Biological Forum – An International Journal **15(2): 1075-1085(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Isolation and Characterization of Bioactive Phytoconstituents in the Medicinal Plant *Amaranthus viridis* L.

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(Received: 02 January 2023; Revised: 14 February 2023; Accepted: 18 February 2023; Published: 23 February 2023)
(Published by Research Trend)

ABSTRACT: Owing to the fact that plant extracts exist as a mixture of bioactive compounds with dissimilar polarities, their segregation remained a great challenge for the process of identification and characterization of bioactive compounds. The current study was designed to isolate and identify bioactive phytoconstituents in the medicinal plant *Amaranthus viridis* L. The leaf methanol and stem ethyl acetate extracts were subjected to TLC, where chloroform: methanol (95:5) showed spot with minimum Rf value 0.3. Maximum antidiabetic activity was exhibited by LMF8 fraction and highest antiurolithiatic activity was observed in SEAF12 fraction of stem ethyl acetate fractions obtained from column chromatography. The GC-MS chromatograms of fraction LMF8 contained Decane, 1,1-Diethoxy-(100%) and the stem ethyl acetate fraction SEAF12 exhibited the presence of Epibromohydrine (100%). Decane, 1,1-Diethoxy- and Epibromohydrine compounds obtained from the fractions have been confirmed by NMR spectroscopy analysis. The mass spectrum of compound LMF8 showed a strong molecular ion peak at m/z between 230-231 with molecular weight of 230.392 which corresponds to the molecular formula $C_{14}H_{30}O_2$ of Decane 1,1-Diethoxy while compound SEAF12 contained Epibromohydrine. In this study, two compounds namely Decane 1,1-Diethoxy and Epibromohydrine had been isolated and identified from the bioactive fractions from *A. viridis*, which has no reports of its toxicity to human, based on literature. Decane 1, 1-Diethoxy and Epibromohydrine have also proved its potential bioactivity against diabetes and urolithiasis respectively.

Keywords: *Amaranthus viridis,* GCMS, TLC, GC-MS, Decane 1,1-Diethoxy and Epibromohydrine.

INTRODUCTION

Natural products, such as plants extracts, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Simon *et al*., 2022). Extraction and characterization of many active phytocompounds from these green factories have given birth to some high activity profile drugs (Ivanova *et al*., 2005).

A crude plant extract may have up to hundreds of different secondary metabolites of considerably differing chemical and spectroscopic nature (Adelia *et al.,* 2021). Therefore, chromatography, purification, isolation or separation is critical prior to detection, identification and quantification (Homans and Fuchs 1970). Many bioactive compounds have been isolated and purified using paper, thin layer and column chromatographic techniques. Column chromatography and thin-layer chromatography (TLC) are still mostly used due to their convenience, economy and availability in various stationary phases (Altemimi *et al*., 2017). Silica, cellulose, polyamide and alumina were the most best for separating the phytochemicals. Plant materials contain surprising quantities of complex phytochemicals, which make a good separation difficult. Therefore, increasing polarity using multiple mobile phases is useful for highly valued separations (Zang *et al.*, 2005).

Determination of the structure of certain molecules uses data from a wide range of spectroscopic techniques such as UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR) and mass spectroscopy (Ingle *et al*., 2017). NMR is used to elucidate the molecular structure based on the chemical environment of the proton and carbon magnetic nuclei even at low concentrations. This is one of the most powerful non-destructive techniques in elucidating the molecular structure of biological and chemical compounds (Dash *et al*., 2022). The phases to exploit a biologically active compound from plant resources comprise extraction, pharmacological screening, isolation and characterization of the bioactive compound, its toxicological evaluation and clinical assessment (Sasidharan *et al*., 2011).

The current study was designed to separate the phytoconstituents in leaf methanol and stem ethyl acetate extracts of *Amaranthus viridis* L. by thin layer chromatography, to fractionate the phytoconstituents in bioactive crude extracts (leaf-methanol extract and stem-

ethyl acetate extracts) of *A. viridis* by column chromatography, to check for the antidiabetic and antiurolithiatic potential of column chromatography fractions from leaf-methanol extract and stem-ethyl acetate extracts of *A. viridis* in vitro, to recognize the major components in column chromatography fractions from leaf-methanol extract and stem-ethyl acetate extract of *A. viridis* L. by GC-MS and to identify the isolated compounds from LMF8 and SEAF12 fractions of *A. viridis* by NMR and mass spectrometry.

MATERIAL AND METHODS

Extraction of phytoconstituents. 100g of dried leaf powder of *A. viridis* was packed in a muslin cloth tied at one end to form a bag and placed in the body of a Soxhlet extractor containing 500ml of methanol and extracted for 8 hours at 60°C. Similarly 100g of dried stem powder of *A. viridis* was placed in 500ml of ethyl acetate and extracted for 8 hours at 55°C. The extracts were passed through Whatman no.1 filter paper and placed in a hot air oven at 40° C to obtain dry extractives.

Separation of phytoconstituents by chromatographic methods

(a) Thin-Layer Chromatographic Investigation

(i) Preparation of TLC plates: TLC plates were prepared as per the method adopted by (Asha and Kumar 2015).

(ii) Mobile phases used: The different solvent systems used as mobile phases(10mL each) used are listed in Table 1.

(iii) Separation of phytoconstituents:10µl volumes of leaf methanol and stem ethyl acetate extracts of *A. viridis* were placed on the TLC plates prepared, at 2cm above its bottom with the help of capillary tubes. The plates with sample were inserted into TLC glass chambers containing 10mL of different mobile phases and were let to move up the plate by capillary action through adsorbent phase (Silica gel) up to 3/4th of the plate. The number of spots and their diameters were measured and labelled. The Rf values were calculated according to the following formula:

Rf = Distance from start to centre of substance spot
Distance from start to solvent front

(iv) Check for qualitative phytochemicals in the TLC spots: The individual spots on TLC plates were scrapped and suspended in 10mL of their respective mobile phase solvents and centrifuged. The supernatant was screened qualitatively for the phytochemicals present using the standard protocols.

(b) Separation of phytoconstituents using Column chromatography

(i) Preparation of the Column (Stationary phase): The procedure adopted by (Hussain, 2020) was used in column preparation.

(ii) Preparation of samples: The samples used were leaf methanol extract powder and stem ethyl acetate powder. The samples were mixed with a minimum amount of their respective solvents (methanol/ethyl acetate). The samples were transferred to the top of their columns through the funnel using Pasteur pipette.

(iii) Gradient solvent systems (mobile phase): The column loaded with stem ethyl acetate extract was then eluted with different polarity of solvents as listed in Table 1.

