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CHEMICAL CONSTITUENTS, FREE RADICAL SCAVENGING AND ENZYME INHIBITORY POTENTIAL OF SELECTED NIGERIAN BEE (*Apis mellifera*) PROPOLIS

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ABSTRACT

Extracts of propolis from southwest Nigeria (Ondo and Ife) were analysed using GC-MS and were evaluated *in vitro* for free radical scavenging potentials and inhibitory activities on alpha-glucosidase and porcine lipase enzyme. Crude methanolic extracts of both propolis were separately defatted with *n*-hexane to give *n*-hexane fractions and defatted fractions. Each fraction was analysed using GC-MS. Diphenyl-2-picrylhydrazyl radical scavenging potentials of the crude and defatted extracts were evaluated while *in vitro* antihyperglycemic and antihyperlipidemic were also determined using spectrophotometric method. The GC-MS analysis indicated 18 (88.38 %) and 20 (99.99%) compounds for *n*-hexane fraction of propolis from Ife (BPIH) and Ondo (BPOH) respectively. For the defatted extracts propolis from Ife (BPID) and Ondo (BPOD), 21 compounds each were identified, accounting for 100% composition in each extracts. The IC_{50} DPPH radical scavenging activities of all the propolis samples were moderate compared to the standard (32.61 ± 2.60 μg/mL) however, BPOD demonstrated highest radical scavenging activity (141.49±0.29 μg/mL) among the propolis fraction tested. All the extracts demonstrated better inhibition than the standard acarbose, however, BPOD inhibited the enzyme the most with IC_{50} value of 25.35±0.48 μg/mL. The anti-lipase activities of the propolis were fairly moderate but much lower compared to the standard orlistat (0.88 ± 0.12 μg/mL). The excellent inhibitory performance of the propolis extracts on alpha-glucosidase enyzme gives credence to its traditional use in treatment of diabetic patients, also the moderate free radical scavenging and antipase activities make bee propolis an important bioresource for discovery of lead drugs.

Keywords: Bee propolis; Terpenoids; Antidiabetic; Antioxidant; Porcine lipase.

INTRODUCTION

Propolis is a sticky resinous substance produced by the honeybees to shut the cracks, ward off invaders and regulate the humidity and temperature in the hive (Bankova, 2005). Extracts of propolis have been reported to exhibit a wide range of pharmacological activities which includes antioxidative, anti-inflammatory, antitumoral and antibacterial (Isla *et al.*, 2001; Jose *et al.*, 2011; Shittu *et al.*, 2015; Babatunde *et al.*, 2015; Balogun *et al.*, 2021). Averagely, propolis is composed of 30% wax, 50% resin, 10% essential oils, 5% pollen and 5% various organic residues (Silici *et al.*, 2007).

The propolis from Nigeria has received little attention with respect to its chemistry and pharmacological applications. Lawal *et al.* (2016) reported 14-methyl pentadecanoate (22.39%), oleic acid (14.70%), 11-octadecenoic acid, methyl ester (11.53%) and *n*-hexadecanoic acid (5.04%) as the principal constituents of propolis from Ondo State Nigeria. Likewise, phenolic compounds, calycosin, liquiritigenin, pinocembrin, vestitol, medicarpin, 8-prenylnaringenin, 6-prenylnaringenin, propolin D and macarangin were isolated from propolis obtained from Rivers State, Nigeria (Omar *et al.*, 2016).

It was reported in our previous study on volatile oils from propolis from Osun and Ondo state, Nigeria that both oils were rich in monoterpenes and sequisterpenes, and they both exhibited α-glucosidase, antibacterial and DPPH scavenging activities better than the positive standards (Balogun *et al.*, 2021). In this study, non-volatile extracts of propolis from locations which are used for agricultural activities in Ondo and Osun States were analysed using GC-MS and were evaluated for free radical scavenging potentials and inhibitory activities on alpha-glucosidase and porcine lipase enzyme.

