

## PHARMACOGNOSTIC AND PHARMACOLOGICAL EVALUATION OF *Withania somnifera* HERBAL FORMULATIONS

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### ABSTRACT

Microscopic, physicochemical and pharmaceutical parameters of herbal formulations of *Withania somnifera* were evaluated. Both qualitative and quantitative phytochemical analyses were conducted on the extracts prepared from these formulations. The anti-stress and locomotor activities of these formulations were also investigated. Results from the study showed that the root powder, powdered preparation and capsules contain withanolides including withaferin A as active constituents. Microscopic examination of the capsule formulation, which was labeled as dry extracts of *W. somnifera*, revealed that it contains phloem fibers, starch and parenchyma cells indicating that the capsules were not prepared from only genuine extracts of *W. somnifera*.

**Key words:** *Withania somnifera*; herbal formulations; anti-stress activity; withanolides; HPLC analysis

### Introduction :

Exploration of medicinal properties of plants had created, through careful observation, trial and error, a vast heritage of knowledge and expertise in different cultures and civilizations<sup>1</sup>. About 300,000 species of higher plants exist in nature. In China, documentary evidence shows that herbal medicines have been used for at least 7,000 years<sup>2</sup>. Medicinal plants also offer therapeutic agents for age related disorders like memory loss, osteoporosis, immune disorders etc. for which no modern medicine is available<sup>3</sup>. It has been estimated that there are about 7,000 firms in the small scale manufacturing of traditional medicines. However, none of these firms standardizes herbal medicines using active compounds as markers linked with confirmation of bioactivity of herbal drugs in experimental models<sup>3</sup>. To induce the required therapeutic effect, medicines should contain the correct amount of active substances. The assurance is given, both to the doctors prescribing the medicament and the patients by the pharmaceutical company producing it that

different batches of the medicine are released for use into the market only after they have been tested for quality and quantity by the quality control laboratories of the company, a process known as standardization<sup>4</sup>.

*Withania somnifera* is an erect, evergreen, tomentose shrub, 15-30 cm high. The commercial drug consists of the dried roots of *W. somnifera*. The root is known to contain steroids and alkaloids. The total alkaloid content of the Indian roots has been reported to vary between 0.13 and 0.31 percent<sup>5</sup>. The leaves contain steroidal lactones, which are commonly known as "withanolides". The various activities reported for *W. somnifera* include antimicrobial, antitumor<sup>6</sup>, immunomodulating, anti-inflammatory, antiarthritic, hepatoprotective, antioxidant, antifeedant and antistress<sup>7</sup>. The herbal formulations of *W. somnifera* are sold in different dosage forms such as tablets and capsules, and there are at least 50 different brands available in India. In the present paper the evaluation of herbal formulations of *W. somnifera* is reported.

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## Materials and Methods

### Procurement of plant material and drugs

*Withania somnifera* roots were procured from the Chowdapudi shop No. 8 - 8 -7 St. Road, Warangal, A.P and recorded as B. *W. somnifera* roots were procured from Srisailam, (A.P) India and recorded as A.

Powder preparations having three brands and capsules manufactured by three different companies were procured from Devarakonda Chandramouli and Son's, Ayurvedic Shop, Chowrastha, Warangal, (A.P) India. They were labeled as powder B, D and Z (Baidyanath, Dabur, Zandu,), Capsule B, D and G (Gufic, India).

### Macroscopic examination

Physical observation of root A, root B, the powder preparations and capsules of *W. somnifera* formulations were recorded.

### Microscopic examination

Each of the samples of root A and B, powder preparations B, D and Z, and capsules B, D and G were ground into fine powder and taken onto three clean glass slides. The first slide is treated with a solution of chloral hydrate and gently heated on an open flame. To the second slide a drop of iodine solution was added for observation of starch grains. To the third slide phloroglucinol and concentrated hydrochloric acid each one drop were added for observation of fibers. Each of the slides were then mounted with addition of a drop of glycerin (the excess glycerin was removed carefully with a filter paper) and observed under microscope.

### Measurement of diameter of starch grains and width of phloem fibers

The measurements were done using eyepiece micrometer. Prior to measurement, the eyepiece micrometer was calibrated using stage micrometer. The slides were observed under the microscope, the number of units on the eyepiece micrometer was measured and the units were multiplied with calibration factor to get the diameter of starch grains and width of phloem fibers in microns. Twenty observations were made and the mean was calculated.

