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ORIGINAL ARTICLE

Various Histological Assay and Molecular Analysis Evaluating Neuroprotective Effect of *Pongamia pinnata* Leaf on Cerebral Ischemia -Preclinical Study

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ABSTRACT
Cerebral ischemic/stroke is the most common cause of disability worldwide. The concept of Neuroprotection is gaining a lot of attention in the hunt for innovative therapies that could protect the brain tissue and enhance the cognitive and motor abilities. The aim of the study is to compare and determine the efficacy of 70 % ethanolic extract of Pongamia pinnata (P.pinnata) leaves on stroke induced model of Wistar rats. On the experimental rodents, novel stroke induction technique was used and characterized. In male Wistar rats, ischemia/reperfusion (I/R) was incorporated in the brain by occlusion of BCCA method for 60 minutes followed by reperfusion for 72 hours. The effect of the P.Pinnata leaf extracts on the brain sample was examined using various histological staining assays such as TTC for locating the infarct area, Cresyl violet staining for quantification of normal neurons, acridine staining for detecting apoptopic cells present in and around the motor and subventricular zone of the brain and finally H&E staining was used to understand the changes occurred in the brain infarct and its penumbra. The objective of the present investigation is to determine the effects of pretreatment with 400 mg of P.pinnata leaf extract on functional motor recovery. Rats were randomly divided into 4 groups; G1, G2, G3 and G4, with 6 animals in each group. Control (G1-Positive Control,with 0.9% Normal Saline (NS)support); G2-Sham (Sh + NS), G3- ischemia induced but treated and G4- (Treated with 400 mg ethanolic extract of PP (400mg + NS). The dosed rats per the group descriptions were maintained in their respective state for three weeks (21 Days) Neurological examination was performed after 24 hours of reperfusion, animals were sacrificed, and their brains were used for histopathological & molecular studies. To access the cerebral injury and recovery, the Cresyl Staining was used to demonstrate the Nissl substance in the neurons and cell nuclei, Additionally, acridine orange staining was done to detect the live and dead cells with the help of fluorescent microscopy and also H & E To confirm the same the gene expression of BDNF & GDNF is also done by RTPCR. Images of the all the groups were documented for evaluating the patterns of the group's 1 to 5 based on the treated and untreated groups. When compared to the G3 & the treated group(G4) showed a significant motor recovery. The MRNA level is almost same in Normal control(G1) and Sham control(G2) group. On histological examination, the cresyl violet stained images showed the neuro protective changes on ischemic models exposed to P.Pinnata leaf extract. Results of Cresyl violet, Acridine orange & H&E, P.Pinnata leaf therapy demonstrated significant antioxidant effects by preventing lipid peroxidation in ischemic condition & resting the glutathione peroxidase enzymes to considerable levels. After I/R in animal groups, the motor function deficit is comparatively less to a significant extent when given at a dose of 400 mg for 21 subsequent days. The study concludes that the element of P.pinnata leaf extract has neuroprotective potential in ischemic injury, by increasing normal neurons and vascularity.
Key words; P.pinnata leaf, cresyle violet for brain, Acredine orange for brain tissue, Neuro protective.

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INTRODUCTION

Cerebral Ischemia has become increasingly common in recent years due to various reasons. Stroke has become a third leading cause of death worldwide next to the cancer and the cardio vascular diseases. Cerebral ischemia/Stroke can result in sudden cerebral death or severe, long-lasting disability if prompt,

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effective management is not provided [1]. Ther e are two primary forms of stroke: hemorrhagic stroke (cerebral and subarachnoid) and ischemic stroke (thrombotic and embolic). Even though hemorrhagic stroke is more common, more frequently fatal, cerebral ischemic stroke has the highest frequency and incidence rates when compared to hemorrhagic stroke [1,2].

As a result of this injury, degeneration, oxidative stress inflammation, cellular death another events cascade occurs [3, 4]. Lack of effective therapy is the fundamental problem in treating ischemia. Considering this situation, there is a necessity to address and focus alternative therapeutic options for ischemic stroke. In recent years, the researchers have concentrated heavily on researching medicinal plants for their neuro protective properties.

P.Pinnata pierre is a part of Fabaceae (=Leguminosae) family that includes the promising oilseed crop known as karanja (Millettia pinnata (L.) Panigrahi). Other names for this medium-sized tree include *Pongamia glabra Vent.,P. pinnata* (L.) Pierre and Derris indica (Lam.) Bennett . 'Karum Tree' or 'Poonga Oil Tree' are the common, well-known names worldwide. A glabrous, perennial tree known as karanja can be found in Australia and South-Eastern Asia and is thought to have Indian origin [5].

