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Molecular Basis behind the Neuroprotective Potential of Beta Sitosterol in Lipopolysaccharide-Induced Wistar Albino Rats

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Abstract

Neurodegenerative disorders are on the rise globally. β-Sitosterol shows potential therapeutic benefits, but its neuroprotective mechanisms remain largely unexplored. This study aimed to assess the neuroprotective effects of β-Sitosterol on pro-inflammatory (NFκB) and antioxidant (NRF-2/KEAP-1) pathways in an in vivo in LPS-induced neurodegeneration model in albino rats. The rats were divided into four groups: normal control, LPS-induced, LPS-induced treated with β-Sitosterol (20 mg/kg/day for 4 weeks), and normal treated with β-Sitosterol. Neurotransmitters (dopamine and serotonin) and antioxidant enzymes (GSH and CAT) were measured by ELISA, and gene expression of NFκB, NRF-2, KEAP-1, IL-6, and IL-18 was assessed by Real-Time RT-PCR. Histopathology of brain tissues was performed. LPS induction significantly decreased neurotransmitters and antioxidant enzymes and upregulated NFκB while downregulating NRF-2 and KEAP-1 mRNA expression. β-Sitosterol treatment normalized these levels ($p<0.05$) and reduced hyperchromatic pyknotic changes in neuronal nuclei observed in LPS-induced rats. Normal rats treated with β-Sitosterol showed no significant alterations, indicating its safety. These findings suggest β-Sitosterol can reduce neuroinflammation by modulating antioxidant signaling, providing a potential therapeutic approach for neurodegenerative diseases.

Keywords: *Health and well-bring, Neurodegenerative disorders NFκB, NRF2-KEAP-1, β-Sitosterol, Neurotransmitters, Novel Methods, Phyto therapeutics*.

Introduction

The evolution of humans from their primates have resulted in highly complex and intricate network of neuronal circuitry within our central nervous system. There exists billion of neuronal connections and

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communications within our central nervous system. The chemical milieu of the neurons should have an intact free radical cleansing mechanism to maintain the integrity of neurons. Any flaw in the expression of antioxidant enzymes or the lack of their concentration due to their increased demand can shift the scale towards the increased quantum of reactive oxygen species. Innumerable reduction-oxidation reactions occur in a non-terminating way within the neuronal habitat. There is a delicate balance between the reactive oxygen species and antioxidant factors and disruption of this balance can lead to oxidative injuries within the neuron which can cause turbulences in the neuronal connections within the brain and central nervous system. The slow hampering of the neuronal circuits due to oxidative insults can result in the manifestations of neurodegenerative disorders [1].

Neurodegenerative disorders like Alzheimer's disease, Parkinsonism are a growing trend in the present generation due to the pile-up of stress in day-to-day life and dietary habits. Genetic background is the main etiologic factor behind these diseases but the influence of the environment also plays a part [2]. Human race is in the urge of consuming a diet rich in antioxidants and leading a stress-free life to increase their longevity in the planet. We are under the exploration of newer phytoconstituents, which can be beneficial for the human race to postpone the incidence of neurodegenerative disorders. β-Sitosterol is a plant-derived sterol which has found applications in medicine and its mitigating effect in neuroinflammation is explored in this study. Neuroinflammation occurring due to oxidative stress seems to be the main culprit for neurodegenerative disorders [3]. Hence, in this study we have explored the antioxidant potential of β-Sitosterol through the NRF2-KEAP pathway.

In this study we have chosen an entity that regulates oxidative processes – KEAP – NRF2 (Kelch ECH Associating Protein – Nuclear factor erythroid 2 related factor) and a key molecule that regulates inflammation – NFκB as indicators of neuroinflammation. Normally NRF2 is bound with KEAP, which is a repressor protein that promotes the degradation of NRF2, by proteosome pathway. During oxidative stress, NRF2-bound KEAP is inactivated and freshly originated NRF2 proteins gets liberated from KEAP and migrate to the nucleus. In the nucleus NRF2 associate with antioxidant response element (ARE) and facilitates the expression of NRF2 target genes like heme oxygenase, glutathione -S-transferase, NAD(P)H quinone oxidoreductase which are critical in detoxication and eradication of reactive oxygen species [4].

