Continental J. Applied Sciences
Fatokun and Okwute (2020) 15 (2): 54 - 71

Research Article

Two Antimicrobial Compounds from the Leaf and Root Extracts of *Waltheria indica* Linn

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Abstract

Waltheria indica Linn is a plant used for medicinal purposes to treat several diseases like cough, diarrhea, fever, hemorrhage, malaria, stomach ache and wounds. In this study, two compounds were isolated from the plant leaves and roots solvent fractions using chromatographic techniques. The antimicrobial activities of the compounds were evaluated on clinical microbial test strains associated with respiratory diseases. The chromatographic elution revealed two previously known compounds from the polar fractions of ethanol extract of leaf and the ethyl acetate fractions of the roots. The compounds were identified based on spectral analyses (IR, ^{1}HNMR and ^{13}CNMR) and physical characteristics as well as reported literature to be n- tetraicosanol and stigmasterol. The antimicrobial evaluation of the compounds using well diffusion method against eight pathogens of Gram positive/negative bacteria and fungi such as (S. aureus, S.pneumonia, S.pyrogens and K.pneumonia) and fungi strains (C.ulcerans, C.albicans, C. krusei and C. tropicalis) revealed susceptibility against all the test organisms, showing inhibition zones ranging from 27 -31 mm for n- tetraicosanol and 28-35 mm for stigmasterol against the test strains except against Streptococcus pyrogens and Candida albicans. However, the compounds were less active than the reference drugs, ciprofloxacin and fluconazole. This is the first report for the presence of stigmasterol and tetraicosanol from Waltheria indica plant. These two antimicrobial compounds confirm the ethno-medicinal use of the plant as an anti-infective agent and may serve as leads in the development of new antibiotics.

Keywords: *Waltheria Indica* Linn, antimicrobial activity, well diffusion. IR, ^{1}HNMR and ^{13}CNMR, n- tetracosanol and stigmasterol.

ISSN: 1597 – 9928.

Science and Education Development Inst., Nigeria

Introduction

Respiratory tract infections are widespread and significant cause of diseases and death worldwide especially in developing countries. This is mainly due to the development of resistance to routine anti-microbial therapies in microbes such as bacteria, virus, fungi and their associated opportunistic infections. Apart from death, these diseases irrespective of their origin greatly diminish the reproductive capabilities of livestock as well as the overall effectiveness of humans (Davies and Davies 2010; Beltrame *et al*., 2007; Ngwu *et al*.,2016). The re-occurrence of opportunistic infections associated with respiratory tracts prolongs the prevalence of more lethal conditions such as tuberculosis and other acute respiratory complications. Microbial infections commonly associated with respiratory tract includes, but are not limited to asthma, bronchitis, common cold, cough and whooping cough etc. (Thomas, 2017; Ellis 2008). Prolonged exposure and reoccurrence of these nosocomial infections creates a platform for invasion by more lethal pathogens (Ahameethunisa and Hoper, 2010). Such phenomenon necessitates the search and investment for more effective drug alternatives against these microbial diseases (Richard and Yitzhak, 2014).

Indigenous therapeutic plants are the key source of natural medicinal drugs and are widely used as treatment agents that have been used for disease control since ancient times and form the core component of primary health care because of consistency, acceptability, effectiveness and affordability (Sofowora *et al*., 2013; Oladeji, 2016). The people depend on the indigenous plant resources to treat various respiratory disorders as herbal remedies are common practice in many parts of the world and thus showing that people are encouraging indigenous production and processing of these medicinal plants (Alamgeer *et al*., 2018; Aziz *et al.,* 2019).

A number of *in-vitro* clinical studies have shown the therapeutic potential of medicinal herbs in the treatment of respiratory diseases. Examples include M*enthapiperita* (peppermint) that contains menthol and exhibits antibacterial and antiviral properties as well as an antitussive effect (McKay and Blumberg 2006). *Origanum syriacum* contains the active ingredients of thymol and carvacrol and possesses antimicrobial and antifungal properties (Baydar *et al*., 2004; El-Gendy *et al.,* 2013).

