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Chemical Profiling, Spectroscopic Characterization and Biological Evaluation of a novel polyherbal formulation with natural binders- Ayurgreen Natura Pain Gel

Sakkariya KN[1], Habeebulla MV[1], Jisha Dev[1], Jinitha TV[2], Hashim NK[3], Muhamned Kutty PV[4]

[1] Research and Development Division, Ayurgreen, Edappal, Malappuram-673635, Kerala, India
[2] Department of Chemistry, University of Calicut, Malappuram-673635, Kerala, India
[3] Department of Physics, WMO Arts and Science College, Muttil, Wayanad-673122 Kerala, India
[4] Department of Physics, GHSS Agali, Palakkad, Muttil- 673122, Kerala, India

ABSTRACT

Introduction: Herbal compositions are becoming increasingly important in today's world of raw material scarcity. Polyherbal formulations exhibit high efficacy due to the presence of active phytochemicals which may enhance their potency due to the synergetic interaction of active ingredients of different plants. Ayurgreen Natura Pain Gel is an important Ayurvedic polyherbal formulation prepared using specified plant parts of dried aloe vera and fresh aloe vera pulp, frankincense, myrrh, ferula asafetida. **Methods:** The phytochemistry of Ayurgreen Natura Pain Gel has been evaluated using a liquid chromatography-mass spectrometer and their bioactive functional groups were characterized using Fourier Transform Infrared Spectroscopy and UV-Visible spectroscopy. Moreover, the thermal analysis was performed using differential scanning calorimetry. Further, invitro studies were used to evaluate its anti-inflammatory, antioxidant and anti-cancerous activities. **Results:** The LCMS results revealed the presence of 40 phytoconstituents. It shows the presence of Manumycin A which helps wound healing by binding it with RAS protein. The thermogram results revealed the presence of volatile ingredients, melting, and degradation temperature. The formulation showed remarkable anti-inflammatory (IC50 119.8 μg/mL) and antioxidant (IC50 200 μg/mL) activities. The formulation showed potent cytotoxic effect towards Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cell lines with IC 50 values 62 mg/mL and 20 mg/mL respectively. **Discussion/Conclusions:** The formulation can be considered as a potent anti-inflammatory cum anti-cancerous Natura Pain Gel with antiproliferative activity. Fascinatingly, the wild habitat contained some anticancerous phytoconstituents which might be responsible for enhanced anti-cancerous activity in mice cancer cell lines (EAC) and (DLA) cell lines.

Keywords: Polyherbal formulation, Ayurgreen Natura Pain Gel, Chemical Profiling.

INTRODUCTION

Ayurveda is the oldest science that promotes healthy living and maintains a sound mind in a sound body. Though it originated in India, it is now acknowledged worldwide as a medical system that provides solutions to the majority of ailments and provides satisfactory solutions to all the problems the world is facing today. The pledged purpose of Ayurveda as an everlasting supreme science is to ensure a longer and healthier life for humankind by promoting the use of herbal compounds, and unique health practices. Though foundations of Ayurveda, were laid down by ancient sages, this healing method has proven its worth through time-tested ideas and formulas devised by the ancient Acharyas. However, the treasures of formulations mentioned for many disease conditions must be subjected to scientific research not only to confirm its validity but also to enrich it with contemporary advances to the existing knowledge. Ayurvedic formulations encompass a variety of botanicals as constituent materials, some may comprise of minerals, metals, and animal-derived substances, and each of them has several chemical compounds that, when combined, may offer the anticipated action [1]. Herbal compositions are becoming increasingly important in today's world of raw material scarcity. Herbal medicine contains several active ingredients for a variety of ailments, but proper knowledge must be necessary for the manufacture of herbal formulations [2]. Polyherbal formulations exhibit high efficacy due to the presence of active phytochemicals which may enhance their potency due to the synergetic interaction of active ingredients of different plants. reported the presence of tannins, flavonoids, phenolics, saponins, terpenoids, ascorbic acids, carbohydrates and many other phytochemicals [5]. The qualitative analysis of *P. niruri* leaf extract was made and reported the availability of phytochemicals like saponins, alkenes, phenolics, flavones and terpenes [6]. It is reported as phenols and flavonoids have significant antioxidant properties and has ability to inhibit the growth of microbial pathogens.

***Corresponding author:**
Dr. Sakkariya KN
Research and Development Division, Ayurgreen, Edappal, Malappuram-673635, Kerala, India
Email: dr@ayurgreenhospitals.com

Ayurgreen Natura Pain Gel is a novel polyherbal formulation, that can be used as an anti-inflammatory drug. Inflammation is a common pathological condition of 30-40% of the world population and can be considered as a defensive mechanism triggered by damage, infection, or irritation in the body to eradicate consequent necrosed cells and tissues to limit the spread of agents. Despite the fact that it is a defensive mechanism complex actions and mediators involved in the inflammatory response can induce, maintain or aggravate many diseases. As a normal response, inflammation causes pain, swelling, and erythema in the human body. Anti-inflammatory drugs, such as NSAIDs, work on various inflammatory pathways to relieve pain, but they can have unfavorable side effects such as stomach ulcers and, less commonly, myocardial infarction and stroke. People are fed up with the side effects and after effects of today's most potent and fast-acting contemporary medications, which reduce human immunity while suppressing illness. It is true that modern science has grown up considerably, but it continues to face serious challenges in the face of a number of vexing issues. In such situations, we can depend ayurvedic medical formulations which have least side effects compared to other treaties.

In this work, a novel Ayurvedic herbal formulation; Ayurgreen Natura Pain Gel, is introduced. It is a polyherbal formulation prepared by using natural binders like Magnesium silicate and a clay mineral (hydrous aluminum silicates) that can be used for ailment situations of inflammation. Polyherbal formulations include different plant parts of dried aloe vera and fresh aloe vera pulp, frankincense, myrrh, ferula asafetida as shown in Figure 1. The plant Aloe vera is used in Ayurvedic, Homoeopathic and Allopathic streams of medicine, and not only tribal com-munity but also most of the people for food and medicine. The plant leaves contain numerous vitamins, minerals, en-zymes, amino acids, natural sugars and other bioactive compounds with emollient, purgative, antimicrobial, anti in-flammatory, anti-oxidant, aphrodisiac, anti-helmenthic, antifungal, antiseptic and cosmetic values for health care. This plant has potential to cure sunburns, burns and minor cuts, and even skin cancer [3]. Frankincense, as shown in Figure 1. a, is a traditional medicine of the East, that is believed to have anti-inflammatory, expectorant, antiseptic, and even anxiolytic and anti-neurotic effects. The frankincense is constitutes 60 % oil , 13% mono-terpenes 40% diterpenes , 21.4 % ethyl acetate, 13.4 % octyl acetate and 7.6 % methylanisole [4]. Myrrh is a resinous exudate obtained from the tree Commiphora myrrha. It has been widely used in traditional medicines due to its variant biological applications against diseases including ulcerative colitis, fever, gall bladder, skin infections, dysmenorrhea, amenorrhea, tumors, chest ailments and in burn treatment [5]. Myrrh contains about 3% –8% of essential oils, 25% –40% alcohol-soluble resin, and 30% –60% water-soluble gum The phytochemical constituents of Myrrh are monoterpenes, sesquiterpenes, aromatic compounds, triterpenoids, diterpenoids, steroids, and lignans. Myrrh resin has been shown to have antimicrobial, anticancer, and anti-inflammatory effects [6]. Sahasravedi (Ferula asafoetida) is herbaceous plant of the umbelliferae family. It is oleo gum resin obtained from the rhizome and root of plant. It is used in modern herbalism in the treatment of hysteria, some nervous conditions, bronchitis, asthma and whooping cough. The gum resin is antispasmodic, carminative, expectorant, laxative, and sedative. It also thins the blood and lowers blood pressure [7]. The main chemical constituents of Ferula asafoetida are as follows: coumarin (ferulenol, galbanic acid and umbelliprenin), coumarin esters (ferulone A, B), sesquiterpenes (germacranes, himachalanes, carotanes, humulanes, guaianes, daucane estersfarnesiferol A and B, and sinkiangenorin C and E), sesquiterpene lactones, monoterpene (α-pinene, β-pinene), monoterpene coumarins (auraptene), prenylatedcoumarins (ferprenin), sulfur-containing derivatives, phytoestrogen (ferutinin), flavonoids, carbohydrates (galactose, glucuronic acid, arabinose, rhamnose) [8].

