E-ISSN:2456-3110

Research Article

Liv.52

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Journal of Ayurveda and Integrated Medical Sciences

2025 Volume 10 Number 7 JULY

Publisher

Maharshi Charaka Ayurveda

www.maharshicharaka.in

Qualitative and Quantitative Standardization of Liv.52: A Polyherbal Formulation

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DOI:10.21760/jaims.10.7.9

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Standardization of a polyherbal formulation is essential for establishing the authenticity, quality and efficacy of finished herbal product. World Health Organization (WHO) has given the preliminary guidelines for standardizing herbal formulations. The present work is an attempt to standardize Liv.52, using simple, non-expensive spectrophotometric method, including phytochemical screening and determination of various physicochemical parameters. Liv.52 is a polyherbal ayurvedic formulation, is the world's best-selling liver support formula, used for liver strengthening and repairing the damaged liver cells. Phytochemical screening of Liv.52 showed the presence of phenols, flavonoids, steroids and alkaloids. The formulation found to contain total ash (7.8 % ± 0.2), water-soluble ash (3.0 % ±0.3) and acid-insoluble ash (1.4% ±0.1) within the prescribed standard limits. The alcohol extractive value of the formulation was found to be more, 38.62% compared to the water-soluble extractive value, 28.44%. The total phenolic and flavonoid content of the formulation was found to be 9.8% and 5.6%, equivalent to gallic acid and rutin respectively.

Keywords: Liv.52, Standardization, UV-Visible spectrophotometric, total phenols, total flavonoids

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How to Cite this Article

Metri S, Gopari S, Mathew C, Qualitative and Quantitative Standardization of Liv.52: A Polyherbal Formulation. J Ayu Int Med Sci. 2025;10(7):66-72. Available From https://jaims.in/jaims/article/view/4533/

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Manuscript Received	Review Round 1	Review Round 2	Review Round 3	Accepted
2025-05-24	2025-05-31	2025-06-07	2025-06-14	2025-06-28

Conflict of Interest	Funding	Ethical Approval	Plagiarism X-checker	Note
None	Nil	Not required	11.98	

Introduction

The basic resources of medicines come from nature and they are used as medicaments from ancient time to present day. People around the world possess unique knowledge of the natural resources on which they depend, including tremendous botanical expertise. The traditional medicines cater about 85% of the world population for their health needs. Indian healthcare consists of medical pluralism and Ayurveda still remains dominant compared to modern medicine, particularly for treatment of a variety of chronic disease conditions. In the present scenario according to World Health Organization (WHO) about 80% of the world population uses herbs, herbal and other traditional medicines for their primary health care needs. Tremendous raise in the use of herbal medicine is leading to a fast-growing market of polyherbal formulations worldwide. Thus, standardization in recent years, has been great demand in developed countries to maintain safety, quality and efficacy of the plant and their products to avoid and serious health problems.**[1,2]**

Standardization of herbal drugs means confirmation of its identity, quality and purity throughout all phases of its cycle by various parameters like morphological, microscopical, physical, chemical and biological observations. In order to have a good coordination between the quality of raw materials, in process materials and the final products, it has become essential to develop reliable, specific and sensitive quality control methods using a combination of classical and modern instrumental method of analysis. Specific standards are worked out by experimentation and observations, which would lead to the process of prescribing a set of characteristics exhibited by the particular medicines. Hence standardization is a tool in the quality control process.**[3-5]**

