Original Research Paper

Volume - 12 | Issue - 08 | August - 2022 | PRINT ISSN No. 2249 - 555X | DOI : 10.36106/ijar

Chemical Science

GAS CHROMATOGRAPHY-MASS SPECTROMETRY INVESTIGATION OF BIOACTIVE COMPOUNDS AND BIOASSAY OF LEAVES OF VIGNA MUNGO (L.) HEPPER.

Author	Affiliation
Jaysing Mahavirsing Dinore*	Assistant Professor, Department of Chemistry, Indraraj Arts, Commerce and Science College, Sillod. District: Aurangabad, Maharashtra, India. *Corresponding Author
Ali Alrabie	Research Scholar, Post Graduate and Research Centre, Maulana Azad College, Aurangabad. Maharshtra, India.
Samreen Farooqui	Assistant Professor, Post Graduate and Research Centre, Maulana Azad College, Aurangabad. Maharshtra, India.
Vidya Pradhan	Associate Professor. Dr. Rafiq Zakaria College for Women, Aurangabad. Maharshtra, India.
Mazahar Farooqui	Professor, Post Graduate and Research Centre, Maulana Azad College, Aurangabad. Maharshtra, India.

ABSTRACT Medicinal plants are used as traditional medicines but yet remain unexplored in terms of their bioactive metabolites. This study targeted identifying bioactive metabolites from Vigna mungo leaves by gas chromatography-mass spectrometry and assessing their efficacy in the antimicrobial and anti-oxidant assay. Gas chromatography chromatogram was analysed systematically, which described the phytochemical profile of the leaves. GC-MS analysis revealed the presence of valuable bioactive compounds in the sample like essential oils, fatty acids, sesquiterpenes, alkaloids, and steroids. The prevalent constituents found in extracts were dasycarpidan-1-methanol acetate (21.91%), octadecane, 3-ethyl-5-(2-ethylbutyl) (18.55%), Z-5-Methyl-6-heneicosane-11-one (9.26%), 5,7,9-(11)-Androstatriene, 3-hydroxy-17-oxo (8.15%), 14-hydroxy-14-methylhexadec-15-enoic acid, methyl ester (6.45%), and 12,15-octadecadienoic acid, methyl ester (4.85%). Leaves extract showed a rational antibacterial activity against E. coli (MIC: 25 µg/ mL) and significant anti-fungal action against C. albicans (MIC: 25 µg/ mL). Besides, the extract demonstrated reasonable free radical scavenging activity with IC50 of 39.60±0.07%.

KEYWORDS : *Vigna mungo,* soxhlet extraction, gas chromatography mass spectrometry, antimicrobial activity, DPPH.

INTRODUCTION:

Legumes are appreciated for their protein contents and a fabulous source of nutrients for the majority of developing nations. Pulses have taken on significant place in diet in Indian subcontinent due to their high protein contents and low glycemic index. *Vigna mungo* (L.) Hepper [VM] is one of the important legume crops that originated, extensively cultivated and occupying a unique position in Indian agriculture. [1] It is recognised as black gram in English, Masha in Sanskrit and Urad in Hindi. *Vigna mungo* belongs to the family *Fabaceae*. It is fast growing erect legume, with long twining branches that reaches 30-100 cm in height. It has well-developed taproots and its stems diffusely branched from the base. The trifoliate leaves have ovate leaflets that are 4-10 cm long and 2-7 cm wide. Its seeds are used in cooked dhal and seed flour is utilised in the preparation of food items like papad, dosa, idli, vada, hopper and waries. [2] The nutritional value of seeds of black gram reflected by its rich content of valuable nutrients like carbohydrates (56.6%), proteins (26.2%), fat (1.2%), vitamins-vitamin-B, vitamin-B2 and niancin and minerals such as Ca (185 mg/100g), Fe (8.7 mg/100g) and P (345 mg/100g). There are many ethanobotanical uses of black gram. The seeds are reported to protect DNA and erythrocytes from free radical oxidative damage and possess significant lipid-lowering activity. It is used to treat many ailments like rheumatism, asthama, abscesses and liver diseases. In addition to these black gram has been mentioned to possess analgesic, anti-inflammatory, anti-osteoarthritic and anti-cancer activity.

