FORM-F

COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH
Human Resource Development Group
(Extra Mural Research Division)
CSIR Complex, Library Avenue, Pusa, New Delhi – 110012

PROFORMA FOR PREPARING FINAL TECHNICAL REPORT

(Five copies of the report must be submitted immediately after completion of the research scheme)

CSIR Scheme no: No. 37 (1543) / 12/ EMR-II
SCTIMST Scheme no: 8066

1. Title of the scheme

To investigate the effects of short and long term administration of alpha asarone on oxidative stress and anxiety alleviation in insomnia model in rats.	Scheme No.: **No. 37 (1543) / 12/ EMR-II** Date of Commencement : **14/11/2012** Date of termination **:** **13/11/2015**

2. Name and address of Principal Investigator

Dr. Kamalesh K Gulia
Scientist-E
Division of Sleep Research
Biomedical Technology Wing, Poojappura
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3. Name of Sponsoring laboratory of CSIR (If applicable)

None

4. Total grant sanctioned and expenditure during the entire tenure

	Amount Sanctioned	Expenditure
Staff	5,28,000	4,70,941
Contingency +OH	9,28,157	7,73,509.4
Equipment	8,00,000	7,97,703
Total	22,56,157	20,42,153.6

5. Equipment(s) purchased out of CSIR grant

Name of Equipment	Cost in Rs	Supplier

1. EEG amplifiers (6); EMG (4); EOG (2) from Biopac, USA	7,29,664.00	Gentech Co., Delhi
2. Fluke 287/FVF model multimeter	54,113.00	Avante Global Services, Delhi
Minor Equipments		
2a. UPS	6,090.00	Cyber Corp., Tvm
3. Luxmeter	3,284.00	Valli Aqua & Process Ind.
4. Cyclomixer REMI CM 10	4,552.00	Inlab Equipments
5. Homogenizer	11,717.00	Inlab, Kochi
6. Guillotine	20,610.00	Holmarc, Kochi
7. Rotating wheel for sleep deprivation	33,663.00	Thermosystems, Trivandrum

6. Research fellows associated with scheme

Name& Designation	Date of Joining	Date of leaving
Dr. Lakshmy R, Research Associate	20.01.2014	09.01.2015
Dr. Baskaran R, Research Associate	27.01.2015	31.07.2015

7. Name(s) of the fellow(s) who received Ph.D. by working in the scheme, along with the Title(s) of thesis:

Ms Arathi R, PhD title "Study of sleep-wake properties of the medicinal plant, *Acorus calamus* Linn, in animal insomnia model", Thesis submission is due in 2017.

8. List of research papers published/communicated, based on the research work done under the scheme (Name(s) of author(s), Title, Journal, Volume number, Year and Pages should be given for each paper published and a copy of each of them should be enclosed; reprints/copies of papers appearing after submission of FTR should also be sent to CSIR):

Abstracts in Journal (Proceeding of Conferences)

Radhakrishnan R, Kumar VM, Gulia KK (2016). Alpha-asarone mediated improvement in sleep and anxiety in rat model of insomnia. Proceeding of the Indian Academy of Neurosciences 2016.

Gulia KK, Radhakrishnan R, Kumar VM (2015). Pre-clinical evaluation of 'α-asarone', an active principle of Acorus calamus for management of insomnia and anxiety. Proceedings of the International Training Workshop on Herbal Medicine: Drug Discovery from Herbs- Approaches, Innovations and Applications conducted by Centre for Science and Technology of the Non-Aligned and Other Developing Countries (NAM S&T Centre) at JSS University.

Radhakrishnan A, Ramachandran L, Jayakumari N, Kumar VM, Gulia KK (2014). Effect of alpha-asarone on sleep, brain and body temperature during acute total sleep deprivation. Sleep Biological Rhythms, Volume 12; Issue 4: 260

Ramachandran L, Radhakrishnan A, Kumar VM, Gulia KK (2014). Alpha-asarone alleviates anxiety and memory deficits induced by acute total sleep deprivation in rats. E-proceedings of the 8th ASRS-2014 Conference.

Book chapter:

Invited Chapter in Book: **"**Approach to sleep disorders in the traditional school of Indian medicine Complementary and alternative medicine" authored by Kamalesh K Gulia, Arathi Radhakrishnan, Velayudhan Mohan Kumar, in **Sleep Disorders Medicine**: Basic Science, Technical Considerations and Clinical Aspects. (4th Edition) In Press

9. Details of new apparatus or equipment designed or constructed during the investigation:

Rotating wheel for sleep deprivation (Fabricated). This was fabricated to achieve chronic sleep deprivation in rat model.

10. The likely impact of the completed work on the scientific/technological potential in the country (this may be attached as Enclosure-I):

Enclosure -I

11. Is the research work done of some industrial or agricultural importance and whether patent(s) should be taken? Yes/No; if yes, what action has been/should be taken:

Yes. We are trying for patent.

12. How has the research work complemented the work of CSIR Laboratory that sponsored your scheme?

Not applicable

13.Detailed account of the work carried out in terms of the objective(s) of the project and how for they have been achieved; results and discussion should be presented in the manner of a scientific paper/project report in about 5000 words; and this should be submitted as Enclosure-II to this report.

Enclosure-II

14. An abstract of research achievements in about 200-500 words, suitable for publication.

Insomnia is a very common medical problem in the current 24h civilization. As all the available medicines have several side effects, there is a dire need to find a relatively safe substitute medication. The present study was aimed to investigate the hypnotic potential of an active principle from the herb *Acorus calamus Linn*, alpha-asarone.

In this investigation, rat model for acute/chronic insomnia were used to examine the action of alpha-asarone. To tease out its mechanism of action, sleep-wakefulness (S-W) of animals were monitored simultaneous with brain and body temperature recordings before and after the drug injection since sleep and thermoregulation have close correlation. Sleep-wakefulness (S-W) was assessed electrophysiologically taking EEG and EMG in adult male Wistar rats which is the gold standard for sleep evaluation. A pre-calibrated thermocouple was also implanted stereotaxically in the hypothalamus, to measure brain temperature (T_{hy}). A radio-transmitter was implanted intra-peritoneally for the assessment of body temperature (T_{body}). After post-surgical recovery, baseline readings of all the signals were acquired for 8h (9 am to 5 pm). The rats were also tested using elevated plus maze at 2 pm, on different days, for assessing their anxiety levels. One set of animals

was subjected to 5 h sleep deprivation (SD) from 9 am 2 pm on five consecutive days. In another set of animals, alpha-asarone (10 mg/kg) was given in the beginning of sleep deprivation on five consecutive days. The data for sleep parameters, changes in the T_{hy} and T_{body} and the anxiety levels between the two groups were compared statistically. Results indicated that alpha-asarone enhanced the sleep quality and quantity, and reduced anxiety in insomniac rats. These findings suggest that alpha-asarone could be a potential candidate in the management of insomnia. In another set of animals, chronic sleep deprivation was achieved (SD for 3 weeks) and effects of administration of alpha-asarone were tested on S-W, brain and body temperature. The results indicated that prolonged use of alpha-asarone (10 mg/kg) improved the sleep in chronic insomnia rats.

In this study, we have produced a strong model to further investigate the hypnotic potential of various other herbal plants and to evaluate their pharmacogenic potential. It is challenging task to test the sleep enhancing property of a drug in free moving organism. This would be instrumental in scientific testing of the traditional medicines, which are not used effectively due to lack of evidences of safety, efficacy and quality due to the general lack of research.

15. Mention here whether or not the unspent grant has been refunded to CSIR: Yes
(Cheque enclosed for an amount of INR 2,14,003/)

Date: 24.12.2016 Signature of PI

Enclosure-I

Insomnia is a very common medical problem in the current 24h civilization all over the globe. As all the available medicines do have several side effects, there is a dire need to find a relatively safe substitute medication. The present study provided evidences for the hypnotic potential of an active principle from the herb *Acorus calamus Linn*, alpha-asarone. The evaluation of sleep-wakefulness by electrophysiological parameters of EEG and EMG, which is gold standard for sleep evaluation, provided the appraisal of effects of drug on sleep. This will open the avenues for testing various other herbal drugs/ products for their hypnotic potential electrophysiology which is a difficult and complex technique requiring special expertise.

In India, there are a very few laboratory for sleep testing. Our lab can be used to conduct further testing of potential herbals products for getting detailed pharmaceutical potentials of each herb/ product/ its active principals. Plants, herbs and ethno-botanicals are still used throughout the world for health promotion and treatment of diseases. Information from ethnic groups on indigenous traditional herbal medicines had always played a vital role in the discovery of novel chemotherapeutic agents from plants. In India, about 70% of the population depend on traditional medicines to help meet their healthcare needs. Further research will help in achieving appropriate safety, efficacy, good quality control of traditional medicines. We have made a strong model to further investigate the hypnotic potential of various other herbal plants and to evaluate their pharmacogenic potential. It is challenging task to test the sleep enhancing property of a drug in free moving organism.

Enclosure-II

Detailed account of the work carried out

The study was aimed to investigate the effects of short and long term administration of α-Asarone on oxidative stress and anxiety alleviation in insomnia rat model.

Objectives:

The effects of α-Asarone on the biochemical, physiological and cognitive components were investigated in acute and chronic sleep-deprived rats with the following objectives:

1. To find the effects of acute (single and multiple-5 days) total sleep deprivation (TSD) of 5 h on changes in brain and body temperature, antioxidant levels in the brain and anxiety.

2. To evaluate effects of short-term effects of administration of α-Asarone on spontaneous sleep-wake architecture, the brain-body temperatures, and anti-oxidant levels in acute sleep deprived rats (TSDX5 h, 5 days).

3. To assess the long-term effects of administration of α-Asarone on sleep-wake architecture, the brain-body temperatures, and anti-oxidant levels in chronic partial TSD (20 h X 1 day; 3 days; 7 days; 15 days; 21 days) rats.

Plan of work, methods and techniques used:

Adult male Wistar rats weighing between 220 - 250 g, bred and reared in the Animal Facility of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum were used for the study. These were housed in separate cages in an animal room having lights on from 6:00 to 18:00 hours (12:12 light: dark cycle) and controlled temperature. Food and water were provided *ad libitum*. The effect of α-asarone, an active principle of *Acorus calamus* Linn (AC), on oxidative parameters, anxiety alleviation was investigated in sleep deprived rats.

All procedures were conducted in accordance with the rules of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Institutional Animal Ethics Committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology had approved the study (B 1342009 VIII).

<u>Objective 1</u>: Effects of single and multiple (5 days) of TSDX5h on spontaneous changes on sleep-wakefulness, brain and body temperature, the levels of antioxidants in the brain, and anxiety levels.

