African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper　　　　　　　　　　　　　　　　　**Open Access**

EXPLORATION OF *INDIGOFERA PROSTRATE* AGAINST OXIDATIVE STRESS AND EVALUATION FOR NEUROPROTECTION IN CHEMICALLY INDUCED NEUROTOXIC RATS

Neelam Injeti[1], Kumar Shiva Gubbiyappa[2*]

[1]Research Scholar, GITAM Deemed to be University, Gandhi Nagar, Rushikonda, Visakhapatnam, Andhra Pradesh, India, 530045

[2]GITAM School of Pharmacy, GITAM Deemed to be University, Rudraram, Patancheru Mandal, Hyderabad, Telangana, India, 502329

Corresponding address:

*Address for correspondence:
Dr. G. Shiva Kumar,
Professor, Principal Gitam School of Pharmacy, GITAM Deemed to be University, Rudraram, Hyderabad campus, Telangana - 502329.India
Email: sgubbiya@gitam.edu
Contact no. : +91-7997490070

Abstract

Objective: The goal of the current study was to assess the effect of *Indigofera prostrate* on memory and spatial learning induced by a Bisphenol A (BPA) in experimental rats and how *I. prostrate* helps to mitigate these effects.

Methods: The methanolic seed extract of *I. prostrate* was prepared, and subjected to phytochemical constituents' analysis. Wistar Albino rats weighing 150 + grams were divided into 5 groups –Control, positive control, standard drug and test drug treated groups (200 & 400 mg/kg). *In vitro* and *in vivo* anti-oxidant methods were employed to evaluate the anti-oxidant property of *I. prostrate*. The behavioural paradigm of animals was analysed by using models like elevated plus maze, Y maze and actophotometer. At the end of the experiment, the brain tissue was isolated and evaluated for anti-oxidant enzymes.

Results: The *in vitro* anti-oxidant enzyme activity was evaluated by DPPH, ABTS and metal chelating assay and the free radical scavenging property of the test extract was proven with the IC_{50} values and the % inhibition. There was an improved behavioural pattern with the treatment of the test drug in the above-mentioned models, all the values were found to be significant (**p<0.001). Furthermore, when the oxidative stress and anti-oxidant level was assessed by biochemical tests like MDA (Malondialdehyde), SOD (Superoxide dismutase) and catalase, there was an increased levels of anti-oxidant enzymes in a significant manner (**p<0.001). **Conclusions:** It was concluded that *I. prostrate* showed a remarkable effect on behavioural pattern, exhibited anti-oxidant effect on oxidative stress induced by BPA.

Key words: Anti-oxidative enzymes, Bisphenol A*, Indigofera prostrate,* oxidative stress, phytocomponents

1. Introduction

Endogenous increased intracellular reactive oxygen species (ROS) that are deleterious to lipids, proteins, and DNA tend to end up with oxidative stress[1]. A plethora of diseases have been linked to oxidative stress. Elevated ROS, however, also serve as signalling molecules in redox biology, which preserves physiological processes. As a consequence of aerobic metabolism and defence mechanisms free radicals are generated normally in the biological system, these free radicals form as a subset of ROS[2]. There are various reasons of creation of free radicals such as radiation, smoke and environmental hazards. It was identified that ROS has positive effects such as supporting immune system, promotes apoptosis and inhibits carcinogenesis[3]. However, it also causes harmful effects like, carcinogenesis, heart-related diseases, brain-related disorders and neurotoxicity. Mechanism-based therapeutics for oxidative stress is sceptical, hence thorough investigation needed to analyse the pharmacodynamic characteristics of a drug so that it gains therapeutic efficacy[4]. Pertaining to pathophysiological aspects of neurogenerative disorders, there is always an intricate interaction of various general and disease-specific variables in the neuronal degeneration process. Key elements in the genesis of neuropsychiatric disorders include an increase in oxidative and nitro-oxidative stress as well as a decrease in the brain's antioxidant capacity[5]. To combat the oxidative stress and subsequently prevent the neurodegenerative diseases, anti-oxidants are used adequately both as a part of treatment and also stand as a source of protection for the neuro-based ailments. The effect of anti-oxidants might be because of free radical trapping, thus delivering neuroprotection. Suppression of oxidative stress and neuroinflammation by anti-oxidants could find a way for the emergence of novel therapies, also motivates research in this aspect so that a promising treatment and prevention could be possible[6].

Since ancient times, herbal medicine and its active components have been a reliable source of healthcare. Concerns have been expressed about the need to investigate the best source of medicine using contemporary research, technology, and concepts due to the rise in diseases, treatment resistance, and desire for medications with minimized side effects[7]. Plants are the finest option for innovative medicinal sources that multinational pharmaceutical corporations are searching for. Over the past few decades, herbal products have become more and more popular all over the world. These days, preclinical and clinical investigations favour herbal medicines with well-defined components over crude forms because of their consistency[8].A lifelong dependency on medication becomes mandatory in chronic ailments and the majority of them belong to neuro-related ailments. Hence, it is always better to choose such a medication which is safe, with minimum side effects and used for long time. Long-term usage of medicinal plants in conventional medicine, including folk remedies, has been shown to be a reliable source of active chemicals and is usually regarded as safe and effective in preventing a wide range of ailments[9].

