Medicinal Chemistry

Research Article Open Access

LC-MS Analysis, Total Phenolics Content, Phytochemical Study and DPPH Antiradical Scavenging Activity of Two Cameroonian Propolis Samples

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Abstract

Two samples of Cameroonian propolis from Nyambaka (Adamawa) and Mveg (Nord-West) were collected for some analysis. Six extracts were obtained by simple maceration with solvents of increasing polarity: hexane, ethyl acetate (EtOAc) and the MeOH/H_2O (80:20, v/v). Liquid Chromatography coupled with Mass Spectrometry (LC-MS) analysis revealed the presence of compounds such as: *p*-coumaric acid, cinnamyl ester, dimethylkuradine, galangin, pinocembrin inside the both propolis. The total phenolics content (TPC) was evaluated using Folin-Ciocalteu method on the EtOAc and MeOH/H_2O (80:20, v/v) extracts of each sample. Nyambaka propolis has the major results which ranged from (200.06 ± 0.17) mgGAE/100 g RM for EtOAc extract to 847.73 ± 0.15 mgGAE/100 g RM for MeOH/H_2O (80:20, v/v). The antiradical activity of the different extracts was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical inhibition method and MeOH/H_2O extract of Nyambaka propolis has the highest antiradical activity with an IC_{50} of 0.34 mg/mL.

Keywords: Propolis; Phenolics; Anti-radical

Introduction

Propolis is a natural substance of viscous consistency that bees make from resins collected on certain plant parts and mixed with bee's enzyme [1]. Propolis has been mentioned as one of the most fascinating product of the hive because of its various biological activities such as: antimicrobial, anti-inflammatory, antinociceptive, antioxidant and anti-ulcer [2-6]. Chemical studies revealed the presence of more than 300 compounds inside propolis and the most represented are: phenolics, coumarines, anthraquinones flavonoids, fatty acids, lignans, sugars, amino acids, steroids and triterpenes [7].

In Cameroon, honey is the best known and used product of the hive, but propolis remains one of the most interesting, because of its biological properties [4]. The Adamawa and North-west regions are the major productions of propolis in cameroon and the population of these regions is still unaware about the medicinal properties of this natural substance [8]. That is the reason why we opted to work on propolis of Nyambaka (Adamawa) and Mveg (North-West) where the researches have not yet been done.

Reactive oxygen species (ROS) such as free radicals, released by the human body are responsible of certain diseases such as: cancer, diabetes, arthritis and cardiovascular disease [9]. Antioxidants are moleculars able to slow down, prevent or inhibit the oxidation of a chemical substance [10]. The most well-known antioxidants are: *β*-carotene (provitamins A), ascorbic acid (vitamin C), tocopherol (vitamin E), flavonoids (very common in plants), and tannins (condensed polyphenolic compounds). The high content of phenolics and flavonoids in Cameroonian propolis is responsible of its antiradical activity [11]. The purpose of this article is to do LC-MS analysis in order to know the different compounds present and also determine total phenolics, flavonoids content and DPPH antiradical scavenging activity of Cameroonian propolis.

Material and Methods

Sample collection

Two samples of propolis were harvested at the same time in April 2018 with the help of a bee keeper. 1 kg of Nyambaka propolis and 500 g of Mveg were kept in a black plastic in the absence of heat and light.

Extraction

Propolis from Nyambaka (1 kg) was extracted three times (5 L × 3) sequential y using hexane, EtOAc and MeOH/H_2O (80:20, v/v) during one week (48 h × 3). Filtration was done using Whatmann No.1 filter paper, the solvent was evaporated under reduced pressure with a rotary evaporator. The MeOH/H_2O (80:20, v/v) extract was dried using a lyophilizer to evaporate all the residual water. 500 g of propolis of Mveg was extracted following the same method.

Qualitative analysis

Phytochemical screening: Phytochemical screening was carried out to identify the different family of compounds present in the extracts: flavonoids, alkaloids, triterpenoids, tannins, anthraquinons, glycosides, saponins, coumarins and fatty acid were screened according to the standard methods [12].

Protocol of liquid chromatography coupled with mass spectrometry (LC-MS): For LC separation coupled with simultaneous mass spectrometry, samples were separated on an Agilent 1200 HPLC system (Agilent technologies, Santa Clara, USA) consisting of an autosampler, pump, column oven and diode array detector. LC flow was directly injected to an agilent 6220 ESI time-of-flight mass spectrometer, in extended dynamic range mode equipped with a

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Received December 14, 2019; **Accepted** December 19, 2019; **Published** December 26, 2019

Citation: Balingui CF, Noel NJ, Emmanuel T, Benoit NM, Gervais HM, et al. (2019) LC-MS Analysis, Total Phenolics Content, Phytochemical Study and DPPH Antiradical Scavenging Activity of Two Cameroonian Propolis Samples. Med Chem (Los Angeles) 9: 100-099.

