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In vitro Susceptibility of Some Bacteria Strains to Ethanol Extracts of Propolis Collected from Two Different Geographic Origins in Cameroon

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Abstract: Antibacterial activity of fifteen samples of Ethanol Extracts of Propolis (EEP) prepared from propolis collected in two different geographic regions of Cameroon were investigated. Those antibacterial properties were determined by the well diffusion method on agar medium and by evaluating the Minimal Inhibitory Concentration (MIC) according to the macrodilution method. The activities of EEP were tested against seven strains of bacteria including four gram positive strains and three gram negative strains. All the samples of EEP studied were active only against gram positive bacteria. The most active samples were the EEP1 and EEP12 ($p<0.05$). Considering the MIC values, the most susceptible strains to the most active EEP tested were *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus subtilis* with a MIC$\leq$18.60 μg mL^{-1}, the least susceptible strain was *Enterococcus faecalis* to the EEP11 with a MIC value equal to 36.20 μg mL^{-1}. Considering the Principal Component Analysis (PCA), the areas of the minor and the major peaks of the phenolic compounds obtained by HPLC showed a relationship with antibacterial activities of the EEP. The EEP antibacterial properties were also linked to their geographic origins.

Key words: Ethanol extracts of propolis, antibacterial activity, minimal inhibitory concentration, *in vitro*, phenolic compounds, HPLC

INTRODUCTION

Propolis is a resinous substance collected by honeybees from various plant sources around their hive. Marcucci (1995) has noted that the compounds in raw propolis originate from three sources: plants exudates collected by bees, secreted substances from bee metabolism and materials which are introduced during propolis elaboration. In general, crude propolis is composed of 50% resin and vegetable balsam, 30% wax, essential and aromatic oils, 10% salivary secretion of bees, 5% pollen and 5% various other substances including amino acids, minerals, ethanol, vitamins A, B complex, E and bioflavonoids (Monti *et al*., 1983; Cirasino *et al*., 1987; Marcucci, 1995).

Propolis contains a variety of chemical compounds such as polyphenols (favonoid aglycones, phenolic acids, phenolic aldehydes, alcohols and ketones), terpenoids, steroids and inorganic compounds (Dimov *et al*., 1991; Volpert and Elstner, 1993; Moreno *et al*., 2000). Chemical composition of propolis is linked to its geographical and botanical origins (Kujumgiev *et al*., 1999; Moreno *et al*., 2000; Kumazawa *et al*., 2004). The presence of propolis within the hive provide an environment not suitable for the growth of bacteria and other micro-organisms (Kartal *et al*., 2003). Among the biological properties of propolis, its antibacterial activities have been reported by Ghisalberti (1979), Mochida *et al*. (1985), Velikova *et al*. (2000), Castaldo and Capasso (2002), Stepanovic *et al*. (2003), Sonmez *et al*. (2005), Choi *et al*. (2006). Others biological activities have been established such as anti-inflammatory (Miyataka *et al*., 1997), anticancer (Burdock, 1998), antioxidant (Sun *et al*., 2000; Isla *et al*., 2001; Choi *et al*., 2006), antifungal (Ota *et al*., 2001; Choi *et al*., 2006), antihepatotoxic (Banskota *et al*., 2001), antiviral (Amoros *et al*., 1994; Gekker *et al*., 2005); dental care (Koo *et al*., 2002; Santos *et al*., 2003). The mechanism of antimicrobial activity of propolis is attributed to a

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synergism between phenolic and other compounds in the resin (Burdock, 1998). The present research consists to investigate the *in vitro* susceptibility of some bacteria strains to Ethanol Extracts of Propolis (EEP) samples collected in Cameroon and to determine the eventual linkage of their activity to their geographic origin after statistical analysis.

