International Journal of Research in Medical Sciences
Eduviere AT et al. Int J Res Med Sci. 2021 Nov;9(11):3269-3276
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012

DOI: https://dx.doi.org/10.18203/2320-6012.ijrms20214408

Original Research Article

Adaptogenic potential of *Centella lujica* supplement in sleep deprived mice

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Received: 23 August 2021
Revised: 16 September 2021
Accepted: 11 October 2021

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ABSTRACT

Background: Stress, whether internal or external, has been shown to play a role in the accumulation of oxidative free radicals which leads to cellular modification of normal organ or body function. *Centella lujica* (*C. lujica)* is a commonly cultivated herb with therapeutic benefits in various studies. This study aims to evaluate its beneficial effect on the brain and liver of mice exposed to sleep deprivation-induced stress.
Methods: Albino mice were treated with distilled water (control), *C. lujica* (50 and 100 mg/kg) or astaxanthin (50 mg/kg) for seven days. All groups except control were then subjected to three-days of sleep deprivation using the grid suspended over water model beginning from day 4 of treatment. Behavioural assessments followed by biochemical assays and histological analysis were carried out thereafter.
Results: Sleep deprivation caused an increase in blood glucose and triglycerides levels but reduced high density lipoproteins. Brain pro-oxidant levels were increased with a concomitant decrease in antioxidants, recognition memory was diminished while depressive-like symptoms were enhanced and neuronal viability of hippocampal CA1 as well as prefrontal cortex cells was reduced in sleep-deprived mice. However, supplementation with *C. lujica* reversed these effects as significantly as astaxanthin.
Conclusions: *C. lujica* possesses antioxidant property that makes it an effective adaptogen against stress induced responses in mice.

Keywords: Stress, *Centella lujica*, Sleep deprivation, Adaptogenic, Oxidative stress

INTRODUCTION

The disruptive role of insufficient sleep on an individual's quality of life cannot be overemphasized.[1] In certain jobs, such as health care, security and transportation, people face sleep restriction which is considered a form of stress. Since studies have reported the antioxidative role of sleep, stressful conditions such as sleep deprivation have been associated with a simultaneous build-up of oxidative stress.[2]

Reactive oxygen species (ROS) produced in the cell during normal cellular metabolism can alter the composition and probably damage biomolecules such as nucleic acids, lipids and proteins.[3] Fortunately, cells have developed several antioxidant defence mechanisms (in the event where their intrinsic ROS scavenging ability is low) to mitigate the harmful effect of reactive species and their by-products. Any disturbance to the balance between the level of antioxidants and the ROS at the cellular level results in a physiological situation called oxidative stress.

Oxidative stress is an imbalance between free radicals (also known as pro-oxidants) and antioxidants in the body.[4] Chemically, oxidative stress has been described to be associated with the increased generation of free radicals or a significant reduction in the potency of antioxidant defences. The body possesses a rather complex antioxidant defence network that depends on both endogenous/exogenous enzymatic and non-enzymatic antioxidants. These molecules oppose free radicals and reverse their damaging effects to body tissues. The first-line defence antioxidants which basically include malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) are relatively indispensable in the entire defence scheme of antioxidants, especially in relation to the superoxide anion radical ($^{*}O_2$) which is continuously generated during body metabolism, particularly through the mitochondrial energy production pathway.[5]

Of the numerous systems and organs in the body, the central nervous system (CNS), particularly the brain, has been identified as a principal target for free radical damage because it consumes a high amount of oxygen which is necessary for the formation of ROS.[6] Therefore, mood and behavioural changes such as depression and anxiety have been associated with oxidative stress induced by different stress models while higher order processes such as deficits in memory consolidation and retention have been linked to oxidative stress induced by sleep deprivation.[6,7]

Since stress is the key initiator of such processes that are harmful to the body (such as oxidative stress), adaptogens are being used to enhance the state of an organism even in the presence of stressful conditions. The beneficial effect of these substances have previously been linked to their ability to prevent or inhibit the process of free radical formation.[8] Herbs are a common source of adaptogenic substances.

