Evaluation of Antimicrobial Potential and GC-MS Profiling of Bioactive Compounds conferring antimicrobial potential in *Achyranthes aspera* L.

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Abstract
Achyranthes aspera L., commonly known as prickly chaff flower or devil's horsewhip, is an annual or perennial herb of the Amaranthaceae family that grows up to a height of 1 meter. Achyranthes aspera has a wide geographical distribution across the globe. It is found in tropical and subtropical regions of Asia, Africa, Australia and the USA. The plant thrives in a variety of habitats including grasslands, wastelands, roadsides and agricultural fields. Even though the plant has been categorized as a weed, it possesses a range of pharmacological properties. Various parts of the plant such as the roots, leaves and seeds, have been used to treat a range of ailments including inflammation, gastrointestinal issues and skin conditions. The aim of the current study was to assess the potential antimicrobial activities of the methanolic inflorescence extract of A. aspera by determining the susceptibilities of various strains of microbes, Gram-positive bacteria: Bacillus cereus, Staphylococcus aureus; Gram negative bacteria: Escherichia coli and Pseudomonas aeruginosa and fungal strains Candida albicans, Fusarium oxysporum, Aspergillus niger, Alternaria alternata assessed by disc-diffusion assay.

The bioactive compounds of the methanolic inflorescence extract were profiled using gas chromatography-mass spectrometry (GC-MS). The methanolic extract exhibited relatively significant antifungal activity compared to antibacterial activity. The highest antimicrobial activity was exhibited against fungal strain C. albicans. The GC-MS profile of the extract revealed the presence of bioactive constituents like cis-9-hexadecenal (4.63 %), n-Hexadecanoic acid (3.07 %), 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro- (2.26 %), acetophenone (2.22 %), stigmasterol (1.47 %), gamma-sitosterol (1.07 %). The presented research provides evidence to substantiate the use of the plant as a nutraceutical agent against tested microbial strains.

Keywords: *Achyranthes aspera* Linn., inflorescence, methanol extract, antimicrobial, disc diffusion assay, GC-MS.

Introduction

Achyranthes aspera is a perennial or annual herb belonging to the family Amaranthaceae. It is commonly known as prickly chaff flower or devil's horsewhip. The plant is found to be native to tropic and subtropic regions of Asia and Africa but has also been introduced to other regions around the world. It is a hardy plant that thrives in a variety of habitats including disturbed areas, roadsides, waste lands and agricultural fields. It prefers well-drained soils and can tolerate extreme drought conditions. It is considered a weed in many regions due to its aggressive growth habit and ability to outcompete native vegetation. Despite being classified as a weed, the plant has been used in traditional medicine for its remarkable medicinal potential.

In India, it is found in abundance at an altitude of 210 m. Its occurrence has also been reported from southern Andaman Islands. It is often seen growing along roadsides or in wastelands, plains, foothills, abandoned gardens, crops, grasslands and forest margins of tropical and warmer regions. It is reportedly found distributed in various States like Uttar Pradesh, Bihar, West Bengal, Tamil Nadu, Karnataka and Maharashtra. Rajasthan is one of the states in India where the plant has been found as a weed found in the vicinity of agricultural crops or growing naturally in the wild. The distribution of this plant in such an extremely arid and semi-arid climate of Rajasthan is attributed to its tolerance to drought conditions. In Rajasthan, this plant is known by local names such as "Apamarga" or "Chirchita" and is valued for its therapeutic uses in traditional medicine practices.[9,44]

Traditionally, the plant has been reported as a potent pharmacological intervention in treatment of asthma, dysentery, fever and possesses therapeutic activities such as antileprosy[52], abortifacient[54], contraceptive[86], antibacterial[84], thyroid-stimulating, antiperoxidative[79], cancer chemopreventive[10], stimulates reproductive functions[66], anti-inflammatory, anti-arthritic[20] and hepatoprotective[8]. In different folklore, different plant part extracts of *A. aspera* were used in treating hydrophobia[22]. Adding *A.aspera* decoction to the drugs obtained from plants leads to an increase in the efficacy of plant originated drugs as claimed by the traditional healers[24].

