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HPLC-DAD and GC-MS characterization of Cameroonian honey Samples and Evaluation of their antibiofilm, Anti-quorum sensing and Antioxidant activities

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ABSTRACT

HPLC-DAD was used to detect and quantify fumaric acid and chrysin in honey from Adamawa(AD) and North-West (NW) regions. GC-MS showed that most abundant volatile compound inAD and NW samples were α-terpineol acetate and 1-(2-furanyl)-ethanone respectively. The honey samples inhibited the growth of microorganisms, C. albicans, S. aureus and E. coli with MIC values between 0.5 and 1 mg/mL. AD exhibited the highest antibiofilm activity ranging from 13.9±0.2% (MIC/4) to 34.7±2.4% (MIC) for C. albicans and from 11.3±0.6% (MIC/8) to46.2±1.8% (MIC) for E. coli. NW showed highest biofilm inhibition on S. aureus from 20.7±4.0% (MIC/4) to 52.3±1.5% (MIC). NW had best anti-quorum sensing inhibition zone of 7.5±2.0 (MIC/4) to 15.0±1.5mm (MIC) using C. violaceumCV026 and AD had best violacein inhibition of 14.72±2.50% (MIC/8) to 100±0.00% (MIC and MIC/2) using C. violaceumCV12472. Antioxidant activities (DPPH, CUPRAC, β-carotene, Metal chelation) were moderate compared to the standards.

Key words: *Honey, HPLC-DAD, GC-MS, antibiofilm, anti-quorum sensing, antioxidant activity*

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INTRODUCTION

Despite advances in food safety, foodborne diseases still occur around the world caused by major food borne pathogens which have developed multidrug resistance with time as a result of misuse of antibiotics[1].The efficacy of existing conventional antimicrobials need to be revisited because of the observed occurrence of multidrug microbial resistant strains. Antipathogenic medicines that can target major bacterial systems responsible for regulating the expression of virulence factors is a novel therapeutic approach that can help to circumvent the emergence of bacterial resistance [1,2,3]. Investigations concerning cooperative or coordinated behavior in communities of microorganisms have recently been shown that bacteria do communicate to coordinate the activities of their colonies usually by a process of guided social behavioral patterns that is called quorum sensing[4]. QS is achieved when the bacteria releases autoinducers up to the critical threshold concentration. At this concentration, they autoinducers bind to and activate receptors consequently triggering genes encoding information that concerns several characteristics such as sporulation, biofilm formation, motility, plasmid conjugation, exopolysaccharide production, toxin production, siderophore synthesis and bioluminescence[5,6]a

majority of which contribute to the pathogenicity of the bacteria. For very longtime, mankind has been using traditional medicine as a remedy to infection and this practice involves honey produced by *Apis mellifera* (*A. mellifera*) which is amongst the oldest traditional remedies that has been of considerable importance to humans as they use it in treating several ailments[7].

Honey produced from some botanical sources exhibits high antimicrobial activity and therefore can find applications in clinical practice for the treatment of infections, hence afield of intensive research[8].Development of research that can maximize the exploitation of the properties of honey against microorganisms and address the obstacles that arise from the in vivo use can produce antimicrobial agents that can find applications in clinical practice. It is interesting to note that no honey-resistant bacterial strains are reported and this can be as a consequence of the fact that the antimicrobial properties of honey samples is highly multifactorial in nature [9].Besides the important antibacterial activities of honey, the antioxidant properties of honey usually associated to its polyphenols (e.g., flavonoids and phenolic acids), vitamins (e.g., vitamin C), antioxidant enzymes (e.g., catalase and peroxidase), Maillard reaction products (e.g., melanoidins), and carotenoids and amino acids (e.g., proline)is also considered important[10].However, the therapeutic effects of honey are usually associated to the presence of other compounds other than sugar for example flavonoids, phenolic acids,enzymes, peptides and free amino acids, organic acids, vitamins and minerals [11].

MATERIAL AND METHODS

Honey samples and solid phase micro extraction

The samples of honey were collected from the Adamawa Region (AD) and from North-West Region (NW) of Cameroon during the month of November 2018.Eachof the honey samples (2.5 g) was dissolved in water (20 mL) and subjected to solid phase micro extraction according to the method described elsewhere [12].

HPLC-DAD profiling of the samples

RP-HPLC-DAD system was used for the profiling of compounds. A total of 31 standard compounds were used namely: fumaric acid, gallic acid, p-benzoquinone, protocatechic acid, theobromine, theophylline, catechin, 4-hydroxybenzoic acid, 6,7-dihydroxycoumarin, vanillic acid, caffeic acid, vanillin, chlorogenic acid, p-coumaric acid, ferulic acid, cynarin, prophylgallate, rutin, trans-2-hydroxycinnamic acid, ellagic acid, myricetin, fisetin, quercetin, trans cinnamic acid, luteolin, rosmarinic acid, kaemferol, apigenin, chyrsin, 4-hydroxylresorcinol and 1,4-diclorobenzene.A Stock solution of each honey sample was dissolved in water/methanol (80/20) at concentration of 8 mg/mL and filtered with an Agilent 0.45 µm disposable LC filter disk for HPLC–DAD. Separation was achieved on an Intertsil ODS-3 reverse phase C18 column (5 µm, 250 mm × 4.6 mm i.d) thermostatted at 40 ^{o}C. Samples were prepared in methanol. The solvent flow rate was 1.5 mL/ min. The sample volume injection was 20 µL. The mobile phases used were: (A) 0.5% acetic acid in water, (B) 0.5% acetic acid in methanol. The elution gradient was as follows: 0–20% B (0–0.01 min); 20–60% B (0.01–2 min); 60–80% B (2–15 min); 100% B (15–30 min); 100–10% B (3–35 min); 10–0% B (35–40 min). Detection was carried out photodiode array detector (PDA) in the range 230–350 nm wavelength for different standard compounds. The phenolic compounds were characterized according to their retention times, and UV data were compared with commercial standards. Three parallel analyses were performed, and results were expressed as milligrams per gram of honey.

