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Research Article

Resazurin based microtitre assay and comparison of DLA and EAC cell lines in *Amaranthus viridis* L.

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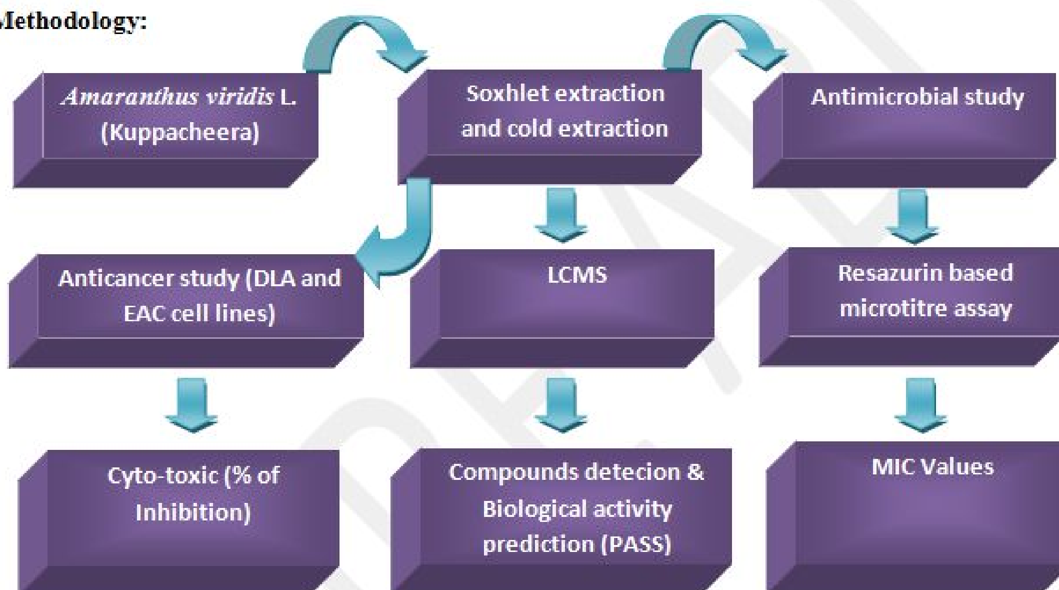
Objective: To evaluate the pharmacological aspect of *A.viridis* as a vegetable drug empolying LCMS studies to findout the active components in the drug and predict their activity using PASS software.

Methodology:



Duration taken for the research: 4 months

Conclusion: The finding shows that *A. viridis* has antimicrobial and tumour suppressing activity. Compounds confirmed using LCMS analysis were predicted using Biological activity prediction (PASS) software for presence of active biological compounds.

Applicable Industries: Pharmaceutical industries

Applicable geographical area: Tropical regions

Expected outcome: Good pharmacological activity, new compounds detected in the plant has not been reported elsewhere.

Research Article

Resazurin based microtitre assay and comparison of DLA and EAC cell lines in *Amaranthus viridis* L.

Cherian and Sheela

Abstract

Amaranthus viridis L. a medicinal plant belonging to family Amaranthaceae, is used for bactericidal activity to kill infectious diseases and has reported to be antioxidant and anticancer though it contains glycosides, phenols and flavanoids. The study conducted to evaluate the anticancer ability using the cell lines and antimicrobial activity using resazurin dye. The leaf extract were made by cold extraction using different solvent based on polarity. Antimicrobial activity was determined using resazurin based microtitre assay and MIC and anticancer study was conducted in DLA and EAC cell lines. The result showed ethanol and methanol extract posses antimicrobial activity (1.25-0.625 mg/mL). DLA cell line showed good percentage of activity than EAC cell line. *Amaranthus viridis* L. extract showed antibacterial and antifungal activity against human pathogenic strains and has got anticancer property.

