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Phytochemical Analysis, Antimicrobial Efficacy and Determination of Bioactive Components from Leaves of *Justicia Adhatoda* (Linn)

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ABSTRACT

To determine the antibacterial, antifungal and phytochemical properties of petroleum ether, ethyl acetate, chloroform, methanol extracts and to evaluate the bioactive components from leaves of Justicia adhatoda (Linn). The extracts were tested against bacteria and fungus through disc diffusion assay. The phytochemical compound screened by qualitative and GC-MS method. Qualitatively analyzed as alkaloids, anthraquinones, flavonoids, saponins, phytosterols, triterpenoids and polyphenols gave positive results. Carbohydrates and cardio glycosides gave negative results. In the GC-MS analysis 9 major bioactive components were identified in the Petroleum ether extract of Justicia adhatoda. The four crude extracts of Justicia Adhatoda where investigated for their potential anti-bacterial and anti-fungal activities. All extracts showed anti-bacterial and anti-fungal activities against all the organisms tested except Pseudomonas aeruginosa and Proteus vulgaris. This study provides scientific evidence on the traditional use of Justicia Adhatoda leaf extract in treating microbial diseases. Further, the leaf extract can possibly be used to produce alternative forms of antimicrobials.

Key words: *Justicia Adhatoda,* Phytochemical, Antibacterial, Antifungal, GC-MS, Leaf Extracts.

INTRODUCTION

Medicinal plants which from the backbone of traditional medicine have in the last few decades been the subject of very intense pharmacological studies. This has been brought about by the acknowledgement of the value of medicinal plant as potential source of new compounds of therapeutic value and as source of new compounds in drug development. In many parts of the world medicinal plants are used for antibacterial, antifungal and antiviral activities a plant derived drugs serve as a prototype to develop more affective and loss toxic medicinal. Tribal medicine has not been studied extensively [1]. Several screening studies have been carried out in different parts of the world. There are several reports on the antimicrobial activity of different herbal extracts in different regions of the world [2].

Plant extracts have great potential as antimicrobial compound against microorganisms [3]. The medicinal value of plants lies in the bioactive compounds such as alkaloids, flavonoids, tannins, and phenolic compounds that produce a definite physiological action on the human body. The increasing use of plant extracts in the food, cosmetic, and pharmacological industries suggests that in order to extract active compounds, a systematic study of medicinal plants is very important [4]. Thus, this study aimed to evaluate the potential antimicrobial activities of *Justicia adhatoda* leaves.

Justicia Adhatoda is a species of plant in the Acanthaceae family. It is a shrub growing throughout India especially in the lower Himalayan regions. The name, J. adhatoda L. and Adhatoda zeylanica Medic are used synonymously. It is commonly known as Vasaka or Malabar nut. It is a perennial, evergreen and highly branched shrub (1.0 m to 2.5 m height) with unpleasant smell and bitter taste. It has opposite ascending branches with white, pink or purple flowers.

The plant has potent anti-periodic, astringent, diuretic and purgative action. It is a highly valued Indian medicinal plant which is used in the treatment of respiratory diseases like asthma, cough, bronchitis and tuberculosis [5, 6]. The flowers, leaves and root have antispasmodic property. The activities against tuberculosis were reported by many researchers quite early [7, 8]. It has been used extensively as an important herbal drug in treating a wide variety of diseases and the leaves of the plant are the main source of drug formulation. For instance, the source of the drug 'vasaka' is well known in the indigenous system of medicine for its beneficial health effects, particularly in treating bronchitis [9]. The different parts of the plant is used in the Indian traditional medicine for the treatment of various diseases like asthma, joint pain, lumber pain and sprains, cough, eczema, malaria, rheumatism, swellings, venereal diseases [10 - 12]. In homeopathy, *Justicia Adhatoda* or *A. vasica* has been used in the treatment of cold, cough, pneumonia, spitting of blood, fever, jaundice, catarrh, whooping cough and asthma [13]. This study provides scientific proof on the use of these plants which are being utilized traditionally as herbal medicines.

