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Floral Origin Influences the Antibacterial Components and Activities of Commonly Produced Honey in Ethiopia

Ofijan Tesfaye[1*]

[1]Oromia Agricultural Research Institute, Addis Ababa, Ethiopia. E-mail: apistesfaye@gmail.com

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Abstract

Honey has been used as a traditional medicine while its antibacterial components and activities primarily rely on geographical and blooming origin. The study aimed to investigate the antibacterial composition and activities among the honey collected from different areas and blooming origin. The blooming origin was identified using melissopalynology. The antibacterial components and zone of inhibition were determined by the Harmonized method of the International Honey Commission and the agar well diffusion method, respectively. Six different blooming-based honey, namely: *Guizotia scabra* (75.3%), *Coffea arabica* (68.6%), *Vernonia amygdalina* (90.5%), *Schefflera abyssinica* (100%), *Croton macrostachyus* (64.4%), and *Eucalyptus globules* honey (100%) were harvested in December, February, February, April, May and June 2022/2023 respectively. The result depicted the mean content of pH (4.27), Moisture (20.12 %), water activity (0.53), Phenol (49.23 mgGAE (milligram gallic acid equivalent) 100g of honey and Flavonoid (40.09 mgQE (milligram of quercetin equivalent) 100g of honey). The *S. abyssinica* honey had the highest pH (5.54 ± 0.19), moisture (22.46 ± 1.00), water activity (0.61 ± 0.01), lowest phenol (24.10 ± 0.43), and flavonoid (18.60 ± 2.67) content. However, *C. macrostachyus* honey contained more acidic (3.72 ± 0.03), low moisture content (18.06 ± 0.80) and water activity (0.47 ± 0.01). The *V. amygdalina* honey had the highest phenol (77.20 ± 0.74) and flavonoid (65.02 ± 3.76). The large median zone of inhibition ranged from 17.01 mm for *C. macrostachyus* honey to 10.51 mm for *S. abyssinica* honey against all bacterial strains. This finding showed an excellent content of the tested antibacterial composition and strong antibacterial activities of honey samples, varying with blooming source and collection area.

Keywords: *Antibacterial activities, Coffea arabica honey, Croton macrostachyus honey, Eucalyptus globules honey, Guizotia scabra honey, Schefflera abyssinica honey*

1. Introduction

Infectious disease is one of the major public health problems leading to morbidity and mortality (WHO, 2002) and worldwide, more than 50 million people died each year in society and healthcare settings (Andersson and Hughes, 2011). In Ethiopia, WHO line reported that lower respiratory tract infections caused by human pathogens are one of the top three reasons for disability-adjusted life years (Laxminarayan *et al.*, 2013). To solve this problem drugs have been introduced in the area of pharmacology as they intensely reduced human illness and mortality. Besides, WHO (Moges *et al.*, 2014) estimated, 70-80% of the world's population rests on traditional medicines as the main health care, alternative, and balancing medicines However, through time, pathogenic microbes evolve multidrug and extended resistance

* *Corresponding author: Ofijan Tesfaye, Oromia Agricultural Research Institute, Addis Ababa, Ethiopia. E-mail: apistesfaye@gmail.com*

mechanisms against commonly available drugs owing to indiscriminate use of it, and on the other hand, consuming a drug has a side impact on human health (Moges *et al.*, 2014). Furthermore, the new generations of drugs are less available and are expensive for resource-poor societies (Habtamu *et al.*, 2010). Thus, the resistance or slow killing of antibiotics calls for an urgent search for more effective sources of natural products to tackle problems owing to drugs' failure to treat infectious diseases.

