

Research Article

Neuroprotective role of *Celastrus paniculatus* Willd and *Sida cordifolia* Linn on kainic acid-induced neuronal damage in neurodegenerative diseases

Gajanand R Pujari^{1*}, Vijayalakshmi Subramanian², Suresh Rangogi Rao³

¹ Srinivas Institute of Medical Sciences and Research centre, Department of Anatomy, Mukka, Managalore Karnataka, India

² Saveetha Institute of Medical and Technical Sciences, Department of Anatomy, Poonamallee High Road, Chennai, Tamil Nadu, India

³ University of West Indies, Department of Preclinical Sciences and Faculty of Medical Sciences, Trinidad and Tobago

ABSTRACT

Neurodegenerative diseases (NDs) are caused by the dysfunction of neurons. Neuronal death is associated with the aggregation of proteins in neurons and glial cells. The aggregated proteins impede mitochondrial function and induce oxidative stress. Increased oxidative stress produces more reactive oxygen species (ROS) which is detrimental to cells in the brain causes neuronal degeneration. There are no treatments for NDs other than reducing disease progression. Hence, the treatment strategies, which reduce oxidative stress and neuronal damage are in demand. *Celastrus paniculatus* Willd (CP) and *Sida cordifolia* Linn (SC) have been extensively used in the indigenous therapeutic systems for treating various brain-related ailments. The present investigation was carried out to examine the biochemical and histological alterations of seed oil of CP (SOCP) and aqueous root extract of SC (ARESC) on the hippocampus of the brain in Kainic acid (KA)-induced NDs. The extracts of SOCP and ARESC were administered for 14 days and KA was administered by i.p. on the 14th day to all the groups except the vehicle control group. At the end of the study, the rat brain was removed, the hippocampus was separated, and the homogenate was prepared to estimate the antioxidant parameters (SOD, catalase, and LPO). LDH assay, dopamine (DA) level, α -synuclein immunohistochemistry, and ROS assays were conducted. The results revealed that the treatment with SOCP and ARESC increased the levels of antioxidant enzymes, reduced oxidative stress, decreased α -synuclein protein aggregation, and elevated the levels of DA neurotransmitters.

Keywords:

Antioxidant, Kainic acid, Neurodegeneration, Reactive oxygen species

1. INTRODUCTION

Neurodegenerative diseases (NDs) are caused by the dysfunction of neurons. They are also associated with the abnormal aggregation of proteins in neurons and glial cells¹. NDs are incurable, increasing in the aged population. The common NDs are amyloidosis (Alzheimer's disease (AD)), α -synucleinopathies (Lewy body disorders-Parkinsonism disease [PD], multiple system atrophy [MSA]), tauopathies (Pick's disease), transactivation response DNA binding protein 43 (TDP-43), and proteinopathies (Amyotrophic lateral sclerosis)². The abnormal protein aggregation (amyloid- β , tau, α -synuclein, prion

and TDP-43) and their distribution in the brain and other parts of the body in these disorders are essential in the diagnosis of specific NDs and development of therapies². The aggregated proteins impede mitochondrial function and induce oxidative stress. Increased oxidative stress produces more reactive oxygen species (ROS) which is detrimental to cells in the brain causes neuronal degeneration³⁻⁴. Some evidence suggest that aggregation of amyloid- β , tau, α -synuclein, prion and TDP-43 proteins can interact with each other like; α -synuclein and tau interact in PD and dementia with Lewy bodies (DLB), α -synuclein can modulate amyloid- β , and amyloid- β stimulates α -synuclein and tau in AD and DLB5-12. Therefore

*Corresponding author:

*Gajanand R Pujari Email: gajanand4025@gmail.com



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therapies against one protein can regulate aggregation of other¹³. α -synuclein is a 14 kDa, consisting of 140 amino acids. It consists of three domains: N-terminal lipid-binding α -helix, an acidic C-terminal tail and non-amyloid component (NAC)¹⁴. They regulate synaptic activities¹⁵. The aggregation of α -synuclein was identified in PD, PD with dementia, DLB, MSA and recently in AD¹⁶⁻¹⁸. Therefore therapies that reduce aggregation of this protein may help in resolving key symptoms of these NDs.

It is well understood that dopaminergic neurons are depleted in PD¹⁹. Recently a study found an association between depleted dopaminergic neurons and AD. Dopaminergic neurons are important in cognition and emotion²⁰. These neurons undergo neuropathological changes due to age and increased oxidative stress²¹. Many NDs share common symptoms. Therefore therapies which reduce protein aggregation and oxidative stress are essential to prevent neurodegeneration.

This research work aimed to study traditionally used neuroprotective medicinal plants to treat NDs. We selected *Celastrus paniculatus* Willd (CP) and *Sida cordifolia* Linn (SC). A study reported the neuroprotective activity of CP seed extract on neurons of the forebrain against hydrogen peroxide-induced oxidative injury and glutamate-induced injury. CP reduced neuronal death and also showed regulation of functions of glutamate receptors²².

