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Antiradical activity and polyphenol content of ethanolic extracts of *Propolis*

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Abstract

Propolis is a natural substance produced by honeybees from resinous products collected from plants. Its ethanolic extract is currently commercialised in Cameroon under the brand name *Promax C*. This study investigated the total polyphenols, tannins and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of *Promax C* (6 samples) compared to the freshly ethanol extracted *propolis* (15 samples). The results revealed that all *Promax C* samples tested showed evidence of radical scavenging properties with values ranging from 28 to 70 %. Although the ethanolic samples had lower phenol contents (8.6 – 17.0 g/100 g), their anti-oxidant activities (38.8 - 85.9 %) were systematically higher than those of *Promax C*. In addition the *Promax* C manufactured in 2006 systematically exhibited the highest scavenging activity (67.3%) and polyphenol contents (772.8 mg/L) compared to those manufactured in 2004 (mean scavenging activity 43.7 %; mean polyphenol contents, 227.8 mg/L). While there was a linear relationship between the radical scavenging activity and the polyphenols or tannin content in the *Promax* samples, this was not the case with the fresh ethanolic extract. The *Cameroonians propolis* exhibited higher scavenging activity which could justify their commercialisation and role in the management of some chronic diseases. However, the activity of *Promax* tends to decrease with aging, and this needs to be investigated.

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Introduction

Propolis is a natural substance produced by honeybees from resinous products collected from plants. It has been shown to have a very complex chemical composition. At least 300 different compounds have been identified in *propolis* (Bankova *et al.,* 2000). *Propolis* has been used since the ancient time in folk medicine. Its utilization in the treatment and prevention of numerous diseases has been documented (Castaldo and Capasso, 2002). This beneficial role is partially attributed to its biological activities such as antibacterial (Sforcin *et al.,* 2000), antitumor (Banskota *et al.,* 2002), and immunomodulatory (Murad *et al.,* 2002), among others. The disease-prevention activity of propolis may be attributed to its anti-oxidant activity (Ozen *et al.,* 2004).

The anti-oxidative action is meant to protect living organisms from oxidative damages, thus helping in the prevention of various diseases such as cancer, cardiovascular diseases, and diabetes (Gutteridge and Halliwell, 1994). The importance of protective defence systems in living cells, against damages caused by reactive oxygen, is well known. Free radicals and other oxidants are of great importance in the mechanism of action of many toxins. Their involvement in the aging process and diseases has been investigated (Ceruti, 1994, Dean *et al.,* 1993). These oxygen radicals induce oxidative damages in bio-molecules such as carbohydrates, proteins, lipids, and nucleic acids that would lead to damage of cell organelles, thus causing, aging with or without diseases (Ceruti, 1994; Dean *et al.,* 1993)

Because of the recrudescence of chronic diseases in the world, and particularly in Cameroon, there has been a renewed interest in the study of honey products and *propolis* composition and biological properties. A commercialised form of *propolis*, called *Promax*, has been developed in Cameroon. However, there has been no research conducted to determine the phenolic content and anti-oxidant activity of Cameroonian *propolis*. It is believed that the anti-oxidant activity of *propolis* varies widely, depending on the floral source, and hence the origin. In the case of honey, it has been proved that its botanical origin has a great influence on its antioxidant activity, while processing, handling and storage affect honey anti-oxidant activity only to a minor degree (Al-Mamary *et al.,* 2002).

Several investigations on *propolis* in Eastern Europe and South America have indicated that phenols concentrated in *propolis* are powerful anti-oxidants which are able to scavenge free radicals (Banskota *et al.,* 2002). Because of lack of knowledge about anti-oxidant activity of Cameroonian *propolis*, this study was designed to measure the scavenging activity of the ethanolic extract of Cameroonian *propolis* using the DPPH assay as an easy to use and inexpensive method. The general purpose of this study was to evaluate the scavenging activity, the total polyphenols and tannins content of ethanolic extract of some Cameroonian propolis, compared to that of the commercialised form, Promax C.

