IJPSR (2022), Volume 13, Issue 2 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 20 April 2021; received in revised form, 22 June 2021; accepted, 28 June 2021; published 01 February 2022

QUALITATIVE PHARMACOGNOSTIC AND PHYTOCHEMICAL EVALUATION ON *ACHYRANTHES ASPERA* LINN. LEAF

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Keywords:

Achyranthes aspera, Pharmacognosy, Phytoconstituents, *Staphylococcus aureus.*

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ABSTRACT: Medicinal plants and their investigation towards the phytoconstituents is much interested in today's research. The plant *Achyranthes aspera* Linn. is one of the most use full drugs in Siddha and Ayurvedha medicines for treating various diseases in different formulations. Here the aim of this study is to evaluate the pharmacognostic parameters and identify the phytoconstituents. A preliminary phytochemical study helps identify the phytoconstituent present in different extracts, and Column Chromatography was used to isolate the phytoconstituent principle present in the methanol extract. The structure of the isolated compound was elucidated by Spectroscopic methods like UV spectroscopy, IR spectroscopy, NMR spectroscopy, MASS spectroscopy. A yellow crystalline compound was isolated from the methanol extract of *Achyranthes aspera* Linn. in the fraction of Chloroform: Ethyleacetae in (80:20) ratio. From the spectroscopic method, the structure of the isolated compound was elucidated, and the proposed structure may be (E)-2-(E)-tetracos-21-enoyloxy) ethyl pentacos-22-enoate. The antimicrobial activity of the methanol and aqueous extract of *Achyranthes aspera* Linn. has also been screened towards bacterial and fungal organisms like *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes* and fungi *Candida albicans* and *Aspergillus flavus*, *etc.* Both extracts show antimicrobial activity, but the methanol extract shows better activity than the aqueous extract.

INTRODUCTION: Medicinal plants are used locally in the treatment of infections caused by fungi, bacteria, viruses, and parasites. The interest in the scientific investigation of these medicinal plants from rural areas is based on. The claims of their effective use for the treatment of many diseases [1]. Therefore, research into the effects of these local medicinal plants is expected to enhance the use of these plants against diseases caused by the test depend on the traditional medicine for the treatment of their ailments [2, 3].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.13(2).793-02

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.13(2).793-02

Different plants have been used as a source of inspiration in the development of novel drugs. Therefore, basic phytochemical investigation of these extracts for their major phytoconstituents is also vital [4]. However, most of these plants used in folk medicine have not been evaluated for their

phytoconstituents and also screened for their pharmacological activities and antimicrobial activity [5, 6]. Plant-derived medicines are widely used because they are relatively safer than synthetic alternatives; they are easily available and cheaper. The stability study of the herbal extracts and their formulations are very important [7]. *Achyranthes aspera* Linn. belonging to the family - Amaranthaceae, commonly called as "Prickly chaff flower" is an erect, ligneous herb [8]. The plant is widespread in the world as a weed; it is distributed throughout India up to an altitude of 3000ft [9, 10]. The plant and seeds are rich in carbohydrates, proteins, and certain constituents such as flavonoids, tannins, saponins that help maintain the overall health of an individual [11]. According to Ayurveda, taking *Achyranthes aspera* powder with honey helps improve digestion due to its Deepan (appetizer) and Pachan (digestive) properties. Regular consumption of a handful of *Achyranthes aspera* seeds helps manage weight by reducing excess fat accumulation, which results in a reduction of bodyweight. Directly applying the juice of *Achyranthes aspera* leaves at the affected area might help in wound healing due to its astringent and anti-inflammatory [12].

It can also be used to relieve from ulcers due to its anti-ulcer and gastroprotective activity. It is advisable to use the leaves or root paste of *Achyranthes aspera* with water or milk while applying on the skin as it may lead to skin rashes and skin irritation due to its hot potency [13]. Here this plant has been selected to study and evaluated for its phytochemical nature. Bioactive agents like saponins, glycoside flavonoids, phytosterols, tannins, and alkaloids were detected. The methanol and water extracts were screened for the antimicrobial activity towards bacteria and fungi organisms like *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes* and fungi *Candida albicans* and *Aspergillus flavus*, *etc.* The zone of inhibition produced by the extracts was compared with the zone produced by the standards.

