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Research Article

Chemical Composition and Antimicrobial Potential of *n*-hexane and Methanol Extracts of *Benue Propolis*

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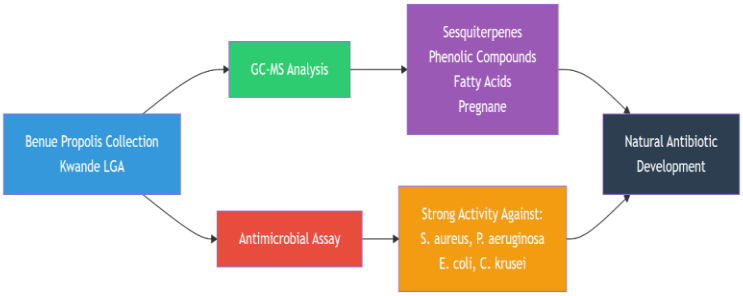
Abstract

Propolis is a resinous material made by honey bees for protecting their hives against microbial attack. *Propolis* sample was collected from Kwande Local Government Area of Benue State. This study investigated the chemical constituents and antimicrobial activity of the crude extracts using gas chromatography–mass spectrometry (GC–MS) and in vitro susceptibility assays. *Propolis* sample was extracted using *n*-hexane, ethyl acetate and methanol. Antimicrobial evaluation against nine bacterial and four fungal strains demonstrated broad-spectrum activity significantly for the *n*-hexane and methanol extracts, with significant inhibition of *Staphylococcus aureus* (25±0.03 mm, 20±0.04 mm) and *Pseudomonas aeruginosa* (21±0.03 mm, 17±0.01 mm) by *n*-hexane, and methanol extracts, respectively. Moderate activity was observed against *Escherichia coli* (18±0.01 mm, 20±0.04 mm). The minimum inhibitory and bactericidal concentration (MIC/MBC) of the crude extracts of the inhibited bacteria ranged from 1.57 – 25 mg/mL. Significant antifungal activity was recorded against *Candida krusei* (24±0.02 mm) with the minimum fungicidal concentration of 1.57 mg/mL. GC–MS analysis of the extracts revealed a complex mixture of hydrocarbons, fatty acids, esters, alcohols, terpenoids, phenolics, and steroidal compounds. The methanol extract showed majorly 9,12-octadecadienoic acid (51.65%), alongside hexadecanoic acid (14.30%), glycerin (7.73%) and hydroquinone. Batilol and pregnane were reported for the first time in Nigerian *Propolis*. Hexane extract was characterized by hydrocarbons and sesquiterpenes. These findings demonstrate that Benue *Propolis* possesses a phytochemical profile with significant antimicrobial potential, which can serve as promising source of bioactive compounds for therapeutic and drug development applications.

Graphical Abstract



1. Introduction

Propolis is a complex resinous material produced by honeybees through the mixing of plant exudates with beeswax and salivary secretions collected primarily from saps, buds, bark, and exudates of botanical sources [1]. The various plant species include birch, poplar, pine, alder, willow, palm, *Baccharis dracunculifolia*, and *Dalbergia [2,3]*. The *Propolis* conventionally was used by the bees for hive defense against intruders, contributes to colony immunity which is supported by bee-derived antimicrobial peptides and plant-derived bioactive constituents [4]. Its characteristic aroma is attributed to volatile compounds present in the glue [5]. The chemical composition varies significantly with botanical

origin, geographic location, season, and bee species [6,7] as over 500 compounds have been identified in *Propolis*, highlighting its remarkable chemical diversity [8,9].

Extensive phytochemical investigations across Europe, South America, Asia, and the Middle East have revealed that *Propolis* is rich in flavonoids, phenolic acids and their esters, phenylpropanoids, terpenoids, stilbenes, and prenylated derivatives [10] For instance, Brazilian green *Propolis* is characterized by prenylated phenylpropanoids derived from *Baccharis dracunculifolia* [11], while European *Propolis* commonly contains flavonoids associated with *Populus* species [12]. Studies from countries such as Brazil, China, Turkey, and Poland have consistently reported strong antimicrobial, antioxidant, anti-inflammatory, antitumor, and immunomodulatory activities, often correlating these biological effects with specific phenolic and flavonoid constituents [13,14,15,16]. These underscore the therapeutic potential of *Propolis* and demonstrate how geographical and botanical factors influence both composition and bioactivity [17].