Materials and methods

Source of propolis

Unmanufactured *propolis* and commercially available *propolis* extracts, Promax C, (GAA, Cameroon) originated from the NGO "ABEILLES-FLEUR-HOMME" located in Ngaoundere-Cameroon, and organized in the production and manufacture of bees products. All the *propolis* samples were harvested in Bamendjou town, located in the west region of Cameroon.

Preparation of propolis ethanolic extracts

Promax samples: Two batches of *Promax* production each containing three bees hives were used. The first batch was harvested in December 2003 and manufactured in January 2004 (*Promax* samples coded P41, P42 and P43) while the second was harvested in April 2004 and manufactured in February 2006 (Promax samples coded P61, P62 and P63). The

only information obtained from the manufacturing process was that the *propolis* was subjected to ethanol extraction for 6 days.

Preparation of ethanolic extract of unmanufactured *propolis*: Fifteen samples were harvested from fifteen bee hives in April 2004. Bee hives were situated in three different locations of the town: five hives in location Bn (coded Bn1, Bn2, Bn3, Bn4, Bn5), five in location Mn (Mn1, Mn2, Mn3, Mn4, Mn5) and five in location Mt (Mt1, Mt2, Mt3, Mt4, Mt5). Samples obtained from locations Bn and Mn were black in colour, while those from location Mt were dull in colour. For the preparation of ethanolic extract, 12.5 g of dried *propolis* (40°C for 1 h) were extracted with 30 ml ethanol 70% at room temperature for 24 h. The ethanol suspension was separated by centrifugation at 1000 rpm for 10 min at room temperature, and the supernatant was poured in a 50 ml dark volumetric flask and the volume completed with 70% ethanol. The extracts were stored under dry conditions at 4°C until needed for analysis.

Determination of total polyphenol and tannins contents

Total polyphenol contents in *propolis* extracts and *Promax* were determined according to the Folin–Ciocalteu colorimetric method described by Kumazawa *et al.* (2002) with some modifications. In this procedure, 0.02 mL of sample was mixed with 0.2 mL of the Folin–Ciocalteu reagent (Kanto Chemicals, Tokyo, Japan) and 0.4 mL of 20% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h of incubation at room temperature. The total polyphenol contents were expressed as milligrams per gram of gallic acid equivalents.

Tannins were determined in extracts according to the method of Hagerman *et al.* (2000b) with some modifications. In the procedure, gelatin was used to precipitate tannins in the extract; the polyphenols in the supernatant was determined by the Folin-Ciocalteu method as described above; and tannins content, expressed as mg gallic acid/ ml, were determined by difference from the total polyphenol.

Evaluation of DPPH Free radical scavenging activity

The reaction mixture contained 0.25 mL of DPPH 1 M in methanol, and 0.25 mL of test samples. After 30 min incubation at room temperature, the absorbance was recorded at 517 nm. Control solution contained only methanol and DPPH. Results were expressed as percentage decrease of absorbance with respect to control values (Okada and Okada, 1998). Gallic acid was used as the reference samples.

Statistical analysis

All the chemical analyses were done in triplicate. The results obtained were expressed as means ± standard deviation and also subjected to one way analysis of variance and Duncan multiple range test when there was a significant ($p < 0.05$) difference using the Statgraphics 3.0 (Manugistics, Rockville, Maryland, USA) statistical software.

