RESEARCH ARTICLE

The antioxidative role of *Aegle marmelos* Linn. on colchicine-induced experimental rat model of Alzheimer's disease

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ABSTRACT

Antioxidants play a crucial role in managing neurodegenerative diseases, including Alzheimer's disease. Many Indian medicinal plants have been used in the traditional system of medicine (Ayurveda) for the management of neurodegenerative diseases, including Alzheimer's disease. Some of these plants have already been reported to possess strong antioxidant activity. *Aegle marmelos* (AM) Linn., a fruit of common use, is rich in vit-E, beta-carotene, flavonoids and flavonols. The effect of chronic oral treatment of aqueous pulp extract of AM (250 mg/ kg BW) was studied in Holtzman strain adult albino rats of both sexes. The behavior study, antioxidant level superoxide dismutase (SOD), catalase (CAT), reduced glutathione level (GSH) and lipid peroxidation level were studied in different brain areas - cerebral cortex (CC), cerebellum (CB), midbrain (MB), caudate nucleus (CN), and pons medulla (PM) in colchicine induced experimental Alzheimer rat model with and without treatment with AM. The results revealed that chronic treatment with AM pulp extract markedly increased the number of correct choices in the radial Y arm maze task, significantly decreased lipid peroxidation level, and significantly increased SOD, CAT, and reduced glutathione level in the different parts of the brain. The present study demonstrated that the antioxidant property of AM may be beneficial for managing the colchicine-induced rat model of Alzheimer's disease.

Keywords: Antioxidant, Neurodegenerative diseases, Alzheimer's disease, *Aegle marmelos,* Colchicine, Radial Y maze.

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INTRODUCTION

Aegle marmelos (AM) *Linn.*, commonly known as bael (family Rutaceae), is widely cultivated in India and many Southeast Asian countries, including Myanmar, Bangladesh, and Thailand.[1] Its fruit, leaf, root, and stem have long been used in traditional medicine because of their medicinal uses, such as antidiarrheal, antidysenteric, demulcent, antipyretic, and anti-inflammatory activities.[2] The leaves, roots, bark, seeds, and fruits are edible and have medicinal value. Eugenol ($C_{10}H_{12}O_2$), present in AM leaf extract, has potent antioxidant properties[3-5] and inhibits lipid peroxidation.[5,6] AM leaf extract is identified as a potential antioxidant drug that lowers blood sugar levels in alloxan-induced diabetic rats.[7] The neuroprotective effect of *A. marmelos* pulp extract in scopolamine-induced cognitive impairment in mice was reported.[8] AM contains various phytochemicals such as carotenoids, alkaloids, pectins, tannins, phenolics, coumarins, flavonoids, terpenoids, etc.[9-11] Several vitamins, such as vitamin A, C, and E, have been isolated from AM.[12] Inflammatory injury is associated in part with increased generation of ROS. Alzheimer's disease (AD) is a neurodegenerative disorder that is associated with excessive loss of memory.[13-15] The principal neuropathological features observed in the cortex and hippocampus of the brain are the formation of neurofibrillary tangles and neuritic plaques.[15-17] The tangles and plaques seem to result from degenerative processes in neuronal perikarya and neuritis. It has been shown that AD afflicts about 8 to 10% of the population over 65 years of age, and its prevalence doubles every 5 years thereafter.[18]

The presence of amyloid plaques and neurofibrillary tangles in the brain characterizes AD.[19] Amyloid-β (Aβ) peptides and phosphorylated tau proteins are the key components of extracellular amyloid plaques and intracellular neurofibrillary tangles, respectively. Microtubules are conspicuous components of the neuronal cytoskeleton. It is important in various cellular occurrences, including growth, differentiation, and 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 resembles microtubule dysfunction in AD.[15,20,23] It has been observed that colchicine binds to tubulin and disrupts its microtubule polymerization. Moreover, blockage of axonal transport[24] and induction of neurofibrillary degeneration[25] have also been observed after colchicine treatment. Recently, colchicine has been neurotoxic and destroys certain neuronal cells selectively.[26]

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It has been observed that oxidative stress, which means an imbalance between free radical production and endogenous antioxidant defense mechanisms, plays an important role in the pathogenesis of AD.[27,28] Oxidative stress was first observed as an increase of oxidation products of proteins and lipids in brain tissue of AD patients.[29] Especially markers of lipid peroxidation (LPO) are elevated not only in brain tissue [30] but also in plasma[31,32] and cerebrospinal fluid (CSF)[33] of AD patients.