Dual-ESI source operating with a spray voltage of 2.5 Kv. Spectra were recorded in range from *m/z* 100-2500 with an accumulation of spectra per s. The solvent system was in gradient. Eluent A: 94. 9% water/5% acetonitrile/0.1% formic acid, Eluent B: 94.9% acetonitrile/5% water/0.1% and the flow rate: 0.30 mL/min.

Quantitative analysis

Determination of total phenolic content (TPC): Folin-ciocalteu reagent was used to quantify total phenolic compounds in the extracts according to the method of Talla et al. with few modifications [11]. Indeed, 100 µL of extract (0.2 mg/mL) was added to 200 µL of Folin-Ciocalteu and 1380 µL of distil ed water. The mixture was stirred and left to stand for 3 minutes. After that, 1000 µL Na_2CO_3 (20%) was added to the mixture which was incubated at room temperature and protected from light for 1 hour. The absorbance was measured at 760 nm on the spectrophotometer against a solution of MeOH used as white. The control preparation, gallic acid (0.2 mg/mL) was made under the same conditions. The results obtained were expressed in grams of gallic acid equivalent per 100 g of raw material (gEAG/100 gRM). The quantification of total phenolic compounds was made according to a linear calibration curve made by different concentrations (50 to 200) µg/mL of Gallic acid by following the equation below (1):

$$X=(y-b)V_1/V_2am \times 100\ g\ EX \quad (1)$$

x: Content of phenolic compounds;

y: Calibration curve equation

Determination of total flavonoids content (TFC): The TFC was based on a colorimetric test using aluminum chloride. The TFC was performed according to the method described by Betoloum et al. with slight modifications [13]. To 100 µL of each extract diluted in MeOH, 400 µL of distilled water, 30 µL of Na_2NO_2 5% were added. 5 min later, 20 µL of a solution of $AlCl_3$ 10%, 200 µL of Na_2CO_3 1 M along with 25 µL of distilled water were also added. After standing for 5 minutes, the mixture obtained was shaked in vortex and 1 mL of this mixture was taken to read the absorbance against the requested MeOH. The flavonoid content was calculated by the equation below (2):

$$F=0.05 \times A_{ext}/A_rC_{ext} \times 100 \quad (2)$$

F: Content of total flavonoids; *Aext*: absorbance of the extract; *Aq*: absorbance of quercetin;

Cext: Concentration of propolis extract

Antiradical activity

The antioxidant test was carried out according to the method of Talla et al. with slight changes. A stock solution of concentration 1 mg/mL of the extracts and vitamin C (reference compound) from which dilutions were prepared respectively at the following concentrations: 100, 200, 400, 600, 800, and 1000 µg/ml [11]. Then, 1000 µL of DPPH was prepared at a concentration of 0.5 mM and added to the previous solution. The mixture was incubated in the dark at room temperature for 45 minutes. After incubation, absorbances were read at 517 nm. The inhibition percentage (%IP) was calculated according to the equation below (3):

$$\%IP=Absorbance\ control-Absorbance_{extract}/Absorbance\ control \times 100 \quad (3)$$

Results and Discussion

Extraction yields of propolis crude extracts

Extraction yields of propolis from Nyambaka and Mveg are shown in Tables 1 and 2.

Phytochemical screening

Table 2 revealed the lack of compounds such as: flavonoids, tannins, anthocyanins, phenolics, anthraquinones and the presence of terpenoids in the hexane extracts of samples. This can be justified by the fact that propolis of tropical countries is very rich in terpenoids and steroids [4]. MeOH/H_2O 80:20 extract of samples contained alkaloids, flavonoids, phenolic, steroids, triterpenes, anthraquinones, saponins. In the EtOAc extract of Nyambaka propolis, we observed the presence of alkaloids, flavonoids, phenolic compounds, steroids, triterpenes, anthraquinones and the absence of saponins. This result corroborated with the one of Talla et al. for propolis of Tekel (Adamawa, Cameroon) [14].

LC-MS analysis

The mass spectrum obtained after analysis and the database found in the literature enabled us to identify the different compounds present in each sample [15,16]. The results of LC-MS analysis of EtOAc and MeOH/H_2O extracts of Nyambaka propolis were consigned in Table 3. We noted from Tables 3 and 4 that EtOAc and MeOH/H_2O extract of Nyambaka were rich in *p*-coumaric acid cinnamyl ester (*m/z*=279) found in all extracts. The flavonoids as: dimethylkuradine, Pinobanksin-3-*O*-propionate, Chrysin-5,7-dimethylether and also diterpene as cupressic acid were identified. These results were in agreement with those obtained by Bankova et al. for the propolis of the black Poplar type from *populus nigra* [3]. In MeOH/H_2O extract we noticed the presence of 1,3 dicoumaroylglycerol, quercetin-3-*O*- glucuronic found also in Portuguese propolis [16].

