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USHIRASAVA-PHYSICOCHEMICAL STANDARDIZATION AND QUANTITATIVE ESTIMATION OF SOME BIOACTIVES

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ABSTRACT: Ushirasava is a commonly used ayurvedic formulation reported to be effective in Raktapitta (Bleeding disorders), Pandu (Anaemia), Kustha (Skin diseases), Prameha (Urinary disorders), Arsa (piles), Krmi (Worm infestation) and Sotha (Inflammatory diseases). It is a polyherbal hydroalcoholic product comprising of more than twenty-five ingredients. An exhaustive literature survey revealed that standardization of Ushirasava is not reported to date. The research work described here aims to standardize some marketed formulations of Ushirasava with respect to their physicochemical (Organoleptic properties, phytochemical, and microbial parameters. It also aims to develop and validate a new HPTLC method for the quantitative estimation of luteoin and rubiadin present in the test formulations of Ushirasava. The standardization of Ushirasava involved a determination of organoleptic and physicochemical properties, phytochemical screening, identification and quantitative analysis of some bioactive constituents by HPTLC. The 'protocol for testing Ayurveda, Siddha, and Unani medicines' was used as a reference. Luteolin and rubiadin were selected as the chemical markers for quantitative analysis. An HPTLC method was developed and validated as per the International Conference on Harmonization ICH-Q2 (R1). The results of physicochemical and microbial tests were compared with the specifications mentioned in the 'Ayurvedic Pharmacopoeia of India. The selected marketed samples of Ushirasava were subjected to AYUSH screening protocol and quantitative estimation by HPTLC. This HPTLC method was validated and used for the simultaneous estimation of rubiadin and luteolin in some selected marketed Ushirasava formulations. The results of this work will serve as a valuable tool for routine quality control of Ushrasava formulations.

INTRODUCTION: Ayurveda, often called "mother of all healing' is an ancient science practiced widely in India and has also gained tremendous attention globally for treating a wide variety of disease conditions. Asavas are one of the many types of formulations used in the Ayurvedic system of medicine [1].

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Ushirasava is one of the ancient, commonly used polyherbal Ayurvedic formulations. It comprises of *Nelumbo nucifera*, *Vetivera zizanioides*, *Gmelina arborea*, *Nymphaea stellata*, *Rubia cordifolia*, and 22 other plant ingredients.

It is prescribed for the treatment of in Raktapitta (Bleeding disorders), Pandu (Anaemia), Kustha (Skin diseases), Prameha (Urinary disorders), Arsa (Piles), Krmi (Worm infestation) and sotha (inflammatory diseases) [2]. Asavas and Arishtas are medicinal preparations made by soaking the herbs (drugs), either in powder form or in the form of decoction (Kasaya), in a solution of sugar or jaggery, as the case may be, for a specified period

of time, during which it undergoes fermentation generating alcohol, thus facilitating the extraction of active principles contained in the herbs. The self-generated alcohol also serves as a preservative in these formulations. Asavas have been used as medicines for over 3000 years to treat various disorders, including the treatment of diseases related to the digestive system. An extensive literature survey revealed that standardization of some ayurvedic formulations is reported.

The 'Protocol for testing Ayurveda, Siddha, and Unani medicines' describes the determination of organoleptic properties, physicochemical properties, and analytical method development and validation studies for the quantitative estimation of the bioactive present in them.

Various methods are used for the standardization of herbal drugs. Due to the complexity of most Ayurvedic formulations, the use of only conventional methods for standardization is not adequate for their evaluation. The Ayurvedic Pharmacopoeia of India and Pharmacopoeial standards of ayurvedic formulations mentions the study of physicochemical parameters and thin layer chromatography of raw materials and formulations, which are not sufficient for proper standardization in present era.

Over the last few years high-performance thin layer chromatography (HPTLC) has been extensively used for analysis of ayurvedic pharmaceuticals, plant extracts, bioanalytical samples and biomacromolecules. A major advantage of HPTLC is its ability to analyze polyherbal samples simultaneously utilizing a very small quantity of mobile phase, thereby reducing the cost of analysis. There were no reports on standardization of Ushirasava; hence, the present study was undertaken to standardize Ushirasava and also develop and validate a simple, accurate, precise, and robust analytical method for the simultaneous estimation of rubiadin and luteolin in Ushirasava by HPTLC. The composition of Ushirasava as mentioned in the Ayurvedic Pharmacopoeia of India is given below **(Table 1)** [3-5].