Despite the growing research on *Propolis* in different countries, African *Propolis*, particularly Nigerian *Propolis* remains underexplored. Preliminary reports suggest that Nigerian *Propolis* may possess unique phytochemical profiles linked to indigenous flora, yet comprehensive chemical characterization and biological evaluation are still limited [18]. This knowledge gap restricts the comprehensive literature on the understanding of African *Propolis* diversity and its potential pharmaceutical relevance [19]. Therefore, the aim of the present study is to investigate the chemical composition and antimicrobial activities of *Propolis* collected from Kwande Area of Benue State, Nigeria. By characterizing its bioactive constituents and evaluating its antimicrobial potential, this work seeks to contribute to the growing global database on *Propolis*, highlight the uniqueness of Nigerian *Propolis*, and provide scientific evidence supporting its potential applications in medicine and natural product research.

2. Materials and Methods

2.1. Sample collection and preparation

Propolis samples were collected from the wild beehive located in Kwande Local Government Area of Benue State, Nigeria (Latitude. 6.801° N; Longitude. 9.4702° E) during the dry season (December, 2019). *Propolis* sample was harvested by placing sterile plastic mats over the external surfaces of beehives this is to ensure the deposit excess resinous material onto the mats, facilitating non-destructive collection of crude *propolis* without damaging hive structure [20]. After removal, samples were visually inspected to ensure absence of visible contaminants such as soil, wax, bee parts, and hive debris. *Propolis* samples were air-dried in a well-ventilated room at ambient temperature (25-30 °C) for 7 days to reduce water content and improve extraction efficiency [21]. Drying was conducted on clean aluminum foil to prevent cross-contamination. After drying, samples were homogenized manually using a sterile mortar and pestle to yield uniform *propolis* powder. Homogenized samples were transferred into labeled, airtight amber glass containers to protect against light-induced degradation of bioactive compounds and stored at 4 °C until further analysis.

2.2. Extraction

Extraction of the *propolis* was done by cold maceration [22]. About 60.0 g of *propolis* was stored in the refrigerator, frozen *propolis* was grated and dissolved in 400 mL of *n*-hexane. This mixture was kept for 24 hours in a tightly closed bottle. This was filtered using Whatman No.1 filter paper and the filtrates were collected and the residues were dried using Genlab incubator at 40 °C. This residue was successively extracted in 400 mL of ethyl acetate and methanol in the same manner and the filtrates were collected [22]. The filtrates obtained from all the solvents were concentrated using a rotatory evaporator and the resulting extracts were kept for analysis.

2.3. GC-MS analysis

The *n*-hexane and methanol extracts of the samples were investigated and GC–MS analysis was performed using a Shimadzu GCMS-QP2010SE equipped with a DB-5MS capillary column (30 m × 0.25 mm x 0.25 μm film thickness interfaced with a BG mode analytical spectrometer. Helium at a flow rate of 1ml/min was used as a carrier gas. Initial column temperature was 120 °C held for 5 minutes and increased at 5 °C/minute to 230 °C and held for 5 minutes [23]. For the MS, electrons impact ionization was carried out at 70 eV. Identification of the constituent compounds was with the Chem-office software [23] along with the MS library [23].

