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Int. J. Biol. Chem. Sci. 18(3): 982-990, June 2024

ISSN 1997-342X (Online), ISSN 1991-8631 (Print)

International Journal of Biological and Chemical Sciences

Original Paper http://ajol.info/index.php/ijbcs http://indexmedicus.afro.who.int

Preliminary phytochemical analysis, determination of phenolic contents and evaluation of antioxidant activity of propolis collected in Koumra (Southern Chad)

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Received: 11-04-2024 Accepted: 11-06-2024 Published: 30-06-2024

ABSTRACT

Propolis is a resinous substance manufactured by bees to protect their hive. It is used in folk medicine in Chad for the treatment of many microbial and inflammatory pathologies. The objective of this study was to determine the chemical composition and the antioxidant activity of different extracts of propolis from Koumra (Southern Chad) obtained by the Soxhlet extractor using solvents with increasing polarity. Phytochemical screening revealed the presence of alkaloids, coumarins, flavonoids, polyphenols, saponins, tannins and triterpenes. The total polyphenol content was evaluated using Folin-Ciocalteu reagent and gallic acid as standard. The total flavonoid content was assessed by ferric chloride and quercetin standard. The methanolic extract had a higher amount of polyphenolics (6.150±0.008 gEAG/100gMB) and flavonoids (2.143±0.006 gEQc/100gMB). The DPPH free radical assay showed that the methanolic extract exhibited antiradical activity with the lowest IC_{50} value of 0.0379 g/mL. All extracts revealed a chelating power to iron with a maximum effect at 71.53% for the methanol extract. Significant amounts of antioxidants could justify the use of propolis in the traditional medicine for the treatment of many diseases.

Keywords: Propolis, Phytoconstituents, Antioxidant, Polyphenols, Flavonoids, Chad.

INTRODUCTION

Propolis, is a complex resinous substance collected by bees from various plant to which they add their own secretions (Kim et Yoo, 2016). This substance is really a chemical compounds substances source (Huang et al., 2014). Bees use propolis as a chemical weapon to fight pathogenic microorganisms in the hive. Several authors have reported the uses of propolis as anti-inflammatory (Dongmo et al., 2020), analgesic (Dongmo et al., 2020), antiviral (Vilhelmova-Ilieva et al., 2023), antimicrobial (Checalla et Sanchez-Tito, 2021), vasodilator (Ntchapda et al., 2015), estrogen (Zingue et al., 2017), antioxidant (Betoloum et al., 2022), anticancer (Alanazi et al., 2021), anti-diabetic (El Adaouia et al., 2020) and antibacterial (Bankova et al., 2016). In Chad, propolis is traditionally used for wound healing and for the treatment of inflammation. Previous study indicated that this substance consists of resins (50%) made of flavonoids, polyphenolic compounds, coumarins, and terpenoids, waxes and fatty acids (30%), essential oils (10%), pollen (5%), and other organic and inorganic compounds

DOI : https://dx.doi.org/10.4314/ijbcs.v18i3.21

(5%). It should be noted that plant sources, geographical area and bee species are factors effecting the chemical compositions of propolis. Despite the variability of its chemical composition, it has a higher potential biological activity. Thus, propolis from unexplored regions of the world offers the potential to provide important bioactive secondary metabolites (Talla et al., 2016a). Hence, the present study aimed at characterizing the major structural groups of chemical compounds and to evaluate in vitro the antioxidant activity of propolis collected in Koumra (Chad).

MATERIALS AND METHODS

Collection of propolis sample

The propolis sample was collected in April 2022 in a village near Koumra, called Kotkouli (located between 8° 54' 45" North latitude and 17° 33' 14" East longitude) in the Eastern Mandoul Department, Province of Mandoul, Southern Chad.

Extraction

To extract secondary metabolites from propolis, the Soxhlet extractor was used. Thus, 250 g of propolis was introduced into a cartridge, then inserted into the extractor and the whole was placed on a flask containing two liters (2 L) of hexane. The operation was carried out for 6 hours in order to extract as many secondary metabolites as possible. The resulting mixture was filtered and the filtrate was concentrated by evaporating the solvent by distillation until the crude hexane extract was obtained. This same operation was carried out on the residual powder with solvents and solvent system (mixing) of increasing polarity: Hex/AcOEt (1:1, v/v), AcOEt, MeOH and MeOH/water (7:3, v/v). Five (05) extracts were obtained and stored at room temperature.

