ORIGINAL ARTICLE

Role of *Ocimum sanctum* in the experimental model of Alzheimer's disease in rats

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Alzheimer's disease (AD), a neurodegenerative disorder incapacitating elderly people towards the end of their life, accounts for approximately 70% of dementia. It affects 17-25 million elderly people worldwide. In spite of the remarkable increase in scientific knowledge about the pathobiology of AD, attempts other than modifying the cholinergic neurotransmission have proved futile. In Ayurveda a number of agents are in use, since ancient times, for chronic debilitating disorders. One such preparation, *Ocimum sanctum* (OS) has been found to possess anti-ulcerogenic, anti-inflammatory and neuroprotective activities. Because of the nonavailability of proper curative therapy for AD, the present study has been undertaken to evaluate the possible role of OS in experimental AD in rats. Experimental AD in rats was produced by a nucleus basalis magnacellularis lesion with ibotenic acid (IB) and intracerebroventricular (i.c.v) administration of colchicine (Col). Various behavioural tests and biochemical analysis were performed to explore the possible role of OS in AD. OS exhibited anxiolytic activity in an open field test. In an elevated plus maze test, OS significantly alleviated IB, and Col induced anxiety and depression in the Porsolt's swim test. In Morris' water maze test, OS pretreatments improved reference memory, working memory and spatial learning. Both IB and Col induced deficits in active avoidance learning and retention of learned behaviour, which were significantly reversed by OS. IB and Col induced increased lipid peroxidase activity, which was significantly reversed by OS (as seen from the reductions in the malondialdehyde level) and stabilized the rise in superoxide dismutase activity, but it had no effect on the acetylcholinesterase activity. OS might be effective in clinical AD by virtue of its cognition enhancement, antidepressant and antianxiety properties, which are the primary needs to be addressed in AD.

Key words: Alzheimer's disease, eugenol, *Ocimum sanctum*

INTRODUCTION

Alzheimer's disease (AD), an age-dependent, progressive, neurodegenerative disorder characterized by multiple cognitive deficits is often accompanied by behavioural disorders and mood changes. AD is the most common form of dementia, accounting for approximately 70% of the dementia cases in most industrialized countries and affecting an estimated 17 to 25 million people world wide.[1-3] With increasing longevity of most populations, the prevalence of AD is likely to increase, imposing greater social and economic burdens on society and healthcare systems.[4] Since no cure for patients with AD is currently available, symptomatic treatment for AD focuses on the restoration of cholinergic function.[5-7] Reports indicate that memantine and donepezil are more effective drugs in improving cognitive impairment in patients with AD.[8-10] However, two clinical trials have shown no improvement in the cognitive deficit or reduction in the institutionalization rate.[11,12] In view of the above shortcomings in the drugs used for the treatment of AD there has been an increased interest in herbal products as a source of treatment.[13]

The etiology and pathogenesis of AD have not been fully determined, although several factors have been postulated to contribute to the development of the disease.[14-16] Oxidative stress is one of the critical determinants in the stimulation of neuronal cell death and plays a key role in AD-associated degenerative neuronal changes.[17-19] Several herbal drugs with antioxidant properties have been reported to have beneficial effects in AD.[20,21] For example, clinical studies in AD patients have already shown that Ginko biloba is as effective as donepezil in improving dementia.

Ocimum sanctum Linn (Labiatae), a popular indigenous plant in India, and is commonly grown in households here. In Ayurveda, it is described as 'Rasayana'.[22] Rasayanas are plants that possess attributes of adaptogens and are used to promote physical/mental health, increase nonspecific resistance of body and augment cognition and physiological functions.[23,24] OS has been shown to possess anti antioxidant and anti-stress properties.[25-35] Eugenol forms the major constituent of OS, even though other minor constituents like fixed oils and flavones have also been reported to have pharmacological activities.[36,37] The *Ocimum sanctum* L. has also been suggested to possess antifertility, anticancer, antidiabetic, antifungal, antimicrobial, hepatoprotective, cardioprotective, antiemetic, antispasmodic, analgesic, adaptogenic and diaphoretic

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actions. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituent present in *Ocimum sanctum* L., has been found to be largely responsible for the therapeutic potential of Tulsi (Ocimum sanctum).[38,39] Furthermore, the standardized extract of OS significantly alleviated the chronic hypoperfusion-induced cognitive deficits and ischemia-reperfusion-induced oxidative stress in rats.[40,41] Some recent studies have revealed the anti-genotoxic effect of *Ocimum sanctum* L.[42,43]

Ocimum sanctum has earlier been proved to possess cognition enhancing and antioxidant attributes in other models of cerebrodegenerative disorders. As AD is also associated with cognitive deficits and increased oxidative stress, the effect of OS in Alzheimer's disease was evaluated in well-validated models, using neurotoxins like ibotenic acid and colchicine. Ibotenic acid is a structural analogue of glutamate and causes neuronal necrosis by excitotoxicity stimulating glutamate receptors. Injections of ibotenic acid into the medial septum in rats lead to a profound deficit in spatial learning and memory in the Morris water maze test, a model that represents the advanced stage of AD. It has been reported that intradentate infusion of colchicine induces memory deficits, by destroying granule cells in the dentate gyrus of the hippocampus.[44]

In view of the above observations, the present study was undertaken to explore the effect of the standardized extract of OS in the experimental models of AD.

MATERIALS AND METHODS

Animals

After approval from the Institutional Ethics Committee, the experiments were conducted on in-bred male Charles-Foster rats (150-200 g). The animals were maintained in colony cages, under an ambient temperature of 25 ± 2°C and 45-55% relative humidity, with a 10-hour light/14-hour dark cycle. They were allowed food and water ad libitum; however, food was withdrawn 18 hours prior to surgery. Principles of Laboratory Animals Care and Use guidelines were followed throughout.[45]

Chemicals and Reagents

Ibotenic acid, colchicine, acetylcholine chloride, thiobarbituric acid (TBA), Nicotinamide Adenine Dinucleotide plus Hydrogen (NADH), Nitroblue tetrazolium (NBT) and Phenazine methosulfate (PMS) were obtained from Sigma, USA. 1,1,3,3-Tetraetoxypropane (TEP) was obtained from Merck, Germany. All other chemicals and reagents were purchased locally and were of the highest analytical grade. Donepezil (DOZ) and Imipramine (IMI) were purchased from Sun Pharma, India and Diazepam (DPZ) was purchased from Glaxo, India.

