Journal of Advanced Zoology
ISSN: 0253-7214
Volume 45 Issue 2 Year 2024 Page 1825:1841

Unveiling the Anxiolytic Potential of *Epimedium* Extract in Sleep-Deprived Mice

Zoya Khan[1*], Sakshi Panwar[2], Shriya Samant[3], Ayush Mishra[4]

[1*,2]*HIMT College of Pharmacy, Greater Noida*
[3,4]*IIMT College of pharmacy, Greater Noida*
Email id:- sakshipanwar0704@gmail.com

***Corresponding Author:** Sakshi Panwar*
[*]*HIMT College of Pharmacy, Greater Noida*

Abstract

Aim: To investigate the anxiolytic potential of Epimedium extract in sleep-deprived mice compared to diazepam.

Objective: To evaluate the anxiolytic effects of Epimedium extract in sleep-deprived mice, using behavioral and biochemical assays, and compared the effects with those of diazepam.

Background: Anxiety disorders and sleep deprivation commonly coexist, worsening each other's symptoms and impacting overall well-being. Epimedium L, a traditional herbal remedy, is known for its adaptogenic properties, which may offer relief from stress-related conditions.

Method: Male Swiss albino mice were divided into five groups: control, sleep-deprived, two treatment groups of Epimedium and one standard group of diazepam. The effects of treatment were assessed using various behavioral tests, such as actophotometer, elevated zero maze, and light/dark test. Followed by biochemical assays measuring levels of oxidative stress markers, neurotransmitters, and enzymes associated with anxiety

Result: The study found that Epimedium extract significantly reduced anxiety-like behavior in sleep-deprived mice, with effects comparable to diazepam in a dose dependent manner. The extract also influenced oxidative stress markers and neurotransmitter levels, suggesting a multifaceted mechanism of action.

Conclusion: Epimedium extract has demonstrated significant potential as an effective anxiolytic agent, offering a safer alternative to diazepam due to its reduced side effect profile. This positions it as a promising option for managing anxiety disorders.

Keywords: Epimedium extract, Anti-anxiety activity, Sleep deprivation, Mice, Anxiety disorders, Behavioral assays

1. Introduction

Anxiety is a kind of psychological reaction that individuals have to situations that threaten their spiritual and financial well-being. Since anxiety disorders rank highest among mental illnesses, their prevalence is believed to be between 10 and 30%.[1] Drugs that are sedative and anti-anxiety are thus often employed, due to their]calming, hypnotic, and relaxing effects on the muscles and organs, the majority of anti-anxiety medications lead to both physical and mental addiction.[2]

Diazepam is a well-known anti-anxiety medication that has been shown to have effects on the central nervous system. Since, diazepam interacts with brain GABA receptors, notably in the midbrain reticular formation, it has palliative and anti-anxiety properties.[3] Memory loss, extreme sleepiness, slurred speech, bradycardia, dyspnea, ataxia, unusual bleeding or bruises, and mouth ulcers are among the side effects of diazepam.[4] When it is abruptly stopped, it results in withdrawal syndrome. Seizures, tremors, anxiety, sleeplessness, and irritability are some of the symptoms of this condition. However, herbal medications offer several benefits over chemical treatments since their active components are combined and balanced with other substances, do not build up in the body, and have no negative side effects.[5]

The World Health Organization [WHO] has repeatedly stressed the need for traditional medicine and herbal remedies to be used in a scientific and cost-effective manner. One of the most significant problems facing the globe in recent decades, particularly in emerging nations, is this strategy.[6] The use of medicinal plants has increased in the modern era due to the discovery of several of their physiological characteristics, including anti-cancer, anti-sensitivity, and anti-diabetic effects. There are reports on how flavonoids affect benzodiazepine receptors.[7] The active flavonoids found in medicinal plants influence the binding of benzodiazepine to GABA-A receptors, which has a calming and anti-anxiety effect.[8]

Epimedium is a plant that is often used in Traditional Chinese medicine (TCM) and herbal teas. The perennial forest plants of the genus Epimedium are found between 650 and 3000 meters above sea level in thickets and on slopes under woodlands. In the Berberidaceae family, Epimedium is the largest herbaceous genus.[9] The disjunctively and extremely unevenly distributed old World species Epimedium inhabits woodlands and scrubs in the Mediterranean region, western Asia, and eastern Asia. Japan, Korea, north-eastern China, far-eastern Russia, five species in Algeria and the Caucasus, and around fifty-one species in central-southeastern China.[10] China is the distribution and diversification center of the Epimedium.[11] The dried aerial parts of Epimedium wushanense T.S. Ying. (E. wushanense), Epimedium sagittatum Maxim (E. sagittatum), Epimedium brevicornum Maxim (E. brevicornum), Epimedium koreanum Nakai (E. koreanum), and Epimedium pubescens Maxim (E. pubescens) are used to make herbal epimedium in the Chinese provinces of Sichuan, Shanxi, Liaoning, and Gansu.[12] To process Herbal Epimedium therapeutically, mixtures of butter, wine, suet, and fire moxibustion are added. Stir-frying with suet is the most often used technique among them.[13] Flavonoids make up the main constituent of Epimedium; the most prevalent kind are called Epimedium flavonoids (EFs), and icariin (ICA; molecular formula C33H40O15) is the active monomer.[14] Prenyl flavonoids, plant polysaccharides, alkaloids, phytosterols, terpenoids, chlorogenic acid, and other bioactive compounds are also present in epimedium. [15] Important functions are played by EFs and their progeny in the genus Epimedium. In 17 distinct Epimedium species, more than 141 flavonoids have been discovered, including chalcone, flavone, flavonol, flavonol glycoside, and flavanone. Only six phenethyl alcohol glycosides have been discovered so far from the genus Epimedium, twelve ionones and their derivatives, and thirty lignans and their corresponding glycosides have been detected. Apart from the constituents mentioned before, other compounds including acids, alkaloids, xanthones, and aldehydes were also isolated from Epimedium plants. Epimedins A, B, C, and ICA comprise at least 52% of the flavonoids found in the herbal Epimedium and are considered significant bioactive components [16].

The current study examined the effects of Epimedium extract and diazepam on anxiety reduction in mice, considering the lack of previous research on the anti-anxiety properties of Epimedium as well as the prevalence of anxiety in the population, the adverse effects of chemical drugs, and the superiority of herbal drugs.

2. Material and Method

2.1. Animals:

Swiss male albino mice (25-35 g, 3-5 months old) were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary & Animal Science (LUVAS), Hisar (Haryana). According to (Zárate et al., 2017), “Female mice were excluded from the study since estrogens (female sex hormone) have been reported to possess neuroprotective effect”. Animals were housed under controlled conditioning (22±1°C, 55–65% relative humidity and 12 h dark/light cycles). After 7 days of acclimatization to laboratory conditions, animals were randomly assigned in 5 groups, 8 mice each. Food and water were allowed ad libitum during the study period. But food was not provided 2 h before and 2 h after drug administration, to rule out the effect of food on absorption of drugs. All tests were performed between 9:00 a.m. and 3:00 p.m. to minimize circadian

influences on anxiolytic activity. The experimental protocol was approved by IAEC (Institutional Animals Ethics Committee) in its meeting held 6th August, 2023.

2.2. Drugs and chemicals

All the drugs solutions and suspensions were freshly prepared before use. Epimedium was procured from Sigma-Aldrich,USA, and Diazepam injection was procured from LORI ®, NEON laboratories Ltd.; India. Reagents used in this study were analytical grade. Drugs were administered Intraperitoneally (i.p.). All solutions were administered in a volume of 0.1ml/10g of body weight.

