CHAPTER – 4

RESULTS

RESULTS

4.1. Pilot methodological cum dose finding experiments

Recently reported observations made with a commercially available *Withania somnifera* extract (standardized to contain 2 to 3% total withanolides), often used in our laboratories as a reference drug, have reaffirmed that its 100 mg/kg daily oral doses afford almost complete protection against diverse stress responses in laboratory rodents [A. Shakya et al., 2014; A.K. Thakur et al., 2014c]. Therefore, two pilot experiments were designed and conducted to estimate and reaffirm the minimally effective daily oral doses ranges of another *Withania somnifera* root extract (containing 2.7% total withanolides) in stressed mice. Speculating that such extracts could as well be herbal alternatives for prevention and cure of environmental stress triggered central hypersensitivity to pain, the potential anti-nociceptive effects of repeated daily doses of the extract was also tested in the first of these two experiments. The results of that experiment are described first in the following sub-sections.

4.1.1. The first pilot experiment

In this experiment, the effects of a single and ten daily oral doses (10, 20 and 40 mg/kg) of an analytically well standardized *Withania somnifera* root extract against foot shock stress triggered transient hyperthermia and hotplate test for centrally acting analgesics in male mice were quantified. Body weights and basal rectal temperatures of animals were recorded on all observational days and on the 11th and 12th day of the experiment, all animals were subjected to tail suspension and pentobarbital hypnosis tests respectively. Analogous test procedure are often used in our research groups for estimating pharmacologically interesting dose ranges and dosing regimens of herbal extracts and other test agents. It must be noted though, that the animals preselected for their reaction times in hot plate test (i.e. mildly stressed on earlier occasions) were used in this experiment. Such pre-selection was necessary

for more reliably estimating the effects of treatments on foot shock stress triggered central hypersensitivity to pain in mice.

4.1.1.1. Body weights: Mean body weight, and rate of changes in body weights of different experimental groups, recorded during the course the experiment are shown in the **Figures 4.1A & 4.1B respectively**. Animals of the stressed control group steadily lost their body weights, whereas those of the non-stressed one gained weights during the course of the experiment. Such losses of the body weights were not observed in any of the WSR treated and foot shock stress groups. Like the mean body weight values of the non-stressed control group, those of the WSR treated groups continued to increase during the course of the experiment after the 6th treatment day. Numerically, the mean values of the body weight changes in the WSR treated groups were always somewhat higher than those of the control non-stressed group. It was interesting to note also that on the last two observational days, these mean values of the 40mg/kg/day extract treated group were significantly higher than those recorded for the 10 mg/kg/day WSR treated one. These results reveal that 10 mg/kg/day WSR is high enough for affording protections against occasional exposures of laboratory mice to very short durations (< 1min) of foot shock stress and hot plate tests, and could indicate that higher doses of the extract could have growth promoting effects in stressed rodents. Growth promoting and other beneficial of *Withania somnifera* and other plants in broilers and other farm animals have often been reported indeed during more recent years [K. Dhana, 2015]. It has often been suggested and experimentally verified that such effects of regular consumptions of plants is due to structurally and functionally diverse bactericidal substances biosynthesized and stored by terrestrial plants.

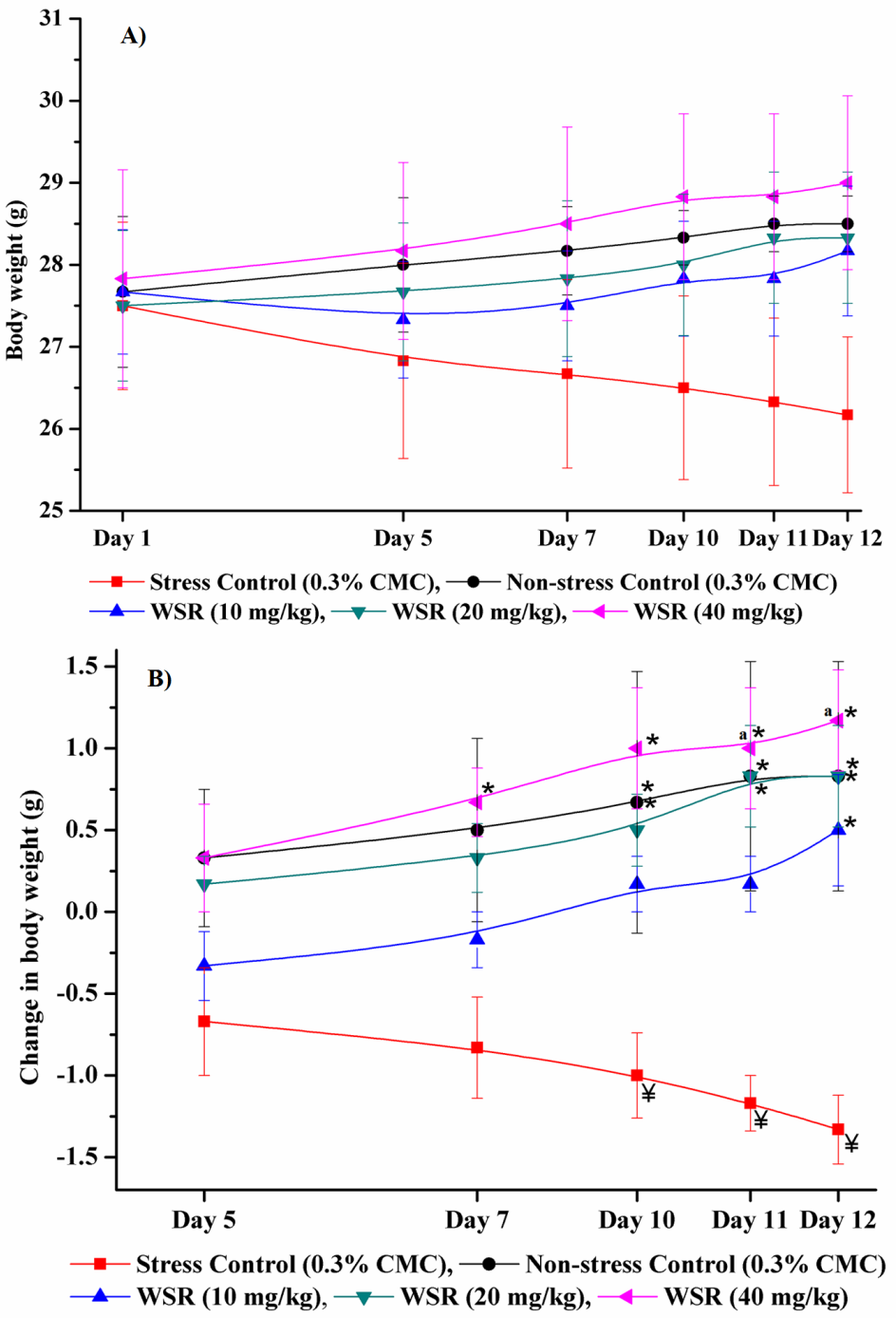

Figure 4.1: Effect of occasional exposures to short durations of foot shock stress on **(A)** mean body weights, and **(B)** gains or losses in body weights of male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR). Values are mean ± SEM (n=6). [a]=p<0.05 vs. WSR (10 mg/kg), *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.1.1.2. Basal core temperatures: No statistically significant differences between the mean basal core temperatures of the vehicle treated stressed or un-stressed control groups and of the WSR treated stressed groups were observed **o**n the first day of the experiment. The results summarised in the **Figure 4.2** revealed that from the 5th experimental day onwards, mean basal core temperatures of the vehicle treated stressed control group were slightly higher, than that recorded for the group on the 1st observational day, whereas those of the non-stresses group steadily decreased only very slightly during the course of the experiment. Mean basal core temperatures of all WSR treated stressed groups on all observational days were quite similar to those of the non-stressed vehicle treated control group. Therefore, it is apparent that 10 mg/kg daily WSR dose is high enough also for protecting the animals against occasional foot shock stress triggered and longer lasting mild elevations in their basal core temperatures, evolving slowly and steadily in occasionally foot shock stressed animals.

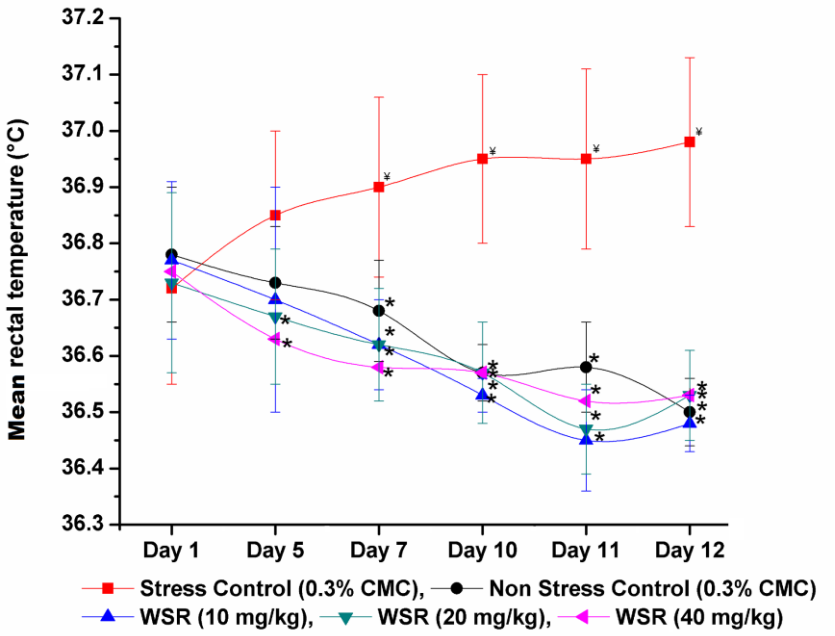

Figure 4.2: Effect of occasional exposures to short durations of foot shock stress on mean rectal temperature of male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.1.1.3. Foot shock stress-triggered transient hyperthermic response: Transient hyperthermic responses triggered by exposures of laboratory rodents to environmental conditions are often quantified for estimating their anxiety states [J.A. Bouwknecht et al., 2007; T.J. Zethof et al., 1995]. In humans, excessive or longer lasting stressors can lead to chronic hyperthermia, which is recognized as a psychosomatic symptom called "psychogenic fever [K. Nakamura, 2015; R.B. Harris, 2015]. Since benzodiazepines and other anxiolytic drugs effectively suppress stress induced hyperthermic responses, diverse versions of the so called "stress induced hyperthermia" test are often used for detecting anxiollytics likes activities of test agents [B. Olivier et al., 2003].

The results summarized in **Figure 4.3** revealed that the core temperatures of the stressed control group recorded 10 minutes after exposures to short duration for unpredictable foot shock stress of fairly short durations (<1 min.) were significantly elevated on all test days, and that such responses of the non-stressed control group were much lower, or almost negligible. On the first test day, such foot shock stress induced hyperthermic responses of all WSR treated groups were of the same order of magnitude as those observed in the stressed control group. However, clear dose dependant antagonistic effects of the extract on stress-triggered hyperthermic responses were observed after repeated daily treatments. This effectiveness of WSR continued to increase also with increasing numbers of treatment days. On the 10th observational day, this response of even the 10 mg/kg daily WSR treated group was significantly lower than recorded for the stressed control group, and statistically significant such effects of the highest WSR dose tested (40 mg/kg/day, p.o.) was observed only after 7 daily treatments. Such foot shock stress triggered transient hyperthermic responses were not completely absent even after 10 daily highest tested doses of the extract.

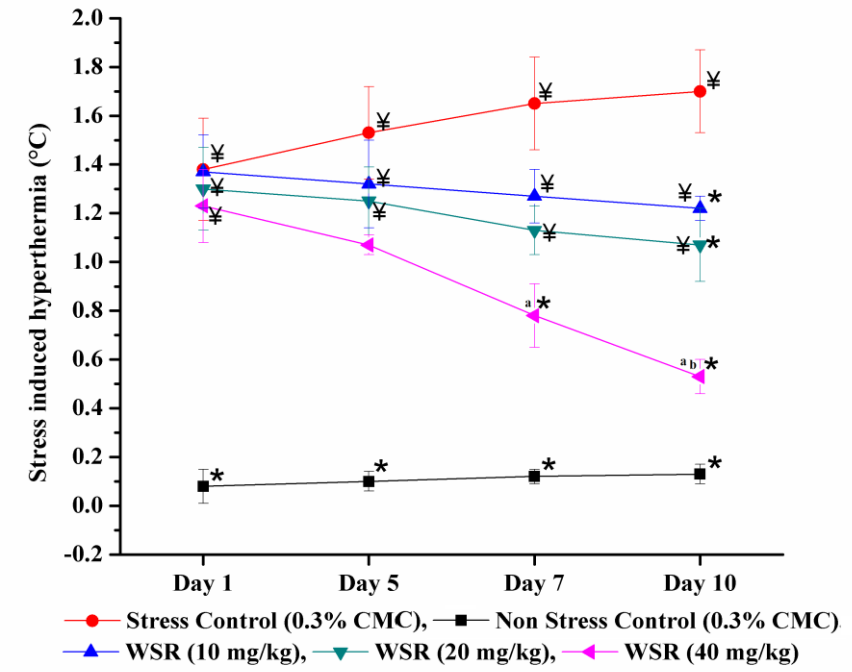

Figure 4.3: Effect of occasional exposures to short durations of foot shock stress on stress induced hyperthermia in male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR). Values are mean ± SEM (n=6). [a]=p<0.05 vs. WSR (10 mg/kg), [b]=p<0.05 vs. WSR (20 mg/kg), *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.1.1.4. Hot plate reaction time: Mean reaction times of foot shock stressed and unstressed control groups in the hot plate test remained almost constant on all observational days. Unlike for the 40 mg/kg WSR treated group on the first day of the experiment, these mean values for the two lower dose (10 or 20 mg/kg) WSR treated ones were not statistically significantly different from those of the vehicle treated stressed or unstressed control groups. Quantitatively, centrally acting analgesics like effectiveness of the highest tested WSR dose continued to increase with increasing numbers of treatment days, and such were also the observations for the 10 or 20 mg/kg/day WSR treated groups (see **Figure 4.4**). After 10 daily treatments, the mean reaction time of the 10 mg/kg/day (p.o.) WSR treated group was also

statistically significantly higher than that of the stressed control group. These observations reveal that WSR possess centrally acting analgesics like nociceptive activity after its single oral doses higher than 20 mg/kg, and that its daily oral 10 mg/kg/day doses in mice is high enough also for suppressing their central hypersensitivity to pain induced by repeated exposures to unpredictable foot shock stress.

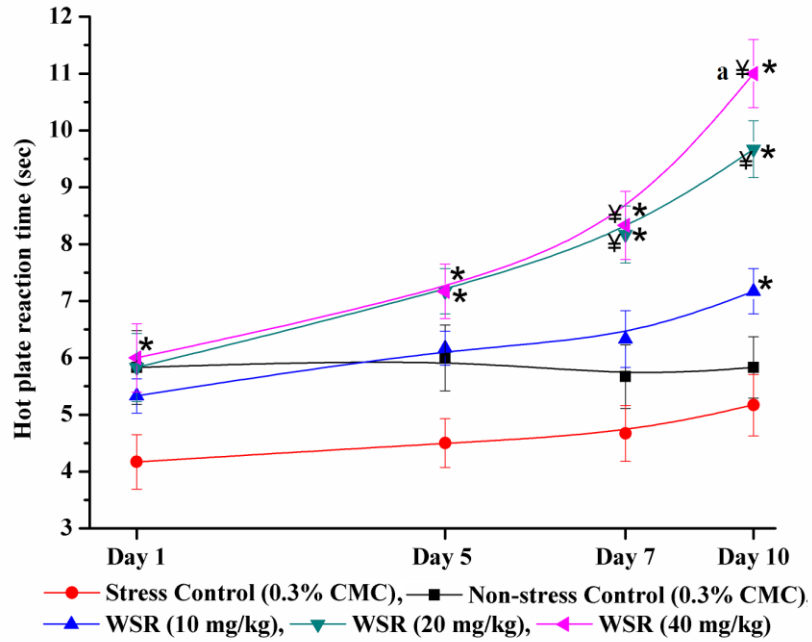

Figure 4.4: Effect of occasional exposures to short durations of foot shock stress on hot plate reaction time in male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR). Values are mean ± SEM (n=6). [a]=p<0.05 vs. WSR (10 mg/kg), *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.1.1.5. Tail suspension test: This test for potential antidepressants initially proposed in 1985 [L. Steru et al., 1985], is now often used also for assessing depressive states of laboratory rodents [J.F. Cryan et al., 2005]. Results of this test summarised in **Figure 4.5** These results revealed that mean immobility time of the stressed control group was significantly higher than that of the non-stressed one, and that repeated daily 10 mg/kg/day

oral doses of WSR is high enough also for counteracting exaggerated depressive state of mice induced by occasional exposures to unpredictable foot shock stress. Such antidepressants like effectiveness of WSR in stressed mice also increased dose dependently up to its highest daily dose tested (40 mg/kg/day, p.o.).

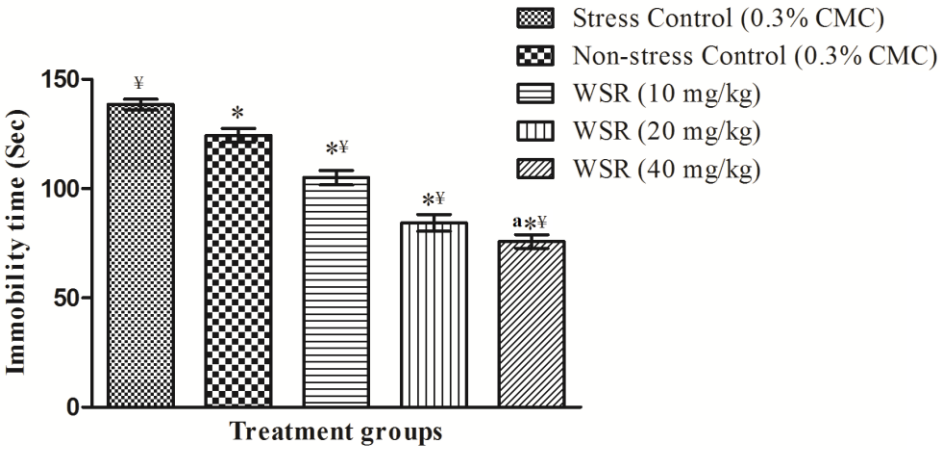

Figure 4.5: Effect of occasional exposures to short durations of foot shock stress on tail suspension test in male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR). Values are mean ± SEM (n=6). [a]=$p<0.05$ vs. WSR (10 mg/kg), *=$p<0.05$ vs. stress control group and [¥]=$p<0.05$ vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.1.1.6. Pentobarbital induced hypnosis: It has since long been well recognized that alterations in pentobarbital induced sleep-induction time and duration of sleep are altered in stressed animals [D.P. Lovell, 1986]. Therefore, the pentobarbital induced hypnosis test was conducted 24 hours after the last oral treatment day of the experiment. Results of the test summarized in **Table 4.1,** revealed that the although the mean sleep induction time of the stressed control group was significantly shorter than that of the non-stressed control group, there were no statistically significant differences between the mean duration of sleep recorded for the two control groups. Although numerically the mean values of durations of sleep in all WSR treated groups were almost identical to that of the control group, these values for onset

of sleep were dose dependently shortened in the stressed WSR treated groups. These observations could indicate that twelve daily oral doses up to 40 mg/kg of the tested extract do not have any residual effects on pentobarbital metabolizing enzymes, and that its lower daily doses can have stimulating effects. However, for more definitive inferences further effort will be necessary. It seems certain though, that repeated daily oral 10 mg/kg WSR doses are high enough also for affording protection against repeated testing and foot shock stress-induced longer lasting alterations in physiological mechanisms and processes regulating sleep induction by pentobarbital.

Table 4.1: Effect of occasional exposures to short durations of foot shock stress on pentobarbital induced hypnosis on day 12 in male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR).

Treatment groups	Onset of sleep (sec)	Duration of sleep (min)
Stress Control (0.3% CMC)	240.0±0.60$^{¥}$	47.50±2.00
Non-Stress Control (0.3% CMC)	150.0±0.22*	52.17±3.23
WSR (10 mg/kg)	229.8±0.30*$^{¥bc}$	53.33±1.90
WSR (20 mg/kg)	199.8±0.33*$^{¥ac}$	57.00±1.81
WSR (40 mg/kg)	190.2±0.31*$^{¥ab}$	59.83±1.74

Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group, $^{¥}$=$p<0.05$ vs. non-stress control group, ^{a}=$p<0.05$ vs. WSR (10 mg/kg), ^{b}=$p<0.05$ vs. WSR (20 mg/kg), ^{c}=$p<0.05$ vs. and WSR (40 mg/kg) (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.1.2. The second pilot experiment

This experiment was designed and conducted to reaffirm the dose response relationship of WSR in stressed mice not preselected for their response times in hot plat test, and to verify whether the marble tests could also be used for estimating the dose effect relationship of *Withania somnifera* extracts in affording protection against obsessive compulsive behavior in

stressed mice. Historically, diverse versions of this test have often been used for pharmacological screening of potential anxiolytic and antidepressant drug leads. However, it is now well recognized that this test is one of the cost effective and most straightforward procedure for quantifying drug effects on obsessive compulsive behavior of rodents and also for quantifying the effects of anxiolytics and antidepressants on this behaviour [N. Albelda and D. Joel, 2012; A. Thomas et al., 2009]. Observations made during efforts to standardize two versions of the test used in the experiment have reaffirmed that marble burring behavior of non-stressed mice can be reproducibly quantified in both versions to the test conducted on two consecutive days of an experiment. Like in the other pilot cum dose finding experiment, groups of treated mice either with *Withania somnifera* root extract, or with its vehicle, were subjected to a stress induced hyperthermia test on the 1^{st}, 5^{th}, 7^{th} and 10^{th} day of the experiment. On the 11^{th} and 12^{th} treatment days, all experimental groups were subjected to one of the two versions of marble burying test for reaffirming brain function modulating, or antidepressants and anxiolytic like, effects of WSR after its repeated daily dose. Results of this experiment are described in the following sub-sections.

4.1.2.1. Body weights and basal core temperatures: These results summarised in the **Figures 4.6A and 4.6B** were quite analogous to those observed in the first dose finding experiment. They reaffirm that the minimally effective daily oral WSR dose in counteracting stress triggered body weight losses and elevations in basal core temperatures is 10 mg/kg or lower. The rates of changes in mean body weights of the two higher WSR treated stressed groups were also slightly higher than that of the unstressed control group. Therefore, they indicate again that its higher daily oral doses can have growth promoting effects in mice not preselected in hot plate test. Since the maintenance and protection of core body temperature through tight control of metabolism is a defining element of mammalian physiology, these

observations suggest again that WSR is a fairly potent regulator of metabolic process regulating body weights and basal core temperatures.

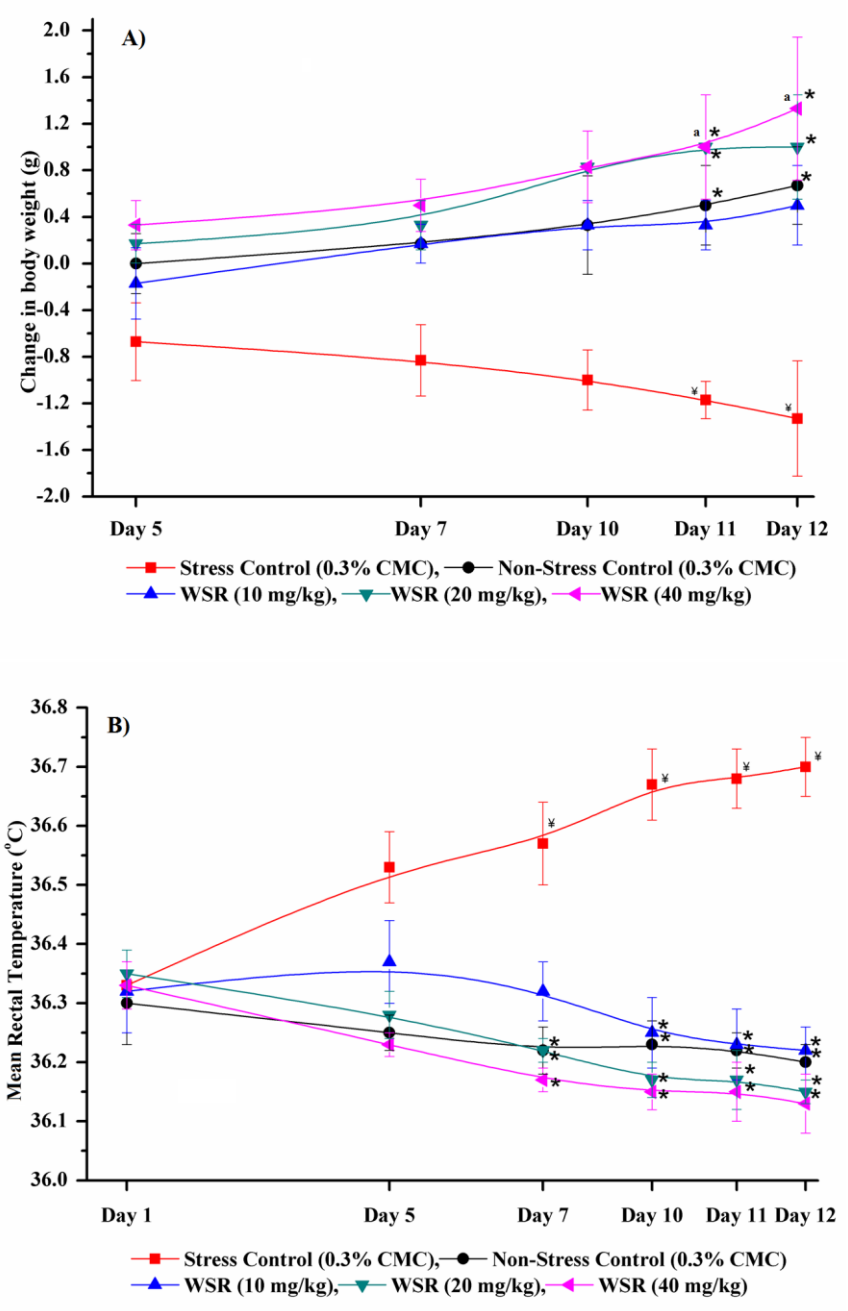

Figure 4.6: Effect of occasional exposures to short durations of foot shock stress on **(A)** gains or losses in body weights and **(B)** mean rectal temperature of male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR). Values are mean ± SEM (n=6). [a]=p<0.05 vs. WSR (10 mg/kg), *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.1.2.2. Stress induced hyperthermic responses: These results summarized in the **Figure 4.7**. revealed also that 10 or 20 mg/kg daily oral doses of WSR for 7 or more days statistically significantly suppresses foot shock stress triggered transient hyperthermic responses, and that a single 40 mg/kg oral WSR dose is high enough for significantly suppressing this response in mice. Analogous to the observations made in the first pilot experiment, effectiveness of all tested WSR doses in this test continued to increase with increasing numbers of treatment days. But the transient hyperthermic responses were not completely absent in animals treated daily with its 40 mg/kg oral doses for 10 consecutive days. These observations are in agreement with the working hypothesis that homeostatic processes and mechanisms involved in thermoregulation are most probable involved in the mode of action of the extract.

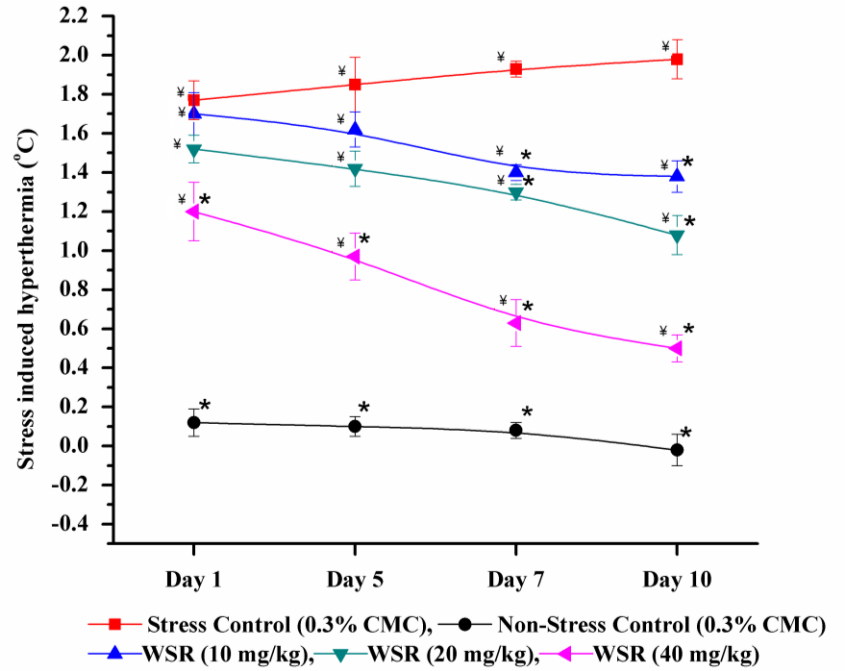

Figure 4.7: Effect of occasional exposures to short durations of foot shock stress on stress induced hyperthermia in male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.1.2.3. Marble burying tests: Mean numbers of marbles buried by the animals of different groups in two versions of the test are shown in the **Figures 4.8A & 4.8B.** This number for the stressed control group in the standard version of the test conducted one day after last exposures to foot shock stress (**Figure 4.8A**) was statistically significantly higher than those of the non-stressed control one. Although, numerically this number of the non-stressed group in the two-zone version of the test conducted on the following day of the experiment was also lower than that of the stressed control group (**Figure 4.8B**), there were no statistically significant differences between these two values in this version of the test. The numbers of marbles buried were dose dependently lowered further by daily oral treatments with the extract, but the observed mean values of the 10 mg/kg/day WSR treated group in the standard version of the test was not statistically significantly different from that of the stressed control group. Since such were not the observations in the two zone version of the test, it seems that the standard version of the test to be more sensitive and specific for quantifying the effects of repeated exposures to unpredictable foot shock stress. However, the two zone version of the test could also be used for estimating the effects of the extract on obsessive compulsive behaviour, or for detecting its anxiolytics and antidepressants like activities.

