

COMPARATIVE EFFECT OF *WITHANIA SOMNIFERA* AND *PANAX GINSENG* ON SWIM-STRESS INDUCED IMPAIRED ENERGY STATUS OF MICE

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Summary

Withania somnifera and *Panax ginseng* are well-known for their energy augmenting effect. In this study, *Withania somnifera* and *Panax ginseng* standardized extracts were evaluated for their energy augmenting activity in an experimental model using the forced swimming test (FST) in Swiss albino mice. *Withania somnifera* standardized extract (WSE) dose dependently attenuated ATP-depletion and other energy related indices during both short term (7 days) and long term (30 days) treatment periods. The treatment doses were 50 mg/kg and 100 mg/kg. *Panax ginseng* (PGE), on the other hand, did not elicit similar energy-restoring effect when compared with that of WSE at the lower dose level and on prolonged administration (50 mg/kg for 30days). This latter finding would seem to project WSE as a better adaptogen. Also, WSE may be considered as a better agent for stress management in view of the well documented adverse effects of Ginseng (known as the Ginseng abuse syndrome). To achieve this objective, the need for standardization of *Withania somnifera* extract is emphasized.

Keywords: *Withania somnifera*, *Panax ginseng*, ATP, TAN, AEC, energy augmenting effect, adaptogen.

Introduction

Withania somnifera Dunal (Family: Solanaceae), known as *Ashwagandha* in Ayurveda, is used as a *rasayan* for the improvement of stamina, mental concentration, resistance to stress and disease, and also to attenuate cerebral function deficits in geriatric population and to augment immune surveillance (non-specific host defence), [1-4]. However, wild-crafted plants of *Ashwagandha* differ considerably from those of cultivated varieties morphologically [5] and also in respect of chemical constituents and, hence in respect of bioactivities [4]. In India, there is evidence for the presence of more than one chemotype of this plant. Differences in chemotypes affect the bioactivities of the corresponding extracts [2, 4, 6].

Hence, it is necessary to use a specific chemotype to ensure reproducible therapeutic efficacy of the extract.

Using a patented procedure, a standardized extract powder of *Ashwagandha*, named Sensoril[®], was prepared from a cultivated chemotype of *Withania somnifera* [3, 4]. It contains the required levels and proportions of the major types of bioactives, *viz.* glycowithanolides (*syn.* sitoindosides), withanolide aglycones (e.g. Withaferin A,); oligosaccharides (molecular weight < 2000) and only traces of alkaloids and polysaccharides. This extract is thus devoid of undesirable constituents, e.g. the dementia-producing scopolamine-type alkaloid(s) of the family Solanaceae to which *Withania somnifera* belongs and toxic polysaccharides.

Several investigations were conducted by earlier workers with standardized *Withania somnifera* extracts where anti-inflammatory, anti-tumour and anti-stress activities were established [7]. Also, standardized *Withania somnifera* extracts elicited immuno-modulatory action [8]; inhibited cognitive defects in animal models of Alzheimer's disease [9], increased antioxidant activity and augmented oxidative free radical scavenging activity [10]. The bioactive constituents occurring in standardized *Withania somnifera* extracts were also evaluated for their anti-stress effect [11]. However, its direct impact on systemic energy synthesis and transduction, e.g., ATP (and equivalents), has not been evaluated before. Mitochondrial function is impaired by oxidative stress induced by exercise [12]. This in turn leads to depletion of energy, reflected by decrease in ATP content during forced exercise. Adaptogens can protect the mitochondria against oxidative onslaught and can restore the impaired energy levels.

The Korean medicine *Panax ginseng* C.A. Meyer (Araliaceae) has been widely used in China and Japan for thousands of years to fight fatigue [13] and for enhancement of resistance to many diseases [14]. The active components of *Panax ginseng* are considered to be ginsenosides, a group of tetracyclic triterpenoidal saponins responsible for many of the important effects of *Panax ginseng* [15].

Forced swimming test (FST) is perhaps one of the most commonly used animal models of behavioral despair and has been used extensively as a pre-diagnostic tool for the assessment of novel anti-depressants [16]. FST has also been used as an endurance test and also to examine whether certain agents have anti-fatigue effects [14].

The present investigation was carried out to evaluate the ATP level and other related energy indices on treatment with a standardized extract of WS in Swiss albino mice using swimming exercise as a means of depletion of energy. The energy-augmenting effect of WS was further compared with a standard adaptogenic agent, *viz.* South Korean red-*Panax ginseng*.

