Research Article

# Natural herbal supplements – Studies on phytochemical constituents and Estimation of their nutritional values

**Kruti Pandya[1*], Bhavna Solanki[1], Nilesh Gurav[1], Surendra Bhatt[1]**
*[1]Vasu Research Centre, A Division of Vasu Healthcare Pvt Ltd, 896/A, GIDC Makarpura, Vadodara, India.
***Corresponding author's E-mail:** krutishah85@gmail.com

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**ABSTRACT**

Nutritional deficiency is almost impossible to avoid in these modern times, thus supplements help us to overcome the nutritional deficiencies. It also helps us to boost our immune system. Nutritional supplements are also useful in getting rid of the toxins that are accumulated in our body. Thus the five natural supplements that are mentioned below are tested for the various parameters that include the basic Quality Control Parameters, the phytochemical analysis, Microbial Analysis that includes the testing for the presence of pathogens along with the total bacterial and fungal count. It is also tested for the presence of heavy metals in them, followed by Aflatoxin and Pesticide analysis. The Nutritional Value for each of them were determined and reported in the form of mg/capsule. The actives of Ashwagandha Capsule and Bacopa Capsule were confirmed by the HPLC method.

**Keywords:** Heavy Metal analysis, High Performance Liquid Chromatography, Microbial analysis, Nutritional Value, Phytochemical analysis.

## INTRODUCTION

### *Withania somnifera* (Ashwagandha) Capsule

It belongs to the Family Solanaceae. The roots of the wild variety contain somniferin which is hallucinogenic in nature.[1] The main active constituent is alkaloids and steroidal lactones. Among the various alkaloids, withanine is the main constituent.

Withaferin A It is claimed to possess aphrodisiac, sedative, rejuvenative and life prolonging properties. Some of its benefits include chronic fatigue, dehydration, bone weakness, impotency, debility, constipation, nervous exhaustion etc.[2,3] Ashwagandha soothes and calms down the stressed mind and nerves. It relieves nervous exhaustion, brain fag, loss of memory, and loss of muscular energy. It also helps to normalize the sympathetic tone that is increased in Stress, Anxiety and Depression. It is a good adaptogenic, immunity booster and an essential additive in herbal formulation for hypertension due to its effects on nerves, stress and brain and sleep.

### *Emblica officinalis* (Amla) Capsule

It belongs to the family Euphorbiaceae**.** The fruit is a rich source of Vitamin C. It has mild depressant action of CNS and spamolytic action. Amla is an extensively used herb in making ayurvedic medicines. Amla is supposed to rejuvenate all the organ systems of the body, provide strength and wellness. It is the richest natural source of vitamin C. It is a strong rejuvenative, balances stomach acid and improves food absorption. It is an anti-oxidant. It is effective in the treatment of peptic Ulcer[4-7] and in dyspepsia.[8]

### *Andrographis paniculata* Capsule

It belongs to the family Acanthaceae. Andrographis and its various components have demonstrated a variety of effects in the body. Aspects stimulate the general immune activities. The active chemical, Andrographolide, helps to stop the clumping of blood platelets which is the clotting process that can lead to heart attacks. It is said to possess Anti-inflammatory activity.[9-11] The major constituents include Lactones; Diterpene lactone andrographolide (0.5-0.9%)[12-14] Andrographolide and related diterpenes are immunostimulant[15] and antiinflammatory activities.[16]

### *Terminalia arjuna* (Arjuna) Capsule

It belongs to the Family Combretaceae. It is a prominent cardiotonic drug in the Indian medicine and is effective on heart and blood vessels. It increases the relaxation period of the heart and acts as a cardiac tonic. It increases the force of contraction of the heart and regulates its rhythm, thus acting as a cardiac stimulant. The bark is useful as a cardio stimulant, tonic, cooling, astringent and febrifuge, lithintropic, chalagogue and vulnerary. The major Chemical Constituents are Hydrolysable and condensed tannins, ellagic acid, arjunin, arjunic acid, arjunetin, arjunolitin, friedelin, terminoic acid, arjungenin, arjunglucoside I, II[17-20] Others include Sitosterol[21] and a flavone arjunolone.[22]

