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Research Article

Chemical Fingerprint by HPLC-DAD-ESI-MS, GC-MS Analysis and Anti-Oxidant Activity of Manasamitra Vatakam: A Herbomineral Formulation

Srikalyani V[1], Ilango K[1,2,*]

ABSTRACT

Background: Manasamitra Vatakam is a classical ayurvedic herbo mineral formulation used for the treatment of neurodegerative properties and epileptic disorders. The wide range mixture of herbal extracts and minerals were used in the formulation. **Aim:** The aim of the study implies in performing the chemo-profiling, chromatographic fingerprint analysis by HPLC-DAD-ESI-MS for the selected formulations of Manasamitra Vatakam followed by the identification of bioactive compounds by Gas Chromatography – Mass Spectrometric (GC-MS) analysis, to evaluate the diffusion and dilution methods for the determination of anti-bacterial activity in the methanolic extracts of Manasamitra Vatakam (MMV). **Materials and Methods:** The antibacterial activity was performed by both diffusion and dilution methods whereas the antioxidant activity was performed by free radical scavenging of 2,2-diphenyl-1-picrylhydrazy and hydrogen peroxide scavenging assay method. **Results:** The estimation of bioactive constituents showed positive results by qualitative analysis. Antibacterial activity of MMV was evaluated against two-gram positive *Staphylococcus aureus* and *Bacillus cereus*, two gram negative *Escherichia coli* and *Klebsiella pneumonia* by disk diffusion (0.078-10µg mL^{-1}), broth dilution (0.078-10µg mL^{-1}) and broth micro dilution method (0.39-50µg mL^{-1}) respectively. The bioactive constituents were analysed by GC-MS analysis for the methanolic extract of the formulation. **Conclusion:** To conclude, the formulation was found abundant with phenolic and flavonoid compounds by HPLC-ESI-MS analysis, the bioactive compounds identified are responsible for the anti-bacterial activity. The broth microdilution method performed by resazurin method was observed as the fast screening, sensitive and accurate method for the quantitative determination of anti-bacterial activity.

Key words: Classical formulation, Phytochemicals, MIC, Diffusion and dilution methods, Heavy metals.

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INTRODUCTION

Globally, India is the largest producer of medicinal herbs used for enormous aliments in day to day practise. In the traditional medicinal systems, the whole plant is used as the alternative and complementary therapy.[1] Medicinal plants have been the mainstream of traditional herbal medicines worldwide since the antiquity to date.[2] About 1/4^{rth} of the pharmaceutical industries have structural modifications of natural products for the advancement of drug discovery and development. The non-nutrient compounds or the bioactive constituents referred to as "phytochemicals" are responsible against various microbial infections and hence the study of natural products is known as "Phytochemistry".[3] Even, the pharmacological activity of the crude extract is determined by the nature of phyto constituents present in the plant species and these compounds are responsible for the potential activity are known as "Secondary metabolites" like alkaloids, flavonoids, isoprenoids, saponins exerts antioxidant, steroidal activity, modulation of hormones and detoxification of enzymes and anti-cancer properties. Hence, plants are the major source of mankind throughout the history.[4,5] With the rise in the clinical toxicity, the use of ayurvedic formulation is enhancing significantly over the decade.[6] The standardization of botanicals for the specific markers was confirmed with the fingerprinting analysis. Ayurveda is considered as "Mother of healing", is the integral part of the traditional medicine claims to aid the various metabolic and chronic disorders by enhances the longevity.[7]

Manasamitra Vatakam (MMV) is a herbomineral formulation, a Classical traditional medicine from the antique Ayurvedic formulations cited in Sahasrayogam, potentially used for memory impairment, neuroprotective activity and manic illness.[8] MMV contains a composite mixture of 55 plant species of varying therapeutic activities like anxiolytic, anti-depressant, Antioxidant and anti-epileptic activities.[9] The minerals present in MMV are Pravala pishti, Tamrachuda Padika, Rajata Bhasma, Swarna Bhasma, Mrigashringa Bhasma, Makshika Bhasma, Mukta Pishti, Loha Bhasma and Shilajat. 70-80% of the nutrient consumptions were proven to be obtained from the vegetable crops in the form herbal supplements claims to contain the heavy metal and the trace elements in it. The macro and

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microelements are used for various biochemical processes in the human body reveals to have a significant effect on combating various ailments. In parallel to which, with the increase in adverse drug events from the synthetic drugs many of the populations finds the herbal drugs are of safe and intoxic with lesser amount side effects. But, upon the regular usage of these herbal formulations results in the nephrotoxicity because of the contaminants, heavy metals and adulterants used.

Chemo-profiling and marker analysis plays a critical role in the herbal drug for ensuring the therapeutic efficacy, pharmacokinetic profile.[10,11] The profiling and fingerprinting of phytoconstituents explores the plant analysis better and is the best alternative for the classical analytical methodology. Nevertheless, it helps to differentiate the species are of authentic or adulterated by its origin and allows for the proper discrimination between the species or the herbal medicines. LC-MS fingerprinting analysis can be laid as a platform for the additional testing to identify the fingerprinting analysis as it emphasizes on the systematic characterization of herbal compositions. No methodology has stated for the qualitative or quantitative estimation of the MMV for the quality control of the herbs, hence from the quality aspects of the classical formulation the fingerprinting analysis was performed to be the emerging need for its identification. The HPLC-DAD-ESI-MS analysis was performed for the fingerprinting analysis and further confirmation of the constituents were determined qualitatively. GC-MS is the versatile analytical technique which is unsuited to rapid high sensitivity analysis of specific compounds, represents the mass of specific particle (Da) to number (z) of electrostatic changes (e).[12] The inborn error of metabolism in new-born are detectable, isotopic labelling of metabolites helps in metabolic activity. In traditional medicine, GC-MS is the feasible technique for the analysis of the liposoluble compounds like volatile or essential oils.[13]

