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Estimation of Quality, Safety and Efficacy of Ashwagandhadi Leha Using Chromatographic Technique

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Abstract:

Background: Ashwagandhadi leha falls under the category of avaleha-paka group of Ayurvedic formulations. If the raw material to be used in a formulation and routine checking stage by stage processes of manufacture are standardized, the resulted product can be expected to confirmed uniform standard, therefore present study envisaged to develop quality control parameters in order to evaluate the quality, safety and efficacy of the formulation (Ashwagandhadi leha).

Method: The three batches of avaleha were prepared in laboratory as per procedure given in Ayurvedic formulary of India. All the crude drugs, formulation were evaluated on basis of its quality and purity. The extractive values were performed for alcohol and water was for crude drugs,

Result: These formulations were evaluated for its organoleptic property, pH, ash values, loss on drying, phytochemical evaluation, fat content, sugar content, total solid, stability studies, phytochemical evaluation, fat content, sugar content, total solid, stability studies, UV Spectroscopy studies, Chromatographic studies (HPLC, TLC), antioxidant activity carried out for free radical activity done by DPPH method also performed. Safety analysis and heavy metals analysis also carried out safety purpose.

Conclusion: The scientific confirmation of the therapeutic utility of time tested formulation was supplemented by good efforts for the development of pharmacopoeial standard for quality control to ensure uniformity and consistency in the quality of product.

Keywords: Ashwagandhadi leha, Chromatographic Technique, Avaleha, Ayurvedic formulary, Extractive values, DPPH method.

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1. Introduction

Ayurveda is an ancient and popular form of holistic medicine system originated in India and now spreading worldwide. In ancient time when the Ayurvedic medicines were developed, preparation, standardization and quality control of Ayurvedic formulation maintained by fully committed professionals. Process of preparation hold significant role in effectiveness and quality of Ayurvedic formulations[1].

In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing as well as in developed countries owing to its natural origin and lesser side effect. Traditional/herbal medicines, which formed the basis of health care throughout the world since the earliest days of mankind are still widely used, and have considerable importance in international trade[2]. Ashwagandhadi leha is a well-known Ayurvedic formulation described in Ayurvedic formulary of India. It is a potent Ayurvedic medicine used in the treatment of cough. The main components Draksha (*Vitis vinifera),* Pippali *(Withania somnifera),*Sugar and Honey. The prescribed dose of Ashwagandhadi leha is 25 gm daily in divided doses[3].

Ashwagandhadi leha falls under the category of avaleha-paka group of Ayurvedic formulations. If the raw material to be used in a formulation and routine checking stage by stage processes of manufacture are standardized, the resulted product can be expected to confirmed uniform standard, therefore present study envisaged to develop quality control parameters in order to evaluate the quality, safety and efficacy of the formulation (Ashwagandhadi leha). The scientific confirmation of the therapeutic utility of time tested formulation was supplemented by good efforts for the development of pharmacopoeial standard for quality control to ensure uniformity and consistency in the quality of product.

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2. Material and Method

2.1 Plant Material

The crude drugs were purchased from local market of Ujjain and Dewas (M.P), and identified at Department of Botany, Vikram University Ujjain (M.P). All above drugs were shade dried, reduced to coarse powder, weighted separately and sieved through sieve no. 20 (for coarse powder). The voucher specimen numbers were submitted at Department of Pharmacy M.I.P.S. Ujjain (M.P.)

- Vitis vinifera: MIPS/V/05/2011
- Withania somnifera: MIPS/P/06/201

2.2 Method

2.2.1 Physico-Chemical Evaluation of Crude Drugs

A) Determination of morphological characters of crude drugs

Colours of crude drugs were examined by visual inspection under diffuse daylight. Length, width and thickness of crude materials were measured by graduated ruler in millimeters. For examination of odour small portions of drugs were placed in the palm of the hand and slowly and repeatedly inhaled the air over the materials[4]. Morphological characters of crude drugs are tabulated in table no.1.

B) Determination of foreign matter

100-500 gm of the drug sample to be examined was weighed, and spread out in a thin layer. The foreign matter was detected by inspection with an unaided eye or by the use of a lens (6x). It was then separated and weighed and calculated the percentage [5]. Foreign matters of crude drugs are tabulated in table no. 2.

C) Determination of loss on drying

10 gm of the drug (without preliminary drying) after accurately weighing was placed in a tarred evaporating dish. it was dried at 105^0C for 5 hours and was weighed. Then again the tarred dish was dried in oven for one hour and subsequently cooled in desiccators and then again weight of

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the dish was taken. This procedure was continued until difference in weight was not more than 0.01 gm [6]. loss on drying of crude drugs are tabulated in table no.3.