Numerous herbal treatments, either used singly or in combination, are suggested for the treatment of various ailments and are popular in giving effective cures. The curative effects of the herbal derivatives are commented to be due to the presence of number of phytoconstituents such as Alkaloids, Carbohydrates, Phytosterols, Saponins, Tannins, Flavonoids etc. Phytochemical components include Furano flavonoids and chalcones which are capable of exerting neuroprotective activity in various neurodegenerative diseases [6, 7]. This study focuses on evaluating the possibility of restorative effect on stroke rat models with the use of ethanolic extracts of leaves from *P. pinnata* [8].

The other parts of the tree are also utilized for various diseases in a different way. The seeds are utilised for keloid tumours in Sri Lanka, the fruits and sprouts are used in traditional Indian and Sri Lankan treatments for abdominal tumours, and a plant-derived powder is used for neoplasms in Vietnam. To treat skin conditions, seeds were employed in Sanskrit-era India. The oil is still used today as a rheumatism liniment. The juice from the leaves is used for colds, coughs, diarrhoea, dyspepsia, flatulence, gonorrhoea, and leprosy & Micrococcus. Roots can be used to clean teeth, gums, and ulcers. For bleeding piles, bark is ingested. The idea to use *P.pinnata* as a therapeutic option to treat Stroke occurred with the beneficial effects of the tree and its parts for various complications. Additionally, the phytochemistry has provided a strong hypothetical confidence that can support its beneficial effects to neurological conditions such as stroke.

This study focuses on evaluating the possibility of protective effect on stroke animal model of rat.[5] Histopathological evaluation was done by using Cresyl violet as the characteristic and differentiating stain. Cresyl violet is reliable and widely used for studies carried out in the field of brain research.[6] Cresyl violet when it is used in the evaluation of histopathological specimens, it is possible to scrutinize the level of neuronal damage present, degree of pathological changes in the infarct area and also the effect of *P.pinnata* leaf extract at the site of ischemic lesion.[6] The another important staining is acredine orange (AO), which is a nucleic acid dye with unique spectral properties, enables real-time measurement of RNA and DNA as proxies for cell viability during exposure to various noxious stimuli. This tool illuminates spectral signatures unique to various modes of cell death, such as cells undergoing apoptosis versus necrosis.[9,10.]

A comparison between Cresyl violet, Acredine orange, TTC and hematoxylin eosin staining, gives an increased clarity in the neuronal structure in normal, pathological, and treated brain sections. Being the gold standard of histopathology, hematoxylin and eosin staining highlights neurodegeneration to a wider extent when compared to other stains, and has been used as a part of this study. Similarly a simultaneous molecular analysis of the neurotrophic factors (GDNF, BDNF), can help to correlate the levels of each factors, the probability of the incidence of a ischemia to occur and the neuroprotective property of *P.Pinnata* leaf extract.

MATERIAL AND METHODS

Plant material:

The green matured *P.pinnata* leaves were collected from BMCRI Campus @ Bangalore at the month of march 2022.The voucher specimen of P.pinnata (no B-00010) was placed in Vishwesaraiya College of science, in Department of Botany at Bangalore. The processing of plant material and extraction was done in Govt. college of Pharmacy, at department of pharmacognosy, in Bangalore. Hydroalcoholic extracts were made from shade-dried plant portions of leaves using 500 gm of coarse *P. Pinnata* leaf powder (Borosil) with the help of Soxhlet apparatus, [14]. The filtrates were individually concentrated using rotary vacuum evaporation at temperatures over 45 °C and freeze drying at temperatures below 20 °C to get solid residue [11].

The ethanolic extract of *P.Pinnata is* carried for the preliminary phytochemical screening Which gave positive results for glycosides, sterols, tannins, and flavonoids. The extract was utilized by dissolved in sterile water [12].

Experimental animal:

The experiment was carried out after receiving the approval from the Institutional Animal Ethical Committee at SDC, CHENNAI, India (BRULAC/SDCH/SIMATS/IAEC/08-2022/138). Male *Wistar* albino rats weighing 250-300 grams was used for this study. Rats were fed a commercial rat pellet diet provided by MASS BIOTECH, CHENNAI, and were provided with water *ad libitum*. They were kept at a natural light and dark cycle under a temperature of 20 -25 degrees C, at a humidity of 50 to 55 percent.

