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Research Article

Preclinical Evaluation of Neuroprotective Activity of *Piper nigrum* L. in Cerebral Ischemic Reperfusion Induced Oxidative Stress

Keval Raval[1], Pravin Tirgar[2], Milan Patel[3], TR Desai[4]

[1]Assistant Professor, [2]Professor, [3]Research Scholar, [4]Director, School of Pharmacy, RK University, Rajkot, Gujarat, India.
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I N F O

Corresponding Author:
Keval Raval, School of Pharmacy, RK University, Rajkot, Gujarat, India.
E-mail Id:
keval.raval@rku.ac.in
Orcid Id:
https://orcid.org/0000-0003-4114-0352
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A B S T R A C T

Introduction: Stroke is a potentially fatal condition that is defined by the fast development of clinical symptoms of ischemia. Numerous flavonoids have been demonstrated in animal models to ameliorate brain ischemia-reperfusion damage. Piperine is a flavonoid derived from *Piper nigrum* L. that exhibits a variety of pharmacological effects. The purpose of this research was to determine if *Piper nigrum* L. has a protective effect against the brain damage caused by bilateral common carotid artery occlusion (BCCAO) in rats.

Materials and Methods: The animal study was certified by Institutional Animal Ethics Committee (IAEC) under research project no. RKCP/COI/RP/12/28. Numerous parameters were evaluated to ascertain the extent of oxidative stress and eventual protection of *Piper nigrum* L. including glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), lipid peroxidation (LPO), brain protein, and calcium levels in brain homogenate. The preventive effect of *P. nigrum* was evaluated and compared with quercetin as a standard using histopathology and the region of cerebral infarction.

Results: In our research, we observed a substantial rise in superoxide dismutase (SOD), catalase, glutathione, and brain protein levels and a fall in lipid peroxidation and calcium levels in the *P. nigrum* and quercetin treated groups with the level of significance (p value) less than 0.05, confirming the protective effect against brain injury. Additionally, *P. nigrum* was shown to provide less protection compared to quercetin.

Conclusion: As a result of these data, we hypothesise that *P. nigrum* may have a considerable neuroprotective effect in the brain against ischemic/ reperfusion-induced oxidative damage.

Keywords: Ischemic Reperfusion, Flavonoid, Oxidative Stress, *Piper Nigrum* L

Background

Cerebrovascular illnesses include a number of the most prevalent disorders, including ischemic stroke, haemorrhagic stroke, and cerebrovascular abnormalities.[1] As a life-

threatening illness, stroke shows clinical indications of localised or global disturbances in brain function within 24 hours or up to weeks. In addition to vascular sources, it can also lead to unexpected death.[2] In cerebral ischemia, there is insufficient blood flow to satisfy metabolic requirements in the brain. Because of the excessive neuronal loss and glia in ischemic brain regions, cerebral infarcts may cause moderate to serious neurological problems and even death in certain cases.[3] Poor blood flow to the brain causes cerebral hypoxia, which results in brain tissue loss, ischemic stroke, or cerebrovascular disease. Stroke is a major health problem in the world after cardiovascular disease and cancer. Every year in India, there are between 1.44 and 1.64 million new cases of acute strokes, with a frequency of 55.6 per 100,000 people of all ages.[4]

The process of restoring blood flow to ischemic tissue is known as reperfusion. As a result of tissue's vulnerability to ischemia-reperfusion injury (IRI), both effective infarct reperfusion and organ transplantation are difficult.[5] Although, there is currently no clinically effective treatment for acute stroke other than tissue-type plasminogen activator (t-PA), there are several drugs that are used for treating stroke due to their potential neuroprotective activity. Various natural products have attracted attention recently because of the neuroprotection that they offer. In particular, some natural products act as neuroprotective agents by reducing the death and apoptosis of neurons, thereby helping their survival.[6]

