Scientific Research Publishing

American Journal of Analytical Chemistry, 2022, 13, 241-254
https://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

Phytochemical Tests, Assessment of Antioxidant Properties and Isolation of Two Compounds of Ethyl Acetate Extract of Chadian Propolis: Case of Bebotho (Southern Chad)

Salomon Madjitoloum Betoloum[1*], Abel Mbaiogaoun[1], Severin Mbaihougadobe[1], Emmanuel Talla[2]

[1]Department of Chemistry, Faculty of Exact and Applied Sciences (FSEA), University of N'Djamena, N'Djamena, The Republic of Chad
[2]Department of Chemistry, Faculty of Science, University of Ngaoundéré (UN), Ngaoundéré, The Republic of Cameroon
Email: *mbaiogaouabel@yahoo.fr

How to cite this paper: Betoloum, S.M., Mbaiogaoun, A., Mbaihougadobe, S. and Talla, E. (2022) Phytochemical Tests, Assessment of Antioxidant Properties and Isolation of Two Compounds of Ethyl Acetate Extract of Chadian Propolis: Case of Bebotho (Southern Chad). *American Journal of Analytical Chemistry*, **13**, 241-254.
https://doi.org/10.4236/ajac.2022.137017

Received: March 11, 2022
Accepted: July 25, 2022
Published: July 28, 2022

Abstract

The present work deals with the research of chemical constituents and evaluation of antioxidant properties of Bebotho propolis. From the ethyl acetate extract, we isolated, using various chromatographic techniques, a mixture of two identical compounds (isomers) indexed PBy4a and PByb. The structures of these compounds were elucidated by means of spectroscopic analysis techniques (MS, IR, ^{1}H-NMR, ^{13}C-NMR, HMBC and HSQC) and by comparison of the spectral data with those described in the literature. Thus, these compounds were identified to a mixture of two chromones namely 5,7- dihydroxy-2-methylchromone-6-C-α-D-glucopyranoside and 5,7-dihydroxy-2-methylchromone-8-C-β-D-glucopyranoside, first reported in propolis. The study of the antiradical power, chelating power and the quantification of phenolic compound of these same extracts, showed interesting properties that propolis extracts have to scavenge free radicals.

Keywords

Isolation, Propolis, Chelating Power, 5,7-Dihydroxy-2-Methylchromone-6-C-α-D-Glucopyranoside and 5,7-Dihydroxy-2-Methylchromone-8-C-β-D-Glucopyranoside

1. Introduction

Propolis or bee glue is a complex mixture of several organic and inorganic com-

pounds, used by bees as glue, coating and antibiotic. Since ancient times, man has been interested in bees and their products: honey, pollen, wax, venom, royal jelly and finally Propolis [1]. Among these products from the hive, only one is the subject of this study: propolis.

At the end of the 21st century, an important market for propolis exists all over the world since it was a popular remedy that claimed to cure all ailments. It was mainly used externally as an anti-infective, healing, soothing and anti- inflammatory agent in the form of ointment, plaster, lotion and fumigation [2]. During the last world war, propolis was experimented in Soviet clinics [3].

Considering the capital importance of propolis, several researchers have focused their attention to search for the secondary metabolites responsible for therapeutic activities. The applications of this famous product, propolis, are also very interesting in several areas such as health, food, cosmetics, crafts, ... [4].

In Cameroon, propolis has been used in a traditional way to treat various diseases such as dysentery, stomach ache, asthma, sterility, ulcers, dental caries, fevers and different types of inflammation. This product of the hive is very valuable because of its anti-oxidant [5], antibacterial [6] [7], analgesic [4], antiviral, anticancer [8] and antiradical [9] properties and therapeutic properties related to its composition in polyphenols and flavonoids. In Mexico, Jiménez, O. *et al.* showed that propolis from native bees (Plebeia frontalis) has a statistically significant antiviral effect in both treatments of distemper virus, when administered one hour before and simultaneously to the infection, although it is slightly better when applied one hour before the viral infection [10].

For this purpose, propolis is extensively used in the food industry, medicine, cosmetology and veterinary medicine. Within the limits of our knowledge, it is time to discover the virtues of Chadian propolis. Thus, we have oriented our study on one of the propolis collected in Chad: Propolis of Bebotho. Our contribution to the realization of this work concerned the phytochemical tests of the extracts, the isolation of two stereoisomers of the ethyl acetate extract of this propolis and the evaluation of their antioxidant activities.

2. Materials and Methods

Material

The raw material of our study is a propolis harvested in April 2020. It was bought with the beekeepers in a site of sale of the apiarian products of the locality of Bebotho, in the Department of Khou-Ouest, Province of the Logone oriental in the south of Chad. A powder of this material was obtained after trituration of the raw propolis. This powder is then used for the extractions.