Determination of volatile composition of the honey sample

Gas Chromatography with Flame Ionization Detector (GC-FID)

GC analyses of the honey samples were performed using a Shimadzu GC-17 AAF,V3, 230V LV Series (Kyoto, Japan) gas chromatography, equipped with a FID and a DB-1 fused silica column [30 m × 0.25 mm (i.d.), film thickness 0.25µm]; the oven temperature was held at 60 °C for 5 min, then programmed to 200 °C at 4 °C/min and held isothermal for 5 min; injector and detector temperatures were 250 °C and 270 °C respectively; carrier gas was helium at a flow rate of 1.3 mL/min; Sample size, 1.0 µL; split ratio, 50:1. The percentage composition of each honey was determined with a Class-GC 10 computer program.

Gas chromatography–mass spectrometry (GC–MS)

The analysis of each honey sample was performed using a Varian Saturn 2100(Old York Rd., Ringoes, NJ, USA), ion trap machine, equipped with aDB-1 MS fused silica non-polar capillary column [30 m × 0.25 mm(i.d.), film thickness 0.25µm]. Carrier gas was helium at a flow rate of 1.4 mL/min. The oven temperature was held at 60 °C for 5 min, then increased up to 200 °C with 4 °C/min increments and held at this temperature for 5 min. Injector and transfer line temperatures were set at250 and 180 °C, respectively. Ion trap temperature was 270 °C. The injection volume was 0.2µL and split ratio was 1:30. EI-MS measurements were taken at 70 eV ionization energy. Mass range was from m/z 28–650 amu. Scan time was 0.5 s with 0.1 s inter scan delays. Identification of components of AD and NW was based on GC

retention indices and computer matching with the Wiley, NIST-2005 and TRLIB Library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature.

Antimicrobialand Anti-biofilmactivity

BacterialandFungalStrains

Bacterial and fungal strains *Staphylococcus aureus* (ATCC® 25923™), *Escherichiacoli*(ATCC® 25922™)and *Candida albicans* (ATCC® 10239™) were selected for the in vitro antimicrobialand anti-biofilmactivities. The above-mentioned bacteria except *C. albicans* were grown in NutrientBroth (NB, Difco); *C. albicans* was grown in Sabouraud dextrose broth (SDB, Difco). The cultures of microorganisms were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Determination of minimal inhibitory concentration (MIC)

MICs were determined by a microtitre broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) 2006 [13]. The MIC was defined as the lowest honey sample concentration that yielded no visible growth. The test medium was Mueller–Hinton Broth (MHB) and the density of bacteria was 5×10^5 colony-forming units (CFU)/mL. Cell suspensions (100 µL) were inoculated into the wells of 96-well microtitre plates in the presence of honey samples with different final concentrations (0.25, 0.5, 1, 2.5, 5, 10mg/mL). The wells containing only MHB and MHB with inoculum were employed as negative and positive controls, respectively. The inoculated microplates were incubated at 37 °C for 24 h. The absorbance was measured at 550 nm. The lowest concentration of the tested samples, which did not show any visual growth of tested organisms after macroscopic evaluation, was determined as MIC, which was expressed in mg/mL. Each assay was performed in triplicate for all bacteria.

Biofilm inhibition assays

The effect of the honeysamplesat concentrations of 1, 1/2, 1/4 and 1/8 MIC on biofilm-forming ability of the bacterial and fungal strains selected was tested using a microplate biofilm assay [14]. Briefly, 1% of overnight cultures of the selected strains was added into 200 µL of fresh Tryptic Soy Broth (TSB) supplemented with 0.25% glucose were diluted in growth medium to 5 x 10^5 colony-forming units (CFU)/mL and 100 µl and dispensed into each well of 96-well polystyrene flat-bottomed microtitre plates in the presence of 100 µl of honey sample and incubated without agitation for 48 h at 37 °C. The wells containing TSB + cells were used as control. After incubation, the wells were washed with water to remove planktonic bacteria or yeast cells. The remaining bacteria or yeast were subsequently stained with 0.1% crystal violet solution for 10 min at room temperature. Wells were washed once again to remove the crystal violet solution that had not specifically stained the adherent bacteria. Microplates were inverted and gently tap on paper towels to remove any excess liquid then air dried. 200 µL of 95 % ethanol were filled into the plates containing *E. coli* and *C. albicans* while 33 % glacial acetic acid were filled into the wells of the plates containing *S. aureus*. Biofilm stains solubilized at room temperature. After shaking and pipetting of the wells, 125 µL of the solution from each well was transferred to a sterile tube and volume made up to 1 mL with distilled water. Finally, the optical density of each well was measured at a wavelength of 550 nm. Each strain was tested for biofilm production in triplicate and the mean deduced. Percentage of inhibition of NW and AD was calculated using the formula given below.

$$\text{Biofilm inhibition (\%)} = \frac{ODcontrol - ODsample}{ODcontrol} X100$$

Anti-quorum sensing activity

Bioassay for QSI activity using ChromobacteriumviolaceumCV026

The quorum sensing inhibition potential of honey samples were performed by following the method specified by Koh and Tham [15]. The limit of detection of activity was also determined by applying serial dilutions of thehoneysamples (MIC, MIC/2, MIC/4, MIC/8, using LB broth as the diluent) against *Chromobacterium violaceum* CV 026. Each experiment was repeated and the assay plates were incubated at 30°C for 3 days. Each sample was tested in triplicate.

