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In-vitro free radical scavenging and antimicrobial activity of honeybee propolis extracts from North-Western, Nigeria

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ABSTRACT

Honeybee propolis extracts and its soluble sub-fraction from Apismellifera (Linn); a propolis use in ethno medicine as an emollient in the treatment of infectious diseases; ringworm, measles and chickenpox in Northern Nigeria were subjected to chemical, antioxidant and antimicrobial screening using standard procedures. Preliminary chemical screening of ethanol, hexane, chloroform, ethyl acetate and butanol extracts revealed the presence of flavonoids, phenolic compounds, saponins, steroids and triterpenes. The antioxidant activity using 1,1- Diphenyl-2-Picrylhydrazyl (DPPH) revealed ethyl acetate fraction (EE) having significant antioxidant activity of IC_{50} value of 1.78 ± 0.01µg/mL compared to other extracts and standard ascorbic acid of 2.54 ± 0.01µg/mL at $p \leq 0.05$. The antimicrobial activity of the propolis extracts using Agar diffusion and Broth dilution methods against Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtillis, Candida krusei and Candida tropicalis revealed zone of inhibition between 20 to 27mm with a MIC result ranged from 1.25 to 2.5 mg/mL while, the MBC/MFC values ranged from 5 to 10mg/mL. The result of the propolis extracts and its soluble sub-fractions indicates it contains compounds that possess antioxidant and antimicrobial activity and provides credibility of its use for the treatment of infectious diseases and as an immune booster.

Keywords: Propolis, Antioxidant, Antimicrobial, MIC

INTRODUCTION

Natural occurringpropolis from honey bee is a salivary enriched enzymatic resinous secretion collected by the worker bee *Apismellifera* Linn from pollens, nectars, bark of trees and leaves of plants [1]. It plays a significant role as a building and insulating material, to seal the holes in their honeycombs, smooth out the internal walls and protect the entrance from intruders like ants, insect and bacteria [1]. Hence, these properties have been achievable by the bee, because been a social and pollinating insect it possess theability to travel as far as 8km in search ofdiverse melliferous plant nectar, pollens, cracks of bark of trees globally and also in the Northern Guinea Savannah of Nigeria. These melliferous plants around each locality have medicinal value and accounts for the ethno medicinal potential of their honey and propolis[2].

In addition, several review studies have shown that the chemical composition of propolis varies quantitatively and qualitatively, depending on the vegetation in the area from which it was collected and species of the bee [3]. Therefore, as a result of the variability of the chemical constitute in thepropolis, this has steered research into propolis from North-Western, Nigeria.Propolis in Nigeria has been use traditionally as an emollient in treatment of measles, ringworm, chickenpox disease[2, 4], and different researchershave reported antitumour, anti-inflammatory, antimicrobial, immunomodulatory[1, 5] antioxidant activity [6] of the propolis, and these are attributed to a number of polyphenolic compounds, mainly flavonoids, steroids, triterpenes, phenolic acids and esters present in the it. The antimicrobial activity of prenylated flavonoids from Egyptian propolis was identified and reported by Ashraf, (2009). Although numerous studies have reported the biological activity of propolis collected worldwide, information about North-Western Nigeria propolis are still lacking, Therefore, this study tends to investigate the

antimicrobial and antioxidant activity of propolis from ZangoKataf, Kaduna State (North-Western Nigeria). In order to added new knowledge and confirmed the rationale of the ethno medicinal use of the propolis.

MATERIALS AND METHODS

Chemicals and Reagents

Ascorbic acid powder (Merck Co.), Distilled water, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma-Aldrich Co.), Dimethyl sulfoxide (DMSO) and all other chemicals were of analytical grade BDH Chemical laboratory (England, UK).