Honey is a natural sweetener and viscous food synthesized from the nectar of flowering plants through regurgitation, enzymatic activity, and water evaporation by bees (Alvarez-Suarez *et al*., 2009). Honey has been used as an antimicrobial agent to treat ulcers, bedsores, skin infections, wounds, and respiratory tract infections (Abdullah *et al.,* 2012) due to the peroxide and non-peroxide factors present in the product. The peroxide (hydrogen peroxide) produced by the oxidation of glucose by glucose oxidase plays an important role in the antimicrobial activity of honey (Vukovic *et al*., 2011). As hydrogen peroxide breaks down, it creates highly reactive free radicals that react and kill the bacteria. The non-peroxide factors have a high osmotic effect; low water activity, acidic environment; low pH, phenolic acids, flavonoids, low protein, high carbon to nitrogen ratio and low redox potential; high content of reducing sugars (Ramos *et al.*, 2018; Molan, 1992). Its properties mainly depend on botanical, entomological, and geographical origins, storage conditions, environmental factors, and processing methods (Silva *et al.*, 2013). The differences in the level of bactericidal and bacteriostatic activity between different types of honey are directly related to the botanical and geographic roots of the floral sources, leading to variations in peroxide and non-peroxide factors (Saranraj *et al.*, 2016). Ethiopia has great potential for developing both quantity and quality of honey as it is endowed with a great diversity of bee flora, over 8000 bee flora and a high bee population density; over 10 million bee colonies (Adgaba, 2007). This makes the country the leading honey producer in Africa and 10th worldwide (Estevinho *et al.*, 2014).

Sources of natural honey in the country vary from region to region depending on agro ecology, soil types, bee feed, and bee species. However, there is insufficient data identifying antibacterial potency based on the type of nectar used to make honey. The current investigation aimed to screen the antibacterial potential between floral sources and geographical origin from different parts of Ethiopia.

2. Materials and Methods

2.1. Study Site and Collection of Honey Samples

The study was conducted in different parts of Oromia region of Ethiopia. Honey samples were collected based on monofloral honey availability (plant abundance and dominance). For this purpose, the Kellem Wollega zone; Dale Sedi district (*Guizotia scabra, Coffea arabica,* and *Croton macrostachyus* honey), West Shoa Zone; Caliya district (*Vernonia amygdalina* honey), West shoa zone; Walmara district (*Eucalyptus globules* honey) and Guji Zone; Bore district (*Schefflera abyssinica* honey were collected from an apiary site and assigned as a source of honey type. 10 kg of honey samples for each honey type from 10 different farm beekeepers (about 1 kg per farmer; sample volume), a total of 60 kg (sample size) of all honey types were collected. The collected honey samples were taken to the Oromia Agricultural Research Institute for antibacterial susceptibility test, Holota Bee Research Center for melissopalynological and antibacterial component analysis, Ethiopia in 2022 to 2023. During harvest, in addition to face-to-face communication with experienced beekeepers in each study area, visual observation of the apiary environment was conducted to identify the flowering source of honey samples and bee forage growing in the area.

2.2. Determination of Botanical Origin

Pollen analysis of honey was carried out using harmonized method of melissopalynology (Louveaux *et al*., 1978; Von Der Ohe *et al*., 2004). For this, ten grams of honey was dissolved in 20 mL of warm distilled water in a centrifuge tube at temperatures that ranged from 20-40°C. The solution was centrifuged at 3800 rpm for 10 minutes and the supernatant was decanted. The distilled water of 20 mL again was added to completely dissolve the remaining sugar crystals and centrifuged at 3800 rpm for 5 minutes and the supernatant was removed completely. The sediment was spread evenly using a sterile micro spatula on a microscope slide and the sample was dried for a while. Thereafter, one drop of glycerin jelly was added to the coverslip, and the pollen grains morphology from honey were identified using a pollen atlas prepared from the local flora of Ethiopia (Adgaba, 2007). In addition to the pollen analysis harvest time, and geographic regions were considered to differentiate the honey types. In addition to face-to-face communication with highly experienced beekeepers in each study area, visual observation of the apiary environment was conducted during harvest to identify the flower source of honey samples and bee forages growing in the area. The percentage of pollen types in each honey sample was calculated based on the total number of different types of pollen grains counted in each sample. Then pollen was categorized as predominant pollen (monofloral honey), if the relative frequency of the pollen of that *taxon* exceeds

45%, secondary pollen (16–45%), important minor pollen (3–15 %), and minor pollen (<3 %) according to standard reference by Louveaux et al., 1978. The pollen count was done under a light microscope (Swift instrument international, serial number 8750038, Japan, high power 400x) linked to a computer. Pollen grain analysis of the honey samples was done at the Laboratory of Holota Apiculture Research Center and replicated three times.

