PHYTOCHEMICAL PROFILE, ANTIBACTERIAL, AND ANTIOXIDANT ACTIVITIES OF APIS MELLIFERA PROPOLIS FROM SELECTED REGIONS IN KENYA

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Phytochemical Profile, Antibacterial, and Antioxidant Activities of Apis Mellifera Propolis from Selected Regions in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

Signature……Date…….……

Timothy Mugodo Kegode

This thesis has been submitted for examination with our approval as the University Supervisors

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DEDICATION

This research thesis is dedicated to my beloved family.

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First, I honor the Almighty God for giving me life and the opportunity to realize my scientific ambitions, as nothing can come to pass without the will of God.

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ACRONYMS AND ABBREVIATIONS

ROS	Reactive Oxygen Species
H^+	Hydrogen ion
FRAP	Ferric Reducing Antioxidant Power
DPPH	2, 2 – diphenyl -1- picrylhydrazyl
TFC	Total Flavonoid Content
$AlCl_3$	Aluminum trichloride
GAE	Gallic Acid Equivalents
QE	Quercetin Equivalents
MHB	Mueller Hinton II Broth
w/v	Weight per Volume
OD	Optical Density
CFU	Colony Forming Units
GC-MS	Gas Chromatography Mass Spectrometer

ABSTRACT

Currently, there is an increase in demand for natural products like propolis, yet little information is currently available on the chemical composition of African propolis and their biofunctional properties. Therefore, this study was aimed at quantifying the phytochemicals, determining the antioxidant, and anti-microbial properties of *Apis mellifera* propolis sourced from various selected regions in Kenya. The samples were analysed by colourimetric assays and Gas Chromatography-Mass Spectrometry (GCMS) for phytochemicals profiles and antioxidant activities. While disc diffusion assays were used to determine the antibacterial properties. The phytochemical analysis of propolis samples from different regions in Kenya revealed significant variations in the levels of flavonoids, phenolics, terpenoids, and alkaloids ($p < 0.05$). Flavonoid content was highest in samples from Marigat (2,131.4 mg QE) and lowest in Taita Hills (102.2 mg QE). Phenolic compounds were most abundant in samples from icipe, Nairobi (3,711.8 mg GAE), while the lowest levels were observed in Taita Hills (522.6 mg GAE). Terpenoid levels were generally higher in Marigat and South Kitui, with the lowest recorded in icipe. Alkaloid content showed the greatest variability, with the highest concentrations found in Lower Taita (8,767.5 mg CE) and the lowest in Taita Hills (3,014 mg CE). Propolis samples also demonstrated stronger activity against *B. thuringiensis* than *E. coli*. Likewise, the antioxidant activity varied significantly across the different locations. The highest antioxidant activity was observed in samples from Marigat and the least from Taita Hills. The propolis samples collected from hotter climatic conditions not only contained relatively higher composition of total phytochemicals, but they also displayed higher antioxidant and anti-microbial activities than those obtained from cooler climatic conditions. Some of the compounds identified included triterpenoids alpha- and beta-amyrin, oleanen-3-yl-acetate, urs-12-en-24-oic acid, lanosta-8,24-dien-3-one, and hydrocarbons tricosane and nondecane, which have been reported to have either anti- microbial or antioxidant activities. Key findings of this study demonstrate the occurence of relatively higher phytochemical content in Kenya's propolis, which has both antioxidant and anti-microbial properties; hence this property of propolis could be harnessed for disease control. The limitations of the study were that the samples may not have represented all possible climatic regions of the country and lack of resources to do other analysis for better chemical profile results.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Disease-causing microbes are evolving to adapt to the constant exposure to antimicrobial agents leading to the emergence of multidrug-resistant pathogens (Emacar *et al*., 2010). This has made the treatment of diseases to be difficult and costly. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an example, which could cause disfiguring of the affected body part, or loss of life if left untreated (Konya *et al*., 2019; Al-Gadaa *et al*., 2015; Durgad *et al*., 2014). These challenges have inspired revitalized research in search of better alternatives that are efficient, leading to a re-evaluation of the therapeutic properties of natural products exhibiting antimicrobial activity such as propolis (Boateng & Diunase, 2015). The potency of propolis as a natural bioactive agent against both Gram-positive and Gram-negative microorganisms like multidrug-resistant bacteria was demonstrated by Anjum *et al*., (2019b), indicating its efficacy. Turkish propolis was also efficacious against tuberculosis, and the results pointed out its potential activity against different species of mycobacteria (Das *et al.*, 2020). These properties result from diverse antimicrobial factors such as flavonoids, phenols, terpenes, tannins, alkaloids and even hydrocarbons (Al-Waili *et al*., 2012). The clinical antibacterial properties of propolis have been supported due to myriad of bioactive compounds present in it (Sforcin *et al*., 2005). Therefore, propolis has attracted much interest in its application to treat various maladies. Consequently, propolis has been used to develop new drugs through biotechnology (Sforcin *et al*., 2005).

Lifestyle diseases, including but not limited to arthritis, cardiovascular disease, cancer, diabetes, Parkinson's, and Alzheimer's disease, are affecting an increasing number of people (Thorburn *et al*., 2014). This is attributed to free radicals, chemical, and physical factors leading to cell ageing, the genesis of pathophysiological processes (Lagouri *et al*., 2014a). Propolis is a source of natural antioxidants, scavenging and acting as secondary defensive factors against free radicals produced in the human body (Kuropatnicki *et al*., 2013). Enzymes serve as the primary defence system against

oxidative stress because of normal physiological processes. These enzymes include catalase, glutathione peroxidase, and superoxide dismutase (Hasan *et al*., 2014). When the generation of free radicals overcomes the primary buffer capacity of the human body, the second line of defence comprises vitamins (Mayne, 2003). These vitamins, for example vitamin C and E, play antioxidant roles by scavenging free radicals and inactivating them (Oryan *et al*., 2018). Exhaustion of these two defence mechanisms leads to severe cellular damage in the body (Morrissey *et al*., 1998). Phenolic compounds, flavonols, flavones, flavanones, isoflavanones and terpenoids are important bioactive constituents of propolis with proven ability to scavenge free radicals, and thus shield vitamin C and lipids from denaturation by the resulting oxidative physiological process (Traber & Stevens, 2011).

Propolis is a natural resinous mixture comprising of plant resins, bee saliva, and wax, which is utilized by wild and feral bees as a building material for sealing unwanted spaces in the hive, hence smoothening it (Anjum *et al*., 2019a). In addition, propolis plays a crucial role in chemical defence against invading microorganisms, thus making the hive environment sterile (Simone *et al*., 2009). Furthermore, bees use propolis to embalm dead and live intruders such as small hive beetles (Neumann *et al*., 2001), and other organisms that are too large to be removed (Cuthbertson *et al*., 2013; Ellis, 2005).

Further, the beneficial therapeutic effects of propolis have been recognized and applied since ancient times. Historical records suggest that its use dates back to ancient Greeks and Romans (Hernández *et al*., 2018). The bioactive properties of propolis, such as anti-inflammatory, anticancer, antioxidant, antimicrobial, and immune-stimulating activities, have all been demonstrated by previous studies (Bailão *et al*.,; 2015Aliyazcoglu *et al*., 2013). The chemicals responsible for these bioactive properties have been shown to include flavonoids, phenols, alkaloids, saponins, terpenoids, and hydrocarbons (Huang *et al*., 2014). In recent years, modern societies have recognized and appreciated natural propolis-based treatments for disease control and management, and are therefore embracing the development of these products (Oryan *et al*., 2018).

Environmental factors influence the quality and quantity of propolis produced (Ribeiro

et al., 2009). Majorly, seasonal variation in tree resin production leads to variation in the chemical composition of propolis. Different geographical locations have reported different kind of propolis such as the red propolis from Brazil (Daugsch *et al*., 2008). The chemical composition of propolis from other parts of the world have been reported (Huang *et al.*, 2014, Das *et al.*, 2020). Currently, information on the phytochemical composition and the biological activities of propolis from Kenya is limited. In this study, the phytochemicals composition, antioxidant, and antibacterial activities of *Apis mellifera* propolis collected from seven regions in Kenya that are geographically distinct, with different climatic and vegetation parameters were analysed. These counties are Baringo, Murang'a, Nairobi, Kakamega, Taita Taveta, Kitui and Kilifi. The results may help understand how propolis' properties vary according to regions and climatic conditions that influence it.

1.2 Statement of the Problem

Currently, many disease-causing microorganisms are developing resistance to antibiotics. This could result to antibiotic resistant super bugs which will render the use of antibiotics ineffective (Levy & Bonnie, 2004). Antibiotic resistance problem is more pronounced in patients with HIV/AIDS because of the prolonged exposure to antimicrobials that they use regularly for prevention and treatment of opportunistic infections which can lead to increased HIV co-morbidity and associated mortality rates in these patients (Emacar *et al*., 2010). AMR is considered a significant threat to the public health systems not just in developing countries but throughout the world (Founou *et al*., 2017) because it increases health-care costs, morbidity and mortality in both developed and developing countries (Gulen *et al*., 2015). If this is not addressed, it is estimated that 10 million deaths will be caused by drug resistance by 2050, with approximate loss of 100 trillion USD of the world's economic (WHO, 2017). The problem of antimicrobial resistance to the existing manufactured molecules has made exploration of alternative sources of antimicrobials which include the natural sources to be next direction of advancement (Boateng & Diunase, 2015). These antimicrobials are thought to be more effective, with limited side effects and readily available (Topliss *et al*., 2002).

Lifestyle diseases are on the rise, and it is estimated that 71% of all deaths are attributed to these diseases (WHO, 2017). Redox reactions occur in biological systems, which are detrimental to human health due to the release of reactive oxygen species (ROS). These affect cell components including lipids which are key to cell integrity (Hsu & Hsieh, 2014). These reactive oxygen species include hydrogen peroxide (H_2O_2) and hydroxyl radicals. When the production of these radicals exceeds antioxidant defense in the body it leads to oxidative stress which has effects on many cellular processes (Burton & Jauniaux, 2011). The exploitation of natural botanical sources for the phytochemicals can lead to deforestation and hence more environmentally friendly sources must be explored.

1.3 Justification

The role of natural products in medicine and their contribution to health has been invaluable (Afrouzan *et al*., 2018). Propolis has been used as a natural remedy in traditional medicine. It has not only been found to be an effective antimicrobial, antioxidant and anticancer agent (Pasupuleti *et al*., 2017). Many studies have shown propolis to possess antioxidant properties which is attributed to the presence of the flavonoids and phenolics that are able to scavenge free radicals (Oryan *et al*., 2018).

The chemical composition of propolis is determined by the plant source, which varies with different geographic regions (Wilson *et al*., 2015). Since there are diverse sources of propolis in Kenya, this research sought to establish the phytochemical and bioactivity characteristics from different regions of the country.

1.4 Research Questions

(i) What are the quantities of phytochemicals (phenols, flavonoids, terpenoids, and alkaloids) in *Apis mellifera* propolis from selected regions in Kenya?

(ii) What is the activity of propolis extracts against Gram-Negative bacteria (*E.coli*) and Gram-Positive Bacteria (*B. thuringiensis*)?

(iii) What is the antioxidant activity of propolis extracts?

(iv) What are the volatile organic compounds present in propolis?

1.5 Objectives

1.5.1 General Objective

To establish phytochemical profiles, antibacterial, and antioxidant activities of *Apis mellifera* propolis from selected regions in Kenya.

1.5.2 Specific Objectives

(i) To quantify the phytochemical compounds in the raw propolis extracts (phenols, flavonoids, terpenoids, and alkaloids) from different regions.

(ii) To determine the *in vitro* antibacterial activity of propolis extracts against *E.coli* and *B. thuringiensis*

(iii) To determine *in vitro* antioxidant activity of propolis extracts

(iv) To establish the volatile organic compounds, present in the propolis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Propolis

Propolis is one of the bee products of plant origin that is produced by transformation of resins and exudates from plants (Zabaiou *et al*., 2017). It is resinous in nature giving it a sticky characteristic hence the name "bee-glue". Propolis has important pharmacological properties and it has been used for a wide range of use as, anti-inflammatory, bacteriostatic, bactericidal, anti-fungal, tumor suppressant and antioxidant (Huang *et al*., 2014). All such applications have increased their pharmaceutical demand and rendered it an interesting subject of this study. Its biological activity is attributed to its complex chemical composition contributed by the plant phytochemicals obtained from the botanical sources of propolis (Anjum *et al*., 2019b). It's fairly complex chemical composition includes, phenols, tannins, aromatic compounds, flavonoids among others (Park *et al*., 1998). Propolis has lipophilic nature, hard and brittle material and it becomes soft, pliable, gummy, and very sticky when heated. It possesses a characteristic and pleasant aromatic smell and varies in colour from yellow green to red and to dark brown depending on its source and age (Bankova *et al*., 2014). But even transparent propolis has been reported (Trusheva, *et al.*, 2014). The use of propolis in folk medicine continues to increase (Castaldo & Capasso, 2002). Chemical studies have revealed chemical variability among propolis according to plant sources (Sforcin *et al.*, 2005).