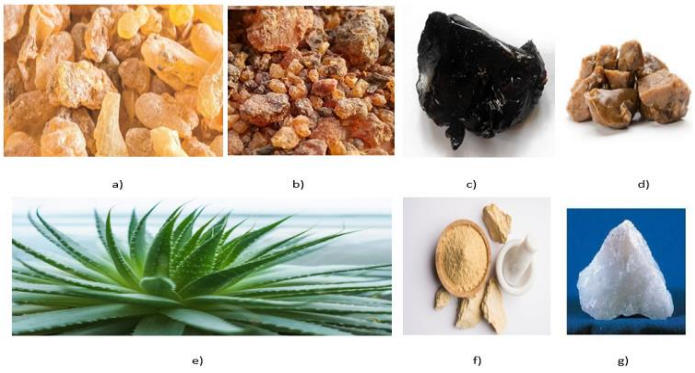


Figure 1: Photographic images of ingredient plants in the polyherbal formulation. a) Frankincense b) Myrrh c) Chenninayakam (Dried Aloe vera) d) Sahasravedi (ferula asafoetida) e) Aloe vera f) Multhanimitti g) kannaram (magnesium silicate)

Additionally, some natural binders like Magnesium silicate and Fuller's earth were added to the polyherbal formulation to articulate in a cream form and to enhance its adhesive preoperty. Magnesium silicate, locally called as Kannaram, is a compound of magnesium oxide (MgO) and silicon dioxide (SiO2) [9]. Fuller's earth is an adsorbent clay consisting essentially of calcium montmorillonite [10].

Although herbal plants include numerous active chemicals that are used to treat a variety of ailments, there is a chance for the creation or annihilation of bioactive molecules during the manufacturing of polyherbal formulations. Because the preparation technique is often endothermic, it necessitates a significant amount of heating and mechanical stirring. Hence there is an inevitability for the identification of bioactive compounds, physical and biological evaluation to confirm the chemical reactivity and biological activity of a finished polyherbal product. The current study describes a detailed confab on chemical profiling, physical characterization, and biological evaluation of a novel herbal formulation. Chemical profiling has been carried out using liquid chromatography-mass spectrometry (LC-MS) coupled to gas chromatography-mass spectrometry (GC-MS) for the identification of bioactive compounds above mentioned formulation. Physical characterization was done with different spectroscopic tools including Fourier Transform Infrared Spectroscopy (FT-IR) and UV-Visible spectroscopy and the thermal stability was evaluated using differential scanning calorimetry (DSC). Further, the synergistic antioxidant capacity was investigated in terms of its radical scavenging activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide dismutase (SOD) radicals. The anti-inflammatory activity was evaluated towards nitric oxide scavenging radical in RAW – cells. Finally, the anti-cancer potential of the herbal formulation was screened against mice cancer cell lines (Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA).

MATERIALS AND METHODS

Chemicals

Ingredients	Quantity (in gm)
Frankincense	800gm
Dried aloe vera	875gm
Myrrh	125gm
Magnesium silicate	300gm
Ferula asafetida	100gm
Fuller's earth	375gm
Aloe vera	1250gm

Preparation of Ayurgreen Natura Pain Gel

A mixture of frankincense and dried aloe vera is boiled with juice of aloe vera to form a melt. All other ingredients were grinded to form fine powder and added to the melt while stirring to form a homogeneous mixture. The stirring will be continued for 2 -3 days without having fermentation and contamination.

LC-MS analysis

LC–MS/MS experiments were performed on Agilent 6520 accurate mass MS Q-TOF coupled with Agilent LC 1200. The MS analysis was performed with dual AJS ESI ion source in positive and negative mode. Maas spectral data analysis was done by Agilent molecular ion extraction algorithm. The general conditions for mass spectrometry were drying gas (nitrogen) flow 13 L/min; nebulizer pressure 35 psig; drying gas temperature 250oC; capillary voltage 3500V; fragmentor volt 750 V; Oct RF Vpp. A gradient of water (95%) and acetonitrile (5%) was used as mobile phase for ESI ionization mode at constant flow of 0.3 ml/min.

The mobile phase was fixed as gradient of acidified methanol (A) and water (B) system for ESI ionization mode. Gradient elution was performed at a constant flow rate of 0.9 ml/min and 1200.00 bar pressure.

UV-Vis Spectroscopy

UV–Vis spectra of polyherbal formulation was recorded in ethanol on Jasco UV–Visible Spectrophotometer model V-550. The base line was corrected using ethanol solvent before analysis.

FT-IR Spectroscopy

The FTIR spectra of polyherbal formulation was recorded by JASCO FTIR-4100 spectrometer at room temperature. The measurements were taken in the range from 400 to 4000 cm-1 using KBr pellet.

Differential Scanning Calorimetry

The DSC of the samples was carried out at a temperature range of 30–350℃ at a heating rate of 10° min-1 using Perkin Elmer DSC 4000 series.

Nitric Oxide Scavenging Assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions (Ebrahimzadeh *et al*., 2010).

Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different c oncentrations of the methanolic extract and incubated at 25℃ for 3 hours. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. A volume of 150 µL of the reaction mixture was transferred to a 24 well plate. The absorbance was measured at 546 nm using a UV/Visible spectrophotometer, Gallic acid was used as the positive control. The percentage inhibition of the extract and standard was calculated and recorded.

Percentage nitrite radical scavenging activity:

$$Nitrite\ oxide\ scavenged\ (\%) = \frac{Abs.of\ Control\ -\ Abs.of\ test}{Abs.of\ Control}\ x\ 100$$

Where Abs is absorbance.

DPPH Radical Scavenging Assay

The antioxidant activities of the ethanol extract of the lepam against the free radical present in towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) were investigated at various drug concentrations. Diluent part of DPPH solution in methanol (187 μl) were added to 24 well plates with various drug concentration to make up 2 mL in each well. The reaction mixture was incubated in the dark condition for 20 minutes and the absorbance was measured at 517 nm. The results were evaluated as percentage scavenging of radical.

$$\%\ scavenging\ of\ DPPH\ = \frac{Abs.of\ Control\ -\ Abs.of\ sample}{Abs.of\ Control}\ x\ 100$$

Results were plotted in a graph of the double integral intensity versus extract concentration. By graphical analysis, it was possible to determine the concentration that reduces the initial signal intensity by 50% (IC_{50}).

