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Evaluation and method development for quantification of Piperine in *Hutabhugadi Churna* by RP- HPLC

Research Article

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Abstract

Aim and Objective: The current work was aimed at preparing the *Hutabhugadi Churna* in the laboratory and evaluating the same including the method development for the estimation of a marker compound Piperine by using RP-HPLC. Methods: Prepared *Hutabhugadi churna* was subjected for macroscopic, physical, and chemical evaluation considering WHO guidelines. The methanolic extract was subjected for estimation of Piperine as marker using RP-HPLC. Results: The macroscopic characteristics like colour, odour and taste are recorded. The physical characteristics like loss on drying, ash value, extractive value, swelling index, foaming index, powder properties like angle of repose, bulk density, tapped density, compressibility index etc. were determined. Total phenolic content, total flavonoid content, preliminary phytochemical screening was also carried out. The results are compared with marketed formulation of *Hutabhugadi churna*. The retention time of the standard Piperine was found to be 5.517, while the Piperine in extracts of laboratory and marketed formulations was found to be was found to be 5.554 and 5.562 respectively. The concentration of Piperine in laboratory and marketed formulation was found to be 0.17 %w/w and 0.18 % w/w respectively. The method developed was also validated. Conclusion: The laboratory made *Hutabhugadi churna* and marketed formulation of *Hutabhugadi churna* was comparatively evaluated. The resulting data will be useful for the standardization of the Hutabhugadi churna, an Ayurvedic formulation.

Keywords: *Hutabhugadi churna,* Total phenolic content, Total flavonoid content, Preliminary phytochemical screening, Marker compound, RP-HPLC.

Introduction

Hutabhugadi churna is an Ayurvedic formulation reported in The Ayurvedic Formulary of India (1). It is also mentioned in *Sahasra yoga* and *Churnaprakarana*. The ingredients of *Hutabhugadi churna* (HC) include *Hutabhugadi* (*Plumbago zeylanica* L.), *Marica* (*Piper nigrum* L.), *Pippali* (*Piper longum* L.), *Ajamoda* (*Trachyspermum roxburghianum* (DC).), *Saindava lavanam* (Rock salt), and *Haritaki* (*Terminalia chebula* Retz). As per Ayurvedic Pharmacopoeia of India, it is an important formulation useful in treating digestive impairment (*Agni mandya*), oedema (*Sopha*), anemia (*Pandu*), and haemorrhoids (*Arsa*) (1).

Literature survey revealed the lack of standardisation data related to Hutabhugadi churna. The churna was screened for various pharmacological activities. Hence in the present study, the formulation was prepared in the laboratory and subjected to standardisation using various organoleptic, physical, and chemical evaluations. Further, the formulation was subjected to estimation of marker compound analysis using Piperine as a marker by RP-HPLC method. Compared the results with the marketed formulation of *Hutabhugadi churna*.

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Materials and methods

Preparation of *Hutabhugadi churna*:

Hutabhugadi Churna consists of 6 ingredients:

- *Plumbago zeylanica* L. (Chitrak),
- *Piper nigrum* L. (Marica),
- *Piper longum* L. (Pippali),
- *Trachyspermum roxburghianum (DC)* (Ajamoda),
- *Terminalia chebula* Retz (Haritaki), and
- Rock salt (Saindava lavana).

All the ingredients were procured from the local Ayurvedic shops. The identity of the drugs *Marica, Pippali,* and *Haritaki* was carried out in the laboratory as per Ayurvedic Pharmacopoeia of India. *Chitrak* was authenticated by Dr Madhava Chetty, a professor at the Department of Botany, Sri Venkateswara University Tirupathi. *Ajamoda* was authenticated at the National Institute of Science Communication & Policy Research (NISCPR), New Delhi. All the specimens are deposited in the college's Pharmacognosy laboratory for future reference.

All the ingredients are adequately cleaned and powdered individually. The fine powders are passed through the sieve number 80. One part each of *Plumbago zeylanica* L., *Piper nigrum* L., *Piper longum* L., *Trachyspermum roxburghianum* DC., and Rock salt is mixed thoroughly with five parts of *Terminalia chebula* Retz. to obtain a homogenous blend. The marketed formulation was collected from the online market.