TIC chromatogram and mass spectrum of EtOAc extract of Nyambaka propolis

The base peak of the molecular ion at *m/z* 425 was corresponding to Dimethylkuraridine. The Figures 1 and 2 illustrate the TIC chromatogram of EtOAc extract and the mass spectrum of the compound which has the most intense peak.

TIC chromatogram of MeOH/H_2O extract

The Figures 3 and 4 illustrate the TIC chromatogram of MeOH/

Solvants of extraction	Nyambaka Propolis		Mveg propolis	
	Mass of extract (g)	Yield (%)	Mass of extracts (g)	Yield (%)
Hexane	542.83	54.3	281.01	56.20
EtOAc	128.27	28.71	51.56	25.96
MeOH/H_2O	14.76	4.66	10.34	7.16
Total	685.86	87.67	342.91	89.32

Table 1: Percentage yield of extracts of propolis from both regions.

Structural group	Hexane		EtOAc		MeOH/H_2O (80:20, v/v)	
	Nyambaka	Mveg	Nyambaka	Mveg	Nyambaka	Mveg
Alkaloïds	+	-	+	+	+	+
Phenolics compound	-	-	+	+	+	+
Flavonoïds	-	-	+	+	+	+
Steroïds and terpenoïds	+	+	+	+	+	+
Saponins	+	-	-	+	+	+
Tannins	-	-	+	-	-	+
Anthocyanins	-	-	-	-	-	-
Glycosids	-	-	-	-	-	-
Anthraquinones	-	-	+	+	+	-

Table 2: Phytochemical screening of the extract.

Retention time (min)	*m/z* (+ESI)	% Fragments of the peaks	Compounds
4.8	279	248 (100); 120 (31); 110 (15)	*p*-Coumaric acid cinnamyl ester
5.1	231	189 (100); 271 (61); 129 (38)	*p*-Coumaric acid isoprenyl ester
11.1	281	281 (100); 263 (30)	Chrysin -5,7-dimethylether
11.3	261	279 (100); 261 (61)	Ferulic acid prenyl ester
12.3	425	425 (100); 311 (38); 124 (15)	Dimethylkuraridin

Table 3: LC-MS Analysis of EtOAc extract of Nyambaka propolis.

Retention time (min)	*m/z* (+ESI)	%Fragments of the peaks	Compounds
4.3	279	190 (100); 144 (77); 213 (46); 110 (23)	*p*-Coumaric acid cinnamyl ester
4.6	303	214 (100); 279 (15)	Mucronulatol
5.2	383	383 (100); 279 (23); 110 (8)	1,3-dicoumaroylglycérol
6.9	147	-	Cinnamic acid
7.4	477	-	Quercetin-3-*O*-glucuronide
9.0	515	533 (100); 279 (23); 124 (11)	Dicaffeoylquinic acid
9.7	327	415 (100); 279 (15)	Pinobanksin-3-*O*-propionate
11.8	149	149 (100); 279 (11); 205 (8)	3-phenylpropanoic acid
12.0	313	-	Kaempferol-dimethyl-ether
12.6	425	425 (100); 295 (46); 409 (38)	Dimethylkuraridine

Table 4: LC-MS analysis of the MeOH/H_2O extract of Nyambaka propolis.

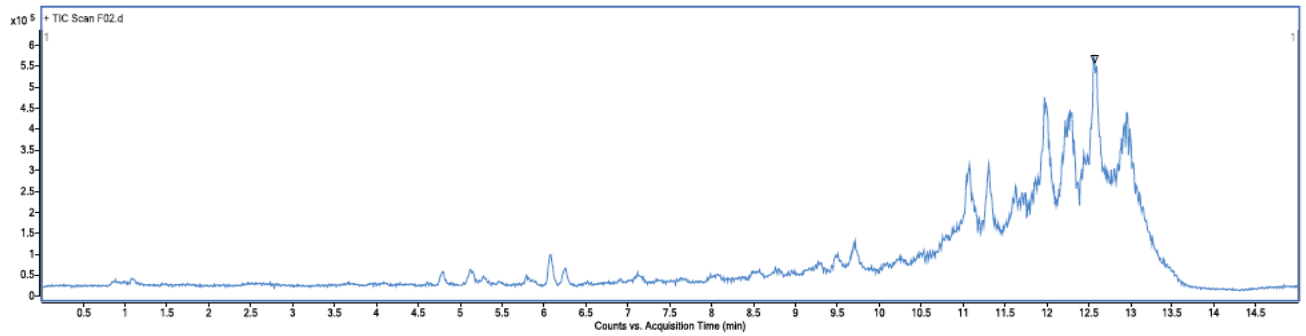

Figure 1: TIC chromatogram of EtOAc extract of Nyambaka propolis.

