Isolation and characterization of active fraction of *pergularia tomentosa* against dermatophyte

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Abstract: Pergularia tomentosa is reported to have antifungal (Hassan et el., 2007), anti-bacterial, (Dangoggoet el., 2002) and anti-dermatophyte activity (Gill, 1992). This work was aimed at isolating the most active fraction against some dermatophyte isolate, phytochemical screening of crude, n-hexane, chloroform and ethanol extracts indicate the presence of alkaloid, flavonoid, tannin, glycoside, saponin, and phenols in all fractions but volatile oils are absent. The main work was to isolate the active fraction, So, fractionation of was done by suspending 100g of the dried plant sample in 500ml n-hexane, chloroform and then ethanol.the minimum percentage inhibition of the three fractions (n-hexane, chloroform and ethanol) were assayed by Agar well dilution method, which shows that, chloroform fraction ha highest percentage inhibition of 58.15, and 55.42% against malassezia and Trichoptyton than n-hexane 7.40 and 15.11% and ethanol extract 20.32 and 25.13%respectively. The chloroform extract was then fractioned using column chromatography, dichloromethane, ethyl acetate and ethanol were used as eluent, and five fractions was obtained. All the column chromatographic fractions were also tested for its antidermatophytic activity using the same method above, dichloromethane fraction shows highest percentage inhibition against the dermatophyte (malassezia and Trichoptyton) with the percentage inhibition 82.22 and 92.32%

respectively than other five fractions of ethyl acetate: (12.22 and 34.36), (20.56 and 30.13) and ethanol fractions (17.78% and 27.03%), and (10.00%and 19.22%) respectively. The dichloromethane fraction were then subjected to: physical examination(color, physical state, TLC analysis and melting point examination), and spectroscopic techniques (IR, GC- MS) were utilized.

Keywords: Isolate, Chloroform,n-hexane, Malassezia, Trichoptyton

Received for Publication on 2 November 2018 and Accepted in Final Form 22 December 2018

INTRODUCTION

Knowledge of chemical constituents of plant is important for the discovery of therapeutic agent (Ubani 2002). Plants have the ability to synthesize wide variety of chemical compounds that are used to perform important biological functions, and to defend the plant against attack from predators such as insect, fungi and herbivorous mammals, (Abdullah M.A. 2006). Many of these phytochemicals have beneficial effects on long term health when consumed by humans, and can be used to effectively cure human disease, (Allison Onyechere and et al, 2008).

In developing countries, infectious diseases remain the main cause of the high mortality rates, majority of rural dwellers have limited access to formal and adequate health services and thus heavily resort to traditional healers (WHO 1996). Indigenous herbal medicines are widely used against many infectious diseases, but only few of them have been studies chemically and biologically in order to identify their

bioactive constituent (Longanga 2000, in Ubani 2012). The chemistry of plant is very important as it involves both plant biochemistry and natural product chemistry. It is concerned with many varieties of organic compounds that are accumulated, their metabolism and biological function. To fully understand the mode of action of a particular plant, one needs to study its chemical constituents that lead to phytochemical study of plant which is very essential, (Sumner and Judith, 2000).

Phytochemicals are non-nutritive plant chemicals that have protective or diseases preventive properties. It is well known that plant produces these chemicals to protect itself, but recent research demonstrate that they protect humans against diseases, (Brian FH, Thomas-Bigger J, Goodman G, 2001). Phytochemistry is a scientific discipline used to describe medicinal plant consisting on the study of how to extract, concentrate, analyse, and standardize and preserve herbal plant (Evans, W C, 2009)

Plants are rich in wide variety of secondary metabolites, the most important of these metabolites include: alkaloid, tannins, steroids, terpenoids, saponins, flavonoids and glycosides which have been found in-vitro to have anti-microbial property, (Banso2001 and Cowam 1999) and (Irobi O N and et al. 1993). *Pergularia tomentosa* (milk weed) belongs to the family *Asclepiadecea*, it is a perennial plant, and is found mostly in the Sahara and sub-Sahara Desert (Gill, 1992). The local name of the plant is "fatakko" or "malaiduwa" in Hausa, and it is mostly found in northern part of Nigeria, (Husain, H.I, and et al. 1999). It is a perennial shrub about 50-60cm high, reaching 1m in good conditions, with older woody stems around which the younger once

wind. The stems are covered with erect green hairs. The leaves are opposite, entire, 1–2 cm long, heart-shaped, with long stalks that are also covered with green hairs. There are small flowers with 5 yellow-whitish free petals. The fruits are oblong, globulous follicules, covered in fleshy bristles. At the slightest touch the plant secretes a white sticky fluid from the leaves and fruits. It flowers in spring in the northern Sahara and any time of year in the central Sahara.