Endocrine-disrupting chemicals (EDCs) such as bisphenol-A (BPA) are widely used in dental sealants, plastics, thermal sheets, food packaging, and other products. Although BPA is widely distributed in the air, soil, water, and dust, food is the main way that people are exposed to it. Additionally, metabolites are found in over 90% of urine samples from general people across

various nations and regions. An epidemiologic data has demonstrated a link between BPA exposure and the risk of several illnesses, such as autoimmune or inflammatory response-related diseases, brain and nervous system abnormalities[10].

The blood-brain barrier is easily crossed by BPA. The detrimental effects of BPA on the neurological system are widely known. It acts by affecting the hippocampus in the limbic system, thus causing deficit in learning and memory, along with the spatial navigation. Besides this effect, it results in a range of behavioural alterations linked to increased aggression, hyper reactivity, learning impairments, and increased drug dependency[11]. A study on rats revealed that at low dose there was no effect on the F1 and F2 developmental landmarks, open-field behaviour, cognitive tasks and brain weights. However, few research studies explored the learning and memory deficits in SD rats during their embryonic development on exposure to the BPA administration, but still the mechanism needs an elucidation. A broad category of naturally occurring substances having the ability to reduce or scavenge radicals is referred as plant-derived antioxidants. Pharmacologists, doctors, and scientists alike pay close attention to these substances because of their powerful medicinal and preventative properties[12].

Indigofera prostrate belongs to Fabaceae family, has spread branches. Indigofera is a broad genus that has shown unique features that make it a promising candidate for perennial crops. Different species differ greatly from one another, this variation include differences in fruit type, flowering shape, and pericarp thickness[13]. The present study included *in vitro* anti-oxidant and neuroprotective activity of methanolic extraction of *I. prostrate* in experimental rats.

2. Materials and Methods

2.1.Plant Material

From the local place of Tirupati town, Andhra Pradesh, seeds of *Indigofera prostrata* were obtained and were authenticated by Dr. K. Madhava Chetty, Department of Botany.

2.2.Extraction by Maceration

About one kg of fresh seeds of *I. prostrate* were cleaned and shade dried, and grounded, kept 7 days in methanol for maceration with occasional stirring. The content was filtered and dried in a desiccator on 8th day, then used for further analysis.

2.3.Preliminary Phytochemical Analysis

The phytochemical constituents like carbohydrates, amino acids, proteins, and lipids, and secondary metabolites, such as alkaloids, tannins, phenols, flavonoids, saponins, steroids, glycosides, and resins were analyzed in methanolic extract of *I. prostrate* by means of standard procedures[14].

2.4.Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Using a 7890A gas chromatograph system (GCMSQP2010, SHIMADZU) and mass spectrophotometer, fixed with a HP-5 MS fused silica column (5% phenyl methyl siloxane 30.0m ×250μm, film thickness 0.25μm), interfaced with 5675C Inert MSD with Triple-Axis detector was

used. Helium was used as carrier gas with a1.0ml/min flow, temperature of 250°C; interface temperature at 300°C; pressure16.2 psi. Split mode injector of 1µl with split ratio of 1:50, injection temperature of 300 °C was maintained. Temperature in the column was raised to 4 °C per minute after five minutes at 150 V, was maintained up to 250°C, elution time was 37 minutes. Average peak area was compared to total area and relative percentage was calculated[15].

2.5.Identification of Compounds

Database of National Institute of Standards and Technology (NSIT) which had > 62,000 patterns of known compounds was utilized for the interpretation of mass spectrum, and identification of components according to retention indices. The spectra of the unknown components of *I. prostrate* fraction obtained were compared with the standard mass spectra of known components.

2.6.Analysis of MEIP by *In vitro* Antioxidant Assays

2.6.1. DPPH Radical Scavenging Assay

In this assay, to 6 ml of methanolic solution of DPPH (33 mg/l) in a test tube, various concentrations of the plant extract or standard (2 ml) were added, then the reaction mixture was incubated for one hour at 25^0C.Triplicate values of absorbance were measured at 517 nm in a UV-Visible Spectrophotometer. Ascorbic acid was used as standard drug. The % inhibition (I %) was calculated by formula[16].

$$I \% = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \text{ X } 100$$

2.6.2. ABTS Radical Scavenging Activity

ABTS 2 mM (0.0548 gm in 50 ml) and potassium per sulphate70 mM (0.0189 gm in 1ml) were prepared in distilled water. Then, mixed with 200 ml of potassium per sulphate and 50 ml of ABTS, left aside for 2 h. To 0.6 ml of ABTS radical cation and 3.4 ml of phosphate buffer (pH 7.4) were added, various concentrations of plant extract (or) standard were added, and triplet values of absorbance was recorded at 734 nm. Ascorbic acid was used as a standard. The percentage of inhibition (I %) was calculated using following formula[17].

$$I \% = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \text{ X } 100$$

2.6.3. Metal Chelating Assay

In this assay, with continuous shaking, 0.2 ml of 2 mM ferric chloride and 0.4 ml of ferrozine solution were added to various concentrations of extract, and kept aside for 10 minutes at room temperature. Triplicate values of absorbance were recorded at 562 nm. EDTA was used as standard. The percentage inhibition (I %) was calculated using following formula[18].

$$I \% = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \text{ X } 100$$

2.6.4. Acute Toxicity Tests

Wistar Albino rats (200-250gm) of either sex were split in to 8 groups of 6 animals each. A minimal dose of 50mg/kg up to maximum of 2000mg/kg of the test extract was administered orally according to the guidelines. There were no visible symptoms of toxicity or mortality. A dose of $1/5^{th}$ and $1/10^{th}$ of the maximum concentration was chosen for the study[19].

