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PHYTOCHEMICAL ANALYSIS AND *in vitro* ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF ETHANOLIC LEAF EXTRACT OF *Achyranthes aspera* L.

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ABSTRACT

The present study was aimed to evaluate the phytochemicals present in the ethanolic extract of *Achyranthes aspera* leaves (EEAA) and identify the bioactive compounds by GC-MS analysis. *In vitro* antioxidant activity was measured by using DPPH, metal chelating and superoxide radical scavenging assays. The anti-bacterial activity was assayed by disc diffusion method. The results revealed the presence of phenols, flavonoids, and tannins in significant amount in EEAA [372 ± 2 mg GAE g^{-1}, 22.6 ± 1.34 mg CE g^{-1} and 242 ± 2 mg TAE g^{-1}, respectively. GC-MS analysis revealed the presence of 20 major bioactive compounds with 74.36% total leaf extract, dominated by phytol (10.67%), palmitic acid (6.56%), pseudo-ephedrine (6.06%) and hexacosane (5.76%). EEAA exhibited a maximum DPPH radical scavenging activity of 46.3%. The metal chelation activity in EEAA was 58.8%. The EEAA exhibited a maximum of 46.51% superoxide scavenging activity. *In vitro* antibacterial activity of EEAA assayed against three human pathogenic bacteria *viz., Salmonella typhi, Bacillus subtilis* and *Escherichia coli*. EEAA showed maximum inhibition against *B. subtilis* (16.33 ± 0.577 mm). The study revealed that *A. aspera* is a potential source for developing health-oriented products.

Keywords: *Achyranthes aspera*, antibacterial, antioxidants, flavonoid, GC-MS analysis, leaf extract, phenols, phytoconstituents

INTRODUCTION

In recent past the frequent increase in the use of plant-based products has been observed in well-developed countries resulting in the growth of herbal products globally. As per WHO about 80% people depend on herbal medicines for their health (Sardesai *et al.*, 2002). The main advantage of plant-based products is that these are less expensive and safe for human than modern synthetic drugs. The traditional knowledge about the herbal products has led to the discovery of drugs, therefore, documentation of traditional knowledge and conservation of medically important plants is essential strategy to develop proactive policies and implement action plans which may strengthen the use of traditional medicine to keep the population healthy (Kushwaha, 2019).

Achyranthes aspera L. (prickly chaff flower), also known as Latjeera (Chirchita), is a multi-medicinal plant of family Amaranthaceae and used as ethnomedicine for treating various diseases (Manandhar *et al*., 2021). All the parts of plant including leaves, seeds and roots possess high medicinal properties. Different parts of *A. aspera* are used in the treatment of piles, renal dropsy, pneumonia, kidney stone, cough, skin eruption, snake bite, dysentery, gonorrhoea, etc. The distinct-

iveness of fragrance and aroma in many chaff flower species is due to the presence of essential oil in leaves and flowers, as these contain oleanolic acid and linalools (Rehman *et al*., 2018).

The plant has antibacterial, anti-inflammatory, antifertility and antitumor properties, increases pituitary and uterine weight in ovariectomized rats, abortifacient activity (Tanveer *et al*., 2019). The plant possesses wide range of biological properties including anti-phlegmatic, diuretic, antiperiodic, laxative, and purgative, and also beneficial for the treatment of oedema, piles, boils, and damage to the skin also in the treatment of pneumonia, while the fluid of the root is useful for the treatment of mild astringent during bowel complaints (Nadkarni, 2009). The extract of *A. aspera* also shows spermicidal activity, hepatoprotective activity, hypoglycemic activity and cancer-preventive activity (Hasan, 2014). The pharmacological properties of *A. aspera* have been attributed to the presence of active constituents like D-glucuronic acid, β-D-galactopyranosyl ester of D-glucuronic acid, oleanolic acid, amino acids, betaine, p-benzoquinone, hydroquinone, spathulenol, hentriacontane, sapogenin, ecdysterone, nerol, α-ionone, asarone, and eugenol (Devi *et al.*, 2017). The antioxidant defense system protects the cells against free radicals. Once the formation of free radicals overtakes the antioxidant defense system, the free radicals begin attacking the cells leading to several physiological disorders. The leaf extract of *A. aspera* has shown potential antioxidant activity (Awasthi *et al*., 2021; Manandhar *et al*., 2021). The leaves of *A. aspera* are rich in flavonoids and tannins (Dey, 2011) and the flavonoids have the ability to prevent the development of some cancers (Lanjwani *et al.*, 2021; Selsa *et al*., 2020; Awasthi *et al.*, 2021). The potential antioxidant activity could be correlated to their high flavonoid and phenolic compounds or may be due to the presence of other phytochemicals like alkaloids and saponins (Priyamvada *et al*., 2021). The ethanolic extract of *A. aspera* seeds significantly controlled oxidative stress in the pancreas (Radha *et al*., 2017). The plant also possesses potential antimicrobial activity against several human pathogenic bacteria. Methanolic extract of *A. aspera* exhibited potential antimicrobial activity against *Klebsiella pneumonia, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus* (Selsa *et al*., 2020). Chloroform- and methanol-based root and shoot extracts of *A. aspera* showed significant antibacterial activity against *Klebsiella* sp. (Kaur *et al.*, 2005).

