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FORMULATION AND STANDARDIZATION OF LOHASAVA: AN AYURVEDIC AASAVA FORMULATION

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ABSTRACT
Background: Plant solutions or decoctions are fermented with added sugar to create alcoholic remedies like arishta and aasava. Standardizing ayurvedic formulations is essential to assessing the quality of medicine. **Objective**: Standardisation of Lohasava, which contains iron as a metal and is used as hematinic, has been carried out in the current study. **Methods**: The standard ayurvedic procedure was used to prepare the aasava formulation. Modern scientific control processes have standardized the formulation for the final products. Organoleptic analysis, phytochemical assessment, and physicochemical characteristics such as pH, specific gravity, viscosity, acid values, total solid content, alcohol content, heavy metal content, and stability studies were used to standardize asava. Additionally, formulations were examined for the presence of pesticides. Using UV-visible spectrophotometric analysis, the iron content was determined. Animal studies were carried out to evaluate pharmacological activity. **Results**: Physical and chemical parameters were found within limits. The alcohol content of formulations was within limits and indicated good fermentation. **Conclusion**: The study's findings have revealed good formulation quality and provide a standard for aasava and ayurvedic formulations.

INTRODUCTION

Herbal medicine, also called phytomedicine, involves medicinal constituents from plant roots, leaves, flower seeds, barks, etc. can be used to treat health-related problems. Ayurveda, an ancient medicinal system of India, has been in practice for several years. Ayurvedic pharmacopoeia contains more than twelve hundred species from plant origins and approximately a hundred from minerals and animal origin. Ayurvedic medicines frequently contain ingredients derived from plants, minerals, metals, and animals. The practice of raising a herbal medicine preparation's concentration of one or more components with known therapeutic benefits to a predetermined level is known as standardization [1]. The liquid preparation known as asava facilitates extracting the active components. It consists of alcoholic solutions containing all the active ingredients in the

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prepared drugs. Arishta and Aasava are considered the best formulations in Ayurveda because they preserve better, maybe because of fermentation's role in preservation [2]. Ayurvedic medications come in various forms, but their fermented counterparts, known as Arishta (fermented decoctions) and Aasava (fermented infusions), are valued for their medicinal properties and pleasant effects [3]. Because of their effectiveness and distinct qualities, two of the many Ayurvedic medicinal forms—fermented decoctions, or Arishta—and fermented infusions, or Aasava—are recognized as excellent medicines. These preparations are fermented by adding dhataki (Woodfordia fruticosa Kurz) flowers as a source of sugar [4].

The alcohol content of these formulations ranges from 12% by volume to 15%, and they have a pleasant, sweet taste with a hint of acidity and a pleasant aroma. Because alcohol is present in these preparations, they have superior therapeutic qualities, better-maintaining quality, and better medication distribution in human body sites. The uneven content of herbal remedies and the sporadic instances of intoxication by adulterants and/or hazardous ingredients have long been causes for concern. Quality control for herbal medications aims to guarantee their efficacy, safety, and consistency [5]. Understanding the molecular makeup of the complex formed between metallic ions and organic phytoconstituents found in medicinal plants is crucial when using metal-containing Ayurvedic medicines [6]. Determining the quality standards and comprehending the mode of action of such significant, powerful, and distinctive Ayurvedic/Siddha medications requires understanding the nature of the complex compounds created while processing metals, minerals, and medicinal plant material. Given the possibility of heavy metal contamination, the safety of such formulations should also be assessed [7]. The essence of standardization is ensuring that each medicine packet sold contains the appropriate amount of ingredients and will provide its intended therapeutic effect [8, 9]. Patients' and doctors' acceptance of medicinal plants and their preparations rises with standardization and quality control of the raw material or completed product [10, 11]. **Lohasava** is an iron-containing Aasava formulation used in anemia. The present study aimed to formulate lohasava in laboratories and standardize prepared formulations according to guidelines to set typical quality control standards. Formulating ayurvedic formulations in laboratories according to procedure was challenging, and good manufacturing practices were followed to assure the safety and efficacy of formulations. Lohasava formulations were assessed for several phytochemical components and quantitative factors like pH, acid value, and alcohol concentration. Toxic heavy metal levels were assessed; stability studies, active ingredient content determination, and pharmacological activity evaluation were also done in aasava formulations.

