

SPINACEA OLERACEA L. EXTRACT PROTECTS AGAINST LPS INDUCED OXIDATIVE STRESS, INFLAMMATORY RESPONSE AND ENSUING BIOCHEMICAL, NEUROCHEMICAL AND NEUROBEHAVIORAL IMPAIRMENT IN MICE

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ABSTRACT

Objective: Spinacia oleracea L. extract has been reported to possess potent antioxidant activity in several animal models of neurodegenerative disease but its anti-inflammatory role has not been established much. Thus, the present study had been undertaken to evaluate the possible neuroprotective mechanism of S.oleracea against Lipopolysaccharide (LPS) induced PD.

Methods: LPS, a bacterial endotoxin was injected intraperitoneally (5mg/kg b.wt) in balb c mice and various neurobehavioral tests, biochemical, RT-PCR and neurochemical studies were performed at day 21.

Results: LPS injection in mice produced PD like symptoms as evident from impaired motor functions total locomotor activity, catalepsy and rotarod, increased oxidative burden (MDA, GSH, SOD), enhanced mRNA expression of proinflammatory cytokines and decreased dopamine turnover in striatum. However, spinach leaf extract (50mg/kg, i.p) administration intraperitoneally for 21 days significantly attenuated the LPS induced behavioural, biochemical, neurochemical and cellular alterations. Enhanced mRNA expression of proinflammatory cytokines (TNF- α and IL-1 β) have also been observed following LPS injection which was significantly attenuated by the S.L.E (50mg/kg, i.p) administration for 21 days.

Conclusion: Collectively, these observations suggest a possible anti-inflammatory effect of S.L.E against LPS induced neurodegenerative conditions.

Keywords: Lipopolysaccharide, Motor dysfunction, Neuroinflammation, Oxidative stress.

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by hypokinesia, rigidity and tremor, primarily due to the loss of dopaminergic neurons in the substantia nigra pars compacta. Although the pathogenesis of PD remain to be obscure, many interacting pathological processes appear to contribute to dopaminergic neurodegeneration including presence of activated microglia, inflammatory cytokines and increased oxidative stress etc [1,2]. Typically, microglia exist in a resting state but its sustained, uncontrolled activation can lead to an excess production of several deleterious factors such as nitric oxide, pro-inflammatory cytokines (IL-1 β , TNF- α) [3], reactive oxygen species (ROS) [4] and glutamate [5] which collectively leads to neurodegeneration. The current methods used to treat PD are focused mainly on replacing dopamine in the nigrostriatal system with L-Dopa and its analogues. As the entire nigrostriatal environment is altered in PD, targeting only the dopaminergic system does not solve the problems associated with the multifactorial disease.

In order to study the anti-inflammatory properties of S.Oleracea we employed an inflammation driven experimental model induced by the administration of the bacterial endotoxin, LPS which provides a valuable tool for the invivo modeling of the characteristics of progressive dopaminergic neurodegeneration associated with neuroinflammation. LPS is a Gram-negative bacterial endotoxin and is a potent microglial cell activator [6,7]. LPS has been well established as an effective initiator of SN dopaminergic neuronal loss via microglial activation [8] resulting in parkinson like symptoms in rats [7-9]. LPS injected in microgram quantities to the SN region of Wistar, Fischer or Sprague Dawley rats indeed leads to a marked loss (50-85%) of SNpc DA neurons [8-10] which is irreversible and permanent. Exposure to endotoxin like LPS is often associated with systemic or localized gram negative bacterial infection from exogenous sources or via bacterial translocation from gut.

Extensive epidemiological and experimental studies carried out within the last half century indicates that life style plays a major role in the development of chronic diseases and interaction of life style

factors with genetic factors contribute to the progression of the disease. Food is perhaps one of the most important life style components that could modulate the courses of various diseases. Currently proclaimed by Hippocrates almost 25 century ago: "Let food be thy medicine and medicine be thy food. Among the World's Healthiest vegetables, spinach ranks the list as it is abundantly rich in vitamins and minerals. Health-promoting phytonutrients such as carotenoids (beta-carotene, lutein, and zeaxanthin) and flavonoids have been reported [11] to be present in spinach which offers powerful antioxidant protection. Even though virtually all vegetables contain a wide variety of phytonutrients (flavonoids and carotenoids) spinach grabs a special place among vegetables in terms of its phytonutrient content.

More than a dozen different flavonoid containing compounds have been identified in spinach that functions as anti-inflammatory and anti-cancer agents. The methylenedioxy flavonol in spinach has been reported to provide anti-inflammatory as well as antioxidant benefits. Given the fact that spinach is an excellent source of other antioxidant nutrients such as vitamin C, vitamin E, beta-carotene, manganese, selenium, it's no wonder that spinach helps lower risk of numerous health problems associated with enhanced oxidative stress.

