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Studies on the Antioxidant Role of B-Sitosterol on Neuronal Cell Line (IMR32) *In Vitro*: Role of NRF2-Keap Pathway

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Abstract

β-Sitosterol is a phytosterol which is universally present in most of the plant seeds and nuts. Its anti-inflammatory property has been studied in vitro in cell line studies and some in vivo studies have also been conducted but few studies have been done to establish its antioxidant property. In this study, we have attempted to explore the antioxidant property through the NRF2-KEAP pathway in the IMR 32 neuronal cell line. Initially, cell viability was checked for varying concentrations of sitosterol using an MTT assay. There was a decline in viability for increased concentration and time factors. Reactive oxygen species (ROS) assay revealed the tendency of sitosterol to inhibit lipid peroxides and hydrogen peroxides. An antioxidant activity assay depicted activating antioxidant enzymes, superoxide dismutase, and nonenzymatic antioxidants, such as reduced glutathione. In the gene expression analysis using Real Time-PCR NRF2 and KEAP expression was found to be observed maximum for concentration ranges of 10 µM concentration of β-Sitosterol. Current studies have substantiated the antioxidant property of β-Sitosterol through the NRF2- KEAP signalling pathway.

Keywords: *NRF2-KEAP Pathway, β-Sitosterol, Health and Well-being, Novel Methods, Phyto therapeutics.*

Introduction

β-Sitosterol is a phytosterol ubiquitously present in vegetable oil, plant seeds & nuts. Its medicinal properties are investigated in various fields of medicine and its usefulness is well established. It has sufficient anti-inflammatory property which has been

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pragmatically tested by scientists in various animal models. In a zebrafish model of inflammation, pretreatment with β-sitosterol before $CuSO_4$ exposure blocked recruitment of neutrophils and caused damage to lateral line neuromasts, reflecting a potent anti-inflammatory effect derived from inhibitions in the expressions of il-8 and myd88 [1]. In a study conducted in BV2 murine microglial cell line the pretreatment with β-Sitosterol minimized the LPS induced expression of inflammatory mediators' interleukin-6 (IL-6), inducible nitric oxide (iNOS), Tumour Necrosis Factor-α (TNF-α) and Cyclooxygenase-2 (COX-2) by blocking the LPS-induced activation of p38, ERK and NF-κB pathways [2]. There seems a direct association between ILs like IL-18 and NFκB which is involved in the expression of adhesion molecules and acts as a mediator for the process of chemotaxis involved in inflammation [3]. It also has the inherent capacity to control the phosphorylation and degradation of inhibitor of nuclear factor kappa B (IκB) and inhibited the phosphorylation of nuclear factor kappa B (NF-κB) which are significant molecules to regulate different chemical mediators in inflammatory pathways [4]. In another study done in rats, β-Sitosterol alleviates neuropathic pain by modulating the TLR receptor and NFκB signaling pathways [5].

The antioxidant effect of β-Sitosterol was demonstrated in DMH-induced colon carcinogenesis in the Wistar rat model where it caused suppression of reactive oxygen species and upregulated antioxidant factors [6]. The antioxidant potency of β-Sitosterol had been proven in another study where the compound caused a rise in pancreatic antioxidants with a decrease in thiobarbituric acid derivatives in streptozotocin-induced diabetic rat model [7]. Its anti-inflammatory property has been already studied in cell lines however its antioxidant nature has to be investigated at the molecular level, especially through the NRF2-KEAP pathway [2]. This NRF2-KEAP pathway has a significant role in regulating oxidative processes within a cell. Oxidants and electrophiles modify critical cysteine thiols of KEAP and NRF2 thereby activating the proteosomal degradation of Kelch like erythroid cell derived protein (KEAP). This results in the ubiquitination of KEAP and dissociation of NRF2 which is a key transcription factor in inducing the manufacture of antioxidant enzymes to combat the oxidative stress [8].