2.7.Animals

Wistar albino Rats weighing 150±5 g were procured from National Institute of Nutrition, Hyderabad, Telangana, India. The animals were kept in a temperature-controlled (22 ± 5 °C) environment with a 12-hour light-dark cycle, 45–55 percent humidity, and a normal pellet meal and water available to them at all times.

2.8.Experimental Protocol

The animals were divided into 5 groups of 6 in each. Group I serve as control was administered with 2 ml of normal saline. Group II was administered with BPA 50 µg/kg for 21 days, Groups III-V was also administered with BPA. Standard drug treated group was group III (piracetam 200mg/kg, i.p) Test drug treated groups were Group IV and V, treated with MEIP (200 and 400 mg/kg orally). After the treatment, behavior performance in rats was analyzed and sacrificed for further estimations.

Table 1. Grouping of animals

Group I	Control
Group II	BPA (50 µg/kg)
Group III	Piracetam (200 mg/kg)
Group IV	MEIP (200 mg/kg)
Group V	MEIP (400 mg/kg)

2.9.Assessment of Neurobehavioral Activity

2.9.1. Elevated Plus Maze Method (EPM)

In this model, anxiogenic property was examined with the animal spending more time in closed spaces. The '+' shaped apparatus had two enclosed and open arms uplifted above the ground. At the junction of the four arms each rat was placed individually and allowed to explore all the four arms. The time spent in both the arms as an indication of anxious nature of an animal was recorded[20].

2.9.2. Y MAZE Spontaneous Alternation Test

Learn and remember was analyzed by 'Y' shaped apparatus with 120° angle. Exploration of the animal was done by placing at the junction for 5 minutes; animal with good remembering ability was analyzed and recorded. The number of entries and alterations were recorded and percentages of alteration were calculated[21].

2.9.3. Actophotometer Test (Locomotor Count)

Using this apparatus, the mental alertness (or) wakefulness of the animals was evaluated. It possesses photoelectric cells and light beam passed through the photo cell was recorded by the animal as it passes, also indicated as a count of locomotor activity[22].

2.10. Estimation of Biochemical Parameters and Enzyme Activity

After the animals were sacrificed, brain tissues were isolated, washed with ice-cold saline at the end of the experiment and preserved at -80°C.

2.10.1. Analysis of Anti-oxidative Enzymes

The brain tissue (hippocampus and cortex) was homogenized in about 10 ml of ice cold 0.1 M phosphate buffer solution (pH 7.4) in a homogenizer. As Malondialdehyde (MDA) remains as a marker for lipid peroxidation, MDA on reaction with thiobarbituric acid reactive substances (TBARS), delivers pink color at an absorbance of 540 nm as described by Ohkawa et al in 1979. By means of method described by Aebi (1983), catalase (CAT) estimation was done, reduced glutathione was measured by method of Ellman[23,24,25].

2.10.2. Estimation of Acetylcholinesterase Activity in Brain Homogenate

With few modifications Ellman et al method was followed. A mixture of 0.1 ml supernatant (obtained from brain homogenate), sodium phosphate buffer (2 ml of 0.1 M at pH 8.0) with 0.1% BSA was prepared, then added 0.1 ml dithio-bis-nitrobenzoic acid (DTNB) and 0.05 ml of acetylthiocholine iodide (AChI). Absorbance at 412 nm was noted using UV-VIS Spectrophotometer. Acetylthiocholine iodide hydrolyzed per min per mg protein in micromoles was considered as the concentration of acetylcholinesterase activity[26].

2.11. Statistical Analysis

Each group (n=6) value was represented in Mean ± SEM. One way ANOVA followed by Dunnett's test was performed to find out the statistical significance. **$P<0.01$ denoted significant statistically.
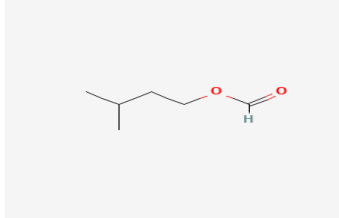
3. Results and Discussion