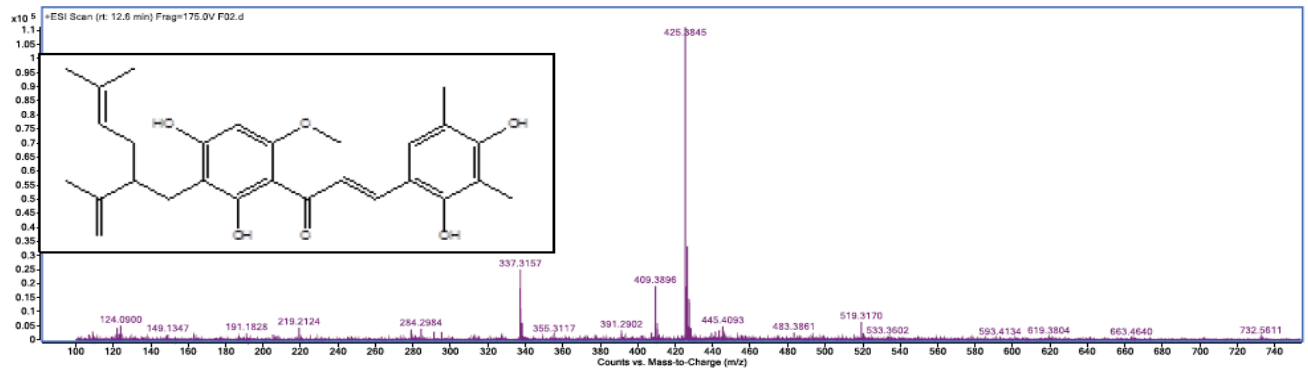

Figure 2: Mass spectrum of Dimethylkuraridine at m/z: 425.

H_2O extract and the mass spectrum of 3-phenylpropanoic acid at *m/z:* 149. The results of LC-MS analysis of EtOAc and MeOH/H_2O extracts of Mveg propolis were consigned in the Tables 5 and 6. We observed in these tables the presence of identical compounds such as p-cinnamyl coumarate (*m/z*=269), p-coumaric acid (*m/z*=163), dimethylkuradine (*m/z*=425) and mucronulatol (*m/z*=303) in both samples. Whereas, in Mveg propolis, MeOH/H_2O 80/20 extract revealed the presence of flavonoid such as: galangin (*m/z*=269) and pinocembrin (*m/z*=255). These compounds were also found in poplar propolis [3].

TIC chromatogram and mass spectrum of EtOAc extract of Mveg's propolis

The Figures 5 and 6 illustrate the TIC chromatogram of EtOAc extract and the mass spectrum of mucrolunatol at *m/z*=303

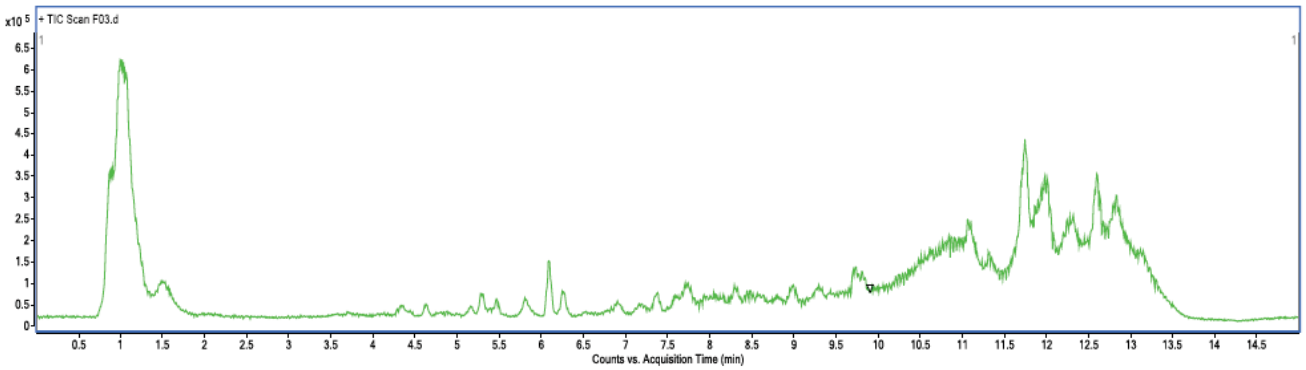

Figure 3: TIC chromatogram of MeOH/H_2O extract of propolis from Nyambaka.