TABLE 1: COMPOSITION OF USHIRASAVA

S. no	Ingredients	Botanical name	Part used	Quantity (g)
1	Ushira	*Vetivera zizanioides*	Rt.	48
2	Balaka (Hrivera)	*Coleus vettiveroides*	Rt.	48
3	Kasmari (Gambhari)	*Gmelina arborea*	Fr.	48
4	Nilotpala (Utpala)	*Nymphaea stellata*	Fl.	48
5	Manjistha	*Rubia cordifolia*	Rt.	48
6	Padma	*Nelumbo nucifera*	Fl.	48
7	Priyangu	*Callicarpa macrophylla*	Fl.	48
8	Padmaka	*Prunus cerasoides*	St.	48
9	Lodhra	*Symplocos racemosa*	St. Bk.	48
10	Dhanvayasaka	*Fagonia cretica*	Pl.	48
11	Patha	*Cissampelos pareira*	Rt./ Pl.	48
12	Kiratatikta	*Swertia chirata*	Pl.	48
13	Nyagrodha	*Ficus benghalensis*	St. Bk.	48
14	Udumbara	*Ficus racemosa*	St. Bk.	48
15	Sati	*Hedychium spicatum*	Rz.	48
16	Parpata	*Fumaria parviflora*	Pl.	48
17	Pundarika (Kamala)	*Nelumbo nucifera*	Fl.	48
18	Patola	*Trichosanthes dioica*	Lf./Pl.	48
19	Kancanaraka	*Bauhinia varigata*	St. Bk.	48
20	Jambu	*Syzygium cumini*	St. Bk.	48
21	Salmaliniryasa	*Salmalia malabarica*	Exd.	48
22	Draksha	*Vitis vinifera*	Dry Fr.	960
23	Dhataki	*Woodfordia fruticose*	Fl.	Qs
24	Jala	Water		24.576
25	Sarkara	Sugar		768
26	Ksaudra (Madhu)	Honey		4.8kg
27	Marica	*Piper nigrum*	Fr.	q.s.

Rt = Root; Fr = Fruits; Fl = Flowers; St = Stem; St. Bk = Stem bark; Pl = Whole Plant; Lf = Leaf; Exd = Exudate

MATERIALS AND METHODS:

Reagents and Test Samples of Ushirasava: The reference standards of rubiadin and luteolin were procured from Natural Remedies, Bangalore, and Yucca Enterprises, Mumbai, respectively. The solvents and chemicals used in this research project

were of analytical grade. Baidyanath, Sandu and Patanjali Ushirasava (B-1, S-1 and P-1) were procured from local shops in Mumbai.

Sample Preparation: The test formulations (25 mL) each were heated on an electric water bath at 60-70 °C to evaporate the self-generated alcohol and obtain a residue (pasty in nature). 10-15 mL of methanol was added to this residue and allowed to stand for some time. This sample was centrifuged at 1000 rpm and filtered to obtain the sample solution for HTLC analysis.

Physicochemical Studies:

Organoleptic Properties: The marketed formulations of Ushirasava were evaluated for their organoleptic properties *viz*. color, odor, appearance, and taste.

pH: The pH of the formulations was measured using a calibrated pH meter.

Specific Gravity: The specific gravity of the test formulations was determined (in triplicate) using 10 ml specific gravity bottle and calculated using the formula:

Specific gravity of liquid (Formulation) = (Weight of 10 ml of liquid / 10) / (Weight of 10 ml of water / 10)

Total Solid Content: The total solid content of the test formulations was determined by taking 10 ml of the test formulation in a porcelain evaporating dish and heating it on an electric water bath at 60–70 °C followed by heating in an oven at 105 °C to constant weight.

Ethanol Content: 25 ml of each test formulation was taken in a 500 ml round bottom flask, diluted with 150 ml of distilled water, and was distilled, and not less than 90 ml of distillate was collected in 100 ml volumetric flask and diluted to volume with distilled water. The relative density of this solution was determined, and the alcohol content was reported as per the table given in Indian Pharmacopoeia 2014 [5].

Reducing and Non-reducing Sugar Content: This was determined by Lane Eymon's method of classical titration using Fehling's solution [6].

