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Spectra of Antibacterial Activity of Propolis (Promax-C) Samples from Two Localities of Adamaoua Province (Cameroon)

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Abstract: Fifteen samples of Promax-C, ethanolic extracts of propolis collected from different hives situated in two localities of the Adamaoua Province of Cameroon were tested each against seven strains of bacteria namely *Samonella enterica*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Pseudomonas fluorescens* and *Bacillus subtilis*. The aim of this study was to evaluate the antibacterial activity of those Promax-C samples. Antibacterial activity essays were investigated by the determination of the zones of growth inhibition using the well diffusion method on agar medium and the evaluation of the Minimal Inhibitory Concentration (MIC) using the macrodilution method. All the Promax-C samples were active against the Gram positive bacterial strains except *E. faecalis*. On the other hand, there was no activity of those samples on the Gram negative bacterial strains studied. Considering the diameter of the inhibitory zones and the MIC values, the susceptibility of bacterial strains to the Promax-C samples decreased as follows: *L. monocytogenes*>*S. aureus*>*B. subtilis*. The most active sample was Promax-C8 from the Martap locality and the most susceptible bacteria was *L. monocytogenes*. The areas of the minor and major peaks of the phenolic compounds obtained by HPLC analysis were more important for the Promax-C8 sample, showing that the greatest activity of these antimicrobial components was probably linked to their higher contents in the samples.

Key words: Antibacterial activity, propolis, minimal inhibitory concentration, phenolic compounds, HPLC

INTRODUCTION

Since thousands of years, natural products have been used in folk medicine to treat several diseases. Among them, propolis has got an increased interest because of its antimicrobial activity spectra against a wide range of pathogenic micro-organisms (Sonmez *et al*., 2005). The word propolis is derived from the Greek pro which means for or in defense and polis for city, referring to a substance used to defend the city or the hive (Santos *et al*., 2002). Propolis is a complex resinous mixture collected by honeybees (*Apis mellifera*) from buds and exudates of certain plants. This resin

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is masticated, salivary enzymes are added and the partially digested material is mixed with wax and used by bees to seal cracks and crevices, smooth out the internal walls and protect the entrance of the hive against intruders (Molan, 2001; Sonmez *et al*., 2005). The chemical composition of propolis varies according to the plants that can be found in a specific region (Ghisalberti, 1979 ; Markham *et al*., 1996). More than three hundred constituents were identified in different propolis samples by Bankova *et al*. (2000). Flavonoids, aromatic acids, diterpenic acids and phenolic compounds appear to be the principal components responsible for the biological activities of propolis samples.

In general, propolis is used in a crude form or as ethanolic extracts. Many researchers reported the pharmacological properties of ethanolic extract of propolis such as antibacterial (Kujumgiev *et al*., 1999; Sforcin *et al*., 2000; Sorkun *et al*., 2001; Borelli *et al*., 2002; Kartal *et al*., 2003; Silici and Kutluca, 2005) antifungal (Kujumgiev *et al*., 1999; Ota *et al*., 2001; Sawaya *et al*., 2002; Kartal *et al*., 2003; Choi *et al*., 2006) antiviral (Manolova *et al*., 1985; Amoros *et al*., 1994; Gekker *et al*., 2005) anti-inflammatory (Miyataka *et al*., 1997), local anaesthetic effect (Paintz and Metzner, 1979), antioxidant (Volpert and Elstner, 1993; Orhan *et al*., 1999; Choi *et al*., 2006), immunostimulating (Dimov *et al*., 1991; Sforcin, 2007) and cytostatic effects (Banskota *et al*., 1998). Egyptians, Greeks and Romans used propolis to cure some lesions of the skin. In Cameroon, Promax-C is a new natural product prepared as ethanolic extract of propolis that is used by population to treat wounds, burns, respiratory and dental infections, stomach ulcer, etc.

The aim of the present study is to describe the antibacterial activity spectra of Promax-C samples, prepared from propolis collected in two localities of Adamaoua Province (Cameroon), in order to confirm the validity of their popular use as an antibiotic agent.

MATERIALS AND METHODS

This study was conducted from February 2006 to July 2007 in the Laboratory of Microbiology of the National Advanced School of Agro-Industrial Sciences, University of Ngaoundere (Cameroon) and the Laboratory of Science and Food Engineering of the National Polytechnic Institute of Lorraine (Nancy, France).

Characteristics of Promax-C Samples

Propolis origins and other properties of Promax-C samples analyzed are indicated in the Table 1.

Promax-C samples are propolis extracts in 70% (v/v) ethanol prepared and provided by AFH Association of Ngaoundere and kept in amber flasks. The 70% ethanol used for extraction of the Promax-C samples showed no bactericidal activity on bacteria tested.