The Minimum inhibitory concentration (MIC) is the minimum (lowest) concentration of a test compound that will inhibit the growth of a bacterial strain. It can be done in several methods like micro dilution or macro dilution test, extended break point sensitivity test and e-strip test methods. Conventionally, this is determined using a series of doubling dilutions of the antibiotic in liquid culture medium, to produce a range of concentrations in test tubes (macro dilution) or in a microtiter tray (micro dilution). After inoculation of the test strain into each antibiotic concentration, bacterial growth is determined by visible turbidity after 18–24h of incubation. The MIC is the lowest concentration of compound with no visible bacterial growth.[14,15]

In the present study, four different marketed formulations of MMV were chosen, and analyzed for the estimation of bioactive constituents, heavy metal analysis for the determination of elemental and trace analysis, HPLC-DAD-ESI-MS fingerprinting analysis. Based on the results, comparative profiling was studied for all the four formulations of MMV, of all the formulations the formulation found to be comparatively better was subjected to GC-MS analysis to study the possible volatile compounds present and also the evaluation of antibacterial activity was studied by both the diffusion and dilution techniques in the herbo mineral formulation.

MATERIALS AND METHODS

Materials

All the chemicals of analytical grade were procured and used for the analysis. HPLC grade Methanol 99.9% (Merck Specialties' Pvt. Ltd., Mumbai, India), Milli-Q water (Milli-Q10 TS, Millipore water Purification system, Merck) was used. Formulations A, B, C and D of MMV were prescribed by Ayurvedic Physician and procured from Ayurvedic pharmacy. Quercetin, glucose, gallic acid, ursolic acid, diosgenin were purchased form M/s Natural remedies, Bangalore. Cefotaxime susceptibility test discs (SD040-1VL) were procured from HIMEDIA lab Pvt. Ltd.

Sample preparation

A pooled mix of tablets of twenty was finely weighed and powdered. 1 g of powdered mix was transferred into a 10 mL standard flask. Sample extraction was exerted by adding 5 mL of methanol followed by sonication for about 30min and made to the mark with the diluent. The sample matrix prepared was then subjected to prior filtration with Whatmann filter paper and followed by 0.2 µm membrane filter. The filtered solution was used to perform the HPLC-DAD-ESI-MS and GC-MS analysis.

Estimation of bioactive constituents

Bioactive compounds often termed as secondary metabolites are essential for eliciting the pharmacological effects. Besides the primary metabolites, secondary metabolites are the compounds regarded as "biological side tracks" functioning of the plant. Irrespective of the pharmacological activity they themselves help in shielding the plant as a whole i.e., flavonoids protect against free radicals during photosynthesis, terpenoids attract pollinators and inhibit the competing plants, and alkaloids rid the phytoalexins and herbivore animals. However, exceptionally few plants are highly poisonous due to the increased concentration of secondary metabolites. The determination of the bioactive contents i.e., alkaloids, flavonoids, glucoside, phenolic, saponins, sterol saponins, sterols and terpenoids were estimated in all the four formulations of MMV.[16-20]

In vitro antioxidant activity

The Antioxidant activity was performed by DPPH (2,2-diphenyl-1picrylhyrazyl) free radical scavenging activity. 0.1mM of DPPH was prepared using methanol as vehicle from which 1mL was added to the formulation extract diluted to various concentrations. The aliquots were incubated in the dark at room temperature for 30min and the absorbance was recorded at 517nm, where ascorbic acid was kept as positive control.[21] The samples were performed in triplicated and expressed as % incubation percentage. The IC50 value was calculated using Graph pad prism.

Elemental analysis

The glassware and Teflon tube used for digestion were soaked in dilute nitric acid and rinsed with Milli-Q-water. All the standard solutions were made by diluting 1000 mg/L of stock solutions (Merck). For the estimation of Hg, As and Se 5%v/v HCL was used for both standards and sample preparations, Whereas Milli-Q-water was used as diluent for Zn, Cr, Ni, Cu, Fe, Na, Pb and Cd standards and sample analysis.[22] Elemental analysis was carried out by using atomic absorption spectroscopy (AAS) system (AA 7000 AAS, Lab India Instruments Pvt Ltd).

The sample was subjected to digestion in microwave digestor as per EPA Method 3052,[23] 1996 with slight modification in the methodology. Briefly, 0.5 g of the formulation in powder form was taken in a digestor holder of 100 mL capacity to which 5 mL of concentrated HCl and 5 mL of HNO_3 was added, mixed well and digested for a period of 30 min. The mixture was then transferred to a 100mL volumetric flask, diluted with small volumes of Milli-Q-water to avoid splashes of acid matrix, filtered with Whatmann No.1 and then made up to mark with Milli-Q-water. The Sample obtained was taken up for analysis and also a blank digest was conducted in the similar way. Flame absorption method (FAM) was used to determine the concentration of Zn, Cr, Ni, Cu, Fe, Na, Ca, Pb and Cd using air-acetylene as carrier gas. Hg, As and Se concentration was estimated by hydride generation method (HG-AAS) equipped with AAA7000. 5% v/v HCl was used as carrier liquid

2% sodium borohydride ($NaBH_4$) in 0.5 % (w/v) sodium hydroxide (NaOH) was used as reducing agent. Milli-Q-water and 5% v/v HCl was used as diluents for FAM and HG-AAS method respectively.

Instrument calibration was performed with a blank solution made to autozero followed by standardization with different working standards of different calibration sets. Standard samples were analysed prior to the samples to plot the calibration curve. The analysis was carried out in triplicate and the absorbance was measured.