D) Determination of extractive matter

4 gm of coarsely ground air-dried material was weighed accurately in glass-stopper 250 ml iodine flask. 100 ml of solvent was added in the flask. It was shaken occasionally for 6 hours. The flask was allowed to stand for 18 hours. Contents of the flask were filtered. Meanwhile, tarred evaporating dish was weighed and 25 ml of the filtrate was transferred to the tarred dish. Filtrate was evaporated on a water bath and contents were dried for 6 hours in an oven. The filtrate was then cooled in desiccators for 30 minutes, and was weighed again without delay. The amount of extractable matter in mg/gm of the air-dried material was calculated. The same procedure was applied with other solvents such as water, alcohol [7]. Extractive matter are tabulated in table no.4.

E) Determination of ash

- Total ash

  2-4 gm of accurately weighed ground drug material was incinerated in a tared silica dish at a temperature not exceeding 450 ^{0}C until free from carbon, cooled and then weighed. Sometime carbon free ash could not be obtained in this way, than 2 ml of water was added and dried on a boiling water bath then on a hot plate and ignited to constant weight. The residue was allowed to cool in desiccators for 30 minutes, and then weighed without delay. The amount of total ash in mg/gm of the air-dried material was calculated.

- Acid-insoluble ash

  To the crucible containing the total ash, 25 ml of hydrochloric acid was added, covered with a watch-glass and boiled gently for 5 minutes. The watch glass was rinsed with 5 ml of water and this liquid was added to the crucible. The insoluble matter was collected on ash less filter- paper and washed with hot water until the filtrate was neutral. Filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to constant

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weight. The residue was allowed to cool in desiccators for 30 minutes and then weighed without delay. The amount of acid insoluble ash in mg/gm of the air-dried material was calculated [8]. Ash values of crude drug are tabulated in table no. 5.

F) Determination of foaming index

The foaming ability of an aqueous decoction of plant material and their extracts is measured in terms of a foaming index (WHO, 1998). 1 gm of powder was reduced to fineness by passing through a sieve no.100. The fine powder was weighed, and transferred to 500 ml conical flask containing 100 ml of boiling water, maintained at moderate boiling for 30 minutes, the flask was cooled and the contents were filtered in 100 ml volumetric flask and sufficient water was added to make up the volume. The decoction was poured into 10 stopper test tubes in successive portions of 1, 2, 3 ml etc. up to 10 ml, and the volume of the liquid in each tube was adjusted with water to 10 ml. The tubes were stopper and shaken in a lengthwise motion for 15 seconds. Those were allowed to stand for 15 minutes and the height of the foam was measured. When the height of the foam in every tubes less than 1 cm [9]. so the foaming index will be less than 100, foaming index of crude drugs is tabulated in table no.6.

2.2 Preparation of Formulation

Three Batches were encoded ASLI, ASLII, ASLIII made by strictly confirmed to Ayurvedic formulary of India.

A) Method

All ingredients were taken pharmacopoeial quality the Draksha were washed two or three times with water and drained compleletely. The seeds were removed and crushed to fine paste. Pippali and Sarkara were powdered dried separately and passed through sieve no. 85. All the ingredients were triturated to a homogeneous mixture by adding required amount of Honey, to form a semisolid mass [10]. Formula for Ashwagandhadi leha is maintained in table below.

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2.3 Evaluation of Formulation

A) Thin layer chromatography

20 gm of the avaleha was extracted with a combination of 50 ml of a mixture of diethyl ether: chloroform (2:1) and 5 ml methanol. It was concentrated to 10 ml and filtered .the thin layer chromatography was carried out.10 µl of the extracts applied on TLC plate and developed the plate to a distance of 8 cm using toluene : ethyl acetate: formic acid (4: 2.5:0.7) as mobile phase. Allow the plate to dry in air and examine under ultraviolet light (254 nm). The plate showed major spots at R_f 0.41, 0.58, 0.64 (piperine), 0.74. Under ultraviolet light (366 nm) the plate shows major spots at R_f 0.45 (blue), 0.55 (brown), 0.64 (blue)(piperine),0.84 (red),0.88 (red) and 0.93(blue). the plate sprayed with anisaldehyde sulphuric acid reagent followed by heating at 110^0C for about 10 min. It showed major spots at R_f 0.40 (brown), 0.52(purple), 0.58 (yellow), 0.64 (blue, piperine), 0.68 (purple) and 0.75 (violet) under visible light [11].

B) Determination of pH

The test solution were prepared by dissolving 1 gm drug in 100 ml of carbon dioxide free water (5% w/v), and 10 gm in 100 ml of carbon dioxide free water(10% w/v) [12].

Carbon dioxide free water: The distilled water was boiled for few minutes vigorously and was used immediately the electrodes were immersed in the solution to be examined and pH was measured which is tabulated below in table no. 12.

C) Loss on drying of formulation

The same procedure was followed as given in 2.2.1 (C) [13]. loss on drying of formulations are tabulated in table no.13.

D) Ash values of formulation

The same procedure was followed as given in 2.2.1 (D) [14]. Ash values of formulations are tabulated in table no. 14.