Determination of Fehling's Factor: 1 g of sucrose was hydrolyzed by adding 1 ml of concentrated hydrochloric acid (HCl), and volume was made up to 100 ml with water. This was kept for 24 h, followed by titration against Fehling's solution (5 ml of each Fehling A and Fehling B solution) using methylene blue as an indicator. Fehling factor was determined using the formula:

Fehling factor (For invert sugar) = (Titre × Weight of sucrose (g) / 100

Determination of Reducing Sugar Content: 10 ml of the test formulation was taken in 100 ml volumetric flask and volume made up with water. This solution was titrated against Fehling's solution (5 ml of each of Fehling A and Fehling B solution) using methylene blue as an indicator in triplicate and the average titer value was used to calculate the percent reducing sugar content using the formula:

Percent reducing sugar (% w/v) = (Fehling factor × Dilution factor × 100) / (Average titer value (ml)

Determination of Total Sugar Content: 10 ml of the test formulation was hydrolyzed by adding 1 ml of concentrated hydrochloric acid, and volume was made up to 100 ml with water. This was kept for 24 h, followed by titration against Fehling's solution (5 ml of each of Fehling A and Fehling B solution) using methylene blue as an indicator in triplicate and the average titer value was used to calculate percent total sugar content using the formula:

Percent total sugar (% w/v) = (Fehling factor × Dilution factor × 100) / (Average titer value (ml)

Determination of Non-reducing Sugar Content: The non-reducing sugar content value was determined using the formula:

Percent non-reducing sugar (% w/v) = (Percent total sugar (% w/v)) – (Percent reducing sugar (% w/v)

Phytochemical Studies: Phytochemical evaluation of the methanol extract of the test formulation residues was conducted (for the presence of plants primary and secondary metabolites like alkaloids, glycosides, flavonoids, steroids, tannins and phenolics, carbohydrates and proteins) using qualitative tests as the standard text [7].

Microbial Studies: This involved determination of total bacterial count, total fungal count, and test for specific pathogens *viz. P. aeruginosa*, *E. coli*, and *S. aureus*. Each of the microbial experiments was

performed in a sterilized laminar airflow chamber. This experiment was performed on a sample taken from a newly opened bottle. The sample stock solution was prepared by taking 10 ml of formulation and making up the volume to 100 ml with Soyabean Casein Digest (SCD) broth. 1 ml of this stock solution was used as a test sample for all the microbial tests. Four control petri plates viz. medium control, diluent, *i.e.*, broth solution (negative) control, positive control (bacteria/fungus culture), and environment control were set for each experiment. For total bacterial count, the test and control samples were incubated in Soybean Casein Digest Agar (SCDA) medium at 35 °C for 2-3 days. For total fungal count, the test and control samples were incubated in sabouraud dextrose agar (SDA) medium at 25 °C for 5-7 d. The test for the presence of specific pathogen *viz*. *P. aeruginosa*, *E. coli*, and *S. aureus* were determined by its characteristic growth of green, pink, and light yellow colored colonies when incubated in characteristic cetrimide agar, mac conkey agar, and vogel Johnson agar medium respectively at 35 °C for 2-3 days 5.

HPTLC Studies:

Development and Optimization of Mobile Phase: A series of mobile phase systems reported for individual determination of luteolin and rubiadin were explored in order to achieve good resolution of the selected phytoconstituents. However, modification and optimization of the mobile phase were required as none of these preliminary studies gave a good resolution of luteolin and rubiadin.

Preparation of Reference Standard Solutions: The stock solutions of the reference standards were prepared in methanol. An accurately measured quantity of each reference standard was dissolved in methanol to get 1000 ppm of luteolin and rubiadin, respectively. Working standard solutions of 100 ppm of luteolin and rubiadin each was prepared from the stock solutions, respectively.

Preparation of Sample Solution: The test formulations (25 mL) each were heated on an electric water bath at 60-70 °C to evaporate the self-generated alcohol and obtain a residue (pasty in nature). 10-15 mL of methanol was added to this residue and allowed to stand for some time. This sample was centrifuged at 1000 rpm and filtered to obtain the sample solution for application to the TLC plates.

HPTLC Method Validation Studies: The chromatographic conditions used for the validation of the analytical method were.

Application Mode: Camag Linomat 5 Applicator.

Filtering System: Whatman filter paper.

Stationary Phase: MERCK- TLC/ HPTLC Silica Gel 60F254 on aluminum sheets.

Mobile Phase: Toluene: ethyl acetate: methanol: formic acid (9:8.5:1.5:1).

Applications(Y axis) Start Position: 8 mm.

Development Distance: 80 mm.

Band Length: 8 mm.

Development Mode: CAMAG TLC twin trough chamber

Saturation Time: 20 min.