Keywords: DLA (Dalton's Lymphoma ascites), EAC (Ehrlich Ascites Carcinoma)**,** MIC (Minimum Inhibition Concentration), Resazurin, *Amaranthus viridis,* Anticancer

Introduction

Dreadful diseases are prevailing in the world in wide variety of strains and representative class of organism. Many pathogenic micro-organisms are becoming more virulent and cannot be controlled by synthetic molecules derived from chemicals. The progressive resistance of pathogenic microorganisms to multiple drugs has encouraged the search for new agents, especially those derived from natural products (Ma *et al*., 2006; Antunes *et al*., 2006; de Oliveir *et al*., 2006). World is a rich source of floral diversity which were created for the betterment of mankind.

Antimicrobial compounds of plant origin with a restricted action against specific bacteria species and/or strains are desired not only due to their efficacy in the infection control, but also because they permit the maintenance of the normal microbiota. *Amaranthus* L. species are rich source of phenolic components which may have direct role in producing these antimicrobial and anticancer effects. Phenols, flavonoids are common interacting factor for the pharmacological activity of the plant drugs. Thus the presence of phenolic acids in the extract suggests a direct relationship with its antimicrobial activity since an antimicrobial action of these compounds has been attributed to their ability to form complexes with extracellular polysaccharides and proteins, rupturing the bacterial cell wall and inhibiting the enzymatic systems responsible for the synthesis of cell wall components (Ying-Shan Jin *et al*., 2013). Since the

pharmacological action of a plant species employed as a phyto-therapeutic agent involves the interaction between its different chemical components (Houghton *et al*., 2007).

A significant number of modern pharmaceuticals drugs are based on or derived from medicinal plants. Herbal medicines are usually in the aim of vegetable drugs or their extract which primarily serves for the treatment of diseases or to maintain a state of improved health. Many researchers reported ethno-pharmacological and nutritional importances of *Amaranthus* L. species in their works (Mitra Noori1 *et al*., 2015).

Amaranthus viridis L. commonly called as Kuppacheera in Malayalam, are widely distributed i.e., cosmopolitan in distribution, considered as underutilized plant used in traditional medicine for the treatment of diseases. The plant is used as diuretic, anti-inflammatory, antipyretic, analgestic, emollient and lactogogue (Vaidyaratnam, 1996). The utilization of liquid chromatography coupled to single (LC – MS) and tandem (LC – MS/MS) mass spectrometry has grown rapidly and is now widely recognized as powerful tool for a comprehensive General Unknown Screening (GUS) of organic molecules in a multiple component mixture (Sauvage *et al*., 2009). In general, finding of new leads among existing drugs provides significant advantages for a pharmaceutical company because their general pharmacology, toxicity and pharmacokinetic properties are already studied in more detail. In the present study we have investigated the possibilities of utilizing computer-aided prediction to estimate the general biological potential of molecules under study.

Materials and methodology

The plant materials were collected from grow bags grown in garden and was authenticated. Voucher specimen deposited in KFRI, Peechi, Thrissur. Resazurin dye (Sigma Aldrich), Microtitre plates (Tarsons 96 well), Himedia nutrient broth and Luria broth, Dimethyl sulphoxide (Merk). Bacterial and fungal strains were procured from Microbiology Department, St Xavier's College, Aluva, India. The tools used are Laminar air flow chamber (LABLINE) for the sub-culturing of microbes, Incubator (Rotex RJSS 10 AC) and Spectrophotometer (104 Systronics).

Extraction

Leaves of *Amaranthus viridis* L. were collected and air dried by keeping in shade for 3 weeks. Afterwards, they were transferred to oven at 40°C for 20-24 hrs. The properly dried plant leaves were grinded to fine powder with the help of electronic grinder. Sixty gram leaves powder of each plant was

used for obtaining extracts through cold extraction and maceration. Solvents such as chloroform, acetone, methanol, ethanol and water were used depending upon the polarity.

