TANG [HUMANITAS MEDICINE]

Original Article

A bioassay system for pharmacological standardization of *Withania somnifera* derived herbal remedies

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ABSTRACT

Background: Contents of bioactive substances extractable from different parts of terrestrial plants vary enormously. Aim: To ascertain that parts of *Withania somnifera* other than its roots can also be used for prevention and cure of unavoidable stress triggered central hypersensitivity to pain. Material and Methods: Groups of male or female mice treated either with *Withania somnifera* extracts or with metformin, aspirin, imipramine, diazepam and niacin for 11 consecutive days were subjected to "foot-shock stress-induced hyperthermia" and "hot plate" tests on the 1st, 5th, 7th, and 10th days of the experiments. On the 11th day, they were subjected to tail suspension test and on 12th day pentobarbital hypnosis test. Results: Except for diazepam and imipramine, protective effects of all other tested drugs as well as of the *Withania somnifera* extracts against stress-induced central hypersensitivity to pain were accompanied by their preventive effects against foot-shock stress-induced body weight losses. All observed stress response suppressing effects of all test agents increased with increasing numbers of treatment days. However, mean duration of pentobarbital-induced sleep was shorter in the extracts treated groups and longer in the diazepam treated ones only. Conclusions: Reported observations reveal that pharmacological activity profile of *Withania somnifera* extracts in male and female mice are almost identical, and are not like those of several drugs currently often prescribed for the treatment of diabetes-associated comorbidities. Withanolides are not the only extractable bioactive constituents of *Withania somnifera*. The described bioassay system is well suited for pharmacological standardization of diverse types of *Withania somnifera* extracts.

Keywords *Withania somnifera*, Stress, Hyperalgesia, Depression, Bioassay.

INTRODUCTION

Roots of *Withania somnifera* ("Ashwagandha" in Sanskrit) are often used as rejuvenators or as tonics in Ayurvedic system of medicine and health care widely practiced in India and other Asiatic countries (Balasubramani et al., 2011; Murthy et al., 2008; Singh et al., 2011). Numerous preclinical and clinical reports published during the past few decades have continued to reveal and reaffirm stress response modulating and other therapeutically interesting bioactivity profiles of diverse types extracts obtained from its roots and other parts of the plant as well (Rayees and Malik, 2017; Pratibha et al., 2013). Due to high commercial demand, *Withania somnifera* is now often cultivated and diverse types of extracts obtained from different cultivars and parts of the plant are now often used for commercializing Ayurvedic and other traditionally known herbal remedies. Since several plants mentioned in classical Ayurvedic texts have now become rare or extinct, Ashwagandha is now also often used as a substitute for some of them (Virk et al., 2015). However, appropriateness of such substitutions has been questioned, and necessity of better standardization methods for Ayurvedic and other herbal formulations has often been pointed out during more recent years (Mathur and Velpandian, 2009; Virk et al., 2017; Ameh et al., 2014; Andrew and Izzo, 2017).

Modern medical practitioners and researchers consider *Withania somnifera* to be a pharmacologically unique adaptogenic plant with stimulating effects on mental functions (Winston and Maimes, 2007; Dar et al., 2015; Kumar et al., 2015a). Formulations containing the roots of the plant (Ashwagandha) as an active ingredient are now often prescribed also for prevention and cure of diabetes and other metabolic disorders associated mental health problems, pain, sleep disorders, hypertension and other cardiovascular diseases. Such medicinal uses of *Withania somnifera* extracts is well supported by numerous preclinical and several clinical reports. Experimental evidence now available on medicinal values of the plant strongly suggest that appropriately standardized extracts of the plant alone can also be used as herbal alternatives for combinations of metformin and other drugs potentially useful for prevention and cure of diabetes associated co-morbidities (Kumar et al., 2017). Results of a recently reported dose range finding study conducted with a currently commercialized *Withania somnifera* root extract has revealed that its 400 mg/kg daily oral doses for more than a week are well tolerated, and that its 50 mg/kg/day oral dose is high enough for protecting diabetic rats against stress triggered gastric ulcers, alterations in glucose and insulin homeostasis, and other organ pathologies (Thakur et al., 2015).

Analogous observations made in our research groups with several food plants often recommended by Ayurvedic medical

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practitioners for prevention of obesity and metabolic disorders associated comorbidities (Kumar et al., 2015b) strongly suggest that diverse combinations of food phytochemicals encountered in *Withania somnifera* extracts could also contribute to their adaptogenic and other therapeutically interesting bioactivities. That such is indeed the case is well support and reaffirm by numerous observations made in our laboratories and elsewhere (Chatterjee and Kumar, 2017). A few such phytochemicals more extensively studied in our research groups and reported to be present in *Withania somnifera* extracts (Chatterjee et al., 2010; Bhatia et al., 2013) are structurally diverse organic acids (fumaric, salicylic, 4-hydroxybenzoic, lactic and ascorbic acids), quercetin, and phloroglucinol, i.e. a metabolically unstable transient mammalian metabolite of quercetin, and other food flavonoids (Rauniyar et al., 2015). Fairly low daily oral doses (between 1 to 5 mg/kg/day) of many of them afford almost complete protection against stress triggered body weight losses and hyperthermia, and their somewhat higher daily oral doses (10 to 20 mg/kg) also possess antidepressants and centrally acting analgesics like activities (Chatterjee and Kumar, 2017). Analogous stress response suppressing and antidepressant and analgesics like activities of a *Withania somnifera* root extract was also observed after its daily oral doses between 10 and 40 mg/kg/day (Dey et al., 2016a; Dey et al., 2016b).

Triethylene glycol is a relatively new addition to the long list of bioactive substances encountered in *Withania somnifera* (Wadhwa et al., 2013; Kaushik et al., 2017). Since like quercetin, fumaric acid and many other food phytochemicals triethylene glycol also possess bactericidal and antiviral activities, its adaptogenic or stress response protective potentials was also quantified in one of the bioassay system standardized and often used in our laboratories for identifying bioactive constituents of adaptogenic plants (Shrivastava et al., 2015). Although fumarates or quercetin like anti-stress activity of low daily oral doses (< 5 mg/kg/day) of triethylene glycol could be reaffirmed in the bioassay system, as yet we have not been able to detect fumaric acid or triethylene glycol in *Withania somnifera* root extract used in our studies. Analogous observations made with other adaptogenic plant extracts and some of its known or proposed bioactive constituents reaffirm that therapeutically interesting bioactivity profiles of herbal extracts are due to biological interactions between diverse bioactive substances present in them. They indicate also that quantification of chemotaxonomic markers of plant material used, or chromatographic fingerprints of their extracts, cannot guarantee pharmacological equivalence of herbal extracts obtained from different cultivars and parts of the same plants.

Like for all plant extracts, the contents of extractable bioactive constituents in diverse types of extracts obtained from different parts and cultivars of *Withania somnifera* vary enormously (Chatterjee et al., 2010; Kumar et al., 2007; Sangwan et al., 2004). Since chemotaxonomic markers of the plant structurally defined as withanolides are encountered in all parts and cultivars of *Withania somnifera*, for commercial or experimental purposes extracts of the plant are now often standardized on their contents in withanolides and chromatographic fingerprints only. However, such practices often neglect that activity profiles not all withanolides are identical and that synergistic and antagonistic activities between them (Rai et al., 2016) or with other substances present in the extracts, can influence their bioactivity profiles. It is now well recognized that *Withania somnifera* extracts devoid of withanolides also possess stress response-modulating or adaptogenic activities (Singh et al., 2001; Singh et al., 2003; Wadhwa et al., 2013), and that structurally and functionally diverse plant phenolics with anti-oxidative, bactericidal, and other therapeutically interesting bioactivities activities also contribute to therapeutically interesting bio-activity profiles and traditionally known medicinal uses of the plant (Alam et al., 2011; Pal et al., 2015).

