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Beta-sitosterol enhances motor coordination, attenuates memory loss and demyelination in a vanadium-induced model of experimental neurotoxicity

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ABSTRACT

Environmental discharge of vanadium causes cognitive and behavioral impairments in humans and animals via production of reactive oxygen species leading to lipid peroxidation and alteration in antioxidant defence system. The current study was carried out to investigate the cognitive-enhancing ability of β-sitosterol in vanadium-induced neurotoxicity.

Forty eight mice were randomly assigned into 4 groups (A–D) with the following treatments: group A; distilled water, B; α-tocopherol + sodium metavanadate (NaO_3V), C; β-sitosterol + NaO_3V and D; only NaO_3V. NaO_3V was administered intraperitoneally while other treatments were administered through gavage for 7 consecutive days.

Neurobehavioral parameters measuring cognition, locomotion, anxiety and grip strength were evaluated at day 8. Following sacrifice, brain levels of catalase, superoxide dismutase, glutathione, malonaldehyde (MDA) and hydrogen peroxide (H_2O_2) were measured. Immunohistochemical expression of Myelin Basic Protein (MBP) in the brain was also investigated.

The results showed that deficits in spatial learning, locomotor efficiency, and motor coordination, induced by acute vanadium neurotoxicity were mitigated by beta-sitosterol. Significantly ($\alpha \leq 0.05$) decreased *in vivo* antioxidant enzyme activities, increased brain levels of MDA and H_2O_2, structural damage to myelin sheaths and decreased expression of MBP were also observed in the NaO_3V group (D), however, co-administration of β-sitosterol reduced these pathologic features.

It is concluded that β-sitosterol alleviates vanadium-induced neurotoxicity by enhancing cognition and improving motor co-ordination via its antioxidant and myelo-protective activities.

1. Introduction

Vanadium is a heavy metal with industrial and technological applications leading to its discharge in the environment [1]. When in low concentrations, it maintains various biochemical and physiological functions in living organisms but becomes harmful when it exceeds certain threshold concentrations [2]. Despite its adverse effects and persistence in the environment, vanadium exposure is on the increase in several parts of the world [3,4] especially in oil fields generating plants, petrochemical, steel and mining industries. Vanadium accumulates in soil, groundwater, and plants that are consumed by animals and humans [5,6]. It has been reported that vanadium damage cell membranes via free radical injury leading to oxidative stress [3]. The polyunsaturated fatty acid side chains, high oxygen tension and poor antioxidant capacity of the central nervous system (CNS) make it vulnerable to free radical damage [7]. Furthermore, vanadium crosses the blood brain barrier [8] inducing alterations in levels of biogenic amine neurotransmitters in the brain [9]. Vanadium neurotoxicity causes neurobehavioral impairments such as: tremor, decreased visuo-

Abbreviations: SEM, standard error of mean; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; MDA, malondialdehyde; P, escape latency; VitE, α-tocopherol; V, sodium metavanadate; Sito, β-sitosterol.

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spatial abilities, decreased locomotion as well as attention deficits [10–14]. Damage to myelin tracts, degeneration of the Purkinje cell layer of the cerebellum and lipid peroxidation in discrete brain regions have also been reported [15,16], leading to impairments in cognition.

Synthetic antioxidants such as ascorbic acid and α-tocopherol have yielded improvement in enzymatic activities and sensorimotor dysfunctions but their impact on axonal degeneration and segmental demyelination in brain hypothalamic region is limited [14,17]. Phenolic and other endogenous metabolites in plants have been documented to possess wide varieties of free radical scavenging and antioxidant activities [18,19]. Thus, increasing attention has been given to plants in a bid to discover more effective and cheaper antioxidant sources [20,21], for instance, *Ginkgo biloba* (EGb 761) is known for its excellent antioxidant properties that restrict brain damage and neurodegeneration [22]. Also, EGb 761 improves cognitive activities and neuronal function in Alzheimer's disease [23].

Grewia species are used in folk medicine worldwide [24]. Previous phytochemical studies of *G.carpinifolia* showed the presence of tannins, saponins, flavonoids, glycosides, phenols, steroids and the absence of alkaloids in the leaf and stem bark [25]. In addition, ethanol crude extract of *G. carpinifolia* leaf possesses considerable CNS and antioxidant activities [25–27]. Consequently, since antioxidant and chelating agents have been proposed for the treatment of vanadium poisoning, the current study investigated the neuroprotective ability of an isolated bioactive compound of *Grewia carpinifolia* following acute vanadium toxicity.