2.4. Antimicrobial screening

The antimicrobial activities of methanol, ethyl-acetate and *n*-hexane extracts were determined using some pathogenic microbes obtained from the Department of Medical Microbiology Ahmadu Bello University, Zaria. Mueller Hinton agar was the medium used as the growth medium for the microbes [24]. The medium was prepared according to the manufacturer's instructions, sterilized at 121 °C for 15 minutes, poured into sterile petri dishes and allowed to cool and solidify [24]. The sterilized medium was seeded with 0.1 mL of the standard inoculum of the test microbe. The inoculum was spread evenly over the surface of the medium using a sterile swab. Agar well diffusion method was used for screening the extracts [24]. Using a standard sterile cork borer of 6 mm in diameter, a well was cut at the centre of each inoculated medium. The extracts (0.5 g each) were weighed and dissolved in 10 mL of DMSO to obtain a concen-

tration of 50 mg/mL Thereafter, 0.1 mL of the solution of the extract at the concentration of 50 mg/mL was introduced into the well on the inoculated medium. The plates were incubated at 37 °C for 24 h after which the plates of the media were observed for the zone of inhibition of growth. The zone was measured with a transparent ruler and the result recorded in millimetres [24]. While the sabaroud dextrose agar will be seeded with 0.1 mL of the test fungi. The inoculum was spread evenly over the surface of the medium by use of a sterile swab. By the use of a standard sterile cork borer of 6mm in diameter, a well was cut at the center of each inoculated media. Also, 0.1 mL of solution of the extract of concentration 50 mg/mL was then introduced into the well on the inoculated medium. Incubation was done at 37 °C for 1-7 days for fungi after which plates of the media were observed for zone of inhibition of growth. The zones of inhibition were measured with a transparent ruler and result recorded in millimeters [24].

2.5. Identification of compounds

The compounds were identified by comparison with chromatograph retention characteristics and mass spectra of authentic standards [25], literature mass spectra [25] and mass spectral library [25] of the GCMS data system.

3. Results and Discussion

Results from the laboratory experiment and analysis are presented, compared and discussed in this section.

3.1. Extraction and identification of compounds

The GC-MS chromatograms of the *n*-hexane and methanol extracts of Benue *propolis* revealed numerous peaks corresponding to different compounds (Figures 1 and 2). The gas chromatogram represents the relative abundance of compounds eluted as a function of retention time (RT**)**, where the peak heights and peak areas correspond to the relative concentration of the detected constituents. These mass spectra serve as molecular fingerprints which allow compound identification by comparison with spectral libraries such as the National Institute of Standards and Technology (NIST) database [26,27].

GC-MS analysis revealed that the *n*-hexane and methanol extracts of *Benue propolis* contain a diverse mixture of hydrocarbons, fatty acids, alcohols, esters, terpenoids, phenolics, and steroidal compounds (Table 1). The phytochemical profile reflects the influence of solvent polarity on compound extraction, where non-polar solvents such as *n*-hexane preferentially extract lipophilic hydrocarbons and terpenoids, while methanol extracts relatively polar constituents such as phenolics, fatty acids, and alcohols. Similar solvent-dependent extraction patterns have been reported in *propolis* studies from Brazil, Turkey, and China [28-30].

Several compounds were detected in both extracts which were mainly hexadecanoic acid, 9,12-octadecadienoic acid, tetracosanol, heptacosanol, methyl stearate, hexadecanoic acid methyl ester, hexadecanoic acid ethyl ester, and 9-octadecenoic acid ethyl ester. Among these, hexadecanoic acid exhibited the highest relative abundance (14.30% area; 11.67% height) in the methanol extract which is lower in the hexane extract, suggesting it is a major component of *Benue Propolis*. These compounds are commonly reported constituents of *propolis* and are known to contribute to antimicrobial, antioxidant, and anti-inflammatory activities [31-33].

The *n*-hexane extract showed several non-polar hydrocarbons and terpenoid constituents including eicosane, octacosane, pentatriacontane, cycloheptane, and 9-tricosene, as well as sesquiterpenes such as caryophyllene, caryophyllene oxide, and humulene. This agrees with the work of [34]. These compounds are frequently detected in non-polar extracts because of their high hydrophobicity.

In contrast, the methanol extract is characterized by very high abundance polyunsaturated fatty acid (PUFA; 9,12-Octadecadienoic acid (51.65%), which has shown to be the principal constituent of the methanol extract which is known for its anti-inflammatory activity, antioxidant properties and nutritional/therapeutic relevance [35]. Other notable compounds were also identified such as hexadecanoic acid (14.30%) and glycerin ((7.73). Phenolic compounds such as hydroquinone and catechol are particularly significant because phenolics are recognized as major contributors to the antioxidant and antimicrobial activities of *Propolis* [36]. Batilol and the steroid pregnane were detected in *propolis*, a first time report in Nigerian *propolis* sample.