The preliminary phytochemical screening was analyzed in the methanolic extract of *I. prostrata* (MEIP) as depicted in the Table 2. Table 3 consists of bioactive compounds of MEIP.
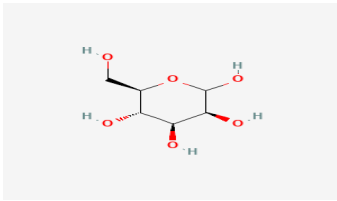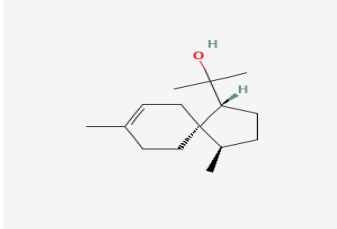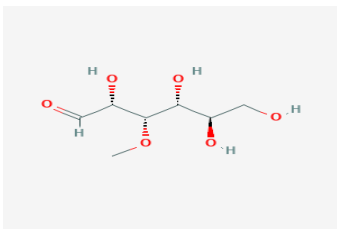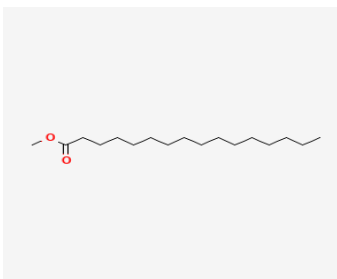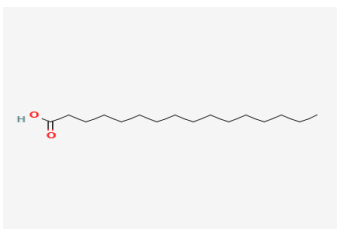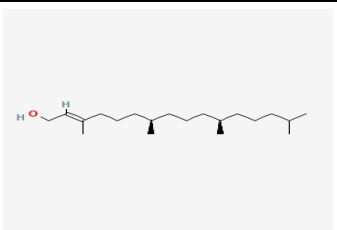
Table 2. Phytochemical constituents of methanolic extract of *Indigofera prostrata* (MEIP)
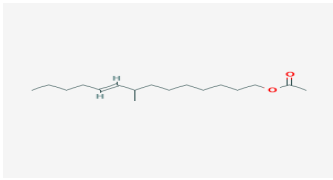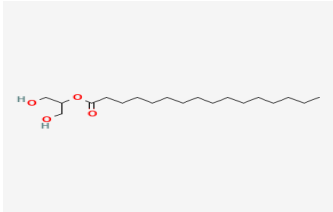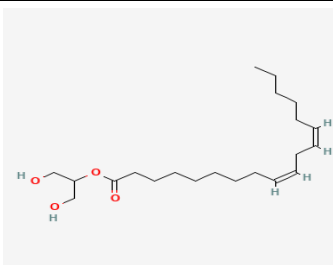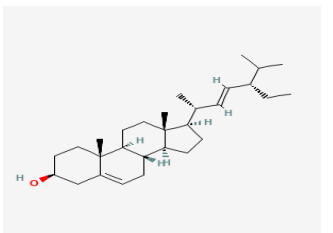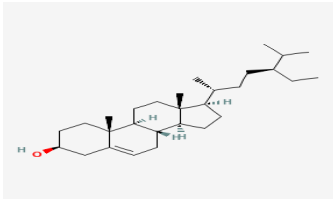
Name of the Phytochemical constituent	MEIP
Carbohydrates	+
Amino acids	+
Proteins	+
Alkaloids	+
Cardiac glycosides	+
Triterpenoids	+
Saponins	+
Flavonoids	+
Phenolic compounds	+
Tannins	+
Steroids	+
Gums	-
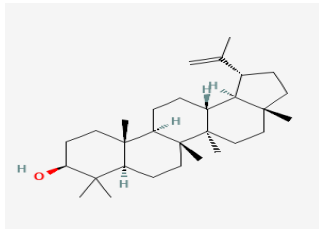
+ means present and - means absent

Table 3. Bioactive compounds of Methanolic extract of *Indigofera prostrate* (MEIP)

S. No	R. Time	Area (%)	Name of the component	Molecular Formula	M.W g/mol	Compound structure
1	6.494 min	19.34	1-Butanol, 3-methyl-, formate	$C_6H_{12}O_2$	116.16	

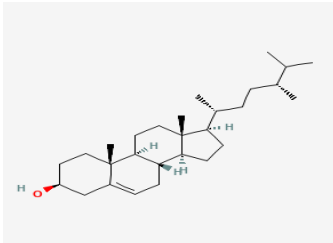| 2	15.246 min	8.06	d-Mannose	$C_6H_{12}O_6$	180.156	
3	19.621 min	0.59	β-Acorenol	$C_{15}H_2O_6$	222.37	
4	24.797 min	7.55	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	194.18	
5	26.091 min	1.56	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.5	
6	27.191 min	6.49	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	
7	rt: 30.623 min	4.70	Phytol	$C_{20}H_{40}O$	296.5	

8	rt: 33.580 min	0.79	E-8-Methyl-9-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268.4	
9	rt: 37.950 min	4.11	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.5	
10	40.719 min	5.43	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{21}H_{38}O_4$	354.5	
11	47.989 min	1.21	Campesterol	$C_{28}H_{48}O$	400.7	
12	48.395 min	6.42	Stigmasterol	$C_{29}H_{48}O$	412.7	
13	49.133 min	12.59	γ-Sitosterol	$C_{29}H_{50}O$	414.7	

| 14 | 50.214 min | 8.35 | Lupeol | $C_{30}H_{50}O$ | 426.7 | |

The chromatogram of GC-MS displayed in Figure 1. Component name, Retention Time (RT), atomic equation, Molecular weight (MW) and Area (%) in MEIP was displayed in Table 3. The following bioactive compounds were present in the GC-MS analysis carried on methanolic fraction of *I. prostrta* was found the following bio active compounds 1-butanol, 3-methyl-, formate, d-mannose, β-acorenol, 3-O-methyl-d-glucose, hexadecanoic acid, methyl ester, n-hexadecanoic acid, phytol, E-8-methyl-9-tetradecen-1-ol acetate, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, 9,12-octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester, campesterol, stigmasterol, γ-sitosterol, and lupeol**.**

<<Insert Figure 1 here>>

Figure 1. Analysis of components in methanolic extract of *Indigofera prostrata*

3.1.Acute Toxicity Tests

These particular studies revealed the innocuous nature of MEIP. At a dose of 2000 mg/kg, neither fatality nor significant adverse effects were detected, which suggests that the plant extract is safe.