MATERIALS AND METHODS

Collection, Extraction and Silylation of Propolis

Bee (*Apis mellifera*) propolis samples were collected from apiaries in Ile-Ife (BPI) and Ondo town (BPO). Both BPI and BPO were brown in colour and were preserved under refrigeration at 4 °C prior to extraction. Five gram each of the propolis were separately extracted with 200 mL of 95% aqueous ethanol under sonication at 35 °C and 100 W for 2 h. The crude extract of from Ife (BPIC) and Ondo (BPOC) were reduced to 100 mL *in vacuo* and thereafter deffated using *n*-hexane. Both the *n*-hexane (BPIH and BPOH) remaining defatted mother liquor (BPID and BPOD) were concentrated *in vacuo*.

Five milligram each of *n*-hexane and deffated extract were dissolved in 50 μL pyridine (water-free) and heated at 80 °C for 20 min with 60 μL of N, O-bis-(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (silylating agent). The silylated product was dissolved in chloroform and analysed on TSQ 8000 Evo GC (Thermo Scientific) system with a Mass Selective Detector (MSD).

GC/MS Analysis

The silylated extracts were analysed on TSQ 8000 Evo GC (Thermo Scientific) system with a Mass Selective Detector (MSD) equipped with a HP-5MS (5% phenyl methyl siloxane) capillary column (length 30 m, inner diameter, 0.25 mm and film thickness 0.25 μm). The GC oven initial temperature was 40 °C for 1 min and was taken through two successive ramps. Ramp 1 was maintained at 15.0 °C/min to a final temperature of 180.0 °C, with 1 min hold time while ramp 2 was set at 5.0 °C/min to final temperature of 300.0 °C, with hold time of 8 min. Helium gas, at flow rate of 1.00 mL/min was used as carrier gas and 1.0 μL of the sample was injected in the splitless mode. The splitless flow and splitless time were was 33.3 mL/min and 1.0 min respectively. The GC was coupled to MS transfer line heater maintained at 250 °C with EI (positive) ionisation mode. The mass range was 30-600 amu at scan time of 0.2 s. Qualitative identification of different constituents was based on comparisons of the relative retention indices and mass spectra with those of the Wiley and NIST library of the GC/MS through the use of probability merge search software and the NIST MS spectra search program. Also, comparisons were made using retention indices and mass spectra reported in literature (Adam, 1989). The relative amount (% composition) of individual component of the oil was expressed as percentages of their peak areas relative to the total peak area.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

Forty microliter of methanolic solution of DPPH (0.2 mg/mL) was mixed with 40 μL of crude and defatted extracts separately at different concentrations (50-400 μg/mL), and made up with methanol to 200 μL in 96-well plates. Due to solubility problem, the *n*-hexane fractions, BPIH and BPOH were not used in the experiment. The plate was incubated at 23 °C for 30 min in darkness and thereafter absorbance was recorded at 515 nm. The positive and negative controls used were ultra-pure water and ascorbic acid respectively. All measurements were taken in triplicates and percentage inhibition was calculated using the equation:

$$\text{Inhibition (\%)} = \{(OD_{control} - OD_{sample})/OD_{control}\} \times 100 \quad (1)$$

Lipase Inhibition Assay

Measurement of optical density of coloured *p*-nitrophenol formed by the enzymatic hydrolysis of substrate pNP laurate was used to assess the inhibitory potentials of the propolis extracts on porcine pancreas lipase (McDougall, 2009). Lipase at 0.50 mg/mL in Tris-HCl buffer solution was centrifuged at 10,000 rpm for 5 min and the resulting supernatant was used as enzyme solution. A 4.5 mM *p*-nitrophenyl laurate in Tris-HCl buffer (pH 8.2) solution containing 1% Triton X-100 heated in water bath at 50 °C for 5 min for proper dissolution was used as substrate. Forty microliter each of varying concentrations of the propolis extracts, buffer and enzyme were added in 96-well plate and then incubated at 37 °C for 5 min. Thereafter, 80 μL of the substrate was added to start the reaction and the mixtures were then incubated for 30 min at 37 °C. The reaction was then terminated using sodium acetate buffer (pH 4.2). Absorbance were recorded on Tecan GENios multifunctional microplate reader (Männedorf, Switzerland) at 405 nm. The positive and negative controls used were buffer solution

and orlistat respectively. The experiment was performed in triplicates and the inhibition (%) was calculated using equation (1). Due to solubility problem the *n*-hexane fractions, BPIH and BPOH were not used in the experiment.