### *Bacopa monnieri* (Bacopa) Capsule

It belongs to the Family Scrophulariaceae. It is a traditional treatment for epilepsy and asthma.[23] It has antioxidant properties. It is listed as a nootropic, a drug that enhances cognitive ability. Recent studies suggest Bacopa may improve intellectual activity[24-26] Its active compounds include alkaloids (brahmine and herpestine), saponins (d-mannitol and hersaponin, acid A,

and monnierin), flavonoids (luteolin and apigenin) also in significant amounts betulic acid, stigmastarol, beta-sitosterol, bacopasaponins. The triterpenoid saponins and their bacosides are responsible for Bacopa's ability to enhance nerve impulse transmission. The bacosides aid in repair of damaged neurons by enhancing the kinase activity, neuronal synthesis, and restoration of synaptic activity, and ultimately nerve impulse transmission. Loss of cholinergic neuronal activity in the hippocampus is the primary feature of Alzheimer's disease. The major chemical constituents include glycosides - Saponin glycosides – triterpenoid (tetracyclic).

## MATERIALS AND METHODS

### Quality Control Parameters

The herbal supplements were tested for their basic Quality control parameters that included tests like pH, Moisture, bulk density, disintegration and dissolution time. The presence of actives in the respective samples was detected with the help of sophisticated instruments. The results are as tabulated in Table 1.

**Table 1**: Quality Control parameters & Phytochemical Analysis

| **Quality Control parameters** | | | | | |
|---|---|---|---|---|---|
| **Test Parameters** | **Amla Capsule** | **Andrographis Capsule** | **Arjuna Capsule** | **Ashwagandha Capsule** | **Bacopa Capsule** |
| **pH 2 % Solution** | 5.47 | 7.71 | 4.60 | 4.82 | 4.59 |
| **Moisture** | 1.89 % | 1.26 % | 1.24 % | 2.56 | 2.20 |
| **Bulk Density g/mL** | 0.675 | 0.746 | 0.625 | 0.793 | 0.609 |
| **Disintegration Time** | 15 38” | 11 05” | 24 00” | 20 30 “ | 9 10” |
| **Dissolution test** | 73.70 % | 96.01 % | 48.27 % | 81.14 % | 72.80 % |
| **Phytochemical Analysis** | | | | | |
| **Tannin %** | 41.30 ±0.33 | NA | NA | NA | NA |
| **Andrographolide (HPLC)** | NA | 11.90 ±0.30 | NA | NA | NA |
| **Tannin %** | NA | NA | 41.12± 0.33 | NA | NA |
| **Withanolides** | NA | NA | NA | 3.86 % | NA |
| **Bacoside (HPLC)** | NA | NA | NA | NA | 25.75 % |

**Table 2**: Test for Contaminants

| **Microbial Analysis** | | | | | | |
|---|---|---|---|---|---|---|
| **Name of the Sample** | **TBC** | **TFC** | ***E.c*** | ***P.a*** | ***S.a*** | ***S.spp*** |
| **Limits** | **$10^5$ cfu/g** | **$10^3$ cfu/g** | **Ab** | **Ab** | **Ab** | **Ab** |
| Amla Capsule | 16 x $10^2$ | Ab | Ab | Ab | Ab | Ab |
| Andrographis Capsule | 5 x $10^2$ | Ab | Ab | Ab | Ab | Ab |
| Arjuna Capsule | 22 x $10^2$ | Ab | Ab | Ab | Ab | Ab |
| Ashwagandha Capsule | 20 x $10^2$ | Ab | Ab | Ab | Ab | Ab |
| Bacopa Capsule | 12 x $10^2$ | Ab | Ab | Ab | Ab | Ab |
| **Heavy Metal Analysis** | | | | | | |
| | **Lead** | | **Cadmium** | **Arsenic** | **Mercury** | |
| **Limits** | **10 ppm** | | **0.3 ppm** | **10 ppm** | **1 ppm** | |
| Amla Capsule | ND | | ND | 0.156 | 0.030 | |
| Andrographis Capsule | 2.517 | | ND | 0.056 | 0.030 | |
| Arjuna Capsule | 1.809 | | 0.144 | 0.256 | 0.020 | |
| Ashwagandha Capsule | 0.896 | | 0.072 | 0.194 | 0.540 | |
| Bacopa Capsule | 1.534 | | 0.216 | 0.213 | 0.020 | |
| **Aflatoxin & Pesticides** | | | | | | |
| | **Aflatoxin** | | | **Pesticides** | | |
| Amla Capsule | Not Detected | | | Not Detected | | |
| Andrographis Capsule | Not Detected | | | Not Detected | | |
| Arjuna Capsule | Not Detected | | | Not Detected | | |
| Ashwagandha Capsule | Not Detected | | | Not Detected | | |
| Bacopa Capsule | Not Detected | | | Not Detected | | |

