Effects of *Sargassum wightii* Methanolic Extract on L-Methionine Induced Experimental Vascular Dementia like Syndrome in Albino Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Sargassum wightii is brown seaweeds with high nutritive value with natural bioactive compounds having diverse therapeutic activity. In recent years, research on *Sargassum wightii* has gain much interest in neuropharmacological field. Basing on neuroprotective effect of *Sargassum wightii* against Parkinson's disease animal models, the possible protective effect on dementia is hypothesised. Addressing this question the present study was designed to investigate the effect of *S. wightii* on L-methionine induced vascular dementia like syndrome in male wistar albino rats. Vascular dementia was induced by oral administration of L-methionine (1.7 g/kg) per body weight for 4weeks *S. wightii* was given from day1 to day32. The neurobehavioral assessment was performed by Morris water maze test for learning and memory ability and blood sample was collected for serum nitrite and homocysteine analysis. The whole brain content was subjected to a

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series of biochemical analysis that included brain cholinesterase, malondialdehyde, reduced glutathione and histopathological examination. L-methionine impaired learning and memory, increased serum homocysteine and decreased serum nitrite level significantly ($p<0.001$). It also increased brain thiobarbituric acid reactive substances, reduced glutathione and increased acetylcholinesterase activity significantly ($p<0.001$). *Sargassum wightii* at 200mg/kg and 400mg/kg body weight reversed the L-methionine induced learning and memory deficits ($p<0.01$) and ($p<0.001$) respectively. The L-methionine induced biochemical alterations in serum and brain as well as histopathological aberration in cortex and hippocampus were restored by *Sargassum wightii* ($p<0.01$). This study concluded that *Sargassum wightii* ameliorated L-methionine induced vascular dementia like syndrome and provided a new concept in anti-dementia therapy as a neuroprotectants.

1. INTRODUCTION

Vascular dementia is a type of chronic progressive neurodegenerative disorder caused by cerebrovascular lesions that restrict the blood flow to different part of the brain. Persistent decrease in blood flow to the brain region causes hypoxia and nutrition supply to the brain tissue leading to death of neuronal tissues that is responsible for progressive deterioration of cognitive abilities and learning memory function. The clinical relevance of vascular dementia in forms lacunar infracts have a relation with cognitive impairment in cerebrovascular diseases also support this phenomenon [1]. Vascular dementia is the second common type of dementia, and it caused due to endothelial dependent relaxing factor, endothelial nitric oxide synthesis and reduced vascular tone in vascular system [2]. Evidence supported that the vascular oxidative stress and endothelial dysfunction is the major functions for the pathogenesis of dementia of vascular origin [3]. Homocysteine, a metabolic by product of L-methionine, causes neurodegeneration similar to vascular dementia like syndrome. Hence elevation of total plasma homocysteine level is an important indicator for cardiovascular disease and vascular dementia. It has also been reported the homocysteine induced endothelial dysfunction by reducing bioavailability of nitric oxide (NO) and increasing oxidative stress [4]. The increased level of reactive oxygen species bring a structural and functional change in cerebral blood vessel causing cerebrovascular lesion that is recognized as an important factor in the pathogenesis of dementia. Only few therapeutic agents are available to manage dementia in vascular origin [5,6]. Polyphenolic compounds like flavonoids present in marine macro algae being potent antioxidants. The antioxidant, anti-inflammatory and neuroprotective effects of *Sargassum wightii* were well established [7,8]. But there is paucity of data regarding neuroprotective effect of *Sargassum wightii* especially of Indian coasts origin. Evidence on the protective effect of *S wightii* in Alzheimer's disease in an *in-vitro* [9] test guided us to hypothesize the protective effect of *S. wightii* in vascular dementia model. Therefore in this work, we took interest to search the possible neuroprotective effects of methanolic extract of *Sargassum wightii*, in L-methionine induced vascular dementia in rats.

2. MATERIALS AND METHODS

2.1 Plant Material

The plant material *Sargassum wightii* was collected as a gift sample from Prasad Biotech limited, Gujarat India. To prepare a methanolic extract of *Sargassum wightii,* 40grams of powdered sample was extracted with 400ml of methanol by using Soxhlet's apparatus for 72 hours. The extract obtained was dried in the evaporator and stored at 4°C for further use. Different concentration of drug solution was freshly prepared on the day of experiment using 0.5% carboxy methyl cellulose suspension [10].