Antioxidant property of this compound can be used to alleviate neuroinflammation potentiating its use in the management of neurodegenerative disorders. The beneficial effect of β-Sitosterol in the management of Alzeimer's disease in mice model was studied which produced the result that the compound can subdue cognitive deficits and prevent amyloid plaque deposition in the brain of the animals [9]. In the HT22 hippocampal cell line study the neuroprotective effect of the compound was proven when there was an increased availability of soluble neuroprotective soluble amyloid precursor protein – s APP α than amyloid β-Aβ [10]. More studies are a primary requisite to substantiate the neuroprotective property of β-Sitosterol in animal models which later can be extended to the human race in future.

Recently CNS disorders due to degeneration are up the ramp due to oxidative stress in day-to-day activities therefore a phytocompound which could reduce oxidative damage is in high demand. Globally prevalence of neurodegenerative disorders may reach 2 % in persons aged 65 years & older. There is an increasing trend seen recently [11]. In India, 30 million people suffer from neurological disorders with an average of 2394 per 1 lakh population [12]. Neurological disorders occur due to genetic influence or environmental factors or due to the interaction of both factors. Postponement of the signs of neurodegenerative disorders is a

necessity for smooth and easy conduct of life. Implementation of lifestyle changes and a nutritious diet rich in antioxidants may help in tiding over the crisis of ill health. Nature has given us lots of phytoconstituents which are inherently powerful antioxidants but however the uniqueness of phytocompounds has to be substantiated by experiments. Hence, in this study we have analyzed the antioxidant potency of β-Sitosterol in its activity against the cell line IMR 32 through the NRF2-KEAP pathway. If the antioxidant property of β-Sitosterol is proven through this pathway then in vivo studies can be conducted so that the experiments will open up new horizons of applications in the near future.

Materials and Methods

Procurement, Maintenance and Treatment of Cell lines

The neuronal cell line was obtained from NCCS, Pune, India. The cells were cultured in T-75 culture flask, containing DMEM supplemented with 10% FBS under 5%CO2, 95% air at 37 degrees Celsius. Upon reaching confluence (80 %) the cells were trypsinized and passaged. The dose of β-sitosterol was estimated from cell viability assay.

Cell Viability Assay

The cell viability assay was performed by MTT assay, which determines mitochondrial activity in living cells. Cells were seeded in a 96-well plate at a density of 5 × 104 cells/well and incubated for 24 h at 37°C, 5% CO2 incubator. After attachment, cells were washed with PBS and then incubated with serum-free medium for 6–12 h. β-Sitosterol was dissolved in DMSO with different concentrations β-Sitosterol (1.56, 3.12,6.25,12.5, 25, 50, 100 µM) and added to the cells. After the treatment period, 20 ml medium was removed and 20 ml of MTT was added and incubated for 30 min. Then, 100 ml of DMSO was added to solubilize the crystals and was kept in dark for 10 min. The intensity of color development was measured at 570 nm in ELISA reader. The cell viability was calculated as follows: Cell viability = absorbance of treated cells/absorbance of control cells ×100%.

Assessment of Lipid Peroxidation

Lipid peroxidation (LPO) was measured in liver tissue homogenate as in accordance to the study by Devasagayam and Tarachand. The malondialdehyde (MDA) content was expressed as nmoles of MDA formed/min/mg protein. Hydrogen peroxide generation was done in accordance to the method of Pick and Keisari and the values were expressed in µmoles/min/mg protein.

Assessment of Antioxidant Enzymes

Rotruck et al. method was used to assess GPx and the activity was expressed as µg of utilized glutathione / per min per mg protein in liver tissue homogenate. The method of Habig et al. was used to assess GlutathioneS-transferase (GST) activity and values were expressed in the form of µmoles of CDNB utilized per min per mg protein. The method of Staal et al.37 was used to analyse Glutathione reductase (GR) and values for activity of GR and GSH were expressed in the form of nmoles of GSSG reduced per min per mg protein and nmoles of GSH per mg protein respectively.