After harvesting seeds of a black gram, a significant quantity of the stem and leaves is produced as a byproduct. Literature survey revealed that researchers have concentrated mainly on seeds only and published reports on it. Traditionally *Vigna mungo* leaves have been reported to possess impressive anti-inflammatory, analgesic and ulcerogenic properties. The leaves have been shown to display a potent cytotoxic effect. [3] The plant root is used to treat abscesses, inflammations, paralysis, rheumatism and hemoptysis.

On the onset of increasing incidents of fatal diseases, researchers diverted their attention towards the use of traditionally used medicinal plants in the search for a novel potential therapeutic agent. It is noted that research work mainly concentrated on seeds of pulses and its vegetative parts were remained under explored. In our previous work we reported phytochemical constituents of leaves of *Cajanus cajan* [4] and *Vigna unguiculata* [5]. The existing literature on black gram showed studies of the chemical composition of *Vigna mun*go had not systematically studied in the Marathwada region of Maharashtra, India. Despite its immense nutritive and medicinal values, *Vigna mungo* is underexplored for its bioactive components. The present study was designed with objectives to evaluate antimicrobial, antioxidant activities and to investigate unexplored phytochemicals abound in Vigna mungo leaves using Gas chromatography mass spectrometry. [GC-MS]

Experimental

Chemicals and Reagents

All the chemicals and reagents used in this study were of analytical grade and purchased from Sigma Aldrich, Loba Chemie and Merck. Methanol used for extraction purposes was of HPLC grade.

Plant Material

The fresh leaves of *Vigna mungo* were collected from the adjoining farms from Sillod City (Latitude N 20° 19', Longitude E 75° 39') Dist: Aurangabad, Maharashtra, India. The botanical identification of species was carried by late Dr. Narayan Pandhure Associate Professor Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Where herbarium specimen was deposited voucher number MF.2019.VML. Leaves were shade dried until it attains the constant weight and milled into a fine powder (to increase a surface area for extraction process) with the help of an electric blender. Next obtained powder was labeled and stored within a dried, sealed container.

Preparation of Vigna mungo leaves extract

A weighed quantity (40 g) of powder of leaves of *Vigna mungo* was then subjected to continuous hot extraction in the Soxhlet apparatus with methanol (200 ml) at 60°C for 6-8 hrs. The extract was collected and concentrated by evaporation under reduced pressure using a rotary evaporator. The concentrated extracts were stored at 4°C in an airtight container prior to GC-MS analysis.

Preliminary phytochemical screening of extract

After completion of the extraction process extract was subjected to

preliminary phytochemical screening by following standard method. The various phytochemical screening tests were performed in order to establish the chemical profile of the extract.

GC-MS experimental system and measurements

The analyses of phytoconstituents were performed using an instrument Thermo Scientific TSQ-800 GS-MS which was equipped and coupled with silica capillary column TG-5-MS (dimensions-30 m × 0.25 mm, film thickness 0.25 μm). The run time for GC take place 24 minutes. Helium gas with a flow rate of 1 ml/min was used as a carrier. Prior to the commencement of phytochemical analysis procedure the oven temperature was initiated at 60°C for 2 min. After end of this preparatory phase it programmed to increase up to 280°C at the rate of 5°C/min, and later maintained isothermally for 10 min. The temperature of the injector port, ion source, and detector were set at 250°C, 260°C and 280°C respectively. The mass-spectrometric detector was operated in the form of electron impact ionization mode at a fragment of 70eV with a scanning mass range was set at 50-700 (m/z). The database of the National Institute of Standards & Technology (NIST) Library was used to retrieve the names, molecular weight and structures of the components.

Evaluation of antibacterial and antifungal activity

In an antimicrobial study, Microbroth dilution method was employed to determine the minimum inhibition concentration (MIC) of *Vigna mungo* leaves methanolic extract (VMLME). A total of seven pathogens were employed to evaluate the antimicrobial potential of plant extract. All MTCC strains were procured from the Institute of Microbial Technology, Chandigarh. Anti-bacterial activity of plant extract was evaluated using the four bacterial strains two Gram-negative bacteria (*Escherichia coli* MTCC 443 and *Pseudomonas aeruginosa* MTCC 444) and two Gram-positive bacteria (*Staphylococcus aureus* MTCC 96 and *Streptococcus pyogenes* MTCC 442). Five other standard drugs Gentamycin, Ampicillin, Chloramphenicol, Ciprofloxacin and Norfloxacin were also studied for comparing the antimicrobial potential of the extract. The plant extract was analysed for its antifungal activity against three fungus strain namely *Candida albicans* MTCC227, *Aspergillus Niger* MTCC282 and *Aspergillus clavatus* MTCC1323. Two standard drugs nystatin and greseofulvin were applied for the comparison purpose. The experimental design includes growth media; sample preparation and MIC determination were performed as per standard method [6]. To determine the MICs, the plant extract were serially diluted ranging from 6.250 μg/ml to 1000 μg/ml. Muller Hinton Broth and DMSO were used as nutrient medium diluent respectively. The MIC was determined after microscopic evaluation of the culture tube which did not witness any visual growth of the tested organisms and expressed in μg/ml. Each assay was carried out in triplicate and data is expressed as mean value.