(iv) Development of the Chromatogram: The stopcock of the column was opened carefully and the flow rate of the solvent was adjusted to 5-7 drops/minute. The components of the mixture ran down the column forming different coloured bands.

(v) Recovery of fractions: Fractions were collected in separate test tubes on the basis of the colour of the bands developed or every 10mL fractions were collected when the fractions were colourless and pooled together on the basis of similar results.

(vi) Tests for Bioactivity of the fractions: The antidiabetic and antiurolithiatic activities of each fraction collected were tested by the same procedure used by Helen and Bency (2019).

Identification of the phytoconstituents by spectroscopic methods

(i) GC-MS (Gas Chromatography-Mass Spectrometry) analysis: The phytochemical investigation of the 8 bioactive fractions obtained from 4 fractions of leaf methanolic extract (LMF1, LMF5, LMF7, LMF8) and 4 fractions from stem ethyl acetate (SEAF5, SEAF8, SEAF10, SEAF12) was performed on a GC-MS equipment (Thermo Scientific Co.) Thermo GC-TRACE ultra ver.: 5.0, Thermo MS DSQ II in Kerala forest research institute, Trissur.

(ii) Fourier-transform infrared spectroscopy (FTIR) analysis: FTIR analysis was also performed in Kerala forest research institute, Trissur. 1mg of fractional powders LMF8 and SEAF12 reported to contain single phytoconstituents in their fractions namely Decane, 1,1-Diethoxy-and Epibromohydrine respectively upon GC-MS analyses were encapsulated in 10mg of KBr pellet discs and loaded in FTIR spectroscope (Shimadzu, Japan) in the region 4000-400cm-1. The results were analysed using Spectra Manager software.

(iii) NMR spectroscopy analysis: 10mg of fractional powders LMF8 and SEAF12 reported to contain single phytoconstituents in their fractions namely Decane, 1,1-Diethoxy-and Epibromohydrine respectively upon GC-MS analyses were dissolved in CDCl3. 1H and 13C NMR experiments were carried out at processional frequencies of 300 MHz and 75 MHz, respectively in CDCl3 at 25°C temperature on BRUKER NMR (Switzerland). The results were interpreted using Mest ReNova software.

(iv) High Resolution Mass Spectrometry analysis: 1mg of fractional powders LMF8 and SEAF12 reported to contain single phytoconstituents in their fractions namely Decane, 1,1-Diethoxy-and Epibromohydrine respectively upon GC-MS analyses was subjected to high resolution mass spectrometric analysis. The MS system consisted of API 2000 Q-Trap MS (Perkin Elmer, Germany). The mass to charge (m/z) ratio was in the range of 50-800 m/z. Capillary and Rf voltage were 80 V, with nebulizer gas as air at 35 psi pressure and curtain gas as nitrogen 10 psi. The data was collected and processed using ANALYST software.

RESULTS AND DISCUSSION

Separation and isolation of phytoconstituents from leaf methanol and stem ethyl acetate extracts of *Amaranthus viridis* L. by chromatographic methods

The leaf methanol and stem ethyl acetate extracts that were found to exhibit the highest antidiabetic and antiurolithiatic activity were subjected to TLC and column chromatography to separate and isolate bioactive compounds.

(a) Thin-Layer Chromatographic Investigation:

TLC chromatogram of leaf methanol and stem ethanol extracts produced different number of spots of various diameters, Rf values and colours when different solvent systems as shown in Fig. 1 and Table 1. Most of the solvents produced a single spot with colours ranging from grey, yellow, yellow-green and green. Leaf methanol extract in Ethyl acetate: Methanol (75:25) produced the spot with maximum Rf value 0.48 while stem ethyl acetate extract in Chloroform: Methanol (95:5) exhibited spot with minimum Rf value 0.3 (Table 1).

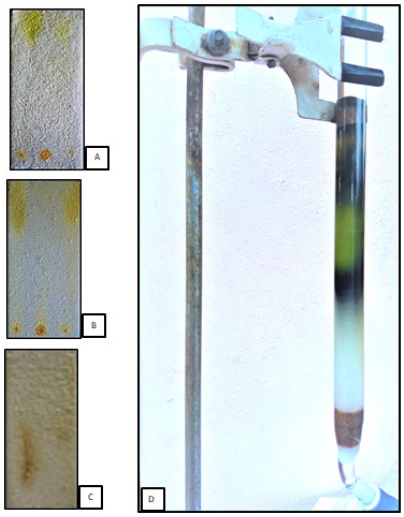


Fig. 1. Fractionation of bioactive crude extracts (leaf-methanol extract and stem-ethyl acetate extract) of *Amaranthus viridis* L. using silica gel TLC (A-C) using different solvent systems stained with Iodine and D-Column chromatography of *A. viridis* Leaf methanol extract by elution with Hexane (75): Ethyl acteate (25) solvent.

Qualitative check for phytochemicals in the TLC spots: The spots after elution with their respective solvents were tested for qualitative phytochemical tests and the results are tabulated in Table 1. TLC of Leaf methanol extract contained more flavonoid spots while stem ethyl acetate extract had more phenols and saponins followed by flavonoids and alkaloids.

(b) Separation of phytoconstituents using Column chromatography

Dry extracts (leaf-methanol extract and stem-ethyl acetate extract) of *Amaranthus viridis* L. through column chromatography yielded several fractions of varying colours when different solvents were used in different ratios as listed in Table 2.

Tests for Bioactivity of the fractions: The column chromatography fractions of the leaf-methanol and stem-ethyl acetate extracts of *Amaranthus viridis* L. were tested for antidiabetic and antiurolithiatic activities and the results were tabulated (Table 3). Maximum antidiabetic activity was exhibited by LMF8 fraction followed by LMF1 fraction of leaf methanol extract, while highest antiurolithiatic activity was observed in SEAF12 fraction of stem ethyl acetate fraction.

Isolation and identification of the phytoconstituents by spectroscopic methods

(i) GC-MS (Gas Chromatography-Mass Spectrometry) analysis

Among the 24 fractions obtained from column chromatography, 8 bioactive fractions alone were subjected to GC-MS analysis. Among the 8 fractions, 4 fractions from leaf methanol extract namely LMF1, LMF5, LMF7 and LMF8 and 4 fractions from stem ethanol extract namely SEAF5, SEAF8, SEAF10 and SEAF12 were found to be most active against invitro antidiabetic and antiurolithiatic activities respectively. The complete list of phytochemicals present in each fraction was identified by GC-MS analysis and is listed in Table 4. The GC-MS chromatogram of the fractions from leaf-methanol extract is shown in Fig. 2 and the chromatogram obtained by GC-MS analysis of the fractions from stem-ethyl acetate extract is shown in Fig. 3.

