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Anti-Bacterial, Anti-Oxidant and Cytotoxic Activities of Nimbin Isolated from African Azadirachta Indica Seed Oil

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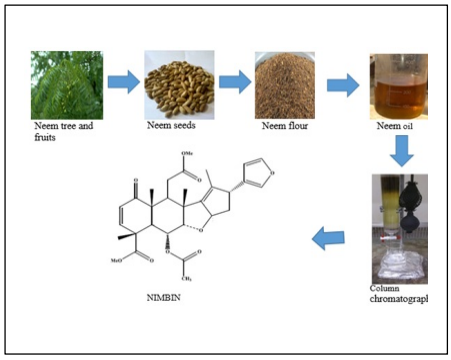

Keywords:
Azadirachta indica;
Anti-Bacterial;
Anti-Oxidant;
Cytotoxic;
Nimbin.

ABSTRACT

Azadirachta indica (neem), belonging to Meliaceae family and native to Asia, has been used therapeutically in traditional Ayurveda, Unani, several local systems within Africa and Chinese medicine to treat different diseases for long times. The phytochemical screening of the seed oil of these plants grown in Nigeria resulted in the isolation of nimbin from the methanol soluble fraction of the hexane extract. The structure of this compound was established on the basis of nuclear magnetic resonance (NMR), Infrared (IR) and mass (MS) spectroscopy. The obtained ^{1}H and ^{13}C NMR data of the compound was compared with those reported already in the literature. Finally, the identity of the compound was further substantiated by employing the X-ray crystallography. The compound was also subjected to antioxidant, antibacterial and brine shrimp lethality bioassay for further analyses. The pure compound showed poor antioxidant activity and was only moderately active against Escherichia coli among all the organisms tested. Moreover, the compound shows no cytotoxicity.

Introduction

Azadirachta indica (A. indica), also known as neem, belongs to the Meliaceae (mahogany) family native of Nepal, India, Bangladash, Thailand and Pakistan [1]. The neem tree is an evergreen, or deciduous, fast-growing tree that can reach a height of 30 meters. The trunk is stout and the branches are wide and spreading, in severe drought may shed most or nearly all of its leaves. The flowers and fruits are borne in axillary clusters and when ripe the smooth ellipsoidal drupes are greenish yellow and comprise a sweet pulp enclosing a seed. The seeds consist of a shell with 1-3 kernels [2] but in Nigeria, one-seeded drupe type of fruits grow wild and are planted all over the northern part of the country. All parts of neem tree have been used traditionally in Ayurveda, Unani, Homeopathy, Chinese, several local systems within Africa and modern medicine for the treatment of many infectious, metabolic or

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cancerous diseases [3-5]. Therapeutically, its crude extracts from the leaves, root and stem bark have been used in folk medicine to control diseases such as leprosy, malaria fever, smallpox, diarrhoea, cholera, intestinal helminthiasis and respiratory system [6-8]. Furthermore, numerous biological and pharmacological activities of this plant parts extracts have been studied and was reported to include antimalarial, antidiabetic, antibacterial, antifungal, anti-inflammatory, antioxidant, antiarthritic, antipyretic, hypoglycemic, antigastric ulcer, antiseptic, antiparalitic and antitumour activities [7-13]. Similarly, the oil extracted from the neem seeds has been reported to be effective as an antioxidant, insecticides, miticides, fungicide, nematocides and as an insect antifeedents and repellents [9, 14]. More than 135 compounds have been isolated from different parts of neem tree and these compounds have been group into two; isoprenoids including deterpenoids, azadirone, gedunin, nimbin, salanin and azadirachtin and non-isoprenoids such as flavonoids, alkaloids and steroids [15].

Isolation of nimbin (Fig. 1) from different parts of neem tree and their biological activities have been reported [16-18]. Several studies have supported the views that secondary metabolites are adaptive traits that have been diversified during evolution by natural selection and are greatly influenced by the environmental factors [19, 20]. Hence, it is necessary to phytochemicaly screen all plants found in any local community to ascertain their therapeutic action, and compounds responsible for such action should be isolated and identified. The isolated compounds should also be subjected to all biological tests so as to ascertain their potency on different ailments. Although it is very much important to investigate the structural features of natural products, but this is the first report on the antibacterial, antioxidant and cytotoxic effect of nimbin to the best of our knowledge.

