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Research Article

PHYTOCHEMICAL AND PHARMACOLOGICAL STUDY OF *ASHWAGANDHA* (*WITHANIA SOMNIFERA*) WILD AND CULTIVATED VARIETIES

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ABSTRACT

Ashawagandha is herb used for various kinds of disease especially as a nervine tonic. Considering these facts many scientific studies were carried out and its memory, anti-stress activities were studied in detail. **Aims and Objectives:** To study the Phytochemical analysis and Pharmacological Study of *Ashwagandha* (*Withania somnifera*) varieties and *Withania somnifera* (L.) Dunal wild purified with milk steam (WSWM) root powder. To study the efficacy of Wild and Cultivated varieties of *Ashwagandha* on rats through Elevated Plus Maze test and Morris Water Maze (MWM) model. **Materials and Methods:** The formulations *Withania somnifera* (L.) Dunal wild (WSW) root powder, *Withania somnifera* (L.) Dunal *Nagori* (WSN) root powder, *Withania somnifera* (L.) Dunal wild purified with milk steam (WSWM) root powder, PG (Wheat powder placebo) were subjected to preliminary phytochemical screening for the detection of various chemical constituents present. Animal experimentation was done on Wistar Albino Rats obtained from the animal house attached and are divided into three groups consisting of 6 rats per group. Nootropic agents are effectively screened using this paradigm in scopolamine-induced dementia. Elevated Plus Maze and Morris Water Maze (MWM) model are based on this phenomenon. **Results:** By performing phytochemical analysis, *Withania somnifera* (L.) Dunal *Nagori* (WSN) showed the presence of alkaloids, carbohydrates, glycosides, phytosterols, saponins, proteins and amino acids. *Withania somnifera* (L.) Dunal wild purified with milk steam (WSWM) showed the presence of alkaloids, carbohydrates, glycosides, proteins and amino acids and wheat powder placebo (PG) showed the presence of alkaloids, carbohydrates, glycosides, saponins, phenolic compounds, tannins, proteins amino acids and flavonoids. **Conclusion:** The formulation group 3 (WSWM) showed remarkable reduction in the transfer latency time (in elevated plus maze test) from the acquisition day to the retention day and therefore considered Group 3 is statistically significant. The formulation group 3 (WSWM) showed remarkable reduction in the latency scores in Morris water maze and hence Group 3 (Ashwagandha wild purified with milk steam (WSWM) root powder) is statistically significant.

KEYWORDS: *Ashawagandha*, *Withania somnifera* (L.) Dunal wild (WSW), *Withania somnifera* (L.) Dunal *Nagori* (WSN), Phytochemical analysis, Elevated Plus Maze test and Morris Water Maze (MWM) model.

INTRODUCTION

Withania somnifera (L) Dunal is a well known Indian medicinal plant widely used in the treatment of many clinical conditions in India. It is an important drug, which has been used either single or in combination with other drugs in Unani as well as Ayurvedic system of medicine for centuries. *Withania somnifera* has various therapeutic actions such as anti-inflammatory, sedative, alterative and aphrodisiac.[1]

Withania somnifera herb has been studied as adaptogenic, antioxidant, anticancer, anxiolytic, antidepressant, cardioprotective, thyroid modulating, immunomodulating, antibacterial, antifungal, anti-inflammatory, neuroprotective, cognitive enhancing and hematopoietic agent. *Ashwagandha* contains a range of constituents like withanolides, sitoindosides and other alkaloids that are pharmacologically and medicinally important. These chemicals protect cells from oxidative damage and disease.[2]

Withania somnifera (*Ashawagandha*) is very revered herb of the Indian Ayurvedic system of medicine as a *Rasayana* (tonic). It is used for various kinds of disease processes and especially as a nervine tonic. Considering these facts many scientific studies were carried out and its adaptogenic/anti-stress activities were studied in detail. In experimental

models it increases the stamina of rats during swimming endurance test and prevented adrenal gland changes of ascorbic acid and cortisol content produce by swimming stress.[3]

The formulations *Withania somnifera* (L.) Dunal wild (WSW) root powder, *Withania somnifera* (L.) Dunal *Nagori* (WSN) root powder, *Withania somnifera* (L.) Dunal wild purified with milk steam (WSWM) root powder, PG (Wheat powder placebo) were subjected to preliminary phytochemical screening for the detection of various chemical constituents present. The term qualitative analysis refers to the establishing and providing the identity of a substance. The pharmacological actions of crude drugs are determined by the nature of their constituents. The phyto-constituents are responsible for the desired therapeutic properties. To obtain these pharmacological effects, the plant materials itself or extract in a suitable solvent or isolated active constituent may be used.

