Molecular Biology

Alemu and Ahmed, Mol Biol 2017, 6:1
DOI: 10.4172/2168-9547.1000180

Research Article OMICS International

Antimicrobial Activities and Chemical Characterization of Propolis Collected from Three Different National Areas of Ethiopia

Fisseha Alemu[1]*, Awol Ahmed[2], Daniel Seifu[2] and Solomon Gebre[2]

[1]*Addis Ababa University, College of Natural Science, Institute of Biotechnology, Ethiopia*
[2]*Jig-jiga University, College of Natural Science and Computational Science, Department of Chemistry, Jig-jiga, Ethiopia*

Abstract

Background: Propolis has revealed versatile and valuable biological and pharmacological activities such as antibacterial, antimitotic and antiviral. It has other less obvious effects such as cytotoxic, antioxidant, anti-inflammatory and immunomodulation. The variation in the chemistry of propolis from different locations and plant sources has made it a source of new biologically active molecules, mainly antioxidative and antibacterial agents.

Aim of the study: The purpose of the present study was primarily to establish and assess the variation (if any) in the antimicrobial activities of propolis samples from three different local areas of Ethiopia. The study also incorporates identification and characterization of the major antimicrobial component(s) of propolis that are responsible for the antimicrobial activity.

Materials and methods: Pure cultures of two gram positive (*S.aureus* and methicillin resistant *S.aureus (MRSA)*), three gram negative (*E. coli*, *P. aeruginosa*, *S. typhi*) bacterial species and one fungal species (*C. albicans*) were obtained from the department of Microbiology and Bacteriology Laboratory center, Addis Ababa University. Three propolis samples were collected from South Wollo, Tembain and Holeta regions of Ethiopia during the summer season. 70% ethanolic extract preparation for each propolis sample was made by using maceration procedure. Agar plate disk diffusion using paper disks and Agar well diffusion methods were employed to study antimicrobial activity of ethanolic extract of propolis from Tembain (EEPT) and ethanolic extract of propolis from Holeta. Thin layer chromatography (TLC) phytochemical screening method using different TLC spray reagents (vanillin, aluminum chloride, diazotized sulfanilic acid and *p*-anisaldehyde), TLC agar overlay bioautography method and column chromatography were employed to partially identify and characterize the antimicrobial active components of propolis from Holeta and Tembain regions.

Conclusion: Ethanolic extract of propolis from Holeta and ethanolic extract of propolis from Tembain showed antibacterial activity against *S. aureus* and *MRSA* only without showing effect on other bacterial or the fungal strain. A variation in the chemical composition between EEPH and EEPT was noted. The antimicrobial activities of propolis could not be correlated with any single components of propolis therefore the bactericidal effects of propolis from this region may be as a result of the sum antimicrobial components.

Keywords: Propolis; EEPT; EEPH; Antibacterial; Antifungal; Bioassay

Introduction

There are about 500 plant species in Ethiopia (400 herbs and shrubs and 100 trees) that have been chosen to be important to honey bees [1]. The availability of these diverse and unique plant species in the country is suitable for sustaining a large number of honey bee colonies and a long practice of beekeeping [2]. However, propolis collection is a rare activity of honey bees and often it takes place high up in the trees that it is difficult to be observed. According to Deffar as reported by Bankova et al., plants which are thought to be chosen by bees as sources of propolis could be recognized based principally on observations of bee behaviour and only in a few cases on comparative chemical analysis of propolis and plant materials. On the basis of this fact, there is no documented evidence in Ethiopia for the list of plants which are the favorite choice for bees to make propolis [3,4].

Interest in the correlation of chemical composition of propolis with its pharmacological activities started only 40 years ago. Biological activities were always present in propolis but they were associated with different chemical profile in samples collected from different geographic and climatic zones [5-7]. Several studies concerning chemistry of propolis allowed researchers to realize that its chemical composition is not only complex but also highly variable, depending on the season in which it is collected and local flora as well as on the type of bees prepare propolis [4,6,8].

As stated above the chemical composition of propolis responsible for its biological activities was highly variable depending on the different geographic and vegetation sources. It is therefore important to study the chemical compositions of propolis from different locations both quantitatively and qualitatively and correlate the findings with its various aforesaid mentioned biological activities [8,9]. When scanning the literature, it was noted that the antimicrobial activities of different type of propolis was due to the presence of chemicals such as flavonones, flavones, phenolic acids and their esters in European propolis, while such activities were due to the presence of prenylated p-coumaric acids and diterpenes for Brazilian propolis [8,10,11].

Although several studies on propolis have been done in many countries, there is no documented evidence about the antimicrobial effect of propolis and characterization of its chemical composition in Ethiopia.

In the present study, the propolis samples collected from three different zones of Ethiopia were investigated for its antibacterial and

***Corresponding author:** Fisseha Alemu, Addis Ababa University, College of Natural Science, Institute of Biotechnology, Addis Ababa, Ethiopia, Tel: +251913806336; E-mail: fishalemu1@gmail.com

Received December 12, 2016; **Accepted** December 19, 2016; **Published** December 26, 2016

Citation: Alemu F, Ahmed A, Seifu D, Gebre S (2017) Antimicrobial Activities and Chemical Characterization of Propolis Collected from Three Different National Areas of Ethiopia. Mol Biol 5: 180. doi: 10.4172/2168-9547.1000180

antimycotic activity. Further efforts were made to characterize the bioactive component of the propolis which may be responsible for its antimicrobial activity. So that, this research has been done to determine the *in vitro* antimicrobial effect of Ethiopian raw hive propolis collected from different local and floral origins of South Wollo, Tembain and Holeta, and to identify as well as quantify the antimicrobial bioactive components using analytical methods.