2.3. Antibacterial Components

2.3.1. Moisture Content

The moisture content of honey samples was determined using an Abbée refractometer (ABBE- 5 Bellingham Stanley.Ltd, United Kingdom) that can be thermo-stated at 20ºC and regularly calibrated with distilled water. Honey samples were homogenized and placed in a water bath until all the sugar crystals were dissolved. After homogenization, the surface of the prism of the refractometer was covered with honey and after 2 minutes refractive index for moisture was determined. The value of the refractive index of the honey sample was determined using standard table designed for this purpose (Bogdanov, 2009).

2.3.2. Water activity

The water activity of honey was measured at 25 ± 0.2°C using Lab Master-aw (CH8853 Lachen, Novasina, Switzerland) (Gleiter *et al.*, 2006). The duration of the measurement is set at 5 minutes. To shorten measurement time, honey samples in plastic sample racks were first equilibrated at 25°C by placing them in an incubator (BOD Incubator HN-BI025, Hankuk Scientific Technology, co. Kyonggi, S. Korea).

2.3.3. pH and Free Acidity

From each honey sample, ten gram of honey was dissolved in 75 mL of distilled water in 250 mL beaker and stirred using magnetic stirrer. The electrode of pH meter (METTLER TOLEDO, CHINA) was immersed in thesolution and the pH of honey was recorded. For measurement of free acidity, the solution was further titrated with 0.1M sodium hydroxide (NaOH) solution to pH 8.30. For precision, the reading to the nearest 0.2 mL was recorded using a 10 mL burette. Free acidity is expressed as a Millimole of acid/kg honey and is equal to mL of 0.1M NaOH x 10. The result is expressed to one decimal place following the procedure of Bogdanov (2009). Acidity=10 V, Where: V = the volume of 0.1N NaOH in 10 g of honey.

2.3.4. Phenolic Contents

To analyze and compare the total phenol content between honey samples, Folin-Ciocalteu method was used (Chua *et al.,* 2013). Honey stock solution was prepared by mixing 5 g of honey sample in 50 mL of distilled water and filtered through Whatman no.1 filter paper. From this stock solution, 0.5 mL aliquot was mixed with 2.5 mL of 0.2N Folin-Ciocalteu reagent and incubated for 5 min. A 2 ml of 75 g/L sodium carbonate solution was added into the solution. Finally, after the solution was incubated for 2 h at 25°C, the absorbance of the reaction mixture was measured at 765 nm using UV (PerkinElmer Lambda 950 UV /VIS/NIR Spectrophotometer). Gallic acid (0-200 mg/L) was used as a standard chemical to produce calibration curve and finally, the total phenol content was expressed as milligram of gallic acid equivalent (mgGE) in 100 grams of honey from the mean value of triplicate data derived from a standard calibration curve.

2.3.5. Flavonoid Contents

The total flavonoid content of each botanical sources of honey samples were determined (Chua *et al.*, 2013). The stock solution was prepared by diluting five grams of honey sample in fifty milliliters of distilled water and filtered through Whatman no.1 paper. Five milliliters from honey stock solution was pipetted and mixed in five milliliters of 2% aluminum chloride ($AlCl_3$) solution. After incubation for 10 minutes, the absorbance of the reaction mixture was measured at 415 nm by using Spectrophotometer (Perkin Elmer Lambda 950 UV/VIS/NIR Spectro photometer). Quercetin (0-200 mg/L) was used as a standard chemical to produce calibration curve and finally, the total flavonoid content was reported as mean value of triplicate assays and expressed as milligram of Quercetin equivalent (mgQE) per100 grams of honey from the mean value of triplicate data derived from calibration curve.

2.4. Antibacterial Assay

2.4.1. Test Bacteria and Inoculum Preparation

The human bacterial strains namely *Eschericia coli (ATCC 25922), Staphylococcus aureus* (*ATCC 25923*), *Pseudomonas aeruginosa* (*ATCC 27853*), *Klebsiella pneumonia* (ATCC 43816), *Acinetobacter baumannii* (ATCC 17978), and

Enterobacter cloacae (*ATCC 13047*) were obtained from Ethiopian Public Health Institute. Then bacterial verification, incubation and preservation was done by standard bacteriological methods (Andrews, 2006).