2.3. Anxiety effect:

Effect of the drugs on locomotor activity was evaluated after the administration of drugs on the last day (14th day) of the curement, for sleep deprivation mouse was placed in polypropylene cages (29x15x7cm) filled with water up to 2 cm from the bottom of cages and covered with top wire lid. The rods of top wire lid were l cm apart from each other. Mouse will try to hold the rods and as soon as the mouse will try to sleep, they will lose their muscle tone and thus grip over the rods. This will make the mouse fall in water and thereby keep them awakened. Hence, sleep induced anxiety were caused.

2.4. Experimental design

Treatment schedule

Following 5 groups of mice were constituted (n=8 each group)

Unstressed mice:
Group 1: Vehicle (0.9 % saline) administered i.p, for 14 consecutive days.
Group 2: Sleep deprived group, for 72 hours
Group 3: SD +Epimedium (25 mg/kg) administered i.p, for 14 consecutive days.
Group 4: SD +Epimedium (50 mg/kg) administered i.p, for 14 consecutive days.
Group 5: SD +Diazepam (2mg/kg) administered i.p, for 14 consecutive days.

In this study, the effect of Epimedium on anxiety due to sleep deprivation was investigated. All the animals were divided into 5 groups. Group I was vehicle treatment normal control group, which only vehicle was injected. Group II was inducing group in which animals were sleep deprived for 72 hours. Group III & IV were the treatment group which was sleep deprived for 72 hours and then treated with Epimedium in 2 doses (25 mg/kg;i.p. and 50 mg/kg;i.p., respectively) for 14 consecutive days. The last Group V was positive control group in which mice were sleep deprived for 72 hours and then Diazepam (2mg/kg) was administered for 14 consecutive days.

2.5. Behavioral parameter:

2.5.1. Actophotometer

The mice locomotor behavior was monitored using actophotometer. Mice were placed in actophotometer individually, and basal activity score was recorded over the period of 5 min. Each mouse was treated with respective drug, and activity score was recorded after 30 min and 1 h. Decreased activity score was taken as index of CNS depression. [17]

The spontaneous locomotor activity of each mouse was recorded in square arena of actophotometer (Inco, Ambala, India) individually for ten minutes [18]. Actophotometer registers of the number of times IR photobeams of light were interfered, as the mouse moved inside the cage. Each mouse was placed in the centre of the metal cage of actophotometer and ambulatory activity was measured. The arena was cleaned with dilute alcohol and dried between trials [17] to avoid any experimental interference.

The locomotor activity was measured on innate pretreated mice by an actophotometer. Actophotometer functioned on photoelectric cells that were attached in circuit with a counter. When the ray of light dropping on the photocell was cut off by the mouse, a count was noted. These cutoffs were calculated for a period of 10 min and the number was used as a degree of the locomotor activity of the mouse.[19]

2.5.2. Elevated zero maze:

The elevated zero maze (Zero) was created to eliminate the center region of the Plus and has also been pharmacologically validated with anxiolytic.[20] Unfortunately, no direct comparison of these two mazes has been reported in mice. The Zero is an elevated ring-shaped runway with the same amount of area devoted to adjacent open and closed quadrants. The Zero has not been used as extensively as the Plus but has seen increased use in recent years. In mice, a recent study found the Zero was more sensitive to benzodiazepines than the Plus however no adjustment for center time in the Plus was made.[21]

The maze was constructed of black acrylic in a circular track 10 cm wide, 105 cm in diameter, and elevated 72 cm from the floor (San Diego Instruments, San Diego, CA). The maze was divided in four quadrants of equal length with two opposing open quadrants with 1 cm high clear acrylic curbs to prevent falls and two opposing closed quadrants with black acrylic walls 28 cm in height. A 5 min trial under the same lighting conditions as in the Plus began with the mouse placed in the centre of a closed quadrant. Dependent measures were the same as for the Plus except that there was no centre region. Between trials, the maze was cleaned with 70% ethanol.[22]

2.5.3. Light/Dark test:

The light/dark (LD) test is based on an approach-avoidance conflict between exploration of novel environments and avoidance of brightly lit, open spaces.[23] The test was developed by Crawley and colleagues, who observed that anxiolytic drugs increased the number of crossings between compartments.[24] Later studies showed that time in the light compartment and distance traveled in the light also reflect anxiety-like behavior and expanded the use of the LD test in mice.[25] The LD test has been widely used to assess anxiety-like behavior in adult rodents, and a few studies have utilized this test in younger animals.

Mouse was tested in Kinder locomotor boxes (Kinder Scientific, Inc. Poway, CA) (40 cm × 40 cm × 30cm) with black plastic inserts that occupied half of the locomotor box. Both age groups were tested in the same locomotor boxes. The two compartments were connected by a small opening (7.5 cm × 8.5 cm) that was covered by a sliding door. The room was lit by two incandescent lamps so that brightness of the light side of each box averaged 65 lx. Mouse was placed in the dark half of the box and testing began as the door to the light side of the box was raised. Time (s) and distance travelled (cm) in each compartment were measured for 15 min using infrared photobeams and software from the manufacturer. We have successfully used Kinder locomotor boxes to measure activity in adolescent and adult rats as well as adult mice, and do not anticipate any confounding effect of the size difference in adult and adolescent mice on measurement of activity.[26] The latency to emerge into the light compartment was determined by dividing the session into 5 s bins and determining the bin of first light entry. The following behavioural measures were recorded: time spent in the light compartment, distance travelled in the light and in the dark compartments, total distance travelled, the percent of total distance travelled in the light compartment, the number of entries into the light compartment, the number of pokes into the light compartment, the latency to emerge into the light compartment, and total rearing across both compartments.[27]

2.6. Biochemical estimation:

2.6.1. MDA

Malondialdehyde (MDA) levels can be quantified using the thiobarbituric acid reactive substances (TBARS) assay, a widely used method for assessing lipid peroxidation and oxidative stress. The process begins with the preparation of biological samples, such as serum or brain tissue, which are homogenized in a suitable buffer to ensure consistency and accuracy. Following homogenization, thiobarbituric acid (TBA) is added to the sample. The mixture is then heated, which promotes the reaction between MDA and TBA, resulting in the formation of a colored complex. This reaction is key to the assay, as the intensity of the color produced is directly proportional to the concentration of MDA in the sample. The absorbance of the resultant solution is measured at 532 nm using a spectrophotometer. By comparing the absorbance values to a standard curve, the MDA concentration in the samples can be determined, providing insight into the degree of oxidative stress and lipid peroxidation present in the biological system.[28]

2.6.2. Plasma

Plasma, the liquid component of blood, is essential for transporting cells, nutrients, hormones, and waste products throughout the body. It plays a crucial role in physiological processes, particularly those related to stress and anxiety. Changes in the plasma levels of certain hormones and neurotransmitters, such as cortisol—a primary stress hormone—can significantly impact anxiety. Elevated cortisol levels in plasma are commonly linked to anxiety disorders, making the analysis of plasma an important tool for understanding these conditions. To measure these components, blood samples are first collected via standard venipuncture. The samples are then centrifuged at high speeds to separate the plasma from the blood cells. Finally, specific hormones or neurotransmitters in the plasma are quantified using techniques such as enzyme-linked immunosorbent assay (ELISA) or mass spectrometry, providing valuable insights into the biochemical factors influencing anxiety.[29]

2.6.3. GABA

GABA (gamma-aminobutyric acid) is the primary inhibitory neurotransmitter in the brain, playing a vital role in reducing neuronal excitability and maintaining balance in neural activity. Low levels of GABA are closely linked to increased anxiety and various mood disorders, as the lack of sufficient inhibitory control can lead to heightened neural activity and stress responses. As a result, enhancing GABAergic activity is a key target for many anxiolytic medications, which aim to increase GABA levels or mimic its action to alleviate symptoms of anxiety.