The compositions of all the lab and marketed formulations are represented in Table 1.

Macroscopic evaluation

The macroscopic characteristics such as colour, odour, taste, and fineness are noted.

Physical evaluation

Physical parameters such as extractive values, ash values, loss on drying, swelling index, foaming index, and pH were evaluated according to methods as described in World Health Organisation Guidelines (2).

Determination of moisture content:

About 5 g of the formulations were weighed into a flat weighing bottle previously dried and tared and dried in an oven at 105°C.

Drying was continued until two consecutive weightings did not differ by more than 5 mg. The difference in the weight of the drug before and after drying was noted, and the loss of drying was calculated concerning the air-dried material (2).

Table 1: Ingredients of laboratory and marketed formulations of *Hutabhugadi churna*

S. No	Drug	Lab Formulation	Marketed Formulation	Part	Quantity
1	*Hutabhuga (Chitraka)*	*Plumbago zeylanica* L.	*Plumbago zeylanica* L.	Root	1 part
2	*Marica*	*Piper nigrum* L.	*Piper nigrum* L.	Fruits	1 part
3	*Pippali (Magadha)*	*Piper longum* L.	*Piper longum* L.	Fruits	1 part
4	*Ajamoda*	*Trachyspermum roxburghianum* DC	*Trachyspermum ammi* L.	Fruits	1 part
5	*Saindhava lavana*	*Rock salt*	*Rock salt*	-----	1 part
6	*Pathya (Haritaki)*	*Terminalia chebula* Retz	*Terminalia chebula* Retz	Pericarp	5 parts

Determination of water-soluble extractive value

4 g of each formulation was macerated with 100 ml of water in a glass stoppered conical flask for 24 h, shaking first 6 h frequently and allowed to stand for 18 h. The solution was filtered rapidly, and the solvent was evaporated in a tared flat-bottomed dish in a water bath. Dried the residue obtained for 6 h at 105°C and weighed. The content of extractable matter was calculated based on air-dried material (2).

Determination of alcohol soluble extractive value

4 g of each of the formulations were macerated with 100 ml of 95% ethanol in a glass stoppered conical flask for 24 h shaking first 6h frequently and allowed to stand for 18 h. The solution was filtered rapidly, and the solvent was evaporated in a tared flat-bottomed dish in a water bath. Dried the residue obtained for 6 h at 105°C and weighed. The content of extractable matter was calculated based on air-dried material (2).

Determination of ash value

The weighed amount (2-3 g) of each formulation was incinerated in a silica crucible at a temperature not exceeding 450°C in a muffle furnace until the formulation was free from carbon. It was cooled and weighed. The total ash values for the air-dried drugs were calculated. (2).

Determination of swelling index

It is the volume in ml taken by the swelling of 1 g of the plant material under specified conditions. Its determination is based on adding water or a swelling agent as defined in the test procedure for each plant material. Swelling index of the laboratory and marketed formulations was determined as per the method mentioned in Quality control methods for medicinal plant materials by World Health Organisation (2)

Determination of foaming index

The foaming ability of an aqueous extracts of plant materials is measured using a foaming index. The foaming index, which is almost constant for crude drugs, can be used as a physical parameter in the evaluation of crude drugs. The foaming index of the laboratory and marketed formulations was determined as per the reported methods (2)

Determination of powder parameters

The powder parameters like angle of repose, bulk density, tapped density, compressibility index etc. were determined by using reported methods (3).

a) Angle of repose: The fixed funnel and the free-standing cone method was used. The funnel is secured with its tip at a given height (h), above the glass plate that is placed on a horizontal surface. Formulation powder was carefully poured through the funnel, until the apex of the conical pile.

