RESEARCH
www.commprac.com
ISSN 1462 2815

PRECLINICAL STUDY EVALUATING NEUROPROTECTIVE EFFECT OF *PONGAMIA PINNATA* LEAF ON CEREBRAL ISCHEMIA - HISTOLOGICAL ASSAYS

Suganitha Balasundaram [1]*, Lavanya Prathap [2], Shyamala Ganesan [3], Senthil Kumar S [4], Vijaya Raghavan [5]

[1,4,5] Department of Research and Development, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences University, Chennai.
[2] Department of Anatomy, Saveetha Dental College and Hospital, Saveetha University, Poonamalle High Road, Velappanchavadi, Chennai, Tamilnadu.
[3] Department of Clinical Anatomy and Medical Imaging, American University of College of Medicine, Antigua and Barbuda.
[4] Department of Biosciences, Institute of Biotechnology, Saveetha School of Engineering, SIMATS, Chennai.
*Corresponding Author Email: nithakeyan@gmail.com

DOI: 10.5281/zenodo.12580840

Abstract

Cerebral ischemic stroke is a leading cause of disability globally. There is growing interest in the concept of Neuroprotection as researchers seek new therapies to safeguard brain tissue and improve cognitive and motor functions. [1] The study aims to assess and compare the effectiveness of 70% ethanolic extract from Pongamia pinnata (P.pinnata) leaves on a stroke-induced model using Wistar rats. Male Wistar rats were used to create the stroke models and evaluate the impact of the leaf extract. The research focused on examining the effects of 400 mg of P.pinnata leaf extract on functional motor recovery. The rats were divided into four groups; G1, G2, G3, and G4, with six animals in each group. Rats were divided into 4 groups; G1, G2, G3 and G4, with 6 animals in each. Control (G1-Positive Control, with 0.9% Normal Saline (NS) support; G2-Sham control (NS), G3- induced control group (NS) and G4- Treated with 400 mg ethanolic extract of PP (400mg + NS). Cresyl Staining was utilized to illustrate the presence of Nissl substance in neurons and cell nuclei, fluorescent microscopy was used for acridine orange staining to identify live and dead cells, and H & E staining was carried out to verify the histologic changes. The application of cresyl violet staining revealed neuroprotective changes in ischemic models treated with P.Pinnata extract. The results from cresyl violet, acridine orange, and H&E staining indicated that P.Pinnata leaf therapy exhibited significant antioxidant effects by preventing lipid peroxidation during ischemic conditions and restoring glutathione peroxidase enzymes to substantial levels.

Keywords: P. Pinnata, Cresyl Violet, Acridine Orange, Neuro Protective, Stroke, Cerebral Ischemia.

INTRODUCTION

In recent years, cerebral ischemia has become more prevalent for various reasons. Stroke ranks as the third leading cause of death globally after cancer and cardiovascular diseases. Without prompt and effective management, cerebral ischemia/stroke can lead to sudden death or long-lasting severe disability [1].

There are two main types of stroke: hemorrhagic (cerebral and subarachnoid) and ischemic (thrombotic and embolic). Although hemorrhagic stroke is more common and often fatal, ischemic stroke has the highest frequency and incidence rates when compared to hemorrhagic stroke [1,2]

After sustaining this injury, a sequence of degeneration, oxidative stress, inflammation, cellular death, and other events takes place. [3,4] The lack of effective therapy poses a significant challenge in treating ischemia. Given this scenario, there is a need to explore and emphasize alternative therapeutic options for ischemic stroke.

In recent times, there has been significant research focus on investigating the neuroprotective properties of medicinal plants. P.Pinnata pierre belongs to the Fabaceae (=Leguminosae) family, which includes the promising oilseed crop known as karanja (Millettia pinnata (L.) Panigrahi).

