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Research Article

EVALUATION OF ANTIBACTERIAL POTENTIAL OF HONEY AGAINST SOME COMMON HUMAN PATHOGENS IN NORTH GONDAR ZONE OF ETHIOPIA

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ABSTRACT

The use of honey as a medicine has continued into present-day medicine. It has been shown that natural unheated honey has some broad-spectrum of antibacterial activity when tested against pathogenic bacteria. Different types of honey vary substantially in the potency of their antibacterial activity. The objective of this study is to evaluate the variations in the antibacterial potential of honey derived from North Gondar Zone against bacterial species commonly causing disease in human. Honeys were obtained from four districts of North Gondar Zone such as Chilga, Dembia, Debark and Gondar Zuria. The antibacterial potential of these honeys were assayed against five bacterial species (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and *Klebsiella pneumonia*) using different concentrations (10v/v, 20v/v, 40v/v, 80v/v, and the undiluted honey). The standard disc diffusion methods were entertained. Undiluted and two-fold serial dilutions of honeys were tested to determine MIC using broth tube dilution methods through visual inspection and MBC were determined by sub culturing tubes showing no visible sign of growth/turbidity in MIC. Results showed that the zones of inhibition ranged from 0-21mm and honey from Gondar Zuria showed the highest antibacterial activity. There was a noticeable variation in the antibacterial potential of honeys at different concentrations (6.25-100%). All collections of honeys showed varied bacteriostatic and bactericidal activities and none of the honeys produced any effect on *Klebsiella pneumoniae*.

Keywords: Disk diffusion, Honey, MIC, MBC, Pathogens.

INTRODUCTION

Honey is nectar collected by bees from a wide variety of plants which is concentrated by evaporation of water to form a saturated or supersaturated solution of sugars, consisting typically of 17% water, 38% fructose, 31% glucose, 10% other sugars, and a wide range of micronutrients (White, 1975). Honey has been used in medicine of by ancient communities, including the ancient Egyptians. The ancient Chinese and Sumerians were the first to provide written prescriptions relating to the medical use of honey, found as clay tablets, dating back to 2000 B.C (Molan, 2006). The major components of honey are sugars, in which they possess antibacterial activity due to their osmotic effect (Hayam and Dalia, 2011). The enzyme glucose oxidase introduced to the honey during nectar collection acts on glucose and produces gluconic acid and hydrogen peroxide upon dilution (White and Subers, 1963). Many other substances occur in honey, but the sugars are by far the major components and the principal physical characteristics and behavior of honey are due to its sugars, but the minor constituents – such as flavoring materials, pigments, acids, and minerals are largely responsible for the differences among individual honey types (Molan, 1992a).

Laboratory studies and clinical trials have shown that honey is an effective broad-spectrum antibacterial agent and it has marvelous inhibitory effect on several bacteria including, aerobes and anaerobes, gram-positive and gram-

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negative (Cooper *et al.,* 1999; Allen *et al.,* 2000; Kingsley, 2001, Cooper *et al.,* 2002; Al-Waili *et al.,* 2005). Research has been conducted on manuka (*L. scoparium*) honey (Visavadia *et al.,* 2006), which has been demonstrated to be effective against several human pathogens, including *Escherichia coli, Enterobacter aerogenes, Salmonella typhimurium, S. aureus* (Visavadia *et al.,* 2006). The antibacterial potency of honey has been attributed to its strong osmotic effect, naturally low pH (Kwakman and Zaat, 2012), the ability to produced hydrogen peroxide which plays a key role in the antimicrobial activity of honey (Kacaniova *et al.,* 2011) and phytochemical factors, which include its content of tetracycline derivatives, peroxides, amylase, fatty acids, phenols, ascorbic acid, terpenes, benzyl alcohol and benzoic acid (Bogdanov, 1989; Molan, 1992b). Honey produced by honeybees (*Apis mellifera*) is one of the oldest traditional medicines considered to be important in the treatment of various diseases and ailments including gastrointestinal infection, respiratory ailment, wound infections and other diseases and it has been used effectively as a dressing for wound, (including surgical wounds), burns and skin ulcers to reduce pain and odor quickly (Andargarchew *et al*., 2004). Honey is produced from many sources, and its antimicrobial activity varies greatly with origin and method of processing (Molan, 1992a). Although it is recognized that honey has antibacterial activity, it is generally realized that there is a very large variation in the antibacterial potency of different types of honeys which this hampered its acceptance in modern medicine (Kwakman, 2008).

