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Evaluation of the neuroprotective potential of bioactive fraction of *Rosmarinus officinalis* leaves on Cerebral stroke in rats

Bijankari Balaji[1], GV. Parameshwar[1], Shaik Ayesha[1]

Correspondent Author Email: bijankarbalaji123@gmail.com
Mobile no: 9110788818

ABSTRACT:

Stroke is a clinically defined syndrome of acute, focal neurological deficit attributed to vascular injury (infarction, haemorrhage) of the central nervous system. This Study aimed to Evaluate the neuroprotective potential of *Rosmarinus officinalis* leaves on Cerebral stroke in rats. Methodology: The neuroprotective potential of the bioactive fraction of *Rosmarinus officinalis* leaves was evaluated in normal rats using behavioural models such as the Elevated Plus Maze, Y-Maze, Wire Hanging Test, and Beam Walking Test, following daily IV administration of 250 mg/kg and 500 mg/kg for 14 days. Subsequently, cerebral ischemia was surgically induced in the same rats, and the tests were repeated to assess the protective effects of the extract following ischemic injury. Histopathological studies of brain tissues were also conducted to support behavioural findings and confirm neuroprotection. Results: Animals treated with the bioactive fraction *of Rosmarinus officinalis at 500mg/kg normalized the altered behavioural biochemical, and histopathological parameters in ischemic rats.* Conclusion: The study demonstrated significant neuroprotective potential in Albino Wistar Rats treated with bioactive fraction of *Rosmarinus officinalis* leaves. The neuroprotective effects of n-hexane fraction were found to be dose-dependent. The combined effect of antioxidant and anti-inflammatory biomolecules of the n-hexane fractions was responsible for the protective effects against post-stroke complications in rats.

Key words: Bilateral Carotid Arteries occlusion (BCAO), n-hexane bioactive fraction of *Rosmarinus Officinalis (NFRO),* neuroprotective.

Introduction:

Cerebrovascular strokes are the third largest disease in India. Based on past studies, the incidence rate is 119- 145/100,000, but at present, around 1.54 million Indians are affected by strokes every year in India [2,3]. If a brain's circulating arteries are blocked, then the brain cells cannot get enough energy and cannot carry the fresh blood from the heart and lungs. Interference of blood flow to the brain reduces functions in the lateral aspect of frontal, temporal and parietal lobes, the corona radiata, globus pallidus, caudate and putamen [4,5.] So, it eventually stops working within a few minutes the brain cells are damaged, long periods of disability or death. It produces symptoms like paralysis, severe headache, difficulty speaking, weakness, visual problems etc. Stroke rehabilitation is not well developed in India due to a lack of employees. Although India is a leading generic drug manufacturer still many people can't afford the commonly used secondary prevention drugs [3]. Stroke is a wide word that refers to any brain impairment caused by a vascular event. The most frequent kind of stroke is ischemic stroke, which accounts for 87 per cent of all strokes. Acute ischemia of a brain region fed by a single artery (focal ischemia) causes them and they are a primary cause of disability and death. The *rosemary* herb was used as a food seasoning and natural medicine for over a million years. The species is widely used for cooking, consumed as an herbal tea, preparation of cosmetics, as well as traditional and modern medicine system. Due to the presence of several secondary metabolites species

In recent years, many research studies have been conducted to document the traditional uses of *R. officinalis* and to find new biological effects for this plant. These studies have revealed a wide range of pharmacological activities including anticancer, anti-inflammatory, anti- nociceptive, antioxidant, antimicrobial, antimutagenic, antidementia, hypoglycaemic, and hypolipidemic, effects. In this review, an effort has been made to discuss all pharmacological findings that have been frequently reported for *Rosmarinus officinalis.* Also, chemical constituents responsible for the biological effects of this plant are presented and discussed. And the study is aimed to extract and fractionate the bioactive constituents from the leaves of *Rosmarinus officinalis*, estimate their antioxidant activity using the DPPH method, assess the effect of the bioactive fraction on behavioural and biochemical changes in cerebral stroke-induced animals, and analyze the associated histopathological changes.

Materials and methods:

1. instruments:

UV- Visible spectrophotometer - Lab India UV/VIS 3000+spectrophotometer. Centrifuge - Remi

Analytical balance - Shimadzu 220g capacity

Deep-frozen apparatus - Venchal scientific -40oc freezer

Rotary evaporator - BVK TEC (AICTE – RPS)

2. Chemicals

5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), Ethylene diamine tetra acetic acid (EDTA), 1,1,3,3, tetra ethoxy propane, L-Glutathione reduced, 2,2-Diphenyl-l-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich company. Trichloroacetic Acid (TCA), Adrenaline bitartrate 98% AR, Thiobarbituric acid (TBA), 2,3,5-Triphenyl tetrazolium chloride (TTC) were purchased from LOBA Chemie Pvt ltd, ketamine, xylazine, normal saline, potassium chloride, tricarboxylic acid, ethanol, sodium azide, pyrogallol, hydrochloric acid, hydrogen peroxide, sodium hydroxide pellets and monopotassium phosphate.