Lipopolysaccharide has been used to induce inflammation in the brain tissue of rats by induction of NF-κB signalling pathway due to activation of Toll -like receptor (TLR-4). The stimulation of NF-κB in turn leads to spike in levels of TNF-α, IL-1β, NO & PGE2 which are found to be neurotoxic factors causing neuronal degeneration [5]. NF-κB stimulation leads to activation of astrocytes by microglia ultimately leading to amplification of inflammation precipitating cellular dysfunction [2]. There exists evidence that there should be an interplay between Nrf2 and NFκB signalling systems. At the molecular level there seems to be a crosstalk between the molecular entities that bolster anti-oxidative processes and inflammatory cascade. NFκB inhibits Nrf2 pathway at the transcription level and viceversa [6]. The oxidative damage gets coupled with neuronal inflammation and contributes to the incidence of neurodegenerative disorders.

During the changes that are evolving within the CNS, there might be alterations in the levels of certain neurotransmitters like dopamine and serotonin [7]. There are animal studies showing that sitosterol derivatives have the potential to modify the actions of monoaminergic neurotransmitters like

dopamine and serotonin in the brain [8]. In this study we have explored the change in the neurotransmitter constitution in the serum especially dopamine and serotonin observed during LPS induction and post intervention with β-Sitosterol. We have substantiated the fact that phytosterols like β-Sitosterol has the tendency to boost up the levels of these essential neurotransmitters in the serum and helps in its regulation within the brain thus contributing to its neuroprotective effect. Post induction β-sitosterol was given which may reduce the oxidative damage and inflammatory processes in the brain which can be deducted by monitoring the levels of the regulatory molecules KEAP-NRF2 and NFκB respectively.

Methodology

Ethical Approval

The outline of the study was presented to the members of the Institutional Animal Ethical Committee (IAEC) and approval was obtained. (Approval No. BRULAC/SDCH/SIMATS/IAEC/04-2022/097).

Animals

Albino Rats weighing 150-200 gms with good access to food and water maintained under stable environmental conditions. (22 ˚ C and 12 hr dark-light regimen). The number of animals approved were 24 which were segregated into 4 groups each containing 6 animals.

Experimental Design

Animals categorized into 4 groups. Group 1- Control –olive oil was given orally for 4 weeks; Group 2- LPS induced group – 0.25 mg/kg/day of LPS given by i.p for 1 week; Group 3- LPS+ β-Sitosterol - 20 mg/kg body wt./day of β-Sitosterol was given for 4 weeks (post LPS induction); Group 4- Control+ β-Sitosterol -20 mg/kg body wt./day of β-Sitosterol was given for 4 weeks (without LPS induction). At the end of the experiment blood was obtained from the animals from the retroorbital vein and serum was stored for further investigations. Later animals were euthanized using chloroform and organs were dissected and removed and the required organs were preserved for molecular analysis.