A major approach to treating AD has involved attempts to augment the cholinergic function of the brain.[33] Four inhibitors of acetylcholinesterase (AChE) - tacrine (1,2,3,4-tetrahydro-9-aminoacridine), donepezil 34, rivastigmine, and galantamine - were used in the management of AD. The side effects of these drugs include nausea, vomiting, diarrhea, insomnia, etc. These side effects have prompted the scientific world to search for alternative herbal remedies for AD. AM has no side effects.

The current study aims to elucidate AM's antioxidative role in the colchicine-induced experimental rat model of Alzheimer's disease.

MATERIALS AND METHODS

Animal Use and Maintenance

Twenty-four male Holtzman strain adult albino rats weighing between 200 to 250 gm were selected throughout the experiment. The rats were kept in standard laboratory conditions (room temperature 27 ± 1°C, humidity 60%, and 12 hours light/dark cycle) in accordance with the Institutional Ethical Committee rules and regulations, S. N. Pradhan Centre for Neurosciences, University of Calcutta. Holtzman strain adult albino rats have been selected throughout the experiment for two primary reasons. First, rats develop very slowly. They, therefore, made features of physiological, neural, and psychological development accessible to the experimental method at a time when its application to the phenomena of development remained controversial.

They were allowed free access to a standard laboratory diet, which supplemented the necessary proteins, carbohydrates, and minerals. Drinking water was supplied *ad libitum*. The rats' body weights were recorded daily and maintained in the laboratory throughout the experiment. The behavioral procedure was carried out between 12:00 and 14:00 hours.

Preparation of aqueous extract from the pulp of AM

The pulp of AM fruit was used throughout the experimental study. The fruits of AM were cut into pieces, sun-dried, and ground with the help of an electrical grinder to get a free-flowing powder. This powder was extracted with water (1:3) at room temperature for 48 hours. The extract obtained was filtered through Whatman filter paper and vacuum dried at 40 to 50°C to get a dry powder dissolved in double-distilled water for final use.[35] Ethanolic extracts often contain a higher concentration of certain bioactive compounds like flavonoids and phenolic acids compared to aqueous extracts, which may lead to stronger biological activity in some cases.

Treatment

The control animal was treated with normal saline. The AM pulp extract was given orally through an orogastric cannula at the standard dose of 250 mg/kg BW for fourteen consecutive days (between 10:00 and 11:00 hours). The dose was standardized in our laboratory. After 14 days, the animals were sacrificed by cervical dislocation, and the different parts of the brain, such as the CC, CB, CN, PM, and MB, were isolated for antioxidant estimation.

Grouping of Animal

The animals were divided into four groups. Each experimental group contained six (6) animals: Control rats, a colchicine-induced Alzheimer's rat model, control rats treated with AM pulp extract, and a colchicine-induced Alzheimer's rat model treated with AM pulp extract.

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 anesthetized with anesthetic ether (Kobra Drugs Ltd, India). The anesthetized animals were placed on a stereotaxic instrument (INCO, India Ltd.) equipped with a custom-made ear bar, which prevents damage to the tympanic membrane. The head was fixed so 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 coordinates of the stereotaxic atlas36 (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 to 3 minutes following the infusion.

Postoperative Care

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

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-day habituation trials in which food pellets (chocolate chips) were scattered throughout the maze, and the rats were allowed to explore it for 5 minutes freely. Following habituation sessions, the animals were trained for 10 daily trials on RAM tasks (10 trials/day). In this task, an animal was placed in the maze's center and allowed to visit each of the four arms baited with a 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.[37]

Biochemical Estimation

Tissue preparation

Rats were sacrificed by cervical dislocation on day 14 immediately after the behavior study. The CC, CB, CN, PM, and MB were dissected. The tissues were weighed and homogenized in ice-cold phosphate buffer and prepared for biochemical estimation.