Similarly, SC has numerous neuroactive, anti-inflammatory, and antioxidant molecules. In the traditional medicine system, it has been used as *rasayana* to treat NDs. The rasayanas are rich in antioxidant activity. Its traditional use needs validation²³.

Kainic acid (KA) is an agonist of the ionotropic glutamate receptor. It has been used to create excitotoxicity models. KA increases oxidative stress and ROS formation, this leads to neuronal death and memory loss²⁴.

In the present condition, there are no treatments for neurodegenerative diseases. Hence, there is a need to develop therapies that reduce oxidative stress and prevent neuronal damage and disease progression.

2. MATERIALS AND METHODS

2.1. Plant collection and extract preparation

The SC roots were obtained in and around the locality of Kerala. The root of the plant was thoroughly rinsed with tap water for avoiding contaminants accumulated on the roots from their natural setting. The roots (dried) were powdered with the help of an electric blender and the extraction was carried out in succession conferring the Soxhlet extraction method. Every extract was prepared solvent-free and concentrated. The seed oil of CP was procured online. Verification was done by

a taxonomist (Regional Ayurveda Research Institute for Metabolic Disorders, Bengaluru, India).

2.2. Experimental animals

Thirty albino Wistar rats weighing 180-200g were used for the study. All the animals were kept in polypropylene cages maintained under controlled temperature ($23^{\circ}\text{C}\pm 2^{\circ}\text{C}$), and relative humidity (45-55%). The rats were accustomed to laboratory conditions for 48 hours before starting the experimental protocol for minimizing non-specific stress if any. The approval for the experiment was taken from the Institutional Animal Ethics Committee (IAEC) and the experiment was conducted conferring Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India.

2.3. Experimental design

2.3.1. Animal grouping

Animals were classified into five groups each having six animals. Group I was vehicle control. Group II was KA-treated. Group III was seed oil of CP (SOCP)+KA. Group IV was aqueous root extract of SC (ARESC)+KA. Group V is SOCP+ARESC+KA.

2.3.2. KA induction and treatment

Group I (Vehicle control) rats were given 0.5% carboxymethyl cellulose orally during the course of 14 days. Group II rats were administered a single dose of KA (25 mg/kg b.w) i.p on the day 14. Group III rats were given SOCP (500 mg/kg b.w) i.p for 14 days and a single dose of with KA was administered i.p on the day 14. Group IV animals were given ARESC (250 mg/kg b.w) p.o for 14 days and a single dose of KA was administered i.p on the day 14. Finally, Group V received SOCP+ARESC+KA at a similar test dose. A single dose of 25 mg/kg b.w of KA was given according to previously published results²⁵. About 250 mg/kg dose was selected for ARESC as per the results of previous acute oral toxicity study²⁶ and 500 mg/kg i.p dose of SOCP was used. CP was solubilized in 1% Tween 20 and 5% dimethyl sulfoxide, and the solution was prepared freshly²⁷. The volume of extract administered to the experimental animals was not greater than 1 ml/100 g.

Overnight fasted animals were sacrificed after the experimental procedure by an overdose of ketamine anaesthesia (150 mg/kg b.w i.p) as per CPCSEA norms. Rat hippocampus was separated from all the groups, weighed, and was immediately kept in the solution containing an ice-cold solution of 0.25 M sucrose-10 mM, Tris-0.5 mM, EDTA, pH 7.4. The tissue was sectioned by continuously washing the tissue with an ice-cold

isolation medium; its half portion was homogenized with cold isolation medium using a homogenizer. Homogenate was centrifuged. The obtained supernatant was centrifuged at 10,500 rpm for 20 minutes at 4°C to get the post mitochondrial supernatant (PMS) used and for the assay of antioxidant parameters.

2.3.3. Estimation of hippocampus tissue catalase

The tissue homogenate (approximately 0.1 mg protein) was added to 1.9 ml of the phosphate buffer (0.5 M, pH 7). The samples were kept in the reference cuvette having 2.9 ml of buffer, 1 ml of H₂O₂, and 0.1 ml of tissue homogenate. Catalase activity was measured using the mMoles (mM) extinction coefficient 40 cm⁻¹μmoles of H₂O₂ decomposed/min/mg protein²⁸.

2.3.4. Estimation of hippocampus tissue Super Oxide Dismutase (SOD)

To the 0.2 ml PMS hippocampal homogenate, 1.3 ml of 0.1 mM of EDTA, 0.65 ml of 50 mM of sodium carbonate, 0.5 ml of 96 μM of Nitro Blue Tetrazolium, 0.1 ml of solution C, and 0.1 ml of 20 mM Hydroxylamine Hydrochloride were added (pH 6). The rate of NBT reduction was recorded for one minute at 560 nm using a Shimadzu spectrophotometer²⁸.

