STUDY OF SLEEP-WAKE PROPERTIES OF α-ASARONE, AN ACTIVE PRINCIPLE OF ACORUS CALAMUS LINN, IN ANIMAL INSOMNIA MODEL

A THESIS PRESENTED BY

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DECLARATION BY THE STUDENT

I, **Arathi R,** hereby certify that I had personally carried out the work depicted in the thesis entitled, "**study of sleep-wake properties of α-Asarone, an active principle of acorus calamus linn, in animal insomnia model**". No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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Clearance was obtained from the Institutional Animal Ethics Committee for carrying out the study.

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LIST OF IMPORTANT ABBREVIATIONS

α-Asarone	Alpha-Asarone
AP	Antero-Posterior
CAT	Catalase
DV	Dorso-Ventral
EEG	Electroencephalogram
EMG	Electromyogram
EPM	Elevated Plus Maze
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSH-R	Glutathione reductase
i.p.	Intra-peritoneum
i.m.	Intra-muscular
MDA	Malondialdehyde
ML	Medio-Lateral
NREM	Non-Rapid Eye Movement
OFT	Open Field Test
REM	Rapid Eye Movement
SD	Sleep Deprivation
SOD	Superoxide dismutase
S-W	Sleep-Wakefulness
T_{body}	Body temperature
T_{hy}	Hypothalamic temperature
Veh	Vehicle

SYNOPSIS

This thesis includes seven chapters. An overview of the purpose and significance of the study is described in Introduction as Chapter 1. Detailed Review of Literature is the provided in the Chapter 2. Summary and comparison of the main developments and the current debates in the field related to the topic of thesis are described along with the research problem, and the proposal to solve the problem containing the aim and the objectives set for validating the hypotheses are given below.

Sleep is a reversible behavioral state of perceptual disengagement from and unresponsiveness to the environment. Sleep is broadly classified into two stages, non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. Importance of sleep becomes evident when it is disturbed. It is noted that any perturbation in sleep and its regulation adversely affects the quality of life. Out of the various sleep disorders classified by International Classification of Sleep Disorders-3 (ICSD-3), insomnia is considered as the most common sleep disorder observed in one-third of the general population. Insomnia is characterized by "a persistent difficulty with sleep initiation, duration, consolidation, or quality that occurs despite adequate opportunity and circumstances for sleep, and results in some form of daytime impairment". Sleep disturbances in insomniacs includes prolonged sleep latency, reduced sleep duration, frequent arousals and fragmentation of sleep and high frequency EEG activity during NREM sleep. Apart from sleep disturbances, insomnia also alters thermoregulation. Lack of sleep or insomnia reduces the ability to dissipate body heat from distal areas

thereby preventing the normal decline in core body temperature leading to hyperthermia. Insomnia is also linked bi-directionally to anxiety. Insomniacs are more likely to have an anxiety disorder compared to those without insomnia and generalized anxiety disorder patients have sleep problems than those without an anxiety disorder. It is also emphasized that the sleep is an antioxidant for the brain. Consequently loss of sleep may lead to oxidative damage in various regions of the body.

Out of the several treatment options for insomnia and associated problems, the most commonly used is the pharmacological treatment which includes drugs like benzodiazepines and non-benzodiazepines which are found to be efficient only for short-term use. Chronic usage produces side-effects like rebound insomnia, drug dependence and tolerance, amnesia, psychomotor impairment, residual daytime drowsiness etc with very low benefit to risk ratio. In *Ayurveda, Acorus calamus* Linn. (*Vacha*), an aromatic rhizome commonly known as sweet flag, is considered to produce calming and cooling effect on the nerves and thereby curing tension, stress and insomnia. α-Asarone (a trans-isomer), is one of the key components (active principle) of this herb that contributes to many of its pharmacological properties. The percentage of α-Asarone is relatively lower than the other major active principles (β-Asarone) and is considered for therapeutic purpose. α-Asarone, a phenylpropene, is lipophilic and is sparingly soluble in water. Because of high lipophilicity, this compound gets rapidly distributed in different regions of brain. α-Asarone is found to have hypnotic-potentiating,

hypothermic, anxiolytic and antioxidant properties making it a potential candidate for the treatment of insomnia.

Based on the literature review, the aim of the study was defined as 'to assess and validate scientifically the effectiveness of α-Asarone, an active principle from *A. calamus* on sleep and insomnia'. The effectiveness of the hypothermic property of α-Asarone in regulating the sleep wakefulness (S-W), hypothalamic (T_{hy}) and body (T_{body}) temperature during normal and sleep deprivation conditions was assessed. To fulfill the aim of the present study, five objectives were defined as i) evaluation of the effect of administration of various doses of α-Asarone, on S-W, T_{hy} and T_{body} in normal rats, ii) investigation of the effect of chronic administration (21 days) of optimal dose of α-Asarone on S-W, T_{hy} and T_{body} in normal rats, iii) investigation of the effect of optimal dose of α-Asarone on S-W, T_{hy}, T_{body}, anxiety and brain antioxidant levels in rats acutely sleep deprived by gentle handling (5 h/ 5 days), iv) investigation of the effect of optimal dose of α-Asarone on S-W, T_{hy}, T_{body}, anxiety and brain antioxidant levels in rats chronically sleep deprived in rotating wheel (5 h/ 21 days) and v) investigation of the mechanism of action of α-Asarone by examining the relationship of T_{hy} and T_{body} with sleep.

Materials and Methods described in Chapter 3 provided details of the study design, methods and analysis techniques chosen. The study was conducted in adult male Wistar rats (N=60). The rats were surgically implanted with EEG-EMG electrodes, thermocouple and transmitter to record S-W, T_{hy} and $T_{body.}$ S-W

was recorded using Biopac systems, T_{hy} using Fluke multimeter and T_{body} using DSI telemetric systems simultaneously. Quantity of S-W was analyzed by calculating the duration of these stages in percentage. Improvement in the quality of sleep is defined on the basis of increase in NREM bout duration, decrease in NREM bout frequency and arousal index (marker of sleep fragmentation), increase in delta power and decrease in high frequency beta activity during NREM sleep and increased theta power during REM sleep. Change in T_{hy} and T_{body} and their association with stages of sleep was also assessed. Anxiety-like behavior was measured using elevated plus maze (EPM) and open field tests. Antioxidant markers like malondialdehyde (MDA) and glutathione (GSH) levels and catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GSH-R) and glutathione peroxidase (GSH-Px) activities were also measured.

Results (Chapter 4) provided the objective-wise actual statements of observations, including statistics, tables and graph. Results as per the Objective 1, pertains to dose-response study, wherein the effect of administration of various doses of α-Asarone on S-W, T_{hy} and T_{body} in normal rats were investigated. Out of 2, 10, 40, 80 and 120 mg/kg α-Asarone given intra-peritoneally, dose 10 mg/kg improved the quality and depth of NREM sleep without much alteration in the duration of sleep. Slight reduction in T_{hy} and T_{body} (<0.5 ˚C) was observed at this dose. Higher doses (40, 80 and 120 mg/kg) not just reduced the quality but also the quantity of sleep. Severe hypothermia was observed at doses 80 and 120

mg/kg (>1 ˚C). The results obtained from objective 1 proved that 10 mg/kg α-Asarone was the optimum dose for sleep and thermoregulation.

The effect of chronic administration (21 days) of optimal dose of α-Asarone i.e. 10 mg/kg on S-W, T_{hy} and T_{body} in normal rats was investigated as per Objective 2. The effect was measured on day 7 and 21. The quality of sleep was improved for 21 days without any change in the quantity. Increase in the depth of NREM sleep was observed for 21 days. Slight lowering of T_{hy} and T_{body} was observed only till day 7. The results obtained from objective 2 proved that 10 mg/kg α-Asarone given chronically was optimum and safe for sleep and thermoregulation.

In objective 3, the effect of optimal dose of α-Asarone i.e. 10 mg/kg on S-W, T_{hy}, T_{body}, anxiety and antioxidant levels in acutely sleep deprived (SD) rats was investigated. Acute SD was done using gentle handling methods for 5 h for 5 consecutive days. The effect was compared with a positive control midazolam (2 mg/kg). Sleep rebound was observed in all the groups. REM sleep was reduced only in the midazolam group. Sleep quality and depth was improved and fragmentation was lowered only in the α-Asarone-treated SD rats. No withdrawal effect was observed in the α-Asarone group unlike midazolam group. T_{hy} and T_{body} was quickly normalized in the α-Asarone group after SD and the difference between T_{hy} and T_{body} was less than <0.2 ˚C unlike midazolam-treated rats in which the difference was >0.5 ˚C. Anxiety was lowered in the α-Asarone-treated

rats and the antioxidant level did not change much since there was an adaptive response observed after acute SD.

As per objective 4, the effect of optimal dose of α-Asarone i.e. 10 mg/kg on S-W, T_{hy}, T_{body}, anxiety and antioxidant levels in chronically SD rats was investigated. Chronic SD was done in rotating wheel for 5 h for 21 consecutive days. The effect was compared with a positive control midazolam (2 mg/kg). Sleep rebound was observed in the α-Asarone group for 21 days. REM sleep was reduced only in the midazolam group. Improvement in the quality of sleep and the effect on T_{hy} and T_{body} was similar to what was observed in the acute model. No withdrawal effect was observed 24, 48 and 72 h post 21 days SD in the α-Asarone group unlike midazolam group. Anxiety was lowered in the α-Asarone-treated rats and the antioxidant levels were improved in the α-Asarone group after chronic SD. As per the objective 5, the association of NREM and REM sleep with T_{hy} and T_{body} was assessed by linear regression analysis.

In the Discussion (Chapter 5) interpretation and comparison of the results are carried out in terms of the already existing knowledge in the area to provide plausible mechanism of actions of α-Asarone. α-Asarone 10 mg/kg improved the association of NREM and REM sleep with both T_{hy} and T_{body} in normal and SD rats. Higher doses of α-Asarone and midazolam reduced the association of sleep with T_{hy} and T_{body}. It was observed that the longer bouts of sleep occurred only when T_{hy} and T_{body} was moderately reduced and the difference between T_{hy} and T_{body} was ≤0.1 ˚C. Higher doses of α-Asarone resulted in >1˚C reduction of T_{hy}

and T_{body} and the difference between T_{hy} and T_{body} was >0.3 ˚C. α-Asarone 10 mg/kg moderately reduced T_{hy} and T_{body} and the difference between T_{hy} and T_{body} was ≤0.1 ˚C. This resulted in the improvement in the quality of sleep and increased association of sleep with T_{hy} and T_{body}.

Conclusion of the study (Chapter 6) summarized the highlight of the study. This study confirmed that α-Asarone at dose 10 mg/kg improved the quality and depth of sleep without producing withdrawal effect through its hypothermic, anxiolytic and antioxidant properties. This compound may be a potential candidate for the treatment of insomnia and associated problems.

Bibliography is given as last chapter (Chapter 7). This chapter includes the references cited in the thesis.

Chapter I: Introduction

Sleep is a reversible behavioral state of perceptual disengagement from and unresponsiveness to the environment (Carskadon & Dement, 2005). Sleep is indispensable and is found to be heterogeneous across the animal kingdom. It is an active process with multiple brain regions involved in its generation, maintenance and regulation. It is proposed that sleep is an auto-regulated global phenomenon (Kumar, 2012). Based on electrophysiological measures mainly electroencephalogram (EEG), the electromyogram (EMG) and the electrooculogram (EOG), sleep is broadly classified into two stages, non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. Human NREM sleep is further subdivided in to three stages based on various electrophysiological markers. Both NREM and REM sleep are unique in all aspects which include the electrophysiological and physiological changes observed during these stages, the areas involved in their generation, maintenance and regulation, their functions and their ontogeny and phylogeny.

Importance of sleep becomes evident when it is disturbed. It is noted that any perturbation in sleep and its regulation adversely affects the quality of life. Disorders associated with sleep have become a very common life-style related health issue in the current world. It is reported that about ten percent of the studied population suffer from various sleep disorders (Ohayon, 2011). Out of the various sleep disorders classified by International Classification of Sleep Disorders-3 (ICSD-3), insomnia is considered as the most common sleep disorder (AASM, 2014), observed in one-third of the general population (Ohayon, 2011). Insomnia is characterized by “a persistent difficulty with

sleep initiation, duration, consolidation, or quality that occurs despite adequate opportunity and circumstances for sleep, and results in some form of daytime impairment" (AASM, 2014). Insomnia may be chronic, short-term or other variant types according to the recent classification of ICSD-3 (AASM, 2014). Insomnia is a disorder of hyperaousal which is explained by two models, physiologic and cognitive (Perlis et al, 2005). The physiologic model suggests that chronic insomnia may be understood as a condition in which the patient has a trait level of arousal or a level of arousal prior to or during the preferred sleep period that is incompatible with good sleep continuity (Perlis et al, 2005). On the other hand, cognitive model talks about three factors; the cognitive arousal in the form of rumination and worry predisposes the individual to insomnia, precipitates acute episodes and perpetuates the chronic form of the disorder (Perlis et al, 2005).

Insomnia is also observed to be co-morbid with other chronic medical conditions like cardiovascular diseases, metabolic disorders, musculoskeletal disorders, gastro-intestinal problems, upper airway diseases, rheumatic diseases, pain due to injuries, infection, obesity, allergy, autoimmune disorders, psychiatric disorders and other sleep disorders (Ohayon, 2010). A bi-directional relationship is observed between insomnia and various psychiatric disorders like anxiety, depression, substance abuse and schizophrenia. Poor sleep hygiene, shift work, irregular sleep-wake schedule, lifestyle stress and use and abuse of psychoactive drugs (like sedatives, anxiolytics, alcohol, caffeine etc) also increases the risk of insomnia (Ohayon, 2010).

Insomnia negatively affects the social life, task performance and daytime functioning of an individual with increase in the chance of committing errors, falls and accidents (Roth & Roehrs, 2003). It also increases the risk of obesity, hypertension, altered metabolic/endocrine profile (such as diabetes), coronary heart disease and increased risk of mortality (Roth & Roehrs, 2003; Ohayon, 2010). Insomnia impairs initiation and maintenance of sleep and reduces sleep duration and quality. Insomnia makes an individual increasingly fatigue with impaired psychomotor skills, memory problems, mood swings, irritability, increased sensitivity to external stimuli etc (Roth & Roehrs, 2003).

Research on insomnia may be conducted in humans as well as in animal models. Since there are limitations in using humans as a research model, animal models mainly rodents may serve as a good candidate (Toth & Bhargava, 2013). This model when subjected to various sleep deprivation (SD) procedures and other stressful conditions shows sleep disturbance similar to those observed in insomnia patients. Sleep disturbances includes prolonged sleep latency, reduced sleep duration, frequent arousals and fragmentation of sleep and high frequency EEG activity during NREM sleep (Cano et al, 2008).

Apart from sleep disturbances, insomnia also alters several other physiological processes taking place in the body namely thermoregulation. A vast survey of literature has clearly indicated the association of sleep with thermoregulation (Gilbert et al, 2004; Mallick & Kumar, 2012). Brain mechanisms controlling sleep are anatomically and functionally coupled with thermoregulatory mechanisms. In humans, sleep is initiated in the dark phase

when the core body temperature (CBT) is low and arousal is observed during the light period when the CBT is high. In nocturnal animals like rats, CBT is high during dark period and *vice-versa*. Sleep becomes deep during low CBT and total duration of NREM sleep reduces when the CBT increases. Lack of sleep or insomnia reduces the ability to dissipate body heat from distal areas thereby preventing the normal decline in CBT (Lack et al, 2008; Van Den Heuvel et al, 2004). Sleep loss leads to increase in CBT or hyperthermia (Lack et al, 2008).

Insomnia is also linked bi-directionally to anxiety (Alvaro et al, 2013). Insomniacs are more likely to have an anxiety disorder compared to those without insomnia (Ford & Kamerow, 1989) and generalized anxiety disorder (GAD) patients have more sleep problems than those without an anxiety disorder (Monti & Monti, 2000). It is also suggested that the sleep functions as an antioxidant for the brain (Reimund, 1994). Consequently loss of sleep may lead to oxidative damage in various regions of the body (Gopalakrishnan et al, 2004; Ramanathan et al, 2002; Reimund, 1994). These changes may lead to several metabolic, hormonal, immunological and cognitive deficits (Cirelli, 2006; Durmer & Dinges, 2005).

There are several treatment options for insomnia and associated problems. However, each of these approaches has their own disadvantages. For example, the most commonly used pharmacological treatment in the clinical practice i.e. use of drugs like benzodiazepines and non-benzodiazepines are found to be efficient only for short-term use. Chronic usage produces side-effects like rebound insomnia, drug dependence and tolerance, amnesia, psychomotor

impairment, residual daytime drowsiness etc with very low benefit to risk ratio (Ashton, 1994; Chouinard, 2004; Gunja, 2013). Moreover, these drugs produce poor quality sleep. Other drugs which include anti-depressants, orexin antagonists (suvorexant) and anti-histamines are minimally effective in treating insomnia. Alternative therapy using the hormone melatonin also has been found to induce sedation, lower core body temperature, reduce sleep latencies and increase total sleep time (Erman et al, 2006). However, mixed outcome among the insomniac population has rendered it unreliable. Non-pharmacological therapeutic techniques like sleep hygiene, cognitive behavioral therapy, stimulus control therapy, sleep restriction therapy etc are found to be effective for long-term results. These methods are more durable as they address causes contributing to insomnia. They are safer in comparison to pharmacological treatment. However, these methods require a lot of patience and are time-consuming (Ebben & Spielman, 2009).

Traditional/Herbal treatment for insomnia made from herbs like valerian, withania (*Ashwagandha*), hops, ginseng, chamomile, passion flower, kava kava, skull cap etc or their combination have been used world-wide (Attele et al, 2000; Cao et al, 2010; Davies et al, 1992; Kaushik et al, 2017; Mowrey, 1986; Ngan & Conduit, 2011; Perharic et al, 1994; Rhee et al, 1990; Schulz & Jobert, 1994; Schulz et al, 1998). However, except for valerian, none of the other herbs have undergone systematic clinical or scientific validation for their sedative or hypnotic properties. Pre-clinical assessment of some herbs like passion flower and withania is incomplete.

In traditional school of medicine *Ayurveda*, seven herbs *Tagara, Ashwagandha, Brahmi, Jatamanasi, Sarpagandha, Shankhupushpi and Vacha* are given alone and in combination to treat insomnia (http://ayurvedanextdoor.com/herbs-for-insomnia/). Even though there are reports on their effectiveness in humans, complete scientific studies are still lacking. More studies are required to validate the sedative property of these herbs along with the isolation of the active component contributing to this property (Gulia et al, 2017).

In *Ayurveda, Acorus calamus* Linn. (*Vacha*), an aromatic rhizome commonly known as sweet flag, is considered to produce calming and cooling effect on the nerves and thereby curing tension, stress and insomnia. In scientific terms, however, there are no reports on its effect on insomnia. There are some studies reporting the *A. calamus*-induced potentiation of hypnotic activity of various hypnotics like pentobarbital, hexobarbital and ethanol (Dandiya et al, 1959b; Dandiya & Sharma, 1962; Menon & Dandiya, 1967). Nevertheless, these studies do not have standard assessment procedures and provide no information on the influence of herb or its active components on normal or altered sleep.

Out of the many components isolated from *A. calamus*, there are two main components α- and β-Asarone (trans- and cis-isomer), contributing to many of the pharmacological properties of this herb (Dandiya et al, 1959a; Sharma et al, 1961). Due to the toxicity observed in the animal studies, β-Asarone and β-Asarone-containing herbs are banned by US Food and Drug Administration (FDA) (Keller & Stahl, 1983; Taylor et al, 1967). However, the other potent

active component α-Asarone is less toxic and is still considered for therapeutic purpose.

α-Asarone, a phenylpropene, is the second major component isolated from *A. calamus*. This component is lipophilic and is sparingly soluble is water. Because of high lipophilicity, this compound gets rapidly distributed in different regions of brain and other vital organs (Lu et al, 2014). α-Asarone is found to be short-acting (Kim et al, 2015) and doses above 300 mg/kg were found to be lethal in rodents (www.caymanchem.com/product/11681). Hypnotic-potentiating property of α-Asarone is similar to that observed in its parent herb (Sharma et al, 1961). Moreover, this component also shows hypothermic, anxiolytic and antioxidant properties, like its parent herb, making it a potential candidate for the treatment of insomnia (Kumar et al, 2012; Limón et al, 2009; Liu et al, 2012; Pages et al, 2010; Shin et al, 2014).

Even though there are studies validating many of these properties in various models, there are none which reports its effectiveness in sleep and sleep disorders. Henceforth, the present study was aimed to assess the effectiveness of α-Asarone on sleep and insomnia and the objectives defined validated the hypnotic property of α-Asarone in the normal rats and in the acute and chronic rat models of insomnia.

Firstly, the dose-response study was conducted to identify the optimal dose facilitating sleep followed by tests evaluating the effects of administration of optimal dose for short term and long term in normal and SD animals. Finally the mechanism of action of α-Asarone with respect to sleep and thermoregulation was identified.

In chapter 2 (Literature review) the main developments and the current debates in the field of sleep, thermoregulation, insomnia and associated problems, therapeutic interventions for insomnia, herbal interventions, works on *Acorus calamus* Linn. (*Vacha*) and α-Asarone are summarized, evaluated and compared. The lacunae and the objective of the study are discussed on the basis of the second chapter. In chapter three (Materials and Methods) the study design, methods and analysis techniques chosen for the study are discussed. In the fourth chapter (Results) the actual statements of observation, including statistics, tables and graph, are described objective-wise and in the fifth chapter (Discussions), interpretation and comparison of the results are done in terms of the already existing knowledge in the area. Chapter six (Conclusions) summarizes the main observations of the study and chapter seven (Bibliography) includes the references cited in the thesis.

Chapter II: Review of Literature

1. Sleep and Wakefulness (S-W)

Sleep is a behavioral state of perceptual disengagement from and unresponsiveness to the environment (Carskadon & Dement, 2005). It is a state of reversible unconsciousness which is a highly complex and organized amalgam of physiologic and behavioral processes. Sleep is studied on the basis of certain behavioral observations and also by identifying various physiological and electrophysiological variables. Behavioral assessment of sleep of an individual is attained by maintaining sleep diaries and logs that gives preliminary details about the sleep profile of an individual which is relatively less reliable due to subjective variations. Physiological monitoring of sleep, on the other hand, is more reliable and quantifiable. It is defined using polygraphic/ electrophysiological measures primarily the EEG, EMG and EOG. These electrophysiological measures are found to more reliable and are considered as the gold standard for sleep assessment. However, both physiological and behavioral variables of sleep are highly correlated.

During S-W, EEG records the voltage variation of the neuronal membrane, EMG records the movement of muscles and EOG records the movement of eye. These three measures are simultaneously recorded and their variations are noted to identify the states of vigilance. W is a state of consciousness in which we can perceive and interact with the environment. State of W is further divided in to active and quiet wakefulness.

In humans, during active W, EEG shows low voltage (20-40 μV) high frequency (25-40 Hz) desynchronized brain waves, EMG is high due to

increased muscular tone and shows increased artifacts due to body movements and EOG shows high amplitude spiky waves produced by eye movements. During quiet W, the EEG shows desynchronized brain waves with slightly increased voltage and decreased frequency (8-12 Hz). EEG also shows increased alpha activity. EMG is still high with no movement artifacts and spiky waves in EOG are absent.

Sleep, on the basis of various physiological parameters, is divided into 2 stages, REM and NREM sleep. Human NREM sleep is further differentiated in to 3 stages (N) 1, 2 and 3 as per the new classification (AASM, 2007). The major differences among the NREM sleep stages are identified based on the EEG patterns. During NREM sleep, EMG fluctuates from moderate to low on the basis of muscle tone and EOG remains low due to decreased eye movements. Stage 1 (N1) is characterized by low voltage mixed frequency activity with decreased alpha activity and stage 2 (N2) is characterized by occurrence of 12-14 Hz sinusoidal waves called “sleep spindles” and “K-complexes” (high amplitude negative sharp waves followed by positive slow waves) along with brain waves of moderate voltage and mixed frequency. Stage 3 (N3) known as deep sleep or slow wave sleep (SWS) is characterized by high voltage (> 75 μV) low frequency (0.5-4 Hz) brain waves. Since the frequency falls in the delta range, the waves are also called as delta wave. The arousal threshold gradually falls from N 1 to 3.

REM sleep is characterized by bursts of eye movements visualized in the EOG. EMG shows muscle atonia due to reversible muscle paralysis, however, occasional muscle twitches are observed against the low background. EEG

shows desynchronized low voltage brain waves with mixed frequency (theta predominance). In animals, EEG pattern during REM sleep shows apparent similarity with the EEG observed during wakefulness; hence, this stage is also called as the “paradoxical sleep”. REM sleep-specific physiological signs are myoclonic twitches, pronounced fluctuations in cardio-respiratory rhythms and core body temperature, penile erection (male) and clitoral tumescence (females). Other characteristic features of REM sleep are theta rhythm in the hippocampal EEG and spiky field potentials in the pons (P-waves), lateral geniculate nucleus, and occipital cortex (called as ponto-geniculo-occipital or PGO spikes). In addition, occurrence of vivid dreaming is an important mental experience of REM sleep.

Sleep in humans is marked by alternations of NREM and REM sleep in cycles of about 90 min which is repeated over 3 to 6 times. Duration of each component varies across night. First onset of REM sleep occurs 80 min after NREM sleep and it last for around 10 min. As night progresses, the proportion of REM sleep increases and SWS decreases. Stages 1 and 2 constitute 55 %, stage 3 cover 20 % and REM sleep makes for 25 % of total sleep time.

Apart from these electrophysiological measures, NREM and REM sleep can also be differentiated based on some motor, metabolic and autonomic correlates. Thermoregulation, for example, is intact during NREM sleep but is found to be altered during REM sleep. Brain temperature decreases during NREM sleep whereas it increases during REM sleep (Krueger & Takahashi, 1997). Muscle tone and the reflex activities are reduced during the NREM sleep, whereas it is suppressed during the REM sleep. Heart rate, respiratory

rate and blood pressure fluctuates during REM sleep along with an increase in the cerebral blood flow. Dreaming is found frequently during the REM sleep and rarely during the NREM sleep.

Sleep is indispensable, but is found to vary phylogenetically across the animal kingdom. Out of the various classes of animal kingdom, sleep of mammals is extensively studied (Lesku et al, 2008). Behavioral and electrophysiological studies indicate that mammals differ greatly in their sleep duration, habits, habitats, postures, pattern etc.

Rodents are the most widely used model for sleep studies due to its availability, cost and also ease in handling. However, the sleep profile of rats is considerably different from that of humans. Rodents are predominantly nocturnal and have a polyphasic sleep with shorter NREM-REM cycles distributed around 24 h. They sleep less and are relatively more active during night (dark period) and during day time (light period) they are less active and sleep most of the time. It is suggested that they have increased NREM sleep for energy conservation (Berger & Phillips, 1995). Unlike humans, the recording of sleep in rodents is an invasive procedure which includes chronic implantation of electrodes on the skull, nuchal muscles and external canthus for recording EEG, EMG and EOG respectively. The electrophysiological quantification of sleep and wakefulness is also slightly different in these animals.

Figure 1 represents the EEG and EMG traces of S-W in rat. In rats, NREM sleep is subdivided in to 2 stages: light slow wave sleep SWS (S1) and deep SWS (S2). S1 is characterized by the presence of synchronized low

frequency (8-14 Hz) high voltage (150-200 μV) EEG with spindles (with a duration of 1.5-2.5 s) appearing at a rate of 5-12 per min. This stage is comparable to Stage 1 and 2 in humans. S2 is identified by the presence of high amplitude (200-300 μV) low frequency (0.1-4 Hz) waves in the cortical EEG. This stage is comparable to Stage 3 in humans.

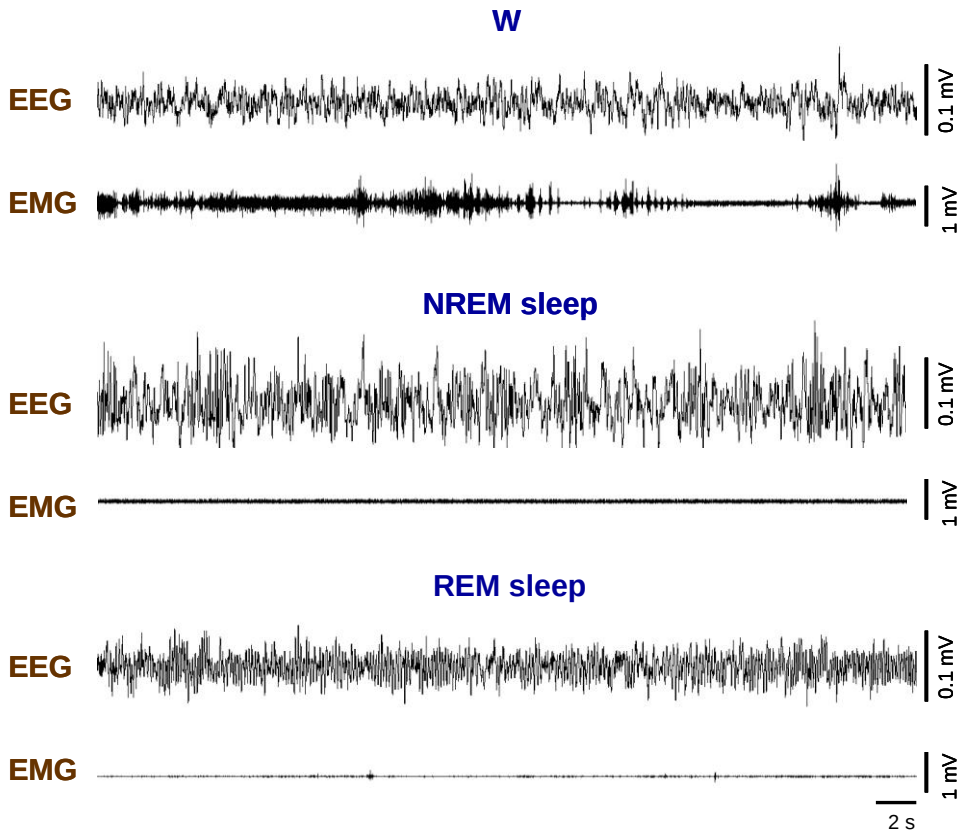

Fig. 1 Representative EEG and EMG traces during W, NREM and REM sleep in rats

The regions and the mechanism in brain promoting sleep and W are distinct. NREM sleep promoting region is the preoptic area of the anterior hypothalamus, whereas W promoting regions are posterior hypothalamus,

basal forebrain, and mesopontine tegmentum (Nauta, 1946; Kumar et al, 1993; John et al, 1994; Kumar, 2004; Sakai & Crochet, 2003). Pontine tegmentum and adjacent regions are involved in the generation of REM sleep. The involvement of these regions was confirmed by several lines of animal experiments, including stimulation, lesion, single-unit recording and the c-fos method. A mutual inhibitory interaction is observed between these sleep and W promoting regions (Saper et al, 2001). In addition, the inhibitions on the sleep promoting region observed during W are removed for sleep onset and maintenance.

Sleep is regulated as a result of the interaction between the homeostatic drive (process S) and the circadian drive (process C) (Borbely & Tobler, 1989). Homeostatic drive is due to the accumulation of some chemical substances during the process of wakefulness (Borbely & Tobler, 1989). Adenosine, produced as a product of ATP metabolism, is the most well studied substance that produces sleep drive (Porkka-Heiskanen et al, 2002). Process C, however, is primarily driven by an endogenous hypothalamic pacemaker suprachiasmatic nucleus which is independent of any environmental cues (Borbely & Tobler, 1989). The light entrained circadian cues from suprachiasmatic nucleus interact with the environmental time cues (feeding, temperature, social cues etc) provided by the dorsomedial nucleus and the subparaventricular zone to regulate the sleep-wake cycles (Borbely & Tobler, 1989).

2. Sleep and thermoregulation

Brain mechanisms that control sleep are anatomically and functionally coupled with thermoregulatory mechanisms. Thermoregulation is the process of maintaining the core body temperature (CBT) at an optimal level for normal physiological functioning. Like the regulation of sleep, there are two key systems which control the process of thermoregulation; one is the homeostatic system and the other is the circadian system. Studies in humans have identified that the homeostatic thermoregulatory system controls the behavior necessary to optimize and maintain the CBT with minimum deviation (Rogers & Ferguson, 2009). This system is explained by a 'core-shell model' which emphasizes that the core always remain stable (37 °C) in contrast to the shell which varies over a much wider range of temperatures. Temperature changes in shell maintain a stable core (Rogers & Ferguson, 2009). Thermoreceptors at the peripheral skin receptors (especially the hand, feet and face) detect the change in the temperature and relay them to the spinal cord through various ascending neural pathways. Final regulation of temperature takes place in the brain regions mainly the pre-optic area of anterior hypothalamus (involved in sleep generation), medial forebrain and thalamus.

The core is maintained stable by both heat loss and production (Van Someren et al, 2002). Heat loss mechanisms include radiation, convection, conduction and evaporation. Vasodilation of tiny blood vessels arteriovenous anastomoses in the distal skin sites carrying warm blood promotes heat loss

through radiation. In contrast, vasoconstriction prevents the loss of heat from the body, thereby maintaining the core during cold temperature (Rogers & Ferguson, 2009). Heat production occurs as a result of normal cellular functions, shivering muscular activity, non-shivering thermogenesis in brown adipose tissue and behavioral choices.

Like sleep, CBT is also regulated by an endogenous system known as circadian rhythm which fluctuates over a period of approximately 24 h in the absence of environmental cues. Suprachiasmatic nucleus is the central clock controlling and regulating the bodily physiological functions and maintaining a rhythm in the absence of external cues. In suprachiasmatic lesioned rodents, 24 h rhythm in both sleep and T_{body} is abolished; however, the total amount of sleep remains unchanged (Baker et al, 2005). The CBT is found to be higher during the light period/day in comparison to the dark period/night. The CBT rises in the morning till the midday and then plateau briefly and again peaks in the evening. After entering in to the dark period, the CBT starts to lower forming a trough till the next light period (Dijk et al, 2001). Loss of heat through periphery reduces the CBT during dark period. Heat generation during morning is due to the decrease in the shell temperature thus preventing the heat loss (Campbell & Broughton, 1994).

Sleep and thermoregulation are closely linked (Gilbert et al, 2004; Mallick & Kumar, 2012). In humans, sleep is initiated during the dark period when the CBT is low and arousal is observed during the light period when the CBT is high. In nocturnal animals like rats, CBT is high during dark period and *vice-versa* (Rogers & Ferguson, 2009). Depth of the sleep becomes higher when it

occurs at the time when CBT is lower and the total duration of NREM sleep reduces when CBT increases. The proximal skin temperature follows the CBT pattern whereas the distal skin temperature follows an inverse time course. An elevated distal to proximal skin temperature gradient during the onset of sleep indicates an increase in the peripheral blood flow (vasodilation) and heat loss. This is associated with short sleep latency, decreased arousals and increase in the SWS (Kräuchi, 2007).

Sleep onset in mammals is associated with a fall in CBT which varies in different species and is controlled by circadian rhythm and environmental variables like ambient temperature. This lowering of thermal set point during sleep onset is accompanied by decrease in metabolic heat production and increase in heat loss by peripheral vasodilation and/or sweating (Rogers & Ferguson, 2009). Any deviation from the set point is adjusted by thermoregulatory responses. For instance, during fall in CBT, cold defense mechanisms (shivering, piloerection, peripheral vasoconstriction) are activated and when it goes above, heat defense response (sweating, panting, peripheral vasodilation) is activated. The lowering of set point is also an evidence for the energy conservation function of sleep. Lowering of CBT leads to lower CBT-ambient temperature gradient which in turn results in minimum heat loss. This is an important regulatory mechanism in small mammals (rats) with high surface to volume ratio which are prone to excessive heat loss (Rogers & Ferguson, 2009). During NREM sleep, thermoregulation is relatively diminished in comparison to the W and during REM sleep thermoregulatory responses are further reduced. So the

vulnerability to thermal stress is more during sleep especially the REM sleep (prominent in infants and small animals). Even though the thermal set point reduces during sleep, the animal tends to seek a warmer ambient temperature (Ray et al. 2004). However, lowering of metabolic rate and T_{body} during sleep along with lowering of thermal set point is beneficial for energy conservation (Rogers & Ferguson, 2009). Circadian phase relationship between sleep and T_{body} at sleep onset determines sleep amount and composition. The changes in temperature that are normally under circadian control, act to reinforce the direct effect of the circadian system on sleep initiation (Gilbert et al, 2004).

Sleep episodes initiated on the falling phase of the temperature rhythm are associated with shorter sleep latencies, longer sleep episode durations and increased slow wave sleep. Sleep episodes initiated on the rising phase of the temperature rhythm are associated with longer sleep latencies, shorter sleep episode duration and increased REM sleep. Sleep onset during the rising phase of T_{body} along with increased heat production and heat conservation disrupts sleep and promote waking (Czeisler et al, 1980).

Temperature-sensing neurons in the preoptic and anterior hypothalamus (POAH) are involved in thermoregulation. Warm-sensing neurons (WSN) are activated by an increase in the temperature and inhibited by cooling whereas the cold-sensing neurons (CSN) are excited by local decrease in temperature and inhibited by local warming. These neurons receive inputs from their corresponding thermoreceptors in the skin (McGinty & Szymusiak, 1990). Skin temperature which is sensitive to the changes in the ambient temperature sends signals to the central thermoregulatory system. The level of activation

of POAH WSN versus CSN determines the thermal set point. Excitation of WSN and inhibition of CSN results in reduced metabolic heat production, increased heat loss from the periphery and stable lowering of T_{body}. Activation of CSN will increase metabolic heat production and conservation and hence T_{body}. It is shown that central warm receptors can produce changes in sleep at different ambient temoperature, even in absence of peripheral warm receptors (Gulia et al., 2005). It is assumed that the circadian rhythm in T_{body} is driven by the relative activation of WSN and CSN regulated by the output of SCN, with WSN activated on the descending phase of the rhythm and CSN predominating on the rising phase (McGinty & Szymusiak, 1990). Activation of POAH WSN is observed during sleep onset and NREM sleep and is as a result of inhibition of wake-promoting neurons. The CSN are activated during waking and exhibit decline during sleep onset and NREM sleep. These changes follow the same pattern of thermoreceptor activity to lower the thermal set point accompanying sleep onset.

Apart from this, subtle changes in the POAH/cortical temperature are also observed in association with various stages of sleep. NREM sleep is associated with decrease in POAH/cortical temperature and REM sleep produces increase in the POAH/cortical temperature (Krueger & Takahashi, 1997). Decrease associated with the NREM sleep points towards the energy conservation function of sleep whereas the increase associated with REM sleep is due to hemodynamic changes. It is suggested that a decrease in the common carotid artery blood flow and the associated increase in the cerebral

blood supply through the vertebral artery is the reason behind the increase in the brain temperature during REM sleep (Calasso & Parmeggiani, 2008).

Henceforth, it is emphasized that the S-W is closely associated with thermoregulation and any perturbation in one process invariable affects the other.

3. Sleep disorders

Any alteration in sleep and its regulation adversely affects the quality of life. In the present world, sleep is highly compromised and hence disorders associated with sleep loss have become very common. It is reported that about ten percent of the studied population suffer from various sleep disorders clinically recognized. According to ICSD-3 (AASM, 2014), sleep disorders are classified in to six major categories:

- **Insomnia:** A persistent difficulty with sleep initiation, duration, consolidation, or quality that occurs despite adequate opportunity and circumstances for sleep, and results in some form of daytime impairment.

- **Sleep Related Breathing Disorders:** Includes several conditions characterized by disordered respiration during sleep which may be arising from disorders of the respiratory apparatus and airway or due to inadequate neural drive for breathing or both. These includes obstructive sleep apnoea

disorders, central sleep apnoea disorders, sleep related hypoventilation disorders and sleep-related hypoxaemia disorder.

- **Central Disorders of Hypersomnolence:** Daytime sleepiness, i.e. the inability to stay awake and alert during the major episodes of wakefulness during the day, resulting in periods of incoercible sleep or involuntary bouts of drowsiness or sleep. Out of nine subcategories, narcolepsy, idiopathic hypersomnia and Kleine-Levin syndrome are the most common.

- **Circadian Rhythm Sleep-Wake Disorders:** Disorder caused by alterations of the circadian time-keeping system, its entrainment mechanisms or a misalignment of the endogenous circadian rhythm and the external environment. This includes delayed sleep-wake phase disorder, advanced sleep-wake phase disorder, irregular sleep-wake rhythm disorder, non-24 h sleep-wake rhythm disorder, shift work disorder, jet lag disorder and circadian sleep-wake disorder not otherwise specified.

- **Parasomnias:** Disorders characterized by the occurrence of complex motor or behavioral events or experiences at sleep onset, within sleep or during arousal from sleep. Parasomnias may occur during any sleep stage, NREM, REM or during transitions to and from sleep. During parasomnia events abnormal sleep-related complex movements, behaviors, emotions, perceptions, dreams and autonomic nervous system activity may occur which are potentially

harmful and can cause injuries, sleep disruption, adverse health consequences and undesirable psychosocial effects.

- **Sleep Related Movement Disorders:** Occurrence of stereotyped repetitive movements during sleep or at its onset which may be brief, singular or even myoclonic. Restless leg syndrome in association with periodic limb movement disorder is one such disorder characterized by an urge to move the limbs with or without unpleasant sensations.

Out of these categories, insomnia is the most common sleep disorders with their prevalence increasing with age.

