Solvents Fractionation, Characterization and Evaluation of Antimicrobial Activities of Beeswax from *Apismelifera*

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Abstract: **- The variations in the compositions and properties of the unfractionated beeswax and its fractions were investigated with a view to widening the scope of applications of beeswax. Beeswax from *Apismeelifera* was fractionated using solvents and Methanol, n-hexane, dimethylsulphoxide and diethyl ether were employed. The chemical parameters, antibacterial and antifungi activities as well as FTIR analyses of the beeswax and its fractions were carried out. The chemical parameters of the unfractionated beeswax were within the international standards ranges but most for the fractions were outside the ranges. The n-hexane and diethyl ether fractions were found to be richer in esters and hydrocarbon; while the methanol and dimethylsulphoxide fractions were richer in unsaturated free fatty acids and fatty alcohols. The methanol and dimethylsulphoxide fractions showed stronger antimicrobial activities than unfractionated wax, with the dimethylsulphoxide fraction giving the best inhibitory effects. The results showed that fractionating beeswax into its simpler constituents would add values to this underutilised material.**

Keywords: **Beeswax, honeycomb, fractionation, FTIR, chemical parameters, antimicrobial activity**

I. INTRODUCTION

Beeswax (white or yellow) is a product made from the honeycombs of the bees. It is a natural wax produced in wax glands located in the abdomen of worker bees and used in the construction of combs. A honeycomb is a mass of hexagonal wax cells built by honey bees in their nests to contain their larvae, stores of honey and pollen. Generally, beeswax consists of a complex mixture of saturated and unsaturated aliphatic hydrocarbons, mono-, di- and poly esters, hydroxy esters, free fatty acids, free fatty alcohols and minor of other compounds. Crude beeswax contains a large number of minor components (e.g. terpenoids and flavonoids), most of which appear to be plant-derived ([1], [2]). Reference [2] reported that there are more than 300 individual components in beeswax from various species of honeybees and more than 100 of them are volatile. The colour of the wax depends to some extent on the type of flora visited by the bees and volatile products have also been detected at low levels ([1], [3], [4]). Oxygenated compounds produced by the bees are also present, amongst which decanal, 1-decanol, nonanal, octanal, furfural, and benzaldehyde are responsible for the bouquet of the wax [3]. Many authors reported that the chemical composition of beeswax depends on its origin, age, and climatic conditions ([5] - [8]).

In the earliest time it was exploited for various purposes such as preservation of mummies, square wax writing tablets, bending agent, and for sealing and waterproofing. Although beeswax is now partly replaced by synthetic or fossil products, it plays an important role in a number of fields such as polymer technology, symbolic and artistic fields, preparation of cosmetics or medicinal commodities, food, pharmaceutical, and pesticides ([7], [9] –[12]).

Literature survey revealed that most published research works on beeswax and its applications had been centred on the whole (unfractionated) material and that its compositions vary from one region or locality to another. Fractionation of beeswax from different regions into its simpler constituent components and characterisation of the fractions may further extend the applications of beeswax and make it value added material.

II. MATERIALS AND METHODS

A. Materials and their Procurement

Raw beeswaxes (honeycombs from which honey has been extracted) samples from *Apismelifera* were collected from beekeepers in Ondo City, Nigeria. The chemicals used were obtained from certified manufacturers and suppliers through registered chemical suppliers in Nigeria. These include: n-hexane (GFS Chemicals), Diethyl ether (INDENTA Chemicals), Dimethylsulphoxide (KERMEL), Silica gel of 60-120mesh and Sodium hydroxide (OXFORD), Potassium hydrogen phthalate (AR China), Iodine chloride, Starch indicator, Acetone, Hydrochloric acid, Methanol, Ethanol, and Chloroform (British Drug House (BDH)), Muller Hinton Agar and Potato Dextrose Agar (Biotec), Nutrient Agar (Oxoid), Potassium Hydroxide (LOBA Chemie), Sodium Thiosulphate (Kermel).