Measurement of superoxide dismutase (SOD)

Superoxide dismutase (SOD) was estimated by the method as described elsewhere.[38,39] Tissue samples were homogenized with 5 mL of ice-cold 0.1 M phosphate buffer (pH-7.4). The homogenates were then centrifuged at 3000 rpm for 10 minutes. Then, 0.1 mL of the sample was mixed with 0.8 mL of Trehalose- 6, 6 dibehenate (TDB). The reaction was started by adding 4 µL of nicotinamide adenine dinucleotide phosphate (NADPH). Then, 25 µL of ethylenediaminetetraacetic acid manganese chloride (EDTA-$MnCl_2$) mixture was added. Thereafter, spectrophotometric readings were recorded at 340 nm. After recording the spectrophotometric readings, 0.1 mL of mercaptoethanol was added to this mixture, and again, spectrophotometric readings were recorded at 340 nm.

Measurement of catalase (CAT)

Catalase (CAT) activity was estimated by the method of Cohen *et al*.[39,40] Tissue samples were homogenized with 5 mL of ice-cold 0.1 M phosphate buffer (pH-7.4). The homogenates were then centrifuged at 3000 rpm for 10 minutes. The precipitate was then stirred with the addition of 15 mL of ice-cold 0.1 M phosphate buffer and allowed to stand in cold conditions with occasional shaking. The shaking procedure was repeated three times. About 1-mL of the sample was added to 9 mL of H_2O_2. The decomposition rate of H_2O_2 was measured spectrophotometrically from the changes in absorbance at 350 nm. The activity of CAT was expressed as a % inhibition unit.

Measurement of Glutathione (GSH)

Reduced glutathione (GSH) was measured using Ellman's method.[41,39] Equal quantity of homogenate was mixed with 10% TCA and centrifuged to separate the proteins. To 0.01 mL of this supernatant, 2 mL of phosphate buffer (pH- 8.4), 0.5 mL of 5, - 5- dithiobis- (2-nitrobenzoic acid), and 0.4 mL of double-distilled water were added. The mixture was vortexed, and the absorbance was read at 412 nm within 15 minutes. The concentration of GSH was expressed as µg/g of tissue.

Measurement of Lipid Peroxidation (LPO)

Lipid peroxidation (LPO) was measured according to the method of Roy *et al*. and Hazra *et al*.[39,42] Tissue samples were homogenized with 5 mL of ice-cold 0.1 M phosphate buffer (pH-7.4). The homogenates were then centrifuged at 3000 rpm for 10 minutes. Then, 0.5 mL of the sample was mixed with 1-mL of TDB and incubated at 37°C for 1-hour. To this, 0.5 mL of trichloroacetic acid (TCA) was added, vortexed and the absorbance was read at 350 nm. After recording the spectrophotometric reading, a one mL sample was mixed with 500 ml mercaptoethanol, and again, the absorbance was read at 350 nm.

Statistical Analysis

The data were expressed as mean ± SD and analyzed statistically using one-way analysis of variance (one-way ANOVA) followed by multiple comparison 't' test. In addition, a two-tailed Student 't' test was performed to determine the significance level between the means. A difference below the probability level of 0.05 was considered statistically significant.

RESULTS

Behavioral Analysis by RAM Training

Prior to surgery, all rats acquired the RAM task and were making 8 to 9 correct choices (>90% accuracy) in their first four arms selections (acquisition). Intracerebroventricular infusion of colchicine (15 µg/5 µL of ACSF) produced significant impairments in the RAM performance (reacquisition) after 3 days of surgery compared to that of the control group. The correct choices out of 10 daily trials were significantly decreased (p <0.05), and the latency time was significantly increased (p <0.05) 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 <0.05) the correct choices and significantly decreasing (p <0.05) the latency time. The correct choices out of 10 daily trials were significantly increased (p <0.05), and the latency time was significantly decreased (p <0.05) in AM-treated groups rather than control groups. The result is shown in Table 1.

