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**Effect of *Indigofera tinctoria* on  $\beta$ -amyloid (25–35) mediated Alzheimer's disease in mice: Relationship to anti-oxidant activity**

## Effect of *Indigofera tinctoria* on $\beta$ -amyloid (25-35) mediated Alzheimer's disease in mice: Relationship to anti-oxidant activity

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### Abstract

The oxidative stress reducing effect of methanol extract of *Indigofera tinctoria* leaves (250 and 500 mg/kg) was investigated on  $\beta$ -amyloid (25-35) peptide-induced Alzheimer's disease in mice. All the anti-oxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) in brain were reduced significantly ( $p < 0.001$ ) in the  $\beta$ -amyloid peptide injected group, whereas lipid peroxidation was increased significantly ( $p < 0.001$ ). The reduced enzyme level were restored significantly ( $p < 0.01$ ;  $p < 0.001$ ) by the administration of extract at the tested dose levels. A significant ( $p < 0.001$ ) reduction in lipid peroxidation was observed in the groups of animals administered with extract. Histopathological sections of the hippocampal region showed the extent of neuronal loss and its restoration upon administration of extract. Treatment with extract at the tested doses moderately prevented the neuronal loss.

### Introduction

Oxidative stress has been implicated as a major cause of neurotoxicity in a number of neurodegenerative disorders including Alzheimer's disease. Oxidative damage in Alzheimer's disease may be a direct result of amyloid beta (AP). Markers of oxidative DNA damage, including mitochondrial DNA damage, have been localized to amyloid plaque affected areas in the Alzheimer's disease brain (Mecocci et al., 1994); the generation of lipid peroxidation products and the lipid peroxidation of membranes is also noted in amyloid plaques (Matsuoka et al., 2001).

The mechanism of AP mediated oxidative stress may be direct or indirect, functionality of mitochondrial electron transport chain (ETC) is particularly susceptible to inhibition by AP, which is a major source of reactive oxygen species (ROS) within the cell and may therefore represent an indirect source of oxidative

stress. Alternatively, AP can cause neurotoxicity by direct production of ROS (Behl et al., 1992), the mechanism which is directly related to biometal dyshomeostasis as evident in the brain of Alzheimer's disease (Lovell et al., 1998). Cell culture studies have provided further support for AP-ROS production as a potential mechanism for AP mediated neurodegeneration (Huang et al., 1999). The constant assault of oxidative stress during the aging process contributes towards neurodegeneration in Alzheimer's disease (Bush, 2003).

*Indigofera tinctoria* Linn (Fabaceae) is an evergreen shrub native to entire India, especially in Southern India (Santapav and Henry, 1994). The entire plant is a stimulant, alternative and purgative. It is used in the treatment of liver and spleen enlargements. Root of the plant is used to cure hepatitis and urinary complications. The dried whole plant is used in phobia, delusion and disturbed mental states (Khare, 2007;



Asolkar et al., 1992; Ambasta, 1994; Chopra et al., 1996).

The leaf extract of the plant is reported to possess hepatoprotective effect (Singh et al., 2001; Singh et al., 2006; Sreepriya et al., 2001), strong hypolipidemic activity (Puri et al., 2007; Narender et al., 2006), anticancer activity and anticonvulsant activity (SaravanaKumar et al., 2009). Phytochemical investigations on the plant revealed the presence of Indican (a glucoside), Indigo, and Indicine, flavanoids such as apigenin, kaempferol, luteolin and quercetin maximum in the leaves and minimum in the roots (Rastogi and Mehrotra, 1995; Khare, 2007; Chatterjee and Prakash, 1992). The leaves contain a number of useful phytoconstituents but not evaluated for its anti-oxidative properties in neurodegenerative disorders. Hence, the work was carried out to explore the anti-oxidative effect of methanol extract of *I. tinctoria* leaves against  $\beta$ -amyloid mediated Alzheimer's disease in mice model.

## Materials and Methods

### Plant material and extraction

*I. tinctoria* leaves was collected from Thirunelveli, Tamil Nadu and was identified and authenticated from Botanical Survey of India, Southern circle, Tamil Nadu. A voucher specimen was deposited at BSI (Voucher No: BSI/ SC/5/23/09-10 Tech-1101). Extract of *I. tinctoria* was obtained by macerating dried, powdered leaves with methanol for 72 hours. The filtrate was reduced to a molten mass by evaporating in vacuum. For experimental study doses of 250 and 500 mg/kg of extract dissolved in distilled water was used.

### Experimental animals

Male Swiss albino mice (20-25 g) were procured from the animal house. The animals were kept at  $25 \pm 2^\circ\text{C}$  in polypropylene cages under standard laboratory conditions. They had free access to pellet diet (Amruth feeds, India) and water *ad libitum*. The study was approved by the Institutional Animal Ethical Committee.