MATERIALS AND METHODS

Materials

All raw materials were purchased in powder form at a local market and were authenticated from Ayurveda Seva Sangh, Nasik, Maharashtra. All the experiments for pharmacological evaluation were approved by the Institute Animal Ethics Committee (IAEC) constituted under the provisions of the committee for control and supervision of experiments on Animals (CPCSEA), Ministry of Environmental and Forests, Government of India. Ethical guidelines were strictly followed during the experimental study (IAEC (MGV/PC/CPCSEA/ XXXVI/01/2018/18).

Methods

The prescribed quantity of jaggery was dissolved in hot water (Approx. 250 ml) contained in the cleaned fermentation flask (glass; 350ml). It was boiled for half an hour and then cooled to room temperature. All ingredients (table 1)were accurately weighed (Powder form) and passed through sieve no 44, and ingredients except Dhataki Pushpa were added to the final mixture. The mixture was stirred for 15 minutes, and then Dhatakipusha (*Woodfordia fructicosa*) was added. The flask was closed with a cleaned lid wrapped around using clay-smeared cloth. The vessel was then fermented in a dark place for 40 days [12]. After 40 days, the vessel was removed from the dark. Formulations were filtered through muslin cloth and placed in amber-colored bottles. Three batches were prepared using the same procedure.

Standardization of Aasava formulation

Preliminary Evaluation: Prepared formulations were observed for odor, taste, color, and clarity.

Assessment of phytochemicals: Formulations were tested chemically for carbohydrates, saponins, and tannins [13].

Determination of physicochemical parameters

A calibrated digital pH meter was used to assess the pH of formulations. Other physicochemical parameters, such as specific gravity, viscosity, and acid value, were determined [12, 14, 15].

Determination of Alcohol content

Method 1: Prepared aasava (25 ml) was taken in a distillation flask and diluted with distilled water (150 ml). Pumice powder was added to it and then connected for distillation. Distillate (approx. 90 ml) was collected and diluted up to 100 ml with distilled water at 24.9° to 25.1°C. Relative density at 24.9° to 25.1°C was calculated and the alcohol content was calculated from the table [14].

Gas chromatography (Method 2): The amount of ethanol in prepared formulations was determined using FID and a straightforward, quick, accurate, and exact GLC method. For GC analysis, Carbowax 20M (stationary phase) was packed into a steel column with an internal diameter of 2 mm. As the carrier gas, nitrogen was employed at a flow rate of 1 kg/cm^2/min [16].

Quantitative determination of heavy metals

The principal heavy metals were quantitatively evaluated using a Perkin Elmer-400 Atomic Absorption Spectrometer (ASS), with argon serving as the carrier gas and a flow rate of 1 milliliter per minute. The method was adopted as given in the literature. Standard and sample were aspirated in flame absorbance recorded, and % of heavy metals were determined [17, 18].

Determination of pesticides in formulations

Aasava sample (10 gm) was taken in a round bottom flask, and sodium sulfide was added to it with 100 ml n-hexane. It was refluxed for 1 hour. The filtrate was taken in a separating funnel and extracted with 50ml and 25ml of acetonitrile. The acetonitrile layer was mixed with 500 ml de-mineralized water with 2.5 ml saturated sodium sulfide, shaken in a separating funnel with an n-hexane layer, and evaporated on a water bath. The residue was used to analyze organochlorine, organophosphate, and carbamate pesticides [19, 20].

Determination of iron content by colorimetry

Iron (II) reacts with 1, 10 phenanthroline to form an orange-red complex ion. The intensity of the colored species is measured using a UV-visible double-beam spectrophotometer (UV-2450PC Shimadzu) [21].

Preparation of standard solution

Ferrous sulfate 0.2482 gm was weighed and dissolved in water using dilute sulphuric acid. It was diluted to 100 ml with distilled water. Using a 25 ml volumetric flask, a standard series ranging from 2 to 10 µg/ml was prepared. Sodium acetate 0.2 M (4.0 ml) and 1, 10-phenanthroline 0.25% (2 ml) were added. Distilled water was used to mark the volume.

Preparation of sample solution

For each formulation (A, B, C), 1 ml was diluted to 100ml with distilled water.1 ml from the diluted solution was treated the same as above. The absorbance of all the solutions was noted at 515 nm. The absorbance was screened in the visible range. λmax was observed at 515 nm. The calibration curve (absorbance versus concentration) is constructed by taking the absorbance at 515 nm. The blank was water, 2 ml 0.25% 1, 10 phenanthroline, and 4 ml of 0.2 M sodium acetate. The concentration of the unknown iron formulation was determined from the calibration equation [22].

