Research Article

Investigation of Phytochemical Profile and other Safety Parameters for A Hepatoprotective Proprietary Polyherbal Formulation (Liverem) by Using Advanced Instrumentation

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ABSTRACT

India has long heritage in the use of plant products as medicines. Globally WHO and Nationally AYUSH both specify and encourage doing quality analysis of herbal raw materials and finished products which ensure quality and safety. The main objective of the present study was to determine the phytochemical profile in selected polyherbal formulation by using GC-MS technique and also screen the safety parameters such as microbial load, pesticide residues, heavy metals and Aflatoxins by using appropriate protocol specified in WHO, AYUSH, (API and SPI) guidelines. With the help of GC-MS totally 19 compounds were identified and safety parameters such as microbial load and heavy metals are found within the limits. Other parameters like pesticide residues (DL - 0.00001mg/kg) and aflatoxins (DL – 0.20µg/kg) are found below detectable limits. The results indicate safety and quality of selected polyherbal formulation (Liverem). Further, LC-MS analysis, Toxicological and Pharmacological studies are in progress.

Keywords: Phytochemical profile, GC-MS, Pesticide residues, Heavy metals, Aflatoxins.

INTRODUCTION

In spite of the tremendous advances made, no significant and safe hepatoprotective agents are available in modern therapeutics. Therefore, importance has been given globally to develop plant based hepatoprotective drugs effective against a variety of liver disorders.[1] Even though herbal medicines are become popular and effective in the treatment of several ailments, they are still unacceptable in the treatment modalities due to (i) lack of standardization (ii) lack of identification of active ingredient(s) / principle(s) (iii) lack of randomized controlled clinical trials (RCTs) and (iv) Lack of toxicological evaluation. Recent years, most of the population showing interest to consume medicinal plants in the form of dietary supplements and herbal medicines. With help of advanced / hyphenated instrumentation it's becoming easy to analyse the formulations and we are able to know the composition of phytochemicals or active principle compounds. This hyphenated instrumentation also useful for analysis of safety parameters such as heavy metals and pesticides which are causes many complications if entered into the human physiological system.

In this research work, a marketed hepatoprotective polyherbal ayurvedic formulation 'Liverem' has been taken to determine the phytochemical profile by using GC-MS technique and also screen the safety parameters such as microbial load, pesticide residues, heavy metals and Aflatoxins by using appropriate protocol specified in WHO[2], AYUSH, (API[3] and SPI[4]) guidelines. The formulation is manufactured by Rumi Herbals Pvt.Ltd, a GMP certified, 22 years old company and is marketed by Rohini Global Marketing Pvt Ltd. Chennai.

MATERIALS AND METHODS

Sample

The selected marketed formulation was received from Rumi Herbals Pvt. Ltd as gift sample. All the quality control parameters was assessed and reported in previously published research article.[5]

Preparation of sample for GC-MS analysis

One gram of selected polyherbal formulation was soaked overnight in 10 ml of Ethanol. Filter through double layered whatmann no.1 filter paper and the extract was passed through anhydrous sodium sulphate. 2 µl of extract was injected to GC-MS and analysed by Triple Quadrupole Acquisition Method by using Agilent Mass Hunter Workstation Software – 7000 Series.

Sample Preparation for screening pesticides

Take 2.0g of sample in centrifuge. Add 20ml of dichloromethane. Shake well and Vortex ten minutes. Remove the organic layer. Repeat extraction twice. Pool organic layer and pass through anhydrous sodium sulphate layer to remove moisture. Evaporate this to dryness using nitrogen evaporator at 40◦C. Rediscover the residue in 1 ml of Acetonitrile and use for GC-MS & LC-MS/MS analysis.