Plant Material and Standardization of Extract

The standardized methanolic extract of the leaves of OS was prepared as per the procedure reported earlier.[46,47] Fresh, tender leaves of OS were collected in the month of December from the Ayurvedic garden of our Institute. The leaves were authenticated by Prof. V.K. Joshi, Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University, Varanasi. The leaves were size reduced and macerated with methanol for seven days. The extract was filtered, vacuum dried and stored in a refrigerator until further use. The yield was 6.04%. The methanolic extract of OS was quantified for the essential oil, eugenol, by High-Performance Thin Layer Chromatography (HPTLC) using a CAMAG assembly (evaluation software© 1990 TLC system; Scanner II. V, 3.14/PC/CTS version), Toluene : ethyl acetate (93 : 7) as a developing solvent, eugenol as standard and quenching at 260 nm. The percentage of eugenol was 5.1%. The animals received this extract orally in dosages of 200 mg/kg/day suspended in 0.3% carboxymethyl cellulose (CMC). This particular dose was selected on the basis of our earlier reports that assessed the ability of the OS extract to attenuate the cerebral reperfusion injury.[40]

Experimental Procedure

Drug treatment

The animals were pretreated with the standardized extract of OS (200 mg/kg/day, p.o) for seven days. On the seventh day after drug dosing, experimental AD was induced in the rats. The day of induction of AD was considered as day 0. Thereafter, neurobehavioural cognitive and biochemical experiments were performed on the seventh, fourteenth and twenty-eighth days, 1 hour after the administration of the respective drug schedule. The standard drugs, donepezil (DOZ; 2 mg/kg/day, p.o), diazepam (DZP, 1 mg/kg/day, p.o) and Imipramine (IMI; 10 mg/kg/day, p.o) were administered from day 1, 1 hour before the experiments, until the end of the study period.

Experimental models for Alzheimer's disease

Colchicine-induced AD: Colchicine was administered via the intacerebroventricular route. Briefly, the right lateral ventricle was cannulated in rats under pentobarbitone sodium (40 mg/kg i.p.) anaesthesia, using stereotaxic coordinates, 0.6 mm posterior to the bregma, 1.8 mm right lateral and 2.7 mm below the cortical surface. Colchicine (15 µg/rat), dissolved in 5 µL of artificial cerebrospinal fluid (ACSF; in nM: NaCl 147, KCl 2.9, MgCl2 1.6, CaCl2 1.7 and dextrose 2.2), was slowly injected into the cannulated right lateral ventricle using a 10 µL Hamilton syringe and the needle was held in place for 2 minutes for proper dispersal of the drug from the tip. Sham control groups were subjected to the same surgical procedure and received artificial cerebrospinal fluid.

Ibotenic acid-induced AD: Ibotenic-induced lesion of nucleus basalis magnacellularis (nbm) was performed based on earlier reports. Bilateral nbm lesions were induced in rats by injecting ibotenic acid (10 μg/rat), dissolved in 5 μL of ACSF with the help of a 10 μL Hamilton syringe, under pentobarbitone sodium (40 mg/kg i.p.) anaesthesia, using stereotaxic coordinates 1.0 mm posterior to bregma, 2.6 mm right/left lateral and 7.9 mm below the cortical surface. The needle was held in position for 2 minutes so that the drug would be properly dispersed.

Behavioural Procedures

Open-field test

Exploratory behaviour was evaluated in an open-field paradigm. The open field was made of plywood and consisted of a floor (96 × 96 cm) with high walls. The entire apparatus was painted black except for 6-mm-thick white lines that divided the floor into 16 squares. Each animal was placed at one corner of the apparatus and for the next 5 minutes they were observed for their ambulations (number of squares crossed), total period of immobility (in seconds), number of rearings, groomings and fecal pellets.

Elevated plus-maze test

The method of Pellow *et al*., 1985, was followed.[48] Briefly, the apparatus consisted of two open arms (50 × 10 cm each), two enclosed arms (50 × 10 × 20 cm each) and a central platform (10 × 10 cm), arranged in such a way that the two arms of each type were opposite each other. The elevation of the maze was 100 cm above the floor. On the seventh day, approximately 1 hour after administration of the standardized OS extract or DZP or the solvent, each animal was placed at the centre of the maze facing one of the enclosed arms. During the 5-minute test period, the number of open and enclosed arm entries plus the time spent in the open and enclosed arms were recorded. Entry into an arm was defined as the point when the animals place all four paws onto the arm. Animal behaviour was taped by using a video camera located above the maze. After the test the maze was carefully cleaned with a wet tissue paper (10% ethanol solution).

Porsolt's swim test

The rat was placed in a cylinder (45 × 20 cm) containing 38 cm of water (25 ± 2°C), so that the rat could not touch the bottom of the cylinder with its hind limb or tail, or climb over the edge of the chamber. Two swim sessions were conducted, with an initial 15 minute pretest, followed by a 5 minute test 24 hours later. Drugs were administered after the pretest. The period of immobility (remained floating in water without struggling and making only those movements necessary to keep its head above water) during the 5 minute test period was noted.[49]

Learned helplessness test

(a) Inescapable shock pretreatment: Electric foot shock was delivered in a 20 × 10 × 10 cm chamber with plexiglass walls and cover. The floor was made of steel grids to deliver electric shock. A constant current shocker was used to deliver 60 scrambled, randomized, inescapable shocks (15-second duration, 0.8 mA, every minute + 15 seconds to grid floor. Control rats were placed for 1 hour in identical chambers, but no shocks were administered. Inescapable shock pretreatment was performed in the morning.[50]