Scavenging activity by DPPH assay

DPPH radical scavenging activities for extract were carried out by using a slight modification of the reported method**.** [7] A stock solution of 0.1mM DPPH in methanol was prepared and 1ml of extract solution in water at different concentration (5-50 μg/ml) added to the 0.1 mM DPPH solution. The mixture was shaken vigorously and left in the dark for 30 minutes. The reduction of the DPPH radical was determined by measuring the absorbance at 517 nm. The ascorbic acid was used as standard reference antioxidant. Each assay was carried out in triplicate and data is expressed as mean ± SD, where control group contain no sample. The decrease in optical density of DPPH by addition of tests sample in relative to the control was used to calculate the antioxidant activity. The capacity to scavenge DPPH radical was calculated by following equation:

$\% \text{ Inhibition} = 1 - (A_S/A_0) \times 100.$

Where, A_0 is the absorbance at 517 nm of control and A_S is the absorbance in presence of the sample extract.

RESULT AND DISCUSSION:

Determination of phytoconstituents

Phytochemical analysis of *Vigna mungo* leaves methanolic extract [VMLME] was evaluated and the active ingredients found positive in the phytochemical analysis included sesquiterpenes, fatty acid esters, steroids and alkaloids. In response to this, we selected methanolic extract for further isolation studies using GC-MS.

GC-MS analysis of VMLME

GC-MS remain a valuable tool in natural product research and earned reputation for high-resolution separation and identification of isolated compounds. Volatile components like essential oils, sesquiterpenes are the best candidates of choice and well suited by GC-MS analyses. [8] The GC-MS analyses provided the tentative overall composition of *Vigna mungo* leaves and conveyed valuable information about the most probable major components. The phytochemicals from leaves extract were estimated by GC chromatogram (Figure 1) and MS mass spectra data of the contents is presented in Table 1. VMLME exhibited 20 unique signals and putative empirical formulas of 20 compounds were acquired. The height of the peak that appeared in the GC-MS chromatogram is a reflection of the concentration of a particular compound present in the extract. The chromatogram presented 6 predominant compounds with a high peak as illustrated in Table 1. The prevalent constituents found in extracts were dasycarpidan-1-methanol acetate (21.91%), octadecane, 3-ethyl-5-(2-ethylbutyl) (18.55%), Z-5-Methyl-6-heneicosane-11-one (9.26%), 5,7,9-(11)-Androstatriene, 3-hydroxy-17-oxo (8.15%), 14-hydroxy-14-methylhexadec-15-enoic acid, methyl ester (6.45%), and 12,15-octadecadienoic acid, methyl ester (4.85%). The structures of most abundant bioactive compounds are depicted in Figure 2. Whereas benzaldehyde, 2-nitro-4-trimethylsilyl (0.81%), β-D-Galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic butylboronate (0.95%) and 2, 7-Diphenyl-1, 6-dioxopyridazino-[4,5:2',3']-pyrrolo-[4',5'd]-pyridazine (0.98%) were least abundant in occurrence. The major bioactive compounds recognized belong to the class essential oils. In addition, two alkaloid compound-1 and compound-4 and two sesquiterpenoids compound-5 and compound-15 were also identified. The medicinal value of the plant is the assets of its bioactive compounds present in them. The identified compounds like perhydrocyclopropa[e]azulene-4,5,6-triol, 1,1,4,6-tetramethyl, 9,12,15-octadecatrienoic acid, 2,3-bis-[(trimethylsilyl)oxy]-propyl ester, (Z,Z,Z) (Oleic acid) and monolinoleoylglyceroltrimethylsilyl ether possess anticancer, anti-inflammatory and hepatoprotective functions. [9] The previous study of GC-MS spectrum of root nodule of black grams also reported oleic acid in the plant. [10] Very limited literature is available for the identification of bioactive compounds by GC-MS analyses of *Vigna mungo* leaves.