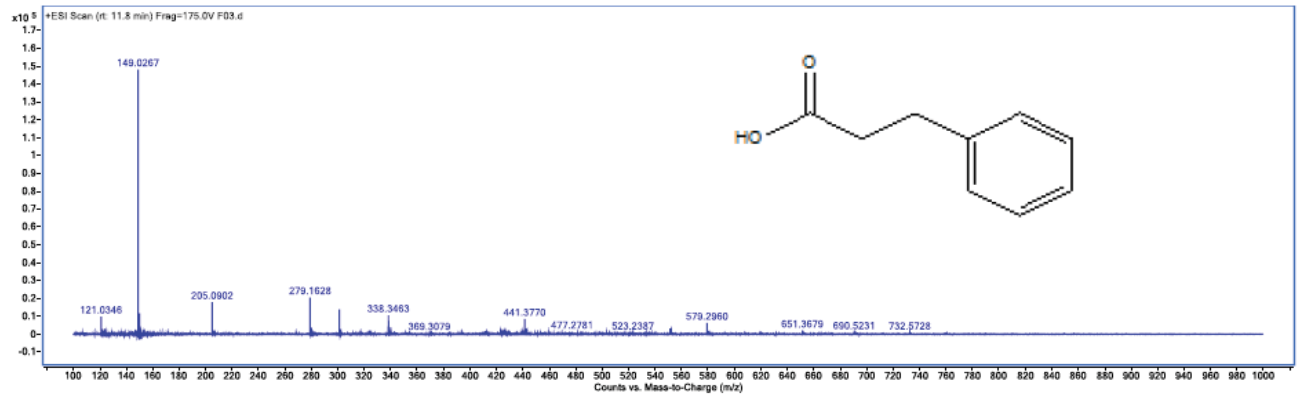

Figure 4: Mass spectrum of 3-phenylpropanoic acid.

RT (min)	*m/Z* (+ESI)	% Fragments of peaks	Compounds
3.9	279	114 (100); 159 (6)	*p*-Coumaric acid cinnamyl ester
9.9	303	303 (100); 279 (23)	Mucronulatol
11.5	425	425 (100); 439 (38); 457 (30)	Dimethylkuraridine

Table 5: LC-MS analysis of EtOAc extract from Mveg propolis.

RT (min)	*m/z* (+ESI)	% Fragments	Compounds
4.0	355	355 (100); 114 (40)	*P*-coumaric acid
4.9	163	360 (100); 135 (11)	2-methylbutyrate
5.2	433	433 (100); 136 (15) ;	Pinocembrin-5-*O*-3-hydroxy -4-methoxyphenylpropionate
10.6	255	255 (100); 237 (23) ;	Pinocembrin
12.3	269	269 (100); 237 (15)	Galangin

Table 6: LC-MS analysis of MeOH/H_2O extract.

TIC chromatogram of MeOH/H_2O extract propolis of Mveg

The Figures 7 and 8 illustrate the TIC chromatogram of MeOH/H_2O extract and the mass spectrum of Galangin at *m/z*=269

Quantification of phenolic and flavonoids

The results obtained from screening and LC-MS analysis showed that phenolics and flavonoids compounds were present in samples. So it was important to quantifying them. Nyambaka's propolis was richer in phenolics and flavonoids than Mveg's propolis. In fact, EtOAc extract of Nyambaka propolis has a content of 200.06 ± 0.17 mgGAE/100 g RM which was greater than EtOAc extract of Mveg (3.42 ± 0.017) mgGAE/100 g RM. The MeOH/H_2O extract of Nyambaka also has higher phenolics content (847.73 ± 0.15) than Mveg propolis (114.6 ± 0.07) mgGAE/100 g RM. In conclusion, the MeOH/H_2O 80:20 of Nyambaka extract had higher phenolics compounds than the other extracts. It also has a greater content of flavonoids (22.83 ± 0.005) mgQE/100 g RM than the other extracts. This result was inferior to those obtained by Mohammadzadeh et al. for Iranian propolis which contained more phenolic compounds (3080 ± 0.02) mgGAE/100 g RM than Cameroonian propolis [17] (Tables 7 and 8).

