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INHIBITION OF OXIDATIVE STRESS AND INFLAMMATION ON PUMPKIN SEED EXTRACT IN NEURO 2A AND RAW 264.7 MACROPHAGE CELL LINES

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Multiple sclerosis, *Cucurbita maxima*, anti-oxidant, anti-inflammatory, demyelination.

ABSTRACT

Multiple sclerosis is a demyelinated disease of the CNS. Abundant evidence demonstrates that oxidative stress and inflammation may not only an early event in this disease but also a key factor in the pathogenesis of Multiple sclerosis. *Cucurbita maxima* seed extract has a strong ability to scavenge oxygen free radicals and inhibits pro-inflammatory mediators. The present study aim to investigate the effect of *Cucurbita maxima* seed extract on Neuro2A cells and RAW264.7 macrophage cells for inhibiting oxidative stress and inflammation. The oxidative stress was assessed by measuring superoxide dismutase, the level of Malondialdehyde and inflammation assessed by LPS-induced TNF-α and IL-6. This study showed that *Cucurbita maxima* seed extract treatment reduced production of free radicals and pro-inflammatory mediators. These findings suggest that *Cucurbita maxima* seed extract can reduce oxidative stress and inhibit pro-inflammatory mediators by enhancing antioxidant and anti-inflammatory status further leading to reduced demyelination in Multiple sclerosis. *Cucurbita maxima* seed might be a potential therapeutic agent for Multiple sclerosis.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease in humans whose key pathology is demyelination and axonal loss as the result of immune cell production of pro inflammatory molecules and active attack of both the myelin sheaths and the cells producing them, the oligodendrocytes. The hallmarks of the pathology are plaques which are found in myelin-rich white matter regions of the central nervous system (CNS) in both brain and spinal cord. Destruction of myelin, oligodendrocytes, and ultimately axons is mediated by activated T cells, auto antibodies directed self-antigens, enzymes, and free radicals secreted by macrophages microglia[1,2]. Oligodendrocytes progenitors reside throughout the CNS from birth well in old age in the human brain[3, 4]**.** Reactive oxygen species (ROS) are produced in many aerobic cellular metabolic processes. They include, but are not limited to, species such as superoxide and hydrogen peroxide which react with various intracellular targets, including lipids, proteins, and DNA[5]. Although ROS are generated during normal aerobic metabolism, the biological effects of ROS on these intracellular targets are dependent on their concentration and increased levels of these species are present during oxidative stress. Increased levels of ROS are cytotoxic, while lower levels are necessary for the regulation of several key physiological mechanisms including cell differentiation[6],apoptosis[7], cell proliferation[8] and regulation of redox-sensitive signal

transduction pathways[9] The hydro peroxyl radical ($HO._2$) plays an important role in the chemistry of lipid peroxidation. This protonated form of superoxide yields H_2O_2 which can reacts with redox active metals including iron or copper to further generate $HO\cdot_2$ through Fenton or Haber-Weiss reaction. The $HO\cdot_2$ is a much stronger oxidant than superoxide anion-radical and could initiate the chain oxidation of poly unsaturated phospholipids, thus leading to impairment of membrane function[10–12]. Chronic inflammation is known to be an important etiological condition for various chronic diseases, including atherosclerosis, diabetes, and arthritis. Furthermore, increasing evidence suggests that innate immune activation is a major component of age-related neurodegenerative diseases, such as Alzheimer's and Parkinson's disease[13, 14]. Inflammatory cells, including macrophages and microglia, play a major role in the body's response to immunogenic challenges, byre-establishing tissue homeostasis, producing large amounts of superoxide, nitric oxide (NO) and pro-inflammatory cytokines that aggravate and propagate inflammation and disrupt the normal function of cells NO is a signalling molecule with diverse cellular roles; it is released by macrophages, neurons and endothelial cells and can be either protective or toxic to cells depending on the cellular context [15,16]. The toxicity of NO is attributed to its ability to bind to proteins that contain iron, copper or organic side groups, such as thiols, resulting in protein disruption and alterations in cellular activities. As a result of reactivity with a wide range of cellular components, high levels of NO are toxic to neurons, while low levels can be neuro protective[17]. Cucurbita maxima (Family: Cucurbitaceae) known as Dadhiphala in Sanskrit, Red Gourd in English and Kashiphala in Hindi, widely available in temperate regions such as North America and Australia [18, 19]. The chemical constituents from seeds contain 30% unsaturated fixed oil (linoleic and oleic fatty acids), triterpenoids, flavonoids, coumarins, saponins, cucurbitacins, vitamins, minerals notably zinc, amino acid known as cucurbitin[20] which has anthelmintic effect, high amount of carotenoid content which include lutein and beta-carotene. It has also been suggested that phytosterols present in the seed may play some role in the treatment of prostate problem. Long chain hydrocarbons and fatty acids present in fruits, spinasterol present in flowers have been reported. Pulp is applied to burns, scalds, inflammations, abscesses, boils and is remedy for migraine, neuralgia, haemoptosis & hemorrhages [21, 22]. Therefore we undertook the present investigation to examine the antioxidant activity and anti-inflammatory activity.

