

Anti stress effect of *Centella asiatica* leaf extract on hippocampal CA3 neurons – a quantitative study

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ABSTRACT

Background: *Centella asiatica* (CeA) is a small perennial herbaceous creeper, growing in moist soil belonging to the umbelliferae family. In Ayurvedic medicine, CeA is popular as a brain tonic to promote brain growth and enhance memory. In addition to its memory enhancing and anxiolytic effects it also has anti-thrombotic activity, antibacterial effects, antinociceptive and anti inflammatory properties. However its neuroprotective effect has not been reported so far. In the present study the neuroprotective effect of CeA leaf extract on hippocampal CA3 neurons was investigated.

Materials and Methods: Three months old albino mice were divided into four groups. Group (i) was normal control, Group (ii) was saline control, Group (iii) was stress group, Group (iv) was stress + CeA treated group. Group (iii) mice were stressed in a wire mesh restrainer for 6 hours/day for 6 weeks. Group (iv) mice were also stressed like group (iii) but they received orally CeA leaf extract throughout the stress period. After 6 weeks, brain was removed, hippocampus was dissected and processed for Golgi staining. Hippocampal neurons were traced using camera lucida focused at 400X magnification. The concentric circle method of Sholl was used to quantify the dendrites.

Results: There was a decrease in the number of dendritic branching points and dendritic intersections in the hippocampal CA3 neurons in group (iii). However, there was a sharp contrast observation in group (iv) which was subjected to restraint and treated with CeA leaf extract.

Conclusion: CeA leaf extract has neuroprotective effect against the stress induced neuronal atrophy in mice.

Key words: Restraint stress, dendritic branching points and dendritic intersections, hippocampus.

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INTRODUCTION

Hippocampus is one of the important areas of the brain concerned with learning, memory and emotional behaviour of the individual.^[1] It is also involved in the control of secretion of the adrenocorticotrophic hormone (ACTH) through the hypothalamo pituitary adrenal (HPA) axis.^[2] Damage to the neurons of the hippocampus has been observed in some of the neurological disorders like Alzheimer's disease and epilepsy.^[3,4]

Neurons are the structural and functional unit of the nervous system. The structural organization of the neurons, neurochemical composition and functional integrity is known to be altered by various factors. The structural

organization particularly the dendritic arborization and synaptic junctions respond to various factors like stress, ultrasound and pesticides.^[5-7]

However, various experimental studies on mice and rats have shown that there is an improvement in dendritic arborization by the intake of certain plant extracts like *Centella asiatica*, *Clitoria ternatea* and *Ocimum sanctum*.^[8-10]

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Chronic exposure to stress results in hippocampal neuronal death.^[5] The effect of restraint stress on the hippocampal CA3 neurons has been reported to dendritic atrophy.^[11] *Centella asiatica* (CeA) leaf extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization in rats.^[12,13] Fresh leaf extract of CeA at a dose of 4 ml/kg/day and 6 ml/kg/day for 6 weeks has shown significant improvement in learning ability of rats. This was correlated with increased dendritic branching points and dendritic intersections in hippocampal CA3 neurons and amygdala neurons.^[14] But the anti-stress effect of CeA on the dendritic branching points and intersections of the hippocampal CA3 neurons has not yet been reported. Thus, the aim of the study was to determine the anti-stress effect of CeA leaf extract on the dendritic branching points and intersections of hippocampal CA3 neurons.

MATERIALS AND METHODS

Experimental animals

The study procedure was approved by the Institutional Ethics Committee of KMC, Manipal University, India. Male and female albino mice of 3 months of age weighing 30-36 g were included in the present study. The mice were bred and maintained in the central animal house. The mice were maintained in light and dark cycles of 12 h duration each, in a well-ventilated room. Four to six mice were housed in each polypropylene cage, with paddy husk as the bedding material, changed on alternate days. Mice were fed with food and water *ad libitum* except during stress period of the experimental study.

Extraction procedure

Fresh *Centella asiatica* leaves were collected, cleaned, sunshade dried and powdered. Dry powder was mixed with distilled water at 1:10 ratio and boiled over a low flame for 30 minutes. The solution was cooled and decanted. The procedure was repeated twice. Clear super-

natant obtained each time was decanted and then centrifuged (300 rpm for 5 minutes). The supernatant was evaporated on a low flame to get a thick paste like extract, which was later dried in a desiccator.