(b) Conditioned avoidance training: In order to evaluate the escape and avoidance performance, avoiding training was initiated 48 hours after the inescapable shock pretreatment in the Sidman jumping box (Techno, Lucknow, India). The jumping box was divided into two equal chambers (27 × 29 × 25 cm) by a plexiglass partition, with a gate providing access to the adjacent compartment through a 14 × 17 cm space. The animals were placed singly in one of the chambers of the jumping box and were allowed to habituate to the test environment for 5 minutes (for the first session only) and then were subjected to 30 avoidance trails (intertrial intervals being 30 seconds). During the first 3 seconds of each trial, a light signal was presented, allowing the animals to avoid shocks. If a response did not occur within this period, a 0.8 mA shock (3 second duration) was applied via the grid floor. In case no escape response occurred within this period, the shock and "light conditioned stimulus" were terminated. Avoidance sessions were performed for three consecutive days (day 3, 4, and 5) in the morning, and the number of escape failures, referred to as 'no crossing response during shock delivery' was recorded.

Morris' water maze test

Spatial learning and memory was tested in a water maze following the method of Pappas *et al*. 1996.[51] The maze consisted of a black circular pool (diameter 2.14 m, height 80 cm) filled to a depth of 44 cm with water (25°C). On post surgical day 14, rats received habituation (exposure in water maze for 1 minute) in which there was no platform present. Then, on day 15, a circular platform (9 cm in diameter) was kept hidden 2 cm below the water level in the centre of one of the quadrants. The platform remained in the same position during all the sessions. At the beginning of each session, a random sequence of four starting poles, along the perimeter of the pool, was generated. All animals followed this sequence for that session. Each rat was placed in the water facing the wall at the start location and was allowed 90 seconds to find the hidden platform. The animal was allowed a 20-second rest on the platform. The latency to reach the platform was recorded. If the rat was unable to locate the hidden platform, it was lifted out and placed on the platform for 20 seconds. The procedure was repeated for all the four start locations.

Two sessions of four trials each were conducted on the first day of testing separated by 4 hours, and one session of four trials was conducted on the next day. Following that, the platform was removed and a probe trial (without platform) was conducted 4 hours later. Each rat was placed in the pool at the same, randomly selected starting pole; the swimming path was observed and time spent in the quadrant of the pool, which initially contained platform, was measured.

Biochemical Analysis

Assessment of oxidant-antioxidant status

An antioxidant study was performed on the forebrains of ibotenic acid- and colchicine -induced AD in rats on the seventh, fourteenth and twenty-eighth days after induction of AD with ibotenic acid and colchicine. The levels of lipid peroxidation (LPO) and superoxide dismutase (SOD) activities were measured. Estimation of lipid peroxidation was done by measuring the levels of malondialdehyde (MDA), a by-product of lipid peroxidation. The rat forebrain regions were rinsed with ice-cold normal saline. To 1 g of wet tissue, 9 ml of 1.12% KCl was added. The mixture was then homogenized with the help of a Teflon homogenizer and 200 µl of the whole homogenate was taken for the assay. The concentration of MDA in brain homogenates was expressed in terms of nM MDA/mg protein. 1, 1′, 3, 3′,-tetraethoxypropane was used as a reference compound. Evaluation of the SOD activity was done on the rat brains, which were homogenized in ice-cold sodium pyrophosphate buffer (pH 8.3), in a ratio of 50 mg/ml, and 200 µl of this homogenate was used for the assay. The inhibition by SOD of reduction of NBT to blue-coloured chromogen in the presence of PMS and NADH was measured at 560 nm. One unit of enzyme activity was defined as the enzyme concentration required to inhibit the absorbance at 560 nm of chromogen production by 50% in 1 minute, under an assay condition, and expressed as the specific activity in milliunits per milligram of protein.[52]

Acetylcholinesterase Activity

The anti cholinesterase activity was measured on the seventh, fourteenth and twenty-eighth days after AD was induced by ibotenic acid and colchine. Rat brains were homogenized with the Teflon homogenizer in an M/15 ice-cold phosphate buffer (pH 7.2) and 10 µL of this homogenate was used for further assay. The colour developed was read at 540 nm and the results were expressed as µmol of ACh hydrolysed/g of brain tissue.[53]

Statistical Analysis

Statistical analysis of data (using GraphPad InStat, Version 3.05, 32 bit for Win95/NT, GraphPad Software, San Diego, CA, USA) was performed by applying one-way analysis of variance (ANOVA) followed by Tukey test for biochemical parameters and Newman Kueles's test for behavioural observations. A value, <0.05, was considered statistically significant.

RESULTS AND OBSERVATIONS

Effect of OS on Learning and Memory in AD Rats

The results of Morris' water maze are summarized in [Table 1]. In the first session of the escape latency trail, the latency to locate the submerged platform was significantly increased compared to sham controls in both IB- and Col-lesioned rats during the fourteenth and twenty-eighth day, but not on day 7. In the second and third session, the same trend was observed, but on all the experimental days, the OS treatment significantly decreased time to locate the submerged platform in IB- and Col-lesioned rats during all the sessions except on day 7, in Col-lesioned rats. In the probe trial a significant difference in the spatial bias of animals towards the quadrant of the pool that contained the submerged platform during the escape latency was observed. The animals of both IB and Col control groups swam for lesser time on day 7 [$P < 0.05$] in the quadrant where the platform was kept earlier. However, a change in swimming time was not altered on days 14 and 28. Day 14 and day 28 comparisons of IB OS with IB control and Col OS with Col control show that the OS-treated group swam for a significantly longer time [$P < 0.05$] compared to the corresponding control groups.

In a new platform trial, the animals of the IB control and Col control groups took more time to find the platform kept in a different quadrant than that of the sham-operated animals, which reached a significant level [$P < 0.05$] on day 14 and day 28. OS treatment in IB-lesioned animals made them reach the new platform much earlier than the IB control group animals, which was significant on day 14 and day 28. OS treatment in Col lesion animals reduced the time requirement on all days [$P < 0.05$].