TBC: Total Bacterial Count; TFC: Total Fungal Count; *E.coli: Escherichia coli*; *P.a: Pseudomonas aeruginosa*; *S.a: Staphylococcus aureus; Sal: Salmonella;* ND: Not Detected.

***Microbial Analysis***

Microbial analysis was carried out for all the samples as per procedure of Indian pharmacopoeia 2010 and WHO Guideline. It included Total Bacterial Count, Total Fungal Count, presence of pathogens like *Escherichia coli, Salmonella spp, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* Pure culture of *Escherichia coli* (NCIM: 2065; ATCC: 8739), *Salmonella ebony* (NCIM: 2257 NCTC: 6017), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6358) were obtained from NCIM Pune. The media used for the microbial limit test were of HiMedia Pvt. Ltd.[27-29] The results are as tabulated in Table 2.

***Heavy Metal Analysis***

Heavy metal analysis was performed using Shimadzu AA-6300, HVG and digestion was done on CEM MARS Express microwave digestive system. Sample amount 0.5g and 8ml of 69% Nitric acid was taken in the Teflon PFA 75ml vessels. Parameters used for the digestion were Power 400 W with 100%, Ramp 20 minutes to attempt temperature 150$^{O}$C and held for 10 minutes. After the digestion process was completed the sample was diluted up to 50ml by distilled water and filtered through Whatman filter paper No. 1. The standards of Lead (Pb), Cadmium (Cd), Arsenic (As) and Mercury (Hg) were prepared and the calibration curve was developed for each of them. The samples were analyzed by using these standard curves. The permissible limit for Heavy metal in herbal drugs is consider as per describe limit given by Department of Ayush. The results are as tabulated in Table 2.[28]

***Aflatoxin and Pesticide Analysis***

The analysis was also carried out as below

Sample Preparation

500 mg of the sample was dissolved in 10 ml of Methanol. It was then concentrated on water bath to approximately 7-8 ml. This is then used as the test solution.

**Analytical parameters**

| | |
|---|---|
| **Instrument** | : GC-MS |
| **Model** | : Auto system XL with Turbo mass |
| **Make** | : Perkin Elmer |
| **Column** | : PE-5MS (30 meters capillary column) |
| **Carrier gas** | : Helium |
| **Flow** | : 1ml / min |
| **Injection Temp** | **:** 250°C |
| **Oven Temp** | : 70$^{0}$C and held for 5 minutes; |
| **Rate** | : 10° C/min up to 290°C and held for 30 minutes |
| **EI Source Temp** | : 250°C |
| **Scan range** | : 30-650 amu. |

The results are as tabulated in Table 2.

***Nutritional Value***

When one has to take herbal supplement as a part of their daily diet it is very important to estimate the amount of nutrition present per capsule. Thus they were tested for 10 important parameters that included Total Carbohydrate Content, Total Protein Content, Assay of Calcium, Thiamine Estimation, Estimation of Niacin Content, Iron Estimation, Riboflavin Estimation, Total Fat Content, Cholesterol Content, and Vitamin C Estimation.