$$\tan \alpha = h/r$$

$$\alpha = \tan^{-1} (h/r)$$

Where h=height, r =radius

b) Bulk density determination: 5 g of the powder was taken and poured into the measuring cylinder. The initial volume was noted. Then the cylinder was dropped onto the hard wooden surface 3 times from height of 1 inch at 2 second intervals. The cylinder was tapped for about 500 times. This procedure is repeated 3 times. The bulk density was calculated with the formula

Bulk density = weight of the powder/bulk volume of the powder.

c) Tapped density: The tapped density is an increased bulk density attained after mechanically tapping a container containing powder sample.

d) Compressibility index: Carr and Neumann developed a simple test for evaluating the flow ability of a powder by comparing the tapped density and poured density of a powder and the rate at which it is packed by a useful empirical guide given by Carr's Compressibility Index.

Chemical evaluation

Estimation of total phenolic Content

The total phenolic content of the formulations' ethanolic extracts was estimated using the Folin ciocalteu method (4) and prepared the stock solution (10mg/10ml) of the extract in ethanol. 1 ml of the extract was taken from this stock solution and added to a 25 ml volumetric flask. Then added 10 ml of water and 1.5 ml of Folin ciocalteu reagent. The mixture was kept aside for 5 minutes, added 4 ml 20% sodium carbonate solution, distilled water added to make the volume to 25 ml and kept aside for 30 minutes. The absorbance of the blue colour that developed was recorded at 765 nm. To prepare the calibration curve, the solutions of standard gallic acid were prepared in the concentration range of 50 to 250 µg/ml.

Estimation of total flavonoid content

The ethanol extract of each formulation was assessed for total flavonoid content using the aluminum chloride colorimetric method, following the procedure outlined by Chang et al (5). Quercetin was employed to establish the calibration curve. Different volumes (0.1, 0.2, 0.3, 0.4, and 0.5 ml) were drawn from the standard stock solution to obtain concentrations of 10, 20, 30, 40, and 50 µg/ml, respectively. These diluted standard solutions (0.5 ml) were mixed separately with 0.1 ml of 10% aluminum chloride, 1.5 ml 95% ethanol, 0.1 ml 1M potassium acetate, and 2.8 ml of distilled water. Mixture was incubated for 30 minutes. The reaction mixture's absorbance was measured at 415 nm. In the blank, the amount of 10% aluminum chloride was substituted with an equal volume of distilled water. Likewise, 0.5 ml of ethanol extracts from each formulation (2 mg/ml) were subjected to the aluminum chloride reaction for flavonoid content determination.

The method by Chang et al. (5) was used for the estimation of flavanones. Naringenin was used as a reference standard to prepare the calibration curve. 20 mg of naringenin was dissolved in methanol and then diluted to give concentrations of 250, 500, 1000, 1500, and 2000 µg/ml. 1 ml of each of these diluted standard solutions was mixed with 2 ml of 1% 2, 4-dinitro phenyl hydrazine reagent and 2 ml of methanol separately at 50°C for 50 min. Upon cooling to room temperature, the mixture was mixed with 5 ml of solution of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. Then 1 ml of the mixture was taken, mixed with 5 ml of methanol and centrifuged at 1000 rpm/min to remove the precipitate formed. The supernatant was collected and adjusted to 25 ml. The absorbance of the supernatant was measured at 495 nm. Similarly, 5 ml each of ethanolic extracts of the formulations (5 mg/ml) were treated with 2, 4-dinitro phenyl hydrazine reagent for determination of flavonoid content.

The sum of the values obtained from these two methods was considered as total flavonoid content.

Preliminary phytochemical screening

The successive solvent extracts obtained by extracting the *Hutabhugadi churna* successively with the solvents of increasing polarity were subjected to various qualitative tests to determine the presence of various phytoconstituents using reported methods (6). Hydro-distillation process was used to detect the presence of volatile oil in the drugs.

Alkaloids were tested by Dragendorff, Wagner, and Mayer tests; cardenolides were tested by Kedde's, Legal, and Raymond tests; while phenolics and flavonoids were tested by Ferric chloride, Shinoda, and Lead acetate tests. Chlorosulphonic acid and Salkowski tests were used for the detection of tri-terpenoid saponins. Vanillin sulphuric acid and Liebermann-Burchard tests are used to detect the Terpenoids and Steroids. Phytosterols in an unsaponifiable matter of petroleum ether extract were detected by Liebermann-Burchard tests. Fixed oils and fats were detected by spot and saponification tests. Carbohydrates were detected by Molisch and Fehling tests; while amino acids were by Ninhydrin tests.