The data of active avoidance study shows that normally, in sham-operated and OS per se animals there was a decreasing trend in the number of trials required to achieve 100% avoidance from day 7 to day 28, which reached a significant level [$P < 0.05$] only on the twenty-eighth day [Table 2]. No significance was found between these groups when the comparison was done for the corresponding days. Comparison of sham versus IB control showed that from day 7 there was an increase in the number of trials required to achieve 100% learning. On day 7 it was significant at $P < 0.05$, but on day 14 and day 28 the significance level was $P < 0.01$. Intra group comparison of the IB control group showed an increasing trend in the number of trials required, but they were not significant. Comparison between the sham and Col control groups showed a significant increase in the number of trials required on all days [$P < 0.05$]. OS

Table 1: Effect of OS on Morris' water maze test in Ibotenic acid-and Colchicine-induced AD in rats

Groups	Day 7				Day 14				Day 28			
	Escape latency 1	Escape latency 2	Escape latency 3	Probe trail	New platform	Escape latency 1	Escape latency 2	Escape latency 3	Probe trail	New platform	Escape latency 1	Escape latency 2	Escape latency 3	Probe trail	New platform
Sham	56.00 ± 1.71	33.00 ± 2.13	16.00 ± 2.65	34.50 ± 1.61	18.00 ± 1.51	51.80 ± 2.31	33.80 ± 1.66	21.40 ± 1.50	27.60 ± 2.58	14.60 ± 1.72	52.00 ± 1.77	32.67 ± 2.08	20.00 ± 1.83	23.00 ± 2.39	15.33 ± 1.91
OS *per se*	46.00 ± 2.96	25.00 ± 1.81	20.00 ± 2.02	34.00 ± 1.71	14.00 ± 1.69	55.00 ± 1.84	32.00 ± 2.32	18.00 ± 1.59	28.00 ± 1.91	17.00 ± 0.82	50.00 ± 1.39	29.00 ± 2.03	17.67 ± 1.82	31.00 ± 1.98	16.00 ± 2.03
IB control	69.33 ± 3.38[a]	48.33 ± 2.79[a@]	32.33 ± 2.23[a@]	15.50 ± 2.26	23.67 ± 1.61	74.67 ± 3.03[a]	47.83 ± 2.24[a@]	37.67 ± 2.43[a@]	22.17 ± 1.54	34.67 ± 3.00[a]	74.17 ± 2.27[a]	45.33 ± 1.74[a@]	38.67 ± 1.96[a@]	25.33 ± 2.11	41.17 ± 2.74[a]
Col control	63.33 ± 3.09	44.50 ± 3.02[a@]	30.67 ± 2.19[a@]	18.33 ± 2.79	27.33 ± 2.08	68.17 ± 2.88[a]	48.83 ± 2.83[a@]	35.83 ± 1.78[a@]	17.17 ± 2.27	30.83 ± 1.92[a]	71.50 ± 1.84[a]	52.00 ± 1.77[a@]	37.67 ± 2.23[a@]	14.17 ± 2.14	35.00 ± 2.28[a]
IB OS	63.50 ± 2.57[b]	35.33 ± 3.00[b@]	14.33 ± 2.23[b@]	29.33 ± 2.16	14.67 ± 1.58	54.33 ± 1.72[b]	28.17 ± 1.96[b@]	16.83 ± 1.80[b@]	34.83 ± 2.18[b]	24.50 ± 2.43[b]	50.67 ± 1.54[b]	27.33 ± 1.73[b@]	14.83 ± 1.76[b@]	30.67 ± 2.16	21.67 ± 1.14[b]
IB DOZ	57.33 ± 2.75[b]	28.50 ± 2.01[b]	24.33 ± 2.11[b]	29.33 ± 1.82	16.50 ± 1.93	60.17 ± 2.36[b]	30.50 ± 1.18[b]	24.67 ± 2.80[@]	29.50 ± 2.39	19.00 ± 2.67[b]	68.83 ± 2.79	23.50 ± 1.93[b@]	17.83 ± 1.35[b@]	34.83 ± 2.68b	21.67 ± 1.52[b]
Col OS	63.17 ± 1.85	31.33 ± 1.69[c@]	18.50 ± 1.31[c@]	30.17 ± 1.17	18.67 ± 1.02	58.17 ± 1.51[c]	25.33 ± 1.20[c@]	19.83 ± 0.87[c@]	28.67 ± 1.89[c]	16.33 ± 0.89[c]	57.50 ± 2.36	27.17 ± 1.83[c@]	19.17 ± 1.62[c@]	28.83 ± 1.51	10.50 ± 0.96[c]
Col DOZ	59.33 ± 2.36	26.00 ± 1.39	22.83 ± 1.28[c]	30.33 ± 2.01	14.00 ± 1.15	59.83 ± 2.29[c]	28.33 ± 1.56[c@]	19.50 ± 1.59[c@]	31.67 ± 2.04[c]	16.83 ± 1.38[c]	60.17 ± 2.43[c]	24.50 ± 1.75[c@]	16.67 ± 0.95[c@]	26.50 ± 0.89	22.67 ± 1.36[c]

[a] $P < 0.05$ compared to sham control; [b]$P < 0.05$ compared to IB control; [c]$P < 0.05$ compared to Col control during the same day. *$P < 0.05$ seventh day compared to fourteenth and twenty-eighth day; #$P < 0.05$ fourteenth day compared to twenty-eighth day. @$P < 0.05$ compared between latancy 1, 2 and 3. (n = 6, all data are expressed as mean ± SEM)

Table 2: Effect of OS on active avoidance paradigm in Ibotenic acid and Colchicine induced AD in rats

Groups	Day 7	Day 14	Day 28
Sham control	5.00 ± 0.68	3.67 ± 0.42	2.00 ± 0.37*
OS *per se*	4.83 ± 0.31	3.83 ± 0.54	2.50 ±0.43
IB control	8.33 ± 0.76[a]	9.67 ± 0.88[a]	10.66 ± 0.72[a]
Col control	7.83 ± 0.60	8.83 ± 0.54[a]	9.50 ± 0.62[a]
IB OS	5.83 ± 0.70[b]	4.33 ± 0.62[b]	3.67 ± 0.56[b]
IB IMI	4.83 ± 0.60[b]	3.67 ± 0.33[b]	2.50 ± 0.34[b]
Col OS	5.50 ± 0.56	3.83 ± 0.60[c]	3.17 ± 0.48[b]
Col IMI	3.67 ± 0.33	2.83 ± 0.31[c]	1.83 ± 0.31[c]