### Extractive values<sup>10</sup>

Accurately weighed 500 mg of root A and B, powder preparation B, D and Z and capsules B, D and G were taken in a separate test tube and 5 ml of water, alcohol or ether. The test tubes were shaken for six hours and allowed to stand for 18

hours. The resultant solutions were filtered and the residues obtained on the filter paper were dried at 105 °C and weighed to get water-, alcohol- and ether soluble extractive values respectively. The extractive values were calculated and expressed in terms of % w/w with reference to air dried drug.

### Solubility<sup>10</sup>

A small amount of *W. somnifera* roots A and B, powder preparations B, D and Z and capsules B, D and G were taken in separate test tubes and all the samples were tested for solubility in water, methanol, ethanol, acetone, chloroform and ether. The solvent was added and the tubes were kept on rotary shaker for 6 hours.

### Qualitative chemical examination<sup>12</sup>

Each of the root samples and formulations were extracted with ethanol (90% v/v). Dilute HCl was added to the extract and tested carefully with Dragendorff's reagent for the presence of alkaloids. To the chloroform extract of each of the formulations, magnesium oxide pellets and concentrated HCl were added to test for the presence of flavonoids. Similarly, Lieberman-Burchard reagent was used for the detection of steroids. Water or chloroform extract (1 ml) derived from each of the samples was diluted separately with distilled water to 20 ml. The resulting solution was shaken in a graduated cylinder for 15 minutes. and the foam layer was measured to confirm the presence of saponins. The presence of phenolic substances was tested by the addition of 5% ferric chloride solution. Addition of  $\alpha$ -naphthol solution and concentrated sulfuric acid to alcoholic extract at slanted position was carried out for testing the presence of carbohydrates.

### Loss on Drying<sup>10</sup>

It is the amount of volatile matter of any kind (including water) that can be driven off. One gram each of the samples was weighed accurately, spread and dried in hot air oven at 105 °C for 8 hours. The samples were weighed immediately after removing from the oven. Loss on drying is expressed as the loss in weight in percent w/w.

### Determination of pH<sup>10</sup>

The pH value represents the acidity or alkalinity of an aqueous solution. 50 g of each of the accurately weighed samples was taken into conical flasks and added 200 ml of distilled water to each. The pH of the water-soluble portion was measured with calibrated pH meter at 25 °C.

### Ash values<sup>10</sup>

Eight grams of each of the samples was weighed separately in a Gooch crucible and incinerated at a temperature not exceeding 450 °C until the formation of ash. The sample was then cooled and weighed. The charred mass was extracted with hot water and the residue collected on ashless filter paper. The residue and filter paper were further incinerated until the ash was white in color. The ash was boiled with water for 5 minutes to obtain water-soluble ash. The ash was boiled with 2N HCl for 5 minutes to obtain acid insoluble ash. The amount of sulfated ash was determined by moistening with sulfuric acid followed by heating gently and ignition at 800 °C until disappearance of black particles. The resultant powder was cooled and weighed. The total ash, acid insoluble ash and sulphated ash were calculated as the percentage with reference to the air dried drug.

### Thin layer chromatography (TLC)<sup>12</sup>

Each of the samples was extracted with methanol separately and a chromatogram was developed using benzene: diethylamine: methanol (94:5:1) as a solvent system using cholesterol as standard. Vanillin-sulfuric acid was used as a detecting agent. The  $R_f$  values were calculated for each spot.

### Extraction of total steroids and withaferin A<sup>15</sup>

An accurately weighed 5 g of powdered drug was homogenized and macerated for 24 hours in methanol (100 ml). The methanolic extract was filtered and marc was re-extracted with 10 ml of methanol. The alcoholic layer was pooled and dried. The residue was dissolved in methanol (20 ml) and diluted with 20 ml of water containing small quantity of sodium chloride for the prevention of emulsion formation. The mixture was defatted with hexane (10 ml) and the hexane layer discarded. The alcohol was removed from the hydroalcoholic under reduced pressure and the aqueous layer partitioned three times with dichloromethane (20 ml). The dichloromethane extracts were mixed and divided into two equal parts and evaporated to dryness. One part was used for estimation of total steroids content and the other was used for the estimation of withaferin A.

### Estimation of steroids<sup>12</sup>

The dichloromethane extract was dissolved in 20 ml dichloromethane of which 1 ml was taken in a test tube and diluted to 20 ml with

dichloromethane and evaporated. To the residue 10 ml of Lieberman-Burchard reagent was added and the reaction mixture kept aside for 2 hours. The absorbance of the solution was measured using colorimeter at 405 nm.