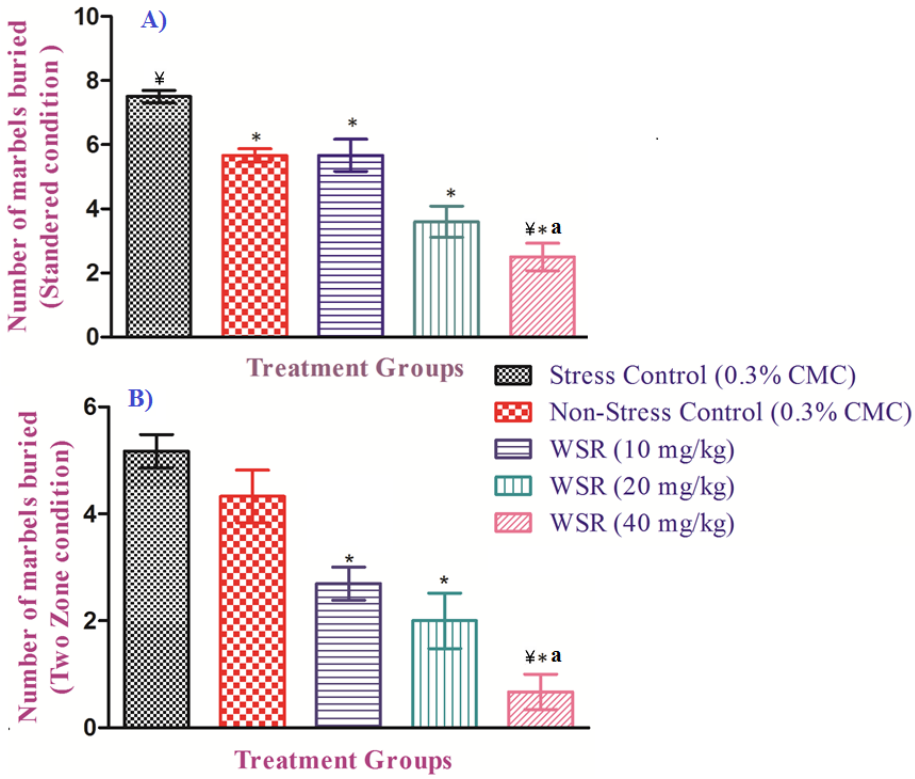

Figure 4.8: Effect of occasional exposures to short durations of foot shock stress on marble burying test: **A)** Standard condition on day 11 and **B)** Two-zone condition on day 12 in male mice daily treated (orally) with a *Withania somnifera* roots extract (WSR). Values are mean ± SEM (n=6). [a]=p<0.05 vs. WSR (10 mg/kg), *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.2. Aadaptogenic potentials of three types of *Withania somnifera* extracts

Since qualitatively as well as quantitatively the contents of withanolides and other bioactive substance in different part of the plant vary considerably, these experiments were conducted to compare the activity profiles of three *Withania somnifera* extracts obtained from its roots, areal parts, and only stems of the plant obtained by the same extraction procedure. They were prepared, and generously supplied (together with their HPLC finger prints and analytical certificates) by the research laboratories often supplying us commercialized samples of

extracts from *Withania somnifera* roots and other plants for experimental purposes. According to the analytical data supplied by them, total contents of withanolides in the *Withania somnifera* extracts (as quantified by HPLC) uses in all described experiments were 2.7% (w/w) in the roots extract (WSR), 3.0% (w/w) in the aerial parts extract (WSA) and 1.5% (w/w) in the stem parts extract (WSS). Their HPLC chromatograms are shown in the following **Figures 4.9, 4.10** & **4.11.**

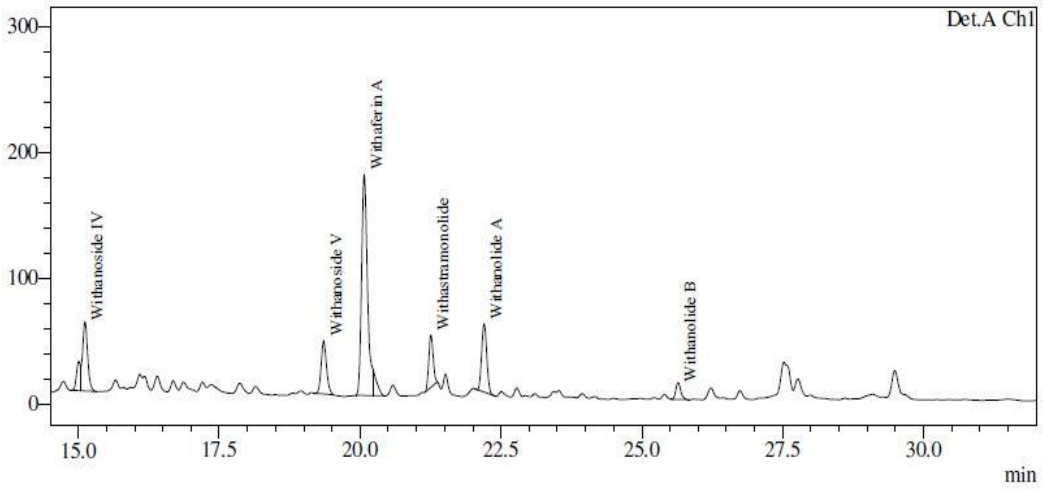

Figure 4.9: HPLC chromatogram of roots extract of *Withania somnifera* (WSR) used in all described experiments.

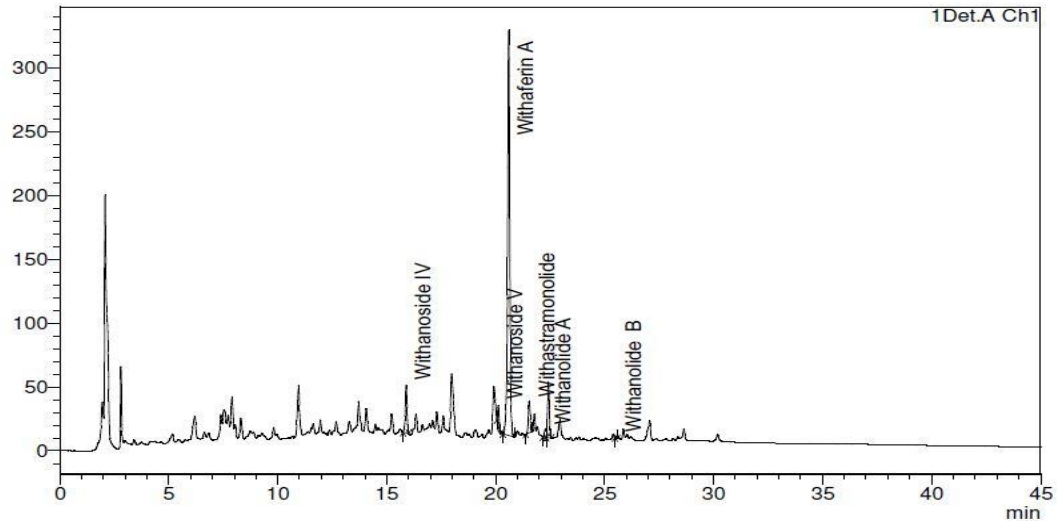

Figure 4.10: HPLC chromatogram of aerial parts extract of *Withania somnifera* (WSA) used in the experiments.

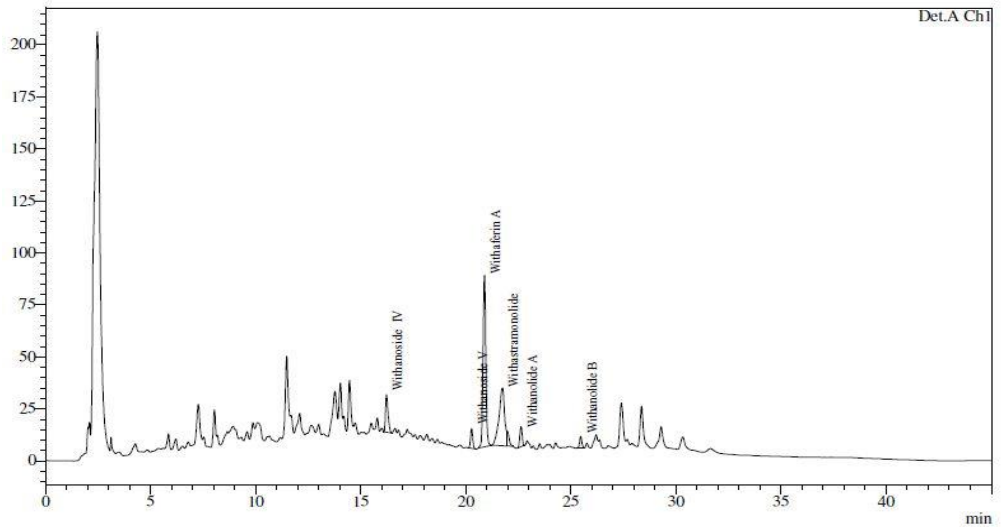

Figure 4.11: HPLC chromatogram of stem parts extract of *Withania somnifera* (WSS) used in the experiments.

Choices of the doses, treatment regimen, experimental procedures, and tests used in the two sets of experiments conducted were based on the observations made in the pilot methodological cum dose finding experiments. Aim of the first of these two sets of experiments was to compare the activity profiles of WSR, WSA, and WSS with those of some plant derived and other drugs currently often used for prevention and cure of metabolic disorders and their co-morbid psychopathologies in stressed mice. Choices of the doses of these drugs were based on earlier observations made in our laboratories during efforts to pharmacologically validate the foot shock stress paradigm for assessing pharmacologically interesting and toxicologically safe dose ranges of herbal extracts and their known bioactive constituents [A.J. Langstieh et al., 2014]. Results of these sets of experiments will be described first.

4.2.1. The first set of comparative experiments

In the first of the two sets of comparative experiments, effectiveness of a fairly high single and repeated daily well tolerated oral doses (50 mg/kg/day, p.o.) of WSR, WSA and WSS were compared with those of diazepam (5 mg/kg), imipramine (15 mg/kg), metformin (50 mg/kg), nicotinic acid (1 mg/kg), and aspirin (100 mg/kg) in both male and female Swiss albino mice. As judged from earlier observation in our laboratories, these doses of the drugs used in the experiments are almost their highest ones as modulators of diverse foot shock stress triggered response in mice. The test procedures used in these experimenst were identical to that used in the first pilot methodological cum dose finding experiment.

4.2.1.1. Body weights: Mean body weights of different groups of animals recorded for different experimental groups are summarized in **Figure 4.12.** As expected from earlier observations, the vehicle treated male and female control groups steadily lost their body weights during the course of the experiments, whereas all three *Withania somnifera* extract treated ones had started gaining weights from 7th treatment day onwards. Effectiveness of the tested daily oral doses (50 mg/kg/day) of all three extracts in affording such protections were almost equal in magnitude, whereupon such effects of WSR in female mice were somewhat more prominent than those of WSA or WSS (**Figures 4.12A and 4.12C**).

Results summarized in the **Figures 4.12B and 4.12D** revealed that in both male and female mice, such effects of daily oral treatments with the tested doses of metformin, or aspirin, or nicotinic acid (i.e. niacin or Vitamin B3) were quite analogous to those of the three *Withania somnifera* extracts tested. 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 and hot

plate tests. These observations suggest that the homeostatic processes and mechanisms involved in the observed effects of the tested extracts and the other three reference drugs (with structural similarities with plant metabolites) on stress triggered body weight losses are most likely not the same as those involved in their anxiolytic or antidepressant like effects observed in tail suspension and other tests for synthetic anxiolytics and antidepressants like diazepam and imipramine.

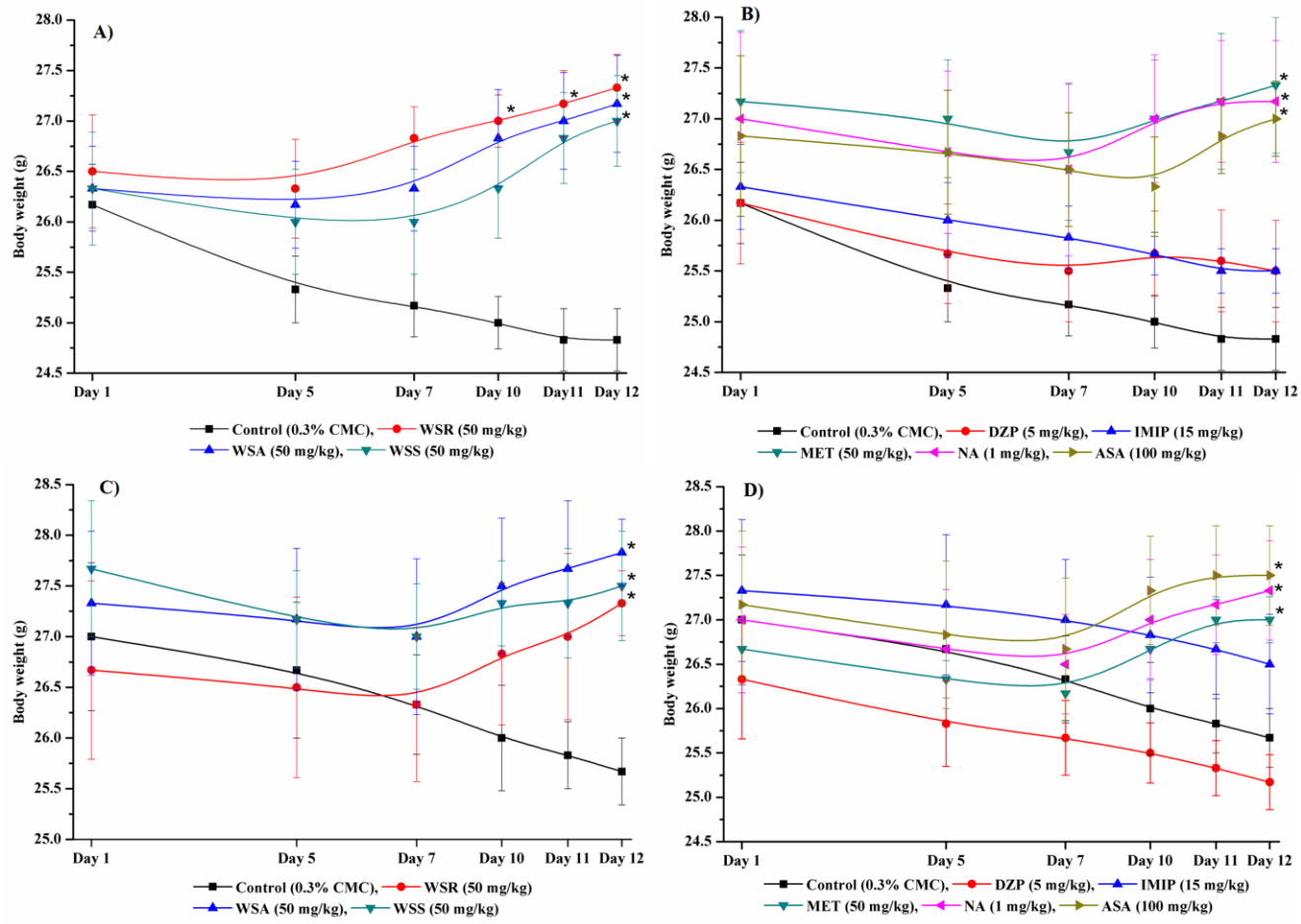

Figure 4.12: Effect of occasional exposures to short durations of foot shock stress on mean body weight of male mice daily treated (orally) with **A)** *Withania somnifera* extracts or **B)** reference drugs (DZP: diazepam, IMIP: imipramine, MET: metformin, NA: nicotinic acid and ASA: aspirin), and of female mice daily treated similarly with the **C)** *Withania somnifera* extracts or **D)** the same reference drugs. Values are mean ± SEM (n=6). *=$p<0.05$ vs. control group (Two way ANOVA followed by Bonferroni post hoc test).

4.2.1.2. Basal core temperatures: These mean values of the preselected male or female control groups on the first observational day were almost identical and were within the average values of the animal colony used the experiments. Except for the male WSR treated group, these man values of all other test groups subjected to foot shock stress on this day of the experiments were also not statistically significantly different than those of the corresponding control ones. During the course of the experiments mean basal core temperatures of the male and female control groups steadily increased somewhat, also remained within physiological ranges, during the course of the experiments (**Figure 4.13**). Such were not the observations made for all reference drug or *Withania somnifera* extract treated groups, the mean vales of all of which steadily decreased with increasing numbers of days of the experiments. These observations revealed that although acute oral doses of WSR tested (50 mg/kg/day) can increase core temperature of mice with prior experiences in hot plate test (i.e. occasionally exposed to very short duration of thermal stimuli or stress), such effects of the extract disappear also after its repeated daily doses. From these results it can be inferred also that like all reference drugs tested, after daily oral doses of 50 mg/kg/day WSR, WSA, or WSS also down regulates physiological thermoregulatory processes and mechanisms involved in the observed longer lasting effects of repeated stressful experiences on basal core temperatures of male or female mice.

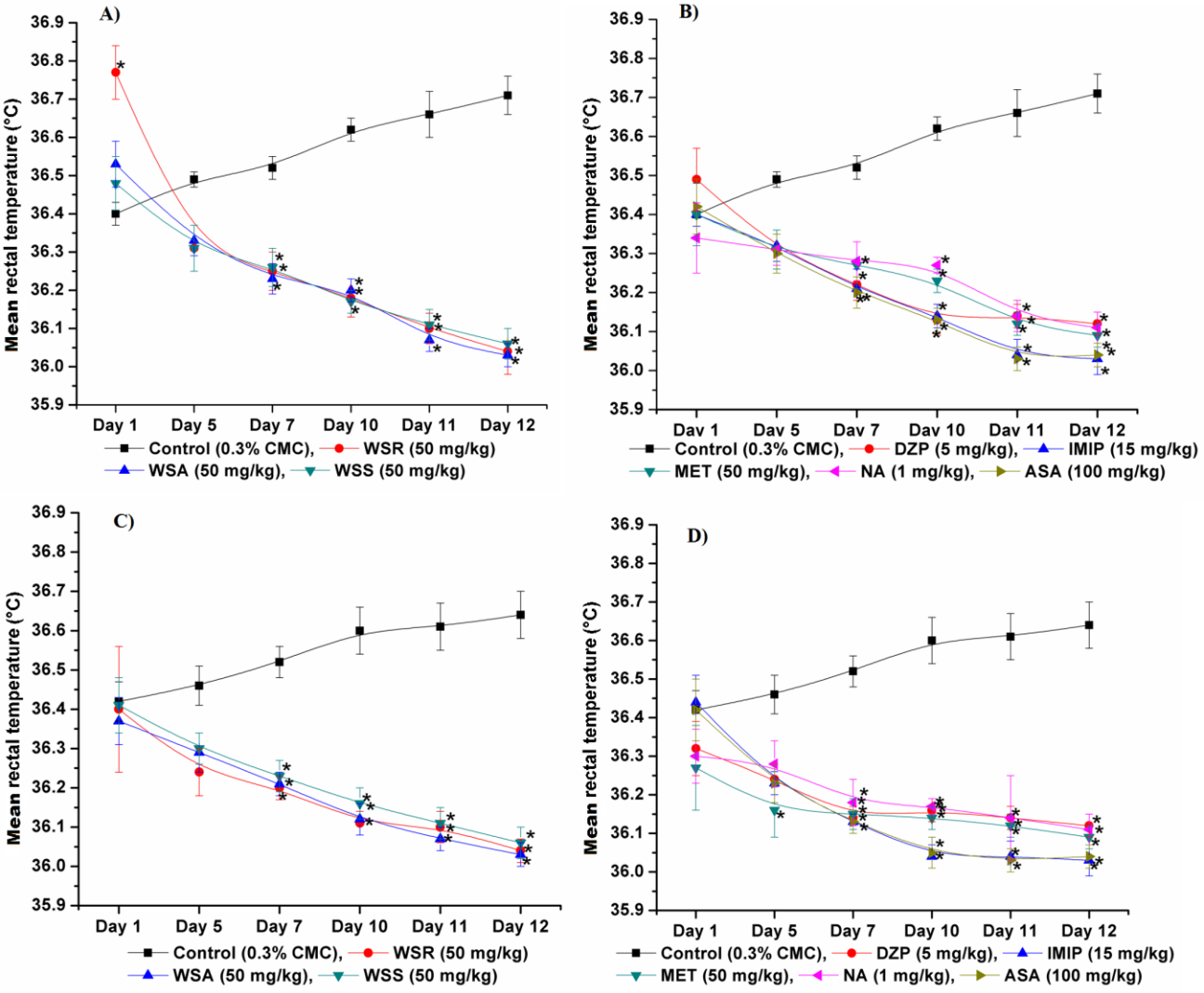

Figure 4.13: Effect of occasional exposures to short durations of foot shock stress on mean rectal temperatures of male mice daily treated (orally) with **A)** *Withania somnifera* extracts or **B)** reference drugs (DZP: diazepam, IMIP: imipramine, MET: metformin, NA: nicotinic acid and ASA: aspirin), and of female mice treated similarly with the **C)** *Withania somnifera* extracts or **D)** the same reference drugs. Values are mean ± SEM (n=6). *=p<0.05 vs. control group (Two way ANOVA followed by Bonferroni post hoc test).

4.2.1.3. Foot-shock stress induced hyperthermic responses: These results summarized in **Figure 4.14** revealed that after their single fairly high oral doses (50 mg/kg/day), all three extracts significantly suppressed the stress triggered transient hyperthermic responses in both male and female mice.

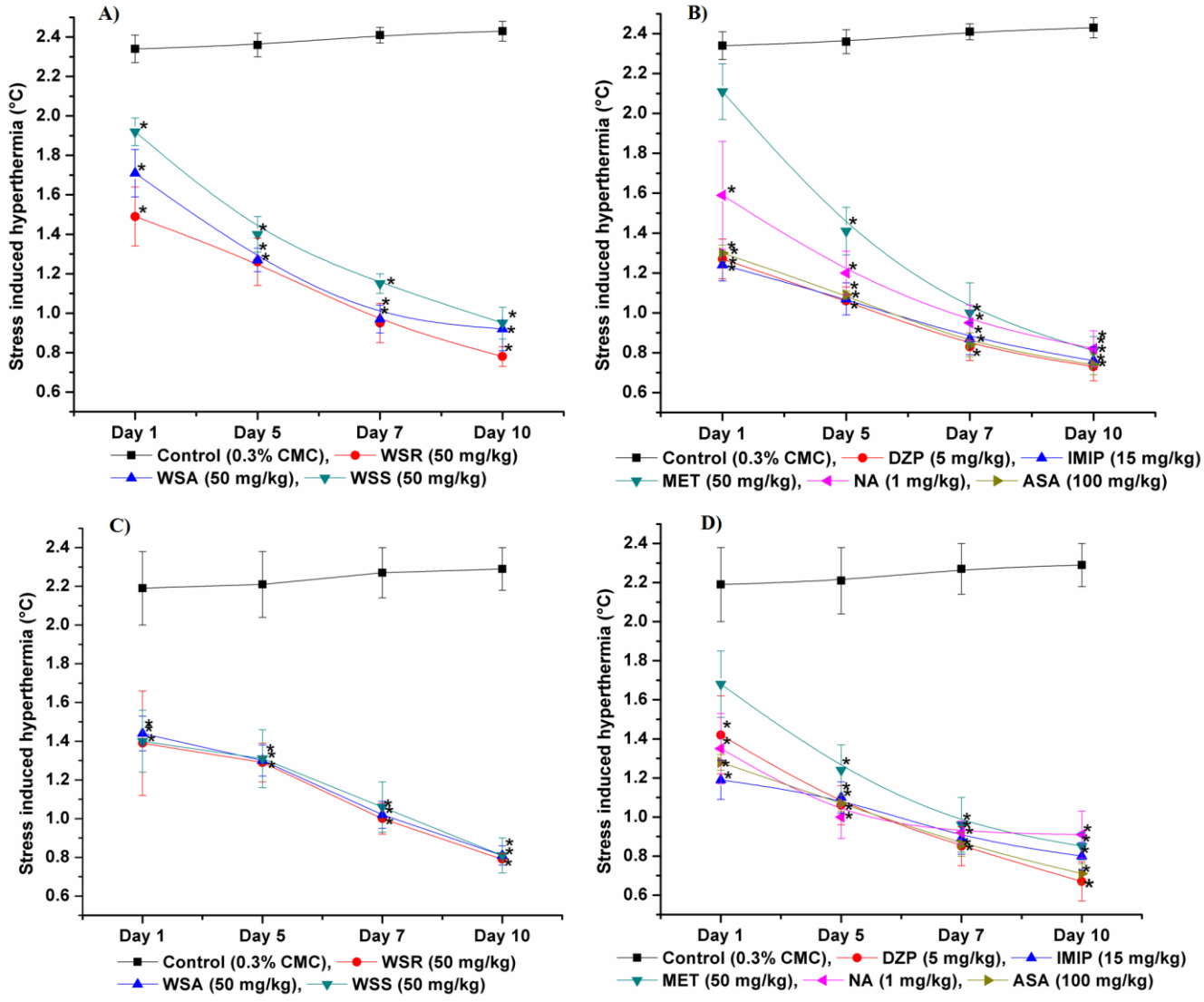

Figure 4.14: Effect of occasional exposures to short durations of foot shock stress on stress induced hyperthermia of male mice daily treated (orally) with **A)** *Withania somnifera* extracts or **B)** reference drugs (DZP: diazepam, IMIP: imipramine, MET: metformin, NA: nicotinic acid and ASA: aspirin), and of female mice treated similarly with the **C)** *Withania somnifera* extracts or **D)** the same reference drugs. Values are mean ± SEM (n=6). *=p<0.05 vs. control group (Two way ANOVA followed by Bonferroni post hoc test).

Quantitatively, these observed effects of all tree extracts in both male and female mice continued to increase steadily with increasing numbers of treatment days. Although in males this mean value of the WSA treated group on this day was somewhat lower and higher than

those for WSS and WSR treated ones respectively (**Figure 4.14A and 4.14C**), there were no statistically significant differences between the mean values of these three male groups. Except for metformin, statistically significant effects of all other tested standard or reference 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 (**Figure 4.14B and 4.14D**). On the first observational day, the mean values of metformin treated male or female groups were statistically not significantly different from the corresponding stressed control groups. However, in both males and females its statistically significant effects in the test increased also after its five or more daily doses. Mean values of all test agents on the 10^{th} observational day were statistically not significantly different from each other.

4.2.1.4. Hot plate reaction time: Results of this test are graphically summarized in **Figure 4.15**. On the first observational day, neither aspirin (a peripherally acting analgesic drug with anti-inflammatory activity), nor any of the tested reference drugs or extracts had any nociceptive effects in this test for centrally acting analgesics. Mean reaction times of both male and female control groups decreased somewhat on the subsequent observational days, whereas those of all drug or extract treated ones continued to increase with increasing numbers of treatment days. Statistically significant effects of all three tested extracts were observed in both male and female mice after 5 or more treatment days, and their observed effects in the test were also quantitatively similar to each other. Such were also the observations made with aspirin, diazepam, and niacin. Statistical significance of the differences between the mean reaction time values of metformin treated male and female groups and the corresponding control ones were observe on the 7^{th} and 10^{th} treatment days

only. In male mice, statistically significant effects of imipramine were also observed on these two observational days, whereas in males its significant effects were observed after its ten daily doses only.

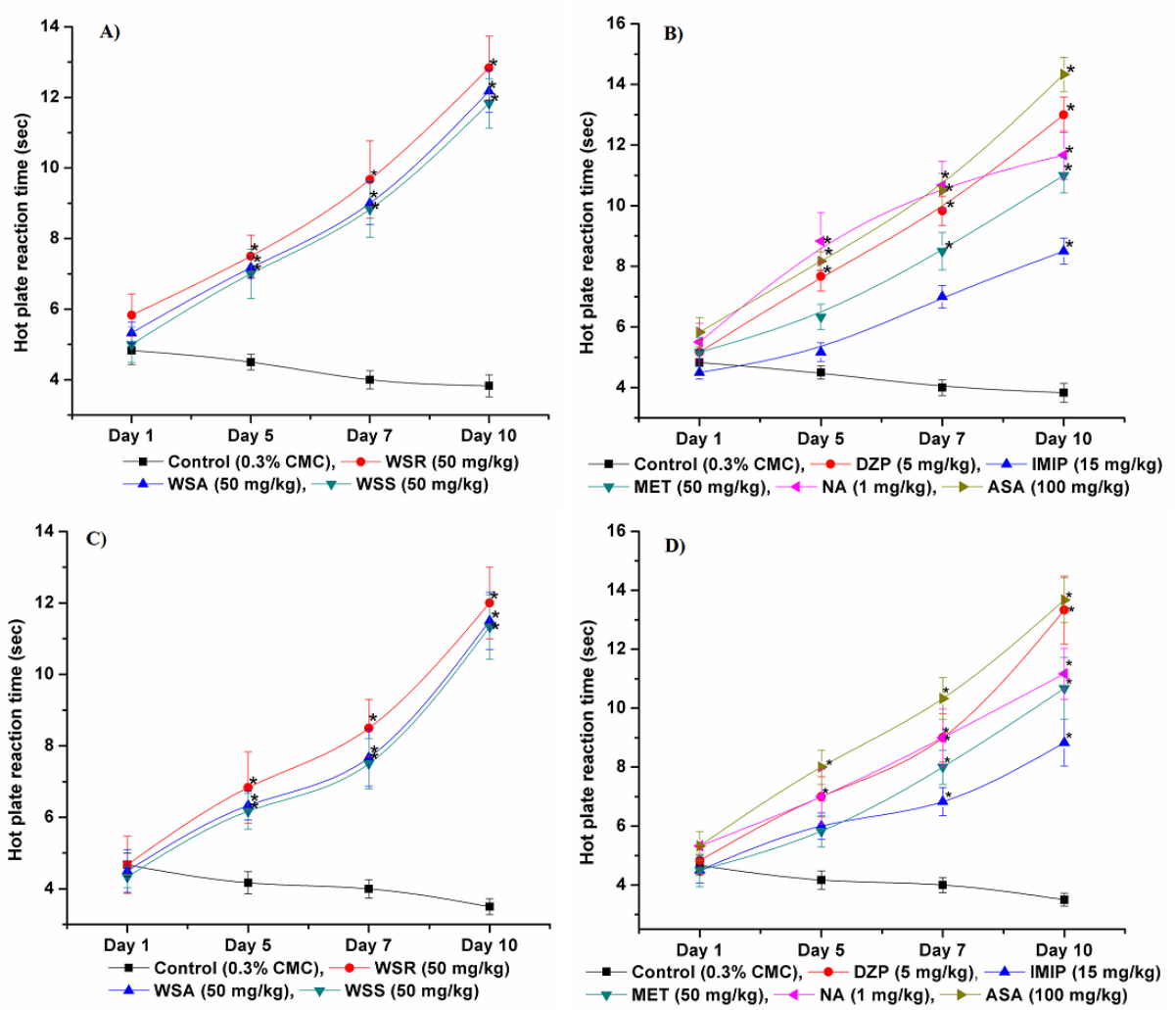

Figure 4.15: Effect of occasional exposures to short durations of foot shock stress on hotplate reaction time of male mice treated daily with **A)** *Withania somnifera* extracts or **B)** reference drugs (DZP: diazepam, IMIP: imipramine, MET: metformin, NA: nicotinic acid and ASA: aspirin), and of female mice treated with **C)** *Withania somnifera* extracts or **D)** the same reference drugs. Values are mean ± SEM (n=6). *=p<0.05 vs. control group (Two way ANOVA followed by Bonferroni post hoc test).

4.2.1.5. Tail suspension test: This test was conducted on the 11th day of the experiments to compare the effects of repeated daily oral treatments on 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 4.16** revealed that after the tested daily oral doses, all three *Withania somnifera* extracts were almost equiactive in both male and female mice, whereupon they were somewhat more effective in males than in females. After their tested daily oral doses, both imipramine and diazepam were almost equally active 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 effects of aspirin treatments were also observed in both males and females, whereas such were the observations for metformin and niacin in males only.

