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Original Article

Thymol Improves Brain Functional Outcomes after Cerebral Ischemia-Reperfusion Injury by Regulating p38 MAPK Associated Apoptosis Signalling Pathway

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ABSTRACT

Background: Thymol, a monoterpene phenolic compound and an essential oil extracted from *Tachyspermum ammi* (ajwain) has been shown to possess multiple therapeutic potentials as anti-inflammatory, antioxidant, antihyperlipidemic, antiapoptotic, antitumor agent against neurological, cardiovascular, gastrointestinal and malignant diseases. **Objectives:** The current investigation aimed to determine whether thymol could protect rats from cerebral ischemia reperfusion injury by preventing Bilateral Common Carotid Artery Occlusion (BCCAO). **Materials and Methods:** After 14 days of pretreatment with thymol at the doses of 50 mg/kg, *p.o* and 100 mg/kg, *p.o.* respectively, Albino Wistar rats (200-250 g) were subjected to BCCAO for 1 hr followed by 22 hr reperfusion (I/R). The levels of protein carbonyl content, Myeloperoxidase (MPO) were measured in brain tissue homogenate. Utilising western blotting, the expression levels of TNF-α, Interleukin 10 (IL-10) anti-inflammatory cytokines, pro-apoptotic mediators Bax and Caspase 3 and anti-apoptotic mediator Bcl-2 were assessed. **Results and Discussion:** Reduction in levels of protein carbonyl content and Myeloperoxidase (MPO) was observed in thymol groups. Thymol pretreated rats showed significant (p<0.01) downregulation in the expressions of TNF-α and inhibited phosphorylation of p38 MAPK. In parallel, the expressions of IL-10 were upregulated significantly (p<0.01). Thymol exerted anti-apoptotic effect as reflected by decreased expression of the key downstream executioner caspase-3 and Bax, which is probably mediated by significant upregulation of expression of Bcl-2. **Conclusion:** Thymol is endowed with neuroprotection against global cerebral ischemia reperfusion injury which may be probably mediated by attenuation of inflammatory and apoptotic mechanism via p38 MAPK signaling pathway.

Keywords: Anti-inflammatory activity, p38 MAPK, Anti-apoptotic activity, Ischemic reperfusion injury, Thymol.

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INTRODUCTION

Cerebral Ischemia Reperfusion Injury (CIRI) is documented as leading cause of mortality and morbidity worldwide.[1-4] It is a syndrome of neurological deficit developed after acute circulatory failure to blood vessels in the brain, known as either ischemic or hemorrhagic stroke.[5] Early reperfusion with thrombolytics such as recombinant tissue plasminogen activator is the best therapeutic intervention for stroke. Reperfusion aggravates the condition through oxidative stress, inflammation and apoptosis in the neurons. Thus, challenging the researchers for the development of safe and effective therapeutic intervention targeting prognostic mechanisms.[6-9]

In the molecular mechanisms underpinning the pathology of apoptosis, oxidative stress is caused by the dysregulation of mitochondrial oxidative phosphorylation, which decreases ATP production and raises the production of ROS, or reactive oxygen species. ROS have the potential to cause immediate damage to cell components.[10-12] Apoptosis is regulated by both internal and external factors including several genes, enzymes and signal transduction pathways.[13,14] Among these factors, Mitogen-Activated Protein Kinase (MAPK) has been implicated in the molecular mechanisms of ischemia-reperfusion injury. Numerous physiological cellular activities, including differentiation, adaptation, proliferation, survival, and apoptosis,

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are mediated by the MAPK cascade. Signals are transferred from the cellular surface to the nucleus by it.[15,16] p38 MAPK is a crucial member of the MAPK family that can be phosphorylated in response to various extracellular stimuli, which in turn activates the MAPK signaling pathway.[17] p38 MAPK has been linked to cellular differentiation, cell cycle progression, and apoptosis. Additionally, it has been linked to the regulation of inflammatory responses, which is associated with the synthesis of cytokines that promote inflammation such as TNF-α and interleukin 1β.[18] During CIRI, p38 MAPK signalling has been reported to be activated since it has been found to navigate from the cytoplasm into the nucleus, initiate further downstream kinases and transcriptional proteins, and change the level of gene expression associated with apoptosis, so triggering cellular apoptosis.[9,19,20]

Herbal medicine rich in essential oils have been widely used for the treatment of cardio-cerebro vascular disorders including cerebral ischemia in many countries.[21] One such essential oil is Thymol, also known chemically as 2 isopropyl-5-methylphenol, is a compound found in *Trachyspermum ammi* that has a strong flavour and a pleasant, aromatic smell. Pharmacological characteristics such as anticancer,[22] anticonvulsant,[23] antifungal,[24] antioxidant, antihyperlipidemic[25] antispasmodic, anti-inflammatory,[26] antiseptic, antiepileptogenic, and radioprotective effects[27] have been observed. Here we intended to determine the potential of thymol as a therapy against apoptosis and inflammation during cerebral ischemia reperfusion injury.

MATERIALS AND METHODS

Animals

Male Wistar albino rats weighing 200-250 g were kept in established laboratory settings with a 12 hr light/dark cycle and unrestricted access to food and drink and *ad libitum*. The research facility environment was habituated to the animals seven days in advance of the trial. The CPCSEA's recommendations were followed for handling and maintenance. The Institutional Animal Ethics Committee of Sri Padmavathi school of Pharmacy, Tirupati, Andhra Pradesh gave its consent for the experimentation ("Institutional Animal Ethics Committee, approval Number: 1016/PO/Re/S/06/CPCSEA/2019/010").