### Estimation of withaferin A<sup>16</sup>

The remaining dichloromethane layer was evaporated to dryness and the residue dissolved in 5 ml methanol (HPLC grade) and mixed thoroughly on cyclomixer. The sample (20 µl) was injected into HPLC injecting system. The HPLC column used was C18 reverse phase (Nucleosil) with particle size of 5 microns, and mobile phase was methanol-water (53:47) and the sample was detected using UV (Photodiode array detector) at 218 nm. The injection volume was 5 µl with a flow rate of 0.7 ml/min. The peak areas corresponding to withaferin A were noted. A standard graph was plotted using pure sample of withaferin A.

### Locomotor activity<sup>13</sup>

Healthy Albino male and female rats weighing between 70-100 g were used for this study. They were divided into three groups of 6 rats each. One group was used as a control and the other groups were employed as test animals. The animals were fasted for 24 hrs before the experiment. Accurately weighed (300 mg) of each of the samples was triturated with acacia (1 g) while adding normal saline solution and the final volume was adjusted to 6 ml with normal saline. The volumes corresponding to 10 mg and 20 mg per kg body weight were given to the group II and III rats, respectively. Mixture of acacia and normal saline was used as a control and the root of administration was i.p. The locomotor activity was studied half an hour after the administration of the test compounds for 10 min.

### Results and Discussion

Root A of *W. somnifera* has light yellow color while root B was yellowish white. Microscopic analysis of root A and root B powder showed the presence of abundant cork cell, oval, round, many simple and few compound dark blue color starch grains, non-lignified, numerous large, fusiform, striated walls and conical pex phloem fibres. Vessels, which are fairly large, usually occur in small groups. Reticulated thickened and pitted, lignified pink color, non-lignified with brown matter parenchyma cells were also observed in both root A and B. The diameter of starch grains and phloem fibers was found to be in the range of

15.20-35.26-60.80  $\mu$ , 5.20-21.88-30.40  $\mu$ , (root A) and 15.20-29.79-45-64, 15.2-21.88-45.60 $\mu$  (root B). All the characteristics observed in root A, B were in line with powdered drug derived from root and wood of *W. somnifera*.

The label on the capsules states that it contains dry extract of *W. somnifera*, which upon microscopic analysis show the presence of starch grains, phloem fibers, parenchyma and vessels. However, the extract is not supposed to contain any of these characters because extraction damages the cells. This clearly shows that there is a misclaim on the label of these herbal formulations.

The powdered drug and the different formulations had much more water-soluble extract than either ether or alcohol. The maximum water-soluble extract was found to be 38% for root B. However, there is no significant difference between the water-soluble extract of all the root powders and formulations except powder preparation B and capsule G, which contain less water-soluble extractives. There is no significant difference in the amount of ether and alcohol soluble extracts between the root powders and the different formulations. It complies with standard monograph of *W. somnifera* (more than 20% w/w)<sup>11</sup>.

With regard to the solubility profile of the formulations, it was found that capsule D is more soluble in chloroform (78%) than all the other formulations whereas Cap B has maximum solubility in acetone. Root A and B have similar solubility in methanol (about 22%). Furthermore, 18% of root A was found to be soluble in ether while root B has only 15% alcohol solubility. The general solubility profile indicates that all the formulations and the crude drugs of *W. somnifera* used in the present study contain more polar components than the non-polar ones.

Qualitative phytochemical analyses carried out on the crude drugs and the various formulations indicate that they contain steroidal glycosides. Powdered root A, powder preparations B, D, Z and Cap B showed moisture content of more than 44%, whereas the moisture contents of root B, capsule D and Z were found to be less than 44% indicating the possibility of other substances. All the preparations showed very high moisture content and do not comply with the standards specified for the herbal formulation which is less than 8%<sup>11</sup>.

All the samples showed pH below 7 indicating that the plant contains acidic components. Among all the formulations the maximum total ash value (20% w/w) was obtained for capsule B and the minimum for root B. Similarly, capsule B contained the maximum acid insoluble ash value (66.6%), whereas the lowest value (3.5%) was obtained for powder preparation Z. The maximum sulphated ash value was found in powder preparations B (80%), D (92%), and Z (91%). On the other hand, root A and root B gave values corresponding to 7.33% and 8%, respectively. All the powder preparations showed high sulfated ash values an indication for the presence of inorganic salts such as oxalates, carbonates, phosphates etc. This was not observed in the capsules.

