

Full Length Research Paper

Anti-dermatophytic activity of hexane extracts of *Azadirachta indica* A. Juss.

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Dermatophytes cause superficial fungal infections that pose public health problem to man and animals. Long term treatment with antifungal agents is required to control these infections. Various parts of *Azadirachta indica* are claimed to have significant medicinal value in treatment of infections especially ringworm. To determine the antifungal activity of the oil and fractions against dermatophytes isolated from clinical cases. Seeds of *A. indica* were collected, dried, grinded and extracted with hexane using soxhlet and cold maceration. Physicochemical analysis of the oil was carried out as described by Association of Official Analytical Chemists methods (AOAC, 1990). Fractionation of the oil was subjected using column chromatography and Infra-Red (I.R) analysis using spectrophotometer. Swab samples were collected from pupils in Kudan, Kaduna State with suspected cases of *Tinea corporis* (ringworm). The causative fungal organisms were isolated and identified by routine mycological and biochemical procedures. The antifungal activities of the extracts were evaluated by determining the minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and diameter zones of inhibition. Hexane extract from soxhlet method of extraction showed higher diameter zone of inhibition against isolated dermatophytes (*Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Microsporium canis*), ranging from 14.33 – 17.33 mm. The MIC and MFC values of the extract range from 3.13 to > 50% v/v and 50 to >50%v/v respectively. The oils and fractions recorded class of compounds which include alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide. Hexane extract of seed of *A. indica* extracted by soxhlet and cold maceration methods had inhibitory activities against the causative agents of *T. corporis* (ringworm) when tested.

Key words: Dermatophytes, *Azadirachta indica*, antifungal, Fourier-transform infrared (FT-IR).

INTRODUCTION

Dermatophytes are fungi that require keratin for growth. These fungi can cause superficial infection of the skin, hair and nails. Dermatophytes are spread by direct contact from other people, animals and soil.

Dermatophytes which comprise a group of closely related fungi made up of three genera; *Trichophyton*, *Microsporium*, and *Epidermatophytes*, have the ability to invade the stratum corneum of the epidermis and

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keratinized tissues derived from it, such as skin, hair, and nail of humans and other animals. They cause superficial fungal infections that pose public health problems to man and animals. Dermatophytes infections can be disfiguring and recurrent and generally need long-term treatment with antifungal agents (Tortorano et al., 2014).

The use of plants in the alleviation and cure of bodily ills goes as far back as the history of the human race itself. The universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine irrespective of the underlying philosophical premise (Evans, 2002).

Use of herbal medicines in the developed world has continued to rise because they are rich source of novel drugs and their bioactive principles form the basis in medicine, pharmaceutical intermediates and lead compounds in synthetic drugs (Akula et al., 2003). More importantly in Africa, particularly West Africa, new drugs are often beyond the reach of the poor such that up to 80% of the population uses medicinal plants as remedy against infections and diseases (Thomford et al., 2015).

Various parts of neem tree are claimed to have significant medicinal importance: which include uses in leprosy, malaria, asthma, intestinal worms. Topical application of neem seed oil can cure dermatological diseases within 3-4 days (Akula et al., 2003). Neem oil used in cosmetic industries is a product of the neem tree and is employed in soap making (Edwards, 2015). Nimbolide, gedunin and nimbin (triterpenoids) are chemical compounds with antifungal activity from neem seed oil (Hashmat et al., 2012). The objective of this research is to determine the *in-vitro* antifungal activity of n-hexane extracts and fractions of *Azadirachta indica* against clinical isolates. The work aimed at a therapeutic alternative against dermatophytes and the functional groups responsible for such activity.

MATERIALS AND METHODS

Media used were: Sabouraud Dextrose Agar (Oxoid, Basingstoke, U.K.), Sabouraud Dextrose Liquid Medium (SDLM: Oxoid, Basingstoke, U.K.) and Sabouraud Dextrose Agar + Chloramphenicol + Cycloheximide (Cat No.21089.00 Deben Diagnostics Limited, U.K). The media were prepared according to the manufacturer's instruction.