Phytochemical screening

Extracts were evaluated qualitatively for the presence of flavonoids, alkaloids, polyphenolic compounds, coumarins, saponins, tannins and terpenoids/steroids while quantitatively, total polyphenolic compounds and total flavonoids were determined. The qualitative screening included:

Test for flavonoids

In a test tube, 1 mg of extract, 3 mL of methanol, five pieces of magnesium metal and 1 mL of concentrated hydrochloric acid were successively placed. Orange coloration produced indicates the presence of flavonoids (Ano et al., 2018).

Test for alkaloids

The test was performed by a precipitation reaction with Dragendorff's reagent (Ano et al., 2018). One mL of extract and 5 drops of Dragendorff's reagent were mixed. A red precipitate was observed, which indicates the presence of alkaloids.

Test for polyphenolic compounds

Total phytochemical phenolic contents were evaluated using the reaction with ferric chloride ($FeCl_3$) (Ano et al., 2018). Two mL of extract (0.5 mg/mL) and a drop of 2% ferric chloride were mixed to form a $[Fe(OAr)_6]_3$-type complex. Observation of a precipitate or a green coloration indicates the presence of phenolics while a blue coloration indicates the presence of polyphenols.

Test for terpenoids and steroids

Sterols and triterpenes were observed according to the Liebermann-Burchard reaction (Talla et al., 2016b). In a test tube, 2 mL of extract (0.5 mg/mL), 2 mL of acetic anhydride and 0.5 mL of concentrated sulphuric acid were successively added. Blue-green or purple-pink colour indicates the presence of steroids or triterpenes.

Test for tannins

The tannin test was performed by mixing 1 mL of 1% ferric chloride ($FeCl_3$) solution to 5 mL of extract. Greenish or blackish-blue colour indicates the presence of tannins (Talla et al., 2016b).

Test for saponins

Five ml of the extract and 5 ml of distilled water were placed in a test tube and shaken thoroughly for 15 seconds. If foam produced persists for several minutes, it indicates the presence of saponins (Talla et al., 2016b).

Test for coumarins

A few milliliters of the extract (0.5 mg/mL) were placed in a test tube and then 0.5 mL of 1M NaOH was added. The test tube was

heated to boil for 3 minutes. After cooling, 4 ml of distilled water was added. Observation of yellow fluorescence colour under UV light indicates the presence of coumarins (Shaista et al., 2023).

Quantitative analyses

Total polyphenols and total flavonoids content of the extracts of propolis were evaluated. All measurements were performed in three replications.

Determination of total polyphenols

This assay was performed using the method described by Kouamé et al. (2021) with slight modifications. Total phenolic phytochemical content was measured using the Folin-Ciocalteu. Briefly, 100 µL of the extract (0.2 mg/mL), 2000 µL of distilled water were added to 200 µL of Folin-Ciocalteu reagent, stirred and left to stand for 2 minutes to allow for the reagent to react completely with the oxidable substances or phenolates. After this time, 2000 µL of sodium carbonate (20%) were added to destroy the residual reagent. Absorbances were measured at 760 nm with a spectrophotometer (Genesys 10 UV-Vis spectrophotometer) after incubation at room temperature in the dark for 15 minutes against distilled water as a blank. Preparation of gallic acid at different concentrations was carried out under the same conditions. Results obtained were expressed in grams of gallic acid equivalent per 100 g of raw material (gEAG/100gMB). Total phenolic contents of the samples were calculated from a linear calibration curve performed by different concentrations of gallic acid whose equation is $Y = 2.8390X + 0.0058$ with the correlation coefficient $R^2 = 0.9989$.

Determination of total flavonoids

Total flavonoid content of the extracts was determined using UV spectroscopic method (Talla et al., 2016b). According to the method, a volume of 1 mL of propolis extract (10 mg/mL) was mixed with 1 mL of $AlCl_3$ (2%). The whole solution was allowed to incubate for 1 hour at room temperature in the dark. Then, absorbances were measured at 430 nm. A calibration curve has been previously performed with standard solutions of quercetin prepared for different concentrations (500 to 5000 µg/mL). Results, determined from the calibration curve equation ($Y = 0.6420X - 0.0659$, $R^2 = 0.9989$) were expressed in grams of Quercetin Equivalents (QE)/100g dry material (DM)