MATERIALS AND METHODS

Plant Collection and Authentication

The fresh leaves of *Justicia Adhatoda* (L) Linn of acanthaceae family were collected from local areas of Vellore district of Tamilnadu, India and authenticated by professor P. Jayaraman, Botanist, Director, Plant anatomy research centre, Tambaram, Chennai, India in the month of May 2014 and registered Number of the Specimen is PARC/2014/2074.

Preparation of Extracts

Five hundred grams of coarse powder of shade dried leaves of *Justicia Adhatoda* was extracted successively with petroleum ether (60-80°C), chloroform, ethyl acetate and methanol in soxhlet extractor for 48 h. dark green residues were obtained after concentrating the extract under reduced pressure (Yield 8.20%, 3.80%, 2.15% and 14.30% respectively). The obtained extracts were stored in desiccators for further phytochemical and antimicrobial investigations. The dried material was tested for its constituents by standard methods [14 - 16]. The plant extracts were diluted with respective solvents to the final concentration of 20 mg/ml. Microorganisms like *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Candida albicans* were used for testing.

Preliminary Phytochemical Screening

Test for alkaloids

To 2ml of plant extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

Test for anthraquinones

To 1ml of plant extract few drops of 10% ammonia solution was added, appearance pink color precipitate indicates the presence of anthraquinones.

Test for carbohydrates

To 2ml of plant extract, 1ml of Molisch's reagent and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.

Test for cardiac glycosides

To 0.5ml of extract, 2ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was under layered with 1 ml of concentrated sulphuric acid. Formation of brown ring at the interface indicates presence of cardiac glycosides.

Test for flavonoids

To 2ml of plant extract, 1ml of 2ml sodium hydroxide was added. Presence of yellow color indicates the presence of flavonoids.

Test for saponins

To 2ml of plant extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

Test for phytosterols

To 1ml of plant extract equal volume of chloroform is added and subjected with few drops of concentrated sulphuric acid appearance of brown ring indicates the presence of steroids.

Test for Triterpenoids

10mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of Conc.H2SO4. Formation of reddish violet colour indicates the presence of triterpenoids.

Test for Polyphenols

To 1ml of the extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols or polyphenols.

Physicochemical Screening

Total Ash Content

Accurately weigh a quantity of the Test Sample, representing 2 to 4 g of the air-dried material, in a tarred crucible, and incinerate, gently at first, and gradually increase the temperature to 675 ± 25 ºC, until free from carbon, and determine the weight of the ash. If a carbon-free ash cannot be obtained in this way, extract the charred mass with hot water, collect the insoluble residue on an ash less filter paper, and incinerate the residue and filter paper until the ash is white or nearly so, then add the filtrate, evaporate it to dryness, and heat the whole to a temperature of 675 ± 25ºC. If a carbon-free ash cannot be obtained in this way, cool the crucible, add 15ml of alcohol, break up the ash with a glass rod, burn off the alcohol, and again heat the whole to a temperature of 675 ± 25ºC. Cool in desiccators, weigh the ash, and calculate the percentage of total ash from the weight of the drug taken.

Acid-Insoluble Ash

Boil the ash obtained as directed under *Total Ash*, above, with 25 ml of 3 N hydrochloric acid for 5 minutes, collect the insoluble matter on a tarred filtering crucible or ash-less filter, wash with hot water, ignite, and weigh. Determine the percentage of acid-insoluble ash calculated from the weight of drug taken.

Water-Soluble Ash
Boil the ash obtained as directed for *Total Ash* with 25 ml of water for 5 minutes. Collect the insoluble matter in a sintered-glass crucible or on an ash-less filter paper. Wash with hot water, and ignite for 15 minutes at a temperature not exceeding 450ºC. Subtract the weight of this residue, in mg, obtained under *Total Ash*, and calculate the percentage of water-soluble ash with reference to the weight of sample as determined under *Total Ash*.