Methodology

Extraction and isolation of compound

1.2 kg of propolis powder were introduced into a container with a volume of 6 liters of distilled hexane, the whole is closed and left to rest (maceration), after 48 hours at room temperature assumed 25˚C, the mixture was filtered and the filtrate is then concentrated in the rotary evaporator. Then the residue of the

hexane extract was subjected to the same treatment with ethyl acetate, and finally the residue with ethyl acetate was subjected to the same treatment with methanol. Note that for the same solvent this operation is repeated three times. The yield of the extraction thus obtained expressed in percentage compared to the mass of the starting propolis powder is calculated by the formula below:

$$\text{Yiel} = \frac{\text{Mass of extract}}{\text{Mass of propolis powder}} \times 100 \quad (1)$$

Phytochemical screening

Phytochemical screening was done on propolis extracts to show the presence if possible of alkaloids, polyphenols (flavonoids, anthocyanins, tannins), saponosides, steroids, coumarins, sterols, terpenes, cardiotonic heterosides, essential oils, reducing sugars according to the protocol of Preeti *et al.* [11].

Separation of the extract

Phytochemical screening of the extracts shows that both ethyl acetate and methanol extracts contain compounds of biological interest. However, we chose the ethyl acetate extract because of its yield to submit to the chromatographic column. 50 g of the acetate extract were dissolved in ethyl acetate and then fixed on silica were evaporated to dryness using a rotary evaporator. The powder obtained was introduced into a column containing silica gel packed with the apolar solvent at room temperature 25˚C. This column was eluted (first with hexane, hexane-ethyl acetate, ethyl acetate-methanol, then pure methanol) in increasing polarity. 392 fractions of 250 mL were collected, concentrated and pooled on the basis of analytical Thin Layer Chromatography (TLC) in fifteen series.

Quantification of organic compound

Quantification of total polyphenols content

We quantified total polyphenols using the Folin-Ciocalteu reagent, a method described as early as 1965 by Singleton and Rossi revised by Boizot and Charpentier [12]. The results are expressed in grams of gallic acid equivalent/100g of raw material (g GAE/100g MB).

Protocol: The dry extract is diluted in methanol. A standard range in aqueous medium (6 concentration points from 0 to 20 $g{\cdot}L^{-1}$) is performed with a control polyphenol, gallic acid. To perform the assay, 200 µL of Folin-Ciocalteu reagent (diluted 1/16) is added to 20 µL of diluted extract or range point. Shake and let stand for 3 minutes. Then 400 µL of Na_2CO_3 (20%) is added. The blank of the reaction containing no polyphenol is made as the 0 $\mu g{\cdot}ml^{-1}$ point of the range. The reaction mixtures, corresponding to each range point and sample, are shaken and incubated 40 min at 40˚C in a water bath. Tubes were prepared in duplicate for the standard range and samples. The optical density reading was taken at 760 nm using a spectrophotometer (UV Rayleigh Vis-723N spectrophotometer).

Quantification of total flavonoids content

The estimation of total flavonoid content in propolis extracts is performed by the method described by Hariri *et al.* [13].

Procedure: One gram of ground plant material is placed in the presence of 100

ml of 80% methanol. After shaking and sonication, 2 ml of the extract is mixed with 100 µl of Neu (1% in pure methanol). The absorbance is determined at 404 nm and compared with that of standard quercetol (0.05 mg/ml) treated with the same amount of reagent. The percentage of total flavonoids is then calculated in grams of quercetin equivalent per 100 grams (g QE/100g) according to the following formula:

$$TF = \frac{0.05 \times A_{ext} \times 100}{A_q \times C_{ext}} \quad (2)$$

with *TF*: Total flavonoids; A_{ext}: Absorption of extract; A_q: Absorption of quercetin and C_{ext}: Concentration of the extract in plant material *i.e.* 10 mg/mL.

Evaluation of the antioxidant activity

The antioxidant activity of bee propolis extracts is evaluated by two different methods: the reducing power of iron and the anti-radical activity of Diphenyl-PicrylHydrazyl (DPPH).

Anti-radical activity to the DPPH radical

The demonstration of the antioxidant power of propolis extracts via the DPPH test described by Laskar [14].

Procedure: In 1 mL of each extract at different concentrations, 1 mL of DPPH of concentration 20 mg/L is added. We let incubate in the dark for 15 min at room temperature 25˚C and the optical density reading is done at 517 nm. The percentage of DPPH radical inhibition is calculated by the formula:

$$\%\ \text{d'inhibition} = \frac{A_c(0) - A_e(t)}{A_c(0)} \quad (3)$$

with $A_c(0)$ = Absorbance of the control and $A_e(t)$ = Absorbance of the sample.

Chelating power of ferrous ions

The chelating power of iron ions was measured according to the method of Suter and Richter [15] with slight modifications.

Procedure: The reagent solution contained 100 µL at 2×10^{-3} mol·L^{-1} of ferric chloride ($FeCl_3$), 400 µL at 5×10^{-3} mol·L^{-1} of potassium hexacyanoferrate ($K_3[Fe(CN)_6]$), and 200 µL of sample at different concentrations from 50 to 200 mg/mL in 25 mg/mL steps. Distilled water was added to have a total volume of 1 mL. The reaction mixture was incubated at 20˚C for 10 min.