4. Insomnia

The three general traits of patients with insomnia is persistent sleep difficulty, adequate sleep opportunity and associated daytime dysfunction. Patients are usually tired, suffer poor concentration, and are more irritable and exhausted. Insomnia has a significant negative impact on social life, task performance and daytime functioning with higher odds of errors or accidents. It has been linked to obesity, hypertension, altered metabolic/endocrine profile, coronary heart disease and increased mortality risk. According to ICSD-3 (AASM, 2014), insomnia is further categorized in to six diagnoses:

1. Chronic insomnia disorder

2. Short-term insomnia disorder
3. Other insomnia disorder
4. Isolated symptoms and normal variants
5. Excessive time in bed
6. Short sleeper

4.1. Epidemiology of insomnia

In the previous classifications like ICSD-1 & 2, DSM-IV (Diagnostic and Statistical Manual of Mental Disorders-IV), ICD-10 (International Classification of Diseases-10), diagnosis of insomnia was extensively sub typed. Insomnia disorder was divided in to several nomenclatures for instance, acute and chronic insomnia, primary and secondary/comorbid insomnia, organic and inorganic insomnia etc. Primary insomnia was further subcategorized in to psychophysiologic, idiopathic, and paradoxical insomnia. However, due to overlapping symptoms and therapeutic approaches, lack of proper diagnostic distinction, and due to uncertainty existing with respect to the "nature of associations and the direction of causality, all the subtypes were consolidated in to one chronic insomnia disorder (Macêdo et al, 2016). DSM-V launched in 2013 is much like ICSD-3 with primary and secondary insomnia replaced by insomnia disorders.

Prevalence of insomnia varies across different population due to difference in the lifestyle. The prevalence rate also varies across different studies due to differences in case definitions, assessment procedures, sample characteristics and length of assessment intervals. When considering only one

insomnia symptom i.e. difficulty in initiating or maintaining sleep, about one-third of adults (30-48 %) is found to be insomniacs as per the meta-analysis performed on a population-based data. When frequency modifiers were included, this rate went down to 12-16 %. This rate further reduced to 9-15 % when daytime consequences are added to the case definition. Rates of subjective sleep dissatisfaction without any sleep diagnosis, also vary widely (10-25 %) in the adult population. Based on DSM and ICSD criteria, prevalence rates of insomnia is between 6-10 % (Ohayon, 2002). Some of the prominent epidemiological studies based on symptoms, complaints and diagnoses of insomnia in the general population across the world are extensively reviewed by Ohayon (2002).

In Indian scenario, acknowledgement of sleep disorders and its consequences happened only in recent years. Recently Panda et al (2012) reported that 18.6 % of apparently healthy adults from South Indian states have insomnia out of which 18 % had difficulty initiating sleep, 18 % had problem maintaining sleep and 7.9 % had early morning awakening. 2.5 % had anxiety, 11.7 % had depression and 42.6 % had hypertension co-morbid with insomnia. In North Indian population (2475 subjects of 30-60 yr), 28.1 % of the subjects reported various insomnia complaints and in an elderly population (1240 subjects), 59 % had problem with initiating and maintaining sleep (Suri et al, 2009). A hospital-based study in North India reported that 32.5 % of the geriatric study population had insomnia which was co-morbid with other health issues (Gambhir et al, 2014). A study conducted on school children in Delhi (2475 subjects) reported that 17.3 % of them suffered

various insomnia-associated problems (Suri et al, 2008). In a retrospective chart review-based study on patients seen over an 8 year period, 15.34 % patients had difficulty in falling asleep and 22.73 % had frequent awakenings after falling asleep in the first 4 years formed group. In the later 4 years formed group 13.6 % had difficulty falling asleep and 31.4 % had frequent awakenings due to other sleep disorders (Sharma et al, 2013). Insomnia is also seen prevalent in shift workers and patients having circadian sleep disorders. Shift workers from New Delhi was found to be sleepier and exhausted in comparison to their control counterpart even after getting equal amount of sleep. They had higher incidence of depression, anxiety, substance abuse and sleep disturbance (Suri et al, 2007). Insomnia is also seen in people suffering from metabolic disorders like diabetes mellitus and those undergoing chronic hemodialysis (Bhattacharya et al, 2013). Demographic studies have shown that insomnia is more prevalent among the females and the geriatric population. Females especially in their third trimester pregnancy and post-menopausal stage are pre-disposed to insomnia. The reports on the geriatric population are mixed with few associating age with insomnia and few others concluding that age per se does not contribute to insomnia. Factors like occupations, socio-economic status, marital status and mental and physical health have significant impact on the precipitation and perpetuation of insomnia (Bhattacharya et al, 2013).

Highest prevalence of insomnia is found to be in people with other chronic medical conditions. Many studies have shown bi-directional relationship of

insomnia with various psychiatric disorders mainly anxiety, depression, substance abuse and schizophrenia.

4.2. Pathophysiology of insomnia

Insomnia is considered as a disorder of hyperarousal. This hyperarousal is explained by four models, physiologic, cognitive, behavioral and neurocognitive (Perlis et al, 2005). The physiologic model suggests that chronic insomnia is a condition in which the patient has a trait level of arousal or a level of arousal prior to or during the preferred sleep period that is incompatible with good sleep continuity. This model assumes that physiologic arousal and sleep are mutually exclusive (Perlis et al, 2005). On the basis of measurements like whole body metabolic rate (measured by oxygen consumption VO_2), heart rate variability, neuroendocrine measures and functional neuroimaging, physiological arousal is evaluated. Insomnia patients exhibit higher metabolic rates, higher heart rate and lower heart rate variability (Bonnet & Arand, 1998). Increased levels of plasma cortisol and adrenocorticotropic hormone in the insomniacs suggest that the hypothalamic-pituitary-adrenal axis is associated with the pathology (Vgontzas et al, 2001). Based on the positron emission tomography imaging, patients with insomnia showed a greater cerebral glucose metabolism during W and NREM sleep states (Nofzinger et al, 2004).

According to the cognitive model, the cognitive arousal in the form of rumination and worry predisposes the individual to insomnia, precipitates acute episodes, and perpetuates the chronic form of the disorder (Perlis et al,

2005). Patients with chronic insomnia report that their life stress events often precede and precipitate their insomnia. Furthermore, these worries and rumination strengthens and become perpetuating factor for insomnia (Perlis et al, 2005).

Behavioral model of insomnia suggests that “although a variety of biopsychosocial factors may precipitate acute insomnia, chronic insomnia results from behaviors that disrupt sleep” (Perlis et al, 2005). There are three behavioral models, sleep hygiene, stimulus control and the Spielman models. Sleep hygiene models states that “specific kinds of behavior are conducive to or incompatible with sleep and that modifying behavior may alleviate insomnia”. These behaviors include sleep duration, bedtime rituals, sleep surface, ambient temperature, sleep satiety and body position. Stimulus control model “is based on the behavioral principle that one stimulus may elicit a variety of responses, depending on the conditioning history”. In the case of patients with insomnia, the normal cues associated with sleep (e.g., bed, bedroom, bedtime) are often paired with activities other than sleep which lead to stimulus dyscontrol lowering the probability that sleep-related stimuli will elicit the desired response of sleepiness and sleep (Perlis et al, 2005). According to the Spielman’s model or the three factor model or the three-P model, “insomnia occurs acutely in relation to both traits (predisposing factors) and life stresses (precipitating factors) and that the chronic form of the disorder is maintained by maladaptive coping strategies (perpetuating factors)” (Spielman et al, 1987). Predisposing factors may be biological, psychological or social. Precipitating factors include the triggers like medical

and psychiatric illness and stressful life events. Perpetuating factors refer to the strategies that the patient adopts to compensate for sleep loss like the practice of staying in bed while awake and the tendency to extend sleep opportunity. These lead to mismatch between sleep opportunities and sleep ability (Spielman et al, 1987).

Neurocognitive model of insomnia states that "the acute insomnia occurs in association with cognitive and behavioral factors and chronic insomnia is a reversible central nervous system disorder that occurs in part in relation to behavioral factors and in part as a result of classical conditioning" (Perlis et al, 2005). Unlike the cognitive model, neurocognitive model suggests that rumination and worry are not responsible for extended wakefulness but the *vice-versa* is true. Apart from somatic and cognitive arousals as mentioned in the previous models, this model also includes a cortical arousal. Cortical arousal leads to abnormal levels of sensory and information processing with increased formation of long-term memory. This in turn is linked to sleep continuity disturbance and sleep state misperception. Enhanced sensory processing makes the individual vulnerable to perturbations by environmental stimuli, which interferes with sleep. Enhanced information processing during NREM sleep makes the distinction between sleep and wakefulness unclear and enhanced long-term memory around sleep onset and during NREM sleep may interfere with the subjective experience of sleep initiation and duration. In patients with primary insomnia, cortical arousal occurs due to classical conditioning and is observed as high-frequency EEG activity (14 Hz to 45 Hz) at or around sleep onset and during NREM sleep. EEG during NREM sleep in

patients with insomnia exhibit more beta activity than good sleepers and beta activity which is a marker of electrophysiological arousal is negatively associated with the perception of sleep quality and is positively associated with the degree of subjective-objective discrepancy (Perlis et al, 2005).

Till date, the neurocognitive model is considered to be the most valid of all models since there are evidences substantiating the claims (Perlis et al, 2005) However, none of the models explaining the cause of insomnia have studied the circadian and homeostatic regulation of sleep.

4.3. Causes and consequences of insomnia

Insomnia may be classified in to three categories based on its origin (Ohayon, 2010).

- Comorbid with another physical and/or mental illness.
- Induced by use of psychoactive substances.
- Caused by lifestyle or without apparent cause.

Insomnia (4-11 %) is found to be comorbid with medical conditions like upper airway diseases, rheumatic diseases, chronic pain due to injuries, infection, gastro-intestinal problems, obesity, allergy, autoimmune disorders and cardiovascular diseases. Neurological and psychological conditions like Parkinson's disease, migraine, Alzheimer's disease, bipolar disorders, anxiety disorders, depression, and psychotic disorders are also major contributors (30-40 %) of insomnia. Other sleep disorders like sleep apnea, periodic limb movement disorder, narcolepsy, circadian rhythm disorder and

hypoventilation also results in insomnia (5-15 %). Poor sleep hygiene, shift work, irregular sleep-wake schedule and lifestyle stress are responsible for 10 % of the insomnia complaints and use and abuse of psychoactive drugs (like sedatives, anxiolytics, alcohol, caffeine etc) are responsible for 3-7 % of insomnia complaints (Ohayon, 2010).

Effect of sleep loss and fragmentation has been documented in various studies and is found to affect the sleep pattern and the psychomotor skills of an individual. Insomniacs have impairment in initiation and maintenance of sleep, have reduced sleep duration and have associated poor quality sleep. Effect of sleep on day time sleepiness is clearly demonstrated using multiple sleep latency tests and in other sleepiness and mood scales. Also with an increase in the degree of insomnia, progressively deteriorating psychomotor skills (reaction time, attention, concentration, vigilance) are observed. Increase in the number of lapse which is defined as a period of non-responsiveness on the part of the subject, believed to be a manifestation of a microsleep, is a daytime impairment observed after acute sleep loss. Increased appearance of daytime fatigue along with impaired psychomotor skills is observed in chronic insomniacs co-morbid with other medical conditions. Memory problems, mood swings, irritability, increased sensitivity to external stimuli etc are also observed in insomniacs. Insomniacs underestimate their subjective sleep latency (increased latency to sleep) and have subjective fatigue associated with it (Bonnet & Arand, 1998).

In patients with severe insomnia, sleep disturbance is the major problem faced and it is reported that 88.2 % of patients have this issue for 5 yrs after

the onset of insomnia (Mendelson, 1995). Sleep disturbance has been shown to last for 4 years (Chevalier et al, 1999) and 56 % of individuals have remission after 10 years (Janson et al, 2001).

Insomniacs are reported to have decreased life quality as assessed by the 36-item Short Form Health Survey of the Medical Outcomes Study (SF-36). SF-36 measures the quality of life under eight domains which includes physical functioning, role limitation due to physical health problems (role physical), bodily pain, general health perceptions, vitality, social functioning, role limitations due to emotional health problems (role emotional) and mental health (Katz & McHorney, 2002). Severe insomniac patients had more pain and increased emotional and mental health problems in comparison to the congestive heart failure patients. Insomniacs are more prone to accidents due to their lack of attention. They show poor job performance, lower physical and social functioning and an overall lower quality of life comparable to that of individuals with chronic medical conditions (Katz & McHorney, 2002). Insomnia is an important risk factor for several psychiatric disorders. Anxiety, mood disorders and depression are the most commonly seen co-morbid conditions with chronic insomnia (Roth, 2007). There are studies reporting reciprocal relationship of these psychiatric disorders with insomnia. In patients with depression, improvement in sleep is found to be positively correlated with antidepressant response (Roth, 2007).

4.4. Methods to assess insomnia

Insomnia is diagnosed subjectively on the basis of patient history and sleep diary maintenance and quantified using actigraphy and polysomnography techniques. As a part of patient history evaluation, clinicians enquire about the 24 h S-W pattern and habits, sleep environment, medications taken, regularity and variability of sleeping hours, daytime activities, bed partner's habit and presence of other disorders (Buysse et al, 2006). Actigraphy, a motion sensitive wrist-worn device, is also used to assess the rest-activity pattern in a patient. For more precise measurement, polysomnography which primarily uses EEG, EMG and EOG to assess S-W, is a gold standard for identifying and quantifying sleep disturbances including insomnia. Once the cause and the symptoms are identified, the patients are categorized based on the various classifications.

4.5. Animal models of insomnia

An animal model which shows the four characteristic features representing the pathophysiologic mechanism of insomnia may be considered for studying insomnia. These features include disruption of sleep homeostatic regulation, disruption of the circadian clock and intrinsic systems responsible for the expression of sleep and enhancement of extrinsic systems that can alter normal S-W regulation. Modeling insomnia in animals requires creating situations in which the animals cannot initiate sleep at the appropriate circadian phase, despite being given enough opportunity to sleep, subsequently developing sleep debt and need for recuperative sleep (Toth &

Bhargava, 2013). Rat which is exposed to a stressful condition (for example, immobilization, social stress, isolation, fear and fear conditioning, sensory stimulation) is a good model to study insomnia as it shows sleep disturbances observed in humans suffering from insomnia. These disturbances include prolonged sleep latency, reduced sleep duration, frequent arousals and fragmentation of sleep and high frequency EEG activity during NREM sleep (Cano et al, 2008). Simultaneous activation of W and sleep promoting regions in the brain is associated with the insomnia in rats (Cano et al, 2008). Rodent genetic model for example DBA/2J mice spent relatively more time awake with high fragmentation and low delta power which make it a good candidate to study insomnia (Franken et al, 1999).

Other genetic models which include the *Drosophila* insomnia-like (ins-1) flies may also be useful to identify the genes contributing to sleep and its disorders (Seugnet et al, 2009). Caffeine also produces transient insomnia as its intake increase arousal, prolong latency to sleep onset, reduce sleep duration and sleep pressure and reduce sleep quality in animals and humans (Bonnet & Arand, 1992). Animals with lesions in brain areas specific to sleep also show temporary reduction in sleep.

To study insomnia in animals, various SD procedures are commonly used. All SD procedures involve either partial or complete removal of sleep in organism (Colavito et al, 2013). In partial SD, a specific sleep state (most commonly the REM sleep or paradoxical SD) is selectively targeted for SD and in total SD all the stages are prevented for a desired amount of time (Colavito et al, 2013). To create insomnia in rodents there are 29 distinct

methods of SD (Revel et al, 2009). This includes stress-related models (cage change, introduction of aversive odors, immobility etc), discomfort (immobility, exposure to cold or hot temperatures, current or pain etc), pharmacological models (administration of caffeine, psychostimulants etc), and genetic models (DBA/2J mice, clock gene mutants, orexin overexpression etc).

The present study mainly focuses on the total SD and its effects in rats. Two procedures of total SD are used in this study. One is by gentle handling method for creating acute insomnia model and the second using rotating wheel for creating chronic insomnia model. Gentle handling method is the most popular method for short term SD in rodents. It involves direct interaction of rodents with the experimenter, who actively monitors the animals with or without the support of EEG and EMG recordings. The moment the animals become drowsy, the experimenter will stimulate the animal either passively or actively. Passive stimulation includes making mild noise, tapping or shaking the cage or directly contacting the animal with soft bristled brush. Active stimulation includes introducing novel objects in to the cage, changing the nesting materials etc. Gentle handling method is found to be the least stressful out of all 29 SD protocols (Colavito et al, 2013; Rechtschaffen et al, 1999) and is found to reduce an average of 92 % of NREM sleep and 100 % of REM sleep (Franken et al, 1991). Continuously moving rotating wheel (automated motor device which has a rotating drum moving at a specified rotation per minute) produces total SD by forcing specific patterns of locomotion in rats (Borbely & Neuhaus, 1979). Forced

locomotion using rotating wheel is found to be an additional stress factor along with SD, however, one study has reported that the forced locomotion is just a minor factor in comparison to the sleep deprivation and hence may not contribute to the studies on sleep deprivation and its effects (Borbely & Neuhaus, 1979). Also, this method is more efficient for chronic SD and it provides standardized and equal stimulation to all experimental rats which cannot be achieved using gentle handling method (Colavito et al, 2013).

4.6. Insomnia and thermoregulation

Sleep is most conducive in the minimum phase of temperature and is inhibited before and after the minimum phase which represents the 'wake maintenance zone' (Lack et al, 2008). Different symptoms of insomnia and abnormalities in thermoregulation are closely associated (Lack et al, 2008). Sleep onset insomnia is associated with a delayed temperature rhythm for more than 2 h, early morning awakening insomnia is associated with phase advance temperature rhythm, sleep maintenance insomnia is associated with nocturnally elevated CBT and combination of onset and maintenance insomnia is associated with a 24 h elevation of CBT. Insomniacs attempt their sleep within the 'wake maintenance zone' when the CBT is close to maximum (Lack et al, 2008). Moreover, insomniacs have difficulty sleeping in the night due to reduced ability to dissipate body heat from distal areas thereby preventing the normal decline in core temperature (Lack et al, 2008; Van Den Heuvel et al, 2004).

Reduction in sleep quality, difficulty initiating and maintaining sleep, increased W after sleep onset, longer latency to sleep, reduced slow wave sleep and increased stage 1 sleep is associated with elevated T_{body} minimum, decreased responsiveness to thermal changes and decreased amplitude of the temperature rhythm (Stepanski et al, 1988). Sleep deprivation reduces the magnitude of the 24 h peak-trough difference in the T_{body} (Czeisler et al, 1980). Total SD showed an initial rise and subsequent decline and partial SD produced a decline in the intra-peritoneal temperature in the rats (Rechtschaffen & Bergmann, 2002). An increase in the cortical temperature was observed in rats subjected to 12 h and 24 h SD due to increased thermal load as a result of enforced waking (Franken et al, 1991).

4.7. Insomnia and anxiety

Insomnia and anxiety have a complex bidirectional interaction with each other (Alvaro et al, 2013; Taylor et al, 2005). 60 % of people with social phobia and 68 % of people with panic disorder have been reported to have insomnia (Stein et al, 1993). People with chronic insomnia were six times more likely to have an anxiety disorder compared to those without insomnia (Ford & Kamerow, 1989). Similarly, patients with generalized anxiety disorder (GAD) have also reported difficulties with initiating and maintaining sleep than people without an anxiety disorder (Monti & Monti, 2000). Insomnia symptoms occurred prior to a first episode of an anxiety disorder only 18 % of the time, while they occurred at the same time in 39 % of the

cases and insomnia symptoms occurred after the anxiety disorder in the another 44 % of cases (Ohayon & Roth, 2003).

Polygraphic recordings of sleep in anxious patients have consistently shown increased sleep latency and a reduced total sleep time, less slow-wave sleep, a greater arousal index and an increased duration of wakefulness during sleep (Bourdet & Goldenberg, 1994). People with insomnia and co-morbid GAD also have higher levels of pre-sleep cognitive activity and sleep problems like middle of the night insomnia, poor sleep quality and nightmares than those without insomnia (Marcks et al, 2010). In children and adolescents with anxiety disorders, 88 % of them had at least one sleep-related problem, and over half had three or more sleep disturbances like insomnia, nightmares, and reluctance/refusal to sleep alone (Alfano et al, 2007).

Normal reduction in arousal and alertness at bedtime is attenuated in insomniacs leading to anxiety. Increased anxiety is linked to increase in sympathetic nervous system activation, thereby lowering of distal temperature in insomniacs thus contributing to increased sleeplessness (Lack et al, 2008). Central nervous system hypervigilance and hyperarousal, which are found to be the actual symptoms of GAD, lead to nocturnal insomnia, which in turn may cause diurnal tiredness as a consequence of sleep pressure (Saletu-Zyhlarz et al, 1997). Furthermore, $GABA_A$ (gamma amino butyric acid) receptor deficits contribute to both insomnia and anxiety (Möhler, 2008).

4.8. Insomnia and oxidative stress

According to the free-radical flux theory by Reimund (1994), cerebral free radicals accumulate during wakefulness and are removed during sleep. Removal of excess free radicals during sleep is accomplished by decreased rate of formation of free radicals and increased efficiency of endogenous antioxidant mechanisms. Thus, sleep functions essentially as an antioxidant for the brain (Reimund, 1994). Consequently loss of sleep or insomnia may lead to oxidative damage in various regions of the body (Gopalakrishnan et al, 2004; Ramanathan et al, 2002; Reimund, 1994). These changes lead to several metabolic, hormonal, immunological and cognitive deficits (Cirelli, 2006; Durmer & Dinges, 2005). Patients with primary insomnia had significantly lower glutathione peroxidase (GSH-Px) activity and higher malondialdehyde (MDA) levels when compared with the controls (Gulec et al, 2012). Oxidative damage associated with sleep loss may be caused by the generation of reactive oxygen or nitrogen species and other oxidative stress markers and cellular impairments in multiple organs. Brain is found to be the most susceptible area for oxidative stress and cell damage because of the increased presence of polyunsaturated fatty acid, increased utilization of oxygen and decreased levels of antioxidants (Gutteridge & Halliwell, 2000). Oxidative stress as a result of both total as well as partial/paradoxical SD in rodents is extensively reviewed in Villafuerte et al (2015).

Importance of understanding and treating insomnia has still not received its due respect in the current world. Research on various aspects of insomnia

has just started gaining momentum. Considering the severity of insomnia and its after effects, more studies focusing on the treatment options are required.

5. Various therapeutic approach to insomnia

Conventional treatment for insomnia is broadly classified in two, pharmacological and behavioral or psychological treatments. Apart from these two, there are some alternative therapies which include the herbal and other traditional treatment methods (Attele et al, 2000).

5.1. Pharmacological treatment

Contemporary FDA approved pharmacological treatment includes $GABA_A$ receptor agonists (benzodiazepines and non-benzodiazepines), sedating antidepressants and melatonin agonists.

5.1.1. Benzodiazepines (BDZ) and non-benzodiazepines (nBDZ)

BDZ and nBDZ are the most commonly prescribed medications for insomnia, and have demonstrated efficacy in short-term treatment. The first FDA approved drugs for insomnia were BDZ (estazolam, quazepam, triazolam, flurazepam and temazepam) and nBDZ, also known as z-drugs (zaleplon, zolpidem, and eszopiclone). All these drugs are effective for short term usage except eszopiclone which has efficacy up to 6 months. Both drug groups are $GABA_A$ receptor agonist with binding site located between α- and γ-subunits of α- and γ-subunit containing $GABA_A$ receptors. While BDZ bind

to subunits of the α1, α2, α3, and α5 classes, nBDZ preferentially bind to the α1 subclass. These drugs hyperpolarize the resting potential via GABA activation, thus inhibiting or reducing the activity of neurons. The $GABA_A$ channel opens quickly contributing to the early part of the inhibitory post-synaptic potential (Equihua et al, 2013). These drugs produce several unpleasant side-effects like altered sleep architecture, anterograde amnesia, psychomotor impairment, withdrawal effect or rebound insomnia, drug dependence, tolerance, residual daytime drowsiness, muscle relaxation, respiratory distress, accidents etc (Ashton, 1994; Chouinard, 2004; Gunja, 2013). Furthermore, these drugs promote only stage 1 NREM sleep with very little slow wave sleep and REM sleep. Even though these drugs decrease the latency to sleep, the resultant sleep has very poor quality (Aeschbach et al, 1994; Lancel et al, 1996).

BDZ and nBDZ may be short, intermediate or long acting based on their half-lives. For elderly and those with renal or hepatic problems, short half-life drug (triazolam) which does not form active metabolites is preferable to avoid oversedation and accumulation of active metabolites. A BDZ with a longer half-life (flurazepam) is appropriate for patients with daytime anxiety. An intermediate acting BDZ (temazepam or estazolam) may be a reasonable compromise for patients with early morning awakenings (Ringdahl et al, 2004).

5.1.2. Tricyclic antidepressants

Tricyclic antidepressants are also prescribed for insomnia in doses sub-threshold for the treatment of depression. In 2010, the FDA approved the tricyclic antidepressant doxepin for the treatment of sleep maintenance insomnia (frequent nighttime or early morning awakenings). Doxepin is classified as a serotonin and norepinephrine reuptake inhibitor along with antihistaminergic properties. Therapeutic effects of doxepin are observed at very low dosages (3–6 mg/day), improving sleep maintenance without rebound insomnia or physical dependence (Equihua et al, 2013). Side-effects include anticholinergic effects (urinary retention, dry mouth, and constipation), cardiac toxicity, orthostatic hypotension, sexual dysfunction nasopharyngitis, gastrointestinal effects, and hypertension (Equihua et al, 2013).

5.1.3. Antihistamines and other over-the-counter medications

Antihistamines are active agents in many over-the-counter medications. This drug antagonizes central histamine H1 receptors (Attele et al, 2000). They are only minimally effective in inducing sleep, and may reduce sleep quality. While these medications are generally safe, they may have anticholinergic side-effects (dry mouth, blurred vision, constipation, tachycardia, urinary retention and memory deficits) and daytime impairment. Other over-the-counter medications include alcohol and L-tryptophan (Equihua et al, 2013). L-tryptophan, though banned in 1989 because of its association with eosinophilia-myalgia syndrome, is used as a sleep aid. The

safety and efficacy of the lower dose has not been studied. Alcohol is also used to produce drowsiness. In one study 28 % used alcohol to help them sleep, and 67 % found it effective. However, alcohol can act as a central nervous system (CNS) stimulant leading to increased nocturnal awakenings and has a potential to become substance abuse (Ringdahl et al, 2004).

5.1.4. Melatonin agonists

Melatonin is a neurohormone secreted by the pineal gland following a circadian rhythm. The production of melatonin peaks when the lights go out, which signals the organism that it is nighttime. In humans, melatonin has sleep-promoting effects as it has been found to induce sedation, lower CBT, reduce sleep latencies and increase total sleep time (Erman et al, 2006).

Ramelteon is an FDA approved melatonin agonist that acts upon MT1 and MT2 receptors improving sleep-onset latency at a recommended dose of 8 mg/day. The side-effects observed include headache, somnolence, dizziness and sore throat. However, ramelteon is well tolerated, and does not show residual effects such as cognitive and psychomotor impairments (Equihua et al, 2013).

5.1.5. Orexin antagonists

Orexin antagonist suvorexant (MK-4305) reduces active wake time by increasing NEM and REM sleep in rats, dogs, and monkeys. Suvorexant is a dual orexin receptor antagonist. This molecule is also in phase III clinical trials and is currently under evaluation for approval by the FDA. In healthy

humans, the lowest dose (10 mg) reduced the number of awakenings after sleep onset; and at higher doses (50 mg) it reduced sleep latency, while increasing sleep efficiency and total sleep time. High doses (50 and 100 mg) elicit narcoleptic symptoms such as an increase in reaction time, difficulty waking up and reduced alertness following awakening. In addition it leads to mild complaints of headaches, somnolence, dizziness and abnormal dreams, all of which occurred in a dose dependent manner (Equihua et al, 2013).

5.2. Non-pharmacological treatment

Behavioral or psychological treatments, which address mechanisms contributing to insomnia, are found to be as effective as sedative hypnotic medications with benefits more durable. Sedative hypnotics do not presume the mechanism responsible for insomnia, therefore symptoms typically return once treatment is discontinued (Kales et al, 1974). Non-pharmacologic treatment is less expensive and has fewer side effects compared with pharmacologic treatment. Non-pharmacologic treatments for insomnia are considered effective if they decrease sleep onset latency or increase total sleep time by 30 minutes. Criteria used include total sleep time, sleep-onset latency and number of nocturnal awakenings (Ringdahl et al, 2004). Behavioral treatments for insomnia includes stimulus control, sleep restriction, cognitive-behavior therapy, progressive muscle relaxation and paradoxical intention. Sleep hygiene (used in conjunction with other techniques) and biofeedback are other relatively less effective methods (Ebben & Speilman, 2009). Furthermore, to treat sleep onset insomnia, morning bright light is used to

phase advance the circadian rhythms and to treat early morning awakening, evening bright light therapy is often used (Lack et al, 2008). Phase advance method is sometimes used in combination with melatonin (Ebben & Spielman, 2009). However, the non-pharmacological methods are found to be subjective, time-consuming and demands lot of patience in carrying out.

5.3. Traditional/Herbal treatment

Traditional and herbal therapies like *Ayurveda* have offered wide range of medications reasonably giving relief from insomnia. Some of these are used as a single drug or in combination with others. Some of them, for example valerians, are available as over-the-counter drugs. Description on few of the well established herbal hypnotics is given below. Except for valerian, none of the other herbs have undergone systematic clinical or scientific validation for their sedative or hypnotic properties.

5.3.1. *Valeriana officinalis* (valerian)

The root and the rhizome of the valerian have sedative and anxiolytic properties. This herb is considered as safe by FDA. Sedation is produced by valepotriates, valerenic acid and unidentified aqueous constituents of valerian (Wagner et al, 1998). However, these active compounds do not produce sedation when given alone. Clinically, valerian was found to improve sleep quality and delta sleep and decrease stage 1 sleep and sleep latency and reduce the number of night awakenings (Schulz et al, 1998). Interaction of valerian constituents with central $GABA_A$ and adenosine A_1 receptors leads to sedation

(Houghton, 1999). Long term usage of valerian produces withdrawal effects, central nervous system depression, muscle relaxation, cytotoxicity, cardiac complications and cancer (Houghton, 1999).

5.3.2. *Withana somnifera* (Withania)

Withania roots and leaves are considered to have mild hypnotic property. Triethylene glycol is found to be the sleep-inducing component extracted from leaves (Kaushik et al, 2017). Other potential components include withanolide and withaferin. Withania has GABA mimetic activity and is also found to be a good anxiolytic.

5.3.3. *Matricaria recutita* (German Chamomile)

Flower head of German Chamomile has sedative effects due to the presence of benzodiazepine-like compound. Chamomile causes allergy and at high dose produces vomiting. A reduction in anxiety was observed in 57 subjects after 8 weeks treatment with chamomile extract (Amsterdam et al, 2009).

5.3.4. *Passiflora incarnata* (passion flower)

Even though there is lack of strong evidence, passion flower is used for the treatment of insomnia. Active components of passion flower include harmala-type indole alkaloids, maltol and ethyl-maltol and flavonoids. Subjective improvement in sleep quality was observed after having passion flower tea for 3 weeks before sleep in a random control trial on 41 subjects

(Ngan & Conduit, 2011). Passion flower extract prolonged sleeping time in rats.

5.3.5. *Piper methysticum* (kava kava)

Kava kava rhizome is used to treat anxiety, stress and restlessness associated with insomnia. The sedative property of kava kava is due to a group of resinous compounds known as kava lactones or kava pyrones which induce sleep and muscle relaxation in animals. Kava kava is thought to act on GABA and benzodiazepine binding sites in the brain (Davies et al, 1992). CNS depressants potentiate the effects of kava.

5.3.6. *Humulus lupulus* (Hops)

The dried strobile of hops induces sleep (Attele et al, 2000). Active ingredients in hops include a volatile oil, valerianic acid, estrogenic substances, tannins and flavonoids. Hops in tea have a calming effect within 20-40 minutes of ingestion and is used as a treatment for insomnia (Mowrey, 1986). Hops modulate melatonin receptor M_1 and M_2. Allergy, hormonal variation and potentiation of other sedatives and alcohol are some of the side-effects (Attele et al, 2000).

5.3.7. *Panax species* (ginseng)

Panax ginseng (Korean or Asian ginseng), Panax quinquefolius (American ginseng), and Panax vietnamensis (Vietnamese ginseng) are reported to have sleep-modulating effects. Active component ginsenosides Rb1, Rb2 and Rc

mixture prolonged the duration of hexobarbital-induced hypnosis in mice. Amount of slow wave sleep increased during light period after intake of ginseng extract (Rhee et al, 1990). Ginsenosides compete with agonists for binding to $GABA_A$ and $GABA_B$ receptors. Long term usage leads to side-effects like nervousness, excitation and hormonal variation.

5.3.8. *Hypericum perforatum* (St John's wort)

St John's wort is commonly used herb for psychiatric disorders. Hypericin and pseudohypericin are the main active ingredients of St John's wort. The extract and hypericin modulates deep and REM sleeps (Schulz & Jobert, 1994). They have high affinity for $GABA_A$ and $GABA_B$ receptors. Side-effects include sedation, dry mouth, dizziness, gastrointestinal upset, restlessness and hypersensitivity.

5.3.9. *Scutellaria laterifolia* (Skullcap)

Leaves and flowers of skullcap are found to be sleep-inducing. Clinical studies on their effect on insomnia are lacking. Side effects include dizziness, confusion and seizures, and hepatoxicity (Perharic et al, 1994).

5.3.10. *Zizyphus jujube* (Sour date)

Sour date active component jujubosides (saponin) increased total and NREM sleep time in rats. Circadian rhythm and the serotonergic system influence the hypnotic effect of jujubosides. Jujubosides inhibits glutamate-

mediated pathway in hippocampus modulation of central monoamines and limbic system interaction (Cao et al, 2010).

According to *Ayurveda*, imbalances in the body lead to insomnia. Insomnia or *anidra* is an imbalance in *Tarpaka Kapha*, *Sadhaka Pitta* and *Prana Vayu* (Patil et al, 2014). *Ayurveda* evaluates these imbalances and prescribes treatment to restore balance (https://www.banyanbotanicals.com/). The imbalances broadly include:

1. Toxins accumulating in tissues and blocking circulation
2. Poor nutrition
3. Poor digestion
4. Imbalance of the nervous system
5. Accumulation of physical and mental stress
6. Lowering of natural resistance and immunity
7. Disruption of natural biological rhythms

Ayurveda mentions about few useful herbs and their combinations for the treatment of insomnia (adapted from http://ayurvedanextdoor.com/herbs-for-insomnia/).

- ***Tagara (Valeriana wallichi)*:** *Tagara* rejuvenates and relaxes nerves and clear out toxins from blood, blood vessels, joints etc. *Tagara*, also known as Indian Valerian is always used along with other herbs as it may have some dulling effect when used alone.

- ***Ashwagandha (Withania somnifera)*****:** *Ashwagandha* vitalizes mind and improves memory. It is also good for insomnia as it refreshes nerves and relaxes them.

- ***Brahmi (Bacopa monnieri)*****:** *Brahmi* is a brain tonic which rejuvenates the brain and brain cells. It provides vitality and longevity. *Brahmi* is an effective herb in case of insomnia, tension, fatigue, depressions etc. For ages it has been used as a tranquilizer to cure patients.

- ***Jatamanasi (Nardostachys jatamansi)*****:** *Jatamanasi* is a sedative herb, which is used to tranquilize a patient. It helps in relaxation of nervous system and is very effective in case of neurosis. It increases neurotransmission and is good for memory too.

- ***Sarpagandha (Rauwolfia serpentina)*****:** *Sarpagandha* is used for curing obesity, hypertension, insomnia, stress etc. It is a powerful tranquilizer and induces sleep. It clears toxins from the blood, blood vessels, digestive system and destroys poisons. It is also good for the heart and cures insanity.

- ***Shankhupushpi (Convolvulus pluricaulis)*****:** *Shankhupushpi* is used for brain rejuvenation for ages. The herb cures insomnia by clearing the nerve cells of toxins and opening capillaries for better blood circulation. It prevents mental fatigueness and gives rest to the brain. It boosts brain and cures hypertension, insomnia and depression.

- ***Vacha (Acorus calamus)*:** *Vacha* is an efficient mind calming herb which cures tension and insomnia. The herb has a coolant property which relaxes the nerves thereby inducing sleep. *Vacha* has a speeding effect on human mind, taking away tensions, emotional stress and depressions. It is a nervous tonic for the mind and body.

Even though there are multiple treatment approaches to insomnia, there still exist one or more disadvantages with respect to efficacy, safety, long term usage, reliability and cost. Conventional and clinically approved pharmacological treatment, even though efficient and cost effective, may not be safe for long term usage. Non-pharmacological options, even though safe for long term usage, have proven its efficiency only when given in combination with other treatment approaches. Also, this method provides subjective therapeutic results. One the other hand, the herbal treatment which is reported to be safe, does not have enough scientific validation for use in clinical practice. Moreover studies on few herbs, for example valerian, have been found to produce only subjective improvement in insomniacs with low reliability. More studies need to be conducted to validate the properties of herbs commonly used in the traditional school of medicines.

In the present study, we have scientifically evaluated the hypnotic potential of an active component from the herb *Acorus calamus* Linn *(vacha).* We chose this herb on the basis of its calming and cooling effect on the nervous system. Since insomnia leads to hyper-activation of brain along with

hyperthermia, this herb may be a good candidate for the treatment of insomnia. We specifically chose α-Asarone, an active component from *Acorus calamus*, for our study in view of developing a new drug for the treatment of insomnia.

6. *Acorus calamus* Linn.

6.1. Features and distribution

A. calamus Linn. *(vacha, vayambu)* is an aromatic rhizome commonly referred to as calamus, sweet flag, rat root or flag root. This perennial semi-aquatic herb is a member of the family Araceae and is found in moist areas such as swamps and marshes throughout North America, Europe and Asia. It has sword-shaped leaves with small, yellowish-green flowers and may grow to six feet (Fig. 2). Its leaves and rhizomes are scented (Fig. 2). The genus *Acorus* comprises about 40 species, however, only few species like *A. calamus* (Linn.), *A. christophii*, *A. tatarinowii* (Schott.) and *A. gramineus* (Solandin Ait.) have been investigated for their chemical composition and bioactivities. *A. calamus* is the most extensively studied species due to its medicinal and pharmacological significance (Ganjewala & Srivastava, 2011).

A. calamus is a native of Central Asia and Eastern Europe, and is indigenous to the marshes of the mountains of India. It is cultivated throughout India, ascending to an altitude of about 2200 m. It is found and cultivated in the states of Jammu Kashmir, Himachal Pradesh, Manipur, Nagaland, Uttarakhand, Uttar Pradesh, Tamil Nadu, Andhra Pradesh,

Maharashtra and Karnataka (Rajput et al, 2014). According to the Red Data Book list of threatened species, *A. calamus* has been identified as the vulnerable species (Sharma et al, 2014).

Fig. 2 *Acorus calamus* Linn. (A); spadix (B); wet rhizome (C); dry rhizome (D)

6.2. Chemical compositions

A wide variety of chemical constituents have been reported from the herb *A. calamus*. Monoterpene hydrocarbons, Sequestrine Ketones, *trans*- or α-Asarone, *cis*- or β-Asarone and Eugenol were also identified (Balakumbahan et al, 2010). Other constituents such as alkaloids, flavanoids, gums, lectins

mucilage, phenols, quinine, saponins, sugars, tannins and triterpenes are also recorded from this plant.

From Indian *A. calamus*, 93 volatile compounds and some amino acids were detected out of which α-Asarone, β-Asarone and γ-Asarone was found to be the major components. The other constituents include Methyl-isoeugenol, Isoeugenol, Eugenol, Calameone, Asaronaldehyde, Terpinolene, Camphor, α-Caryophyllene, β-Pinene, Azulene, Diterpene, α-Pinene, Acoramone, Isoshyobunone, Elemene, Esocalamendiol, Ehyobunone and Ecorone. The major constituents in the rhizome and leaf essential oils of *A. calamus* are β-Asarone (83.2 and 85.6 %). On the other hand, α-Asarone (9.7 %) was recorded as the second major constituent in the rhizome oil, while it was linalool (4.7 %) in the leaf oil (Raina et al, 2003).

Oil from dried rhizomes of an Indian specimen yielded 2.8 % oil that contained 82 % Asarone, 5 % Calamenol, 4 % Calamene, 1 % Calameone, 1 % Methyl-eugenol and 0.3 % Eugenol. Two bitter principle compounds, Acorin and Acoretin, are also reported (Sharma et al, 2014). Besides essential oil, *A. calamus* rhizomes have also been examined for other chemical constituents such as protein, carbohydrates, sugars, fatty acids, amino acids, iron, fat and calcium. Various sugars such as maltose (0.2 %), glucose (20.7 %) and fructose (79.1 %) are reported (Balakumbahan et al, 2010).

6.3. Pharmacological properties

Sweet flag has a very long history of medicinal use in Chinese and Indian herbal traditions. In China, the root is used as a restorative tonic for both body

and mind, while in *Ayurveda*, it is highly valued for its ability to bring clarity to the consciousness and to counter the side-effects of all hallucinogens. In *Ayurveda*, the use of sweet flag is effective against wide varieties of illnesses. The word '*acorus*' is originated from the Greek word '*acoron*' used by the Dioscorids which is derived from the word '*coreon*' meaning ‘pupil’ based on its use in the treatment of eyes diseases and inflammation. *Acorus* is considered as a *lekhaniya*-reducing herb, *asthapanopaga* (an adjunct to decoction enemas), *sitaprasamana* (relieves cold sensation on the skin), *samjnathe sthapana* (restores consciousness), *vaya sthapana* promotes longevity), *arsoghna* (anti-hemorrhoidal) and *siro virecana* (cleansing nasal therapy) (Singh et al, 2011).