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E) Determination of Fat contents:

50 gm of Ashwagandhadi leha was suspended in 100 ml of water and extracted with 200 ml of petroleum ether applying slow stirring for about half an hour and then refluxing at 40^0C for 6 h, the content was allowed to cool and two layers were separated out, The petroleum ether extract was passed through a bed of anhydrous sodium sulphate to remove any traces of water, the solvent was allowed to evaporate at low temperature and then at 105^0C in oven until constant weight [14]. The total fat content (% w/w) was calculated and tabulated in table no. 15.

F) Determination of sugar contents:

The Fehling's reagent (10 ml) was titrated against test solution in similar way as with standard invert sugar solution above and the end point was noted, the reducing sugar content of the formulation was calculated [15].

$$\% \text{ Total sugar} = [250 \times S / H \times M] \times 100$$

Where, S = Strength of Fehling's reagent(0.00486 dextrose / ml)

H= Volume of sample required for titration

M= Weight of the sample taken

Sugar content of formulations is tabulated in the table no. 16.

G) Determination of total solid content

Ashwagandhadi leha (5 gm) from each batch was extracted separately with 100 ml distilled water by maceration, 50 ml of these samples were taken in a previously dried and weighed evaporating dish, evaporated on water bath and further dried in an oven at 105^0C till constant weight, From the weight of the residue obtained the percentage of total solid content (% w/v) in the sample was determined [15]. and tabulated in table no.17.

H) Phytochemical Screening

Phytochemical Screening for alcoholic extract of crude drugs and Ashwagandhadi leha are tabulated in table bo. 18.

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I) U V Spectroscopic Estimation of Withanolidefrom Ashwagandhadi leha[16]

- Preparation of extract of ashwagandhadi leha and Pippli churna : The Ashwagandhadi leha (1gm) and powdered Pippali churna (1gm) refluxed with 60ml methanol for 1 h. the extract was filtered and refluxed again with the marc left with 40 ml of methanol for another 1 h. Filtered and combined the filtrate. the methanol extract concentrated under vacuum till the semisolid mass was obtained. the residue dissolved in 75 ml methanol and filtered through sintered glass funnel (G-2) by vacuum filtration assembly. The filtrate was centrifuged at 2000 rpm for 20 minutes, the supernatant was collected in 100 ml volumetric flask and volume was made with methanol. The same procedure was performed for each batch of the Ashwagandhadi leha and solution (100 ml) of their withanolide extract were prepared.
- Preparation of standard solution of withanolide: An accurately weighed withanolide (100 mg) was dissolved in methanol and volume was made up to 100ml with methanol in volumetric flask. Two ml of this solution was diluted with methanol up to 100 ml in volumetric flask to give 20µg/ml withanolide solution.
- Calibration curve of withanolide : A series of calibrated 10 ml volumetric flask were taken and appropriate aliquots of the working standard solution of withanolide were withdrawn and diluted up to 10 ml with methanol. The absorbance was measured at absorption maxima (λ max) 343 nm, against the reagent blank prepared in similar manner without the piperine. The absorption maxima and Beer's law limit were recorded and data that prove the linearity and obey Beer's law limit were noted. The linear correlation between these concentrations (x-axis) and absorbance(y-axis) were graphically presented and slope(b), intercept(a), and correlation coefficient (r^2) were calculated for the linear equation (Y = bx +a) by regression using the methanol of the least square and Calibration curve of withanolideprepared and tabulated below.
- Estimation of withanolidein Ashwagandhadi leha : The appropriate aliquots from extract of each batch of Ashwagandhadi leha and separately powdered fruits of *Withania somnifera*

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(Pippali) were withdrawn in 10 ml volumetric flask separately absorbance for aliquots of each was noted at 343 nm. The corresponding concentration of withanolideagainst respective absorbance value was determines using the withanolidecalibration curve and estimation of withanolidein Ashwagandhadi leha are tabulated .

J) Estimation of Gallic acid of formulation by UV [17].

- Instrumentation

  Spectroscopic analysis was carried out using Shimadzu UV-1700 1601UV/Vis double beam spectrophotometer with Spectra treaties software. Spectrophotometer with spectral width 2 nm, wavelength accuracy of 0.5 nm and a pair of 10 mm matching quartz cells was used to measure absorbance of the resulting solutions.

- Preparation of standard stock solution

  Standard stock solution (A) gallic acid was prepared by dissolving 100mg of drug in 100 ml volumetric flask by using methanol. From the stock solution final concentration (100µg/ml) of the individual working standards were prepared with methanol. Working standards solution was scanned in the range of 200 to 600 nm to determine the λ_{max} of drugs using methanol as a blank. The λ_{max} of gallic acid was found to be 227nm. From the stock solution (A) 10 ml was taken and diluted to 100 ml with methanol (B), form this solution (B) 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml were taken and volume is made up to 10 ml in volumetric flask to get a concentration of 10, 20, 30, 40 and 50 µg/ml. The absorbance of the resulting solution was measured at 227 nm respectively and a calibration curve were plotted and tabulated at these wavelengths.