Chemical and reagents used include: Tween 80 (Sigma Aidrich-Missouri, U.S.A.), Dimethylsulfoxide (DMSO: Sigma Aidrich-Missouri, U.S.A.), n-Hexane (Sigma Aidrich- Missouri, U.S.A.), Chloroform (Sigma Aidrich- Missouri, U.S.A.), Christensen's urea agar medium (Sigma Aidrich- Missouri, U.S.A.) and Lactophenol cotton blue (Sigma Aidrich- Missouri, U.S.A).

Antifungal used: Terbinafine powder (Cat No. F8929, Sigma Aidrich, U.S.A.).

Instrument

Spectrophotometer, Single-beam, Spectronic 20D; Milton Roy Company, Madrid, Spain.

Collection, extraction and fractionation

Plant seeds were obtained from National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria. Identification and authentication was done 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, sun-dried and foreign materials removed by winnowing. The cleaned neem seeds were oven dried at 50°C and then grinded into powder using milling machine at National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State.

Oil extraction

Soxhlet extraction: Soxhlet extraction method was used to extract the oil from the processed seeds. One thousand five hundred (1500 g) of the neem seed powder was packed inside a muslin cloth and placed in a thimble of Soxhlet extractor. A round bottom flask containing n-hexane was fixed at the end of the extractor and a condenser was tightly fixed at the bottom end of the extractor. Extraction was done each time with n- Hexane. The flask was then heated at 60°C with the use of a heating mantle. The solvent was vaporized and condensed into the evaporator. The process continued for 4 h. Oil was recovered from the mixture (oil and solvent) by the use of rotary evaporating process. The oil was obtained and stored in a bottle for further processes (Awolu et al., 2011).

Cold maceration: In this process, 1500 g of the coarsely powdered crude plant seed was placed in a stoppered container (conical flask) with 1000 ml of n- hexane and was allowed to stand at room temperature for a period of 3 days with intermittent agitation (stirring) until the soluble matter dissolved. The mixture was then strained, the marc (the damp solid material) pressed, and the oil extract was clarified by filtration after standing. The oil was recovered from the mixture (oil and solvent) by the use of rotary evaporator.

Physicochemical analysis

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

Fractionation of oil extract

A portion (50 ml) of crude n-hexane oil extract was subjected to column chromatography using silica gel G as an absorbent. The column was successively eluted with hundred milliliters (100 ml) of n-hexane (100%), hexane: chloroform mixture (75:25 and 50:50%) and 100% chloroform.

Spectroscopic analysis of *A. indica* oil and its fractions

Infra-red (I.R) analysis

Infra-red (I.R) analysis of the absorption spectra of *A. indica* oil and the fractions that have activity against dermatophytes was conducted at National Institute for Chemical Technology (NARICT), Basawa, Zaria, Kaduna state of Nigeria. A Fourier- transform infrared (FTIR) spectrometer was used in which the sample was

placed. The spectrometer directed beams of IR at the sample and measured how much of the beam and at which frequencies the sample absorbs the infrared light. The molecular identities were determined through a reference database which houses thousands of spectra, so samples can be identified. 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 an FT-IR spectrophotometer (Coates, 2000).

Isolation and identification of dermatophytes

Thirty swab samples of *Tinea corporis* (ringworm) were collected from infected skins of pupils at Kudan, Kaduna State using sterile scalpels. Affected areas were cleansed with 70%v/v ethanol, allowed to dry and light scrapings from the edge of the lesions were taken using a blunt sterile scapel blade. The specimens were placed in well labelled clean white envelopes. Mycological analysis of the 30 specimens from the suspected infected sites of the participants was carried out in the Pharmaceutical Microbiology Laboratory at the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Kaduna State, Nigeria.

The specimens were inoculated into 10 ml Sabouraud Dextrose Liquid Medium (SDLM) and incubated for 48 h. Growth from the broth was then streaked on Sabouraud Dextrose Agar containing chloramphenicol and cycloheximide and incubated at 30°C for 14 days. Cultures were examined weekly for sporulation. Colonies of dermatophytes were later subculture on respective petri-dishes containing SDA and incubated at 30°C for 21 days. The isolates were identified by routine mycological and biochemical procedures (Urease Test, Corn meal test and Nutritional test) as modified by Refai et al. (2013).