MATERIALS AND METHODS:

Plant Materials Collection and Authentication: The leaf of *Achyranthus aspera* L is available locally and was collected in and around Coimbatore. The botanical identity has been authenticated by the Director, Botanical Survey of India, Coimbatore, No: BSI/SRC/5/23/2012-13/Tech/496. The voucher specimen has been submitted and preserved in the herbarium for future reference.

Processing of Plant Material: The plant materials were collected, and shade dried at room temperature, and were subjected to size reduction to get course powder of desired particle size. This powdered material was subjected to successive extraction. One kilogram powdered drug was extracted with methanol and water separately by the cold maceration method for 7 days. Then the extracts were filtered, and the solvent was evaporated under reduced pressure in a rotary evaporator to get the dry extract. The yield of the dry extracts was calculated and stored in desiccators and used for further experiments.

Microscopical Evaluation of the Plant Leaf: The required sample leaf was cut and removed from the plant and fixed in FAA (Farmalin-5 ml +Acetic acid -5 ml+ 70 % Ethyl alcohol-90 ml). The standard procedure was followed as per the procedure given by Sass, 1940 [14, 15]. Photographs of different magnifications were taken with NIKON Lab photo 2 microscopic Unit. For normal observations, a bright-field was used. For the study of Crystals, Starch grains, and lignified cells, Polarized light was employed [16, 17]. Under polarized light, they appear bright against a dark background. Descriptive terms of the anatomical features are as given in the standard Anatomy books [18].

Preliminary Phytochemical Identification: The methanol and aqueous extract of the plant material was separately prepared and subjected to a preliminary phytochemical identification test to identify its chemical constituents using standard procedures [19, 20].

Fluorescence Analysis: The drug powder was treated with different chemical reagents, acids, and alkalies. Then these solutions made a spot on a TLC plate. The developed colours were observed under UV-Fluorescent light at 254 and 366 nm [21].

Column Chromatography: Column chromatography was used to separate the compounds. For this purpose 10 gm of methanol extract of the plant was taken and placed in a

column (column size 90 cm × 2.5 cm), Packed with Silica Gel (100-200 mesh) are the adsorbent used to complete separation of the component of the sample, and various organic solvents in various proportions were used. Silica gel was made into homogenous suspension by shaking with petroleum ether (first eluent). The bottom of the column was plugged with a little cotton to prevent pass put, and then the silica gel suspension was poured into the column, set aside for 10 min, and used. Methanol extract of the sample was subjected to column chromatography over silica gel. The column was eluted with solvents of increasing polarity. They were Petroleum ether, Chloroform, Ethyl acetate, Acetone, Ethanol. The column was run with organic solvents in various proportions to isolate compounds from the plant extract [22].

Spectral Study: The isolated compound was examined under visible and UV light for proximate analysis. For UV and FTIR spectrophotometer analysis, the sample was scanned in the wavelength ranging from 260-900 nm using Perkin Elmer Spectrophotometer, and the characteristic peaks were detected. FTIR analysis was performed using the Perkin Elmer Spectrophotometer system, which was used to detect the characteristic peaks ranging from 400-4000 cm^{-1} and their functional groups. The peak values of the UV and FTIR were recorded. NMR 30 and MASS spectral studies were also performed and recorded. Each and every analysis was repeated twice for the spectrum confirmation [23, 24].

Evaluation of Antimicrobial Activity: The antimicrobial activity of the test sample (leaf) extracts were carried out by the standard disc diffusion method (Kirby Bauer method). The bacterial strains used were *Staphylococcus aureus* (+ve) (NCIM 2079), *Bacillus subtili*s (+ve) (NCIM2063), *Pseudomonas aeruginosa* (-ve) (NCIM 2036), *Klebsiella aerogenes* (-ve) (NCIM 2098), and fungi *Candida albicans* (NCIM 3102) and *Aspergillus flavus* (NCIM105) were obtained from National Chemical Laboratory (NCL), Pune and maintained by periodical subculturing on Nutrient agar and Sabourad dextrose agar medium for bacteria and fungi respectively. From the culture obtained, using sterilized Pasteur loop, one loop full of each of the microorganisms was transferred into the test tubes containing sterile nutrient broth for screening studies. The pH of the above media was maintained at 7.2; it is then sterilized by autoclaving at 121 °C at 15 lbs pressure for 15 min, in which the nutrient broth was used for subculturing and MHA media was used for screening studies [25, 26]. Nutrient broth with standard modification was prepared and sterilized by autoclaving at 120 °C (151b/in2); about 30 ml of nutrient agar medium was transferred aseptically into every sterilized petri plates to get the thickness of 5 to 6 nm. The plate was allowed to solidify and upturned to prevent the condensate from declining on the agar surface. The plates were dried at 37 °C sooner than organisms were inoculated in the plates prepared prior, by dipping sterilize swab in the previously standardized inoculums and spreading the organism by shacking the swab all over the surface the medium. The plates were left at room temperature. Reference standard disc (6 mm diameter) was used as positive antibacterial and antifungal (Ciprofloxacin 5 µg/disc for bacteria; Nystatin 100 µg/disc for fungi) as control. Each leaf extract was reconstituted with solvents and tested at the concentration of 200 µg /ml, 400 µg /ml.