EXPERIMENTAL DESIGN:

Distribution of Animals:

4 distinct categories of Wistar albino rats with a total of seven rats each were randomly assigned to them For 21 days.

G1: (positive control- Cont+NS) The rats were administered with 0.2 ml normal saline orally for 21 days. no induction of ischemia.

G2 (Sham + NS) received a 0.2 ml of saline orally for 21 days before the procedure and undergone for surgery & no ischemia is induced.

G3 (Negative Control – Stroke Induced Model)**-** received a 0.2 ml of saline orally for 21 days before the procedure and undergone for surgery & Ischemia is induced.

G4 (Treated groups) **-** Rats were treated with *P.pinnata* leaf extract 400 mg per/ kg of body weight prepared in 0.2 ml of saline for 21 days as a pretreatment respectively before the surgery & ischemia is induced .

Table No. 1 – Distribution of Groups

Groups	Details	No. of Animals
G1	Positive Control + NS	6
G2	Sham Cont+ NS	6
G3	Negative Control – Stroke Induced Model	6
G4	Experimental Study 1 (Dosage 1 – 200 mg/kg)	6
	Total Number of Animals	24

Bilateral common carotid occlusion model:

Rats were anaesthetized by injecting 0.3 of ketamine & xylazine, followed by crown of head was shaved and cleaned with 70 % alcohol. Skin incision was done on the ventral aspect of neck reflecting the skin and the superior belly of omohyoid muscle to find the carotid artery which ran along with the Vagus nerve. The Vagus nerve was divided from the carotid artery to avoid injury & 3.0 ethilon was used to ligate the both common carotid artery and it left for 60 minutes. under the aseptic condition the incision was sutured & were placed in a dry cage separately with assistance of heat pad. Temperature was monitored periodically. The neurological examination was performed for each rat 24 h after procedure accordingly [13,14]. (FIGURE A)

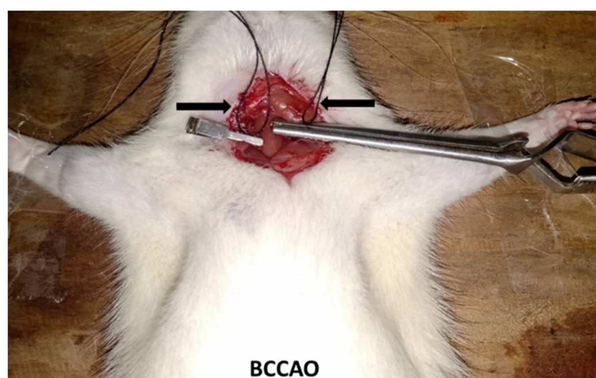

Figure A; Shows Bilateral Common Carotid Occlusion Of Artery

Histological study:

For Cresyl violet staining, the procedure was done with the reference of (Kudret Türeyen el). Slices were 40m thick when cryo sectioned, mounted on slides, stained with CV, and then scanned. The slides were examined with microscope (Olympus CX31 light microscope). CV staining showed a high degree of correlation in infarct area and volume indicating that both methods are suitable for producing accurate measurements of cerebral experimental infarcts [15].

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To find the apoptotic cells in a better way the acredine orange staining was carried as per (ref Sarah Beckman et al's) procedure [16]. Accordingly, Tissues were put in 10 g/mL of AO (Sigma, St. Louis, MO) in E3 medium to conduct the test and rinsed after 60 minutes of staining. Tissues were stained 3 times in E3-related media, and then moved to 96-well plates with a flat bottom for imaging [12].
According to Feldman AT, Wolfe D, the H & E staining procedure was carried out. The foundation of anatomical pathology diagnosis is the tissue sections stained with hematoxylin and eosin (H&E). To easily distinguish cellular components, the H&E technique uses contrasting colours to stain the nucleus and cytoplasm. The effective specimen processing method which includes tissue preservation, dehydration, cleaning, and paraffin infiltration is necessary for proper outcomes [37].
The percentage of positive neurons present in the motor cortex was done with, cresyl violet, acredine orange, H & E staining techniques. After completing the behavioural test, the rats were euthanized using CO_2 chamber. Then the whole brain tissue was taken out and transferred to 10 % formalin followed by embedded in paraffin.

Statistical Analysis:

The method used was ANOVA test (a multiple variance test) Probability criteria- values less than 0.005 were considered significant ($P< 0.005$).