3.2.*In vitro* Antioxidant Assays

3.2.1. DPPH Radical Scavenging Assay

The reaction between DPPH and odd electron initiates purple color, with an absorption at 517 nm. An anti-oxidant reacts with DPPH and forms DPPHH, with a lower absorbance, causes decolorization (identified by yellow color), as evident with an increase in number of electrons. The test extract produced 61.24 % of inhibition as compared to the ascorbic acid. The IC_{50} value was recorded as 40.04 µg/ml for the test extract while 27.18 µg/ml was observed for standard drug Ascorbic acid (Table 4, Figure 2).

Table 4. Effect of MEIP on DPPH free radical scavenging assay

Name of the drug	Concentration(µg/ml)	% Inhibition	IC_{50} Value
Methanolic extract of *Indigofera prostrata*	25	30.16±1.32	
	50	35.72±1.54	

	100	41.85±2.65	40.04 µg/ml
	200	50.59±2.42	
	400	61.24±2.43	
Ascorbic acid	25	14.16±1.42	
	50	21.22±1.65	
	100	28.88±2.75	27.18 µg/ml
	200	39.95±2.64	
	400	54.18±2.43	

<<Insert Figure 2 here>>

Figure 2. Free radical scavenging effect of MEIP in DPPH assay

3.2.2. *ABTS Radical Scavenging Assay*

On addition of potassium per sulfate, ABTS got converted to its radical cation. The ABTS radical cation counteracted with anti-oxidant nature of the test extract. During this reaction, the blue clor ABTS radical was converted to colorless neutral form. The IC_{50} values of the MEIP (Methanolic extraction of *I. prostrata)* was found to be 58.01 µg/ml. IC_{50} value for the Vitamin C (Standard) was found to be 49.76 µg/ml, presented in Table 5 & Figure 3.

Table 4. Effect of MEIP on ABTS radical scavenging assay

Name of the Compound	Concentration (µg/ml)	% Inhibition	IC_{50}
Methanolic extract of *Indigofera prostrata*	25	45.25 ±2.43	
	50	52.81±2.65	
	100	59.75±2.62	58.01 µg/ml
	200	71.21±2.76	
	400	91.77±3.87	
Ascorbic acid	25	36.73±2.43	
	50	42.63±3.21	
	100	51.69±2.76	49.76 µg/ml
	200	62.18±2.85	

	400	78.11±2.97

<<Insert figure 3 here>>

Figure 3. Free radical scavenging effect of MEIP in ABTS assay

3.2.3. Metal Chelating Assay

Excess free iron was linked to the production and stimulation of free radicals in biological systems. Metal chelating assay was employed to test the medicinal plant extracts. Different concentrations from 25 – 400 µg/ml of test extract were tested and the % inhibition was recorded, the test concentrations showed a potent chelating property in concentration-dependent manner. The IC_{50} values of the MEIP was found to be 36.16 µg/ml as compared to the standard (32.47 µg/ml), presented in Table 5 & Figure 4.

Table 5. Metal chelation assay of MEIP

Name of the compound	Concentration (µg/ml)	% Inhibition	IC_{50}
MEIP	25	18.64±2.42	
	50	23.19±2.54	
	100	31.48±2.76	36.16 µg/ml
	200	39.09±3.25	
	400	51.21±3.87	
EDTA	25	21.23±1.42	
	50	27.11±1.87	
	100	33.39±2.23	32.47 µg/ml
	200	42.73±1.71	
	400	54.18± 1.23	

<<Insert figure 4 here>>

Figure 4. Metal chelation assay of MEIP

3.3.Evaluation of Test Extract in the Behavioral Paradigm

The locomotor activity of the test extract was evaluated in the Actophotometer. The number of crossings, denoted by locomotor score made by each animal was recorded. Decreased locomotor

score was found in the disease – induced animals, taken as indication of CNS depression. The test drug at the dose of 200 & 400 mg/kg showed a significant (**P<0.01) in the promotion of locomotor score in seconds as displayed in Table 6 & Figure 5.

Table 6. Effect of MEIP on Spontaneous locomotor activity in Actophotometer

Groups	Treatment & Dose	Locomotive score (sec) in days		
		3rd day	5th day	7th day
I	Control	380±3.9	392±1.32	384.33±1.34
II	Disease control	184±2.13**	160±1.05**	142.41±0.24**
III	Standard	164±2.41**	215±0.04**	242.38±0.73**
IV	MEIP 200mg/kg	149±2.19**	181±2.19**	209.32±1.29**
V	MEIP 400mg/kg	162±2.17**	210±2.31**	236.24±1.28**

Values were interpreted in Mean ± SEM; **p<0.01 was considered as significant when the test drug treated groups were compared with the disease control

<<Insert figure 5 here>>

Figure 5. Effect of MEIP on Spontaneous locomotor activity in Actophotometer

In the elevated plus maze, disease induced group animals spent reduced in time in open arm while time spent in closed arm was raised, because of dislike of animal to get explored to open arms. On treatment with the test drug at the dose of 200 and 400 mg/kg, there was an increased time spent in open arm with a declined fear, as compared to the time spent in closed arms. All the values were found to be significant (**P<0.01).