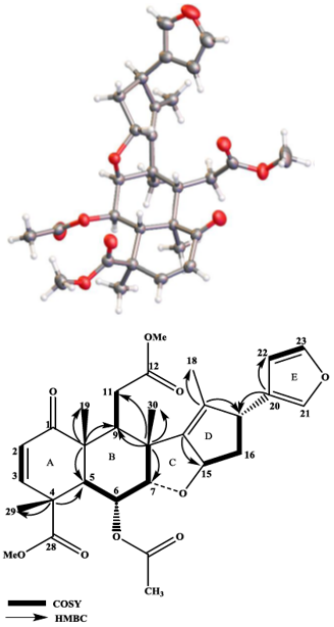

Fig. 1. The x-ray crystal structure of nimbin (up) and HMBC and COSY correlation of nimbin (down).

Materials and Methods

Apparatus

Column chromatography (CC) and thin layer chromatography (TLC) were performed on (silica gel 60 F254, 25 Glass plates 20 × 20 cm, E. Merck, Germany). NMR spectra were recorded on a Bruker Avance (400 MHz) spectrometer with internal references of δH 7.27 and 77.0 ppm for CDCl3 using tetramethylsilane (TMS) as an internal standard. Thermo Instruments HPLC system mass spectrometer with electron spray ionization (ESI) source was used for recording of the mass spectra. The IR spectrum (KBr) was recorded on a Perkin Elmer 100 FT-IR spectrometer. The melting points were determined by a Stuart instrument and is uncorrected.

Sample Collection and Preparation

The fruits of A. indica were collected in Shabu-Lafia, Nasarawa State, Nigeria, and authenticated by Dr. J. A. Alanana of Nasarawa State University Keffi. A voucher specimen No. 1723 was deposited in the herbarium of Ahmadu Bello University Zaria, Nigeria. The fresh fruits were thoroughly washed under tap water and later rinsed with distilled water to remove mud and dust particles, air-dried

for two weeks and the seeds manually removed. The dried seeds was pulverized using mortar and pestle to fine particles and stored in nylon containers and kept away from moisture for further analysis.

Extraction and Isolation

The flaked Azadirachta indica seeds (1.8 kg) was extracted four times with 8 L of n-hexane for 4 days at room temperature. The hexane extracts were combined and concentrated using rotary evaporator at 40 °C to produce 317 ml of brownish oil extract. The extract (100 ml) was added 150 ml of n-hexane and extracted with 95% methanol (4 × 150 ml). The methanol extracts were re-extracted with hexane (4 × 150 ml) to give a 4 × 4 partition. On the basis of their TLC profiles, the first two methanol extracts were pooled together and evaporated to give a brown gum (5.32 g). The methanol extract (5 g) was subjected to silica gel column (100 g, 4 × 50 cm) chromatography using hexane and ethyl acetate (100:0 to 0:100 v/v) gradient elution system to yield 32 fractions and was allowed to stand for 12 hours. Fractions 17 and 18 obtained on elution with hexane: ethyl acetate (1:1) yielded white crystals, which crystallized from methanol to yield nimbin (41 mg).

DPPH-Free Radical Scavenging Activity

The analysis of the DPPH radical scavenging activity of the compound was performed according to the method described by Koleva and co-workers [21]. Stock solution was prepared by dissolving 100 mg of the compound in 1 ml of methanol and seven, two fold serial dilutions was made to obtain a concentration of 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 mg/ml. 0.5 ml of each of the concentrations was measured into separate test tubes and 0.3 ml of 0.5 mM DPPH was added. The reaction mixtures were vigorously shaken for 30 s in a Vortex apparatus and allowed to stand in the dark at room temperature for 30 minutes. Ascorbic acid was used as a standard for the investigation of the antiradical activity and was prepared in a similar manner. The absorbance was read using spectrophotometer at 517 nm against the blank. The blank was prepared by mixing 0.5 ml of the extract or ascorbic acid with 3.3 ml of methanol. Similarly, the control solution was prepared by mixing 3.5 mL of methanol and 0.3 ml of DPPH radical solution. The percentage of scavenging activity (X %) was calculated according to eq. (1):

$$X\% = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of control}} \times 100 \quad (1)$$

Bioassays

The antibacterial activity of the compound was determined by the disc diffusion method [22]. The isolated compound was dissolved in chloroform and applied to sterile filter paper discs at a concentration of 500 ug/ disc. Ampicillin disc (10ug/disc) was used as standard in the study. For cytotoxicity screening, dimethyl sulfoxide (DMSO) solution of the compounds was applied against Artemia salina for 24 hours in a simplified in vivo simplified assay [23]. In this experiment, 1 mg of the isolate was dissolved in 1 ml of DMSO and seven, two-fold serial dilutions was made to obtain varying concentrations between 500 and 0.032 µg/ml. Then 0.5 ml each of these standard concentrations were added to the test tubes containing 10 shrimps in simulated brine water. After 24 h, the media lethal concentration (LC_{50}) of the test sample was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration. Vincristine sulphate was used as positive control in this assay to compare the cytotoxicity of the compound.