MATERIALS AND METHODS

Propolis origins: Samples characteristics of propolis analysed are shown in the Table 1. Propolis samples were collected with hand and 10 g of each were kept dry in the dark at room temperature in appropriate bags. Samples were analysed on may 2006 in the Laboratory of Science and Food Engineering (Nancy, France). Thus, the samples collected on december 2003 were stored 2.5 years before analysis while the samples collected on March or April 2005 were stored 1 year before analysis. About 3 g of crude propolis were ground in a mortar and extracted in amber flasks with 10 mL of 70% (v/v) ethanol (a final suspension of 30% (w/v) propolis was obtained) by moderate shaking at 210 rounds min^{-1} (Grant GLS400 shaker) for 7 days at room temperature (22°C). At the end of extraction, the mixture was centrifuged at 5000 rounds min^{-1} for 15 min (refrigerated Eppendorf Centrifuge 5804R), the supernatant was collected and kept in dark at room temperature until use as EEP.

Bacterial strains: All the seven bacterial strains tested were provided by the Laboratory of Science and Food Engineering of ENSAIA-INPL (Nancy, France). They are:

- *Salmonella enterica* sp. *enterica* CIP 81.3
- *Staphylococcus aureus* CIP 7625
- *Escherichia coli* CIP 54.8
- *Enterococcus faecalis* CIP 76117
- *Listeria monocytogenes* CIP 82110
- *Pseudomonas fluorescens* CIP 6913
- *Bacillus subtilis* CIP 6624

Susceptibility tests: Qualitative test were investigated by the well diffusion method on agar medium. About 100 mL of TSA-YE medium (Trypcase Soja Agar-Yeast Extract)+tween 80 inoculated with 1 mL of an 18 h pre-culture of the *Bacillus subtilis* strain or 0.1 mL of an 18 h pre-culture of others bacterial strains obtained in TSB-YE medium (Trypcase Soja Broth-Yeast Extract) were poured in Petri dishes (15 mL of agar medium per dish). After solidification of the medium, six well were created in the agar per dish using a sterile Durham test tube. About 20 µL of each EEP sample were introduced per well and then, 20 µL of 70% ethanol were introduced in a well per Petri dish and used as negative control. All the dishes were placed for 24 h in a refrigerator at 4°C. Dishes containing *Pseudomonas fluorescens* strain were incubated at 30°C and dishes containing others strains at 37°C during 18 h. After incubation, the antibacterial activity of EEP was evaluated by measuring the inhibitory zone (total diameter of inhibition zone around each well-diameter of the well (6 mm)). An inhibitory zone with a diameter equal to zero corresponds to the lack of the activity of the EEP. Ampicillin 10 µg mL^{-1} was used as antibiotic control.

For quantitative test, the Minimal Inhibitory Concentration (MIC) was determined by the macrodilution method according to the National Committee of Clinical Laboratory Standard guidelines (NCCLS, 2000). A bacterial inoculum of 10^4 UFC mL^{-1} was prepared with an 18 h pre-culture of the bacterial strains in a double concentration TSB-YE medium. Then, two fold serial dilutions of EEP were prepared in hemolysis tubes as follows: to 1 mL of bacterial inoculum was added sterile distilled water and/or 70% ethanol to yield a total volume of 2 mL per tube in order to obtain final concentrations of 0.5, 1, 3, 4, 5, 6, 8, 9, 10, 11, 13 and 14% (v/v). Several controls such as: TSB-YE not inoculated+70% ethanol, TSB-YE inoculated+sterile distilled water+70% ethanol and TSB-YE inoculated+sterile distilled water were prepared. All test tubes were incubated at 37°C for 24 h. After incubation, 50 µL of each tube contain were inoculated in TSA-YE medium with a WASP 2 DW Scientific Limited spiral inoculator (Whitley Automatic Spiral Plater). Two Petri dishes were prepared per dilution and incubated at 37°C for 24 h. The MIC values were defined as the lowest concentration of EEP inhibiting completely the bacterial growth in Petri dishes culture. The expression of quantitative test (MICs values in µg mL^{-1}) was done after taking in consideration the dry extracts values.