DPPH radical scavenging

The antiradical activity was evaluated spectrophotometrically following the reduction of DPPH which was accompanied by its passage from the violet color to the yellow color measurable at 517 nm [4]. This

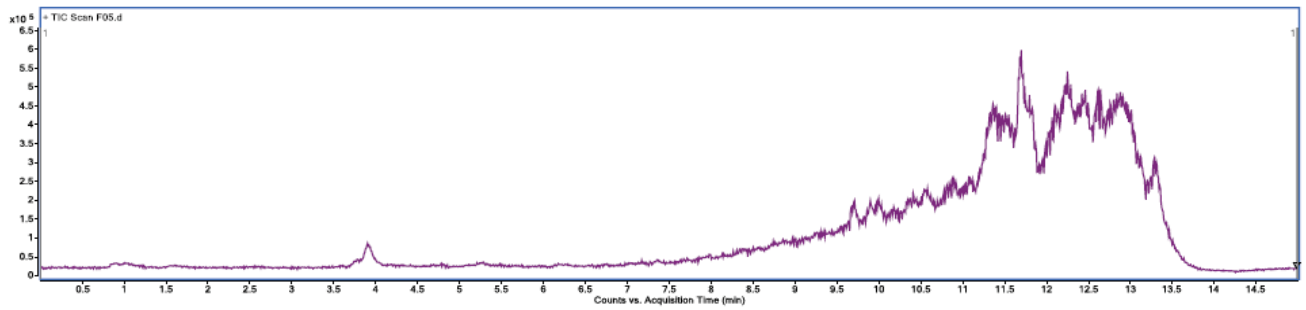

Figure 5: TIC chromatogram of EtOAc extract of Mveg propolis.

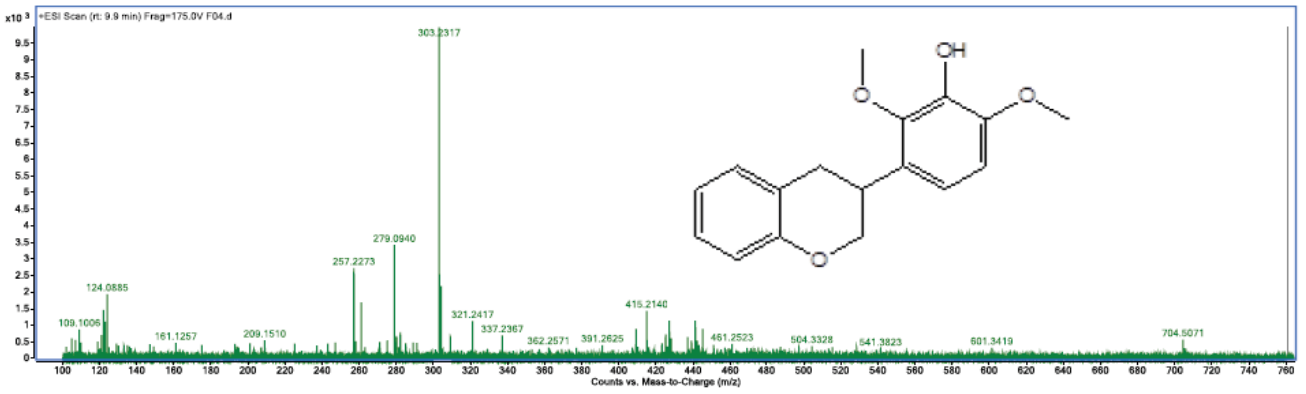

Figure 6: Mass spectrum of Mucronulatol at m/z=303.

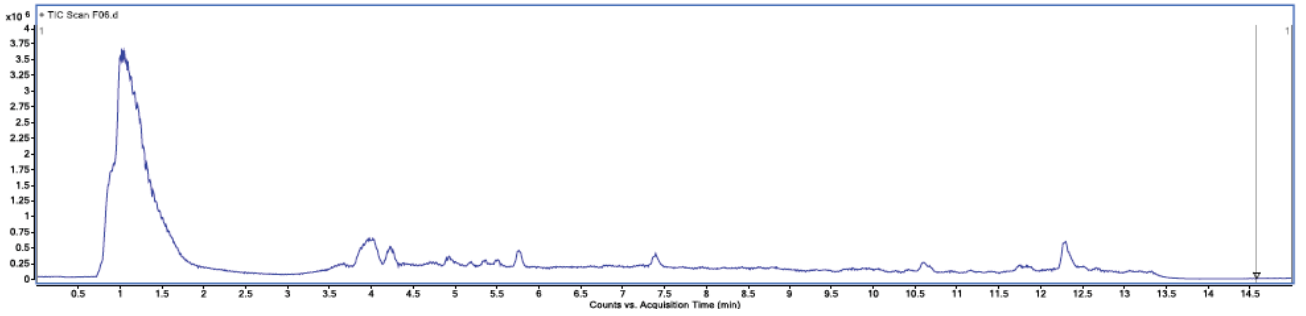

Figure 7: TIC chromatogram of MeOH/H_2O extract propolis of Mveg.

activity was evaluated by determining the concentration corresponding to 50% inhibition IC_{50}. The different percentage inhibition and the IC_{50} values are shown in the table below (Tables 9 and 10).