Piper nigrum (black pepper) is among the most widely used herbal medications worldwide due to its exceptional pharmacological properties recognised by Ayurveda. *Piper nigrum* is known as Maricha and is recognised for its pacifying activity on Vata and Kapha. It is commonly known as black pepper and is traditionally used as an analgesic, antipyretic, anti-inflammatory, antispasmodic, antioxidant, and nerve tonic or antiseptic. It is an aphrodisiac as well as a digestive aid and diuretic. It is also a febrifuge, laxative, and rubefacient.[7] It possesses a high level of antioxidant activity, which is attributed to flavonoids, polyphenolic, vitamin B complex and antioxidants like vitamin A and vitamin C.[8] Recently, different parts of *Piper nigrum* are reported to have hepatoprotective,[9] antifungal,[9] antidepressant,[10] anxiolytic,[10] antipyretic,[10] thrombolytic,[10] antischistosomal[11] and antimutagenic[12] activity.

As reported, black pepper possesses a high level of antioxidant and anti-inflammatory activity, which is attributed to the antioxidants and numerous phenolic and flavonoid constituents. The effect of black pepper extracts on cerebral stroke has not been evaluated yet. Hence, we have endeavoured to explore the neuroprotective effect of *Piper nigrum* on the cerebral ischemic reperfusion-induced cerebral injury.

Method

Plant Materials

Fruits of *Piper nigrum* L. were collected from the local market of Rajkot, Gujarat and identified in the Agriculture Department, RK University. The fruits were washed and dried in the shade until completely dry.

Extraction Process

Dried fruits were powdered to 40 mesh and stored at 25°C till further use. The coarsely powdered dried fruit of *Piper nigrum* was defatted with petroleum ether in the Soxhlet apparatus. Furtherly, the powder was extracted with alcohol by the Soxhlet apparatus; the alcoholic solvent was removed under reduced pressure in a rotary vacuum evaporator. After residue extraction, the solvent was evaporated and any remaining solvent was fully eliminated to yield a pure product. The purified product was authenticated at the Department of Biology, Christ College, Rajkot with specimen No. RKCOP/RE/SP.No.05. The solution was then thoroughly dried in a freeze dryer and kept in a sealed container at 4°C.

Phytochemical Screening

The resultant extract was used to determine the presence of phenol using the ferric chloride test, the Folin-Ciocalteu test, the alkaline reagent test, the Shinoda test, and the lead acetate test.[13,14]

Animals and Ethical Consideration

Wistar albino rats of either sex weighing 250-300 gm were used for the study. The animals were procured from Animal House, Department of Pharmacology, School of Pharmacy, Rajkot, India. The research was authorised and carried out in accordance with the Institutional Animal Ethics Committee's (RKCP/CO/RO/12/28) guidelines. The rats were housed in cages at an ambient temperature of 25°C and relative humidity of 45-55%, with a 12 h:12 h dark and light cycle and free access to a conventional mouse pellet food and drinking water.

Experimental Protocol

The Wistar albino rats were split into four groups of six rats each. Animals of each group were subjected to the respective treatment for ten days before the surgery. The experimental procedure was performed in accordance with the standard protocol.[15]

Group I: Sham-operated - surgical procedure except bilateral common carotid artery occlusion (BCCAO) for 30 min followed by reperfusion for 45 min.

Group II: Disease control - BCCAO for 30 min, followed by reperfusion for 45 min.

Group III: Test-methanolic extract of *P. nigrum* (30 mg/kg, orally), BCCAO for 30 min, followed by reperfusion for 45 min.

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Group-IV: Standard (Quercetin-10 mg/kg, orally), BCCAO for 30 min, followed by reperfusion for 45 min.