Results and discussion

Polyphenols and tannins

Table 1 shows the total polyphenols and tannin contents of *propolis* extracts and *Promax*. The amounts of total polyphenols and tannin contents in Cameroonian *propolis* varied widely, ranging from 8.60 to 16.97 g of gallic acid equivalent/100g of extract. Mohammadzadeh *et al.* (2007) and Ahn *et al.* (2007) previously reported that the polyphenols content of ethanolic extract of *propolis* from Iran and China was approximately 3.02-30.8 g gallic acid equivalent/100g. Hence, the total polyphenols contents in Cameroonian *propolis* fell within the range of values reported for *propolis* from other regions. *Propolis* contains a wide variety of phenolic compounds. Lots of studies have revealed that the main polyphenols in *propolis* are flavonoids (Mohammadzadeh *et al.,* 2007; Ahn *et al.,* 2007). Very little information exists on the tannin contents of *propolis*, although it does play a role in the

bitterness of *propolis*. The variation in the polyphenols and the tannin contents of *propolis* has been mainly attributed to differences in the preferred regional plants visited by honeybees. In the present study, significant variations in total polyphenols and tannin contents were observed in *propolis* samples originated not only from bee hives of similar areas, but also from different zones.

Table 1. Polyphenol contents and anti-oxidant activity in ethanolic extract of propolis samples.

Propolis sample	Antiradical activity (%)	Total polyphenols (g/100g)	Tannins (g/100g)
Bn1	82.4±0.1^{ab}	9.36±0.01^{b}	0.62±0.02^{c}
Bn2	85.9±0.1^{a}	14.06±0.01^{a}	2.16±0.05^{b}
Bn3	83.4±1.1^{a}	8.60±0.20^{c}	0.53±0.01^{c}
Bn4	80.0±0.2^{b}	9.10±0.04^{b}	0.67±0.01^{c}
Bn5	85.2±0.1^{a}	13.81±0.51^{a}	3.89±0.01^{a}
Means±SD	**83.4±2.3**	**10.99±2.56**	**1.57±1.62**
Mt1	54.9±6.5^{bc}	9.94±0.04^{e}	0.17±0.01d
Mt2	58.0±0.3^{b}	12.30±0.28^{c}	0.87±0.03c
Mt3	51.4±0.4^{c}	10.56±0.12^{d}	0.25±0.01^{d}
Mt4	48.6±1.7^{c}	13.78±0.24^{b}	2.41±0.02^{b}
Mt5	68.5±0.4^{a}	16.97±0.40^{a}	4.17±0.02^{a}
Means±SD	**56.3±7.7**	**12.71±2.66**	**1.57±1.52**
Mn1	64.8±0.7^{a}	14.06±0.63^{a}	3.28±0.10^{ab}
Mn2	38.8±2.9^{d}	13.64±0.35^{a}	3.25±0.01^{b}
Mn3	47.1±0.1^{c}	13.98±0.12^{a}	4.09±0.07^{ab}
Mn4	60.4±0.7^{b}	9.44±0.20^{g}	1.43±0.12^{d}
Mn5	43.6±1.9^{c}	9.78±0.28^{b}	2.32±0.04^{c}
Means±SD	**50.9±11.2**	**12.17±2.24**	**2.87±1.05**

Means±standard deviation; n=3; Means in each group of samples, and in the same column followed by different superscripts are significantly different at P<0.05.

Bn1, Bn2, Bn3, Bn4 and Bn5 propolis samples harvested in different bees hives located at the Bn position of the Ngaoundere town; Mn1, Mn2, Mn3, Mn4, Mn5 propolis samples harvested in different bees hives located at the Mn position of the Ngaoundere town; Mt1, Mt2, Mt3, Mt4 and Mt5 propolis samples harvested in different bees hives located at the Mt position of the Ngaoundere town.

Levels of total polyphenols and tannins are shown in Table 2. The concentration of total polyphenols in *Promax* samples varied from 186 to 1084 mg/L. A significant difference was observed between the two batches of samples, P6 being 3 to 6 times richer than P4. Based on this difference, it can be assumed that the composition of manufactured ethanolic extract varied widely from one production to another. In a comparative basis, the content of polyphenols in *Promax* samples was similar or less than that in fresh ethanolic extract. In fact, the levels of total polyphenols expressed in mg/L were 318±66, 275±64 and 304±56 mg/L for Mt, Bn and Mn respectively. Similar observation was made on tannins samples which

varied from 39±40 mg/L for Mt and Bn samples to 71±26 mg/L for Mn sample.