INSTRUMENTATION AND CHROMATOGRAPHIC CONDITIONS

HPLC-DAD-ESI-MS analysis

The HPLC system comprises of a Shimadzu binary solvent delivery module (LC10ADVP), SPD M20A PDA detector and manual Rheodyne injector with the loop capacity of 0.02mL loop volume and column oven CT0-20A on the whole controlled by the communication module CBM-20A. The chromatographic separation was achieved using Phenomenex C_{18} column (25cm x 4.6mm; 5µ) and the solvent system of water-formic acid (A; 100:0.1% v/v) and methanol (B) at 45:55 v/v with an isocratic flow of 0.8mL/min. The detection was set at 254nm.[24-26].

The separation was made on an (G1) LC-MS 2020 system equipped with single quadrupole mass spectrometer with electrospray ionization ESI (+) source with the positive ionization mode. The temperature was set at 280°C and 320°C for curved desolvation line (CDL) and heat block. The interface parameters as; nitrogen gas was used to assist nebulization with a flow rate of 1.5 mL/min. The drying gas flow was maintained at 15L/min.

GC-MS analysis

For the identification of the chemical compounds, the methanolic extract was subjected to analysis on Agilent GC-MS system (GC 7890B equipped with Agilent 5977A MSD mass detector) using HP_5 Phenyl Methyl Silox (60°C-325°C) 30m×250mm×0.2µm capillary as stationary phase. The oven temperature programming employed was with the initial temperature of 80°C for 1min, raised for 70°C linearly to 220°C and held for 5min, followed with the linear raise of 10°C/min to 290°C and held for 10min. the injector port was maintained at 290°C, helium gas was used as carrier gas with a flow rate of 1.2mL min^{-1}. Samples were given through split less mode of 1µL injection volume i.e., in the ratio of 1:10. The ionization voltage of 70ev and ion source temperature was kept at 280°C. The injection was performed by split mode with a split ratio of 10: 1. Solvent delay time was set for 3 min for all samples generated by different methods. MSWS V 8.0 workstation was used to process data. Interpretation on mass spectrum of GC-MS was done using the database of in-built libraries like NIST 8 (National Institute of Standards and Technology) and WILEY 9 having more than 62,000 patterns.[27]

Bacterial strain and growth conditions

The test bacterial strains include the gram positive *Staphylococcus aureus* and *Bacillus cereus,* gram negative *Escherichia coli* and *Klebsiella pneumonia*. The test microorganisms were obtained from MTCC. The bacteria were revived and maintained in nutrient broth at 35°C in a shaking incubator.

Disk diffusion method

To perform the diffusion assay method, nutrient agar medium and petri plates were sterilized in autoclave and followed by solidification of the medium in the laminar air flow hood. The assay for the antibacterial activity of each culture was diluted with agar medium to 10^{-5} to 10^{-6} CFU mL^{-1}. 1mL of each bacterial suspension was uniformly spread on the growth medium in the petri plate to which sterile paper disk 6mm were placed on the agar plate and were impregnated with 10ul of the sample. The plates were incubated for 24h. Disk impregnated with the antibiotic cefotaxime 30µg/disc serves as positive control and replicate for each concentration was performed.[28]

Broth dilution assay

5mL of sterile nutrient broth medium was taken into the test tubes for the proper growth of the medium. Serial dilutions in the nutrient broth were performed using the sample concentration of 10 mg/mL to 0.078mg/mL to which loop full of microbial suspension was inoculated. All the inoculated tubes were incubated at the temperature of 37°C for 24h. Following the incubation of the broth tubes, were examined for the presence of microbial growth. The antibacterial activity in terms of MIC was the lowest concentration of the suspension where no viability was observed after 24h of incubation. The MIC was recorded by taking the optical density values of each concentration of the test inoculum at which the bacteria failed to grow or the lowest concentration which inhibits the growth of bacteria from the cell suspension after the incubation of 37°C for 24h. The broth tubes were interpreted by measuring the OD values using UV-Spectrophotometer. The entire test was performed in duplicate.[29]

Broth microdilution assay

The resazurin based dilution method is the most appropriate method for the determination of MIC values for the anti-bacterial activity, as the possibility was enhanced for two-fold dilution of the anti-bacterial agent with the smaller volumes using 96-well microtituration plate. *Staphylococcus aureus* and *Bacillus cereus* (Gram-positive), *Escherichia coli* and *Klebsiella pneumonia* (Gram-negative) were grown on Nutrient agar by streak plate technique. After 48h, the individual colonies were picked and inoculated in the nutrient broth. Both the cultures were monitored for growth using a UV-1800 spectrophotometer (Shimadzu, Japan) after 24h at a wavelength of 600 nm to obtain a final OD 1.0. These liquid cultures were used for further inoculation on 96-well microtituration plate. After well mixing without any agitation the 96-well microtituration plate was cautiously incubated under 35 ± 2° C for 20h (Table 3). Eventually, the resazurin solution was prepared with the concentration of 6 mg mL^{-1} with sterile water as vehicle and the mixture was vortexed to ensure for the homogenous solution.

RESULTS AND DISCUSSION

Phytochemical analysis

Nonetheless, in all the four formulation A, B, C and D the composition of the plants is similar to as cited in Sahasrayogam, but the manufacturing and processing varies results in change in the presence of active constituents thereby results in the variation of the active principles present in the formulation. The methanolic extracts of the formulation A, B, C and D were subjected to bioactive estimations i.e. total alkaloid, flavonoid, glycoside, saponin, steroidal saponin and terpenoid content and the results were given in Figure 1 and depicted in the Table 1. From the phytochemical analysis, the determination of the major bioactive content was identified for all the formulations. Indeed, the formulation B has shown better availability of the bioactive contents followed by formulation D, A and C. The long-term utilization of the herbal medicines results in the deposition of the trace elements and the heavy metals in the human body resulting in the potential risk and it also depends on the daily dietary intake of the herbal medicines. Hence, the heavy metal and trace elemental analysis was performed and was present to be in varied amounts in all the formulations but Was found to be present in permissible limits and the results were listed in Table 2. From, the HPLC chromatograms of all the four formulations were submitted to LC-MS analysis.