Standard 0.5 McFarland was prepared according to the method (Moussa *et al.*, 2012). Then the standard was dispensed into a sealed test tube of the same size and volume as those used to prepare the test inoculum. Inoculums were then prepared by picking 2-3 colonies from a 24-hours-old culture grown on their selective media and suspended in 5 mL saline (0.85% NaCl). The suspended inoculums were vortexed for 15 seconds and their turbidity was adjusted by adding colony or saline solution into the microbial stock and compared visually with standardized 0.5 McFarland (10^8 CFU/mL) against a white paper background with contrasting black lines.

2.4.2. Inhibition Zone Susceptibility Test

Agar well diffusion assays were used to detect an observable zone of inhibition (Boateng and Diunase, 2015). For this purpose, Muller-Hinton agar plates were evenly seeded with the bacterial pathogen using a sterile cotton swab from an already adjusted inoculated saline solution. Wells 6 mm in diameter and 4 mm depth were then made in the inoculated agar medium using a sterile cork borer. Using a micropipette, 60 mL of the 75% concentration honey sample (3 g honey in 1 mL sterile distilled water) of each sample was added to the wells in the plates. Positive controls (chloramphenicol (30 g/60 mL) and sterile distilled water as negative control were filled in equal parts into the wells. The plates were incubated at 37°C for 24 h. The diameters of inhibition zones including the wells were measured with calipers. All Assays were repeated three times.

3. Data Analysis

The computer package SAS version 9.1.3 was used to calculate the mean and standard deviation. Determination of the significant differences between each honey type was performed using one-way ANOVA at 5% probability level. The antibacterial component parameters and inhibition zone of the honey types tested were used to separate the mean values. Multiple pair wise comparisons between means were performed using the least significant difference (LSD).

4. Results and Discussion

The present study identified the most commonly used monofloral honey based on the honey harvest calendar and geographic origin from Ethiopia. Pollen grains were counted and categorized based on abundance or pollen grain frequency in percentage (Table 1). All honey had a predominant pollen source as their pollen grain count was >45%. Each of the six floral based honey types with their pollen grain morphology showed in Figure 1. These were *G. scabra* (75.3% pollen grain frequency) from the Nedjo honey sample, *V. amygdalina* (90.5%) from the Chaliya honey sample, *C. arabica* (68.6 %) *and C. macrostachyus* (64.4 %) from the Dale Sedi honey sample, *S. abyssinica* (100 %) from the Bore honey sample, and *E. globules* (100 %) from Walmara honey sample. No secondary pollen source plant was observed in any of the honey samples, while important minor pollen source (3-15 %) and minor pollen source (<3 %) plants were recorded in each honey type.

Similarly, a study by Bareke and Addi (2019) showed that *S. abyssinica* honey is harvested from April to May and is whitish with an aroma and very pleasant properties, while *V. amygdalina* honey is harvested in February with a dark to a black color and has a very strong flavor and bitter test (Admassu *et al.*, 2014). The *Guizotia* honey is mainly harvested from November to December and its honey is very viscous and yellow. The predominant pollen of a specific plant in honey sample is due to its abundantly occurrence at the study area, and nectar and pollen releasing potential of the plant (Adgaba *et al.*, 2020).

Table 2 summarizes the antibacterial parameters (mean ± standard deviation) among the tested honey types. The pH value ranged from 3.72 ± 0.03 (*C. macrostachyus*) to 5.54 ± 0.19 (*S. abyssinica*), moisture content (%) ranged from 22.46 ± 1.00 (*S. abyssinica*) to 18.06 ± 0.80 (*C. macrostachyus*). The water activity was found between 0.47 ± 0.01 (*C. macrostachyus*) to 0.61 ± 0.01 (*S. abyssinica*) honey. The TPC ranged from 77.20 ± 0.74 (*V. amygdalina*) to 24.10 ± 0.43 mgGAE/100g of *S. abyssinica* honey while total flavonoid content (TFC) found between 65.02 ± 3.76 mgQE/100g (*V. amygdalina*) to 18.60 ± 2.67 mgQE/100g by *S. abyssinica* honey.