The increased recognition of the role of neuroinflammation and oxidative stress against the onset and progression of the neurodegenerative diseases has markedly influenced the number of researchers studying the anti-inflammatory effects of the present flavonoids in altering or forestalling the neurodegenerative changes [12]. Keeping in mind the reports mentioned in literature, the present study has been designed to evaluate the possible neuroprotective mechanisms involved in the neuroprotective effect of S.Oleracea against LPS induced behavioural abnormality, oxidative damage, and alteration in inflammatory cytokine levels.

MATERIALS AND METHODS

Healthy Male BALB/c Mice (30-35gms) were procured from the animal house of the university and were housed in polypropylene cages under hygienic conditions and were provided standard animal feed and water ad libitum throughout the treatment period. All

procedures were performed in accordance with ethical guidelines on the care and use of laboratory animals which were approved by Institutional Animal Ethics Committee (IAEC).

Chemicals

Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO). Spinach Leaf extract was received as a gift sample from BLP Pharmaceutical & Chemical Limited, China. Other chemicals were purchased from Sigma (St. Louis, USA), Merck, Sisco Research Laboratories Pvt Ltd (Mumbai, India) and Hi-Media Chemicals.

Detailed specifications of the Spinach Leaf Extract Table 1.

Experimental Methodology

Animals were divided in four different groups containing 6-8 animals in each group. (n=6/8).

The Grouping of experimental animals is as follows:

Group I: Control animals

Animals were given single injection of normal saline (0.9%) (5ml/kg, i.p).

Group II: LPS treated animals

The animals in this group were given single injection of LPS (5mg/kg, i.p).

Group III: LPS+ Spinach leaf extract (S.L.E) treated animals

Animals in this group were administered with single injection of LPS (5mg/kg, i.p) and spinach leaf extract (50mg/kg, i.p) for a period of 21 days.

Group IV: Spinach leaf extract treated (S.L.E) animals

Animals in this group were administered with spinach leaf extract (50mg/kg, i.p) for a period of 21 days.

Experimental Design

Behavioural assessment was carried out before the LPS administration and after that regularly at an interval of 7 days till day 21. Biochemical (oxidative stress parameters) studies, neurochemical estimation (Neurotransmitter levels estimation by HPLC/ECD) and histological studies were done at day 21.

Behavioral studies

Measurement of locomotor activity

Total Locomotor activity (ambulations and rearing) was measured by using a computerized Actophotometer (IMCORP, India). Locomotion was expressed in terms of total photo beam counts per 5 minutes per animal [13].

Catalepsy

The bar test was used for measuring catalepsy according to the method of Costall and Naylor [13]. The maximum cutoff for bar test was fixed at 180 sec.

Rotarod Test

The effects of LPS and Spinach leaf extract on muscle performance were evaluated using a Rota-rod (**Techno**). The time each animal remained on the rotarod was recorded; animals not falling off the rotarod were given a maximum score of 0 seconds [14].

Neurotransmitter's estimation

Biogenic amines i.e dopamine was estimated by HPLC with electrochemical detector by the method of Church [15]. On the day of experiment frozen samples of mid brain were homogenized in the homogenizing solution containing 0.1 M perchloric acid. After the samples were centrifuged at 12000xg for 5 minutes. The supernatant was further filtered through 0.25 micron nylon filters before injecting in the HPLC injection pump. Data was recorded and analyzed with the help of Empower software [15].

Biochemical Parameters

Protein estimation

Protein contents in various samples were estimated by the method of Lowry [16].

Lipid Peroxidation

Malondialdehyde levels were determined by following the method described by Wills [17].

Catalase

Catalase was estimated by using the method as described by Luck [18].

Superoxide Dismutase

Activity of superoxide dismutase (SOD) was estimated by the method of Kono [19].

Reduced Glutathione

Reduced glutathione content was estimated according to the method of Ellman [20].

Total Glutathione Content

The total glutathione content was measured by the method of Zahler and Cleland [21].

Determination of Oxidized Glutathione

Oxidized glutathione was calculated by subtracting the value of glutathione reduced from total glutathione.

Determination of Glutathione Peroxidase

Glutathione peroxidase activity was estimated by the method of Paglia and Valentine [22].

mRNA Expression studies for pro-inflammatory cytokines :-

Reverse transcriptase polymerase chain reaction (RT-PCR) kit was used (Qiagen, Inc., Germany). cDNA synthesis and PCR were carried out sequentially in the same tube (Labware Scientific, Inc., USA). Procedure was performed according to the manufacturer's instructions. RNA (3 µg) was used for each reaction. Master mix was first of all prepared: 59 Qiagen RT-PCR buffer, 10 µl; dNTP mix, 2 µl; Qiagen one-step RT-PCR enzyme mix, 2 µl; and RNase inhibitor, 1 µl. The master mix was mixed thoroughly, and 15 µl of it was added to each PCR tube.

