

EVALUATION OF HERBAL FORMULATION (CAPSULE) CONTAINING ASHWAGANDHA AS A SINGLE HERB WITH THEIR NUTRITIONAL VALUE DETERMINATION

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ABSTRACT: The most important challenges faced by herbal formulations arise because of their lack of complete evaluation. Evaluation is necessary to ensure quality and purity of the herbal product. For evaluation of capsule containing single herb various parameters were tested. These parameters for raw material include powder characteristic study, organoleptic, physicochemical, phytochemical parameters etc., and assay of active constituent. Parameters for finished product (capsule) include uniformity of weight, pH, moisture content, disintegration time and dissolution study. HPTLC study, heavy metal analysis, microbial analysis and Nutritional value were carried out as a part of evaluation. Results indicate that Ashwagandha capsule has passed through all organoleptic and physicochemical parameters. Active constituent was present in adequate amount in the Ashwagandha capsule. Data of HPTLC finger printing indicates that extract was derived from genuine plant or parts of the plant and also capsule contain the same extract. Concentration of lead, arsenic and cadmium in capsules passed the limit of heavy metal. Mercury was absent in capsule and in their extract. Capsules have <10 cfu/gm total bacterial count. Total yeast and mould was absent in capsules. The pathogens like *E. coli*, *Salmonella*, *P.aeruginosa* and *S.aureus* were also absent in capsule. Carbohydrate, protein and cholesterol content for Ashwagandha capsule is 84.56%, 8.29% and 2.32% respectively.

Data suggested that capsule and its extract were consistent with various identity, quality and purity parameters such as organoleptic characters, physico-chemical parameter, HPTLC fingerprinting, heavy metal and microbial analysis. Nutritional assessment of the each capsule indicates their dietary supplement value.

Keywords: *Withania somnifera*, Herbal Formulation, HPTLC fingerprinting, Nutritional value, Heavy metal & Microbial analysis.

INTRODUCTION

In herbal medicine plant based formulations are used to alleviate the diseases. But the most important challenges faced by these formulations arise because of their lack of complete evaluation. So evaluation is necessary to ensure quality and purity of the herbal product. It is very important to establish a system of evaluation for every plant medicine in the market, since the scope for variation in different batches of medicine is enormous. Nutrition is the provision, to cells and organisms, of the materials necessary (in the form of food) to support life. A poor diet can have an injurious impact on health, causing deficiency diseases. Herbal nutritional supplements provide essential nutrients that are not present or present in less amount in diet (Tamizhmani et al., 2003; Mohapatra et al., 2008).

MATERIALS & METHODS

Composition of capsule

Each 500 mg capsule contains

Concentrated extract of *Withania somnifera*..... 400 mg

Excipients..... q.s.

(A) Powder study

The shade dried roots of the plant were powdered and pass through sieve 60# and used for the observation of microscopical characters. The powder drug was separately treated with phloroglucinol:hydrochloric acid (1:1), lactophenol and iodine solution to determine the presence of lignified cells, calcium oxalate crystals and starch grain respectively (Khandelwal, 2005).

(B) Evaluation of quality control parameters for raw material (extract)

(a) Organoleptic parameters

Organoleptic parameter like color, odor and taste of all extracts were carried out.

(b) Physicochemical parameters

The physicochemical parameters like loss on drying, ash value (total ash, acid insoluble ash and water soluble ash) and extractive matter (alcohol soluble extractive and water soluble extractive) were determined as per WHO guideline, 2002.

(c) Determination of pH

The pH value of a solution was determined potentiometrically by means of a glass electrode, a reference electrode and a digital pH meter. The pH meter was operated according the manufacturer's instructions. First the apparatus was calibrated using buffer of 4, 9 and 7 pH. 1 g powdered extract was taken and dissolved in 100 ml demineralized water. The electrodes were immersed in the solution and the pH was measured (Anonymous, 1996).