2.2 Phytochemicals

Plants are the primary producers in any ecosystem and are found across all terrestrial ecosystems in the world. Phytochemicals are bioactive compounds produced by plants to adapt to their environment. These substances can cause significant physiological changes in humans and offer various health benefits (Brintha S *et al*., 2017; Babich *et al*., 2011). The most important classes of these bioactive constituents of plants are alkaloids, tannins, terpenoids, flavonoids and phenolic compounds (Bailão *et al*., 2015). The secondary metabolites perform specialist functions in plants such as

attracting insects, killing microbes and discouraging herbivores that exploit the plants (Gershenzon & Dudareva, 2007).

2.3 Propolis and Phytochemicals

Honeybees collect plant resins and exudate from plant wounds to make propolis and hence propolis is a good reservoir for these phytochemicals in a more refined form. The chemical composition of propolis is determined by the plant species and indirectly by the location due to difference in vegetation (Huang *et al*., 2014). The chemical composition will be determined by the kind of vegetation in the region where the bee colonies are foraging (Zabaiou *et al*., 2017). Phenolic compounds are the most ubiquitously distributed phytochemicals found in many plant tissues and are secondary metabolites synthesized through shikimic acid and phenylpropanoid pathways (Cianciosi *et al*., 2018). They exist as either soluble or bound to plant cell walls (Jara *et al*., 2013). Based on their chemical structures, phenolic compounds can be divided into various sub-groups which include; phenolic acids, flavonoids, tannins, lignans, coumarins, stilbenes and quinones (Mulyati *et al.*, 2020). Phenolic compounds are responsible for antioxidant, anti-carcinogenic, anti-mutagenic and anti-inflammatory properties of propolis (Di Marco *et al.*, 2018).

2.3.1 Flavonoids

Flavonoids are the largest naturally occurring polyphenolic compounds in plants responsible for the many colours of flowers and fruits (Bylka *et al.*, 2004). Chemically, they are C6-C3-C6 compounds with C6 being two benzene rings and the C3 being an aliphatic chain with a pyran group (Rice-Evans *et al*., 1996). They have O- or C-glycosidic linkages which make them highly reactive (Bylka *et al*., 2004). As the major constituents of propolis, flavonoids contribute greatly to the pharmacological activities of propolis. The quantity of flavonoids is used as a criterion to evaluate the quality of propolis (Simone *et al*., 2009). Flavonoids have a broad spectrum of biological properties, such as antibacterial, antiviral and anti- inflammatory effects (Bankova, 2005). According to the chemical structure, flavonoids are classified into flavones, flavonols, flavones, flavanonols, chalcones, dihydrochalcones, isoflavones, isodihydroflavones, flavans, isoflavans and neoflavonoids (Ramnath *et al.*, 2015).

Flavonoids have been identified in different type of propolis in the past. In addition, flavonoid glycosides that are very rare in propolis have also been identified, and they include isorhamnetin-3-*O*-rutinoside and flavone *C*-glycoside (Talla *et al*., 2014)

2.3.2 Phenolics

Propolis has been reported to be rich in phenylpropanoids including cinnamic acid, caffeic acid, ferulic acid and their derivatives (Marcucci, 1995). These contributes on the antimicrobial activity of propolis (Hasan *et al*., 2014). In recent years, researchers identified a series of phenylpropanoid derivatives in Brazilian propolis (Kocot *et al*., 2018). Flavonoids and phenolic compounds are important components of propolis, both substances have proven their ability to remove or deactivate free radicals, on top of being able to protect lipids and vitamin C from being destroyed in the oxidative process (Zabaiou *et al*., 2017). By this characteristic, propolis has gained popularity among consumers. Nowadays it is added to drinks, foods, cosmetics, and even to chewing gum and toothpaste (Ahangari *et al*., 2018). Its broad-spectrum activities, biological properties and multiple applications have given rise to interest in investigating their characteristics according to their origin. A lot of studies indicate that flavonoids and phenolics in propolis can be able to scavenge free radicals in the human body (Mohdaly *et al*., 2015). There are colorimetric methods used to determine the content of phenols and flavonoids in propolis. These methods are used to estimate the content of active compounds in propolis (Bankova *et al*., 2019).

2.3.3 Alkaloids

Alkaloids are among the largest classes of natural products and are found particularly in plants. They are classified based on the structure of the nitrogen-containing moiety such as the quinolizidine (Muhammad & Abubakar, 2016). They are widely distributed in species belonging to several families within angiosperms. They are versatile nitrogen compounds with reported antimicrobial activity against fungal or bacterial phytopathogens (Cushnie *et al*., 2014). They are most common in herbaceous plants, but some occur in woody plants, chiefly tropical species. Alkaloids commonly are concentrated in particular organs such as the leaves, bark, or roots (Ani *et al.*, 2022).

2.3.4 Terpenoids

Plants produce a high diversity of volatile organic compounds (VOC). The emission of these secondary metabolites can be strongly increased as a result of certain biotic or abiotic stresses (Ayala-Jara *et al.*, 2018). Plants are capable of accumulating and emitting VOC from some of their organs, including leaves, flowers, fruits, which are collected by the honey bees, while foraging (Pino *et al*., 2006). These characteristics allow plants to have adequate interaction with other organisms and their environment in general. These volatile organic compounds comprise of, esters, terpenoids and other plant derived volatiles (Bankova *et al*., 2014). Terpenoids, even though present in only 10% of propolis, are responsible for the odour, because they are volatile components of plants, and contribute to the biological properties of propolis. Terpenoids are the largest group of plant secondary metabolites and they include monoterpenes, diterpenes, triterpenes and sesquiterpenes (Bankova, 2005). They have various biological activities including antiviral, antiprotozoal and antibacterial activities (Ullah et al., 2019).

The analysis of terpenoids involves headspace extraction, which is a simple, non-destructive and solvent-free technique used to extract volatile compounds. The technique has been widely used in the food and flavour industries to generate flavour profiles for a variety of foods and spices. Solid phase micro-extraction has also been used to determine the volatile constituents of propolis (Xie *et al*., 2013).

2.4 Propolis as an Antibacterial Agent

Many researchers have investigated the antibacterial activity of propolis and its extracts against gram-positive and gram-negative strains and they found that propolis had antibacterial activity against a wide range of gram-positive rods but had a limited activity against gram-negative bacilli (Aga *et al*., 1994). Extracts of propolis have been shown to potentiate the effect of certain antibiotics (Zabaiou *et al*., 2017). The antibiotic action against *S. aureus* and *E. coli* was increased by the addition of propolis to nutrient medium. The presence of propolis prevented or reduced any gradual build-up in tolerance of *Staphylococci* to antibiotics (Park *et al*., 1998). The antibacterial activity of propolis is reportedly due to flavonoids, aromatic acids, and esters present

in the resins (Banskota *et al*., 2001). Galangin, pinocembrin, and pinostrobin have been recognized as the most effective flavonoid agents against bacteria (Patil & Gaikwad, 2010). Ferulic and caffeic acid also contribute to the bactericidal action of propolis (Popova *et al*., 2005).

2.5 Antioxidant Activity of Propolis

Free radical, chemical and physical factors are partly responsible for cell aging, which is linked to several pathological conditions such as cardiovascular disease, cancer, diabetes, arthritis, Parkinson's, and Alzheimer's disease, among others (Lagouri *et al.*, 2014b). Antioxidant agents can serve as defensive factors against free radicals in the human body (Kuropatnicki *et al*., 2013). Enzymes such as superoxide dismutase, catalase, and glutathione peroxidase comprise the main defense system against oxidation (Hasan *et al*., 2014). . Flavonoids and phenolic compounds are important components of propolis, both substances have proven their ability to remove free radicals, on top of being able to protect lipids and vitamin C from being destroyed in the oxidative process (Traber & Stevens, 2011)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

An experimental laboratory-based design with qualitative and quantitative analysis of the collected propolis was utilised. The samples were analysed at ICIPE at the Bee Health Laboratories in Nairobi. All the analyses were done in triplicate.

3.2 Chemicals

Mueller Hinton Agar (MHA) was purchased from Himedia Laboratories Pvt. Ltd., (F&S Scientific, Nairobi, Kenya). Sodium nitrite ($NaNO_2$), 1,10-phenanthroline, aluminium chloride ($AlCl_3$), gallic acid, linalool, quercetin, sodium carbonate (Na_2CO_3), Folin–Ciocalteu's reagent, sodium hydroxide (NaOH), hydrochloric acid (HCl), chloroform, sulphuric acid (H_2SO_4), colchicine, ferric-III-chloride ($FeCl_3$), absolute ethanol, Streptomycin, Methanol and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were all purchased from Sigma-Aldrich (Kobian Kenya Ltd.). All the analyses utilized chemicals of analytical grade

3.3 Sample Size

The sample size for this study was determined based on the need to detect statistically significant differences in phytochemical content, antioxidant potential, and antibacterial activity of *Apis mellifera* propolis collected from different ecological regions in Kenya. A power analysis using G*Power 3.1 was conducted with three groups representing the three climatic conditions covered. With a medium effect size ($f = 0.25$), a significance level of $\alpha = 0.05$, and a desired statistical power of 0.80. The analysis indicated a minimum of 20 samples per group to detect meaningful differences among groups which gave a total of 60 samples.

Samples were collected from different regions representing three climatic conditions in Kenya of hot and dry, hot and humid and cold and wet influenced by the altitude, waterbodies and the winds. This was also informed by the availability of farmers based

on the previous projects by ICIPE.

3.4 Propolis Samples

Sampling was done by randomly selecting two hives per apiary from each of the selected locations as indicated in table 1. Propolis samples were collected directly from the hives by scrapping them off the walls and top covers with a hive tool and wrapping them with aluminium foil. Propolis samples were transported to ICIPE laboratory in Nairobi in a cooler box where they were stored at -80 ^{o}C, awaiting further analysis.

3.5 Solvent Extraction of Propolis

The individual propolis samples were briefly immersed in liquid nitrogen and crushed using a mortar and pestle into a fine powder. Extracts for phytochemical analysis and radical scavenging activity were prepared by weighing 0.5 g of each crushed sample in 10 mL of 50% (v/v) ethanol in 15 mL Falcon tubes and left to stand at room temperature for 72 h. They were vortexed at 3,000 rpm for 3 min, followed by the second vortexing step after 24 h of incubation at room temperature. After the extraction of propolis, they were centrifuged at rpm. 600 for 2 min, and the supernatant decanted and stored at -80 ^{o}C. For testing the antibacterial activity assays, the extractions were conducted by dissolving 2 g of the propolis sample in 10 mL of absolute ethanol. The mixture was vortexed at 3,000 rpm for 2 minutes and then incubated for 24 h at room temperature. The subsequent extraction followed the same steps described above and concentration done in an Eppendorf concentrator plus (Eppendorf, Hamburg, Germany) to complete dryness. The residue was weighed on an analytical plus scale (OHAUS Corporation Parsippany, New Jersey) and dissolved in absolute ethanol to make a concentration of 80 mg/mL. The extracts were stored in amber bottles at 4 ^{o}C away from the sunlight until they were used for further analysis.

3.6 Quantification of Phytochemicals in Propolis

3.6.1 Flavonoid Content

The total flavonoid content (TFC) of propolis samples was done following the aluminium chloride ($AlCl_3$) colorimetric assay (Popova *et al*., 2005). To 1 mL extract of each sample, 4 mL of distilled water was added, followed by 0.3 mL of 5 % (w/v) $NaNO_2$ and mixed. After 5 min, 0.3 mL of 10% $AlCl_3$ was added to the mixture and left for 1 min prior to adding 2 mL of 1 M NaOH, followed by a top-up step with 2.4 mL of distilled water. The absorbance of the mixture was measured at 510 nm using a spectrophotometer (BioSpec, Bartlesville, USA) against a reagent blank containing all above reagents, except the sample that was replaced with 50% ethanol. Quercetin was used as standard ($y = 0.0006x + 0.0028$, $R^2 = 0.9981$) to generate a calibration curve (20 – 200 μg/mL) and TFC expressed as mg quercetin equivalent per 100 g propolis (mg QE/100 g propolis).

3.6.2 Phenolics Content

Folin–Ciocalteu method (Popova *et al*., 2005) was used to determine the total phenol content (TPC). To 1 mL extract, 5 mL of 0.2 N Folin–Ciocalteu reagent was added and left at room temperature for 5 min. After adding 4 mL of 75 g/L sodium carbonate, the mixture was incubated at room temperature for 2 h. The absorbance of this reaction mixture was read at 760 nm against an ethanol blank instead of methanol. Gallic acid was used as a positive standard ($y = 0.0073x + 0.0233$, $R^2 = 0.9992$) to yield a calibration curve (0 – 250 μg/mL). All the assays were done in triplicates, and the mean obtained was used to calculate the total phenol content, TPC, in propolis samples. The TPC was expressed as mg gallic acid equivalents (mg GAE/100 g propolis).