It is essential to mention the materials and methods used for the study based on which the clinical work has been carried out. The materials used for the study is categorized into following three headings.

Collection of Material

According to *Bhavaprakasha nighantu*, *Nagori Ashwagandha*[4] is used for *Vajikarana, Balya, Brmhana* properties and root of wild variety is useful for *Vatashamaka guna, Bahya lepa* and *Apasmara* etc diseases. Wild variety has *Avasadaka, Swapnajanaka, Mutrajanaka* etc properties. So for internal use it must be purified with milk steam (*Swedana* in milk)[5]. The cultivated variety of *Ashwagandha* which is thin and lean is mainly brought from Nagori district of Madhya Pradesh, hence the name "Nagori variety". Along with these classical texts of Ayurveda, Sanskrit dictionaries, books related to western science, Articles published in reputed journals and also from the various media like Internet etc., followed by retrospective study of related research works.

DRUGS AND GROUPS

Collection of Drugs (Wild and Cultivated *Ashwagandha*)

The roots of Wild and Cultivated *Ashwagandha* are collected from Seshachalam forest and TTD's Sri Srinivasa Ayurvedic Pharmacy, Srinivasa Mungapuram, Tirupati respectively.

They are well cleaned and stored in a place where there is no much moisture or heat.

MATERIALS AND METHODS

Drugs: 1. *Ashwagandha* Nagori
2. *Ashwagandha* wild
3. *Gokshira*

Method of Purification and Preparation of wild variety of *Ashwagandha*

Apparatus

- Stainless Steel Vessel
- Heating Device
- Iron Mesh
- Steel plate

Ingredients

1. *Ashwagandha* wild variety: 500gms
2. *Gokshira*: 2 litres

Figure 1: Pieces of wild variety of *Ashwagandha*

Figure 2: *Gokshira*

Figure 3: *Swedana* of *Ashwagandha*

After that mesh is placed on the vessel. Over this mesh pieces of *Ashwagandha* are kept for purification.

Figure 4: *Swedana* of *Ashwagandha* root

Figure 5: Colour change of *Ashwagandha* root

Figure 6: *Swedana* of *Ashwagandha* by using a clean cloth

Swedana of *Ashwagandha* root with milk vapour, Then it is continuously boiled for about 1 hr on *Mandagni*. This vessel is closed by steel plate so that the vapors will not go out. This is done until hard pieces of *Ashwagandha* becomes soft.

Figure 7: Process of doing *Swedana* of *Ashwagandha*

Figure 8: Purified wild variety of *Ashwagandha*

Tests for Alkaloids

1. **Dragendroff's Test:** To 1ml of the extract, 1ml of Dragendroff's reagent was added, formation of orange red precipitate indicated the presence of alkaloids.
2. **Wagner's Test:** To 1ml of the extract, 2ml of Wagner's reagent was added, the formation of a reddish brown precipitate indicated the presence of alkaloids.
3. **Mayer's Test:** To 1ml of the extract, 3ml of Mayer's reagent was added, the formation of full white precipitate confirmed the presence of alkaloids.
4. **Hager's Test:** To 1ml of the extract, 3ml of Hager's reagent was added, the formation of yellow precipitate confirmed the presence of alkaloids.

Test for Carbohydrates

1. **Molisch Test:** To 2ml of the extract, 1ml of α-naphthol solution was added, and concentrated sulphuric acid through the sides of test tube. Purple or reddish violet colour at the junction of the two liquids revealed the presence of carbohydrates.
2. **Fehling's Test:** To 1ml of the extract, equal quantities of Fehling's solution A and B were added, upon heating formation of a brick red precipitate indicated the presence of carbohydrates.
3. **Benedict's test:** To 5ml of Benedict's reagent, 1ml of extract solution was added and boiled for 2 minutes and cooled. Formation of a red precipitate showed the presence of carbohydrates.

