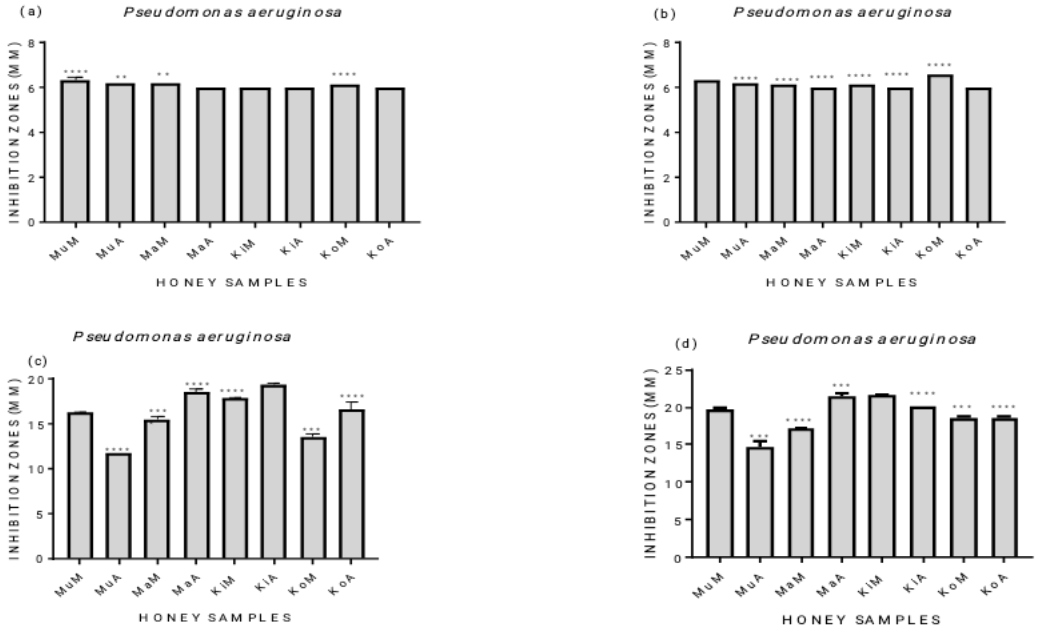



Figure 4.11: Mean zones of inhibition for both honey and stingless bee honey samples against Pseudomonas *aeruginosa* at 10x10$^4$µg/ml, 20x10$^4$µg/ml, 50x10$^4$µg/ml and 75x10$^4$µg/ml. The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05,**P<0.01, ***P<0.001 and ****

P<0.0001) (KEY: MaM – Maoi Meliponin, MaA – Maoi Apis, MuM – Mukutani Meliponin, MuA - Mukutani Apis, KiM – Kibigor Meliponin, KiA- Kibingor Apis, KoM- Koriema Meliponin, KoA - Koriema Apis.

***Klebsiella pneumoniae***: *Klebsiella* organisms are resistant to multiple antibiotics due to possessing plasmids as the primary source of resistant genes. Hence, the findings of this study indicated an average inhibition of 6.01 ± 0.04 to 6.04 ± 0.05 mm at $10x10^4$µg/ml, 6.025 ± 0.05 to 6.075 ± 0.12 mm at $20x10^4$ µg/ml, 10.07 ± 0.39 to 19.65 ± 0.38 mm at $50x10^4$ µg/ml and 14.19 ± 0.19 to 22.96 ± 0.00 mm at $75x10^4$ µg/ml as indicated in figure 4.12.

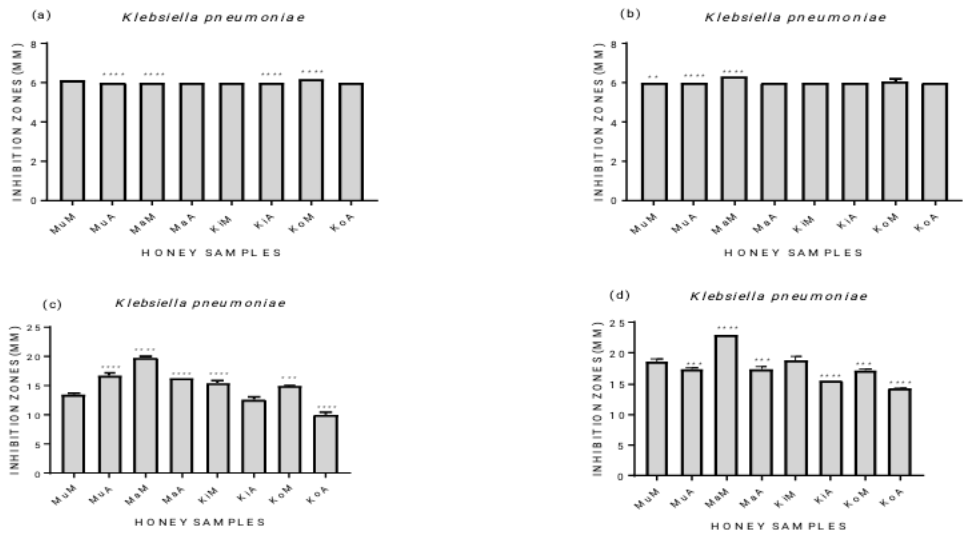



Figure 4.12: Mean zones of inhibition for both honey and stingless bee honey samples against *Klebsiella pneumonia* at $10x10^4$µg/ml, $20x10^4$µg/ml, $50x10^4$µg/ml and $75x10^4$µg/ml. The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are

represented by stars on the bars (*P< 0.05,**P<0.01, ***P<0.001 and **** P<0.0001) (KEY: MaM – Maoi Meliponin, MaA – Maoi Apis, MuM – Mukutani Meliponin, MuA - Mukutani Apis, KiM – Kibigor Meliponin, KiA- Kibingor Apis, KoM- Koriema Meliponin, KoA - Koriema Apis.

*Escherichia coli:* It indicated a mean inhibition of 6.03 ± 0.05 mm to 6.08 ± 0.12 mm at $10x10^4$ µg/ml, 6.04 ± 0.07 to 6.09 ± 0.15 mm at $20x10^4$ µg/ml, 6.49 ± 1.09 to 12.55 ± 0.22 mm at $50x10^4$ µg/ml and 14.19 ± 0.19 to 22.96 ± 0.00 mm at $75x10^4$ µg/ml. The mean inhibition variations recorded for the honey samples showed the differences across the regions and the two bee species as shown in Figure 4.13.

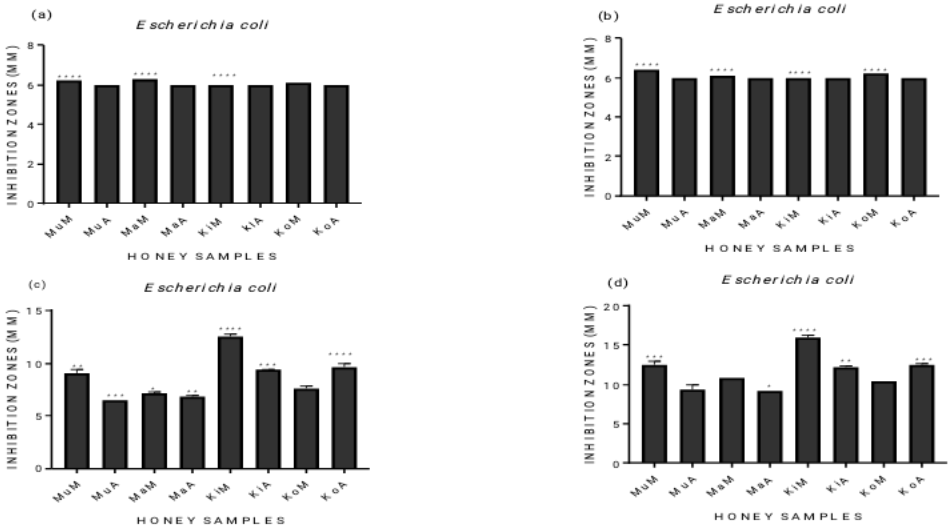


Figure 4.13: Mean zones of inhibition for both honey and stingless bee honey samples against *Escherichia coli* at 10x10$^4$µg/ml, 20x10$^4$µg/ml, 50x10$^4$µg/ml and 75x10$^4$µg/ml. The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05,**P<0.01, ***P<0.001 and **** P<0.0001) (KEY: MaM – Maoi Meliponin, MaA – Maoi Apis, MuM – Mukutani Meliponin, MuA - Mukutani Apis, KiM – Kibigor Meliponin, KiA- Kibingor Apis, KoM- Koriema Meliponin, KoA - Koriema Apis.

According to the findings of this study, higher antibacterial inhibition activity was noted in samples collected from the medium altitude regions (Kibingor and Koriema) and the stingless bee honey samples which compared well to honeybee samples against each bacterial isolate. The findings of this study revealed that the honey being analyzed possesses some level of antibacterial activity, which tends to increase with the concentration of the honey tested. As a result, parallel antibacterial inhibition results to those of the honey dilutions were also observed with the bacterial control strains (ATCC) as depicted in table 4.7 and table 4.8.

Table 4.7: Mean zones of inhibition of the honey (stingless and honey bee samples) dilutions (10, 20, 50 and 75 x 10$^4$ µg/ml) against the bacterial isolates

| | | HONEY SAMPLES (Stingless and Honey bee) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *ISOLATES/CONC. (x10$^4$ µg/ml)* | | *Mum* | *MuA* | *MaM* | *MaA* | *KiM* | *KiA* | *KoM* | *KoA* |
| *S. aureus* | 10 | 6.4 ± 0.00 | 6.0 ± 0.00 | 6.1 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.1 ± 0.00 |
| | 20 | 6.4 ± 0.00 | 6.0 ± 0.00 | 6.2 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.1 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 |
| | 50 | 12.8 ± 2.17 | 15.28 ± 0.01 | 20.08 ± 0.67 | 13.47 ± 0.01 | 14.62 ± 0.3 | 12.62 ± 0.19 | 21.73 ± 0.37 | 18.38 ± 0.01 |
| | 75 | 15.29 ± 0.31 | 18.45 ± 0.02 | 22.9 ± 0.00 | 18.58 ± 0.60 | 17.56 ± 0.53 | 15.90 ± 0.29 | 25.22 ± 0.49 | 23.60 ± 0.54 |
| *E. coli* | | | | | | | | | |
| | 10 | 6.2 ± 0.00 | 6.0 ± 0.00 | 6.3 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.1 ± 0.00 | 6.0 ± 0.00 |
| | 20 | 6.4 ± 0.00 | 6.0 ± 0.00 | 6.1 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.2 ± 0.00 | 6.0 ± 0.00 |
| | 50 | 9.00 ± 0.37 | 6.49 ± 1.09 | 7.11 ± 0.19 | 6.84 ± 0.07 | 12.55 ± 0.22 | 9.35 ± 0.01 | 7.59 ± 0.19 | 9.66 ± 0.29 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 12.48 ± | | 10.89 ± | | 15.87 ± | 12.18 ± | 10.48 ± | 12.47± |
| | 75 | 0.52 | 9.43 ± 0.58 | 0.00 | 9.24 ± 0.00 | 0.37 | 0.12 | 0.00 | 0.23 |
| *K. pneumoniae* | | | | | | | | | |
| | 10 | 6.1 ± 00 | 6.0 ± 0.00 | 6.3 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.2 ± 0.00 | 6.0 ± 0.00 |
| | 20 | 6.1 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.1 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.6 ± 0.00 | 6.0 ± 0.00 |
| | | 13.48 ± | 16.74 ± | 19.65 ± | | | 12.58 ± | 14.88 ± | 10.07 ± |
| | 50 | 0.27 | 0.31 | 0.38 | 16.25 ± 0.0 | 15.43± 0.46 | 0.48 | 0.22 | 0.39 |
| | | 18.67 ± | 17.34 ± | 22.96 ± | 17.35 ± | 18.86 ± | 15.53 ± | 17.14 ± | 14.19 ± |
| | 75 | 0.39 | 0.33 | 0.00 | 0.51 | 0.69 | 0.00 | 0.16 | 0.19 |
| *P. aeruginosa* | | | | | | | | | |
| | 10 | 6.3 ± 0.00 | 6.2 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.1 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 |
| | 20 | 6.3 ± 0.00 | 6.0 ± 0.00 | 6.1 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.0 ± 0.00 | 6.6 ± 0.00 | 6.0 ± 0.00 |
| | | 16.28 ± | 11.80 ± | 15.45 ± | 18.60 ± | 17.89 ± | 19.42 ± | 13.52 ± | 16.65 ± |
| | 50 | 0.06 | 2.18 | 0.35 | 0.26 | 0.03 | 0.07 | 0.41 | 0.70 |
| | | 19.75 ± | 14.67 ± | 17.19 ± | 21.46 ± | 21.63 ± | | 18.48 ± | 18.63 ± |
| | 75 | 0.12 | 0.83 | 0.03 | 0.39 | 0.08 | 20.2± 0.00 | 0.35 | 0.17 |

KEY: MuM – Mukutani meliponin, MuA – Mukutani apis, MaM – Maoi meliponin, MaA – Maoi apis, KiM – Kibingor meliponin, KiA – Kibingor apis, KoM – Koriema meliponin, KoA – Koriema apis.