Thin layer chromatography of *Hutabhugadi churna*

The results obtained by the qualitative tests in preliminary phytochemical screening are supported by thin-layer chromatography.

The screening of the formulations was carried out using thin-layer chromatography to confirm the presence of various phytoconstituents as detected by qualitative tests. Different extracts of the formulation were subjected to thin-layer chromatographic studies (7) using Silica gel 60F254 pre-coated plates to confirm the presence of various constituents.

Alkaloids were detected by Wagner's reagent using Chloroform: Acetone: Diethyl amine (78:12:10) as mobile phase. Flavonoids were developed using Toluene: Ethyl formate: Formic acid (50: 40: 10)) as mobile phase and detected using Natural product-polyethylene glycol reagent (NP-PEG) as visualization agent. Terpenoids were developed using Ethyl acetate: Toluene (7:93) as the mobile phase and Vanillin-sulphuric acid as the detecting agent. Cardenolides were developed using Chloroform: Methanol (90:10) as mobile phase and detected using Kedde's reagent as a visualization agent. Triterpenoids were detected by antimony trichloride reagent using Chloroform: Acetic acid: Methanol: Water (64:32:12:8) as mobile phase.

These results were compared with the results of the qualitative tests.

Estimation of Piperine content in formulation by HPLC

A simple method was developed using RP-HPLC and validated for the estimation of marker

compound Piperine in the laboratory and marketed formulations of *Hutabhugadi churna*.

Preparation of mobile phase

An 80:20 ratio of Methanol and 0.1% Formic acid was selected as a mobile phase based on various trials performed.

Preparation of sample solution

Each 1 g of formulations (lab formulation and marketed) in triplicate was refluxed with 15 ml of methanol for 1 hr. The solutions were filtered, and the marc was again refluxed with 15 methanol for 30 min. The extracts were combined, filtered and concentrated using a Rota evaporator. The concentrated extracts were dissolved in 10 ml of mobile phase.

Preparation of standard solution of Piperine

A stock solution of standard Piperine was obtained by dissolving Piperine 10 mg in 10 ml of the mobile phase and subjecting the same for sonication. This solution was used to prepare the dilutions to give the solutions of 5, 10, 15, 20, and 25 µg/ml.

Determination of Analytical wavelength

1 ml of the standard Piperine solution (10 mg/ml) was diluted to 10 ml using mobile phase and subjected to scanning in a UV spectrophotometer between 200 to 400 nm to find out the λ max.

System suitability

To find out the system's suitability six replicates of 15 µg/ml concentrations were prepared from standard stock solution, filtered, and injected. The peak areas, Retention time, tailing factor, and theoretical plates of all the prepared concentrations were noted and compared with limits.

Linearity

Solutions of piperine in the concentration range of 5 - 25 µg/ml, in triplicate, were injected into the HPLC system. Peak responses were noted, and the calibration curve was plotted using concentration on the X-axis and peak area on the Y-axis. Used the method of linear regression for data evaluation.

Precision

Intra-day precision was performed within a day at different time intervals. The optimised concentration of 15µg/ml was injected into the HPLC system, and six replicates of peak responses were noted. Inter-day precision was performed on different days. The optimised concentration of 15µg/ml was used from the stock solution, and six replicates were injected into HPLC for three days consecutively.

Accuracy

Accuracy was carried out by the standard addition method. The optimised standard solution spiked to solution of Piperine at three different concentration levels of 50%, 100%, and 150% respectively. The prepared sample solutions was injected into the HPLC system and the mean recovery of the sample was calculated.

Limit of detection & Quantification

The solutions for a limit of detection & quantification were prepared based on the signal-to-noise ratio (S/N) obtained from standard deviation and slope, filtered, and injected.