2.3.5. Estimation of hippocampus tissue Lipid peroxidation (LPO)

LPO was determined by the amounts of malonaldehyde (MDA) produced. SDS (8.1%; 0.2 ml), acetic acid (20%; 1.5 ml) and TBA (8%; 1.5 ml) were added to hippocampus homogenate (0.1 ml). The 4 ml mixture volume was prepared with 4 ml distilled water and kept in the water bath (95°C×1h). Lastly, under tap water, the reaction mixture was cooled, 5 ml of butanol: pyridine (15:1) mixture was added and mixed and centrifuged (3000 rpm×10 mins), the upper pink organic layer was taken and its OD was measured (532 nm) against a suitable blank. The LPO was measured as nanomoles of MDA reactive substances per min in mg protein²⁸.

2.3.6. Estimation of DA in striatal region

Dopamine was measured in BAS 460 MICROBORE-HPLC (Bio-analytical Systems Inc USA). Waters standard system had a sample injector valve (20 μl), a high-pressure isocratic pump, an electrochemical detector, and C18 reverse-phase column. The mobile phase consisted of 2% KHPO₄, 1 mM EDTA, 2% citric acid, 70 mg/ml of sodium octyl sulphate, and the pH of the mobile phase was adjusted to three with HCl (6N). The electrochemical set up for the trial was +0.800 V, and sensitivity ranged from 5~50 nA. Separation was done at a flow rate of 1

ml/min and the samples (20 μl) were administered manually²⁹.

2.3.7. Assay of Lactate dehydrogenase (LDH) activity

Cellular damage was measured by the activity of LDH in plasma. Blood was collected in heparinized plain tubes instantly after sacrificing for assays. Blood plasma was separated by centrifugation (2000 x g, 15 min, 8°C) and immediately used for LDH activity measurement.

The LDH activity was measured in a spectrophotometer by following the Bergmeyer and Brent procedure, by measuring the conversion of NADH to NAD⁺, at 340 nm³⁰.

2.3.8. ROS assay

To study the ROS formation in the cells, 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA probe, Invitrogen, Cat No: 399) was used in the study. The hippocampus of brains were cut at 5 μm, non-fixed sections were used for the study. The samples were incubated for 30 mins. The procedures were performed according to Wilhelm J et al³¹.

2.3.9. Alpha-synuclein protein expression by immunohistochemistry

Immunohistochemical analysis was done by using rat monoclonal anti-α-syn (Becton Dickinson Biosciences #610787). Slides were deparaffinized and rehydrated. Antigen retrieval was done using tris ethylene diamine tetraacetic acid (Tris EDTA, pH 9) buffer. Then the slides were washed three times in tris buffer saline (TBS, pH 7.2-7.5) and peroxidase block was done for 10 minutes. Then the slides were incubated with primary antibody for one hour. After that, the slides were washed with three changes of TBS and were incubated with horseradish peroxidase (HRP) for 30 minutes. Then the slides were washed with three changes of TBS and DAB solution was added and counterstained. After that slides were washed, dipped in xylene and mounted. All immunoreactions were observed using a compound light microscope and these results were quantified using the Image J software³².

2.3.10. Histopathological study of hippocampus tissue

The portions of the hippocampus of the rats were immersed in formalin solution (10%). About five μm thick sections from the hippocampus were obtained using a rotary microtome and paraffin embedding was done. After dehydration with different series of alcohol and then stained by haematoxylin and eosin. Slides were observed for structural changes and number of death neurons per area of brain tissue was quantified using Image J software. The counting field was defined as a

square 0.25 mm wide grid (objective, 40) in the eyepiece of a microscope. In the $\times 40$ objective, this grid corresponds to an area of 0.0625 mm². We counted 9 fields located at the hippocampus per section and 3 sections/animal for all experimental groups. The nuclei are counted in those thin sections, then one extrapolates for the sections in between the ones used for counting, and applies correction factors to account for the fact that larger particles appear in multiple sections.

2.4. Statistical analysis

All the values were measured as mean \pm SD. Statistical analyses were done by ANOVA test followed by Dunnett's post hoc test using of Graph Pad Prism version 5.0. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ was considered as significant compared to disease control.

3. RESULTS

3.1. Antioxidants assay

We found a significant decrease in the hippocampus tissue catalase activity in ND control compared to the

normal vehicle group. The SOCP, ARESC, and SOCP+ARESC treated ND rats exhibited a significant increase in the hippocampus tissue catalase activity when compared to group II rats (Table 1).

The SOD activity was reduced among the KA-induced neuronal damage rats in contrast to the normal vehicle group. The SOCP, ARESC, and SOCP+ARESC treated ND rats showed a significant increase in the hippocampus tissue SOD activity when compared to group II rats (Table 2).

The LPO level was increased significantly in the KA-induced ND rats when compared to the normal vehicle group. The SOCP, ARESC, and SOCP+ARESC treated ND rats markedly showed a reduction in hippocampus tissue LPO level when compared to group II rats (Table 3).

