Research Article

Development and Standardization of Novel Herbal Formula for the Management of Liver Disease

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ABSTRACT

Ethanomedicinal plant like *Gymnosporia montana* belonging to the family Celeastraceae commonly known as Vikalo in Gujarat. Ethanomedicinally fresh leaves of Vikalo are chewed in trible regions of Gujarat to cure jaundice. To develop drugs from these new sources, additional work is required for preclinical and clinical results. Since ancient times, different plant part extracts have been used as traditional medicines against liver diseases. This knowledge may be useful in developing future powerful drugs. The present study deals with the development of novel herbal formula comprising of the Hydro-alcoholic (70%) extract of Gymnosporia montana. The Preformulation parameters and parameters for finished product (hard gelatin capsule) include uniformity of weight, disintegration time, moisture content, pH, phytochemical estimation were performed. Hepatoprotective activity of the finished product was performed By MTT assay against HepG2 cell line. The prepared novel herbal formula showed significant hepatoprotective activity against HepG2 cell line.

Keywords: Liver disease, novel herbal formula, hard gelatin capsule, HepG2 cell line.

INTRODUCTION

Plants have been used worldwide in traditional medicines for the treatment of various diseases and it is estimated that even today approximately 65-75% of the World's population rely only on medicinal plants as their primary source of medicines. India is one of the few countries in the World which has unique wealth of medicinal plants and vast traditional knowledge of use of herbal medicine for cure of various diseases [1, 2].

Herbal medicine is the oldest form of health care known to mankind. It is an integral part of the development of modern civilization. In herbal medicine plant based formulation are used to alleviate diseases. But the most important challenges faced by these formulations arise because of their lack of complete evaluation. So evaluation is necessary to ensure the quality and purity of the herbal product. It is very important to establish a system of evaluation for every plant medicine in the market, since the scope for variation in different batches of medicine is enormous[3, 4].

Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorder. Most of the hepatotoxic chemicals damage liver cells mainly by lipid peroxidation and other oxidative damages. Liver is the most important organ where drugs are structurally altered; resulting biologically inactive or active metabolites and some of these are toxic. Liver is exposed to drugs in higher concentration as whole of the drug pass through liver to reach systemic circulation. Thus, the liver is a vulnerable target of injury from various chemicals and drugs and disordered hepatic function is an important cause of abnormal drug handling. Further liver has capacity to recover from acute injury by hepatocellular regeneration with the production of new cells, which restore liver functions and normal tissue structure. Chronic liver injury, however often leads to fibrosis, scar formation and distortion of normal tissue architecture [5, 6].

Supporters of herbal medicine claim that herbs may treat and prevent diseases. This adds to a deep belief that these treatments are safe as they are natural and fit into the image of a gentle and therefore, harmless alternative to conventional medicine. Herbal remedies support natural healing phenomena through blocking the progression of the degenerative pathological processes. Modern medicine offers limited success in providing effective cure and there is a severe need to develop new drugs capable of healing toxic liver damages. In traditional systems of medicine, plants were claimed to be effective and used successfully to alleviate multiple liver disorder. But, evidence for efficacy is sparse. In spite of limitations, a number of herbal show promising effects, either experimentally in cell culture (in-vitro models), animal studies (in-vivo models), or even in clinical trials [7].

Gymnosporia montana (known as Vikro), occurring throughout the arid, dry areas of India, is traditionally claimed to be useful in various ailments. Very few reports on pharmacological activity of Gymnosporia montana are available. On the basis of its traditional and folk-lore claim of being useful in jaundice and inflammation, researchers have evaluated its leaf extracts for possible antiinflammatory and hepatoprotective activities. [8]

In poly-herbal preparations it will be very difficult if we want to estimate each and every ingredient in

term of their chemical constituent. But if few major constituents having particular therapeutic action indicated in the labelled can be pinpointed then these constituents should be estimated quantitatively along with the other parameters through which presence of all ingredients can be confirmed[9].

MATERIALS AND METHODS

Selection of plant material

Selections of plants for formulation are on the basis of their hepatoprotective activity previously studied using MTT assay against HepG2 cell line.