Chemicals

Thymol was procured from Sigma Aldrich. 2,3,5 Triphenyltetrazolium chloride (CAS no:298-96-4) was purchased from Hi media. Antibodies of Bcl-2 (cat. no. ab19645), p38 MAPK, Interleukin-10 (cat. no. ab133575), Tumor necrosis factor alpha (TNF α) (cat. no. ab1793), Bax (cat. no. ab53154), Caspase 3 (cat. no. ab4051), Horseradish Peroxidase (HRP) were purchased from Abcam Inc. All other chemicals were of analytical grade.

BCCAO

Cerebral ischemia reperfusion injury was carried out as per the methodology of Iwasaki *et al.*[28] The rats were given an Intramuscular (I.M.) ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthetic. The procedure involved cutting through the sternocleidomastoid and sternohyoid muscles to expose the carotid arteries, which were then sealed for an hour using sterile cotton thread. After an hour, the cotton thread was taken out and the blood was allowed for 22 hr reperfusion. Using a thermal statistically regulated infrared lamp, the temperature of the body was kept at around 37°C±0.5°C during the surgical procedure. The rats in the sham-operated group were not subjected to occlusion or reperfusion. The rats were assessed for motor coordination and their infarct size, p38 MAPK, TNF-α, IL-10, Caspase 3, Bcl-2, and Bax levels were measured after they were euthanized after 22 hr after reperfusion.

Experimentation

Albino rats were divided into 4 groups randomly of nine rats in each group. Group I: Sham-control (subjected to surgical procedure without BCCAO and received 2% tween 80 *p.o*), Group II: BCCAO control (subjected to BCCAO 1 hr accompanied by 22 hr reperfusion and received 2% tween 80 *p.o*) Group III: Thymol 50 mg/kg *p.o* (subjected to BCCAO 1 hr accompanied by 22 hr reperfusion) and Group IV: Thymol 100 mg/kg *p.o* (subjected to BCCAO 1 hr accompanied by 22 hr reperfusion).[29,30] Prior to BCCAO, the animals were treated for 14 days with each treatment.

Infarct volume

Rats were anesthetized, and their brains were removed and frozen for 15 min at -4°C. Frozen brains were cut into 2 mm thick slices and incubated for 15 min at 37°C in a 2% 2, 3, 5 triphenyl tetrazolium chloride solution. The brain slices were captured digitally, and total infarct volume was calculated using image J analysis software.[31]

Brain water content

Rats were sacrificed under overdose of ketamine (100 mg/kg) and xylazine (10 mg/kg) anaesthesia after reperfusion for 22 hr and the brains were removed and their wet weights were noted. After subjecting the brains at 120°C for 24 hr, dry weights of the brains were assessed. The brain water content was estimated using the equation.[32]

$$\text{Percentage of brain water content} = \frac{\text{Wet weight - Dry weight}}{\text{Wet weight}} \times 100$$

Biochemical Estimation

Brain homogenization

After 22 hr of reperfusion, the animals were sacrificed by under overdose of ketamine and xylazine. The brains were isolated and homogenized to produce a 10%w/v homogenate in 5 mm phosphate buffer with 0.1 mm EDTA. After centrifuging

this homogenate for 15 min at 10,000 rpm, the supernatant was collected, kept in a refrigerator at -5°C, and utilized for biochemical assessment.

Estimation of Myeloperoxidase (MPO) levels

Myeloperoxidase present in brains were measured by adding phosphate buffer having O-dianisideine dihydrochloride (0.167 mg/mL), hydrogen peroxide and 0.5% of hexadecyltrimethyl ammonium bromide to 0.1 mL of brain tissue supernatant. This mixture was agitated and the absorbance was measured at a wavelength of 460 nm at an interval of 1 min upto 3 min and represented as one unit. MPO levels were represented as Units/mL.[33]

Estimation of protein carbonyl content

Brain homogenate supernatant is mixed with 1% streptomycin sulfate solution, incubated for 15 min, then centrifuged at 3600 g for 10 min. After removing the brain supernatant, it was vortexed for 30 min at 10 min intervals while incubated with 10 mM DNPH (in 2 mol/L HCl). After adding an equivalent amount of 20% TCA to precipitate the protein, the mixture was centrifuged for 5 min at 8600 g. To get rid of the extra 2,4-Dinitrophenylhydrazine, three rounds of washing the residue with ethanol: ethyl acetate in a 1:1 ratio were conducted. A 6 mol/l guanidine solution was used to re-dissolve the precipitated protein. At 370 nm, the optical density was observed. Protein carbonyl content was measured in nmol/mg.[34]

Estimation of TNF-α, IL-10, p38 MAPK, Bcl-2, Bax, Caspase 3 by western blotting

The isolated brain tissues were cleaned and homogenized by using Radio Immuno Precipitation (RIPA) buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.5% Sodium deoxycholate, Sodium dodecyl Sulphate (0.1% SDS), 1mM Sodium orthovanadate, 1 mM Sodium flouride, 10 µL per mL Protease inhibitors]. This solution was heated for 5 min at 70°C and this was used for separation of proteins on 4% SDS-PAGE gel using electrophoresis. 15 µL of the samples and 6 µL of Tris HCl loading gel buffer were loaded in each well and allowed to run for one hr. The proteins were transferred on to the Polyvinylidene Flouride (PDVF) membrane and the quality of transfer was checked by using Ponceau stain. Blocking was performed in 3% skim milk in 20 mM Tris buffer, 150 mM saline with 0.1% Tween 20 (TBST) in 1XPBS for 1hr. It was washed 3 times using PBST solution for 5 min by maintaining constant temperature. Incubation was carried out with primary antibody (1:1000 of TNF α, p38 MAPK, IL10, Bax, caspase 3 and Bcl-2) in 1XPBS overnight at 4°C with continuous shaking. Membranes were further incubated with horseradish peroxidase-conjugated secondary antibody for about 2 hr and washed with 1XPBST thrice for 5 min. This membrane was visualized using chromomeric substrate- 3, 3'-Diaminobenzidine (DAB). Visible bands were photographed and quantitative densitometry analysis was done by image J analysis software. β-actin antibody was used as loading control.[35]

Statistical Analysis

Data were expressed as Mean±Standard Error of Mean (SEM). Analyzed by one-way Analysis of Variance (ANOVA) with *post hoc* test of Dunnet's analysis using GraphPad Prism version 5.0. A value of $p<0.05$ value was considered as statistically significant.