MATERIALS AND METHODS

Extraction procedure: The selected plant part was shade dried and powdered. The 1 kg each of the course powders of all the three plants on dry weight basis were subjected for cold maceration using petroleum for 72 hrs. At the end of each respective extraction, the extract was filtered and the filtrate was concentrated under reduced pressure in vacuum at 30°C using the rotary evaporator (Buchi, Switzerland). The concentrated extracts were further dried in desiccators to obtain dry extracts. The percentage yield of all the dry extracts were calculated and used for the qualitative phytochemical analysis.

Anti – oxidant activity:

Determination of Superoxide Dismutase (SOD)[23]

Maintenance of cell lines: N2a (Neuroblastoma) cells (kindly supplied from NCCS Pune ATCC- 346) were maintained at 37 ° C in Dulbecco's modified Eagle's media, containing 20% (vol/vol) fetal bovine serum, 1%nonessential amino acids, 1 mM L-glutamine, 10 000 U/ml penicillin and 10 000 µg/ml streptomycin in an atmosphere of CO2: air (1:19) at 90 to100% relative humidity. Cells were grown in 100-ram petridishes and were supplied with culture medium every 48 h. Cells from Passages 42 to 46 were confluent 10 days after being seeded onto 100-mm petriplates. At 72-h intervals during 16 days of confluence, three dishes of cells were trypsinized, washed with phosphate buffered saline (PBS) (17 mM

NaC1, 3mM KCI, 2 mM NaH2PO 4, 10 mM Na2HPO4), resuspended in 1.0 ml PBS and frozen at -20 ° C until enzyme determinations were performed.

Enzyme assay: SOD was assayed according to the method of Paoletti et al. N2a (Neuroblastoma) cells were homogenized in 4 vol of PBS with 2.0 gm phenyl methyl sulfonyl fluoride (Sigma) using a Wheaten tissue grinder. The homogenate was centrifuged at 30,000 Xg for 15 min at 4 ° C. The supernatant was poured over a G-25 column. The oxidation of NADH was followed spectrophotometrically at 340 nm after the addition of 10 mM mercapto ethanol/mg protein. One unit of enzyme activity is the amount of SOD capable of inhibiting by 50% the rate of NADH oxidation by mercaptoethanol. Results are expressed as the mean + SEM.

Determination of lipid peroxidase by measuring (MDA) level[24]

Maintenance of Cell lines: N2a(Neuroblastoma) cells (kindly supplied from NCCS Pune) were maintained at 37 ° C in Dulbecco's modified Eagle's media, containing 20% (vol/vol) fetal bovine serum, 1%nonessential amino acids, 1 mM L-glutamine, 10 000 U/ml penicillin and 10 000 µg/ml streptomycin in an atmosphere of CO_2:air (1:19) at 90 to 100% relative humidity. Cells were incubated at 37°C for 24 h.