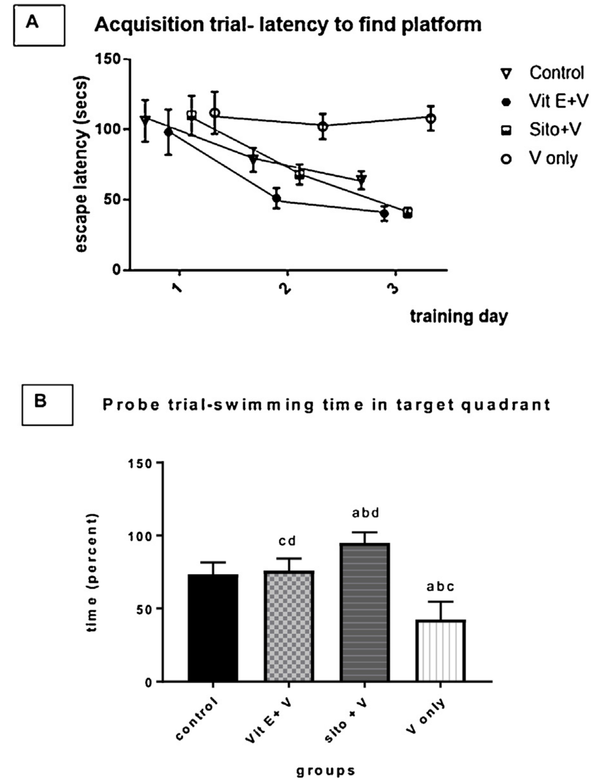

Fig. 1. Effect of β-sitosterol on (A) escape latency in the acquisition trial (B) mean percentage of total time spent swimming in the target quadrant in the probe trial of Morris water maze.
n = 12, Vit E= α-tocopherol; V = sodium metavanadate; sito= β-sitosterol; a = significantly different from the control at $\alpha<0.05$, b = significantly different from the α-tocopherol + sodium metavanadate group, c = significantly different from the β-sitosterol + sodium metavanadate group; d = significantly different from the sodium metavanadate group.

2. Materials and methods

2.1. Animals

Forty eight weaned (juvenile) BALB/c male mice of about 4 weeks old were obtained and housed at the Animal House, Department of Veterinary Physiology and Biochemistry, University of Ibadan, Nigeria and randomly divided into four groups (A–D). The animals were housed under standard conditions of temperature, (25 ± 2 °C) and light, (approximately 12/12 h light-dark cycle), relative humidity 45 ± 5%, fed on standard rodent laboratory diet (Animalcare® Feeds Ltd., Nigeria) and unlimited supply of water. All the animals were acclimatized to laboratory conditions for two weeks prior to the experiment. All the experimental protocols and investigations complied with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Animal Care and Use Research Ethics Committee, University of Ibadan (UI-ACUREC/App/2016/025).

2.2. Isolation of β-sitosterol from Grewia carpinifolia

β-sitosterol isolated from *G. carpinifolia* as earlier described [28] was used for the present study.

2.3. Experimental design

Animals in group A were given distilled water throughout the experiment and served as control. Group B: the standard group, was administered vitamin E (500 mg/kg) orally every 72 h and a daily dose of sodium metavanadate (NaO_3V) (pH, 7.8) (Sigma-Aldrich, St. Louis, USA) at 3 mg/kg [14,29] intraperitoneally (i/p) for 7 consecutive days. Group C: the test group was administered 100 μg β-sitosterol orally, and NaO_3V (3 mg/kg) i/p for 7 consecutive days while group D: the model group, was administered NaO_3V (3 mg/kg) i/p for 7 consecutive days.

2.4. Behavioral tests

Twenty four hours after the administration of the last dose of the extract, Morris water maze (MWM), open field and Hanging wire tests were performed on each animal.

2.5. Morris water maze

The maze was 100 cm in diameter and 40 cm high and divided into four quadrants. A height adjustable circular platform (6 cm diameter) was placed in one quadrant. The tank was filled with water to about 1 cm above the platform. This behavioral task consists of an acquisition phase and a probe trial. The protocol was similar to that described by Morris [30] with minor modifications [31]. In the acquisition phase, mice were subjected to daily sessions of four trials per day for 3 days to find the submerged platform that was located at the centre of the south east (SE) quadrant of the pool and remained at the same position throughout the whole experiment. At the beginning of the experiment, animals were placed on the platform for about 20 s after which they were gently placed in the water in the North West (NW) quadrant with their heads facing the wall. Timing was started simultaneously to record the time it takes the animal to find the platform once it is placed in the water. The learning curve for each animal was constructed by plotting the trial number on x-axis and time to find the platform on y-axis. The probe trial was performed 24 h after the last acquisition trial, in which the platform was removed. The mice were allowed to swim freely for 120 s. The number of crossings over the position at which the platform had been located and the swimming time in the quad-