Four types of *A. calamus* are used in herbal medicine: type I *A. calamus* L. var. americanus, a diploid American var.; type II var. vulgaris L. (var. calamus), a European triploid; type III and type IV var. augustatus Bess. and var. versus L., subtropical tetraploids (Khare, 2007). *A. calamus* L. var. calamus, syn. var. vulgaris L. is sterile distributed throughout Europe, temperate India, and the Himalayan region. *A. calamus* var. angustatus Bess variety is found in eastern and tropical Southern Asia.

Both roots and leaves have shown strong antioxidant, antimicrobial, anti-asthmatic, antimutagenic, antispasmodic, anti-ulcer, antidiarrhoeal, anticancer, anti-inflammatory, antidiabetic, antioxidant, antifungal, antibacterial and insecticidal activities (Sharma et al, 2014). Sweet flag and its active components have the following medicinal properties.

1. **Antipyretic**, i.e. it reduces fever.
2. **Anodyne**, i.e. it can relieve or soothe pain by lessening the sensitivity of the brain or nervous system. Chewing the root alleviates toothache. Sweet flag is also used externally to treat rheumatic pains and neuralgia.
3. **Carminative**, i.e. it prevents formation of gas in the gastrointestinal tract or facilitates its expulsion, thereby combating flatulence.
4. **Stomachic,** i.e. it tones the stomach, improving its function and normalizing the appetite. In small doses, it reduces stomach acidity, while larger doses increase stomach secretions and is therefore recommended in the treatment of anorexia nervosa, dyspepsia, disorders of the gall bladder, and other digestive disorders, including dysentery in children.
5. **Diaphoretic**, i.e. it increases perspiration.
6. **Sedative**, i.e. it reduces irritability or excitement.
7. **Antiseptic**, i.e. it reduces the possibility of infection, sepsis (blood poisoning), or putrefaction, and is used externally to treat skin eruptions.
8. **Hypotensive**, i.e. it reduces blood pressure.
9. **Expectorant**, i.e. it dissolves thick mucus and helps relieve respiratory difficulties, and is used internally in the treatment of bronchitis, sinusitis, and asthma.
10. **Emmenagogue**, i.e. it can stimulate blood flow in the pelvic area and is used as an aphrodisiac. It is also used as an abortive herb.
11. **Vermifuge**, i.e. it expels parasitic worms from the body, by either stunning or killing them.

12. **Hypocholesterolemic and cholelitholytic**, i.e. it is useful in reducing total and serum-LDL (low density lipoprotein) cholesterol levels in the body.

6.4. Effect of *A. calamus* on central nervous system

The volatile oil obtained from the roots and rhizomes of an Indian indigenous plant *A. calamus* Linn possess sedative properties (Dandiya et al, 1959a; Dandiya et al, 1959b). The volatile fraction of the petroleum ether extract potentiated the sedative activity with pentobarbital, hexobarbital and ethanol in mice (Dandiya et al, 1959b). The essential oil showed sedative-tranquilizing action in rats, mice and dogs and high dose of oil inhibited monoamine oxidase activity (Dhalla & Bhattacharya, 1968). Doses 10, 25 and 50 mg/kg of herbal extract antagonized spontaneous motor activity, and also exerted sedative and tranquilizing action (Panchal et al, 1989). The 100 mg/kg dose decreased both treadmill performance and locomotor activity, caused hypothermia and potentiated pentobarbital-induced sleep (Pages et al, 2010). Ethanolic extract of *A. calamus* (0.5 ml/kg, i.p.) potentiated pentobarbitone-induced sleeping time in rats and the time of loss of righting reflex was prolonged in mice. Rats also exhibited hypothermia for 4 h at a latency of 30 min with the peak effect after 60 min (Vohora et al, 1990).

Methanol extracts of *A. calamus* roots when administered orally at doses of 100 and 200 mg/kg exhibited protective effect against the pain models in mice (Jayaraman et al, 2010). Methanolic extract of *A. calamus* displayed significant acetyl cholinesterase (AChE) inhibition at a concentration 200

µg/ml (Mukherjee et al, 2007). Because cognitive performance and memory are related to acetylcholine levels, the AChE-inhibitory effect of the plant may account for its traditional use.

The anticonvulsant effect against the pain models in mice was observed when methanol extract of *A. calamus* root was administered orally at the doses of the 100 and 200 mg/kg. This study suggested that the anticonvulsant effects might be potentiated by the activity of GABA (Jayaraman et al, 2010). The steam volatile fractions of rhizome of *A. calamus* exacerbated tonic seizures provoked by metrazol in rats and also potentiated the action of reserpine in reducing amphetamine toxicity in aggregated mice (Dandiya et al, 1959b).

70 % hydro-ethanolic extract of 500 mg/capsule twice a day given after meals to 33 human adults (20 male and 13 female) attenuated anxiety related disorders and reduced stress phenomenon and its correlated depression investigated on the basis of standard questionnaires on different physiological rating scale (Bhattacharyya et al, 2011). In models of depression, when the animal was subjected to *A. calamus* administration, the behavioral deficit was prevented as compared to stressed group (Tripathi & Singh, 2010). *A. calamus* given for 6 weeks in 50 individuals with depression showed reduction in the degree of severity and better rehabilitation and also a significant improvement in assessment based on the rating of symptoms on the Hamilton depression rating scale.

Ischemic rats treated with *A. calamus* (25 mg/kg/p.o. for 5 days) exhibited a significant improvement in neuro-behavioral performance in Rota-Rod test and grid walking as compared to the control group (Shukla et al, 2006).

Hydroalcoholic extract of *A. calamus* (100 and 200 mg/kg/p.o.) attenuated chronic constriction injury (CCI) of sciatic nerve (peripheral neuropathy)-induced development of painful behavioral, biochemical and histological changes in a dose dependent manner (Muthuraman & Singh, 2011).

In *Ayurveda*, *A. calamus* is considered as a *Rasayana* herb possessing strong antioxidant activity (Govindarajan et al, 2005). As reported in *in-vitro* DPPH antioxidant assay, *A. calamus* extract have very high antioxidant capacity (IC_{50}= 46.75 ± 3.34 µg/mL) exhibiting DPPH radical-scavenging activity, chelation of ferrous ions, reducing power and strong superoxide anion-scavenging activity (Ahmeda et al, 2009). In rats subjected to noise and chronic restraint stress, *A. calamus* has been found to normalize the levels of antioxidants and other metabolites in brain (Manikandan & Devi, 2005; Manikandan et al, 2005; Reddy et al, 2014). Antioxidant property of *A. calamus* was also observed in regions like kidney, liver and heart in various models (Palani et al, 2009; Sandeep & Nair, 2012).

6.5. Dose in *Ayurvedic* literature

The dose of *Vacha* as per *Ayurvedic* Pharmacopoiea of India is 120 mg per day. The dose for experimental animals was calculated by extrapolating the human dose to animals based on the body surface area ratio by referring to the standard table of Paget and Barnes (1964). On this basis, the rat dose of *Vacha* samples was found to be 10.80 mg/kg body weight.

6.6. Toxicity

Chemical isolation studies have led to the discovery of two stereoisomers, α- and β-Asarone, which have psychoactive effects (Motley, 1994). These are the major active components in the extracts of different plant parts and essential oils. However, studies in rats of the active ingredient, β-Asarone isolated from the Indian Jammu strain of *A. calamus* demonstrated an increase in malignant duodenal tumor. It is demonstrated that β-Asarone is potentially toxic and carcinogenic (Taylor et al., 1967). In this study, rats were fed with diets containing various concentrations of β-Asarone for two years. The tumors were identified as leiomyosarcomas of the small intestine and were found in 800 ppm (0.08 %) and 2000 ppm (0.2 %). Also thrombosis within the chambers of the heart was observed in the 800 and 2000 ppm (Taylor et al., 1967). This led to the removal of *A. calamus* as a food or food additive in the US in 1968 by the FDA.

Later studies have demonstrated that the North American species of *A. calamus* lacks the carcinogenic agent. *A. calamus* is poisonous under certain conditions, causing disturbed digestion, gastroenteritis, persistent constipation, followed by diarrhoea and passage of blood into the feces. The carcinogenic agent, β-Asarone, appears only to be present in the triploid and tetraploid varieties of *A. calamus*. Due to the toxicity of β-Asarone, attempts are being made to reduce its concentration in herbal medicines. The diploid varieties such as American diploid varieties lack β-Asarone and are always preferred for therapeutics and other industries.

In the present study, α-Asarone was chosen as the study material as it exhibits all the major properties (hypnotic-potentiating, hypothermic, anxiolytic and antioxidant) observed in the parent herb with relatively less toxicity.

7. α-Asarone

Trans- or α-Asarone belongs to the family of phenylpropenes (Fig. 3). These are compounds containing a phenylpropene moiety, which consists of a propene substituent bound to a phenyl group. Molecular weight of α-Asarone is 208.257 g/mol and the molecular formula is $C_{12}H_{16}O_3$. IUPAC name is *trans*-1,2,4-Trimethoxy-5-(1-propenyl)benzene. It is available in the form of crystalline solid. α-Asarone is practically insoluble in water and soluble in alcohol, ether, glacial acetic acid, carbon tetrachloride, chloroform and petroleum ether.

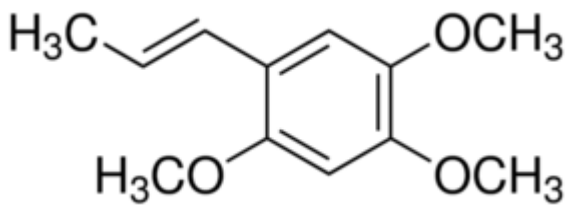

Fig. 3 Chemical structure of α-Asarone

7.1. Pharmacokinetics of α-Asarone

Because of high lipophilicity, the oral bioavailability of α-Asarone in rats is poor (Lu et al, 2014). However, the same property allows α-Asarone to get rapidly distributed in to the brain regions by passing through blood-brain barrier (Lu et al, 2014). This makes it a good drug candidate for various central nervous system disorders. The plasma half-life of both α-and β-asarone are short (~1-1.5 h) with rapid distribution to vital organs such as liver, spleen, heart, kidney, lung, and brain (Kim et al, 2015). The rate of metabolism of α-Asarone is directly proportional to the CYP450 concentration (Cartus & Schrenk, 2016).

7.2. Toxicity of α-Asarone

Studies on toxicity of α-Asarone have shown mixed observations. In mice and rats, α-Asarone is found to be safe at lower doses (Chellian et al, 2017). LD50 intra-peritoneum in mouse is found to be 310 mg/kg and LD50 oral in mouse is 417.6 mg/kg (https://www.caymanchem.com/msdss/11681m.pdf). Intravenous lethal dose low in man is found to be 66 mg/kg and intraperitoneal toxic dose low in mouse is found to be 50 mg/kg (https://www.caymanchem.com/msdss/11681m.pdf). Sub-chronic treatment of α-Asarone (50 and 100 mg/kg for 28 days) produced no behavioral changes and no loss of righting reflex (Chen et al, 2013). However, α-Asarone at a higher dose (200 mg/kg, for 28 days) significantly decreased spontaneous locomotor activity; but no mortality was observed (Chen et al, 2013).

The morphology of adult rat hepatocytes was altered *in vitro* when exposed to high dose of α-Asarone for 1 or 2 weeks. Short-term treatment (not sub-chronic) of α-Asarone (5-30 mg/kg, i.p. for 5 days) produced germinal mutation in rodents (Chamorro et al, 1999). α-Asarone (50 mg/kg, p.o.) pretreatment in normal mice did not cause DNA damage or chromosomal abbreviation in the bone marrow samples (Sandeep & Nair, 2011), indicating the absence of genotoxicity. The eggs treated with α-Asarone survived in the chicken embryo test indicating absence of teratogenicity (JECFA, 1981). α-Asarone did not produce developmental defects in zebrafish embryos (Cai et al, 2016) or teratogenic effect in organogenesis of pregnant rats (Chamorro et al, 1999).

7.3. Effect of α-Asarone on central nervous system

α-Asarone potentiated the hypnosis induced by pentobarbital, hexobarbital and ethanol, lower T_{body} of mice, block conditioned response of rats and exert a calming influence on hostile cats (Dandiya & Sharma, 1962). Like the herb, α-Asarone (10 mg/kg/i.p.) also completely abolished motor activity and caused hypothermia even in low doses (Dandiya & Menon, 1963). The animals were less responsive to tactile and auditory stimuli (Dandiya & Menon, 1963). α-Asarone significantly enhanced the anesthetic activity of pentobarbitone, hexobarbitol, and ethanol in mice (Sharma et al, 1961). Sedative effect of α-Asarone was dependent on the depression of the ergotropic division of the hypothalamus (Menon & Dandiya, 1967). α-

Asarone also produced a prolonged calming effect in monkeys (Dandiya & Menon, 1964).

Houghton et al, (2006) reported an *in-vitro* acetylcholinesterase inhibitory effect of α-Asarone similar to the parent herb. Furthermore, α-Asarone showed a tendency to protect against metrazol convulsions and modified electroshocks (Sharma et al, 1961). Measurement of tonic GABA currents and miniature spontaneous inhibitory postsynaptic currents indicated that α-Asarone enhanced tonic GABAergic inhibition without affecting phasic GABAergic inhibition (Huang et al, 2013). In both pentylenetetrazole and kainate seizure models, α-Asarone suppressed epileptic activity of mice by prolonging the latency to clonic and tonic seizures and reducing the mortality as well as the susceptibility to seizure by activating $GABA_A$ receptors (Huang et al, 2013).

In light dark test, α-Asarone 7 mg/kg increased the time spent in the light area and the number of transitions between the two compartments (Liu et al, 2012). In the marble burying test also, α-Asarone inhibited marble burying at doses of 14 and 28 mg/kg, as did diazepam. These observations indicated that α-Asarone exhibits anxiolytic-like effect (Liu et al, 2012). Treatment with 30 mg/kg α-Asarone attenuated the loss of CA1 neurons, increased the TUNEL-labeled cells, and upregulated BACE1 expression-induced by lipopolysaccharide in the hippocampus (Shin et al, 2014). Behaviorally, α-Asarone increased the number of target heading and memory score in the Morris water maze (Shin et al, 2014). Treatment with α-Asarone (3, 10, 30 mg/kg/i.p.) also attenuated scopolamine-induced cognitive deficits (Kumar et

al, 2012). The memory impairment correlated with nitric oxide overproduction and neuronal damage caused by the injection of amyloid beta peptide (25-35) was reduced by the administration of α-Asarone 10 mg/kg orally for 16 days (Limón et al, 2009)

α-Asarone attenuated the activity of AchE, normalized MDA (lipid peroxidation marker) levels and superoxide dismutase (SOD) activity in hippocampus and cortex in the scopolamine-treated amnesic mice (Kumar et al, 2012). In rats subjected to noise and chronic restraint stress, α-Asarone, like the parent herb, normalized the levels of antioxidants and other metabolites in brain (Manikandan & Devi, 2005; Reddy et al, 2014). A reversal of increased nitrate levels in the hippocampus and temporal cortex was observed in the rats injected with amyloid-β (25-35) after 16 days administration of 10 mg/kg α-Asarone (Limón et al, 2009). Furthermore, α-Asarone was found to increase the levels of antioxidants in different brain regions in various epileptic seizure models and was also observed in regions like kidney, liver and heart in various models (Palani et al, 2009; Sandeep & Nair, 2012).

8. Lacunae

Extensive literature search revealed the following lacunae in insomnia research

- The current interventions have multiple disadvantages and a therapeutic intervention with minimum side-effects to cure insomnia is lacking.
- Scientific evaluation of majority of apparently safe herbs or active components used in traditional medicine is lacking.
- No studies have investigated the sleep-inducing property of α-Asarone, using objective measure of polysomnography.
- There are no reports on effects of α-Asarone on sleep and thermoregulation and their association under normal and sleep deprived conditions (acute or chronic).
- There are no studies on the anxiolytic and antioxidant properties of α-Asarone under sleep deprived conditions (acute or chronic).

9. Aim and objectives

Henceforth the aim of the present study was "**to assess and validate scientifically the effectiveness of α-Asarone, active principle from *A. calamus* on sleep and insomnia**". Since sleep is closely associated with thermoregulation, we have simultaneously assessed the effectiveness of the hypothermic property of α-Asarone in regulating the sleep wakefulness (S-W), hypothalamic (T_{hy}) and body (T_{body}) temperature during normal and altered sleep. This study aims to identify the sleep-promoting dose of α-Asarone, the effect of chronic administration of α-Asarone on sleep in normal rats, the effect of α-Asarone in acute insomnia, the effect of α-Asarone in chronic insomnia and the mechanism of action of α-Asarone with respect to sleep and thermoregulation.

To fulfill the aim of the present study, five objectives were formulated as follows

1. To evaluate the effect of administration of various doses of α-Asarone, an active principle of Acorus calamus on S-W, T_{hy} and T_{body} in normal rats.

2. To investigate the effect of chronic administration (21 days) of optimal dose of α-Asarone on S-W, T_{hy} and T_{body} in normal rats.

3. To investigate the effect of optimal dose of α-Asarone on S-W, T_{hy}, T_{body}, anxiety and brain antioxidant levels in rats acutely sleep deprived by gentle handling (5 h/ 5 days).

4. To investigate the effect of optimal dose of α-Asarone on S-W, T_{hy}, T_{body}, anxiety and brain antioxidant levels in rats chronically sleep deprived in rotating wheel (5 h/ 21 days).

5. To understand the mechanism of action of α-Asarone by examining the relationship of T_{hy} and T_{body} with sleep.

Chapter III: Materials and Methods

1. Animal model

The experimental model of this study was adult male Wistar rats weighing 250-350 g. The rats were individually housed in polystyrene cages (40X25X15 cm), kept in controlled temperature (26 ± 1 °C) and light-dark schedule of 12 h (lights on at 6:00 h). Food (rat/mice pellet, VRK Nutritional Solutions, India) and water were provided *ad libitum*. All the surgeries and procedures employed in this study were approved by the Institutional Animal Ethics Committee of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala, India (SCT/TAEC-019/June/2012/77).

2. Sampling

Wistar rats were randomly distributed to carry out various experiments under specified objectives as mentioned in Fig. 4.

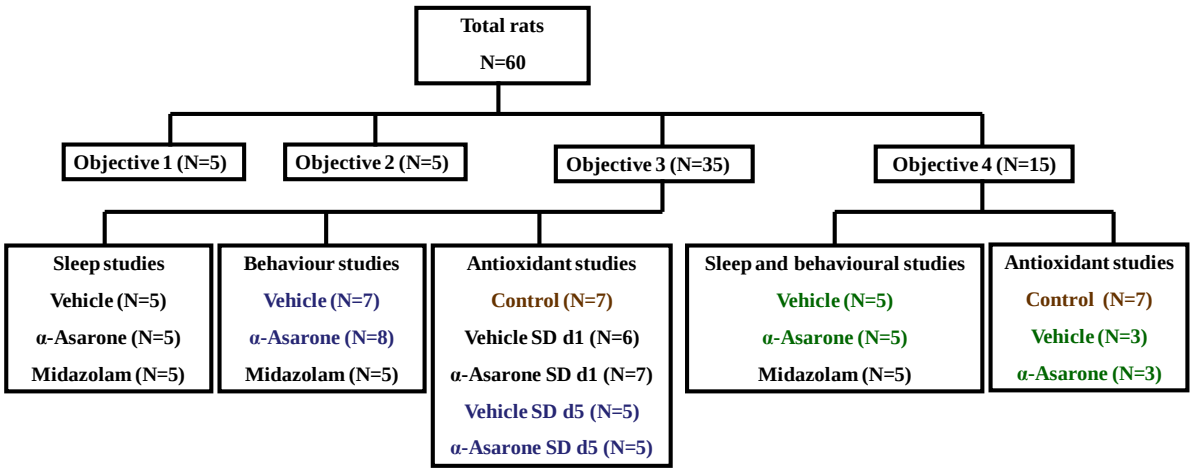

Fig. 4 Schematic representation showing objective-wise distribution of rats; N represents sample size, d represents day. Colour coding indicates same rats used for different studies.

3. Procedures to assess S-W, T_{hy} and T_{body}

3.1. Chemicals and instruments used

3.1.1. Chemicals used

Ketamine hydrochloride (50 mg/ml, Aneket, Neon Laboratories Ltd., India), Xylazine hydrochloride (2 ml, Indian Immunologicals Ltd., India), sterile normal saline (0.9 %), dental cement (DPI-RR cold cure, Dental Products of India, The Bombay Burmah Trading Company, India), betadine (Povidone-Iodine Solution IP 5 % w/v, Win-Medicare Pvt. Ltd., India), Cidex OPA (*ortho*-Phthaldehyde solution, Johnson & Johnson, USA), Ampicillin (Roscillin 500 mg, Sun Pharmaceutical India Ltd., India), Meloxicam (Melonex 5 mg/ml, Intas Pharmaceuticals Ltd, India) and Neosporin (Neomycin and Polymyxin B sulfates and Bacitracin Zinc Powder, GlaxoSmithKline, India).

3.1.2. Instruments used

Kopf small animal stereotaxic instrument (David Kopf instruments, CA), hair clipper (Philips, Netherlands), microdrill with burrs (K1070 Rotary micromotor kit, Foredom, USA), stainless steel screw electrodes (1.3 mm diameter, 3.5 mm length; soldered to radio wires, for EEG), stainless steel loop electrodes (loop soldered to radio wires, for EMG), pre-calibrated K-type thermocouple (chromal/alumel type with tip connected; Range Enterprises, India), pre-calibrated radio-transmitters (type: TA10TA-F40, serial no. 46159

and 59389, Data Sciences International, USA), soldering iron and lead, 8 pin machine tooled integrated circuit socket (ElectronComponents, India) and surgical tools (suture threads, needles, scalpel, scissors, forceps, arterial forceps etc.).

3.2. Surgical Procedure

All the animals were chronically implanted under anesthesia (Ketamine 60 mg/kg and Xylazine 5 mg/kg body weight, i.m.) to record S-W, T_{hy} and T_{body}. Implantations were performed with the Kopf small animal stereotaxic instrument (Fig. 5A) and the coordinates were chosen as per the rat brain atlas (Paxinos and Watson, 1997) as per standardized procedures (Gulia et al., 2004; Gulia et al., 2008).

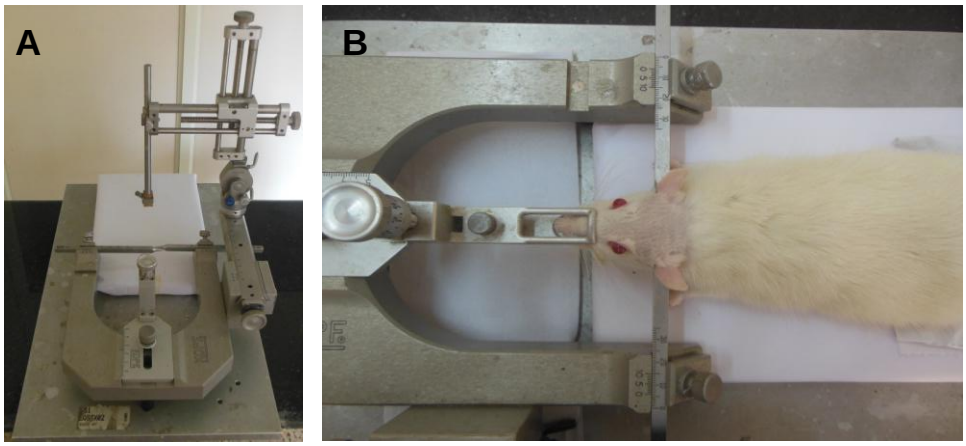

Fig. 5 (A) Stereotaxic instrument used for the implantation of EEG-EMG electrode and thermocouple in rats; (B) Anaesthetized rat fixed in the stereotaxic instrument

After clipping the scalp hair, the rat's head was firmly fixed using the ear plug in such a way that the skull is placed horizontally with bregma and lambda positioned on the same plane (Fig. 5B). Incision bar was kept 3.3 mm below the ear bar.

After fixing the head of the rat firmly (Fig. 6A), a 2 cm sagittal incision was made on the scalp starting from the position behind the eyes to expose the skull (Fig. 6B). The underlying connective tissue (membranous fascia) was cleaned using normal saline and betadine to expose the stereotaxic landmarks to define coordinates (Fig. 6B). Taking bregma as the reference point, concerned areas were marked on the skull as per the coordinates in stereotaxic instrument (Fig. 6B). Using the microdrill, the marked areas were drilled without puncturing the dura to implant the electrodes. To assess S-W, screw electrodes were implanted bilaterally on the parietal cortex (AP: -3 mm and ML: 2 mm) for recording EEG and loop electrodes were sutured bilaterally on either side of the nuchal muscles for measuring EMG. Ground electrode was placed bilaterally on the frontal cortex (Fig. 6C).

To measure T_{hy}, a pre-calibrated K-type thermocouple was implanted, 1 mm anterior to the hypothalamus (AP: -0.26 mm, ML: 3 mm, DV: 6 mm) at an angle of 25° (connected tip facing hypothalamus) (Fig. 6D). The electrodes and the thermocouple were soldered to an integrated circuit socket and the whole assembly was fixed on the skull using dental cement (Fig. 6E&F).

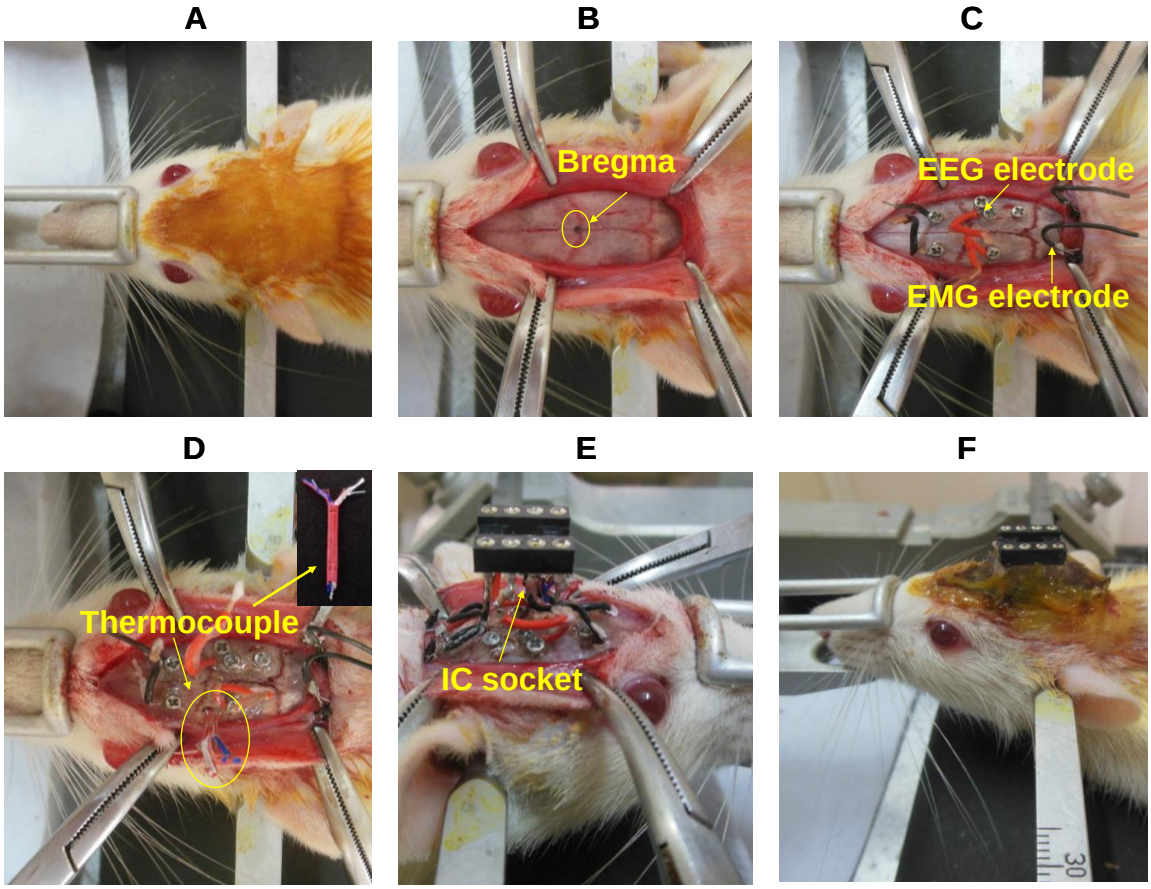

Fig. 6 Implantation of EEG and EMG electrodes and thermocouple to measure S-W and T_{hy} respectively in rats; (A) Anaesthetized and fixed the rat in stereotaxic instrument; (B) Exposed the skull and located the coordinates from bregma; (C) Implanted the ground screw electrodes, EEG screw electrodes and EMG loop electrodes; (D) Implanted the thermocouple (inset) (E) Soldered the electrodes and thermocouple to the IC socket; (F) Secured the socket using dental cement and applied betadine near the implant

For the assessment of T_{body}, a 2 cm mid-sagittal incision was made to expose the peritoneal cavity and a radio-transmitter was placed inside the cavity (Fig. 7A-C). The incision was sutured using cotton thread and betadine and neosporin was applied to the wound until it healed (Fig. 7D-F). Care was taken not to damage the peritoneal organs. Radio-transmitters were reused in multiple animals after cleaning thoroughly with Cidex.

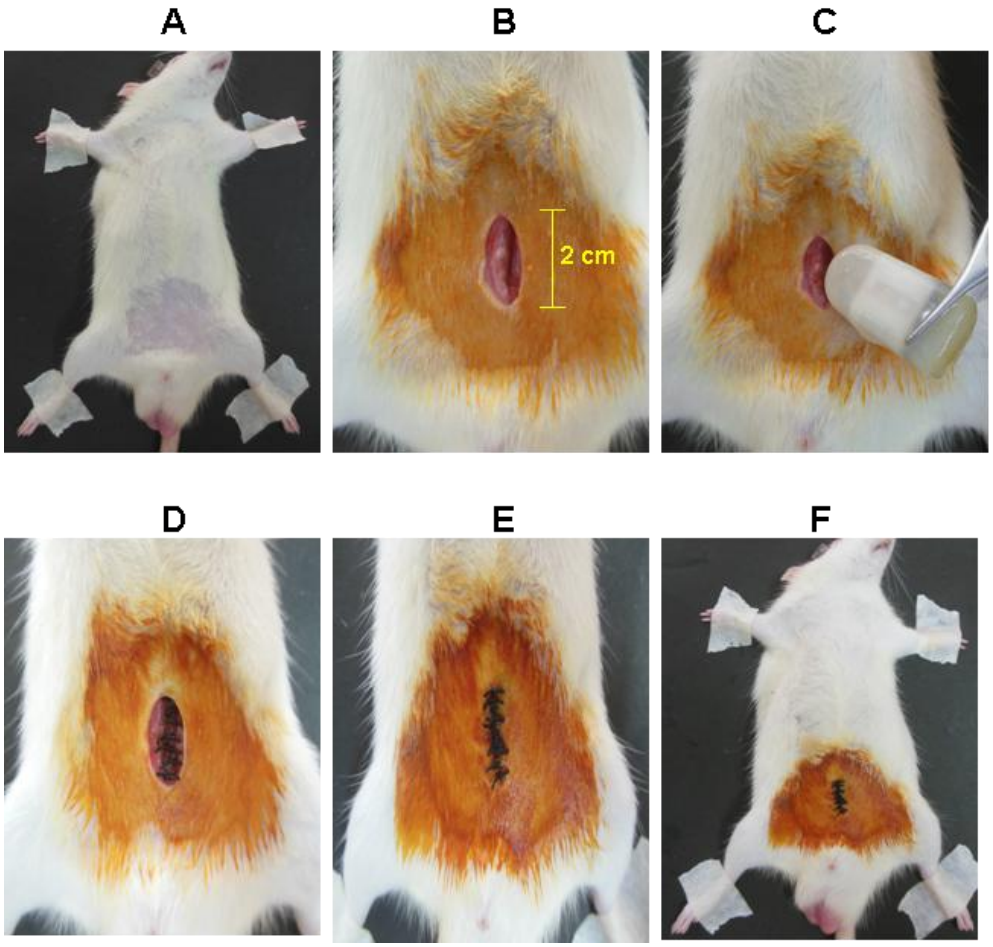

Fig. 7 Implantation of radio-transmitter to measure T_{body} in rats; (A) Anaesthetized and exposed the ventral side of the rat; (B) Made a 2 cm sagittal incision to expose the peritoneal cavity; (C) Inserted the radio-transmitter in to the cavity; (D) (E) Sutured the incision internally and externally; (F) Applied betadine and neosporin on the sutured incision.

3.3. Post-surgical procedures

After surgery the animals were treated with antibiotic ampicillin (100 mg/kg/i.m.) for six days. Analgesic meloxicam (mg/kg/i.m.) was given for three to four days after the surgery. Neosporin was applied peripherally near the surgical areas for ten days. The animals were singly housed in the clean cages with food and water *ad libitum* (Fig. 8). The animals were provided with additional nutrition which includes carrots and soaked chick pea until complete recovery. A recovery period of minimum two weeks was given to the animals.

Fig. 8 Implanted rat singly housed in polystyrene cage with food and water provided *ad libitum*.

4. Acquisition and analysis of S-W, T_{hy} and T_{body} signals

4.1. Instruments and software's used

EEG and EMG signals for assessing S-W were acquired using EEG and EMG modules of data acquisition system MP 150 (BIOPAC systems, Inc). The acquired EEG and EMG signals were amplified 5000 times, filtered

(EEG: 0.1-35 Hz; EMG: 1-500 Hz) and digitized at 1 kHz. Acknowledge software installed in the computer was used for the acquisition and analysis of EEG and EMG signals.

T_{hy} was acquired using a digital multimeter (True RMS Multimeter Model 287/289, FLUKE Technologies Pvt. Ltd.) which has an inbuilt memory to store the data. Data acquisition cable (FLUKE Technologies Pvt. Ltd.) was used to acquire the stored data. To acquire the T_{body}, a telemetric DSI system comprising of a receiver (PhysioTel Model RPC-1, serial number 021668 and 026462) and a computerized data acquisition system Dataquest via data exchange matrix (Data Sciences International, USA). Radio waves transmitted by the implanted transmitter were acquired using the receiver kept in the vicinity. The Dataquest is meant for the conversion of received signals in to T_{body} data. DSI 'acquisition' software installed in the computer was used to acquire the data with online display and DSI 'analysis' software was used to analyze the T_{body} data. Schematic representation of the experimental setup is given in Fig. 9.

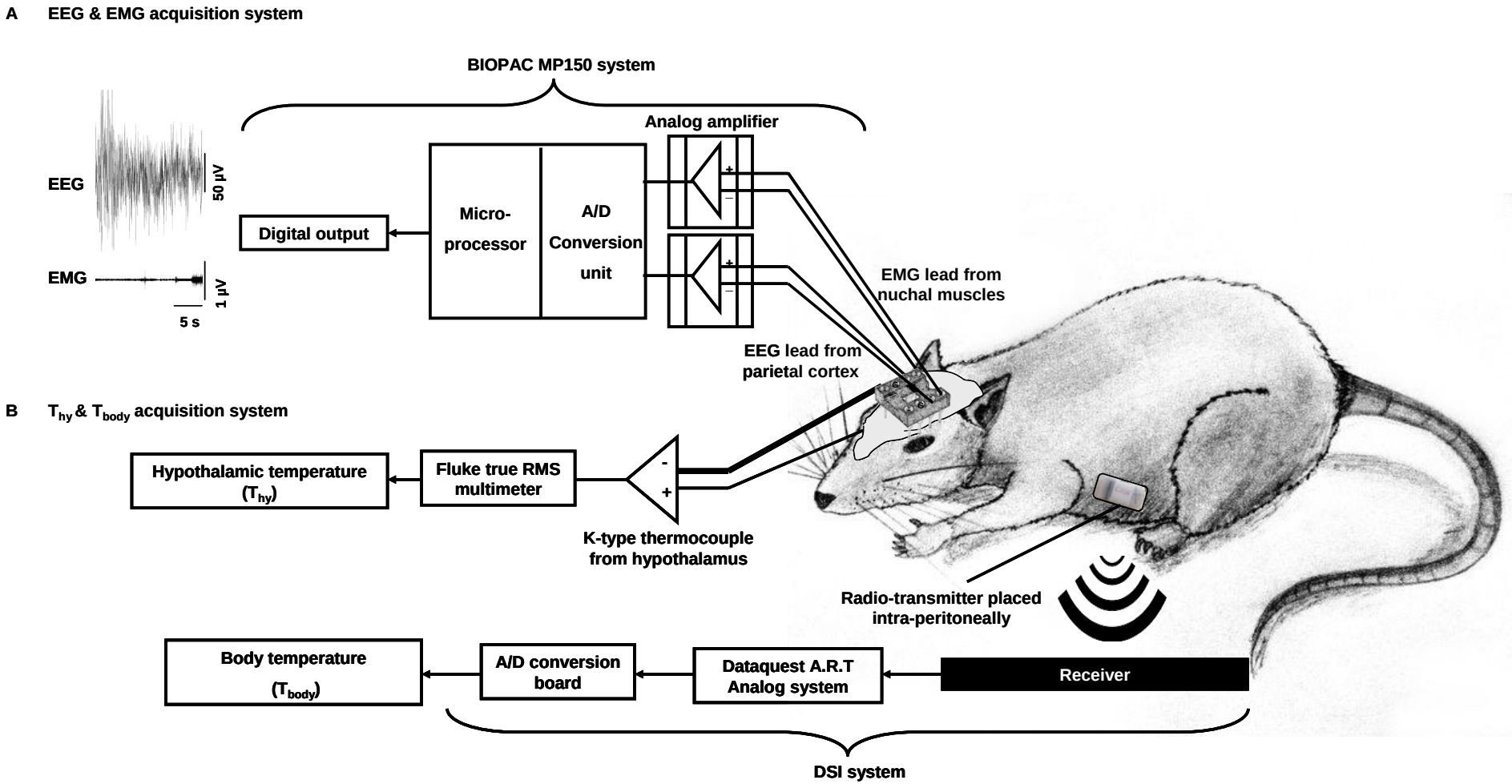

Fig. 9 Schematic representation of experimental set up and acquisition

4.2. Procedure

After complete recovery from the surgical trauma, the rats were kept for overnight habituation in the recording cage. A head cable made of 3 pairs of insulated wires (1 pair for collecting EEG, 1 for EMG and 1 for grounding) and a thermocouple compensatory wire was fixed in to the implanted socket of the rats. This cable transfers the signals from the rats to the data acquisition systems (FLUKE) via probes.

On the day of recording, the rats were connected to the data acquisition systems via head cables to record S-W and T_{hy}. To record T_{body}, a magnet (Data Sciences International, USA) was used to switch on the implanted transmitter. After recording, the same process with the magnet switched off the transmitter. Before all recordings in this study, the rats were given an overnight habituation in the recording cage with the head cables connected to prevent acclimatization issues. Before subjecting the rats to various experiments under specified objectives, baseline or control recordings of S-W, T_{hy} and T_{body} were taken on three days for 8 h (from 9:00 to 17:00 h).

Once acquired, the signals were subjected to offline analysis. S-W data acquired were visually staged in the Acknowledge software, taking 10 s epochs, and were classified into three stages, W, NREM sleep and REM sleep. T_{hy} and T_{body} data were also acquired for every 10 s in such a way that every stage of S-W was having a corresponding T_{hy} and T_{body} values. All signals were time matched.

Quantity of S-W was assessed by measuring the percentage total time spent in these stages and quality of sleep was assessed by measuring the

average bout duration and bout frequency of NREM and REM sleep and W. Quality was further confirmed by calculating the arousal index (number of 3-10 s wake bouts per hour of sleep) during sleep which is an indicator of sleep fragmentation (Bonnet et al, 2007). Additionally, power at various frequency bands of EEG during sleep was calculated using power spectral density analysis to measure the depth of sleep. From each 10 sec epochs of EEG signals, 0.5–30 Hz frequencies were filtered and the power spectral density of delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz) and beta (12–30 Hz) bands were analyzed. The relative power (in percentage) of each frequency bands per epoch were calculated from the total power of EEG.

5. Acquisition and analysis of anxiety data

5.1. Instruments and software's used

Anxiety in rats was measured using custom made acrylic elevated plus maze (EPM) test and open field test (OFT). The acrylic EPM having two open arms (50X10 cm) and two closed arms (50X10X50 cm), arranged in plus shape, was kept at a height of 45 cm. The arms of same type faced each other and were connected through an open centre zone (10X10 cm) (Fig. 10A).

The acrylic OFT maze chamber of dimension 100X100X40 cm was demarcated into three concentric zones, namely outer, middle and inner zone (Mikaelsson et al, 2013) (Fig. 10B).

The behavior on the maze was video monitored, acquired and analyzed using AnyMaze video tracking system (version 4.82 from Stoelting Co. USA)

which is a system comprising of a camera/video source (FireWire with USB cable) focusing the underlying maze (~1.5 m below the camera) and a software which detects the rat present on the maze and analyze and provide output on various behavioral parameters as per the experimenter's input.