3. Collection of plant

The fresh leaves of *Rosmarinus officinalis* were obtained from a botanical garden in Ooty, Tamil Nadu. The plant material was identified by Dr. S. Rajan, Field Botanist, Survey of Medicinal Plants & Collection unit, Department of AYUSH, Govt. of India, Ooty, Tamil Nadu. The plant material was shade-dried and then crushed into a powder using a mechanical grinder. The powdered material was stored in an airtight container.

4. Extraction of plant material by maceration method

The 3 kg of powdered plant materials of each Rosmarinus officinalis were soaked in 6 liters of methanol in a glass container for a period of 1 week with intermittent mixing. After 1 week, the mixture was filtered through muslin cloth, obtained filtrate was concentrated in a rotary evaporator under reduced pressure and concentrated liquid was further evaporated in a glass container to obtain final semisolid residue. This crude methanol extract was stored in an airtight container. This procedure was repeated several times whenever necessary. [1]

5. Bioassay guided fractionation

The obtained crude methanol extract was dissolved in hot water in a glass container. This water-soluble extract was transferred into a separating funnel and fractionated using solvents from non-polar to polar, such as n-hexane, dichloromethane, ethyl acetate fractions, and the remaining aqueous fraction. All the fractions were further concentrated using a rotary evaporator under reduced pressure. The concentrated fractions were then stored in an airtight container

5. Evaluation of Anti-oxidant activity by *In vitro* methods

Antioxidant potential of crude methanol extract and its fractions such as n-hexane, dichloromethane, ethyl acetate and aqueous fractions were evaluated by following *in vitro* methods [3].

5.1. DPPH Radical Scavenging Assay:

DPPH radical quenching activity of methanol extract and its fractions of *Rosmarinus officinalis* (methanol and its fractions such as n-hexane, DCM, ethyl acetate, aqueous), was tested at different concentrations such as 250, 500, 750, 1000, 1250 µg/ml. The test sample (4.0 mL) was mixed with 1.0 mL DPPH (0.2 mM) solution. The mixture was shaken vigorously and left to stand for 30 minutes, and the absorbance was measured at 517 nm. The percentage of DPPH decolorization of the sample was calculated according to the equation: % Inhibition= [1-(ABS sample/ABS control)] X 100

6. Animals

Male albino Wistar rats (250 to 300g) were used for the present study procured from Sri Raghavendra Enterprises, Bangalore, India. The rats were kept in an air-conditioned room (24± l°C) with 12/12 hours of light and dark cycle. Animals received enough standard pellets and mineralized water for daily consumption. The protocol was carried out with the approval of the Institutional Animal Ethical Committee of Raghavendra Institute of Pharmaceutical Education and Research (IAEC/XVII/01/RIPER/2021)

6.1. Induction of stroke:

Acute ischemic stroke was induced in rats by bilateral common carotid artery occlusion model. The animals were anesthetized with a combination of ketamine (60mg/kg,i.p) and xylazine (10mg/kg, i.p). The anesthetized rats were then placed on the surgery table, and their body temperature was maintained at 37.0°C with a heating lamp. A small incision was made in the middle of the neck up to 1 inch, and the skin was retracted. The both carotid arteries were identified and carefully separated from the vagus nerve without damaging the glands and nerves. Both arteries were blocked with cotton thread for the period of 30 minutes ischemic fallowed by 24 hours reperfusion. The animals recovered spontaneously through breathing, and post-surgical care was provided under standard conditions with free access to food and water [6].

6.2 Acute toxicity study:

The acute toxicity of the n-hexane fraction was determined according to the guidelines of OECD 423. The n-hexane fraction was administered in a single dose of 2000 mg/kg orally to a group of 3 rats. The rats were carefully observed for a period of 14 days to detect any signs of toxicity. Dose levels of 250 ($1/8^{th}$) and 500 mg/kg ($1/4^{th}$) were selected for the animal study as no signs of toxicity were observed [7].

6.3. Study design:

All the experimental animals were divided into five groups, each consisting of eight animals. Animals in the test groups received the n-hexane fraction of Rosmarinus officinalis for a period of 14 days.

Table:1 Study design:

S. No	Group	Treatment
1.	Normal	Animals received only vehicle and water and pellet diet *ad libitum*
2.	Sham control	Animals received only vehicle and water and pellet diet *ad libitum*
3.	Ischemic reperfusion injury(I/R Injury)	Carotid artery occlusion for 30 minutes followed by 24 reperfusion
4.	n-hexane fraction	Rats were received n-hexane fraction of *Rosmarinus officinalis* suspended in carboxy methyl cellulose at the dose of 250 mg/kg, p.o with carotid artery occlusion for 30 minutes followed by 24 reperfusion.
5.	n-hexane fraction	Rats were received n-hexane fraction of *Rosmarinus officinalis* suspended in carboxy methyl cellulose at the dose of 500 mg/kg, p.o with carotid artery occlusion for 30 minutes followed by 24 reperfusion.