[a]$P < 0.05$ compared to sham control; [b]$P < 0.05$ compared to IB control; [c]$P < 0.05$ compared to Col control during the same day. *$P < 0.05$ seventh day compared to fourteenth and twenty-eighth day day; #$P < 0.05$ fourteenth day compared to twenty-eighth day. (n = 6, all data are expressed in s as mean ± SEM)

treatment in both IB- and Col-lesioned groups decreased the number of trials required to reach 100% acquisition when compared to the corresponding control groups [$P < 0.05$]. In both IB- and Col-lesioned groups, DOZ significantly reduced the number of trials required [$P < 0.01$].

Effects of OS on Anxiety in AD Rats

Ocimum sanctum pretreatment reversed the anxiogenic responses in IB- and Col-induced AD rats. The effect of OS on IB- and Col-induced experimental AD rats in an open field test is summarized in [Table 3]. No differences were observed between sham operated animals, normal, or OS per se groups on all days, for all parameters. IB-induced reduction in ambulations was observed on the seventh day compared to sham [$P < 0.05$], but these changes were not observed in the following 14 or 28 days. In contrast there were no significant changes in ambulations in the Col-treated groups compared to the controls. OS did not increase ambulations per se, but significantly increased the ambulations in IB- and Col-lesioned rats. This effect was comparable was comparable with that of the DZP treatment [$P < 0.05$] throughout the experimental period. Immobility increased in both IB and Col groups compared to sham rats [$P < 0.05$]. OS treatment in both IB- and Col-lesioned animals significantly reduced immobility, which was comparable with that of the DZP group [$P < 0.05$] on all the experimental days. The number of rearings were reduced only on the seventh day in IB- and Col-lesioned rats [$P < 0.05$] compared to the sham-operated animals. This effect was reversed only in the IB, but not the Col-lesioned rats, with OS treatment [$P < 0.05$], on all days.

The effects of OS on IB- and Col-induced experimental AD in the elevated plus maze test are summarized in [Table 4]. The time spent and the entries were decreased in the open arm and a subsequent increase was observed in the closed arm on day 7, with IB- and Col-lesioned animals [$P < 0.05$] indicating anxiogenic response. However, this trend was observed even during days 14 and 28 with time spent, but not on entries in the open arm or closed arm. OS treatment

Table 3: Effect of OS on ambulations, immobility and rearing in the open-field test in Ibotenic acid- and Colchicine-induced AD in rats.

Group	Ambulation (no.)			Immobility (sec.)			Rearing (no.)		
	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28
Sham control	60.83 ± 2.24	61.83 ± 2.60	61.67 ± 2.29	37.33 ± 3.41	40.67 ± 3.89	42.00 ± 2.84	17.83 ± 1.35	19.5 ± 0.99	20.67 ± 1.56
OS *per se*	66.33 ± 2.40	66.5 ± 2.20	67.33 ± 2.10	47.00 ± 4.96	42.50 ± 4.46	40.50 ± 3.61	18.00 ± 1.31	18.17 ± 1.64	19.17 ± 0.70
IB control	49.33 ± 2.04[a]	53.00 ± 2.34[a]	52.83 ± 2.55	72.33 ± 3.01[a]	79.67 ± 4.52[a]	79.83 ± 5.74[a]	10.83 ± 1.01[a]	9.83 ± 0.95	9.83 ± 0.98
Col control	54.50 ± 2.54	56.83 ± 2.65	55.00 ± 2.90	79.17 ± 4.9[a]	82.67 ± 3.16[a]	80.67 ± 2.95[a]	12.50 ± 1.34[a]	11.67 ± 0.84	10.50 ± 0.67
IB OS	78.33 ± 1.67[b]	81.00 ± 1.65[b]	78.00 ± 1.67[b]	36.67 ± 2.68[b]	44.00 ± 2.39[b]	45.50 ± 1.98[b]	17.17 ± 1.30[b]	17.67 ± 1.17[b]	17.00 ± 1.29[b]
IB DOZ	57.00 ± 2.54	49.83 ± 1.64	52.17 ± 2.73	75.00 ± 6.04	79.33 ± 5.8$_0$	78.83 ± 6.24	9.33 ± 0.96	10.17 ± 1.08	10.67 ± 0.83
IB DZP	75.17 ± 3.42[b]	79.16 ± 2.24[b]	79.83 ± 1.56[b]	40.00 ± 3.2[b]	39.00 ± 2.60[b]	40.17 ± 2.90[b]	18.33 ± 1.54	17.17 ± 1.54	19.00 ± 0.58[b]
Col OS	66.17 ± 2.73[c]	67.50 ± 3.23	68.67 ± 1.93[c]	44.00 ± 4.65[c]	40.83 ± 3.10[c]	44.83 ± 4.03[c]	11.67 ± 1.31	12.17 ± 1.40	13.33 ± 1.26
Col DOZ	47.33 ± 1.63	47.0 ± 2.41	48.33 ± 1.63	81.17 ± 6.82	85.83 ± 6.07	78.17 ± 5.70	11.00 ± 1.16	12.17 ± 0.87	11.67 ± 1.36
Col DZP	72.50 ± 3.82[c]	72.00 ± 3.71[c]	72.17 ± 4.18[c]	40.33 ± 3.84[c]	41.00 ± 2.80[c]	41.33 ± 3.38[c]	17.33 ± 1.09	15.50 ± 1.91	16.50 ± 1.84[c]

[a]$P < 0.05$ compared to sham control; [b]$P < 0.05$ compared to IB control; [c]$P < 0.05$ compared to Col control during the same day; [*]$P < 0.05$ on the seventh day compared to the fourteenth and twenty-eighth days; [#]$P < 0.05$ on the fourteenth day compared to the twenty-eighth day. (n = 6, all data are expressed as mean ± SEM)

reversed these changes only in Col, but not in IB-lesioned animals. The open arm to closed arm ratio was decreased compared to the controls indicating loss of spontaneous motor activity. The ratio was reversed by OS treatment only in the Col-lesioned rats and not in IB-lesioned rats [$P < 0.05$].