The GC-MS chromatograms of the fractions showed the presence of several peaks. The compounds pertaining to the peaks were identified by comparing the NIST library data of the peaks. The leaf methanol extract fraction LMF8 contained a single compound identified as Decane, 1,1-Diethoxy-(100%) and the stem ethyl acetate fraction SEAF12 also exhibited the presence of a single compound namely Epibromohydrine (100%). Both these compounds also exhibited the highest antidiabetic and antiurolithiatic activity respectively.

(ii) Fourier-transform infrared spectroscopy (FTIR) analysis

FT-IR spectroscopy was carried out to ascertain functional groups. In the FT-IR spectrum of Decane 1,1-Diethoxy, dominant IR absorption bands were observed in the high wave region at 3504 cm^{-1} and 3000 cm^{-1}; attributed to –OH(alcohol) and O-H(carboxylic acids) asymmetric and symmetric stretching vibrations respectively. In the finger print region, the FT-IR spectrum pre-sets dominant bands at 1429, 1267, 1221, 1182, 949, 846, 757 and 666 cm^{-1} and many other bands of medium to weak intensity. In the FT-IR spectrum of Epibromohydrine, dominant IR absorption bands were observed in the high wave region at 2926 cm^{-1} and 2856 cm^{-1}; attributed to O-H (carboxylic acids) asymmetric and symmetric stretching vibrations respectively. In the finger print region, the FT-IR spectrum pre-sets dominant bands at 1467, 1344, 1127 and 1063 cm-1 and many other bands of medium to weak intensity. Theoretical wave numbers responsible for functional groups were compared with observed wave numbers and presented in Table 5.

Table 1: Rf values of spots obtained by fractionation of bioactive crude extracts (leaf-methanol extract and stem-ethyl acetate extract) of *Amaranthus viridis* L. using silica gel TLC using different solvent systems.

Solvent system (ratio)	Bioactive crude extracts									
	Leaf-methanol extract					Stem-ethyl acetate extract				
	No. of spots	Diameter of spots (mm)	Rf value	Colour of spot	Phytochemical	No. of spots	Diameter of spots (mm)	Rf value	Colour of spot	Phytochemical
Hexane:Ethyl acetate (25:75)	1	2	0.86	Dark Green	Saponin	1	2	0.83	Light Green	Phenol
Hexane:Ethyl acetate (50:50)	1	3	0.86	Yellow	Flavanoid	1	2	0.85	Grey	Flavanoid
Hexane:Ethyl acetate (75:25)	1	1	0.81	Yellow	Flavanoid	1	1	0.8	Yellow	Phenol
Hexane:Dichloro methane (75:25)	1	3	0.9	Light Green	Flavanoid	1	3	0.9	Pale Yellow	Saponin
Ethyl acetate (100)	1	2	0.93	Yellow	Flavanoid	1	4	0.93	Grey	Phenol
Dichloromethane: Methanol(50:50)	1	12		Light Grey	Phenol	1	13	0.66	Yellow	Alkaloid
Methanol(100)	1	15	0.61	Green	Saponin	1	21	0.35	Yellow	Flavanoid
Ethyl acetate:Methanol (75:25)	1	15	0.48	Brown	Cardiac Glycoside	1	12	0.75	Light Brown	Saponin
Ethyl acetate:Methanol (50:50)	1	9	0.65	Grey	Flavanoid	1	16	0.56	Yellow	Alkaloid
Ethyl acetate:Methanol (25:75)	1	14	0.53	Grey	Flavanoid	1	16	0.41	Light Brown	Saponin
Chloroform:Methanol (95:5)	1	14	0.36	Green	Phenol	1	1	0.3	Dark Green	Saponin
Hexane:Ethyl acetate (9:10)	1	3	0.86	Grey	Flavanoid	1	2	0.86	Brown	Cardiac Glycoside
Chloroform:Methanol(85:15)	1	2	0.85	Yellow	Phenol	1	2	0.88	Green	Phenol

Table 2: Isolation of compounds from dry extracts (leaf-methanol extract and stem-ethyl acetate extract) of *Amaranthus viridis* L. through column chromatography.

Stem-Ethyl acetate extract powder					
Sr. No.	**Mobile phase**	**Ratio**	**Fractions**	**Colour**	**Volume**
1.	Hexane: Ethyl acetate	75:25	SEAF1	Pale yellow	10mL
2.			SEAF2	Colourless	10mL
3.	Dichloromethane: Methanol	50:50	SEAF3	Colourless	13.75mL
4.			SEAF4	Pale yellow	15mL
5.			SEAF5	Pale yellow	15mL
6.			SEAF6	Pale yellow	15mL
7.	Ethyl Acetate : Methanol	25: 75	SEAF7	Pale yellow	15mL
8.			SEAF8	Pale yellow	15mL
9.			SEAF9	Pale yellow	15mL
10.			SEAF10	Colourless	15mL
11.	Ethyl Acetate: Methanol	75: 25	SEAF11	Pale green	13mL
12.			SEAF12	Pale green	13mL
13.			SEAF13	Pale green	13mL
14.			SEAF14	Pale green	17mL
15.			SEAF15	Pale green	13mL
16.			SEAF16	Pale green	13mL
Leaf-methanol extract powder					
Sr. No.	**Mobile phase**	**Ratio**	**Fractions**	**Colour**	**Volume**
1.	Hexane: Ethyl acetate	75:25	LMF1	Pale yellow	15mL
2.			LMF2	Dark green	15mL
3.			LMF3	Yellow	15mL
4.			LMF4	Light green	15mL
5.			LMF5	Dark green	15mL
6.			LMF6	Light green	15mL
7.	Dimethy ether: Methanol	50: 50	LMF7	Dark green	15mL
8.	Ethyl Acetate: Methanol	75: 25	LMF8	Dark green	15mL

Table 3: Antidiabetic and Antiurolithiatic activity of column chromatography fractions from leaf-methanol extract and stem-ethyl acetate extract of *Amaranthus viridis* L.

