

Triethylene glycol-like effects of *Ashwagandha* (*Withania somnifera* (L.) Dunal) root extract devoid of withanolides in stressed mice

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Abstract

Background: The objective of the study is to compare stress resistance-promoting effect of triethylene glycol (TEG) and root extract of *Ashwagandha* (*Withania somnifera*) i.e. withanolide-free root extract of *Withania somnifera* (WFWS). **Materials and Methods:** Mice groups treated orally with 10 mg/kg TEG or WFWS (3.3, 10, 33.3, or 100 mg/kg) for 12 consecutive days were subjected to foot shock stress-triggered hyperthermia test on the 1st, 5th, 7th and 10th day and to marble-burying test on the following 2 days. Effects of treatment on stress-triggered alteration in body weight, core temperature, blood glucose, insulin and cortisol level were quantified and statistically analyzed. **Results:** WFWS doses up to 10 mg/kg/day were as effective as TEG in affording protection against stress-triggered alteration in body weight, core temperature and marble-burying behavior. Protection against stress-triggered alteration in blood glucose and insulin level, as well as antidepressants or anxiolytic-like activities in the behavioral test, were observed in the higher two WFWS doses (33.3 and 100 mg/kg) treated groups only. **Conclusion:** *Ashwagandha* metabolites other than withanolides contribute to its stress resistance increasing effects. The observations suggest that modulation of physiological functions of gut microbiota may be involved in the mode of action of *Withania somnifera* root extracts.

Keywords: *Ashwagandha*, body weight regulation, marble-burying test, *Rasayana* therapy, stress resistance, triethylene glycol, withanolides

Introduction

Dried mature roots of *Withania somnifera* (L.) Dunal (*Ashwagandha*), a plant of the *Solanaceae* family (genus *Withania*), are used in many Ayurvedic formulations prescribed for a variety of inflammatory conditions (e.g., arthritis and rheumatism) and as a general tonic to increase energy, improve overall health and longevity and prevent diseases.^[1] *Ashwagandha* is classified in Ayurveda as a *Rasayana* drug useful for promoting physical and mental health, increasing stress resistance against diseases and environmental challenges and prolonging healthy life span.^[2-5] The Ayurvedic Pharmacopoeia of India also describes an analytical method for identifying and quantifying the contents of withanolides (a group of structurally analogous steroidal lactones) and alkaloids in *Ashwagandha*.^[6] Withaferin-A was the very first withanolides type constituent isolated from the plant in the

1960s and since then, a number of withanolides encountered in different parts of *Withania somnifera* and other plants as well as of reports on their antimicrobial and other therapeutically interesting bioactivities have continued to increase.^[7-9]

The contents of total withanolides quantified by modern analytical methods are seldom >3%–5%, which vary considerably in different parts and cultivars of the plant. It is now well recognized also that several bioactive substances other than withanolides are also biosynthesized and stored in different amounts in different parts of the plant and that many of them also possess bactericidal, anticancer, brain function modulating and other therapeutically

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interesting bioactivities.^[10,11] Triethylene glycol (TEG) is a relatively new addition to the long list of microbicidal substances with cytostatic or anticancer and sleep-inducing activities.^[12,13] However, the presence of TEG has been detected only in the leaves of the plant cultivated under the experimental condition and that too by the same Japanese research group. Although we have not yet been able to identify TEG in crude extracts of *Withania somnifera* root extracts, observations in laboratories have revealed that its fairly low daily oral doses (10 mg/kg/day) are effective in increasing stress resistance and that its such effectiveness continues to increase with increasing numbers of treatment days.^[14] These observations, taken together with our earlier ones made with structurally diverse bactericidal phytochemicals and some plant-derived drugs,^[15-21] led us to the working hypothesis that their modulating effects on gut microbiota composition and functions may also contribute to therapeutically interesting bioactivity profiles of *Withania somnifera*-like adaptogenic herbs often used in *Rasayana* therapies or as herbal remedies.^[22,23] Results of an exploratory experiment added further preclinical evidence in favor of this working hypothesis and suggested that combinations of microbicidal substances other than withanolides are also involved in the traditionally known and more recently, rediscovered medicinal uses of *Withania somnifera* roots. This experiment was conducted in the realm of our psychopharmacological studies with extracts and bioactive constituents of Ayurvedic *Rasayana* drugs^[24] currently often used for the treatment of obesity and diabetes associated physical and mental health problems.^[25,26] Choice of the doses and experimental procedures used in the experiment were based on our earlier observations made with diverse types of *Withania somnifera* extracts and some of their already known constituents.^[25-29] The aim of the experiment was to verify whether or not the presence of withanolides in *Withania somnifera* root extracts is essential for their obsessive-compulsive behavior suppressing effects observed in rodents and patients.^[28,30,31]