B. Purification of Beeswax

The honeycombs were packed in a clean cotton cloth (felt) and dipped in hot water regulated at 65 – 70°C on a thermostatic hot plate. The beeswax melted out through the felt onto the hot water leaving the impurities behind. The floated hot beeswax was allowed to cool to room temperature and the solid was then removed from the water. The process was repeated until very pure beeswax was obtained.

C. Solvents Fractionation of Beeswax Samples

Beeswax sample of 24.0 g was dissolved in 400 mL of n-hexane. The resulting solution was mixed with 50 mL methanol in a separating funnel, shook vigorously for 15 minutes, allowed to partition and then collected separately into flasks. The n-hexane fraction was further extracted for four more times with 50 mL methanol per batch. Each of the fractions was then concentrated using a rotary evaporator RE 52A. In a similar manner, same mass of beeswax was fractionated using diethyl ether as solvent and dimethylsuphoxide as extractant.

D. Determination of Chemical Parameters

The chemical parameters were determined for both the whole and the fractionated samples. For the Saponification value (SV), accurately measured 25.0 mL of 0.5 M alcoholic KOH was added to 3.0 g of each sample and the mixture was refluxed for 120 minutes at 70°C. The solution was cooled to room temperature and titrated against 0.5 M HCl to phenolphthalein end point. Bank determination was also carried out. The saponification value was calculated using the following equation:

$$SV = \frac{(B - S)x\ M\ x\ 56.1}{Weight\ of\ sample} \qquad 1$$

B and *S* are the respective volume of titrant (mL) for the blank and sample titrations. M is the molarity of HCl and 56.1 is molecular weight of KOH.

For the Free Fatty Acid Value (FFAV), 0.25 g sample was dissolved in 20 mL chloroform and titrated against 0.5 M KOH to phenolphthalein indicator end point. The free fatty acid was calculated as follows:

$$FFAV = \frac{\text{titre value (mL)}\ x\ M\ x\ 56.1}{Weight\ of\ sample} \qquad 2$$

M is the molarity of KOH solution. The difference between the saponification value and the free fatty acid value gave the ester value.

For the iodine value (IV), 20 mL chloroform was added to 0.3 g of each sample followed by addition of 25 mL of 25% ICl solution. The mixture was left in a dark place for an hour and after that 25 mL of 25% KOH solution was added. The resulting solution was titrated against 0.1 M $Na_2S_2O_3$ solution till the colour changed to a pale yellow after which 2-3 mL of starch solution was added (as indicator) and the titration was continued till the blue colour just disappeared. Bank determination was also carried out. The iodine value was calculated by using the following equation:

$$IV = \frac{(B—S)\ x\ M\ x\ 126.9\ x\ 10^3}{Weight\ of\ sample} \qquad 3$$

B and *S* have same definitions in given equation 1. *M* is the molarity of $Na_2S_2O_3$ and 126.9 is the molecular weight of iodine.

For the peroxide value (PV), Chloroform of 20 mL and glacial acetic acid of 15 mL were added to 0.25 g of beeswax sample. Accurately measured 5 mL of 10% KI was added to the mixture and the resulting solution was left in dark cupboard for one hour. Titration against 0.1 M $Na_2S_2O_3$ solution was carried out till the colour changed to pale yellow and then 2 mL of starch indicator was added and the titration was continued till the disappearance of the blue colour. A similar procedure was repeated for the blank solution. The peroxide value was calculated using the following equation:

$$PV = \frac{(S — B)\ x\ M\ x\ 1000}{Weight\ of\ sample} \qquad 4$$

B and *S* have same definitions in given equation 1. M is the molarity of $Na_2S_2O_3$ solution.

The whole beeswax and its fractions were also analysed for compositions using SHIMADZU FT-IR-8400S.

E. Antimicrobial Screening of the Beeswax and its Fractions

The anti-microbial analysis was use to evaluate the bioactivity the whole beeswax and its fractions samples. The bacteria used were clinical isolates, obtained from General Hospital Akure, Nigeria and ObafemiAwolowo University Teaching Hospital, Ile-Ife, Nigeria; while the fungi used (*Beauveriabassiana*, *Cordycepsmilitais*and *Candida albicans*) were gotten from Brazil (soil) Pelotas, Rio Grande do Sul and South Africa, Mooreesburg, Western Cape Province. All the bacterial cultures were checked for purity and maintained on Mueller Hinton agar slant for bacteria and Potato dextrose agar for fungi.