Foreign Matter
Spread the sample out in a thin layer, and separate the foreign organic matter by hand as completely as possible. Weigh it, and determine the percentage of foreign organic matter in the weight of drug taken.

Moisture Content
Hot extraction method under alcohol soluble extractives, except to use water in place of alcohol and Cold extraction method under alcohol soluble extractives, except to use water in place of alcohol.

Anti-bacterial Screening
Disc diffusion method was adopted for the antibacterial study [17, 18]. Ciprofloxacin at conc of 10mcg/disc was used as a standard. The filter paper impregnated with extracts (separately in each extracts at a concentration of 20 mgml-1) and ciprofloxacin disc were placed aseptically on the seeded agar medium (Bio-zone Research Technologies Pvt. Ltd., Chennai) which was already swabbed with the test organisms and incubated at 37°C for 24h. The zone of inhibition in mm was measured.

Anti-fungal Screening
The antifungal activity of the crude extracts was determined against *Proteus vulgaris* and *Candida albicans* by disc diffusion method [17, 18]. Ketoconazole (10 mcgdisc-1) was used as standard. The filter paper disc impregnated with various extracts (20 mgml-1) individually and ketoconazole disc were placed aseptically on the seeded sabouraud dextrose agar medium (Bio-zone Research Technologies Pvt. Ltd., Chennai) which was already swabbed with the test organism and incubated at 37°C for 48 h. The zone of inhibition (in mm) was measured and recorded.

Gas Chromatography Mass Spectrum (GC-MS) Analysis
GC-MS technique was performed using GC SHIMADZU QP2010 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column (Length : 30.0 m, Diameter : 0.25 mm, Film thickness : 0.25 is Composed of 100% Dimethyl poly siloxane). An electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.51ml/min and an injection volume of 2ìl was employed (split ratio: 20). Injector temperature 200°C: Ion-source temperature 200°C. The oven temperature was programmed from 70°C (isothermal for 2 min.), with an increase of 300°C for 10 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 – 1000 m/z. Total GC running time was 35 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was GC MS solution ver. 2.53.

Identification of components
Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technique (NIST08s), WILEY8 and FAME having more patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST08s, WILEY8 and FAME library. The Name, Molecular weight, Molecular formula and Structure of the component of the test material were identified.

RESULTS AND DISCUSSION

Phytochemical Analysis
The phytochemical analysis of leaves extract of *Justicia Adhatoda* revealed the presence of various components such as alkaloids, anthraquinones, flavonoids, saponins, phytosterols, triterpenoids and poly-phenols (Table 1.0). Comparing the extraction solvents triterpenoids were present whereas, carbohydrates were absent in all the extracts. Methanol leaf extracts depicted the presence of all the phytochemicals when compared with other extracts.

Physicochemical and Proximate Analysis

Physicochemical parameters and extractive value of leaves were studied and results were shown in Table 2.0 respectively. The moisture content was 15.20% (leaves) and the total ash content, acid insoluble ash, Water soluble ash and foreign matter values which were determined to be not more than 11.40%, 1.50%, 3.85%, 1.68% respectively. While study of extractive values can serve as a valuable source of information and provide suitable standards to determine the quality of plant material in future investigation. The proximate analysis value of the present data indicated that methanol (14.30%) extract showed higher extractives value when compared to other solvents (Table 3.0).

Inhibition Activity on Micro-organisms

The four crude extracts of *Justicia Adhatoda* were investigated for their potential anti-bacterial and anti-fungal activities. Petroleum ether extract was slightly affective for the organism tested. Standard antibiotics ciprofloxacin (10mcg/disc) and ketoconazole (10mcg/disc) showed good inhibitory action on the micro-organisms tested. Methanolic extract indicated significant activity only against Staphylococcus aureus, Klebsiella pneumonia and Candida albicans. Pseudomonas aeruginosa and *Proteus vulgaris* did not specify any antibacterial and antifungal activities against all the organisms tested (Table 4.0).