The paper discs were impregnated appropriately labeled and evenly spaced sides over the inoculated plates. On incubation, the bacteria grow on the area of the plate, excluding those approximately the inhibitory compound of the plant, which they are sensitive. In the duration of overnight, the phytocompound present in the plant, extract prevents the development of visible growth, which indicates the extract has antibacterial action. The inhibition measured the inhibition zone's diameter after prior incubation, and the experiment was executed two-fold, and the average determination was recorded. The effect produced by the sample was compared with the effect produced by the positive control (Reference Standard Ciprofloxacin 5 µg/disc for bacteria; Nystatin 100 µg/disc for fungi).The antimicrobial action was evaluated by measuring the width of inhibition zone [27].

RESULTS AND DISCUSSION:

Microscopical Studies:

Anatomy of Leaf: Transverse section of the leaf revealed that the epidermal cells consist of straight anticlinal walls. The mesophyll consists of the adaxial part of two. The bundle is surrounded by a

single ring of large hyaline bundle sheaths parenchyma with adaxial bundle sheaths extension. Beneath every upper epidermal cell, there are about 3.54 ± 0.40 palisade parenchyma cells present; they are very green and tightly packed. Spongy parenchyma cells are present beneath the palisade parenchyma cells. The midrib is thick and prominent. The adaxial part of the midrib is tall, conical, and flat at the apex. The abaxial part is wide and semicircular. The midrib is 750 mm thick. The epidermal layer of the midrib is thin, and the cells are small and spindle-shaped. Inner to the epidermis is a similar layer of small spindle-shaped cells. The outer zone of adaxial cone includes narrow and short palisade cells, which is extensive of the palisade layer of the lamina. The remaining ground tissue consists of thin-walled compact angular parenchyma cells. The vascular system of the midrib is multi-stranded. These are three top-shaped collateral bundles placed in a shallow arc in the abaxial bulged part of the midrib; of the three bundles the median bundle is smaller than other laterals. There is another solitary collateral bundle located beneath the adaxial bundles located beneath the adaxial cone. All bundles have wide thick-walled and angular, diffused, distributed Xylem elements and a thick pad of phloem attached to the outer part of the Xylem element seen abutting the phloem [28]. Calcium oxalate clusters and widespread in the mesophyll tissue of the lamina. They may be located either in the palisade zone or spongy mesophyll zone. The ducts are present 25 mm in diameter in the midrib. Uni- and multi-cellular trichomes are present both on the upper and lower epidermis. The leaf provided the diacytic type of stomata. Stomata are present only in the lower surface of the leaf whereas, no stomata were present on the upper surface. Microscopic observation revealed that the leaf provided the diacytic type of stomata. Stomata are present only in the lower surface of the leaf whereas, no stomata were present on the upper surface. The transverse section of the leaf revealed that the epidermal cells consist of straight anticlinal walls. Beneath every upper epidermal cell, there are about 3.54 ± 0.40 palisade parenchyma cells present; they are very green and tightly packed [28].