Antimicrobial activity studies of the whole beeswax and its fractions (dimethylsulphoxide, Methanol, n-Hexane and diethyl ether fractions) were carried out using Agar diffusion techniques [13]. The inoculum was prepared by inoculating the test organism in mullerhinton broth and incubated for 24 hours at 37°C for bacteria; while for fungi, potato dextrose broth was used and incubated for 48 hours at 25°C. After incubation, the mirco-organisms were well grown.

Muller Hinton agar was used as the growth medium and was prepared according to manufacturer's instruction, sterilized at 121°C for 15 minutes. A volume of 20 mL 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-stick. The seeded plates were allowed to dry in an incubator at 37°C for 30 minutes. A standard cork-borer of 8mm in diameter was use to cut cups (well) at the centre of each inoculated medium and 0.1 mL of both extracts solution was introduced separately into each well on the medium, the plates were incubated at 37°C for 24 hours for bacteria and 25°C for 48 hours for fungi after which the plates were observed for zones of inhibitions of growth. The zones were measured and the result recorded to the nearest millimetres. Filter paper disc containing solvent of extraction served as a negative control, the antimicrobial activities were tested in duplicate and the mean zone of inhibitions was calculated for each.

III. RESULTS AND DISCUSSION

A. Characterisation

Table 1 presents the fractional percentages of beeswax in the solvents, while Table 2 displays the mean values of the chemical parameters of the beeswax samples and their fractions. Only about 10% were in the dimethylsulphoxide layer in the diethyl ether-dimethylsulphoxide fractionation, while about 20% were in the methanol layer in the n-hexane-methanol fractionation. The chemical parameters for the unfractionated beeswax were within the international standard ranges. The acid value (17.882 mg KOH/g) is at the lower end of the standard range; thus very low level of rancidity could result on storage. The beeswax samples and their fractions contained no peroxide value. This showed that both the whole and the fractions are stable to oxidation processes, during storage and transportation.

The dimethylsulphoxide fraction has the highest iodine and acid values. It is followed by methanol fraction; while n-hexane and diethyl ether fractions were high in ester and saponification values. These results show that most of the unsaturated and free fatty acid components of the wax were in these polar solvents layers, leaving most of the esters and saturated hydrocarbons in non-polar n-hexane and diethyl ethers layers. None of the fractions had ester to acid ratio within the international standard range for unfractionated beeswax (Table 1).

Table I. Percentage Fractional Yields

Fraction	M_F	nH_F	$DMSO_F$	DEE_F
Yield (%)	19.86	80.14	10.22	89.78

M_F = methanol fraction; nH_F = n-hexane fraction; DEE_F = diethyl ether fraction; $DMSO_F$ = dimethylsulphoxide fraction

Table II. The Mean Chemical Parameters of the Beeswax Samples and their Fractions

Parameter	BW_U	M_F	nH_F	DEE_F	$DMSO_F$	International Standard* (BW_U)
PV (meq/kg sample)	0.000	0.000	0.000	0.000	0.000	Not specified
IV (g I_2/100g sample)	8.621	10.018	7.598	7.942	10.694	Not specified
AV (mg KOH/g sample)	17.882	25.015	11.301	9.800	31.680	17 – 24
SV (mg KOH/g sample)	92.525	66.881	89.569	88.144	58.088	83 – 103
EV (mg KOH/g sample)	75.315	41.866	78.268	78.344	26.408	66 – 82
EAR	4.212	1.674	6.926	7.994	0.834	3.00 – 4.30

BW_U = unfractionated beeswax; M_F = methanol fraction; nH_F = n-hexane fraction; DEE_F = diethyl ether fraction; $DMSO_F$ = dimethylsulphoxide fraction; PV = peroxide value; IV = iodine value; AV = acid value; SV = saponification value; EV = ester value; EAR = ester to acid ratio

