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Chemical and Biological Profiling of the N-Hexane Extract of Crude Honeybee Residue

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ABSTRACT

The chemical and biological profiling of the n-hexane extract, TM of crude honeybee residue, CHR has been investigated. TM was obtained from crude honeybee by selective separation technique, soxhlet's extraction, concentrated by simple distillation and dried under vacuum. The extract was profiled for its chemical characteristics: thin layer chromatography, TLC, Fourier Transform- Infra Red, FT-IR characterization, antioxidant and phytochemical screenings and biologically for its anti-infective effectiveness, using standard procedures. TM had a yield of 5.76%, multiple spots observed from its TLC when developed from two different solvent systems (EtOAc:n-hexane, 0.5/6 and 100% n-hexane); -O-H, -C=O, -C-Hsp^3 and –C-O functional group units were obtained from the FT-IR analysis at the wave numbers (cm^{-1}): 3516, 1736, 2918-2849 and 1173 respectively. It has shown a relative antioxidant activity when compared to those of the dark brown viscous crude honeybee and standards (natural and synthetic). The phytochemicals present include: steroids (terpenoids), quinones, saponins and alkaloids. TM showed relative antibacterial activities against strains of *Staphylococcus aureues*, *Eschericia coli*, *Bacillus subtillis*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Klebsiella pneumonae*, in a dose concentration gradient when compared to the positive control: gentamycin. The highest and lowest activities were observed at 100 and 12.5 mg/mL of TM against the test organisms respectively. The study has established a preliminary investigation on the n-hexane extract of crude honeybee residue.

***Keywords*:** Crude honeybee, crude honey residue, n-hexane extract, chemical and biological profiles

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1. INTRODUCTION

The global drug industry is faced with the menace of drug resistance, due to pathogenic microbes' abilities to manipulate substances that were once suicidal or inhibitory to their collective bio-economic significance. This trend of challenges has being a serious concern to research and development, R&D. Community disease transmission in the twenty first century is a global challenge. There is need to urgently source alternative therapeutic treatment. More potent therapeutic substances are needed to combat the global public health challenge and nature could just the way forward.

Nature in its kindness has offers enormous reservoirs for better health sustainability. These reservoirs have proven inexhaustible, reliable and alternative sources of drug discovery. One of such depots nature has offered is the crude honeybee composition, with limited scholarly studies especially in the Northern part of Nigeria. Crude honeybee compositions differ from one location to another. This varying disparity is due to some associated ecological factors with the locality of interest [1, 2].

Locally, it has been reported that crude honeybee since ancient civilization, has some medicinal properties which include: treatment of wound [3], gastrointestinal [4], cardiovascular and liver related ailments [5, 6], maintaining a healthy gums and teeth and as a topical ointment [3, 6]. Pharmacologically, crude honeybee has been reported to exhibits the following activities: antioxidant [6, 9, 11, 12, 17], antimicrobial [6, 13-17], anti-inflammatory [6, 18, 19], antineoplastic [6, 20], anti-leishmanial [6, 21] and as food preservatives [10].

Secondary metabolites such as steroids (terpenoids), flavanoids, phenolics, coumaric acids and essential oils have been reported to be present in crude or wild honeybee [7-12]. These set of organic compounds in therapeutics have been of profound benefits to public health. The presence of these organic compounds is an established justification for the numerous ethno-medicinal and pharmacological claims associated with crude honeybee discuss.

The study is designed to evaluate the chemical and biological profiling of the n-hexane extract of a crude honeybee residue.

2. EXPERIMENTAL

2. 1. Materials

The materials employed in the course of this study were obtained in line with established standard scientific procedures. Crude honey was collected from Kashimbila, in Takum Local Government Area of Taraba State, Northern Nigeria.

Chemicals and reagents used to carry out the experimental of this work were of analytical grade (products of BDH and Sigma-Aldrich).

Some of the equipment/apparatus used, include: Fourier Transform Infra Red (FT-IR) spectrophotometer (spectro UVD-2960), HH-S water bath, High vacuum pump, thin layer pre-coated F_{254} chromatography plate, filter papers (grade: no.1 Whatmann), simple distillation set-up, soxhlet's apparatus, vaccum dessicator and other glasswares.

2. 2. Methods

Standard methods as reported in literatures were employed for the purpose of this study.