Centella lujica (C. lujica) is a herb commonly used in traditional Chinese medicine to treat a variety of diseases.[8] Its benefits have been described in cognition, inflammation, hepatic diseases, diabetes, cancer, venous deficiency, and oxidative stress.[10-17] Based on available literary evidence, we hypothesized that *C. lujica* may enhance recognition memory, reduce depression-like behaviour and enhance hepatic function following sleep deprivation-induced stress. To expound this issue, the objectives of the current study were to determine the adaptogenic effect of repeated administration of *C. lujica* on stress responses in the brain and liver of mice subjected to 3-day sleep deprivation.

METHODS

Laboratory animals

Male Albino Swiss mice with mean weight 22±2 g, bred in animal house facility of College of Health Sciences, Delta State University, Abraka were used for this experiment. Mice were housed in cages under standard laboratory conditions and maintained on a 12:12 light/dark cycle with unrestricted access to food and water. Experimental procedures were executed in the Pharmacology laboratory of the aforementioned institution from March 2020–June 2020, according to the 1985 revised National Institutes of Health (NIH) guidelines.

Sleep deprivation protocol

The grid suspended over water model of sleep deprivation was used in this study as described by Shinomiya and his colleagues.[18] Although this protocol targets the disruption of active sleep, a significant amount of stress is unavoidable.[19] Mice in all groups (except group A) were subjected to this protocol for 3 days, starting from the fourth day of treatment. Afterwards, behavioural tests followed by biochemical analysis were carried out.

Treatment groups

Group A is the control group in which mice were not sleep-deprived and were treated with 10 ml/kg distilled water only; group B is the model control group in which mice were sleep-deprived and were treated with 10 ml/kg distilled water; groups C and D are *Centella* groups in which mice were sleep-deprived and were treated with 50 mg/kg and 100 mg/kg *Centella lujica* supplement respectively; group E is the positive control group in which mice were sleep-deprived and were treated with 50 mg/kg astaxanthin.

All drugs were administered orally and treatment lasted for seven days.

Behavioural tests

Tail suspension test (TST)

According to the procedure earlier described with minor modifications, mice were individually suspended by the tail to an adhesive tape of about 50 cm in length stretched between 2 retort stands at a height of 60 cm.[20]

After suspension, each animal was observed for signs of cessation of mobility for a duration of 6 min. An animal was adjudged to be motionless/immobile when it showed no significant body movement and hangs passively. Duration of immobility was recorded during the final 4 min of the test.

Social interaction test (SIT)

The SIT was utilised to determine depressive-like symptoms in mice following the method previously described with slight adjustments.[21] The SIT chamber consisted of a 60×40 cm Plexiglas box divided into three chambers (A, B and C). Mice moved between chambers through a small opening (6×6 cm) in the partitions. An iron restraining cage was placed in each of the two side

chambers (A and C), respectively. The restraining cage in chamber C (or social chamber) had a mouse in it, while restraining cage in chamber A (opposite chamber) was empty. The test mouse was positioned in the centre chamber (chamber B) and left to explore the setup for 6 min. At 6 min post-exploration, the total time spent (in seconds) by the test mouse in exploring chamber A and C were measured separately. The index of social preference was defined as the result of the percentage time spent in the opposite chamber subtracted from the percentage time spent in the social chamber.

Forced swimming test (FST)

The FST was carried out in line with the method earlier described with minor adjustments.[22] The apparatus comprised of a transparent water-filled Plexiglas cylinder (37×37×30 cm) to a depth of 25 cm at 25°C in which mice were forced to swim individually. Each mouse was considered immobile when it ceased struggling and floats passively in water. Duration of immobility was noted during a 6 min test session.