2.1.1 Phytochemical screening of *Sargassum wightii* extract

The phytochemical screening for phytochemical constituents of methanolic extract of *S. wightii* was tested in my earlier study and reported by us. [10].

The patterns of composition of alkaloids, steroids, tannins, saponins, flavonoids, phenols, proteins and glycosides were performed with methanol extracts of *Sargassum wightii* differed considerably in their varied degree. Saponins did not show any positive result for its presence in the extract.

2.2 TLC Analysis

The phenolic and tannins present in methanolic extracts of *Sargassum wightii* were qualitatively detected by using TLC. The chloroform, methanol and n-hexane (6:3:1 ratio) was used as mobile phase for phenolic compounds and toluene and acetone (7:3 ratio) was used for tannins. Folin ciocalteau reagent was used as spying agents to detect the phenolic compound. The appearance of blue colour spot in the TLC plate indicated the presence of phenolic compounds. Three distinct phenolic spots were detected in methanolic extract of *S. wightii* with different Rf values of 0.270, 0.531 and 0.821.The 3% ferric chloride was served as spying agent to detect tannin. The occurrence of dark black zone in the TLC plate indicated the presence of tannins. Tannins profile was displayed only one distinct spot with the Rf value of 0.0710.

2.3 Quantitative Analysis

Phenolics, flavonoids and tannins contents of *Sargassum wightii* were estimated quantitatively varied according to solvents used in extraction processes [10]. The highest total phenolics (2.74 ± 0.17 mg GAE/g dry wt), tannins (2.65 ± 0.43 mg CAE /g dry wt) and flavonoids (1.97 ± 0.03 mg RUE/g dry wt) was recorded in *S. wightii* extract.

2.4 Drugs and Chemicals

L-methionine from Sigma-Aldrich, Inc., MI, USA, Nitrobluetetrazolium chloride, 5,5-dithiobis-(2-nitrobenzoic acid), thiobarbituric acid trichloro acetic acid from HiMediaLaboratories Pvt. Ltd. Mumbai, India. All other chemicals were used with analytical grade and procured from standard manufacturers.

2.5 Animal

Adult male Wistar albino rats (weighing between 100-150 grams) were employed in this study. All animals were maintained in central animal house of Roland Institute of Pharmaceutical Sciences, Berhampur, were used in the present experiment. Rats were allowed to acclimatize in standard laboratory environment for 7 days prior to the experimental procedure. Animals were fed with standard chow diet and had free access to water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC), (Regd. No: 926/PO/Re/S/06/CPCSEA) and care of the animal has taken as per the Experiments on Animals (CPCSEA) formulated by the Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

2.6 Experimental Design

Twenty male Wistar rats were divided into four groups (n=5). L-methionine was dissolved in 0.5% (w/v) CMC and administered (1.7g/kg, *p.o.*) for 4weeks to induce endothelial dysfunction and vascular dementia. All drugs and vehicle were given orally for 32 days [11,12].

Group-I: Normal control (vehicle) rats were administered with 0.5% (w/v) CMC for 4weeks and the subjected to Morris water maze (MWM) test. The vehicle was administered 45 min before acquisition trial conducted from day 1 to day 4 and retrieval test conducted on day 5.Group-II: L-methionine (1.7g/kg, *p.o.*) in order to induced vascular dementia. The animals were administered with L-methionine (1.7 g/kg/day, *p.o.*) for 4weeks the subjected to MWM test and continued 45 min before acquisition trial conducted from day 1 to day 4 and animals were administered with vehicle during retrieval test conducted on day 5. For group Group-III: L-methionine + *S. wightii* (200mg/kg) and Group-IV: L-methionine + *S. wightii* (400mg/kg), all animal were subjected to MWM test on 28th day and L-methionine continued 45 min before acquisition trial conducted from day 1 to day 4 and animals were administered with vehicle during retrial test conducted on day 5.

On 32th day animals were sacrificed by cervical dislocation. Just before sacrificing, the blood samples were collected for determination of serum nitrite and homocysteine concentration. Brains were removed, hippocampus and cortex was dissected from forebrain and subjected to biochemical analysis (Brain cholinesterase activity, TBAR and GSH level) and histopathological assays. Two brains from each group were preserved in 10% formalin and sent for histopathological examination of hippocampus and cortex [13].