Induction of Ischemia

After ten days, the animals of group-I received the vehicle and served as the sham-operated control. The surgical procedure that they underwent was similar to that of the other groups except for the fact that their arteries were not occluded under ketamine anaesthesia (45 mg/kg, intraperitoneal).[16] As groups II, III, and IV were treated for ischemia, mainly two carotid arteries were revealed and severed from the vagus via lateral incisions. Ischemia was produced for 30 minutes by bilaterally clamping the common carotid arteries. Reperfusion was accomplished after cerebral ischemia by de-clamping the arteries and reestablishing circulation for 45 minutes. Throughout the surgical process, the temperature was kept at about 37 ± 0.5 °C and respiratory support (95% O_2 and 5% CO_2) was supplied through an artificial respirator.

Preparation of Post-mitochondrial Supernatant

After the treatment period of 10 days, the brain was removed after decapitation, and was washed with cooled 0.9% saline, kept on ice, and afterwards collected on filter paper before being quantified and homogenised in chilled phosphate buffer at a concentration of 10% w/v (0.05 M, pH 7.4). The homogenates were centrifuged at 10,000 rpm for 10 minutes at 4 °C, and the post-mitochondrial supernatant (PMS) was immediately frozen until analysis.[17] Prepared post-mitochondrial supernatant was used for the biochemical estimation.

Biochemical Estimations

Protein Content

The protein content was determined as per the recommendation of Lowry OH et al.[18] 0.2 mL homogenate was diluted with 1 mL alkaline copper sulfate solution followed by the addition of dilute Folin phenol reagent. After 30 minutes, the absorbance of samples was determined using a spectrophotometer at 620 nm.

Lipid Peroxidation

The procedure was performed in accordance with Prabhakar KR et al.[19] 0.2 mL of homogenate was reacted with 0.2 mL of 4% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid, and 1.5 mL of 0.5% thiobarbituric acid. The reaction mixture was heated at 95 °C for 60 minutes. After the centrifugation, the absorbance of the supernatant was determined at 532 nm.

GSH Levels

GSH levels of the homogenate were determined following the procedure given by Sedlak J and Lindsay RH.[20] 1 mL of homogenate was reacted with 1 mL of 10% trichloroacetic acid and centrifuged. 0.5 mL of supernatant was furtherly reacted with 2 mL of Ellman's reagent. The final volume was increased using 3 mL of phosphate buffer and analysed at 412 nm by colorimetry.

SOD Levels

The procedure was undertaken in accordance with Misra HP and Fridovich I.[21] 0.1 mL supernatant was reacted with 0.1 mL EDTA, 0.5 mL carbonate buffer (pH 9.7), and 1 mL epinephrine (3×10^{-3} M). The absorbance was recorded at 480 nm.

Catalase Levels

Catalase levels were determined using the method given by Bogomolets BB.[22] Buffer solution of homogenate was prepared and enzyme solution was added. Phosphate buffer was considered as a blank and the absorbance was recorded at 240 nm.

Calcium Levels

Blank, standard and test samples were prepared in accordance with the standard procedure.[23] Prepared samples were mixed well and incubated at room temperature for 5 minutes. The absorbance of samples was determined at 624 nm.

Infarct Area Assessment

The Infarct area assessment protocol was performed in accordance with the standard protocol.[24,25] The brain was removed from the sacrificed animal and placed in a brain blocker. The sample was sliced into a 2 mm thick coronal section. The section was stained in 0.5% 2,3,5 triphenyltetrazolium chloride (TTC) solution and kept at 37 °C for 20 minutes. The stained section was scanned and analysed using ImageJ software. The brain infarction was determined from the captured image.

Histopathology

A brain histopathological evaluation was conducted to determine the influence of the test extract on degenerative changes associated with oxidative stress. The tissue was embedded in melted paraffin in wooden blocks to allow sectioning. Several sections of 3 µm thickness were taken from each tissue, and histopathology was performed under 10x magnification using a phase-contrast microscope on chosen sections with uniform shape and size.