The current study revealed the antibacterial composition of honey samples from different blooming sources. *C. macrostachyus, V. amygdalina* and *G. scabra* had statistically similar pH values and more acidic than other honey types. In contrary, *S. abyssinica* honey was significantly different ($p < 0.01$) in pH values and relatively less acidic from the other tested monofloral honey. Even though the pH values of honey limit has not yet been indicated by the

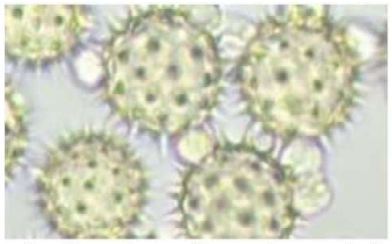
Table 1: The Characteristics of Identified Honey Plants with Their Pollen Frequency Class from Each Honey Type

Zone	District	Altitude	Agroecology	Month	Predominant Pollen Source (>45%)	Secondary Pollen Source (16-45%)	Important Minor Pollen Source (3-15%)	Minor Pollen Source (<3%)
West Wollega	Nedjo	1892	Midland	December	*G.scabra* (100 %) (Monofloral honey type)	-	*Bidens spp, Trifolium* spp and Grass type	*Plantago lanceolate, Sesamum indicum, Allophylus abyssinicus, Lipidium sativum, Brassica carinata* & *Satureja paradoxa*
West Shoa	Chaliya		Highland	February	*V.amygdalina* (90.5 %) (Monofloral honey type)	-	*Guizotia abyssinica*	*Eucalyptus camaldulensis, Brassica carinata, Caroluus ayasanyu, Plantago lanceolate*
Kellem Wollega	Dale Sedi	1461	Lowland	February	*C. arabica* (68.6 %) (Monofloral honey type)	-	*V.amygdalina, Terminalia* & *Guizotia* spp.	*Apodytes dimidiate,* Bidens spp, Eucalyptus spp, *Hypoested trifolia, Galiniera saxifrage, Justicia schimperiana, Bersama abyssinica,Rumex nervosus, Euphorbia ampliphylla* and *Dobiya torida*
Guji	Bore	2800	Highland	April	*S. abyssinica* (100 %) (Monofloral honey type)	-	*Syzium guineense, E.globules&Acacia* spp	*Justicia schimperiana, Rumex nervosus, Coffea arabica,* Guizotia spp & Bidens spp
Kellem Wollega	Dale Sedi	1524	Midland	May	*C. macrostachyus* (64.4 %) (Monofloral honey type)	-		
West Shoa	Walmara	2700	Highland	June	*E.globules* (100 %)(Monofloral honey type)	-		

Regulatory Committees, a pH level from 3.2 to 4.5 has been reviewed by a researchers (Belay *et al.*, 2017; da Silva *et al.*, 2016). The findings of this study showed that all blooming based honey had an acidic characteristics. The acidic property of honey sample is determined by its blooming source (Tura and Admassu, 2018; Belay et al., 2017).

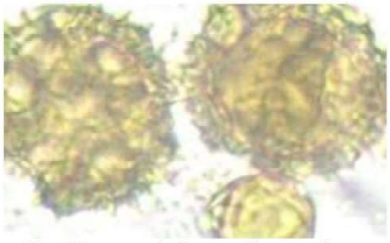
The *S. abyssinica* honey demonstrated a significant difference ($p < 0.01$) in MC and water activity with other honey types. Comparable with the current result, the highest MC (20.54 g/100g) and water activity (0.6) of *S. abyssinica* honey is reported by Belay *et al.* (2017). MC and water activity are largely influenced by geographical location from where the

A1; *G. scabra* honey sample



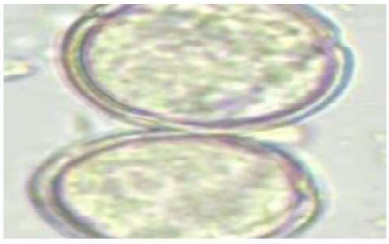
A2; *G. scabra* pollen grain morphology

B1; *V. amygdalina* honey sample



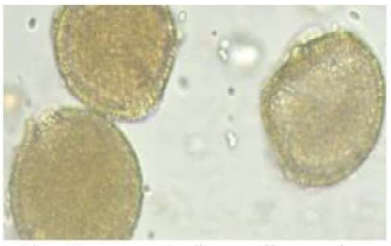
B2; *V. amygdalina* pollen grain morphology

