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Neuroprotective role of *Moringa oleifera* (MO) Linn. on colchicine induced experimental rat model of Alzheimer's disease: Possible involvement of antioxidants

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Abstract

The present study was designed to undertaken the role of *Moringa oleifera* (MO) Linn. leaf extract on colchicine induced experimental rat model of Alzheimer's disease (AD) with possible involvement of antioxidants. The antioxidant enzyme activities such as, superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and lipid peroxidation (LPO) level were studied in different parts of the brain such as Cerebral cortex (CC), Cerebellum (CB), Caudate nucleus (CN), Pons and Medulla (PM) and Midbrain (MB) in MO leaf extract treated colchicine induced experimental Alzheimer's rat model. MO significantly increased the superoxide dismutase (SOD) and catalase (CAT) activity along with increased the reduced glutathione (GSH) level in CC, CB, CN, MB and PM respectively whereas it significantly decreased the lipid peroxidation (LPO) level in all those mentioned portions of the rat brain. MO protects rat neurons against oxidative stress as is evidenced from our results of LPO, CAT, SOD and GSH possibly by vitamin E, C and beta carotene which are present in MO leaf extract.

Key Words: *Moringa oleifera, Colchicine, Alzheimer's disease*

1. Introduction

Moringa oleifera (Family: Moringaceae) Linn. or MO, commonly known as Drumstick tree in English, is cultivated throughout India, mainly in West Bengal. Leaves have been also reported to possess hypotensive, antispasmodic, diuretic abortifacient and anti-microbial properties. Seed oil and gum plays a vital role in the treatment of rheumatism and dental caries. Flower possesses antibacterial, anti-ulcer, anti-tubercular, antiviral, anti-fertility, depressant, anti-inflammatory and anti-cancer property (Chatterjee and Pakrashi, 1992). A number of Indian medicinal plants have been used for thousands of years in the traditional system of medicine (Ayurveda) for the management of neurodegenerative diseases such as Alzheimer's disease (AD). Some of these plants (rasayana) have already been reported to possess strong antioxidant activity (Scartezzini and speroni, 2000). MO leaves contain vitamins and antioxidants. It contains good amount of proteins, minerals, essential amino acids, vitamin A, vitamin C, vitamin B complex and a high content of vitamin E (Das, 1965). These compounds not only have antioxidant property but also have memory facilitating effect (Drazkiewicz et al., 2003). Vitamin E, which is found in membranes and on lipoprotein particles is considered to be the major lipophilic antioxidant in

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humans (Neuzil et al., 2001) and, especially, is essential for normal brain function (Vatassery et al., 1998). Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which is associated with excessive loss of memory (Terry & Davies, 1980; Wisniewski and Iqbal, 1980; Roy et al., 2007). The principal neuropathological features observed in cortex and hippocampus of the brain is the formation of neurofibrillary tangles and neuritic plaques (Ball, 1976; Tomlinson et al., 1970; Roy et al., 2007). The tangles and plaques seem to be the result of degenerative processes in neuronal perikarya and neuritis. The average course of AD is a decade, but the rate of progression is variable. It has been shown that AD afflicts about 8-10% of the population over 65 years of age and its prevalence doubles every 5 years thereafter (Irizarry and Hyman, 2001).

Microtubules are conspicuous components of the neuronal cytoskeleton. It has an important role in a wide range of cellular occurances, including growth and differentiation, axonal and dendritic transport. In animals, it has been observed that central administration of microtubule disrupting agents can result in cell death associated with cognitive impairment, which resemble the microtubule dysfunction in AD (Flaherty et al., 1989; Sofroniew et al., 1986; Tilson and Peterson, 1987; Tilson et al., 1988; Roy et al., 2007). It has been observed that colchicine binds to tubulin and disrupts its microtubule polymerization. Moreover, blockage of axonal transport (McClure, 1972) and induction of neurofibrillary degeneration (Wisniewsky & Terry, 1967) also have been observed after colchicine treatment. Recently colchicine has been shown to be neurotoxic and to destroy certain neuronal cells selectively (Goldschmidt & Steward, 1982; Goldschmidt & Steward, 1980).