The formation of potassium hexacyanoferrate complex was measured at 700 nm using a spectrometer (Raleigh). The experiment was performed at 20˚C to prevent Fe^{2+} oxidation. The lower absorbances indicated a high chelating capacity of iron. Diamine Ethylene Tetra Acetic (DETA) was used for comparison. The percentage of iron ion chelating power (PCIF) was determined according to the formula:

$$\%\text{IICP} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{extracted}}}{\text{DO}_{\text{controle}}} \times 100 \quad (4)$$

with IICP: Iron Ion Chelating Power and OD: Optical Density.

3. Results and Discussion

Yield of extraction

Table 1 below shows the extraction yield of propolis according to the different organic solvents used.

It appears from this table that the polarity of the solvent used and the yield obtained are inversely proportional. We see here that hexane, apolar solvent presents an extraction yield of 50.38% whereas methanol, the most polar solvent presents only a yield of 5.21%. This explains that propolis is rich in apolar compound. The work of Trusheva *et al.* [16] gave a yield of 13.87% using petroleum ether to extract propolis from Iran. This low yield would be due to the absence of some apolar compounds in this propolis sample that petroleum ether could extract them unlike our studied sample. The yield of the ethyl acetate extract, 23.96% is comparable to that of Petrova *et al.* [17] who, extracting under the same conditions with ethyl acetate, obtained a yield of 20.03% on propolis from Mwingi in Kenya.

In general, our sample studied is mostly composed of compounds of low polarities compared to those found in the literature due to the high extraction yield with hexane. Also, the extraction yields vary according to the propolis, the nature, the extraction method and the physicochemical characteristics of the solvents used, in particular their polarity. Indeed, the solubility of the substances contained in the vegetable matter in a given solvent depends on these properties. It follows that the extraction yields and the composition of the extracts vary from one solvent to another.

Results of phytochemical screening of extracts

The analysis method used allowed us to know the chemical composition of the different propolis extracts from the locality of Bebotho (South of Chad) in Table 2.

From the results obtained, we notice the presence of alkaloids in ethyl acetate and methanol extracts. This result is in agreement with the work of Preeti *et al.* [11] on Indian propolis. We also notice the presence of O-heterosides and C-heterosides in ethyl acetate and methanol extracts. Similarly, flavonoids show their presence in the methanol extract. These results also corroborate those of Bankova *et al.* and Xu *et al.* [18] [19].

The presence of terpenes in all extracts confirms the work of Bankova *et al.* [18] that triterpenes are characteristic of propolis from tropical regions. Free anthracenes, sterols, saponins and coumarins were not detected in the different extracts.

Table 1. Extraction yield.

Extracts	Mass of extracts (g)	Extraction yield (%)
Hexane	606	50.38
Ethyl acetate	247.52	21.76
Methanol	60.35	5.11

Table 2. Phytochemical screening.

Family of compound	Results		
	Hexane extract	Ethyl acetate extract	Methanol extract
Alkaloids	−	+	+
Triterpenes	+	+	+
Sterols	−	−	−
Flavonoids	−	−	+
Polyphenols	+	+	+
Tannins	+	+	+
Coumarins	−	−	−
Free anthracenes	−	−	−
Saponins	−	−	−
Combined Anthracenes:			
O-heterosides	−	+	+
C-heterosides	−	+	+

+: presence of the family of compounds; −: absence of the family of compounds.

Total phenols and total flavonoids content

Based on the absorbance values of the various extract solutions, reacted with the Folin-Ciocalteu reagent and compared to the standard solution in gallic acid equivalence as described above, the colorimetric analysis values of total phenolics and total flavonoids are summarized in Table 3 and Figure 1.

Total phenolics content

The total phenolics contents of the different propolis samples show great differences, the variation goes from 0.71 ± 0.07 g EAG/100g to 2.59 ± 0.23 g EAG/100g of raw propolis. Considering the results obtained, we notice that the total polyphenol content is different from one sample to another. Similarly these results are comparable to those of Bornes (329 mg EAG/g DM) and Fundậo (159 mg EAG/g DM) in Portugal [5]. These variations in phenolic compound content from one region to another or from one country to another confirm the influence of plant material origin on the results.

Total flavonoids content

Table 3 shows that the total flavonoid content varies from 0.19 ± 0.03 g QE/100g of the hexanic extract to 1.01 ± 0.02 g QE/100g of the acetic extract. This content is different from that detected in Iranian propolis, which is 77.9 ± 0.39; 31.1 ± 0.08 and 12.2 ± 0.33 mg QE/mg of Khorsan propolis [5]. This result shows that our studied sample consists of the compounds of low polarity.