α-Glucosidase Inhibition Assay

The alpha-glucosidase inhibitory test was performed using method previously described with slight modifications (Balogun, 2016). Forty microlitre the propolis extracts at varied concentrations were incubated at 37 °C for 5 min with 20 µL of 100 mM phosphate buffer (pH 6.8) and 40 µL of 0.2 U/mL α-glucosidase in phosphate buffer, followed by 100 µL of 2.5 mM of the substrate, *p*-nitrophenyl-α-D-glucopyranoside in phosphate buffer to start the reaction. The mixture was incubated for another 30 min at 37 °C. The reaction was stopped by adding 100 µL of 0.2 $mol.L^{-1}$ sodium carbonate, and absorbance was measured at 405 nm. The positive and negative controls were phosphate buffer solution and acarbose, respectively, which were used in place of sample. The experiment was performed in triplicates and the inhibition (%) was calculated using equation (1). Due to solubility problem, the *n*-hexane fractions, BPIH and BPOH were not used in the experiment.

RESULTS AND DISCUSSION

The constituents of both the *n*-hexane and defatted extracts were classified into fatty alcohols and esters, fatty acids, hydrocarbons, aromatics and steroidal/terpenoidal compounds (Table 1). In the *n*-hexane extracts, 88.38 % and 99.99% of the chemical constituents of BPI and BPO were identified respectively. Except for steroidal and terpenoidal components, other chemical constituents were more in BPI. The waxy components (36.72%) and aromatics (20.82%) of BPI were almost twice as much as those of BPI (17.62% and 10.82%). However, unlike BPI, *n*-hexane extract of BPO was rich in bioactive steroid and terpenoids such as β-amyrone (11.73%), olean-12-en-3-ol, acetate, (3β)-(10.12%), lup-20(29)-en-3-one (7.44%) and α-amyrin (6.32%).

An ether lipid precursor, 1-O-hexadecylglycerol (5.18%) in BPO was not detected in BPI. The alkyl glycerol has been shown to protect HEp-2 cells against Shiga toxin and Shiga toxin 2 which produce *Escherichia coli* bacteria that causes haemorrhagic colitis and haemolytic uremic syndrome in humans. Also, 1-O-hexadecylglycerol protected endothelial cell lines HMEC-1 and HBMEC are against Shiga toxins suggesting its role in cancer therapy (Bergan *et al.*, 2019). The principal fatty acids common to both propolis were palmitic (BPI, 3.63%; BPO 8.31%), stearic (BPI, 3.64%; BPO 4.26%) and eicosanoic acid (BPI, 7.56%; BPO 6.20%) while heptadecanoic acid (4.92%) was detected only in BPI.