Determination of Leaf Constant: The leaf constant like a stomatal number, stomatal index, vein islet number, vein termination number, and palisade ratio of the *Achyranthes aspera* leaf was studied and recorded. Stomatal no. Epidermal cells, vein islet (10 ×), Vein termination (10 × s) counts per 1 mm square area (1 mm 2), Stomatal length in µm. (all the readings are taken in 40 ×). The quantitative leaf parameters are listed in **Table 1.**

TABLE 1: LEAF CONSTANT OF *ACHYRANTHES ASPERA* LINN

Parameters	Range	Mean ± SE
Palisade ratio	3.21-3.86	3.54±0.40
Stomata number lower surface	43.15-54.54	49.00±2.5
Stomata index lower surface	20.42-25.11	22.77±0.60
Vein islet number	30.54-35.49	33±3.50
Vein termination number	24.85-31.34	28.10±2.00
Epidermal cells Upper surface	131.57-139.42	135.50±1.50
Epidermal cells lower surface	136.26-144.13	140.20±3.50
Stomata length	20.45-24.54	22.50±1.25
Stomata Breadth	14.93-17.56	16.25±1.25

TABLE 2: FLUORESCENT ANALYSIS OF ACHYRANTHES ASPERA LINN

S. no.	Treatment	Fluorescent at 254 nm	Fluorescent at 366 nm
1	Powder as such	----	----
2	Powder + Dilute Nitric acid	Yellow orange	Light Or
3	Powder + 10% Sodium hydroxide	Dark yellow	Yellow
4	Powder + 1N hydrochloric acid	Light yellow	Orange Yellow
5	Powder + 50% Nitric acid	Light yellow	Yellow Orange
6	Powder + Acetic acid	Dark yellow	Yellow Orange
7	Powder + Picric acid	Dark yellow	Orange
8	Powder + 50% Ferric chloride	S red	Reddish Orange
9	Powder+N/50 Iodine Solution	Blue	Fluorescent Blue
10	Powder + 50% Sulphuric acid	Yellow	Light Orange
11	Powder + Ethanol	Dark yellow	Greenish Yellow

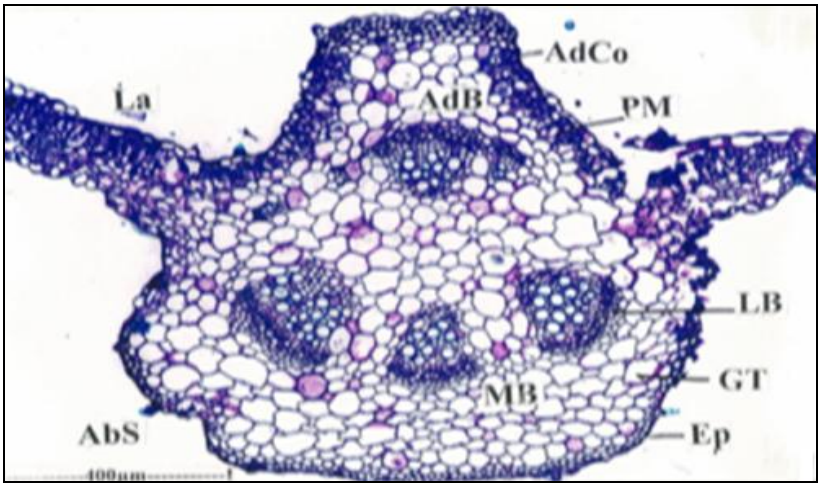


FIG. 1: T.S OF THE LEAF OFACHYRANTHESASPERALINN AD. CO-ADAXIAL COLLENCHYMA, ABS-ABAXIAL SIDE EP-EPIDERMIS, GT-GROUND TISSUE PM-PALISADE MESOPHYLL, LA-LAMINA, ADB–ADAXIAL VASCULAR BUNDLE, MB -MIDDLE BUNDLE, LB -LATERAL BUNDLE

Fluorescent Analysis: The leaf powder was treated with a different reagent to find out their fluorescent character of colour development at 254 and 366 nm. It was tabulated in **Table 2.**

TABLE 2: FLUORESCENT ANALYSIS OF ACHYRANTHES ASPERA LINN.

S. no.	Treatment	Fluorescent at 254 nm	Fluorescent at 366 nm
1	Powder as such	----	----
2	Powder + Dilute Nitric acid	Yellow orange	Light Or
3	Powder + 10% Sodium hydroxide	Dark yellow	Yellow
4	Powder + 1N hydrochloric acid	Light yellow	Orange Yellow
5	Powder + 50% Nitric acid	Light yellow	Yellow Orange
6	Powder + Acetic acid	Dark yellow	Yellow Orange
7	Powder + Picric acid	Dark yellow	Orange
8	Powder + 50% Ferric chloride	S red	Reddish Orange
9	Powder+N/50 Iodine Solution	Blue	Fluorescent Blue
10	Powder + 50% Sulphuric acid	Yellow	Light Orange
11	Powder + Ethanol	Dark yellow	Greenish Yellow