Materials and Methods

Chemicals and Reagents: All chemicals and reagents were of analytical grade and obtained from SRL, India. The solvents used for chromatographic techniques were of HPLC grade, obtained from Merck, India

Plant material and preparation of the extract: *Withania somnifera* roots and leaves were collected from the plants of a specific chemotype, cultivated in central and northern provinces of India (Madhya Pradesh, Uttaranchal and some places of Himachal Pradesh, up to 5000ft).

For preparation of the extract, GMP norms were adopted. Briefly, the roots and leaves of freshly harvested plants were washed with water, air-dried and powdered. The powder was extracted with water: methanol (40:60) by constant percolation. The pooled extract was filtered and the filtrate was evaporated to dryness in a spray dryer. The extract was designated as WSE. The extract was then analyzed by HPTLC and HPLC, using authentic markers; any deficiencies in the contents of bioactives were made up by adding the respective enriched fraction(s) obtained from an earlier operation.

Authentic *Panax ginseng* root (red) powder was obtained as a gift to Prof. Shibnath Ghosal from Ginseng Plant Laboratory, Govt. of Korea, Seoul. The powder was extracted with aqueous methanol and the extract was designated as *Panax ginseng* extract (PGE).

Chromatographic Techniques:

HPTLC Conditions: HPTLC was performed on Merck KGaA; 1.05554.0007 precoated TLC plates. Standard solution of glycowithanolides, Withaferin A and oligosaccharides along with the extracts of *Withania somnifera* roots and leaves were applied. The plates were developed in a twin trough chamber with *n*-butanol-acetic acid- water 4:1:2 (v/v/v) as mobile phase. Densitometric evaluation of the plates was performed at $\lambda = 225$ nm by means of a Camag TLC Scanner 3 in reflection / absorption mode. The scanned data were processed by means of Camag winCATS software, version 1.3.4. The plates were subsequently scanned to determine the UV reflectance spectra of each spot between 200 and 400 nm to identify the bioactives of *Withania somnifera* present in the extracts.

HPLC conditions:

Estimation of WS bioactives: HPLC analysis of glycowithanolides and withaferin-A were performed using Waters HPLC system with PDA detector and Empower software with a Merck-Hibar[®] pre-packed column (RT 250-4, LiChrosorb[®] RP-18, particle size 5 μ m, 4 x 250 mm cartridge column) fitted with a reverse phase guard column and acetonitrile: water-1: 1 (v/v) as the mobile phase, with a run time of 20 minutes and flow rate 0.6 ml/min in an isocratic mode.

Oligosaccharides were determined using Waters HPLC system with a RI detector and Empower software with a carbohydrate analysis column [Waters] 300 x 3.9 mm; acetonitrile: water- 80:20 (v/v) was used as the mobile phase; the run time was 10 minutes and flow rate was 2 ml/min in an isocratic mode.

Estimation of adenine nucleotides: Unclotted blood was mixed with equal volume of 1.3 M perchloric acid and the mixture was vortexed for one minute and centrifuged at 10000 rpm for 10 minutes at 4 °C. Supernatants were neutralized with saturated K₂CO₃ solution [17]. Then the clear supernatant was subjected to HPLC analysis using Waters HPLC system comprising with PDA detector and μ BondaPak RP C₁₈ [300 x 3.9 mm] (Waters) column; 30 mM phosphate buffer [pH 6.00] was used as the mobile phase. The flow rate was 1.0 ml/min (isocratic mode) and detection was done at 260 nm for ATP, ADP and AMP using respective standards [18].

Experimental Animals: Male Swiss albino mice (25-28 gms.) were fed with standard rodent pellets and water *ad libitum* and pre-acclimatized under standard laboratory conditions (temperature 26± 2°C).

Dosage of test compound and forced swimming exercise: The animals were divided into four groups-with six animals in each group. The doses for the animals were calculated in correlation to human doses [19]. Two groups of animals (Groups A and B) were administered 0.8% CMC suspension in water for 7 days. Two doses of WSE were administered [50mg/kg, p.o. and 100 mg/kg, p.o.] for the same period to the animals of Groups C and D, respectively. On the 6th and 7th day (marked respectively as 1st and 2nd day of swimming exercise), two hours after WSE and vehicle(control animals) administration, the animals of all the groups except Group A were subjected to swimming exercise for 30minutes [16]. Blood samples were collected immediately after 30 minutes of swimming exercise on both the days for estimation of the adenine nucleotides. From Group A animals, who received the vehicle control (0.8% CMC suspension in water for 7 days) but were not subjected to the swim exercise, blood was collected for estimation of adenine nucleotides on the seventh day for comparison purpose as the unstressed control.