Sr. No.	Fractions	Antidiabetic activity (% Alpha amylase inhibition) Mean+SE	Antiurolithiatic activity Stone weight in mg after 25days Mean +SE(Initial weight=15mg)
1.	LMF1	73.12±0.84^{e}	14.56±0.56^{g}
2.	LMF2	24.13±0.02^{b}	14.98±0.87^{g}
3.	LMF3	61.18±0.38^{q}	13.26±0.09^{fg}
4.	LMF4	67.08±0.07^{o}	14.89±0.36^{g}
5.	LMF5	59.47±1.23^{mn}	12.97±0.98^{ef}
6.	LMF6	63.64±0.59^{p}	13.68±0.93^{fg}
7.	LMF7	69.68±0.01^{p}	13.45±0.48^{fg}
8.	LMF8	82.6±0.62^{r}	9.10±0.36^{b}
9.	SEAF1	55.87±0.68^{l}	11.94±0.98^{de}
10.	SEAF2	44.24±0.09^{i}	11.19±0.36^{de}
11.	SEAF3	36.98±0.57^{g}	11.08±0.76^{de}
12.	SEAF4	60.11±0.46^{no}	7.50±0.87^{fg}
13.	SEAF5	21.01±0.89^{a}	9.87±0.46^{bc}
14.	SEAF6	28.10±0.76^{d}	14.46±0.87^{g}
15.	SEAF7	37.21±1.54^{g}	12.25±0.56^{ef}
16.	SEAF8	44.60±0.24^{i}	8.20±0.5^{cd}
17.	SEAF9	58.82±0.10^{m}	7.38±0.27^{g}
18.	SEAF10	26.18±0.36^{c}	13.85±0.79^{fg}
19.	SEAF11	46.27±0.09^{j}	12.27±0.84^{ef}
20.	SEAF12	51.58±1.75^{k}	5.96±0.57^{a}
21.	SEAF13	23.00±0.46^{b}	14.40±0.34^{g}
22.	SEAF14	35.41±0.34^{f}	14.35±0.13^{g}
23.	SEAF15	41.02±0.20^{h}	11.87±0.20^{de}
24.	SEAF16	32.00±1.72^{e}	12.56±0.07^{ef}

Table 4: Major components in column chromatography fractions from leaf-methanol extract and stem-ethyl acetate extract of *Amaranthus viridis* L. identified by GC-MS analysis.

Fractions	Main components	Retention time(min)	Area %	Height %	Base m/z
LMF1	2-Nonen-1-Ol, (E)-	26.523	1.37	4.91	68.05
	2,2-Dimethyl-Propyl 2,2-Dimethyl-Propanesulfinyl Sulfone	31.874	1.59	2.49	71.10
	Furan, 3-(Chloromethyl)-	40.708	10.37	7.24	55.10
	Decycloxyethanol	41.050	5.53	6.5	57.10
	1-(2-Hydroxyethoxy)Tridecane	41.154	13.30	8.99	57.10
	Threo-3,4-Epoxy-2-Octanol	43.052	0.67	1.40	69.10
	Docosyl 2-Methylbutanoate	46.875	18.10	14.07	103.10
	Decane, 1,1-Diethoxy-	46.947	8.87	14.94	103.10
	Tetracosane	47.636	24.58	29.69	57.10
LMF5	Butane, 2,2-Dimethyl-	12.065	5.71	10.13	57.10
	2,2-Dimethyl-Propyl 2,2-Dimethyl-Propanesulfinyl Sulfone	15.800	3.98	6.13	57.10
	Oxalic Acid, CyclobutylHeptyl Ester	16.492	5.75	10.96	55.10
	3-Buten-2-Ol	17.995	2.85	4.86	57.10
	5-Hepten-3-One, 5-Ethyl-4-Methyl-	19.052	8.19	14.72	55.10
	Neopentyl Trifluoroacetate	22.476	5.04	10.08	57.10
	Butanamide, 3,3-Dimethyl-	23.312	45.93	15.44	59.10
	3-Methyl-3-Oxetanemethanol	23.450	9.05	7.96	57.10
	Benzodioxol-2-One, Hexahydro-, Trans-	23.527	5.15	8.11	57.10
LMF7	Glycinamide, N-[(1,1-Dimethylethoxy)Carbonyl]Glycylthio-	19.211	23.28	12.55	191.10
	Bis-(3,5,5-Trimethylhexyl) Ether	25.660	23.77	23.31	55.05
	2-Nonen-1-Ol, (E)-	26.552	8.82	15.87	68.10
	Allyloxymethylacrylamide	36.817	3.62	6.11	57.05
	Epibromohydrine	47.675	5.93	3.65	57.10
LMF8	Decane, 1,1-Diethoxy-	46.947	100	100	103.10
SEAF5	2-Tert-Butyl-4-(1,1,3,3-Tetramethylbutyl)Phenol	19.209	38.33	28.98	191.15
	Oxalic Acid, CyclobutylHeptyl Ester	25.662	13.17	19.59	55.10
	N-Allyloxymethylacrylamide	29.711	17.30	20.19	55.05
	Ethanol, 2-Butoxy-	44.309	17.50	20.75	57.10
	Epibromohydrine	47.662	13.71	10.48	57.10
SEAF8	(Z)-Cis-9,10-Epoxyheptadec-6-Ene	16.604	3.15	6. 17	68.10
	Hexahydrofarnesylacetone	16.704	16.70	26.75	58.05
	2-Hexylallyl Alcohol	17.876	2.05	3.00	58.05
	2,2-Bis[4-(2-Cyano)Phenyl]Propane	17.986	1.71	2.68	57.10
	Nonanoic Acid, Methyl Ester	18.072	1.93	2.67	74.05
	Phytol	22.306	20.39	20.36	71.05

	4-Tridecen-2-Ynal, (Z)-	25.547	7.00	7.65	57.10
	1,10-Dicarbamoyldecane	27.617	2.25	2.04	59.05
	Bis(2-Ethylhexyl) Ester Of Hexanedioic Acid	28.021	3.51	4.00	129.10
	DioctylPhtalate	30.531	9.22	9.72	149.05
	Glycerine 2-Monostearyl Ether	33.408	13.42	5.21	57.10
	1,7-Octadien-3-Ol	33.592	9.47	3.97	57.10
	4,8-Dimethyl-3(E),7-Nonadienyl Thioacetate	34.915	1.25	1.63	69.10
SEAF10	Ethyl 4-Bromohexanoate	31.768	2.75	10.12	85.05
	Dodecanamide	36.504	61.50	45.88	59.05
	Ethanone, 1-Cyclobutyl-	36.750	8.81	22.87	55.00
	Epibromohydrine	44.422	13.26	11.87	57.05
SEAF12	Epibromohydrine	44.667	100	100	57.15