It is used in Ayurvedic toxicology, in treating poisoning that destroys it or is eliminated from the body[22]. The root extract of plant was reported to have potent insect hormonal molting

activity[26]. The saponins obtained from the plant extract exhibit *in vitro* phosphorylase activity on heart[61]. The root extract of this plant has been found to be effective in conditions like high blood pressure, malaria, respiratory conditions like asthma[50] and diabetes[2]. On the other hand, the complete plant decoction has been utilized in pneumonia treatment and also shows diuretic effects[49,50]. The application of *A. aspera* leaves as a principal constituent of one of the medications from the Siddha School of Medicine, Naayuruvi kuzhi thailum, has been reported to be quite beneficial in the management of asthma. Preliminary phytochemical screening reveals the existence of diverse classes of phytochemicals including saponins, flavonoids, alkaloids, terpenoids, steroids, quinones, acyl quinic acid, essential oils, phenols, glycosides, fatty acids and esters.[15,18,85]

The current study aims at evaluation of the antimicrobial potential of the inflorescence extract against various antibacterial and antifungal strains as well as detailed profiling of the bioactive phytoconstituents of the extract using GC-MS.

Material and Methods

Collection and authenticity of plant material: *A. aspera* fresh plant material was collected from Chandli village, Deoli tehsil of district Tonk in Rajasthan, India in July, 2021. The plant specimens were identified and authenticated by the experts from Department of Botany, University of Rajasthan, Jaipur, Rajasthan (India). Voucher specimen of the plant (RUBL-21548) was deposited in the herbarium of the same department for future reference. The collected plant material was thoroughly rinsed first in running tap water and then with distilled water in order to remove the dirt and soil particles, followed by blot drying in order to remove any excess moisture. The inflorescence from the collected plants was clipped, collected and shade dried for a week and grounded into uniform powder. Fig. 1 illustrates the plant found in the wild as well as the spike inflorescence of the plant.

Chemical Reagent: All the chemicals employed in the analysis procedure were of analytical grade. The distilled water used in the current research procedure was obtained from the steam distillation unit installed at the laboratory facility.

Extraction of *A. aspera* L. inflorescence: The methanol extract was prepared by dissolving dried inflorescence powder (20 mg) using 100 mL methanol via Soxhlet extraction apparatus. The solvent was allowed to evaporate at room temperature spontaneously and the remaining dry crude extract from each of the solvent was weighed and then diluted in 100% DMSO at a concentration of 10 mg/ml. Fresh samples of the whole plant have been collected.

Fig. 1: a: Whole plant of *A. aspera* L.; b. Inflorescence on the tip of the plant; c. Close up picture of separated inflorescence

Different plant parts were manually separated with precision and they were allowed to get completely dried at room temperature separately under shade and then they were mashed into fine powder using a grinder.

Test Microorganism: The antimicrobial activity of the methanolic inflorescence extract was tested against various antimicrobial strains (antibiotic as well as antifungal). The strains of bacteria tested for susceptibility are Gram-positive bacteria: *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 3160); Gram negative bacteria: *Escherichia coli* (MTCC 1652) and *Pseudomonas aeruginosa* (MTCC 741) and fungal strains *Candida albicans* (MTCC 3958), *Fusarium oxysporum* (MTCC 3004), *Aspergillus niger* (MTCC 282) and *Alternaria alternata* (MTCC 2060). These bacterial strains were collected from S.M.S. Medical College, Jaipur, Rajasthan, India. These strains were maintained in sterile nutrient agar (HiMedia) slants and were stored at 4°C until further use.