In another set of experiments, the duration of drug administration was increased to 30 days. Five groups of animals with six animals in each group were taken. The control group (Group E) received the vehicle (0.8% CMC suspension in water) for 30 days. Two groups of animals were administered, respectively, two doses [50mg/kg, p.o. (Group F) and 100mg/kg, p.o. (Group G)] of WSE, for the same period (30 days). Another two groups of animals were administered PGE [50mg/kg, p.o. (Group H) and 100mg/kg, p.o. (Group I)] for 30 days. On the 29th and 30th day (marked respectively, as 1st and 2nd day of swimming exercise), the animals of all the groups were subjected to swimming for 30 minutes, two hours after the test compound administration. The mice were made to swim in glass cylinders (45x40x30cm), half filled with water (23± 2°C). Blood samples were collected immediately after the swimming session for estimation of the adenine nucleotides on the 1st and 2nd day of the swimming exercise. The values of the energy indices were expressed as the percentage difference between the 1st and 2nd swimming exercise (negative values reported to indicate the extent of depletion of energy indices).

Statistical Analysis: In the first set of experiments (Groups A to D, mean ± SEM values of unstressed control animals were compared with the stressed control and WSE treated groups by one-way ANOVA followed by post hoc analysis of pair-wise comparisons of different groups using the least significant difference (LSD) method. In the second set of experiments, (Groups E to I), mean SEM values of stressed controls were compared with different drug treated groups using similar statistical methods. The values were considered significant when *p* value was less than 0.05.

Results

Assay of the active principles by HPTLC and HPLC:

Analysis of WSE for the chemical ingredients using HPTLC revealed the presence of three distinctly separated regions for the bioactive compounds of *Withania somnifera* (Fig 1).

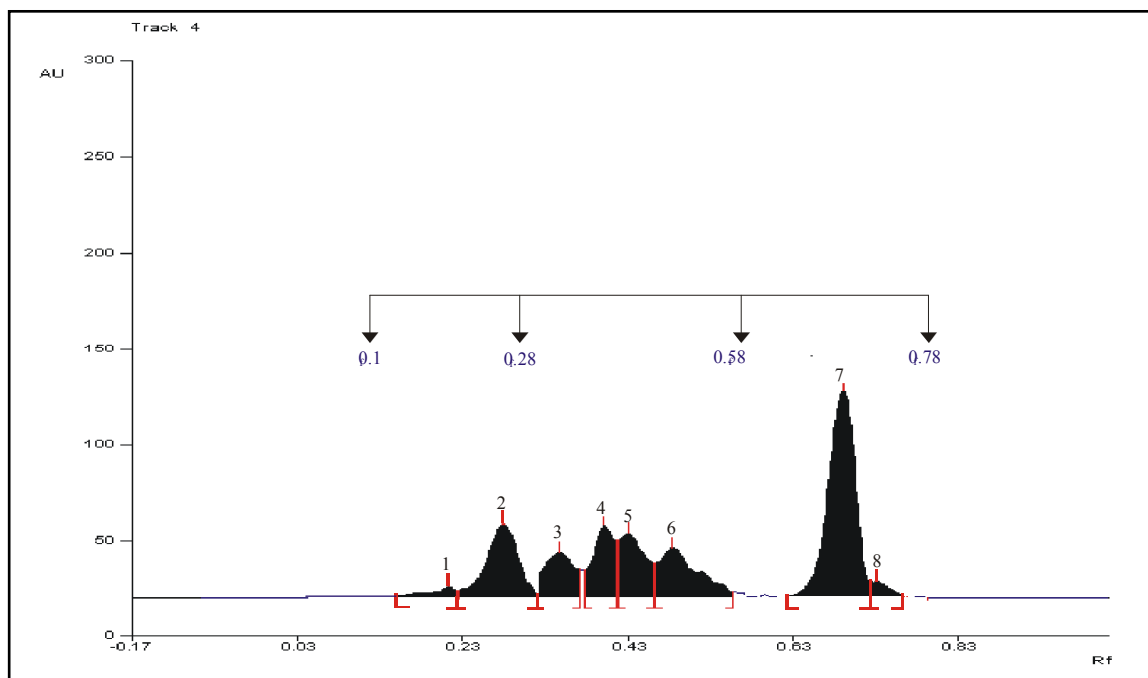


Fig.1. HPTLC chromatograms of WSE showing the three zones of bioactive compounds separated at three different R_f. The respective zones are marked by arrows. R_f 0.1 to 0.28 = oligosaccharides; R_f 0.28 to 0.58 = glycowithanolides and R_f 0.58 to 0.78 = withanolide aglycones (e.g Withaferin A)

