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Cycloeucalenol and P-Coumaric Acid from Nigerian Propolis and their Antimicrobial Activity

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ABSTRACT

Propolis is a honeybee product employed in folkloric medicine and as a source of various bioactive constituents. Chromatography is a proven method for isolating natural compounds and the ethyl acetate extracts of propolis from Owerri in Imo State and Umuahia in Abia State Nigeria were subjected to column chromatography and the isolated compounds were identified by proton nuclear magnetic resonance (^{1}H-NMR) analysis. The compounds isolated were cycloeucalenol and para-coumaric acid. Antimicrobial assay of the isolated compounds was carried out by agar well diffusion and broth dilution methods using local clinical isolates. The isolated compounds showed good antibacterial and antifungal activities with zones of inhibition ranging between 25-34 mm. The MIC/MBC/MFC values were between 5 mg/mL and 2.5 mg/mL. This study confirmed the folkloric use of propolis and its varying phytochemical composition.

Keywords: Nigerian propolis, Cycloeucalenol, Para-coumaric acid, ^{1}H-NMR, Antimicrobial.

Introduction

Propolis is one of the products of the honeybees which has been utilized in folkloric medicine over time. It is a resinous material made up of beeswax, resin, sap and other materials collected by honeybee as a building material for their nest, to seal holes and to serve as protective agents against bacterial and fungal attack in their nest. [1] It is becoming a popular remedy and is available in either a pure form or combined with other natural products in cosmetics and as a constituent of health foods.[2] All species of honey bees produce propolis, but the amount and type of propolis produced is determined by the genus or species and vegetation type in the region. *Tetragonula* is a group of stingless bees of the *Meliponini* tribe, they produce large amounts of propolis, they have a non-functioning stinger to defend themselves against nest invaders but use their jaws to bite them. The *Apis mellifera* bees are the stinger bees and are larger than their *Tetragonula* counterparts. There are several hundred species worldwide, which differ significantly in colour, body size and colony size. [3,4] Propolis contains a variety of compounds such as polyphenols, flavonoids, phenolic acid and its esters, terpenoids, steroids, amino acids, wax acids, and sugars.[5] Cycloartane triterpenoids are widespread in the plant kingdom.[6] Hundreds of families of plants containing cycloartane triterpenoids have been reported, but the types and contents vary. [7 ,8] Propolis has been reported to be rich in triterpenes and some of their botanical sources have been identified.[9, 10] This study was carried out on two propolis samples from southeastern Nigeria. The isolation of cycloeucalenol and para-coumaric acid and their antimicrobial activities are hereby reported for these samples.

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Materials and Methods

General experimental procedure

NMR spectra were obtained on a Bruker AVIII-400 NMR spectrophotometer operating at 400 MHz for ^{1}H-NMR and spectra were processed using MestReNova. Chemical shifts are expressed in δ parts per million (ppm) and chemical shifts were referenced to the residual solvent peak at δ_H 7.26 for $CDCl_3$ while coupling constants are expressed in Hertz. Column chromatography was carried out using silica gel (40-60 µm, 60A, thermos scientific) and hexane-ethyl acetate gradients. The column used was 600 mm × 30 mm, TLC analysis was performed with Machery-Nagel precoated silica gel 60 F_{254} plates. The antimicrobial assay was carried out using an agar well diffusion and serial dilution method on some local clinical isolates including Methicilin Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Enterococci* (VRE), *Helicobacter pylori, Campylobacter jejuni, Salmonella typhi, Escherichia coli,, Candida albicans, Candida krusei , Candida tropicalis.*

Propolis samples were obtained from an apiary in Abia (*5° 31' 29.8308" N and 7° 29' 32.0676" E*) and Enugu (*6° 27' 30.1176" N and 7° 32' 47.0004" E*) in April 2022. Samples were confirmed by Prof. John Igoli of the Center for Natural Products Chemistry Research, Joseph Sarwuan Tarka University, Makurdi, Nigeria

Extraction and Isolation Procedure of Compound EPE (47 and 50), ABPE 37

Two propolis samples (50 g and 48.1 g) were extracted with 500 mL each of hexane, ethyl acetate and methanol in succession for 24 hours by maceration, after filtration, the ethyl acetate extract was concentrated using rotatory evaporator at 40^{o}C to yield 8.5 g brown and 12.7 g greenish-brown extracts respectively. About 1.0 g each of the ethyl acetate extracts were separated by column chromatography on a silica gel packed column eluted with hexane-ethyl acetate gradients (90:100 to 100% ethyl acetate). Fractions were collected and monitored by thin layer chromatography. Fractions EPE-47, EPE-50 and ABPE-37 were subjected to proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy to determine their constituents.