Propolis sample and extraction

Honeybee propolis samples were collected from Zangokataf local government area of Kaduna State, Nigeria, from Honeybee cultivators, propolis was pressed to remove stored honey from it, sizes reduce with scissors and stored for use. The raw honey bee propolis (2500g) was extracted with 4 Litres of 95% ethanol using cold maceration method for 7days. The extract was filtered using Whatman No. 1 filter paper and concentrated in vacuo to yield a brown semi solid residue (350g) referred to as honeybee propolis ethanol extract (CR). The ethanol extract (300g) was suspended in distilled water and partitioned successively with n-hexane, chloroform, ethyl acetate and n-butanol to obtain n-hexane fraction 18.5g (HH), chloroform fraction 4.6g (CC), ethyl acetate fraction 7.1g (EE), n-butanol fraction 27.8g (BB), and the residual aqueous fraction 230.7g (AF) respectively.

Chemical screening of Propolis extracts

Chemical screening was carried out on the ethanol extracts and soluble sub-fraction to detect the presence of secondary metabolites such as flavonoids, alkaloids, steroids and triterpenes according to standard procedures[7].

Qualitative antioxidant activity

The ethanol extracts and soluble fractions (100µg/mL) was spotted on a TLC plate and developed using the mobile phase in a chromatographic tank. Developed chromatogram plate was sprayed with DPPH (0.15 % w/v) in methanol solution using an atomizer. The colour change (yellowish colour development on pinkish background on the TLC plate) is an indicator for the presence of antioxidant substances [8].

Estimation of free radical scavenging activity using DPPH method

The free radical scavenging activity (antioxidant capacity) of the propolis extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) were evaluated according to the reported method[9]. In this assay, a volume of 1.5ml of methanol solution of the extracts at different concentrations was mixed with 0.5ml of the methanol solution of DPPH (0.1mM). An equal amount of methanol and DPPH without sample served as a control. After 30mins of reaction at room temperature in the dark, the absorbance was measure at 518nm against methanol as a blank using a UV-Visible spectrophotometer (UVmini-1240). The percentage free radical scavenging activity was calculated according to the following equation:

% scavenging activity = [(Ac-As) / Ac] x 100

Where Ac = absorbance of control and As = absorbance of sample.

Antimicrobial Assay

Test organism

The test organisms were *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtillis*, *Candida krusei and Candida tropicalis*were all obtained from the Medical Microbiology Department, Ahmadu Bello University Teaching Hospital Zaria, Nigeria (ABUTH). All isolates were maintain on nutrient agar slants and check for purity.

Inoculums preparation of test organisms

The turbidity organisms suspension were made in sterile distilled water and compared with the McFarland turbidity standard, until the opacity matched with the scale number 0.5 standard by visual comparism, which corresponded to 1.5 x 108 cfu/mL [10]

Antimicrobial Susceptibility Test of Propolis Extracts

The propolis ethanol extract (CR) and soluble sub fraction; hexane (HH), chloroform (CC), ethyl acetate (EE) and n-butanol (BB) were each screened for antimicrobial activity against clinical isolates from(ABUTH) using Agar diffusion method [11]. The inoculum were prepared by inoculating the test organism in mullerhinton broth and incubating for 24 hours at 37^0C for bacteria and while fungi, saboraud dextrose broth was used and incubated for 48 hours at 25^0C. After incubation, the broth cultures were diluted to 1:1000 for Gram-positive bacteria and 1:5000 for the Gram- negative bacteria. Each extracts 0.1g were weighted and dissolved in 10ml of DMSO to obtain a concentration of 10mg/mL. Muller Hinton agar was used as the growth medium and was prepared according to

manufacturer's instruction, sterilized at 121^{0}C for 15mins, 20ml of the sterile medium was poured into a sterilized petri dishes allowed to cool and solidify. The sterile medium was seeded with 0.1ml of the standard inoculum of the test microorganisms; the inoculum was spread evenly over the surface of the medium with a sterile swab. The seeded plates were allowed to dry in an incubator at 37^{0}C for 30 mins. A standard cork borer of 6mm in diameter was use to cut cups (well) at the centre of each inoculated medium and 0.1ml of both extracts solution were introduced separately into each well on the medium, the plates were incubated at 37^{0}C for 24 hours for bacteria and 25^{0}C for 48 hours for fungi after which the antimicrobial activities were expressed as the diameter to the nearest millimetres of zone of inhibition by the extracts. Filter paper disc containing solvent of extraction without any extract served as a negative control. Standard antibiotic (5μg/mL) of (Sparfloxacin, Ciprofloxacin and Fluconazole) were use as positive control.

