

Chemical Screening and Antibacterial Activity of Honey Produced in Benin

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Abstract

Objectives: This study aimed to evaluate the antibacterial activity of honeys produced in Benin against selected pathogenic strains and to determine their composition in plant secondary metabolites with known antibacterial activity. **Methods:** A total of 60 honey samples were collected in the country's three phyto-geographical zones during the dry and rainy seasons. Chemical screening for secondary metabolites was performed on raw undiluted honey. Undiluted honey (100%) and honey diluted at 75%, 50% and 25% were used to assess their antimicrobial effect *in vitro* on six reference strains (ref-S) and six meat isolated staphylococcal strains (meat-S). MIC and MBC were also determined. **Results:** Chemical screening of undiluted honeys revealed that tannins, flavonoids, leuco-anthocyanins, alkaloids, coumarins and reducing compounds were preponderant irrespective of season of production. Only the undiluted honeys were effective at inhibiting some of the strains, namely four ref-S (*P. mirabilis*, *S. aureus*, *S. epidermidis* and *S. oralis*) and three meat-S (*S. aureus*, *S. lentus*, *S. xylosus*). Season of honey production had a significant effect on mean Inhibition Zone Diameter (IZD) for ref-S. For these strains, greater IZD were found for honeys produced during the dry season, except for *S. aureus*. For meat-S, in contrast, zone of production had a significant effect, with greater IZD for honeys from the Sudanian zone. Strains differed significantly in their sensitivity assessed by IZD: across all honeys, the greatest IZD were against *P. mirabilis* and meat-S: *S. aureus* and *S. lentus*. MIC varied greatly among honeys and strains. For meat-S, the effects of production zone and season were significant and the highest MIC were found for honeys from the Sudanian zone. **Application/Improvements:** Benin honey has bacteriostatic properties against several pathogens, and influenced by season and zone of production. Further studies are warranted to substantiate these new findings and identify the active principles.

Keywords: Antimicrobial Activity, Benin, Honey, Human Pathogens, Phyto-Geographical Zone, Season of Production

1. Introduction

Antimicrobial agents have a therapeutic effect on the pathogenic microorganisms involved in microbial and/or

non-microbial diseases. Microorganisms are currently found in the environment (air, water) in food products, human outside and inside body. There are useful species on one hand and pathogenic causing infectious diseases and

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food borne illness in the other.¹ It has been that food-borne diseases are public health problem and an important cause of mortality in most of developing countries. So, the three pathogens most commonly causing food-borne diseases are: *Staphylococcus aureus*, *Salmonella* and *Clostridium perfringens*. Furthermore species like *Escherichia coli*, *Bacillus cereus*, *Vibrio cholerae*, *Shigella*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Campylobacter jejuni*, *Campylobacter coli*, *Arcobacter spp*, *Listeria monocytogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Rhanelia aquatilis* may also be responsible for gastroenteritis a few hours or days after food ingestion.² The pathogenicity of germs in the case of foodborne infections is manifested either by a higher loading or by the release of toxins. Thus, for the treatment of bacterial infections, the abusive and often uncontrolled use of antibiotics by self-medication increases the resistance selection pressure.³ Indeed, many cases of multidrug-resistant bacteria are reported in Benin, Côte d'Ivoire and elsewhere.^{4,6} So, to face the resurgence of increasing infectious diseases worldwide and the failure of most conventional antibiotics, it important to investigate natural products for their antimicrobials properties. Among the product to be considered for those investigations, we can mention honey.

Produced by from the nectar of flowers or exudates of trees *Apis mellifera*, honey is the natural sweet substance.⁷ Indeed, the therapeutic properties of honey have been investigated.^{8,9} The antimicrobial property depends on the combined presence of several factors that may have redundant activity, be mutually dependent, or have additive or synergistic activity depending on the target bacterial species.¹⁰ The origins of the antibacterial properties of honey are multiple such as hydrogen peroxide, polyphenols, acid pH, defensin-1 and methylglyoxal.¹¹⁻¹³ Nevertheless, various vegetable inhibitors (lysozymes, flavonoids, aromatic and volatile substances) also possess antibacterial properties.¹⁴ The nature of the antimicrobial compound depends on the type of flower, source of nectar.¹⁵ So, the geo-climatic characteristics may probably influence the antibacterial activity of the honeys.