7. Assessment of Parameters

After 24 hours of reperfusion, animals in all groups were assessed for motor and cognitive dysfunction by various methods [8].

7.1. Evaluation of cognitive dysfunctions

7.1.1. Elevated Plus Maze:

The elevated plus maze consists of two open and two closed arms, each 50 cm above the floor. Before inducing ischemic stroke, animals were trained by placing them at the end of one of the open arms facing towards the center of the maze. The time taken for the rats to enter one of the closed arms was recorded as the transfer latency time, with a cut-off of 90 seconds before the experimental procedure. After 24 hours of reperfusion, the transfer latency time was evaluated again to assess the retention of trained memory.

7.1.2. Y Maze:

The Y-maze helps assess the spatial memory of animals. It contains three arms with equal dimensions. After the end of the reperfusion period, all groups of rats were kept individually in the center of the maze and observed for 5 minutes. During this period, rats were observed, and spontaneous alterations were noted down. Animals with a higher number of spontaneous alterations were found to have good spatial memory.

Percentage spontaneous alterations (SA %) calculated with the following formula.

%SA= Actual number of alterations – maximum number of alterations×100

Maximum number of alterations= Total number of entries-2

7. 2. Evaluation of Motor dysfunction

7.2.1. Beam walking test

The beam walking test is used to assess the balance and motor coordination of animals. Animals were allowed to train to walk on a wooden bar (120 cm length, 2.3 cm diameter) placed between two poles. The time required to reach from one end to the other end is recorded. The cutoff time for the beam walking test is 120 seconds.

7.2.2. Wire hanging test:

This test is used to measure the grip strength of the forelimbs of a rat. During this test, animals were allowed to hang with their forelimbs on a wire (45 cm long, 0.3 cm diameter) suspended between two posts above the floor at a height of 40 cm. Individual animals were tested for the time taken to fall down from the wire. The cut-off time was set at 90 seconds.

8. Estimation of Antioxidant Parameters in the Brain Tissue

Rats were euthanized after a 24-hour period of reperfusion. The rats' brains were immediately removed, cleaned, centrifuged in ice-cold PBS (pH 7.4) at 7000 rpm for 15 minutes. These homogenates were used to estimate biochemical parameters in the rat brain.

8.1. Estimation of Superoxide Dismutase

Reagent preparation

Preparation of pyrogallol (10 mM): Prepare the reagent by dissolving 0.0635g of pyrogallol in 50ml of water.

Procedure

The Superoxide dismutase (SOD) activity test determines the extent to which an enzyme can inhibit pyrogallol oxidation. The protocol for this test was described by Soon and Tan (2002) and was first published by Marklund and Marklund in 1974. In the test, a mixture of phosphate buffer, brain homogenate, and distilled water is prepared. Then, 0.02ml of pyrogallol solution is added to start the reaction. In a typical 3 ml assay, the amount of enzyme needed to reduce pyrogallol oxidation by 50% is determined and expressed as Units/min/g of tissue .[9]

$$\% \text{ Inhibition of pyrogallol autoxidation} = \frac{\Delta A\ test}{\Delta A\ control} \times 100\%$$

$$\text{SOD activity (U/g of tissue)} = \frac{\%\ \text{Inhibition of pyrogallol autoxidation}}{50\%}$$

8.2. Estimation of Catalase assay:

Reagent preparation

Preparation of Hydrogen peroxide (H2O2) (0.019M): Prepare the reagent by dissolving 0.065ml of H2O2 in 100ml of water.

Procedure

Catalase converts H2O2 into H2O and O2, and its activity was tested in the supernatant. Using Beer and Sizer's approach (1952), the rate of H2O2 breakdown was determined spectrophotometrically at 240 nm. H2O2, brain homogenate, and sodium phosphate buffer made up the assay combination. For five minutes, the absorbance was measured at 240 nm once every minute. Catalase activity was expressed with an extinction coefficient of 43.6 M^{-1} cm^{-1}.[9]

$$\text{Catalase activity (U/gm of tissue)} = \frac{Absorbance\ at\ 240\ nm}{L \times E} \times D$$

Where,

L= light path = 1cm

E = Extinction coefficient = 43.6 M^{-1} cm^{-1}

$$D = \frac{Total\ volume\ in\ ml}{volume\ of\ sample\ taken}$$

8.3. Estimation of Reduced glutathione:

Reagent preparation

Preparation of 10% Trichloroacetic acid (TCA): Prepare the reagent by dissolving 10ml of TCA in 100ml of water.

Preparation of 0.1% Sodium citrate: Prepare the reagent by dissolving 0.1ml of Sodium citrate in 100ml of water.

Preparation of 5, 5-dithio-bis-(2-nitrobenzoic acid (DTNB): Prepare the reagent by dissolving 19.8mg of DTNB in 100ml of 0.1%Sodium citrate.