- Procedure for analysis of avaleha

  1.5 g avaleha subjected to refluxing with 100 ml methanol for 1 hour, semisolid mass transferred to 100 ml of volumetric flask and volume is adjusted with 100ml with methanol. The solution was centrifuged for five minutes at 3000 rpm. Centrifugation was found to be faster and more effective than filtration. Centrifugation forms a residue at the bottom of the test tube, which is not disturbed while drawing out the supernatant solution. This supernatant solution was pipette

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out and diluted appropriately with methanol to obtained the concentration of 10 µg/ml concentration of gallic acid. the solution was scanned from 200-600 nm. The absorptivity value at 227 nm the drug were determined by checking the absorbance values of over a concentration range 10-50 µg/ml for working standards.

K) Estimation of Withanolide by HPLC

- Chromatographic conditions: The method developed involved the use of Younglin system. The column used was Varian (25×4.6). A flow rate of 1.5 ml/min was maintained. The optimized mobile phase was found to be Methanol: Water (69:31v/v) was passed through a 0.45µm membrane filters and degassed by ultrasonication under vacuum before use. The injection volume was 20µL and the effluent was monitored for UV absorption at 343 nm was used for quantitative estimation. All separations were performed at ambient temperatures. The optimized method was then validated for limits of detection, linearity, range, precision and accuracy and specificity[18]. Analytical parameters of HPLC procedure for the withanolideestimation tabulated in table no.27.

L) Safety Analysis

The total viable aerobic and fungi count of different batches of Ashwagandhadi leha were determined by plate count method. Sample (1%) after treatment with sodium chloride-peptone buffer solution (pH-7.0) was inoculated. The samples were incubated at 30-35 ^{0} C for 24 hrs, number of colonies formed were counted after 24 h.For safety analysis, 1% sample was taken and dissolved in distilled water, then this solution was inoculated on the nutrient broth medium and incubated at 30-35 ^{0}C for 24 h [18]. Safety analysis are tabulated in table no.28.

M) Heavy Metal Estimation of formulation

- Determination of heavy metals

5gm of sample was ignited in a preheated silica crucible at 400ºc, cooled at room temperature and 0.5 ml of concentrated sulphuric acid was added it. Further heated on heating mantle and ignited again muffle furnace for constant weight at 600c, the ash was dissolved in 100 ml of 5%

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Hydro chloride acid. The solution was subjected to Atomic Absorption spectrometric analysis for the determination of heavy metals[19]. Determinations of Heavy metals are tabulated in table no. 29.

N) Stability studies

The International Conference on Harmonization (ICH) Guidelines titled "Stability Testing of New Drug substance and Products" (QIA) describes the stability test requirements for drug registration applications in the European Union, Japan and the United States of America. ICH specifies the length of study and storage conditions.

Long-term Testing: 25^0 C □ 2^0 C / 60 % RH □ 5 % for 12 Months.

30^0 C □ 2^0 C / 65 % RH □ 5 % for 12 Months

Accelerated Testing: 40^0 C □ 2^0 C / 75 % RH □ 5 % for 6 Months.

Stability studies were carried out at 40^0 C / 75 % RH for the selected formulation for the period of 2 months.

● Method

The selected formulations were packed in Ambered coloured glass containers. They were then stored at 40^0 C / 75 % RH for 2 months and evaluated for their physical appearance and drug release at specified intervals of time [20].

3. RESULT AND DISCUSSION

The macroscopic parameters were performed for crude drugs *Vitis vinifera* and *Withania somnifera* which include shape was berry and cylindrical, size were 1.5 to 2.5 cm long, 0.5 to 1.5 cm thick and 2.5 to long, 0.4 to 1 cm thick, colour was dark brown to black and greenish to black, odour was sweetish, pleasant and aromatic and taste was sweet and pungent for *Vitis*

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vinifera and *Withania somnifera* respectively, the drugs showed good results in all macroscopic parameter.

Loss on drying (% w/w) in *Vitis vinifera* and *Withania somnifera* was found to be 2.9±0.2 and 1.3±0.1 respectively which indicates that all data were within in specific limits and chances of microbial contamination was less.

The extractive value is used to indicate presence of constituents in particular solvents, it was performed in solvents like alcohol and water. The extractive value (% w/w) for alcohol was found to be 28.5±0.3 and 16.0±0.6 for *Vitis vinifera* and *Withania somnifera* respectively and the extractive value in water was found to be 76.6±0.5 and 23.7±0.7 for *Vitis vinifera* and *Withania somnifera* respectively, thus indicate presence of more polar compound on moving on polarity scale.

The total ash value (% w/w) was found to be 2.5±0.2 and 6.8±0.4 for *Vitis vinifera*, and *Withania somnifera* respectively and acid insoluble ash value (% w/w) was found to be 0.19±0.4% and 0.4±0.1 for *Vitis vinifera* and *Withania somnifera* respectively, which show the presence of less inorganic matter in drugs.

Foaming index was not found in *Vitis vinifera* and *Withania somnifera*, this indicating abesence of Saponin content (Table 5.6). Swelling index for *Vitis vinifera* and *Withania somnifera* 1.4±0.3, and 4.8±0.2 respectively, this indicating the presence of mucilage respectively.