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined on the organisms that were sensitive to the extracts, and was carried out using broth dilution method [11-12]. Two fold serial dilutions of variable concentrations of the extracts (10-0.625mg/mL) were prepared. The organisms (0.1 mL) were inoculated into each tube containing the extracts. The tubes were incubated at 37ºC for 24 hours for bacteria and 48hours at 25ºC for fungi. The lowest concentration of the extract showing no visible growth (turbidity) of the test organism was considered to be the MIC.

Minimum bactericidal and fungicidal concentration (MBC/MFC)

The contents of the MIC tubes in the serial dilution were sub cultured onto appropriately labelled Mueller Hinton andSaboraud dextrose agar plates, and incubated at 37ºC for 24 hoursand 25^{0}C for 48 hours for bacteria and fungi respectively, then they were observed for colony growth. The lowest concentration of the sub culture with no growth was considered as the MBC and MFC [12-13].

Statistical analysis

Triplicate of all experiments were conductedand results were given as the mean ± standard deviation. The data in all the experiments were analysed (Microsoft Excel 2010) for statistical significance using Students t-test and differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The result of the preliminary chemical screening of the ethanol extract of honey bee propolis and soluble sub-fractions are as shown in Table 1.0. Flavonoids, saponins, carbohydrate, steroids and triterpenes were found in the ethanol extract (CR), whilephenolic nucleus, steroid and triterpenes were present in n-hexane soluble fraction (HH). The chloroform fraction (CC) revealed the presence of phenolic nucleus, steroids and triterpenes, flavonoids, the ethyl acetate fraction (EE) revealed the presence of phenolic nucleus, flavonoids and while phenolic nucleus, steroids and triterpenes, flavonoids, carbohydrate and saponins were found in the n-butanol fraction (BB).The usefulness of these metabolites in phytomedicines or treatment of ailments has been documented. Saponins are used for gastro-intestinal infections; Flavonoids are free radical scavengers and therefore useful in management of inflammatory diseases; tumour and oxidative stress- related diseases[14][15][16]. Steroids and triterpenes have analgesic, anti-inflammatory, anti-malaria, anti-microbial and anticancer activities, some compounds with phenolic nucleus have antiseptic and antioxidant property[16][14][15]. Therefore, the presence of these metabolites in the propolis supports their uses in the treatment of ailments traditionally [2, 4].

Table 1.0: Result of preliminary chemical screening of CR, HH, CC, EE and BB

Chemical Constituent	CR	HH	CC	EE	BB
Steriods/ Triterpenes	+	+	+	-	+
Flavonoids	+	-	+	+	+
Phenolic nucleus	+	+	+	+	+
Saponins	+	-	-	-	+
Anthraquinone	-	-	-	-	-
Alkaloids	-	-	-	-	-
Carbohydrate	+	-	-	-	+

Key: *= + present, - absent*

The qualitative antioxidant test using hexane: ethyl acetate (9:1) as solvent system for (Plate 1, 2) and hexane: ethyl acetate: methanol (4:2:1) for (Plate 3, 4) revealed that the ethanol extract (CR) and all soluble fractions (HH, CC, EE and BB) possess antioxidant activity with ethyl acetate showing more of distinct yellow spots of compounds with antioxidant property than the other fractions.

