Neuroprotective Outcome of Thymol against Global Cerebral Ischemia Reperfusion injury in Albino Rats

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Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The purpose of the present study was targeted to explore the possible role of thymol against global cerebral ischemia-reperfusion injury in albino rats.

Study Design: Healthy Albino Wistar rats (200–250 gm) were divided randomly into 5 groups (n=6). Group I and II were considered as normal control and sham control, received 2% tween 80 orally, group III was ischemic reperfusion (disease control) and received 2% tween 80 orally and Group IV and V received thymol at doses of 50 mg/kg, per oral and 100 mg/kg, per oral respectively.

Place and Duration of Study: Department of Pharmacology, Sri Padmavathi School of Pharmacy, Tiruchanur, Tirupati in between Sept 2019 to March 2020.

Methodology: Group I and II were considered as normal control and sham control, received 2% tween 80 orally, group III was ischemic reperfusion (disease control) and received 2% tween 80 orally and Group IV and V received thymol at doses of 50 mg/kg, per oral (p.o) and 100 mg/kg, per oral (p.o). respectively. After pretreatment with thymol for 2 weeks, rats were subjected to
bilateral common carotid artery occlusion for 1 hour accompanied by 22 hours reperfusion (I/R). After 22 hrs of reperfusion, motor coordination, hanging wire test, despair swim tests were studied. Antioxidant levels of reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and pro-oxidant level of Malondialdehyde (MDA) were analyzed in brain tissue homogenate. Changes in cerebral infarct size and histopathology were studied.

**Results:** Pretreated groups of thymol (50 and 100 mg/kg p.o) showed significant improvement in neurobehavioral changes and attenuated oxidative damage as indicated by reduced LPO, restored GSH, SOD and CAT levels and decreased infract size when compared to ischemic reperfusion group.

**Conclusion:** This study suggests that thymol may have a beneficial role against global ischemia reperfusion induced damage caused by excessive free radicals and behavioral alterations in rats.

**Keywords:** Global ischemia; thymol; oxidative stress; behavioral alterations; infarct size.

**ACRONYMS**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.O</td>
<td>Per oral</td>
</tr>
<tr>
<td>BCCAO</td>
<td>Bilateral common carotid artery occlusion</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced Glutathione</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemic reperfusion</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>I.M</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5 triphenyl tetrazolium chloride</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis-[2-nitrobenzoic acid]</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
</tbody>
</table>

**1. INTRODUCTION**

Ischemic stroke happens because of the abrupt impediment of the blood vessel by a thrombus or embolism, is a typical reason for death and inability around the world. [1] Ischemia is a limitation in the supply of blood to tissues, gives rise to the absence of glucose and oxygen required for the metabolism of cell. Reperfusion is the reclamation of bloodstream to an organ or tissue in the wake of having been blocked. Ailments like high blood pressure, alcohol smoking, abuse, and stress may also bring insufficient blood supply to the brain. Impairment in cognition capacity and behavioral outcomes also occurs due to ischemia reperfusion injury.

Stroke is positioned as the subsequent driving reason for death worldwide with a yearly death pace of about 5.5 million. Stroke recurrence is 150-200/100,000 individuals/ year. It is estimated that each year nearly 6 million people die from stroke. It is estimated that in the midcentury in the older population (> 65 years old) stroke incidence is tripled from 516 million in 2009 to 1.50 billion in 2050. [2] Various components are engaged with the pathogenesis of cerebral stroke incorporates programmed cell death, oxidative pressure, inflammation and necrosis that causes the demise of neuronal cells. Despite of advances in the comprehension of the pathophysiology of cerebral ischemia, an ideal treatment for intense ischemic stroke stay constrained. [3]. Improvement of neuroprotective medications with numerous consequences for the ischemic course is possibly more engaging than drugs following up on a single drug of the course. Numerous neuroprotective drugs indicated gainful impacts in preclinical investigations either flopped in clinical preliminary, showed extreme symptoms or declined the result of stroke. The purpose behind this is obvious disparity between tests on animals and clinical application which are multifactorial.[4,5] One of reason for the disappointment of evolved drugs can focus on single sub-atomic pathway, while a couple of pathways are included spatially and transiently in the pathophysiology of stroke.