Treatment of Cells: After 24 h, the cells were treated with 1, 5, 10, 20, 50, 100, 150 and 200 µM of hydrogen peroxide and were kept in the incubator at 37°C for another 24 and 48 h. Simultaneously untreated and negative controls (200µL distilled water) were also checked.

Preparation of buffers: Dissolve0.064g of 1-methyl-2-phenylindole into 30 mL of acetonitrile and makeup the volume to 40mL with 10mL of methanol and label it as Reagent 1 (R1). 37% HCl served as the reagent R2.

Preparation of Standard: The standard (S2) was prepared by dissolving 16.5µl of 1,1,3,3-tetramethoxypropane in 10 ml of 20mM TrisHCl (0.242g of TrisHCl in 100ml H2O DW). The solution S2 was diluted to 1:100 in H2O (DW) (i.e. 20 µL of S2 was added to 2 ml of H2O). The final volumes were measured into 2 ml micro centrifuge tubes, as shown in Table. The tubes were vortexes and 300 µL of R2 was added to each tube. The vortexed tubes were further incubated at 45°C for 40 min. After incubation, tubes were cooled in ice and centrifuged at 15000 g for 10 min. All samples were read on a double beam spectrophotometer at 586 nm.

After incubation for 24 h, the treated samples were centrifuged at 3000 g for 20 min, and subsequently the supernatants were collected. In a micro centrifuge tube 1300 µL of R1 was taken. 1 ml of supernatant was diluted 10 times with TrisHCl and 200 µL of this diluted supernatant from each culture was added to 200 µL of distilled water and vortexed. 300 µL of R2 is added to each tube, which was then vortexes and incubated at 45°C for 40 min. After incubation, the tubes were cooled in ice and centrifuged at 15000 g for 10 min at 4°C. All samples were read on a double beam spectrophotometer at 586 nm. The same procedure was adopted for the treated cells for 48 h duration.

Anti-inflammatory activity

Maintenance of Cell lines: RAW264.7 macrophage cells (kindly supplied from NCCS Pune ATCC - 2564) were maintained at 37 ° C in Dulbecco's modified Eagle's media, containing 20% (vol/vol) fetal bovine serum, 1% nonessential amino acids, 1 mM L-glutamine, 10 000 U/ml penicillin and 10 000 µg/ml streptomycin in an atmosphere of CO2:air (1:19) at 90 to 100% relative humidity.

Determination of Anti-Inflammatory Activity (TNF– α) [25]

As a biomarker of inflammatory response, TNF-α production was determined, a

stable TNF-α oxidation product, from RAW264.7 macrophage cells. Cells were plated at a density of 2×105 cells/well in 96-well culture plate and incubated at 37℃ for 3-4 h. Plated cells were treated with lipopolysaccharides (LPS, 1 mg/ml) for stimulation of TNF-α production and the indicated concentrations of each sample, followed 24 h, 48 h and 72 hrs incubation. LPS-stimulated TNF-α production from macrophage cells was measured by the Griess reaction.100 ml of each supernatant was mixed with 100 ml of Griess reagent (1% sulfanilamide in 5% phosphoric acid and0.1% N-1-naphthylethylenediamine di-hydrochloride in distilled water), and the absorbance of the mixture was measured at 540 nm by a micro plate reader.

IL-6 Assay[26]: Bio-Plex cytokine assay for simultaneous quantitation of interleukin IL-6 was employed according to the recommended procedure. In brief, the premixed standards were reconstituted in 0.5 ml of culture medium, generating a stock concentration of 50 000 pg/ml for each cytokine. The assay was performed by using 96-well plate supplied with the assay kit. The plate was shaken for 30 s and then incubated at room temperature for 30 min with low-speed shaking. After incubation and washing, premixed detection antibodies (50 µl) were added to each well. The incubation was stopped after shaking for 10 min at room temperature. After washing three times, resuspend the beads in 125 µl of Bio-Plex assay buffer after washing three times. Read the beads on the Bio-Plex suspension array system, and the data were analyzed by using Bio-Plex Manager™ software.