Gas Chromatography Mass Spectrum (GCMS) Analysis

Phytochemical components in petroleum ether extract of *Justicia Adhatoda* by GC-MS report. The GC-MS analysis revealed the presence of nine compounds from the petroleum ether leaf extract of *Justicia Adhatoda* (Figure 1). The major constituents were *[E]-9-octadecenoic acid ethyl ester (RT: 18.6)* (Figure 1a)*; Heptadecanoic acid, 15-methyl-ethyl ester (RT: 18.8)* (Figure 1b)*; 16-octadecenoic acid, methyl ester (RT: 18.2)* (Figure 1c) along with other minor constituents were also present. The GC-MS chromatogram shows the peak area separation of the components.

Table 1.0 Phytochemical screening of *Justicia Adhatoda* leaf extracts

Test	PE	EE	CE	ME
Alkaloids	-	+	+	+
Anthraquinones	-	+	-	+
Carbohydrates	-	-	-	-
Cardiac glycosides	-	-	-	+
Flavonoids	-	+	+	+
Saponins	-	+	-	+
Phytosterols	+	+	-	+
Triterpenoids	+	+	+	+
Polyphenols	-	+	-	+

+ve – Present; -ve – Absent; PE – Petroleum Ether Extract; EE - Ethyl Acetate extract; CE - Chloroform Extract; ME - Methanol extract;

Table 2.0 Physicochemical screening of *Justicia Adhatoda* leaf extracts

S. No.	Parameters	Values
1	Total ash content	21.40%
2	Acid insoluble ash	0.92%
3	Water Soluble ash	4.85%
4	Foreign matter	0.28%
5	Moisture content	18.20%

Table 3.0 Proximate analysis of *Justicia adhatoda* leaf powder

S. No.	Parameters	Values
1	Petroleum Ether	2.10%
2	Chloroform	3.80%
3	Ethyl acetate	6.15%
Solubility		
4	Methanol	14.30%

Table 4.0 Evaluation of antibacterial and antifungal activity of *Justicia Adhatoda* Linn

S. No.	Micro-organisms	Zone of inhibition (mm)				
		Std.	PE	CE	EE	ME
1.	*Escherichia coli*	25	09	09	10	NS
2.	*1. Klebsiella pneumonia*	24	05	05	14	18
3.	*Staphylococcus aureus*	30	16	14	20	20
4.	*Pseudomonas aeruginosa*	23	NS	NS	NS	NS
5.	*Proteus vulgaris*	30	NS	NS	NS	NS
6.	*Candida albicans*	22	12	19	12	14

PE – Petroleum Ether Extract; EE - Ethyl Acetate extract; CE - Chloroform Extract; ME - Methanol extract; NS = Not Specified; Std. – Standard;