Statistical Analysis

All data are provided as the standard error of the mean (SEM). The significant heterogeneity in means among all groups of animals for various parameters was evaluated using a one-way analysis of variance (ANOVA) accompanied by Dunnett's multiple comparison test. P value < 0.05 was considered statistically significant. The results were analysed using GraphPad Prism 8.0.2.

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Results

Evaluation of Extract of *Piper nigrum* L

Table 1 elucidates the evaluation parameters of *Piper nigrum* extract including the chemical nature of extract, percentage yield, physical appearance, and consistency.

Table 1.Evaluation of Extract of *Piper nigrum L*

Evaluation Parameter	Result
Extract	Methanolic
Yield (%W/W)	6.3
Colour	Brown
Consistency	Semisolid

Phytochemical Screening

The obtained extract by Soxhlet extraction was subjected to various qualitative chemical tests to determine the presence of essential phytoconstituents responsible for possible neuroprotective activity such as phenol and flavonoids and the results were recorded and have been elucidated in Table 2.

Effect of Methanolic Extract of *Piper nigrum* L. (MEPN) against Ischemic Reperfusion-induced Cerebral Injury in the Rat

The concentrations of glutathione, SOD, catalase, lipid peroxidation, brain protein, and calcium for experimental groups were collected and have been recorded in Table 3.

Table 2.Phytochemical Screening Extract of *Piper nigrum L*

Chemical Constituents	Name of Test	Observation	Inference
Phenol	Folin-Ciocalteu test	Blue colour	Phenol was present
Flavonoids	Shinoda test	Red colour	Flavonoids were present
	Alkaline reagent test	Yellow colour	Flavonoids were present
	Zinc hydrochloride test	Red colour	Flavonoids were present

Table 3.Effect of Methanolic Extract of *Piper nigrum* L. (MEPN) against Ischemic Reperfusion-induced Cerebral Injury in the Rat

Treatment Group	Glutathione	SOD	Catalase	Lipid Peroxidation	Brain Protein	Total Calcium
Shamcontrol	3.30 ± 0.09	16.69 ± 0.06969	0.30 ± 0.04	4.82 ± 0.11	620.00 ± 8.01	2.07 ± 0.14
Diseasecontrol	1.20 ± 0.07*	10.82 ± 0.079944*	0.08 ± 0.00*	8.11 ± 0.08*	111.68 ± 2.09*	9.20 ± 0.09*
Quercetin + IR	3.80 ± 0.06**	20.34 ± 0.067082**	0.65 ± 0.06**	1.73 ± 0.10**	395.00 ± 4.87**	6.34 ± 0.13**
30 mg/kg PN+ IR	2.95 ± 0.09$^{**\#}$	13.12 ± 0.153854$^{**\#}$	0.92 ± 0.04$^{**\#}$	1.40 ± 0.10$^{**\#}$	344.05 ± 2.95$^{**\#}$	3.40 ± 0.08$^{**\#}$

Values are mean ± SEM, n = 6, One-way analysis of Variance (ANOVA) followed by multiple comparisons Dunnett test, IR- Ischemic/ Reperfusion
*Indicates a statistically significant difference in the data compared to the sham control group, with a significance level of $p < 0.001$.
**Indicates a statistically significant difference in the data compared to the disease control group, with a significance level of $p < 0.001$.
#Indicates a statistically significant difference in the data compared to the quercetin + IR group, with a significance level of $p < 0.001$.