Methods and techniques used:

Surgery for placement of electrodes for recording sleep, transmitter for body temperature and thermocouple for brain temperature: To assess sleep-wakefulness (S-W), the electrodes for electroencephalogram (EEG) and electromyogram (EMG) were implanted under anesthesia (Ketamine 60 mg/kg and Xylazine 5 mg/kg body weight, i.p.). A pre-calibrated thermocouple was implanted 1 mm anterior to the hypothalamus at an angle of 25 ° (AP: 1.2 mm, ML: 3 mm, DV: 5.83 mm as per Paxinos and Watson atlas) to measure the brain temperature (T_{hy}). The electrodes and thermocouple were connected to an IC socket. The whole assembly was fixed on the skull using dental cement. A radio transmitter (TA10TA-F40, DSI, USA) was implanted in the peritoneum for the assessment of body temperature (T_{body}). After post-operative recovery of 10 days, the rats were habituated to the recording chamber and baseline recordings for S-W, T_{hy} and T_{body} were taken.

Experimental protocol: All the recordings were performed in an environmental chamber, maintained at the ambient temperature of 27°C. After post-operative recovery, the rats were habituated to move freely in the recording cage. The S-W, T_{hy} and T_{body} were simultaneously recorded for 8 h from 9 am to 5 pm on 3 days to obtain a pre-treatment control data. The MP 150 system (BIOPAC systems) was used for acquisition of S-W. T_{hy} was recorded using Fluke multimeter (FLUKE True RMS Multimeter Model 287/289) and the data was stored every 10 sec. T_{body} was recorded using telemetric system (Data Sciences International system, USA). The rats were then subjected to acute TSDX5h for 5 h (9 am to 2 pm) with simultaneous recording of three parameters for 8 hrs. TSDX5h was achieved by gentle handling of animal prior to onset of sleep on the basis of the electrophysiological and behavioral parameters. To compare the effects of TSDX5h, 5 days, these parameters were calculated separately for initial 5 h (from 9 am to 2 pm) and from 6^{th} to 8^{th} h (2 pm to 5 pm).

Analysis of sleep-wake data and temperatures: The acquired S-W recordings were scored and were confirmed by manual scoring, taking 10 sec epoch duration. The S-W was classified into wakefulness, NREM sleep and REM sleep. Rebound sleep pattern after TSDX5h, 5 days was compared in hourly bin. T_{hy} and T_{body} were also noted at every 10 sec interval and the values were averaged, and plotted in hourly bins. The average bout duration of the sleep stages was calculated taking the ratio of total duration and the number of bouts. Arousal index, measure of sleep fragmentation, representing wake bouts of 3 to 10 s duration per hour of sleep was also calculated.

Tests for behavioral anxiety: Anxiety was assessed through behavioral test using elevated plus-maze (EPM) and open field test (OFT) in animals on different days of TSDX5h. EPM was tested on day 1 and 4 and OFT on day 1 and 5. ANY-maze software with a video tracking system was used to measure variables.

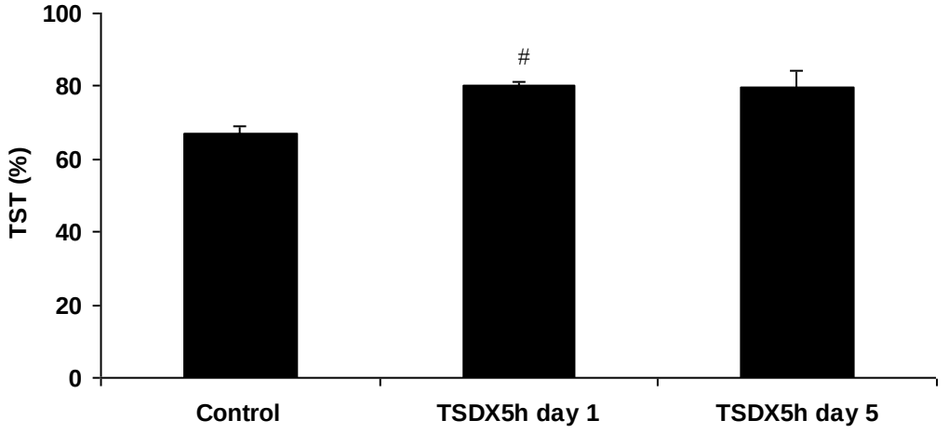
Biochemical estimation of oxidative stress parameters: On day 1 and 5, the animals were sacrificed by decapitation using guillotine at 2 pm. The brain were quickly removed and rinsed in pre-chilled saline and rapidly dissected on ice to separate 3 brain regions i.e. cortex, subcortex and brainstem, and processed to measure for estimation of oxidative stress markers. The total glutathione (GSH) and antioxidant enzymes like glutathione reductase (GSH-R), glutathione peroxidase (GSH-Px) superoxide dismutase (SOD), catalase (CAT) was measured by the methods previously employed in our biochemical laboratory (Jayakumari et al., 1992, 1998). Malondialdehyde (MDA), a biomarker for lipid peroxidation, was estimated by the TBA assay method (Deepa et al., 2008)

Results:

1.1. Effect of TSDX5h, 5 days on S-W, T_{hy} and T_{body}

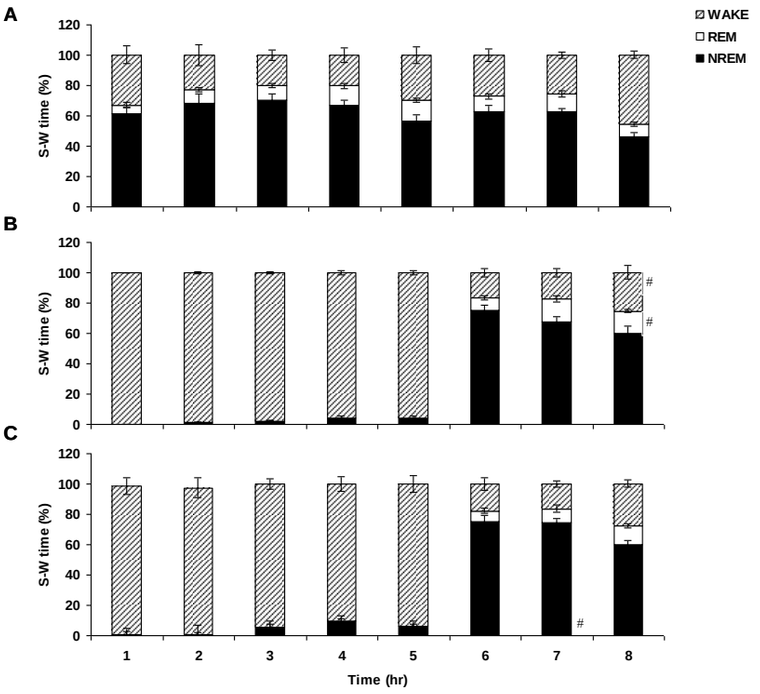
The sleep deprivation was achieved efficiently (above 95%) on all days (evident from % time in wake). Latency to sleep after the sleep deprivation protocol was 4.8 ± 1.9 min on day 1 and 3.5 ± 1.3 min on day 5 of TSDX5h. In comparison to the control, TSD resulted in an increased total sleep time after TSDX5h on day 1 (Fig. 1). Changes in NREM sleep, REM sleep and wakefulness are shown in Fig. 2.

Figure 1: Percentage total sleep time during in control, TSDX5h day 1 and TSDX5h day 5



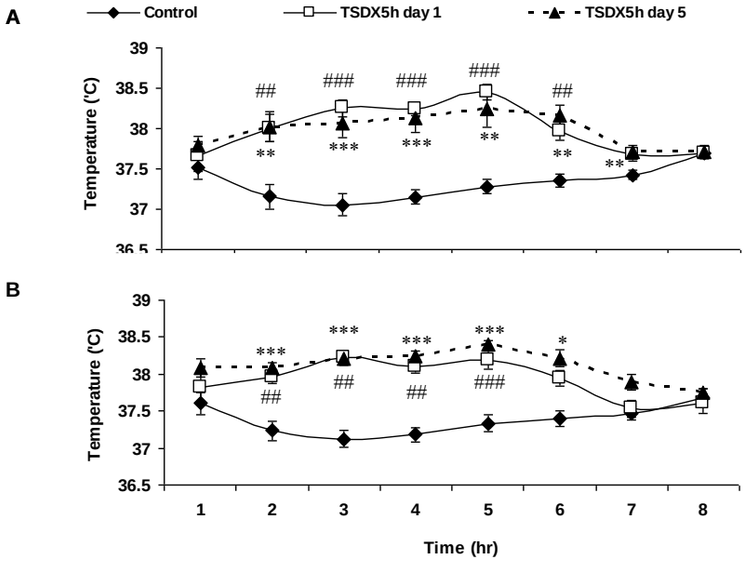
Change in the percentage total sleep time during (A) control, (B) TSDX5h, 1 day and (C) TSDX5h, 5 days for 3 h post TSD. The data is represented as mean ± SEM. $^{\#}$ indicates the comparison of groups with their respective control value of same time bin. Level of significance $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$. N= 5

Figure 2: Percentage sleep time of W, NREM and REM sleep during control, TSDX5h, 1 day and TSDX5h, 5 days



Change in S-W across 8 h during (A) control, (B) TSDX5h, 1 day and (C) TSDX5h, 5 days plotted in hourly bin for 8 h. The data is represented as mean ± SEM. # represents bin-wise comparison of stages of S-W with the respective control value. Level of significance # $p<0.05$. N= 5.

Figure 3: Change in T_{body} and T_{hy} in hourly bin across 8 hrs during control, TSDX5h, 1 day and TSDX5h, 5 days



Change in (A) T_{body} and (B) T_{hy} plotted in hourly bins during control, TSDX5h, 1 day and TSDX5h, 5 days plotted in hourly bin for 8 h. The data is represented as mean ± SEM. * represents bin-wise comparison of TSDX5h day 5 values with control, # represents bin-wise comparison of TSDX5h day 1 values with control. Level of significance * $p<0.05$, ** ## $p<0.01$, *** ### $p<0.001$. N= 5

During TSDX5h, there was a gradual increase of T_{body} and T_{hy} with progression of sleep deprivation on day 1 and 5. Increase in T_{hy} was observed on day 5 of TSDX5h (Fig. 3). Post-TSDX5h (6 to 8 h), T_{body} started to decrease and was found to attain normalcy by 6^{th} and 7^{th} h on day 1 and 5 respectively. T_{hy} was found to attain normalcy by 6^{th} hr on day 5 of TSDX5h (Fig. 3).

Fig. 4A shows an increase in NREM mean bout length after TSDX5h on day 1 and 5. The increase was significant in the 6^{th} h on day 1 and 7^{th} h on day 5 of TSDX5h. No change was observed in the arousal index after TSDX5h when compared to control (Fig. 4B).