Effects of OS on Depression in AD Rats

The result of OS on Porsolt's swim test is summarized in [Table 5]. The immobility period compared to sham animals was increased both in IB and Col control groups indicating increased depression with neurotoxins [$P < 0.01$]. A temporal progression of depression was observed and behavioural despair was significantly different on day 28 compared to day 7 [$P < 0.05$]. OS treatment in both IB- and Col-lesioned animals decreased the immobility period on all days, which was statistically significant [$P < 0.05$], but although there was a decreasing trend in the immobility time as the days progressed, the difference was not significant within the respective groups. The antidepressant effect of OS was comparable to those observed with IMI on the corresponding days.

Change in Biochemical Parameters

Effect of OS on lipid peroxidation in AD rats

Change in MDA level

Effect of OS on MDA level in IB- and Col-induced experimental AD is summarized in [Table 6]. An intergroup comparison between normal/OS per se versus sham-operated animals, showed that there was no statistical significance between these groups. In both IB and Col control groups there was a significant increase in MDA levels when compared to sham-operated group [$P < 0.05$]. Pretreatment with OS in both IB- and Col-lesioned animals decreased MDA levels on all days compared to their corresponding control groups [$P < 0.05$].

Change in SOD level

Effect of OS on SOD level in IB- and Col-induced experimental AD is summarized in [Table 6]. An intergroup comparison between normal / OS perse vs sham-operated animals showed no statistical significance between these groups. In IB and Col control groups there was a significant increase [$P < 0.01$] in SOD activity with respect to sham-operated group. Pretreatment with OS in both IB- and Col-lesioned groups reduced SOD activity on all days when compared with their corresponding control [$P < 0.05$]. These alterations were of significance on the same days (inter group), but not on different days (intra group).

Acetylcholinesterase activity

Both IB and Col control groups showed a decrease in the values of Ach hydrolysed when compared with the sham operated group on corresponding days, but were not significant. OS pretreatment in IB- and Col-lesioned groups did not significantly alter the values of Ach hydrolysed with respect to their corresponding control animals [Table 6].

DISCUSSION

The present study, for the first time, demonstrates the beneficial effects of the standardized extract of *O. sanctum* in IB

Table 4: Effect of OS on time spent and number of entries elevated plus maze test in Ibotenic acid-and Colchicine-induced AD in rats

Group	Time spent								Number of entries							
	Day 7			Day 14			Day 28			Day 7			Day 14			Day 28		
	OA	CA	Ratio	OA	CA	Ratio	OA	CA	Ratio	OA	CA	Ratio	OA	CA	Ratio	OA	CA	Ratio
Sham control	46.5 ± 4.14	194.00 ± 23.19	0.24 ± 0.02	47.30 ± 2.99	198.00 ± 34.91	0.24 ± 0.02	44.00 ± 3.96	195.50 ± 35.12	0.23 ± 0.03	7.83 ± 0.70	3.83 ± 0.79	2.52 ± 0.54	6.83 ± 0.60	4.67 ± 0.88	1.86 ± 0.55	8.66 ± 1.31	3.00 ± 0.73	3.75 ± 0.92
OS *per se*	38.17 ± 3.34	201.00 ± 13.69	0.19 ± 0.02	36.33 ± 3.93	201.50 ± 34.37	0.18 ± 0.02	45.00 ± 2.78	199.17 ± 33.47	0.23 ± 0.01	6.83 ± 1.17	5.50 ± 0.62	1.37 ± 0.36	5.33 ± 0.76	5.66 ± 0.67	1.02 ± 0.22	5.50 ± 0.76	4.50 ± 0.76	1.48 ± 0.36
IB control	30.17 ± 3.07[a]	219.83 ± 23.22[a]	0.14 ± 0.02[a]	33.67 ± 2.11[a]	220.50 ± 33.01[a]	0.15 ± 0.01[a]	31.33 ± 1.67	222.33 ± 43.64[a]	0.14 ± 0.05[a]	4.00 ± 0.37[a]	7.00 ± 0.86	0.62 ± 0.10[a]	3.83 ± 0.40	2.67 ± 0.33	0.50 ± 0.06	4.00 ± 0.44[a]	8.17 ± 0.60[a]	0.49 ± 0.05[a]
Col control	28.33 ± 1.69[a]	233.33 ± 14.82[a]	0.12 ± 0.01[a]	28.67 ± 2.12[a]	232.83 ± 45.13[a]	0.13 ± 0.01[a]	28.33 ± 2.36[a]	232.50 ± 43.02[a]	0.12 ± 0.01[a]	5.17 ± 0.60	7.33 ± 0.76	0.73 ± 0.09	5.33 ± 0.42	.17 ± 0.91	0.80 ± 0.11	3.83 ± 0.47[a]	7.83 ± 0.98[a]	0.52 ± 0.10[a]
IB OS	38.33 ± 2.49	212.67 ± 33.48	0.18 ± 0.01	37.67 ± 2.10	213.83 ± 32.98	0.17 ± 0.01	36.83 ± 1.70	215.83 ± 23.67	0.17 ± 0.01	10.5 ± 0.92[b]	3.50 ± 0.67[b]	3.03 ± 0.74[b]	10.33 ± 0.61[b]	3.50 ± 0.72[b]	3.77 ± 0.81[b]	10.17 ± 0.87[b]	3.17 ± 0.60[b]	3.83 ± 0.69[b]
IB DOZ	20.00 ± 1.46	251.00 ± 32.44	0.08 ± 0.01	19.17 ± 1.33[b]	251.83 ± 42.21[b]	0.07 ± 0.01	22.17 ± 1.40	250.83 ± 53.92[b]	0.08 ± 0.01	4.00 ± 0.73	8.50 ± 1.06	0.50 ± 0.09	5.50 ± 0.81	8.17 ± 1.28	0.71 ± 0.11	3.00 ± 0.37	7.50 ± 1.34	0.47 ± 0.09
Col OS	41.50 ± 3.47[c]	203.00 ± 23.43[c]	0.21 ± 0.02[c]	44.50 ± 2.49[c]	201.67 ± 12.93[c]	0.22 ± 0.01[c]	2.33 ± 3.64[c]	203.17 ± 23.09[c]	0.21 ± 0.01[c]	10.0 ± 0.97[c]	3.67 ± 0.42[c]	2.95 ± 0.47[c]	9.00 ± 0.73[c]	.33 ± 0.56[c]	2.78 ± 0.41[c]	.17 ± 0.70[c]	4.33 ± 0.56[c]	2.12 ± 0.37[c]
Col DOZ	15.83 ± 2.09	249.50 ± 23.49	0.06 ± 0.01	15.33 ± 1.82	251.50 ± 43.77[c]	0.06 ± 0.01	17.33 ± 1.63	250.83 ± 43.38[c]	0.07 ± 0.01	4.67 ± 0.49	8.33 ± 0.42	0.56 ± 0.05	3.83 ± 0.48	9.17 ± 0.60	0.41 ± 0.06	33 ± 0.56	9.83 ± 0.60	0.35 ± 0.06