Identification of compounds

Our objective was to isolate compounds that could present interesting antioxidant properties. As in our case, we had quantified only the ethyl acetate

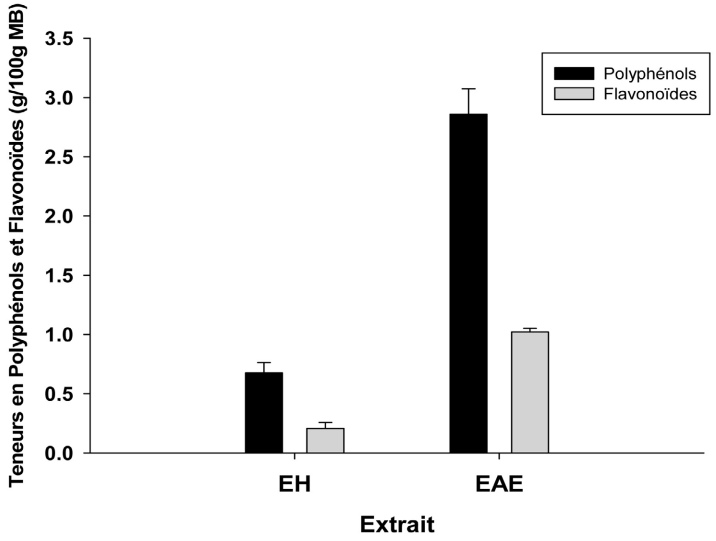


Figure 1. Total phenolics and flavonoids contents (g/100g MB).

Table 3. Total phenols and flavonoids contents.

Extracts	Total Phenols (g GAE/100 g DM)	Total flavonoids (g QE/100 g DM)
HE	0.71 ± 0.07	0.19 ± 0.03
EAE	2.59 ± 0.23	1.01 ± 0.02

EH: Hexane extract; EAE: ethyl acetate extract.

extract and the hexane extract, we chose to fractionate the ethyl acetate extract. At the end of this roughing column, a mixture of almost identical compounds (isomers) was isolated PBy4 (Propolis Bebotho fraction y4).

Identification of the compound PBy4 (Figure 2)

The compound PBy4 crystallizes as a yellow powder in ethyl hexane-acetate (8:2). Soluble in dimethylsulfoxide (DMSO), it melts between 183˚C and 184˚C and reacts positively to ferric chloride ($FeCl_3$, blue coloration) test suggesting the presence of phenolic hydroxyls.

Its IR spectrum shows characteristic vibrational bands of free hydroxyls at λ_{max} = 3299 cm^{-1}, a chelated hydroxyl at 2917 cm^{-1}; carbon-carbon double bonds of aromatics and olefins between 1656 and 1584 cm^{-1}.

Mass spectrum of the compound PBy4

Its mass spectrum under ESI-TOF (Electrospry Ionisation- Time Of Fly) ionization at high resolution and in positive mode shows the peak of the protonated molecular ion $[M + H]^+$ at m/z 355.2 (calculated 355.309 for $C_{16}H_{19}O_9$). Then, another peak at m/z 377.2 corresponding to the pseudomolecular ion $[M + Na]^+$ at m/z 377.2 (calculated 377.309 for $C_{16}H_{18}O_9Na$). In addition, we observe a peak at m/z 731.1 corresponding to the $[2M + Na]^+$ ion. All these data are compatible with the crude formula $C_{16}H_{18}O_9$ containing 8 degrees of unsaturation.