Real time PCR analysis

Traditionally RT-PCR involves two steps: the RT reaction and PCR amplification. CFX96 Touch Real Time PCR Detection system was used for this analysis. Genes Forward primer Reverse primer Amplicon size (bp) are given in Table 2.

Table No. 2: Forward and reverse primers

Markers	Forward Primers	Reverse Primers
BDNF	AATAATGTCTGACCCCAGTGCC	CTGAGGGAACCCGGTCTCAT
GDNF	GCGCTGACCAGTGACTCCAA	GCGACCTTTCCCTCTGGAAT

The first stage included designing primers for the target gene, which was accomplished using primer synthesis, followed by extraction of the RNA. The total RNA was extracted from the brain section by a standard methodology using Trizol, and continued according to the reference [17]. It helped to compare the levels of mRNA expression in the treated groups to determine the impact of *P.Pinnata* [18, 19].

RESULTS

Figure 1A shows the Cresyl violet staining of G1-Positive control group G1 showed histological picture of normal rat brains, G2-Sham control group showed histological picture of normal rat brains as well. G3-Induced control group & G4 -Treated group (by P.pinnata leaf extract 400 mg). The rats treated with P.Pinnata leaf extract (G4) lost less cortical tissue than the ischemia induced control rats(G3). Further examination found that P.Pinnata treatment decreased dead cells in the cortex and the subventricular zone estimated by Cresyl violet staining.

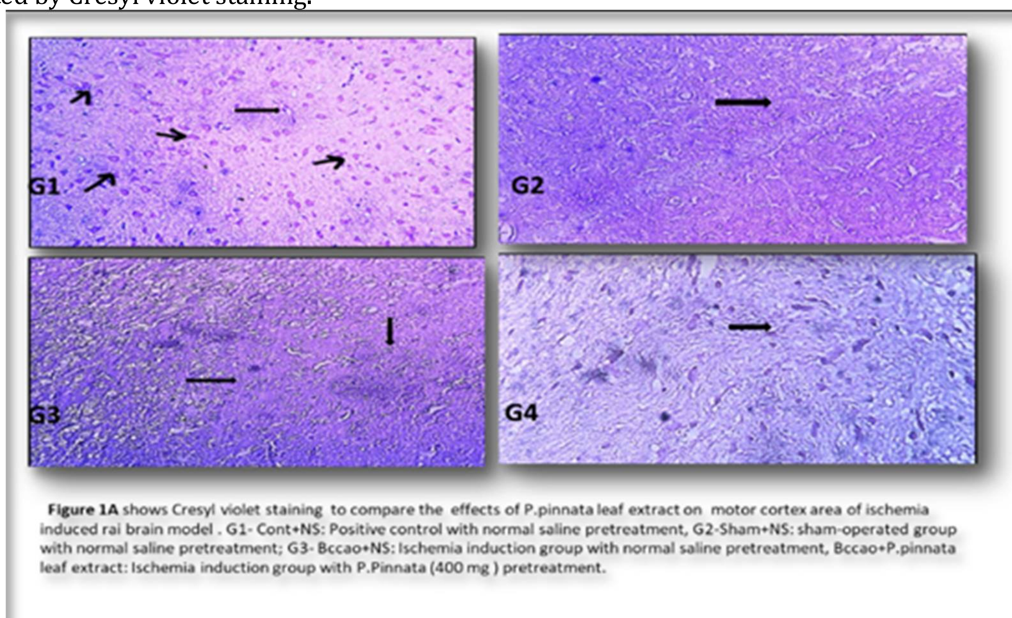

Figure 1A shows Cresyl violet staining to compare the effects of P.pinnata leaf extract on motor cortex area of ischemia induced rai brain model . G1- Cont+NS: Positive control with normal saline pretreatment, G2-Sham+NS: sham-operated group with normal saline pretreatment; G3- Bccao+NS: Ischemia induction group with normal saline pretreatment, Bccao+P.pinnata leaf extract: Ischemia induction group with P.Pinnata (400 mg) pretreatment.

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CV staining is based on the fact that early on in an area of infarction under light microscopy, the earliest neuronal alteration after ischemia is micro vacuolation of the cytoplasm. The infarct area of the section contains less intact cells than the normal brain. The resulting CV staining shows dark blue color in a normal region, but light blue color in the infarct area of the brain[15.] This was showing swollen astrocytic processes surrounding the damaged neurons .CV stains normal neuronal cytoplasm with blue color.