2.7 Behavioural Assessment

2.7.1 Morris water maze (MWM)

Morris water maze test was employed to assess learning and memory of the experimental animal. The MWM principle based on when the animal places in large pool of water as they dislike swimming, their tendency to escape from the water being accomplished by finding the escape platform.

The water maize consists of a circular pool (150 cm in diameter and 45 cm height), filled with up to 30 cm water at temperature 28±1°C. The water was opaque with non- toxic white colour dye. The pool was divided hypothetically into four equal quadrants by help of two threads; fixed at right angle to each other on the rim of the pool. A platform of (10 cm^2) was submerged 2 cm below the surface of water and the position of the platform remains unaltered throughout the training session. Each rat was given four consecutive trials on each testing day with gap of 5 min. The rat was gently placed in the water of the pool between the quadrants facing wall of the pool by changing the location for each trial and allowed them for 120 seconds to locate the submersed platform. Then animal was allowed to stay on the platform for 20 seconds, if it was failed to find the hidden platform, it was guided manually on to the platform for 20 seconds for rest. The escape latency time to locate the hidden platform in the maze was taken as an index of acquisition or learning. Animal was subjected to four acquisition trial daily for four consecutive days. On the fifth day, the platform was removed from the pool and each rat was allowed to explore for 120 seconds, the mean time spent in all four quadrant was note and mean time spent by the animal in the target quadrant searching for the hidden platform was noted as an index of retrieval or memory Morris RGM [14,15].

2.7.2 Biochemical analysis

After the behavioral assessment, the blood sample was collected by retro-orbital puncture and pooled in to Eppendorf tube, permitted to coagulate for 15 min at room temperature then centrifuged the sample at 3000 rpm for 15 min to separate the serum. The partitioned serum was used for estimation of nitrite and homocysteine level. Afterwards the animals were sacrificed by cervical dislocation, the brain tissue were carefully removed and subjected to various biochemical analysis. After removing the brains of the animal homogenised with 10% w/v phosphate buffer (pH 7.4) using homogenizer and centrifuged at 3000 rpm, the supernatant fluid was used for estimation of acetyl cholinesterase activity (AChE), thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH). Histopathological studies were performed on the brain tissue of rats randomly selected from each group. Brains were removed and immediately isolate cerebral cortex and preserved in 10% formalin and stained with cresyl violet and viewed under light microscope.

2.7.3 Estimation of serum nitrite concentration

Determination of serum nitrite concentration was done by method of Sastry et al. [16]. 400 µL of carbonate buffer (pH 9.0) was added to 100 µl of serum and standard sample followed by addition of small amount of copper-cadmium alloy (≈ 0.15 gram). Then all the samples were incubated for 1hour to reduce nitrate to nitrite. Then the reaction was stopped by adding 100µl of 0.35M sodium hydroxide and followed by addition of 400 µl of 120mM zinc sulfate solution was added to deproteinate the samples. The samples were allowed to stand for 10 min and again centrifuged at 4000 ×g for 10 min. Greiss reagent (250µL of 1% sulfanilamide in 3N HCl and 250µL of 0.1% N-naphthylelediamine in water) was added to the clear supernatant (0.5mL) then serum nitrite was measured spectrophotometrically at 545nm. The standard curve of sodium nitrite (5-50 µM) was plotted to calculate concentration of serum nitrite.

2.7.4 Estimation of serum homocysteine concentration

Determination of homocysteine (Hcy) of blood serum was carried out by Dimitrova et al.[17] using HPLC (Prominence HPLC with FD) attached with RTXL-10 fluorescence detector (Shimadzu SPD 20 AD).100 µL of serum sample was added to 1.5 mL of eppendorf containing 10µL of water and 5 µL of n-amyl alcohol and gently vortexed. 35 µL of sodium borohydrate reagent (35 µL of 1.43 M sodium borohydride in 0.1 M sodium hydroxide), 35 µL of 1 M of HCl and 50 µL of 10 mM monobromobimane in 4mM sodium EDTA (pH 7) were added to eppendorf containing serum sample mixed well and incubated at 42 °C for 12 minutes. Then it was cooled, vortexed and maintained at room temperature for 10 min. The sample was centrifuged at 12000×g for 10 min to remove protein, and then the supernatant was adjusted to pH 4 using 25 µl of 2 M Trtis-HCL. The sample was then centrifuged at 12000 ×g for 1 min and 100 µl of supernatant was taken for analysis. The calibration curve for Hcy as L-homocysteine was plotted using 10 µl (1-1000 µM) solution. The calibration curve was regressed and the concentration of serum Hcy was calculated. Animal with serum Hcy levels more than 10 µM were considered to be hyperhomocysteinemic. [17,18].