Table 7. Effect of MEIP on anxiogenic behavior in Elevated plus maze

Groups	Treatment& Dose	Time spent in closed arm in sec	Time spent in open arm in sec
1	Normal control	221.6±1.47	20.0±1.01
II	Disease control	146.6±1.67**	53.3±1.34**
III	Standard	127.8±1.46**	81.7±1.9**
IV	MEIP 200mg/kg	131.62±1.34**	61.3±1.44**
V	MEIP 400mg/kg	129.53±1.53**	78.23±2.09**

Values were interpreted in Mean ± SEM; **p<0.01 was considered as significant when the test drug treated groups were compared with the disease control

<<Insert figure 6 here>>

Figure 6: Effect of MEIP on anxiogenic behavior in Elevated plus maze

In the Y maze test, each rat was placed individually and the entry of rat in the three different arms was noted which was called as spontaneous alternation (Table 8). The percentage of alternation was calculated using the below formula;

$$Percentage\ of\ alteration = \frac{Spontaneous\ alteration}{Total\ number\ of\ arm\ entries - 2} X\ 100$$

During the recording of alternations each time, each rat was placed for 5 minutes after a thorough cleaning with 5% ethanol to avoid any bad odor.

Table 8. Effect of MEIP on spatial learning in Y maze

S. No	Treatment	% of alternations
1	Normal control	13.5±2.86
2	Disease control	5.1±0.59
3	Standard	1.62±0.24**
4	MEIP 200mg/Kg	2.13±0.72**
5	MEIP 400mg/Kg	3.41±1.26*

Values were interpreted in Mean ± SEM; **p<0.01 was considered as significant when the test drug treated groups were compared with the disease control

Acetyl Cholinesterase (AchE) enzyme in brain homogenate of the disease induced animals exhibited increase in the concentrations significantly (**p<0.001). With the treatment of test extract at 200 and 400 mg/kg produced decreased levels significantly, thus overcome the damage by free radicals (Table 9).

Table 9. Effect of MEIP on Acetyl Cholinesterase enzyme activity in brain

Treatment & Dose	Acetyl cholinesterase enzyme activity in brain (moles x10^{-6}/min/ g of tissue)
Normal control (Distilled Water)	30.31 ± 0.76

Disease control	43.56 ± 0.612
Standard control	14.23 ± 0.542**
MEIP 200mg/kg	21.16 ±0.428**
MEIP 400mg/kg	15.72 ±0.577**

Values were interpreted in Mean ± SEM; **p<0.01 was considered as significant when the test drug treated groups were compared with the disease control

In the determination of anti-oxidant enzymes in the brain homogenate, it was noticed that there was an increase in the Malondialdehyde (MDA) levels, which is considered as a lipid peroxidation marker in disease induced animals. Also, the levels of reduced glutathione and catalase enzyme were reduced in the positive control rats. After the treatment with the test drug at the dose of 200 and 400 mg/kg, there was a decrease in the MDA levels, rise in reduced glutathione and catalase enzymes significantly (**p<0.01, Table 10 & Figure 7).

Table 10. Effect of MEIP on Anti-oxidant enzymes - Lipid peroxidation, reduced glutathione and Catalase

Groups and Treatment	MDA (µm/mg tissue)	Reduced glutathione (µm of GSH/mg tissue)	Catalase (units/mg protein)
Normal control	2.853±0.97	4.22±0.54	6.72±0.49
Disease control	5.233±0.24	2.90±0.13	3.45±0.31
Standard control	2.965±0.22**	3.61±0.45**	5.63±0.15**
MEIP 200mg/kg	3.674±0.63**	3.34±0.14**	6.59±0.54**
MEIP 400mg/kg	3.112±0.35**	3.43±0.45**	7.62±0.74**

Values were interpreted in Mean ± SEM; **p<0.01 was considered as significant when the test drug treated groups were compared with the disease control

<<Insert figure 7 here>>

Figure 7. Effect of MEIP on anti-oxidant enzyme levels in brain homogenate

4. Discussion

The process of neurodegeneration is connected to both brain aging and neuropathological disorders. Brain pathology, namely neurodegenerative and cerebrovascular diseases, is recognized as a major global cause of death, accounting for 8% of all deaths[27]. In the twenty-first century, cognitive dysfunction is considered as a significant health issue with a number of neuropsychiatric and neurodegenerative disorders, including depression, schizophrenia, dementia from Alzheimer's

disease (AD), cerebrovascular impairment, parkinsonism, and others, can have a severely debilitating effect on one's ability to function[28]. There exist few techniques and corresponding mechanisms that safeguard and rescue central nervous system (CNS) from harmful aspects such as neuropathological conditions and aging of brain. There is a dissatisfaction in an individual and also family who are suffering from deficiency of alternative therapeutic options[29]. This drives investigations into the causes of neuronal death and the development of novel drugs to regulate it.