Procedure

Ellman's (1959) method of measuring total reduced glutathione is based on the formation of a yellow color with a maximum absorbance at 412 nm when 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reacts with substances containing sulfhydryl groups. After deproteinizing the brain homogenate (0.5 ml, 10% w/v) with 5% TCA (3.5 ml), the mixture was centrifuged for 30 minutes at 2000 rpm. The supernatant (0.5 ml), newly made Ellman's reagent (0.5 ml, 19.8 mg DTNB in 100 ml of 0.1% sodium citrate), and 0.2M phosphate buffer (3.0 ml, pH 8.0) were then added. The reaction product was measured at 412 nm. The results are expressed as µm (micromole) GSH/g protein [9].

$$\text{GSH activity (µm/gm of tissue)} = \frac{Absorbance\ at\ 412\ nm}{L \times E} \times D$$

Where,

L= light path = 1cm

E = Extinction coefficient = 1.36 × 104 M^{-1} cm^{-1}

$$D = \frac{Total\ volume\ in\ ml}{volume\ of\ sample\ taken}$$

8.4. Estimation of lipid peroxidase (LPO):

Reagent preparation

Preparation of 10% Trichloroacetic acid (TCA): Prepare the reagent by dissolving 10ml of TCA in 100ml of water.

Preparation of 0.67% Thiobarbituric acid (TBA): Prepare the reagent by dissolving 0.335gm of TBA in 50ml of water.

Procedure

Using Buege & Aust's 1978 approach, lipid peroxidation was quantified by measuring the amount of malondialdehyde (MDA) formed. Lipid peroxidation produces MDA, which is recognized by its interaction with TBA to form a chromogen that absorbs at 535 nm. The assay mixture was incubated in a water bath for 30 minutes and then cooled. It contained 10% brain homogenate (0.1 ml), 10% tricarboxylic acid (TCA) (2 ml), and 0.67% thiobarbituric acid (TBA) (2 ml). The absorbance of the supernatant was measured at 535 nm. Using a molar extinction coefficient of 1.56x105 M-1 cm-1 for MDA, the extent of LPO was represented as n moles of MDA generated per gram of tissue [9].

$$\text{MDA activity (nm of MDA/gm of tissue)} = \frac{Absorbance\ at\ 535\ nm}{L \times E} \times D$$

Where,

L= light path = 1cm

E = Extinction coefficient = $1.56 \times 105\ M^{-1}\ cm^{-1}$

$$D = \frac{Total\ volume\ in\ ml}{volume\ of\ sample\ taken}$$

9. Assessment of cerebral stroke area:

The rats were euthanized with pentobarbital (40 mg/kg, i.p.) and their brains were removed, then cooled to -4°C after a 72-hour period of reperfusion. The brains were cut into 2 mm pieces and submerged in a 1% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate-buffered solution (PBS) at pH 7.4. These sections were then incubated for 30 minutes at 37°C in a humid, dark environment. The sections were examined after cleaning. Dead brain tissue remains white, while living brain tissue reacts with TTC to produce a red pigment through the action of dehydrogenase enzymes and cofactors [10].

10. Histopathological studies:

A portion of the brain was cut into 5-µm-thick pieces in semi-automatic cryostat microtome. Brain sections were stained with hematoxylin and eosin dye for histological analysis.

11. Statistical analysis:

The findings were displayed as Mean ± SD. Tukey's multiple comparison test was performed after one-way analysis of variance (ANOVA) in GraphPad Prism V 7.01 statistical analysis. P-values less than 0.05 were taken into consideration for statistical significance.

RESULTS AND DISCUSSION

1. Results of DPPH Radical scavenging activity of *Rosmarinus officinalis*

The DPPH radical scavenging activity of methanol extracts and their fractions demonstrates dose-dependent scavenging activity. The n-hexane fraction showed the highest antioxidant activity, as indicated by the highest percentage inhibition. (Results are provide in Tables 2 and 3).

Table 2. Results of DPPH radical assay

Con. µg/ml	**DPPH radical scavenging assay (% Inhibition)**				
	Methanol	**n-hexane**	**DCM**	**EA**	**Aqueous**
250	48.73± 2.52	45.87±8.70	44.04±5.21	43.76±4.22	38.04±0.68
500	59.8±3.04	68.66±3.45	55.05±2.58	62.96±3.95	45.36±3.39
750	63.07± 4.78	73.99±2.11	57.01±4.10	71.94±3.20	53.31±3.53

1000	69.12±5.97	84.42±2.40	70.94±5.28	78.34±1.82	66.95±2.19
1250	77.73±3.49	86.56±0.75	79.19±1.01	85.2±1.74	69.02±0.98

Table 3. Results of IC_{50} values DPPH radical assay

	DPPH radical scavenging assay (IC_{50})				
	Methanol	**n-hexane**	**DCM**	**EA**	**Aqueous**
IC_{50} (µg/ml)	225.12	186.16	423.5	280	614.64

2. Effect of n-hexane fraction Rosmarinus officinalis (NFRO) on cognitive dysfunctions

2.1. Elevated plus maze

In I/R injury group, there was a significant (****P < 0.0001) increase in the transfer latency period, indicating impairment in cognitive functions due to ischemic reperfusion. Specifically, the transfer latency period increased by 65 seconds. However, pretreatment with varying doses of NFRO (250 & 500 mg/kg) for 14 days prior to ischemic reperfusion injury notably reduced (****P < 0.0001) the transfer latency period compared with the I/R injury group. The reduction in transfer latency period compared to I/R injury group was 24.667 seconds for the NFRO (250 mg/kg) treatment and 19.50 seconds for the NFRO 500 mg/kg treatment (Figure 1).