According to EHBPEA (2011) report, Ethiopia is the leading producer of honey and beeswax in Africa. Globally, the country stands fourth in beeswax and tenth in honey production. This is due to ecology and presence of diverse honey plants in different flowering seasons. In turn, this contributes in the production of fresh honey throughout the year. There are both mono-floral and multi-floral honey products in the country that could be supplied according to the buyers need. There are research works regarding the antimicrobial potential of honey worldwide including Ethiopia, but research papers explaining both the antimicrobial potential and the variations in the antimicrobial potential of different honeys are limited. This study, therefore, not only highlights the antibacterial potential of the honeys but also gives information about the variations in the antibacterial potential of different honeys from different districts in North Gondar Zone. The aim of this study is to evaluate the variations in the antibacterial potential of honeys derived from North Gondar Zone of Ethiopia against clinically important bacterial species.

MATERIAL AND METHODS

Study districts

This study was conducted from August 2012 to June, 2013 in Microbiology laboratory of University of Gondar. The study districts are located in North Gondar Zone, which lies between 12-14^{o} N latitude, and 35-39^{o} E longitude in the North Western part of Ethiopia bordering the Sudan. The study was undertaken on some districts of North Gondar Zone; comprising three agro-ecologies (Debark from highland, Gondar Zuria and Dembia from midland and Chilga from lowland). According to IPMS (2005), the altitude range of the study districts were less than 1500 m.a.s.l (lowland), 1500-2000 m.a.s.l (midland) and 2500-3200 m.a.s.l (highland) with a mean temperature of 26.4°C, 17.5°C and 12.5°C respectively. Mean annual rainfall ranges from 850 mm to 1100 mm and has unimodal pattern.

Sample collection and processing

The honey samples were obtained from field aseptically by purchasing from farmers in sterile screwed-cup container and kept in cool and dry place at room temperature overnight before they were finally transported to the laboratory for processing. The samples were first filtered with a sterile mesh to remove debris and checked for purity by streaking on blood agar plates and incubated at 37ºC for 24 hours. Sample that showed no contaminations were stored at refrigeration temperature of about 2-8ºC until used. A 100% (w/v) solutions were prepared by adding 10 ml of sterile distilled water to 10g of well-mixed honey from each types of honey and then each honey samples were further diluted thorough homogenization with sterile distilled water to (10, 20, 40, 50, 80 % and the undiluted

honey (100.0%) referred to as net (Roland *et al.*, 2007).

Bacterial isolates

The clinical isolates of bacterial species most commonly causing disease in human such as gastroenteritis, wound infections and urinary tract infections were obtained from Gondar University hospital. The bacterial species include *S. aureus, E. coli, S. typhi, P. aeruginosa* and *K. pneumoniae.* Pure cultures of each of the isolates were prepared by sub-culturing the isolates on their selective media. Briefly, a colony was picked from the nutrient agar slant containing *S. aureus* obtained from the University and streaked onto Mannitol salt agar and incubated at 37ºC for 24 hours. The organism fermented the Mannitol. Similarly, a colony was picked from nutrient agar slant containing *E. coli*. This was introduced into Mac Conkey agar and incubated at 37ºC for 24 hours. Smooth pink colonies were seen on the plate overnight incubation, this was confirmed using Eosin Methylene Blue (EMB) agar on which the organism gave a blue-black, dark centered colony with green, metallic sheen; an indication of *E. coli*.

Standard morphological techniques were similarly carried out for the other organisms (*S. typhi, P. aeruginosa and K. pneumonia*). Biochemical tests were performed on each isolates to confirm the identity of the organisms according to standard procedures (Cheesbrough, 2000; Barrow and Feltham, 2004).