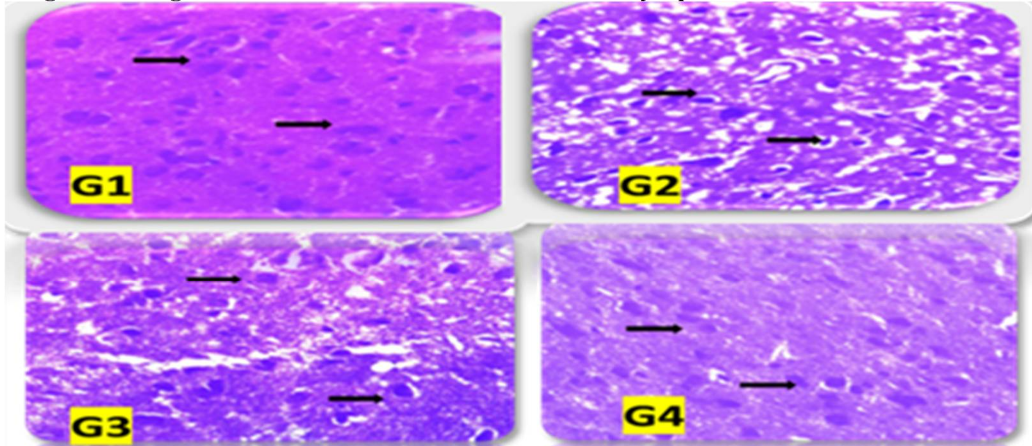

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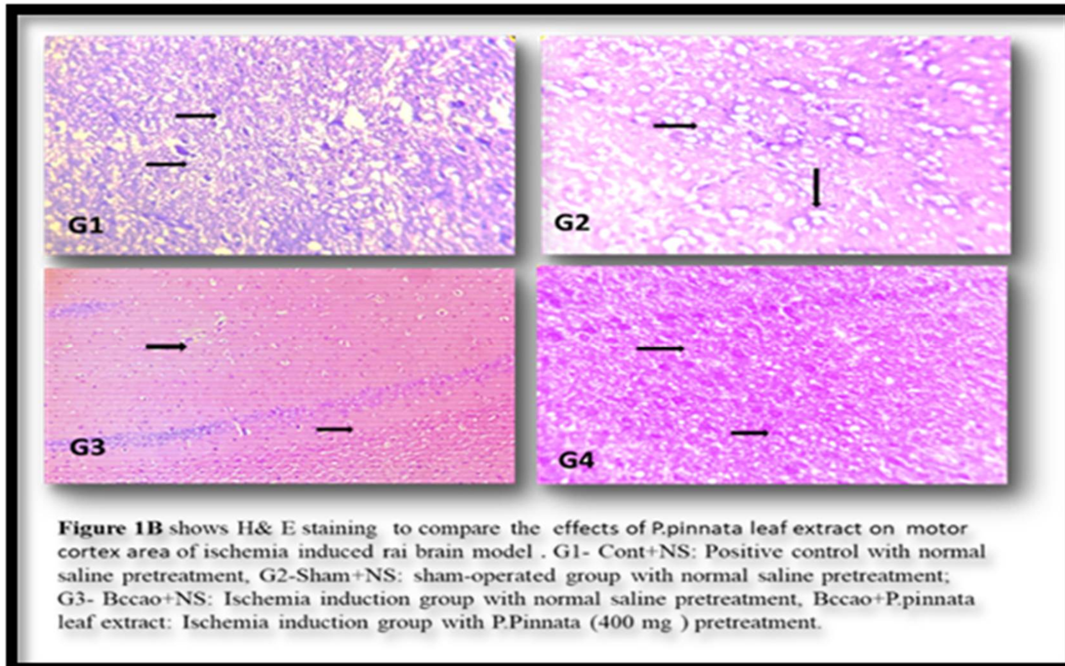
Figure 1A1; shows the cresyl violet staining of 4 groups (G1-G4) animals in 100x

Figure 1B; shows the H&E staining of all 4 groups. **G1 – Positive** control rats showing the normal histological structure of the rat brain. **G2 -** Sham control rat showing the normal histological structure of the rat brain as well. **G3-** Induced control group showed, neurons were found to present shrinkage, nuclear pyknosis, and showed increased intercellular space (thick arrow). **G4** rat treated with 400 mg showing reduced numbers of degenerated and necrosed neurons.