Estimation of haematinic activity

Estimation of haematinic activity by Phenyl hydrazine-induced anemia

Adult male rats weighing 140-155 g were divided into six groups, each containing six animals. The rats received phenyl hydrazine (4 mg/ 100 g for 6 days) with 1.0 ml of vehicle, formulation A, B, C, or Dexorange daily for 5 days. One group received the vehicle alone. On the seventh day, the rats were lightly anesthetized with anesthetic ether, and blood was withdrawn by tail nibbling. The erythrocyte count was measured using Neubauer's counting chamber [23].

Stability study

The stability study was assessed for 2 months at 400°C and a relative humidity of 75. After 2 months, changes in color, odor, taste, and pH were observed [24].

RESULTS

Preliminary evaluation

All Formulations were examined for color, odor, and taste: all three batches' formulations were brown with an alcoholic smell and astringent and slightly bitter taste.

Phytochemical evaluation

A preliminary phytochemical screening was conducted [25] to investigate the presence of significant metabolites such as flavonoids, tannins, carbohydrates, saponins, steroids, and phenols, as well as glycosides, alkaloids, proteins, free amino acids, oils, and fats. For this, a standard chemical test is

frequently employed. Phytochemical tests of Lohasava showed the presence of carbohydrates, saponins, and Tannins (Table 2)

Table 2: Phytochemical Investigation of Formulations of Lohasava

Test	A	B	C
I . Carbohydrates:			
A) Molisch Test	+	+	+
B) Fehling's Test	+	+	+
II. Phytosterols.	–	–	–
III Proteins a) Millon's Test	–	–	–
IV. Tannins.			
A)5%$Fecl_3$ Solution	–	–	–
B)Dil.$KMNO_4$	+	+	+
V. Saponins	+	+	+
VI. Flavonoids	-	-	-

(+) indicates present & (-) indicates absent

Determination of pH, specific gravity, viscosity, and acid value

Aasava is an acidic formulation, so the pH of the formulations was analyzed. The pH of the formulations from all three batches A, B, and C after the fermentations was found to be 4.803± 0.005, 3.893±0.0008, and 3.79±0.007respectively. It revealed that formulations from batches A and B (higher pH values) fermented better than batch C. This may be attributed to alcohol content after fermentation. Other parameters like specific gravity viscosity and acid values were also measured (Table 3).

Table 3: pH, specific gravity, viscosity, and acid value of Lohasava formulations (n=3)

Batch	pH	Specific gravity	Viscosity (cp)	Acid value
A	4.803± 0.005	1.077 ± 0.002	1.033 ± 0.002	1.215±0.116
B	3.893 ±0.0008	1.092 ± 0.004	1.052 ± 0.005	1.529±0.031
C	3.79± 0.007	1.033 ± 0.004	1.072 ± 0.002	1.775±0.114

Two methods were used to detect the alcohol level in formulations: simple distillation and a specific GLC method. Both methods revealed comparable values and were found within limits (Table 4).

Table 4: Alcohol content

Batch	% Alcohol content (± SEM) Method 1	% Alcohol content (± SEM) Method 2
A	2.12±0.005	3.13 ±0.05
B	1.59±0.005	1.35±0.017
C	1.516±0.006	1.30 ±0.06

In GLC method calibration plot was used to calculate alcohol content in formulations (Figure1)

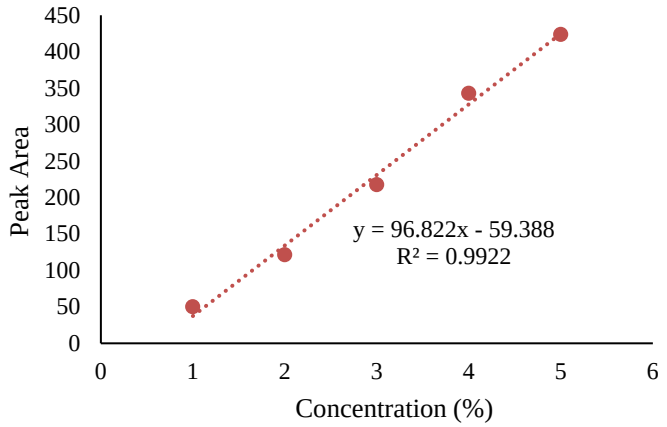

Figure 1: Linearity graph of area of GC peak versus concentration

Evaluation of heavy metals of formulations by AAS

Quantitative determination of heavy metals in formulations was done by atomic absorption spectroscopy, and the results were within WHO's prescribed limits (Table 5).