Preliminary Phytochemical Screening: The leaf extract with different solvent systems were studied, and the presents of different phytoconstituents were identified and tabulated in **Table 3.**

TABLE 3: PRELIMINARY PHYTOCHEMICAL SCREENING WITH DIFFERENT SOLVENT EXTRACTS

S. no.	Chemical Test	Acetone	Ethyl Acetate	Petroleum Ether	Chloroform	Methanol	Water
1	Alkaloids	+	+	+	+	+	+
2	Carbohydrates	+	+	+	+	+	+
3	Proteins	_	-	-	-	-	+
4	Free Amino acids	+	-	-	-	-	-
5	Glycosides	-	-	-	-	-	-
6	Tannins and Phenolic Compounds	+	+	+	+	+	+
7	Phytosterols	+	+	+	+	+	+
8	Flavanoids	+	+	+	+	+	+
9	Saponins	+	+	-	-	-	+

Column Chromatography: The phytoconstituents from the methanol extract of the leaves of *Achyranthes Aspera* Linn. were isolated by using column chromatography. The column was eluted with solvents of increasing polarity. They were Petroleum ether, Chloroform, Ethyl acetate, Acetone, Ethanol. The column was run with organic solvents in various proportions. A yellow crystalline compound was isolated from the methanol extract of *Achyranthes Aspera Linn*. in the fraction of Chloroform: Ethyle acetate in (80:20) ratio. It is Hygroscopic in nature [29].