This medium-sized tree is also referred to as Pongamia glabra Vent., P. pinnata (L.) Pierre, and Derris indica (Lam.) Bennett. It is globally recognized by common names such as 'Karum Tree' or 'Poonga Oil Tree.' The glabrous, perennial karanja tree is native to Australia and Southeast Asia, and it is believed to have originated in India [5]. Many herbal remedies are recommended for the treatment of different illnesses and are well-known for their ability to provide successful cures, whether they are administered alone or in combination.

It is suggested that the presence of many phytoconstituents, including alkaloids, carbohydrates, phytosterols, saponins, tannins, flavonoids, and others, is what gives herbal derivatives their therapeutic benefits. Furano flavonoids and chalcones are examples of phytochemical components that have the ability to provide neuroprotective effect in a variety of neurodegenerative disorders [6, 7] This work aims to assess if ethanolic extracts of P. pinnata leaves might have a restorative impact on stroke rat models [8].

The various parts of the tree are utilized for treating different ailments. In Sri Lanka, the seeds are used for keloid tumours, while traditional Indian and Sri Lankan treatments make use of the fruits and sprouts for abdominal tumours. In Vietnam, a plant-derived powder is utilized for neoplasms. In ancient India, seeds were used to treat skin conditions, and today the oil is still used as a rheumatism liniment. The juice from the leaves is employed for treating colds, coughs, diarrhoea, dyspepsia, flatulence, gonorrhoea, and leprosy & Micrococcus.

The roots can be used for cleaning teeth, gums, and ulcers. P.pinnata bark is taken orally to address bleeding hemorrhoids. The potential of using P.pinnata as a treatment for stroke was inspired by the tree's beneficial properties for various ailments. Furthermore, the plant's phytochemistry has generated strong theoretical support for its positive impact on neurological conditions like stroke.

This research aims to investigate the potential protective effects of P.pinnata in a rat model of stroke. Histopathological assessment was conducted using Cresyl violet, a widely utilized stain known for its reliability in brain research studies. [6]. When Cresyl violet is utilized for examining histopathological samples, it allows for the assessment of neuronal damage levels, extent of pathological changes in the infarct area, and the impact of P.pinnata leaf extract at the site of ischemic lesion.

Another significant staining method is acredine orange (AO), which is a nucleic acid dye with distinct spectral properties. It facilitates real-time assessment of RNA and DNA as indicators of cell viability when exposed to various harmful stimuli. This technique reveals characteristic spectral patterns associated with different forms of cell death, such as cells undergoing apoptosis versus necrosis. [9,10].

A comparison of Cresyl violet, Acredine orange, TTC, and hematoxylin eosin staining reveals improved visualization of neuronal structure in normal, pathological, and treated brain sections. Hematoxylin and eosin staining, known as the gold standard of histopathology, effectively highlights neurodegeneration to a greater extent compared to other stains and has been utilized in this study.

Similarly, concurrent molecular analysis of neurotrophic factors (GDNF, BDNF) can aid in establishing correlations between the levels of each factor, the likelihood of ischemic events, and the neuroprotective properties of P. Pinnata leaf extract.

MATERIALS & METHOD

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 pharmaco cognosy, 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. 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 Animals:

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.

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	4
G2	Sham Cont+ NS	4
G3	Negative Control – Stroke Induced Model	4
G4	Experimental Study 1 (Dosage 1 – 400 mg/kg)	4
	Total Number of Animals	16

Rats were given anesthesia with a combination of 0.3 ketamine and xylazine, and then the top of their heads was shaved and cleaned with 70% alcohol. A skin incision was made on the front of the neck, and the skin and the upper part of the omohyoid muscle were moved aside to locate the carotid artery, which ran alongside the Vagus nerve. To prevent injury, the Vagus nerve was separated from the carotid artery, and both common carotid arteries were tied off with 3.0 ethilon for 60 minutes. The incision was sutured under sterile conditions, and the rats were placed in separate dry cages with the help of a heat pad.

Their temperature was checked regularly. 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]

To find the apoptotic cells in a better way the acredine orange staining was carried as per (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] Feldman AT and Wolfe D conducted the H&E staining procedure.