Violac in inhibition assays using Chromobacterium violaceum CV12472

Both honey samples were subjected to qualitative analysis to find their violacein inhibition potentials against *Chromobacterium violaceum* ATCC 12472 [16]. Overnight culture (10µl) of *C. violaceum* (adjusted to 0.4 OD at 600nm) was added into sterile microtiter plates containing 200µL of LB broth and incubated in the presence and absence of various concentrations of tested agents (MIC-MIC/16). LB broth containing *C. violaceum* ATCC 12472 was used as a positive control. These plates were incubated at 30°C for 24h and observed for the reduction in violacein pigment production. Each experiment was performed in triplicate. The absorbance was read at 585nm. The percentage of violacein inhibition was calculated by following formula:

$$\text{Violacein inhibition (\%)} = \frac{ODcontrol - ODsample}{ODcontrol} X100$$

Swarming and swimming motility assays using Pseudomonas aeruginosa PA01

The swimming and swarming motility assays were performed using *P. aeruginosa* PA01. In swimming assay, 3 µl overnight cultures of the uropathogens (0.4 OD at 600 nm) were point inoculated at the center of the swimming agar medium consisting of 1% tryptone, 0.5% NaCl and 0.3% agar with increasing concentrations of honey (50, 75 and 100 µg/ml). For swarming assays, 5 µl (0.4 OD at 600 nm) overnight cultures of uropathogens were inoculated at the center of the swarming agar medium consisting of 1% peptone, 0.5% NaCl, 0.5% agar and 0.5% of filter-sterilizedD-glucose with increasing concentrations of honey (50, 75 and 100 µg/ml). The plates were then incubated at 30 °C in upright position for 16 h. The reduction in swimming and swarming migration was recorded by measuring the swim and swarm zones of the bacterial cells after 16 h.

Antioxidant activity assay

DPPH free radical scavenging assay

The free radical scavenging activity of AD and NW was determined by the DPPH assay. In its radical form DPPH absorbs at 517 nm, but on reduction by an antioxidant or a radical species its absorption decreases. Briefly, a 0.1 mmol L^{-1} solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL of samples solution in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical of an antioxidant was calculated using the following equation:

$$DPPH\ radical\ scavenging\ (\%) = \frac{Acontrol - Asample}{Acontrol} X100$$

where $A_{Control}$ is the initial concentration of the DPPH,A_{Sample} is the absorbance of the remaining concentration of DPPH in the presence of the extract and positive control. BHT and a-tocopherol were used as antioxidant standards, for comparison of the activity. The sample concentration providing 50% free radical scavenging activity (IC_{50}) was calculated from the graph of DPPH Scavenging effect percentage against sample concentration.

β-Carotene/linoleic acid assay

The antioxidant activity of AD and NW was evaluated using the β-carotene-linoleic acid test system. β-Carotene (0.5 mg) in 1 mL of chloroform was added to 25 µL of linoleic acid, and 200 mg of Tween-40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, were added by vigorous shaking. 4 mL of this mixture was transferred into different test tubes containing different concentrations of AD and NW. As soon as the emulsion was added to each tube, the zero-time absorbance was measured at 470 nm using a 96-well microplate reader (SpectraMax 340PC, Molecular Devices, USA). The emulsion system was incubated for 2 h at 50 °C. A blank, devoid of β-carotene, was prepared for back ground subtraction. BHA and α-tocopherol were used as standards. The bleaching rate (R) of β-carotene was calculated according to the following equation:

$$R = \frac{ln\frac{a}{b}}{t}$$

where: ln=natural logarithm, a=absorbance at time zero, b=absorbance at time t (120 min)

The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation below

$$AA\ (inhibition\ \%) = \frac{Rcontrol - Rsample}{Rcontrol} X100$$

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity was determined according to the Cupric reducing antioxidant capacity (CUPRAC) assay method. To each well, in a 96 well plate, 50 µL10 mM Cu (II), 50 µL 7.5 mM neocuproine, and 60 µL NH_4Ac buffer (1 M, pH 7.0) solutions were added. 40 µL of AD and NW at different concentrations were added to the initial mixture to make the final volume 200 µL. After 1 h, the absorbance at 450 nm was recorded against a reagent blank by using a 96-well microplate reader. Results were recorded as absorbance compared with the absorbance of BHT (Butylated hydroxytoluene) and α-tocopherol were used as antioxidant standards for comparison in the study.

Ferrous ions chelating activity

The chelating activity of the extracts on Fe^{2+} was measured. The extracts solution (80 µL dissolved in ethanol in different concentrations) were added to 40 µL 0.2 mM $FeCl_2$. The reaction was initiated by the addition of 80 µL 0.5 mM ferene. The mixture was shaken vigorously and left at room temperature for 10 min. After the mixture reached equilibrium, the absorbance was measured at 593 nm. EDTA was used as antioxidant standard for comparison of the activity. The metal chelation activity was calculated using the following equation:

$$Metal\ chelating\ activity\ (\%) = \frac{Acontrol - Asample}{Acontrol} X100$$

where $A_{Control}$ is the absorbance without of sample and A_{Sample} is the absorbance of sample in the presence of the chelator.