Standardization of the inoculum

Trichophyton mentagrophytes, *Trichophyton rubrum*, *Microsporum canis*, *Candida albican* and *Aspergillus niger* fungal spores were harvested from 7 day old Sabouraud dextrose agar (SDA) slant cultures by washing with 10 ml sterile normal saline containing 3% v/v Tween 80 with aid of sterile glass beads to help in dispersing the spores. Thereafter, the spore suspension were standardized to 1.0×10^5 spores / ml by using a single-beam spectrophotometer at 530 nm (OD530) adjusted to 70-72% transmittance for *T. mentagrophytes*, *T. rubrum* and *M. canis*. All adjusted suspensions were quantified by spreading 100 μ l on Sabouraud dextrose agar plate and incubated at 30°C for 72 h for dermatophytes (Aberkane et al., 2002). Standardized isolates were maintained at 4°C (in the refrigerator) until required for use.

Determination of antifungal activity of *A. indica* A. Juss

The plates were allowed to dry at 30°C temperature in a sterilized incubator. Using the agar diffusion cup plate method, a sterile cork borer (6 mm) was used to bore wells in the agar plates. The bottoms of the wells were each sealed with a drop of molten SDA. Using micropipette, 0.1 ml each of the different graded concentrations of the extract (soxhlet and cold maceration) were dispensed into the wells marked 100 and 90%v/v and 10% DMSO (used in diluting the extracts and a negative control). These were allowed to diffuse into the agar at room temperature for an hour before incubation at 30°C for 72 h. The diameter of zones of inhibition of the test organisms were measured to the nearest millimetre using a well-calibrated meter ruler. The experiment was carried out in triplicate.

Table 1. Biochemical and mycological tests of dermatophytes.

Test	TM	MC	TR
Urease test	+	-	-
Corn meal	-	-	+
Nutritional test	-	+	-

TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*, Urease test, + = Pink colour, Corn meal, + = Dark red colour, Nutritional test, + = Growth.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined by agar dilution method as modified by Serban et al. (2011). Ten millilitres of the graded concentration of the crude extracts was mixed with 10 ml of double strength SDA supplemented with 0.5%v/v Tween-80 and poured aseptically into sterile plates. The plates were allowed to set. Ten microlitres of the standardized organisms containing 10^6 CFU ml^{-1} was inoculated on the equidistantly placed sterile filter paper disc. The plates were allowed to stand for one hour and then incubated at 30°C for 48 h. The same procedure was repeated using Terbinafine. The lowest concentration of the agent that inhibits the visible growth of the test organisms was taken as the MIC. The experiment was carried out in triplicate.

Determination of minimum fungicidal concentration (MFC)

The filter paper discs showing visible growth at two test concentrations below MIC, discs at MIC and discs at two concentrations above MIC were aseptically removed with the aid of a sterile forceps and transferred into 5 ml sterile SDLM supplemented with 0.3%v/v Tween 80 and incubated at 30°C for 48 h. Minimum fungicidal concentrations were determined as the lowest concentration resulting in no growth on subculture.

Statistical analysis

All the data obtained from the studies were expressed as mean \pm standard deviation (SD).

RESULTS

Dermatophytes isolated with *T. corporis* infections

A total of 18 dermatophytes consisting of *Trichophyton species* (16 isolates) (Table 1).

<i>T. mentagrophytes</i>	10 Isolates
<i>T. rubrum</i>	6 Isolates
<i>M. canis</i>	2 Isolates

Extraction of oil from *A. indica* A. Juss seeds

The percentage yields of the oils from *A. indica* seeds using n-hexane and extraction methods are shown in Table 2. Soxhlet method of extraction yielded more oil than the cold maceration method; about 4% higher than

Table 2. Percentage yield of oil extracts using various solvents and methods of extraction.

Solvent	Methods	Yield (ml)	Percentage yield
Hexane	Soxhlet	220	13.33
	Maceration	154	09.33

Table 3. Physicochemical characteristics of the extracted seed oil.