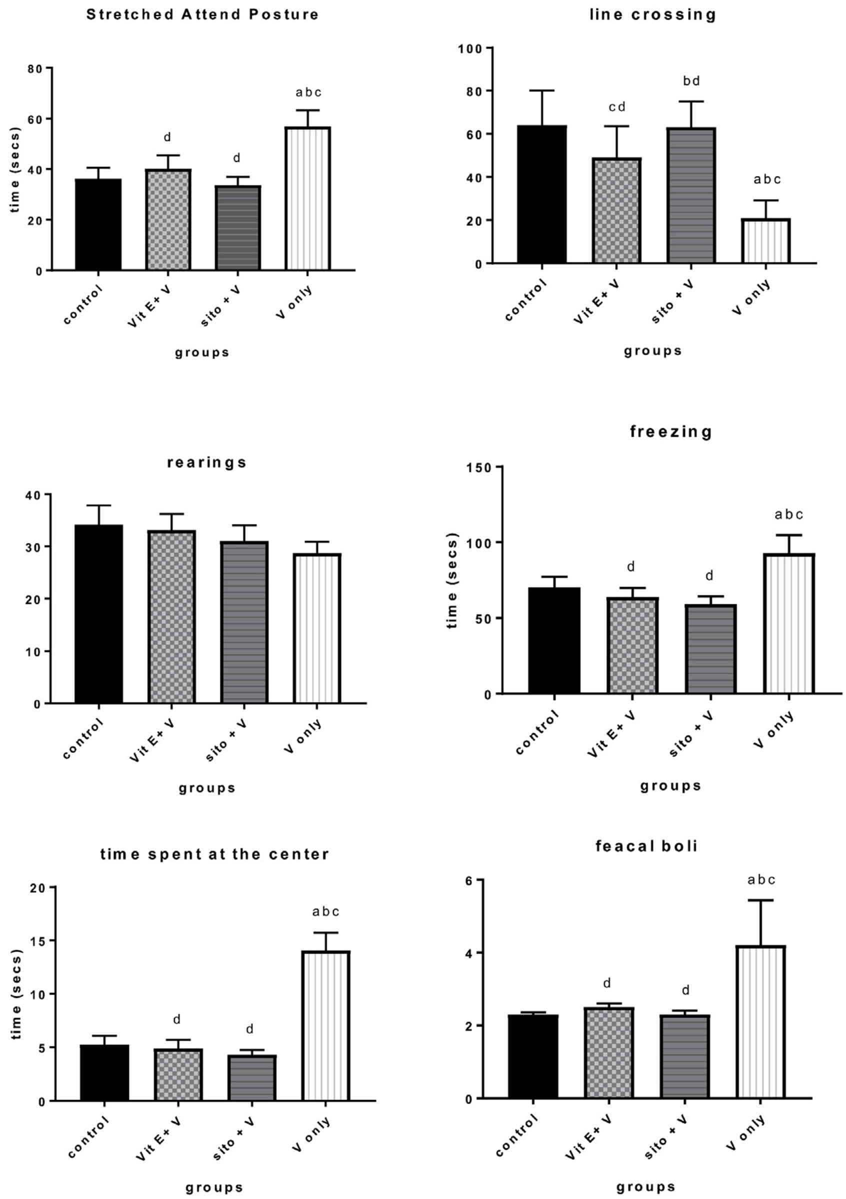

Fig. 2. Stretch attend posture, number of line crossings and rearing, freezing, time spent at the centre and faecal boli in the open field test following concurrent administration of β-sitosterol and vanadium.
n = 12, Vit E= α-tocopherol; V = sodium metavanadate; sito= β-sitosterol; a = significantly different from the control at α<0.05,b = significantly different from the α-tocopherol + sodium metavanadate group, c = significantly different from the β-sitosterol + sodium metavanadate group; d = significantly different from the sodium metavanadate group.

rant of the former platform position were recorded as measures for spatial memory.

2.6. Open-field test (OFT)

Each mouse was placed in the centre of a square box (120 x 120 cm). The floor was divided into 20 cm squares drawn in black ink. At the beginning of each test, every animal was introduced to the centre square and allowed to explore the box freely for 5 min. The following observations were recorded:

Stretch attend Posture- Frequency with which the mouse demonstrated forward elongation of the head and shoulders followed by retraction to the original position

Line crossing- number of times a mouse crossed from one square to another entering with at least its two front paws

Number of rearing - number of times mouse stood on its hind legs

Number of grooming- a set of heterogeneous constituents comprising face washing, body licking, paw licking, head and body shaking, scratching and genital licking.

Freezing- Duration with which the mouse was completely stationary

The open field box was cleaned with 30% alcohol solution before placing subsequent animals in it to avoid possible bias due to odour clues left by previous mouse.

2.7. Hanging wire test

The testing procedure was as previously described [32]. Each mouse was suspended with both forepaws on a horizontal steel wire 80 cm long, 2 mm in diameter. The animal was held in a vertical position and its front paws were placed in contact with the wire. When the mouse grasped the wire, it was released, and the latency to fall was recorded with the aid of a stopwatch. Each mouse was given two trials with a 6 h rest interval [33].

Animals were sacrificed on the eight day at the end of the behavioral test.

2.8. In vivo *antioxidant assay*

The brain was weighed and homogenized in ice-saline to prepare a 10% (w/v) tissue homogenate. Catalase (CAT), Superoxide Dismutase (SOD) and Glutathione (GSH) levels in the homogenates were determined using the methods of Sinha [34], Marklund and Marklund [35] and Ellman [36] respectively. Malondialdehyde (MDA) and H_2O_2 were measured using methods described by Ohkawa et al. [37] and colorimetric hydrogen peroxide (H_2O_2) assay kit (#CS0270 Sigma).

