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RESEARCH ARTICLE

Kalayanaka ghrita Ameliorates Okadaic Acid Induced Memory Deficits in Wistar Rats

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Abstract

Background: Alzheimer's disease depicts the characteristic features of intracellular neurofibrillary tangles and extracellular amyloid plaques. *Kalyanaka ghrita* (KG) is an *Ayurvedic* formulation used to treat impaired learning and memory such as *Manasmandata* or *Buddhimandyata*. KG is traditionally used to enhance memory. The constituents present in KG are also reported to be memory-enhancing, anti-inflammatory, and antioxidant and KG is reported to be effective in neurodegeneration in rats induced by *β*-amyloid. **Aim:** This study is aimed to validate the effect of KG on memory deficit, tauopathy and neurodegeneration induced by intracerebroventricular administration of okadaic acid. **Methods:** Okadaic acid was administered intracerebroventricularly on day 7 to the Wistar rats. KG was administered orally or intranasal from day 14 to 35 to the respective groups. The behavioural parameters on spatial memory, social recognition, and novel object recognition tests were determined. The molecular parameters such as brain acetylcholinesterase activity, protein phosphatase 2A, antioxidant parameters, monoamine levels and the brain histopathology were studied. **Results:** KG treatment significantly improved cognition, as evidenced by a decrease in escape latency, path length, and social and novel object recognition tests. KG treatment also increased the brain Protein phosphatase 2A, reversed the oxidative stress, and decreased brain acetylcholinesterase. The brain monoamines were reversed upon treatment with KG. Further, the molecular and histological studies confirmed the prevention of neuronal damage. **Conclusion:** These findings imply the traditional nootropic property of KG. The neuroprotective properties and decreased neurofibrillary tangles upon treatment with KG suggest KG to be a potential therapy for AD-like neurodegeneration.

Keywords: Alzheimer's Disease, Brain Monoamines, *Kalyanaka ghrita*, Neuroprotection, Okadaic Acid

Abbreviations: AD: Alzheimer's Disease; OKA: Okadaic Acid; KG: *Kalyanaka ghrita*; API: *Ayurvedic Pharmacopoeia of India*; HPLC: High-Performance Liquid Chromatography; MWM: Morris Water Maze; PP2A: Protein Phosphatase 2A; ICV: Intracerebroventricular; NORT: Novel Object Recognition Test; SRT: Social Recognition Test; AChE: Acetylcholinesterase; MDA: Malondialdehyde; NO; Nitric Oxide; GSH; Reduced Glutathione; SOD: Superoxide Dismutase; CAT: Catalase; BSA: Bovine Serum Albumin; 5HT: 5-Hydroxy Tryptamine; DA: Dopamine; NE: Norepinephrine; Aβ: Amyloid *β*; CCSEA: Committee for the Control and Supervision of Experiments on Animals; SIT1: Social Interaction Time1; SIT2: Social Interaction Time2; RI: Recognition Index; PCA: Perchloric Acid; H and E: Haematoxylin and Eosin stain; CA3: *Cornu Ammonis*; h-tau: Hyperphosphorylated tau; GSK3*β*: Glycogen Synthase Kinase 3*β*; NFT: Neurofibrillary tangles

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1. Introduction

Alzheimer's Disease (AD) leads to dementia has marked by extreme neurodegenerative changes with cerebral atrophy, detriment of synapses, neurons and memory. The presence of intracellular Neurofibrillary Tangles (NFTs) and extracellular amyloid plaques are key factors in AD. NFTs are formed when normal phosphorylation events are disrupted, resulting in abnormally hyperphosphorylated tau protein deposits[1]. Protein phosphatases 1/2A (PP2A) prevent the *in vivo* hyperphosphorylation of tau. Reduced activity of PP2A enhances *in vivo* tau hyperphosphorylation and leads to the generation of neurofibrillary tangles. Okadaic acid (OKA) is a powerful serine/threonine phosphatase 1 and 2A inhibitor, that results in the onset of tau hyperphosphorylation[2]. It is also reported to inhibit acetylcholine (ACh) and increase oxidative stress as evidenced by increased lipid peroxidation, decreased reduced glutathione (GSH), and reduced anti-oxidative enzyme activity. This increase in oxidative stress by oxygen and nitrogen-centred free radicals causes activation of microglia that causes cognitive deficits and neurodegeneration in AD. Many processes of the brain like fear, memory, learning, pain and neuropathologies involve neurotransmitters. The disturbances in these neurotransmitter systems partly are responsible for memory impairment, depression and attention deficits, which are symptoms of AD[3]. The altered monoamine levels in AD may be attributed to both age and disease. The monoaminergic system of dopamine (DA), serotonin (5-HT), and noradrenaline (NA) govern attention, perception and learning that are features of cognitive functions. This system also modulates motivation, mood and emotions[4]. Targeting the Aβ pathway alone did not prevent neurodegeneration in AD which necessitates the development of tau-directed therapies and multitarget therapies to address the monoamine changes. *Kalyanaka ghrita* (KG), an *Ayurvedic* formulation, is considered as *Medhyarasayana*. KG is used traditionally to treat memory deficit in all three components of memory as per the *Ayurvedic* principle – *dhi* (perception), *smriti* (memory) and *dhriti* (memory retention). It is used traditionally in the treatment of psychological and physical developmental disability[5]. A clinical study reported that *Kalyanaka ghrita* causes cognitive deficits in *Manasmandata* or *Buddhimandyata* in youngsters, including subnormal general intellectual performance, decreased learning, social adjustment, and maturation. KG treatment has been also reported to improve thinking, perception, memory, language, motivation and skilled movements[6].

The presence of *shalaparni*, *haridra*, *dadima* and *devadaru* in KG makes it a potential formulation to act on several mechanistic pathways involved in AD since these compounds have antioxidant, anticholinesterase, Aβ aggregation inhibition and stabilizing of microtubule- activities[5]. Ghee is one of the key components of the formulation, which helps carry ingredients to the brain. Standardization of the KG formulation, used in the present study, for its active constituent curcumin, chebulagic acid, berberine, gallic acid, and tannic acid by HPLC is reported in our earlier study. The neuroprotective and cognitive enhancing effects of KG in neurodegeneration induced by the administration of β-amyloid are reported. A1-42-induced neurotoxicity in rats was markedly reversed by KG, which also raised brain BDNF and antioxidant status and dramatically enhanced cognition and memory[7]. However, the effect of KG on the tau pathway of AD was not explored in the earlier studies.

The prime cause of tau deposition by the formation of neurofibrillary tangles can be targeted by stabilizing the microtubules[2]. Curcumin, one of the constituents of KG, has microtubule stabilizing properties along with a reduction in brain oxidative stress, inflammatory cytokines, acetylcholinesterase, and thus preventing neuronal damage without any adverse effects[8]. Considering these facts, this study evaluated the role of KG in OKA-induced cognitive and memory deficits. The study also attempted to determine the brain monoamines, oxidative stress, acetylcholinesterase, protein phosphatase 2 A and deposition of neurofibrillary tangles induced by OKA in Wistar rats.

2. Experimental Design

2.1 Animals

The research proposal was sanctioned by the Institutional Animal Ethics Committee (IAEC) of Bharati Vidyapeeth Poona College of Pharmacy registered with the Committee for Control and

Supervision of Experiments on Animals (CCSEA) with approved protocol number IAEC/PCL15/2020-2021. The animals, male adult Wistar rats of 200–230 g were procured from Global Bioresearch, Pune. The animals were housed in a 12:12 h light: dark cycle with the supply of feed (Nutrivet Feeds, India) and water was provided for the entire study. The rats were quarantined and acclimatized for one week before the study. All the studies took place between 9 a.m. and 2 p.m.

2.2 Materials

Okadaic acid was procured from Sigma Aldrich, St. Louis, MO, USA (Catalogue No. O9381- 25UG, Lot#SLBV0493). Memantine was procured from Sun Pharma Laboratories India Private Limited. (Batch no 2HX2752A). A Serine Threonine phosphatase kit manufactured by Upstate Cell Signalling Solutions, USA was procured. Dopamine (Catalogue No. 17-313 Lot#22051), norepinephrine (Cas no. 62-31-7), and 5-HT (Cas no. 51-41-2) were sources of Sigma Aldrich. The reagents and chemicals used in the experimentation with analytical grade. OKA was dissolved in 0.29 mM dimethyl sulfoxide (DMSO) and diluted with phosphate buffer saline to the desired concentration[9].