2. 3. Separation technique

Crude honey was subjected to selective separation technique using pre-treated sieves and muslin sheets of varying mesh sizes. This was done to obtain crude honeybee residue, CHR for extraction and a refined crude viscous liquid honey.

2. 4. Extraction

Hot method (soxhlet) of extraction at the boiling point range of n-hexane was used to obtain n-hexane extract, TM from 100 g of CHR, concentrated by simple distillation, retained in an open vial, dried and stored under vacuum to a constant weight. The percentage yield was calculated using the relationship:

$$Percentage\ yield = \frac{mass\ of\ dried\ TM}{mass\ 0f\ CHR} \times 100\% \qquad (1)$$

where: TM- n-hexane extract, CHR- crude honeybee residue

2. 5. Chemical Profiling

Thin layer chromatography, fourier transform infra red, antioxidant and phytochemical were investigated to evaluate the chemical profiling of the hexane fraction, TM of the residue of crude honey, using standard protocols.

2. 5. 1. Thin Layer Chromatography, TLC Profiling

0.1 ml of TM was dissolved in a suitable solvent system, spotted unto TLC pre-coated plates and then eluted using different solvent systems (100% N-hexane and EtOAc : n-hexane, 0.5:6). The developed TLC plates were dried and viewed under a UV lamp.

2. 5. 2. Fourier Transform Infra Red (FT-IR) Profiling

Infra red spectrum profiling was carried out on TM with the aid of a Fourier Transform Infra Red (FT-IR) spectrophotometer (spectro UVD-2960) at the Department of Chemistry, University of Ibadan, Nigeria. 1.0 mg of TM was pelletized with 100 mg of KBr and the IR spectrum was recorded between 4000-400 cm^{-1}.

2. 5. 3. Antioxidant Profiling

The antioxidant profiling of both CHR and TM were carried out using the 2, 2-diphenyl-1-picrylhydrazyl, DPPH method [22, 23] with absorbance recorded at 517 nm and both vitamin C (ascorbic acid) and butylated hydroxylanisole, BHA were used as natural and synthetic standards respectively, in a triplicate concentration dose range. The percentage inhibition, %I was calculated using the relationship:

$$\%I = \frac{A_{BLANK} - A_{TM}}{A_{BLANK}} \qquad (2)$$

where: %I – percentage inhibition, A_{BLANK} – Blank absorbance, A_{TM} – TM absorbance

2. 5. 4. Phytochemical Profiling

Standard analytical techniques as reported by scholars were used in the qualitative profiling of TM [22, 26]. The phytoconstitutions investigated include: steroids, diterpenes, flavonoids, quinones, saponins, tannins, alkaloids, cardiac glycosides, phenols, coumarins and anthocyanins using the procedures outlined in Table 1. The phytochemical profiling was carried out in the Department of Pharmaceutical Chemistry, University of Ibadan, Nigeria.

Table 1. Procedures for the phytochemical profiling of TM.

Phytochemicals	Test
Steriods (Salkowski test)	2 mL TM + 2 mL $CHCl_3$ + 2 mL conc. H_2SO_4
Diterpenes (Copper acetate test)	2 mL TM + drops of copper acetate
Flavanoids (NaOH test)	2 mL TM + 1 mL 2M NaOH
Quinones (Sulphuric acid test)	1 mL TM + 1 mL conc. H_2SO_4
Saponins (Froth's test)	5 mL aqueous TM + boiling for few minutes
Tanins (Braymer's test)	2 mL TM + 10% alcoholic ferric chloride
Alkaloids (Hager's test)	2 mL TM + few drops of Hager's reagent
Cardiac glycosides (Legal's test)	TM + 2 ml of Glacial acetic acid + drops of $FeCl_3$
Phenols (Ferric chloride test)	TM + 4 drops of ferric chloride solution
Coumarins (Reaction with 10% NaOH)	2 mL TM + 3 mL 10% NaOH
Anthocyanins (Reaction with acid and ammonia)	2 mL + 2mL 2M HCl + NH_3 solution

*TM- N-hexane extract

2. 6. Biological Profiling

2. 6. 1. Anti-infective Profiling

Antibacterial analysis was carried out to evaluate the biological activity profiling of the n-hexane fraction, TM against the following microbes: *Staphylococcus aureus, Escherichia coli, Bacillus subtillis, Pseudomonas aeruginose, Salmonellae typhi* and *Klebsiella pnuemonae*. The pour method was employed in the antibacterial profiling as reported in literatures [23, 27].