*Source: [6], [8], [14-[16]

Figure 1-5 show the SHIMADZU FT-IR spectra of the unfractionated beeswax and the fractions in the wavenumber range of 4000−500 cm^{-1}. That for whole beeswax (Figure 1) has highest number of detectable peaks, which confirms its complexity. All the samples contain absorption peaks at around 2918 to 2914 cm^{-1} (CH_2 asymmetric stretch), 2850 to 2848 cm^{-1} (CH_2 symmetric stretch), 1464 to 1414 cm^{-1} (CH_2 and CH_3 bending vibration), 1377 to 1315 cm^{-1} (CH_3 bending vibration), 1290 to 1026 cm^{-1} (C-O stretch of alcohol, ester, free fatty acid), 3300 to 3500 cm^{-1} (OH stretch of alcohol)and 719 to 705 cm^{-1} (long-chain (CH_2) band).

The spectrum for the DMSO fraction (Figure 5) is distinct while those for the unfractionated beeswax and for methanol, n-hexane and diethyl ether fractions are similar (Figure 1-4). The DMSO fraction has strong and broad peak at around 3450 cm^{-1} and strong band at 1026 cm^{-1}representing OH stretch and C-O stretch of alcohol and free fatty acid. Other characteristic features include medium but broad band at about 1645 cm^{-1} for C=O stretch (fatty acid) and C=C stretch (olefin), and a distinct peak at around 3001 cm^{-1} for olefinic C-H stretch which for others fractions appears as shoulder slightly below 3000 cm^{-1} (possibly due to conjugation). These showed that the DMSO fraction was rich in unsaturated fatty alcohols and fatty acids. These results corroborated the high iodine value (high degree of unsaturation) and high acid value in the DMSO fraction (Table 2).

Though having similar spectra, there were variations in the relative abundance of the peaks of the spectra in Figure

1-4 as reflected in the variations of their peak areas and corresponding areas (Table 3). Again, the whole unfractionated wax had highest peak areas and corresponding areas for most of the bands. The low ester value of the methanol fraction compared to n-hexane and diethyl ether fractions (Table 2) is also shown in their peak areas and corresponding areas C=O stretch for ester (Table 3) where methanol fraction had the least. Furthermore, the n-hexane and diethyl ether fractions showed no detectable peak areas and corresponding areas for C=O stretch for free fatty acid, hence their low acid values (Table 2). Also, notable distinction in abundance were observed for 1413 – 1416 cm^{-1} band (CH_2 bending –hydrocarbon) where n-hexane and diethyl ether fractions were more in abundance; 1629 – 1638 cm^{-1} band (C=C stretch) where methanol fraction took the lead; 2850 and 2918.4 cm^{-1} (symmetry and asymmetry C-H stretch) where n-hexane and diethyl ether fractions predominate; 3430 – 3451 cm^{-1} (OH stretch for alcohol, free fatty acid) in which methanol fraction was the largest. These results were in agreement with those for chemical parameters (Table 2) and showed that the n-hexane and diethyl ether fractions were richer in esters and hydrocarbon; while the methanol and dimethylsulphoxide fractions were richer in unsaturated free fatty acids and fatty alcohols.