2.3 Formulation of *Kalyanaka ghrita*

The plant ingredients; *Terminalia chebula* Retz. (Black or chebulic Myrobalan) [*Combretaceae*], *Terminaliabellirica* (Gaertn.) Roxb. (Behada) [*Combretaceae*], *Phyllanthus emblica* L. (*Amla*) [*Phyllanthaceae*], *Curcuma longa* L. (Turmeric) [*Zingiberaceae*], *Elettaria cardamomum* (L) Maton (Cardamom) [*Zingiberaceae*], *Rubia cordifolia* L. (Indian *maddar*) [*Rubiaceae*], *Hemidesmus indicus* (L.). R. Br. (Anantamul) [*Apocynaceae*], *Amomum subulatum* Roxb. (*Badi elaichi*) [*Zingiberaceae*], *Punica granatum* L. (Pomegranate) [*Lythraceae*] and *Valeriana jatamansi* Jones ex Roxb. (Synonym: *Valeriana wallichi* DC.) (Tagara) [*Caprifoliaceae*] were authenticated at Agharkar Research Institute, Pune, Maharashtra with the authentication numbers, AUTH20-145, 146, 147, 148, 149, 150, 151, 152, 153 and 154, respectively. [Whole plant of *Desodium gangeticum* (L) DC (*Fabaceae*)], [*Abies spectabilis* leaves (D. Don) Spach (*Pinaceae*)], [*Jasminum officinale* Linn. (*Oleaceae*), whole plant], [root *of Saussurea lappa* C.B. Clarke (*Asteraceae*)], [*Embelia ribes* Burm. (*Myrsinaceae*). Fruit], [*Solanum indicum* Linn. (*Solanaceae*), whole plant], [Fruit, *Citrullus colocynthis* (L.) Schrader (*Cucurbitaceae*)], [wood of *Pterocarpus santalinus* Linn. (*Fabaceae*)], [*Cedrus deodara* (Roxb.) Loud. (*Pinaceae*), wood], underwent authentication in the National Institute of Siddha, Tamil Nadu, Ministry of AYUSH (Government of India) with the identification numbers NISMB4692021-1-9, respectively. The traditional method as per API was followed for the KG preparation as per the steps outlined in the previous study[7].

2.4 Injection of Intracerebroventricular (ICV) Okadaic Acid

The combination of propofol (85mg/kg i.p) and xylazine (5 mg/kg i.p) is used as anaesthesia for animals. The scalp was shaved, cleansed and sliced, incised with a midline sagittal region. The skull was exposed and placed in a stereotaxic frame at the co-ordinates lateral ventricles: 0.8 mm posterior to the bregma, 1.5 mm lateral to the sagittal suture, and 3.6 mm, from pointing out the bregma[10]. At these coordinates on both sides of the bregma, burr holes were drilled. Okadaic acid (100 ng in 10µL) was injected per side, to administer 200 ng per animal[11]. After the injection, Hamilton's syringe remained in position for 5min for the prevention of backflow. The skin was sutured and antiseptic ointment (Povidone iodine) was applied daily. The rats were administered oral amoxicillin, and oral glucose for four days after surgery as postoperative care. The regular diet was provided after 4 days of surgery. Sham animals were operated following the above procedure and 10µl of water for injection was administered bilaterally instead of okadaic acid[12].

2.5 Design for Experiments

2.5.1 Grouping

A total of 80 animals were used in the study out of which 64 animals were induced with okadaic acid, 8 animals were kept as control and 8 animals were operated on with sham. The okadaic acid-induced animals were grouped into 8 groups with 8 animals in each: Neurodegeneration control, Memantine (standard), *Kalyanaka ghrita* oral at doses of 1g/kg, 2g/kg, and 4g/kg OD, *Kalyanaka ghrita* twice daily, at nasal doses of 25µl, 50µl, and 100µl. Naïve animals (n=8) were allotted to the vehicle control group. The

control groups (sham and vehicle) were administered cow ghee. Standard group Memantine (1 mg/kg) p.o. once daily was administered to the rats[13].

2.5.2 Study Design

The training on Morris Water Maze (MWM) was conducted for the first 5 days. The rats underwent okadaic acid induction on day 7. The animals were kept for recovery protocol for a week. The respective treatment was administered from day 14 to day 35. The spatial memory in MWM was conducted on days 7, 14 and 35. Novel object recognition tests and social recognition tests were conducted on days 19 and 20, respectively. On day 35, all groups of rats were humanely killed, and the brains were isolated quickly, and cleaned with ice-cold saline. The brains were divided into 3 portions - one portion was homogenized in 0.1mol/L ice-cold perchloric acid for determination of monoamines, the second part of the brain was used for estimation of biochemical, and the third portion was used for histopathology.

2.6 Estimation of Parameters of Memory

2.6.1 Morris Water Maze

In the animals, the spatial memory was evaluated using the Morris water maze[14]. This apparatus consists of a water tank with a diameter of 150 cm and 45 cm height consisting of water to 40 cm height at 25°C. A platform of 15 cm diameter was located inside the tank, kept hidden 2 cm under the water level. Vividly coloured cues, which can be seen from the pool for guiding rats, were placed on the outside of the maze at the same place throughout the study. For the initial 5 days, the animals practised reaching the platform with 4 training trials per day for 60 s each with a duration of 15 s between each trial. The animals were placed in the maze at one of the 4 different beginning positions, the order of which was chosen at random. The rat was allowed to be located on the platform for 10 s once the animal reached. The animal which was not able to reach within 60 s was directed to the platform with a cane and was made to settle in the platform for 10 s. The spatial memory was evaluated on day 7 (before induction), day 14 (after 7 days of induction, before treatment) and day 35 (after 21 days of treatment). During evaluation, each animal was kept at the same beginning location of the maze. The animal was permitted to swim until it reached and climbed onto the submerged platform. The path length (cm) was the distance and escape latency was the time taken to approach the platform[15] which was determined using a video tracking system and Maze master software.

2.6.2 Social Recognition Test

On day 19, a social interaction procedure was conducted for the evaluation of short-term memory. The animals were housed separately on day 17 in a home cage for habituation. On day 19, a 25-30 days-old rat was introduced for a 5 min session in the home cage. After 2 h, another session of 5 min 2nd interaction was observed. The time duration in which the rat spent in active social investigations such as biting, jumping, sniffing and dragging over or under the juvenile rats was noted. The first and second sessions were marked as SIT1 and SIT2, respectively[16]. % Reduction in Social Interaction Time (% RSIT) was measured using formula (% RSIT) = [(SIT1- SIT2) x 100 / SIT1].

2.6.3 Novel Object Recognition Test (NORT)

The short-time memory was evaluated on day 20 using NORT. The animals had access to explore 2 identical objects during the familiarization phase of 5 min. After 2 h, another unfamiliar object was placed with one of the identical objects. The preference of the animal to explore the unfamiliar object was recorded as time expended with it. The olfactory cues were cleaned by using 0.1 % sodium bicarbonate[17].

The formula for the Recognition index is Recognition Index (RI) = Time spent by novel object X 100/(Time spent with novel object + Time spent with familiar object)

2.7 Biochemical Evaluation

2.7.1 Brain Homogenate Preparation

The rats were humanly killed on day 35, their brains were isolated with ice-cold saline, kept in (pH 7.4) ice-cold phosphate buffer, homogenized then centrifuged (12000 rpm, 10 min, and 4°C). The acetylcholinesterase activity, PP2A activity, lipid peroxidation and antioxidant activity were determined in the homogenate[18].

2.7.2 Determination of Brain Acetylcholinesterase Activity

Acetylcholinesterase (AChE) activity was determined by Ellman's assay. In this procedure, the hydrolysis of

ACh occurred due to the reaction of thiocholine with Ellman's reagent. The % inhibition was calculated using the mean of change in absorbance of 5 intervals at 412nm[19].