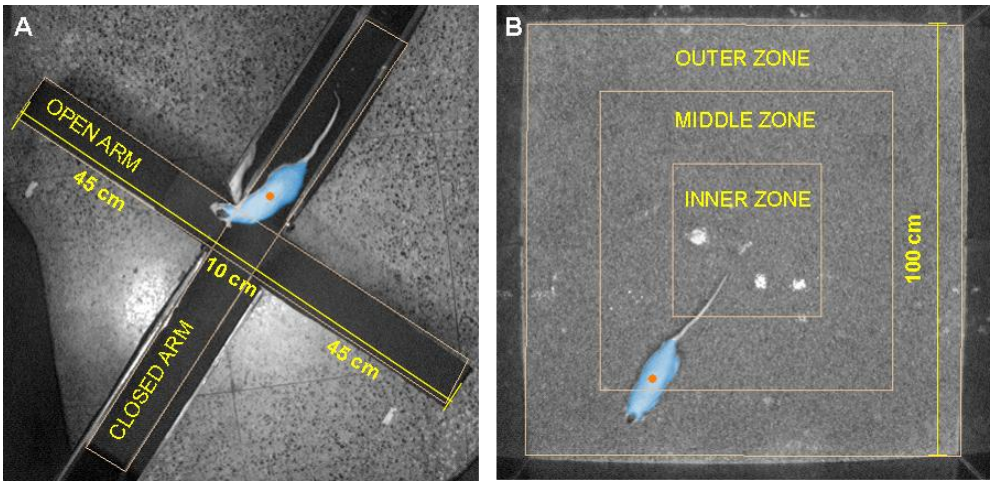

Fig. 10 EPM (A) and OFT (B) for anxiety measurement in rats

5.2. Procedure

Before starting the experiment, the rats were placed in transporting cage for 5 min and taken in to the recording room with minimum disturbance. The mazes were cleaned with 70 % rectified spirit 15-20 min before each recording. After completion of test, the rats were placed back in to the transporting cage and taken away from the recording room.

5.2.1. Elevated Plus maze (EPM)

At the beginning of the experiment the rats were placed in the centre zone facing the opposite open arm and the behavior of rats on the maze was tested

for 5 min. The number of entries in to the open and closed arm, time spent in the open and closed arm, total distance traveled on maze, average speed on maze and total time mobile on the maze were assessed. Ethologically-derived parameters which include head dipping, rearing and grooming were noted. Fall from the open arm of the maze was counted and the rats were placed back at the same spot from where it falls. If the animal falls more than 3 times, the test was aborted and re-conducted after 24 h.

5.2.2. Open field test (OFT)

At the beginning of the experiment, the rats were placed in the centre zone and allowed to explore the chamber for 5 min. Number of entries and time spent in the inner and outer zones, duration of mobility on the maze, total distance traveled, average speed and ethologically-derived parameters like rearing and grooming were measured.

6. Brain antioxidant estimation

6.1. Chemicals and instruments used

6.1.1. Chemicals used

Normal saline (0.9 %), DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), GSH (Glutathione), NADPH (Nicotinamide adenine dinucleotide phosphate), GSSG (Glutathione disulfide), pyrogallol, Folin-Ciocalteau reagent and TBA (Thiobarbituric acid) were obtained from Sigma-Aldrich Co. LLC. All other

chemicals were of analytical grade procured from reputed manufacturers (Merck Millipore, Merck KGaA, Darmstadt, Germany and Sisco Research Laboratories Pvt. Ltd., India).

6.1.2. Instruments used

Guillotine (Holmarc Opto-Mechatronics Pvt. Ltd, India), Motor homogenizer (REMI High speed Homogenizer-RQ 127 A/D), centrifuge (R-8C, REMI laboratory centrifuge), low temperature centrifuge (Eppendorf 5415R), microcentrifuge (Eppendorf 5415R), UV spectrophotometer (Shimadzu UV-1601PC), pH meter (Cyberscan 510 pH), -80 °C freezer (U410 Premium, Ultra-Low temperature Freezer, New Brunswick), water bath (Julabo SW22), vortex mixer (REMI cyclomixer) and laboratory equipments (test tubes, micro-pipettes, eppendorf's tube, beakers, flasks etc).

6.2. Procedure

6.2.1. Extraction of brain regions

The rats were decapitated using guillotine after the completion of various experiments. Immediately after decapitation, the skull and dura were removed without damaging the underlying brain. The brain was carefully removed from the skull cavity till the region where the spinal cord begins by cutting the optic chiasm mid-level. The brain was put in to ice-cold saline (0.9 %) and the two hemispheres were separated from each other. Cerebellum was removed from the brain tissue. From each hemisphere, the cortical layer was carefully

removed leaving the subcortex and brain stem intact. Then the brain stem (which includes midbrain, pons and medulla) was carefully removed leaving behind the subcortical layer intact (includes thalamus, hypothalamus, limbic structures and basal forebrain). Each of these regions were separately weighed and stored in Eppendorf's tube at -80 °C until further processing. The entire procedure till storing was performed in 10 min.

6.2.2. Preparation of tissue homogenates

Tissues were thawed to room temperature and homogenates (10% w/v) were prepared in ice cold 0.1 M phosphate buffer (pH 7.4) in a motor homogenizer and centrifuged in microcentrifuge at 10,000 rpm for 10 min at 4 °C. The supernatants were used for the biochemical analysis. Absorbance was read using a UV spectrophotometer.

6.2.3. Analysis of total protein levels

Protein content in the tissue was determined according to the method of Lowry et al, (1951). The tyrosine and tryptophan residues of proteins cause reduction of the phosphomolybdate and phosphotungstate components of Folin-Ciocalteau reagent in an alkaline medium to give a bluish purple color with absorbance at 660 nm in spectrophotometer. Protein content was calculated from the standard graph plotted using bovine serum albumin (BSA).

0.1 ml homogenate was mixed and incubated for 10 min at room temperature with 0.9 ml distilled water and 5 ml Lowry's reagent (solution A

containing 2 % sodium carbonate in 0.1 N sodium hydroxide solution mixed with solution B containing 0.5 % copper sulphate in 1 % sodium potassium tartarate). To this mixture, 0.5 ml Folin-Ciocalteau reagent was added, mixed and incubated in dark for 30 min. OD was measured at 660 nm. Protein content per sample was calculated from the standard graph.

6.2.4. Analysis of malondialdehyde (MDA) levels

The level of lipid peroxidation was measured as MDA according to the method of Buege and Aust, (1978). MDA is formed mainly from the peroxidation of polyunsaturated fatty acids. MDA is a Thiobarbituric acid (TBA) reacting substance (TBARs) and the product formed between the reaction of MDA and TBA is estimated at 532 nm in spectrophotometer. The lipid peroxidation values are expressed as nanomoles MDA/ mg protein. 1, 1, 3, 3-tetraethoxypropane was used as the standard.

1 ml TBA agent (TBA + Trichloroacetic acid + Hydrochloric acid + Ethylene diamine tetra acetic acid) was added to 100 µl homogenate. The mixture was made up to a final volume of 2 ml with distilled water and kept in boiling water bath for 30 min. The mixture was cooled and centrifuged for 15 min at 1800 rpm and the supernatant was read at 532 nm. MDA content per sample was calculated from the standard graph.

6.2.5. Analysis of catalase (CAT) activity

CAT activity was assayed by measuring the decomposition of hydrogen peroxide (H_2O_2) (Aebi, 1984). H_2O_2 has absorption maxima at 240 nm in

spectrophotometer and absorption decreases with the decomposition of H_2O_2. One unit is defined as the amount of enzyme that decomposes 1 µ mol of H_2O_2 per min at pH 7.0 at 25°C. The extinction was measured at 15 sec intervals for 1 min immediately after adding 1 ml of 30 mM H_2O_2.

To 0.1 ml homogenate, 1.9 ml 0.1 M phosphate buffer (pH 7) was added. H_2O_2 was immediately added to the mixture before taking the reading at 240 nm against the blank (without H_2O_2). OD was taken for every 15 sec interval for 1 min at 240 nm.

$$\text{Activity (U/ml)} = \frac{(\Delta \text{ OD sample} - \Delta \text{ OD blank})}{0.0436 \text{ (extinction coefficient of } H_2O_2)} \times \frac{\text{Total volume}}{\text{Sample volume}}$$

6.2.6. Analysis of glutathione reductase (GSH-R) activity

GSH-R activity was determined following the procedures of Smith et al, (1988) by measuring the disappearance of Nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm in spectrophotometer at 1 min intervals for a total time of 5 min. Enzyme activity (U) calculated as µmol NADPH oxidized/min/mg protein using its molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

To 100 µl homogenate was added 500 µl ethylene diamine tetra acetic acid (10 mM), 500 µl oxidised glutathione (12 mM) and 1 ml sodium phosphate buffer (1 M, pH 7). The mixture was made up to a final volume of 3 ml using distilled water. 200 µl of NADPH (2 mM) was added just before

reading the absorbance at 340 nm. Absorbance was read for 5 min in 1 min intervals.

$$\Delta OD_{min} = \left[\frac{OD\ @\ 1\ min - OD@\ 5\ min}{4\ min}\right]_{sample} - \left[\frac{OD\ @\ 1\ min - OD@\ 5\ min}{4\ min}\right]_{blank}$$

$$\text{Activity (mU)} = \frac{(\Delta OD_{min})}{6.22 \times 10^{-3}\ \text{ml/nmol}} \times \frac{\text{Total volume}}{\text{Sample volume}}$$

(extinction coeffiecient of NADPH)

6.2.7. Analysis of superoxide dismutase (SOD) activity

SOD activity was estimated by the method of Marklund and Marklund, (1974). The degree of inhibition of autoxidation of pyrogallol at an alkaline pH by SOD was used as a measure of the enzyme activity. Initially, the rate of autoxidation of pyrogallol was noted at 1 min intervals for 3 min. The rate of inhibition of pyrogallol autoxidation after the addition of the tissue homogenate was noted at 420 nm in UV spectrophotometer. The enzyme activity (U) was expressed in terms of units/mg protein in which one unit corresponds to the amount of enzyme that inhibited the autoxidation reaction by 50 %.

100 µl homogenate was mixed and centrifuged at 5000 g for 15 min with 25 µl ethanol and 15 µl chloroform. 50 µl supernatant was then mixed with 1 ml Tris-hydrochloric acid buffer with Diethylenetriaminepentaaceticacid DETAPAC (pH 8.2). To this 250 µl of pyrogallol (2 mM) was added and the

whole mixture was made up to a final volume of 2 ml using distilled water. OD was read at 420 nm for 1 to 3 min at 1 min interval.

$$\% \text{ inhibition} = [1- (OD_{sample}/OD_{blank})] \text{ X } 100$$

$$\text{Activity (U/ml)} = \frac{\% \text{ inhibition}}{50\ \%} \text{ X } \frac{\text{Total volume}}{\text{Sample volume}}$$

6.2.8. Activity of glutathione peroxidase (GSH-Px) activity

GSH-Px activity was determined according to the method of Rotruck et al, (1973). The absorbance of the yellow coloured complex was measured at 412 nm in spectrophotometer after incubation for 10 min at 37 °C. Enzyme activity (U) was calculated as µ moles of glutathione utilized/min/mg protein.

To 25 µl homogenate was added 50 µl reduced glutathione (5 mM), 50 µl sodium azide (25 mM), 250 µl hydrogen peroxide (2.5 mM), 0.1 M sodium phosphate buffer (pH7) and 200 µl ethylene diamine tetra acetic acid (0.8 mM). This mixture was incubated at 37 °C for 6 min followed by addition of 1 ml of 10 % trichloro acetic acid. The mixture was then centrifuged for 15 min at 1500 g. To 1 ml supernatant, 1 ml sodium phosphate buffer (0.4 M) and 0.5 ml DTNB (5,5'-dithio-bis-[2-nitrobenzoic acid] or Ellman's reagent) was added and incubated at 37 °C for 10 min. OD was read at 412 nm.

$$\text{Activity (U/ml)} = \frac{\text{OD X sample dilution}}{14.15 \text{ X } 10^{-3} \text{ ml/nmol (molar extinction coefficient of TNB)}}$$

6.2.9. Analysis of reduced glutathione (GSH) levels

GSH in tissue homogenate was determined by the method of Moron et al, (1979). Reduced glutathione forms a yellow coloured complex with DTNB with an absorbance at 412 nm in spectrophotometer. The GSH content of the sample was arrived at from the standard graph and expressed as µmol/mg protein.

To 100 µl homogenate, 900 µl distilled water and 2 ml trichloroacetic acid (5 %) was added and incubated for 5 min. The mixture was centrifuged for 10 min at 1800 rpm. 100 µl supernatant was mixed with 900 µl sodium phosphate buffer (0.2 M, pH 8) and 2 ml DTNB (0.6 mM). Absorbance was read at 412 nm. GSH levels per sample were calculated from the standard graph.

7. SD procedure

In the present study SD was performed using two methods. Method of gentle handling was used to acutely deprive (5 h/5 days) the rats from sleep and custom made rotating wheel was used to conduct chronic SD (5 h/21 days).

Gentle handling method was done by keeping the rats awake by introducing new objects into the cage, making noise with and changing the

bedding material, tapping the cage and mildly touching the animal with soft bristled brush (Rechtschaffen et al., 1999). Food and water was provided *ad libitum*. In the implanted animals, this method was done in the recording cage using electrophysiological monitoring.

For chronic SD, custom made motorized rotating wheel consisting of a geared motor unit (24 V DC driven by DC drive with step down transformer having 230 V AC primary with 24 and 30 V secondary tapings) and a wheel (diameter: 365 mm x length: 150 mm; max capacity: 1 kg) moving at 2.5 rotations per minute (~ 0.05 m/s) was used (Fig. 11).

Fig. 11 Custom made rotating wheel for conducting chronic SD in rats

Water was supplied continuously to the rats through a pipe permanently attached to the wheel and enough food was placed inside the wheel during SD.

Fecal matter and urine was collected in a plate placed below the wheel (Fig. 11).

8. Positive control for SD studies

Sleep deprivation studies was conducted on three groups of rats, where the hypnotic property of the optimal dose of α-Asarone, was compared with vehicle and a positive control midazolam. Midazolam is a benzodiazepine primarily used as a hypnotic-sedative and an anxiolytic drug, which enhances the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at the GABAA receptor (Lancel et al, 1996).

9. Experimental design

9.1. Objective 1: To evaluate the effect of administration of various doses of α-Asarone on S-W, T_{hy} and T_{body} in normal rats.

9.1.1. Chemicals used

α-Asarone (trans-1,2,4-Trimethoxy-5-(1-propenyl)benzene) and Tween80 (Polyoxyethylene (20) sorbitan monooleate) were obtained from Sigma-Aldrich Co. LLC. α-Asarone was freshly prepared in the base containing 5 % Tween80 and normal saline. The base containing 5 % Tween80 was taken as the vehicle.

9.1.2. Procedure

After taking baseline recordings, rats (N=5) were given intra-peritoneal injection of vehicle and various doses of α-Asarone (2, 10, 40, 80 and 120 mg/kg) at 10:00 h. S-W, T_{hy} and T_{body} were then recorded simultaneously for 7 h (10:00 to 17:00 h) (Fig. 12). Five doses of α-Asarone were tried on the same animal on different days using a counterbalanced repeated measure design. An interval of five days was given between each dose in order to prevent the effect of repeated drug administration. The behavior of the animals was also monitored for 7 h after drug administration.

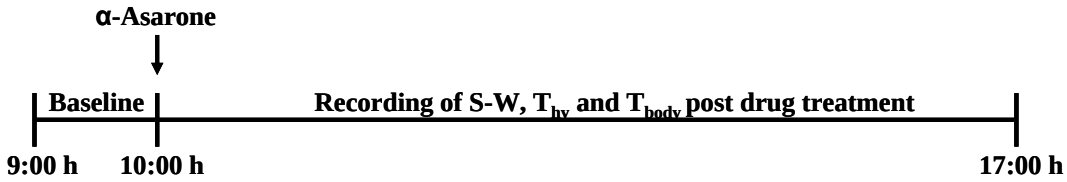

Fig. 12 Schematic representation of the objective 1 experimental schedule; Baseline represents pre-drug state

9.1.3. Analysis

Changes in the percentage time, bout duration and frequency of NREM and REM sleep and W for 7 h after the administration of vehicle, and 2, 10, 40, 80 and 120 mg/kg α-Asarone were calculated to find out the differences in the effects of various doses of the drug and the vehicle. Arousal index after the administration of vehicle and α-Asarone was also calculated.

Changes in T_{hy} and T_{body}, after administering various doses of α-Asarone, were compared with the vehicle and the pre-injection baseline (0 h) values.

NREM and REM sleep EEG, after administration of vehicle and various doses of α-Asarone were analyzed for their spectral profile.

9.1.4. Statistics

All values were expressed as mean ± SEM. $P \leq 0.05$ were considered as statistically significant. All statistical analysis was done in SPSS (version 16.0). Repeated measures ANOVA followed by Bonferroni's correction were used to compare the effect of different doses of drug on S-W and temperature.

9.2. Objective 2: To investigate the effect of chronic administration (21 days) of optimal dose of α-Asarone on S-W, T_{hy} and T_{body} in normal rats.

9.2.1. Chemicals used

α-Asarone was freshly prepared in the base containing 5 % Tween80 and normal saline. The base containing 5 % Tween80 was taken as the vehicle.

9.2.2. Procedure

After taking baseline recordings, rats (N=5) were given intra-peritoneal injection of vehicle at 10:00 h for three consecutive days. S-W, T_{hy} and T_{body} were recorded simultaneously for 7 h after injection (10:00 to 17:00 h). The rats were then given repeated injection of α-Asarone (10 mg/kg) intra-peritoneally at 10:00 h for 21 consecutive days. S-W, T_{hy} and T_{body} were recorded simultaneously for 7 h (10:00 to 17:00 h) on days 7 and 21. On days 7 and 21, a 1 h pre-drug baseline was taken from 9:00 to 10:00 h (Fig. 13).

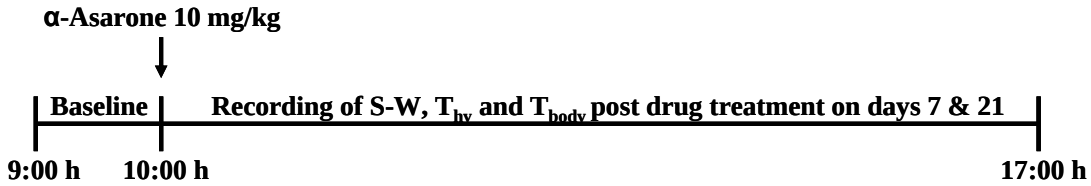

Fig. 13 Schematic representation of the objective 2 experimental schedule; Baseline represents pre-drug state

9.2.3. Analysis

Changes in the percentage time, bout duration and frequency of NREM and REM sleep and W for 7 h after the administration of vehicle and 10 mg/kg α-Asarone (day 7 and 21) were calculated to find out the effect of chronic administration of the drug in normal rats. Arousal index after the administration of vehicle and α-Asarone (day 7 and 21) was also calculated. Changes in T_{hy} and T_{body}, were compared with the vehicle and the pre-injection baseline (0 h) values. NREM and REM sleep EEG, after administration of vehicle and 10 mg/kg α-Asarone (day 7 and 21) were analyzed for their spectral profile.

9.2.4. Statistics

All values were expressed as mean ± SEM. $P \leq 0.05$ were considered as statistically significant. All statistical analysis was done in SPSS (version 16.0). Repeated measures ANOVA followed by Bonferroni's correction were used to compare the effect of drug on different days.

9.3. Objective 3.1: To investigate the effect of optimal dose of α-Asarone on S-W, T_{hy} and T_{body} in rats acutely sleep deprived by gentle handling (5 h/ 5 days).

9.3.1. Chemicals used

α-Asarone was freshly prepared in the base containing 5 % Tween80 and normal saline. The base containing 5 % Tween80 was taken as the vehicle. Midazolam (Neon Laboratories Ltd.) at a dose of 2 mg/kg was taken as the positive control.

9.3.2. Procedure

This study was conducted on 15 rats randomly distributed into three groups of 5 rats each. After baseline recording of all parameters for 1 h (8:00 to 9:00 h), the rats received intra-peritoneal injection of vehicle or drug at 9:00 h followed by SD for 5 h (9:00 to 14:00 h) for 5 consecutive days by gentle handling method. The first group received vehicle, the second received 10 mg/kg α-Asarone and the third group received 2 mg/kg midazolam injection.

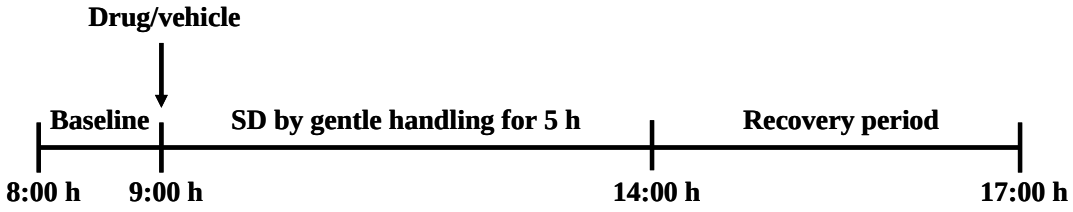

Fig. 14 Schematic representation of the objective 3.1 experimental schedule; **Baseline represents pre-drug state**

After SD, the recording of S-W and T_{hy} and T_{body} was continued further for another 3 h (14:00 to 17:00 h) on day 1 and 5. This 3 h recording (recovery period) was analyzed as described below (Fig. 14).

9.3.3. Analysis

The latency to sleep (time for the onset of NREM sleep) was calculated for all groups on days 1 and 5 of SD. Changes in the percentage time, bout duration and frequency of NREM and REM sleep and W before, during and after SD were calculated on day 1 and 5 to find out the differences in the effects of the drugs and the vehicle. Arousal index after the administration of vehicle and drugs was also calculated. Changes in T_{hy} and T_{body} during SD and recovery period after injection of vehicle, α-Asarone and midazolam were compared with the control values obtained during the same time bin. The change in these temperatures during SD (9:00 to 14:00 h) and 3 h recovery period (14:00 to 17:00 h) were also analyzed. NREM and REM sleep EEG during recovery period were analyzed for their spectral profile. Spectral analyses of baseline sleep (8:00 to 9:00 h) after four days of SD was performed to study the withdrawal effect.

9.3.4. Statistics

All values were expressed as mean ± SEM. $P \leq 0.05$ were considered as statistically significant. All statistical analysis was done in SPSS (version 16.0). Two-way ANOVA with repeated measures on one factor was done to

compare across time bin (repeated measures) and among three groups (independent).

9.4. Objective 3.2: To investigate the effect of optimal dose of α-Asarone on anxiety in rats acutely sleep deprived by gentle handling (5 h/ 5 days).

9.4.1. Chemicals used

α-Asarone was freshly prepared in the base containing 5 % Tween80 and normal saline. The base containing 5 % Tween80 was taken as the vehicle. Midazolam at a dose of 2 mg/kg was taken as the positive control.

9.4.2. Procedure

After testing baseline anxiety levels, the un-implanted rats (N=20) were randomly distributed into 3 groups. First group received vehicle (N=7), second (N=8) received 10 mg/kg α-Asarone and the third (N=5) received 2 mg/kg midazolam (positive control) intra-peritoneally at 9:00 h for 5 days, before 5 hours of SD by gentle handling method. EPM test was conducted on days 1 and 4, and OFT on days 2 and 5 from 13:30 to 14:00 h (Fig. 15).

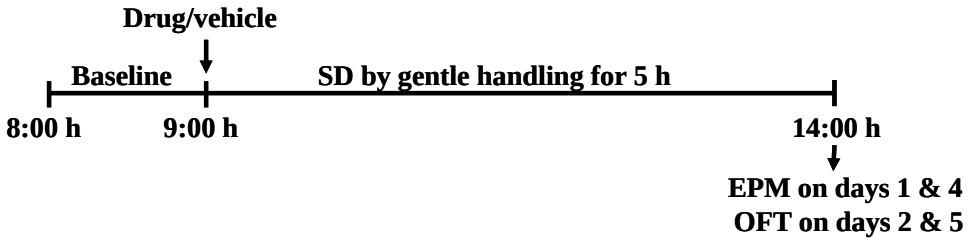

Fig. 15 Schematic representation of the objective 3.2 experimental schedule; Baseline represents pre-drug state

For EPM test, the parameters such as entries to open and the closed arm, average speed, time spent in the open and the closed arm, total distance traveled, time spent in mobility and ethologically-derived parameters like head dipping, rearing, grooming and stretch-attend posture were assessed.

For OFT, entries and time spent in the inner and outer zones were noted. Parameters including average speed, time spent in moving around, total distance traveled and ethologically-derived parameters like rearing and grooming were also measured.

9.4.3. Statistics

Shapiro-Wilk test was done to check the normality of the data. All values expressed as mean ± SEM. $P \leq 0.05$ were considered as statistically significant. All statistical analyses were done in SPSS (version 16.0). For EPM and OFT parameters, Kruskal Wallis test was done to compare among the groups and Wilcoxon Sign Rank test was used for comparison with the baseline.

9.5. Objective 3.3: To investigate the effect of optimal dose of α-Asarone on antioxidant levels in the brain of rats acutely sleep deprived by gentle handling (5 h/ 5 days).

9.5.1. Chemicals used

α-Asarone was freshly prepared in the base containing 5 % Tween80 and normal saline. The base containing 5 % Tween80 was taken as the vehicle.

9.5.2. Procedure

The un-implanted rats were randomly distributed into 5 groups. First group was left untreated and served as control (N=7). Group 2 and 3 received vehicle (N=6) and 10 mg/kg α-Asarone (N=7) respectively at 9:00 h before starting the SD procedure for five hours. Group 4 and 5 (N=5 each) received vehicle and 10 mg/kg α-Asarone respectively at 9:00 h for five consecutive days before starting the SD procedure for five hours. Animals of group 2 and 3 were decapitated after 5 h of SD and animals in group 1, 4 and 5 were decapitated after 5 days of SD using guillotine at 14:00 h (Fig. 16).

SD was done by gentle handling method. Different brain regions (Cortex, Subcortex and Brainstem) were dissected out in ice-cold saline and stored at -80 °C for the biochemical analysis of oxidative stress markers. Levels of MDA and GSH and activities of CAT, SOD, GSH-Px and GSH-R were measured.

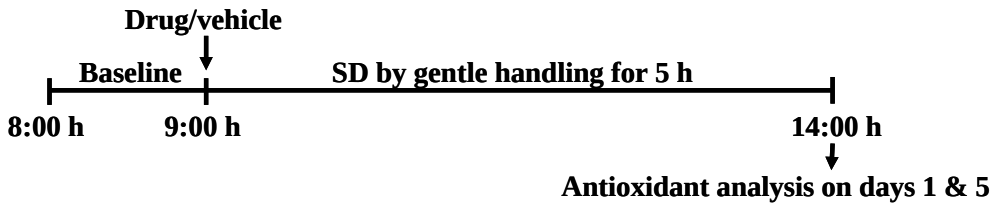

Fig. 16 Schematic representation of the objective 3.3 experimental schedule; Baseline represents pre-drug state

9.5.3. Statistics

Various parameters were compared among five groups using one way ANOVA with Tukey's post hoc test.

9.6. Objective 4: To investigate the effect of optimal dose of α-Asarone on S-W, T_{hy}, T_{body}, anxiety and brain antioxidant levels in rats chronically sleep deprived in rotating wheel (5 h/ 21 days).

9.6.1. Chemicals used

α-Asarone was freshly prepared in the base containing 5 % Tween80 and normal saline. The base containing 5 % Tween80 was taken as the vehicle. Midazolam at a dose of 2 mg/kg was taken as the positive control for S-W and anxiety tests.

9.6.2. Procedure

This study was conducted on 15 rats randomly distributed into three groups of 5 rats each. After baseline recording of all parameters for 1 h (8:00 to 9:00 h), the rats received intra-peritoneal injection of vehicle or drug at 9:00 h followed by SD for 5 h (9:00 to 14:00 h) for 21 consecutive days in rotating wheel rotating at 2.5 rpm (~0.05 m/s). The first group received vehicle, the second received 10 mg/kg α-Asarone and the third group received 2 mg/kg midazolam injection. After SD, the recording of S-W and T_{hy} and T_{body} was continued further for another 3 h (14:00 to 17:00 h) on days 1, 7, 14 and 20 (Fig. 17). This 3 h recording (recovery period) was analyzed as described below.

On days 1, 7, 14 and 20, EPM test was taken and on days 2, 8, 15 and 21, OFT was taken from all three groups at 14:00 h immediately after SD (Fig. 17). On day 21, rats which received vehicle (N=3) and α-Asarone (N=3) was

decapitated using guillotine; their brains were dissected in to 3 regions (cortex, sub-cortex and brainstem) and stored at -80 °C for antioxidant estimation (Fig. 17). Levels of MDA and GSH and activities of CAT, SOD, GSH-Px and GSH-R were measured.

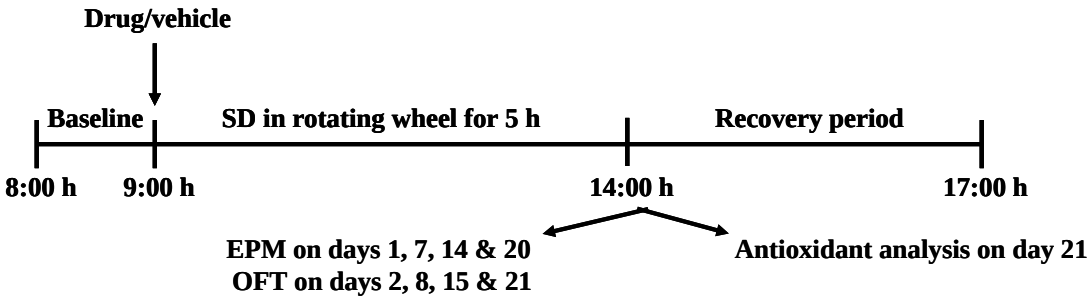

Fig. 17 Schematic representation of the objective 4 experimental schedule; Baseline represents pre-drug state

S-W of few rats which received vehicle (N=2), α-Asarone (N=2) and midazolam (N=5) were taken 24, 48 and 72 h after 21 days SD. S-W was recorded for 8 h (9:00-17:00 h) on all three days to study the withdrawal effect of drugs.

9.6.3. Analysis

The latency to sleep (time for the onset of NREM sleep) was calculated for all groups on days 1, 7, 14 and 20 of SD. Changes in the percentage time, bout duration and frequency of NREM and REM sleep and W before, during and after SD on days 1, 7, 14 and 20 were calculated to find out the differences in the effects of the drugs and the vehicle. Arousal index after the administration of vehicle and drugs were also calculated. S-W was also

analyzed 24, 48 and 72 h after termination of SD to check the withdrawal effect of drugs.

Changes in T_{hy} and T_{body} during SD and recovery period after injection of vehicle, α-Asarone and midazolam were compared with the control values obtained during the same time bin. NREM and REM sleep EEG during 3 h recovery sleep were analyzed for their spectral profile. Spectral analysis was also performed in NREM and REM sleep observed 24, 48 and 72 h after termination of SD to check the withdrawal effect of drugs

For EPM test, the parameters such as entries to open and the closed arm, average speed, time spent in the open and the closed arm, total distance traveled, time spent in mobility and ethologically-derived parameters like head dipping, rearing, grooming and stretch-attend posture were assessed. For OFT, entries and time spent in the inner and outer zones were noted. Parameters including average speed, time spent in moving around, total distance traveled and ethologically-derived parameters like rearing and grooming were also measured.

9.6.4. Statistics

All values were expressed as mean ± SEM. $P \leq 0.05$ were considered as statistically significant. All statistical analysis was done in SPSS (version 16.0). For S-W, T_{hy} and T_{body}, two-way ANOVA with repeated measures on one factor was done to compare across time bin (repeated measures) and among three groups (independent). For EPM and OFT parameters, Kruskal Wallis test was done to compare among the groups and Wilcoxon Sign Rank

test was used for comparison with the baseline. And for antioxidant analysis, various parameters measured from vehicle and α-Asarone groups were compared between and with control group using one way ANOVA with Tukey's post hoc test.

9.7. Objective 5: To understand the mechanism of action of α-Asarone by examining the relationship of T_{hy} and T_{body} with NREM and REM sleep.

Scatter plots were prepared to show the correlation between the sleep bout durations and the T_{hy} and T_{body}. The correlation of T_{hy} and T_{body} with the bout duration of NREM and REM sleep was assessed by taking bouts showing clear transition to NREM stages from W and REM sleep, and to REM stages from NREM sleep. Stages with minimum bout duration of 30 s, preceding and succeeding the transition, were only taken for analysis. T_{hy} and T_{body} during NREM and REM sleep was calculated as the percentage change from the preceding stage (taken as 100 %). Linear regression analysis was done to quantify the relationship between sleep duration and temperature after drug administration.

Chapter IV: Results

1. **Objective 1: To evaluate the effect of administration of various doses of α-Asarone on S-W, T_{hy} and T_{body} in normal rats.**

Percentage time spent in NREM and REM sleep was unaltered after administering 2 and 10 mg/kg α-Asarone (Fig. 18; Table 1). On the contrary, doses 40, 80 and 120 mg/kg α-Asarone considerably reduced the quantity of sleep (Fig. 18). Doses 80 and 120 mg/kg α-Asarone reduced the percentage NREM sleep time for 2 and 3 h respectively and a dose-dependent decrease in the percentage REM sleep time was observed after administration of 40, 80 and 120 mg/kg α-Asarone, for 3, 5 and 6 h respectively (Table 1).

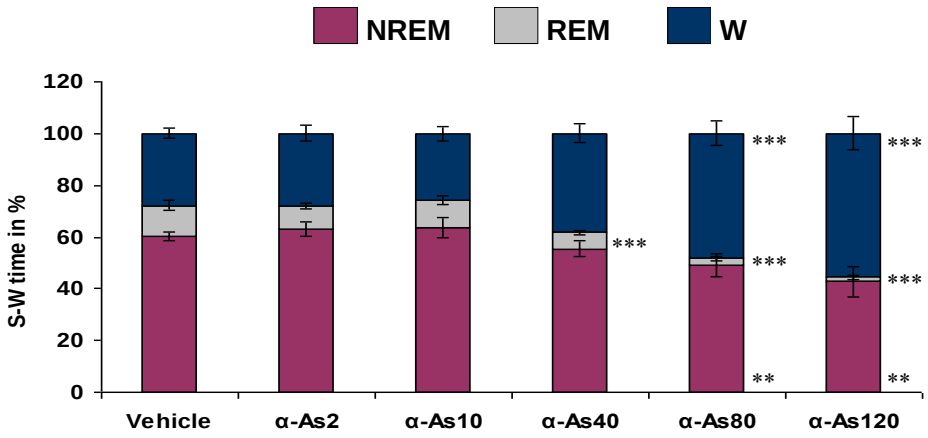

Fig. 18 Effect of various doses of α-Asarone on the percentage S-W time

Changes in the percentage time of NREM and REM sleep and W for 7 h 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 vehicle. Level of significance ** $p \leq 0.01$ and *** $p \leq 0.001$.

Time (h)	Vehicle		α-As2		α-As10		α-As40		α-As80		α-As120	
	NREM	REM	NREM	REM	NREM	REM	NREM	REM	NREM	REM	NREM	REM
1	57.8 ± 4.0	07.3 ± 2.0	58.3 ± 5.5	05.3 ± 2.3	65.0 ± 3.8	02.2 ± 1.3	40.1 ± 6.7	02.0 ± 1.3 *	27.4 ± 8.9 *	00.0 ± 0.0 *	26.9 ± 7.2 *	00.0 ± 0.0 *
2	67.6 ± 4.1	12.6 ± 3.4	70.8 ± 4.1	09.5 ± 3.3	73.9 ± 5.0	12.9 ± 4.9	52.9 ± 9.2	02.5 ± 1.7 *	37.6 ± 8.1 *	00.0 ± 0.0 *	23.2 ± 11.9 *	00.0 ± 0.0 *
3	61.6 ± 6.7	10.3 ± 2.6	64.5 ± 4.0	10.3 ± 1.0	59.1 ± 3.2	09.4 ± 2.5	50.3 ± 4.0	04.2 ± 1.6 *	54.7 ± 6.7	00.0 ± 0.0 *	35.7 ± 7.7 *	00.0 ± 0.0 *
4	59.9 ± 5.7	14.5 ± 2.7	68.0 ± 5.7	06.7 ± 1.7	70.0 ± 5.4	11.7 ± 4.5	63.7 ± 6.8	06.6 ± 2.0	54.5 ± 6.2	03.2 ± 2.2 *	44.8 ± 8.8	00.0 ± 0.0 *
5	63.4 ± 2.1	14.6 ± 2.8	58.8 ± 4.7	11.7 ± 3.3	67.6 ± 5.5	12.9 ± 2.6	62.5 ± 3.1	14.6 ± 4.9	57.6 ± 3.7	03.8 ± 2.0 *	49.6 ± 2.7	00.9 ± 0.9 *
6	52.1 ± 6.0	12.4 ± 2.9	60.7 ± 3.5	07.9 ± 1.7	59.8 ± 7.1	16.6 ± 2.4	61.3 ± 4.9	10.2 ± 1.6	54.7 ± 9.0	04.7 ± 1.8	61.0 ± 7.7	03.3 ± 2.3 **
7	59.9 ± 2.2	10.8 ± 2.0	60.5 ± 9.0	10.9 ± 3.8	49.3 ± 5.5	08.8 ± 3.2	56.9 ± 6.1	09.5 ± 1.0	56.8 ± 9.5	06.9 ± 2.0	59.0 ± 8.7	07.1 ± 3.5

Table 1 Effect of different doses of α-Asarone on the percentage NREM and REM sleep in hourly bin

Values are represented as mean ± SEM and are shown in hourly bins for 7 h (1-7) after administration of vehicle and α-Asarone (α-As) 2, 10, 40, 80, 120 mg/kg in rats (N=5). * indicates the significant difference from vehicle treatment. Level of significance * $p \leq 0.05$, ** $p \leq 0.01$.

An increase in the NREM sleep bout duration and decrease in the NREM sleep bout frequency was observed only after the administration of 10 mg/kg α-Asarone (Table 2). In order to see how long the NREM sleep promoting effect of 10 mg/kg α-Asarone persists after administration, hourly bin changes in NREM bout duration and frequency was assessed. For 2 h, the increase in NREM bout duration and decrease in NREM bout frequency were found to be significant (Fig. 19). Higher doses, especially 80 and 120 mg/kg, showed increased W bout duration and decreased NREM and REM sleep bout duration for 7 h after the administration (Table 2).

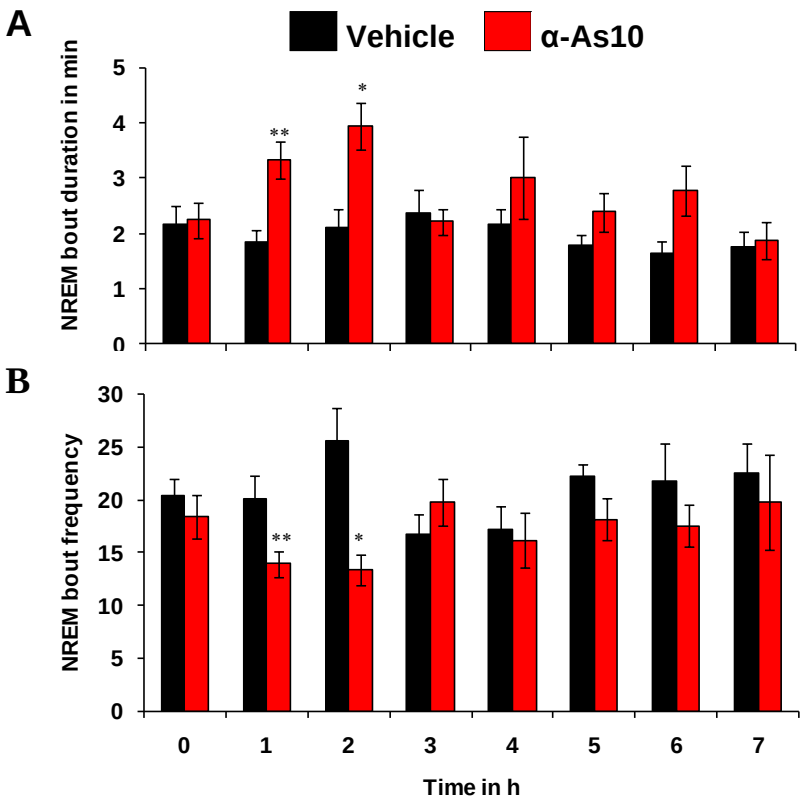

Fig. 19 Changes in the average duration and frequency of NREM sleep bouts after administration of 10 mg/kg α-Asarone and vehicle

Change in the average bout duration (A) and frequency (B) of NREM sleep after the administration of vehicle and 10 mg/kg α-Asarone (α-As10), plotted in hourly bins for 7 h (N=5). 0 represents pre-injection baseline. The bars represent mean ± SEM. * indicates the difference from vehicle. Level of significance * $p \leq 0.05$ and ** $p \leq 0.01$.

Arousal index was significantly decreased [F (1.10, 4.41)=15.63, p=0.033] at 10 mg/kg α-Asarone in comparison to the vehicle treatment (Table 2). The quality of REM sleep remained unaltered at doses 2 and 10 mg/kg α-Asarone (Table 2). Doses 80 and 120 mg/kg reduced bout durations of both NREM [F(1.83, 62.16)=5.67, p=0.007] and REM sleep [F(1.84, 62.64)=25.20, p=0.001] along with simultaneous increase in the W bout frequency [F(1.49, 50.54)=5.73, p=0.010] and reduction in the REM sleep bout frequency [F(1.98, 67.29)=21.59, p=0.000] (Table 2). Furthermore, higher doses increased arousal index with significance observed at 120 mg/kg α-Asarone (Table 2).