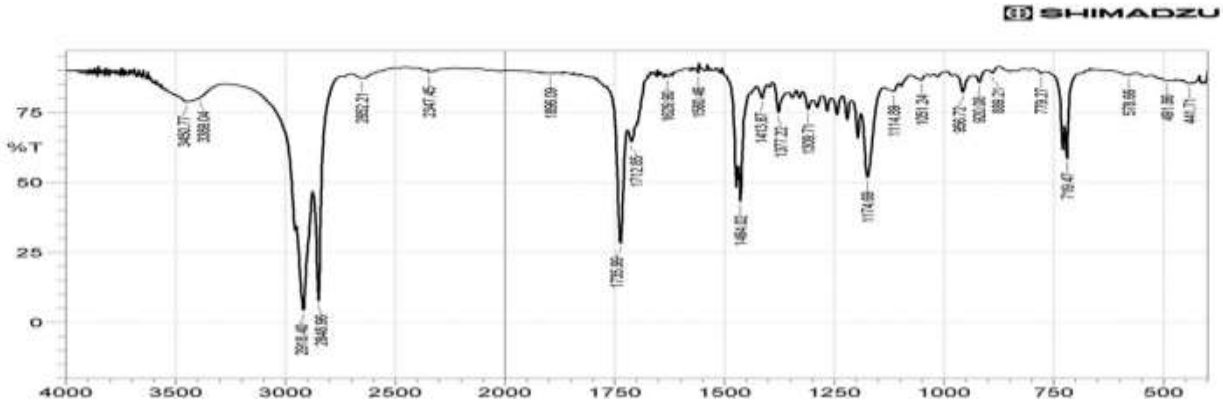

Fig 1. FTIR spectrum for the unfractionated beeswax sample

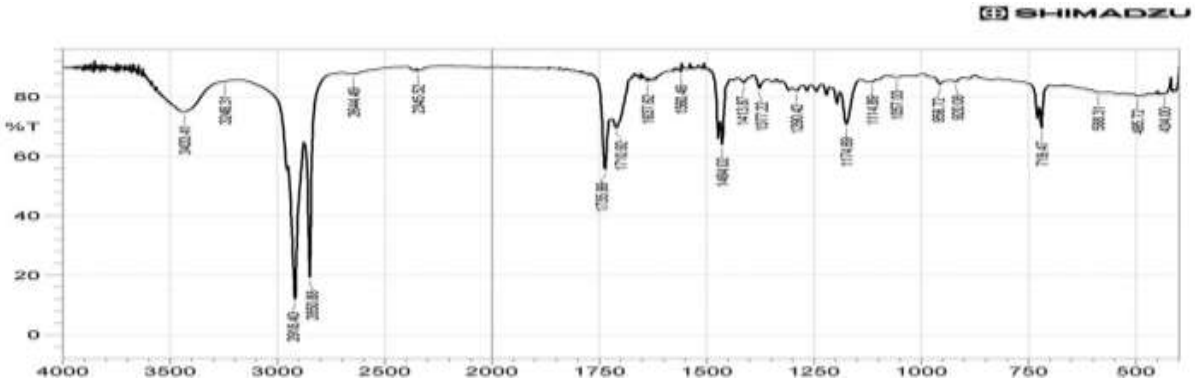

Fig 2. FTIR spectrum for the methanol fraction

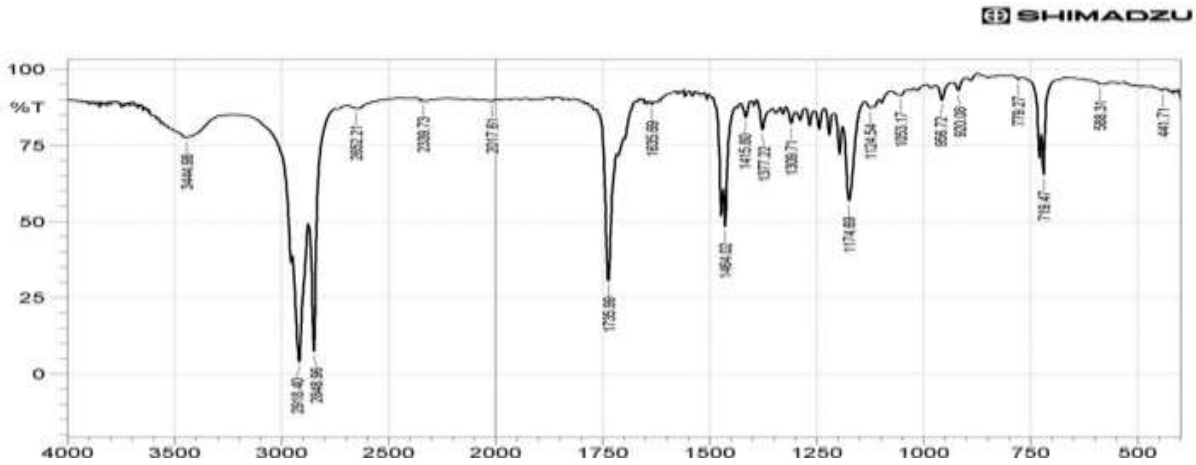

Fig 3. FTIR spectrum for the n-hexane fraction

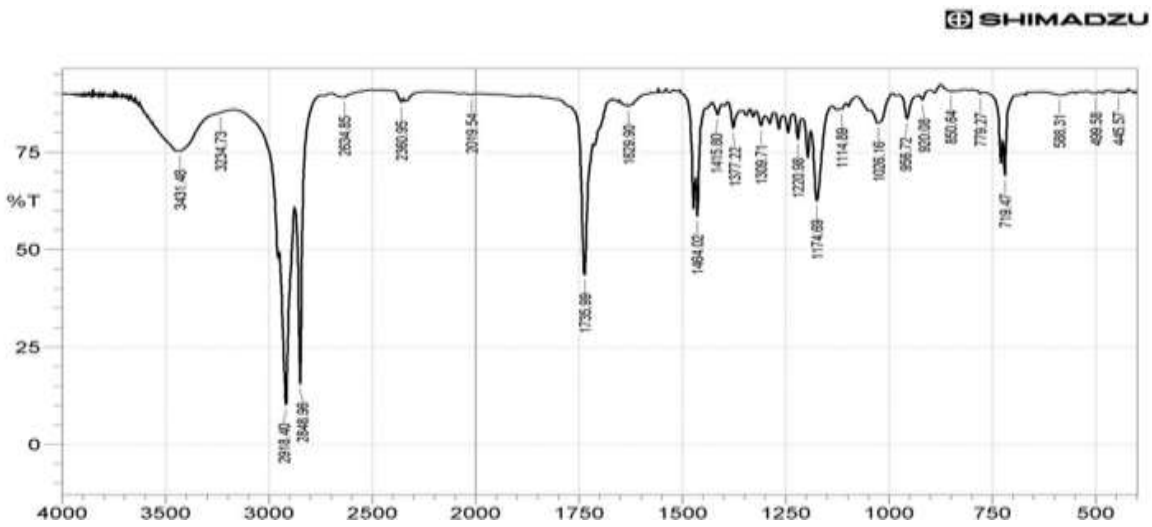