2. Review of Literature

It has been observed that oxidative stress, which means imbalance between free radical production and endogenous antioxidant defence mechanisms, plays an important role in the pathogenesis of AD (Smith et al., 1996; Markesbery, 1997). Oxidative stress was first observed as an increase of oxidation products of proteins and lipids in brain tissue of AD patients (Smith et al., 1991; Lovell et al., 1995). Especially markers of Lipid peroxidation (LPO) have been found to be elevated not only in brain tissue (Montine et al., 1997; Sayre et al., 1997) but also in plasma (Schippling et al., 2000; McGrath et al., 2001) and cerebrospinal fluid (CSF) (Schippling et al., 2000; Bassett et al., 1999) of AD patients.

A major approach to the treatment of AD has involved attempts to augment the cholinergic function of the brain (Johnston, 1992). Four inhibitors of acetyl cholinesterase (AChE) *tacrine* (1,2,3,4-tetrahydro-9-aminoacridine; COGNEX), *donepezil* (ARICEPT) (Mayeux and Sano, 1999), *rivastigmine* (EXCELON) and *galantamine* (REMINYL) were used in the management of AD. The side effects of *donepezil*, *rivastigmine*, *tacrine* and *galantamine* are nausea, vomiting, diarrhea, insomnia etc. These side effects have prompted the scientific world for the search of alternative herbal remedies of AD. MO has no side effects.

3. Objective of the Paper

So, the objective of our study is to elucidate the antioxidant neuroprotection of *Moringa oleifera* (MO) Linn. leaf extract on the colchicine induced experimental rat model of Alzheimer's disease with possible involvement of antioxidants.

4. Sample source and Methodology

4.1 *Animal used and Maintenance*

Twenty-four male Holtzman strain adult albino rats weighing between 200-250gm were selected throughout the experiment. The rats were kept in standard laboratory conditions (room temperature 27 ± 1^0C, humidity 60% and 12h light/dark cycle) in accordance with 'Institutional Ethical Committee' rules and regulations. They were allowed free access to standard laboratory diet, which supplemented the necessary proteins, carbohydrates and minerals. Drinking water was supplied ad libitum. Body weight of the rats were recorded every day and maintained in the laboratory throughout the experimental period. The behavioral procedure was carried out between 12:00 and 14:00 h.

4.2 *Preparation of water extract from leaves of MO*

The leaves of MO were purchased from the local market and the identity of the plant was authenticated by the Botanical Survey of India, Howrah and kept in S. N. Pradhan Centre for Neurosciences, University of Calcutta. Fresh, Young, healthy leaves of MO were shaded, dried and grinded with the help of on electrical grinder to get a free flowing powder. This powder was subjected to extraction with water at room temperature for 24 hours. The extract obtained was filtered through Whatman filter paper and vacuum dried at 40^0-50^0C to get a dry powder, which was dissolved in double distilled water for final use (Tahiliani & Kar, 2000).

4.3 *Treatment*

The control animal was treated with normal saline. The MO leaf extract was given orally through orogastric cannula at the standard dose of 250mg/kg b.w. for fourteen consecutive days (between 10:00 and 11:00 hrs). The dose was standardized in our laboratory.

After fourteen days, the animals were sacrificed by cervical dislocation and the different parts of the brain like Cerebral cortex (CC), Cerebellum (CB), Caudate nucleus (CN), Pons and Medulla (PM) and Midbrain (MB) were isolated for antioxidant estimation.

4.3.1 *Grouping of Animals*

The animals were divided into four groups.

1. Control rats
2. Colchicine induced Alzheimer's rat model
3. Control rats treated with MO leaf extract
4. Colchicine induced Alzheimer's rat model treated with MO leaf extract.