Then to this, 5 µl of each sense and antisense gene-specific primers (from 10 µM stock) was added. Then, 3 µg template RNA was added, and the final volume was adjusted to 50 µl with RNase-free water provided in the kit. All the reactions were carried out on ice. The PCR tubes were gently vortexed and centrifuged in order to settle all the components at the bottom. PCR amplification was performed using 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and extension at 72 °C for 1 min. The primer sequences, predicted productsizes, and GenBank accession numbers (where available) for the amplified genes were as follows: IL-1β(F) 5'-AGCTGCACTGCAGGCTTCGAGATG-3' (R) 5'-GAACTGTGCAGACTCAAACCTCCAC-5' NM_008361.3, TNF-α(F) 5'-ACTGAACTTCGGGGTGATTG-3' (R) 5'-GTGGGTGAGGAGCAGGTAGT-3' NM_013693.2. PCR products were separated on 2 % agarose gels containing ethidium bromide and visualized under ultraviolet light. Densitometric analysis of bands was done by using the Image J software (NIH). The cycle time values of genes first normalized with beta-actin of the same sample and, then, the relative differences between control and treatment groups were calculated and expressed as relative change

Light Microscopic Examination

To assess histoarchitectural changes if any, sections of mid brain from each of the normal control and different treated animals were taken, washed with ice-cold 0.9% NaCl and were fixed in the buffered formalin (10%) for about 24–48 h. After the fixation, tissues were dehydrated in ascending grade of alcohol, embedded in

wax following the standard technique. 5–7 μm thick paraffin sections were cut and then were subjected to hematoxylin–eosin staining as described by Humanson [23].

Statistical Analysis

For analyzing the data, one way analysis of variance (ANOVA) followed by Newman Keul's test was performed using the statistical software package "SSPS v 11 for windows". The post-hoc comparison of means from different treatment groups were made by the method of least significant difference (LSD). Results corresponding to a p value of 0.05 or less were considered statistically significant.

RESULTS

Brain weight/ body weight

Single i.p. administration of LPS (5 mg/kg) resulted in significant decrease in brain wt. and body weight whereas S.L.E (50 mg/kg) administration for 21 days attenuated the decrease in brain and body weight (fig 1). However, in LPS treated animals decrease in brain/body wt. ratio was observed but the change in brain/body weight ratio was found to be non-significant between all groups.

Behavioral parameters (Total locomotor activity, catalepsy and rotarod)

Total locomoter activity was measured by digital Actophotometer. In control animals there was no significant change in the mean counts/3 min at subsequent weeks, whereas in LPS treated animals there was significant decrease in the total locomoter activity on day 21 (fig 2(A)).

Administration of S.L.E significantly improved the total locomoter activity on 21st day of treatment. As compared to LPS treated animals, there was no significant alteration in the total locomoter activity when compared to control animals.

Catalepsy: In control animals (group I) the fall off time remained almost same with a small variation at subsequent weeks whereas LPS treated animals (group II) demonstrated significant increase in fall of time significant as compared to the control group at day 21 (fig 2B).

Administration of S.L.E (50 mg/kg) significantly improved the fall off time when compared with the LPS treated animals. No significant change in group IV was observed when compared to control animals throughout the protocol.

Motor Co-ordination: In LPS treated animals (group II) fall off time from rotarod was found to be decreased significantly ($p < 0.001$) as compared to control animals (group I) at all intervals on day 7, day 14 and day 21 as compared to control animals (fig 2C). However, administration with S.L.E (group III) significantly improved the fall off time as compared to LPS treated animals as demonstrated by improved motor coordination.

Dopamine

Significant ($p < 0.05$) decrease in dopamine level was observed in the brain of LPS treated animals (group I) when compared to Control (group I) animals whereas treatment with S.L.E (50 mg/kg) significantly increased the Dopamine level following LPS exposure showing neuroprotective effect. In S.L.E treated animals, increase in dopamine level was observed as compared to control group animals (fig 3).

Biochemical parameters

Lipid peroxidation: MDA formation, Superoxide Dismutase (SOD), Catalase

LPO: Significant elevation in the level of MDA was observed in LPS treated animals as compared to control animals whereas significant reduction in MDA formation was observed on S.L.E (50 mg/kg) administration for 21 days (Table 2).

SOD: S.L.E administration for 21 days (50 mg/kg) significantly improved the altered SOD activity in LPS treated showing the anti-oxidative potential of S.L.E (Table 2).

Catalase: Catalase activity was found to increase following LPS injection whereas S.L.E treatment for 21 days reduced the activity levels near to that of control (Table 2).

Total glutathione, GSH, GSSG (oxidised glutathione) and redox ratio (GSH/GSSG)

Different enzymes involved in Glutathione cycle was estimated by biochemical analysis (Table 3):

Glutathione peroxidase (GPx): Specific activity of GPx was found increase significantly in LPS treated animals (group II) as compared to control whereas S.L.E administration (50mg/kg) increased the GPx activity.

Reduced Glutathione (GSH): The level of reduced glutathione was found to decrease significantly in LPS treated animals (group II) when compared with control animals whereas S.L.E administration (50mg/kg) for 21 days extract improved the level of GSH.

Total Glutathione (TG): Increased total glutathione level was observed LPS treated animals (group II) when compared with control animals (group I) whereas with S.L.E (50mg/kg) a decrease in total glutathione level was observed.