Earlier studies have shown that due anti-bacterial activity the *A. aspera* leaf-based ointment can be applied externally (Singh and Samanta, 2022). The roots of plant have strong antibacterial sensitivity (Lanjwani *et al*., 2021). The antibacterial activity of plant attributed to the presence of tannins, saponins, and flavonoids (Samanta *et al.*, 2010). Alkaloids are significant for the protection and survival of plants because they ensure their survival against bacteria and fungi (Mishra, 2018). *A. aspera* reportedly is able to achieve substantial secondary treatment and significant primary and tertiary treatments of greywater in a clean and inexpensive fashion which is key to prevent the dysfunction of ecosystem (Abbasi and Tauseef, 2019). The *A. aspera* plant has several medicinal properties; however, its antioxidant and antibacterial activities are not fully validated and authenticated. Hence, the present study was aimed to assess the potential valuable phytochemicals in the ethanolic leaf extract of *A. aspera*, and also validate its antioxidant and antibacterial potential.

MATERIALS AND METHODS

Sources of chemicals and plant material

All the chemicals and solvents were purchased from Merck, India, and all solvents used were of Good and analytical grade and nutrient agar from Hi-Media, India. *A. aspera* were collected from a different location in Pantnagar, Uttarakhand. The plant was identified by taxonomist Dr. D.S. Rawat, Department of Biological Sciences, GBPUAT, Pantnagar, India.

Preparation of leaf extract of A. aspera

The leaves of *A. aspera* were washed with tap water and then dried at room temperature for at least 15 days. The dried plant material was grounded in a grinder to powdered form. Then 5 g powdered

sample was soaked in 50 mL ethanol and incubated at 37°C for 24 h at 120 rpm in a centrifuge (model: LAB-i-FUGE C series; Lab India Instrument). The solution was filtered through Whatman filter paper No. 1 and the filtrate collected was evaporated and concentrated at 37°C. The dried extract stored at 4°C in the refrigerator till use for analysis.

GC-MS analysis of ethanolic leaf extract

The 0.5 g crude leaf extract of *A. aspera* was dissolved in 50 mL ethanol. The ethanolic extract was analyzed for chemical constituents by a combined gas chromatography (GC) HP 6890 with mass selective detector MS 5973 (Agilent Technologies, USA), fitted with a DB-5 silica column (30 m X 0.25 µm thick film), with split/splitless injector and electronic pressure control. Helium was used as a carrier gas at the flow rate of 1.0 mL min^{-1}. The injector was operated at 250°C and the oven temperature was programmed at 60°C for 15 min. Detection was performed in full scan mode from m/z 41 to 450. The data was analyzed by using software Calibur 4.0, and the chromatograms were analyzed and identified by matching their mass spectral fragmentation patterns with a database deposited at the National Institute of Standards and Technology (NIST 2.2) library on the basis of mass spectral database. The KI (Kovats index) values of constituents were compared with those recorded in authentic literature (Araoud *et al.*, 2007).

Phytochemical screening

Total phenolic content: The quantitative phenol analysis was done as per the standard procedures of Adams (2007). The diluted test sample (0.5 mL) was first reacted with 0.2 mL Folin Ciocalteau reagent and then neutralized with 0.5 mL solution of sodium carbonate. This reaction mixture was kept in dark for 1 h for colour development. The varying concentrations (100-500 ppm) of standard gallic acid solution were used and the readings expressed in terms of mg GAE (gallic acid equivalent) 100 mg^{-1}. Absorbance was measured at 760 nm in a UV-Vis spectrophotometer (GENESYS™; Thermo-Fisher Scientific). All the experiment was conducted in triplicates.

Estimation of flavonoids: Flavonoid contents in ethanolic leaf extract were assayed as per the method of Singleton and Rossi (1965). In brief, 250 µL extract was treated with 1.25 mL distilled water, followed by the addition of 75 µL of 5% sodium nitrite. After 5 min incubation, 150 µL of 10% aluminium chloride was added to the mixture. Then after 6 min, 500 µL of 1M sodium hydroxide was added and the pink colour development was observed and measured at 560 nm. Standard curve for flavonoid content was made using varying concentrations (100-500 ppm) of standard catechin solution. Total flavonoid content was expressed in terms of mg CE (catechin equivalent) 100 mg^{-1}.

Estimation of tannins: The tannin content was determined by using the standard method (Choi *et al*., 2006). In brief, to 0.5 mL leaf extract 0.1 mL Folin-Ciocalteau reagent was added and incubated for 15 min. Then 2.5 mL saturated sodium carbonate solution was added and the solution left as such for 30 min. The absorbance was measured at 760 nm using a UV spectrophotometer. The standard curve for tannin contents was prepared by using standard tannic acid solution (100-500 ppm) and the values expressed in terms of mg TAE (tannic acid equivalent) 100 mg^{-1}.