Phenolic substances have been suggested to play a preventive role in the development of chronic diseases such as cancer and heart disease (Kahkonen *et al.,* 1999). This has probably informed the manufacture of Promax C for health treatments (wound healing, tissue regeneration, bums and herpes).

Table 2. Polyphenol contents and anti-oxidant activity in Promax samples

Promax samples	Antiradical activity (%)	Total polyphenols (mg/L)	Tannins (mg/L)
P41	49.5±4.1^{c}	250.7±2.0^{c}	21.0±0.1^{c}
P42	28.0±2.5^{d}	186.3±9.9^{d}	2.1±1.0^{d}
P43	53.7±0.5^{c}	246.5±5.9^{c}	25.9±5.0^{c}
Means±SD	**43.7±13.8**	**227.8±36.0**	**16.3±12.6**
P61	68.9±0.3^{a}	607.8±45.6^{b}	247.2±56.4^{b}
P62	63.8±0.2^{b}	626.0±15.8^{b}	243.0±20.8^{b}
P63	69.2±0.2^{a}	1084.7±5.0^{a}	871.2±2.0^{a}
Means±SD	**67.3±3.0**	**772.8±270.2**	**453.8±361.5**

Means±standard deviation; n=3; Means in each group of samples. and in the same column followed by different superscripts are significantly different at P<0.05.

P41. P42. P43 propolis samples harvested in december 2003 and transformed to promax in january 2004; P61. P62 and P63 propolis samples harvested in april 2004 and manufactured in february 2006.

DPPH free radical-scavenging activity of various propolis samples

Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity (Kahkonen *et al.,* 1999). The free radical-scavenging activities of various samples were evaluated at their initial concentration. All propolis and promax samples showed free radical scavenging activity (Tables 1 and 2). The unmanufactured extract from Bn had strong DPPH free radical-scavenging activities (above 80%). This was unexpected since Bn had the lowest polyphenol content. The polyphenols content has widely been shown to correlate positively with DPPH scavenging activity. The opposite results observed here probably highlighted the differences in the phenolic profiles, some being more active than others. Recent studies by Ahn *et al.* (2007) revealed that the composition of propolis varied a lot from one sample to another. In addition these authors found that *propolis* with strong anti-oxidant activity contained large amounts of caffeic acid, ferulic acid and caffeic acid phenethyl ester.

Promax samples which had high total polyphenols contents, exhibited weak DPPH free radical-scavenging activity. It was expected that *Promax* samples P61, P62 and P63, with high levels of total polyphenols, could exhibit higher anti-oxidant activity. This was not the case, suggesting that either some polyphenols can be more active than others, or anti-radical activity of phenols in solution can decrease with storage time, but this still needs to be investigated. In addition the anti-radical activity is not always a linear relation of the polyphenols content even for diluted solution. As shown in Fig. 1, the decreased anti-radical activity with the dilution factor followed a Z shape, highlighting the non-linear relationship suggested above.

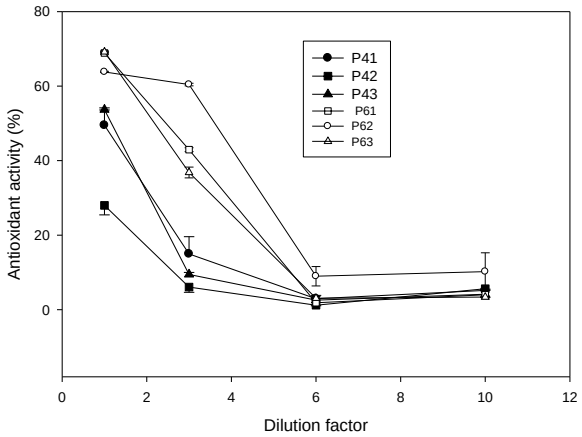

Fig. 1. Effect of dilution on the antioxidant activity of Promax samples. Means±standard deviation; n=3; P41. P42. P43 propolis samples harvested in december 2003 and transformed to promax in january 2004; P61. P62 and P63 propolis samples harvested in april 2004 and manufactured in february 2006.