Antioxidant activities

Methods for evaluation of antioxidant activities of the propolis extracts were as follows:

Free Radical Activity by DPPH

The potential antioxidant activity of the extracts was assessed on the basis of the scavenging activity of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method described by Talla et al. (2016b). For 500 µL of the sample solutions prepared at different concentrations, 1000 µL of a methanolic solution of DPPH (20 mg/L) was added. For the control, 1 mL of DPPH was added to 6 mL of methanol. Preparation of ascorbic acid, a reference standard, and samples was performed as follows: the stock solution at 200 µg/mL was obtained by dissolving 8 mg of extract in 40.00 mL of methanol. Subsequently, nine additional concentrations were obtained by dilution of the stock solution for each sample. The resulting mixture was incubated at room temperature for 15 minutes. Then, the absorbance was measured at 517 nm using the spectrophotometer and then the percentage of inhibition (%IP) was calculated according to the following formula:

$\%IP = [(A_{t0}-A_{t1})/A_{t0}]\times 100$

A_{t0} = control reaction absorbance

A_{t1} = testing specimen absorbance

Reduction of ferric ion

In order to confirm the antioxidant activity of the extracts, the reducing power of these extracts by iron ions (FRAP test) was also evaluated. The method is based on the

reduction of ferric ions (Fe^{3+}) into ferrous ions (Fe^{2+}). The protocol used for the test is as described by Betoloum et al. (2022), The chelating power of the ions was measured as follows: A reactive solution containing 100 µL (2 mM) of ferric chloride ($FeCl_3$) and 400 µL (5 mM) potassium hexacyanoferrate ($K_3[Fe(CN)_6]$) was prepared. Then, 200 µL of sample at different concentrations (10 to 1 mg/mL) were added to the solution. Distilled water was added to complete the total volume to 1 mL for each extract concentration. EDTA solution was used as a reference standard, the reducing power of the samples were comparable with the reference standard. The reaction mixture was then left to stand in the darkness for 10 minutes at room temperature. Absorbance of the solutions was measured using a spectrophotometer at 700 nm, after incubation. The percentage of iron ion chelating power (ICP) has been calculated according to the following formula:

$$\%ICP = (A_{control}-A_{extract})/ A_{control}\times 100$$

ICP: Iron Ion Chelating Power;
A: Absorbance.

RESULTS

The yield extractions were 54.96%, 10.22%, 10.68%, 11.30% and 2.50% respectively for Hexane, Hex/AcOEt(1:1, v/v), AcOEt, MeOH and MeOH/water (7:3, v/v) (Table 1).

Phytochemical screening revealed the presence of various phytocompounds such as alkaloids, triterpenes and coumarins in hexane and hexane/ethyl acetate (1:1, v/v) extract. Flavonoids, polyphenols and coumarins were found to be present in ethyl acetate extract, in methanolic extract and in hydromethanolic extract, while tannins and saponins were present in methanolic extract and in hydromethanolic extract (Table 2).

Figure 1 summarized total polyphenol and total flavonoid contents. It can be seen that polyphenols and flavonoids are present in all extracts. However, the lowest amounts were observed for the hexane extract (0.501±0.040 gEAG/100gMB and 0.110±0.003 gEQc/100gMB). The methanolic extract contains a higher amount of total polyphenols and total flavonoids (6.150±0.008 gEAG/100gMB and 2.143±0.006 gEQc/100gMB) than the hydromethanolic extract (5.131±0.006 gEAG/100gMB and 1.732±0.005 gEQc/100gMB), followed by the AcOEt extract (4.881±0.004 gEAG/100gMB and 1.512±0.003 gEQc/100gMB) and the Hex/AcOEt extract (1:1, v/v) (2.1331±0.013 gEAG/100gMB and 1.090±0.003 gEQc/100gMB).

Results of the free radical test (Table 3) revealed that all samples show antiradical activity. Antiradical activity increases with increasing concentration. The IC_{50} values are respectively 0.4253, 0.1810, 0.1152, 0.0732, 0.0379 and 0.0198 for extract of hexane, Hex/AcOEt, AcOEt, MeOH/water, methanol and ascorbic acid (Vitamin C) used as standard.

Results for reducing properties indicated that all samples showed ferric ion reducing power. The absorbances of all extracts increase with increase in concentrations. This is in good agreement with the antioxidant activity. Maximum chelating effects with 33.12%, 38.46%, 52.12%, 64.15%, 71.53% and 75.67% were obtained for hexane, Hex/AcOEt, AcOEt, MeOH/water, methanol and EDTA extract respectively, at 10 mg/mL.