Formula for Polyherbal formulation:

The polyherbal formulation (capsules) contained the 70% Hydro-alcoholic extracts of *Gymnosporia montana* (leaves) and *Long pepper* (Fruits).

Preformulation studies

Preformulation parameters such as bulk density, tap density, Compressibility index, Hausner's ratio, and angle of repose were determined for the prepared herbal formula and the best trial batch were taken for capsule filling and further studies [10, 11].

Preformulation parameters

Bulk density, tap density and Carr's index [12, 13]

A weighed quantity (15g) of powdered material was taken in a 50ml measuring cylinder and recorded the initial volume (vo). tapped the contents and recorded the powdered volumes after 50 taps (v50).

Fluff density = w/vo g/cc

Tapped density = w/v50 g/cc

Carr's index = Tapped density- Fluff density/ Tapped density * 100

Value for Carr's index below 15 indicate excellent flowing material and value over 20-30 suggested poor flowing material.

Angle of repose [14]

A funnel was fixed at a particular height (1.5, 2.5, 3.5 cm) on a burette stand. A white paper was placed below the funnel on the table. The powdered drug passed slowly through the funnel until it forms a pile. The radius of the pile was noted down.

Angle of repose of the powder material was calculated by using the formula:

$\tan\theta = h/r$

$\theta = \tan (h/r)$

Where, h = height of the pile, r = radius.

Values for angle of repose 30^{o} usually indicate a free flowing material and angle 40^{o} suggest a poor flowing material.

Preparation of novel herbal formula by wet granulation method

The formulation preparation began with trials by adding a different ratio of binders and selecting the quantity of lubricants and preservatives, and finally the procedure was optimized. The polyherbal formulation (capsules) contained the hydro-alcoholic extracts of *Gymnosporia montana* (leaves) and *Long pepper* (Fruits). Preparation of Hard gelatin capsules by wet granulation technique using 5% starch paste as a binder. The wet mass was passed through sieve number 22 to obtain granules. The granules were dried at 45°C in a tray [15].

Standardization of polyherbal formulation (hard gelatin capsule)

Capsule evaluation

The hard gelatin capsules were evaluated for their description, average weight, weight variation, moisture content, disintegration time, pH and microbial load and compared with Indian pharmacopoeial standards [16].

Average weight

Twenty capsules were individually weighed and the average weight of the capsule was calculated.

Weight variation

The individual weights of the each capsule should be within the limits of 90% and 110% of the average weight.

Moisture content

Moisture content was determined by using automatic Karl Fischer titration apparatus.

Disintegration time

Disintegration test was performed using the digital microprocessor based disintegration test apparatus. One capsule was introduced into each tube and a disc was added to each tube. The assembly was suspended in water in a 1000 ml beaker. The volume of water at its highest point was at least 25 mm below the surface of the water and at its lowest point was at least 25 mm above the bottom of the beaker. The apparatus was operated and maintained at a temperature of 37 ± 2ºC.

pH value

pH of 1% solution was determined by using a digital pH meter.

Dissolution

Dissolution is considered as a tool for predicting rate of absorption and bioavailability in some cases, replacing clinical studies to determine bioequivalence of drug. We were added six capsules in the basket type dissolution apparatus containing distilled water as a dissolution media. The speed was set on 50 rpm for 1 hour and the sample was drawn at every 10 minutes and the amount of dissolved active ingredient in the solution was calculated as percentage dissolved in 1 hour.

Stability

Pharmaceutical products are generally studied for stability profile at accelerated temperature, humidity and also at different intensities of light. The studies were performed to determine the physical, chemical, and therapeutic changes occurring in the polyherbal capsule by extrinsic factors [17, 18].

(a) **Light**: Sample was stored in different intensities of light i.e. sunrays, fluorescent (tube) light, UV and infrared light for detection of degradation of powder material.

(b) **Temperature:** The effect of temperature on the stability of polyherbal capsule was checked by keeping all the capsule at different temperatures i.e. ambient, 35^{0}C, 50^{0}C, 55^{0}C, 65^{0}C for 30 minutes, 1, 3, and 6 hours.