### Chemicals

Amyloid 3-protein Fragment 25-35 was obtained from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals and reagents used in the study were from S. D. Fine Chemicals (Mumbai, India).

### Treatment protocol

The animals were randomly divided into four groups of 10 animals each. Group I received distilled water 1 mL/kg; Group II, the Alzheimer group, received  $\beta$ -amyloid; Group III, treatment group, received  $\beta$ -amyloid and extract (250 mg/kg); Group IV, treatment group, received  $\beta$ -amyloid and extract (500 mg/kg). The extracts were administered to all the animals by oral gavage

once a day for 21 days prior to  $\beta$ -amyloid injection and continued for 7 more days.

### ICV injection of $\beta$ -amyloid (25-35) peptide

Alzheimer disease was induced by intracerebroventricular injection of aged preparation of  $\beta$ -amyloid (25-35) peptide (prepared in normal saline and incubated for 4 days at  $37^\circ\text{C}$ ) to Groups II, III and IV on day 21 after pretreatment with the extract and the drug treatment was continued for another 7 days. Control healthy animals were injected with normal saline by intracerebroventricular injection route. The administration of A $\beta$  25-35 was performed by identifying the bregma point in the skull, each mouse was injected at bregma with a 50  $\mu\text{L}$  Hamilton micro syringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. In brief, the needle was inserted unilaterally 1 mm to the right of the middle point equidistant from each eye slightly angled towards  $45^\circ$  perpendicular to the plane of the skull. Mice exhibited normal behavior within 1 min after injection and the injection volume was 10  $\mu\text{L}$ /animal (Muralidharan et al., 2010).

### Estimation of brain anti-oxidant status

All the animals were sacrificed by cervical dislocation on day 29 post treatment and brains were dissected out. The brains were washed with ice-cold normal saline for biochemical assay. The tissues were homogenized in Tris HCl and then centrifuged for 10 min at  $10,000 \times g$  at  $4^\circ\text{C}$ .

Anti-oxidant enzymes were estimated to detect the level of neuroprotection offered by methanol extract by enhancing their concentrations. The enzymes were estimated calorimetrically by employing standard procedures for superoxide dismutase (SOD) (Marklund and Marklund, 1974), Catalase (CAT) (Aebi, 1974), glutathione peroxidase (GSHx) (Lawrence and Burk, 1976), glutathione reductase (GSHr) (Dobler and Anderson, 1981) and Lipid peroxidation (LPO) (Ohkawa et al., 1979).

### Histopathology

The mice from each group were anesthetized by intraperitoneal injection of thiopentone sodium. The brain was carefully removed without any injury after opening the skull. The collected brain was washed with ice cold normal saline and fixed in 10% formal saline (10 mL of formaldehyde in 90 mL of physiological saline). Paraffin embedded sections were taken 100  $\mu\text{m}$  thickness and processed in alcohol-xylene series and stained with haematoxylin-eosin dye. The sections were examined microscopically for histopathological changes in the hippocampal zone.

### Statistical analysis

Statistical analysis was carried out using GraphPad

Prism version 5.0. Groups of data were compared with one-way ANOVA followed by Dunnett's t-test. Values were considered statistically significant when  $p < 0.05$ .

## Results

There was a significant reduction in the SOD level of the Alzheimer group animals when compared with the control ( $p < 0.001$ ; Table I). Treatment with methanol extract (250 and 500 mg/kg) increased the SOD levels significantly in comparison with Alzheimer group ( $p < 0.01$  and  $p < 0.001$ , respectively). The level of catalase in Alzheimer group was reduced significantly ( $p < 0.001$ ) in comparison with control. Treatment groups exhibited a higher level of CAT in comparison with Alzheimer group ( $p < 0.001$ ).

Lipid peroxidation was found to be increased in brain of Alzheimer group significantly ( $p < 0.001$ ) when compared with control. Lipid peroxidation was reduced in treatment groups significantly ( $p < 0.001$ ). There was a significant reduction in the GSHx level in the Alzheimer group. Treatment with methanol extract increased the GSHx levels significantly in comparison with Alzheimer group ( $p < 0.01$  and  $p < 0.001$  respectively). The GSHr level in the Group II animals were reduced significantly when compared with the Group I animals ( $p < 0.001$ ). Treatment with methanol extract increased the GSHr level.

There was a noticed increase in neuronal degeneration and decrease in the number of neuronal cells in hippocampal region in  $\beta$ -amyloid (25-35) induced group with respect to group I animals. Whereas, treatment with methanol extract exhibited decreased degeneration and improved neuronal configuration than Alzheimer group (Figure 1).