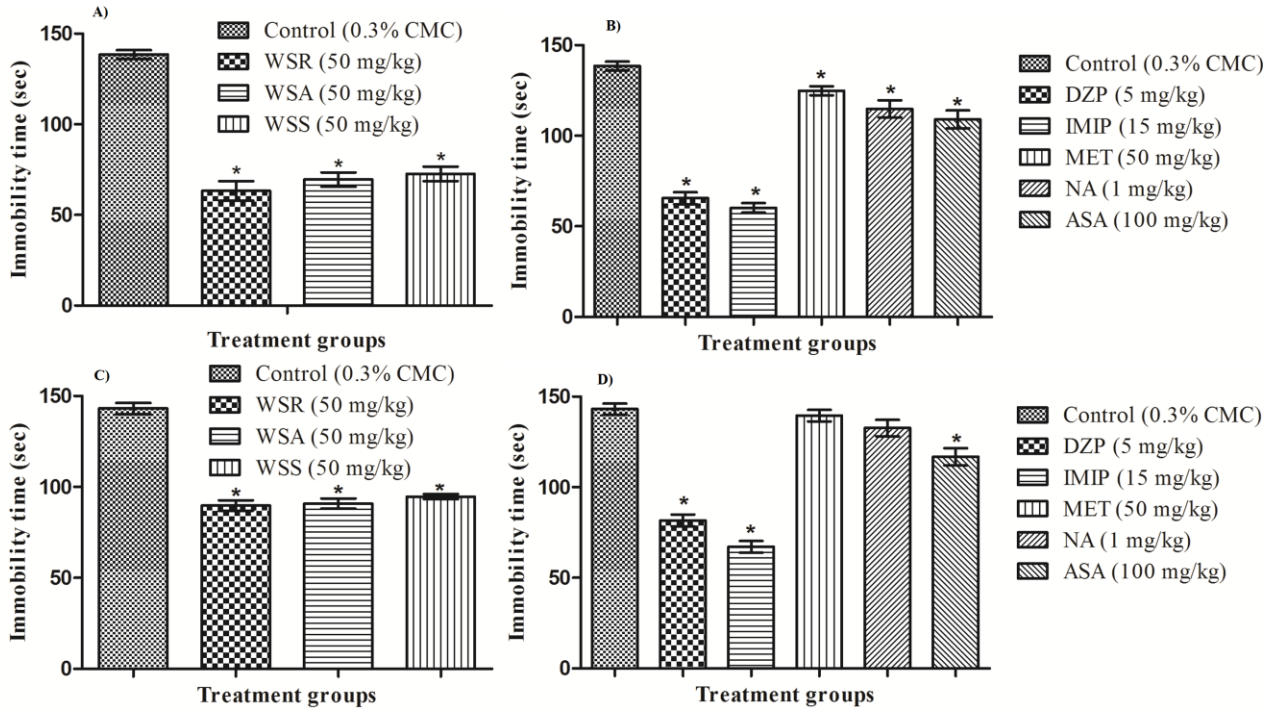

Figure 4.16: Effect of occasional exposures to short durations of foot shock stress on tail suspension test in male mice daily treated (orally) with **A)** *Withania somnifera* extracts or **B)** reference drugs (DZP: diazepam, IMIP: imipramine, MET: metformin, NA: nicotinic acid and ASA: aspirin), and in female mice similarly treated with the **C)** *Withania somnifera* extracts or **D)** the same reference drugs. Values are mean ± SEM (n=6). *=$p<0.05$ vs. control group (Two way ANOVA followed by Bonferroni post hoc test).

4.2.1.6. Pentobarbital induced hypnosis test: This test was conducted on the 12th day 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 test summarized in **Tables 4.2** (for males) and **Tables 4.3** (for females) 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 females. 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 duration of sleep induced by pentobarbital in both males and females, whereas in both males and females the observed effects of all tested extracts on this parameter were in directions opposite to those observed for diazepam. Unlike the sedative and anxiolytic drug diazepam (which prolonged sleep duration), all three extracts shortened the durations of sleep in both males and females. Quantitatively, there were no statistically significant differences in their shortening effects on duration of sleep induced by pentobarbital observed 24 hours after their last oral doses.

Table 4.2: Effect of occasional exposures to short durations of foot shock stress on pentobarbital induced hypnosis on day 12 in male mice daily treated (orally) with *Withania somnifera* extracts and reference drugs.

Treatment groups	Onset of sleep (sec)	Duration of sleep (min)
Stress Control (0.3% CMC)	179.17±1.40	60.67±1.78
DZP (5 mg/kg)	117.83±1.42*	79.17±2.39*
IMIP (15 mg/kg)	133.17±1.01*	65.17±2.77
MET (50 mg/kg)	161.67±1.48*	65.00±2.89
NA (1 mg/kg)	144.67±1.67*	59.17±2.39
ASA (100 mg/kg)	179.33±1.36	54.33±2.46
WSR (50 mg/kg)	152.50±1.10*	45.67±2.40*
WSA (50 mg/kg)	158.33±1.20*	48.33±2.50*
WSS (50 mg/kg)	160.83±1.54*	49.67±1.33*

DZP - Diazepam, IMIP – Imipramine, MET- Metformin, NA – Nicotinic Acid, ASA – Acetyl salicylic acid (Aspirin). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group (One way ANOVA followed by Student-Newman-Keuls test).

Table 4.3: Effect of occasional exposures to short durations of foot shock stress on pentobarbital induced hypnosis on day 12 in female mice daily treated (orally) with *Withania somnifera* extracts and reference drugs.

Treatment groups	Onset of sleep (sec)	Duration of sleep (min)
Stress Control (0.3% CMC)	183.67±1.28	63.67±1.28
DZP (5 mg/kg)	123.33±1.31*	80.50±1.63*
IMIP (15 mg/kg)	135.50±1.38*	63.00±1.91
MET (50 mg/kg)	170.00±1.34*	66.67±2.11
NA (1 mg/kg)	142.17±1.30*	61.33±1.20
ASA (100 mg/kg)	185.67±1.38	57.50±2.38
WSR (50 mg/kg)	153.67±1.30*	47.17±2.10*
WSA (50 mg/kg)	159.83±1.70*	50.67±1.70*
WSS (50 mg/kg)	163.17±1.50*	52.00±1.00*

DZP - Diazepam, IMIP – Imipramine, MET- Metformin, NA – Nicotinic Acid, ASA – Acetyl salicylic acid (Aspirin). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group (One way ANOVA followed by Student-Newman-Keuls test).

4.2.2. The second set of comparative experiments

In these two experiment, effectiveness of single and repeated daily lower oral doses (10 mg/kg/day, p.o.) of the three *Withania somnifera* extracts (WSR, WSA and WSS) to suppress foot shock stress triggered transient hyperthermic response and other activities were compared in Swiss albino male mice. In the first of them, the experimental procedures, tests used and quantified parameters were the same as those used in the first pilot cum dose finding experiment. The other one was conducted for reaffirming the effects of the lower doses (10 mg/kg/day) of the three extracts against foot shock stress triggered alterations in body weights and temperatures observed in the first experiment, and to compare their repeated daily low oral dose effects in two versions of the marble burying test used in the second pilot cum dose finding experiment. The results of the first of these two experiments will be described at first, and thereafter the results of the other one are summarized.

4.2.2.1. Body weights: Mean body weights of different experimental groups recorded during the course the experiment are summarized in **Figure 4.17A & 4.17B**. As expected the animals of the stressed control group steadily lost their body weights during the course of the experiment, whereas those of the non-stressed control group gained weights during the 12 observational days. Like for the no-stressed vehicle treated control group, mean body weights of all three extract (10 mg/kg/day) treated and foot shock stresses groups increased during the 12 days of the experiment. Although the net weight gain of the WSR treated group during the last few observational days were somewhat higher than those of the other two extract treated ones, there were no statistically significant differences between the mean weight gain values of any of the three stressed extract treated groups and corresponding ones of the non-stressed control group.

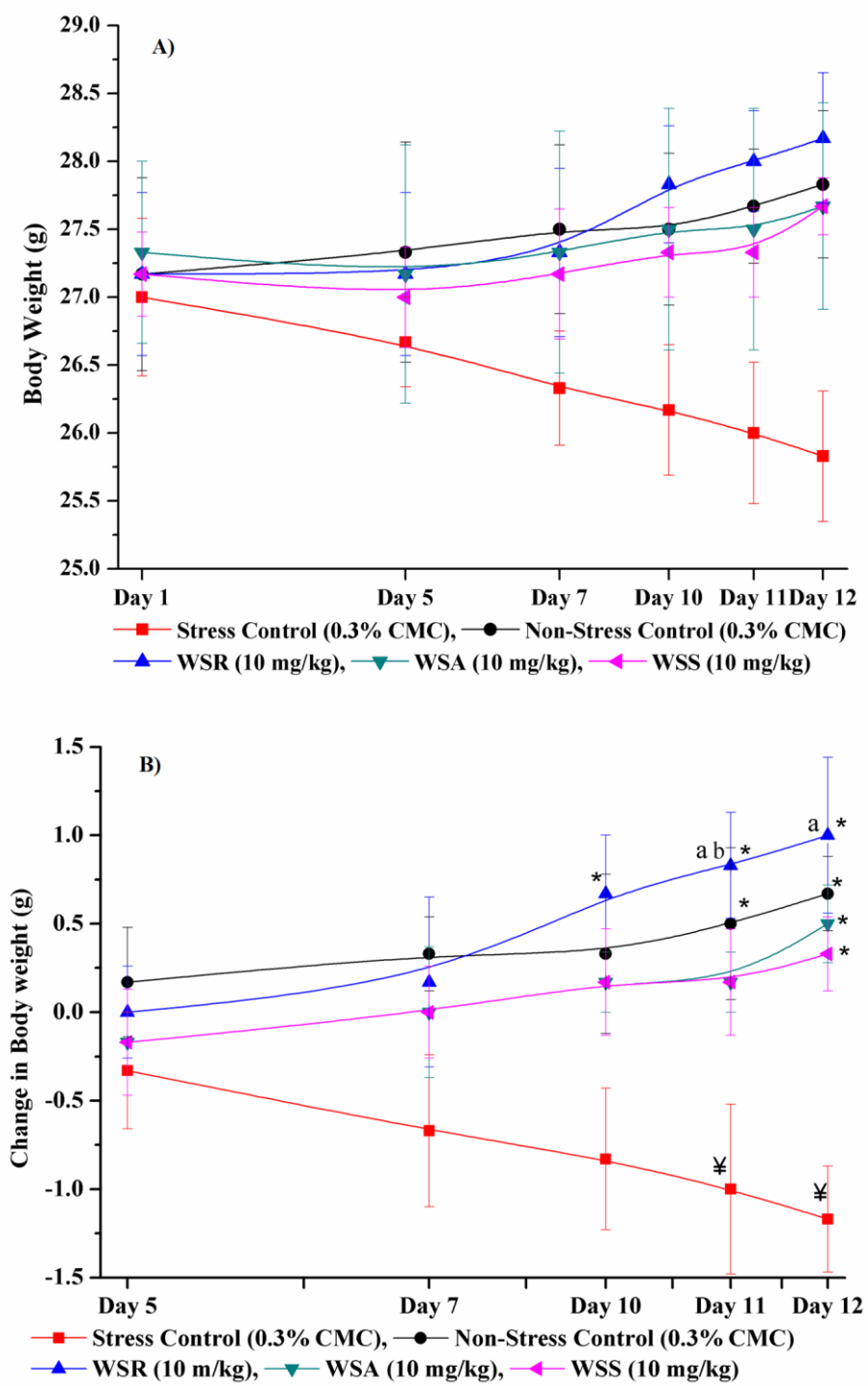

Figure 4.17: Effect of occasional exposures to short durations of foot shock stress on **A)** mean body weights, and **B)** gains or losses in body weights of male mice daily treated (orally) with three different *Withania somnifera* extracts (WSR, WSA and WSS). Values are mean ± SEM (n=6). [a]=p<0.05 vs. WSA (10 mg/kg), [b]=p<0.05 vs. WSS (10 mg/kg), *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.2.2.2. Basal core temperatures: Mean values of all five experimental groups recoded one hour after the first oral treatments were not statistically significantly different from one another. The results summarized in **Figure 4.18** revealed that from the 5th oral treatment day onwards these values of the stressed control group were slightly higher than that recorded for the group on the first treatment day, whereas the mean core temperature of the non-stressed control group decreased steadily on subsequent oral treatment days. Decreases in the mean basal core temperatures of all three extract treated groups observed from 5th day onwards of the experiments were quite similar in magnitude to those recorded for the non-stressed control group. It was interesting to note though, that the mean basal core temperatures of the non-stressed control and the WSR or WSS treated stressed groups were statistically significantly different from the stressed control one on the last two observational days (days 11 and 12 of the experiment), whereas such statistically significant difference between the stressed WSR treated and the stressed control group were observed already on the 7th treatment day and one hour before the treatment.

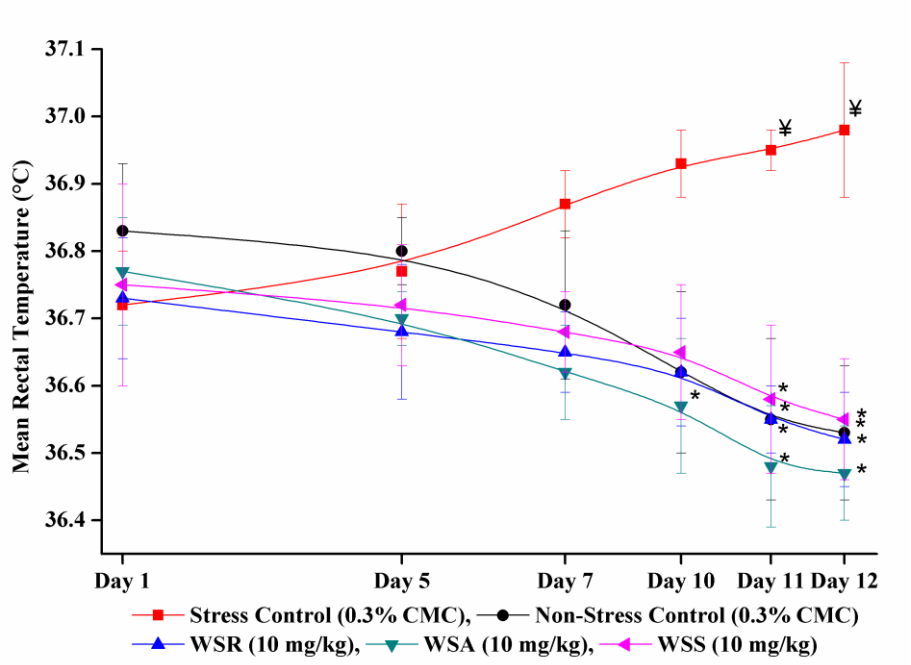

Figure 4.18: Effect of occasional exposures to short durations of foot shock stress on mean rectal temperature in male mice daily treated (orally) with three different extracts of *Withania somnifera* (WSR, WSA and WSS). Values are mean ± SEM (n=6). *=p<0.05 vs. stress control group and ¥=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.2.2.3. Foot-shock stress induced transient hyperthermic response: In this experiment stress triggered transient hyperthermic responses of the vehicle treated control group increased only slightly but steadily on the 5th and subsequent testing days. The results summarized in **Figure 4.19** reaffirmed that a single 10 mg/kg oral WSR dose of do not have any statistically significant effects on this response, and revealed that such is also true for similar treatments with WSA or WSS. Statistically significant suppressing effects of WSS on this response was observed already on the 5th treatment day, whereas those of the other two extracts tested (WSR and WSA) were observed first on the 7th treatment day. However, stress induced transient hyperthermic response suppressing effects of 10 mg daily oral doses

of all three extracts tested on all observational days were not statistically or significantly different from one another.

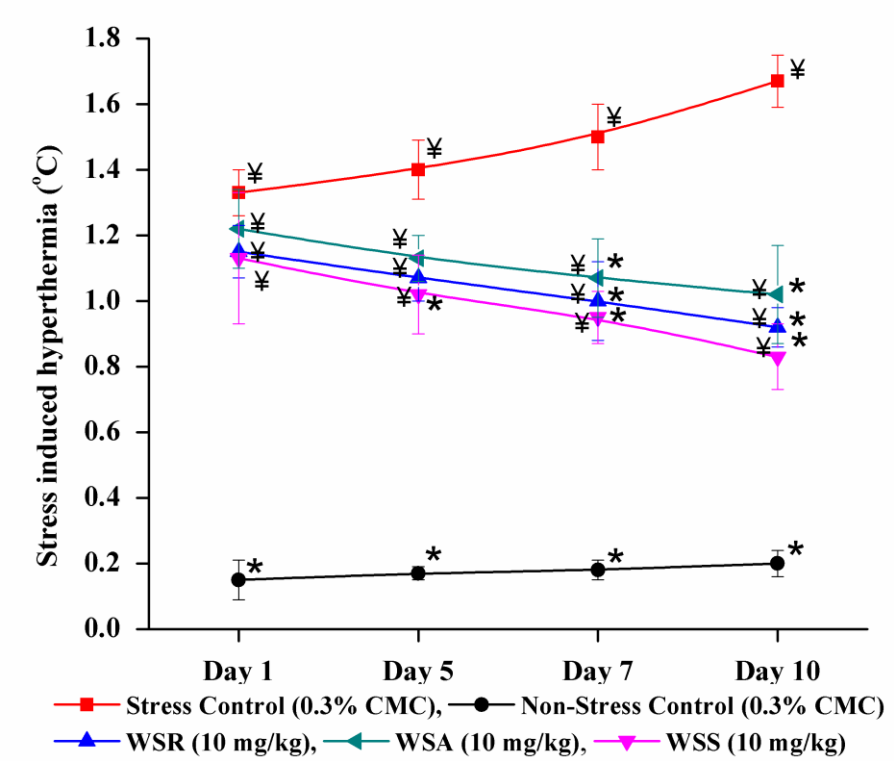

Figure 4.19: Foot shock stress triggered hyperthermic responses in male mice treated daily (orally) with three different extracts of *Withania somnifera* (WSR, WSA and WSS). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.2.2.4. Hot plate reaction time: Although not statistically significantly different, the mean numerical values of reaction times of all stressed groups (extract treated or not) in hot plate test on the first treatment day were somewhat higher than that recorded for the non-stressed group. Such were also the observations on the 5th treatment day. As shown in **Figures 4.20**, mean reaction times of the stressed WSS treated group was statistically significantly higher than the stressed controls on the 10th treatment day only, whereas these mean values of WSA or WSR treated stressed groups were statistically significantly higher than the stressed or unstressed control groups.

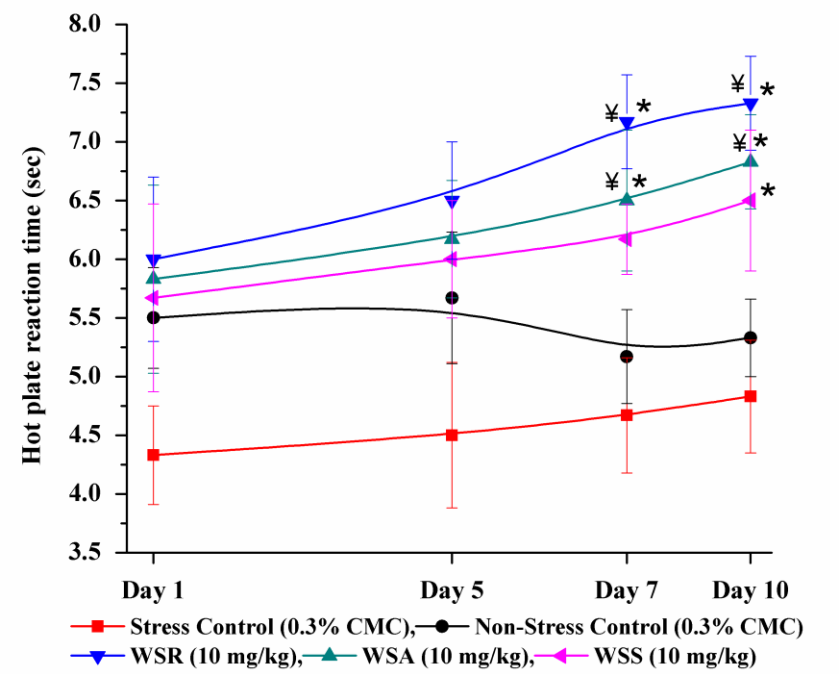

Figure 4.20: Effect of occasional exposures to short durations of foot shock stress on hot plate reaction time in male mice treated daily with three different types *Withania somnifera* extracts (WSR, WSA and WSS). Values are mean ± SEM (n=6). *=p<0.05 vs. stress control group and ¥=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.2.2.5. Tail suspension test: Results of this test performed on the 11th day of the experiment are summarized in **Figures 4.21**. Mean immobility time of the stressed control group was significantly higher than that of all three *Withania somnifera* extracts (10 mg/kg/day, p.o.) treated groups. Although, there were no statistically significant differences between the mean values of the three groups, this mean numerical value of the 10 mg/kg/day WSA treated group was somewhat higher than those of the groups treated with the same daily oral doses of WSR or WSS.

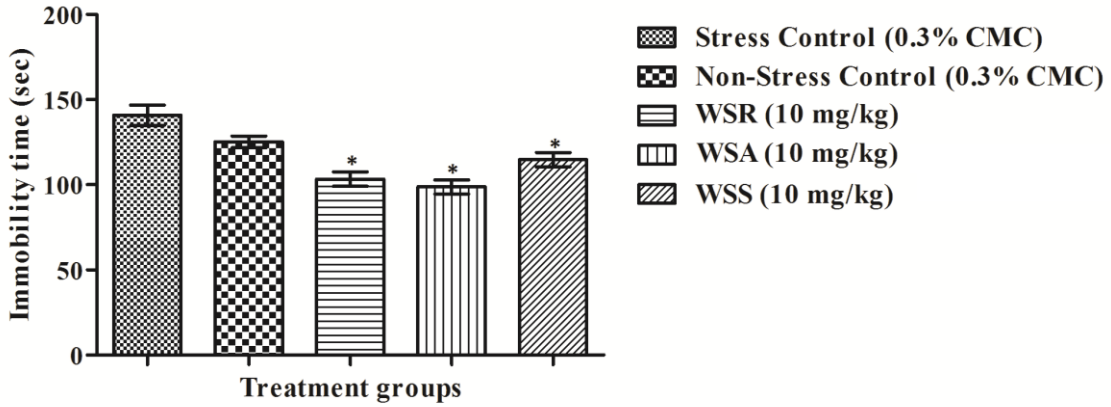

Figure 4.21: Effect of occasional exposures to short durations of foot shock stress on tail suspension test in male mice daily treated (orally) with three different extracts of *Withania somnifera* (WSR, WSA and WSS). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.2.2.6. Pentobarbital induced hypnosis test: Results summarized in **Table 4.4** revealed that mean onset time of sleep induced by pentobarbital (40 mg/kg; i.p.) in the two control groups were statistically significantly different from each other. As compared to this mean value of non-stress control group, that of the stressed control group was higher. However, the mean values for the duration of sleep in both control groups were statistically not significantly different from one another. Daily treatments with 10 mg/kg doses of any of the three *Withania somnifera* extracts did not have any effects on mean duration of sleep induced by pentobarbital. These mean values of the extract treated groups were of the same order of magnitude as those recorded for the non-stress control group. However, the time elapsed before sleep induction in all three *Withania somnifera* extract treated groups were statistically significantly different than those of the stressed or unstressed control groups.

Table 4.4: Effect of occasional exposures to short durations of foot shock stress on pentobarbital induced hypnosis on day 12 in female mice daily treated (orally) with three different extracts of *Withania somnifera* (WSR, WSA and WSS).

Treatment groups	Onset of sleep (sec)	Duration of sleep (min)
Stress Control (0.3% CMC)	180.0±0.40[¥]	44.33±3.10
Non-Stress Control (0.3% CMC)	130.2±0.31*	55.83±2.01
WSR (10 mg/kg)	160.2±0.42*[¥bc]	57.50±2.38
WSA (10 mg/kg)	139.8±0.33*[¥ac]	55.33±2.20
WSS (10 mg/kg)	169.8±0.30*[¥ab]	56.67±2.95

Values are mean ± SEM (n=6). *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group, [a]=p<0.05 vs. WSR (10 mg/kg), [b]=p<0.05 vs. WSA (10 mg/kg), [c]= p<0.05 vs. and WSA (10 mg/kg) (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.2.3. Results of the second sets of comparative experiments:

In the first of these experiments, the animals were preselected for their similar response time in hot plate test, and they were occasionally exposed to both unpredictable foot shock stress as well as noxious thermal stimuli in the hot plate test on days 1, 5, 7, and 10 of the experiment. Therefore, another experiment was conducted to verify the protective effects of the tested low doses of the extracts against occasional exposures to very short durations of foot shock stress only, and to compare their effectiveness in two versions of marble burying tests after their 11 or 12 daily oral doses in only in foot shock stressed mice. Observed protective effects of all three extracts against stress triggered alterations in body weights, basal core temperatures, and transient hyperthermic responses were qualitatively and quantitatively very similar to those observed in the first of these comparative experiments. Therefore, only the results of the marble burying tests are summarised here. They are graphically shown in the **Figures 4.22A & 4.22B.**

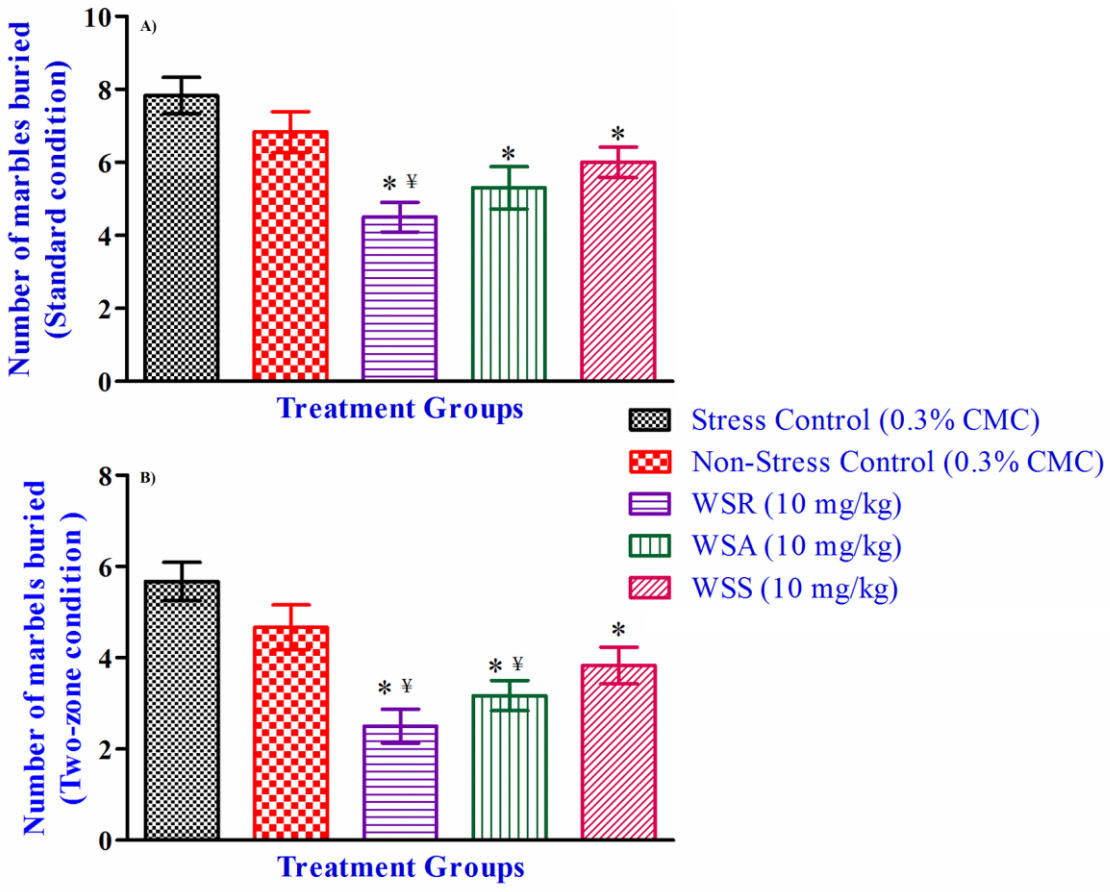

Figure 4.22: Effect of three different types of *Withania somnifera* extracts in mice (occasionally exposed to foot shock stressed only), and subjected to: **A)** Standard marble burying test on the 11th treatment day, and **B)** Two-zone version of marble burying test on the 12th treatment day. Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

Although mean numbers of marbles buried by the animals of the non stressed control group in both versions the test were lower than those of the stressed control one, there were no statistically significant effects of foot shock stress exposures on these numbers in both the test versions. These mean numbers of the WSR, WSA, and WSS were statistically significantly lower than those of the stressed control group in both versions of the test. These mean values for the WSR treated group only were statistically significantly lower than those of non-

stressed control group in both versions of the test. Such were the observations for WSA treated group in the two-zone version of the test only. These mean values for the stressed WSS treated group in both version of the test were statistically not significantly different from those of the non-stressed control. Although the mean values of WSR treated group was numerically lower than the WSA or WSS treated ones in both versions of the test, there were no statistically significant differences between these three mean values.

4.3. Adaptogenic activity of a *Withania somnifera* extract devoid of withanolides

It is now apparent that structurally and functionally diverse phytochemicals, other than withanolides, are also encountered in *Withania somnifera*, and several recent reports have revealed and reconfirmed Adaptogenic or stress response modulating effects of its extracts devoid of withanolides [B. Singh et al., 2001; 2003; S. Chatterjee et al., 2010; A. Bhatia et al., 2013]. Varying combinations of numerous such phytochemicals are biosynthesized and stored in terrestrial plants for defending themselves against predators and environmental stress [D. Maag et al., 2015]. Several of them are also well known for their bactericidal, antiviral, and anti-mitotic activities and for their ability to modulate the functions of diverse enzymes, receptors, and biological process regulating brain functions [D.O. Kennedy, 2014a; 2014b; V. Murugaiyah and M.P. Mattson, 2015; S. Davinelli et al., 2016].

During efforts of our research groups to better understand psychopharmacology of some Ayurvedic medicinal and food plants [S.S. Chatterjee and V. Kumar, 2012], a report suggesting that triethylene glycol (TEG) is a bioactive constituent of aqueous *Withania somnifera* root extracts had appeared [R. Wadhwa et al., 2013]. Since as yet only one report revealing the presence of TEG in a plant extract has appeared, the question whether or not it was an environmental contaminant in the root samples used in the reported study cannot yet

be answered with any certainty. However, the results of dose finding and other experiments conducted in our laboratories have suggested that like fumaric acid, quercetin, and other bactericidal and virucidal *Withania somnifera* extracts, repeated daily fairly low oral TEG doses (5 mg/kg/day) is also effective in suppressing diverse adverse stress responses [N. Shrivastava et al., 2015].

These observations, and numerous others made in our research groups with structurally diverse bactericidal virucidal phytochemicals often encountered in terrestrial plants [V. Yadav et al., 2015; A.J. Langstieh et al., 2014; S.A. Khan et al., 2016; A. Shakya et al., 2015b], strongly suggest that their modulating effects on gut microbial ecology are also involved in adaptogenic activities of *Withania somnifera* extracts and many of their bio-active constituents [A.K. Thakur et al., 2014b].

Although adaptogenic and other therapeutically interesting bioactivities of *Withania somnifera* rot extracts devoid of withanolides have been reported and reaffirmed, as yet only very little efforts have been made to better define their quantitative systems phytopharmacology and medicinal phytochemistry. Therefore, this experiment was conducted for assessing the therapeutically interesting doses and dosing regimen of a *Withania somnifera* root extract devoid of withanolides (WFWS), and to verify whether or not its stress response suppressing effects can also be quantified by estimating its repeated dose effects on circulating cortisol, glucose, and insulin levels. Since TEG one of the more potent and toxicologically safer antiviral agent with bactericidal activity [S.N. Rudnick et al., 2009; W. Lester et al., 1952], and it has been reported to be another bioactive constituent of *Withania somnifera* root extracts, it was used as a reference standard in the experiment. The results of this experiment are described in the following.

4.3.1. Body weights and basal core temperatures: Results graphically summarized in **Figures 4.23A & 4.23B** revealed that even the 3.3 mg/kg/day daily oral doses of WFWS is effective in preventing body weight losses of occasionally stressed mice, and that the rate of body weight gain in the group treated with its 100 mg/kg/day oral doses was almost identical to that of the unstressed control group. Such were also the observations made for the 10 mg/kg/day TEG treated group, the maximally effective daily doses of which in suppressing stress triggered alterations in body weight and basal core temperature in the recently reported dose finding experiment [N. Shrivastava et al., 2015] was also less than 5 mg/kg/day.