Preparation of bacterial culture

Using aseptic techniques, six 100 mL bottles of Luria broth were inoculated with six bacterial strains (each bottle contain only one type of bacteria) and kept in the incubator overnight for 12-18 h at 35° C. After incubation, all bacterial cultures were centrifuged at 4000 rpm for 5 min. Supernatants were discarded and pellets were re-suspended in 20 mL of sterile normal saline and re-centrifuged at 4000 rpm for 5 min. The pellet was then dissolved in 20 mL of sterile normal saline and was labeled as bacterial solution. Optical densities (OD) of the bacterial solutions were measured at 600 nm and serial dilutions were made until the OD was in the range of 0.5-1.0. The viability graph was used to calculate the actual number of colony forming units. The dilution factor was calculated and the dilution was performed to obtain a final concentration of 5 x 10^6 CFU/mL (Sarkar *et al.*, 2007).

Preparation of Resazurin Dye Solution (RDS)

Resazurin dye (300 mg) was dissolved in 40 mL sterile water. Vortex mixer was used to homogenize the solution**.** This solution was then referred as Resazurin dye solution. Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays (McNicholl *et al.*, 2006). It is purple non-fluorescent and non-toxic dye which becomes pink and fluorescent when reduced to resorufin by oxidation reduction within viable cells. Resorufin is further reduced to hydroresorufin (uncoloured). Resazurin reduction test has been used from decades to demonstrate bacterial and yeast contamination of milk (Bigalke, 1984).

Resazurin based Microtiter Dilution Assay (RMDA)

Under aseptic conditions, 96 well microtitre plates (Tarson) were used for Resazurin based Microtitre Dilution Assay. The first row of microtitre plate was filled with 100 µl of test materials in 10% (v/v) DMSO or sterile water. All the wells of microtitre plates were filled with 100 µl of nutrient broth. Two fold serial dilution (throughout the column) was achieved by transferring 100 µl test material from first row to the subsequent wells in the next row of the same column in the microtritre plate. 10 µl of resazurin solution as indicator was added in each well. Finally, a volume of 10 µl was taken from bacterial suspension and then added to each well to achieve a final concentration of 5×10^6 CFU/mL. Each

microtitre plate had a set of 3 controls: (a) a column with Streptomycin as positive control, (b) a column with all solutions with the exception of the test extract and (c) a column with all solutions except bacterial solution replaced by 10 µl of nutrient broth. The plates were incubated in temperature controlled incubator at 37° C for 24 h. The lowest concentration of plant leaf extract at which colour change occurred was recorded as the MIC value. All the experiments were performed in triplicates. The average values were calculated for the MIC of test material.

Anti-cancer activity

In-vitro cytotoxicity study

Dalton's Lymphoma ascites cells (DLA)

The test compounds were studied for short term *in vitro* cytotoxicity using Dalton's Lymphoma ascites cells (DLA).The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS or normal saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension was added to tubes containing various concentrations of the test compounds and volume was made up to 1mL using phosphate buffered saline (PBS) control tube contained only cell suspension. These assay mixture were incubated for 3 hour at 37°C, further cell suspension was mixed with 0.1 mL of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye. The number of stained and unstained cells was counted separately (Subramoniam and Shylesh, 2005).

$$\% \text{ cytotoxicity} = \frac{\text{No.of dead cells x 100}}{\text{No.of live cell + No.of dead cell}}$$

Ehrlich Ascites Carcinoma (EAC) cells

The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS or normal saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension (1 x10^{6} cells in 0.1 mL) was added to tubes containing various concentrations of the test compounds and the volume was made up to 1 mL using phosphate buffered saline (PBS). Control tube contained only cell suspension. These assay mixture were incubated for 3 hour at 37°C. Further cell

suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye. The numbers of stained and unstained cells were counted separately (Shapiro, 1988).

$$\% \text{ cytotoxicity} = \frac{\text{No.of dead cells x 100}}{\text{No.of live cell + No.of dead cell}}$$

Liquid-chromatograph-mass spectra (LC-MS)

Extract was subjected to chromatographic separation on Phenomenex Reverse phase 18 column (dimension 25 cm × 2.5 cm) and operated at a column temperature of 25°C with a flow rate of 2 mL/min. Injection volume was 10 µl. Electronic spray ionization (ESI) mode was used with m/z range of 50–1000 for negative and 50–980 for positive. Class VP integrated software was used and identification of isolated compounds was based on the comparison of the mass spectral data with METWIN 2.0. Eluent was water: Methanol (50:50) .Estimation of general biological potential for drug like compounds on the basis of their structural formulae is performed with a computer program PASS (Prediction of Activity Spectra for Substances) (Filimonov *et al*., 2014).