Many studies suggested that plant derived natural compounds showed beneficial role in various ailments and were used in alternative therapy. [6] Due to increasing in undesirable side effects, resistance and high cost much attention has been given to traditional medicines for the development of new compounds naturally. One of such compounds are thymol that has gained
consideration due to its pharmacological properties.

Thymol, a white crystalline monoterpenic phenol, known as 2-isopropyl-5-methylphenol having strong flavor and pleasant aromatic odor. It seemed to have many pharmacological properties containing anticonvulsant,[7] antifungal,[8] antihyperlipidemic, antioxidant,[9] antispasmodic, anti-inflammatory,[10] antiseptic, antiepileptogenic, radioprotective[11] and antitumor activities[12] . It is one of the most significant constituents in Trachyspermum ammi and many other thyme species. Hence this research was focused on thymol, a naturally occurring phytochemical against the global cerebral ischemia reperfusion model in albino rats.

2. MATERIALS AND METHODS

2.1 Animals

The animals utilized for this study were healthy albino rats of 200-250 gm. Rats were housed under standard conditions of laboratory, kept on 12h light dark and had free access to water and food. Animals were habituated to research facility environment for 7 days preceding to study.

2.2 Chemicals

Thymol was procured from Sigma-Aldrich, Bangalore, India. Trichloroacetic acid, 2-Thiobarbituric acid, epinephrine, 2,3,5 triphenyl tetrazolium chloride (TTC). Chemicals of analytical grade were used for the research study.

2.3 Method of Experimental Design

Cerebral ischemia reperfusion injury was prompted by the strategy for Iwasaki et al. [13] [14] Induction of ischemia was performed under ketamine (100 mg/kg/L.M.) and xylazine (10 mg/kg/L.M.) anesthesia. Surgery was done by making an incision between the sternocleidomastoid muscle and sternohyoid muscle, both carotid arteries were exposed and occlusion was done by using bulldog clamps for one hour. The clamps were removed after one hour and allowed for recirculation for duration of 22 hours. During surgery the body temperature was maintained around 37°C±0.5 °C with a thermo statistically controlled infrared lamp. The rats were returned to separate cages after suturing. Surgery was not performed to the rats of sham-operated group. After 2 weeks, 1 hour occlusion followed by 22 hours of reperfusion rats were evaluated for neurological and behavioral outcome and were sacrificed for the assessment of oxidative stress markers, infarct size and histopathological changes.

2.4 Study Protocol

Healthy albino rats were randomly separated into 5 groups and each group consisted of 6 rats.

Group I: Normal group served as control group and was given 2% tween orally. Group II: Sham-operated control groups (received only identical procedure without BCCA occlusion), were given 2% tween orally. Group III: Ischemic reperfusion (Disease control) received 2% tween oral and the animals were undergone to BCCA occlusion for 1 hour followed by 22 hrs reperfusion Group IV: Pretreatment with thymol (50 mg/kg, p.o) and Group V: Pretreatment with thymol (100 mg/kg, p.o) of thymol for 2 weeks respectively. [15,16] After pretreatment with thymol for 2 weeks, rats were undergone to BCCA occlusion for 1 hour followed by 22 hrs reperfusion. The animals were subjected to behavioral studies.

2.5 Effect of Thymol on Behavioral Testing

2.5.1 Beam walking test

Motor coordination was evaluated by the beam walking test. Individual rats were kept on a wooden beam suspended at an inclination of 60º from the platform. The performance of animals was recorded by using a 4 point scale. Animals that easily cross the beam were given score 0; Animals that had mild, moderate and severe impairment were given scores as 1, 2 and 3 respectively. Animals showed a complete inability to walk were given score 4. [17]