These were identified using authentic markers (Fig 2). HPLC analysis also revealed the presence of withanolide aglycones, glycowithanolides and oligosaccharides (Figs 3 and 4). The following percentage of ingredients was determined in the extract:

- Glycowithanolides ($\geq 8\%$): are composed of fully characterized withanolide glycosides (also called Sitoindosides, e.g., Sitoindosides VII to X & XV)
- Withaferin A ($\leq 2\%$): a withanolide aglycone (in combination with oligosaccharides, it elicits immunopotentiating effect, while per se it is immunosuppressive).
- Oligosaccharides (MW < 2000, $\geq 32\%$)
- Alkaloids ($\leq 0.1\%$), tropine and pseudotropine (and no dementia-producing scopolamine)
- Polysaccharides ($\leq 4.0\%$).

The structures of the major bioactive compounds are presented in Fig. 5.

The low levels of the adverse compounds (alkaloids and polysaccharides) in standardized WSE make it free from producing any toxic effect. The standardized formula is used to ward off stress and increase the energy status by modulating body homeostasis.

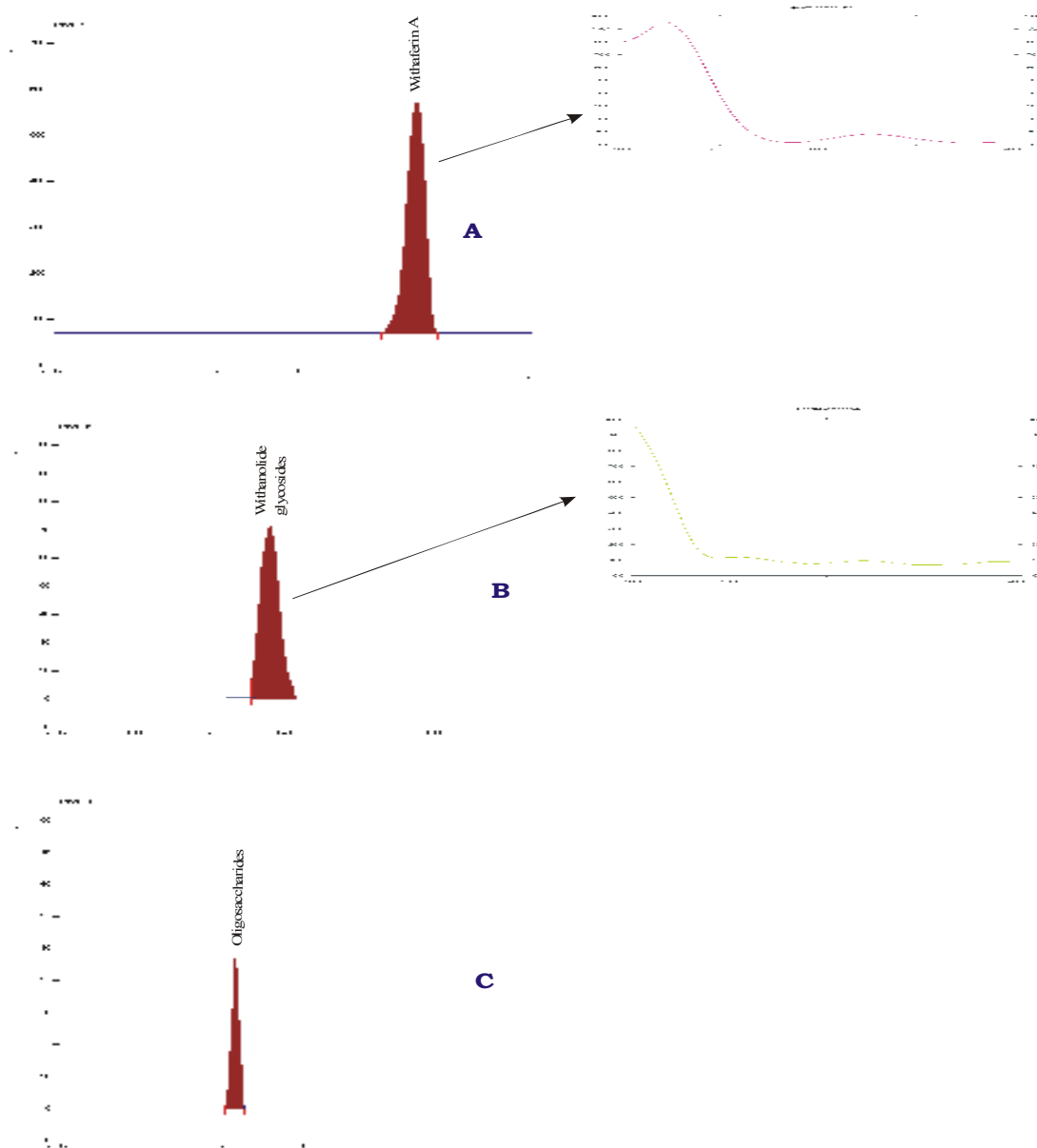


Fig.2. HPTLC chromatograms of different standard compounds of *Withania somnifera*. A. Standard Withaferin A with spectrum; B. Standard glycowithanolide with spectrum; C. Standard oligosaccharides.