A significant increase in the relative delta power and decrease in the relative alpha power [F(1.804,7.218=5.477, p=0.038] was observed during the NREM sleep after administering 10 mg/kg α-Asarone in comparison to the vehicle treatment (Fig. 20A). No alteration was seen in the EEG power spectrum during REM sleep at doses 2 and 10 mg/kg α-Asarone (Fig. 20B). However, at doses 40, 80 and 120 mg/kg, a decrease in the relative theta power was observed with an increase in the relative delta power during REM sleep (Fig. 20B).

	Vehicle	α-As2	α-As10	α-As40	α-As80	α-As120
W bout duration (min)	01.3 ± 0.1	01.3 ± 0.2	01.3 ± 0.1	01.5 ± 0.2	01.7 ± 0.3	01.7 ± 0.4
NREM bout duration (min)	02.0 ± 0.2	02.2 ± 0.2	02.8 ± 0.2 **	01.7 ± 0.1	01.5 ± 0.1 **	01.3 ± 0.2 **
REM bout duration (min)	01.1 ± 0.1	01.0 ± 0.1	01.0 ± 0.2	00.7 ± 0.1 **	00.5 ± 0.1 **	00.2 ± 0.1 **
W bout frequency	13.7 ± 1.6	13.4 ± 0.8	12.1 ± 0.9	16.9 ± 1.1	19.2 ± 1.3 **	20.4 ± 4.0 **
NREM bout frequency	20.9 ± 1.7	18.7 ± 1.7	17.0 ± 1.5	21.4 ± 0.9	20.5 ± 1.6	22.7 ± 4.8
REM bout frequency	08.1 ± 2.3	06.2 ± 0.7	06.4 ± 1.9	05.3 ± 1.7	01.8 ± 0.5 ***	01.5 ± 0.9 ***
Arousal index	07.5 ± 1.2	07.0 ± 0.5	05.7 ± 0.5 *	09.7 ± 1.5	11.8 ± 1.5	18.3 ± 4.2 *

Table 2 Effect of various doses of α-Asarone on S-W quality parameters

Values are represented as mean ± SEM. * indicates the difference from vehicle. Level of significance * $p \leq 0.01$, ** $p \leq 0.01$, *** $p \leq 0.001$.

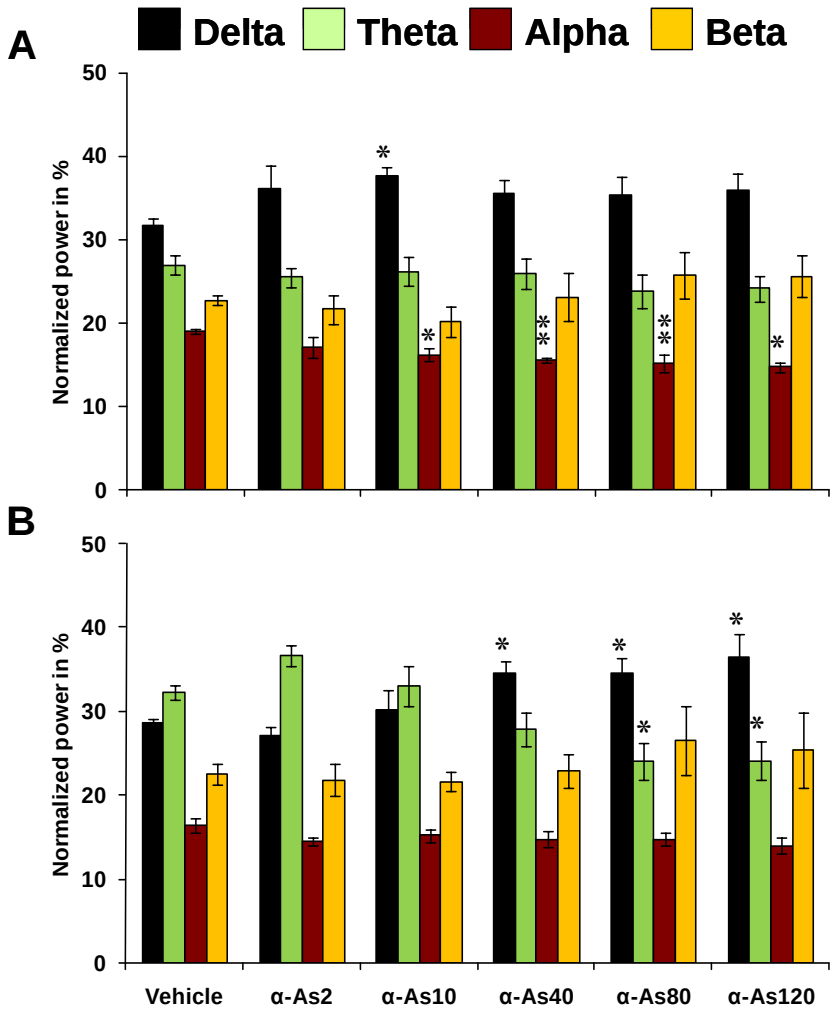

Fig. 20 EEG power spectrum at various doses of α-Asarone

The relative power in % at delta (0.5-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (12-30 Hz) frequency ranges during NREM (A) and REM (B) sleep in normal rats (N=5) administered with vehicle and 2, 10, 40, 80 and 120 mg/kg α-Asarone (α-As). The data points represents mean ± SEM. * indicates the difference from vehicle. Level of significance * $p \leq 0.05$, ** $p \leq 0.01$.

Abnormal behaviors like hyperventilation, piloerection, ataxia, crawling and jerky movements were observed after administration of 80 and 120 mg/kg

α-Asarone. At 120 mg/kg, these abnormal behaviors were accompanied by paroxysmal activity in EEG for 40.00 ± 10.79 min (Fig. 21).

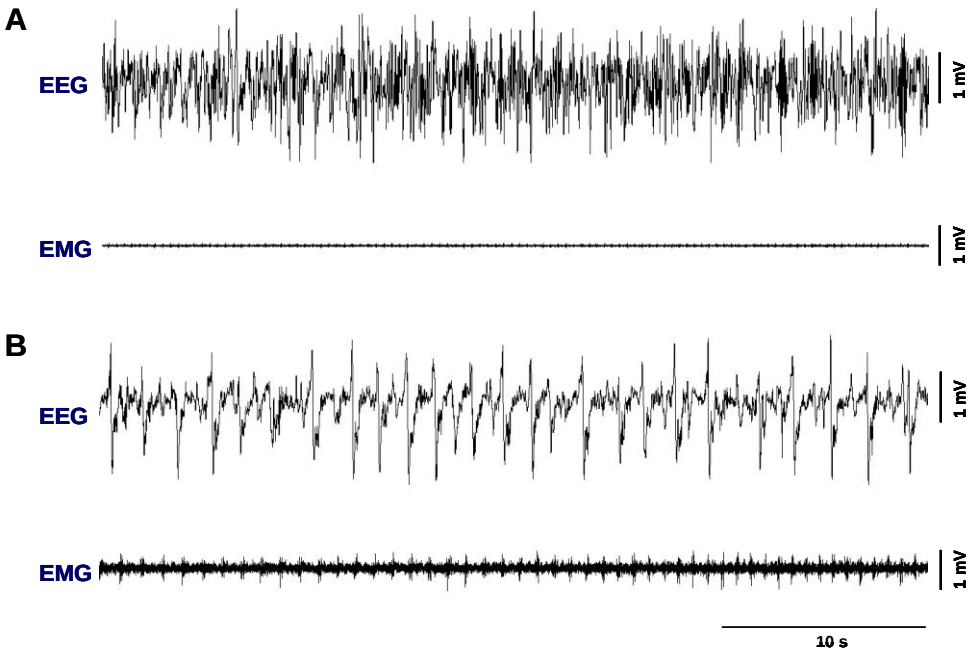

Fig. 21 Paroxysmal activity in EEG at dose 120 mg/kg α-Asarone

EEG activity observed before (A) and after (B) the administration of 120 mg/kg α-Asarone.

A dose-dependent decrease was observed in T_{hy} and T_{body} after administration of α-Asarone (10 to 120 mg/kg) with the maximum effect observed during the 2nd h after treatment (Table 3). In the 2nd h, the dose of 10 mg/kg reduced both T_{hy} and T_{body} by 0.5-0.6 ºC from the baseline and the vehicle treatment (Table 3). After administration of 40, 80 and 120 mg/kg α-Asarone, the reduction ($p=0.000$) in the T_{hy} and T_{body} was more than 1 ºC, in comparison to the vehicle group and the hypothermic response persisted for more than 5 h at doses 80 and 120 mg/kg (Table 3).

Time (h)	Vehicle		α-As2		α-As10		α-As40		α-As80		α-As120	
	T_{hy}	T_{body}	T_{hy}	T_{body}	T_{hy}	T_{body}	T_{hy}	T_{body}	T_{hy}	T_{body}	T_{hy}	T_{body}
0	37.7 ± 0.2	37.6 ± 0.2	37.5 ± 0.3	37.3 ± 0.2	37.5 ± 0.2	37.4 ± 0.2	37.7 ± 0.2	37.5 ± 0.2	37.6 ± 0.2	37.5 ± 0.2	38.0 ± 0.3	37.7 ± 0.2
1	37.7 ± 0.1	37.5 ± 0.1	37.4 ± 0.2	37.3 ± 0.2	37.2 ± 0.2 $^{\#\#}$	37.0 ± 0.2 $^{\#}$	37.2 ± 0.1 $^{\#\#}$	36.8 ± 0.2 $^{\#\#}$	36.5 ± 0.1 $^{*\#\#\#}$	36.4 ± 0.1 $^{*\#\#}$	36.6 ± 0.1 $^{**\#\#\#}$	36.7 ± 0.1 $^{*\#\#}$
2	37.5 ± 0.1	37.4 ± 0.1	37.3 ± 0.2	37.2 ± 0.1	36.8 ± 0.1 $^{*\#}$	36.8 ± 0.1 $^{*\#}$	36.5 ± 0.3 $^{**\#\#}$	36.5 ± 0.2 $^{**\#\#}$	35.8 ± 0.2 $^{**\#\#}$	35.5 ± 0.1 $^{**\#\#}$	35.9 ± 0.2 $^{**\#\#}$	35.5 ± 0.3 $^{**\#\#}$
3	37.5 ± 0.2	37.3 ± 0.1	37.4 ± 0.1	37.3 ± 0.1	37.0 ± 0.2	37.0 ± 0.1	36.8 ± 0.4 $^{\#}$	36.9 ± 0.2 $^{\#}$	35.8 ± 0.3 $^{**\#\#}$	35.6 ± 0.3 $^{**\#\#}$	36.0 ± 0.3 $^{*\#\#}$	35.6 ± 0.4 $^{*\#\#}$
4	37.6 ± 0.1	37.4 ± 0.1	37.5 ± 0.1	37.3 ± 0.1	37.0 ± 0.2	37.1 ± 0.1	37.0 ± 0.3	37.0 ± 0.1	36.1 ± 0.3 $^{**\#}$	35.9 ± 0.3 $^{**\#\#}$	36.3 ± 0.3 $^{*\#\#}$	35.9 ± 0.3 $^{*\#\#}$
5	37.6 ± 0.2	37.4 ± 0.1	37.6 ± 0.1	37.5 ± 0.1	37.2 ± 0.1	37.3 ± 0.1	37.2 ± 0.2	37.2 ± 0.2	36.6 ± 0.3 $^{*\#}$	36.4 ± 0.2 $^{*\#}$	36.6 ± 0.3 $^{*\#\#}$	36.2 ± 0.3 $^{*\#\#}$
6	37.8 ± 0.1	37.6 ± 0.1	37.7 ± 0.1	37.6 ± 0.2	37.4 ± 0.1	37.4 ± 0.1	37.5 ± 0.2	37.4 ± 0.2	36.8 ± 0.3 $^{\#}$	36.8 ± 0.2	37.0 ± 0.2 $^{*\#\#}$	36.6 ± 0.3 $^{*\#\#}$
7	37.9 ± 0.1	37.7 ± 0.2	37.6 ± 0.2	37.6 ± 0.2	37.6 ± 0.1	37.6 ± 0.2	37.6 ± 0.2	37.6 ± 0.2	37.0 ± 0.2 $^{\#}$	37.1 ± 0.1	37.3 ± 0.2 $^{\#\#}$	36.9 ± 0.2 $^{*\#\#}$

Table 3 Effect of various doses of α-Asarone on T_{hy} and T_{body} profile

Values in ° C are represented as mean ± SEM and are shown in hourly bins for 7 h (1-7) after administration of vehicle and α-Asarone (α-As) 2, 10, 40, 80, 120 mg/kg in rats (N=5). 0 represents the pre-injection baseline hour. * indicates the significant difference from vehicle, $^{\#}$ indicates the significant difference from the pre-injection baseline (0). Level of significance $^{*\#}$ $p \leq 0.05$, $^{**\#\#}$ $p \leq 0.01$, $^{\#\#\#}$ $p \leq 0.001$.

2. Objective 2: To investigate the effect of chronic administration (21 days) of optimal dose of α-Asarone on S-W, T_{hy} and T_{body} in normal rats.

Chronic administration of α-Asarone in normal rats did not bring any significant change in the percentage NREM and REM sleep in comparison to the vehicle treatment (Table 4). However, an increase in the percentage NREM sleep time was observed in the 2^{nd} h ($p=0.014$) after 3 weeks of α-Asarone treatment (Table 4). REM sleep was marginally reduced immediately after drug injection (Table 4).

A significant increase in the NREM sleep bout duration [$F(1.513, 6.051)=20.616$, $p=0.003$] and a significant decrease in the NREM sleep bout frequency [$F(1.419, 5.677)=12.429$, $p=0.011$] and arousal index [$F(1.497, 5.989)=10.337$, $p=0.014$] was observed on both days 7 and 21 after α-Asarone administration (Fig. 22).

A significant increase [$F(1.436, 5.744)=8.741$, $p=0.022$] in the relative delta power was observed during the NREM sleep on both day 7 and 21 of α-Asarone administration (Fig. 23A). A moderate non-significant decrease in the theta power was also observed during NREM sleep after α-Asarone administration (Fig. 23A). EEG power spectrum remained unaltered during the REM sleep (Fig. 23B).

	Time in W (%)			Time in NREM (%)			Time in REM (%)		
Time (h)	**Vehicle**	**Day 7**	**Day 21**	**Vehicle**	**Day 7**	**Day 21**	**Vehicle**	**Day 7**	**Day 21**
1	28.10 ± 2.7	38.00 ± 2.7	34.72 ± 4.4	67.95 ± 1.3	61.00 ± 3.2	63.11 ± 3.0	04.04 ± 1.5	01.00 ± 0.1 *	02.17 ± 2.1
2	17.84 ± 4.6	19.00 ± 6.5	08.52 ± 4.0 *	73.46 ± 4.6	75.33 ± 7.8	85.98 ± 4.8 *	08.69 ± 0.7	05.67 ± 1.7	05.50 ± 1.4
3	18.19 ± 2.1	22.78 ± 3.5	19.00 ± 6.5	70.94 ± 1.9	67.89 ± 4.5	74.11 ± 7.3	10.86 ± 1.6	09.33 ± 1.5	06.91 ± 2.3
4	24.01 ± 1.7	15.00 ± 4.0	14.67 ± 3.0	66.98 ± 2.0	77.50 ± 4.2	78.17 ± 2.3	09.06 ± 1.8	07.50 ± 1.0	07.17 ± 1.3
5	16.06 ± 1.7	29.56 ± 5.3	15.28 ± 6.6	72.73 ± 2.1	61.33 ± 4.8	77.33 ± 6.9	11.20 ± 1.5	09.11 ± 1.0	07.39 ± 0.6
6	38.37 ± 5.5	27.67 ± 6.5	17.00 ± 4.6	55.12 ± 4.3	64.28 ± 6.2	72.11 ± 2.4	06.51 ± 1.3	08.06 ± 1.4	10.89 ± 2.8
7	33.16 ± 6.1	30.17 ± 7.5	34.33 ± 7.0	59.32 ± 5.3	61.61 ± 6.9	57.33 ± 5.7	07.52 ± 1.0	08.22 ± 2.4	08.33 ± 2.0

Table 4 Effect of chronic administration of 10 mg/kg α-Asarone on percentage S-W time

The data shown is the change in percentage time spent in S-W from 10:00 to 17:00 h (7 h) after 7 and 21 days of α-Asarone 10 mg/kg administration and after vehicle administration (average of 3 recordings). Values are represented as mean ± SEM. * represent difference from the vehicle. Level of significance * $p \leq 0.05$.

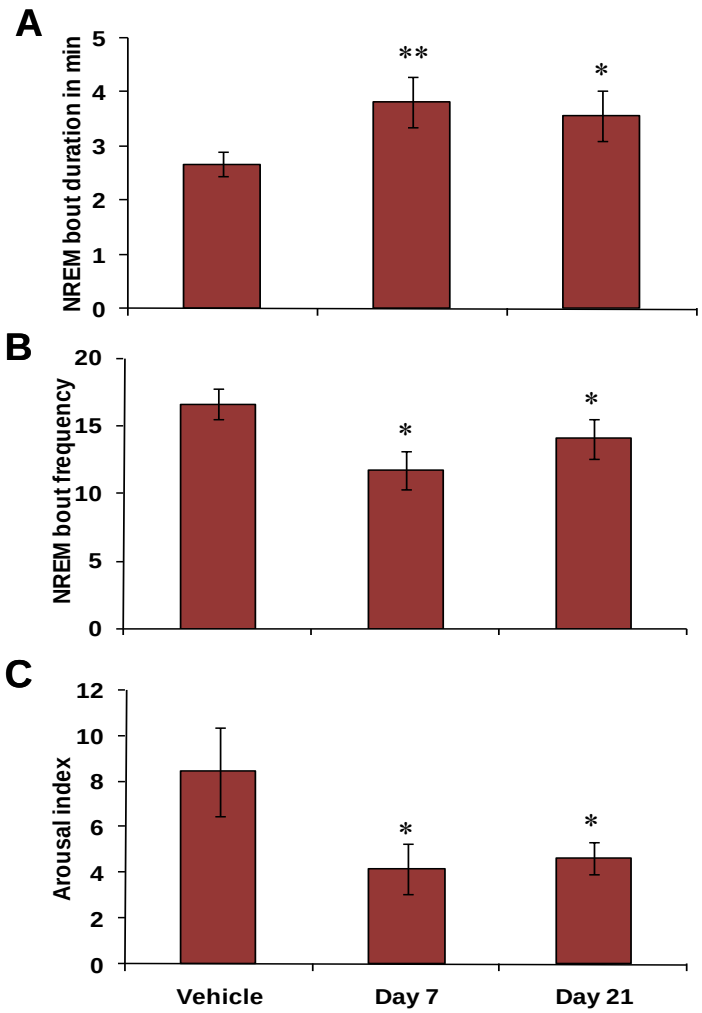

Fig. 22 Effect of chronic administration of 10 mg/kg α-Asarone on S-W quality parameters

Average NREM sleep bout duration in min (A), average NREM sleep bout frequency (B) and arousal index (C) in normal rats (N=5) administered with 10 mg/kg α-Asarone on days 7 and 21. The bars represents mean ± SEM. * indicates the difference from the vehicle group. Level of significance * $p \leq 0.05$ and ** $p \leq 0.01$.

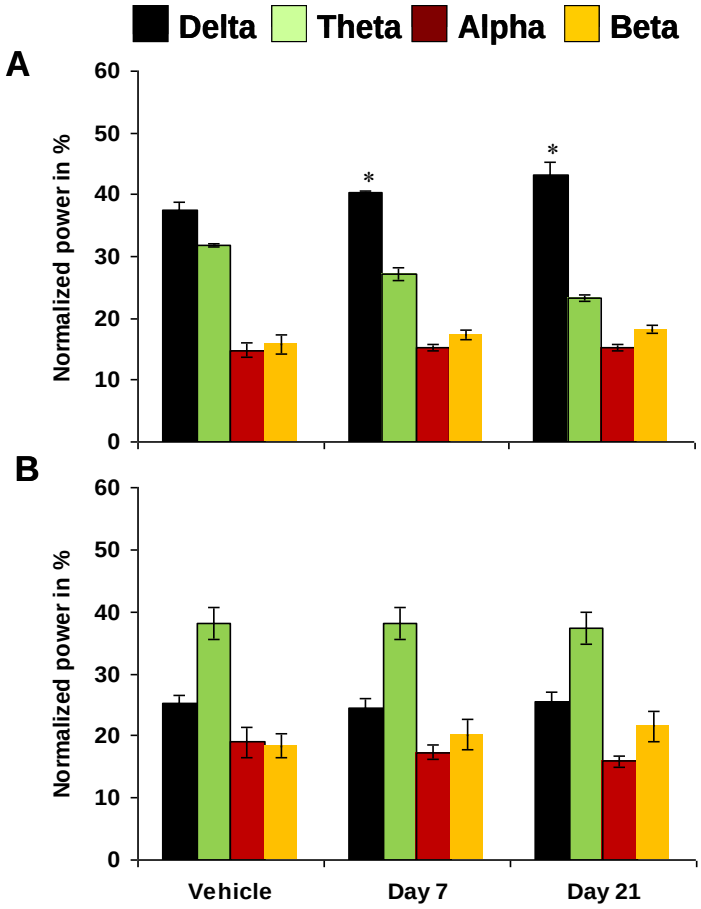

Fig. 23 Effect of chronic administration of 10 mg/kg α-Asarone on EEG power spectra of NREM and REM sleep

The relative power in % at delta (0.5-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (12-30 Hz) frequency ranges in NREM (A) and REM (B) sleep in normal rats (N=5) administered with 10 mg/kg α-Asarone on days 7 and 21. The data points represents mean ± SEM. * indicates the difference from the vehicle group. Level of significance * $p \leq 0.05$.

In comparison to the vehicle, a significant ($p<0.05$) decrease in T_{hy} and T_{body} was observed on day 7 of α-Asarone administration (Table 5). However, only a marginal reduction was observed in T_{hy} and T_{body} on day 21 of administration (Table 5).

	Vehicle		Day 7		Day 21	
Time (h)	**T_{hy}**	**T_{body}**	**T_{hy}**	**T_{body}**	**T_{hy}**	**T_{body}**
0	37.28 ± 0.1	37.09 ± 0.1	37.20 ± 0.2	36.85 ± 0.1	37.33 ± 0.2	37.04 ± 0.0
1	36.96 ± 0.1	36.96 ± 0.1	36.71 ± 0.1 $^{*\ \#}$	36.54 ± 0.2	37.00 ± 0.1	36.89 ± 0.1 $^{**\ \#}$
2	36.97 ± 0.1	36.94 ± 0.1 $^{\#}$	36.65 ± 0.1 $^{*\ \#}$	36.46 ± 0.2 $^{*\ \#}$	37.00 ± 0.1	36.84 ± 0.1 $^{\#}$
3	36.97 ± 0.1	36.95 ± 0.1	36.49 ± 0.1 $^{**\ \#\#}$	36.53 ± 0.1 $^{*\ \#\#}$	36.94 ± 0.1 $^{\#}$	36.88 ± 0.1
4	37.06 ± 0.1	37.10 ± 0.1	36.77 ± 0.1 *	36.84 ± 0.1 *	37.08 ± 0.1	37.02 ± 0.1
5	37.07 ± 0.1	37.15 ± 0.1	36.97 ± 0.1	37.02 ± 0.1	37.14 ± 0.1	37.24 ± 0.1
6	37.23 ± 0.1	37.33 ± 0.1	37.13 ± 0.1	37.06 ± 0.1	37.25 ± 0.1	37.30 ± 0.1
7	37.26 ± 0.1	37.47 ± 0.1	37.20 ± 0.1	37.30 ± 0.1	37.28 ± 0.1	37.41 ± 0.1

Table 5 Effect of chronic administration of 10 mg/kg α-Asarone on T_{hy} and T_{body} profile

The data shown is the change in T_{hy} and T_{body} (in °C) from 10:00 to 17:00 h (7 h) after 7 and 21 days of α-Asarone 10 mg/kg administration and after vehicle administration (average of 3 recordings). Values are represented as mean ± SEM. 0 represents the pre-injection baseline hour. * indicates the significant difference from vehicle and $^{\#}$ indicates the significant difference from the pre-injection baseline (0). Level of significance *, $^{\#}$ $p \leq 0.05$ and **, $^{\#\#}$ $p \leq 0.01$.

3. **Objective 3.1: To investigate the effect of optimal dose of α-Asarone on S-W, T_{hy} and T_{body} in rats acutely sleep deprived by gentle handling (5 h/ 5 days)**

SD by gentle handling for 5 h reduced sleep by 96-97 %. A significant reduction in the latency to sleep (p= 0.045) was observed in the α-Asarone group (0.4 ± 0.0 min) in comparison to the vehicle (3.5 ± 1.3 min) and the midazolam (3.4 ± 1.5 min) group by day 5 of SD. Rebound NREM sleep observed during the 3 h recovery period after SD was similar [F(2,24)=26.658, p=0.000] in all three groups (Table 6). However, in the midazolam group, REM sleep was marginally reduced [F(2,12)=3.693, p=0.05] after SD (Table 6).

α-Asarone significantly increased the NREM sleep bout duration [F(2,12)=21.809, p=0.000] simultaneously with a decrease in NREM sleep bout frequency [F(2,12)=7.314, p=0.000] in SD rats (Fig. 24A & B). Quality of REM sleep was unaltered after α-Asarone administration. Furthermore a decrease in the arousal index during the recovery period [F(2,12)=15.427, p=0.000] was observed in the α-Asarone-treated SD group in comparison to both vehicle and midazolam group (Fig. 24C).

	Time in W (%)			Time in NREM sleep (%)			Time in REM sleep (%)		
	Control	**SD day 1**	**SD day 5**	**Control**	**SD day 1**	**SD day 5**	**Control**	**SD day 1**	**SD day 5**
Veh	32.9 ± 2.1	19.9 ± 0.9 ###	20.5 ± 4.6 ###	57.1 ± 2.5	67.4 ± 1.2 ###	69.9 ± 4.0 ###	10.0 ± 1.7	12.8 ± 1.0	09.6 ± 1.1
α-As	30.5 ± 2.4	19.6 ± 1.6 ###	16.0 ± 2.8 ###	61.0 ± 2.1	71.5 ± 1.9 ###	73.6 ± 1.7 ###	08.5 ± 1.1	09.4 ± 0.8	10.5 ± 2.1
MDZ	32.1 ± 3.4	23.4 ± 3.5 ###	22.9 ± 3.5 ###	56.5 ± 2.9	69.6 ± 3.0 ###	70.0 ± 3.6 ###	11.4 ± 1.4	07.0 ± 0.9 *	07.1 ± 1.5 *

Table 6 Effect of acute SD by gentle handling on percentage S-W time after administration of vehicle (Veh), α-Asarone (α-As) and midazolam (MDZ)

The data shown is the percentage time spent in S-W for 3 h from 14:00 to 17:00 h during the recovery period on day 1 and 5 of SD and during the control recordings (average of 3 control recordings). Values are represented as mean ± SEM. * represents significant difference from the vehicle group and # represents significant difference of each group from their respective control values. Level of significance * $p \leq 0.05$, ### $p \leq 0.001$.

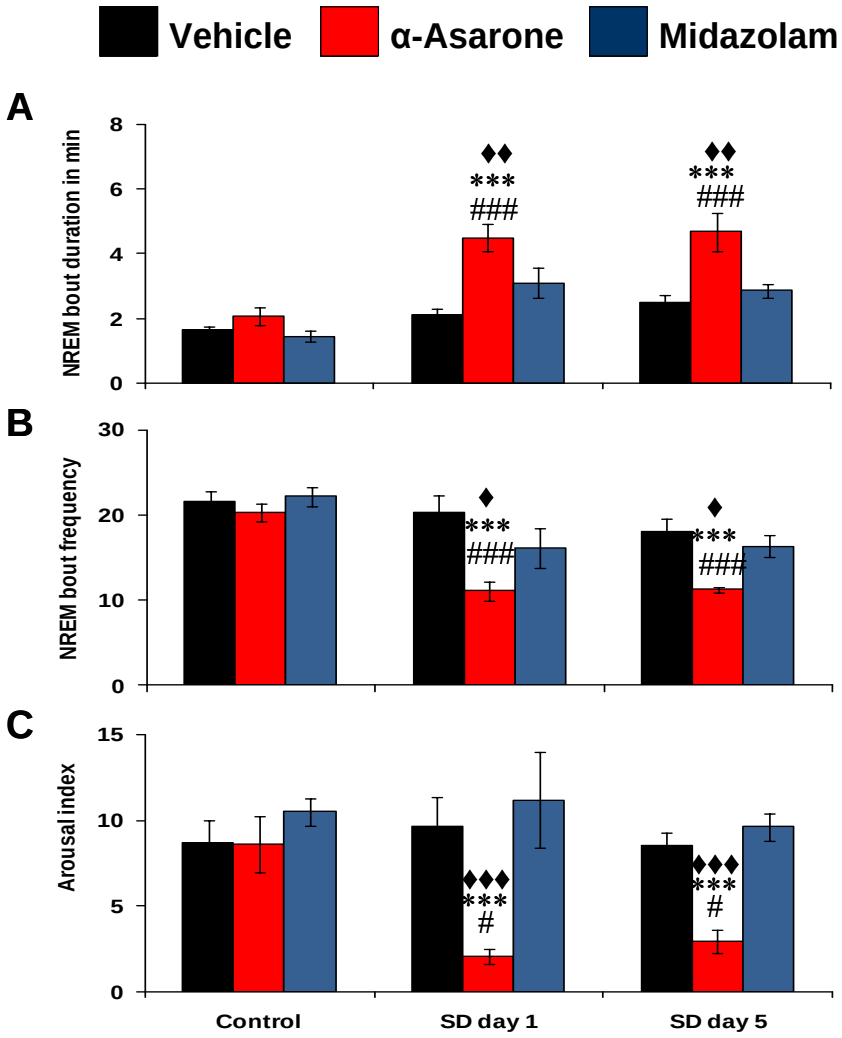

Fig. 24 Effect of acute SD by gentle handling on S-W quality parameters after administration of vehicle, α-Asarone and midazolam

Average NREM sleep bout duration in min (A), average NREM sleep bout frequency (B) and arousal index (C) during recovery period in SD rats (N=15) treated with vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam on day 1 and 5. The bars represents mean ± SEM. * indicates the difference from the vehicle group, # indicates the bin-wise difference from their respective control value of same time bin and ♦ indicates the difference from the midazolam group. Level of significance # ♦ $p \leq 0.05$, ♦♦ $p \leq 0.01$ and *** ### ♦♦♦ $p \leq 0.001$. N=5 for each group.

In comparison to the vehicle and midazolam group, a significant increase in the relative delta power during the NREM sleep was observed for the entire 3 h recovery period [$F(2,12)=15.954$, $p=0.000$] in the α-Asarone group after 5 days of SD (Fig. 25A). Furthermore, relative alpha [$F(2,12)=4.018$, $p=0.046$] as well as beta power [$F(2,12)=5.690$, $p=0.018$] were significantly reduced after α-Asarone administration in comparison to the midazolam group during the recovery period (Fig. 25A). Power spectral profile was unaltered during REM sleep (Fig. 25B).

Furthermore, during the 1 h baseline (8:00-9:00 h), 23 h after the fourth drug injection (before beginning the fifth day of SD), a decrease in the relative delta power [$F(2,12)=4.763$, $p=0.031$] and increase in the relative beta power [$F(2,12)=4.949$, $p=0.027$] was observed in the midazolam group in comparison to the α-Asarone and vehicle groups and from its own control (Fig. 26).

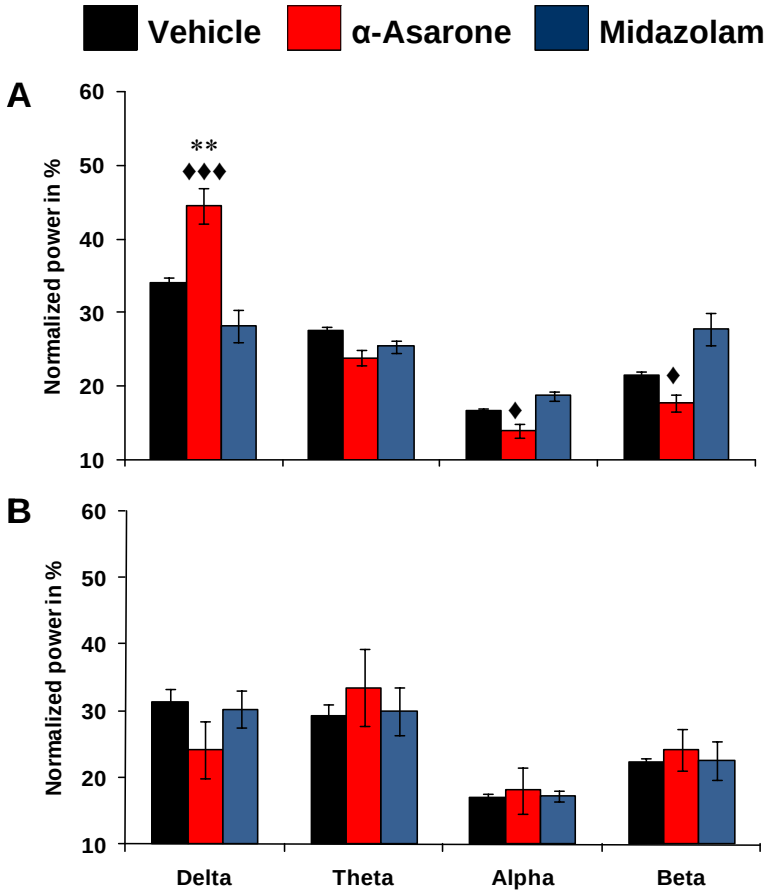

Fig. 25 Effect of acute SD by gentle handling on EEG power spectra of NREM and REM sleep after administration of vehicle, α-Asarone and midazolam

The relative power in % at delta (0.5-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (12-30 Hz) frequency ranges in NREM (A) and REM (B) sleep during the 3 h recovery period after 5 days of SD in rats administered with vehicle, α-Asarone 10 mg/kg and midazolam 2mg/kg. The data points represents mean ± SEM. * indicates the difference from the vehicle group and ♦ indicates the difference from the midazolam group. Level of significance ♦ $p \leq 0.05$, ** $p \leq 0.01$ and ♦♦♦ $p \leq 0.001$. N=5 for each group.

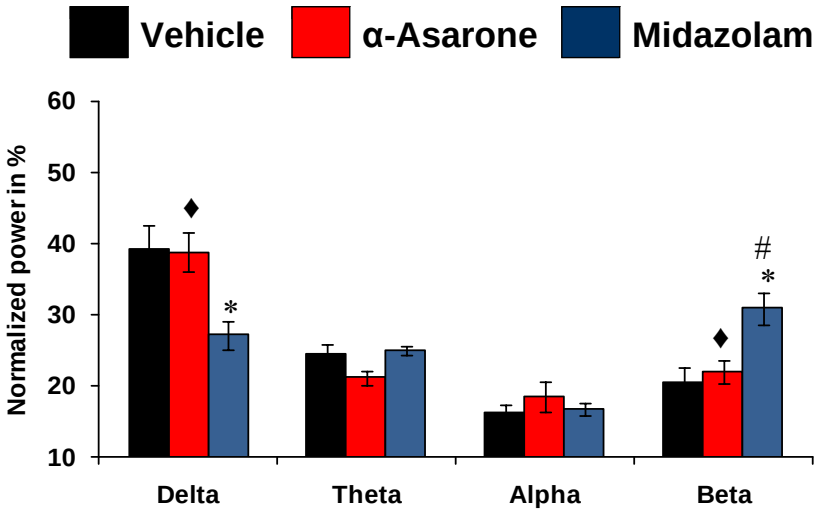

Fig. 26 EEG power spectra of NREM sleep after 23 h of fourth injection of vehicle, α-Asarone and midazolam: Withdrawal effect

The relative power in % at delta (0.5-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (12-30 Hz) frequency ranges in NREM sleep during the 1 h (8:00-9:00 h) after 23 h of fourth injection of vehicle, α-Asarone 10 mg/kg and midazolam 2mg/kg. The data points represents mean ± SEM. * indicates the difference from the vehicle group, # indicates the difference from their respective control value and ♦ indicates the difference from the midazolam group. Level of significance * # ♦ $p \leq 0.05$. N=5 for each group.

T_{hy} and T_{body} during SD on day 1 and 5 was consistently higher (p=0.000) in all the three groups in comparison to control (Fig. 27). However, after SD, during the recovery period, the T_{hy} was lower (p=0.011) in α-Asarone and midazolam groups. T_{body}, on the other hand, still remained higher in the midazolam group during the recovery period (Fig. 27). Also, T_{body} was significantly higher ($p<0.01$) than T_{hy} in the midazolam-treated SD group during the recovery period on day 1 and 5 (Fig. 27).

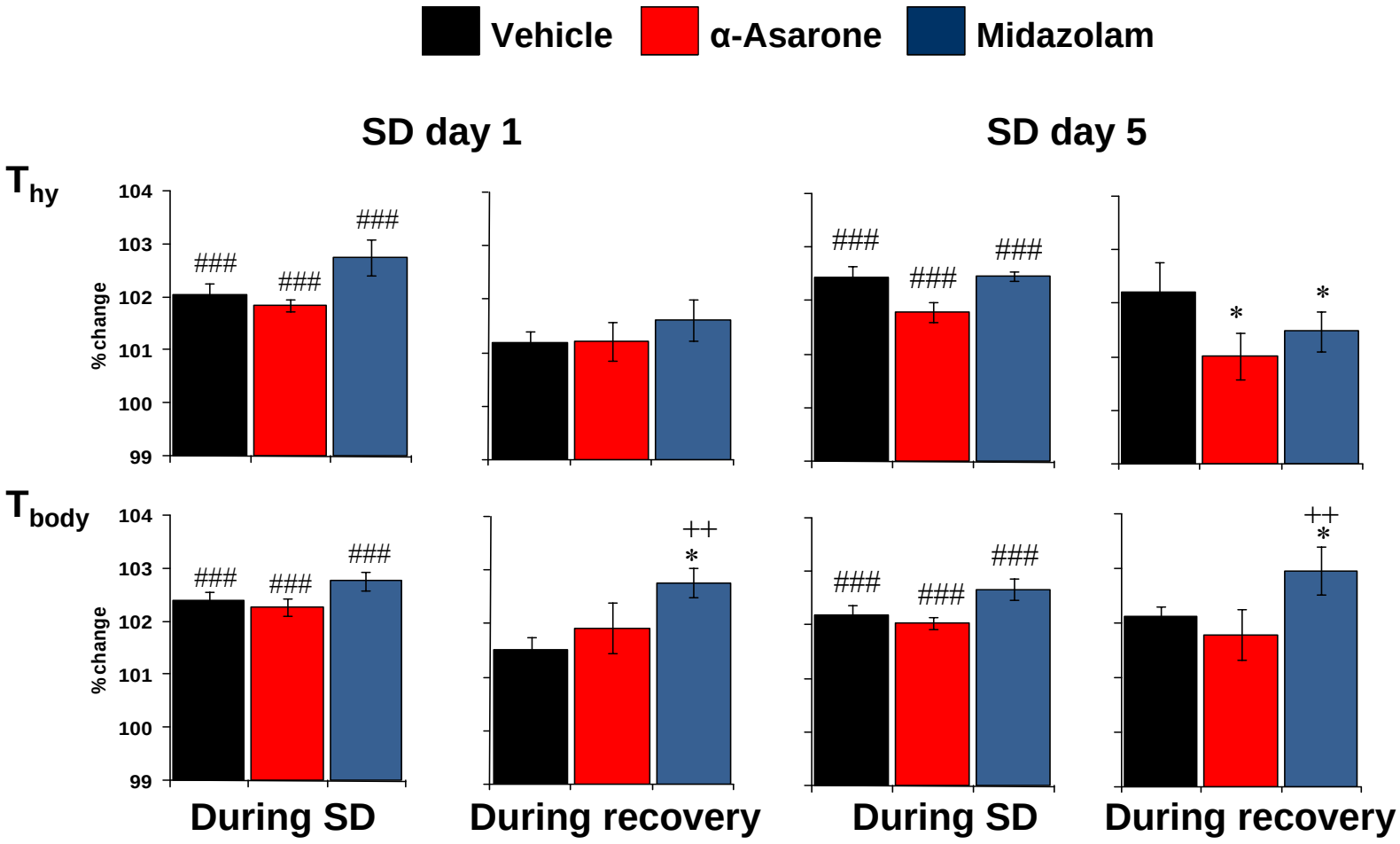

Fig. 27 Effect of acute SD by gentle handling on T_{hy} and T_{body} profile after administration of vehicle, α-Asarone and midazolam

Change in T_{hy} and T_{body} during SD (5 h) and recovery period (3 h) in rats treated with vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam on day 1 and 5 represented as the percentage change from the control values of the same time bin (taken as 100 %). The data is represented as mean ± SEM. * represents bin-wise significant difference from the vehicle group, $^{\#}$ represents the difference from the control values taken as 100 %, and $^{+}$ represents difference between T_{hy} and T_{body}. Level of significance * $p \leq 0.05$, $^{++}$ $p \leq 0.01$ and $^{\#\#\#}$ $p \leq 0.001$. N=5 for each group.