Figure 4: Average NREM bout duration and arousal index during control, TSDX5h, 1 day and TSDX5h, 5 days

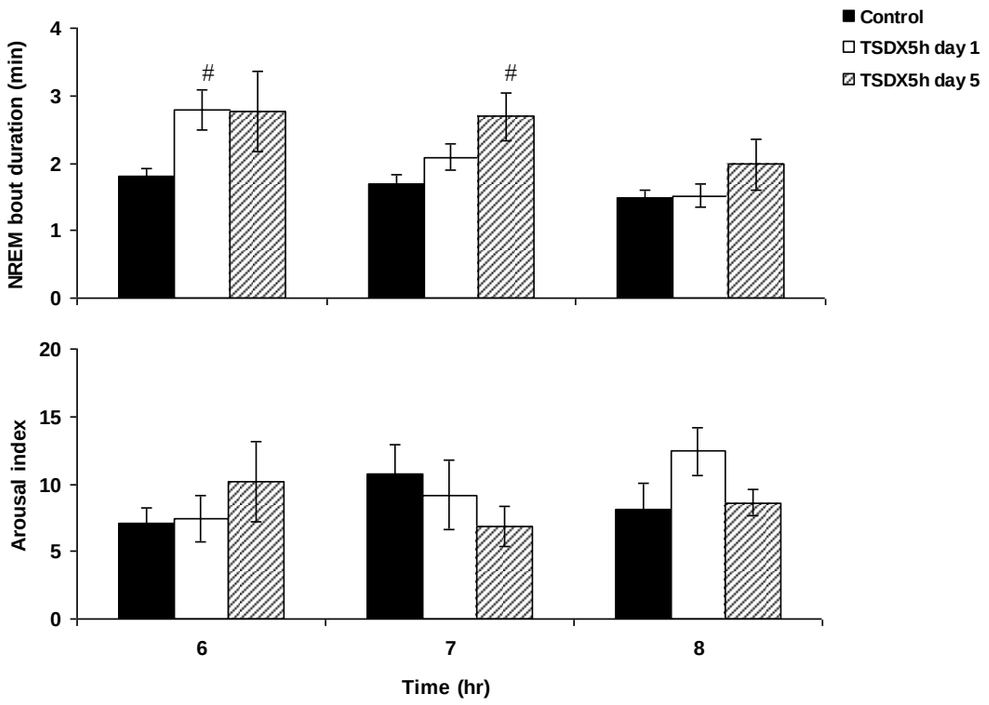
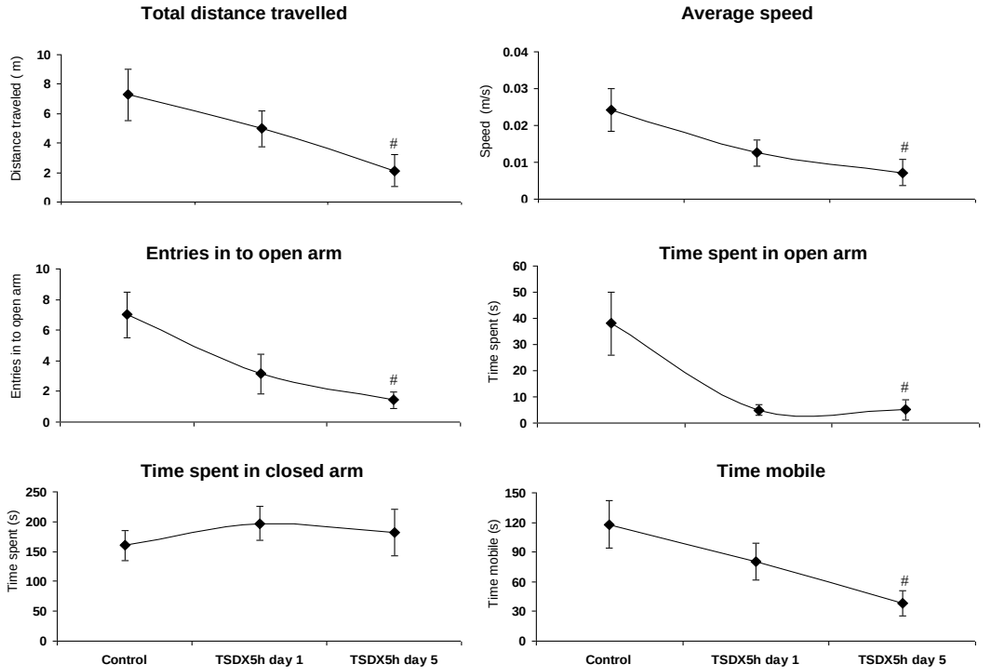


Fig. 4 (A) Average bout duration of NREM sleep and (B) arousal index after TSDX5h, 1 day and TSDX5h, 5 days plotted in hourly bin for 3 h (2 pm to 5 pm). The data is represented as mean ± SEM. # represents bin-wise comparison of TSDX5h values with control. Level of significance # $p<0.05$. N= 5

1.2. Effect of TSDX5h, 1 day and TSDX5h, 5 days on anxiety

Figure 5 and 6 shows a significant increase in anxiety in EPM and OFT tests after TSDX5h. In elevated plus maze test, the time spent in the open arm was declined on day 1 and 5 of TSDX5h (Fig. 5). By day 5, entries in to open arm, distance traveled and mobility on the maze was also decreased significantly (Fig. 5).

Figure 5: Anxiety levels tested in EPM during control, TSDX5h, 1 day and TSDX5h, 5 days



Changes in various EPM parameters indicating the anxiety levels in rats during control, TSDX5h, 1 day and TSDX5h, 5 days. The data is represented as mean ± SEM. # represents bin-wise comparison of TSDX5h values with control. Level of significance # $p<0.05$. N= 5

In open field test, TSDX5h resulted in significant decrease in the time spent in inner zone and increase in the time spent in outer zone (Fig. 6). Furthermore, entries in to inner zone and total mobility over the maze were found decreased indicating increased anxiety in animals subjected to TSDX5h (Fig. 6).

Figure 6: Anxiety levels tested in OFT during control, TSDX5h, 1 day and TSDX5h, 5 days

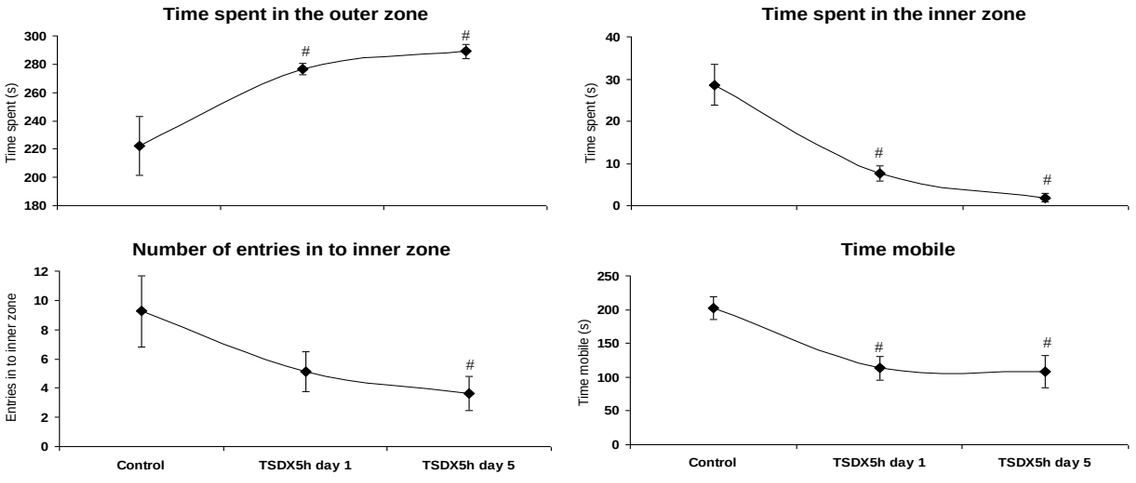
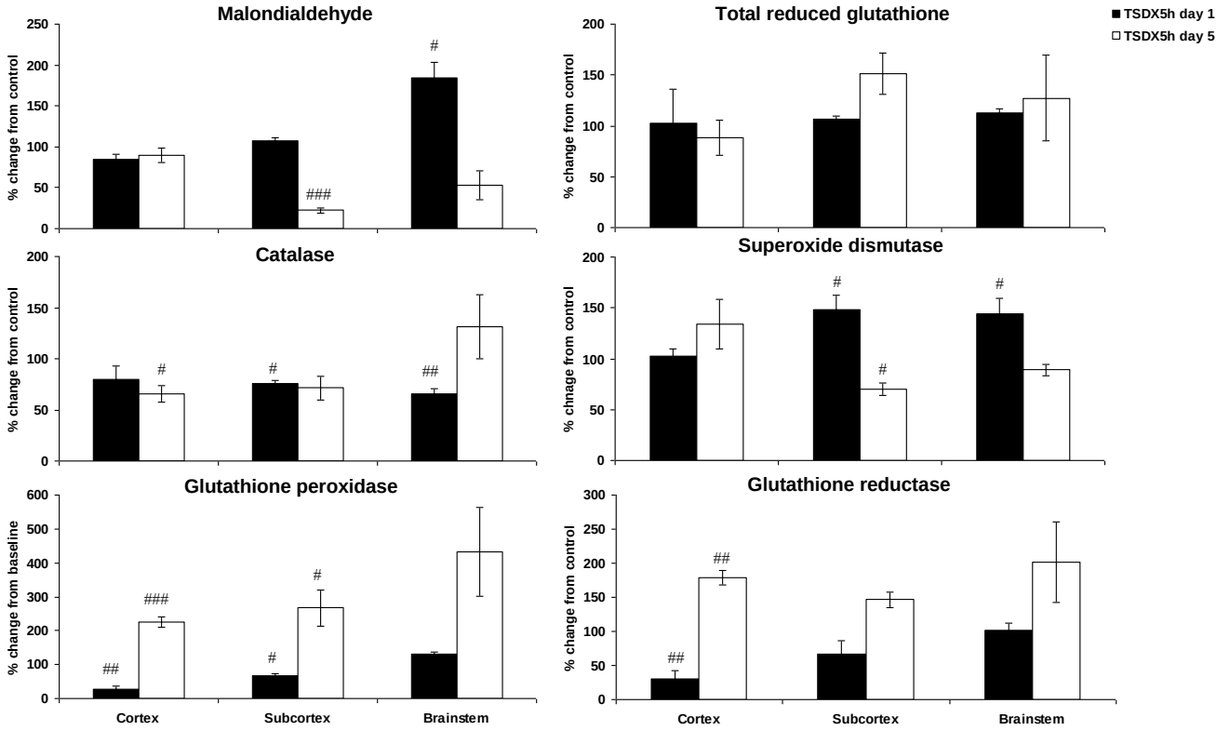


Fig. 6 Changes in various OFT parameters indicating the anxiety levels in rats during control, TSDX5h, 1 day and TSDX5h, 5 days. The data is represented as mean ± SEM. # represents bin-wise comparison of TSDX5h values with control. Level of significance # $p<0.05$. N=5

1.3. Effect of TSDX5h, 1 day and TSDX5h, 5 days on oxidative stress markers in brain

Changes in various oxidative markers after 1 and 5 days of TSDX5h are given in Figure 7.