2.7.5 Measurement of brain acetylcholinesterase concentration

The quantitative measurement of brain acetylcholinesterase (AChE) was performed as

method described by Ellman *et al.*,. The reaction mixture was prepared by using 0.05ml of iodide and 0.10 ml of DTNB (Ellman's reagent). The change in absorbance of prepared solution was measured for 2 min after every 30 seconds at 412 nm spectrophotometrically. The enzymatic activity of the supernatant solution expressed as nmol/min/g of tissue [19,20].

2.7.6 Measurement of brain lipid peroxide concentration

The lipid peroxides were estimated as thiobarbituric acid reactive substances (TBARS) using malondialdehyde (MDA). The quantitative analysis of lipid peroxidation was assayed by measuring the level of MDA in the brain tissue following the method of Ohkawa *et al.*,(1996). The MDA activity was determined by measuring the absorbance of thiobarbituric reactive species spectrophotometrically (Shimadzu UV 1800) at 532 nm [21].

2.7.7 Measurement of brain reduced glutathione concentration

The levels of reduced glutathione (GSH) in brain tissue homogenate were estimated by the method described by Ellman *et al.,* 1959. The supernatant 1ml of the tissue homogenate was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4 °C for 1 hour then the sample centrifuged at 1200×g for 15 min. An aliquot of supernatant was diluted with 2.7ml of phosphate buffer (0.1M, pH 8), and 0.2 ml of DTNB. The yellow color reaction mixture was measured immediately at 412 nm using spectrophotometer. The assessed reduced glutathione was expressed as mg/g wet tissue [22,23].

2.8 Histopathological Studies

Histopathological studies were performed on the brain tissue of rats randomly selected from each group. Brains were removed and immediately and isolate cerebral cortex and preserved in 10% formalin and stained with cresyl violet and viewed under light microscope.

2.9 Statically Analysis

The results were expressed as mean ± standard error. And the results were analyzed for statistical significance using one way ANOVA followed by Tukey's multiple comparison tests using graph pad prism. A probability value less than 0.05 (p<0.05) was considered as minimum level of significance.

3. RESULTS

3.1 *Sargassum wightii* Enhanced Learning and Memory Ability in Morris Water Maze Tests

The animal in each group were trained for 4consecutive days in MWM apparatus started from 28th day of the drugs administration. The rats was received L-methionine showed a significant increase in mean escape latency time (ELT) during the 4 days and time spent in target quadrant during day 5 retrieval trial as compare to the normal control group (Table-1). Administration of *S. wightii* 400mg/kg along with L-methionine treated animal significantly decreased the day 4 ELT (p<0.01) as well as increase in time spent in target quadrant (p<0.001)in contrast to L-methionine alone treated group, that indicates reversal of loss of memory of the rats as compared to rats only treated with L-methionine .

3.1.1 *Sargassum wightii* attenuated L-methionine induced change in serum Hcy and serum nitrite concentration

Administration of L-methionine at 1.7g/kg, *p.o.* amplified the serum Hcy level and diminished the serum nitrite level as compared to the normal control. *Sargassum wightii* at 200 mg/kg and 400 mg/kg reverse the L-methionine induced amplified the serum Hcy level and marked reduction in serum nitrite as compared to the L-methionine treated group (Fig.1).