Trypan Blue Assay

Daltons Lymphoma Ascites (DLA) cell line was aspirated freshly from peritoneum of tumour bearing female Swiss albino mice as transplantable ascites tumors. Cells were washed 3 times with phosphate buffer solution (PBS) and the concentration was confirmed at 1 x 10^7 cell/ml using hemocytometer. The viability of the cells was ensured using 1 % of trypan blue in saline cell to be above 98%. The assay mixture was made up to 1ml using varying drug concentration and PBS with 1 x 10^6 cells and incubated at 35℃ for about 3 hours. The number of dead cells was counted after adding the number of dead

cells was counted using Heamocytometer [11,12]. The same procedure was followed for Ehrlich Ascites Carcinoma (EAC) cell lines, which is aspirated from peritoneal cavity of mice to check the antiproliferative effect of the synthesized drugs. The percentage of cytotoxicity was calculated using the formulae.

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

RESULT AND DISCUSSION

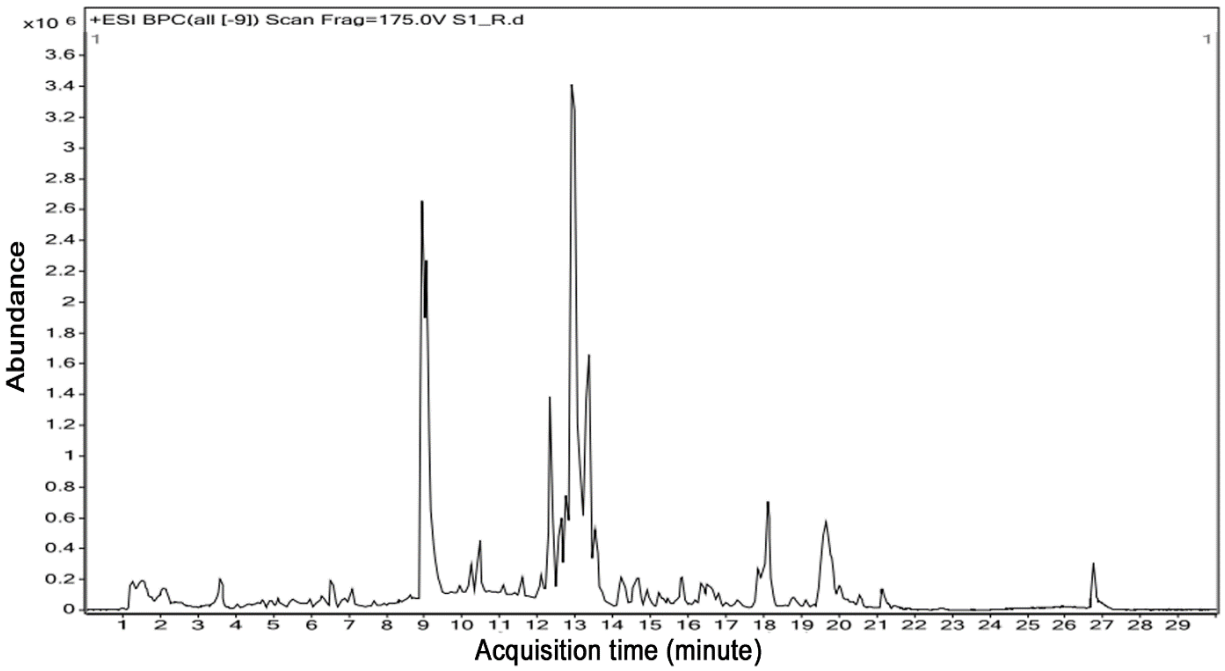
Chemical profiling

LC/MS analysis of polyherbal formulation was carried out with ESI ionization in both positive and negative modes and information regarding the presence of chemical compounds was extracted by Agilent Mass Hunter software. 25 molecular ions were detected in negative mode and 15 molecular ions peaks were exhibited in positive mode based on the abundance of ions were further fragmented in auto ms/ ms analysis with varying collision energy. The consistency of fragments was confirmed by targeted ms/ms analysis with fixed collision energy based on the auto ms/ms analysis. The chromatogram in positive and negative mode were depicted in Figure 2.

The ESI-MS fingerprint of the sample in positive mode (Fig. 2.a, Table 1) presented the ions of m/z 138.05 - 4-fluoro-l-threonine, 268.10 - prinomide, 433.11 - 6-hydroxydaidzein 4'-glucoside, 274.27 - sphinganine, 318.30 - phytosphingosine, 573.25 - muricinine, 354.13 - papaveraldine, 411.27 - sterol 3-beta-d-glucoside, 147.04 - phenylpropiolic acid, 369.13 - glycyrrhizaisoflavone C, 573.25 - manumycin A, 336.12 - gamma-glutamyl-S-methylcysteinyl-beta-alanine, 274.14 - piperlonguminine, 286.14 - piperine and 354.98 – idoxuridine.

The ESI-MS fingerprint of the sample in negative mode (Fig. 2.b, Table 2) presented the ions of m/z 191.05 - quinic acid, 447.09 - quercitrin, 197.04 - syringic acid, 331.06 - glucogallic acid, 289.07 - catechin, 483.08 - 2,6-digalloylglucose, 321.02 - digallate, 435.13- phenethyl 6-galloylglucoside, 431.10 - genistein 8-C-glucoside, 197.04 - syringic acid, 301.07- homoeriodictyol, 187.09 - nonate, 593.13 - 2''-O-trans-p-coumaroylastragalin, 515.12 - b-D-glucuronopyranosyl-(1->3)-a-D-galacturonopyranosyl-(1->2)-L-rhamnose, 665.39 , 785.41 - mycinamicin VI,II, 367.12 - 7-O-methylluteone, 763.43 - 28-glucosylsiaresinolate 3-arabinoside, 763.43 - digitoxin, 367.12 - 7-O-methylluteone, 337.11 - gualenate, 367.12 - mollicellin H, 329.03 - 2, 408.99 - 8-di-O-methylellagic acid, 967.49 - imibenconazole, 967.49 - goyasaponin III, 609.478 - annotemoyin 1.

It is fascinating to note that the polyherbal formulation contains a variety of chemical compounds including phenolics, flavanones, furans, gallotannin, glucoside, oligosachiride, acids with different biological activities like anti-inflammatory, anti-bacterial, ant-fungal, anti-viral and anti-cancerous. More interestingly it shows the presence of Manumycin A which helps wound healing by binding it with RAS protein. It contains phytosphingosine that enhance the permeability of chemical compounds through skin's barrier.