Visualization: 278 nm and 284 nm.

Drying Mode: TLC plate heater preheated the developed HPTLC method was validated as per ICH Q2 (R1) guidelines for the following parameters [8].

Linearity and Range Studies: The linearity studies were conducted in the concentration range of 100-500 ng/band for luteolin and 30-80 ng/band for rubiadin. The data obtained (peak area versus concentration) was subjected to least-square linear regression analysis, and the slope, intercept, and regression value for the calibration was determined.

Limit of Detection (LOD) and Limit of Quantitation (LOQ) Studies: The LOD and LOQ for each marker compound (luteolin and rubiadin) were determined using the formula:

$$LOD = (3.3 \times \sigma) \div S \text{ and } LOQ = (10 \times \sigma) / S$$

Where, σ is the standard deviation, and S is the slope of the calibration curve.

Accuracy Studies: The accuracy of the developed method was evaluated by calculating the percent recovery of known amounts (80%, 100%, and 120%) of each marker (luteolin and rubiadin) spiked into the sample solution. The percent recovery was calculated using the formula:

Percent recovery = (Practical recovery / Theoretical recovery) × 100

Precision Studies: The precision of the developed method was studied at three levels- repeatability, intraday precision and inter-day precision. Luteolin and rubiadin at concentrations of 3 µg/band and 0.5 µg/band respectively were applied seven times on three different plates and percent coefficient of variation (% CV) between the peak area values were calculated by repeating the same experiment three times in a day (intraday precision studies) and on three different days (inter-day precision studies).

Specificity Studies: Specificity studies were conducted by densitometric scanning of samples of the standards, samples, mobile phase, and diluent at 284 nm. The chromatograms were checked for presence of interference peaks if any, from the diluent and mobile phase.

Robustness Studies: Robustness studies were carried out by deliberate variations in analytical method parameters, variation in two chromate-graphic parameters (mobile phase composition and saturation time). The influence of these variations was evaluated on Rf values, peak area, and peak shape of each standard. The mobile phase composition of toluene: ethyl acetate: methanol: formic acid (9:8.5:1.5:1) was altered to toluene: ethyl acetate: methanol: formic acid (8.5:9:1:1). The saturation time was altered from 15 min to 25 min and the densitometric scans were recorded at 284 nm.

Quantitation of Marker Compounds (Luteolin and Rubiadin): The quantitation of rubiadin and luteolinwas conducted through multi-level calibration mode, and the developed and validated analytical method was applied for quantitative estimation of the same in the test formulations.

Antioxidant Assays: Ushirasavais composed of medicinal plants that contain luteolin (a flavonoid) and rubiadin (anthraquinone glycoside). These classes of phytoconstituents are known to possess significant antioxidant activity; hence we thought it prudent to evaluate the test samples of Ushirasava for potential antioxidant activity.

Reducing Power Assay: Reducing power of the methanol extract of the formulation was determined on the basis of the ability of their antioxidant principles to form a colored complex with potassium ferricyanide, trichloroacetic acid (TCA) and ferric chloride. The complex formed by this method was measured using the following method.

1 mL of different concentrations (20, 40, 60, 80, 100 pg/m1) of the methanol extract was mixed with potassium ferricyanide (2.5 mL, 1%), 2.5 mL of phosphate buffer (pH 6.6). The mixture was incubated at 50 °C for 20 min. To this solution, 2.5 mL TCA (10%) was added and centrifuged at 3000 rpm for 10 minutes. After centrifugation, 2.5 mL of the supernatant was withdrawn, and to this 2.5ml distilled water and 0.5 ml ferric chloride was added, and absorbance was measured at 700 nm. A higher absorbance value of the reaction mixture indicated higher reducing power. The results were compared to ascorbic acid (reference standard for antioxidant activity) using the same concentration as that of methanol extract under identical experimental conditions [9].

Hydroxyl Radical Scavenging Assay: Hydroxyl radical scavenging activity was measured as the ability of different concentrations of methanol extract to scavenge the hydroxyl radicals generated by the Fe- ascorbate-EDTA-H20, system (Fenton reaction). The hydroxyl radical scavenging capacity was measured according to the method given below. Stock solutions of EDTA (1 mM), $FeCl_3$, (10 mM), ascorbic acid (1 mM), H2O2 (10 mM), deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml H_20_2, 0.36 ml of deoxyribose, 1.0 ml of the extract at different concentrations (10, 20, 30, 40,, 50, 60 mg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH-7.4), 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37 °C for 1 hr. A 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of

0.5% thiobarbituric acid to develop the pink chromogen measured at 532 nm. The same procedure was carried out using curcumin as a reference standard (20, 40, 60, 80, 100 µg/ml). The hydroxyl radical scavenging activity of the extract was reported as % inhibition of deoxyribose degradation is calculated by using the following equation:

Percentage inhibition = 1 - absorbance of test / absorbance of control × 100

A graph of percentage inhibition versus conc. was plotted for both sample and curcumin. The IC_{50} of the sample was calculated from the graph and compared to the IC_{50} value of Curcumin [10].