RESULTS

Brain infarction volume

The infarction volume was considerably ($p<0.001$) higher in the BCCAO-induced ischemia-reperfusion group compared to the sham control group, and significantly ($p<0.001$) lower in the thymol pretreatment groups compared to the ischemic reperfusion group, as illustrated in Figures 1a and 1b.

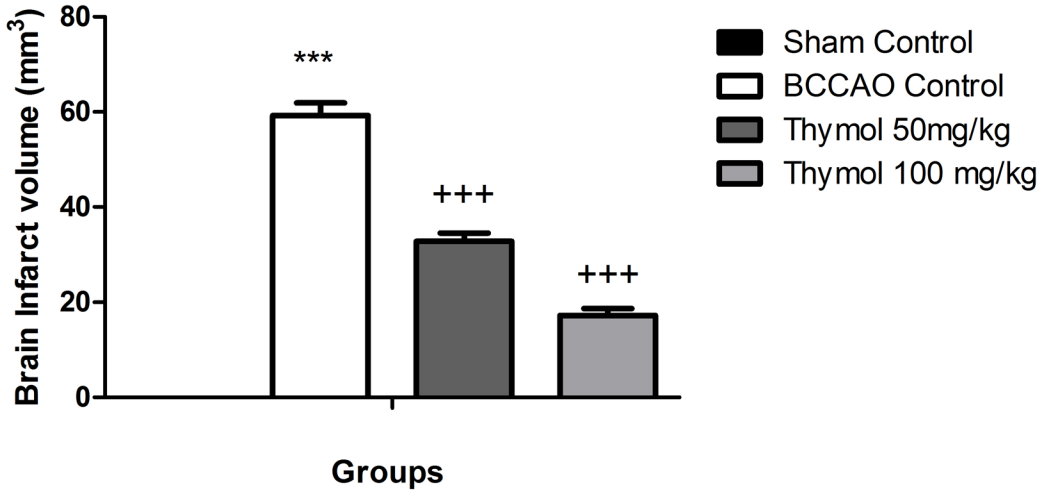

Figure 1a: Effect of thymol on brain infarct volume. Data were expressed as mean±SEM. ***($p<0.001$) BCCAO control as compared to sham control. +++ ($p<0.001$) Thymol 50 and 100 mg/kg as compared to BCCAO.

Brain water content

The brain water content was measured 22 hr after the reperfusion period, the BCCAO-induced ischemia reperfusion group showed a significant ($p<0.001$) increase in brain water content compared to the normal and sham-operated groups, when thymol was administered prior to BCCAO group treatment, the edema was significantly ($p<0.001$) reduced in a dose-dependent manner (Figure 2).

Effect of thymol on myeloperoxidase levels

BCCAO group showed significantly ($p<0.001$) increased levels of myeloperoxidase as compared to sham group. Whereas Thymol pretreated group 50 mg/kg ($p<0.01$) and 100 mg/kg ($p<0.001$) significantly prevented the increase in myeloperoxidase levels when compared to sham group (Figure 3).

Effect on thymol on protein carbonyl content

When ischemia reperfusion injury occurs, the amount of oxidative brain damage is measured in terms of protein carbonylation. When compared to the sham group, the BCCAO group's protein carbonyl concentration was significantly ($p<0.001$) higher. However, thymol pretreatment at 50 mg/kg and 100 mg/kg, respectively, significantly ($p<0.05$) avoided an increase in protein carbonyl content (Figure 4).

Effect of thymol on proinflammatory mediator TNF α levels

As shown in Figure 5 the expressions of proinflammatory mediator TNF-α in BCCAO control were up-regulated markedly ($p<0.001$) compared to sham control and was found as down-regulated markedly in thymol pretreated groups compared to BCCAO group.

Effect of thymol on proinflammatory mediator p38 MAPK

As shown in Figure 6, Phosphorylation of p38 MAPK was observed in disease control and was found as significantly increased expressions when compared to sham control and dephosphorylation of p38 MAPK was observed in thymol pretreated groups compared to BCCAO control.