Waltheria indica Linn is an important medicinal plant and widely used around the world. It belongs to the family *Sterculiaceae* and is a common weed in dry places in the settled areas of most tropical countries. It is used as a traditional herbal medicine

ISSN: 1597 – 9928.

in Bangladesh, China, Hong Kong, India, Pakistan, Philippines, Malaysia, Indonesia, Nigeria and Thailand (Ayantunde *et al*., 2009). It is ethnobotanically used in the management of inflammation, diarrhea, dysentery, abscess conjunctivitis, epilepsy, and erectile dysfunction of the bladder, anti-inflammatory, cough and asthma (Olowokudejo *et al*., 2008; Zongo *et al*., 2014; Zongo *et al*., 2013; Saunders *et al*., 2007). Previous study on extracts of ethanolic crude extracts and fractions of leaves and roots of the plant showed remarkable inhibitory activity against microbial pathogens (Okwute *et al*., 2017; Fatokun *et al*., 2017). The present study aimed at isolating compounds from leaf and roots of *Waltheria indica* and evaluate their antimicrobial activity for the purpose of confirming the ethno-medicinal uses of the plant as well as determining its chemical constituents.

Plate 1: Pictures of *Waltheria indica* Linn

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Experimental

Methods

All the solvents used such as ethanol, methanol, chloroform, ethyl acetate and n-hexane were analytical grade. The column chromatography utilisedc was Whatman silica gel (Purasi 60A (230-400mesh size). Thin- layer chromatography (TLC) used was pre-coated Merck Kieselgel 60F 254 with 0.25 mesh. The spots were visualized by exposure to UV light at 254/366 nm and to iodine vapour (TLC). The equipment used were Soxhlet apparatus (Borosilicate) and UV-visible spectrophotometer (7500 CECIL UV-Vis). The infrared (IR) spectrophotometer (Thermofisher Spectrum 100). The 1H, 13C and 2D nuclear magnetic resonance (NMR) (BrukerAvance III 400 spectrometer).

Plant materials and preparation of extract

The *Waltheria indica* fresh leaf and root were collected from the village of Cukuku, Kwali, Federal Capital Territory (FCT), Nigeria. The plant was identified and authenticated by the botanist at the Department of botany, Nigeria Pharmaceutical Research and Development (NIPRID), Abuja, with Voucher specimen number (Herbarium no NIPRD/H/6738). The *Waltheria indica* leaf and root were washed and filled into Soxhlet apparatus and extracted with 3000ml ethanol solvent at a specific boiling point of the solvent for 6–7 h. The extracts were filtered through Whatman filter paper No 1 (24 cm) and the filtrate was concentrated under reduced pressure at a specific temperature. The extracts were dried, weighed, and yield percentages were calculated. The ethanol crude leaf extract was subjected to bioassay-guided fractionation (Figure 1) to give the basic, acidic, non-polar neutral and polar neutral fractions (Okwute *et al.*,2017) and root bark extract were successfully fractionated using solvents using 2.5L each of ethyl acetate, chloroform and chloroform: methanol (ratio 1: 1) (Fatokun *et al.*, 2017).