2.7.3 Estimation of Brain PP2 Activity

ELISA method of Malachite green phosphatase was used to evaluate PP2A activity. Malachite green phosphatase was prepared by adding 1 1ml of solution A, with 10 µl solution B, which was placed aside for 30 min at room temperature. 100 µl of this solution was used for the assay. 12.5 µl of tissue supernatant was added to 12.5 µl of phosphopeptide and kept at room temperature for 30 min. 100 µL of Malachite green solution was added to stop the reaction. The plate was kept for 15 min for colour development at room temperature and the absorbance was taken at 620nm [As per kit instructions Catalogue No. 17-313 Lot#22051].

2.7.4 Estimation of Brain Tissue Oxidative Stress

The Malondialdehyde (MDA) and nitric oxide (NO)[20], the anti-oxidants like reduced Glutathione (GSH), Superoxide Dismutase (SOD)[21] and catalase[22] contents of tissue were determined adapting reported methods.

2.8 Estimation of Monoamines

The monoamine levels of the brain were measured by the HPLC method. The tissues were homogenized with 2 mL cysteine (30 ng/mL at 0.1 mol/L PCA) that were centrifuged for 15 min, 12000 rpm at 4 °C. A 0.22 µm syringe filter was used to filter the supernatant. HPLC system with a fluorescence detector (Jasco), Thermosil C-18 250 X 4.6 mm column (5 m) and C- Phenomenex guard column (18, 4X3 mm ID) were utilized to record peaks at room temperature with Borvion software (Jasco, Japan). The HPLC column was loaded with 20µL of test sample and the excitation wavelengths of 280 nm and emission wavelengths of 315 nm were used. The concentration of norepinephrine (NE), dopamine (DA), 5-hydroxytryptamine (5-HT), and cysteine were measured, with cysteine as an internal standard[4].

2.9 Histopathology of the Rat Hippocampus

The brains were isolated and stored in neutral buffered formaldehyde of 10% and processed for histopathology. The rat hippocampus was set in paraffin and sliced into 5 µm thick sections for histostaining. Changes in the morphology of the hippocampus were assessed by H and E stain and the hippocampus was stained using silver stain for assessingneurofibrillarytangles[23].

2.10 Statistical Analysis

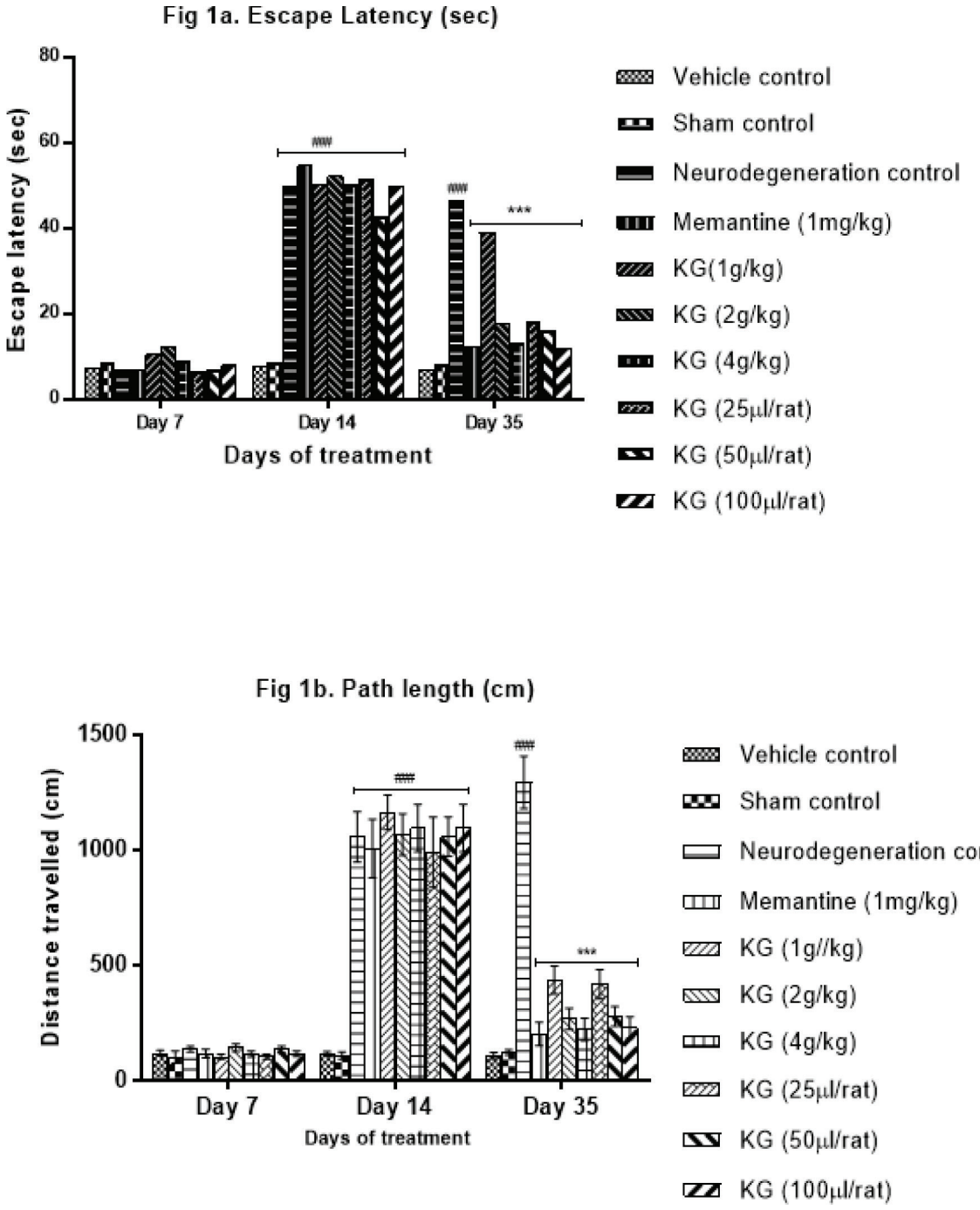
The results were represented using mean ± S.E.M. The data was analyzed with two-way ANOVA concerning Bonferroni's test for behavioural assessment tests and One-way Analysis Of Variance (ANOVA) with Dunnett's tests for biochemical parameters as well as for monoamines estimation. The Graph Pad Prism® demo software version was used. $p<0.05$ statistical significance.

3. Results

3.1 Effect of KG in MWM on Spatial Memory

KG has a role in spatial memory was tested by MWM. The investigation consisted of a training phase and retention trials. On day 6, all the groups of animals reached the platform in 1 min. On day 14, after 7 days of induction with okadaic acid, a significant increase in escape latency ($F(9,70)=20.09$, $p<0.001$) with a failure to reach the platform in 1 min in all the induced animals was observed. A similar increase in escape latency was observed with the neurodegeneration control group on day 35 (Figure 1a). However, treatment with KG for 21 days resulted in a significant ($p<0.001$) decrease in escape latency in KG-treated animals (Figure 1a) except for KG 1 g/Kg treated rats.

The path taken to arrive at the platform in MWM was represented by the path length. There was a significant improvement in the path length ($F(9,70) = 26.89$, $p<0.001$) in all animals induced with okadaic acid compared to sham control as observed on day 14. Further, a similar increase in path length was found in neurodegeneration control on day 35 (Figure 1b). From day 14 the animals were treated with KG which resulted in a significantly ($p<0.001$) decreased path travelled in all doses of KG (oral and nasal) treatment compared to neurodegeneration control as observed on day 35 (Figure 1b). Further, treatment with memantine for 21 days significantly ($p<0.001$) lowered the time taken and distance travelled compared with neurodegeneration control.



Data was expressed as Mean ± SEM. (n=8). Data were analysed using two-way ANOVA followed by the Bonferroni post-test. ### p<0.001 as compared with the sham control group. ***p<0.001 as compared to neurodegeneration control.

Figure 1. Effect of *Kalyanaka Ghrita* on **(a).** Escape latency (sec), **(b).** Pathlength (cm) in Morris water maze in Oka-induced AD in rats.