**Table 4.8: Mean zones of inhibition of the honey (stingless and honey bee samples) dilutions (10, 20, 50 and 75 x $10^4$ µg/ml) against the bacterial control strains**

| | HONEY SAMPLES (Stingless and Honeybee) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CONC.(x104 µg/ml) | MuM | MuA | MaM | MaA | KiM | KiA | KoM | KoA |
| *S.aureus*(ATCC 25923) | | | 12.96 ± | 12.84 ± | 16.98 ± | | 17.84 ± | 16.34 ± |
| 10 | 13.04 ± 0.01 | 11.8 ± 0.05 | 0.03 | 0.23 | 0.03 | 14.99 ± 0.08 | 0.56 | 0.06 |
| | | 12.01 ± | 13.42 ± | 13.13 ± | 17.52 ± | | 18.41 ± | 17.29 ± |
| 20 | 13.97 ± 0.04 | 0.15 | 0.10 | 0.05 | 0.10 | 15.81 ± 0.16 | 0.04 | 0.05 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 50 | 14.27 ± 0.02 | 12.44 ± 0.04 | 14.4 ± 0.02 | 13.68 ± 0.09 | 18.49 ± 0.07 | 16.36 ± 0.00 | 19.66 ± 0.08 | 18.01 ± 0.16 | | | |
| | 75 | 14.92 ± 0.05 | 12.98 ± 0.02 | 14.98 ± 0.11 | 14.12 ± 0.05 | 18.61 ± 0.12 | 16.94 ± 0.13 | 20.09 ± 0.03 | 18.9 ± 0.02 | | | |
| *E. coli* (ATCC ) | 10 | 9.74 ± 0.06 | 6.88 ± 0.09 | 10.86 ± 0.02 | 6.46 ± 0.03 | 7.58 ± 1.09 | 7.60 ± 0.08 | 12.97 ± 0.09 | 8.82 ± 0.01 | | | |
| | 20 | 9.86 ± 0.00 | 7.68 ± 0.03 | 11.84 ± 0.04 | 7.46 ± 0.02 | 7.86 ± 0.01 | 9.13 ± 0.05 | 13.46 ± 0.02 | 9.98 ± 0.01 | | | |
| | 50 | 9.98 ±0.10 | 8.78 ± 0.10 | 12.08 ± 0.03 | 7.78 ± 0.02 | 8.13 ± 0.03 | 9.78 ± 0.03 | 13.88 ± 0.08 | 11.24 ± 0.05 | | | |
| | 75 | 10.85 ± 0.034 | 9.41 ± 0.05 | 12.89 ± 0.21 | 8.02 ± 0.06 | 8.98 ± 0.09 | 10.07 ± 0.04 | 14.62 ± 0.08 | 12.81 ± 0.04 | | | |
| *K. pneumoniae* (ATCC 27736) | 10 | 12.19 ± 0.03 | 12.08 ± 0.03 | 13.89 ± 0.04 | 12.81 ± 0.03 | 17.06 ± 0.03 | 15.31 ± 0.04 | 18.78 ± 0.01 | 13.93 ± 0.02 | | | |
| | 20 | 13.39 ± 0.04 | 12.96 ± 0.08 | 14.96 ± 0.05 | 13.62 ± 0.03 | 17.81 ± 0.05 | 16.22 ± 0.02 | 19.01 ± 0.04 | 14.99 ± 0.04 | | | |
| | 50 | 13.96 ± 0.02 | 13.48 ± 0.05 | 15.79 ± 0.04 | 14.29 ± 0.06 | 18.88 ± 0.09 | 17.04 ± 0.09 | 19.86 ± 0.02 | 15.11 ± 0.00 | | | |
| | 75 | 14.01 ± 0.03 | 14.06 ± 0.02 | 16.83 ± 0.02 | 15.19 ± 0.03 | 19.91 ± 0.00 | 17.82 ± 0.02 | 10.6 ± 0.08 | 15.86 ± 0.04 | | | |
| *P. aeruginosa* ( ATCC 27853) | 10 | 15.07 ± 0.02 | 16.03 ± 0.01 | 16.68 ± 0.05 | 15.01 ± 0.04 | 16.99 ± 0.03 | 17.08 ± 0.01 | 18.66 ± 0.01 | 18.04 ± 0.03 | | | |
| | 20 | 16.14 ± 0.03 | 16.84 ± 0.02 | 17.04 ± 0.03 | 15.81 ± 0.04 | 17.02 ± 0.02 | 17.84 ± 0.06 | 19.01 ± 0.00 | 18.81 ± 0.09 | | | |
| | 50 | 17.0 ± 0.12 | 17.49 ± 0.01 | 17.89 ± 0.04 | 16.73 ± 0.02 | 17.79 ± 0.02 | 18.62 ± 0.08 | 19.89 ± 0.04 | 19.36 ± 0.04 | | | |
| | 75 | 17.81 ± 0.02 | 18.79 ± 0.00 | 18.92 ± 0.01 | 17.84 ± 0.01 | 18.88 ± 0.07 | 19.51 ± 0.00 | 20.84 ± 0.02 | 20.58 ± 0.06 | | | |

KEY: MuM – Mukutani meliponin, MuA – Mukutani apis, MaM – Maoi meliponin, MaA – Maoi apis, KiM – Kibingor meliponin, KiA – Kibingor apis, KoM – Koriema meliponin, KoA – Koriema apis.

The study's findings also showed that, at equivalent doses, the two kinds of honey—which came from different bee species and geographical locations—exhibited different inhibitory effects against the same bacterial isolates and the control strains. As shown in Table 4. 9, stingless bee honey demonstrated much greater antibacterial activity against isolates of both Gram-positive and Gram-negative bacteria than honeybee samples.

Table 4.9: An overview of the comparison of zones of inhibition between bacterial and control isolates against honey samples from stingless and honeybee at $10x10^4$, $20x10^4$, $50x10^4$ and $75x10^4$ µg/ml

| ISOLATES/ PC | HONEY BEE SAMPLE (APIS) | STINGLESS BEE SAMPLES (MELIPONIN) |
|---|---|---|
| *S. aureus* | 16.98 ± 2.89 | 18.79 ±4.51 |
| *S. aureus* ATCC 25923 | 14.68 ± 2.44 | 16.21 ± 2.66 |
| *E. coli* | 9.48 ± 1.69 | 10.76 ± 2.48 |
| *E. coli* ATCC 25922 | 8.89 ± 1.41 | 11.00 ± 2.46 |
| *K. pneumoniae* | 14.52 ± 2.09 | 17.55 ± 2.50 |
| *K. pneumoniae* ATCC 27736 | 14.68 ± 1.48 | 17.00 ± 2.47 |
| *P. aeruginosa* | 17.19 ± 1.78 | 18.81 ± 1.26 |
| *P. aeruginosa* ATCC 27853 | 17.81 ± 1.28 | 17.85 ± 1.30 |

The bacterial isolates showed different degrees of susceptibility to the various antibiotics under test as being resistant, intermediate, or susceptible with the following mean inhibition diameters: 9.50 ± 0.23 mm to 25.0 ± 0.62 mm, 6.0 ± 0.0 mm to 24.0 ± 0.56 mm, 6.0 ± 0 0 mm to 19.0 ± 0.50 mm and 6.0 ± 0.0 mm to 18.0 ± 0.46 for *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* respectively. Despite significant resistance to most antibiotics under investigation, the organism that exhibited the least resistance to the therapy was Staphylococcus aureus with a mean inhibition of 17.68 ± 5.10 mm was the highest. In comparison, *Escherichia coli* was the least with a mean inhibition diameter of 11.50 ± 7.31mm, then *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with a mean inhibition diameter of 9.33 ± 4.89 mm and 8.92 ± 4.65 mm respectively. The findings of this study also showed that the bacterial isolates were susceptible to Gentamicin (10µg), Chloramphenicol (30µg) and Levofloxacin (5µg), as presented in Figure 4.14.

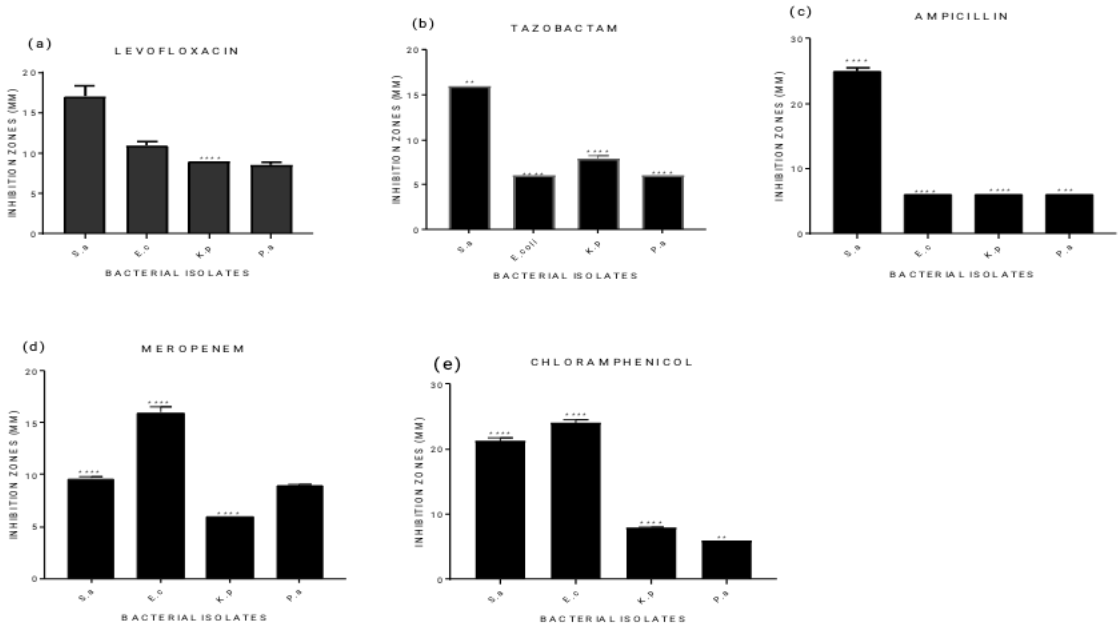



Figure 4.14: Antibacterial activity of commonly used antibiotics against bacterial isolates from burns and cutaneous wounds. Levofloxacin (5 µg), Tazobactam (110 µg), Ampicillin (10 µg), Meropenem (10 µg), Gentamicin (10 µg) and Chloramphenicol (30 µg). The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05, **P<0.01, ***P<0.001 and **** P<0.0001. (KEY: S. a- *Staphylococcus aureus*, E. c. - *Escherichia coli*, K. p. - *Klebsiella pneumoniae* and P.a. - *Pseudomonas aeruginosa*).

Further to the study, most antibiotics could inhibit all the control bacterial isolates (ATCC) with relatively lower mean inhibition zones than those created by honey discs. Chloramphenicol indicated the highest inhibition with a mean inhibition zone of 15.29 ± 6.65 mm, followed by Gentamicin (15.24 ± 5.22 mm), Levofloxacin (11.52 ± 3.85 mm), Ampicillin (10.56 ± 8.37 mm), Meropenem (10.05 ± 3.35 mm)

and finally Tazobactam (9.00 ± 4.39 mm). The microorganisms indicated varying susceptibility to the antibiotics with considerable resistance for *Pseudomonas aeruginosa* against Tazobactam and ampicillin, as shown in Figure 4.15.

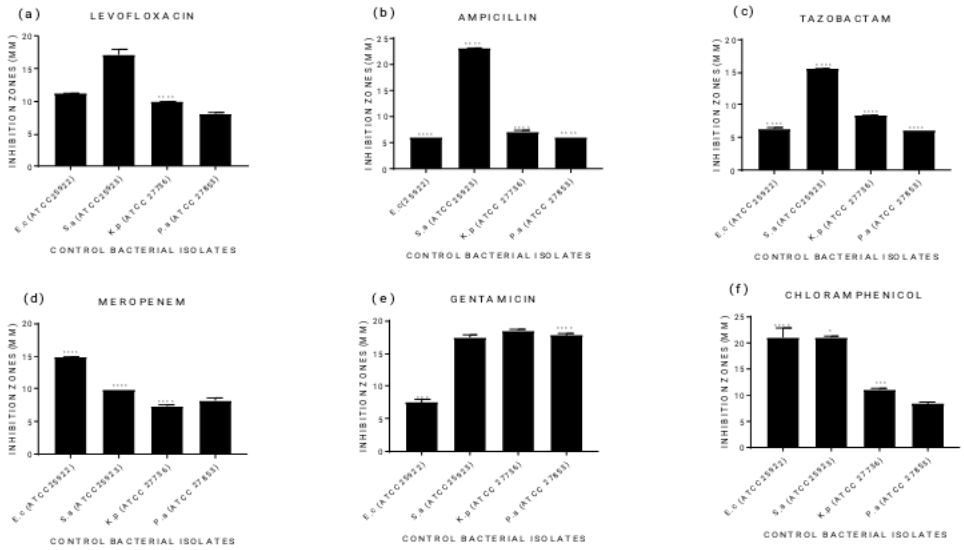



Figure 4.15: Antibacterial activity of commonly used antibiotics against control bacteria Levofloxacin (5 µg), Ampicillin (10 µg), Tazobactam (110 µg), Meropenem (10 µg), Gentamicin (10 µg) and Chloramphenicol (30 µg). The values are represented in mean ± SD as error bars represent Standard deviation (SD). Significant values (P<0.05) are represented by stars on the bars (*P< 0.05, **P<0.01, ***P<0.001 and **** P<0.0001. (KEY: S. a- *Staphylococcus aureus*, E. c. - *Escherichia coli*, K. p. - *Klebsiella pneumoniae* and P.a. - *Pseudomonas aeruginosa*).

