Protective effects of *Nymphaea lotus* Linn. (Nymphaeaceae) aqueous extract against chronic unpredictable mild stress induced testicular lipid peroxidation

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ABSTRACT :
Stress has been associated with various diseases such as harms the heart system, nervous system, affect the level of hormone, but also morphology and function of testis. The purpose of the present study was to investigate protective properties of *Nymphaea lotus* (*N. lotus*), against deleterious effects induced by unpredictable chronic mild stress (CMS) on testes of albino male rats. Adult male Wistar rats were divided into five groups as one non-stressed group and four stressed groups (n = 5 in each group). The stressed groups were exposed to 14 days of CMS, receiving concomitantly and respectively, distilled water (10 mL/kg), Yohimbine (2 mg/kg) and *N. lotus* at the level of 75 and 200 mg/kg. The reproductive organs weight, oxidative stress markers, and histoarchitecture of the testis were examined. Exposition to CMS to the male rats caused no significant changes in the relative weight of testis, but affects its histoarchitecture, that is preserved in stressed rats treated with *N. lotus*. Further CMS significantly declined the amount of Nitrites ($P<0.05$), GSH ($P<0.05$) and SOD ($P<0.001$) levels and increase up to 16.25% the level of testicular lipid peroxidation when compared to unstressed rats. *N. lotus* at the both doses significantly reduced ($P<0.001$ for 75 mg/kg and $P<0.01$ for 200 mg/kg) MDA levels in comparison to stressed rats, suggesting an lipid peroxidation inhibitory effect of our plant. The unpredictable chronic mild stress paradigm used in our study enhanced oxidative stress in testis and causes histological damages that *N. lotus* treatment significantly minimize.

Keywords: *Nymphaea lotus*, Chronic Unpredictable Mild Stress, Lipid peroxidation, testis histology.

INTRODUCTION:

Stress may be defined as a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological or behavioural responses [1]. In this modern world, stress is the more common situation a human can experiment. Stress is implicated in the statement of many diseases; its can harms the heart system, nervous system and hearing system [2,3], affects the level of insulin [4], morphology [5] and function of testis [6]. Effectively, Chronic stress exposure has been reported as a potential risk factor for reproductive function [7,8].

In males, physical and psychological stressors might inhibit reproductive function. Chronic unpredictable mild stress (CMS), one of the most clinically relevant stress paradigms in rodents, mimics a number of behavioral characteristics observed in patients with anxiety, depression and related mood disorders [9]. Adverse effects of stress on male reproductive system have already been described. Immobilisation stress, as example, is known to decrease the activities of catalase, glutathione peroxidase, glutathione transferase, and glutathione reductase in the interstitium of testes. Stress also increases reactive oxygen species (ROS) generation in the male reproductive tract. High ROS levels may be linked to low sperm quality and male infertility. Effectively, ROS are reported to damage almost all macromolecules of the cell, including polyunsaturated fatty acids of membranes, thus causing impairment of cellular functions [10]. Testicular membranes are extremely rich in polyunsaturated fatty acids therefore; thus the organ is highly susceptible to oxidative stress [11]. Lipid peroxidation has been considered as a serious consequence of free radical toxicity leading to profound changes in the membrane structure and function that may cause even cell death [12,13].

To reduce the effects of stress, which cause many diseases, substances showing antioxidant properties were actively sought after [14]. Plants have been used as valuable resources in the development of novel drugs [15] and many have shown potentials to be used as antioxidant agents. *Nymphaea lotus* Linn. (Nymphaeaceae) is a water-plant with white flowers, generally wide spread in tropical Africa. Traditional uses of the plant include treatment of mental illnesses like depression [16], gastric ulcers [17], and recent studies have revealed that the aqueous flowers extract of *Nymphaea lotus* Linn. contains various pharmacological active compounds like saponins, flavonoids which are famous for their antioxidant potential [18]; and alkaloids, glycosides, terpenes and steroids which are well known for their fertilizing and anti-depressant properties [19,20,21,22]. These secondary metabolites may exert a significant physiological effect on the mammalian

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system [23,24]. But despite the widespread uses of this plant, there are no literature reports on the scientific evaluation of its influence on reproductive function.

In this study, unpredictable model of chronic stress was chosen in order to avoid habituation to the stressor and to maintain robust the stress response. Thus the purpose of the present study was to determine whether exposure to chronic unpredictable stressors applied to adult male rats induces changes in reproductive parameters and the possible inhibitory effect of *N. lotus* aqueous extract.