2.5.2 Locomotor activity

The locomotor action was performed by Actophotometer. Animals were placed on Actophotometer separately and allowed for habituation for three min, once habituation the locomotor action was analyzed for 5 min by using Actophotometer. Wandering movement was noted and indicated as complete photo beam counts / 5 min. [18]
2.5.3 Hanging wire test
After ischemia-reperfusion brain injury, grip strength and forelimb strength of the rats were studied. This methodology was done by extending a wire for a distance of 60 cm between two edges over a froth cushion, both disease control and drug treated animals were allowed to suspend by the forelimbs on a wire. The time was noted in sec until the animal fell. The cessation time was considered about 90 sec. [17]

2.5.4 Despair swim test
The rats were kept separately in water containing chamber so that the rats could not contact the base of the chamber with its tail or hind limbs or climb over the edge of the chamber. Two swim sessions were directed, an underlying 15 min pre-test followed by a 5 min test after 24 hours. The time of idleness (period of floating in the water without battling and making only those movements necessary to keep its head above the water level), during 5 min trial was noted. [19]

2.6 Brain Edema
After reperfusion for 22 hours, under xylazine (10 mg/kg I.M.) ketamine (100 mg/kg I.M.) the rats were decapitated and brains were taken out and estimate the water content. Brains were allowed to dry for one day by preserving at 120 ºC. [20]

The water content of brain was estimated using the equation:

\[
\text{Percentage of brain water content: } \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100
\]

2.7 Estimation of Biochemical Parameters
2.7.1 Preparation of brain homogenate:
After behavioral studies rats were sacrificed by cervical dislocation under ketamine and xylazine. The brains were taken out and homogenized in 50 Mm cold phosphate buffer containing 0.1 Mm EDTA and were centrifuged at 10,000 ×g for 15 min to yield 10% W/V brain tissue homogenate and the supernatant was separated and kept in a refrigerator at -80 ºC. Separated supernatant was used for biochemical estimations. [21]

2.7.2 Superoxide Dismutase (SOD)
SOD activity was determined by adding a mixture of 20 µl of 30 Mm epinephrine in 880 µl of 0.05 M carbonate buffer (having PH 10.2, 0.1 Mm EDTA) to 100 µl of brain tissue homogenate supernatant. Absorbance was measured at 480 nm for 4 min on UV Visible Spectrophotometer. SOD activity was calculated based on the quantity of enzyme that inhibits 50% oxidation of epinephrine which is equal to 1 unit. [22]

2.7.3 Catalase (CAT)
Catalase activity was assayed by adding 0.1 ml of brain tissue homogenate supernatant to 10 µl of ethanol and cooled. To 50 µl of the above mixture, 10 µl of Triton X 100 200 µl of phosphate buffer and 250 µl of 0.066 M H2O2 was added. Absorbance was measured at 240 nm for 60 sec on UV–Visible Spectrophotometer. The molar extinction coefficient of 43.6 M/cm was used to express catalase activity which is equal to the moles of H2O2 degraded/mg protein/min. [23]

2.7.4 Reduced Glutathione (GSH)
Glutathione was determined by mixing 0.75 ml of brain tissue homogenate supernatant with 0.75 ml of 4% sulpho salicylic acid then centrifuged at1200 rpm for 5 min at 4 °C. Add 4.5 ml of 0.01 M DTNB to 0.5 ml of above supernatant, then absorbance was measured at 412 nm by UV–Visible Spectrophotometer. [24]

2.7.5 Lipid peroxidation estimation (LPO)
MDA levels were measured by adding 50 µl of 8.1% sodium dodecyl sulphate, 375 µl of thiobarbituric acid (0.6%) to 0.2 ml of supernatant and shaken vigorously. The mixture was incubated for 10 min and then heated on a bubbling water bath for 60 min and samples were allowed to cool. Add 1.25 ml of butanol: pyridine (1.5:1) to samples, agitated vigorously and centrifuged at 1,000 rpm for 5 min, separate the colored layer and absorbance was measured at 532 nm against reference blank and also the values were expressed in nM of MDA formed form g protein/min. [25]

2.7.6 Assessment of Calcium levels
Total levels of calcium were estimated by commercially available kits according to instructions of the manufacturer (Erba diagnostic Ltd., India).