Thiobarbituric Acid Assay: 1 ml of different concentrations (20, 40, 60, 80, and 100 µg/ml) of the methanol extract were mixed with 2 mL of 20% TCA and 2 mL of 0.67% of thiobarbituric acid. The mixture was placed in a boiling water bath for 10 min and then centrifuged after cooling at 3000rpm for 20 min. The same procedure was carried out using curcumin as standard (20, 40, 60, 80, and 100 µg/ml). The absorbance of the supernatant was measured at 552 nm. The percentage inhibition of the sample and standard was calculated from the absorbance using the following formula.

Percentage inhibition = 1 - absorbance of test / absorbance of control × 100

A graph of percentage inhibition versus conc. was plotted for both sample and curcumin. The IC50 of the sample was calculated from the graph and compared to the IC50 value of curcumin [11].

Stability Studies: The test samples of Ushirasava were kept under accelerated stability conditions (40 °C, 75% RH) for 3 months. They were subsequently analyzed for organoleptic and physicochemical properties. The stability samples were subjected to HPTLC studies to compare with the control samples.

RESULTS AND DISCUSSION:

Physicochemical Studies: The results of physicochemical tests conducted on the test samples of Ushirasava are given in **Table 2**.

pH: The pH of the test formulations was found to be within the specified range (3.5-4.5) as per the Ayurvedic Pharmacopoeia of India.

Specific Gravity: The specific gravity of the test formulations was found to be within the specified range (1.0-1.10) as per the Ayurvedic Pharmacopeia of India.

Total Solid Content: The total solid content of the test formulations was found to be within the specified range (NLT 7%), indicating that all the test formulations complied with the specifications mentioned in the Ayurvedic Pharmacopoeia of India.

Ethanol Content: The ethanol content of two test formulations was found to be within the specified range (5-10 %), indicating that one of the test formulations (S-1) did not comply while B-1 and P-1 complied with the specifications mentioned in the Ayurvedic Pharmacopoeia of India.

Reducing and Non-reducing Sugar Content: The test samples of Ushirasava showed mixed results in this test. One test sample (P-1) did not comply with the specifications for reducing sugar content (NLT 5%), while two test samples (B-1 and S-1) did not comply with the specifications for no-reducing sugar content (NMT 0.65%).

Organoleptic Properties: The organoleptic evaluation of test samples of Ushirasava indicated that all the formulations were brown to blackish brown in color with an alcoholic odor and sweet taste. **Table 3** depicts the results of the organoleptic evaluation of the test samples.

Phytochemical Evaluation: The methanol extract of the test formulation residue was found to contain steroids, flavonoids, and glycosides as secondary metabolites, a result of the polyherbal composition of the test samples of Ushirasava.

Microbial Evaluation: The test formulations of Ushirasava were found to comply with the specification limit for the total bacterial count, i.e., NMT 1×10^5 Colony Forming Unit per ml (CFU/ml) and total fungal count, *i.e.*, NMT 1×10^3 CFU/ml as mentioned in the 'Protocol for testing Ayurveda, Siddha, and Unani medicines'. *P. aeruginosa* and *E. coli* were absent in all three formulations. In the test for the presence of *S. aureus,* the test formulations showed the presence of characteristic light-yellow colored bacterial growth. However, the red color of Vogel Johnson

agar medium didn't turn yellow as observed in positive control petri plate. On performing gram staining of the bacterial growth observed in both the samples, gram-positive rod-shaped bacteria were observed in B-1 sample petri plate while gram positive cocci-shaped bacteria were observed in P-1 sample petri plate. As *S. aureus* is gram positive cocci-shaped bacteria, P-1 might contain these bacteria.