Oxidized Glutathione (GSSG): After 21 days of LPS injection significant increase in the level of GSSG was observed as compared with control animals whereas S.L.E (50mg/kg) administration to LPS treated animals significantly lowered the GSSG level.

Pro-inflammatory cytokines: IL-1 β and TNF- α

The mRNA expression of pro-inflammatory cytokines i.e TNF- α and IL- 1 β increased significantly ($p < 0.05$) in LPS treated group as compared to control group whereas 21 days treatment with S.L.E significantly decreased the mRNA expression as compared to LPS treated group (fig 4).

Light microscopy studies

In control and spinach extract treated group, the intact neurons were observed, indicating no oxidative stress whereas in LPS treated group dead and shrunken neurons were observed (marked by open arrow). S.L.E administration reduced the number of dead neurons (marked by open arrow) (fig 5).

DISCUSSION

Studies pertaining to free radicals, antioxidants and the phytochemical-rich whole foods in human medicine are producing revolutionary data that promises a new age of health and neurodegenerative disease management. For years, medical interest in the relationship between nutrition and neurodegenerative disorders has been focused largely on etiology and the influence of dietary macronutrients on the rate and severity of disease, while the cause of these disorders remains to be unknown and the influence of macronutrients is unclear. Recent studies on antioxidant intake from foods and oxidative stress in neurodegenerative disorders are strengthening the rationale in support of a nutritionally sound, antioxidant and phytochemical-rich, whole food-based eating regime. Spinach diet has found to be effective in reducing neuronal vulnerability to oxidative stress in comparison to vitamin E, even though both diets have equal antioxidant capacity in terms of Trolox equivalents [24].

It has also been speculated that there are other effects of the phytochemicals present in spinach and strawberries in addition to antioxidant protection. The anti-inflammatory potential of spinach leaf extract was confirmed by Nager [25]. These observations emphasized the fact that phytochemicals present in spinach leaf extract possess antioxidant, antiallergic, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic properties [26]. Further, observations made by Joseph [27] confirmed the antioxidant properties in selected whole foods do play a role in protection against several neurodegenerative disorders.

Table 1: Detailed specifications of Spinach leaf extract. Analysis Specifications Results

Analysis	Specifications	Results
Spinach extract (UV)	10:1	10:1
Solvent	Water	Water
Solubility	90%	90%
Ash	≤ 8.0 %	5.28 %
Heavy metal	<10ppm	corresponding
Pesticide	<2ppm	corresponding
Appearance	Light green powder	corresponding
Loss on drying	≤ 5.0 %	3.56 %
Total Plate	<10000 CFU/g	corresponding
Yeast & Moulds	<1000 CFU/g	corresponding
Salmonella	Negative	Negative
E.Coli	Negative	Negative

Table 2: Effect of S.L.E on Lipid peroxidation level (LPO), Catalase and Superoxide dismutase (SOD) activity in mid brain of LPS subjected rats

	LPO nmoles of MDA/mg protein	Catalase (nmol of H ₂ O ₂ hydrolyzed /min /mg protein)	SOD (Units/min/mg protein)
Control	3.04±0.80	0.251±0.07	3.669±0.10 ^{bx}
LPS	9.859±1.14 ^c	1.417±0.51 ^b	1.846±0.16 ^b
LPS+SLE	6.123±1.92 ^{ax}	0.563±0.15 ^{ax}	2.307±0.60 ^a
SLE	3.044±0.61 ^z	0.691±0.10 ^{bx}	3.846±0.11 ^z

Data is mean ± S.D of 6 animals.

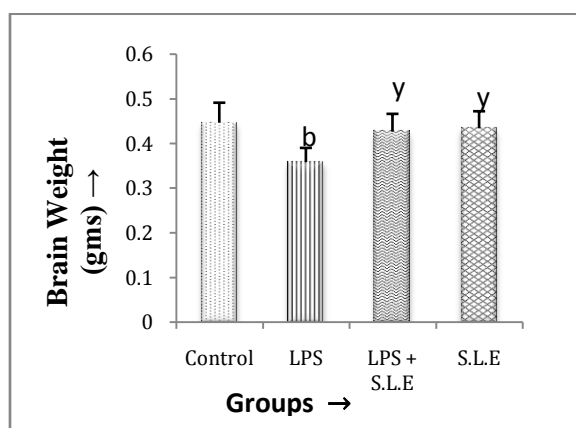
^ap<0.05, ^bp<0.01, ^cp<0.001 by Newman –Keuls test when the values are compared with normal control group. ^xp<0.05, ^yp<0.01, ^zp<0.001 by Newman –Keuls test when the values of LPS+S.L.E are compared with LPS treated group.

Table 3: Effect of S.L.E on glutathione status i.e Total Glutathione, GSH, GSSG, GSH/GSSG and GPx in mid brain of rats subjected to LPS.