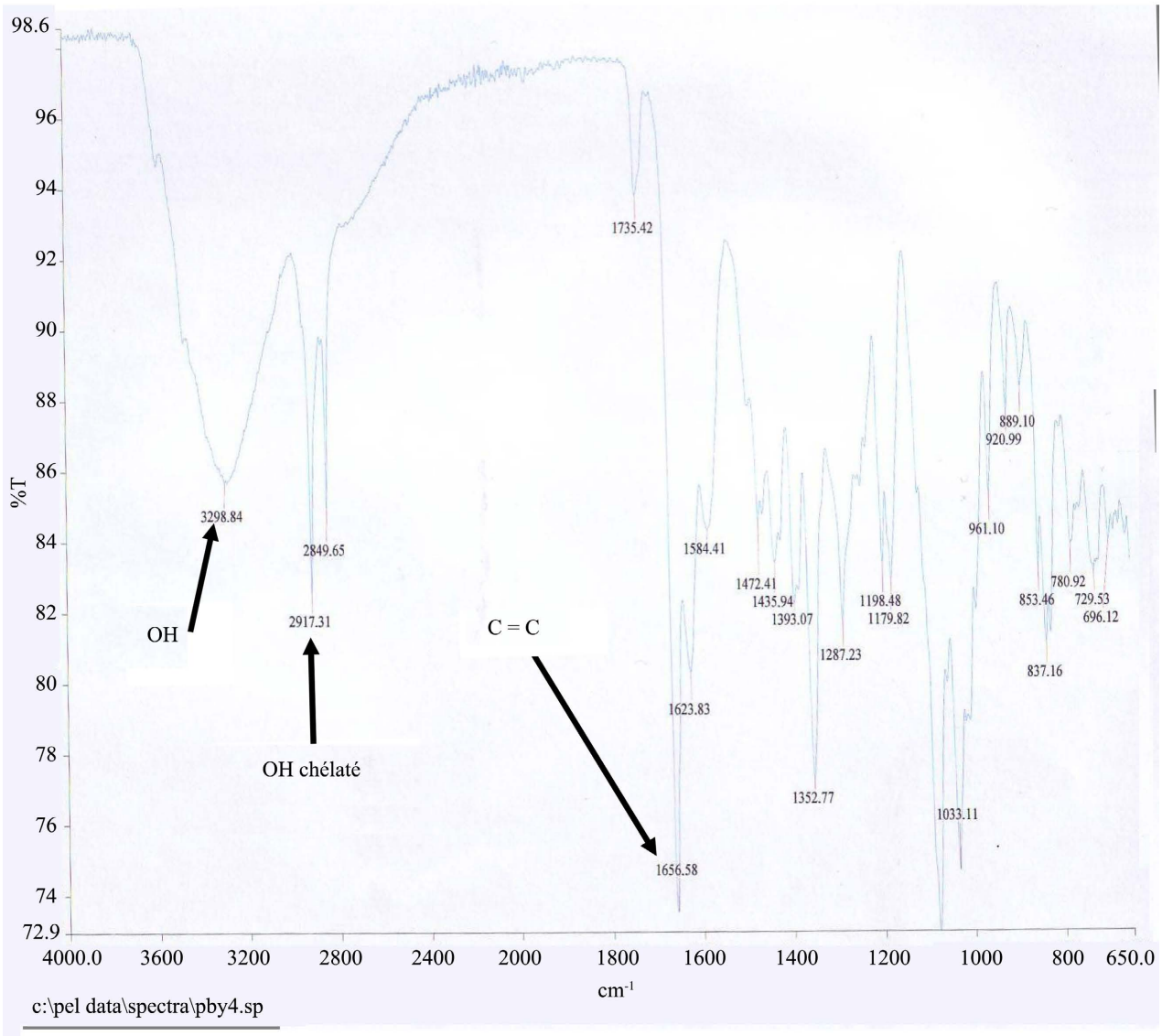


Figure 2. Infrared spectrum of the compound PBy4.

Spectrum Nuclear Magnetic Resonance of the 1H proton (^{1}H-NMR) of the compound PBy4

In its ^{1}H-NMR spectrum, we observe two signals from the chelated protons with one having a medium intensity at δ_H 13.47 (s) attributable to the hydroxyl group of the minority isomer and the other having a long intensity at δ_H 13.02 (s) attributable to the hydroxyl group of the majority isomer. We also observe two signals of different intensities of the proton carried by carbon C-3 between δ_H 6.09 (s) and δ_H 6.19 (s). At the end we observe at 4.94 ppm a doublet, characteristic of a proton carried by an anomeric carbon.

Nuclear Magnetic Resonance spectrum of carbon ^{13}C (^{13}C-NMR) of compound PBy4

Its broad band decoupled ^{13}C-NMR spectrum shows the presence of two phloroglucinol type aromatic rings at δ_C [108.7 (s), 99.5 (d); 104.5 (s), 103.1 (s);

156.7 (s), 156.3 (s); 160.7 (s), 160.4 (s); and 163.2 (s), 162.7 (s)]; double bond at δ_C [108.7 (d), 107.8 (d) and 167.4 (s), 167.5 (s)]; a two-carbonyl group at δ_C [181.9 (s), 182.1(s)] and a two-methyl group appearing at the same location at δ_C [19.8 (q), 19.8 (q)]. However, we note the absence of the normally expected signals in the 188 - 129 ppm region that may correspond to the chemical shifts of three carbons in ring B. In comparison with literature data and referring to the unsaturation number, we believe that PBy4 would have a 2-methyl-5,7- dihydroxychromone type of skeleton [20]. Furthermore, the appearance of the aliphatic carbon signals at δ_C 81.5 (d), 81.3 (d); 78.9 (d), 78.5 (d); 72.9 (d), 72.5 (d); 70.9 (d), 70.6 (d); 70.4 (d), 70.1 (d), and 63.3 (t), 61.4 (t), whose chemical shifts are closely related to those of the C-glucosyl residue of 6-C-glucosylquercetin [δ_C 73.0 (C-1), 70.5 (C-2), 78.9 (C-3), 70.3 (C-4), 81.3 (C-5), and 61.4 (C-6)] [21], indicates that PBy4 is a mixture of two C-glucoside isomers of 2-methyl-5,7- dihydroxychromone. We have compared the ^{13}C-NMR spectral data of PBy4 with those of 2-methyl-5,7-dihydroxychromone 8-C-β-D-glucopyranoside from the literature in the following Table 4.

Table 4. Comparative ^{13}C-NMR spectral data of PBY4 with that of 2-methyl-5,7-dihydroxychromone 8-C-β-D-glucopyranoside from literature.