2.9. *Immunohistochemistry*

Immunohistochemistry protocol optimized for paraffin-embedded brain samples was performed as described by Todorich et al. [38]. Briefly, brain sections were immersed in 4% phosphate buffer formalin. Antigen retrieval was done in 10 mM citrate buffer for 25 min, with subsequent peroxidase quenching in 3% H_2O_2/methanol. All the sections were blocked in 2% skimmed milk overnight and probed with anti-MBP rat monoclonal antibody 1:200 (Abcam, Cambridge, UK) for 16 h at 4 °C. Detection of bound antibody was done using appropriate HRP-conjugated secondary antibodies in VECTASTAIN kit (Vector Labs, Burlingame, USA) according to manufacturer's protocol. The reaction was enhanced with diaminobenzidine for 6–10 min, with subsequent dehydration in ethanol, cleared in xylene and mounted on frosted glass slides. Images were acquired with digital microscope (Leica DM 750 HD & ICC50 E, Leica Microsystem®). The intensity of expression of the myelin basic protein was thereafter quantified using the ImageJ® software called Fiji. Briefly, individual images of eight sections acquired from at least five animals per group were analyzed by identifying the region of interest and then defined with the freehand tool. The channels of the selected area were split to obtain one image per channel; a constant threshold of intensity was established in control animals which were then applied to all the experimental groups and the staining intensity of all images within the experiments was measured. The threshold intensity was maintained constant during the comparison and measurement of all experimental and control images.

2.10. *Statistical analysis*

Data are presented as mean ± SEM (standard error of mean). The open field, hanging wire and the probe trial of the MWM task results were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test for multiple comparisons. Group differences in the escape latency of the acquisition trial in the MWM task were analysed using two-way ANOVA with repeated measures. P-values ≤ 0.05 were considered significant.

3. Results

3.1. *Behavioral tests*

3.1.1. *Morris water maze*

In the acquisition trial, two-way repeated measure ANOVA (day × group) revealed that the group had a significant eff ;ect on escape latency. Also a significant day eff ;ect on escape latency was observed, which indicated that learning in mice improved over the course of training trails in all groups except the group treated with only NaO_3V. Subsequent comparisons showed that no diff ;erence in escape latency was observed between the control and β-sitosterol treated groups. Mice injected with only NaO_3V took longer time to locate the platform than mice in the β-sitosterol (Fig. 1A), but this became significant on days 2 and 3. β-sitosterol attenuated the spatial learning deficits observed following NaO_3V as indicated by a reduction of the escape latency (P) (day 1, P = 110 s; day 3, P = 41 s) in comparison to the group administered with only NaO_3V (day 1, P = 112 s; day 3, P = 108 s).

In the probe trail, the swimming time in the target quadrant was significantly lower ($\alpha \leq 0.05$) in the model group treated with only NaO_3V. By contrast, β-sitosterol increased swimming time in the target quadrant markedly ($\alpha \leq 0.05$) when compared to all other treated groups including the control (Fig. 1B).

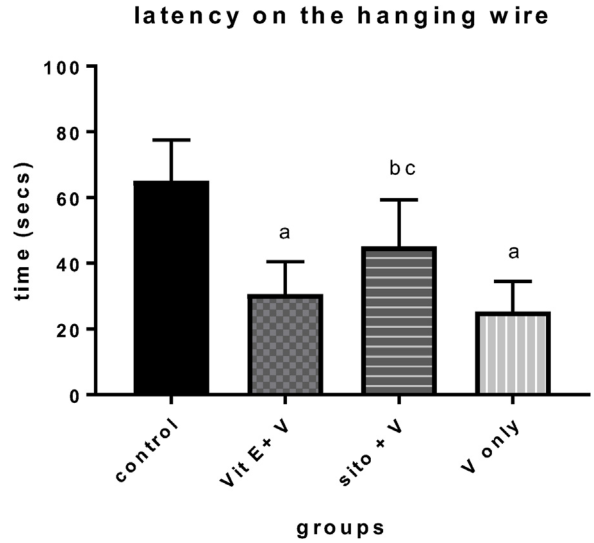

Fig. 3. Time spent on the hanging wire following concurrent administration of β-sitosterol and vanadium.
n = 12, Vit E= α-tocopherol; V = sodium metavanadate; sito= β-sitosterol; a = significantly different from the control at α<0.05,b = significantly different from the α-tocopherol + sodium metavanadate group, c = significantly different from the β-sitosterol + sodium metavanadate group; d = significantly different from the sodium metavanadate group.

3.1.2. *Open fields test*

In the open field test, frequency of line crossed by animals in the control and β-sitosterol groups was not significantly different; this parameter was however, significantly ($\alpha \leq 0.05$) lower in the model group administered with only NaO_3V (Fig. 2). Mean values recorded for the stretch-attend posture frequency of animals were 36.31 ± 5.95, 40.09 ± 6.75, 29.16 ± 6.09 and 56.91 ± 7.02 for control, α-tocopherol, β-sitosterol and NaO_3V, respectively (Fig. 2). The grooming and rearing frequency of experimental animals were lower in the β-sitosterol group when compared with control, although this was not statistically significant except in the model group administered with only NaO_3V.