In Benin, there is a lack of data on the biological potential of the honeys produced in the different localities. The aim of this study was to draw the chemical composition and evaluate the antimicrobial activity of homey collected in Benin.

2. Material and Methods

2.1 Study Area

Samples of honey were collected in the three climatic (Guinean, Sudano-Guinean and the Sudanian) zones of the Republic of Benin (Figure 1).

The Republic of Benin presents a varied range of climates characterized by relatively low annual precipitation ranging from 900 to 1300 mm per year. The combination of these seasons gave rise to three climatic zones spreading from south to north: the Guinean zone, the Sudano-Guinean zone and the Sudanian zone.¹⁶ The Guinean zone has four seasons and extends from the coast (6 ° 25 N) to the latitude of 7 ° 30 N. It has an average rainfall of 1200 mm per year with on average 250 days of rains. It records an average daily temperature ranging from 25 ° to 29 ° C. The humidity in the air varies between 69% and 97%. The Sudano-Guinean zone is located between 7 ° 30 'N and 9 ° 45' N. The rainfall regime in the Sudano-Guinean zone is uni modal (May-October) and the average annual rainfall varies from 900 mm to 1110 mm distributed mostly on average 113 days. The relative humidity varies from 31% to 98% in this area. The average insolation is 2305 hours per year. Temperatures range from 25 ° C to 29 ° C in this area. The Sudanian zone is located between 9 ° 45 N and 12 ° 25 N. The rainfall in this zone varies from 900 to 1100 mm per year, distributed on average over 145 days. Air humidity ranges from 18% during the harmattan (December to February) to 99% in August during the rainy season. The average monthly temperature varies from 24 ° C to 31 ° C in this area. The total number of sunny hours is 2862 per year.

2.2 Samples Collection

The honey samples (60) were collected both in rainy (30 samples) and dry (30 samples) season from beekeepers of the three phyto-geographical zone (10 samples per zones/season). Once collected in sterile labeled falcon tubes, the honey samples were stored at laboratory room temperature (25±2°C) for further analysis.

2.3 Microorganisms used for the Antimicrobial Activity

The tested microorganisms were composed of 6 references strains (*Staphylococcus aureus* ATCC 29213, S.