Effect of thymol on anti-inflammatory mediator IL 10

IL-10 expressions were significantly decreased in BCCAO group as compared to sham control. Whereas pretreated with 100mg/kg

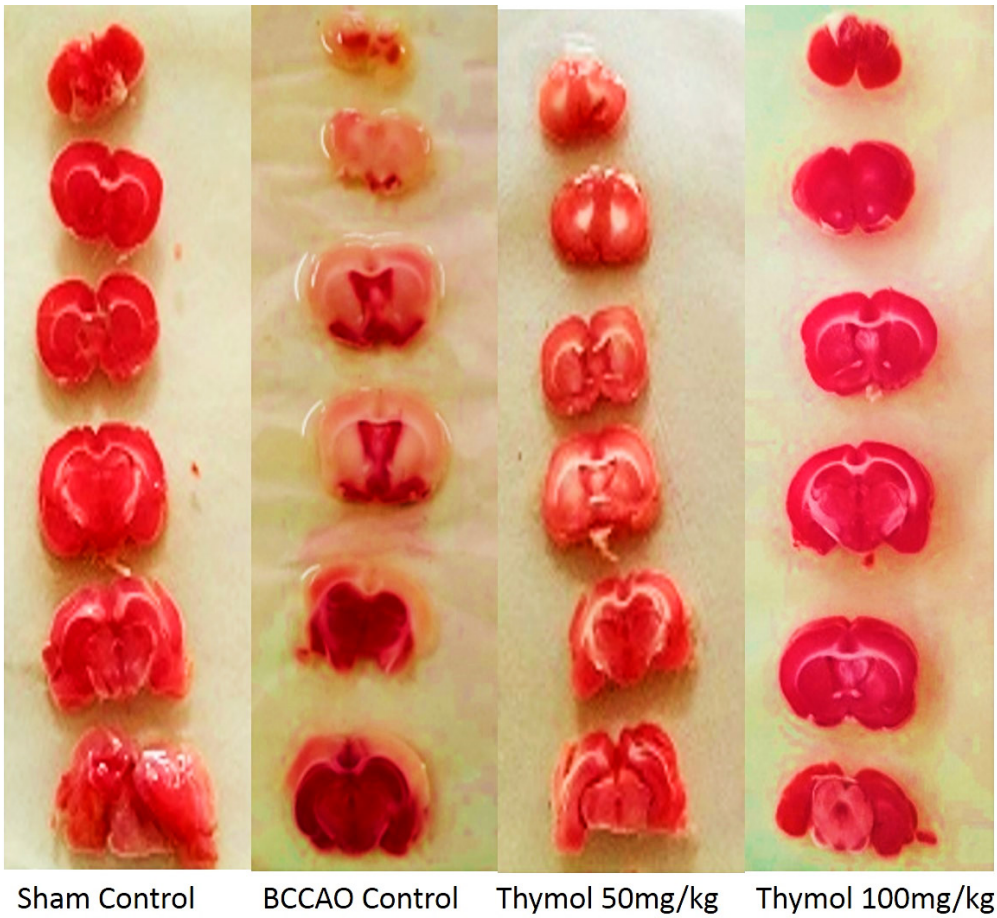

Figure 1b: Pictorial representation of effect of thymol on brain infarct volume. Brain sections of sham control, BCCAO control, Thymol 50 mg/kg and Thymol 100 mg/kg treatment groups.

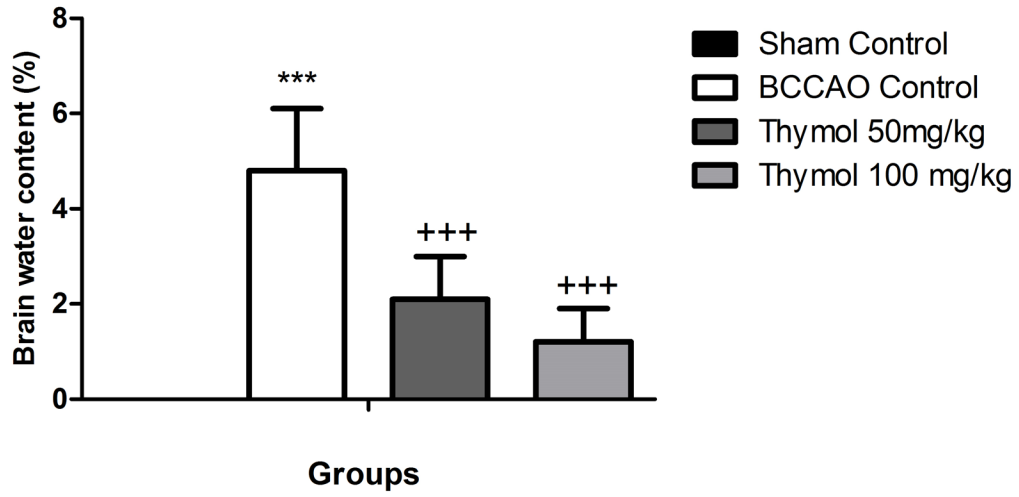

Figure 2: Effect of thymol on brain water content. Data was expressed as Mean±SEM. ***(p<0.001) BCCAO control as compared to sham control. $^{+++}$(p<0.001) thymol 50 and 100 mg/kg compared to BCCAO control.