RESULTS AND DISCUSSION

HPLC-DAD profiles of the honey samples

The chemical profiles of the honey samples were determined by HPLC-DAD using 31 internal standards and reported on table 1. Amongst the standard compounds used in the HPLC-DAD profiling, only fumaric acid and chrysin were detected and quantified in the two honey samples. Fumaric acid was detected in the AD sample and NW sample in the amounts of 0.728 mg/g of honey and1.117 mg/g of honey respectively meanwhile chrysin was detected and quantified in the AD honey and NW honey in amounts of 0.023 mg/g of honey and 0.017 mg/g of honey respectively. Though the same compounds are detected in the two honey samples, they are in different amounts and this effect could be due to the difference in the floral and geoclimatic origins.The presence of fumaric acid isrelated to citric acid content, indicates aging, authenticity and purity of honey and contributes to honey acidity and flavour[17,18,19].Chrysin (5,7-dihydroxyflavone) is a natural compound that occurs in bee products mainly propolis and honey. Previously, the chrysin contents were determined to be in the range of 0.10 mg/kg in honeydew and 5.3 mg/kg in forest honey samples [20].

Table 1. HPLC-DAD profiles of AD and NW honey samples

Compound	AD mg/g honey	NW mg/g honey
fumaric acid	0.728	1.117
Gallic acid	nd	nd
p-benzoquinone	nd	nd
Protocatechic acid	nd	nd
Theobromine	nd	nd
Theophlline	nd	nd
Catechin	nd	nd
4-hydroxybenzoic acid	nd	nd
6,7-dihydroxycoumarin	nd	nd
Vanilic acid	nd	nd
Caffeic acid	nd	nd
Vanillin	nd	nd
Chlorogenic acid	nd	nd
p-coumaric acid	nd	nd
Ferulic acid	nd	nd
Cynarin	nd	nd
Prophylgallate	nd	nd
Rutin	nd	nd
trans-2-OH cinnamic acid	nd	nd
Ellagic acid	nd	nd
Myricetin	nd	nd
Fisetin	nd	nd
Quercetin	nd	nd
trans cinnamic acid	nd	nd
Luteolin	nd	nd
Rosmarinic acid	nd	nd
Kaemferol	nd	nd
Apigenin	nd	nd
Chyrsin	0.023	0.017
4-hydroxylresorcinol	nd	nd
1,4-diclorobenzene	nd	nd

nd=not detected

Volatile composition of honey samples

GC-MS analysis of the two honey samples enabled the detection and identification of 15 volatile compounds in the AD sample and 14 in the NW sample as represented on table 2 showing their respective percentage compositions. The most abundant compound in AD and NW samples was α-terpineol acetate

and 1-(2-furanyl)-ethanone respectively. 1-(2-furanyl)-ethanone, n-octanal, cis-linalool oxide, pentyl pentanoate, 2-decen-1-ol and naphthalene were present in both honey samples in different amounts. Furfural, eucalyptol, 3-(1-cyclopentenyl) furan, isopinocampheol, benzoic acid ethyl ester and 6,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran were found exclusively in the NW honey sample while 6-nonenoic acid methyl ester, (E)-2-octenal, octanoic acid ethyl ester, 2-propyl-1-heptanol, (Z)-2-decenal, α-terpineol acetate, ethyl-5-methylnonanoate, n-dodecane and dodecanoic acid ethyl ester where exclusive contained in the AD honey sample. Common volatile compounds in honey are of diverse chemical groups notably monoterpenes, C13-norisoprenoid, sesquiterpenes, benzene derivatives and to some extent fatty alcohols, esters, fatty acids, ketones and aldehydes [21,22]. These volatile compounds are responsible for the aromatic properties of the honey samples which makes them attractive to consumers. Some of themare present in the nectar or honeydew collected by bees and could give information about plant origin and characteristics while others come about during honey collection, processing or storage. cis-linalool oxide, nonanol and decanal were identified in acacia honey [23]. cis-linalool oxide was present in high amounts in both honey samples and linalool derivatives have been reported in significantly high amounts in lychee and longan honeys honey samples. Many esters have been described to confer particular aroma to honey types[12]. Naphthalene was present in both samples while n-dodecane was found in AD sample and these hydrocarbons are believed to come from wax. However, naphthalene is believed to come from hives treated with moth repellents.

Some furan derived compounds were identified notably in NW honey sample. These furan compounds possibly result from inadequate thermal and storage conditions [24]. The smoke or fire used to chase the bees and access the hive during honey harvest could account for the occurrence of furan-derived compounds. Some researchers reported the presence of certain compounds such as 1-(2-furanyl)-ethanone found in our honey samples as also present in the smoke or the common fuels (pine needles, cypress leaves, fungus, sawdust...) used during honey harvest and the amount and type of compounds generated[25]. Proper honey collection and practices are necessary to minimize this type of contamination and unpleasant odours in honey. The volatile composition of the honey samples varies according to the floral origin and honeybee species that produced the honey. Honeybees foraging different floral sources produce honeys with different quality and quantity of volatiles [12].Some of the volatile compounds can be used to determine the floral sources and geographical origins of honeys samples.