3.1.2 *Sargassum wightii* attenuated L-methionine induced change in AChE, MDA and GSH

Administration of L-methionine (1.7g/kg, *p.o.*), there was a highly significant increase in brain AChE activity and TBARS, respectively as compare to normal control group (p<0.001). Treatment with both the doses of *S. wightii* 200 mg/kg and 400 mg/kg significantly (p<0.01), (p<0.001) attenuated L-methionine-induced rise in brain AChE activity and TBARS. Similarly L-Methionine (1.7g/kg/day, *p.o.*) significantly decreased the GSH level (p<0.001) as compare to normal control, where as *S. wightii* both 200mg/kg and 400mg/kg significantly reverse the change in oxidative stressed biomarker enzyme reduced glutathione (GSH), (p<0.01), (p<0.001) respectively (Table-2).

Table 1. *Sargassum wightii* on L-methionine induced change in escape latency time, using Morris water maze test

Serial no.	Group	Dose of treatment	Day 1ELT in second	Day 4 ELT in second	Time spent (second) in target quadrant
I	Normal control (0.5% CMC)	10ml/kg	82.0±2.98	23.33±1.20	5.4±0.57
II	L-methionine	1.7g/kg	75.41±2.97	63.79±2.31[#]	3.19±0.61[#]
III	*S. wightii*+ L-methionine	200mg/kg + 1.7g/kg	91.2±1.98	51.83±2.1	7.47±0.36**
IV	*S. wightii*+ L-methionine	400mg/kg + 1.7g/kg	81.0±2.54	37.13±1.95**	8.21±0.28***

*Values are expressed mean ± Standard error ***=P<0.001 vs. DAY 4 control rats; **=P<0.01 vs. day 4 ELT in tests vs L-methionine treated rats [#]= P<0.001 control vs L-methionine control using one way ANOVA followed by Tukey's multiple comparisons test.*

Table 2. *Sargassum wightii* on L-methionine-induced change in biochemical parameters

SI no.	Group	Dose	AChE (nM/min/g of tissue)	MDA (nM/g of tissue)	GSH (mg/g of tissue)
I	Normal control(0.5% CMC)	10ml/kg	4.81±0.45	3.12±0.29	16.37±0.21
II	L-methionine	1.7g/kg	12.15±0.19[#]	11.14±0.59[#]	6.12±0.27[#]
III	*S. wightii*+ L-methionine	200mg/kg+ 1.7g/kg	6.18±0.81**	4.65±0.38**	11.78±0.44**
IV	*S. wightii*+ L-methionine	400mg/kg+ 1.7g/kg	5.61±0.93***	3.94±0.14***	15.62±0.21***

*Values are expressed mean ± Standard error; [#]=P<0.001 control vs L-methionine control, ***= P<0.001**= P<0.01 test group vs L-methionine treated group, using one way ANOVA followed by Tukey's multiple comparisons test.*
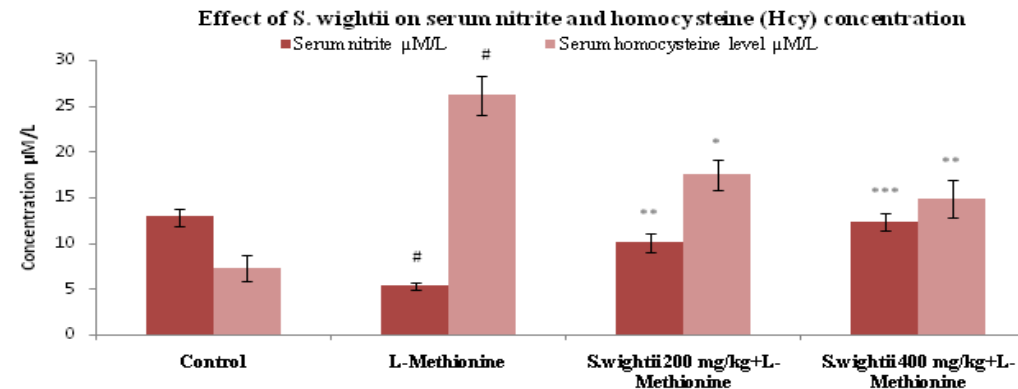

Fig. 1. Effect of *Sargassum wightii,* One-way ANOVA followed by Tukey's multiple comparison tests was applied for analysis ***= P<0.001, **= P<0.01 tests vs L-methionine treated rats, #; P<0.001 vs L-methionine control using one way ANOVA followed by Tukey's multiple comparisons test

