ORIGINAL ARTICLE

Comparative Evaluation of Physical Endurance Enhancing Ability of *Withania somnifera* Stem Powder Versus Spent Root Powder

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ABSTRACT

Introduction: *Withania somnifera* (WS)/Ashwagandha is well-known as an anti-stressor. Although the active constituents are present in the entire plant, only roots are used. Thus, our aim was to evaluate the physical endurance enhancing capacity of the stem extract versus root extract post extraction. **Methods:** A total of 18 healthy, adult, male Swiss albino mice of age 6 – 8 weeks were randomized into three groups of 6 each: (1) WS stem extract 100mg/kg (2) WS spent root extract 100mg/kg and (3) 0.5% carboxymethyl cellulose. The animals were dosed orally once daily for 7 days and were subjected to the swim endurance test. **Results:** There was no significant difference in weight between the three groups (p=0.196). Difference in mean swim time across the 3 groups was significant (p=0.006). The mean difference (MD) in swim time between the spent root group and the control group was statistically significant (MD (95% CI) = 113. 64 (48.22, 179.06); p = 0.002). However, the other two comparisons (stem vs spent root and stem vs control) were not significant. **Conclusion:** The stem extract of WS showed physical endurance enhancing actions not significantly different from that of spent root. However, when compared with the control, spent root extract significantly improved physical endurance. The stem extract although improved physical endurance, did not achieve statistical significance. Thus, the stem of the plant may also be explored for use in therapeutics which otherwise is merely discarded after harvesting the roots.

KEY WORDS: Withania somnifera, Ashwagandha, Stem, Physical endurance, Forced swim test.

Introduction

Withania somnifera (WS) also referred to as Ashwagandha or Winter Cherry is a green shrub that belongs to the Solanaceae family and is known to be a potent 'adaptogen' and anti-stressor.[1] It increases the body's ability to tackle resistance against harmful factors/stressors which may be physical, chemical, biological and/or psychological in nature, thereby achieving homeostasis within individuals.[2] Different parts of this plant have been used for many years in the traditional medical system to treat various ailments.[3] Extracts from the root, stem, leaf, fruit, etc. of WS contain steroidal bioactive compounds and lactones such as Withaferin A, Sitoindosides VII-X and likewise which have anti-inflammatory, anti-oxidative, anticancer, antistress and immunomodulatory properties.[4] To confirm its medicinal properties, WS has been extensively studied. For instance, in two double-blind, randomized controlled trials (RCTs) in humans, WS root extract helped reduce symptoms in adult patients who presented with generalized anxiety disorder in comparison to placebo.[5] Few other RCTs conducted in adults with chronic stress with either normal bodyweight[6] or those who were overweight or obese[7], have also confirmed that WS root extract has anti-stress and cortisol-lowering effects. All these studies further concluded that WS was well tolerated with only minimal safety concerns.

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WS has also been proven to enhance physical endurance in multiple animal experimental studies. Bhattacharya et al. concluded that the alcohol-based root extract of WS demonstrated an increased swimming endurance and significantly reduced cold restraint stress induced changes in mice.[8] This is probably because, the root extract reduced lipid peroxidation, thereby relieving oxidative stress in the mice and thus improving the physical endurance.[8] Since, the root extract has been extensively studied, they are routinely used for extraction of active ingredients in commercial products. However, the other parts of the plant are wasted resulting in overuse of the plants only for its roots and endangering WS species. Given this background, medicinal products manufactured using WS leaves and stems have long been thought to offer useful alternatives to the root products. A study conducted by Maitha et al. reported the presence of active substances like withaferin in stem and leaves of Withania though in concentrations lesser than the roots.[9] Thus, with an aim to optimize the use of plant resources, we had conducted a similar study in the recent past comparing commercial root powder with the spent root post extraction wherein we found that the latter still produced a notable biological activity.[10] Subsequently, this study was proposed with an objective to evaluate the efficacy of the stem extract in comparison to a spent root extract that is also likely to have lower concentrations of the active ingredients after the initial extraction, using the swim endurance test model by estimating the duration of swim by the test animals.