4. **Objective 3.2: To investigate the effect of optimal dose of α-Asarone on anxiety in rats acutely sleep deprived by gentle handling (5 h/ 5 days).**

In EPM test, the time spent and entries in the open arm after SD was increased by day 4 in α-Asarone-treated rats (Fig. 28 and Table 7). An improvement in the distance travelled and the average speed of mobility was also observed in α-Asarone group in comparison to the vehicle group (Fig. 28 and Table 7). The effects of α-Asarone on most of the parameters were on par with that of midazolam in the sleep deprived rats (Fig. 28 and Table 7). In OFT, the α-Asarone-treated rats entered the inner zone more frequently even after five days of SD (Fig. 29 and Table 8).

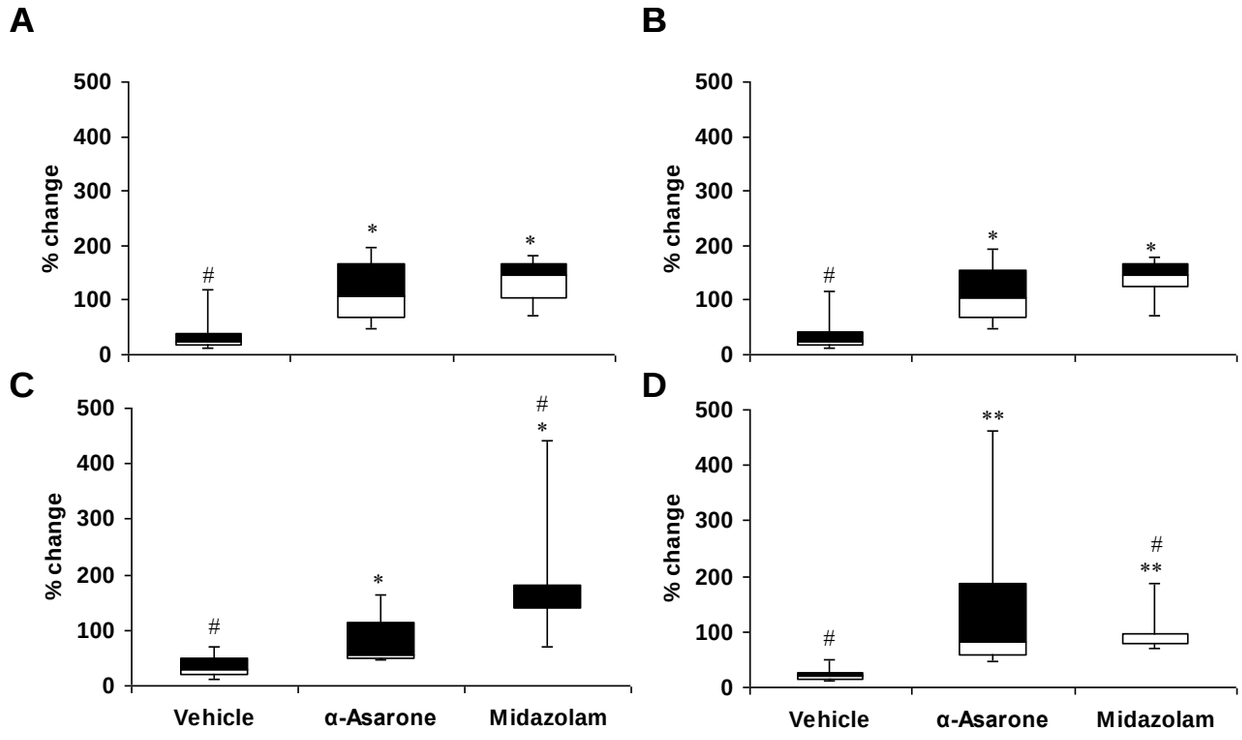

Fig. 28 Effect of acute SD by gentle handling on EPM test parameters after administration of vehicle, α-Asarone and midazolam

Change in the EPM parameters on day 4 in SD rats that received vehicle, α-Asarone and midazolam. The box-whisker plot shows the total distance traveled (A), average speed (B), the entries into the open arms (C) and the time spent in the open arms (D) on day four of SD as compared to the control values (taken as 100 %) taken before SD. * indicates significant change from vehicle group and # indicates significance from the control. Levels of significance *, # $p \leq 0.05$ and ** $p \leq 0.01$. N= 7 for vehicle group, N= 8 for α-Asarone group and N= 5 for midazolam group.

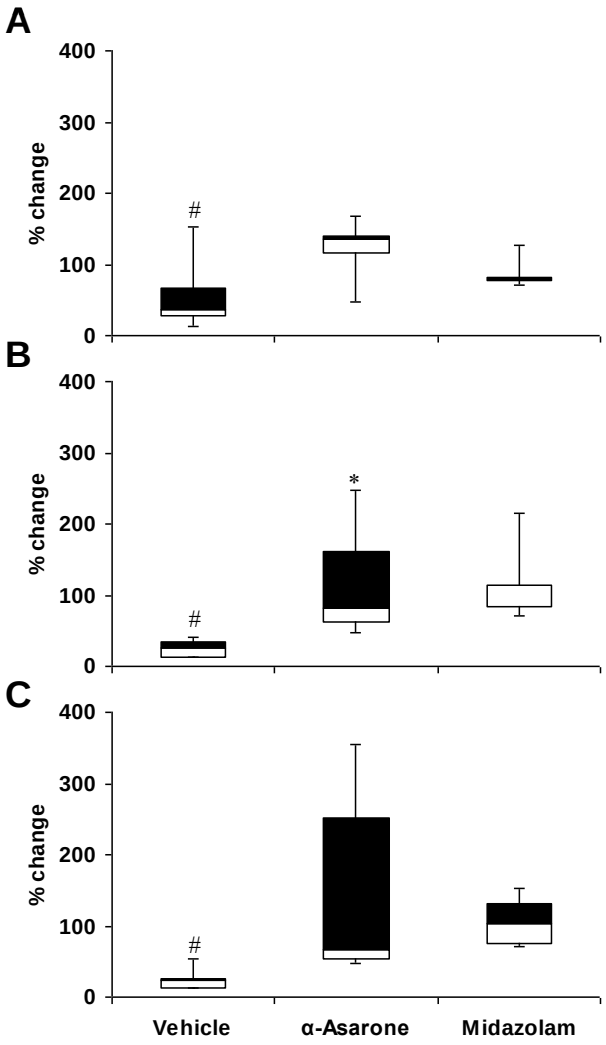

Fig. 29 Effect of SD by gentle handling on OFT parameters after administration of vehicle, α-Asarone and midazolam

Change in the OFT parameters on day 5 in SD rats that received vehicle, α-Asarone and midazolam. The box-whisker plot shows the time spent in the outer zone (A), entries in to inner zone (B) and the time spent in the inner zone (C) on day five of SD as compared to the control values (taken as 100 %) taken before SD. * indicates significant change from vehicle group and # indicates significance from the control. Levels of significance *, # $p \leq 0.05$. N= 7 for vehicle group, N= 8 for α-Asarone group and N= 5 for midazolam group.

	Vehicle			α-Asarone			Midazolam		
Parameters	**Control**	**SD day 1**	**SD day 4**	**Control**	**SD day 1**	**SD day 4**	**Control**	**SD day 1**	**SD day 4**
Total distance traveled (m)	7.3 ± 1.7	5.0 ± 1.3	2.1 ± 1.1 #	9.0 ± 1.1	5.2 ± 0.7 #	7.1 ± 1.3 *	7.2 ± 1.3	5.7 ± 0.8	6.7 ± 1.0 *
Average speed (m/s)	0.02 ± 0.0	0.01 ± 0.0	0.01 ± 0.0 #	0.03 ± 0.0	0.02 ± 0.0 #	0.02 ± 0.0 *	0.02 ± 0.0	0.02 ± 0.0	0.02 ± 0.0 *
Time mobile (s)	93.3 ± 20.1	82.1 ± 26.9	40.8 ± 17.5	113.1 ± 26.0	51.1 ± 7.4	52.0 ± 8.8 #	72.9 ± 18.2	53.5 ± 5.7	49.9 ± 8.5
Time spent in open arms (s)	38.0 ± 11.9	5.0 ± 2.1 #	5.1 ± 3.9 #	31.5 ± 12.7	15.2 ± 4.4 ♦	23.9 ± 4.2 **	22.6 ± 4.4	30.2 ± 3.9	29.8 ± 4.8 ** #
Time spent in closed arms (s)	165.7 ± 36.6	213.4 ± 35.4	144.1 ± 48.9	233.7 ± 16.2	250.1 ± 9.1	231.2 ± 21.3	247.4 ± 4.7	242.8 ± 4.3	248.9 ± 5.1
Time spent in center zone (s)	96.3 ± 34.3	81.6 ± 35.7	150.8 ± 50.6	34.8 ± 7.7	34.7 ± 5.9	45.0 ± 19.5	36.1 ± 7.2	33.0 ± 7.1	21.2 ± 3.3
No. of entries in open arms	7.0 ± 1.5	3.1 ± 1.3	1.4 ± 0.5 #	7.8 ± 1.9	4.3 ± 0.9	04.6 ± 1.0 *	4.2 ± 1.6	5.8 ± 1.9 #	8.6 ± 1.9 * #
No. of entries in closed arms	8.6 ± 1.8	12.0 ± 2.6	6.3 ± 1.8	12.6 ± 1.5	9.4 ± 1.6	9.1 ± 1.4 #	13.8 ± 3.6	15.2 ± 3.2	14.8 ± 3.4

Table 7 Values of all parameters of EPM after administration of α-Asarone, midazolam and vehicle in acutely SD rats

The data is represented as mean ± SEM. # indicates significance from the control, * indicates significance between vehicle and α-Asarone group on days 1 and 4 of SD and ♦ indicates significance between α-Asarone and midazolam group on days 1 and 4 of SD. Levels of significance *, #, ♦ $p \leq 0.05$ and ** $p \leq 0.01$. N= 7 for vehicle group, N= 8 for α-Asarone group and N=5 for midazolam group.

	Vehicle			α-Asarone			Midazolam		
Parameters	**Control**	**SD day 2**	**SD day 5**	**Control**	**SD day 2**	**SD day 5**	**Control**	**SD day 2**	**SD day 5**
Total distance travelled (m)	28.8 ± 3.6	17.5 ± 4.5 ##	11.8 ± 3.0 ##	25.6 ± 3.7	21.7 ± 4.0	13.3 ± 3.2 ##	23.2 ± 4.6	26.3 ± 4.1	22.6 ± 4.6
Average speed (m/s)	0.1 ± 0.0	0.1 ± 0.0 ##	0.0 ± 0.0 ##	0.1 ± 0.01	0.1 ± 0.0	0.0 ± 0.0 ##	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Time mobile (s)	208.8 ± 18.2	114.3 ± 19.7 ##	87.1 ± 18.0 ##	186.4 ± 21.9	146.4 ± 20.3	103.2 ± 18.9 ##	163.5 ± 41.1	172.3 ± 25.9	155.6 ± 36.0
Time spent in outer zone (s)	222.5 ± 20.7	276.3 ± 4.0 #	289.1 ± 5.0 #	254.6 ± 10.5	267.1 ± 7.4	245.9 ± 29.3	266.8 ± 20.2	277.9 ± 8.9	285.6 ± 6.3
Time spent in inner zone (s)	28.6 ± 14.8	7.6 ± 1.8	1.9 ± 0.9 #	17.7 ± 6.7	11.4 ± 3.2	16.4 ± 9.7	14.9 ± 10.0	3.8 ± 2.6	2.2 ± 0.70
No. of entries in outer zone	18.0 ± 3.2	10.3 ± 2.6	5.7 ± 1.6 #	14.3 ± 1.6	11.0 ± 2.7	8.5 ± 1.5 #	10.0 ± 2.8	12.2 ± 2.4	8.4 ± 2.8
No. of entries in inner zone	11.1 ± 2.5	5.3 ± 1.2	1.0 ± 0.4 #	8.4 ± 1.7	6.4 ± 1.8	4.6 ± 1.9 *	11.8 ± 8.6	3.4 ± 1.4	2.0 ± 0.4

Table 8 Values of all parameters of OFT after administration of α-Asarone, midazolam and vehicle in acutely SD rats.

The data is represented as mean ± SEM. # indicates significance from the control and * indicates significance between vehicle and α-Asarone group on day 2 and 5 of SD.

Levels of significance *, # $p \leq 0.05$ and ## $p \leq 0.01$. N= 7 for vehicle group, N= 8 for α-Asarone group and N=5 for midazolam group.

5. Objective 3.3: To investigate the effect of optimal dose of α-Asarone on antioxidant levels in the brain of rats acutely sleep deprived by gentle handling (5 h/ 5 days).

5.1. MDA levels

The MDA levels in the brainstem as well as the subcortex lowered after 5 days of SD (Table 9). In comparison to the vehicle treated rats, the MDA levels in the subcortex decreased on day 1 and 5 in SD rats when treated with α-Asarone (Table 9). In brainstem, the MDA levels decreased in α-Asarone treated group only after 5 days of SD (Table 9). No changes were observed in the MDA levels in cortex in any groups after SD (Table 9).

5.2. CAT activity

CAT activity was significantly decreased in the cortex and subcortex of the vehicle treated group after 5 days of SD in comparison to the control group (Table 9). In comparison to the vehicle treated rats, the CAT activity in the subcortex and the brainstem region increased on day 1 in SD rats when treated with α-Asarone (Table 9). In cortex, no changes were observed in the CAT activity in any groups after SD (Table 9).

5.3. GSH-R activity

GSH-R activity was significantly increased after 1 and 5 days of SD in the cortex of vehicle treated group in comparison to the control (Table 9). In the α-Asarone treated rats, GSH-R activity was increased only in the subcortical

region after 5 days of SD. GSH-R activity in none of the other areas was affected by α-Asarone treatment.

5.4. SOD activity

In the subcortical region, a significant increase in the SOD activity was observed after 5 h of SD in rats treated with vehicle in comparison to the untreated control (Table 9). No change was observed in SOD activity after α-Asarone administration in SD groups on day 1 or 5 (Table 9).

5.5. GSH-Px activity

In cortex and subcortex, in comparison to the untreated control, the GSH-Px activity was decreased on day 1 and increased on day 5 of SD (Table 9). No change was observed in GSH-Px activity after α-Asarone administration in SD groups on day 1 or 5 (Table 9).

5.6. GSH levels

No changes were observed in the GSH levels after α-Asarone administration in SD groups on day 1 or 5 (Table 9).

	CORTEX					SUBCORTEX					BRAINSTEM				
		SD day 1		SD day 5			SD day 1		SD day 5			SD day 1		SD day 5	
	Control	Veh	α-As	Veh	α-As	Control	Veh	α-As	Veh	α-As	Control	Veh	α-As	Veh	α-As
MDA (nmol/mg protein)	6.0 ± 0.4	5.0 ± 0.4	5.2 ± 0.3	5.3 ± 0.5	4.9 ± 1.3	3.8 ± 0.2	4.1 ± 0.1	3.8 ± 0.1 *	0.9 ± 0.1 ###	0.6 ± 0.0 * ###	3.1 ± 0.9	5.3 ± 0.6 #	4.2 ± 0.6	1.6 ± 0.6 ###	0.8 ± 0.1 * ##
CAT (U/mg protein)	1.7 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.1 ± 0.1 #	1.5 ± 0.1	1.4 ± 0.1	1.1 ± 0.0 #	1.3 ± 0.1 *	1.0 ± 0.1 #	2.4 ± 0.3 * #	3.1 ± 0.3	2.0 ± 0.2 #	3.1 ± 0.5 *	3.1 ± 0.2	2.7 ± 0.1
GSH-R (U/mg protein)	2.6 ± 0.4	0.8 ± 0.3 ##	1.3 ± 0.3 ##	4.6 ± 0.2 ##	4.5 ± 0.5 #	1.9 ± 0.4	1.3 ± 0.4	1.4 ± 0.2	2.8 ± 0.4	3.6 ± 0.3 * ##	1.6 ± 0.2	1.6 ± 0.2	1.4 ± 0.2	2.3 ± 0.3	2.2 ± 0.1
SOD (U/mg protein)	11.6 ± 1.9	11.2 ± 0.8	10.1 ± 1.5	15.8 ± 2.3 #	13.7 ± 0.6	8.7 ± 0.3	15.0 ± 1.4 #	12.8 ± 0.9	11.2 ± 1.4 #	6.5 ± 0.5 *	10.1 ± 1.9	13.2 ± 1.5	11.8 ± 1.4	8.4 ± 0.6	7.9 ± 0.5
GSH-Px (U/mg protein)	0.03 ± 0.0	0.01 ± 0.0 ##	0.02 ± 0.0 #	0.08 ± 0.0 ##	0.07 ± 0.0 ##	0.02 ± 0.0	0.01 ± 0.0 #	0.01 ± 0.0 #	0.05 ± 0.0 #	0.04 ± 0.0 ##	0.02 ± 0.0	0.02 ± 0.0	0.01 ± 0.0	0.05 ± 0.0	0.03 ± 0.0
GSH (nmol/mg protein)	103.1 ± 35.3	106.11 ± 34.2	120.9 ± 37.8	91.4 ± 17.8	68.1 ± 5.8 #	187.0 ± 11.1	199.2 ± 5.5	187.8 ± 7.4	282.2 ± 38.1	204.1 ± 6.6	357.9 ± 29.8	402.8 ± 13.4	356.6 ± 15.4	310.7 ± 52.9	306.4 ± 33.5

Table 9 Effect of acute SD by gentle handling on antioxidant levels after administration of vehicle and α-Asarone

# indicates significance from untreated control and * indicates significance between vehicle (Veh) and α-Asarone (α-As) group. Levels of significance *, # $p<0.05$, ## $p<0.01$ and ### $p<0.001$.

N= 7 for Control, N=6 for Vehicle SD day 1, N=7 for α-Asarone SD day 1, N=5 for Vehicle SD day 5 and N= 5 for α-Asarone SD day 5.

6. Objective 4: To investigate the effect of optimal dose of α-Asarone on S-W, T_{hy}, T_{body}, anxiety and brain antioxidant levels in rats chronically sleep deprived in rotating wheel (5 h/ 21 days).

6.1. Effect of optimal dose of α-Asarone on S-W, T_{hy} and T_{body} after chronic SD in rotating wheel.

In comparison to the vehicle group, a significant reduction in the latency to sleep was observed in the α-Asarone group by day 14 and 20 of SD (Fig. 30).

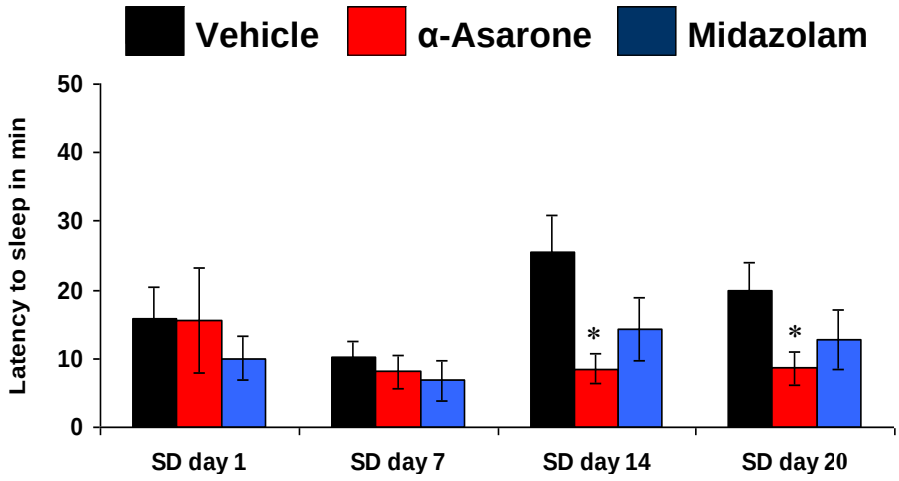

Fig. 30 Effect of chronic SD in rotating wheel on latency to sleep after administration of vehicle, α-Asarone and midazolam

Latency to sleep in min during the 3 h recovery period after 1, 7, 14 and 20 days of SD for 5 h in rats administered with vehicle, α-Asarone 10 mg/kg and midazolam 2 mg/kg. The data is represented as mean ± SEM. * represents significant difference from the vehicle group. Level of significance * $p \leq 0.05$. N=5 for each group.

	Vehicle			α-Asarone			Midazolam		
	NREM	**REM**	**W**	**NREM**	**REM**	**W**	**NREM**	**REM**	**W**
Control	59.5 ± 0.9	10.3 ± 2.1	30.3 ± 2.3	62.6 ± 2.9	10.4 ± 1.5	25.8 ± 3.1	56.6 ± 4.3	12.6 ± 1.0	30.8 ± 4.4
SD day 1	67.5 ± 1.9 #	09.9 ± 1.8	24.9 ± 2.9	74.1 ± 3.1 ##	06.2 ± 1.4 #	21.1 ± 4.1	74.8 ± 2.1 #	09.4 ± 2.4	15.8 ± 3.7 #
SD day 7	68.4 ± 1.7 ##	09.1 ± 2.1	23.0 ± 2.9 #	70.9 ± 2.0 ##	08.0 ± 1.3	23.2 ± 3.3	66.5 ± 3.8 #	05.8 ± 1.2 ##	27.7 ± 4.5
SD day 14	58.7 ± 1.2	11.1 ± 1.9	30.6 ± 2.7	70.2 ± 3.5 # *	05.9 ± 0.6	24.5 ± 3.6	63.4 ± 3.3	07.6 ± 1.2 ##	29.1 ± 3.5
SD day 20	61.0 ± 2.1	09.2 ± 2.3	30.1 ± 2.3	70.2 ± 2.9 # *	07.6 ± 1.5	23.7 ± 2.7	58.9 ± 4.5	07.6 ± 1.1 ##	33.6 ± 4.9

Table 10 Effect of chronic SD in rotating wheel on percentage S-W time after administration of vehicle, α-Asarone and midazolam

The data is represented as mean ± SEM. # indicates significance from the control and * indicate significance from vehicle group. Levels of significance # * $p \leq 0.05$ and ## $p \leq 0.01$.

N= 5 for all groups.

Table 10 shows the change in S-W in SD rats administered with vehicle, α-Asarone and midazolam. In comparison to the vehicle and midazolam group, α-Asarone significantly increased the NREM sleep bout duration simultaneously with a decrease in NREM sleep bout frequency on all days of SD (Fig. 31A & B). Quality of REM sleep was unaltered after α-Asarone administration. Furthermore a decrease in the arousal index during the recovery period was observed in the α-Asarone-treated SD group on days 7, 14 and 20 in comparison to both vehicle and midazolam group (Fig. 31C).

A significant increase in the relative delta power during the NREM sleep was observed for the entire 3 weeks in the α-Asarone group (Fig. 32A). Furthermore the relative delta power was significantly lower in the midazolam group (Fig. 32A). Similar to the vehicle group, the relative theta power during NREM sleep was reduced in the α-Asarone group (Fig. 32B). Theta power during NREM sleep was always higher in the midazolam group (Fig. 32B). By day 20, relative theta power in both α-Asarone and midazolam group NREM sleep increased in comparison to the vehicle group (Fig. 32B). Reduction in the relative alpha and beta power was also observed in the α-Asarone group (Fig. 32C&D). However, in the midazolam group, both alpha as well as beta power was significantly higher on all days of SD (Fig. 32C&D). REM sleep EEG spectrum remained unaltered after 3 weeks of SD.

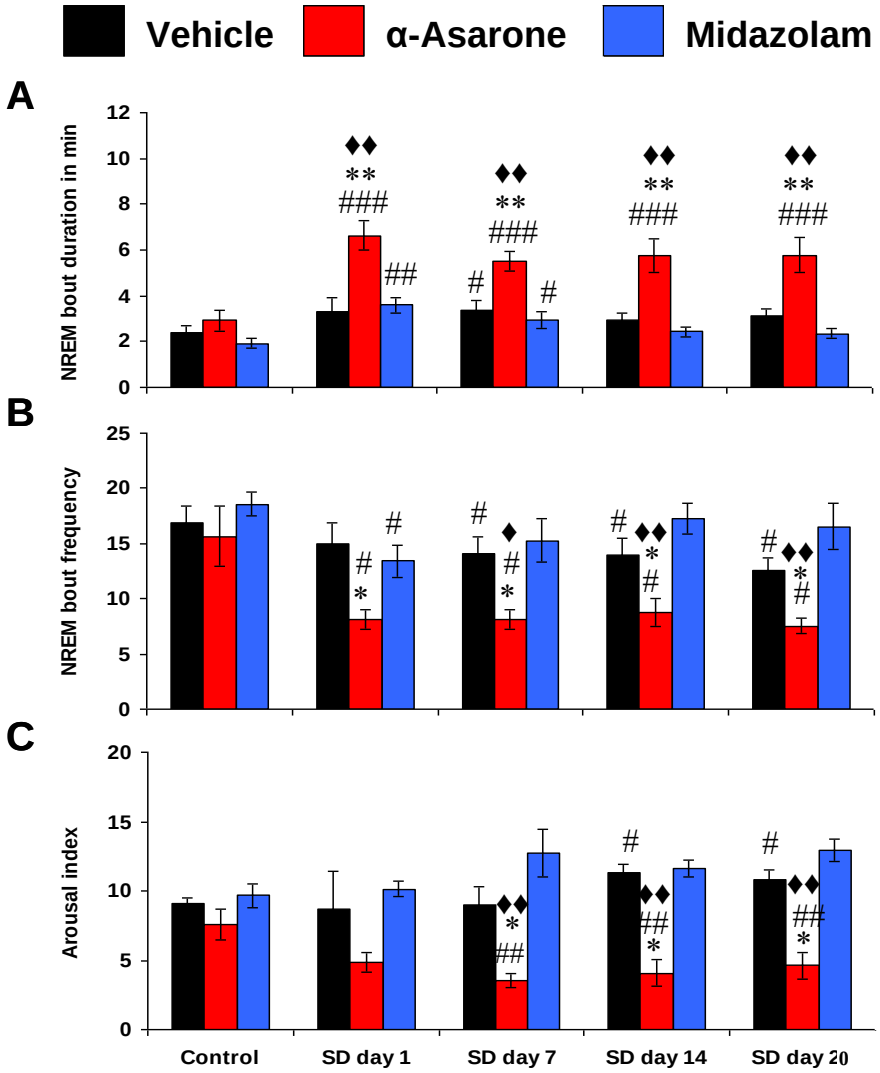

Fig. 31 Effect of chronic SD in rotating wheel on S-W quality parameters after administration of vehicle, α-Asarone and midazolam

Average NREM sleep bout duration in min (A), average NREM sleep bout frequency (B) and arousal index (C) during recovery period in SD rats treated with vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam on day 1, 7, 14 and 20. The bars represents mean ± SEM. * indicates the difference from the vehicle group, # indicates the bin-wise difference from their respective control value of same time bin and ♦ indicates the difference from the midazolam group. Level of significance # ♦ $p \leq 0.05$, ♦♦ $p \leq 0.01$ and *** ### ♦♦♦ $p \leq 0.001$. N=5 for each group.

During the 3 h recovery period, the T_{hy} and T_{body} remained high in both vehicle and midazolam groups (Fig. 33A&B). However, in the α-Asarone group, the T_{hy} was on par with that of the control values and was significantly lower than the vehicle SD group on all days (Fig. 33A). By day 20, the T_{body} also showed significant reduction (Fig. 33B).

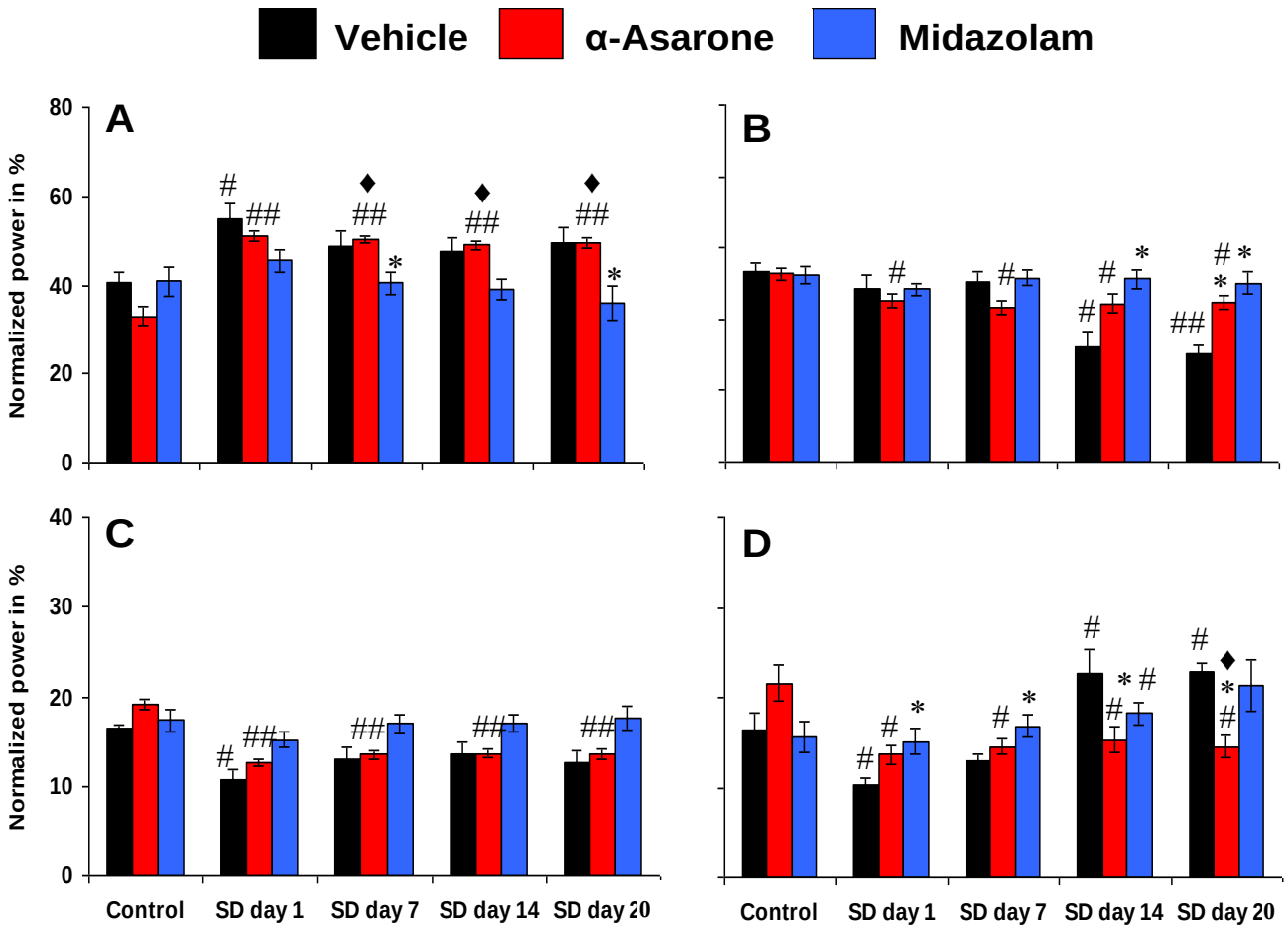

Fig. 32 Effect of chronic SD in rotating wheel on EEG power spectra of NREM sleep after administration of vehicle, α-Asarone and midazolam

The relative power in % at (A) delta, (B) theta, (C) alpha and (D) beta frequency ranges in NREM sleep during the 3 h recovery period after 20 days of SD in rats administered with vehicle, α-Asarone 10 mg/kg and midazolam 2mg/kg. The data points represents mean ± SEM. * indicates the difference from the vehicle group, # indicates the difference from the control and ♦ indicates the difference from the midazolam group. Level of significance ♦,*, # $p \leq 0.05$, ## $p \leq 0.01$. N=5 for each group.

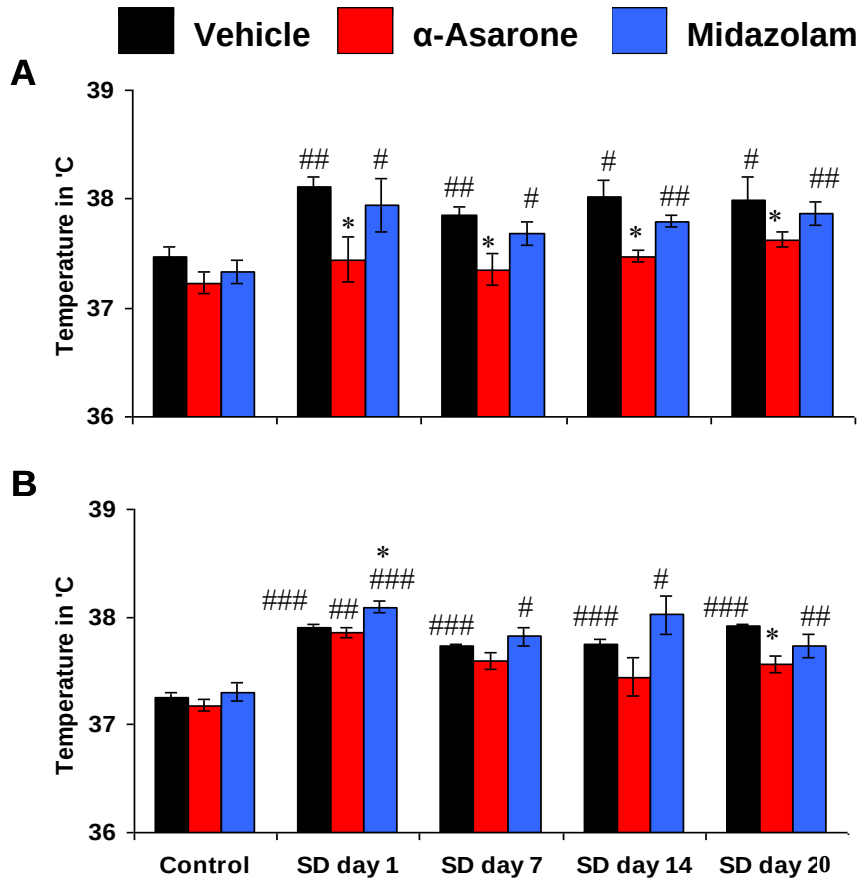

Fig. 33 Effect of chronic SD in rotating wheel on T_{hy} and T_{body} profile after administration of vehicle, α-Asarone and midazolam

Change in T_{hy} (A) and T_{body} (B) during the 3 h recovery period in SD rats treated with vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam on days 1, 7, 14 and 20. The data is represented as mean ± SEM. * represents bin-wise significant difference from the vehicle group, # represents the difference from the control. Level of significance *, # $p \leq 0.05$, ## $p \leq 0.01$ and ### $p \leq 0.001$. N=5 for each group.

Observation from the recovery studies post 21 days SD conducted on few rats is explained below. Percentage NREM sleep gradually lowered across

72 h after SD in the vehicle group. Midazolam group also had a reduced NREM sleep post 21 days SD (Table 11). However, NREM sleep percentage in the α-Asarone group was on par with its control values (Table 11). REM sleep duration was gradually increasing in all the three groups after SD for 21 days (Table 11). The NREM sleep bout duration remained unaltered and the arousal index was still minimal in the α-Asarone group in comparison to the vehicle and midazolam groups (Table 11).

EEG spectral profile was significantly modified in all the three groups after 24, 48 and 72 h after SD (Fig. 34). During NREM sleep, the relative delta power remained higher and relative beta power remained lower in the α-Asarone groups for 72 h, in comparison to the vehicle and the midazolam group (Fig. 34). During REM sleep, the relative theta power was higher and relative delta and beta power was lower in the α-Asarone group in comparison to the vehicle and the midazolam group (Fig. 34).

T_{hy} and T_{body} values observed 24 h after 21 days SD was on par with the control values in all the three groups.

		24 h recovery			48 h recovery			72 h recovery		
	Control	**Vehicle**	**α-Asarone**	**Midazolam**	**Vehicle**	**α-Asarone**	**Midazolam**	**Vehicle**	**α-Asarone**	**Midazolam**
NREM sleep duration (%)	61.5 ± 1.0	54.8 ± 0.6	62.4 ± 0.4	52.4 ± 1.8	46.4 ± 9.0	61.8 ± 0.2	55.1 ± 4.0	43.8 ± 0.3	64.2 ± 3.8	53.3 ± 3.9
REM sleep duration (%)	11.6 ± 0.8	08.4 ± 1.0	10.9 ± 0.3	10.6 ± 1.5	12.8 ± 0.2	14.4 ± 0.5	12.5 ± 1.6	18.1 ± 1.7	15.0 ± 0.1	14.2 ± 1.3
NREM sleep bout duration (min)	02.2 ± 0.1	01.8 ± 0.1	02.3 ± 0.1	01.3 ± 0.2	01.3 ± 0.3	02.1 ± 0.2	01.9 ± 0.5	01.2 ± 0.0	02.2 ± 0.4	01.7 ± 0.4
NREM sleep bout frequency	18.5 ± 1.1	20.0 ± 0.1	16.6 ± 1.1	21.4 ± 2.7	21.4 ± 0.6	18.3 ± 1.9	19.1 ± 2.7	23.1 ± 1.1	18.5 ± 1.7	20.6 ± 2.9
Arousal index	09.7 ± 1.5	10.0 ± 3.7	05.3 ± 0.0	13.6 ± 2.6	09.0 ± 1.7	05.8 ± 0.2	12.0 ± 1.2	09.1 ± 1.6	05.2 ± 0.9	10.2 ± 1.4

Table 11 Changes in S-W parameters 24, 48 and 72 h after chronic SD in rotating wheel for 21 days

The data is represented as mean ± SD

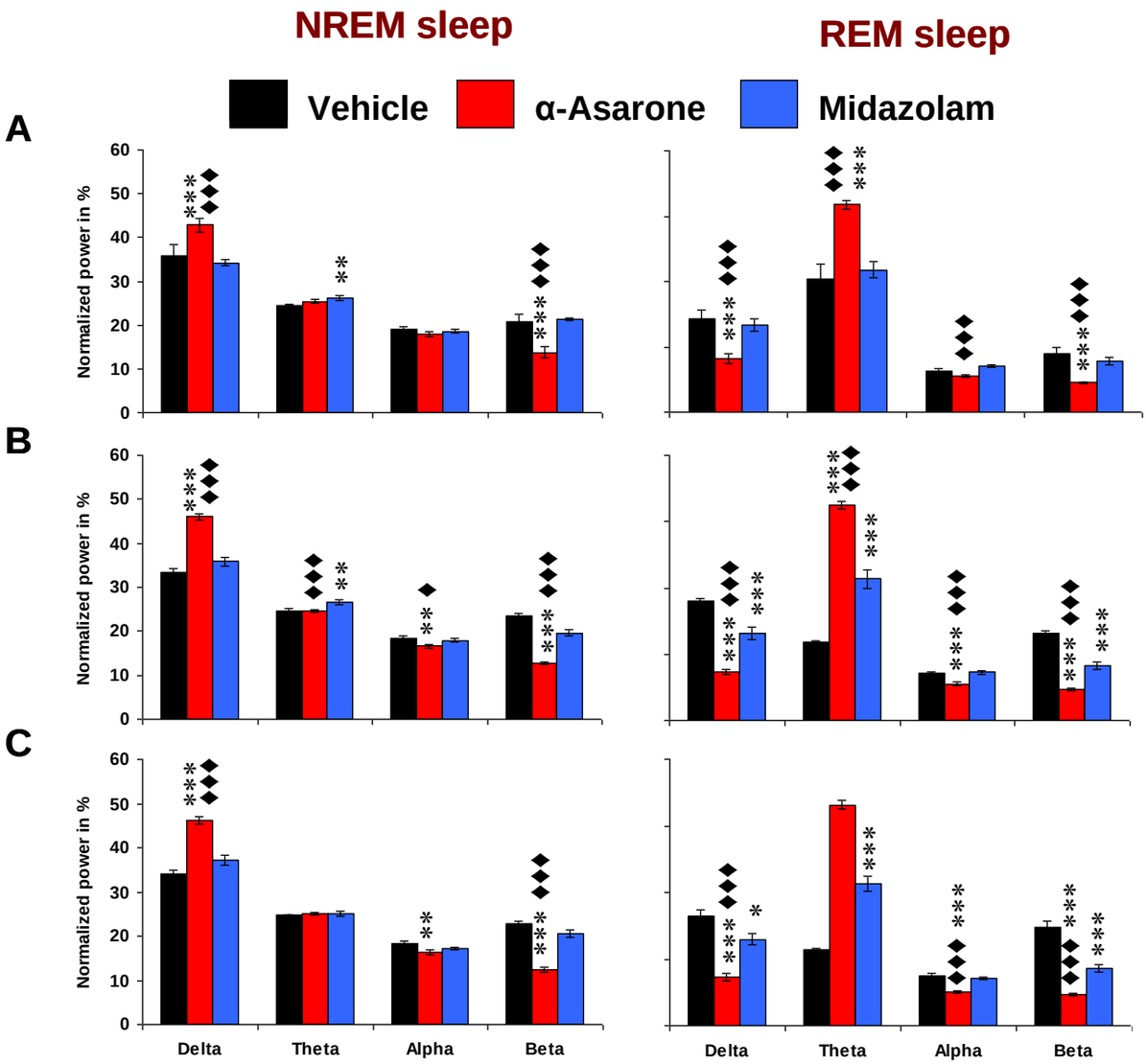

Fig 34 Changes in EEG spectrum of NREM and REM sleep 24, 48 and 72 h after chronic SD in rotating wheel for 21 days

The relative power in % at delta (0.5-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (12-30 Hz) frequency ranges in NREM and REM sleep 24 h (A), 48 h (B) and 72 h (C) after SD for 21 days in rotating wheel in rats treated with vehicle, α-Asarone 10 mg/kg and midazolam 2mg/kg The data points represents mean ± SEM. * indicates the difference from the vehicle group and ♦ indicates the difference from the midazolam group. Level of significance * p≤ 0.05, ##, ** p≤ 0.01, ###, *** p≤0.001.