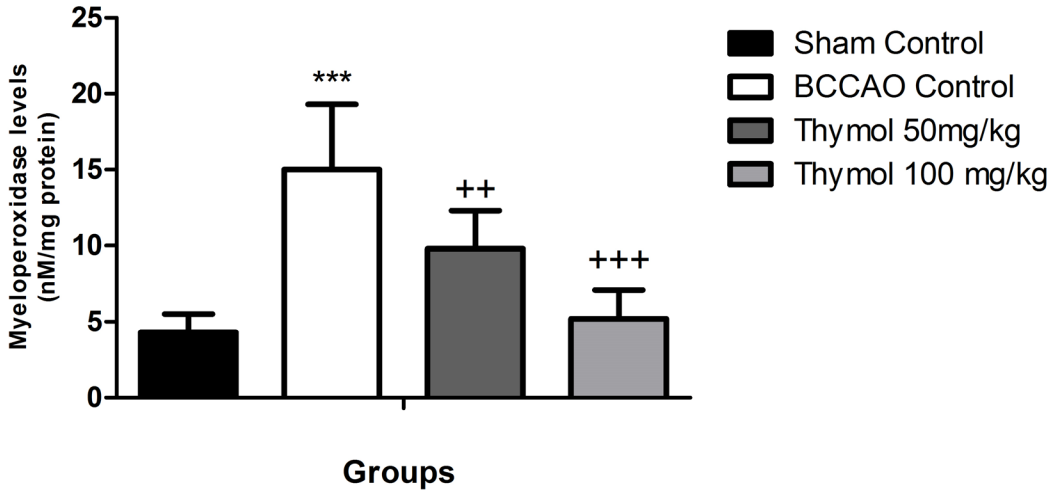

Figure 3: Effect of thymol on myeloperoxidase levels. Data was expressed as Mean ±SEM. ***(p<0.001) BCCAO control as compared to sham control. $^{++}$(p<0.01), $^{+++}$(p<0.001) thymol 50 and 100 mg/kg compared to BCCAO control.

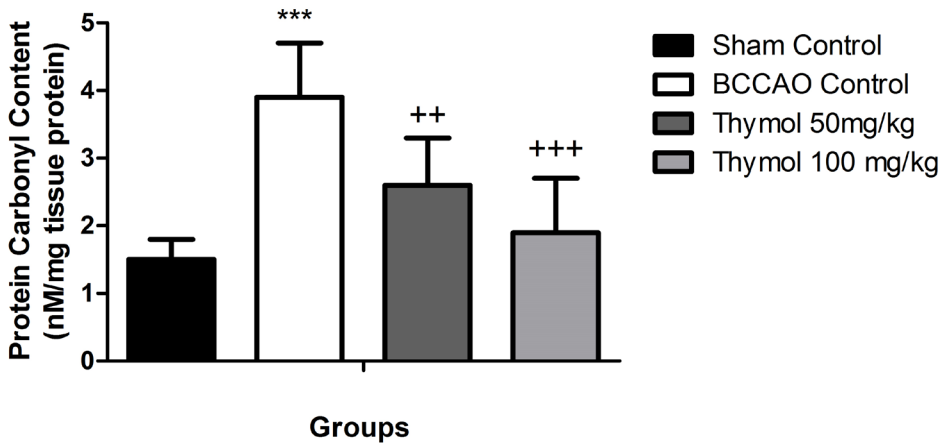

Figure 4: Effect of thymol on protein carbonyl content. Data was expressed as Mean±SEM. ***(p<0.001) BCCAO control as compared to sham control. $^{++}$(p<0.01) thymol 50mg/kg; $^{+++}$(p<0.001) thymol 100 mg/kg as compared to BCCAO control.

of thymol showed significant increase in IL-10 expression when compared with BCCAO group (Figure 7).

Effect of thymol on pro-apoptotic (Bax, Caspase-3) and antiapoptotic (Bcl-2) mediators

As shown in Figure 8, after reperfusion phase for 22 hr, BCCAO control group showed significant downregulation in BCl-2 expression when compared with sham group. Prophylactic treatment with thymol showed significantly prevented the downregulation of BCl-2 expression as compared to BCCAO control. Reversing it, appearance of caspase 3 and Bax were upregulated significantly in BCCAO control as compared to sham control which was considerably declined by the prophylactic treatment with thymol 50 mg/kg and 100 mg/kg respectively.

DISCUSSION

Reperfusion injury after cerebral ischemia is the contributing factor for mortality and morbidity in most of the stroke victims. Underlying pathological mechanisms are associated with events like excitotoxicity, oxidative stress, apoptosis and inflammation as evident from most of the literature. These events limiting the therapeutic application of most of the interventions and remain challenging for development of novel, safe and effective drugs for stroke treatment.[4,9,13]

All across the world, Essential Oils (EO) have been utilized to treat a variety of illnesses in traditional, national, and folk medicine (Bastos *et al.*, 2010). In several different countries, herbal remedies high in EO have been utilized extensively to treat CVDs. According to recent research, EO is significant prospective treatments for CVDs that may also improve their prognosis. The pathogenic components of CVDs are numerous and intricate. Patients must take a range of combination medications, which leads to low medication compliance, and the molecular mechanisms of existing drugs are more focused on treating a single target than multi-target harmony. EO possesses the ability to control many targets in a systematic manner and have multiple components and targets.[36] One such essential oil is thymol, which comes from the plant *Trachyspermum ammi*. It has been shown to possess pharmacological attributes such as antioxidant, antihyperlipidemic, anticonvulsant, antifungal, antispasmodic, anti-inflammatory, antiseptic, antiepileptogenic, radioprotective, and anticancer effects. We revealed in the present investigation