Materials and Methods

Animals

Albino male mice of Wistar strain weighing 25 ± 5 g used in this study were procured from Central Animal House of Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration number: 542/AB/CPCSEA). They were randomly selected and group housed (six animals per cage) in polypropylene cages provided with husk bed at an ambient temperature ($25 \pm 1^\circ\text{C}$) and relative humidity ($50 \pm 10\%$) with a 12:12 h light/dark cycle. All animals were acclimatized to laboratory conditions for at least 1 week before the start of the experiment. They were always fed with standard rodent diet and water *ad libitum*. Ethical clearance for animal experimental work was obtained from the Central Animal Ethical Committee of the University (Dean/2014/CAECI/604, dated 30/05/2014) before the commencement of experiments. All experimental

groups were always tested in parallel (i.e., on the same day of the experiment) and handled, weighed and observed by a blinded observer.

Plant extract, chemicals, and test kits used

Withania somnifera root extract freed from withanolides (WFWS) used in this study was prepared and generously supplied together with its analytical certificate by the Research Department of Natural Remedies Private Limited, Bengaluru, India. In short, *Withania somnifera* root extract prepared according to the manufacturing procedure used by the company (published elsewhere)^[32] for manufacturing and commercializing them was chromatographically subfractionated. Subfractions of the extract devoid of withanolides were combined and evaporated to obtain WFWS (Batch No. RD15052). Traces of any known withanolides encountered in the plant could be detected in WFWS by the chromatographic techniques used by the company for analytically standardizing *Ashwagandha* extracts commercialized by the company and many others.

Carboxymethyl cellulose (CMC) was procured from Central Drug House (New Delhi, India) and TEG was from Spectrochem (Mumbai, India). Plasma glucose level was estimated by biochemical test kit (ERBA Diagnostics Mannheim GmbH, Germany); plasma insulin level was estimated using enzyme-linked immunosorbent assay (ELISA) test kit (Chemux BioScience Inc., USA) and plasma cortisol was estimated using ELISA test kit (DSI S.r.l., Italy).

Animal grouping and drug treatments

Six randomly selected experimentally naive mice were allotted to each of the seven experimental groups. i.e., group I: stressed control (vehicle treated); group II: nonstressed control (vehicle treated); group III: stressed + TEG (10 mg/kg); group IV: stressed + WFWS (3.3 mg/kg); group V: stressed + WFWS (10 mg/kg); group VI: stressed + WFWS (33.3 mg/kg) and group VII: stressed + WFWS (100 mg/kg). The animals of the stressed and nonstressed control groups were treated once daily with 0.3% CMC (p. o) for 12 consecutive days. For similar oral treatments, TEG and WFWS were suspended in 0.3% CMC and oral application volume for all treatments was 10 ml/kg/day. On each experimental day, body weights and mean rectal temperature of the animals were recorded 1 h before the oral drug administration. All animals were closely observed for apparent behavioral abnormalities during the entire course of the experiment. Further detail of the experimental procedure used are graphically summarized in Figure 1.

Foot shock stress-induced hyperthermia test

This test was conducted on the 1st, 5th, 7th and 10th days of the experiment and 60 min after the day's oral treatments. The experimental procedure used for this test was the same as those described in detail in other reports from our research group.^[15-21,27-29,33] Temperature was measured using a rectal probe and a calibrated digital thermometer (Easy Care, Mumbai, India).