Antioxidant assays

DPPH free radical scavenging activity: DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity was estimated as per Yen *et al*. (1993). In brief, fresh stock solution of 0.004% DPPH was prepared in methanol and stirred well in dark. The standard solutions of ascorbic acid, gallic acid and BHT (butylated hydroxytoluene) were also prepared. Then 1 mL test filtrate (10-50 µg mL^{-1}) were put in a series of volumetric flasks, 0.004% DPPH added to it before making the final volume of 3 mL by ethanol. The solution was kept in dark for 30 min and absorbance measured at zero time at 517 nm using UV-visible spectrophotometer. The decrease in absorbance of DPPH scavenging activity in test sample was measured after 15 min at 517 nm. Percentage inhibition in DPPH radical scavenging activity was determined as per the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [1 - A_t/A_0] \times 100$$

Where A_t is the absorbance of sample and A_0 is the absorbance of control.

Metal chelating method: The metal chelating method is principle mainly based on chelating ability of Fe^{2+} ion of antioxidants was estimated based on the method (Hsu *et al.*, 2003). In brief, 0.1 mL 2 mM $FeCl_2.6H_2O$ [iron (III) chloride hexahydrate] reagent was prepared and added in a 1 mL sample followed by 0.2 ml of 5 mM solution of ferrozine. A test sample of different concentrations (10-50 μg mL^{-1}) was taken. The whole reaction mixture makes up of 5 mL of ethanol. EDTA (ethylene diamine tetraacetate) and citric acid were taken as a standard. The reaction mixture was shaken vigorously and kept at room temperature for 10 min. Absorbance was measured at 562 nm and compared Fe^{2+} chelating activity with the concentration of standards.

$$\text{Chelating activity (\%)} = [1 - A_t/A_0 \times 100]$$

Where A_t is the absorbance of the sample, and A_0 is the absorbance of control at 562 nm.

Superoxide radical scavenging activity: The superoxide radical scavenging activity was assayed as per nitroblue tetrazolium (NBT) reduction method (Fu *et al.*, 2010). A mixture of NBT 156 Mmol in 100 mM phosphate buffer (pH, 7.4) solution was prepared; and 1 mL NBT and 1 mL NADH (nicotinamide adenine dinucleotide) was added to the ethanolic test extract (10-50 μg mL^{-1}), followed by the addition of 100 μL phenazine. The reaction mixture was kept as such for 5 min at 25°C. Two standards *viz.*, ascorbic acid and quercetin were taken as positive standards. The absorbance was measured spectrophotometrically at 560 nm and superoxide radical scavenging activity determined as per the formula:

$$\text{Superoxide radical scavenging activity (\%)} = [1 - A_t/A_0 \times 100]$$

Where A_t is the absorbance of methanolic extract or standard and A_0 is the absorbance of control.

Antibacterial activity

For antibacterial screening, human pathogenic bacteria *viz*., *Escherichia coli, Salmonella typhi,* and *Bacillus subtilis* were procured from the Department of Microbiology, GBPUAT, Pantnagar (India). Antibacterial activity was assayed by disc diffusion method (Singh *et al*., 2005). The bacteria were maintained on nutrient agar (Hi-Media, India) at 4ºC and sub-cultured before use. These bacterial strains clinically cause several infections. Bacterial inocula were prepared using Luria Bertani broth (Hi-Media) for *E. coli,* buffered peptone water broth for *S. typhi* and nutrient broth for *B. subtilis*. The sterile growth media were poured into sterile plates under aseptic conditions using a laminar flow. The plates were incubated at 37°C overnight. Then 100 μL of each test bacterial inoculum were added to the plates containing specific growth medium and spread uniformly. The sterilized paper disc of 5 mm dipped in test ethanolic leaf extracts (10, 20, 30, 40 and 50 μL) were placed in plates and incubated overnight at 37°C. Sterile paper discs dipped in sterile solvent (ethanol) alone served as a negative control and antibiotic (gentamicin,10 μg $disc^{-1}$) served as a positive control. The antimicrobial potential of leaf extract was determined in terms of inhibition zone sizes. The diameters of zones were measured and compared with controls. The relative susceptibility of each organism was worked out.

Statistical analysis

Each assay was performed with a completely randomized design, with each treatment replicated three times. All the activities were expressed as mean ± standard error and result with $P < 0.05$ were considered statistically significant.

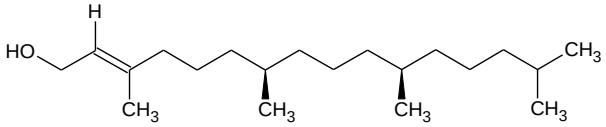
RESULTS AND DISCUSSION

GC-MS analysis of A. aspera leaf extract

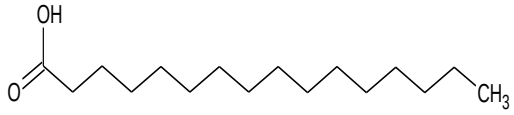
On extraction by cold percolation method, the ethanolic leaf extract of *A. aspera* yielded primary and secondary metabolites through GC-MS analysis. The bioactive compounds were identified on the basis of their peak area, molecular formula and molecular weight. GC-MS analysis revealed the presence of 20 major bioactive compounds in the ethanolic extracts of *A. aspera* (Fig. 2). So, the results were 74.36% of the total extract. The NIST and PubChem database search for these bioactive compounds