Position	PBy4 (a) δ_C (500 MHz DMSO-d6) δ_C (a) (ppm)	PBy4 (b) δ_C (500 MHz DMSO-d6) δ_C (b) (ppm)	2-methyl-5,7-dihydroxychromone-8-*C*-β-D-glucopyranoside δC (125 MHz, pyridine-d5)
2	167.4	167.5	167.3
3	107.8	107.5	107.5
4	181.9	182.1	182.0
5	160.7	160.4	160.4
6	108.7	99.5	98.4
7	163.2	162.7	162.6
8	93.4	103.6	104.4
9	156.7	156.3	156.2
10	104.5	103.1	103.5
1'	72.9	72.5	73.1
2'	70.4	70.9	70.8
3'	78.9	78.5	78.5
4'	70.6	70.1	70.4
5'	81.3	81.5	81.2
6'	61.4	63.3	61.3
1"	19.8	19.8	19.7

Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectra of the compound PBy4

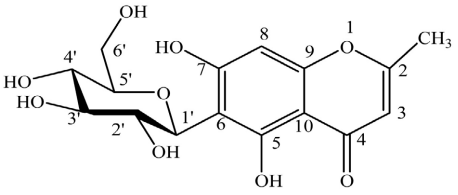
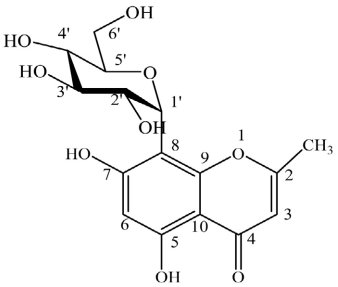
The location of the C-glucosyl moiety is determined by examining the HSQC and HMBC 1H-13C spectra of the compound PBy4.

In the HSQC spectrum, we observe a direct coupling between the δ_C 93.4 (C-8) carbon and the δ_H 6.41 (H-8) proton, followed by a direct coupling between the δ_C 103.6 (C-6) carbon and the δ_H 6.25 (H-6) proton.

In the HMBC spectrum, we observe long-range coupling between the anomeric protons at δ_H 4.94 - 4.74 (d); δH 4.56 - 4.63 (d, J = 9.9 Hz) and carbons bearing an aromatic hydroxyl at δ_C 163.2 (C-7a), δC 162.7 (C-7b); then between carbons at δC 160.7 (C-5) for the PBy4a isomer and at δC 156.3 (C-9) for the PBy4b isomer. Then, a similar correlation is observed between the C-5 carbon and the aromatic proton at δH 6.25 (s, H-6); then between the C-7 carbon and the aromatic proton at δ_H 6.41 (s, H-8). These observations clearly indicate that the aromatic protons at δ_H 6.41 (s, H-8); δ_H 6.25 (s, H-6), are attached to the C-8 and C-6 carbons of the chromone backbone, respectively. Thus, the location of the C-glucosyl moiety is attached to the C-6 positions for the **PBy4a** isomer and C-8 for the **PBy4b** isomer.

Structures of the PBy4a and PBy4b isomers

The compound PBy4 is thus the mixture of 2-methyl-5,7-dihydroxychromone 8-C-β-D-glucopyranoside already isolated from the leaves of Syzygium aromaticum Merr. et Perry by Tanaka *et al.* [21] and 2-methyl-5,7-dihydroxychromone 6-C-α-D-glucopyranoside. To our knowledge these isomers are for the first time isolated from propolis. The structures of its isomers have been proposed as follows.

Compound	Structure	Name
PBy4a		2-methyl-5,7-dihydroxychromone-6-C-β-D-glucopyranoside
PBy4b		2-methyl-5,7-dihydroxychromone-8-C-α-D-glucopyranoside

Evaluation of the antioxidant power

We used a method based on the reducing potential of iron and another based on the anti-free radical activity at DPPH.

Iron chelating power

The chelating power is often used as an indicator of the capacity of a compound to bind metal cations by forming a stable complex [22]. The chelating capacity test highlights the capacity of a molecule to reduce an oxidant by giving up an electron, thus allowing the anti-oxidant activity of the tested sample to be assessed. This antioxidant activity is based on the reduction of Iron (III) present in the $K_3Fe(CN)_6$ complex to Iron (II) [23]. The results of the chelating activity of the hexane and acetate extracts of the propolis studied are shown in **Figure 3**.

We find that the chelating capacity is proportional to the increase of the concentration of the extracts of propolis and Ethylene Diamine Tetraacetic Acid (EDTA), a synthetic antioxidant used for the reference. For a concentration of 0 to 50 mg/ml, all the extracts present the same chelating power including also EDTA. But beyond this concentration, the ethyl acetate extract shows a very high chelating power compared to the hexane extract. This can be explained by the majority presence of phenolic compounds found in the acetic extract; because being good electron donors, phenolic compounds have this ability to fix metal cations by forming a stable complex and thus show a high chelating power.

Anti-radical power of DPPH

The antioxidant activity of the extracts is expressed in IC50, this parameter was used by several groups of researchers to present their results. It defines the effective concentration of the substrate which causes the loss of 50% of the activity of the DPPH radical (color). It is 0.69 mg/ml for the hexane extract, 0.38 mg/ml for the ethyl acetate extract and 0.28 mg/ml for the methanol extract. This last value is close to IC50 of BHT and Vitamin C which are reference molecules (**Table 5**).