Therefore, the experiments described in this report were conducted to verify whether the bioassay strategy often used in our laboratories for estimating therapeutically interesting dose ranges and dosing regimen of test agents could also be used for pharmacological standardization of diverse types of extracts of the plant. In these experiments, the activity profiles of the extracts of stems and areal parts of *Withania somnifera* were compared with that obtained from its roots and with some other drugs often prescribed for treatments of diabetes associated comorbidities. Since activity profiles of test agents can vary considerably in males and females (Hankin et al., 2001; Olff et al., 2007), two sets of experiments using either male or female mice were conducted as well. Results of these experiments reaffirming that the experimental strategy and bioassay procedure used in this study is well suited for pharmacological standardization of *Withania somnifera* extracts will be described and discussed in this report

MATERIALS AND METHODS

Animals

Male and female albino mice of Wistar strain (25±5g) 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, and were maintained at an ambient temperature (25±1°C) and relative humidity (50±10%). All animals used in this study were acclimatized to laboratory conditions for at least one week prior to the start of the experiments. Except during the observation periods, they always had free access to standard rodent diet and water *ad libitum*. Wastes in the cages were removed daily to ensure hygienic condition and maximum comfort for the animals. Prior to the commencement of experiments, ethical clearance was obtained from the Central Animal Ethical Committee of our University (BHU; Dean/2014/CAECI/604, dated 30/05/2014). All experimental groups of a given experiment were always tested in parallel (i.e. on the same day of an experiment) and were handled, weighed, and observed by blinded observers and under the same laboratory conditions.

Plant extracts

Together with their analytical certificates and HPLC fingerprints, the analytically characterised hydro-alcoholic extracts of the *W. somnifera* roots (WSR), stems (WSS) and aerial parts (WSA) used in this study were generously supplied by Natural Remedies Private Limited, Bengaluru, India. As quantified by HPLC, total contents of withanolides (chemotaxonomic markers of *Withania somnifera*) in WSR, WSA and WSS were 2.7 %, 3.0%, and 1.5 % (w/w) respectively (Figure 1). The extraction procedure used for obtaining the tested extracts was the same as that used for obtaining *Withania somnifera* root extracts commercialised by the company and reported elsewhere (Orrù et al., 2016). The yields of the WSR,

WSA and WSS obtained from the starting materials were 10, 12, and 12% (w/w) respectively.

Drugs and reagents

Diazepam (Calmpose Tablets, Sun Pharmaceutical Industries Ltd., Mumbai), imipramine (Sun Pharmaceutical Industries Ltd., Mumbai), carboxymethyl cellulose [Central Drug House (P), New Delhi], nicotinic acid (SD Fine-Chemical Ltd., India) and metformin hydrochloride (Sigma-Aldrich, USA). All other chemicals and reagents used were from other laboratory suppliers and of highest analytical quality available in India.

Animal grouping and drug treatments

Groups of male or female mice used in the two sets of experiments were preselected by their similar reaction times (< 15 s) in the hot plate test. They were randomly allotted to the nine groups of six animals each in the two experiments conducted. The experiments were started two days after the last pre-selection day.

For oral administrations, the extracts and the standard drugs were suspended in 0.3% CMC and the volume of administration was always 10 ml/animal/day. The control group of an experiment was treated orally with the vehicle (0.3% CMC, 10 ml/kg/day) for 11 consecutive days. The three extract treated groups of an experiment were similarly treated with 50 mg/kg oral doses of either WSR, or WSA, or WSS. The five others were also similarly treated either with the anxiolytic drug diazepam (5 mg/kg), or with the antidepressant imipramine (15 mg/kg), or with the antidiabetic drug metformin (50 mg/kg), or with nicotinic acid (1 mg/kg), or with the anti-inflammatory drug aspirin (100 mg/kg). Animals of all experimental groups were subjected to foot-shock stress-induced hyperthermia test on the 1st, 5th, 7th and 10th days of the experiment. During the course of the experiment, they were closely observed for apparent behavioural abnormalities. On all observational days, the body weights and rectal temperatures of the animals were recorded one hour before the oral administrations, and all tests on all observational days were conducted one hour after the day's oral treatments. Further details of the experimental procedure used are graphically summarised in Figure 2.

Foot-shock stress-induced hyperthermia test

The test procedure now often used in our laboratories for stress response suppressing studies with herbal extracts and their bioactive constituents have often been described in details (Thakur et al., 2015; Dey et al., 2016b; Khan et al., 2016). In short, an individual mouse of a test group was place in a black box (24 x 29 x 40 cm) with a steel grid floor for 1 min. During this period, electric foot shock through the grid floor (2 mA, 50 Hz of 2 ms duration) was deliver for stress induction. After 10 s of their stay in the box, five consecutive foot shocks of 2 mA at 10 s intervals were delivered through the grid floor. Immediately thereafter, the animals were place back in their home cages, and 10 min thereafter their rectal temperatures were record again. Numerical differences between the basal rectal temperature of a given animal on the test day and 10 min after the foot shock session was calculated and used as an index for the stress-triggered transient hyperthermic response. All core temperatures were record by using a calibrated rectal probe coupled with a digital thermometer.

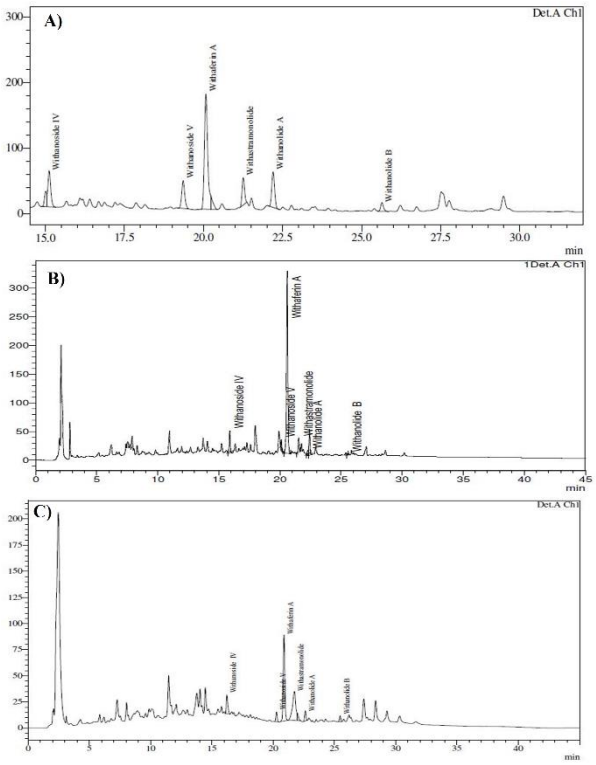

Fig.1 HPLC fingerprints of **A)** WSR (total content of Withanolides = 2.7%), **B)** WSA (Total content of Withanolides = 1.5%), and **C)** WSS (total contents of Withanolides = 3.0%).