### 4.6.3 Bacteriostatic and bactericidal activity of honey

*i. Honeybee samples*

To ascertain the bacteriostatic minimum inhibitory concentration (MIC) and bactericidal minimum bactericidal concentration (MBC), a series of dilutions was also generated utilizing honeybee samples. These concentrations were used on bacterial isolates that were taken from burns and cutaneous lesions that were infected. Starting with an unadulterated sample, the dilutions were done in steps

of 500, 250, 125, 62.5, and 31.5µg/ml. A honey-negative control (HC) and a Gram-positive bacterial positive control (GC) were the two control tubes that were set up. The former was made up of Muller Hinton media without any bacterial microorganisms and honey, whereas the latter was made up of a medium with a bacterial microorganism but no milk. The minimum inhibitory concentration (MIC) activity of the honeybee samples against each isolate was 62%. The minimum inhibitory concentration (MIC) activity was determined to be 5 µg/ml and the minimum bactericidal concentration (MBC) activity to be 250 µg/ml, based on the data shown in Table 4.10.

**Table 4.10: Minimum bacteriostatic and bactericidal concentration –MIC/MBC (µg/ml) of honey bee samples against bacterial isolates**

| | *S. aureus* | | *E. coli* | | *P. aeruginosa* | | *K. pneumoniae* | | GC | | HC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC (µg/ml) | MBC (µg/ml) | MIC (µg/ml) (µg/ml) | MBC | MIC (µg/ml) | MBC (µg/ml) | MIC (µg/ml) (µg/ml) | MBC | MIC (µg/ml) (µg/ml) | MBC | MIC (µg/ml) | MBC (µg/ml) |
| MuA | 125 | 250 | 250 | 250 | 125 | 250 | 125 | 250 | 500 | ND | ND | ND |
| MaA | 62.5 | 62.5 | 250 | 250 | 125 | 250 | 250 | 250 | 500 | ND | ND | ND |
| KiA | 125 | 125 | 250 | 250 | 125 | 250 | 250 | 250 | 500 | ND | ND | ND |
| KoA | 125 | 250 | 250 | 250 | 125 | 125 | 250 | 250 | 250 | 500 | ND | ND |

**KEY**: MIC – Minimum inhibition concentration, MBC – Minimum Bactericidal concentration. MuA - Mukutani Apis, MaA – Maoi Apis, KiA- Kibingor Apis, KoA - Koriema Apis). (**S.a** – Staphylococcus aureus, **E.c** – Escherichia coli, **P.a** – Pseudomonas aeruginosa, **GC** – Positive control, **HC** – Negative control)

### *ii. Stingless bee honey samples*

Stingless bee honey samples showed higher antibacterial activity at lower

concentrations (125µg/ml) than the honeybee samples (250µg/ml), as shown in Table 4.11. There were notable inhibitory effects on the growth of all the bacterial isolates. Importantly, higher activity was observed against *Klebsiella pneumoniae,* inhibited for growth entirely at the lowest concentration of 62.5µg/ml, while lower susceptibility was observed against *Pseudomonas aeruginosa.*

**Table 4.11: Minimum bacteriostatic and bactericidal concentration – MIC/MBC (µg/ml) of the stingless bee honey against bacterial isolates**

| | S. *aureus* | | *E. coli* | | *P. aeruginosa* | | *K. pneumoniae* | | GC | | HC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC µg/ml | MBC µg/ml | MIC µg/ml | MBC µg/ml | MIC µg/ml µg/ml | MBC | MIC g/ml µg/ml | MBC | MIC µg/ml µg/ml | MBC | MIC µg/ml | MBC µg/ml |
| MuM | 125 250 | | 250 | 125 | 62.5 | 125 | 125 | 125 | 500 | ND | ND | ND |
| MaM | 125 | 250 | 250 | 250 | 125 | 125 | 62.5 | 62.5 | 500 | ND | ND | ND |
| KiM | 125 | 125 | 125 | 125 | 62.5 | 62.5 | 62.5 | 62.5 | 250 | 500 | ND | ND |
| KoM | 62.5 | 125 | 125 | 250 | 62.5 | 125 | 62.5 | 125 | 500 | ND | ND | ND |

KEY: MIC – Minimum inhibition concentration, MBC – Minimum Bactericidal concentration. MaM – Maoi Meliponin, MuM – Mukutani Meliponin, KiM – Kibigor Meliponin, KoM- Koriema Meliponin). GC- Growth control tube, HC – Honey control tube

### *iii. Reference bacterial microorganisms*

Thus, the antibacterial function of honey is much broader for this study; it demonstrated that honey can both immobilize and kill the control bacterial microorganisms (ATCC). The honey and stingless bee honey samples exhibited

inhibitory effects at dilutions ranging between 31.25 µg/ml and 125 µg/ml; it was found that the average MICs value was less in *Staphylococcus aureus* (ATCC 25923) isolates that were 31.25µg/ml to 62.5µg/ml while *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 27736) and *Pseudomonas aeruginosa* (ATCC 27853). Stingless bees sampled at Koriema and Kibingor had a higher inhibitory effect than the other honey samples in the study, as presented in Table 4.12.

**Table 4.12: Minimum bacteriostatic and bactericidal concentration – MIC/MBC (µg/ml) of the honey and stingless bee honey against control bacterial isolates (ATCC)**

| | *S. a* (ATCC25923) | | *E. c* (ATCC25922) | | *P. a*(ATCC27853) | | *K. p* (TCC27736) | | GC | | HC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC (µg/ml) | MBC (µg/ml) | MIC (µg/ml) | MBC (µg/ml) | MIC (µg/ml) | MBC (µg/ml) | MIC (µg/ml) | MBC (µg/ml) | MIC (µg/ml) | MBC (µg/ml) | MIC (µg/ml) | MBC (µg/ml) |
| MuA | 31.2 | 6.25 | 62.5 | 62.5 | 62.5 | 125 | 125 | 250 | 250 | 250 | ND | ND |
| MuM | 31.2 | 62.5 | 62.5 | 125 | 62.5 | 125 | 62.5 | 125 | 250 | 250 | ND | ND |
| MaA | 62.5 | 62.5 | 125 | 125 | 125 | 250 | 125 | 125 | 250 | 500 | ND | ND |
| MaM | 31.2 | 62.5 | 62.5 | 62.5 | 62.5 | 62.5 | 62.5 | 125 | 500 | ND | ND | ND |
| KiA | 62.5 | 62.5 | 62.5 | 125 | 125 | 250 | 125 | 250 | 500 | ND | ND | ND |
| KiM | 31.2 | 62.5 | 62.5 | 125 | 62.5 | 62.5 | 62.5 | 62.5 | 250 | 250 | ND | ND |
| KoA | 62.5 | 62.5 | 62.5 | 125 | 125 | 125 | 62.5 | 125 | 250 | 250 | ND | ND |
| KoM | 62.5 | 125 | 62.5 | 125 | 62.5 | 125 | 62.5 | 125 | 250 | 250 | ND | ND |

KEY: MIC – Minimum inhibition concentration, MBC – Minimum Bactericidal concentration of control bacterial isolates. MaM – Maoi Meliponin, MaA – Maoi Apis, MuM – Mukutani Meliponin, MuA - Mukutani Apis, KiM – Kibigor Meliponin, KiA- Kibingor Apis, KoM- Koriema Meliponin, KoA - Koriema Apis).

# CHAPTER FIVE

# 5.0 DISCUSSION

## 5.1 Isolation, characterization and identification of bacteria

The study's findings show that burn and wound swab samples, which were taken from the lesions of adult outpatients and the surgical ward at The Nakuru County Referral Hospital, contained a total of 15 Gram-positive cocci and 28 Gram-negative rods. In clinical and research laboratories, the Gram staining method is a commonly used diagnostic tool that depends on the permeability properties of the cell wall and cell membrane. Microorganisms are classified as either Gram-positive or Gram-negative using this method. Gram-negative bacteria experience severe cell injury as a result of solvent decolorization, in contrast to Gram-positive bacteria (Oya et al., 2022). With a few notable exceptions, Gram staining can be

used to detect or image almost all clinically significant bacteria. According to Boyanova (2018), these bacteria are mostly found inside cells, have no cell wall structure, and cannot be solved under a light microscope. It is imperative to recognize that almost all clinically relevant bacteria can be detected or visualized via Gram staining.

As Oya et al. (2022) noted, in bacteria, the cell wall is located externally to the cytoplasmic membrane, thus giving them shape and hardness and protecting them from lysis by the pressure from inside. It is a large macromolecule belonging to a mesh-like network of polymerized peptidoglycan containing long glycan chains linked with short oligopeptide-N-acetyl muramic acid and N-acetyl glucosamic acid moiety. They also postulated that while those Gram-positive bacteria have a thick envelop (20-35nm), Gram-negative have a thin one (2-7nm); that there is a virulence factor in the presence of capsule and lipopolysaccharides that amplify the inflammation resulting in septic shock.

This study also documents various morphologies on the culturing of these isolates. For instance, the presence of a characteristic yellow colony pigment uniform in colour and a wide zone of beta (complete) haemolysis on blood agar as documented previously (Krumkamp et al., 2020), was reported in this study. These types of colonies are suggestive of *Staphylococcus aureus*. Also, the presence of red /pink donut-shaped colonies on MacConkey agar was highly suggestive of *Escherichia coli* isolate. Yellow to whitish-blue and exceptionally mucoid colonies on CLED agar suggest *Klebsiella pneumoniae*. In contrast, greenish colonies with typical matted surface and rough periphery on MacConkey agar suggest

*Pseudomonas aeruginosa* as documented by Vakayil et al. (2020).

Biochemical characterization was used as a set of confirmatory tests to further group and identify the microorganisms upon culturing and Gram staining (Mahdi et al., 2020; Kaiser, 2019). According to these tests, 35% tested positive for the coagulase test, which indicates the presence of *Staphylococcus aureus* isolates. Other biochemical tests like Triple sugar iron agar (TSI) were also used to test for members of the Enterobacteriaceae family, of which (13.9%) indicated the presence of *Escherichia coli* as they turned out to be producing gas and carbohydrate fermentation indicated by a change in colour of the pH indicator. *Klebsiella pneumoniae* (23.26%) was found to be Methyl red test negative. However, a positive finding on the methyl red test was found amongst the *Escherichia coli* strains (13.95%), which produced a cherry red color, confirming their presence. On the other hand, 28% of the isolates tested positive for oxidase which presented itself as a production of a purple color, hence confirming the presence of *Pseudomonas aeruginosa* as documented by (Hasan et al., 2020). Therefore, a total of 43 bacteria were isolated from 29 cutaneous wound swabs as well as 5 swabs from burn wounds, which were successfully identified *Escherichia coli* 6 (13.9%), *Staphylococcus aureus* 15 (35%), *Klebsiella pneumonia* 10 (23.3%) as well as *Pseudomonas aeruginosa* 12 (27.9%). This was based on their Gram staining reaction, their shapes (bacilli/cocci), cultural characteristics and biochemical tests. Control strains: *Klebsiella pneumoniae* (ATCC 27736), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923) were obtained from Nakuru Veterinary

Laboratories and used as for quality control.

Furthermore, the outcomes aligned with the findings previously published by Abera et al. (2020) concerning the identification of lesion pathogens. The outcomes confirmed each isolate's status. With the use of Gram stain and biochemical tests, Hasan et al. (2020) and Afroz et al. (2020) have both reported the isolation and identification of the causative organisms of wound infections from wound swabs taken from patients in a tertiary care hospital in Kathmandu, Nepal, and the Niger Delta University Teaching Hospital, respectively. The same results have been recorded. Both of these investigations were carried out in 2020. Comparable results about the isolates acquired from the same samples as those utilized in this inquiry have also been confirmed and reported by other investigations (Abebe et al., 2019; Gebissa et al., 2021; Hassan et al., 2019; Kasif and Avcioglu, 2018; Mama et al., 2019; Misha et al., 2016). These findings have been confirmed and recorded by both of these studies.

A total of 4 burn and 24 cutaneous wound swab samples yielded significant bacterial growth. In comparison, 1 burn and 5 cutaneous wound swab samples were bacteriologically sterile since they did not produce substantial growth. This could be attributed to swabs not capturing bacteria that could be protected by biofilms which have been documented to be in chronic wounds (Razdan et al., 2022). Bacteria with slow growth adapted to biofilm may fail to grow using standard culture technique, and wound swabs collected through instant skin in acute cellulitis may fail to isolate any pathogen due to their low concentration (Gajula et al., 2020). The results of this study indicated that 28 swabs were

infected, with some showing polymicrobial growth and the prevalence of the isolated pathogens were *Escherichia coli* 6 (14.0%), *Klebsiella pneumoniae 10* (23.3%), *Pseudomonas aeruginosa 12 (*27.9%) and *Staphylococcus aureus* 15 (34.8%). The obtained results agreed with those of Sisay et al. (2019), who documented a 70.0% overall prevalence of infections in wounds, with *Staphylococcus aureus* being the utmost prime etiological agent at 36.0% from wound samples acquired from patients pursuing Medicare in an Ethiopian hospital. Also, Edrees et al. (2021) documented similar findings that indicated that 191 (28.2%) of the isolates obtained from wound swabs were *Staphylococcus aureus*. This was in comparison to *Pseudomonas aeruginosa* - 170 (25.2%), *Escherichia coli* - 53 (7.8%), *Staphylococcus epidermidis* 48 - (7.1%) and *Enterococcus faecalis* 38 - (5.6%).

