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Anti-dermatophytic activity and FTIR analysis of Petroleum ether extracts of *Azadirachta indica A. Juss* seed (Meliaceae)

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Abstract

Plant derived medicines have made massive contributions to the humans over the years. *Azadirachta indica* is used in ethnomedicine to treat ailments such as; eczema, ringworm, sore throat, respiratory tract infection, and scabies. Seeds of *A. indica* were collected, shade dried, pulverized and extracted with petroleum ether using soxhlet and cold maceration. Physicochemical analysis of the seed oil was carried out as described by the Association of Official Analytical Chemists methods. The oil was fractionated using column chromatography, and then Infra-Red (IR) analysis was carried out using a spectrophotometer. The antifungal activities of the oil and Terbinafine standard compound were evaluated by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), using the Agar diffusion method. The petroleum ether oil recovered from the soxhlet method of extraction showed higher percentage of yield, whereas the oil obtained from cold maceration exhibited higher diameter of inhibition zones against several dermatophytes including; *Trichophyton mentagrophytes*, *T. rubrum* and *Microsporum canis*, ranging from 12.00- 16.33 mm. The MIC and MFC values of the oil ranged from 6.25 to > 50% v/v, and 12.5 to > 50% v/v, respectively. Moreover, the seed oil and its fractions recorded classes of several compounds which included; alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide, and alkyl halide. The seed oil had inhibitory potential against a dermatophyte which causes ring worm (*T. corporis*), thus confirmed the claims of using the *A. indica* extract for treatment of such disease. This study aimed to evaluate the antidermatophytic activities of the oil obtained from *A. indica* seeds using petroleum ether, and their fractions.

Keywords: *Azadirachta indica*, Seed oil, Antifungal potential, Fourier-transform infrared

1. Introduction

The history of the relationship between man and the plants is as old as the history of the creation of the world, as man used plants for different purposes (White, 2016). A previous study of Rist, (2014) reported that the use of plants in the alleviation and cure of body ills goes as far back as the history of the human race itself. Arya *et al*., (2016) added that the universal role of plants in the treatment of diseases is exemplified by their employment in all the major systems of medicine, irrespective of the underlying philosophical premise. The use of herbal medicines in the developing world has continued to rise because they are rich sources of novel drugs and their bioactive principles form the basis of medicine such as; pharmaceutical intermediates, and lead compounds used in synthetic drugs (Demain, 2014).

The Neem tree (*A. indica*) which belongs to the family Meliaceae (Mahogany) has a broad dark stem and wide spreading branches. It is a tall evergreen tree with clear foliage originally native of India (Abubakar, 2016). This species is one of the most successful introduced trees in Nigeria. Tiwari *et al*., (2017) added that it is extensively naturalized in drier parts of Nigeria, a successful shade, and used as a fuel plantation tree. The study of Sivasankari *et al*., (2014) reported that various parts of the Neem tree have significant medicinal uses including their uses in the treatment s of leprosy, ringworm, malaria, asthma, and intestinal worms. Nde *et al*., (2015) added that Neem oil is used in cosmetic industries, and is employed in soap making. The current study reports the antidermatophytic potential of the petroleum ether extract of *A. indica* seeds, and the possible functional groups responsible for such activity.

2. Material and methods

2.1. The source of the Dermatophytic strains

The strains of *T. mentagrophytes*, *T. rubrum*, and *M. canis* were provided by the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria.

2.2. The *A. indica* seeds

The *A. indica* seeds were obtained from National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria. Identification and authentication of these seeds were carried out in the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria with a voucher specimen number (900151).

The seeds were washed and foreign materials removed by winnowing. They were oven dried at 50°C, pulverized to powder using milling machine, and then stored at room temperature till further use (Mounguengui *et al*., 2013; Tesfaye *et al*., 2018).