Tests for Proteins and Amino Acids

1. **Biuret Test:** To 1ml of the extract add 1ml of 40% sodium hydroxide solution was added followed by 2 drops of 1% copper sulphate solution. Formation of a violet colour showed the presence of proteins.
2. **Xanthoprotein Test:** To 1ml of the extract 1ml of concentrated nitric acid was added. A white precipitate is formed, it is boiled and cooled. 20% of sodium hydroxide or ammonia is subsequently added, orange colour indicated the presence of aromatic amino acids.
3. **Lead Acetate Test:** To the extract, 1ml of lead acetate solution is added. Formation of a white precipitate indicated the presence of proteins.
4. **Ninhydrin Test:** Two drops of freshly prepared 0.2% ninhydrin reagent were added to the extract solution and it was then heated. Development of blue colour revealed the presence of proteins, peptides or amino acids.

Tests for Phytosterol

1. **Libermann Burchard Test:** The extract was dissolved in 2ml of chloroform in a dry test tube. 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid were added. The solution became red, then blue and finally bluish green, indicated the presence of steroids.
2. **Salkowski Test:** Dissolve the extract in chloroform and equal volume of concentrate sulphuric acid was added. Formation of bluish red to cherry red colour in chloroform layer and green fluorescence in the acid layer represented the steroid components in the tested extract.

Tests of Glycosides

1. **Legal Test:** The extract was dissolved in pyridine and sodium nitro prusside solution was added to make it alkaline. The formation of pink red to red colour showed the presence of glycosides.
2. **Baljet Test:** To 1ml of the test extract 1ml sodium picrate solution was added and the yellow to orange colour revealed the presence of glycosides.
3. **Borntrager's Test:** A few ml of dilute HCl was added to 1ml of the extract solution. It was then boiled, filtered and the filtrate was extracted with chloroform. The chloroform layer was then treated with 1ml of ammonia. The formation of red colour showed the presence of anthraquinone glycosides.
4. **Keller Killiani Test:** The extract was dissolved in acetic acid containing traces of ferric chloride and it was then transferred to a test tube containing sulphuric acid. At the junction, formation of a reddish brown colour, which gradually became blue, confirmed the presence of glycosides.

Test for Saponins

1. About 1ml of methanol extract was diluted separately with distilled water to 20ml, and shaken in a graduated cylinder for 15 minutes. A 1% 1cm layer of foam indicated the presence of saponins.

Test for Flavonoids

1. **Shinoda Test:** To 1ml of the extract, magnesium turnings were added followed by 1-2 drops of concentrated hydrochloric acid. Formation of red colour showed the presence of flavanoids.

Test for Tannins and Phenolic compounds

1. To 1ml of the extract, ferric chloride was added, formation of a dark blue or greenish black colour product showed the presence of tannis.

2. To the extract, potassium dichromate solution was added, formation of a precipitate showed the presence of tannins and phenolic compounds.

Test for Triterpenoids

1. Two or three granules of tin metal were dissolved in 2ml thionyl chloride solution and 1ml of the extract was then added into the test tube. The formation of a pink colour indicated the presence of triterpenoids.

Test for Fixed Oils

1. **Spot Test:** A small quantity of extract was pressed between two filter papers. Oil stains on paper indicated the presence of fixed oils.
2. **Saponification Test:** To 1ml of the extract few drops of 0.5 N alcoholic potassium hydroxide was added along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. The formation of soap or partial neutralization indicated the presence of fixed oils.