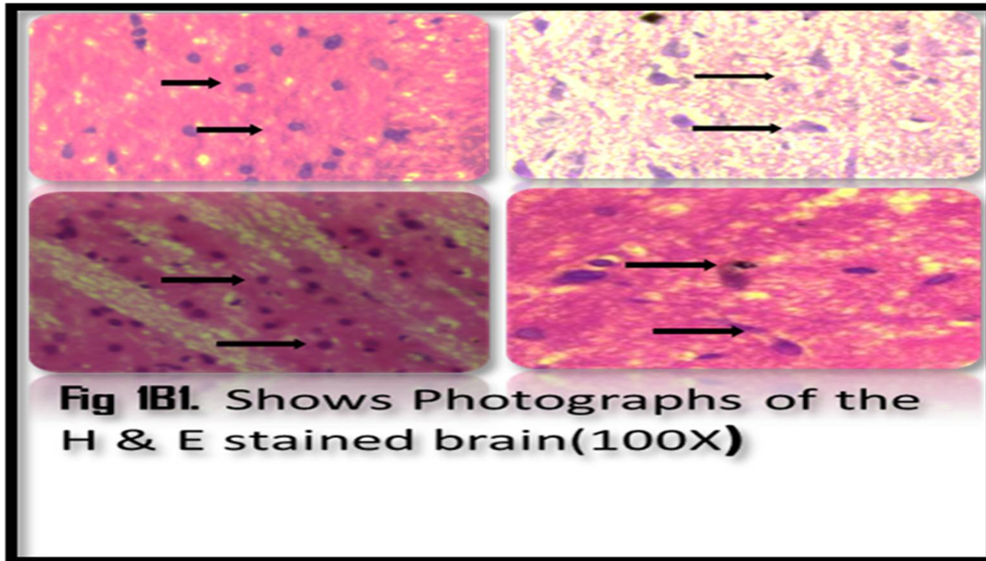


Figure 1B1; shows the H & E staining of 4 groups (G1-G4) animals in 100x

FIGURE 1C shows Acredine orange staining images for 4 groups. **G1-** positive control and **G2-** sham control shows Normal rat brain structures. **G3-** Induced control group more of florescence stain indicates more of cell death than the treated group **G4** -(treatment group with 400 mg P.Pinnata leaf extract.

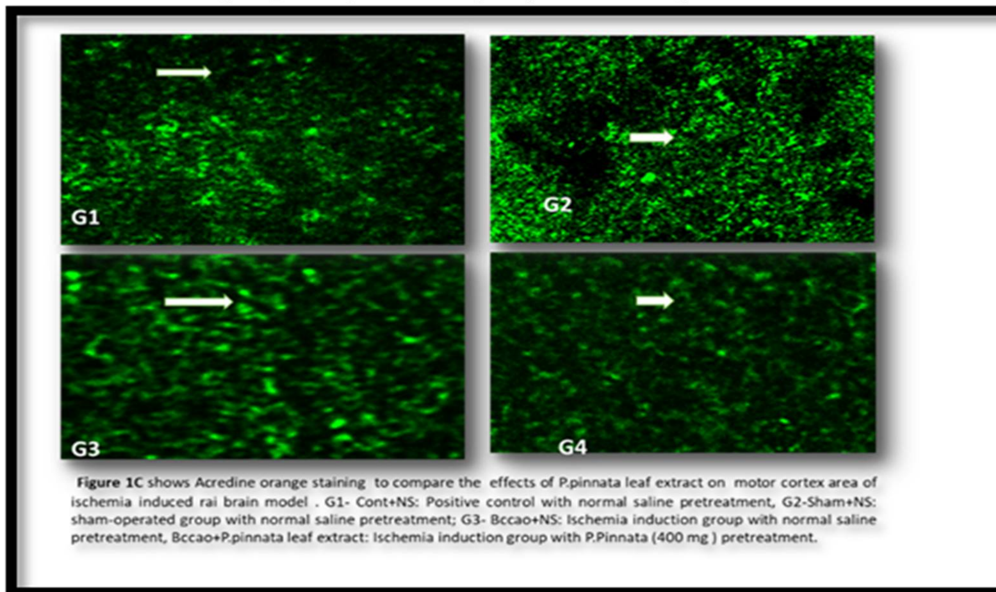


Figure 1C shows Acredine orange staining to compare the effects of P.pinnata leaf extract on motor cortex area of ischemia induced rai brain model . G1- Cont+NS: Positive control with normal saline pretreatment, G2-Sham+NS: sham-operated group with normal saline pretreatment; G3- Bccao+NS: Ischemia induction group with normal saline pretreatment, Bccao+P.pinnata leaf extract: Ischemia induction group with P.Pinnata (400 mg) pretreatment.