2.3. Extraction of oil from the *A. indica* seeds

2.3.1. The Soxhlet extraction method

According to Awolu and Odedeji, (2011), approximately 1500 g of the neem seed powder was packed inside a muslin cloth, and then placed in a thimble of a soxhlet extractor. The process of extraction was carried out several times with petroleum ether (Sigma Aidrich- Missouri, U.S.A.). The extractor flask was heated at 60°C with the use of a heating mantle; the solvent was vaporized and then condensed into the evaporator. This process continued for 4 h. The seed oil from petroleum ether using soxhlet method of extraction (PES) was recovered from the mixture (oil and the solvent) by the use of rotary evaporator. The obtained oil was stored in a dark bottle at room temperature for further processes.

2.3.2. The cold maceration method

In this process, about 1500 g of the coarsely powdered seed was placed in a conical flask with 1000 ml of petroleum ether, and then allowed to stand at room temperature for a period of 3 d with intermittent agitation (stirring) until the soluble matter dissolve. This mixture was then strained, the damp solid

material was pressed, and then the oil extract was purified by filtration after standing. The seed oil obtained from maceration method of extraction (PEM) was recovered from the mixture (oil and the solvent) by the use of rotary evaporator at room temperature (Jadeja *et al*., 2011).

2.4. The physicochemical analysis of the seed oil extract

Several physicochemical analysis including; the organoleptic properties, density, viscosity, saponification value, iodine value, acid value, peroxide value, and the free fatty acid of the neem seed oil were carried out, as described by the Association of Official Analytical Chemists methods (AOAC. 1990).

2.5. Fractionation of the oil extract

About 50 ml of the crude Pet. ether oil from soxhlet method of extraction (PES) and maceration method of extraction (PEM) were subjected to column chromatography using silica gel G as an absorbent. The column was successively eluted with 100 ml of Pet. ether (100 % v/v), Pet. ether: chloroform mixture (3:1 and 1:1) ratio, and chloroform (100 % v/v) (Sigma Aidrich- Missouri, U.S.A.). The fractions were collected and their antifungal activities were detected using agar well diffusion method according to Deng *et al*., (2012).

2.6. Analysis of the *A. indica* oil and its fractions using the Infra-red (IR) spectroscopy

The oil of *A. indica* seed and its fractions were subjected to spectroscopic analysis (i.e. Infra-red spectroscopy) using Fourier- transform infrared (FTIR) spectrometer (Single-beam, Spectronic 20D; Milton Roy Company, Madrid, Spain). According to Coates and Sanders, (2000), the functional groups present in the oil and its fractions were determined by comparing the vibration frequencies in wave numbers of the samples spectrograph obtained from the FT-IR spectrophotometer.

2.7. Preparation of the dermatophytes inocula

The fungal conidia of *T. mentagrophytes*, T. *rubrum* and *M. canis* were harvested from 7 d old cultures of SDA by washing with 10 ml of sterile normal saline containing Tween 80 (3% v/v) (Sigma Aidrich- Missouri, U.S.A.), in the presence of sterile glass beads to help in dispersing the conidia. Thereafter, these conidial suspensions were adjusted to (1.0×10^5 cfu/ml) by using a single-beam spectrophotometer at OD of 530 nm (Aboh *et al*., 2014).

2.8. Determination of antifungal potential of the *A. indica* seed oil and its fractions

Using the agar diffusion cup plate assay, approximately 100 µl of the conidial suspensions of each test fungus was spread individually on SDA plates, and then a sterile cork borer (6 mm) was used to form wells in these SDA plates. Approximately 0.1 ml of each concentration (100% v/v and 90% v/v) of the oil (PES and PEM) was dispensed individually into the wells; however, the negative control wells were dispensed with 10% DMSO (Sigma Aidrich- Missouri, U.S.A.). These plates were then incubated at 30°C for 72 h. The diameter of zones of inhibition of the tested dermatophytes was measured using a well-calibrated meter ruler. All the tested concentrations were used in triplicate and the assay was repeated twice, in reference to Vorobets and Yavorska, (2016).