Table 2. Volatile composition of AD and NW showing percentage composition by volume

RT	Compound Name	AD	NW
3.033	6-Nonenoic acid methyl ester	2.47	-
3.078	Furfural	-	3.87
4.509	1-(2-furanyl)-ethanone	1.42	25.03
8.405	n-Octanal	1.62	0.43
9.358	(E)-2-Octenal	2.40	-
9.580	Eucalyptol	-	2.09
9.757	3-(1-Cyclopentenyl) furan	-	1.23
10.007	Benzene acetaldehyde	-	21.27
11.124	cis-Linalool Oxide	15.68	26.53
12.491	Isopinocampheol	-	2.78
12.597	2-Decen-1-ol	15.80	6.87
12.857	Pentyl pentanoate	4.68	3.07
15.277	Benzoic acid ethyl ester	-	1.44
15.729	Naphthalene	1.95	1.81
16.427	Octanoic acid ethyl ester	3.90	-
16.568	2-propyl-1-heptanol	3.19	-
18.891	(Z)-2-Decenal	2.47	-
21.958	α-Terpineol acetate	25.67	-
23.712	Ethyl-5-methylnonanoate	3.17	-
23.937	n-Dodecane	5.86	-
27.267	6,7-dimethoxy-2,2-dimethyl-2H-1-Benzopyran	-	3.58
30.174	Dodecanoic acid ethyl ester	9.70	-

- **= not detected**

Besides conferring the aromatic properties to honey, the volatiles and their amounts in honey is known to influence the therapeutic properties such as antioxidant and. For instance, linalool and linalool oxide have been described as antibiotic volatiles which show broad-spectrum antimicrobial activity and antioxidant activity [26,27] and linalool oxide is found in both honey samples studied.

Antimicrobial and Anti-biofilm activities

The antimicrobial activity of the honey samples AD and NW were tested against *S. aureus*, *E. coli* and *C. albicans* and the results are given in Table 3. The honey samples inhibited the growth of all microorganisms tested between 0.5 and 1 mg/mL.*C. albicans* was the most susceptible microorganism showing a MIC of 0.5 µg/mL for both honey samples. *C. albicans* and *S. aureus* were more susceptible to NW sample with MIC 0.5 mg/mL as against 1 µg/mL for AD sample while AD showed higher activity on *E. coli* with MIC of 0.5 mg/mL as compared to NW with MIC of 1 mg/mL.

Diverse honeys differ in the potency of their antibacterial activity depending on plant source, geographical location, harvesting, processing and storage conditions[28]. Studies carried out so far have mainly investigated the antimicrobial activity of honey in relation to wound infections and can be concluded from in vitro studies that honey has powerful antimicrobial activity against dermatologically relevant microbes. Some of the bacteria used in this study are known to infect wounds and being susceptible to our honey types implies that theses honey could be applied externally for wound treatment. In a previous study, honey samples from Cameroon were shown to possess antibacterial activity which was variable according to the bacterial type and the source of honey sample [29].The antibacterial potency among different honey types is variable, primarily depending on its botanical, seasonal and geographical source, although harvesting, processing and storage conditions may affect the antibacterial properties of honey [10]. Besides these factors, the chemical composition of honey samples also depends on its chemical composition. Some researchers have equally demonstrated that honey samples of different botanical origins possess antibacterial activity which is mainly attributed to their H_2O_2 contents [30].

All the honey samples were tested for their ability to inhibit biofilm formation on *S. aureus* (Gram +), *E. coli* (Gram -), and *C. albicans* (yeast) within concentration range from MIC – MIC/16. All samples inhibited biofilm formation of all microorganisms tested in various percentages at MIC and MIC/2. AD exhibited the highest antibiofilm activity against *C. albicans* and *E. coli* with percentage inhibitions ranging from 13.9±0.2 (MIC/4) to 34.7±2.4 (MIC) for *C. albicans* and from 11.3±0.6 (MIC/8) to 46.2±1.8 (MIC) for *E. coli*. NW showed highest biofilm inhibition on *S. aureus* as the percentage inhibition varied from 20.7±4.0 (MIC/4) to 52.3±1.5 (MIC). NW showed relatively lower biofilm inhibition than AD and in all tested microorganisms there was no biofilm inhibition at MIC/16 for both samples.

Honeys inhibit biofilm production or disrupt preformed biofilms by many microorganisms but without complete biofilm eradication[28]and has been proven to be effective against biofilm forming clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*[31]. Considering the topical applications of honey, its antibiofilm activity is clinically important, especially since most traditional antibiotics do not inhibit biofilm formation. Although antimicrobial activity of Cameroonian honey against planktonic bacteria have been reported, no previously reported information on its antibiofilm activity study exists. Antibiotics may have only minimal long-term effects on preventing or treating established biofilms, as most antibiotics are designed to target metabolically active planktonic bacterial cells, while bacterial cells embedded in an extracellular polymeric substance matrix are unresponsive. Hence, there is an urgent need to introduce novel or re-emerging effective approachesto combat bacterial biofilms[32].The most logical approach to prevent bacterial biofilm formation is by inhibiting the initial binding of the bacterium to the tissue or biomaterial[33].

Table 3. MIC values(mg/mL) and anti-biofilm activity of AD and NW

	NW					AD				
	Planktonic	% inhibition on biofilm formation				Planktonic	% inhibition on biofilm formation			
Microbe	MIC	MIC	MIC/2	MIC/4	MIC/8	MIC	MIC	MIC/2	MIC/4	MIC/8
S. aureus	0.5	52.3±1.5	39.7±0.2	20.7±4.0	-	1	38.6±1.6	36.8±4.1	25.5±0.4	-
E. coli	1	29.1±3.0	15.5±0.9	4.0±0.71	-	0.5	46.2±1.8	40.0±2.1	26.7±0.5	11.3±0.6
C. albicans	0.5	22.7±1.0	12.3±2.5	-	-	0.5	34.7±2.4	26.4±1.3	13.9±0.2	-

- : no inhibition

Anti-quorum sensing activity using *C. violaceum* CV026(antimicrobial and anti-quorum sensing zones in mm)