Taken together, the results demonstrate that both extracts share several fatty acid derivatives and alkanols, while hydrocarbons and sesquiterpenes are more characteristic of the *n*-hexane extract and phenolic constituents are more prominent in the methanol extract. This complementary chemical profile highlights the complex phytochemical nature of Benue propolis and is consistent with reports that propolis composition varies with botanical origin and extraction solvent [37]. The presence of these bioactive chemical classes further supports the pharmacological potential of Nigerian propolis and its relevance for future natural product and drug discovery studies.

Table 1. Chemical constituents of *n*-hexane crude *propolis* extracts as identified and characterized by the GC-MS

S/No	Compound	m/z	RT (min)	Area % (RA)	MF	Classification
1	Benzeneacetaldehyde, α,2,5-trimethyl-	162	6.796	0.47	$C_{11}H_{14}O$	Aldehyde
2	Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethyl-	146	7.089	0.75	$C_{11}H_{14}$	Terpenoid
3	Caryophyllene	204	11.82	0.44	$C_{15}H_{24}$	Sesquiterpene
4	Humulene	204	12.386	0.39	$C_{15}H_{24}$	Sesquiterpene
5	Caryophyllene oxide	220	14.034	0.32	$C_{15}H_{24}O$	Oxygenated sesquiterpene
6	Hexadecanoic acid, methyl ester	270	16.799	3.12	$C_{17}H_{34}O_2$	Fatty acid ester
7	n-Hexadecanoic acid	256	17.172	4.42	$C_{16}H_{32}O_2$	Fatty acid
8	Hexadecanoic acid, ethyl ester	284	17.264	4.42	$C_{18}H_{36}O_2$	Fatty acid ester
9	9-Octadecenoic acid (Z)-, methyl ester	296	17.984	2.37	$C_{19}H_{36}O_2$	Fatty acid ester
10	Methyl stearate	298	18.128	0.94	$C_{19}H_{38}O_2$	Fatty acid ester
11	9,12-Octadecadienoic acid (Z,Z)-	280	18.356	8.45	$C_{18}H_{32}O_2$	Polyunsaturated fatty acid
12	(E)-9-Octadecenoic acid ethyl ester	310	18.398	5.08	$C_{20}H_{38}O_2$	Fatty acid ester
13	Ethyl 14-methyl-hexadecanoate	298	18.537	3.29	$C_{19}H_{38}O_2$	Fatty acid ester
14	Eicosane	282	19.158	5.28	$C_{20}H_{42}$	Alkane
15	1-Docosanol, acetate	354	19.765	1.92	$C_{24}H_{50}O_2$	Fatty acid ester
16	n-Tetracosanol-1	354	20.131	4.9	$C_{24}H_{50}O$	Alcohol
17	Octacosane	394	20.793	1.67	$C_{28}H_{58}$	Alkane
18	Batilol	330	20.923	0.82	$C_{21}H_{44}O_3$	Ether lipid
19	1-Heptacosanol	396	21.253	3.1	$C_{27}H_{56}O$	Alcohol
20	Pentatriacontane	492	21.381	4.9	$C_{35}H_{72}$	Alkane

RT: Retention Time. **RA**: Relative Abundance. **MF**: Molecular Formula

Table 2. Chemical constituents of methanol crude *propolis* extracts as identified and characterized by GC-MS.