Tissue sections stained with hematoxylin and eosin (H&E) form the basis of anatomical pathology diagnosis. The H&E technique utilizes contrasting colors to stain the nucleus and cytoplasm, making it easy to distinguish cellular components. Proper outcomes require an effective specimen processing method that includes tissue preservation, dehydration, cleaning, and paraffin infiltration. This method is essential for accurate results. [17] 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 CO2 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).*

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.

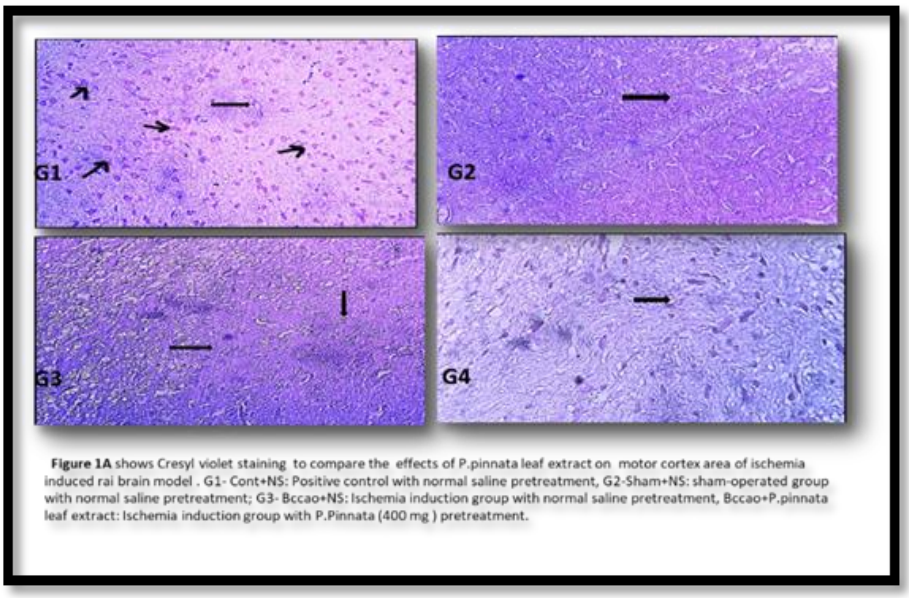

Figure 1A: Shows the Cresyl Violet Staining of 4 Groups (G1-G4) Animals in 10x

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 colour in a normal region, but light blue colour 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 colour.

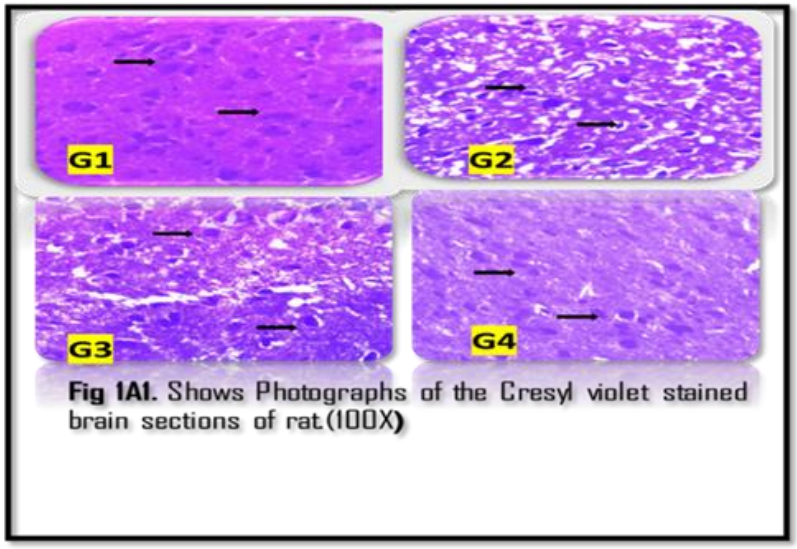

Figure 1A1: Shows the Cresyl Violet Staining of 4 Groups (G1-G4) Animals in 45x

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.