Results graphically summarized in **Figure 4.24** revealed that unlike the stressed control group, mean basal core temperatures of all others decreased steadily during the course of the experiment. On the last observational day this mean value of all WFWS treated group were statistically not significantly different from those of the non-stressed control group. These results reveal that although 3.3 mg/kg/days is high enough also for suppressing unpredictable foot shock stress triggered and longer lasting elevation in core temperature, its much hier daily oral doses do not induce hypothermia in stressed mice

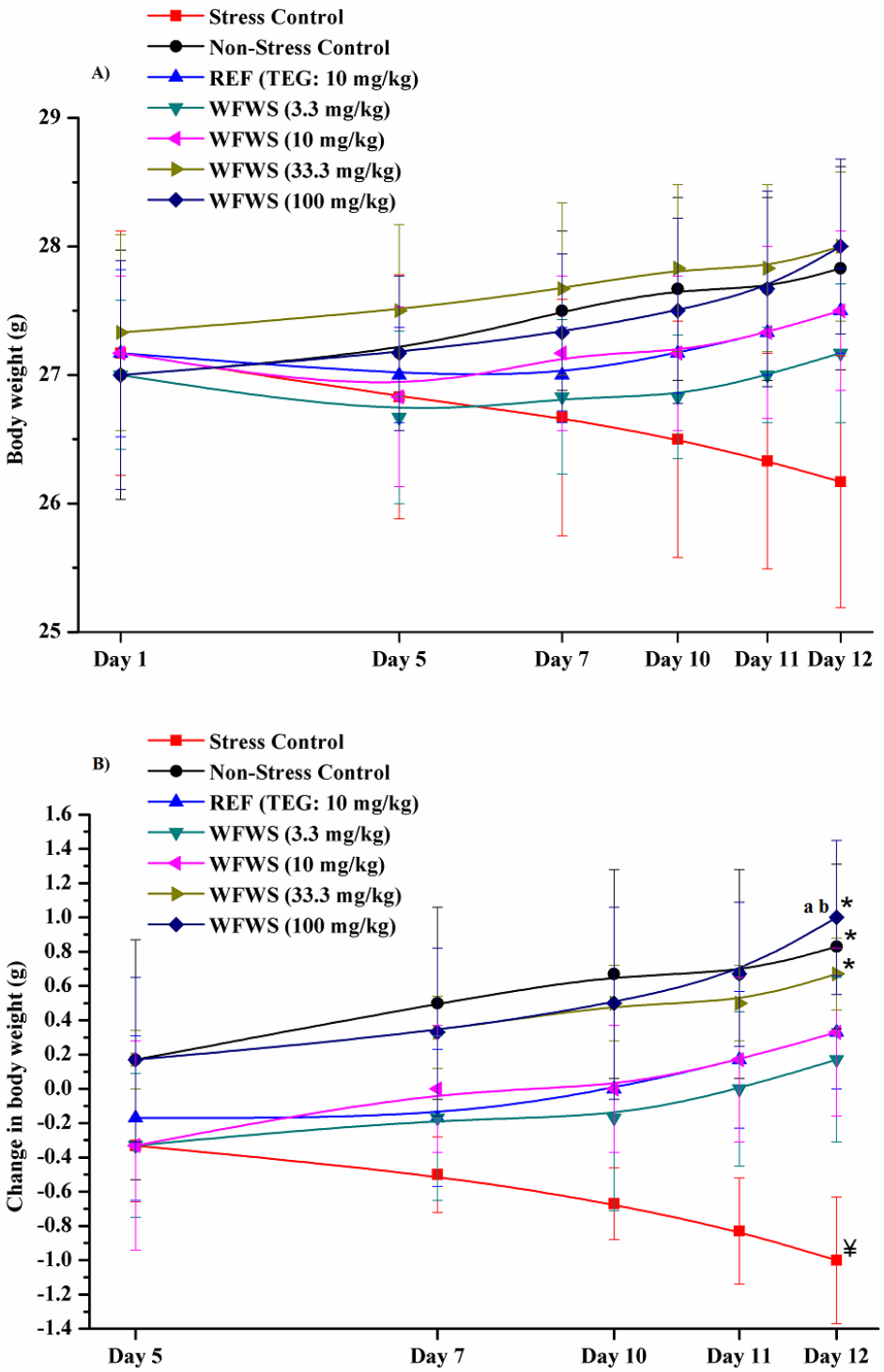

Figure 4.23: Effect of occasional exposures to short durations of foot shock stress on **A)** body weight and **B)** change in body weight of male mice treated with withanolides free *Withania somnifera* extract. Values are mean ± SEM (n=6). [a]=p<0.05 vs. WFWS (3.3 mg/kg), [b]=p<0.05 vs. WFWS (10 mg/kg), *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

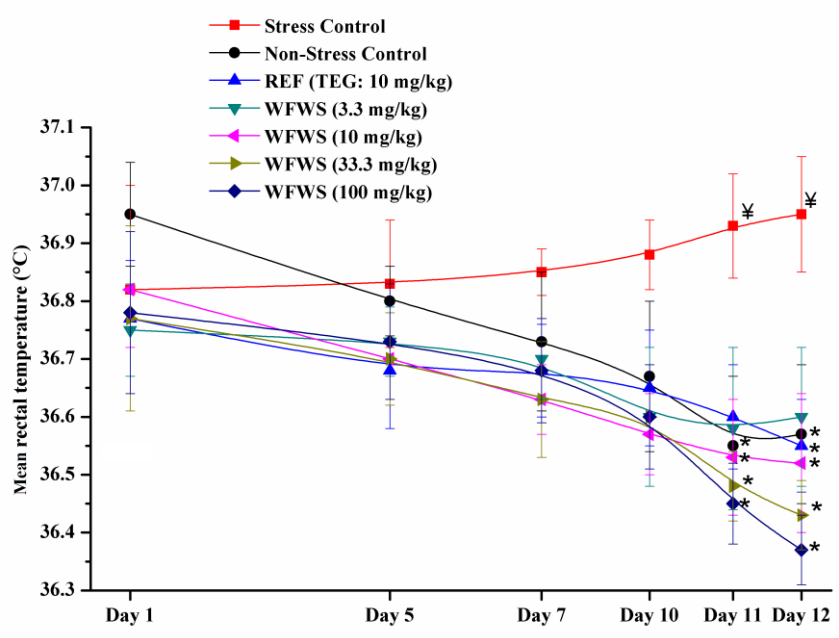

Figure 4.24: Effect of occasional exposures to short durations of foot shock stress on mean rectal temperatures of male mice treated with withanolides free *Withania somnifera* extract. Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.3.2. Stress triggered transient hyperthermic response: Results summarized in **Figure 4.25A** revealed that although numerically these values of the TEG or WFWS treated group on the first observational day were lower than that of the stressed control group, no statistically significant effects of TEG or WFWS were observed on this day. However, after twelve daily oral 3.3 or 10mg/kg doses of the extract, stress triggered transient hyperthermic response were statistically significantly lower than that of the stressed control group. Observe effectiveness of these two doses of the extract on this day were almost identical, and were somewhat lower than that of 10 daily oral doses of 10 mg/kg TEG. Like that observed for the TEG treated group, statistically significant effects of the 33.3 or 100 mg/kg WFWS doses were also observed on the 5th treatment day. Such effectiveness of these two WFWS treated groups was almost identical on all test days. From the dose response curves of WFWS in this

test shown in **Figure 4.25B** it is apparent that its effective daily dose range in suppressing foot shock stress induced hyperthermic responses is between 3 and 33.3 mg/kg, and that effectiveness of given daily oral dose of the extract increases also with increasing numbers of treatment days.

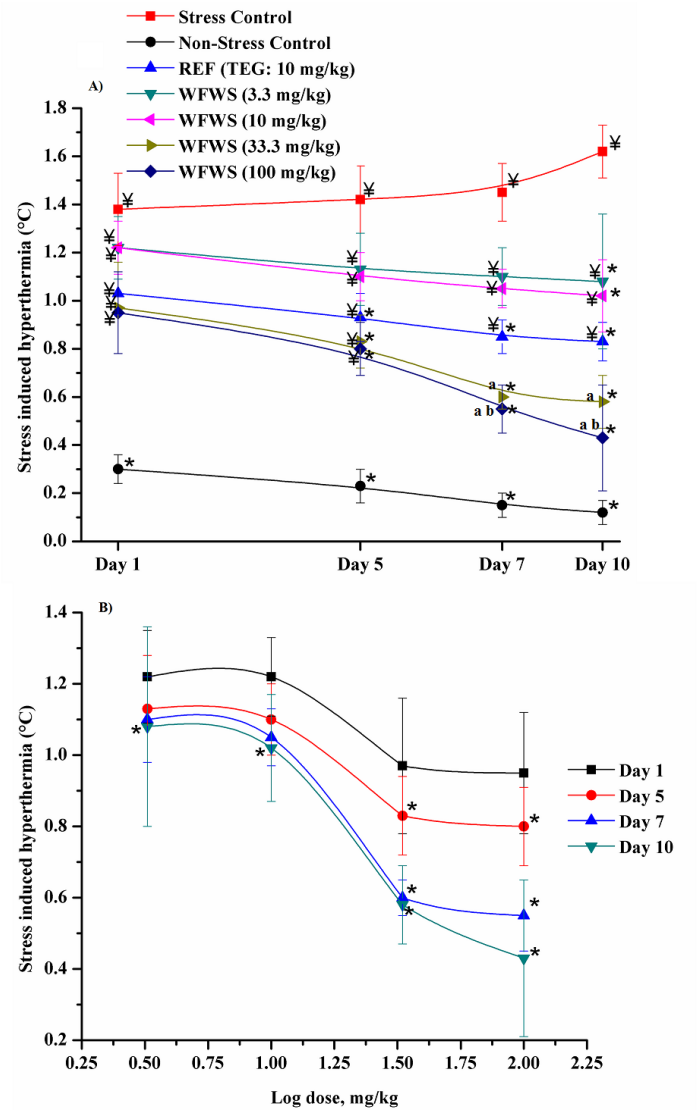

Figure 4.25: Effects of a *Withania somnifera* extract devoid of withanolides (WFWS) on foot shock stress triggered hyperthermic response after its single and repeated daily oral doses (A). Values are mean ± SEM (n=6). [a]=p<0.05 vs. WFWS (3.3 mg/kg), [b]=p<0.05 vs. WFWS (10 mg/kg), *=p<0.05 vs. stress control group and [¥]=p<0.05 vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test). Log dose response curves drawn from these values on the 1st, 5th, 7th and 10th test days (B). *=p<0.05 vs. the corresponding values on the first test day (Two way ANOVA followed by Bonferroni post hoc test).

4.3.3. Marble burying tests: Results of the standard and the two-zone version of the test conducted one hour after 11 and 12 daily oral treatment days are shown in the **Figures 4.26A** and **4.26B.** They revealed again that unlike in the standard version of the test the mean numbers of marbles buried by the stressed control group in the two-zone version test was statistically not significantly different from the number recorded for the non-stressed control group. These numbers for the TEG (10 mg/kg/day) or the two lower tested WFWS dose (3.3 and 10 mg/kg/day) treated stressed groups in both versions of the test were not statistically significantly different from those of the vehicle treated non-stressed group. However, these numbers for the 33.3 or 100 mg/kg/day WFWS treated groups in both the test versions were statistically significantly lower than those of the stressed control group. It was interesting to note though that these values of the two higher tested doses of WFWS were also statistically significantly lower than the non-stressed control group in the standard version of the test, and that this value of these two WFWS doses in both version of the test were almost the same. These observations reveal that like many adaptogenic herbal extracts and phytochemicals, daily oral doses of WFWS effective as anxiolytics or antidepressants in stressed mice are several folds higher (more than 10 mg/kg/day) than those necessary for suppressing stress triggered alterations in body weight and physiological thermoregulatory processes.

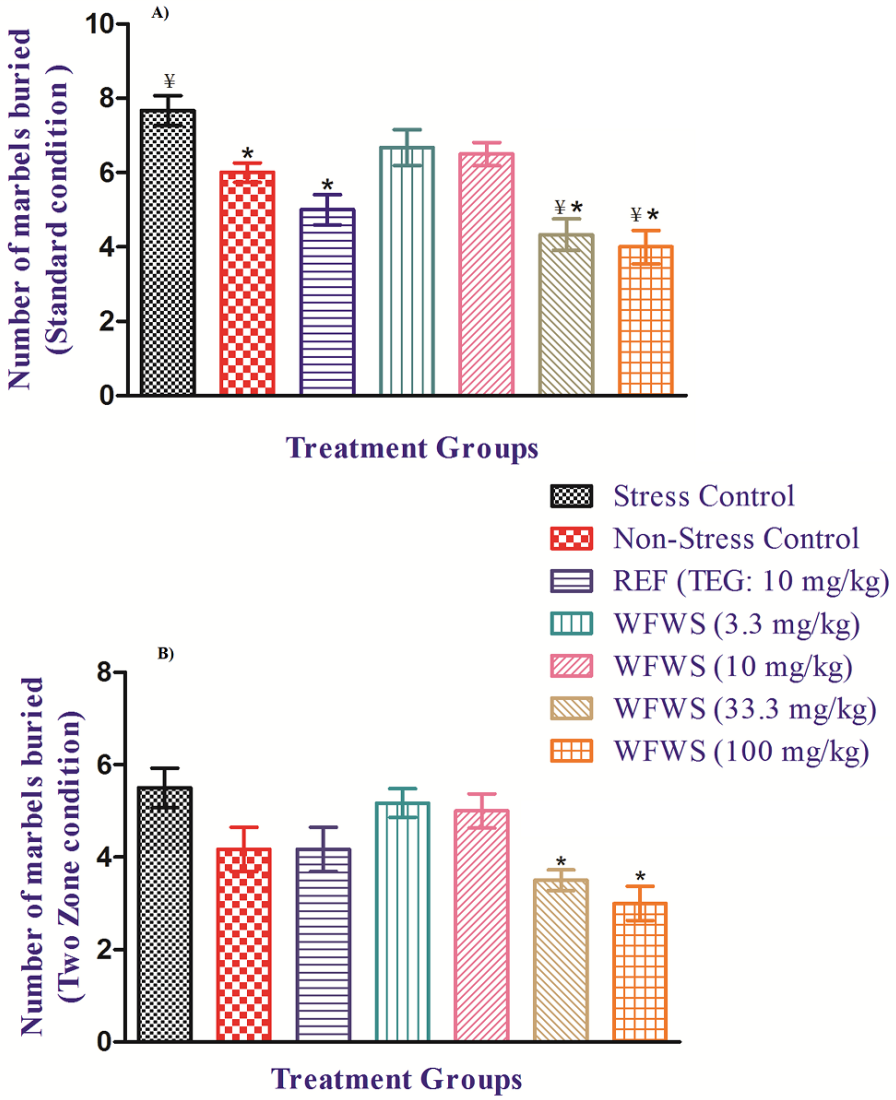

Figure 4.26: Effect daily treatments with withanolides free *Withania somnifera* extract in mice marble burying test: **A)** Standard condition on day 11 and **B)** Two-zone condition on day 12. Values are mean ± SEM (n=6). *=p<0.05 vs. stress control group and ¥=p<0.05 vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5.5. Organ weights and plasma glucose, insulin and corticosterone levels: The possibility that WFWS is a stress resistant promoting agent is also inferred from its observed preventive effects against stress triggered alterations in adrenal gland weight (**Table 4.5**) and plasma corticosterone level (**Table 4.6**). It was interesting to note also that mean plasma

glucose levels of stressed control group was slightly but significantly higher than the non stressed group, and that no such stress triggered hyperglycemic responses were observed in the 33.3 or 100 mg/kg/day WFWS treated stressed animals (**Table 4.6**). Since antidepressants or anxiolytic like effects of the extract were also observed after these two higher doses, this extract could as well be another therapeutic alternative for prevention of mental health problems of obese hyperglycemic patients. The observations that plasma insulin levels of neither the TEG nor WFWS treated groups were not statistically significantly different from that of the stressed control group indicate that its observed anti-stress activity is not due their effects on physiological processes regulating insulin homeostasis.

Table 4.5: Effects of a withanolides free *Withania somnifera* extract (WFWS) and triethylene glycol (TEG) on the weights of spleen and adrenal glands in foot shock stressed male mice**.**

Treatment groups	**Organ weights (mg)**		**Relative organ weights (mg/g of body weight)**	
	Spleen	**Adrenal glands**	**Spleen**	**Adrenal glands**
Non-Stress Control	92.80±2.07	14.03±0.60*	3.73±0.18	0.51±0.03*
Stress Control	96.78±1.53	19.45±0.85$^{¥}$	3.35±0.12	0.75±0.05$^{¥}$
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). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and $^{¥}$=$p<0.05$ vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

Table 4.6: Effects of a withanolides free *Withania somnifera* extract (WFWS) and triethylene glycol (TEG) on the plasma glucose, insulin and corticosterone levels in foot shock stressed male mice.

Treatment groups	**Glucose (mg/dl)**	**Insulin (µIU/ml)**	**Corticosterone (ng/ml)**
Non-Stress Control	82.81±1.60*	19.40±1.27*	87.50±2.04*
Stress Control	97.00±1.47¥	14.55±0.84¥	96.57±1.49¥
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 100g of body weight)**		
	Glucose (mg/dl)	**Insulin (µIU/ml)**	**Corticosterone (ng/ml)**
Non-Stress Control	299.27±13.02*	69.89±4.65	315.56±11.09*
Stress Control	373.81±17.48¥	56.39±4.87	370.87±10.12¥
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). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.4. Effects of co-administration of triethylene glycol with *Withania somnifera* root extract.

The antiviral agent triethylene glycol (TEG) is a minor impurity of commercially available emulsifier polyethylene glycol often used in preclinical studies for suspending water insoluble test agents or for preparing their pharmaceutical formulations for clinical studies. The reports suggesting that TEG is a bioactive constituent of *Withania somnifera* root extract [R. Wadhwa et al., 2013], and observations in our laborites revealing that oral 5 mg/kg/day TEG doses is high enough for suppressing foot shock stress triggered alteration in bodyweight and core temperature [N. Shrivastava et al., 2015], triggered interest to verify the

possibility whether co-administration TEG and *Withania somnifera* root extracts could be a more effective option for increasing stress tolerance or not. For such purposes, three different sets of experiments were conducted, in which one of the three graded doses of TEG (4, 12, 36 mg/kg) were used in one set of experiment. In these experiments, one of the selected dose was administered in combination with either 1 or 3 or 10 mg/kg daily oral doses of *Withania somnifera* root extracts (WSR) for 12 consecutive days. Except for the non-stressed control group of the experiment, all others were subjected to a stress induced hyperthermia test on the 1st, 5th, 7th and 10th day of the treatments, and on the 11^{th} and 12^{th} treatment days, they were subjected to two different versions of marble burying tests. Effects of treatments on chronic unpredictable foot shock stress triggered alterations in body weights and basal rectal temperatures during the course of the experiment were quantified. The effect of treatments on blood glucose, insulin and corticosterone were evaluated and the adrenal glands and spleen weights were also quantified. Results of this experiment are described in the following.

4.4.1. Body weights: Results summarized in the **Figure 4.27A** revealed that even 1 mg/kg daily oral doses of WSR antagonizes foot shock stress triggered body weight losses and that protective effects of this low dose were more pronounced when co-administration with 12 mg/kg or more daily doses of TEG (**Figure 24.27A**). Analogous were the effects of co-administration of TEG and 3 mg/kg daily oral doses of the extract (**Figure 24.27B**). However, the rates of body weight gains in the 10 mg/kg/day WSR treated co-administered with all tested TEG doses were almost equal and not more than those recorded for the unstressed control group (**Figure 4.27C**).

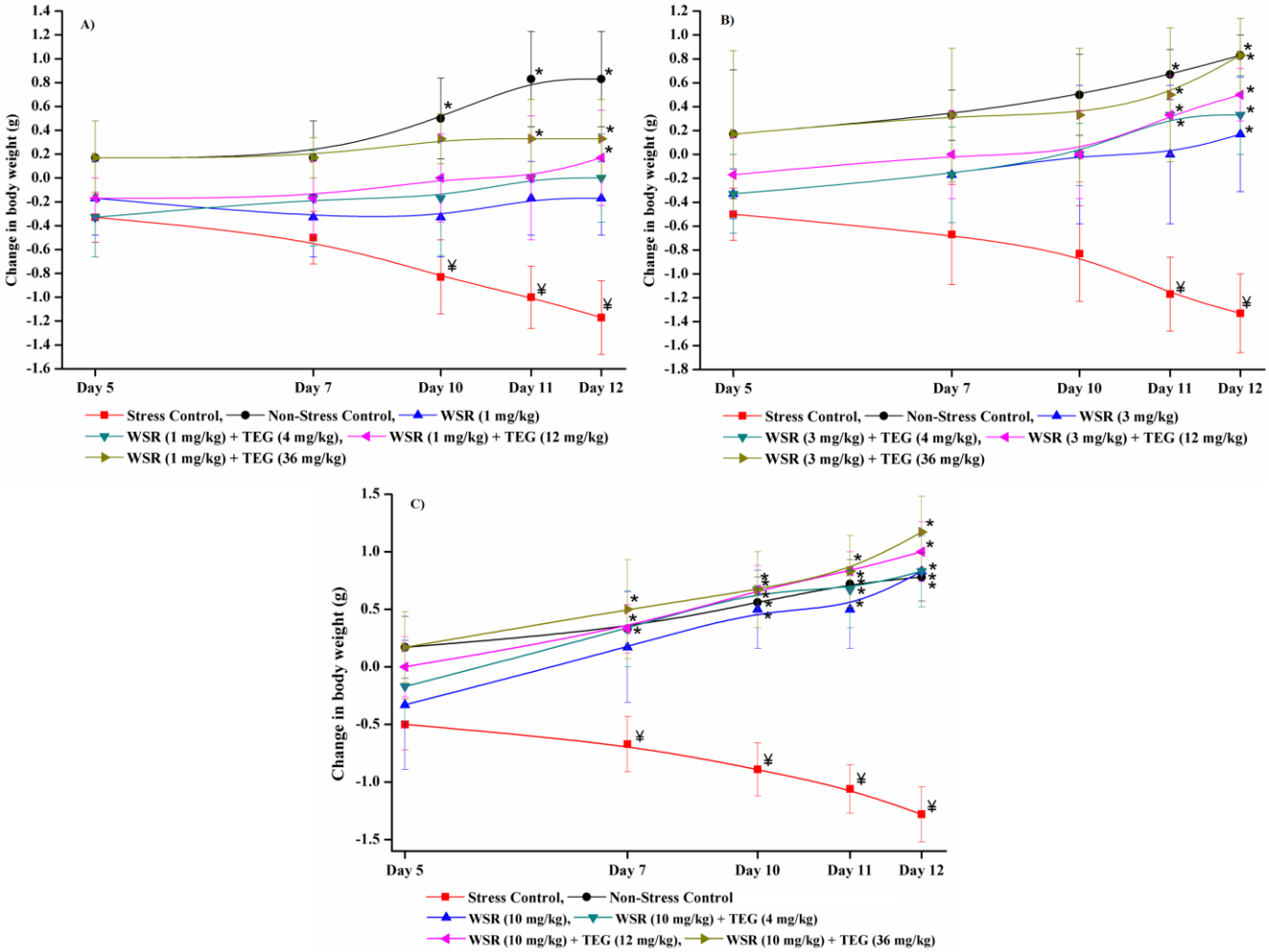

Figure 4.27: Effect of occasional exposures to short durations of foot shock stress on change in body weight of male mice treated with combinations of graded oral doses of TEG (4, 12, 36 mg/kg/day, p.o.) and **A)** 1 mg/kg/day of WSR, or **B)** 3 mg/kg/day of WSR, or **C)** 10 mg/kg/day of WSR. Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.4.2. Basal Rectal temperatures: These results are summarized in the **Figure 4.28.** As expected, mean basal core temperature of the stressed control groups increased steadily during the course of the experiment, whereas those of the non-stressed ones continued to decreases. Mean basal core temperatures of all WSR treated groups, co-administered with TEG or not, on all observational days were statistically not significantly different from those of the non-stressed and vehicle treated groups.

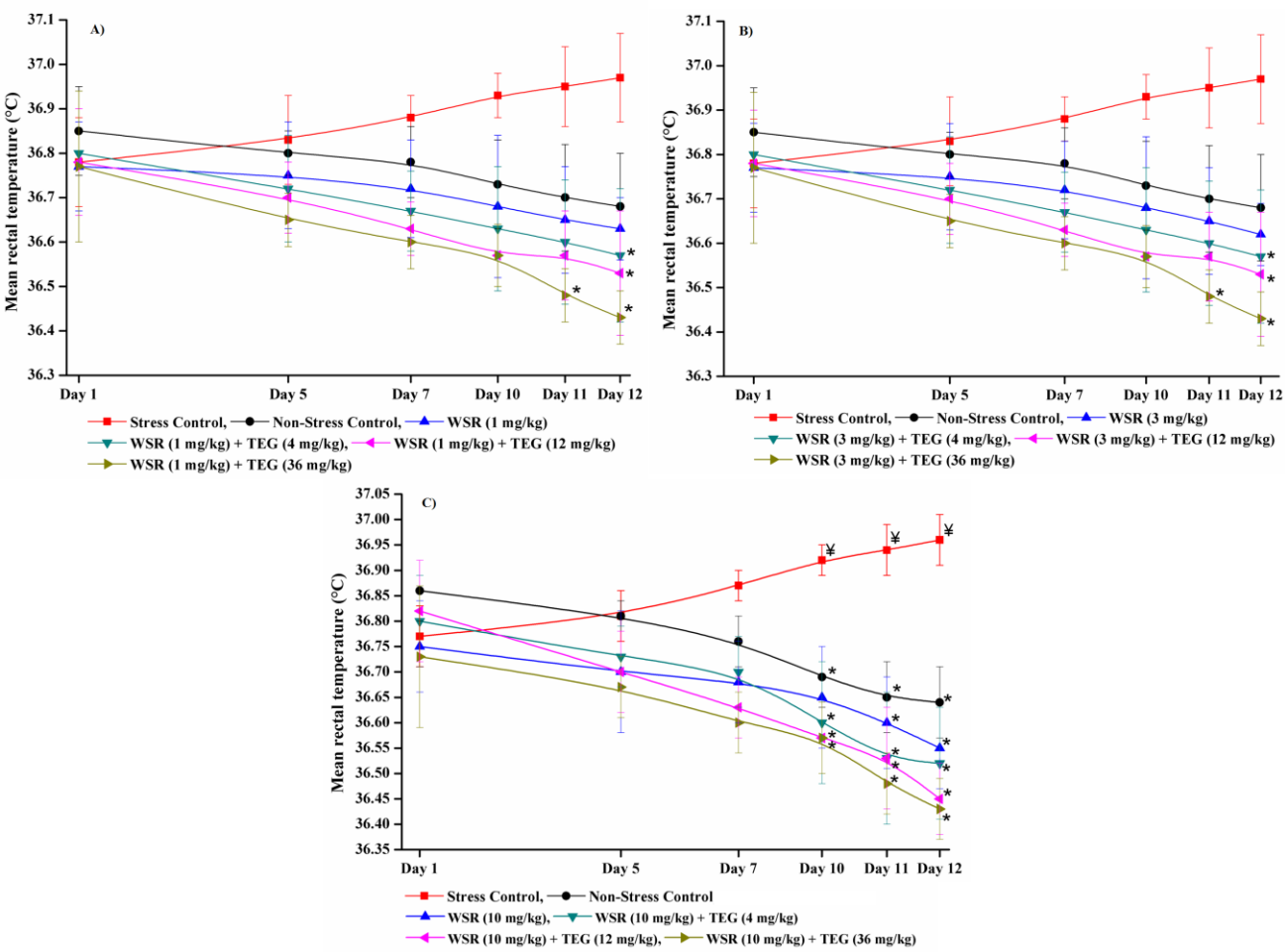

Figure 4.28: Effect of occasional exposures to short durations of foot shock stress on mean rectal temperature of male mice treated with combination of graded oral doses of TEG (4, 12, 36 mg/kg/day, p.o.) and **A)** 1 mg/kg/day of WSR;, or **B)** 3 mg/kg/day of WSR, or **C)** 10 mg/kg/day of WSR. Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.4.3. Foot shock stress induced hyperthermia: These results are summarised in **Figure 4.29**. In these experiments, daily dose and number of daily doses dependant inhibitory effects of WSR against stress triggered transient hyperthermic responses were observed after more than 5 days of treatments. After 10 daily treatments, its statistically significant inhibitory effects in this test were observed even after its 3mg/kg daily doses, and numerically the mean values of the of the TEG and WSR co-administered groups were always lower than those of

the extract only treated groups. However, statistically significant potentiating effects of TEG co-administration were observed only after co-administration with in the 1 mg/kg/day WSR doses.

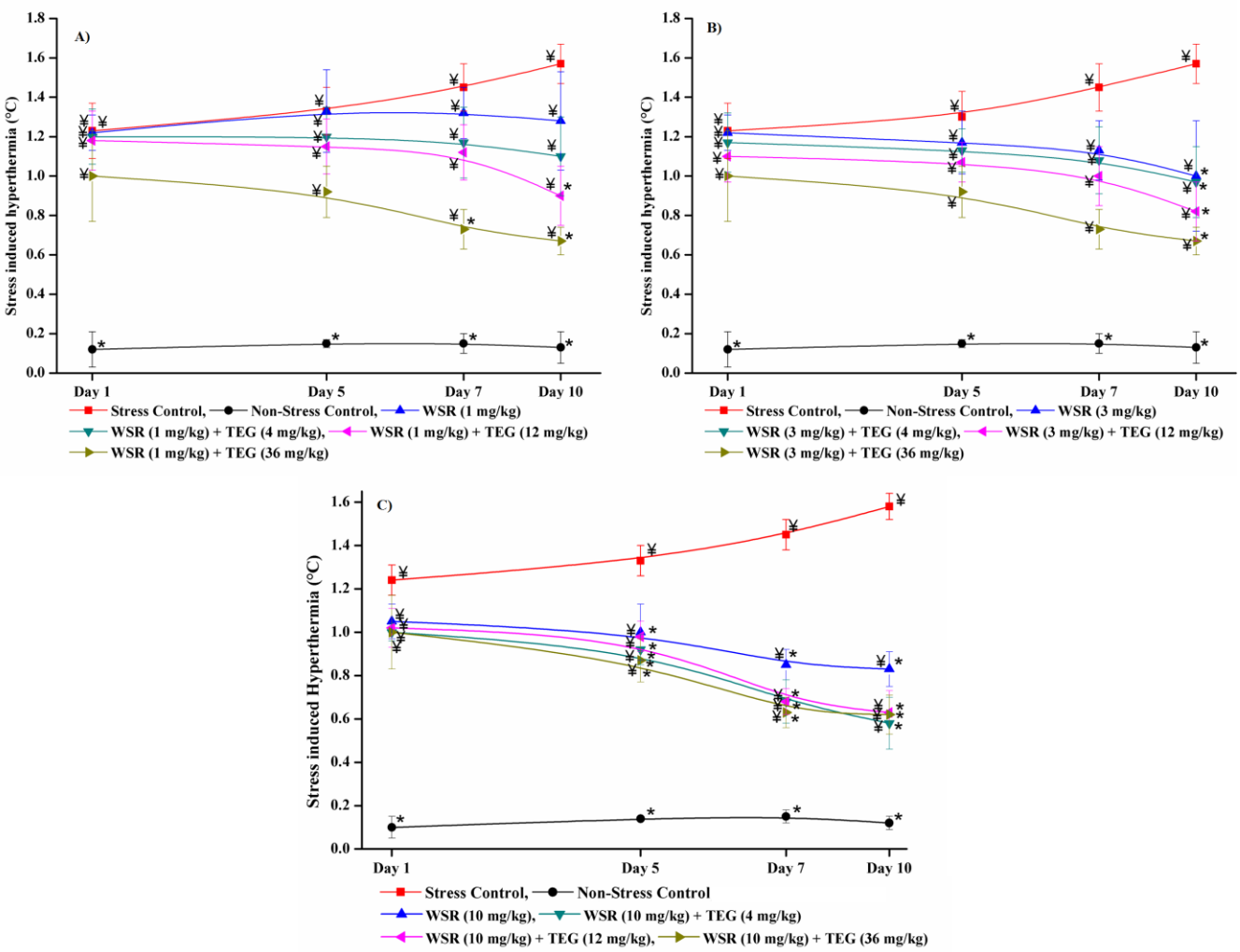

Figure 4.29: Effect of occasional exposures to short durations of foot shock stress on stress induced hyperthermia in male mice daily treated with combination of graded oral doses of TEG (4, 12, 36 mg/kg/day, p.o.) and **A)** 1 mg/kg/day of WSR, or **B)** 3 mg/kg/day of WSR, or **C)** 10 mg/kg/day of WSR. Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (Two way ANOVA followed by Bonferroni post hoc test).