3.6.3 Alkaloid Content

The propolis samples' total alkaloid content (TAC) was measured using the 1,10-phenanthroline method (Pandey *et al*., 2014). 1 mL of 0.025 M $FeCl_3$ in 0.5 M HCl was mixed with 1 mL propolis extract, followed by addition of 1 mL of 0.05 M 1,10-phenanthroline in 50% (w/v) ethanol. The mixture was incubated for 30 min in a water

bath at 70ºC. The absorbance of the red coloured complex was measured at 510 nm against a reagent blank containing the reagents only without the sample. Total alkaloid content was estimated from the standard curve plotted using 0.1 - 1.5 mg/mL colchicine ($y = 1.866x + 0.2332$, $R^2 = 0.9844$). Thus, 10 mg of colchicine was dissolved in 10 mL of 50% ethanol (w/v) to generate seven data points conducted in triplicates to allow computation of the means for plotting the standard curve. The TAC was expressed as mg colchicine/100 g propolis.

3.6.4 Terpenoid Content

The total terpenoid content (TTC) was quantified using the colorimetric method (Malik, 2017). First, 1.5 mL chloroform was added to 200 µL propolis sample, and the mixture was vortexed at 3,000 rpm for three minutes. Then, 100 µL of concentrated sulphuric acid was added to the mixture and incubated in the dark for 90 minutes. The supernatant was decanted gently to leave the reddish-brown precipitate forming at the bottom. Next, 1.5 mL of absolute methanol was added to the precipitate and the resulting mixture vortexed again to completely dissolve the precipitate. The same procedure was repeated for the linalool as the positive standard ($y = 0.0009x - 0.0158$, $R^2 = 0.9914$) using different concentrations of linalool (10 – 500 mg/mL). The absorbance was measured at 538 nm with methanol as the blank. TTC was calculated using the linalool standard curve and expressed as linalool equivalents in 100 g propolis (mg LE/100 g propolis).

3.7 GC-MS Analysis of Propolis Samples

3.7.1 Liquid-liquid Extraction and Analysis

Based on their phytochemical profiles and bioactivities, representative samples with highest activity and phytochemicals from hot and dry climatic conditions and cooler climatic conditions were chosen for Gas Chromatography-Mass Spectrometry (GC-MS) analysis. This was done using Gas chromatography (HP-7890A, Agilent Technologies, USA) coupled with Mass spectrometry (MS-597C, Agilent Technologies, USA). The original propolis samples were extracted using absolute ethanol, and the supernatant evaporated to complete dryness at 45°C living a solid

residue. The residue was dissolved in dichloromethane to make one part per million and subjected to the GC-MS analysis. Chromatographic separations were achieved by a HP-5MS capillary column, 30 m × 0.25 mm i.d., 0.25 μm thick (J & W Scientific, USA) immobilized with 5% phenylmethyl silicone as the stationary phase.

In the splitless mode, 1 μL of the propolis sample was injected into the GC-MS instrument using an auto sampler (7683B series, Agilent Technologies, USA). The sample was then transported by helium gas (99.99% purity) as the carrier gas at a flow rate of 1.2 mL per min. The oven temperature was programmed at 35°C, where it was held for 5 min followed by a gradual increase at the rate of 10°C/min to 280°C, where it was held at an isothermal state for 30 min.

3.8 Antimicrobial Activity of Propolis Samples

3.8.1 Bacterial Growth and Inoculum Preparation

Single, well-isolated colonies of *Escherichia coli* and *Bacillus thuringiensis* were selected from overnight cultures grown on Mueller-Hinton Agar (MHA) at 37 °C. Each colony was aseptically suspended in sterile distilled water and vortexed to ensure uniformity. The bacterial suspensions were adjusted to a turbidity of 0.5 McFarland standard (≈ 1×10^8 CFU/mL), verified by measuring the optical density (OD) at 600 nm with OD of 0.132, in accordance with Clinical and Laboratory

Standards Institute (CLSI) guidelines. The standardized inocula were then used for antimicrobial assay.

3.8.2 Disc Diffusion Assay (Kirby-Bauer Test)

This assay was performed in sterile MHA prepared in separate sterile petri dishes (diameter 90 mm). From an overnight culture of *E. coli* prepared as mentioned above, 25 μL were spread on each agar plate using ten silica beads for even spreading. Circular filter paper discs (Whatman, Maidstone, England) of 8 mm diameter were cut using an improvised metallic borer that was sterilized by autoclaving and placed on the surface of the agar plates containing the bacteria, four in each plate. To the discs, 25 μL of 80 mg/mL propolis sample extracts dissolved in absolute ethanol were introduced.

The plates were incubated for 24 h at 37°C alongside negative control using the solvent (ethanol) instead of propolis, and a positive control containing streptomycin at the same concentration of 80 mg/L and volume of 25 µL as used for the samples. Digital pictures of petri dishes with zones of inhibition were recorded using a digital camera, and the zone diameters were measured using ImageJ software (Alonso *et al*., 2017). Each sample was assayed in triplicate. The above procedure was repeated for *B. thuringiensis.*

3.9 Antioxidant Activity of Propolis Samples Using DPPH

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was performed (Lagouri *et al.*, 2014a) with minor modifications. Briefly, to 1.5 mL of the propolis sample solution, 3 mL of DPPH ethanolic solution (2 mg/100 mL ethanol) were added. The mixture was incubated for 45 min at 37ºC in the dark, and the absorbance was measured at 517 nm. Ethanol was used as negative control instead of propolis. For positive control, Quercetin was used to prepare a standard curve (10-100 µg/mL) (Figure S1) and the results tabulated as quercetin equivalent ($y = 0.591x + 38.413$, $R^2 = 0.9988$) (Table S2). Each sample was assayed in triplicate, and the results were averaged and used to calculate the antioxidant activity as free radical scavenging activity expressed as a percentage of inhibition, using the following formula:

$$\textit{\% inhibition} = [(\textit{control absorbance} - \textit{sample absorbance}) / \textit{control absorbance}] \times 100$$

3.10 Data Analysis

R working environment v3.5.0 (R Core Team 2019) and the *factoextra* 1.0.5 and *ggplot2* 3.1.1 packages were used to perform principal components analysis (PCA) to analyse and visualize the phytochemical composition of propolis from the different geographical regions. A Kruskal-Wallis test was used to compare phytochemical contents and the biological activities of propolis samples from different locations at $p < 0.05$, with Dunn's test for pair-wise comparison between the regions. In addition, a Spearman's rank correlation was performed to evaluate relationships between the studied parameters.

Extract chemical components were identified by comparing the retention time of the chromatographic peak with the WILEY8 database combined with NIST library ver.2.0. name and area under the peak of the components. Prediction of biological activity of the compounds was based on Dr. Duke's Phytochemical and Ethnobotanical Databases created by Dr. Jim Duke of Agricultural Research Service/USDA-ARS 20.

CHAPTER FOUR

RESULTS

4.1 Quantification of Phytochemicals in Propolis

4.1.1 Samples

A total of fifty-nine propolis samples for the study were collected from various regions as indicated in the table below.

Table 4.1: Sampling Regions, Locations, and their Different Climatic Conditions

Region	Location	Climatic conditions	Propolis samples (N)
Baringo	Marigat	hot, dry	9
Murang'a	Murang'a	cold, wet	8
Kitui	South Kitui	hot, dry	5
Kakamega	Kakamega	hot, wet	10
Nairobi	Karura	cold, wet	6
	icipe	hot, dry	3
Kilifi	Gede	hot, wet	4
	Mtwapa	hot, wet	6
Taita- Taveta	lower Taita	hot, dry	3
	Taita Hills	cold, wet	5

The samples from each region were collected from different apiaries and based on the climatic conditions. Hot and dry samples were 20, Hot and wet were also 20 except the cold and wet regions with 19 samples.

4.1.2 Phytochemical Yield

The four quantified total phytochemicals based on the colorimetric assays, showed significant variation between all the locations (Table 2) with $P \leq 0.05$. The total content of individual phytochemicals varied in each sample, with alkaloids being most abundant, then phenols, flavonoids, and terpenoids with confidence interval (CI) of 16.7%, 9.4%,4.0% ,8.3% respectively.

Table 4.2: Phytochemical Components of Studied Propolis in mg/100 g Propolis.

Region	Location	Flavanoids (mg QE)	Phenolics (mg GAE)	Terpenoids (mg LE)	Alkaloids (mg CE)
Baringo	Marigat	2,131.4± 1132.4^{c}	2,732.7± 672.1^{cd}	471.1±270.5^{b}	4,651.5± 138.3^{abc}
Murang'a	Murang'a	412.2± 276.0^{a}	1,712.4± 496.6^{bc}	272.0±90.0^{a}	4,672.2± 181.7^{abc}
Kitui	South Kitui	1,388.9± 1065.6^{bc}	3,161.2± 2339.1^{cd}	475.7±93.0^{b}	7,494.7± 4100.1^{c}
Kakamega	Kakamega	1,153.3± 488.1^{bc}	853.0± 506.3^{ab}	347.7±61.5^{b}	4,196.4± 426.6^{a}
Nairobi	*icipe*	1,717.8± 48.4^{bc}	3,711.8± 429.7^{d}	202.8±59.5^{a}	4,453.1± 98.4^{ab}
	Karura	1,112.2± 143.6^{bc}	2,218.5± 380.1^{cd}	301.4±57.3^{a}	4,435.7± 131.1^{a}
Kilifi	Gede	456.7± 124.9^{ab}	1225.4± 190.4^{abc}	407.3±134.6^{b}	7698.7± 835.1^{d}
Taita-Taveta	lower Taita	825.3± 361.1^{abc}	2,813.8± 323.3^{cd}	353.1±22.9^{b}	8,767.5± 257.4^{d}
Kilifi	Mtwapa	1,391.1± 658.1^{bc}	1,726.1± 1283.9^{abc}	319.4±104.5^{a}	7,614.7± 2807.2^{bc}
Taita - Taveta	Taita Hills	102.2± 68.3^{a}	522.6± 169.3^{a}	258.5±21.40^{a}	3,014± 2692.7^{a}
	p-value	0.0002	0.00006	0.0005	0.002

Table of Means ± Standard Deviation of the four phytochemicals in propolis showing variations among regions and individual locations. Different superscript letters in each column show variations in samples collected from different locations as revealed by the pairwise comparisons (Dunn's test). QE - Quercetin Equivalent, GAE - Gallic Acid Equivalent, LE - Linalool Equivalent, CE - Colchicine Equivalent.

A pairwise comparison by Dunn's test showed how samples from each location differed with different superscript letters showing the difference. The locations had different climatic conditions (Table 1), and those with hot and dry climatic conditions exhibited high total phytochemical content compared to the other locations. The terpenoid content indicates the apparent difference of the locations, categorizing them only into two groups.

There was a separation of propolis phytochemicals according to locations indicated by the PCA plot (Figure 4.1). Primary separation is on Dim 1, which separates all the regions into two, with Kitui, Gede, *icipe*, Mtwapa, lower Taita, and Marigat on the

negative side while Karura, Murang'a, Kakamega, and Taita Hills are on the positive side. The regions on the negative side experience hot climates while the others have cold climates. Dim 2 separates the components based on phytochemical content with flavonoids and terpenoids on the positive side and alkaloids together with phenols on the opposing side.