mRNA Expression Analysis by RT-PCR

Total RNA, 2 µg was used for reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. RT-PCR was carried out using a two-step RT-PCR kit. In the first step, complementary DNA (cDNA) was made 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 conversion, standard PCR was carried out using gene-specific oligonucleotide primers by the initial PCR activation at 95ºC for 5 min. The three-step PCR cycles include denaturation at 95ºC for 2

min, annealing at 60ºC 30 s, and extension at 73 ºC for 30 s. The amplification of PCR was extended for 30 cycles and to ensure that the products are extended completely, a final extension at 73ºC for 5 min was carried out. Amplification of gene-specific oligonucleotide primers for the house-keeping gene was conducted after adding to the same PCR reaction vial. The product of RT-PCR 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. Molecular weight DNA marker (100 bp ladder) was simultaneously resolved in the first lane. The densitometric scanning was done for the gel after electrophoresis and the band intensity of cDNA fragment of each gene of interest was normalized against the band intensity of cDNA fragment of the house-keeping gene, β-actin, using quantity one software (Bio-Rad, USA) and further amplified by PCR.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. The Statistical Package for Student version 17.0 (SPSS Inc., Chicago, IL) software was used to analyse the statistics performed using one-way ANOVA which was followed by Duncan's tests to compare between treatment and control values. $P < 0.05$ was considered to be statistically significant.

Results

Effect of β-Sitosterol on Cell Viability

In MTT assay, neuronal cells were treated with ascending concentration ranges of β-sitosterol and its viability was checked for 3 consecutive days. Almost 50% of the cells were viable in the concentration ranges between 3 and 12 µM of β-sitosterol in the first 24hrs followed by a mild decline in viability in the next 24 and 48 hrs respectively. There was a decrease in cell viability with respect to the increase in concentration of the compound β-Sitosterol and also with respect to time which can be seen in Figure 1. Thus 3,6 and 10 µM concentrations were used for investigation. The cell inhibitory nature of β-Sitosterol can be attributed to its antioxidant effect which was demonstrated by increase in expression of regulatory molecules NRF2 and KEAP in PCR analysis. When a graph was plotted between log dose and response there seems to be a nonlinear regression between the two factors (Figure 2).

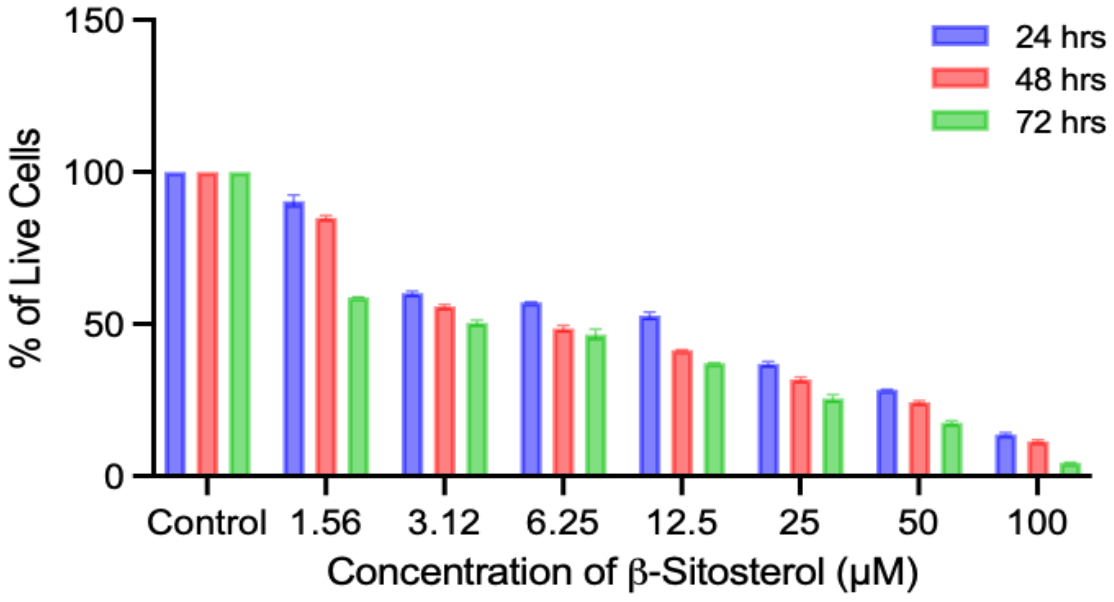

Figure 1. Effect of β-Sitosterol on Cell Viability in Neuronal Cell Line (IMR32)