Table 1: Showing the Phytochemical analysis results of *Aswagandha*[6]

S.No.	Test	WSW	WSN	WSWM	PG
I	Alkaloids	+	+	+	+
II	Carbohydrates and glycosides	+	+	+	+
III	Phytosterols	-	+	-	-
IV	Fixed oil and fats	-	-	-	-
V	Saponins	-	+	-	+
VI	Phenolic compounds and tannins	+	-	-	+
VII	Protein and Amino Acid	+	+	+	+
VIII	Gum and Mucilage	-	-	-	-
IX	Test for flavonoids	-	-	-	+

+ Positive; - Negative

Results and observations

The above table showed that alkaloids, carbohydrates and glycoside, Phenolic compounds and tannins, Protein and Amino Acid, are present in *Withania somnifera* (L.) Dunal wild (WSW)

Withania somnifera (L.) Dunal *Nagori* (WSN) showed the presence of alkaloids, carbohydrates, glycosides, phytosterols, saponins, proteins and amino acids

Withania somnifera (L.) Dunal wild purified with milk steam (WSWM) showed the presence of alkaloids, carbohydrates, glycosides, proteins and amino acids

Wheat powder placebo (PG) showed the presence of alkaloids, carbohydrates, glycosides, saponins, phenolic compounds, tannins, proteins, amino acids and flavonoids.

PHARMACOLOGICAL STUDY

Introduction to Animal Experimentation:

Evidence based health sciences are order of the day today. Any small evidence shown logically creates confidence and promotes practice. Ayurveda means to search ornamentation of evidences to become popular. Animal experimentation provides evidences provided properly designed. The study here is designed in accordance with Ayurvedic methods of application. No synthetic drug is used to create a pathological condition in the animal. The study has provided interesting results.

MATERIALS AND METHODS

Drug Material

The test drugs are used for experimental purpose and administered to the experimental animals according to the dose calculated.

- *Ashwagandha* wild (WSW) root powder
- *Ashwagandha* Nagori (WSN) root powder
- *Ashwagandha* wild purified with milk steam (WSWM) root powder

Animals

Wistar albino Rats obtained from the animal house attached to Sree Vidyanikethan College of Pharmacy, Tirupati were divided into four groups consisting of 6 animals per group. The animals were maintained with rat pellets feed and tap water is given. The animals were maintained under normal ambient conditions.

Figure 9: Showing Experimental Animals

Figure 10: Showing cages of Experimental animals

Figure 11: Showing Drug administration in animals

Instruments Used:

1. Rat Feeding Needles (No.18 & 20)
2. Syringe (Tuberculin)
3. Flask
4. Pipettes

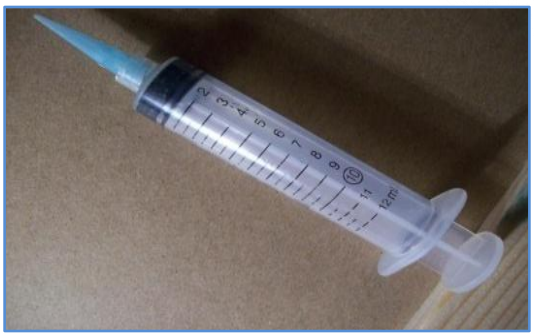

Figure 12 : Showing syringe

Figure 13: Showing Rat Oral feeding needles

Methods

Mazes are traditional tools for assessing learning and memory performance in laboratory animals. Conventionally, maze consists of open and enclosed arms. The rodents have a natural inclination towards enclosed area and spend more time there in comparison to open area. On the basis of this, the transfer latency of the animal is recorded. The animal learns to avoid open arms and shortens transfer latency to enclosed area. Nootropic agents are effectively screened using this paradigm in scopolamine-induced dementia. Elevated plus maze, and Mirror Water Maze model are based on this phenomenon.

The plus maze consists of two opposite open arms (50cm*10cm), crossed with two closed arms of the same dimensions with 40cm high walls. The arms were connected with a central square (10cm*10cm). Rats are placed individually at one end of an open arm, facing away from the central square. Time taken for the rat to move from the open arm and enter into one of the closed arms is recorded and termed as “Initial Transfer Latency”

(ITL). Animals are allowed to explore the maze for 30s after recording transfer latency. Retention Transfer Latency (RTL) is recorded by placing the rats similarly on the open arm at specified intervals.[7]

Groups

- Group 1 *Ashwagandha* wild (WSW) root powder
- Group 2 *Ashwagandha* Nagori (WSN) root powder
- Group 3 *Ashwagandha* wild purified with milk steam (WSWM) root powder