According to values described above all parameters were within limits .so, three batches of Ashwagandhadi leha formulations were prepared in laboratory according to methods described in Ayurvedic Formulary of India.

Formulation was evaluated for its organoleptic property, pH, ash values, loss on drying, phytochemical evaluation, fat content, sugar content, total solid, stability studies etc.

Organoleptic properties indicated dark brown colour, characteristic odour and pungent in taste of formulation.

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Thin layer chromatography done for formulation shows R_f values 0.4 and 0.43 which shows that confirms of withanolide.

pH values of formulations was found between 4-6 due to presence of acidic components.

Loss on drying (% w/w) of formulations was found to be 4.7±1.5, 7.08±1.13, 6.03±1.5 for ASLI, ASLII, ASLIII respectively.

The total ash values (% w/w) was found to be 0.2±0.01, 0.15±0.20, 0.26±0.21 and total insoluble ash was found to be 0.05±0.01, 0.01±0.03, 0.03±0.015 for ASLI, ASLII, ASLIII respectively showing presence of less amount inorganic matter in the formulation.

Fat content (% w/w) was found to be 0.01±0.15, 0.03±0.3, 0.10±0.1 for ASLI, ASLII, ASLIII respectively which indicating that highest amount was present in ASLIII.

The total sugar content was estimated titrimatically compared to standard invert sugar solution as dextrose. The sugar contents (% w/w) was found to be 70.0±0.13, 71.0±0.6, 70.5±0.14 for ASLI, ASLII, ASLIII respectively which indicating that highest amount present in ASLII (Table 5.14).

The total solid content (% w/w) was found to be 2.50±0.20, 3.11±0.1, 3.50±0.15 for ASLI, ASLII, ASLIII respectively which indicating that highest amount present in ASLIII (Table 5.15).

Phytochemical screening indicates the presence of chemical constituents in the drugs and formulations. It contained the tests for carbohydrate, tannins, saponin, glycoside, alkaloid, flavonoid etc. Thus *Vitis Vinifera* and *Withania somnifera, contain* chemical constituents like corbohydrate, glycoside, tannins, terpenoids, triterpenoids and flavonoids.

UV Spectroscopy studies was carried out to develop the spectrum of the formulation and validated by linearity study. The Withanolide follows Lambert-Beer's law limit in the concentration range of 10-50 µg/ml at range 343 nm. The amount of Withanolidei was found be 1.25±0.070 and 0.065±0.006, 0.059±0.003, 0.054±0.007.for *Withania somnifera* and ASLI, ASLII, ASLIII respectively which indicating that highest amount present in ASLI (Table 5.22).the reading were taken in the triplicate manner to reduce the error. The recovery studies of Withanolidewere found to be 95.15±0.2 and 94.3±0.3 which was repeated six times at all levels.

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The gallic acid also follows Lambert-Beer's law limit in the concentration range of 10-50µg/ml at range 227 nm. The amount of gallic acid in the formulation is found be 1.30±0.91, 0.87±0.011, 0.91±0.014, 0.95±0.01. The reading were taken in the triplicate manner to reduce the error (Table 5.26). The recovery studies of gallic acid were found to be 98.55±0.3 and 98.68±0.4 which was repeated six times at all levels. Result was concluded that formulation was stable under specific temperature and humidity condition.

HPLC method was used for the quantification of constituents as withanolidein ASLI, ASL II , ASLIII. C_{18} column and methanol: water in 69 :31 ratio was used as mobile phase. Absorbance was detected at 343 nm. The amount of withanolidein ASL III was quantified by using a calibration curve where peak areas were plotted against different concentrations of standard withanolide (2-10 µg/ml) by comparing the peaks areas, The amount of Withanolidewas found be 1.28±0.060 and 0.067±0.005, 0.060±0.001, 0.058±0.003.for *Withania somnifera* and ASLI, ASLII, ASLIII respectively which indicating that highest amount present in ASLI (Table 5.22) The readings were taken in triplicate manner to reduce error. The recovery studies of withanolide were performed and values were found to be 95.15±0.2 and 94.3±0.3 which were repeated six times at all levels(Table 5.23) The HPLC method was validated by defining the linearity, peak purity, limit of quantification and detection, precision, accuracy, specificity and robustness. The peak purity was studied in the major peaks. Linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated for quantification purposes. The LOD and LOQ was found to be 0.6 and 2 mg/ml (Table18) respectively which suggest full capacity for quantification of withanolide content in ASLIII. R^2 value for the regression equation of the withanolide is- 0.997 this confirms the linearity of the method. Finally the robustness of the method was also assessed.

The total viable aerobic and fungi count of different batches of Ashwagandhadi leha were determined by plate count method. Microorganism are absent in ASLI, ASLII, ASLIII, so. the safety analysis for formulations shows good results and it is safe to use.

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Heavy metals analysis was carried out for presence of lead and arsenic cause harmful effect. By use of atomic absorption spectroscopy lead and arsenic was determine. lead and arsenic was not detected in the ASL III.