Figure 7: Changes in various oxidative stress markers in brain during TSDX5h, 1 day and 5 days



Changes in oxidative stress markers in rat brain subjected to TSDX5h for 1 day and 5 days. The data is represented as mean ± SEM. $^{\#}$ represents bin-wise comparison of TSDX5h values with control. Level of significance $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$. N= 5

On day 1, the activities of GSH-Px and GSH-R were decreased in cortex, the activities of CAT and GSH-Px decreased and SOD increased in subcortex and level of MDA and activity of SOD increased in brainstem (Fig. 7). By day 5 of TSDX5h, MDA levels came down in both subcortex and brainstem, activity of SOD declined in subcortex, and activities of glutathione peroxidase and glutathione reductase were increased in cortical and subcortical areas (Fig. 7).

Discussion and conclusion for objective 1:

Acute TSDX5h for 5 days was altered the S-W pattern and temperature profile of rats in this study. NREM sleep was found to increase marginally after acute sleep deprivation. A slight increase in the NREM bout duration was also observed after sleep deprivation. No change was observed in REM sleep quality or quantity. In a previous report, shorter sleep deprivation (3 h or 6 h) increases NREM sleep without affecting REM component (Tobler and Borbely, 1986, 1990). The suppression of NREM sleep was found to produce an immediate NREM sleep rebound in the first recovery hour. However, acute reduction of NREM sleep resulted in an inferior NREM sleep rebound (Endo et al., 1997). Total

sleep deprivation experiments of different durations have previously shown that the level of NREM sleep during recovery is a function of prior waking (Tobler and Borbely, 1986, 1990). An increase in T_{hy} and T_{body} associated with sleep deprivation was found in this study. Previously, T_{hy} was found increased from the baseline by 0.4 °C in sleep-deprived rats and T_{body} showed an initial increase followed by decrease during prolonged sleep deprivation (Everson, 1995).

Rats acutely deprived of sleep were found to have increased anxiety and had altered antioxidant levels in brain. There is no earlier report that had studied the anxiety levels of rats in EPM and OFT after acute sleep deprivation by gentle handling method. However, in some studies, sleep deprivation was found to increase anxiety (Kumar et al., 2009, Silva et al., 2004, Vollert et al., 2011). Loss of sleep or insomnia has been found to produce oxidative damage in the body (Reimund, 1994). On day 1, increased levels of MDA and activity of SOD and decreased activities of CAT and GSH-Px shows the sleep loss associated oxidative stress. However, by day 5 of sleep deprivation, the cellular adaptive response was observed in the regions studied. A natural tendency to normalize the oxidative stress induced by acute sleep loss was seen in cortex, subcortex and brainstem. Ramanathan et al., (2010) proposed that in acute or short term sleep deprivation increased production of free radicals is balanced by the increased antioxidant responses whereas in chronic or long term sleep loss this balance is disrupted leading to decreased antioxidant responses. They also reported that this differential effect of acute and chronic sleep deprivation varies across brain regions (Ramanathan et al., 2002).

<u>Objective 2</u>: To evaluate effects of short-term effects of administration of α-Asarone on spontaneous sleep-wake architecture, the brain-body temperatures, and antioxidant levels in acute sleep deprived rats (TSDX5h, 5 days).

Methods and techniques used: Implanted rats were kept for recording S-W, T_{hy} and T_{body} (as mentioned in objective 1). The S-W, T_{hy} and T_{body} were simultaneously recorded for 8 h from 9 am to 5 pm on 3 days to have a pre-treatment control data. Recording setup and acquisition done as mentioned in objective 1.

2.1. Dose-response effects of α-Asarone in S-W, T_{hy} and T_{body} of normal rats

For the dose-response study, after taking pre-drug recording from 9 am to 10 am, rats were intraperitoneally administered with vehicle or different doses of α-Asarone (2, 10, 40, 80 and 120 mg/kg) at 10 am on different days (interval of 5 days) followed by a post-drug recording till 5 pm. Analysis for S-W, T_{hy} and T_{body} was done as mentioned in objective 1.

2.2. Effect of α-Asarone on TSDX5h, 5 days-induced changes in S-W, T_{hy} , T_{body}, anxiety and antioxidant levels

The rats were subjected to TSDX5h, 5 days (9 am to 2 pm) with one group receiving vehicle and the other receiving α-Asarone at 9 am intraperitoneally. After TSDX5h, the recording was continued for 3 h (2 pm to 5 pm) on day 1 and 5. Gentle handling method was used for depriving sleep as mentioned in objective 1. Analysis for S-W, T_{hy} and T_{body} was done as mentioned in objective 1. Immediately after TSDX5h, Elevated plus maze (EPM) and Open field test (OFT) were performed to test the anxiety level in rats. EPM was tested on day 1 and 4 and OFT on day 2 and 5. Soon after EPM and OFT, the rats were decapitated, brains quickly removed, rinsed in pre-chilled saline, rapidly dissected on ice (cortex, sub-cortex and brainstem) and immediately transferred to −80°C for analysis of biochemical parameters (as mentioned in objective 1).

Results:

2.1. Dose-response effects of α-Asarone on S-W, T_{hy} and T_{body} in normal rats

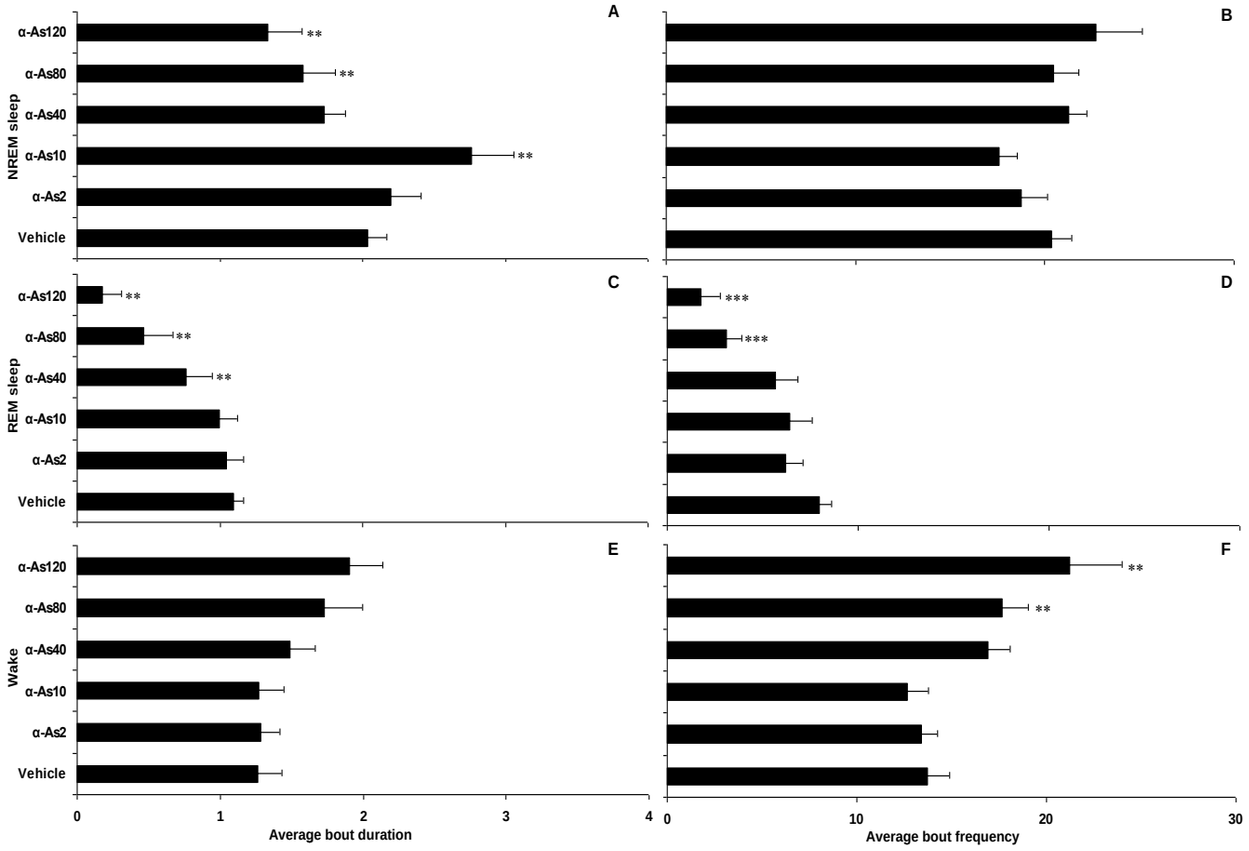
Out of the various doses studied, α-Asarone 10 mg/kg was found to improve the quality of sleep as indicated by increased average NREM bout duration (Fig. 8). However, no change was observed in the quantity of NREM or REM sleep at lower doses (Fig. 9). Unlike lower doses, sleep quality and quantity was significantly reduced after administration of 40, 80 and 120 mg/kg α-Asarone. Decrease in the NREM sleep time and bout duration and an increase in wake time were observed after administration of 80 and 120 mg/kg α-Asarone (Fig. 8 and 9). After administration of 40, 80 and 120 mg/kg α-asarone, a decrease in the percentage REM sleep time was observed (Fig. 9). In addition, the bout duration of REM sleep was also reduced after the administration of these doses (Fig. 8).

Changes in T_{body} and T_{hy} in hourly bin after the administration of various doses of α-Asarone are shown in Table 1. For all the doses of α-Asarone, minimal peak of T_{body} and T_{hy} was observed in the 2^{nd} h (Table 1). The decrease in the T_{body} and T_{hy} was evident from the 1^{st} hr after administration of various doses of α-Asarone, however, this change persisted for 7 h at doses 80 and 120 mg/kg (Table 1). In comparison to the vehicle group, a significant reduction in the T_{body} and T_{hy} was observed after administration of 80 and 120 mg/kg α-Asarone (Table 1). A marginal reduction in the T_{body} and T_{hy} was also observed after administration of 40 and 10 mg/kg α-Asarone (Table 1).