Fig. 1: The *Achyranthes aspera* plant

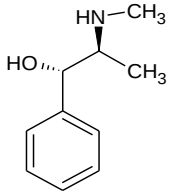
indicated that they were documented for antioxidant, antimicrobial, anticancer and other pharmacological activities and herbicidal properties. The dominant compounds were a) phytol 10.67%**,** b) palmitic acid 6.56%, c) pseudoephedrine 6.06%, d) hexa-cosane 5.76%, e) lanosta-8,24-dien-3-ol 4.9%, f) viminalol (α-amyrin) 4.80%, g) piperidinone,n-(4-bromo-n-butyl) 4.16%, h) stigmasterol 3.6%, i) squalene 3.32%, and j) vitamin E 2.89%. The composition of ethanolic *A. aspera* leaf extract showing Kovats index (K.I), percent peak area, molecular formula, and molecular mass is given in Table 1 and GC-MS chromatogram in Fig. 3 presents the pictures of peaks of individual mass spectrum of identified bioactive compounds. In present study phytol was most dominant in ethanolic leaf extract in the form of phytol acetate. The compound has antiinflammatory, antioxidant, antiallergic, antituberculosis, antimicrobial and antinociceptive activities (Muthuswami, 2020). The 2nd major component was palmitic acid (an unsaturated fatty acid) that could be used in future cosmetic and food industry. The other fatty acid derivatives found in traces too have anti-microbial, antineoplastic and antifungal activity



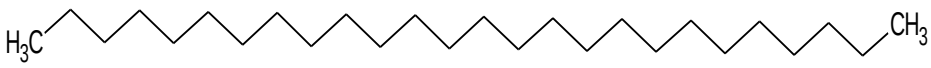
a) Phytol



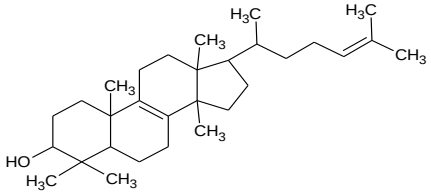
b) Palmitic acid



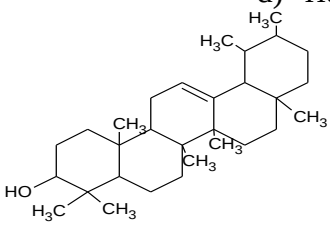
c) Pseudophedrine



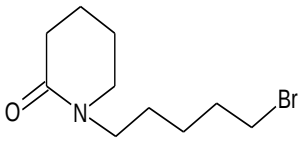
d) Hexacosane



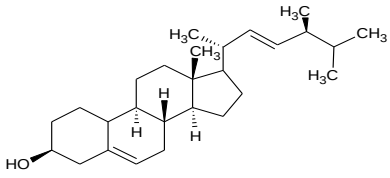
e) Lanosta-8,24-dien-3-ol (Parkeol)



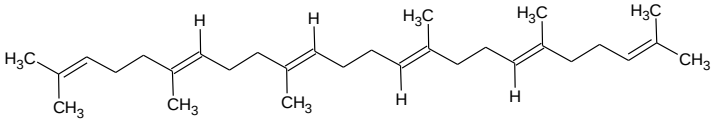
f) Viminalol(α-Amyrin);



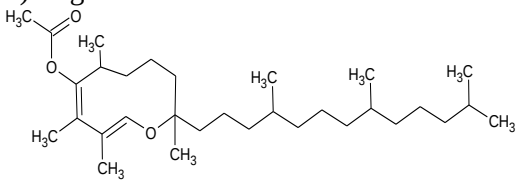
g) 2-Piperidinone,n-(4-bromo-n-butyl)



h) Stigmasterol



i) Squalene



j) Vitamin E

Fig. 2: Chemical structure of major phyto-constituents in the ethanolic leaf extract of *Achyranthes aspera*

Table 1: GC-MS analysis of AAEE (ethanolic extract of *Achyranthes aspera* leaves)

S. No.	Constituents detected	Kovats index (KI)	Peak area (%)	Molecular formulae	Molecular mass (g mol^{-1})
1.	Isoxazolidine,5-hexyl-,(+)-	1292	3.50	$C_9H_{19}NO$	157
2.	1,2,4-Butanetriol, trinitrate	1728	3.48	$C_4H_7N_3O_9$	241
3.	N-Ethyl-N-nitroguanidine	-	3.49	$C_3H_8N_4O_2$	132
4.	Phytol	2105	10.67	$C_{20}H_{40}O$	296
5.	Pseudophedrine	1360	6.06	$C_{10}H_{15}NO$	165
6.	2-Piperidinone, N-[4-bromo-n-butyl]	1635	4.16	$C_9H_{16}BrNO$	233
7.	Vitamin E	3131	2.89	$C_{29}H_{50}O_2$	430.7
8.	Lanosta-8,24-dien-3-ol,(3β)	2882	4.9	$C_{30}H_{50}O$	426.7
9.	Viminalol	2873	4.80	$C_{30}H_{50}O$	426.73
10.	Linoleic acid	2108	2.56	$C_{18}H_{32}O2$	280.45
11.	Stigmasterol	3332	3.6	$C_{29}H_{48}O$	412.69
12.	Hexacosane	2606	5.76	$C_{26}H_{54}$	366.71
13.	Palmitic acid	1927	6.56	$C_{16}H_{32}O_2$	256.2
14.	Squalene	2790	3.32	$C_{30}H_{50}$	410.72
15.	6-Pentatriacontane	3500	3.69	$C_{35}H_{72}$	492
16.	7-Methyl-7H-dibenzo(b,g)carbazole	2586	0.689	$C_{21}H_{15}N$	281
17.	5,6-Dihydrobenz[A,H]anthracene	2592	0.57	$C_{22}H_{16}$	280
18.	Isopropalin	2379	0.379	$C_{15}H_{23}O_4N_3$	309
19.	7,10-Dimethylbenzo(A)pyrene	2761	2.06	$C_{22}H_{16}$	280
20.	2-Methoxy-6-nitro-4-phenyl quinazoline	2490	1.23	$C_{15}H_{11}O3N_3$	281
	Total		74.36%		