4.4.4. Marble burying tests: Results of the standard version of the marble burying test conducted one hour after eleven daily oral treatments revealed that the mean numbers of the marbles buried by the animals of the stressed control group was statistically significantly higher than that of the non-stressed control group (**Figure 4.30**). Statistically significant protective effects of WSR against stress triggered exaggerated marble burying behavior was observed only after its 10 mg/kg daily doses. However, in combination with 12 or 36 mg/kg/day TEG, such statistically significant effectiveness of WSR were also observed after its co administered 1 or 3 mg/kg daily doses (**Figure 4.30A and 4.30B**).

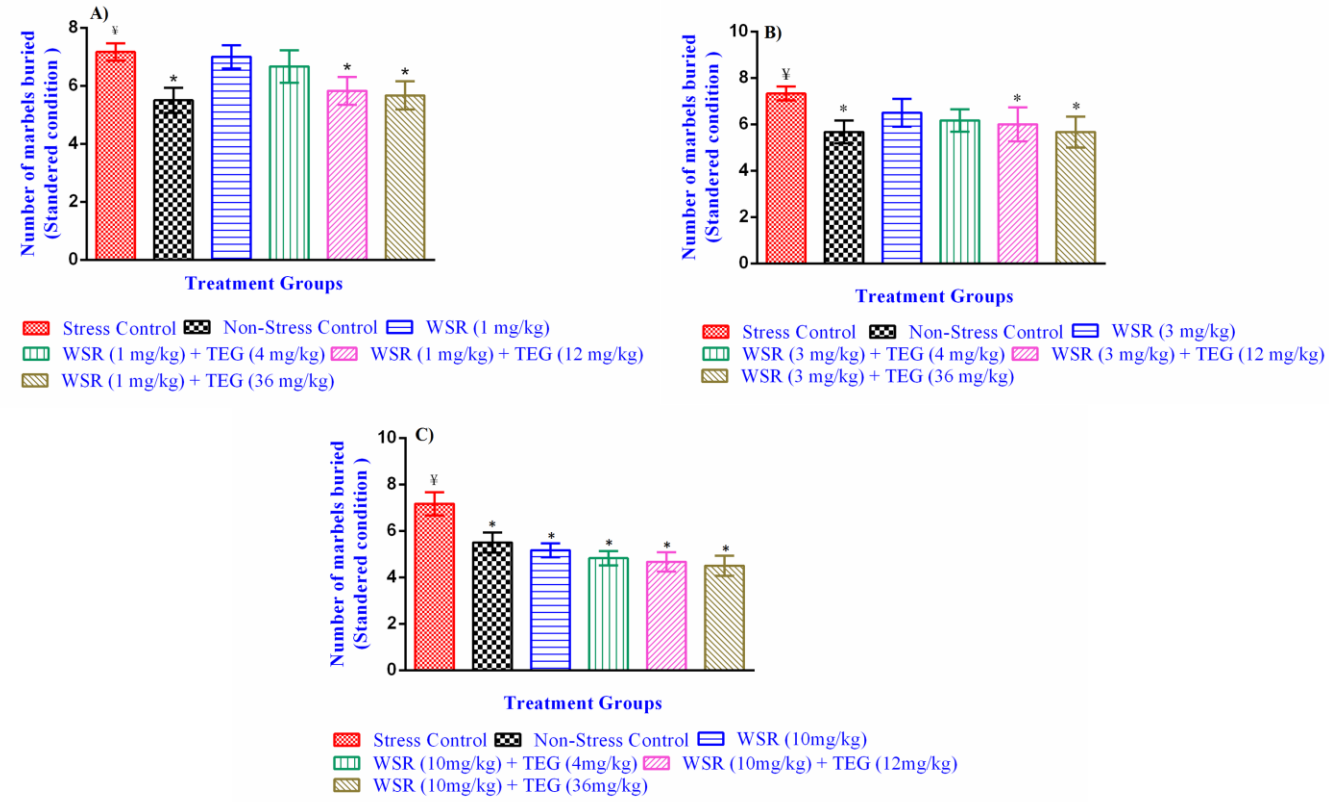

Figure 4.30: Effect of combination of graded oral doses of **A)** TEG (4, 12, 36 mg/kg/day, p.o.) and 1 mg/kg of WSR or **B)** TEG (4, 12, 36 mg/kg/day, p.o.) and 3 mg/kg of WSR or **C)** TEG (4, 12, 36 mg/kg/day, p.o.) and 10 mg/kg of WSR in mice (occasionally exposed to foot shock stressed only), and subjected to standard version of marble burying test on day 11. Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

Results of the two zone version of the test conducted on the last days of the experiments are summarised in **Figure 4.31**. In this version of the test, statistically significant stress triggered alterations in marble burring behaviour of vehicle treated control groups were observed only in one experiment, and in this experiment the mean values of the 10 mg/kg/day WSR only treated group was numerically almost identical to those of the non-stressed control group (**Figure 4.31C**). Although co-administration of this dose WSR dose with TEG increased the effectiveness of the extract, such potentiating effects of TEG did not increase with its co-administered daily doses. These results reaffirm our earlier observations [N. Shrivastava et al., 2015] suggesting that the standard version of marble burying test is more sensitive and reproducible for quantifying stress triggered alterations in compulsive behaviour of rodents that the two zone version.

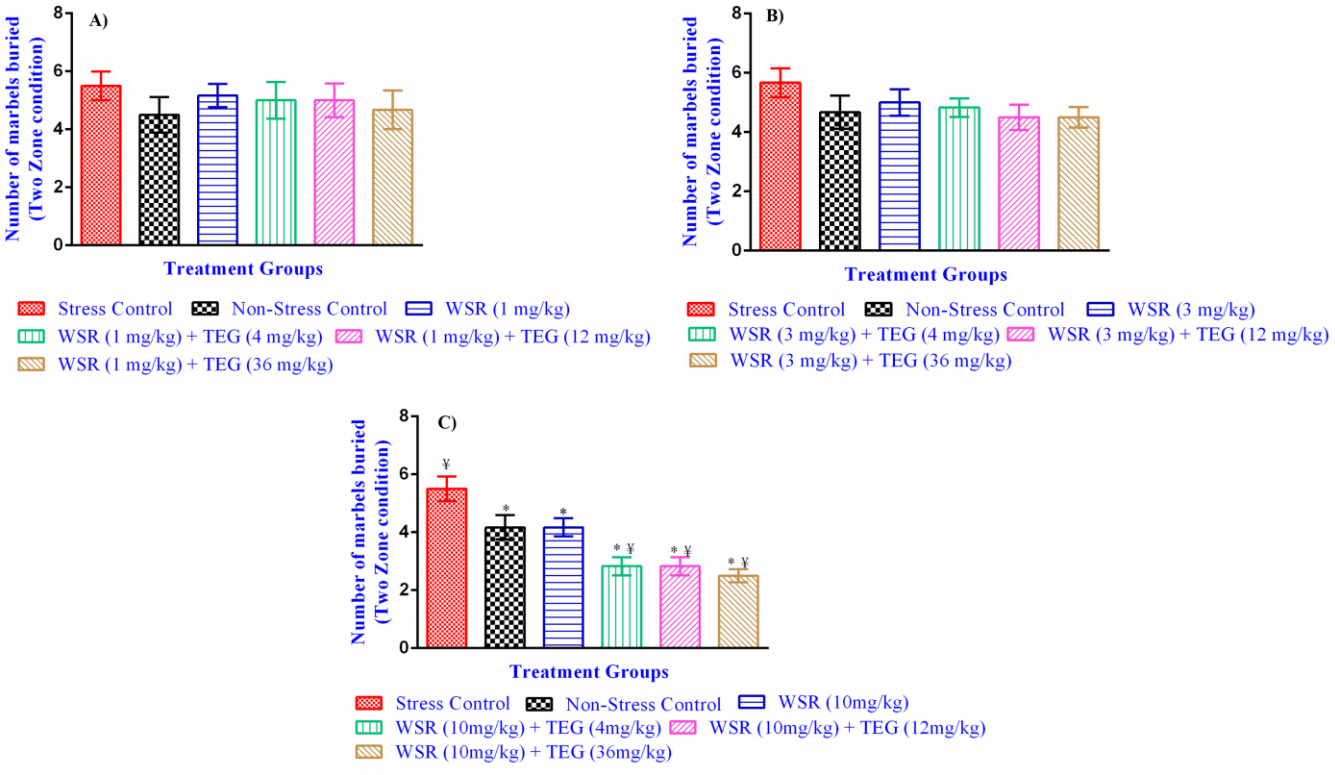

Figure 4.31: Effect of combination of graded oral doses of **A)** TEG (4, 12, 36 mg/kg/day, p.o.) and 1 mg/kg of WSR or **B)** TEG (4, 12, 36 mg/kg/day, p.o.) and 3 mg/kg of WSR or **C)** TEG (4, 12, 36 mg/kg/day, p.o.) and 10 mg/kg of WSR in mice (occasionally exposed to foot shock stressed only), and subjected to two zone version of marble burying test on day 12. Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.4.5. Organ weights and plasma glucose, insulin, and corticosterone levels: The possibility that TEG could potentiate stress resistant promoting effects of WSR is suggested also by the observed effects of its co-administrations on organ weights, and plasma glucose, insulin and corticosterone levels. Adrenal gland or spleen hypertrophy observed in stressed control groups were of same magnitude in all WSR alone or WSR + 4 mg/kg/day TEG treated groups (**Table 4.7**). However, quantitatively such hypertrophies were statistically significantly less pronounced in all WSR+ 12 or 36 mg/kg/day TEG treated ones. Such

potentiating effects of TEG were more consistent against stress triggered adrenal gland hypertrophy, which is a more specific indicator of stress responses in laboratory rodents.

Table 4.7: Effects of combination of graded oral doses of triethylene glycol (TEG) and *Withania somnifera* root extract (WSR) on the weights of spleen and adrenal glands in foot shock stressed male mice.

Treatment groups	Organ weights (mg)		Relative organ weights (mg/g of body weight)	
	Spleen	Adrenal glands	Spleen	Adrenal glands
Experiment A: TEG (4, 12, 36 mg/kg/day, p.o.) and 1 mg/kg of WSR				
Non-Stress Control	91.27±1.78*	15.58±1.09*	3.31±0.10*	0.57±0.04*
Stress Control	97.94±1.93¥	20.85±1.70¥	3.80±0.13¥	0.81±0.07¥
WSR (1 mg/kg)	94.70±1.18	18.32±2.33	3.58±0.06¥	0.69±0.08
WSR (1 mg/kg) + TEG (4 mg/kg)	94.58±1.13	16.48±1.78	3.60±0.09	0.62±0.06
WSR (1 mg/kg) + TEG (12 mg/kg)	93.60±1.39	16.32±1.18	3.49±0.02*	0.61±0.05*
WSR (1 mg/kg) + TEG (36 mg/kg)	93.35±1.28	15.70±1.20*	3.51±0.09*	0.59±0.05*
Experiment B: TEG (4, 12, 36 mg/kg/day, p.o.) and 3 mg/kg of WSR				
Non-Stress Control	92.94±1.17*	16.58±1.25*	3.32±0.07*	0.59±0.05*
Stress Control	98.27±1.99¥	21.28±1.49¥	3.91±0.20¥	0.85±0.06¥
WSR (3 mg/kg)	97.70±1.67	19.48±2.17	3.62±0.13	0.72±0.09
WSR (3 mg/kg) + TEG (4 mg/kg)	96.45±1.47	18.82±2.24	3.49±0.08	0.68±0.08
WSR (3 mg/kg) + TEG (12 mg/kg)	96.93±1.50	18.32±0.90	3.56±0.07¥	0.68±0.05
WSR (3 mg/kg) + TEG (36 mg/kg)	96.68±1.25	16.60±0.97*	3.42±0.09*	0.62±0.03*
Experiment C: TEG (4, 12, 36 mg/kg/day, p.o.) and 10 mg/kg of WSR				
Non-Stress Control	93.60±1.88*	16.58±1.25*	3.33±0.11*	0.59±0.05*
Stress Control	98.87±1.05¥	21.62±0.96¥	3.96±0.08¥	0.85±0.04¥
WSR (10 mg/kg)	96.70±2.70	18.98±2.23	3.45±0.14	0.67±0.07
WSR (10 mg/kg) + TEG (4 mg/kg)	95.23±2.85	18.52±2.31	3.36±0.10	0.66±0.09
WSR (10 mg/kg) + TEG (12 mg/kg)	94.93±1.05	17.65±1.11	3.36±0.10	0.63±0.05*
WSR (10 mg/kg) + TEG (36 mg/kg)	94.18±2.12	16.13±1.17*	3.29±0.10	0.60±0.04*

Values are mean ± SEM (n=6). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

Stress triggered hyperglycemia and hyper-corticosteronemia and hypoinsulinemia were apparent in the vehicle treated stressed control groups (**Table 4.8**). Statistically significant protective effect of WSR against hyper-cotricosteronemia was observed only after its 10 mg/kg/day doses, and such were also the observations made for the WSR + 12 or 36 mg/kg/day TEG treated groups. Although stress triggered alterations in blood glucose (hyperglycemia) and insulin (hypoinsulinemia) levels of any of the WSR alone treated groups were statistically not significantly different than the vehicle treated stressed control groups, these mean values of all WSR alone or WSR+TEG treated ones were always lower (glucose) and higher (insulin) than the corresponding stressed control ones. These observations could indicate that WSR itself or its combinations with TEG could also have modulatory effects on physiological processes and mechanisms regulating glucose and insulin homeostasis, and that their observed protective effects on stress triggered alterations in bodyweight and thermoregulatory process are most probably independent of their antistress activities.

Table 4.8: Effects of combination of graded oral doses of triethylene glycol (TEG) and *Withania somnifera* root extract (WSR) on the plasma glucose, insulin and corticosterone levels in foot shock stressed male mice.

Treatment groups	**Relative values (per 100g of body weight)**		
	Glucose (mg/dl)	**Insulin (µIU/ml)**	**Corticosterone (ng/ml)**
Experiment A: TEG (4, 12, 36 mg/kg/day, p.o.) and 1 mg/kg of WSR			
Non-Stress Control	300.09±13.77*	77.66±6.54*	324.27±13.26*
Stress Control	372.99±9.77¥	58.96±3.21¥	380.47±10.38¥
WSR (1 mg/kg)	358.10±10.79¥	64.94±2.34	355.05±6.28
WSR (1 mg/kg) + TEG (4 mg/kg)	356.03±7.32¥	68.08±4.48	355.49±9.67
WSR (1 mg/kg) + TEG (12 mg/kg)	349.29±11.52¥	69.77±3.23*	346.16±3.83*
WSR (1 mg/kg) + TEG (36 mg/kg)	343.26±9.40¥	71.11±2.44*	342.78±12.85*
Experiment B: TEG (4, 12, 36 mg/kg/day, p.o.) and 3 mg/kg of WSR			
Non-Stress Control	298.67±12.04*	75.42±6.43*	311.69±8.01*
Stress Control	383.14±12.78¥	58.91±3.45¥	386.79±13.79¥
WSR (3 mg/kg)	356.76±15.56¥	60.84±4.81	354.15±13.01¥
WSR (3 mg/kg) + TEG (4 mg/kg)	344.43±8.32*¥	59.43±2.66¥	343.44±8.04*¥
WSR (3 mg/kg) + TEG (12 mg/kg)	349.91±14.54¥	60.95±3.90	347.43±13.45*¥
WSR (3 mg/kg) + TEG (36 mg/kg)	335.32±11.29*	62.34±4.12	334.13±6.78*
Experiment C: TEG (4, 12, 36 mg/kg/day, p.o.) and 10 mg/kg of WSR			
Non-Stress Control	291.17±12.75*	68.82±5.49*	304.05±10.16*
Stress Control	357.04±9.80¥	58.37±2.58¥	359.67±8.81¥
WSR (10 mg/kg)	325.48±10.33¥	55.82±4.02	322.36±6.12*
WSR (10 mg/kg) + TEG (4 mg/kg)	318.77±6.84¥	57.42±2.61	310.89±4.48*
WSR (10 mg/kg) + TEG (12 mg/kg)	318.85±8.33¥	57.61±4.45	308.30±10.04*
WSR (10 mg/kg) + TEG (36 mg/kg)	311.01±10.25¥	59.79±4.49	301.41±5.00*

Values are mean ± SEM (n=6). Values are mean ± SEM (n=6). *=$p<0.05$ vs. stress control group and ¥=$p<0.05$ vs. non-stress control group (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5. Metformin like activity profile of withanolides rich and withanolides free roots extracts of *Withania somnifera* in stressed diabetic rats

The described dose finding experiments had consistently revealed and reaffirmed that 10 mg/kg daily oral doses of diverse types of *Withania somnifera* extracts are high enough for preventing stress triggered alterations in body weight and temperature, and that their such or higher daily doses are also effective as centrally acting analgesic, anxiolytics, and/or antidepressants like and anti-hyperglycemic agents in stressed mice. Such low dose activity profiles of several other food plants often used for prevention and cure of diabetes and obesity in Ayurvedic and other traditionally known systems of medicine and health care have also often been observed and reaffirmed in our laboratories and elsewhere [V. Kumar et al., 2015b; S.S. Chatterjee, 2015]. Analogous activity profiles of 50 mg/kg/day or higher doses of the anti-diabetic drug metformin in foot shock stressed mice have also been observed in our research groups [S. Verma et al., 2015; 2017]. Therefore, three further experiments were conducted for comparing the stress response protective potentials of 50 mg/kg/day metformin with those of 10 mg/kg daily oral doses of the *Withania somnifera* extracts containing withanolides (WSR) or devoid of them (WFWS) in diabetic rats. Choices of biochemical and other parameters quantified in these experiments were base on our current knowledge on the modes of actions of metformin and *Withania somnifera* extracts evolving from observations made in diverse *ex vivo*, *in vitro* and other cellular models now often used for such purposes, or for better understanding of biological processes involved in diabetes associated comorbidities. Results of these three sets of experiments will be described in the following.

4.5.1. Anti-stress activity: In this experiment**,** effects of ten daily oral doses of the test agents (WSR, WFWS and metformin) were compared in diabetic rats using the foot-shock stress paradigm often used in our laboratories for dose finding studies or for identifying stress

response modulating components of herbal extracts. Apart from bodyweights and body temperature, the effects of treatments on chronic hyperglycaemia, blood lipid profiles, alterations in body weights and basal rectal temperatures and level of inducible nitric oxide synthase (iNOS) and nitric oxide and during the course of the experiment were quantified also.

4.5.1.1. Body weights: Mean body weights of the experimental groups on different treatment days, and calculated mean changes in body weights of the groups on the 5th, 7th, 10th, 11th and 12th day of the treatments are summarized in **Figures 4.32A & 4.32B**. These mean values of the stressed control groups (both diabetic and non-diabetic) decreased continuously during the course of the experiments, whereas those of the non-stressed non-diabetic control group continued to increase. Such intermittent foot shock stress-triggered body weight losses in the stressed diabetic groups were compensated after seven daily oral doses (10 mg/kg) of both withanolides rich and withanolides free *Withania Somnifera* extracts and metformin (50 mg/kg) as well. Rate of body weight changes of 10 mg/kg/day WFWS or WSR treated groups observed during the course of the experiment were quite analogous, and their observed protective effectiveness were like that observed in the 50 mg/kg/day metformin treated one (**Figure 4.32B)**. These observations made in diabetic rats were analogues to those observed in male or female non-diabetic mice with diverse types of *Withania somnifera* extracts, and using the same foot shock stress paradigm and treatment regimen. Thus it can safely be said that physiological processes and mechanisms involved in stress triggered alterations in body weights of diabetic rats are most probably the same, or analogous, as those in mice.

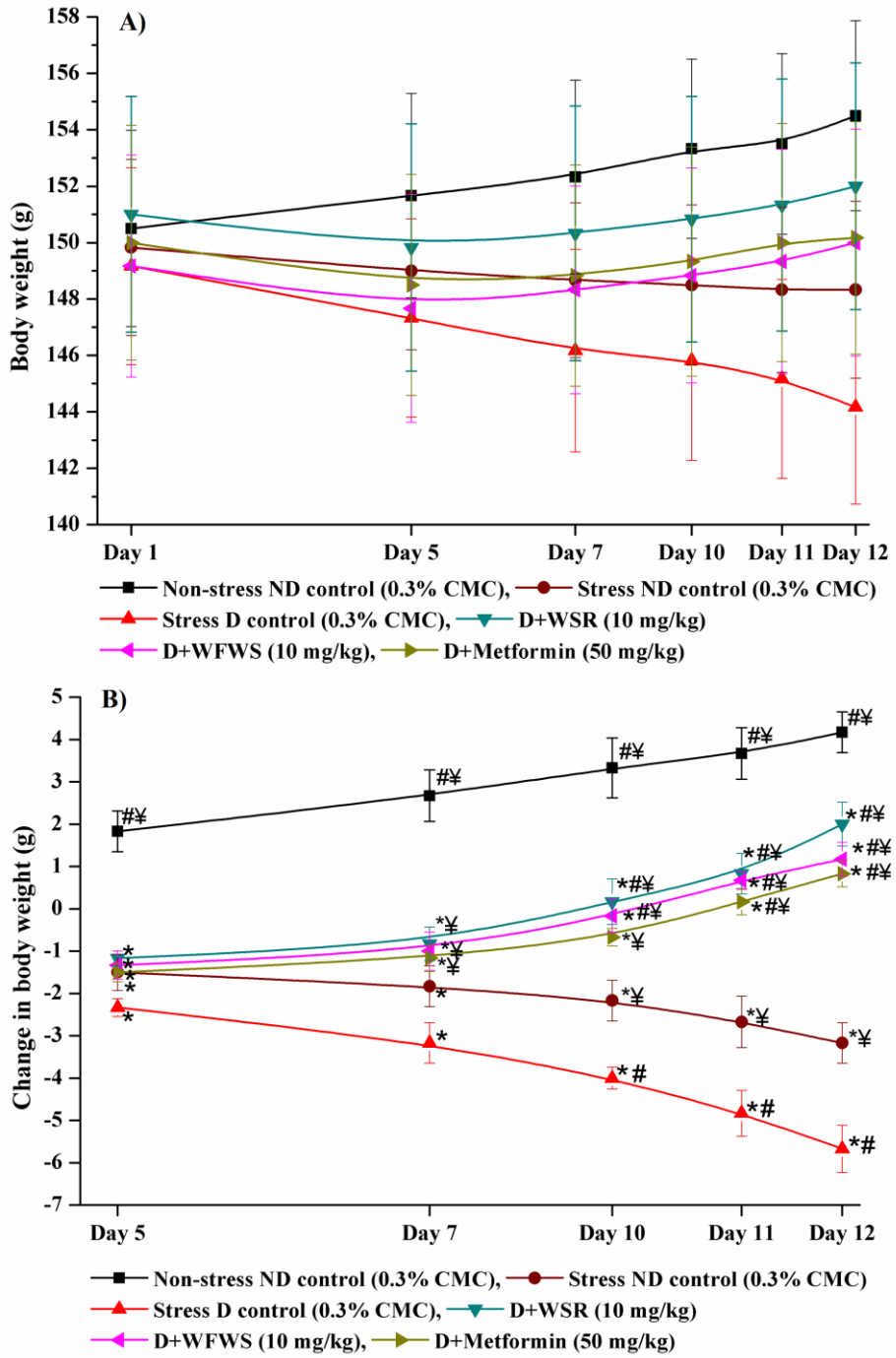

Figure 4.32: Effect of occasional exposures to short durations of foot shock stress on **A)** mean body weight and **B)** change in body weight of normal and diabetic rats daily treated with withanolides containing and withanolides free *Withania somnifera* extracts and metformin. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress non-diabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress nondiabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.1.2. Basal core temperatures: Results summarised in **Figure 4.33** revealed that like in mice, mean rectal temperatures of both the stressed control rat groups continued to increase slightly from the fifth observational days onwards, whereas slight but continuous decrease in basal core body temperature of non-stressed non-diabetic control group were observed during the course of the experiment. These values for the WFWS or WSR or metformin treated diabetic groups on all observational days were statistically not significantly different from those of the non-stressed non-diabetic control group. Therefore, it is apparent that the tested daily oral doses of both the extracts (10 mg/kg/day) and metformin (50 mg/kg/day) are their highest effective one for suppressing the stress triggered hyperactivity of thermoregulatory processes in regulating basal core temperature in hyperglycaemic rats, and that these doses of all three test agents do not induce hypothermia in diabetic rats.

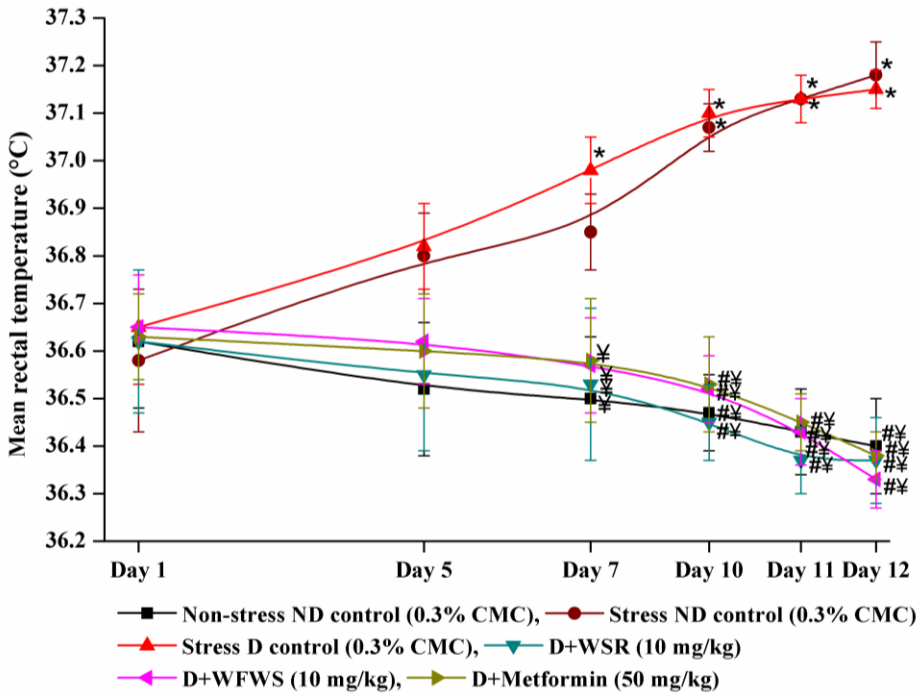

Figure 4.33: Effect of occasional exposures to short durations of foot shock stress on mean rectal temperature of normal and diabetic rats treated with withanolides rich and withanolides free *Withania somnifera* extracts or metformin. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.1.3. Foot shock stress induced transient hyperthermic responses: Like in mice, transient hyperthermic responses of diabetic rats were also suppressed by five or more daily oral doses of both the extracts and metformin, and their effectiveness also increased with increasing numbers of treatment day. Results summarized in the **Figure 4.34** revealed that in this respect the 10 mg/kg daily oral doses of both the tested extracts and 50 mg/kg/day metformin are almost identical. It was interesting to note also that like in mice, even after 10 daily treatments the tested extracts and metformin do not completely suppress foot shock stress triggered transient hyperthermic responses.

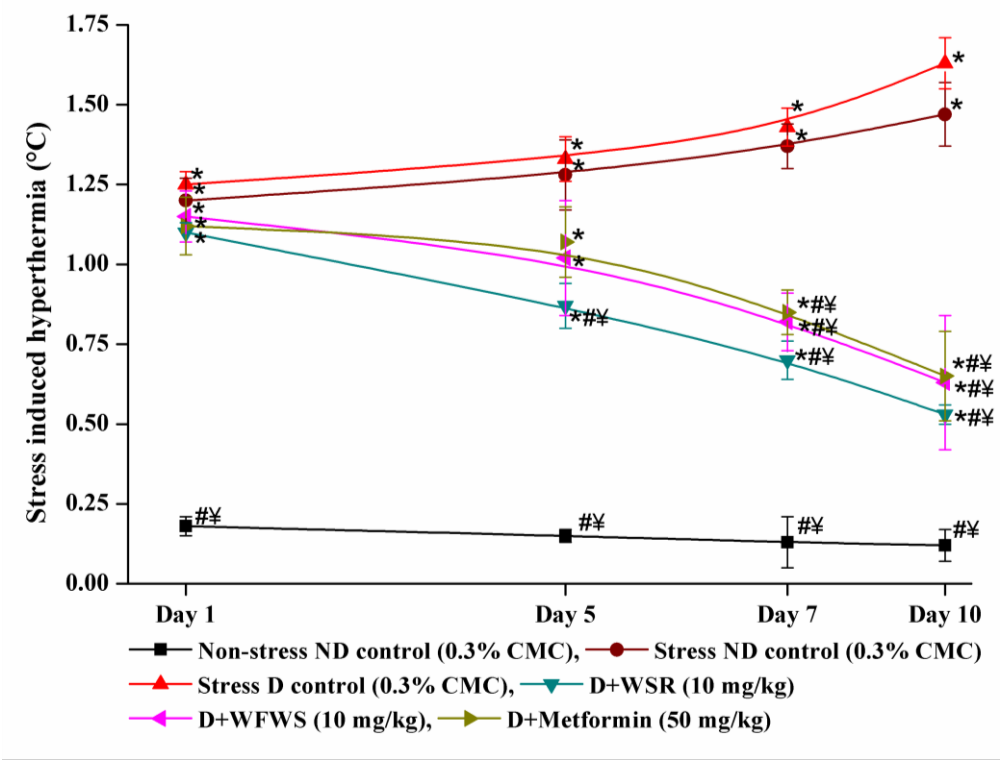

Figure 4.34: Effect of occasional exposures to short durations of foot shock stress on stress induced hyperthermia of normal and diabetic rats daily treated with withanolides rich and withanolides free *Withania somnifera* extracts or metformin. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.1.4. Plasma glucose insulin and corticosterone levels: These results summarized in **Table 4.9** reveal that the blood levels of glucose and corticosterone are higher and that of insulin is lower in stressed diabetic rats than in stressed or non-stressed non-diabetic rats. Blood corticosterone levels of the metformin or WSR or WFWS treated groups were much lower than that of the stressed diabetic control group. These mean values normalized for the body weights of animals (relative mean values) of the drug treated groups were almost identical, and of same magnitude as that observed in the stressed non-diabetic group. Therefore it can safely be said that as anti-stress agents 50 mg/kg/day metformin is as effective as 10 mg/kg/day WSR or WFWS in affording protection against stress triggered alteration in corticosterone homeostasis in diabetic rats. Qualitatively the observed effects of 10 mg/kg/day WSR or WFWS on blood glucose level were quite analogous to those of 50 mg/kg/day metformin. Quantitatively though, the observed antihyperglycemic effectiveness of WSR and WFWS were somewhat lower than that of metformin.