2.2. Effect of α-Asarone (10 mg/kg) on TSDX5h, 1 day- and 5 days-induced changes in S-W, T_{hy} and T_{body}

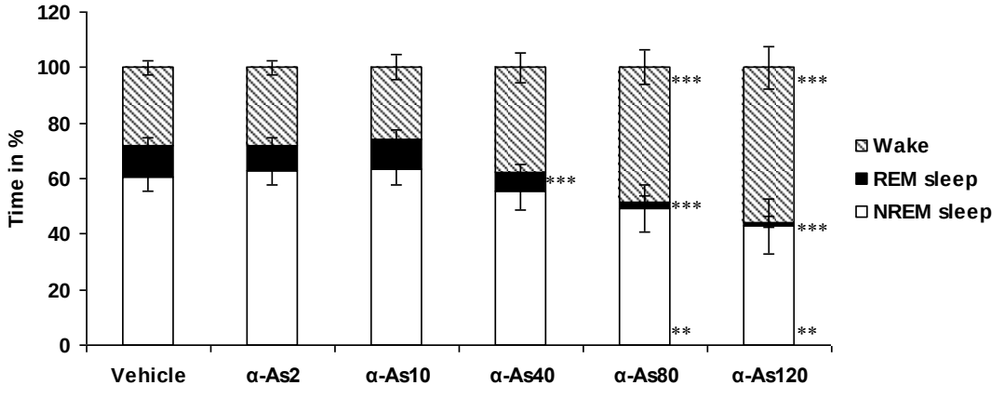
The latency to sleep during the recovery period after sleep deprivation was found to be significantly lesser ($p=0.045$) in α-Asarone-treated group in comparison to the group which received vehicle treatment on day 5. In comparison to their respective control, both groups showed an increase in the total sleep time after 5 h of SD (Fig. 10). However, only in the α-Asarone-treated sleep deprived group, the total sleep time was increased from the control level after 5 days of SD (Fig. 10).

Figure 8: Effects of various doses of α-Asarone on the average bout duration of W, NREM and REM sleep



The average bout duration of NREM, REM and Wake bouts for 7 hr after the administration of vehicle and 2, 10, 40, 80 and 120 mg/kg α-Asarone (α-As) in rats (N=5). The bars represent mean ± SEM. * indicates the difference from the vehicle. Level of significance ** p<0.01 and *** p<0.001

Figure 9: Effect of various doses of α-Asarone on the percentage NREM, REM and Wake time



Changes in the percentage NREM and REM sleep time and wakefulness for 7 hr after the administration of vehicle and 2, 10, 40, 80 and 120 mg/kg α-Asarone (α-As) in rats (N=5). The bars represent mean ± SEM. * indicates the difference from the vehicle. Level of significance ** p<0.01 and *** p<0.001.

Figure 10: Percentage total sleep time during recovery period after administration of vehicle and α-Asarone in sleep deprived rats

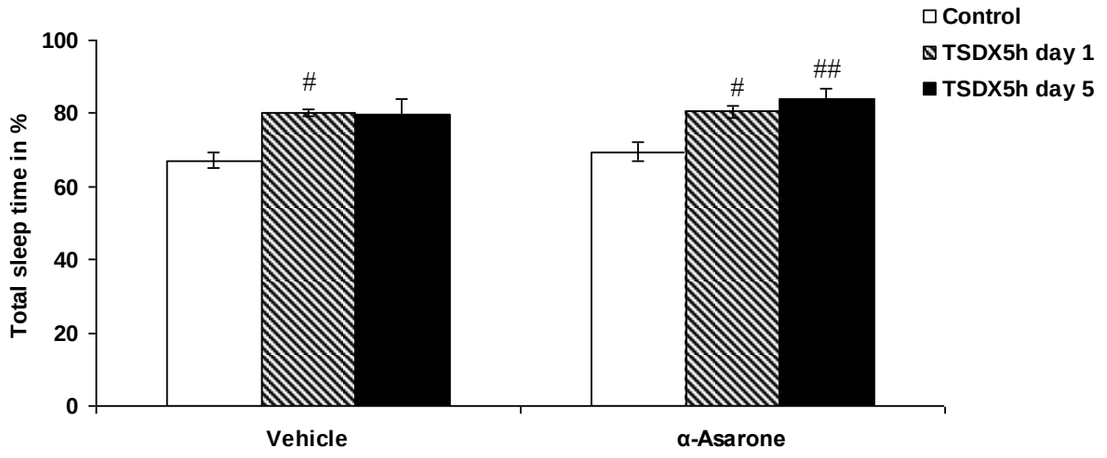


Fig. 10 Change in the percentage total sleep time from 2 pm to 5 pm after treatment of vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam on day 1 and 5 in sleep deprived rats. The data is represented as mean ± SEM and TSD indicates total sleep deprivation. $^{\#}$ indicates the comparison of groups with their respective control value of same time bin. Level of significance $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$. N= 5 for each group.

Fig. 11 shows change in the percentage S-W, T_{body} and T_{hy} during control, TSDX5h day 1 and TSDX5h day 5 in the three groups plotted in hourly bin for 3 h. In comparison to the vehicle-treated group, a marginal increase in the NREM sleep time ($p=0.05$) was observed in the α-Asarone-treated group in the 3rd h of recovery period (Fig. 11).

In the same hour a decrease in the REM sleep time was also observed in the α-Asarone-treated group in comparison to its vehicle counterpart (Fig. 11). However, after 5 days of TSDX5h, the NREM and REM sleep time in the α-Asarone-treated group was found similar to the vehicle group (Fig. 11). In both groups, a significant increase in the T_{body} and T_{hy} was observed during TSDX5h on both day 1 and 5 in comparison to the control. On both day 1 and 5 of sleep deprivation, T_{body} and T_{hy} was found to normalize soon after the TSDX5h by 1st h of recovery period in the α-Asarone-treated group (Fig. 11B). T_{hy} drop in the 1st and 2nd h after TSDX5h indicates faster recovery to normalcy in comparison to the vehicle-treated group (Fig. 11B).

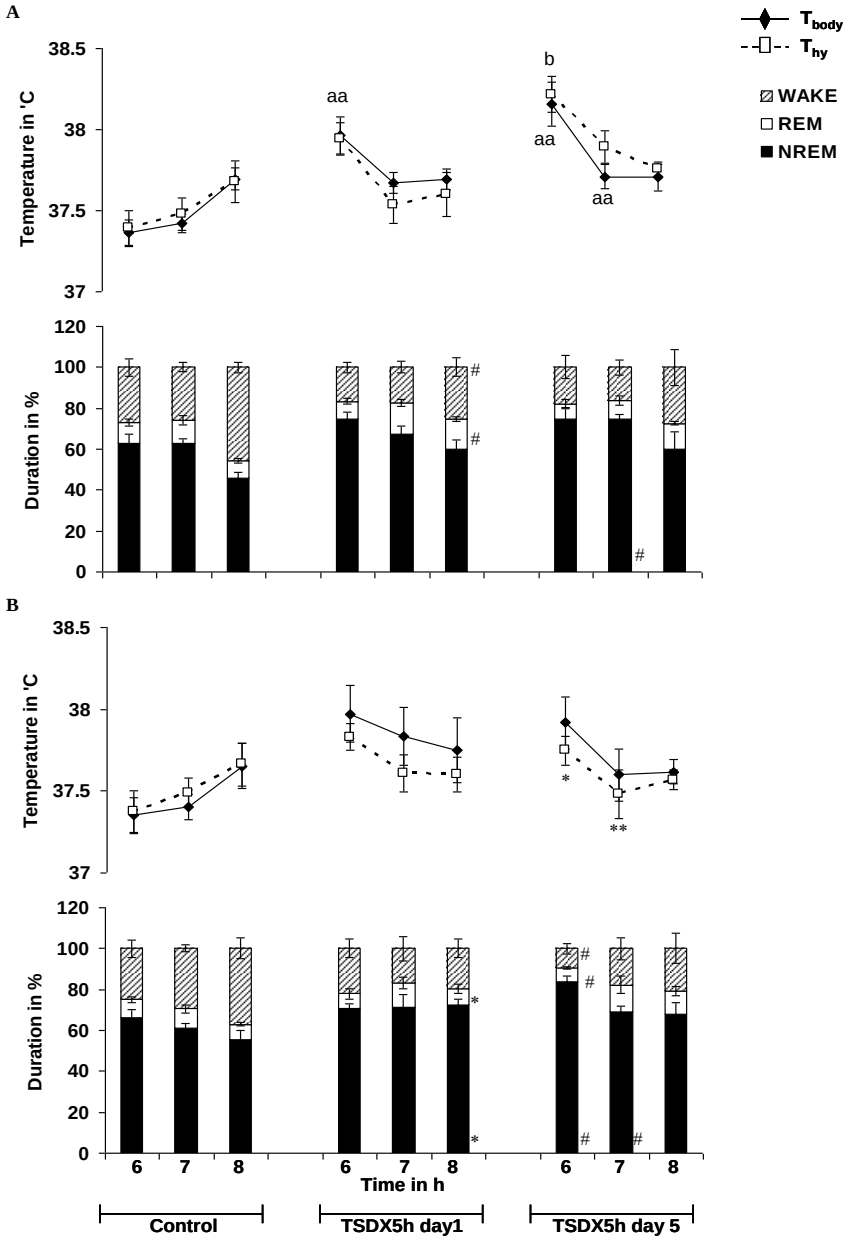
Table 1 Effects of different doses of α-Asarone on T_{body} and T_{hy} in normal rats