Stability studies of were performed by keeping the formulation at 40°C ± 2°C/ 75 ± 5 % RH in stability chamber for 2 months. Results showing slightly changes in the physical and chemical parameters of formulation, thus it could be observed that formulation was stable (Table 5.32). data shows that slightly changes in the physical and chemical parameters of formulation after stability studies at different periods of time.

4. Conclusion

Ayurvedic medicine may be used for maintained the general health and to reduce the chances of infection. it is widely used due to lesser toxic effects, because it is one of the polyherbal formulation containing more than two drugs, Mriwikadi leha composed of two herbs and useful in the treatment of cough.

According to WHO guidelines crude drugs subjected to quality control parameters such as morphological and physiochemical characters (extractive value, ash value, swelling index, foaming index). Three batches of Ashwagandhadi leha formulations were prepared in laboratory and these formulations subjected to evaluated for its organoleptic property, pH, ash values, loss on drying, phytochemical evaluation, fat content, sugar content , total solid, stability studies, UV Spectroscopy studies, Chromatographic studies (HPLC, TLC), antioxidant activity carried out for free radical scavanging activity done by DPPH method also performed. Safety analysis and heavy metals analysis also carried out safety purpose. The scientific confirmation of the therapeutic utility of time tested formulation was supplemented by good efforts for the development of pharmacopoeial standard for quality control to ensure uniformity and consistency in the quality of product.

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Table 1: Formula of Ashwagandhadi leha

S no	Local name	Biological source	Quantity prescribed in API
1.	Sarkara	-	1.356 kg
2.	Ashwagandha	Withania somnifera	192 g
3.	Sariva	Hemidesmus indicus	192 g
4.	Jiraka	Cuminum cyminum	192 g
5.	Madhusnuhi	Smilax glabra	192 g
6.	Draksa	Vitis vinifera	192 g
7.	Ghrita	-	226 g
8.	Honey	-	452 g
9.	Ela	Elettaria cardamomum	24 g
10.	Water	-	452 g

Figure 2: Ashwagandhadi leha Laboratory formulation.

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Table 3: Morphological characters of crude drugs

S no	Morphological characters	Vitis vinifera fruits	Withania somnifera fruits
1.	Shape	Berry	Cylindrical
2.	Size	1.5 to 2.5 cm long and 0.5 to 1.5 cm thick	2.5 to 5cm in long, 0.4 to 1 cm thick
3.	Colour	Dark brown to black	Greenish to black
4.	Odour	Sweetish and pleasant	Aromatic
5.	Taste	Sweet	Pungent

Foreign matter was not found in *Vitis vinifera* and *Withania somnifera* indicates that the presence of impurities was absent.

Table 4: Foreign matter for crude drugs

S no.	Drugs	Foreign Matter	
		Std	Obd
1.	Vitis vinifera	NMT 2%	Nil
2.	Withania somnifera	NMT 2%	Nil

Table 5: Loss on drying of crude drugs (% w/w)

S no	Drugs	Loss on Drying
1.	Vitis vinifera	2.9±0.2
2.	Withania somnifera	1.3±0.1

All values a Mean (n) ± SD, n=3 and SD= Standard Deviation.

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Table 6: Extractive value for crude drugs (% w/w)

S no	Drugs	Alcohol Soluble Extractive		Aqueous Soluble Extractive	
		Std	Obd	Std	Obd
	Vitis vinifera	NLT 25%	28.5±0.3	NLT 70%	76.6±0.5
	Withania somnifera	NLT 5%	16.0±0.6	NLT 7%	23.7±0.7

All values are mean (n) ± SD, n=3 and SD= Standard Deviation.

Table 7: Ash value of crude drug (% w/w)

S no	Drugs	Total Ash		Acid Insoluble Ash	
		Std	Obd	Std	Obd
	Vinifera vinifera	NMT 2%	2.5±0.2	NMT 0.2%	0.19±0.4
	Withania somnifera	NMT 7%	6.8±0.5	NMT 0.5%	0.4±0.1

All values are mean (n) ± SD, n=3 and SD= Standard Deviation.

Table 8: Foaming index of crude drugs

S no	Drugs	Foaming Index
	Vinifera vinifera	<100
	Withania somnifera	<100

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Table 9: Organoleptic characteristics of Ashwagandhadi leha

S no	Organoleptic Characteristics	Observation			
		Std	**Obd in ASLI**	**Obd in ASLII**	**Obd inASLII**
1	Colour	Dark brown	Dark brown	Dark brown	Dark brown
2	Odour	Melleable	Characteristics	Characteristics	Characteristics
3	Taste	Pungent, slightly sweet and sour	Pungent	Pungent	Pungent

Figure 10: TLC of Ashwagandhadi leha

Table 11: R_f values of Piperine

S no	Solvent system	Spot no.	Detecting agent	R_f values
1.	toluene : ethyl acetate:formic acid (4 : 2.5 : 0.7)	1	anisaldehyde-sulphuric acid reagent	0.4