Methods

Nymphaea lotus flowers aqueous extract preparation

The fresh flowers of *Nymphaea lotus* Linn. were collected in Yaounde (Cameroon). Botanical identification was done at the National Herbarium in comparison with the Herbarium Voucher specimen N° 8647/HNC. The flowers were cut into small pieces, dried, and powdered using an electric grinder. The dried powder (50 g) was infused in 1 L of boiled water. Then, extract was filtered and the filtrate was evaporated at 45 °C. This aqueous extract was administered orally at two different doses of 75 and 200 mg/kg, once per day, for 14 days.

Experimental animals

Adult male Wistar rats weighing around 150 - 180 g were used. The animals were kept in polypropylene cages (five in each) at an ambient temperature, relative humidity and under natural light and dark cycle. Animals have free access to food and water, except the days when food or water deprivation was applied as a stressor. All experimental procedures and animal maintenance confirmed to the strict guidelines of Institutional Ethics Committee.

Experimental design

Adult Male Albino Wistar rats were randomly divided into five groups of five each. Group I served as control and was kept away from others groups. Group II, stressed-rats, Group III, stressed rats concomitantly treated with Yohimbine at the dose of 2 mg/kg, Groups IV and V, stressed rats concomitantly treated with aqueous extract of *Nymphaea lotus* flowers at the respective doses of 75 and 200 mg/kg. Administration of the extract, yohimbine and distilled water was by orogastric gavages using a metal cannula.

Unpredictable Chronic mild stress procedure (CMS)

The CMS procedure was performed as described by [25], with slight modifications. Briefly, CMS consisted of exposure to a variety of unpredictable stressors (randomly); as follows: (1) 24 h food deprivation, (2) 12 h Intermittent white noise + overnight illumination, (3) 22 h soiled cage (250ml water in few sawdust bedding), (4) 22 h cage tilt (45°), (5) 12h Novel odor exposure, (6) Shaker stress (30 seconds, 3 time) + Water deprivation, (7) exposure to a empty bottle (18h) + Stroboscopic illumination, (8) Cold + Heat stress (4°C and 60°C water bath respectively, 30 min each) and (9) 30 minutes physically restraint. These stressors were randomly scheduled over a 1-week period and repeated throughout the 2-week experimental period. Control animals were undisturbed except for necessary housekeeping procedures.

Body and organ weights

The initial and final body weights of the animal were recorded. The reproductive tract was trimmed off of fat, and each organ was weighed separately on electronic balance. The reproductive organs taken into account for study in male included testes, epididymis, ventral prostate, seminal vesicles, the *elevator ani* muscle and penis.

Tissues processing for histology

The testes and epididymis were removed and dissected free from adjacent connective tissue. Left testis was put in 10% formaldehyde for one week to be fixed, dehydrated in alcohol, and embedded in paraffin. The 5 µm thick sections were cut onto slides. The slides were stained with Hematoxylin & Eosin, for histomorphological analysis.

Biomarkers of oxidative stress

The other piece of right testis was used for biochemical tests. Testis was washed with cold normal saline and homogenized with sodium phosphate buffer. The homogenized testis was used for measurement of scavenging enzymes SOD [26], CAT [27] with hydrogen peroxide as the substrate, and lipid peroxidation using MDA levels. MDA level was measured as an indication of lipid peroxidation using the colorimetric method as described by [28]. The GSH was measured as described by [29] and the absorbance was measured at 412 nm. The concentration of reduced glutathione was expressed as µg/mg of protein.

Protein estimation and Testicular cholesterol level

The protein content was measured in all testis samples for oxidant and antioxidant activity by the biuret method [30] using bovine serum albumin as standard.

Statistical analysis

Statistical test was performed using SPSS Version 21 for Macintosh system. Data are reported as the mean ± SEM. Differences between means were evaluated by one-way analysis of variance (ANOVA) followed by *Tukey* hoc test. The level of significance was set at $p < 0.05$.

Results

Body weight

CMS reduced the body weight in rats, which were increased by Yohimbine and *N. lotus* at the both doses when compared to stress rats receiving only distilled water (**Table 1**).

Table 1: Effect of *Nymphaea lotus* on body weight in control and stressed rats after 2 weeks

Groups	Initial weight (g)	Final weight (g)	Weight gain/loss (g)
Control	158.60 ± 2.38	182.00 ± 7.67	14.75 ± 0.22
CMS	166.20 ± 6.41	149.40 ± 2.71	-10.11 ± 0.58
CMS + Yohimbine (2)	164.60 ± 8.13	161.00 ± 8.70	-2.19 ± 0.07
CMS + *N. lotus* (75)	167.40 ± 10.8	160.00 ± 10.92	-4.42 ± 0.01
CMS + *N. lotus* (200)	163.40 ± 4.20	156.40 ± 2.79	-4.28 ± 0.34

Reproductive organs relative weight

Table 2 shows that the weight of the organs like prostate, penis and *elevator ani* muscle were significantly (P <0.05) increase in stressed animals receiving concomitantly aqueous extract of *N. lotus* at the both doses compared to normal or stressed rats, while the weight of testis, epididymis an seminal vesicles remain in normal range in stressed rats.