Table 1: Estimation of phytochemical constituents in Manasamitra Vatakam of various formulations.

Quantitative parameters	Formulation A	Formulation B	Formulation C	Formulation D
Alkaloid	41.7 ± 0.0009	50.3 ± 0.0024	35.7 ± 0.0007	40.1 ± 0.0003
Flavonoid	39.60 ± 0.003	47.40 ± 0.003	31.80 ± 0.003	45.2 ± 0.003
Glycoside	10.7 ± 0.0052	23.1 ± 0.0140	6.6 ± 0.0048	12.9 ± 0.0083
Phenolic	88.90 ± 0.0014	86.10 ± 0.0031	67.90 ± 0.0010	81.1 ± 0.0017
Saponin	106.9 ± 0.0032	149.8 ± 0.0038	94.2 ± 0.0026	136.2 ± 0.0056
Sterol Saponin	54.9 ± 0.2117	86.65 ± 0.0510	35.5 ± 0.0328	78.4 ± 0.0746
Terpenoid	23.3 ± 0.0091	44.6 ± 0.0129	17.7 ± 0.0212	32.6 ± 0.0146

Values are in mean ± SD, n=3, phenolic equivalent to gallic acid, flavonoid equivalent to quercetin, glycoside equivalent to glucose, saponin, sterol saponin equivalent to diosgenin, terpenoid equivalent to ursolic acid.

Table 2: Concentration range (mg kg^{-1}) of Heavy metal and trace elements present in MMV of various formulations.

Elements	Formulation A	Formulation B	Formulation C	Formulation D	Limits
Arsenic (As)	ND	ND	ND	ND	NMT 3 ppm
Cadmium (Cd)	0.027	0.032	0.040	0.039	NMT 0.3 ppm
Mercury (Hg)	ND	ND	ND	ND	NMT 0.5 ppm
Lead (Pb)	1.231	1.744	1.979	2.681	NMT 10 ppm
Calcium (Ca)	2.385	1.184	2.210	1.557	NMT 15ppm
Copper (Cu)	0.275	0.074	0.865	0.115	NMT 40 ppm
Iron (Fe)	12.25	9.48	21.177	12.074	NMT 55 ppm
Potassium (K)	0.955	0.497	1.566	0.686	NMT 20 ppm
Selenium (Se)	ND	ND	ND	ND	NMT 2 ppm
Sodium (Na)	2.872	1.151	2.863	1.308	NMT 10 ppm

HPLC-DAD-ESI-MS fingerprinting analysis

LC-MS fingerprinting analysis and sown in Figure 3; the process of selection includes the identification of common peaks, normalization of their retention times and determination of the mass value for all the common peaks present in the formulations. Out of all the peaks, 11 peaks are termed as common peaks as they are present in all the four formulations. ESI-MS was performed to further confirm the fingerprints of the peaks identified. Analysis was performed with both the positive and negative ionization modes. On comparison with the m/z values all the peaks were unambiguously identified and were detected as Berberine, jatrorrhizine, curcumin, vitexin, rutin, quercetin, catechin, piperine, palmatine, naringenin, and ellagic acid respectively and the compounds were listed in the Table 3. All the formulations have shown similar fingerprinting pattern and molecular mass. But, formulation B has showed higher peak abundance and the variations caused may be difference in the preparation, manufacturing, drying process and the storage conditions. Upon the comparative note, the order of phytochemical, heavy metal analysis and HPLC-DAD-ESI-MS for the formulation Manasamitra Vatakam analysis was found to be Formulation C < Formulation A < Formulation D < Formulation B.

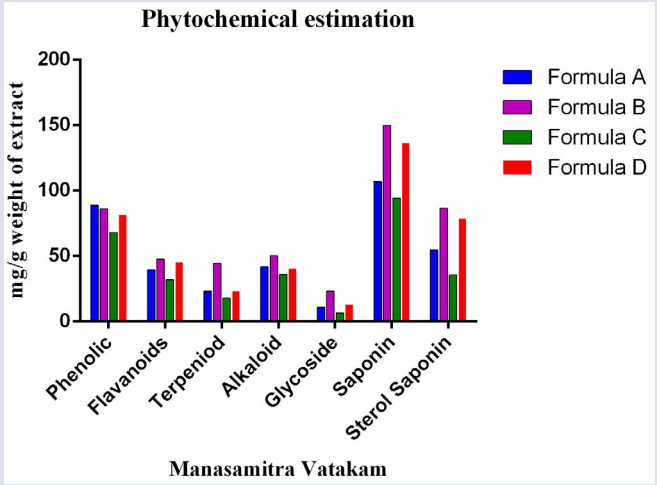

Figure 1: Comparative Profiling of Bioactive constituents in MMV of various formulations.

GC - MS analysis

For the better understanding of the secondary metabolites present, formulation B was subjected to GC MS analysis and the GC MS chromatogram was depicted in Figure 4 and tabulated in Table 4. The results appertain to GC MS analysis for the methanolic extract of formulation B, Manasamitra Vatakam lead to the identification of numerous compounds. The compounds interpreted from the mass spectra are Cyclopropyl carbinol, Camphor (levo), Isobornyl alcohol, Safranal, Decanoic acid, Dodecanoic acid, Ascaridole epoxide, Cuminic acid, Undecanoic acid, 10-methyl-, methyl ester, Benzoic acid, Lauric acid, 4-Octadecenal, Diethyl Phthalate, α- Asarone, 2-Naphthalenemethanol, Ar-tumerone, β-bisabolol, β-Asarone, Methyl tetradecanoate, Isocalamendiol, 3,4,5-trimethoxyphenyl-2-propanone, Tetradecanoic acid, Tricyclo(5.1.0.0(2,4))oct-5-ene-5-propanoic acid, 3,3,8,8-tetramethyl-, β-Eudesmol, n-Pentadecanoic acid, 13-Heptadecyn-1-ol, 5(1H)-Azulenone, Phthalic acid, Phytol,