2.7.7 Assessment of Glutamate levels
In brief, 2 ml of perchloric acid was added to 1 ml supernatant of brain tissue homogenate and pH
was adjusted to 9 by using phosphate buffer. The resulting mixture was allowed for centrifugation at 1500×g for 15 min and left undisturbed for 10 min, then filtered. Absorbance was recorded at 340 nm. The levels of glutamate are expressed as µmol/g tissue. [26]

2.7.8 Estimation of Sodium potassium ATPase (Na+K+ATPase)

Na+K+ATPase assay was performed by dividing the brain homogenate supernatant into two parts. The first part considered as a test mixture and the second part served as a standard mixture. First part contained 0.1 ml of brain homogenate supernatant, 0.2 M Tris HCl buffer (PH 7.4), 0.1 M MgCl2, 1M NaCl, 0.2 M KCl, and made up to 2ml volume. Second part consisted of 0.1 ml of brain homogenate supernatant, 0.1 M MgCl2, 1.0M NaCl, 0.2 M Tris HCl buffer (PH 7.4), 10 Mm Ouabain and made up to 2 ml. 0.2 M 25Mm ATP was added to both test mixture and standard mixture and allowed to incubate at 37ºC for 15 min. After 15 min, the reaction was stopped by adding 1 ml of chilled 10% Trichloroacetic acid and was centrifuged 1000×g for 15 min. [27] Inorganic phosphate (pi) was estimated by taking 0.5 ml of the above supernatant. [28]

2.8 Infarct area measurement

Brain infarct area measurement was done by TTC staining method. After behavioral tests, rats of sham-operated, disease control and pretreated groups were decapitated and brains were removed, frozen at -4 ⁰C for 15 min. Frozen brains were sliced with 2mm thickness and kept in 2% 2,3,5 triphenyl tetrazolium chloride solution at 37 ⁰C for 15 min. The slices were then digitally photographed. As infarcted cells deficient to nicotinamide adenine dinucleotide (NAD), they remained unstained. Unstained pale necrotic infarcted part was removed, weighed and determine percentage infarction. [29]

2.9 Histopathological Examination

Histopathology was done to observe changes in brain regions. Rats were sacrificed and the brains were taken out and kept in 10% formalin for overnight. The coronal brain sections were prepared and stained by hematoxylin and eosin dye. The prepared stained slices were observed under the microscope.

2.10 Statistical Analysis of Data

Results were expressed as Mean± Standard error of the Mean. All parameters were assessed by using One way ANOVA followed by the post hoc test of Dunnett’s test. Statistical analysis was done by Prism software (version 5.03). Value of P<0.05 was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Effect of thymol on neurobehavioral outcome

The animals were scored for deficit in motor coordination after 22 hrs of reperfusion using 4 point scale. As shown in Table 1, the BCCAO group showed a significant (P<0.05) increase in neurological score as compared to normal control group. Thymol (50 and 100 mg/kg, p.o) pretreated groups showed significant (P<0.001) improvement in neurologic score as compared to the ischemic reperfusion group. Motor coordination done by locomotor activity and hanging wire test were significantly modified with thymol pretreatment as compared to ischemic-reperfusion injury group.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Beam walking test</th>
<th>Locomotor activity Counts / 5min</th>
<th>Hanging wire test time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0.00</td>
<td>205.2±9.32</td>
<td>85.62±3.08</td>
</tr>
<tr>
<td>Sham control</td>
<td>0.00</td>
<td>203.2±8.49</td>
<td>84.62±0.64</td>
</tr>
<tr>
<td>Ischemic reperfusion</td>
<td>3.68± 0.18*</td>
<td>95.8±7.137*</td>
<td>21.35±0.37*</td>
</tr>
<tr>
<td>Thymol (50mg/Kg, p.o)</td>
<td>2.41±0.75***</td>
<td>154.4±2.83***</td>
<td>72.68±0.43***</td>
</tr>
<tr>
<td>Thymol (100mg/Kg, p.o)</td>
<td>1.36± 0.48***</td>
<td>188.2± 3.62***</td>
<td>59.43± 0.51***</td>
</tr>
</tbody>
</table>