FIGURE 2A: shows the Graphical representations of RT-PCR analysis of the Tremendous increase in BDNF values as shown is seen after the exposure to *P.pinnata*, can be related with its prognosis. The higher the levels, the better the prognosis in an after-stroke episode.

FIGURE 2B: shows the Graphical representations of RT-PCR analysis of. Glial derived factors were analysed to be increased even after exposure to ethanolic *P.pinnata* leaf extract. This primarily because the increased levels by GDNF is directly proportional to the neuroprotective effects such as neuronal survival and decreasing the oxidative stress levels [20].

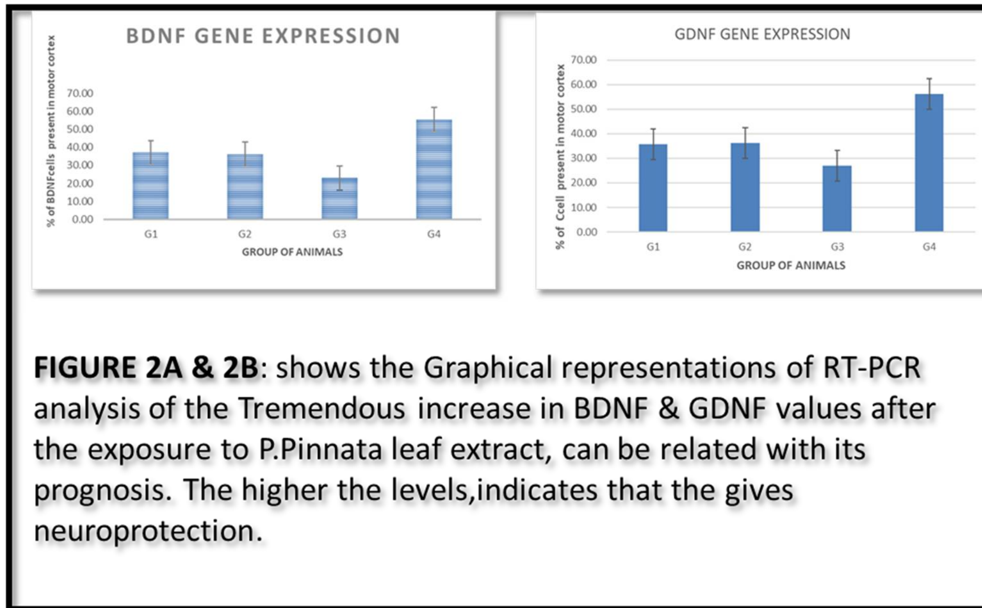


FIGURE 2A & 2B: shows the Graphical representations of RT-PCR analysis of the Tremendous increase in BDNF & GDNF values after the exposure to *P.Pinnata* leaf extract, can be related with its prognosis. The higher the levels, indicates that the drug gives neuroprotection.

Alterations of cell population happen in the central nervous system during the pathogenesis of Cerebral ischemia/ ischemic stroke. Morphological changes are quite common in the neurons whereas in the case of axons and cell bodies, they get disintegrated in the system. Disappearance of nucleolus can be seen when the Glial cells and neurons undergo cytoplasmic swelling. Significant changes are appreciable in penumbra with the ischemic neurons showing changes in the Nissl's bodies' disintegration and endoplasmic reticulum. One or the other morphological difference is displayed in the other cells such as glial cells, astrocytes and microglia [14]. During the sequence of stroke incidence, BBB shows exceedingly increased permeability that leads to infiltration of immune cells like macrophages, leukocytes and monocytes in the site of ischemic lesions. There occurs a rapid influx of neurotoxic and neurotropic factors exerting the neuroprotective or detrimental effects on ischemic brain tissues [21, 22].

Brain tissues depict a depleted or loss in the storage of oxygen and glucose which is caused due to an abrupt interruption in the cerebral blood flow, which in consequence would derange the level of ion homeostasis, ATP synthesis, and acid-base imbalance, subsequently altering a highest level of energy deficiency. The effect of energy deficiency results in activating or dysregulating various signalling pathways during the pathological transitions [23, 24] which may occur either separately or simultaneously in the stroke sequence.