C1; *C. arabica* honey sample



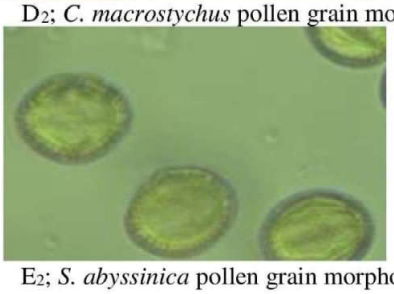
C2; *C. arabica* pollen grain morphology

D1; *C. macrostychus* honey sample



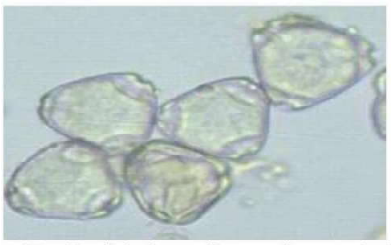
D2; *C. macrostychus* pollen grain morphology

E1; *S. abyssinica* honey sample



E2; *S. abyssinica* pollen grain morphology

F1; *E. globules* honey sample



F2; *E. globules* pollen grain morphology

Figure 1: Honey Sample with its Pollen Grain Morphology of Each Honey Type

Table 2: Antibacterial Components (Mean ± Standard Deviation) Among Honey Types

Honey Type	pH	Moisture Content (%)	Water Activity	Phenolic Content (mgGAE/100g of honey)	Flavonoid Content (mgQE/100g of honey)
C. macrostachyus	3.72 ±0.03d	18.06± 0.80d	0.47± 0.01e	70.32± 1.58b	53.44± 0.51a
V. amygdalina	3.76 ±0.03d	19.20± 1.31cd	0.49± 0.01d	77.20± 0.74a	65.02± 3.76b
G. scabra	4.01± 0.20cd	20.56± 0.73bc	0.54± 0.01c	57.59±0 .28c	31.47± 0.75c
C. arabica	3.97± 0.09c	19.40± 0.80cd	0.50± 0.01d	47.87± 2.23c	39.73± 2.16c
E. globules	4.61± 0.11b	21.06± 0.50ab	0.58± 0.01b	26.21± 0.19d	26.27± 1.13d
S. abyssinica	5.54± 0.19a	22.46± 1.00a	0.61± 0.01a	24.10± 0.43e	18.60± 2.67e
Grand mean	4.275	20.12778	0.532222	49.23	40.09
Least significant difference	0.2353	1.595	0.0168	2.1232	3.823
p-Value	<0.0001	0.0009	<0.0001	<0.0001	<0.0001

Note: Means followed by different letters (a, b, c, d, e) within the row are statistically significant difference at $p \leq 0.05$.

bee plant was found, maturity of honey during harvesting, botanical origin, and harvesting techniques (Adgaba *et al.*, 2020). If the value of water activity is less than 0.61/0.62, it will limit the growth of microorganisms, including osmophilic yeasts. The total phenolic and flavonoid contents of the honey samples recorded in the current study were high and varied among floral based honey types. A Comparable phenolic and flavonoid result were reported for Burkina Fasan honeys (Meda *et al.*, 2005), Cuban honeys (Alvarez-Suarez *et al.*, 2010a) and Ethiopian honey (Adgaba *et al.*, 2020).

The mean diameter of the zone of inhibition (mm) of honey of botanical origin against the human pathogenic strain was shown in Table 3. The activity ranged from 9.3 ± 0.6 mm for the *S. abyssinica* honey against the *P. aeruginosa* (ATCC 27853) strain to 19.3 ± 0.6 mm in *C. macrostachyus* honey against the *S. aureus* (ATCC 25923) strain. The *S. abyssinica* honey was statistically ($p < 0.01$) less effective against all bacterial pathogenic strains tested compared to other monofloral honey. Of all pathogenic strains tested, *S. aureus* was the most susceptible to all monofloral honey types ranging from 12.0 ± 1.0 mm for *S. abyssinica* to 19.3 ± 0.6 mm for *C. macrostachyus* honey. In contrast, *P. aeruginosa* was relatively the least susceptible bacterial strain, ranging from 9.3 ± 0.6 mm in *S. abyssinica* to 14.6 ± 1.5 mm in *C. macrostachyus* honey. The large mean zone of inhibition of the strain tested varied from 17.0 ± 0.9 mm for *C. macrostachyus* to 10.5 ± 0.9 mm for *S. abyssinica*.