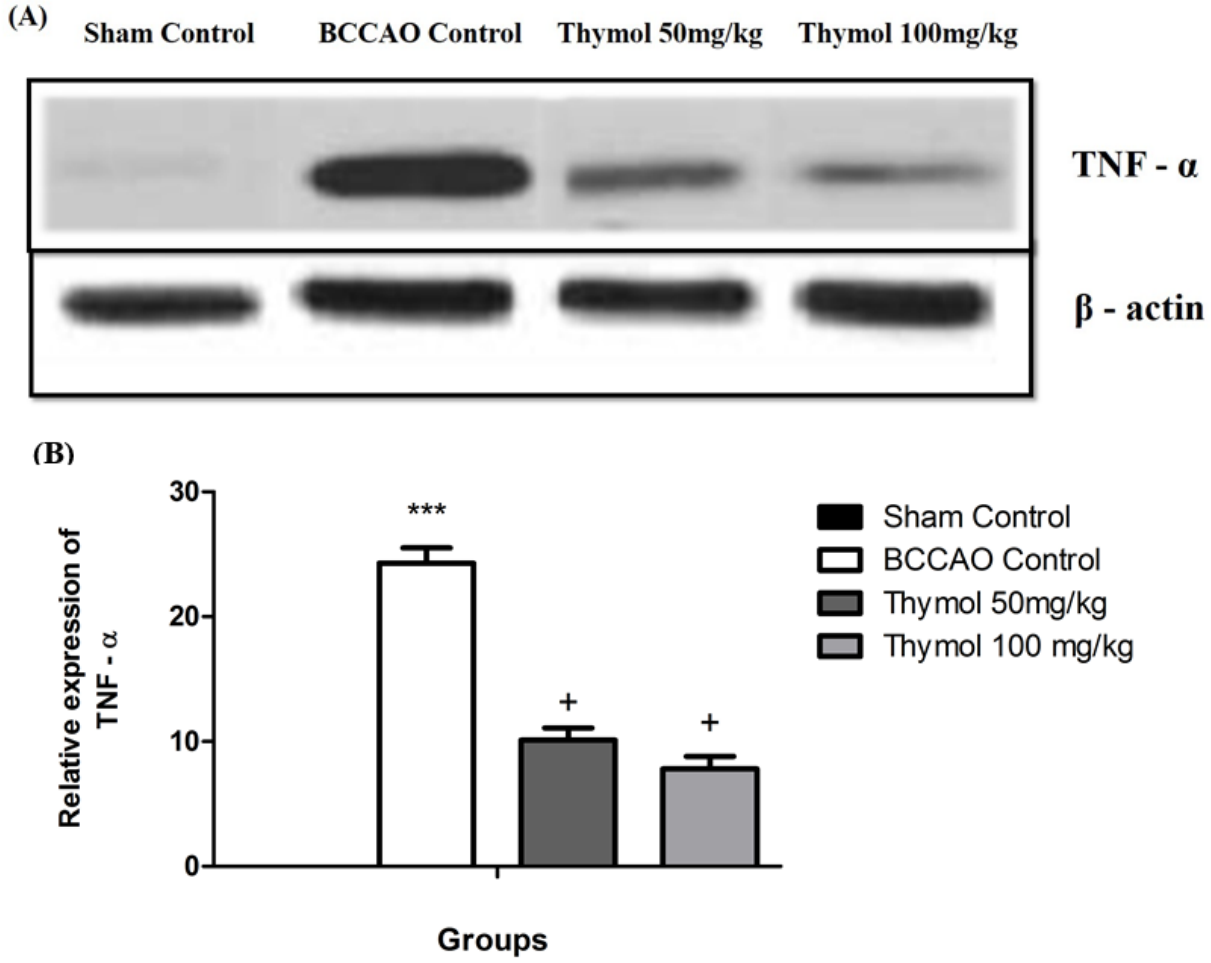

Figure 5: Effect of Thymol on TNF α levels. Data were expressed as Mean±SEM. ***(p<0.001) BCCAO control as compared to sham control. + (p<0.05) Thymol 50 and 100 mg/kg as compared to BCCAO.

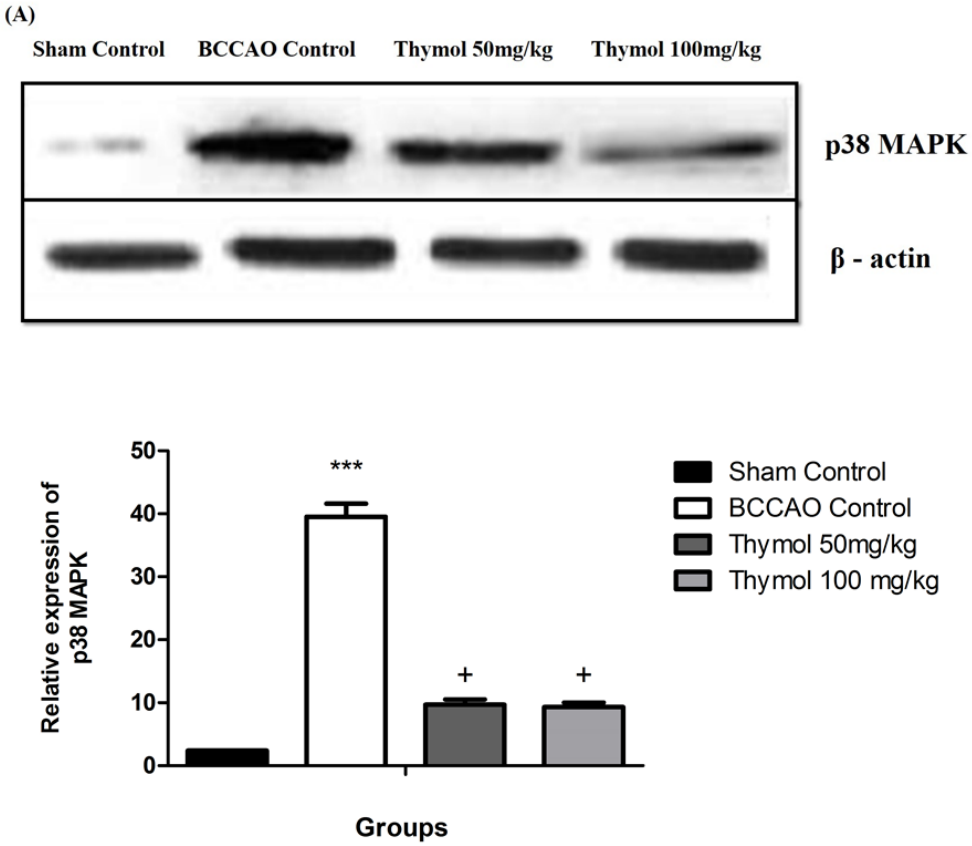

Figure 6: Effect of Thymol on p38 MAPK expressions. Data were expressed as Mean±SEM. ***(p<0.001) BCCAO control as compared to sham control. $^{+}$ (p<0.05) Thymol 50 and 100 mg/kg as compared to BCCAO.

