

Neuroprotective Effects of a Polyherbal Formulation on Oxidative and Nitrosative Stress in a Rat Model of Transient Cerebral Ischemia

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Abstract

Background: Cerebral ischemia–reperfusion injury is strongly associated with oxidative and nitrosative stress, which contributes to neuronal damage and disruption of redox homeostasis. Polyherbal formulations may provide multi-target antioxidant effects against ischemia-induced injury. This study evaluated the effect of a polyherbal formulation on oxidative and antioxidant parameters in a rat model of transient cerebral ischemia.

Methods: Adult Sprague–Dawley rats were randomized into three groups (n = 6/group): healthy control, transient middle cerebral artery occlusion (tMCAO), and tMCAO treated with the polyherbal formulation. Focal cerebral ischemia was induced by 2 h of MCA occlusion followed by reperfusion. At 24 h after ischemia, cortical brain tissue was collected for assessment of malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH).

Results: tMCAO produced a marked oxidative imbalance, characterized by increased MDA (43.33 ± 12.24) and NO (54.81 ± 15.22), together with reduced SOD (54.55 ± 41.25) and GSH (0.0750 ± 0.0211) compared with healthy controls. Catalase activity was increased following ischemia (0.4700 ± 0.0408), consistent with an altered antioxidant response. Polyherbal treatment significantly reduced MDA to 11.24 ± 4.84 ($p = 0.0023$) and NO to 23.07 ± 2.74 ($p = 0.0018$). Treatment also increased SOD activity to 315.00 ± 176.72 ($p = 0.0241$) and GSH to 0.3283 ± 0.1154 ($p = 0.0002$), while reducing CAT activity to 0.0952 ± 0.0919 ($p < 0.0001$).

Conclusion: The polyherbal formulation attenuated ischemia-associated oxidative and nitrosative stress and improved several components of endogenous antioxidant defense in the acute phase following tMCAO. These findings support further investigation of the formulation as a potential neuroprotective intervention for ischemic stroke.

Keywords: Polyherbal formulation; cerebral ischemia; tMCAO; oxidative stress; nitrosative stress; antioxidant defense; neuroprotection.

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1. Introduction

Ischemic stroke is a major cause of death and long-term neurological disability and occurs when cerebral blood flow is interrupted, resulting in a cascade of cellular and molecular events that contribute to neuronal injury (Salaudeen et al., 2024). Cerebral ischemia followed by reperfusion involves excitotoxicity, ionic imbalance, inflammation, mitochondrial dysfunction, and excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Among these mechanisms, oxidative and nitrosative stress play important roles in the progression of ischemia–reperfusion injury by disrupting cellular redox homeostasis and promoting neuronal damage (Qin et al., 2022).

Reperfusion can markedly increase the production of reactive species, including superoxide anion, hydrogen peroxide, and nitric oxide. Excessive ROS can initiate lipid peroxidation, protein oxidation, and nucleic acid damage, whereas increased nitric oxide production can contribute to nitrosative stress and formation of highly reactive nitrogen-derived species (Lin et al., 2023). Lipid peroxidation is particularly relevant to ischemic brain injury because neuronal membranes are rich in polyunsaturated fatty acids. Malondialdehyde (MDA), a major product of lipid peroxidation, is therefore commonly used as an indicator of oxidative membrane damage (Ayala et al., 2014).

The brain possesses endogenous antioxidant systems that counteract excessive reactive species. Superoxide dismutase (SOD) converts superoxide radicals into hydrogen peroxide, while catalase (CAT) contributes to hydrogen peroxide detoxification. Reduced glutathione (GSH) provides an important intracellular redox buffer and participates in the neutralization of ROS and maintenance of cellular antioxidant capacity. During ischemic injury, excessive reactive species generation can overwhelm these defense systems, resulting in depletion or dysregulation of antioxidant capacity and further progression of neuronal injury (Jurcau and Ardelean, 2022; Nandi et al., 2019). Thus, simultaneous assessment of oxidative damage and endogenous antioxidant defenses may provide a more comprehensive indication of redox disturbances following cerebral ischemia.