Oil/ parameter	Extraction method	
	Soxhlet	Cold Maceration
Colour	Light brown	Dark brown
Smell	Pungent	Pungent
Taste	Bitter	Bitter
Viscosity (mm^2S^{-1}) /60	492.8	449.4
Relative density (g/ml)	0.917	0.914
Density (g/ml)	0.911	0.908
Refractive Index	1.4652	1.4647
Acid value (mgKOH/g)	6.311	7.013
Peroxide value (mEq KOH/g)	0.10	0.26
Iodine value (mgI_2/g)	154.18	172.58
Saponification value (mgKOH/g)	207.54	223.03
Free fatty acid (mgKOH/g)	4.982	8.648

cold maceration.

Physicochemical characteristics of *A. indica* seeds oil

The physicochemical and organoleptic characteristics of the oils obtained via the two methods of extractions (soxhlet and maceration) using n-Hexane is shown in Table 3. Hexane oil from maceration recorded higher acid, iodine, saponification and free fatty acid values compared to values for the other oil. Oil obtained by soxhlet method was generally lighter in colour; more viscous, denser with lower peroxide and acid values. Oil obtained by maceration was associated with lower viscosity higher acid, iodine, and free fatty acid values. The variations can be attributed to the different methods involved in the oil extraction as reported by Jessinta et al. (2014).

Antifungal activities of the oil fractions

A total of 8 fractions were collected from the elution of the n-hexane and chloroform of the two oil extracts. The results of their antifungal test are presented in Table 4. Antifungal activities of the fractions varied among the fractions. None of the fractions obtained from oil extracted by soxhlet method using Hexane as solvent

had inhibitory activity against the test isolates. On the other hand, fractions from the oil obtained by maceration had activity only against the Trichophyton species (*T. mentagrophytes*).

Determination of the functional groups present in the oils and its fractions

The spectroscopic analysis (FT-IR) interpretation detecting functional groups of the oils and the fractions having antifungal activity is shown in Tables 5 and 6 respectively. Both the oils and fractions recorded class of compounds which include alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide. However, there was a significant difference in FT-IR of the oil extract and its fractions, where the oil extract recorded the presence of aromatic compounds.

A strong and broad absorption band or frequency from 2500- 3500 cm^{-1} showed the presence of O – H stretch from carboxylic acid as seen in all the spectra, a strong absorption band from 1740- 1755 cm^{-1} revealed the presence of C = O bond for a five member cyclic ketone. A weak absorption band from 3400-3500 showed the presence of N – H stretch from an amides. The absorption frequency with a strong band from 1160-1210 cm^{-1} revealed the presence of O = C – O – C from an aliphatic esters. A medium absorption band 1450-1600 cm^{-1} showed the presence of C = C ring from an aromatic

Table 4. Antifungal susceptibility profiles of test fungal isolates to fractions of *A. indica* oil.

Test organisms Solvent systems	Zone of inhibition (mm)		
	TM	MC	TR
A 1	0	0	0
A2	0	0	0
A3	0	0	0
A4	0	0	0
C1	0	0	0
C2	16.33 ± 0.58	0	0
C3	0	0	0
C4	12.33 ± 0.58	0	0

*Values are mean inhibition zone (mm) ± S.D of three replicates, cork borer- 6 mm, A = Extract from soxhlet, C = Extract from cold maceration, 1= 100% Hexane and 0% chloroform solvent system, 2 =75% Hexane and 25% chloroform solvent system, 3= 50% Hexane and 50% chloroform solvent system, 4 = 0% Hexane and 100% chloroform solvent system.

Table 5. Functional groups present in the oil fractions.

Sample	Absorption (cm ⁻¹)	Class of compound
C2	412.78	Alkyl halide
	718.51	Alkenes
	1743.71	Ketone
	2862.46	Carboxylic acid
	2924.18	Alkyl
	3471.98	Amide
C4	384.81	Alkyl halide
	715.61	Alkenes
	1743.71	Ketone
	2865.35	Carboxylic acid
	2929	Alkyl
	3472.95	Amide

C2 = 75% Hexane and 25% chloroform solvent system, C4 = 0% Hexane and 100% chloroform solvent system.

Table 6. Functional groups present in the oil extract.