Preparation of bacterial isolates

Fresh isolates of the morphologically identical pure culture of each test organism was picked with an inoculating wire loop from the selective medium. Specifically, three to five well-isolated colonies of each of the isolates were picked with an inoculating wire loop, suspended in 4-5 ml of nutrient broth and incubated at 37ºC for 24 hours, to reactivate the organisms. The bacteria suspension thus obtained were compared with the 0.5 Mc Farland standard. 0.5 Mc Farland standard was prepared by adding 0.5ml of a 1.175% w/v barium chloride anhydrate ($BaCl_2.2H_20$) solution to 99.5 ml of 1% v/v sulfuric acid (H_2S0_4) with constant stirring to maintain a suspension (CLSI, 2006).

The bacteria suspension was then diluted with sterile distilled water until a turbidity that matched 0.5 Mc Farland Standards (105 – 106 cfu / ml) was reached. This was done by adding more organisms (incubating the organism) when the suspension is too light or diluting with sterile distilled water when it is too heavy. The turbidity of each suspension was compared by holding both the standard and the inoculums tubes side by side in front of a white paper with black lines. The resulting suspensions were further diluted 1:100 in sterile nutrient broth (Oxoid, UK) to set an inoculums density of 1 × 104 cfu / ml used in this study (Woods and Washington, 1995; Miles and Amyes, 1996). Turbidity of overnight-incubated tubes by matching with the growth control was used. From the tubes showing no visible sign of growth/turbidity in MIC determination, were sub-cultured onto fresh sterile nutrient agar (Oxoid, UK) plates by streak plate method. Then the plates were then incubated at 37ºC for 24 h. The least concentration that did not show growth of test organisms was considered as the Minimum Bactericidal Concentration (MBC). Then, each the plates were scored according to the assessment of Payveld (1986). These include; no growth (bactericidal); light to moderate growth (bacteriostatic); heavy/luxuriant growth (no effect).

The processes were repeated until all the isolates were similarly prepared. These dilutions were stored at 2-8ºC until used same day and the purity was checked by culturing some on blood agar and incubate them at 3-5% of CO_2 at 37ºC (CLSI, 2006).

Antibacterial evaluation

The antibacterial evaluations were carried out using the disc diffusion methods (Bauer *et al.*, 1966). Mueller Hinton agar (Oxoid, UK) prepared by suspending 39 gram of the powder in 1 liter of distilled water and brought to boil to dissolve the medium completely, sterilized by autoclaving at 121ºC for 15 minutes and held in water bath (45-50ºC). Mueller Hinton agar plates (with a uniform agar depth of approximately 4 mm) were prepared aseptically, allowed to set and dried and the surface of Mueller Hinton agar plates were uniformly inoculated in individual Petri dishes with the reactivated broth culture of

each of the bacterial species prepared in a nutrient broth medium previously. The bacteria were adjusted with 0.5 Mc Farland standard (105-106 cfu / ml). A sterile cotton swab was dipped into the standardized bacterial suspension rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculums from the swab. The swab was streaked over the entire surface of the Mueller Hinton agar plate with repeated streaking motion to evenly inoculate the Mueller Hinton agar plates. The plates were allowed to dry for 3-5 minutes.

Whatman No 1 filter paper discs (Whatman International Ltd., UK), 6 mm in diameter were used to know the inhibition zone. Five filter paper discs were soaked in the different honey dilutions or net honey, dried at room temperature, carefully and aseptically placed into the petri dishes seeded with the bacteria together with one 5µg Ciprofloxacin disc (a positive control) and one disc without honey impregnation (a negative control). Thereafter, all discs were placed on the plates pressed gently to ensure complete contact with the agar. A distance of at least 15 mm was maintained from the edges of the plates to prevent overlapping of inhibition zones. These processes were repeated for each honey samples and for each of the bacterial isolates. Plates were kept at 4ºC for 2-6 hours to provide sufficient time for the test material to diffuse into the medium and finally incubated at 37ºC for 24 hours. The resulting zone of inhibition produced around the discs were measured, including the diameter of the disc using sliding calipers, which was held on the back of the inverted petri plate, as index of antibacterial potential and recorded for the different concentrations of the honeys and net preparation.

MIC and MBC determination

The minimum inhibitory concentration of three honeys Dembia, Debark and Gondar Zuria were determined by using the broth tube dilution method (Fakruddin, 2006; Barrow and Feltham, 2004). Overnight nutrient broth (Oxoid, UK) cultures of *S. aureus, E. coli, S. typhi, P. aeruginosa,* and *K. pneumoniae* 37ºC were prepared. The cultures were adjusted to obtain a turbidity that matched 0.5 Mc Farland Standards (105 – 106 cfu / ml) was reached by holding both the standard and the inoculums tube side by side in front of a white paper with black lines.