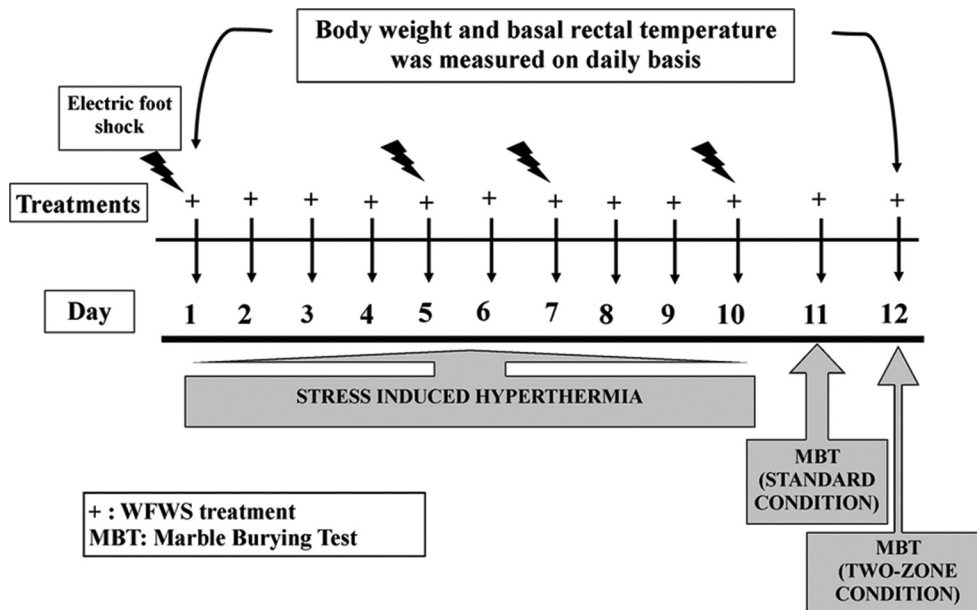


Figure 1: Summary of experimental procedures used

Marble-burying test

On the 11th day and 60 min after the day's oral treatment, individual mouse from each group was placed in a polypropylene cage (30 cm × 23 cm) provided with husk bed, where 12 glass marbles (color and size of marbles were kept constant) were evenly spaced for standard marble-burying condition test. On the 12th day, 60 min after the oral treatment, they were tested again in the two-zone marble-burying condition, whereupon 8 glass marbles (color and size of marbles were kept constant) were evenly spaced but only on one half of the polypropylene cage. After 15 min (standard condition) or 30 min (two-zone condition) of exposure, the animals were placed back to their home cages and the number of marbles at least two-thirds covered by husk was counted.^[34]

Determination of plasma glucose, insulin, cortisol level and organ weight

Immediately after the last observation made in two-zone marble-burying test on the 12th day of the experiment, all animals were sacrificed by decapitation and blood was collected by direct cardiac puncture in EDTA-coated tubes kept in ice. Immediately after blood collection adrenal glands and spleen of the animals were dissected out, washed under slowly running tap water and weighed after removing adhered water by gently drying them on sheets of filter papers.^[17,35] Blood glucose, insulin and cortisol levels in the plasma of the blood samples were quantified using commercially available test kits and following the instruction manual of the kits supplied by their manufacturers.

Statistical analysis

Mean ± standard error of mean was calculated for the observed values in each experimental group. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test and

two-way ANOVA followed by Bonferroni *post hoc* test unless otherwise stated. GraphPad Prism-5 (GraphPad Software Inc., La Jolla, California, USA) was used for statistical analysis. Origin Pro 8 software (Origin Lab Corporation, Massachusetts, USA) was used for graph representation. $P < 0.05$ was considered as statistically significant.

Results

Body weight

Unlike for the nonstressed control group, mean body weight of the stressed control group decreased during the course of the experiment [Figure 2a and b]. Such body weight losses triggered by occasional exposures to very short duration (50 s) of foot shock stress were not observed in the TEG or any of the WFWS-treated groups. The rate of change in the mean body weights of the 3.3 or 10 mg/kg/day WFWS-treated groups were almost identical to that observed in the 10 mg/kg/day TEG-treated group, whereas those of the 33.3 and 100 mg/kg/day extract-treated groups were like those of the vehicle-treated nonstressed group. These results indicate that 33.3 mg/kg daily oral does of the tested extract is its maximally effective one in affording complete protection against body weight changes caused by repeated exposures to unavoidable stressful stimuli and that its minimally effective ones are 3.3 mg/kg/day or lower.

Mean rectal temperatures

Mean rectal temperature of the stressed control group increased slightly and that of the nonstressed one continued to decrease somewhat during the course of the experiment [Figure 3]. Mean rectal core temperature of TEG- or WFWS-treated and stressed groups also continued to decrease during the course of experiment. On the last 2 observational days, these mean values of the nonstressed group were statistically significantly higher than most of the stressed groups. Therefore, it is apparent that