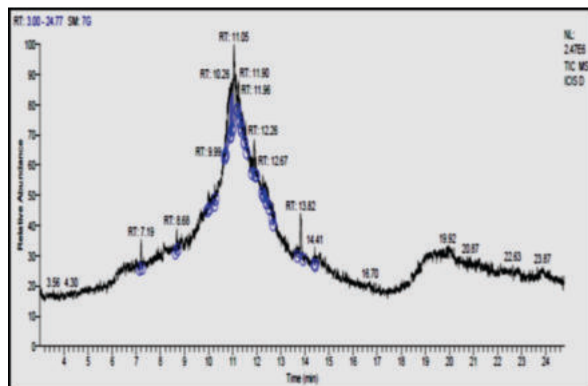
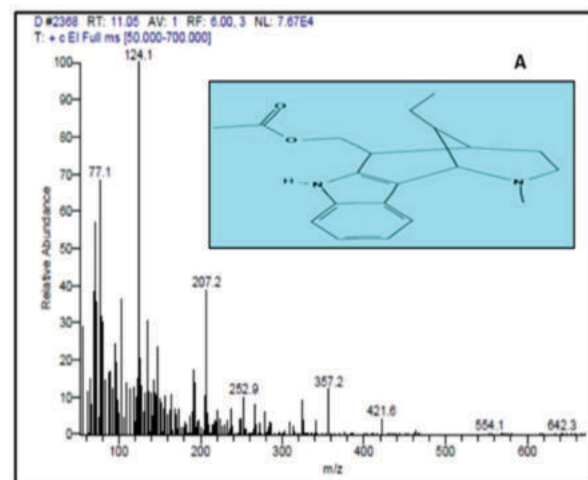
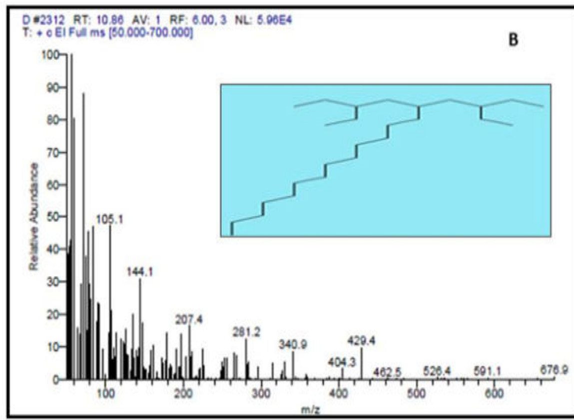


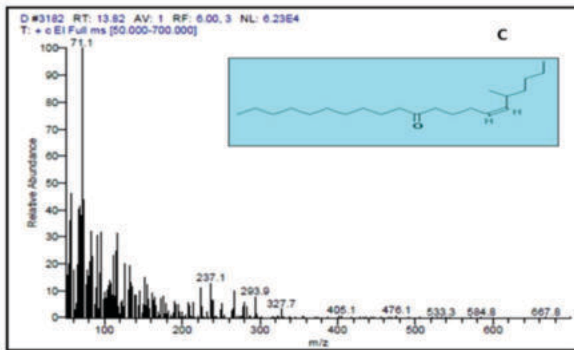
Figure 1: **GC chromatogram of *Vigna mungo* leaves methanolic extract.**



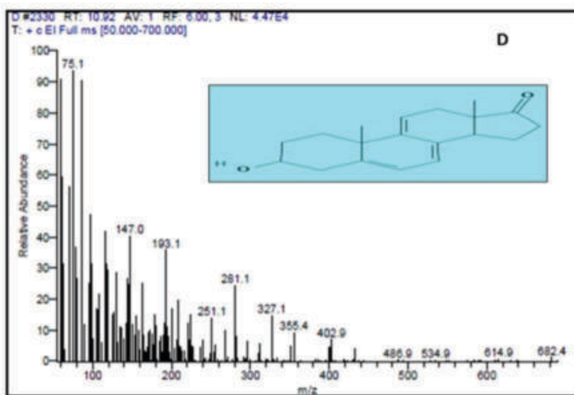
A = Dasycarpidan-1-methanol acetate (ester)