Elevated Plus Maze Test

The elevated plus maze is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents and steroid hormones, and to define brain regions and mechanisms underlying anxiety-related behavior. Briefly, rats or mice are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded by a video-tracking system and observer simultaneously for 5 min. Other ethological parameters (i.e., rears, head dips and stretched-attend postures) can also be observed. An increase in open arm activity (duration and/or entries) reflects anti-anxiety behavior. In our laboratory, rats or mice are exposed to the plus maze on one occasion; thus, results can be obtained in 5 min per rodent.[8]

Figure 14: Elevated Plus Maze Model

Elevated plus maze was described as a tool for testing memory by the investigator working in the field of psycho pharmacology. Elevated plus maze served as exteroceptive behavioral model to evaluate learning and memory in rats. The elevated plus maze consists of two open arms and two closed arms (50cmX10cmX40cm) with an open roof arranged so that the two arms are opposite to each other. The maze was elevated to a height of 50cm. First group served as a vehicle control. On the 14th day, each rat was placed at the end of the open arm, facing away from the central platform.

Transfer latency was time taken by the rats to move into covered arm with all its four paws, transfer latency was recorded. If the animals did not enter into one of the covered arms within 90s, it was gently pushed into one of the two covered arms and transfer latency was assigned as 90s. The rat was allowed to explore the maze for 10s and returned to the home cage. Twenty four hours later i.e. on 15th day transfer latency was recorded again. The measurement of transfer latency on the day 14 served as a parameter for acquisition and those on day 15 served as a parameter for retention of memory.

Morris Water Maze (MWM) Model

The Morris water maze (MWM) is a test of spatial learning for rodents that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform. Spatial learning is assessed across repeated trials and reference memory is determined by preference for the platform area when the platform is absent. Reversal and shift trials enhance the detection of spatial impairments. Trial-dependent, latent and discrimination learning can be assessed using modifications of the basic protocol. Search-to-platform area determines the degree of reliance on spatial versus non-spatial strategies. Cued trials determine whether performance factors that are unrelated to place learning are present. Escape from water is relatively immune from activity or body mass differences, making it ideal for many experimental models. The MWM has proven to be a robust and reliable test that is strongly correlated with hippocampal synaptic plasticity and NMDA receptor function.[9]

Figure 15: Showing Morris Water Maze Model

It is the most popular task in behavioral neuroscience. In its most basic form, it assesses spatial learning and stress, anxiety along with non spatial discrimination learning. The rodents are placed in a large circular pool of water from where they can escape onto a platform to avoid swimming. In this way, the animal learns the spatial location of

the platform. The latency to escape from water onto the platform is measured.

The neuro-endocrine mechanisms involved in this case may be responsible for the rapid learning capability of animals. It is useful for studying, working and reference memory processes also along with assessing stress related phenomena. Motor, motivational or sensory factors can be easily eliminated. The animals are trained faster, easily and with reliability using water maze experiment.

Procedure

Spatial memory of the rats was tested using Morris water maze test.

The water maze apparatus consists of a water tank of 1.83 meters in diameter. There was a 4"x4" size platform submerged in water tank one of the fixed quadrant. The rats were trained in the water maze in 4 sessions on 7 days. During each training session the rat was placed in water so that it faced the wall of the pool. In training session, the latency (time) to escape onto the platform was recorded. Once a mouse locates the platform, it is allowed to remain there for 30sec before being removed from the tank.

In this test, the rats that were treated with the drug formulations for 7 days learned the platform location faster than the controls.

Route of Administration

Administration of compound plays a large part in experimental design using animals. Before administrating any substance (therapeutic or experimental) to an animal subject, make appropriate decision on the dose to be administered, frequency of administered volume to be administered, the solvent (if necessary) and route of administration.

Drugs are to be administered orally in Ayurveda because the drugs here follow the food pathway in the body. The oral administration of solution of drugs or test substances to experimental rats is often necessary in various pharmacological, toxicological and other biomedical researches. Oral ingestion is the most common method of drug administration to humans. It is also the safest, most convenient and most economical route in the animals.

It is scientifically sound and preferable to administer test substances to experimental animals by the same route(s) by which it is taken or meant to be taken by humans. As systemic bioavailability, pharmacokinetics and toxicological parameters obtained for the substance will depend markedly on the route used to administer it.