Table 2: Effect of *Nymphaea lotus* on relative weight of reproductive organs in control and stressed rats

Groups	Reproductive organs relative weight					
	Testes	Epididymis	Prostate	Penis	Seminale vesicles	Elevator ani muscle
Control	1.69±0.03	0.55±0.03	0.33±0.04	0.19±0.01	0.49±0.05	0.45±0.02
CMS	1.69±0.08	0.50±0.02	0.19±0.02*	0.14±0.01*	0.43±0.06	0.29±0.05*
CMS+ Yohimbine (2)	1.69±0.10	0.56±0.04	0.24±0.01	0.19±0.01	0.38±0.05	0.38±0.05
CMS+ *N. lotus* (75)	1.86±0.14	0.60±0.05	0.25±0.04	0.18±0.01[a]	0.48±0.05	0.40±0.02
CMS+ *N. lotus* (200)	1.71±0.08	0.62±0.02[a]	0.27±0.03	0.22±0.01[b]	0.52±0.02	0.44±0.04[a]

Biochemical parameters and Oxidative status in testis homogenate

In **Table 3**, total protein contents of testes did not change between groups. In *N. lotus* both doses groups, testicular cholesterol level showed a significant increase when compared with that of stress group and also when compared with that of unstressed control group (P<0.01 and P<0.05 for 75 mg/kg and 200 mg/kg respectively). Whereas many oxidative stress markers in testis of stressed rats also increased significantly compared to normal rats.

In comparison to control rats a significant decrease in the Nitrites levels was observed in testis (P<0.05) of CMS rats. Besides, testis proteins levels remain unaltered by stress.

CMS did not demonstrate any significant change in catalase levels in testis. It produces depleted (P<0.001) SOD activity and GSH levels (P<0.05) in CMS rats testis, except in CMS rats treated with aqueous extract of *N. lotus* at the level of 200 mg/kg, which contrarily exhibited a significant increase (P<0.05) in SOD level and (P<0.01) in GSH level compared to CMS rats receiving with distilled water. CMS increase up to 16.25% the amount of lipid peroxidation in testis compared to control group and *N. lotus* at the both doses significantly reduced MDA levels (P<0.001 for 75 mg/kg and P<0.01 for 200 mg/kg) in comparison to CMS rats. Such significant reduction is also observed in CMS rats treated with yohimbine (P<0.01).

Table 3: Effect of *Nymphaea lotus* on biochemical parameters in testis of control and stressed rats

Groups	Oxidative stress and biochemical parameters in testis						
	Proteins level	Total Cholesterol	SOD (U/g of proteins)	GSHx (µmol/mg of proteins)	Catalase (U/mmol H2O2)	Nitrites (Nitrites, M)	MDA (µmol/g of ogan)
Control	1.42± 0.21	11.89± 1.39	34.50± 1.30	35.40± 4.63	3.90± 0.38	0.09± 0.01	23.38± 0.87
CMS	1.42± 0.16	10.26± 0.81	07.50± 0.71***	18.53± 2.35*	3.56± 0.28	0.07± 0.00*	27.18± 0.36
CMS+ Yohimbine (2)	1.38± 0.05	15.20± 2.21	16.50± 1.14***b	31.80± 1.16	2.68± 0.09*	0.07± 0.01*	21.06± 0.38*b
CMS+ *N. lotus* (75)	1.15± 0.28	25.05± 1.97**b	11.00± 0.89***	31.10± 4.46	3.06± 0.22	0.07± 0.00*	18.84± 0.75c
CMS+ *N. lotus* (200)	1.42± 0.37	20.88± 2.44 *a	67.50± 2.55***c	43.64± 5.78b	3.91± 0.25	0.11± 0.01c	21.56± 1.99b

Effects of *N. lotus* on CMS-induced changes in testis and epididymis histology

Control group: the seminiferous tubules were bounded together by intertubular connective tissue, which contained fibroblasts, collagen fibers, blood vessels, and groups of interstitial cells or leydig cells. The capillaries were infiltrated among the clumps of leydig cells (Figure 1A).