*P< 0.05 significant from normal control, +++P< 0.001 significant from ischemic-reperfusion group; Mean±SEM = mean values ± standard error of means of six experiments
Table 2. Effect of thymol on despair swim test after ischemic-reperfusion injury in rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Immobility (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>40.45±7.34</td>
</tr>
<tr>
<td>Sham control</td>
<td>39.32± 9.85</td>
</tr>
<tr>
<td>Ischemic reperfusion</td>
<td>91.66± 8.36***</td>
</tr>
<tr>
<td>Thymol (50 mg/Kg, p.o)</td>
<td>67.67±10.36+++</td>
</tr>
<tr>
<td>Thymol (100 mg/Kg, p.o)</td>
<td>49.92± 9.24+++</td>
</tr>
</tbody>
</table>

***P< 0.001 significant from normal control, +++P< 0.001 significant from Ischemic-reperfusion group.

Mean ± S.E.M = Mean values ± Standard error of means of six experiments

Despair swim test reflects a measure of behavioral evaluation. After 1 hr occlusion followed by 22 hrs reperfusion, the ischemic-reperfusion group showed significant (P<0.001) immobility when compared with normal control. Pretreatment with thymol significantly (P<0.001) reversed the increased time as compared to ischemic reperfusion group. Pretreatment with thymol (50 and 100 mg/kg p.o) markedly improved mobility activity as compared to ischemic reperfusion group as shown in Table 2.

3.2 Effect of Thymol on Brain Edema

As shown in fig 1, after 22 hours period of reperfusion brain water content was assessed and ischemic reperfusion group showed significant (P<0.001) increase in water content which indicates edema formation when compared with normal and sham-operated. The edematous condition was significantly (P<0.001) attenuated by pretreatment with thymol.

3.3 Effect of Thymol on Antioxidant and Pro-oxidant Levels

As shown in table 3, the BCCAO ischemic-reperfusion group markedly decreased antioxidant levels (SOD, CAT and GSH) and potentiated the levels of MDA in the injured brain tissue of rats as compared with normal control. However SOD, CAT and GSH levels in pretreated groups with thymol (50 and 100 mg/kg, p.o) were notably increased compared to ischemic-reperfusion group. In contrast MDA levels were decreased significantly (P<0.001) in thymol pretreated groups compared to ischemic reperfusion group.

3.4 Effect of Thymol on Excitotoxicity Mediators and Na+K+ ATPase

Table 4 shows, Significant (P<0.001) elevated levels of calcium and glutamate were observed in ischemic-reperfusion group as compared to normal control. Pretreated groups of thymol showed significant (P<0.001) attenuation of calcium and glutamate levels when compared to ischemic-reperfusion group. Substantial reduction in sodium-potassium ATPase activity was observed in ischemic-reperfusion group as compared to sham-operated. Pretreatment with thymol (50 and 100 mg/kg, p.o) markedly ameliorated (P<0.001) in sodium-potassium ATPase activity as compared to ischemic reperfusion group.

![Fig. 1. Effect of thymol on brain water content after ischemic reperfusion injury](image)

***P< 0.001 significant from normal control, +++P< 0.001 significant from ischemic reperfusion group.

Mean±SEM = mean values ± standard error of means of six experiments
Table 3. Antioxidant and pro-oxidant levels in brain tissue homogenate in all groups

<table>
<thead>
<tr>
<th>S.no</th>
<th>Group</th>
<th>SOD (U/mg protein)</th>
<th>CATALASE (µ M/min/mg)</th>
<th>GSH (µm of GSH/mg protein)</th>
<th>MDA (n moles/mg/protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal control</td>
<td>43.36±5.170</td>
<td>34.35±4.595</td>
<td>19.18±2.174</td>
<td>11.38±2.095</td>
</tr>
<tr>
<td>2</td>
<td>Sham control</td>
<td>40.63±4.165</td>
<td>32.83±4.212</td>
<td>18.81±2.696</td>
<td>10.65±1.691</td>
</tr>
<tr>
<td>3</td>
<td>Ischemic reperfusion</td>
<td>15.90±3.141***</td>
<td>9.22±1.987***</td>
<td>5.368±1.824 ***</td>
<td>32.00±4.685***</td>
</tr>
<tr>
<td>4</td>
<td>Thymol (50 mg/Kg p.o)</td>
<td>27.76±3.26+++</td>
<td>20.85±2.380++</td>
<td>10.29±1.775++</td>
<td>22.98±4.080++</td>
</tr>
<tr>
<td>5</td>
<td>Thymol (100 mg/Kg p.o)</td>
<td>34.93±4.033+++</td>
<td>27.02±3.602+++</td>
<td>16.83±2.431+++</td>
<td>14.8±2.206+++</td>
</tr>
</tbody>
</table>