Co-chromatography of the methanolic extract derived from root A with cholesterol using solvent system, benzene–diethylamine-methanol (94:5:1) showed two spots with  $R_f$  values of 0.2 and 0.42 when vanillin–sulphuric acid was used as a spraying reagent. The spot with the higher  $R_f$  value gave a violet colour and corresponded to cholesterol. Root B showed 3 spots with  $R_f$  values of 0.4, 0.45, and 0.62. Among these spots the one with  $R_f$  value of 0.4 corresponded to that of the standard substance (cholesterol). Similarly, all the formulations showed a spot on TLC plates corresponding to cholesterol. These results clearly demonstrated the presence of cholesterol-like molecule in roots A and B as well as in all the formulations.

The percentage of total withanolides in the methanolic extracts of the crude drugs and all the formulations as estimated by colourimetric assay is shown in Figure 1

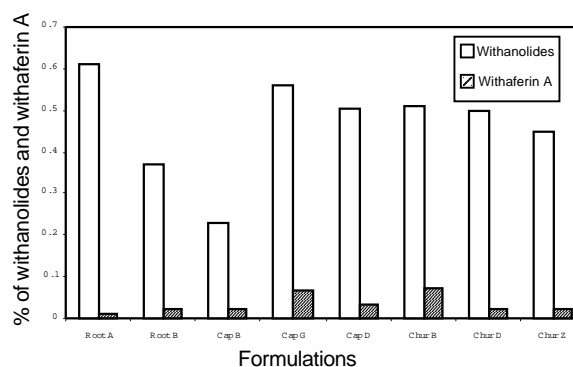


Fig. 1 : Total steroids and Withaferin A in *W. somnifera* and its formulations

The maximum amount of withanolides in root A was found to be 0.608% whereas root B contained 0.368% indicating root A has a better quality than root B. All the formulations had total withanolide content in the range of 0.44-0.56% except capsule B, (0.22%). These results clearly demonstrate that except capsule B all the formulations used a standard grade *W. somnifera* root, which complies standards set for the monograph of *W. somnifera*<sup>11</sup>.

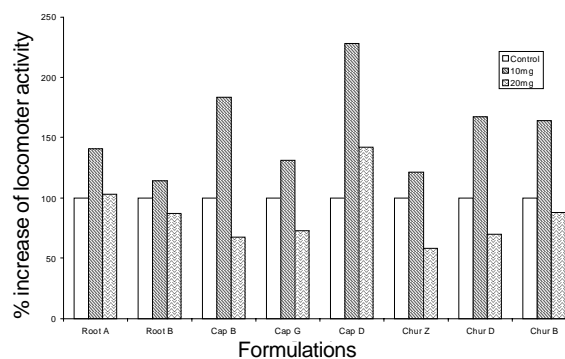
The amount of withaferin A present in Root B powder was found to be 0.022% on dry weight basis, whereas root A had 0.09% on dry weight basis. All the capsule formulations and powdered preparations showed the presence of withaferin A as detected by HPLC. The withaferin A content was shown to vary from 0.021-0.071% on dry weight basis. The content of all the formulations was similar to that of root B powder with the exception of powdered preparation D and capsule G. This could be due to the interaction of withaferin with the other substances present in these formulations. HPLC analysis of the capsules B, D and G indicated that they contain 350 µg, 355 µg and 230 µg of withaferin A, respectively. It has been reported that withaferin A acts as anti-stress agent in a very small dose. However, the dose verses the amount of withaferin A should be verified before fixing the dose of herbal formulations for the intended activity.

Investigation of the crude drugs and all the formulations on locomotor activity of rats showed that all the test substances in a dose of 10 mg increased locomotor activity (Table 1).

**Table 1.** Effect of different formulation of *Withania somnifera* and root A & B on Locomotor activity.

Formulation	Control	10 mg Test I	20 mg Test II	Control	% increase 10mg	20mg
Root A	60	85	62	100	141.6	103
Root B	69	79	60	100	114.4	86.9
Cap B	49	90	33	100	183.6	67.3
Cap G	96	126	70	100	131.2	72.9
Cap D	50	114	71	100	228	142
Chur Z	70	85	41	100	121.4	58.4
Chur D	60	72	42	100	167.4	70
Chur B	50	82	44	100	164	88.1

However, the activity exerted by capsule D was much higher than all the other products (Figure 2).



**Fig.2 :** Effect of different formulation of *W. somnifera* and root A, B on locomotor activity

At a higher dose (20 mg/kg), all the products exhibited sedative effect.

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