Fig 4. FTIR spectrum for the diethyl ether fraction

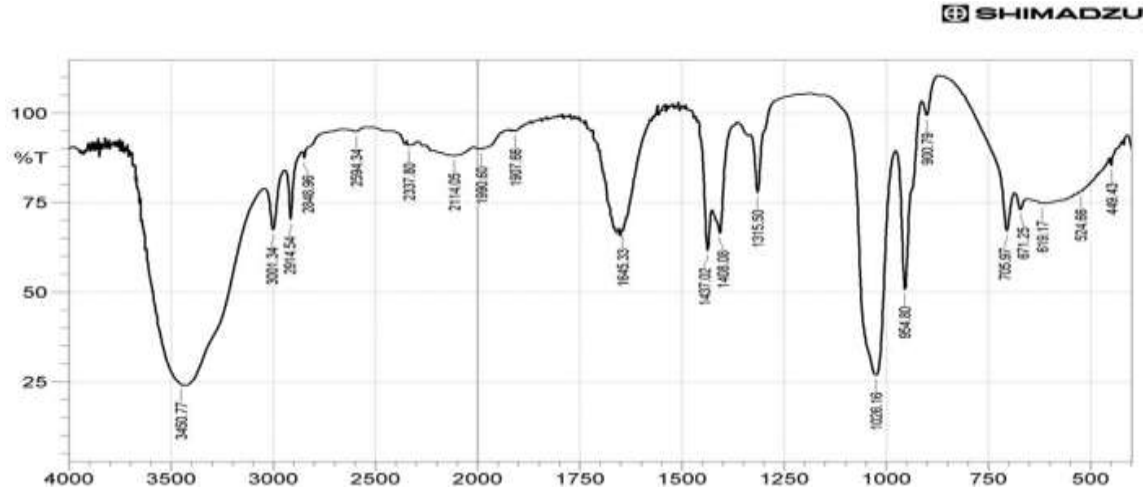

Fig 5. FTIR spectrum for the dimethylsulphoxide fraction

Table III. Variation in Area and Corresponding Area of FTIR Spectral Peaks of Unfractionated Beeswax and Methanol, n-Hexane and Diethyl ether Fractions