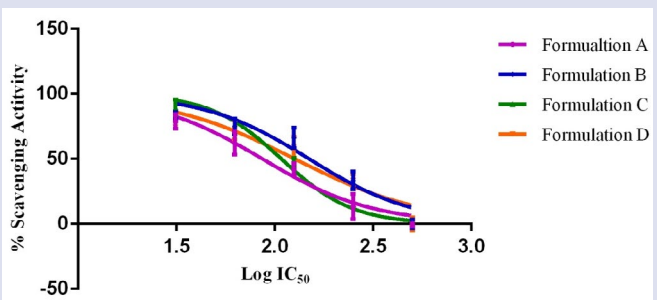

Figure 2: Antioxidant activity with DPPH assay.

α-Copaen-11-ol, Naphthalene, Benzenepropanoic acid, Eudesma-5,11(13)-dien-8,12-olide, naphthalenone, n-Hexadecanoic acid, 18-Hydroxy-10-pentyl-11-oxa-1,5-dithia-spiro(5.13)nonadec-15-yn-12-one, Hexadecanoic acid, Heptadecanoic acid, 9,12-Hexadecadienoic

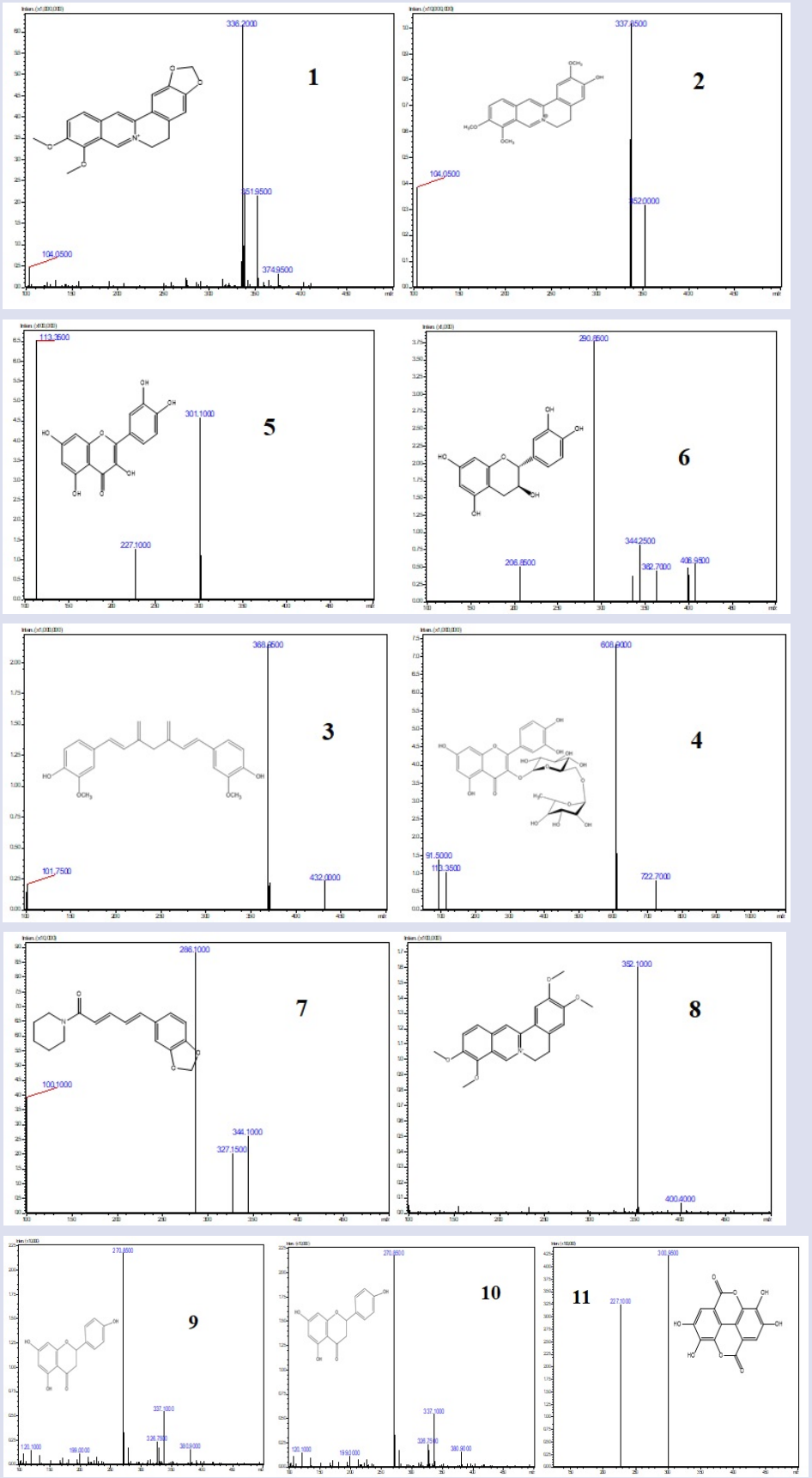

Figure 3: The characteristic compounds identified through mass spectrum by HPLC-DAD-ESI-MS analysis were bereberine (1), jatrorrhizine (2), curcumine (3), vitexin (4) rutin (5), quercetin (6), catechin (7), piperine (8), palmatine (9), naringenin (10) and ellagic acid (11).