Materials and Methods

Ethics

The Institutional Animal Ethics Committee granted ethics approval to conduct this study [IAEC/ NR – PCL/04/11.16]. Animals were cared for and handled as per the guidelines from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) which were followed throughout experimental procedure.

Animals

Healthy, adult, male Swiss albino mice of age 6 – 8 weeks were utilized in this study. Animals were randomly assigned to three groups, each group consisting of six animals. A sample size of six was chosen based on the resource equation approach (minimum n per group = 10/k+1 and maximum n per group = 20/k+1, k being the number of groups) and accordingly for 3 groups, rounding off to the closest higher integer the minimum n = 5 and maximum n = 7 per group.[11] All animals were maintained in a room at 25°C ± 2°C, and relative humidity maintained between 30 and 70%. A light-dark cycle of 12 hours each was set using an artificial light source. UV-treated water and conventional laboratory rodent diet were offered *ad libitum*. Animals were allowed to adapt to this environment for at least one week before the experiments.

Design of the Experiment

A total of 18 animals were randomized into three groups (control, standard, and test groups) of six animals each as follows:

Group 1: Control - Carboxy Methyl Cellulose (CMC-0.5%) at 10 ml/kg was administered.

Group 2: Standard - Spent root powder of WS administered at the dose of 100mg/ kg, prepared in 0.5% of CMC as vehicle.

Group 3: Test - Stem powder extract of WS administered at the dose of 100 mg/ kg, prepared in 0.5% of CMC.

Animals were randomized based on their body weights. At the time of study commencement, the weight variation of the animals was less than 20% of the mean body weight.

Preparation of Test substance (Stem Powder)

WS stems were cleaned, sorted, ground and passed through size #40 mesh. Material above the mesh was reground till such time that <5% of stem material remained in the mesh. The material that passed through the mesh were steam sterilized after being filled in high density polyethylene bags. This material was then dried until the moisture content returned back to ±1% of the moisture content of the pre-sterilized material and it was delumped, milled, sieved and magnetically separated from contaminants. The test substance was then blended in a 500 kg/1 MT double cone blender before it was finally packed in single ply laminated polycovers (polyester 12 micron and polyethylene 100 micron) and screened using metal detectors.

Preparation of Standard (Spent Root)

WS roots were cleaned, sorted and ground into coarse particles. After having the particle pass through size #10 mesh, aqueous extraction was carried out using a large-scale extractor (Miscella SS316) by

circulating hot water at 75°C – 85°C over the ground root particles for 3h. This extraction process was repeated three times and the resulted root material was the spent root post extraction. The spent root was then finely ground, steam sterilized, and spray dried. The resultant material was then delumped, milled, sieved, and had its contaminants separated magnetically. It was then blended, packed and passed through metal detectors in a similar manner to that of the stem material.

Drug Authentication

The test substances were obtained from Natural Remedies Private Limited, Bengaluru. WS plants used in this experiment were sourced from the firm's subcontracted farmers in Kurnool, Andhra Pradesh (Kurnool) who cultivate high quality of WS under the firm's supervision. The raw material used for engineering the spent root and stem powder identified using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). WS TLC profile was then compared with that of the botanical reference standard of the WS root and HPLC was performed on the test and standard to check the presence of withanolides and confirm the identity of WS.

Details of the chromatography performed

High Performance Thin Layer Chromatography was utilized. The conditions maintained for the procedure were as follows: Chromatographic silica gel with an average particle size of 5 mm was used as absorbent. A relative humidity of 33% and ambient temperature not to exceed 30^0C was maintained. The developing distance was 6 cm and the developing solvent system was toluene, ethyl acetate and glacial acetic acid (55:45:3) and derivatization agent was 20 mL of sulfuric acid combined with 180mL of ice-cold methanol. The complete details of the analysis are given in the supplementary file.

Preparation of solution of test substances

The CMC solution was first homogenized through a magnetic stirrer and 100 mg of the test solution was added to 10ml of a 0.5% CMC. This was again homogenized in a similar manner for a minimum period of 10 minutes thereby giving a solution of concentration 10mg/ml. The same procedure was followed to prepare spent root and stem powder solutions.