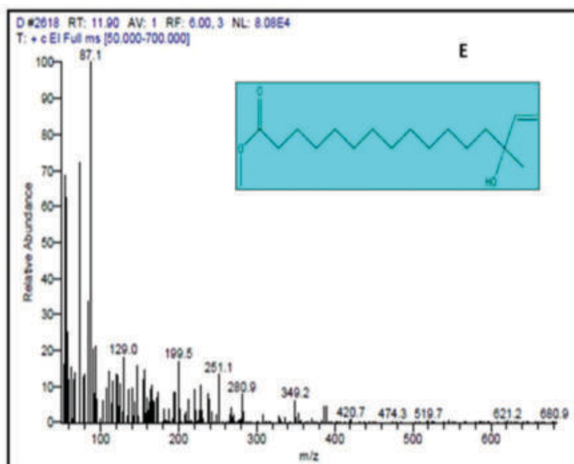
B = Octadecane, 3-ethyl-5-(2-ethylbutyl)



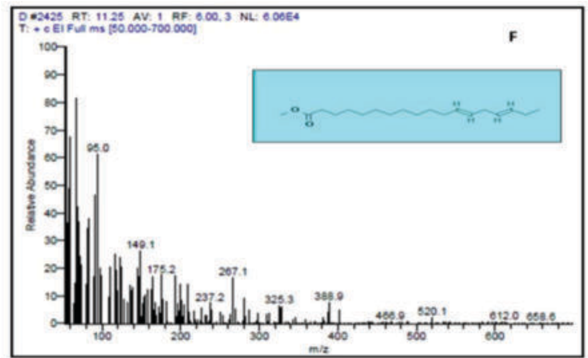
C = Z-5-Methyl-6-heneicosane-11-one



D = 5,7,9-(11)-Androstatriene, 3-hydroxy-17-oxo



E = 14-hydroxy-14-methylhexadec-15-enoic Acid, Methyl Ester



F = 12,15-Octadecadienoic acid, methyl ester

Figure 2: Most Abundant Compounds Found In Vmlme By Gc-ms Analysis.

Anti-microbial analysis

Anti-bacterial activity analysis

The VMLME demonstrated varying anti-bacterial activities by broth dilution method as shown in Table 2. The minimum inhibition concentration (MIC) value of methanol extract (µg/ mL) mentioned in Table 2. There was no significant difference in activity against Gram-positive and Gram-negative bacterial strains. The extract showed the most remarkable antibacterial activity against *E. coli* (MIC: 25 µg/ mL). This was detected to be four times and two times more potent than standard drugs ampicillin and chloramphenicol respectively. Further, showed substantial effect against *P. aeruginosa* (MIC: 100 µg/ mL) and *S. aureus* (MIC: 125 µg/ mL). It was found mild active against S. pyogenes (MIC: 250 µg/ mL). The graph for anti-bacterial activity with MIC and minimum bactericidal concentration (MBC) values of VMLME and standard drugs against studied organisms illustrated in Figure 3.

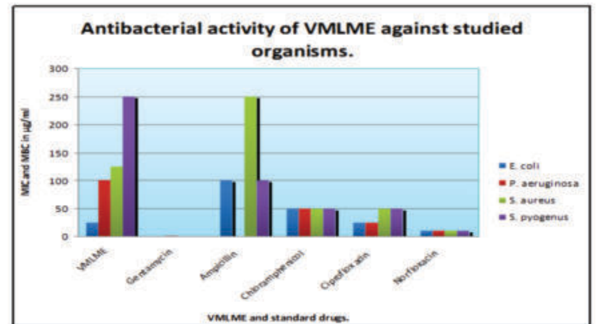


Figure 3 : Graph of MIC and MBC values of VMLME and standard drugs against studied organisms.

Anti-fungal activity analysis

The result revealed that VMLME exhibited notable anti-fungal activity against *C. albicans* with MIC 250 µg/ mL. It was found to be more efficient even in half concentration than standard drug Greseofulvin with a minimum fungicidal concentration (MFC) of 500 µg/ mL. It displayed very weak activity against *A. niger* and *A. clavatus* with MFC 1000 µg/ mL for each. MIC value of extract and MFC values of standard drugs tabulated in Table 3. The graph for anti-fungal activity is shown in Figure 4. Moreover, the presence of identified compounds might be responsible for the anti-microbial activity.