LOD= 3.3 x standard deviation/ slope

LOQ= 10 x standard deviation/ slope

Robustness

15µg/ml solution was prepared and injected into the HPLC system by making changes like flow rate ± 0.2 ml/min (1.2 ml/min), wavelength ± 2nm (343 nm), and temperature ± 5°C (30°C), concerning optimised chromatographic conditions.

Assay of Piperine

Each 1 g of formulations (lab formulation and marketed) in triplicate was refluxed with 15 ml of methanol for 1 hr. The solutions were filtered, and the marc was again refluxed with 15 methanol for 30 min. Both the methanolic extracts are combined, filtered, and made into residue using a rotary evaporator. Dissolved the residue in in 10 ml of mobile phase, i.e. Methanol: 0.1% Formic acid (80:20). Transferred 0.4 ml of this stock solution into a 10 ml volumetric flask, and made the volume to 10 ml with mobile phase. Injected the 20 µl of this solution into HPLC to record the chromatogram. The extracts are prepared in triplicate. The peak areas were determined, and the amount of Piperine was calculated.

Results and Discussion

The formulation was prepared in the laboratory as per Ayurvedic Pharmacopoeia of India as mentioned in Table 1, and the marketed formulation was procured from the market. Both the formulations were subjected to comparative evaluation.

Macroscopic evaluation

Laboratory and marketed formulations exhibited brown colour, with a slightly pungent taste and acrid taste. Both the formulations pass through sieve no 80.

Physical evaluation

Determination of various physical parameters like moisture content, ash value, extractive values, pH value, swelling index, foaming index and various powder parameters (angle of repose, bulk density, tapped density, compressibility index etc.) was carried out and the results were represented in Table 2. The physical parameter values for plant drugs are more or less constant and hence used for the evaluation of crude drugs and herbal formulations.

The loss on drying equal to moisture content is an indicative of moisture present which can be controlled and determined always. The ash values represent the amount of inorganic matter present in the formulation

which is adhering to it and added deliberately. The extractive values are indicative of the number of soluble constituents for the solvent selected, present in the formulation. Swelling index denotes the presence of mucilaginous matter in the formulation. The information related to triterpenoid saponins will be provided by foaming index. The powder parameters provide the various powder properties like flow, compressibility etc. of the powder particles of the formulation.

Table 2: Physical parameters of the laboratory and marketed formulations of *Hutabhugadi churna*

Parameters	Lab formulation	Marketed formulation
Loss on drying (% w/w)	8.16 ± 0.120	9.00 ± 0.001
Water soluble Extractive value (% w/v)	0.27 ± 0.021	0.50 ± 0.018
Ethanol soluble extractive value (% w/v)	0.25 ± 0.009	0.16 ± 0.004
Total ash value (% w/w)	0.14 ± 0.005	0.13 ± 0.005
pH (10% aqueous solution)	3.84 ± 0.012	3.94 ± 0.012
Swelling index	2.6 ± 0.002	2.7 ± 0.012
Foaming index	< 100	<100
Bulk density (gm/cc)	0.44 ± 0.003	0.41 ± 0.002
Tapped density (gm/cc)	0.67 ± 0.006	0.66 ± 0.011
Angle of repose (°)	42.18 ± 0.637	42.79 ± 0.890
Compressibility index	34.63 ± 0.653	38.48 ± 0.610

Chemical evaluation

Determination of total phenolic content, total flavonoid content

Total phenolic content was calculated by the Folin ciocalteu reagent method using gallic acid as standard. The values for lab formulation and marketed formulation were found to be 2.2528±0.0018 % w/w and 1.6061±0.0007 % w/w respectively representing various phenolic compounds like poly phenols, flavonoids, phenolic acids etc. The total flavonoid content was determined by Aluminium chloride and 2,4 Di nitro phenyl hydrazine methods using quercetin and naringenin as markers. The flavonoid content of the laboratory and marketed formulations were found to be 1.0353±0.008 % w/w and 1.237±0.012 % w/w respectively representing the presence of flavonols, flavones and flavanones etc.