Novel object recognition test (NORT)

The NORT was carried out in the open-field chamber (60×50×40 cm) as previously described with slight modifications.[23]

The NORT consists of two phases; the trial phase and the test phase. The trial phase involved placing each animal in-between two duplicate objects (1 and 2) on opposite sides of the open-field chamber, 8 cm (from the walls) and 34 cm (from each other) for 5 min. Afterwards, each animal was returned to its home cage for an interval of 1 hr. In the test phase, object 2 was replaced with object 3 (discriminate object), which was novel to the mice and different from either object 1 or 2. Mice were then left to explore objects 1 and 3 for a period of 5 min. The apparatus was cleaned before and after each test. The duration of time spent (s) in with each object was recorded in both phases.

The percentage preference, which was used as a measure of memory, particularly non-spatial/recognition memory function, was calculated as the total time spent by a mouse in exploring object 3 divided by the summation of total time spent exploring both objects 2 and 3 multiplied by 100%.

Measurement of blood glucose

Blood glucose level was measured using a blood glucose monitoring meter (AccuCheck meter) and blood glucose strip (AccuCheck). The strip was placed into the meter and a drop of blood obtained via ocular puncture was dropped at the tip of the strip and drawn up into the meter. Immediately the blood glucose reading was displayed on the meter and recorded.

Assessment of lipid profile

Determination of serum triglyceride levels

Serum triglyceride level was determined by enzymatic colorimetry following the method earlier described.[24]

Determination of high-density lipoproteins (HDL)

HDL level was determined in mouse serum by enzymatic colorimetric method according to the protocol formerly described.[24]

Determination of biomarkers of oxidative stress

Determination of reduced glutathione (GSH) levels

Glutathione level was determined in brain supernatant using a previously described method and expressed as μmol/g tissue.[25]

Determination of malondialdehyde (MDA) in brain tissues

Determination of MDA in brain tissue homogenate was done following the technique earlier described in which MDA, an end-product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to give a coloured complex.[26] MDA values were expressed as μmol/g tissue.

Determination of superoxide dismutase (SOD) activity

The brain activity of SOD was determined by a previously described method[27] and expressed as units/minute/mg protein.

Estimation of catalase (CAT) activity

Catalase activity in brain supernatant was estimated according to the protocol described beforehand and expressed in μmol of H_2O_2 decomposed per minute/mg protein.[28]

Estimation of brain level of nitric oxide production

Brain nitrite concentration was determined using Greiss reagent – a marker of nitric oxide production – following the method described earlier.[29]

Histology of brain regions

Brain tissue obtained from the mice in each group was fixed in 10% formaldehyde. Hippocampal transverse sections of 5–6 μm thickness were then obtained by slicing with a microtome and processed by the routine method for paraffin wax embedment. The slides were then stained using Hematoxylin and Eosin (H&E) staining pattern. Thereafter, the CA1 of the hippocampus and the prefrontal cortex were examined under the binocular light

microscope. Photomicrographs were thereafter taken with the aid of a digital camera (Japan).

Determination of neuronal density of hippocampal regions

Hippocampal neuronal cells were considered viable if they were round-shaped, had intact cytoplasmic membrane and had no distortions. The neuronal cells were counted in a particular section within a given square area of the circular view of the microscope at X400 magnification.[30]

Statistical analysis

The data were statistically analysed using One-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test and were expressed as mean±S.E.M. Values were considered statistically significant at $p<0.05$.

RESULTS

Effect of Centella lujica supplement on depression-like behaviour in mice subjected to sleep deprivation-induced stress

Sleep deprivation-induced stress significantly increased duration of immobility in the FST (Figure 1) and TST (Figure 2) compared with the control group. Also, the SIT revealed that social interaction/social preference was significantly reduced in the sleep deprived mice when compared with the control group (Figure 3). These suggest the onset of depression-like behaviour. However, pre-treatment with *C. lujica* (50 and 100 mg/kg, p. o.) significantly attenuated this behaviour as effectively as astaxanthin when compared to the model control group.

