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Syzygium malaccense L. EXTRACT ATTENUATES INFLAMMATION AND IMPROVES ANTIOXIDANT DEFENCES IN A RAT MODEL OF STROKE

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ABSTRACT
The study was taken to investigate the protective role of hydroalcoholic extract of *Syzygium malaccense* L. (HASM) against brain damage and functional outcome after cerebral ischemia/reperfusion (I/R) injury in rats. Briefly, male rats of wistar strain were randomly divided into sham control, disease control, HASM-I (200 mg/kg, b.wt., p.o), HASM-II (400 mg/kg, b.wt., p.o) groups, treated with their respective treatment for 14 days cerebral I/R injury was induced by middle cerebral artery occlusion (MCAO) for 2 h, followed by reperfusion for 24 hrs. After 24 h of reperfusion, the rats were tested for neurological deficits using five point scale, morris water maze and grip strength test. In addition, the brain tissues were isolated for measurement of levels of tumor necrosis factor α (TNF-α), interleukin (IL)-6 and PGE_2, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and glutathione peroxidase (GPx). The results revealed that the neurological deficit scores were significantly (p<0.001) attenuated upon treatment with HASM in a dose dependent manner when compared with sham group. HASM treatment also reversed MCAO induced changes in inflammation, as evident with a significantly (p<0.001) decreased levels of TNF-α, IL-6 and PGE_2 expression. Moreover, HASM significantly (p<0.001) prevented oxidative damage and improved the antioxidant enzymes of SOD, GSH, GPx with a significant (p<0.001) decline in prooxidant enxymes of MDA, CAT. Together the results, it can be concluded that HASM attenuated MCAO induced brain damage and improved function outcome, probably due to its antioxidative and anti-inflammatory properties.
Key Words: Cerebral Ischemia, *Syzygium malaccense* L., Neuroprotection, oxidative damage, inflammation

INTRODUCTION

Stroke is one of the leading cause of mortality and long-term disability worldwide [1, 2]. Cerebral ischemia, a major type of stroke, accounting for almost 80% of stroke cases. Ischemic stroke causes a reduction in blood flow, alters normal cellular function sufficiently and subsequent restoration of blood flow resulted in reperfusion injury. Reperfusion is a therapeutic strategy in treatment of ischemic stroke which is critical and remains challenging. However, post-reperfusion pronounced large amounts of reactive oxygen species (ROS), leading to oxidative damage, apoptosis and inflammation, which are frequently associated with a blood brain barrier (BBB) disruption and brain edema [4, 5]. Recombinant tissue plasminogen activator (r-tPA) is the only FDA-approved thrombolytic therapy for acute ischemic stroke treatment which could be beneficial if administered within 3 hrs after onset of stroke and the efficacy decline with the time window. The therapeutic time constraint of rtPA challenges the researchers to discover and develop novel and alternative therapeutic lead for ischemic stroke.

Syzygium malaccense (L.) Merr. & L. M. Perry, a small plant indigenous to Malaysia and South East Asian regions, well-known as 'Malay Apple' extensively employed in traditional medicines for mouth infections, throat infections, stomach ache and abdominal ailments [9, 10]. Crushed leaves are used as antiemetic, purgative and also to treat bronchitis, tongue inflammation, dysentery, constipation, diabetes, cough, pulmonary cataract, headache and other ailments [11]. Phytochemical screening was performed on the crude hydro alcoholic extract of Syzygium malaccense using standard procedures to identify the active phytoconstituents. It revealed the presence of saponins, flavonoids, tannins, steroids and glycosides [12]. However, the investigation in Nigeria showed that leaves of this plant contain hydrodistilled essential oil of about 61.1% monoterpenes and 30.8% sesquiterpenes. Middle cerebral artery occlusion (MCAO) is the most commonly used model of ischemic stroke and still results in high death rates of 40% to 80% [3].

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MATERIALS AND METHODS

Plant Material

The plant material of ***Syzygium malaccense*** was collected and authenticated (Voucher No.: 2349) by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh.

Preparation of extract

The peel of the fruit was removed and shade dried and pulverised to coarse powder. 500 gm of coarse powder was extracted by percolation method using soxhlet apparatus and solvent hydro alcohol (70%, 60–80˚C) in 1:4 ratio. The extract was dried and the percentage yield of the extract was calculated and is found to be 12% (60gm) and is used for the investigation [13-15].