Results and discussion

Plant determination

The plant materials were authenticated and identified as *Amaranthus viridis* L. Voucher specimen deposited in KFRI, Peechi, Thrissur, Kerala, India.

Drying and Extraction

About 30 gm of plant were extracted with chloroform, acetone, methanol, ethanol and water by cold extraction. For anticancer study the powder were refluxed below 40^0 C and solvent were vapourized and mixed with DMSO (Dimethyl Suphoxide). The phytochemical analysis of aerial parts of *Amaranthus* L. showed the presence of active constituents like alkaloids, flavonoids, phenols, saponins and glycosides (Kavita and Puneet, 2017). Presence of all these phytochemicals has direct or combined action for the pharmacological effects of the plant.

Antimicrobial studies

Five different crude extracts the plant were screened for their antibacterial potential. From the Antimicrobial studies using Resazurin dye *A. viridis* showed better MIC value with respective extract ie., methanol and ethanol extract showed minimum inhibition concentration range from 1.25-0.625 mg/mL in Table 1. Many reports supported that methanolic extract of another species of *Amaranthus lividus*. *A. spinosus* showed good antimicrobial activity in the methanol extract (Amabye, 2015).

Another report is indicating that *A. viridis* shows better inhibition against *E. coli* and *S. aureus* than *A. lividus* (Iqbal *et al*., 2012). In order to confirm the validity of the results obtained in the study and medicinal application *in-vivo* studies have to be done which can address the issue regarding the safety and toxicity (Malekpoor *et al*., 2015).

Table 1: MIC value of *A. viridis*

Bacteria	Chloroform	Acetone	Ethanol	Methanol	water	control
K.pneumonia	1.25±0.72	1.25±0.72	0.625±0.36	0.625±0.36	1.25±1.90	1.25±0.36
S.aureus	1.25±0.72	0.625±0.36	1.25±0.72	0.625±0.36	0.625±0.36	1.25±0.36
P.aeruginosa	2.5±0.72	2.5±1.44	2.5±0.72	2.5±0.72	2.5±0.72	1.25±1.90
E coli	2.5±0.72	2.5±1.44	2.5±0.72	5±1.44	2.5±0.72	0.312±0.47
B.subtilis	1.25±0.72	1.25±0.72	1.25±1.90	1.25±0.36	1.25±1.90	0.625±0.36
Fungus						
A. flavus	2.5±1.44	2.5±1.44	2.5±0.72	2.5±0.72	2.5±0.72	1.25±0.72
A.niger	2.5±1.44	2.5±0.72	2.5±0.72	2.5±1.44	2.5±0.72	0.625±0.36
R.oligoporus	2.5±0.72	2.5±0.72	2.5±1.44	1.25±1.90	2.5±1.44	1.25±0.72
P. notatum	2.5±1.44	2.5±1.44	2.5±1.44	2.5±1.44	2.5±1.44	1.25±0.36
Candida	1.25±0.72	1.25±1.90	1.25±0.36	1.25±1.90	1.25±0.72	0.625±0.36

