Effect of *Capparis spinosa* Linn. extract on lipopolysaccharide-induced cognitive impairment in rats.

Ashish Goel\(^1\), Digvijaya\(^1\), Arun Garg\(^2\) & Ashok Kumar\(^3\)*

\(^1\)Department of Pharmacology, PDM College of Pharmacy, Bahadurgarh, Haryana-124 507
\(^2\)School of Medical Allied Sciences, K R Mangalam University, Gurgaon, Haryana-122 003, India
\(^3\)College of Medicine, Department of Physiology and Functional Genomics, University of Florida, Gainesville, USA 32608

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Cognitive disorders in mankind are not uncommon. Apart from neurodegenerative diseases such as Alzheimer’s (AD), various stresses also affect cognitive functions. Plants are known to be potential source of compounds that ameliorate several diseases including cognitive impairment. Here, we evaluated effect of aqueous extract of caper (*Capparis spinosa*) buds on lipopolysaccharide-induced cognitive impairment in rats using two different oral doses i.e. 10 (pre-treatment) and 30 mg/rat(post-treatment) through assessment of behavioural (Morris Water maze test and Y maze test), biochemical (Cholinesterase assay) and histopathological (H&E staining) parameters. Lipopolysaccharide (from *E. coli*) administration resulted in an increased neurodegeneration and time taken to reach the platform (in Morris water maze). The increased neurodegeneration in CA1 region of hippocampus was significantly reduced in animals which received caper bud extract; they showed marked reduction in time taken to reach the platform at both the dose levels. The experiment demonstrated that caper bud extract exhibits potential protective effect against learning and memory damage induced by chronic administration of lipopolysaccharide (175 µg/kg) for 7 days. The results suggest that the caper bud extract could be explored for its use in the treatment of cognitive disorders.

Keywords: Caper Bush, Cholinesterase assay, Learning and Memory, Neurodegeneration, Stress.

Alzheimer’s disease (AD) is an age related neurodegenerative disease characterized by a progressive decline in cognitive functions\(^1,2\). Cognitive functions are mainly categorized into attention, creativity, intelligence and memory. Recently, Pande *et al*.\(^3\) attributed the cognitive ability to estimate short intervals in humans to an endogenous circadian rhythm. Several neurotransmitters and secondary messenger systems such as cAMP and cGMP are directly involved in signal transduction pathways in brain, which has been reported to be modified due to aging\(^4\).

Reactive oxygen species (ROS) cause neuronal damage during stress resulting in cognitive disorders. Oxidative stress (including hypoxic stress and ischemic injury) causing free radical toxicity, radical induced mutations, autoimmunity, mineral and nutrient deficiencies etc., have all been implicated in cognitive disorders\(^5\). Flavonoids have been shown to activate key enzymes in mitochondrial respiration and to protect neuronal cells by acting as antioxidants, thus breaking the vicious cycle of oxidative stress and tissue damage\(^6\). Flavonoid intake inhibits certain biochemical processes of brain aging, and might thus prevent to some extent the decline of cognitive functions with aging as well as the development or the course of neurodegenerative diseases. It also possesses neuroprotective effect, mainly by scavenging the amyloid beta plaques\(^7\).

Plants are natural resources of active components used in the treatment of various disorders. An ayurvedic formulation, *Panchagavya Ghrita* comprising natural ingredients has been recently shown to attenuate seizures, cognitive impairment and oxidative stress in pentylenetetrazole induced seizures\(^8\). The wild shrub *Gardenia jasminoides* J. Ellis\(^9\) and the common Ginger (*Zingiber officinale* Roscoe)\(^10\) are known for their neuroprotective nature. Extracts of *Argemone mexicana* (L.)\(^11\), *Boerhaavia diffusa* L.,\(^12\) *Coriandrum sativum* L.,\(^13\) have been shown to have potential for treatment of epileptic disorders, depression and cognitive dysfunction, respectively. The common Caper Bush *Capparis spinosa* is known as a rich source of flavonoid content, especially flavonols (quercetin, kaempferol, myricetin, etc.)\(^14,15\). A polyherbal

\(^*\)Correspondence:
Phone: +1 3527454810
E-mail: akb_bits@yahoo.com
formulation consisting of *C. spinosa* has been reported to exhibit hepatoprotective ability in CCl₄-induced liver toxicity. In the present study, we evaluated the effect of herbal extract of *Capparis spinosa* on pathogenesis of LPS-induced animal model of AD.