Animals and Grouping

Forty male Wistar rats weighing 200-220 gms were housed under standard laboratory conditions with an ambient temperature of 23 ± 2°C, under a 12 hrs light/12 hrs dark cycle and access to food and water ad libtium. The experiment was carried out after prior approval from Institutional Animal Ethical Committee (Approval no. SHCP/IAEC/20-01). Rats were divided into Sham control (treated with normal saline), disease control (MCAO was performed for rats and treated with saline), HASM-I (MCAO + treated with HASM 200mg/kg/p.o) and HASM-II (MCAO + treated with HASM 400mg/kg/p.o). Rats were received their respective treatment once daily for 14 days. On 14th day the animals were subjected to surgical procedure of middle cerebral artery occlusion.

Middle Cerebral Artery Occlusion (MCAO) surgery

Rats were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) [16] and fixed in the supine position on the operation table. A modified middle cerebral artery occlusion (MCAO) was taken to accomplish focal cerebral ischemia on a heating lamp at 37°C [17]. Briefly, a 2 cm midline incision performed on the neck, and the right external and internal carotid arteries was dissected carefully. A standard 4–0 nylon filament with a heat blunted tip inserted into the right internal carotid artery from the external carotid artery to block the origin of the MCA for 2 hrs. After 2 hrs of ischemia, the cerebral obstruction was withdrawn carefully to allow MCA reperfusion. Sham group received the same surgical operations except the occlusion / reperfusion. Sham and I/R rats were administered with equivalent volume of saline.

The animals were subjected to the above surgical procedure on day 14 for 2 hrs occulusion followed by 24 hrs reperfusion [17].

Assessment of neurological deficit

After reperfusion, the behavioural parameters like neurological deficit score, Morris water maze test and Grip strength test were assessed in the animals. 6 rats of each group were evaluated for neurological deficits by the method of Longa and co-workers [17]. The five-point scale was as follows: **Grade 0** = no neurological deficits; **Grade 1** = failure to extend the contralateral forepaw fully when held by the tail; **Grade 2** = circling to the ipsilateral side; **Grade 3** = falling to the contralateral side of brain damage; **Grade 4** = did not walk spontaneously and depressed level of consciousness. Spatial learning and memory was assessed by Morris water maze test and the grip strength test was measured in the left and right forelimbs of each rat using a grip strength meter [18].

Estimation of TNF- α, IL-6 and PGE_2 levels

After behavioural assessments, animals were sacrificed under euthanasia by decapitation under mild anaesthesia. The brains were removed carefully and each brain tissue sample was weighed using an analytical balance, and 100 mg tissue of each sample was homogenized in 0.01M PBS buffer (pH 7.2). After the homogenate centrifugation at 12,000 × g for 30 min at 4°C, the supernatant was collected and quantitatively assayed for tumor necrosis factor α (TNF-α), interleukin (IL)-6 and PGE_2 using ELISA kits [19-24]. The brain tissues were homogenized in ice-cold phosphate buffer with pH 7.4. The brain homogenate was centrifuged at 800 ×g for 30 min at 4°C to remove the nuclear debris. The supernatant was used for estimation of MDA content. The remaining supernatant liquid was again centrifuged at 12,000 rpm for 15 min and was used to estimate reduced GSH, SOD, CAT and GPx [25-29].

Histological Examination

Brain samples from the control and the experimental group were obtained and immediately immersed in 10% buffered formalin fixative for 48 hrs. The specimens were processed to obtain paraffin blocks, from which 5-µm-thick sections were prepared and processed for histopathological studies. Haematoxyline and eosin stain was used

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[30]. To assess the histopathological change, the sections were further subjected to hematoxylin eosin staining and will be examined by light microscopy.