The measurement of GABA levels involves a few precise steps. First, samples are collected from brain tissue or plasma, depending on the focus of the study. High-Performance Liquid Chromatography (HPLC) with fluorescence detection is commonly used to separate and quantify GABA due to its sensitivity and specificity. Alternatively, mass spectrometry can be utilized for more precise measurements, offering detailed insights into the concentration of GABA in the samples. These methods are crucial for understanding the role of GABA in anxiety and for assessing the efficacy of treatments aimed at enhancing GABAergic function.[30]

2.6.4. SOD

Superoxide dismutase (SOD) is a critical enzyme that protects cells from oxidative stress by catalyzing the dismutation of superoxide radicals into oxygen and hydrogen peroxide. This enzymatic action is essential for mitigating oxidative damage in cells, which is particularly important in maintaining cellular health and preventing stress-related conditions. Reduced SOD activity has been associated with heightened levels of oxidative stress, which can contribute to increased anxiety and other stress-related disorders. By neutralizing superoxide radicals, SOD plays a protective role in the body, making its activity an important marker for assessing oxidative stress and its potential impact on mental health.

The activity of SOD can be measured through a series of precise steps. First, tissue or cell samples are homogenized in a suitable buffer to prepare them for analysis. The SOD activity is then measured using a spectrophotometric assay, which typically involves a colorimetric method that quantifies the enzyme's ability to inhibit the reduction of a tetrazolium salt by superoxide radicals. The change in absorbance during the reaction is monitored, and these changes are compared to a standard curve or control samples to calculate the SOD activity. This method provides a reliable assessment of SOD's ability to protect cells from oxidative damage, which is crucial in understanding its role in anxiety and stress-related disorders.[31]

2.6.5. CAT

Catalase (CAT) is a vital enzyme that decomposes hydrogen peroxide into water and oxygen, thus playing a crucial role in protecting cells from oxidative damage. By breaking down hydrogen peroxide, a potentially harmful byproduct of cellular metabolism, catalase helps to maintain cellular integrity and prevent oxidative stress. Dysregulation or reduced activity of catalase can lead to an accumulation of hydrogen peroxide, which contributes to oxidative stress—a condition often associated with the development and progression of anxiety disorders. Understanding and measuring catalase activity is, therefore, important in exploring its connection to oxidative stress in mental health conditions.

The activity of catalase can be accurately measured using a spectrophotometric assay. First, tissue or plasma samples are prepared to ensure that the enzyme remains active for analysis. The catalase activity is then measured by monitoring the rate at which it decomposes hydrogen peroxide. This reaction is observed by

measuring the decrease in absorbance at 240 nm over time using a spectrophotometer. The rate of this decrease is directly related to the activity of catalase in the sample. By calculating the rate of hydrogen peroxide decomposition, researchers can determine the catalase activity, providing insights into the enzyme's role in protecting against oxidative stress and its potential involvement in anxiety disorders.[32]

2.6.6. Nitrite

Nitrite is a stable end product of nitric oxide metabolism and plays a significant role in various physiological processes, including neurotransmission. It serves as an indicator of nitric oxide production in the body. Elevated nitrite levels are often associated with increased nitric oxide production, which has been linked to anxiety and stress responses. Nitric oxide, while essential for normal cellular functions, can contribute to oxidative stress and inflammation when produced in excess, thereby influencing mood and anxiety disorders. Measuring nitrite levels in biological samples can provide insights into the nitric oxide pathways involved in these conditions.

Nitrite levels can be accurately measured using the Griess reagent assay. The process begins with the collection of biological samples, such as plasma or urine, where nitrite concentration is to be determined. The Griess reagent, which reacts specifically with nitrite, is added to the samples. This reaction produces a colored azo dye, the intensity of which is directly proportional to the nitrite concentration in the sample. The resulting color change is measured by determining the absorbance at 540 nm using a spectrophotometer. By comparing the absorbance values to a standard curve, the exact concentration of nitrite in the sample can be quantified, providing valuable information about nitric oxide production and its potential impact on anxiety and stress responses.[33]

3. Results

3.1. Behavioral parameters:

3.1.1. Actophotometer:

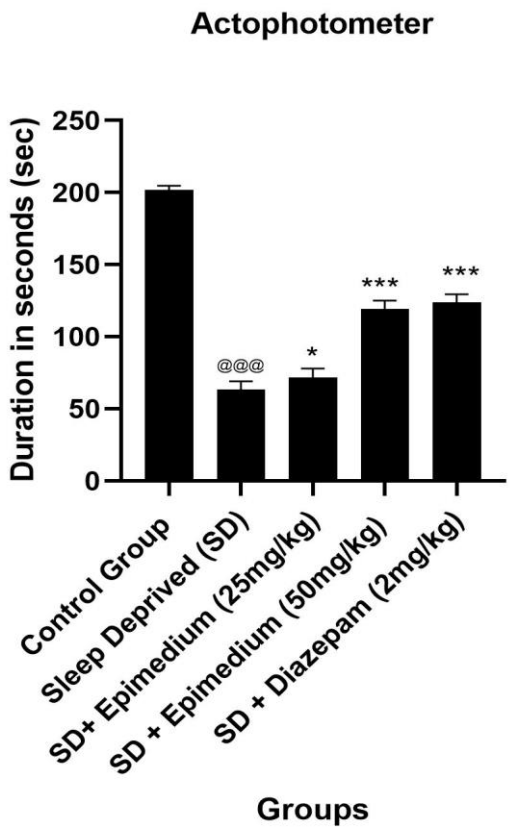

Fig: 1. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced locomotion impairment in Actophotometer. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. @@@p<0.001; @

represents control group vs sleep deprived group; *p<0.05; **p<0.01; ***p<0.001; * represents sleep deprived group vs Epimedium groups and Diazepam.

The study evaluated the effects of Diazepam and Epimedium on locomotion impairment due to sleep deprivation using an actophotometer. Epimedium at both 25 mg/kg comparison with the sleep-deprived group showed a (P value of 0.0007), however when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) substantially increase locomotion activity was observed and P value in comparison to the sleep-deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract , as reflected in the data presented in Figure 1. This suggests that Epimedium has a potent anxiolytic effect, comparable to Diazepam, in alleviating locomotor deficits induced by sleep deprivation.