Drug dosage

Dry CeA leaf extract was prepared and stored in air tight bottles. The CeA leaf extract of 500 mg/kg body weight was administered to mice, orally throughout the experimental period (6 weeks) in separate groups. The dose selected was the standardized dose from our preliminary study.^[12,13] Plant extract was dissolved in saline to get the appropriate dilution to administer orally just before the stress exposure on each day.

Oral administration of drugs

The required dose of drug was taken in a syringe attached with a capillary tube and tube was introduced gently into the oral cavity of the mice to ensure slow delivery of the drug.

Restrainer and stress procedure [Figure 1]

A wire mesh restrainer, fabricated locally consisting of 12 compartments was used for restraint stress. Each compartment had 2" (length) X 1.5" (breadth) X 1.4" (height) dimension. Mice were stressed individually by placing within the restrainer for 6 h/day for 6 weeks. Stress induction and its severity were assessed by measuring the suprarenal gland weight at the time of sacrifice.

Figure 1: Wire mesh restrainer with mice.



Experimental design

Group (i) or the normal control (NC) remained undisturbed in their home cage. Group (ii) or the saline control group (SC) received equivolume of normal saline during the experimental period. Group (iii) was stressed (S) in a wire mesh restrainer 6 h/day for 6 weeks. Group (iv) was stressed in the same way as group (iii), but treated with CeA aqueous extract of 500 mg/kg/day throughout the stress period (S + CeA). Drug was administered orally just before the stress exposure on each day.

A day after the last dose or equivalent day in control group, mice in all the groups were sacrificed with ether anaesthesia. Brain was removed, hippocampus was dissected and processed for rapid Golgi staining (n=8 in each group). Number of dendritic branching points and dendritic intersections were quantified.

Quantification of neurons

Slides were viewed by using a compound microscope attached with the camera lucida apparatus. Tracing of neurons were made using the camera lucida focused at 400 X magnification. The concentric circle method of Sholl was used to quantify the dendrites. Concentric circles were drawn at 20 μ m intervals on a transparent sheet and used for dendritic analysis. The center of the cell body was taken as the reference point. Using the camera lucida tracings of neurons following analysis were done.

Dendritic branching points

It is the measure of the nature of dendritic arborization. The number of dendritic branching points within each concentric circle (between adjacent concentric circles) was counted.

Dendritic intersections

It is the measure of the total length of dendrites. It is a point where a dendrite

touches or crosses concentric circle of the Sholl's grid placed over a traced neuron.

Statistical analysis

The data was represented as mean \pm SEM. Results obtained from the above experiments were correlated and analysed using one way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Student's t-test was applied wherever applicable using statistical software package (Graph pad in Stat). A p value of ≤ 0.05 was considered as statistically significant.

RESULTS

Dendritic Branching Points (Figure 2)

Apical dendritic branching points at different concentric zones (Figure 3)

Apical dendritic branching points were decreased in group (iii) at all concentric zones compared to normal control group. In group (iv), dendritic branching points at 40-60 μ m, 60-80 μ m, 80-100 μ m and 100-120 μ m concentric zones were significantly increased ($p \leq 0.05$) compared to group (iii).

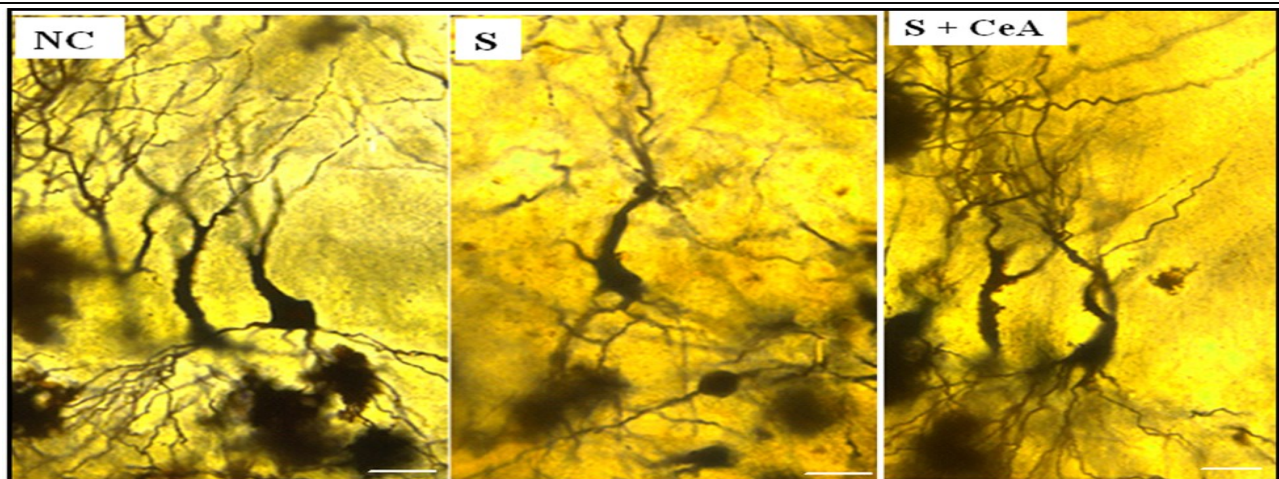
Basal dendritic branching points at different concentric zones (Figure 4)