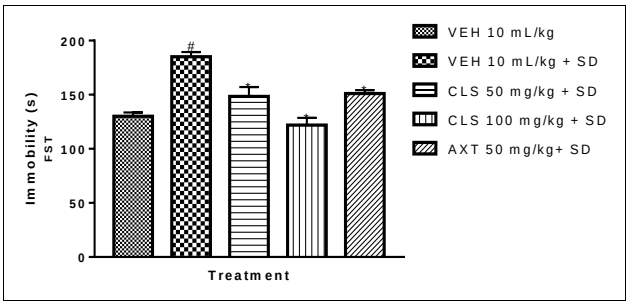

Figure 1: Effect of *Centella lujica* supplement on depression-like behaviour in mice subjected to sleep deprivation-induced stress in the forced swimming test.

VEH: vehicle, AXT: astaxanthin, CLS: *Centella lujica* supplement, SD: sleep deprivation

Effect of Centella lujica supplement on blood glucose in mice subjected to sleep deprivation-induced stress

Sleep deprivation caused a significant increase in mice blood glucose levels when compared with the control group. However, both doses of *C. lujica* significantly reduced blood glucose levels towards normal when compared to the model control group (Figure 4).

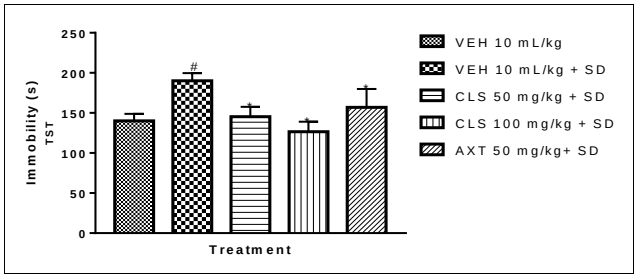

Figure 2: Effect of *Centella lujica* supplement on depression-like behaviour in mice subjected to sleep deprivation-induced stress in the tail suspension test.

VEH: vehicle, AXT: astaxanthin, CLS: *Centella lujica* supplement, SD: sleep deprivation

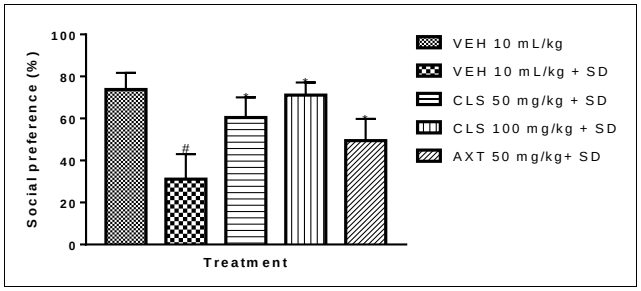

Figure 3: Effect of *Centella lujica* supplement on depression-like behaviour in mice subjected to sleep deprivation-induced stress in the social interaction test.

VEH: vehicle, AXT: astaxanthin, CLS: *Centella lujica* supplement, SD: sleep deprivation

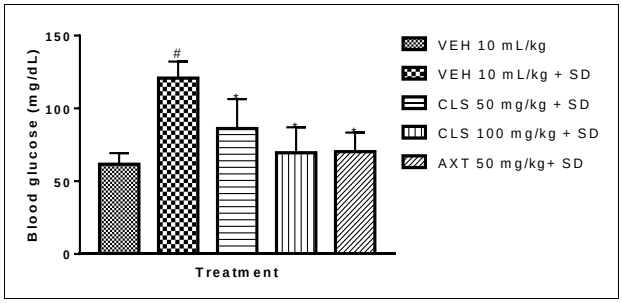

Figure 4: Effect of *Centella lujica* supplement on blood glucose in mice subjected to sleep deprivation-induced stress.

VEH: vehicle, AXT: astaxanthin, CLS: *Centella lujica* supplement, SD: sleep deprivation

Effect of Centella lujica supplement on brain oxidative parameters in mice subjected to sleep deprivation-induced stress

Brain concentration of antioxidants (i.e. GSH, CAT, and SOD) was significantly diminished with a simultaneous

increase in pro-oxidants (i.e. MDA and nitrites) in the sleep deprived group when compared with the control group. These effects were reversed upon administration of *C. lujica* supplement (Table 1).