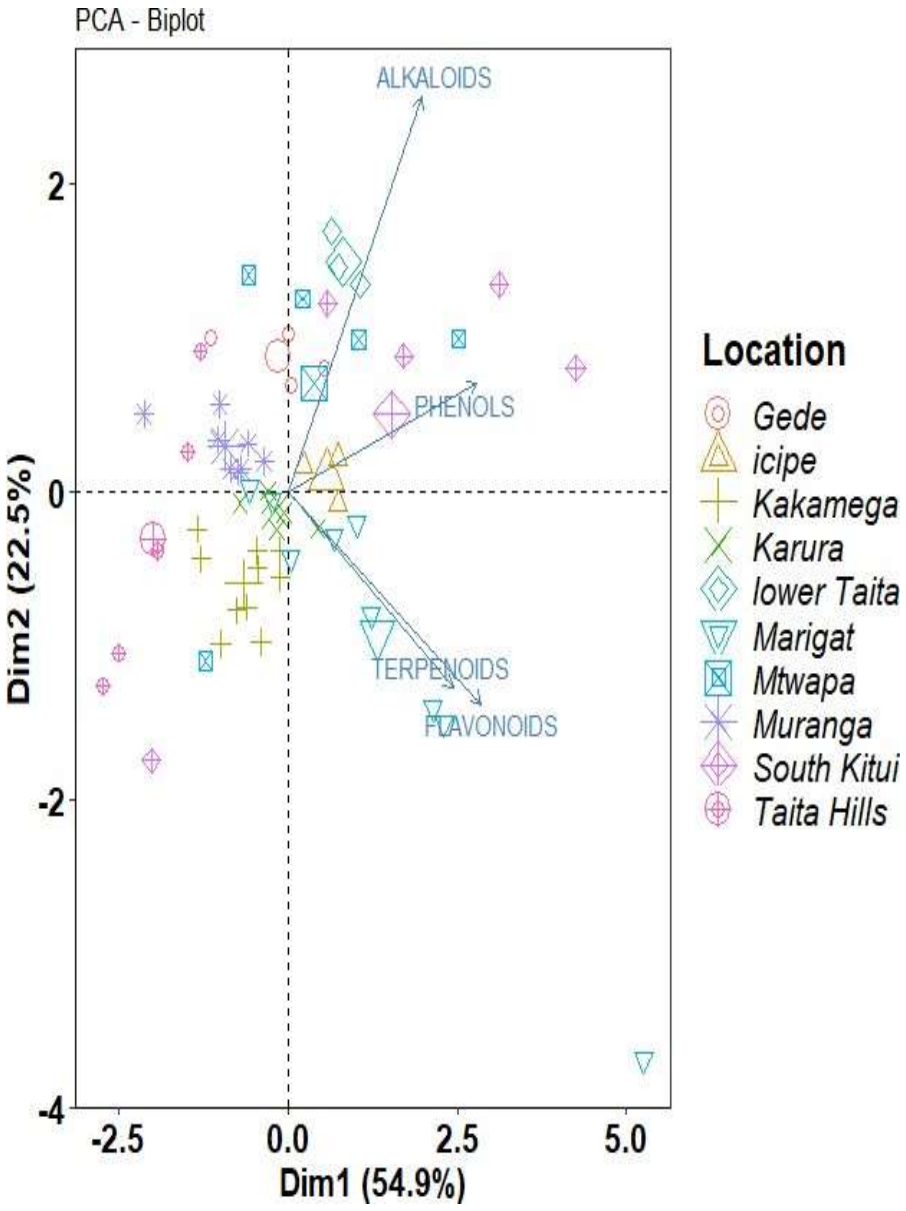

Figure 4.1: PCA-Biplot Graph of Kenyan Propolis Samples Based on their Phytochemical Content

4.1.3 Antioxidant Activity

The samples had an antioxidant activity based on their ability to scavenge DPPH free radicals leading to a colour change from purple to colourless. There is a difference in antioxidant activity of propolis ranging from 15 to 74% RSA for 250 mg/L of propolis extract (Figure 4.2). The quercetin equivalent from the standard curve indicates the average inhibition at 0.25µg/mL (250mg/L) to be equivalent to be 23.0 µg/mL of ability quercetin. The samples with lower equivalence are because of very low concentration of our samples which means at same concentration with the standard they will have a stronger inhibition. There is a variation in regions with *icipe*, Marigat, and Murang'a having all samples with high antioxidant activity, while Kitui and Mtwapa had samples with considerable variations, with some having the highest levels compared to others. Karura and Kakamega had samples with almost the same antioxidant activities, with the same pattern exhibited by Mtwapa and Gede. Taita had the lowest antioxidant activity.

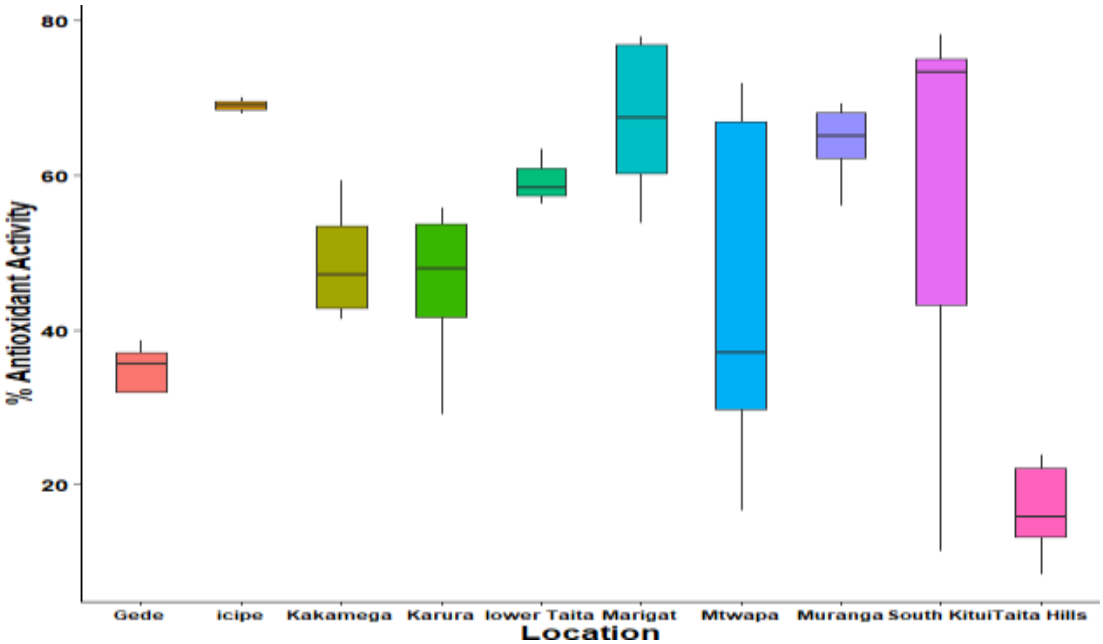

Figure 4.2: Antioxidant Activity of Kenyan Propolis Samples according to their Respective Sampling Locations

4.2 Antimicrobial Activity

All the tested propolis samples exhibited broad-spectrum antibacterial activity. They inhibited the growth of *E. coli* and *B. thuringiensis*, which are Gram-negative and Gram-positive bacteria, respectively (Figure 4.3). They have elevated activity against

B. thuringiensis compared to *E. coli*. Propolis from Gede exhibited the highest inhibition against Gram-positive bacteria, while propolis from Kitui, Mtwapa, lower Taita, and Marigat had the highest activity against Gram-negative bacteria.

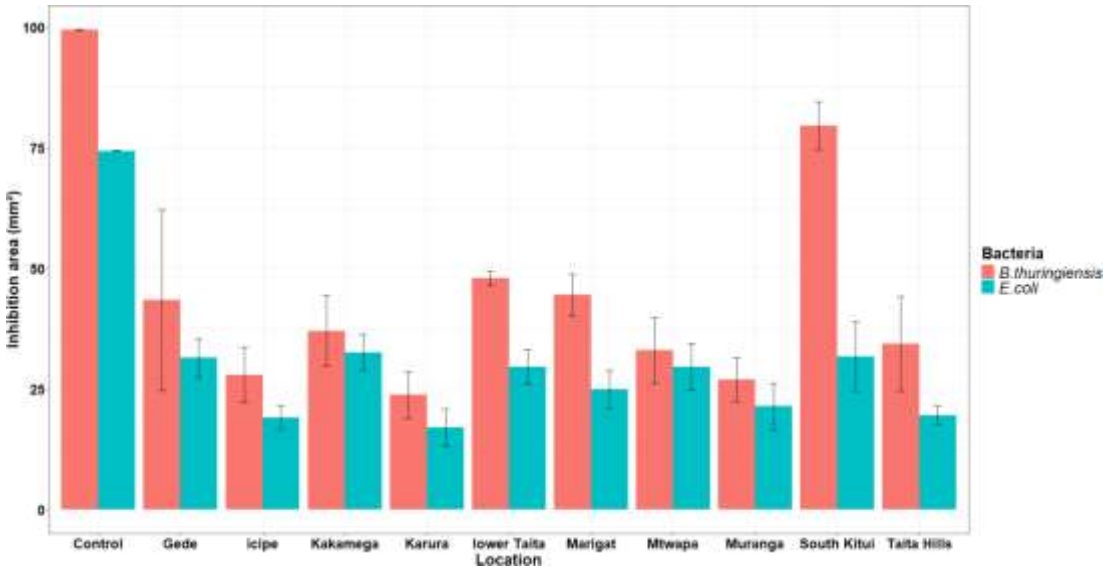

Figure 4.3: Antibacterial Activity of Kenyan Propolis Samples.

There is a significant correlation between phytochemicals and the biological activities of propolis, e.g., phenols and antioxidant activity (r = 0.78), and alkaloids and antibacterial activity against *E. coli* (r = 0.61), indicated by the Spearman rank correlation plot (Figure 4.4).

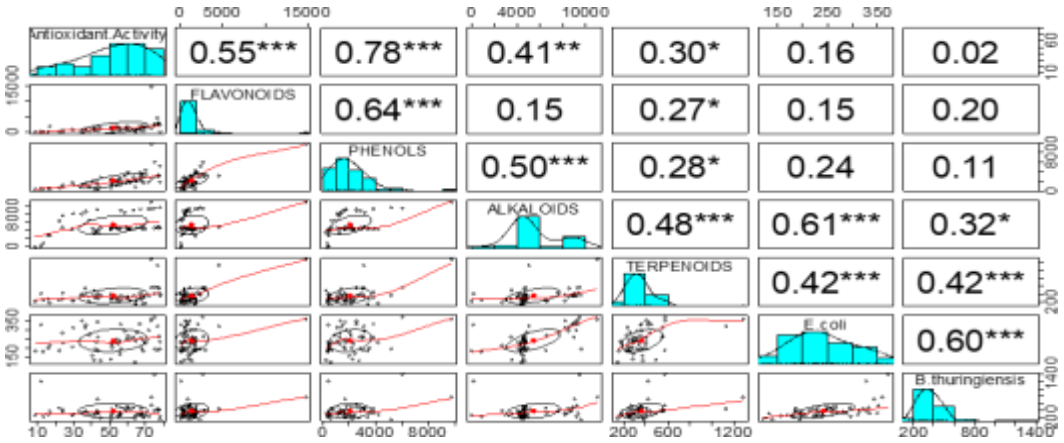

Figure 4.4: Spearman Rank Correlation Plot for All Pair-Wise Comparisons of Phytochemicals (Alkaloids, Flavonoids, Phenols, and Terpenoids) and Biological Activity (Antimicrobial and Antioxidant Activity). * 0.05, ** 0.01, ***0.001

4.3 GC-MS Analysis

Analysis of GC-MS chromatograms of the selected propolis samples showed more than a hundred peaks indicating the presence of various compounds including hydrocarbons, terpenoids, diterpenoids, triterpenoids, and phenols (Table 3). Terpenoids appeared to be the most abundant probably due to their higher volatility thus more easily detected by GC-MS analysis. Hydrocarbons also are volatile but with long chains hence they were also detected in moderate abundance. The flavonoids and phenol classes of compounds were the least. In all the locations there was clear difference on the compounds present with those from hot and dry having more compounds compared to the other regions. Climatic condition contributes to the diverse chemical composition with only two compounds being present in all the samples, octadecahydro-2H-picen-3-one and eicosane. The compounds present in all samples have varying abundance. All the compounds reported have biological activities except nonadecane, which is a hydrocarbon.

Compounds in the propolis samples have been reported to possess antioxidant and/or antibacterial activity (Figure 4.5).

Table 4.3: Compounds Present in Propolis Identified by GC-MS Analysis

Compound	Class	Retention time (min)	Hot and dry		Hot and wet		Cold and wet		Biological activity
			South Kitui	Marigat	Gede	Kakamega	Murang'a	Taita Hills	
Octadecahydro- 2H-picen-3-one	Flavonoid	40.1	216.0	211.0	81.0	57.0	3.0	19.0	Antioxidant (Malik *et al*., 2016)
Squalene	Flavonoid	31.1	-	-	10.0	-	-	-	Antioxidant (Güneş, 2013)
Sempervirol	Terpenoid	26.8	-	-	9.0	10.0	-	-	Antioxidant (Hussain *et al*.,2019)
Lup-20(29)-en- 3-ol	Diterpenoid	43.1	-	29.0	-	-	-	-	Antioxidant (Usman *et al*., 2022)
Rosadiene	Diterpenoid	26.9	-	10.0	-	-	-	-	Antibacterial (Zanin *et al*.,2012)
Cubitene	Diterpenoid	23.6	-	5.0	-	-	-	-	Antibacterial (Ailli *et al*.,2023)
Lupenone	Triterpenoid	31.7	-	40.0	52.0	107.0	89.0	209.0	Antibacterial (Andrade *et al*., 2020)
Amyrin	Triterpenoid	40.7	23	-	-	-	-	-	Antibacterial (Bata *et al.,* 2023)
Ursenoic acid	Triterpenoid	43.2	4.0	-	-	-	-	-	Antibacterial (Attallah *et al*., 2021)
Eicosane	Hydrocarbon	28.4	8.0	10.1	20.0	40.0	7.0	19.0	Antibacterial (Chuah *et al*., 2018)
Heneicosane	Hydrocarbon	34.2	24.0	62.0	23.0	-	-	-	Antibacterial (Vanitha *et al*., 2020)
Sugiol	Phenol	29.6	-	-	25.0	7.0	-	-	Antimicrobial and antioxidant (Bajpai *et al.,* 2021)

Different compounds result in the antioxidant and antibacterial activities of propolis as indicated in PCA (Figure 4.5) because of their separation and indication of specific compounds having a more pronounced effect on either antioxidant activity or antibacterial activity. Amyrin and ursenoic acid are seen to be responsible for activity against *E. coli* and *B. thuringiensis* while cubitene, squalene and sugiol are responsible for the antioxidant activity.