Sample	Absorption cm ⁻¹	Class of compounds
HS	416.64	Alkyl halide
	1165.04	Aliphatic esters
	1458.25	Aromatic compounds
	1743.71	Ketone
	2924.18	Carboxylic acid
	3471.98	Amide
HCM	1165.04	Aliphatic ester
	1458.23	Aromatic compound
	1743.71	Ketone
	2924.18	Carboxylic acid
	3464.27	Amide

Key: HS = Oil obtained by n-Hexane using soxhlet, HCM = Oil obtained using n-Hexane using cold maceration.

Table 7. Susceptibility of the test fungi to the extracts of *A. indica* at 100 and 90% v/v.

Organism	Zone of inhibition (mm)					
	100%w/v		90%w/v		DMSO	TBF (32 µg/ml)
	Soxhlet	Cold maceration	Soxhlet	Cold maceration		
TM	17.33 ± 0.58	14.00 ± 0.00	14.67 ± 0.58	13.33 ± 1.16	0	34.67 ± 0.58
MC	15.33 ± 0.58	11.67 ± 0.58	16.00 ± 0.00	12.00 ± 0.00	0	39.67 ± 0.58
TR	14.33 ± 0.58	14.00 ± 0.00	16.33 ± 0.58	14.00 ± 0.00	0	32.67 ± 0.58

*Values are mean inhibition zone (mm) ± S.D of triplicate cork borer- 6 mm. DMSO = 10% Dimethylsulfoxide, TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*, TBF = Terbinafine

Table 8. Minimum inhibitory/fungicidal concentrations (%v/v).

Parameter	M.I.C		M.F.C		TBF (µg/ml)
	Soxhlet	Cold maceration	Soxhlet	Cold maceration	
TM	50.00 ± 0.00	50.00 ± 0.00	50.00 ± 0.00	> 50.00 ± 0.00	< 2.00 ± 0.00
MC	3.13 ± 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 zone (mm) ± S.D of triplicate determination.

compounds and frequency bands of 200-500 cm^{-1} revealed the presence of an alkyl halide.

Antifungal activities of n-hexane extract of *A. indica*

Table 7 shows the results of the susceptibility of the isolates to the undiluted and diluted with DMSO in a ratio of 90 to 10%v/v extracts of *A.indica*. Hexane extract from soxhlet method of extraction had shown higher diameters of zones of inhibition against the isolated dermatophytes. Activity was exerted mostly on *T. mentagrophytes* and *M. canis*. However, the levels of inhibition against the dermatophytes were far less compared those exerted by terbinafine. The level of inhibitory also changed following dilution of the n-hexane extracts with DMSO. The results of the susceptibility of the isolates from soxhlet showed higher activities against isolated dermatophytes.

Minimum Inhibitory Concentration and Minimum Fungicidal Concentration values

The MIC of n-hexane extract of *A. indica* is as shown in Table 8. The MIC against *T. mentagrophytes* and *T. rubrum* was 50%v/v by both extraction methods but *M. canis* was as low as 3.13%v/v (by soxhlet) and 12.5%v/v (by cold maceration). The MFC against *T. mentagrophytes* and *T. rubrum* is 50%v/v and above by both extraction methods while against *M. canis*, it was as low as 12.5%v/v (by Soxhlet and 25%v/v (by cold maceration).

DISCUSSION

Plants have been known to contain bioactive constituents with inhibitory substances against bacteria and fungi (Kadhim et al., 2016). Results from this study reveal a degree of antifungal activities of the plant seed oil which varied from one clinical isolate to another. The gradual increase in the diameter zones of inhibition with increase in concentration of the oil shows that the inhibitory action on the fungi clinical isolates is dependent on the amount of drug used (Table 8). Generally, oil obtained from soxhlet method of extraction had better antifungal activity on *T. rubrum*, *T. mentagrophytes*, *M. canis*. The zones of inhibition of the oil against all the clinical isolates tested at 100% v/v was comparable to the standard drugs (Terbinafine) used. The antifungal activity of the oil and its fraction can be linked to the presence of secondary metabolites which have been shown to possess bioactive properties. The seed oil which is usually extracted by steam or solvents from crushed seed consists mainly of triglycerides and large amounts of triterpenoids (Takase et al., 2015). The oil extracts and some fractions revealed an antifungal activity against dermatophytes, the classes of compounds present include, alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide and aromatic compounds (Supplementary Figures 1 and 2). The presence of ketone C = O functional group from a five membered cyclic ring and O = C - O - C functional group from an aliphatic esters revealed that gedunin, nimbin, nimbinin and nimbolide constituents are likely present in the oil extracts and its fractions. Thus, these functional groups of ketone, hydroxyl, carboxyl and