Measurement of Parameters of Oxidative Stress

About 14 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 <0.05) in lipid peroxidation levels in the colchicine-treated group as compared to the control

Table 1: Role of *A. marmelos* on behavioral parameters (RAM test)

Animal Group	*Acquisition*		*Reacquisition*	
	No. of trials (out of 10)	*Latency (in secs)*	*No. of trials (out of 10)*	*Latency (in secs)*
Control	8.55 ± 0.49	105.00 ± 8.37	8.67 ± 0.32	110.50 ± 12.51
Colchicine	9.02 ± 1.05	107.00 ± 11.43	1.59 ± 0.05*	212.50 ± 23.37*
AM	7.04 ± 0.34	112.50 ± 11.81	9.62 ± 0.29*	72.08 ± 6.49*
AM + Colchicine	8.70 ± 0.46	109.00 ± 10.29	5.56 ± 0.10#	135.50 ± 23.47#

Values are mean ± SD of six observations, * and # indicate significant differences ($p < 0.05$) with the control and colchicine-treated groups, respectively.

Table 2: Changes in SOD activity in different brain areas - Cerebral cortex (CC), Cerebellum (CB), Caudate nucleus (CN), Pons and Medulla (PM), and Midbrain (MB)

Animal Group	*SOD (% inhibition unit)*				
	CC	*CB*	*CN*	*MB*	*PM*
Control	12.07 ± 0.34	12.59 ± 0.39	11.81 ± 0.34	12.88 ± 0.17	12.33 ± 0.34
Colchicine	21.74 ± 0.51*	21.65 ± 0.39*	19.66 ± 0.59*	20.96 ± 0.37*	21.46 ± 0.61*
AM	10.24 ± 0.20*	9.88 ± 0.22*	8.21 ± 0.51*	10.52 ± 0.27*	10.41 ± 0.29*
AM + Colchicine	15.24 ± 0.15#	15.21 ± 0.37#	13.27 ± 0.34#	15.04 ± 0.39#	14.34 ± 0.22#

Values are mean ± SD of six observations, * and # indicate significant differences ($p < 0.05$) with the control and colchicine-treated groups, respectively.

Table 3: Changes in CAT activity in different brain areas - CC, CB, CN, PM, and MB

Animal Group	*CAT (% inhibition unit)*				
	CC	*CB*	*CN*	*MB*	*PM*
Control	14.34 ± 0.17	13.29 ± 0.36	13.24 ± 0.29	13.32 ± 0.27	12.97 ± 0.09
Colchicine	22.89 ± 0.20*	22.33 ± 0.24*	21.70 ± 0.29*	21.32 ± 0.29*	20.94 ± 0.27*
AM	11.56 ± 0.34*	9.98 ± 0.10*	9.94 ± 0.07*	10.32 ± 0.15*	10.35 ± 0.22*
AM +Colchicine	16.39 ± 0.24#	15.32 ± 0.20#	16.21 ± 0.24#	15.52 ± 0.15#	14.95 ± 0.07#

Values are mean ± SD of six observations, * and # indicate significant differences ($p < 0.05$) with the control and colchicine-treated groups, respectively.

Table 4: Changes in reduced glutathione levels in different brain areas - CC, CB, CN, PM, and MB

Animal Group	*Reduced glutathione (µg/g of tissue)*				
	CC	*CB*	*CN*	*MB*	*PM*
Control	39.44 ± 0.29	43.18 ± 0.55	30.44 ± 0.74	28.62 ± 0.58	31.32 ± 0.54
Colchicine	1.68 ± 0.49*	2.33 ± 0.26*	2.66 ± 0.26*	1.68 ± 0.14*	2.18 ± 0.14*
AM	45.04 ± 0.51*	51.77 ± 0.68*	39.76 ± 0.34*	35.42 ± 0.27*	40.04 ± 0.26*
AM +Colchicine	30.44±0.53#	27.98 ± 0.74#	15.32 ± 0.68#	19.81 ± 0.88#	24.12 ± 0.55#