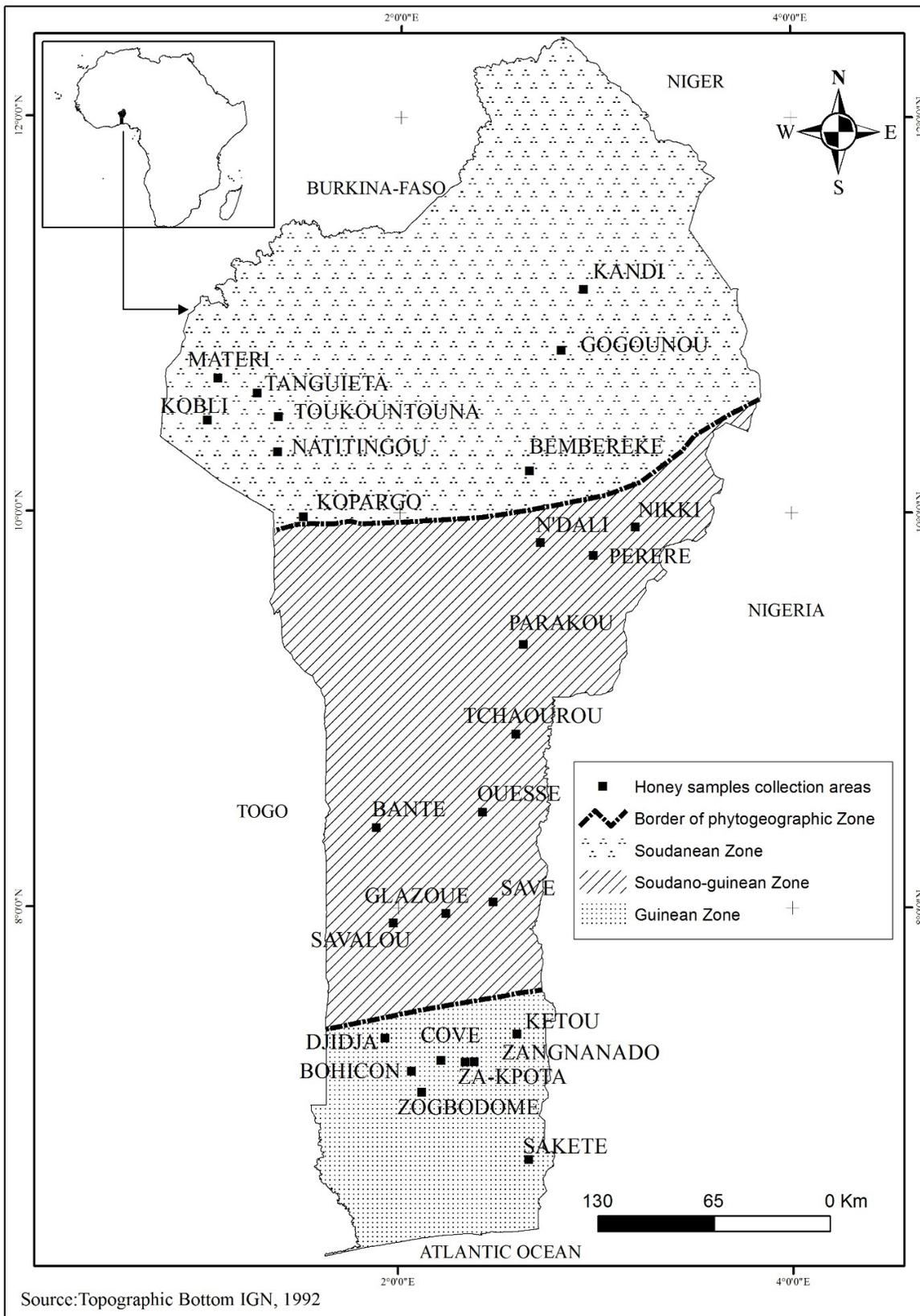


Figure 1. Map showing areas and locations of honey samples collection.

epidermidis T22695, *Streptococcus oralis*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* A24974 and *Escherichia coli* ATCC 25922) and 6 previously isolated food staphylococcal strains (*S. lentus*, *S. xylosus*, *S. aureus*, *S. sciuri*, *S. equorum* and *S. simulans*).¹⁷

2.4 Chemical Screening of Honeys

The major chemical groups (alkaloids, polyphenols, saponosides, coumarins, alkaloids, polyphenols, saponosides, coumarins, sterols, Terpenes, mucilages, proteins and reducing sugars) of the honey samples was explored using to the method previously describe by the article.¹⁸

2.5 Antimicrobial Activity of Tested Honey

The antimicrobial activity of four fraction (A: 1V honey +3 V sterile distilled water, B: 2V honey+2 V sterile distilled water, C: 3V honey+1 V sterile distilled water, and D: pure honey) honey samples was evaluated by disk diffusion method.¹⁹ Briefly, 100 µl of 10⁶ CFU/ml appropriate microbial suspensions were used to inoculate Mueller Hinton agar by flooding.²⁰ Sterile paper discs impregnated with 30 µl of the appropriate honey fraction (A, B, C or D) were then deposited on the medium. For each test, the experiment is duplicated and a negative control was performed using sterile distilled water. Plates were then left for 15-30 min at room temperature before being incubated at 37°C for 24 to-48 h. Diameters of inhibition zones were then measured using a sliding graduated scale after incubation times (24 h and 48 h).