CMS group: it stay to observe a decrease in spermatogenesis. The architecture of the testis was maintained, but the germinal epithelium showed disorganization as well as marked degenerative changes (pycnosis) and vacuolisation. There was cell debris in the lumen of tubules as a result of infusion of degenerated germ cells. The capillary network was well defined (Figure 1B).

CMS+ Yohimbine group: we observed that the shape and diameter of seminiferous tubules were the same as those of control group (Figure 1C).

CMS+ *N. lotus* 75 group: Testicular histology showed increased spermatogenesis and seminiferous tubules full of sperm in the treated group compared to the untreated controls The germ cells in the tubules were arranged in an order like control group. The degenerations seen in stress group were disappeared (Figure 1D).

CMS+ *N. lotus* 200 group: Normal general aspect of testis (Figure 1E).

No significant structural changes have been observed in the histoarchitecture of epididymis between different groups (Figure 1F, 1G, 1H, 1I and 1J).

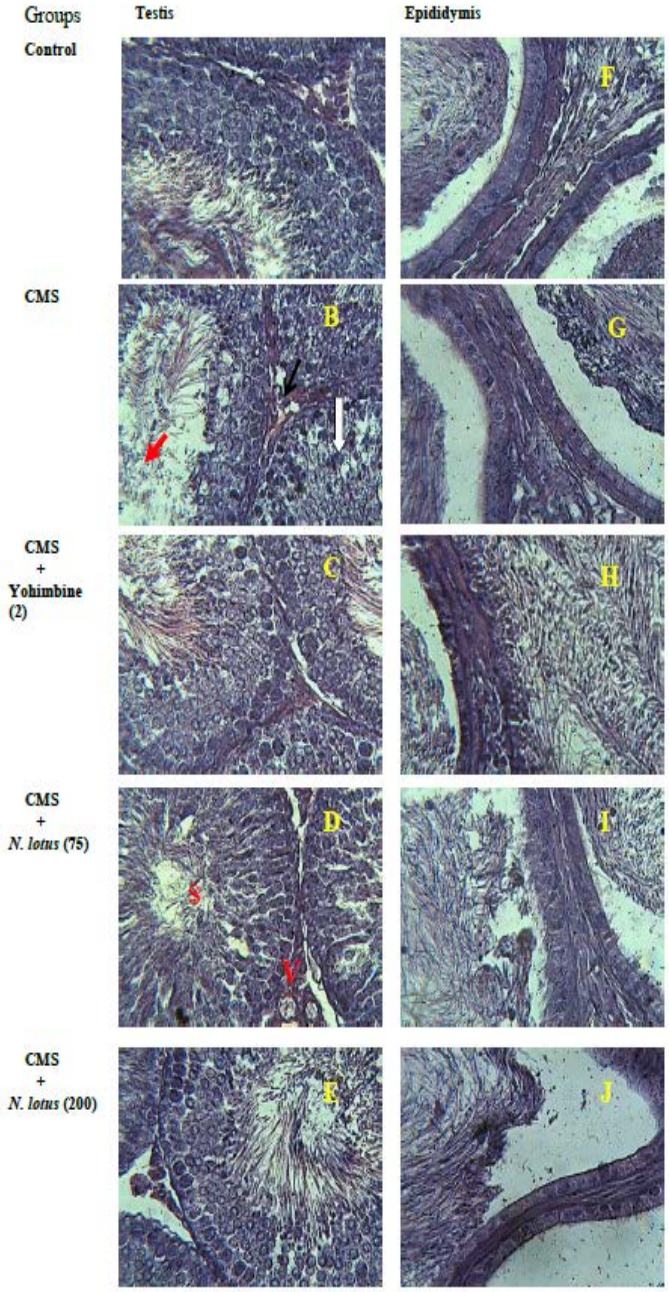

Figure 1: Effect of *Nymphaea lotus* on testis and epididymis of control and stressed rats. White arrow: vacuolisation and dgeneration; Black arrow: pycnosis; Red arrow: cell debris in the lumen of tubules; S: spermatozoids; V: capillaries and leydigs cells

Discussion

Stress is an internationally recognized phenomenon fortified by advancement of industrialization and a demanding civilization. Thus, every person faces stressful situations in day-to-day life [31]. Chronic unpredictable mild stress (CMS), one of the most clinically relevant stress paradigms in rodents, mimics a number of behavioral characteristics observed in patients with anxiety, depression and related mood disorders [9], this type of stress was used in the present study.

The exposure to CMS for 14 days resulted in a reduction of body weight (Table 1) in all stressed animals. This observation is in line with the literature. Stress might have increased the metabolic demands, reduced digestion, and hampered the utilization of food consumed (*unpublished results*) during the stress period, thereby causing decrease in body weight. Increases in various reproductive organ weights after chronic mild stress exposure observed in this study confirm other previous reports [32]. In addition to that, treatment with *N. lotus* and with Yohimbine reduced the body weight loss in the stress-loaded animals.