(d) Qualitative phytochemical evaluation

Qualitative chemical tests for identifying various phytoconstituents present were carried out on various extracts and powder of capsule (Khandelwal, 2005; Harborne, 1973; Finar, 1975).

(1) Tests for Alkaloids

Tests for alkaloids were carried out using Mayer's test, Dragendroff's test, Wagner's test and Hager's test.

(2) Tests for Glycosides

General test: Extract 200 mg of the drug using 5 ml of dilute (10%) sulphuric acid and boil on water bath. After boiling add equal volume of water to the volume of NaOH used in the above test. Add 0.1 ml of Fehling's A and B until alkaline (red litmus changes to blue) and heat on water bath for two minutes. Note the red precipitate formed represents the glycoside after acid hydrolysis.

(3) Tests for Flavanoids

Shinoda test was used for detection of flavanoids.

(4) Test for Saponins

Frothing test: The sample drug was vigorously shaken with distilled water and was allowed to stand for 10 minutes. Stable froth more than 1.5 cm indicates presence of Saponin.

(5) Test for Tannins

With FeCl₃: The water extract of the sample drug was treated with alcoholic FeCl₃. Blue color indicates presence of tannins.

With lead acetate: With 5% lead acetate solution tannins give precipitate which turns red on addition of KOH solution, on excess addition precipitate is dissolved.

(6) Tests for Sterols and Triterpenoids

Liebermann-Burchard test and Salkowski test were used for detection of sterols and triterpenoids.

(7) Tests for Proteins

Biuret test was used for detection of proteins.

(8) Tests for Carbohydrates

Molisch's test was used for detection of carbohydrates.

(e) Estimation of total alkaloid in Ashwagandha root extract

Accurately weighed 3gm of the sample was taken. To the sample 5 ml of ammonia was added & shaken for some time then mixture of 75 ml of ether and 25 ml of alcohol was added. This solution was shaken continuously for 1 hour. The solution was filtered in the separator through cotton plug. The residue was washed from conical flask with mixture of 75 ml of ether and 25 ml of alcohol. The total solution was extracted with 25ml dilute sulphuric acid. Then extracted with mixture of 25 ml dilute sulphuric acid, 55 ml distilled water and 20 ml ethanol. Entire acid layer was collected and washed with 25 ml of chloroform. Acid layer was collected and made it alkaline with ammonia. It was extracted with chloroform (25x4). Water washing given to chloroform (20 ml). The chloroform layer was filtered in a weighed beaker and evaporates to dryness (Anonymous, 1996).

(C) Evaluation of Quality Control Parameters for Finished Product (Capsule)**a) Description**

Size, shape, colour etc were evaluated (Lachman et al., 1987).

b) Uniformity of weight

Test for uniformity of weight was performed as per Indian pharmacopoeia, 1996.

c) Determination of pH

The test was performed same as earlier in the case of pH determination for extract. Here powder of one capsule was used.

d) Disintegration test for capsule

Disintegration test was performed using the digital microprocessor based disintegration test apparatus by VEEGO.

One capsule was introduced into each tube and added a disc to each tube. The assembly was suspended in the water in a 1000 ml beaker. The volume of water was such that the wire mesh at its highest point is at least 25 mm below the surface of the water, and at its lower point was at least 25 mm above the bottom of the beaker. The apparatus was operated and maintained the temperature at $37^{\circ} \pm 2^{\circ}\text{C}$. Noted down the time require to all capsules to disintegrate and pass through wire mesh (Anonymous, 1996).

e) Dissolution test for capsule

The dissolution test was performed for capsule using USP dissolution apparatus 2 by VEEGO. The 900 ml of the 5 N HCl as dissolution medium was introduced into the vessel of the apparatus. The dissolution medium was warmed to 36.5^o-37.5^oC. For the capsules basket type dissolution apparatus was used. The apparatus was operated immediately at the speed of 50 rpm for two hrs. After two hrs a 25 ml specimen was withdrawn from a zone midway between the surface of the dissolution medium and top of the rotating blade or basket, in evaporating dish. Filter and evaporate the specimen. For the each of the capsule tested, the amount of dissolved active ingredient in the solution was calculated as a percentage dissolved in two hrs (Anonymous, 1996).

f) Determination of Moisture content

The test was performed using Karl Fischer instrument by VEEGO.