3.1.2. Zero maze test:

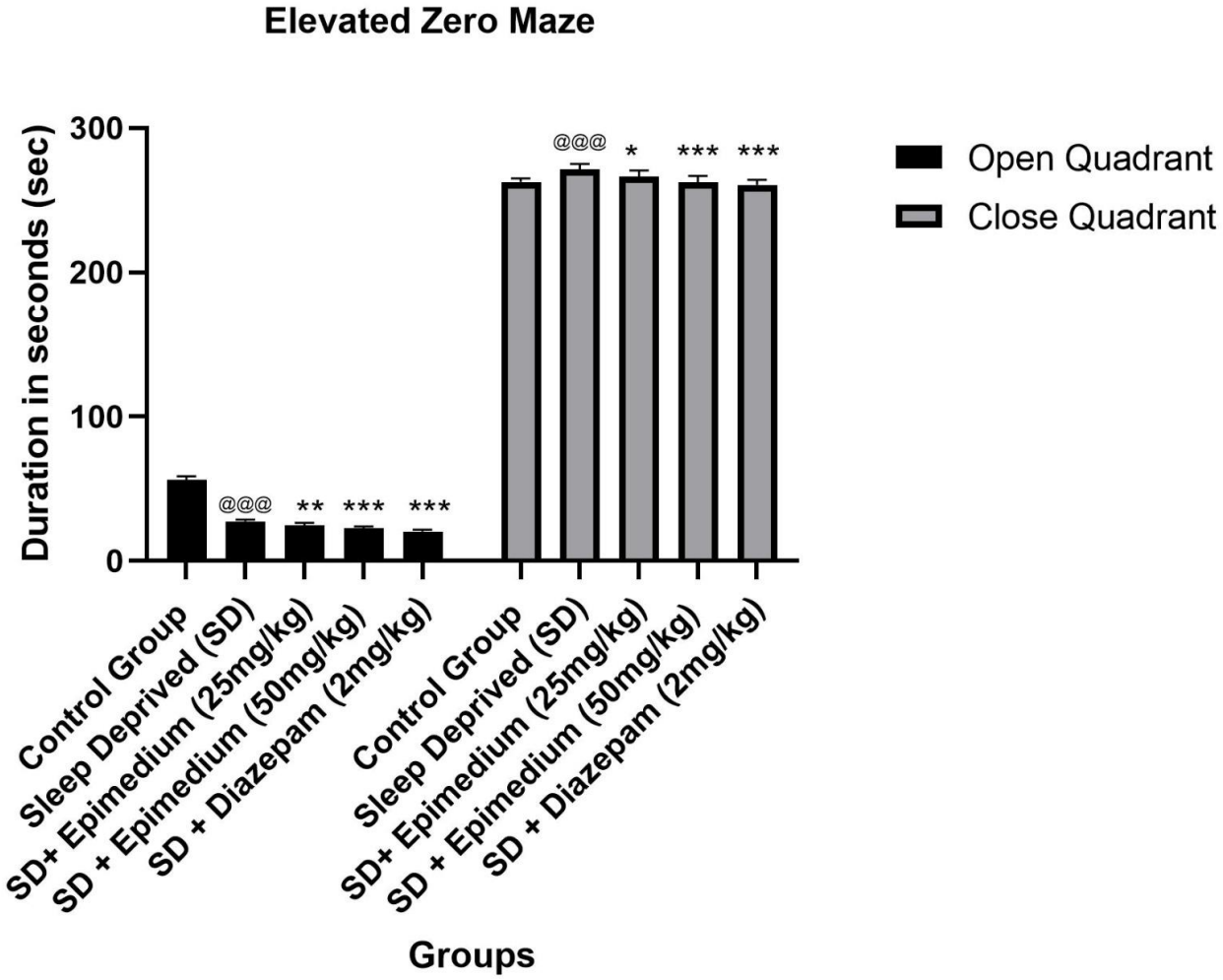

Fig: 2. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced impairment in thinking ability in Elevated zero maze. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. @@@p<0.001; @ represents control group vs sleep deprived group; *p<0.05; **p<0.01; ***p<0.001; * represents sleep deprived group vs Epimedium groups and Diazepam.

In the elevated zero maze test, sleep deprivation significantly increased anxiety-related behaviors, as evidenced by the increased time spent in the closed quadrants and the decreased number of entries into the open quadrants. Treatment with Epimedium at both 25 mg/kg comparison with the sleep-deprived group showed a (P value of 0.0007), however when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) substantially reduction in thinking impairment was observed and P value in comparison to the sleep-

deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract, but the results from Epimedium at 50 mg/kg were nearly identical to those of Diazepam, highlighting its anxiolytic efficacy. These findings are detailed in and Figure 2.

3.1.3. Light dark test

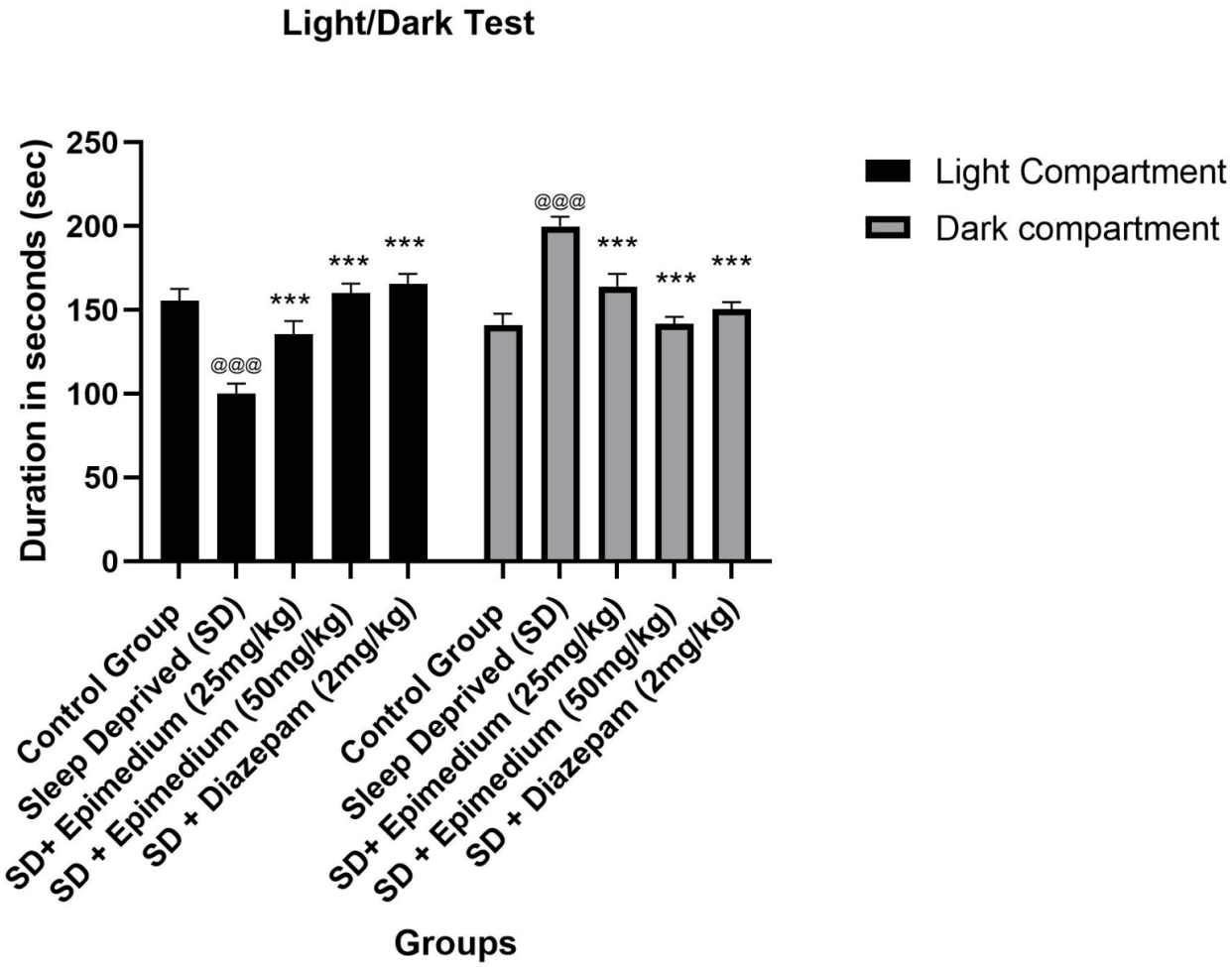

Fig: 3. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced impairment in their nocturnal vision and further ability to react in Light/Dark test. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. $^{@@@}p<0.001$; $^{@}$ represents control group vs sleep deprived group; $^{***}p<0.001$; * represents sleep deprived group vs Epimedium groups and Diazepam.