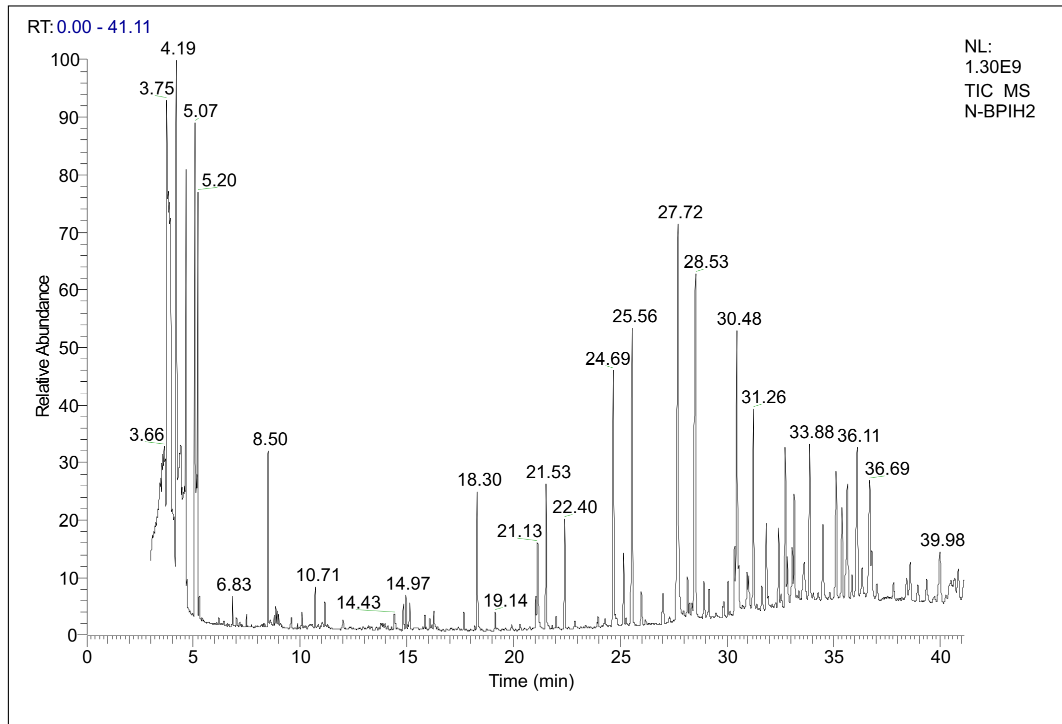

Figure 1: GC chromatogram of *n*-hexane fraction of propolis from Ile-Ife (BPIH).

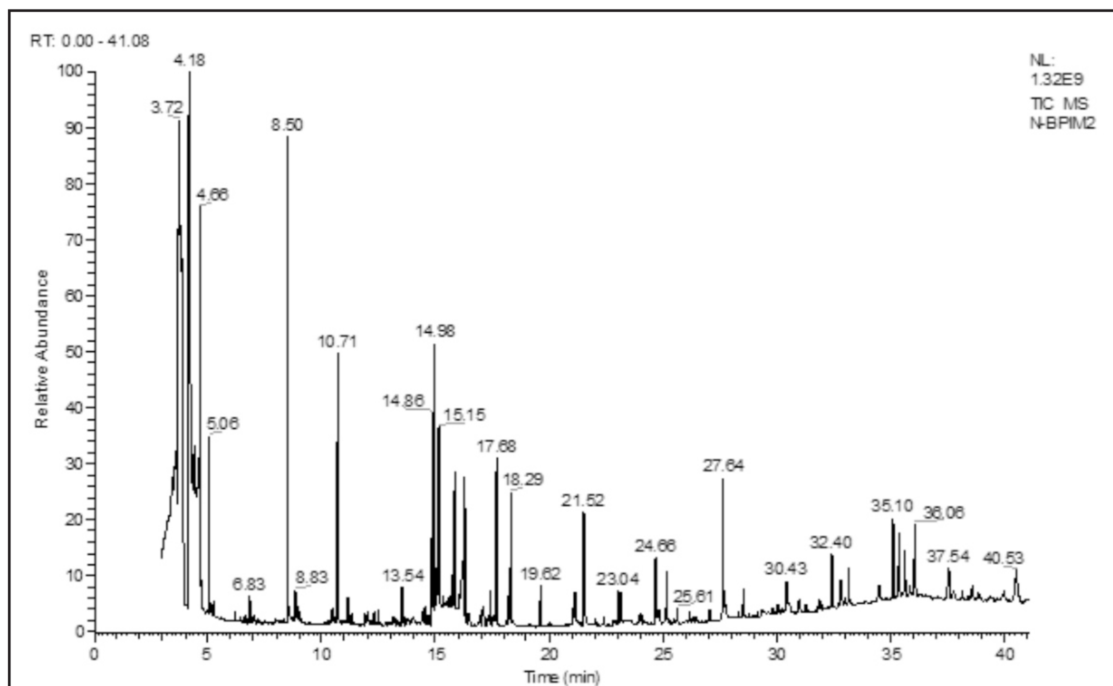

Figure 2: GC chromatogram of defatted fraction of propolis from Ile-Ife.