Hot plate reaction time in mice

Immediately after the second temperature measurement in foot-shock stress-induced hyperthermia test on 1st, 5th, 7th and 10th days of the experiment, an individual mouse of a test group was place on the hot plate of an analgesiometer maintained at 55±1°C. For preventing any thermal injury, the maximum time a mouse was allow to stay on the hot plate was 30 seconds (Khan et al., 2016). The latency until a mouse showed the first sign of discomfort (paw licking, hind paw lifting or jumping) on the hot plate was record as its reaction time (Turner, 1965).

Tail suspension test

This test for assessing the depressive state of animals was conduct on the 11th day of an experiment. The test procedure used has also been described elsewhere (Thakur et al., 2015;. Can et al., 2012) In short, an individual mouse of a group was hung by its tail in its head down posture by an adhesive tape placed approximately 1 cm from the tip of the tail on a horizontal wire placed 50 cm above the table floor. After initial vigorous movements, the mouse assumes an immobile posture and the total period of immobility during 5 min observation period were record.

Pentobarbital sleep test

This test was conduct on the 12th day of an experiment on all

animals of all test groups for estimating residual the effects of foots-hock stress and treatments on pentobarbital-induced sedation and sleep. On this day, no treatments were given, and core temperatures and body weights of animals were recorded immediately before the pentobarbital challenge (40 mg/kg; i.p.) for sleep induction. Time taken for sleep onset (loss of righting reflex) and duration of pentobarbital-induced sleep were recorded (Ojima et al., 1995).

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test and one-way ANOVA followed by Student-Newman-Keuls test. GraphPad Prism-5 (GraphPad Software Inc., California, USA) software was used for statistical analysis and Origin Pro 8 software (Origin Lab Corporation, Massachusetts, USA) was used for making graphs. A p value less than 0.05 was considered as statistically significant.

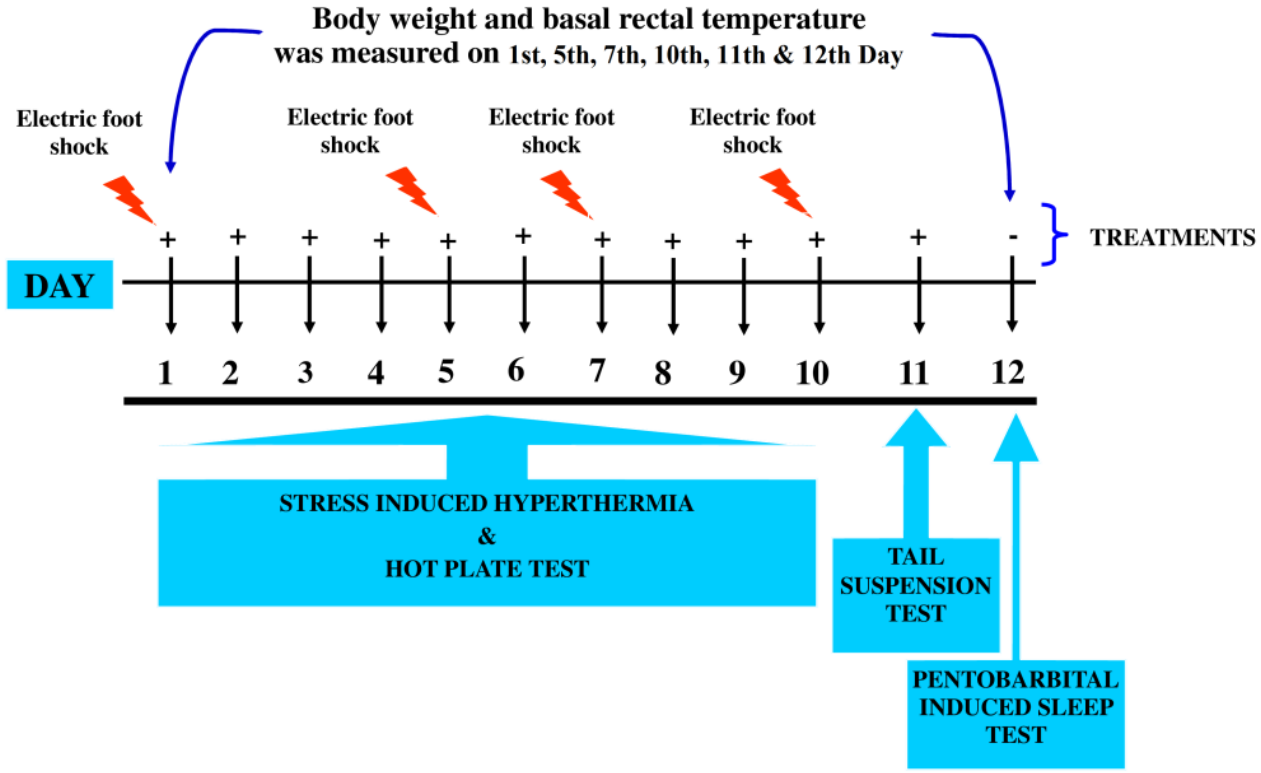

Fig.2 Summary of the experimental procedures and tests used.

RESULTS

Body weight

Mean body weights of different groups of animals recorded during the experiments are summarized in Figure 3. As expected from our earlier observations, the vehicle-treated male or female control groups steadily lost their body weights during the course of the experiments, whereas all *Withania somnifera* extract treated ones gained body weights from 7th treatment day onwards. Observed effectiveness of the tested daily oral doses (50 mg/kg/day) of all three extracts in affording such protections was almost equal in magnitude (Figures 3A and 3C). In both male and female mice, this stress response suppressing effect of daily oral treatments with the tested doses of metformin, aspirin, and nicotinic acid (i.e. niacin or Vitamin B3) were quite similar to those of the extracts tested (Figures 3B and 3D). However, neither diazepam (5 mg/kg/day) nor imipramine (15 mg/kg/day) had any such protective effects against body weight losses triggered by occasional exposures to only 50 seconds durations of unpredictable foot-shock stress followed by hot plate tests. These observations indicate that the effects of the tested extracts and other three reference drugs are most probably not due to their anxiolytic or antidepressant like effects observed in tail suspension test.

Basal core temperature

These mean values of the preselected male or female control groups on the first observational day were almost identical and within the average values of the animal colony used in our laboratories. Except for the male WSR treated group, these values of all other test groups on this day were also not statistically significantly different than those of the corresponding control ones (Figure 4). Mean basal core temperatures of the male and female control groups steadily increased, but still remained within physiological ranges during the course of the experiments. Such were not the observations made in the reference drugs or *Withania somnifera* extract treated groups. The mean values of all such

groups decreased steadily with increasing numbers of treatment and observational days. These observations revealed that an acute oral dose of WSR tested (50 mg/kg/day) increases core temperature of male mice with prior experiences in hot plate test (i.e. occasionally exposed to very short duration of thermal stimuli or stress). However, this acute dose hyperthermic effect of the extract disappeared after its repeated daily doses. Like the two other tested extracts and all reference drugs, repeated daily treatments with WSR also completely suppressed stress triggered and longer lasting elevations in basal core temperatures in both male and female mice.