mRNA Expression Analysis by RT-PCR

In RT-PCR analysis two µg of RNA was utilized for reverse-transcriptase polymerase chain reaction. In a first step, complementary DNA (cDNA) was produced from an mRNA template using OligodT, dNTPs, and reverse transcriptase. The components were combined with a DNA primer in a reverse transcriptase buffer for an hour at 37ºC. After cDNA synthesis, standard PCR was initiated using gene-specific oligonucleotide primers by the preliminary PCR activation at 95ºC for 5 min. The three-step PCR cycles includes denaturation at 95ºC for 2 min, annealing at 60ºC 30 s, and extension at 73 ºC for 30 s. The PCR amplification was manipulated up to 30 cycles and to ensure that the products are extended completely, a final extension at 73ºC for 5 min was conducted. Gene-specific oligonucleotide primers for the housekeeping gene was included in the same PCR reaction vial and co-amplified. RT-PCR product was taken from each reaction tube, mixed with gel loading dye, and resolved in a standard 2 % agarose gel containing ethidium bromide (0.5 mg/ml) under an electrical field (60 mA and 80 V) for 2 h. The molecular weight DNA marker (100 bp ladder) was simultaneously resolved in the first lane. After electrophoresis, the gel was subjected to densitometric scanning and the band intensity of cDNA fragment of each gene of interest was normalized against the band intensity of the cDNA fragment of the housekeeping gene, β-actin, using quantity one software (Bio-Rad, USA) and further amplified by PCR. Gene expression analysis was done for NFκB, NRF-2, KEAP-1, IL- 18 and IL-6.

Estimation of Antioxidant Enzymes

Reduced glutathione activity was checked (GSH) by the method of Sedlak and Lindsay (1968) and results were expressed as nanomoles of GSH/mg protein [9]. The activity of catalase (CAT)was assessed by the method of Takahara et al. (1960) and the results for the same was expressed as µmoles of H_2O_2 consumed /min/mg protein [10].

Histological Evaluation

According to the method described by Gabe (1968), a part of the brain was fixed in 10% neutral buffered formalin embedded in paraffin, sectioned and stained with Haematoxylin and Eosin for histological examination. Then semi-thin sections (0.5-1 microns) were prepared by using LKB ultra microtome. The sections were stained with toluidine blue, examined with a light microscope and photographed [11].

Statistical Analysis

Data was analyzed statistically using one-way analysis of variance and Duncan's multiple range test to determine the significance of individual variations between the control and treatment groups using a computer-based software (SPSS 23 for Windows student version) and expressed mean ± standard error of the mean. In Duncan's test, the threshold of significance was considered at $p< 0.05$.

Results

Effect of β-Sitosterol on Keap-1 mrna Levels in Brain

Figure 1 depicts the suppression of KEAP-1 mRNA levels in LPS induced group which was later rectified by β-Sitosterol and near maximum levels was attained in the treated group. There was not much variation found in the β-Sitosterol treated control group.

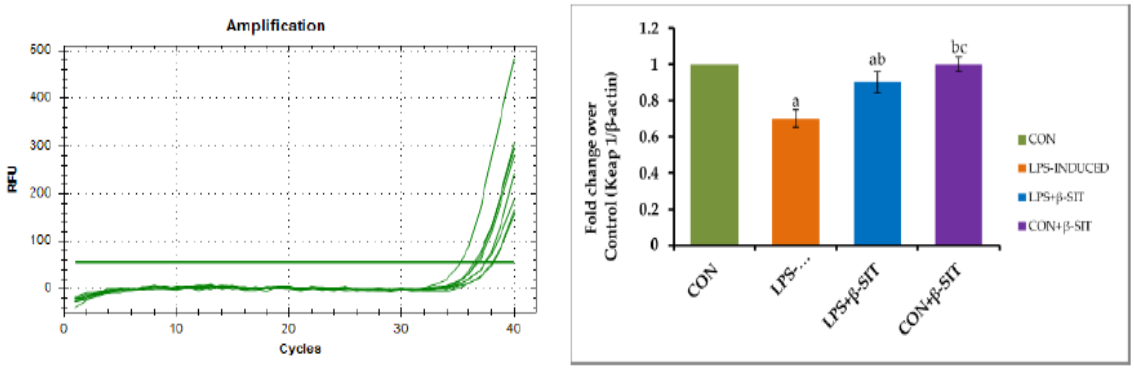

Figure 1. Elucidating Changes in the Expression of Keap1-Mrof Following LPS and B-Sitosterol Administration

Effect of β-Sitosterol on NRF2 Levels in Brain

From the above figure 2, we can understand that LPS induction caused more than a 50% decline in the expression of NRF2 whereas β-Sitosterol resumed the levels of NRF2 in the treated group and not much variation has been observed in the treated control group.