The mean overall diameter of the zone of inhibition of this study was larger than that of Ofijan et al. (2022) who reported inhibition zone of 10.80 ± 2.63 mm for *A. mellifera* honey purchased from market to 14.83 ± 2.45 mm from *A. mellifera* honey harvested directly from the hive at 75% w/v honey concentration. A study by Yalemwork Ewnetu *et al.* (2013), however, reported a higher mean diameter of inhibition zone (18.07 to 21 mm) at 50% v/v honey concentration of *A. mellifera* yellow and *A. mellifera* white honey in the same bee species but different plant species and honey concentration. In addition, a higher than present result was obtained from Cameroonian *A. mellifera* honey from the market, which was 29.3 0.6 mm against *E. coli* to 37.0 ± 0.0 mm against *P. aeruginosa* at a concentration of 75% (w/v) using the agar well diffusion method (Boateng and Diunase, 2015). The variation of this finding with results reported elsewhere could be due to the methods and materials used. For example, the turbidity of the inoculum suspension, incubation time and temperature, depth of the poured medium, the medium used for the susceptibility test, the diameter of the well and the measurement of the diameter of the zone of inhibition (Coyle, 2005). In addition, honey qualities and freshness, botanical and entomological origin, geographic sources, and honey's plant biotypes and chemotypes imply disparity in its antimicrobial and biological activities (Alvarez-Suarez *et al.*, 2010b).

The present finding indicated honey types didn't equally inhibit all the tested pathogenic bacterial strains, which could probably be attributed to their bioactive compounds they contained. For instance, *C. macrostachyus* and *V. amygdalina* had more acidic, low moisture content and water activity, and high phenolic and flavonoid compounds.

Table 3: Inhibition Zone of Different Honey Types at 75% (w/v) Honey Concentration Against Human Bacterial Pathogenic Strain by Agar Well Diffusion

Pathogenic Strains	Inhibition Zone of Honey Type (Mean ± SD)						Chloramphenicol	Least Significant Difference	*p*-Value
	C. macrostachyus	*G scabra*	*V. amygdalina*	*C. arabica*	*E. globules*	*S. abyssinica*			
S.aureus (ATCC 25923)	19.3±1^{b}	18±1^{b}	18±1^{b}	17±3^{b}	14±1^{c}	12±1^{d}	26.3±1^{a}	2.4	<.0001
E.coli (ATCC 25922)	15.3±1^{b}	14.3±1^{b}	14±0^{b}	12.3±1^{c}	11±1^{cd}	9.6±1^{d}	22±1a	1.4	<.0001
P.aeruginosa (ATCC 27853)	14.6±2^{b}	13.6±2^{bc}	14±1b^{c}	12.3±1^{c}	12±1^{c}	9.3±1^{d}	23.3±0a	2.1	<.0001
E. cloacae (ATCC 13047	17.6±2^{b}	16.3±1^{b}	16±1^{b}	15.3±2^{b}	11.3±2^{c}	11±1^{c}	24.6±1a	2.4	<.0001
K. pneumonia (ATCC 43816)	16.3±1^{bc}	17±1^{b}	15±1^{bc}	15.4±2^{bc}	11±1^{d}	10±1^{d}	23.6±1a	1.6	<.0001
A.baumannii (ATCC 17978)	18.6±1^{b}	16±1^{c}	18±0^{bc}	17±2^{bc}	13±1^{d}	11±1^{e}	25.3±1a	1.7	<.0001
Grand mean	17.0±1	15.9±1	15.8±1	14.9±2	12.05±1	10.5±1	24.2±1		

Note: Means followed by different letters (a, b, c, d, e) within the row are statistically significant difference at $p \leq 0.05$.

The acidity, high phenolic and flavonoid content, and strong antimicrobial properties of honey from *C. macrostachyus* and *V. amygdalina* plant were investigated (Adgaba *et al.,* 2020). The susceptibility of gram positive bacterium from the present study to all types of honey compared to all gram-negative bacteria was related to the finding of Adgaba *et al.* (2020) and Tesfaye *et al.* (2022). The complex cell wall with an additional outer membrane protects the gram-negative bacteria from external influences by excluding toxic molecules and forming an additional stabilizing layer around the cell (Coyle, 2005).