Stress-induced hyperthermic responses

Results of the foot-shock stress-induced hyperthermia test are summarized in Figure 5. After their single oral doses, all three extracts significantly suppressed the stress triggered transient hyperthermic responses in both male and female mice, and such effectiveness of all three tested extracts was almost identical (Figure 5C). Although the mean value of the WSA treated males on this day was somewhat higher than those for WSS and WSR treated ones (Figure 5A and 5C), there were no statistically significant differences between the mean values of these three groups. Such anti-stress effectiveness of all three extracts continued to increase with increasing numbers of treatment days in both males and females, whereas the mean values of the control groups remain almost constant on all observational days. Except for metformin, statistically signifycant effects of all other tested drugs in the test were also observed after their single tested doses in both male and female mice (preselected for their responses in hot plate test after repeated testing). Their effectiveness in the test also continued to increase with increasing numbers of treatment days in both males and females (Figures 5B and 5D). On the first observational day, the mean values of the metformin treated male or female groups were statistically not significantly different from the corresponding control groups. However, its statistically significant effects in the test increased also after its five or more daily doses. The mean values of all test agents on the 10th observational day were statistically not significantly different from each other.

Hot plate test

Results of this test for centrally acting analgesics are graphically summarized in Figure 6. Mean reaction times of both male and female control groups decreased somewhat in the subsequent observational days, whereas those of all drug or extract treated ones continued to increase with increasing numbers of treatment days. However, on the first observational day, no statistically significant effects of aspirin (a peripherally acting analgesic drug with anti-inflammatory activities) or of any other tested drugs and extracts were observed in the test. Statistically significant effects of all three tested extracts were observed in both male and female mice after 5 or more treatm

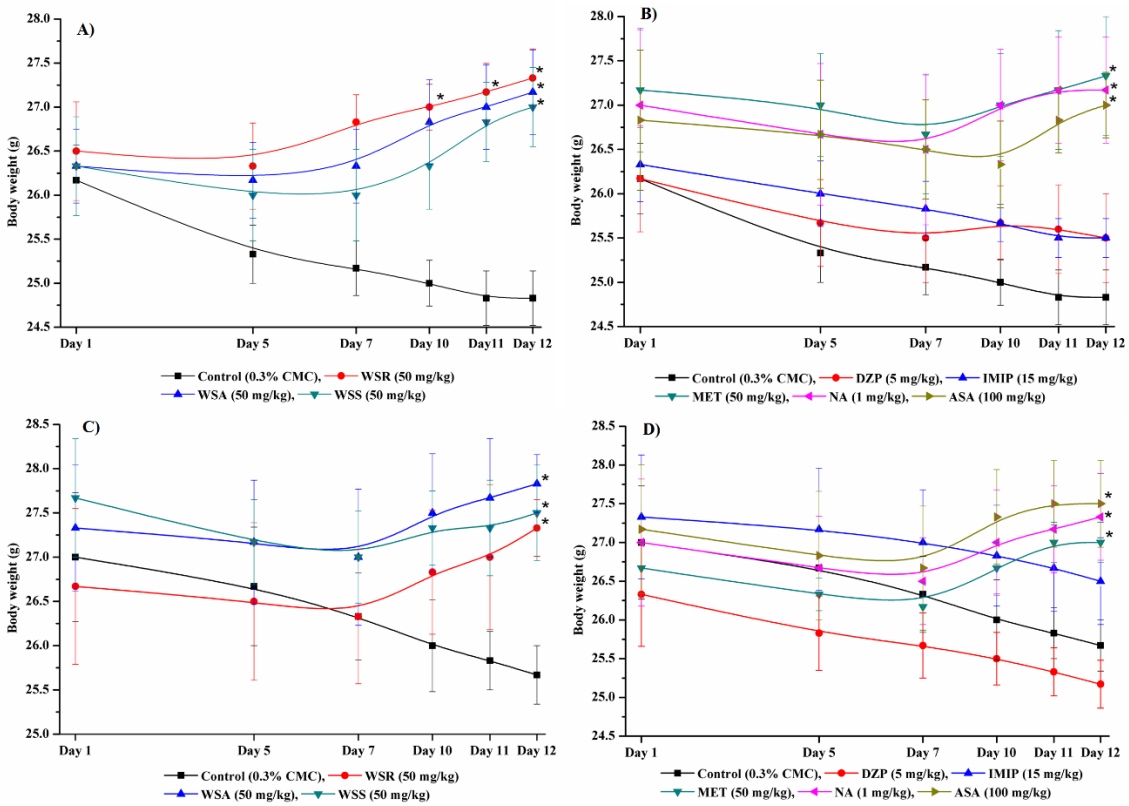

Fig.3 Effect of occasional foot shock stress (duration 50 seconds) on mean body weight of male (**A** and **B)** and female mice (**C** and **D)** treated either with the extracts of roots (WSR), stems (WSS) or areal parts (WSA) of *Withania somnifera*, or with diazepam (DZP) imipramine (IMIP), metformin (MET), nicotinic acid (NA), or aspirin (ASA) during the course of the experiments. All values are mean ± SEM (n=6). *=p<0.05 vs. the corresponding vehicle (0.3% CMC) treaded control groups (Two-way ANOVA followed by Bonferroni post hoc test).

-ent days, and their observed effectiveness in the test were quantitatively similar to those of aspirin, diazepam, and niacin. Statistical significance of the differences between the mean reaction time values of metformin treated male group and the corresponding control one was observed on the 7th and 10th treatment days, whereas such effects of the antidiabetic drug in females were observed after its ten daily doses only.

Tail suspension test

This test was conducted on the 11th day of the experiments for comparing the effects of repeated daily oral treatments on the depressive state of male and female mice stressed by occasional exposures to foot-shock stress and other experimental procedures used in the experiments. Results summarised in Figure 7 revealed that all three tested extracts were almost equally active in both male and female mice, whereupon they were somewhat more effective in males than in females. Both imipramine and diazepam were also almost equally effective in both males and females, whereupon the observed effect of diazepam in females was somewhat less pronounced than that of imipramine. Slight, but statistically significant antidepressants or anxiolytics like effects of aspirin treatments were also observed in both males and females, whereas those of metformin and niacin were observed in males only.

Pentobarbital sleep test

This test was conducted on the 12th days of the experiments for comparing the residual effects of treatments on sedative state, or on pentobarbital metabolizing enzymes, in animals stressed by the experimental procedures used in this study. Results of the tests summarized in Figure 8 revealed that the man time taken for sleep onset or duration of sleep induced by pentobarbital in male or female mice groups treated with aspirin were statistically not significantly different from those of the corresponding control ones, and there were no such differences between these values of males and female control groups. Mean sleep onset time of all other drugs or extract treated male or female groups were statistically significantly lower than the corresponding control ones. Mean duration of sleep of all three extract treated male or female groups were significantly shorter than the corresponding control ones, whereas those of diazepam treated ones were much longer. It is apparent from these data, that imipramine, metformin, niacin, and aspirin had no significant effects on pentobarbital-induced sleep on males or females, whereas effects of all tested extracts on this parameter were opposite to those of the anxiolytic drug diazepam in both males and females.