Sample Preparation for screening of Aflatoxins

Weigh 2.0 gm of homogenized sample in Petri dish and transfer to Blender jar. Add 2 gm of sodium chloride and

25.0 ml of extraction solvent (80:20 Methanol: water) and wash the Petri dish. Blend 2 min at high speed and centrifuge at 6000 rpm for 10 min. Filter through pre folded paper. Adjust pH 7.4 using 2 M sodium hydroxide. Pipette 10 ml of filtrate and dilute with 100 ml Phosphate Buffered Saline Solution (PBS) and mix thoroughly. Adjust the IAC columns to room temperature prior to conditioning. Remove the cap from the top of the column and fix in the vacuum manifold. Pass the whole 100 ml of diluted filtrate through the column at a flow rate of 2 - 3 ml per minute. A slow, steady flow rate is essential for the capture of the toxin by the antibody. Wash the column by passing 10 ml of water through at a flow rate 6 ml per minute repeat with another 10 ml PBS. Discard water washings. Pass air through the column to remove residual liquid. Elute the bounded aflatoxins from the column at a flow rate of 1 drop per second using 1.0 ml of 100% methanol and following elution pass 1.0 ml of Millipore water through the column and collect in a amber glass vial. The extract is ready for analysis by HPLC-FLD system.

Preparation of samples by acid digestion method for analysis of heavy metals

Accurately weighed 2 g of sample was taken in Kjeldahl flask. Acid mixture of Nitric acid: Perchloric acid (4:1) was added in the flask and heated continuously till the solution became colourless. The sample was then transferred to a 25 ml volumetric flask and the volume was made-up with distilled water. Reagent blank was synchronously prepared according to the above procedure. The standards of Lead, Cadmium, Arsenic and Mercury were prepared as per the protocol in the manual. The samples were analyzed for the presence of Pb, Cd, As and Hg using Atomic Absorbance Spectrophotometer (AAS) (SHIMADZU).[6]

Microbial safety profile

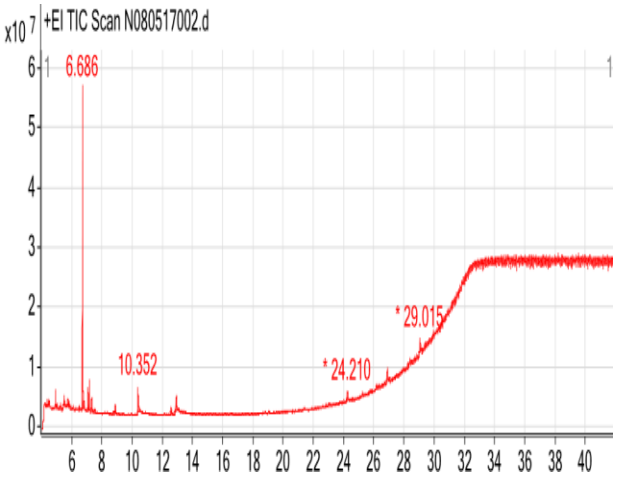
Microbial screening was carried out to estimate the number of viable microorganism present in the formulation. Various differential and selective medias are utilized for screening microbial contamination. For Total viable count (Casein soyabean digest agar), Total *yeast* and *moulds* (Sabouraud dextrose agar with antibiotics), *E.coli* (MacConkey agar and EMB agar), *Salmonella* (Brilliant Green agar) *Staphylococcus* sp. (Mannitol salt agar) *Pseudomonas aeruginosa*, (Cetrimide agar) were used to screen the organisms as per the AYUSH guidelines.

RESULTS AND DISCUSSION

The polyherbal formulation was formulated as hepatoprotective agent by using traditional medicinal plants. In the earlier research work, the formulation was screened for hepatoprotective activity in pre-clinical model[7] and also the formulation was prescribed by Ayurvedic and Siddha Physicians for several liver diseases.

Identification of phytoconstituents

Interpretation of mass spectra of GC-MS was performed utilizing the database of National Institute Standard and Technology (NIST) having more than 62,000 designs. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight (MW), and structure of the components of the test materials were ascertained.