aliphatic ester may be responsible for the antidermatophytic activity of neem seed oil. The constituents nimbinin, gedunin, nimbin and nimbolide are terpenoid compounds and a work done by Nuzhat and Vidyasagar (2014) revealed that terpenoid compounds are responsible for antidermatophytic activity.

The predominance of *Trichophyton species* as the causative agent of *Tinea corporis* (Ringworm of the body) is not unexpected. Most studies found *T. rubrum* and *T. mentagrophytes* as the commonest etiological agents of dermatomycosis (Hayette and Sacheli, 2015). *Trichophyton* spp accounted for 76.2% of *T. corporis* in India (Harinath, 2016). *T. mentagrophytes* has been cited as the major causative agent for *T. corporis* and is known to account for as much as 47.6- 69.5% of all dermatophytic infections (Pranab et al., 2003).

The key discovery that has emerged from this research is that the oil extracted from *A. indica* A Juss seeds using both soxhlet and maceration methods of extraction inhibited the growth of clinical isolates of *dermatophytes* (*T. mentagrophytes*, *T. rubrum* and *M. canis*). This study has established the *in-vitro* activity of *A. indica* seed oil against dermatophytes isolated from clinical sample. It has also shown that the oil extract can be used to carry out further studies (bioassay guided) so as to isolate lead compounds responsible for such activity and enable its redeployment as potent antidermatopytic agent.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Figures