3.2. Histological findings

Characteristic features of control rats showed pyramidal cells, in CA1 region of the hippocampus (arrow mark) (Figure 1A). KA-induced rat displayed pyknotic, hyperchromatic nuclei with vacuolated and deteriorated neurons (arrow, Figure 1B). Glial proliferation was also

Table 1. Effects of ARESC and SOCP on hippocampus tissue catalase.

Groups	Catalase (Units/mg protein)
Normal control	105.4 \pm 3.75
ND control	28.7 \pm 4.20***
ND + SOCP	67.8 \pm 5.35 ^b
ND + ARESC	74.8 \pm 7.10 ^b
ND + SOCP + ARESC	82.3 \pm 7.85 ^b

Results are expressed as Mean \pm SD (n=6); *** $p < 0.001$ (Significant difference compared to normal control), ^b $p < 0.001$ (Significant difference compared to ND control). ND: Neurodegenerative disease, SOCP: Seed oil of *Celastrus paniculatus*, ARESC: Aqueous root extract of *Sida cordifolia*.

Table 2. Effects of ARESC and SOCP on hippocampus tissue SOD activity in control and experimental rats.

Groups	SOD (units/mg protein)
Normal control	0.66 \pm 0.006
ND control	0.45 \pm 0.012***
ND + SOCP	0.57 \pm 0.008 ^b
ND + ARESC	0.54 \pm 0.008 ^b
ND + SOCP + ARESC	0.63 \pm 0.008 ^b

Results are expressed as Mean \pm SD (n=6); *** $p < 0.001$ (Significant difference compared to normal control), ^b $p < 0.001$ (Significant difference compared to ND control). ND: Neurodegenerative disease, SOCP: Seed oil of *Celastrus paniculatus*, ARESC: Aqueous root extract of *Sida cordifolia*.

Table 3. Effects of ARESC and SOCP on hippocampus tissue LPO levels in control and experimental rats.

Group Name	LPO (nM/mg protein)
Normal control	15.90 \pm 0.81
ND control	48.68 \pm 1.10***
ND + SOCP	32.82 \pm 1.96 ^b
ND + ARESC	23.38 \pm 1.28 ^b
ND + SOCP + ARESC	14.55 \pm 0.49 ^b

Results are expressed as Mean \pm SD (n=6); *** $p < 0.001$ (Significant difference compared to normal control), ^b $p < 0.001$ (Significant difference compared to ND control). ND: Neurodegenerative disease, SOCP: Seed oil of *Celastrus paniculatus*, ARESC: Aqueous root extract of *Sida cordifolia*.