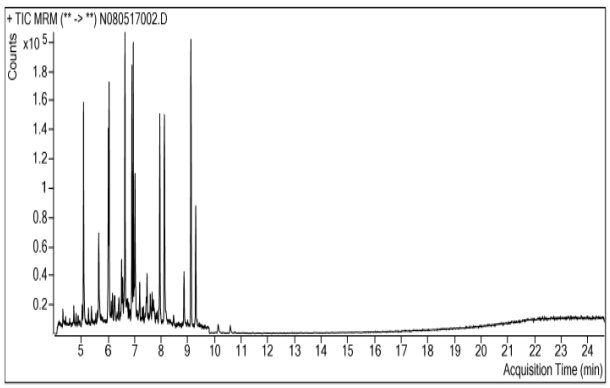
Picture: 1. Gas chromatography and mass spectroscopy chromatogram of Liverem

Screening of Pesticides

The worldwide use of pesticides in food production has been the cause for increasing concern. Consumers are looking for the assurance that the food they eat is safe. Chemical diversity of these pesticides also presents a considerable challenge to food safety chemists, who require a range of analytical techniques to extract pesticides from food samples, and then to accurately determine their identity and concentration at trace levels. In the present work both GC-MS and LC-MS/MS was used to screen the pesticide residues

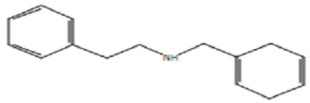
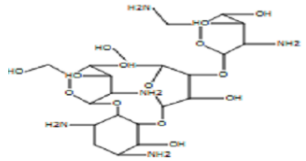
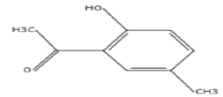
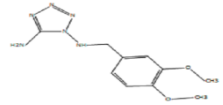
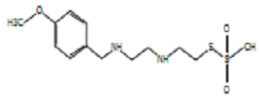
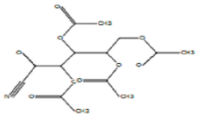
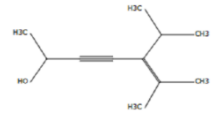
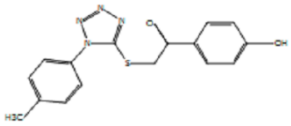
GC-MS analysis of pesticides

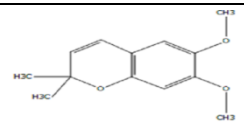
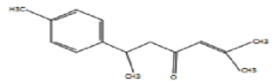
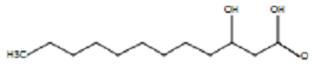
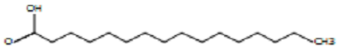
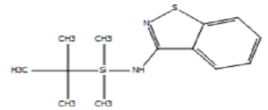
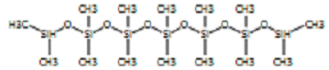
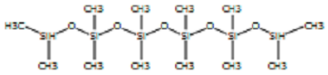
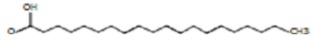
The pesticide residues was analysed in the formulation by using Agilent Technologies, 5977 single quadrupole GC/MS.



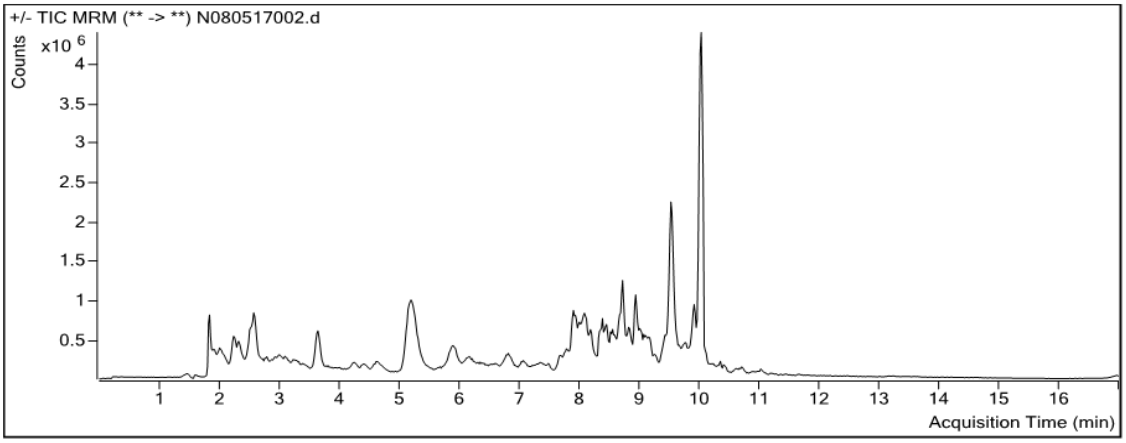
Picture 2: GC-MS chromatogram of pesticides residues