***P< 0.001 significant from normal control, ++P<0.001, +++P<0.001 significant from ischemic reperfusion group; Mean±SEM = mean values ± standard error of means of six experiments

Table 4. Effect of thymol on Excitotoxicity mediators and Na⁺/K⁺ATPase after ischemic reperfusion injury in rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Calcium (µg/mg protein)</th>
<th>Glutamate (µg/mg protein)</th>
<th>Na⁺/K⁺ATPase (µM of pi liberated/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>9.92±1.89</td>
<td>8.93±1.53</td>
<td>7.52±0.853</td>
</tr>
<tr>
<td>Sham control</td>
<td>9.1±0.184</td>
<td>7.64±0.117</td>
<td>7.68±0.428</td>
</tr>
<tr>
<td>Ischemic reperfusion</td>
<td>35.8±0.954**</td>
<td>24.7±0.215**</td>
<td>2.15±0.262**</td>
</tr>
<tr>
<td>Thymol (50 mg/Kg, p.o)</td>
<td>29.2±0.307***</td>
<td>18.7±0.213***</td>
<td>3.71±0.352***</td>
</tr>
<tr>
<td>Thymol (100 mg/Kg, p.o)</td>
<td>16.7±0.282**++</td>
<td>11.2±0.234++</td>
<td>5.95±0.271++</td>
</tr>
</tbody>
</table>

**P< 0.01 significant from normal control, +++P< 0.001 significant from Ischemic-reperfusion group. Mean±SEM = mean values ± standard error of means of six experiments

3.5 Effect of Thymol on Infarct Size

As shown in fig 2 and 3, the infarction area was significantly (P<0.001) increased in ischemic reperfusion group when compared to normal control and significantly decreased (P<0.001) in thymol pretreated groups when compared to ischemic reperfusion group. Infarct size was assessed by Image J software analysis.

Divide the infarct area by brain area to obtain percentage infarction area. [30]

“Protection of cerebral cells infarction after ischemia reperfusion injury in rats is calculated by:

\% of Protection in cerebral infarction = (Control – Sample) / Control x100”.

Fig. 2. Effect of thymol on percentage infarction area after ischemic reperfusion injury in rats.

***P< 0.001 significant from normal control, +++P< 0.001 significant from ischemic reperfusion group.

Mean±SEM = mean values ± standard error of means of six experiments
In recent years much attention has been focused on traditional medicine. Numerous studies indicated that the plant parts like seeds, leaves, stem, flowers and phytochemicals that are of natural origin promotes health benefits against various diseases in humans especially CNS disorders including brain stroke. The phytochemicals like naringin, Shikonin, curcumin, rutin, ginseng tea, limonene, punicalagin [31-36] showed neuroprotection due to their antioxidant mechanisms.

Photographs of brain sections were stained with Haemotoxylin and Eosin dye. In histopathological studies ischemic-reperfusion group showed notable neutrophilic infiltration, increased intracellular space, numerous vacuoles were observed and density of cells was decreased followed by edema when compared with normal control. A significant decrease neutrophilic infiltration, intracellular space, density of cells, edema and recovered architecture was observed in pretreated thymol groups (50 mg/kg, p.o and 100 mg/kg, p.o) when compared with ischemic reperfusion group as shown in Fig 4.

In recent years much attention has been focused on traditional medicine. Numerous studies indicated that the plant parts like seeds, leaves, stem, flowers and phytochemicals that are of natural origin promotes health benefits against various diseases in humans especially CNS disorders including brain stroke. The phytochemicals like naringin, Shikonin, curcumin, rutin, ginseng tea, limonene, punicalagin [31-36] showed neuroprotection due to their antioxidant mechanisms.