Pearson correlation among pH, moisture content, water activity, phenol and flavonoid with inhibition zone of Ethiopian blooming based honey types were depicted in Table 4. Significant negative correlations ($p < 0.01$) were observed between pH and inhibition zone (r = -0.939), moisture content and inhibition zone (r = -0.757), and water activity and inhibition zone (r = -0.892). This study indicated that as pH, water activity and moisture content in honey sample increased, the inhibition zone or the diameter of inhibiting bacterial growth decreased and vice versa. On the other hand, statistically positive significant correlations ($p < 0.01$) were observed between phenol and inhibition zone (r = 0. 868), and flavonoid and inhibition zone (r = 0.777) and this indicated that when phenol and flavonoid content increased, the inhibition zone also increased.

The correlation between moisture and water activity, in this study, was in line with the findings of Portuguese honey (r = 0.75) (Gomes, 2010) and Ethiopian honey (r = 0.959) (Belay *et al.*, 2017). A strong and significant positive correlation between phenol and flavonoid (r = 0.771) of this study was in agreement with the study carried out in Algerian honey (Khalil *et al.*, 2012) and Brazilian honey (Salgueiro *et al.*, 2014). The difference in levels of antibacterial properties between the different honeys have been associated to their botanical origin, geographical location, climatic conditions and the plant biochemistry (Belay *et al.*, 2017; Özkök *et al.*, 2010). The hydrogen peroxide, high osmotic effect (low water activity) and acidic environment (low pH) that varies between honey samples plays an important role in the antimicrobial activity of honey (Vukovic *et al.,* 2011; Ramos *et al.*, 2018). Water activity in honey that falls between 0.562 to 0.62, and pH 3.2 to 4.5, is a very marked characteristic of its antibacterial efficacy (Almasaudi, 2021), and at low pH, the bacterial cell membrane become more acidic and resulting cell death (Coyle, 2005). Moreover, the sugar present in honey exerts osmotic pressure on bacterial cells, causing dehydration, and are unable to survive in the hypertonic sugar solution (Isla *et al.*, 2011). Furthermore, the more antimicrobial properties of honeys due to their higher phenolic content and antioxidant properties were investigated (Adgaba *et al.*, 2020) which is comparable with the current finding.

Table 4: Correlation Between pH, Moisture Content, Water Activity, Phenol, and Flavonoid with Inhibition Zone

	pH	Moisture Content	Water Activity	Phenol	Flavonoid	Inhibition Zone
pH	1	0.802(**)	0.893(**)	-0.813(**)	-0.793(**)	-0.939(**)
Moisture content (%)		1	0.879(**)	-0.568(**)	-0.728(**)	-0.757(**)
Water activity			1	-0.741(**)	-0.873(**)	-0.892(**)
Phenol				1	0.771(**)	0.868(**)
Flavonoid					1	0.777(**)
Inhibition zone						1

Note: * Correlation is insignificant at the 0.05 level; ** Correlation is significant at the 0.01 level.

5. Conclusion

Six monofloral honey, namely: *G. scabra, C. arabica* and *C. macrostachyus* from Dale Sedi District were harvested in December, February and April respectively while a honey from *V. amygdalina*, *S. abyssinica* and *E. globules* were harvested in February(from Caliya district), May(from Bore district) and June (from Walmara district) respectively.In this study, the effects of blooming origin of Ethiopian honey on antibacterial components and *in vitro* antibacterial properties were reported for the first time. All types of honey were not the same in antibacterial composition and inhibiting bacterial growth. The *C. macrostachyus* honey demonstrated more acidic, less moisture content and water activity whilst *S. abyssinica* honey contradicted this result. The *V. amygdalina* honey produced more phenol and flavonoid content than others. A strong associations were identified between pH, moisture content, water activity, phenol and flavonoid content with the inhibition zone of honey sample. Comparatively, honey from *C. macrostachyus, V. amygdalina, G. scabra*, and *C. arabica* plants were more effective, while *S. abyssinica* honey was the least effective against all bacteria tested. Gram-positive bacteria were more susceptible to all types of honey than gram-negative bacteria. Therefore, the current finding indicated that, it is the contribution of biological compounds found in honey that accounts for its antibacterial activity. Moreover, blooming of honey and area of honey collected greatly influences the antibacterial components and properties of that honey sample.

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