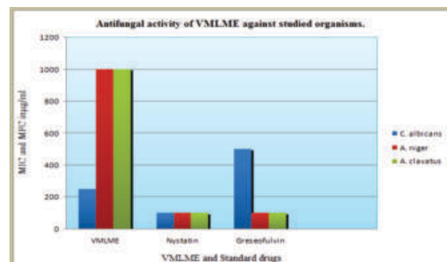


Figure 4 : Graph of MFC and MBC values of VMLME and standard drugs against studied organisms.

Anti-oxidant assay

Free radicals are very reactive species that possess the capabilities to trigger various diseases like cardiovascular, neural disorder, Perkinson's disease, atherosclerosis and aging. [11] Hence, there is an immediate need to explore substances with free radical scavenging activity. In vitro 2,2-Diphenyl-1-picrylhydrazil (DPPH) quenching assay was designed to study the anti-oxidant activity of *Vigna mungo* leaves extract. The free radical scavenging activity is depicted in Table S4. The dose-response curve of DPPH for VMLME was equated with standard reference Ascorbic acid Figure 5. The DPPH method revealed the scavenging of the free radicals was found to be 12.42±0.12 %, 18.63±0.23%, 28.89±0.26%, 38.51±0.18%, 48.65±0.26% and 60.89±0.33% at 5, 10, 20, 30, 40 and 50 µg/ ml. In the DPPH assay, the IC_{50} value of Ascorbic acid was 10.45±0.15% while that of VMLME was 39.60±0.07%. The IC_{50} value of VMLME was found to be satisfactory which is in support of its biological activity.

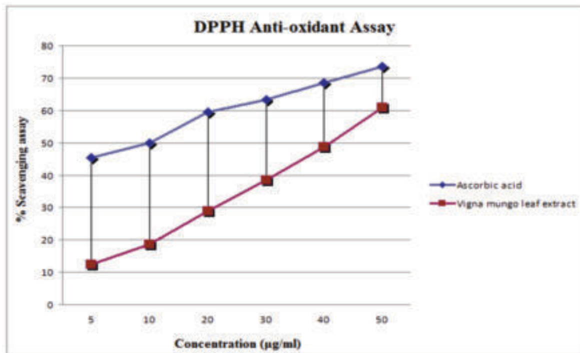


Figure 5: DPPH scavenging activities of sample. Data is expressed as Mean±SD. (n=3)

Table 1: Tentatively identified metabolites from *Vigna mungo* leaves by GC-MS analysis.

Sr. No	Probable compound	RT (Minutes)	Molecular Mass (g/mol)	Molecular Formula	Peak area %	Compound nature
1	(5β)Pregnane-3,20-β diol,14-α,18-α-[4-methyl – oxo-(1-oxa-4-azabutane1,4-diyl)]-,diacetate	7.19	489.6	$C_{28}H_{43}NO_6$	2.87	alkaloid
2	2,5-Dihydroxyacetophenone, bis(trimethylsilyl) ether	8.68	297	$C_{14}H_{24}O_3Si_2$	2.26	ether
3	9,12,15-Octadecatrienoic acid, 2,3-bis-[(trimethylsilyl)oxy]-propyl ester, (Z,Z,Z)	9.99	497	$C_{27}H_{52}O_4Si_2$	2.43	fatty acid
4	2, 7-Diphenyl-1, 6-dioxopyridazino-[4,5:2',3']-pyrrolo-[4',5'd]-pyridazine	10.26	355.3	$C_{20}H_{13}N_5O_2$	0.98	alkaloid
5	5-H Cyclopropa-[3,4]-benz-[1,2e]-azulen-5-one,9,9-abis(acetyloxy)-1,1α,1β,2,4α,7α,7β,8,9,9-adecahydro-2,4a,7-btrihydroxy-3-(hydroxymethyl) 1,1,6,8-tetramethyl,[1αR(1αβ,1βá,2,4αβ,7αβ,7βà,8β,9β,9αβ)	10.70	464	$C_{24}H_{32}O_9$	1.40	sesquiterpenes
6	Octadecane, 3-ethyl-5-(2-ethylbutyl)	10.86	367	$C_{26}H_{54}$	18.55	essential oil
7	5,7,9-(11)-Androstatriene, 3-hydroxy-17-oxo	10.92	284	$C_{19}H_{24}O_2$	8.15	steroid
8	Dasycarpidan-1-methanol acetate (ester)	11.05	326.4	$C_{20}H_{26}N_2O_2$	21.91	acetate ester
9	12,15-Octadecadienoic acid, methyl ester	11.25	294.5	$C_{19}H_{34}O_2$	4.85	fatty acid
10	10,12-Tricosadiynoic acid, trimethylsilyl ester	11.31	419	$C_{26}H_{46}O_2Si$	2.15	fatty acid
11	Decanoic acid, 10-bromo, methyl ester	11.41	265	$C_{11}H_{21}BrO_2$	3.98	essential oil
12	Deoxyspegualin	11.54	387	$C_{17}H_{37}N_7O_3$	2.58	--
13	14-Hydroxy-14-methylhexadec-15-enoic acid, methyl ester	11.90	249	$C_{18}H_{34}O_3$	6.45	fatty acid
14	Benzaldehyde, 2-nitro-4-trimethylsilyl	11.96	223	$C_{10}H_{13}NO_3Si$	0.81	carbonyl compound
15	Perhydrocyclopropa[e]azulene-4,5,6-triol, 1,1,4,6-tetramethyl	12.26	254	$C_{15}H_{26}O_3$	1.72	sesquiterpenes
16	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]1[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)	12.36	497	$C_{27}H_{52}O_4Si_2$	4.29	essential oil
17	Pregnane-3,20-dione,11-[(trimethylsilyl)oxy],bis(Omethyloxime),(5β,11β) 2-Phenanthrenol	12.45	462	$C_{26}H_{46}N_2O_3Si$	3.19	steroid
18	1-Monolinoleoylglycerol trimethylsilyl ether	12.67	498	$C_{27}H_{54}O_4Si_2$	1.25	fatty acid
19	Z-5-Methyl-6-heneicosen-11-one	13.82	322	$C_{22}H_{42}O$	9.26	carbonyl compound
20	β-D-Galactopyranoside, methyl -2,3-bis-O-(trimethylsilyl)-, cyclic butylboronate	14.41	404	$C_{17}H_{37}BO_6Si_2$	0.95	--