Agar well diffusion assay: *In vitro* antimicrobial potential of methanolic extract of inflorescence was tested using agar well diffusion method[56]. The provided protocol was followed and the medium used for bacterial growth was Mueller Hinton agar medium. The agar media was first melted and then cooled to 48-50°C before pouring into plates. The assay plates were prepared using freshly prepared agar media and 25 ml of it was poured in autoclave sterilized 100 mm x15 mm Petri plates. They were allowed to solidify. The solidified agar plates were aseptically inoculated with suspensions of various microbial strains. The target bacterial strain suspensions were freshly prepared by diluting the microbial culture to achieve a concentration of 10^8 CFU/ml. Wells of radius of 3 mm were created in the inoculated agar plates.

The analyte or test material (60 µl per well) was then introduced into these wells. These plates were incubated for the period of 24 hrs at 37°C. A solution of 10% (v/v) DMSO was used as negative control whereas antibiotic ciprofloxacin 10 mg/ml (40 µl per well) was used as positive control. In the agar well diffusion assay, diameter of zone of inhibition (mm) was measured to determine the antibacterial activity. Zones of inhibition formed around the wells filled with sample extracts, standard and DMSO were measured and recorded.

GC-MS analysis: The methanol extracts of *A. aspera* inflorescence were analyzed for the identification of phytoconstituents using Shimadzu gas chromatography-mass spectrometry (QP-2020) with a mass spectrometer operating in electron impact (EI) mode with MS voltage 0.96 kV with the specifications provided in table 1. Immediately prior to putting the sample extract in GC-MS vials, the *A. aspera* inflorescence methanol extract was filtered two times using Whatmann filter paper no. 1 in order to remove any suspended solids from the sample extract that could possibly disrupt or damage the GC-MS column injections.

Table 1
GC- MS Instrumentation Parameters

Instrument	GC-MS Shimadzu QP 2020
GC Conditions	
Carrier Gas	Helium; Constant flow
Column Volume	Column SH-RXI-5silMS (30 × 0.25 mm × 0.25 µm thickness)
Column Oven temperature	50 ºC
Injection Temperature	250 ºC
Injection Mode	Split
Split Ratio	1: 50
Flow Control Mode	Linear Velocity
Linear Velocity	40.1 cm/sec
Flow Pressure	52.7 kPa
Total Flow	16.3 ml/min
Column Flow	0.99 ml/min
Purge Flow	3.0 ml/min
Rate	At 50°C; held isothermal for 3 mins. and then increased the column temperature up to 320°C at the rate of 30°C/min with intermediate hold time at 200°C (2 min.), 250°C (4 min), 300°C (4 min.), 320°C (1 min). The duration of one complete operation was 30 min.
MS-parameter	
Transfer line Temperature	250 ºC
Ionization mode	Electron impact (EI) mode
MS Voltage	0.96 kV
Scan Range	35 - 500 m/z

About 1µl volume of the tested extract was injected into GC and the chromatogram was obtained in the time range of 30 min. The mass spectral survey and identification of the phytoconstituents were performed using the NIST library of mass spectral search program.

Identification of Phytoconstituents: The relative abundance of each compound present in the extract was determined by comparing the area of its peak to the total peak area, expressed as relative percentage. Turbo mass software was used for handling mass spectra and chromatograms. Phytoconstituents were identified by matching their retention times in the solvent and their recorded mass weights were compared with the authentic samples from GC records, National Institute of Standards and Technology, US database that keeps the record of approximately 62,000 patterns and the Wiley pesticide library 3rd edition.

The findings were cross-verified using other research articles and the PubChem online database for compound activities and structures. The gas chromatogram reports obtained of the resultant extracted phytoconstituents were further checked for their potential pharmacological activity.

