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Original Research Article

Chemical composition and evaluation of anticholinesterase activity of essential oil from Cameroonian propolis

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Limited studies exist on tropical propolis volatile constituents. Hydrodistillation of a Cameroonian propolis sample afforded a light-yellow oil with a yield of 0.04% (v/w). Chemical characterization using gas chromatography flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS) led to the identification of 73 compounds representing 98.3% of total oil constituents. The most abundant components were α-calacorene (21.61%), 4,5,9,10-dehydroisolongifolene (5.56%), δ-cadinol (5.36%), 8,8,9-trimethyldeca-3,5-diene-2,7-dione (4.98%), 2-hydroxymethylene-6-isopropyl-3-methylcyclohexanone (4.90%) and γ-himachalene (4.11%). Ellman's colorimetric assay was used to evaluate anticholinesterase activity of propolis essential oil. Acetylcholinesterase inhibition was moderate with IC_{50} value of 54.35±1.01 µg/mL compared to 5.01±0.09 µg/mL for galantamine while butyrylcholinesterase inhibition was profoundly high with IC_{50} value 27.63 ± 0.62 µg/mL compared to the standard drug galantamine with IC_{50} 53.9±0.56 µg/mL. These results show that anticholinesterase activity of propolis volatiles could be a potential solution to Alzheimer's disease.

Key words: Propolis, essential oil, hydrodistillation, GC-MS, α-calacorene, anticholinesterase activity

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that is responsible for cases of dementia and death within a large number of aging people (Malileh et al., 2019). The major physiological evidence of AD involves the degradation of cholinergic neurons and reduction in acetylcholine. For this reason, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitors such as galantamine, donepezil and rivastigmine are used in the management of AD and the inhibition of the two types of cholinesterase enzymes (AChE and BuChE) is remedial for such treatment (Malileh et al., 2019). Recently, there is a growing interest about finding new AChE inhibitors from natural sources due to the draw backs of synthetic AChE inhibitors such as gastro intestinal disorders, moderate to low effectiveness, high cost and short half-life (Owokotomo et al., 2015). These natural sources or natural products involve natural compounds, essential oils, extracts from plants and other natural substances like propolis. Propolis (bee glue) is a sticky dark-coloured material that honeybees collect from buds and exudates of plants in their

environment and carry to the hive, mix with pollen as well as enzymes and other bee secretions (Bankova et al., 2014; Tamfu et al., 2016). Many papers reporting chemical composition of various propolis samples exist but chemical analysis of volatile components (essential oils) of propolis remains under-researched and since the bioactivities of propolis are related to its composition in non-volatile and volatile components, studies on essential oils of propolis becomes very necessary (Piotr et al., 2018). Though existing studies on Cameroonian propolis report predominantly triterpenes (Tamfu et al., 2016; Talla et al., 2017), no study reporting the chemical composition of essential oils of Cameroonian propolis exists to the best of our knowledge. The aim of this study is to obtain essential oil from a Cameroonian propolis sample by hydro-distillation, determine its chemical composition by gas chromatography mass spectrometry (GC-MS) and evaluate its anticholinesterase activity.

MATERIALS AND METHODS

Hydrodistillation of Propolis

The propolis was collected from bee hives in Babanki village (6° 7' 0" N and 10° 15' 0"E), North-West region of Cameroon in January 2018. 20 g of the propolis sample were subjected to hydrodistillation according the method described by Garcia et al., (2019) with slight modifications. The essential oils were obtained by heating a mixture of 20 g of propolis sample in 1000 mL of distilled water for 3 hours using a Clevenger-type apparatus. The steam mixed with the essential oils were condensed forming two layers which were carefully separated on a separatory funnel to yield a light-yellow oil. The oil was dried over anhydrous sodium sulfate and stored in a refrigerator at 4° C until it was used.

Analyses of essential oil

GC-FID analysis

The dried essential oil from propolis, diluted with Et_2O, was subjected to GC-FID thermal gradient analysis, on an Agilent model 6280 gas chromatograph, fitted with an HP-5® (30 m × 0.25 mm, 0.25 µm film thickness) capillary column. The initial temperature of the column was kept at 50°C for 5 min and was heated to 300°C at a rate of 4°C/min and then kept at final temperature for 20 min. The temperature of the detector and injector were kept at 250°C and 280°C respectively. Helium, as carrier gas, was used at flow rate of 1 mL/min. 1 µl of sample was injected with a split ratio of 1:20.

GC-MS analysis

The GC-MS analysis was performed on similar conditions and parameters as described for GC-FID, using Perkin-Elmer Clarus 500 gas chromatograph equipped with an HP-5MS® (30 m × 0.25 mm, 0.25 µm film thickness) capillary column. The MS was operated at standard conditions 70 eV, and 250°C. The processed mass spectra of components from essential oil of propolis were identified by mass fragmentation patterns and by comparison with the electronic MS NIST library and the calculated retention indices (RI) with literature data (Table 1).