TABLE 2: PHYSICOCHEMICAL PROPERTIES OF MARKETED SAMPLES OF USHIRASAVA

Physicochemical attributes	Result			Specifications	Conclusion		
	B-1	S-1	P-1		B-1	S-1	P-1
pH	4.44	4.24	4.29	3.5- 4.5	Complies	Complies	Complies
Specific gravity (g/cc)	1.03	1.07	1.04	1- 1.10	Complies	Complies	Complies
Alcohol content (% v/v)	6.2	10.3	9.4	5 - 10%	Complies	Does not comply	Complies
Total solid content (% w/v)	9.93	18.04	21.41	NLT 7%	Complies	Complies	Complies
Reducing sugar (%w/v)	6.25	6.84	3.19	NLT 5%	Complies	Complies	Does not comply
Non-reducing sugar (%w/v)	1.07	1.04	0.75	NMT 0.65%	Does not comply	Does not comply	Complies
Methanol extractive (%w/v)	0.50	0.67	1.27	---	---	---	---
Total bacterial countCFU/mL	94.56	198.22	212.26	NMT 1×10^5CFU/mL	Complies	Complies	Complies
Total fungal countCFU/mL	102.64	77.65	122.90	NMT 1×10^5 CFU/mL	Complies	Complies	Complies

TABLE 3: ORGANOLEPTIC PROPERTIES OF TEST SAMPLES OF USHIRASAVA

Marketed formulations	Appearance	Odour	Taste
Ushirasava (B-1)	Dark brown	Alcoholic	Sweet
Ushirasava (S-1)	Brownish black	Alcoholic	Sweet
Ushirasava (P-1)	Light brown	Alcoholic	Sweet

TABLE 4: PHYTOCHEMICAL SCREENING OF THE MARKETED USHIRASAVA SAMPLES

Phytoconstituents	Observations		
	B-1	S-1	P-1
Reducing sugar	+	+	+
Monosaccharides	+	+	+
Pentose sugars	-	-	-
Hexose sugars	+	+	+
Proteins	-	-	-
Amino acids	-	-	-
Steroids	+	+	+
Glycosides	+	+	+
Flavonoids	+	+	+
Alkaloids	-	-	-

(+) indicates presence (-) indicates absence

Phytochemical Evaluation: The methanol extract of the formulations was tested for the presence of reducing sugars, pentose sugars, hexose sugars, proteins, amino acids, steroids, glycosides, flavonoids and alkaloids using standard chemical tests for detection of primary and secondary plant metabolites. The results indicated the presence of reducing sugars, steroids, glycosides, and flavonoids extracted in the formulations of Ushirasava **Table 4**.

HPTLC Studies of Ushirasava:

HPTLC Method Development Studies: A series of compositions of the mobile phase were evaluated in an effort to arrive at an optimum mobile phase that resolves the markers from other phytoconstituents in the methanol extract of the Ushirasava formulations. The optimum mobile phase for determination of luteolin was found to be toluene: ethyl-acetate: methanol: formic acid in the ratio 8: 9.3: 0.4: 1. Similarly, the optimum mobile phase for rubiadin was found to be toluene: ethyl acetate: methanol: formic acid in the ratio 9: 8.5: 1.5: 1. HPTLC fingerprinting indicated that the R_f value of rubiadin was 0.73, and that of luteolin was 0.63 **Fig. 1** and **2**. HPTLC-UV scans were recorded for rubiadin **Fig. 3** and luteolin **Fig. 4** and the λ_{max} was found to be 284 nm and 278 nm, respectively.

HPTLC Method Validation Studies: The developed HPTLC method was validated for parameters stated in the International Conference on Harmonization (ICH) guidelines. The method was validated for linearity, range, the limit of detection, the limit of quantitation, specificity, robustness, accuracy, and precision. The results of the validation study are presented in **Table 5**.