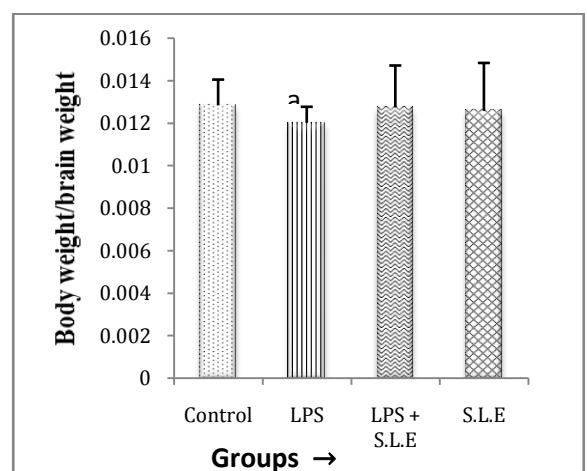
	Total Glutathione (nmol GSH mg/ protein)	Reduced Glutathione (nmol GSH mg/ protein)	Oxidised Glutathione (nmol GSSG mg/ protein)	Redox ratio (GSH/GSS G)	Glutathione Peroxidase (nmol of NADPH used min/ mg/ protein)
Control	0.169±0.018	0.191±0.0148	0.15±0.0179	1.27	1.071±0.126
LPS	0.253±0.063 ^a	0.084±0.031 ^b	0.24473±0.06 ^a	0.35	3.155±0.277 ^c
LPS+S.L E	0.201±0.033	0.141±0.016 ^{bz}	0.186±0.033	0.758	2.148±0.79 ^{cy}
S.L.E	0.143±0.032 ^a	0.34±0.0345 ^{by}	0.108±0.035 ^x	3.14	1.862±0.179 ^{bz}

Data is mean ± S.D of 6 animals.

^ap<0.05, ^bp<0.01, ^cp<0.001 by Newman –Keuls test when the values are compared with normal control group. ^xp<0.05, ^yp<0.01, ^zp<0.001 by Newman –Keuls test when the values of LPS+S.L.E are compared with LPS treated group.



Data is mean ± S.D of 6 animals. ^ap<0.05, ^bp<0.01, ^cp<0.001 by Newman –Keuls test when the values are compared with normal control group. ^xp<0.05, ^yp<0.01, ^zp<0.001 by Newman –Keuls test when the values of LPS+S.L.E are compared with LPS treated group.

**Fig. 1: Effect of S.L.E on brain weight and brain weight /body weight ratio of LPS treated rats.**

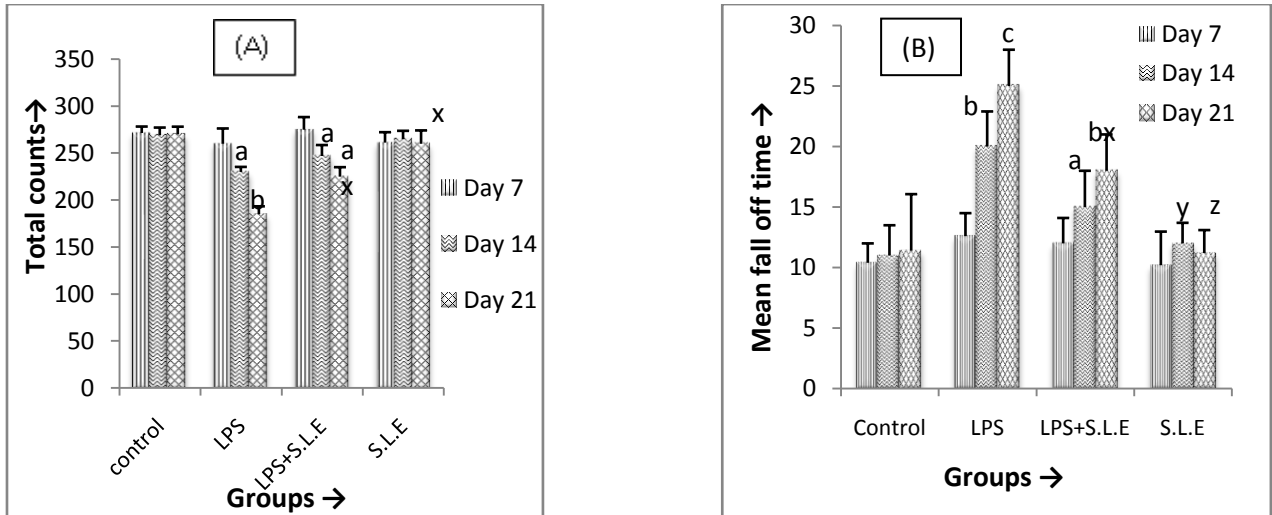


Fig. 2: Effect of S.L.E on (A) Total locomoter activity (using Actophotometer) (B) Catalepsy (C) Rotarod behaviour of LPS treated rats at various time intervals during the disease progression:

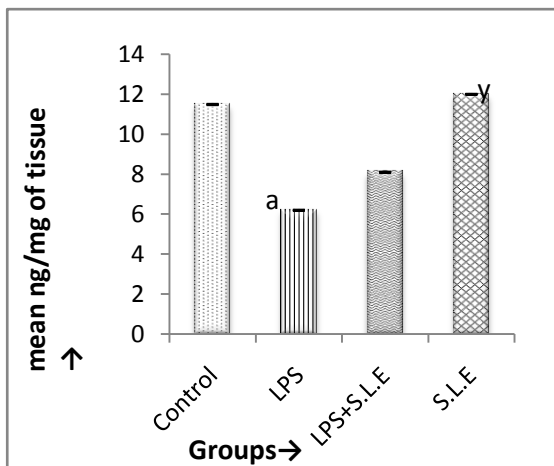
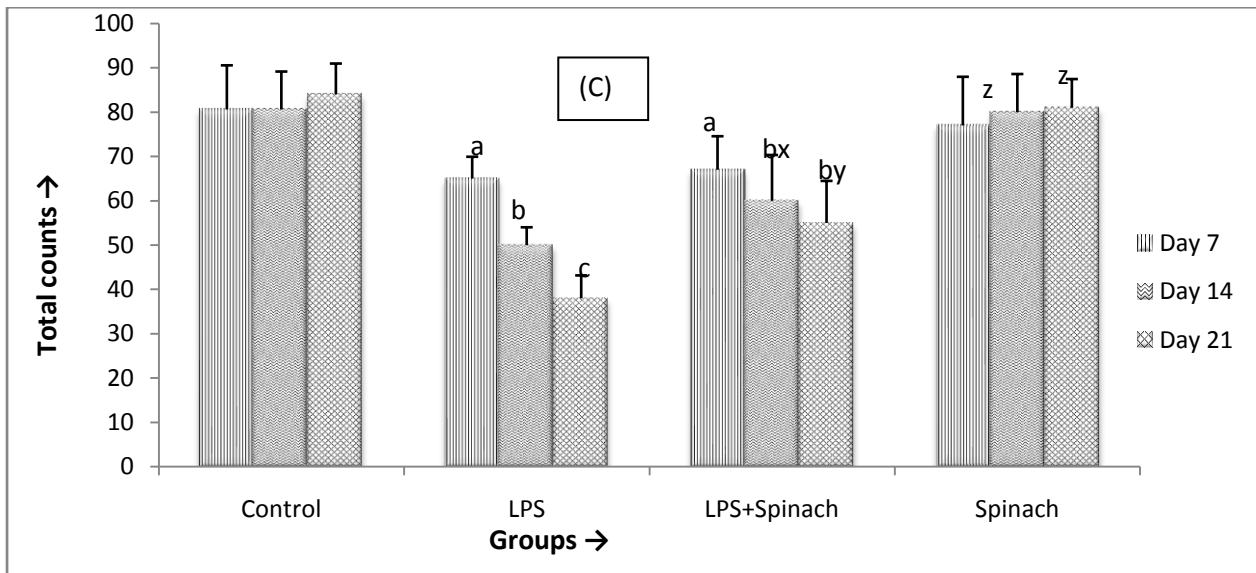


Fig. 3: Effect of S.L.E treatment on the dopamine level of LPS treated rats.

Data is mean \pm S.D of 4 animals. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ by Newman -Keuls test when the values are compared with normal

control group. ^x $p < 0.05$, ^y $p < 0.01$, ^z $p < 0.001$ by Newman -Keuls test when the values of LPS+S.L.E are compared with LPS treated group. Data is mean \pm S.D of 6 animals. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ by Newman -Keuls test when the values are compared with normal control group. ^x $p < 0.05$, ^y $p < 0.01$, ^z $p < 0.001$ by Newman -Keuls test when the values of LPS+S.L.E are compared with LPS treated group.

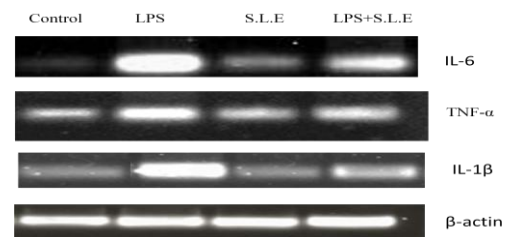
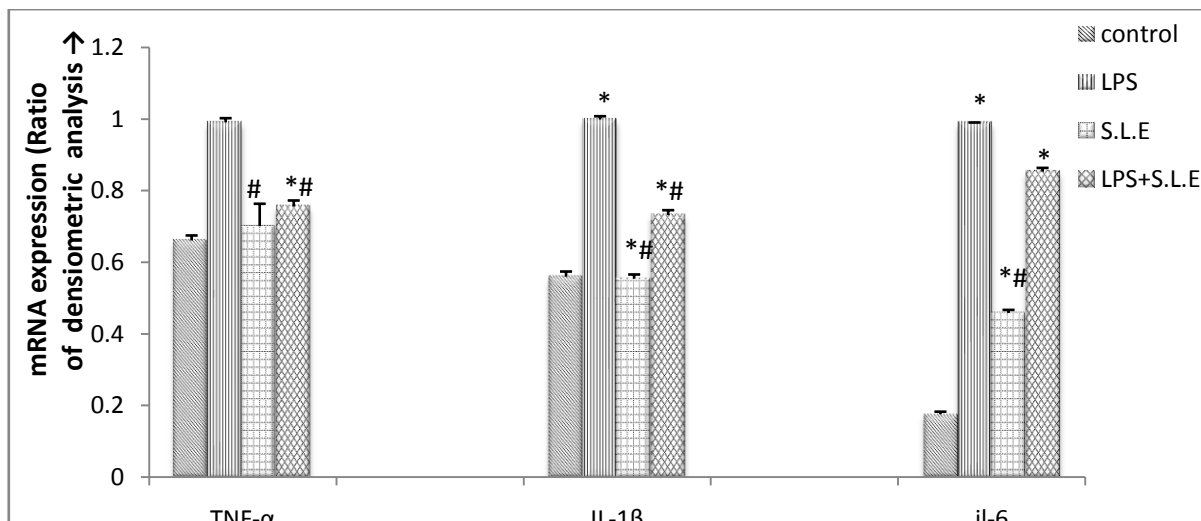