HPLC analysis of phenolic compounds of propolis extracts: The phenolic compounds of EEP were analysed by injection of 50 µL of each sample in a chromatograph (SHIMADZU 10A) equipped with a LichroChart PUROSPHER RP18 column of 250 mm length; internal diameter, 4 mm and particle size, 5 µm. The column was eluted by using a linear gradient of water (solvent A) and methanol (solvent B) starting with 30% B (0-15 min) and increasing to 90% B (15-75 min) held at 90% B (75-95 min) and decreasing to 30% B (95-105 min) with a solvent flow rate of 1 mL min^{-1} at 30°C. The detection was done with a diode array detector (SHIMADZU SPD-M10). Chromatograms were recorded at 268 nm for phenolic compounds quantification (Markham *et al*., 1996).

Statistical analysis: The test of antibacterial activities of EEP (measurements of the diameters of the inhibition zones expressed in mm) was made in duplicate and subjected to Analysis of Variance (ANOVA) using STATGRAPHICS Plus 5.0 program. Results were expressed as mean standard deviation and the level of $p<0.05$ was used as the criterion for statistical significance. The Principal Component Analysis (PCA) of the EEP samples was also done using XLSTAT program.

RESULTS AND DISCUSSION

Dry extracts yield, inhibitory growth zone and Minimal Inhibitory Concentration (MIC) of the EEP samples: According to the values of dry extracts (Table 1), the lowest dry extracts yield were those of EEP3 (3.03%) and EEP13 (3.05%) while EEP15 (7.24%) and EEP7 (7.57%) gives the best yield.

Table 2 shows the antibacterial activity of the different EEP. Four strains of gram positive bacteria and three strains of gram negative bacteria were tested. All the EEP samples studied showed activity only against gram positive bacteria. No activity against all the bacteria tested was not detected when the 70% ethanol were used as solvent control. Gram positive bacteria showed an intermediary susceptibility comparatively to the control antibiotic while gram negative strains were resistant to all the EEP tested.

Table 1: Samples characteristics of propolis analysed

Propolis number	Sample location	Collection date	Colour	Hardness	DE in% (w/w)
1	Meiganga*	December 2003	Dark	+++	3.27
2	Meiganga	December 2003	Dim	+++	4.00
3	Meiganga	December 2003	Dim	+++	3.03
4	Bahouan**	April 2005	Dark± yellowish	++	5.88
5	Bahouan	April 2005	Dark	++	3.63
6	Bahouan	April 2005	Dark	++	4.07
7	Bahouan	April 2005	Dark	++	7.57
8	Bahouan	April 2005	Dark	++	3.65
9	Bahouan	April 2005	Dark	++	6.43
10	Bahouan	April 2005	Dark	++	3.58
11	Bahouan	April 2005	Dark	++	3.44
12	Meiganga	April 2005	Dark	++	4.20
13	Meiganga	April 2005	Dark	+++	3.05
14	Meiganga	April 2005	Dim	+++	5.00
15	Dang*	March 2005	Dim	+	7.24*

*localities of Adamaoua region (Cameroon); **locality of West region (Cameroon). +less hard; ++hard; +++: very hard; DE = Dry Extracts

The greatest inhibition zones were observed for EEP12 against *L. monocytogenes* (5.0±0.1 mm), *S. aureus* (4.8±0.2 mm), *B. subtilis* (3.8±0.0 mm) and *E. faecalis* (3.6±0.1 mm). The highest inhibition zone (5.0±0.1 mm) was that of EEP12 against *L. monocytogenes* while EEP9 and EEP15 showed the lowest activity against all the bacteria tested. The susceptibility of gram positive bacteria against active EEP decreased with the following order: *L. monocytogenes*>*S. aureus*>*B. subtilis*>*E. faecalis*.

The MICs of the most active EEP against the most susceptible bacteria are shown in the Table 3. As can be seen, the most susceptible bacterial strains against the most active EEP were *L. monocytogenes*, *S. aureus* and *B. subtilis* with a MIC ≤18.60 μg mL^{-1}. The EEP11 was less active on *E. faecalis* with a MIC of 36.20 μg mL^{-1}, value that was more than two time the value of the same EEP sample on *L. monocytogenes* and *B. subtilis.*

The MIC values of the EEP samples obtained showed that the susceptibility of bacterial strains decreased as follows: *L. monocytogenes*>*S. aureus*>*B. subtilis*>*E. faecalis*. These results are in agreement with those of the qualitative tests.