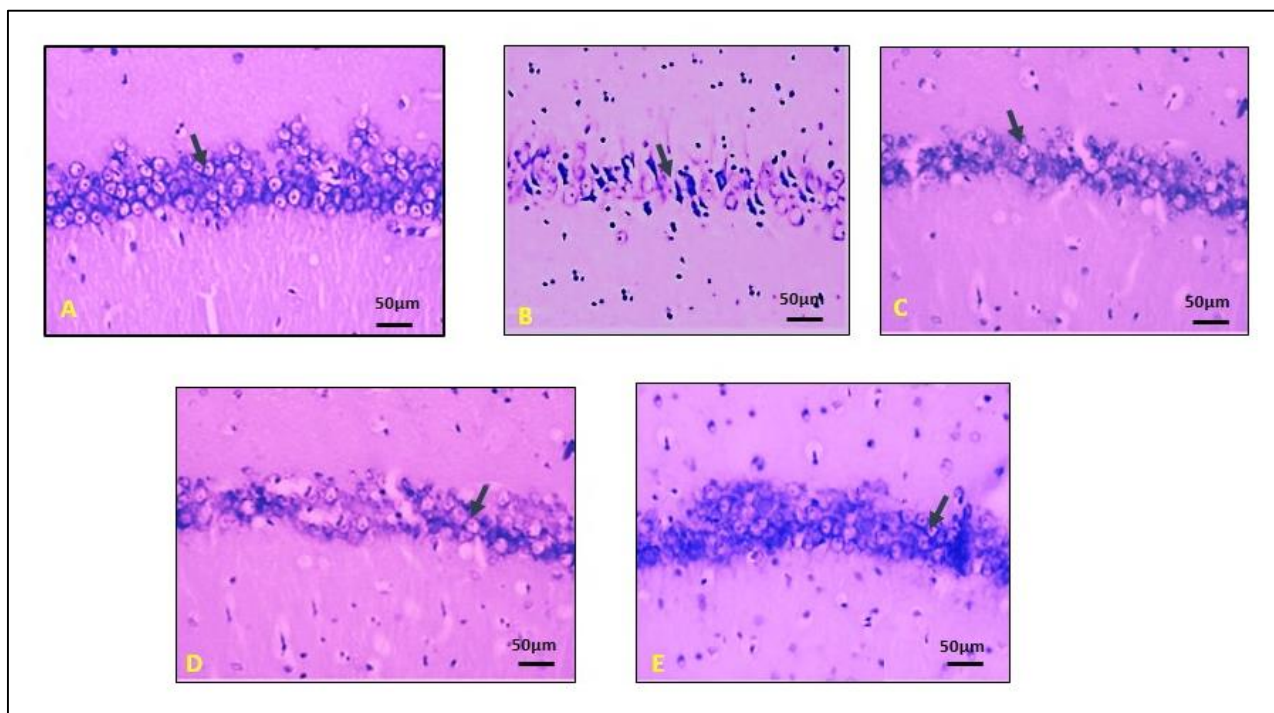


Figure 1. Showing histopathological changes in hippocampus tissue (CA1 region) in ND induced rat and treatment groups of neurodegenerative diseases. (A) Normal control (B) KA-induced rats (C) KA+SOCP (D) KA+ARESC (E) KA+ARESC+SOCP. Scale bar -50µm.

observed. The pyramidal cell layer exhibited decreased thickness of the pyramidal layer and in neuronal number (arrow, Figure 1B). KA and SOCP and ARESC treated rats showed considerable improvement in the structure of neurons and the thickness of the pyramidal layer (arrow, Figure 1C), and most of them appeared normal (Figure 1C & Figure 1D). The hippocampus of rats treated with KA and ARESC+SOCP showed reduced neuronal damage. Most of the neurons appeared normal and the thickness of the pyramidal layer was normal (arrow, Figure 1E).

The number of damaged neuronal cells in area of

0.0625 mm² of brain was increased significantly ($P < 0.001$) in the KA-induced group when compared with control rats. SOCP+ARESC treated group showed more reduction in damaged neuronal cells in comparison with a KA-induced group, which was statistically significant ($P < 0.001$) (Figure 2).

3.3. DA estimation

The levels of DA were significantly reduced in the brain striatal region after KA injection. An increase in striatal DA levels was observed in the treatment groups.

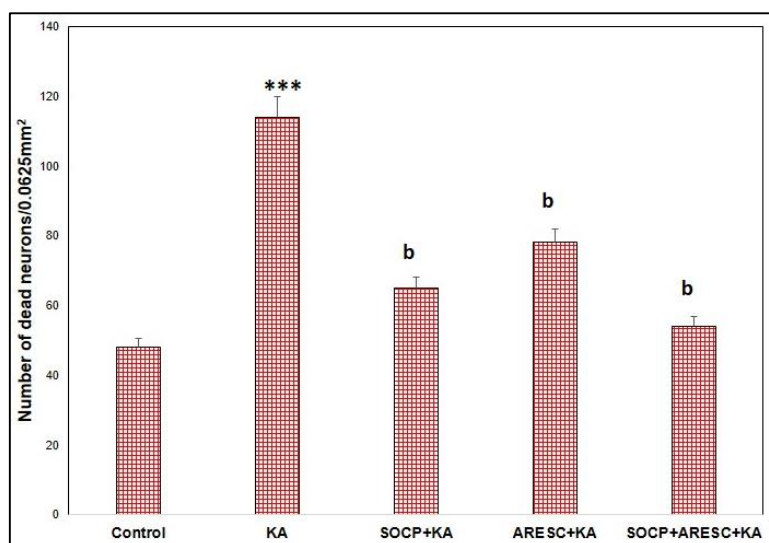


Figure 2. The number of damaged neuronal cells per 0.0625 mm² area of brain. The results were represented as Mean±SD. Results are expressed as Mean±SD (n=6); *** $p < 0.001$ (Significant difference compared to normal control), ^b $p < 0.001$ (Significant difference compared to ND control). ND: Neurodegenerative disease, SOCP: Seed oil of *Celastus paniculatus*, ARESC: Aqueous root extract of *Sida cordifolia*.

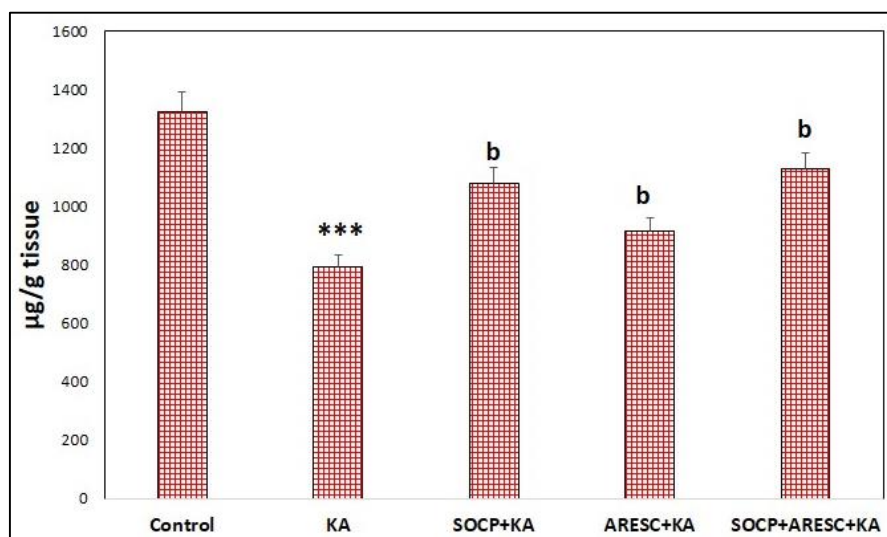


Figure 3. Levels of DA levels in brain striatal region. Results were expressed as mean±SD. *** $p < 0.001$ (Significant difference compared to normal control), ^b $p < 0.001$ (Significant difference compared to ND control). ND: Neurodegenerative disease, SOCP: Seed oil of *Celastrus paniculatus*, ARESC: Aqueous root extract of *Sida cordifolia*.