Ecologically, *Pergularia tomentosa* is a hardy chamaephyte that shows rather wide amplitude for soil from sandy, clayey to gravelly stony sites. It is found on wadi beds as well as on gravelly plateau (regs). The plant thrives in hot deserts where the rainfall does not exceed 100 mm. (Salima and *et el 2005*).

Traditional uses of plant

The parts of the plant used include latex leaves and roots. These are collected in spring in the northern Algerian Sahara and any time after a rainy period in the central Algerian Sahara. They are prepared as an infusion, decoction or powder and mixed with other plants, and taken by mouth or used externally, (Salima and *et el 2005*).

The information gathered from traditional healers of the northern part of Nigeria has shown that for over hundred years the juice extract from the plant leaves has been used in the treatment of skin infections, such as tinea capitis. A number of researches have also been carried out on the antifungal activity of this plant and results obtained shows that the plant has antifungal effect against *Aspergillus niger* (Gill, 1992). The plant was reported to have mulluscidal activity and persistent hypoglycemic effects, (Hussein et al., 1999; Shabana et al.,

1990) and its isolated cardenolides have been shown to cause apoptotic cell death of Kaposis sacorma cells. Also toxicity study shows that the plant has antidermatophytic activities against *T. rubrum T. mentagrophyte M. gpyseum,* (Shinkafi S.A 2014).(Hassan S.W and *at el* 2007*),* Revealed that the plant has significant nutritive value.

Pharmacological studies have confirmed that the different parts of the plant have had a wide application in different folk medicine remedies, such as in case of diarrhea, bronchitis, constipation, skin diseases, tuberculosis and used as poultice, depilatory, laxative, anthelmintic, and abortifacient. (Mossallam, and BaZaid, 2000), (Abou- Zeid et el., 2008; Clauzel, 2006). (Hammiche and Maiza. 2006).

Crude alkaloids were extracted from aerial part of the plant. Newly emerged fifth instar larvae were treated by forced ingestion, results showed that the test compound has Insecticidal activity, exhibited a considerable larvicidal effect with a dose-dependent mortality(Fatma A. And *et al.* 2013). On the other hand, it also caused antifeeding effect, weight loss of larvae with a reduction in protein and carbohydrate contents. These results indicated that *P. Tomentosa* is a promising naturally occurring agent for locust larval control, (Fatma A. And *et al.* 2013)

Pergularia tomentosa represent a source of antifungal (Hassan et al., 2007) antibacterial (Dangoggo et el., 2002), enzymes screening (Lahmar I. 2015).

MATERIAL AND METHODO

Sample Collections and preparation

The plant sample ware obtained from Wamakko town in Wamakko Local Government, Sokoto state and was identified and confirmed at Usmanu Danfodiyo University, Sokoto herbarium (Botany Unit, department of Biological science). The sample was washed with tap water and air dried, and was pulverized in to fine powder using pestle and mortar and then sieved to removed coarse parts, the powder was stored in an air tight polythene bag, (Trease GE, Evans WC, 1978).

Test Organism

The dermatophyte fungal strains used was clinical isolate obtained from the mycology laboratory of usmanu Danfodiyo university Sokoto; Malassezia (dandruff causing organism), Trichophyton.

Sample extractions

The plant extracts were prepared using the solvents: n-Hexane, chloroform and ethanol. 100g of the sample were taken and homogenized with 500cm^3 of n-Hexane in 1000cm^3 conical flask, shaken for several minutes and allowed to stand overnight at room temperature, the resulting liquid were first decanted and then filtered using What man filter paper (no 1), the filtrate was evaporated on water bath. The residue was air dried in the laboratory and extracted again with the same procedure above with chloroform and then ethanol, the extract in each case was evaporated to dryness, and taken to mycology lab for anti-dermatophyte test.

Determination of extraction yield (% yield)

The yield (%, w/w) from the dried extracts was calculated as:

$$\text{Yield (\%)} = (W1 / W2) \times 100$$

Where W1 is the weight of the extract after evaporation of solvent, and W2 is the weight of the plant powder.

Phytochemical analysis of Crude extracts.

