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Research Article

Antioxidant Effect of *Hibiscus sabdariffa* Calyces Aqueous Extract Against Sleep Deprivation-induced Pituitary, Testicular and Epididymal Oxidative Damage in Wistar Rats

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ABSTRACT
Sleep deprivation can significantly impact male fertility. Ensuring sufficient and quality sleep is crucial for maintaining optimal reproductive health. This study evaluated the effect of *Hibiscus sabdariffa* calyces against sleep deprivation-induced testicular and epididymal toxicity in Wistar rats. Thirty male Wistar rats were divided into six groups of seven animals each, with group I as the control receiving distilled water, group II as sleep-deprived and untreated, and Groups III-V receiving varying doses of HS (100, 200, and 400 mg/kg), respectively, while being sleep-deprived. Group VI received vitamin C (100 mg/kg) and was sleep-deprived. Administration of HS significantly (P< 0.05) reduced testicular and epididymal malondialdehyde (MDA) compared to the sleep-deprived-untreated group. Antioxidant enzymes; SOD, GSH CAT and GSH were significantly increased (P< 0.05) with HS treatment compared to the sleep-deprived-untreated group. These results support the potential of *Hibiscus sabdariffa* as a natural therapeutic agent in mitigating sleep deprivation-induced reproductive toxicity.

Keywords: *Hibiscus sabdariffa L;* Oxidative stress; Sleep deprivation; Sperm; Spermatogenesis

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INTRODUCTION

Infertility is a widespread problem that affects many men globally, with prevalence rates estimated to be between 2.5% and 12% (Agarwal *et al*., 2016). This issue is a significant public health concern, impacting around 9% of couples worldwide, as reported by the World Health Organization (WHO) (Ombelet, 2020). Sleep, a fundamental biological process characterized by a rhythmic pattern, is essential for the restoration of both bodily and mental functions (Pehlivan, 2022). Disruptions to the natural circadian rhythm, particularly those caused by work schedules with rotating shifts, can significantly impact sleep quality and contribute to sleep deprivation (McDowall *et al*., 2017). Research has indicated a potential link

between insufficient sleep duration and poor sleep quality with semen quality, although findings have been inconsistent (Liu *et al*., 2017). Oxidative stress can damage testicular cells and impair spermatogenesis (Asadi *et al*., 2017). Many plants are rich in antioxidants, which protect cells from damage caused by sleep deprivation. Roselle, for instance, exhibits potent antioxidant activity. Unlike synthetic drugs, natural plant-based remedies typically pose fewer side effects and are considered safer for prolonged use. There is a growing interest in alternative infertility treatments, particularly those involving medicinal plants, as evidenced by recent studies (Abdi *et al*., 2017). Traditionally, these plants have been used to either enhance or reduce male fertility, but modern research is now focusing on the scientific basis for these effects (Roozbeh *et al*., 2021). The calyx of the roselle plant is the most commonly utilized part, and it has been the subject of numerous studies (Maurice *et al*., 2020; Diantini *et al*., 2021). Research has shown that the calyx exhibits antioxidant effects (Agunbiade *et al*., 2022; Efosa *et al*., 2023). The World Health Organization (WHO) also encourages the exploration of plants with medicinal properties for potential new interventions (Nantia *et al*., 2009). Therefore, this study was designed to evaluate the effect of *Hibiscus sabdariffa* calyces against sleep deprivation-induced testicular toxicity in adult Wistar rats.

MATERIALS AND METHODS

Materials

Ascorbic acid (Sigma Aldrich, Germany) [CAS Number: 010112]. Trichloroacetic acid, thiobarbituric acid, and hydrochloric acid. All other reagents were of analytical grade and were obtained from the British Drug House (Poole, Dorset, UK). Weighing machine (model: XY100C, number: 1404273, Changzhou Xingyun). GSH Colorimetric Assay Kit (Elabscience, China, E-BC-K030). Vital feeds, NAFDAC: A9-0744, 5 ml syringes, dissection tool kit, phosphate buffer (PBS, pH 7.4), ketamine, xylazine, Filter paper.