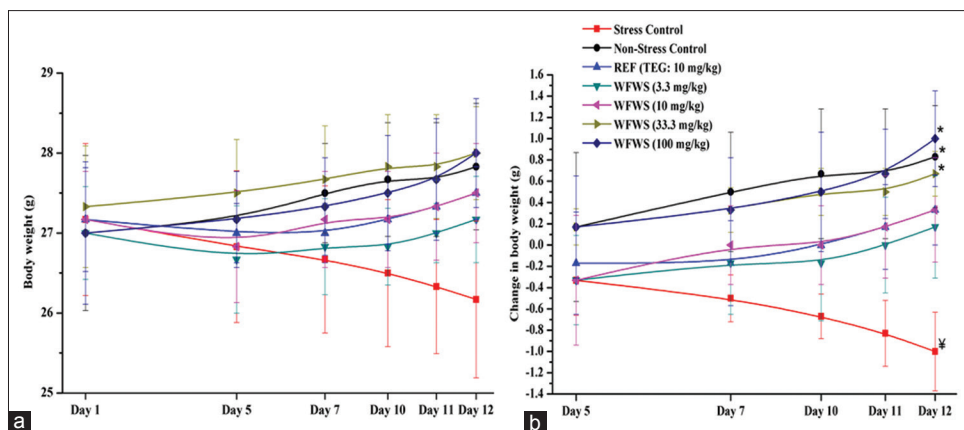


Figure 2: Effect of stress on (a) mean body weight and (b) change in body weight of male mice treated with withanolide-free *Withania somnifera* extract. WFWS: Withanolide-free *Withania somnifera* extract, TEG: Triethylene glycol. Values are mean \pm standard error of mean ($n = 6$). *denotes statistically significant difference (two-way ANOVA followed by Bonferroni *post hoc* test) relative to stress control group ($*P < 0.05$). †denotes statistically significant difference (two-way ANOVA followed by Bonferroni *post hoc* test) relative to nonstress control group ($†P < 0.05$)

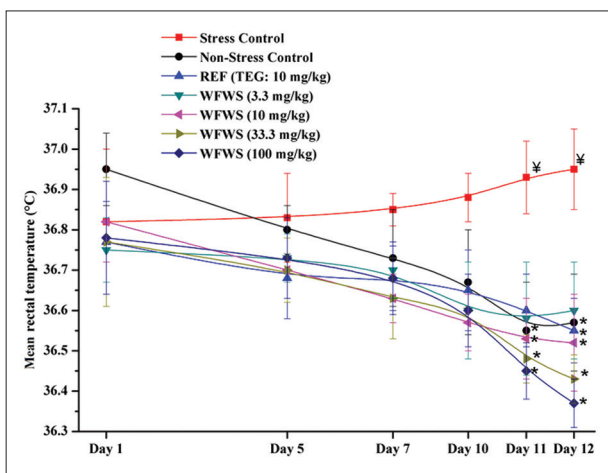


Figure 3: Mean rectal temperature of male mice treated with withanolide-free *Withania somnifera* extract. WFWS - Withanolide-free *Withania somnifera* extract, TEG - Triethylene glycol. Values are mean \pm standard error of mean ($n = 6$). *denotes statistically significant difference (two-way ANOVA followed by Bonferroni *post hoc* test) relative to stress control group ($*P < 0.05$). †denotes statistically significant difference (two-way ANOVA followed by Bonferroni *post hoc* test) relative to nonstress control group ($†P < 0.05$)

the minimally effective daily oral doses of the tested extract in affording protection against stress-triggered alterations in mean body core temperature of stressed mice are also 3.3 mg/kg or lower. Except for the last 2 observational days, mean rectal temperature of all WFWS- or TEG-treated groups were not statistically significantly different from the nonstressed one. These observations indicate that the observed effects of the test agents in stress-triggered alteration in mean rectal core temperature are due to their stress response suppressing effects evolving slowly during the course of the experiment.

Stress-induced hyperthermia

Mean transient hyperthermic response of the stressed control group remained almost constant on all observational days

[Figure 4a]. Although numerically these values of the TEG- or WFWS-treated group on the 1st observational day were lower than that of the stressed control group, no statistically significant effect of TEG or WFWS were observed on this day. However, after 12 daily oral 3.3 or 10 mg/kg doses of the extract, this transient hyperthermic response was statistically significantly lower than that of the stressed control group. Observed effectiveness of these two doses of the extract on this day was almost identical and was somewhat lower than that of 10 daily oral doses of 10 mg/kg TEG. Such statistically significant effect of the tested TEG daily dose and of 33.3 or 100 mg/kg WFWS doses were observed even after their 5 daily doses. Effects of 33.3 or 100 mg/kg daily WFWS doses observed from the 5th observational day onward were always almost identical. These observations reveal that the effective daily dose range of the extract in foot shock stress-induced hyperthermia test lies between 3 and 33.3 mg/kg and reaffirm that its efficacy in this test depends also on its daily dose as well as number of days of treatment given [Figure 4b].