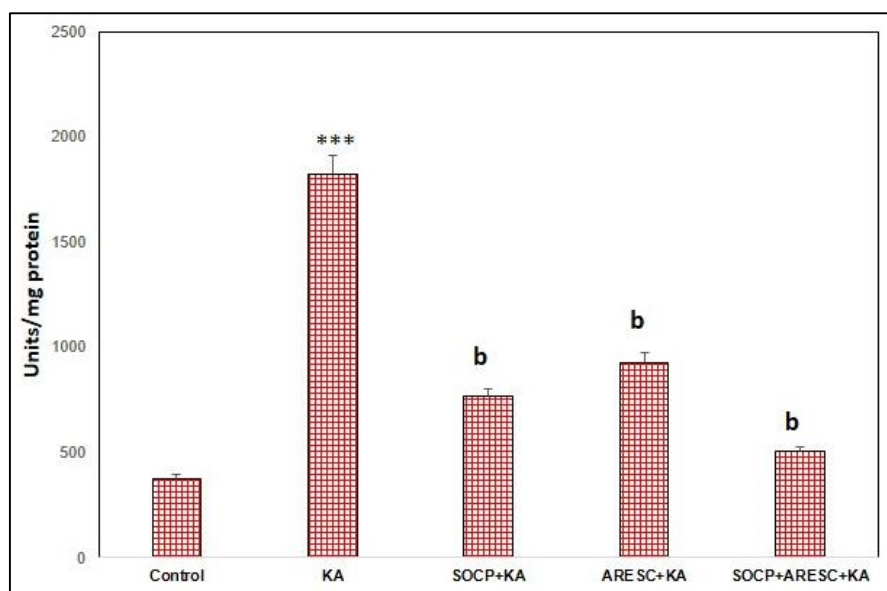


Figure 4. This figure depicts LDH activity in serum. Results were expressed as mean±SD. *** $p < 0.001$ (Significant difference compared to normal control), ^b $p < 0.001$ (Significant difference compared to ND control). ND: Neurodegenerative disease, SOCP: Seed oil of *Celastrus paniculatus*, ARESC: Aqueous root extract of *Sida cordifolia*.

SOCP+ARESC treated group showed a significant increase ($P < 0.001$) in DA levels in comparison with the KA-induced group (Figure 3).

3.4. Assay of LDH activity

The LDH activity was increased significantly ($P < 0.001$) in the KA-induced group when compared to the control group. Serum LDH activity was significantly ($P < 0.001$) reduced in SOCP+ARESC treated rats when compared with KA-induced rats (Figure 4).

3.5. ROS assay

ROS formation was observed in all the areas of

hippocampal tissue with green fluorescence. The KA-induced rats showed more ROS formation (Figure 5B) compared to normal (Figure 5A). After SOCP and ARESC treatment, the ROS formation was remarkably decreased compared with KA-induced groups (Figure 5C and Figure D). SOCP+ARESC combination showed more reduced ROS formation in comparison with KA-induced groups (Figure 5E).

3.6. Alpha-synuclein protein expression by immunohistochemistry

The immunoreactivity for alpha-synuclein was present in the brain-damaged cells exhibited a positive reaction. In KA-induced rats, alpha-synuclein was