2.6 Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MIC) of tested honey fractions was determined by micro-dilution method with visual appreciation of the growth of the microorganisms after 24 h of incubation.²¹ A 40-well micro plate was used for mix preparation (1 ml of the culture medium + 1 ml honey fraction). So the tested concentrations were: 50%, 37.5%, 25% and 12.5% of pure honey concentration. Culture medium without honey fractions and without microorganisms was used as controls. Thus, after gently mix, the content of the plate was incubated at 37 °C for 18-24 h and growth was indicated by turbidity. The lowest concentration of the honey at which the tested microorganism does not demonstrate visible growth was considered as minimum inhibitory concentrations.

2.7 Determination of the Minimum Bactericidal Concentration

The minimum bactericidal concentration of the tested microorganisms was determined by sub culturing method. For this, the content of each test well used in the minimum inhibitory concentration assay without microorganism growth after incubation were streaked on Muller Hinton agar plate and then incubated at 37°C for 24 h. The starter's lowest concentration without bacterial growth was identified and taken as Minimum Bactericidal Concentration.²²

2.8 Statistical Analysis

The results of all experiments were reported on the bench sheet and then inserted into an Excel 2010 database. The data were subjected to analysis of variance (ANOVA) using the SAS 9.2 Software. The Student Newman Keuls test ($p < 0.05$) was used to compare the means of inhibition zones between seasonal samples of the three phyto-geographical zones.

3. Results

3.1 Chemical Screening

The quantitative evaluation of homey samples shows a moderate contain of chemical compounds such as tannins, catechuic tannins, flavonoids and reducing compounds. Compounds such as Alkaloids, leuco anthocyanin, coumarins and the quinone derivate are slightly present in the tested honeys samples whereas anthocyanin, saponins, triterpenoids, cardenolidescyanogenic derivatives, O-glycosides and C-glycosides are completely absent (Table 1).

3.2 Inhibition Effect of the Tested Honey Samples on Reference Strains

The recorded data shows that only the undiluted honey (Faction D) inhibited the growth of microorganisms while the three diluted honey fractions (A, B and C) had no effect on the growth of the tested reference strains. Thus, our results reveals that the undiluted honey samples had antibacterial effect on some reference strains (Table 2). The dry season samples of the three collection localities, exhibited antibacterial effect on 3 (*P. mirabilis*, *S.*

Table 1. Chemical content of the tested homey samples

Class of chemical substance	Subgroups	Test Results	
		Dry season	Rainy season
Alkaloids		+	+
Polyphenolic compound	tannins	++	++
	catechic tannins	++	++
	gallic tannins	+	+
	flavonoids	+	+
	anthocyanin	-	-
	Leucoanthocyanes	+	+
Quinone derivatives		±	+
Saponins		-	-
Triterpenoids		-	+
Steroids		±	+
Cardenolites		-	-
Cyanogenic compound		-	-
Mucilages		-	+
Coumarins		+	+
Reducing compound		+	+
Anthracene derivatives	Free anthracene	±	±
	Combine anthracene:	±	+
	-O- glycosides	-	+
	-C- glycosides	-	-

Legend: ++: Fair Presence; +: Low presence; ±: trace,-: Absence

Table 2. Antimicrobial activity of collected honeys on the reference strains

Strains	Dry season			Rainy season		
	Sudanian	Sudano-Guinea	Guinean	Sudanian	Sudano-Guinea	Guinean
<i>P. mirabilis</i>	+	+	+	+	+	+
<i>P. aeruginosa</i>	-	-	-	-	-	-
<i>S. oralis</i>	+	+	+	+	-	-
<i>E. coli</i>	-	-	-	-	-	-
<i>S. epidermidis</i>	+	+	+	+	+	+
<i>S. aureus</i>	-	-	-	+	+	-