	Vehicle		**α-As2**		**α-As10**		**α-As40**		**α-As80**		**α-As120**	
	T_{body}	**T_{hy}**	**T_{body}**	**T_{hy}**	**T_{body}**	**T_{hy}**	**T_{body}**	**T_{hy}**	**T_{body}**	**T_{hy}**	**T_{body}**	**T_{hy}**
0	37.6 ± 0.1	37.7 ± 0.2	37.3 ± 0.2	37.5 ± 0.3	37.4 ± 0.2	37.5 ± 0.2	37.5 ± 0.2	37.7 ± 0.2	37.5 ± 0.2	37.57 ± 0.2	37.7 ± 0.2	37.9 ± 0.3
1	37.5 ± 0.1	37.7 ± 0.1	37.3 ± 0.2	37.4 ± 0.2	37.0 ± 0.2 $^{\#}$	37.2 ± 0.2 $^{\#\#}$	36.8 ± 0.2 $^{\#\#}$	37.2 ± 0.1 $^{\#\#}$	36.4 ± 0.1 $^{*\,\#\#}$	36.49 ± 0.1 $^{*\,\#\#}$	36.7 ± 0.1 $^{*\,\#\#}$	36.6 ± 0.1 $^{**\,\#\#\#}$
2	37.4 ± 0.1	37.5 ± 0.1	37.2 ± 0.1	37.3 ± 0.2	36.8 ± 0.1 $^{*\,\#}$	36.8 ± 0.1 $^{*\,\#}$	36.7 ± 0.2 $^{*\,\#\#}$	36.8 ± 0.3 $^{*\,\#}$	35.5 ± 0.1 $^{**\,\#\#}$	35.77 ± 0.2 $^{**\,\#\#}$	35.5 ± 0.3 $^{**\,\#\#}$	35.9 ± 0.2 $^{**\,\#\#}$
3	37.3 ± 0.1	37.5 ± 0.2	37.3 ± 0.1	37.4 ± 0.1	37.0 ± 0.1	37.0 ± 0.2	36.9 ± 0.2	36.8 ± 0.4	35.6 ± 0.3 $^{**\,\#\#}$	35.8 ± 0.3 $^{**\,\#\#}$	35.6 ± 0.4 $^{*\,\#\#}$	36.0 ± 0.3 $^{*\,\#\#}$
4	37.4 ± 0.1	37.6 ± 0.1	37.3 ± 0.1	37.5 ± 0.1	37.1 ± 0.1	37.0 ± 0.2	37.0 ± 0.1	37.0 ± 0.3	35.9 ± 0.3 $^{**\,\#\#}$	36.1 ± 0.3 $^{**\,\#}$	35.9 ± 0.3 $^{*\,\#\#}$	36.3 ± 0.3 $^{*\,\#\#}$
5	37.4 ± 0.1	37.6 ± 0.2	37.5 ± 0.1	37.8 ± 0.1	37.3 ± 0.1	37.2 ± 0.1	37.2 ± 0.2	37.2 ± 0.2	36.4 ± 0.2 $^{*\,\#}$	36.6 ± 0.3 $^{*\,\#}$	36.2 ± 0.3 $^{*\,\#\#}$	36.6 ± 0.3 $^{*\,\#\#}$
6	37.6 ± 0.1	37.8 ± 0.1	37.6 ± 0.2	37.7 ± 0.1	37.4 ± 0.1	37.4 ± 0.1	37.4 ± 0.2	37.5 ± 0.2	36.8 ± 0.2	36.8 ± 0.3 $^{\#}$	36.6 ± 0.3 $^{*\,\#\#}$	37.0 ± 0.2 $^{*\,\#\#}$
7	37.7 ± 0.2	37.9 ± 0.1	37.6 ± 0.2	37.6 ± 0.2	37.6 ± 0.2	37.6 ± 0.1	37.6 ± 0.2	37.6 ± 0.2	37.1 ± 0.1	37.0 ± 0.2 $^{\#}$	36.9 ± 0.2 $^{*\,\#\#}$	37.3 ± 0.2 $^{\#\#}$

Values are represented as mean ± SEM and are shown in hourly bins for 7 hr after the administration of vehicle and α-Asarone (α-As) 2, 10, 40, 80 and 120 mg/kg in rats (N=5). 0 represents the pre-injection baseline, T_{body} indicate body temperature and T_{hy} indicate hypothalamic temperature.

* indicates the difference from the vehicle and # indicates the difference from the pre-injection baseline (0). Level of significance * # $p<0.05$, ** ## $p<0.01$, ###$p<0.001$

Figure 11: Change in S-W, T_{body} and T_{hy} after administration of vehicle and α-Asarone in sleep deprived rats



Change in S-W, T_{body} and T_{hy} from 2 pm to 5 pm after treatment of (A) vehicle and (B) 10 mg/kg α-Asarone on day 1 and 5 of TSDX5h plotted in hourly bin. The data is represented as mean ± SEM. * represents bin-wise comparison with the vehicle-treated group, # represents bin-wise comparison of stages of S-W with the respective control value, a represents the bin-wise comparison of T_{body} with the respective control value and b represents the bin-wise comparison of T_{hy} with the respective control value. Level of significance * # b $p<0.05$, ** aa bb $p<0.01$. N= 5 for each group.

Figure 12: Average bout duration of NREM sleep and arousal index during recovery period after administration of vehicle and α-Asarone in sleep deprived rats

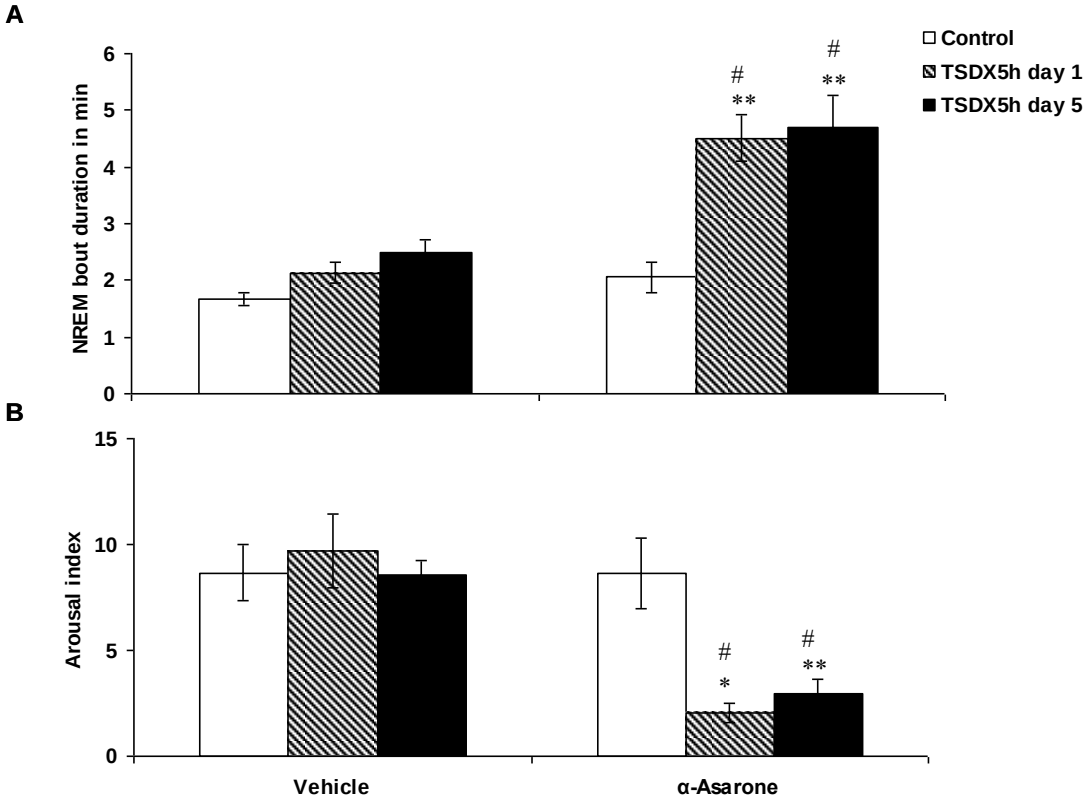


Fig. 12 Changes in the (A) average NREM bout duration in min and (B) arousal index after treatment of vehicle and 10 mg/kg α-Asarone on day 1 and 5 in sleep deprived rats. The data is represented as mean ± SEM and TSD indicates total sleep deprivation. * represents comparison with the vehicle group, # indicates the comparison of groups with their respective control value of same time bin. Level of significance * # $p<0.05$, ** $p<0.01$. N= 5 for each group.

NREM bout duration was significantly increased in the α-Asarone-treated group in comparison to vehicle group on day 1 and 5 of TSDX5h (Fig. 12A). The increase in NREM bout duration was found to be significantly different in comparison to its respective control of the same time bin (Fig. 12A). No significant change was observed in the bout duration and frequency of REM sleep. Decrease in the arousal index during the recovery period on day 1 and 5 further confirms the improvement in sleep quality in α-Asarone-treated sleep deprived group (Fig. 12B). An increase in the mean delta power was observed in both groups after 5 h of TSDX5h (Fig. 13). In comparison to the vehicle-treated group, mean delta power was found to be higher in the 1st h of recovery period in the α-Asarone-treated group after 5 h of sleep deprivation (Fig. 13).

Figure 13: Mean delta power during recovery period after administration of vehicle and α-Asarone in sleep deprived rats

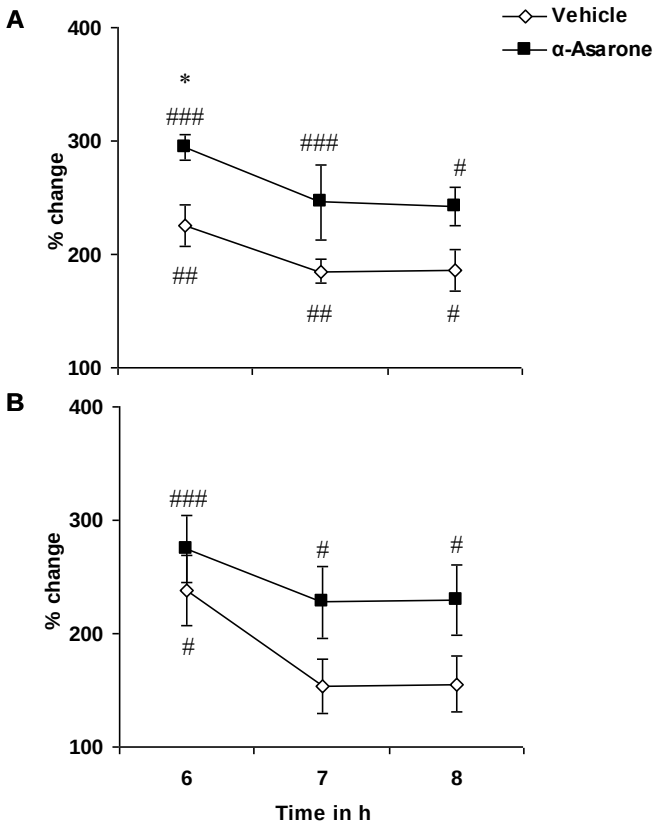


Fig. 13 Change in mean delta power in percentage after treatment of vehicle and 10 mg/kg α-Asarone on (A) day 1 and (B) day 5 in sleep deprived rats. The data is represented as mean ± SEM and is represented by taking the percentage change from the representative control values (taken as 100 %). * represents comparison with the vehicle group and # indicates the comparison of groups with their respective control value of same time bin. Level of significance $^{*\ \#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$. N= 5 for each group.

2.3. Effect of α-Asarone (10 mg/kg) on TSDX5h-induced anxiety

In comparison to the vehicle-treated group, α-Asarone-treated group showed decreased anxiety in elevated plus maze test. Alpha-asarone treatment increased the time spent in the open arm, entries in to open arm, distance traveled and average speed on the elevated plus maze (Fig. 14).

Decreased level of anxiety after α-Asarone treatment was further observed in the open field test. The rats spent more time in the inner zone and less time in the outer zone. Also the entry in to inner zone increased (Fig. 15).