Values are mean ± SD of six observations, * and # indicate significant differences ($p < 0.05$) with the control and colchicine-treated groups, respectively.

group and correspondingly a significant decline (p <0.05) in the reduced glutathione levels in the colchicine treated group as compared to the control group. Also, there was a significant decline (p <0.05) in the SOD and CAT levels in the colchicine-treated group as compared to the control group (Tables 2 and 3). Besides this, there was a significant increase (p <0.05) in lipid peroxidation levels in the colchicine-treated group compared to that of AM pretreated colchicine-infused group (Table 4) and correspondingly a significant decline (p <0.05) in the reduced glutathione levels in the colchicine treated group as compared to the AM treated colchicine infused group (Table 5). The levels of reduced glutathione were significantly increased (p <0.05) in only AM-treated animals rather than the control (Table 5).

Table 5: Changes in lipid peroxidation level in different brain areas - CC, CB, CN, PM, and MB

Animal Group	*Lipid peroxidation (nmol of TBARS/gm mol of tissue)*				
	CC	*CB*	*CN*	*MB*	*PM*
Control	4.21 ± 0.59	3.77 ± 0.22	3.24 ± 0.14	3.34 ± 0.28	3.32 ± 0.10
Colchicine	9.46 ± 0.12*	9.67 ± 0.18*	10.23 ± 0.05*	10.54 ± 0.14*	10.22 ± 0.07*
AM	2.11 ± 0.10*	2.56 ± 0.10*	2.11 ± 0.10*	1.82 ± 0.10*	1.87 ± 0.14*
AM +Colchicine	5.49 ± 0.22#	5.54 ± 0.10#	6.14 ± 0.07#	6.12 ± 0.10#	5.65 ± 0.14#

Values are mean ± SD of six observations, * and # indicate significant differences ($p < 0.05$) with the control and colchicine-treated groups, respectively.

Also, there was a significant decline (p <0.05) in SOD and CAT levels in the colchicine-treated group as compared to the AM-treated colchicine group (Tables 2 and 3). The CAT activity was significantly increased (p <0.05) in AM-treated control groups rather than control groups (Table 3). The SOD activity was significantly increased (p <0.05) in AM-treated animals compared to that of control groups (Table 2). The LPO activity was significantly decreased (p <0.05) in AM-treated control groups rather than control groups (Table 5).

DISCUSSION

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.[43] Free radicals play an important role in a complex interplay of different mechanisms in normal aging and neurodegenerative diseases.[44] 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.[45]

Both vitamin E and β carotene were found to protect rat neurons against oxidative stress.[46] 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 AM pulp extract for fourteen consecutive days (dose: 250 mg/kg BW) 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 the cortex of mice brains.[47] The relative increase in the GLU activity exerts a neurotoxic effect by producing hydroxyl radicals.[48] From our present observation, in an 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 and cell membranes. So, colchicine administration into the lateral cerebral ventricle (LCV) produced ROS, initiating the LPO reactions. Subsequently, a chain reaction is started by the oxidation of neighboured PUFA. Oxidized PUFAs are further degraded to toxic products, including 4-hydroxy-2-nonenal (HNE), acrolein, malondialdehyde (MDA), and other short-chain aldehydes, which in part have been shown to evolve neurotoxic action.[49] 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 decline 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 observed 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.[50,51]

Treatment with AM pulp 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.[52] In an animal model, vitamin E has been shown to accumulate in the brain and decrease lipid peroxidation.[53] It was previously discussed that AM contains many proteins, minerals, essential amino acids, vitamin A, C, B complex, and a high vitamin E content. Besides this, AM contains flavonoids and flavonols. So, AM pulp 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 AM pulp extract significantly increased the correct choices and significantly decreased the latency period 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, AM pulp help to improve memory by

enhancing the activity of SOD and CAT, reducing glutathione levels, and depleting the LPO level, which is evident from behavioral studies like RAM performance.

So, from the present observation, it may be concluded that *A. marmelos,* containing vitamins A, C, and E, Flavonols, and Flavonoids, provides antioxidant neuroprotection against colchicine-induced Alzheimer's disease by free radical scavenging action.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.