Results and Discussion

Antimicrobial analysis: The results of the agar well diffusion assay revealed that the tested extract has relatively stronger antimicrobial potential as antifungal agent, with maximum growth inhibitory activity exhibited against fungal strain *C. albicans* with an activity index of 0.57, followed by another fungal strain *A. alternata* with activity index of 0.56 with the zone of inhibition of 12 $\pm$ 0.07 mm and 10 ± 0.63 respectively. Although the tested extract exhibited significant antibacterial potential against both Gram positive and Gram-negative bacteria. The extract exhibited maximum antibacterial potential against *B. subtilis* as well as *P. aeruginosa* with zone of inhibition of 17 $\pm$ 0.14 mm and activity index of 0.47 for both the bacterial strains (Fig. 2). Table 2 enlists the activity index exhibited by the test extract against different microbial strains.

Methanol extract of leaves has been tested for potent antimicrobial activity but on the contrary to the current findings, it has been reported to exhibit no significant activity against the fungal strain *C. albicans*[40]. Previous antimicrobial analysis report on the aerial extract of plant has been reported to be effective against *B. subtilis*, *E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa* at the concentration of 1000 µg mL^{-1}.[23]

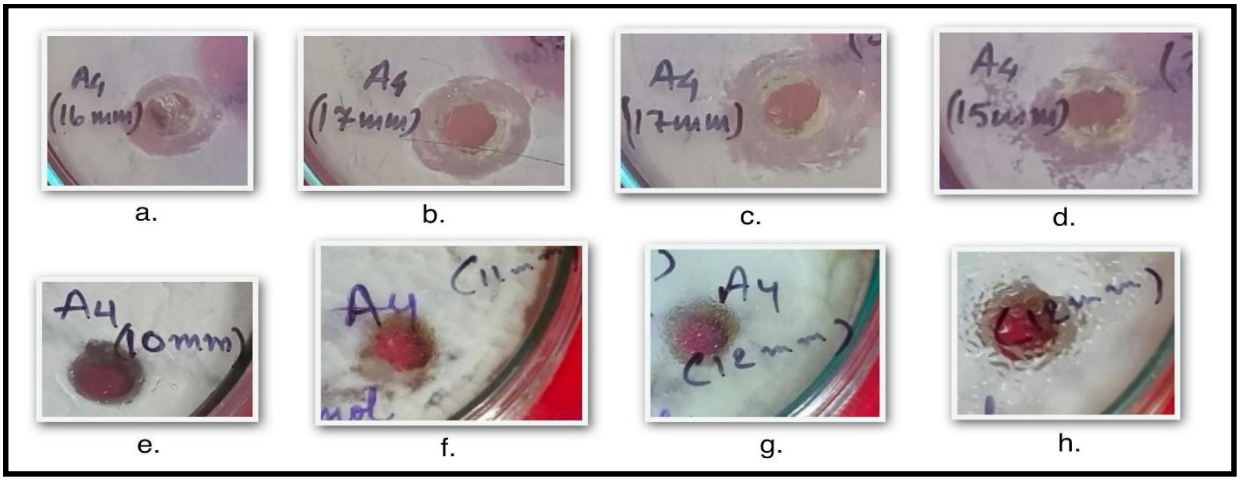

Fig. 2: Antimicrobial agar well diffusion plates with measured ZI of methanol extract: a. *E. coli*; b. *P. aeruginosa*; c. *B. subtilis*; d. *S. aureus*; e. *A. alternata*; f. *A. niger*; g. *C. albicans*; h. *F. oxysporum*

Table 2
Agar well diffusion assay results

Microbial Strains	Activity Index (AI)
Bacterial strain	
E.coli	0.429 ± 0.057
P. aeruginosa	0.472 ± 0.061
B. subtilis	0.472 ± 0.040
S. aureus	0.405 ± 0.023
Fungal strain	
A.alternata	0.561 ± 0.015
A. niger	0.522 ± 0.062
C. albicans	0.572 ± 0.038
F. oxysporum	0.502 ± 0.048

GC-MS analysis of *A. Aspera* inflorescence extract: The phytoconstituents present in the *A. aspera* methanol extract of inflorescence analyzed by GC-MS are listed in table 1. Fig. 1 represents the GC-MS chromatogram of the same. The analysis results revealed the identification of 31 compounds in total from the extract with presence of a range of compounds like phytol, fatty acid, fatty acyl esters, sterols etc. The compounds such as cis-9-hexadecenal (4.63 %), n-Hexadecanoic acid (3.07 %), 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro- (2.26 %), acetophenone (2.22 %), stigmasterol (1.47 %) and gamma-sitosterol (1.07 %) are identified as the prominent bioactive phytoconstituents of the *A. aspera* inflorescence.