S/No	Compounds	m/z	RT (min)	Area% (RA)	MF	Classification
1	Cycloheptane	98	5.88	0.28	C_7H_{14}	Alkane
2	Catechol	110	10.19	1.12	$C_6H_6O_2$	Phenolic
3	Hydroquinone	110	11.41	0.81	$C_6H_6O_2$	Phenolic
4	9,12-Octadecadienoic acid	280	18.35	4.30	$C_{18}H_{32}O_2$	Fatty acid
5	17-Octadecynoic acid	280	19.60	2.77	$C_{18}H_{32}O_2$	Fatty acid
6	Hexadecanoic acid	256	17.26	14.30	$C_{16}H_{32}O_2$	Fatty acid
7	Glycerin	92	8.678	7.73	$C_3H_8O_3$	n-alkanol
8	Hexadecanoic acid methyl ester	270	16.79	1.65	$C_{17}H_{34}O_2$	Fatty acid methyl ester
9	Pregnane	288	19.64	2.41	$C_{21}H_{36}$	Steroid
10	9,12-Octadecadienoic acid methyl ester	294.47	17.97	4.30	$C_{19}H_{34}O_2$	Fatty acid methyl ester
11	7-Octadecenoic acid methyl ester	296.49	18.01	3.55	$C_{19}H_{36}O_2$	Fatty acid methyl ester
12	Cyclopentanetridecanoic acid methyl ester	296.49	18.14	1.26	$C_{19}H_{36}O_2$	Fatty acid methyl ester
13	9,12-octadecadienoic acid	280.45	18.51	51.65	$C_{18}H_{32}O_2$	Fatty acid
14	17-octadecynoic acid	280.45	18.58	2.77	$C_{18}H_{32}O_2$	Fatty acid
15	Hexadecanoic acid, 2-hydroxy-1-(hydroxyme-thyl)ethyl ester	330.50	20.43	1.35	$C_{19}H_{38}0_4$	Fatty acid
16	Diisooctyl phthalate	390.56	20.51	0.84	$C_{24}H_{38}O_4$	Plastic
17	Propylene glycol monooleate	340.54	21.50	3.67	$C_{21}H_{40}O_3$	Emulsifier

RT: Retention Time. **RA**: Relative Abundance. **MF**: Molecular Formula

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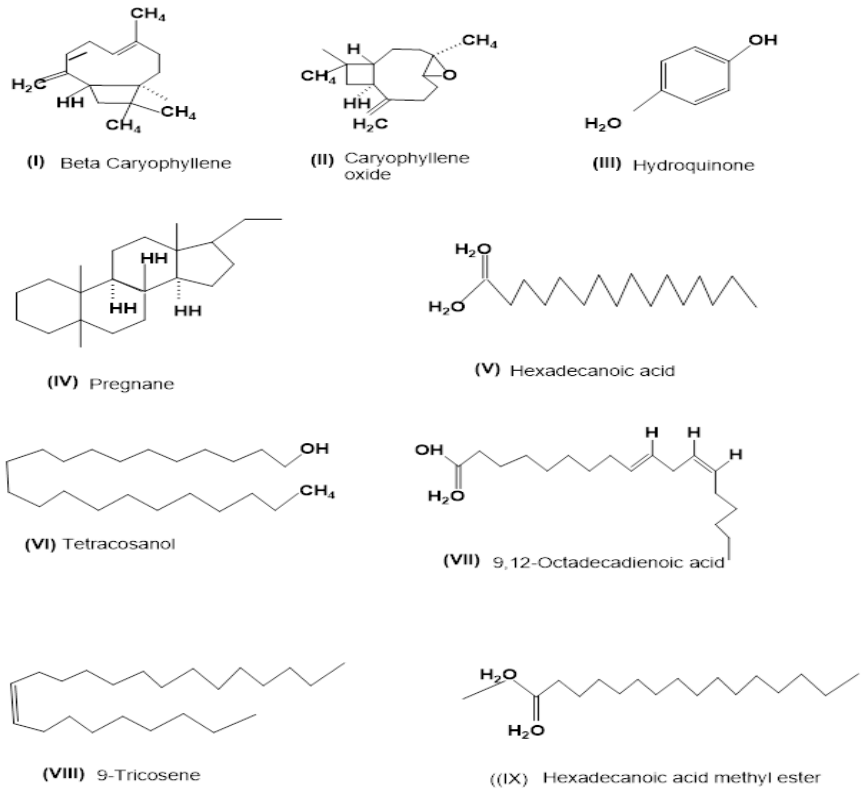

Figure 1. Selected chemical constituents identified by GC-MS.