These observations reveal that the antidiabetic drug metformin also possess stress resistance increasing properties and that both WSR and WFWS posses metformin like therapeutically interesting modulating effects on glucose and insulin homeostasis. As potential antihyperglycemic drug, WSR seems to be slightly more potent than WFWS (as judged by blood glucose and insulin levels in stressed diabetic rats).

Table 4.9: Effects of ten daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on the plasma levels of glucose, insulin and corticosterone in diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	Glucose (mg/dl)	Insulin (µIU/ml)	Corticosterone (ng/ml)
Non-stress ND control (0.3% CMC)	94.20±3.99$^{¥}$	20.37±1.13$^{\#¥}$	98.35±2.64$^{\#¥}$
Stress ND control (0.3% CMC)	99.85±2.19$^{¥}$	14.64±1.10*$^{¥}$	113.01±2.05*$^{¥}$
Stress D control (0.3% CMC)	354.58±4.72*$^{\#}$	9.37±0.86*$^{\#}$	145.33±3.67*$^{\#}$
D+ WSR (10 mg/kg)	234.22±2.83*$^{\#¥}$	18.33±1.46$^{¥}$	119.20±4.04*$^{¥}$
D+ WFWS (10 mg/kg)	253.72±3.51*$^{\#¥}$	14.88±1.24*$^{¥}$	123.03±2.49*$^{¥}$
D+ Metformin (50 mg/kg)	173.37±3.58*$^{\#¥}$	12.93±0.71*$^{¥}$	119.65±1.29*$^{¥}$

Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress nondiabetic (ND) control, $^{\#}$=p<0.05 vs. Stress non-diabetic (ND) control and $^{¥}$=p<0.05 vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.1.5. Blood lipid profiles: Results summarized in **Table 4.10** revealed no statistically significant differences in blood cholesterol and triglyceride levels between the stressed and unstressed non-diabetic control rat groups, whereas high-density lipoproteins (HDL) blood levels were lower and that of low-density lipoproteins (LDL) was higher in stressed non-diabetic groups. As compared to the stressed non-diabetic group, mean cholesterol, triglyceride, and LDL levels in stressed diabetic group were higher and that of HDL lower in stressed diabetic group. All these stress triggered and longer lasting stress responses in diabetic animals were much less pronounced in WSR or WFWS or Metformin treated groups. Hereupon the observed effectiveness of the tested extracts was almost equal, and always somewhat lower than those observed for the metformin treated group.

Table 4.10: Effects of ten daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on the plasma lipid profile in normal and diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	HDL/LDL
Non-stress ND control (0.3% CMC)	89.13±2.09$^{¥}$	62.47±2.18$^{¥}$	49.45±1.77$^{\#¥}$	58.28±3.77$^{\#¥}$	0.84±0.46$^{\#¥}$
Stress ND control (0.3% CMC)	92.38±2.25$^{¥}$	65.72±3.40$^{¥}$	38.32±2.61*$^{¥}$	103.32±3.30*$^{¥}$	0.37±0.78*$^{¥}$
Stress D control (0.3% CMC)	177.30±3.93*$^{\#}$	128.97±2.87*$^{\#}$	24.38±1.68*$^{\#}$	144.62±2.90*$^{\#}$	0.16±0.58*$^{\#}$
D+ WSR (10 mg/kg)	117.47±4.47*$^{\#¥}$	85.47±2.28*$^{\#¥}$	41.93±1.44*$^{¥}$	60.15±3.58$^{\#¥}$	0.69±0.39$^{\#¥}$
D+ WFWS (10 mg/kg)	126.88±5.90*$^{\#¥}$	96.88±3.47*$^{\#¥}$	37.23±1.70*$^{¥}$	65.53±2.14$^{\#¥}$	0.56±0.79$^{¥}$
D+ Metformin (50 mg/kg)	103.70±3.98*$^{¥}$	80.37±4.03*$^{\#¥}$	43.82±1.54*$^{¥}$	59.07±2.60$^{\#¥}$	0.74±0.59$^{\#¥}$

Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.1.6. Inducible nitric oxide synthase (iNOS) activity, nitric oxide (NO) levels, and expression of nuclear factor kappa beta (NF-κβ)

These results summarized in the **Figures 4.35, 4.36** and **4.37** reveal that both NO levels and iNOS activity in all three studied brain regions of non-diabetic rats were significantly higher in the stressed group, and that such stress triggered responses in the diabetic animals are more pronounced than in non-diabetic animals. Levels of the nuclear transcription factor NF-κβ in all brain regions were also higher in stressed control animals, and this stress triggered effect in the brain tissue of diabetic control group were more pronounced than in non-diabetic control group. These observations strongly suggest that the observed elevated NO levels in the brain tissue of stressed animals is due to elevated synthesis if iNOS [J.L. Madrigal et al.,

2001; F. Aktan, 2004], and that hyperglycemic state of the animals facilitate the biosynthesis of the enzyme.

It was interesting to note that the extremely elevated levels of the transcription factor in all three brain regions were almost completely absent in WSR and WSWF treated as well as in metformin treated groups. These observations, taken together with the observed protective effects of WSR, or WFWS, or Metformin treatments on the NO levels and enzymatic activity of iNOS, indicate further that the observed protective effects of the test agents against stress triggered deteriorations in brain functions is due to suppression of the expression of NF-κβ, which regulates both glial and neuronal functions [L.A. O'Neill and C. Kaltschmidt, 1997]. Quantitatively, these observed effects of WFWS were of the same order of magnitude as those of WSR.

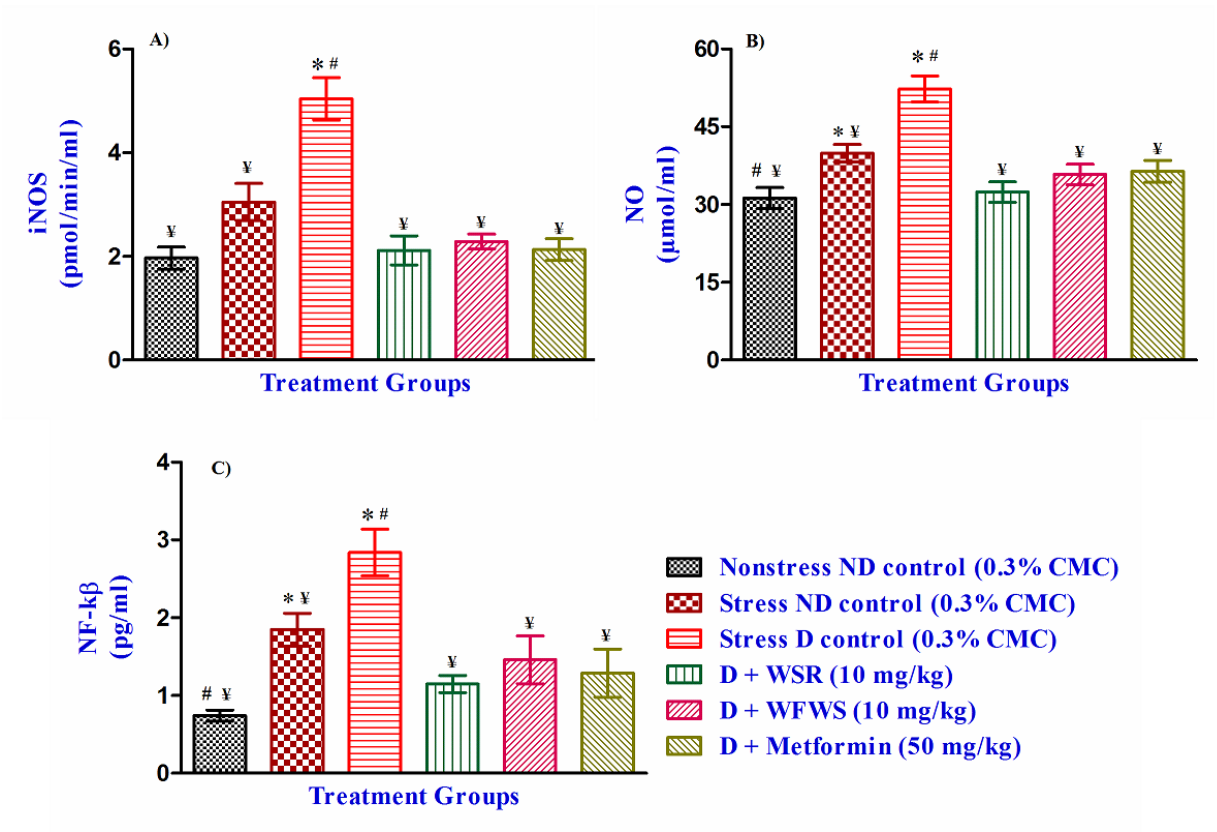

Figure 4.35: Effect of ten daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on **A)** inducible nitric oxide synthase (iNOS), **B)** nitric oxide (NO) and **C)** expression of nuclear factor kappa beta (NF-kB) activities in blood plasma of normal and diabetic rats occasionally exposed to short durations of foot shock stress. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

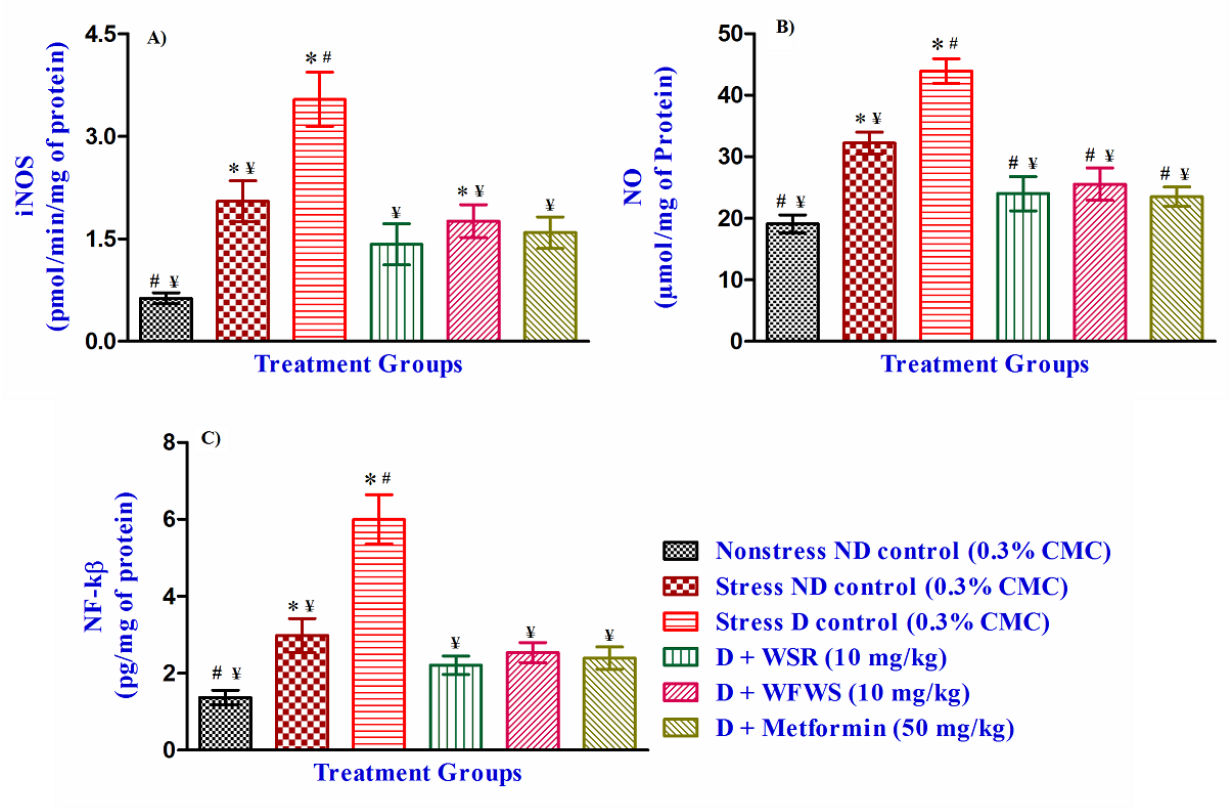

Figure 4.36: Effect of ten daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on **A)** inducible nitric oxide synthase (iNOS), **B)** nitric oxide (NO) and **C)** expression of nuclear factor kappa beta (NF-kB) activities in frontal cortex of normal and diabetic rats occasionally exposed to short durations of foot shock stress. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

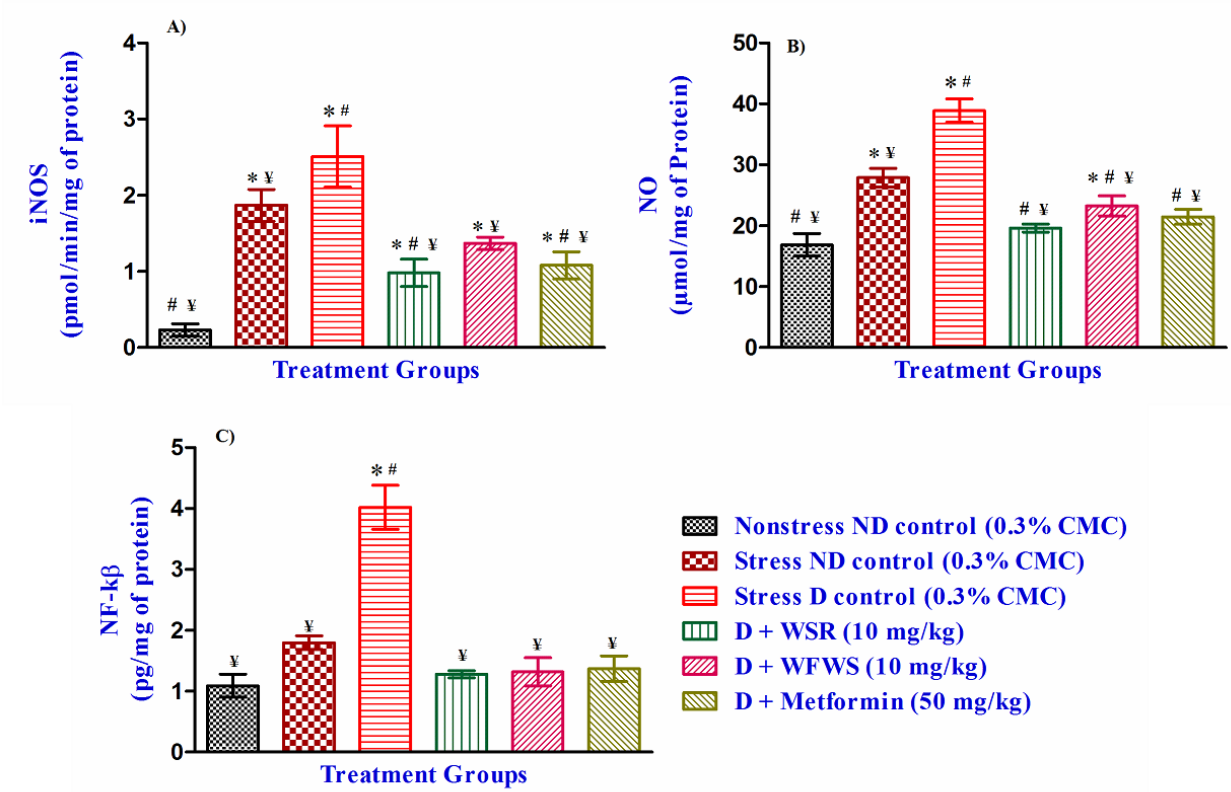

Figure 4.37: Effect of ten daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on **A)** inducible nitric oxide synthase (iNOS), **B)** nitric oxide (NO) and **C)** expression of nuclear factor kappa beta (NF-kB) activities in hippocampus of normal and diabetic rats occasionally exposed to short durations of foot shock stress. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5.2. Antidepressant like activities in diabetic rats: Aim of this experiment was to compare the effectiveness of WSR, WFWS and metformin as antidepressants and gastric ulcer proactive agents in stressed diabetic rats and to verify whether oxidative processes are involved in their modes of action.

4.5.2.1. Body weight and basal core temperatures: These results summarised in the **Figure 4.37** were quite analogous to those observed in the first anti-stress experiments conducted in stressed diabetic rats. Protective effects of tested daily oral doses of WSR, WFWS, and metformin against stress triggered alterations in body weights (**Figure 4.38A**) and basal core temperatures (**Figure 4.38B**) were qualitatively as well as quantitatively very similar to those observed in the first experiment. These results reaffirm that 10 mg/kg daily oral doses of both WSR and WFWS are high enough also for counteracting metabolic as well as physiological stress triggered body weight losses and elevations in basal core temperatures in diabetic rats, and that such is also the case for metformin (50 mg/kg/day).

4.5.2.2. Foot shock stress induced transient hyperthermic responses: Results summarised in **Figure 4.39** revealed that the magnitude of this response in stressed diabetic rat is similar in magnitude as those observed in stressed non-diabetic ones, and reaffirm that the tested daily oral doses (10 mg/kg) of WSR and WFWS are as effective in suppressing this responses as 50 mg/kg/day metformin. Like in the first experiment their statistically significant protective effects were observed after 5 or more daily treatment days and their effectives continued to increase with increasing numbers of treatment days.

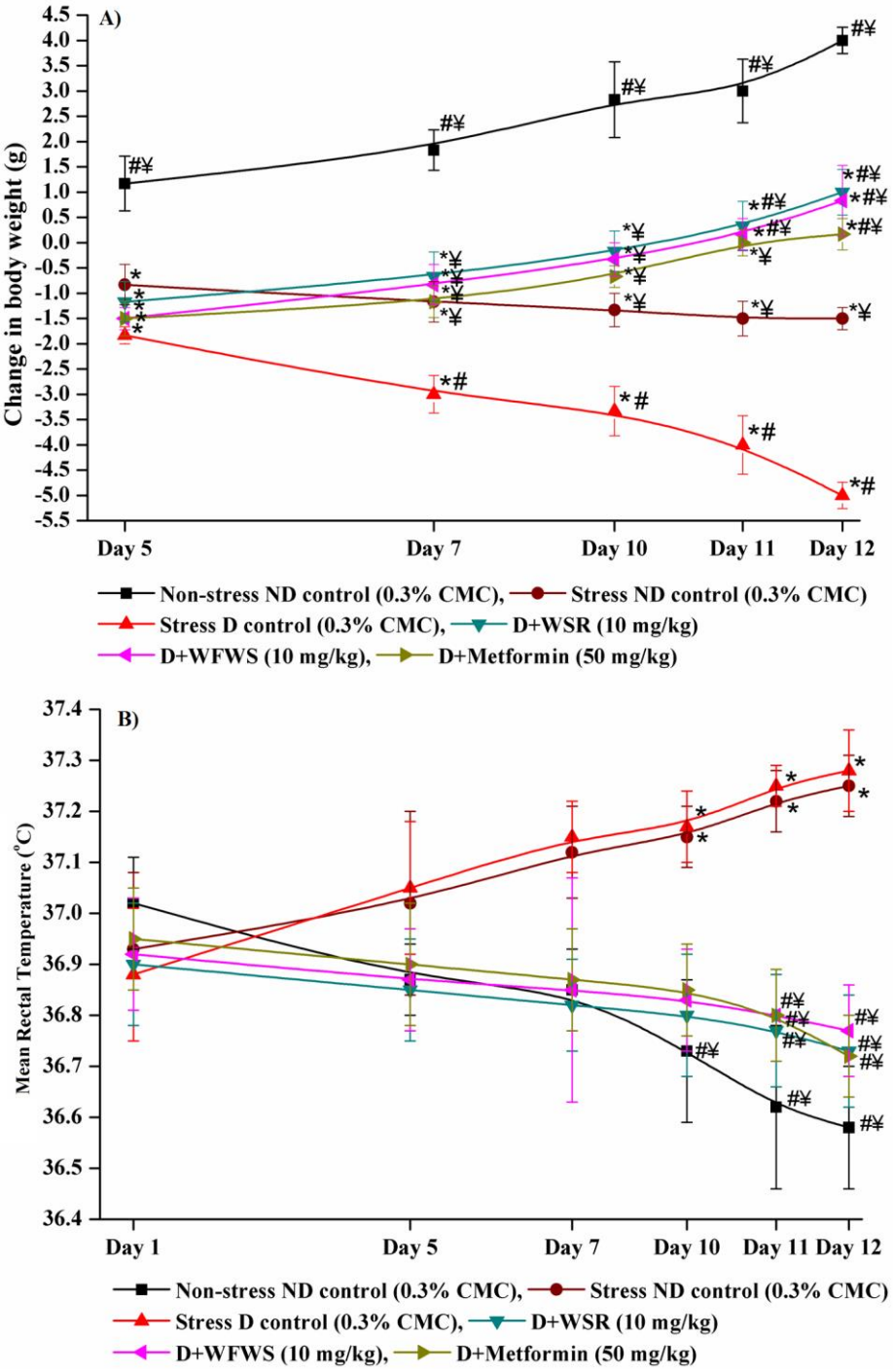

Figure 4.38: Effect of occasional exposures to short durations of foot shock stress on **A)** change in body weight and **B)** mean rectal temperatures of normal and diabetic rats daily treated with withanolides containing and withanolides free *Withania somnifera* extracts and metformin. Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress non-diabetic (ND) control, #=p<0.05 vs. Stress nondiabetic (ND) control and ¥=p<0.05 vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

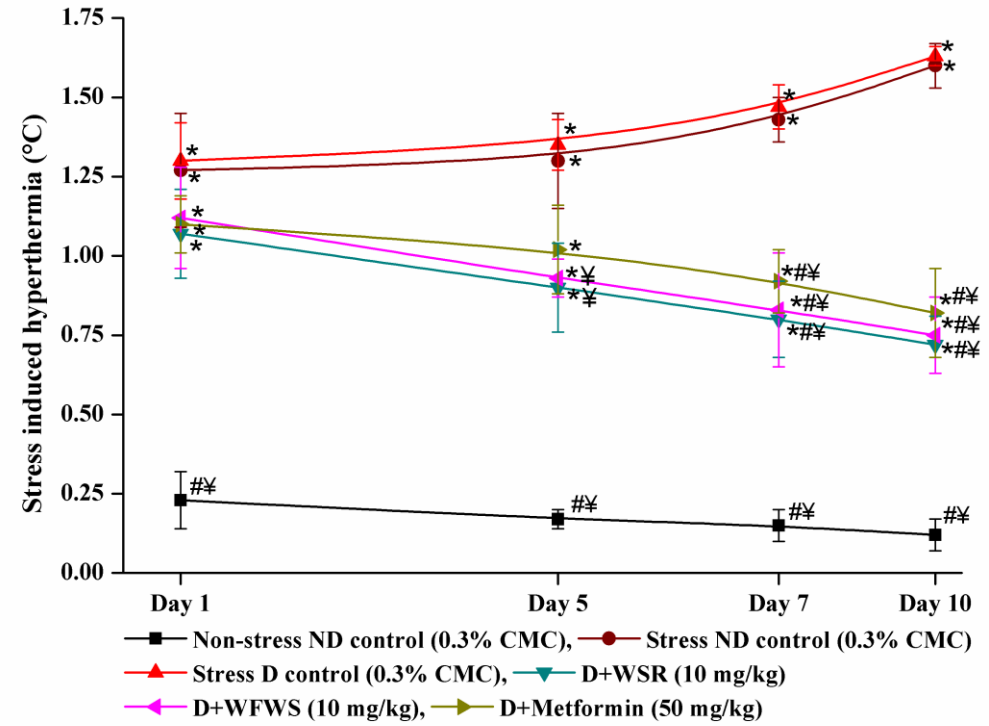

Figure 4.39: Effect of occasional exposures to short durations of foot shock stress on stress induced hyperthermia of normal and diabetic rats daily treated with withanolides rich and withanolides free *Withania somnifera* extracts or metformin. Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress nondiabetic (ND) control, $^{\#}$=p<0.05 vs. Stress non-diabetic (ND) control and $^{¥}$=p<0.05 vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.2.3. Marble burying test: Mean numbers of the marbles buried by the animals of both stressed non-diabetic and diabetic control groups were statistically significantly higher than that of the non-stressed non diabetic control group (**Figure 4.40**). These mean values of the 10 mg/kg/day WSR or WFWS treated stressed diabetic rats were significantly lower than the vehicle treated stressed diabetic or non-diabetic control groups. Quantitatively, the observed effects of both the extract were almost equal in magnitude, and somewhat higher than that of the tested metformin dose (50 mg/kg/day). However, no statistically significant effect of 50 mg/kg/day metformin was observed when compared with stressed diabetic and non-diabetic groups.

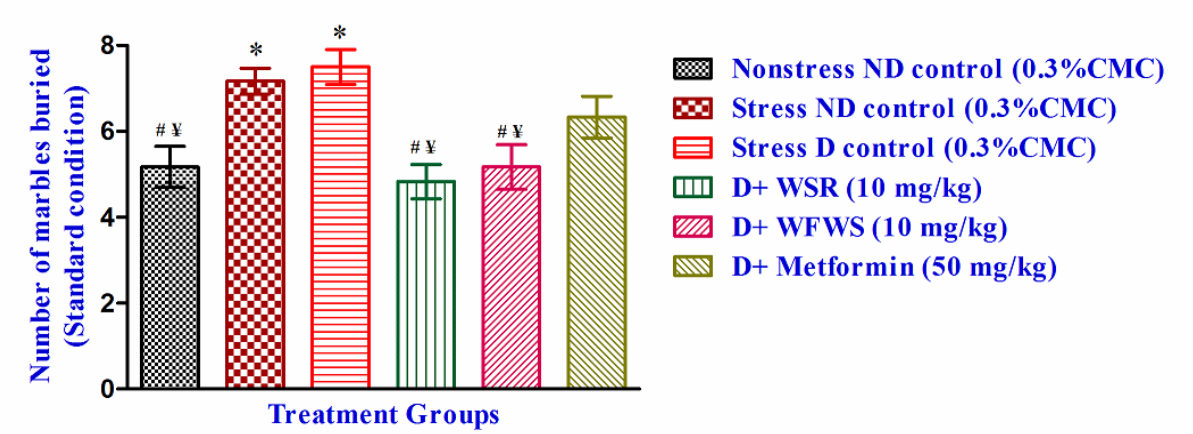

Figure 4.40: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on marble burying test in normal and diabetic rats. Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress nondiabetic (ND) control, $^{\#}$=p<0.05 vs. Stress non-diabetic (ND) control and $^{¥}$=p<0.05 vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5.2.4. Forced swimming test: Results of forced swimming test are summarized in **Figure 4.41**. The mean immobility period of the stressed diabetic or non-diabetic control groups were statistically significantly higher than than that of the non-stressed non diabetic control group. In comparison to the mean values of the stressed diabetic control group, these values for the WSR or WFWS, or metformin treated groups were significantly lower (p<0.05). Statistically, this mean value of the WSR treated group was significantly lower than that of the metformin treated or non-stressed non-diabetic control group, and that of the WSWF treated one was almost equal to that of the non-stressed non-diabetic group. These results suggest that the tested doses of metformin (50 mg/kg/day) or of WFWS (10 mg/kg/day) are effective in suppressing stress triggered exaggerated depressive state of diabetic rats, and that WSR is more effective as an antidepressant than WFWS or metformin.

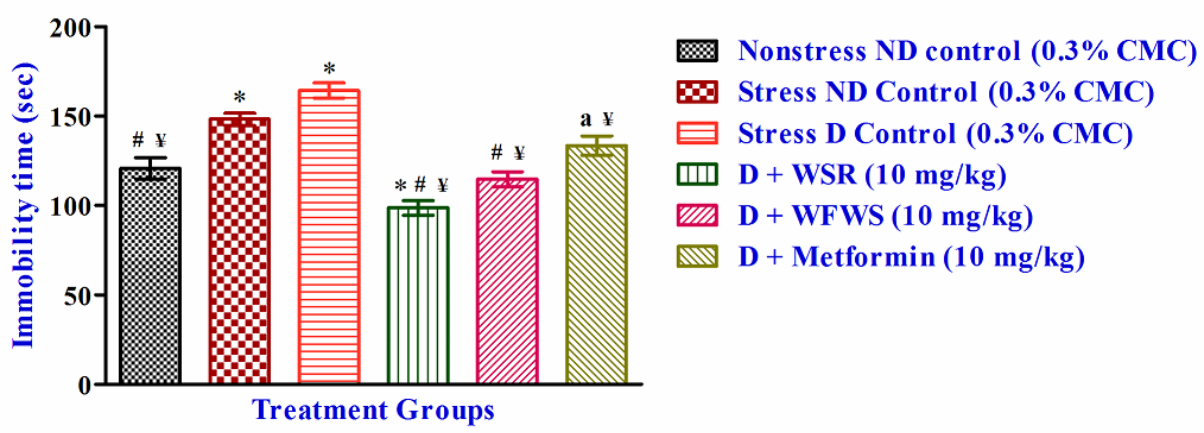

Figure 4.41: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on forced swimming test in normal and diabetic rats. Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress nondiabetic (ND) control, $^{\#}$=p<0.05 vs. Stress non-diabetic (ND) control and $^{¥}$=p<0.05 vs. Stress diabetic (D) control ^{a}=p<0.05 vs. WSR (10 mg/kg) (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5.2.5. Organ weights and plasma glucose, insulin, and corticosterone levels:

Mean weights of the spleen, adrenal glands and liver of different groups, and their calculated relative values, are summarized in the **Table 4.11.** Statistically significant adrenal gland hypertrophy, and spleen hypotrophy were observe in stressed non-diabetic control groups, and these stress triggered responses were more pronounced in the vehicle treated diabetic control group. Mean as well as relative liver weights of the stressed diabetic control group were also significantly lower than in stressed non-diabetic control one. All these stress triggered alterations in organ weights were much less pronounced, or almost absent, in the WSR or WFWS or metformin treated diabetic rats. These observed effects of the same daily oral doses (10 mg/kg/day) of WSR were numerically somewhat more pronounced than those of WFWS, and the in this respect metformin (50 mg/kg/day) was the most effective one.

Table 4.11: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on the weights of spleen, adrenal glands and liver in diabetic rats occasionally exposed to short durations of unpredictable foot shock stress.