Table 12: pH value

S no	Concentrations	pH value
1.	1%w/v	4.86±0.2
2.	10%w/v	5.12±0.3

All values are Mean (n) ± SD, n=3 and SD= Standard Deviation

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Table 13: Loss on drying

S no	Formulations	Loss on drying (%w/w)
1.	ASL I	4.7±1.5
2.	ASL II	7.08±1.13
3.	ASLIII	6.03±1.5

All values are Mean (n) ± SD, n=3 and SD= Standard Deviation

Table 14: Ash values

S no	Formulations	Ash values (%w/w)			
		Total ash		Acid insoluble ash	
		Std	Obd	Std	Obd
1.	ASL I	NMT 1%	0.2±0.01	NMT 0.2%	0.05±0.01
2.	ASL II	NMT 1%	0.15±0.20	NMT 0.2%	0.01±0.03
3.	ASLIII	NMT 1%	0.26±0.21	NMT 0.2%	0.03±0.015

All values are Mean (n) ± SD, n=3 and SD= Standard Deviation

Table 15: Fat content

S no	Formulations	Fat content(% w/w)
1.	ASL I	0.01±0.15
2.	ASL II	0.03±0.3
3.	ASLIII	0.10±0.1

All values are Mean (n) ± SD, n=3 and SD= Standard Deviation

Table no. 16: Sugar content

S no	Formulations	Sugar content(% w/w)
1.	ASL I	70.0±0.13
2.	ASL II	71.0±0.6
3.	ASLIII	70.5±0.14

All values are Mean (n) ± SD, n=3 and SD= Standard Deviation

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Table 17: Total solid content

S no	Formulations	Total solid content(% w/w)
1.	ASL I	2.50±0.20
2.	ASL II	3.11±0.1
3.	ASLIII	3.50±0.15

All values are mean (n) ± SD, n=3 and SD= Standard Deviation

Table 18: Phytochemical screening of alcoholic extracts of crude drugs and formulation

S no	Test	Alcoholic extract of *Withania somnifera*	Alcoholic extract of *Vitis vinifera*	Formulations (Alcoholic extract of Ashwagandhadi leha)		
				ASL I	ASL II	ASL III
1.	Carbohydrate	+	-	+	+	+
2.	Glycosides	-	-	-	+	+
3.	Alkaloids	-	+	+	-	+
4.	Tannins	+	+	+	+	+
5.	Flavonoids	+	-	+	+	+
6.	Saponins	+	-	-	-	-

Here, + indicates presence, - indicates absence

Table 19: Analytical parameters for U V Spectroscopic Estimation of Withanolide

S no	Parameters	Value
1.	Absorption maxima	343 nm
2.	Beer's law limit	100-500 µg/ml
3.	Regression equation (y=mx + c)	y = 0.074x + 0.028
4.	Intercept (a)	0.028
5.	Slope (b)	0.014
6.	Correlation coefficient (r^2)	0.992

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Table 20: Calibration curve of withanolide

S no	Concentration(µg/ml)	Absorbance
1.	2	0.184
2.	4	0.318
3.	6	0.463
4.	8	0.608
5.	10	0.717

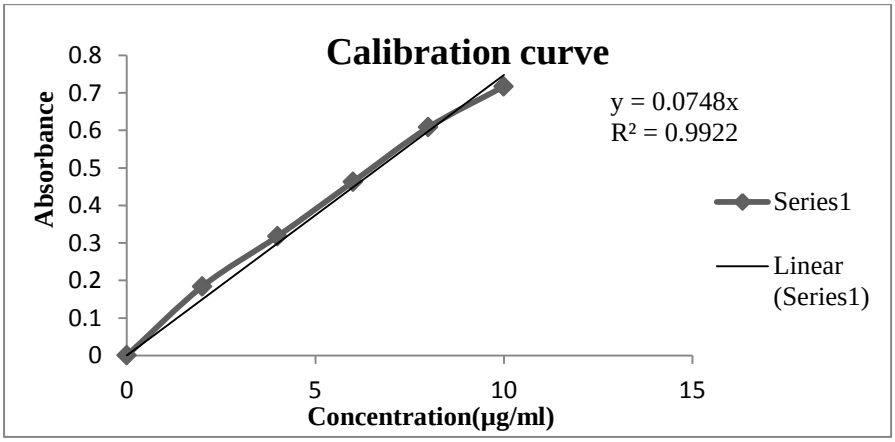

Figure 21: Calibration curve of withanolide

Table 22: Results of estimation for withanolide content

S no	Name	Withanolide content(%w/w)
1.	*Withania somnifera*	1.25±0.070
2.	ASL I	0.065±0.006
3.	ASL II	0.059±0.003
4.	ASL III	0.054±0.007

Mean ± SD of six determinations

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Table 23: Analytical parameters for UV spectroscopic estimation of gallic acid

S no	Parameters	Value
1.	Absorption maxima	227 nm
2.	Beer's law limit	100-500 µg/ml
3.	Regression equation (y=mx + c)	y = 0.019x + 0.053
4.	Intercept (a)	0.028
5.	Slope (b)	0.014
6.	Correlation coefficient (r^2)	R^2 = 0.989
7.	LOD	0.6
8.	LOQ	2.0
9.	Precision(n=6,% RSD)	0.35
10.	Accuracy (%)	99.3