Statistical analysis

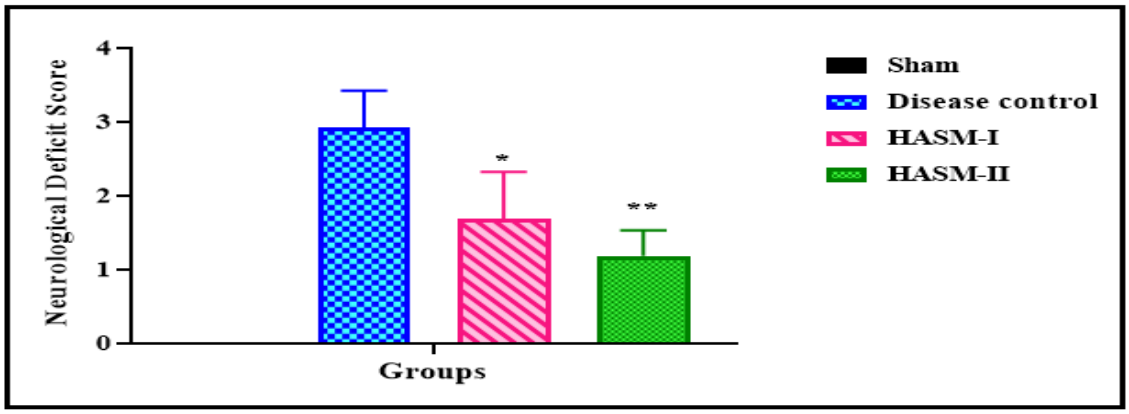
All the data were expressed as Mean ± Standard error mean (SEM). The difference between groups analysis will be performed with one-way ANOVA followed by Dunnett's test using Graph Pad Prism Software (Version 8).

RESULTS

❖ **Effect of HASM on neurological deficit score of rats**

The neuroprotective effect of HASM was assessed based on Neurological severity scores. The experiment was carried on day 15 after I/R. Table 1 exhibits that the Disease control group rats showed the highest neurological deficit score and HASM treatment significantly decreased the score

Graph 1: Effect of HASM on neurological deficit score of rats

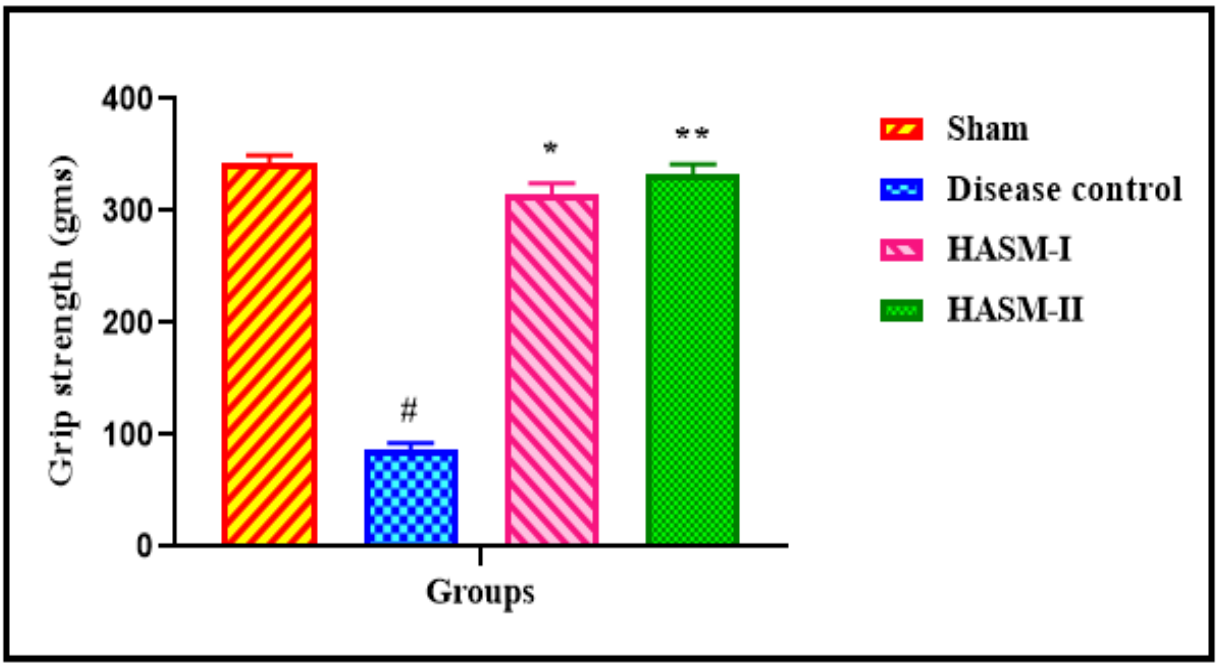


Data was expressed as Mean ± SEM. Treatment groups were compared with Disease control *p < 0.05; ** p < 0.01; *** p< 0.001 using one-way ANOVA following Dunnett's test.