HPLC profiles of phenolic compounds of propolis extracts: Figure 1 and Table 4 show HPLC chromatograms and areas of minor and major peaks of phenolic compounds of some EEP, respectively. These results showed that areas of minor and major peaks were less

Table 2: Antibacterial activity of EEP (mean values of the diameters of the inhibition zones in mm*)

Bacteria	Sample number of EEP															
	C	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
S.a	7.5±0.7^{g}	4.5 ±0.1^{f}	3.0 ±0.2^{c}	3.5 ±0.1^{de}	3.0 ±0.0^{c}	3.0 ±0.1^{c}	2.3 ±0.3^{b}	2.1 ±0.1^{b}	2.2 ±0.2^{b}	1.6 ±0.1^{a}	3.0 ±0.0^{c}	2.8 ±0.1^{c}	4.8 ±0.2^{f}	3.6 ±0.1^{e}	3.1 ±0.2^{cd}	2.0 ±0.0^{ab}
S.e	5.0 ±0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.c	5.2 ±0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.f	5.8 ±0.1^{g}	3.0 ±0.3^{e}	2.5 ±0.1^{cd}	2.0 ±0.2^{b}	1.1 ±0.1^{a}	2.2 ±0.2^{bcd}	1.0 ±0.0^{a}	1.0 ±0.1^{a}	1.2 ±0.2^{a}	1.0 ±0.1^{a}	2.1 ±0.1^{bc}	2.6 ±0.0^{de}	3.6 ±0.1^{f}	2.0 ±0.0^{b}	2.0 ±0.0^{b}	1.0 ±0.0^{a}
L.m	8.0 ±0.4^{h}	4.8 ±0.0^{fg}	4.5 ±0.3^{def}	3.5 ±0.1^{d}	4.2 ±0.2^{de}	4.1 ±0.3^{e}	4.6 ±0.1^{efg}	1.6 ±0.1^{b}	2.6 ±0.1^{c}	1.0 ±0.0^{a}	2.0 ±0.0^{b}	4.7 ±0.2^{fg}	5.0 ±0.1^{g}	3.5 ±0.0^{d}	2.6 ±0.1^{c}	1.1 ±0.1^{a}
Ps.f	6.5 ±0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B.s	6.6 ±0.1^{e}	3.6 ±0.2^{d}	3.0 ±0.0^{c}	1.0 ±0.3^{a}	1.0 ±0.0^{a}	1.2 ±0.2^{a}	1.0 ±0.0^{a}	1.1^{a} ±0.1	1.0 ±0.0^{a}	1.0 ±0.0^{a}	2.5 ±0.0^{b}	3.2 ±0.2^{c}	3.8 ±0.0^{d}	1.0 ±0.0^{a}	1.0 ±0.0^{a}	1.0 ±0.0^{a}

Values that have not the same letter in superscripts on the same line are significantly differents ($p<0.05$). C = Control (ampicillin 10 μg mL^{-1}); S.a = *Staphylococcus aureus*; S.e = *Salmonella enterica*; E.c = *Escherichia coli*; E.f = *Enterococcus faecalis*; L.m = *Listeria monocytogenes*; Ps.f = *Pseudomonas fluorescens*; B.s = *Bacillus subtilis*. -= Diameter of the inhibition zone lower or equal to 6 mm. *The tests were done in duplicate

Table 3: Minimal Inhibitory Concentration (MIC) of the most active EEP against the most susceptible bacteria

	MIC of EEP (μg mL^{-1})				
Bacteria	EEP1	EEP2	EEP10	EEP11	EEP12
L. monocytogenes	11.10	ND	18.60	16.30	10.40
S. aureus	12.60	ND	ND	ND	ND
B. subtilis	ND	17.10	ND	14.20	12.50
E. faecalis	ND	ND	30.40	36.20	ND