Table 1: Propolis origins and other properties of Promax-C samples analyzed

Promax-C No.	Propolis origin	Propolis collection date	Promax-C sample fabrication date
1	Meiganga*	December 2003	January 2004
2	Meiganga	December 2003	January 2004
3	Meiganga	April 2004	August 2004
4	Meiganga	April 2004	August 2004
5	Martap*	August 2004	February 2005
6	Martap	August 2004	February 2005
7	Martap	August 2004	February 2005
8	Martap	August 2004	February 2005
9	Meiganga	April 2005	August 2005
10	Meiganga	April 2005	August 2005
11	Meiganga	April 2005	August 2005
12	Meiganga	April 2005	August 2005
13	Martap	August 2005	February 2006
14	Martap	August 2005	February 2006
15	Martap	August 2005	February 2006

*Localities of Adamaoua Province (Cameroon)

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

Antibacterial Tests

Assay for Inhibition of Bacterial Growth

The well diffusion technique on agar medium was used to test the Promax-C samples against bacteria. To Petri dishes containing 15 mL of TSA-YE medium (Trypcase Soja Agar-Yeast Extract)+ tween 80 was added 0.15 mL of an 18 h pre-culture of the *Bacillus subtilis* strain or 0.015 mL of an 18 h pre-culture of others bacterial strains obtained in TSB-YE medium (Trypcase Soja Broth-Yeast Extract) and thoroughly mixed. After solidification of the medium, six wells of 6 mm diameter were created in each Petri dish and five of them loaded with 20 μL of different Promax-C samples. Twenty microliter of the solvent control (70% ethanol) were introduced in the remaining well per dish. Dishes were left in a refrigerator at 4°C for 24 h. The plates were incubated at 30°C for *Pseudomonas fluorescens* strain and 37°C for others bacterial strains during 18 h. After incubation, the diameter of the zone of growth inhibition (mm) around each well was measured. An inhibitory zone with diameter less than 6 mm corresponds to lack of activity of the sample. The solvent control (ethanol) did not show any antibacterial activity. All determinations were made in duplicate.

Determination of Minimal Inhibitory Concentrations (MICs)

The MICs were determined by the macrodilution method according to the National Committee of Clinical Laboratory Standard guidelines (Jorgensen *et al*., 1997). An 18 h pre-culture of the bacterial strains in a double concentration TSB-YE medium corresponding to an inoculum of approximately 10^4 cfu mL^{-1} (0.5 Mc Farlands) was prepared. Serial concentrations of Promax-C from different samples ranging from 0.5 to 14% (v/v) were achieved in test tubes with sterile distilled water and/or 70% ethanol to yield a total volume of 2 mL per tube. Each antibacterial assay also included tubes containing the culture medium inoculated or not and/or ethanol, in order to obtain controls of the solvents antibacterial effect. The test tubes were incubated at 30°C for *Pseudomonas fluorescens* strain and 37°C for others bacterial strains during 24 h. After incubation, plates were inoculated with 50 μL of each tube by a multipoint inoculator and incubated at 30°C for *Pseudomonas fluorescens* and 37°C for others strains during 24 h. The MIC endpoints were read as the lowest concentration of Promax-C that resulted in no visible growth on the surface of the culture medium. All tests were made in duplicate.

HPLC Analysis

The phenolic compounds of Promax-C samples were analyzed in a chromatograph (SHIMADZU 10A) equipment. The chromatographic conditions were reverse phase column (LichroChart PUROSPHER RP-18; 25.0×0.4 cm, particle diameter of 5 μm (Merck)). The mobile phase was water (solvent A) and methanol (solvent B), at a flow rate of 1 mL min^{-1} at 30°C using a

linear gradient, 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). The time of analysis was 50 min and 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).

RESULTS AND DISCUSSION

All the Promax-C samples were active against all the Gram positive bacteria tested except *E. faecalis* (Table 2). On the contrary, there was no activity of the same propolis samples against all the Gram negative bacteria studied. The most susceptible bacteria strain to the Promax-C samples was *L. monocytogenes* for which was recorded the greatest inhibitory zone (5.8±0.1 mm) due to the most active sample namely Promax-C8. The most active propolis sample against *S. aureus* and *B. subtilis* was also the Promax-C8 with inhibitory zones of 5.6±0.2 mm and 4.6±0.3 mm, respectively. The susceptibility of the bacterial strain against the Promax-C samples tested decrease in the following order *L. monocytogenes*>*S. aureus*>*B. subtilis*.

Results of the susceptibility of the Gram positive bacteria (except *E. faecalis*) to the most active Promax-C samples are represented in Table 3 and show that the most susceptible strain to the Promax-C samples was *L. monocytogenes* and the least susceptible was *B. subtilis*. The most active propolis sample against two of the three most susceptible bacteria strains tested was Promax-C8 with a MIC<1% (v/v) for *L. monocytogenes* while the least active sample was Promax-C2 against *B. subtilis* with a MIC equal to 9% (v/v).