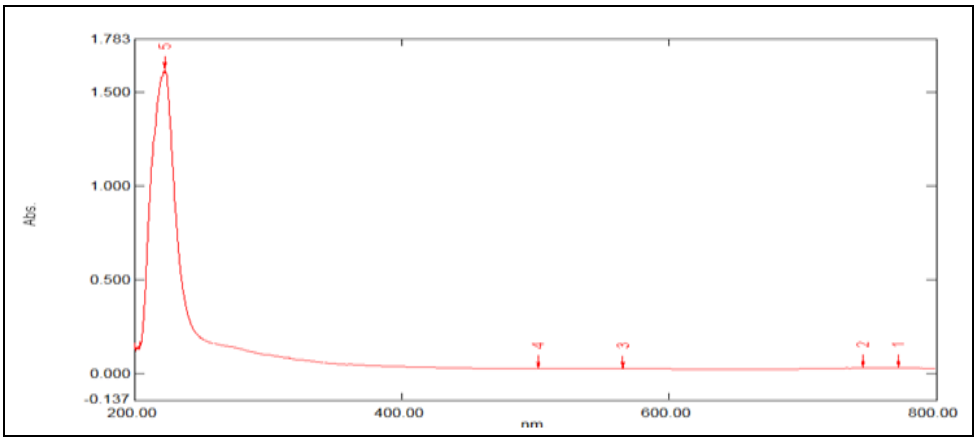


FIG. 2: UV-VIS SPECTRUM OF THE COMPOUND ISOLATED FROM METHANOL EXTRACT

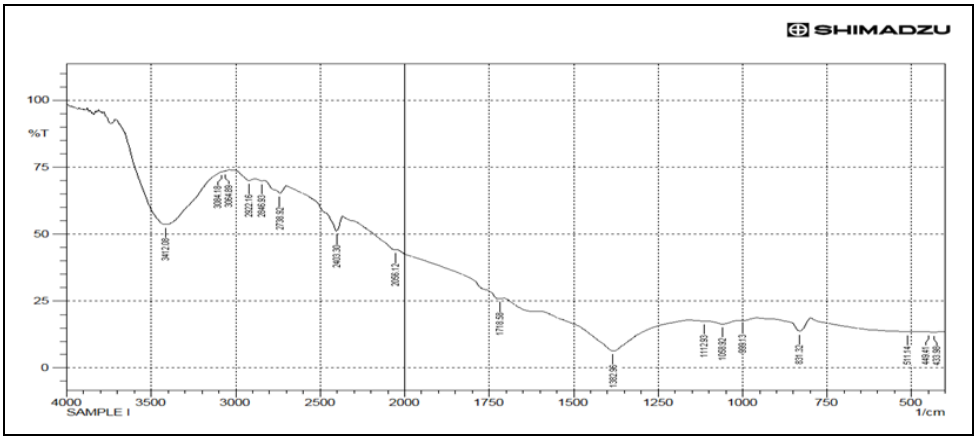


FIG. 3: IR SPECTRUM OF COMPOUND ISOLATED FROM *ACHYRANTHESASPERA* LINN

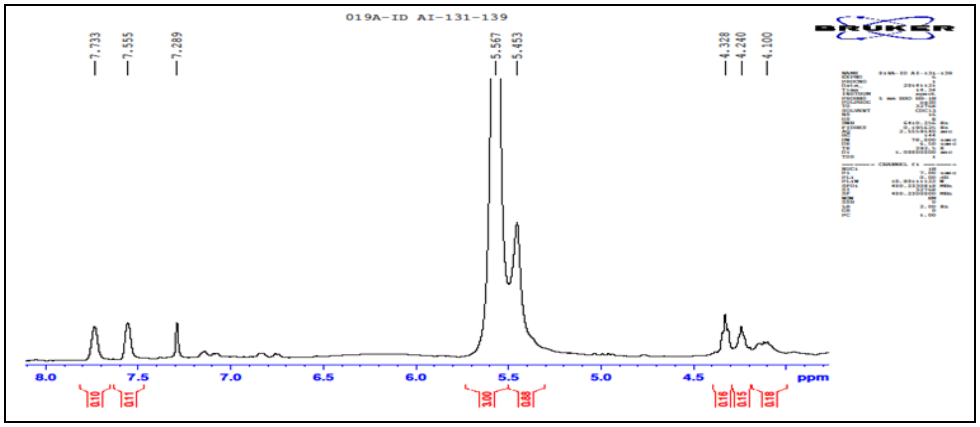


FIG. 4: NMR SPECTRUM OF COMPOUND ISOLATED FROM *ACHYRANTHES ASPERA* LINN

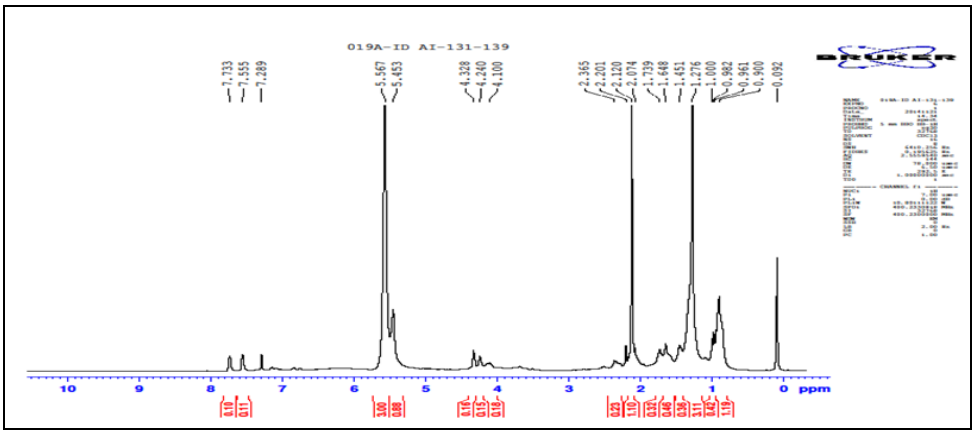


FIG. 5: NMR SPECTRUM OF COMPOUND ISOLATED FROM *ACHYRANTHES ASPERA* LINN

TABLE 5: FTIR FREQUENCY PEAK VALUES OF THE ISOLATED COMPOUND [29]

S. no.	Peak Values absorption	Functional groups	Compound class
1	433.98	-	-
2	449.14	-	-
3	511.14	C-Br	-
4	831.32	C=C	Alkanene
5	999.13	C-C	Alkane
6	1058.92	C-F	Fluro compound
7	1112.93	C-F	Fluro compound
8	1382.96	CH_3 -,-CH_2-	Alkane
9	1718.58	C=O	Ketone Ester
10	2056.12	-	-
11	2403.3	-	-
12	2730.92	C-H	Aliphatic
13	2846.93	C-H	Aliphatic
14	2922.16	C-H	Aliphatic
15	3064.89	O-H stretching	Phenolic
16	3084.18	O-H stretching	Phenolic
17	3412.08	O-H stretching	Phenolic

TABLE 6: NMR SPECTRUM - CHEMICALS SHIFT OF THE ISOLATED COMPOUND [30, 31]