Profile of extracts after spraying with 10% aqueous H_2S0_4

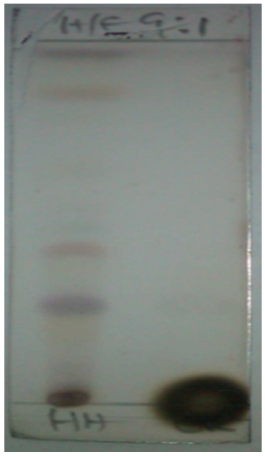

Plate 1

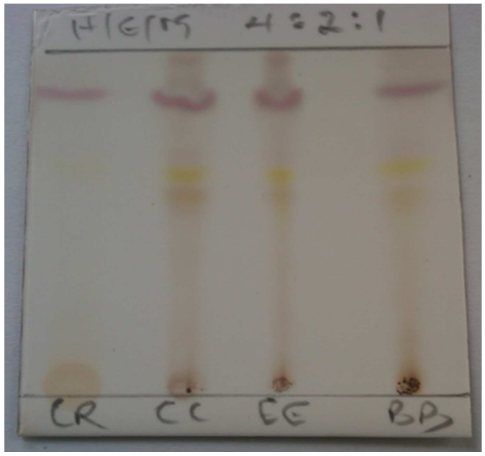

Plate 3

Profile of extracts after spraying with 0.15% DPPH in methanol

Plate 2

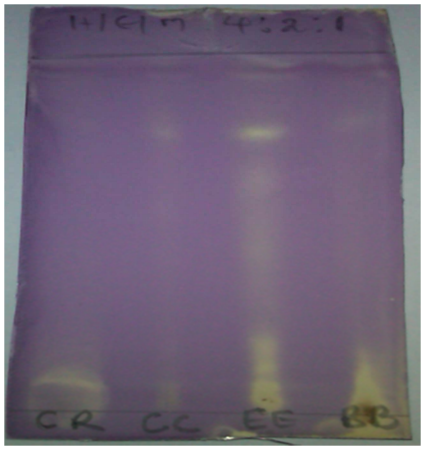

Plate 4

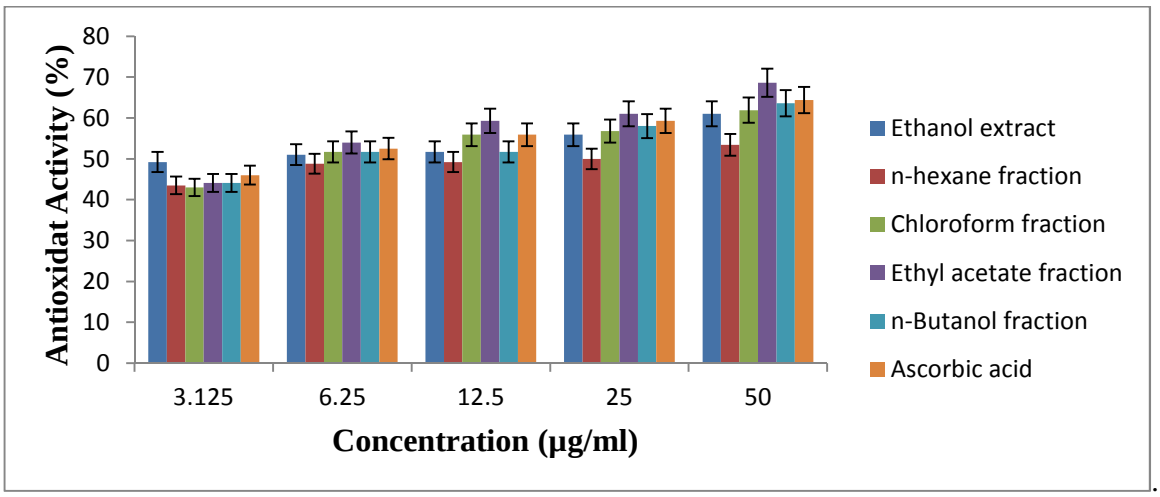

Figure 1: DPPH Free radical scavenging activity of Honeybee Propolis extract and its partition fractions presented as the mean value ± standard deviation SD (n=3)