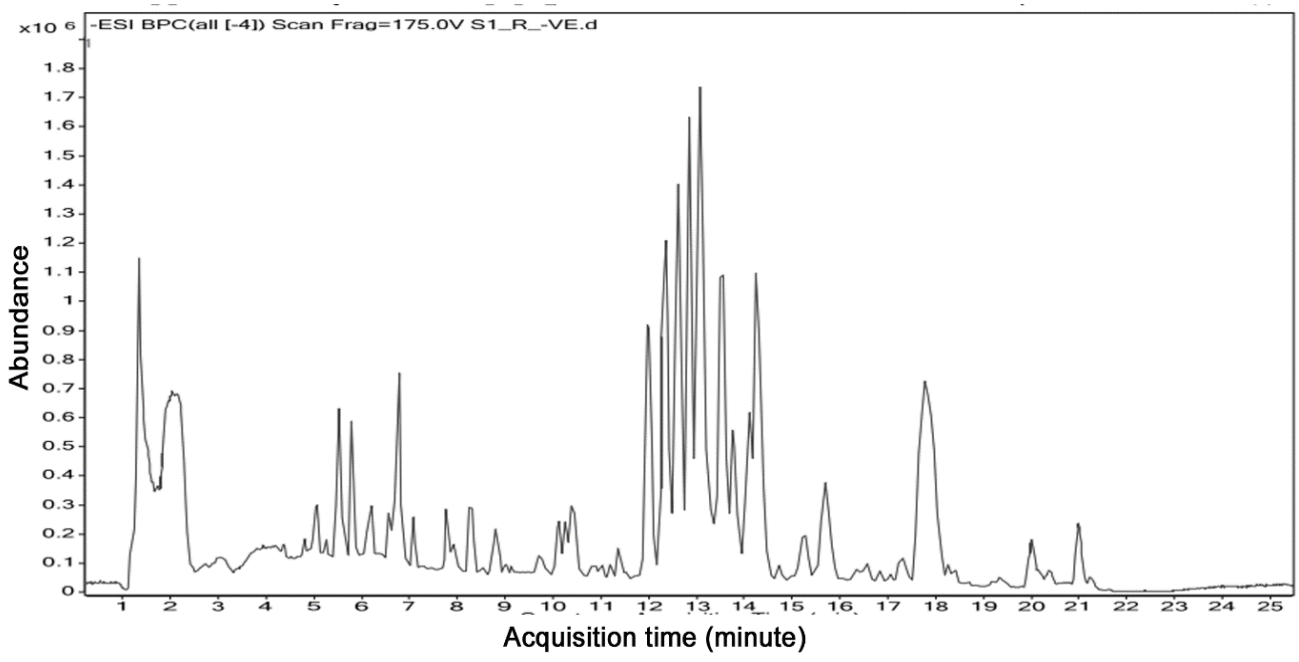


a) Positive ESI mode

Table 1: HR–LC-MS/MS analysis of polyherbal formulation in positive mode

Sl.No:	m/z	MS/MS	Tentative Identification	Type of Compound	Molecular Formula	Biological Activity
1.	138.05	137.04	4-Fluoro-L-threonine	Amino Acid	$C_4H_8FNO_3$	Anti-bacterial
2.	268.10	267.09	Prinomide	anilide	$C_{15}H_{13}N_3O_2$	anti-bacterial, anti-fungal,

						anti-viral, anti-inflammatory
3.	433.11	432.10	6-Hydroxydaidzein 4'-glucoside	isoflavonoids	$C_{21}H_{20}O_{10}$	antioxidation **activity**
4.	274.27	273.26	Sphinganine	aminoalcohols	$C_{16}H_{35}NO_2$	Anti-fungal, anti-cancerous
5.	318.30	317.29	Phytosphingosine	aminoalcohols	$C_{18}H_{39}NO_3$	skin's barrier function
6.	573.25	550.2662	muricinine		$C_{18}H_{19}NO_4$	
7.	354.13	353.12	Papaveraldine		$C_{20}H_{19}NO_5$	
8.	411.27	410.26	Sterol 3-beta-D-glucoside		$C_{23}H_{38}O_6$	
9.	147.04	146.03	Phenylpropiolic acid	phenylpropanoid	$C_9H_6O_2$	
10.	369.13	368.12	*Glycyrrhizaisoflavone C*	flavonoids	$C_{21}H_{20}O_6$	antibacterial, antifungal, antineoplastic
11.	573.25	550.26	*Manumycin A*	polyketide	$C_{31}H_{38}N_2O_7$	Binds with RAS protein for wound healing
12.	336.12	335.11	gamma-Glutamyl-S-methylcysteinyl-beta-alanine		$C_{12}H_{21}N_3O_6S$	
13.	274.14	273.13	Piperlonguminine		$C_{16}H_{19}NO_3$	Anti-cancerous, to inhibit melanin production in melanoma B16 cells
14.	286.14	285.13	piperine		$C_{17}H_{19}NO_3$	
15.	354.98	353.97	Idoxuridine		$C_9H_{11}IN_2O_5$	



b) Negative ESI mode

Figure 2: APCI-MS-base peak chromatogram of polyherbal formulation in a) positive and b) negative ionization

Table 2: HR–LC-MS/MS analysis of polyherbal formulation in negative mode

Sl.No:	m/z	MS/MS	Tentative Identification	Type of Compound	Molecular Formula	Biological Activity
1.	191.0572	192.0645	Quinic acid	cyclohexanecarboxylic acid.	$C_7H_{12}O_6$	
2.	447.0959	448.1034	Quercitrin	flavanoid	$C_{21}H_{20}O_{11}$	Anti-oxidant, osteoporosis, lung cancer,

						and cardiovascular disease
3.	197.0469	198.05	Syringic acid	phenolic compound		
4.	331.0686	332.0759	Glucogallic acid	methyl group	$C_{13}H_{16}O_{10}$	keratolytic
5.	289.0735	290.0808	Catechin	Catechin	$C_{15}H_{14}O_6$	
6.	483.0805	484.0878	2,6-Digalloylglucose	gallotannin	$C_{20}H_{20}O_{14}$	Anti-cancer, anti-diabetic
7.	321.0269	322.0342	Digallate	Polyphenol	$C_{14}H_{10}O_9$	Anti-viral, Anti-oxidant
8.	435.1324	436.1396	Phenethyl 6-galloylglucoside	galloyl esters	$C_{21}H_{24}O_{10}$	
9.	431.1005	432.108	Genistein 8-C-glucoside	*glucoside*	$C_{21}H_{20}O_{10}$	Anti-proleferative and apoptotic
10.	197.0469	198.0546	Syringic acid	phenolics	$C_9H_{10}O_5$	
11.	301.0735	302.0808	Homoeriodictyol	flavanones	$C_{16}H_{14}O_6$	
12.	187.0991	188.1064	Nonate	Succinic acid	$C_9H_{16}O_4$	
13.	593.1343	594.1413	*2''-O-trans-p-Coumaroylastragalin*	flavanones	$C_{30}H_{26}O_{13}$	
14.	515.1235	516.1307	b-D-Glucuronopyranosyl-(1->3)-a-D-galacturonopyranosyl-(1->2)-L-rhamnose	oligosachiride	$C_{18}H_{28}O_{17}$	
15.	665.3954, 785.4184	666.4025, 726.4043	Mycinamicin VI,II		$C_{35}H_{57}NO_{11}$	Anti-biotic
16.	367.1216	368.1288	7-O-Methylluteone	flavanones	$C_{21}H_{20}O_6$	
17.	763.4319	7643.4427	28-Glucosylsiaresinolate 3-arabinoside		$C_{41}H_{66}O_{13}$	
18.	763.432	764.4401	Digitoxin		$C_{41}H_{64}O_{13}$	
19.	367.1221	368.1294	7-O-Methylluteone	flavanones	$C_{21}H_{20}O_6$	
20.	337.1116	278.098	Gualenate		$C_{15}H_{18}O_3S$	Anti-inflammatory
21.	367.1224	368.1297	Mollicellin H		$C_{21}H_{20}O_6$	Antibacterial
22.	329.0331	330.0404	2,8-Di-O-methylellagic acid		$C_{16}H_{10}O_8$	antibacterial activity
23.	408.9906	409.998	Imibenconazole		$C_{17}H_{13}Cl_3N_4S$	Anti-fungal
24.	967.4971	968.50	Goyasaponin III		$C_{49}H_{76}O_{19}$	
25.	609.4783	564.4801	Annotemoyin 1	furans	$C_{35}H_{64}O_5$	antibacterial activity

Physiochemical characterization

UV-Vis Spectroscopy

UV-Vis spectroscopic analysis can be used as a quality control in ayurvedic formulations, since spectra can be considered as fingerprint of each authenticated standard formulations. It can be also used for quantitative analysis for evaluating adulterants in the formulations in terms of specific markers of standard authenticated samples. UV/Vis spectroscopic method is based on electronic absorption caused by the compound present in the plants and compound formed in chemical reactions during manufacturing. The total spectral analysis help us to investigate concentration of different chemicals through multiple peaks over broad frequency range from 200 to 900 nm [13].