Several agents have the potential of inducing dementia, e.g. scopolamine, streptozotocin, dizocilpine (MK-801), etc. But none of them are capable of producing neuritic plaques and neurofibrillary tangles (NFTs) located in the cortical areas and medial temporal lobe structures of the brain, the signature lesions in AD. Lipopolysaccharide (LPS), on the other hand, on intraperitoneal administration results in neuronal death in the mouse model, increase Amyloid precursor protein (APP) processing and intracellular accumulation of amyloid beta (Aβ), and alter Blood Brain Barrier (BBB) transport activity. Inflammation induced by LPS could contribute to the development of AD pathology by altering Aβ transport at the BBB. Hence, in this study we used LPS-induced animal model of AD.

**Materials and Methods**

**Extract preparation**

Ten gram of caper flower buds were crushed using a pestle motor and mixed with water (100 cm³). The pH was adjusted to 6-8 using 1 M NaOH, and the mixture was boiled (for conventional extraction). The mixture was filtered and the filtrate was acidified (pH 5-6) using 1 M HCl to precipitate the rutin and left to stand overnight. The precipitate was collected by vacuum filtration and dissolved in water and further purified. The pH was adjusted to 6-8 and the solution was boiled for 30 min. It was filtered under vacuum and the filtrate was acidified and left to stand overnight. The resulting precipitate was collected by vacuum filtration and dried in an oven at 70°C. The aqueous extracts were prepared daily, just before administration. The extracts obtained were then given orally to different groups of rats at a dose of 10 and 30 mg/rat.

**Animals**

Young male Sprague-Dawley rats weighing 260±20.5 g obtained from the animal house of the PDM College of Pharmacy were kept under a controlled temperature of 25±2°C and 12 h light/dark cycle and fed with commercial rodent pelleted diet and water *ad libitum*. The animals were allowed to adapt to the laboratory conditions for 2 wk before the experiment started. The experiments were performed after clearance from the Institutional Animal Ethical Committee. About 30 animals were divided into 5 groups of 6 each, for behavioural, biochemical and histopathological studies. Doses of *Capparis spinosa* bud extract (10 mg/rat for pre-treatment and 30 mg/rat for post-treatment) were selected and administered orally for 21 days. In pre-treatment group, lipopolysaccharide (175µg/kg) was given for 7 days i.e. from day 22 to day 28. In post-treatment group, the same LPS dose was given for first 7 days and then from day 8 to day 28 caper extract was given. Vehicle control group was administered normal saline in place of extract. Neurotoxin group was administered lipopolysaccharide for the first seven days. Standard group was administered with AD drug memantine (10 mg/kg, s.c) for 21 days after the LPS injection for the first 7 days. On day 28 behavioural tests (Y maze test and Morris water test) were conducted for another 5 days i.e., 29-33 and then the animals were sacrificed by cervical decapitation under light anaesthesia for biochemical and histopathological assessment on day 33. Figure 1 illustrates the design of the experimental study.

**Morris Water Maze test**

After the treatment for 28 days, the spatial learning and memory were evaluated by the Morris water maze test. The water-maze task of was carried out...
from day 29 to day 33. The experimental apparatus consisted of a white circular water tank (140 cm in diameter and 45 cm high). A transparent platform (10 cm in diameter and 25 cm high), which could not be seen by rats, was set inside the tank, which was filled to a height of 27 cm with water of temperature approximately 23°C; the surface of the platform was 2 cm below the surface of the water. The water tank was located in a test room (15 m$^2$), in which there were many cues outside the maze. The room had adjustable indirect illumination, and a camera was fixed to the ceiling. The position of the cues remained unchanged throughout the water maze task.