The light/dark test further confirmed the anxiolytic properties of Epimedium. The sleep-deprived group spent significantly less time in the light compartment, indicating heightened anxiety levels. However, when treated with Epimedium at both 25 mg/kg comparison with the sleep-deprived group showed a (P value of 0.0007), however, when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) substantially reducing impairment in the nocturnal activity was observed and P value in comparison to the sleep-deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract. Notably, the effects of Epimedium at 50 mg/kg were almost identical to those of Diazepam, underscoring its potential as an effective anxiolytic agent. The relevant data are presented in Figure 3.

3.2. Biochemical estimation:

3.2.1. MDA

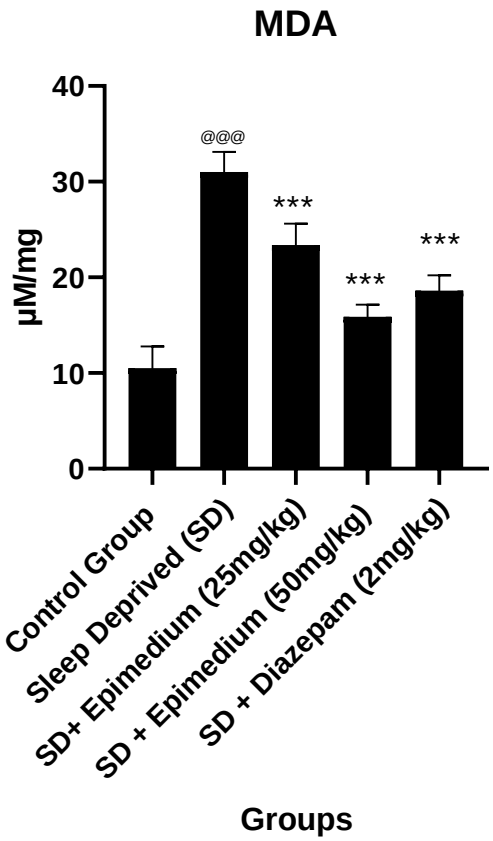

Fig: 4. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced elevation on MDA levels. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. $^{@@@}p<0.001$; $^{@}$ represents control group vs sleep deprived group; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; * represents sleep deprived group vs Epimedium groups and Diazepam.

Malondialdehyde (MDA) is a marker of oxidative stress. The results indicated that sleep deprivation significantly elevated MDA levels, reflecting increased lipid peroxidation and oxidative damage. However, treatment with Epimedium at both 25 mg/kg and comparison with the sleep-deprived group showed a (P value of 0.0007), however when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) substantially reduced MDA levels was observed and P value in comparison to the sleep-deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract. The reduction was most pronounced in the group treated with the higher dose of Epimedium (50 mg/kg), which produced effects comparable to Diazepam. This suggests that Epimedium has strong antioxidant properties, mitigating the oxidative stress caused by sleep deprivation. Shown in fig 4.

3.2.2. Plasma Cortisol

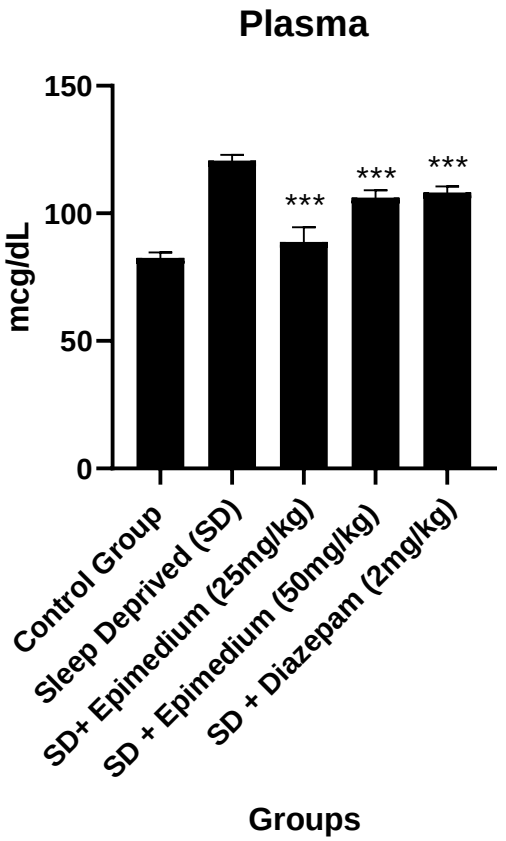

Fig: 5. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced lower level of Plasma Cortisol levels. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. $^{@@@}p<0.001$; $^{@}$ represents control group vs sleep deprived group; $^{***}p<0.001$; * represents sleep deprived group vs Epimedium groups and Diazepam.

Plasma levels were measured to assess the general physiological response to sleep deprivation and the effects of the treatments. Sleep deprivation led to significant alterations in plasma markers, indicative of systemic stress. Treatment with Epimedium at both 25 mg/kg and comparison with the sleep-deprived group showed a (P value of 0.0007), however when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) substantially helped restore plasma levels towards those observed in the control group, suggesting a protective effect and the P value in comparison to the sleep-deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract helped restore plasma levels towards those observed in the control group, suggesting a protective effect. The higher dose of Epimedium (50 mg/kg) was particularly effective, demonstrating results nearly identical to those of Diazepam. Shown in fig 5.

3.2.3. GABA

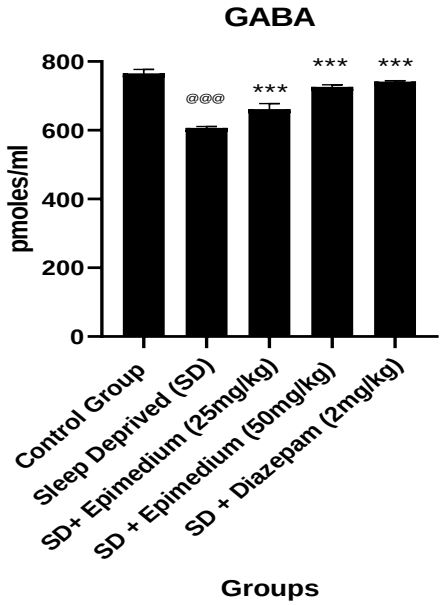

Fig: 6. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced elevated level of GABA levels. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. $^{@@@}p<0.001$; $^{@}$ represents control group vs sleep deprived group; $^{***}p<0.001$; * represents sleep deprived group vs Epimedium groups and Diazepam.

GABA is a major inhibitory neurotransmitter in the brain, often associated with anxiolytic effects. The study showed that sleep deprivation significantly decreased GABA levels, contributing to increased anxiety. Treatment Epimedium at both 25 mg/kg and comparison with the sleep-deprived group showed a (P value of 0.0007), however when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) not significantly elevated GABA levels in sleep-deprived animals but the P value in comparison to the sleep-deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract. The increase was dose-dependent, with the 50 mg/kg dose of Epimedium producing an effect similar to that of Diazepam, highlighting its potential in modulating neurotransmitter levels and reducing anxiety. Shown in fig 6.