This is further reaffirmed by an*in vitro* DPPH free radical scavenging activity of the ethanol extract and its sub fraction in (Figure 1) showing a persistent increase in antioxidant activity with increase in concentration of extract, 50µg/ml of each extract showed a higher antioxidant activity than the lowest concentrations. It also revealed that all fractions possess a free radical scavenging activity with ethyl acetate fraction (EE) having significant antioxidant activity of with IC_{50} value of 1.78 ± 0.01µg/ml compared to other extracts and standard ascorbic acid of 2.54 ± 0.01µg /ml at $p \leq 0.05$. The order of decreasing antioxidant activity of the extracts was EE>CR>CC>BB>HH. This may be due to high polyphenolic compounds in ethyl acetate fraction [9, 17]. In addition, the low antioxidant

activity of the ethanol crude extract (CR) may be associated with complex functional groups interactions in the ethanol crude extract use for the studies. This method which is simple, rapid, sensitive and reproducible shows that propolis extracts are apparently good free radical scavengers and can inhibit autoxidation of lipids and thus be beneficial in the treatment of disease in which lipid peroxidation is there mechanism of pathogenesis[9]. Therefore, as free radical scavenger they may act by terminating the peroxide chain reaction process either reacting directly or converting free radicals to less reactive species[18].

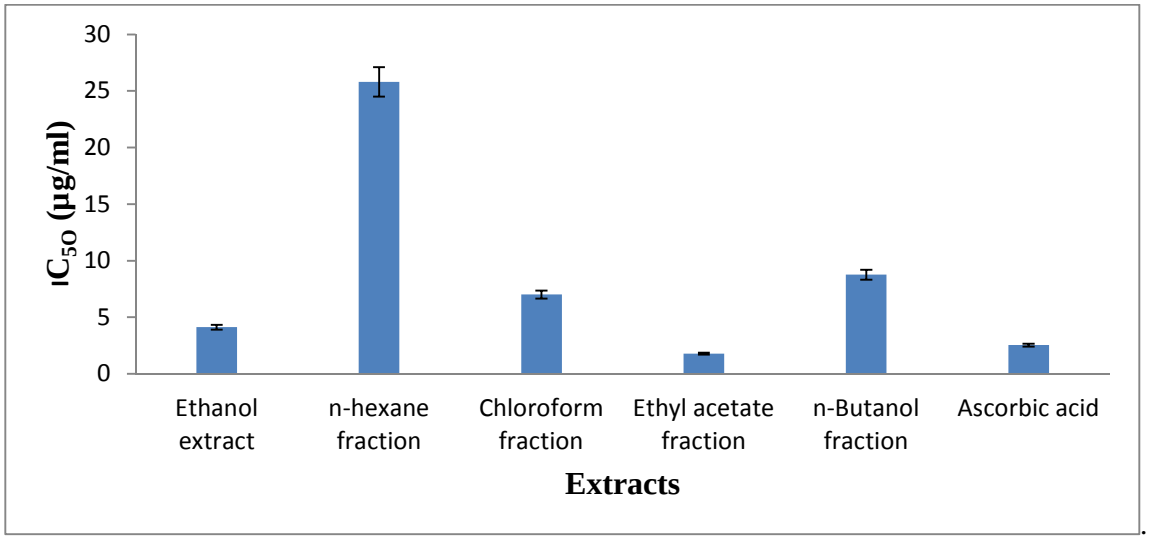

Figure 2: Graphical presentation of IC_{50} of various extracts and standard ascorbic acid as mean value ± SEM (n=3) using student, t-test: statistical significant at p≤0.05