Current treatment strategies for acute ischemic stroke are largely dependent on timely reperfusion, with thrombolytic therapy and mechanical thrombectomy being important therapeutic approaches. However, treatment eligibility, therapeutic time windows, and the risk of complications limit their applicability in some patients (Baig and Bodle, 2023). Consequently, increasing attention has been directed toward plant-derived compounds and polyherbal formulations as potential complementary approaches because their multiple bioactive constituents may act on several components of the ischemic injury cascade. Experimental studies have reported antioxidant and neuroprotective effects of plant-derived flavonoids, polyphenols, and other phytochemicals in models of neurological injury (Bellavite, 2023; Alsawaf et al., 2022).

Evidence from experimental studies further suggests that polyherbal interventions can influence oxidative and inflammatory pathways associated with cerebral ischemia. Nakym et al. (2022) reported neuroprotective effects of a polyherbal formulation containing ginger, Chinese date, and wood ear mushroom in an ischemic stroke model, with effects associated with modulation of oxidative stress and inflammation. Similarly, studies of polyphenolic and plant-derived interventions have reported improvements in antioxidant defenses and reductions in oxidative damage, supporting the potential of multi-component herbal approaches in ischemic brain injury (Liu et al., 2019; Martemucci et al., 2022).

Despite these findings, the effects of a polyherbal formulation on a coordinated panel of oxidative and antioxidant biomarkers following transient cerebral ischemia remain insufficiently characterized. In particular, simultaneous evaluation of lipid peroxidation, nitric oxide, SOD, CAT, and GSH can provide a broader assessment of treatment-associated changes in cerebral redox status. Therefore, the present study investigated the effect

of a polyherbal formulation on oxidative and nitrosative stress and endogenous antioxidant defenses in rats subjected to transient middle cerebral artery occlusion (tMCAO). The study specifically evaluated MDA, NO, SOD, CAT, and GSH in cortical brain tissue following ischemia–reperfusion.

2. Materials and Methods

For the shortened manuscript, I recommend removing the Rota-Rod and TTC/infarct-volume procedures from Methods because those outcomes are no longer part of the primary focus. The Methods below retains the experimental design and all procedures directly relevant to the oxidative/nitrosative stress analysis. The wording is based on your uploaded manuscript.

2.1. Experimental Animals and Study Design

Adult Sprague–Dawley (SD) rats weighing 250–310 g were used in the study. Animals were randomly allocated into three experimental groups ($n = 6$ per group): healthy control, transient middle cerebral artery occlusion (tMCAO), and tMCAO + polyherbal formulation-treated groups. Both male and female rats were included. Animals were housed under controlled environmental conditions (25 ± 3 °C; relative humidity $60 \pm 5\%$; 12-h light/dark cycle) with free access to standard rodent chow and water. The polyherbal formulation was freshly prepared in sterile saline and administered intraperitoneally at the time of MCA occlusion and again following reperfusion. Anaesthesia was induced using thiopental sodium (5 mg/kg, i.p.). All experimental procedures were performed in accordance with institutional ethical requirements and were approved by the Institutional Animal Ethics Committee (IAEC), Faculty of Pharmacy, Smriti College of Pharmaceutical Education (Approval No. IAEC/SCOPE/2024/006).

2.2. Induction of Transient Middle Cerebral Artery Occlusion

Transient focal cerebral ischemia was induced using a modified intraluminal filament technique. Briefly, the left common carotid artery was exposed through a midline cervical incision and traced to its bifurcation into the external and internal carotid arteries. The occipital artery was ligated and transected to facilitate access to the external carotid–internal carotid junction. A flame-rounded, poly-L-lysine-coated 3-0 nylon monofilament (0.35 mm diameter) was introduced through the external carotid artery and advanced approximately 19–21 mm along the internal carotid artery until resistance at the origin of the middle cerebral artery indicated occlusion. The filament was maintained in position for 2 h and subsequently withdrawn to initiate reperfusion. Following surgery, 1 mL sterile saline was administered subcutaneously (0.5 mL per flank) to maintain hydration and circulatory volume. The procedure was performed according to previously

described methods (Morris et al., 2016; Kobayashi et al., 2019).