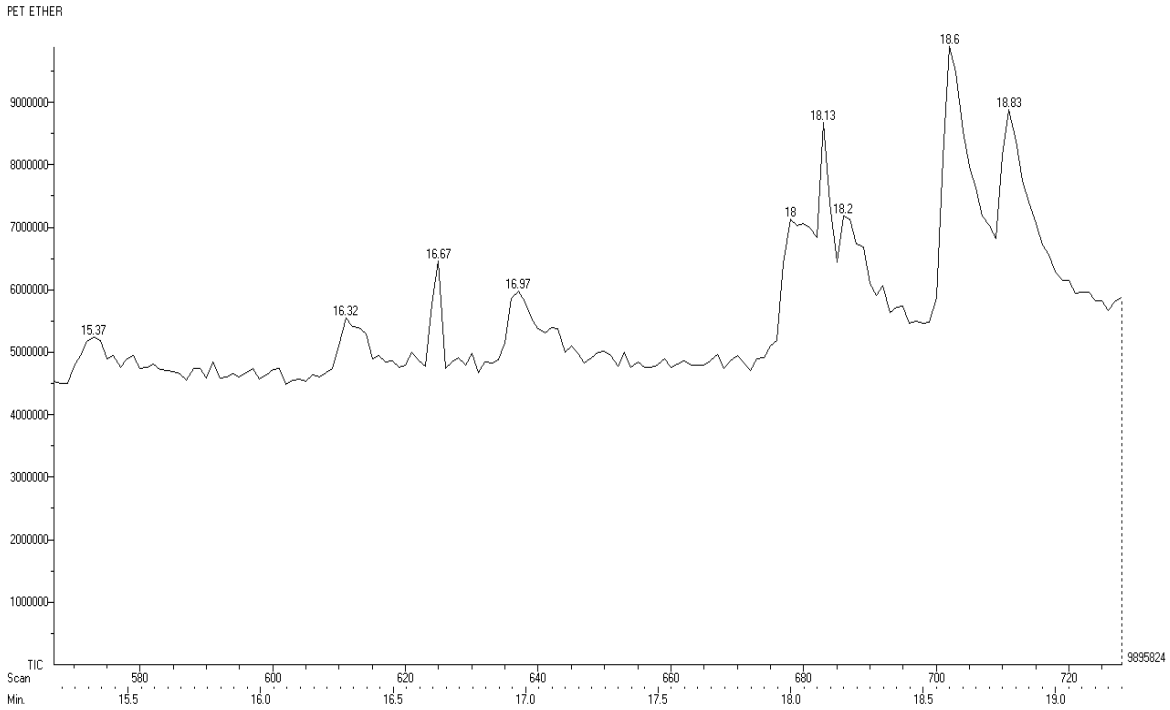

Figure 1 Gas Chromatography Mass Spectrum – Ms Profile.