ND = Not Determined

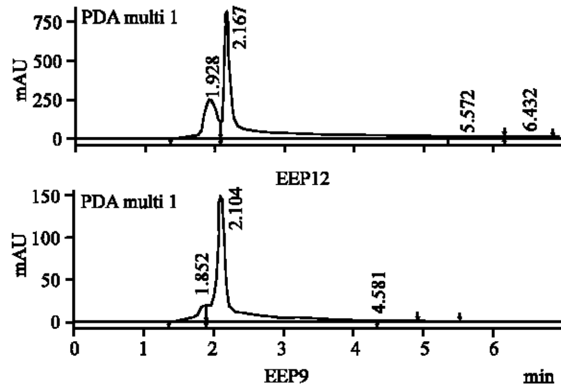

Fig. 1: HPLC chromatograms of phenolic compounds of the most and the least active Ethanolic Eextracts of propolis (EEP) sample

Table 4: Peaks area (minor and major) obtained after HPLC analysis of phenolic compounds of some EEP

	Minor peak			Major peak		
Samples	RT (min)	λ (nm)	Area	RT (min)	λ (nm)	Area
EEP1 (20x)	1.851	268	774 065	2.100	268	3 764 572
EEP2 (20x)	1.852	268	712 815	2.096	268	3 314 778
EEP10 (20x)	2.085	268	199 632	2.306	268	1 729 591
EEP9 (20x)	1.852	268	188 768	2.104	268	1 718 892
EEP12 (20x)	1.928	268	793 544	2.167	268	3 904 107
EEP15 (20x)	1.852	268	202 148	2.100	268	1 723 168
EEP11 (20x)	1.891	268	728 428	2.133	268	3 358 336

20x = dilution rate; RT = Retention Time in min; λ = wavelength innanometer

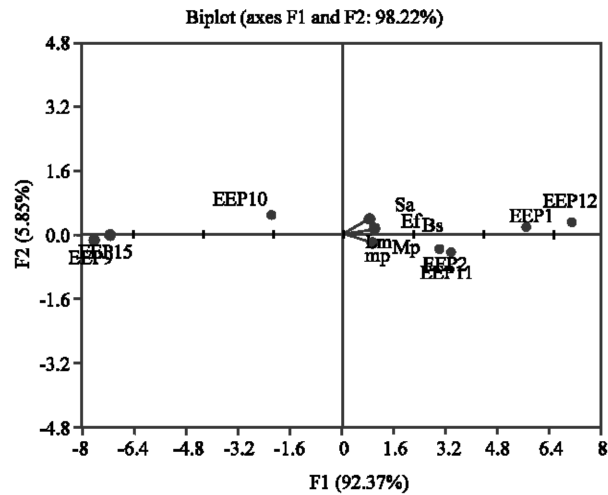

Fig. 2: Principal Component Analysis (PCA) of the most and the least active EEP samples; Sa = *Staphylococcus aureus*; Ef = *Enterococcus faecalis*; Lm = *Listeria monocytogenes*; Bs = *Bacillus subtilis*; EEP = Ethanolic Extracts of Propolis; mp = minor peak ; Mp = Major peak

important for the least active samples (EEP9, EEP15) than those of the most active EEP samples (EEP1, EEP12). In general, samples from the Adamaoua region were more active than those from the West region. Furthermore, the most active samples were EEP1 and EEP12 all of them from Meiganga in the Adamaoua region.

Statistical analysis: Statistical analysis of results of Table 2 show that there is a significant difference ($p<0.05$) between propolis samples considering their activity against each strain of bacteria tested. Thus, the most active EEP samples are in a decreasing order EEP12>EEP1>EEP2>EEP11>EEP10 while the least active sample is EEP9 ($p<0.05$). The susceptibility of bacterial strains to EEP samples decrease in the following order *L. monocytogenes*>*S. aureus*>*B. subtilis*>*E. faecalis*, these results confirm those of experiments.

The Principal Component Analysis (PCA) of the most and the least active EEP samples is shown in Fig. 2. The results obtained show that the variability is quite homogeneous, F1 and F2 axes explain >98% of the total variance and F1 is the more significative axis of the analysis. The Pearson (n) matrix of correlation allows to affirm that the correlations between the bacterial strains (variables) are positive and high. All the variables are well represented to F1-F2 plan and they are highly correlated to F1 axis since their correlation coefficients are all superior to 0.9 and positive. With regard to F2 axis, there is a weak correlation between the variables; *S. aureus*, *E. faecalis* and *B. subtilis* are in opposition to *L. monocytogenes*, minor peaks (mp) and Major peaks (Mp); *S. aureus* and mp contribute more to F2 axis.