Table 1: Effect of *Centella lujica* supplement on brain oxidative parameters in mice subjected to sleep deprivation-induced stress.

Treatment	GSH (µmol/g tissue)	CAT (units/mg protein)	SOD (units/mg protein)	MDA (µmol/g tissue)	Nitrite (µ M)
VEH 10 ml/kg	16.09±1.45	33.47±1.94	16.91±0.62	2.58±0.20	47.37±4.56
VEH 10 ml/kg + SD	6.21±0.66$^{\#}$	15.87±1.95$^{\#}$	10.32±0.96$^{\#}$	7.64±0.45$^{\#}$	76.59±6.37$^{\#}$
CLS 50 mg/kg + SD	12.31±1.71*	26.46±2.37*	16.48±0.48*	5.28±0.56*	49.66±4.58*
CLS 100 mg/kg + SD	15.46±0.99*	32.19±3.64*	19.55±0.37*	3.19±0.27*	40.78±5.88*
AXT 50 mg/kg+ SD	13.05±0.88*	27.10±1.88*	15.61±1.07*	5.00±0.48*	42.34±5.14*

Each result is expressed as mean±S.E.M of grouped mice (n=6); #indicates significant difference ($p<0.05$) compared to the vehicle (not sleep deprived) group; *indicates significant difference ($p<0.05$) compared to the vehicle + SD group (one-way ANOVA followed by Student-Newman-Keuls post-hoc test)

Effect of Centella lujica supplement on serum triglyceride levels in mice subjected to sleep deprivation-induced stress

Triglyceride levels was significantly increased in the model control when compared with the control group. Administration of *C. lujica* (in both doses) attenuated this effect (Table 2).

Table 2: Effect of Centella lujica supplement on serum levels of triglycerides and high density lipoproteins in mice subjected to sleep deprivation-induced stress.

Treatment	Triglycerides (mg/dl)	High density lipoproteins (mg/dl)
VEH 10 ml/kg	110.0±2.68	118.3±2.11
VEH 10 ml/kg + SD	166.3±5.76$^{\#}$	88.00±4.03$^{\#}$
CLS 50 mg/kg + SD	143.5±7.83*	105.2±2.61*
CLS 100 mg/kg + SD	136.3±5.89*	112.5±3.22*
AXT 50 mg/kg + SD	149.9±3.41*	104.0±2.37*

Each result is expressed as mean±S.E.M of grouped mice (n=6); #indicates significant difference ($p<0.05$) compared to the vehicle (not sleep deprived) group; *indicates significant difference ($p<0.05$) compared to the vehicle + SD group (one-way ANOVA followed by Student-Newman-Keuls post-hoc test)

Effect of Centella lujica supplement on high density lipoproteins in mice subjected to sleep deprivation-induced stress

Sleep deprivation caused a significant decrease in the level of high density lipoproteins in the serum of mice in the model control group which was absent in the control group. However, administration of *C. lujica* significantly increased the level of serum high density lipoprotein when compared to the model control group (Table 2).

Effect of Centella lujica supplement on recognition memory in mice subjected to sleep deprivation-induced stress

Figure 5 reveals that mice subjected to sleep deprivation had significantly lower percentage preference for the discriminate/novel object than mice in the control group. The reverse was observed in mice pre-treated with *C. lujica* when compared with the model control group (Figure 5).

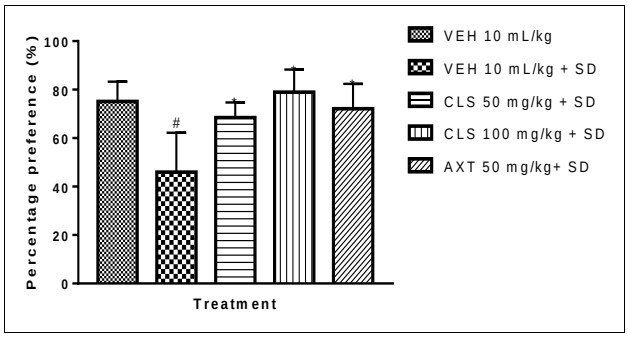

Figure 5: Effect of *Centella lujica* supplement on recognition memory in mice subjected to sleep deprivation-induced stress.