(c) **Humidity**: The effect of humidity on the stability of capsule was checked by keeping the entire capsule at four different humidity percentage i.e. 30%, 50%, 70% and 90%.

Composition of capsule

Each 250 mg capsule contains:

Gymnosporia montana (leaves)	200mg
Long pepper (Fruits)	50mg

***In-Vitro* hepatoprotective activity of prepared polyherbal formulation**

MTT ASSAY[19, 20]

Microculture tetrazolium (MTT) assay

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3(4, 5dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically.

Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 3-lakh cells/ml using medium containing 10% fetal bovine serum.

Cells were seeded in a flat-bottomed 96-well plate and incubated for 24 hour at 37°C and in 5% CO2. Vero cell line was treated with different plant extracts at various concentrations (1000µg/ml, 500µg/ml and 100µg/ml) for 48 hours. Isoniazid +Rifampicin were used as a Positive Standard and The DMSO treated cells served as control.

Cells were then treated with MTT reagent (0.5 mg/ml as final concentration, i.e. 20µl/well of stock) for 4 h at 37°C. All the media and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; thiazolyl blue) reagent was removed from the wells and DMSO (200 µl) was added to each well to dissolve the formazan crystals.

The optical density (OD) was recorded at 570 nm in a Microplate (ELISA) reader.21

Percentage of cell viability was determined as (Avg. OD of treated cells/Avg. OD of control cells) ×100.

% Growth inhibition = 100 - [Mean OD of individual test group/Mean OD of control group × 100].

RESULTS

The most important part of any formulation is standardization which ensures the quality, safety and reproducibility. It involves the complete process of bioprospection right from the collection of raw materials to development of finished product. In the present study, standardized polyherbal mixture was formulated in hard gelatin capsule.

Preformulation studies

Preformulation parameters like bulk density, tap density, Carr's index and angle of repose were obtained for the laboratory granules. The granules showed excellent flow property.

Table 2: Preformulation parameters

S. No.	Parameters	Results
1	Bulk density	0.7
2	Tap density	0.5
3	Carr's index	19.8
4	Angle of repose	15.26

As per the standards, the flow property of the blend to be filled in the capsule should be in good range and was confirmed by the above parameters. Trail batch III showed excellent flow characters and batch III was taken for capsule filling.

The trial III flow properties were Excellent and all parameters were within the Specified limits. So, Third trial was chosen for further studies.

Table 3: Evaluation of in process Parameters:

Parameter	Trial I	Trial II	Trial III	Trial IV
Flow property	Poor flow	Poor flow	Good	Fair
Uniformity of Filling	-	-	Uniform	Uniform
Uniformity of Weight	-	-	Uniform	Less weight

Standardization of formulation

Capsule evaluation

Description "light brown" coloured granules packed in "0" size blue capsules. The polyherbal capsules were evaluated for organoleptic characters which include colour, odour, taste and nature.

Table 4: Organoleptic Characters of Capsules

Parameters	Observation
Description	Light green granule in blue cap and body "0" size capsule
Colour	Light green granule
Odour	Characteristic odour
Taste	Characteristic taste

Table 5: Evaluation of capsules

Parameter	Observation
Average weight	Within limits
Weight variation	Within limits
Moister content(LOD)	2.96%
Disintegration time	10 mins 4 secs
pH(1% aqueous solution)	5.82± 0.17

Result (n=3) are reported as Mean ± Standard deviation

Table 6: In Vitro Dissolution Studies

Time (min)	Abs	Conc. (µg/ml)	Amt (mg/5ml)	Amt (mg/ml)	Amt (mg/900ml)	CDR	%CDR
0	0.038	9.72	0.0486	0.00972	8.748	8.75	3.49
5	0.296	136.81	0.68405	0.13681	123.129	123.13	45.36
10	0.375	183.43	0.91715	0.18343	165.087	165.10	64.21
15	0.483	217.18	1.0859	0.21718	195.462	195.45	75.79
20	0.512	245.62	1.2281	0.24562	221.058	221.10	86.00
25	0.634	279.89	1.39945	0.27989	251.901	251.9	93.71
30	0.756	283.37	1.41685	0.28337	255.033	255.0	98.99