(D) HPTLC finger-printing of raw material and finished product**Selection of plate and adsorbent**

Precoated aluminium plates with Silica Gel 60F254 (E. Merck, India) of 10 x 10 cm and 0.2 mm thickness, were used for the detection. The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography.

Sample solution

Accurately weighed 1 g of extract and finished product equivalent to 1 g of extract were taken in separate iodine flask. Then 50 ml methanol was added into each flask and refluxed for 1 hour. Filter the solution. Then the filtrate was concentrated to 1-2 ml. This solution was used for HPTLC finger-printing.

Application of sample

The most recent automatic device “CAMAG LINOMAT V” was used to apply 1 band of 6 mm width with different concentration of extract and finished product solution viz. 5 and 10 µl.

Development

The plate was developed in CAMAG glass twin-through chamber (10-10 cm) previously saturated with the solvent for 60 min (temperature 25.2 °C, relative humidity 40%). The development distance was 8 cm. Subsequently scanning was done.

Solvent system: Chloroform: Methanol (9:1)

Detection

The plate was scanned at UV 366 nm and 254 nm using CAMAG TLC Scanner-3 and LINOMAT-V. R_f value of each compound which were separated on plate and data of peak area of each band were recorded.

(E) Heavy Metal Analysis**Preparation of samples by acid digestion method**

Accurately weighed 2 g of sample was taken in Kjeldahl flask. Acid mixture of HNO₃:HClO₄ (4:1) was added in the flask and heated continuously till the solution is colorless. The sample was then transferred in a 25 ml volumetric flask and the volume was made-up with distilled water.

Reagent blank was synchronously prepared according to the above procedure. The standards of Lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg) were prepared as per the protocol in the manual and the calibration curve was developed for each of them.

Detection

Then samples were analyzed for the presence of Pb, Cd, As and Hg using Atomic Absorbance Spectrophotometer (AAS) 6300 (by SHIMADZU) (Lohar, 2007).

(F) Microbial analysis

Microbial analysis was carried for capsule as per procedures of Indian pharmacopoeia 2007 and WHO Guideline. The test included total bacterial count, total yeast and mould count, Identification of specified organisms such as *Escherichia coli*, *Salmonella sp.*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

(G) Nutritional Value Determination

(a) Carbohydrate estimation

Test stock solution preparation

1 g of capsule powder was taken and hydrolyzed with 10 ml of 2.5 N HCl solution by keeping in a boiling water bath for 3 hours. Then neutralization was done with solid Na_2CO_3 until effervescences ceases. Volume was made upto 100 ml with distilled water. Then filter the solution.

Protocol

Glucose was used as standard for preparation of standard graph within ranges of 0 to 250 μg concentration. 0.1 ml of sample was taken from test stock solution. Then volume was made up to 3 ml with distilled water in case of all standard and test sample solutions. Then 4 ml of anthrone reagent (200 mg anthrone in 100 ml ice cold solution of 95% H_2SO_4) was added into each solution. Then the solution was heated on water bath for 8 minutes and cooled. Readings were taken at optical density 630 nm in order to estimate the concentration of carbohydrate in the sample by comparison with the known standard graph of carbohydrate (Hedge & Hotreiter 1962).