Basal dendritic branching points were decreased significantly in group (iii) at 0-20 μ m, 20-40 μ m, 40-60 μ m, and 60-80 μ m concentric zones compared to normal control. In group (iv), the dendritic branching points at all concentric zones were significantly increased compared to group (iii).

Dendritic Intersections

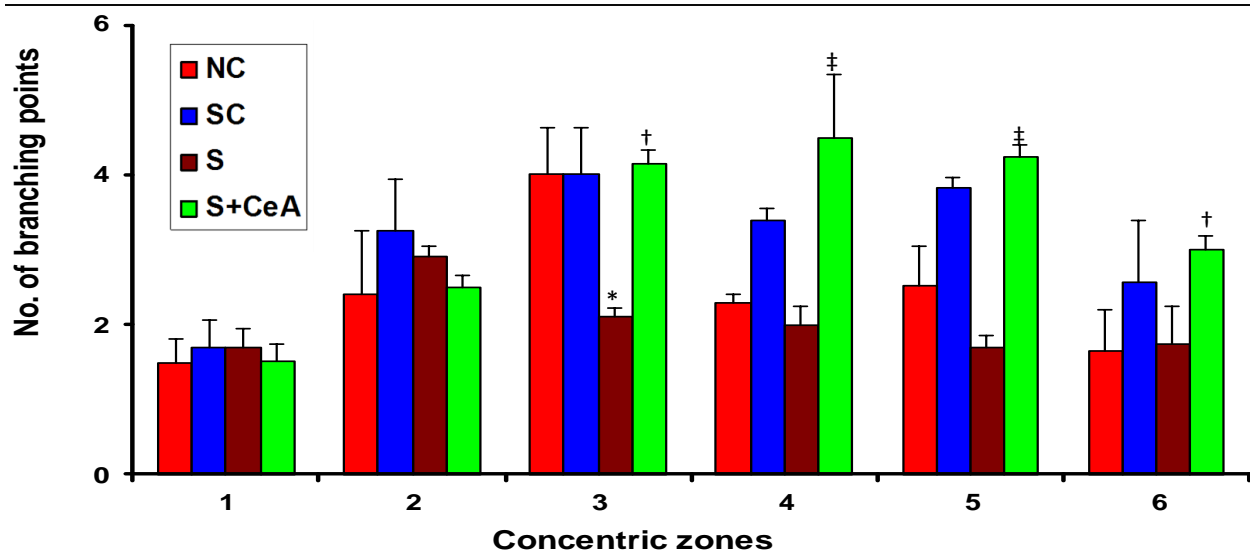
Apical dendritic intersections (Figure 5)

Apical dendritic intersections were decreased at 60 μ m, 80 μ m, 100 μ m, 120 μ m distance from soma in group (iii) mice compared to group (i) and (ii) mice ($p < 0.001$). The dendritic intersections were increased significantly at the same radial distance in group (iv) mice ($p < 0.001$).