Considering the MIC values, the susceptibility of bacteria strains to the Promax-C samples decreased in the following order *L. monocytogenes*>*S. aureus*>*B. subtilis* and confirmed the results of the qualitative tests.

Results of HPLC analysis of phenolic compounds of the least active and the most active Promax-C samples are shown in Fig. 1a-f and Table 4. These results showed that areas of the minor peaks and major peaks of phenolic compounds were less important for the least active Promax-C samples (Promax-C1, Promax-C2) than those of the most active Promax-C samples (Promax-C7, Promax-C8 and Promax-C13).

The well diffusion method on agar medium has been used to determine the inhibitory zones of four Gram positive bacteria strains and three Gram negative bacteria strains due to the activity of different Promax-C samples. All the Promax-C samples studied showed an activity against *S. aureus*, *L. monocytogenes*, *B. subtilis* except *E. faecalis* concerning the Gram positive bacterial strains. These results are in agreement with those of Choi *et al*. (2006), who showed an antibacterial activity of propolis against *S. aureus* and *B. subtilis*. On the contrary, the same Promax-C samples showed no activity against the Gram negative bacterial strains studied namely *E. coli*, *S. enterica*

Table 2: Antibacterial activity of Promax-C samples*

	Promax-C sample														
Bacteria	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
S.a	2.5±0.2	2.5±0.0	2.0±0.1	3.6±0.1	3.7±0.2	3.0±0.3	4.7±0.4	5.6±0.2	2.6±0.1	3.0±0.4	3.0±0.1	3.6±0.2	3.2±0.2	3.5±0.1	2.0±0.0
S.e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L.m	1.0±0.0	5.0±0.1	4.0±0.0	2.1±0.2	3.5±0.0	5.0±0.0	4.8±0.3	5.8±0.1	5.2±0.2	2.5±0.0	3.6±0.2	3.6±0.2	4.8±0.3	4.0±0.0	3.0±0.0
Ps.f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B.s	1.6±0.1	1.0±0.0	1.1±0.1	1.0±0.0	1.1±0.1	3.6±0.1	3.2±0.2	4.6±0.3	3.5±0.0	2.0±0.3	3.0±0.0	1.0±0.0	3.6±0.1	2.0±0.0	1.0±0.0

Diameter of the inhibitory zone±SD (mm); SD: Standard deviation; 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*; -: No inhibition; *Mean values of two measurements

Table 3: Susceptibility of the Gram positive bacteria (except *E. faecalis*) to the most active Promax-C samples

	MIC of Promax-C in (% v/v)					
Bacteria	Promax-C2	Promax-C6	Promax-C7	Promax-C8	Promax-C9	Promax-C13
L. monocytogenes	nd	4	nd	<1	4	4
S. aureus	nd	nd	5	nd	nd	nd
B. subtilis	9	5	nd	4	5	5

nd: Not determined

Table 4: Minor and major peaks area of phenolic compounds of the least active and the most active Promax-C samples obtained by HPLC

	Minor peak			Major peak		
Samples	RT(min)	λ(nm)	Area	RT(min)	λ(nm)	Area
Promax-C1 (20x)	1.913	268	68 434	2.281	268	151997
Promax-C2 (10x)	1.931	268	330 489	2.202	268	829946
Promax-C6 (20x)	1.908	268	476 982	2.174	268	1497101
Promax-C7 (20x)	1.938	268	532 020	2.197	268	1604099
Promax-C8 (20x)	2.082	268	670 716	2.296	268	2090971
Promax-C9 (20x)	1.930	268	452 287	2.187	268	1381802
Promax-C13 (20x)	1.947	268	485 527	2.192	268	1601301

10x and 20x: Dilution rate; RT: Retention time in min; λ: Wavelength in nm

and *Ps. fluorescens*. The *E. coli* resistance to ethanolic extracts of propolis was described by Drago *et al*. (2000) and Popova *et al*. (2005). Similar results were obtained by Grange and Davey (1990), Keskin *et al*. (2001), Ugur and Arslan (2004) and Silici and Kutluca (2005), who showed that propolis was more active against Gram positive bacterial strains than Gram negative strains.