[a] $P < 0.05$ compared to sham control; [b] $P < 0.05$ compared to IB control; [c] $P < 0.05$ compared to Col control during the same day. * $P < 0.05$ on the seventh day compared to the fourteenth and twenty-eighth days; # $P < 0.05$ on the fourteenth day compared to the twenty-eighth day. (n = 6, all data are expressed as mean ± SEM)

Table 5: Effect of OS on Porsolts' swim test in Ibotenic acid and Colchicine

Groups	Day 7	Day 14	Day 28
Sham control	37.33 ± 2.99	43.00 ± 2.06	41.00 ± 1.84
OS *per se*	43.00 ± 2.13	41.00 ± 2.53	40.00 ± 0.58
IB control	91.00 ± 2.58[a]	110.0 ± 3.48[a]	114.0 ± 3.12[a*]
Col control	99.00 ± 3.99[a]	108.0 ± 3.47[a]	119.0 ± 3.97[a*]
IB OS	71.33 ± 1.76[b]	74.00 ± 2.16[b]	66.00 ± 2.46[b]
IB IMI	64.00 ± 2.9[b]	67.00 ± 3.20[b]	70.00 ± 3.08[b]
Col OS	69.00 ± 2.85[c]	67.00 ± 3.06[c]	72.00 ± 2.52[c]
Col IMI	61.33 ± 1.99[c]	64.00 ± 2.16[c]	69.00 ± 3.99[c]

[a] $P < 0.05$ compared to sham control; [b] $P < 0.05$ compared to IB control; [c] $P < 0.05$ compared to Col control during the same day. * $P < 0.05$ on the seventh day compared to the fourteenth and twenty-eighth days; # $P < 0.05$ on the fourteenth day compared to the twenty-eighth day

and Col-induced AD in rats. OS significantly ameliorated the cognitive deficit induced by the above neurotoxins in rats.

Rats treated with OS showed shorter swimming latencies to the goal platform than the placebo groups, indicating improved reference, i.e., spatial memory performance. OS-treated rats also showed enhanced working memory in probe trials, indicating consolidation of memory. The ability to find the new platform kept in a different opposite quadrant was significantly compromised in both IB and Col-treated rats. OS treatment reversed the above memory inability and thereby increased reversal learning.

This indicates that OS augments learning *per se,* as the animal is able to learn a new aspect of the given task, resulting in a better performance. Hence, OS not only improves cognition, but also facilitates acquisition of new information. OS also improves memory deficits in the active avoidance task. OS treatment reverses the increased escape latencies with IB and Col treatment, indicating improvement of memory with regard to negative reinforcement to electric shock. Although both the paradigms are based on negative reinforcement they are different from each other, particularly with the nature of information acquired through negative reinforcement. The active avoidance paradigm is influenced by anxiety parameters, while the Morris water maze is more sensitive to spatial learning, dependent on the extra-maze cues.[54] OS is able to alleviate learning disabilities induced by IB and Col in the active avoidance test, which is more influenced by psychopharmacological parameters. Hence, OS should also influence the psychological parameters associated with learning disabilities. To test this hypothesis the effect of OS on anxiety and depression parameters with IB and Col treatment was evaluated.

OS has reversed the decrease in ambulations induced by IB and ameliorated the decrease in immobility induced by IB and Col in the open-field test indicating anxiolytic activity. It is interesting to note that although OS showed anxiolytic activity in neurotoxin-treated rats, it did not show any anxiolytic activity *per se*. In another validated

Table 6: Effect of OS on lipid peroxidation and acetylcholinesterase activity in Ibotenic acid and Colchicine induced AD in rats. The data represents MDA levels in nM/mg of brain protein, SOD m units/mg of protein, and n mols of acetylcholine hydrolysed/g of brain tissue as mean ± SEM, (n = 6)