Plant extract ware tested for the presence of active principles such as Triterpenoids, Steroids, Glycosides, Saponins, Alkaloids, Flavonoids, Tannins, and phenol compound carbohydrate. (Harbone J.B 2005), in (Kumar S.B 2012), Following standard procedures.

Antifungal Assays

The effect of various plant extracts on the fungal strains ware assayed by Agar well dilution method. The minimum concentrations of the plant extracts to inhibit the microorganisms ware also determined by micro dilution method using plant fractions serially diluted in sterile nutrient broth.

Reagents

Savoured Dextrose Agar medium: The commercially available (HiMedia) Savoured dextrose agar medium (39g) was suspended in 1000ml of distilled water. The medium ware dissolved completely by boiling and was then autoclaved at 15 lbs pressure (121°C) for 15 minutes.

Procedure

The fungal species was maintained in savored dextrose agar (SDA) medium in 90mm Petri dishes. 10, 20 and 30mg of ethanol extract was reconstructed using water and was mixed with 15cm^3 of SDA. The Hexane, Chloroform and ethanol extract ware also prepared using the same procedure above. The 90mm Petri-dishes was filled with 20cm^3

final volume of SDA containing the requisite amount of diluted extract solution, the SDA was allowed to solidify in the Petri-dishes at room temperature and ware then inoculated at the centre with (2x2mm) cut from the periphery of a 14 days dermatophyte fungal colonies. $15cm^3$ of water instead of extract and $15cm^3$ of agar was mixed together as a negative control. The treated and control Petri-dishes ware incubated at room temperature for 14days for superficial mycosis. Growth was observed each day to the last day. From these, the percentage inhibition ware calculated using the relation, (Shinkafi S A. 2014);

$$I(\%) = \frac{dc - dt}{dc} \times 100$$

Where;

I; Inhibition

DC; diameter of colony of control culture

DT; diameter of colony of treated culture

The result ware presented as mean ± standard deviation of the percentage inhibitions obtained.

Chromatography

The Column chromatography was done to isolate and purify the constituents present in the active (chloroform) extracts.

Packing of the Column:

Column preparation: The lower end of the clean dried glass column ware plugged with glass wool. The columns were packed with slurry of silica gel in dichloromethane by pouring the silica gel slurry into the column in a stepwise manner. The side of the column ware taped gently. As the silica gel settles, the column outlet was adjusted. 0.5g of sample was dissolved in a small quantity of ethanol and mixed with

small amount of silica gel to adsorbed the sample, the mixture ware then loaded on to the silica gel in the column, followed by small amount of glass wool and then eluted with Dichloromethane, Ethyl acetate and then Ethanol. Five fractions ware obtained, antifungal activity ware carried out on each fraction.

Thin Layer Chromatography

TLC is a method for analyzing mixtures by separating compounds in the mixture. It is use to determine the number of component Small amount of the dichloromethane chromatographic fraction was dissolved in ethanol; using micro pipette, a small amount of dissolved substance was transferred to one end of the plat coated with silica gel, and allowed to evaporate leaving behind a small spot of dry substance. The bottom of the plate was placed into a shallow pool (TLC tank) of a development Solvent (dichloromethane/ethanol) in the ratio 60:40, the lid ware covered, the solvent then travels up the plate by capillary action to about ¾ of the plate. The plate was removed and line ware drowned with pencil at front of the solvent. The visualization of the movement of spot was done directly.

The Rf value ware calculated by dividing the distance travelled by the substance by the distance travelled by the solvent.

RESULT AND DISUSSION

RESULTS

Table 1: Result of phytochemical content of crude and organic solvent extract of *pergulariatomentosa.*

Fraction	Crude extract	n-Hexane fraction	Chloroform fraction	Ethanol Fraction
Alkaloid				
Dragendorff's test	++	++	+++	++
Mayer's test	++	++	++	++
Hager's test	++	++	++	–
Wagner's	++	++	++	++
Flavonoid				
Shinoda's test	++	++	+++	++
Tannin				
Alkaline test	++	–	+	+
Glycoside				
Fehling solution test	++	–	+	+
Saponin				
Foam Test	++	++	++	+
Phenol				
Iron III Chloride test	++	–	++	++
Volatile Oil Dilute HCl Test	–	–	–	–

(+) Presence, (-) absence of the respective phytochemicals

Table 2: Result of percentage mass of extract obtained.