RT = Retention time

Table 2: Antibacterial activity of *Vigna mungo* Leaves Methanolic Extract [VMLME]. [MIC and MBC expressed in µg/ml]

Sr.No.	Drug	E. coli	P. aeruginosa	S. aureus	S. pyogenus
	-	MTCC 443	MTCC 441	MTCC 96	MTCC 442
		µg/ml			
1	Gentamycin	0.05	1	0.25	0.5
2	Ampicillin	100	--	250	100
3	Chloramphenicol	50	50	50	50
4	Ciprofloxacin	25	25	50	50
5	Norfloxacin	10	10	10	10
6	(VMLME)	25	100	125	250

Table 3: Antifungal activity of *Vigna mungo* leaf methanolic extract (VMLME). [MFC and MIC expressed in µg/ml]

Sr. No	Drug	C. albicans	A. niger	A. clavatus
		MTCC 227	MTCC 282	MTCC 1323
		μg/ml		
1	Nystatin	100	100	100
2	Greseofulvin	500	100	100
3	(VMLME)	250	1000	1000

Table 4: % DPPH free radical scavenging activity of *Vigna mungo* leaf methanolic extracts. Data is expressed as Mean±SD. (n=3)

Sr. No. \	Concentration (μg/ml)	% Scavenged (Ascorbic acid)	% Scavenged (Vigna mungo leaf methanolic extract)
1.	5	45.46±0.15	12.42±0.12
2.	10	49.96±0.17	18.63±0.23
3.	20	59.48±0.28	28.89±0.26
4.	30	63.26±0.05	38.51±0.18
5.	40	68.47±0.21	48.65±0.26
6.	50	73.44±0.17	60.89±0.33
IC50 Value (μg/ml)		10.45±0.15	39.60±0.07

CONCLUSION

GC-MS analysis revealed that the *Vigna mungo* leaves are rich source of bioactive compounds. In conclusion, vital phytochemicals like essential oils, terpenes, fatty acids, steroids and alkaloids were analysed in the methanolic extract. The extract exhibited excellent anti-microbial activities against *E. coli* and *C. albicans* and displayed satisfactory anti-oxidant potential. This finding validates the therapeutic value of *Vigna mungo*. However, further extensive research is necessary for the enrichment of literature on this plant.

Acknowledgement

Authors are thankful to SAIF Chandigarh for GC-MS analysis of the sample.

Disclosure statement

There was no conflict of interest reported by authors.

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