It was observed that maximum absorption peaks of lepam, were found in the region of 232 nm which may be ascribed to the primary

oxidation products like conjugated dienes with rich orbitals primed for electronic transition in the formulation [14]. The deconvoluted graph reveals three more absorptions at 268, 293, and 403 nm. Normally, the absorptions of radiation in between 260-280 nm are meant for double bonds C=C, C=O and N=N of the aromatic or unsaturated components of humic substances [15]. As the chemical profiling using LCMS indicated the polyherbal formulation is abundant with such hydroxy acids and phenolics, amino acids etc., A small absorbance peak at 293 nm associated to flavonoids present in the formulation from different herbs like 6-hydroxydaidzein, 4'-glucoside glycyrrhizaisoflavone c, quercitrin, homoeriodictyol, 2''-O-trans-p-coumaroylastragalin, 7-O-Methylluteone, 7-O-methylluteone [16]. A light jump at 403 nm is credited to the presence of peroxide compounds, which may be derived from moringa olifera [17].

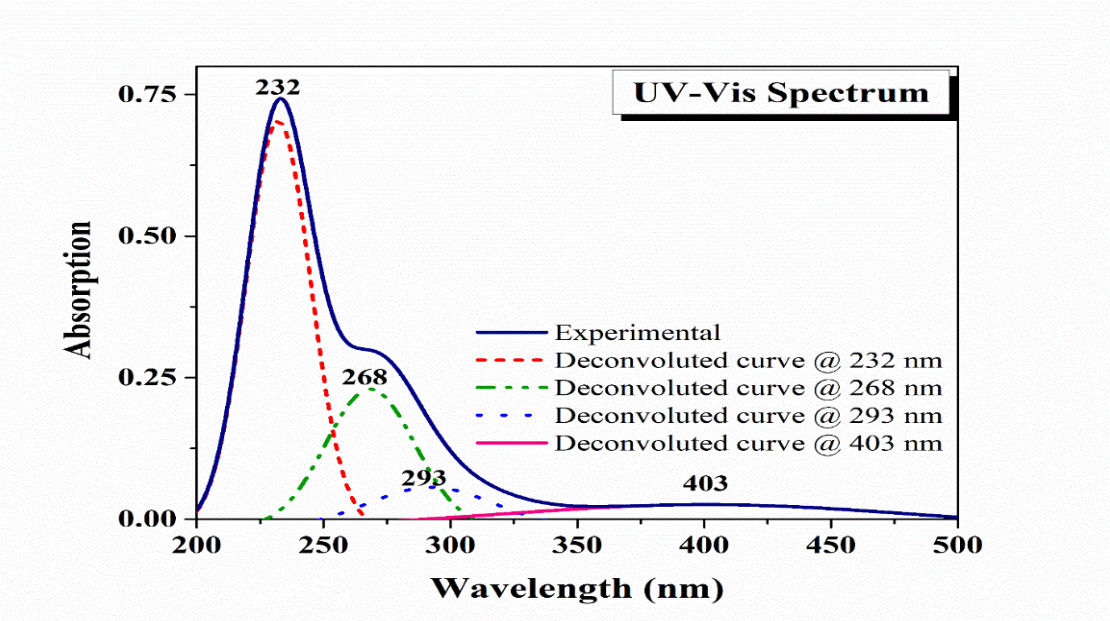


Figure 3: UV-Visible spectrum and their deconvoluted gaussian peaks of polyherbal formulation

FTIR Spectroscopy

To our knowledge from chemical profiling the polyherbal formulation has the presence of 40^{+} chemical constituents. For each molecules have N atoms and each will have 3N-6 normal modes of vibration. So, it is not that much easy to trace the origin of harmonic vibrations for observed wave numbers. Thus, a detailed vibrational assignment of fundamental modes along with IR intensities will be a tedious job, the only possibility is to interpret in terms of fundamental modes of vibrations of functional groups like NH stretching, CH stretching and bending vibrations. The observed FT-IR spectra of the polyherbal formulation were shown in Figures 4 and their corresponding assignments were tabulated in table 3.

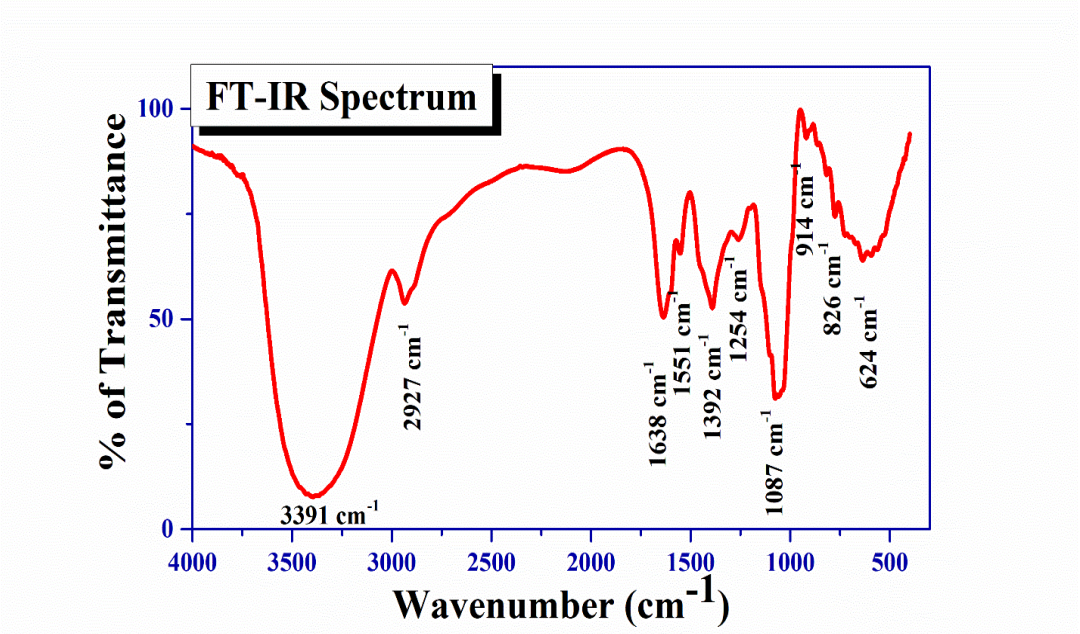


Figure 4: Infrared spectra of polyherbal formulation

Table 3: Observed vibrational frequencies for the polyherbal formulation and their tentative assignment