Peak (cm^{-1})	Sample	Area	Corr. Area	Bond vibration type
889 – 851	BW_U	1.132	0.070	=C-H out-of-plane bending
	M_F	-	-	
	nH_F	-	-	
	DEE_F	1.150	0.010	
920.08	BW_U	1.571	0.118	
	M_F	1.810	0.030	
	nH_F	0.570	0.120	
	DEE_F	1.260	0.060	
956.72	BW_U	2.481	0.387	
	M_F	2.360	0.100	
	nH_F	1.130	0.290	
	DEE_F	2.630	0.460	
1026 – 1057	BW_U	2.243	0.159	C-O stretch (alcohol, ester and fatty acid)
	M_F	1.900	0.050	
	nH_F	0.630	0.030	
	DEE_F	3.660	0.660	
1124.54 – 1114.89	BW_U	8.170	3.099	
	M_F	5.090	1.200	

	nH_F	6.470	3.000	
	DEE_F	5.980	2.080	
1174.69	BW_U	8.170	3.099	
	M_F	5.090	1.200	
	nH_F	6.470	3.000	
	DEE_F	5.980	2.080	
1290 – 1310	BW_U	2.356	0.221	C-O-H bending
	M_F	2.200	0.080	
	nH_F	1.760	0.210	
	DEE_F	1.800; 1.780	0.230; 0.150	
1377.22	BW_U	2.763	0.531	CH_2 bending
	M_F	2.150	0.220	
	nH_F	1.930	0.440	
	DEE_F	2.100	0.320	
1413 – 1416	BW_U	1.923	0.233	
	M_F	1.050	0.040	
	nH_F	1.190	0.230	
	DEE_F	1.590	0.140	
1464.02	BW_U	5.194	0.287	CH_3 bending
	M_F	2.530	0.090	
	nH_F	3.920	0.060	
	DEE_F	3.070	0.010	
1629 – 1638	BW_U	0.321	0.009	C=C stretch
	M_F	0.760	0.040	
	nH_F	0.590	0.020	
	DEE_F	0.610	0.020	
1710 – 1713	BW_U	5.881	0.964	C=O stretch (free fatty acid)
	M_F	5.400	0.750	
	nH_F	-	-	
	DEE_F	-	-	
1735.99	BW_U	10.654	4.943	C=O stretch (ester, fatty acid)
	M_F	4.740	1.260	
	nH_F	9.780	5.180	
	DEE_F	8.220	3.490	
≈ 2850	BW_U	31.673	11.882	C-H (SP^3) symmetric stretch
	M_F	20.280	7.050	
	nH_F	30.900	11.820	
	DEE_F	22.150	8.520	
2918.4	BW_U	51.726	23.466	C-H (SP^3) asymmetric stretch
	M_F	31.190	15.590	
	nH_F	50.180	24.480	
	DEE_F	35.830	17.240	
3230 – 3390	BW_U	7.666	0.009	=C-H stretch
	M_F	0.940	0.000	
	nH_F	-	-	
	DEE_F	1.470	0.000	

3430 – 3451	BW_U	3.474	0.059	OH stretch (alcohol, free fatty acid)
	M_F	15.520	0.650	
	nH_F	3.370	0.020	
	DEE_F	15.330	0.210	

BW_U = unfractionated beeswax; M_F= methanol fraction; nH_F = n-hexane fraction; DEE_F = diethyl ether fraction