The honey samples were screened on *C. violaceum* CV026 which is a Gram-negative bacterium that produces purple violacein pigment that acts as an antioxidant protecting the bacterial membrane against oxidative stress through a QS-mediated process[34]. Prior to this, the MIC of the honey samples were determined so that anti-quorum sensing activity is evaluated at MIC andsub-MIC concentrations. The MIC values of the honey samples NW and AD on *C. violaceum* CV026 were 0.5 mg/mL and 1 mg/mL, respectively. On the test plates, a white or cream-colored ring around the well against a purple lawn of

activated CV026 bacteria was an indication of QS inhibition while a clear halo indicated antimicrobial (AM) activity and both QS and AM zone diameters were measured in millimeters and reported on table 4. NW showed higher QS activity with QS inhibition zone of 15.0±1.5 mm at MIC and 7.5±2.0 mm at MIC/4, and AM activity with AM zone of 10.0±1.5 mm at MIC. The QS zone of NW varied from 7.5±2.0 (MIC/4) to 15.0±1.5 mm (MIC) while that of AD varied from 6.0±2.0 (MIC/4) to 12.5±1.5 mm (MIC) and no inhibition was observed at MIC/8 for both samples.

Anti-quorum sensing activity using *C. violaceum* CV12472(percentage violacein inhibition)

The bacterial strain *C. violaceum*ATCC 12472 was used in qualitative screening of the inhibition of violacein production exhibited by absence of violet colour and thus demonstrating quorum sensing (QS) inhibitory potential in *C. violaceum* by the honey samples. Prior to this, the MIC values of NW and AD on *C. violaceum* CV12472 were determined as 1 mg/mL and 0.5 mg/mL, respectively. At the concentration MIC, all compounds showed percentage inhibition of violacein formation of 100 %. At MIC/2 concentration, the sample AD showed 100 % inhibition still and no inhibition was observed at MIC/16 for both honey samples as shown on table 4. The sample AD showed highest percentage violacein inhibition ranging from 14.72±2.5 (MIC/8) to 100±0.00 (MIC and MIC/2). The sample NW had percentage violacein formation inhibitions ranging from 32.95±0.5 (MIC/4) to 100 (MIC).

Honey's ability to counter bacterial infections involves bactericidal aspects and QS inhibition. Since honey highly complex substance there may be other contributing factors or interdependent components. Thus, working at sub-MIC concentrations, the hypothesis of bactericidal effect of honey that occurs at high concentrations is eliminated giving way for QS investigation. Compounds such as *N*-acyl-homoserine lactones (AHLs) are signaling molecules produced by microorganisms to control cell-to-cell communication through a process called quorum sensing (QS) which mediate cellular processes and virulence factors such as population density biofilm formation. Some honey sample showed QS inhibition, and this involved both inhibition of AHL production and its degradation and the bacteria growth were not affected meanwhile biofilm formation was [22].Some results have shown that chemical compounds in honey responsible for QS inhibition may be found in all honey types regardless of plant source and is not affected by heat treatment. The most probable being sugar, which is the largest component in all types of honey [35].At diluted concentrations. the bioactive components of honey may be in little amounts but are still capable of inhibiting the QS related genes.

Table 4. Anti-quorum sensingactivityresults of AD and NW

Concentration	NW			AD		
	AM zone against CV026 (mm)	QS zone against CV026 (mm)	Violacein inhibition against CV 12472 (% inh.)	AM zone against CV026 (mm)	QS zone against CV026 (mm)	Violacein inhibition against CV 12472 (% inh.)
MIC	10.0±1.5	15.0±1.5	100±0.00	9.5±2.5	12.5±1.5	100±0.00
MIC/2	-	10.5±0.5	71.6±1.5	-	9.0±0.5	100±0.00
MIC/4	-	7.5±2.0	32.9±0.5	-	6.0±2.0	36.1±1.0
MIC/8	-	-	-	-	-	14.7±2.5

- : No activity

Swimming and swarming motility inhibition using *P. aeruginosa* PA01

Motility of microorganisms is implicated QS-mediated biofilm formation. Swimming and swarming motility of *P. aeruginosa* PA01 strain was evaluated at three concentrations of 50, 75 and 100 µg/ml and results presented on table 5. The honey samples inhibited the *P. aeruginosa* PA01 bacterial swimming and swarming motility at the three tested concentrations (50, 75 and 100 µg/ml) in a dose-dependent manner. The extents of inhibition of motility migration was relatively higher in the swarming model than in the swimming model for all samples. The sample NW exhibited the highest percentage inhibition of motility of 19.4±1.0 and 29.02±1.5 in swimming model and swarming model respectively at 100 µg/ml while AD showed the lower percentage inhibition of 17.79±0.5 and 18.60±1.0 in swimming and swarming respectively at 100 µg/ml. At the lowest tested concentration of 50 µg/ml, AD showed no inhibition of swimming motility but showed swarming motility inhibition of 4.91±0.5. At 50 µg/ml, NW exhibited inhibition of 0.72±0.5 and 3.57±1.0 in swimming and swarming motility respectively.

Table 5. Swarming and swimming motility percentage inhibiton of AD and NWsamples against *P.aeruginosa* PA01.

Concentration (µg/mL)	NW		AD	
	Swimming motility inh. (%)	Swarming motility inh. (%)	Swimming motility inh. (%)	Swarming motility inh. (%)
100	19.4±1.0	29.02±1.5	17.79±0.5	18.60±1.0
75	9.35±0.5	15.63±0.5	6.55±1.0	12.95±0.5
50	0.72±0.5	3.57±1.0	-	4.91±0.5

Antioxidant activity (IC_{50} values in µg/mL)

The antioxidant potential of NW and AD were evaluated using four different methods: DPPH radical scavenging assay, cupric reducing antioxidant capacity (CUPRAC), metal chelation and β-carotene-linoleic acid assay and the results are shown on table 6. Although no sample showed better activity than the standards BHT and α-tocopherol in the DPPH, metal chelation and β-carotene-linoleic acid assays, their values were moderate. NW sample was more active in the DPPH, CUPRAC and β-carotene-linoleic acid assays than AD. NW was more active showing IC_{50} of 136.58±1.81 than standard quercetin (IC_{50} 250.09±0.87) in metal chelation assay.