Marble-burying tests

Results of the standard version of the marble-burying test conducted 1 h after 11 daily oral treatment revealed that the mean number of the marbles buried by the animals of the stressed control group was statistically significantly higher than that of the nonstressed control group [Figure 5a]. These values of the 10 mg/kg/day TEG or of the 33.3 or 100 mg/kg/day WFWS-treated groups were statistically significantly lower than the vehicle-treated control group. Effectiveness of these two higher doses of the extract was almost equal and somewhat higher than that of the tested TEG dose. Since no statistically significant effects of 3.3 or 10 mg/kg/day WFWS doses were observed, it seems reasonable to assume that its behaviorally effective daily oral doses are >10 mg/kg. This inference is reaffirmed by the results of the two-zone marble-burying test summarized in Figure 5b. It must be noted, that unlike in the standard version of the test, no statistically significant difference between the mean number of marbles buried by the

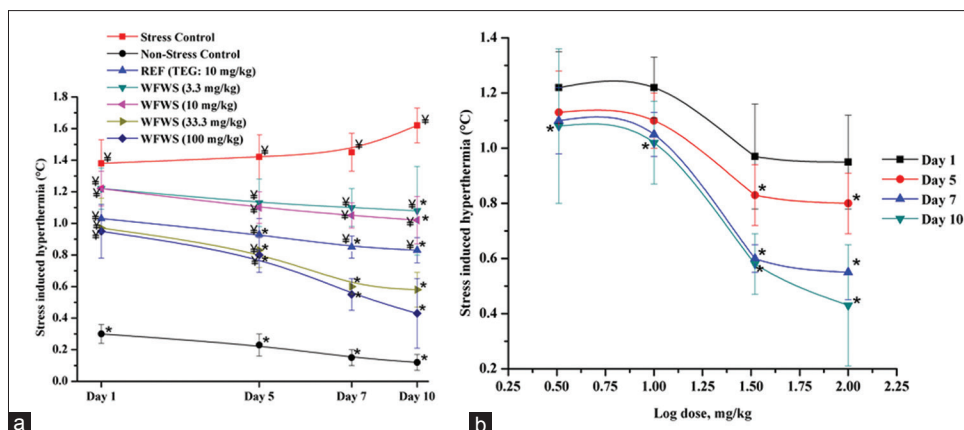


Figure 4: Stress-induced hyperthermia of male mice treated with graded doses of (a) WFWS and (b) log dose-response curve of single and daily repeated treatment with WFWS in the foot shock stress induced hyperthermia test. Values are mean \pm standard error of mean ($n = 6$). *denotes statistically significant difference (two-way ANOVA followed by Bonferroni *post hoc* test) relative to stress control group ($*P < 0.05$). \forall denotes statistically significant difference (two-way ANOVA followed by Bonferroni *post hoc* test) relative to nonstress control group ($\forall P < 0.05$)

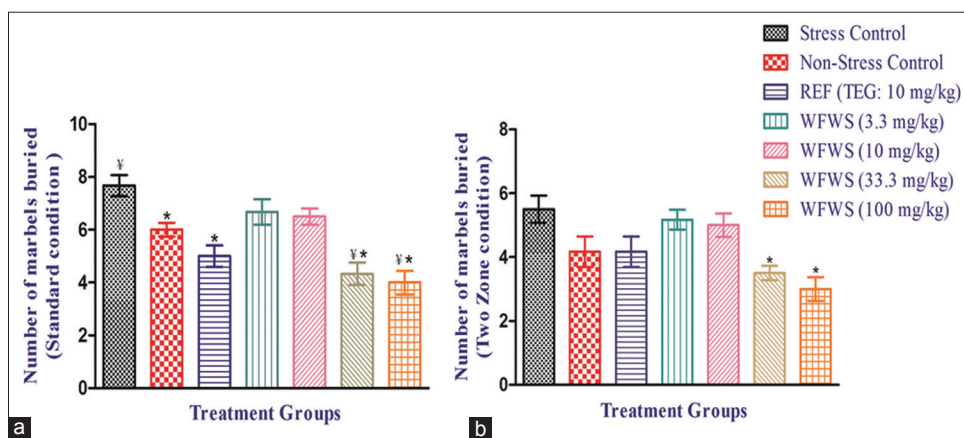


Figure 5: Effect of withanolide-free *Withania somnifera* extract on (a) standard condition marble-burying test in male mice on day 11 and (b) two-zone condition marble-burying test in male mice on day 12. Values are mean \pm standard error of mean ($n = 6$). *denotes statistically significant difference (one-way ANOVA followed by Student–Newman–Keuls multiple comparison test) relative to stress control group ($*P < 0.05$). \forall denotes statistically significant difference (one-way ANOVA followed by Student–Newman–Keuls multiple comparison test) relative to nonstress control group ($\forall P < 0.05$)