Polyherbal formulation Wavenumbers (cm-1)	frankincense Wavenumbers (cm-1)[22]	Myrrh Wavenumbers (cm-1[23]	Aloe vera Wavenumbers (cm-1)[19]	Ferula asafetida Wavenumbers (cm-1)[24]	Magnesium silicate Wavenumbers (cm-1)[25]	Multani mitti Wavenumbers (cm-1)[26]	Bond Assigned	Functional groups
	3510	3449					O-H(s)	Alcohol and phenol
3391			3287	3287			O-H(v)	Polysaccharide
						3290		
2927	2980	2925 2855	2966 2930 2922 2874	2924		2916 2848	C-H(S) O-H(S	Carboxylic acid, alkane and aldehyde
	1750		1700 1716				COO^-(S)	
1638		1635	1624		1610		O-H(S) N-H(V)	Aromatic ring
1551	1510		1585	1598		1514	COO^-(S) C=O	Uronic acid
1392	1385	1438 1445	1392			1469 1411	C=C(S)	Aldehyde,ketone,alcohol,phenol,carboxylic acid
1254	1240		1278 1255 1235	1200	1240		C-O-C(S) Si-O(V)	Alcohol and ether
1087		1045		1030	1000		C-O(S) C-C Si-O(S)	Carbohydrate
914	950				980	961	O-H(S) Si-O(S) P-OR esters	Alcohol, ether
826				820			C-C-O C-O-C	
					750	717	Si-O Si	
624					620		Si-O(SV)	

N H vibration

The broad peak observed at 3399 cm−1 in FT-IR spectrum corresponds to N H stretching vibration. The N H stretching frequencies are usually observe in the region at 3500 to 3300 cm−1. Here there are large number of molecules with NH bonds in the polyherbal formulation which made the harmonic vibration due to NH stretching mode intense. The NH stretching mode was observed at 3399 cm−1 in polyherbal formulation those harmonic vibrations were present in the constituent ingredients like dried aloe vera and fresh aloe vera pulp, frankincense, myrrh, ferula asafetida. The sharp peak observed at 1638 cm−1 corresponds to N H in-plane bending vibration. The same N H in-plane bending was reported earlier in dried aloe vera at the wavenumbers 1624 cm−1[18], in Myrrh at 1635 and in Magnesium silicate 1610 cm−1.

Pyrrole ring vibration

The C H stretching frequencies arisen from the pyrrole rings were found as a sharp peak at 2827 cm-1. These modes of vibrations observed at 2895 cm-1 may be due to the presence of alkane, aromatic, hydroxyl, methylene, aromatic ring and alkyl group in the polyherbal formulations [19]. These absorptions may arise from the constituent ingredients like aloevera at 2895 cm-1 due to the presence of pyranose CH and glycoside –OCH2 moieties [19]. may also due to the presence of alkanes in frankincense at 2980 and in Myrrh at 2925 cm-1 [20]. In Aloe vera symmetrical and asymmetrical stretching of the C-H and CH2 group were observed at 2966, 2930,2922 and 2874 cm-1 due to the presence of in Carboxylic acid, alkane and aldehyde [21].

Aldehyde group vibrations

The C=O stretching vibrations appear as a strong band with high intensity and identified at the wavenumber 1638-1392 cm-1¬. The red shifting of carbonyl stretching mode is attributed to the fact that the carbonyl group chelate with the other nucleophilic group, thereby forming both intra- and inter-molecular hydrogen bonding between the molecules in the formulation.

DSC Thermogram

The thermal behavior of the polyherbal formulation was investigated by differential scanning calorimetric analyses. DSC provides information regarding glass transition, melting and crystallization behavior of the sample in addition to associated enthalpy for each process [27,28,37,38,29–36]. This will help in understanding apparently homogeneous formulation junctions that might play the key role behind the properties like release rate and diffusion. Figure 5 depicts the DSC curves of polyherbal formulation in the range of temperature from 30℃ up to 300℃. The thermogram of the formulation displays

three endothermic peaks in addition to some weight loss due to volatile substances present in the formulation. The first peak at 57.5℃ is assigned as a thermal effect due to the presence of natural volatile oils in the formulation also may be due to a glass transition, second peak at 109℃ was related to moisture evaporation or free water release from the sample [27–31]. A sharp endothermic melting transition at 223℃ is attributed to 100% melting of sample.

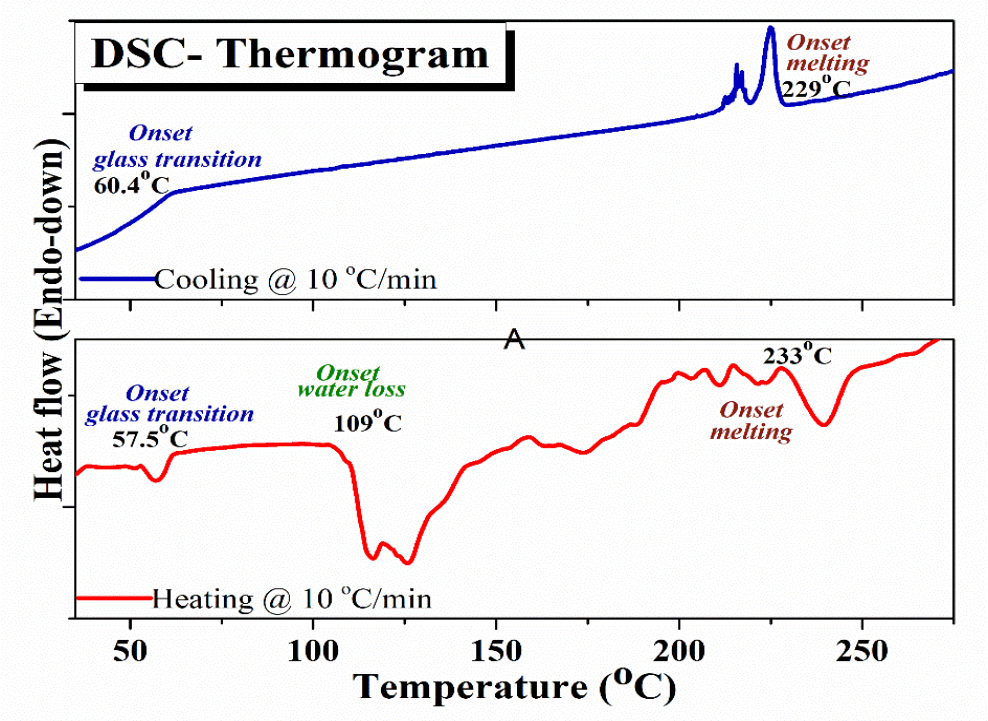


Figure 5: DSC thermogram of polyherbal formulation

Biological Evaluation

Anti-Oxidant Assay

DPPH (1,1, -diphenyl-2- picrylhydrazyl) is a well known antioxidant assay based on electron-transfer that produces a violet solution (Aquino *et al*., 2001). This free radical is stable at room temperature and is reduced in the presence of an antioxidant molecule, from which they accept electrons or hydrogen radical to become a stable diamagnetic molecule, giving rise to colorless methanol solution. The free radical, DPPH was scavenged by ethanolic extract of polyherbal formulation with an IC_{50} value of 200 µg/mL. The graph is shown in figure 8.

While Superoxide scavenging activity is another easy way to evaluate the antioxidant activity of the molecules by determining nitro blue tetrazolium (NBT) reduction method by McCord and Fridovich, 1969. It is based on the ability of a drug to inhibit the reduction of NBT by superoxide, which is generated by the reaction of photoreduction of riboflavin within the system. The scavenging ability of lepam was found to be dose dependent with an IC_{50} value of 64.12 µg/mL (Fig. 6 b).