Anticholinesterase activity

The inhibition activity of Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured by the Elman's method with slight modification (Ozturk et al, 2011), using 96-well microplate reader (SpectraMaxPC340, Molecular Devices, USA). The substrates of there action of both enzymes were acetylthiocholineiodide (0.71 mM) and butyrylthiocholine chloride (0.2mM). In a 96 well plate, 10 µL of sample (EOP) were mixed with 150µL sodium phosphate buffer100mM (pH = 8) and 20 µL of enzymes [AChE (5.32×10^{-3}U) or BChE (6.85×10^{-3}U)]. After 15 minutes incubation at 25 °C, 10 µL of Ellman's Reagent (DTNB 0.5 mM) and 10 µL of substrates were added to make the final volume200 µL. The absorbance was measured at 412 nm. Percentage inhibition of AChE or BChE was determined by comparison of reaction rates of samples relative to control using the formula:

$(E - S)/E \times 100$

Where: E: the activity of the enzyme with control.

S: the activity of the enzyme with the sample.

The experiments were carried out in triplicate. Galantamine was used as the standard. MeOH was used as solvent to dissolve the essential oil and control.

RESULTS

Chemical composition of essential oil

The hydrodistillation afforded a light-yellow oil with a yield of 0.04% (v/w). The volatile compounds identified by GC-MS in the essential oil of propolis (EOP) are shown in Table 1, along with their percentage compositions and retention index. Seventy-three compounds of the volatile contents were identified, and this represents 98.3% of the EOP components. The most abundant components were α-Calacorene (21.61%), 4,5,9,10-dehydro-isolongifolene (5.56%), and δ-Cadinol (5.36%), followed by 8,8,9-Trimethyl-deca-3,5-diene-2,7-dione (4.98%), 2-Hydroxymethylene-6-isopropyl-3-methylcyclohexanone (4.90%) and γ-Himachalene (4.11%).

Anticholinesterase activity of essential oil

The results of AChE and BChE inhibitory activities of the EOP compared with that of Galantamine used as a standard drug showed that the EOP has high activity as shown in

Table 1. Chemical constituents of essential oil of Cameroonian propolis

Number	Compound	RI	%Composition
1	Tricyclene	922	0.11
2	Camphene	943	0.15
3	β-Pinene	970	0.04
4	γ-Terpinene	998	0.03
5	3-Carene	1005	0.68
6	α-Phellandrene	1007	0.54
7	α-Terpinene	1008	1.31
8	Eucalyptol	1023	0.29
9	o-Cymene	1042	0.04
10	cis-Pinen-3-ol	1070	0.12
11	1-Isopropenyl-4-methyl-1,3-cyclohexadiene	1085	0.27
12	2,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol	1088	1.33
13	3-Isopropenyl-1,2-dimethylcyclopentanol	1099	0.29
14	1,3,3-Trimethyl-2-vinyl-1-cyclohexene	1105	0.12
15	4-Isopropyl-1-methyl-2-cyclohexen-1-ol	1106	0.12
16	trans-p-Mentha-2,8-dienol	1113	0.33
17	Camphor	1121	2.82
18	Camphol	1148	0.26
19	1-Isopropyl-4-methyl-3-cyclohexen-1-ol	1161	0.04
20	trans-2-Caren-4-ol	1176	0.15
21	p-Menth-8-en-2-ol	1178	0.54
22	(+)-Sabinol	1179	2.00
23	Fenchyl acetate	1207	0.37
24	cis-Geraniol	1215	0.29
25	3-Carvomenthenone	1228	0.06
26	Isogeraniol	1237	0.12
27	2-methyl-3-phenyl-Propanal	1244	0.16
28	Thymol	1266	0.49
29	4-Isopropenyl-1-methylcyclohexyl acetate	1267	0.64
30	2-Camphanol acetate	1269	0.25
31	Eucarvone	1287	0.27
32	Terpinene 4-acetate	1332	1.75
33	δ-Elemene	1334	0.07
34	2-Hydroxymethylene-6-isopropyl-3-methylcyclohexanone	1372	4.90
35	α-Bourbonene	1374	0.33
36	Isoledene	1388	1.72
37	Copaene	1397	0.08
38	Thujopsene	1435	1.41
39	α-Gurjunene	1436	3.42
40	8,8,9-Trimethyl-deca-3,5-diene-2,7-dione	1453	4.98
41	(+)-Epi-bicyclosesquiphellandrene	1470	0.46
42	γ-Himachalene	1479	4.11
43	Guaiene	1483	0.57
44	Eremophilene	1486	0.63
45	Cadinene	1491	1.46
46	cis-α-Bisabolene	1521	0.10
47	4,5,9,10-dehydro- Isolongifolene	1544	5.56
48	α-Calacorene	1547	21.61
49	Diepicedrene-1-oxide	1551	1.79
50	Caryophyllene oxide	1576	0.51
51	Ent-Spathulenol	1577	0.21
52	Cedrol	1595	0.92
53	1-Methyloctyl butyrate	1600	0.53
54	δ-Cadinol	1605	5.36
55	1β-Cadin-4-en-10-ol	1646	1.27
56	Cubenol	1651	0.99
57	Cadalene	1655	3.05
58	Aromadendrene oxide	1678	0.54
59	Drimenol	1685	0.28
60	9-Methoxycalamenene	1686	3.11
61	6-Methoxy-4,4-dimethyl-2-chromanone	1716	1.77