3.2.4. SOD

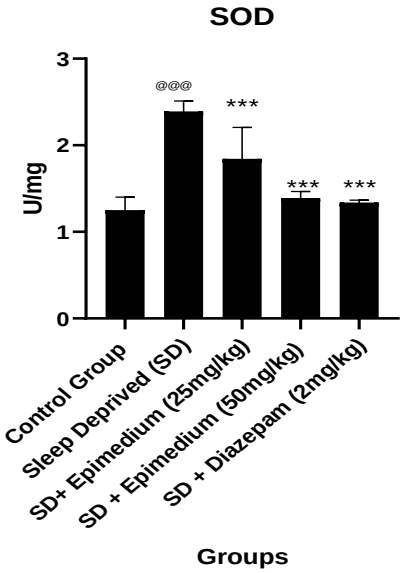

Fig: 7. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced elevation of SOD levels. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. $^{@@@}p<0.001$; $^{@}$ represents control

group vs sleep deprived group; *p<0.05; **p<0.01; ***p<0.001; * represents sleep deprived group vs Epimedium groups and Diazepam.

Superoxide dismutase (SOD) is an important antioxidant enzyme that protects against oxidative stress. Sleep deprivation significantly reduced SOD levels, indicating impaired antioxidant defenses. Epimedium at both 25 mg/kg and comparison with the sleep-deprived group showed a (P value of 0.0007), however, when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) the P value in comparison to the sleep-deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract. This suggests that Epimedium enhances the antioxidant capacity in sleep-deprived animals, protecting against oxidative damage. Shown in fig 7

3.2.5. CAT

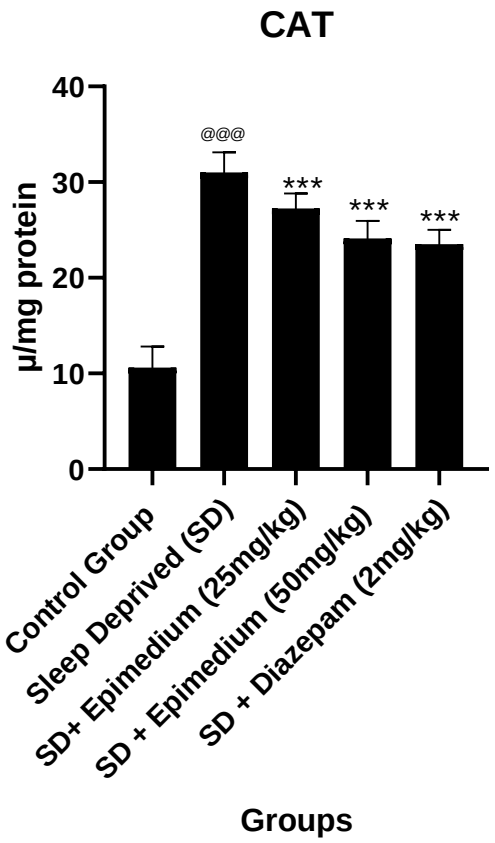

Fig: 8. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced elevation in CAT levels. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. @@@p<0.001; @ represents control group vs sleep deprived group; ***p<0.001; * represents sleep deprived group vs Epimedium groups and Diazepam.

Catalase (CAT) is another crucial antioxidant enzyme that helps break down hydrogen peroxide into water and oxygen, reducing oxidative stress. Sleep deprivation resulted in a significant decrease in CAT levels, reflecting increased oxidative stress. Epimedium at both 25 mg/kg and comparison with the sleep-deprived group showed a (P value of 0.0007), however, when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) not significantly elevated CAT levels in the sleep-deprived groups but the P value in comparison to the sleep-deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract. The 50 mg/kg dose of Epimedium restored CAT levels to near those seen with Diazepam, indicating its strong antioxidant effects in counteracting sleep deprivation-induced oxidative stress. Shown in fig 8.

3.2.6. Nitrite

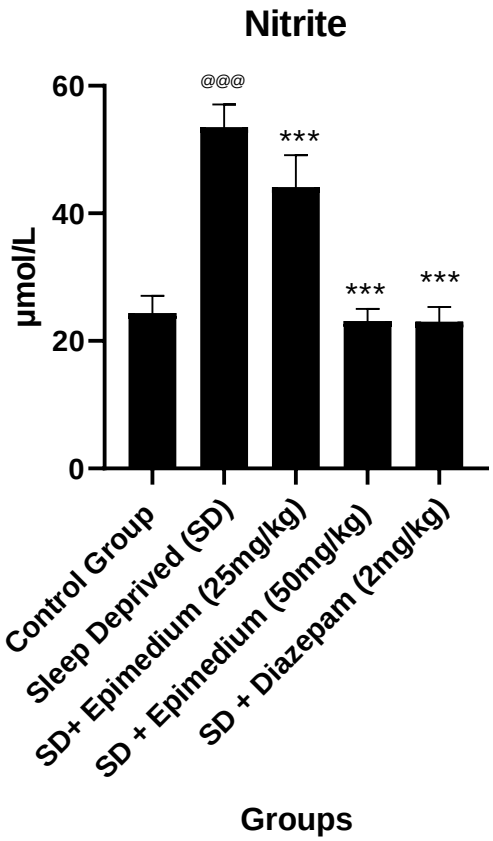

Fig: 9. Effect of Epimedium against anxiolytic effect due to sleep deprivation induced elevation in Nitrite levels. Data represented as mean±sd, using unpaired t-test and one-way ANOVA. $^{@@@}p<0.001$; $^{@}$ represents control group vs sleep deprived group; $^{***}p<0.001$; * represents sleep deprived group vs Epimedium groups and Diazepam.

Nitrite levels were measured as an indicator of nitric oxide production, which can contribute to oxidative stress and inflammation. Sleep deprivation significantly increased nitrite levels, suggesting enhanced nitric oxide production. Epimedium at both 25 mg/kg and comparison with the sleep-deprived group showed a (P value of 0.0007), however, when the dose was increased to 50mg/kg and further comparison was done with a sleep-deprived group it showed the (P<0.0001) suggesting it dose depended on the activity and at Diazepam (2 mg/kg) not significantly elevated nitrite levels in the sleep-deprived groups but the P value in comparison to the sleep-deprived group was found to be <0.0001 which further shows almost similar dose dependency with the 50mg/kg epimedium extract. The reduction was most notable at the higher dose of Epimedium (50 mg/kg), which showed similar effects to Diazepam. This indicates that Epimedium effectively reduces nitrite levels, thereby potentially mitigating the inflammatory and oxidative effects of sleep deprivation. Shown in fig 9.

4. Discussion

Sleep deprivation anxiety is a condition where due to lack of sleep, anxiety is likely to occur. The condition is initially characterized by poor decision making and in extreme cases loss of coordination and further reaction time. The main reason for such characteristics is still not very clear. However, some researchers suggest that due to changes in the biochemicals present in the brain such characteristics occur. In the present world due to changes in lifestyle sleep deprivation anxiety cases are increasing at an alarming rate which the WHO considers as a major challenge. However, the condition can be controlled by utilizing medication like Diazepam which helps in reducing anxiety and its further symptoms.

However, the main issue with the anti-anxiety drugs has several adverse effects. Therefore, a better safer option is required. Hence, in our present study, we have utilized the extract of Epimedium a plant which said to have antioxidant and anti-inflammatory action. It can cross the blood-brain barrier and therefore can be utilized in treating conditions like sleep-induced anxiety. In the present study, Epimedium extract was used in two doses

25 and 50mg/kg; p.o. to assess the dose-dependent effect. The Sleep-induced anxiety was caused by forcefully not letting the mice to sleep. A controlled group was also prepared in which the mice were perfectly healthy and received sleep. A negative controlled sleep deprived group was prepared in which the mice were not administered any drug. For the sake of comparison, we administered diazepam as a standard drug of choice in one mice group. The mice were further, evaluated on behavioral parameters and later biochemical tests were performed.