B. Antimicrobial activity

The antimicrobial activities of the beeswax extracts and whole beeswax against different bacteria and fungi (moulds and yeasts) are shown in Table 4. The inhibition zones varied amongst the beeswax extracts and whole beeswax. According to the analyses among the tested bacteria, *Streptococcus pneumonia* (16.5 mm) was the most sensitive in beeswax dimethyl sulphoxide (DMSO) fraction, and the sensitivity of the bacteria decreased as follows: *Staphylococcus epidermis* (15.5 mm) >*Streptococcus pyogenes* (15 mm). *Streptococcus pyogenes* was the most sensitive in beeswax n-Hexanefraction (9 mm) and beeswax diethyl ethyl (DEE) fraction (4 mm) while *Klebsiella pneumonia* (14.5 mm) was the most sensitive in beeswax methanol fraction. *Staphylococcus aureus* (5 mm) was the most sensitive in unfractionated beeswax followed by *Staphylococcus epidermidis* and *Streptococcus pyogenes* with zones of inhibitions of 4.5 mm.

According to the analyses among the tested fungi, *Candida albicans* was the most sensitive in all the beeswax fractions and unfractionated beeswax sample with the highest zone of inhibition of 19 mm in beeswax DMSO fraction, followed by *Cordycepsmilitaris* which was sensitive to beeswax DMSO and methanol fractions only.

The variation in the antimicrobial activities of tested beeswax fractions may be due to the different bioactive compounds/ constituents present in each of the fractions. The beeswax fractions, especially the DMSO and methanol fractions used in the study were found effective against most of the test microorganisms and showed a pronounced inhibitory effect on *Candida albicans*. The finding was also reported by [17]. In accordance with the present results, previous studies have demonstrated that beeswax extracts had antimicrobial activities against *S. aureus*, *C. albicans*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus epidermidis*([17], [18]). According to [19], the effectiveness of beeswax extracts against pathogenic microorganisms suggests a potential future use of beeswax extracts in food processing as a preservative agent.

Table IV. Inhibitory Effects of Beeswax Fractions and Unfractionated Beeswax against Bacteria and Fungi (Inhibition Zone Diameter in mm)

Microorganisms	**Control**				**Fractions**				**BW_U**
	$DMSO_F$	**DEE_F**	**nH_F**	**M_F**	**$DMSO_F$**	**DEE_F**	**nH_F**	**M_F**	
Streptococcus pneumonia	10	-	-	8	16.5	-	-	13	-
Klebsiella pneumonia	8	-	-	10	14.5	-	-	14.5	-
Staphylococcus aureus	6	-	-	2	10	-	-	8	5
Staphylococcus epidermidis	6	-	-	3	15.5	2	2	10.5	4.5
Escherichia coli	7	-	-	5	12.5	-	-	9	2
Pseudomonas aeruginosa	8	2	-	6	14	2	-	9	
Streptococcus pyogenes	6	4	7	7	15	4	9	12.5	4.5
Beauveriabassiana	4.5	-	-	-	6	-	-	-	-
Cordycepsmilitaris	6	-	-	10	12.5	-	-	15.5	-
Candida albicans	9.5	-	-	8	19	6	5	17	15

$DMSO_F$ = dimethylsulphoxide fraction; DEE_F = diethyl ether fraction; nH_F= n-Hexane fraction; M_F = methanol fraction; BW_U = unfractionated beeswax

IV. CONCLUSION

The result show that the chemical parameters of the unfractionated beeswax were within the ranges of international standards but most for the fractions were outside the ranges for the unfractionated beeswax. There were variations in the properties and compositions of the fractions. The n-hexane and diethyl ether fractions were richer in esters and hydrocarbon; while the methanol and DMSO fractions were richer in unsaturated free fatty acids and fatty alcohols. It could be inferred that the beeswax sample and its fractions contain different functional groups (various chemical

compounds) and in different proportions which account for their structural complexity.

The research has demonstrated that beeswax fractions studied possess antimicrobial activities against bacteria and fungi and some of the fractions have stronger activities than the unfractionated wax. The inhibitory effect of the beeswax extracts was found to be solvent-dependent. Among the tested extracts, the best inhibitory effects were shown by the beeswax DMSO fraction. The overall most sensitive microorganism was *Candida albicans* which has the highest zone of inhibition in all the beeswax extracts and whole beeswax sample. *Beauveriabassiana* was the most resistant microorganism that was only inhibited by beeswax DMSO fraction.

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