Liv.52 (Figure 1) is a hepatoprotective polyherbal Ayurvedic medicine manufactured by Himalaya Wellness Company, Bengaluru. It has been licenced by the Drug Regulatory Authority of the Government of India and the Ministry of Health and Family Welfare's Ayurveda, Yoga, Naturopathy, Unani, Siddha and Homoeopathy (AYUSH) department of (New Delhi, India).**[6]** It consists of many herbs including *Arjuna (Terminalia arjuna*), *Kakamachi* (*Solanum nigrum*), *Kasamarda* (*Cassia occidentalis*), *Tamarisk* (*Jhavuka -Tamarix gallica*), Yarrow (*Biranjasipha - Achillea millefolium*), caper bush (*Himsara - Capparis spinose*), wild chicory (*Kasani - Cichorium intybus*), *Mandur Bhasma* - Ferric Oxide. Liv.52 restores functional efficiency of liver by protecting hepatic parenchyma and promoting hepatocellular regeneration and indicated in management of jaundice, hepatitis a (infectious hepatitis), alcoholic hepatitis, early cirrhosis or pre-cirrhotic conditions, fatty liver disease orsteatohepatitis etc.**[7,8]** Keeping this in view, present study was carried out to standardize the polyherbal formulation-Liv.52.

Figure 1: Marketed formulation of Liv.52

Aims and Objectives

1. Preliminary phytochemical screening of Liv.52 for the identification of chemical groups.
2. Determination of physicochemical parameters of Liv.52 such as ash value and extractive values and heavy metals
3. Spectrophotometric determination of Total Phenolic Content and Total Flavonoid Content of Liv.52.

Materials and Methods

Preparation of extract

Liv.52 was procured from online pharmacy, PharmEasy, and 10 tablets equivalent to 5g were crushed into powder. The powder was subjected to extraction with ethanol on water bath at 40°C and filtrated. Filtrate was subjected to the chemical tests for the identification various phytoconstituents and determination total phenolics and flavonoids.

Phytochemical study

Extract was subjected to preliminary phytochemical investigation to identify presence of various phytochemical constituents in formulation.

Test for alkaloids

Mayer's test: To the filtrate few drops of Mayer's reagent was added. A cream- colored precipitate indicates the presence of alkaloids.

Dragendorff's test: To extract, 1 mL of Dragendorff's reagent was added. Formation of orange or orange red precipi. indicates presence of alkaloids.

Test for phenols:

Ferric chloride test: The extract was treated with 10% ferric chloride solution and observed for formation of deep blue colour, which indicates the presence of phenols.

Test for flavonoids

Alkali test: To small volume of filtrate few drops of sodium hydroxide solution was added. Intense yellow colour indicates the presence of flavonoids.

Shinoda test: To extract few fragments of magnesium ribbon & conc. HCL, drop wise, were added. Pink scarlet colour indicates presence of flavonoids.

Test for proteins

Biuret Test: Extract was treated with Biuret reagent for 4–5 min. If there is appearance of bluish-violet colour, it indicates presence of protein.

Test for carbohydrates

Molisch's Test: To the filtrate Molisch's reagent was added followed by addition of con H_2SO_4, formation of blue ring at junction of solutions, indicate the presence of carbohydrates.

Test for steroids

Liebermann–Burchard reaction: The extract was dissolved in chloroform and treated with few drops acetic anhydride followed by addition of concentrated sulfuric acid from the side of the tube, Brown ring with green colour in the upper layer/formation of deep red colour, indicates the presence of steroids/triterpenoids.

Sulphur powder test: Add small amount of sulphur powder to the test solution. It sink at the bottom indicates the presence of steroids.

Test for saponins

Froth formation test: Place 2 mL of solution of drug in water in a test tube, shake well. Stable foam is formed.**[9-11]**

Determination of physicochemical parameters

Ash value

The tablets were weighed equivalent to 2g and total ash value was determined according to the standard procedure. The total as than utilized to determine water soluble ash and acid-insoluble ash following the standard protocols.

Extractive Value

5 gm of the tablet powder was subjected to the maceration with alcohol and water separately with frequent shaking for 24 hrs, to determine respective alcohol and water soluble extractive values.**[12-14]**

Determination of heavy metals

Preparation of sample Solution:

Weigh 1g of sample and dissolve the sample in 23mL of water add 2mL of dilute acetic acid, adjust the PH 3 to 4 by using acetic acid solution, transfer to Nessler cylinder and dilute 35 mL of water. Now add 10mL hydrogen sulphide solution, mix well and finally make the volume to 50mL with water.