Table 5: Heavy metal content

Batch code	Arsenic (10.0 ppm)	Lead (10ppm)	Mercury (1.0ppm)	Cadmium (0.30ppm)
A	0.017	0.092	0.009	0.0004
B	0.014	0.081	0.004	0.003
C	0.016	0.072	0.012	0.015

Determination of pesticides in formulations

Pesticide of formulations were evaluated qualitatively by different chemical tests (Table 6).

Estimation of iron content

Iron in the Fe+2 state forms a colored complex with 1,10 phenanthroline, which shows absorbance at 515 nm. Standard solutions (3-15 µg /ml) formed an orange-colored complex with 1,10 Phenanthroline and scanned at 515nm (Figure 2). Iron content from formulations (Table 7) was determined by calibration plot and line equation Y=0.0296X+0.8133 (Figure 3)

Table 6: Pesticide evaluation by chemical tests

Batch code	Test	Observation	Result
A	Organochloro	No colour	Dichloropropone (-)
	Organophosphate	No colour	Phosphate (-)
	Carbamate	No colour	Amide group (-)
B	Organochloro	No colour	Dichloropropone (-)
	Organophosphate	No colour	Phosphate (-)
	Carbamate	No colour	Amide group (-)
C	Organochloro	No colour	Dichloropropone (-)
	Organophosphate	No colour	Phosphate (-)
	Carbamate	No colour	Amide group (-)

(-) indicates absent

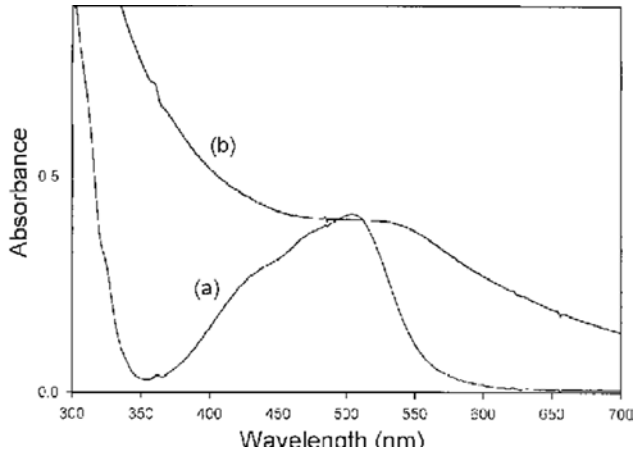

Figure 2: Absorption spectrum of iron and 1,10 phenanthrene complex

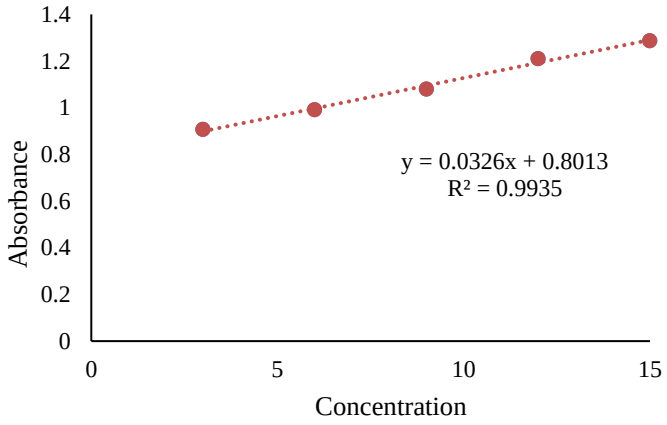

Figure 3: Calibration plot of absorbance vs concentration

Estimation of haematinic activity by Phenyl hydrazine-induced anaemia

The haematinic activity of Lohasava formulations was performed on Phenyl hydrazine-induced anemia in rats. A maximum rise in RBC content (106/mm^3) was observed for brand B (Table 8).

Table 7: Estimation of iron in Lohasava formulation

Batch code	Absorbance	Concentration (µg/ml) (n=5)
A	0.974	4.19±0.002
B	0.985	5.07±0.031
C	0.941	3.42±0.007

Table 8: Effect of Lohasava on Phenylhydrazine-induced decrease in RBC count in rats

Group	RBC content (10^6/mm^3)
Vehicle:	5.64 ± 0.3 x 10^6
Phenylhydrazine (4 mg/100 g for 6 days)	3.1 ± 0.4 x $10^{6\#}$
Phenylhydrazine + A	4.84± 0.13 x 10^{6*}
Phenylhydrazine + B	4.9 ± 0.3 x 10^{6*}
Phenylhydrazine + C	4.80 ± 0.18 x 10^{6*}
Phenylhydrazine + Dexorange	5.01 ± 0.5 x 10^{6*}

F4, 20 = 7.38, P = 0.0001, n=6, * p<0.05 (One-way ANOVA followed by Dunnett's test)

Stability studies

The stability study of prepared aasava was evaluated (Table 9). For 60 days, no significant change in color, odor, or taste was noticed in all batches. Slight changes were noticed in the pH of all batches at the prescribed temperature during storage.

