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Cerebroprotective Potential of *Punica granatum* and *Nigella sativa* Against Global Ischemia/Reperfusion Induced Cerebral Infarction in Wistar Rats

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ABSTRACT
In the world today, cerebral ischemia is now second only to ischemic heart disease in terms of the frequency of deaths and disabilities. As per the WHO reports, around the world, 15 million people were experienced with cerebral stroke every year. A reduction in cerebral blood flow affects the entire brain in global cerebral ischemia. It is well recognized that cerebral ischemia and reperfusion cause the production of reactive oxygen species, which can cause oxidative damage to proteins, membrane lipids, nucleic acids and initiation of lipid peroxidation through free radical chain reaction. This study's main goal was to determine whether Punica granatum and Nigella sativa seed powder (PNSP) together could protect male Wistar rats' brains from ischemia reperfusion injury. Bilateral common carotid artery occlusion was used to cause ischemia. For 14 days, several PNSP mixtures were given to rats. After PNSP therapy, brain homogenate was used to determine infarction size, oxidative stress markers like catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), and reduced glutathione (GSH) levels. Statistics were used to examine each and every outcome. The degree of activity was more in PNSP in combination treated group rats, as these plant products have an excellent source of antioxidants and also acts as cerebroprotective agent. Because PNSP contains the corresponding active ingredients, such as thymoquinone and punicalagins, which are responsible for significant reduction in infarction size and oxidative damage in wistar rats.
Keywords: *Global cerebral ischemia, Carotid artery occlusion, Cerebro-protective, Punica granatum, Nigella sativa, Oxidative stress.*

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INTRODUCTION

Cerebral ischemia is a condition in which brain tissue is deprived of oxygen and nutrients due to insufficient blood flow, causing damage or death of the tissue [1]. Brain solely depends on oxygen and glucose from the blood circulation, therefore interruption of blood supply exacerbates the irreversible damage to the brain tissue. Stroke continues to be the second largest cause of death and the third greatest cause of death and disability combined (as measured by disability-adjusted life-years lost—DALYs) in the world, according to the most recent estimates from the Global Burden of Disease (GBD) for 2019. From 1990 to 2019, there were an additional 125.0 million stroke-related DALYs as a result of risk factors [2,3]. Stroke can be divided into two types those are hemorrhagic stroke and ischemic stroke. Thrombotic and embolic ischemic strokes are the two most typical forms. If a blot clot develops in any one of the arteries, which delivers blood to the brain is a thrombotic stroke occurs [4]. The blood clot enters the bloodstream, lodges, and prevents blood flow. When a blood clot or other piece of debris forms in another area of the body and then moves to the brain, it is known as an embolic stroke. Hemorrhagic stroke has subcategories such as intra-cerebral and subarachnoid stroke. Stroke patients need to see a doctor right away. Early intervention is essential to avert death, permanent disability, and brain damage [5]. The arteries providing blood to the brain constrict are blocked during an ischemic stroke. Blood clots or drastically diminished blood flow are the main causes of these obstructions. They may also be brought on by plaque fragments from atherosclerosis that separate and clog a blood artery [6]. Stroke symptoms include paralysis, numbness or weakness in the arm, face, or leg, especially on one side of the body, difficulty in speaking or understanding speech, confusion, slurry

speech, vision problems like double vision or difficulty in seeing in one or both eyes, difficulty in walking, loss of balance or coordination, dizziness, and severe, sudden headaches with no known cause [7]. A powerful antioxidant is an organic compound that is stable enough to eliminate an uncontrolled free radical by giving it an electron, hence reducing the radical's risk of damage. Delays or inhibits cellular damage primarily because these antioxidants may scavenge free radicals[8]. These antioxidants' low molecular weight allows them to engage with free radicals in a safe way to halt the chain reaction before it damages crucial components. The body's normal metabolic processes produce some of these antioxidants, including glutathione, ubiquinol, and uric acid. Antioxidants are also included in the diet[9]. Oxidative stress plays an important role in cerebral ischemia reperfusion injury. So, the agents having antioxidant property are very useful to control the injury induced during reperfusion[10]. Based on this concept, we have selected the *Punica granatum* and *Nigella sativa* seed powder (PNSP) from plant origin[11]. The prime aim of the current study is to evaluate the cerebroprotective potential of PNSP against ischemia and reperfusion induced cerebral injury in wistar rats.