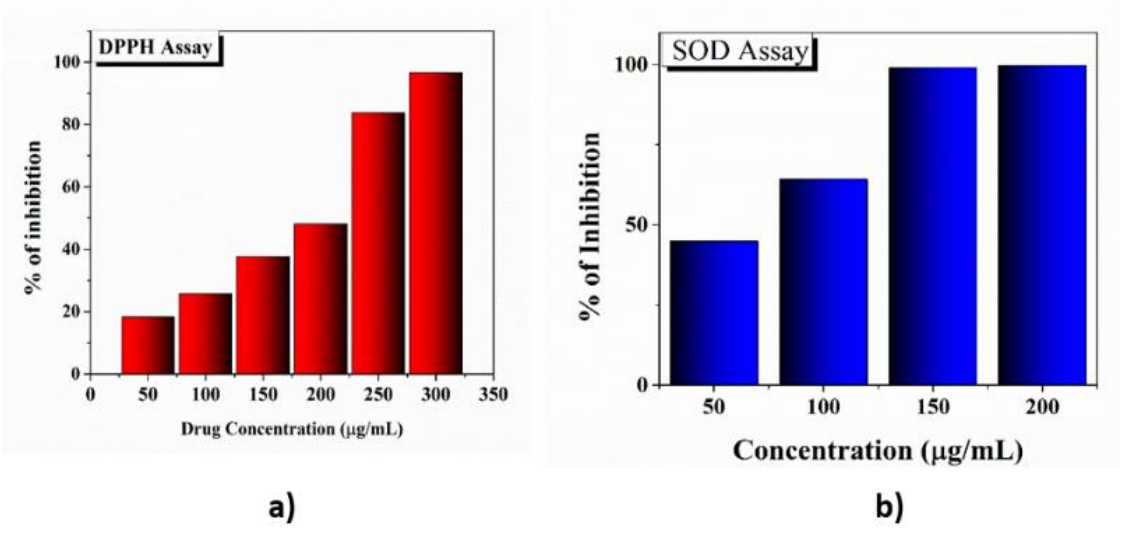


Figure 6: Anti-oxidant assay a) DDPH and b) SOD assay of polyherbal formulation

Anti-inflammatory activity-NO Scavenging Assay

The investigation of anti-inflammatory potential of MS in LPS-stimulated RAW264. 7 cells, shows significant reduction in NO production. The LPS treatment increased the production of NO, which was significantly reduced in the cells treated with MS in a dose-dependent manner. The inhibitory concentration compared to control was found to be 119.8 μg/mL (Fig. 12).

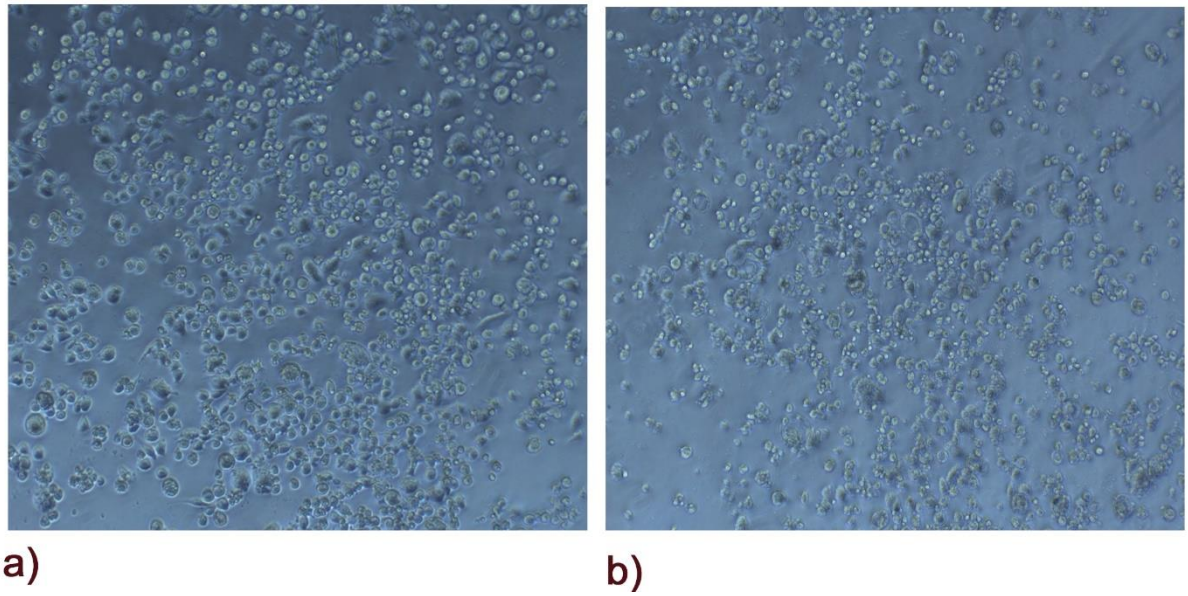


Figure 7: The nitric oxide radical scavenging activity of methanolic extract was explored and showed to have effectively scavenged NO with an IC_{50} value of 50 μg/mL (Fig. 13)

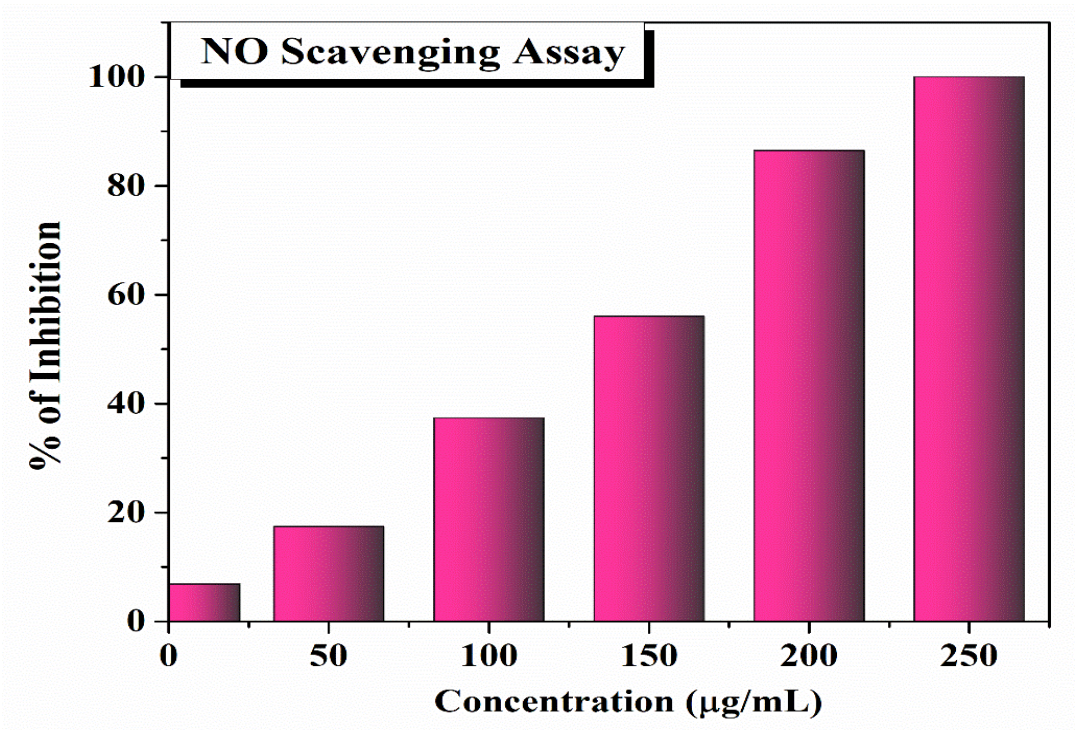


Figure 8: Anti-inflammatory activity of polyherbal formulation

This result emphasizes the enhanced cytotoxic nature of the prepared polyherbal formulation, which was dose dependent. Therefore, the IC_{50} profiles of the polyherbal formulation in DLA cell lines were determined by trypan blue assay for different concentration as shown in Figure 9. The dose-response curve was fitted with Hill Equation as follows [39].

$$\frac{E}{Emax} = \frac{1}{1 + \left(\frac{EC\ 50}{|A|}\right)^{n}}$$

where E_{max} is the maximum percentage of inhibition, EC_{50} is the 50% inhibition, n is the Hill coefficient A is the drug concentration. It was found the polyherbal formulation has very low IC_{50} value of 20 mg/mL that emphasis its high apoptotic effect against cancer cell lines.