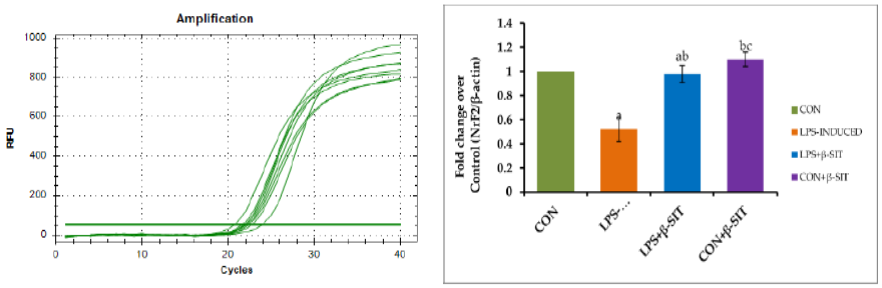

Figure 2. Showing the Variations in nrf2 mRNA Post β-Sitosterol Administration

Effect of B-Sitosterol on Nfκb in Brain

Above figure 3 depicts that the stimulation of NFκB by LPS is brought down by β-Sitosterol and not many changes are observed in the treated control group.

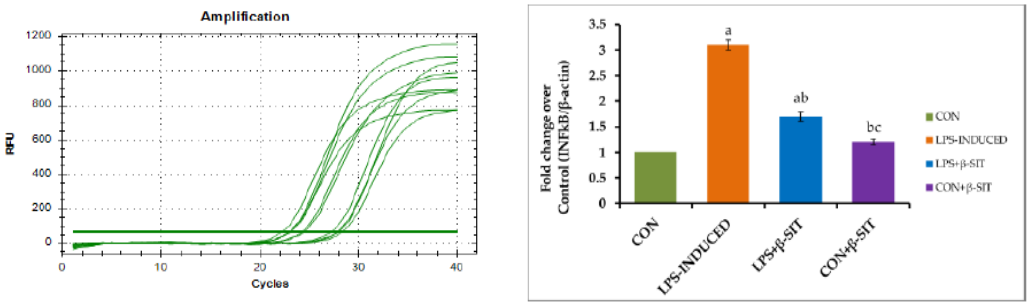

Figure 3. Indicating Suppression of nfκb by β-sitosterol

Effect of β-Sitosterol on Interleukin-6 Levels

The above figure 4 illustrates that the rise in mRNA expression of IL-6 after LPS induction is brought down by β-Sitosterol administration. This substantiates the anti-inflammatory effect of β-Sitosterol through suppression of NFκB.

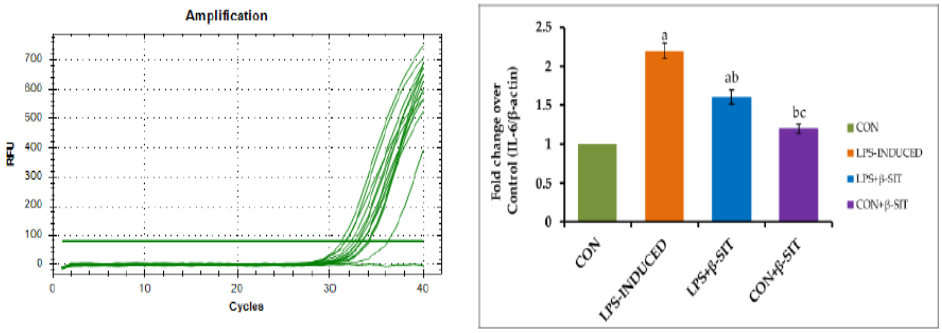

Figure 4. Illustrating the Changes in IL-6 Post β-Sitosterol Administration

Effect of β-Sitosterol on IL-18 Levels

The above figure 5 shows the increased mRNA expression of IL-18 due to LPS induction is reverted to the normal range by β-Sitosterol, which can be seen in the sitosterol-treated group. There is not much change in the IL-18 levels in the β-sitosterol-treated control group.