4.4 *Preparation of experimental Alzheimer's model by colchicine*

Prior to surgery, all the animals were subjected to overnight fasting though drinking water was not withdrawn. The rats were anaesthetized with anesthetic ether (Kobra Drugs Ltd, India). The anaesthetized animals were placed on stereotaxic-instrument (INCO, India Ltd.) equipped with a custom-made ear bar**,** which prevents the damage of the tympanic membrane. Head was fixed in such a position that lambda and bregma sutures were in the same horizontal plane by introducing the incisor bar properly attached to the mouth. For aseptic surgery, absolute alcohol or rectified spirit was applied. The scalp was incisioned in the midline and the pericranial muscles and fascia were retracted laterally. After retracting the nuchal musculature the overlying bone was drilled at the specific loci in the lateral ventricle following the coordinanates of the stereotaxic atlas (Pellegrino and Cushman, 1967) (Coordinates for the lateral ventricles were: 0.6 mm posterior to

bregma, 1.8 mm lateral to the midline and 2.7 mm below the cortical surface). Colchicine (15 μgm of colchicine/ 5 μl of artificial CSF or ACSF) was then slowly infused (0.125 μl / min) into the lateral ventricle using a 10 μl Hamilton syringe. A total volume of 1 μl was delivered to the injection site and the injection cannula was left in place for 2-3 min following infusion.

4.4.1 *Postoperative care*

After surgery, all aseptic measures and care were taken for feeding until recovery from surgical stress. Penicillin or PCN (10,000 IU) was given post operatively to all animals for 3 consecutive days by intramuscular route. After 3 days of surgery, experiment was started and continued routinely until sacrificed. Similar procedure was repeated thrice, each at an interval of two days.

4.5 *Behavior study by radial Y–arm maze training*

Radial Y – arm maze study was used to assess cognitive function. The apparatus is a four Y – arm connected together in which the animals were trained to perform a standard radial arm maze (RAM) task. Rats were given 7 days habituation trials in which food pellets (chocolate chips) were scattered throughout the maze and the rats were allowed to freely explore inside it for 5 mins. Following habituation sessions, the animals were trained for 10 daily trials on RAM task (10 trials/day). In this task, an animal was placed in the centre of the maze and allowed to visit each of the 4 arms, which were baited with single food pellet. Entry into an arm previously visited within any daily trial was scored as an error. Animals not reaching this criterion were discarded from the study (Dwaine & Thomas, 1990).

4.6 *Biochemical Estimation*

4.6.1 *Tissue preparation*

Rats were sacrificed by cervical dislocation on day 14 immediately after behavior study. The Cerebral cortex (CC), Cerebellum (CB), Caudate nucleus (CN), Pons and Medulla (PM) and Midbrain (MB) were dissected out. The tissues were weighed and homogenized in ice-cold phosphate buffer and prepared for biochemical estimation.

Estimation of SOD, CAT, GSH activity and LPO level

Catalase activity was estimated by the method of Cohen *et al.* (1970), Superoxide Dismutase (SOD) was estimated by the method of Mishra and Fridovich (1972), Reduced glutathione (GSH) level was measured according to the method of Ellman (1959) and Lipid Peroxidation (LPO) was estimated by the method of Bhattacharya *et al.* (2001). Detailed procedures of the estimation of SOD, CAT, GSH and LPO level has been described in methodology section.

Statistical analysis: The data were expressed as MEAN ± S.E.M. and were analyzed statistically using one way analysis of variance (one way ANOVA) followed by multiple comparison 't' test. In addition to this, two-tailed Student't' test was performed to determine the level of significance between the means. Difference below the probability level 0.05 was considered statistically significant.