## Discussion

The present study was carried out to investigate the

anti-oxidative effects of methanolic extract of *I. tinctoria* on Ap (25-35) induced neurotoxicity. In our study, there was a significant increase in the MDA content in the TBARS assay in animals treated with Ap indicating increased lipid peroxidation. The LPO reaction is initiated by ROS, e.g. the highly reactive hydroxyl radical. LPO can be especially deleterious because one free radical is able to damage several polyunsaturated fatty acid (PUFA) molecules. Oxidized PUFAs are further degraded to toxic end products including MDA, HNE, acrolein etc, which in part have been shown to evolve neurotoxic action (Neely et al., 1999; Picklo and Montine, 2001). Because of the high concentration of easily oxidizable lipids combined with high oxygen consumption, the brain is particularly vulnerable to oxidative stress and lipid peroxidation. Due to their restricted regeneration capacity, nerve cells require highly efficient protection mechanisms. Chain-braking anti-oxidants, such as the lipophilic anti-oxidants vitamin E are capable of aborting the LPO chain reaction, supported by co-anti-oxidants like vitamin C of ubiquinol (Stocker, 1994).

In our study, administration of Ap decreased the activity of SOD in brain which could result from inactivation of SOD by H<sub>2</sub>O<sub>2</sub>. This result suggested a compensatory response to oxidative stress due to an increase in endogenous H<sub>2</sub>O<sub>2</sub> production. Thus the elevated level of SOD in methanol extract treatment group predicts that *I. tinctoria* may contain free radical scavenging activity, which could exert a beneficial action against the pathological alteration caused by the presence of O<sub>2</sub><sup>-</sup> and OH (Thatcher et al., 2005).

Glutathione (GSH) is the major non-protein thiol anti-oxidant in mammalian cells and is considered the main intracellular redox buffer. One of the most important GSH-dependent detoxifying processes involves glutathione peroxidase (GSHx), which plays a central role in the removal of hydrogen and organic peroxides and leads to the formation of oxidized glutathione but is reduced back to its thiol form (GSH) by the ancillary

Table I

### Effect of methanol extract of *Indigofera tinctoria* on anti-oxidant enzymes

Parameters	Group			
	I	II	III	IV
Lipid peroxidase (nmol MDA/mg protein)	2.3 ± 0.1	3.9 ± 0.2a <sup>c</sup>	2.9 ± 0.1b <sup>c</sup>	2.3 ± 0.1b <sup>c</sup>
Superoxide dismutase (Units/min/mg protein)	7.3 ± 0.6	3.0 ± 0.7a <sup>c</sup>	6.3 ± 0.6b <sup>b</sup>	7.1 ± 0.6b <sup>c</sup>
Catalase (nmol H <sub>2</sub> O <sub>2</sub> decomposed/min/min protein)	60.2 ± 0.6	40.0 ± 0.4a <sup>c</sup>	48.1 ± 0.5b <sup>c</sup>	59.6 ± 0.5b <sup>c</sup>
GSHx (nmol GSH oxidized/mg protein)	113.0 ± 4.7	69.1 ± 4.9a <sup>c</sup>	94.9 ± 4.5b <sup>b</sup>	103.8 ± 4.7b <sup>c</sup>
GSHr (NADPH oxidized/min/mg protein)	7.9 ± 1.0	2.3 ± 0.5a <sup>c</sup>	6.7 ± 0.5b <sup>b</sup>	7.6 ± 0.8b <sup>b</sup>

GSHx, Glutathione peroxidase; GSHr, Glutathione reductase; Each value is mean ± SEM, n=10; aGroup I Vs group II; bGroup II Vs group III and IV; <sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 (One-way ANOVA followed by Dunnett's t-test)