***Total Carbohydrate Content***

The standard Anthrone method was used to check for the amount of Total Carbohydrate in the given sample. The sample to be tested was accurately weighed in boiling tubes and dissolved in 10 ml of 2.5 N HCl solution. It was then hydrolyzed by keeping it in a boiling water bath for 3 hours and cooled down to room temperature. It was then neutralized with solid $Na_2CO_3$ until the effervescence ceases and made up the volume to 100 ml with distilled water. It is then centrifuged and the supernatant was collected and two different aliquots were prepared. Glucose was used as a standard for the preparation of the standard graph with ranges of 0µg - 200µg concentration (0µg served as a blank). Make up the volume to 2 ml with Distilled water then add 4 ml of anthrone reagent to all the tubes and heat for 10 minutes on a boiling water bath. Cool the tubes and their absorbance was read at 630 nm on a UV Spectrophotometer.

***Total Protein Content***

The protein estimation was carried out by the Lowry's method. Bovine serum albumin (BSA) was used as a standard for preparation of the standard graph with ranges from 0µg - 250µg concentration (0µg served as a blank). The sample to be tested was weighed accurately and dissolved in distilled water and filter and use as the sample. Take different aliquotes and make up the volume with distilled water and add reagent C (Alkaline copper solution: Mix 50 ml of Reagent A and 1 ml of Reagent B) and incubate at room temperature for 10 minutes, after which 0.5 ml of Folin- Ciocalteau reagent was added and incubated at dark for 20 minutes and the absorbance was read at 660 nm on a UV Spectrophotometer.

(Note: Reagent A- 2 % Sodium carbonate in 0.1 N NaOH, Reagent B- 0.1 % Na-K tarterate and 0.5 % $CuSO_4$.)

***Estimation of Calcium***

Accurately weigh the sample and dissolve it in a 150 ml conical flask containing 3 ml dilute HCl and 10 ml distill water. Boil for 10 minutes to dissolve the sample and cool down to room temperature. Dilute it with 50 ml of Distilled water. Titrate against 0.05 N disodium EDTA solution nearing the end point and then add 8 ml of 20 % NaOH solution with the addition of 0.1 g calcon mixture which acts as an indicator. Continue the titration till the end point is achieved. The percentage of Calcium is then calculated according to the formula below.

$$\%\text{ of Calcium} = \frac{\text{Burette reading} \times \text{Factor} \times \text{Actual Normality of EDTA} \times 100}{\text{Weight of the sample} \times \text{normality of EDTA}}$$

Where Factor =0.005004

***Thiamine (Vitamin B12) Estimation***

Accurately weigh the sample and dissolve it in 100 ml of 0.1 N HCl and mix well. Incubate it overnight. Filter the solution and the filtrate is used as the sample for further analysis. Thiamine is used as standard for preparation of the standard graph which ranges from 0µg - 250µg concentration (0µg served as a blank). Make up the volume to 5 ml with Distilled water. Add 2.5 ml of Buffer solution having a pH 6.6 followed by 2.5 ml 4% Cyanogen bromide solution and shake well. Incubate for 30 minutes at room temperature and take the reading at 366 nm on a UV Spectrophotometer.

***Niacin Estimation***

The sample was accurately weighed and dissolved in 30 ml of 4 N $H_2SO_4$. It was boiled for 30 minutes, cooled down to room temperature and the volume was made up to 50 ml with distilled water. 60 % lead acetate was added and the pH was adjusted to 9.0 and centrifuged. 2 ml of concentrated H2SO4 was added to the supernatant and incubated at room temperature for 1hour. It is then centrifuged again and the supernatant was collected and 5 ml of 40 % $ZnSO_4$ was added and the pH was adjusted to 8.4 and centrifuged again and the supernatant was collected again and pH is now adjusted to 7.0 and then is used as the sample. Niacin was used as the standard for preparation of the standard graph which ranges from 0µg - 250µg concentration (0µg served as a blank). The volume was made up to 5 ml with distilled water after which 5 ml of 1 % Cyanogen bromide was added and set aside at room temperature for 10 minutes. 1 ml of 4 % aniline is added to all the tubes and then the absorbance was read at 420 nm on a UV Spectrophotometer.

***Estimation of Iron***

Accurately weigh the sample and dissolve it in 50 ml of distilled water and mix well. Filter the solution and the filtrate is used as the sample for further analysis. $Fe^{3+}$ is used as standard for preparation of standard graph which ranges from 0µg - 250µg concentration (0µg served as a blank). Make up the volume to 2.5 ml with Distilled water. Add 0.5 ml of Hydroquinone solution followed by 2.5 ml of Acetate buffer having a pH of 5.0. Then add 0.5 ml of ά0.1% ά - ά di pyridine and incubate at room temperature for 30 minutes and take the reading at 540 nm on a UV Spectrophotometer.