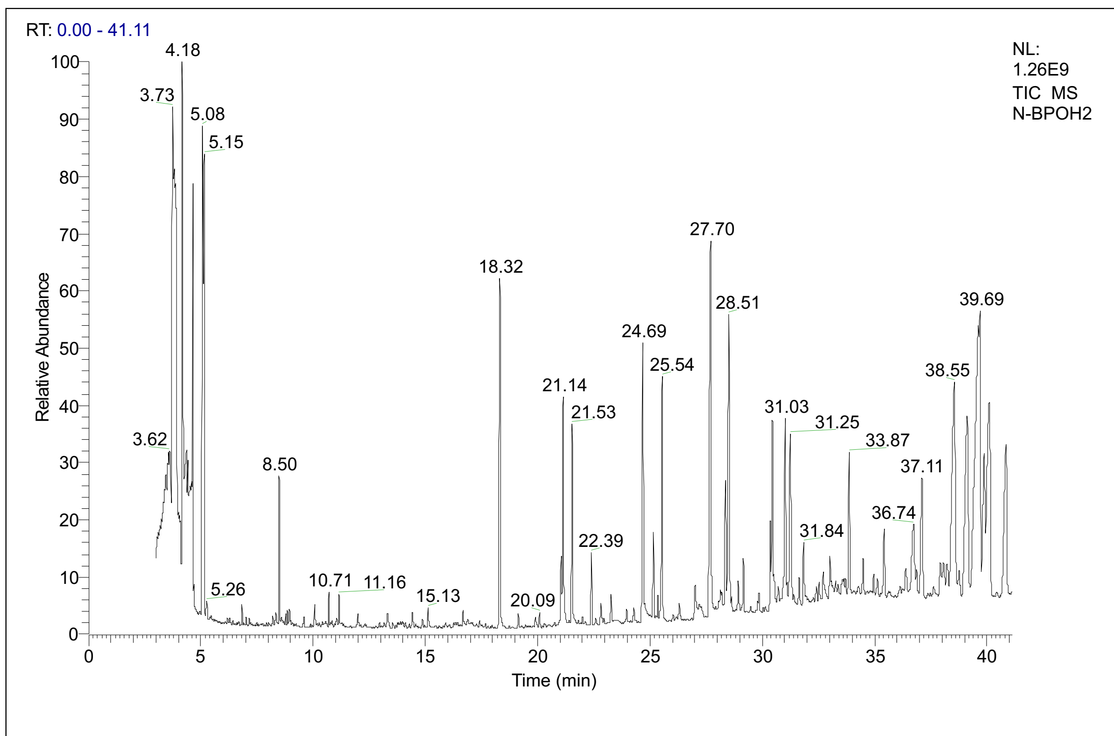

Figure 3: GC chromatogram of *n*-hexane fraction of propolis from Ondo (BPOH).