Results and Discussion

The compound, nimbin (41 mg) was isolated as white crystalline solid. It has a molecular formula of $C_{30}H_{36}O_9$ which was established on the basis of ESI-HRMS at m/z 541.2441 $[M + H]^+$ (Calcd for 541.2438) (Fig. 2). The IR spectrum (Fig. 3) showed

a band at 2953 cm^{-1} (saturated C-H stretching), 1735 (C=O stretching), 1685 (C=C stretching), 1435 (C-H deformation), 1234 and 1030 (C-O stretching) and 754 (C-H out of plane deformation) cm^{-1}.

The DEPT spectrum (Fig. 4) showed thirty carbon signals, which consisted of ten quaternary, eleven methine, two methylene, seven methyl group. It also showed signals for the carbon atoms of the keto group (C-1) at (δc 201.62), two methyl esters carbonyl carbon atoms C-12 and 28 (δc 173.66 and 174.64), the acetate carbon atom at C-6 (δc 170.58), the β-substituted furan ring resonates at C-21 (δc 139.01), C-22 (δc 110.49) and C-23 (δc 143.02). The carbon atoms in the tetrahydrofuran ring linked to oxygen occured at C-7 (δc 84.58) and C-15 (δc 87.12).

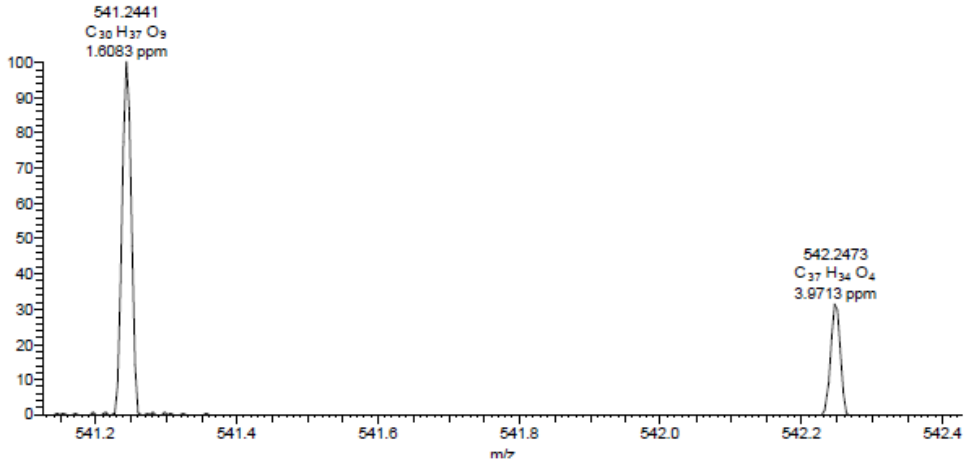

Fig. 2. MS spectrum of nimbin.

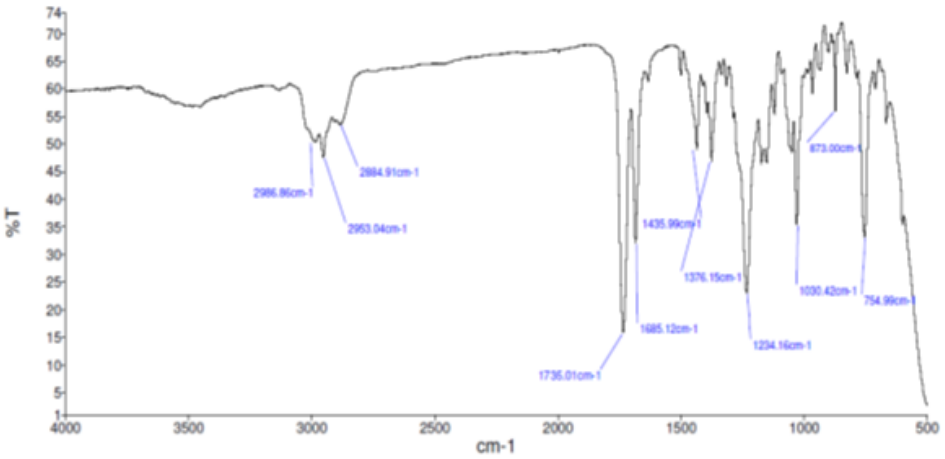

Fig. 3. Infrared spectrum of nimbin.