Not much phytochemical analysis was done on the phytochemical profiling of the inflorescence extract of the plant. Previous phytochemical analysis of *A. aspera* seeds methanol extract has reported the presence of a triterpenoid saponin viz. β-D-Glucopyranosyl oleanolate[67]. Several phytochemical studies on methanolic extract of leaves of the same plant have been reported to contain different classes of saponins that function in making plant larvicidal in nature[19,71,76]. On the contrary, there are no such saponins reported from the tested inflorescence extract. However, compounds have been identified that have been reported to exhibit nematicidal, antimicrobial properties like n-Hexadecanoic acid, hexadecanoate, methyl ester and larvicidal properties by tetradecanoic acid[75].

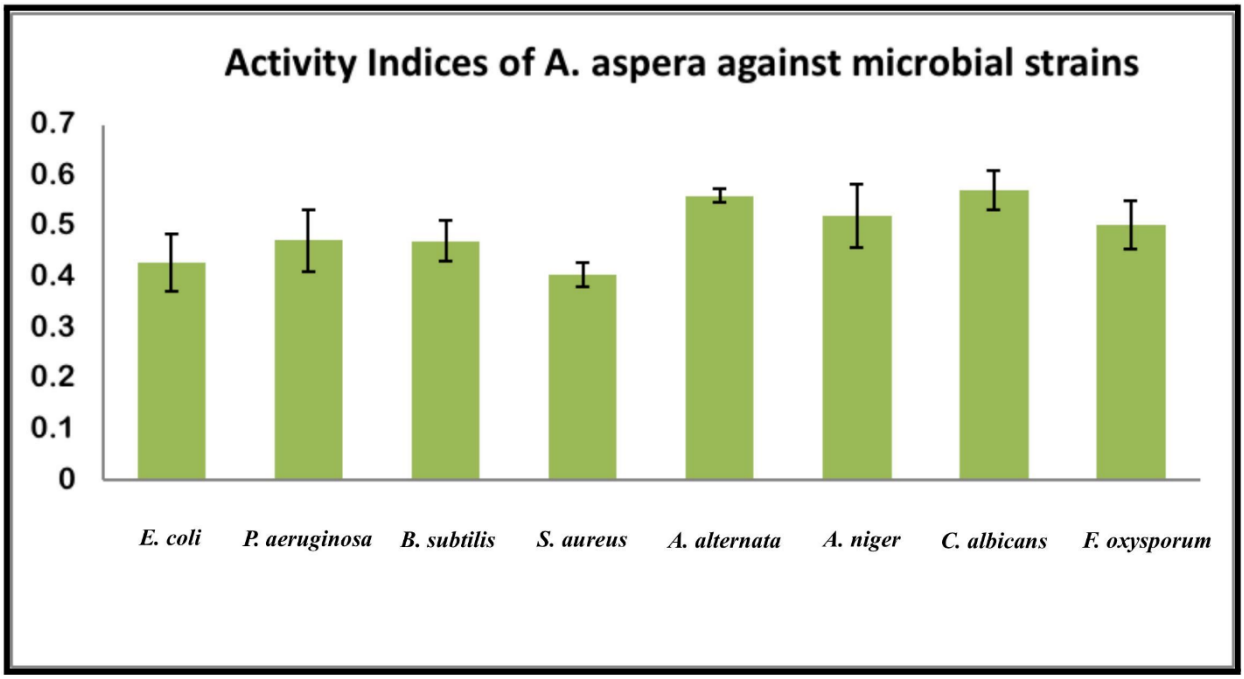

Fig. 3: Relative Activity Index of *A. aspera* methanol extract of inflorescence against microbial strains