The isolates were further subjected to molecular characterization to predict the existence of the most important virulence genes of interest. This study aimed at identifying hla and 16SrRNA virulence genes of *Staphylococcus aureus* isolated from infected cutaneous wounds that were observed in outpatient and surgical wards of County Referral Hospital – Nakuru. The presence of these small RNA (sRNA) genes is essential in regulating the expression of an immune invasion molecule. These are secreted by *Staphylococcus aureus* as its key virulence trait in impairing the host immune responses (Patel & Nair, 2021). The findings did show the presence of these two genes, 16SrRNA (756bp) and hla (209bp), in all the fifteen isolates, which confirms the presence of *Staphylococcus aureus* isolates. This is a clear indication that *Staphylococcus aureus* is one of the skin

floras that can simply take advantage of the underlying conditions to be pathogenic (Wan & Chen, 2020). These findings do concur with those of Ogonoswska & Nakonieczma (2022), Tomic-Canic et al. (2020) and Matademi et al. (2018), both of whom did document the presence of 16SrRNA genes in addition to (hemolysin encoding) hle genes from ready to eat (RTE) food samples collected from different food outlets located in the Klang valley in Malaysia. The presence of such genes has been found to enhance tolerance against oxidative stress and epithelial barrier disruption, which gives Staphylococcus aureus high potential in producing cytotoxins, enhancing their virulence in the target host. This could easily explain their up-surging resistance patterns, more so to tetracycline antibiotics (Wu et al., 2019).

Also, the *Escherichia coli* isolates were subjected to molecular characterization and the hly (1177bp) and cnf1 (498bp) genes whose presence was also determined. These genes have been documented to be responsible for toxin production and biofilm formation and decreasing the rate of bacterial phagocytosis and were amplified in 2 (33.3%) and 1 (17%) of the six isolates, respectively. The distribution of genes among the studied isolates was by the reports that were made by Rodis et al. (2020), Martinez-Medina, (2021), Abd El-Baky et al. (2020) and Raeispour and Ranjbar (2018) who did also report the presence of Hly and Cnf1 genes from *Escherichia coli* isolates obtained from faecal and urinary tract infection samples. Cnf2, unlike Cnf1 and hlyA, was not magnified; hence, no amplicons were formed. The presence of these genes clearly exhibits the ability of the Escherichia coli isolates to be more virulent and, if they

are not properly managed, may lead to more devastating conditions at the wound level. This is because hly and cnf1 genes have been documented to enhance Escherichia coli's pathogenicity in wounds (Tayh et al., 2021).

Also mapped in this study were MagA (121bp) and rmpA (106bp) *Klebsiella pneumoniae* genes, which produce a profuse amount of polysaccharide capsules resistant to serum killing and phagocytosis as well as production of extracellular polysaccharide synthesis, respectively. From the study findings, MagA (19%) and rmpA (40%) were present in the obtained clinical isolates. This concurred with a previously carried out study by Parrott et al. (2021), who also did document the presence of rmpA and magA genes from *Klebsiella Pneumoniae* isolates obtained from patients with bacteraemia at the prevalence of 48% and 17%, respectively, Khe gene, unlike rmpA and magA, was not magnified in this analysis. The findings were also in tandem with the study done by Shankar et al. (2018), who were able to isolate and identify *Klebsiella pneumoniae* from urinary tract infections in Rasht (Iran) and was able to identify the presence of magA and rmpA genes at a frequency of 2(3.07%) and 10 (15.38%) respectively. The MagA gene has also been associated with pathogenicity cases, meningitis and endophthalmitis (Choby et al*.,* 2020). On the other hand, rmpA gene involved in extracellular polysaccharide synthesis has been associated with the hypermucoviscous phenotype and the clinical syndrome attributed to invasive strains (Parrott et al. , 2021).

This study also deduced the presence of the lasI and gyrB *Pseudomonas aeruginosa* genes, which encode the production of toxins by producing the signaling molecules known as autoinducers (AIs). The $AI_s$ regulate cell

communications, enhancing bacterial survival and colonization by organizing phenotypic alterations through quorum sensing (QS) (Jones et al., 2021) and were amplified, producing amplicons at 600bp and 222bp, respectively. The gyrB gene encodes the subunit B of DNA gyrase, is responsible for catalyzing the negative supercoiling of DNA and is a reliable PCR target for *Pseudomonas aeruginosa* detection (Skariyachan et al., 2018), while LasI and RhlI are AHL (Acyl-homoserine lactones) signals produced by AHL synthase that are commonly used by this isolate in quorum sensing (Jones et al., 2021).

In the concept of quorum sensing, as the bacterial density increases during infection due to multiplication, it leads to an increase in autoinducer (signal molecule) concentration/production, which, once it reaches a particular threshold, the autoinducer will subsequently bind onto transcriptional activator (LasR/RhIR) forming a complex which stimulates genes involved in the formation of biofilm. These metabolic inactive biofilm cells form a layer that accommodates the slow infiltration and break of antibiotics and immune response thus giving the bacteria enough time to produce resistance genes. This is succeeded by motility twitching, microcolony formation and quorum sensing (cheering/screaming) signals that start to build up among the bacterial cells. Microcolonies at a certain developmental stage are invested in a protective matrix known as the extracellular matrix (Ahmed et al., 2019). The results of the present work were also convergent with the works done by Keikha & Karbalaei (2022), who identified *Pseudomonas aeruginosa* with the help of a triplex polymerase chain reaction on lasI and gyrB genes.

## 5.2 Antibacterial efficacy of the honey samples

Antibiotics are naturally occurring antimicrobial compounds created by microorganisms such as fungi. They exert antimicrobial activities by chemically denaturing enzymes and membrane proteins, causing cellular death (Smith et al., 2020). The findings of this study indicated the susceptibility of most of the test microorganisms to the antibiotics used, with a few isolates showing resistance and no inhibition zones around the antibiotic discs.

The study outcomes indicated a significant statistical difference ($p<0.0001$) between the inhibition zones around the antibiotic discs used to estimate the rate of the bacteria's susceptibility to the relative antibiotic. From the study findings, the Gram-positive (*Staphylococcus aureus*) isolate was more vulnerable than the Gram-negative isolates (Escherichia coli, Klebsiella pneumoniae, and *Pseudomonas aeruginosa*). This finding is not a surprise as Gram-positive isolates are more susceptible to most antimicrobial agents since they possess a thick single-cell wall that is the outer shell of the cells. At the same time, Gram-negative cells have relatively thin cell wall layers but harbor an additional outer membrane. These differences in the cell envelope convene diverse possessions to the cell, particularly responses to antibiotics (Anuj et al., 2019). This could also be the purpose for the high resistance of *Escherichia coli* to most antibiotics, as noted in this study and concurs with various studies that have been done before (Bucekova et al., 2019; Guan et al., 2021; Masound et al. 2021) *Staphylococcus aureus* is the utmost shared organism related with surgical wound infections by providing virulence factors that are very potent responsible for maintaining the infection as

well as causing delay in the wound healing progression.

In support of the findings of the present study is the study conducted by Misha et al. (2021) who also reported high levels of antibiotic susceptibility of *Staphylococcus aureus* to a higher generation of antibiotics. The cross-sectional study evaluated bacterial isolates and their resistance patterns to antibiotics in inpatients and outpatients with pus/wound discharge at the University of Gondar, Ethiopia. Among the Gram-negative bacteria, *Escherichia coli* was found highly susceptible to Chloramphenicol but lesser sensitivity to Ampicilin, Tazobactum and Gentamicin. This finding also concurs with findings from another study by Kadhim (2022) that involved patients with infected wounds visiting Jimma University Specialized Hospital. However, almost all bacterial isolates from this study were highly susceptible to Gentamicin and Chloramphenicol. This high susceptibility pattern might support Gentamicin and Chloramphenicol as suitable antibiotics to treat wound infections.

When the various doses of honey were bio-assayed for their bioactivity, the lower dilutions indicated a more inhibitory effect than honey samples with a higher dilution concentration. However, unlike for antibiotics, none indicated resistance. This supported the finding that pure honey can stagnate bacterial growth with other possibilities including the high fructose content exerting osmotic pressure on bacterial cells thereby forcing water to move out of bacterial cells through osmosis as explained earlier (Ismail et al., 2021). This causes dehydration of the cells, making them incapable of growing and thriving in hypertonic sugar solution, but this action will be minimized at the site of infection upon dilution (Albadiri,

2019). *Staphylococcus aureus* also indicated the highest susceptibility to the honey discs at a mean inhibition zone of 14.00 to 23.35mm (mean average 17.91 ± 3.66mm) followed by *Pseudomonas aeruginosa* 13.10 to 20.00mm (mean average 17.56 ± 2.37mm), *Klebsiella pneumoniae* 12.13 to 21.60mm (mean average 16.05 ± 2.64mm) and finally *Escherichia coli* 8.00 to 10.90mm (mean average 10.14 ± 2.08mm). These findings were in tandem with those of Suganthi & Saranraj (2018), who examined the antimicrobial activity of commercial honey samples collected from Chetheri, India. They identified various inhibition ranges of the bacterial isolates studied as *Staphylococcus aureus* (22mm), *Pseudomonas fluorescens* (18mm), *Klebsiella pneumoniae* (16mm) and *Escherichia coli* (17mm).

This clearly demonstrates that honey can be a potential candidate in managing Gram-positive isolates as its inhibitory effects are higher compared to those produced by the commonly used antibiotics. It is also a clear indication that its activity mode could be attributed to the action on the bacteria's cell wall and cell membrane, though more scientific validation is needed. The honey inhibition zones indicated a statistically significant difference ($p<0.0001$) between them, with both the stingless bee and honey bee samples producing mean inhibition zones of 16.48 ± 3.85mm and 14.54 ± 3.58 mm, respectively, on all the isolates as well as the control strains. Both stingless bee and honeybee samples from the Kibingor and Koriema regions indicated the highest inhibitions, which could confirm many studies that have proved that not all honey samples possess a similar degree of antibacterial activity (Cilia et al., 2021). These study outcomes also agree with the study done by Masound et al. (2021) which reported the

inhibitory effectiveness of nine Tualang and Acacia honey samples in Malaysia against antibiotic-resistant pathogenic bacteria. The target bacterial isolates (*Staphylococcus aureus, Escherichia coli as well as Pseudomonas aeruginosa*) showed high resistance to several antibiotics (Bacitracin, Gentamicin, Tetracycline, Penicillin and Chloramphenicol) with a mean inhibition range of 2.17 ± 3.75mm to 11.17 ± 9.83mm. All nine honey samples showed variable inhibitory activities with a mean inhibition range of 14.83 ± 1.76mm to 16.5 ± 3.28mm. The high rates of isolated multiple drug-resistant pathogens are, therefore, necessary to suitably monitor the choice of antibiotics.

The tested honey samples in this study were found to have both bacteriostatic and bactericidal properties with growth obstruction and complete inhibition on all the test organisms detected at a concentration of 6 to 2500 µg/ml, while all the microorganisms were inhibited at 600 to 5000µg/ml concentration. It is worth noting that the Gram-positive isolate had a lower inhibitory concentration than the Gram-negative isolates, which concurs with the disc diffusion findings. The study also deduced that honey has both bactericidal and bacteriostatic properties and depends again on the pathogen of interest. The stingless bee honey samples indicated bacterial inhibition at lower concentrations than the honeybee samples, indicating its high antimicrobial efficacy. According to the study findings, relatively higher concentrations of honey inhibited both *Escherichia coli* and *Klebsiella pneumoniae* compared to other bacterial isolates. This possibility could be due to the bacterial nature of lower cell wall permeability and its ability to regulate resistant genes through expressing resistant mechanisms and mutation in

chromosomes (Li et al., 2020).

The findings of this study are consistent with those of earlier research projects undertaken by Tesfaye et al. (2022). Eighty percent of the pathogens examined, including *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*, had a minimum inhibitory concentration (MIC) of 625µg/ml for stingless bee honey from Northern and Northwest Ethiopia, according to the researchers' findings. On the other hand, white and yellow Apis mallifera honey had a minimum inhibitory concentration (MIC) of just 40%. The findings of the investigation showed that the minimum bactericidal concentration (MBC) of each type of honey against every organism tested was 1250µg/ml. Furthermore, Mama et al. (2019) reported that honey from Ethiopia's Gamo Gofa zone demonstrated a range of 938 to 3750µg/ml for both mean minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) activity against Methicillin-resistant *Staphylococcus aureus*.

Regarding the honey antibacterial activity, control bacterial organisms (ATCC) also indicated an inhibition activity relative to the test bacterial isolates. The findings of this study indicated a lower MIC (312µg/ml to 625µg/ml) for *Staphylococcus aureus,* but a higher value (625µg/ml-1250µg/ml) was observed for *Escherichia coli* (ATCC 25922*), Klebsiella pneumoniae* (ATCC 27736) and *Pseudomonas aeruginosa* (ATCC 27853). These findings were in tandem with the findings of Cebrero et al. (2020) on Ulmo honey against *Staphylococcus aureus* (310µg/ml-630µg/ml) and equivalent MICs for manuka honey (1250µg/ml) for the *Escherichia coli* and *Pseudomonas* strains. Subsequently, a study by Jemberie et al. (2020) on

the use of stingless collected from the North-West region of Ethiopia in the treatment of wounds and respiratory infections found the honey samples having MIC of 625µg/ml and MBC of 1250µg/ml against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922).