Figure 14: Changes in EPM parameters after administration of α-Asarone and vehicle in sleep deprived rats

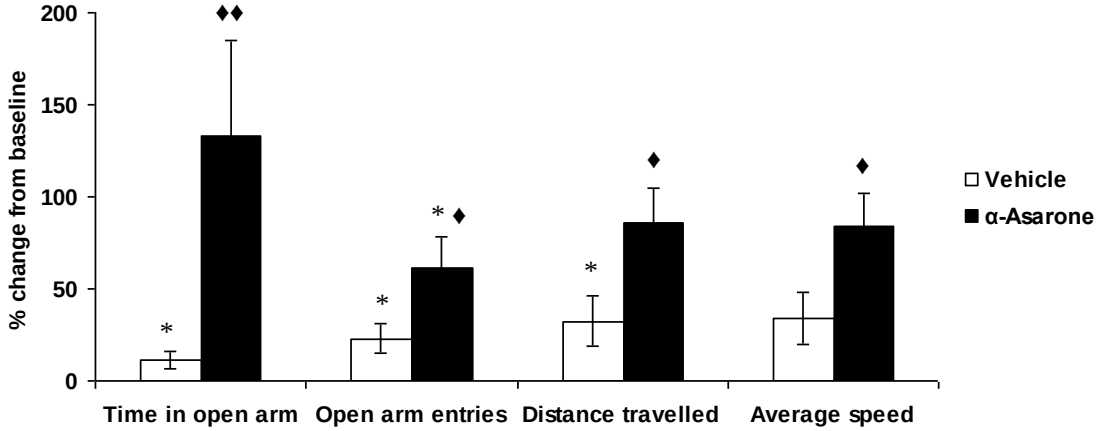


Fig. 14 Changes in the parameters in EPM test show decreased levels of anxiety in those rats that received α-Asarone before sleep deprivation. The graph shows variables on day 4 of sleep deprivation as compared to the baseline values (taken as 100%). * indicates significant change from the baseline and ♦ indicates significant difference between vehicle and α-Asarone groups on day 4 of sleep deprivation. The data is represented as mean ± SEM. Levels of significance * ♦ $p<0.05$ and ♦♦ $p<0.01$. N= 7 for vehicle group and N= 9 for α-Asarone group.

Figure 15: Changes in OFT parameters after administration of α-Asarone and vehicle in sleep de

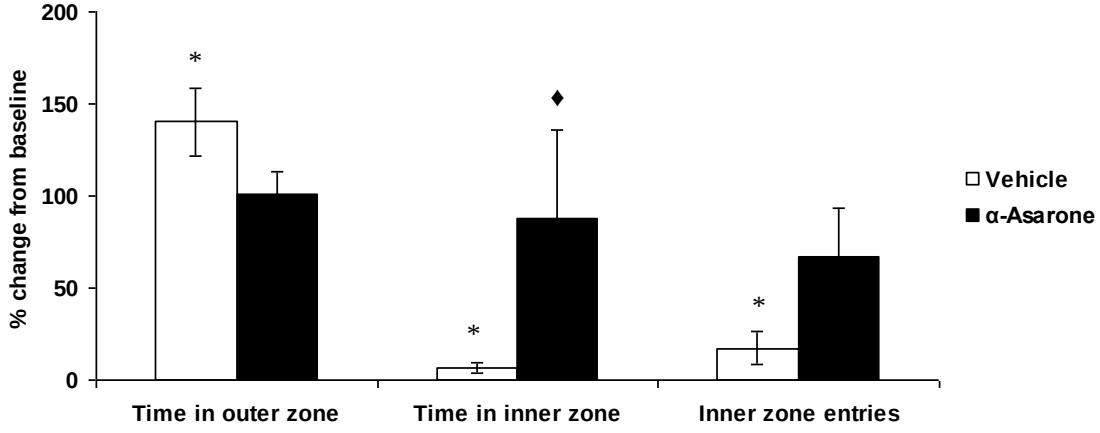
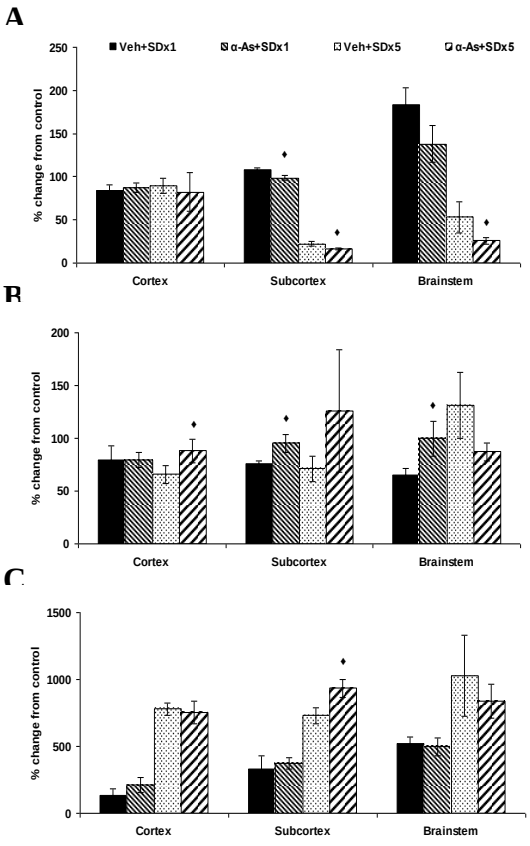


Fig. 15 Changes in the parameters in OFT shows decreased levels of anxiety in those rats that received α-Asarone before sleep deprivation. The graph shows variables on day 5 of sleep deprivation compared to the baseline values (taken as 100%). * indicates significance from the baseline and ♦ indicates significant difference between vehicle and α-Asarone groups on day 5 of sleep deprivation. Levels of significance * ♦ $p<0.05$. N= 7 for vehicle group and N= 9 for α-Asarone group.

2.4. Effect of α-Asarone (10 mg/kg) on TSDX5h-induced changes in oxidative stress markers

In subcortical region, a decrease in the level of MDA and an increase in the activity of CAT were observed in α-Asarone-treated group in comparison to vehicle-treated group (Fig. 16).By day 5 of TSDX5h, an increase in the GSH-R level was also observed in the subcotex of α-Asarone-treated group. CAT activity was higher on day 1 and MDA level was lowering on day 5 in the brainstem region of α-Asarone-treated sleep deprived group (Fig. 16).

Figure 16: Changes in the (A) level of MDA and (B) activity of CAT and GSH-R after administration of α-Asarone and vehicle in sleep deprived rats.



Changes in the (A) level of MDA, (B) activity of CAT and (C) activity of GSH-R in cortex, subcortex and brainstem after administration of α-Asarone and vehicle in sleep deprived rats as compared to the control mean value (taken as 100%).♦ indicates significant difference between vehicle and α-Asarone groups of sleep deprivation. The data is represented as mean ± SEM. Levels of significance ♦ $p<0.05$. Groups: Veh + SDx1 (N= 6), α-As + SD x1 (N= 7), Veh + SDx5 (N= 5) and α-As + SDx1 (N= 5).

Discussion and conclusion for objective 2:

Alpha-asarone 10 mg/kg was found to be optimum for sleep. This dose improved the quality of sleep and did not alter the S-W pattern and thermoregulation in normal rats. A mild hypothermia within physiological ranges was produced by this dose which favored sleep. A complete abolition of motor activity with no response to tactile or auditory stimuli along with decrease was observed in the rectal temperature was observed after administering 10 mg/kg α-Asarone in mice (Dandiya and Menon, 1964). Alpha-asarone 10-20 mg/kg was observed to attenuate the oxidant/inflammatory events taking place between epileptic episodes (Pages et al., 2010).

Alpha-asarone improved the quality of sleep in acute sleep deprivation condition. Administration of α-Asarone increased the NREM bout duration and decreased the number of arousals during sleep. Increase in the homeostatic sleep pressure was also higher in α-Asarone-treated rats after sleep deprivation. Increase in $T_{hy,}$ associated with sleep deprivation was lesser in this group. There are no previous reports which scientifically validated the effect of α-Asarone on S-W stages and thermoregulation during normal and sleep deprived conditions. However, there are reports which indicate its sedative and sedation-potentiating activity assessed by behavioral observation and monitoring for changes in body temperature using rectal temperature measurement (Dandiya and Menon, 1963, 1964; Menon and Dandiya, 1967).

The animals treated with 10 mg/kg of α-Asarone were less anxious when compared to their vehicle treated counterparts after being subjected to acute sleep loss. The rats subjected to restraint, or treated with corticosterone to increase their anxiety, showed decrease in their anxiety by making increased entries into the open arms and spending more time there after daily administration α-Asarone or *Acorus calamus* for 21 days (Reddy et al., 2015; Shukla et al., 2002). In addition, α-Asarone treatment (200 mg/kg) in the corticosterone-treated rats reduced their anxiety as was evident from the increased number of line crossings in the OFT (Lee et al., 2014). A dose of 3.5 and 7 mg/kg α-Asarone in mice increased their entries into the open arms and the time spent there. According to them, doses lower than 1.75 mg/kg or higher than 14 mg/kg did not elicit any anxiolytic effects when tested in normal mice (Liu et al., 2012).

Administration of α-Asarone was found to selectively repair the oxidative stress and was also found to be area and marker specific. The effect was seen mainly on the levels of MDA and the

activities of CAT and GSH-R in cortex, subcortex and brainstem regions. Increase in the MDA levels observed after 5 h of sleep deprivation was eliminated because of increased activity of CAT. Previously, Limón et al., (2009) reported an enhancement of the CAT activity due to sub-chronic treatment with α-Asarone 10mg/kg, i.o. for 16 days in the rat brain treated with amyloid-β (25–35). After 5 days of α-Asarone administration in sleep deprived rats, MDA levels were dramatically brought down in subcortex and brainstem and the activity of CAT in cortex and GSH-R in subcortex was found to improve when compared to their vehicle administered counterpart. Previously, in mice, treatment of α-Asarone for a week increased the level of GSH-R in striatum, hippocampus and to lesser extent in cortex (Pages et al., 2010). A reversal of the increased MDA levels were observed in the hippocampus and cerebral cortex of scopolamine treated mice administered with α-Asarone 10 mg/kg for 15 days (Kumar et al., 2012). By day 5 of sleep deprivation, α-Asarone enhanced the cellular adaptive response in the regions studied.

<u>Objective 3</u>: To assess the long-term effects of administration of α-Asarone on sleep-wake architecture, the brain-body temperatures, and anti-oxidant levels in chronic partial TSD (20 h X 1 day; 3 days; 7 days; 15 days; 21 days) rats.

Implanted rats were subjected to chronic sleep deprivation of 5 h for 3 weeks after administering vehicle and α-Asarone intraperitoneally.