The ^{1}H NMR spectrum (Fig. 5) revealed the characteristic signals due to a β-substituted furan ring (ring E) protons at δH 7.34 (m, H-21), 6.35 (m, H-22) and 7.25 (m, H-23). These protons signals were seen to be coupled to each other in the COSY spectrum (Fig. 6). The HSQC spectrum (Fig. 7) correlated the proton signals at δH 7.34, 6.35 and 7.25 to the carbon resonance at δc 139.01, 110.49 and 143.02 attributable to position (C-21), (C-22) and (C-23) respectively. The quaternary carbons atom resonance at δc 126.82 were assigned to C-20.

In ring A, the ^{1}H NMR spectrum displayed resonances ascribable to enone protons at (H-2) and (H-3). These vinylic protons split each other resulting in a doublet at δH 5.86 and 6.34 (d, 10.1 Hz, each) assigned to position H-2 and H-3. The COSY spectrum showed these protons to be

coupled to each other. The HSQC spectrum correlate H-2 to C-2 (δc 125.98) and H-3 to C-3 (δc 147.59). The keto and methyl ester carbonyl carbon atom occurred at C-1 and C-28, while the quaternary carbon atom resonance at δc 47.77 was assigned to C-4.

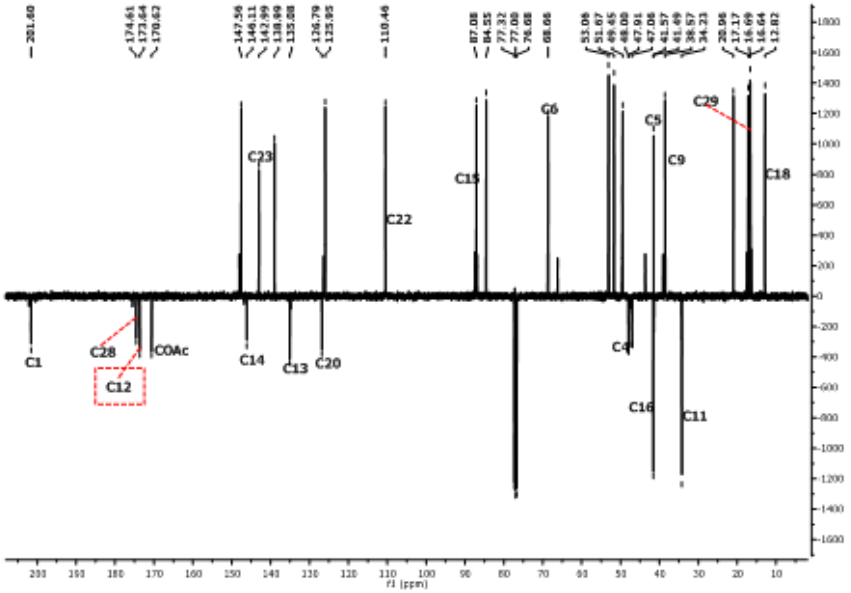

Fig. 4. DEPT spectrum of nimbin.