Plant identification and extraction

Fresh samples of the red calyces of *Hibiscus sabdariffa* were collected from Samaru market, Zaria, Kaduna State, Nigeria. The sample was identified at the herbarium of the Department of Biological Sciences, Ahmadu Bello University, and voucher number (V/N) 01056 was deposited. Extraction was carried out as described by Aliyu *et al* (2014).

Experimental animals and grouping

Thirty animals weighing between 150 and 180 g were used in the study and were housed in plastic cages in a well-ventilated area. The animals were provided with chow (vital feeds, NAFDAC: A9-0744) and had access to water. Thirty male Wistar rats were divided into six groups of five animals each, with Group I as the control receiving distilled water, Group II as sleep-deprived and untreated, and Groups III-V receiving varying doses of HS (100, 200 and 400 mg/kg) respectively while being sleep-deprived. Group VI received vitamin C (100 mg/kg) and was also sleep-deprived. The experiment lasted fourteen days, with 20 hours of sleep deprivation daily. The dosages of the extract and vitamin C in this research were determined from prior studies (Sireeratawong *et al*., 2013; Umosen *et al*., 2018). The administration of the extract and vitamin C was done orally, with the animals receiving them after sleep deprivation each day at 10:00 am for 14 days. The experimental procedures were conducted per the approved guidelines of Ahmadu Bello University's Ethical Committee on Animal Use and Care [ABUCAUC/2023/004].

Sleep deprivation protocol

Rats were acclimatized to the glass tank for 1 hour per day over three consecutive days before water was added to the tank. The water level was maintained 3 cm below the surface of the platforms. Sleep deprivation was induced using the column-in-water method (Choi *et al*., 2016; Rizk *et al*., 2020).

Animal sacrifice and tissue sample collection

Rats were anaesthetized with intraperitoneal ketamine 90 mg/kg plus xylazine 3 mg/kg (Kumar and Clover, 2015). Blood samples were obtained by cardiac puncture and centrifuged at 3,000 g for 10 minutes. The pituitary gland was carefully excised, weighed, and stored on dry ice in a plastic tube (Tzou *et al*., 2010). It was later homogenized in a phosphate buffer solution (PBS, pH 7.4). The testes and epididymis were removed, dried with filter paper, weighed, and then homogenized in 50 mM Tris-HCl buffer (pH 7.4) for further analysis (Adedara *et al*., 2018).

Testicular, epididymal, and pituitary oxidative stress biomarker assessment

Lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) to determine malondialdehyde (MDA) levels in the tissue, following the procedures outlined by

Farombi *et al*. (2000). Superoxide dismutase activity was determined according to the method described by Azevedo *et al*. (2001), while reduced glutathione levels were estimated following the procedure established by Jallow *et al*. (1974). Glutathione peroxidase activity was assessed based on the method outlined by Rotruck *et al*. (1973). Catalase activity was estimated using the protocol developed by Hadwan (2018). Protein concentration was quantified using the Bradford method with Coomassie Brilliant Blue G-250, as described by Kielkopf *et al*. (2020).

Statistical analyses

Data obtained from the study are expressed as mean ± SEM and statistical analysis was carried out using version 23 of the IBM Statistical Package for Social Sciences (SPSS). A one-way analysis of variance (ANOVA) was carried out, followed by *Tukey*'s *post hoc* test, to determine the differences among the groups.

RESULTS

Pituitary gland oxidative stress biomarkers

Figure 1 shows the effect of HS on pituitary gland oxidative stress biomarkers: MDA [1a], SOD [1b], GPx [1c], GSH [1d], and CAT [1e]. MDA concentration in the SD-untreated group was significantly higher ($p< 0.05$) compared to the control. Treatment with HS at 400 mg/kg and vitamin C at 100 mg/kg significantly ($p< 0.05$) lowered MDA concentration compared to the SD untreated group. The activity of SOD was significantly ($p< 0.05$) increased in the HS 400 mg/kg and vitamin C 100 mg/kg groups compared to the SD untreated group. There was no significant GPx activity ($p> 0.05$) observed. The activity of GSH was significantly reduced ($p< 0.05$) in the SD-untreated group compared to the control. Treatment with HS at 200 and 400 mg/kg and vitamin C significantly ($p< 0.05$) increased GSH activity compared to the SD untreated group. More so, GSH activity was significantly higher in the groups given HS at 400 mg/kg and vitamin C compared to HS 100 mg/kg treated group. Although there was no significant change ($p> 0.05$) observed in the SD untreated group compared to the control, treatment with HS, 200 and 400 mg/kg, and vitamin C significantly increased the activity of CAT (see figure 1e).