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Results and Discussion

Characterization of Fractions EPE-47 and EPE-50 as Cycloeucalenol (1)

Fractions EPE-47, EPE-50 were obtained as yellow crystals (mp 141 ^{o} C) and were both eluted by hexane: ethyl acetate 80:20 mixtures. They had Rf value 0.62 in hexane: ethyl acetate 60:40 solvent system. Their proton spectra gave the following chemical shifts:

EPE 47: ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 4.71 (*s*, 1H), 4.66 (*q*, *J* = 1.5 Hz, 1H), 3.21 (*td*, *J* = 9.9, 9.1, 4.5 Hz, 1H), 2.30 – 2.18 (m, 1H), 1.67 (*d*, *J* = 9.2 Hz, 1H), 1.49 – 1.38 (m, 2H), 1.04 (*d*, *J* = 2.2 Hz, 3H), 1.02 (*d*, *J* = 2.2 Hz, 2H), 0.99 (*s*, 1H), 0.90 (*s*, 1H), 0.89 (*d*, *J* = 2.2 Hz, 5H), 0.83 (*s*, 1H), 0.45 – 0.29 (*m*, 1H), 0.14 (*d*, *J* = 4.1 Hz, 1H).

EPE 50: ^{1}H NMR (400 MHz, Chloroform-d) δ 4.72 (*s*, 1H), 4.66 (d, *J* = 1.7 Hz, 1H), 3.22 (td, *J* = 9.9, 9.1, 4.6 Hz, 1H), 2.23 (q, *J* = 6.9 Hz, 0H), 2.06 – 2.02 (*m*, 5H), 1.67 (*d*, *J* = 9.2 Hz, 3H), 1.15 (s, 0H), 1.04 (*d*, *J* = 2.2 Hz, 4H), 1.02 (*d*, *J* = 2.2 Hz, 3H), 0.99 (*s*, 1H), 0.97 (*d*, *J* = 1.4 Hz, 4H), 0.91 (s, 1H), 0.83 (s, 0H), 0.39 (*d*, *J* = 4.1 Hz, 1H), 0.14 (*d*, *J* = 4.1 Hz, 1H).

^{1}H NMR spectra data are given in appendix 1-3 and values of chemical shift presented in Table 1. Olefinic protons occurred as a singlet and quartet at *δ* 4.71 (*s*, 1H), 4.66 (*q*, *J* = 1.5 Hz, 1H), *δ* for EPE 47 while EPE 50 showed olefinic protons as a singlet and doublet at *δ* 4.72 (*s*, 1H), 4.66 (*d*, *J* = 1.7 Hz, 1H). methylene protons of cyclopropane ring as triplets at 3.21 (*td*, *J* = 9.9, 9.1, 4.5 Hz, 1H), 3.22 *(td*, *J* = 9.8, 9.3, 4.7 Hz, 1H) for EPE 47 and EPE 50 respectively, the ^{1}H NMR spectral data were consistent with literature data for [9,10]. Based on literature reports and comparison of the ^{1}H NMR data with spectra of previously isolated compounds in our lab the fractions were identified as cycloeucalenol (**1**). The compound has recently been isolated from a propolis sample from Papua New Guinea [9]. The presence of cycloeucalenol could be attributed to the plants growing in the vicinity of the hives and foraged by the bees producing the propolis. Cycloartane triterpenes are ubiquitous in plants and trees growing in rainforest regions such as *Musa sapientum, Mangifera indica, Manihot esculenta, Zea mays* and *Capsicum annum*.[11] Cycloartane triterpenes have been reported to have antitumor, anti-inflammatory, antidiabetic, splasmolytic, and cytotoxic properties. [10, 12, 13]

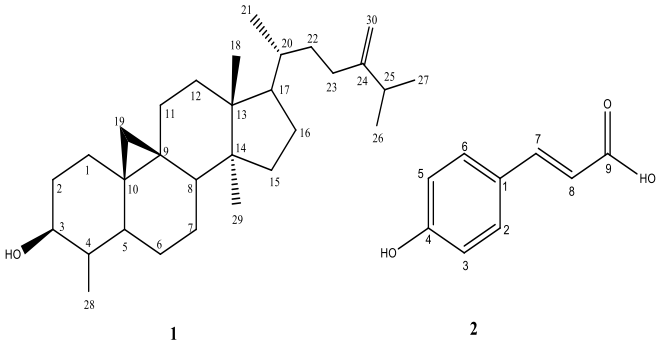

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Figure 1: Structure of Cycloeucalenol (1) and p-Coumaric acid (2)

Table 1: Results of ^{1}HNMR for ABPE 37 experimental, in comparison with the data from literature