Ten sterile tubes were placed in rack, labeled each 1 through 8 and first one labeled as HC and last one was labeled as GC. One ml of freshly prepared nutrient broth was added to each tube, sterilized and cooled. One ml of undiluted honey solution 100 (w/v) was added to test tube no 1 and HC with a sterile micropipette and tips, two-fold serial dilutions were performed by transferring 1 ml undiluted honey (100%) into the second tubes with separate sterile micropipette and tips and vortexed for homogenization. After a through mixing, 1 ml was transferred with another sterile micropipette from tube no. 2 to tube no. 3. These procedures continued until eighth tubes with a dilution of 1:128 were reached and 1 ml was removed and discarded from tube 8 (using fresh pipette for each dilution).

The last tube (tube GC) received no honey was served as a growth control whereas the first (tube HC) received no inoculums was served as a honey control. Each tube was inoculated (including the growth control except honey control) with 1 ml of the culture of respective organism prepared previously. The entire procedures were repeated for all the organisms to each of the honeys. The tubes were incubated at 37ºC for 24 hours and observed by visual inspections.

RESULTS

In this study honeys from districts of North Gondar Zone were tested for their antibacterial potentials on five clinically isolated bacteria. The zones of inhibition ranged from 0-21mm. Honeys from Gondar Zuria and Debark districts showed inhibitory effect against *S. aureus*. Honeys from Gondar Zuria and Dembia districts inhibited *P. aeruginosa. S. typhi* were inhibited by honeys from Debark and Dembia districts. Honey from Gondar Zuia district inhibited *P. aeruginosa* at 100% and 80%. Only honey from Gondar Zuria district inhibited E. coli while honey from Chilga district showed partial inhibitory activities to all the tested bacterial isolates at any concentrations (Table 1).

None of the honey from the districts showed antibacterial potential on *K. pneumoniae.* Honey

from Gondar Zuria district showed bacteriostatic against *P. aeruginosa* at 6.25%, *E. coli* at 6.25%, *S. aureus* at 12.5% and *S. typhi* at 12.5% concentrations. Honey from Dembia district showed bacteriostati against *S. typhi* at 25% *E. coli* at 25%, *S. aureus* at 12.5% and *P. aeruginosa* at 12.5% concentrations. While honey from Debark district showed bacteriostatic against *S. typhi* at 12.5%, *E. coli* at 12.5%, *P. aeruginosa* at 25% and *S. aureus* at 6.25% concentrations (Table 2).

Honey from Debark and Gondar Zuria districts showed bactericidal effects at 100% concentration against *E. coli* whereas honey from Dembia district showed bactericidal potential against *S. aureaus, S. typhi and P. aeruginosa* at 100% concentration however, it has also bactericidal potential on *E. coli* at 50% after 24 hours of incubation. Even though honey from Gondar Zuria and Debark districts have no bactericidal effect on *S. typhi* but

Gondar Zuria district had bactericidal against *P. aeruginosa* and *S. aureaus* at 50% concentration and Debark district formed similar effect on *S. aureaus* and *P. aeruginosa* at 50 % concentration too. General none of the honeys purchased from farmers in the districts were not effective at concentration below 50% (Table 3).

Table 1. Antibacterial potential of honeys from districts of North Gondar zone against some common human pathogens (in millimeters).

Districts	Honey dilutions	Test organisms			
		S. aureus (in mm)	*E. coli* (in mm)	*S. typhi* (in mm)	*P.aeruginosa* (in mm)
Chilga	Undiluted	16	13	21	21
	80%	15	13	20	NI
	40%	11	10	13	NI
	20%	8	NI	10	NI
	10%	8	NI	NI	NI
	Control(+)	15	17	10	21
	Control(−)	NI	NI	NI	NI
Dembia	Undiluted	17	19	**9+**	**7+**
	80%	15	NI	11	NI
	40%	10	NI	8	NI
	20%	8	NI	NI	NI
	10%	7	NI	NI	NI
	Control(+)	16	20	10	19
	Control(−)	NI	NI	NI	NI
Gondar Zuria	Undiluted	**15+**	**15+**	11	**9+**
	80%	13	16	11	**7+**
	40%	10	11	8	9
	20%	7	7	5	6
	10%	9	NI	NI	NI
	Control(+)	16	18	11	20
	Control(−)	NI	NI	NI	NI
Debark	Undiluted	**13+**	17	**8+**	15
	80%	12	11	10	7
	40%	12	7	10	7
	20%	NI	8	NI	NI
	10%	NI	NI	NI	NI
	Control(+)	15	19	10	19
	Control(−)	NI	NI	NI	NI