+ : Inhibition ; - : No inhibition

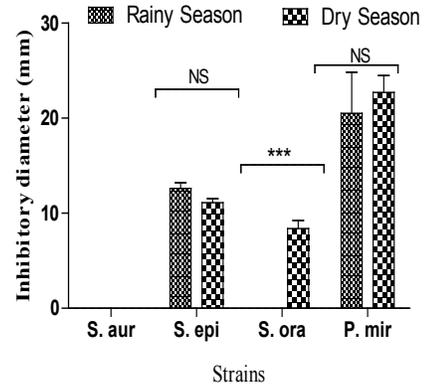
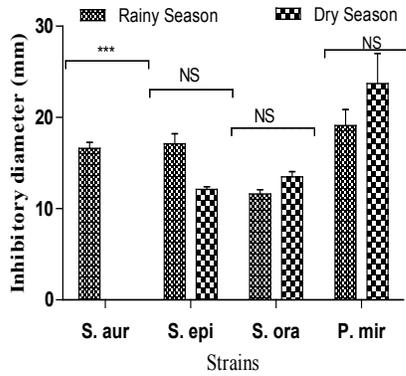
oralis and *S. epidermidis*) of the 6 tested reference strains. In addition of the three strains that displays sensitivity to the dry season honeys, *Staphylococcus aureus* were also sensitive to rainy season's honeys.

The variance of the mean inhibition diameter of the *S. aureus* revealed very high ($P < 0.001$) influence of the geographical area, season and their interaction (Table 2). Whereas only the season have an effect on the inhibition of *P. mirabilis*. Also, there was a very high difference between the median diameters of the inhibition diameter

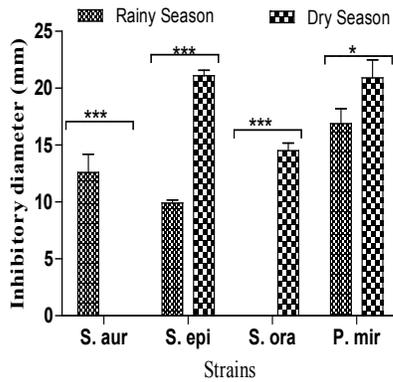
of the different geographical areas for *S. oralis* whereas the median diameter for *S. epidermidis* was not influence by the geographical areas and season (Figure 2).

The Figure 3 shows the compilation of inhibition diameter measured after 24 hours of incubation. Globally, considering the sensitive strains, the mean inhibitory diameter zones vary from 8.4 mm (*S. oralis* with dry season's honey collected in Guinean zone) to 23.70 mm (*P. mirabilis* with dry season's honey collected in Sudanian zone). The activity of the honey samples highly varies

according to the tested bacterial ($p < 0.0001$). Thus, independently to the samples collection zones, our data shows subsequent activity against *P. mirabilis* (Figure 2).