Testicular oxidative stress biomarkers

Figure 2 shows the effect of HS on testicular MDA, SOD, GPx, GSH, and CAT, designated 2a, 2b, 2c, 2d, and 2e, respectively. In Figure 2a, MDA was significantly ($p< 0.05$) higher in the SD untreated group compared to the control. Treatment with HS at 200 and 400 mg/kg significantly reduced ($p< 0.05$) MDA compared to the SD untreated group. MDA in the HS 400 mg/kg and Vitamin C group was significantly ($p< 0.05$) reduced compared to the group treated with HS 100 mg/kg. The activity of SOD in Figure 2b, GPx in Figure 2c, GSH in Figure 2d, and CAT in Figure 2e was significantly decreased ($p< 0.05$) in the SD untreated group compared to the control. In the HS-treated groups, SOD was significantly increased ($p< 0.05$) compared to the SD untreated group. Treatment with the extract HS significantly increased ($p< 0.05$) the activities of the antioxidant enzymes compared to the SD untreated groups.

Epididymal oxidative stress biomarkers

Figure 3 shows the effect of HS on epididymal oxidative stress biomarkers: MDA [3a], SOD [3b], GPx [3c], GSH [3d], and CAT [3e]. The level of MDA was significantly ($p< 0.05$) higher in the SD untreated group compared to the control. Treatment with HS at 400 mg/kg and vitamin C significantly ($p< 0.05$) decreased MDA compared to the SD untreated group. The activities of the antioxidant enzymes; SOD, GPx, GSH, and CAT were significantly decreased ($p< 0.05$) in the SD-untreated group compared to the control. Treatment with HS significantly ($p< 0.05$) increased the activity of these antioxidants compared to the SD-untreated groups. At 200 and 400 mg/kg, the activities of SOD and GSH were significantly ($p< 0.05$) higher compared to the group treated with HS 100 mg/kg.