It was also noticeable that once neurodegenerative diseases develop, they last long and permanent. There are currently no effective treatments for neurodegenerative illnesses that both assist the body's afflicted parts repair and recover from ongoing neurodegeneration. Therapeutic herbs in Indian system of medicine have demonstrated an encouraging and promising neuro-psycho-pharmacological activity. Long term use is acceptable as the traditional medicines possess minimum side effects and drug-drug interactions[30]. Correspondingly, many medicinal plants have unique therapeutic effects without adding nourishment to the diet and can be utilized either temporarily or permanently to treat a variety of health issues. Hence, might be a reduced risk for few neuro-related diseases that cause neuronal dysfunction for those who include and consume higher consumption of fruits and vegetables. While the exact mode of action of herbal medicines is still unknown, many of them have been demonstrated to exhibit anti-oxidant and/or anti-inflammatory properties[31].

In the current study, the focus was on the anti-oxidant activity exhibited by herbal drug oxidative stress stands as one of the contributing factors in the etiopathogenesis of both neurodegenerative and neurological diseases. As a consequence of oxidative stress, neuro-inflammation precedes resulting from neuroglial activation at the level of neurons, microglial cells, and astrocytes. Antioxidants derived from phytochemicals may play both neurodegenerative (by decreasing the loss of neurons) and neuroprotective (by preventing apoptosis) functions by minimizing or reversing cellular damage[32].

BPA is an endocrine disrupter, presents a structural similarity with human oestrogen, alters learning and memory, as it crosses blood brain barrier causes cognitive impairment and aggressiveness. In the present study, BPA was administered to the experimental rats, and were investigated by treatment with the herbal drug, also aided to prove the efficacy of the test drug[33]. The toxic effects of BPA lead to decreased expression of NMDA receptors. In the assessment of anti-oxidant effect of test extract, the test drug was proven to scavenge the free radicals thus attained antioxidative potential[34,35].

In the behavioural paradigm, the anxiogenic behaviour and locomotor activity of animals was reduced in test drug treated rats, as evident with the screening in Actophotometer, elevated plus maze and Y maze respectively. In the evaluation of anti-oxidant enzymes by *in vivo* techniques, the levels of MDA, catalase and SOD of test extract presented efficiency in scavenging the amplified ROS generated in brain tissue. Thus, the involvement of oxidative stress in BPA induced cognitive impairment was assessed, in evaluation of levels of MDA, catalase and SOD in the brain. In the stress condition, increased levels of MDA, which subsequently lead to remarkable increased

LPO levels, was observed in the BPA intoxicated rats as compared to control. This aspect was consistent with several studies that showed increased production of reactive oxygen species (ROS) in the brain and lower levels of endogenous antioxidants in the liver and epididymal sperm upon exposure to BPA[36]. On treatment with the test drug, the test drug produced alleviating effect on the oxidative stress as proven by the levels of anti-oxidant enzymes in the brain tissue. Thus, MEIP acted as a potent anti-oxidant agent, regulating the level of endogenous antioxidants, which are usually depleted as a result of aggravated oxidative stress. The anti-oxidant effect of the methanolic extract of *I. prostrate* was due to the presence of flavonoids and phenolic compounds, also due to the functional groups, configuration and hydroxyl groups that was attributed to the free radical scavenging property.

5. Conclusion

According to the study, giving *I. Prostrate* helped to improve the behavioural abnormalities brought on by BPA. In addition, treatment with *I. prostrate* may have upregulated NMDA receptors in the hippocampal region and demonstrated antioxidant properties while enhancing the brain's natural antioxidant enzyme levels. On basis of these, *I. prostrate* dramatically reduced BPA-induced oxidative stress, which may have therapeutic benefits for cognitive dysfunction, one of the very common symptoms of several neurodegenerative illnesses.

6. Conflicts of Interest

No conflict of interest.

7. Author Contributions

N.I conducted the investigation, collected data, and wrote the manuscript following statistical analysis. KSG helped develop the topic, design, supervise, correct, and approve the text.

8. Reference

1. Rao PS, Mohan GK. *In vitro* alpha-amylase inhibition and *in vivo* antioxidant potential of *Momordica dioica* seeds in streptozotocin-induced oxidative stress in diabetic rats. Saudi J Biol Sci. 2017;24(6):1262-1267.

2. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010;4(8):118-126.

3. Kovacs GG. Current concepts of neurodegenerative diseases. Eur Med J Neurol. 2014; 1:78-86.

4. Baquero M, MartinN. Depressive symptoms in neurodegenerative diseases. World J Clin Cases. 2015; 3:682–93.

5. Johannes Brettschneider J, Tredici KD, Lee VMY, Trojanowski J Q. Spreading of pathology in neurodegenerative diseases: a focus on human studies. Nat Rev Neurosci. 2015;16:109–20.

6. Yu Tang. Differential roles of M1 and M2 microglia in neurodegenerative diseases. Mol Neurobiol. 2016; 53:1181–94.

7. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol. 2014;4:177.

8. Singh N, Pandey BR, Verma P. An overview of phytotherapeutic approach in prevention and treatment of Alzheimer's syndrome and dementia. Int J Pharm Sci Drug Res. 2011; 3:162-72.