Preparation of standard solution:

Take Nessler cylinder add 2mL ((1mL= 10ug/mL of lead) standard lead solution add 2mL dilute acetic acid solution and enough water to make 25mL. Adjust the PH of solution between 3to 4 using acetic acid or ammonia solution, transfer to Nessler cylinder and dilute to 35mL with water. Add 10mL of hydrogen sulphide solution. mix well and finally make the volume to 50mL with water.

Both solutions were kept aside for 10min and compared the colors of sample and standard against white background.**[15]**

Estimation of total phenolic content

Folin Ciocalteu's reagent method was used to determine Total Phenols Content (TPC) in the formulation.

Preparation of standard gallic acid stock solutions:

Gallic acid was used to make a standard calibration curve. Accurately weighed 10 mg of gallic acid was transferred to 100 mL of volumetric flask and ¾th of 50% alcohol was added and sonicated for 10 mins and the volume made up to the mark (100µg/mL).

Preparation of standard working solution:

The stock solution 1, 2, 3, 4, and 5mL pipetted out in 10 mL volumetric flask and diluted with 50% of alcohol to get concentrations of 10, 20, 30, 40 and 50 µg/mL.

1 mL solution of each dilution was pipette out into 10 mL of volumetric flask and diluted to 3 mL with distilled water. Then 0.5 mL of 1:1 diluted Folin Ciocalteu's reagent and 2 mL of Na_2CO_3 (2.0% w/v) were added in each volumetric flask and vortexed. Final volume was adjusted to 10 mL with distilled water. Each volumetric flask was warmed and absorbance was measured at 765 nm using UV/VIS spectrophotometer (Jasco V630) against blank, containing distilled water

Preparation of sample solution

Take 10 tablet (5g) and extract with 50 mL70% alcohol on water bath for 15 min and filtered. The filtrate was evaporated to dryness. The residue was dissolved in 70% alcohol and filtered. 1 mL filtrate was taken in a volumetric flask and proceed with the similar steps as mentioned preparation of standard solution.**[16]**

Estimation of total phenols was done on the basis of calibration curve of gallic acid and the results were expressed as percentage W/W.

$$\text{Total Phenolic Content (\% W/W)} = \frac{\text{GAE x V x D x}10^{-6}}{\text{W}} \text{ x } 100$$

GAE - Gallic acid equivalent (µg/mL), V - Total volume of sample (mL), D - Dilution factor,

W - Sample weight (gm).

Estimation of total flavonoid content

Aluminium chloride method was used to determine Total Flavonoid Content in the formulation.

Preparation of standard rutin stock solutions:

Rutin was used to make standard calibration curve. Accurately weighed 10 mg of rutin was transferred to 100 mL of volumetric flask & ¾th of 70% alcohol (diluent)was added & sonicated for 10 mins & volume made up to mark (100µg/mL).

Preparation of standard working solution:

From the stock solution 0.5, 1, 1.5, 2 and 2.5mL pipetted out in 10 mL volumetric flask and diluted with 70% of alcohol to get concentrations of 05, 10, 15, 20 and 25 µg/mL.

1 mL from each of solution was pipetted out in 10 mL volumetric flask. To this solution 1.5 mL of 95% methanol, 0.1 mL of 10% aluminium chloride & 0.1 mL of 1 M potassium acetate were added & mixed well. Finally, solutions were diluted to 10 mL with distilled water & incubated at room temperature for 30 min. Absorbance of resulting solution was measured at 415 nm with UV/ VIS spectrophotometer.

Preparation of sample solution

Take 10 tablet (5g) and extract with 50mL 70% alcohol on water bath for 15 min and filtered. The filtrate was evaporated to dryness. The residue was dissolved in 70% alcohol and filtered. 1 mL filtrate was taken in a volumetric flask and proceed with the similar steps as mentioned preparation of standard solution. The blank solution was prepared by replacing the amount of 10% aluminium chloride with same amount of distilled water.**[17, 18]**

The determination of total flavonoid content was done on the basis of calibration curve of rutin and the results were expressed as percentage w/w.