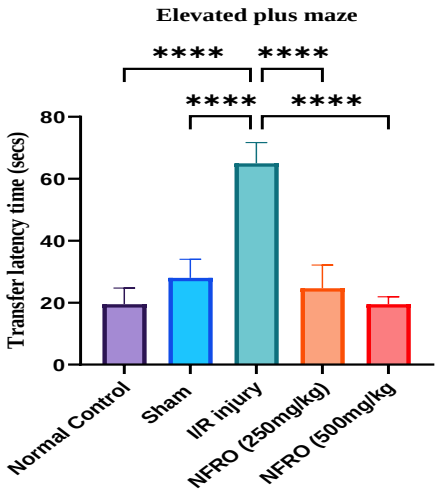

Figure 1. Effects of NFRO on transfer latency in the elevated plus maze

All data are presented as mean ± SEM for n=6 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01,*** p<0.001, ****p<0.0001.

2.2. Y Maze

In the Y-maze test, spontaneous alterations were evaluated. The I/R injury group displayed a notable (****P < 0.0001) decrease in the number of alterations, indicating a decline in cognitive function. However, administration of NFRO groups led to an improvement in this aspect, resulting in a significant (*p<0.05 for 250 mg/kg and **<0.01 for 500 mg/kg) increase in spontaneous alterations indicating an improvement in spatial memory compared to I/R injury group. These findings suggest that NFRO consumption could potentially enhance learning and memory abilities, countering the decline observed in the I/R injury group (Figure 2).

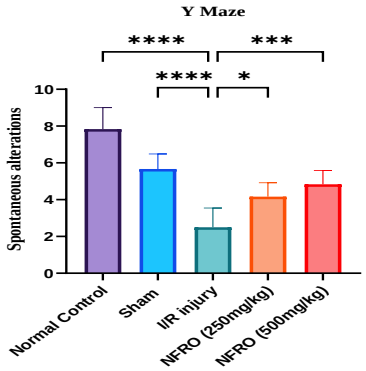

Figure 2. Effects of NFRO on spontaneous alterations in the Y maze

All data are presented as mean ± SEM for n=6 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01,*** p<0.001, ****p<0.0001.

3. Effect of n-hexane fraction of Rosmarinus officinalis on motor dysfunctions

3.1. Wire hanging test

In the I/R injury group, there was a significant (****P < 0.0001) decrease in hanging latency time, indicating a decline in motor function. Specifically, the decrease in hanging latency time amounted to 5.333 seconds. However, pretreatment with different doses of NFRO (250 & 500 mg/kg) for 14 days prior to ischemic reperfusion injury led to a notable increase (****P<0.0001) in hanging latency periods when compared to the I/R injury group. The increase in hanging latency time compared to the I/R injury group was 17.1667 seconds for the NFRO (250 mg/kg) treatment and 19.8333 seconds for the NFRO 500 mg/kg treatment, indicating an improvement in motor functions (Figure 3).

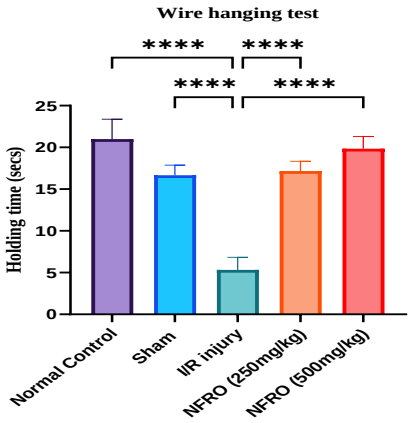

Figure 3. Effects of NFRO on holding time in Wire hanging test

All data are presented as mean ± SEM for n=6 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01,*** p<0.001, ****p<0.0001.

3.2. Beam walking test

In the I/R injury group, there was a significant increase (****P<0.0001) in beam walking latency, suggesting impairment of motor functions due to carotid artery occlusion. Specifically, the increase in beam walking latency amounted to 71.5 seconds. However, pretreatment with varying doses of NFRO (250 and 500 mg/kg orally) for 14 days prior to ischemic reperfusion injury led to a significant decrease (****P<0.0001) in beam walking latency period when compared to the I/R injury group. The reduction in beam walking latency compared to the I/R injury group was 30.1667 seconds for the NFRO (250mg/kg) treatment and 26.667 seconds for the NFRO 500 mg/kg treatment (Figure 4)

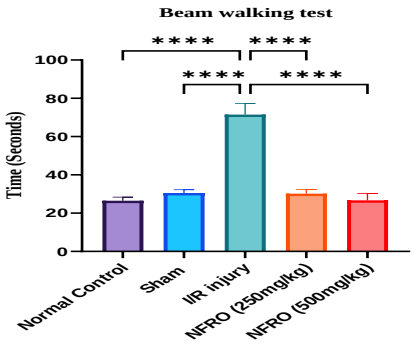

Figure 4. Effects of NFRO on Beam walking test

All data are presented as mean ± SEM for n=6 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001.