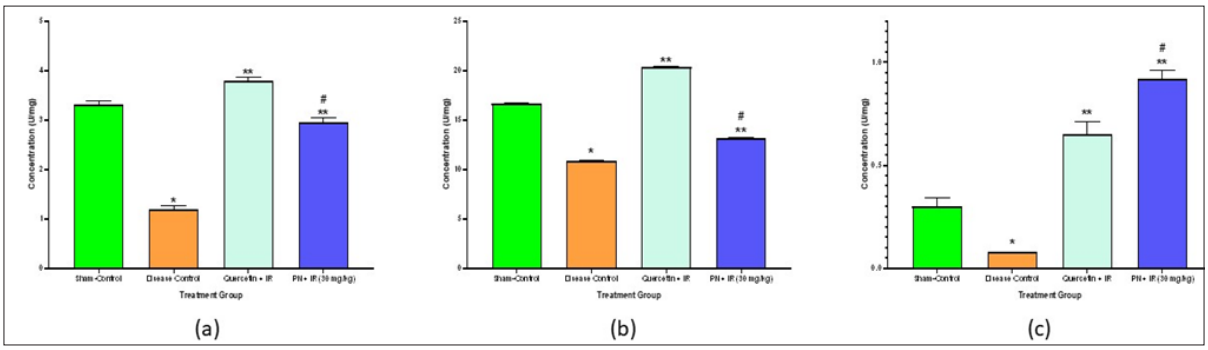

Figure 1.Estimation of Biomarkers in the Brain Homogenate of Rats (a) Glutathione (b) SOD (c) Catalase

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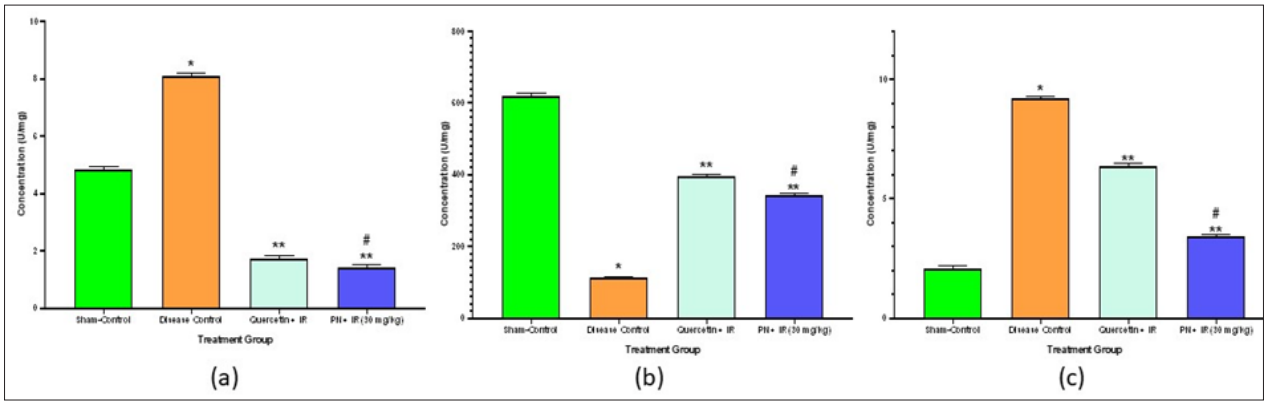

Figure 2.Estimation of Biomarkers in the Brain Homogenate of Rats
(a) Lipid Peroxidation (b) Brain Protein (c) Total Calcium

*Indicates a statistically significant difference in the data compared to the sham control group, with a significance level of $p < 0.001$.
**Indicates a statistically significant difference in the data compared to the disease control group, with a significance level of $p < 0.001$.
#Indicates a statistically significant difference in the data compared to the quercetin + IR group, with a significance level of $p < 0.001$.

TTC (2,3,7-Triphenyltetrazolium chloride) Staining of Brain Coronal Slices Indicated Infarction Area

In the disease control group section, a large infarction area was observed in the hippocampal region as given in Figure 3(a). A small infarction area was observed in the hippocampal region of the sham control group section as compared to the disease control group elucidated in Figure 3(b). There was a marked reversal of brain damage and cerebral infarction in the quercetin group section was reduced up to a normal as shown in Figure 3(c). There was a marked reduction of infarction area in the hippocampus region of *Piper nigrum* L. group section as compared to disease control, and compared to quercetin, there wasa further reduction of infarction area as given in Figure 3(d).