S. no.	Type of Hydrogen	Chemical Shift
	R-CH_3,Alkyl[Methyl]	0.092,0.900, 0.961,0.982 , 1.000
	R-CH_2-R,Alkyl[Methlene]	1.276, 1.451
	Alkyl[Methine]	1.148, 1.739
	Alpha To Carbonyl	2.074, 2.120, 2.201
	Benzylic	2.3
	Or-C-Nhr [Amide]	5.453,5.367
	Ar-H AROMATIC	7.289
	Or-C-Nhr [Amide]	7.555,7.733

As per the spectral studies, the isolated compound has two carbonyl groups that appeared at δ 167.86 & 177.14. Unsaturated carbons appeared at δ 128.85 & 132.26 **Fig. 2** [32, 33].

The unsaturated protons appeared between δ 5.56., Two -CH_2-O- groups appeared at δ 65.63 & 68.19 **Fig. 3** [34, 35]. The corresponding protons at δ 5.45. Two methyl carbons appeared at δ 14.14 & 19.19. The methyl protons appeared at δ 0.98., **Fig. 4** [36, 37]. The long-chain methylene carbons appeared between δ 20.58 & 38.70.

The long-chain methylene protons appeared at δ 1.29 as a broad singlet **Fig. 5** [38]. The isolated compound may be (E)-2-(E)-tetracos-21-enoyloxy) ethyl pentacos-22-enoate.

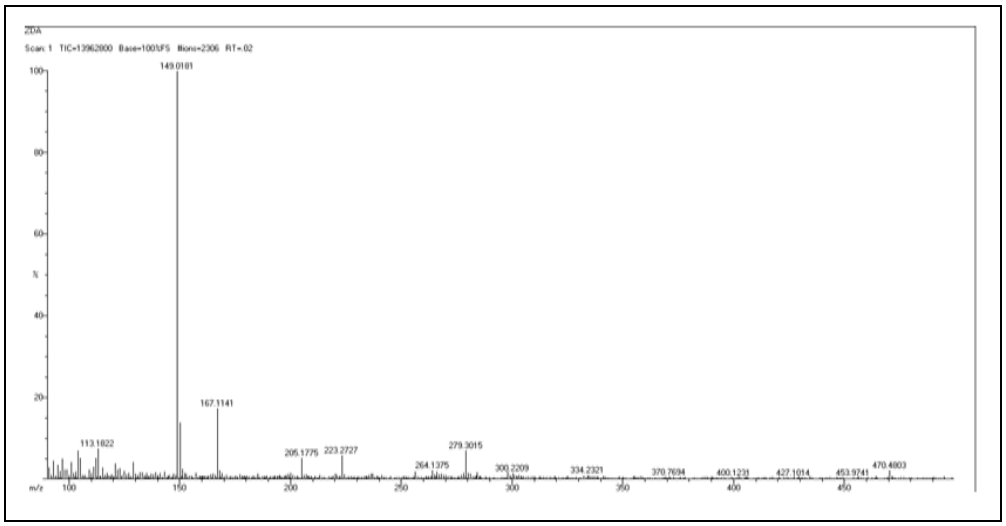
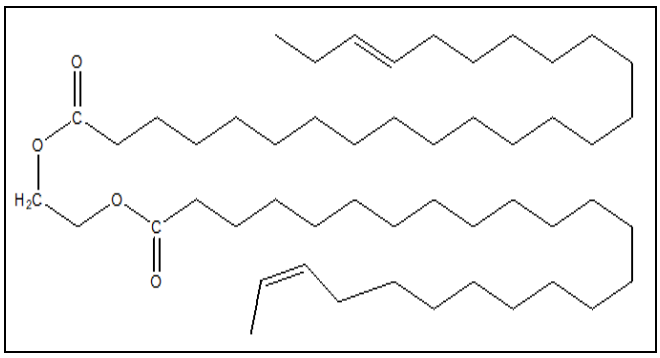


FIG. 6: MASS SPECTRA OF COMPOUND ISOLATED FROM *ACHYRANTHES ASPERA*. LINN

Proposed Chemical Structure:



(E)-2-(E)-TETRACOS-21–ENOYLOXY) ETHYL

Antimicrobial Activity: Methanol and water extract of the plant was tested for their antibacterial and antifungal activity. The effect produced by the sample was compared with the effect produced by the positive control (Reference standard Ciprofloxacin 5 µg/disc for bacteria; Nystatin 100 µg/disc for fungi). The inhibition of microbial growth measured the diameter of the inhibition zone after prior incubation, and the experiment was done twice, and the average determination was recorded and included. The obtained results are tabulated.