5. Results and Discussion

5.1 *Behavioral analysis by RAM training*

Prior to surgery, all rats acquired the RAM task and were making 8 – 9 correct choices (>90% accuracy) in their first 4 arms selections (acquisition). Intracerebroventricular infusion of colchicine (15 μg/ 5μl of ACSF) produced significant impairments in the RAM performance (reacquisition) after 3 days of surgery compared to that of control group. The correct choices out of 10 daily trials were significantly decreased ($p<0.001$) and the latency time was significantly increased ($p<0.001$) in colchicine treated groups as compared to the control group. Pretreatment with MO leaf extract for 14 days improved RAM performance significantly 3 days after surgery by significantly increasing ($p<0.001$) the correct choices and significantly decreasing ($p<0.001$) the latency time. The correct choices out of 10 daily trials was significantly increased ($p<0.001$) and the latency time was significantly decreased ($P<0.02$) in MO treated groups rather than control groups. The result is shown in Table – 1.

5.2 *Measurement of parameters of oxidative stress*

Fourteen days after Intracerebroventricular (ICV) infusion of colchicine, the SOD, CAT, reduced glutathione levels and lipid peroxidation levels were estimated. There was a significant rise ($p<0.001$) in lipid peroxidation levels in the colchicine treated group as compared to the control group (table 3) and correspondingly a significant decline ($p<0.001$) in the reduced glutathione levels in the colchicine treated group as compared to the control group (table 5). Also, there was a significant decline ($p<0.001$) in the SOD and CAT levels in the colchicine treated group as compared to the control group (table 2 and 4). Besides this, there was a significant increase ($p<0.001$) in lipid peroxidation levels in the colchicine treated group compared to that of MO pretreated colchicine infused group (table 3) and correspondingly a significant decline ($p<0.001$) in the reduced glutathione levels in the colchicine treated group as compared to the MO treated colchicine infused group (table 5). The levels of reduced glutathione were significantly increased ($p<0.001$) in only MO treated animals rather than control (table 5). Also, there was a significant decline ($p<0.001$) in SOD and CAT levels in the colchicine treated group as compared to the MO treated colchicine group (table 2 and 4). The CAT activity was significantly increased ($P<0.001$) in MO treated control groups rather than control groups (table 4). The SOD activity was significantly increased ($P<0.001$) in MO treated animals compared to that of control groups (table 2). The LPO activity was significantly decreased ($P<0.001$) in MO treated control groups rather than control groups (table 3).

Table 1. Role of Moringa oleifera on behavioral parameters (RAM test) of colchicine (15 μgm of colchicine/5μl of ACSF) infused rats.

	Acquisition		Reacquisition	
	No. of trials (out of 10)	Latency (in secs)	No. of trials (out of 10)	Latency (in secs)
Control	8.59 ± 0.19	105.5 ± 3.89	8.54 ± 0.12	111 ± 5.05
Colchicine	9 ± 0.47	106 ± 4.54	$1.64 \pm 0.03^{***}$	$210.5 \pm 10.34^{***}$
MO	7 ± 0.12	111.7 ± 4.67	$9.51 \pm 0.15^{***}$	$72 \pm 2.67^{**}$
MO+Colchicine	8.61 ± 0.18	108.5 ± 4.23	$5.50 \pm 0.07^{\#}$	$134.17 \pm 9.52^{\#}$

Values are mean ± SEM, n = 6;, $^{***}p < 0.001$, $^{**}p < 0.02$ when compared with control group. $^{\#}p < 0.001$ when compared with colchicine treated group. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison t – test.
Source: Computed by the authors

Table 2. Changes in SOD activity in different brain areas (CC, CB, CN, MB and PM) of MO treated colchicine (15μg/ 5 μl of ACSF) induced Alzheimer rat model.

	SOD (% inhibition unit)				
	CC	CB	CN	MB	PM
Control	12.09±0.16	12.68±0.20	11.86±0.18	12.97±0.08	12.37±0.11
Colchicine	21.79±0.26***	21.77±0.22***	19.75±0.32***	21.06±0.21***	21.53±0.30***
MO	10.13±0.10***	9.97±0.13 ***	8.27±0.26***	10.45±0.12***	10.34±0.15***
MO+Colchicine	15.37±0.14$^{\#}$	15.13±0.19$^{\#}$	13.19±0.21$^{\#}$	15.00±0.19$^{\#}$	14.28±0.15$^{\#}$

Values are mean ± SEM, n = 6; $^{***}p < 0.001$ when compared with control group. $^{\#}p < 0.001$ when compared with colchicine treated group. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison t – test.
Source: Computed by the authors

Table 3. Changes in LPO level in different brain areas (CC, CB, CN, MB and PM) of MO treated colchicine (15μg/ 5 μl of ACSF) induced Alzheimer rat model.