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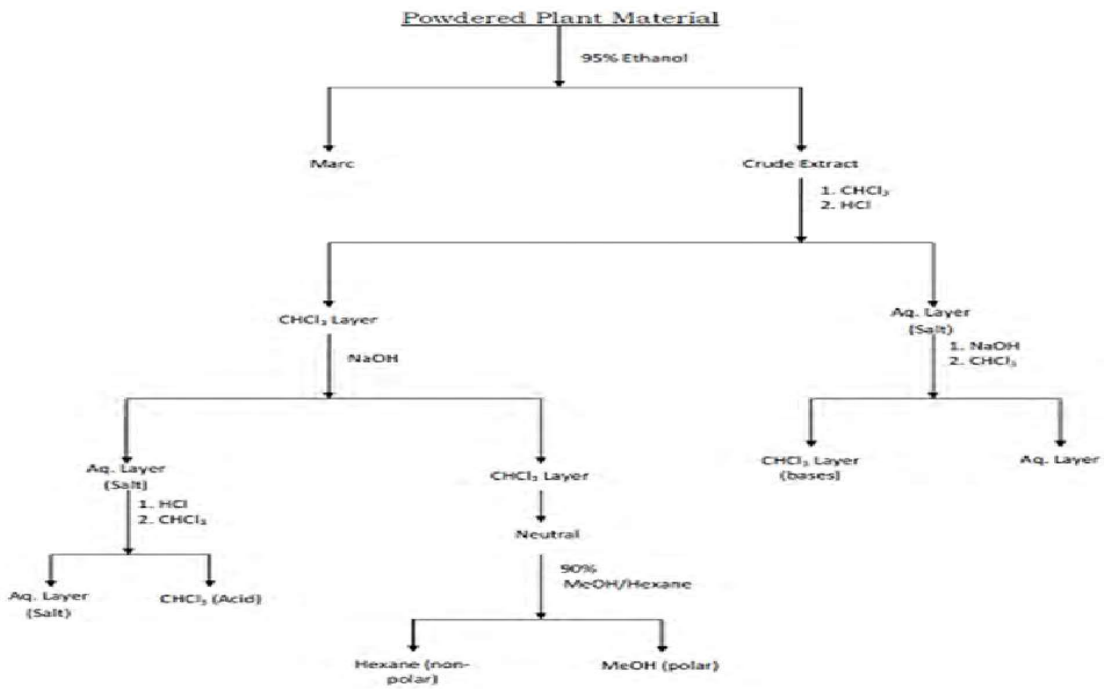

Figure 1: Flowchart of key fractionation steps

Chromatography separation of extracts

The column, 40cm length and 3cm in diameter, was parked with 150g of silica gel (Purasil 60A) to the height of 23cm under reduced pressure. The column was washed with n-hexane to facilitate compact packing. The neutral polar (14.0g) fraction of the leaf extract was loaded on the silica gel column using the wet method and eluted with n-hexane: ethyl acetate (100:1(v/v)) to give 58 fractions of 100ml each. Fractions 19 and 20 with similar TLC profiles were combined and washed with n-hexane to give compound A, a white wool-like crystal (14mg). The ethyl acetate fraction (15g) of the root extract was subjected to column chromatography using n-hexane, ethyl acetate: methanol as solvent to give 50 fractions. Fractions 4-5 were combined, washed with acetone and filtered to give compound B, a colorless wool-like solid (30mg).

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Thin layer chromatography.
Thin layer chromatography of the compounds A and B were carried out using solvent system n-hexane; ethyl acetate (50:50, 80:20 and 90:10) used as mobile phase. The TLC plates (Merck-silica gel 60 F254).

Antimicrobial activity.
Microbial strains.
The antimicrobial activity of the compounds A and B from the solvent fraction of *Walthreria indica* (leaf and root) were tested individually on Gram-positive / Gram-negative bacterial and fungi strains. The well diffusion method of antibacterial test was performed on four tests was performed on four obtained from Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Kaduna state. Bacterial strains were maintained on nutrient agar at 4 °C and sub-cultured every month in the laboratory.

Culture media and plates preparation.
Muller Hinton (MH) was used in antibacterial activity as nutrition for bacterial growth while Saboraud agar was used for fungi growth. For the preparation of the media, the powder Muller Hinton/ Saboraud agar (weight 38 /68 g) and dissolved it in 1000 ml distilled water. The media was autoclaved and after some time poured in Petri plates and left for solidification. Keep all plates in a cool, clean, and dry place when ready to use for experiments.

Standard preparation.
The antibiotic ciprofloxacin and fluconazole were used as the referenced drug for the antimicrobial activity. 0.1mg powder of ciprofloxacin and 0.3mg fluconazole were added to 10 ml distilled water to obtain concentration of 10µg/ml (ciprofloxacin) and 30µg/ml (fluconazole).