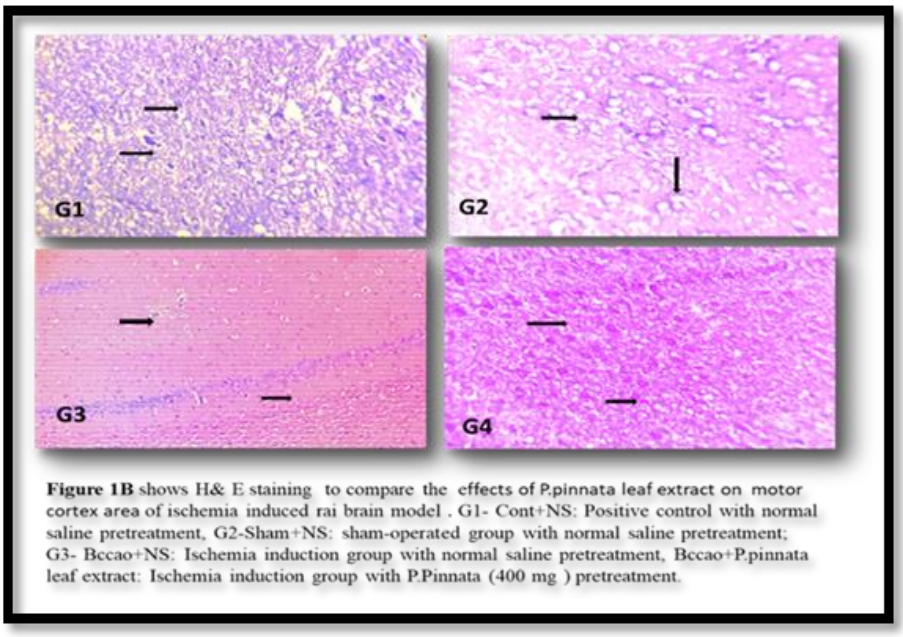

Figure 1B: Shows H&E of 4 Groups (G1-G4) Animals in 10x

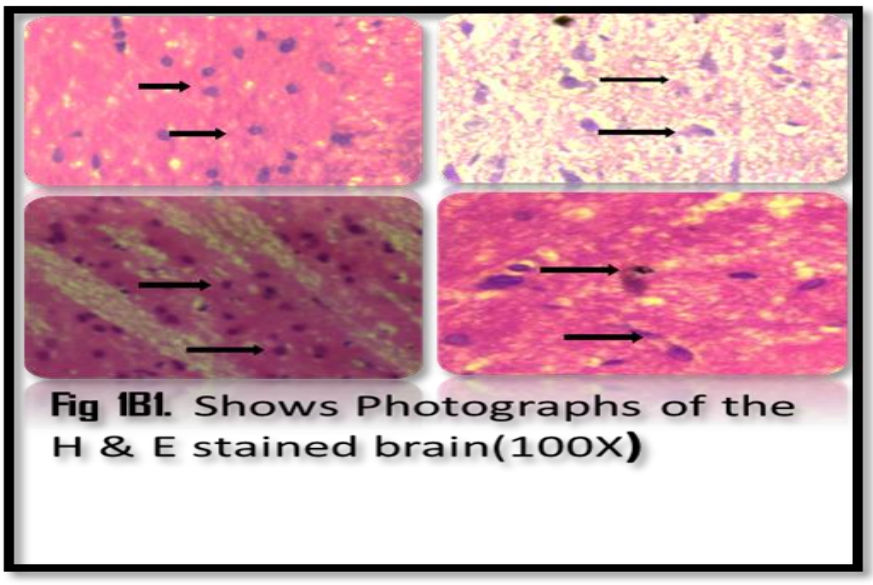

Figure 1B1: Shows the H & E Staining of 4 Groups (G1-G4) Animals in 45x

FIGURE 1C shows Acridine 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.