Table 24: Calibration curve values of gallic acid

S.No.	Concentration (µg/ml)	Absorbance (abc)
1.	10	0.28
2.	20	0.47
3.	30	0.62
4.	40	0.83
5.	50	0.97

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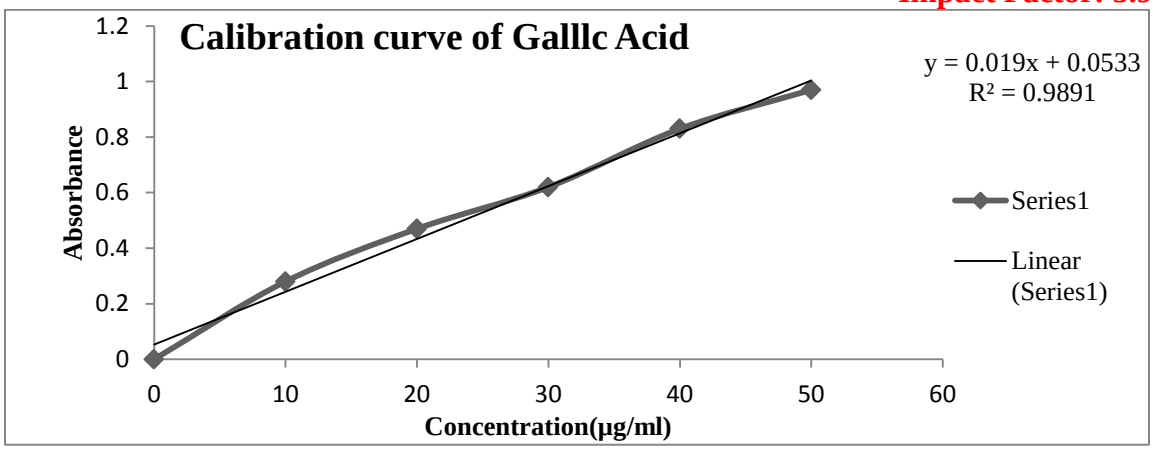
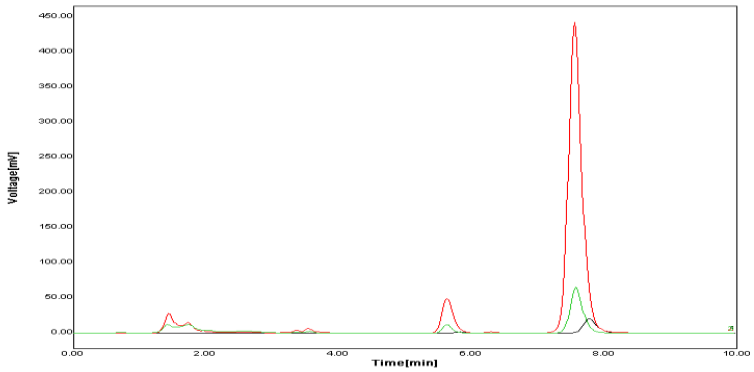


Figure 25: Calibration curve of Gallic acid



Standard **Raw Material** **Laboratory Formulation**

Figure 26: HPLC Chromatogram

Table 27: Analytical parameters of HPLC procedure for the withanolide estimation

S no	Parameters	Value
1.	Absorption maxima	343 nm
2.	Beer's law limit	100-500 µg/ml
3.	Regression equation (y=mx + c)	y = 0.014x + 0.028
4.	Intercept (a)	0.028

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5.	Slope (b)	0.014
6.	Correlation coefficient (r^2)	0.997
7.	LOD	0.6
8.	LOQ	2.0
9.	Precision(n=6,% RSD)	0.35
10.	Accuracy (%)	99.3

Table 28: Safety analysis

S no.	Formulations	Colonies formed CFU/gm	Microorganism Present		
			S. aureuos	***P.areugenosa***	***C.albicans***
1.	ASL I	10 ± 0.12	-	-	-
2.	ASL II	11 ± 0.23	-	-	-
3.	ASL III	11 ± 0.26	-	-	-

Table 29: Quantitative estimation of heavy/toxic metals present in the formulation

S.No.	Formulation	Heavy metals	
		Lead	**Arsenic**
1.	ASL III	ND	ND
2.	Prescribed limit	10 ppm	10 ppm

Here, ppm means parts per million and ND means not detectable

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Table no 30: Stability study

Sno.	Batch code	After 1month	After 2month	After 3month
1.	Color	NC	NC	NC
2.	Consistency	Good	Good	Good
3.	pH	4.86±0.2	4.90±0.25	4.96±0.4
4.	% of solid content	3.21±0.2	2.78±0.3	2.68±0.1
5.	LOD	4.7±0.15	4.50±0.15	4.25±0.25

All values are mean (n) ± SD, n=3 and SD= Standard Deviation