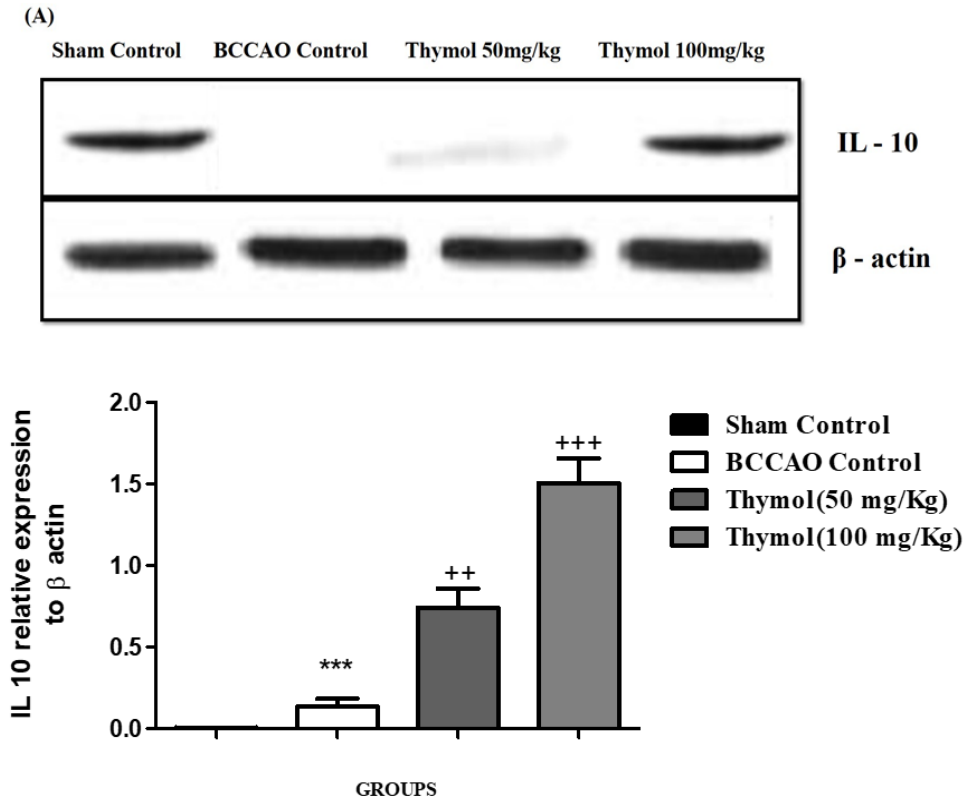

Figure 7: Effect of Thymol on IL-10 levels. Data were expressed as Mean±SEM. ***(p<0.001) BCCAO control as compared to sham control. $^{+++}$ (p<0.001), $^{++}$ (p<0.01) Thymol 50 and 100 mg/kg compared to BCCAO.

that Thymol protects against apoptosis and inflammation in a BCCAO model of cerebral ischemia reperfusion damage.

An increasing number of research indicates that after cerebral I/R, the inflammatory response causes subsequent brain injury.[37-39] The p38 MAPK signaling pathway is the primary signaling mechanism that controls neuroinflammation, and the MAPK signaling pathway is also implicated in the inflammatory processes of cerebral I/R injury.[40-42] Pro-inflammatory factors like TNF-α, myeloperoxidase, protein carbonyl compounds, IL-1β, and IL-6 are produced excessively by activated MAPK through phosphorylation of p38 and immune effector cells from the cerebral cortex, and the release of the anti-inflammatory factor IL-10 is inhibited.[43] These will intensify the inflammatory reaction and cause more brain injury.[44] Thus, one efficient way to mitigate cerebral I/R impact is to decrease the synthesis of pro-inflammatory factors and increase the release of anti-inflammatory factors. Additionally, it is thought that one of the primary goals for fostering the development of novel stroke therapies is to focus on inflammation.[45] Existing evidences of research have reported that dephosphorylation of p38 significantly reduced the activation of inflammatory response and can protect the neuronal cell from inflammatory damage.[38,43,46,47] In this study, we found that Thymol treatment significantly decreased expression levels of TNF-α and increased IL-10 expressions induced by BCCAO/R. We also found significantly decreased levels of myeloperoxidase and protein carbonyl content in thymol pretreated rats. This could be because of dephosphorylation of p38 MAPK signalling pathway and which might have prevented the upregulation of TNF-α and downregulation of IL-10. These results show that thymol has neuroprotective effects against I/R injury by preventing proinflammatory cytokines through regulation of MAPK signal transduction pathway.

Neurotoxicity caused by the aberrant release of excitatory amino acids, abnormalities in neuronal metabolism, enhancement in generation of oxygen free radicals and ROS-mediated impairment, intracellular Ca^{2+} overload, damage mediated by cytokines, and brain damage from cerebral ischemia reperfusion injury have all been linked to these cellular processes.[48-50] In the rat hippocampus CA1 region after cerebral ischemia, it has been observed that inhibiting p38/MAPK signalling reduces neuronal apoptosis, suggesting the role of p38 MAPK stimulation in neuronal apoptosis. Once phosphorylated, p38 MAPK can activate several transcription factors and downstream kinases. It can also control the expression of target genes and play crucial roles in neuronal apoptosis during ischemia-reperfusion damage.[45,47,51] The current findings showed that thymol effectively hindered the activation of bax and caspase-3, perhaps through blocking the activation of p38 MAPK.