❖ **Effect of HASM on muscular coordination skill in rats**

Middle cerebral artery occlusion leads a significant depletion in motor coordination as compared with sham group and significantly recovered in HASM pretreated I/R group (HASM + I/R) as compared with Disease control group. The grip strength was decreased significantly in the Disease control group animals as compared with the sham group animals. Treating the animals with HASM followed by I/R has protected grip strength as compared with Disease control group.

Graph 2: Effect of HASM on muscular coordination skill in rats



Data was expressed as Mean ± SEM. Disease control group compared to Sham # p< 0.001. Treatment groups were compared with Disease control *p < 0.05; ** p < 0.01; *** p< 0.001 using one-way ANOVA following Dunnett's test.

❖ **Effect of HASM on the escape latency time using Morris Water Maze test in rats**

The escape latency was measured on day 15 after ischemia. Animals in the sham group performed better than Disease control animals where increase in the escape latency time was observed in Disease control group. Significant differences were revealed in escape latency time between the sham and Disease control groups. This result confirmed that I/R elicited significant deficits in spatial learning. HASM dose dependently decreased the escape latency time, and the two doses (200 mg/kg and 4000 mg/kg) caused a significant reduction in the escape latency time as compared to Disease control animals.

Graph 3: Effect of HASM on the escape latency time using Morris Water Maze test in

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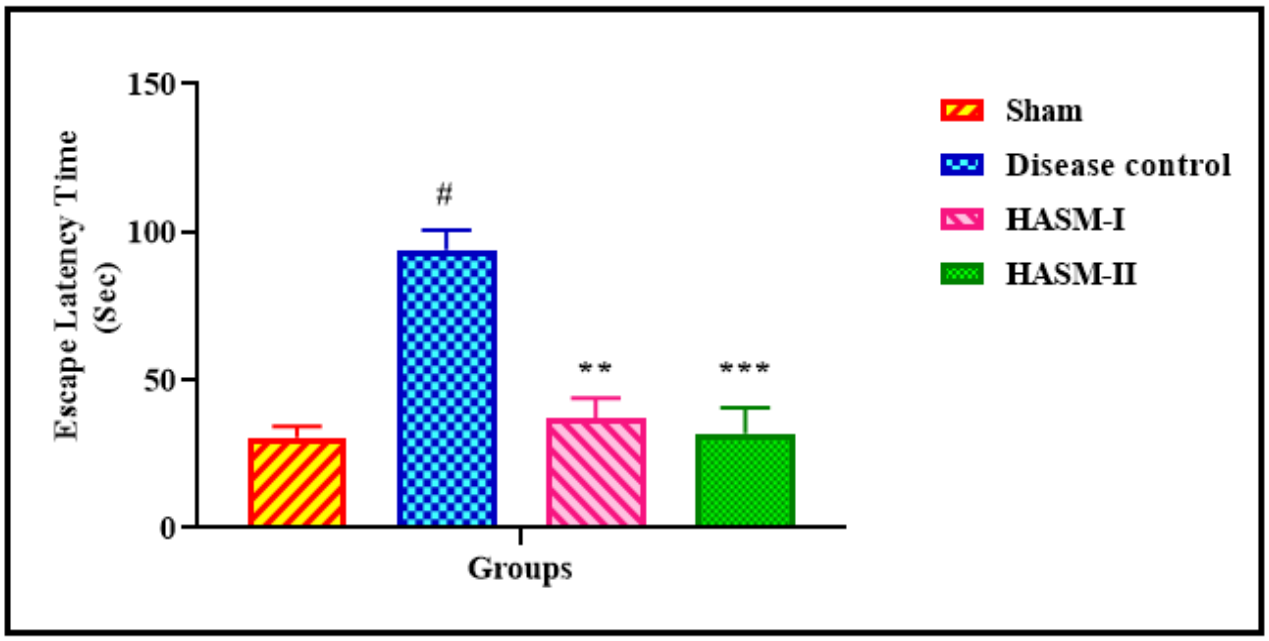
rats