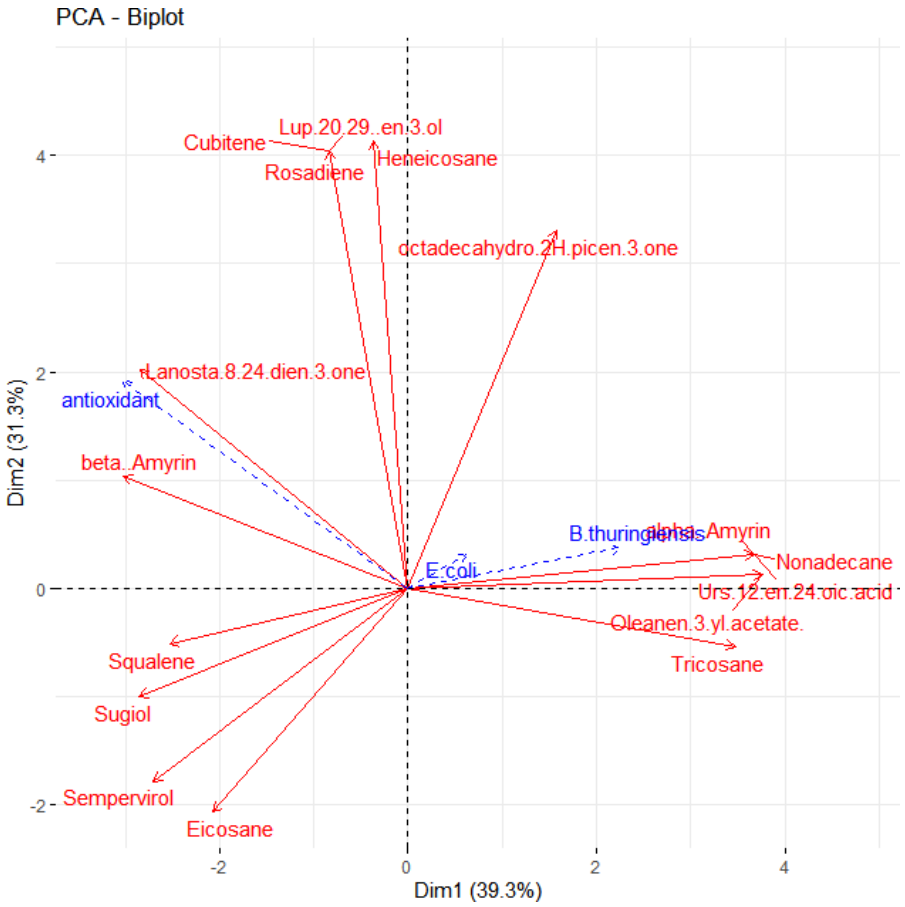

Figure 4.5: PCA-Biplot Graph of Kenyan Propolis Samples Based on Their Bioactivities and GCMS Compounds Identified.

The dotted lines in the PCA-Biplot are the bioactivities while the continuous lines are the various compounds present in propolis

CHAPTER FIVE

DISCUSSION

5.1 Quantification of Phytochemicals in Propolis

Varying amounts of phytochemicals illustrate the complex chemical composition of propolis. The concentration of phytochemicals is dependent on the geographical origin and its associated vegetation, which is influenced by the local climatic conditions (Bankova *et al*., 2014). The extracting solvent also affects the concentration of the compounds in different extracts, but water–ethanol as a solvent is preferred (Emacar *et al*., 2010). The total phenol and flavonoids content agree with the range recorded by (Sun *et al*., 2015) of 6.68 to 164.20 mg GAE /g and 4.07 to 282.83 mg RE/g, which also agrees with quantities recorded (Bankova *et al.*, 2019).

Alkaloids are biologically active compounds with at least one N atom and have basic properties (Debnath *et al*., 2018). They are among the largest classes of natural products and are found particularly in plants, ingested foods and beverages such as coffee, tea leaves, and cocoa (Aniszewski, 2015). Of importance is that most of the plant-derived pharmaceutical products used as antimalaria, antibacterial and anticancer are alkaloids (Ozansoy & Küplülü, 2017). The total alkaloids and terpenoids content in propolis has not been reported before, and this is the first work to report the same. Studies have reported these compounds through GC-MS analysis (Kalsum *et al*., 2016). Terpenoids are the largest class of naturally occurring products with classification based on isoprene units with a regular arrangement of these units; some are irregular (Ludwiczuk *et al*., 2017). Hemiterpenoids are found in the leaves of many plants, with monoterpene being found as complexes in plant essential oils. Terpenoids are responsible for the characteristic aroma of propolis.

5.2 Antioxidant Activity

The DPPH is a stable free radical widely used to evaluate the antioxidant activity of extracts and pure substances. The effect of antioxidants on the DPPH is due to its two mechanisms of action, single electron transfer and hydrogen ion transfer (Mulyati *et*

al., 2020). Propolis displays antioxidant activity due to phytochemicals, which are secondary plant metabolites and are known to act as natural antioxidants. The antioxidant activities of terpenoids, phenols, and flavonoids are well documented (Das *et al*., 2020). The concentration of 250 mg/L in our study promoted varying antioxidant activity, with the highest being 74%. This is low compared with the reported 92.4% (Cristina *et al*., 2015) for the same propolis concentration using only absolute ethanol as a solvent compared to our 50% ethanol solvent The results were close to those reported by Bonamigo *et al* (2017) . The difference is attributed to the solvent used and the geographical location of the propolis collection. Phytochemicals contribute substantially to the antioxidant activity indicated by the positive correlation.

5.3 Antimicrobial Activity

Propolis demonstrated broad-spectrum antibacterial activity as shown by activity against *E. coli* and *B. thuringiensis*, which are Gram-negative and Gram-positive bacteria, respectively. This is in accordance with other studies that have shown the similar activity of propolis (Kaur *et al.*, 2020). Phytochemicals present in propolis could be responsible for its antibacterial activity. The GC-MS analysis identified compounds that are known to exhibit antibacterial activity, such as amyrin (Balingui *et al*., 2019), ferruginol (Kalsum *et al*., 2016), and totarol (Bankova *et al.*, 2014). The variation in antibacterial activity is due to the presence of these compounds and their concentration in the samples. These results make propolis a potential source of antibacterial compounds for further analysis.

5.4 GC-MS Analysis

In this study, the propolis samples had varying compounds reported by others (Kalsum *et al*., 2016). There was a difference in chemical composition from different climatic conditions because the composition of propolis is directly affected by vegetation (Kuropatnicki *et al*., 2013). The presence of compounds as squalene, picenone, sempervirol, amyrin, sugiol, and long-chain hydrocarbons such as eicosane have been reported to be components of propolis (Nina *et al.*, 2015; Pino *et al*., 2006). There are three sources of organic compounds from propolis: compound derived from the honey bees metabolism, plant origin, and materials used to form propolis (Kaur *et al*., 2020).

The bioactivites for these compounds include amyrin as antibacterial, squalene as antioxidant, sugiol as antibacterial and antioxidant, and lupenone as an antioxidant (Balingui *et al*., 2019; Georgieva *et al*., 2019; Kalsum *et al*., 2016; Cristina *et al*., 2015)

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The outcome of this study suggests that *Apis mellifera* propolis from Kenya has varying amounts of phytochemicals. This is dependent on the geographical and climatic conditions of different sampling locations. Propolis consisting of plant exudates, buds, and resins implies that the vegetation type directly influences its chemical composition. Apart from widely studied phenols and flavonoids, alkaloids were the most abundant phytochemicals with noticeable effects on the bioactivities of propolis samples. Terpenoids were also abundant in the propolis, and they influenced the antibacterial activities of propolis.

Propolis is a rich source of biologically active compounds and GC-MS analysis can be used to screen these compounds resulting in identification of different compounds. The bioactivities of propolis are ascribed to the presence of reported compounds, sugiol, lupenone, and squalene which are phytochemicals identified through GC-MS analysis. Propolis is a natural source of antioxidants with high potency compared to honey (Mouhoubi *et al*., 2016).

6.2 Recommendations

While this study established antibacterial and antioxidant activities, further research is recommended to elucidate the mechanisms of action of key bioactive compounds in propolis and involve the determination of minimum bactericidal concentrations assays. Future studies should include a broader range of ecological zones in Kenya for sampling of propolis. This would help identify regions with the highest bioactive potential and give general representation.

Furthermore, isolation, purification, and characterization of specific bioactive compounds responsible for the observed effects, using techniques such as LC- MS/MS for non-volatile compounds is also needed.And finally, propolis and its utilization should be encouraged by adopting innovative strategies for its production and harvesting.

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APPENDICES

Appendix I: Table of Sample Collection Sites Dates and Coordinates

Region	Collection date	latitudes	longitudes
Marigat	Feb-20	0.473938	35.996275
Marigat	Feb-20	0.473938	35.996275
Marigat	Feb-20	0.473938	35.996275
Marigat	Feb-20	0.474997	35.995616
Marigat	Feb-20	0.474997	35.995616
Marigat	Feb-20	0.474997	35.995616
Marigat	Feb-20	0.469840	35.994537
Marigat	Feb-20	0.469840	35.994537
Marigat	Feb-20	0.469840	35.994537
Murang'a	Feb-20	-0.864550	37.215790
Murang'a	Feb-20	-0.902930	37.253440
Murang'a	Feb-20	-0.893840	37.129270
Murang'a	Feb-20	-0.893840	37.129270
Murang'a	Feb-20	-0.855830	37.140380
Murang'a	Feb-20	-0.855830	37.140380
Murang'a	Feb-20	-0.852730	37.122270
Murang'a	Feb-20	-0.817850	37.066880
Kakamega	Mar-20	0.15983	34.85622
Kakamega	Mar-20	0.159830	34.856220
Kakamega	Mar-20	0.257690	34.751080
Kakamega	Mar-20	0.257690	34.751080
Nairobi	May-20	-1.223605	36.896230
Nairobi	May-20	-1.223605	36.896230
Nairobi	May-20	-1.223605	36.896230
Nairobi	May-20	-1.233629	36.834411
Nairobi	May-20	-1.233629	36.834411
Nairobi	May-20	-1.234103	36.835092
Kitui	Sep-20	-1.950955	38.200683
Kitui	Sep-20	-2.100528	38.149983
Kitui	Sep-20	-2.102603	38.118071
Kitui	Sep-20	-2.125337	38.082460
Kitui	Sep-20	-2.126058	38.037653
Mtwapa	Sep-20	-3.930117	39.750286
Mtwapa	Sep-20	-3.902308	39.736809
Mtwapa	Sep-20	-3.902308	39.736809
Mtwapa	Sep-20	-3.874129	39.739584
Mtwapa	Sep-20	-3.874129	39.739584
Mtwapa	Sep-20	-3.918448	39.720189
Gede	Sep-20	-3.305335	40.010460
Gede	Sep-20	-3.298525	40.021981
Gede	Sep-20	-3.306953	40.017968

Gede	Sep-20	-3.306953	40.017968
Taita	Sep-20	-3.434800	38.343130
Taita	Sep-20	-3.434800	38.343130
Taita	Sep-20	-3.434800	38.343130
Taita	Sep-20	-3.475362	38.339951
Taita	Sep-20	-3.475362	38.339951
Taita	Sep-20	-3.457503	38.392729
Taita	Sep-20	-3.457503	38.392729
Taita	Sep-20	-3.457503	38.392729

Appendix II: Published Manuscript

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Research

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Phytochemical composition and bio-functional properties of *Apis mellifera* propolis from Kenya

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There is an increased demand for natural products like propolis, yet little information is available about the chemical composition of African propolis and its bio-functional properties. Therefore, in this study, we aimed to quantify the phytochemicals and determine the antioxidant and antimicrobial properties of *Apis mellifera* propolis (n = 59) sourced from various regions in Kenya. Principal component analysis (PCA) showed that the sampling region had a remarkable impact on the propolis's composition and bio-functional properties. Generally, the propolis contained high amounts of phytochemicals, particularly alkaloids (5.76 g CE/100 g) and phenols (2.24 g GAE/100 g). Furthermore, analysis of propolis by gas chromatography–mass spectrometry (GC-MS) revealed various compounds with varying bio-functional activities. These compounds included triterpenoids alpha- and beta-amyrin, oleanen-3-yl-acetate, urs-12-en-24-oic acid, lanosta-8,24-dien-3-one, and hydrocarbons tricosane and nondecane, which have been reported to have either antimicrobial or antioxidant activities. The propolis samples collected from hotter climatic conditions contained a higher composition of phytochemicals, and additionally, they displayed higher antioxidant and antimicrobial activities than those obtained from cooler climatic conditions. Key findings of this study demonstrate the occurrence of relatively high phytochemical content in Kenya's propolis, which has antioxidant and antimicrobial properties; hence this potential could be harnessed for disease control.