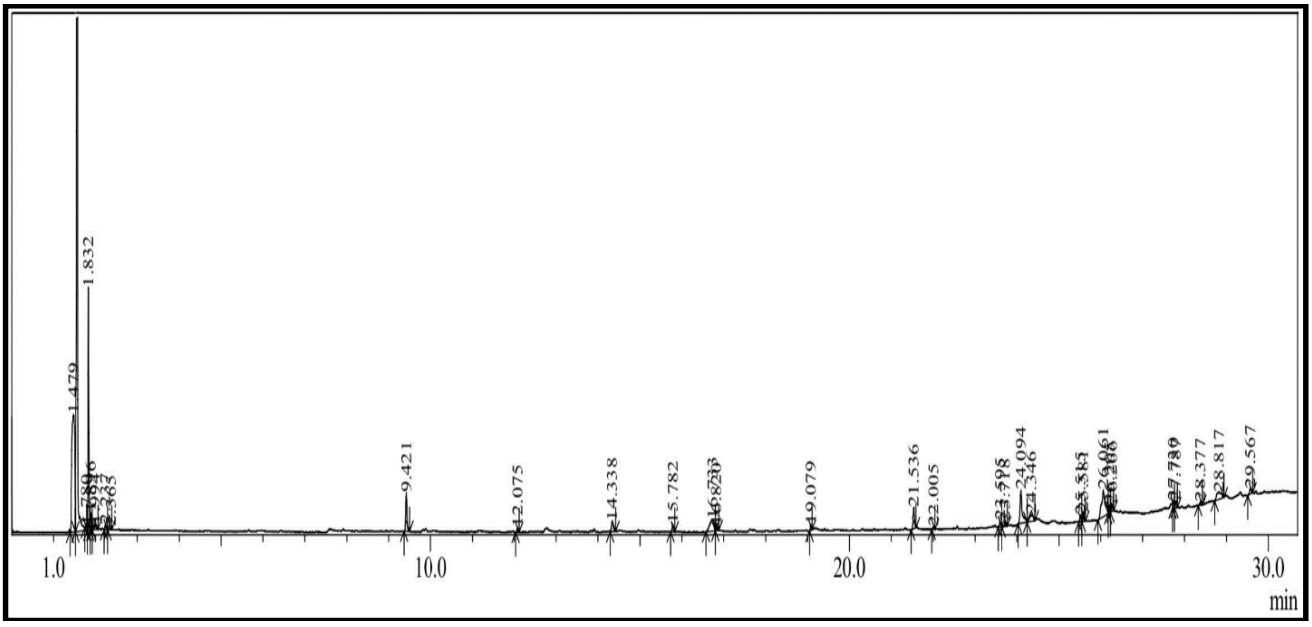

Fig. 4: GC-MS chromatogram of inflorescence methanol extract of *A. aspera*

Table 3
GC-MS Compounds with RF and peak area %

Compound name	Retention Time	Area %	Pharmacological Activity
Hydrazinecarbothiamide	2.365	0.13	Antimicrobial[65], antiradical[68] anti-inflammatory[55]
Acetophenone	9.421	2.22	Selectively antimycobacterial[60]; antifungal[21], antimicrobial[43,53], antibacterial[74], anticancer[5]
Dodecane	12.075	0.25	Antimicrobial[4,34]
2-methoxy-4-vinylphenol	14.338	0.81	Anti-inflammatory[7], antimicrobial[64], anticancer
Tetradecane	15.782	0.39	Antifungal and antibacterial[42]
2-(hydroxymethyl)-2-nitro-1,3-Propanediol	16.733	2.26	Antimicrobial[14], Microbicidal, bacteriostat in disinfectants[57,72]
Tetradecamethyl- cycloheptasiloxane,	16.820	0.31	Antifungal[45], anti-inflammatory[51]
Hexadecane	19.079	0.29	Antibacterial[35,37]
Tetradecanoic acid	21.536	1.34	Larvicidal[75], Antioxidant[77]
Octadecane	22.005	0.13	Antioxidant, antityrosinase, antimicrobial[46], antifungal[48]
Hexadecanoic acid, methyl ester	23.595	0.31	Active antimutagenic, antibacterial[17], nematicidal[78], Antifungal[1], cytotoxic, anticancer, antioxidant, antimicrobial, anti-inflammatory[70,81,82]
Benzenepropanoic acid, 3,5-bis(1,1-dimethyl)-4-hydroxy-methyl ester	23.718	0.25	Antifungal and antioxidant[80]
n-Hexadecanoic acid	24.094	3.07	Anti-inflammatory[6], antioxidant, hypocholestrolemic, nematicidal, pesticide, anti-androgenic, haemolytic, 5α- reductase inhibitor[33], potent mosquito larvicide[59], antibacterial and antifungal[11]
Gamma-sitosterol	24.346	1.07	Anticancer, cytotoxic, antihyperlipidemic, antidiabetic, antifungal[13,69]
9, 12- octadecadienoic acid (z,z)- ester	25.515	0.36	Hepatoprotective, antihistamines, antieczemic, hypocholestrolemic[38,62,87]
9-octadecanoic acid methyl ester	25.581	0.49	Antiviral[30,39,88]
cis-9-hexadecenal	26.061	4.63	Anti-inflammatory[32,47], antimicrobial and antifungal[25,27]
Ethyl oleate	26.266	0.45	Antimicrobial[2]
Oxazole	27.787	0.31	Antimicrobial, antidiabetic, anticancer, antiobesity, anti-inflammatory[28]
Hexanedioic acid bis (2-ethylhexyl) ester	28.377	0.18	Antimicrobial[29]