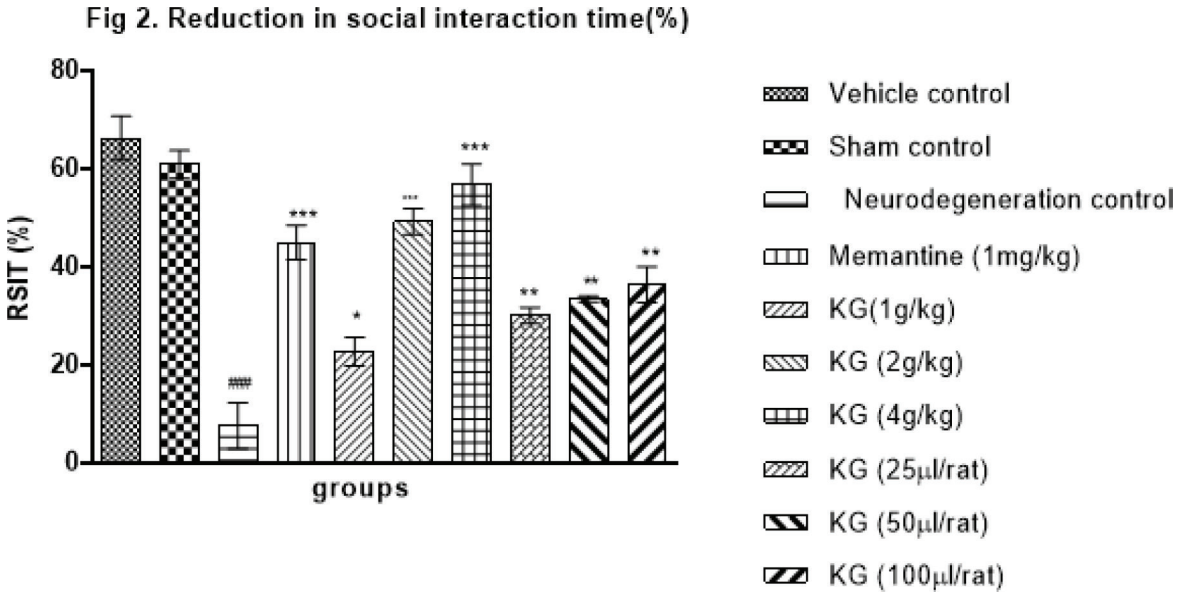
3.2 Role of KG in SRT (% RSIT)

SRT was performed to evaluate the ability of test animals to recognize the unfamiliar juvenile rat when exposed for the second time in a shorter time and is expressed as % RSIT. The % RSIT of the sham and vehicle control rats did not change in SIT2. The % of RSIT was considerably lower in the neurodegenerative control animals ($F(9,70) = 29.12$, $p<0.01$). In comparison to the neurodegenerative control group, KG therapy (oral 2 g/kg and 4 g/kg) causes a significant ($p<0.01$)

improvement of % RSIT (Figure 2). Further, intranasal KG also resulted in a ($p<0.01$) significant reduction in % RSIT. However, KG (1g/kg oral) treatment significantly ($p<0.05$) improved %RSIT marginally. Treatment with Memantine (1 mg/Kg) likewise produced the substantial ($p<0.001$) advancement of a %RSIT when compared to neurodegenerative control.

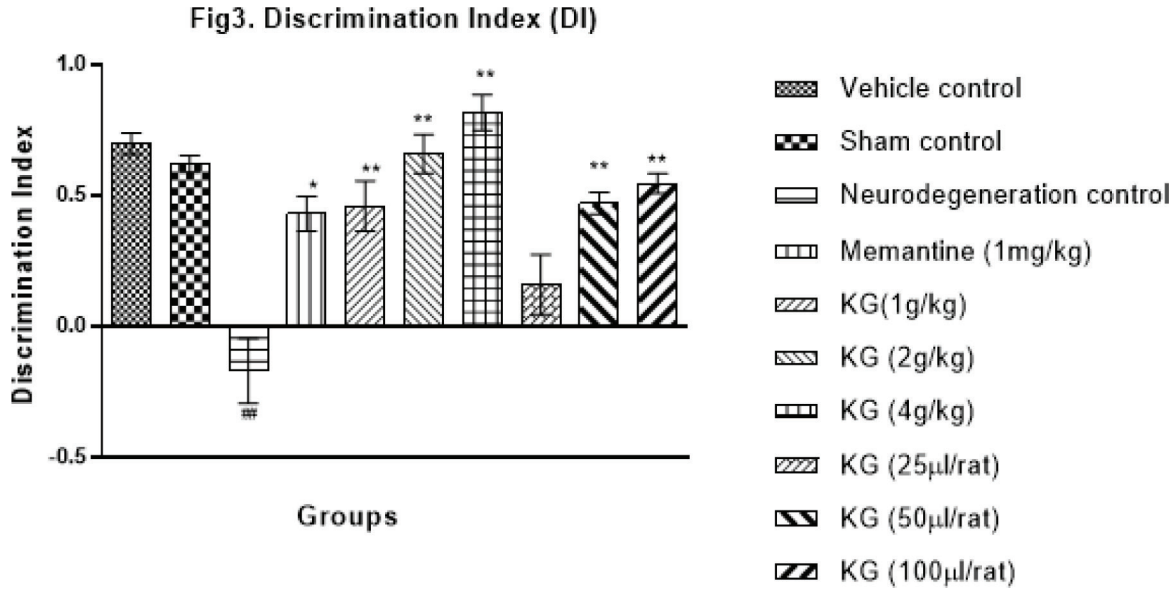
3.2.1 Role of KG in Discrimination Index (DI)

NORT was performed to estimate short-term memory and was expressed as a discrimination index (Figure 3). Induction with okadaic acid, in the neurodegeneration control group, caused a marked ($p<0.001$) decrease ($F(9, 70) = 14.85$) in the discrimination index as opposed to the control group.



Data was expressed as Mean ± SEM. (n= 8). Data were analysed using one-way ANOVA followed by Dunnett's test. ###p<0.001 as compared with the sham control group.*p<0.05 and **p<0.01, ***p<0.001, as compared with neurodegeneration control.

Figure 2. Effect of *Kalyanaka ghrita* on social interaction time (%) in OKA-induced AD in rats.



Data was expressed as Mean ± SEM. (n= 8). Data were analysed using one-way ANOVA followed by Dunnett's test. ##p<0.01 as compared with the sham control group.*p<0.05 and **p<0.01 as compared with the Neurodegeneration control group.

Figure 3. Effect of *Kalyanaka ghrita* on Discrimination index in OKA-induced AD in rats.

The memory in neurodegeneration control was impaired as proven by the increase in time explored with familiar objects compared to novel objects. KG treatment (oral and intranasal medium and high doses) caused a marked ($p<0.01$) improvement in memory related to short-term as opposed to neurodegeneration control. However, KG at lower doses did not improve short-term memory.

3.3 Effect of KG on Biochemical and Molecular Parameters

3.3.1 Role of KG on AChE

The AChE significantly ($p<0.001$) increased ($F(9,70)= 40.67$) in the hippocampus of okadaic acid control rats compared to sham control. KG at higher doses markedly decreased ($p<0.001$) the brain acetylcholinesterase activity when compared with the sham control group. Further, KG (oral 1g/kg, 2g/kg, and intranasal 25, 50 and 100 µl) treatment significantly ($p<0.01$) decreased acetylcholinesterase activity, compared with neurodegeneration control. This confirms the AChE inhibition property of KG (Figure 4).