To establish that epimedium extracts have anti-anxiety effects the test like actophotmeter, zero-maze test and light and dark test were performed, and the results were evaluated. The study it was observed that the group receiving plant extract 25mg/kg showed some improvement in behavioral parameters during the actophotometer experiment the result obtained suggests that 25mg/kg dose in sleep-deprived mice increased the locomotion compared to only the sleep-deprived group, However, the better results were observed when the dose increase to 50mg/kg as this dose have comparable effect with the diazepam 2mg/kg. Hence, this suggests that the epimedium indeed has some positive effect in sleep-deprived mice (See figure 1.).

In the elevated zero maze test, sleep deprivation led to a significant increase in anxiety-related behaviors, as indicated by the increased time spent in the closed quadrants and a reduced number of entries into the open quadrants. This behavioral pattern is consistent with heightened anxiety levels. The administration of Epimedium at 25 mg/kg showed a statistically significant reduction in these anxiety-related behaviors, with a P value of 0.0007 when compared to the sleep-deprived group. When the dosage was increased to 50 mg/kg, the anxiolytic effect became even more pronounced, as reflected by a P value of <0.0001. This dose-dependent response strongly suggests that Epimedium's anxiolytic activity increases with dosage. Furthermore, the behavioral outcomes at 50 mg/kg of Epimedium were nearly identical to those observed with diazepam (2 mg/kg), which also significantly reduced anxiety-related behaviors ($P < 0.0001$). These findings highlight the potential of Epimedium as an alternative anxiolytic, with efficacy comparable to that of diazepam, particularly at higher doses (See figure 2).

The light/dark test further corroborated these findings. Sleep-deprived mice exhibited a clear preference for the dark compartment, a behavior indicative of increased anxiety. Treatment with Epimedium at 25 mg/kg led to a significant increase in time spent in the light compartment, as demonstrated by a P value of 0.0007, suggesting a reduction in anxiety. Again, increasing the dosage to 50 mg/kg resulted in an even stronger anxiolytic effect, with a P value of <0.0001, highlighting a dose-dependent relationship. Diazepam (2 mg/kg) also produced a similar anxiolytic effect, with nearly identical behavioral outcomes to those seen with 50 mg/kg of Epimedium, further underscoring the extract's potential as an effective anxiolytic agent (See figure 3).

The behavioral tests suggest that epimedium extracts have some significant activity in brain biochemical release as it further reduce the anxiety caused by sleep deprivation. Hence, to further understand the underlying biochemicals the mice were sacrificed, and their brain biochemicals like MDA, SOD, CAT, Nitrite, and plasma cortisol level were studied. As, these biochemicals are thought to play role in causing anxiety.

A notable increase in MDA levels was observed in the sleep-deprived anxiety group, indicating elevated oxidative stress during anxiety. However, administration of Epimedium at 25 mg/kg significantly reduced MDA levels compared to the anxiety group, while a higher dose of 50 mg/kg brought the results almost on par with diazepam (2 mg/kg). This suggests that Epimedium can cross the blood-brain barrier and modulate brain function by regulating MDA levels (See figure 4).

Similarly, plasma cortisol levels were markedly higher in the anxiety group, but significantly reduced following treatment with Epimedium at 25 mg/kg. At 50 mg/kg, Epimedium's effect on cortisol levels was comparable to that of diazepam. This indicates Epimedium's ability to influence brain function by regulating cortisol levels (See figure 5.).

Anxiety was associated with an increase in GABA levels in the sleep-deprived group. Treatment with Epimedium at 25 mg/kg significantly lowered these levels, and at 50 mg/kg, the results were nearly equivalent to those observed with diazepam. This suggests that Epimedium may modulate brain function by influencing GABA levels (See figure 6).

The study also found elevated SOD levels in the anxiety group. Epimedium administration at 25 mg/kg significantly reduced these levels, and at 50 mg/kg, the effect was almost identical to that of diazepam. This implies that Epimedium can regulate SOD levels, potentially contributing to its anxiolytic effects (See figure 7).

CAT levels were significantly higher in the anxiety group but were notably reduced with Epimedium treatment at 25 mg/kg. A dose of 50 mg/kg resulted in CAT levels similar to those seen with diazepam, suggesting Epimedium's capability to alter brain function through CAT regulation (See figure 8).

Finally, the study observed elevated nitrite levels in the sleep-deprived anxiety group, which were significantly lowered by Epimedium at 25 mg/kg. At 50 mg/kg, Epimedium's effect on nitrite levels was almost equivalent

to that of diazepam, indicating its potential to modulate brain function by regulating nitrite levels (See figure 9).
Hence, biochemical tests like MDA, SOD, CAT, Nitrite, GABA, and Plasma cortisol levels were performed as these biochemicals are somewhere responsible for triggering anxiety. The results suggest that the plant extract had comparable activity to diazepam in the sleep-induced anxiety mice group at 50mg/kg. The levels of MDA, SOD, CAT, Nitrite, and GABA were significantly at the normal range after the administration while plasma cortisol levels were also in range (shown in figure 4-9). These findings suggest that Epimedium extract can help in reducing sleep-induced anxiety in mice by regulating or altering the biochemicals.

Conclusion

Sleep deprivation is one of the major conditions in the present world which is generally caused by stress. Hence, the WHO also called it as one of the serious health problems in the world. Additionally, anti-anxiety medications even though can give good results are a concern due to their adverse effects. Therefore, in the present study, we evaluated the extract of the plant Epimedium for its anti-anxiety effect in sleep-deprived mice and compared the results with standard drug diazepam.
Behavioral tests like actophotometer, zero maze test, and Light and dark test were performed to check the behavior of the mice and then was further estimated for biochemicals like MDA, SOD, CAT, Nitrite, GABA, and Plasma cortisol levels.
Interestingly, the data obtained from the results suggest that the mice treated with Epimedium extract at 50mg/kg had comparable results with the diazepam 2mg/kg. During the sleep deprivation state the test mice showed confusion and poor decision-making ability in their behavioral pattern. Also, during the Biochemical estimation, it was observed that in sleep-deprived mice the levels of MDA, SOD, CAT, Nitrite, and GABA were high, and a low level of plasma cortisol was observed. Hence, it can be said that the change in behavioral patterns was due to the change in these biochemicals in mice. However, after treating with 25 and 50mg/kg they showed better results in 50mg/kg Epimedium extract.
Therefore, it can be concluded that the Epimedium extract has anti-anxiolytic activity and can be further studied and utilized.

Ethical Approval

Ethical approval no. CPCSEA Registration No. 436/PO/ReBi/S/2001. The animals were sacrificed, and further studies were done to collect the valuable results.