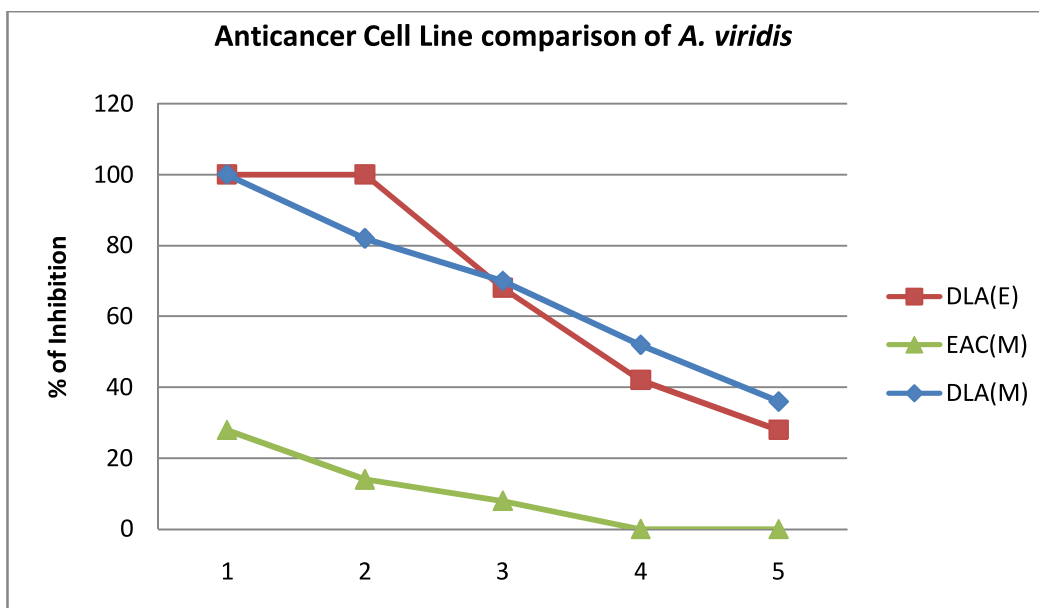
Mean±S.D.

From the MIC (Minimum inhibitory Concentration) studies, methanol extract showed higher MIC value followed by ethanol extract for antibacterial and antifungal activity with better action against human pathogenic microbial strains.

Anticancer activity

Cancer was considered as a disease called silent killer and it appears across each and every being in the dynasty. Ever changing food habits of the people and depending more on junk food has created a mess for emerging lifestyle diseases. Free radicals produced inside the body can be removed by consuming medicinal plants that has free radical scavenging activity, which can purify blood and the organs, finally detoxify those unwanted waste present inside the body.

Figure 1: Anticancer cell line activity



Previous studies showed that the Ethyl acetate extract exhibited higher antioxidant ability than ethyl ether extract of *A. viridis* and can act as a potent source of antioxidant molecules (Ying-Shan Jin *et al*., 2013). In the present study methanol extract showed better cyto-toxic condition in both DLA and EAC cell line. The study basically showed the comparison of two cell lines that cause cancer in human body. Methanol and ethanol extract of the plant were subjected for the study and about 100% inhibition is seen in DLA cell line and there is good percentage of cyto-toxicity. Methanol extract of leaves of *Amaranthus viridis* L. has shown effective antioxidant activity in DPPH assay technique. This indicates that the leaf of *Amaranthus viridis* L. was a potential source of natural antioxidants (Vrushali and Biradar, 2016). Generally, flavonoids and tannins are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers (Polterait, 1997). Phenolic compounds, flavonoids are found in detectable level in the plant and other species related to the taxa (Korlepara *et al*., 2010). From the present work carried out the IC_{50} for methanol extract was 4.1 µg/µl

while it was 3.8 μg/μl for ethanol extract.The IC_{50} value shows the ability of the drug to scavenge the free radicles about 50% and it depends up on the concentration of the sample.

Thus considerable interest has been given to the use of natural antioxidants which may also have nutritional properties (Jadhav *et al*., 1996). Though the plant was used as a dietary supplement across the country, natural herbs can provide greater health benefits, counteract oxidative stress or damage than synthetic medicines.

Liquid-chromatograph-mass spectra (LC-MS) Studies

From the LCMS studies, the methanolic extract of *A. viridis* showed different pharmaceutical important compounds detected in the sample that can be used for the preparation of medicines and treat variety of diseases. Figure 2 and 3 show the spectrum of compounds with m/z values in both negative and positive mode helps in identifying the compounds present in the methanolic extract of *A.viridis*. The compounds found to possess antimicrobial, antiseptic, antioxidant activity as listed in Table 2 with their respective activity using PASS, activity prediction software.