VEH: vehicle, AXT: astaxanthin, CLS: *Centella lujica* supplement, SD: sleep deprivation

Photomicrograph of the hippocampal CA1 region and prefrontal cortex of mice subjected to sleep deprivation-induced stress

Histology of the CA1 region of the hippocampus (Figure 6) and prefrontal cortex (Figure 7) of mice subjected to sleep deprivation revealed neuropathological changes such as significant neuronal necrosis of hippocampal cells when compared with the control group. This effect was however attenuated by *C. lujica* supplementation as effectively as astaxanthin in both regions.

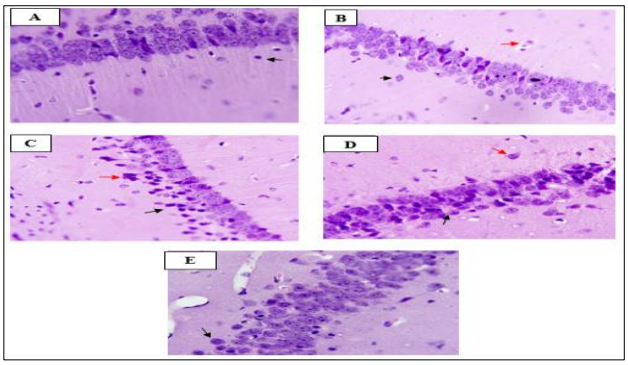

Figure 6: Photomicrograph of the hippocampal CA1 region of mice subjected to sleep deprivation-induced stress.

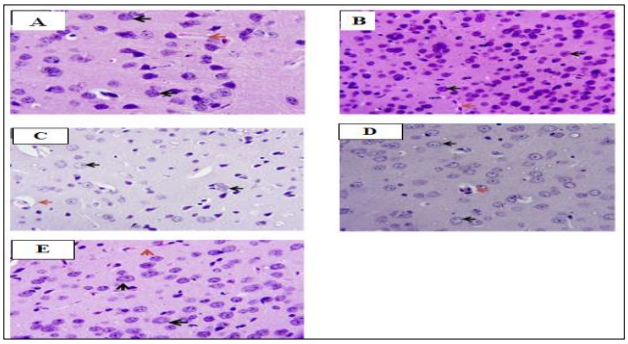

Figure 7: Photomicrograph of the prefrontal cortex of mice after sleep deprivation (A) vehicle only, (B) vehicle+SD, (C) CLS 50 mg/kg+SD, (D) CLS 100 mg/kg+SD, and € AXT 50 mg/kg+SD.

Black arrow: Normal neuronal cells, red arrow: neuronal cells undergoing necrosis, magnification: X400

Effect of Centella lujica supplement on viable hippocampal CA1 and prefrontal cortex neurons in mice subjected to sleep deprivation-induced stress

Furthermore, sleep deprivation significantly decreased the population of viable neuronal cells of the CA1 region (Figure 8) and prefrontal cortex (Figure 9) of the hippocampus when compared to the control group, suggesting neurodegeneration. However, administration of *C. lujica* attenuated this loss of neuronal cells.

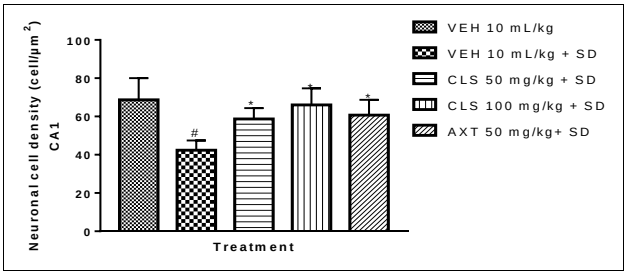

Figure 8: Effect of *Centella lujica* supplement on viable hippocampal CA1 neurons in mice subjected to sleep deprivation-induced stress.