2. 6. 1. 1. Preparation of Gradient Concentration of TM

1.0 g of TM was weighed and dissolved into 10 mL of an appropriate solvent, from which 0.5 mL was serially transferred and made up with solvent for gradient concentration of the

extract, TM. The negative control was dimethyl sulfoxide while the positive control was gentamycine dissolved in the solvent.

2. 6. 1. 2. Pour Plate Method

Inoculation of each bacterium was done on a sterile nutrient broth and incubation lasted for 24 hrs at 37 °C. 0.1 mL solution of each of the test bacterium (*Staphylococcus aureus, Escherichia coli, Bacillus subtillis, Pseudomonas aeruginose, Salmonellae typhi* and *Klebsiella pnuemonae*) was prepared by transferring 0.1 mL of cultured bacterium into 9.9 mL of disinfected distilled water so as to obtain 1:100 bacterium solutions. 0.2 mL bacterium solution was transferred into a prepared sterile agar nutrient with temperature maintained above room temperature aspectically poured into sterile petri dishes, which on cooling solidified. Wells were bored with the aid of an 8 mm diameter disinfected cork borer on the solidified test organism and then exposed separately to the test sample, TM of different concentration gradient and that of the positive and negative controls. This was done in triplicate, allowed to stay on the bench for 2 hrs after which the plates were incubated uprightly for 24 hrs at 37 °C. Feasible inhibition zones were measured, average taken and reported in table 4 [23, 27].

3. RESULTS AND DISCUSSION

3. 1. Separation technique

The physical composition of the crude honey solid residue, CHR obtained had an obvious mixture of death bees, pollen and waxy substances amongst other constituents. The viscous liquid component was dark brown in coloration.

3. 2. Extraction

5.76 g of n-hexane fraction, TM was obtained from 100 g of crude honey residue, CHR with a percentage yield of 5.76%.

3. 3. Chemical Profiling

3. 3. 1. Thin Layer Chromatography, TLC Profiling

The TLC profiling result (Table 2) suggests the presence of secondary recipes from the number of spots observed from viewing the TLC plates under the UV lamp. Varying the gradient of the developing solvent systems (mobile phases) from non polar to that of moderate polarity witnessed an additional spot. This implies the n-hexane fraction is a mixture of phytochemicals that are worth investigating.

Table 2. TLC profiling result of TM.

Solvent system, mL	No. of spots
EtOAc : n-C_6H_{14} , 0.5/6	4
100% n-C_6H_{14}	3

*EtOAc – ethyl acetate

3. 3. 2. Fourier Transform Infra Red (FT-IR) Profiling

The result of the FT-IR profiling of TM (Table 3) shows the presence of functional groups whose chemistry is pivotal to the synergistic structural relationship activities of the active phytochemicals making up the matrix, TM under study. A broad band around 3516 cm^{-1} is typical of an alcohol stretch while the sharp peak observes around 1736 cm^{-1} depicts a carbonyl stretch. These two moieties: alcohol, ~3516 and carbonyl, ~1736, are very vital to the chemistry of TM. They constitute the more important organic functional groups present in the n-hexane extract of the crude honeybee under profiling. The electron density of the polar –OH and the dipolar –C=O is unique to the nature of the candidates present in TM. The other functional signatures present include: an aliphatic saturated –CH stretch and a –C-O- unit. The FT-IR profiling of TM has shown a system whose functional group composition, is relevance to the combine (synergistic) effect of the active metabolites.

Table 3. Result of FT-IR profiling of TM

Wave number, cm^{-1}	3516 (broad)	2918 – 2849	1736	1173
Suspected functional group	Alcohol, -OH	Sp^3 –CH moiety	Carbonyl, -C=O	-C- O- unit

3.3.3. Antioxidant Profiling

The result of the antioxidant profiling (fig.1.) of TM shows a relatively low free radical scavenging potentials as compared to the crude honey residue and those of the standards (vitamin C and butylated hydroxylanisole). Free radical scavengers (antioxidants) are substances that inhibit oxidative processes either as radical scavengers or by modifying free radicals to less reactive species [28].