3.1.3. *Hanging wire test*

Animals treated with only NaO_3V had significant ($\alpha \leq 0.05$) decrease in grip strength and hanging latency when compared to normal control animals. Co-treatment with β-sitosterol improved significantly ($\alpha \leq 0.05$) the grip strength and hanging latency when compared to the standard group (Fig. 3).

3.1.4. Ex vivo *antioxidant study*

In the present study, vanadium toxicity in mice resulted in significant reduction ($\alpha \leq 0.05$) in activities of superoxide dismutase

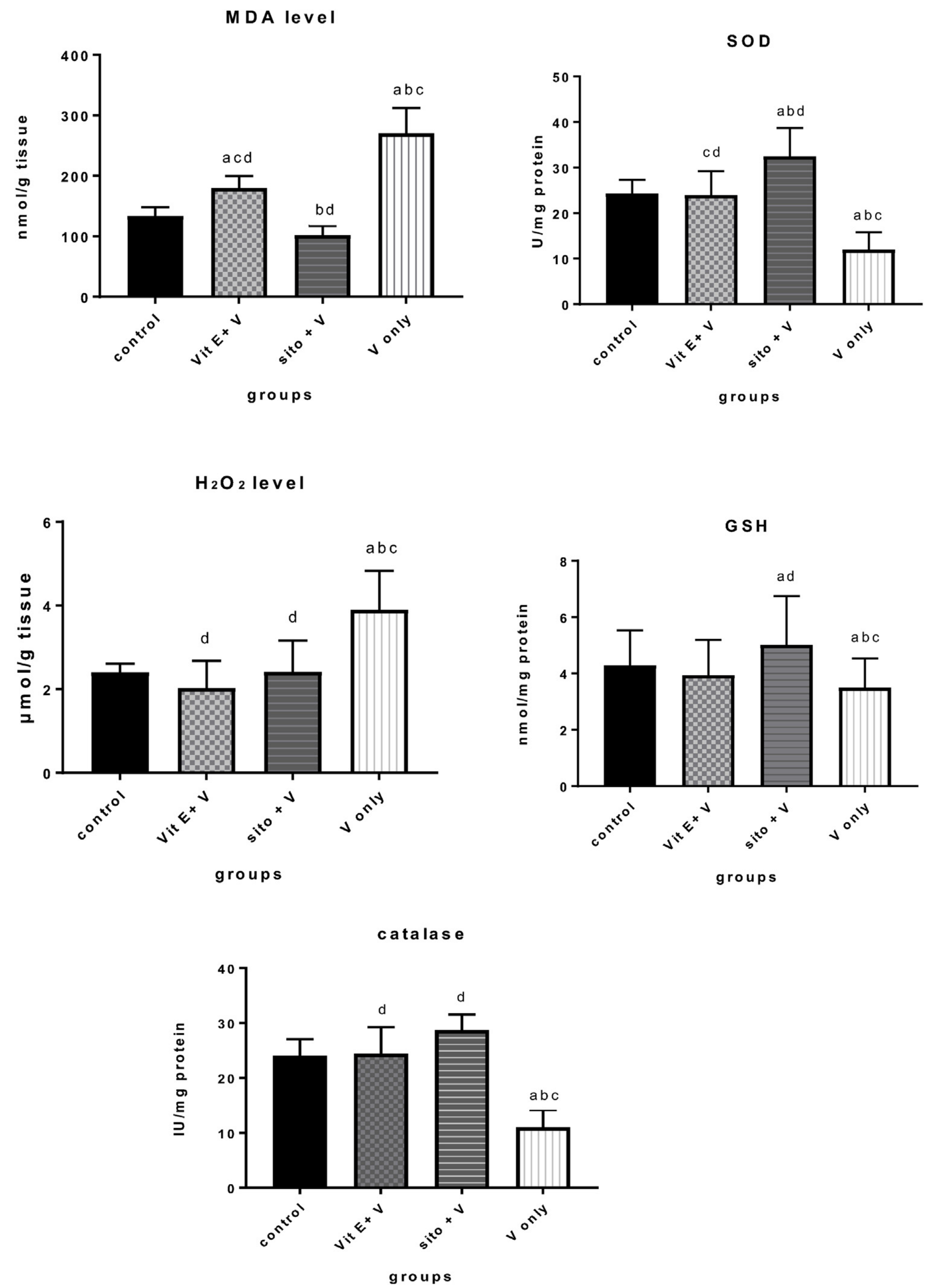

Fig. 4. Levels of MDA and H_2O_2, activities of SOD, catalase and GSH in the brain following concurrent administration of β-sitosterol and vanadium.
n = 5, Vit E= α-tocopherol; V = sodium metavanadate; sito= β-sitosterol; a = significantly different from the control at α<0.05,b = significantly different from the α-tocopherol + sodium metavanadate group, c = significantly different from the β-sitosterol + sodium metavanadate group; d = significantly different from the sodium metavanadate group.

(SOD), catalase (CAT), glutathione (GSH) and an increase in malondialdehyde (MDA) levels in the brain (Fig. 4). These anomalies were prevented with concurrent treatment with β-sitosterol causing a reduction in elevated brain MDA. Furthermore, SOD, CAT and GSH activities were similar in the test and control groups.