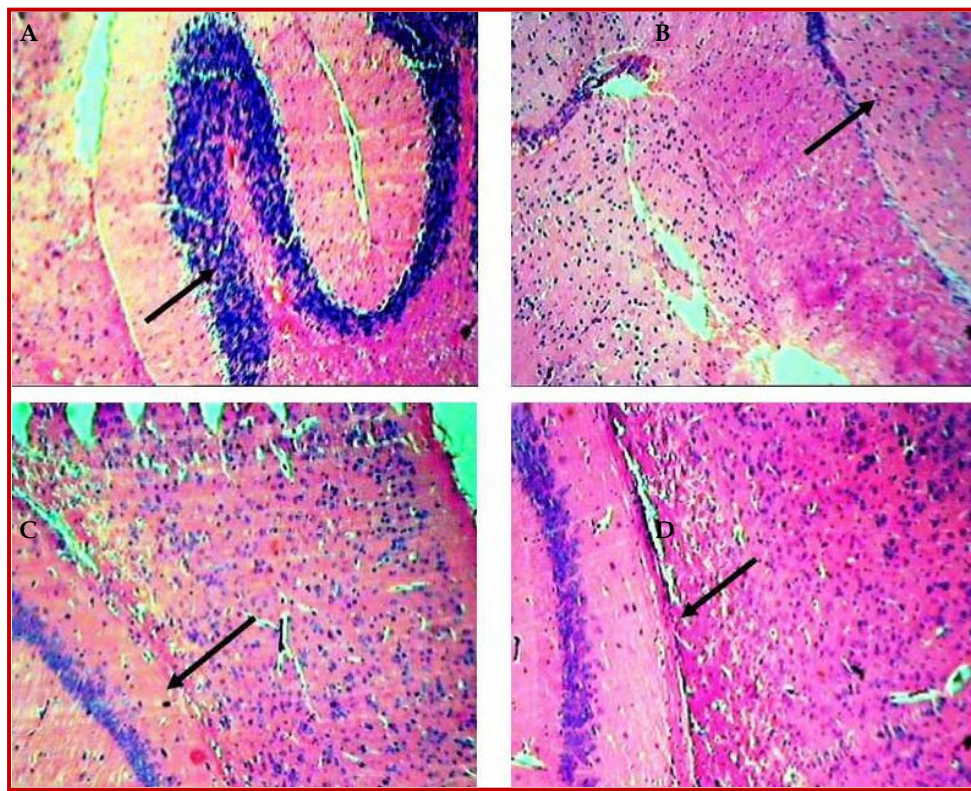


Figure 1: Photomicrographs of brain hippocampal sections stained with haematoxylin and eosin (x100). (A) Normal group showing normal neuronal cells, (B) neuronal cell loss due to  $\beta$ -amyloid, (C) and (D): prevention of neuronal loss by methanol extract (250 and 500 mg/kg)

enzyme glutathione reductase (GSHr), leading to the consumption of NADPH, which is mainly produced in the pentose phosphate pathway. GSH also takes part in xenobiotic conjugation with the assistance of several glutathione S-transferase isoenzymes. GSH conjugates or its oxidized form can be eliminated from the cell by the family of ATP-dependent transporter pump. The inhibition of GSH synthesis leads to an increase in Ap induced cell death and intracellular Ap accumulation (Hayes et al., 2005).

Many clinical studies have reported strong evidences that memory impairment produced in rodents are associated with altered levels of GSH in the brain and with the activities of anti-oxidant enzymes (El-Sherbiny et al., 2003). Elevation of brain oxidative status of amnesic mice resembled the clinical pathology observed in Alzheimer's disease patients (Palmer, 1999). In this experiment condition, the treatment of p amyloid resulted in a significant decrease of glutathione reductase, glutathione peroxidase and SOD activities. The administration of methanol extract significantly preserved the activity of glutathione reductase and glutathione peroxidase in the hippocampus to a similar level observed in normal control mice. The restoration of activities of glutathione reductase and glutathione peroxidase by methanol extract might promote scavenging of free radicals using recycled GSH from GSSG (Jeong et al., 2009).

ging of free radicals using recycled GSH from GSSG (Jeong et al., 2009).

The SOD and GSHx constitute a mutually supportive team of defense against reactive oxygen species. SOD is a metallo protein and is the first enzyme involved in the anti-oxidant defense by lowering the steady-state level of  $O_2^-$  (Balamurugan and Muthusamy, 2008). The most remarkable effect of methanol extract is the increased activity of SOD in brain. Treatment with methanol extract preserved the reduced SOD activity to that of normal control. Although there are conflicting reports associated with the SOD activities in Alzheimer's disease, most recently, SOD mimetic have come to the forefront of anti-oxidative therapeutics of neurodegenerative diseases (Pong, 2003).

It has also been suggested that anti-oxidant might contribute to the prevention of Alzheimer's disease. Anti-oxidant such as beta-carotene and vitamins C, E and A may protect cells from the type of damage that leads to aging in the brain and tissues. Both vitamin C and E are anti-oxidant, which are likely to reduce oxidative stress and injury in the central nervous system; this may reduce the Ap plaque deposition in the neuronal cells (Zandi, 2004).

It appeared from the results of the study that

administration of aged peptide produced significant neuronal loss in the hippocampal region due to oxidative stress. It remains possible that the amnesic action of  $\beta$ -amyloid (25-35) is related to marked neurotoxic effects ant to more subtle inflammatory changes (Rush et al., 1992). The reduction in the neuronal cell loss as evident from the histology of the treatment group is a possible approach for the reduction of oxidative stress in the preventive and or curative potential of *I. tinctoria*.

## Conclusion

The potential of *I. tinctoria* leaves as an anti-oxidant agent in Alzheimer's type of neurodegenerative disorder is shown.

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