Propolis of Nyambaka

The Figure 9 illustrates the percentage inhibition of each extract and vitamin C according of the concentrations

Propolis of Mveg

The Figure 10 illustrates the percentage inhibition of each extract and vitamin C according of the concentrations. We should note that all the extracts have free radical scavenging effect on DPPH• and hence antiradical activities. MeOH/H_2O extract of Nyambaka has a greater antiradical activity with IC_{50} (0.34) mg/mL compared to other extracts. The results obtained could be justified by the fact that MeOH/H_2O extract of Nyambaka propolis has a higher total phenolic compounds (TPC) (847.73 ± 0.15) mgGAE/100 gRM compared to the MeOH/H_2O extract of Mveg propolis. It could be possible that a correlation existed between the antiradical activity and TPC because the more they were present in the extract, the better they trapped the free radicals [14]. Odiba et al. showed that propolis of Nigeria has a higher antiradical activity than Cameroonians propolis with IC_{50} value of 0.026 mg/mL [18]. This variation was explained by the influence of geographical area on the composition of propolis and his activity [19].

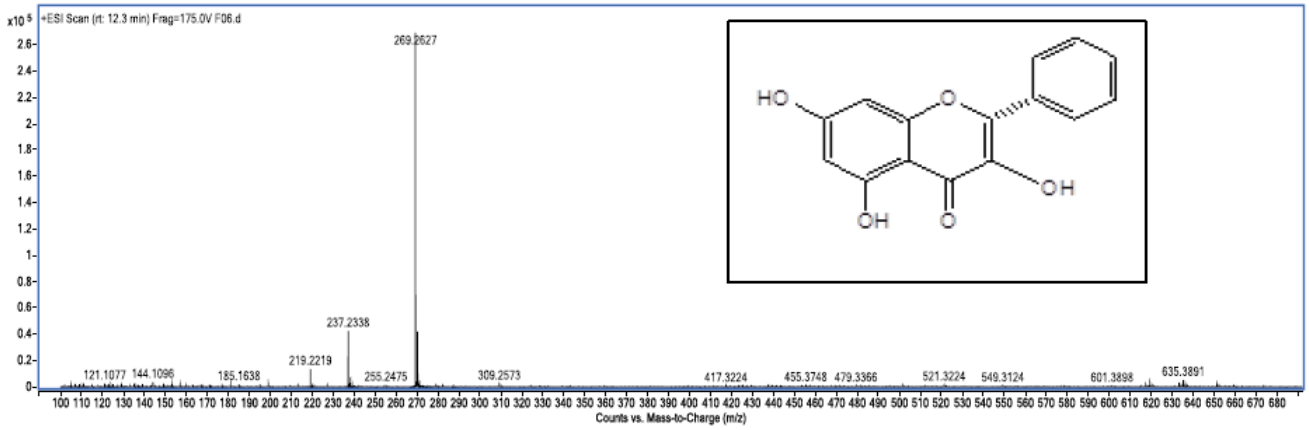

Figure 8: Mass spectrum of Galangin at m/z = 269.

Extracts	Nyambaka propolis	Mveg propolis
	Total phenolic content mgGAE/100 g RM	Total phenolic content mgGAE/100 g RM
EtOAc	200.06 ± 0.17	107.6 ± 0.02
MeOH/H_2O 80:20	847.73 ± 0.15	114.6 ± 0.07

Table 7: Total phenolics content of Nyambaka and Mveg propolis.

Extracts	**Nyambaka propolis** Total flavonoids content (mg QE/100 g RM)	**Mveg propolis** Total flavonoids content (mg QE/100 g RM)
EtOAc	3.42 ± 0.017	2.12 ± 0.045
MeOH/H2O 80:20	22.83 ± 0.005	7.55 ± 0.012

Table 8: Total flavonoids content.

Extract and standard	Inhibition Percent (%)	IC_{50} (mg/mL)	Correlation coefficient (R^2)
Vit.C	71.76 ± 0.13	0.049	0.97
MeOH/H_2O 80:20. E.	45.58 ± 0.23	0.34	0.99
EtOAc. E.	37.05 ± 0.19	0.45	0.97

Table 9: Percentage inhibition (%) and IC_{50} (mg/mL) values of Nyambaka extracts and vitamin C.