Stigmasterol	28.817	1.47	Anti-inflammatory, antioxidant, antimicrobial, anti-hepatotoxic, antiviral, antioxidant, cancer preventive, hypocholestrolemic[16,31,63,73]
Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl)ethyl ester	29.567	0.41	Antioxidant[73]

The present findings on these extracts were found in agreement with the previous phytochemical screening work on the leaf ethanol extract of the plant that reported the presence of oleate, benzene (1-methyl-propyl), cyclohexane, octadecanoic acid as the major phytochemical[41]. Further findings on the phytoconstituents from the methanolic leaf extract were also found concurrent to the current phytochemical profile revealing the presence of major chemical constituents: phytol, 9,12 octadecanoic acid (z,z) and 9,12,15- octadecanoic acid methyl ester (z,z,z), hexadecanoic acid, ethyl ester[58,83].

Conclusion

Medicinal plants constitute a significant category of economically important flora, acting as essential sources of raw materials necessary for pharmaceutical applications. Achyranthes aspera is particularly notable for its novel bioactive compounds which demonstrate a range of properties including anti-inflammatory, antimutagenic, anticancer, antiviral, antimicrobial, nematicidal and larvicidal, antioxidant, antiviral activities etc. In modern times, plant-derived materials continue to play a crucial role in primary healthcare as therapeutic interventions, especially in developing regions. Additionally, they offer a promising alternative source of pharmacological agents.

The phytoconstituents present in *A. aspera* methanol extract of inflorescence were identified by the GC-MS technique and their antibacterial properties against 8 microbial (bacteria and fungi) strains were studied. In total, 31 compounds were recognized from these extracts with dominant bioactive phytoconstituents as cis-9-hexadecenal, n-Hexadecanoic acid, 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-, acetophenone, stigmasterol, gamma-sitosterol. Hence, the perceived biological property of the plants and their application which has been reported in traditional medicinal practices, may be due to the presence of a wide range of constituents present in the plant, observed and analyzed in the current research.

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