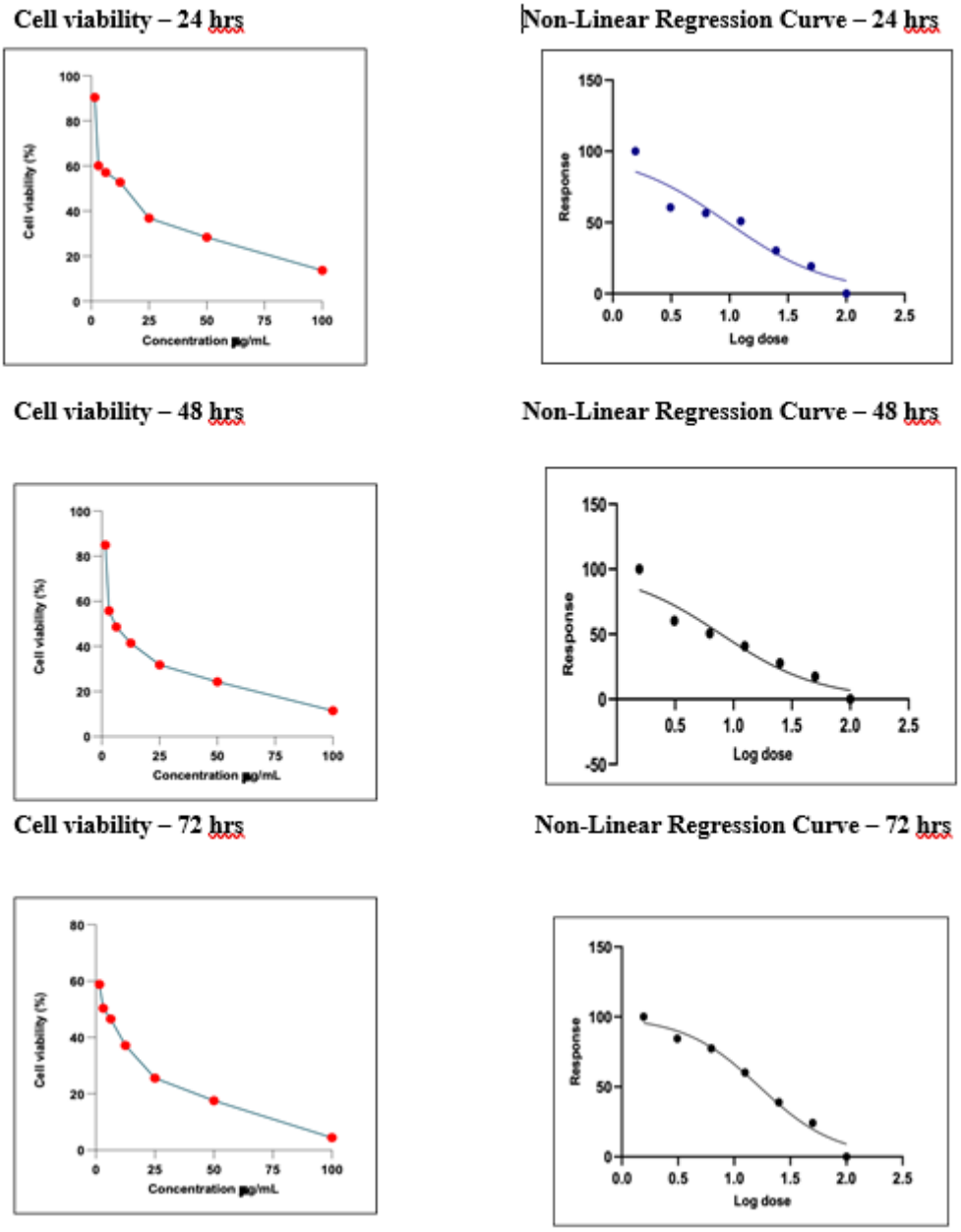

Figure 2. Effect of β-sitosterol on Cell Viability on a Duration Dependent in Neuronal Cell Line (IMR32).