animals of the stressed and unstressed groups was observed and that unlike in the standard version of the test, no statistically significant effect of the test TEG dose were observed in the two-zone version of the test. These observations indicate that unlike for TEG, beneficial effect of WFWS as an anxiolytic or antidepressant-like bioactive agent can be detected in both versions of the test.

Organ weight and plasma glucose, insulin and cortisol levels

The possibility that WFWS is a stress-resistant promoting agent is inferred also from its observed preventive effect against stress-triggered alteration in adrenal gland weight [Table 1] and plasma cortisol level [Table 2]. It was interesting to note that mean plasma glucose level of stressed control group was slightly but significantly higher than the nonstressed group and that no such stress-triggered hyperglycemic response was observed in the 33.3 or 100 mg/kg/day WFWS-treated stressed animals [Table 2]. The observations that plasma

insulin level of these two extract-treated groups was not statistically significantly different from that of the stressed control group could indicate that observed anti-stress and/or antihyperglycemic activities of the higher tested doses of WFWS are independent of its effect on insulin homeostasis. However, further more detailed mechanistic studies will be necessary for reaffirming this possibility.

Discussion

Reported observations are the very first one revealing the effect of TEG and *Withania somnifera* root extract devoid of withanolides on marble-burying behavior of stressed rodents. They suggest that their 10 mg/kg daily oral doses are high enough for preventing stress-triggered alteration in obsessive–compulsive behavior of mice and reveal that even extremely low daily doses (3.3 mg/kg or lower) of WFWS are highly effective in protecting stress-triggered losses in body weight and alterations in thermoregulatory process. However,

Table 1: Effect of withanolide-free *Withania somnifera* extract on the weight of adrenal glands and spleen in male mice

Treatment groups	Organ weights (mg)		Relative organ weights (mg/g of body weight)	
	Spleen	Adrenal glands	Spleen	Adrenal glands
Stress control	96.78±1.53	19.45±0.85 [‡]	3.35±0.12	0.75±0.05 [‡]
Nonstress control	92.80±2.07	14.03±0.60*	3.73±0.18	0.51±0.03*
REF (TEG: 10 mg/kg)	94.30±1.74	17.07±1.24 [‡]	3.43±0.05	0.62±0.05
WFWS (3.3 mg/kg)	96.43±1.50	18.58±1.05	3.57±0.03	0.69±0.04
WFWS (10 mg/kg)	94.32±1.37	16.75±0.74	3.44±0.12	0.61±0.04
WFWS (33.3 mg/kg)	93.17±1.23	15.27±0.69*	3.33±0.08	0.55±0.03*
WFWS (100 mg/kg)	92.78±1.78	14.30±1.13*	3.33±0.11	0.52±0.05*

Values are mean±SEM (n=6). *Statistically significant difference (one-way ANOVA followed by Student-Newman-Keuls multiple comparison test) relative to stress control group (*P<0.05). [‡]Statistically significant difference (one-way ANOVA followed by Student-Newman-Keuls multiple comparison test) relative to nonstress control group ([‡]P<0.05). *W. somnifera*: *Withania somnifera*, SEM: Standard error of mean, ANOVA: Analysis of variance, TEG: Triethylene glycol, WFWS: Withanolides free *W. somnifera* extract, REF: Reference Control Group

Table 2: Effect of withanolide-free *Withania somnifera* extract on the plasma glucose, insulin and cortisol in male mice

Treatment groups	Glucose (mg/dl)	Insulin (μIU/ml)	Cortisol (ng/ml)
Stress control	97.00±1.47 [‡]	14.55±0.84 [‡]	96.57±1.49 [‡]
Nonstress control	82.81±1.60*	19.40±1.27*	87.50±2.04*
REF (TEG: 10 mg/kg)	91.46±1.74 [‡]	15.86±1.14 [‡]	89.73±1.62*
WFWS (3.3 mg/kg)	92.74±1.60 [‡]	14.75±0.65 [‡]	94.16±2.26
WFWS (10 mg/kg)	91.50±1.45 [‡]	14.79±0.76 [‡]	93.56±1.11
WFWS (33.3 mg/kg)	88.87±1.46* [‡]	14.84±0.43 [‡]	89.36±1.40*
WFWS (100 mg/kg)	89.19±2.03* [‡]	15.08±0.58 [‡]	88.13±2.13*