3.3.2 Effect of KG on PP2A Activity

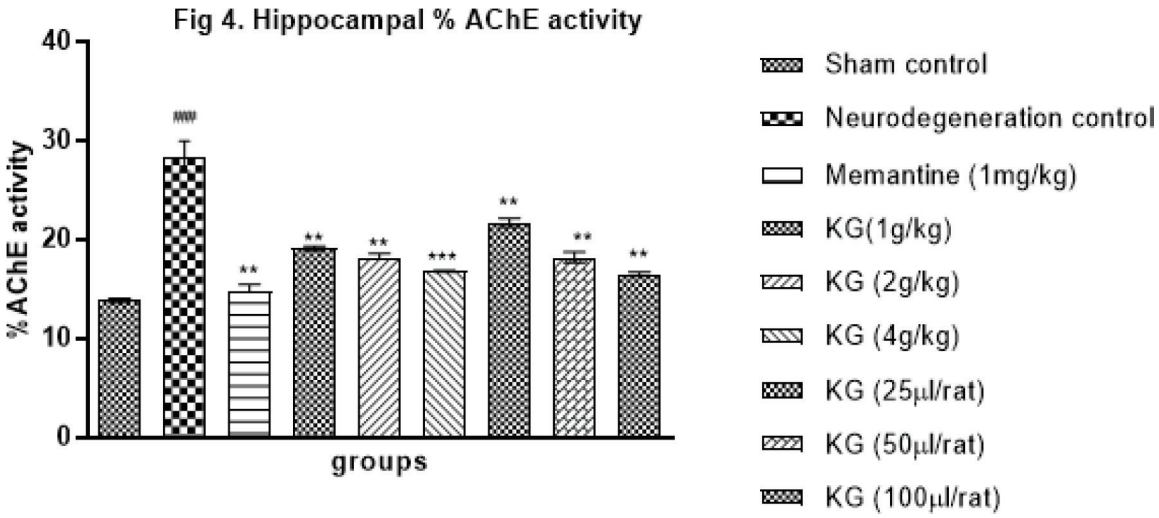
The hippocampal PP2A levels of the neurodegeneration control group were significantly ($p<0.001$) decreased ($F(9,70) = 176.7$) in comparison to the sham group. The KG oral (all groups) as well as nasal (50, and 100 µl/rat) treatment significantly improved ($p<0.01$) the PP2A levels in comparison with neurodegeneration control. However, KG (25 µl intranasal) and memantine (1 mg/Kg) caused minimally ($p<0.05$) increased PP2A activity in comparison to neurodegeneration control (Figure 5).

3.3.3 Effect of KG on Brain Monoamines

There was a significant ($p<0.01$) downregulated brain DA, NE, and 5-HT levels in the neurodegeneration control group. KG treatment resulted in significant ($p<0.01$)increased DA levels ($F(9, 70)= 233.3$) compared to neurodegeneration control (Figure 6a). KG oral and intranasal (medium and high doses) treatment represent the marked significantly ($F(9,70) = 38.78$, $p<0.01$) upregulated NE levels with comparison to neurodegeneration control (Figure 6b). Further, KG oral low dose and nasal low dose caused minimal ($p<0.05$) increase in brain norepinephrine. KG (oral 2, 4 g/kg and 100 µl/rat intranasal) treatment causes a moderately significant ($p<0.01$) increased 5-HT when compared to neurodegeneration control. Further KG (oral 1g/kg and 25 and 50 µl intranasal) caused a minimal ($p<0.05$) increase of 5-HT levels as compared to okadaic acid injected rats (Figure 6c).

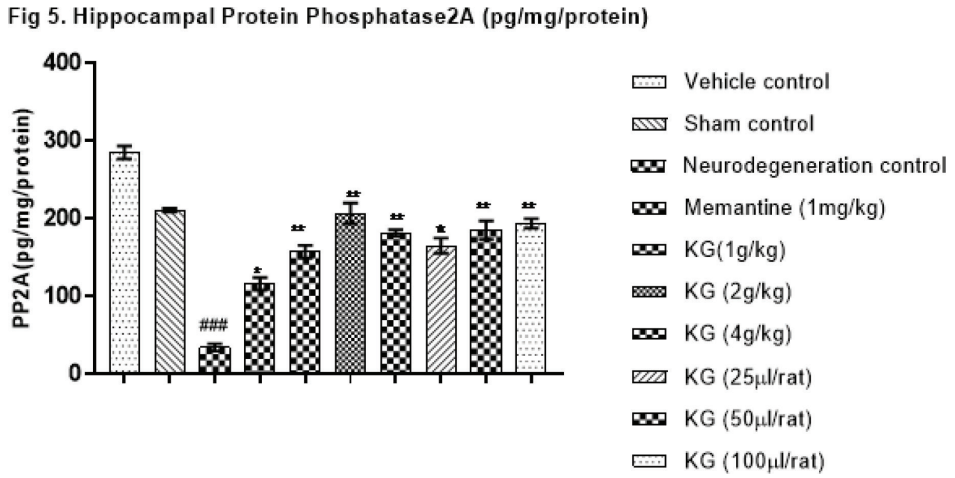
3.4 Effect of KG on Lipid Peroxidation

The brain MDA as well as NO levels (nmol/mg protein) were observed as significant ($p<0.001$) up-regulation of neurodegeneration control as compared to sham



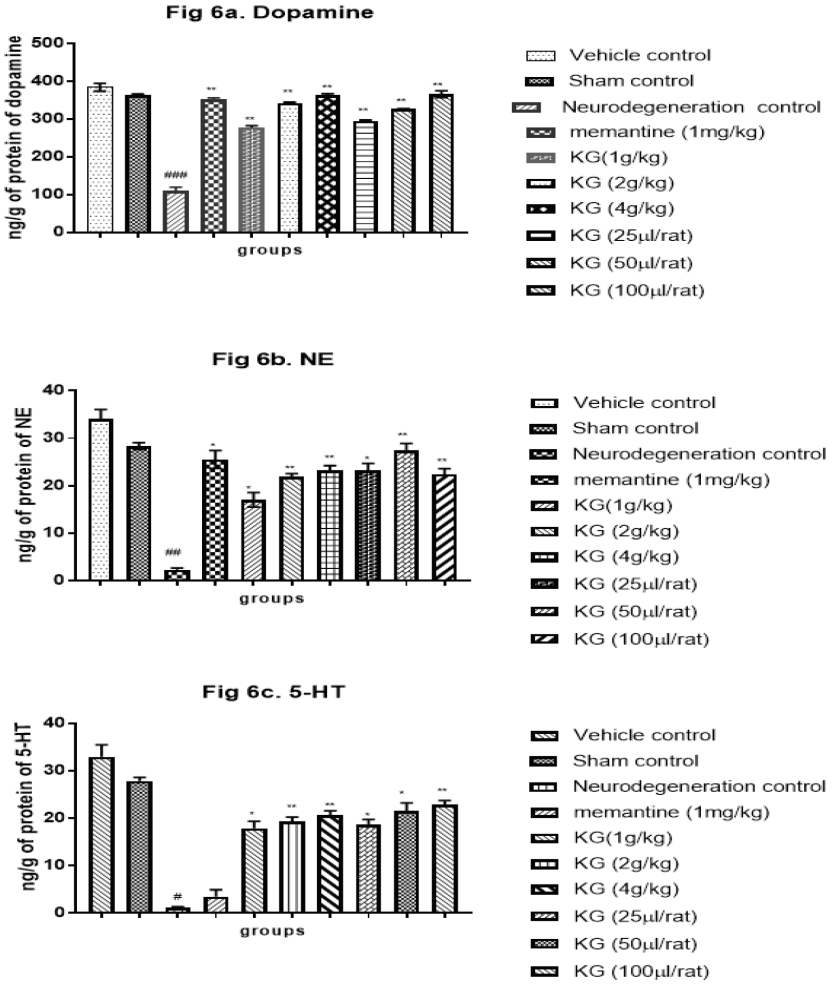
Data was expressed as Mean ± SEM. (n= 8). Data were analysed using one-way ANOVA followed by Dunnett's test. ###p<0.001 as compared with the sham control group. **p<0.01, and ***p<0.001, as compared with the Neurodegeneration control group.

Figure 4. Effect of *Kalyanaka ghrita* on % AChE activity in OKA-induced AD in rats.



Data was expressed as Mean ± SEM. (n= 8). Data were analysed using one-way ANOVA followed by Dunnett's test. ###p<0.001 as compared with the sham control group. *p<0.05 and **p<0.01 as compared to the neurodegeneration control group.

Figure 5. Effect of *Kalyanaka ghrita* on PP2A in OKA-induced AD in rats.



Data was expressed as Mean ± SEM. (n = 8). Data were analysed by one-way ANOVA followed by Dunnett's test #p<0.05, ##p<0.01 and ###p<0.001 as compared with the sham control group. *p<0.05 and **p <0.01 as compared with the Neurodegeneration control group, respectively.