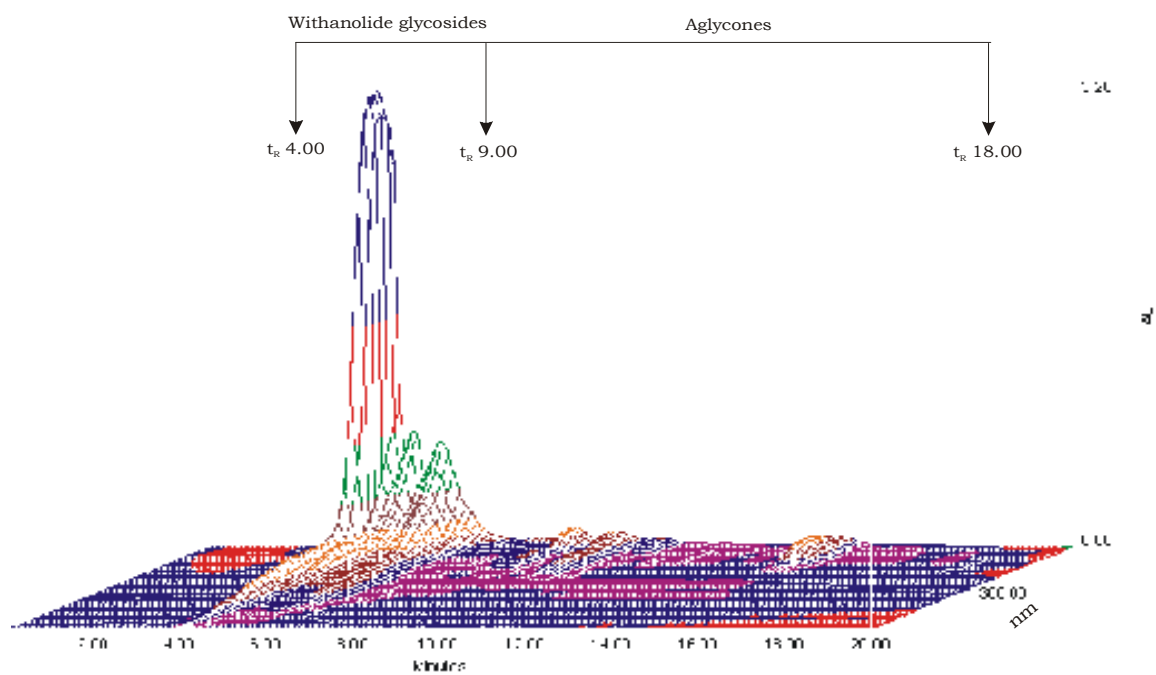


Fig.3. Three dimensional HPLC chromatogram of WSE

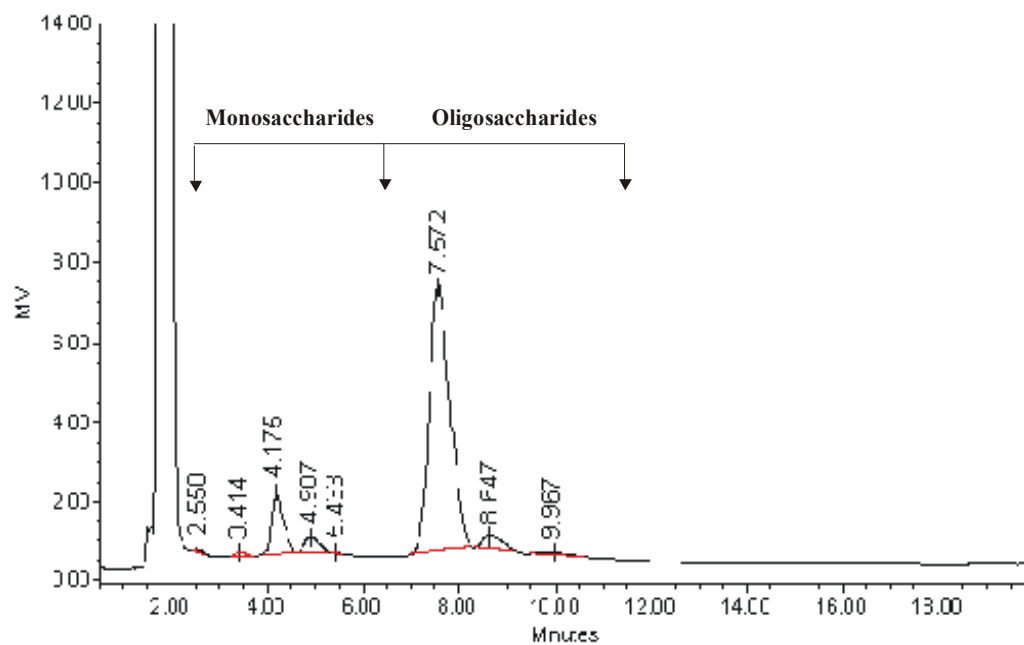
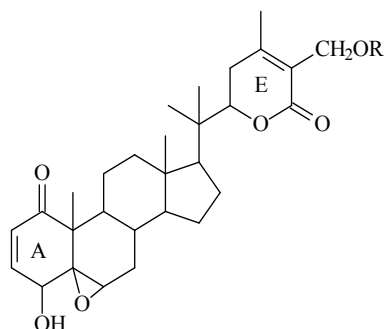
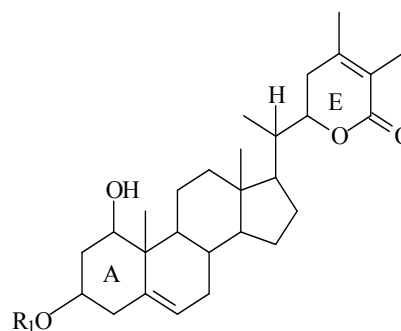


Fig.4. HPLC chromatogram of WSE showing the oligosaccharide region

**Sitoinoside-IX:** R=D-glucose**Sitoinoside-X:** R=6-O-palmitoyl