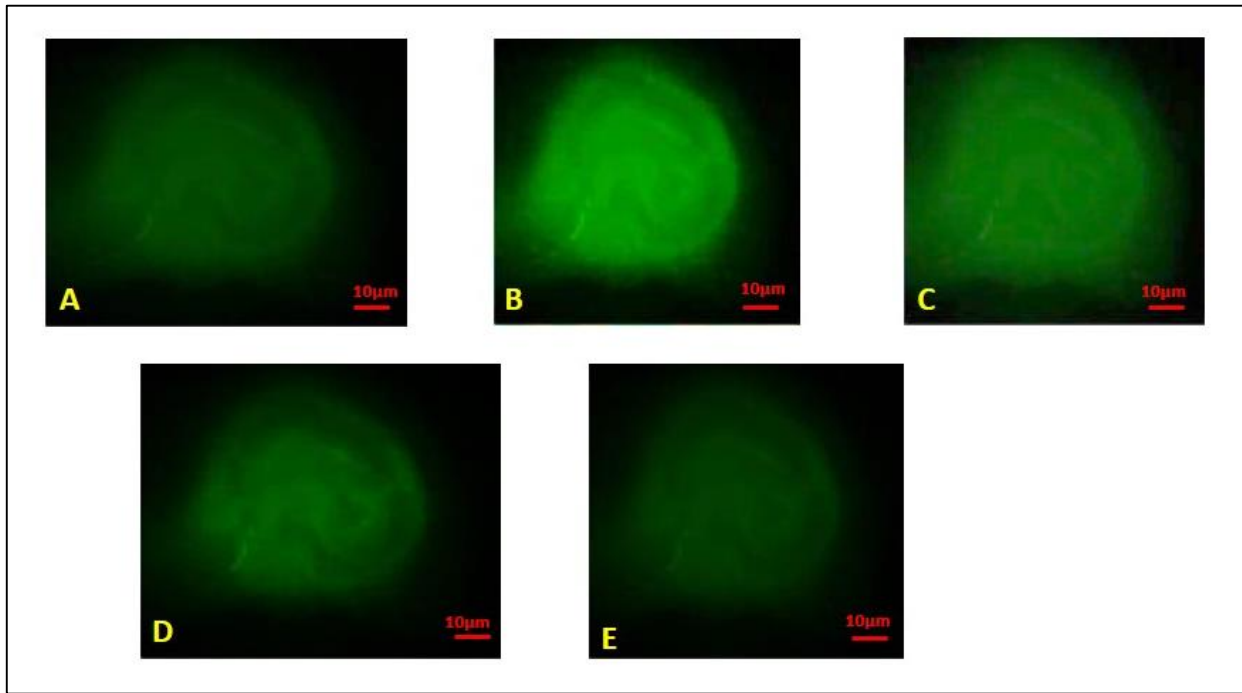


Figure 5. Figure shows ROS formation in ND rats and treatment groups. In control (A); Kainic acid (KA) induced (B); SOCP+KA (C); ARESC+KA (D); SOCP+ARESC+KA (E). Scale bar -10µm.