3.1.5. Immunohistochemistry

The intensity of myelin basic protein (MBP) expression, in the cerebellum depicts the myelination status of the traversing axons. MBP expression was less intense in NaO_3V exposed group (Fig. 5C, F) when compared with the control groups (Fig. 5A, D). An intense expression was evident in the group that received β-sitosterol along with NaO_3V (Fig. 5B, E).

4. Discussion

4.1. *Effects of β-sitosterol on vanadium-induced behavioral alterations*

The Morris water maze is a hippocampal dependent learning task that can be used to detect deficits produced by lesions of the hippocampus. Spatial learning was assessed during the acquisition trials and reference memory was determined by preference

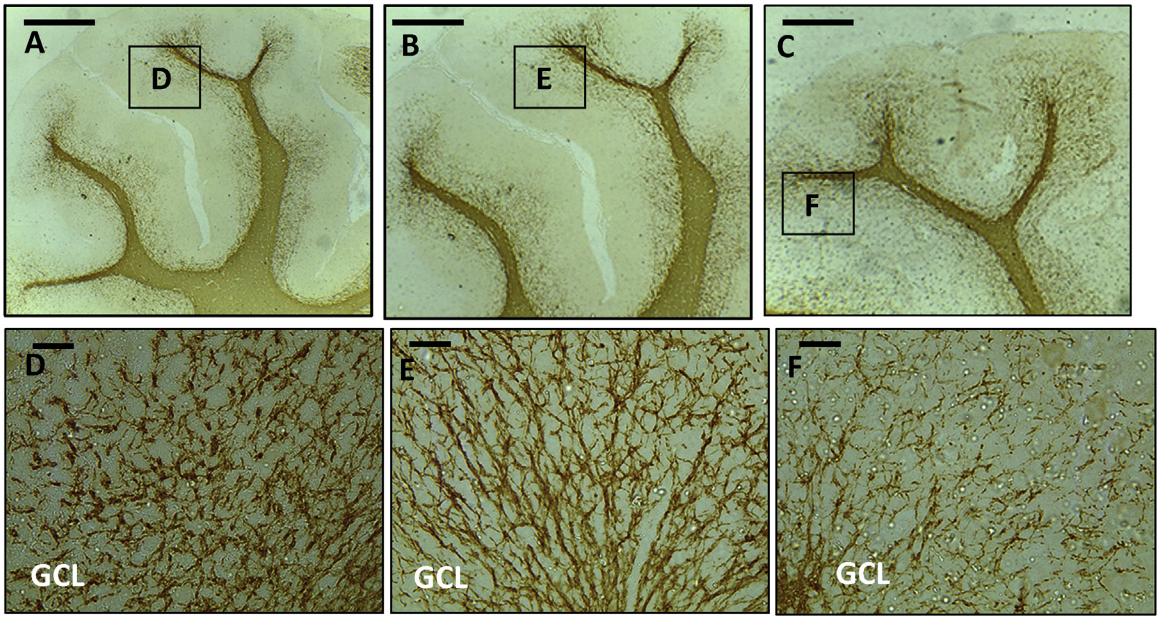

Fig. 5. MBP-immunolabelled myelin fibres in the cerebellum.
A,B,C (cerebellum of control, sodium metavanadate + β-sitosterol and sodium metavanadate only groups respectively) D,E,F (granular cell layer (GCL) of the cerebellum of control, sodium metavanadate + β-sitosterol and sodium metavanadate only groups, respectively)
D&E: MBP-immunolabelled myelin fibres arranged densely and with high staining intensity. F: MBP-immunolabelled myelin fibres arranged loosely and with low staining intensity
D,E,F are higher magnifications of boxed areas in A, B and C. Scale bars in A, B and C = 200 μm Scale bars in D, E and F = 50 μm

for the platform area when the platform was absent. In the present study, mice injected with only NaO_3V showed significant learning and memory impairments as depicted by significantly extended escape latency. This is similar to findings by Folarin et al. [39] that vanadium administration led to memory deficit in mice. However, β-sitosterol significantly reduced the escape latency prolonged by NaO_3V injection after 3 days of training. Interestingly, using the escape latency as an index of learning and memory, the β-sitosterol treated group showed better improvement ability compared to α-tocopherol. In addition, during the probe trial session, β-sitosterol similarly increased swimming time in the quadrant where the platform was previously placed as did the α-tocopherol treated group. This indicates that β-sitosterol improved learning and memory.

The open field test provides an opportunity to assess novel environment exploration, general locomotor activity and screen for anxiety-related behavior in rodents [40]. In this study, results showed that β-sitosterol from *Grewia carpinifolia* extract significantly improved locomotion by increasing line crossing and frequency of rearing which were decreased in the NaO_3V only group. In addition, β-sitosterol increased the number of entries into the central portion of the arena and reduced the number of faecal boli deposits which are indices of anxiety. This finding indicates a relationship between anxiety and locomotion as administration of β-sitosterol led to an increase in alertness resulting in increased locomotor activity, which is suggestive of its anxiolytic property. Furthermore, Gadekar et al. [41] had reported that phytosterols have anxiolytic activity. Sedative properties of a drug or plant extract are said to be carried out by GABA (Gamma- amino-butyric-acid), the major inhibitory neurotransmitter in the CNS [42] which may be the mechanism through which β-sitosterol acts. This may lead to a decrease in the rate of firing of major neurons in the brain or may directly activate GABA receptors [43].