2.3. Brain Tissue Collection

Twenty-four hours after tMCAO and treatment, animals were euthanized under anaesthesia. Intracardiac perfusion was performed with 100 mL ice-cold 1% heparinized saline to remove blood from the cerebral vasculature. The brains were rapidly removed, and the ipsilateral cortical region was dissected on an ice-cold platform. Approximately 100 mg tissue was used for each biochemical assay. Samples were processed immediately or flash-frozen in liquid nitrogen and stored at -80°C until analysis.

2.4. Assessment of Oxidative Stress and Antioxidant Parameters

2.4.1. Malondialdehyde (MDA)

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) using a modified thiobarbituric acid reactive substances (TBARS) assay. Approximately 100 mg cortical tissue was homogenized in 5 mL Hanks' balanced salt solution (pH 7.4) using three 30-s homogenization bursts at 3000 rpm. The homogenate was centrifuged at 3000 rpm for 10 min at 25°C . The resulting sample was reacted with SDS, acetic acid, thiobarbituric acid, and distilled water and heated in a boiling water bath for 60 min. After cooling, *n*-butanol:pyridine (15:1, v/v) was added, followed by vigorous mixing and centrifugation at 4000 rpm for 15 min. Absorbance of the resulting chromogen was measured at 532 nm. MDA concentration was calculated from a 1,1,3,3-tetraethoxypropane standard curve and expressed as $\mu\text{mol}/\text{mg}$ tissue (Mateos et al., 2005).

2.4.2. Nitric Oxide (NO)

Nitric oxide levels were estimated indirectly by measuring nitrite using a modified Griess reaction. Cortical tissue (100 mg) was homogenized in 5 mL cold phosphate buffer (0.1 M, pH 7.4) for two 30-s periods at 3000 rpm. A 300- μL aliquot of homogenate was mixed with 100 μL freshly prepared Griess reagent containing sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride. Samples were incubated for 30 min at room temperature in the dark, after which 2 mL phosphate buffer was added. Absorbance was measured at 548 nm, and nitrite concentration was determined using a sodium nitrite standard curve. Results were expressed as mM/mg tissue (Sun et al., 2003).

2.4.3. Superoxide Dismutase (SOD)

SOD activity was determined by measuring inhibition of pyrogallol autoxidation. Approximately 100 mg cortical tissue was homogenized in 4 mL ice-cold Tris-EDTA buffer (50 mM Tris, 1 mM EDTA, pH 8.2), followed by addition of 1% Triton X-100. Samples were maintained on ice for 30 min and centrifuged at 10,000 rpm for 30 min at 4°C . The supernatant was used for the assay. Absorbance was recorded at 420

nm at 1-min intervals for 10 min. SOD activity was calculated from the inhibition of pyrogallol autoxidation and expressed as units/mg protein (Weydert and Cullen, 2009; Senthilkumar et al., 2021).

2.4.4. Catalase (CAT)

Catalase activity was determined by monitoring the decomposition of hydrogen peroxide. Cortical tissue (100 mg) was homogenized in 5 mL phosphate buffer (50 mM, pH 7.0) using three 30-s bursts at 1800 rpm and subsequently diluted 1:5 with the same buffer. One milliliter of diluted sample was mixed with 2 mL freshly prepared 100 mM H_2O_2 . The decrease in absorbance at 240 nm was recorded at 30-s intervals for 10 min. Catalase activity was expressed as units/min/mg tissue.

2.4.5. Reduced Glutathione (GSH)

Reduced glutathione was measured using Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid), DTNB]. Approximately 100 mg cortical tissue was homogenized in 5 mL ice-cold phosphate buffer (0.1 M, pH 7.4). Trichloroacetic acid (5%) was added to precipitate proteins, followed by centrifugation at $3900 \times g$ for 10 min at 25°C . One milliliter of supernatant was mixed with 1 mL DTNB (0.01 M) and 1 mL phosphate buffer. After a 5-min incubation at room temperature, absorbance was measured at 412 nm. GSH concentration was calculated from a reduced-glutathione standard curve and expressed as $\mu\text{M}/\text{mg}$ tissue.

2.5. Statistical Analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM), with six animals per group. The original manuscript reports analysis using ANOVA with multiple-comparison testing for the biochemical endpoints. Statistical significance was considered according to the reported pairwise *p* values.