Preliminary phytochemical screening

The results of phytochemical screening of various successive extracts of the laboratory and marketed formulations are represented in Table 3. The formulations revealed the presence of various secondary metabolites like, alkaloids, phenolics, flavonoids, terpenoids, sterols and triterpenoids.

Table 3: Qualitative evaluation of successive extracts

Constituents	Laboratory formulation				Marketed formulation			
	P	C	E	W	P	C	E	W
Alkaloids	-	+	-	-	-	+	-	-
Cardenolides	-	-	-	-	-	-	-	-
Phenolics and flavonoids	-	-	+	+	-	-	+	+
Anthracene glycosides	-	-	-	-	-	-	-	-
Terpenoids	+	-	-	-	+	+	-	-
Sterols	+	-	-	-	+	-	-	-
Triterpenoids	-	+	-	-	-	+	-	-
Amino acids	-	-	-	+	-	-	-	+
Carbohydrates	-	-	+	+	-	-	+	+
Fixed oils and fats	+	-	-	-	+	-	-	-
Volatile oils	Absent				Absent			

P: Petroleum ether, C: Chloroform, E: Ethanol, W: Chloroform water, -: Negative, +: Positive.

Thin layer chromatography of *Hutabhugadi churna*

The screening of the formulations was carried out using thin layer chromatography in order to confirm the presence of various phytoconstituents as detected by qualitative tests.

Alkaloids when developed with Chloroform: Acetone: Diethyl amine (78:12:10) as mobile phase, showed revealed four spots with Rf values of 0.13, 0.68, 0.86 and 0.91 in laboratory formulation, while marketed formulation showed two spots with Rf values of 0.11 and 0.87. terpenoids when developed with Toluene: Ethyl acetate (93:7) as mobile phase revealed 5 spots with Rf values of 0.09, 0.15, 0.25, 0.50, and 0.86 in the case of laboratory formulation, while marketed formulation revealed 4 spots with Rf value of 0.09, 0/19, 0.23, 0.41 and 0.82. Flavonoids, when developed using Toluene: Ethyl formate: Formic acid (50: 40: 10) as mobile phase, revealed 2 spots with Rf values of 0.56 and 0.72, while the marketed formulation showed 2 spots with 0.29 and 0.59. Phenolics were detected by developing with Toluene: Ethyl acetate: Acetic acid (2.25: 2.5: 0.25) and revealed 2 spots with Rf values of 0.58 and 0.72, while the marketed formulation also revealed two spots with similar Rf values. Cardenolides and anthracene glycosides are not detected in thin-layer chromatography.

Estimation of Piperine content in formulation by HPLC

The Piperine solution has shown the maximum response at 343 nm in UV region. Based on the various trials conducted for HPLC the optimised mobile phase was found to be 80: 20 ratio of Methanol and 0.1% Formic acid. The λ max of the Piperine in UV spectroscopy was represented in Figure 1 The HPLC chromatogram of the Piperine was represented in Figure no 2. The optimal conditions for the experiment were represented in table no 4.

Figure 1: λ max of the Piperine in UV Spectroscopy

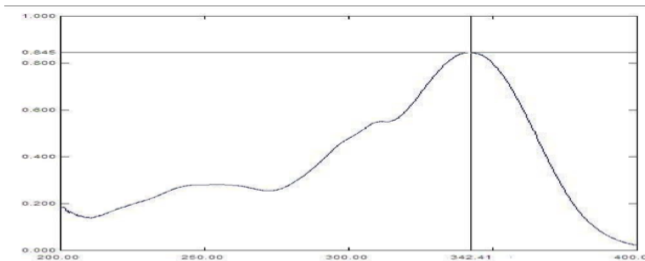


Figure 2: Optimised HPLC chromatogram of standard Piperine (15 µg/ml)