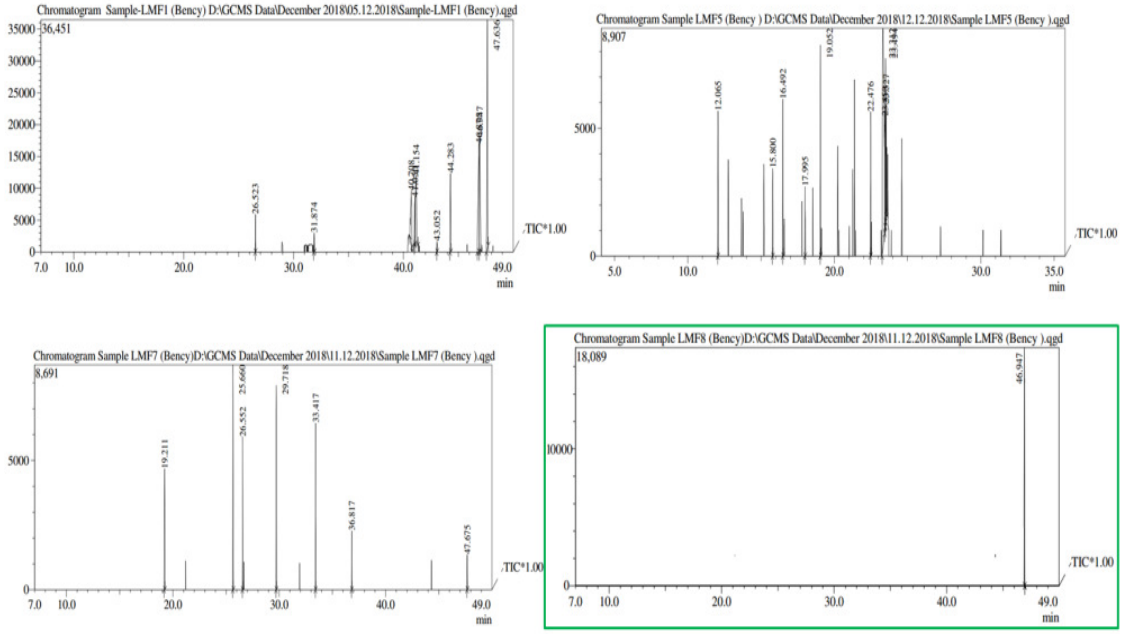


Fig. 2. Chromatogram obtained by GC-MS analysis of fractions from leaf-methanol extract of *Amaranthus viridis* L.

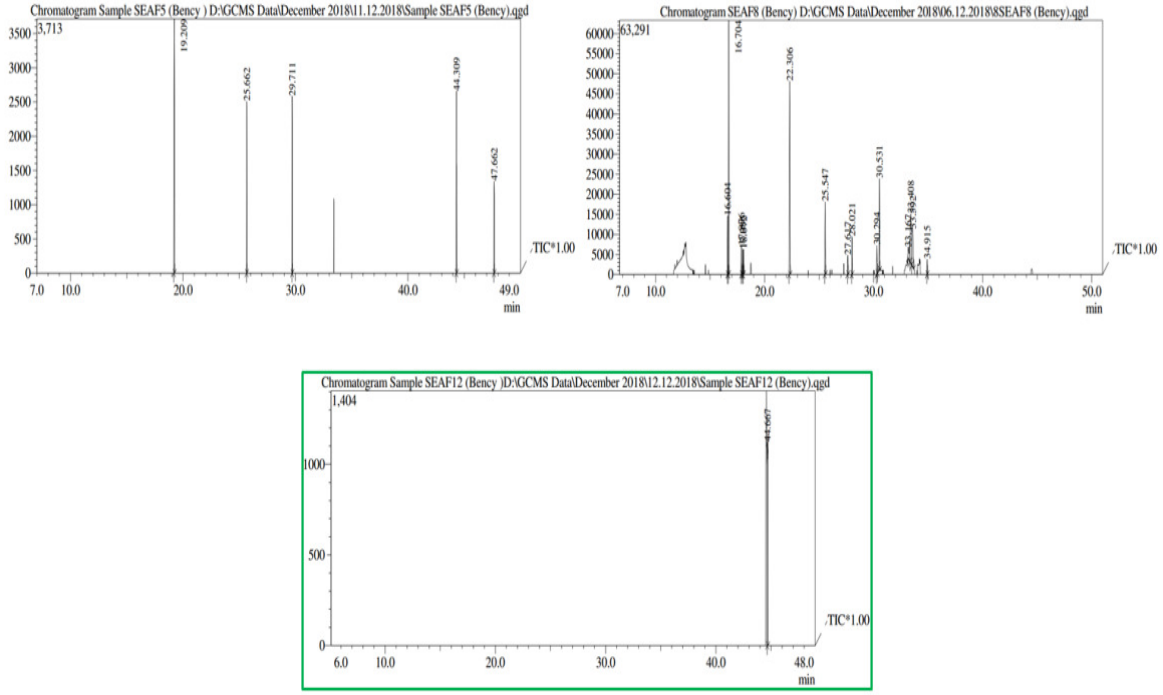


Fig. 3. Chromatogram obtained by GC-MS analysis of fractions from stem-ethyl acetate extract of *Amaranthus viridis.*

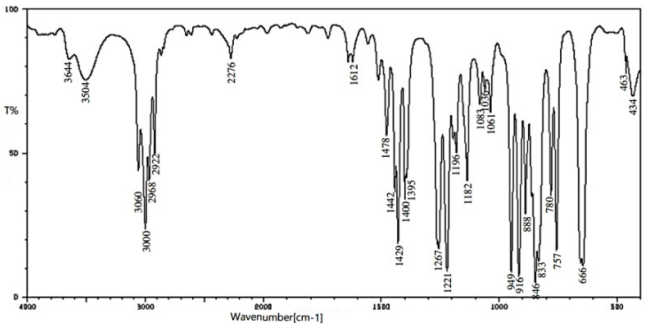


Fig. 4A. FT-IR Spectrum of the isolated compound LMF8 from fraction of *Amaranthus viridis* L.

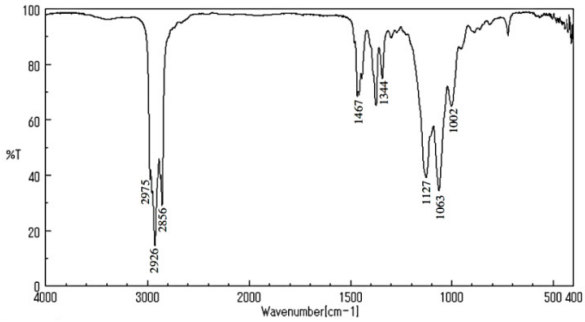


Fig. 4B. FT-IR Spectrum of the isolated compound SEAF12 from fraction of *Amaranthus viridis.*

Table 5: Important frequencies obtained in FT-IR spectra of Decane 1,1-Diethoxy from LMF8 fraction and Epibromohydrine from SEAF12 fraction of *A. viridis* L.