Treatment groups	Organ weights (mg)		Liver (g)	Relative organ weights (mg/g of body weight)		Relative Liver weights (g/100g of body weight)
	Spleen	Adrenal glands		Spleen	Adrenal glands	
Non-stress ND control (0.3% CMC)	336.10±3.43$^{\#¥}$	50.68±1.93$^{\#¥}$	4.73±0.14$^{¥}$	2.18±0.04$^{\#¥}$	0.33±0.02$^{\#¥}$	3.07±0.07$^{¥}$
Stress ND control (0.3% CMC)	269.60±4.47*$^{¥}$	62.23±1.55*$^{¥}$	4.92±0.17$^{¥}$	1.82±0.06*$^{¥}$	0.42±0.02*	3.31±0.07$^{¥}$
Stress D control (0.3% CMC)	219.37±1.85*$^{\#}$	70.28±1.43*$^{\#}$	3.57±0.16*$^{\#}$	1.53±0.05*$^{\#}$	0.49±0.02*	2.48±0.11*$^{\#}$
D+ WSR (10 mg/kg)	296.67±4.14*$^{\#¥}$	55.48±2.98$^{¥}$	4.40±0.11$^{¥}$	1.96±0.06*$^{¥}$	0.37±0.03$^{¥}$	2.91±0.11$^{¥}$
D+ WFWS (10 mg/kg)	288.60±3.93*$^{\#¥}$	57.42±1.36$^{¥}$	4.18±0.12$^{¥}$	1.93±0.05*$^{¥}$	0.38±0.01$^{¥}$	2.80±0.14$^{¥}$
D+ Metformin (50 mg/kg)	303.57±4.66*$^{\#¥}$	55.93±1.69$^{¥}$	4.55±0.16$^{¥}$	2.05±0.05$^{\#¥}$	0.37±0.02$^{¥}$	3.06±0.19$^{¥}$

Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

Stress triggered elevations in circulating glucose and corticosterone levels in the diabetic control group were more pronounced than in the stressed non-diabetic control group, and the circulating insulin level in the stressed diabetic group were much lower than in stresses non-diabetic control group (**Table 4.12**). Like in the earlier described experiments, the magnitude of hyperglycemia and elevated corticosterone level observed in the stressed diabetic control group were much less pronounced in the extracts or metformin treated groups. All three test agents also afforded protections against hypoinsulinemia observed in diabetic rats. Hereupon

the observed protective effects of both the tested extracts were more pronounced than that observed for the 5 times higher daily oral doses of the antidiabetic drug metformin. These observations strongly suggest that WSR or WFWS are more effective in regulating insulin homeostasis than metformin, and add further experimental evidences in favor of the conviction that presence of withanolides in *Withania somnifera* extracts is not necessary for obtaining therapeutic benefits from them in patients prone to hypoinsulinemia.

Table 4.12: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on the plasma levels of glucose, insulin and corticosterone in normal and diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	**Glucose (mg/dl)**	**Insulin (µIU/ml)**	**Corticosterone (ng/ml)**
Non-stress ND control (0.3% CMC)	91.58±2.56$^{¥}$	22.84±1.71$^{\#¥}$	99.51±2.58$^{\#¥}$
Stress ND control (0.3% CMC)	100.01±3.88$^{¥}$	14.98±0.84*$^{¥}$	113.85±2.76*$^{¥}$
Stress D control (0.3% CMC)	353.08±4.01*$^{\#}$	9.71±0.94*$^{\#}$	145.00±3.52*$^{\#}$
D+ WSR (10 mg/kg)	201.55±4.57*$^{\#¥}$	17.41±0.50*$^{¥}$	111.55±2.78*$^{¥}$
D+ WFWS (10 mg/kg)	238.22±4.82*$^{\#¥}$	16.21±0.60*$^{¥}$	116.38±3.02*$^{¥}$
D+ Metformin (50 mg/kg)	181.04±3.34*$^{\#¥}$	13.43±0.76*	114.51±2.88*$^{¥}$

Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.2.6. Anti-oxidant activity in liver tissues of diabetic rats: In comparison to the vehicle (0.3% CMC) treated non-stressed non-diabetic control group, lipid peroxidation (LPO) activity in stressed non-diabetic or diabetic animals were significantly higher. The magnitude of LPO activity in stressed diabetic rats was more severe than stressed non-diabetic rats (**Table 4.13**). However, such elevations of LPO activity were significantly less pronounced in

the three drugs treated groups. The observed effects of 10 mg/kg/day WSR or WFWS on LPO activity were almost equal to that of the 50 mg/kg/day metformin treated ones.

Similarly, the levels of anti-oxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in stressed diabetic rats were significantly lower than that of the non-diabetic control rats. Such falls in the levels of both the anti-oxidative enzymes in diabetic rats were reversed with eleven daily oral treatments with metformin or with WSR or WFWS. However, the tested metformin daily dose had higher efficacy in increasing catalase (CAT) activity than either of the tested extracts. WSR as well as WFWS seems to be slightly more potent than metformin in elevating SOD enzyme level.

Table 4.13: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on liver anti-oxidative status in normal and diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	LPO (nmol MDA/mg protein)	SOD (IU/mg protein)	CAT (µmol H_2O_2/min/mg protein)
Non-stress ND control (0.3% CMC)	$4.68\pm0.82^{\#¥}$	$10.55\pm0.89^{\#¥}$	$17.09\pm1.31^{\#¥}$
Stress ND control (0.3% CMC)	$9.42\pm0.85^{*¥}$	$5.15\pm0.44^{*¥}$	$10.69\pm0.94^{*¥}$
Stress D control (0.3% CMC)	$14.14\pm1.52^{*\#}$	$2.79\pm0.23^{*\#}$	$7.12\pm1.22^{*\#}$
D+ WSR (10 mg/kg)	$7.08\pm0.83^{¥}$	$7.22\pm0.48^{*¥}$	$15.10\pm1.19^{\#¥}$
D+ WFWS (10 mg/kg)	$7.94\pm0.75^{¥}$	$6.74\pm0.57^{*¥}$	$14.06\pm1.40^{\#¥}$
D+ Metformin (50 mg/kg)	$7.80\pm0.73^{¥}$	$5.84\pm0.77^{*¥}$	$17.03\pm0.65^{\#¥}$

Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.2.7. Glyoxalase 1 and Paraoxonase 1 (PON1) activities:

Estimated glyoxylase-1 activity in the livers of non-diabetic rats subjected to foot shock test or not was almost identical. As compared to the mean values of the non-diabetic groups, this activity of the intra-cellular enzyme in the liver of the diabetic control group was significantly

lower (**Figure 4.42**). Repeated daily oral treatments with metformin (50 mg/kg/day) or with 10 mg/kg/day WSR or WFWS in diabetic rats significantly increased glyoxalase-1 activity in stressed diabetic rats. Hereupon, effectiveness of WFWS seems to be a bit lower than that of WSR, and that of the tested daily dose of metformin seems to be its highest effective ones.

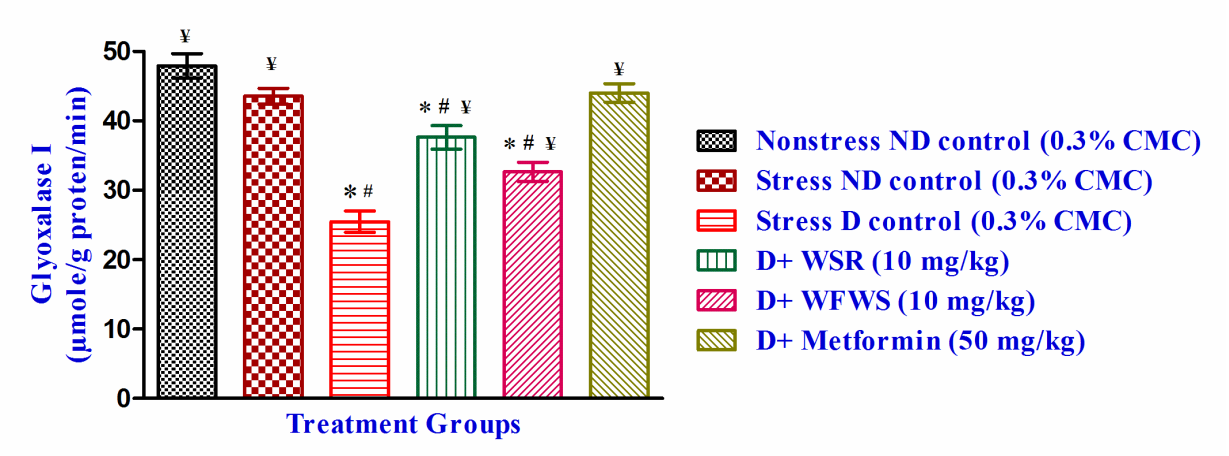

Figure 4.42: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on liver Glyoxalase-1 enzyme activity in normal and diabetic rats occasionally exposed to short durations of foot shock stress. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

Unlike Glyoxylase-1 activity, PON-1 activities in the liver of stressed non-diabetic rats was much lower, and mean PON-1 activity in the liver as well as in circulating blood of stressed diabetic rats were statistically not significantly different from those of the stressed non-diabetic animals (**Figure 4.43A & 4.43B**). Estimated levels of this anti-oxidative enzyme, often considered to be a biomarker of infections and hepatitis [A.K. Pyati et al., 2015], in stressed diabetic rats treated with all three test agents were much higher than in stressed control animals. Quantitatively the observed effects of WSR and WFWS on circulating PON-

1 levels were almost identical to that of metformin, whereas in the liver this effect of WFWS were somewhat less pronounced than those observed for WSR or metformin.

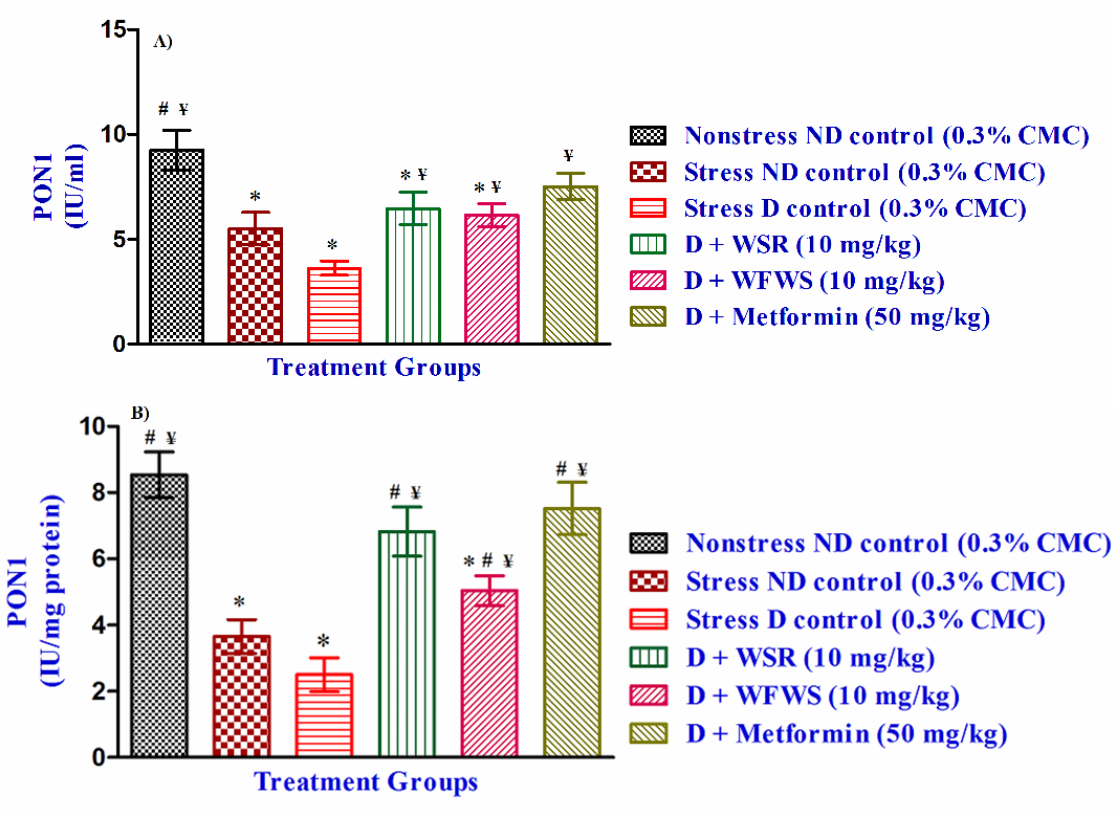

Figure 4.43: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on Paraoxonase 1 (PON1) enzyme activity [**A)** in blood plasma and **B)** in liver] in normal and diabetic rats occasionally exposed to short durations of foot shock stress. Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress nondiabetic (ND) control, #=p<0.05 vs. Stress non-diabetic (ND) control and ¥=p<0.05 vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5.2.8. Anti-oxidant activity in diabetic rat brain: The results are summarized in **Table 4.14**. As observed in the diabetic liver tissues, the LPO activity in stressed diabetic rat brains was also higher than that of the stressed non-diabetic control groups. Such elevations of LPO activity in diabetic rat brains were less severe in the metformin as well as in both the extract treated groups. Similarly, the levels of SOD and CAT activities in stressed diabetic rat brains were also significantly lower than that of the corresponding non-diabetic control rats. Such

reductions in the levels of both the anti-oxidative enzymes were also less pronounced in both the extract treated as well as in the metformin treated groups. Unlike the observation made in the livers of diabetic rats, the observed effects of WSR in reducing brain LPO activity and elevating brain activity levels of both the anti-oxidative enzymes were quite higher than that observed for the metformin treated group. These observed anti-oxidative effects of WFWS treatments was quite similar, but not equal to that of the metformin treated ones.

Table 4.14: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on brain (frontal cortex) anti-oxidative status in normal and diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	LPO (nmol MDA/mg protein)	SOD (IU/mg protein)	CAT (µmol H_2O_2/min/mg protein)
Non-stress ND control (0.3% CMC)	7.37±0.70$^{\#¥}$	17.22±1.11$^{\#¥}$	44.86±2.93$^{\#¥}$
Stress ND control (0.3% CMC)	15.88±1.70*$^{¥}$	7.65±0.85*	27.36±2.87*
Stress D control (0.3% CMC)	22.94±2.84*$^{\#}$	4.73±0.81*	22.12±1.15*
D+ WSR (10 mg/kg)	11.84±0.85*$^{¥}$	14.87±1.13$^{\#¥}$	37.43±1.33*$^{\#¥}$
D+ WFWS (10 mg/kg)	14.94±0.98*$^{¥}$	12.22±1.48*$^{\#¥}$	32.39±1.42*$^{¥}$
D+ Metformin (50 mg/kg)	16.35±1.01*$^{¥}$	11.26±0.77*$^{\#¥}$	35.36±1.88*$^{\#¥}$

Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.2.9. Monoamine neurotransmitter levels in diabetic rat brains: Concentration of all three quantified neurotransmitters (norepinephrine, dopamine and 5-hydroxytryptamine) in the stressed non-diabetic rat brains were much lower than the unstressed non-diabetic control group, and their levels were further reduced in the stressed diabetic control group (**Figure 4.44**). As compared to the vehicle treated diabetic control group, the levels of all three quantified neurotransmitters were much higher in the 10 mg/kg/day of WSR or WFWS treated groups, and such were the observations made for the 50 mg/kg/day and metformin treated one. Quantitatively, the observed effects of the tested extracts were quite similar to

that of the metformin (50 mg/kg). Numerically, the mean values of the WSR treated group were always somewhat higher than the WFS or metformin treated groups.

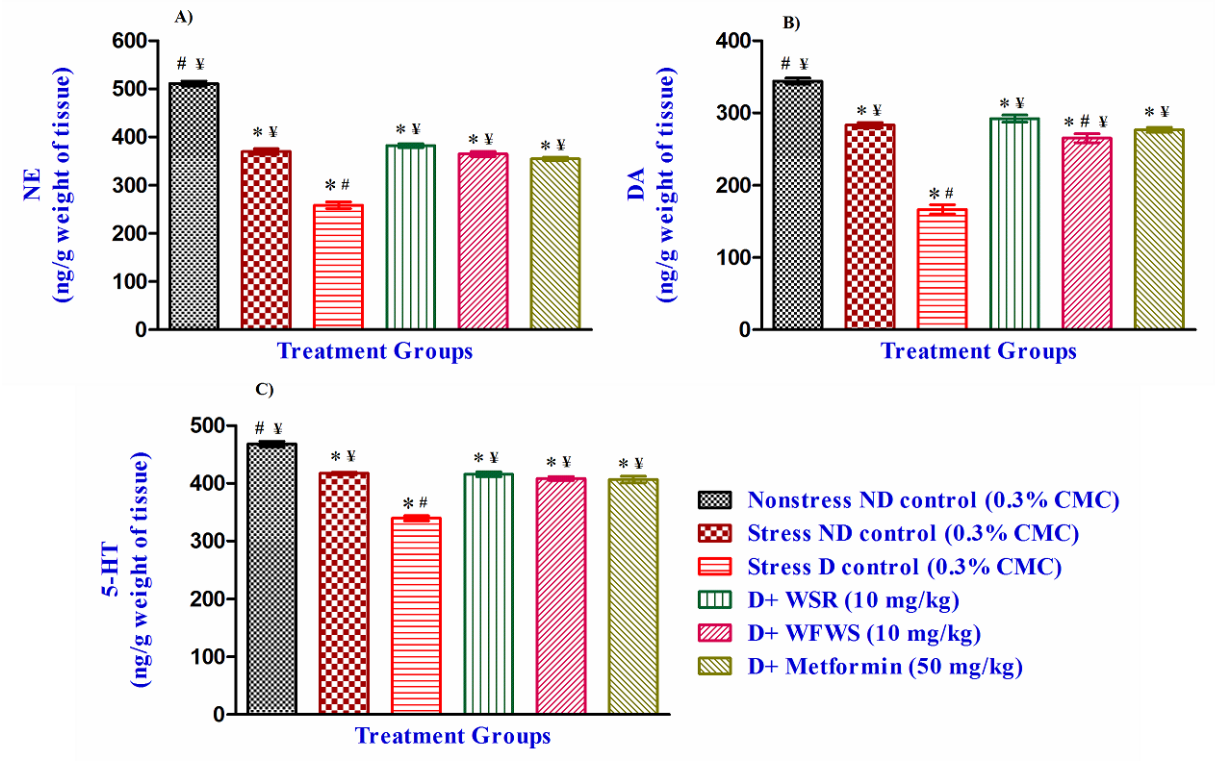

Figure 4.44: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on monoamines level in normal and diabetic rat brains occasionally exposed to short durations of foot shock stress. **A)** Norepinephrine (NE), **B)** Dopamine (DA) and **C)** 5-hydroxytryptamine (5-HT). Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5.2.10. Monoamine oxidase (MAO) activity in diabetic rat brains: Results of the MAO-A and MAO-B assays performed with mitochondrial preparations from diabetic and non-diabetic rat brain (hippocampus) are summarized in **Figures 4.45A & 4.45B**. In stressed vehicle treated diabetic rats, activities of both monoamines oxidizing enzymes assayed in the brain region were significantly higher than in stressed non-diabetic ones. Observed protective effects of daily 50mg/kg metformin treatment on the activities of both the enzymes were less

than those of quantified for the 10 mg/kg/day WSR or WFWS treated ones. Hereupon the effectiveness of WSR was somewhat higher than those observed for WFWS. Such were also the observed effectiveness of the extracts and metformin in the forced swimming test for antidepressants in foot shock stressed diabetic rats.

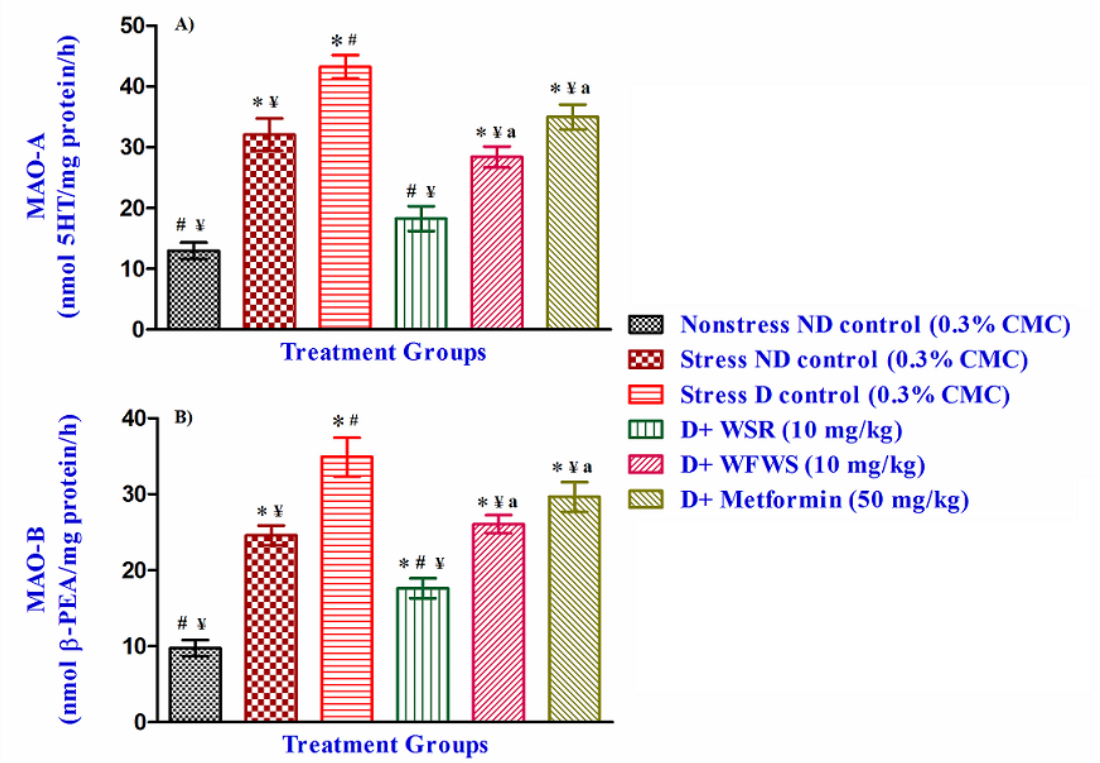

Figure 4.45: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on monoamine oxidase activity in normal and diabetic rat hippocampus occasionally exposed to short durations of foot shock stress. **A)** Monoamine oxidase - A (MAO-A) and **B)** monoamine oxidase - B (MAO-B). Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress nondiabetic (ND) control, $^{\#}$=p<0.05 vs. Stress non-diabetic (ND) control, $^{¥}$=p<0.05 vs. Stress diabetic (D) control and ^{a}=p<0.05 vs. WSR (10 mg/kg) One way ANOVA followed by Student-Newman-Keuls multiple comparison test.

4.5.2.11. Gastric ulceration: Results are summarized in **Table 4.15,** and representative pictures of stomachs of animal of different groups are shown in the **Figure 4.46.** Results summarized in the table revealed that mean gastric ulcer score of the stressed diabetic control

group was higher than that for the stressed non-diabetic ones, and that daily treatments with 50 mg/kg metformin or with 10 mg/kg WSR, or WFWS, afford complete protection against stress triggered gastric damages in diabetic rats.

Table 4.15: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on stomach ulcer index in normal and diabetic rats.

Treatment groups	Stomach ulcer index
Non-stress ND control (0.3% CMC)	$0.00\pm0.00^{¥}$
Stress ND control (0.3% CMC)	$0.42\pm0.15^{¥}$
Stress D control (0.3% CMC)	$1.58\pm0.25^{*\#}$
D+ WSR (10 mg/kg)	$0.00\pm0.00^{¥}$
D+ WFWS (10 mg/kg)	$0.00\pm0.00^{¥}$
D+ Metformin (50 mg/kg)	$0.00\pm0.00^{¥}$

Values are mean ± SEM (n=6). Statistically significant difference (One way ANOVA followed by Student-Newman-Keuls multiple comparison test) were denoted with *=$p<0.05$ vs. Non-stress non-diabetic (ND) control; $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control; $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control.

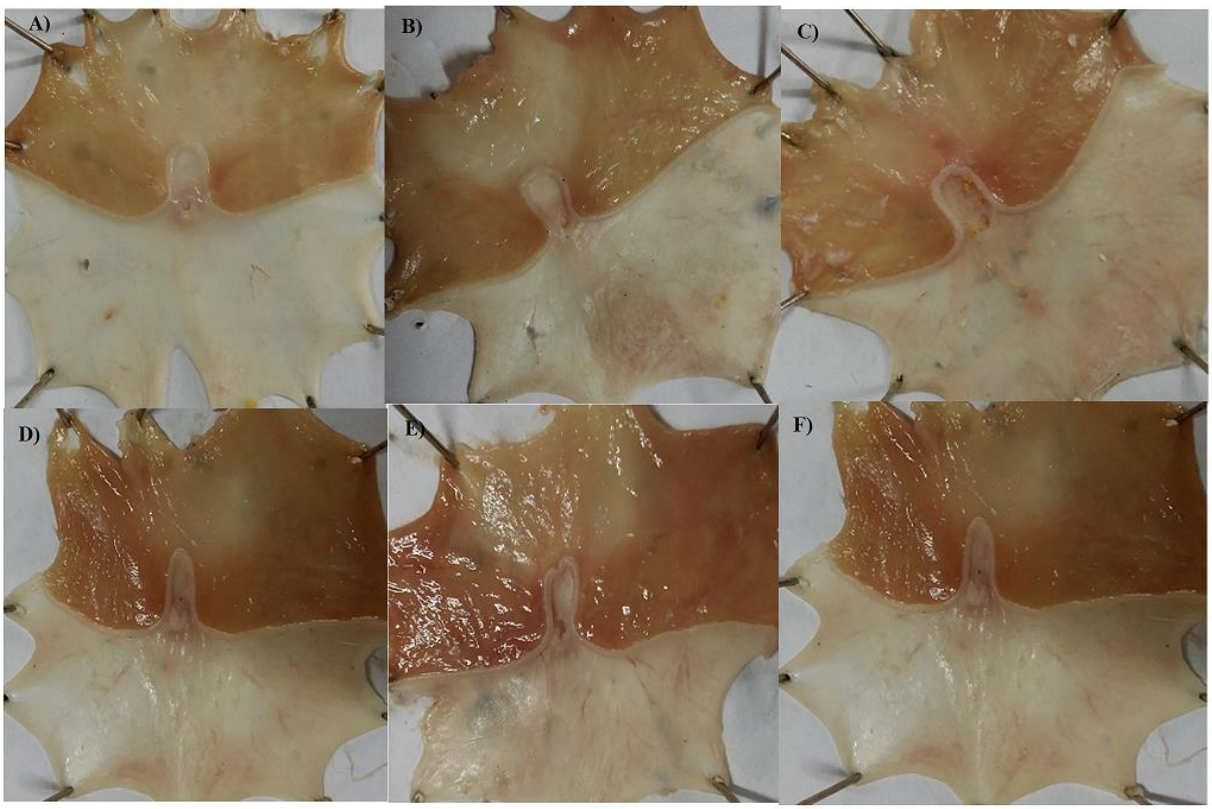

Figure 4.46: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin stomach ulceration in normal and diabetic rats. **A)** Non-stress non-diabetic (ND) control, **B)** Stress non-diabetic (ND) control, **C)** Stress diabetic (D) control, **D)** Diabetic + WSR (10 mg/kg), **E)** Diabetic + WFWS (10 mg/kg) and **F)** Diabetic + metformin (50 mg/kg).

4.5.3. Anxiolytics like activities in diabetic rats: In this experiment**,** effects of eleven daily oral dose of test agent in the conventionally known elevated plus maze test for benzodiazepine like anti-anxiety drugs were quantified in occasionally foot shock stressed male diabetic rats. Spontaneous locomotors activities of the experimental groups were also quantified occasionally during the first ten treatment days. Blood and organ samples were collected 24 hours after the last oral treatment day.

4.5.3.1. Body weights and basal core temperatures: These results summarised in the **Figure 4.47** are quite analogous to those observed in the first two experiments with diabetic rats. The protective effects of low oral doses (10 mg/kg) of both WSR and WFWS against stress triggered alterations in body weights were observed after their seven or more oral doses. The rate of body weight changes of WFWS treated animals observed during the course of the experiment were quite analogous to that of the metformin treated ones (**Figure 4.47A**). Observed effectiveness of all three test agents against occasional exposure to short duration foot shock stress-triggered increment in basal core temperatures (**Figure 4.47B**) were also very similar to those observed in the first anti-stress or antidepressant experiments. These results reaffirm that the tested daily oral doses of WSR or WFWS (10 mg/kg/day) as well as of metformin (50 mg/kg/day) are their highest effective one for suppressing the stress triggered hyperactivity of thermoregulatory processes in regulating basal core temperature in hyperglycaemic rats.

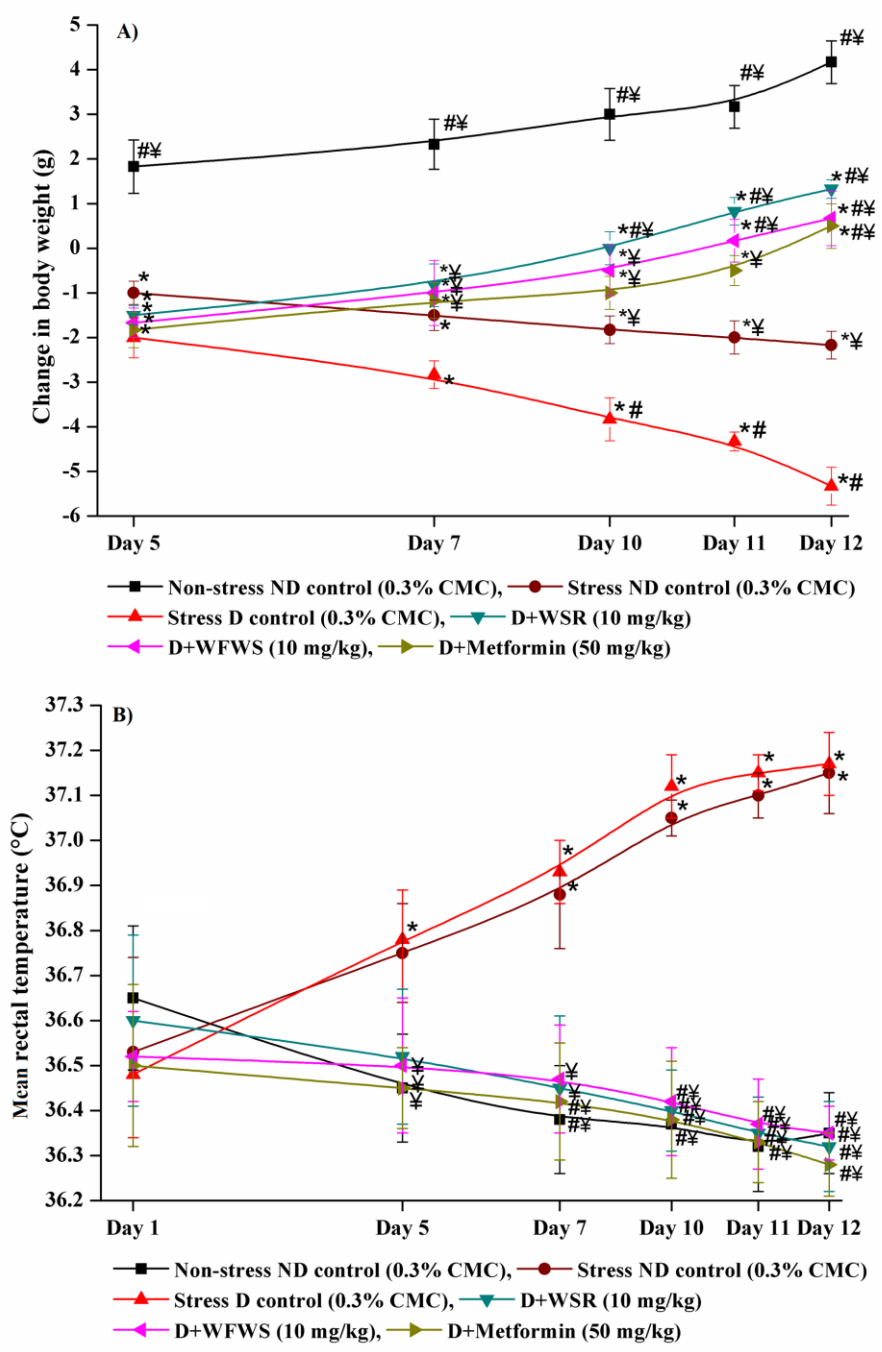

Figure 4.47: Effect of occasional exposures to short durations of foot shock stress on **A)** mean change in body weights and **B)** mean rectal temperatures of normal and diabetic rats daily treated with withanolides containing and withanolides free *Withania somnifera* extracts and metformin. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress non-diabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress nondiabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.3.2. Foot shock stress induced transient hyperthermic responses: Like in the first two rat experiments, transient hyperthermic responses of diabetic rats were also suppressed by five or more daily oral doses (10 mg/kg) of WSR or WFWS and metformin (50 mg/kg/day). Results summarized in the **Figure 4.48** revealed that in this respect the tested daily doses of the WFWS and metformin were qualitatively and quantitatively almost identical, and reaffirm that even 10 daily treatment days are not long enough for complete suppression of foot shock stress triggered transient hyperthermic responses in diabetic rats as well.