From this table, we notice that all the extracts have an anti-radical power towards DPPH. The smaller the IC50 value, the more the extract is considered as a powerful antioxidant. These results corroborate well those of Ferhoum [3] who evaluated the anti-free radical activity of propolis from different regions of Algeria. On the other hand, the study conducted on ethanolic extracts of propolis from Portugal showed that the IC50 values obtained are about 0.006 mg/ml and

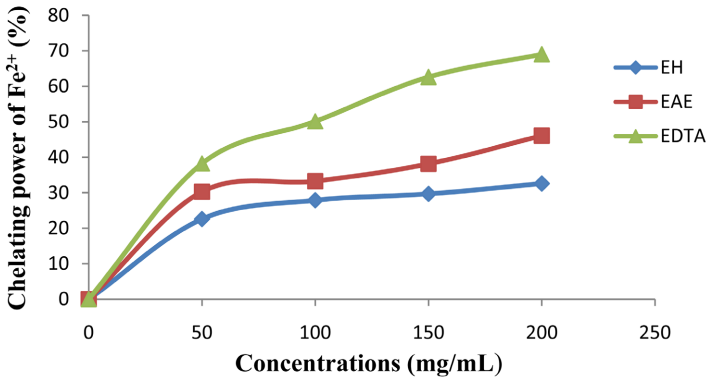


Figure 3. Variation of chelating capacity as a function of propolis concentration.

Table 5. Comparison of DPPH radical inhibition by propolis extracts and reference compounds.

Extracts	Inhibitory concentration IC_{50} (mg/mL)
HE	0.69
EAE	0.38
ME	0.28
BHT	0.26
Vitamine C	0.24

HE: Hexane extract; EAE: Ethyl acetate extract; ME: Methanol extract.

0.025 mg/ml respectively for propolis from Bornes and Funddao [23]. Results, by far, are inferior to those found in our study, so the propolis of Portugal would have a more important antiradical power than our analyzed samples.

4. Conclusions

This work on the ethyl acetate extract allowed us to isolate, using the usual chromatographic methods, two compounds indexed PBy4a and PBy4b. The structures of PBy4a and PBy4b were elucidated on the basis of the interpretation of one and two dimensional NMR spectral data (1H NMR, 13C NMR, HSQC, HMBC) compared to those of the literature, of its Infrared spectrum and of its mass spectrum under electrospray ionization. It is a mixture of two chromones namely 5,7-dihydroxy-2-methylchromone-6-C-β-D-glucopyranoside and 5,7-dihydroxy-2-methylchromone-8-C-β-D-glucopyranoside reported for the first time from propolis.

Quantification of phenolic compounds gave the content that varied from 0.71 ± 0.07 to 2.59 ± 0.23 g EAG/100g MB for total phenols and content of 0.19 ± 0.03 to 1.01 ± 0.02 g EQ/100g MB for total flavonoids for hexanic and acetic extracts respectively.

The study of the anti-free radical power of the propolis extracts by the DPPH test gave IC50 values which varied from 0.69 mg/mL for the hexanic extract and 0.38 mg/mL for the ethyl acetate extract to 0.28 mg/mL for the methanolic extract.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Donadieu, Y. (1991) Les produits de la ruche. 3ème Edition, Dangles, Paris, 73.

[2] Debuyser, E. (1984) La propolis. Thèse pour le diplôme d'état de docteur en pharmacie. Université de Nante, Faculté de pharmacie, 106.

[3] Ferhoum, F. (2010) Analyses physico chimiques de la propolis locale selon les étages

bioclimatiques et les deux races d'abeille locales. Magister en technologie alimentaire, Alger, 174.

[4] Talla, E., Dabolé, B., Taiwe, G.S., Ngo Bum, E., Mbafor, J.T., Acthadé, A.D.T., Malik, R., Zulfiqar, A., Sidiki, N., Nguimbou, R.M. and Coudhari, M.I. (2013) Antinoceptive Pentacyclic Triterpenoids from the Cameroonian Brown Propolis. *Pharmacologia*, **4**, 218-227. https://doi.org/10.5567/pharmacologia.2013.218.227

[5] Mohammdi, Z. (2004) Etude de pouvoir antimicrobien et antioxydant des huiles essentielles et flavonoïdes de quelques plantes de la région de Telmcen. Thèse de magistère, option: produit naturels, activités biologiques et synthèse, Alger, 147.