Extract and standard	Inhibition percentage (%)	IC_{50} (mg/mL)	R2
EtOAc. E.	41.8 ± 0.062	1.233	0.9960
MeOH/H_2O 80/20. E.	61 ± 0.074	0.788	0.9912
Vit. C	94.5 ± 0.003	0.089	0.9965

Table 10: Percentage inhibition (%) and IC_{50} (mg/mL) of Mveg propolis extracts and vitamin C.

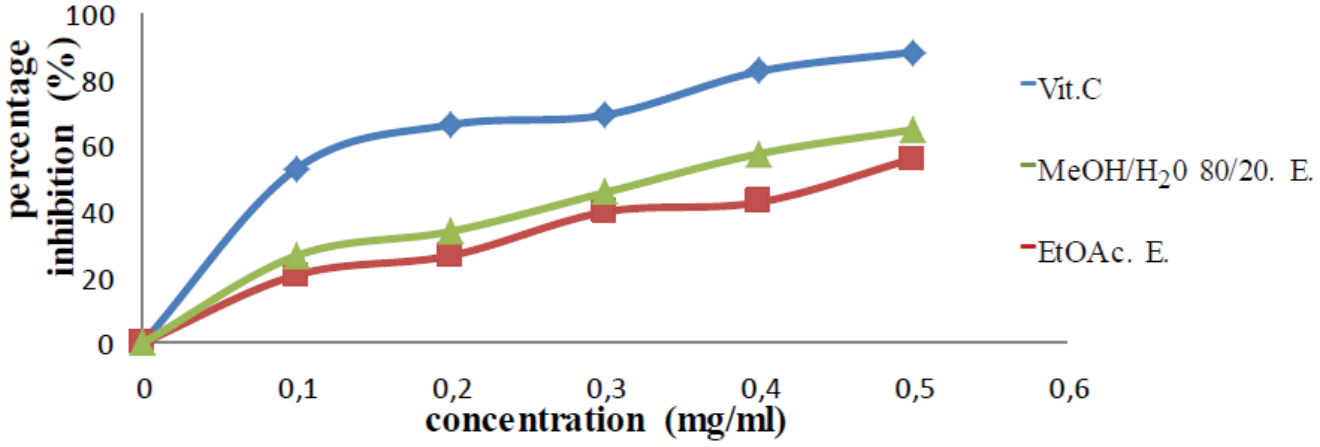

Figure 9: Percentage inhibition of Nyambaka propolis extracts and Vit C.

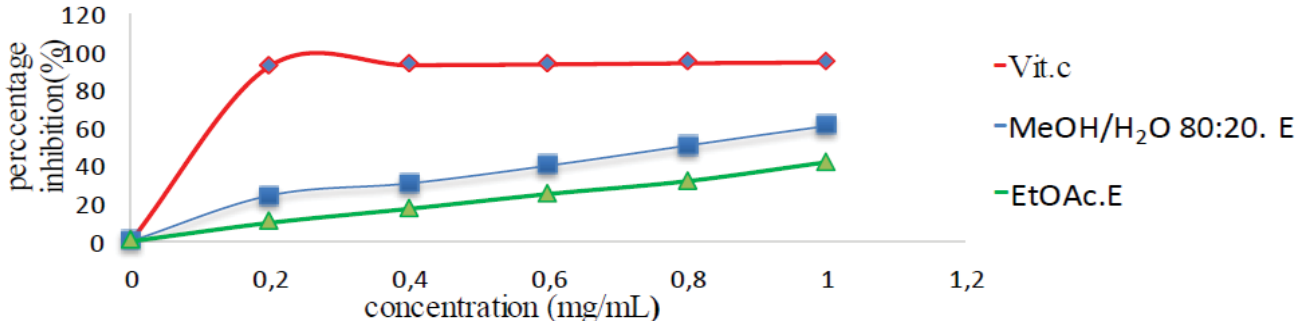

Figure 10: Percentage inhibition of Mveg propolis extracts and Vit C.

Conclusion

Phytochemical study of Cameroonian propolis revealed that the hexane extracts of the two regions did not have phenolics compounds, flavonoids and tannins. Nevertheless, EtOAc and MeOH/H_2O 80:20 extracts of each sample contained: phenolics, alkaloids, flavonoids, anthraquinones, steroids, triterpenes and tannins. The results obtained from LC-MS revealed that phenolics acids, subclasses of flavonoids (chalcone, flavones, glycosidic flavonol) also fatty acids, triterpene and carboxylic acid were present in each propolis. Furthermore, MeOH/H_2O 80:20 extract of Nyambaka propolis had a higher content of phenolics compounds and flavonoids compared to extracts of Mveg propolis. These was resulting in a higher antiradical activity of Nyambaka propolis with an IC_{50} (0.34) mg/mL.

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