Dose calculation

Previous studies used 100 mg/kg/day of WS commercial preparation of root powder (derived from recommended human dosage on body weight basis).[12] So, for an animal weighing 30g, 3.0mg/day dose will be required. Given that the solutions that were prepared were of concentrations 10mg/ml each, we required 0.30ml of the test and standard solutions respectively, for a 30g animal.

Dosing

On the first day, the animals were allowed to acclimatize to the laboratory environment. From the 2^{nd} day onward until the 8^{th} day, test, control and standard preparations were administered once daily to the mice via oral gavage. On the 8th day, the swim endurance test was conducted using the animals.

Swim endurance test

The forced swimming test model in mice was developed by Porsolt et al. in 1977 and is a widely accepted model for studying stress and depression in animals.[13] This method is based on the observation that stress can be induced in an animal when subjected to swim continuously with no apparent escape. After an initial period of vigorous activity, the animal eventually develops a state of despair and ceases to move altogether except for those movements that are mandatory to keep its head above the water. Precaution to be taken such that the animal's tail does not touch the base of the tank, as this would in balancing and sustaining its head above the water surface. The end point of the experiment is considered when the snout of the test mouse goes below the level of the water at least three times which signifies near drowning. The total time spent by the mice swimming can be calculated and is referred to as forced swim time. This can be used as a parameter to study physical endurance and the modification of the forced swim test is considered as the swim endurance test.[14]

In the current study, a propene tank of dimension 40 cm* 25 cm* 15 cm was filled with water at ambient room temperature (30^0C) to a height of 25 cm and the animals were subjected to swim until exhaustion. The swimming time was recorded in minutes for each animal until it exhibited loss of coordinated movements and failed to return to the water surface within 10s for three consecutive times.

Statistical analysis

The weight of the mice and swim time in each group were summarized using descriptive statistics. The difference in mean swim time between the three groups was analyzed using the Kruskal Wallis test. Post Hoc analysis was done using Mann Whitney U test and P value was adjusted for multiplicity testing using Bonferroni's correction. The level of significance was set at $P < 0.05$ except when Bonferroni's correction was used wherein, the revised level of significance was $P < 0.017$.

Results

A total of 18 animals were used and their mean (SD) weight was 32.61 (3.85) g. The group-wise descriptive statistics for weight (g) and swim time (s) is summarized in Table 1. There was no significant difference between the three groups (Control vs spent root vs stem) in terms of weight [H(df=2) = 3.263, p = 0.196] with mean rank 7.08, 12.50 and 8.92 respectively. Difference in mean swim time across the 3 groups (Control vs spent root vs stem) was significant [H(df=2) = 10.257, p = 0.006] with mean rank 4.33, 14.17, and 10.00 respectively. There was no significant difference between the test (stem) group and the control group [Mean difference [MD] (95% Confidence Intervals [CI] = 68.90 (3.48, 134.32) p = 0.041] as well as the test (stem) group and the standard (spent root) group [MD (95% CI) = -44.74 (-110.16, 20.68); p = 0.132]. However, the mean difference in swim time between the standard (spent root) group and the control group was statistically significant (MD (95% CI) = 113. 64 (48.22, 179.06); p = 0.002).

Table 1: Weight and swim time of mice in 3 groups

Groups	Weight (g)		Swim time (minutes)	
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)
Control	34.67 (1.63)	34.50 (33.00, 36.25)	151.12 (17.32)	155.68 (138.97, 164.80)
Spent Root (Standard)	35.67 (5.32)	38.00 (33.25, 38.25)	264.76 (57.08)	289.41 (195.10, 308.96)
Stem Powder	35.17 (2.64)	35.50 (33.25, 37.25)	220.03 (46.38)	238.39 (173.47, 257.77)

The chromatograms of unprocessed raw root material, pure Withanolides extract, the post-extraction (spent) root material and the stem powder are depicted in Figures 1, 2, 3 and 4 respectively. The pattern obtained in all the four chromatograms were comparable. The retention times and the area under the curve of the pure Withanolides extract, spent root material and the stem material are tabulated in Table 2.