Majority of the compounds from METWIN 2.0 library after analysis through LCMS showed that the compounds have anticancer and antiseptic property. Liquid Chromatography – Mass Spectrometry (LC – MS) is proved to be a useful technique for plant metabolite profiling which allows the quantification of a large variety of plant metabolites in a single chromatogram (Payal *et al*., 2013). Identification is done with the aid of Computer assisted evaluation of the resulting data by searching against the spectral library. Some of the identified compounds are reported to have great pharmacological importance, while some compounds are novel and yet to be studied.

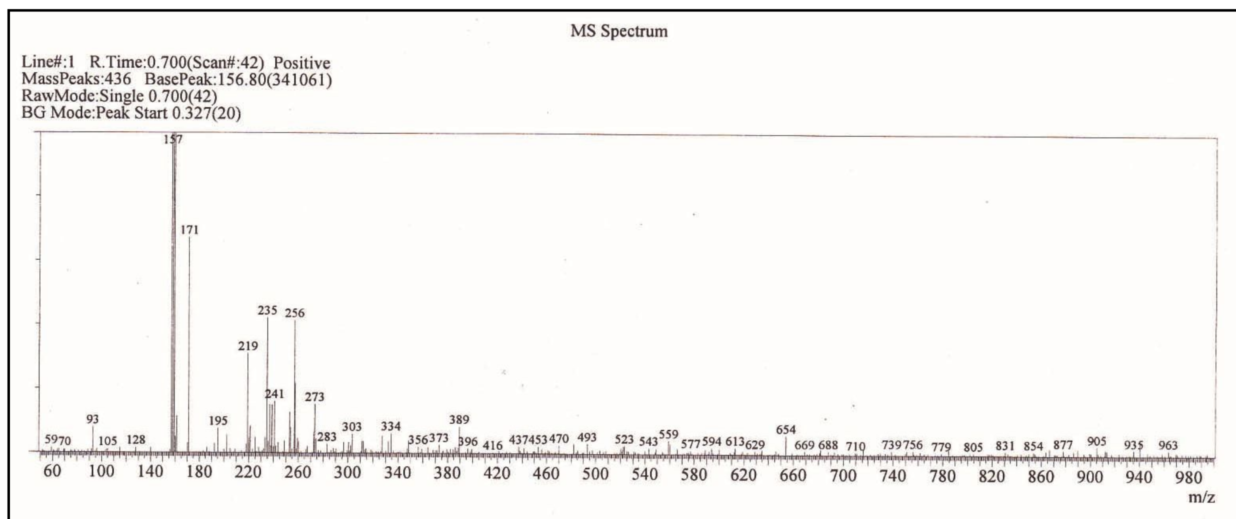
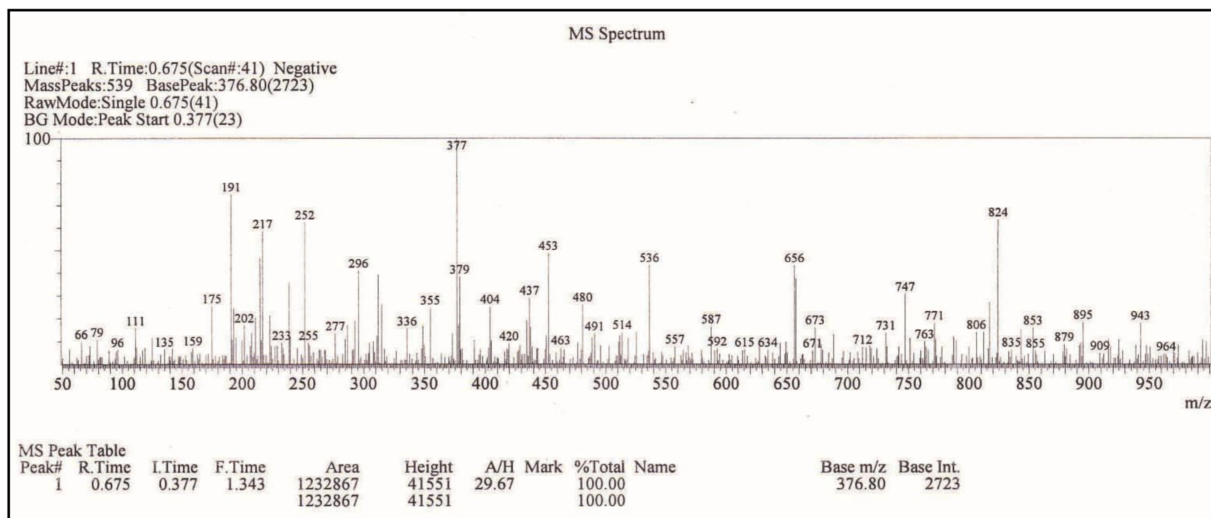