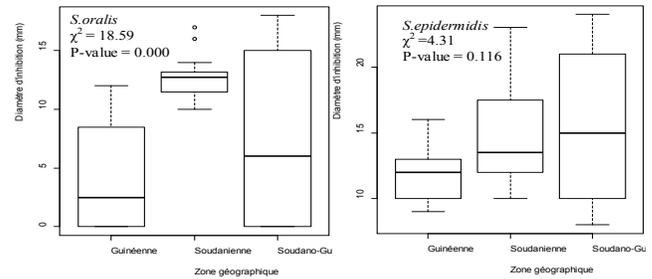
a. Sudanian



c. Guinean

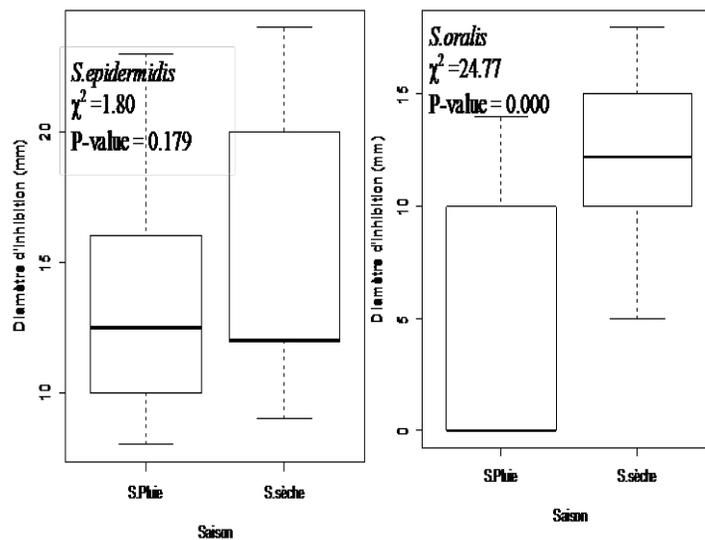
S. aur: *Staphylococcus aureus*, **S. epi:** *Staphylococcus epidermidis*, **P. mir:** *Proteus mirabilis*, **S. or:** *Streptococcus oralis*, ***: $p < 0.0001$, *: $p < 0.05$, NS: Non-Significant.

Figure 2. Mean inhibition zones' diameter of honey samples collected on some references strains after 24 h.



Geographical effect

b. Sudano- Guinea



Season effect (with S. Pluie means Rainy season and S. sèche means Dry season)

Figure 3. Boxplot showing the effect of the season and the phyto-geographical zone.

Table 3. Antimicrobial activity of collected honeys on the meat isolated *Staphylococcus* strains

<i>Staphylococcus</i> Strains	Dry season			Rainy season		
	Sudanian	Sudano-Guinea	Guinean	Sudanian	Sudano-Guinea	Guinean
<i>S. lentus</i>	+	+	+	+	+	+
<i>S. xylosus</i>	+	+	+	+	+	+
<i>S. aureus</i>	+	+	+	+	+	-
<i>S. sciuri</i>	-	-	-	-	-	-
<i>S. equorum</i>	-	-	-	-	-	-
<i>S. simulans</i>	-	-	-	-	-	-

+ : Inhibition ; **-** : No inhibition

Table 4. Effect of the phyto-geographical zone and season on the mean diameter of inhibition of meat isolated *Staphylococcal* strains

Source de variation	<i>S. lentus</i>			<i>S. xylosus</i>		<i>S. aureus</i>	
	DL	F-value	P-value	F-value	P-value	F-value	P-value
Season	1	1.26	0.267 ns	1.23	0.272 ns	7.06	0.010 **
Zone	2	8.31	0.001***	3.54	0.036**	35.44	0.000***
Season-Zone	2	1.08	0.347 ns	0.68	0.511 ns	0.36	0.700 ns

ns : $p > 0,05$ (no significant) **: $p < 0,01$ (highly significant) *** : $p < 0,001$ (very highly significant)

Table 5. SNK test result on the average inhibitory diameter of meat isolated *Staphylococcus* strains according to phyto-geographical zone

zones	<i>S. lentus</i>		<i>S. xylosus</i>		<i>S. aureus</i>	
	m	cv	m	cv	m	cv
Guinean	15.03 ^a	21.29	13.66 ^{ab}	14.55	12.32 ^b	17.08
Sudanian	17.25 ^a	25.16	15.80 ^a	29.94	20.20 ^a	18.21
Sudano-Guinean	12.88 ^b	21.15	12.67 ^b	26.69	13.83 ^b	22.12

In a same column, values with the same letters are not significantly different.

Table 6. Student test result on the Mean inhibition diameter of meat isolated *Staphylococcus* strains according to seasons

Season	<i>S. lentus</i>		<i>S. xylosus</i>		<i>S. aureus</i>	
	m	cv	m	cv	m	cv
Rainy season	14.57	25.34	14.51	25.36	14.39	28.25
Dry season	15.53	26.07	13.58	27.85	16.51	29.18

Considering sudanian zones' honey samples, dry seasons' were highly active on *S. aureus* in comparison to the rainy seasons' ($p < 0.0001$). There was no significant difference ($p > 0.05$) between the samples of rainy and dry season for *S. epidermidis*, *S. oralis* and *P. mirabilis* (Figure 2).