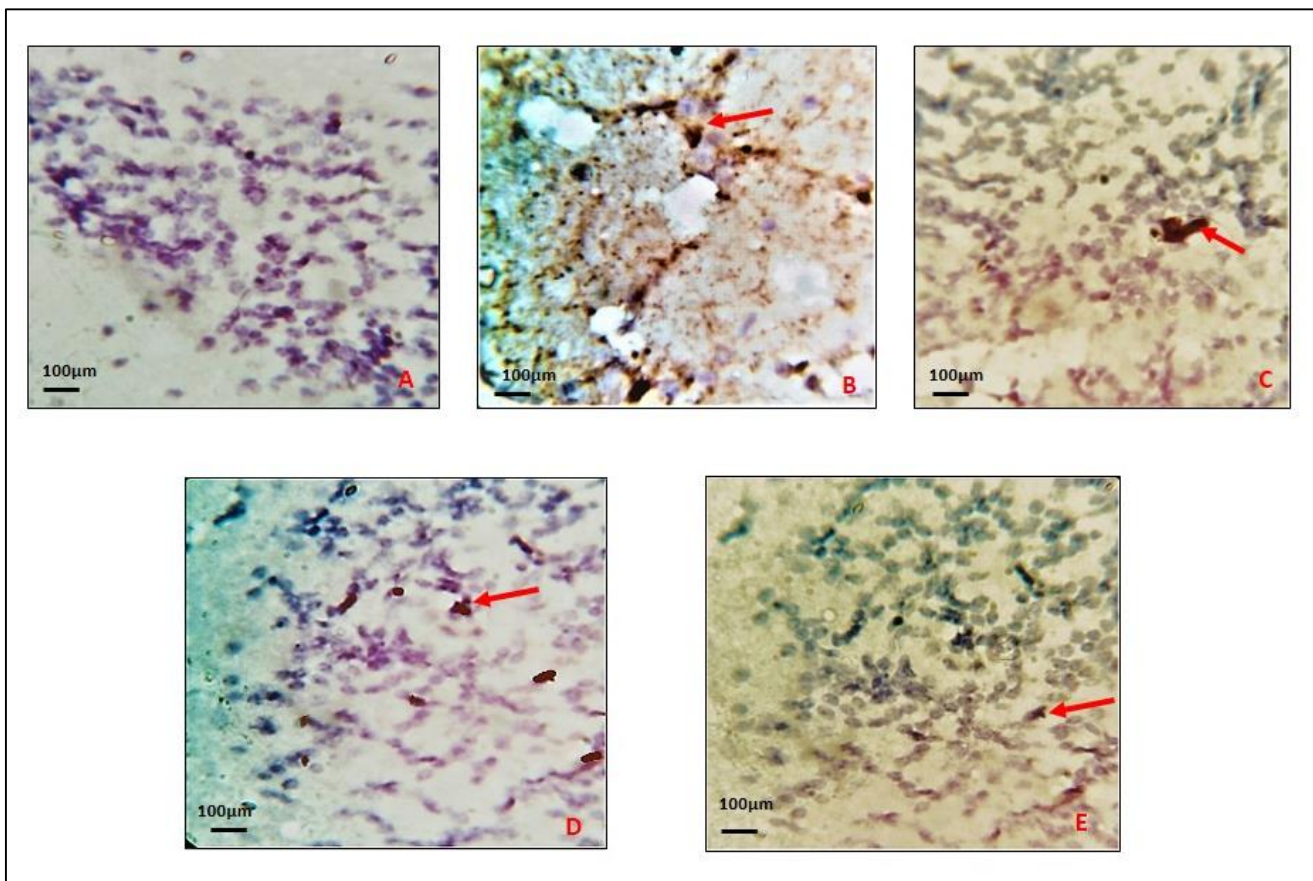


Figure 6. Figure shows immunohistochemical study of alpha-synuclein protein expression in brain tissue of ND rats and treatment groups of neurodegenerative disease. Control (A); Kainic acid (KA) induced (B); SOCP+KA (C); ARESC+KA (D); SOCP+ARESC+KA (E). Scale bar -100 µm. Magnification-100x.

increased in the in the cerebral cortex. The protein expression was reduced in SOCP+ARESC treated group (Figure 6A-E).

4. DISCUSSION

In the present study, we demonstrated the neuro-protective effect of SOCP and ARESC by studying their antioxidant properties. SOD is an antioxidant that forms an important defence against ROS-mediated injuries by catalyzing the reaction of superoxide ions to less reactive hydrogen peroxide³³. Moreover, oxidative damage may also cause damage to components of the antioxidant system. The present study established that oxidative damage reduced the SOD activity in rat hippocampal tissue. ND rats with supplementation of SOCP and ARESC showed a significant increase in SOD levels compared to ND control rats.

Hippocampus lipid peroxidation (LPO) was increased in ND control rats significantly shows that LPO induced oxidative damage. ND rats with supplementation of SOCP and ARESC reduced the LPO. A study by Elharram *et al.*, showed that reduced LPO by Deuterium-reinforced D-PUFAs supplementation and reduced oxidative stress, and improved memory in AD³⁴. Studies also showed that increased SOD reduces LPO³⁵, which will help in the recovery of neuronal damage and in planning the treatment³⁶.

Hippocampal tissue catalase activity was reduced in ND control rats with supplementation of SOCP and ARESC increased the tissue catalase activity. This study showed that the antioxidant property of SOCP and ARESC can reduce the formation of free radicals and neuronal damage.

H2DCFDA is the most extensively used probe for identifying intracellular H₂O₂ and oxidative stress. Rats treated with KA showed increased ROS. After treatment with SOCP and ARESC, the ROS formation was reduced but significantly more reduced in the SOCP+ARESC combination. Formation ROS is the source of oxidative stress which damages the neurons and glial cells. The reduced ROS formation confirms reduced oxidative stress and neuronal death in this study. The histopathological findings and reduced LDH confirms this observation.

In the present study, we showed reduced DA neurotransmitters in the striatal region in KA-induced ND. The reduced DA causes synaptic disarrangement, aggregation of amyloid protein and cognitive impairment, and predementia-like symptoms³⁷. With SOCP and ARESC treatment showed a significant increase in DA, which was more in SOCP+ARESC group. Therefore these drugs can be administered to prevent the depletion of dopaminergic neurons in NDs.

In this study, we observed aggregation of more α -synuclein in KA-induced ND rat's brain. NDs are

characterized by the aggregation of proteins. Treatments that reduce or prevent this aggregation are in demand. The NAC domain of α -synuclein can form cross β sheets and has the most hydrophobic pattern, and the phosphorylation of Serine 129 at the C-terminal makes it more prone to aggregation³⁸⁻⁴¹. This aggregation leads to Lewy body (LB) formation and subsequent neurodegeneration⁴². The aggregation of protein occurs due to overexpression of proteins, genetic multiplication, and failure to clear the protein normally. With the SOCP and ARESC treatment α -synuclein was reduced.

Roots of SC consist of saponins, flavonoids, phyto-sterols, proteins, tannins, alkaloids, lignin, phenolic compounds, fixed oils, and fats. A study also showed roots of SC consist of more flavonoids⁴³⁻⁴⁴. The seeds of CP consist of more flavonoids and phenol compounds⁴⁵. The flavonoids and phenolic compounds exert antioxidant activity which, reduces oxidative stress and cell damage⁴⁶. The presence of more phenolic and flavonoids compounds in the ARESC and SOCP is may be the reason for increased antioxidant enzymes and reduced formation of free radicals and neuronal cell death in the present study. Therefore we could observe that the combination of ARESC and SOCP produced better results than the individual plants.

5. CONCLUSION

This study showed that treatment of SOCP and ARESC reduced alpha-synuclein aggregation, and increased DA level by reducing oxidative stress in KA- induced neuronal damage.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

None to declare.

Ethics approval

Approval taken from the IAEC (Approval No. PESCP/IAEC/62/2016).

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