As a result, the presence of a range of phytochemicals with the potential to function as antioxidants and antibacterial agents can be used to partially explain the antibacterial effect that was seen in this study for both types of honey that were bio-assayed against bacterial isolates of wounds and burns. Phytochemicals include nitrogenous substances such alkaloids, amino acids, chlorophyll derivatives, and amines, as well as phenolic compounds like tocopherols, flavonoids, and phenolic acids, in addition to tannins and polyphenols. It is also important to keep in mind that other factors, like the temporal and spatial changes in nectar supplies, may cause honey's antibacterial activity to change (Cebrero *et al.*, 2020). It is a matter that warrants consideration.

However, from this study, it is demonstrated that the two types of honey (honey and stingless bee) sampled form a classification as a broad-spectrum antimicrobial due to its demonstrated ability against various Gram-positive and Gram-negative bacterial cells; hence, it is necessary to incorporate honey among other antibacterial therapies bacterial infections management since it has both antiseptic and antimicrobial effects and can save a patient’s life. Therefore, honey can be an important medicinal product with established antibacterial properties that can be used to treat diseases and infections (Kushagri and Kativa, 2019).

## 5.3 Physico-chemical properties of Honey

### *5.3.1 Sugar content*

The honey samples analyzed in this study had total sugar content ranging from 80.83 ± 1.6 to 95.2 ± 5.0 g/100g. All studied samples had sugar content above the minimum requirement of the Codex Alimentarius Commission (1999) of not less than 65g/100g, EU standard and KEBS guidelines for quality assurance of bee products (2007) of not less than 65g/100g (WHO, 1999). Higher sugar levels were documented in samples from Maoi and Mukutani locations, regions grouped as lower altitude ecological zones. Honey with high sugar levels has been demonstrated to possess high carbohydrate composition (65 – 75%), which has been attributed to the presence of rich floral sources, geographical origin, climatic conditions and temperature (Wang *et al.,* 2023). Therefore, the lower the moisture content, the higher the glucose content of honey, and the faster the crystallization rate (Czipa et al., 2019). The results showed that the honey samples with high sugar levels were found in *Melipponine* bee species as compared to *Apis* species. The results from this study also showed a statistically significant difference ($p<0.0001$) in the samples collected in all the diverse areas under investigation.

These findings do confirm well the findings reported previously by Upreti et al. (2018), who documented a range of 62.80 ± 0.68g/ml to 70.00 ± 0.80g/ml from Algerian honey samples and Azonwade et al. (2018) who also documented a mean content of 82.30 ± 2.03g/100g in honey samples from the Northern sub-region of Nigeria. Other samples that compared to this test's findings are Roby et al., 2020 and Johnston et al. (2018), who indicated a range of total sugar of between 45.3 to

86.0g/100g. The presence of high sugar molecules in honey has been found to cause a strong interaction with water molecules, leaving very little water accessible for the growth of microorganisms. This interaction leads to a high osmolarity, which is also responsible for antibacterial activity (Majtan et al., 2021).

### *5.3.2 Moisture content*

Moisture is one of the physicochemical properties affecting honey's climate and its degree of maturation. In this case, it is necessary to specify the honey's shelf life because there is a correlation between water content and yeast count. The results of this study showed moisture content levels ranged from 71.70 ± 2.25mg/g to 147.33 ± 2.08mg/g, The Codex Alimentarius Commission has set the standard for honey moisture content ranging from 6.5 mg/g to 210mg/g (WHO, 2023). Moisture content signifies contamination and as such the results obtained revealed that the honey samples used in this study were of high quality (Chen 2019). The honeybee samples analyzed in this scholarship indicated a higher mean moisture content (114.33 ± 26.61mg/g) related to the stingless bee samples (81.83 ± 10.44mg/g), which is less dense when naturally observed. The honey samples from Koriema showed a significant difference ($p<0.001$), while samples from Kibingor were more significant at $P<0.0001$.

Singh & Singh (2018) reported a high honey moisture content of up to 233.6mg/g, which was found to accelerate the microbial fermentation process of honey, reducing its shelf life. It could also cause increased viscosity, leading to increased container pressure upon packaging. Low moisture content causes osmosis due to

the elevated sugar content level, which can stop the development of bacterial cells through dehydration. Still, upon honey dilution by body fluids at the site of infection, this antibacterial action is reduced (Albadiri, 2019).

### *5.3.3 The pH content*

All the honey samples analyzed in this study indicated a low pH ranging from 3.70 ± 0.1 to 4.14 ± 0.47, whereby samples from stingless bee honey recorded lower PH values (3.88 ± 0.12) than the honeybee samples (4.07 ± 0.08). The values identified did not show a significant difference between them. They were comparable to those previously reported by Johnston et al. (2018), who documented pH values ranging from 3.7 to 4.4 from Indian honey samples. Other researchers who reported correlated pH values include Upreti et al. (2018) and Muresan & Buttstedt (2019), with ranges of 3.84 to 4.42 and 3.28 to 4.67, respectively, reported on honey samples from Algeria and Mauritius. Other studies that recorded low pH values on stingless bee honey samples collected from Thailand and Brazil were by Avila et al. (2019) and Omar et al. (2019) and documented pH values between 3.60 to 5.84 and 3.33 to 6.56, respectively.

According to the National Honey Board, honey is characteristically acidic, with pH values between 3.2 and 4.5 (Mohammed et al., 2019). These values are low enough to inhibit several bacterial pathogens since the minimum values for growth are 4.0 – 4.4, and thus, in undiluted honey, the acidity level is a noteworthy antibacterial factor (Almasaudi, 2021). The low pH enhances changes in osmolarities, leading to lysis of microbial cell walls (Morroni et al., 2018).

### *5.3.4 Free acidity*

The free acid is one of the basic characteristics underlying honey quality control defined within the European Composition Standards for this food product (Roby et al., 2020). The free acid of honey is obtained through all the free acids and low pH in bacterial inhibition. It increases with time and changes its flavor during yeast fermentation of sugars and alcohols. Free honey is highly hygroscopic, and its moisture content may vary depending on air humidity during storage (Aroucha et al., 2019).

According to the outcomes of this study, the average amount of free acidity ranged from 0.03 ± 0.02 to 0.15 ± 0.03 meq/ kg with an individual average value of 0.062 ± 0.01 and 0.063 ± 0.06meq/kg for honey bee and stingless bee honey samples respectively; this is within the limits of <50meq/kg by Condex Alimentarius Commission, (2001). The findings of this study do concur with the findings of Balos et al. (2018), who analyzed honey samples from Northern Serbia and documented a mean free acidity range of 1.50 ± 30.0 meq/kg to 5.0 ± 29.0 meq/kg. But in contrast to these, there is the study by Maringgal et al. (2019), who documented Malaysian stingless bees' mean free acidity content ranging from 64.50 meq/kg to 207.67 meq/kg. This large difference in the free acidity value could be as a result of differences in floral content and bee species. All the honey samples from honeybee and stingless bee samples were not significantly different from each other, however, the stingless bee sample from Maoi was significantly different at p<0. 0001.

### *5.3.5 Hydroxyl methyl furfural (HMF) content*

Hydroxyl methyl furfural (HMF) and its products have demonstrated the potential of triggering genotoxic, mutagenic, carcinogenic, DNA damaging, organotoxic as well as enzyme inhibitory effects of human health. Specifically, as an antioxidant, it possesses a dose-dependent action to scavenge the free radical and offers significant protection on erythrocytes against the impairment caused by ROS (Braghini et al., 2019).

Most of the honey samples analyzed in this study gave an average of 4.7 ± 0.85mg/kg – 42.3 ± 0.38mg/kg, within the recommended maximum of 60mg/kg according to The Codex Alimentarius Commission (2001). Those samples collected from the Mukutani region indicated a higher value due to the hot weather during honey collection, which accelerates the production of undesirable substances while decreasing the amount of reducing sugars, thus increasing the honey's tendency to crystallize (Shapla et al., 2018). The stingless bee samples also had higher HMF values (27.9mg/kg) than the honeybee samples (15.7 mg/kg), thus indicative of their higher antioxidant activity. Such variation in the concentrations of the HMF in honey collected from different kinds of bees could be attributed to the species, the flowering, and the climate of the honey sampling areas. In addition to the temperature, the formation of 5-HMF concentration depends on the kind of sugar, pH, and water content. Some of the studies have indicated that honey samples from Stingless bees contained higher sugar and moisture content as compared to honey from Apis mellifera (Brown et al., 2020). Thus, the results of the present study revealed a highly significant variation

(p<0.0001) between the samples obtained from the various areas of study.

HMF has been described by former scholars like Mesele (2021), Azonwade et al. (2018) & Osaili et al. (2023), who analyzed honey samples from Central, Eastern, and Northern Tanzania, Nigerian as well as Pakistan honey and documented an HMF range of 8.6mg/kg to 45.0mg/kg, 5.0 to 17.22mg/kg and 27.69 to 36.08 mg/kg respectively. Similar comparison studies were also carried out by Edo et al. (2022) and Xu et al. (2022), who analyzed HMF concentration of different species of *Meliponina* from Brazil and Nigeria, documenting a higher mean range of 0.4 to 78.4mg/kg and 16.58 to 72.34mg/kg respectively compared to *Apis mellifera*. This, therefore, acted as a positive finding as it demonstrates that both honey samples could have potential antioxidant properties that have been found to show a disease-dependent free-radical scavenging capacity as well as substantial defensive properties on erythrocytes besides ROS-prompted mutilation (Mancuso *et al.,* 2021).

### *5.3.6 Hydrogen peroxide content*

In this scholarship, the average concentration of hydrogen peroxide in the honey samples ranged between 0.50 ± 0.0mM – 1.00 ± 0.41mM, with a mean average of 0.69 ± 0.26mM and a higher mean concentration of 1.00 ± 0.41mM for stingless bee honey sample compared to 0.65 ± 0.25mM for honey bee samples. These findings were within the recommended range of 0.146 to 2.93mM (Almasaudi, 2021). All the honey samples indicated the presence of hydrogen peroxide, with the samples from ecological zone IV (Maoi and Mukutani) indicating a higher concentration, and the findings showed no significant difference between them.

Hydrogen peroxide generally prevents microbes' putrefaction of unripe honey when they have deficient sugar concentrations. Glucose oxidase is secreted by honeybees during honey production and is added to nectar; this enzyme converts glucose to gluconic acid and hydrogen peroxide ($H_2O_2$), which has documented effective antimicrobial activity (Bucekova et al., 2019). Its presence in honey and other phytochemical compounds has also been documented to enhance bacterial DNA degradation, a possible mode of its activity against microorganisms. It is a significant oxidizing and sanitizing agent produced enzymatically by glucose oxidase when honey is diluted (Albadiri, 2019). Therefore, its presence in honey samples could also add more value to the antimicrobial activities that have been documented.

## 5.4 Phytochemical and Antioxidant Properties of Honey

Phenolic acids and flavonoids are other phytochemicals that are often present in low concentrations in honey and have been credited with the honey flavour, colour and its bioactivities (Talebi et al., 2020). The complementary and additive mode of action is provided through antioxidant, antibacterial, and antiviral effects and immunostimulatory and enzyme-inducing effects on detoxifying enzymes and cholesterol synthesis (Nguyen et al., 2019).

### *5.4.1 Total Phenolic Content*

The total phenolic content of all the honey samples analyzed in this study was at an average of 40.87 ± 1.95 to 155.83 ± 7.06 mgGAE/100g with 80.81 ± 36.25mg GAE/100g and 90.17 ± 51.18mgGAE/100g for honey and stingless bee samples respectively. From the findings of the study, the mean total phenolic substances

were higher in Koriema and Kibingor compared to Mukutani and Maoi and are slightly below the recommended level of 161 to 186 mgGAE/100g due to the differences in the floral sources, environmental and seasonal factors in the regions. The findings of this study concurred with Suleiman et al. (2020), who documented a range of 44.8 to 241.4 mgGAE/100g in Acacian honey samples.

Phenolic compounds are linked with the stability of free radical scavengers since they produce less toxic and stable molecules after releasing hydrogen from one of the hydroxyl groups (Talebi et al. , 2020). Thus, it is recorded as the primary source of honey's antioxidant potential (Cianciosi et al. , 2018). By these, they can prevent many diseases through various mechanisms have many diseases mechanisms such as regulation of a specific gene expression or other metabolic pathways that are involved in disease development such as cell intracellular pathways for cell proliferation and apoptosis among others (Zulkhairi Amin et al. , 2018).

### *5.4.2 Total Flavonoid Content*

The samples analyzed in this study indicated a significant presence of total flavonoids with a mean concentration of 17.57 ± 0.76 to 31.63 ± 1.27 mgRE/100g. An uppermost mean value of 23.66 ± 5.87 mgRE/100g was documented from stingless bees compared to the honeybee samples that had a mean of 21.83 ± 6.16 mgRE/100g. The overall honey flavonoids depend on the floral source, geographical origin of honey, plant origin, certain climatic conditions and most of the bee species (Chin & Sowndhararajan, 2020). This was evident by the variation in the mean content obtained from the regions under study.

The total flavonoid contents acquired in this study were within the recommended range of 15.6 to 54.23 mgRE/100g by the Codex Alimentarius Commission, 2001. Also, they corresponded to the positive control mean of 20.86 ± 1.66 mgRE/100g. The findings concurred with those of Mohammed et al. (2020), who documented a range of 16.13 to 28.90 mgRE/100g on honey samples from six honey-producing regions in the Sultanate of Oman, Burkina Faso. It also corresponded to another study by Upreti et al. (2018), which documented a range from 4.80 to 22.80mgRE/100g on Turkish and Malaysian kinds of honey but differed from Algerian honey samples' range of 27.07mgRE/100g to 71.78mgRE/100g.