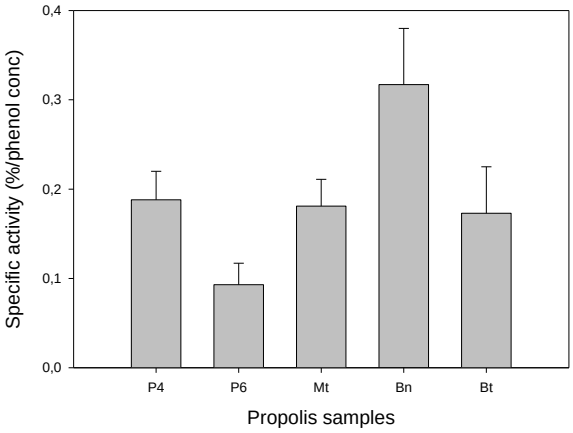

Fig. 2. Relative radical DPPH scavenging activity of *propolis* and *Promax* samples. means±standard deviation; n=9; P4 is mean of samples P41. P42. P43 *Promax* samples manufactured in January 2004; P6 is mean of samples P61. P62 and P63 *Promax* samples manufactured in February 2006; Mt. Bn and Bt are means of *propolis* samples harvested at the different locations in the town.

In order to compare the activity of the different extracts, the specific anti-radical activity was calculated by dividing the antiradical activity value (%) by the total polyphenols concentration (mg/L). Samples of similar areas were pooled and the results showed that there was a wide variation between the groups of samples. In fact, while groups P4, Mt and Bt samples possessed similar specific anti-radical activity, P6 exhibited the lowest activity and Bn the highest (Fig. 2). The difference in the specific activity suggests that either there is a difference in the activity of the polyphenols involved, or there are some molecules, other than polyphenols that are responsible for the activity in sample P6. It can also be suggested that the polyphenols in P6 lost their activities during storage. According to Mohammadzadeh *et al.* (2007) and Nivea Morena *et al.* (2000), the strong anti-oxidative activity occurred in *propolis* with high amounts of phenolic compounds and weak activity with low amounts. These authors also recognized that other non-flavonoids scavengers such as enzymes, anti-oxidant vitamins in *propolis* were also involved. In addition Ahn *et al.* (2007) reported that the composition of *propolis* varied not only qualitatively, but also quantitatively with the geographical and botanical origins. Some of the observed biological activities might be attributed to the identified chemical constituents and partially from its high content of flavonoids. In this respect, it was reported that *propolis* samples from Europe, South America and Asia have different chemical compositions (Ahn *et al.,* 2007). *Propolis* from Europe and China contains many flavonoids and phenolic acid esters (Bankova *et al.,* 2000). In contrast, the major components in *propolis* of Brazilian origin are terpenoids and prenylated derivatives of p-coumaric acids. As a consequence of variation in composition, the biological activity of *propolis* samples varies. Studies by Chen *et al.,* (2007) revealed that *propolins* are the major phenolics in Taiwan propolis, with *propolin D* scavenging free radicals better than *propolins C* and *F*. The phenolic profiles of Cameroonian *propolis* will probably tell more about the discrepancy of the anti-radical activities of our samples.

Conclusion

Radical scavenging activity was demonstrated in all *propolis* ethanolic extracts including the commercialized form, *Promax C*. The radical scavenging activity of ethanolic extract of propolis varies significantly ($p<0.01$) from one harvesting location to another, while in the same location little but significant ($p<0.05$) difference is observed from one bees-hive to another. The commercialized form of ethanolic extract, Promax C, exhibited low radical scavenging activity compared to the fresh extracted ethanol samples. The low radical scanvenging activity observed in commercialized forms, *Promax*, suggests a progressive lost in activity during storage, and this needs to be investigated.

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