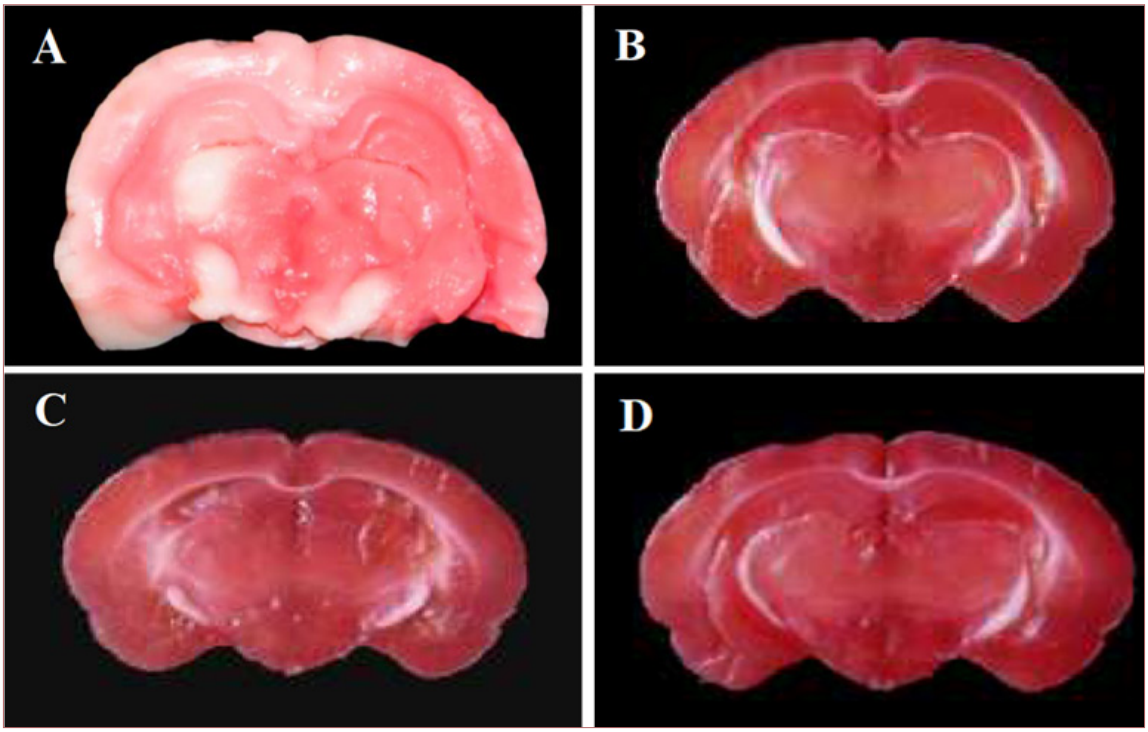

Figure 3.Brain Coronal Slice stained with 2,3,7-triphenyltetrazolium chloride (TTC) (A) Disease Control: BCCAO for 30 minutes followed by Perfusion for 45 minutes (B) Sham control: Surgical Procedure except BCCAO for 30 minutes followed by Perfusion for 45 minutes (C) Quercetin (10 mg/kg orally): BCCAO for 30 minutes followed by Perfusion for 45 minutes (D) *Piper nigrum* L.: (30 mg/kg orally) BCCAO for 30 minutes followed by Perfusion for 45 minutes