Decane 1,1-Diethoxy			
Wave numbers (cm^{-1})	**Intensity**	**Shape**	**Functional groups**
3504	medium	broad	O-H (alcohol)
3000	strong	sharp	O-H(carboxylic acids)
2276	weak	sharp	C≡C(alkyne)
1612	medium	sharp	C=C(alkene)
1429	medium	sharp	C-H(alkane)
1267	strong	sharp	N-O(nitro)
1196	medium	sharp	C-H(haloalkane)
1221	strong	sharp	C-N(amines)
1182	medium	sharp	C-H(haloalkane)
1061	weak	sharp	C-O(esters)
949	strong	sharp	O-H(carboxylic acid)
846	strong	sharp	N-H(1"&2" amine)
780	medium	sharp	C-H(alkene)
757	strong	sharp	N-H(1"&2" amine)
666	strong	sharp	N-H(1"&2" amine)
434	weak	broad	C-Cl(alkyl chloride)
Epibromohydrine			
2975	medium	broad	O-H(carboxylic acid)
2926	strong	sharp	O-H(carboxylic acid)
2856	strong	sharp	O-H(carboxylic acid)
1467	medium	broad	C-C(aromatics)
1344	weak	sharp	C-O(esters)
1127	strong	broad	C-N(aliphatic amine)
1063	strong	sharp	C-N(aliphatic amine)
1002	medium	broad	C-O(esters)

(iii) NMR spectroscopy analysis

The 1H and 13C NMR analyses of LMF8 and SEAF12 compounds were carried out and the results obtained are represented in Fig. 5 and 6. The 1H and 13C observed NMR spectral assignments for LMF8 and SEAF12 compounds are tabulated in Table 6.

(iv) High Resolution Mass Spectrometry analysis

Mass spectroscopy (MS) was carried out to determine the molecular weight of the two isolated phytoconstituents present in LMF8 and SEAF8 fractions of *Amaranthus viridis* L. The mass spectrum of the compound LMF8 showed a strong molecular ion peak at m/z between 230-231(Fig. 7A) which confirms the molecular weight of LMF8 at 230.392 which corresponds to the molecular formula $C_{14}H_{30}O_2$ of

Decane 1,1-Diethoxy. The mass spectrum of the compound SEAF12 showed a strong molecular ion peak at m/z almost 136 (Fig. 7B) which confirms the molecular weight of SEAF12 at 136.976 which corresponds to the molecular formula C_3H_5BrO of Epibromohydrine.

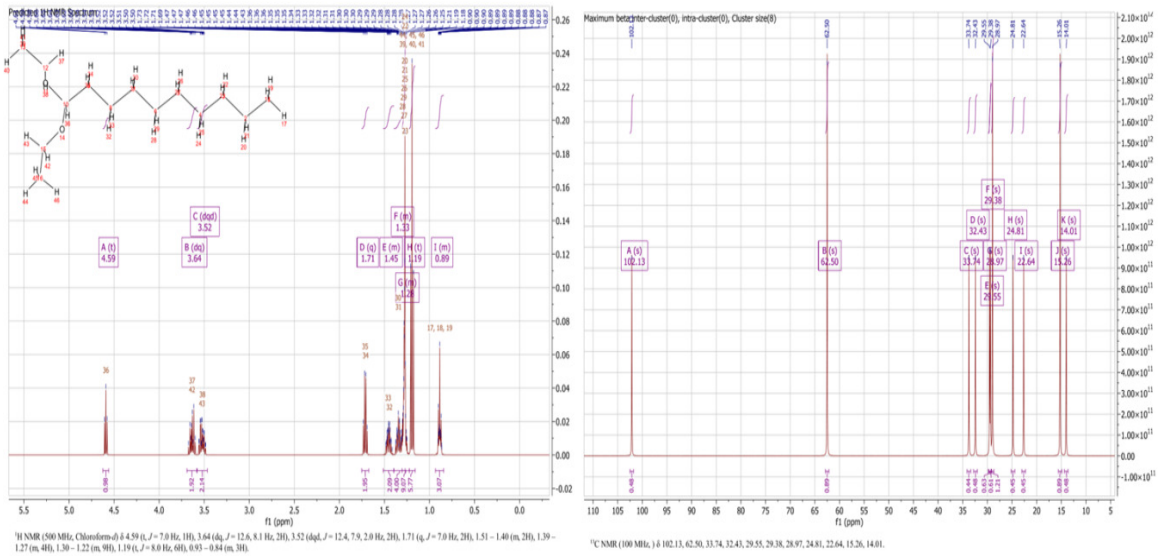


Fig. 5. 1H NMR and 13C NMR spectra of the isolated compound from LMF8 fraction of *Amaranthus viridis* L. identified as Decane 1,1-Diethoxy.

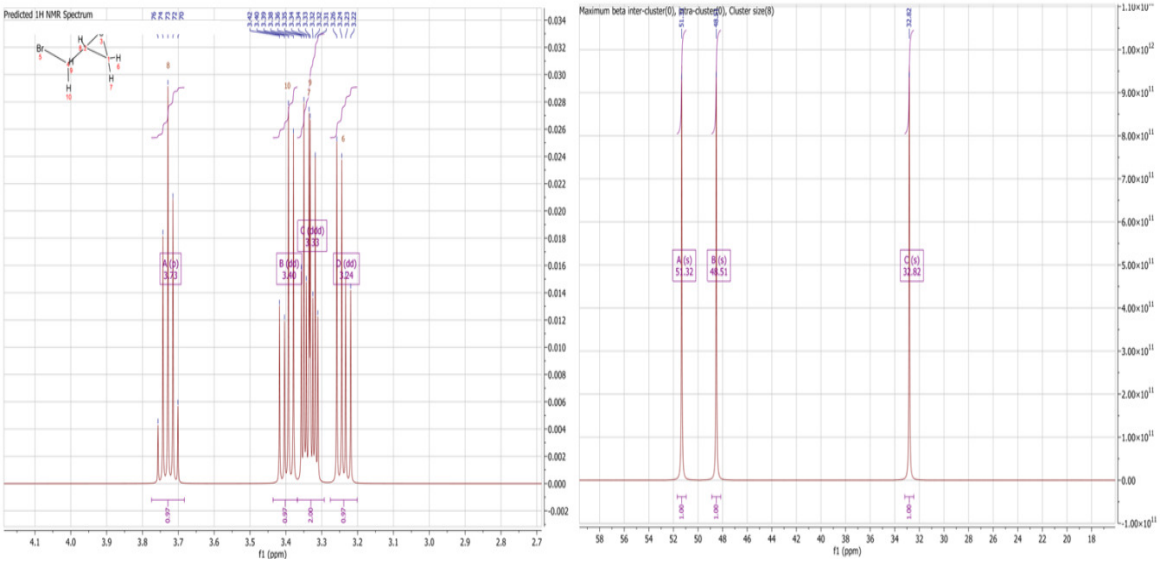
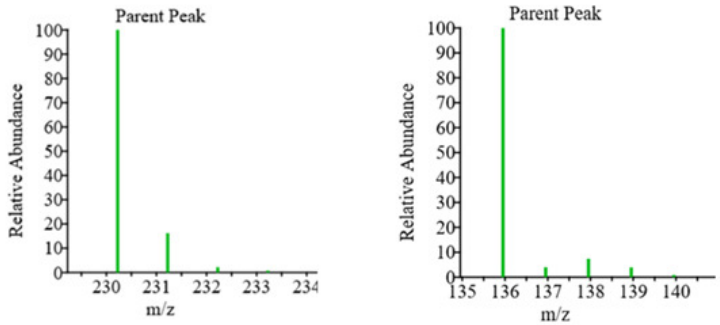