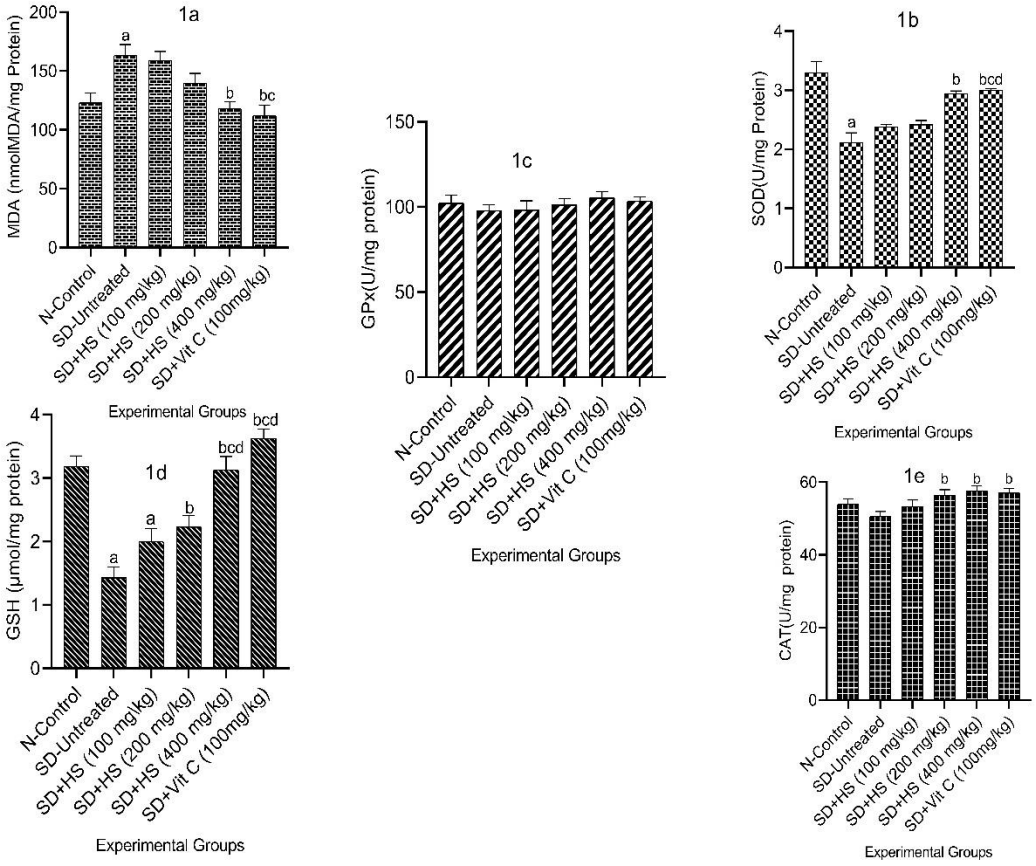

Fig. 1. Effect of an aqueous extract of *Hibiscus sabdariffa calyces* on pituitary gland

MDA [1a], SOD [1b], GPx [1c], GSH [1d], and CAT [1e]. N-control = normal control (1 ml/kg distilled water), SD = sleep-deprived, HS = *Hibiscus sabdariffa,* and Vit C (vitamin C). Each bar in the figure represents the mean ± SEM of 5 rats per group. Superscripts: a: $p < 0.05$ against control; b: $p < 0.05$ against SD untreated; c: $p < 0.05$ against SD+HS (100 mg/kg); and d: $p < 0.05$ against SD+HS (200 mg/kg)