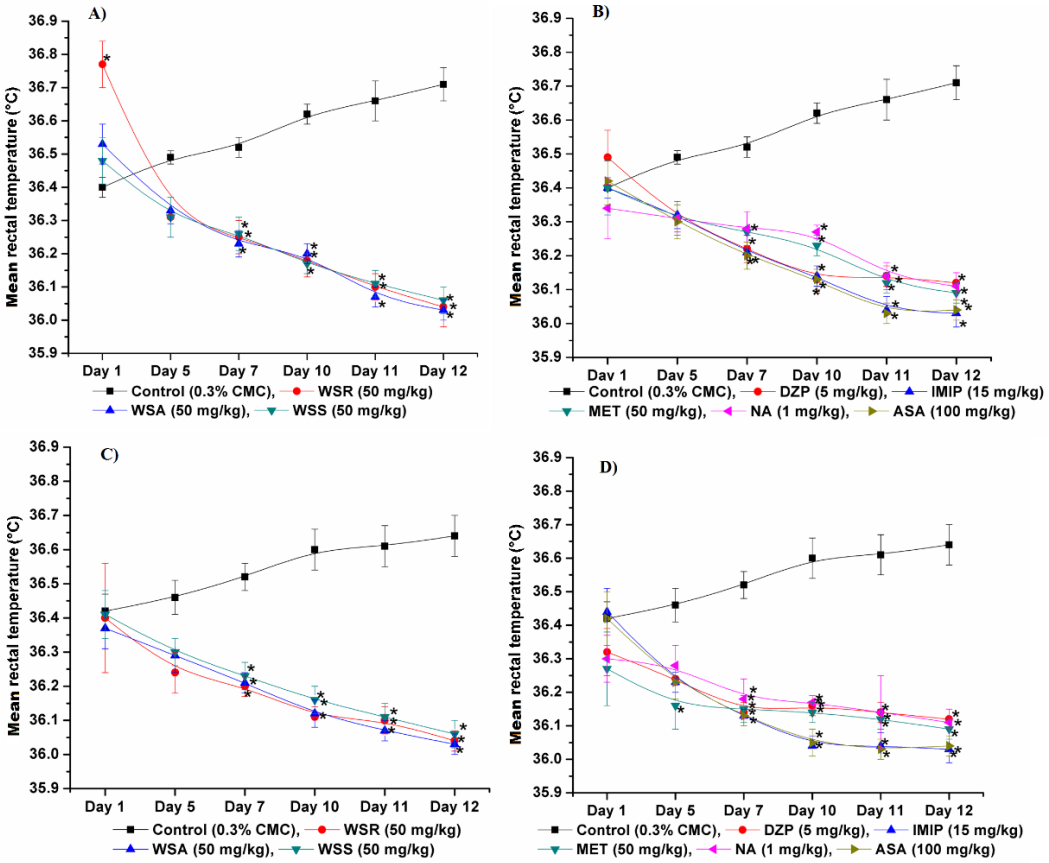

Fig.4 Effect of occasional foot shock stress (duration 50 seconds) on mean basal core temperatures of male (**A** and **B)** and female mice (**C** and **D)** treated either with the extracts of roots (WSR), stems (WSS) or areal parts (WSA) of *Withania somnifera*, or with diazepam (DZP) imipramine (IMIP), metformin (MET), nicotinic acid (NA), or aspirin (ASA) during the course of the experiments. All values are mean ± SEM (n=6). *=p<0.05 vs. the corresponding vehicle (0.3% CMC) treaded control groups (Two-way ANOVA followed by Bonferroni post hoc test).

DISCUSSION

Reported results reveal that the observed activity profile of WSR as a stress response modifier with analgesic and antidepressants or anxiolytic like activities is qualitatively quite similar to those of WSS and WSA in both male and female mice. The only qualitative difference between the effects of WSR and those of the other two tested extracts observed in this study was that the mean basal core temperature of the male group treated only once with the extract was higher than those of the WSS or WSA or the vehicle treated male groups. Otherwise, the activity profiles observed after 50 mg/kg daily oral doses of all three tested extracts were almost their maximally effective ones as stress response modulators or adaptogens. However, the question concerning the pharmacological equivalence of lower daily oral doses of the tested extracts still remains open. It can safely be said though, that the bioassay system used in this study is well suited for pharmacological as well as analytical standardization of diverse types of extracts obtained from different parts and cultivars of *Withania somnifera* extracts with stress resistance increasing and brain function regulating activities.

It was interesting to note also that unlike the tested extracts and the plant derived drugs aspirin, metformin, and the vitamin nicotinic acid, the two psychoactive drugs imipramine and diazepam were ineffective in affording protection against body weight losses triggered by very short durations (less than one minute) of exposures to unpredictable aversive stimuli. Amongst many other phytochemicals and plant derived vitamins tested to date in our laboratories, nicotinic and fumaric acids and phloroglucinol are the more potent ones in affording protection against stress triggered body weight losses (Chatterjee and Kumar, 2017). Although as yet no reports revealing the presence of nicotinic acid in a *Withania somnifera* extract have appeared, the presence of its N-methyl derivative trigonelline in *Withania somnifera* (Chatterjee et al., 2010; Bharti et al., 2011) and in many other medicinally used food plants (Kumar et al., 2015b) has been reported. Apart from trigonelline, salicylic acid (a mammalian metabolite of aspirin) and many other organic acids, alkaloids, plant phenolics, and their metabolic precursors with anti- hypergly