2.9. Determination of the minimum inhibitory concentration (MIC) of the seed oil

The MIC was determined by the agar dilution method as modified by Serban *et al*., (2011). About 10 ml of the concentration (50, 25, 12.5, 6.25, 3.125, 1.56 % v/v) of the seed oil was mixed with 10 ml of double strength SDA supplemented with Tween-80 (0.5% v/v), and then poured aseptically into sterile plates. Approximately 10 ml of each fungal conidial suspension (10^5 cfu\ ml) was inoculated individually into 2 sterile filter paper discs placed equidistantly in the petri plates. The plates were allowed to stand for 1 h and then incubated at 30°C for 48 h. The same procedure was repeated using Terbinafine compound

(Sigma Aldrich, U.S.A) as a positive control. The lowest concentration of the crude seed oil that inhibits the visible growth of the tested fungus was considered as the MIC. The experiment was carried out in triplicate (Serban *et al*., 2011).

2.10. Determination of the minimum fungicidal concentration (MFC) of the seed oil

The filter paper discs which showed visible growth at two test concentrations below MIC, discs at MIC, and the discs at two concentrations above MIC were aseptically removed with a sterile forceps and then transferred into 5 ml of sterile SDLM supplemented with Tween 80 (0.3% v/v) followed by incubation at 30°C for 48 h. The MFC were determined as the concentration of the discs which showed no growth on subculture.

2.11. Statistical analysis

All results obtained from this study were expressed as Mean ± SD (Standard Deviation).

3. Results

3.1. Yield of the oil extract and physicochemical analysis

The soxhlet method of extraction yielded higher volume of oil (331ml) with a percentage of yield about 20%, compared to cold maceration (180 ml) with percentage yield of 10.67% (Table 1). The oil from the soxhlet method of extraction (PES) is light brown, denser with higher free fatty acid and low peroxide, iodine and saponification values. On the contrary, the oil from cold maceration (PEM) is dark brown in color with low free fatty acid and relative density, high peroxide, iodine and acid values. Both oils have bitter taste (Table 2).

3.2. Spectroscopic analysis of the *A. indica* seed oil and its fractions

Both of the crude seed oil and its fractions showed classes of several functional groups. The fractions from PES and PEM expressed the presence of alkyl halides, ketone, amide, carboxylic acid, and alkanes, while the crude extracts had additional aromatic compounds and aliphatic esters aside of the previous mentioned functional groups, as clear in Table (3, 4).

3.3. Antidermatophytic activity of the crude seed oil

The antifungal activity of the crude oil from cold maceration at a concentration of 100% v/v recorded 16 mm diameter zone as well as18 mm at a concentration of 90% v/v (Table 5). This is higher than the results obtained for crude oil from soxhlet recording 12- 14 mm diameter zones of inhibition. The activity of the crude oil is prominent on *Microsporum canis* with diameter zones of inhibition of 18 mm. The standard antifungal agent (terbinafine) used had higher diameter zones of inhibition compared to our crude extract recording 34 mm diameter zones of inhibition. The MIC and the MFC of the crude seed oil range from 6.25- 50% v/v (Table 6).

3.4. Antifungal susceptibility profile of the seed oil fractions

The antifungal activity profile of the fractions obtained from column chromatography as presented in Table (7) revealed that the fractions of petroleum ether to chloroform in ratio of (3: 1) and (0: 1) showed significant activity against *T. mentagrophytes*, T. *rubrum* and *Microsporum canis*, with zones of inhibition of diameter of 20.67 mm, while the ratio (1:1) recorded no activity. It is worthy to mention that when the seed oil was fractionated using column chromatography, the fraction has increased activity with higher diameters of zones of inhibition.