Figure 6. Effect of *Kalyanaka ghrita* on monoamines. **(a)** Dopamine, **(b)** Norepinephrine and **(c)** Serotonin (5-HT)) in OKA-induced AD in rats.

control. KG (oral and intranasal) treatments resulted in significantly ($p<0.001$) decreased ($F(9,70)=88.25$) MDA (Table 1). Furthermore, the treatment KG has shown a significant ($p<0.001$) reduction in ($F(9,70)= 156.2$) NO in comparison to the neurodegeneration group (Table 1). The brain anti-oxidants were significantly ($p<0.001$) decreased in the neurodegeneration group. The KG treatment in all tested groups reported a significant ($p<0.01$) increase ($F(9,70) = 1307$) in GSH level compared to neurodegeneration control (Table 1). In addition, KG treatment resulted in a significant ($p<0.01$) increase ($F(9,70) = 579.8$) in SOD levels (Table 1). Further, the level of catalase was also increased ($F(9,70) = 107.4$) significantly ($p<0.01$) upon treatment with KG as compared to the neurodegeneration control group (Table 1). However, the minimal ($p<0.05$) increase of catalase was observed upon treatment of intranasal KG 25 µl/rat, compared to the neurodegeneration control group.

3.5 Histopathology of Rat Hippocampus

The effect of KG on okadaic acid-induced neurodegeneration was investigated by observing the H and E-stained sections of the hippocampus of rats. In the neurodegeneration control group, there were marked degenerative changes at the CA3 regions and *dentate gyrus*. Further, changes in the hippocampus architecture were also observed in neurodegeneration control. KG (oral 4g/kg and intranasal 100 µl/rat) treatment caused moderate reversal of neurodegenerative changes in the *dentate gyrus* as well as the hippocampal CA3 region. However, KG treatment (oral 1 - 2 g/kg and 25 µl – 50 µl) caused minimal reversal of neurodegenerative changes in CA3 regions and dentate gyrus (Table 2). Furthermore, treatment with KG has reversed the okadaic acid-induced changes in the architecture of the hippocampus (Figure 7). Staining with silver stain revealed the formation of the characteristic tangles in the neurodegenerative control compared to the control. KG treatment (4 g/kg and 100 µl/rat) caused a moderate reversal of tangles formation in the hippocampal CA3 region and *dentate gyrus*. However, KG (1g/kg, 2g/kg oral and 25µl, 50µl/rat intranasal) caused a minimal reversal of tangles formation in the CA3 region and *dentate gyrus* (Figure 8).

4. Discussion

In the neurodegenerative disorder, Alzheimer's disease, the main features are β – amyloid ($A\beta$) deposits and neurofibrillary tangles which manifest with cognitive and behavioural impairments[24]. Memantine the noncholinergic drug is an FDA-approved standard drug for the treatment of Alzheimer's disease which antagonizes the NMDA receptor. The antidementic effect of the drug, memantine was investigated on neurotoxicity changes in cholinergic markers to explore clinical relevance[13] and AChE inhibitors such as donepezil, rivastigmine and galantamine and are causing the side effects like diarrhoea, nausea, leg cramps, abnormal dreams and bradycardia. These

Table 1. Effect of *Kalyanaka ghrita* on oxidative stress and anti-oxidant markers in OKA-induced AD in rats

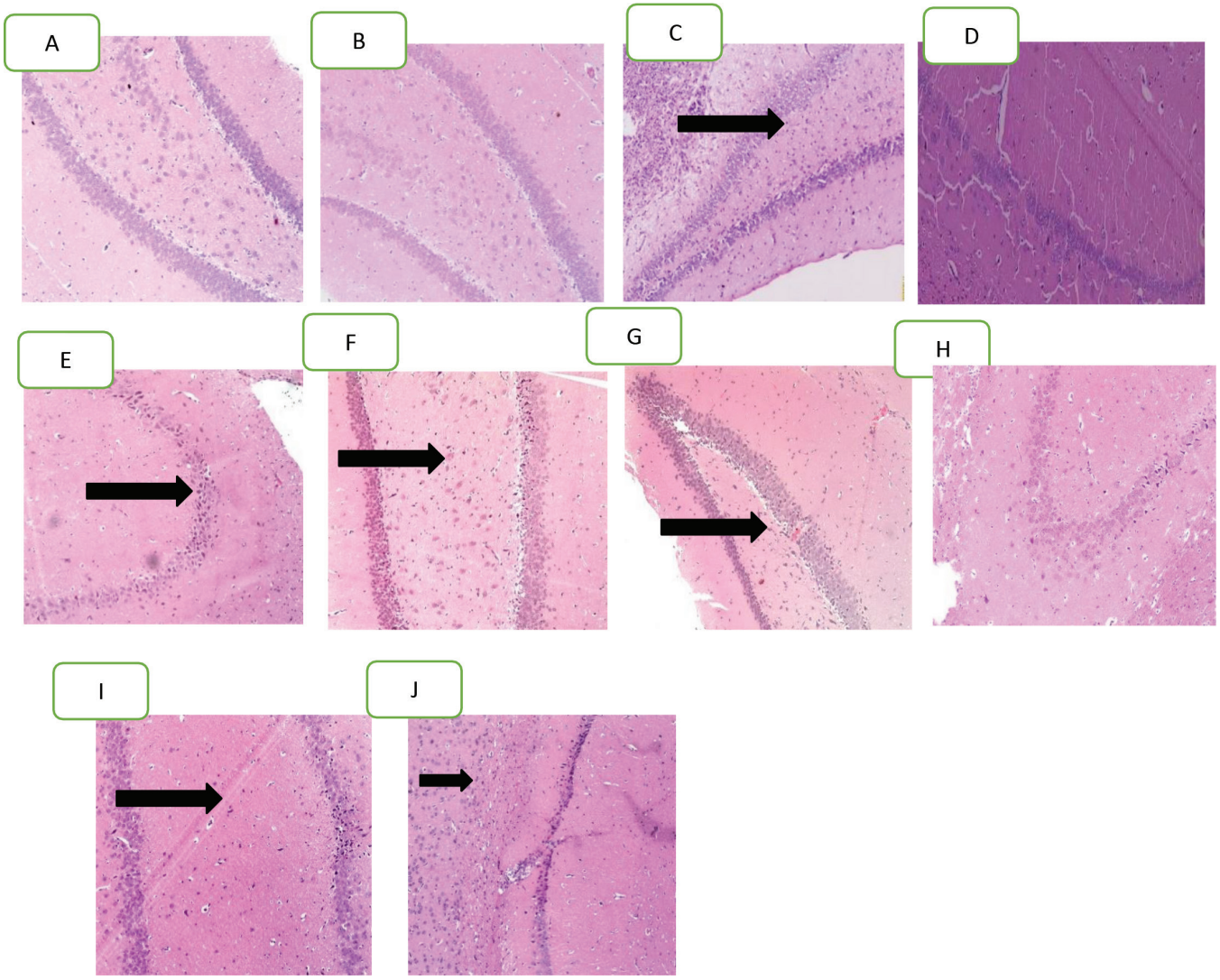
Parameter	MDA (µ/mg protein)	NO (µ/mg protein)	GSH (µ/mg protein)	SOD (µ/mg protein)	Catalase (µ/mg protein)
Vehicle control	0.179 ± 0.013	0.178 ± 0.018	266.7 ± 3.549	3.016 ± 0.077	7.915 ± 0.118
Sham control	0.886 ± 0.021	1.275 ± 0.182	237.8 ± 3.846	2.612 ± 0.028	7.124 ± 0.231
Neurodegeneration control	1.480 ± 0.037###	3.918 ± 0.109###	10.9 ± 1.156###	0.152 ± 0.006###	1.303 ± 0.140###
Memantine (1 mg/kg)	0.751 ± 0.017***	1.202 ± 0.063***	172.9 ± 2.728***	1.871 ± 0.061***	5.768 ± 0.126***
KG (1 g/kg)	0.767 ± 0.009***	1.595 ± 0.019***	65.4 ± 3.055***	0.810 ± 0.023***	2.353 ± 0.186***
KG (2 g/kg)	0.545 ± 0.028***	1.282 ± 0.003***	80.4 ± 1.624***	1.119 ± 0.001***	5.564 ± 0.097***
KG (4 g/kg)	0.663 ± 0.023***	1.715 ± 0.027***	165.9 ± 2.634***	1.956 ± 0.009***	4.196 ± 0.203***
KG (25 µl/rat)	0.780 ± 0.055***	1.636 ± 0.047***	42.9 ± 1.834***	0.629 ± 0.020***	4.469 ± 0.413**
KG (50 µl/rat)	0.719 ± 0.057***	1.613 ± 0.045***	52.45 ± 0.238***	1.256 ± 0.022***	5.229 ± 0.187***
KG (100 µl/rat)	0.718 ± 0.037***	1.838 ± 0.020***	121.7 ± 1.244***	1.294 ± 0.012***	6.497 ± 0.199***

Data was expressed as Mean ± SEM. (n = 8). Data were analysed using one-way ANOVA followed by Dunnett's test. ###p<0.001 as compared with the sham control group. **p<0.01 and ***p<0.001, as compared with Neurodegeneration control, respectively.