1. Introduction

Propolis is a natural resinous mixture comprising plant resins, bee saliva and wax, which is used by wild and feral bees as a building material for sealing unwanted spaces in the hive, hence smoothening it [1]. In addition, propolis plays a crucial role in chemical defence against invading microorganisms, thus making the hive environment sterile [2]. Furthermore, bees use propolis to embalm dead and live intruders such as small hive beetles [3] and other organisms that are too large to be removed [4,5].

Further, the beneficial therapeutic effects of propolis have been recognized and applied since ancient times. Historical records suggest that its use dates back to ancient Greeks and Romans [6]. The bioactive properties of propolis, such as anti-inflammatory, anti-cancer, antioxidant, antimicrobial and immune-stimulating activities, have all been demonstrated by previous studies [7,8]. The chemicals responsible for these bioactive properties have been shown to include flavonoids and phenols, among others [9]. In recent years, modern societies have recognized and appreciated natural propolis-based treatments for disease control and management and are therefore embracing the development of these products [10].

Currently, lifestyle diseases, including but not limited to arthritis, cardiovascular disease, cancer, diabetes, Parkinson's and Alzheimer's disease, are affecting an increasing number of people [11]. This is attributed to free radicals, chemical and physical factors leading to cell ageing, the genesis of pathophysiological processes [12]. Propolis is a source of natural antioxidants, scavenging and acting as secondary defensive factors against free radicals produced in the human body [13]. Enzymes serve as the primary defence system against oxidative stress as a result of normal body physiological processes. These enzymes include catalase, glutathione peroxidase and superoxide dismutase [14]. When the generation of free radicals overcomes the primary buffer capacity of the human body, the second line of defence comprises vitamins [15]. These vitamins, for example vitamins C and E, play roles by scavenging free radicals and inactivating them [10]. Exhaustion of these two defence mechanisms leads to severe cell damage in the body [16]. Phenolic compounds, flavonols, flavones, flavanones and isoflavanones are important bioactive constituents of propolis with a proven ability to scavenge free radicals, apart from shielding vitamin C and lipids denaturation by the resulting oxidative physiological process [17].

Disease-causing microbes are evolving to adapt to the constant exposure to antimicrobial agents leading to the emergence of multidrug-resistant pathogens [18]. This has made the treatment of diseases difficult and costly. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an example that could cause disfiguring of the affected body part or loss of life if left untreated [19–21]. These challenges have inspired research on better and more efficient alternatives. This led to a re-evaluation of the therapeutic properties of natural products exhibiting antimicrobial activity, such as propolis [22]. The potency of propolis as a natural bioactive agent against both Gram-positive and Gram-negative microorganisms like multidrug-resistant bacteria was demonstrated by [1], indicating its efficacy. Turkish propolis was also efficacious against tuberculosis, and the results pointed out its potential activity against different species of mycobacteria [23]. These properties result from diverse antimicrobial factors such as flavonoids, phenols and other compounds [24]. The clinical antibacterial properties of propolis have been supported due to the myriad bioactive compounds present in it [25]. Therefore, propolis has attracted much interest in its application to treat various maladies. Consequently, propolis has been used to develop new drugs through biotechnology [25].

Presently, it is unclear how the vegetation serving as the source of resins in the propolis influences the phytochemical composition and the biological activity of propolis. Often, the plants used for resin collection by bees are unknown. Thus, we can use environmental variables, such as climatic variables, vegetation and land use/land cover (LULC) parameters as proxies to predict propolis's phytochemical composition and biological activity. In this study, we analysed the phytochemicals and determined the antioxidant and antibacterial activities of *Apis mellifera* propolis collected from various geographically distinct regions in Kenya, which differ in climatic and vegetation parameters. The results may help understand how propolis' properties vary according to regions and climatic conditions that influence it.

2. Material and methods

2.1. Chemicals

Mueller–Hinton Agar (MHA) was purchased from Himedia Laboratories Pvt. Ltd (F&S Scientific, Nairobi, Kenya). Sodium nitrite ($NaNO_2$), 1,10-phenanthroline, aluminium chloride ($AlCl_3$), Gallic acid, linalool, quercetin, sodium carbonate (Na_2CO_3), Folin–Ciocalteu's reagent, sodium hydroxide (NaOH),

Table 1. Sampling regions, locations and their different climatic conditions. Sample sizes with respect to apiaries and individual propolis samples are given in the last two columns.

region	location	climatic condition	apiaries (N)	propolis samples (N)
Rift Valley	Marigat	hot, dry	3	9
Central	Murang'a	cold, wet	6	8
Eastern	South Kitui	hot, dry	5	5
Western	Kakamega	hot, wet	5	10
Nairobi	Karura	hot, dry	3	6
	icipe	cold, wet	1	3
Coast	Gede	hot, wet	3	4
	Mtwapa	hot, wet	4	6
	lower Taita	hot, dry	1	3
	Taita Hills	cold, wet	2	5

hydrochloric acid (HCl), chloroform, dichloromethane (DCM), sulfuric acid (H_2SO_4), colchicine, ferric-III-chloride ($FeCl_3$), absolute ethanol and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were all purchased from Sigma-Aldrich (Kobian Kenya Ltd). For all analyses, we used chemicals of analytical grade.

2.2. Collection of propolis

Fifty-nine propolis samples were collected directly from hives by scraping with a hive tool and wrapping them with aluminium foil. Sampling was done by randomly selecting two hives per apiary from each location in the six different regions in Kenya with differing climatic conditions (table 1). The propolis samples were stored at −80°C, awaiting further processing.

2.3. Sample preparation

The individual propolis samples were crushed into a fine powder in liquid nitrogen using a mortar and pestle. Extracts for the analysis of the phytochemical composition and radical scavenging activity were prepared by weighing 0.5 g of each crushed sample in 10 ml of 50% v/v ethanol in 15 ml Falcon tubes and left to stand at room temperature for 72 h. They were vortexed at 3000 r.p.m. for 3 min, followed by the second vortexing step after 24 h of incubation at room temperature. After the extraction, the samples were centrifuged at 600 r.p.m. for 2 min, and the supernatant was decanted and stored at −80°C. For testing the antibacterial activity, the extractions were conducted by dissolving 2 g of the propolis sample in 10 ml of absolute ethanol (100%). The mixture was vortexed at 3000 r.p.m. for 2 min and then incubated for 24 h at room temperature. The subsequent extraction followed the same steps described above, and concentration was done in an Eppendorf concentrator plus (Eppendorf, Hamburg, Germany) to complete dryness. The residue was weighed on an analytical plus scale (OHAUS Corporation Parsippany, New Jersey, USA) and dissolved in absolute ethanol to make a concentration of 80 mg ml^{-1}.

2.4. Quantification of phytochemicals

2.4.1. Flavonoid content

The total flavonoid content (TFC) of propolis samples was done following the aluminium chloride ($AlCl_3$) colorimetric assay described by Popova *et al.* [26]. To 1 ml extract of each sample, 4 ml of distilled water was added, followed by 300 µl of 5% (w/v) $NaNO_2$ and mixed. After 5 min, 300 µl of 10% $AlCl_3$ was added to the mixture and left for 1 min prior to adding 2 ml of 1 M NaOH, followed by a top-up step with 2.4 ml of distilled water. The absorbance of the mixture was measured at 510 nm using a spectrophotometer (BioSpec, Bartlesville, USA) against a reagent blank containing all the above reagents, except the sample that was replaced with 50% ethanol. Quercetin at different concentrations (20–200 µg ml^{-1}) was used as standard to generate a calibration curve ($y = 0.0006x + 0.0028$, $R^2 = 0.9981$) and TFC expressed as mg quercetin equivalent per 100 g propolis (mg QE/100 g propolis).

2.4.2. Phenol content

We used the Folin–Ciocalteu method described by Popova *et al.* [26] to determine the total phenol content (TPC). To 1 ml extract, 5 ml of 0.2 N Folin–Ciocalteu reagent was added and left at room temperature for 5 min. After adding 4 ml of 75 g l^{-1} Na_2CO_3, the mixture was incubated at room temperature for 2 h. The absorbance of this reaction mixture was read at 760 nm against an ethanol blank instead of methanol. Gallic acid at different concentrations (0–250 µg ml^{-1}) was used as a standard to yield a calibration curve ($y = 0.0073x + 0.0233$, $R^2 = 0.9992$). All the assays were done in triplicates, and the mean obtained was used to calculate the total phenol content, TPC, in propolis samples. The TPC was expressed as mg gallic acid equivalents (mg GAE/100 g propolis).

2.4.3. Alkaloid content

The propolis samples' total alkaloid content (TAC) was measured using the 1,10-phenanthroline method as previously described by Pandey *et al.* [27]. One millilitre of 0.025 M $FeCl_3$ in 0.5 M HCl was mixed with 1 ml propolis extract, followed by the addition of 1 ml of 0.05 M 1,10-phenanthroline in 50% (w/v) ethanol. The mixture was incubated for 30 min in a water bath at 70°C. The absorbance of the red-coloured complex was measured at 510 nm against a reagent blank containing the reagents only without the sample. Total alkaloid content was estimated from the standard curve plotted using 0.1–1.5 mg ml^{-1} colchicine ($y = 1.866x + 0.2332$, $R^2 = 0.9844$). Thus, 10 mg of colchicine was dissolved in 10 ml of 50% ethanol (w/v) to generate seven data points conducted in triplicates to allow computation of the means for plotting the standard curve. The TAC was expressed as mg colchicine/100 g propolis.

2.4.4. Terpenoid content

The total terpenoid content (TTC) was quantified using the colorimetric method described by Malik *et al.* [28]. First, 1.5 ml chloroform was added to a 200 µl propolis sample, and the mixture was vortexed at 3000 r.p.m. for 3 min. Then, 100 µl of concentrated H_2SO_4 was added to the mixture and incubated in the dark for 90 min. The supernatant was decanted gently to leave the reddish-brown precipitate forming at the bottom. Next, 1.5 ml of absolute methanol was added to the precipitate and the resulting mixture vortexed again to dissolve the precipitate completely. The same procedure was repeated for the linalool standard using different concentrations of linalool (10–500 mg ml^{-1}). The absorbance was measured at 538 nm with methanol as the blank. TTC was calculated using the linalool standard curve ($y = 0.0009x - 0.0158$, $R^2 = 0.9914$) and expressed as linalool equivalents in 100 g propolis (mg LE/100 g propolis).

2.5. Antimicrobial activity

2.5.1. Bacterial growth and maintenance

Single bacterial colonies of Gram-negative *Escherichia coli* from an overnight culture on Mueller–Hinton Agar (MHA) were inoculated into sterile distilled water to achieve turbidity of 0.5 McFarland (≈1 × 10^8 CFU ml^{-1} as per Clinical and Laboratory Standards Institute) by measuring the optical density (OD) of 0.132 at 600 nm. The same procedure was repeated for the Gram-positive *Bacillus thuringiensis*.

2.5.2. Disc diffusion assay (Kirby-Bauer test)

This assay was performed in sterile MHA prepared in separate sterile Petri dishes (diameter 90 mm). From an overnight culture of *E. coli* prepared as mentioned above, 25 µl were spread on each agar plate using 10 silica beads for homogeneous spreading. Four circular filter paper discs (Whatman, Maidstone, UK) (8 mm diameter) were cut using a sterilized metallic borer and placed on the surface of the agar plates containing the bacteria. To the discs, 25 µl of 80 mg l^{-1} propolis sample extracts dissolved in absolute ethanol were introduced. The plates were incubated for 24 h at 37°C alongside a negative control using the solvent (ethanol) instead of propolis and a positive control containing streptomycin at the same concentration of 80 mg l^{-1} and volume of 25 µl as used for the samples. Digital pictures of Petri dishes with zones of inhibition were recorded using a digital camera, and the zone diameters were measured using ImageJ software [29]. Each sample was assayed in triplicate. The above procedure was repeated for *B. thuringiensis*.