Antimicrobial Screening.
The isolate was screened for antimicrobial using a standard procedure (EUCAST,2012) in which the compound A and B (0.001mg) were diluted with 10ml of dimethylsulphoxide (DMSO) to obtain concentrations of 0.1µg/ml. 0.1ml of the standard inoculum of the test bacteria were spread on Mueller Hinton (MHA) agar and the Sabouraud dextrose agar was seeded with fungi strains. Wells were punched in the agar plates and the samples were loaded in the plates. The samples were then incubated for 24 hours at 37°C for the bacteria and at 30°C for 168 hours for the fungi, antimicrobial activity was determined by measuring zone of inhibition around loaded wells.

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Results and Discussion

Results

The results of IR spectral analyses for compounds A and B are shown in Figures 2 and 3, while the ^{1}H and ^{13}CNMR data are presented in Table 1.

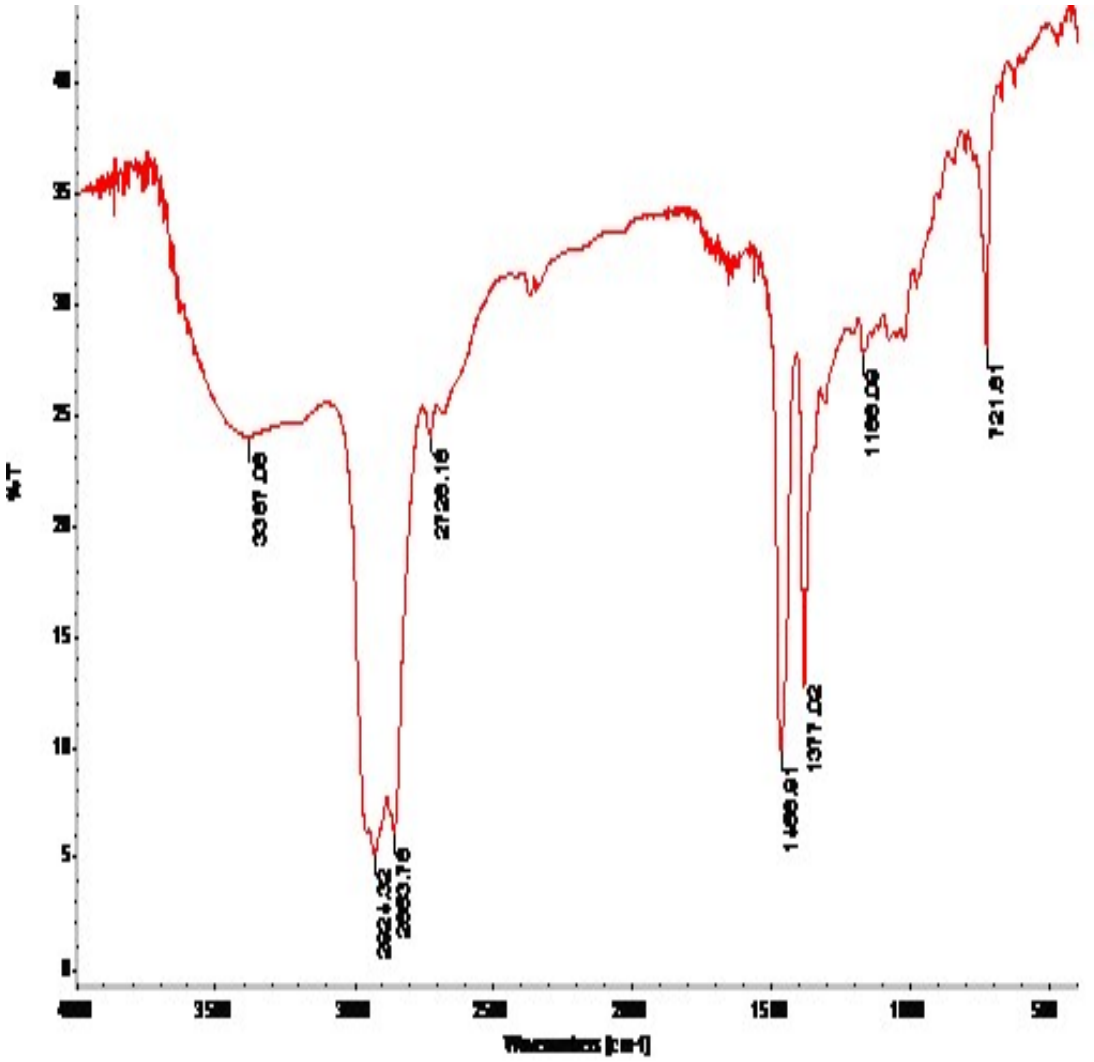

Fig 1: FT-IR spectrum of compound A

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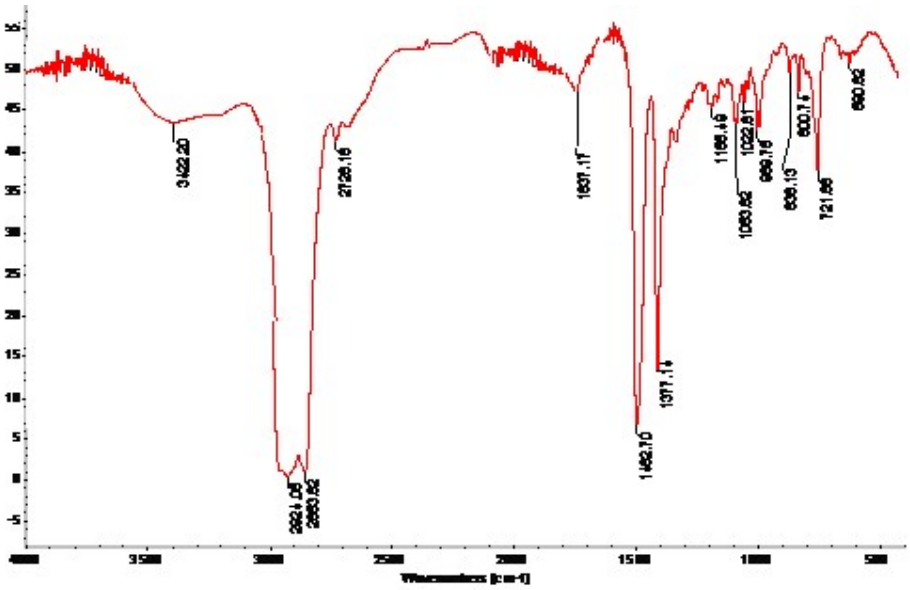

Figure 2: FT-IR Spectrum of compound B

Table 1: ^{1}H and ^{13}C NMR chemical shift values for compound A recorded in $CDCl_3$ (400 MHz).

Carbon atom	^{1}H	^{1}H Literature	^{13}C	^{13}C Literature	Nature of Carbon
C-1	3.63 (2H, t, br, H-1)	3.64	63.12 (t, C-1),	63.1(CH2)	CH2
C-2	1.56-1.51 (2H, m, H-2),	1.56	32.82 (t, C-2),	1.56(m,2H)	CH2
C3-C23	1.23(42H, s, br, H3-H23)	1.25	32..82-22.69(t, C3-C23)	31.9-22.7	(CH2) n
C-24	0.85 (3H, t, H-24)	0.88	14.11(q, C-24)	14.11	CH3

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Table 2: ^{1}H and ^{13}C NMR chemical shift values for compound B recorded in $CDCl_3$ (400 MHz)