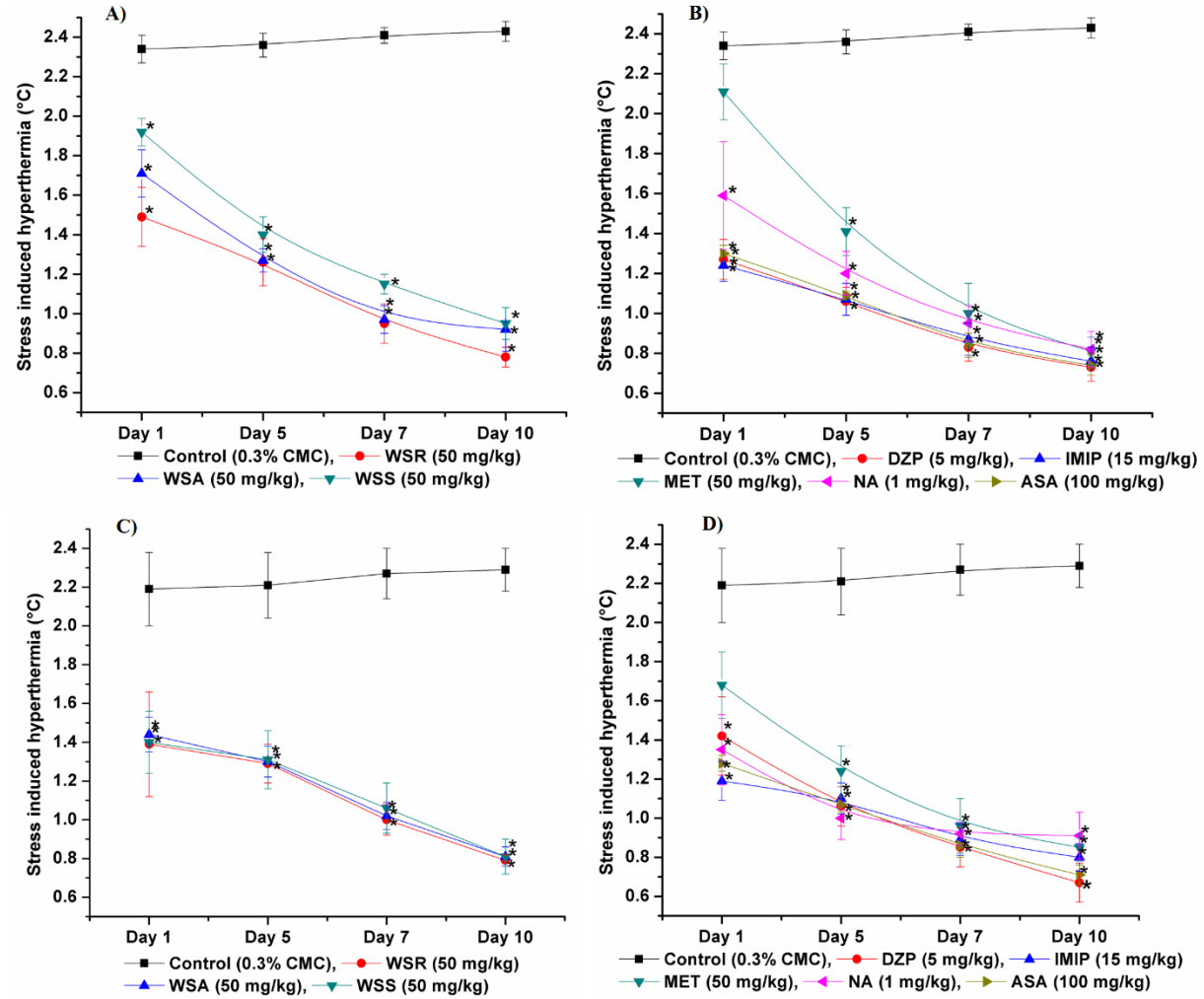

Fig. 5 Effect of occasional foot shock stress (duration 50 seconds) on stress induced hyperthermia of male (**A** and **B)** and female mice (**C** and **D)** treated either with the extracts of roots (WSR), stems (WSS) or areal parts (WSA) of *Withania somnifera*, or with diazepam (DZP) imipramine (IMIP), metformin (MET), nicotinic acid (NA), or aspirin (ASA) during the course of the experiments. All values are mean ± SEM (n=6). *=p<0.05 vs. the corresponding vehicle (0.3% CMC) treaded control groups (Two-way ANOVA followed by Bonferroni post hoc test)

-cemic and other therapeutically interesting bioactivities are encountered in *Withania somnifera*. As mentioned in the introduction, the relative contents of such metabolites, includ -ing those of individual withanolides (i.e. the chemotaxonomic markers of the plant), vary considerably in different parts and cultivars of the plant. Since not all extractable bioactive constituents of *Withania somnifera* still remain unknown, or as yet undefined, and their numbers are very large, analytical procedure currently often used and recommended for standardizing *Withania somnifera* extracts cannot guarantee their pharmacological or pharmaceutical equivalence or similarity. Therefore, the bioassay system used in this study seems to be a more realistic, reliable, and reproducible one for pharmacological quality control of such extracts, or of any other herbal extracts with WSR like stress response modulating activities

The reported observations encourage us suggest that areal parts of *Withania somnifera* can also be used as reliable substitutes for Ashwagandha (*Withania somnifera* roots), and reaffirm that analytical standardization of *Withania somnifera* extracts on their chromatographic fingerprints and contents of withanolides can neither guarantee their bioequivalence nor are very useful for predicting their therapeutic potentials. One interesting observation made during this study was that duration of pentobarbital induced sleep was shortened only in the *Withania somnifera* extracts treated groups and that their antidepressants or anxiolytics like activities in tail suspension test and centrally acting analgesic like effects in hot plate test were similar in magnitude as those of the tested doses of diazepam and imipramine. These observations suggest that unlike all other drugs tested, regular intake of *Withania somn -ifera* extracts can be useful for suppression stress triggered central hypersensitivity to pain and altered states of anxiety and depression as well as for detoxifying or antagonize the effects of pentobarbital like xenobiotics. However, more detailed and dose response studies will be necessary not only for ascertaining the therapeutic relevance of these findings, but also the better understanding of the bioactive constituents and

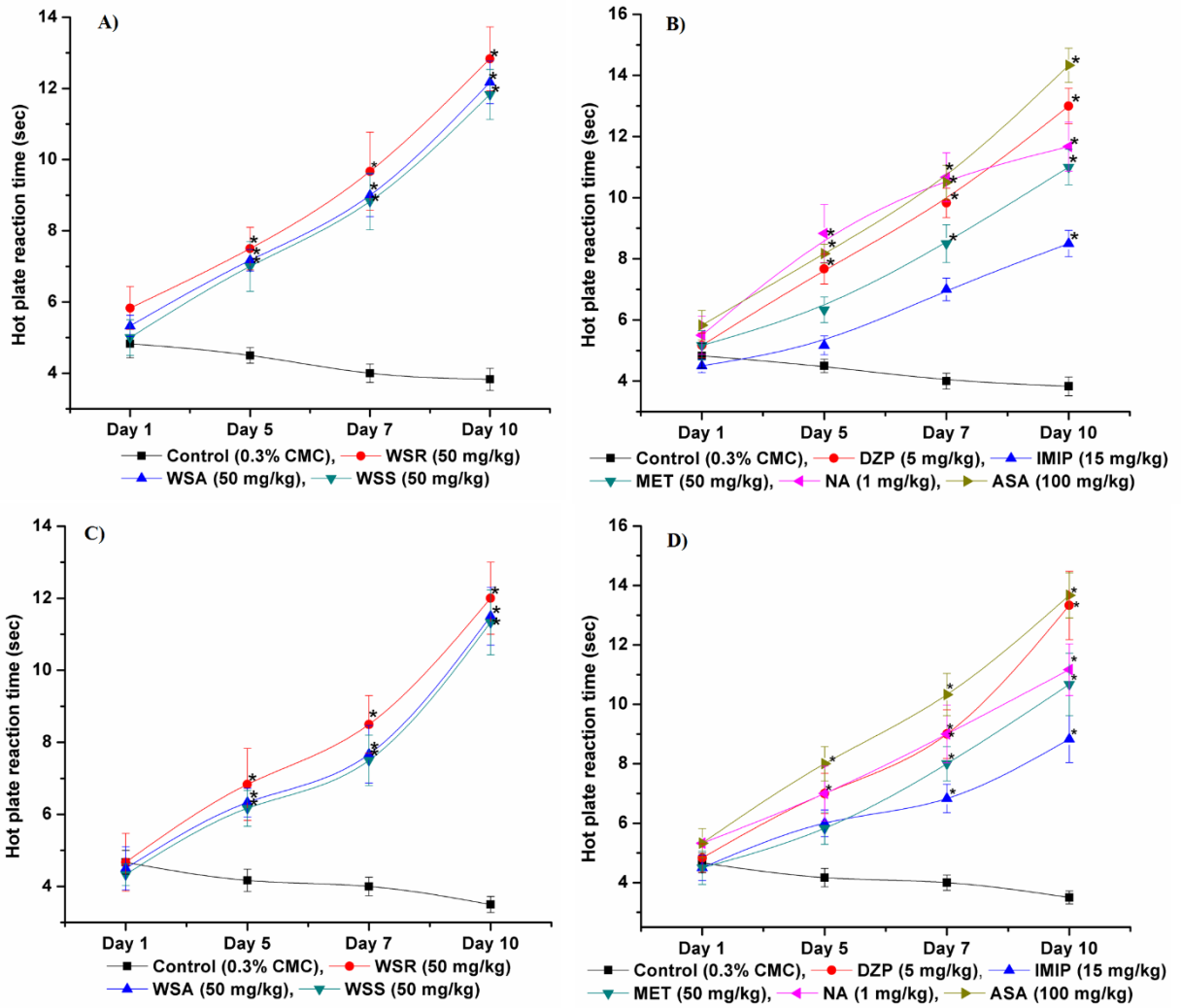

Fig. 6 Effect of occasional foot shock stress (duration 50 seconds) on hot plate reaction time of male (**A** and **B)** and female mice (**C** and **D)** treated either with the extracts of roots (WSR), stems (WSS) or areal parts (WSA) of *Withania somnifera*, or with diazepam (DZP) imipramine (IMIP), metformin (MET), nicotinic acid (NA), or aspirin (ASA) during the course of the experiments. All values are mean ± SEM (n=6). *=p<0.05 vs. the corresponding vehicle (0.3% CMC) treaded control groups (Two-way ANOVA followed by Bonferroni post hoc test).