Table 2. Effect of *Kalyanaka ghrita* on Histopathological reports of the hippocampus (Silver stain) in Oka-induced AD in rats. Overall pathological grade: 0 - No changes; +1 - Minimal changes, +2 - Mild changes, +3 - Moderate changes

Groups	Neurodegeneration in hippocampus	Overall pathological grade	Neurofibrillary tangles in hippocampus	Overall pathological grade
Vehicle control	Dentate gyrus and CA3 regions were normal with no degenerative changes	0	Dentate gyrus and CA3 regions were normal with no abnormal changes	0
Sham control	Dentate gyrus and CA3 regions were normal with no degenerative changes	0	Dentate gyrus and CA3 regions were normal with no abnormal changes	0
Neurode generation control	Dentate gyrus and CA3 regions were with moderate degenerative changes	+3	Dentate gyrus and CA3 regions were with Mild to minimal tangle formation	+2
Memantine (1 mg/kg oral)	Dentate gyrus and CA3 regions were with minimal reversal of neurodegenerative changes	+1	Dentate gyrus and CA3 regions were with mild to minimal tangles formation	+1
Kalyanaka ghrita (KG1 g/kg oral)	Dentate gyrus and CA3 regions were with mild reversal of neuro degenerative changes	+2	Dentate gyrus and CA3 regions were with minimal reversal of tangle formation	+1
Kalyanaka ghrita (KG2 g/kg oral)	A mild reversal of neurodegenerative changes was observed in the Dentate gyrus and CA3 regions of the hippocampus	+2	A mild reversal of tangle formation changes was observed in the Dentate gyrus and CA3 regions of hippocampus	+2
Kalyanaka ghrita (KG4 g/kg oral)	Moderate reversal of neurodegenerative	+3	Mild reversal of tangles formation	+2
	Changes were observed in the Dentate gyrus and CA3 regions of the hippocampus		changes were observed in the Dentate gyrus and CA3 regions of hippocampus	
Kalyanaka ghrita (KG 25 µl/rat)	Dentate gyrus and CA3 regions were a minimal reversal of neuro degenerative changes	+1	Dentate gyrus and CA3 regions were with mild to minimal tangle formation	+2
Kalyanaka ghrita (KG 50 µl/rat)	A mild reversal of neurodegenerative changes was observed in the Dentate gyrus and CA3 regions of the hippocampus	+2	A mild reversal of tangle formation changes was observed in the Dentate gyrus and CA3 regions of hippocampus	+2
Kalyanaka ghrita (KG 100 µl/rat)	Moderate reversal of neurodegenerative changes was observed in the Dentate gyrus and CA3 regions of the hippocampus	+3	Moderate reversal of tangles formation changes was observed in the Dentate gyrus and CA3 regions of hippocampus	+3

drugs are commonly used for AD which relieve the symptoms for a brief period without altering the course of the progression of the disease. A recent drug approved by the FDA - Aducanumab has a serious adverse effect of intracranial bleeding limiting its widespread use[25]. Hence there is the quest for developing a drug to target the multiple targets of AD, and thereby preventing the disease progression exists. *Kalyanaka ghrita* (KG) is under the category of *medhyarasayana* formulation and consists of 27 herbs containing phytoconstituents with proven memory-enhancing, anti-oxidant and anti-inflammatory activities[5]. The formulation is reported to be effective in neurodegeneration induced by injection of β amyloid in rats. One of the constituents of KG is

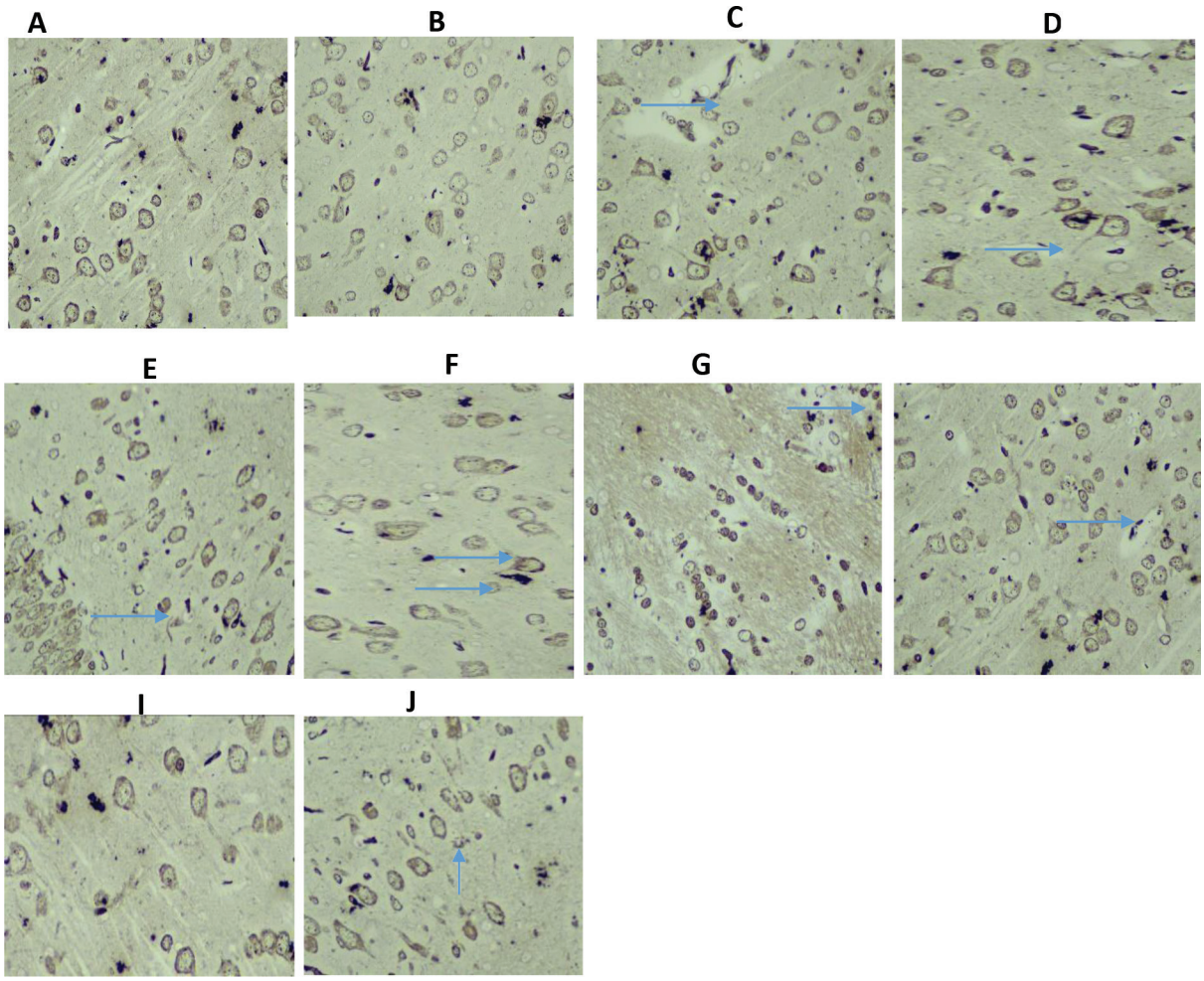


The arrow indicates neurodegeneration in the hippocampus of the animals. (10x magnification). Severe neurodegeneration in CA3 region of neurons in C. Neurodegeneration control. The remaining treatment groups have shown mild changes in neurodegeneration. It may KG prevent the induced severe damage part of the hippocampus.