MATERIAL AND METHODS

Plant Materials used in Study

Pomegranate whole fruit powder was procured from Tirupati Balaji Enterprise and Black cumin seeds powder was Procured from R.S.Inc.

Animals used in Study

The male Wistar rats, which were purchased from Mahaveer Enterprises in Hyderabad, were between 200 and 250 g in weight. Animals underwent two weeks of acclimatisation with a 12-hour light/dark cycle at a constant temperature of 25±2°C and relative humidity levels of 45 to 55%. Animals were given unlimited access to pellet meal and water during the acclimatization process. Animals were grouped for experimentation after the quarantine period according to body weight.

Experimental Protocol

Five groups of eight male wistar rats each (n=8) were used in the study. Animals in group 1 were not subjected to the PNSP treatment and without possessing their carotid arteries blocked as a control group. Animals in the ischemia reperfusion (I/R) control group 2 received saline treatment. The PNSP was administered in varied ratios of 1:1, 2:1, and 1:2 to groups 3, 4, and 5 respectively. According to each animal's specific body weight, PNSP was pretreated once daily orally for up to 14 days. All animal groups except group 1 were subjected to bilateral common carotid artery occlusion (BCCAO) on the fourteenth day for 30 minutes, followed by six hours of reperfusion. The Chebrolu Hanumaiah Institute of Pharmaceutical Sciences' IAEC has given the protocol approval and given the approval number 1529/PO/Re/11/CPCSEA/CHIPS/IAEC 7/PRO-06/2019-20.

Dose Administration

Combinations of *Punica granatum* (PG) and *Nigella sativa* (NS) seed powders were made as per the literature. The combinations (P: N) of 1:1, 2:1 and 1:2, were prepared by using 0.5% Hydroxy Propyl Methylcellulose (HPMC) and given orally once in a day up to 14 days. For each animal, same dose of each proportion to be given by taking utmost care[12]. Each group was divided into two smaller groups. The size of the infarction was measured using the second group (n = 4 rats), while the first subset (n = 4 rats) was used for biochemical estimations. The rats were subsequently sacrificed by cervical dislocation upon reperfusion. The homogenate from isolated brains was used to estimate oxidative stress indicators and biochemical parameters.

Induction of Cerebral Infarction

Rats that had been fasted overnight were put to sleep using a mixture of xylazine (10 mg/kg b.w.) and thiopental sodium (30 mg/kg b.w.)[13]. Right and left common carotid arteries were found and released from surrounding tissue and the vagus nerve using a midline ventral incision. Each carotid artery was run through with a cotton thread, which was then knotted, causing global cerebral ischemia for 30 minutes. The surgical procedure was carried out under sterile conditions at 37˚C[14,15].

Determination of Infarct Size

After 6 hours of reperfusion, animals were euthanized, and their brains were removed and quickly frozen at 4°C. Coronal sections were cut into 1-2 mm thick slices and submerged in 1% 2,3,4-triphenyl tetrazolium chloride (TTC) for 20 minutes at 37°C. Nicotinamide adenine dinucleotide (NAD) and dehydrogenase, which are present in living cells, transform TTC into the red formazone pigment. As a result, viable cells were deeply dyed red. The entire brain was measured. Unstained part's measurement was in mm3.

ESTIMATION OF BIOCHEMICAL PARAMETERS

Preparation of Brain Tissue for Estimation of Oxidative Stress Markers

After completion of 6h reperfusion, cervical dislocation was performed to isolate brain from each animal and washed with cool 0.9% saline, kept on ice cold phosphate buffer (0.1 M, pH 7.4) using a Remi

homogenizer. The homogenization technique was carried out under entirely uniform circumstances as rapidly as possible. One portion of the supernatant from the centrifugation of the homogenate at 1000 rpm for three minutes at 4°C was used to test the amount of malondialdehyde (MDA). The leftover supernatant was once more centrifuged at 12,000 rpm for 15 minutes at 4°C and used to test reduced glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD).