Figure 2: Photomicrographs of Hippocamal CA3 neurons (Golgi staining)

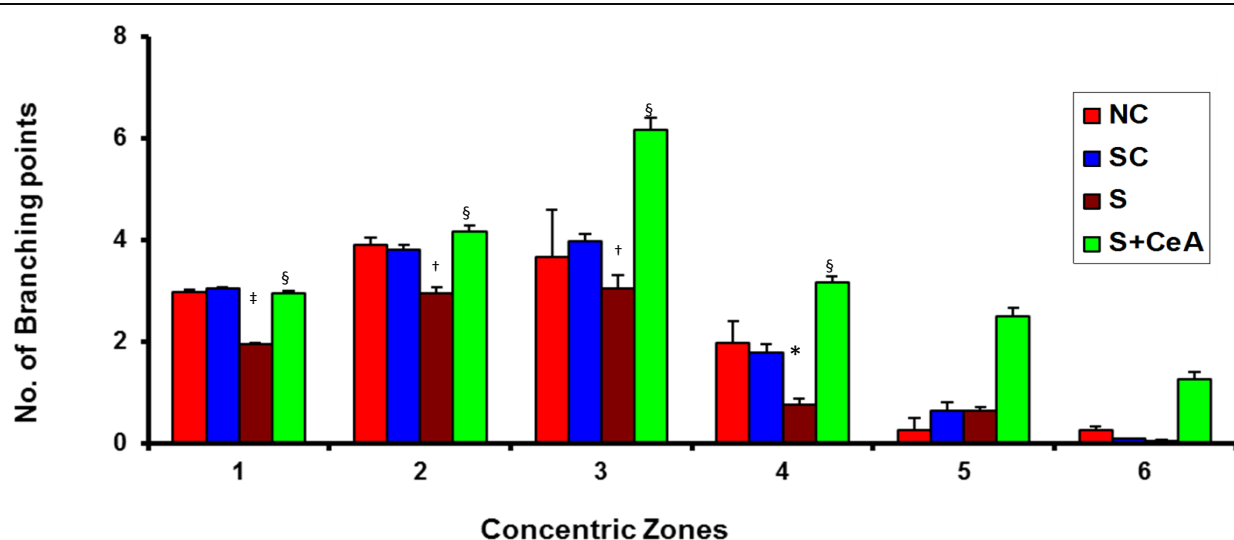
NC: Normal control, S: Stressed, S + CeA: *Centella asiatica* leaf aqueous extract, Scale bar = 40 μ m.

There is increased dendritic arborization of the hippocampal CA3 neurons in S + CeA treated group than stressed group

Figure 3: Apical dendritic branching points in various groups of animals

NC - Normal control, SC- saline control, S - Stressed, S + CeA - *Centella asiatica* extract treated group.

* $p < 0.05$ NC vs. S; † $p < 0.05$ S vs. CeA; ‡ $p < 0.01$ S vs. CeA.

Figure 4: Basal dendritic branching points in various groups of animals

NC - Normal control, SC- saline control, S - Stressed, S + CeA - *Centella asiatica* extract treated group.

* $p < 0.05$ NC vs. S; † $p < 0.01$ NC vs. S; ‡ $p < 0.001$ NC vs. S; § $p < 0.001$ S vs. CeA

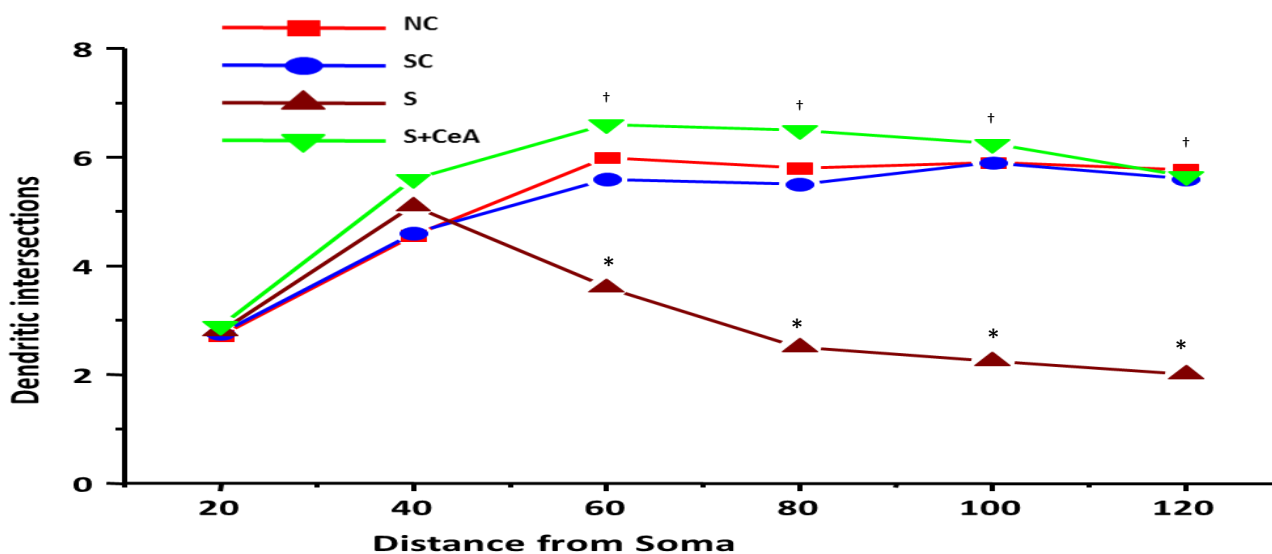
Basal dendritic intersections (Figure 6)