4. Biochemical Estimations in rat brain tissue

Brain tissue is particularly vulnerable to ischemia due to its limited oxygen supply and reliance on aerobic metabolism, unlike many other organs. Ischemic processes in the brain, known as ischemic cascades, occur when blood flow is restricted. Ischemic brain damage resulting from stroke or reperfusion injury leads to the production of oxygen-free radicals and other reactive oxygen species. Free radicals are predominantly generated from various sources, including inflammatory cells, dysfunctional mitochondria, and excitotoxic mechanisms triggered by elevated levels of glutamate and aspartate. Following a 24-hour period of reperfusion, we assessed the levels of antioxidant enzymes such as SOD, CAT, GSH, MDA, and MPO in the rat brain tissue, which served as oxidative indices.

4.1. Effect of NFRO on the levels of superoxide dismutase (SOD):

Brain SOD levels were significantly (***p < 0.001) decreased (0.933 ± 0.01) in I/R injury rats compared to normal group rats (1.794 ± 0.0871) and Sham rats (1.6655 ± 0.0785). However, NFRO (250mg/kg) (1.6768 ± 0.0060) and NFRO 500 mg/kg (1.756 ± 0.0075) treated rats showed a significant increase (***p < 0.001) in SOD levels, respectively (Figure 5).

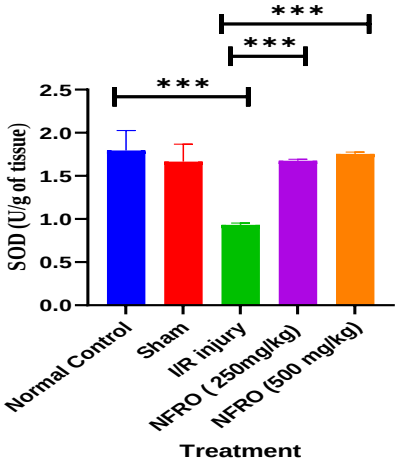

Figure 5. Effects of NFRO on superoxide dismutase (SOD) levels

All data are presented as mean ± SEM for n=4 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001.

4.2. Effect of NFRO on the levels of Catalase:

Brain catalase levels were significantly (***p < 0.001) decreased (0.2120 ± 0.0144) in I/R injury rats compared to normal group rats (0.5490 ± 0.0137) and Sham rats (0.4920 ± 0.0128). However, NFRO 250 mg/kg (0.3390 ± 0.0135) and NFRO 500 mg/kg (0.3970 ± 0.0119) treated rats showed a significant increase (***p < 0.001) in CAT levels, respectively (Figure 6).

All data are presented as mean ± SEM for n=4 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01,*** p<0.001, ****p<0.0001.

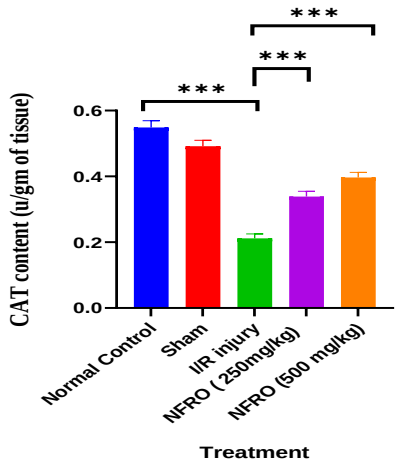

Figure 6. Effects of NFRO on catalase (CAT) levels

All data are presented as mean ± SEM for n=4 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01,*** p<0.001, ****p<0.0001.

4.3. Effect of NFRO on the levels of reduced glutathione:

Brain GSH levels were significantly (**p < 0.01) decreased (0.0004 ± 0.00386) in I/R injury rats compared to normal group rats (0.00073 ± 0.00215) and Sham rats (0.00071 ± 0.00252) respectively. However, NFRO (250mg/kg) treated rats (0.00063 ± 0.00291) and NFRO 500 mg/kg treated rats (0.00067 ± 0.00303) showed a significant increase in GSH levels (*p < 0.05& **p < 0.01) respectively (Figure 7).