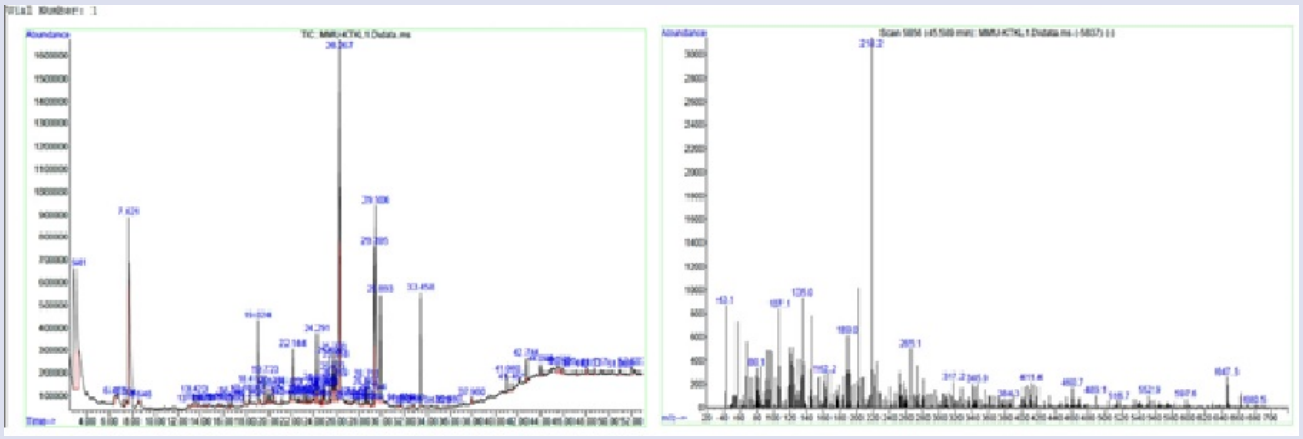

Figure 4: GC-MS chromatogram of Methanolic Extract of MMV (Formulation B).

Table 3: Compounds identified in MMV by HPLC-DAD-ESI-MS.

Peak No	Formulation A	Formulation B	Formulation C	Formulation D	m/z value	Molecular Formula	Identification
01	3.107	3.201	3.112	3.136	336.05	$C_{20}H_{18}NO_4^+$	Berberine
02	3.492	3.492	3. 499	3.480	338.92	$C_{20}H_{20}NO_4^+$	Jatrorrhizine
03	3.607	3.605	3.606	3.597	367.05	$C_{21}H_{20}O_6$	Curcumin
04	4.357	4.283	4.513	4.214	286.21	$C_{15}H_{10}O_6$	Vitexin
05	5.140	5.157	5.103	5.113	609.21	$C_{27}H_{30}O_{16}$	Rutin
06	ND	6.082	ND	6.124	301.26	$C_{15}H_{10}O_7$	Quercetin
07	7.979	7.902	7.972	7.988	289.85	$C_{15}H_{14}O_6$	Catechin
08	9.495	9.610	9.095	ND	286.10	$C_{17}H_{19}NO_6$	Piperine
09	10.028	10.173	ND	ND	357.20	$C_{21}H_{25}NO_4$	Palmatine
10	15.896	16.141	15.504	15.740	271.05	$C_{15}H_{12}O_5$	Naringenin
11	17.266	17.337	17.455	ND	300.25	$C_{14}H_6O_8$	Ellagic Acid

Table 4: Compounds identified in formulation B of MMV by GC-MS.

Peak No	Retention time	Area	Molecular Formula	m/z value	Compound name
1	7.621	4.624	$C_{10}H_{18}O$	154.21	Isobornyl alcohol
2	19.024	2.773	$C_{12}H_{16}O_3$	208.10	Asarone
3	24.291	2.413	$C_{19}H_{28}O_4$	320.19	Phthalic acid
4	25.406	1.288	$C_{17}H_{34}O_2$	270.25	Palmitic acid
5	25.918	1.726	$C_{15}H_{22}O$	218.16	naphthalenone
6	26.267	18.828	$C_{16}H_{32}O_2$	256.24	n-Hexadecanoic acid
7	29.395	6.613	$C_{18}H_{32}O_2$	280.24	9,12-Octadecadienoic acid (Z,Z)-
8	29.506	9.938	$C_{18}H_{34}O_2$	282.25	trans-13-Octadecenoic acid
9	29.893	3.3860	$C_{18}H_{36}O_2$	289.13	Octadecanoic acid
10	32.693	1.215	$C_{29}H_{50}O$	414.38	γ-Sitosterol
11	33.458	4.181	$C_{18}H_{36}O_2$	284.27	Dihydrolycorine

acid, Linolelaidic acid, cis-13-Octadecenoic acid, Octadecane, 3-ethyl-5-(2-ethylbutyl)-, Methyl stearate, 9,12-Octadecadienoic acid (Z,Z)-, trans-13-Octadecenoic acid, Octadecanoic acid, 8-Pregnene, 3-acetoxy-20-hydroxymethyl-4,4,14-trimethyl, Methyl octadec-6,9-dien-12-ynoate, β-Sitosterol, γ-Sitosterol, Dihydrolycorine, 3,19:14,15-Diepoxypregnan-20-one, 3,11,18-triacetoxy-, 3Beta-acetoxy-6-nitroandrost-5-en-17-one, Labda-8(20),12,14-trien-19-oic acid, methyl ester, Isobornyl 3-methylbutanoate, Terephthalic acid, bis(2-ethylhexyl)ester, Squalene, 9-Desoxy-9x-chloroingol 3,7,8,12-tetraacetate, psi-Cholesterol, Tocopherol, Campesterol, Stigmasterol.

Antioxidant activity

The free radical scavenging activity was determined by DPPH assay activity. The activities were analysed statistically by graph pad prism and the IC50 value was calculated. Of all the four formulations, formulation B showed better Antioxidant property on a comparative note. The formulation was evident to have rich in flavonoids and phenolic compounds which have direct inhibitory activity on the free radical mechanism and hence mitigates the Antioxidant nature thereby ceasing the oxidation. The effect free radical scavenging activity was depicted in Figure 2.