The mean flavonoid content obtained in the honey samples analysis in this study is of great significance since they can act as potent antioxidants, free radical scavengers and mental chelators inhibiting lipid peroxidation while exhibiting both inflammatory and antimicrobial physiological activities. Flavonoids in honey cause bacterial cell membrane disruption, leading to cell leakages and biofilm eradication (Cianciosi et al., 2018).

### *5.4.3 Total Carotenoids Content*

Carotenoids are the compounds that contribute to the brilliant colors in fruits and vegetables, related to honey hue. Its total content in fact varies according to the geographical zone considered, the climatic context and more generally the seasons (Boussaid et al. , 2018). The mean total carotenoid value obtained in this study ranged from 1.6 ± 0.07 to 6.73 ± 0.12 mgβ-carotene/kg with the different region's honey samples indicating varying mean concentrations depending on

floral sources environmental and seasonal factors.

The findings of this study concurred with Morroni et al., (2018), who documented mean total carotenoid content on four honey samples from three different countries ( New Zealand, Cuba and Kenya) as; Manuka honey ( 4.63 ± 0.34 mgβ-carotene/kg), African(5.21 ± 0.84 mgβ-carotene/kg), *A. mellifera* (4.78 ± 0.34 mgβ-carotene/kg) and *M. beecheii* (6.24 ± 0.29 mgβ-carotene/kg). Other studies included that of Bazaid et al. (2022), who documented 4.72mgβ-carotene/kg on honey of Tunisian origin, Alvarez –Suarez et al. (2018) who documented a range of 1.17 to 5.57 mgβ-carotene/kg on honey samples from Cuba as well as Souza et al., (2018), who reported a range of 0.56 to 6.19 mgβ-carotene/kg on honey samples from Rio, Grande do Sul Brazil.

Carotenoids in honey have single oxygen-quenching actions, are recognized as common hydrophilic and hipophilic antioxidants and correlate with the pollen extracts' antioxidant activity (Boussaid et al., 2018).

### *5.4.4 Vitamin C*

The mean Vitamin C content in the examined honey samples ranged between 0.73 ± 0.06 to 2.27 ± 0.17mg/kg, with the stingless bee and honeybee samples giving an average of 1.53 ± 0.64 and 1.51 ± 0.71mg/kg, respectively. The mean values obtained were within the recommended range of 0 to 18 mg/kg by the Codex Alimentarius Commission (2001) and corresponded well to the positive control mean value of 1.79 ± 0.06mg/kg. The study findings were in concurrence with those of Pauliuc et al. (2020), who documented the mean Vitamin C

concentration of honey samples produced from different plants in 14 regions of Romania as multifloral honey(0.75mg/kg), Pine tree forest honey (0.89mg/kg) and Acacia honey (2.26mg/kg).

Ascorbic acid (vitamin C) is produced as the end product of the uronic acid pathway produced by honey bees with glucose as its substrate, which is one of the non–enzymatic substances that act as antioxidants through conversion between hydro and dehydroascorbate and also acts as a coenzyme (Mohammed, 2018). The two proposed mechanisms of honey's action include converting Vitamin C into bacterial cell and formation of hydrogen peroxide and production of lactic and acetic acid from Vitamin C. It can be effective to kill and prevent the bacterial pathogens in planktonic and biofilms and its effect in presence of 'free' catalytically active metal ions can contribute for oxidative destruction by formation of hydroxyl and alkoxyl radicals. Although Vitamin C is contained in minute quantities, it forms part of the honey's nutritional profile, encouraging its use as food (Majtan et al., 2020). Total phenolic, total flavonoid and total carotenoid content are strongly correlated to honey's oxidative capacity, proving its therapeutic potential. This correlation stimulates the discharge of a variety of cytokines and activates white blood cells, enhancing wound contraction and reducing the period of epithelization.

### 5.5 Nutritive composition

Honey is also classified as a nutritive and medicinal substance (Yakubu et al., 2021). Nonetheless, heavy metals, including some alkaloids, and HMF derivatives are toxic features of this compound even in trace amounts. Urban honey contains

predominantly sugars and water besides minute quantities of proteins, amino acids, minerals, vitamins, trace elements, and polyphenols, flavonoids derived from pollen to indicate the honey source (Young & Blundell, 2023). According to the nutritional and physiological effects of honey, it is evident that it has an assortment of constructive nourishing and healthiness properties if consumed at greater doses of 50 to 80g per intake (Olas, 2020).

### *5.5.1 Mineral content*

Honey contains different quantities of minerals; the type and amount contained depends on the botanical (the soil composition, floral plant types since they are transported via the roots to the nectar) and geographical (certain minerals cannot be spotted in various honey samples collected from specific regions) origins (Ismail et al., 2021). In this study, the seven elements quantified were magnesium (Mg), calcium (Ca), iron (Fe), sodium (Na), zinc (Zn), Phosphorus (P) and Potassium (K). Among the minerals, potassium was the most prevalent mineral with a mean value of 16.43 ± 2.82 – 20.37 ± 1.67mg/l followed by calcium (1.33 ± 0.02 – 2.32 ± 0.09mg/l), sodium (0.38 ± 0.23 – 2.47 ± 1.02mg/l), Iron (0.37 ± 0.12 – 2.16 ± 0.40mg/l), zinc (0.27 ± 0.06 – 0.44 ± 0.07mg/l), magnesium (0.09 ± 0.02 – 1.19 ± 0.01mg/l) and finally phosphorous (0.06 ± 0.02 – 0.21 ± 0.03mg/l).

These findings were within the recommended ranges and were in tandem with the outcomes by Njokuocha et al., (2019) who did indicate existence of sodium, iron and magnesium on Nigerian bitter and sweet honey samples at 2.80 ± 0.00mg/kg and 3.10 ± 0.00mg/kg, 1.53 ± 0.00mg/kg and 1.25 ± 0.00mg/kg, and 0.22 ± 0.00mg/kg and 1.10 ± 0.00mg/kg respectively. The honey's quantity of

phosphorous in the study stayed equivalent to the content of 0.23 ± 0.02mg/kg in Tualang honey, 0.29 ± 0.03mg/kg in Gelum honey and 0.21 ± 0.02mg/kg in Kelulut honey in a study by Cheung et al., (2019). Contrary to the results obtained in the study, Solayman, 2023) documented mean concentration values of sodium, potassium, calcium, magnesium, phosphorous, iron and zinc as 96.48 ± 80.58mg/kg, 742.43 ± 453.88mg/kg, 84.36 ± 68.34mg/kg, 74.31 ± 163.05mg/kg, 84.16 ± 48.36mg/kg, 30.34 ± 64.99mg. Kg and 9.33 ± 19.55mg/kg of honey samples from Pakistan, Turkey, New Zealand, Romania and Spain.

Such elements serve various functions like bio-indicators of environmental pollution, however, the concentrations of these minerals and metals could vary from one honey sample to another (Pauliuc et al., 2020). For example, it was ascertained in the current research that stingless honey contains more elements than honeybee honey. This could have been due to differences in the vegetation, nectar and the soil mineral content as well as the ability of the stingless bee to pollinate and source honey from the different places it was sourced from according to Njokuocha et al. (2019). Similarly, Olas (2020) notes that minerals are part of the human body, and they work in precise ways and are essential in the metabolism. Other metal-containing enzymes are also important in the protection of the internal cellular structures such as catalase which play a role in preventing oxidative damage. They have antioxidant activity to prevent tissues from damage by free radicals and play a role in the immune response (Sigolo et al., 2019).

### *5.5.2 Crude proteins*

Proteins as a nutritional element were among the contents that were analyzed.

Proteins were detected in all the analyzed honey samples, giving an average concentration of 0.57 ± 0.12 to 2.47 ± 0.35mg/g, within the usual normal range of 2 to 5mg/g. The findings conformed with the findings of Njokuocha et al. (2019), who documented a concentration of 0.85g/100g in Kelulut (Stingless bee) honey and 0.38g/100g in honey bees (*Apis mellifera*) from Nigerian honey samples. Another study by Pang et al. (2016) also documented a relative average protein concentration of 0.66 ± 0.10 g/100g, 0.60 ± 0.05g/100g and 0.85 ± 0.42g/100g in Tualang, Gelam and Kelulut kinds of honey respectively.

They are a major part of and contribute to all cells in the body and serve the purpose of tissue repair and construction. When present at very high concentrations in the wound site, they can inhibit wound healing by degrading the growth factors, and the fibronectin molecules in the wound site that are important in stimulating fibroblast and the migrating epithelial cells (Schuh et al., 2019). It has been written that such proteins are in the form of enzymes that honeybees use to introduce during honey processing from the hypopharyngeal and salivary glands. As for the others, they are found in pollen and nectar. Majority of the antimicrobial activities of honey is associated with the defending-1 protein that has antimicrobial peptide which is cytotoxic to both Gram-positive and some Gram -negative bacteria (Proano et al., 2021).

### *5.5.3 Water-soluble Vitamins*

Water-soluble vitamins were also analyzed in this study and it was established that honey is a non-vitamin-rich food. The most abundant according to our

findings in all honey samples was Vitamin $B_3$ with an average of 0.65 ± 0.06 to 4.19 ± 0.03mg/100g followed by; vitamin $B_2$ (0.72 ± 0.04 – 2.39 ± 0.08mg/100g), vitamin $B_9$ (0.11 ± 0.01 to 1.64 ± 0.11mg/100g), vitamin $B_5$ (0.01 ± 0.00 to 1.22 ± 0.04mg/100g) and finally vitamin$B_1$ (0.22 ± 0.02 to 0.64 ± 0.02mg/100g). Higher concentrations of Vitamin $B_1$ and $B_3$ were obtained from the Maoi region while samples from Kibingor recorded high concentrations of Vitamin $B_2$ and $B_9$; this variation was due to the variety of the floral sources from the regions of honey samples collection. The research findings also concur with the studies done by Alvarez-Suarez et al. (2018), who studied the presence of vitamins in honey samples obtained from different botanical types of Sulla (*Hedysarium coronarium*) and Thistle (*Galactiles tomentosa moench*) in Sardinia, Italy and reported ranges of 0.25 ± 0.00mg/100g, 4.90 ± 0.60mg/100g, 1.75 ± 0.00mg/100g and 1.54 ± 0.08mg/100g for Vitamins $B_2$, $B_3$, $B_5$ and $B_9$ respectively. The outcomes were also in conformity with another study by Olas (2020) on chemical elements found in natural honey from South American stingless bee species, whose findings were documented as 0.00 ± 0.01mg/100g, 0.01 ± 0.02mg/100g, 0.10 ± 0.20mg/100g, 0.02 ± 0.11mg/100g and 0.002 ± 0.01mg/100g for Vitamins $B_1$, $B_2$, $B_3$, $B_5$ and $B_9$ respectively.

In general, vitamins cannot be stored in body tissues and have to be constantly replenished through food intake (Bellows & Moore 2019). They are widely distributed in foods and function as coenzymes in the body, essential in vital functions such as growth, normal metabolism and cell regulation. They are also important in reducing deficiency diseases and have a prophylactic role in

protecting against other diseases. Vitamins in honey also have remarkable antimicrobial activity in vitro by inhibiting bacterial growth and acting as an immune system stimulator in vivo (Majtan et al., 2020). Therefore, the presence of these groups of vitamins clearly indicates the possibility of honey samples being good sources of vitamins.

In general, honey samples from the stingless bee *(Meliponine*) indicated higher levels of the nutritive elements tested and thus prove that it is more nutritional than the samples from honey bee (*Apis*). These outcomes also agree with other scholarship findings that stingless bee honey is rich in nutritive components (Brodschneider et al., 2018; Frey, 2018; Hasam et al*.,* 2020). There is a highly significant relationship (P<0. 01) between vitamins and proteins in honey since both are derived from pollen, particularly polyfloral and are useful in various physiological functions, bee brood development and adult population increase. This positive correlation confirmed the stimulating cell growth property which enhances natural healing properties of wounds. A strong positive correlation existed between protein, vitamins, and glucose. Similarly, it was found that sugars have a significant and positive relationship with moisture content (P<0. 05), and on the other side, sugars have a strong negative relationship with vitamins. This correlation indicated that water is needed to dilute honey to 4% to be added to brood food, hence meeting the energetic expenses of honeybees. It has a high osmolarity, which leads to a moist healing environment that does not adhere to the wound tissues; thus, it prevents bacterial growth (Morroni et al., 2018). Protein and vitamins are essential components of body cells and hence support their

average growth while vitamins decrease epithelialization and granulation which is otherwise stimulated by sugars (Barchitta et al., 2019).

This is an innovation and scientific research towards sustainable development goals (SDGS), the 17 point plan of the world to radically enhance the quality of lives and the earth's health by 2030. Given by all the member countries of the United Nations in the year 2015 to preserve poverty, inequality, and foster more positive society with less conflict. This research work will help advance goal 3 in the enhancement of healthy lives and promoting the well-being of everyone. The UNICEF, the government and other UN agencies collaborate for the purpose of ensuring that the goals deliver (UNDP, 2023).

# CHAPTER SIX

# 6.0 CONCLUSIONS AND RECOMMENDATIONS

## 6.1 CONCLUSIONS

Millions of people worldwide are diagnosed with chronic lesions that show clinical signs of infection every year. As a result, patients who have these infections are more likely to require a lengthy stay in the hospital, pay more for their care, and require more time to heal their wounds. This might be because the incisions are polymicrobial, which creates an environment where bacteria can exchange resistance genes (Manirakiza et al., 2019).