The dry seasons season's honeys of sudano-guinea zone were highly inhibited the growth of the *S. epidermidis*, *S. oralis* ($p < 0.0001$) and *P. mirabilis* ($p < 0.05$) (Figure 2).

The honeys from the Guinean zone were active on three strains in dry season and two in rainy season (Figure 2). The inhibitory diameters were similar for the two strains both in dry and rainy season ($p > 0.05$).

3.3 Inhibition Effect of Tested Honey Samples on Meat Isolated Strains

As mentioned above, the recorded data shows that only the undiluted honey inhibited the growth of three (*S. aureus*, *S. xylosus* and *S. lentus*) food isolated *Staphylococcus* strains during the two collection season (Table 3).

The analysis of meat isolated strains revealed that the area of honey samples significantly influenced ($P < 0.000$)

their inhibition diameter except for *S. aureus*, in which the season also significantly impacted the diameter of inhibition (Table 4). Also, the results of the SNK test revealed that the Sudanian zone displays the largest diameter of inhibition in all meat isolated strains (Table 5). There was no significant difference in season for *S. lentus* and *S. xylosus* over the season, but *S. aureus* gave the largest diameter of inhibition in the dry season (Table 6).

3.4 Minimum Inhibitory and Bacterial Concentrations of Honey on References and Food Isolated Strains

3.4.1 Minimum Inhibitory Concentrations

On the reference bacterial stains, the recorded MIC varies depending not only on the types of strains but also on the collection period and the zone of the honey (Table 7). The MIC varies from 50% to 12.5% of pure collected honey samples. Thus, considering the rainy season samples, the highest MIC (50%) was recorded with *P. mirabilis* (honey samples collected from Sudanian zone) and *S. epidermidis* (Guinea zone). The smallest MIC (25%) was obtained on the same strains with honey samples from the same areas.

Table 7. Minimum Inhibitory Concentration of the honey samples on some references strains

Zones	CMI (%)					
	Rainy season			Dry season		
	<i>P. mirabilis</i>	<i>S. epidermidis</i>	<i>S. oralis</i>	<i>P. mirabilis</i>	<i>S. epidermidis</i>	<i>S. oralis</i>
Sudanian	50	12.5	>50	50	12.5	>50
Sudano-Guinean	25	25	25	50	37.5	37.5
Guinean	12.5	50	>50	50	50	>50

Table 8. Minimum Inhibitory Concentration of the honey samples on some meat isolated *Staphylococcus* strains

Zones	CMI (%)					
	Rainy season			Dry season		
	<i>S. lentus</i>	<i>S. aureus</i>	<i>S. xylosus</i>	<i>S. lentus</i>	<i>S. aureus</i>	<i>S. xylosus</i>
Sudanian	50	50	37.5	37.5	37.5	12.5
Sudano-Guinean	>50	50	25	12.5	37.5	25
Guinean	12.5	>50	>50	12.5	37.5	12.5

Considering the food isolated *Staphylococcus* strains, the lowest MIC (12.5%) was recorded with *S. lentus* strain using the rainy season samples whereas the highest was with *S. lentus* (Sudanian) and *S. aureus* (Sudano-Guinean) (Table 8).

3.4.2 Minimum Bacterial Concentrations

There was no CMB recorded during this investigation.

4. Discussion

It appears from the chemical screening the presence of certain secondary metabolites (tannins, catechic tannins, flavonoids and reducing compounds) in honey (Table 1). Several research has been conducted worldwide on the potential antibacterial honey in order to overcome not only the bacteria resistance but also to highlight the therapeutic properties of honey. Thus, the antibacterial activity of honey was reported for the first time since in 1892 by Bogdanow.²³ Later, reported *in vitro* that honey has considerable anti-microbial activity against some pathogenic bacteria.²⁴