	LPO (nmol of TBARS / gm mol of tissue)				
	CC	CB	CN	MB	PM
Control	4.13±0.12	3.73±0.10	3.18±0.09	3.27±0.12	3.25±0.07
Colchicine	9.60±0.10***	9.89±0.06***	10.05±0.02***	10.42±0.09***	10.04±0.04***
MO	2.02±0.05***	2.53±0.08***	2.03±0.05***	1.77±0.08***	1.82±0.08***
MO+Colchicine	5.68±0.11$^{\#}$	5.66±0.05$^{\#}$	6.00±0.04$^{\#}$	6.05±0.03$^{\#}$	5.57±0.08$^{\#}$

Values are mean ± SEM, n = 6; $^{***}p < 0.001$ when compared with control group. $^{\#}p < 0.001$ when compared with colchicine treated group. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison t – test.
Source: Computed by the authors

Table 4. Changes in CAT activity in different brain areas (CC, CB, CN, MB and PM) of MO treated colchicine (15μg/ 5 μl of ACSF) induced Alzheimer rat model.

	CAT (% inhibition unit)				
	CC	CB	CN	MB	PM
Control	14.37±0.08	13.35±0.19	13.21±0.15	13.20±0.10	13.05±0.03
Colchicine	22.84±0.10***	22.42±0.14***	21.79±0.19**	21.25±0.16**	20.86±0.17**
MO	11.50± 0.20***	10.06±0.05***	10.08±0.04**	10.36±0.08**	10.30±0.11**
MO+Colchicine	16.47± 0.12$^{\#}$	15.42± 0.10$^{\#}$	16.29± 0.14$^{\#}$	15.64± 0.09$^{\#}$	15.05±0.09$^{\#}$

Values are mean ± SEM, n = 6; $^{***}p < 0.001$ when compared with control group. $^{\#}p < 0.001$ when compared with colchicine treated group. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison t – test.
Source: Computed by the authors

Table 5. Changes in Reduced glutathione (GSH) level in different brain areas (CC, CB, CN, MB and PM) of MO treated colchicine (15μg/ 5 μl of ACSF) induced Alzheimer rat model.

	Reduced glutathione (μg/g of tissue)				
	CC	CB	CN	MB	PM
Control	40.51±0.14	43.05±0.24	30.56±0.40	28.31±0.30	31.48±0.50
Colchicine	1.48±0.22***	2.16±0.10***	2.67±0.12***	1.62±0.08***	2.12±0.05***
MO	44.90±0.23***	51.69±0.40***	39.77±0.16**	35.28±0.24**	39.98±0.16**
MO+Colchicine	31.79±0.49$^{\#}$	28.68±0.85$^{\#}$	15.47±0.34$^{\#}$	19.79±0.60$^{\#}$	24.02±0.34$^{\#}$

Values are mean ± SEM, n = 6; $^{***}p < 0.001$ when compared with control group. $^{\#}p < 0.001$ when compared with colchicine treated group. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison t – test.
Source: Computed by the authors

Oxidative stress refers to the cytopathologic consequences of a mismatch between the production of free radicals and the ability of a cell to defend against them (Simonian and Coyle, 1996). Free radicals play a crucial role in a complex interplay of different mechanisms in both normal aging and neurodegenerative diseases (Beal, 1995). Strong evidence that oxidative stress is involved in the pathogenesis of AD comes from a clinical study showing that oral vitamin E intake delayed progression in patients with moderately severe impairment from AD (Sano et al., 1997).