It is important to note that the composition of honey is principally responsible for

its antibacterial, antioxidant, and therapeutic qualities, including anti-inflammatory and anti-cancer effects. This depends on the surrounding climate, the kind of pollinators, and the range of nectar compositions found in all plant species that make nectar (Ismail et al., 2021). According to Testa et al. (2019), there is proof that it has been utilized as a medical drug since around 200 BC, and human use of it dates back to 8000 years. During this examination into the composition of honey, it was discovered that the nutritional, physicochemical, phytochemical, antioxidant, and antibacterial properties of honey from stingless bees and honey from honeybees were more equivalent.

The potency of honey, especially stingless bees against microorganisms, proposes its competent potential for use as an alternative therapeutic agent in certain conditions, mostly wound infections. This was ascertained by the high susceptibility of some of the commonly isolated wound infection bacterial isolates to honey samples compared to the conventional antibiotics under study. These microorganisms indicated varying susceptibility to different antibiotics while considerable bacteriostatic and bactericidal activity was recorded by the honey samples, proving its antibacterial competence.

## 6.2 LIMITATIONS AND WEAKNESSES

The methods of extracting honey, especially from the stingless bee is time-consuming and could influence the samples by contamination. These include the location and volume of the honey samples produced. There is a need to put more effort into introducing standardized honey gauzes in Kenyan hospitals in wound management.

## 6.3 RECOMMENDATIONS

### *6.3.1 Institutional Recommendations*

Through the Ministry of Health, the government should install PCR machines in the laboratories to aid in accurate diagnosis and prompt treatment of bacterial infection.

Institutions should implement strategies to train farmers on bee beekeeping practices and create awareness of honey's antibacterial and other properties through community education systems.

### *6.3.2 Recommendations for further research*

More work must be done on using honey as an alternative medicare source. Further investigations on the honey's antibacterial activity against more wound infection-causing isolates should be carried out to ensure a wider scope, thus confirming its bacteriostatic and bactericidal activity.

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## APPENDICES

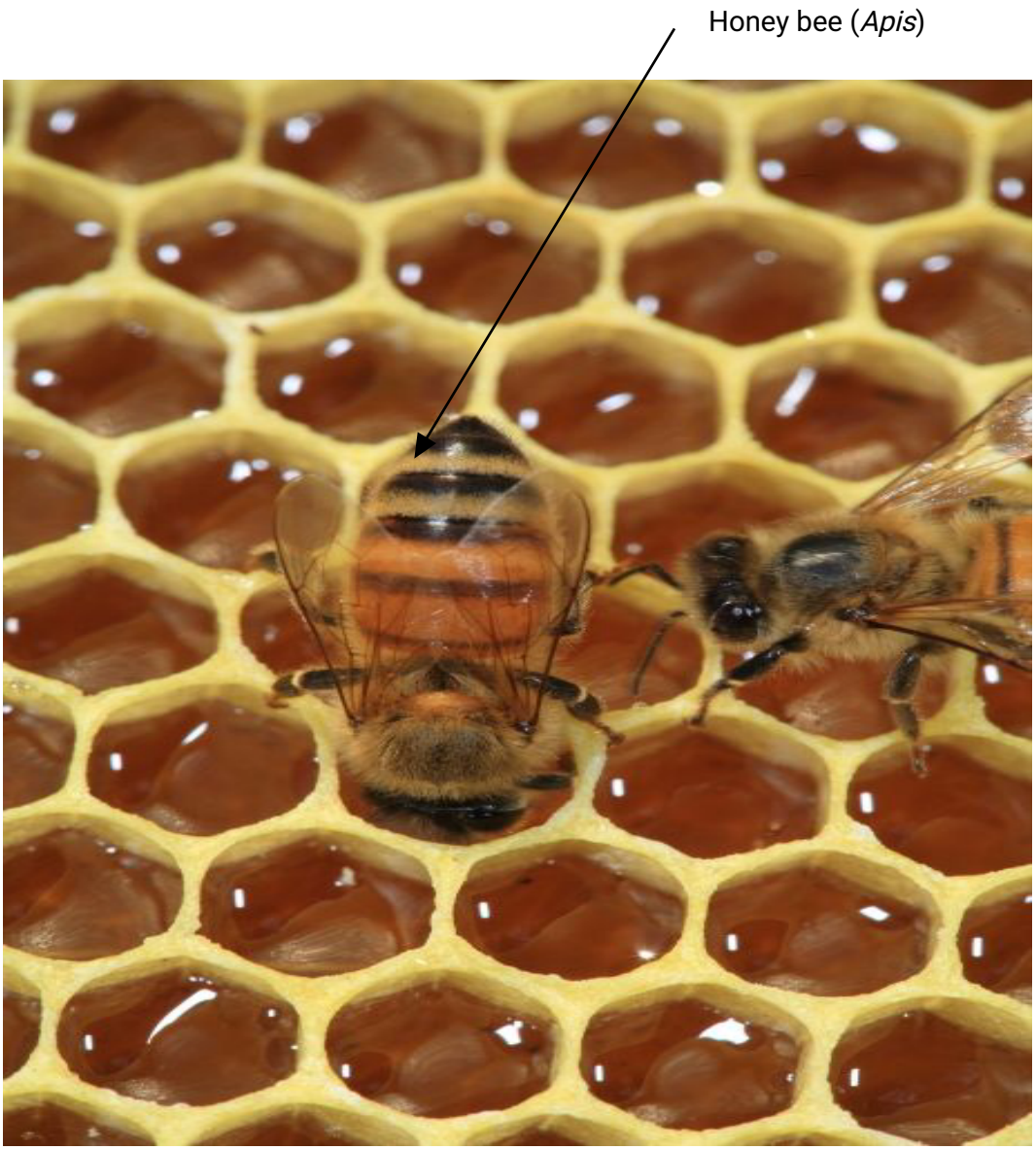
**Appendix I:** Stingless bee oval-shaped honey / pollen pots at Marigat

Honey pots for *meliponin*

## Appendix II:

### *Appendix II: Honey bee hives*

**Appendix III:** Honey bee



**Appendix IV:** Ethics clearance permit (REC)

**OFFICE OF THE DIRECTOR OF GRADUATE STUDIES AND RESEARCH**

**UNIVERSITY OF EASTERN AFRICA, BARATON**

P. O. Box 2500-30100, Eldoret, Kenya, East Africa

January 4, 2017

Miriam Wanjiru Mwangi

Dear Miriam,

Re: ETHICS CLEARANCE FOR RESEARCH PROPOSAL (REC: UEAB/7/12/2016)

Your research proposal entitled *"Antibacterial Efficacy of selected honey from stingless and honey bees in Baringo County"* was discussed by the Research Ethics Committee (REC) of the University and your request for ethics clearance was granted approval.

This approval is for one year effective January 4, 2017 until January 4, 2018. For any extension beyond this time period, you will need to apply to this committee one month prior to expiry date. Note that you will need a clearance from the study site before you start gathering your data.

We wish you success in your research.

Sincerely yours,

Jackie K Obey

Dr. Jackie K. Obey
Chairperson, Research Ethics Committee

University of Eastern Africa, Baraton
07 JAN 2017
Research Ethics Committee

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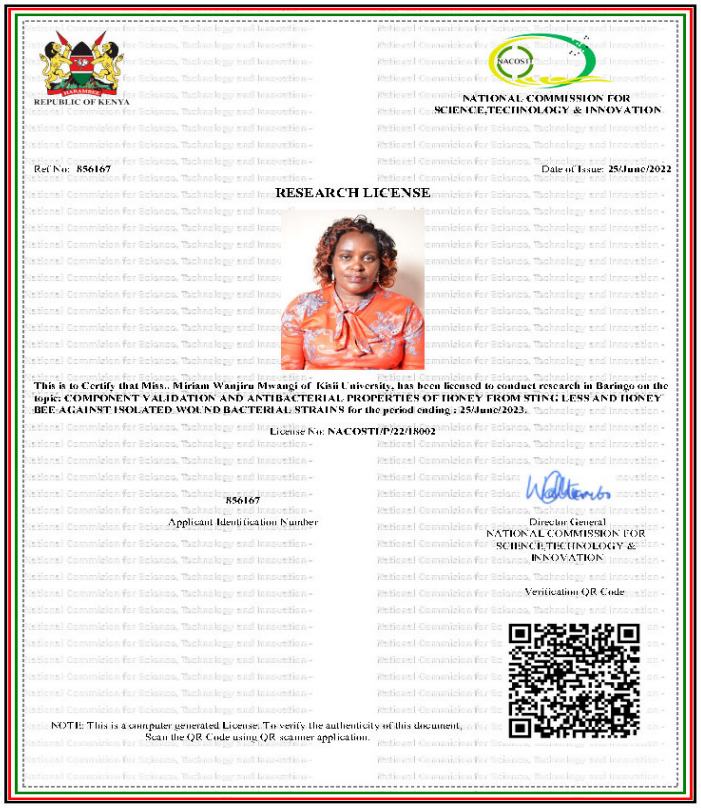
**Appendix V:** Ethics clearance permit (NACOSTI)



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RESEARCH LICENSE

This is to Certify that Miss.. Miriam Wanjiru Mwangi of Kisii University, has been licensed to conduct research in Baringo on the topic: COMPONENT VALIDATION AND ANTIBACTERIAL PROPERTIES OF HONEY FROM STING LESS AND HONEY BEE AGAINST ISOLATED WOUND BACTERIAL STRAINS for the period ending : 25/June/2023.

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**Appendix VI:** Wound swab collection permit

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Telephone: Nakuru 051-2215580-90
When replying please quote
FAX 051 2216497
Email:rvpghnakuru@yahoo.com

PROVINCIAL GENERAL HOSPITAL
RIFT VALLEY PROVINCE
P.O. Box 71
NAKURU

RII/VOL I/08

Date.....16/03/2017.....

To .....MIRRIAM WANJIRU MWANGI.....

.....

.....

Dear .....MADAM.....

**RE: APPROVAL TO UNDERTAKE RESEARCH AT THE RIFT VALLEY PROVINCIAL GENERAL HOSPITAL**

Reference is made to your letter dated .....20TH FEB 2017.....seeking approval to conduct a research on.....

.....ANTIBACTERIAL EFFICACY OF SELECTED HONEY.....

.....FROM STINGLESS AND HONEY BEES.....

.....

Permission has been granted/not granted for the research. It is hoped that you will adhere to the ethics and standards that relate to research at our institution.
Thank you.

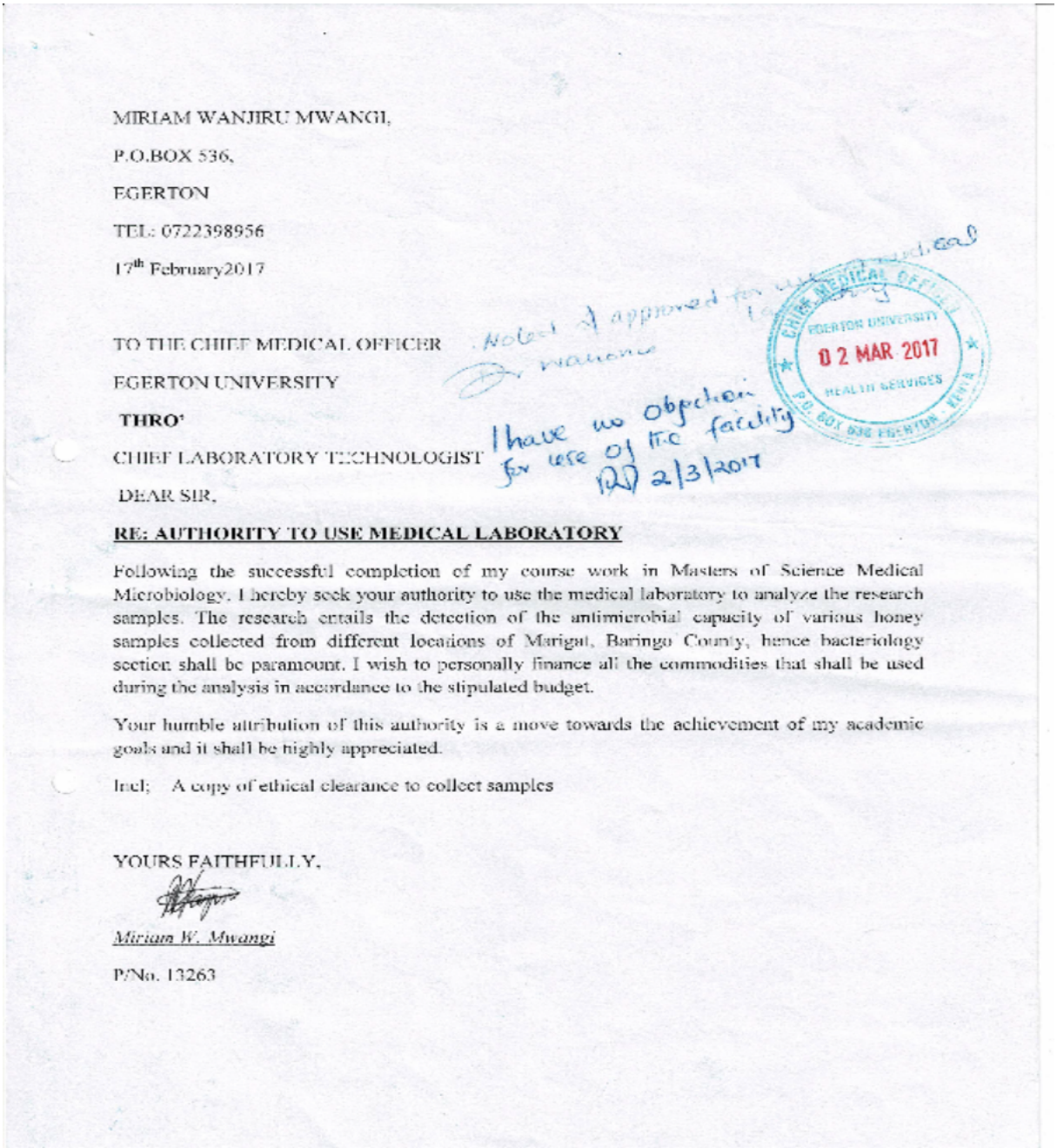
Yours Sincerely,

17 MAR 2017
P. O. Box 71, NAKURU

**MEDICAL SUPERINTENDENT**

**CHAIRPERSON**
**RESEARCH AND ETHICS COMMITTEE**

## *Appendix VII:* Laboratory use authorization



MIRIAM WANJIRU MWANGI,

P.O.BOX 536,

EGERTON

TEL: 0722398956

17th February2017

TO THE CHIEF MEDICAL OFFICER

EGERTON UNIVERSITY

**THRO'**

CHIEF LABORATORY TECHNOLOGIST

DEAR SIR,

**RE: AUTHORITY TO USE MEDICAL LABORATORY**

Following the successful completion of my course work in Masters of Science Medical Microbiology, I hereby seek your authority to use the medical laboratory to analyze the research samples. The research entails the detection of the antimicrobial capacity of various honey samples collected from different locations of Marigat, Baringo County, hence bacteriology section shall be paramount. I wish to personally finance all the commodities that shall be used during the analysis in accordance to the stipulated budget.