Table 1: Extraction of propolis with different solvents.

Solvent	Hexane	Hex/AcOEt (1:1, v/v)	AcOEt	MeOH	MeOH/water (7:3, v/v)
Mass (g)	137.4	11.5	10.8	10.2	2.0
Yield (%)	54.96	10.22	10.68	11.30	2.50

Table 2: Secondary metabolites present in different propolis extracts.

Secondary metabolites	Extracts				
	Hex	Hex/AcOEt (1:1, v/v)	AcOEt	MeOH	MeOH/water (7:3, v/v)
Alcaloids	+	+	+	-	-
Flavonoids	-	-	+	+	+
Polyphenols	-	+	+	+	+
Tannins	-	-	-	+	+
Triterpenes	+	+	-	-	-
Saponins	-	-	-	+	+
Coumarins	+	+	+	+	+

+ = positive reaction; - = negative reaction.

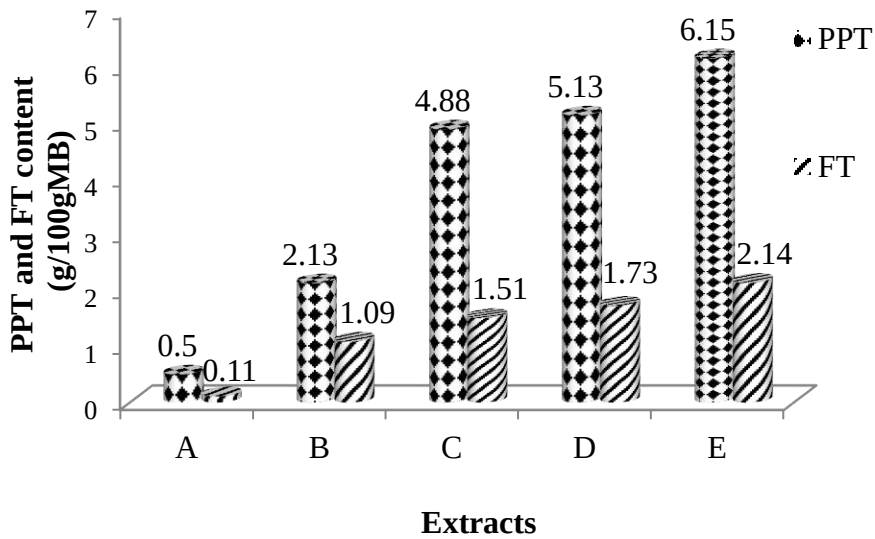

Figure 1: Total polyphenol TPP (expressed as gEAG/100gMB) and total flavonoids contents FT (expressed as gEQc/100gMB): Extract (A) Hex, (B) Hex/AcOEt (1:1, v/v), (C) AcOEt, (D) MeOH and (E) MeOH/water (7:3, v/v).

Table 3: IC_{50} values and correlation coefficients (R^2) of samples.

Samples	logarithmic equations	IC_{50} (mg/mL)	R^2	Inhibition degree (%)
Hexane	y = 14.900ln(x) + 62.737	0.4253	0.9443	41.11
Hex/EtOAc (1:1 v/v)	y = 20.333ln(x) + 84.757	0.1810	0.9605	54.13
AcOEt	y = 22.960ln(x) + 99.614	0.1152	0.9692	64.25
MeOH/water (7:3 v/v)	y = 24.115ln(x) + 113.04	0.0732	0.9904	72.55
Methanol	y = 22.341ln(x) + 123.09	0.0379	0.9985	86.45
Ascorbic acid	y = 19.354ln(x) + 125.86	0.0198	0.9850	92.15

DISCUSSION

Table 1 shows that the highest yield was obtained by the least polar solvent. The yield decreases when the polarity of the solvent increases. This suggests that Koumra propolis is rich in low-polarity organic components. These results are similar to those described by Betoloum et al. (2022), which obtained 50.38% yield from the hexane extract and 5.11% yield from the methanol extract of propolis collected in Beboto, province of Eastern Logone, Chad. This similarity could be justified by the fact that these propolis contain organic compounds of varying degrees of low polarity and in the same proportions. The few differences in yields could be explained by the influence of temperature, the time extraction and the replication number of extractions. However, Oldoni et al. (2011) obtained a lower percent (11.4%) of the hexane extract of propolis from Brazil. So, the brazilian propolis is rich in polar organic compounds. Moreover, Sakava et al. (2014) reported the extract content in Meiganga propolis from Adamawa region, Northern Cameroon, to be 44.8% from the hexane extract and 23.7% from the ethyl acetate extract. Different results explain that propolis sources, geographical area and collecting season are factors effecting the chemical compositions of the material (Bankova et al., 2016).