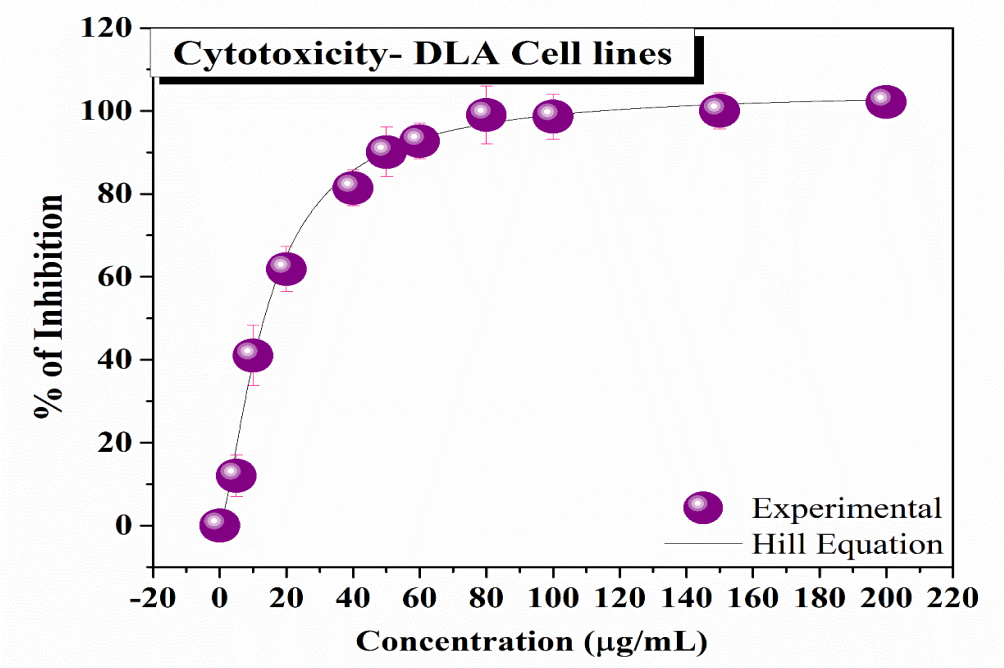


Figure 9: Dose response curve of the drugs towards DLA cells to find out IC_{50}

Cytotoxicity-EAC Cell lines

The same procedure was repeated for EAC cell line and their dose response cure were shown in Figure 10. The dose-response curve of polyherbal formulation was fitted with Hill Equation and it is found that IC_{50} value of 62 mg/mL.

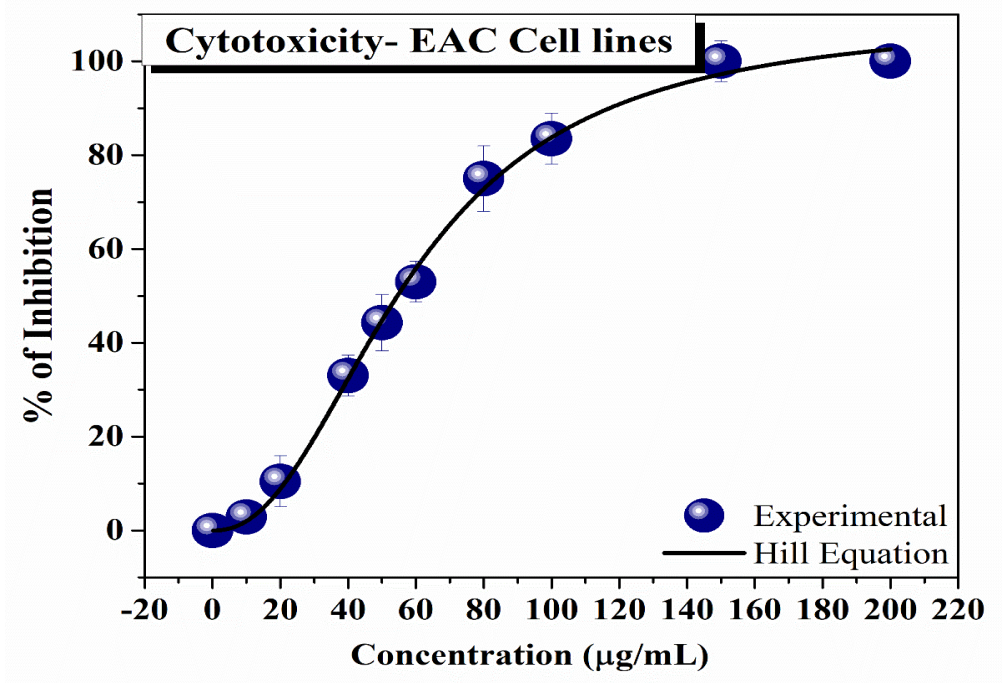


Figure 10: Dose response curve of the polyherbal formulation towards EAC cells to find out IC_{50}

CONCLUSION

A novel polyherbal formulation named Ayurgreen Natura Pain Gel were prepared using specified plant parts of dried aloe vera and fresh aloe vera pulp, frankincense, myrrh, ferula asafetida with natural binders like magnesium silicate and a clay mineral. It is found that the prepared polyherbal formulation exhibited high efficacy due to the presence of active phytochemicals which may enhance their potency due to the synergetic interaction of active ingredients of different plants. The phytochemistry of Ayurgreen Natura Pain Gel has been evaluated using a liquid chromatography-mass spectrometer and revealed the presence of 40 phytoconstituents contains a variety of chemical compounds including phenolics, flavanones, furans, gallotannin, glucoside, oligosaccharide, acids with different biological activities like anti-inflammatory, anti-bacterial, ant-fungal, anti-viral and anti-cancerous. The bioactive functional groups were characterized using Fourier Transform Infrared Spectroscopy and UV-Visible spectroscopy.

Moreover, the thermal analysis was performed using differential scanning calorimetry and revealed the presence of volatile ingredients, melting, and degradation temperature. Invitro anti-inflammatory, antioxidant and short-term cytotoxicity assay were performed to to evaluate the biological activities of the polyherbal formulation. It contains phytosphingosine that enhances the permeability of chemical compounds through the skin's barrier. Further, the anti-inflammatory activities of this polyherbal formulation showed remarkable activity with IC_{50} 119.8 µg/mL along with antioxidant activity with an IC_{50} 200 µg/mL. Fascinatingly, the wild habitat contained some anticancerous phytoconstituents which might be responsible for enhanced anti-cancerous activity in mice cancer cell lines (Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cell lines with IC_{50} 62 and 20 mg/mL.

Conflict of Interest

None declared.

Financial support

None declared.

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