The antimicrobial susceptibility tests results were expressed in terms of MIC,MFC, MBC, and diameter of zones of inhibition of the test organism. The attained results are shown in Tables 2 - 3. The results from the sensitivity test showed that the extracts had remarkable activity against the tested microorganisms with inhibition zones ranging 20mm to 27mm. The ethanol extracts was active against *S. aureus, B. subtilis, P. aeruginosa, C. krusei* and *C. tropicalis*, while hexane fraction (HH) showed activity only on *B. subtilis* and *C. krusei*. The chloroform fraction (CC), ethyl acetate fraction (EE) and n-butanol fraction (BB) showed similar activity on susceptible organism to the ethanol crude extract with exception of *P. aeruginosa* which was not susceptible to only the butanol fraction (BB).The MIC values ranged from 1.25 mg/mL to 2.5 mg/mL while the MBC/MFC values ranged from 5mg/mL to 10mg/mL for all the extracts against the tested microorganisms.

Table 2: Zones of Inhibition of Extracts and Standard Drug against Test Organism Zones of Inhibition (mm)

Test organism	CR	HH	CC	EE	BB	Sparflox	Cipro	Flucoz
S. aureus	24	0.0	21	22	20	37	32	-
B. subtilis	27	20	22	21	21	42	37	-
P. aeruginosa	24	0.0	26	22	0.0	30	-	-
C. krusei	26	20	24	22	21	-	-	40
C. tropicalis	22	0.0	23	20	20	-	-	35

Key: CR – crude ethanol extract HH- n-hexane fraction CC- chloroform fraction EE- ethyl acetate fraction BB- n-butanol fraction Sparflox - sparfloxacin Cipro- ciprofloxacin Flucoz- fluconazole

Furthermore, the zones of inhibitions for all the propolis extracts observed were lower than the standard drugs; Sparfloxacin, Ciprofloxacin and Fluconazole which could be liken to the complexity and inter molecular interaction of secondary metabolites that constitutes the extracts. However, the MIC value of the all the propolis extract suggest an appreciable antimicrobial activity, even though is higher than the recommended value of less than 100µg/mL for compounds [19].

In addition, the CC and EE soluble sub-fraction revealed a broad spectrum of both antimicrobial and antifungal activity against tested organism (Table 2). Although, proposed mechanism of activity of all extract would be difficult to deduce. Hence, many antibacterial agents may exhibit their action through inhibition of nucleic acid,

protein and membrane phospholipids biosynthesis[20]. However, illness such as; wound infections, pneumonia and sepsis caused by pathogenic *S. aureus* and *B. subtilis*[21], as well as candida infection cause by *C. tropicalis*[22] shows susceptibilty to these extracts, indicating their usefulness in the treatment of such diseases. Therefore, propolis extract would serve as a potential source for isolation of bioactive compounds that could be use as immune booster (Antioxidant) andin the management of diseases.

Table 3: MIC and MBC/MFC of Extracts (mg/mL)

	MIC (mg/mL)					MBC/MFC (mg/mL)				
Test organism	**CR**	**HH**	**CC**	**EE**	**BB**	**CR**	**HH**	**CC**	**EE**	**BB**
S. aureus	2.5	-	2.5	2.5	2.5	5.0	-	10.0	5.0	10.0
B. subtilis	1.25	2.5	2.5	2.5	2.5	5.0	10.0	5.0	10.0	5.0
P. aeruginosa	2.5	-	1.25	-	5.0	-	5.0	5.0	-	
C. krusei	1.25	2.5	2.5	2.5	2.5	5.0	10.0	5.0	5.0	10.0
C. tropicalis	2.5	-	2.5	2.5	2.5	10.0	-	5.0	10.0	10.0

CONCLUSION

The studies revealed that information from the chemical screening, antioxidant studies and antimicrobial studies of the Propolis extracts show that the use of extracts in the treatment of infectious diseases and as free radical scavengers is justified, and the ethylacetate and chloroform soluble sub-fractions could be investigated further for the development of antioxidant and antibacterial agents. Further work will be carried out to isolate and elucidate the structures of the most active compounds present in the propolis extracts.

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