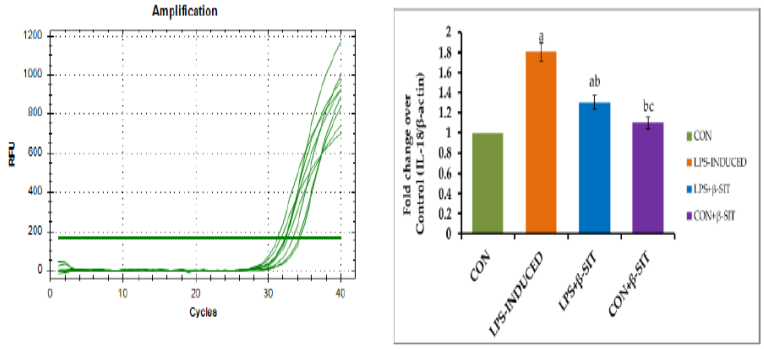

Figure 5. Exhibiting effects of β-Sitosterol over IL-18 levels

Effect of B-Sitosterol on Reduced Glutathione Enzyme Activity

From the above figure 6 we can interpret that the LPS causes suppression of reduced glutathione whereas β-Sitosterol administration shoots up the level of the anti-oxidant factor due to upregulation of NRF2-KEAP1.

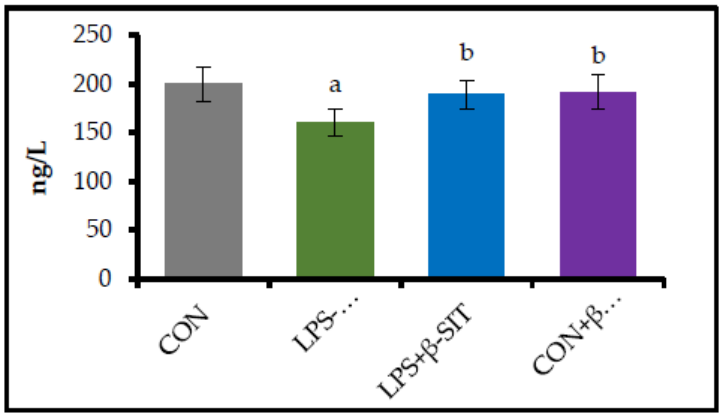

Figure 6. Showing the Changes in Levels of Reduced Glutathione

Effect of β-Sitosterol on Catalase Activity

Figure 7 shows the antioxidant factor escalating potential of β-Sitosterol following the suppression by LPS. β-Sitosterol administration boosts up the level of the anti-oxidant factor due to upregulation of influential factor NRF2-KEAP1.

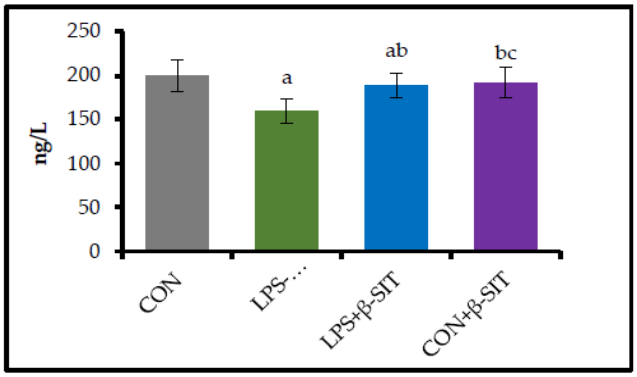

Figure 7. Illustrating the Changes in Catalase Following Administration of β-Sitosterol

Effect of β-Sitosterol on Dopamine Levels

LPS induction caused a dip in dopamine levels which was rectified to near normal level by β-Sitosterol administration. In the control group lagging LPS induction β-Sitosterol caused a further increase in dopamine levels which could be depicted from Figure 8.