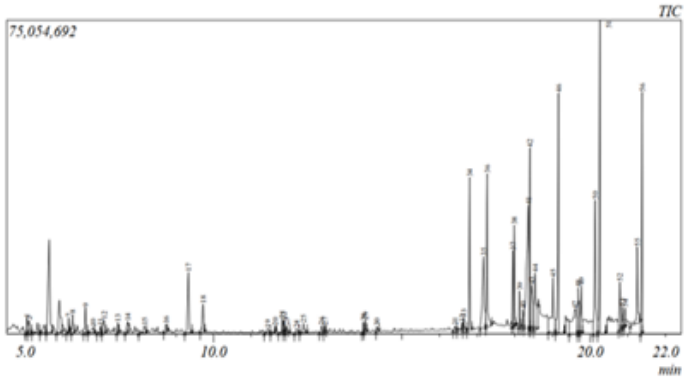

Figure 2. Gas chromatogram of *n*-hexane extract of crude Propolis.

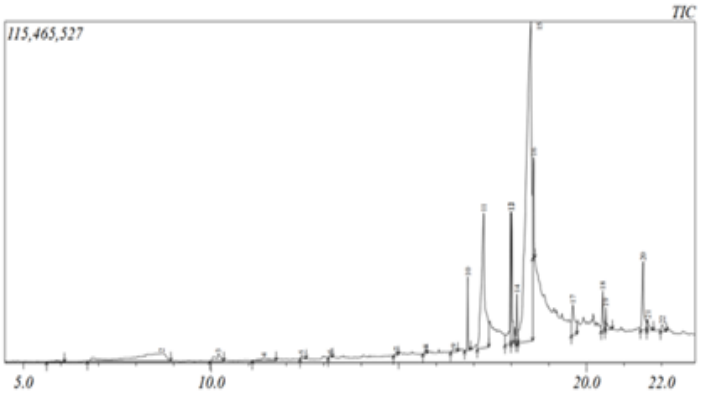

Figure 3. Gas chromatogram of methanolic extract of crude Propolis.

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3.2. Antimicrobial Activities

The antimicrobial activities of the three crude *propolis* extracts (*n*-hexane, ethyl acetate, and methanol) against nine bacterial strains and four fungal species are presented in Tables 2 and 3. The susceptibility studies revealed that most of the tested organisms were sensitive to the extracts, except *Klebsiella pneumoniae*, *Proteus mirabilis*, and vancomycin-resistant *Enterococcus* spp., which showed no observable zones of inhibition. Significant inhibitory effects were recorded against *Staphylococcus aureus* for all three extracts, with zones of inhibition of 18± 0.02 mm, 25± 0.03 mm, and 20 ± 0.04 mm for the ethyl acetate, *n*-hexane, and methanol extracts, respectively. Similarly, inhibitory activity was observed against *Pseudomonas aeruginosa*, with zones of inhibition of 13± 0.03 mm, 21± 0.03 mm, and 17± 0.01 mm for the respective extracts. The minimum inhibitory and bactericidal concentration (MIC/MBC) of the crude extracts of the inhibited pathogens ranged from 1.57 – 25 mg/mL These findings are in agreement with the report of [38], who observed that ethanol extracts of *propolis* completely inhibited the growth of *Staphylococcus aureus* and *Enterococcus* spp., partially inhibited *Pseudomonas aeruginosa* and *Escherichia coli*, and showed no inhibitory effect against *Klebsiella pneumoniae*. These observations suggest that *propolis* may serve as a potential source of bioactive compounds for the treatment of infections associated with these pathogens, including skin infections, urinary tract infections, ventilator-associated pneumonia, and pharyngitis. Moderate inhibitory activity was observed against *Escherichia coli*, with zones of inhibition of 12 ± 0.3 mm, 18 ± 0.01 mm, and 20 ± 0.04 mm for the ethyl acetate, *n*-hexane, and methanol extracts, respectively. The crude extracts also exhibited notable antifungal activity against *Candida krusei*, with inhibition zones of 10 ± 0.01 mm, 24 ± 0.02 mm, and 20 ± 0.04 mm for the ethyl acetate, *n*-hexane and methanol extracts, respectively. The minimum fungicidal concentration (MFC) of the crude extracts of the inhibited fungus is 1.57 mg/mL. This suggests that *Propolis* extracts may possess therapeutic potential in the management of fungal infections such as vaginitis [39]. However, no inhibitory activity was observed against other *Candida* species, including *Candida albicans*, *Candida stellatoidea*, and *Candida tropicalis*.