[6] Alencar, S.M., Oldo, T.L.C., Castro, M.L., Cabral, I.S.R., Costa-Neto, C.M., Cury, J.A., Rosalen, P.L. and Ikegaki, M. (2007) Chemical Composition and Biological Activity of a New Type Brazilian Propolis: Red Propolis. *Journal of Ethnopharmacology*, **113**, 278-283. https://doi.org/10.1016/j.jep.2007.06.005

[7] Mbawala, A., Roger, D., Tchuenguem, F.F-N. and Milliere, J-B. (2010) *In Vitro* Susceptibility of Same Bacteria Strains Collected from Different Geographic Origins in Cameroun. *Journal of Food Technology*, **8**, 217-222. https://doi.org/10.3923/jftech.2010.217.222

[8] Kumazawa, S., Hamasaka, T. and Nakayama, T. (2004) Antioxydant Activity of Propolis of Various Geographic Origins. *Food Chemistry*, **84**, 329-373. https://doi.org/10.1016/S0308-8146(03)00216-4

[9] Njintang, Y.N., Tatsadjieu, N.L., Ngakou, A., Danra, D. and Tchuenguem, F.F.N. (2012) Antiradical Activity and Polyphenol Content of Ethanolic Extracts of Propolis. *International Journal of Biosciences* , **4**, 56-63

[10] Jiménez, O., Pérez, B., Sánchez, T., Tovar, C., Bordes, J. and Zárate, C. (2020) Evaluation de l'activité antivirale de la propolis des abeilles indigènes (*Plebeia frontalis*) contre le virus du Distemper canin. *Open Journal of Veterinary Medicine*, **10**, 207-218. https://doi.org/10.4236/ojvm.2020.1012018

[11] Preeti, K., Neelima, R.K. and Kusum, H. (2012) Phytochemical Screening and Antibacterial Activity of Different Extracts of Propolis. *International Journal of Pharmaceutical and Biological Research*, **3**, 219-222.

[12] Boizot, N. and Charpentier, J. (2006) Méthode rapide d'évaluation du contenu en composés phénoliques des organes d'un arbre forestier dans Méthodes et Outils pour l'observation et l'évaluation des milieux forestiers, prairiaux et aquatiques. *Le Cahier des Techniques de l'Inra*: *Numéro Spécial*, **2006**, 79-82.

[13] Hariri, E.B., Sallé, G. and Andary, C. (1991) Involvement of Flavonoids in the Resistance of Two Poplar Cultivars to Mistletoe. *Protoplasma*, **162**, 20-26. https://doi.org/10.1007/BF01403897

[14] Laskar, R.A., Ismail, S., Roy, N. and Begum, N.A. (2010) Antioxydant Activity of Indian Propolis and Its Chemical Constituents. *Food Chemistry*, **122**, 233-237. https://doi.org/10.1016/j.foodchem.2010.02.068

[15] Suter, M. and Richter, C. (2000) Anti and Pro-Oxidative Properties of PADMA 28, a Tibetan Herbal Formulation. *Redox Reprot*, **5**, 17-22. https://doi.org/10.1179/rer.2000.5.1.17

[16] Trucheva, B., Todorov, I., Ninova, M., Najdenski, H., Daneshmand, A. and Bankova, V. (2010) Antibacterial Mono- and Sesquiterpene Esters of Benzoic Acids from Iranian Propolis. *Chemistry Journal Central*, **4**, 1-4. https://doi.org/10.1186/1752-153X-4-8

[17] Petrova, A., Popova, M., Kuzmanova, C., Tsvetkova, I., Naydenski, H., Mulli, E. and Bankova, V. (2010) New Biologically Active Compounds from Kenyan Propolis.

Fitoterapia, **8**, 509-514. https://doi.org/10.1016/j.fitote.2010.01.007

[18] Bankova, V. (2005) Chemical Diversity of Propolis and the Problem of Standardization. *Journal of Ethnopharmacology*, **100**, 114-117. https://doi.org/10.1016/j.jep.2005.05.004

[19] Xu, Y., Luo, L., Chen, B. and Fu, Y. (2009) Recent Development of Chemical Components in Propolis. *Frontiers of Biology in China*, **13**, 114-117.

[20] Tanaka, T., Orii, Y., Nonaka, G. and Nishioka, I. (1993) Tannins and Related Compound. CXXIII. Chromone, Acetophenone and Phenylpropanoid Glycosides and Their Galloyl and/or Hexahydroxyphenyl Ester from Leaves of Syzygium Aromaticum Merr and Perry. *Chemical pharmaceutical Bulletin*, **28**, 685-687. https://doi.org/10.1248/cpb.41.1232

[21] Shiva, M., Mohammad, S., Manoochehr, H., Reza, A., Narsin, S. and Seyed, N.O. (2006) Chemical Composition, Oral Toxicity and Antimicrobial Activity of Iranian Propolis. *Food Chemistry*, **6**, 201-213.

[22] Wang, H., Zhao, M., Yang, B., Jiang, Y. and Guohuo, R. (2008) Identification of polyphéNols in Tobacco Leaf and Their Antioxydant and Antimicrobial Activities. *Food Chemistry*, **163**, 1399-1406. https://doi.org/10.1016/j.foodchem.2007.09.068

[23] Leandro, M., Luis, G., Diase, J.A.P. and Leticia, E. (2008) Antioxydant Properties, Total Phenol and Pollen Analysis of Propolis Samples from Portugal. *Food and Chemical toxicology*, **46**, 3482-3485. https://doi.org/10.1016/j.fct.2008.08.025