So in the present animal experimentation oral route is elected for drug administration. The test drugs were administered in suitable doses by oral route with the help of rat feeding needle daily for fourteen consecutive days.

Administration of Test Solution (SYT)

1) The rat feeding needle is attached into a 3ml syringe containing the solution of test drug (SYT) and held with the right (dominant) hand.
2) It is introduced into the rat's oral cavity to the left side of the animal's incisor teeth in the midline and maintained in this position throughout the procedure to avoid damage to it from the animal's bite.
3) Carefully advanced down the oral cavity between the tongue and roof of the mouth, occasionally it passes easily straight on into the esophagus at other times a resistance is encountered. This resistance can be relieved by the maintenance of a gentle inward push on the needle while rotating the tip from side to side.
4) This soon stimulates a swallow reflex, which transmits the needle into the esophagus. Occasionally the animal may use any of its strong hands to attempt to push the needle out of the mouth at this stage or during the ingestion.
5) Working alone in this situation, instead of focusing on restraining the tail as explained above it greatly helps to use any of the other free fingers of left hand to hold down the dominant forelimb of the animal preventing any grip on the needle already situated within the esophagus. Once needle in the esophagus the plunger is pushed down the syringe, emptying its content into the esophagus en-route to the stomach.

Dose of the formulations

After taking the experts opinion in the department of Dravyaguna, S.V. Ayurvedic College, the dose is decided as follows (basing on the standard dose of the drug).

- for a 60kg person=60,000mg) → 1gm is the dose
- i.e. for 150gm lab animal →**2.5mg** is the dose

(Animal Dose = $\frac{AW}{60} \times \frac{HD}{1000}$) (AW=Animal weight; HD=Human dose)

Table 2: Showing the Effect of formulations on Transfer Latency of rats using Elevated Plus Maze Paradigm

Treatment Groups	Transfer Latency	
	TL on 1st/7th day	TL after 24 h
Control	15.36±0.53	16.16±0.53
Formulation 1(WSW) (Dose:2.5mg)	8.66±0.79	7.23±0.09
Formulation 2(WSN) (Dose:2.5mg)	7.96±0.34	6.55±0.66
Formulation 3(WSWM) (Dose:2.5mg)	6.55±0.78	6.36±0.47

Results and Observations

From the above table it is clearly evident that the formulation group 3 (WSWM) showed remarkable reduction in the transfer latency time (in elevated plus maze test) from the acquisition day to the retention day. This is visible clearly from the above table which shows that the Group 3 is statistically significant.

Table 3: Effects of Ayurvedic formulations on mean latencies (latency scores) across trail by Morris Water Maze

Treatment	1st day	3rd day	5th day	7th day
Control	48.56 ±1.23	37.66±0.06	34.56±1.05	29.63±0.69
Formulation 1(WSW) (Dose:2.5mg)	32.56±1.26	28.56±0.89	21.09±0.09	18.59±0.06
Formulation 2(WSN) (Dose:2.5mg)	24.55±1.47	19.65±0.89	15.69±0.01	11.26±0.09
Formulation 3(WSWM) (Dose:2.5mg)	21.56±0.56	17.54±1.36	12.56±0.98	9.56±0.78

Values are mean ± SEM of 6 animals per group. Values not sharing a common superscript significantly differ at $P < 0.05$. Statistical significance test was done by ANOVA followed by Dunnet's t test (n=6), Values are mean ± SEM of 6 animals per group.*$P < 0.001$.

Results of Pharmacological study: Groups I (WSW), II (WSN), III (WSWM) showed significant effect when compared to the control group in Elevated plus maze test. Among them Group III (WSWM) showed remarkable effect on transfer latency time when compared to the Groups I (WSW) and II (WSN). Groups I (WSW), II (WSN), III (WSWM) showed significant effect when compared to the control group in Morris Water Maze model.

CONCLUSION

There was a remarkable reduction in the latency scores in Morris Water Maze test in which the formulation WSWM was used. Therefore we can conclude that the Group 3 (*Ashwagandha* wild purified with milk steam (WSWM) root powder) is statistically significant.

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