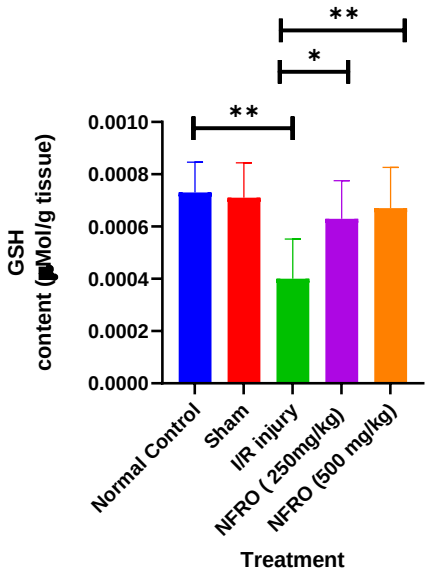

Figure 7. Effects of NFRO on reduced glutathione (GSH) levels

All data are presented as mean ± SEM for n=4 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01,*** p<0.001, ****p<0.0001.

4.4. Effect of NFRO on the levels of lipid peroxidase:

Brain MDA levels were significantly (***p < 0.001) increased (0.000102 ± 0.00046) in I/R injury rats compared to normal group rats (0.000049 ± 0.0008) and Sham rats (0.000059 ± 0.00076), respectively. However, NFRO (250mg/kg) treated rats (0.000077 ± 0.00011) and NFRO 500 mg/kg treated rats (0.00005 ± 0.00116) showed a significant decrease (*p < 0.05, ***p < 0.01) in MDA levels, as shown in Figure 8.

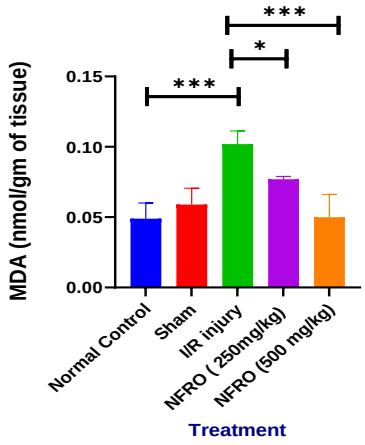

Figure 8. Effects of NFRO on superoxide dismutase (SOD) levels

All data are presented as mean ± SEM for n=4 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001.

4.5. Effect of NFRO on the levels of myeloperoxidase:

Brain MPO levels were significantly (***p < 0.001) increased (12.57 ± 0.06958) in I/R injury rats compared to normal group rats (3.725 ± 0.00496) and Sham rats (4.4 ± 0.0456), respectively. However, NFRO (250mg/kg) treated rats (7.025 ± 0.00449) and NFRO 500 mg/kg treated rats (6.025 ± 0.01182) showed a significant decrease (***p < 0.001) in MPO levels, as shown in Figure 9.

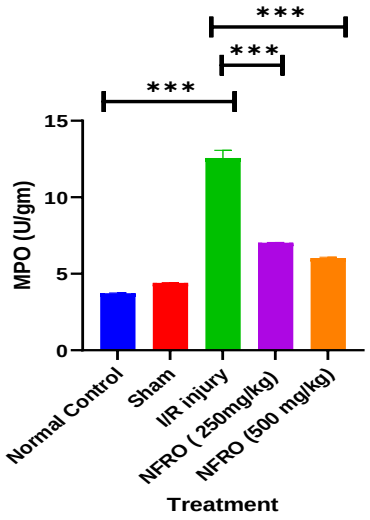

Figure 9. Effects of NFRO on myeloperoxidase (MPO) levels

All data are presented as mean ± SEM for n=4 animals. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. * p<0.05, ** p<0.01,*** p<0.001, ****p<0.0001.

5. Results of TTC staining test:

The percentage of cerebral infarct volume was observed and calculated by Image J software to be increased in rats exposed to ischemia/reperfusion (I/R) injury (13.4%). However, in rats treated with NFRO (250mg/kg) and NFRO 500 mg/kg, the percentage of cerebral infarct volumes was reduced (8.6% & 5%). Specifically, in this study, the percentage of cerebral infarction in the I/R group was increased compared to the normal group (6.3%). Treatment with NFRO 500 mg/kg resulted in a greater reduction in the percentage of cerebral infarction compared to treatment with NFRO 250 mg/kg, indicating the neuroprotective effect of NFRO 500 mg/kg (Figure 10).