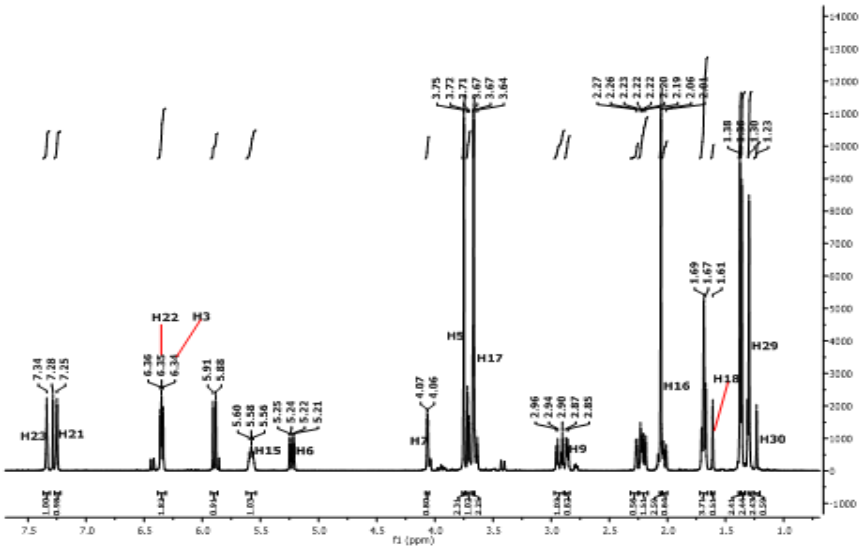

Fig. 5. [1]HNMR spectrum of nimbin.

In ring B, [1]H NMR, H-5 was split by H-6 resulting in a doublet at δH 3.71 (d, 3.3 Hz) in the spectrum. Proton H-6 was split by H-5 and H-7 resulting in a doublet of doublet at δH 5.21 (dd, 3.0, 12.2 Hz) and H-9 was split by H-11a&b resulting in a multiplet at δH 2.85. The COSY spectrum showed H-6 coupled to H-5. The HSQC spectrum correlated H-5 to the carbon atom resonance at δc 41.52 and H-6 to the carbon atom at δc 68.69. The C-10 quaternary carbon atom resonance occurred at δc 48.03.

In ring C, Proton H-7 was split by proton H-6 resulting in a doublet at 4.06 (d, 2.9 Hz). The COSY spectrum also showed H-7 to be coupled to H-6. The HSQC spectrum correlated H-7 to the carbon atom resonance at δc 84.58 (CHO) which was indicative of a carbon atom linked to an oxygen atom. It is noted the results show that the C-8

quaternary carbon atom resonance occurred at δc 47.09.

In ring D, proton H-15 was split by protons H-16a&b and yielded a multiplet at δH 5.56. The protons H-16a&b were split by the H-15 and H-17 δH 3.64 resulted in a multiplet at δH 2.01 and δH 2.19. H-17 was equally split by H-16a&b and occurred as a multiplet at δH 3.64. The COSY spectrum confirmed the coupling between H-16 and H-17. The spectrum of HSQC, also show the coupling of proton resonances H-15, H-16a&b and H-17 to the carbon resonance at δc 87.12 (CHO), δc 41.60 and δc 49.49 (CH). Moreover, the results show that the quaternary carbon atom resonance for C-13 and C-14 occurred at δc 135.11 and δc 146.15.

Table 1: ^{1}H and ^{13}C NMR data of compound Nimbin

Atom No.	Narasimh-an, 2011 $CDCl_3$ δ_C	Johnson and Morgan, 1997 $CDCl_3$ δ_C	Nimbin $CDCl_3$ δ_C (m)	Nimbin $CDCl_3$ δ_H (m, *J* in Hz)	Narasimhan 2011 $CDCl_3$ δ_H (m, *J* in Hz)	Johnson and Morgan, 1997 $CDCl_3$ δ_H (m, *J* in Hz)
1	201.8	201.73	201.62 (C)			
2	126.0	125.96	125.98 (CH)	5.86 (d, 10.2)	5.85 (d, 10.5)	5.58 (d)
3	147.6	147.58	147.59 (CH)	6.34 (d, 10.2)	6.34 (d, 10.4)	6.34 (d)
4	47.0	47.77	47.77 (C)			
5	42.3	41.53	41.52 (CH)	3.71 (d, 3.3)	3.50 (d, 3.0)	3.70 (d)
6	68.3	68.66	68.69 (CH)	5.21 (dd, 3.0, 12.3)	5.19 (dd, 3.0, 12.3)	5.22 (dd)
7	84.55	84.53	84.58 (CH)	4.06 (d, 2.9)	3.80 (d, 2.3)	4.05 (d)
8	49.9	47.08	47.09 (C)			
9	41.3	38.59	38.60 (CH)	2.85 (m)	2.70 (m)	2.87 (m)
10	48.9	47.93	48.03 (C)			
11A	33.2	34.23	34.26 (CH_2)	2.19 (m)	2.13 (m)	2.25 (m)
11B				2.90 (m)	2.70 (m)	2.94 (m)
12	174.9	173.64	173.66 (C)			
13	135.07	135.08	135.11 (C)			
14	146.12	146.16	146.15 (C)			
15	87.08	87.10	87.12 (CH)	5.56 (m)	2.34 (m)	5.57 (m)
16A	41.50	41.59	41.60 (CH_2)	2.01 (m)	5.74 (m)	2.02 (m)
16B	49.46	49.47	49.49 (CH)	2.19 (m)		2.19 (m)
17	12.81	12.83	12.85 (CH_3)	3.64 (m)		3.61 (m)
18	16.96	16.66	16.67 (CH_3)	1.61 (s)	1.66 (s)	1.69 (d)
19	119.2	126.81	126.82 (C)	1.36 (d, 5.8)	1.34 (s)	
20	139.5	139.01	139.01 (CH)			
21	109.4	110.48	110.49 (CH)	7.34 (m)	7.32 (m)	7.32 (m)
22	142.5	143.01	143.02 (CH)	6.35 (m)	6.45 (m)	6.33 (m)
23	174.9	174.59	174.64 (C)	7.25 (m)	7.59 (m)	7.23 (m)
28	17.1	17.19	17.21 (CH_3)			
29	16.67	16.70	16.72 (CH_3)	1.30 (s)	1.29 (s)	1.36 (s)
30	51.8	51.66	51.70	1.23 (s)	0.96 (s)	1.34 (s)
12-OCH3	52.7	53.05	53.09	3.67 (s)	3.76 (s)	3.65 (s)
28-OCH3	170.3	170.58	170.65	3.75 (s)	3.81 (s)	3.73 (s)
OAc	20.62	20.96	20.99	2.06 (s)	1.90 (s)	2.04 (s)