Antibacterial activity

The bacterial strains Staphylococcus aureus (9 mm) and Bacillus cereus (11 mm) and Escherichia coli (7 mm) and Klebsiella pneumonia (9 mm) showed zone of inhibition i.e., the MIC values were found to be 5.0 µg mL-1 for gram positive and 5.0 and 2.5 µg mL-1 for gram negative bacteria respectively and were shown in Figure 5. For the determination of the antibacterial activity of the plant extracts it is evident that the disk

diffusion method could not be persistently reliable method and hence it was performed by agar dilution method where the anti-bacterial activity was shown at the lower concentration on a comparative note. The evaluation of the present study includes disk diffusion, broth dilution assay and broth microdilution assay and was depicted in Figure 6 and 7. Where the broth microdilution assay was performed using resarzurin to enhance the detection of the bacterial growth. The extent of bacterial activity was determined based on the intensity of colour i.e., the blue coloured fluorescence indicated the effect of anti-bacterial nature of the sample whereas the pink indicates mild to moderate activity. The MIC values were found to be 0.390 and 0.781 µg mL-1 for the gram positive stains i.e., *Staphylococcus aureus* and *Bacillus cereus* and the 6..250 and 3.125 µg mL-1 gram negative bacteria i.e., *Escherichia coli and Klebsiella pneumonia* respectively and showed better quantitative determination of the bacterial activity than disk diffusion method and broth dilution assay were shown in Table 5.

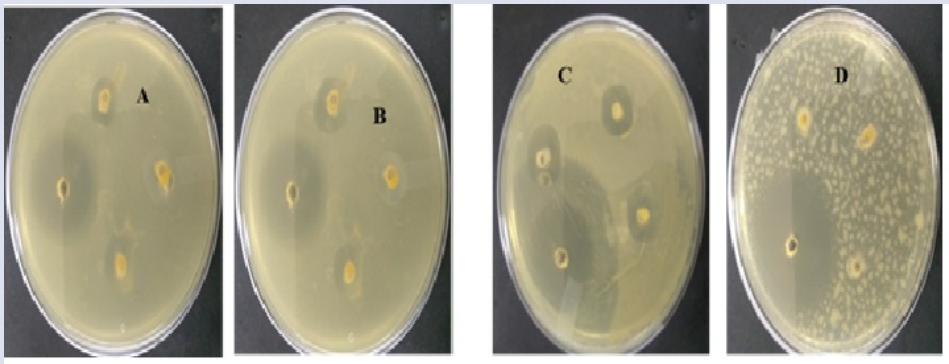

Figure 5: Disk Diffusion method for Gram Positive *Staphylococcus aureus* (A), *Bacillus cereus* (B) *and Gram Negative Escherichia coli* (C), *Klebsiella pneumonia* (D) at 2.5,5 and 10µg mL^{-1} concentration.

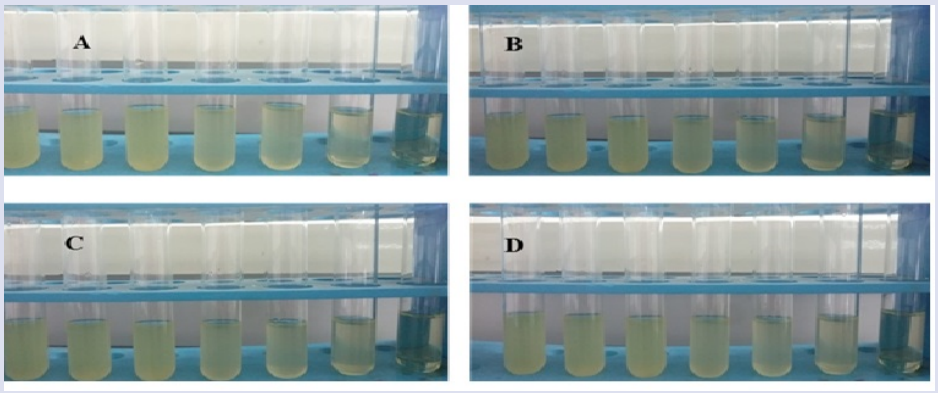

Figure 6: Broth Dilution Assay method for Gram Positive *Staphylococcus aureus* (A), *Bacillus cereus* (B) *and Gram-Negative Escherichia coli* (C), *Klebsiella pneumonia* (D) at 10 mg mL^{-1} to 0.15625mg mL^{-1} concentration.

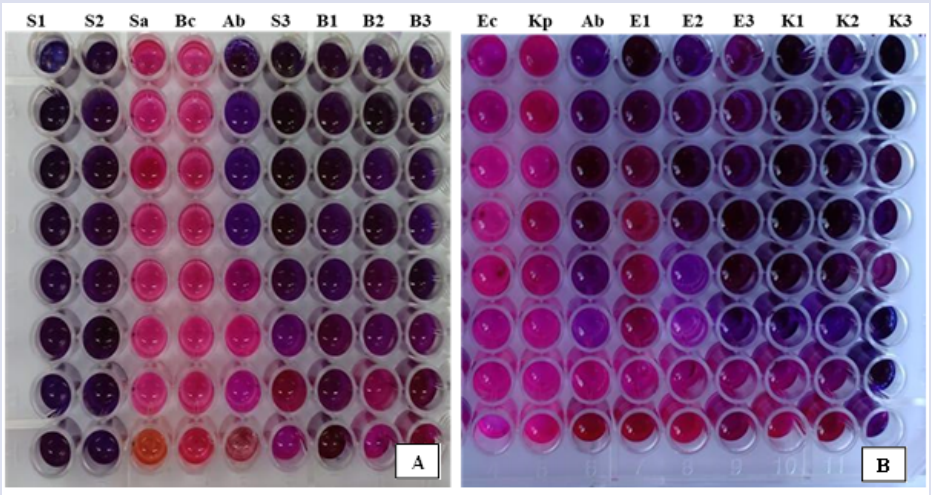

Figure 7: Broth Microdilution Assay method for Gram Positive *Staphylococcus aureus, Bacillus cereus* (A) *and Gram Negative Escherichia coli*, *Klebsiella pneumonia* (B) at 10 mg mL^{-1} to 0.07812 mg mL^{-1} concentration (each in triplicate).