The antioxidant characteristics of TM could be attributed to the hydroxyl and carbonyl functional groups present in the extract under consideration. Active metabolites with characteristic functional moieties such as the hydroxyls and carbonyls are known to exhibit antioxidant properties. This activity can be attributed to the classes of organic compounds screened and obtained from the phytochemical profiling as reported in table 4. The nature of compounds expected to be present in the n-hexane extract are said to be non-polar metabolites. They are usually transferred from plant tissues or any other materials of interest into a non-polar extracting solvent.

The more active candidates are expected to be present in the more polar fractions since the percentage inhibition of the crude honey residue is higher than those of the non-polar n-hexane extract. Several authors [6, 9, 11, 12, 17] have reported the antioxidant potency of crude honeybee.

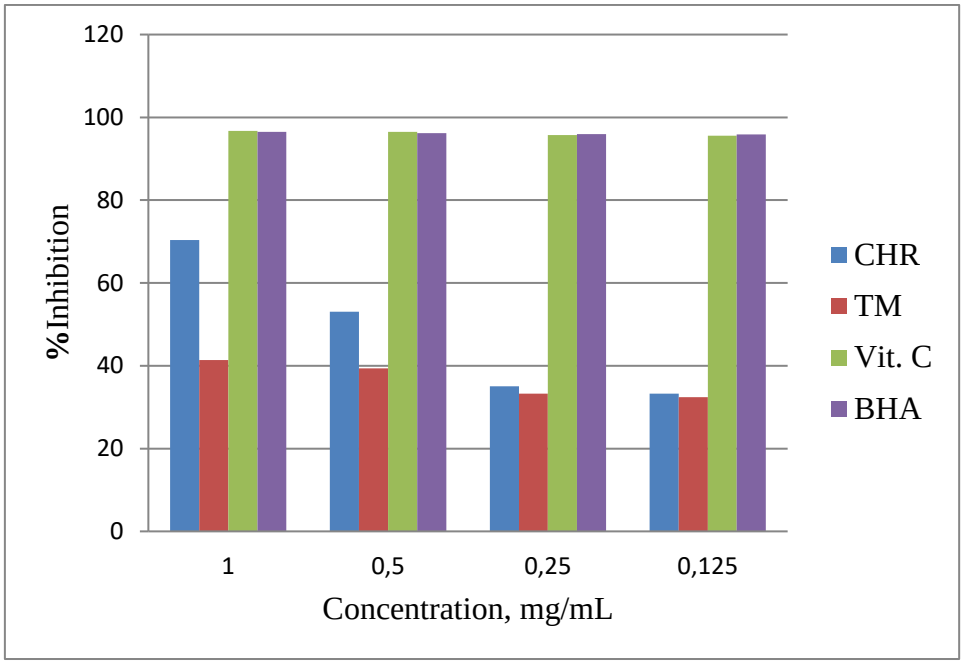

Figure 1. Antioxidant profiling of CHR, TM, Vit. C and BHA. Absorbance measurement of DPPH is 0.952 at 517 nm. *CHR- crude honey residue, TM- n-hexane fraction, Vit. C- Vitamin C, BHA- butylated hydroxylanisole.

Antioxidants have been reported to mediate in the disproportion roles associated with reactive oxygen species, ROS. Examples of ROS include: peroxides, superoxides, hydroxyl radicals and singlet oxygen [47, 48]. They are often products of the redox potential of molecular oxygen with the capacity of initiating and propagating free radicals in biochemical systems, as illustrated in Figure 2.

$O_2 + e^-$ (oxygen molecule) $\xrightarrow{[H]}$ $\cdot O_2^-$ (superoxide radical) 3

$2H^+ + 2\cdot O_2^-$ (proton) $\xrightarrow{[C]}$ $H_2O_2 + O_2$ (hydrogen peroxide) 4

$H_2O_2 + e^- \xrightarrow{[H]} 2\cdot HO^-$ (hydroxyl radical) 5

$.[A]. \xrightarrow{[O]} [A]^{2+} + 2e^-$ (antioxidant) 6

$2H^+ + 2e^- + H_2O_2 \xrightarrow{[H]} 2H_2O$ neutral water molecule 7

[H] - Reduction [O] - Oxidation [C] - Chemical bonding

Figure 2. Equations showing generation of ROS and mode of free radical scavenging.