Fig. 6. 1H NMR and 13C NMR spectra of the isolated compound from SEAF12 fraction of *Amaranthus viridis* L. identified as Epibromohydrine

Table 6: 1H and 13C NMR spectral assignments for LMF8 and SEAF12.

Compound	Observed NMR spectral assignments	
LMF8	1H NMR(δ)	^{1}H NMR (500 MHz, Chloroform-d) δ 4.59 (t, J = 7.0 Hz, 1H), 3.64 (dq, J = 12.6, 8.1 Hz, 2H), 3.52 (dqd, J = 12.4, 7.9, 2.0 Hz, 2H), 1.71 (q, J = 7.0 Hz, 2H), 1.51 – 1.40 (m, 2H), 1.39 – 1.27 (m, 4H), 1.30 – 1.22 (m, 9H), 1.19 (t, J = 8.0 Hz, 6H), 0.93 – 0.84 (m, 3H).
	13C NMR(δ)	^{13}C NMR (100 MHz,) δ 102.13, 62.50, 33.74, 32.43, 29.55, 29.38, 28.97, 24.81, 22.64, 15.26, 14.01.
SEAF12	1H NMR(δ)	^{1}H NMR (500 MHz, Chloroform-d) δ 3.73 (p, J = 7.0 Hz, 1H), 3.40 (dd, J = 12.5, 7.0 Hz, 1H), 3.33 (ddd, J = 12.3, 7.0, 3.7 Hz, 2H), 3.24 (dd, J = 12.5, 7.0 Hz, 1H).
	13C NMR(δ)	^{13}C NMR (100 MHz,) δ 51.32, 48.51, 32.82.



7A 7B

Fig. 7A and 7B. Mass spectrum of the isolated compounds LMF8 and SEAF12 from the fractions of *Amaranthus viridis* L. with parent peaks at 230 and 136 m/z respectively.

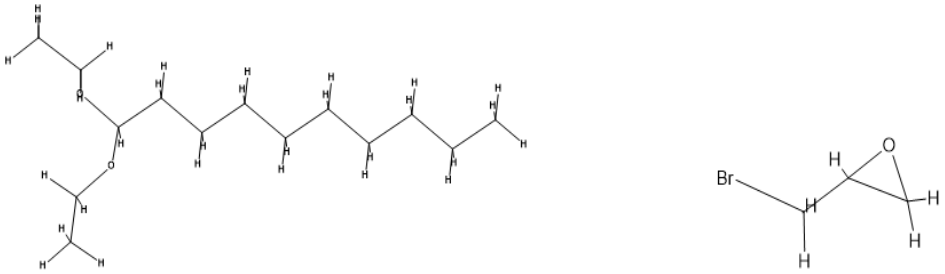
Identification of the isolated compounds. From results of FT-IR, NMR and Mass spectroscopy of the isolated phytoconstituents, it was confirmed through structure in NMR spectroscopy and molecular weight in mass spectroscopy to propose that the compounds may be Decane 1, 1-Diethoxy and Epibromohydrine (Fig. 8A and 8B).

Introduction to Decane 1,1-Diethoxy and Epibromohydrine

(a) Decane 1,1-Diethoxy: Straight-chain primary aliphatic alcohols/aldehydes/acids, acetals and esters with esters containing saturated alcohols and acetals containing saturated aldehydes (CoE, 1992).

(b) Epibromohydrine: Epibromohydrine is an epoxide that is oxirane substituted by a bromomethyl group at position 2 (Doe, 2016).

Alphabets in superscript within same columns are significantly different from each other ($p<0.05$, Tukey's HSD-multiple range post-hoc test) using IBM SPSS package.



8A 8B

Fig. 8A and 8B. The structure of LMF8 and SEAF12 identified as Decane 1,1-Diethoxy and Epibromohydrine respectively.

Table 7: Properties of Decane 1,1-Diethoxy and Epibromohydrine (Source: PubChem).

Sr. No.	Properties	Decane 1,1-Diethoxy	Epibromohydrine
1.	Molecular Formula	$C_{14}H_{30}O_2$	C_3H_5BrO
2.	IUPAC Name	1,1-Diethoxydecane	1-Bromo-2,3-epoxypropane
3.	Appearance	white	Off white
4.	Molecular weight	230.392 g/mol	136.976 g/mol
5.	Solubility	DMSO	water
6.	Boiling point	260ºC at 760 mmHg	273 to 277 ° F at 760 mm Hg
7.	Uses	Flavouring agent, food additive	Used for cross-linking polymers
8.	Toxicity	No reports till date in literature	Toxic, carcinogenic
9.	Storage	Dry, dark and at 0-4°C for short term or -20°C for long term	Away from source of ignition, in tightly closed container, dry, refrigerated.

Defined classes of chemical compounds are responsible for the each manifestation of physiological activity (Steffensen *et al*., 2011). Therefore, the study of their composition and the isolation of physiologically active compounds are exceedingly critical. In this study the leaf methanol and stem ethyl acetate extracts that were found to exhibit the highest antidiabetic and antiurolithiatic activity were subjected to TLC and column chromatography to separate and isolate bioactive compounds. Leaf methanol extract in Ethyl acetate: Methanol (75:25) produced the spot with maximum Rf value 0.48 while stem ethyl acetate extract in Chloroform: Methanol (95:5) exhibited spot with minimum Rf value 0.3. Also, investigation by Ragasa*et al*. (2015) indicated prominent ability of chemical constituents of stems, leaves and roots of *Amaranthus viridis* collected from Manila, Philippines to be separated by TLC. According to the study by Poongothai *et al*. (2011) the methanol extract of *Amaranthus viridis* was subjected to TLC for the characterization of isolated compound –I. It has been already shown by He *et al*. (2002) that four *A. viridis* types grown in China namely

Amaranthus tricolor, *Amaranthus cruentus*, *hypochondriacus* and *A.pumilus* fractions were rich in squalene as detected by TLC. Later they were combined and the solvent was evaporated by rotary evaporation to yield a colourless liquid, with a concentration of 94% and a recovery of 90%.