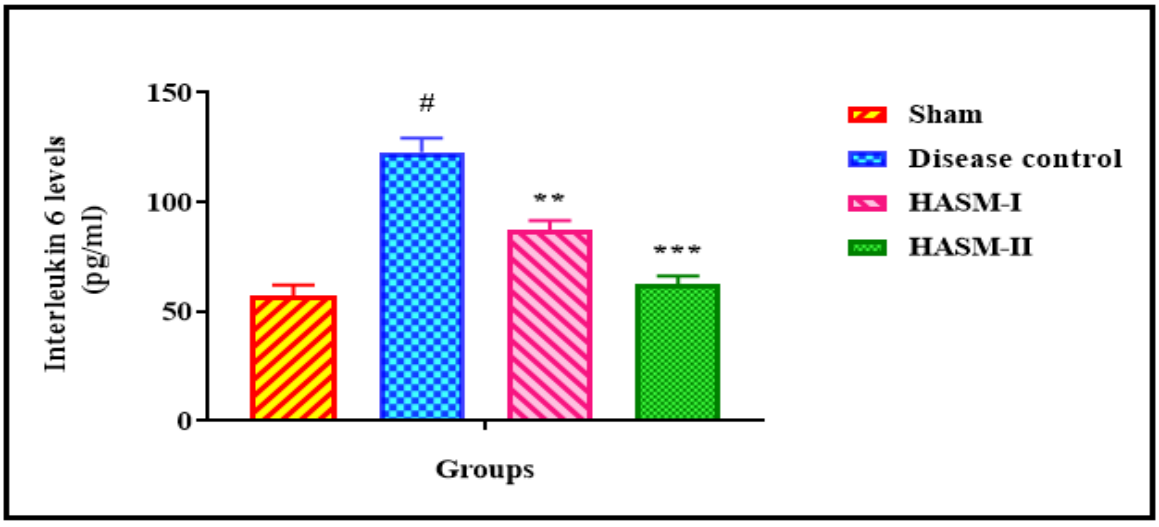


Data was expressed as Mean ± SEM. Disease control group compared to Sham # p< 0.001. Treatment groups were compared with Disease control *p < 0.05; ** p < 0.01; *** p< 0.001 using one-way ANOVA following Dunnett's test.

❖ **Effects of HASM on cerebral inflammatory markers in rats**

The levels of cerebral TNF-α, IL-6 and PGE2 levels significantly (P<0.05) increased in Disease control animals as compared to sham group. The levels of cerebral TNF-α, IL-6 and PGE2 levels in HASM treated groups were significantly (p<0.05) reduced than that of Disease control group. The values of cerebral TNF-α, IL-6, PGE2 are shown in Table 5.4.

Graph 4: Effect of HASM on IL-6 levels in



ra

Data was expressed as Mean ± SEM. Disease control group compared to Sham # p< 0.001. Treatment groups were compared with Disease control *p < 0.05; ** p < 0.01; *** p< 0.001 using one-way ANOVA following Dunnett's test.