Table 1: Phytoconstituents present in the selected polyherbal formulation (Liverem)

S.NO	Name of the compound	RT	% Area	Formula	MW (g/mol)	Structure
1	Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester	4.898	3.88	$C_{26}H_{50}O_2$	394.6740	
2	N-Benzyl-2-phenethylamine	5.477	4.65	$C_{15}H_{17}N$	211.308	
3	Paromomycin	6.686	100	$C_{23}H_{45}N_5O_{14}$	615.634	
4	Ethanone, 1-(2-hydroxy-5-methylphenyl)-	7.054	5.77	$C_9H_{10}O_2$	150.1745	
5	1H-1,2,3,4-Tetrazole-1,5-diamine, N(1)-[(3,4-dimethoxyphenyl)methyl]-	7.14	8.08	$C_{10}H_{14}N_6O_2$	250.257	
6	N-[p-Methoxybenzyl]-N'-[2-thiosulfatoethyl]-1,2-ethanediamine	10.352	14.66	$C_{12}H_{20}N_2O_4S_2$	320.428	
7	Tetraacetyl-d-xylonic nitrile	12.897	16.57	$C_{14}H_{17}NO_9$	343.288	
8	5-Hepten-3-yn-2-ol, 6-methyl-5-(1-methylethyl)-	26.861	7.53	$C_7H_{10}O$	110.156	
9	1-(4-Hydroxy-phenyl)-2-(1-p-tolyl-1H-tetrazol-5-ylsulfanyl)-ethanone	6.788	5.57	$C_{16}H_{14}N_4O_2S$	326.374	

10	2H-1-Benzopyran, 6,7-dimethoxy-2,2-dimethyl-	7.054	6.29	$C_{15}H_{16}O_3$	244.285	
11	Ar-tumerone	7.14	8.77	$C_{15}H_{20}O$	216.324	
12	Dodecanoic acid, 3-hydroxy-	7.288	4.59	$C_{12}H_{24}O_3$	216.321	
13	Z,Z,Z-4,6,9-Nonadecatriene	7.495	1.44	$C_{19}H_{34}$	262.481	
14	Z,Z-2,5-Pentadecadien-1-ol	8.855	3.24	$C_{15}H_{28}O$	224.388	
15	n-Hexadecanoic acid	10.352	18.06	$C_{16}H_{32}O_2$	256.43	
16	1,2-Benzisothiazol-3-amine tbdms	24.21	3.86	$C_{13}H_{20}N_2SSi$	264.462	
17	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	26.855	6.35	$C_{14}H_{44}O_6Si_7$	505.094	
18	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	29.015	12.02	$C_{12}H_{38}O_5Si_6$	430.940	
19	8,11,14-Eicosatrienoic acid, (Z,Z,Z)-	12.897	22.39	$C_{20}H_{34}O_2$	306.49	