Materials and Methods

Study design

Laboratory based experiment, Qualitative.

Study setting

The study was conducted at Biochemistry and Pharmocognosy Laboratory, School of Medicine, Addis Ababa University.

Sample collection and extraction of propolis

A total of three propolis samples of 300 g each were collected from bee hives of three different local areas of Ethiopia during the summer season. The three different regions were South Wollo, Tembain and Holeta. The different vegetation type and climatic conditions in the three local areas are the basis for selection of the samples for the study.

Ethanolic extract of propolis was prepared according to the method described by Hegazi and Abd El Hady [12]. Hand collected Propolis was kept at freezer (20°C) until its processing. Propolis dissolves well in ethanol and is easily extracted in 70 to 80% (v/v) ethanol [13]. 70% (v/v) aqueous ethanol was prepared by adding 700 mL ethanol to 300 mL of water. Each samples of propolis were cut into small pieces using a blender and grounded in a mortar. 10 g of each powdered propolis samples was added into labeled dark bottles and 100 mL of 70% (v/v) ethanol was poured into and the top of the flask was sealed and then shaken briefly. The mixture was then left at room temperature for one or two weeks with moderate shaking repeated once or twice a day. The alcoholic extract was then filtered with Whatman # 1 filter paper to discard the waxes. Afterwards, a new filtered process was applied with a 0.2 µm cellulose acetate filter in order to sterilize the solution. The resulting reddish or brown solution was evaporated in oven at 50°C until it dried. The dry extract for each sample was then re-dissolved in 70% ethanol in order to obtain solutions containing 10%, 20% and 30% (w/v) propolis extracts (Figure 1).

Bacterial strains

The following pure strains of bacterial species such as (*S. aureus*), (*E. coli*), (*P. aeruginosa*) and (*S. typhi*) were obtained from the department of microbiology, Addis Ababa University. Clinical isolates of methicillin resistance *S. aureus* (MRSA) and (*C. albicans*) were obtained from the bacteriology laboratory center of school of medicine, Addis Ababa University.

Antibacterial assays of propolis

In the present study, standard microbiological method that is Agar plate disk diffusion and agar well diffusion methods were used for determination of antibacterial activity of the Propolis extracts of different concentrations against bacteria and *C. albicans*. All tests were performed in duplicate. 70% ethanolic solution without Propolis was used as a control. Antibiotics such as tetracycline (10 mcg), chloramphenicol (10 mcg), penicillin (10 mcg) and gentamcin (10 mcg) were used against the bacteria as reference antimicrobial agents.

Agar plate disk diffusion tests using paper disks: The antimicrobial susceptibility test using agar plate disk diffusion method was performed according to the method of Bauer, as described by Tukmechi [14]. Agar plate disk diffusion method using paper disks was used to qualitatively compare the antibacterial potency of different ethanolic extracts of Propolis.

Figure 1: Photography of propolis sample used for the study.

Whatman No.1 filter paper was used to prepare paper disks used for the study. The filter paper was cut into circular discs using a perforator giving a diameter of 5 mm. The disc was placed into a glass Petri dish and sterilized at 180°C for 30min hot air oven. Sterilized paper discs were soaked in filter sterilized Propolis extracts solution at concentrations of 10%, 20% and 30%. Discs were dried for 5 h at 37°C in a sterile incubator.

Previously prepared Mueller Hinton agar and sabourauds plates for *C. albicans* were used. The surface of the plates were inoculated using a sterile swab containing the saline suspension of bacteria or *C. albicans* adjusted to tube 0.5 Mcfarland (1 to 5×10^8 CFU) and allowed to dry for 2 to 4 min. Previously prepared sterile paper disks containing 10%, 20% and 30% of each propolis extracts were placed in the plates under aseptic conditions. Paper discs containing the solvent alone were used as a negative control. Each disc was pressed down to ensure complete contact with the agar surface. The discs were placed individually with a dispensing apparatus so that they were distributed evenly no closer than 24 mm from center to center.

The agar plates were maintained at room temperature for 2 h allowing for diffusion of the solution. All plates were then incubated at 37°C for 24 h for bacteria and 48 h for fungi and the zones of inhibition were subsequently measured in millimeters using caliper.

Determination of antimicrobial activity by agar well diffusion method: Agar well diffusion method for determination of antibacterial activity was used according to the method of Lofty [15] as described by Nada [6]. Petri plates containing 25 mL of Mueller-Hinton agar for bacteria and Sabouraud dextrose agar for *C. albicans* were used. Agar media was seeded with a 24 h old culture of the microorganism strains by sterile cotton swab dipped into the saline suspension of these microorganisms adjusted to 0.5 Mcfarland (1 to 5×10^8 CFU) standards. Four wells (5 mm diameter) were cut into the agar by using sterile glass-made pipettes attached to a vacuum pump and 0.1 mL of the crude propolis extracts were applied in each well using micro pipette. New pipette tips were used for the application of the test solution or

control solution per well. Incubation was performed at 37°C for 24 h for bacteria and for 48 h for *C. albicans*. Control well containing the solvent alone was also included. The assessment of Antibacterial and antifungal activity was based on measurement of the diameter of the inhibition zone formed around the well using caliper. The inhibitory zone was considered as the shortest distance (mm) from the outside margin of the sample to the initial point of the microbial growth.