Table 7: Effect of different intensities of lights on polyherbal capsules (500 mg)

Light Source	Sun light				Fluorescence				Tube light				UV Light				Infra-Red (IR)				Lamp Light			
Time of Exposure (hours)	1/2	1	3	6	1/2	1	3	6	1/2	1	3	6	1/2	1	3	6	1/2	1	3	6	1/2	1	3	6
500mg polyherbal capsule	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

(-) No change, (+) Degradation

Table 8: Stability test of polyherbal Capsule (500mg) at different Temperature

Storage condition	Testing condition	Time Duration (hours)				Result
		1/2	1	3	6	
Ambient	30°C	-	-	-	-	No change during 6 hours
Warm (30-40 °C)	35 °C	-	-	-	-	No change during 6 hours
Accelerated	50 °C	-	-	-	-	No change during 6 hours
Accelerated	55 °C	-	-	-	+	Degradation start after 3.5 hours
Accelerated	65 °C	-	-	+	+	Degradation start after 3.5 hours

(-) No change, (+) Degradation starts

Table 9: Stability of polyherbal Capsule (250 mg) at different Humidity with respect to different Temperature

Temperature	30% Humidity	50% Humidity	70% Humidity	90% Humidity
30%	-	-	-	-
35%	-	-	-	-
55%	-	-	+	++
65%	-	-	++	+++

(+) Degradation (-) No Change

***In-Vitro* Hepatoprotective activity of prepared polyherbal formulation:**

Polyherbal mixture shows significant hepatoprotective activity which is shown in Table 10.

Table 2: % Viability of Hep G_2 cell line of leaf extracts of *Gymnosporia montana*

Polyherbal mixture	Concentration		
	100 µg/ml	500 µg/ml	1000 µg/ml
Hydro-alcoholic Extract	65.37%	78.45%	**85.17%**

Stability

The stability parameters were analyzed for 30 minutes, 1, 3 and 6 hours of storage at accelerated conditions of temperature, light and humidity were found to be comparable. It was indicating that there gross physical characteristics does not produce any significant change, observation have been tabulated in table 4, 5 and 6 for three Stability parameters

DISCUSSION

Various types of herbal medicines have been used as curative agents in different parts of the world [24]. Drugs derived from traditional herbs may have possible therapeutic relevance in the treatment of illness [25].

Preformulation parameters including angle of repose (a traditional characterization method for pharmaceutical powder flow), porosity (packing geometry), Carr's index and Hausner's ratio (a measure of the interparticulate friction) are useful tools in the development of new formulation. A value of <30° indicates 'excellent' flow whereas >56° indicates 'very poor' flow. Based on this, the flow was rated as 'excellent' (Table-2). The CI was found to be 19.8. A lower CI ratio of a material indicates better flow properties than higher ones. A Carr's index of <10 is considered 'excellent' flow whereas CI>38 is considered 'very very poor' flow [26, 27]. Based on the results obtained (Table-2) flow of selected plant powder was rated as 'good'. Good flow of powder help to avoid the extensive costs and time involved in unloading powders that will not flow out of storage containers. As well as help to achieve the best formulation and improve the quality and consistency of the product.

Both the drugs were approved as quality drug when undergone by phytopharmaceutical evaluation according to the pharmacopoeial standards. 250 mg polyherbal capsules disintegrated in meantime 9.14±67 minutes and in vitro condition we determined the release of a drug from solid dosage form which the substance dissolved in the fluid of gastrointestinal tract. Results indicates that all of six capsules dissolved equal to 90% in 30 minutes and this releasing pattern of drug from their capsule shell in-vitro help in predicting the releasing sequence in-vivo that developing a tool for bioavailability of drug, as well as in some cases, replacing clinical studies to determine bioequivalence. In light of the phytopharmaceutical studies of the polyherbal capsule was found almost stable.

Polyherbal mixtures of selected plants were screened for their hepatoprotective activity. Polyherbal mixure of plant shows maximum hepatoprotective activity against HepG2 cell line. Further studies using more specific methods are required to explore the constituents responsible for the activity and the mechanism of this activity which might prove important and improved therapies for the treatment and prevention of liver diseases.

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