RESULT: SOD activity in N2a cell lines after confluence. SOD is the enzyme that dismutase's the superoxide anion to H202. During the first 4 days after confluence, SOD activity significantly ($P < 0.05$) increased, remained elevated, and then decreased at 16days. The results of the present study reveal a clear dose dependent increase in the production of MDA upon treatment with hydrogen peroxide. Ion radicals generated during oxidative stress leads to a chain reaction called lipid peroxidation. In the present study, a clear dose dependent and significant increase in the concentration of MDA was obtained following H_2O_2 treatment after 24h, though after 48h of treatment, the mean absorbance values were decreased. This may be due to the reduced short half life of H_2O_2 or due to the internal protection mechanism of the cell. The results obtained from the present study shows that Extracts are capable of suppressing various inflammatory mediators. Extracts mediated suppression of LPS induced TNF-α. These cytokines are proinflammatory mediators. It is possible that inhibition of such cytokines by Extracts might regulate the cell mediated inflammatory process.

DISCUSSION

The Production or removal by the antioxidant system will affect the Intracellular concentration of Reactive Oxygen Species. Cells contain a large number of antioxidants to Prevent or repair the damage caused by Free radicals. Three of the primary antioxidant enzymes contained in mammalian cells that are thought to be necessary for life in all oxygen metabolizing cells[6] are superoxide dismutase (SOD), catalase, and a substrate specific peroxidase, glutathione peroxidase (GPx).Superoxide radical converted into hydrogen peroxide and molecular oxygen (O_2) by SOD, while the catalase and peroxidase convert hydrogen peroxide into water and in the case of catalase to oxygen and water. The net result is that two potentially harmful species, superoxide and hydrogen peroxide, are converted to water [27].

S.No	Standard (µM)	Standard (µl)	Reagent (µl)	Distilled Water
1	0	0	1300	400
	2.5	50	1300	350
	5	100	1300	300
	10	200	1300	200
	20	400	1300	0

Table 1: Amount of reagents and standards used for preparation of standard graph

***Cucurbit maxima* Seeds Petroleum Ether Extract:**

S.No	Sample Name	Incubation	IU/Mg/Protein
1	Cucurbit maxima seeds	Day 1	8.3±1.66
		Day 4	10.7±0.85
		Day 7	8.1±1.25
		Day 10	8.1±0.47
		Day 16	6.9±1.89

Table 2: Superoxide Dismutase Activity of *Cucurbit maxima* seeds

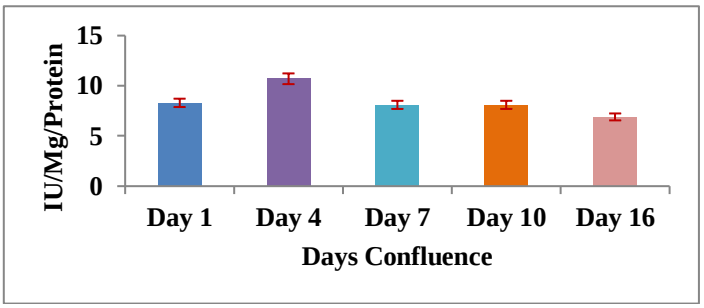

Figure 1: Superoxide dismutase activity of *Cucurbita maxima* seeds as a function of time after confluence in differentiated N2a Cell lines. SOD activity was determined by the inhibition of the oxidation of NADH by mercaptoethanol, measured spectrophotometrically.

Fingolimod:

S.No	Sample Name	Incubation	IU/Mg/Protein
4	Fingolimod	Day 1	9.5±1.32
		Day 4	13.4±1.29
		Day 7	11.2±1.12
		Day 10	11.2±0.89
		Day 16	8.5±0.95

Table 3: Superoxide Dismutase Activity of Fingolimod (Standard)