4. Discussion

In the current study, the soxhlet method of extraction yielded 10.67- 20% as compared to the results obtained by Awolu *et al*., (2013) who had higher yield percentage of 49%. Moreover, Awolu *et al*., (2013) reported that an increase in the mass of the sample (neem seed) will lead to a higher yield value of the oil. The seed particle size might cause the low percentage of yield recorded in our study.

Table 1: Percentage yield (%) of the oil extracts using different methods of extraction

Solvent	Methods	Yield (ml)	Percentage yield (%)
Pet. ether	Soxhlet	331	20.00
	Maceration	180	10.67

Table 2: Physicochemical characteristics of the extracted seed oils

	Extraction method	
Oil/ Parameters	**Soxhlet**	**Cold marceration**
Colour	Light brown	Dark brown
Smell	Pungent	Pungent
Taste	Bitter	Bitter
Viscosity (mm^2S^{-1}) /60	499.7	486.3
Relative Density (g/ml)	0.912	0.891
Density (g/ml)	0.906	0.885
Refractive Index	1.4645	1.4643
Acid value (mg KOH/g)	6.171	6.520
Peroxide value (mg KOH/g)	0.07	0.28
Iodine value (mgI_2/g)	146.57	152.46
Saponification value (mg KOH/g)	211.78	214.52
Free fatty acid (mg KOH/g)	6.768	3.478

Table 3: Spectroscopic analysis to determine the functional groups present in the seed oil fractions

Sample	Absorption spectra (cm^{-1})	Class of the compounds
B2	420	Alkyl halide
	717.54	Alkenes
	1743.71	Ketone
	2863.42	Carboxylic acid
	2933.83	Alkyl
	3470.08	Amide
B4	364.81	Alkyl halide
	716.58	Alkanes/Alkyl
	1164.08	Aliphatic ester
	1744.67	Ketone
	2867.28	Carboxylic acid
	2930.93	Alkyl
	3478.81	Amide
D2	399.28	Alkyl halide
	715.61	Alkanes
	1165.04	Aliphatic ester
	1743.71	Ketone
	2869.21	Alkyl
	2930.93	Carboxylic acid
	3481.63	Amide

Where; B2: 75% Pet. ether and 25% chloroform solvent system, B4: 0% Pet. ether and 100% chloroform solvent system, D2: 75% Pet. ether and 25% chloroform solvent system

Table 4: Spectroscopic analysis to determine the functional groups present in the seed oil extract

Sample	Absorption spectra (cm^{-1})	Class of the compounds
PES	717.54	Alkyl/ Alkanes
	1165.04	Aliphatic esters
	1458.23	Aromatic compounds
	1743.71	Ketones
	2924.18	Carboxylic acid
	3471.98	Amide
PECM	717.54	Alkyl halide
	1165.04	Aliphatic esters
	1458.23	Aromatic compounds
	1743.71	Ketone
	2924.18	Carboxylic acid
	3471.98	Amide

Where; PES= Oil obtained using Petroleum ether and soxhlet method, PEM= Oil obtained using petroleum ether and cold maceration

Table 5: Susceptibility of the tested dermatophytes to the crude seed oil

Organisms	Zone of inhibition (mm)					
	100 % v/v		90 % v/v			
	PES	PEM	PES	PEM	DMSO	TBF (32 µg/ ml)
TM	14.67 ± 0.58	16.33 ± 0.58	12.33± 0.58	14.00±0.58	0	34.67 ± 0.58
MC	13.00 ± 0.00	12.00 ± 0.00	12.67±0.58	18.33±0.58	0	35.67 ± 0.58
TR	12.00 ± 0.00	14.67 ± 0.58	12.33±0.58	14.00±0.00	0	32.67 ± 0.58

*Values are mean inhibition zone (mm) ± S.D of triplicate. Where; DMSO= 10% Dimethylsulfoxide, TM= *Trichophyton mentagrophytes*, MC= *Microsporum canis*, TR= *Trichophyton rubrum*, TBF= Terbinafine, PES= Oil from soxhlet, and PEM = Oil from cold maceration