Estimation of Superoxide Dismutase (SOD)

It was determined spectrophotometrically using a technique created in 1984 by Kakkar[16]. To create a 10% w/v, necrotic tissue was homogenised in an extremely cold phosphate buffer (0.1M, pH 7.4). An aliquot of 0.1 ml of supernatant was added to 1.2 ml of 0.052 M Sodium Pyrophosphate buffer (pH 8.3), then 0.1 ml each of the following solutions were added: 186 mM phenazin methosulphate, 300 mM nitro blue tetrazolium, and 780 mM NADH. The reaction mixture was rapidly stirred with 4.0 ml of n-butanol then performed centrifugation at 4000 rpm for 10 minutes. At 560 nm, the organic layer's absorbance was measured. A control was made using 0.1 ml of pure water and 0.1 ml of homogenate free of any additives. The quantity of enzyme that provided 50% inhibition of Nitroblue tetrazolium colour complex in one minute is considered as one unit of enzyme activity. The units of mg Protein^{-1} were used to represent the SOD level.

$$\text{Formula:}\frac{(\text{Control O.D} - \text{Experimental O.D})}{(\text{control O.D})} \times \frac{1}{(\text{Protein in mg})}.$$

Estimation of Catalase (CAT)

The method developed by Aebi in 1974 was used to measure catalase activity[17]. To generate a homogenate with a 10% (w/v) concentration, tissue was homogenised in ice-cold phosphate buffer. pH 7.2. The homogenate was centrifuged for 15 minutes at 10000 rpm and 4°C. 1.9 ml of 50 mM phosphate buffer was added to 0.1 ml of centrifuged tissue homogenate. After adding 0.1 ml of 30 mM H_2O_2 to this mixture, the changes in absorbance were monitored for 3 min at 240 nm for every 30 sec. A control was created by preparing it with 0.1 ml of distilled water that was free from the homogenate. The amount of enzyme needed to prevent a 50% decrease in absorbance in one minute in the control sample is considered as one unit of enzyme activity. Activity of catalase was expressed as µmoles of H_2O_2 metabolized/mg protein/min.

$$\text{Formula:}\frac{(\text{O.D of the sample})}{(43.6 \text{ X ml of enzyme})} \times \frac{2.5}{(\text{protein in mg/ml})}$$

Estimation of Reduced Glutathione (GSH)

The Ellman method was used to measure GSH activity. To separate the proteins, the homogenate (w/v) and 10% Trichloroacetic Acid (TCA) was combined and centrifuged. 0.5 ml of 5, 5'-dithiobisnitro benzoic acid (DTNB), 2 ml of phosphate buffer (pH 7.4), and 0.4 ml of double-distilled water were added to 0.01 ml of this supernatant. After centrifuging the mixture, the absorbance was measured at 412 nm. GSH values were expressed as µ moles of GSH/mg protein The GSH was calculated as per the formula.

$$\text{Reduced glutathione} = \frac{(Y - 0.0046)}{0.0034};$$

Where, Y= Final reading- Initial reading.

Estimation of Malondialdehyde (MDA)

The technique created by Ohkawa 1979 was used to measure the levels of MDA[18].in a homogenised tissue. The sample of 0.2 ml of tissue homogenate was mixed with 1.5 ml of 20% acetic acid solution, 0.2 ml of 9.1% sodium dodecyl sulphate, and 1.5 ml of 0.9% aqueous Tertiary Butyl Alcohol (TBA) solution. The liquid was subjected to heating using a condenser that was placed on an oil bath for a period of sixty minutes at 95°C after being diluted to 5ml with distilled water. After being cooled with tap water, after adding and shaking, 5 ml of the 15:1 v/v mixture of pyridine and n-butanol was added. The organic layer was removed after 10 minutes of centrifugation at 4000 rpm, and its absorbance at 532 nm was measured. The standard curve was used to calculate the tissue MDA levels, which were then represented in mol/mg of protein.

Statistical Analysis

The data was displayed as (Mean ±S.E.M.). Using a factorial design of one-way ANOVA, differences in reduced glutathione, MDA, SOD, catalase, and infarct size were discovered. Dunnett's test was used to compare different groups. A p-value of 0.05 or higher indicated statistical significance for differences. Software called Graph Pad Prism was used to do the statistical analysis. (Version 5)

RESULT AND DISCUSSION

When compared to the sham group, I/R control group significantly increased infarct volume by 91.3%. At comparison to the I/R group, pre-administration of PNSP in the following ratios (1:1, 2:1, and 1:2) caused a remarked reduction in the size of the infarct in the brain of 30.92%, 41.86%, and 39.85%, respectively.