In this study, isolation of two bioactive compounds from dry extracts (leaf-methanol extract and stem-ethyl acetate extract) of *Amaranthus viridis* was achieved L. from several fractions collected through column chromatography. To the best of our knowledge, isolation of bioactive compounds from *A. viridis* was not reported in the literature till date. In our findings the column chromatography fractions of the leaf-methanol and stem-ethyl acetate extracts of *Amaranthus viridis* L. were tested for antidiabetic and antiurolithiatic activities and the results had proved maximum antidiabetic activity by LMF8 fraction and highest antiurolithiatic activity was observed in SEAF12 fraction of stem ethyl acetate fraction. Limited studies have been reported on the tests for bioactivity of the different fractions collected by column chromatography of *A. viridis* extracts.

Antioxidant activities of fractions of *A. viridis* plants from northeast region of India has been described previously by Kumari *et al*. (2018) where the antioxidant ability of the different fractions of *A. viridis* extracts was determined by the improved DPPH and ABTS+ radical cation scavenging capacity methods, total reduction capability, hydrogen peroxide radical scavenging activity, in vitro lipid peroxidation assay and antigenotoxicity assays. Benabdallah *et al.* (2016) also evaluated the phytochemical and in vitro antioxidant ability of methanolic extract and different fractions of *Amaranthus graecizans* collected from Silvestris, Pakistan.

In the current research, the GC-MS chromatograms of the fractions showed the presence of several peaks. The leaf methanol extract fraction LMF8 contained a single compound identified as Decane, 1,1-Diethoxy-(100%) and the stem ethyl acetate fraction SEAF12 also showed the presence of a single compound namely Epibromohydrine (100%). Both these compounds exhibited the highest antidiabetic and antiurolithiatic activity respectively. The major components identified in the LMF1, LMF5, LMF7, SEAF5, SEAF8 and SEAF10 fractions were Tetracosane (24.58%), Butanamide, 3,3-Dimethyl- (45.93%), Bis-(3,5,5-Trimethylhexyl) Ether(23.77%), 2-Tert-Butyl-4-(1,1,3,3-Tetramethylbutyl)Phenol (38.33%), Phytol(20.39%) and Dodecanamide (61.5%) respectively. Earlier, Salvamani *et al.* (2016) designed a study to investigate the phytochemical components, of *Amaranthus viridis* (*A. viridis*) using Gas Chromatography, Mass Spectrometry (GC-MS/MS) analysis revealed the presence of 30 compounds. Kayarohanam and Kavimani (2015) has already characterized compound –I isolated from the methanol extract of *Amaranthus viridis* by subjecting to FTIR. In a previous study by Verma *et al*. (2016), the phytochemical profiling and GCMS of *Adhatoda vasica* has been reported with isolation of several compounds.

One of the findings in our research was the confirmation of Decane, 1,1-Diethoxy- and Epibromohydrine compounds obtained from the fractions by NMR spectroscopic analysis. Ragasa *et al.* (2015) had recorded on a Varian VNMRS spectrometer in CDCl3 at 600 MHz for 1H NMR and 150 MHz for 13C NMR spectra for the confirmation of the selected compounds of the stems, leaves and roots of *A. viridis* collected from Manila, Philippines. Research had also confirmed the structure and name of the compound –I isolated from the methanol extract of *Amaranthus viridis* using NMR. Minzanova *et al*. (2014) have also confirmed the structure of pectinic polysaccharides from *Amaranthus cruentus* by NMR.

In the present study, the mass spectrum of the compound from LMF8 fraction showed a strong molecular ion peak at m/z between 230-231 which confirms the molecular weight of LMF8 as 230.392 which corresponds to the molecular formula $C_{14}H_{30}O_2$ of Decane 1,1-Diethoxy. The mass spectrum of the compound SEAF12 showed a strong molecular ion peak at m/z almost 136 which confirms the molecular weight of SEAF12 as 136.976 which corresponds to the molecular formula C_3H_5BrO of Epibromohydrine. In an earlier report by Kumari *et al*. (2016), characterization and identification of phenolic compounds present in *A. viridis* was confirmed using ultra performance liquid chromatography tandem mass spectrometer (UPLC MS/MS). Masike *et al*. (2021) defined distinction between isobaric HCA conjugates from *Amaranthus viridis* and *Moringa oleifera,* using mass spectrometry (MS) approaches.

To the best our knowledge, literature on the isolation of the two compounds Decane 1,1-Diethoxy and Epibromohydrine that were isolated from *A. viridis* in the current study was not available. However several other compounds have been previously isolated from other species of *Amaranthus* by Stintzing *et al.* (2004).

CONCLUSIONS

Though isolation of bioactive compounds from their mixtures in plant material and determination is still challenging, practically many of them have been purified by the combination of thin layer and column chromatographic techniques and identified by GC-MS, FTIR, NMR and mass spectroscopy. In this study, two compounds namely Decane 1,1-Diethoxy and Epibromohydrine had been isolated and identified from the bioactive fractions from *A. viridis*, which has no reports of its toxicity to human, based on literature. In the current research, the two isolated compounds Decane 1, 1-Diethoxy and Epibromohydrine have also proved its potential bioactivity against diabetes and urolithiasis respectively. Additionally, this is the first time based on literature that these two compounds have been isolated from *A. viridis*.

FUTURE SCOPE

The promising compounds identified in this study can be exploited commercially to treat diabetes and kidney stones.

Acknowledgement. We are grateful to the staff members of Biotechnology Department, Malankara Catholic College for their encouragement throughout this work. We are also thankful to Rev. Fr. Jose Bright, Correspondent/Secretary and Dr. J. Thampi Thanka Kumaran, Principal, Malankara Catholic College, Mariagiri for their constant encouragement and support.

Conflict of Interest. None.

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How to cite this article: Helen P.A.M. and Bency B.J. (2023). Isolation and Characterization of Bioactive Phytoconstituents in the Medicinal Plant *Amaranthus viridis* L. *Biological Forum – An International Journal, 15*(2): 1075-1085.