Figure 7. Effect of *Kalyanaka ghrita* on brain hippocampus (H and E stain) in Oka-induced AD in rats: **(A).** Vehicle control, **(B).** Sham control, **(C).** Neurodegeneration control through intracerebro ventricular injection, **(D).** Memantine (1 mg/kg oral), **(E).** *Kalyanaka ghrita* (KG 1 g/kg oral), **(F).** *Kalyanaka ghrita* (KG 2 g/kg oral), **(G).** *Kalyanaka ghrita* (KG 4g/kg oral), **(H).** *Kalyanaka ghrita* (KG 25 µl/rat), **(I).** *Kalyanaka ghrita* (KG 50 µl/rat), **(J).** *Kalyanaka ghrita* (KG 100µl/rat).

reported to have microtubule-stabilizing properties, thereby preventing the formation and progression of neurofibrillary tangles. With this background, this study is intended to evaluate KG on okadaic acid-induced neurodegeneration. Okadaic acid injection has been linked to neuropathological changes like neurodegeneration in the hippocampus and paired Helical Filaments (PHF)-like hyper-phosphorylation of tau protein and formation of amyloid plaques, suggesting that the model could be useful to understand the disease progression of AD[26].

In the spatial learning and memory paradigm in MWM, the escape latency of okadaic acid-induced rats increased significantly, implying significant memory impairment after 6 days of induction. These findings of the retrieval trial on day 7 in MWM, support previous reports that okadaic acid induction causes memory loss mediated through oxidative stress, neuroinflammation, and mitochondrial stress all of which are linked with neurodegeneration[27]. KG oral and nasal therapy decreased escape latency in repeated trials which confirmed that the spatial



Blue Arrow indicates mild to minimal neurofibrillary tangles formation in the hippocampus of the animals. (40x magnification).

Figure 8. Effect of *Kalyanaka ghrita* on brain hippocampus (Silver stain) in Oka-induced AD in rats: **(A).** Vehicle control, **(B).** Sham control, **(C).** Neurodegeneration control through intracerebroventricular injection, **(D).** Mementine (1 mg/kg oral), **(E).** *Kalyanaka ghrita* (KG 1 g/kg oral), **(F).** *Kalyanaka ghrita* (KG 2 g/kg oral), **(G).** *Kalyanaka ghrita* (KG 4 g/kg oral), **(H).** *Kalyanaka ghrita* (KG 25 µl/rat), **(I).** *Kalyanaka ghrita* (KG 50 µl/rat), **(J).** *Kalyanaka ghrita* (KG 100 µl/rat).

learning and memory function were intact upon administration of KG.

Memory loss and learning difficulties have been related to low acetylcholine levels in the brain because of the hydrolysis of acetylcholine by acetylcholinesterase which is implicated in neurotransmission control. Acetylcholinesterase inhibition improves cholinergic transmission while simultaneously reducing Aβ-peptide aggregation and formation of neurotoxic fibrils in AD[28]. The amyloid fibrils are formed due to the interaction of AChE with *Aβ* peptides through the amino acids present at the enzyme's anionic binding site[29]. The brain acetylcholinesterase activity of okadaic acid-injected rats significantly increased compared to a vehicle control group, in the current investigation. KG administration dramatically suppressed brain acetylcholinesterase activity, which was consistent with the previous studies on β-amyloid induced neurodegeneration. This is attributed to the presence of phytoconstituents in KG with proven AChE inhibition property[30]. The observed inhibition of acetylcholinesterase in the present study was 16.5 % more effective, than *Terminalia chebula* and *Desmodium gangeticum* as reported[31,32]. The observed activity is attributed to phytoconstituents like curcumin, gallic acid, chebulagic acid, berberine and tannic acid which offer synergistic effects.

In neural cells, oxidative stress causes GSK-3β to become overactive. In the hippocamp of okadaic acid rats, a significantly decreased PP2A activity,

an increased GSK3β and tau phosphorylation are reported. Protein Kinase-C and Nuclear Factor-kB are activated by oxidative stress, which causes the tau hyperphosphorylation complex. In AD, the imbalance between kinases and phosphatases leads to tau hyperphosphorylation (h-tau)[33]. Because h-tau has a lesser affinity for microtubules, it separates from them and forms PHFs, which eventually form NFT. NFT blocks the delivery of nutrients and mitochondria to axons and synaptic terminals, causing retrograde neuronal death[34]. In the present study, OKA induced neurodegeneration control group demonstrated a significant decrease in PP2A activity which indicated the probable increase in GSK3β and tau-hyperphosphorylation[35]. However, KG administration significantly elevated PP2A activity in the hippocampus of okadaic acid-induced rats. This confirms that KG is effective in the prevention of neurofibrillary tangles mediated by increased PP2A and the effect is attributed to the phytoconstituent *Curcuma longa*, which is reported to increase PP2A.

In this study, a 40 % increase in the brain PP2A levels was observed when compared to *Curcuma longa*[36] administration, which is attributed to the other phytoconstituents and lipid-based formulation of KG which contributed to the synergistic effect.

DA, 5-HT and NE are essential for affection, cognition, emotion regulation and neuroprotection. Low NE levels might lead to drowsiness and depression. In rats, motivational behaviour is due to the dopaminergic activity[37]. The 5-HT have a key function of cognition and memory[38]. The levels of neurotransmitters- 5-HT, DA, acetylcholine, and NE decrease as a result of neurodegeneration. In this present study, the okadaic acid-induced groups had decreased NE, DA, and 5-HT levels, which can be linked to memory impairment. There was a dose-dependent reversal of depleted monoamines upon administration of KG, which supports the improvement in cognition attributed to phytoconstituents *Curcuma longa* and *Terminalia chebula*[39].

Superoxide and NO interact to generate peroxynitrite, a dangerous oxidant. It interferes with energy-saving processes, results in oxidative post-translational protein modification, and ultimately kills neuronal cells[40]. In the current investigation also, okadaic acid-induced rats had significantly higher hippocampus NO, and lipid peroxides, with decreased SOD, GSH and catalase activity. This observed increase in NO and decreased SOD could be resulting in the formation of peroxynitrite and resultant neuronal death. KG treatment resulted in decreased brain MDA, and NO and increased SOD, GSH and catalase levels, thus would be a sound antioxidant defence mechanism to combat peroxynitrite-induced neuronal death. The above findings back up the antioxidant impact of KG attributed to its phytoconstituents.

Induction with okadaic acid leads to hippocampal neurofibrillary tangles formation which causes severe damage to hippocampus architecture, neuroinflammation and neurodegeneration along with tangles formation. The presence of these pathological mechanisms along with neurodegeneration is reported to cause cognitive impairment[41]. KG reversed these changes in hippocampal architecture, and decreased neurodegeneration, neuroinflammation and tangles, which is concrete proof that KG attenuates the disease progression of AD. KG also decreased the formation of brain neurofibrillary tangles as evidenced by histopathology. All these conclude the promising effect of *Kalyanaka ghrita* in multiple pathways of AD and prove the KG potential for the attenuation of memory impairment, oxidative stress, monoamine changes and accumulation of neurofibrillary tangles caused by induction with okadaic acid.

5. Conclusion

The findings of this study confirm the traditional cognitive enhancing potential of *Kalyanaka ghrita*. The antioxidant properties of KG may be responsible for its ability to protect against the okadaic acid-mediated mitochondrial dysfunction and neurodegenerative cascade. Along with powerful antioxidant action, inhibition of acetylcholinesterase activity, inhibition of hyperphosphorylation, restoration of brain monoamines levels and prevention of tau filament formation are all contributory factors in preventing cognitive impairment in the okadaic acid paradigm. The fact that *Kalyanaka ghrita* has a wide range of effects on cognition, memory, biochemical and histological results validate its neuroprotective properties against okadaic acid-induced memory impairment. As a result, it is suggested that *Kalyanaka ghrita* is a promising medication for

AD-related neurodegenerative disease treatment. However, exploration of the inflammatory profile of KG would add more value to the current findings.

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