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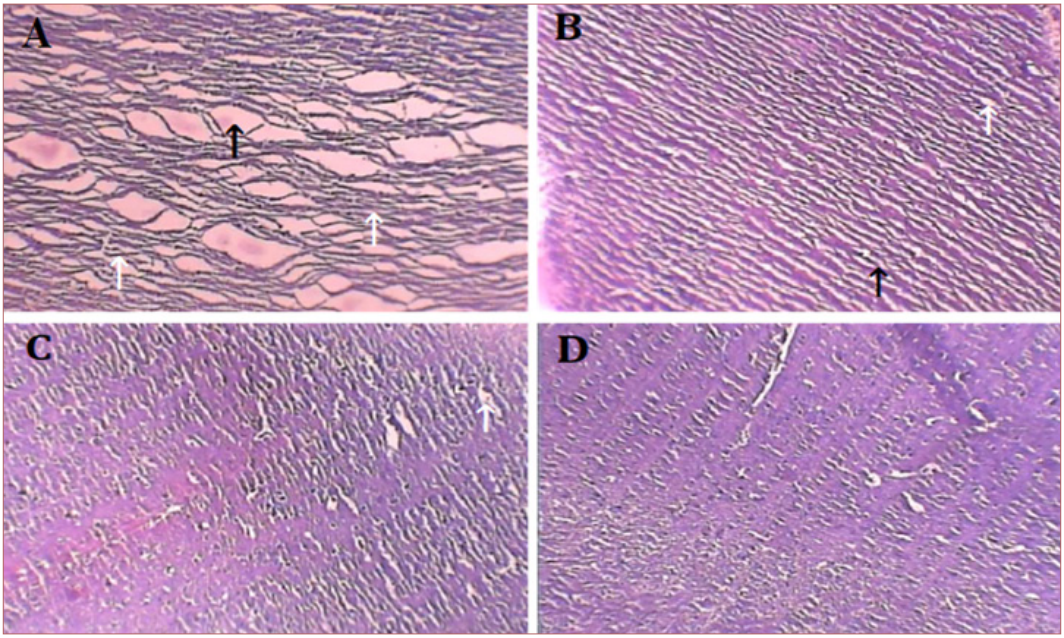

Figure 4.Histopathological Study performed at 10X Magnification for (A) Disease control, BCCAO for 30 minutes followed by Perfusion for 45 minutes: Increased Intercellular Space (Black Arrow), Necrosis (White Arrows) (B) Sham Control, Surgical Procedure except BCCAO for 30 minutes followed by Perfusion for 45 minutes: Compact Intercellular Space (Black Arrow), Necrosis (White Arrow) (C) Quercetin (10 mg/kg orally) BCCAO for 30 minutes followed by Perfusion for 45 minutes: Necrosis (White Arrow) (D) *Piper nigrum* (30 mg/ kg orally) BCCAO for 30 minutes followed by Perfusion for 45 minutes

Histopathological Study of Brain

In the disease control plate, ischemic reperfusion injury resulted in increased intracellular space and disruption of cellular integrity. It was further augmented by reperfusion and there was a marked lymphocytic proliferation and cellular necrosis. A microscopical image of the disease control plate is given in Figure 4(a). The sham-operated group brain hippocampus region showed compact intracellular space, less leukocyte infiltration, and cellular necrosis as given in Figure 4(b). In the quercetin (standard) group section, normal cells were found to be compact and less intracellular space was observed. There was reduced lymphocytic proliferation and cellular necrosis as seen in Figure 4(c). Significant reversal damage caused by ischemic reperfusion injury in the hippocampus was observed in the *Piper nigrum* L. group section. Cells were compact and showed an absence of lymphocytic proliferation and infiltration as given in Figure 4(d).

Discussion

Active constituents of *P. nigrum* such as piperine and alkaloids are reported to have a neuroprotective effect. A study conducted on rats with permanent middle cerebral artery occlusion (pMCAO) injury concluded the neuroprotective activity of piperine.[25] One similar study investigated the neuropeptide effects of *P. nigrum* alkaloids in MPTP-induced mouse models of Parkinson's disease.[26] There is also evidence of the preventive effect of *P. nigrum* dichloromethane fraction against ischemia-induced cellular damage.[27]

In the present investigation, we observed the therapeutic potential of *P. nigrum* on a global model of ischemia for the study of cerebral stroke in rats. We selected a 45-minute reperfusion model. We estimated LPO, SOD, CAT, GSH, brain protein, and calcium levels in the brain tissue as an index to assess the severity of oxidative damage and subsequent protection by *P. nigrum*. Cerebral infarction area and histopathological studies were conducted to assess the protection against the ischemic reperfusion-induced cerebral injury.