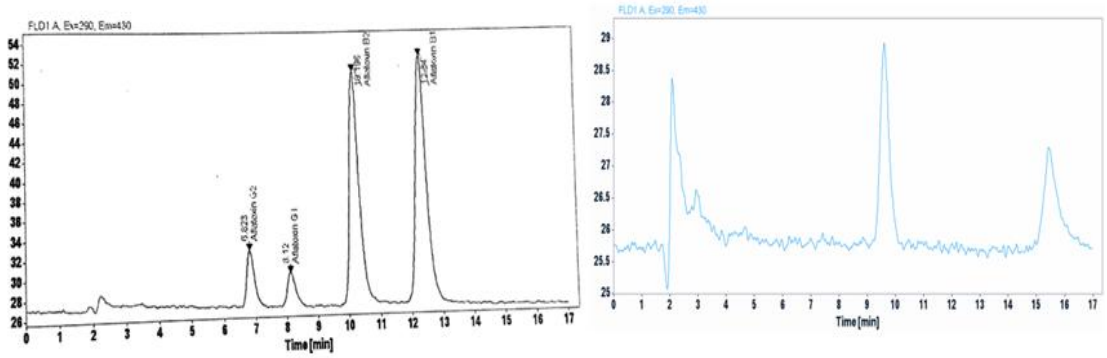
LC-MS/MS analysis of pesticides



Picture 3: LC-MS/MS chromatogram of pesticides residues

Identification of Mycotoxins (Aflatoxins)

Aflatoxins are extremely dangerous to both animals and humans, causing illness and even death when present, and their control in food and animal feed is vitally important. The aflatoxins were screened in the formulation by using HPLC- FLD, Agilent 1260 Infinity system, Column: Zorbax Eclipse Plus C18.



(A) **(B)**

Picture 4: HPLC-FLD chromatogram of aflatoxins (A. Standard, B. Sample)

Table 2: List of standard Aflatoxins and their RT by HPLC-FLD

Name	RT (min)	Area	Height	Amount (ng/µl)
Aflatoxin G2	6.832	101.9671	5.9167	10.192
Aflatoxin G1	8.12	71.8558	3.6322	10.169
Aflatoxin B2	10.198	527.1737	24.4125	10.001
Aflatoxin B1	12.34	626.3959	25.9039	10.043

Table 3: Heavy metal analysis of polyherbal formulation - Liverem

S.No	Name of the heavy metal	Batch: 10008	Batch: 11009	Batch: 12010	Average
A	Mercury (1 ppm)	0.05	0.005	0.055	0.0367
B	Lead (10 ppm)	0.55	0.335	0.678	0.521
C	Cadmium (0.3 ppm)	BDL	BDL	BDL	BDL
D	Arsenic (3 ppm)	0.01	0.004	0.21	0.075

Table 4: Microbial safety limits of polyherbal formulation - Liverem

S. No	Characteristics	Batch: 10008	Batch: 11009	Batch: 12010	Average
1	Total Bacterial count (Limit – $1.0x10^5$)	156 cfu/g	137cfu/g	126 cfu/g	139 cfu/g
2	Total Yeast & Mould (Limit – $1.0x10^3$)	21 cfu/g	36 cfu/g	24 cfu/g	27 cfu/g
3	*Escherichia coli (Absent)*	Absent	Absent	Absent	Absent
4	*Salmonella sp (Absent)*	Absent	Absent	Absent	Absent
5	*Staphylococcus (Absent)*	Absent	Absent	Absent	Absent
6	*Pseudomonas (Absent)*	Absent	Absent	Absent	Absent

SUMMARY AND CONCLUSION

The phytochemical profile was analysed for the selected polyherbal formulation by using hyphenated instrument like GC-MS and found various phytoconstituents. Pesticide residues analysis were done by using GCMS and LC-MS/MS techniques and not found any pesticide residues. Aflatoxins were screened by using HPLC-FLD system and not found any of aflatoxins. Heavy metals was screened by using Atomic Absorption Spectroscopy and found within the limits. Microbial screening was done as per AYUSH guidelines, found TBC and TYM within the limits and not found any toxic bacteria's. Conclusively, present study has become scientific evidence for the safety aspects of a hepatoprotective polyherbal formulation Liverem. Further, Toxicological and pharmacological studies are in progress.

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