Effect of β-Sitosterol on SOD, GSH and Gpx Activity in Neuronal Cell Line (IMR32)

In the antioxidant activity assay upregulation of Superoxide dismutase (SOD) was maximum at 10 µM (Figure 3) and for reduced glutathione it was found to be maximum for 3 µM concentration of β-sitosterol (Figure 4). Upregulation of glutathione peroxidase was also observed (Figure 5).

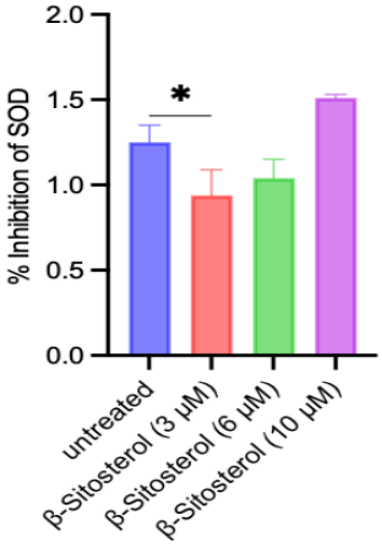

Figure 3. Effect of β-Sitosterol on SOD Activity in Neuronal Cell Line (IMR32)

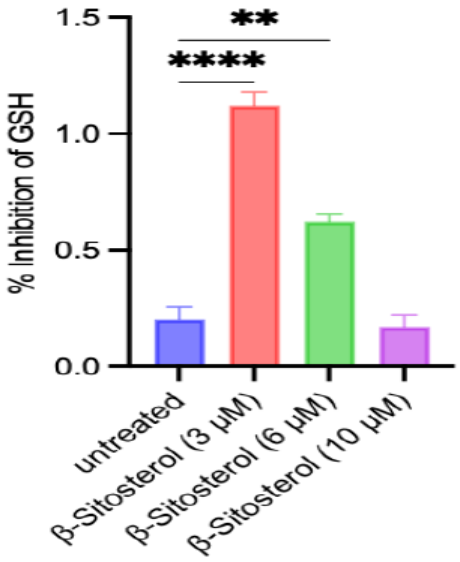

Figure 4. Effect of β-Sitosterol on GSH Activity in Neuronal Cell Line (IMR32)

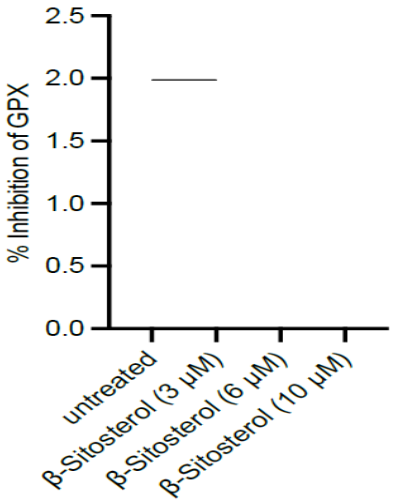

Figure 5. Effect of β-Sitosterol on Gpx Activity in Neuronal Cell Line (IMR32)

Effect of β-Sitosterol on ROS Activity in Neuronal Cell Line (IMR32)

In ROS Assay, inhibition of lipid peroxidases (LPO) was observed (Figure 6).

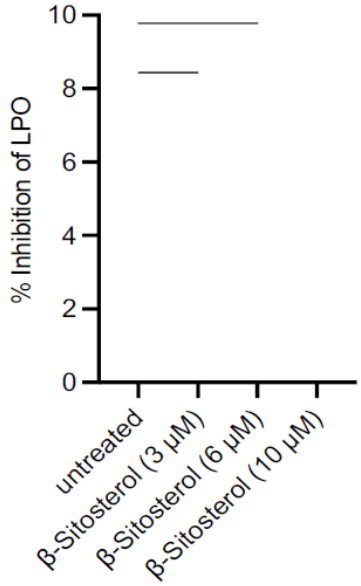

Figure 6. Inhibition of Lipid Peroxides in Neuronal Cell Line (IMR32)