Your humble attribution of this authority is a move towards the achievement of my academic goals and it shall be highly appreciated.

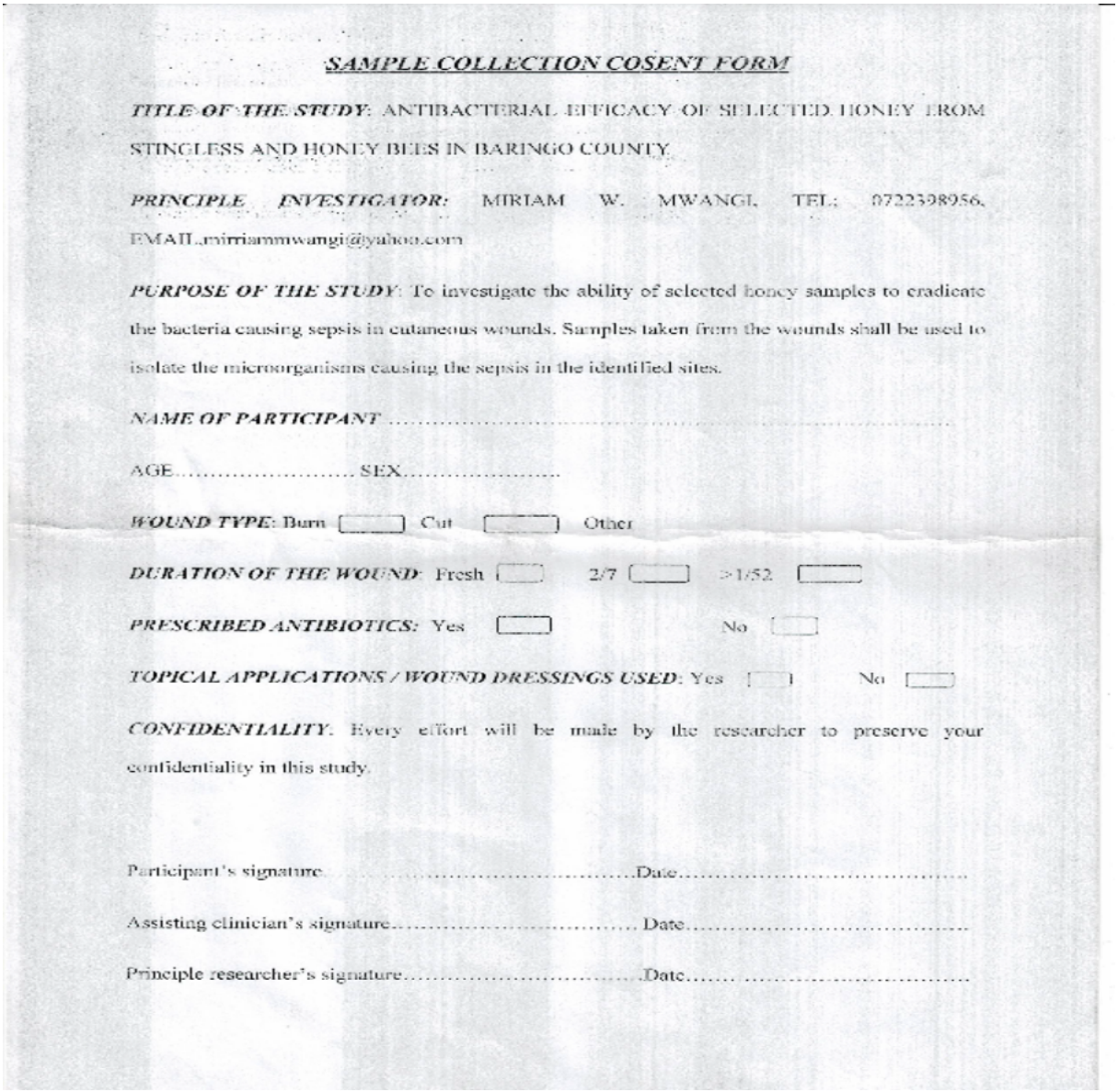
Incl; A copy of ethical clearance to collect samples

YOURS FAITHFULLY,

*Miriam W. Mwangi*

P/No. 13263

**Appendix VIII:** Individual consent form



***SAMPLE COLLECTION COSENT FORM***

***TITLE OF THE STUDY***: ANTIBACTERIAL EFFICACY OF SELECTED HONEY FROM STINGLESS AND HONEY BEES IN BARINGO COUNTY

***PRINCIPLE INVESTIGATOR:*** MIRIAM W. MWANGI, TEL: 0722398956, EMAIL:mirriammwangi@yahoo.com

***PURPOSE OF THE STUDY***: To investigate the ability of selected honey samples to eradicate the bacteria causing sepsis in cutaneous wounds. Samples taken from the wounds shall be used to isolate the microorganisms causing the sepsis in the identified sites.

***NAME OF PARTICIPANT*** .....

AGE..... SEX.....

***WOUND TYPE***: Burn [ ] Cut [ ] Other

***DURATION OF THE WOUND***: Fresh [ ] 2/7 [ ] >1/52 [ ]

***PRESCRIBED ANTIBIOTICS:*** Yes [ ] No [ ]

***TOPICAL APPLICATIONS / WOUND DRESSINGS USED***: Yes [ ] No [ ]

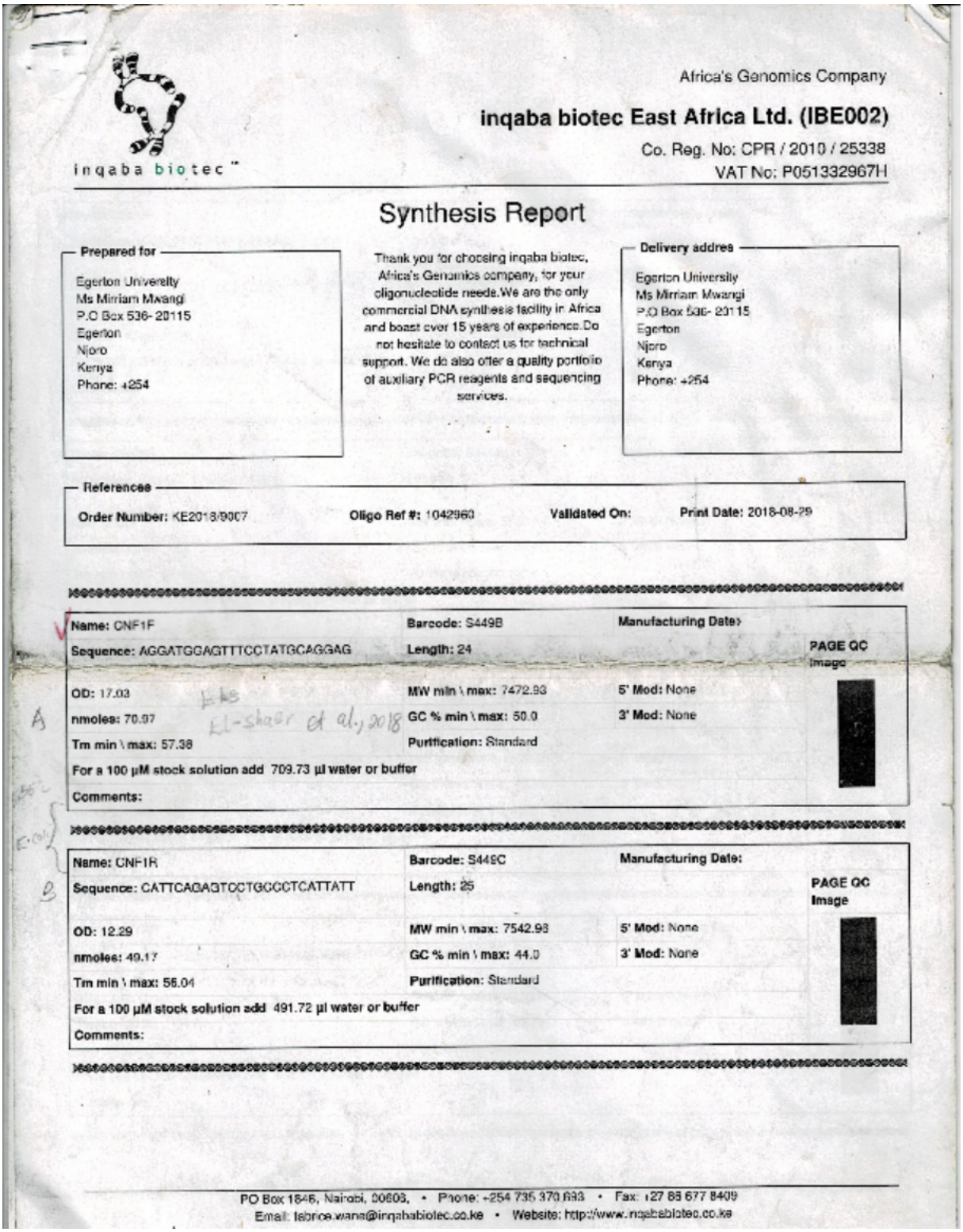
***CONFIDENTIALITY***. Every effort will be made by the researcher to preserve your confidentiality in this study.

Participant's signature.....Date.....

Assisting clinician's signature..... Date.....

Principle researcher's signature.....Date.....

**Appendix IX:** Primers for molecular analysis



inqaba biotec™

Africa's Genomics Company

**inqaba biotec East Africa Ltd. (IBE002)**

Co. Reg. No: CPR / 2010 / 25338
VAT No: P051332967H

## Synthesis Report

**Prepared for**

Egerton University
Ms Miriam Mwangi
P.O Box 536- 20115
Egerton
Njoro
Kenya
Phone: +254

Thank you for choosing inqaba biotec, Africa's Genomics company, for your oligonucleotide needs.We are the only commercial DNA synthesis facility in Africa and boast over 15 years of experience.Do not hesitate to contact us for technical support. We do also offer a quality portfolio of auxiliary PCR reagents and sequencing services.

**Delivery addres**

Egerton University
Ms Miriam Mwangi
P.O Box 536- 20115
Egerton
Njoro
Kenya
Phone: +254

**References**

**Order Number:** KE2018/9007 **Oligo Ref #:** 1042960 **Validated On:** **Print Date:** 2018-08-29

| | | | |
|---|---|---|---|
| **Name:** CNF1F | **Barcode:** S449B | **Manufacturing Date:** | |
| **Sequence:** AGGATGGAGTTTCCTATGCAGGAG | **Length:** 24 | | **PAGE QC Image** |
| **OD:** 17.03 | **MW min \ max:** 7472.93 | **5' Mod:** None | |
| **nmoles:** 70.97 | **GC % min \ max:** 50.0 | **3' Mod:** None | |
| **Tm min \ max:** 57.38 | **Purification:** Standard | | |
| **For a 100 µM stock solution add** 709.73 µl water or buffer | | | |
| **Comments:** | | | |

| | | | |
|---|---|---|---|
| **Name:** CNF1R | **Barcode:** S449C | **Manufacturing Date:** | |
| **Sequence:** CATTCAGAGTCCTGCCCTCATTATT | **Length:** 25 | | **PAGE QC Image** |
| **OD:** 12.29 | **MW min \ max:** 7542.93 | **5' Mod:** None | |
| **nmoles:** 49.17 | **GC % min \ max:** 44.0 | **3' Mod:** None | |
| **Tm min \ max:** 56.04 | **Purification:** Standard | | |
| **For a 100 µM stock solution add** 491.72 µl water or buffer | | | |
| **Comments:** | | | |

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Email: fabrice.wana@inqababiotec.co.ke • Website: http://www.inqababiotec.co.ke