The hanging wire test is performed in order to demonstrate motor neuromuscular impairment and motor coordination in mice [44]. The ability of β-sitosterol to significantly increase latency in the hanging wire test might show its contribution to muscular

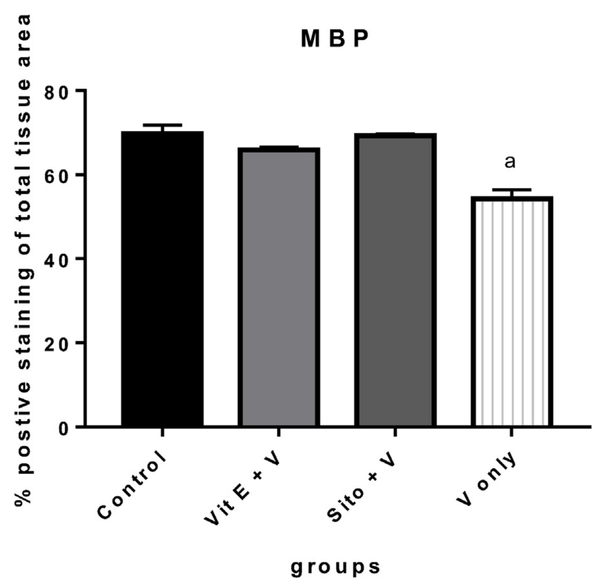

Fig. 6. Densitometric analysis of myelin basic protein (MBP).
n = 5, Vit E= α-tocopherol; V = sodium metavanadate; sito= β-sitosterol; a = significantly different from the control at α<0.05.

strength in animal models. These results from the hanging wire test have conclusively linked the increased locomotor activity and increased swimming speed from the open field test and MWM, respectively indicating the positive role of β-sitosterol in motor coordination.

4.2. Effects of β-sitosterol on ex vivo *antioxidant enzymes and markers of oxidative stress in vanadium-induced toxicity*

The increase in oxidative stress and the overwhelming of *in vivo* antioxidant defence system following vanadium induced toxicity