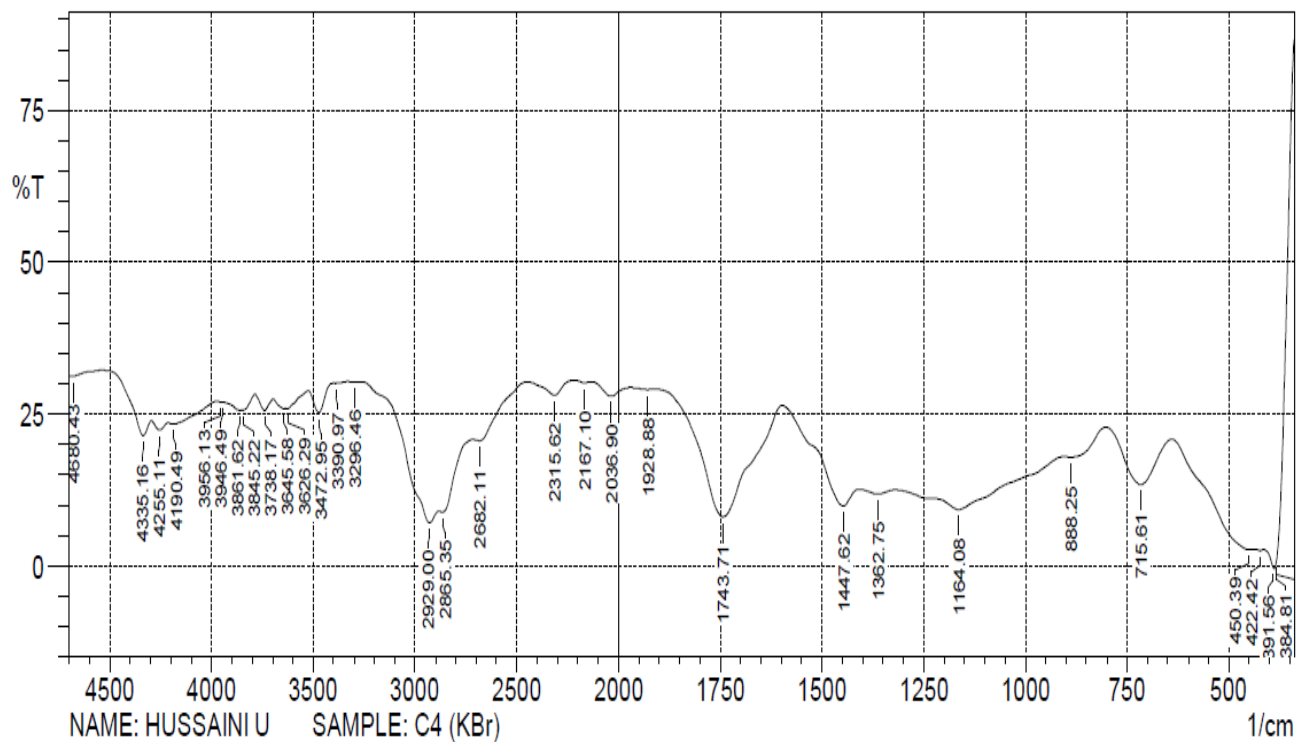
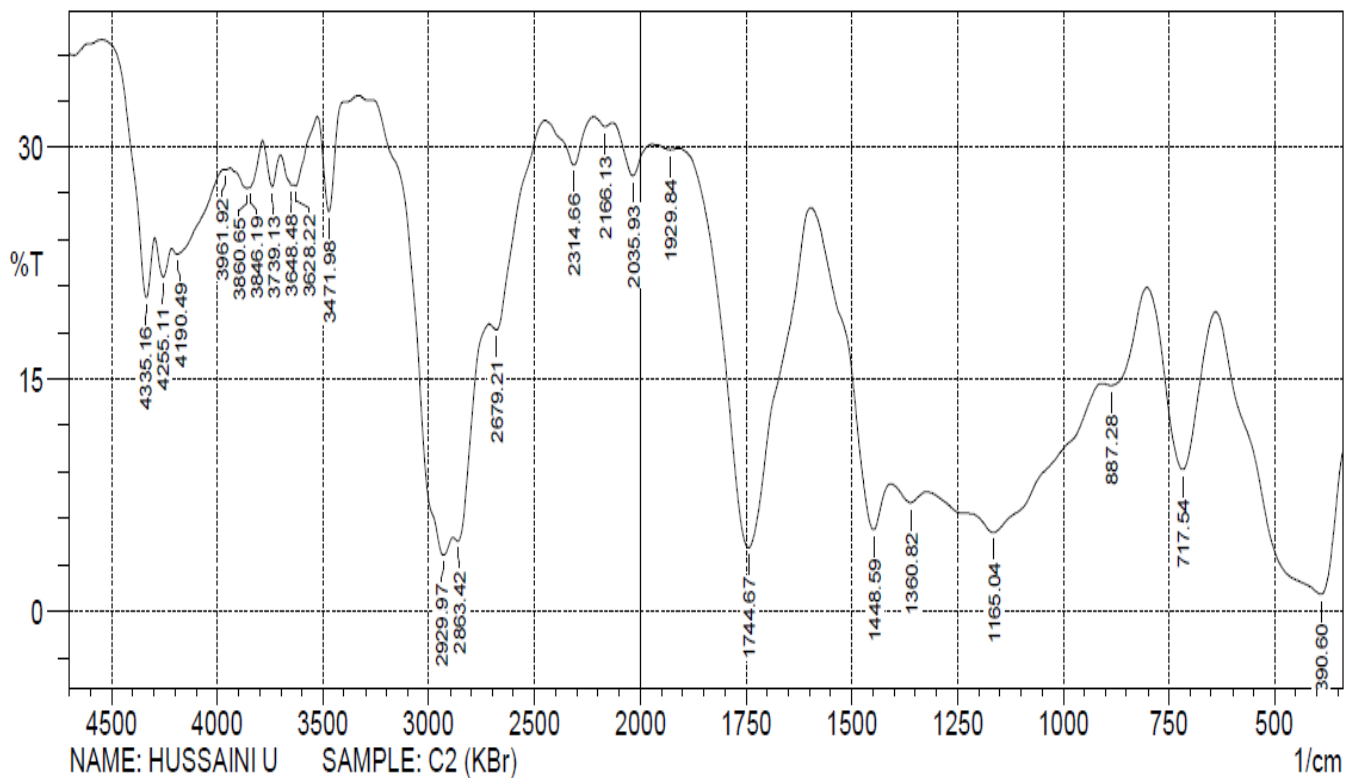


Figure 1. Infra red (IR) absorption spectra of oil fraction having antifungal activity against *T. mentagrophyte*.