***Estimation of (Vitamin B2) Riboflavin***

Accurately weigh the sample and dissolve it in 100 ml of 0.1 N $H_2SO_4$. Boil the sample for 30 minutes and allow it to cool down to room temperature and then add 5 ml of 2.5 M sodium acetate and incubate it at room temperature for 1 hour. Filter the solution and the filtrate is used as the sample for further analysis. Riboflavin is used as a standard for the preparation of standard graph which ranges from 0µg - 250µg concentration (0µg served as a blank). Make up the volume to 2 ml with Distilled water. Add 1 ml of Glacial acetic acid followed by 0.5 ml 4% $KMnO_4$. Incubate for 2 seconds and add 0.5 ml 30 % Hydrogen peroxide. Shake well and read the absorbance at 366 nm on a UV Spectrophotometer.

***Total Fat Content***

The sample is accurately weighed and dissolved in 250 ml of hexane and kept in a thimble of soxhlet apparatus. Then add about 250 ml hexane and keep it for the extraction of fat. Switch on the heating mantle and adjust the temperature at 70°C and cool the solution after 5 hours. Evaporate the solution in a previously weighed evaporating dish and calculate the percentage of fat present in the given sample.

***Total Cholesterol Content***

Accurately weigh the sample and dissolve it in 50 ml of Isopropyl alcohol and mix well. Filter the solution and the filtrate is used as the sample for further analysis. Cholesterol is used as standard for preparation of the standard graph which ranges from 0µg - 250µg concentration (0µg served as a blank). Make up the volume to 2 ml with Isopropyl alcohol. Add 1 ml of $Fecl_3$ – acetic acid and add 2 ml of Conc. $H_2So_4$. Mix well and incubate at room temperature for 10 min and take the reading at 540 nm on a UV Spectrophotometer.

**Table 3:** The nutritional values are as tabulated below and are represented as mg/capsule

| | Ashwagandha Capsule | Amla Capsule | Andrographis Capsule | Arjuna Capsule | Bacopa Capsule |
|---|---|---|---|---|---|
| **Carbohydrate** | 206.97 | 47.36 | 25.27 | 127.09 | 56.02 |
| **Protein** | 1.304 | 31.09 | 0.48 | 838.37 | 8.01 |
| **Calcium** | 2.12 | 1.98 | 12.16 | 4.28 | 2.29 |
| **Thiamine** | 5.01 | 14.48 | 7.25 | 9.86 | 20.75 |
| **Niacin** | ND | 0.174 | ND | ND | ND |
| **Iron** | 0.779 | 1.10 | 1.58 | 2.05 | 0.516 |
| **Riboflavin** | 0.342 | 5.77 | 11.41 | 5.03 | 0.515 |
| **Total Fat** | ND | ND | ND | ND | ND |
| **Cholesterol** | 35.92 | 92.83 | ND | 29.84 | 99.22 |
| **Vitamin C** | 34.73 | 23.42 | 12.55 | 180.78 | 37.5 |