Note: NI= No inhibition**; + =** complete zone of inhibition; Bold numbers indicated complete antibacterial potential. Other values indicated partial antibacterial potential.

Table 2. The MIC of honeys on susceptible test organisms on nutrient broth.

Test organisms	Honey dilutions								Districts	MIC
	net	1/2	1/4	1/8	1/16	1/32	1/64	1/128		
S. aureus	_	_	_	_	+	+	+	+	DB	12.5%
E. coli	_	_	_	+	+	+	+	+	DB	25%
S. typhi	_	_	_	+	+	+	+	+	DB	25%
P. aeruginosa	_	_	_	_	+	+	+	+	DB	12.5%
S. aureus	_	_	_	_	_	+	+	+	DK	6.25%
E. coli	_	_	_	_	+	+	+	+	DK	12.5%
S. typhi	_	_	_	_	+	+	+	+	DK	12.5%
P. aeruginosa	_	_	_	+	+	+	+	+	DK	25%
S. aureus	_	_	_	_	+	+	+	+	GZ	12.5%
E. coli	_	_	_	_	_	+	+	+	GZ	6.25%
S. typhi	_	_	_	_	+	+	+	+	GZ	12.5%
P. aeruginosa	_	_	_	_	_	+	+	+	GZ	6.25%

Note: = No visible growth (not turbid); + = visible growth (turbid) and GZ= Gondar Zuria, DB = Dembia; DK = Debark. NB: None of the honey showed antibacterial potential on *K. Pneumoniae.*

Table 3. The MBC of honeys on susceptible test organisms on nutrient agar.

Test organisms	Honey dilutions					Districts	MBC
	net	½	¼	1/8	1/16		
S. aureus	_	+	++	+++	+++	DB	100%
E. coli	-	-	++	+++	+++	DB	50%
S. typhi	_	+	++	+++	+++	DB	100%
P. aeruginosa	_	+	++	+++	+++	DB	100%
S. aureus	_	_	+	+++	+++	DK	50%
E coli	_	+	++	+++	+++	DK	100%
S. typhi	+	++	+++	+++	+++	DK	0%
P. aeruginosa	_	_	++	+++	+++	DK	50%
S. aureus	_	_	++	+++	+++	GZ	50%
E. coli	_	+	++	+++	+++	GZ	100%
S.typhi	+	++	+++	+++	+++	GZ	0%
P. aeruginosa	_	_	++	+++	+++	GZ	50%

Note: - = Absence of growth (bactericidal); + = Light growth (bacteriostatic); ++ = Moderate growth (bacteriostatic); +++ = Heavy growth (No antibacterial potential).

DISCUSSION

All of the honey exhibited a good antibacterial potential against the bacteria isolates on disc diffusion test except *K. pneuomoniae*. They showed complete antibacterial potential with clear zone of inhibition except honey from Chilga district at 100% at least on one organism. Honeys from Debark and Gondar Zuria districts displayed better bacteriostatic and bactericidal properties on four of the clinical isolated organisms except *K. pneumonia* depending on their dilutions. Of all the honey samples tested honey from Gondar Zuria district had the highest antibacterial activity followed by Debark and Dembia. Especially it showed complete inhibitory effect with clear zone of inhibition against tested organism at 100 except on *S. typhi.*

All honeys showed varied antibacterial potential against the test organism. Even though they showed similar bactericidal effect on *S. aureus* and *P. aeruginosa* and they have different effect on *E. coli* and *S. typhi.* For example honey from Dembia showed bactericidal potential against *E. coli at* 50% concentration but honeys from Debark and Gondar Zuria showed bactericidal potential against the same organism at 100% concentration and honey from Dembia is bactericidal potential against *S. typhi* at 100% but honeys from Debark and Gondar Zuria have no effect on the same organism