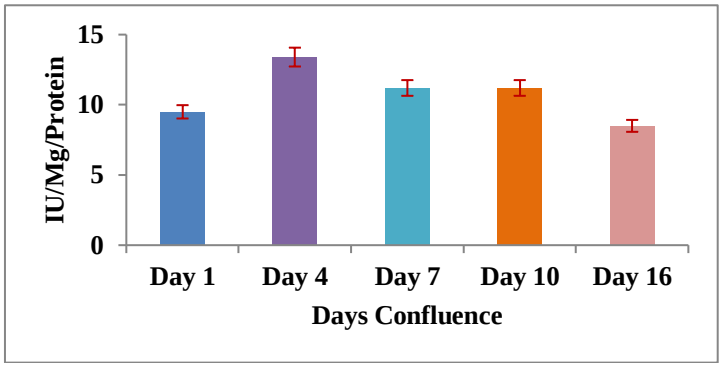

Figure 2: Superoxide dismutase activity of Fingolimod as a function of time after confluence in differentiated N2a Cell lines. SOD activity was determined by the inhibition of the oxidation of NADH by mercaptoethanol, measured spectrophotometrically.

Estimation of MDA by Standard Graph:

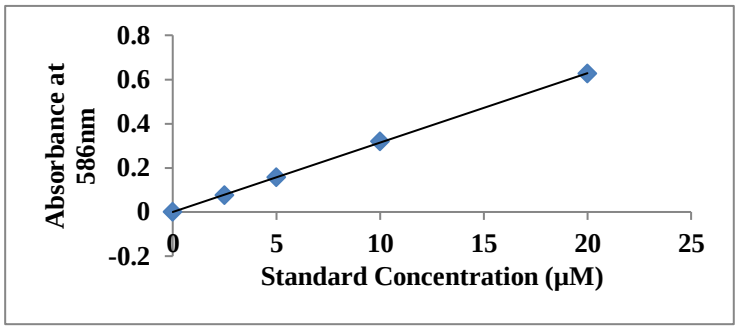

Figure 3: Standard graph for the estimation of MDA

***Cucurbita maxima* seeds petroleum ether extract**

S.No	Sample Name	Concentration (µM)	Absorbance at 586nm (24hours)	Absorbance at 586nm (48hours)
5	*Cucurbita maxima* seeds petroleum ether extract	1	0.0172±0.006	0.0095±0.007
		5	0.0286±0.007	0.0152±0.005
		10	0.0458±0.013	0.0256±0.001
		20	0.0643±0.009	0.0389±0.005
		50	0.0892±0.018	0.0595±0.012
		100	0.1652±0.005	0.0768±0.018
		150	0.2985±0.004	0.1582±0.009
		200	0.4568±0.019	0.2856±0.001
		Untreated	0.0011±0.003	0.001±0.004
		Negative	0.0013±0.008	0.0011±0.015

Table 4: Lipid Peroxidation of *Cucurbita maxima* seeds petroleum ether extract after 24 and 48 hours treatment

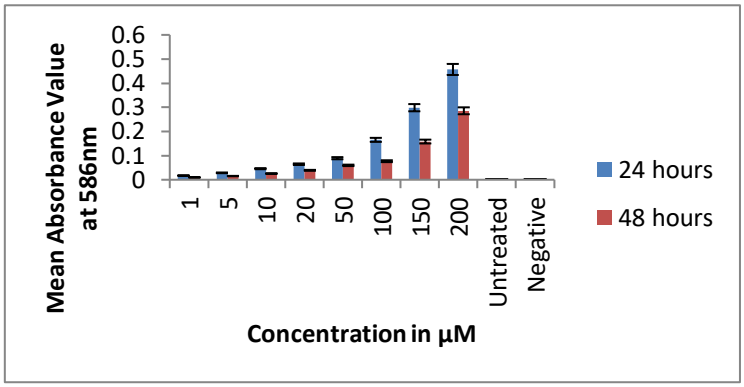

Figure 4: Lipid peroxidation after 24 and 48 h treatment of the H_2O_2 on *Cucurbita maxima* seeds petroleum ether extract Fingolimod