Table 2: Results of the post-hoc analysis for swim time

Groups	Mean difference (minutes)	95% Confidence Interval	p value*
Stem vs Control	68.90	3.48, 134.32	0.041
Stem vs Spent Root	-44.74	-110.16, 20.68	0.132
Spent Root vs Control	113.64	48.22, 179.06	0.002
Standard Error = 25.19			

* New level of significance adjusted for multiplicity testing after Bonferroni's correction = 0.017

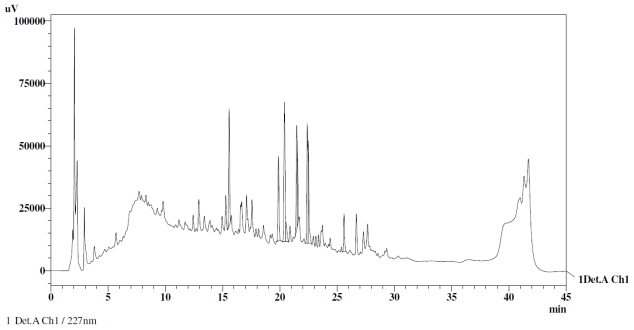

Figure 1: Chromatogram of unprocessed raw root material

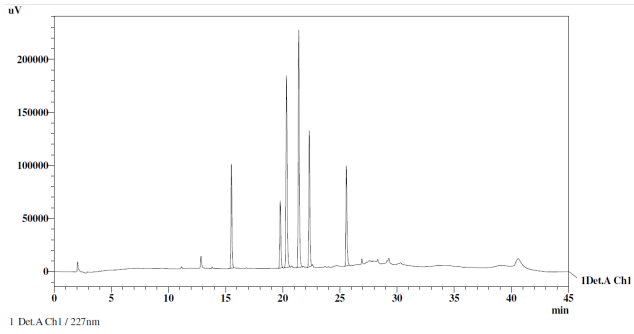

Figure 2: Chromatogram of Pure Withanolides extract

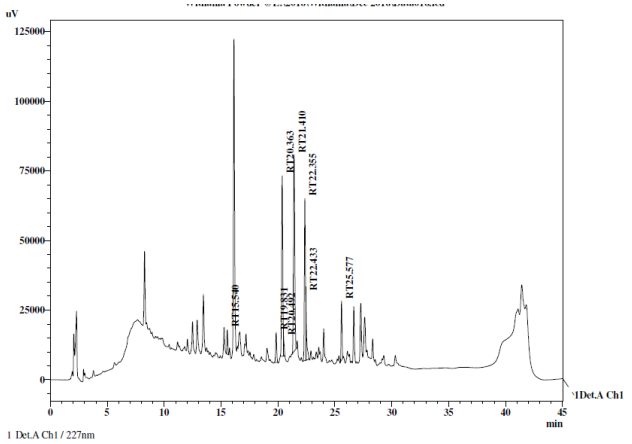

Figure 3: Chromatogram of Post-extraction (spent) root material – Standard

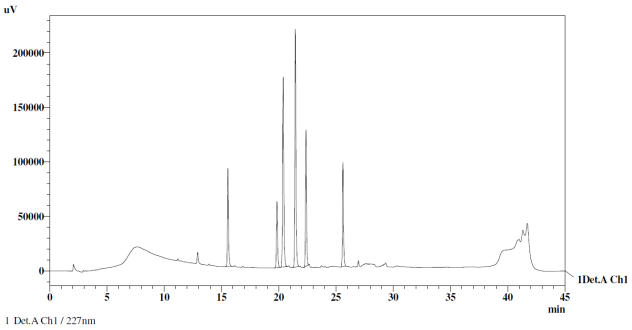

Figure 4: Chromatogram of Stem material - Test

Discussion

The current study evaluated the physical endurance enhancing effect of the stem extract of WS, in comparison to the spent root material of WS and the control using the forced swim test in mice. The physical endurance enhancing capacity of the spent root material was significantly better when compared to that of the control as demonstrated by a longer forced swimming time in the test group [MD (95% CI; p value) = 113.64 (48.22, 179.06; p = 0.002)]. This finding corroborated with the results of our previous study wherein the forced swim time between the spent root material and the control was significantly different [MD (95% CI; p value) = 113.67 (55.23, 172.10; p < 0.001)].[10]