Both vitamin E and β carotene were found to protect rat neurons against oxidative stress (Mitchell et al., 1999). From our present study, in colchicine infused rats, there was a decrease in learning behavior by decreasing the correct choices in 10 daily trials and increasing the latency period along with a decrease in SOD, CAT and reduced glutathione level and an increase in LPO level. Out of 10 daily trials, the correct choices decreased and the latency period increased significantly in colchicine infused rats. Colchicine impaired memory as is evidenced by learning and memory behavior in RAM task, possibly by decreasing the cellular defending enzymes like SOD, CAT and reduced glutathione and by increasing the LPO level. Treatment with MO leaf extract for fourteen consecutive days (dose: 250mg/kg b.w.) helped to improve memory by increasing the correct choices and decreasing the latency period.

It has been reported that central administration of colchicine elevates glutamate/gamma amino butyric acid ratio (GLU/GABA ratio) in cortex of mice brain (Yu *et al.*, 1997). The relative increase in the GLU activity exerts neurotoxic effect through the production of hydroxyl radicals (Hammer *et al.*, 1993). From our present observation, in Alzheimer's rat model induced by intracerebroventricular injection of colchicine, the generation of such free radicals may have occurred and thereby significantly decreased the SOD activity, CAT activity and reduced glutathione level and significantly increased the LPO level. The main substrates for LPO are polyunsaturated fatty acids (PUFA) such as linolenic acid, arachidonic acid or docosahexanoic acid, which are present in lipoproteins as well as in cell membranes. So, colchicine administration into the lateral cerebral ventricle (LCV) produced ROS leading to the initiation of the LPO reactions. Subsequently, a chain reaction is started by the oxidation of neighboured PUFA. Oxidized PUFAs are further degraded to toxic and products including 4-hydroxy-2-nonenal (HNE), acrolein, malonedialdehyde (MDA) and other short chain aldehydes, which in part have been shown to evolve neurotoxic action (Neely et al., 1999; Picklo and Montine, 2001).

Glutathione is an endogenous antioxidant, which is present majorly in the reduced form within the cell. The most robust and significant alteration in the antioxidant defense is a decrease in GSH concentration. During oxidative stress, reduced glutathione is converted to oxidized glutathione (GSSG) by the action of glutathione peroxidase. The decreased level of reduced glutathione seen in our study indicates that there was an increased generation of free radicals and the reduced glutathione was depleted during the process of combating oxidative stress (Reiter, 2000; Schulz *et al.*, 2000).

6. Conclusion

Treatment with MO leaf extract for fourteen days significantly increased the SOD activity, CAT activity and reduced glutathione level and significantly decreased the LPO level. It has been observed that both vitamin C and stable vitamin E levels were decreased in AD (Riviere *et al.*, 1998). In an animal model vitamin E has been shown to accumulate in the brain and decrease lipid peroxidation (Clement & Bourre, 1997; Meydani *et al.*, 1988). It was previously discussed that MO contains good amount of proteins, minerals, essential amino acids, vitamin A, vitamin C, vitamin B complex and a high content of vitamin E. Besides this, MO contains flavonoids, flavonols. So, MO leaf may help to scavenge free radicals either by non-enzymatic defenses like vitamins or by bioactive compounds like flavonoids or both. Further study is required to clarify this mystery.

Treatment with MO leaf extract significantly increased the correct choices and significantly decreased the latency period as compared to colchicine treated groups. The improvement of RAM performance is possibly due to the suppression of LPO level and activation of SOD activity, CAT activity and reduced glutathione level. Thus, MO leaf help to improve memory by enhancing the activity of SOD, CAT and reduced glutathione level and by depleting the LPO level, which is evident from the behavioral study like RAM performance.
So, from the present observation, it can be concluded that *Moringa oleifera* Linn. provides antioxidant neuroprotection against colchicine induced Alzheimer's disease by free radical scavenging action.

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