D-glucose**Sitoinoside-XV:** R₁=Oligosaccharide

=Linear glucose, where, n=2 to 6

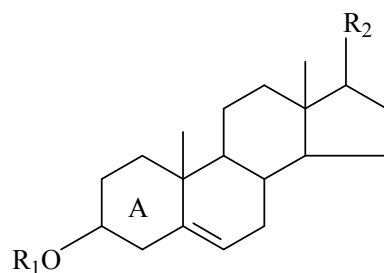
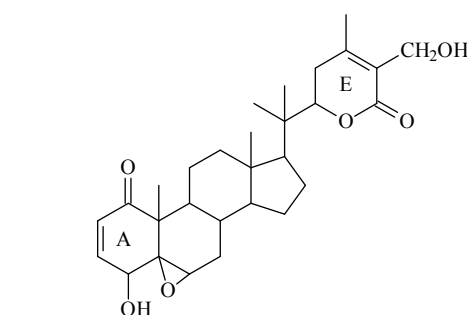
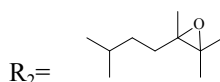
**Sitoinoside-VII:** R₁=6'-O-palmitoyl-β-D-glycoside**Sitoinoside-VIII:** R₁=6'-O-palmitoyl-β-D-glycoside

Fig. 5. Structures of withasteroid bioactives of WSE.