Screening for antibacterial active components of propolis

TLC finger printing: a) Overview: Screening of propolis chemical components using TLC was performed according to the method of Medić-Šarić [16] with modifications. Prepacked analytical thin layer chromatography (silica gel 60 with Aluminum support, 20 cm × 20 cm) was used and the solvent system which gave the highest resolution of the chemical components of propolis was chosen. TLC spray reagent such as aluminium chloride, vanillin, *p*-anisaldehyde and diazotized sulfanilic acid were employed to identify and compare the chemical classes of flavonoids, terpenes and phenolic acids present in propolis samples.

b) Method: TLC analysis of 30% aqueous ethanol extract of Tembain and Holeta propolis were performed on silica gel (Aluminum Merck F254) with mobile phases systems n-Hexane/ethylacetate/glacial acetic acid (6:4:1), Chloroform : Methanol : Formic acid (11:3.5:0.65) and Toluene:ethyl acetate:Formic acid (5:4:1) to determine the maximum number of components. The 3 solvent systems were prepared, each poured in chromatographic chamber after mixing well and allowed to saturate the chamber for about 1 h. 5 µl of 30% EEP solutions of the two samples were applied on a 10 × 20 cm plates 1.5 cm from the bottom and allowed to air dry. The plates were then placed vertically in each TLC chambers primarily saturated with each of the 3 solvent systems with the chambers closed and left until the mobile phase reached 1 cm from the top. The spots were visualized in long and short wave length.

Bio autography and solvent system development: a) Overview: The method of Choudhary [17] and Sarker [18] were used with little modifications for agar overlay bio autographic assay and solvent system development for analysis of antimicrobial active components of propolis. TLC agar overlay antimicrobial assay method was used for *in situ* determination of antimicrobial active components which had been resolved on TLC. The solvent system of Hexane:ethylacetate (6:4) which gave the highest resolution of the chemical components of propolis from Tembain and Holeta was used for the development of the chromatogram.

b) Method: Ethanolic extract of Tembain and Holeta propolis were concentrated at 45°C in an oven. 5 µl 30% crude EEP of the two samples were applied horizontally in 1 cm wide band starting from 1.5 cm from the bottom of the chromatographic plates using micropipette and dried in air. The following solvent system combinations such as Hexane:Ethyl Acetate (9:1, 4:1, 7:3, 6:4) were used for development of chromatograms. Once developed, the plates were visualized under UV lamp of λ=254 nm and λ=366 nm and after spraying with appropriate TLC reagents.

In the case of autobiography method, once the plates were developed, and before they were sprayed with visualizing reagents, they were dried overnight. The plates were placed in petri dish and covered with 20 mL of sterile Mueller-Hinton agar at 45°C inoculated with the saline suspension of *S. aureus*, then incubated for 24 h at 37°C. The bioautograms were sprayed with an aqueous solution (2.5 mg/mL) of MTT and incubated for 4 h at 30°C. Clear inhibition zones were observed against a purple background. The TLC plates were compared and the solvent system that retains the compounds of interest (Retention factor (RF)=0.2 to 0.3) was chosen.

Preparation of spray reagents and detection of spots on TLC: a) Detection of spots: Visualization of the spots were done using UV light of wave length 254 nm and 366 nm after the plates were dried in air for 10 min and RF values of spots recorded. The plates were subsequently sprayed with Aluminum chloride, p-anisaldehyde or dizotized sulfanilic acid reagents followed by heating at 110°C for 10 min and the spots were visualized in UV chamber at 254 nm and 366 nm and their Retention factor (RF) values recorded.

The presence of flavonoids were assessed by the existence of fluorescent yellow zones on TLC at long-wave UV light (λ=366 nm) with $AlCl_3$ reagent. While the presence of terpene, steroid, phenol and sugar were assessed by their characteristic color reactions with p-anisaldehyde reagent on TLC which are red, green and grey respectively. The presences of Phenolic compounds were examined by their characteristic color reactions with diazotized sulfanilic acid reagent on TLC for the existence of an orange or yellow spot.

Column chromatography fractionation of propolis extracts: a) Overview: Fractionation of EEPH using column chromatography was performed according to the method of Trusheva [10] and Sarker [18]. Column chromatography fractionation of propolis sample from Holeta (which demonstrated the highest antimicrobial activity) using normal phase silica gel as a stationary phase was employed to isolate and purify the non-polar and medium polar chemical compounds of propolis. A step gradient of Hexane:Ethyl acetate (100%:0%, 95%:5% to 0%:100% with increment of 10% in each step) and Ethyl acetate: Methanol (100%:0% to 80%:20% with increment of 5% in each step) was employed as a general solvent system for non-polar and medium polar chemical compounds of propolis.

b) Method: A suitable heavy wall glass column was selected, which was about three times the volume of the silica gel required for the separation. About 100 g of silica gel 60 (Merk) was mixed with 233 mL of hexane to form a slurry and stirred using a stirring rod. The column was fixed with clamp in upright position. 20 mL of hexane was added into the column whose vent was plugged with glass frit or a lump of cotton covered with a layer of sea sand (not more than 1 cm) thick when the slurry was packed into the column. The slurry was added slowly until the bed was completed. The column outlet was opened to allow the solvent to flow. When the solvent level reached just above the stationary phase, the flow was stopped by closing the outlet valve. The dried aqueous ethanol extract of Holeta propolis showed the highest antimicrobial activity was weighed and dissolved in a minimum amount Ethyl acetate. A weighed amount of silica gel (double the amount of the sample) was added to the sample solution, mixed with a spatula and the solvent evaporated under vacuum in a rotary evaporator. The dry silica gel containing the sample was transferred to the top of the column bed using a funnel and wetted with a little amount of the initial mobile phase (1.5 mL) to remove air bubbles. Little amount of sand or silica gel to about 1 to 1½ cm of the column height was added.