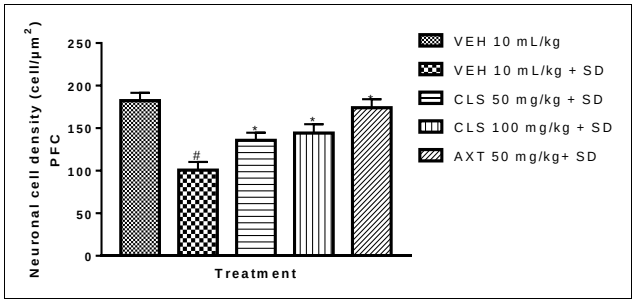

Figure 9: Effect of *Centella lujica* supplement on viable prefrontal cortex neurons in mice subjected to sleep deprivation-induced stress.

Cell counts are based on number of neuronal nuclei in three (3) rectangular boxes per slide, using the pre-calibrated Image J software; each result is expressed as mean±S.E.M for grouped mice (n=3).

DISCUSSION

The liver is the largest visceral organ of the body which plays a role in numerous functions vital for human survival. It is also one of the major organs susceptible to damage by oxidative stress via the mechanism of lipid peroxidation due to its high lipid content.[31] Studies have previously recognized that oxidative stress has a part to play in the development of vascular complications in diabetes (particularly type 2).[32]

This is mainly due to increase in serum glucose and triglyceride levels which is very evident during oxidative stress. It is worthy of note that increased triglycerides levels in the bloodstream have been linked to atherosclerosis, heart disease and stroke in the human body.[33] Supporting, liver damage induced by insufficient sleep in mice was equated to increased triglycerides, reduced high-density lipoproteins and increased glucose production in the present study. However, pre-treatment with *C. lujica* significantly reversed these effects in a dose-dependent pattern, thereby displaying its potential benefit in the management of hepatic diseases.

Also, the brain was adversely affected by oxidative stress in the present study. Endogenous antioxidant levels (measured by GSH, CAT and SOD) were found to be reduced, hence giving way for the accumulation of oxidative species (such as MDA and nitrites). This is in line with existing literature which suggests that the brain consumes a significant amount of oxygen and possesses a reduced antioxidant defence system which makes it susceptible to attack by reactive oxygen species.[2,34]

Furthermore, other central processes were affected by sleep deprivation-induced stress with stressed mice exhibiting depression-like behaviour (assessed using FST, TST and SIT) and recognition memory impairment in the present study. This is in line with previous studies which showed that mice subjected to stress performed poorly in the FST, which is an effective pharmacological tool for the evaluation of depression-like behaviour in rat or mice; and

studies which showed that lapses/deficits in memory were significant in humans exposed to 24 hour sleep deprivation.[6,35] However, an increase in antioxidant activity, a reduction in oxidative species and enhancement of memory and behaviour was observed following pre-treatment with *C. lujica*.[36]

Although the results from this study augment the existing knowledge on *C. lujica* and its potential benefit in alleviating stress, the study was limited by time (i.e. interference by the global lockdown) and financial constraints.

CONCLUSION

The present study has shown that oxidative stress induced by sleep deprivation possesses the ability to impair the normal functioning of the liver and brain. However, a herb such as *C. lujica* with potent adaptogenic property enhanced brain and liver functioning via a mechanism thought to be related to augmentation of antioxidant activity and neuroprotection.

ACKNOWLEDGEMENTS

Authors would like to thank technologists of the Pharmacology laboratory for their assistance throughout the course of the research; and to Mr. Adedamola Fafure, for the histology.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Eduviere AT, Awhin PE, Edje KE, Otomewo LO, Adeoluwa OA, Winter JE. Adaptogenic potential of Centella lujica supplement in sleep deprived mice. Int J Res Med Sci 2021;9:3269-76.