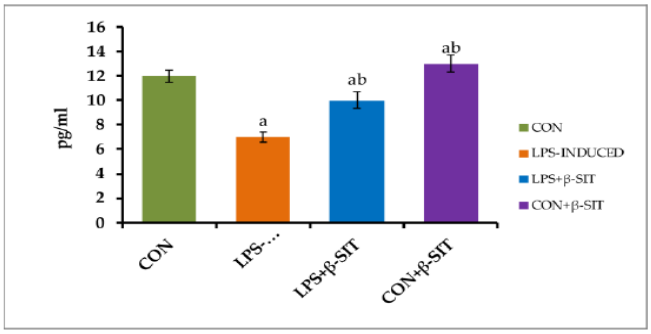

Figure 8. Illustrating the Dopamine Levels in Serum

Effect of β-Sitosterol on Seratonin Levels

LPS induction caused a greater than 50% decline in serotonin levels which was rectified by β-Sitosterol in the treated group to a better value which can be seen from Figure 9. In the β-Sitosterol treated control group the levels were found close to the normal physiological limits.

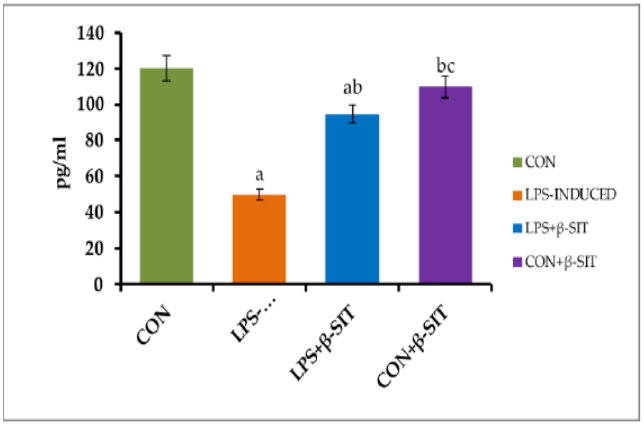

Figure 9. Effects of β-Sitosterol on Serotonin Levels in Serum

Effect of β-Sitosterol on Histopathological Observations of Hippocampus in LPS-Induced Adult Rats by H& E Staining

Row I represent the merged image of hippocampus containing CA1 (Cornu Ammonis), CA2, CA3 and Dentate Gyrus (DG) at 4X Magnification.

Row II represents magnified image of the square mark in first column showing CA3 and Dentate Gyrus (DG). M - Molecular layer, G - Granule cell layer, H – Hilus. Black arrow-heads – Hyperchromatic pyknotic neurons; Red arrow-heads - Hyperchromatic pyknotic neurons. A (Group-1): Control; B-Group-2): LPS -Induced; C-(Group-3): LPS+ β-Sitosterol (20 mg/kg body wt); D-(Group-4): Control+ β-Sitosterol (20 mg/kg body wt)

As depicted in Figure 10 in normal tissue pyramidal cells were seen in the portion of hippocampus. In LPS induced group, we can find hyperchromatic pyknotic neurons which

were a sign of neurodegeneration. In β-Sitosterol treated group, there was a reduction in degenerative neurons and there seems to be a decline in population of inflammatory cells.