The four-methyl proton resonances; H-18, H-19, H-29 and H-30 were assigned to carbon atom resonances at δc 12.85, δc 16.67, δc 17.21 and δc 16.72. Finally, the rings were sustained by long range coupling of quaternary carbon to protons as observed in the HMBC (Fig. 1down and 8), where C-4 correlate with H-5 and H-29; C-8 correlate with H-7, H-9, H-11 and H-30; C-10 correlate with H-5, H-9 and H-19; C-13 correlate with H-17 and H-18; C-14 correlate with H-15 while C-20 correlate with H-17 and H-22 (6.35 m) respectively. Comparison of the 1H and 13C NMR data (Table 1) with those

published by other researchers [16, 17] confirmed nimbin as the compound isolated. Finally, the identity of this compound was further substantiated by X-ray crystallography (Fig. 1).

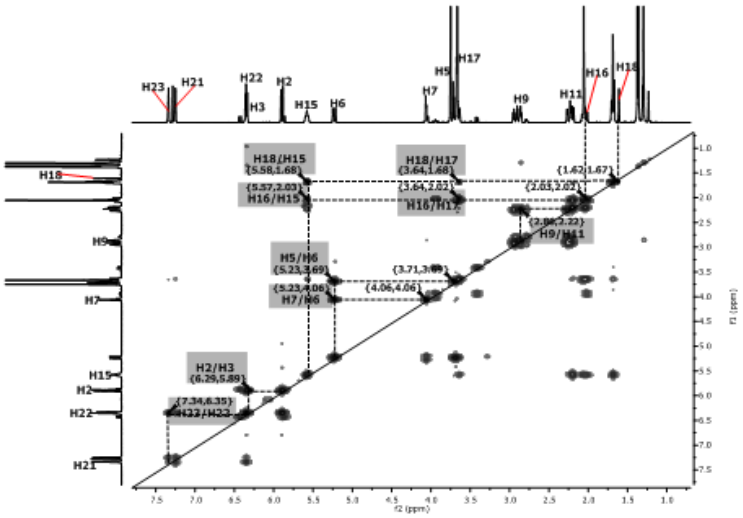

Fig. 6. COSY spectrum of nimbin

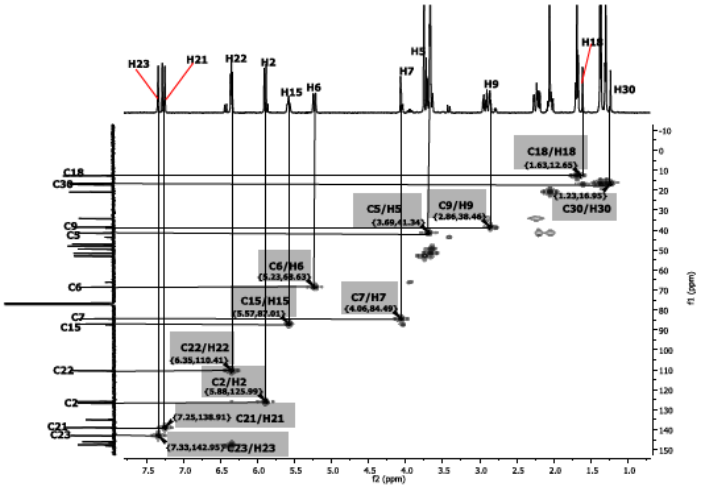

Fig. 7. HSQC spectrum of nimbin

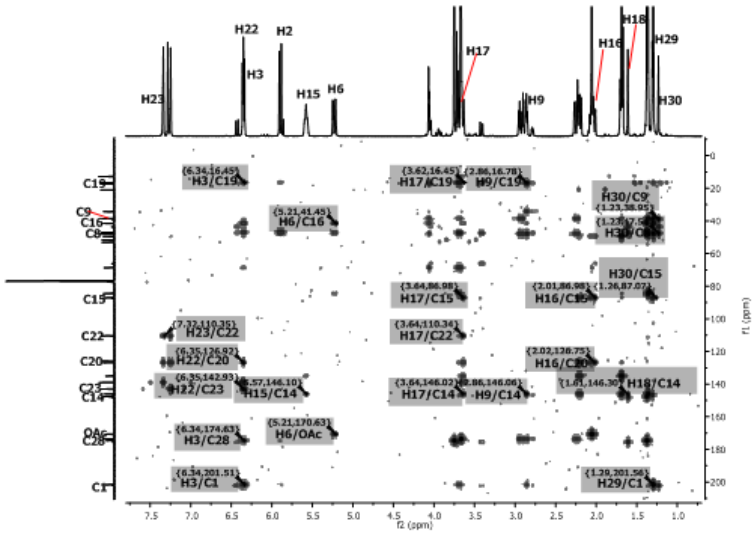

Fig. 8. HMBC spectrum of nimbin.

In the antibacterial screening, the isolated compound exhibited poor antibacterial activity against most of the test organisms cited in Table 2, with the exception of Escherichia coli where it exhibited mild activity with the zone of inhibition of 7 mm at a concentration of 500 µg/disc. Although, Ukaoma and co-workers [29] has reported that neem oil has antibacterial property and this could be due to synergistic effect of other compounds present in the oil.

Table. 2: Antibacterial activity of nimbin 500 µg/disc and ampicillin 10 µg/disc

Test bacterial	Nimbin dissolved in chloroform
Bacillus subtilus	NA
Bacillus cereus	NA
Staphylococus aereus	NA
Salmonella typhi	NA
Escherichia coli	7 mm
Pseudomonas aeruginosa	NA

NA means no activity