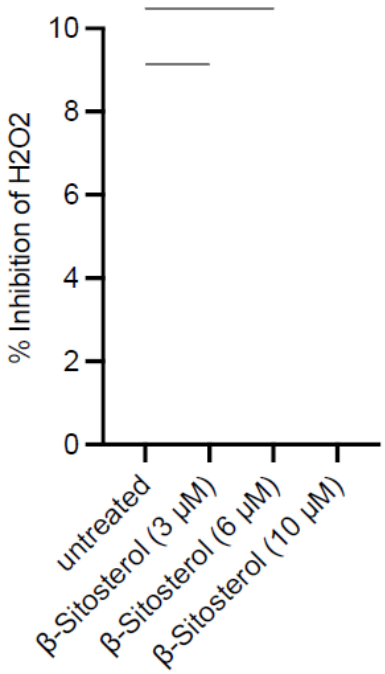

Figure 7. Inhibition of Hydrogen Peroxides in Neuronal cell line (IMR32)

In ROS Assay, inhibition of hydrogen peroxides was observed (Figure 7).

Effect of β-Sitosterol on Gene Expression of NRF2-KEAP

In the gene expression analysis, there was amplification of NRF 2 and KEAP and it was maximum for 10 µM concentration of β-sitosterol. The rise in NRF 2 which is a chief regulator of antioxidant enzymes is responsible for the upregulation of antioxidant compounds like reduced glutathione, superoxide dismutase etc. and fall in levels of reactive oxygen species (Figure 8). The rise in KEAP mRNA was depicted in Figure 9. Thus, the antioxidant effect of the compound

substantiated in neuronal cell line through NRF2 – KEAP pathway.

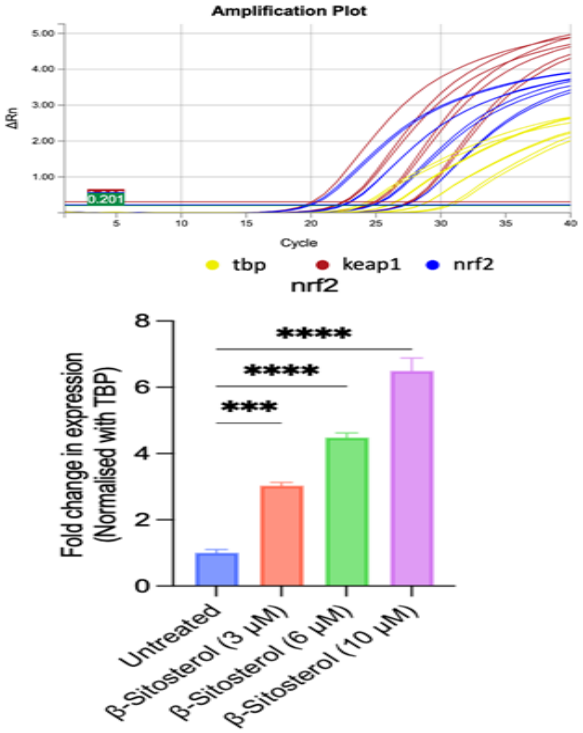

Figure 8. Effect of β-Sitosterol on NRF 2 Expression in Neuronal Cell Line (IMR32)

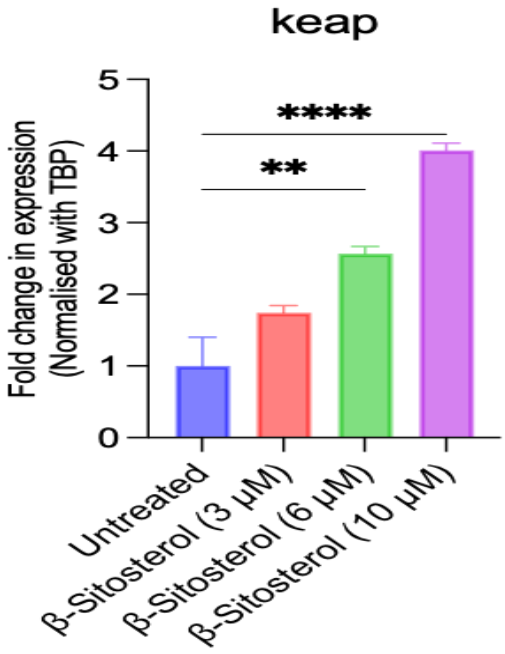

Figure 9 . Effect of β-Sitosterol on KEAP mRNA Expression in Neuronal Cell Line (IMR32)