Control	L-Methionine	Control	L-Methionine

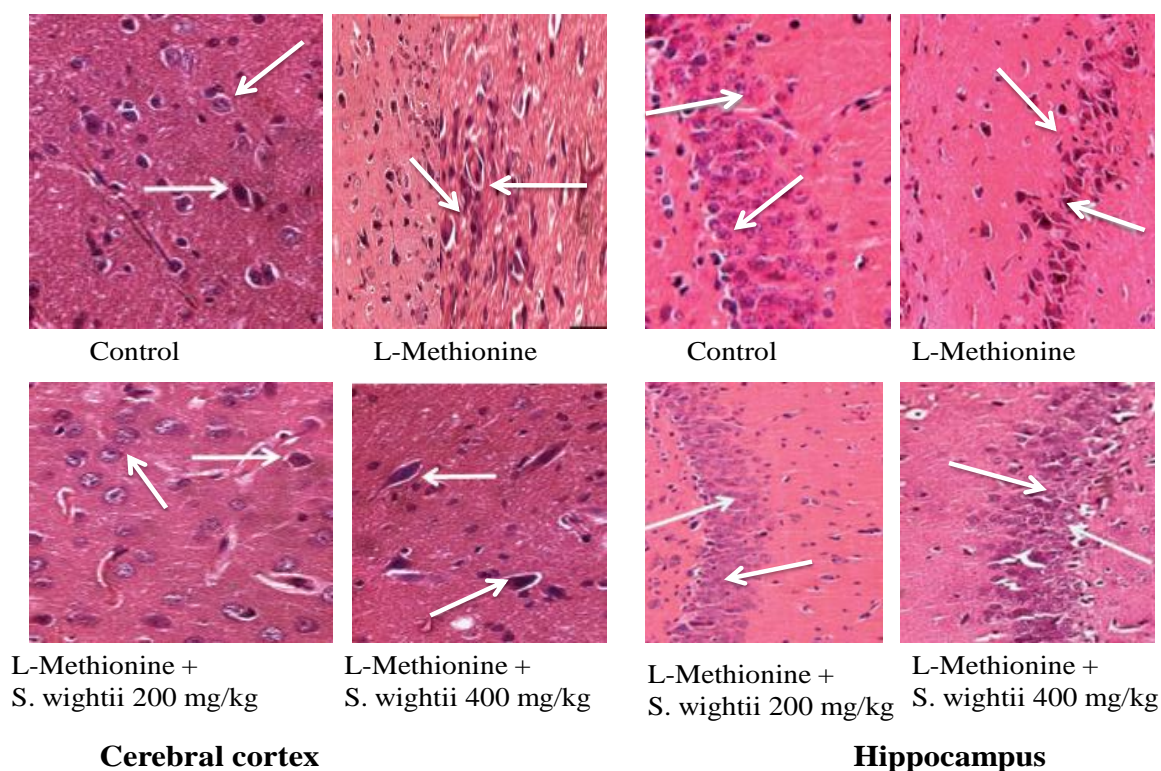| L-Methionine + S. wightii 200 mg/kg | L-Methionine + S. wightii 400 mg/kg | L-Methionine + S. wightii 200 mg/kg | L-Methionine + S. wightii 400 mg/kg |

Cerebral cortex   **Hippocampus**

Fig. 2. Photomicrograph of cerebral cortex showed normal neuronal arrangement in control, necrotic foci in L-methionine and no sign of foci in L-methionine+ *S. wightii*. For hippocampus, neuronal arrangement, loss of structural integrity in L-methionine and normal neuronal arrangement structure in L-methionine+ *S. wightii*

3.1.3 *S. wightii* on histological evaluation

Histological examination of rat brain cortex showed necrotic foci in parenchymal cells and neurodegeneration in L-methionine treated group as compared to the normal control. Pre-treatment with *S. wightii* 200mg/kg and 400mg/kg showed no sign of foci and neurodegeneration in the cortex. Similarly histological examination of hippocampus control showing normal neuron arrangement with normal structural integrity, L-methionine treated group neuronal cells lost the structural integrity and loss of cell outline. *S. wightii* 200 mg/kg and 400 mg/kg showed normal neuronal arrangement and normal neuronal structure as compared to the L-methionine alone treated group (Fig. 2).