Treatment groups	Relative values (per 100 g of body weight)		
	Glucose (mg/dl)	Insulin(μIU/ml)	Cortisol (ng/ml)
Stress control	373.81±17.48 [‡]	56.39±4.87	370.87±10.12 [‡]
Nonstress control	299.27±13.02*	69.89±4.65	315.56±11.09*
REF (TEG: 10 mg/kg)	333.06±8.75	57.82±4.40	326.76±8.18
WFWS (3.3 mg/kg)	343.93±8.81	54.70±2.70	349.24±10.88
WFWS (10 mg/kg)	334.02±12.27	54.14±3.81	341.22±9.91
WFWS (33.3 mg/kg)	318.19±9.16*	53.02±1.34	319.99±9.53*
WFWS (100 mg/kg)	319.06±7.47*	54.11±2.84	315.35±8.40*

Values are mean±SEM (n=6). *Statistically significant difference (one-way ANOVA followed by Student-Newman-Keuls multiple comparison test) relative to stress control group (*P<0.05). [‡]Statistically significant difference (one-way ANOVA followed by Student-Newman-Keuls multiple comparison test) relative to nonstress control group ([‡]P<0.05). *W. somnifera*: *Withania somnifera*, SEM: Standard error of mean, ANOVA: Analysis of variance, TEG: Triethylene glycol, WFWS: Withanolides free *W. somnifera* extract, REF: Reference Control Group

since we have not yet been able to identify TEG in WFWS or any other extract of the plant, the question whether TEG is one of the bioactive constituents of *Withania somnifera* root extracts cannot yet be answered with any certainty.

Results of some clinical studies have revealed that *Withania somnifera* root extract have regulating effect of body weight of stressed and other patients suffering from obsessive-compulsive disorders.^[36,37] Chronic exposure to the stressful environment and life events alter brain function regulating eating behavior and addictive or obsessive habits.^[38-41] Therefore, our observations strongly suggest that Ayurvedic recommendation of *Withania somnifera* could as well be due to their ability to counteract compulsive or addictive eating behavior and food choices regulating body weight, temperature and other bodily and mental functions. It is well known that repeated exposures to unavoidable painful stimuli have longer lasting effects on appetite, eating behavior

and other habits and physiological responses implicated in pathogenesis and progression of obsessive-compulsive or addictive eating behavior.^[38-40] Such behavioral changes lead eventually to diverse malnutrition associated metabolic disorders and diverse spectrums of physical and mental health problems.^[42-45] *Withania somnifera* is also often used by practitioners of Ayurveda for combating addiction, obsession, and diverse so-called “lifestyle disorders”^[46] and numerous preclinical and clinical studies conducted with its different types of extract have continued to justify such traditionally known medicinal uses of different parts of the plant.^[28,30,31,47-50] However, the therapy relevant questions concerning their doses and treatment regimen necessary for prevention and cure of such disorders still remain unanswered or speculative only.

Results of the earlier dose-finding experiments and other studies with *Withania somnifera* extracts (characterized or standardized on their withanolide contents) have revealed

that like numerous adaptogenic herbal extracts, their activity profiles also depend not only on their daily doses but also on the numbers of days of treatment and that their daily dose necessary for observing their anxiolytic or antidepressant-like activities in marble-burying test and other animal models are somewhat higher than those for protecting rodents against stress-triggered alterations in body weight and core temperature.^[27-29] Analogous were also the observations made with several other phytochemicals encountered in *Withania somnifera* extracts as well.^[23,30] Since only 3.3-mg/kg daily oral doses of WFWS afforded almost complete protection against stress triggered in body weight loss and alteration in thermoregulatory process and compulsive behavior, it seems reasonable to assume that TEG-like bioactive substances other than withanolides are also involved in traditionally known medicinal uses of *Ashwagandha* and diverse types of *Withania somnifera* root extract currently commercialized or prepared for research purpose.

It was interesting to note also that like withanolides containing extracts, statistically significant anxiolytic or antidepressant like effects of WFWS in the marble-burying test or on adrenal gland weight and blood glucose and cortisol level in stressed animals were also observed after its higher two tested doses (33.3 and 100 mg/kg/day) only. These observations indicate further that higher doses of the combination of substances other than withanolides encountered in *Withania somnifera* roots are also effective in regulating biological processes and mechanisms involved in unavoidable stress-triggered alterations in mental health accompanying elevated blood glucose and cortisol levels. These observations, taken together with our earlier ones,^[10-12,51-55] strongly suggest that all biological processes and mechanisms involved in antidepressant and anxiolytic-like activities of WFWS and other *Withania somnifera* extract are not necessarily the same as those involved in its protective effect against stress-triggered alterations in body weight and core temperature.