Position	Experimental ^{1}H Chemical shift *δ* (ppm), J (Hz) ABPE 37	Literature^{1}H Chemical shift *δ* (ppm) El-gizawy *et al.*, 2017	Swizlocka *et al.*, 2012
1.	-	-	-
2.	7.43 (*d*, *J* = 8.73)	7. 51 (*d*, *J* = 7.7)	7.49 (*d*)
3.	6.84 (*d*, *J* = 8.60)	6. 83 (*d*, *J* = 7.7)	6.79
4.	-	-	-
5.	6.84 (*d*, *J* = 8.60)	6. 83 (*d*, *J* = 7.7)	6.79 (*d*)
6.	7.43 (*d*, *J* = 8.73)	7. 51 (*d*, *J* = 7.7)	7.49 (*d*)
7.	7.62 (*d*, *J* = 15.99)	7.64 (*d*, *J* = 15.8)	7.52 (*d*)
8.	6.30 (*d*, *J* = 15.94)	6.37 (*d*, *J* = 15.8)	6.29 (*d*)

Table 2: ^{1}HNMR data for EPE 47, 50 and ABPE 37 experimental, in comparison with literature

Position labeled	Experimental ^{1}H Chemical shift *δ* (ppm), J(Hz) EPE 47	EPE 50	Literature^{1}H Chemical shift *δ* (ppm) Alenezi *et al.*,2021	Suttiaporn *et al.*, 2015
1.			1.55(m), 1.28	
2.			1.83(m), .59(m)	
3.	3.21(td,4.5,9.9, 9.0)	3.22(*td* .9,9.1)	3.22(m)	3.20(*m*)
4.			1.32(m)	
5.			1.32(m)	
6.			1.65(m), 0.81(*m*)	
7.		1.15	1.15(m), 1.32(*m*)	

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8.			1.52(m)	
9.			-	
10.			-	
11.		1.08	1.07(*m*), 0.81(*m*)	
12.			1.59(*m*), 1.28	
13.			-	
14.			-	
15.			1.33(*m*), 0.90(*m*)	
16.	-	2.04(*m*)	2.02(*m*), 1.32 (*m*)	
17.	1.65(m)	1.67(*d*)	1.65(*m*)	
18.	0.99(s)	0.97	0.99(*s*)	
19.	0.14(d, 4.07)	0.14(*d*)	0.15(*d*, 4.18)	0.15 (*d)*
	0.38(m)	0.39(*d*)	0.38(*d*, 4.21)	0.38 (*d*)
20.	1.43(*m*)		1.40(*m*)	
21.	0.89(*d*,2.2)	0.97(*d*)	0.92(*s*)	
22.			1.33(*m*)	
23.	2.23(*m*)	2.23(*q*)	2.23(m), 1.65(m)	
24.			-	
25.			2.33(*m*)	
26.	1.02(*d*, 2.2)	1.02(*d*)	1.02(*d*)	
27.	1.04(d, 2.2)	1.04(*d*)	1.03*(s*)	
28.	4.71(s)	4.72(*s)*	4.71(*d*, 2.4)	4.73*(s*)
	4.66 (*q*, 1.5)	4.66(*d*)	4.59(*dd*, 2.4, 3.8)	4.64(*s*)
29.	0.90		0.90	
30.	0.83		0.83	

Table 3: Antimicrobial activities and zone of inhibition of purified fractions of south eastern Nigerian propolis

Microorganism	**Fractions**			**Reference drugs**		
	EPE 47	**EPE 50 (mm)**	**ABPE 37**	**Sparfloxacin**	**Fluconazole**	**Fulcin**
Methicillin Resistant *Staph. aureus*	0	0	26	35	35	0
Vancomycin Resistant *Enterococci*	28	27	25	0	0	0
Staphylococcus aureus	30	30	28	31	31	0
Escherichia coli	31	32	28	30	30	0
Helicobacter pylori	0	0	0	0	34	0
Campylobacter jejuni	27	27	0	0	0	32
Salmonella typhi	30	29	28	0	0	32
Candida albicans	0	0	29	-	35	0
Candida krusei	32	31	0	-	33	34
Candida tropicalis	28	27	-	-	29	34

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Table 4: Minimum inhibitory concentration and minimum bactericidal/fungicidal concentration of pure fractions