In the present study, percentage infarct size was significantly increased in the I/R control group in comparison to sham group. While the treatment group of PNSP 2:1 significantly reduced the % of infarct

size when compared to PNSP 1 & 3 than I/R control group. It indicates that the PNSP 2 showed a potential antioxidant activity and strengthened the oxidative defense mechanisms. Result was demonstrated in Table 1. SOD, CAT, GSH, and MDA levels in the I/R control group were noticeably lowered than those in the sham group, while MDA levels were noticeably higher. Results were demonstrated in graph 1,2,3 and 4. According to WHO, 15 million people worldwide suffer from strokes annually, with high blood pressure being the main cause in those under 40. Approximately 8% of children with sickle cell disease also experience strokes. Stroke ranks second in global death and disability, as per the GBD 2017 study. [19,20]. *Punica granum L* (PG) offers diverse therapeutic benefits including antioxidant, anti-inflammatory, anti-atherosclerotic, anti-cancer, anti-Alzheimer's, anti-diabetic, gastric ulcer, antibacterial, anti-diarrheal, and dermatological uses [21]. *Nigella sativa L* (NS) has therapeutic potential in anti-hyperglycemic, immunopotent, analgesic, antimicrobial, anti-inflammatory, anti-ulcer, antioxidant, and neuroprotective applications[22]. Other study conducted for active constitute isolated from PG as punicalagin also significantly protected the neurons that were against hypoxia induced ischemia brain injury [23,24]. *Nigella sativa* (NS)significantly reduced the intracellular edema as well as neuroprotective activity against global cerebral ischemia reperfusion injury model at the dose of 10mg/kg [25]. The chloroform and petroleum extracts of NS significantly altered the infract volume and neurotoxicity against stroke model in rats via biochemical evaluations. The main constituents of NS seeds, as per the literature, are thymoquinone, p-cymene, carvacrol, and thymol. These compounds may contribute to the demonstrated neuroprotective effects in various studies, including chronic cerebral hypoperfusion in rats, scopolamine-induced memory impairment in rats, rheumatoid arthritis, and clinical trials with healthy volunteers. Additionally, another study highlighted thymoquinone's neuroprotective potential against Alzheimer's and epilepsy [26]. The current study has evaluated the therapeutic efficacy of PNSP on global cerebral ischemic model in male rats, during which free radical production is slightly higher because of decrease in oxygen supply as well the antioxidants production also reduced due to tissue damage during the period of reperfusion for 6 hr. When compared to sham group, PNSP treated rats got effected with less oxidative damage on the strength of antioxidant content that is present in PNSP. PNSP treated rats showed decrease in MDA levels. It suggests that PNSP is efficient in reducing lipid peroxidation, mainly due to the presence of chemical constituent thymoquinone. The current studies significantly shown the reduction of MDA levels compared with the PG and NS alone as stated above. The degree of activity was more in PNSP in combination treated group rats, as these plant products have an excellent source of antioxidants and also acts as cerebroprotective agent [27]. This was confirmed by the increase in the SOD levels, CAT levels and GSH levels as compared to I/R control group. The present study significantly increased the anti-oxidants and decreased free radical production in PNSP treated animal's i.e. 2:1 ratio. The rest of combinations such as 1:1 and 1:2 also significantly reduced the free radical production. This would be strongly suggesting that the combination was effective for treating cerebral ischemia. The mechanism involved in the cerebroprotective activity of PNSP might be due to the presence of major constituents such as punicalagin from PG and thymoquinone from NS. Further molecular studies are required to evaluate the mechanism of cerebroprotection.