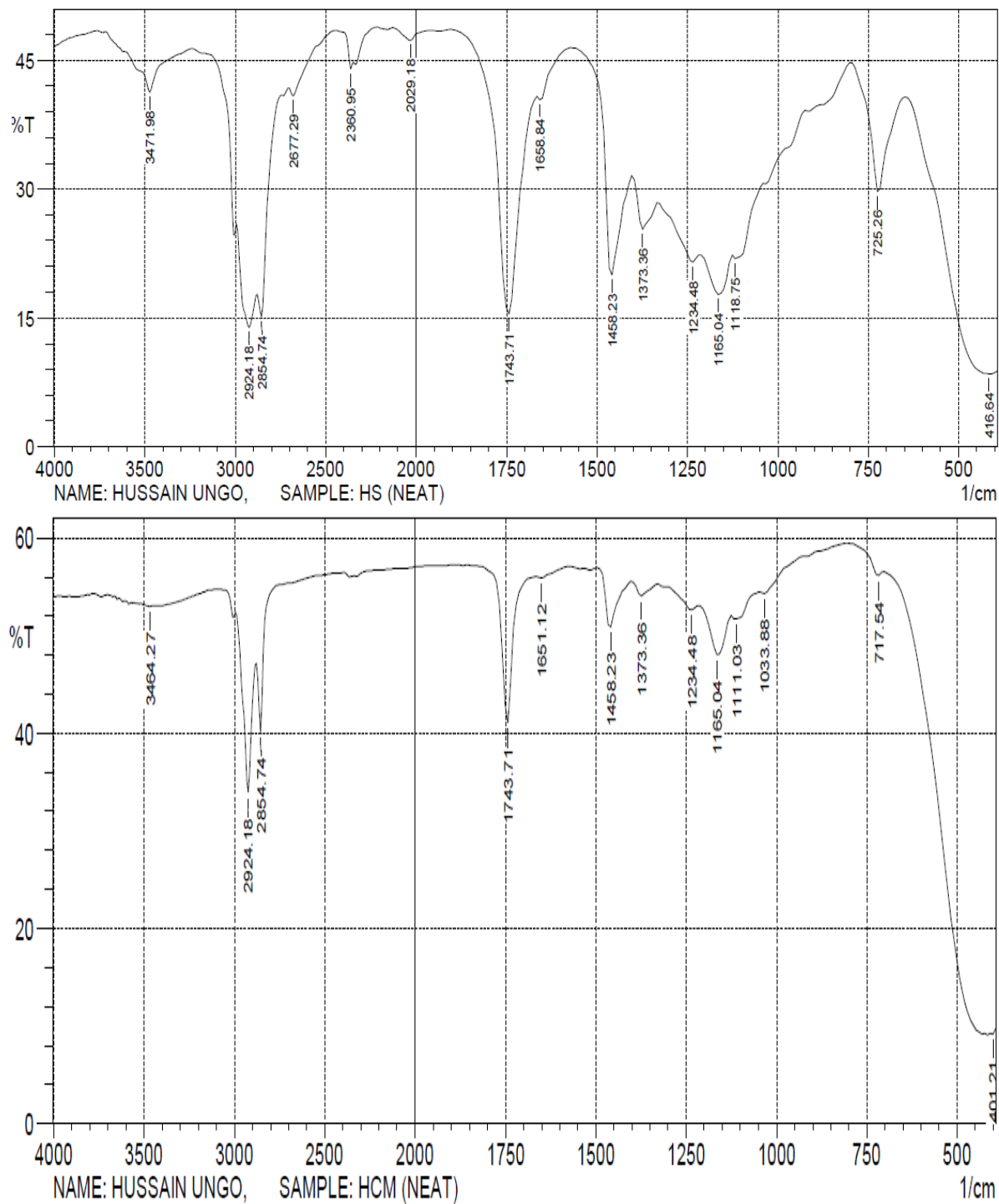


Figure 2. Infra red (IR) absorption spectra of oil extracted from Soxhlet (HS) and maceration (HCM) using hexane.