Table 9: Stability study data

Parameter	Time (days)	Observations		
		A	B	C
Color	60	No change	No change	No change
Odor	60	No change	No change	No change
Taste	60	No change	No change	No change
pH	0	4.803± 0.005	3.893 ±0.0008	3.79± 0.007
	30	4.701±0.002	3.83±0.002	3.80±0.005
	60	4.70±0.004	3.63±0.001	3.65±0.021

DISCUSSION

To push India as a significant player in the global herbal product market, herbal products should be standardized as per WHO guidelines. Three important parameters are required for Standardization and quality control of herbal medicines: Authenticity, Purity, and Assay. Three different batches of Lohasava formulations, as per the documented method, and after the allotted fermentation period, were tried to standardize as per a few guidelines set by WHO. Evaluation of the physicochemical parameters of Aasava was necessary to assess the acidic properties and determine the level of total alcohol generated during storage. pH, acid value, viscosity, and specific

gravity were evaluated after the fermentation process was completed. Acid values were found to be 1.215±0.116, 1.529±0.031and 1.775±0.114 of three batches with pH values 4.803± 0.005, 3.893 ±0.0008and 3.79± 0.007, respectively. It indicates the weak acidic properties of all three Aasava formulations. Aasava is a self-generating alcohol formulation that has acidic properties. Alcohol levels of all Aasava formulations were determined by two methods. One was simple distillation and specific gravity, and the other was a Simple, accurate, and precise GLC method. %Alcohol content by simple distillation and specific gravity method was comparable for all batches. The level of alcohol was found to be within limits and also less than rectified spirit. It indicates that all three Aasava generate alcohol during processing by fermentation, but it was within the limit as per the label claim. Quantitative estimation by atomic absorption spectroscopy resulted in the presence of heavy metals like arsenic, lead, cadmium, and mercury, but was within limits prescribed by WHO. Results of color tests of pesticide evaluation indicated the absence of toxic pesticides like Organochlorine, Organophosphate, and carbamate types of pesticides in all three Aasava formulations. A selective analytical method is recommended for pesticide and Alfatoxins analysis to ensure safety and quality and completely standardize medicinal plants. Iron content was found to be different for three different brands using the colorimetric method. Brand B showed a maximum amount of 5.07±0.031µg/ml; then brand A showed an average of 4.19±0.002 and brand C showed an average of 3.40µg/ml. It indicates that Batch B has a higher iron content than Batch A and Batch C. Lohasava formulations were evaluated for their haematinic activity using an animal model of Phenylhydrazine-Induced anaemia in rats. These results correlate with the colorimetric method of iron estimation of Lohasava formulations. Stability testing was performed at 40°C and 75% relative humidity for 60 days. It showed slight changes in pH. Therefore, Lohasava formulations should be stored below 40°C and in less humid conditions.

CONCLUSION

Strict quality control is necessary to guarantee the efficacy and safety of herbal products. To guarantee the safety of herbal medications, overseeing the caliber of the finished product and customer education regarding herbal remedies is vital. Standardization of Ayurvedic drugs is not merely an analytical process; it also entails comprehensive information and controls, which are essential to ensure the uniform composition of all herbs and the identification of active ingredients. The current investigation involving the formulation and standardization of Lohasava using different physicochemical analytical and pharmacological methods provides an innovative strategy and an attempt to increase the number of analytical techniques that can be easily applied for the quality control of an ayurvedic formulation like asava. Prepared formulation of Lohasava and passed all standard tests confirming the quality of product such as pH, acid value, and alcohol content. All batches were also tested, and the heavy metal and pesticide contents were passed. Iron content was determined with the help of analytical techniques like UV visible spectrophotometry and correlated with pharmacological activity. The study's findings revealed the formulation's safety and efficacy.

FINANCIAL ASSISTANCE

Nil

CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHOR CONTRIBUTION

Manisha Tayde designed the study and experimental work. Sunita Mahale and Yogita Ahire collected and analyzed data. Sarika Patil and Anuja Bhosale reviewed the draft of the manuscript.

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