Although statistically significant effect of TEG in the standard version of marble-burying test was observed, its 10-mg/kg/day oral dose had no effects on the stress-triggered alteration in adrenal gland weight or on circulating blood glucose and cortisol level. These observations could indicate that its therapeutically interesting bioactivity profile is mainly due to its broad spectrum of bactericidal, antiviral and antifungal activities and that it is a fairly potent and specific regulator of the endocrinological function of gut microbiota and gastrointestinal tract involved in digestive and metabolic processes regulating body weight and temperature. Efforts to reconfirm these observations and to define its pharmacological activity profile after its repeated daily higher oral doses will be necessary for judging whether or not it could as well be a substitute or a cheaper therapeutic option of *Withania somnifera* root extract. It can be suggested that TEG is certainly a useful and safe pharmacological tool for better understanding the role of the gut microbiota and gut endocrine functions and in regulating stress-triggered alteration in body weight, temperature and eating behavior.

The very first report on therapeutically interesting bioactivities of TEG in cellular and animal bioassays had revealed that it is a cytostatic agent with cancer growth-inhibiting properties.^[12] Although structurally and functionally diverse cancer chemotherapeutic and antibiotics are effective in suppressing cancerous growth and proliferation, therapeutic responses of currently available treatment against cancer cachexia are often fairly poor or unsatisfactory.^[56,57] Safety margins of TEG and many other microbicidal substances encountered in *Withania somnifera* extracts and several phytochemicals encountered in them are much larger than most anticancer and other drugs currently recommended or prescribed for combating cancer and cancer-associated cachexia. Since many of them are readily available, cheap, pharmaceutically very stable, they could also be considered as the potential therapeutic lead against cancer and associated cachexia and other physical and mental health problems accompanying this and other life-threatening diseases. Appropriate uses of mice models of cancer and other diseases and the bioassay procedure used in this study seem to be a more reliable and time-saving starting point for achieving such goals.

Observations made during such effort will certainly be useful not only for more rational medicinal and commercial uses of *Withania somnifera* but also for better understanding of biological and pharmacological principles behind traditionally known medicinal uses of *Ashwagandha* and other *Rasayana* (rejuvenating) drugs. Although many questions concerning their bioactive constituents and modes of action still remain open, it can now be said that substances other than withanolides with TEG-like microbicidal and other bioactivities extractable from the roots of *Withania somnifera* are also involved in their therapeutically interesting activity profiles. These observations, taken together with current knowledge on the role of the composition and physiological functions of gut microbiota in dictating healthy life span of individuals and their ability to resist against diseases and environmental challenges,^[58,59] strongly suggest that the bioassay procedure used in this study is particularly well suited for pharmacological standardization of Ayurvedic *Rasayana* drugs necessary for obtaining more reliable and reproducible health benefits from them even in the 21st century.

Among all plants used in Ayurveda and other traditionally known systems of medicine and health care, *Withania somnifera* is a unique stress resistance increasing or adaptogenic plant with sedative and sleep-regulating activities,^[60] and it is now well recognized that it is a rich source of naturally occurring bactericidal agent and their combinations.^[61] Therefore, it seems to be a particularly well suited not only for better understanding of biological and pharmacological aspects behind *Rasayana* therapies but also for better understanding of the role of gut microbiota in systems pharmacology of many plants and plant-derived drugs, paradoxical effects of many of which in regulating brain functions have often been reported.^[62] Therefore, an effort to better define Ayurvedic system pharmacology of *Ashwagandha* using the more holistic

experimental strategy used in this and our earlier studies^[23] can be warranted.

Conclusion

TEG is a useful pharmacological tool for Ayurvedic translation research as well as for better understanding of system pharmacology of *Ashwagandha* and other *Rasayana* drugs. However, the question whether this bactericidal molecule is a bioactive constituent of *Ashwagandha* or not still remains open. *Ashwagandha* metabolites other than withanolides contribute to its stress resistance increasing effects. The observations suggest that modulation of physiological functions of gut microbiota are involved in the mode of action of *Withania somnifera* root extracts.

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Conflicts of interest

There are no conflicts of interest.

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