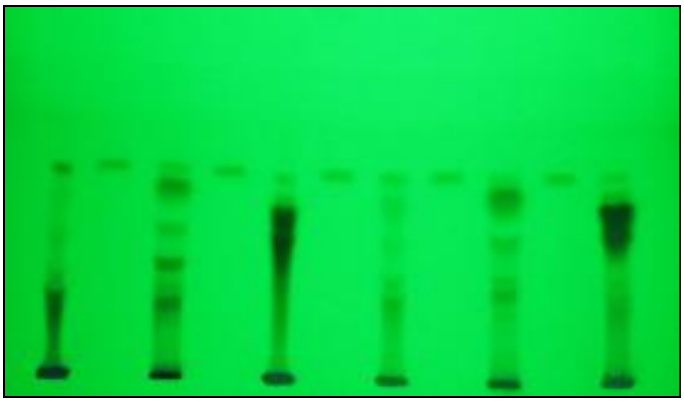

FIG. 1: HPTLC FINGERPRINT OF RUBIADIN, STANDARD AND TEST SAMPLES

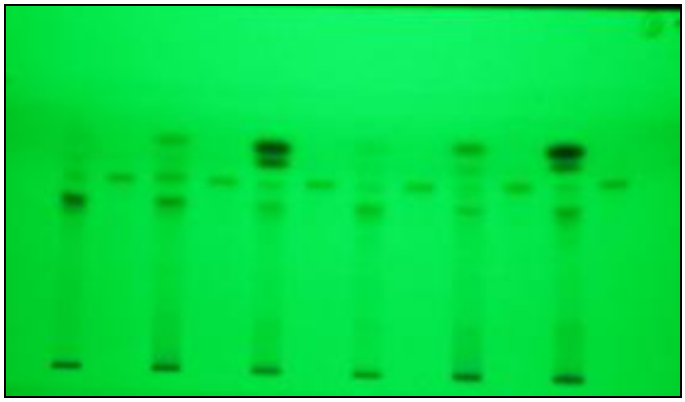

FIG. 2: HPTLC FINGERPRINT OF LUTEOLIN STANDARD AND TEST SAMPLES

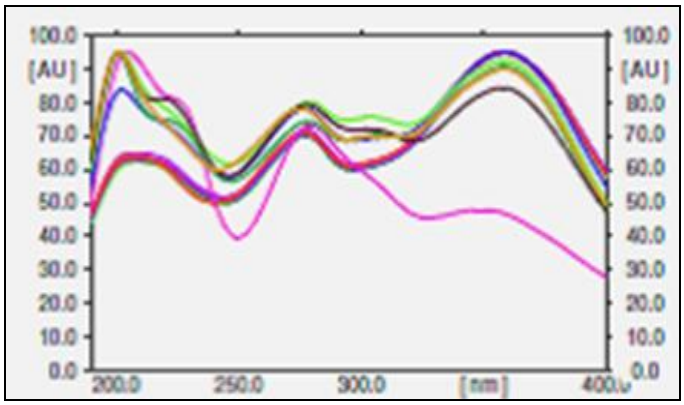

FIG. 3: HPTLC-UV SPECTRUM OF RUBIADIN AND ALL SAMPLES

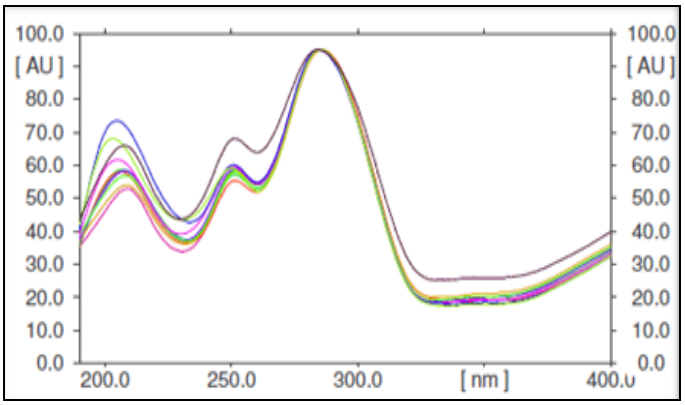

FIG. 4: HPTLC-UV SPECTRUM OF LUTEOLIN AND ALL SAMPLES

TABLE 5: HPTLC METHOD VALIDATION STUDIES OF LUTEOLIN AND RUBIADIN

Validation parameters	Luteolin	Rubiadin
Linearity and range	100-500 ng	30-80 ng
LOD	1.202 ng	0.53 ng
LOQ	3.64ng	1.61 ng
Specificity	Specific	Specific
Robustness	Robust	Robust
Accuracy (% recovery)	88.3%	82.70%
Precision (% RSD) Intraday, Interday, Repeatability	1.065%, 1.811% 1.605%	1.8%, 1.4% 1.1%

Quantitation of Rubiadin and Luteolin in the Test Samples: The calibration curve of rubiadin (1mg/mL) and luteolin (0.2 mg/mL) was plotted. The quantity of luteolin present in Ushirasava manufactured by Baidyanath, Sandu and Patanjali were found to be 24.28 µg/mL, 22.08 µg/mL and 12.82 µg/mL respectively and the quantity of rubiadin present in Ushirasava manufactured by Baidyanath, Sandu and Patanjali were found to be 9.324 µg/mL, 12.6 µg/mL and 3.98 µg/mL respectively.