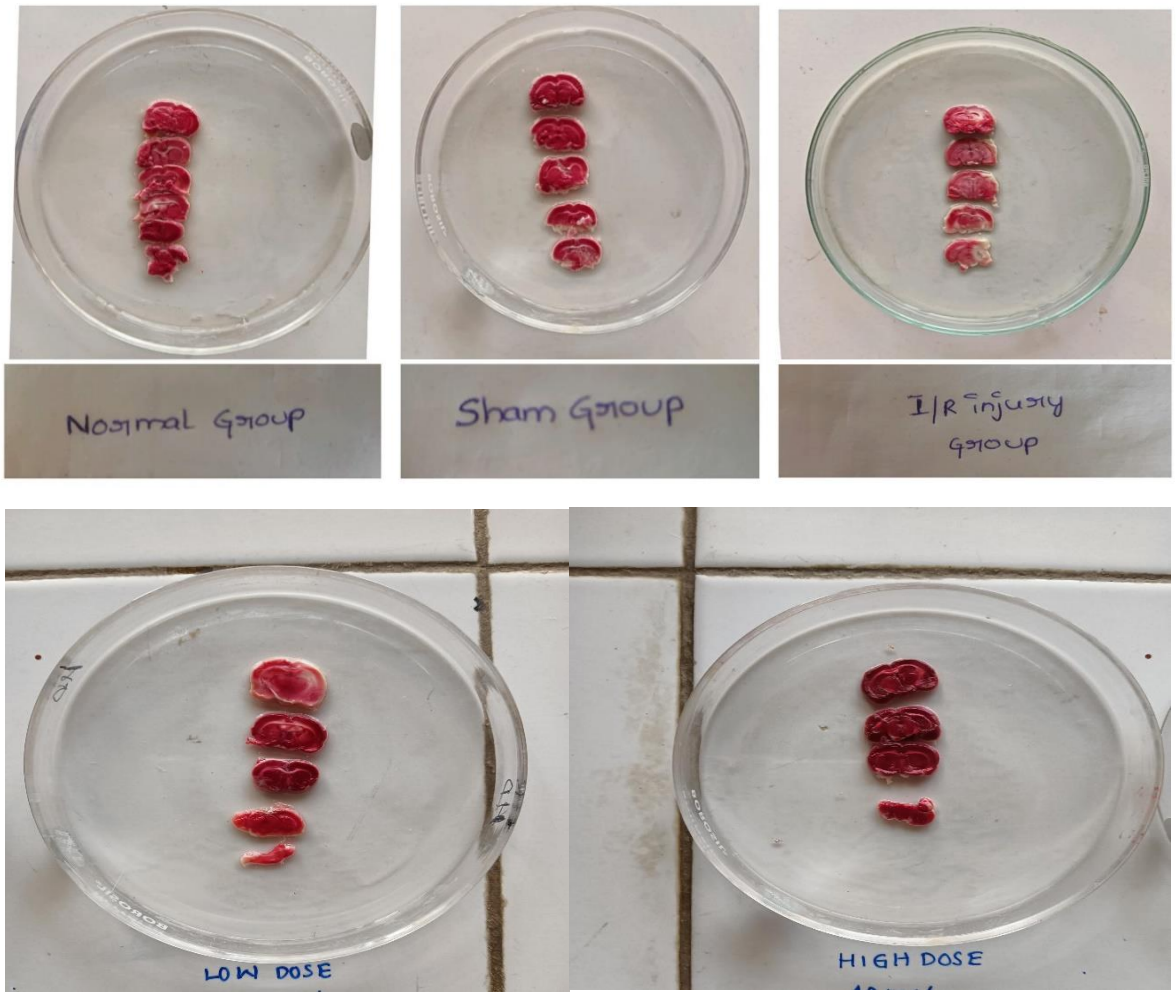

Figure 10. TTC staining of Normal; Sham control; I/R injury; NFRO 250 mg/kg and NFRO 500 mg/kg

CONCLUSION

Stroke is the third leading cause of death and also causes lifelong disabilities such as motor defects and cognitive impairments. The cause of stroke and its complications is a diminished supply of blood to the brain due to the blocking of carotid arteries. The pathological pathways responsible for tissue damage are oxidative stress, activation of inflammatory cascades, and apoptosis. The current study aimed to explore the possibility of using plant extracts to prevent this pathological damage and reduce post-stroke complications. *Rosmarinus officinalis* plants are rich in antioxidant and anti-inflammatory molecules. The leaves were extracted by cold maceration with methanol, followed by the solvent fractionation of water-soluble crude semi-solid methanol extract with various solvents ranging from non-polar to polar, such as n-hexane, dichloromethane, ethyl acetate, and remaining aqueous fractions. In vitro antioxidant and anti-inflammatory activities were performed to identify the bioactive fraction among the extracts or fractions. The study results found that the n-hexane fraction is exhibited the highest antioxidant. Further animal studies were conducted using the n-hexane fraction. The n-hexane fractions were administered to male Wistar rats at doses of 250 mg/kg and 500 mg/kg for a period of 14 days. Experimental stroke was induced by carotid artery occlusion for 30 minutes followed by 24 hours of reperfusion. At the end of the reperfusion period, the experimental animals were assessed for motor and cognitive deficits using tests such as the plus maze test, Y-maze test, beam walking test, and wire hanging test to evaluate post-stroke complications. Biochemical parameters such as lipid peroxidation (LPO), reduced glutathione, superoxide dismutase (SOD), glutathione peroxidase, catalase (CAT), reduced glutathione (GSH), and myeloperoxidase (MPO) levels were estimated in brain tissue. Additionally, TTC staining and histopathology studies were conducted to evaluate neuroprotective activities. In conclusion, n-hexane fraction was found to mitigate neurological deficits and biochemical alterations in brain tissue, infarct size, and histopathological changes induced by ischemic injury in rats. However, the neuroprotective effects of n-hexane fraction was found to be dose-dependent. The combined effect of antioxidant and anti-inflammatory biomolecules of the n-hexane fractions was responsible for the protective effects against post-stroke complications in rats.

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