Energy deficiency in cerebral ischemia causes various other changes such as the mitochondrial dysfunction and the damage caused during oxidative stress. Neuronal cell depolarization and glutamate release is triggered due to oxygen and glucose deficiency [23]. Certain signalling pathways that get triggered in ischemic stroke includes: Phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway wherein synaptic stimulation would result in NMDARs activating the pro-survival PI3K/Akt signalling pathway, that exerts a neuroprotective effect. NMDAR activation at the synaptic junction and Ca2+ influx tends to activate the Ras/extracellular signal-regulated kinase (ERK) signalling pathway and nuclear Ca2+/ calmodulin-dependent protein kinases, which subsequently activates and phosphorylates CREB. In the combination of NMDAR and BDNF, there occurs an activation of numerous pro-neuronal genes through CREB. Extra-synaptic NMDARs are gradually related to the pathways that are associated with cell death and henceforth pavements the effects triggered by synaptic NMDARs [23, 25].

DAPK1 is a known protein that participates in excitotoxicity in ischemic stroke. During ischemia, NMDAR overactivation leads to Ca2+ influx, activates the Ca2+/calmodulin pathway, and stimulates calcineurin phosphatase, which subsequently dephosphorylates and activates DAPK1. DAPK1 is then transferred to the GluN2B subunit of NMDARs, potentially activating ischemic injury [21]. Pro-survival signalling factor of ERK inhibits DAPK1 and thus relates DAPK1-ERK to control the neuro defensive effect of ERK on stroke induced models. The nitric oxide production is heavily supported by Neuronal NMDARs, which is associated with calcium/calmodulin and is regulated by nNOS [23, 25, 36].

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BDNF plays a noteworthy role in the pathogenesis, prognosis, and rehabilitation of stroke. It is well recognized that if there are low levels of circulating BDNF, it is an indication to denote high risk of stroke with poor recovery, as the BDNF expression in the brain is acutely stimulated by stroke [21, 28].
The above-mentioned pathways and interactions were studied to analyse the effect of *P.pinnata*, on stroke. As per the data obtained from the present study, the extract of *P.pinnata* leaves could reverse ischemia-reperfusion injury, that was caused by ischemic stroke. This data obtained in the current study supports and coincides with the findings stated by Annie et al., where in *P.pinnata* flower extracts were shown to exert protective effect in cisplatin and gentamycin induced renal injury, owing to the antioxidative property of the plant [29]. The observed beneficial effect may be contributed by the presence of Flavonoids, which exhibits the antioxidant property.
Cresyl Violet staining depends on the way that from the beginning in a space of localized necrosis under light microscopy, the earliest neuronal modification after ischemia is miniature vacuolation of the cytoplasm. This is in line with the findings of swollen mitochondria, dilatation of the endoplasmic reticulum, an increase in the density of ribosomes and the cytoplasmic matrix, and swollen astrocytic processes that surround the damaged neurons. CV stains the cytoplasm of healthy neurons blue.The infarct region of the segment contains less flawless cells than the typical cerebrum. In a normal area of the brain, the CV staining is dark blue, while in the infarct area, it is light blue [15].
The application of fluorescent techniques is used to the determine nucleic acid concentrations in identifying the desquamated cells AO, that gets bound in response to the concentration of the dye and pH. AO electrostatically binds to RNA's acidic phosphate groups. With respect to complex restricting systems, a quantitative connection between the fluorescence given by Acridine orange. Our outcomes don't uphold in particular restricting AO to DNA, nor does the distinction to the variety that has all the earmarks to be connected directly with one or the other RNA or DNA. Both the nucleolus and edge of the core stained are comparable providing a greenish variety [36].
The present study demonstrated that, the pattern of the neurotrophic factors (BDNF and GDNF) expression has disrupted in motor cortex area after Bccao procedure. Further, pretreatment with *P.pinnata* leaf extract could upregulate the expression of neurotrophic factors (BDFN and GDNF) in the cerebral motor cortex area following brain I/R.

CONCLUSION

Ethanolic extract of *P.pinnata* leaf successfully could retain the expression of BDNF and GDNF firmly showing its efficacy in being neuroprotective against cerebral ischemia/stroke. The study has proven that a herbal compound that is very commonly available in various parts of the world could be a potential alternative to deal with Cerebral Ischemia/stroke. Further to the current study, a clinical evaluation could provide safety and efficacy and thus would prove beneficial to the affected population of stroke which otherwise have limited treatment options with the conservative treatments.

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CONFLICT OF INTERESTS

The authors of this manuscript declare that there are no financial interests associated with this publication and disclose no conflict of interests exists.

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