Curve of rutin and the results were expressed as percentage w/w

$$\text{Total Flavonoid Content (\% W/w)} = \frac{\text{RE x V x D x}10^{-6}}{\text{W}} \text{ x } 100$$

RE–Rutin equivalent (µg/mL), V - Total volume of sample (mL), D - Dilution factor, W - Sample weight (gm)

Statistical analysis

The experiments were conducted in triplicate and the data were analyzed as mean ± S.D. The graphs were plotted using M.S Excel 2007.

Results

Table 1: **Results of preliminary phytochemical analysis**

Phytochemical compound	Observation	Inference
Test for alkaloids	No cream precipitate	+
Test for phenolics	Deep blue colour	+
Test for flavonoids	Yellow colour turns colorless	+
Test for carbohydrates	Blue ring	+
Test for saponins	No foam	-
Test for steroids	Brown ring was formed	+
Test for proteins	No blue colour	-

(+) indicates present, **(-)** indicates absent.

Preliminary phytochemical analysis

The ethanolic extract of Liv.52 was qualitatively tested using various chemical tests. The results listed in Table 1.

Physicochemical analysis

Table 2 reveals the values of different physico-chemical parameters Liv.52.

Table 2: Physicochemical analysis.

SN	Physicochemical analysis	Values (%w/w ± SD, n=3)
Ash values		
1.	Total ash	7.8±0.2
2.	Water soluble ash	3.0 ±0.3
3.	Acid insoluble ash	1.4 ±0.1
Extractive values		
4.	Alcohol soluble extract	38.62 ±0.04
5.	Water soluble extract	28.44 ±0.2

Toxicological Analysis

Heavy metal in the form of arsenical compounds are exceptionally toxic and harmful human health. The formulation passes the limit test for heavy metal.

Determination of total phenols

Total phenols Content of formulation was determined by Folin Ciocalteu reagent method. The standard calibration plot of gallic acid was constructed in the concentration range of 10-50 µg/mL and the co-efficient of determination (R^2) was found to be 0.9996 (Figure 2). Based on standard plot of gallic acid (y= 0.0075x+0.021), the formulation was found to contain 9.8 % total phenols (Table 3).

Table 3: Data for calibration plot of gallic acid and sample

Conc. (µg/mL)	Abs (AM±SD, n=3)
10	0.157 ± 0.003
20	0.341 ± 0.001
30	0.470 ± 0.014
40	0.632± 0.006
50	0.796± 0.005
Sample	0.962± 0.010

Determination of total flavonoids

Quantification of total flavonoid was done on the basis of standard calibration plot of rutin. It was constructed in the concentration range of 5-25 µg/mL and the coefficient of determination (R^2) was found to be 0.9997 (Figure 3).

Based on the standard plot (y=0.003x+0.02), the formulation found to contain 5.6% of flavonoids (Table 4).