Solvents used	% extract(w/w) obtained
n-Hexane	4.33
Chloroform	7.67
Ethanol	5.61

Table 3: Result of percentage inhibition of fungal isolate by organic solvent extract of *Pergulariatomentosa*

Solvent	concentration	malassezia	*Trichoptyton*
N-Hexane	10mg	0.00±0.00%	8.00±0.60%
	20mg	7.00±0.00%	10.21±13.23%
	30mg	7.40±2.31%	15.11±72.51%
Chloroform	10mg	38.08±4.49%	40.34±11.00%
	20mg	40.05±13.8%	43.08±20.15%
	30mg	58.15±0.64%	55.42±33.11%
Ethanol	10mg	0.00±0.00%	11.01±22.10%
	20mg	14.60±30.22%	18.3051.10%
	30mg	20.32±30.06%	25.13±33.0%

Table 4: Result of percentage inhibition of fungal isolate by the chromatographic fractions of Chloroform extract of *Pergulariatomentosa*

Solvent	Fractions	*Malassezia*	*Trichoptyton*
Dichloromethane	Fraction I	82.22±0.22%	92.23±0.11%
Ethyl acetate	Fraction II	12.22±0.38%	34.36±0.10%
,, ,, ,,	Fraction III	20.56±0.81%	30.13±0.15%
Ethanol	Fraction IV	17.78±0.42%	27.03±0.44%
	Fraction V	10.00±0.32%	19.22±0.33%

Table 5: Physical properties of the isolated active fraction

Solvent Property	
Melting point	137^{o}C
Colour	Yellow
State	Paste form

Table 6:TLC Result of the active fraction of *Pergularia tomentosa*

Solvent system	Components		R_f Value
Dichloromethane: Ethanol (70:30)	2	0.83	0.47

Table 7: GC-MS Result for isolated fraction

Peak Number	Retention time	% area	Base peak	Molecular ion peak
1	28.71	3.48	140	270
2	33.27	44.29	157	294
3	33.38	43.37	135	292
4	33.59	8.52	148	298
5	36.14	0.06	519	
6	36.56	0.11	179	326
7	38.08	0.06	105	207
8	38.41	0.12		519

Table 8: IR Result for the isolated compound

Frequency	Assignment	Intensity	Functional group
3420	N-Hstr.	Medium	Amines
2920	C-Hstr.	Strong	Alkanes
2850	CH_3Medium	Alkanes	
2370	C=N	Medium	Nitriles
1725	C=O	Strong	Carboxylic Acid
1650	C=C	Medium/Weak	Alkenes
1430	N-Hbend	Medium/strong	Amines

680	C-X	Strong	Chlorides

DISCUSSION

Phytochemical screening

Table 1 shows the result of phytochemical analysis of *Pergularia tomentosa* leaves crude ethanol extract and organic factions obtained. The result revealed that the crude ethanol extract contains Alkaloid, Flavonoid, Saponin, Tannin, glycoside and phenolic compounds, while volatile oil is absent. In the organic fraction extract, it shows that the n Hexane contains alkaloid, saponin, flavonoid and tannin. While glycoside phenol and volatile oil are absent. In chloroform extract, alkaloid, flavonoid, saponin and phenols are present while tannin, glycoside and volatile oil are absent. In the last ethanol fraction extract, Alkaloid, Flavonoid, Saponin, Tannin, glycoside and phenolic compounds are present, while volatile oil is absent. Phytochemicals found to inhibit bacteria, fungi and pest. The present of Phytochemicals in the leaf of *Pergularia tomentosa* for example, saponins have hypotensive and cardio depressant properties (Olayele MT, 2007). Glycosides are naturally cardio active drugs used in the treatment of congestive heart failure and (1999) cardiac arrhythmia. Flavonoids possess antifungal, antiviral and antibacterial activity, (Neeti 2004).

Result of percentage mass of extract

Table 2 give the result of percentage mass of extract obtained by different solvents, 100g of the plant sample extracted with n-Hexane give 4.33% of the extract, the resulting residue extracted with

Chloroform gives 7.61%, lastly the chloroform residue extracted with Ethanol gives 5.61%. The percentage extract of n-hexane may be due to the law polar nature of hexane compare to chloroform.

Result ofPercentage inhibition of fungal isolate by organic solvent extract of *Pergularia tomentosa.*

The result of antifungal activity of *Pergularia tomentosa* against tested organisms ware presented in table 3. The result revealed that, the chloroform extract has high activity against the tested organism, with the percentage inhibition of 58.15±0.64percent against malassesia and 55.42±33.11 percent against trichophyton at 30mg concentration. Hexane extract shows lowest activity against the tested organisms with the percentage inhibition of 0.00±0.00percent and 8.00±0.60percent at 10mg concentration, and 0.00±0.00percent and 8.00±0.60percent at 20mg, and 7.40±2.31percent and 15.11±72.51percent at 30mg against malassesia and trichophyton respectively.