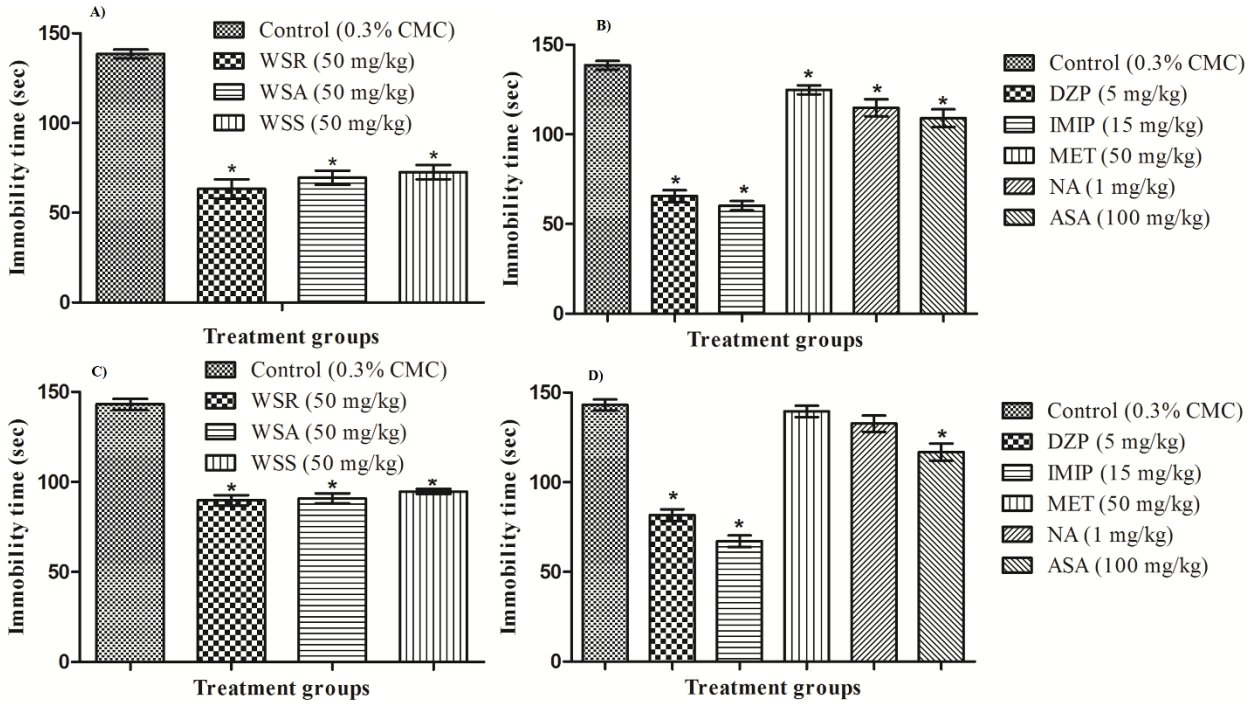

Fig. 7 Effect of occasional foot shock stress (duration 50 seconds) on tail suspension test of male (**A** and **B)** and female mice (**C** and **D)** treated either with the extracts of roots (WSR), stems (WSS) or areal parts (WSA) of *Withania somnifera*, or with diazepam (DZP) imipramine (IMIP), metformin (MET), nicotinic acid (NA), or aspirin (ASA) during the course of the experiments. All values are mean ± SEM (n=6). *=p<0.05 vs. the corresponding vehicle (0.3% CMC) treaded control groups (One-way ANOVA followed by Student-Newman-Keuls multiple comparison test).

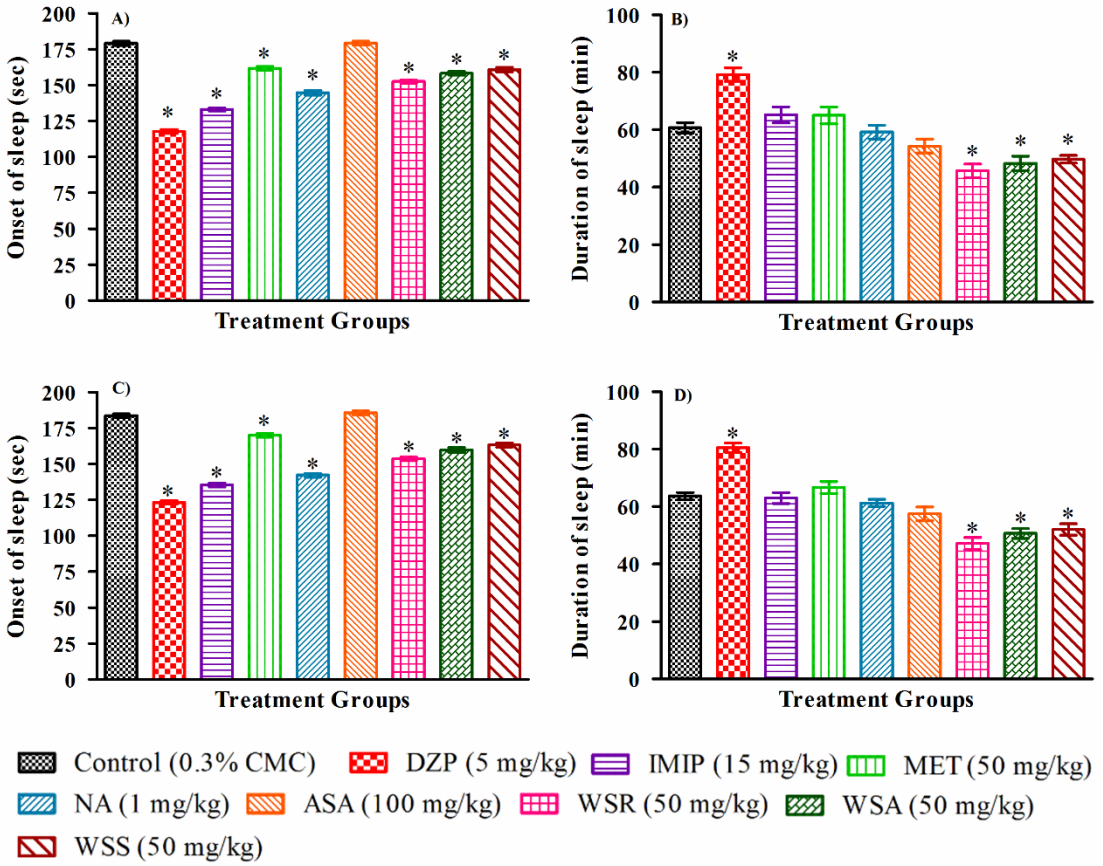

Fig.8 Effect of occasional foot shock stress (duration 50 seconds) on pentobarbital induced sleep test of male (**A** and **B)** and female mice (**C** and **D)** treated either with the extracts of roots (WSR), stems (WSS) or areal parts (WSA) of *Withania somnifera*, or with diazepam (DZP) imipramine (IMIP), metformin (MET), nicotinic acid (NA), or aspirin (ASA) during the course of the experiments. All values are mean ± SEM (n=6). *=p<0.05 vs. the corresponding vehicle (0.3% CMC) treaded control groups (One-way ANOVA followed by Student-Newman-Keuls multiple comparison test).

modes of actions involved in the broad spectrum of therapeuti -cally interesting bioactivities of *Withania somnifera* and other traditionally known medicinal or food plants often used as tonics or rejuvenators in Ayurvedic system of medicine and health care.