S.No	Sample Name	Concentration (µM)	Absorbance at 586nm (24hours)	Absorbance at 586nm (48hours)
8	Fingolimod	1	0.0189±0.008	0.0042±0.005
		5	0.0286±0.015	0.0085±0.019
		10	0.0492±0.009	0.0156±0.014
		20	0.0685±0.025	0.0198±0.025
		50	0.0952±0.030	0.0358±0.006
		100	0.1064±0.004	0.0589±0.018
		150	0.2952±0.028	0.0958±0.009
		200	0.4958±0.018	0.1482±0.023
		Untreated	0.0011±0.014	0.001±0.011
		Negative	0.0013±0.009	0.0011±0.004

Table 5: Lipid Peroxidation of Fingolimod after 24 and 48 hours treatment

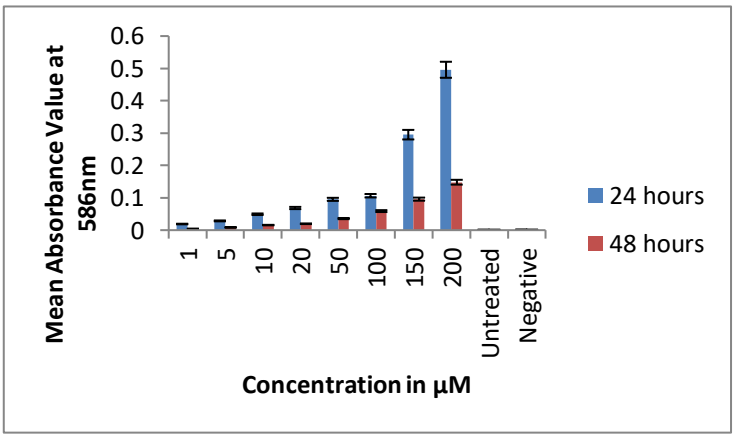

Figure 5: Lipid peroxidation after 24 and 48 h treatment of the H_2O_2 on Fingolimod
Anti-inflammatory activity result

***Cucurbit Maxima* Seeds Petroleum Ether Extract**

Concentration	Absorbance at 540nm			Average	% TNF-α inhibition
Cell Control	0.85	0.852	0.853	0.851	35.585±0.004
Extract (5%) + LPS	1.577	1.579	1.581	1.579	77.20±0.008
Extract (2.5%)+ LPS	1.388	1.39	1.392	1.39	67.94±0.006
LPS Control	2.365	2.365	2.366	2.365	100±0.014
Extract (5%)	0.964	0.966	0.968	0.966	47.15±0.002

Table 6: TNF - α bioassay Of Culture Media of *Cucurbit Maxima* Seeds Petroleum Ether Extract

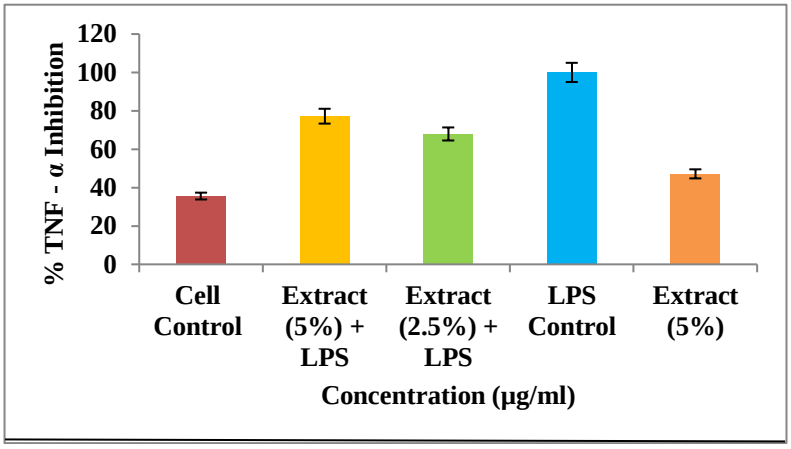

Figure 6: TNF - α bioassay of culture media of *Cucurbit maxima* seeds petroleum ether extract

Fingolimod:

Concentration	Absorbance at 540nm			Average	% TNF-α Inhibition
Cell Control	0.85	0.852	0.853	0.851	35.585±0.018
Extract (5%) + LPS	1.245	1.247	1.251	1.247	51.43±0.014
Extract (2.5%) + LPS	1.209	1.21	1.24	1.219	49.86±0.015
LPS Control	2.365	2.365	2.366	2.365	100±0.006
Extract (5%)	0.989	0.991	0.993	0.991	40.54±0.005

Table 7: TNF - α bioassay of culture media of Fingolimod

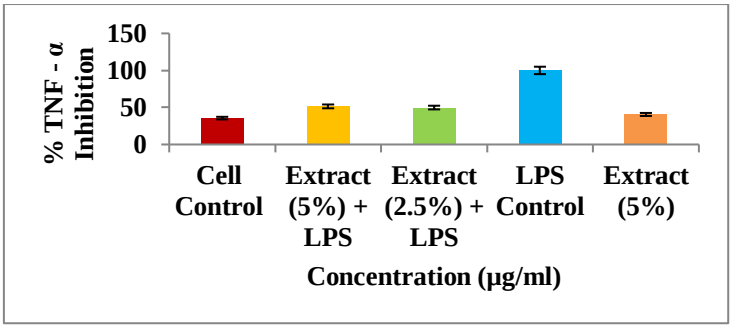

Figure 7: TNF - α bioassay of culture media of Fingolimod

***Cucurbit Maxima* Seeds Petroleum Ether Extract:**

Concentration	Absorbance at 540nm			Average	IL-6(pg/ml)
Cell Control	0.519	0.521	0.523	0.521	26.04±0.019
Extract (5%) + LPS	1.496	1.498	1.499	1.497	75.01±0.046
Extract (2.5%)+ LPS	1.315	1.317	1.319	1.317	65.98±0.058
LPS Control	1.995	1.996	1.995	1.995	100±0.025
Extract (5%)	1.114	1.116	1.118	1.116	55.89±0.051

Table 8: IL – 6 Assay of culture media of *Cucurbita Maxima* Seeds Petroleum Ether Extract

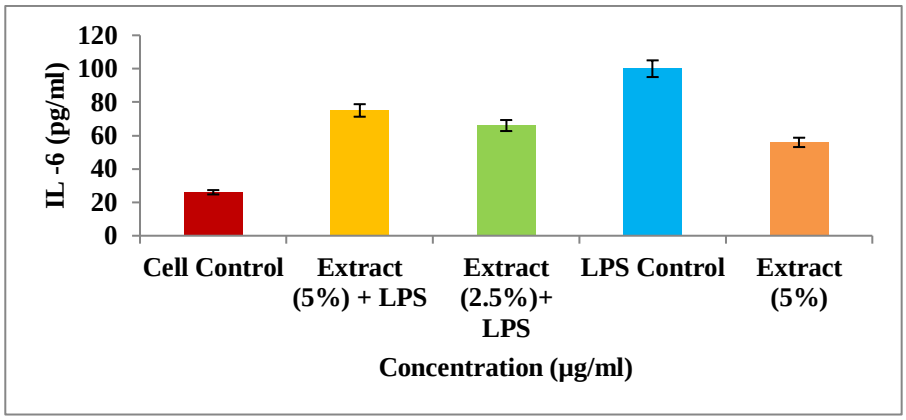

Figure 8: IL – 6 Assay of culture media of *Cucurbita Maxima* Seeds Petroleum Ether Extract

Fingolimod:

Concentration	Absorbance at 540nm			Average	IL-6(pg/ml)
Cell Control	0.519	0.521	0.523	0.521	26.04±0.019
Extract (5%) + LPS	1.264	1.266	1.267	1.265	63.37±0.044
Extract (2.5%)+ LPS	1.099	1.101	1.103	1.101	55.14±0.075
LPS Control	1.995	1.996	1.995	1.995	100±0.025
Extract (5%)	0.842	0.844	0.845	0.843	42.19±0.064