Table 6: Minimum inhibitory (MIC)/ minimum fungicidal concentrations (MFC) (% v/v)

	MIC		MFC		
	Soxhlet	**Cold maceration**	**Soxhlet**	**Cold maceration**	**TBF (µg/ml)**
TM	50.00 ± 0.00	>50.00 ± 0.00	>50.00± 0.00	> 50.00 ± 0.00	< 2.00 ± 0.00
MC	6.25 ± 0.00	12.50 ± 0.00	12.50± 0.00	25.00± 0.00	4.00 ± 0.00
TR	50.00 ± 0.00	25.00 ± 0.00	> 50.00 ± 0.00	>50.00± 0.00	32.00 ± 0.00

*Values are mean inhibition zones (mm) of three replicates, ± S.D of recorded results

Table 7: Antifungal susceptibility profiles of the different *A. indica* seed oil fractions

	Diameter of zones of inhibition (mm)		
	TM	**MC**	**TR**
Solvent systems			
B1	0	0	0
B2	15.33 ± 0.58	0	12.33 ± 0.58
B3	0	0	0
B4	12.33 ± 0.58	11.00 ± 0.00	13.00 ± 0.00
D1	0	0	20.67 ± 1.16
D2	16.67 ± 0.58	0	0
D3	0	0	0
D4	0	0	0

*Values are mean inhibition zone (mm) of three replicates, ± S.E of three replicates. Where; B= Oil from soxhlet, D= Oil from cold maceration, 1= 100% Pet. ether and 0 % chloroform solvent system, 2= 75 % Pet. ether and 25 % chloroform solvent system (3 : 1), 3= 50% Pet. ether and 50 % chloroform solvent system (1 :1), 4 = 0 % Pet. ether and 100 % chloroform solvent system, TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*.

Most vegetable oil has density between 0.876-0.8811 kg/ m3. The density of the recovered neem oil indicated that it has a higher density of 0.906 kg/ m^3.This implies that oils obtained from soxhlet extraction are denser compared to neem oil obtained from cold maceration. This result is in disagreement with Awolu *et al*., (2013) who observed a density of 0.889kg/m3 when heat is applied during extraction. In addition, the saponification value obtained in this study recorded a higher value compared to Awolu *et al*., (2013).

Plants contain bioactive constituents which can have antimicrobial activity against bacteria, fungi and viruses. Current results revealed some classes of compounds with antifungal potential recovered from the test plant which include; aromatic compounds, aliphatic esters, ketone, carboxylic acids etc. Previously, García-Sosa *et al*., (2011) pointed out that secondary metabolites (triterpenes) are responsible for the antimicrobial activity of the plant extracts, thus these metabolites may have some of the aforementioned functional groups.

The oil extracts obtained from petroleum ether using cold maceration tend to have higher activity compared to the other oil extracts, as this method of extraction probably extracted most of the components responsible for the antifungal activity, in agreement with Dube and Tripathi, (1987). This research revealed an antifungal potency as low as 6.25- 50% v/v, in accordance with the findings of Mahmoud *et al*., (2011), who reported that 20% ethyl acetate extract gave the strongest inhibition compared with the activity of the other extracts. Moreover, in accordance with our findings, Natarajan *et al*., (2002) showed that the leaf extract had a potent antifungal activity against *T. mentagrophytes*, *M. nanum* and *Epidermophyton floccosum*.

Conclusion

The Petroleum ether extracts of *A. indica* seed have shown to possess good antidermatophytic potencies against *T. mentagrophytes*, *T. rubrum* and *M. canis*, which are known causative agents of ring worm infections in human. In addition, they also have functional groups responsible for such activities. Findings of this study demonstrated the need for further investigations to exploit the economic value of *A. indica* plant to address fungal ringworm infections, through manipulating Gas Chromatography and Mass Spectroscopy to identify such active compounds responsible for the antidermatophytic potential.

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Conflict of interest

The authors declare no conflict of interests

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