Graph 5: Effect of HASM on TNF-α levels in rats

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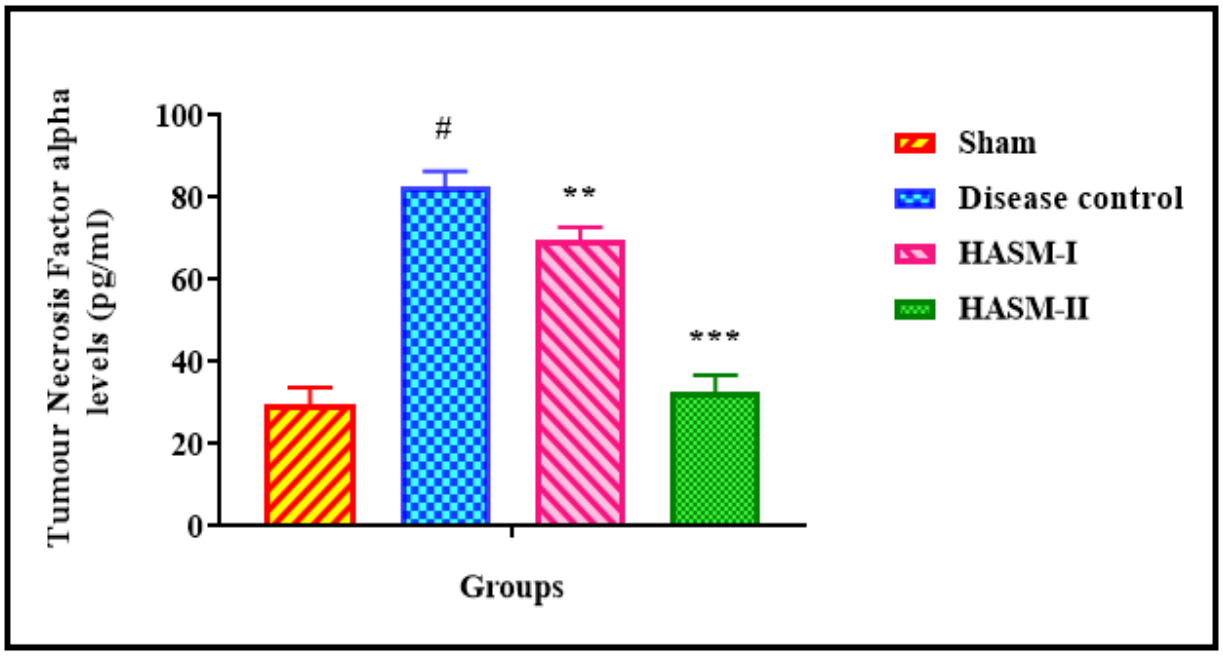
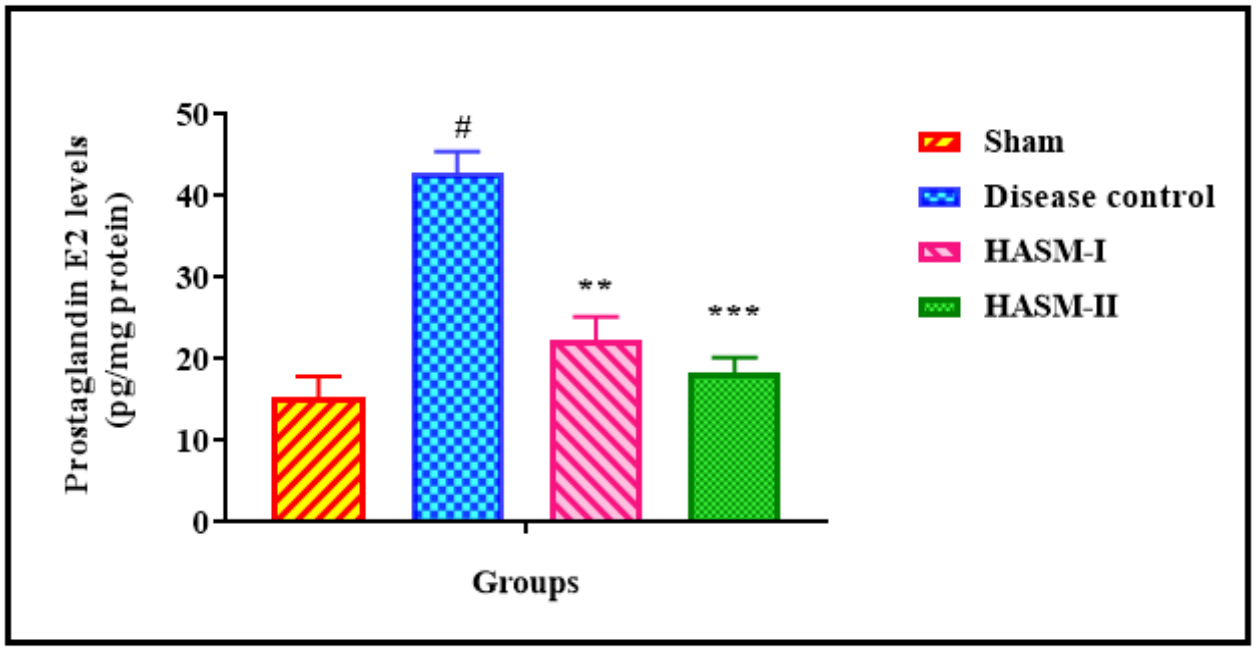


Data was expressed as Mean ± SEM. Disease control group compared to Sham # p< 0.001. Treatment groups were compared with Disease control *p < 0.05; ** p < 0.01; *** p< 0.001 using one-way ANOVA following Dunnett's test.