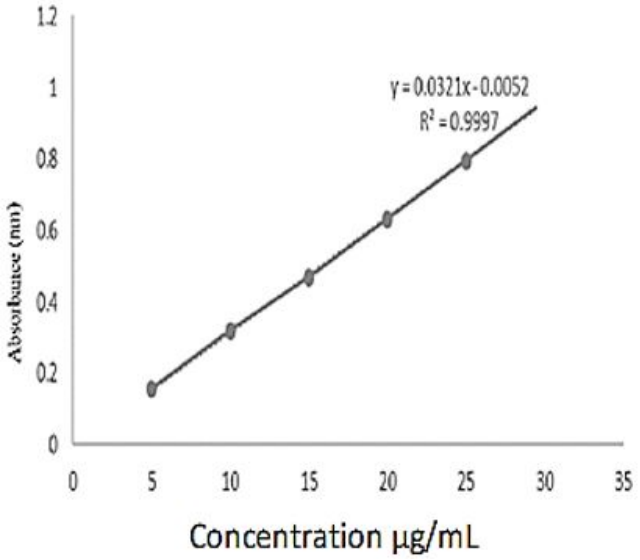

Figure 2: Calibration plot of gallic acid

Table 4. Data for calibration plot of rutin and sample

Con. (µg/mL)	Abs (AM±SD, n=3)
5	0.077 ± 0.002
10	0.151 ± 0.001
15	0.221 ± 0.034
20	0.298 ± 0.047
25	0.372 ± 0.032
Sample	0.372± 0.011

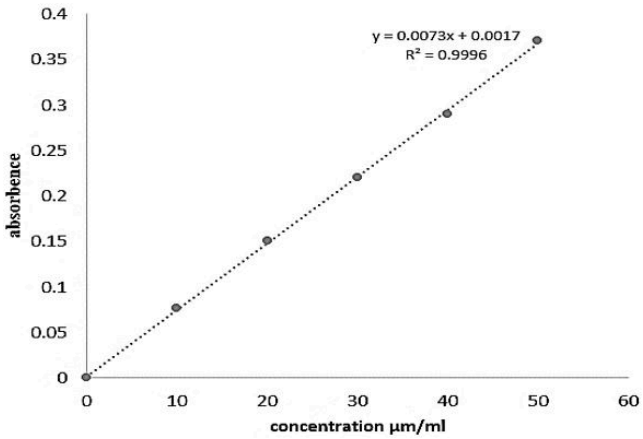

Figure 3: Calibration plot of rutin

Discussion

Phytochemical profile is of special significance as it direct the pharmacological activity of the herbal drugs which influence their therapeutic effects. These compounds, like flavonoids, alkaloids, glycosides terpenoids etc.,

Interact with biological targets to exert pharmacological actions. Variations in phytoconstituents can alter potency, efficacy, and safety of herbal drugs. Thus, standardizing phytochemical composition ensures consistent and predictable drug activity. The metanolic extract of Liv.52, gave a quick reactions to chemical tests used for their identification, showing the richness in the phytoconstituents. The extract showed the presence of alkaloids, glycosides, tannins, flavonoids and phenols.**[19]**

Physico-chemical standards are rarely constant but very important for the evaluation of herbal drugs. It indicates identity, purity, and quality of herbal drugs. Ash values represents the inorganic content naturally present or adhering or deliberately added to it, in as a form of adulteration. Extractive values are indicative of approximate measures of chemical constituents. **[19]** The Liv.52 found to contain, 7.8% of total, 1.4% of acid insoluble and 3.0% water soluble ash falling within the prescribed limit.**[20]**

Naturally occurring polyphenolic chemicals called phenols and flavonoids have a variety of biological activities, including strong free radical scavenging activity due to their capacity to neutralize the free radicals. The presence of these active constituent are quantified using FC reagent (total phenols) and aluminum chloride (total flavonoids) method.[21] In the present investigation the ethanolic extract of Liv.52 showed the presence of 9.8% and 5.6% of total phenols and flavonoids respectively.

Conclusion

The present study attempted to standardize the formulation according to WHO guidelines in terms of qualitative and quantitative parameters. UV-Visible Spectrophotometric determination of Total Phenolic Content and Total Flavonoid Content was carried out to estimate the phenolic and flavonoids content in the formulation. Preliminary phytochemical screening of Liv.52 showed the presence of phenols, flavonoids, steroids and absence of alkaloids. The formulation found to contain total ash, water-soluble ash and acid-insoluble ash was found within the prescribed standard limits. It was concluded that the present study describes the simple spectrophotometric method for the quantitative analysis of phenols and flavonoids in LIV.52, which can be utilized as a quality control method in conjugation with other analytical methods.

The future scope of the study to identify and quantify the suitable chemical/biological marker in the Liv.52 formulation.

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