Combination of metformin, anti-hyperlipidemic drugs, and low dose aspirin are now often used for prevention of obesity-associated diabetes and other metabolic disorders. Exaggerated states of anxiety and depression and central hypersensitivity of pain are often encountered in patients suffering from, or at risk too, such diseases and illnesses. Although beneficial effects of trigonelline (Zhou et al., 2012), salicylic acid (Klessig, 2017; Rahola, 2012; Berk et al., 2013), and numerous other food phytochemicals encountered in *Withania somnifera* against diabesity associated co-morbidities are well recognized, as yet little-concentrated efforts have been made to identify bioassays necessary for quantifying therapeutic potentials of their combinations present in them. Results of the reported experiments reaffirm that the bioassay system used in the present study is well suited for not only for such purposes, but also for better understanding of systems pharmacology of metformin, aspirin and niacin and other plant derived drugs potentially useful for prevention and cure of malnutrition triggered and lifestyle associated physical and mental health problems.

Amongst all phytochemicals with very low molecular weights tested to date for their low dose effects against stress triggered alterations in body weight and core temperatures, fumaric and nicotinic acids are the two most potent ones encountered in many terrestrial plants. Both of them are also biosynthesized and excreted by gut microbial colonies residing inside the digestive tract. Therefore, it seems reasonable to assume that adaptogenic substances are modulators of their endogenous biosynthesis and functions, and that biological processes and mechanisms involved in regulating stress triggered alterations in body weight changes are involved in their modes of actions. Earlier observations in our laboratories have revealed and reaffirmed that pharmacological targets of several bioactive constituents of *Withania somnifera* and other adaptogenic herbs reside inside the gastrointestinal tract and that their oral bioavailability (as often judge by their blood levels after oral intake) is not an essential prerequisite for their effects on brain and other bodily organs (Chatterjee and Kumar, 2017). Therefore, it is apparent that at present pharmacological equivalence of diverse types of adaptogenic herbal extracts containing numerous such ubiquitous food phytochemicals can be ascertained only by comparing their quantitative systems pharmacology and safety profiles in intact animals after their repeated daily oral doses only.

In classical Ayurvedic formulations, powdered roots of *Withania somnifera*, or diverse types of concoctions and extracts obtained from them are often used as an active ingredient. However, the questions concerning bioequivalence of the root powders or extracts obtained from different cultivars and ages of the plant cannot be ascertained by their appearance or morphological characteristics only. This is because the contents of their bioactive constituents and combinations necessary for obtaining health benefits from them vary considerably in their different samples obtained from different geographical regions, and that our current knowledge (both qualitative and quantitative) on their therapy relevant bioactive constituents and their combinations necessary for obtaining health benefits from them still remain far from being satisfactory. The reported observations that bioactivity profiles of extracts obtained from parts of the plant other than its roots (collected randomly, but processed, extracted, and dried under similar conditions) are almost identical in both males and females, strongly suggest that aerial parts of the plant and its roots are almost bioequivalent for similar therapeutic or health care purposes. Thus it can safely be said pharmacologically well standardized *Withania somnifera* extracts obtained from its more conveniently harvestable areal parts could as well be a more realistic and justifiable substitute for prevention and cure of stress triggered and metabolic disorders associated physical and mental health problems.

It cannot be overemphasized that reliable and reproducible therapeutic benefits from *Withania somnifera*, or from any traditionally known medicinal or food plant, can be obtained only when the appropriate quality of starting material are processed, analyzed, and formulated by standardized methods appropriate for obtaining health benefits from them. Although the classical Ayurvedic texts emphasize these facts, as yet no very feasible strategy and experimental procedure has been standardized for obtaining phyto-pharmaceuticals or nutraceuticals that could be used for prevention and cure of diverse lifestyle associated and malnutrition triggered diseases and comorbidities accompanying them. Comorbidities of depression, anxiety and pain are encountered in numerous such diseases associated with, or caused by, alterations in central sensitivity (Yunus, 2007; Yunus, 2015). Despite the availability of numerous drugs and poly-pills their prevention and cure still continue to be one of the major challenges for all healthcare authorities around the globe. Taken together, the observations reported in this and our earlier reports (Chatterjee and Kumar, 2017) reaffirm that bioassay procedure and animal models using rodents occasionally exposed to aversive and unpredictable foot shocks stress for less than one minute are well suited not only for obtaining pharmacologically well standardized extracts from different parts of *Withania somnifera* but also for obtaining aspirin, metformin, or nicotinic acids like multi-targeted drugs from them.

According to Ayurvedic therapeutic principles and pharmacological concepts, appropriate combinations of Ashwagandha and other medicinal and food plants has to be used together with appropriate choices of food and eating habits for balancing the physiological functions of metabolic processes regulating body composition and psychological state (*manas vikruti*) of patients. Ayurvedic medical practitioners (*Vaidyas*) diagnose patients' psychological state and diverse anthropometric and other parameters for judging their personality *(Prakriti*) necessary for prescribing appropriate doses and treatment regimen of drugs according to their health demands. The bioassay strategy used in this and our earlier studies were standardized by paying due attention to these facts, the predictive validity of which is reaffirmed in this study. Our

earlier observations have revealed and reaffirmed that even very low doses (less than 10 mg/kg/day) of *Withania somnifera* root extracts are effective in affording protection against stress triggered abnormalities in body weight and core temperature as well as the central sensitivity of noxious stimuli. Therefore, at present, they are the most effective and safe therapeutic possibilities for coping with environmental and metabolic stress triggered pathologies and their symptoms, the prevalence of which has continued to increase during more recent decades. Since the activity profiles of the extracts of the stems and aerial parts of the plant are almost identical, it seems reasonable to suggest that pharmacologically well standardized *Withania somnifera* extracts obtainable from different parts of the plant can also be used for prevention of diverse spectrums of central sensitivity syndrome accompanying almost all malnutrition triggered metabolic and inflammatory diseases, including obesity and diabetes. However, further, more detailed studies comparing dose-effect relationship and safety profiles of extracts obtained from aerial parts of the plant will still be necessary for reaffirming this possibility.

The bioassay system used in this study is well suited for the better understanding of systems pharmacology of herbal extracts and plant derive drugs with stress response regulating activities. Areal parts of *Withania somnifera* can also be used as substitutes for its roots for prevention and cure of stress triggered diseases and illnesses. Regulating effects of repeated daily oral doses herbal extracts on nicotinic and fumaric acid homeostasis seems to be a characteristic common for numerous bioactive plant metabolites.

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None

CONFLICT OF INTEREST

None

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