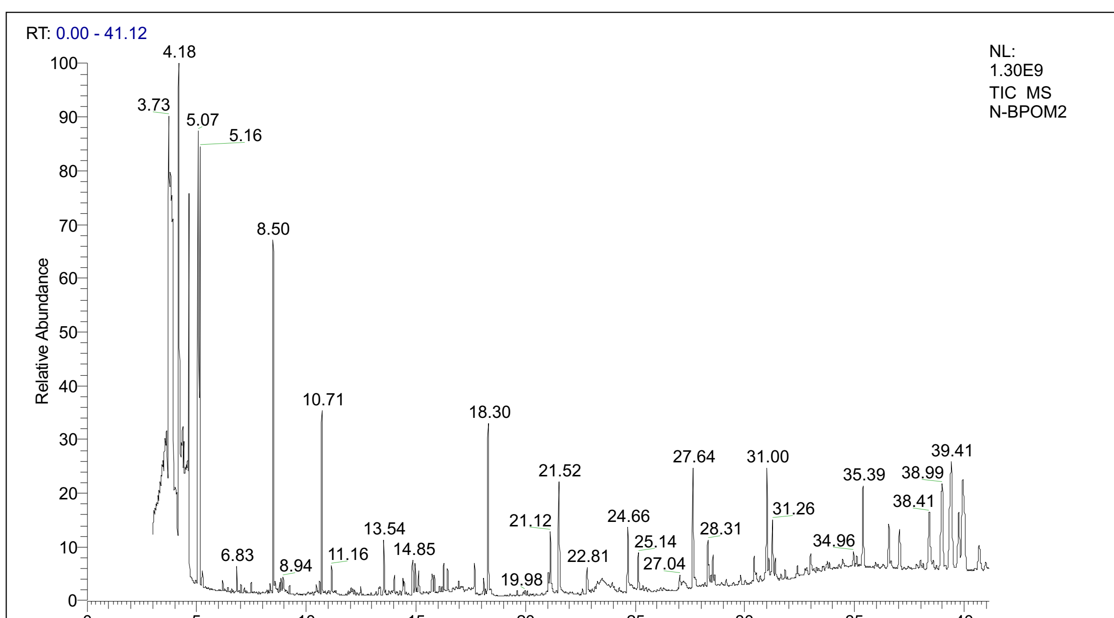

Figure 4: GC chromatogram of defatted fraction of propolis from Ondo (BPOD).

Table 1: Chemical constituents of *n*-hexane fractions of the propolis.

Chemical compound	RT	BPI	BPO
Fatty alcohol and ester			
Glycerol	8.5	3.11	1.97
1-O-hexadecylglycerol - bis-trimethylsilyl ether	31.03	ND	5.18
1-Hexacosanol	31.85	3.08	1.50
1-Octacosanol	34.49	2.81	ND
2,3-Bis((trimethylsilyl)oxy) propyl docosanoate	35.12	4.71	ND
		13.71	**8.65**
Fatty acid			
Palmitic acid	18.30	3.63	8.31
9,12-Octadecadienoic acid (Z,Z)-	21.05	0.24	ND
Oleic Acid	21.13	1.21	2.54
Stearic acid	21.53	3.64	4.26
Eicosanoic acid	24.69	7.56	6.20
Octadecanoic acid	35.65	2.41	ND
Heptadecanoic acid	36.12	4.92	ND
		23.61	**21.31**
Hydrocarbon			
Tetracosane	22.4	2.54	1.18
Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	25.15	1.41	ND
9-Hexacosene	32.73	2.60	ND
2-Methylhentriacontane	33.87	ND	2.55
Tetratriacontane	33.88	27.36	13.89
9-Hexacosene2	36.69	2.81	ND
		36.72	**17.62**
Aromatics			
3,5-di-tert-Butyl-4-hydroxyanisole	25.15	ND	1.96
3-(2-Hydroxyphenyl)-1,1,3-trimethyl-5-indanol	27.7	13.28	8.86
		13.28	**10.82**
Steroid & terpenoid			
Bauer-7-en-3-one	36.69	ND	0.75
Lanosterol	37.11	ND	2.43
β - Amyrone	38.55	ND	11.73
Olean-12-en-3-ol, acetate, (3β)-	39.1	ND	10.12
Lup-20(29)-en-3-one	39.47	ND	7.44
α - Amyrin	39.89	ND	6.32
Cycloartenol	39.98	1.06	1.52
24-Methylenecycloartan-3-one	40.86	ND	1.28
		1.06	**41.59**
Total		88.38	99.99

ND: Not detected

Table 2: Chemical constituents of defatted fractions of the propolis.