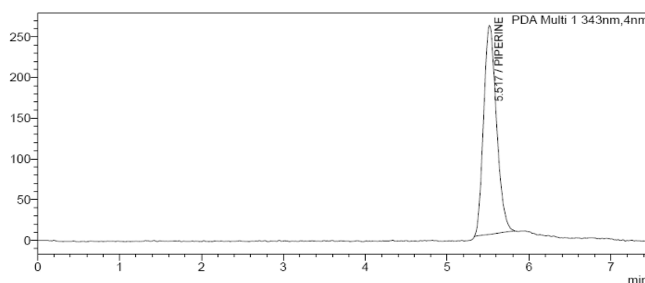


Table 4: HPLC optimised conditions for Piperine estimation

S. No	Parameters	Conclusion
1	Mobile Phase	Methanol: 0.1% Formic acid
2	Column	C-18
3	Wavelength	343nm
4	Column temperature	30^0 C
5	Run time	07 min
6	Retention time	5.517
7	Flow rate	1.2 ml
8	Injection volume	20 µl
9	Concentration	10 µg/ml
10	Theoretical plates	5267

As part of the analytical method validation, system suitability, linearity, precision, robustness, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were carried out.

System suitability

System suitability was carried out by injecting 6 replicates of 15 µg/ml solutions and the results were represented in Table no 5.

Table 5: Data representing system suitability

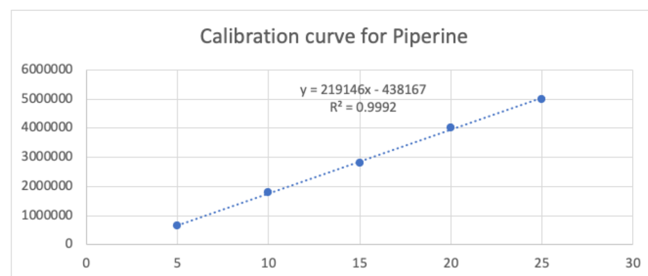
S. No	Peak area	Ret. time	Plate count	Peak height	Tailing factor
1	2812532	5.547	5347	256726	1.212
2	2839167	5.542	5372	258180	1.217
3	2853518	5.532	5351	262003	1.235
4	2856858	5.551	5410	261607	1.197
5	2888840	5.554	5234	257903	1.212
6	2889362	5.535	5280	261890	1.202
Average	2856713	5.54	5332.33	259718.17	1.26
SD	29557.22	0.01	64.15	2371.45	0.01
% RSD	1.03	0.16	1.20	0.91	1.05

Limits: Theoretical plates, >2000; Tailing factor, ≤ 2; % RSD, <2

Linearity

Standard solutions in the concentration range of 5 - 25 µg/ml, in triplicate, were injected into the HPLC system. Peak responses were noted, and the calibration curve was plotted using concentration on the X-axis and peak area on the Y-axis. The calibration curve of Piperine was represented in Figure 3

Figure 3: Calibration curve of Piperine



Precision

Data representing Intra-day precision within a day at different time intervals. and Inter-day precision on different days was represented in Table 6 and 7.

Table 6: Data representing Intra-day precision

S. No	Peak area		
	9.00 am	**1.00 pm**	**5.00 pm**
1	2980076	2981992	2958513
2	2888840	2856856	2880481
3	2910210	2894925	2839169
4	2812662	2889362	2984602
5	2864958	2982871	2897255
6	2853591	2980193	2949412
Average	2885056.17	2931033.5	2918238.67
SD	57115.16	56996.31	54983.38
% RSD	1.98	1.94	1.88
Limits	% RSD < 2.0	% RSD < 2.0	% RSD < 2.0

Table 7: Data representing Inter-day precision

S. No	Peak area		
	Day 1	**Day 2**	**Day 3**
1	2963647	2924629	2862364
2	2962364	2880481	2973003
3	2839167	2921559	2957019
4	2962935	2812532	2897000
5	2927431	2927431	2894925
6	2994421	2853519	2889362
Average	2941660.83	2886691.83	2912278.83
SD	54508.19	46804.53	42989.25
% RSD	1.85	1.62	1.48
Limits	% RSD < 2.0	% RSD < 2.0	% RSD < 2.0

Accuracy

The accuracy was performed by adding the known concentration of the Piperine to the Piperine solution. To the 15 µg/ml solution of Piperine, added in triplicate, 50 %, 100% and 150 % concentration of Piperine and the % recoveries were noted. The mean % recovery for 50%, 100% and 150% added solutions were found to be 101.11, 98.06 and 98.38 respectively.