Table 1. Continue

62	3,9(11)-diene-10-peroxy murolan	1729	2.78
63	6-(p-Tolyl)-2-methyl-2-heptenol	1760	1.28
64	9-Methyl-S-octahydroanthracene	1766	1.13
65	Androst-7-ene	1780	0.27
66	Sclareoloxide	1876	0.30
67	Manoyl oxide	1992	0.23
68	Abieta-8,11,13-triene	2039	0.17
69	3-Ethyl-5-(2-ethylbutyl)octadecane	2413	3.70
70	Mono(2-ethylhexyl) phthalate	2555	0.32
71	n-Heptacosane	2700	0.21
72	n-Hentriacontane	3100	0.14
73	n-Tritriacontane	3300	0.05

Table 2. Anticholinesterase activity of essential oil of Cameroonian propolis

Activity	Concentration / µg/mL	EOP	Galantamine
AChE	6.25	3.5 ± 0.31	52.32 ± 1.20
	12.5	6.02 ± 1.82	62.21 ± 0.09
	25	12.04 ± 0.45	68.36 ± 1.10
	50	46.84 ± 2.89	74.38 ± 0.65
	100	79.69 ± 1.30	78.59 ± 0.47
	200	85.61 ± 1.01	80.4 ± 0.9
	IC_{50}	**54.35 ± 1.01**	**5.01 ± 0.09**
BChE	6.25	14.25 ± 2.15	21.35 ± 0.03
	12.5	29.05 ± 0.61	29.62 ± 0.47
	25	58.10 ± 0.49	40.59 ± 2.88
	50	75.65 ± 0.17	48.73 ± 0.90
	100	82.06 ± 0.17	65.02 ± 0.44
	200	82.42 ± 0.67	82.2 ± 1.6
	IC_{50}	**27.63 ± 0.62**	**53.9 ± 0.56**

Table 2. Using Ellman's colorimetric method in a 96-well plate by a microplate reader, AChE inhibition was moderate with IC_{50} value of 54.35 ± 1.01 µg/mL compared to 5.01 ± 0.09µg/mL for galantamine. However, BChE was profoundly high with IC_{50} value 27.63 ± 0.62 µg/mL compared to the standard drug galantamine whose IC_{50} was 53.9 ± 0.56 µg/mL. It should be noted that the anticholinesterase activity increases with an increase in the dose or concentration of the essential oil.

DISCUSSION

The hydrodistillation afforded a light-yellow oil with a yield of 0.04% (v/w). It should be noted that propolis essential oils are usually extracted in very low yields contrary to outdated information referring to 10% of propolis composed of essential oils. Bankova and co-workers have been able to shed more light on this aspect demonstrating that percentage composition of propolis in volatile oils is usually up to1%, rarely 2 - 3% (Bankova et al., 2014). The volatile compounds identified by GC-MS in the essential oil of propolis (EOP) are shown in Table 1, along with their percentage compositions and retention index. Seventy-three compounds of the volatile contents were identified, and this represents 98.3% of the EOP components. Although volatile compounds are in low concentrations in propolis, they contribute to the aroma and biological properties of propolis which makes them to be of considerable importance. The most abundant components were α-Calacorene (21.61%), 4,5,9,10-dehydro-isolongifolene (5.56%), and δ-Cadinol (5.36%), followed by 8,8,9-Trimethyl-deca-3,5-diene-2,7-dione (4.98%), 2-Hydroxymethylene-6-isopropyl-3-methylcyclohexanone (4.90%) and γ-Himachalene (4.11%). Some corroborations were observed between the chemical composition of Cameroonian propolis essential oil and some reported propolis essential oils of some other regions. The most abundant compound α-calacorene together with cadinol, manoyl oxide, camphor, copaene, bourbonene, cadinene, caryophyllene oxide, cedrol and eucarvone have been detected as a volatile component of Turkish and Anatolian propolis (Hames-Kocabas et al., 2013; Nesrin et al., 2018). Equally, n-alkanes, tricyclene, camphene, terpinene, camphor, bourbonene, copaene, cadinene, α-calacorene, caryophyllene oxide, bisabolene, spathulenol, cedrol, cadinol and thymol have been described in Portuguese propolis volatiles (Falcao, 2013). It is noteworthy to