For the acquisition (hidden platform) test, the hidden platform (10 cm in diameter) was kept constant in the middle of the one certain quadrant and submerged 1 cm below the water surface (30 cm away from the side wall) throughout training. The rats were required to find the hidden platform using spatial clues available in the test room where all conditions were constant throughout the experiment. The rats were subjected to 4 trials per day for 5 consecutive days. In each of the 4 trials, the rats were gently released into the water by facing the tank wall at four different starting positions equally spaced around the perimeter of the pool. They were given 60 s to find the submerged platform. On reaching the platform, the rats were allowed to stay on it for 20 s. If a rat failed to locate the platform within 60 s, it was guided to the hidden platform and allowed to rest on it for 20 s, and a maximum score of 60 s was assigned. The time that an individual rat took to reach the hidden platform was recorded as the escape latency (seconds) for its spatial learning score.

To assess the retention of spatial memory, a probe trial was performed 24 h after the last training trial. In this trial, the platform was removed from the tank, and the rats were allowed to swim freely for 60 s. The time that an individual rat spent in the target quadrant previously containing the platform was recorded as a measure of spatial memory.

Y-Maze test
The Y-maze task was carried out from day 35 to day 36. The experimental apparatus consisted of a Y-maze made of plywood. Each arm of the Y-maze was 49 cm long, 19 cm high, and 8 cm wide and positioned at an equal angle (labelled A, B, and C). The room was filled with distinctive visual cues such as desks, chairs, and pictures. The apparatus was placed on the floor of the experimental room and illuminated with a tube from 60 cm above. Each rat was placed at the centre of maze and allowed to move freely through the maze during a 6-min session. The sequence of arm maze was recorded manually (i.e., ABCBAC)$^{20}$. An actual alternation was determined from successive entries of the three arms on overlapping triplet sets in which three different arms are entered$^{21}$. For example, a sequence of entries of arms ABC that consists of ACBABCAB would yield five alternations, namely, ACB (1), CBA (2), BAC (3), ACB (4) and CBA (5). The number of maximal alternation is counted as the total number of arm entries −2; and the percentage of alternation is calculated as:

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\% \text{ alternation} = \left( \frac{\text{actual alternations} - \text{maximal alternations}}{\text{maximal alternations}} \right) \times 100.
\]

Maze arms were thoroughly cleaned between tasks to remove residual odours. In addition, the total number of arms entered during the sessions was also determined.

Biochemical assessment
Following the behavioural study, the animals were sacrificed by cervical decapitation under light anaesthesia. The whole brain was carefully removed, weighed and transferred to a glass homogenizer. Homogenization of the brain was carried out in an ice bath with 0.1 M phosphate buffer (pH 8). The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant was used for biochemical assessment i.e., AChE activity (Moles thiocholine hydrolyzed/minute/mg protein). The quantitative measurement of acetylcholinesterase in brain was performed according to the method described by Ellman et al.$^{22}$ The assay mixture contained 0.005 mL of supernatant, 3 mL of 0.01 M sodium phosphate buffer (pH 8), 0.10 mL of acetylthiocholine iodide and 0.10 mL of DTNB (Ellman reagent). The change in absorbance was measured immediately at 412 nm spectrophotometrically.

Histology
After the behavioural test, animals were killed on day 37 and brains were removed and dissected into hippocampal and cortex blocks. After the behavioural test, all the rats were anesthetized with ketamine and xylazine and perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer solution (pH 7.4). Then, the brains were removed and fixed in the similar solution for 24 h. Following routine processing in
paraffin, serial coronal sections of the brain were cut at 8 µm thickness in a rotary microtome (Leitz, 1512, Germany). The parts of each rat brain section were stained with H&E. Briefly, sections were deparaffinised through xylene and alcohols into tap water so that hematoxylin (a basic dye) may penetrate well. After washing thoroughly with distilled water, slides were treated with hematoxylin (basic dye for staining nucleus). After staining, slides were washed with 1% acid alcohol for differentiation. After appearance of reddish orange colour the slides were washed in running water followed by treatment with Scott's mixture, which was used for bluing. Further, the slides were washed in running water followed by treatment with 1% eosin (acidic dye) for staining the cytoplasm. Slides were then rinsed in tap water, fixed in DPX (Dibutyl phthalate xylene), and dehydrated through alcohols and xylene. (Note: out of 6 rats in each group used three were sacrificed to carry out AChE activity and remaining three were used for histopathology.)