Table 3. Results of Antibacterial Activities of Crude *Propolis* Extracts.

Test Organisms	Dilution factor (mg/ml)	Minimum zone of Inhibition (mm)			
		***n*-HE**	**EAE**	**ME**	**Sparfloxacin**
Staphylococcus aureus	50	20±0.04	18 ±0.15	25 ±0.12	34 ±0.09
Streptococcus Pyogenes	50	0	14 ± 0.02	21 ±0.044	25 ±0.03
Escherichia coli	50	18 ±0.01	13 ±0.03	20 ±0.035	22 ±0.04
Klebsiella pneumoniae	50	0	0	0	0
Proteus mirabilis	50	0	0	0	0
Salmonella typhi	50	0	15 ±0.03	21 ±0.04	28 ±0.03
Pseudomonas aeruginosa	50	17 ±0.01	13 ±0.03	21 ±0.04	25 ±0.03
Vancomycin resistant E*nterococci spp.*	50	0	0	0	0
Methicillin resistant *Staphylococcus aureus*	50	0	16 ±0.02	22 ±0.044	26 ±0.03

***n-HE**: *n*-hexane Extract. **EAE**: Ethyl Acetate Extract. **ME**: Methanol Extract.

Table 4. Results of Antifungal activities of Crude *Propolis* Extracts.

Test Organisms	Dilution factor (mg/ml)	Minimum zone of inhibition (mm)			
		***n*-HE**	**EAE**	**ME**	**Flucanozole**
Candida albican	50	0	0	0	0
Candida krusei	50	18±0.01	12± 0.02	20±0.04	26±0.02
Candida stellatoidea	50	0	0	0	0
Candida tropicalis	50	0	0	0	0

***n-HE**: *n*-hexane Extract. **EAE**: Ethyl Acetate Extract. **ME**: Methanol Extract.

Table 5. The MIC/MBC and MFC of Benue *Propolis* Crude Extracts

Extracts	Pathogens (mg/mL)						
	S. aureus	***S. Typhi***	***MRSA***	***S. pyogenes***	***E.coli***	***P. aeruginosa***	***C. krusei***
n-Hexane	25	12.5	25	12.5	25	25	3.13
Ethyl- acetate	12.5	12.5	12.5	12.5	12.5	12.5	3.13
Methanol	25	12.5	25	12.5	12.5	12.5	3.13
Sparfloxacin	3.13	1.57	3.13	3.13	1.57	3.13	-
Fluconazole	-	-	-	-	-	-	1.57

Keywords: *S.aureus = Staphylococcus aureus, S. typhi = Salmonella typhirium, MRSA = methicillin resistant staphylococcus aureus, S. pyogenes = Streptococcus pyogenes, E.coli = Escherichia coli, P. aeruginosa = Pseudomonas aeruginosa, C. krusei = Candida krusei*

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4. Conclusions

The study reveals that *propolis* from Benue State, Nigeria contains diverse chemical constituents belonging to classes such as fatty acids, hydrocarbons, terpenoids, phenolics, and related compounds, with variations observed between the *n*-hexane and methanol extracts due to differences in solvent polarity. Methanol extracts were particularly rich in polyunsaturated fatty acids, notably 9,12-octadecadienoic and phenolics. The antimicrobial evaluation demonstrated significant inhibitory activity against selected bacteria and fungi, particularly against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida krusei* indicating that the identified constituents are likely to contribute to the biological activity of the extracts. The identification of batilol and pregnane represents a novel contribution to Nigerian *propolis* chemistry These findings provide important scientific evidence on the chemical composition and antimicrobial potential of Nigerian *Propolis*, highlighting its importance as a natural source of antibiotics or supplements

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