Honey has been shown to possess antioxidant capacity attributed to either its phenolic or volatile constituents. Various in vitro assays show that honey can scavenge different radicals and also reduce ferric cations, chelate metal ions and inhibit β-carotene bleaching [22]. This confirms our findings. Equally in in vivo models, honey has been proven to be able to stimulate the antioxidant defense mechanism in tissues such as pancreas, serum, kidney, and liver of mice thereby improving the potential of cellular antioxidant enzymes, such as superoxide dismutase, catalase etc and increasing the levels of reduced glutathione [36].The antioxidant potential of honey must be considered as the result of a combined effect of several compounds present in honeys, which depend on floral and geographical origins, collection and storage practices among other factors. Though in small amounts, the chrysin that was found in the honey samples has bee shown to be potent antioxidant compound capable of reducing oxidative problems and influencing enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) in tissues[37]. Honey serves as a source of natural antioxidants, which play an important role in food preservation and human health by combating damage caused by oxidizing agents.

Table 6. Antioxidant capacity of AD and NW

Sample	DPPH	Cuprac	Metal chelation	Beta carotene
NW	317.11±0.65	93.22±0.57	136.58±1.81	8.62±1.30
AD	489.85±1.33	83.52±2.10	264.38±0.70	11.70±1.86
BHT	45.37±0.47	3.80±0.00	-	1.34±0.04
α-tocopherol	7.31±0.17	10.20±0.01	-	2.10±0.08
EDTA	-	-	6.50±0.07	-
Quercetin	-	-	250.09±0.87	-

- : Not tested

CONCLUSION

With the emergence of antibiotic-resistant bacterial pathogens which are wide spreading, the effectiveness of the antibiotics is diminished posing a very serious threat to public health. honey has attracted new attention in the fight against drug-resistant bacteria. This has made researchers to develop interest in alternative antimicrobial therapeutics from plants and other natural sources. Since ancient times, honey has been used to control infections. Honey is a chemically complex substance capable of inhibiting bacterial communication known as quorum sensing (QS), a process based on the production and detection of diffusible signal molecules. QS mediates virulence factors such as motility and biofilm formation. The antibacterial properties of honey have been linked to different factors, including its chemical composition which in turn depends on its botanical origin and conditions of processing and storage. Honey from diverse geographical origins have also been shown to possess in vitro and in vivo antioxidant activity and natural antioxidants are important in reducing risks of diseases that involve oxidative stress parameters. No cases of bacterial resistance to honey samples have been reported, hence honey from various regions of the world remains a promising source of antimicrobial agents and need to be studied.