Figure 2: Liquid chromatography-mass spectrometry spectrum (positive mode)



MS Peak Table

Peak#	R.Time	I.Time	F.Time	Area	Height	A/H	Mark	%Total	Name	Base m/z	Base Int.
1	0.675	0.377	1.343	1232867	41551	29.67		100.00		376.80	2723
				1232867	41551			100.00			

Figure 3: Liquid chromatography-mass spectrometry spectrum (negative mode)

Table 2: LCMS analysis of *A.viridis* and compound activity prediction (PASS)

Compound Name & Molecular mass.	Biological Activity
Scopoletin (192.17)	Antimutagenic,Antiseptic, Apotosis agonist, free radical scavenger
Hydroxytremetone (218.3)	Chemopreventive, Antineoplastic, Muco-membranous protector, Apoptosis agonist
Lapachenole (252.3)	Antineoplastic, Spasmolytic, urinary
Phenyl Reservitol (296.4)	Antieczematic, Antihelmintic, Apoptosis agonist, Antimutagenic, Antiseborrheic, Antiseptic
Indole-3-Acetonitrile (156.2)	Pseudolysin inhibitor, Mitochondrial processing peptidase inhibitor
Gallic Acid (170.1)	Antiinfective, Antieczematic, Antimutagenic, Antiseptic
Methyl Caffeate (194.2)	Apoptosis agonist, Antiseborrheic, Antihelmintic, Antiseptic, , Antimutagenic
Alpha Vetivone (218.3)	Immunosuppressant, Antieczematic, Antiseborrheic
Valerenic Acid (234.3)	Antieczematic
Hydrangenol (256.3)	Mucomembranous protector, Antiseborrheic
Dopaxanthin (389.4)	Glutamate-5-semialdehyde dehydrogenase inhibitor

Conclusion

Our country has wide range of natural flora that need to be explored for deriving valuable components necessary for the society. The present work was carried out in order to study the antimicrobial activity to determine the minimal concentration where the plant materials can act as a substitute for the modern synthetic system of medicines. These findings can provide that the vegetable as a drug has better property to inhibit the growth of microbes that are pathogenic for human consumption. Free radical scavenging activity of the plant can act as good dietary supplement for killing the oxidative molecule that can cause

unwanted malignant tumors in human body. From the comparison of the *in-vitro* cell line assay, DLA cell line showed good anticancer activity, though the plant posses better antioxidant activity in DPPH assay. Study can further be analyzed for the active components in the plant which are showing these biological activities.

Social relevance and expected outcome

The work basically focused in evaluating the pharmacological importance and antimicrobial potential of leafy vegetable *A. viridis,* an underutilized medicinal plant. Such plants are considered as weedy plant of cosmopolitan in distribution, so conservation is necessary to study the active components in the plant. PASS, activity prediction software helps the pharmaceutical industry to know the biological activity of the plant and its commercial application quickly. Thus the present work can support Ayurvedic system of medicine in formulating new drug by knowing more about the plant's active component and its biological activity.

Applicable Industry

The work was carried out to derive a new drug for pharmaceutical industry and Ayurvedic formulation preparation.

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