The lethality of nimbin dissolved in chloroform was screened by brine shrimp lethality bioassay for probable cytotoxic activity. The LC50 obtained from the best fit line slope were found to be 0.72 and 15.6 µg/ml for positive control (vincristine sulphate) and nimbin respectively as shown in Table 3. In comparison with the positive control (vincristine sulphate), the cytotoxicity exhibited by nimbin was not significant.

The antioxidant activity expressed as % DPPH inhibition of nimbin is presented in Table 3. From the results, nimbin showed very low percentage DPPH scavenging ability with 12.35%. In comparison with standard ascorbic acid 85.15%, the scavenging activity exhibited by nimbin was insignificant. This contradict the report that nimbolide and azadirachtin isolated from neem seeds showed concentration-dependent antiradical scavenging activity [9]. The result indicate that nimbin will not be able to quench or scavenge free radicals produced in the body or reducing and chelating the transition metal composition of foods [24- 28].

Table 3: Antioxidant activity and cytotoxic effect of nimbin

Test	Sample	Result
% DPPH scavenging activity	Nimbin	12.35
	Ascorbic acid	85.18
Media LC_{50} (µg/ml) of the sample	Nimbin	15.6
	Vincristine-sulphate	0.72

Conclusion

All parts of neem tree contained active ingredients that are responsible for their therapeutic actions. Studies have supported the views that environmental factors have influence on the chemical composition of plants. It is therefore necessary to phytochemicaly screen all plants found in any local environment, and compounds responsible for such activities should be isolated, identified and subjected to different biological tests.

Acknowledgments

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