Groups	Malondialdehyde			Superoxide dismutase			Acetylcholinesterase activity		
	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28
Sham control	2.20 ± 0.20	1.96 ± 0.41	2.05 ± 0.32	313.50 ± 22.46	300.33 ± 23.84	307.00 ± 44.78	38.60 ± 3.94	37.42 ± 4.64	42.48 ± 2.73
OS *per se*	2.17 ± 0.40	2.01 ± 0.43	1.95 ± 0.47	303.50 ± 24.94	318.00 ± 35.13	309.30 ± 34.77	36.66 ± 7.68	36.20 ± 6.83	39.29 ± 4.51
IB control	6.04 ± 0.50[a]	6.52 ± 0.50[a]	6.57 ± 0.61[a]	750.67 ± 45.64[a]	739.00 ± 63.61[a]	763.83 ± 56.42[a]	12.49 ± 3.41[d]	15.38 ± 2.38[d]	10.35 ± 1.73[d]
Col control	5.42 ± 0.50[a]	5.91 ± 0.47[a]	5.90 ± 0.45[a]	697.83 ± 45.81[a]	704.83 ± 53.53[a]	722.33 ± 64.57[a]	10.56 ± 2.25[d]	14.49 ± 3.47[d]	13.78 ± 3.15[d]
IB OS	4.75 ± 0.40[b]	4.91 ± 0.62[b]	4.79 ± 0.40[b]	569.50 ± 34.80[b]	576.33 ± 46.37[b]	542.83 ± 54.92[b]	24.38 ± 6.83	27.74 ± 5.90	23.46 ± 4.47**
IB DOZ	6.12 ± 0.50	6.14 ± 0.67	6.05 ± 0.56	760.50 ± 64.77	737.33 ± 55.44	753.00 ± 66.87	30.02 ± 6.27	33.78 ± 5.81	21.63 ± 4.16**
Col OS	4.25 ± 0.60[c]	4.50 ± 0.80[c]	4.20 ± 0.63[c]	513.33 ± 33.83[c]	504.00 ± 44.25[c]	524.50 ± 33.88[c]	23.09 ± 3.32	29.24 ± 4.29	22.81 ± 6.28**
Col DOZ	5.89 ± 0.40	5.57 ± 0.59	5.97 ± 0.68	718.83 ± 59.17	712.00 ± 68.59	715.17 ± 67.42	29.65 ± 4.49	30.48 ± 4.89	26.28 ± 4.68**

[a]$P < 0.05$ compared to sham control; [b]$P < 0.05$ compared to IB control; [c]$P < 0.05$ compared to Col control during the same day. *$P < 0.05$ seventh day compared to fourteenth and twenty-eighth day; #$P < 0.05$ fourteenth day compared to twenty-eighth day; For Acetylcholinesterase activity: d = Sham control Vs IB control, Col control, **= Sham control Vs all treatments

model of anxiety, the elevated plus maze test OS treatment significantly reversed the decrease in open arm to closed arm ratio induced by IB and Col indicating anxiolytic activity. Similar to the trend observed in the open-field test, OS did not show any anxiolytic activity *per se*.

Ibotenic acid and Colchcine increased the immobility in the Porsalt's swim test, which is indicative of depression.[55] OS significantly alleviated depression, as observed from the decrease in immobility time in the IB and Col-treated group, although OS *per se* did not have any antidepressant activity. From the above observations it is clear that the anxiolytic and antidepressant action is due to the amelioration of psychological effect, induced by IB and Col, rather than a direct anxiolytic or antidepressant activity. Hence, OS may also potentially alleviate secondary psychological disorders associated with Alzheimer's disease.

Oxidative stress is a critical determinant in the stimulation of neuronal cell death, and Aβ toxicity results in an increase in the reactive oxygen species (ROS) and superoxide radicals, which result in oxidative damage within the cell. The toxicity of Aβ is attenuated by treatment with antioxidants such as vitamin E, as well as agents that decrease intracellular superoxide levels.[17] OS treatment significantly attenuated the effects of IB and Col on lipid peroxidation. There was a significant increase in lipid peroxidation in terms of MDA levels in the whole brain, both with IB and Col. Increase in free radical generation results in lipid peroxidation of cell membrane and a subsequent accumulation of its byproduct MDA. Oxidative damage plays a key role in AD-associated cell death, with the level of Aβ-associated neural cell damage being correlated with the extent of lipid peroxidation within the cell. OS pretreatment from day 7 to day 28 significantly protected neurotoxin-induced oxidative stress. Further, it attenuated the upregulation of SOD activity. Increase in SOD activity was due to the activation of self-defence anti-oxidant response to an excessive generation of free radicals. However, the increased SOD activity is overwhelmed by the increased oxidative stress observed in neurodegenerative disorders. Further, the product of SOD activity, H_2O_2, is more toxic and leads to a further generation of toxic hydroxyl radicals in the presence of iron. Hence, increased SOD activity in neurodegeneration can be more deleterious than beneficial. In addition to causing oxidative damage itself, oxidative stress can also serve to potentiate Aβ toxicity, while endogenous antioxidants such as the *bcl-2* proto-oncogene attenuate Aβ toxicity. [56] The effects of oxidative injury, which include damage to proteins, DNA, and lipids, along with membrane lipid peroxidation, may also be partially responsible for other aspects of Aβ -associated cell damage, including the impairment of glucose and glutamate transport systems that occur in the neurons.[57] In view of the above facts the anti-oxidant effect of OS may be one of the important factors responsible for the beneficial effects in the rat models of AD used in the present study. This is in consonant with the earlier studies reporting the anti-oxidant effects of OS, although OS did not show any anti-oxidant effect *per se,* but was able to mitigate the effects of oxidative stress in the animal models used in this study.

The muscarinic receptor G-protein function is disrupted by Aβ and this effect may probably be mediated by the generation of free radicals.[57] The cholinergic loss in AD

is a major component of neuropathology, which has been strongly demonstrated by the fact that cholinesterase inhibitors are effective in alleviating the symptoms of AD.[58] Cholinergic neurotransmission with the acetylcholinesterase inhibitor, physostigmine, reverses scopolamine-induced deficits in nondemented subjects and has been reported to improve the performance of AD patients in tasks that require long-term memory.[59] Treatment with IB and Col significantly reduces acetylcholine esterase activity. However, OS has not shown any significant anticholinesterase activity. The cholinergic system is not the only neurotransmitter system that degenerates in AD. Other systems, such as the serotonergic and noradrenergic systems, are also affected by the disease.[60,61] However, some reports have suggested that the cholinergic therapy may also reduce amyloid accumulation.[62] Although OS did not show anti-cholinesterase activity its effect on the cholinergic system still remains to be investigated.

The standardized extract of OS significantly improved learning and memory deficits associated with IB and Col. Further, OS alleviated the neuropsychological symptoms associated with animal models of AD. The beneficial effect observed with OS can be attributed to its anti-oxidant activity.

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