Percentage inhibition of fungal isolate by the Chromatographic fractions of Chloroform extract of *Pergularia tomentosa*

The Chloroform extract ware separated using column chromatography, each fractions obtained ware tested for its antifungal activity, the result ware given in able 4. Fraction 1 has nearly total inhibition against fungal isolate with total inhibition of 82.22±0.22percent and 92.23±0.11percent against *Malassezia and Trichoptyton* respectively. All other fractions show little inhibition of 10.00±0.32 to 34.36±0.10percent against the fungal isolate.

GC-MS Result.

Table 5 give the summary of GC-MS analysis of the active compound isolated from the leaf of *Pergularia tomentosa.* The give 8 peaks of varying intensities, first four with high intensity and another four with low intensity.

Peak 1of GC chromatogram of retention time 28.71 give mass spectra of unknown compound with molecular ion peak at m/z 270, with base peak at m/z 140, and another fragment at m/z 125, 110 and 95. When compare with NISTO2 Reference spectral library in the MS spectra, it suggested a compound with molecular formula $C_{17}H_{37}O_2$ Hexadecanoic acid, methyl ester. Peak 2 of GC chromatogram of retention time 33.27, give spectra of unknown compound with molecular ion peak at m/z 294.47, with base peak at m/z 137, with another fragments at m/z 124, 109 and 95. When compare with NISTO2 Reference spectral library in the MS spectra, it suggests a compound with molecular formula $C_{19}H_{34}O_2$ 9, 12-Octadecadieonic acid, ethyl ester. Peak 3 of GC chromatogram of retention time 33.28, give spectra of unknown compound with molecular ion peak at m/z 292 with base peak at m/z 135 and another fragments at 140, 125 and 110. When compare with NISTO2 Reference spectral library in the MS spectra, it suggests a compound with molecular formula $C_{19}H_{32}O_2$ 9, 12, 15-Octadecatrienoic acid, methyl ester. Peak 4 of GC chromatogram of retention time 33.59, give spectra of unknown compound with molecular ion peak at m/z 298.72, with base peak at m/z 139, and another fragments at m/z 124 and 109. When compare with NISTO2 Reference spectral library in the MS spectra, it suggests a compound with molecular formula $C_{19}H_{38}O_2$ Octadecanoic acid.

Peak 7 show very low intensity, with GC chromatogram of retention time 38.08, give spectra of unknown compound with molecular ion peak at m/z 326.43, and base peak at m/z 179.5. When compare with

NISTO2 Reference spectral library in the MS spectra, it suggests a compound with molecular formula $C_{12}H_5Cl_5$ 2, 2 3, 4, 5-pentachloro 1, 1-biphenyl.

I R Result.

Table 6 give a summary of the IR result of the active chromatographic fraction isolated from the chloroform extract of *Pergularia tomentosa.*The spectrum shows absorption at 3420cm^{-1}, and2370which represent N-H vibration for amine, C=N vibration for Nitrile respectively. Absorption at 1725 cm^{-1} represent C=O for carboxylic acid. Absorption at 2850 cm^{-1} and 2920 cm^{-1} represent CH3 vibration and C-H vibration for alkane. While 1650 cm^{-1} and 680 cm^{-1} represent C=C vibration for alkenes and chloride respectively.