Several studies have suggested that stress may cause infertility by affecting the gonads [33] and it is also reported that the weight of testis decreases because there is suppression of spermatogenesis and decrease testosterone levels during stress [34]. In the present study contrarily, the weight of the testis do not changes in stressed rats treated with distilled water or with Yohimbine, but significantly increase in stressed rats treated with *N. lotus* at the both doses (Table 2). This significant anabolic effect upon administration of extracts was observable as compared to CMS group, suggesting a testosterone type action of the extracts. Since androgenic effect is attributable to testosterone levels in the blood, it is likely that the plant extracts may have a role in testosterone secretion allowing better availability of hormone to gonads [35,36,37].

Further, It has been demonstrated that in rats, any increase in serum testosterone or treatment with androgens are associated with increased secretory activity and increased weight of the reproductive organs [38,39].

Although non significant, the decrease in the intratesticular cholesterol level of the stressed animals is of physiological importance. Cholesterol is the major substrate for steroidogenesis and its testicular drop may reflect a conversion into testosterone under the control of luteinising hormone (LH) [40]. The decrease of proteins levels observed specifically in the two groups (Yohimbine and *N. lotus* 75 mg/kg) exhibiting the highest cholesterol testis concentrations, may suggest a redirection of metabolic priority of the testis. Indeed, the decreased levels of proteins seen in these two groups are probably due to increase of protein catobolism [41] induced by stress. Therefore, the testosterone synthesis must be a preventive alternative given by androgen mimetic or stimulant compounds in our extract, to restore structural damage occuring in testis under stress conditions. Moreover Yohimbine has already been reported to provide no anabolic effects, to not increase testosterone levels; but to act synergically with testosterone and facilitate its actions [42]. Our plant extract at the level of 75 mg/kg seems to have a similar mechanism of action under stress conditions.

Intensive stress has detrimental effects on organism by causing cellular and tissue injury. The mechanisms underlying stress-induced tissue damages are not yet fully understood. However, accumulating evidence has implied that the production of free radicals plays a critical role in these processes [43,44,45,46,47]. Gao *et al*. [48] showed glucocorticoids secreted massively during stress, control mitosis and apoptosis induction in testicular cells.

In our experiment, CMS produced decline of SOD and GSH levels that indicate that CMS lead to an increased production of free radicals [49]. In fact, GSH plays multiple roles as a cellular antioxidant defense because its main function is to remove hydrogen peroxide and organic peroxides [50]. Further, under normal conditions, there are others natural defense systems provided by several enzymes such as superoxide dismutase (SOD), Nitrites, and catalase that performs a vital role for detoxification of free radicals. The increased Nitrites and SOD levels observed in stressed rats treated with *N. lotus* at the dose of 200 mg/kg suggest that our plant extract possess dose-dependent antioxidant properties.

The decreased levels of Malondialdehyde (MDA) observed in CMS rats treated with *N. lotus* at the both doses suggest that our plant extract may possess inhibition of lipid peroxidation property. Namely, MDA is one of the end products of lipid peroxidation.

Lipid peroxidation has been considered as a serious consequence of free radical toxicity leading to profound changes in the membrane structure and function that may cause even cell death [51]. Saleem *et al*. [52] also reported inhibition of lipid peroxidation property of *Nymphaea lotus*. It is thus possible that the antioxidant property of the extract maybe counteracting oxidative damage caused by CMS. This antioxidant effects appears to contribute to the observed protective activities on testicular morphology.

Exposure to stress leads to the tremendous amount of free radicals. The results obtained in the present study revealed that chronic unpredictable stress procedure significantly increases some oxidative damage biomarkers of lipid peroxidation in the testis and that our plant extract exhibit protective properties.

Conclusion

Overall, the present study report cumulative effects of repeated chronic mild unpredictable stressors on a daily basis for a period of 14 days increase amount of free radicals in testis and thus, induce morphological damages that are counteract by aqueous extract of *Nymphaea lotus* flowers. With the help of derived findings, it is mentioned that *N. lotus* prevented these stress-induced biochemical and structural changes, indicating antioxidant and inhibitory of lipid peroxidation potential of this plant extract.

Authors' contributions

KPM, DDPD contributed to the design of the study, the collection of data and their analysis as well as the writing.

BDC, MNYS, MM, NMC, NR, OCA, DT and KP contributed to the collection and analysis of the data.

All authors read and approved the final manuscript.

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