Basal dendritic intersections are decreased at 20 μm , 40 μm , 60 μm and 80 μm distance from soma in group (iii) compared to group (i) and (ii). The dendritic intersections were increased significantly at the same radial distance in group (iv) mice.

DISCUSSION

In the present study, atrophy in the dendritic arborization of hippocampus CA3 neurons was noted which may affect the various functions of hippocampus. The possible mechanisms of such dendritic atrophy may be due to:

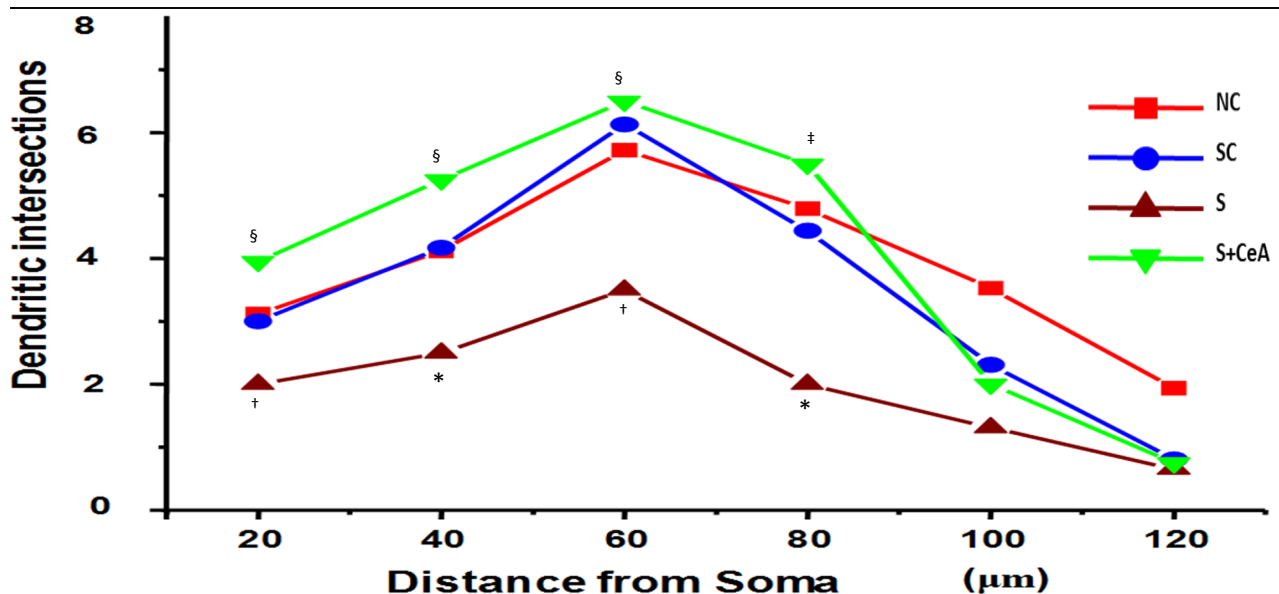
Figure 5: Apical dendritic intersections at 60, 80, 100 and 120 μm distance from soma



NC - Normal control, SC - saline control, S - Stressed, S + CeA - *Centella asiatica* extract treated group.

* $p < 0.001$ NC vs. S; † $p < 0.001$ S vs. CeA

Figure 6: Basal dendritic intersections at 60, 80, 100 and 120 μm distance from soma



NC - Normal control, SC - saline control, S - Stressed, S + CeA - *Centella asiatica* extract treated group.