Discussion

Oxidative insults to the cells of the nervous system seems to be the causative factor for degenerative changes that happen in the cells. Neuroinflammation of the microglial cells coupled with oxidative processes can be the root-cause for the onset of decline of nervous system. Introduction of phytocompounds like phytosterols which possess commendable antioxidant potencies can be an enlightening job to combat the oxidative stress that blossoms within the living systems.

The antioxidant potency of the phytosterol stigmasterol was exhibited in a cell line (SH-SY5Y) study where decline in reactive oxygen species in cells and upwards escalation of antioxidant enzymes like catalase was observed [13]. In this study we have substantiated the antioxidant property of the phytosterol β-Sitosterol through NRF2 – KEAP signaling pathway in human neuroblastoma cell line IMR 32. IMR 32 cell line when differentiated mimics the neurofibrillary mass present in Alzeimer affected brain [14]. NRF2 – KEAP signaling pathway is the key regulatory moiety that influences the synthesis and release of antioxidant enzymes from within the cells [7]. During normalcy KEAP possess negative switch over NRF2 but during oxidative demand this dominance of KEAP is taken off and NRF2 acquires the liberty to migrate towards the antioxidant response element of the nuclear genes to indict the generation of antioxidant factors [15]. The antioxidant enzymes so released scavenge the free radicals which are the causative factors for degenerative changes that happens in the neuronal cells. The antioxidant potency of β-Sitosterol was examined in this in vitro study by measuring the antioxidant parameters like superoxide dismutase, glutathione peroxidase and nonenzymatic parameters like reduced glutathione which are activated through the NRF2-KEAP pathway. The increase in gene expression of NRF2 and KEAP with proportionate increase in concentration of β-Sitosterol seen from the results of PCR proved that one of the key regulatory signaling pathway behind the antioxidant property is NRF2-KEAP pathway. This factor resulted in inhibition of reactive oxygen species like lipid peroxides and hydrogen peroxides with a concomitant increase in upregulation of antioxidant factors.

The antioxidant potential of β-Sitosterol was elucidated in another study where incorporation of β-sitosterol into the membrane increases resistance to oxidative stress and lipid peroxidation via estrogen receptor-mediated PI3K/GSK3β signaling systems [16]. The tendency of β-Sitosterol to regulate one of the key molecules indulging in oxidative processes – the reduced glutathione was demonstrated in RAW 264.7 macrophage culture study where phorbol esters influences the glutathione/oxidized glutathione ratio in reverse direction producing a defect. β sitosterol normalizes the ratio substantiating its antioxidant effect [17]. The antioxidant threshold of β-Sitosterol was proved in transgenic mice model where there was a notable decline in free radical load within the brain tissues in the treated group along with considerable progress in the working memory of the animals in the study [18-20].

Thus, from the present study and the previous researches conducted so far we can understand the antioxidant potential of β-Sitosterol and in the present study we have taken a leap forward by elucidating the molecular basis of antioxidant potential through the NRF2-KEAP pathway. This will lead to furthermore applications of this compound in the field of medicine in the near future. It can also lead to further research and applications in the management and therapeutics of neurodegenerative disorders.

Conclusion

Analysis of antioxidant potencies of phytosterol like β-Sitosterol discovers newer strategies in the management of neurodegenerative disorders in near future. Studies involving human participants are the need of the hour to promulgate the applications of phytocompounds in human community. Choice of right dosage with very negligible side effects have to be discovered and standardized according to the protocol.

Conflict of Interest

The authors declare that there no conflict of interest.

Acknowledgement

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