Graph 6: Effect of HASM on PGE2 levels in rats



Data was expressed as Mean ± SEM. Disease control group compared to Sham # p< 0.001. Treatment groups were compared with Disease control *p < 0.05; ** p < 0.01; *** p< 0.001 using one-way ANOVA following Dunnett's test.

❖ **Effect of HASM on oxidative stress markers in rats**

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The activities of antioxidant enzymes (GSH, GPx, SOD, and Catalase) were decreased and pro-oxidant (MDA) levels increased significantly in frontal cortex of Disease control animals as compared with the sham group and their levels were restored significantly in the frontal cortex of the HASM pretreated animals with both dose levels.

Table 6: Effect of HASM on oxidative stress markers in rats

Groups	Sham [Normal Saline]	Disease control [Saline+I/R]	HASM-I+I/R [200mg/kg, b.wt.]	HASM-II+I/R [400 mg/kg, b.wt.]
MDA (µmol/mg protein)	4.94 ± 0.68	13.21 ± 1.12$^{\#}$	9.88 ± 1.97**	5.74 ± 0.87***
GPx (nmol of NADPH oxidized/min/mg protein)	229.85 ± 9.25	134.75 ± 7.26$^{\#}$	170.26 ± 7.58**	219.71± 9.10***
SOD (U/mg protein)	13.40 ± 1.98	5.10 ± 1.26$^{\#}$	8.22 ± 1.87**	11.15 ± 1.34***
CAT (U/mg protein)	19.85 ± 1.70	8.85 ± 1.38$^{\#}$	10.99 ± 1.43**	17.82 ± 1.56***
GSH (mg/100 g of tissue)	32.66 ± 2.84	11.46 ± 2.63$^{\#}$	23.16 ± 3.12**	30.58 ± 3.45***

Data was expressed as Mean ± SEM. Disease control group compared to Sham $^{\#}$ $p < 0.001$. Treatment groups were compared with Disease control *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ using one-way ANOVA following Dunnett's test.