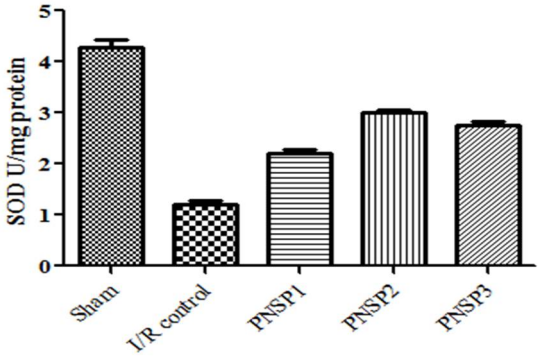
PERCENTAGE OF INFRACT VOLUME AFTER TREATMENT

Table 1: Effect of PNSP on the volume of infarcts as a percentage in rats' cerebral ischemia-reperfusion injury

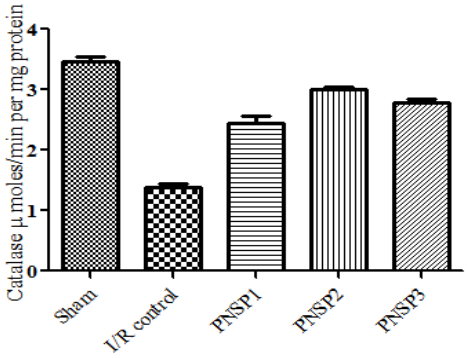
Groups	Infarct volume*	% Infarct volume
Sham	3.805±0.147	10.5
I/R control	43.78±0.106***	91.3
PNSP 1	30.24±0.187***	30.92
PNSP 2	25.45±0.246***	41.86
PNSP 3	26.3±0.196***	39.85

***P Value 0.05 was regarded as statistically significant. *The values are expressed as Mean ±S. E. M.

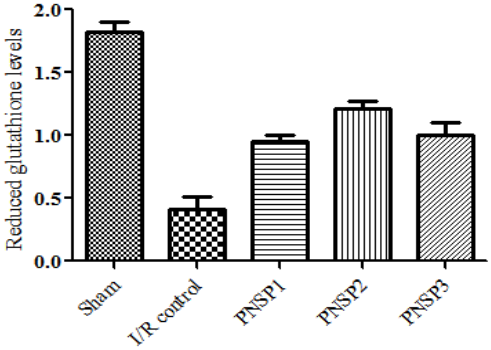
Antioxidant potential of PNSP



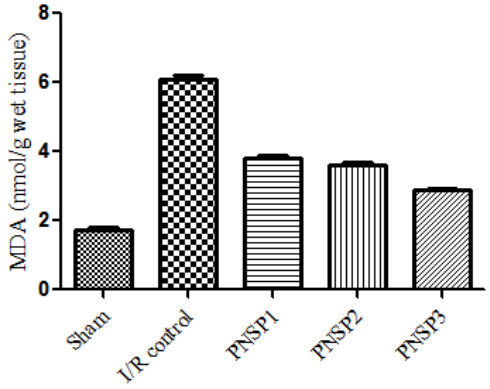
Graph 1: Rats with cerebral ischemia-reperfusion injury and the effect of PNSP on SOD (U/mg protein) levels in infarcted tissue



Graph 2: Effect of PNSP on Catalase levels in infarcted tissue during cerebral ischemia reperfusion damage in rats (µmoles/min per mg protein)



Graph 3: Rats with cerebral ischemia-reperfusion damage and the effect of PNSP on reduced glutathione levels in infarcted tissue



Graph 4: MDA (nmol/g wet tissue) levels in infarcted tissue after cerebral ischemia reperfusion damage in rats are affected by PNSP.

CONCLUSION

Different proportions of PNSP were given orally once in a day up to 14 days. On the 14th day the ischemia was induced followed by reperfusion. After that cerebral infraction was determined for a set of animals using TTC staining technique. Oxidative stress markers and biochemical parameters were estimated for another set of animals. The cerebroprotective potential of PNSP 2 proportion is partially attributed to its antioxidant effect against cerebral reperfusion injury by reducing % infarct volume and increasing SOD, CAT, GSH levels. PNSP 3 proportion is more efficient in reducing lipid peroxidation by reducing MDA levels because of the presence of thymoquinone in *Nigella sativa*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

AUTHOR'S CONTRIBUTION

Sai Reshma Ramineni the guarantor of this study has designed and carried out the process, analyzed the results, prepared and reviewed the manuscript. Sandeep Doppalapudi has carried out the process and reviewed the manuscript. Vidyadhara Suryadevara has supervised the process and reviewed the manuscript. Aruna Kumar Chadalavada has supervised the process and reviewed the manuscript.

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