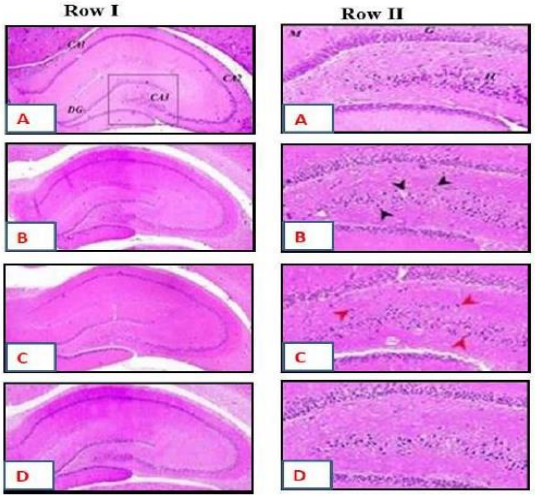

Figure 10. Histopathologic Changes in the Brain Tissue

Discussion

The study has brought to the limelight that the compound β-Sitosterol exhibits its antioxidant effect by modulating the NRF2-KEAP1 pathway. KEAP1 is an electrophilic sensor, and Nrf2 is an effector for cytoprotective gene activity. In the absence of stress, Nrf2 is continuously degraded by KEAP1. However, during oxidative stress Nrf2 is activated vigorously and translocated to the Antioxidant Response Element (ARE) of the genes in the nucleus. Thus, directing the synthesis of antioxidant factors [12]. The treatment of β-Sitosterol has magnified the gene expression for NRF2 and KEAP1 which can be seen from the results of PCR. This elevation of the molecular key induces the synthesis and release of antioxidant factors which includes catalases and reduced glutathione which can be interpreted from the results. The hike in the levels of endogenous antioxidants seen in this study is similar to the results obtained in another study where the anticancer effect of β-Sitosterol is investigated in human hepatocellular cancer cell lines [13].

Nrf2 and its principal negative regulator, the E3 ligase adaptor Kelch-like ECH-associated protein 1 (KEAP 1), play a predominant role in the regulation of intracellular redox homeostasis and inflammation. Recent studies showed an isthmus of connection between the Nrf2/antioxidant response element (ARE) system and the expression of inflammatory mediators, NF-κB pathway and macrophage metabolism [14]. The above finding was proven in our study where β-Sitosterol concomitantly suppresses the release of inflammatory mediators by blocking the expression of NFκB which in turn is the key regulating moiety for the mediators of inflammation. Inhibition of NFκB has led to the suppression of IL-6, IL-18 which are mediators of inflammation. This suppressant effect on inflammatory mediators synchronises with the results obtained from another study where LPS induced inflammation in a microglial cell line was suppressed by β-Sitosterol by modulation of NFκB pathway [15]. Thus, the dual inhibition of inflammation along with oxidation can produce beneficial effects in neuronal degenerative processes.

In another study conducted it was found that LPS induction reduced the viability of neurons, leading to highly condensed nuclei and the absence/retraction of neurites [16]. Similarly, in our study we found that LPS induction inflicted degenerative changes in the morphology of neurons which were

characterized as hyperchromatic pyknotic neurons. However, post-treatment with β-Sitosterol these detrimental impacts on the neurons was moderately reduced. Thus, histopathological findings do substantiate the neuroprotective effect of the compound.

LPS induction results in suppression of the beneficial neurotransmitters namely dopamine and serotonin which in turn is rectified by β-sitosterol. Studies are existing to demonstrate the neurodegenerating effect of LPS in the brain where the pathways in the synthesis of dopamine are inhibited resulting in precipitation of the defect [17]. The disruption of the delicate balance of neurotransmitters in the brain precipitates the manifestations of certain neurodegenerative disorders. The rejuvenation of neurotransmitters to normal levels helps in the recovery from disorders like Alzeimer's and Parkinsonism [18]. The proportionate increase of neurotransmitters like serotonin in the serum can enhance the neurotransmitter turnover within the brain [19-21]. Thus, the phytocompound has the potential to help in the recovery from neurodegenerative disorders at its own pace as it has beneficial effects in the CNS.

Conclusion

The phytosterol β-Sitosterol seems to possess healing effects in neurodegenerative disorders which can be attributed to its neuroprotective potential of enhancing the neurotransmitter action. The inherent antioxidant quality and anti-inflammatory potency give the status to be enrolled in the candidature of phytoconstituents eligible for the therapeutic management of neurodegenerative disorders of mankind in the near future.

Conflict of Interest

The authors hereby declare that there is no conflict of interest.

Acknowledgement

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