6.2. Effect of optimal dose of α-Asarone on anxiety after chronic SD in rotating wheel

In EPM test, the time spent and entries in the open arm after SD was on par with the control values by day 20 in α-Asarone-treated rats (Fig. 35). Moreover, the time spent in the open arm was significantly higher than the midazolam group (Fig. 35). No change was observed in the distance travelled and the time mobile on the maze in α-Asarone group in comparison to the vehicle group (Fig. 35). The effects of α-Asarone on most of the parameters were on par with that of midazolam in the sleep deprived rats (Fig. 35).

In OFT, the α-Asarone group performance was on par with their control values and the midazolam group unlike the vehicle group by day 21 of SD (Fig. 36). Similar trends were observed on days 1, 7 and 14 in EPM test and on days 2, 8 and 15 in OFT.

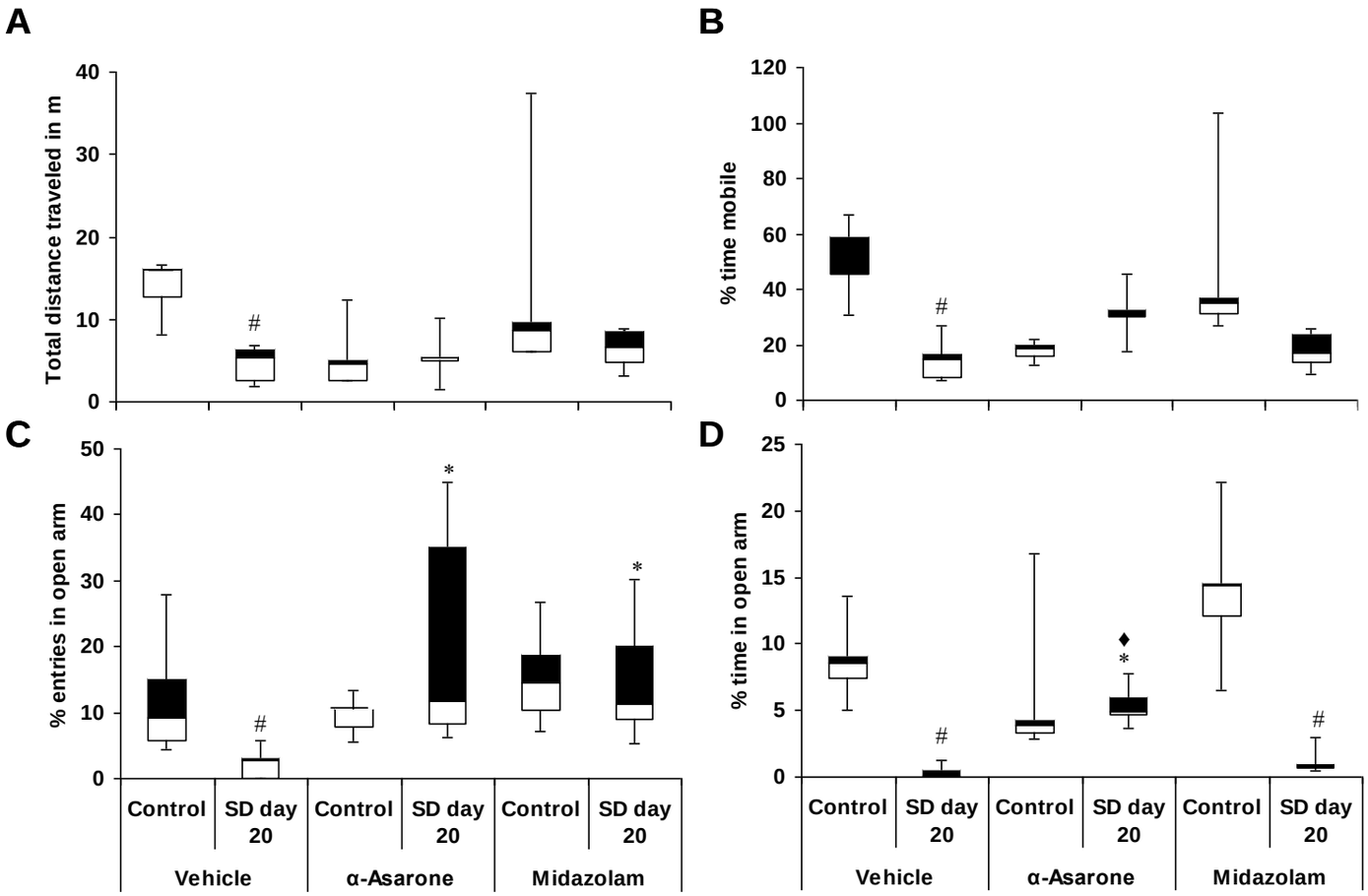

Fig. 35 Effect of chronic SD in rotating wheel on EPM test parameters after administration of vehicle, α-Asarone and midazolam for 20 days

Changes in the EPM parameters after 20 days of SD in rotating wheel in rats treated with vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam. The box-whisker plot shows the total distance traveled in m (A), time mobile in % (B), the entries into the open arms in % (C) and the time spent in the open arms in % (D) on day 20 of SD as compared to the control values taken before SD. * indicates significant change from vehicle group, # indicates significance from the control and ♦ indicates significant change from midazolam group. Levels of significance *, #, ♦ $p \leq 0.05$ N= 5 for all groups.

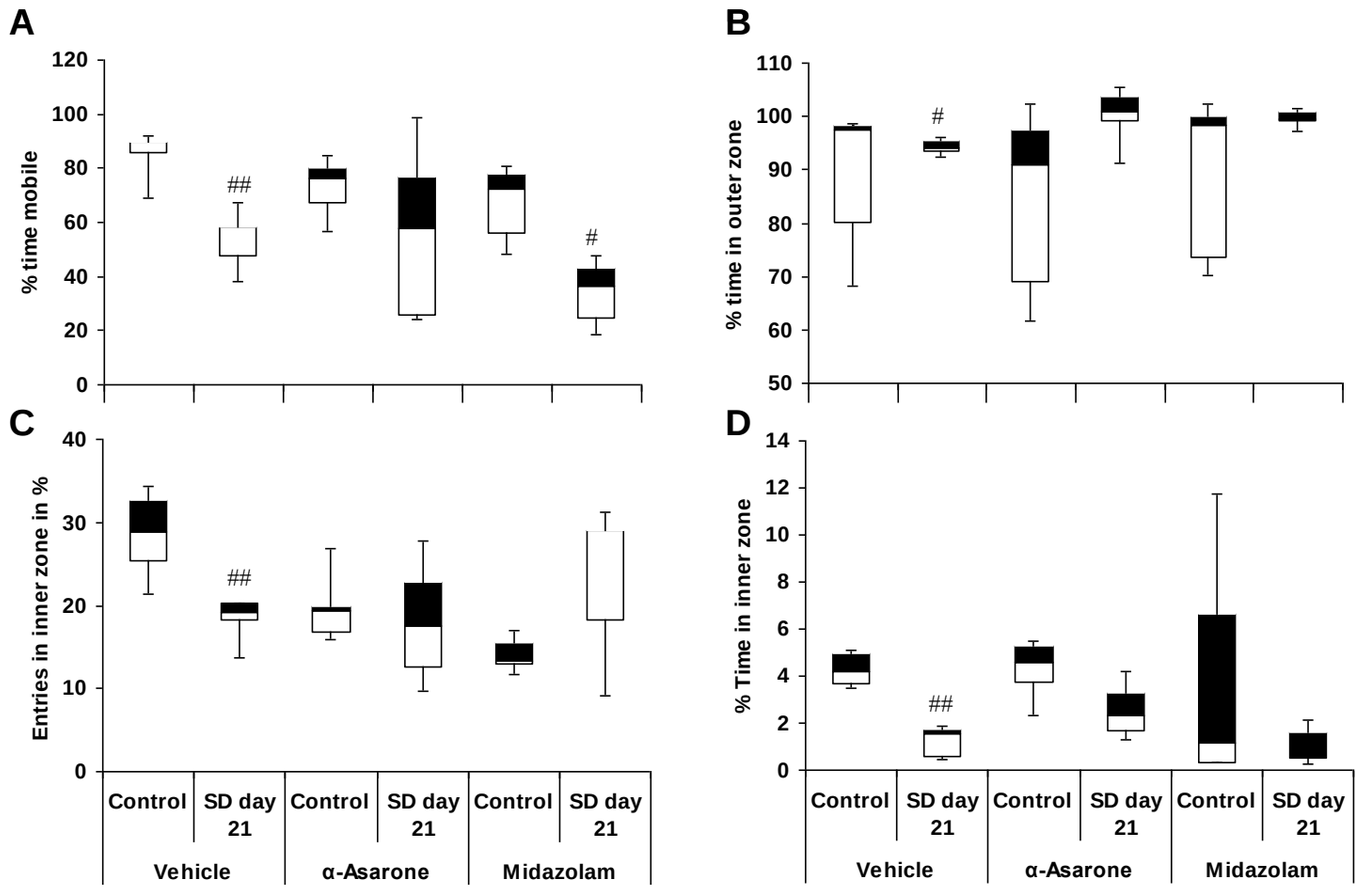

Fig. 36 Effect of chronic SD in rotating wheel on OFT parameters after administration of vehicle, α-Asarone and midazolam for 21 days

Changes in the OFT parameters after 21 days of SD in rotating wheel in rats treated with vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam. The box-whisker plot shows the time mobile in % (A), the time spent in the outer zone in % (B), the entries into the inner zone in % (C) and the time spent in the inner zone in % (D) on day 21 of SD as compared to the control values taken before SD. $^{\#}$ indicates significance from the control. Levels of significance $^{\#}$ $p \leq 0.05$, $^{\#\#}$ $p \leq 0.05$. N= 5 for all groups.

6.3. Effect of optimal dose of α-Asarone on antioxidant levels in brain after chronic SD in rotating wheel

6.3.1. MDA levels

In comparison to the control, an increase in the MDA level was observed after 21 days of 5 h SD in the subcortex ($p=0.05$) and brainstem ($p=0.002$) of the vehicle-treated group (Table 12). In comparison to the vehicle treated rats, the MDA levels lowered in all the three regions on day 21 in SD rats treated with α-Asarone (Table 12).

6.3.2. CAT activity

CAT activity was significantly decreased in all three regions of the vehicle treated group after 21 days of SD in comparison to the control group (Table 12). In comparison to the vehicle group, the CAT activity in the brainstem region increased on day 21 in SD rats when treated with α-Asarone (Table 12).

6.3.3. GSH-R activity

In comparison to the control, the GSH-R activity was significantly decreased ($p=0.05$) only in the cortical region of vehicle treated group after 21 days of 5 h SD (Table 12). No change was observed in the subcortical or brainstem region (Table 12).In the α-Asarone treated rats, GSH-R activity was increased in the subcortical and brainstem region after 21 days of SD (Table 12).

6.3.4. SOD activity

In all the three regions, the activity of SOD was significantly increased after 21 days of 5 h SD in rats treated with vehicle in comparison to the untreated control (Table 12). No change was observed in SOD activity after α-Asarone administration in SD groups after 21 days (Table 12).

6.3.5. GSH-Px activity

In cortex and subcortex, in comparison to the untreated control, the GSH-Px activity was slightly increased after 21 days of 5 h SD in vehicle treated rats (Table 12). No change was observed in the brainstem region (Table 12). No change was observed in GSH-Px activity after α-Asarone administration in SD groups after 21 days (Table 12).

6.3.6. GSH levels

GSH levels were significantly lowered in all the three regions after 21 days of 5 h SD in vehicle treated rats (Table 12). In comparison to the vehicle group, GSH level was increased in all the regions after 21 days in α-Asarone group (Table 12).

	Cortex			Subcortex			Brainstem		
	Control	**Vehicle**	**α-Asarone**	**Control**	**Vehicle**	**α-Asarone**	**Control**	**Vehicle**	**α-Asarone**
MDA (nmol/mg protein)	6.0 ± 0.4	8.9 ± 1.4	5.1 ± 0.4 *	3.8 ± 0.2	10.6 ± 2.4 $^{\#}$	6.5 ± 0.5 *	3.1 ± 0.9	11.0 ± 1.0 $^{\#\#}$	6.1 ± 0.7 $^{* \#}$
CAT (U/mg protein)	1.7 ± 0.1	0.6 ± 0.1 $^{\#\#}$	0.8 ± 0.1 $^{\#\#}$	1.4 ± 0.1	0.5 ± 0.2 $^{\#}$	1.0 ± 0.1 $^{\#}$	3.1 ± 0.3	0.3 ± 0.1 $^{\#\#\#}$	1.4 ± 0.3 $^{* \#\#}$
GSH-R (U/mg protein)	2.6 ± 0.4	1.7 ± 0.2 $^{\#}$	2.1 ± 0.4	1.9 ± 0.4	1.5 ± 0.1	2.2 ± 0.2 *	1.6 ± 0.2	1.5 ± 0.0	2.3 ± 0.0 $^{*** \#}$
SOD (U/mg protein)	10.8 ± 2.2	20.0 ± 1.5 $^{\#\#}$	19.0 ± 0.4 $^{\#\#}$	10.4 ± 1.1	27.5 ± 5.7 $^{\#\#}$	22.5 ± 2.0 $^{\#}$	10.1 ± 1.9	37.1 ± 7.4 $^{\#\#}$	23.0 ± 1.7 $^{\#\#}$
GSH-Px (U/mg protein)	0.03 ± 0.0	0.1 ± 0.0 $^{\#}$	0.1 ± 0.0 $^{\#}$	0.02 ± 0.0	0.1 ± 0.0 $^{\#}$	0.1 ± 0.0 $^{\#\#}$	0.02 ± 0.0	0.2 ± 0.1	0.1 ± 0.0 $^{\#\#}$
GSH (nmol/mg protein)	103.1 ± 35.3	8.2 ± 2.7 $^{\#}$	48.2 ± 12.2 *	187.0 ± 11.1	32.2 ± 28.8 $^{\#}$	206.9 ± 33.9 *	357.9 ± 29.8	13.5 ± 3.1 $^{\#\#\#}$	196.0 ± 31.0 $^{** \#}$

Table 12 Effect of chronic SD in rotating wheel on antioxidant levels after administration of vehicle and α-Asarone

* indicates significance between vehicle and α-Asarone group and # indicates significant difference from the control values.

Levels of significance $^{*,\#}$ $p \leq 0.05$, $^{**,\#\#}$ $p \leq 0.01$ and $^{***,\#\#\#}$ $p \leq 0.001$. N=3 for each group.

7. Objective 5: To understand the mechanism of action of α-Asarone by examining the relationship of T_{hy} and T_{body} with sleep.

7.1. Relationship of T_{hy} and T_{body} with S-W after administration of various doses of α-Asarone

A significant negative correlation was observed between the NREM sleep bout duration and T_{hy} after the administration of doses 2 and 10 mg/kg α-Asarone and vehicle (Fig. 37). The strength of association of NREM sleep bout duration with T_{hy} was found to be higher (61 %) after administering 10 mg/kg of α-Asarone in comparison to the vehicle (Fig. 37). Similarly, the correlation between the T_{body} and NREM sleep bout duration was also improved at this dose (Fig. 37). Moreover, NREM bouts of longer duration were increased after administration of 10 mg/kg α-Asarone (Fig. 37).

A weak positive correlation was observed between the REM sleep bout duration and T_{hy} after administration of vehicle, and α-Asarone at doses of 2 and 10 mg/kg (Fig. 39). After administering 40 and 80 mg/kg α-Asarone, no significant correlation was observed between the NREM and REM bout duration and T_{hy} and T_{body} (Fig. 37 and 39). On the other hand, 120 mg/kg α-Asarone restored the negative correlation between NREM bout duration and T_{hy} and T_{body}, however, with an increase in the NREM sleep bouts of shorter duration (Fig. 37 and 38A).

The average T_{hy} and T_{body} during the NREM bouts of longer duration were 37.01±0.2 and 36.93±0.1 ºC respectively after all treatments (Fig. 38B). T_{body} showed a greater fall at higher doses of α-Asarone. The average difference

between T_{hy} and T_{body} after administering 10 mg/kg α-Asarone was found to be 0.07±0.03 ºC for 7 h. On the other hand, higher doses of α-Asarone produced an increased difference between T_{hy} and T_{body} due to a larger drop in T_{body} reaching up to 0.36±0.04 ºC at 120 mg/kg α-Asarone.

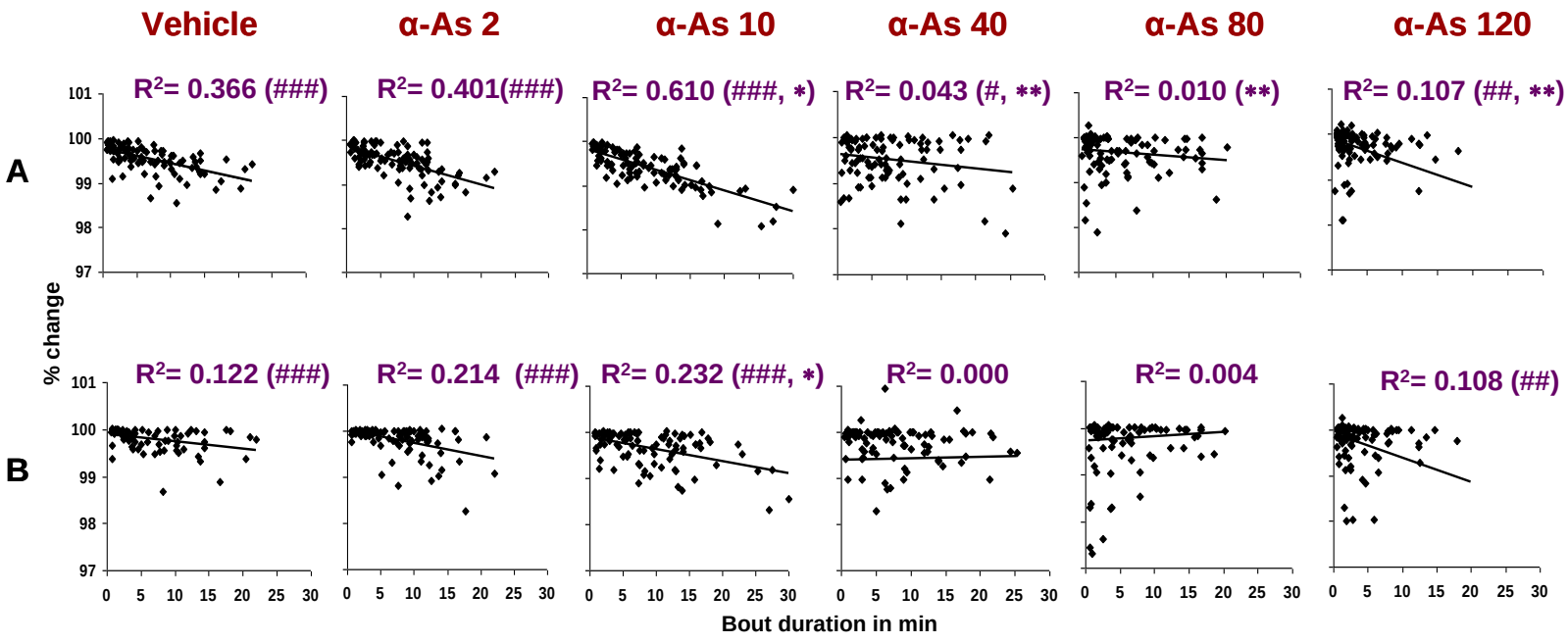

Fig. 37 Scatter plot showing correlation between the NREM sleep bout duration and the T_{hy} and T_{body} during NREM sleep after administration of various doses of α-Asarone

Correlation between the NREM sleep bout duration in min and the T_{hy} (A) and T_{body} (B) during NREM sleep after administration of vehicle and 2, 10, 40, 80 and 120 mg/kg α-Asarone in rats (N=5). T_{hy} and T_{body} was plotted as the percentage change from the preceding stage (taken as 100 %) of the transition to NREM sleep. R^2 represents the coefficient of determination and $^{\#}$ represents the significance of the regression model. * indicates the difference from the vehicle group. Level of significance $^{*\ \#}$ $p \leq 0.05$, and $^{**\ \#\#}$ $p \leq 0.01$, $^{\#\#\#}$ $p \leq 0.01$.

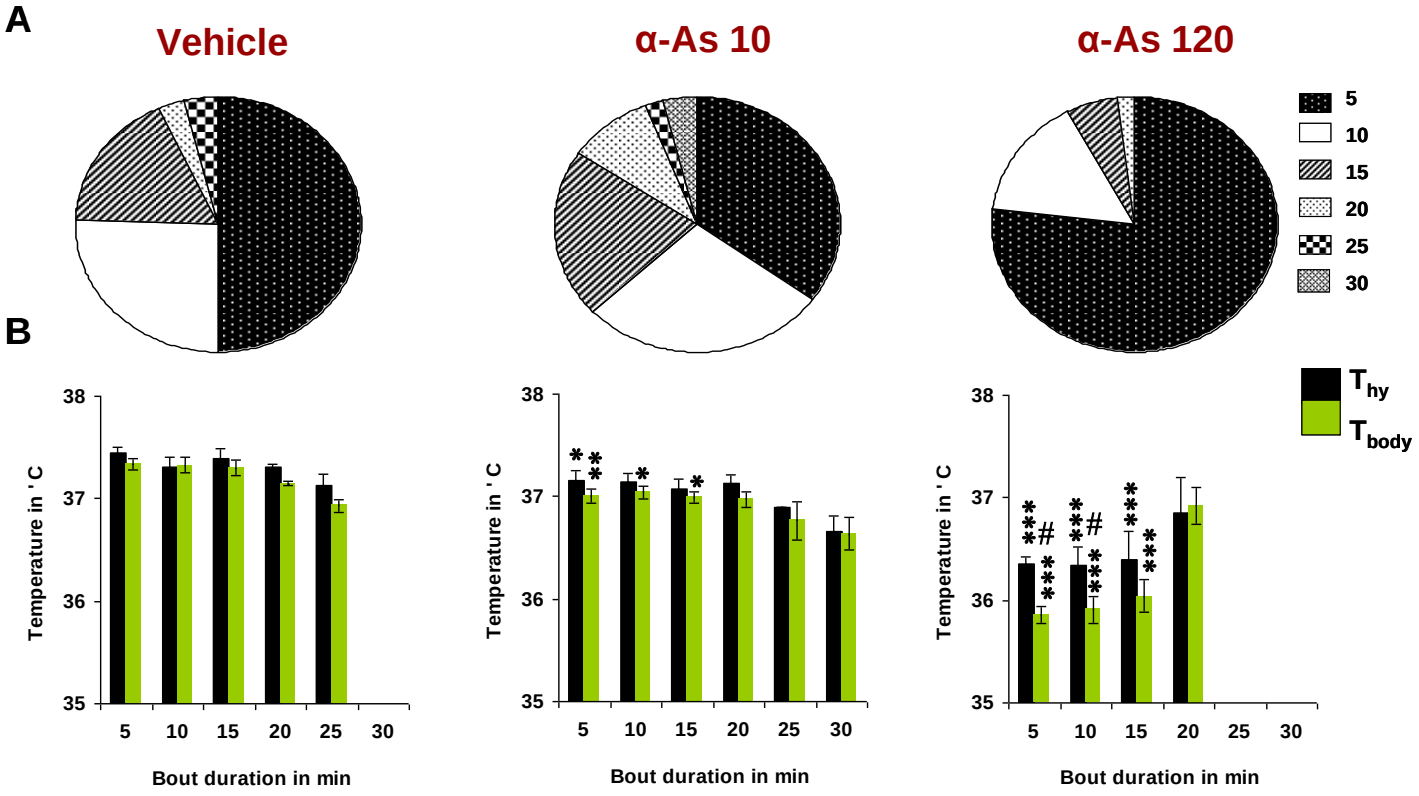

Fig. 38 T_{hy} and T_{body} profile for various NREM sleep bout durations after administration of vehicle, 10 and 120

(A) Count of 5, 10, 15, 20, 25 and 30 min long bouts of NREM sleep and (B) average T_{hy} and T_{body} in ºC observed during 5, 10, 15, 20, 25 and 30 min long bouts of NREM sleep after administration of vehicle, 10 mg/kg α-Asarone and 120 mg/kg α-Asarone (α-As) in rats (N=5). The bars represent mean ± SEM. * indicates the difference from vehicle and $^{\#}$ indicates comparison between T_{hy} and T_{body}. Level of significance $^{*\,\#}$ $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

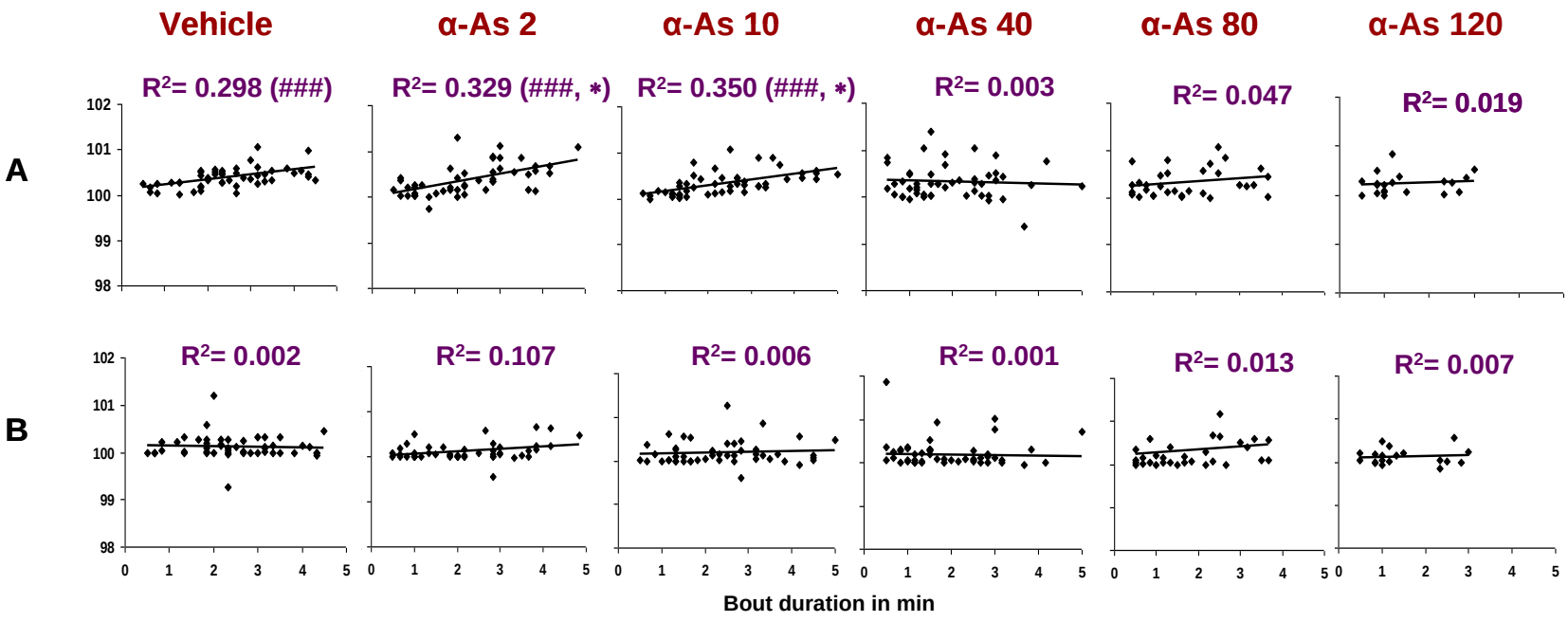

Fig. 39 Scatter plot showing correlation between the REM bout duration and the T_{hy} and T_{body} during REM sleep after administration of various doses of α-Asarone

Correlation between the REM bout duration in min and the T_{hy} (A) and T_{body} (B) during REM sleep after administration of vehicle and 2, 10, 40, 80 and 120 mg/kg α-Asarone in rats (N=5). T_{hy} and T_{body} is plotted as the percentage change from the preceding stage (taken as 100 %) of the transition to REM sleep. R^2 represents the coefficient of determination and $^{\#}$ represents the significance of the regression model. NS indicates non significant, * indicates the difference from the vehicle group. Level of significance * $p \leq 0.05$, $^{\#\#\#}$ $p \leq 0.001$.

7.2. Relationship of T_{hy} and T_{body} with S-W after chronic administration of optimum dose of α-Asarone

A significant negative correlation was observed between the NREM sleep bout duration and T_{hy} after the administration of 10 mg/kg α-Asarone and vehicle (Fig. 40). The strength of association of NREM sleep bout duration with T_{hy} was found to be higher after administering 10 mg/kg of α-Asarone on days 7 (37 %) and 21 (40 %) in comparison to the vehicle (Fig. 40). Similarly, the correlation between the T_{body} and NREM sleep bout duration was also marginally improved after α-Asarone administration (Fig. 40). Positive correlation between REM bout duration and T_{hy} remained unaltered for 3 weeks.

The number of NREM bouts of longer duration (>20 min) was increased after the administration of 10 mg/kg α-Asarone (Fig. 41A). These bouts were observed when the average T_{hy} and T_{body} were 36.91±0.1 and 36.94±0.1 ºC respectively for both the treatments (Fig. 41B). During shorter bouts of NREM sleep (<20 min), a significant difference was observed between T_{hy} and T_{body} after administering vehicle. However, this difference was not significant under α-Asarone treatment for 7 and 21 days (Fig. 41B). The average difference between T_{hy} and T_{body} after administering 10 mg/kg α-Asarone was found to be 0.05±0.01 ºC for 3 weeks and 0.13±0.02 ºC after vehicle treatment.

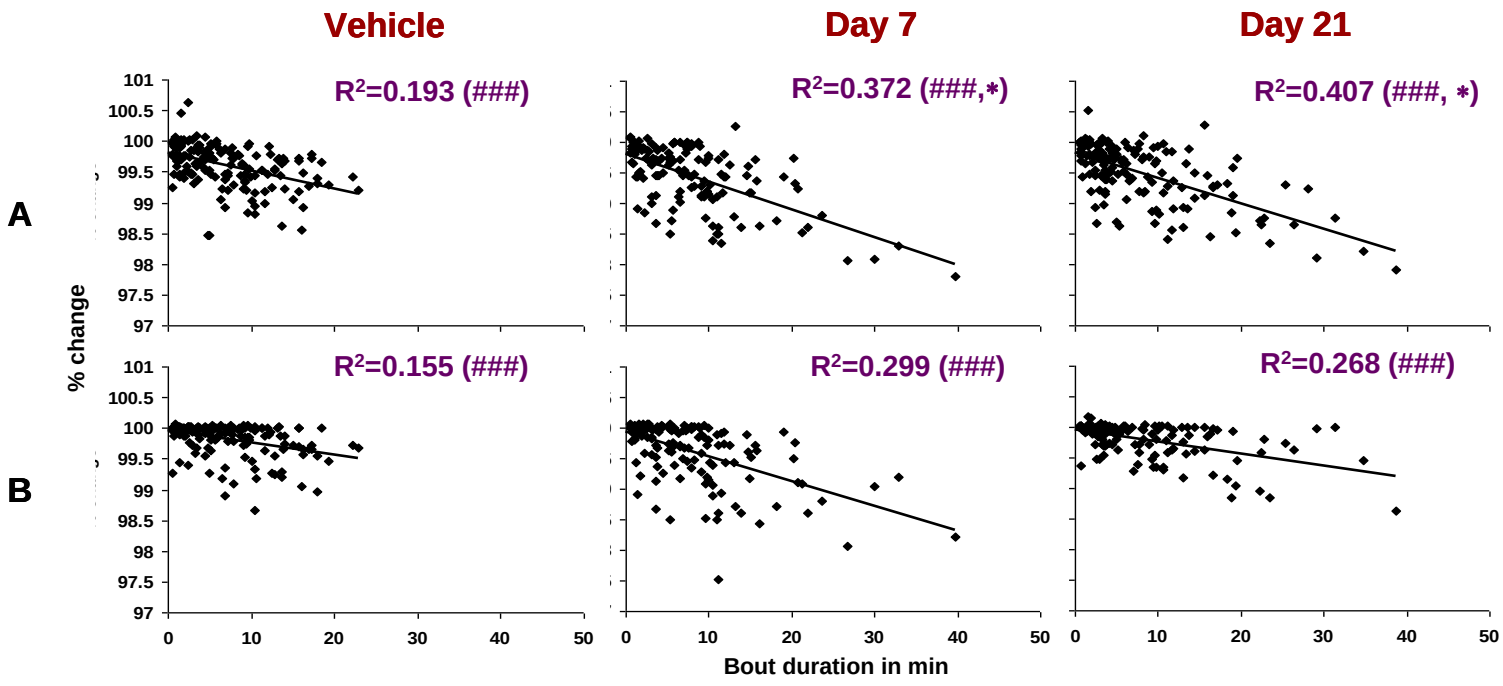

Fig. 40 Scatter plot showing correlation between the NREM sleep bout duration and the T_{hy} and T_{body} during NREM sleep after chronic administration of 10 mg/kg α-Asarone

Correlation between the NREM sleep bout duration in min and the T_{hy} (A) and T_{body} (B) during NREM sleep after administration of vehicle (A) and 10 mg/kg α-Asarone (B) for 7 days and 21 days in rats (N=5). T_{hy} and T_{body} was plotted as the percentage change from the preceding stage (taken as 100 %) of the transition to NREM sleep. R^2 represents the coefficient of determination and $^{\#}$ represents the significance of the regression model. NS represents non significant, * indicates the difference from the vehicle group. Level of significance * $p \leq 0.05$ and $^{\#\#\#}$ $p \leq 0.001$.

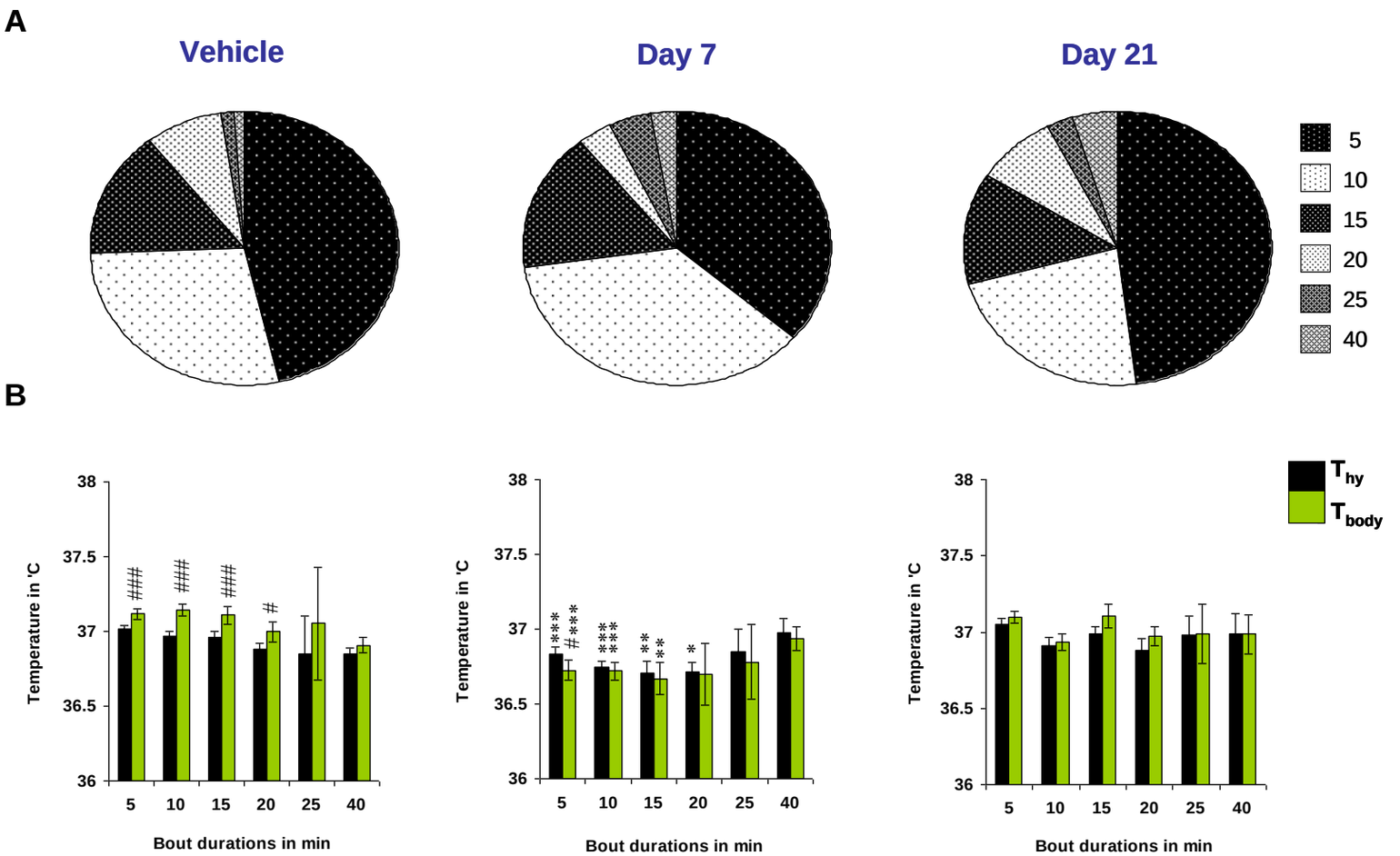

Fig. 41 T_{hy} and T_{body} profile for various NREM sleep bout durations after administration of 10 mg/kg α-Asarone for 7 and 21 days

(A) Count of 5, 10, 15, 20, 25 and 40 min long bouts of NREM sleep and (B) average T_{hy} and T_{body} in ºC observed during 5, 10, 15, 20, 25 and 40 min long bouts of NREM sleep in rats administered with 10 mg/kg α-Asarone on days 7 and 21 (N=5). The bars represent mean ± SEM. * indicates the difference from vehicle and $^{\#}$ indicates comparison between T_{hy} and T_{body}. Level of significance *, $^{\#}$ $p \leq 0.05$, ** $p \leq 0.01$, ***, $^{\#\#\#}$ $p \leq 0.001$.

7.3. Relationship of T_{hy} and T_{body} with S-W in vehicle-, α-Asarone- and midazolam-treated rats acutely sleep deprived by gentle handling

The negative correlation between the NREM sleep bout duration and T_{hy} during the recovery period was the highest ($p< 0.05$) in the α-Asarone group on both days 1 (68 %) and 5 (62 %) of SD in comparison to the vehicle and the midazolam group (Fig. 42). On the other hand a positive correlation (24 %) was observed between the REM sleep bout duration and T_{hy} only in the α-Asarone group during the recovery period on day 5 of SD (Fig. 43). The average T_{hy} and T_{body} observed during the longer bouts (> 20 min) in the α-Asarone-treated SD group was 36.7 ± 0.2 and 36.7 ± 0.2 ºC on days 1 and 36.9 ± 0.2 and 37.1 ±0.1 ºC on day 5.

7.4. Relationship of T_{hy} and T_{body} with S-W in vehicle-, α-Asarone- and midazolam-treated rats chronically sleep deprived in rotating wheel

The negative correlation between the NREM sleep bout duration and T_{hy} during the recovery period was higher in the α-Asarone group after 14 and 20 days of SD in comparison to both vehicle and midazolam group (Fig. 44). Midazolam lowered the negative correlation between the NREM sleep bout duration and T_{hy} (Fig. 44). This persisted even after 21 days post SD (Fig. 44). On the other hand a positive correlation between the REM sleep bout duration and T_{hy} was observed during the recovery period for 3 weeks of SD only in the α-Asarone group (Fig. 45). Midazolam lowered the correlation by day 20 of SD (Fig. 45). Post SD, the positive correlation between the REM sleep bout duration and T_{hy} was restored in all three groups (Fig. 45). The average T_{hy}

and T_{body} observed during the longer bouts (> 20 min) in the α-Asarone-treated SD group was 37.4 ± 0.5 and 37.5 ± 0.5 ºC on day 1, 37.0 ± 0.5 and 37.1 ± 0.3 ºC on day 7, 36.9 ± 0.6 and 37.0 ± 0.1 ºC on day 14 and 37.4 ± 0.3 and 37.5 ± 0.3 ºC on day 20 of SD.