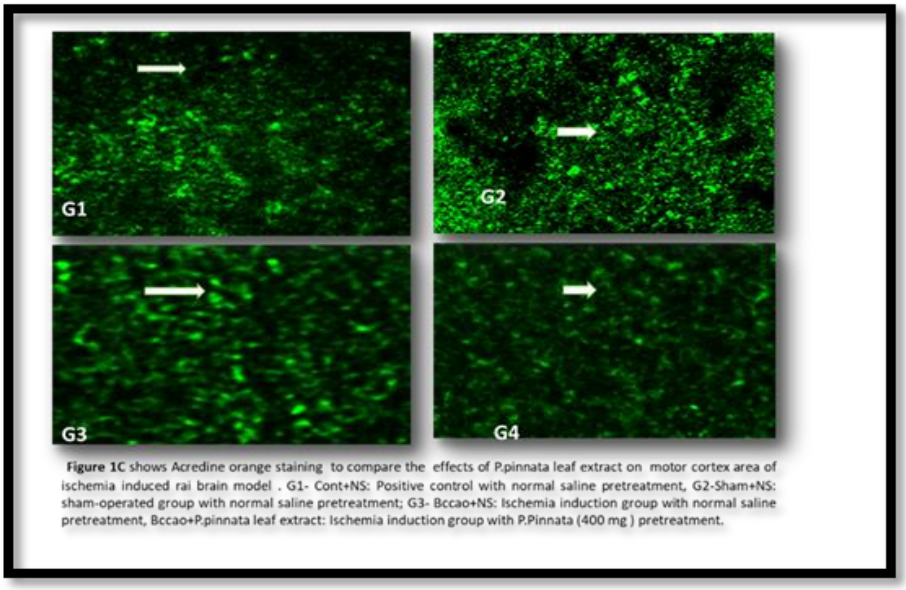

Figure 1C: Shows the Acridine Orange Staining of 4 Groups (G1-G4) Animals in 10x

DISCUSSION

The current study was designed to analyze the neuroprotective potential of *P.pinnata* leaf extract on transient cerebral hypoperfusion and reperfusion by BCCAO in rats. The underlying mechanism of action of *P.pinnata* in protecting neural death in tCHR was assessed by studying the histopathology staining methods such as cresyle violet, acridine orange and H & E.

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 [18]. 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. [19,20]

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 which may occur either separately or simultaneously in the stroke sequence. [20,21]

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. [22] Certain signalling pathways that gets 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. [21]

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.,wherein *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[23]. 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. [15,16]

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. [24,25]

CONCLUSION

The Ethanolic extract from the leaves of P. pinnata demonstrated the ability to maintain more number of normal neurons in treated group of animal compare to control group which indicates its effectiveness as a neuroprotective agent against cerebral ischemia/stroke. This research suggests that a readily available herbal compound from various regions worldwide may offer a potential alternative for managing cerebral ischemia/stroke. Following this study, conducting a clinical assessment could establish the safety and effectiveness of this herbal compound, providing much-needed benefit to stroke patients who have limited treatment options with conventional therapies.

Referrence

1) Wang SW, Liu Z, Shi ZS. Non-coding RNA in acute ischemic stroke: mechanisms, biomarkers and therapeutic targets. Cell Transplant. 2018;27(12):1763-77.

2) Ewida HA, Zayed RK, Darwish HA, Shaheen AA. Circulating lncRNAs HIF1A-AS2 and LINLK-A: role and relation to hypoxia-inducible factor-1α in cerebral stroke patients. Mol Neurobiol. 2021;58(9):4564-74.

3) Ladecola C, Alexander M. Cerebral ischemia and inflammation. Curr Opin Neurol. 2001;14(1):89-94.

4) Langdon KD, Maclellan CL, Corbett D. Prolonged 24-h delayed peripheral inflammation increases short- and long-term functional impairment and histopathological damage after focal ischemia in the rat .J Cereb Blood Flow Metab. 2010;30(8):1450-9.

5) Savita Sangwan et al., A Review on P.pinnata (L.) Pierre: A Great Versatile Leguminous Plant, Nature and Science of Sleep., 2009., 8(11)

6) Manigauha, Ashish & Patel, Sunita & Monga, Jitender & Ali, Huma. (2009). Evaluation of anticonvulsant activity of *P.pinnata* Linn in experimental animals. International Journal of PharmTech Research. 1. 1119-1121.