Methods and techniques used:

Implanted rats were kept for recording sleep, T_{hy} and T_{body} (as mentioned in objective 1). The S-W, T_{hy} and T_{body} were simultaneously recorded for 8 h from 9 am to 5 pm on 3 days to have a pre-treatment control data. Recording setup and acquisition done as mentioned in objective 1. The rats were subjected to TSDX5h, 21 days (9 am to 2 pm) with one group receiving vehicle and the other receiving α-Asarone at 9 am intraperitoneally. After TSDX5h, the recording was continued for 3 h (2 pm to 5 pm) on day 20. Custom made rotating wheel was used for sleep deprivation (Vollert et al., 2011). Analysis of sleep-wake data and temperature done as mentioned in objective 1. Immediately after TSDX5h, Elevated plus maze (EPM) was performed on day 20 to test the anxiety level in rats. The rats were decapitated on day 21 at 2 pm, brains quickly removed, rinsed in pre-chilled saline, rapidly dissected on ice (cortex, sub-cortex and

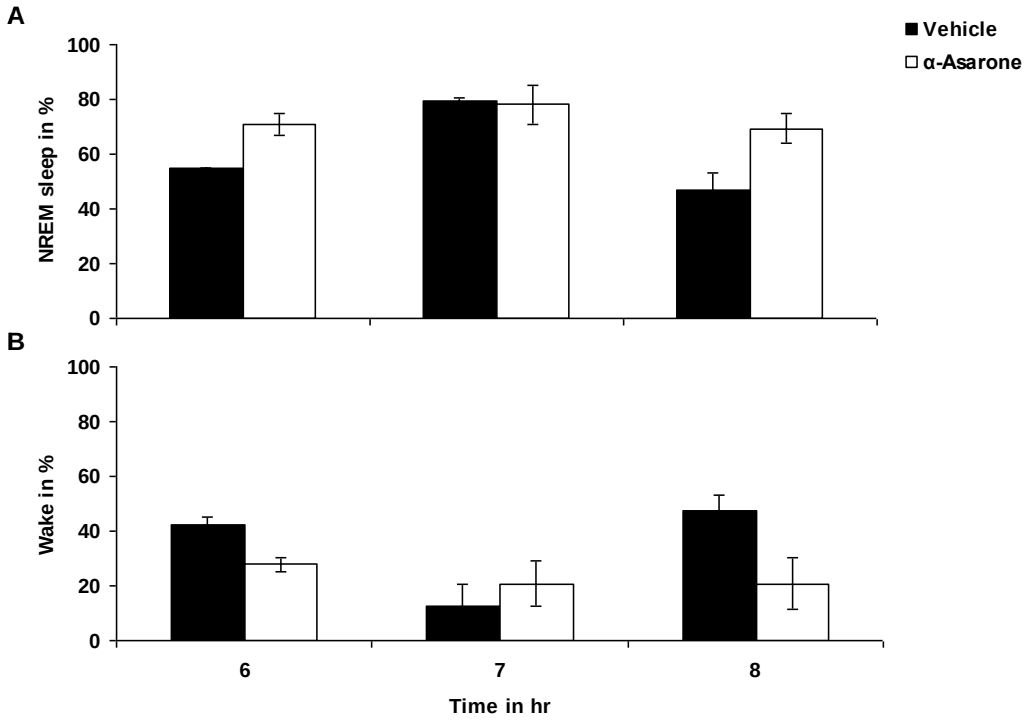
brainstem) and immediately transferred to −80°C for analysis of biochemical parameters (as mentioned in objective 1).

Results:

3.1. Effect of α-Asarone (10 mg/kg) on TSDX5h, 3 weeks days-induced changes in S-W, T_{hy} and T_{body}

Latency to sleep after TSDX5h for 3 weeks was lesser in α-Asarone-treated group. A marginal increase in the NREM sleep time and decrease in the wake time were observed in the α-Asarone group on day 20 (Fig. 17).

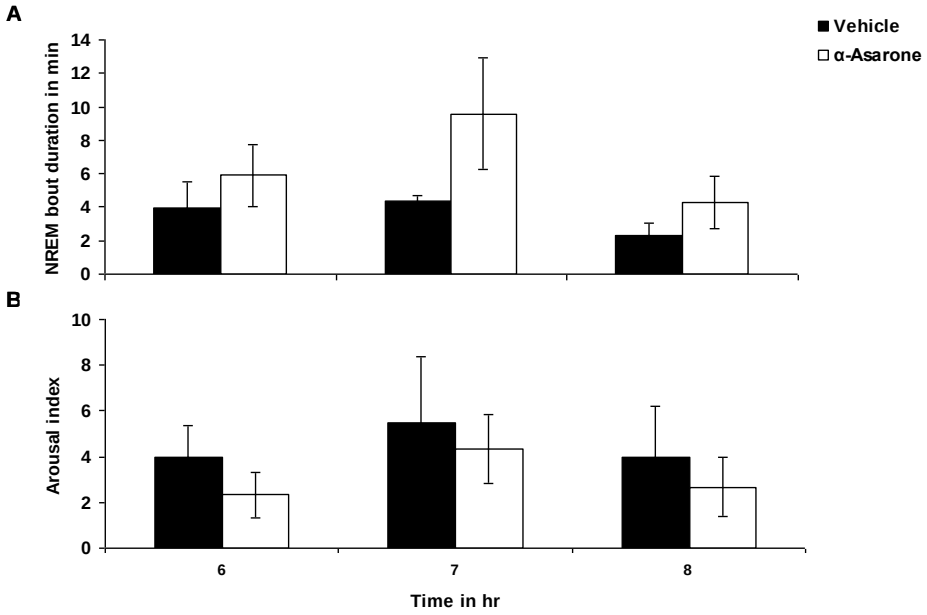
Figure 17: NREM sleep and wake time in percentage after administration of α-Asarone and vehicle in chronic sleep deprived rats



Percentage time of (A) NREM sleep and (B) wakefulness under TSDX5h, 3 weeks plotted for 3 h (2 pm to 5 pm). The data is represented as mean ± SEM.

The average NREM bout duration increased and the arousal index decreased in the α-Asarone group in comparison to vehicle group on day 20 (Fig 18).

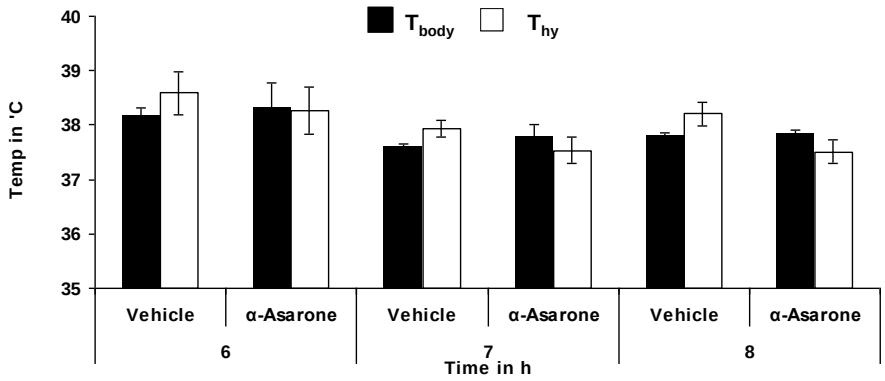
Figure 18: Average NREM bout duration and arousal index after administration of α-Asarone and vehicle in sleep deprived rats



Change in (A) average NREM bout duration in min and (B) arousal index after administration of vehicle (Veh) and 10 mg/kg α-Asarone (α-As10) for under TSDX5h, 3 weeks plotted for 3 h (2 pm to 5 pm). The data is represented as mean ± SEM.

Increase in T_{hy} as a result of TSDX5h was lesser in the α-Asarone-treated rats in comparison to the vehicle-treated rats (Fig 19). However, no change was observed in T_{body} in both groups (Fig. 19).

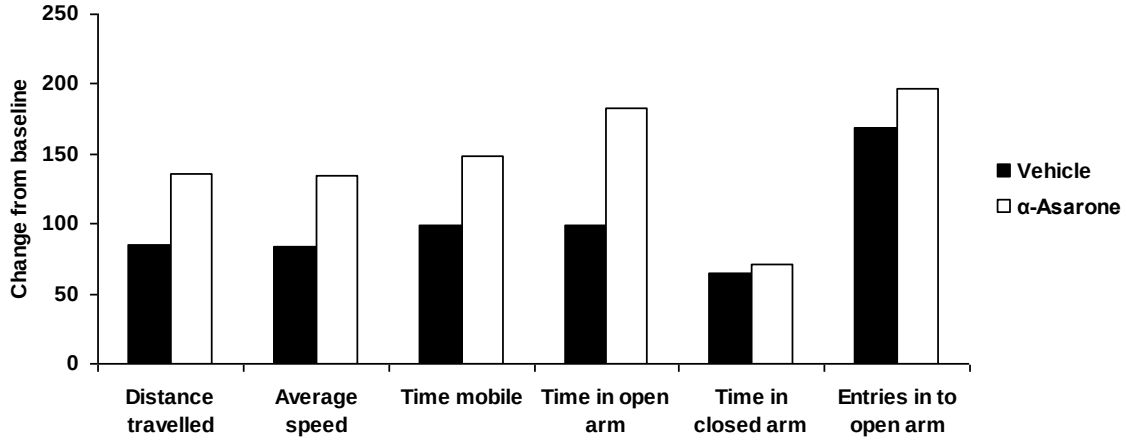
Figure 19: T_{body} and T_{hy} after administration of α-Asarone and vehicle in sleep deprived rats



Change in T_{body} and T_{hy} in ° C after the administration of vehicle and 10 mg/kg α-Asarone for 20 days in chronic sleep deprived rats.

On day 20, α-Asarone-treated rats performed better in the anxiety test. The time mobile and distance travelled on the maze were increased after α-Asarone treatment (Fig. 20). The time spent and entries in the open arm also were higher in these rats (Fig. 20).

Fig. 20: EPM parameters after administration of α-Asarone and vehicle in chronic sleep deprived rats



Changes in the parameters in EPM test shows decreased levels of anxiety in those rats that received α-Asarone before chronic sleep deprivation. The graph shows distance travelled, average speed, time mobile, time spent in the open arms, time spent in closed arm and entries in to the open arms on day 20 of sleep deprivation compared to the baseline values (taken as 100 %) taken before sleep deprivation.

Discussion and conclusion for objective 3:

The general trend of changes in S-W, T_{body} and T_{hy} after administration of α-Asarone in chronically sleep deprived rats (TSDX5h, 3 weeks) was similar to the acute sleep deprivation effect of α-Asarone. Anxiety level was lesser in the α-Asarone-treated chronic sleep deprived group.

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