(b) Protein estimation

Reagents

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Reagent B: 0.1% Sodium Potassium Tartrate and 0.5% CuSO_4 (1:1)

Reagent C: For 50ml preparation, 49ml Reagent A and 1ml Reagent B

Reagent D: FC (Folin Ciocalteu) reagent- (1:1) dilution with water

Test stock solution: 1 gm capsule powder/100 ml distilled water

Protocol

Bovine serum albumin was used as standard for preparation of standard graph within ranges of 0 to 250 μg concentration. 0.1 ml of sample was taken from test stock solution. Then volume was made up to 1 ml with distilled water in case of all standard and test sample solutions. Then 5 ml of reagent C was added into each solution. Incubated at room temperature for 10 minutes. Then 0.5 ml of FC reagent was added. Kept in dark for 20 minutes. Readings were taken at optical density 660 nm in order to estimate the concentration of protein in the sample by comparison with the known standard graph of protein (Lowery et al., 1951).

(c) Cholesterol estimation by Zak's method

Test stock solution: 0.1 g capsule powder/10 ml isopropanol

Protocol

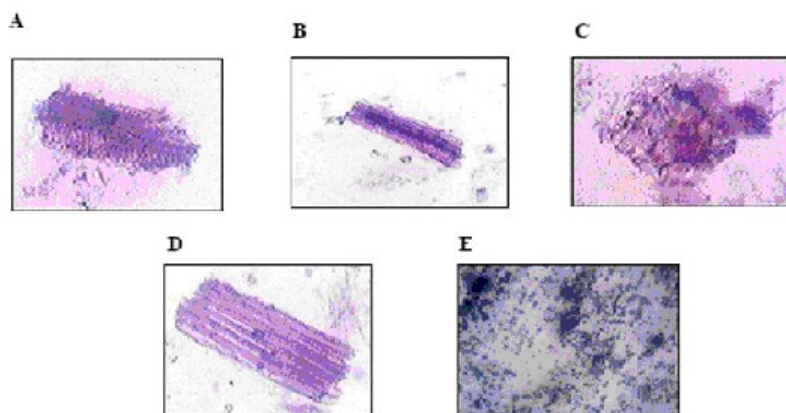
Cholesterol was used as standard for preparation of standard graph within ranges of 0 to 500 µg concentration. 1 ml of sample was taken from test stock solution. Then volume was made up to 2 ml with isopropanol in case of all standard and test sample solutions. Then 1 ml of FeCl₃ reagent (500 mg FeCl₃·6H₂O in 500 ml of glacial acetic acid) was added into each solution. Then 2 ml of concentrated H₂SO₄ was added. Kept at room temperature for 10 minutes. Readings were taken at optical density 540 nm in order to estimate the concentration of cholesterol in the sample by comparison with the known standard graph of cholesterol (Bose, 1963).

Results

The powder study of ashwagandha root (Figure 1) has shown the presence of pitted and annular xylem vessels, tracheids, cork cells, fibres and starch grains.

Color, odor and taste of ashwagandha root extract were light yellowish brown, characteristic and bitter respectively.

Results have shown that loss on drying, pH, water soluble extractive value and alcohol soluble extractive value for ashwagandha extract were 2.4 %w/w, 4.74, 90.74 %w/w and 27.52 %w/w respectively. Total ash, acid insoluble ash and water soluble ash for ashwagandha extract were 5.37 %w/w, 0.76 %w/w and 1.13 %w/w respectively.



(A) xylem vessels, (B) Tracheids, (C) cork cells, (D) fibres and (E) starch grain:

Figure 1. Powder characteristic of *Withania somnifera* root

Ashwagandha extract has shown presence of alkaloid, glycoside, saponin, steroid, carbohydrate and protein during phytochemical evaluation.

Ashwagandha extract showed presence of 0.97 %w/w total alkaloid. Capsule shell was transparent. Size of capsule was “0”. Results have shown that average weight of capsule was 505.75±4.88 mg (mean±SEM, n=3) and capsule passed the test for uniformity of weight.

Results have shown that disintegration time, dissolution, pH and moisture content of ashwagandha capsule were 6.5 minutes, 72.93 %, 5.14 and 3.62 %w/w respectively.