4. DISCUSSION

Vascular dementia is a dynamic and the first usual cases of dementia after Alzheimer's disease. There is no treatment to diminish the pathological process correlated to vascular dementia. Dementia in vascular origin that influences capacities to reduce blood supply to the brain, the patient suffers subjected to impedance like discouragement, memory, learning, recognition, and executive capacities. The cerebral atrophy is a new emerging feature of cerebral small vessel disease and the progressive atrophy of grey matter, recently documented in patients with stroke of the lacunar type with cognitive impairment [24]. Other than cholinesterase inhibitor and amantadine, no other medication until today had been discovered today to manage vascular dementia. Nutritional deficiency, neuro-inflammation, cerebrovascular abnormality and oxidative stress have been widely correlated for the pathogenesis of vascular dementia [25]. Over the decade several scientists have been directed to investigate a specific goal to explore, weather anti-oxidant and neuroprotective agents exert a role in the prevention and treatment of vascular dementia [26,27,28]. Recent years studies had reported experimental hyperhomosteinemia resulting from chronic administration of L-methionine produces cognitive impairment [29]. Hcy is an essential sulphur containing amino acids obtained from

methionine metabolism. The auto oxidation of Hcy results in change in redox homoeostasis and increase oxidative stress leads to vascular changes in cerebrovascular cells and impairs brain energy metabolism causes neuronal damage [30,31]. Hyperhomocysteinemia, serum nitrite concentration have also been reported to induce endothelia dysfunction by decreasing the bioavailability of nitrous oxide (NO) and increasing oxidative stress [11]. NO formed during endogenous reaction is highly unstable and gets converted to nitrite and nitrate. [4] *S. wightii* is a brown algae rich source of bioactive component with own biological activities to study L-methionine induced dementia model in rats. MWM test employed in present study is one of the most widely accepted models to evaluate learning and memory in experimental animals [32,33]. Chronic administration of L-methionine produced a significant impairment of acquisition and retrieval of memory in MWM performance, *Sargassum wightii* exhibited marked improvement to find out the hidden platform and time spent in target quadrant. Enhancement of brain AChE activity and decrease bioavailability of acetylcholine (Ach) in brain. Increase in brain anti oxidant enzyme status such as TBAR, GSH in brain and decrease serum nitrite concentration by L-methionine treatment attenuated by *S. wightii*. Our observations are corroborated with earlier reports [32,11]. Chronic administration of *S. wightii* extract elevated the bioavailability of ACh content by reducing the AChE enzyme activity, and this effect may be one of the mechanisms by which it improves the brain function of animal and similar justification was also reported in the study of Arif Nisha *et al.*, 2013 [9]. Moreover, treatment with Sargassum extract both the dose (200mg/kg and 400mg/kg) significantly improved L-methionine induced endothelial dysfunction as measured in the terms of increased serum nitrite levels and decreased oxidative stress markers (decrease TBARS and increase, GSH). This experimental corroborated with Wang Pian *et al* finding amelioration of *Spirulina platensis* in L-methionine vascular dementia [34]. Meanwhile, the histological evidences showed pre-treatment with *S. wightii* 200mg/kg and 400mg/kg showed no sign neurodegeneration and neuron arrangement with normal structural integrity in the cortex hippocampus. The neuroprotective effect and other neurobehavioral symptoms in L-methionine induced vascular dementia was shown by *S. wightii* may be due to combination of multifactorial mechanisms such as enhancement of cholinergic function, serum nitrite concentration, GSH level, reduced Hcy and oxidative stress activity.

5. CONCLUSION

Sargassum wightii may be regarded as a potential therapeutic strategy to retard the progression of disease in vascular dementia. In this current study conclude that methanolic extract of *Sargassum wightii* rich source of bioactive compounds with promising therapeutic potential exerted by its antioxidant and anti-cholinesterase activity improved endothelial dysfunction on L-methionine induced dementia like syndrome in rats. Further studies are needed to isolate, characterize and determined the structure of the active bioactive molecule and evaluation in different therapeutic and clinical models and safety in long term use.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experiment was conducted upon receiving approval from the Institutional Animal Ethics Committee (IAEC), Roland Institute of Pharmaceutical Sciences, Berhampur, Odisha, India (Ref No: 926/PO/Re/S/06/ CPCSEA). All the experimental procedures strictly adhered to the Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) formulated by the Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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