Chemical Compound	RT	BPI	BPO
Fatty alcohol and ester			
Glycerol	8.5	12.07	12.72
1-O-Tetradecylglycerol	31	ND	7.89
2,3-Dihydroxypropyl icosanoate	33.14	2.33	ND
2,3-Bis((trimethylsilyl)oxy)propyl docosanoate	35.09	2.82	ND
i-Propyl 24-methyl-pentacosanoate	35.62	1.17	ND
Diethyl tartrate, [S-(R*,R*)]-	36.06	2.30	ND
		20.69	**20.61**
Sugar			
meso-Erythritol	10.71	5.91	ND
D-(-)-Fructofuranose	14.98	21.02	1.67
D-(-)-Fructopyranose	15.14	6.32	0.86
Quininic acid	15.75	ND	1.18
D-Pinitol	15.85	2.92	0.62
D-Threitol	16.19	2.27	ND
D-Mannose	17.68	15.13	5.69
Myo-Inositol	19.62	1.91	ND
2-O-Glycerol-α-d-galactopyranoside	23.17	3.35	ND
		58.83	**10.02**
Fatty acid			
Palmitic acid	18.29	3.73	8.23
Oleic acid	21.12	0.55	1.63
Stearic acid	21.53	3.58	5.34
Arachidic acid	24.66	2.20	3.23
Behenic acid	27.64	4.348	5.52
Eicosanoic acid	32.81	0.36	ND
		14.77	**23.95**
Aromatics			
Protocatechoic acid	14.85	ND	2.06
3,5-di-tert-Butyl-4-hydroxyanisole	25.14	2.65	3.11
Bilobol C17:1	31.26	ND	9.89
3-[2-Bromobenzal]-6-trifluoromethylindol-2-one	36.56	ND	5.77
9,10-anthracenedione, 1-amino-4-[(9,10-dihydro-9,10-dioxo-1-anthracenyl)amino]-	40.51	3.06	ND
		5.71	**20.83**
Steroid & terpenoid			
Lanosterol	37.05	ND	1.48
Olean-12-en-3-ol, acetate, (3β)-	39.01	ND	13.34
β-Amyrone	39.41	ND	7.63
9,19-Cyclolanost-24-en-3-ol, (3β)-	39.94	ND	2.09
20-Ethynyl-4-pregnene-20-ol-3-one	40.66	ND	0.05
			24.59
Total		100	100

ND: Not detected

Table 3: IC_{50} of DPPH radical scavenging and enzyme inhibitory activities.

	Radical Scavenging (μg/mL)	α-Glucosidase (μg/mL)	Porcine lipase (μg/mL)
	IC_{50}±SD	IC_{50}±SD	IC_{50}±SD
BPIC	146.31±0.53	94.75±1.30	260.83±3.79
BPID	165.65±0.49	116.05±0.16	100.38±4.17
BPOC	182.88±0.06	79.72±2.44	193.97±3.50
BPOD	141.49±0.29	25.35±0.48	263.78±2.76
Control	32.61 ± 2.60	279 ± 4.21	0.88 ± 0.12

Optimal fatty acid balance at cellular and tissue levels enhances membrane physical properties, facilitates protein palmitoylation, promotes biosynthesis of palmitoylethanolamide and acts as efficient surfactant in the lung (Carta *et al.*, 2017). The use of propolis may argument the endogenous biosynthesis of fatty acids such as palmitic acid which is of physiological relevance in man (Innisand Dyer, 1997). Similarly, stearic acid was found to inhibit the colony-forming ability of 80% of studied rat with mammary carcinoma and two human tumour continuous cell lines *in vitro* indicating its anticancer and anti-tumour potential (Habib *et al.*, 1987). Also, some studies have shown an inverse relationship between the concentrations of odd-chain fatty acids pentadecanoic acid (15:0) and heptadecanoic acid (17:0) in human plasma phospholipids or red blood cells and risk of cardiovascular disease and type 2 diabetes (Jenkins *et al.*, 2015; Pfeuffer and Jaudszus, 2016).

Except cycloartenol, other steroids/terpenoids present in BPO were not detected in BPI. 3β-olean-12-en-3-ol, acetate (13.34%) and β-amyrone (7.63%) were the major terpenoid constituents of BPO. Both terpenoids have been implicated for a number of pharmacological activities such as anti-ulcer (Li *et al.*, 2015), anti-inflammatory (Niu *et al.*, 2014) and hepatoprotective properties (He *et al.*, 1998).