(Nengroo *et al*., 2019). The 3rd major component pseudoephedrine is a drug with long history of medical use and is helpful in treating common cold, asthma, sinusitis and bronchitis (Glowacka *et al*., 2021). Hexacosane has a strong antimicrobial activity (Rukaiyat *et al*., 2015) while lanosta-8,24-dien-3-ol (parkeol) is an uncommon sterol used in biosynthesis of saponins (Y *et al*., 2018). Viminalol (α-amyrin) has antiinflammatory properties (Okoye *et al*., 2014). 2-piperidinone n-(4-bromo-n-butyl) has antimicrobial activity (Al-Salman, 2019). Stigmasterol has pharmacological value like antiosteo-arthritic, antihypercholesterolemic, antioxidant, antiinflammatory, antitumor and hypoglycaemic (Kaur *et al*., 2011). Squalene reportedly suppresses free radicals, tumour cell development and increases WBC count (Gunes, 2013). Vitamin E has several biological and pharmacological roles in body owing to its antioxidant potential and the ability to fight diseases like oxidative atherosclerosis, stress and cancer (Rizvi *et al*., 2014). The mass spectra are the fingerprint of compounds which were identified from Nist library data. The present study showed that the ethanolic leaf extract is rich in diterpene, triterpene, sterols, alkaloids and fatty acids and possesses several biological activities. Their structural elucidation and pharmacological screening may help in drug formulation and development.

Quantification of phytochemicals

Phenolic compounds are key phytochemicals with high free radical scavenging activity and they also possess antimutagenic and antitumour activities. Total phenolic contents in ethanolic leaf extract of *A. aspera* was 372 ± 2 mg GAE 100 g^{-1} (Fig. 4). The concentration of phenolic compounds increased with increase in the dose. The total phenolic content of ethyl acetate extract was reported to be 209.007 µg GAE mg^{-1} (Manandhar *et al*., 2021); while in water and chloroform extracts the highest concentration of phenols were 75.66 and 15.66 mg GAE g^{-1} (Afzal *et al*., 2021). Reportedly ethyl acetate extract of *A. aspera* contains appreciable amount of phenols; while its methanolic extract possesses it in moderate amounts (362.75 ± 10.1 mg GAE 100 g^{-1} (Awasthi *et al*., 2021).

The total flavonoids content in ethanolic leaf extract of *A. aspera* was in the range of 22.6 ± 1.33 mg CE 100 g^{-1} (Fig. 4). Reports reveal 17.59 µg QE mg^{-1} flavonoid contents in the ethyl acetate extract of *A. aspera* (Manandhar *et al*., 2021). Dichloromethane extract (38.48 ± 1.48 mg RE g^{-1}) followed by ethyl acetate extract (29.9 ± 0.71 mg RE g^{-1}) displayed richest total flavonoid content

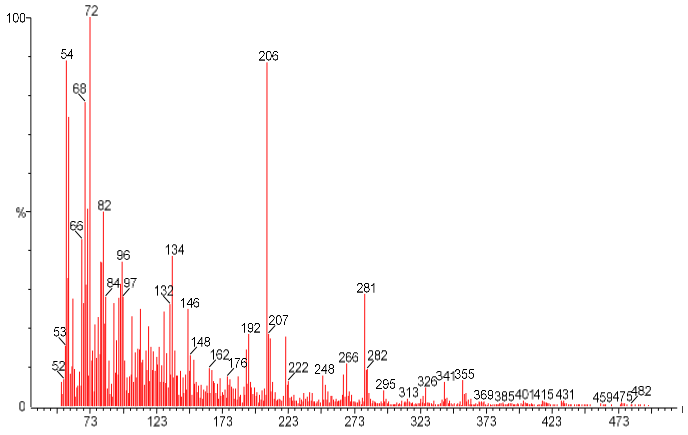


Fig. 3: GC-MS chromatogram of AAEE

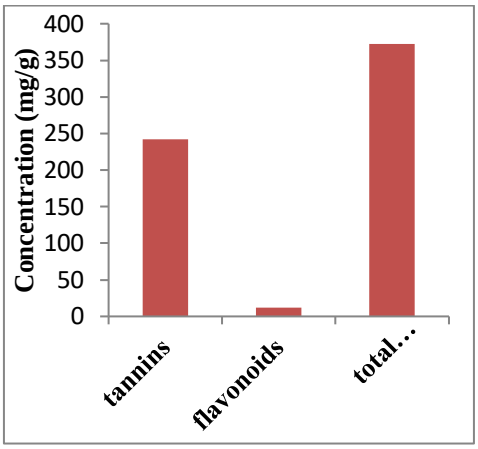


Fig. 4: Tannins, flavonoids and phenolic contents in 1000 ppm of AAEE

(Sinan *et al.*, 2020). Awasthi *et al.* (2021) in ethanolic extract of leaves and ethyl acetate extract of stem of *A. aspera* observed the flavonoid contents of 96.33 ± 7.67 and 69.29 ± 5.98 mg QE g^{-1}). Hydroalcoholic extract of *A. aspera* reportedly has 0.145 mg flavonoid g^{-1} (Brijyog Singh *et al*., 2019).