The resultant outcome of imbalance caused by excessive or moderate concentrations of ROS is referred to as oxidative stress [32]. Several complaints are being linked with oxidative stress. The more recent been the cardiac complication associated with COVID-19 [33]. Others include: cardiac arrest [34], myocardial infarction [32, 35], sickle cell disease [36], cancers [31], Lafora disease [37], Alzheimer's disease [38], Parkinson's disease [39], atherosclerosis [40], vitiligo [41], fragile X syndrome [42], autism [43], depression [44], lichen planus [45] etc. The use of antioxidants in the prevention of some these diseases is still a subject of controversy [46].

3. 3. 4. Phytochemical Profiling

The result of the phytochemical profiling (table 4.) shows the presence of some classes of natural products that are vital to essence of life. The classes of secondary principles present include: steroids, terpenoids, quinones, saponins and alkaloids. These classes of phytochemicals are enough justification for the various ethno-medicinal and scientific claims in literatures.

They are also significant in the biochemical search for new drug discovery. The steroids are special degraded class of triterpenoids often to possess a cyclopentanoperhydrophenanthrene, gonane or sterane nucleus. Some steroids are known sex hormones, e.g. progesterone and testosterone, responsible for both female and male sexual characteristics [29, 30].

Other classes of phytochemicals: quinones, saponins and alkaloids, reportedly present in the extract under examination, are very vital to the course of nature and have been widely reported in scholarly articles. Flavanoids, phenolics, coumaric acids and essential oils have also been reported to be present in crude honeybee [7-13].

These natural products are usually not evenly distributed due to ecological factors [1, 2].The presence of these natural products within the honeybee web should be of interest to researchers with the aim of establishing new candidates with therapeutic potency.

Table 4. The phytochemical profiling of TM

Phytochemicals	Observation
Steriods	+
Diterpenes	-
Terpenoids	+
Flavanoids	-
Quinones	+
Saponins	+
Tannins	-
Alkaloids	+

Cardiac glycosides	-
Phenols	-
Coumarins	-
Anthocyanins	-

*****+: present, - : absent

Table 5. Anti-infective profiling.

Concentration Mg/mL	**Organisms/average inhibition zones**					
	Sa	Ec	Bs	Ps	St	Kp
100	16	14	16	14	14	12
50	14	12	14	12	12	10
25	12	10	12	10	10	-
12.5	10	-	10	-	-	-
6.25	-	-	-	-	-	-
-ve control	-	-	-	-	-	-
+ve control	38	38	38	38	38	38

*Sa- *Staphylococcus aureues*, Ec- *Eschericia coli*, Bs- *Bacillus subtillis*, Ps- *Pseudomonas aeruginosa*, St- *Salmonella typhi*, Kp- *Klebsiella pneumonae*. The negative, - control is dimethyl sulfoxide (DMSO) and the positive control is gentamicin (10 µg/mL) bacterium.

3. 4. Biological Profiling

3. 4. 1. Anti-infective Profiling

The result of the anti-infective profiling (table 5.) clearly reveals the antibacterial efficacies of the n-hexane extract against strains of the following bacteria: *Staphylococcus aureues*, *Eschericia coli*, *Bacillus subtillis*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Klebsiella pneumonae*. The highest activity was observed at 100 mg/mL for all the microbes under study and reduces as the concentration of the extract reduces. Below 12.5 mg/mL no activity was observed for any of the organisms.

This result is obviously in agreement with the antioxidant characteristics and phytochemical constitution of TM. The bacteriostatic potentials of TM on both the gram negative and positive bacteria could be mainly attributed to the synergistic effect of the phytoconstitution amongst other factors as reported in literature [15].

4. CONCLUSIONS

The chemical profiling of n-hexane extract of crude honey obtained from Kashimbila locality in Takum Local Government Area of Taraba State, Northern Nigeria has revealed the presence of more than one compound from its thin layer chromatography. The hydroxyl and carbonyl are the major functional moieties present in the matrix as obtained from the FT-IR profiling. The n-heane extract has antioxidant activity and the phytoconstitution detected includes: steroids (terpenoids), saponins, quinones and alkaloids.The biological profiling shows anti-infective activity against *Staphylococcus aureus, Escherichia coli, Bacillus subtillis, Pseudomonas aeruginose, Salmonellae typhi* and *Klebsiella pnuemonae* at 100 mg/mL of n-hexane extract and reduces as the concentration reduces. The n-hexane extract of the crude honey residue with the reported chemical and biological profiling could be explored for further work on toxicology and as well as an established phytochemistry survey.

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