Phytochemical screening revealed that extracts contain triterpenes, coumarins, alkaloids, Flavonoids, polyphenols, tannins and saponins. Talla et al. (2014) evaluated the phytochemical of propolis from Ngaoundal (Adamawa region, Northern Cameroon) and reported triterpenes from the hexane extract. Betoloum et al. (2022) also studied the propolis from Beboto village (Eastern Logone State, South of Chad) and found presence of triterpenes in the hexane extract. These results matched those obtained for propolis from Koumra. In addition, authors Sakava et al. (2014) and Betoloum et al. (2022) described the presence of flavonoids and polyphenols in the ethyl acetate and methanolic extract of propolis from Meiganga (Adamawa region, northern Cameroon) and Beboto. This confirms the fact that these compounds are present in propolis from central Africa. However, coumarins are found to be present in all extracts of propolis from Koumra while they are absent in extracts from other localities (Odiba et al., 2014; Betoloum et al., 2022). It appears that locations of propolis and his environment have effects on the chemical composition (Bankova et al., 2016).

Total polyphenols and total flavonoid content decreases whereas the polarity of extraction solvents decreases. The high solubility of polyphenols and flavonoids in

polar solvents results in the high content of these compounds in extracts obtained by polar organic solvents. Amounts of polyphenols and flavonoids are higher than those obtained by Betoloum et al. (2022) from the hexane extract and the AcOEt extract of propolis from Beboto (Chad).

For scavenging of DPPH radical, the standard has the lowest inhibitory concentration, therefore it has the highest antiradical activity. Among the five extracts of Koumra propolis, methanolic extract is the most active extract with an IC_{50} value of 0.0379 mg/mL followed by the hyromethanolic extract, then the AcOEt extract, then the Hex/AcOEt extract and finally the hexane extract. The methanolic extract, which has a higher content of polyphenols and flavonoids than the hydromethanolic extract, has a higher activity. It could be noted that polyphenols are responsible for the antiradical activity. Extracts which present higher activities are obtained by the most polar organic solvents. These results show that Koumra propolis is endowed with a higher antiradical activity than that obtained by Betoloum et al. (2022) with extracts of hexane, ethyl acetate and methanol from Beboto propolis collected in the Eastern Logone State, South of Chad.

All extracts showed reducing properties, but lower than EDTA. This activity could be explain that the extracts contain substances able to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). As in the case of DPPH radical assay, the methanolic extract showed higher activity than the hydromethanolic extract correlated with a high content of polyphenols and flavonoids in this extract. In addition, it was shown by several authors including Evenamede et al. (2017) and Amankwaah et al. (2023) that the antioxidant activity of plant products is generally attributed to the radical scavenging activity of phenolic compounds such as flavonoids, polyphenols and others.

Conclusion

The present study shows that Koumra propolis is rich in low polar compounds. Phytochemical investigation of different extracts revealed the presence of alkaloids, coumarins, flavonoids, polyphenols, saponins, tannins and triterpenes. Total polyphenols and total flavonoids contents measured in the methanolic extract were 6.150±0.008 gEAG/100gMB and 2.143±0.006 gEQc/100gMB respectively. Evaluation of the antiradical activity of the different extracts showed that the methanolic extract exhibits the highest inhibition degree at 86.45% with an IC_{50} value at 0.0379mg/mL. Similarly, the chelating power of the ions gave a maximum chelating effect at 71.53% for the methanol extract. These results indicate that Koumra propolis extracts are endowed with a powerful antioxidant activity. This is a potential source of antioxidants of natural origin that justifies its traditional use in the treatment of many diseases.

COMPETING INTERESTS

The authors declare that there is no competing interests.

AUTHORS' CONTRIBUTIONS

YM and AAM designed the research; AAM performed the experiments. AAM and HH analyzed the data. YM and AAM wrote the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors are grateful to the France Embassy in Chad for financial supplies for sample collection.

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