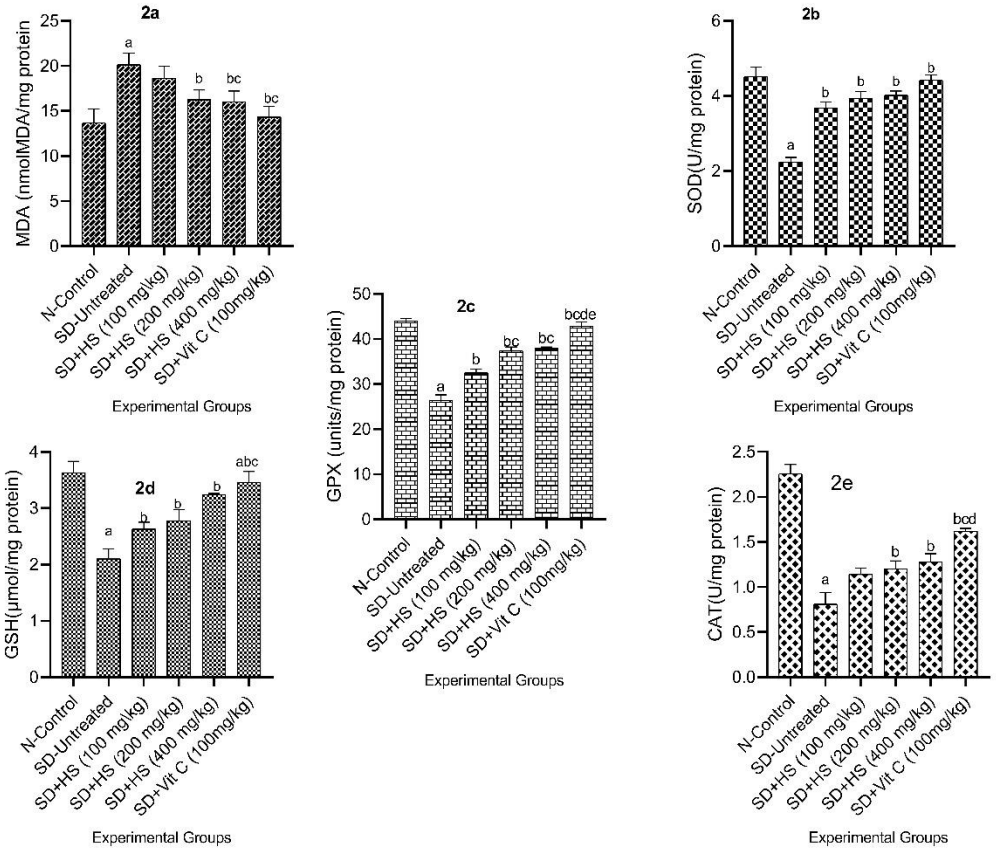

Fig. 2. Effect of an aqueous extract of *Hibiscus sabdariffa calyces* on testicular
MDA [2a], SOD [2b], GPx [2c], GSH [2d], and CAT [2e]. N-control = normal control (1 ml/kg distilled water), SD = sleep-deprived, HS = *Hibiscus sabdariffa,* and Vit C = vitamin C. Each bar in the figure represents the mean ± SEM of 5 rats per group. Superscripts: a: p< 0.05 against control; b: p< 0.05 against SD untreated; c: p< 0.05 against SD+HS (100 mg/kg); d: p< 0.05 against SD+HS (200 mg/kg); e: p< 0.05 against SD+HS (400 mg/kg)