Fig. 4: Effect of S.L.E Co-treatment on mRNA expression of TNF- α , IL-1 β , IL-6 in LPS treated rats as calculated by densitometric analysis by image J software



Data is mean \pm S.D of 4 animals. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ by Newman-Keuls test when the values are compared with normal control group. ^{*} $p < 0.05$, [†] $p < 0.01$, [‡] $p < 0.001$ by Newman-Keuls test when the values of LPS+S.L.E are compared with LPS treated group

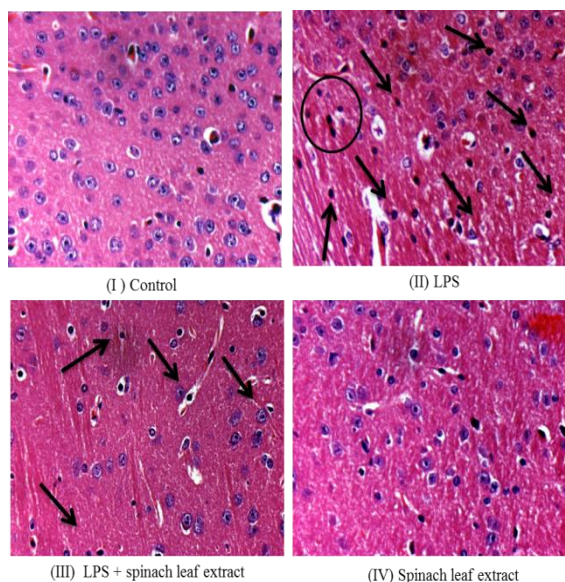


Fig. 5: Photomicrographs of histological sections of mid brain of control (I), LPS treated (II), LPS + spinach leaf extract (S.L.E) treated group (III) and S.L.E treated group (IV) at 40X magnification

Our study has been targeted towards the exploitation of potential components with antioxidant and anti-inflammatory properties present in whole foods like Spinach against endotoxin (LPS) based model of Parkinson's Disease. There always exists a balance between proinflammatory and anti-inflammatory mediators, with microglial cells remaining quiescent [28]. However, in chronic neurodegenerative diseases, microglia are transformed to a primed state leading to the release of inflammatory mediators. Peripheral injection or systemic infection by LPS results in an exaggerated inflammatory response in the brain via the activation of CNS macrophages i.e microglia and astrocytes. Though LPS may not affect the neurons directly (since they lack TLR4 expression) it does indirectly by activation of the microglia [29]. It acts by binding to the LPS binding protein (LBP) and CD14 subsequently leading to the activation of various intracellular signalling pathways, upregulation of gene transcription for a variety of pro-inflammatory factors and free radical-generating enzymes in the microglia [30].

Single i.p LPS administration resulted in the increased mRNA expression of proinflammatory cytokines IL-1 β , IL-6 and TNF- α in LPS treated animals when compared to control animals (Fig 4). The enhanced expression is due to increased binding of nuclear factor- κ B to the TNF- α , IL-1 β and IL-6 gene promoters in microglial cells [31, 32] This increase in the level of various cytokines i.e TNF- α and IL-6 affect the dietary intake [31] and could account for the decrease in body weight as well as motor dysfunctions seen in LPS treated animals. Peripheral cytokines act directly on the glucose-sensitive neurons in hypothalamic nuclei and leads to the suppression of food intake having a direct bearing on the loss in body weight. Following S.L.E treatment, a significant decrease in the expression of proinflammatory cytokines (IL-1 β and TNF- α) in the brain of LPS treated animals was observed, suggesting the efficacy of S.L.E in neuroinflammatory conditions in PD.