Cerebral ischemia is a multi pathophysiological pathway inducing oxidative stress excitotoxicity, ionic imbalance, acidotoxicity, inflammation, peri-infarct depolarization and apoptosis. The ultimate reason of brain ischemia research is the development of new therapeutic strategies by modulating any of the pathways for the treatment of cerebrovascular disease. [37] In the existing investigation, assessed role of thymol in global cerebral ischemia induced by BCCAO in rats for 1 hour followed by 22 hours reperfusion. In this model, due to reperfusion multiple brain regions were affected including cortex, hippocampus and subcortical regions.

The results of the current study revealed that thymol showed neuroprotection against cerebral ischemia. In this study pretreated thymol group showed marked improvement in locomotor activity, grip strength and mobility when compared to ischemic-reperfusion group. Based on above studies pretreatment with thymol may have restorative potential against ischemic-reperfusion injury by enhancing sensorymotor activities. The edema of the brain was significantly decreased in prophylactic treatment groups in comparison with ischemic reperfusion group.

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**3.6 Histopathology**

Photographs of brain sections were stained with 2,3,5 triphenyl tetrazolium chloride (TTC) and represented the TTC stained coronal sections of brain of normal, sham control, ischaemic group and pretreated thymol groups 50 mg/kg p.o. and 100 mg/kg p.o. respectively.

Cerebral ischemia is a multi pathophysiological pathway inducing oxidative stress excitotoxicity, ionic imbalance, acidotoxicity, inflammation, peri-infarct depolarization and apoptosis. The ultimate reason of brain ischemia research is the development of new therapeutic strategies by modulating any of the pathways for the treatment of cerebrovascular disease. [37] In the existing investigation, assessed role of thymol in global cerebral ischemia induced by BCCAO in rats for 1 hour followed by 22 hours reperfusion. In this model, due to reperfusion multiple brain regions were affected including cortex, hippocampus and subcortical regions.

The results of the current study revealed that thymol showed neuroprotection against cerebral ischemia. In this study pretreated thymol group showed marked improvement in locomotor activity, grip strength and mobility when compared to ischemic-reperfusion group. Based on above studies pretreatment with thymol may have restorative potential against ischemic-reperfusion injury by enhancing sensorymotor activities. The edema of the brain was significantly decreased in prophylactic treatment groups in comparison with ischemic reperfusion group.
Several reports suggested that ischaemic reperfusion injury was associated with oxidative stress which leads to the formation of excessive reactive oxygen free radicals. The ROS further increases the brain damage.[38] In the current examination pretreatment with thymol (50 mg/kg, p.o and 100 mg/kg, p.o) revealed reduction in LPO levels and ameliorated SOD, CAT and GSH when compared to ischemic-reperfusion group. Pretreatment with thymol (50 and 100 mg/kg) altogether significantly improved the levels of antioxidants and Na⁺/K⁺ ATPase levels and thus keeping up balance between free radicals and antioxidant safeguard system.

Brain damage is also indicated by changes in histopathology of brain tissue and infarct area. All these changes were diminished in pretreated thymol groups (50 mg/kg, p.o and 100 mg/kg, p.o) when compared with ischemic-reperfusion group. These findings clearly indicate the protective role of thymol against cerebral ischemia.

4. CONCLUSION

Based on the above findings, thymol may have a shielding effect against global cerebral ischemia, evidenced by enhancement in motor coordination, attenuating the brain water content, antioxidant, and pro-oxidant levels and infarct area. Further investigation is required to identify the explicit molecular mechanism for its clinical use.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

Healthy Male Wistar strain rats of about 200–250 gm were used for the study. They were purchased from Biogen laboratory animal facility, Bangalore. The experimental protocol was approved by the Institutional Animal Ethical Committee of Sri Padmavathi School of Pharmacy, Tiruchanoor, Tirupati (No.1016/PO/Re/S/06/CPCSEA/2019/010).
All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS
Authors have declared that no competing interests exist.

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