9. Auerbach BJ, Reynolds SJ, Lamorde M, Merry C, Kukunda-Byobona C, Ocama P, Semeere AS, Ndyanabo A, Boaz I, Kiggundu V, Nalugoda F, Gray RH, Wawer MJ, Thomas DL, Kirk GD, Quinn TC, Stabinski L; Rakai Health Sciences Program. Traditional herbal medicine use associated with liver fibrosis in rural Rakai, Uganda. PLoS One. 2012;7(11):e41737.

10. Fromme H, Küchler T, Otto T, Pilz K, Müller J, Wenzel A. Occurrence of phthalates and bisphenol A and F in the environment. Water Res. 2002;36(6):1429–38.

11. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). Reprod Toxicol. 2007;24(2):139–77.

12. Karimi A, Majlesi M, Rafieian-Kopaei M. Herbal versus synthetic drugs; beliefs and facts. J Nephropharmacol. 2015;4(1):27-30.

13. Elise G, Isabelle G, Jacqueline S, Michel F, Anne GB. A review of traditional uses, phytochemistry and pharmacology of the genus Indigofera. J Ethnopharmacol. 2020; 253.

14. Elezabeth D.V., Subramanian A. Identification of phytochemical constituents and antimicrobial activity of *Indigofera suffruticosa* leaves. Int. J. Curr. Biotechnol. 2013;1:6–10.

15. Pool WG, Leeuw JW, van de Graaf BJ. Automated Extraction of Pure Mass Spectra from Gas Chromatographic / Mass Spectrometric Data. Mass Spectrom. 1997;32:438-443.

16. Oktay M, Gülçin I, Küfrevioglu OI. Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. LWT-Food Sci Technol. 2003;36:263–71.

17. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39(1):44–84

18. Muthuvel R, Venkataraman P, Krishnamoorthy G, Gunadharini D, Kanagaraj P, Stanley AJ, et al. Antioxidant effect of ascorbic acid on PCB (Aroclor 1254) induced oxidative stress in hypothalamus of albino rats. Clin Chim Acta. 2006;365(1–2):297–303

19. Ghosh M, N. 1984. Toxicity studies. Fundamentals of experimental pharmacology.

20. Rabbani M, Sajjadi SE, Vaezi A. Evaluation of anxiolytic and sedative effect of essential oil and hydroalcoholic extract of *Ocimum basilicum* L. and chemical composition of its essential oil. Res Pharm Sci. 2015;10(6):535-543.

21. Lezak KR, Missig G, Carlezon WA Jr. Behavioral methods to study anxiety in rodents. Dialogues Clin Neurosci. 2017;19(2):181-191.

22. Saré RM, Lemons A, Smith CB. Behavior Testing in Rodents: Highlighting Potential Confounds Affecting Variability and Reproducibility. Brain Sci. 2021;11(4):522.

23. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95(2):351-8.

24. Aebi H. Catalase *in vitro*. Methods Enzymol. 1984;105:121-6.

25. Komersová A, Komers K, Cegan A. New findings about Ellman's method to determine cholinesterase activity. Z Naturforsch C J Biosci. 2007;62(1-2):150-4.

26. Shetab-Boushehri SV. Ellman's method is still an appropriate method for measurement of cholinesterases activities. EXCLI J. 2018;17:798-799.

27. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem. 1997; 272(33):20313–6.

28. Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov. 2004;3(3):205.

29. RajaSankar S, Manivasagam T, Sankar V, Prakash S, Muthusamy R, Krishnamurti A, et al. Withania somnifera root extract improves catecholamine's and physiological abnormalities seen in a Parkinson's disease model mouse. J Ethnopharmacol. 2009; 125(3):369–73.

30. Wachtel-Galor S, Benzie IFF. Herbal Medicine: An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs. In: Benzie IFF, Wachtel-Galor S, editors. Herbal Medicine: Biomolecular and Clinical Aspects. 2nd edition

31. Balsano C, Alisi A. Antioxidant effects of natural bioactive compounds. Curr Pharm Des. 2009;15(26):3063-73.

32. Karvandi MS, Sheikhzadeh Hesari F, Aref AR, Mahdavi M. The neuroprotective effects of targeting key factors of neuronal cell death in neurodegenerative diseases: The role of ER stress, oxidative stress, and neuroinflammation. Front Cell Neurosci. 2023;17:1105247.

33. Solleiro-Villavicencio H, Rivas-Arancibia S. Effect of Chronic Oxidative Stress on Neuroinflammatory Response Mediated by $CD4^+T$ Cells in Neurodegenerative Diseases. Front Cell Neurosci. 2018;12:114.

34. Kawato S. Endocrine disrupters as disrupters of brain function: a neurosteroid viewpoint. Environ Sci. 2004;11(1):1–14.

35. Inadera H. Neurological effects of bisphenol A and its analogues. Int J Med Sci. 2015;12(12):926.

36. Xu X, Li T, Luo Q, Hong X, Xie L, Tian D. Bisphenol-A rapidly enhanced passive avoidance memory and phosphorylation of NMDA receptor subunits in hippocampus of young rats. Toxicol Appl Pharmacol. 2011;255(2):221–8.

37. Linillos-Pradillo B, Rancan L, Paredes SD, et al. Low Dose of BPA Induces Liver Injury through Oxidative Stress, Inflammation and Apoptosis in Long-Evans Lactating Rats and Its Perinatal Effect on Female PND6 Offspring. Int J Mol Sci. 2023;24(5):4585.