For the deffated fractions, the GC-MS analysis indicated twenty-one compounds each which were further differentiated into fatty alcohols and ester (BPI, 20.69%; BPO 20.61%), sugar (BPI, 58.83%; BPO 10.02%), aromatics (BPI, 5.71%; BPO 20.83%) and steroidal/terpenoidal compounds. (BPO 24.59%). Sugar was the highest constituent of BPI (58.83%) while steroidal/terpenoidal compounds were not detected, in BPO however, the principal constituents were the steroidal/terpenoidal compounds while sugar components were the lowest (Table 2). Fatty alcohols and esters distributed almost equally in both propolis but the aromatics in BPO was about four-fold of those in BPI (Table 2).

The IC_{50} DPPH radical scavenging activities of all the propolis samples were moderate compared to the standard, however, BPOD demonstrated highest radical scavenging activity (141.49±0.29 μg/mL) among the propolis (Table 3). Alaribe *et al.* (2020) reported similarly result when antioxidant and chelating effects of a Nigerian propolis was evaluated using DPPH radical and ferrous ions respectively, the study indicated IC_{50} of 0.80 mg/mL for the propolis compared to IC_{50} of 0.02 mg/mL for the standard ascorbate, also, the ferrous ion chelating activity was lower (IC_{50} of 1.84 mg/mL) compared to EDTA (IC_{50} of 0.29 mg/mL) indicating moderate activities. Previous *in vivo* studies also showed that propolis decreased lipid peroxidation and the generation of reactive oxygen species on internal organs thus preventing oxidative stress induced metabolic disorders such as atherogenesis in hypercholesterolemic mice and streptozotocin-induced diabetes mellitus in a murine model (Silva *et al.*, 2015; Rivera-Yañez *et al.*, 2018)

In the alpha-glucosidase inhibition assay, all the propolis extracts demonstrated better inhibition than the standard acarbose, however, BPOD

inhibited the enzyme the most with IC_{50} value of 25.35±0.48 μg/mL which was more than 10-fold the value of acarbose. This might be connected to the presence phenolic content of BPOD. The work of Pujirahayu *et al.* (2019) showed the inhibitory effect of some triterpenes such as cycloartenol, ambonic acid, mangiferonic acid and ambolic acid from propolis on alpha-glucosidase. It was shown that mangiferonic acid exhibited the strongest inhibitory effect on the enzyme with an IC_{50} of 3.46 μM/mL. Furthermore, Alaribe *et al.* (2021) established through *in vitro* studies that the phenolic constituents of Nigerian propolis were responsible for its marked antidiabetic effect on α-amylase and α-glucosidase enzymes. A similar study on propolis of Nigerian origin showed evidence of hypoglycemic and hypolipidemic activities on alloxan diabetic. The ethanolic extract of the propolis reduced glycated hemoglobin, fasting plasma glucose, and serum level of very low-density lipoprotein (VLDL), while high-density lipoprotein (HDL) concentrations was elevated.

The anti-lipase activities of the propolis were fairly moderate but much lower compared to the standard orlistat (0.88 ± 0.12 μg/mL). BPID demonstrated the highest activity among the propolis extracts. Hydroalcholic extract of Brazilian red propolis had been reported to effect a hypolipidemic change in a rodent model of dyslipidemia. The extract minimised the effect of a hyperlipidic diet on murine body weight parameters and abdominal fat accumulation (Prata *et al.*, 2022).

CONCLUSION

This study provides insight into the chemical compositions, free radical scavenging ability and inhibitory potentials of selected Nigerian propolis on alpha-glucosidase and lipase enzyme. Though both propolis were collected from rain forest region of the country, a marked chemical diversities were observed in their fractionated samples indicating a nexus between the botanical origins and chemical compositions of propolis. The excellent inhibitory performance of the propolis extracts on alpha-glucosidase enyzme gives credence to its traditional use in treatment of diabetic patients. The free radical scavenging and antilipase activities were moderate but better than some synthetic agents with their attendant side effects. Furthermore, this study provides scientific data on Nigerian propolis which hitherto have been scantily profiled compared to propolis from other regions of the world.

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