Carbon atom	^{1}H	^{1}H Literature	^{13}C	^{13}C Literature	Nature of Carbon
C-1			37.1	37.15	CH2
C-2			31.6	31.56	CH2
C-3	3.15 (1H, m, H-3)	3.51 (, dd, 1H)	71.80(d)	71.71	CH
C-4			43.3	42.19	CH2
C-5			140.75 (s)	140.81	C=C
C-6	5.33 (1H, t, H-6)	5.31 (t, 1H)	121.70 (m)	121.62	C=CH
C-7	2 .04 (2H, m, H-7)		29.7	31.56	CH2
C-8	1.45		31.2.	31.79	CH
C-9			50.1	50.02	CH
C-10			37.2	36.16	C
C-11	1.52		25.4	21.12	CH
C-12	1.58		39.12	39.57	CH2
C-13			42.2	42.10	C
C-14	1.40		56.8	56.76	CH
C-15	1.50		24.3	24.3	CH2
C-16			28.9	28.83	CH2
C-17	1.51		55.9	55.84	CH
C-18	1.06(3H, s, H-18)	1.03 (s, 3H)	12.3	12.15	CH3
C-19	1.06(3H, s, H-19)	0.71 (s, 3H)	19.40 (q)	19.88	CH3
C-20	1.06(3H, s, H-20)	0.91 (d, 3H)	40.4	40.40-40.51	CH
C-21	1.08 (3H, t, H-21)		21.2	20.99	CH3
C-22		5.14 (m, 1H)	138.3	138.23	C=C
C-23	5.02(1H, m, H-23).	4.98 (m, 1H)	129.28 (d)	129.16-129.60	C=C
C-24			51.2	51.13-51.30	CH
C-25			31. 9	31.94	CH
C-26	1.06(3H, d, H-26)'	0.80 (d, 3H)	21.22 (q)	21.23	CH3
C-27	1.06(3H, d, J=7.05 Hz, H-27).	0.82 (d, 3H)	21.0(q)	19.01	CH3
C-28	1.44		25.42	19.01	CH2
C-29	1.06	0.83 (t, 3H)	12.25 (q)	12.25-25.30	CH3

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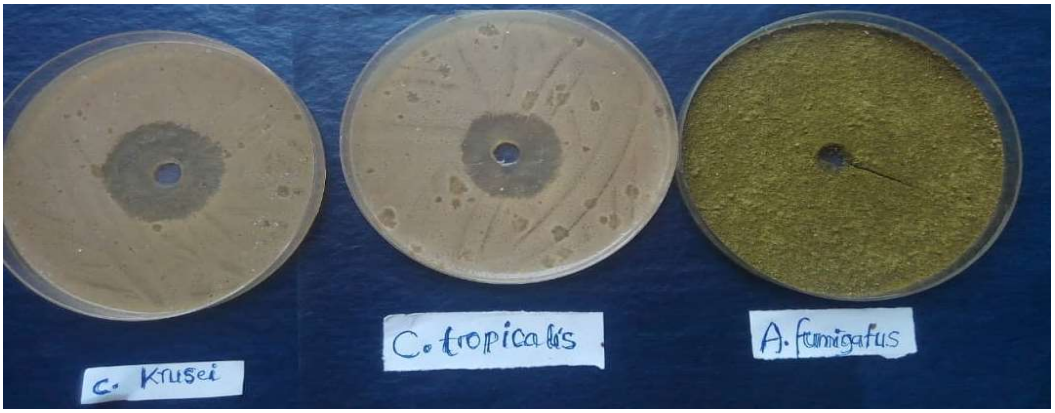

Plate 2: Picture of antimicrobial activity of plates of compound A (n-tetraicosanol) against fungi

Plate 3: Picture of antimicrobial activity of plates of Compound A (n-tetraicosanol) against bacteria

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Plate 4: Picture of antimicrobial activity of plates of Compound B (stigmasterol) against bacteria

Plate 5: Picture of antimicrobial activity of plates of Compound B (stigmasterol) against fungi and bacteria

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Table 3: Zones of inhibition of compounds A and B against test organisms.