The biochemical cascade during damage after cerebral ischemia reperfusion are evidenced by brain infarction and edematous tissue, which was assessed histologically by TTC staining and brain water content. In our study, brain water content and infarction were found more significant in BCCAO induced cerebral injured brains and thymol pretreated animals were found with reduced infarction and brain water content which may support the biochemical evidences.

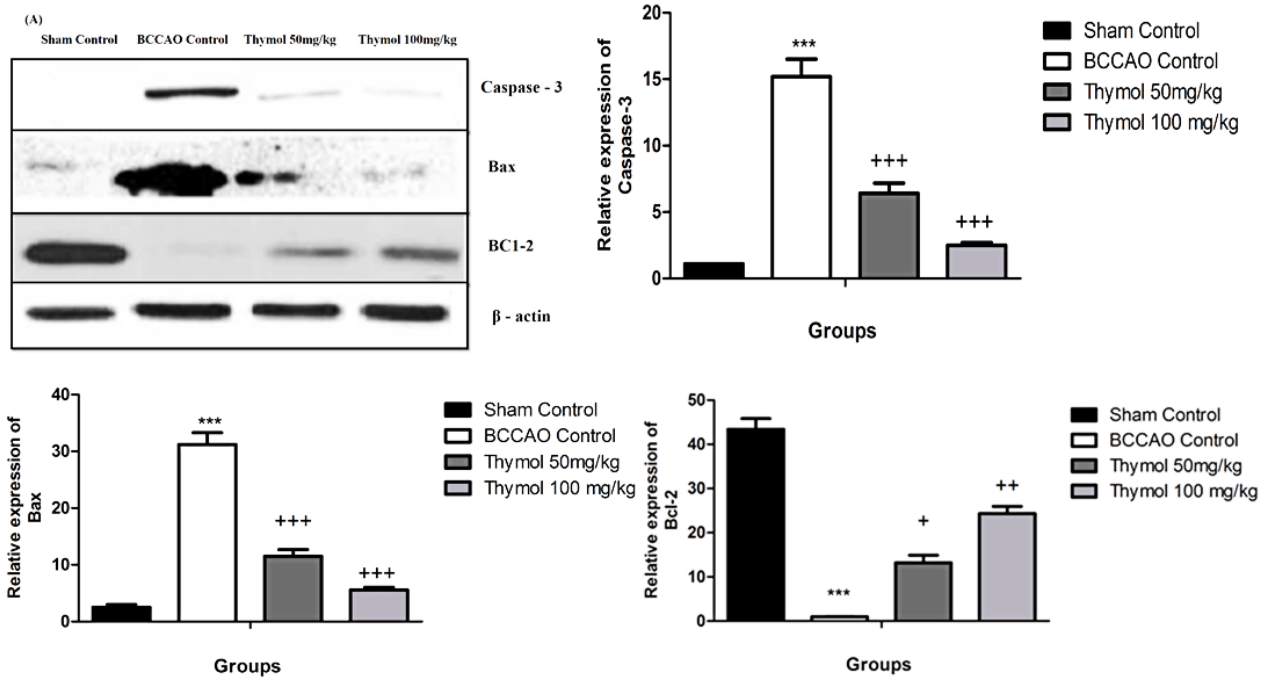

Figure 8: Effect of Thymol on Caspase 3, Bax and Bcl-2 expressions. Data were expressed as Mean±SEM. $^{***}(p<0.001)$ BCCAO control as compared to sham control. $^{++}$ $(p<0.01)$, $^{+}$ $(p<0.05)$ Thymol 50 and 100 mg/kg as compared to BCCAO.

CONCLUSION

Based on the present study's outcomes, taken together, indicate that thymol may prevent brain ischemia-reperfusion injury by inhibiting apoptosis and inflammatory processes, which may be regulated by regulating p38-dependent MAPK signalling pathways.

LIMITATIONS OF THE STUDY

Differences in the level of surgical expertise and methods can affect the degree of ischemia and the result. The amount of brain damage can vary depending on how long the ischemia lasts. The results can be affected by various anesthetic and analgesic regimes. Ischemia may cause diverse reactions in rats of different ages and strains. Results can be affected by humidity and temperature. CIRI affects both men and women, so the findings may not apply to females. Hormonal variations in females may influence CIRI outcomes. Findings obtained in rats might not apply to humans. It's possible that the BCCAO model doesn't exactly mimic human CIRI. Small sample sizes may make it difficult to discover significant differences. Sham-operated controls may not adequately depict healthy animals. The results might not accurately represent the long-term effects of CIRI.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

BCCAO: Bilateral common carotid artery; **MAPK:** Mitogen Activated Protein Kinase; **IL 10:** Interleukin 10; **I/R:** Ischemia Reperfusion; **EO:** Essential Oils; **TNF-α:** Tumor Necrosis Factor-α; **CIRI:** Cerebral ischemia reperfusion injury; **MPO:** Myeloperoxidase.

SUMMARY

Prophylactic treatment with thymol 50 and 100 mg/kg p.o showed significant decrease in MPO and protein carbonyl content, expressed down regulation in the levels of p38 MAPK, TNF-α, Caspase 3, Bax and up regulation of Bcl-2 which were clearly evidenced by western blotting analysis in comparison with BCCAO in dose dependent manner. In comparison to the BCCAO control group, the prophylactic therapy groups also exhibited the anti-inflammatory marker IL-10.

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