All the EEP (observations) are well represented to F1-F2 plan since their cumulated cosinus square (F1+F2) are superior to 0.9 and they are highly correlated to F1 axis. Considering F1 axis, group A (EEP10, EEP1, EEP12 which are the most active according to their origin) is in opposition with group B (EEP9, EEP15, EEP2, EEP11) while group A' (EEP12, EEP1, EEP11, EEP2 all originate from Meiganga locality except EEP11) is opposed to group B' (EEP10, EEP15, EEP9 all collected from others localities) with regard to F2 axis. Considering F1 axis, group A' is positively correlated to all the variables (*L. monocytogenes*, *S. aureus*, *B. subtilis*, *E. faecalis*, mp and Mp) while group B' are negatively correlated to the same variables. With regard to F2 axis, group A is positively correlated to *S. aureus*, *B. subtilis* and *E. faecalis*

variables while group B is negatively correlated to the variables *L. monocytogenes*, mp and Mp. These results suggest that F1 is the axis of EEP effectiveness and F2 is the axis of EEP geographic origins. We can deduce from these considerations that the EEP antimicrobial activity are linked to their peaks area values consequently to their phenolic compounds levels and to their geographic origins.

Well diffusion method on agar medium allowed to determine inhibition zones of bacterial growth by EEP while MIC values of the most active EEP were determined by macrodilution method (NCCLS, 2000).

The values of the diameter of the inhibition zones showed that only gram positive bacterial strains were susceptible to the EEP tested namely *S. aureus*, *E. faecalis*, *L. monocytogenes* and *B. subtilis*. In the other hand, there was no activity of the EEP studied against Gram negative bacterial strains tested that were *E. coli*, *S. enterica* and *Ps. fluorescens*.

These results are in agreement with the findings of Kartal *et al.* (2003) concerning the resistance of Gram negative bacteria to EEP but they are in disagreement with the resistance of *E. faecalis*, a gram positive bacteria to the EEP obtained by the same authors. However, the results are in total agreement with those of Moreno *et al.* (1999), Uzel *et al.* (2005) on the susceptibility of gram positive bacteria to the EEP.

Results of the qualitative analysis of all the EEP and those of quantitative analysis of the most active EEP showed that antibacterial activity of EEP samples from Meiganga in the Adamaoua region were more important when compared with those of EEP samples from the West region.

The variation of antimicrobial activity of propolis with the geographic origin was also described by Moreno *et al.* (2000), Kumazawa *et al.* (2004). Kartal *et al.* (2003) obtained similar results on propolis samples from two different regions of Anatolia in Turkey. Bankova *et al.* (1995) showed that antimicrobial activity of brazilian ethanolic extracts of propolis was attributed mainly to their higher contents in phenolic compounds. HPLC analysis of phenolic compounds showed that EEP1 and EEP12, all of them from Meiganga and which had the most important peak areas were the most active.

The results show that there are a relationship between antibacterial activity of the EEP and their phenolic compounds contents on the one hand, between antibacterial activity of the EEP and their geographic origin on the other hand, findings that are confirmed by PCA interpretations. The EEP1 and EEP12 that had shown their capacity to inhibit the growth of bacteria strains like *E. faecalis*, *S. aureus*, *L. monocytogenes* and *B. subtilis* could justify their eventual use as antibiotic agents.

CONCLUSION

Among the gram positive and the gram negative bacterial strains tested, the EEP studied showed an activity only against Gram positive bacteria. The most susceptible strain was *L. monocytogenes* while the least susceptible strain was *E. faecalis*.

The most active EEP samples were EEP1 and EEP12 while the least active were EEP9 and EEP15. There was a relationship between the phenolic compounds contents of the EEP studied and their antibacterial activity on the one hand, between these constituents amounts and their geographic origin on the other hand.

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