3. Results and Discussion

3.1. Polyherbal Treatment Attenuated Ischemia-Induced Oxidative Stress

Transient middle cerebral artery occlusion produced a clear disturbance in cerebral redox status, as demonstrated by changes in lipid peroxidation and nitric oxide levels. In healthy control animals, MDA concentration was 6.75 ± 2.97 , whereas tMCAO markedly increased MDA to 43.33 ± 12.24 ($p = 0.0009$). Treatment with the polyherbal formulation substantially reduced MDA to 11.24 ± 4.84 , representing a significant reduction compared with untreated tMCAO animals ($p = 0.0023$).

The marked elevation of MDA following ischemia-reperfusion is consistent with enhanced lipid peroxidation and oxidative membrane damage. Because cerebral tissue contains a high proportion of polyunsaturated fatty acids, excessive ROS generation during ischemia-reperfusion can readily initiate lipid peroxidation. MDA is consequently used as an indicator of oxidative damage in biological tissues (Ayala et al., 2014). The reduction

observed after polyherbal treatment suggests attenuation of this oxidative burden. Similar antioxidant effects of plant-derived interventions have been reported in experimental models, where phytochemical-rich preparations reduced lipid peroxidation and improved endogenous antioxidant defenses (Kamal et al., 2023; Li and Xing, 2024).

Importantly, MDA in the treated animals remained higher than the healthy-control value. Thus, the present findings indicate substantial attenuation rather than complete normalization of lipid oxidative damage within the 24-h observation period. This distinction is important when interpreting the neuroprotective potential of the formulation.

3.2. Polyherbal Treatment Reduced Ischemia-Associated Nitrosative Stress

Nitric oxide levels showed a pattern consistent with the oxidative-stress findings. The healthy group exhibited an NO-related value of 16.41 ± 3.89 , which increased markedly to 54.81 ± 15.22 following tMCAO ($p = 0.0005$). Administration of the polyherbal formulation reduced the value to 23.07 ± 2.74 , significantly lower than the untreated tMCAO group ($p = 0.0018$).

The increase in NO following cerebral ischemia is consistent with enhanced nitrosative stress during ischemia-reperfusion. Excessive reactive nitrogen species can contribute to oxidative injury and neuronal dysfunction. The substantial reduction following polyherbal treatment therefore indicates that the formulation may attenuate the nitrosative component of post-ischemic injury.

Previous studies have similarly reported reductions in NO-related oxidative responses following treatment with plant-derived or polyphenol-rich interventions (Uddin et al., 2020; Islam et al., 2022). However, the present experiment measured NO indirectly through nitrite and did not assess nitric oxide synthase expression or activity. Therefore, the observed reduction should not be interpreted as definitive evidence of iNOS inhibition. Further molecular studies would be required to establish the mechanism.

3.3. Polyherbal Treatment Enhanced Endogenous Antioxidant Defense

The oxidative injury observed following tMCAO was accompanied by changes in endogenous antioxidant defenses. SOD activity decreased from 107.50 ± 101.92 in healthy animals to 54.55 ± 41.25 after tMCAO. Treatment with the polyherbal formulation increased SOD activity to 315.00 ± 176.72 , with a significant difference between the treated and untreated ischemic groups ($p = 0.0241$). The reduction in SOD activity after ischemia is consistent with disruption of antioxidant defenses under conditions of excessive ROS generation. Conversely, the marked increase following treatment suggests that the formulation enhanced the enzymatic antioxidant response. Plant-derived polyphenols and other phytochemicals have

previously been associated with enhancement of endogenous antioxidant systems in experimental models of neurological injury (Bellavite, 2023; Nakyam et al., 2022).

The magnitude of the increase in SOD is noteworthy because the treated group exceeded the healthy-control value. However, the present study did not determine whether this increase resulted from enhanced enzyme expression, altered enzyme activity, or other regulatory mechanisms. Therefore, mechanisms such as Nrf2 activation should be regarded as hypotheses for future investigation rather than established mechanisms of action.

Catalase activity demonstrated a complementary pattern. The tMCAO group showed increased CAT activity (0.4700 ± 0.0408) compared with healthy controls (0.0909 ± 0.0552), whereas polyherbal treatment reduced CAT activity to 0.0952 ± 0.0919 . The treated value was reported to be not significantly different from the healthy group ($p \approx 0.999$) and significantly lower than the untreated tMCAO group ($p < 0.0001$).