The energy augmenting effect of Sensoril®:

WSE was administered in two doses (50mg/kg and 100mg/kg, p.o.) for seven days. These doses were calculated on the basis of the human equivalent dose. The vehicle treated stressed control group (Group B) showed a depletion of $-48.0\% \pm 1.2$ ($p < 0.001$) in the ATP level after the second swimming test in comparison to the animals which were not subjected to the swimming exercise (Group A). WSE treatment at both the doses (Group C and D) showed significant improvement in the indices of energy which was reflected in attenuating the depletion of ATP content ($-22.6\% \pm 2.5$ and $-11.4\% \pm 1.7$ respectively) dose dependently after the second swimming test (i.e., 7th day of WSE treatment). These results indicated that WSE treatment attenuated the ATP depletion caused due to swimming exercise and helped to restore the energy status of the body close to normal levels. Repeated swimming exercise increases oxidative stress in several organs including skeletal muscles and brain [20] which, in turn, causes depletion of ATP content and decrease in its regeneration capacity leading to a decrease in TAN values.

Also, intense muscle contraction during exercise is associated with a decrease in the total adenine nucleotide (TAN) pool [21]. AEC (adenylate energy charge), which reflects the relative concentrations of ATP, ADP and AMP [22], also shows reduction with the decrease in ATP level [23]. The TAN and AEC values also showed a significant positive improvement in the WSE treated groups (C and D) compared to those of the stressed control (Group B) (Table 1). The animals in the vehicle treated control group, without exercise (Group A), had energy levels (ATP content: 0.71 ± 0.06 mM, TAN: 0.83 ± 0.05 mM and AEC: 0.9 ± 0.01) much higher than those of Group B, but only marginally higher than those of the treated groups (C and D), reflecting the restoration of energy status on WSE treatment.

Table 1. Percentage difference in the depletion of blood levels^a of ATP and energy related indices in WSE & vehicle treated mice after the second swimming exercise compared to the unstressed control group

Treatment groups	ATP (%)	AEC (%)	TAN (%)	ATP/ADP (%)
Group B	-48.0 ± 1.2	-7.4 ± 0.5	-39.3 ± 1.0	-56.8 ± 1.7
Group C	$-22.6\pm 2.5^{**}$	$-1.3\pm 0.3^*$	$-20.5\pm 2.3^{**}$	$-19.9\pm 2.8^*$
Group D	$-11.4\pm 1.7^{**}$	$-0.4\pm 0.3^*$	$-10.0\pm 1.2^{**}$	$-15.9\pm 2.8^*$

^a determined by HPLC (See Materials and Methods); Values are expressed as mean \pm SEM for 6 animals in each group; * $p<0.01$ and ** $p<0.001$; compared to unstressed control group (Group A)

WSE even at the lower dose (Group C) attenuated the progressive depletion in the energy status after the 1st and 2nd day of swimming. This was reflected in the decrease in the percentage depletion of ATP levels and other related indices in WSE treated groups (Table 2). This above finding prompted us to determine the effect of WSE treatment for a prolonged period of time (i.e. 30days).

Table 2. Percentage difference in the depletion of blood levels^a of ATP and energy related indices in stressed control and WSE treated mice between the second and first swimming exercise

Treatment groups	ATP (%)	AEC (%)	TAN (%)	ATP/ADP (%)
Group B	-38.3 ± 2.9	-5.6 ± 0.9	-29.2 ± 3.3	-48.4 ± 6.2
Group C	$-17.2\pm 2.3^{**}$	$-1.6\pm 0.3^{**}$	$-14.5\pm 2.4^*$	$-16.3\pm 4.2^*$
Group D	$-5.9\pm 2.5^{**}$	$-1.1\pm 0.6^{**}$	$-3.9\pm 3.3^{**}$	$-16.5\pm 9.2^*$

^a determined by HPLC (See Materials and Methods); Values are expressed as mean \pm SEM for 6 animals in each group; * $p<0.01$ and ** $p<0.001$; compared to stressed control group (Group B)

Additionally, the protective and restorative energy-effects of WSE were compared with those of a well-known adaptogen, viz. *Panax ginseng* (PGE). This comparison was deemed necessary especially in view of the fact, that, PGE was reported to suffer from a number of adverse side-effects known as the Ginseng abuse syndrome [24, 25] while WSE is free from these deficiencies [10].