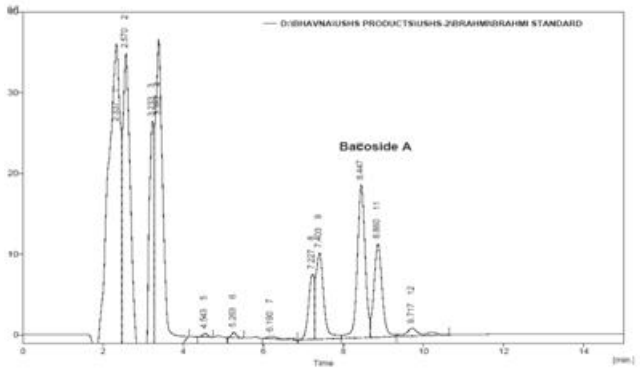


**Figure 1:** Chromatogram of the reference standard of Bacoside A

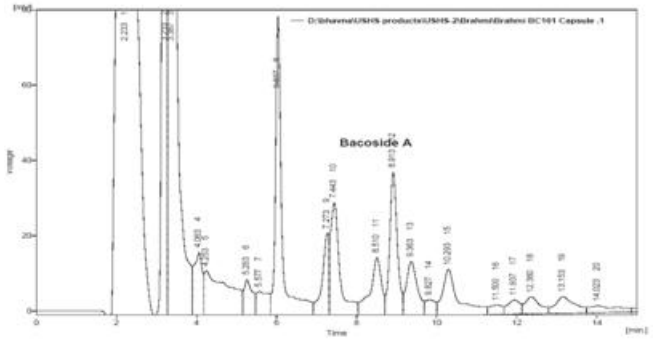


**Figure 2:** Chromatogram of Bacopa Capsule

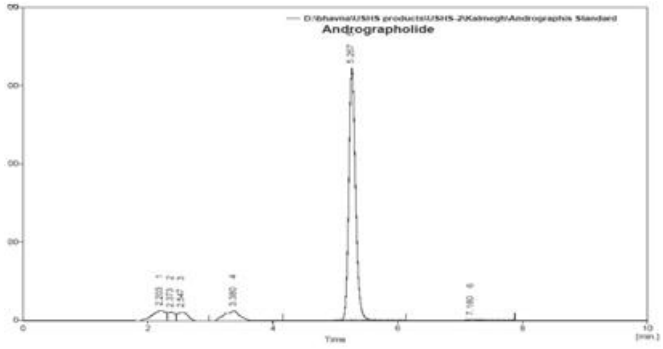


**Figure 3:** Chromatogram of the reference standard of Andrographis

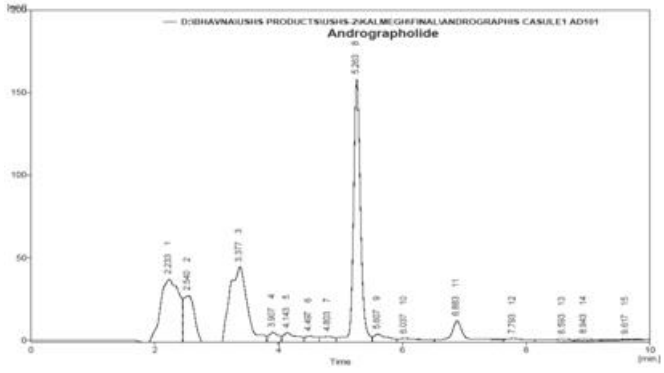


**Figure 4:** Chromatogram of Andrographis Capsule

***Estimation of Vitamin C (Ascorbic acid)***

Accurately weigh the sample and dissolve it in 75 ml of m-Phosphoric acid (m-PA) taken in $SnCl_2$ solution and mix well. Filter the solution and the filtrate is used as the sample for further analysis. Ascorbic acid is used as the standard for preparation of standard graph which ranges from 0µg - 250µg concentration (0µg served as a blank). Make up the volume to 2.5 ml with m-PA. Add 0.5 ml of 2% Dinitrophenyl hydrazine (DNPH). Incubate it for 1 hr at 50°C. Then add 2.5 ml of 85% sulphuric acid and take the reading at 540 nm on a UV Spectrophotometer. The results are as tabulated in Table 3.

**High Performance Liquid Chromatography**

It is a chromatographic technique that is used to separate a mixture of compounds. HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. The pump provides the higher pressure required to propel the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography. The actives of two of the compounds were checked by the HPLC method that included Bacoside of Bacopa capsule and Andrographolide of Andrographis Capsule.The Chromatogram of the reference standard and the chromatogram of Bacopa Capsule are as shown in Figure 1 and 2. The Chromatogram of the reference standard and the chromatogram of Ashwagandha Capsule are as shown in Figure 3 and 4.

## CONCLUSION

Nutritional deficiency is almost impossible to avoid so it is very necessary to consume natural herbal supplements in order to cope up. These natural herbal supplements are prepared in such a way that there are no additives or excipients that have been added and hence are considered to be more nutritive. All the five supplements have good amount of actives, have no presence of any kind of contamination neither traces of heavy metal in them.

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