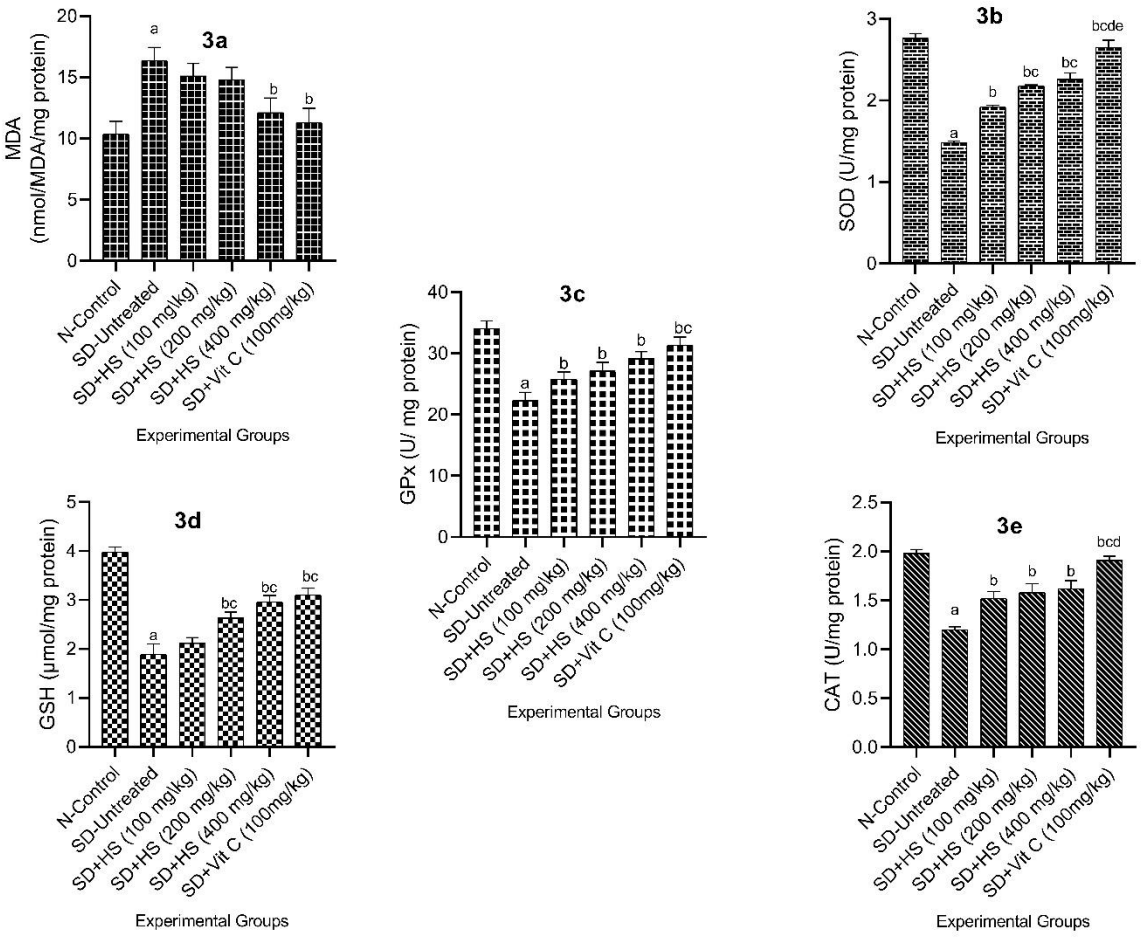

Fig. 3. Effect of an aqueous extract of *Hibiscus sabdariffa calyces* on epididymal
MDA [3a], SOD [3b], GPx [3c], GSH [3d], and CAT [3e]. N-control = normal control (1 ml/kg distilled water), SD = sleep-deprived, HS = *Hibiscus sabdariffa,* and Vit C = vitamin C. Each bar in the figure represents the mean ± SEM of 5 rats per group. Superscripts: a: p< 0.05 against control; b: p< 0.05 against SD untreated; c: p< 0.05 against SD+HS (100 mg/kg); d: p< 0.05 against SD+HS (200 mg/kg); e: p< 0.05 against SD+HS (400 mg/kg)

DISCUSSION

Sleep deprivation is a prevalent form of social stress that impacts various groups and is a powerful trigger of oxidative stress (Rizk *et al*., 2020). The pituitary gland plays a vital role in regulating both sleep and stress reactions (Oyola *et al*., 2019). Various stressors can activate the hypothalamic-pituitary-adrenal (HPA) axis to varying degrees (Moraes *et al*., 2022).

Lipid peroxidation in the pituitary gland of the SD-untreated group may have been caused by the activation of the HPA-axis, leading to cellular oxidative stress as demonstrated by Spiers *et al*. (2015), although the precise mechanism remains unclear. Furthermore, the process of Wakefulness demands a high level of neuronal metabolism to uphold neuronal electrical potentials, necessitating a substantial amount of oxygen, consequently generating a significant number of oxidants as noted by Villafuerte *et al*. (2015). This could potentially explain the observed lipid peroxidation in the SD-untreated group in the current study.

The accumulation of β-Amyloid in the pituitary gland has been linked to sleep deprivation (Shokri-Kojori *et al*., 2018). This β-Amyloid has been found to have prooxidant effects in the brain (Karapetyan *et al*., 2024), which could explain the lipid peroxidation observed in the pituitary gland tissue in the current study. Previous research has also indicated a connection between tissue ROS levels and sleep deprivation (Eisele *et al*., 2015; Morén *et al*., 2017).

The administration of HS extract in the current study resulted in a significant reduction in pituitary gland lipid peroxidation compared to the untreated group. This effect is likely attributed to the

antioxidant properties of the extract, as indicated by the increased activity of antioxidant enzymes in the group that received the HS extract. Previous studies have shown that HS extracts contain bioactive compounds such as delphinidin-3-0-glucoside, delphinidin-3-0-sambubioside, cyanidin-3-0 sambubioside, polyphenolic compounds, and organic acids, which contribute to their significant antioxidant properties (Tahir *et al*., 2016; Subhaswaraj *et al*., 2017).

In the current investigation, the HS extract may have exerted its effects through the presence of secondary metabolites such as flavonoids, as suggested by Formagio *et al*. (2015), Alara *et al*. (2020) and Xiaowei *et al*. (2020). Flavonoids, a diverse group of polyphenols derived from plant sources, are known for their potent antioxidant properties (Krenn *et al*., 2022). Studies have demonstrated that flavonoids can influence key enzymes involved in adrenal steroidogenesis, impacting the synthesis of mineralocorticoids, glucocorticoids, and adrenal androgens (Schloms and Swart, 2014). Therefore, the flavonoids present in the HS extract may have contributed to the reduction in MDA levels observed in this study by regulating glucocorticoid release and reducing the production of reactive oxygen species (ROS). Furthermore, the HS extract may have also acted by down-regulating glucocorticoid receptors in the pituitary gland, thereby decreasing its sensitivity to glucocorticoids and the associated ROS generation (Prevatto *et al*., 2017). Previous research has highlighted the antioxidant properties of HS extract in the brain (Efosa *et al*., 2023).