A step gradient of the solvent systems developed earlier starting from the proportion that showed the highest resolution for each fraction were used with an increment of 5 to 10% of the more polar solvent. For n-hexane fraction, a typical step gradient sequence was as follows: initial composition of 100% n-hexane for column equilibration, followed by at least 1 column volume each of 5% EtOAc in n-hexane, 10–100% EtOAc in n-hexane (increment of 10% in each step) and 5 to 20% MeOH in EtOAc (increment of 5% in each step). Initially added first solvent after the sample application was allowed to flow using

gravity followed by using vacuum pump at lower pressure for stepwise addition of mobile phases on to the column. The out let valve was closed when the solvent level reaches 1 cm above the sea sand level. Sub fractions of approximately 20-30 mL were collected in test tubes labeled with fractionation number, the mobile phase used and its composition. The sub fractions were then concentrated in a rotary evaporator for overnight.

Analytical thin layer chromatography (TLC) and pooling of fractions: Screening of propolis fractions were performed according to the method of Ode [19]. Pre-coated silica gel (F254 on aluminum support) analytical TLC was used. Spots of the fractions obtained after column chromatography was applied on the plate using1.0 λ micro pipette at about 1.0 cm from the edge. It was dried using hot air dryer. The plate was lowered into a small chromatographic jar containing chloroform:methanol (8:2) solvent system. The jar was covered with a glass lid. The solvent was allowed to ascend until the solvent front reached about ¾ of the length of the plate. The plate was removed and dried by a hot air dryer and viewed under UV lamp at 366 and 254 nm to identify the fluorescing spot. The fluorescent spot was marked and then sprayed with spray reagent prepared earlier. The plate was placed in hot oven at 110 °C for 5 s for visibility of fluorescent bands. The color reaction was recorded and the relative Retention factor (RF) value calculated based on the formula described by Stahl:RF=(Distance travelled by the streak from the starting point/Distance travelled by the solvent from the starting point to the solvent front). Similar fractions showing the same RF value and color reactions were pooled together and the fractions kept at 4 °C in the refrigerator for further work.

Further bioassay guided fractionation with preparative chromatography and screening of the fractions: The methods of Trusheva [10] and Ode [19] were used with little modification for further fractionation of propolis fractions. Agar plate disk diffusion method using paper disks was used for the antimicrobial assays of the different pooled fractions obtained from column chromatography procedure. Further bioassay guided fractionation of the pooled fraction(s) using preparative chromatography in solvent systems appropriate for separation and purification of the compounds of interest which were identified in another study as antimicrobial components are the preferred method of choice.

The antibacterial activity of each fraction against *S. aureus* was carried out using paper disk diffusion assay. Fractions containing 3 or more components with antibacterial activity were re-chromatographed on a silica gel column by following the same procedure. Elution of components were performed using the following mobile phase combinations such as n-hexane-diethyl ether gradient (1:0.01/1:1), n-hexane-acetone gradients (gradient 1 (1:0.5/1:0.8), gradient 2 (1:0.05/1:1), gradient 3 (1:0.01/1:1)). The mobile phase gradients were determined by running the fractions on analytical TLC according to the complexity and polarity of the components of the mixtures. Further isolation and purification of the sub fractions that had been obtained from the first column fractionation or second column fractionation procedure were performed on preparative TLC.

Preparations of TLC plates were done as described by Stahl modified by Ode [19]. 50 g of silica gel G (silica gel 60 G, Merck) were mixed with 100 mL of distilled water in a conical flask to form slurry. The conical flask sealed with stopper was vigorously shaken for 30 s to obtain homogenous slurry. The slurry was poured into the trough of a moveable spreader which was adjusted to 0.5 mm. The slurry was spread in a single passage onto five glass plates (20 × 20 cm) placed on an improvised aligning tray. Prior to the spreading of the slurry, the surfaces of the clean glass plates were made grease-free by cleaning them with methanol soaked in cotton wool. The freshly coated chromatographic plates were left on the tray until the transparency of the layer disappeared. The plates were subsequently activated for use in an oven for 1h at 110°C. The coated surface was marked (using a dissecting pin) on the straight edge. A 7 mm margin on both sides of the plate was marked and the area from the edge of the plate to the mark left unstreaked. In the application of fractions, dropping pipettes (10 λ) were used to apply the various pooled fractions on the activated plates. Drops of the pooled fractions were applied in line to form a straight line streak or band. Each streak was dried before another one superimposed on it. The following solvent combinations such as n-hexane-ethyl methyl ketone (1:0.06, 1:0.1), Toluene-Acetone (1:0.2) and Chloroform-Methanol (8:2) were used. 40 mL of each solvent combination was poured in chromatographic tanks. A white blotting paper was placed on each side of the tank to saturate the tank evenly with the vapor of the eluting solvent. The tank was immediately covered after introducing the eluting solvent and the white blotting papers. A smear of Vaseline was applied to the edges of the underside of the coverlid of the tank to make sure that the lid fitted tightly to avoid escape of the vapor. The streaked chromatographic plates were put into the tank, two plates at a time at an angle of 30°C from the edge of the tank. The eluting solvent had been allowed to run for a distance of 15 cm starting from the streaked end. The plates were then removed from the tank and allowed to air dry. The same procedure as described above was used for all the pooled fractions. Each plate containing a single pooled fraction was viewed under ultra-violet lamp at 254 and 366 nm in a dark room. Separated zones were marked and the RF values calculated with the formula previously described.