The honey showed bactericidal activities against the tested organisms up to the dilutions of 50%. This is similar to those reported by Nzeako and Hamdi (2000) who studied on six commercial honeys found that inhibition in agar diffusion of *S. aureus, E. coli* and *P. aeruginosa* did not occur at honey concentrations less than 40% but is at variance with the report of Willix *et al*., 1992. Since honey's antimicrobial property is dependent on its water activity – (the free water molecules in honey, which is usually between 15 and 21%) in part, this will directly affect its osmotic effect. It is then rational to assume that a more diluted honey may have lost its antibacterial ability. This increased dilution factor may also partially explain why honey from Chilga district, does not show full antibacterial activity against any of the organisms tested however, Gondar Zuria district was effective against the tested bacterial organisms.

The bactericidal activity of the honeys on *P. aeruginosa, S. typhi* and *E. coli* was found to be between 50 and 100% concentration for the three honeys Dembia, Debark and Gondar Zuria. The result agrees with the reports of Kingsley (2001) which found honeys to be effective in wound treatment at higher concentrations. The honeys bactericidal effect against *S. aureaus* was at 50% concentration. This organism has been reported to be one of the most sensitive to the effects of honey (Molan, 1992b). Other workers had described the complete inhibition of MRSA at honey concentration of as low as 10% (Willix *et al.,* 1992; Molan and Betts, 2000).

Although Efem (1988) reported that *P. aeruginosa* was resistant to honey, results from this study contradict this assertion since honeys from Gondar Zuria and Debark districts exerted antibacterial potential on *P. aeruginosa* which have been known to be resistant to some common antibiotics such as, penicillin, chloramphenicol, ampicillin etc. Due to a combination of factors including; low permeability of its cell wall, genetic capacity to express resistant mechanisms and mutation in chromosomal genes which regulate resistance genes (Molan and Betts, 2000; Lambert, 2002). The honeys purchased from the districts had no antibacterial potential on *K. Pneumonia*. This is in agreement to those reported by Olawuyi *et al*., (2010) who studied antibacterial activities of honey from different location on Gram positive and Gram negative bacteria but it is in contrast with the report by Molan (1992b), Anyanwu, (2011) and Subrahmanyam (1991) who studied on the nature of antibacterial activity of honey.

In this study the variation in antibacterial potential of North Gondar Zone honey was observed. This variation is mainly due to variations in the content of hydrogen peroxide from each district since this experimental work is performed by diluting each districts honey and in diluted honey hydrogen peroxide is the major

antibacterial factor that brings antibacterial variation among different honeys. This is similar to those reported by Molan (1992b) who studied the variations in the antibacterial activities of different honey varieties. The variation in the antibacterial potentials of honeys used in the present also highlights that the source of the vegetation (nectars and pollens) have contributed to the difference in the antimicrobial activities of honey since flora source determines many of the attributes of honey, for example flavor, aroma, color and composition. The districts assessed in this study have varied crops and vegetation's available for the honeybees in the collection nectar for making honeys, this is in agreement to (Allen *et al.,* 1991a, 1991b) who worked that variation in antibacterial activities of honey can be traced to available nectars and pollens. The variations in sensitivity are also attributed to differences in growth rate of pathogens, nutritional requirements, temperature, inoculums size and the test method itself (Gaill and Washington, 1995).

CONCLUSIONS

In this study attempt was made to assess the antibacterial potential of honeys from some districts within North Gondar Zone as antibacterial therapeutic agents and variation in the antibacterial potential. North Gondar Zone honeys exhibited a good antibacterial activity and shown to prevent the growth of some potential human pathogens and therefore have a broad-spectrum of antibacterial activity when tested in vitro. Specially, honey from Gondar Zuria district has exceptionally high antibacterial potential whereas honey from Chilga district has the least antibacterial potential showing only partial inhibition on all tested organisms; this is due to the variations in hydrogen peroxide, crops and vegetation's in these districts available for honeybees to make honey. In addition, floral source and region together with the hydrogen peroxide and the volatile (phytochemical) components were clearly important in the production and variations in the antibacterial potential of North Gondar Zone honeys, since the level of activity varied among different honeys.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this article.

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