Energy augmenting effect of WSE vis-à-vis PGE:

After 30 days of vehicle treatment, followed by swimming exercise on the 29th and 30th day, the stressed control animals (Group E) showed progressive depletion (2nd day-1st day values) in ATP level(-37.1%±4.2) and TAN values(-28.9%± 6.4).The lower dose of WSE (Group F) on the other hand, attenuated the difference markedly in the level of depletion of ATP content(-6.7% ±1.2) and TAN values (-4.7% ±1.2) when compared to those of the stressed control group (Group E). The same dose of PGE (Group H), however, could not attenuate the depletion of the energy indices to that extent, the depletion being (ATP: -18.2% ±3.2 and TAN: -14.5% ±2.1). Other energy indices, such as, ATP/ADP and AEC values also showed better recovery in the WSE treated group (Group F) than PGE treated group (Group H) (Table 3).Although, PGE (50mg/kg, p.o.) exhibited attenuation in the depletion of energy indices to a reckonable extent but the level of significance (Table 3) in case of WSE at the same dose level was still higher. In the WSE treated group, prolonging the duration of the treatment (from 7 days to 30 days) further improved the attenuation of loss of energy indices (Tables 2 and 3).

In the higher dose groups, both WSE (Group G) and PGE (Group I) showed significant improvement in the blood ATP content, compared to that of the stressed control group (Group E). Once again, the degree of ATP depletion of WSE treated group (-5.6 % ±0.9) was less compared to that of the Ginseng treated group (-11.1% ±3.6) (Table 3). The concomitant decrease in TAN values in Group G, was also markedly reduced (-5.9% ±1.4) compared to that of Group I (-10.7% ± 1.6) (Table 3). AEC values in both WSE and PGE treated groups (Groups G and I), however, remained unaltered after the swimming exercise compared to that of the stressed control (Table 3). These observations support the adaptogenic effects of the two test agents. Thus, on treatment with the two adaptogens, prevention and/or restoration of the energy loss and capacity of energy transduction, during swimming exercise, and systemic improvement in the overall energy status occurred.

Table 3. Percentage difference in the depletion of blood levels^a of ATP and energy related indices in control, WSE and PGE treated (30 days) mice between the second and first swimming exercise

Treatment groups	ATP (%)	AEC (%)	TAN (%)	ATP/ADP (%)
Group E	-37.1±4.2	-5.7±0.9	-28.9±6.4	-47.1±5.8
Group F	-6.7±1.2 ^{**#}	-0.8±0.3 ^{**}	-4.7±1.2 ^{**#}	-15.2±3.9 ^{**}
Group G	-5.6±0.9 ^{**}	0.3±0.2 ^{**}	-5.9±1.4 ^{**}	2.3±2.3 ^{**}
Group H	-18.2±3.2 [*]	-1.8±0.3 ^{**}	-14.5±2.1 [*]	-22.5±0.9 [*]
Group I	-11.1±3.6 ^{**}	-0.1±0.4 ^{**}	-10.7±1.6 ^{**}	-2.5±2.8 ^{**}

^a determined by HPLC (See Materials and Methods)

Values are expressed as mean± SEM for 6 animals in each group

* $p < 0.05$ and ** $p < 0.001$; compared to stressed control group (Group E)

$p < 0.05$ between Group F & Group H

Discussion

The activity of adaptogens seems to be mediated, at least partly, by the increase in the essential energy element, such as ATP, in the muscle-mitochondria in mice that were subjected to repeated swimming exercise [26]. Oxidative stress due to repeated swimming exercise increases the lipid peroxidation levels and decreases glutathione levels in the muscle-mitochondria [20]. On treatment with WSE even at the lower dose level, restoration of the lost glutathione levels occurred along with decrease in the lipid peroxidation levels (data not shown), which ultimately results in augmentation of the endurance capacity of the treated animals. The present investigation thus suggests that WSE, even at a low dose level, can protect the mitochondria against the oxidative onslaught and activate the restoration of lost ATP in mitochondria during and after the repeated swimming exercise. This mechanistic postulate regarding the ATP augmenting effect of anti-stress agents has precedence in the literature [27]. *Panax ginseng*, on the other hand, showed effective adaptogenic activity only at the higher dose level (100 mg/kg) [10]. However, long term administration of *Panax ginseng* at a dose of 100 mg/kg, p.o. might result in a number of adverse side-effects collectively termed the “ginseng abuse syndrome” [24, 25], characterized by high blood pressure, water retention (mineralo-corticoid effect), higher muscle tone, insomnia and hormonal disbalance in women. WSE, on the other hand, composed of the tested bioactives of *Withania somnifera* [4, 8], does not suffer from any of the potential deficiencies of *Panax ginseng*. The present study suggests that sustained use of WSE even at a low dose level would provide energy restoration needed under stressed conditions without any adverse side effects. Administration of higher dose of WSE, which is also devoid of any adverse side effect, would elicit and maintain the energy restoration effects within a short period of time.

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