Tannins are mostly polyphenolic compounds that bind with protein precipitate to form a complex and exhibit antioxidant activity. estimation in various concentrations of was determined. The tannic acid content in the ethanolic extract of *A. aspera* was in the range from 242 ± 2 mg TAE 100 g^{-1} (Fig. 4). Tannin content was higher than flavonoids perhaps due to higher reducing power. Priya *et al*. (2012) reported tannin as a major phytochemical in the methanolic extract of *A. aspera.*

The study demonstrates that the ethanolic extract of *A. aspera* leaves have highest phyto-nutrients in all forms; so is the most preferred option in health and disease. Phenolic compounds and flavonoids display antibacterial and antioxidant properties. Terpenoid are antibacterial, antimalarial, anticancer, anti-inflammatory, antiviral, anthelmintic, insecticidal activities, and inhibition synthesis of cholesterol. The phytochemical screening of ethanol extract of aerial part of *A. aspera* was performed for the screening of antiasthmatic activity (Shukla *et al*., 2016). Tannins were shown to produce anthelmintic activity (Jadhav *et al.*, 2017).

Antioxidant activity

DPPH scavenging activity showed increase with increase in the concentration of EEAA (Table 2). Leaf extract exhibited 46.30 ± 0.10% inhibition in DPPH scavenging activity at 50 µg mL^{-1} which was two-fold lesser than the standard (ascorbic acid, gallic acid and BHT) radical scavenging potential. Pandey *et al*. (2014) found that the DPPH radical scavenging activity of leaf extract was comparable but in-significant to the standard ascorbic acid. Abi *et al.* (2011) reported 24.33, 55.67,71.27 and 86.76% radical scavenging potential in case of hexane-, chloroform-, ethyl acetate- and methanol-based extracts of *A. aspera*, respectively. *A. aspera* extract showed significant antioxidant activity as compared to ethyl acetate and standard (Awasthi *et al.*, 2021). Methanolic leaf extract revealed radical scavenging activity of 68 ± 0.44% at 250 µg mL^{-1}, which was higher than that of petroleum ether extract (63.06 ± 0.56% at 250 µg mL^{-1}) (Priyamvada *et al.*, 2021). The hydro- alcoholic extract of *A. aspera* revealed the IC_{50} value of 105.58 µg mL^{-1}, whereas the fabricated iron oxide nano-

Table 2: Effect of EEAA and standards on scavenging of DPPH free radical

Samples	Percent inhibition of DPPH radical at absorbance of 517 nm				
	10 µg mL^{-1}	20 µg mL^{-1}	30 µg mL^{-1}	40 µg mL^{-1}	50 µg mL^{-1}
AAEE	31.51 ± 0.12	37.9 ± 0.10	40.57 ± 0.11	41.86 ± 0.10	46.30 ± 0.10
Gallic acid	50.89 ± 0.10	61.9 ± 0.10	74.51 ± 0.10	83.18 ± 0.12	88.23 ± 0.15
Ascorbic acid	64.10 ± 0.52	66.8 ± 0.10	68.90 ± 0.10	71.04 ± 0.05	73.07 ± 0.50
BHT	46.98 ± 0.15	56.8 ± 0.10	62.08 ± 5.92	71.10 ± 1.02	80.18 ± 0.10

Values mean ± standard deviation; n = 3 (Tests were done in triplicate)

Table 3: Effect of various concentrations of ethanolic leaf extract (AAEE) and standards on metal chelation activity

Sample	Percent metal chelation at 562 nm absorbance (%)				
	10 µg mL^{-1}	20 µg mL^{-1}	30 µg mL^{-1}	40 µg mL^{-1}	50 µg mL^{-1}
AAEE	46.31 ± 0.17	49.82 ± 6.7	53.02 ± 0.17	54.10 ± 0.11	58.80 ± 0.11
Citric acid	47.09 ± 0.11	54.18 ± 0.17	59.80 ± 0.06	66.78 ± 0.23	77.80 ± 0.11
EDTA	55.02 ± 0.06	65.54 ± 0.11	77.74 ± 0.17	80.07 ± 0.17	88.36 ± 0.12

Values mean ± standard deviation; n=3(Tests were done in triplicate)

particles of leaf extract reportedly have the same activity in the range of 42.7 to 84.9% (Ahmed *et al.*, 2022). Stem-leaves methanolic extract of *A. aspera* revealed the IC_{50} to be 30.5 µg mL^{-1} which was almost 5-fold lesser than ascorbic acid (Raut *et al.*, 2021). The present study revealed appreciable DPPH radical scavenging capacity of EEAA and the extract in different concentrations depicted variable antioxidant activity which has the ability to prevent simple oxidative damage caused by free radicals.