7) Yadav, Rahul & Jain, S. & Alok, Dr. Shashi & Prajapati, Sunil Kumar. (2011). *P.pinnata*: an overview. Journal of Pharmaceutical Sciences and Research. 3. 494-500.

8) Swamy AH, Patel NL, Gadad PC, Koti BC, Patel UM, Thippeswamy AH, et al. Neuroprotective activity of *P.pinnata* in monosodium glutamate-induced neurotoxicity in rats. Indian J Pharm Sci. 2013;75(6):657-63.

9) Jason R. Pleme et al. TTC staining is also a routine method that enables to measure the infarct volume. Journal of Neuroscience Methods., 2004, 139. 203–207.

10) Türeyen k, vemuganti r. kurta. Sailorrobert.j. Dempsey. Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. J Neurosci Methods. 2004., volume 139, issue 2

11) Satish PVV, Sunita K. Antimalarial efficacy of P.pinnata (L) Pierre against Plasmodium falciparum (3D7 strain) and Plasmodium berghei (ANKA). BMC Complement Altern Med. 2017 Sep 11;17(1):458.

12) Byvaltsev VA, Bardonova LA, Onaka NR, Polkin RA, Ochkal SV, Shepelev VV, Aliyev MA, Potapov AA. Acridine Orange: A Review of Novel Applications for Surgical Cancer Imaging and Therapy. Front Oncol. 2019 Sep 24; 9:925. doi: 10.3389/fonc.2019.00925.

13) Speetzen LJ, Endres M, Kunz A. Bilateral common carotid artery occlusion as an adequate preconditioning stimulus to induce early ischemic tolerance to focal cerebral ischemia. J Vis Exp. 2013;75(75):e4387.

14) Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke. 1986 May-Jun;17(3):472-6.

15) Türeyen K, Vemuganti R, Sailor KA, Dempsey RJ. Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques.JNeurosciMethods. 2004 Oct 30;139(2):203-7.

16) Sarah Beckman's et al., Using acridine orange to measure cell death in ethanol-treated zebrafish embryos., Feb 1, 2021. p. 5994-2588EN.

17) Feldman AT, Wolfe D. Tissue processing and hematoxylin and eosin staining. Methods Mol Biol. 2014; 1180:31 -43.

18) Qiu YM, Zhang CL, Chen AQ, Wang HL, Zhou YF, Li YN, et al. Immune cells in the BBB disruption after acute ischemic stroke: targets for immune therapy? Front Immunol. 2021 Jun 23;12:678744.

19) Liu W, Wang X, O'Connor M, Wang G, Han F. Brain-Derived Neurotrophic Factor and Its Potential Therapeutic Role in Stroke Comorbidities. Neural Plast. 2020:1969482.

20) Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol. 2014;4:177.

21) Wang F, Xie X, Xing X, Sun X. Excitatory synaptic transmission in ischemic stroke: A new outlet for classical neuroprotective strategies. Int J Mol Sci. 2022;23(16):9381.

22) Mo Y, Wan R, Zhang Q. Application of reverse transcription-PCR and real-time PCR in nanotoxicity research. Methods Mol Biol 2012 ; 926:99-112 .

23) Annie Shirwaikar, Malini S & Chandrika S, Protective effect of *Pongamia pinnata* flowers against cisplatin and gentamycin induced nephrotoxicity in rats, *Indian J Exp Biol*, 41 (2003) 63.

24) Oruc S, Gönül Y, Tunay K, Oruc OA, Bozkurt MF, Karavelioğlu E, et al. The antioxidant and antiapoptotic effects of crocin pre treatment on global cerebral ischemia reperfusion injury induced by four vessels occlusion in rats. Life Sci 2016;154:79-86.

25) MAI, R. et al, Use of Acridine Orange for Histologic Analysis of the central nervous system,1984. Vol. 32, No. 1, pp. 97-104.