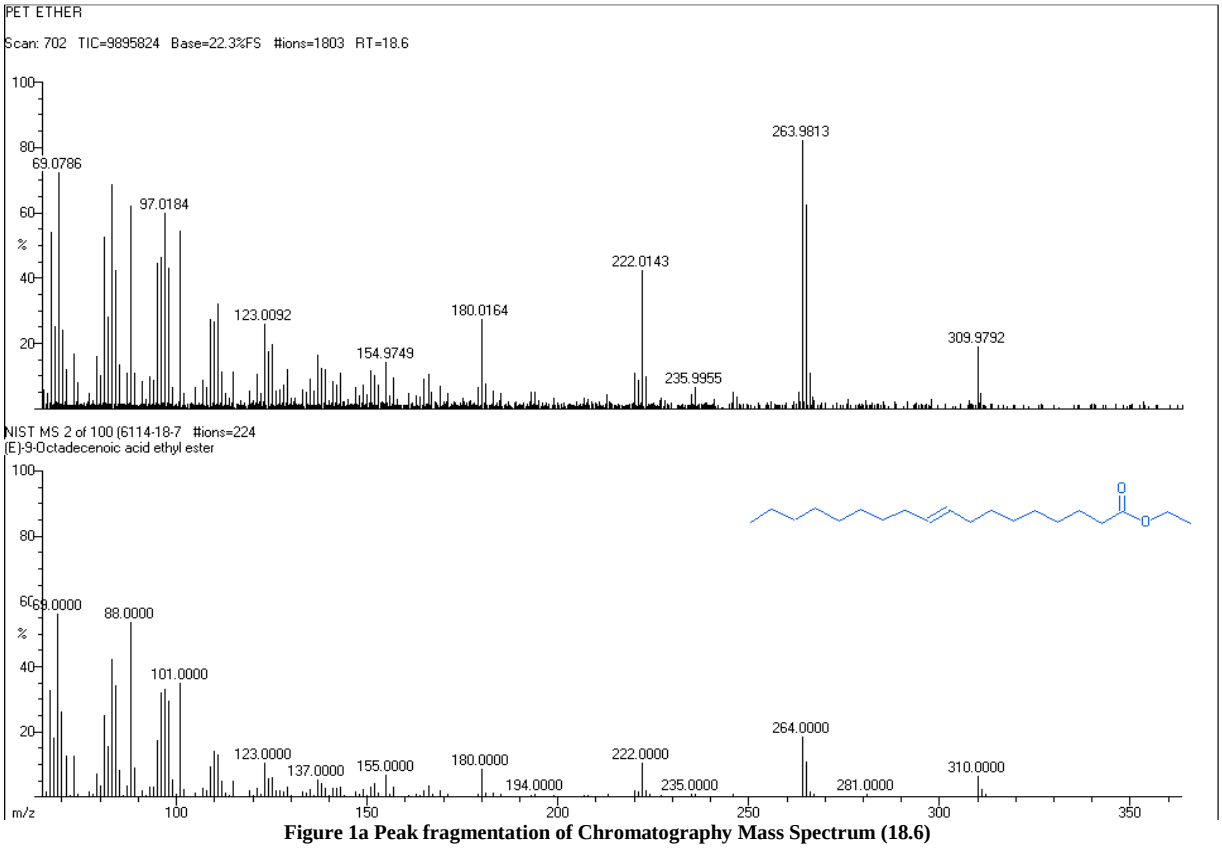

Figure 1a Peak fragmentation of Chromatography Mass Spectrum (18.6)

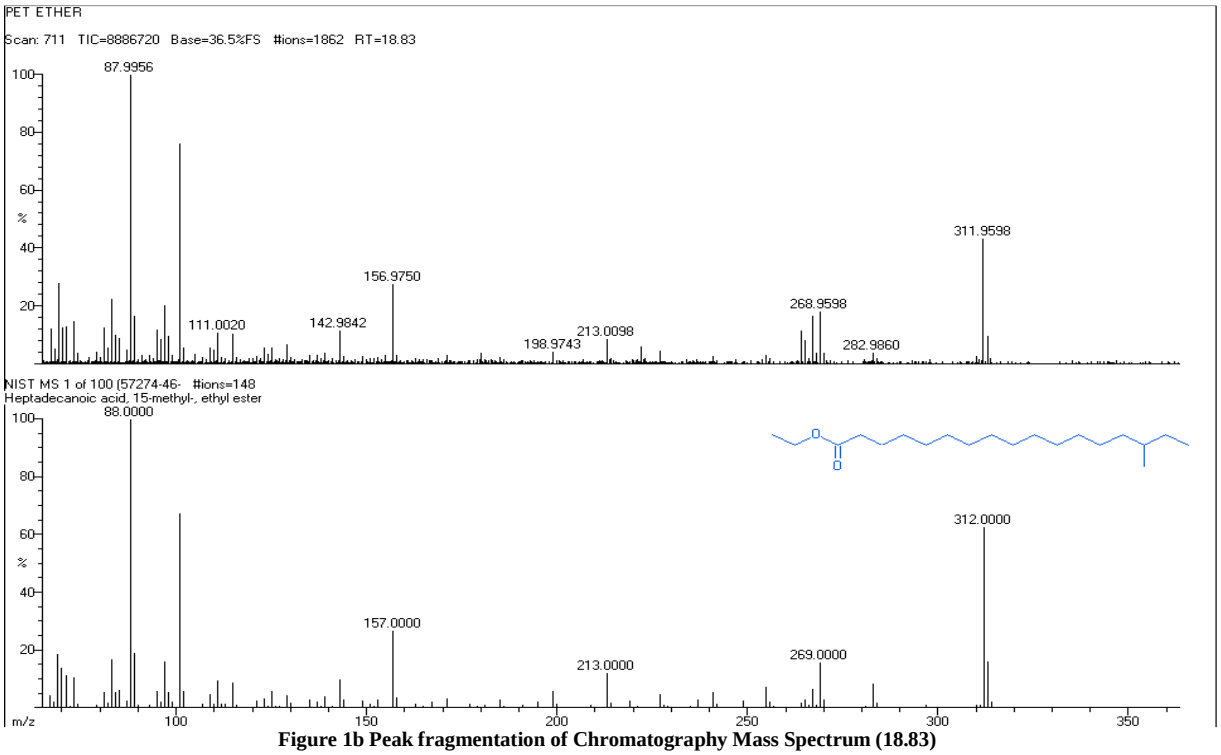

Figure 1b Peak fragmentation of Chromatography Mass Spectrum (18.83)