The metal chelating antioxidant activity was used to estimate the antioxidants present in various concentrations of ethanolic leaf extract (EEAA). Ferrozine forms a complex with Fe^{2+} and the presence of a chelating agent causes disturbance in the complex formation which is helpful in determining the chelating activity. Fe^{2+} has the ability to carry a single electron to avoid the generation of reactive oxygen species (ROS) which is associated with metal catalysis and metal ion chelation. A decrease in red colour shows the ability of extract in metal chelation (Yamaghi *et al.*, 2000). On increasing the concentration of EEAA variable chelating activity was observed in comparison to EDTA and citric acid (Table 3). Compared to the standards, EEAA showed less potential activity (58.8 ± 0.1%) at 50 µg mL^{-1}. Datta *et al.* (2019) found that aqueous ethanolic extracts of *A. aspera* had highest chelating activity (47.9 ± 0.14%). The bioactive compounds with metal chelating potential could have beneficial effects on metal-catalyzed biochemical reactions. The chelating capacity of the studied plant extracts revealed decreased with decreasing polarity. Chelating ability could be attributed to the high phenols and flavonoid contents in ethanol extract. Polyphenols with dihydroxy groups conjugate different heavy metals like Pb, As, Cd and Hg and reportedly produce toxic effects, primarily through damage to the biomolecules including lipid, protein, and nucleic acids (Singh and Sharma, 2020). Polyphenols have various therapeutic effects, including metal chelation, mitochondrial function, and anti-amyloidogenic activity. These therapeutic effects of polyphenols make them strong substitutes for a moderate chelation-based therapy for Alzheimer's disease (Lakey-Beitia *et al*., 2021). Superoxide scavenging activity was maximum (46.51 ± 0.11%) in high concentration solution (50 µg mL^{-1}) of EEAA (Table 4)**.** However, AAEE showed less activity than the standard ascorbic acid. Superoxide anions damage biomolecules by forming H_2O_2, ^{-}OH, peroxy nitrite or singlet oxygen. The antioxidant enzymes (superoxide dismutase and catalase) quench the radicals and thus prevent cell damage (Singh *et al*., 2006). The results showed higher inhibitory effect of AAEE on superoxide anion formation, thus rendering them a potent antioxidant due to the presence of polyphenols. Reduction in rate of lipid peroxidation and enhancement in free radical scavenging activity of *A. aspera* leaf extract is well documented (Khetade *et al*., 2019).

Antibacterial activity

The antibacterial activity of various concentrations of EEAA were studied against human pathogens *Salmonella typhi, Bacillus subtilis* and *Escherichia coli* by disc diffusion technique and the results compared with standard antibiotic gentamicin. The study showed the intensity of antibacterial activity

Table 4: Superoxide anion scavenging activity of different concentrations ethanolic leaf extract (AAEE) and ascorbic acid (standard)

Sample	Percent inhibition superoxide anion radical at 560 nm (%)				
	10 µg mL^{-1}	20 µg mL^{-1}	30 µg mL^{-1}	40 µg mL^{-1}	50 µg mL^{-1}
AAEE	37.59 ± 0.28	38.37 ± 0.16	39.82 ± 0.28	42.44 ± 0.20	46.51 ± 0.11
Ascorbic acid	57.17 ± 0.23	60.46 ± 0.15	64.80 ± 0.15	68.41 ± 0.08	71.29 ± 0.02

Values are mean ± standard deviation; n = 3 (tests were done in triplicate)

was in the order of *B. subtilis* > *S. typhi* > *E. coli*. The inhibitory effect of EEAA was less than standard gentamicin @ 10 µg disc^{-1} (Table 5; Fig. 5 & 6)**.** The inhibition zone of AAEE was high against *B. subtilis* (16.33 ± 0.58%) than *E. coli* and *S. typhi*. Thus *B. subtilis* (Gram +ve bacterium) appeared more susceptible to EEAA than Gram-negative bacteria in ethanol (polar solvent), so EEAA has the potential to replace synthetic drugs and chemicals with no side effects. Khan *et al.* (2009) and

Table 5: Effect of ethanolic leaf extract (AAEE) against human pathogenic bacteria *B. subtilis, S. typhi* and *E. coli*

B. subtilis	250 ppm	500 ppm	750 ppm	1000 ppm
Inhibition zone (mm)	10	11	12	16
	10	10	14	17
	10	12	14	16
Mean ± SD	10.0 ± 0.0	11.0 ± 1.0	13.3 ± 1.2	16.3 ± 0.6
Gentamicin (10 µg disc^{-1})			23 mm	
Ethanol (negative control)			-	
S. typhi	250 ppm	500 ppm	750 ppm	1000 ppm
Inhibition zone (mm)	11	14	14	16
	12	12	15	17
	10	14	14	17
Mean ± SD	11.0 ± 1.0	13.3 ± 1.1	14.7 ± 0.6	16.0 ± 1.0
Gentamicin (10 µg disc^{-1})			21 mm	
Ethanol (negative control)			-	
E. coli	250 ppm	500 ppm	750 ppm	1000 ppm
Inhibition zone (mm)	-	10	12	14
	10	11	13	15
	11	12	14	15
Mean ± SD	10.5 ± 0.5	11.0 ± 0.6	13.0 ± 0.6	14.7 ± 0.2
Gentamicin (10 µg disc^{-1})			23 mm	
Ethanol (negative control)			-	