Elution of separated spot zones: The final isolation and purification process of propolis components were performed according to the method of Sarker [18]. The marked separated zones were scrapped off the glass plates with a spatula onto a clean sheet of paper. The scrapings were transferred into centrifuge tubes containing 5 mL of absolute methanol. The content of the centrifuge tube was shaken manually for 10min. The eluent was separated from the adsorbent by centrifuging at 2500 rpm for 10 min. This process was then repeated satisfactorily that all the eluent was collected as much as possible. The collected eluent was evaporated to dryness using a hot air oven at the temperature of 40°C. The eluents were then subjected to further bioautographic test to confirm biological activity using *S. aureus* as described before.

Statistical analysis: One way ANOVA was used to for analysis of data for growth inhibitions.

Results

Antimicrobial assays of propolis

The results of the antimicrobial activity of three propolis samples collected from three different locations of Ethiopia that is South Wollo, Tembain and Holeta investigated against *S. aureus* and MRSA (gram positive bacteria), three gram negative bacteria (*E. coli*, *S. typhi* and *P. aeruginosa*) and *C. albicans* (fungi) are presented in Tables 1 and 2.

Screening for major antibacterial active components of propolis

The purpose of using TLC screening method for the different propolis samples from the regions of Ethiopia is to identify and characterize the antimicrobial active component(s) which may be responsible for the observed bactericidal effects.

Ethanolic extract of propolis from Tembain and Holeta were run

Microorganisms	Propolis type	IZD (mm) vs. concentration of propolis extract (%)			Control (70% ethanol)	Antibiotic drugs			
	EEPS	10%	20%	30%		Pen G	Cl P	Gent	Tet
S. aureus		NI	NI	NI	NI	NI	NI	NI	NI
MRSA		NI	NI	NI	NI	NI	NI	19 ± 0	11 ± 0
E. coli		NI	NI	NI	NI	NI	NI	NI	NI
P. aruginosa		NI	NI	NI	NI	NI	NI	NI	NI
S. typhi		NI	NI	NI	NI	NI	NI	NI	NI
C. albicans		NI	NI	NI	NI	NI	NI	NI	NI
	EEPT								
S. aureus		NI	NI	12.5 ± 1.9	NI	NI	NI	NI	NI
MRSA		NI	NI	11 ± 0.5	NI	NI	NI	19 ± 0	11 ± 0
E. coli		NI	NI	NI	NI	NI	NI	NI	NI
P. aeruginosa		NI	NI	NI	NI	NI	NI	NI	NI
S. typhi		NI	NI	NI	NI	NI	NI	NI	NI
C. albicans		NI	NI	NI	NI	NI	NI	NI	NI
	EEPH								
S. aureus		NI	NI	13 ± 1	NI	NI	NI	NI	NI
MRSA		NI	NI	12 ± 1	NI	NI	NI	19 ± 0	11 ± 0
E. coli		NI	NI	NI	NI	NI	NI	NI	NI
P. aruginosa		NI	NI	NI	NI	NI	NI	NI	NI
S. typhi		NI	NI	NI	NI	NI	NI	NI	NI
C. albicans		NI	NI	NI	NI	NI	NI	NI	NI

IZD: Inhibition Zone Diameter; NI: No Inhibition was Observed

Table 1: Average zone of inhibition diameter of the bacteria and fungi (in duplicate; mean ± SD) of three different ethanolic extract of each propolis sample at different concentration using disk diffusion method.

Microorganisms	Propolis types	IZD (mm) vs. concentration of propolis extract (%)			Control (70% ethanol)
	EEPS	10	20	30	
S. aureus		NI	NI	NI	NI
MRSA		NI	NI	NI	NI
E. coli		NI	NI	NI	NI
P. aeruginosa		NI	NI	NI	NI
S. typhi		NI	NI	NI	NI
C. albicans		NI	NI	NI	NI
	EEPT				
S. aureus		NI	NI	12 ± 0	NI
MRSA		NI	NI	12 ± 0	NI
E. coli		NI	NI	NI	NI
P. aeruginosa		NI	NI	NI	NI
S. typhi		NI	NI	NI	NI
C. albicans		NI	NI	NI	NI
	EEPH				
S. aureus		12 ± 0	16 ± 2	16 ± 2	NI
MRSA		10 ± 0	12 ± 2	12 ± 0	NI
E. coli		NI	NI	NI	NI
P. aeruginosa		NI	NI	NI	NI
S. typhi		NI	NI	NI	NI
C. albicans		NI	NI	NI	NI

IZD: Inhibition Zone Diameters; NI: No Inhibition was Observed

Table 2: Average zone of inhibition diameter of the bacteria and fungi (in duplicate; mean ± SD) of three different ethanolic extract of each propolis sample at different concentration using agar well diffusion method.

on silica gel packed analytical thin layer chromatography and three different solvent systems were employed for good resolution of chemical compounds. Out of these Toluene:ethyl acetate:acetic acid (5:4:1) was found to be the best and this was used for further experiments. The

different spots obtained in the TLC plates of the two different propolis sample were compared by their RF values and color reactions. The results of thin layer chromatographic analysis of the two propolis samples are presented in Table 3 and Figure 2.

The results presented imply that many of the bioactive components are the same in the two propolis samples. However, there is variation in that some new compounds may be present in either of the samples or some compounds can be seen in one sample but absent in the other (Table 3 and Figure 2). Particularly after spraying with diazotized sulfanilic acid as seen in the difference in two fractions is a clear cut. The phenolic acids are seen in only EEPT but not in EEPH (Table 3).