In the current study, it was found that administration of HS extract enhanced the activity of antioxidant enzymes in the pituitary gland. Superoxide dismutase, catalyzing the conversion of superoxide to hydrogen peroxide, CAT, facilitating the breakdown of H_2O_2 to water, and GPx, involved in reducing lipid hydroperoxides and hydrogen peroxide, all showed increased activity (Crespo *et al*., 2008). Previous research has indicated that polyphenols present in plant extracts can upregulate the expression of genes encoding antioxidant enzymes (Yan *et al*., 2020; Rudrapal *et al*., 2022). Polyphenols typically possess both hydrophilic and hydrophobic regions, enabling them to interact with various membrane components and elicit cellular responses (Erlejman *et al*., 2006). This interaction between polyphenols and membranes can lead to functional changes in the membrane, influencing processes such as signal transduction, ion flux, receptor-ligand interactions, and enzyme activity associated with the membrane (Verstraeten *et al*., 2003).

The antioxidant properties of polyphenols, such as flavonoids, are attributed to their ability to regulate the production of free radicals like RNS and ROS (Sun *et al*., 2018), as well as their capacity to sequester metals and prevent metal-catalyzed free radical formation (Fraga *et al*., 2010). Therefore, the enhanced antioxidant activity observed after administering HS extract in this study may be due to the influence of its phenolic content on gene expressions that increase both the quantity and activity of antioxidants. These results align with previous studies that have shown increased antioxidant enzyme activities following HS extract administration (Efosa *et al*., 2023; Adetunji *et al*., 2023). The impact of ROS on male fertility has been extensively researched (Lafuente *et al*., 2013). Adequate sleep is crucial for overall health and optimal fertility (Rizk *et al*., 2020). The testicular tissue is particularly vulnerable to oxidative stress due to its high rate of cell division and mitochondrial oxygen consumption (Asadi *et al*., 2017), as well as its high levels of unsaturated fatty acids and low oxygen pressure (Guerriero *et al*., 2014). The precise mechanism underlying this susceptibility is not fully understood, but it is thought that disruptions in reactive oxygen species (ROS) levels could result from increased production, decreased clearance, or a combination of both processes (Neculicioiu *et al*., 2023)

The protective effect of HS extract on testicular and epididymal lipid peroxidation observed in this study may be attributed to the elevated levels of testosterone, FSH, and LH. It is known that sex hormones play a role in regulating the genes for antioxidant enzymes (Bellanti *et al*., 2012), and lower FSH levels have been associated with reduced enzymatic activity (Klisic *et al*., 2018). The beneficial effects of HS extract in this study could be linked to the improved levels of FSH, LH, and testosterone, along with the antioxidant enzymes. Previous studies have reported the presence of antioxidant enzymes in the testes (Mruk *et al*., 2002; Singh *et al*., 2008). Anthocyanins, found in HS extract, are known for their antioxidant properties (Aurelio *et al*., 2007) and have been shown to inhibit lipid peroxidation and scavenge free radicals (Tsuda, 2000). The antioxidant activity of anthocyanins has also been demonstrated in the testes (Riaz and Chopra, 2018). Overall, the action of HS extract in mitigating lipid peroxidation in the testes and

epididymis may involve the prevention or inactivation of free radicals through antioxidant enzymes, interruption of lipid peroxidation chain propagation by scavenger molecules, or removal of oxidatively damaged molecules to the synthesis of melatonin, which plays a crucial role in protecting testicles and spermatogonia against oxidative damage and acts as a potent free radical scavenger (Yang *et al*., 2023).

CONCLUSION

In conclusion, this study highlights the protective effects of HS extract against sleep deprivation-induced oxidative damage, particularly in the pituitary gland and reproductive tissues. The findings suggest that HS extract exerts its beneficial effects through antioxidant enhancement. Future research should explore the molecular mechanisms involved and investigate the clinical applications of HS extract in managing sleep deprivation-related reproductive dysfunction.

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