Test microorganisms	Compound A	Compound B	Ciprofloxacin (10 µg/ml)	Fluconazole (30 µg/ml)
S. aureus	29 ± 0.15 (S)	32 ±0.65 (S)	32 ±0.45 (S)	_
S.pneumonia	30 ± 0.35 (S)	30 ±1.20 (S)	30 ±0.25 (S)	_
S.pyrogenes	0 (R)	0 (R)	32 ±0.30 (S)	_
K.pneumonia	28 ±0.60 (S)	28 ±0.90 (S)	35 ±0.10 (S)	_
C. ulcerans	31 ± 0.25 (S)	35 ±0.350 (S)	_	0 (R)
C.albicans	0 (R)	0 (R)	_	35 ±0.95 (S)
C. krusei	30 ±0.50 (S)	_	_	35 ±0.55 (S)
C. tropicalis	27 ±0.25 (S)	31 ±0.70 (S)	_	34 ±0.60 (S)

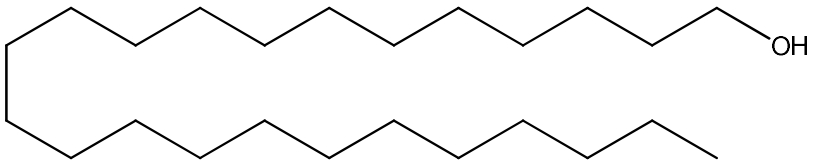
R= Resistant, S= Sensitive, C = Ciprofloxacin, F = Fluconazole

Discussion

The chromatographic separation of neutral polar fraction of leaf and ethyl acetate of root fractions of *Waltheria indica* Linn gave two compounds which were purified and subjected to different spectroscopic analyses for structural elucidation. The compounds a sterol and a long chain fatty alcohol based on their ^{1}H and $^{13}CNMR$ spectra and 2DNMR which were compared to literature. Compound A was obtained as white crystals (14 mg), rf value 0.80;62-68^{o}C. IR spectrum clearly showed the presence of hydroxyl groups at broad band at 3367cm^{-1} (Fig 1). The $^{1}HNMR$ spectrum of compound A (Table 1) suggested that its structure may be n-tetraeicosanol based on the presence of four sets of protons signals at δ3.63ppm, 1.56ppm, 1.23ppm and 0.87ppm corresponding to C-1, C-2, C3- C-23 and C-24, respectively. The $^{13}CNMR$ spectrum had characteristic signals for a fatty acid derivative at δ 63.12ppm (CH2), 31.92ppm (CH2) and 14.11ppm (CH2) corresponding to an oxygenated methylene(C-1), a methylene (C-2) and methylenic chain (C-3-C-23) and methyl (C24) groups respectively.

ISSN: 1597 – 9928.

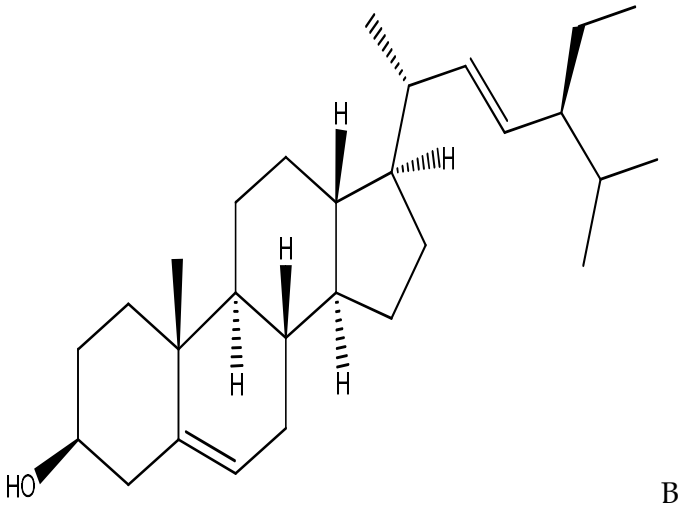
The analysis of the spectroscopic data ^{1}HNMR, ^{13}CNMR, HMQC, HSQC, COSY and HMBC compared. The ^{1}HNMR spectrum are consistent with those reported in the literature (Kamboj, and Saluja, 2011; Muhit *et al*., 2010; Makhafola *et al*., 2017; Olajide and okwute, 2019). Based on the above the compound A has been assigned the name n- tetraicosanol.