The method of TLC agar overlay antimicrobial assay was employed to see if any correlation existed between the chemical components of each propolis sample resolved on TLC and the corresponding bactericidal effects. The result of agar overlay method used for screening of antibacterial active components showed no inhibitions for any of the chemical components of EEPH and EEPT against *S. aureus*.

The use of TLC agar overlay antimicrobial assay method is limited to a smaller sample volume and the bioactive components responsible for antimicrobial activity may probably be subjected to oxidation there by losing potency. Therefore, column chromatography is a method of choice which was used in the present study to further identify and characterize the main antimicrobial components (if any) in propolis sample from Holeta which showed the highest antimicrobial activity. A step gradient of hexane/ethyl acetate (100:0%, 95:5% to 0:100% with an increment of 10%) and ethyl acetate/methanol (100/0, 95/5 to 80/20 with an increment of 5% in each step) were used for fractionation of Holeta propolis using column chromatography. 19 fractions were collected and the fractions analyzed using normal phase silica gel packed analytical thin layer chromatography. Fractions showed similar RF values and, color reactions with vanillin-sulphuric acid were combined. A total of eight fractions were obtained. The results of the analysis for the pooled fractions using TLC are shown in Figure 3.

Antimicrobial assay using agar plate disk diffusion method using paper disks for the pooled fractions obtained from column chromatographic fractionation method were performed to identify the fraction(s) having antimicrobial activities. The results are presented in Table 4.

Discussion

Propolis collected from three different local areas of Ethiopia comprising samples from South Wollo, Tembain and Holeta showed a variation in their antibacterial activities against *S. aureus* and MRSA depending on the geographic locations. In general, ethanol extracts of propolis from Tembain and Holeta exhibited inhibitory action against gram positive bacteria but showed no activity against gram negative bacteria and *C. albicans* at any of the concentrations of 10%, 20% or 30%) used in the experiment. It was noted that the 70% ethanol which was used as a negative control exhibited no inhibitory action against any of the microorganisms studied.

The result of agar disk diffusion test method using paper disks showed that ethanolic extract of South Wollo propolis solution at all concentrations of 10%, 20% and 30% had no activity against all microorganisms used in the present study. According to the information available in the literature, it was found that all propolis types regardless of the variation in their geographic region showed some antimicrobial activities. This variation of the current study may be due to the absence and/or the presence of small amount of bioactive components which are responsible for its antibacterial activity or it may have lost potency during the process of collection and storage.

EEP from Tembain and Holeta had antibacterial activity against *S.aureus* and MRSA but no inhibition zones were observed for the other microorganisms. These propolis samples also did not show activity against the gram negative bacteria. This is in line with the finding of another study reporting that gram negative bacteria has a mechanism that would prevent intracellular entry of propolis or promote their extrusion from the cell due to the presence of their plasma membrane efflux pumps.

The other probable reason for the observed resistance of gram negative bacteria against propolis can be because propolis contains plant resin constituents which are secreted mainly to protect plants from gram positive bacteria [5]. Some studies have also stated that propolis exhibited antibacterial activity against some gram negative bacteria. According to Campos [20], artepllin C in propolis has been found to be more efficient antibacterial compound than the commercial antibacterial substance chloroamphenicol against gram negative bacteria such as *P. aeruginosa*. Pinbanksin-3-O-acetate has also been found to possess antibacterial activity against standard *E. coli* and resistant *E. coli* [21].The isoflavonoids isolated from Brazilian propolis such as the isoflavan isosativan and the pterocarpan medicarpan have been found to have activity against *C. albicans* [10]. The other probable reason that propolis from Tembain and Holeta region did not demonstrate antimicrobial activities against gram negative bacteria and *C. albicans* is because propolis from these regions might not contain the above mentioned antibacterial substances and others which may be responsible for the activity against these microorganisms.

The data obtained from the results of disk diffusion method showed that 30% concentration of EEPT exhibited inhibition zone diameters of 12.5 mm and 11 mm for *S.aureus* and MRSA, respectively. On the other hand, no inhibition zones were observed for this same propolis sample preparations at 10% and 20% against *S. aureus* and MRSA. Inhibition zone diameters of 10 mm, 11.5 mm and 13 mm were recorded for *S. aureus* tested at 10%, 20% and 30% concentrations respectively of Holeta propolis. The data obtained from the disk diffusion test also showed EEPH at concentrations of 20% and 30% against MRSA showed an inhibition zone diameter of 10.5 and 12 mm respectively. The results of the present study is comparative to the finding of other study by Nada [6] who found that the inhibition diameters for *S. aureus* showed by Iraqi propolis at concentration of 10% was 13 mm. The activity of Ethiopian propolis from the two regions against *S. aureus* is highly effective as compared to the findings of Yaghoubi [7] who found that the inhibition showed by Iranian propolis at 67 mg/mL against *S. aureus* was 4.2 mm. Comparison of the inhibition zone diameters exhibited by the ethanolic extract of propolis from Tembain and Holeta with that of standard antibiotic disks against methicillin resistant *S. aureus* showed that the extracts of both samples at higher concentrations are more

Propolis type	RF values of compounds before spray in UV chamber at λ=254nm and λ=366nm	RF values of compounds after sprayed with vanillin	RF values of compounds after sprayed with anisaldehyde	RF values of compounds after sprayed with sulphanilic acid
EEPH	0.27, 0.32, 0.47, 0.73, 0.79	0.36, 0.46, 0.51, 0.64, -, 0.71	0.49, 0.54, 0.71, 0.78	-
EEPT	0.27, 0.32, 0.47, 0.62	0.36, 0.46, 0.61, 0.71	0.71, 0.78	0.57, 0.69

Table 3: RF values for the components of EEPH and EEPT on the TLC plates with the spots in ascending order before and after spraying with different TLC spray reagent.