The results demonstrated that pre-treatment with *P. nigrum* (30 mg/ kg) and quercetin had significantly decreased the MDA level and prevented neuronal injury caused by a chain reaction of lipid peroxidation. The dosage of *P. nigrum* (30 mg/kg) was selected based on the reported activity of antioxidant, anti-inflammatory and in-vitro study.[27] The extracts of *P. nigrum* (30 mg/kg) and quercetin were shown to increase the efficiency of two key oxygen radical scavenging enzymes in ischemic brain regions. The levels of these enzymes were shown to be depleted in the brains of disease control groups.

Interestingly, the rats fed with *P. nigrum* extract and quercetin showed increased levels of GSH, CAT, and SOD. The level of lipid peroxidation was significantly decreased in the *Piper nigrum* and quercetin treated group than that

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of the disease control group. The catalase activity was restored to normal after treatment with *Piper nigrum* and quercetin indicating the antioxidant properties of *Piper nigrum* and quercetin against free radicals.

Similarly, there was a significant decrease in the level of glutathione in IR-induced cerebral injury in the disease control group. Glutathione levels were restored in the *Piper nigrum* and quercetin treated animals. There was a notable increase in intracellular space, lymphocyte proliferation and infiltration in the hippocampus region compared to treated groups with *Piper nigrum* (30 mg/kg) and quercetin, as observed in the histopathological brain sections. The brain coronal section of rat staining with TTC showed a large infarction area in the hippocampal region in the disease group compared to the treated group with *Piper nigrum* (30 mg/kg) and quercetin.

Above biochemical results indicating neuroprotective effects of *Piper nigrum* and quercetin were also confirmed by the histopathological differences between the treatment group, standard treatment group, and disease control group. The results also reveal that *P. nigrum* has significantly less neuro protection activity compared to quercetin.Based on this study, we can conclude that the probable mechanism by which *Piper nigrum* reduces oxidative brain damage is due to its ability to decrease lipid peroxidation or its radical scavenging activity. The collective property of *Piper nigrum* such as antioxidant and radical scavenging activity may be due to the presence of vitamin C, vitamin A, polyphenolic flavonoids, vitamin B-complex, and niacin.

Conclusion

The findings indicate that *Piper nigrum* L. methanolic extract (30 mg/kg) offers substantial neuroprotection against cerebral ischemia-reperfusion caused by bilateral common carotid artery blockage. This effect may be achieved by correcting changes in the brain's metabolic characteristics caused by oxidative stress and histological changes associated with ischemia-reperfusion. *Piper nigrum* L. possesses a potential antioxidant capacity as it decreases lipid peroxidation. In our study, we can conclude that *Piper nigrum* L. has significant neuroprotective activity, but it is less protective than quercetin. With the available data, the precise mechanism of *Piper nigrum* L. in neuroprotection remains obscure. Additional research is needed to elucidate the precise molecular mechanism of action. The current study demonstrates that *Piper nigrum* L. protects the brain against worldwide cerebral ischemia-reperfusion damage caused by oxidative stress.

Abbreviations

- **ANOVA:** Analysis of variance
- **BCCAO:** Bilateral common carotid artery occlusion
- **CAT:** Catalase
- **GSH:** Glutathione
- **IAEC:** Institutional Animal Ethics Committee
- **IRI:** Ischemia-reperfusion injury
- **LPO:** Lipid peroxidation
- **MEPN:** Methanolic extract of *Piper nigrum* L.
- **SOD:** Superoxide dismutase
- **t-PA:** Tissue-type plasminogen activator
- **TTC:** 2,3,5-triphenyltetrazolium chloride

Competing Interests: None

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