**Statistical analysis**

The results are presented as mean±SD. To determine the difference in various groups/treatments, unpaired t test and one way of ANOVA were applied. Values of $P < 0.05$ were considered as significantly different using Graph Pad Prism software, version 6.02.

**Result and Discussion**

**Morris Water Maze**

Comparisons were taken on day 4. The reduction in time over a period of 4 days indicates acquisition of learning and memory in rats. On day 1, lipopolysaccharide (LPS) treated rats didn’t show any significant difference compared to control rats in time to reach platform. On day 4 LPS treated animal exhibited a significantly increased time to reach the platform compared to control rats, indicating loss of memory. This LPS induced loss of memory may be attributed to inflammation produced in brain. LPS can contribute to Alzheimer’s Disease by reducing brain to blood efflux of amyloid beta, which is generally mediated through a transporter. Acquisition and memory which was abolished in case of LPS treated group was restored when LPS injected animals were treated (pre and post) with extract of *Capparis spinosa*. On day 4, pre ($P < 0.001$) and post ($P < 0.001$) treated rats showed significant decrease in time to reach the platform as compared to LPS group. However, there was no significant difference observed between the pre- and post- treated groups. Prevention of loss of memory in pre-treated group may be due to enhancement of immunostimulatory property of *C. spinosa* extract against LPS induced neuroinflammation. However, post-treatment with extract is indicative of curative properties of extract against LPS induced neuroinflammation. Memantine hydrochloride, an NMDA receptor antagonist, didn’t show any improvement in loss of memory caused due to LPS, indicating that loss of memory by LPS is independent of NMDA pathway (Fig. 2).

**Y-Maze test**

Percentage alternation is an indication of an animal responsiveness towards novelty. Therefore, a higher percentage alternation will indicate an animal instinct towards novelty. In comparison to normal control rats, percentage alternation was found to be significantly ($P < 0.001$) decreased in disease control rats (LPS). On the other hand, compared to disease control rats, percentage alternation was significantly increased in both pre-treatment ($P < 0.05$) and post-treatment ($P < 0.05$) groups (Fig. 3A). However, memantine hydrochloride treated group showed significant ($P < 0.01$) increase in percentage alternation compared to disease control group. During Y maze test, an animal (here, rats) exhibits reduced percentage alternation due to LPS treatment. This is probably due to loss of memory as rats did not incline toward new arm (decreased percentage alternation). Percentage alternation also termed as SAB (spontaneous alternation behaviour) should not be considered as strong parameter for memory, especially short term memory, because choice of arm selected for entry doesn’t always reflect the memory, rather...
attention/sensory factors and motivational related novelty preferences also play a major role in it\textsuperscript{21}. However, instinct towards novelty is strong indication of working memory (Fig. 3A). SAB was used as a measure for evaluation of effect of \textit{Capparis spinosa} buds extract on working memory \textsuperscript{27}. An alternation is considered as scored/positive when an animal enters three arms consecutively. LPS administration significantly ($P<0.001$) reduced the positive alternations to about 47% of the control group. Administration of \textit{C. spinosa} buds extract did improve cognition as evidenced by a significant ($P<0.05$) increase in the spontaneous alternation after and before LPS induced impairment in both the treated groups, respectively i.e., pre- and post-treated (Fig. 3A).

**Effect of \textit{Capparis spinosa} bud extract on acetylcholinesterase (AChE) activity**

Although the readings were taken for 10, 20, 30, and 40 µl of substrate, convincing comparisons could be made only at 40 µl as substrate. Cholinesterase is one of the key enzymes that degrade acetylcholine into acetate and choline. LPS didn’t increase AChE activity (Fig. 3B) contrary to the earlier finding on setting of AD model by other reagents (streptozotocin, scopolamine, etc.)\textsuperscript{28}.