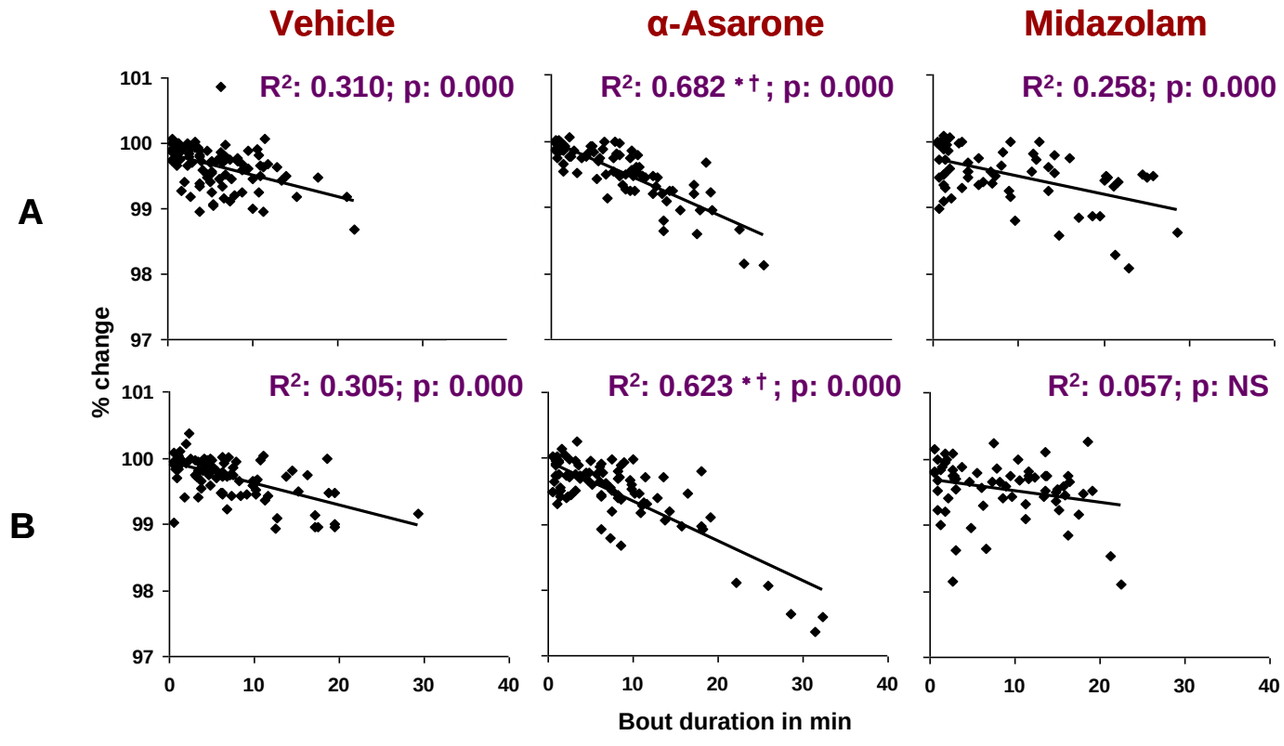

Fig. 42 Scatter plot showing relationship of T_{hy} and T_{body} with NREM sleep in vehicle-, α-Asarone- and midazolam-treated rats acutely sleep deprived by gentle handling

Correlation between the NREM sleep bout duration in min and the T_{hy} during the recovery period in SD rats treated with vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam on day 1 (A) and 5 (B). T_{hy} was calculated as the percentage change from the preceding stage (taken as 100 %) of the transition to NREM sleep. R^2 represents the coefficient of determination and # represents the significance of the regression model. NS indicates non significant, * indicates the difference from the vehicle group and ^ indicates the difference from the midazolam group. Level of significance *, ^ $p \leq 0.05$, ### $p \leq 0.001$.

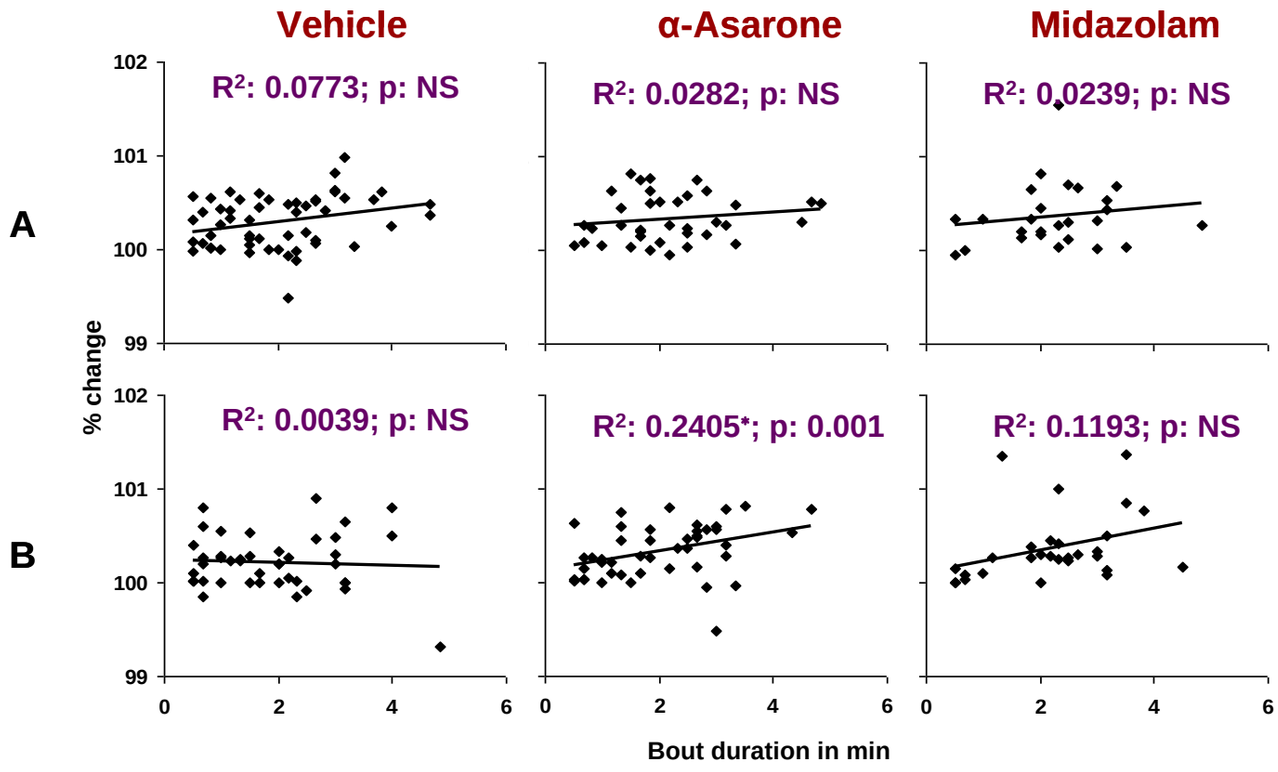

Fig. 43 Scatter plot showing relationship of T_{hy} and T_{body} with REM sleep in vehicle-, α-Asarone- and midazolam-treated rats acutely sleep deprived by gentle handling

Correlation between the REM sleep bout duration in min and the T_{hy} during REM sleep in the recovery period in SD rats treated with vehicle, 10 mg/kg α-Asarone and 2 mg/kg midazolam on day 1 (A) and 5 (B). T_{hy} was calculated as the percentage change from the preceding stage (taken as 100 %) of the transition to REM sleep. R^2 represents the coefficient of determination and $^{\#}$ represents the significance of the regression model. NS indicates non significant, * indicates the difference from the vehicle group. Level of significance * $p \leq 0.05$, $^{\#\#}$ $p \leq 0.01$.

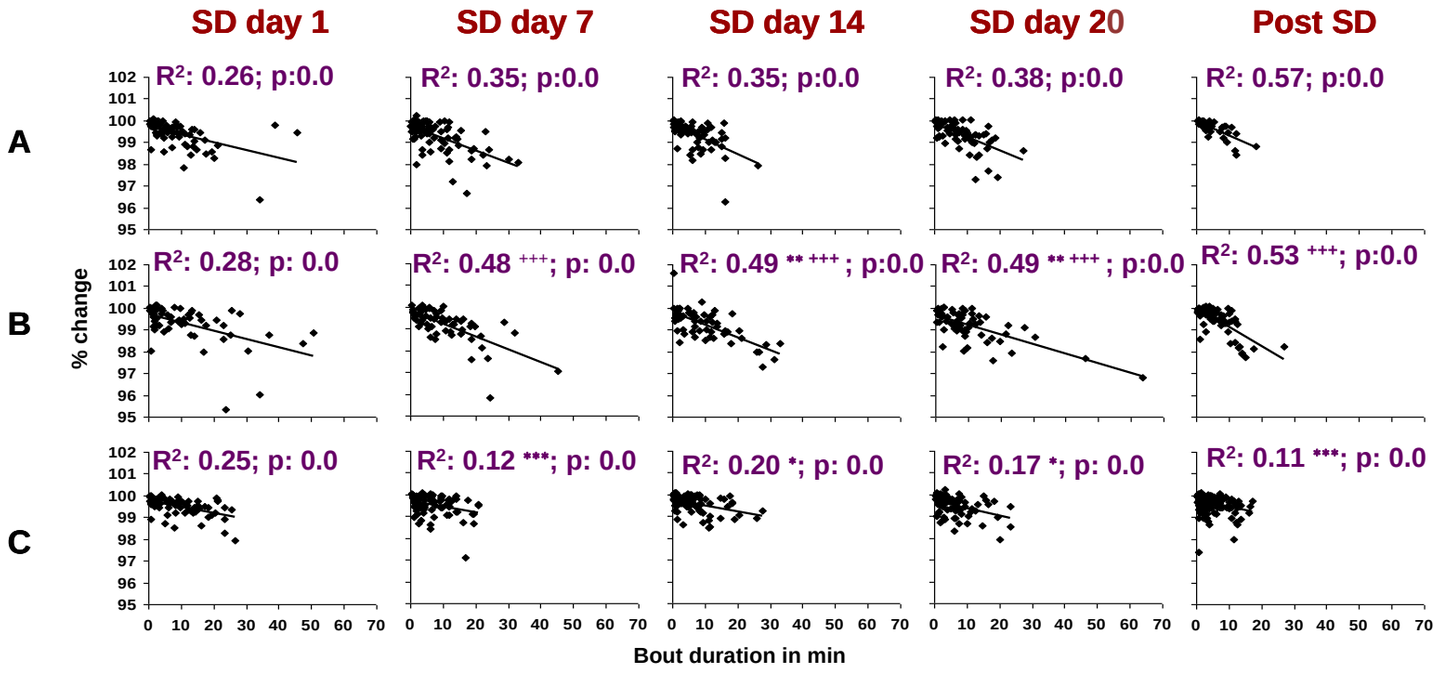

Fig. 44 Scatter plot showing relationship of T_{hy} and T_{body} with NREM sleep in vehicle-, α-Asarone- and midazolam-treated rats chronically sleep deprived in rotating wheel

Correlation between the NREM sleep bout duration in min and the T_{hy} during NREM sleep in the recovery period in SD rats treated with vehicle (A), 10 mg/kg α-Asarone (B) and 2 mg/kg midazolam (C) on days 1, 7, 14 and 20. Correlation after 21 days of SD is also shown. T_{hy} was calculated as the percentage change from the preceding stage (taken as 100 %) of the transition to NREM sleep. R^2 represents the coefficient of determination and p value represents the significance of the regression model. NS indicates non significant, * indicates the difference from the vehicle group and + indicates the difference from the midazolam group. Level of significance *, $^{+}$ $p \leq 0.05$, ** $p \leq 0.01$ and ***, $^{+++}$ $p \leq 0.001$.

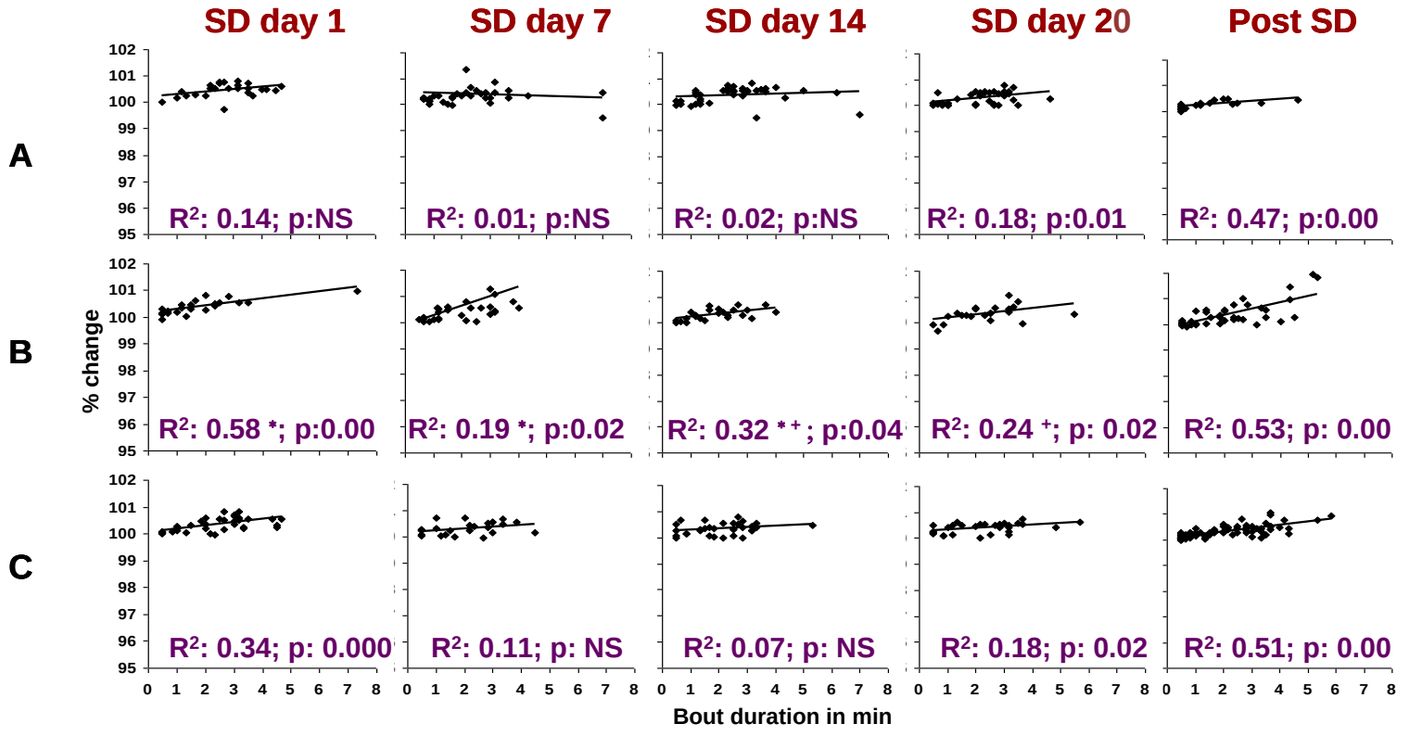

Fig. 45 Scatter plot showing relationship of T_{hy} and T_{body} with REM sleep in vehicle-, α-Asarone- and midazolam- treated rats chronically sleep deprived in rotating wheel

Correlation between the REM sleep bout duration in min and the T_{hy} during NREM sleep in the recovery period in SD rats treated with vehicle (A), 10 mg/kg α-Asarone (B) and 2 mg/kg midazolam (C) on days 1, 7, 14 and 20. Correlation after 21 days of SD is also shown. T_{hy} was calculated as the percentage change from the preceding stage (taken as 100 %) of the transition to REM sleep. R^2 represents the coefficient of determination and p value represents the significance of the regression model. NS indicates non significant, * indicates the difference from the vehicle group and $^{+}$ indicates the difference from the midazolam group. Level of significance *, $^{+}$ $p \leq 0.05$, ** $p \leq 0.01$ and ***, $^{+++}$ $p \leq 0.001$.

Chapter V: Discussion

The sleep-wake property of α-Asarone, an active principle of *Acorus* species, was evaluated for the first time in this study. In the dose response study, 10 mg/kg α-Asarone improved the quality and depth of sleep without altering the total sleep time in normal rats. The improvement in the quality of sleep persisted even after daily administration for 21 consecutive days in normal rats. The hypnotic property of the optimal dose of α-Asarone was evident in both acutely and chronically SD rats in comparison to the vehicle and the positive control midazolam. α-Asarone was also an effective anxiolytic and antioxidant agent under SD conditions. α-Asarone-mediated changes in the T_{hy} and T_{body} facilitated the improvement in sleep quality.

1. Effects of the optimal dose of α-Asarone on S-W, T_{hy} and T_{body}

This study provided crucial evidences to the dose-dependent biphasic effect of α-Asarone on sleep, modulated through changes in T_{hy} and T_{body}. In the present study, daily adminstration of lower doses of α-Asarone especially 10 mg/kg, improved the quality of sleep as indicated by increased NREM bout duration, and decreased NREM bout frequency and sleep fragmentation without altering the total amount of sleep in normal rats for a duration of 21 days. The improvement in the sleep quality at 10 mg/kg was seen in association with a moderate decrease in T_{hy} and T_{body} and a minimum difference between these two parameters. This in turn facilitated a strong negative correlation between the NREM sleep bout duration and T_{hy} in the normal rats. The negative correlation observed is in accordance with the previous reports that indicated a favourable relationship between these two

parameters in a narrow range (Franken et al, 1992). Since the increase in NREM sleep bout duration may also lead to decreased T_{hy} (Franken et al, 1992), α-Asarone-mediated lowering of T_{hy} might be the reason for the increase in the bout duration of NREM sleep, or *vice-versa.* This persisted effect even after daily administration of α-Asarone for 21 days under normal condition probably indicated its sustained effects for long term use.

Doses higher than 10 mg/kg α-Asarone (40, 80 and 120 mg/kg), on the other hand, produced reduced, poor quality fragmented sleep. Severe lowering of T_{hy} and T_{body} and increased gradient between these two parameters resulted in the reduction of sleep quantity and quality. The lowered correlations observed between NREM sleep bout duration and T_{hy} after administration of 40 and 80 mg/kg α-Asarone acted as an adaptive mechanism to prevent the lowering of T_{hy} with NREM sleep, which may further worsen the drug-induced reduction in T_{hy} and T_{body}. Subsequently, the lowered association between sleep and thermoregulation at these doses resulted in poor quality sleep. However, at the dose of 120 mg/kg, the loss of this adaptive mechanism, further contributed to hypothermia, resulting in an arousal effect. This effect in turn produced highly reduced and fragmented sleep along with an increase in the number of shorter bouts. Majority of variations (84 %) in the brain temperature had been attributed to the alteration in sleep and wakefulness (Franken et al, 1992).

Change in the REM sleep duration or any other REM sleep parameters at lower doses of α-Asarone were insignificant but higher doses suppressed REM sleep as a result of hypothermia. A positive correlation of T_{hy} with REM sleep

bout duration (Calasso and Parmeggiani, 2008) remained unaltered at lower doses of α-Asarone (2 and 10 mg/kg). A decrease in the common carotid artery blood flow and the associated increase in the cerebral blood supply through the vertebral artery could be the reason behind the increased brain temperature during REM sleep (Calasso and Parmeggiani, 2008). At higher doses, distinct reduction in REM sleep substantiated the reduced interrelationship of T_{hy} with REM sleep bout duration. This finding is supported by previous reports wherein suppression of REM sleep during hypothermia indicated interplay of thermoregulation and REM sleep (Amici et al, 2014).

2. Effects of optimal dose of α-Asarone on SD-induced changes in S-W, T_{hy} and T_{body}

The observation of SD-induced sleep rebound for 5 days in acute model and for a week in the chronic model is similar to the earlier report (Rechtschaffen et al, 1989). Also the reduction in the quality and depth of sleep by the end of third and the fourth week in the chronic model is also reported earlier (Rechtschaffen et al, 1989). Furthermore, the increase in T_{hy} and T_{body} during acute and chronic SD and its reduction during recovery sleep further strengthens the concept of association of sleep with thermoregulation (Franken et al., 1991; Landis et al, 1992).

Even though the SD-induced increase in the NREM sleep (sleep rebound) for three weeks was similar after administration of both 10 mg/kg α-Asarone and 2 mg/kg positive control (midazolam), however, the enhancement in the

NREM sleep after midazolam administration was at the expense of REM sleep as reported previously (Lancel et al, 1996). Hence, improvement in NREM sleep without altering REM sleep probably makes α-Asarone a better alternative drug for insomnia management.

Moreover, increase in the NREM sleep bout duration and decrease in NREM sleep bout frequency and sleep fragmentation after α-Asarone administration indicates its role in sleep maintenance which was evidently lacking after midazolam administration. Shortening of the latency to sleep after SD by α-Asarone in comparison to the hypnotic midazolam (Lancel et al, 1996) suggests its involvement in the sleep initiation process also.

Increased EEG power at delta frequency range in NREM sleep after SD indicates an increase in the homeostatic drive and intensity for NREM sleep, as reported previously (Franken et al, 1991). Further enhanced relative delta power after α-Asarone administration in comparison to the other treatments in normal and sleep deprived rats clearly indicates that this drug improves the depth of NREM sleep after SD, thereby promoting the quality of sleep. Decrease in alpha and beta power observed in the EEG power spectral analysis further confirms the potential advantage of α-Asarone treatment over midazolam. An increase in the power at beta frequency range (12-35 Hz) during NREM sleep is a common identification in patients with insomnia (Perlis et al, 2001). Increased beta during NREM sleep is negatively correlated with the sleep quality and is considered as an electrophysiological marker for arousal (Nofzinger et al, 2000). Increase in the beta power and decrease in the delta power of EEG during NREM sleep of SD rats after administration of

midazolam or any hypnotics is consistent with previous reports (Aeschbach et al, 1994; Bastian et al, 2003; Feinberg et al, 2000).

During SD, α-Asarone (10 mg/kg) reduced and normalized the T_{hy} and T_{body} rapidly and also retained minimum difference between these two parameters. This concomitantly enhanced the correlation of T_{hy} with both NREM and REM sleep bout duration which facilitated improvement in the quality of sleep without reduction in the REM sleep. However, in the midazolam-treated SD rats, the T_{body} always remained higher along with increased difference between T_{hy} and T_{body}. This in turn reduced the correlation of T_{hy} with both NREM and REM sleep bout duration resulting in reduced sleep quality and quantity (REM sleep).

In the present study observation of withdrawal effect (rebound insomnia) in the midazolam group was similar to a few previous reports in which this was one of the major side-effects seen after discontinuing the usage of short-acting hypnotics (Borbely and Achermann, 1991; Feige et al, 1999; Kales et al, 1974). Decreased total sleep time and EEG delta power and increased high frequency activity especially at beta frequency range are some of the major observations in the present study indicating the withdrawal effect after midazolam discontinuation. Furthermore, midazolam produced withdrawal effects 72 h after its discontinuation. However, peculiar observation of absence of withdrawal effects after discontinuation of α-Asarone 10 mg/kg administration and sustained depth of sleep even 72 h after its discontinuation clearly indicate that α-Asarone is a better alternative drug for insomnia management with minimum side-effects.

3. Effect of α-Asarone on SD-induced changes in anxiety levels

In the present study, anxiety level was increased in both acute and chronic SD models. Increased anxiety after SD, observed in the vehicle-treated group in the present study, is supported by some earlier reports (Cohen et al, 2012; Cortese et al, 2010) and is contrary to some other reports (Berro et al, 2014). On the other hand, chronic SD produces pathological anxiety in rats (Silva et al, 2004).

The animals treated with 10 mg/kg of α-Asarone were less anxious when compared to their vehicle-treated counterparts after being subjected to SD. In this study, the effect of α-Asarone was on par with the benzodiazepine midazolam which is a clinically well established anxiolytic drug (Zangrossi et al, 1999). The increased entries and time spent in the open arms and reversal of SD-induced reduction in the activity of rats observed in the present study after α-Asarone administration are reported in various other studies using different animal models and experimental protocol (Lee et al, 2014; Liu et al, 2012; Reddy et al, 2015; Shukla et al, 2002; Tiwari et al, 2014).

Even though not prominent, the anxiolytic behavior in the rats after administration of α-Asarone was also observed in the OFT. The anxiolysis was on par with the commonly used anxiolytic midazolam (Zangrossi et al, 1999). In contrast to the present study, administration of α-Asarone at doses ranging from 5-20 mg/kg, i.p. did not change the spontaneous behaviour in normal rats when tested in OFT (Han et al, 2013). However, in another study, administration of 200 mg/kg α-Asarone orally in the corticosterone-treated rats

increased the number of line crossings in the OFT indicating anxiolytic activity (Lee et al, 2014).

Apart from rodent models, anxiolytic properties of α-Asarone were studied in monkeys also. A dose of α-Asarone (5 mg/kg, i.p.) administration minimized the signs of hostility in monkeys (Dandiya and Menon, 1964).

4. Effect of α-Asarone on SD-induced changes in brain antioxidant levels

In the present study, oxidative stress level in cortex, subcortex and brainstem was altered in the rats subjected to SD. Even though 5 h SD produced a slight increase in the oxidative stress, by fifth day of SD a cellular adaptive response was observed with respect to the antioxidant profile. This response might be to recoup from the acute stress produced by SD. However, the cellular adaptive response after 5 days of SD was not evident in the behavior of the animals. They showed an increased anxiety even after 5 days of SD. This might be due to the reduction observed in the activity of CAT especially in the subcortical region. Catalase over-expression is reported to be sufficient to reduce anxiety even in the absence of alteration in levels of oxidative stress (Olsen et al, 2013).

On the other hand, chronic SD in the present study produced increased oxidative stress. Ramanathan et al, (2010) proposed that in the acute or short term SD, 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 SD varies across brain regions.

Also, the same group reported that 6 h of SD increased antioxidant responses in the rat cortex, hippocampus, basal forebrain, brainstem and cerebellum, whereas 5-11 days of chronic SD decreased antioxidant responses in the rat hippocampus and brainstem (Ramanathan et al, 2002). It is reported that the acute and chronic sleep loss produce varied transcriptional changes in the rat cerebral cortex and also the brain is capable of responding to the stress associated with acute sleep loss and thereby preventing oxidative stress (Cirelli et al, 2006).

The neuroprotective effect of α-Asarone may be partly due to its antioxidant property. Increase in the MDA levels observed after chronic SD was eliminated in the cortex, subcortex and brainstem of α-Asarone group. Also the activity of CAT and GSH-R and the levels of GSH were found to increase indicating that α-Asarone reduced the oxidative cellular damage induced by sleep loss. The increase in the activity of GSH-R in striatum, hippocampus and to lesser extent in cortex was previously reported in mice after the treatment of α-Asarone (100 mg/kg, i.p.) for a week (Pages et al, 2010). Similarly, enhanced CAT activity was also reported as a result of sub-chronic administration of α-Asarone 10 mg/kg, i.o. for 16 days in Alzheimer's rat model (Limon et al, 2009). Furthermore, administration of α-Asarone reversed the increased MDA levels in various regions of brain in mice treated with scopolamine (α-Asarone 10 mg/kg, i.p. for 15 days) and rats exposed to noise stress (α-Asarone 3-10 mg/kg, i.p. for 30 days) (Kumar et al, 2012; Manikandan and Devi, 2005).

5. Mechanism of action of α-Asarone

It is evident from the present study that the longer NREM sleep bouts indicating improved sleep quality appeared when the range of T_{hy} and T_{body} was narrow (within 37.01±0.2 and 36.93±0.1 ºC) and when their difference was the least (within a narrow range of ~0.1 ºC). Evidently, 10 mg/kg α-Asarone administration moderately reduced both T_{hy} and T_{body} (0.5 to 0.6 ºC) with the difference between T_{hy} and T_{body} maintained to ≤0.1 ºC in comparison to all the other treatments. This probably suggest that this dose was conducive for the appearance of longer bouts along with an increased association between the NREM sleep bout durations and T_{hy} and T_{body}. This further substantiate the possibility that α-Asarone (at 10 mg/kg) improves sleep by modulating T_{hy} and T_{body}.

On the other hand, higher doses of α-Asarone induced a fall in the T_{hy} and T_{body} (> 1 ºC), and produced a huge variation between T_{hy} and T_{body} (> 0.3 ºC) clearly indicating that the higher doses lead to reduced and fragmented sleep. Behavioral abnormalities at higher dose of α-Asarone (80 and 120 mg/kg) and the short-term paroxysmal epileptic-like EEG after administration of 120 mg/kg α-Asarone revealed the importance of an optimal doses for appropriate responses. In a previous study, hypothermia-associated abnormal behavior after injection of α-Asarone at a dose of 60 mg/kg/i.p. was reported in mice (Pages et al, 2010). The dose dependent biphasic effect of α-Asarone is also reported in rodent models of anxiety and depression (Chellian et al, 2016; Han et al, 2013; Liu et al, 2012). Reduced sleep quality with high doses could

probably be due to the decrease in body temperature. This study showed that the low dose of α-Asarone (10 mg/kg/i.p.) is conducive for good quality sleep.

During SD, the minimum difference observed between T_{hy} and T_{body} after α-Asarone administration might have aided in promoting the quality sleep. On the other hand, the reduced sleep quality in the midazolam-treated sleep deprived rats might be due to the increased difference between T_{hy} and T_{body} and the reduced association between NREM bout duration and T_{hy} during the recovery period. There were earlier reports which had expressed doubts about the quality of sleep produced by midazolam (Lancel et al, 1996). Furthermore, it is evident from the present study that the positive correlation between T_{hy} and REM sleep bout duration is lost as a result of SD. However the appearance of the positive association between T_{hy} and REM sleep bout duration confirms the role of α-Asarone in preserving REM sleep. Poor correlation observed between T_{hy} and REM sleep bout duration after midazolam treatment might have resulted in the reduction of REM sleep.

The present study confirmed that the appearance of longer bouts of NREM sleep was facilitated when there is a moderate decrease in T_{hy} and T_{body} and when the difference between these two parameters were minimum. Administration of α-Asarone 10 mg/kg not only reduced T_{hy} and T_{body} moderately but also kept the gradient between T_{hy} and T_{body} minimum in both normal and SD rats. This in turn improved the association between sleep and thermoregulation, thus improving the quality of sleep. Interaction of α-Asarone with the transient receptor potential vanilloid channel 4 (TRPV4) in the hypothalamic region may be responsible for the reduction in T_{hy} and T_{body}.

TRPV4 present in the hypothalamic region is activated at normal body temperature and promotes hypothermic effect (Everaerts et al, 2010).

Apart from modulating T_{hy} and $T_{body,}$ α-Asarone also functioned as an anxiolytic and an antioxidant in the SD model. Increased antioxidant level and decreased levels of lipid peroxidation in the brain after α-Asarone administration is also suggested to be one of the mechanisms for anxiolysis (Kumar et al, 2012; Manikandan et al, 2013; Reddy et al, 2014) which probably facilitated good sleep. Furthermore, SD-induced hyperthermia might have produced anxiety (Shibasaki et al, 2017) which may be associated with an increase in oxidative stress (McAnulty et al, 2005). In α-Asarone-treated rats, the rapid normalization of T_{hy} and T_{body} in the α-Asarone-treated rats and also the minimal gradient between these two parameters with improved association of sleep with thermoregulation might have resulted in reduced oxidative stress which in turn resulted in anxiolysis. However, it may be noted that the hypothermic and anxiolytic properties are not completely dependent on each other since in the midazolam-treated rats anxiolysis was observed even when the association between sleep and thermoregulation was less.

Hence, it can be concluded that all the three properties of α-Asarone (hypothermic, anxiolytic and antioxidant) interacted with each other and eliminated the SD-induced changes i.e. increase in T_{hy} and $T_{body,}$ anxiety and oxidative stress (Fig. 46). The present study thus confirms that α-Asarone at a dose of 10 mg/kg given intra-peritoneally improved the quality of sleep via its cooling, anxiolytic, and antioxidant properties (Fig. 47).

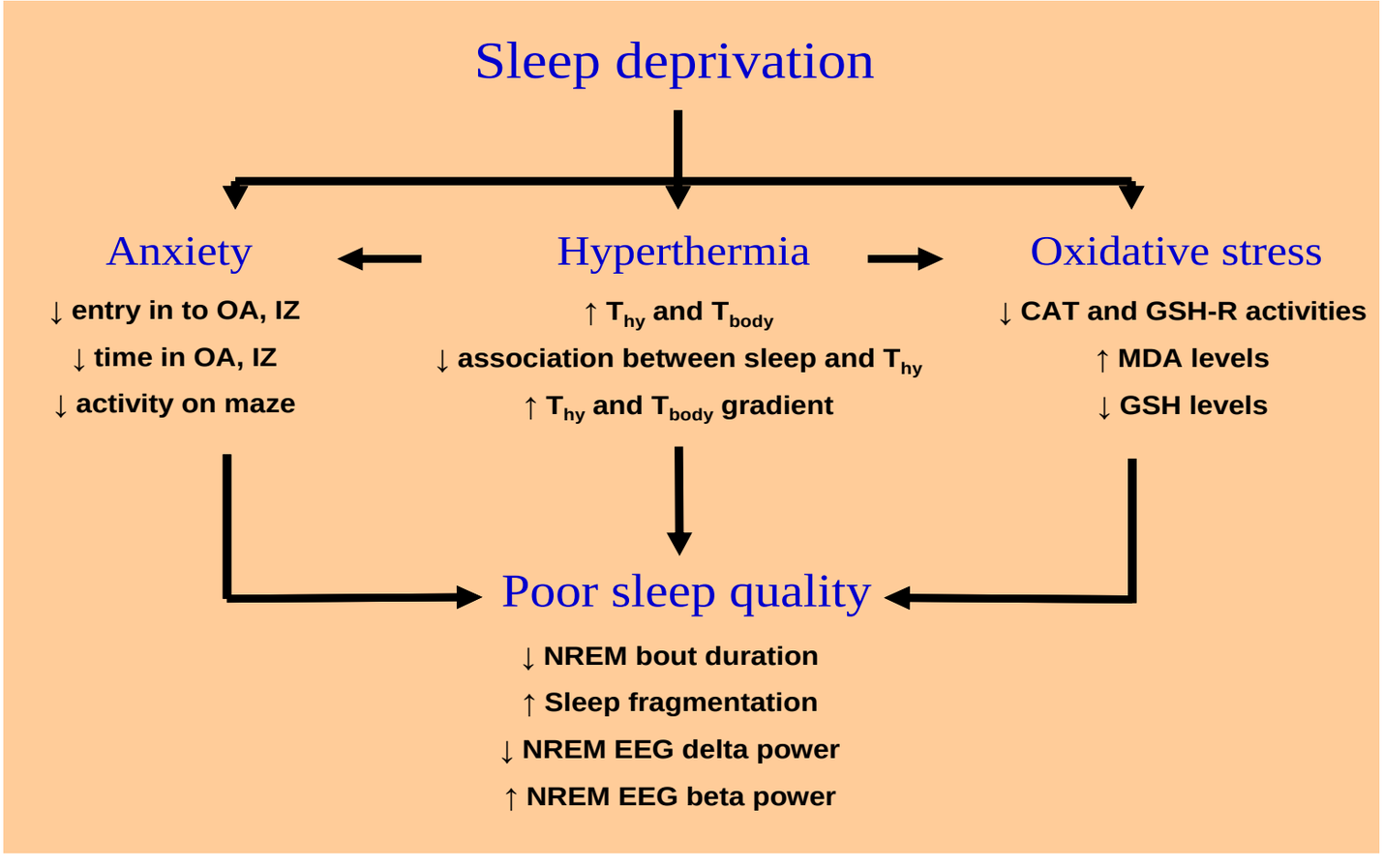

Fig. 46 Changes observed during sleep deprivation; OA represents open arm in EPM, IZ represents inner zone in OFT, T_{hy} represents hypothalamic temperature, T_{body} represents body temperature, CAT represents catalase, GSH-R represents glutathione reductase, MDA represents malondialdehyde and GSH represents total glutathione.

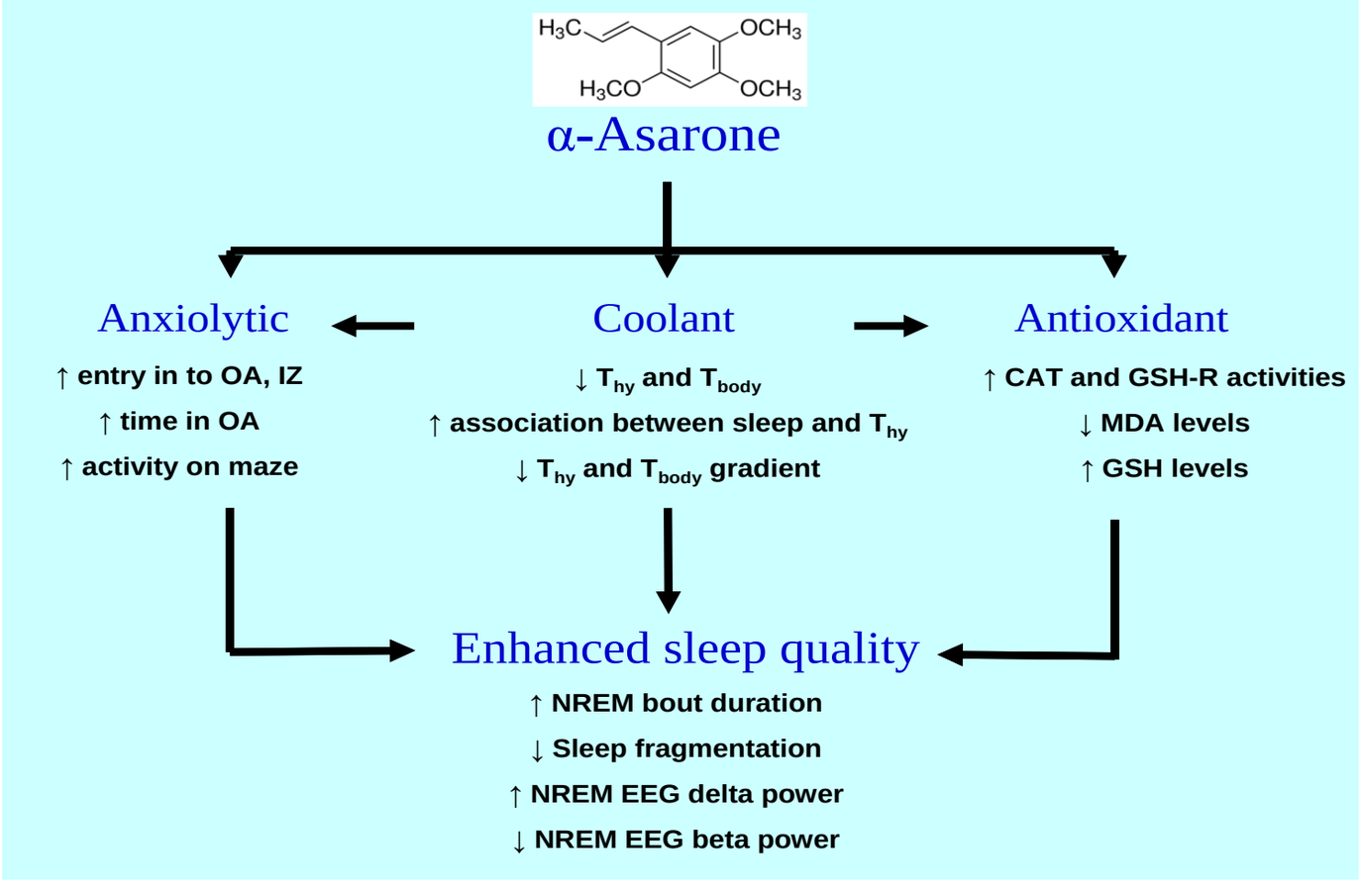

Fig. 47 Mechanism of action of α-Asarone; OA represents open arm in EPM, IZ represents inner zone in OFT, T_{hy} represents hypothalamic temperature, T_{body} represents body temperature, CAT represents catalase, GSH-R represents glutathione reductase, MDA represents malondialdehyde and GSH represents total glutathione.

6. Clinical significance of the study

In the present study α-Asarone at 10 mg/kg has been found to reduce the core body temperature which facilitated an improvement in the sleep especially the quality of sleep. α-Asarone also improved the relationship of sleep with thermoregulation. If we look from the clinical point of view, these properties of α-Asarone will be very important in the patients with insomnia, who have elevated core body temperature leading to severel sleep disturbances. Moreover, the anxiety associated with both acute and chronic SD was alleviated by α-Asarone in the present study. This would be highly beneficial for the insomnia patients who suffer from anxiety. Enhanced antioxidant property of α-Asarone may provide neuroprotection for patients with insomnia. In terms of safety and efficacy we found that α-Asarone at lower dose produced hypnotic-anxiolytic effect without developing tolerance or withdrawal effect unlike any other clinically used medications for insomnia treatment. This is a major breakthrough as there is no therapeutic intervention for insomnia with minimal side-effects and maximum benefits.

7. Future directions

- The effect after oral dosing should be investigated before proceeding to clinical trials.
- Intra-cerebral administration and receptor level activity using patch clamp studies need to be evaluated to understand the target of the drug in brain.
- Newer compounds from α-Asarone may be derived and tested for improving its properties.

Chapter VI: Conclusion

The current study provided strong evidences that α-Asarone at a dose 10 mg/kg/i.p. improves the quality of sleep in normal and SD rats. Moderate reduction and minimum variation between T_{hy} and T_{body}, enhanced the association between sleep and T_{hy}, thereby improving the quality of sleep. Furthermore, anxiolytic and antioxidant properties of α-Asarone also facilitated improvement in the quality of sleep. Hence, α-Asarone may be considered as a potent herbal compound for the treatment of insomnia. This study lays the foundation for developing newer generation of molecules with similar structure but with a better hypnotic property.

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Annexures

LIST OF PUBLICATIONS

From thesis

1. **Radhakrishnan A**, Jayakumari N, Kumar VM, Gulia KK (2017) Sleep promoting potential of low dose α-Asarone in rat model. Neuropharmacology 125: 13-29. **IF: 5.106**

Other publications

1. Sivadas N, **Radhakrishnan A**, Aswathy BS, Kumar VM, Gulia KK (2017) Dynamic changes in sleep pattern during post-partum in normal pregnancy in rat model. Behav. Brain Res. 320: 264-274. **IF: 3.002**

2. Gulia KK, **Radhakrishnan A**, Kumar VM (2017) Approach to Sleep Disorders in the Traditional School of Indian Medicine: Alternative Medicine II. In *Sleep Disorders Medicine*, pp 1221-1231. Springer. **Book Chapter**

3. **Radhakrishnan A**, Aswathy BS, Kumar VM, Gulia KK (2015) Sleep deprivation during late pregnancy produces hyperactivity and increased risk-taking behavior in offspring. Brain Res. 1596: 88-98. **IF: 2.746**

4. Gulia KK, Patel N, **Radhakrishnan A**, Kumar VM (2014) Reduction in ultrasonic vocalizations in pups born to rapid eye movement sleep restricted mothers in rat model. PLoS One 9: e84948. **IF: 2.806**