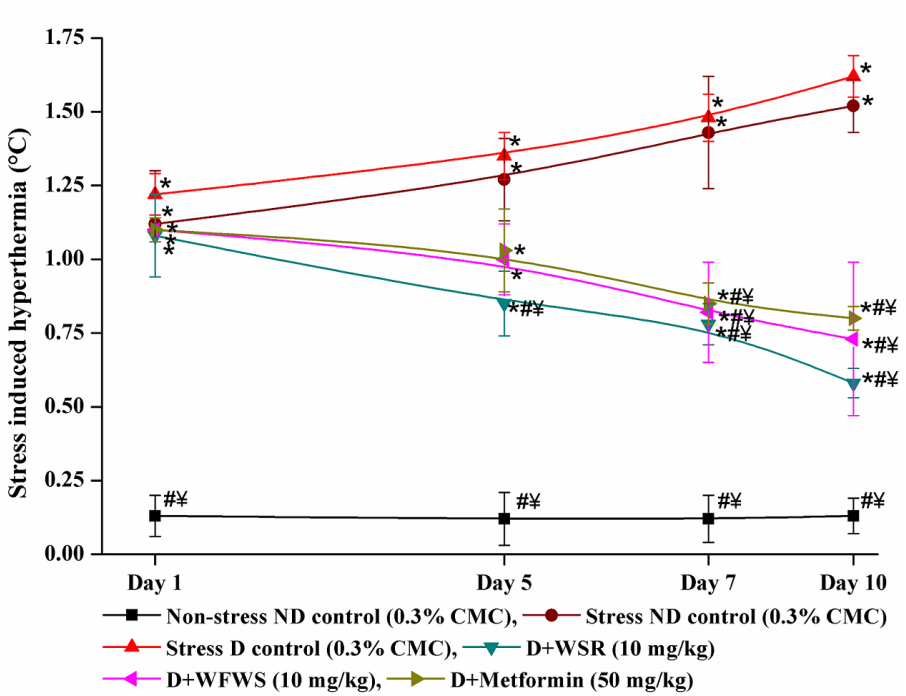

Figure 4.48: Effect of occasional exposures to short durations of foot shock stress on stress induced hyperthermia of normal and diabetic rats daily treated with withanolides rich and withanolides free *Withania somnifera* extracts or metformin. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.3.3. Locomotor activity tests: These results, graphically summarized in the **Figure 4.49,** revealed that mean loco-motor activity counts of the stressed diabetic and non-diabetic control groups on all test days were significantly higher than those of the non-stressed non-diabetic group. These values of the metformin treated group on the first test day were not statistically significantly different from the WFWS or WSR treated groups. Although on this day the mean values of the WFWS and WSR treated groups were numerically lower than those of the stressed diabetic and non-diabetic groups, there were no statistically significant differences between these values. However, after 5 or more treatment days mean loco-motor counts of either WFWS or WSR treated groups were statistically significantly lower than the stressed diabetic and non-diabetic groups. These protective effects of both the extracts increase further on the 7th and 10th test days. Analogous were the observations made for the metformin treated groups on these two days. It was interesting to note that, the anxiolytic effect of WFWS was almost identical to that of the WSR treated group, and this effect was slightly higher than that of the metformin treated rats**.**

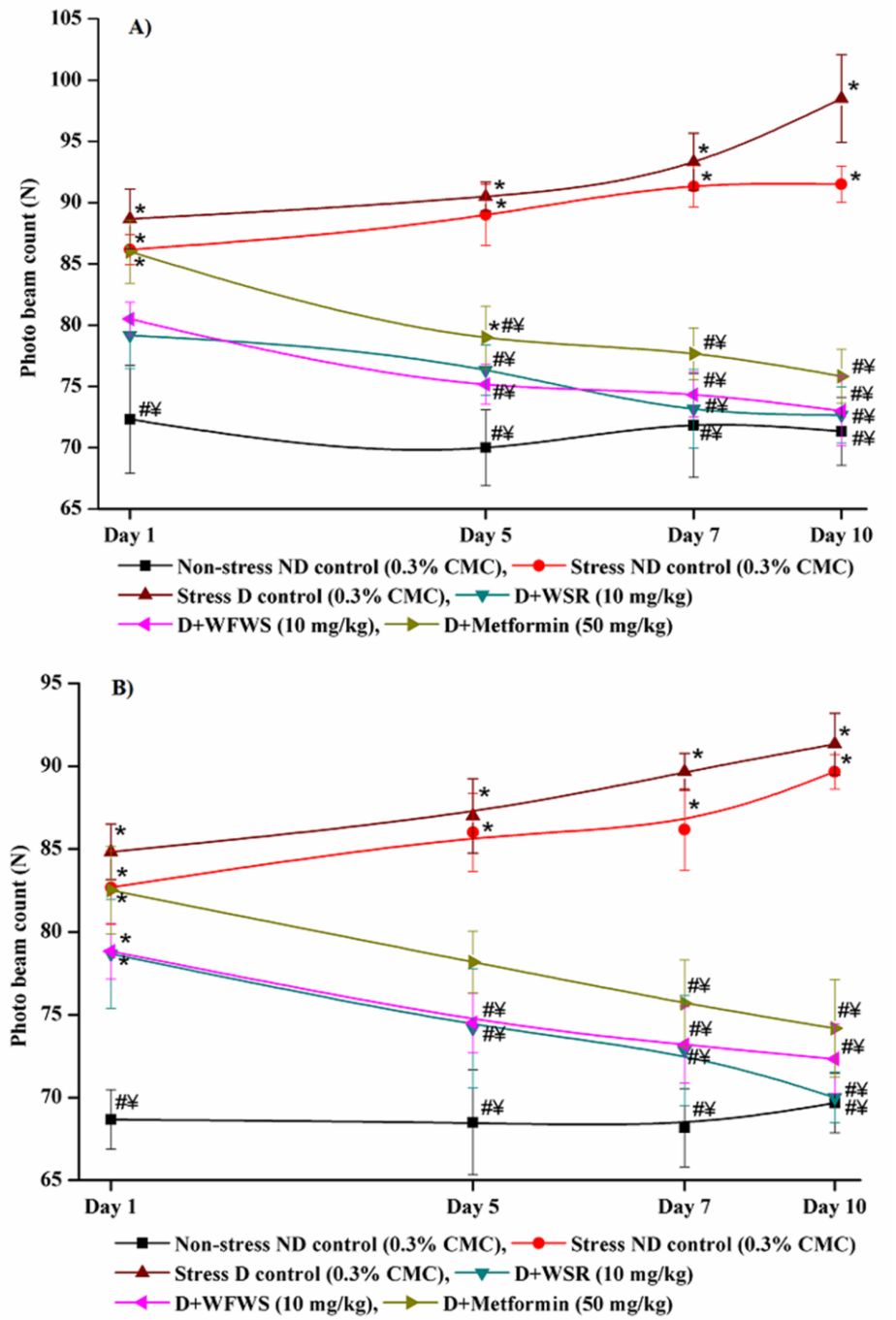

Figure 4.49: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on locomotor activity of diabetic rats. **A)** photo beam count for the first 60 sec and **B)** photo beam count for the last 60 sec of total 10 min period in actophotometer. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stressed nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stressed non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stressed diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.3.4. Elevated plus maze test: This test is one of the more reliable and reproducible one often used in preclinical studies for pharmacological screening and classification of anxiolytics, and is also considered to be well suited for assessing and quantifying the state of anxiety in rodents. Behavior of animals to avoid open arm entries (escaping behavior) after placing them in the middle space of the maze is a reliable index of their state of anxiety [G.K. Singh et al., 2013a]. Results summarized in the **Table 4.16** revealed that anxiety state of occasionally foot shock stressed non-diabetic group was higher than that of the unstressed one, and that the state of anxiety in stressed diabetic group was much higher than in non-diabetic one. Even then, clear anxiolytics like effects of both the tested *Withania somnifera* extracts (10 mg/kg/day) and that of metformin (50 mg/kg/day) were observed in stress diabetic rats. Hereupon, effectiveness of both WSR and WFWS were almost identical and somewhat higher than that of 5 times higher daily oral doses of metformin.

Table 4.16: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on elevated plus maze test in normal and diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	Open arm entries (N)	Closed arm entries (N)	Time spent (Sec)	
			Open arm	Closed arm
Non-stress ND control (0.3% CMC)	4.17±0.31$^{¥}$	9.33±0.42$^{¥}$	37.50±4.72$^{¥}$	150.33±6.91$^{\#¥}$
Stress ND control (0.3% CMC)	3.33±0.49	10.17±0.48$^{¥}$	28.33±1.98	175.50±4.43*$^{¥}$
Stress D control (0.3% CMC)	2.67±0.33*	12.50±0.99*$^{\#}$	21.17±1.64*	189.67±2.78*$^{\#}$
D+ WSR (10 mg/kg)	6.17±0.48*$^{\#¥}$	7.33±0.49$^{¥\#}$	40.50±2.28$^{¥}$	123.17±5.04*$^{\#¥}$
D+ WFWS (10 mg/kg)	5.50±0.34$^{\#¥}$	8.00±0.58$^{¥}$	38.33± 2.23$^{¥}$	129.83±3.21*$^{\#¥}$
D+ Metformin (50 mg/kg)	5.00±0.52$^{\#¥}$	7.67±0.61$^{¥\#}$	36.17±2.52$^{¥}$	135.83±4.04*$^{\#¥}$

Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stressed nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stressed non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stressed diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.3.5. Organ weights and plasma glucose insulin and corticosterone levels: These results summarised in the **Table 4.17 and Table 4.18** was quite analogous to those observed in the first two experiments with diabetic rats. The protective effects of low oral doses (10 mg/kg) of both WSR and WFWS against stress triggered alterations in adrenal gland weights in diabetic rats as well as altered blood glucose; insulin and corticosterone were very similar to those observed in other experiments. Qualitatively these effects of the extracts were quite similar to those of the metformin. These results reaffirm also that both WSR and WFWS are more effective in regulating insulin homeostasis than metformin, and strongly suggest that beneficial effects of metformin on insulin homeostasis is mainly due to its beneficial effects on glucose homeostasis.

Table 4.17: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on the weights of spleen, adrenal glands and liver in diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	Organ weights (mg)		Liver (g)	Relative organ weights (mg/g of body weight)		Relative Liver weights (g/100g of body weight)
	Spleen	Adrenal glands		Spleen	Adrenal glands	
Non-stress ND control (0.3% CMC)	333.60±3.22$^{\#¥}$	42.52±2.20$^{\#¥}$	4.63±0.30$^{¥}$	2.12±0.05$^{\#¥}$	0.27±0.01$^{\#¥}$	2.95±0.20$^{¥}$
Stress ND control (0.3% CMC)	264.60±3.92*$^{¥}$	61.40±2.87*$^{¥}$	4.47±0.35$^{¥}$	1.74±0.04*$^{¥}$	0.40±0.02*	2.94±0.25$^{¥}$
Stress D control (0.3% CMC)	217.20±2.80*$^{\#}$	71.28±2.99*$^{\#}$	3.18±0.25*$^{\#}$	1.45±0.03*$^{\#}$	0.48±0.02*	2.13±0.18*$^{\#}$
D+ WSR (10 mg/kg)	301.67±3.75*$^{\#¥}$	52.82±3.03*$^{¥}$	4.23±0.11$^{¥}$	1.93±0.04*$^{\#¥}$	0.34±0.02$^{¥}$	2.71±0.08
D+ WFWS (10 mg/kg)	290.27±3.65*$^{\#¥}$	55.58±3.55*$^{¥}$	3.93±0.19$^{¥}$	1.85±0.03*$^{¥}$	0.36±0.02*$^{¥}$	2.51±0.10
D+ Metformin (50 mg/kg)	298.23±3.67*$^{\#¥}$	53.47±3.04*$^{¥}$	4.13±0.22$^{¥}$	1.92±0.04*$^{\#¥}$	0.34±0.02$^{¥}$	2.66±0.16

Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

Table 4.18: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on the plasma levels of glucose, insulin and corticosterone in diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	Glucose (mg/dl)	Insulin (µIU/ml)	Corticosterone (ng/ml)
Non-stress ND control (0.3% CMC)	93.37±3.75$^{¥}$	24.04±2.83$^{\#¥}$	102.51±4.16$^{¥}$
Stress ND control (0.3% CMC)	108.18±4.37$^{¥}$	13.98±0.90*	114.85±3.06$^{¥}$
Stress D control (0.3% CMC)	346.75±5.13*$^{\#}$	9.04±0.59*	148.16±3.89*$^{\#}$
D+ WSR (10 mg/kg)	217.55±3.61*$^{\#¥}$	18.67±1.90$^{¥}$	117.36±3.82$^{¥}$
D+ WFWS (10 mg/kg)	243.22±3.43*$^{\#¥}$	15.22±1.94*	119.70±4.78*$^{¥}$
D+ Metformin (50 mg/kg)	180.71±4.94*$^{\#¥}$	12.76±1.00*	118.99±2.59*$^{¥}$

Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.3.6. Anti-oxidant activity in blood samples of diabetic rats: The results summarized in **Table 4.19** are quite analogous to those observed in the anti-depressant experiments with diabetic rats. In stressed diabetic rats, metabolic stresses as well as physical stress causes elevation in the LPO activity in blood plasma, and these effects were statistically higher than that of the non-diabetic control rats. However, such elevations in LPO activity were less severed in the WSR or WFWS treated groups and metformin treated groups. Similarly, reductions in the level of SOD and CAT enzymes in diabetic animals were significantly reversed with repeated daily treatments with fairly low oral doses (10 mg/kg/day) of either WSR or WFWS and with metformin (50 mg/kg/day). The anti-oxidative effects seen with WSR (10 mg/kg/day) were quite higher than that of the metformin treated group, whereas the effects of WFWS treatments is quite similar, but not equal to that of the metformin treated ones.

Table 4.19: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on blood anti-oxidative status in normal and diabetic rats occasionally exposed to short durations of foot shock stress.

Treatment groups	LPO (nmol MDA/ml)	SOD (IU/ml)	CAT (µmol H_2O_2/min/ml)
Non-stress ND control (0.3% CMC)	3.51±0.61$^{\#¥}$	12.22±1.21$^{\#¥}$	18.10±1.82$^{\#¥}$
Stress ND control (0.3% CMC)	9.58±0.59*$^{¥}$	5.78±1.08*	11.63±0.70*$^{¥}$
Stress D control (0.3% CMC)	16.14±1.59*$^{\#}$	2.56±0.37*	6.13±0.90*$^{\#}$
D+ WSR (10 mg/kg)	6.24±1.24$^{¥}$	8.18±1.26*$^{¥}$	16.73±1.54$^{¥}$
D+ WFWS (10 mg/kg)	7.44±0.97*$^{¥}$	6.58±0.99*$^{¥}$	14.22±1.62$^{¥}$
D+ Metformin (50 mg/kg)	7.65±1.07*$^{¥}$	6.05±1.02*$^{¥}$	16.53±1.62$^{¥}$

Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress nondiabetic (ND) control, $^{\#}$=p<0.05 vs. Stress non-diabetic (ND) control and $^{¥}$=p<0.05 vs. Stress diabetic (D) control (Two way ANOVA followed by Bonferroni post hoc test).

4.5.3.7. Plasma levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GTP): Circulating levels of both these liver specific enzymes in the stressed diabetic control group were much higher than in both the non-diabetic control groups (**Figure 4.50**). Eleven daily treatments with low oral doses (10 mg/kg) of WSR and WFWS or metformin (50 mg/kg) significantly compensated the elevated levels of both these these enzymes. Hereupon, the observed effects of WSR or WFWS treatments were almost identical to that of metformin treatments. These results suggest that like *Withania somnifera* extracts, metformin also possess hepatoprotective potentials. ,

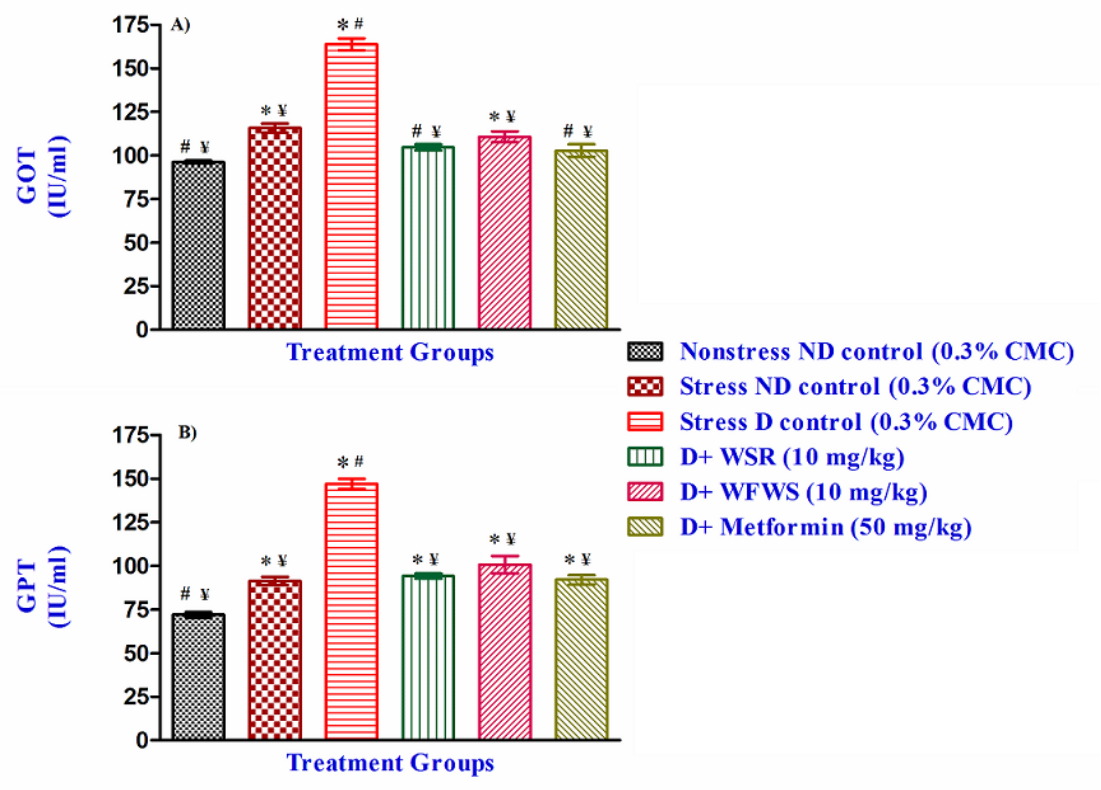

Figure 4.50: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on plasma levels of **A)** glutamic oxaloacetic transaminase (GOT) and **B)** glutamic pyruvic transaminase (GTP) in normal and diabetic rats occasionally exposed to short durations of foot shock stress. Values are mean ± SEM (n=6). *=$p<0.05$ vs. Non-stress nondiabetic (ND) control, $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control and $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5.3.8. Glyoxalase 1 enzyme activity in liver: The results summarized in **Figure 4.51** are quite analogous to those observed in the anti-depressant experiments with diabetic rats. In stressed diabetic rats the level of Glyoxalase-1 enzyme significantly decreased as compared to non-diabetic control groups. Repeated daily oral treatments with metformin (50 mg/kg/day) or with WSR (10 mg/kg/day) or WFWS (10 mg/kg/day) had significantly increased the level of glyoxalase-1 activity in stressed diabetic rats. In this respect, higher effectiveness of the tested daily dose of metformin was observed also.

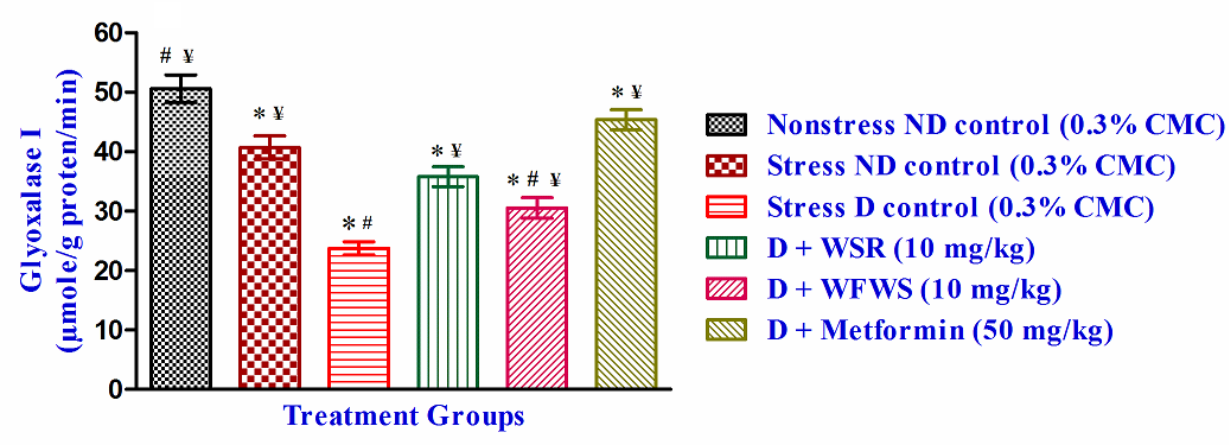

Figure 4.51: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on liver Glyoxalase-1 enzyme activity in normal and diabetic rats occasionally exposed to short durations of foot shock stress. Values are mean ± SEM (n=6). *=p<0.05 vs. Non-stress nondiabetic (ND) control, $^{\#}$=p<0.05 vs. Stress non-diabetic (ND) control and $^{¥}$=p<0.05 vs. Stress diabetic (D) control (One way ANOVA followed by Student-Newman-Keuls multiple comparison test).

4.5.3.9. Cholinesterase activity in diabetic rats: Results summarized in **Figure 4.52** revealed that the levels of both AChE and BChE activities in blood as well as in the two brain regions of stressed diabetic rats studied were much higher than those quantified in the stressed non-diabetic animals. Mean levels of both these putative biomarkers of low grade systemic inflammation [U.N. Das, 2007] in the WSR or WFS or metformin treated groups were always ether equal to, or only slightly higher, than those quantified in the unstressed non-diabetic control group. Quantitatively though, effectiveness of WSR was somewhat higher than that of WFWS, which in turn was also somewhat higher than those observed for the group treated with five times higher daily oral doses of metformin.

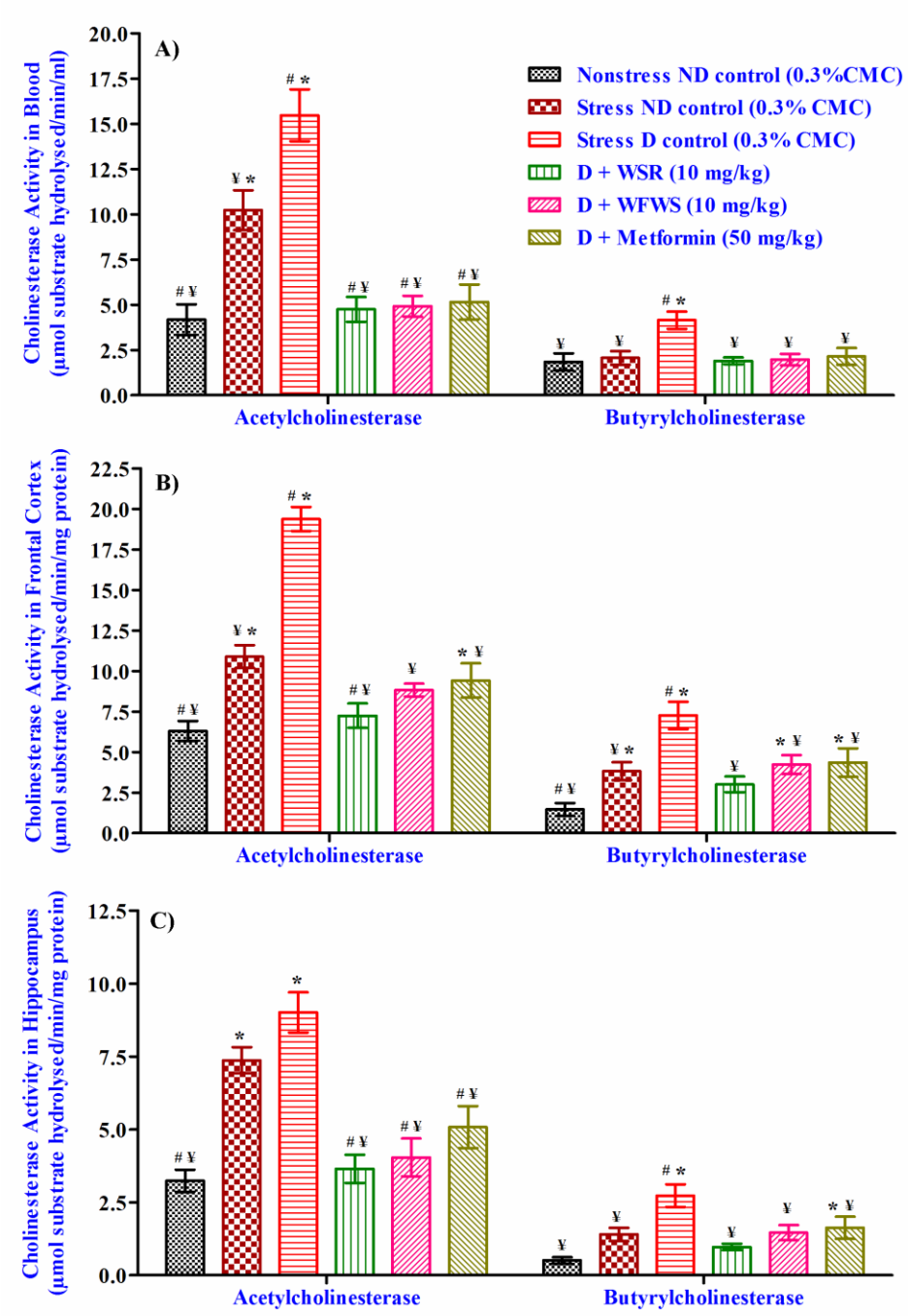

Figure 4.52: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme activity in **A)** blood, B) Frontal cortex and C) Hippocampus of diabetic rats. Values are mean ± SEM (n=6). Statistically significant difference (One way ANOVA followed by Student-Newman-Keuls multiple comparison test) were denoted with *=$p<0.05$ vs. Non-stress non-diabetic (ND) control; $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control; $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control.

4.5.3.10. Gastric ulceration: These results are summarized in **Table 4.20**. Observations made in this experiment were almost analogous to that of the previous described antidepressant experiments. The gastric ulceration induced by metabolic and physical stress were not observed with the WSR treated groups and was less sever with metformin (50 mg/kg) or the WFWS (10 mg/kg) treated groups (**Figure 4.53**). The mean ulcer index of the WFWS or metformin treated groups was much lower than the vehicle (0.3% CMC) treated stressed control groups (ca. 90% protection).

Table 4.20: Effects of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin on stomach ulcer index in normal and diabetic rats.

Treatment groups	Stomach ulcer index
Non-stress ND control (0.3% CMC)	$0.00\pm0.00^{\#¥}$
Stress ND control (0.3% CMC)	$0.92\pm0.17^{*¥}$
Stress D control (0.3% CMC)	$2.33\pm0.25^{*\#}$
D+ WSR (10 mg/kg)	$0.00\pm0.00^{\#¥}$
D+ WFWS (10 mg/kg)	$0.08\pm0.08^{\#¥}$
D+ Metformin (50 mg/kg)	$0.17\pm0.11^{\#¥}$

Values are mean ± SEM (n=6). Statistically significant difference (One way ANOVA followed by Student-Newman-Keuls multiple comparison test) were denoted with *=$p<0.05$ vs. Non-stress non-diabetic (ND) control; $^{\#}$=$p<0.05$ vs. Stress non-diabetic (ND) control; $^{¥}$=$p<0.05$ vs. Stress diabetic (D) control.

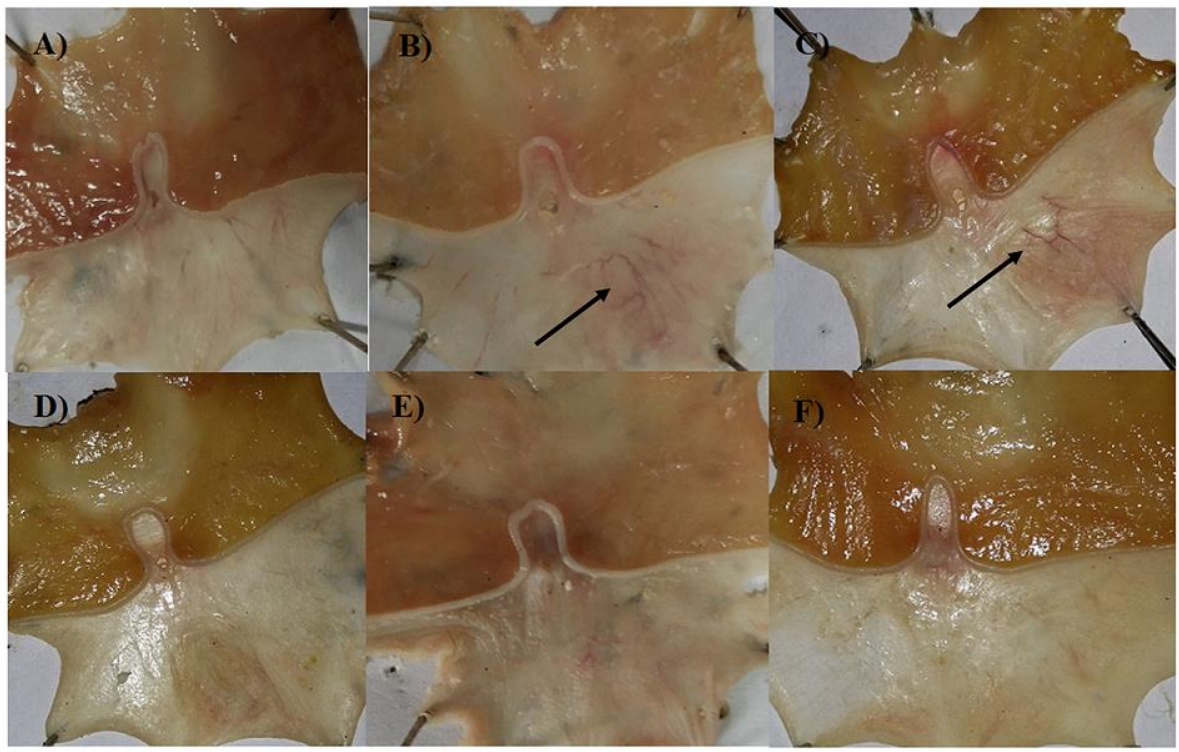

Figure 4.53: Effect of daily treatments with withanolides rich and withanolides free *Withania somnifera* extracts or metformin stomach ulceration in normal and diabetic rats. **A)** Non-stress non-diabetic (ND) control, **B)** Stress non-diabetic (ND) control, **C)** Stress diabetic (D) control, **D)** Diabetic + WSR (10 mg/kg), **E)** Diabetic + WFWS (10 mg/kg) and **F)** Diabetic + metformin (50 mg/kg).