Pathogens	**MIC (µg/mL)**																				**MBC/MFC (µg/mL)**									
	EPE 47					**EPE 50**					**ABPE 37**					**EPE 47**					**EPE 50**					**ABPE 37**				
	100	50	25	12.5	6.25	100	50	25	12.5	6.25	100	50	25	12.5	6.25	100	50	25	12.5	6.25	100	50	25	12.5	6.25	100	50	25	12.5	6.25
MR S. aureus	-	-	-	µ	+						-	-	µ	+	+	-	-	µ	+	+						-	µ	+	+	+
															+					+									+	+
VR Enterococci						-	-	µ	+	+	-	-	-	µ	+						-	µ	+	+	+	-	-	µ	+	+
										+														+	+				+	+
E.coli	-	-	-	µ	+	-	-	-	-	µ	-	-	µ	+	+	-	µ	+	+	+	-	-	µ	+	+	-	µ	+	+	+
															+				+	+					+				+	+
H. pylori											-	-	-	µ	+	-	-	µ	+	+						-	-	µ	+	+
																				+										+
C. jejuni						-	-	µ	+	+	-	-	-	µ	+	-	µ	+	+	+	-	µ	+	+	+	-	-	µ	+	+
										+									+	+				+	+					+
S. typhi	-	-	µ	+	+	-	-	-	µ	+						-	µ	+	+	+	-	-	µ	+	+					
					+														+	+					+					
S. areus	-	-	-	-	µ	-	-	-	µ	+						-	-	µ	+	+	-	-	µ	+	+					
																				+					+					
C. albicans	-	-	-	µ	+						-	-	-	-	µ	-	-	µ	+	+						-	-	µ	+	+
																				+										+
C. krusei	-	-	-	µ	+	-	-	-	-	µ	-	-	-	-	µ	-	-	µ	+	+	-	-	µ	+	+	-	-	µ	+	+
																				+					+					+
C. tropicalis						-	-	µ	+	+	-	-	-	µ	+						-	µ	+	+	+	-	-	µ	+	+
										+														+	+					+

Key- µ= MIC/MBC/MFC, - = no growth, + = scanty growth, ++ = moderate growth

Characterization of Fraction ABPE-37 as p-Coumaric acid

Fraction ABPE-37 was obtained as a yellow solid (mp, 211 ^{o}C) eluted from an ethyl acetate:hexane gradient (80:20), Rf, 0.54 on TLC in hexane: ethyl acetate 60:40 solvent system.

The ^{1}H NMR spectrum showed two aromatic protons at δ 6.84(2H, *d, J* = 8.60, H-3, H-5) and 7.43 ppm (2H, *d, J* = 8.73, H-2, H-6). The trans ethylenic protons were observed as doublets at 7.62 ppm (1H, *d, J* = 15.99) and 6.30 ppm (1H, *d, J* = 15.94). The ^{1}H Similarly, based on literature reports and comparison of the ^{1}H-NMR data with spectra of previously isolated compounds in our lab the fraction was identified as p-coumaric acid (**2**). The compound has been severally isolated from propolis samples. [14,15] The compound is reported to possess antiparasitic, antimicrobial, and cytotoxic properties [16].

Antimicrobial activity

The results of the susceptibility studies of the isolated compounds showed that all organisms were inhibited by the extracts except *Helicobacter pylori* and Methicilin Resistant *Staphylococcus aureus* with a zero diameter zone of inhibition. This is in agreement with the work of Arruda [16] whose study reported that all propolis samples examined showed good activity against *S. aureus.* Their work also identified the susceptibility to propolis extract of *E. coli* and *Staphylococcus aureus* strains that were resistant to various antibiotics. Tosi *et al.,* [17] also noted the antimicrobial activity of ethyl acetate extracts of propolis from Saudi Arabia and Egypt (EEPS and EEPE). EEPS and EEPE inhibited antibiotic-resistant *E. coli*, *S. aureus* and *C.albicans* in single and polymicrobial cultures. *S. aureus* became more susceptible when cultured with *E. coli* or *C. albicans* in single and polymicrobial cultures. The antifungal activity results showed excellent activities against the fungi tested, inhibiting *Candida krusei* and *Candida tropicalis*.

This is consistent with the work of Capoci *et al.,* [19] on the use of propolis extract solution (PES) for the treatment of recurrent vulvovaginal candidiasis (RVVC) and an antibiofilm material for RVVC reported to counteract the biofilm growth of *C. albicans* and resistance to antifungal drugs and this is further supported by Paul (2021) which reported the antibacterial activities of Cameroonian propolis isolated compounds which showed significant activity against *S. typhi* and validated the use of propolis (PROMA-C) in the treatment of infectiouc diseases in Cameroon [20] . The minimum inhibitory concentration of the pure fractions was between 25 µg/mL and 6.25 µg/mL while the minimum bactericidal and fungicidal concentration ranges between 50 µg/mL and 6.25 µg/mL. This is an indication that the compounds were able to inhibit these organisms even at very low concentrations.

Conclusion

It has been reported that triterpenes are a secondary metabolite with extensive biological activity, which are mainly produced by plants to protect against various diseases, so bees pay attention to them and use them to make propolis as a protective chemical agent for nest and members of colonies to build, creating a fantastic chemical interaction between bee and plant. Interestingly, these bioactive compounds present in the resin material have excellent antimicrobial properties, therefore, can serve as a promising starting point for drug and dietary supplement formulation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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