A

Compound B was obtained as white woolen (30 mg); *rf* value 0.77, melting point 162-168^{o}C. IR vmax (cm^{-1}) revealed absorption band at 3422cm^{-1} and a moderately intense band at 1165cm^{-1} that's characteristic of OH bond vibrations. The stretching and bending vibrations of aliphatic C- H were noticed by the intense band at 2924cm^{-1} and 2883cm^{-1}. The out of plane C-H vibrations of unsaturated part was observed at 721 cm^{-1}. The corresponding C=C vibration was shown at 1637cm^{-1} as weakly intense bands. The medium for band at 1432cm^{-1} is characteristic of bending frequency of cyclic (CH2) n while vibration frequency at 1377cm^{-1}were due to the out-of-plane bending vibration. The band observed at 1036cm^{-1} was also due to the C–H asymmetric deformation vibrations from the CH2 group.^{1}HNMR spectrum also showed δ 0.78, δ 0.81, 0.88,0.97, 3.25 (1H, m, H-3), 5.37 5.20 (1H, s, H- 22), 5.01ppm (1H, s, H-23). The proton ^{1}HNMRshowed the proton of H-3 as a multiplet at δ3.25 ppm and revealed the existence of signals for olefinic proton at δ5.33ppm (1H, t, H-6) and 5.02 (1H, m, H-23). Angular methyl proton at 1.06 (each 3H, Me × 6) corresponds to C18, C19, C20, 26, 27 and 29 protons respectively. The ^{13}CNMR has shown recognizable signals three quaternary carbons, eleven methane, nine methylenes and six methyl carbons .C5 and C6 alkene carbons with double bonds revealing distinct signals at 141.01 and 121.65. Angular carbon atom signal (C-19, C-18) was also recognized at 19.41 and 12.06. A signal at 71.80 (C-3) indicated a hydroxyl group. The COSY, HMQC and HMBC correlations aided in the assignments of values of all protons for ^{1}H NMR and ^{13}CNMR. This was supported on the basis of COSY, HMQC and HMBC. Thus, compound B was assigned structure stigmasterol. The physical and spectra data are consistent with the reported literature values (Luhata and Munkombwe, 2015; Kandati *et al*., 2012; Raju *et al*., 2012; Habib *et al*., 2007; Jamal *et al*., 2009; Moghaddam *et al*., 2006).

ISSN: 1597 – 9928.



B

Stigmasterol is known to be a precursor in the synthesis of progesterone and as an intermediate in the biosynthesis of androgen, estrogens, corticoids and in the synthesis of vitamin D3 (Kaur *et al*., 2011). Plant phytosterols such as stigmasterol have been reported to possess other pharmacological attributes such as anti-osteoarthritic, anti-hypercholesterolemic, antitumor, hypoglycemic, antimutagenic, antioxidant, anti-inflammatory and CNS effects (Panda *et al*., 2009). The antimicrobial activities of compounds (A and B) isolated from non-polar fraction of *Waltheria indica* Linn leaf and the ethyl acetate fraction of the root was performed against four bacterial and fungi strains including two-gram negative *S. typhimurium* and *E. coli* and two-gram positive *S. aureus* and *B. subtilis* (Tables 3). The compound A showed significant potency against *S. aureus* (29 mm) while B exhibited higher activities (32 mm), both compounds exhibited same activities against *S.pneumonia* (30mm) and *K.pneumonia* (28mm) respectively. Both isolates displayed no activities against *S. pyrogens* (0) and *C.albicans* (0), however, the two compounds showed susceptibility to *C Ulcerans* (31 mm and 35mm), *C. krusei* (30mm) and *C .tropicalis* (27 mm and 31mm) respectively. The compounds A and B competed favorably with ciprofloxacin (10µg/ml) and fluconazole (30µg/ml).

Conclusion

The present work has shown the antimicrobial screening of isolated compounds from *Waltheriaindica* Linn crude leaf and root fractions to be potent against respiratory disease-causing pathogens and their activities competed favourably against standard drugs fluconazole and ciprofloxacin.

ISSN: 1597 – 9928.

Chemical investigation led to the isolation and identification of tetraicosanol and stigmasterol which are bioactive and indeed possess antimicrobial activity. The research thus justifies the claim by the traditional medical practice.

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