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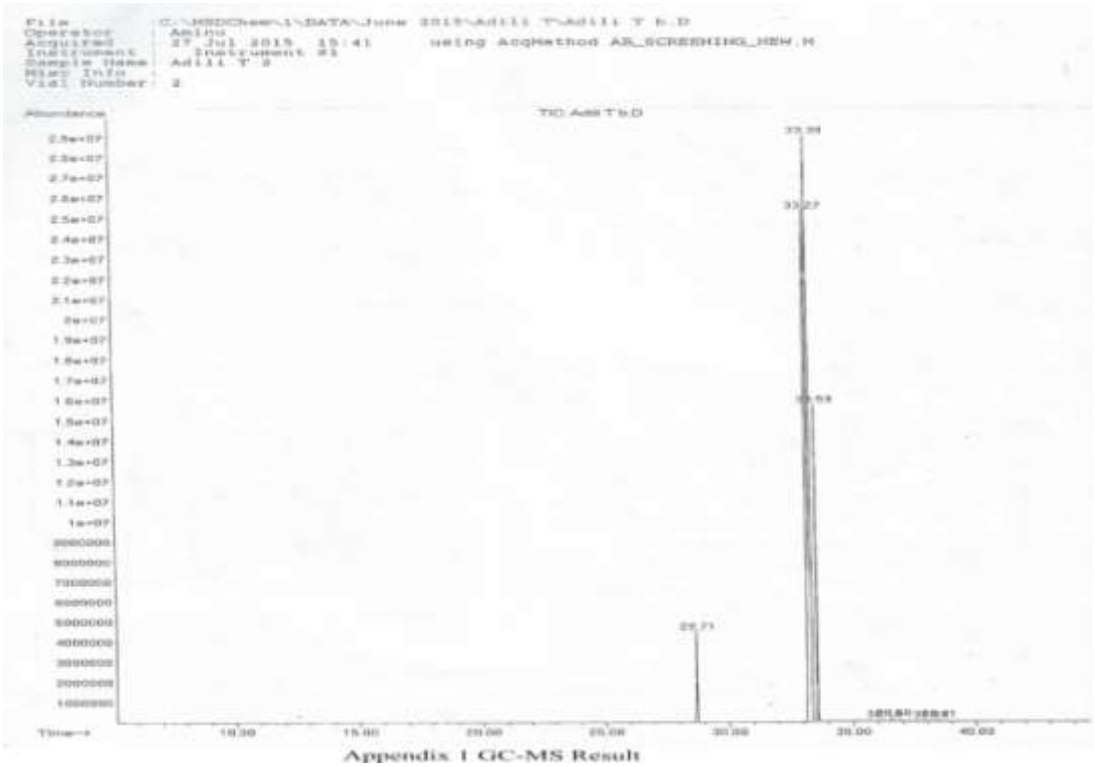
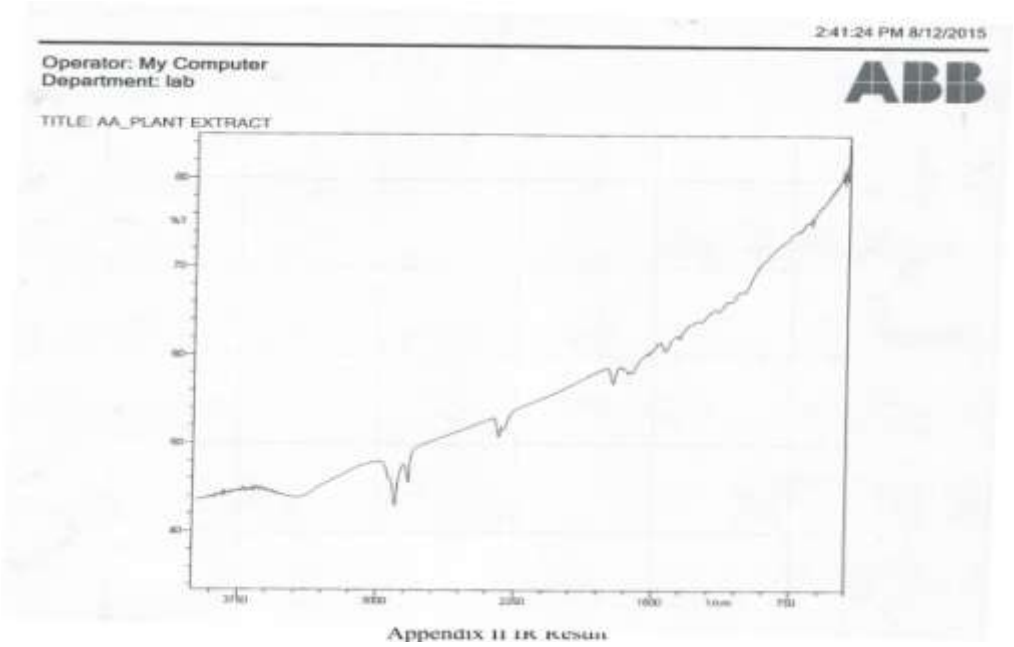
Reference to this paper should be made as follows: Arzika, A.T., et al., (2018) Isolation and characterization of active fraction of *pergularia tomentosa* against dermatophyte. *J. of Engineering and Applied Scientific Research*, Vol. 10, No. 4, Pp. 55-76



Appendix 1 GC-MS Result



Appendix II IR Resin