HPTLC plates under UV 254 nm and UV 366 nm are showed in figure 2. Three dimension image of the fingerprinting *W. somnifera* of is also showed in figure 2.

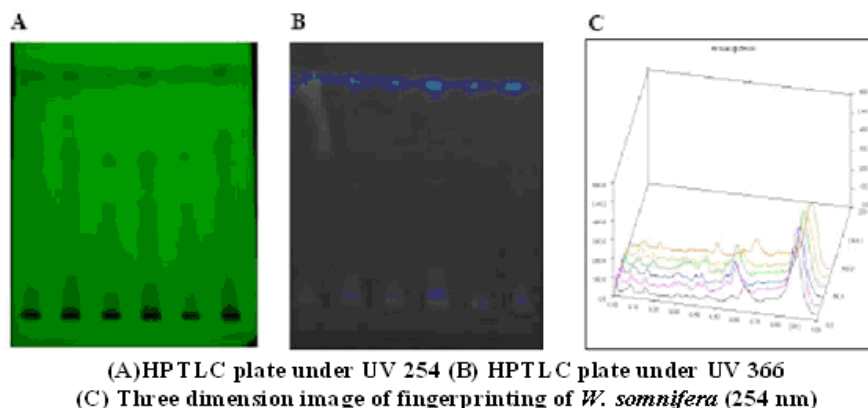


Figure 2. HPTLC fingerprinting of *Withania somnifera*

Track 1: 5 µg/ml of Standard methanol extract of *Withania somnifera*
 Track 2: 10 µg/ml of Standard methanol extract of *Withania somnifera*
 Track 3: 5 µg/ml of raw methanol extract of *Withania somnifera*
 Track 4: 10 µg/ml of raw methanol extract of *Withania somnifera*
 Track 5: 5 µg/ml of methanol extract of ashwagandha capsule
 Track 6: 10 µg/ml of methanol extract of ashwagandha capsule

Results have shown that concentration of lead was 1.998 ppm and 2.278 ppm, concentration of cadmium was 0.005 ppm and 0.01 ppm and concentration of arsenic was 0.069 ppm and 0.25 ppm respectively for ashwagandha extract and ashwagandha capsule. Mercury was absent in ashwagandha extract and ashwagandha capsule.

Ashwagandha capsule has <10 cfu/gm total bacterial count. Total yeast and mould were absent in capsule. The pathogens like *E. coli*, *Salmonella*, *P. aeruginosa* and *S. aureus* were also absent in capsule.

Results have shown that carbohydrate content, protein content and cholesterol content for ashwagandha capsule were 84.56 %, 8.29 % and 2.32 % respectively.

Discussion

Powder study and organoleptic parameters showed identity of the herb. Loss on drying was <5 %w/w which indicates that there is less chances of microbial growth and also it may not cause sticking problem in final processing of formulation. Water soluble extractive value is >90 % which indicates that extract has high water solubility and good quality. Results of ash values are within limit and show that there are less impurities in the extract. Phytochemical evaluation shows presence of constituents in extract. Extract contain good amount of alkaloid. Capsule passed the test for uniformity of weight. All capsules disintegrated within 7 minutes. Dissolution of capsule was >70 %. Moisture content of capsule was <5 % w/w which indicates that there is less chances of microbial growth and capsule will not become soft. Data of HPTLC finger printing indicates that extract was derived from genuine plant or parts of the plant and also capsule contain the same extract. Capsule passed the limit for heavy metals and microbial contamination. Results of nutritional value showed good amount of carbohydrate and protein.

Conclusions

Data suggested that capsule and its extract were consistent with various identity, quality and purity parameters such as organoleptic parameters, physico-chemical parameters, HPTLC fingerprinting, heavy metal analysis and microbial analysis. It also gives surety about the product which is genuinely prepared. Nutritional assessment of the each capsule indicates their dietary supplement value and medicinal role as an herbal supplement. Selected Herbal capsules have passed through all the WHO parameters which were tested.

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