The increase in CAT activity following ischemia may represent a response to increased hydrogen peroxide availability. Restoration toward the control level after treatment, together with the marked increase in SOD, suggests a broader normalization of the antioxidant response. Nevertheless, CAT activity should not be interpreted independently; antioxidant enzymes operate as an interconnected system, and additional parameters such as glutathione peroxidase would help clarify the overall redox response.

3.4. Polyherbal Treatment Partially Restored Reduced Glutathione

Reduced glutathione provided an additional indication of disruption and subsequent recovery of cerebral redox homeostasis. GSH concentration decreased markedly from 1.5814 ± 1.2134 in healthy animals to 0.0750 ± 0.0211 following tMCAO ($p = 0.0274$). Polyherbal treatment increased GSH to 0.3283 ± 0.1154 , significantly higher than the untreated tMCAO group ($p = 0.0002$).

The severe depletion of GSH after tMCAO is consistent with increased consumption of reducing equivalents during oxidative stress. The subsequent increase following treatment suggests improved preservation or recovery of the glutathione-dependent antioxidant system. Similar restoration of GSH has been reported following treatment with antioxidant-rich herbal interventions in experimental models of cerebral ischemia and neurodegeneration (Khazdair et al., 2019; Zhu et al., 2025).

However, GSH levels in treated animals remained below those of healthy controls. Thus, as with MDA, the treatment produced partial rather than complete restoration within the 24-h experimental period. The precise basis for this recovery, whether increased

synthesis, reduced consumption, or enhanced recycling, was not determined in the present study.

3.5. Integrated Effect of Polyherbal Treatment on Cerebral Redox Homeostasis

Taken together, the five biochemical endpoints demonstrate a consistent treatment-associated shift toward improved redox balance following transient cerebral ischemia. tMCAO was characterized by increased MDA and NO, indicating enhanced oxidative and nitrosative stress, together with reduced SOD and GSH and an increase in CAT activity, indicating substantial alteration of endogenous antioxidant responses. Polyherbal treatment reduced MDA and NO while increasing SOD and GSH and bringing CAT activity close to the healthy-control level.

Table 1. Effect of polyherbal treatment on oxidative stress and antioxidant parameters following tMCAO

Parameter	Healthy control	tMCAO	tMCAO + Polyherbal
MDA	6.75 ± 2.97	43.33 ± 12.24	11.24 ± 4.84
NO	16.41 ± 3.89	54.81 ± 15.22	23.07 ± 2.74
SOD	107.50 ± 101.92	54.55 ± 41.25	315.00 ± 176.72
CAT	0.0909 ± 0.0552	0.4700 ± 0.0408	0.0952 ± 0.0919
GSH	1.5814 ± 1.2134	0.0750 ± 0.0211	0.3283 ± 0.1154

Values are reported as mean ± SEM (n = 6), as provided in the original manuscript. Units should be added to the table exactly according to the validated assay calculations before submission.

Overall, these findings support the interpretation that the polyherbal formulation attenuated ischemia-associated oxidative and nitrosative stress and improved several components of endogenous antioxidant defense. The coordinated reduction in MDA and NO together with recovery of SOD and GSH provides biochemical evidence of an improved redox state after treatment. However, because the study assessed these endpoints at a single acute time point and did not directly investigate molecular signaling pathways, the underlying mechanisms remain to be established.

4. Conclusion

The present study demonstrates that the polyherbal formulation attenuated the oxidative and nitrosative

disturbances induced by transient cerebral ischemia in rats. tMCAO was associated with increased MDA and NO levels, reduced SOD and GSH, and altered CAT activity, indicating disruption of cerebral redox homeostasis. Treatment with the polyherbal formulation reduced MDA and NO levels and improved SOD and GSH, while CAT activity was restored toward the healthy-control level. These findings suggest that the formulation may provide neuroprotective effects, at least in part, by reducing oxidative and nitrosative stress and supporting endogenous antioxidant defenses. However, the study was limited to an acute 24-h experimental period and did not directly investigate the molecular mechanisms underlying these effects. Further studies involving longer treatment periods, dose optimization, and molecular assessment of antioxidant and inflammatory pathways are warranted to establish the therapeutic potential of the formulation in ischemic stroke.

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