Limit of Detection & limit of Quantification (LOD & LOQ)

The limit of detection and limit of quantification was calculated by using standard deviation and slope and were found to be 0.060 µg/ml and 0.180 µg/ml respectively.

Robustness

The results of the robustness obtained by varying the parameters, wavelength, flow rate and temperature from optimised conditions were represented in Table 8.

Table 8: Data representing wavelength variation ± 2 mm, Flow rate variation ± 0.2ml and Temperature variation ± 5 °C

S. No	Parameter	Condition	Peak area	Retention time	Standard deviation	% RSD
1	Wave length	341	2809161	5.572	15335.97	0.544
2		343	2864924	5.546	17168.79	0.598
3		345	2830461	5.558	12519.26	0.444
1	Flow rate	1.0 ml	3185781	6.597	55571.90	1.731
2		1.2 ml	2813532	5.547	23284.54	0.815
3		1.4 ml	2427452	4.915	11214.19	0.264
1	Tempe-rature	25°C	2866856	5.661	37390.15	1.346
2		30°C	2883767	5.542	12479.26	0.443
3		35°C	2810210	5.481	31203.4	1.096

Assay of Piperine in *Hutabhugadi churna*

Methanolic extracts of Laboratory and marketed formulations were injected into HPLC and peak areas were noted. The concentration of the Piperine was obtained from the calibration curve using regression equation. The amount of Piperine in laboratory and marketed formulations were found to be 0.17 ± 0.0058 % w/w and 0.18 ± 0.0058 % w/w respectively.

The HPLC chromatograms of the methanolic extract of laboratory and marketed formulations were represented in Figure 4 and Figure 5.

Figure 4: HPLC chromatogram of the laboratory formulation of *Hutabhugadi churna*

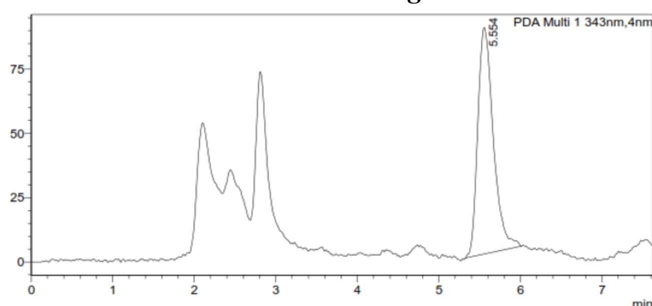
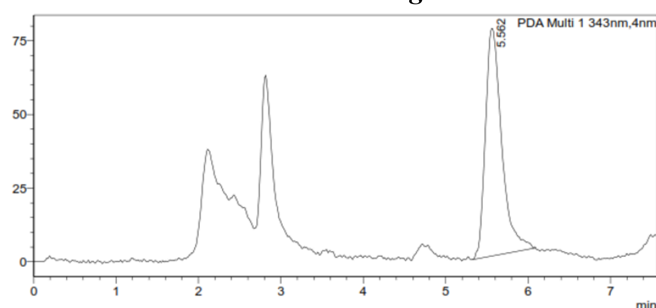


Figure 5: HPLC chromatogram of the marketed formulation of *Hutabhugadi churna*



Conclusion

The *Hutabhugadi churna* was prepared in the laboratory. The same was subjected for macroscopic, physical and chemical evaluations. The marketed formulation was procured from the market and was used for comparative evaluation. The results obtained will help us to establish the analytical profile as part of standardisation of the formulation. A Rapid, simple, economical and sensitive method has been developed and validated for the quantification of Piperine in samples by RP-HPLC. The method was suitable for use in pharmaceutical industry and academic for its regular estimation in quality control and research area. On the whole, these findings offer a standardisation data for the formulation with regard to preparation and use of the formulation in alternative system of medicine.

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