2.6. Antioxidant activity

2.6.1. Analysis of *in vitro* DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH assay was performed as outlined by Lagouri *et al.* [12] with minor modifications. Briefly, to 1.5 ml of the propolis sample solution, 3 ml of DPPH ethanolic solution (2 mg/100 ml ethanol) were added. The mixture was incubated for 45 min at 37°C in the dark, and the absorbance was measured at 517 nm. Methanol was used as control instead of propolis. For positive control, quercetin at different concentrations (10–100 µg ml^{-1}) was used to prepare a standard curve ($y = 0.591x + 38.413$, $R^2 = 0.9988$) and the results tabulated as quercetin equivalent. Each sample was assayed in triplicate, and the results were averaged and used to calculate the antioxidant activity as free radical scavenging activity expressed as a percentage of inhibition, using the following formula:

$$\%\text{inhibition} = [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100$$

2.7. Gas chromatography–mass spectrometry analysis

2.7.1. Liquid–liquid extraction and analysis

Based on the phytochemical contents and level of bioactivity, representative samples from hot and dry climatic conditions and cooler climatic conditions were chosen for GC-MS analysis. This was done using a gas chromatograph (HP-7890A, Agilent Technologies, USA) coupled with a mass spectrometer (MS-597C, Agilent Technologies, USA). The samples were extracted using absolute ethanol, and the supernatant evaporated to complete dryness leaving a solid residue. The residue was redissolved in dichloromethane to make one part per million and subjected to the GC-MS analysis. Chromatographic separations were achieved by a HP-5MS capillary column, 30 × 0.25 mm i.d., 0.25 µm thick (J & W Scientific, USA) immobilized with 5% (phenylmethyl silicone) as the stationary phase.

In the splitless mode, 1 µl of the propolis sample was injected into the GC-MS instrument using an autosampler (7683B series, Agilent Technologies, USA). The sample was then transported by helium (99.99% purity) as the carrier gas at a flow rate of 1.2 ml min^{-1}. First, the oven temperature was programmed at 35°C, where it was held for 5 min, followed by a gradual increase at the rate of 10°C min^{-1} to 280°C, where it was held at an isothermal state for 30 min.

2.8. Data analysis

R working environment v. 3.5.0 (R Core Team 2019) incl. packages *factoextra 1.0.5* and *ggplot2 3.1.1* were used to perform a principal components analysis (PCA) to analyse and visualize the phytochemical composition of propolis from the different geographical regions. A Kruskal–Wallis test was used to compare phytochemical contents and the biological activities of propolis samples from different locations at $p < 0.05$, with Dunn's test for pair-wise comparison between the regions. A Spearman's rank correlation was performed to evaluate relationships between the studied parameters.

Extracted chemical components were identified by name and area under the peak as a proxy for the quantity by comparing the retention time of the chromatographic peak with the WILEY8 database combined with NIST library v. 2.0. The data presented had a similarity structure compound estimate (similarity index) greater than or equal to 90%. Prediction of biological activity of the compounds is based on Dr Duke's Phytochemical and Ethnobotanical Databases created by Dr Jim Duke of Agricultural Research Service/USDA-ARS in 2016.

3. Results

3.1. Quantification of phytochemicals

The four quantified phytochemical groups showed significant variation among all the locations based on the colorimetric assays (table 2) with $p \leq 0.05$. The total content of individual phytochemicals varied in each sample, with alkaloids being most abundant, followed by phenols, flavonoids and terpenoids, respectively. The mean total quantity of phenol content was between 522.6 and 3,711.8 mg GAE/100 g

Table 2. Means ± standard deviation of the four phytochemicals in propolis showing variations between the regions and individual locations (mg/100 g of propolis). Different superscript letters in each column show variations in samples collected from different locations as revealed by the pairwise comparisons (Dunn's test). QE = Quercetin Equivalent, GAE = Gallic Acid Equivalent, LE = Linalool Equivalent, CE = Colchicine Equivalent.

region	location	flavanoids (mg QE/100 g)	phenols (mg GAE/100 g)	terpenoids (mg LE/100 g)	alkaloids (mg CE/100 g)
Rift Valley	Marigat	2131.4 ± 1132.4^{c}	2732.7 ± 672.1^{cd}	471.1 ± 270.5^{b}	$4651.5 \pm 1383^{a,b,c}$
Central	Murang'a	412.2 ± 276.0^{a}	$1712.4 \pm 496.6^{b,c}$	272.0 ± 90.0^{a}	$4672.2 \pm 181.7^{a,b,c}$
Eastern	South Kitui	$1388.9 \pm 1065.6^{b,c}$	$3161.2 \pm 2339.1^{c,d}$	475.7 ± 98.0^{b}	7494.7 ± 4100.1^{c}
Western	Kakamega	$1153.3 \pm 488.1^{b,c}$	$853.0 \pm 506.3^{a,b}$	347.7 ± 61.5^{b}	4196.4 ± 426.6^{a}
Nairobi	*icipe*	$1717.8 \pm 48.4^{b,c}$	3711.8 ± 429.7^{d}	202.8 ± 59.5^{a}	$4453.1 \pm 98.4^{a,b}$
	Karura	$1112.2 \pm 143.6^{b,c}$	$2218.5 \pm 380.1^{c,d}$	301.4 ± 57.3^{a}	4435.7 ± 131.1^{a}
Coast	Gede	$456.7 \pm 124.9^{a,b}$	$1225.4 \pm 190.4^{a,b,c}$	407.3 ± 134.6^{b}	7698.7 ± 835.1^{d}
	lower Taita	$825.2 \pm 361.1^{a,b,c}$	$2813.8 \pm 323.3^{c,d}$	353.1 ± 22.9^{b}	8767.5 ± 257.4^{d}
	Mtwapa	$1391.1 \pm 658.1^{b,c}$	$1726.1 \pm 1283.9^{a,b,c}$	319.4 ± 104.5^{a}	$7614.7 \pm 2807.2^{b,c}$
	Taita Hills	102.2 ± 68.3^{a}	522.6 ± 169.3^{a}	258.5 ± 21.40^{a}	3014 ± 2692.7^{a}
	p-value	0.0002	0.00006	0.0005	0.002

of propolis, flavonoid content ranged from 102.2 and 3324.4 mg QE/100 g, alkaloid content from 2013.9 to 8767.5 mg CE/100 g and terpenoid content from 202.8 to 582.1 mg LE/100 g.

A pairwise comparison by Dunn's test showed how samples differed from each other. The locations had different climatic conditions (table 1), and those with hot and dry climatic conditions exhibited high total phytochemical content compared with the other locations. The terpenoid content indicates the clear difference from the locations, categorizing them only into two groups.

3.2. Antioxidant activity

The samples had an antioxidant activity based on their ability to scavenge DPPH free radicals leading to a colour change from purple to colourless. There is a difference in antioxidant activity of propolis ranging from 15 to 74% RSA for 250 mg l^{-1} of propolis extract (figure 1). The quercetin equivalent from the standard curve indicates the average inhibition at 0.25 µg ml^{-1} (250 mg l^{-1}) to be equivalent to 23.0 µg ml^{-1} of quercetin (electronic supplementary material, table S2). The samples with lower equivalence are a result of the very low concentration of our samples, which means at the same concentration as the standard they will have a stronger inhibition. There is a variation in regions, with *icipe*, Marigat and Murang'a having all samples with high antioxidant activity, while Kitui and Mtwapa had samples with considerable variations, with some having the highest levels compared with others. Karura and Kakamega had samples with almost the same antioxidant activities, with the same pattern exhibited by Mtwapa and Gede. Taita had the lowest antioxidant activity.

3.3. Antimicrobial activity

All the tested propolis samples exhibited broad-spectrum antibacterial activity. They inhibited the growth of *E. coli* and *B. thuringiensis*, which are Gram-negative and Gram-positive bacteria, respectively (figure 2). Compared with the positive control, *E. coli* had an average inhibition of 32.5% and *B. thuringiensis* 39.4% (electronic supplementary material, table S4). Propolis has elevated activity against *B. thuringiensis* compared with *E. coli*. Propolis from Gede exhibited the highest inhibition against Gram-positive bacteria, while Kitui, Mtwapa, lower Taita and Marigat had the highest against Gram-negative bacteria.

From the PCA plot (figure 3), it can be inferred that propolis' phytochemicals are separated according to locations. Major separation is on Dimension (Dim) 1, which separates all the regions into two, with Kitui, Gede, *icipe*, Mtwapa, lower Taita and Marigat on the negative side while Karura, Murang'a, Kakamega and Taita Hills are on the positive side. The regions on the negative side experience hot climates while the others have cold climates. Dim 2 separates the components based on phytochemical content, with flavonoids and terpenoids being on the positive side while alkaloids and phenols are on the negative side.

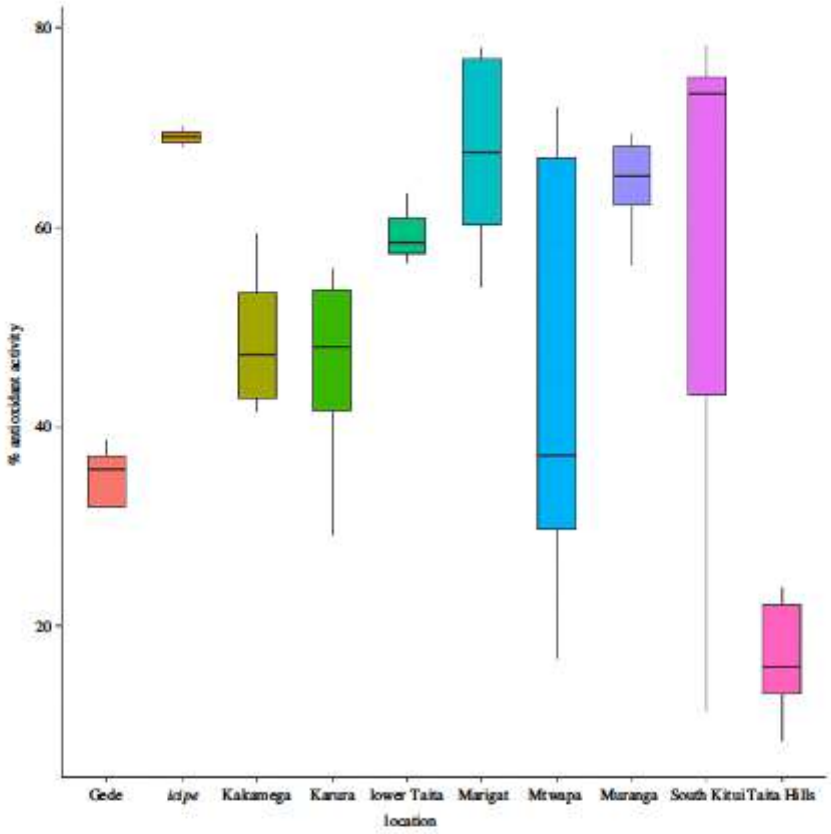

Figure 1. Antioxidant activity of Kenyan propolis samples according to their respective sampling locations.

From the Spearman rank correlation plot (figure 4), we inferred a significant correlation between phytochemicals and the biological activities of propolis with phenols and antioxidant activity ($r = 0.78$), and alkaloids and antibacterial activity against *E. coli* ($r = 0.61$) showing strong correlations.

3.4. Gas chromatography–mass spectrometry analysis

Analysis of GC-MS chromatograms of the selected propolis samples showed more than 100 peaks indicating the presence of various compounds. These compounds belong to different classes: hydrocarbons, terpenoids, diterpenoids, triterpenoids and phenols (table 3). Terpenoids are the most abundant substances as they are volatile organic compounds and hence best to detect in GC-MS analysis. Hydrocarbons also are volatile but most in propolis are long chained; hence they were also detected in moderate abundance. The flavonoid and phenol classes of compounds were the least abundant. There was a clear difference in the compounds present in all the locations, with those from hot and dry climates having more compounds than the other regions. The climatic conditions contribute to the diverse chemical composition, with only two compounds being present in all the samples, octadecahydro-2H-picen-3-one and eicosane, but with varying abundance. All the compounds reported have biological activities except nonadecane, a hydrocarbon. These compounds result in propolis's antioxidant and antibacterial activities (figure 5) because their separation and indication of specific compounds have a more pronounced effect on either antioxidant activity or antibacterial activity. The antibacterial activity is a result of compounds that exhibit broad-spectrum antibacterial activity.