Figure 2: Thin layer chromatography analysis of ethanolic extract of Holeta and Tembain propolis in toluene:ethyl acetate:formic acid (5:4:1) solvent system before and after spraying.

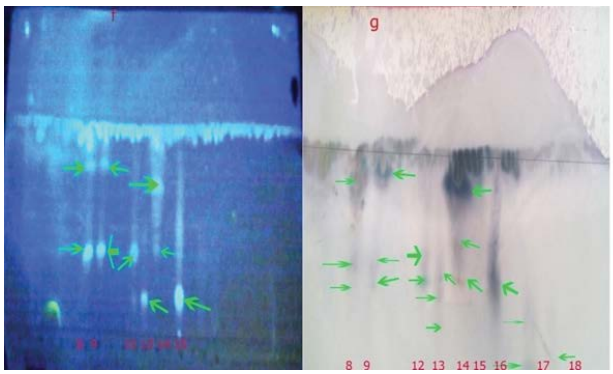

Figure 3: Thin layer chromatography analysis of the propolis pooled fractions from column chromatography in chloroform:methanol (8:2) solvent system before and after spraying. Arrows indicate the compound migration distance on TLC where as the number indicates each of the fractions obtained from column chromatography run.

F=detection of the spots at UV light of wave length 254 nm before spraying, e=detection of spots after spraying with vanillin

Microorganism	Fractions	Antibiotic disk	Inhibition zone diameter
S. aureus	9, 12, 13, 14, 15, 16, 17 and 18	Penicillin G	NI
		Chloroampenicol	NI
		Gentamicin	24
		Tetracyclin	13

NI: No Inhibition was Observed

Table 4: Inhibition zone diameter for antibacterial assay of Holeta propolis fractions obtained from column chromatography fractionation using paper disk diffusion method.

effective than penicillin G and chloramphenicol whereas they are as effective as tetracycline. However, ethanolic extracts of the two samples are not as effective as gentamicin.

Ethanolic extract of propolis solutions of similar concentrations used for disk diffusion were investigated using agar well diffusion method. It was observed that the inhibition zone diameter exhibited by ethanolic extract of Tembain propolis at 30% against *S. aureus* and MRSA were 12, respectively. On the other hand, Ethanolic extract of Holeta propolis at 10%, 20% and 30% against *S. aureus* demonstrated inhibition zone diameters of 12, 16 and 16 mm, respectively.

Ethanolic extract of Holeta propolis at 10%, 20% and 30% against MRSA showed inhibition zone diameters of 10, 12 and 12 mm, respectively. The findings of this study are in agreement with the results of other studies by Silva [22] who found that ethanolic extract of Brazilian commercial propolis of 11% preparations against *S. aureus* showed an inhibition zone diameter of 11 mm. The values for the inhibition zone diameters which were obtained by using the two methods demonstrated that *S. aureus* and MRSA are equally sensitive to both EEPT and EEPH. Statistical analysis showed that there is no significant difference ($P>0.05$) in the inhibition zone diameters between *S. aureus* and MRSA towards ethanolic extract of propolis from Holeta and ethanolic extract of propolis from Tembain. Comparison of the inhibition zone diameters between EEPT and EEPH showed EEPH is more effective in its activity against both *S. aureus* and MRSA than EEPT. Statistical analysis showed the difference between the inhibition zones exhibited by EEPT and EEPH at different concentrations (10%, 20% and 30%) against *S. aureus* and MRSA is significant ($P \leq 0.05$). Comparison of the inhibitions between the two methods (agar well diffusion and paper disk diffusion) showed that there is little difference in the inhibition zones for *S. aureus* and MRSA tested towards EEPH and EEPT. Statistical analysis showed that the difference between agar well diffusion and paper disk diffusion methods is not significant ($P>0.05$). The results obtained from both paper disk diffusion and agar well diffusion methods showed that an increase in concentration of ethanolic extract of propolis from Tembain from 10%, 20% to 30% were associated with the corresponding increase in its inhibitory action against *S. aureus* and MRSA. However, analysis showed there is no significant difference in the inhibition zones for *S. aureus* and MRSA tested towards EEPH at different concentrations (10%, 20% and 30%) ($P \geq 0.05$).

The variation in antibacterial activities between EEPT and EEPH can probably be due to the difference in their chemical components. TLC investigation of the two propolis samples from Tembain and Holeta showed there is difference in chemical components between the two propolis samples seen by different RF value of spots obtained in two chromatograms. The result of the analysis showed two chemical components detected at UV light of 254 and 366 nm (fluorescent active) in EEPH are not found in EEPT where as there is one component which is detected in EEPT but not in EEPH. Two chemical components which are detected in EEPH using vanillin as a general spray reagent are not found in EEPT and one component which is detected in EEPT is not contained in EEPH. Similarly the result of the TLC analysis using anisaldehyde-sulphuric acid as a spray reagent for sterols, phenols, sugars and terpenes demonstrated two chemical components of either of these chemical classes which are found in EEPH are not present in EEPT.

TLC analysis of EEPH and EEPT using diazotized sulfanilic acids as a spraying reagent showed the two samples differ in phenolic acids content. However, thin layer chromatographic analysis using $AlCl_3$ as a spray reagent for flavonoids indicated that none of these chemical classes are found in any of the propolis samples. Several studies indicated that different flavonoids, phenolic acids and their esters are found to be the antimicrobial active compounds in temperate zone whereas

different phenolic acids their esters, mono and sesqueterpenes as well as diterpenes are found to be the antimicrobial active components in propolis from tropical regions.