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REFERENCES

1. Tomadoni B, Moreira MR, Ponce A. (2016). Anti-quorum sensing activity of natural compounds against *Chromobacteriumviolaceum.* Ann. Food Sci. Nut. 1(1): 43-48
2. Fleitas MO, Cardoso MH, Ribeiro SM, Franco OL. (2019). Recent advances in anti-virulence therapeutic strategies with a focus on dismantling bacterial membrane microdomains, toxin neutralization, quorum-sensing interference and biofilm inhibition. Front. Cell Infect. Microbiol. 9:74. doi: 10.3389/fcimb.2019.00074
3. Kirienko NV, Rahme L, Cho Y-H. (2019). Editorial: Beyond antimicrobials: non-traditional approaches to combating multidrug-resistant bacteria. Front. Cell. Infect. Microbiol. 9:343. doi: 10.3389/fcimb.2019.00343
4. Varsha S,Zarine B.(2016). Broad Spectrum Anti-Quorum Sensing Activity of Tannin-Rich Crude Extracts of Indian Medicinal Plants. Scientifica5823013.http://dx.doi.org/10.1155/2016/5823013
5. Gonzalez JE, Keshavan ND.(2006). Messing with bacterial quorum sensing. Microbiol. & Mol. Biol. Rev. 70 (4): 859–875
6. Ren D, Zuo R, González-Barrios AF, Bedzyk LA, Eldridge GR, Pasmore ME, Wood TK. (2005). Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. Appl.& Env. Microbiol. 71 (7): 4022–4034
7. Manisha DM, Shyamapada M.(2011). Honey: its medicinal property and antibacterial activity. Asian Pac. J. Trop. Biomed. 1(2): 154-160
8. Piotr S.(2017). Antimicrobial Activity of Honey. Honey analysis. INTECH Open. DOI: 10.5772/67117
9. McLoone P, Mary W, Lorna F.(2016). Honey: A realistic antimicrobial for disorders of the skin. J. Microbiol. Immunol.& Infect. 49: 161-167
10. Stagos D, Nikolaos S, Christina T, Stamatina P, Charalampos A, Alexandros N, Fani K, SoultanaadamouA, Konstantinos P, Demetrios AS, Demetrios K, Dimitris M.(2018). Antibacterial and antioxidant activity of different types of honey derived from Mount Olympus in Greece. Int.J. Mol. Med. 42: 726-734
11. Terrab A, Gonzalez AG, Diez MJ, Heredia FJ. (2003). Characterisation of Moroccan unifloral honeys using multivariate analysis. Eur. Food. Res. Technol.218:88–95
12. Pattamayutanon P, Angeli S, Thakeow P, Abraham J, Disayathanoowat T, Chantawannakul P. (2017). Volatile organic compounds of Thai honeys produced from several floral sources by different honey bee species. PLoS ONE 12(2): 1-15. doi:10.1371/journal.pone.0172099
13. CLSI (Clinical Laboratory Standards Institute)(2006).Quality Control Minimal Inhibitory Concentration (MIC) Limits for Broth Dilution and MIC Interpretative Breakpoints (M27-s2). Wayne, Pennsylvania, USA
14. Merritt JH, Kadouri DE, O'Toole GA. (2005). Growing and Analyzing Static Biofilms. Current Protoc Microbiol. John Wiley & Sons, Inc., 2005.
15. Koh KM, Tham FY. (2011). Screening of traditional Chinese medicinal plants for quorum sensing inhibitors activity. J. Microbiol. Immunol. Infect. 44: 144–148
16. McLean RJC, Pierson III LS, Fuqua C.(2004). A simple screening protocol for the identification of quorumsignal antagonists. J. Microbiol. Meth. 58: 351–360
17. White JWJr. Honey. In Ed. Board (Eds.). (1978). Advances in Food Research. New York: Academic Press. (vol. 24).
18. Talpay B. (1988). Inhaltsstoffe des Honigs-Citronensaure (Citrat) [Contents of honey-citric acid (citrate)]. Dtsch. Lebensm-Rundsch. 84(2): 41–44
19. Suarez-Luque S, Mato I, Jose FH, Simal-Lozano J, Garcıa-Monteagudo JC. (2003). Different forms of maleic and fumaric acids (cis and trans of 2-butenedioic acid) in honey. Food Chem. 80 215–219
20. Lachman J, Hejtmánková A, Sýkora J, Karban J, Orsák M, Rygerová B. (2010).“Contents of major phenolic and flavonoid antioxidants in selected Czech honey,” Czech J. Food Sci. 28(5): 412–426
21. Da Silva PM, Gauche C, Gonzaga LV, Costa ACO, Fett R. (2016). Honey: chemical composition, stability and authenticity. Food Chem. 196:309–323
22. Miguel MG, Antunes MD,Faleiro ML.(2017). Honey as a Complementary Medicine. Integrative Med. Ins. 12: 1–15
23. Petretto GL, Tuberoso CI, Vlahopoulou G, Alessandro A, Alberto M, Saadia Z, Giorgio P.(2016).Volatiles, color characteristics and other physico-chemical parameters of commercial Moroccan honeys. Nat. Prod. Res. 30:286–292
24. Tanleque-Alberto F, Juan-Borrás M, Isabel E. (2019). Quality parameters, pollen and volatile profiles of honey from North and Central Mozambique. Food Chem. 277: 543–553
25. Tananaki C, Gounari S, Thrasyvoulou A. (2009). The effect of smoke on the volatile characteristics of honey. J. Apicult. Res. 48: 142–144
26. Lee S-J, Umano K, Shibamoto T, Lee K-G. (2005). Identification of volatile components in basil (*Ocimumbasilicum* L.) and thyme leaves (*Thymus vulgaris* L.) and their antioxidant properties. Food Chem. 91 (1):131–137
27. Pripdeevech P, Chumpolsri W, Suttiarporn P, Wongpornchai S. (2010). The chemical composition and antioxidant activities of basil from Thailand using retention indices and comprehensive two-dimensional gas chromatography. J. Serb. Chem. Soc. 75(11):1503–1513
28. Morroni G, Alvarez-Suarez JM, Brenciani A, Simoni S, Fioriti S, Pugnaloni A, Giampieri F, Mazzoni L, Gasparrini M, Marini E, Mingoia M, Battino M and Giovanetti E.(2018). Comparison of the antimicrobial activities of four honeys from three countries (New Zealand, Cuba, and Kenya). Front Microbiol. 9:1378. doi: 10.3389/fmicb.2018.01378

29. Teke GN,Ngienyikeh BE.(2016). Antibacterial activity of three Cameroonian honey types against some pathogenic species. Int. J. Biol. Chem. Sci. 10(6): 2477-2484
30. Farkasovska J, Bugarova V, Godocikova J, Majtan V, Majtan J. (2019). The role of hydrogen peroxide in the antibacterial activity of different foral honeys. Eur. Food. Res. Tech. 245:2739–2744
31. Alandejani T,Marsan J, FerrisW, Slinger R, Chan F.(2009). Effectiveness of honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Otolaryngol Head Neck Surg 141: 114–118
32. Sojka M, Ivana V, Marcela B, Juraj M.(2016). Antibiofilm efficacy of honey and bee-derived defensin-1 on multispecies wound biofilm. J. Med. Microbiol. 65: 337–344
33. Campeau-Michelle EM, Patel R.(2014). Antibiofilm Activity of Manuka Honey in Combination with Antibiotics. Int J Bacteriol. http://dx.doi.org/10.1155/2014/795281
34. Kothari V, Sharma S,Padia D.(2017). Recent research advances on *Chromobacteriumviolaceum.* Asian Pac. J. Trop. Med. 10(8): 744–752
35. Wang R, Melissa S, Ronen H, Laurence GR.(2012). Honey's ability to counter bacterial infections arises from both bactericidal compounds and QS inhibition. Front Microbiol. 3(144): 1-8
36. Erejuwa OO, Sulaiman SA, Ab-Wahab MS.(2012).Honey: a novel antioxidant. Molecules 17:4400–4423
37. Anand KV, Mohamed Jaabir MS, Thomas PA, Geraldine P.(2012)."Protective role of chrysin against oxidative stress in d-galactose-induced aging in an experimental rat model," Ger.Gerontol. Int. 12(4): 741–750

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