Table 9: IL – 6 Assay of culture media of Fingolimod

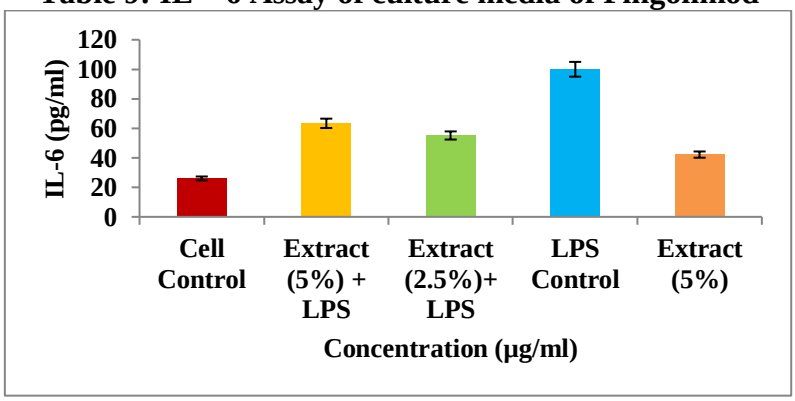

Figure 9: IL – 6 Assay of culture media of Fingolimod

The results of the present study reveal a clear dose dependent increase in the production of MDA upon treatment with hydrogen peroxide. Ion radicals generated during oxidative stress leads to a chain reaction called lipid peroxidation. In the present study, a clear dose dependent and significant increase in the concentration of MDA was obtained following H2O2 treatment after 24h, though after 48h of treatment, the mean absorbance values were decreased. This may be due to the reduced short half life of H2O2 or due to the internal protection mechanism of the cell. The results obtained from the present study shows that Extracts are capable of suppressing various inflammatory mediators. Extracts mediated suppression of LPS induced TNF-α. These cytokines are proinflammatory mediators. It is possible that inhibition of such cytokines by Extracts might regulate the cell mediated inflammatory process.

SOD is an enzyme which dismutase's the superoxide anion to H_20_2. In this SOD activity in N2a cell lines during the first 4 days after confluence, SOD activity significantly ($P < 0.05$) increased, remained elevated, and then decreased at 16days. Lipid peroxidation or reaction of oxygen with unsaturated lipids produces a wide variety of oxidation products. Lipid hydro peroxides (LOOH) are the main Primary products of Lipid peroxidation. Among the many different aldehydes which can be formed as secondary products during lipid peroxidation[28], malondialdehyde (MDA) appears to be the most mutagenic product of lipid peroxidation. The results of the present study reveal a clear dose dependent increase in the production of MDA upon treatment with hydrogen peroxide. Ion radicals generated during oxidative stress leads to a chain reaction called lipid peroxidation. In the present study, a clear dose dependent and significant increase in the concentration of MDA was obtained following H_2O_2 treatment after 24h, though after 48h of treatment, the mean absorbance values were decreased. This may be due to the reduced short half life of H_2O_2 or due to the internal protection mechanism of the cell. In the inflammation of brain proliferation of microglia occurs and is migrated in the inflammatory lesion.[29]. Macrophages cross the blood brain barrier and enter in to the brain are CNS resident microglia and CNS-infiltrating peripheral macrophages.[30]. These two macrophages play a major role and support the neurons. The CNS CNS-infiltrating peripheral macrophages may causes neuro degeneration in Alzheimer's disease but the neurons are protected by activated microglia removing the toxic materials and cellular debris [31].In neuro degeneration the role of macrophages and microglia were demonstrated and clearly understood by using certain cellular models of cell type systemic effects and differences between them [32]. The results obtained from the present study shows that Extract are capable of suppressing various inflammatory mediators. Extract mediated suppression of LPS induced TNF-α. These cytokines are proinflammatory mediators. It is possible that inhibition of such cytokines by Extract might regulate the cell mediated inflammatory process while compared with standard.

CONCLUSION

The findings from this study suggest that pumpkin seed extract has antioxidant and anti inflammatory effect and is capable of down regulating proinflammatory response in cellular models. Our findings provide further evidence of neuroprotective potential of pumpkin seed extract during neuro inflammation.

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Conflict of Interest: -None-

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