mention the absence of the usual eudesmol and its derivatives that are found in most propolis samples (Hames-Kocabas et al., 2013; Falcao, 2013; Nesrin et al., 2018; Piotr et al., 2018), similar observation was made with Indian propolis which equally was shown to be void of eudesmol type constituents (Naik et al., 2013). This absence can be justified by the absence of *P. nigra* in tropical areas since β-eudesmol was found to be the major constituent of propolis volatile oils from France, Hungary, Bulgaria and Northern Italy and it is known that this sesquiterpene alcohol is themajor constituent of essential oils distilled from leaf buds of *P. nigra* (Bankova et al., 2014). The great biodiversity of tropical flora is reflected in the chemical diversity of tropical propolis volatiles (Bankova et al., 2014) which do not show a regular pattern in chemical composition. However, essential oils of propolis from Ethiopia showed similarity with that of Cameroon by the common presence of (+)-Epi-bicyclosesquiphellandrene, 0-cymene, camphor, himachalane, cadinene, cedrol, terpineol acetate and n-alkanes that have been described in Ethiopian propolis previously (Haile et al., 2012). The qualitative and quantitative differences in composition of propolis volatiles from different regions can natural be accounted for by difference in climatic conditions and local flora. Although some of these volatile components are found in smaller concentrations, they play an important role in propolis characterization and can ameliorate the potential uses by virtue of their aroma and significant biological activity and their presence can give valuable information about plant sources in the origin of the propolis (Falcao, 2013).

Research dedicated to bioactivity of propolis volatiles are relatively scarce (Bankova et al., 2014). Natural essential oils exhibit pharmacological properties attributable to the numerous structurally diverse bioactive chemicals they contain which makes them to be increasingly employed for their anti-cholinesterase potential. This is the best therapy for AD by inhibiting the enzymes acetylcholinesterase (AChE) and butrylcholinesterase (BuChE) that causes the breakdown of acetylcholine. This study reports the anticholinesterase activity of essential oil of Cameroonian propolis as well as its chemical composition for the first time. The results of AChE and BChE inhibitory activities of the EOP compared with that of Galantamine used as a standard drug showed that the EOP has high activity as shown in Table 2. Using Ellman's colorimetric method in a 96-well plate by a microplate reader, AChE inhibition was moderate with IC_{50} value of 54.35 ± 1.01 µg/mL compared to 5.01 ± 0.09 µg/mL for galantamine. However, BChE was profoundly high with IC_{50} value 27.63 ± 0.62 µg/mL compared to the standard drug galantamine whose IC_{50} was 53.9 ± 0.56 µg/mL. However, the individual components of essential oils are less active when tested individually (Orhan et al., 2008) and this indicates the complex mixture of the essential oil constituents acts in a synergistic manner on AChE and BChE inhibition. Amongst natural products, essential oils exhibit strong potency to inhibit AChE in brain because of the lipophilicity and small molecular sizes of its volatile constituents which makes them more likely to cross the blood brain barrier and exert their effect. Generally, it is believed that natural product-based medication possesses relatively better penetration of the blood–brain barrier compared to pharmaceutical products and better specificity for human-type AChE (Owokotomo et al., 2015). β-pinene, γ-terpinene, 3-carene, camphor, cis-geraniol and caryophyllene oxide though present in our sample in small amounts have been mentioned in some studies as responsible for AChE inhibition (Orhan et al., 2008). However, taking a keen observation at the results obtained by Saleh et al (2015), the sole essential oil containing α-calacorene had the highest BChE inhibition of 66.1 ± 0.3 % compared to galantamine which showed 88.0 ±0.2 % BChE inhibition when both samples were tested at 1 mg/mL concentration (Saleh et al., 2015). This could be likened to the high content of α-calacorene in the EOP showing a profound BChE inhibition. More investigation will be needed on this aspect.

Conclusion

Few studies exist on chemical composition and biological activities of propolis especially samples from tropical and subtropical zones. However, essential oils have been attracting much attention recently due to their high bioactivities and the chemical complexity of essential oils will warrant a chemical analysis, mostly GC-MS to accompany the bioactivities. The chemical composition of the volatile oils of Cameroonian propolis has been shown to be very rich and containing α-calacorene as the most abundant constituent. The EOP possess potent anticholinesterase activity and was shown to be more active BChE inhibitor than the standard drug galantamine. These results fill the knowledge gap on volatile composition of tropical and subtropical propolis samples and opens promising path on anticholinesterase inhibition substances from natural sources in addressing the AD.

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Conflict of Interests

The authors declare that there is no conflict of interests.

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