With reference to TLC agar overlay method already mentioned on page 36 of the results, no correlation existed between the chemical components of each propolis sample resolved on TLC and the corresponding bactericidal effects. That is this microbiological assay method used for screening of antibacterial active components showed no inhibitions for any of the chemical components of EEPH and EEPT against *S. aureus*.

Conclusion

The results of antibacterial assay using paper disk diffusion assay performed on eight fractions obtained from column chromatography fractionation of Holeta propolis against *S. aureus* showed that no antibacterial activity was observed for any of the fractions. The findings indicated that the antibacterial activity of propolis in this region is due to synergistic effect of the sum of the different antibacterial active component combinations.

In summary, propolises studied from different geographical regions have showed different antimicrobial activities against gram positive, gram negative and fungal species. The antimicrobial effects present in propolis have been associated with completely different chemical profiles in samples from different locations and climatic zones. And their antimicrobial inhibition potency varied with the increasing concentration of the extract.

References

1. Fichtl R, Adi A (1994) Honeybee flora of Ethiopia. Weikersheim, Germany.
2. Khalil ML (2006) Biological activity of bee propolis in health and disease. Asian Pac J Cancer Prev 7: 22-31.
3. Deffar G (1998) Non-wood forest products in Ethiopia. A report presented to EC-FAO Partnership programme (1998-2000). Tropical forestry Budget line B7-6201/97- 15/VIII/ for project GCP/INT/679/EC.
4. Bankova V, Castro SL, Marcucci MC (2000) Propolis: Recent Advances in Chemistry and plant origin. Apidologie 31: 3-15.
5. Fokt H, Aguiar C, Cunha A, Ferreira AM, Pereira A (2010) How do bees prevent hive infections? The antimicrobial properties of propolis. A mendez vilas (ed.) Portugal.
6. Nada K, Habeeb S, Alaa H (2010) *In vitro* antibacterial and antifungal activity of Iraqi propolis. J Medical Plants Res.
7. Ahuja V, Ahuja A (2011) Apitherapy-A sweet approach to dental disease. J academy of dent Dis 2: 1-8.
8. Bankova V (2005) Recent trends and important developments in propolis research. Evid Based Complement Alternat Med 2: 29-32.
9. Sforcin JM (2007) Propolis and the immune system: a review. J Ethnopharmacol 113: 1-14.
10. Trusheva B, Popova M, Bankova V, Simova S, Marcucci MC, et al. (2006) Bioactive constituents of brazilian red propolis. Evid Based Complement Alternat Med 3: 249-254.
11. Yaghoubi SMJ, Ghorbani GR, Soleimanian ZS, Satari R (2007) Antimicrobial activity of Iranian propolis and its chemical composition. DARU 15: 45-48.
12. Hegazi AG, Abd El Hady FK (2002) Egyptian propolis: 3. Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands. Z Naturforsch C 57: 395-402.
13. Selven KA, Prabhu T (2010) Extraction of Propolis from Beehives and Characterization of Its Constituents and Medicinal Properties: A Review. Int J Advanced Eng Tech 1: 50-53.
14. Tukmechi A, Ownagh A, Mohebbat A (2011) *In Vitro* antibacterial activities of ethanol extract of Iranian propolis (EEIP) against fish pathogenic bacteria (*Aeromonas Hydrophila, Yersinia Ruckeri andStreptococcus Iniae*). Brazilian J Microbiol 41: 1086-1092.
15. Khalil ML (2006) Biological activity of bee propolis in health and disease. Asian Pac J Cancer Prev 7: 22-31.
16. Medic-Saric M, Rastija V, Bojic M, Males Z (2009) From functional food to medicinal product: systematic approach in analysis of polyphenolics from propolis and wine. Nutr J 8: 33.
17. Choudhary IM, Rahman UA, Thomsen JW (2005) Bioassay techniques for drug development. Harwood academic publishers, Singapore.
18. Sarker SD, Latif Z, Gray AI (2006) Methods in biotechnology: Natural Product Isolation, 2nd (Ed), Human press, New Jersey.
19. Ode OJ, Asuzu IU, Ajayi IE (2011) Bioassay-Guided Fractionation of the Crude Methanol Extract of Cassia singueana Leaves. J Adv Scient Res 2: 81-86.
20. Campos VAC (2011) Antibacterial Activity of Propolis Produced by Frieseomelitta varia. Ciênc Agrotec Lavras 35: 1043-1049.
21. Darwish RM, Abu Fares RJ, Zarga MHA, Nazer IK (2010) Antibacterial effect of Jordanian propolis and isolated flavonoids against human pathogenic bacteria. African J Biotech 9: 5966-5974.
22. Silva BB, Rosalen PL, Cury JA, Ikegaki M, Souza VC, et al. (2008) Chemical composition and botanical origin of red propolis, a new type of brazilian propolis. Evid Based Complement Alternat Med 5: 313-316.

This article was originally published in a special issue, **Molecules involved in cell signaling pathways** handled by Editor(s). Dr. Masood Alam Khan, Al-Qassim University, Saudi Arabia

Citation: Alemu F, Ahmed A, Seifu D, Gebre S (2017) Antimicrobial Activities and Chemical Characterization of Propolis Collected from Three Different National Areas of Ethiopia. Mol Biol 5: 180. doi: 10.4172/2168-9547.1000180