Several studies have showed that spinach is rich in flavonoids along with several other components such as vitamin E, p-coumaric acid, uridine. Flavonoids have been observed to inhibit NO, IL-1 β and TNF- α production in activated microglia cells [33]

It has been shown by Chen and Swope [34] that flavonols attenuate microglia or astrocyte mediated neuroinflammation. Molecular mechanisms involved in their cytokine-modulating activity, including flavonoid-mediated inhibition of transcription factors NF- κ B and AP-1 (activating protein) and reduction of MAPK activity, have been suggested as relevant anti-inflammatory pathways [35,36].

A growing body of evidences suggest that single intraperitoneal LPS administration evokes oxidative stress and energy disturbances in the brain [36] which is mediated by the activation of NADPH oxidase a multisubunit enzyme [37] present in the plasma membrane of microglial cells, subsequently leading to excess formation of superoxide anion which leads to the loss in enzymatic activity of SOD [37]. In present study specific antioxidant enzyme activities (GSH and SOD) were found to be drastically decreased after 21 days following single intraperitoneal administration of LPS. Lipid peroxidation is a self-propagating process that will proceed until the substrate is consumed or termination occurs. MDA (Malondialdehyde) which is a highly reactive three carbon dialdehyde produced as a by product of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism is considered as an indicator for lipid peroxidation. Single systemic injection of LPS significantly elevated the levels of MDA formed in the brain as compared to control animals as seen from Table 2. Our results are further supported by Cetin [38] and Hanan [39]. Increased levels of lipid peroxidation products have been associated with a variety of

chronic diseases in both humans and model systems as MDA reacts readily with amino groups on proteins and other biomolecules to form a variety of adducts, including cross-linked products. Glutathione system enzymes which have a crucial metabolic importance, their altered levels might play an important role in inducing oxidative stress in brain. A significant decrease in GSH level was observed in the brain of LPS treated animals. S.L.E administration significantly reduced the production of MDA formed in LPS plus spinach treated animals when compared to LPS treated animals. It prevented the increase in MDA levels by virtue of the presence of flavonoids which have antioxidant activity [40] exhibited by detoxifying peroxides lipid membrane by directly interacting with their peroxidated lipids to restore them into lipid alcohol. Similar results were observed by Esposito [41] after flavonoid treatment. Treatment with S.L.E for 21 days resulted in significant improvement in antioxidant enzyme activities suggesting potent antioxidant activity. These findings are further supported by the experimental reports of Nager and his co-workers [37].

Oxidative stress is implicated as a major cause of neuronal injury in PD whether oxidative stress is causal or consequential is still unclear. In either case, oxidative stress contributes to the degeneration of dopaminergic neurons in PD as the brain regions rich in catecholamines such as adrenalin, noradrenalin and dopamine are exceptionally vulnerable to free radical generation [42-43]. Metabolism of dopamine also leads to increased ROS production which further exacerbates inflammation and tissue damage. LPS when injected into different parts of the adult brain stimulates neurodegeneration selectively in the SN, indicating the region specific sensitivity to LPS [9, 44-47] which is most likely attributed to the abundance of microglia in that region [9,45] and is mediated by excess production of cytokines mainly IL-1 β . Further, dopaminergic neurons express the receptors for these cytokines i.e TNF- α . Although cytokines exert toxic effects directly by binding to their receptors and activating second messenger pathway or can also elicit cytotoxic effects directly. It involves four different pathways, such as apoptotic trigger, death receptors, and lack of neurotrophic factors and stress induced caspase activation. All these different events may have the activation of caspase-3 in common, finally leading to DNA fragmentation and apoptotic cell death [48]. Secondly, microglia can become over-activated in response to neuronal damage (reactive microgliosis), which is then toxic to neighboring neurons, resulting in a perpetuating cycle of neuron death. Reactive microgliosis could be an underlying mechanism of progressive neuron damage across numerous neurodegenerative diseases, regardless of the instigating stimuli.

In line with the above mentioned reports in the present study, single intraperitoneal LPS administration resulted in massive decrease in dopamine level in the striatal area. This substantial decrease in DA level may be attributed to the increased expression of pro-inflammatory cytokines and increased oxidative stress. This loss in dopamine level is well correlated with the motor dysfunction depicted by altered locomotor and muscular activity. Further, this decrease in DA content was significantly ameliorated by 21 days treatment with S.L.E which facilitated the recovery from behavioural impairment coordination suggesting a potent neuroprotective effect against dopaminergic neurodegeneration. Finally, the light microscopy data suggest a similar neuroprotective effect of S.L.E (50mg/kg).

CONCLUSION

The present study demonstrates the neuroprotective effect of S.L.E against LPS induced dopaminergic neuronal death resulting from the increased oxidative stress and activated microglia mediated proinflammatory cytokine production leading to increase in dopamine level hence improved motor dysfunction. These findings potentially indicate that S.L.E can be used to mitigate cytokine expression in the brain and can have a beneficial effect on behavioural alterations. Taken together, these data support the idea that pharmacological strategies aimed at decreasing neuroinflammation associated with microglial activation are important for improving and reducing the frequency of neurobehavioral complications.

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