Antioxidant Studies of Marketed Ushirasava Samples: The antioxidant potential of rubiadin from root extract and luteolinfrom fruit extract is reported hence the test formulations of Ushirasava were evaluated using reducing power assay, hydroxyl radical scavenging assay and thiobarbituric acid (TBA) assay. The results of the antioxidant assays are presented in **Table 6**.

TABLE 6: ANTIOXIDANT ASSAYS OF MARKETED USHIRASAVA SAMPLES

Assays Manufacturer	Reducing power assay (increase in absorbance)	Hydroxy radical scavenging assay (IC_{50})	Thiobarbituric acid assay (IC_{50})
B-1	0.514- 0.959	44.47 µg/mL	271.07 µg/mL
S-1	0.468- 0.911	107.37 µg/mL	215.36 µg/mL
P-1	0.319- 0.782	43.41 µg/mL	257.70 µg/mL
Standard	Ascorbic acid	Curcumin	Curcumin
	0.48- 1.63	30.79 µg/mL	84.83 µg/mL

Stability Studies: The three brands of Ushirasava (Test samples) after being kept under accelerated stability conditions (40 °C, 75% RH) for three months were evaluated for organoleptic properties, physicochemical properties and HPTLC finger-printing. The results of organoleptic properties and physicochemical properties are discussed in **Table 7** and **Table 8,** respectively.

TABLE 7: ORGANOLEPTIC PROPERTIES OF STABILITY SAMPLES OF USHIRASAVA

Formulation	Appearance	Odor	Taste
B-1	Dark brown	Alcoholic	Sweet
S-1	Brownish black	Alcoholic	Sweet
P-1	Light brown	Alcoholic	Sweet

TABLE 8: PHYSICOCHEMICAL PROPERTIES OF STABILITY SAMPLES OF USHIRASAVA

Manufacturer Parameters	Result			Specification	Conclusion		
	B-1	S-1	P-1		B-1	S-1	P-1
pH	4.10	4.35	4.29	3- 4.5	Complies	Complies	Complies
Specific gravity	1.07	1.04	1.04	1- 1.10	Complies	Complies	Complies
Alcohol content	7.9	8.8	10.6	5%- 10%	Complies	Complies	Does not comply
Total solid content (% w/v)	19.56	18.33	15.45	10%-20%	Complies	Complies	Complies
Reducing sugar (%w/v)	4.46	3.98	4.23	3.5%- 5.5%	Complies	Complies	Complies
Non-reducing sugar (%w/v)	1.98	1.45	0.95	NMT 1%	Does not comply	Does not comply	Complies

DISCUSSION: Ushirasava is a polyherbal fermented liquid preparation manufactured and marketed by Baidyanath, Sandu, and Patanjali. As a part of the standardization procedure, market samples of Ushirasava were analyzed for physical and chemical parameters. The content of some quantity of actives (Rubiadin and luteolin) was determined using a validated HPTLC analytical method. Analytical methods are the set of techniques that allow us to know qualitatively and/ or quantitatively the composition of any material and chemical present in a formulation. Analytical methods are developed in order to establish the identity, purity, physical characteristics, and potency of the drugs either from the bulk drug from its dosage form. HPTLC is a powerful method for qualitative and quantitative analytical tasks. The qualitative analysis mainly determines the identification of constituents present in a particular product, and quantitative analysis determines the quantity of chemical present in the crude herbal extract. Analysis of marketed herbal formulations for compliance with pharmacopoeial specifications is a crucial factor for establishing and assuring its quality. This data also enhances the credibility of the product and subsequently accentuates consumer belief and satisfaction.

CONCLUSION: Standardisation of three different brands of the ayurvedic formulation Ushirasava was successfully carried out with respect to the physicochemical (organoleptic properties, pH, specific gravity, total solid content, total ethanol content, reducing and non-reducing sugar content), phytochemical and microbial parameters (total bacterial count, total fungal count and test for specific pathogens *viz. P. aeruginosa*, *E. coli*, and *S. aureus*). A new, simple, accurate, precise, and robust HPTLC method was successfully developed for the simultaneous determination of rubiadin and luteolinin in the three marketed Ushirasava formulations. This method was validated as per the ICH Q2 (R1) guidelines.

This method can be used for routine analysis and standardization of Ushirasava and other formulations containing these phytoconstituents, thereby increasing the scientific validity and quality of these herbal formulations.

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