Kujumgiev *et al*. (1993) and Greenaway *et al*. (1998) showed that fatty acid esters, phenolic compounds and cinnamic acid were the main propolis constituents and that some of them had an antibacterial activity. Silici and Kutluca (2005) had attributed the greater activity of the *Apis mellifera caucasica* propolis samples to its varied chemical composition and concentrations of constituents. The mechanism of antimicrobial activity of propolis is complex and could be attributed to a synergism between phenolic and other compounds in the resin (Kedzia, 1990; Krol *et al*., 1993). Popova *et al*. (2005) studied the qualitative and quantitative chemical composition of Turkish propolis and confirmed the importance of its phenolic compounds contents for the different antibacterial activity expressions. These researchers showed that the greater antibacterial activity of propolis samples from Central and Western Anatolia was linked to their high phenolic and flavonoid contents and that the lower activity of other samples against *S. aureus* was related to their low concentrations in these substances. Promax-C8 was the most active sample and its higher phenolic compounds concentrations could explain its greater activity.

S. aureus is a bacterial strain resistant to penicillin-G (Moreno *et al*., 1999). It is interesting to remark that the most active Promax-C samples could be used to treat skin affections due to that bacteria strain.

Promax-C samples studied showed an activity against all the Gram positive bacterial strain tested except *E. faecalis*. On the contrary, the same Promax-C samples showed no activity against the Gram negative bacterial strain tested. The more susceptible bacterial strain to the majority of Promax-C samples was *L. monocytogenes* while the less susceptible bacterial strain was *B. subtilis*. The most active sample namely Promax-C8 had the highest phenolic compounds content while the less active propolis sample, Promax-C1, had the lowest phenolic compounds amount. These findings showed that there is a relationship between the Promax-C phenolic compounds content and their

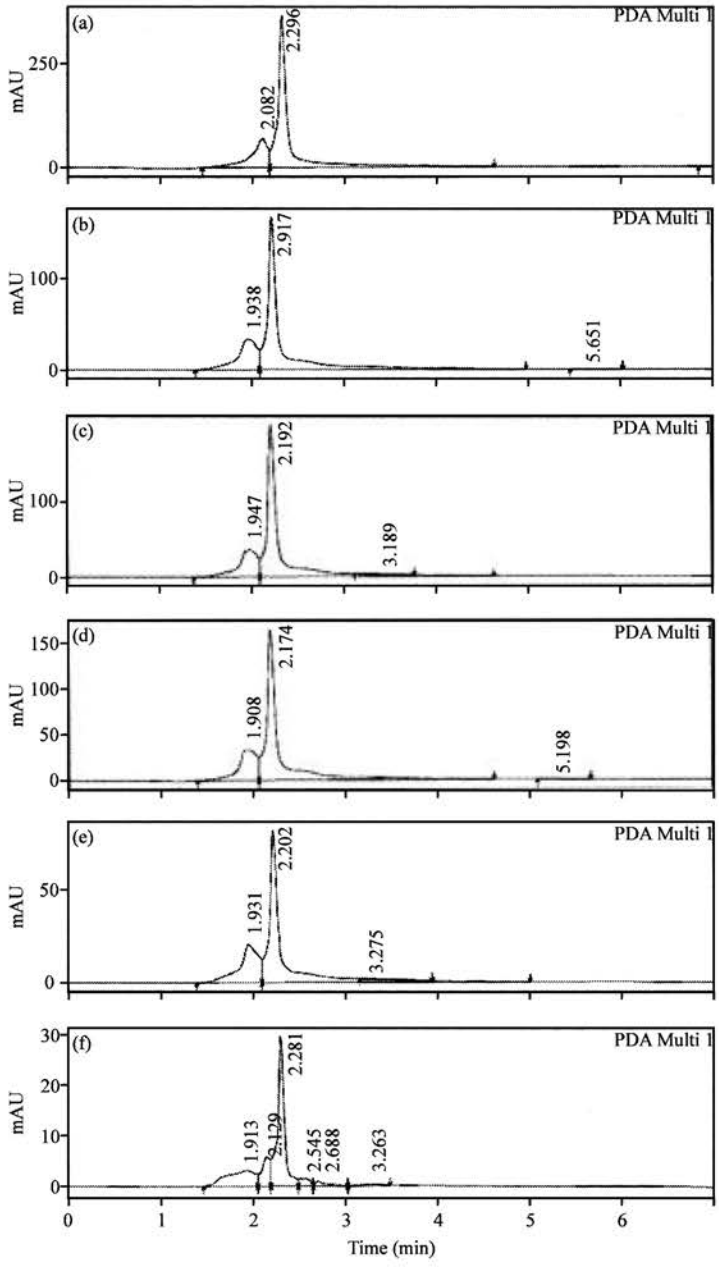

Fig. 1: HPLC chromatograms of phenolic compounds of the Promax-C samples that exhibited weak or high antibacterial activity. (a) Promax-C8, (b) Promax-C7, (c) Promax-C13, (d) Promax-C6, (e) Promax-C2 and (f) Promax-C1

antibacterial activity. The notable antibacterial activity of the most active Promax-C samples obtained in present results could justify their use in the treatment of affections due to some of the bacterial strain tested.

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