However, the stem powder of WS, although showed a trend towards physical endurance enhancing effect as compared to the control, it did not achieve statistical significance probably due to a small sample size. At the same time, the stem powder did not reach a statistically significant difference in its effect when compared to the spent root powder. This suggests that the stem powder indeed has some biological activity although not as high as the spent root material, sufficient enough to reach statistical significance when compared with control irrespective of the small sample size. These results are in line with our HPLC results that showed comparable concentrations of active ingredients in these extracts. This further confirms the findings reported by Singh et al. which states that although the concentration of active ingredients in different parts of the WS varies, the concentration of withanolides and withaferin A in stem, roots and leaves of 5 different species of Withania are largely comparable.[15]

This ability of WS to increase physical endurance can be explained by its ability to significantly increase the maximum oxygen uptake (VO_2 max) as reported in a systematic review and metanalysis by Pérez-Gómez et al.[16] As endurance performance is dependent on mitochondrial function, the improvement in cardiopulmonary fitness with WS can be attributed to the inhibition of succinate dehydrogenase enzyme activity in the mitochondria and benefiting Mg-ATPase activity thereby increasing the energy levels.[17] It is hypothesized that some of the active ingredients found in WS such as the alkaloids, flavonoids, and the steroidal lactones like withanolides or the free radical scavenging antioxidants such as the catalase, superoxide dismutase, and glutathione peroxidase could be responsible for the improvements of VO_2 max.[16] Further, studies have also shown that WS increases the Hemoglobin concentration and the number of red blood cells in both animals and humans which translates into better oxygen carrying capacity to the muscles.[18]These changes could enhance the physical endurance of humans and it has been studied as a nutritional supplement for this indication in athletes and sports persons. In fact, a systematic review of 13 studies concluded that WS supplementation (as against placebo), significantly improved variables related to physical performance such as increase in muscle mass, muscle strength, VO2 max, and hemoglobin, and a decrease in reaction time in healthy men and women.[19]

The current study has a few limitations. The physical endurance enhancing capacity of stem extract was compared only with the spent root extract. Further studies can be planned to compare the efficacy of the stem extract with the unspent root extract and commercially available preparations to further

elicit the potential of stem to substitute roots in commercial preparations. As the stem extract has comparable physical endurance enhancing ability, it could also be studied in other indications. Yet another limitation was the small sample size of just six animals in each group with no biochemical markers being studied as objective measures of stress to corroborate our findings. Thus, larger studies incorporating biochemical markers as one of the outcome measures may be planned in the future to confirm the findings of this study. Lastly, the swim endurance test has its own limitations such as differences between rodent strains, physical stress related affect changes causing difference in swimming behavior, effect due to reversed dark/light cycle, and a possible release of the pheromone by a previous animal tested in the same tank can make the rodents less mobile. Therefore, the biological effect seen in the rodents may not truly translate in its entirety to human therapeutics and human exploratory studies are needed to confirm its use for enhancing physical endurance.[20]

The main strength is that this study, to the best of our knowledge, is the first experiment to use a stem extract to study its biological activity and compare it with spent root extract of WS. This study therefore has opened a new avenue for research in exploring the utilization of the stem as well as the reutilization of spent root commercially thereby avoiding wasting useful resources. If proven of some clinical benefit, this will optimize the use of WS plants which would otherwise become endangered if overused and is likely to reduce the economic burden on the entire process from WS cultivation to extraction.

Conclusions

The stem extract of WS showed physical endurance enhancing actions not significantly different from that of spent root extract. However, when compared with the control, spent root extract significantly improved physical endurance but the stem extract although improved physical endurance, it did not achieve statistical significance probably due to a smaller sample size. The concentration of active ingredients of WS was comparable in the stem, spent root and unprocessed root extracts. We recommend more studies with large sample size to corroborate the findings of the current study and to explore the use of WS stem extract in commercial preparations.

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Nil.

Conflicts of interest

The authors declare no conflicts of interest.

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