Considering the references strains, 3 (*Proteus mirabilis*, *Streptococcus oralis* and *S. epidermidis*) of the 6 were sensitive to dry season honey samples whereas 4 (*P. mirabilis*, *St. oralis*, *S. epidermidis* and *S. aureus*) were sensitive to those of the rainy season. These results clearly show that our honeys are endowed with a broad spectrum of inhibitory activity on the Gram + and - bacterial strains. So, it appears that the Gram + bacteria (*S. epidermidis*, *Str. oralis* and *S. aureus*) are more sensitive to honey. Thus, the effect of honey to microorganisms depends not only on its composition but also on the nature of the target cell. This observation seems different from those reported on *Pseudomonas aeruginosa*.²⁵ However, the composition of honey itself depends on many factors, such as the nature of the soil, bees and the physiological state of the colony.²⁶ However, some honey samples studied were inactive on the reference strains (*P. aeruginosa*, and *E. coli* ATCC 25922) and food strains (*S. sciuri*, *S. equorum* and *S. simulans*). Our results are contrary to those obtained by the presented article who found that in Argentina the honey collected from the southeast region of Buenos Aires province has antibacterial activity against *E. coli* ATCC 25922 at 25% and 50% (w/v) concentrations.²⁷ This difference could be explained not only by the nature of the strains but also by the type (monofloral or multifloral) of honey.

An excellent activity of the undiluted honey samples was observed with the meat isolated *Staphylococcus*

strains particularly on *S. aureus*. These found confirm those of several authors.^{23,24,28,29} Indeed, it was reported that *S. aureus* is the most susceptible bacterial species to honey from collected in Iraq, in Nigeria and Egypt.^{23,24,29} The antimicrobial activity of the honeys may probably be due to the osmotic effect, the acidity, hydrogen peroxide and/or phytochemicals content.^{29,30} During our investigation, only pure honeys displays activities and none of the diluted fraction indicated any antimicrobial effect. This results are not similar to those reporting that the antibacterial activity of honey increased when honey was diluted.^{11,31-33} But our results corroborate those mentioned in studies conducted on *Escherichia coli*, *Staphylococcus aureus*, *Clostridium perfringens* and *Bacillus subtilis*.^{34,35} Indeed, due to its composition, honey is an unfavorable environment for microorganisms grow.¹² So, the inactivity of the diluted honeys could be explained by the fact that the peroxidase secreted by the bee may not be the main antibacterial agent against the tested bacterial strains. Thus, molecules grouped under non-peroxidic inhibitors may be involved, in our case, in the antimicrobial activities. Those non-peroxidic inhibitors have either plant origin or added by bees when making honey. The role of non-peroxidic inhibitors, often underestimated, is very important because they are to a large extent insensitive to light, heat and remain intact after storage of the honey for long periods.

This study revealed that the *in vitro* antibacterial activity of the different honey samples varies from one sample to another, from one zone to another and from one season to another. The results concerning the inhibitory diameter zone are not similar to those performed in Algeria of showing that *Staphylococcus aureus* is the most sensitive strain whereas *Pseudomonasaeruginosa* is moderately sensitive.³⁶ The differences observed between the above mentioned results with our data may be due to the methodology, the composition, origin and the harvest period of the honey that is used.^{37,35} The antibacterial activity was manifested with all the undiluted honeys it is bacteriostatic more than bactericidal. This confirms the work of, which reports that the antibacterial action of honey is essentially bacteriostatic, that is, in the presence of honey, the bacteria do not develop but remain alive.¹³

5. Conclusion

At the end of this study, we can say that the honey samples collected in Benin contain some secondary metabolites namely: tannins, catechial tannins, flavonoids and reduc-

ing compounds. Only the undiluted honey is active on both reference strains and meat isolated Staphylococcus strains. The antibacterial effect varies a samples collection to another and from a season to another. These results clearly indicate that our honey samples are endowed with a broad spectrum of antibacterial action on Gram + and Gram- bacterial strains. These findings could be applied in the treatment of various diseases caused by pathogenic germs. Thus the present work gives some knowledge about certain biological values of the Benin honeys. However, it is not exhaustive and must be completed in order to identify all their therapeutic potentials.

6. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper

7. Acknowledgement

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