Values are the mean ± standard deviation; n = 3 (tests were done in triplicate)

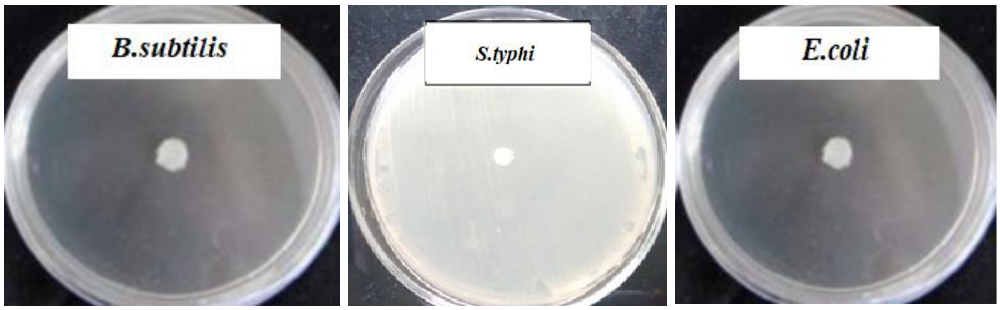


Fig. 5: Antibacterial screening of control against *B. subtilis,* S. *typhi* and *E. coli*

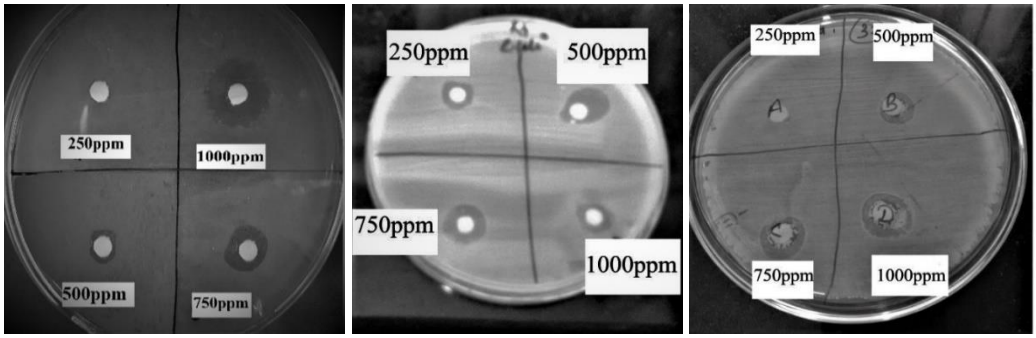


Fig. 6: Antibacterial screening of leaf extract against *B. subtilis* (left), *E. coli* (middle) & *S. typhi* (right)

Singh *et al*. (2022) have reported that the ethanolic extract *A. aspera* leaf or stem exhibit highest zone of inhibition against *S. aureus* (a G +ve bacterium). Yadav *et al*. (2016) found that the aqueous extract of *A. aspera* roots had highest antibacterial activity than its stems. The EEAA reportedly exhibits higher antibacterial activity than other edible and medicinal plants like *Aloe vera* (Sangeetha *et al.*, 2021). The highest activity against *B. subtillis* is supported by Mishra (2018). Rama and Latha (2018) observed *A. aspera* leaf extract to be significantly effective against *E. coli*. Iron oxide nanoparticles biosynthesized by using *A. aspera* leaf extract also showed antibacterial activity against several pathogenic bacteria (Ahmed *et al.*, 2022). The antibacterial activity of *A. aspera* may be attributed to the presence of tannins, a phenolic compound responsible for antibacterial activity which serves as a natural defense against microbial infections **(**Chattopadhyay *et al.*, 2009). Any antimicrobial agent is considered effective, if the given size of inhibition zone produced by it measures $\geq$ 2 mm (Vyas *et al.*, 2008). The results demonstrate that ethanol leaf extract of *A. aspera* possess significant antibacterial properties so confirm their application in traditional medicine. In conclusion, the antioxidant, antibacterial properties and phytochemical investigation of the *A. aspera* are expected to increase the use of leaf in the industry, especially in new human health-oriented products. The present findings are expected to be equally beneficial to everyone dealing with medicinal plants across the world. In the future, we will identify and isolate the active compounds, and elucidate their other pharmacological properties.

Conclusions: The present study revealed that the ethanolic extract of *A. aspera* leaves exert antioxidant and antimicrobial activity against several human pathogens. The extracts showed significant antioxidant activities in various tests like DPPH, superoxide radical scavenging activity and metal chelation activity. The antioxidant properties of phenols, flavonoids, tannins, fatty acids, and other bioactive compounds, as identified in EEAA by GCMS, are responsible for breaking the chain of free radical formation so resisted the pathogenic microbes. The extract of *A. aspera* has application in traditional medicine so the exploitation of AAEE in the development of human health-oriented products is likely to increase.

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