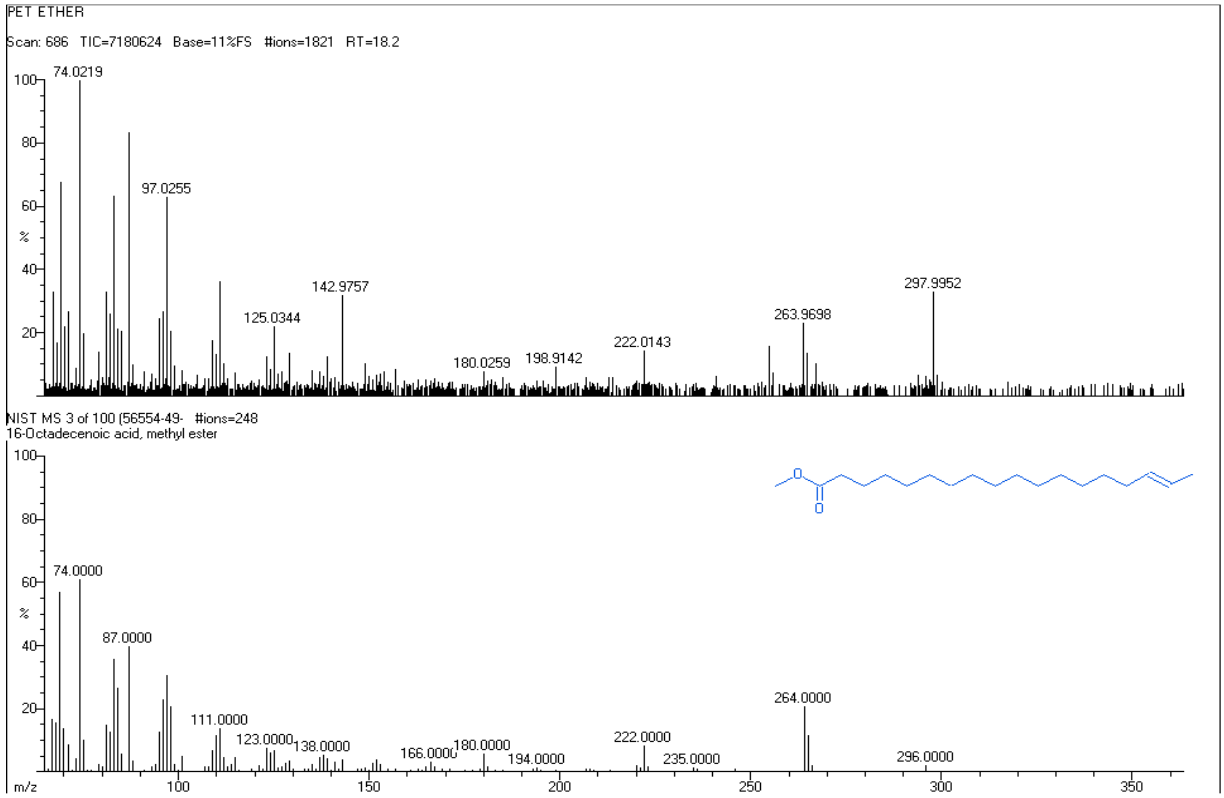

Figure 1c Peak fragmentation of Chromatography Mass Spectrum (18.2)

CONCLUSION

The present study, we can conclude that the traditional use of the plant *Justicia adhatoda* for the treatment of infectious diseases is promising, mainly against bacteria and fungi. Purification of the Bio-active components from the extracts understands the possible antimicrobial activities. GC-MS result signified the presence of nine phytochemical components. This result of the study offers the platform of using *Justicia adhatoda* leaves as herbal alternatives for various diseases and it is highly significant for mosquitocidal activities.

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