Conflict of interest

The authors have no conflict of interest

Reference

1. Patel DR, Feucht C, Brown K, Ramsay J. Pharmacological treatment of anxiety disorders in children and adolescents: a review for practitioners. Transl Pediatr. 2018;7(1):23-35. doi:10.21037/tp.2017.08.05.
2. Seals D.R., Justice J.N., LaRocca T.J. Physiological geroscience: targeting function to increase healthspan and achieve optimal longevity. *J Physiol.* 2016;594:2001–2024. [PMC free article] [PubMed] [Google Scholar]
3. Cui T., Kovell R.C., Brooks D.C., Terlecki R.P. A urologist's guide to ingredients found in top-selling nutraceuticals for men's sexual health. *J. Sex. Med.* 2015;12:2105–2117. [PubMed] [Google Scholar]
4. Zhai M., He L., Ju X., Shao L., Li G., Zhang Y., Liu Y., Zhao H. Icariin acts as a potential agent for preventing cardiac ischemia/reperfusion injury. *Cell Biochem. Biophys.* 2015;72:589–597. [PubMed] [Google Scholar]
5. Colman R.J., Anderson R.M., Johnson S.C., Kastman E.K., Kosmatka K.J., Beasley T.M., Allison D.B., Cruzen C., Simmons H.A., Kemnitz J.W., Weindruch R. Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science.* 2009;325:201–204. [PMC free article] [PubMed] [Google Scholar]
6. Wu Y., Li Y., Liu C., Li E., Gao Z., Liu C., Gu W., Huang Y., Liu J., Wang D., Hu Y. Structural characterization of an acidic Epimedium polysaccharide and its immune-enhancement activity. *Carbohydr. Polym.* 2016;138:134–142. [PubMed] [Google Scholar]

7. Xue L., Jiang Y., Han T., Zhang N., Qin L., Xin H., Zhang Q. Comparative proteomic and metabolomic analysis reveal the antiosteoporotic molecular mechanism of icariin from Epimedium brevicornu maxim. *J. Ethnopharmacol.* 2016;192:370–381. [PubMed] [Google Scholar]
8. Meng F.H., Li Y.B., Xiong Z.L., Jiang Z.M., Li F.M. Osteoblastic proliferative activity of Epimedium brevicornum Maxim. *Phytomedicine.* 2005;12:189–193. [PubMed] [Google Scholar]
9. Zhang G., Qin L., Shi Y. Epimedium-derived phytoestrogen flavonoids exert beneficial effect on preventing bone loss in late postmenopausal women: a 24-month randomized, double-blind and placebo-controlled trial. *J. Bone Miner. Res.* 2007;22:1072–1079. [PubMed] [Google Scholar]
10. Zhang C.Z., Wang S.X., Zhang Y., Chen J.P., Liang X.M. In vitro estrogenic activities of Chinese medicinal plants traditionally used for the management of menopausal symptoms. *J. Ethnopharmacol.* 2005;98:295–300. [PubMed] [Google Scholar]
11. Zhang Y., Li J., Wang Y., Liang Q. Taxonomy of Epimedium (Berberidaceae) with special reference to Chinese species. *Chin Herb Med.* 2022;14:20–35. [PMC free article] [PubMed] [Google Scholar]
12. Pei L.K., Guo B.L., Sun S.Q., Huang W.H. [Study on the identification of some species of Herba Epimedii with FTIR] *Guang Pu Xue Yu Guang Pu Fen Xi.* 2008;28:55–60. [PubMed] [Google Scholar]
13. Jiang J., Li J., Zhang Z., Sun E., Feng L., Jia X. Mechanism of enhanced antiosteoporosis effect of circinal-icaritin by self-assembled nanomicelles in vivo with suet oil and sodium deoxycholate. *Int J Nanomedicine.* 2015;10:2377–2389. [PMC free article] [PubMed] [Google Scholar]
14. Wei J.J., Zhang J.K., Li M., Xie S.S., Tao S.Q., Yang Y. A new flavonoid glycoside from Epimedium sagittatum. *Acta Pharm. Sin.* 2023;58:180–185. [Google Scholar]
15. Wang L., Li Y., Guo Y., Ma R., Fu M., Niu J., Gao S., Zhang D. Herba epimedii: an ancient Chinese herbal medicine in the prevention and treatment of osteoporosis. *Curr Pharm Des.* 2016;22:328–349. [PubMed] [Google Scholar]
16. Dews PB. The measurement of the influence of drugs on voluntary activity in mice. *Br J Pharmacol Chemother.* 1953;8:46–8.
17. Reddy DS, Kulkarni SK. Possible role of nitric oxide in the nootropic and antiamnesic effects of neurosteroids on aging and dizocilipine induced learning impairment. *Brain Res.* 1998;799:215–29.
18. Boissier JR, Simon P. Action of caffeine on the spontaneous motility of the mouse. *Archives Internationales de Pharmacodynamie et de Therapie* . 1965;**158**(1):212–221.
19. Pandey DK, Mahesh R, kumar AA, Rao VS, Arjun M, Rajkumar R. A novel 5-HT2A receptor antagonist exhibits antidepressant-like effects in a battery of rodent behavioural assays: approaching early-onset antidepressants. *Pharmacology Biochemistry and Behavior.* 2010;**94**(3):363–373.
20. Shepherd JK, Grewal SS, Fletcher A, Bill DJ, Dourish CT. Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. *Psychopharmacology (Berl)* 1994;116:56–64.
21. Kulkarni SK, Singh K, Bishnoi M. Elevated zero maze: a paradigm to evaluate antianxiety effects of drugs. *Methods Find. Exp. Clin. Pharmacol.* 2007;29:343–348.
22. Shepherd JK, Grewal SS, Fletcher A, Bill DJ, Dourish CT. Behavioural and pharmacological characterisation of the elevated “zero-maze“ as an animal model of anxiety. *Psychopharmacology (Berl)* 1994;116:56–64.
23. Braun AA, Skelton MR, Vorhees CV, Williams MT. Comparison of the elevated plus and elevated zero mazes in treated and untreated male Sprague-Dawley rats: effects of anxiolytic and anxiogenic agents. *Pharmacol Biochem Behav.* 2011;97:406–415.
24. Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH. Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. *Proc Natl Acad Sci USA.* 1998;95:15049–15054.
25. Cleck JN, Ecke LE, Blendy JA. Endocrine and gene expression changes following forced swim stress exposure during cocaine abstinence in mice. *Psychopharmacology (Berl)* 2008;201:15–28.
26. Cook MN, Crounse M, Flaherty L. Anxiety in the elevated zero-maze is augmented in mice after repeated daily exposure. *Behav Genet.* 2002;32:113–118.
27. Gholamzadeh M, Zare S, Khodagholi F, et al. Anticonvulsant activity of essential oil from leaves of *Zhumeria majdae* (Rech.) in mice: the role of GABA_A neurotransmission and the nitric oxide pathway. *Pharmaceutical Biology*. 2020;58(1):1-10. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7359939/
28. Kolesnikova LI, Zaitseva NV, Gromova EA, et al. Cognitive dysfunction in the offspring of rats with experimental preeclampsia at the early and late stages of ontogeny and its correction with GABA

derivatives. *Semantics Scholar*. 2021; Available from: https://www.semanticscholar.org/paper/3aaab3eeb27ce7bbe72b07f71f70c2c1f2d42441
29. Rajalakshmi M, Sales MS. Antioxidant potentials of active principles from *Terminalia bellarica* fruit: an in-vitro and in-vivo approach. *2015*.
30. Revuelta-Iniesta R, Waterhouse C, Wilson M, Calder N, Ward G, Antal A, et al. Assessment of plasma antioxidants, oxidative stress and polyunsaturated fatty acids in paediatric cancer patients: a prospective cohort pilot study. *2015*.
31. Bortnowska G, Przybylska S, Iwański R. Physicochemical properties, oxidative stability and antioxidant capacity of clean label meat-based sauces: effects of phenolic extracts addition and cold storage. *J Food Sci Technol*. 2021 Jan;58(1):110-120. doi: 10.1007/s13197-020-04519-x. Epub 2020 May 20. PMID: 33500590; PMCID: PMC7813964.
32. Prabhavathi K, Kumari K, Vasavi M, Rao BP. Serum total bilirubin and oxidative stress status in diabetic retinopathy: a hospital-based observational study. *Biomed Pharmacol J*. 2024.
33. Ahluwalia S, Choudhary RK, Ahmed RA, Verma KK, Mehta K, Dixit R, et al. Evaluation of antitumor effect of Russell's viper venom on breast cancer cell line MDA-MB-231. *2015*.