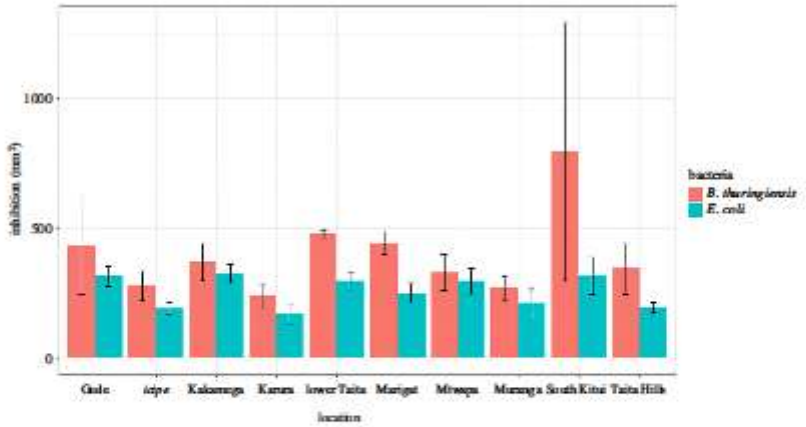

Figure 2. Antibacterial activity of Kenyan propolis samples for the two tested bacteria, *E. coli* and *B. thuringiensis*.

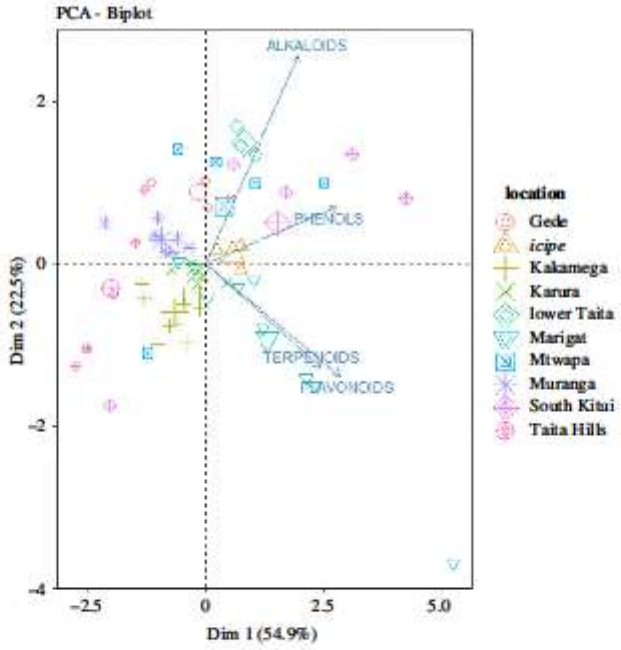

Figure 3. Principal components analysis of Kenyan propolis samples based on their phytochemical content showing clear separation among the sampling locations. Total variance explained: 77.4%.

4. Discussion

4.1. Quantification of phytochemicals

Varying amounts of phytochemicals illustrate the complex chemical composition of propolis. The concentration of phytochemicals is dependent on the geographical origin and its associated

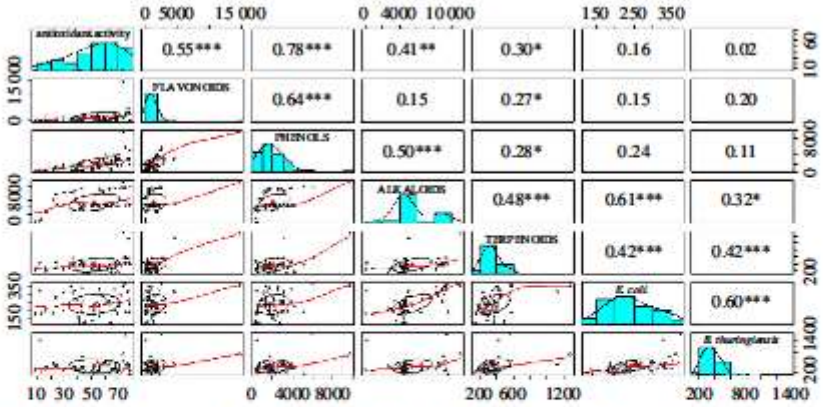

Figure 4. Relationship of phytochemical composition, biological activity, and climatic and landscape variables with correlation coefficients, with corresponding significance levels (no star = not statistically significant, *0.05, **0.01, ***0.001).

vegetation, which is influenced by the local climatic conditions [30]. The extracting solvent also affects the concentration of the compounds in different extracts, but water–ethanol as a solvent is preferred [16]. The total phenol and flavonoid content agree with the range recorded by Sun *et al.* [31] of 6.7 to 164.2 mg GAE g^{-1} and 4.1 to 282.8 mg RE g^{-1}, which also agrees with quantities recorded by Danert *et al.* [32].

Alkaloids are primarily found in plants and are biologically active compounds with at least one N atom [33]. They are generally bicyclic, tricyclic and tetracyclic derivatives of quinolizidine and the major chemicals in commonly ingested foods and beverages such as coffee, tea leaves and cocoa [34]. Of importance is that most of the plant-derived pharmaceutical products against malaria and cancer are alkaloids [35]. The quantification of total alkaloid and terpenoid content in propolis has not been extensively reported, and our results are the first report. Studies have reported these compounds through GC-MS analysis [36]. Terpenoids are the largest class of naturally occurring products with classification based on isoprene units with a regular arrangement of these units; some are irregular [37]. Hemiterpenoids are found in the leaves of many plants, with monoterpenes found as complexes in plant essential oils. Terpenoids are responsible for the characteristic aroma of propolis.

4.2. Antioxidant activity

The DPPH is a stable free radical widely used to evaluate the antioxidant activity of extracts and pure substances. The effect of antioxidants on the DPPH is due to its two mechanisms of action, single electron transfer and hydrogen ion transfer [38]. Propolis displays antioxidant activity due to phytochemicals, which are secondary plant metabolites and are known to act as natural antioxidants. The antioxidant activities of terpenoids, phenols and flavonoids are well documented [39]. The concentration of 250 mg l^{-1} in our study promoted varying antioxidant activity, with the highest being 74%. This is lower than the reported 92.4% [40] for the same propolis concentration using only absolute ethanol as a solvent than our 50% ethanol solvent. The results were close to those posted by Bonamigo *et al.* [41]. The difference is attributed to the solvent used and the geographical location of the propolis collection. Phytochemicals contribute substantially to the antioxidant activity indicated by positive correlations.

4.3. Antimicrobial activity

Propolis demonstrated broad-spectrum antibacterial activity against *E. coli* and *B. thuringiensis*, which are Gram-negative and Gram-positive bacteria, respectively. This is in accordance with other studies that showed a similar activity of propolis [39]. Phytochemicals present in propolis are responsible for its antibacterial activity. The GC-MS analysis identified compounds that are known to exhibit antibacterial activity, such as amyrin [40], ferruginol [34] and totarol [28]. The variation in

Table 3. Compounds present in propolis identified by GC-MS analysis. Values show the relative abundance (* 10^6). Only compounds with similarity score greater than or equal to 90% were considered.

			hot and dry		hot and wet		cold and wet		
compound	class	retention time (min)	South Kitui	Marigat	Gede	Kakamega	Murang'a	Taita Hills	biological activity
octadecahydro-2H-picen-3-one	triterpenoid	40.1	216.0	211.0	81.0	57.0	3.0	19.0	antioxidant
	triterpenoid	31.1	—	—	10.0	—	—	—	antioxidant
sempervirol	diterpenoid	26.8	—	—	9.0	10.0	—	—	antioxidant
lup-20(29)-en-3-ol	triterpenoid	43.1	—	29.0	—	—	—	—	antioxidant
rosadiene	diterpenoid	26.9	—	10.0	—	—	—	—	antibacterial
cubitene	diterpenoid	23.6	—	5.0	—	—	—	—	antibacterial
lupenone	triterpenoid	31.7	—	40.0	52.0	107.0	89.0	209.0	antibacterial
alpha-amyrin	triterpenoid	40.7	23	—	—	—	—	—	antibacterial
beta-amyrin	triterpenoid	39.6	13.0	89.0	197.0	40.0	4.0	—	antibacterial
oleanen-3-yl acetate	triterpenoid	43.2	475.0	—	—	—	—	82.0	antibacterial
ursenoic acid	triterpenoid	43.2	4.0	—	—	—	—	—	antibacterial
lanosta-8,24-dien-3-one	triterpenoid	38.5	—	16.0	24.0	—	—	—	antioxidant
eicosane	hydrocarbon	28.4	8.0	10.1	20.0	40.0	7.0	19.0	antibacterial
heneicosane	hydrocarbon	34.2	24.0	62.0	23.0	—	—	—	antibacterial
tricosane	hydrocarbon	26.7	11.0	—	—	—	10.0	—	antibacterial
nonadecane	hydrocarbon	31.7	4.0	—	—	—	—	—	no reported bioactivity
sugiol	diterpenoid	29.6	—	—	25.0	7.0	—	—	antimicrobial and antioxidant

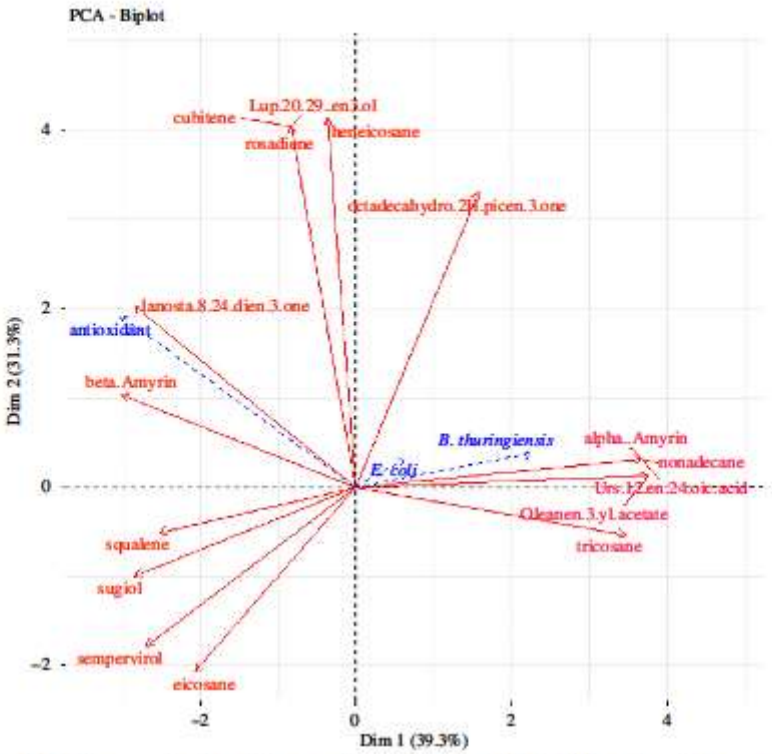

Figure 5. Principal components analysis of Kenyan propolis samples based on their bioactivities and non-volatile compounds identified by GC-MS. Total variance explained: 70.6%.

antibacterial activity is due to the presence of these compounds and their concentration in the samples. These results make propolis a potential source of antibacterial compounds for further analysis.

4.4. Gas chromatography–mass spectrometry analysis

In this study, the propolis samples showed compounds of variable quantity as reported by others [34]. There was a difference in chemical composition from different climatic conditions because the composition of propolis is directly affected by vegetation [11]. The presence of compounds such as squalene, picenone, sempervirol, amyrin, sugiol and long-chain hydrocarbons such as eicosane have been reported to be components of propolis [42,43]. There are three sources of organic compounds of propolis: compounds derived from the honeybees' metabolism, plant origin and structural materials used to form propolis [42]. These compounds have bioactivities that are attributed to the bioactivities of propolis. The bioactivities for these compounds include amyrin as antibacterial, squalene as an antioxidant, sugiol as antibacterial and antioxidant, and lupenone as an antioxidant [33,44–47].

5. Conclusion

The outcome of this study suggests that *Apis mellifera* propolis from Kenya has varying amounts of phytochemicals. This is dependent on the geographical and climatic conditions of different sampling locations. Propolis consisting of plant exudates, buds and resins implies that the vegetation type directly influences its chemical composition. Apart from widely studied phenols and flavonoids, alkaloids were the most abundant phytochemicals with noticeable effects on the bioactivities of

propolis samples. Terpenoids were also abundant in the propolis, and they influenced the antibacterial activities of propolis.

GC-MS analysis can be used to screen bioactive compounds in propolis as it results in different compounds in the propolis samples, and propolis is a rich source of biologically active compounds. The bioactivities of propolis are ascribed to the presence of reported compounds, sugiol, lupenone and squalene, which are phytochemicals identified through GC-MS analysis.

With the exhibited activity of propolis on DPPH free radicals, it is a natural source of antioxidants with high activities in small quantities compared with honey, and this should not be overlooked. Therefore, propolis and its utilization should be encouraged by adopting innovative strategies for its production and harvest.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material [48].

Authors' contributions. T.M.K.: conceptualization, data curation, formal analysis, methodology, writing—original draft; J.L.B.: conceptualization, supervision, writing—review and editing; H.O.M.: data curation, formal analysis, methodology, writing—review and editing; H.M.G.L.: conceptualization, funding acquisition, project administration, resources, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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