TABLE 7: THE ANTIMICROBIAL ACTIVITY OF THE EXTRACTS

S. no.	Name of the Microorganisms	Zone of Inhibition nm				Zone of Inhibition nm			
		Methanol extract		Solv. control	Std 200 µl	Water extract		Solvent control	STD
		200 µl	400 µl			200 µl	400µl		
1.	*Staphylococcus aureus* (NCIM 2079)	14	19	-	35	15	16	-	34
2.	*Bacillus subtilis* (NCIM 2063)	15	20	-	40	16	18	-	36
3.	*Klebsiella aerogenes* (NCIM 2098)	10	15	-	30	09	14	-	32
4.	*Pseudomonas aeruginosa* (NCIM 2036)	13	16	-	40	14	12	-	38
5.	*Aspergillus niger* (NCIM 105)	14	19	-	35	14	18	-	33
6.	*Candida albicans* (NCIM 3102)	13	18	-	32	15	17	-	40

Standard- Ciprofloxacin 5µg /disc for bacteria; Nystatin 100 units / disc for fungi. Solvent- DMSO, Std - standard

Aqueous extract of the plant was tested for its antibacterial and antifungal activity. The effect produced by the sample was compared with the effect produced by the positive control (Reference standard Ciprofloxacin 5 µg/disc for bacteria; Nystatin 100 µg/disc for fungi). The inhibition of microbial growth measured the diameter of the inhibition zone after prior incubation, and the experiment was done twice, and the average determination was recorded and included [40].

The aqueous and methanol extract prepared from the selected plant was tested for their antibacterial and antifungal activity. The effect produced by the sample was compared with the effect produced by the positive control (Reference standard Ciprofloxacin 5 µg/disc for bacteria; Nystatin 100 µg/disc for fungi). The inhibition of microbial growth measured the diameter of the inhibition zone after prior incubation, and the experiment was done twice, and the average determination was recorded and included Standard- Ciprofloxacin 5 µg/disc for bacteria; Nystatin 100 units/disc for fungi [41].

The antibacterial and antifungal activity clearly shows that it is dose-dependent. The methanol extract shows better antibacterial activity than the aqueous extract. The extracts show more activity towards *Staphylococcus aureus*, *Bascillus subtilus* and *Pseudomonas aeruginosa*. In the dose, 200 mg itself shows equal activity same as that of the standard drug. The antibacterial activity is also better in the dose of 400 mg. The extract shows a significant effect on fungal organisms like Aspergillus niger and Candida albicans. As per the literature, a different part of this selected plant is having more pharmacological activities, anti-inflammatory, analgesic, and antipyretic activities [42, 43]. The leaf extracts with methanol and water have antibacterial and antiviral activity. This study also reveals that it has antimicrobial activity.

CONCLUSION: In this study, pharmacognostic parameters like morphological, microscopical and leaf constant were studied and documented. The Pharmacognostical study may be further used for documentation purposes.

The preliminary phytochemical study confirms the presence of alkaloids, terpenoids, tannins, flavonoids, and phytosterols in the different solvent extract. The methanol extract was subjected to column chromatography for the isolation of the active compound and one compound was isolated, and its structural evaluation was done by UV, IR, NMR, and MASS Spectral studies. The proposed structure of the isolated compound may be (E)-2-(E)- tetracos-12-ethyl pentacos-22-enoate.

It may be the intermediate for the production of secondary metabolite or may be the precursor of the secondary metabolite molecule. The methanol and aqueous extracts show antimicrobial activity, but the methanol extract shows better activity than the aqueous extract.

ACKNOWLEDGEMENT: The authors are thankful to PSG College of Pharmacy for providing us to use the laboratory facility to complete this work.

CONFLICTS OF INTEREST: Nil

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How to cite this article:
Christi VEI, Bhatcha MK and Sha NIB Fogarty: Qualitative pharmacognostical and phytochemical evaluation on *Achyranthes aspera* linn. leaf. Int J Pharm Sci & Res 2022; 13(2): 793-02. doi: 10.13040/IJPSR.0975-8232.13(2).793-02.