A memory enhancer is thought to reduce AChE activity. However, in the present study, an increase in AChE was observed in case of pre- and post- treated groups. This resulted in decreased acetylcholine concentration in brain. Acetylcholine and adrenaline are known to exhibit opposite property i.e., an increase in concentration of one will lead to decreased concentration of other and \textit{vice-versa}. Higher activity of AChE in pre- and post- treated groups might have led to reduced concentration of acetylcholine in central nervous system. This may eventually show higher sympathomimetic behaviour, also evident by higher SAB seen in pre- and post- treated groups during Y maze test. Higher sympathomimetic activity may further relate responsiveness to novelty, attentional and motivational activity of rat. Furthermore, AChE has been shown to exhibit neuritogenesis\textsuperscript{29} which could be attributed to non-catalytic behaviour of AChE. The high AChE activity with caper bud extract treatment could be beneficial in dementia, Alzheimer’s and other neurodegenerative disease (Fig. 3B).

**Effect of \textit{Capparis spinosa} bud extract on neurodegeneration**

Sections from the hippocampal area and cerebral cortex of the brain from Wistar rats that had been given LPS only, LPS with \textit{Capparis spinosa} extract and from a normal control group were studied. The neuronal layers in the CA1 region of the hippocampus revealed a minor variation in neuronal densities between the groups (Fig. 4 A and B). There was an apparent difference in neuronal structure; neurons in the control group were large conical shaped cells with well delineated amphophilic cytoplasm and round vesicular nuclei with prominent nucleoli. In the LPS treated group, majority of the CA1 layer neurons showed pronounced shrinkage of neuronal bodies with the nuclei losing their regular outlines and becoming hyperchromatic. The group where herbal extract was administered after LPS i.e., post-treatment group [Fig. 4A(iii)] showed the best results with significantly lesser degree of degenerative changes and only a few neurons showing shrinkage. The pre-treatment group [Fig. 4A(iv)] and the group treated with memantine hydrochloride [Fig. 4A(v)] showed a lesser degree of protective effect against LPS induced damage. In the cerebral cortex too, degenerated neurons were identified in the LPS treated group [Fig. 4B(ii)] and LPS with pre-treatment groups [Fig. 4B(iv)]. While the normal control, LPS with post-treatment and LPS with memantine groups showed only normal neurons [Fig. 4B(ii and v)].
Histopathological examination of hippocampus region, precisely CA1 area, is identified and examined after hematoxylin and eosin (H&E) staining. Hippocampus proper consists of CA1-CA4 region, where CA1 region is primarily responsible for learning and memory process. Any abnormality in the cells of this region indicates alternation in the memory. It is a hallmark of AD. Among the commonly available staining methods, Nissl staining, Bielchowsky staining and Golgi method are widely used to distinguish and examine different types of cells and their abnormalities in hippocampus region. Although some references strongly recommend Congo red staining and immunohistochemistry (IHC) for detection of amyloid plaques to confirm AD, Golgi method helps in identification of dendritic spines in the pyramidal neuronal cells of CA1 region of hippocampus. Reduction in the number of dendritic spines is an indication of pathology of CA1 region which signifies AD. In the present study, H&E staining also known as Nissl staining was used to detect abnormalities in neurons of CA1 region of hippocampus of different treatment groups. Prominent neurodegeneration is observed in LPS induced rats. This neurodegeneration is significantly reduced in post-treated caper group indicating that caper bud extract helped in reversal of CA1 damage caused by LPS. However, it didn’t exhibit effective role when applied before LPS administration as evident by less significant protection in pre-treatment group [Fig. 4A(iv)-4B(iv)]. Fractionation of caper’s extract needs to be carried out in future. Isolated compounds obtained through fractionation need further exploration of neurodegenerative disorders. However, the problem why the caper extract act more efficiently post treatment whereas fail to prevent the LPS induced neurodegeneration remains unaddressed, waning of the active constituents from the circulation perhaps be the reason, which requires further kinetic studies.

References