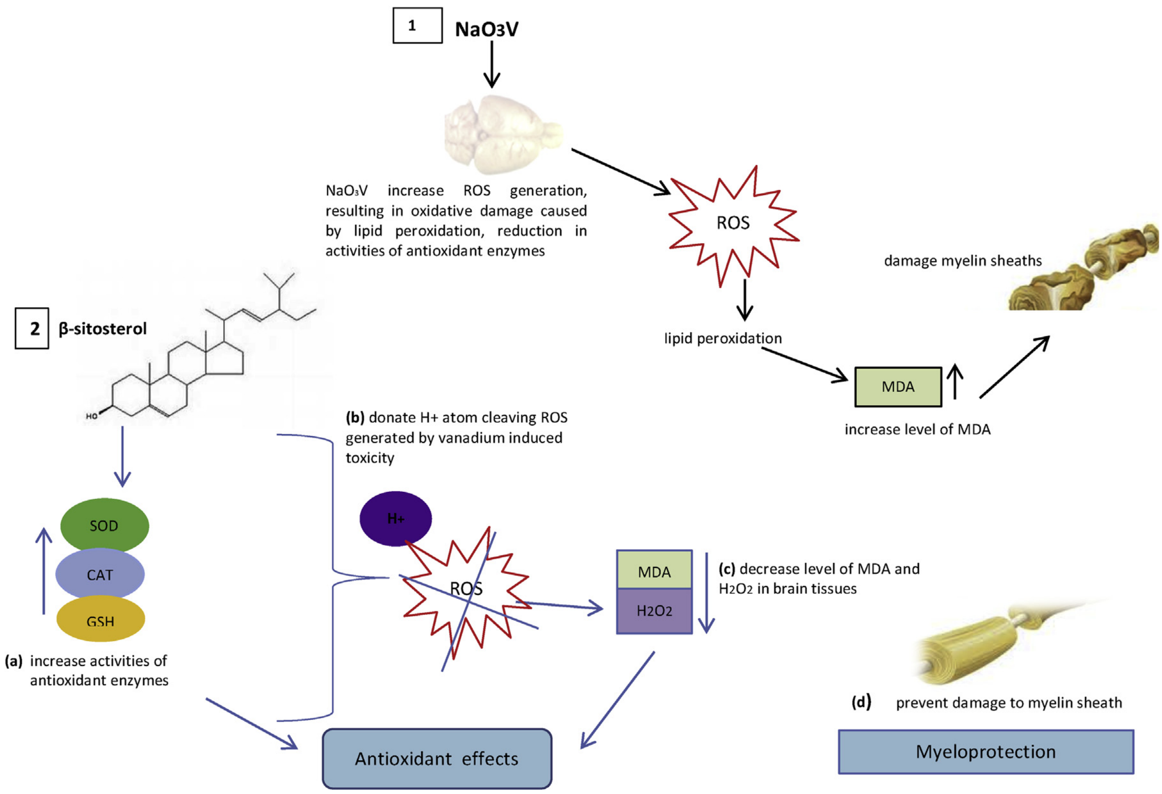

Fig. 7. A proposed model describing the mechanism of action of β-sitosterol following vanadium-induced model of experimental neurotoxicity.
SOD: superoxide dismutase, CAT: catalase, GSH: glutathione, MDA: malondialdehyde, ROS: reactive oxygen species.
(1) Vanadium treatment impairs mitochondrial function with an increase in ROS generation, resulting in oxidative damage caused by lipid peroxidation, reduction in activities of antioxidant enzymes (2) Co-treatment with β-sitosterol (a) increased activities of antioxidant enzymes (SOD, CAT, GSH) (b) and donates H+ atom to free radical cleaving ROS generated by vanadium induced toxicity (c) decreased the level of MDA and H_2O_2 in brain tissues (d) prevent damage to myelin sheaths.

have not been disputed [15,45]. Reactive oxygen species generated in tissues can be efficiently scavenged by enzymatic antioxidants [46] such as SOD; this antioxidant enzyme is responsible for the dismutation of O^2- generated during vanadium metabolism [47] to H_2O_2 which are substrates for peroxisomal catalase and cytosolic glutathione peroxidase enzymes. The observed decrease in both SOD and glutathione (GSH) activities in NaO_3V-treated mice strongly suggests an overwhelming superoxide radical generation and H_2O_2 formation following vanadium administration. This observation is consistent with the findings of Injac et al. [48] and Afeseh *et al.* [49]. Another biomarker for oxidative stress is lipid peroxidation, since free radical gathers electron from lipid molecules present inside the cell membrane, which eventually causes lipid peroxidation [50]. Hence, Malondialdehyde (MDA), an important lipid peroxidation product, indicates the state of oxidative damage of membranes under conditions of oxidative stress [51]. The increase in MDA level in the brain of NaO_3V-treated mice strongly support the hypotheses that increased oxidative stress associated with an impaired antioxidant defence status is one of the mechanisms of vanadium toxicity [49]. In the present study, concurrent treatment with β-sitosterol protected the brain from vanadium-induced injury by increasing the activities of catalase, SOD and GSH while decreasing membrane lipid peroxidation. This could be linked to the ability of this compound in biological system to increase activities of these *in vivo* antioxidant enzymes so as to tackle the increased oxidative stress; this portrays a good signal for neuro-protection.

Hydrogen peroxide is a reactive oxygen metabolic by-product that serves as regulator for oxidative stress-related states [52,53]. The significant increase in the level of H_2O_2 in the brain further contributes to vanadium neurotoxicity and confirmed earlier reports of its mechanism of action. β-sitosterol diminished the levels of H_2O_2 via their reducing properties by donating a hydrogen atom which breaks the H_2O_2 chain as earlier reported [27]. The present findings substantiate reports that neuroprotective effects of phytochemicals are associated with reduced levels of oxidative stress [54].

4.3. Immunohistochemistry

Myelination is required if mammalian neural circuits are to function normally. It has been documented that myelin formation in the brain begins at around postnatal day (PND) 10 in the mice, with the maximal rate of myelin accumulation occurring around PND 20; however, myelin accumulation does continue into adulthood, albeit at a decreasing rate [55]. Toxicants such as vanadium have been documented to be a major leading cause of disruption of myelin [38] before, during and after formation [56]. Since juvenile mice were used in the present study, the altered myelination observed as pallor and discontinuity of myelin fibres could be due to their destruction after synthesis resulting in demyelination. Consequently, the increased myelination density observed in β-sitosterol treated group indicates that concurrent administration of β-sitosterol offers protection in the cerebellum. This is in consonance with findings that β-sitosterol aids repair of damaged neurons by neuronal synthesis, and restoration of synaptic activity and ultimately nerve impulse transmission [57].

Therefore, the finding that mice with decreased MBP-immunostaining intensity in the cerebellum were also impaired in the motor coordination task further supports the important role of the cerebellum in managing coordination of skeletal muscles and body movements in animals (Fig. 6).

5. Conclusion

In summary β-sitosterol improved cognition, motor co-ordination and reduced reactive oxygen species generation in the brain following vanadium induced toxicity. This could be attributed to its *in vivo* antioxidant properties leading to protection against oxidative stress (Fig. 7).

Consent for publication

This manuscript does not contain any individual person's data, so consent for publication is not applicable in this study.

Availability of data and materials

All data and materials of this manuscript are available.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AEO carried out the experiments; AEO, OFO and OJO participated in the design of the study, performed the statistical analysis and drafted the manuscript. NHT provided expertise in the isolation of β-sitosterol, OJO provided expertise in the behavioral study OFO, OJO and NHT participated in the coordination of the study. AEO wrote the manuscript text. All authors read and approved the final manuscript.

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