* $p < 0.05$ NC vs. S; † $p < 0.01$ NC vs. S; ‡ $p < 0.01$ S vs. CeA; § $p < 0.001$ S vs. CeA

Excitotoxicity

The excitotoxicity due to increased release of excitatory neurotransmitter, glutamate may be responsible for the dendritic atrophy of hippocampus CA3 neurons. Elevations in the circulating corticosterone levels can increase basal glutamate levels in the hippocampus.^[15] Restraint stress has been shown to increase glutamate release in the hippocampus.^[16] Glutamate is not the only neurotransmitter involved in dendritic atrophy. Other participating neurotransmitters like GABA and serotonin are also known to cause neuronal damage.^[17,18]

Glucocorticoid toxicity

An increase in stress and glucocorticoids, increase the glutamate concentrations in the hippocampal synapse.^[19,20] Glucocorticoids selectively increase glutamate accumulation in response to excitotoxic insults both in hippocampal cultures and in the hippocampus in vivo.^[21]

Increased levels of Ca^{2+}

Increased levels of Ca^{2+} have been shown to disassemble microtubules^[22] and to activate calcium activated neural proteinase, an enzyme preferentially located more in neurons and responsible for the breakdown of cytoskeletal proteins.^[23]

This may lead to collapse and retraction of dendritic branches, since structural integrity of neuronal processes depends on the presence of stable microtubule. Glucocorticoids are known to increase the cytosolic calcium load in the hippocampus.^[24]

Apoptosis of neurons

Repetitive perforant path stimulation in the rat induces apoptosis and necrosis in different hippocampal neuron populations.^[25]

Brain-derived neurotrophic factor (BDNF)

Stress and glucocorticoids are reported to decrease the expression of BDNF in the

hippocampus and dentate gyrus.^[26] Decreased levels of BDNF in response to stress could lead to loss of normal plasticity and eventually damage and lead to the loss of neurons. Corticosterone induces damage to cultured hippocampal neurons via reducing their BDNF synthesis and this is attenuated by exogenously added BDNF.^[27]

Effects of *Centella asiatica* (CeA) leaf extract on neurons

CeA induces changes in the dendritic morphology of hippocampal neurons. They protect the neurons from death and reduce the dendritic atrophy in stress conditions.

The probable mechanisms involved in increasing the dendritic arborization of hippocampal CA3 neurons and protection against stress induced neuronal injury are:

Neurostimulants and synaptic modulation

CeA leaf extract may contain neurostimulants, which may stimulate the formation of new dendrites or they may influence the hippocampus in releasing corticotrophin releasing factor, which in turn may increase synaptic efficacy in hippocampus. New synapse formation in order to compensate the neuronal loss due to stress^[28] have also been reported.

Growth factors, adhesion molecules and other chemicals

Nerve growth factor (NGF) injection directly into hippocampus improves spontaneous behavior and memory retention, which may be by influencing the formation of new dendrites.^[29] CeA extract may contain NGF like substances.

Neurogenesis

CeA extract may induce neurogenesis in the neural structures involved in stress in the hippocampus. Neurogenesis has been reported in the hippocampus in relation to learning or training a task.^[30,31] Conversely, aversive experience like stress seems to decrease the production of new cells.^[32]

Neuroprotectors and antioxidants

Aqueous extract of CeA may act as an anti-oxidant and have an enhancing effect on cognitive functions.^[33,34] The derivatives of Asiatic acid, a triterpene extracted from CeA are efficacious in protecting neurons from oxidative damage caused by exposure to excess glutamate.^[35] Accordingly, in the present study the cytoprotective and antioxidant property of CeA may be responsible for the neuroprotection against cell death and the deleterious effects of stress.

The study concludes that, oral administration of *Centella asiatica* leaf extract in stressed albino mice lead to the following: a) Increase in the apical and basal dendritic branching points of hippocampal CA3 neurons,

b) Increase in the dendritic intersections of both apical and basal dendrites of hippocampal CA3 neurons.

Significance of the study

This study proves the neuroprotective effect of *Centella asiatica* leaf extract, protecting the neurons against the stress induced neuronal atrophy. Hence this plant extract proves to be beneficial to treat the stress disorders. Its utility to prevent age related, drug induced or spontaneous neurodegeneration remain plausible.

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