Table 5: Minimum inhibitory concentrations of MMV against gram positive and gram-negative stains.

Type of assay	Growth Medium (ug mL^{-1})			
	Bacterial Stains			
	Bacillus cereus	Staphylococcus aureus	Escherichia coli	Klebsiella pneumonia
Disk Diffusion	5.0	5.0	5.0	2.5
Broth Dilution	5.0	5.0	5.0	5.0
Broth Microdilution	0.781	0.3906	1.562	0.781

CONCLUSION

To the best of our knowledge, the study provides the first HPLC-DAD-ESI-MS and GC-MS analysis with antibacterial activity with the major primary and secondary metabolites. The phytochemical analysis proves the formulation B contains major bioactive constituents. The elemental analysis inferred the presence of heavy and the trace elements were found to be in lesser quantity in formulation B on comparison with the others. The HPLC-DAD-ESI-MS analysis revealed the major amounts of alkaloids, flavonoids and the phenolic moiety in all the formulations. In relevance to the phytochemicals identified in HPLC-MS analysis the Antioxidant activity exerted was anticipated due to the presence of phenol and flavonoids .Based on this data, formulation B was selected for GC-MS analysis revealed the major bioactive compounds like asarone, pthalic acid, hexadecanoic acid and octadecanoic acid which are majorly mitigating the anti-bacterial activity. The presences of phytochemicals like α and β asarone, γ-sitosterol, tocopherol may be responsible for the neuroprotective mechanisms. With this inference it was proved that the herbomineral formulation Manasamitra Vatakam showed anti-bacterial activity against both the gram positive and the gram negative stains.

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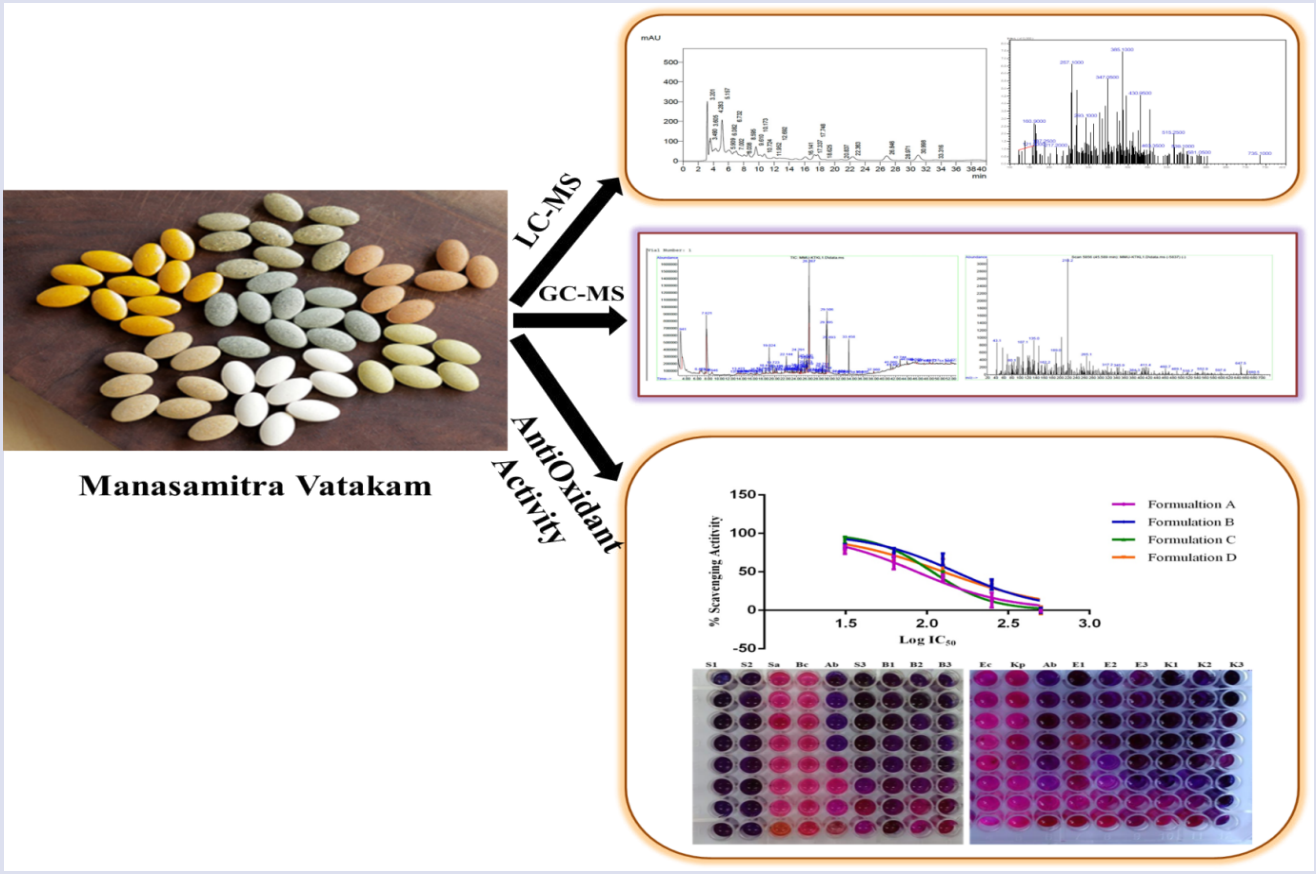
GRAPHICAL ABSTRACT



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