- **Histopathology of Brain**

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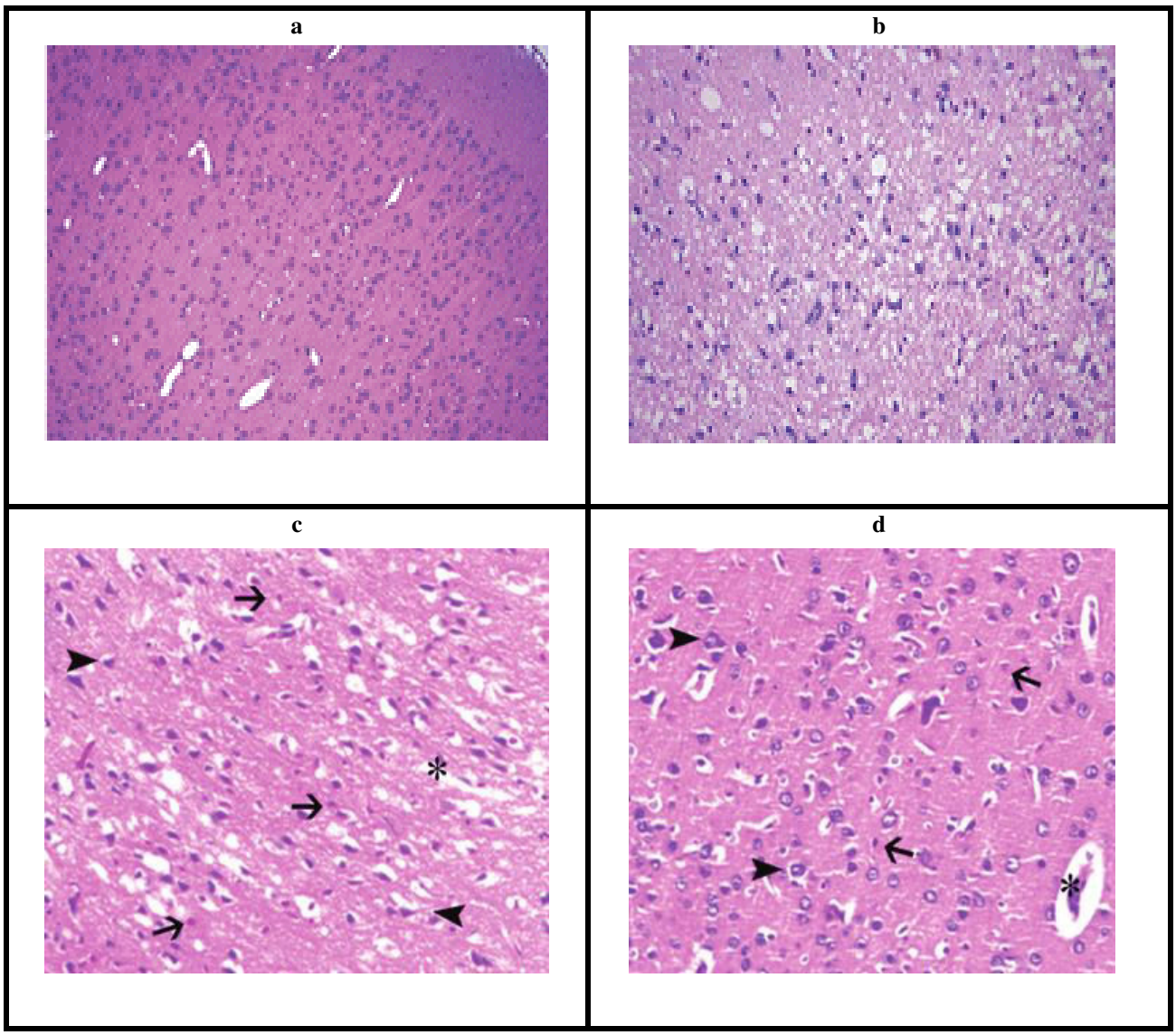

Figure 1: Histopathological studies

a. Sham (Surgery without Occlusion) treated with Vehicle
Normal rat's brain section with normal histoarchitecture

b. Cerebral Ischemic induced Reperfusion tissue
showing cerebral oedema and Infraction

c. I/R+HASM-I [200 mg/kg, b.wt.]

d. I/R+HASM-II [400 mg/kg, b.wt.]

DISCUSSION

Cerebrovascular disease has high morbidity, death rates and disability rates worldwide, posing a serious threat to public health [31]. Approximately 2 million individuals suffer from cerebrovascular disease worldwide and >1.5 million succumb each year [32]. Furthermore, the morbidity is on the increase with improvements in living standards and lifestyle changes [31]. Cerebrovascular disease may be classified as hemorrhagic or cerebrovascular.

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Cerebral atherosclerosis and inflammation of the cerebral artery are major causes of cerebral I/R injury and cause the highest morbidity. The majority of patients who suffer cerebral ischemia have several sequelae [33]. In the present study, the protective effect of HASM on neurological deficits score in rats with cerebral I/R injury was investigated.

Ischemic brain injury is a common cause of permanent disability and is associated with dementia and cognitive decline in the elderly [34]. Certain surgical procedures that involve the reduction or interruption of the blood supply to the brain and events such as strokes, are often accompanied by memory loss that persists for several months during recovery. These changes are believed to be associated with focal cerebral ischemia. The middle cerebral artery occlusion model (MCAO) that was originally developed in rats is considered to be a reliable and reproducible model. The model induces deficits in cognitive function in the rats, which appear to remain fairly stable [35, 36].

The present study evaluated the potential protective effect of HASM on the cerebral ischemia/reperfusion induced brain injury in experimental animals. It was found that HASM significantly decreased the neurological deficit scores in rats with 2 hrs of ischemia followed by reperfusion. In addition, *Syzygium* extract also accelerated the improvement of motor co-ordination functions through grip strength.

The development of mazes to investigate spatial learning and memory has provided a method to determine the protective effects of drugs on the behavioural consequences and the neurological damage to the frontal cortex and other areas involved in the neurotoxic effects of ischemia [37]. The deficits on the learning and memory of rats following I/R injury was therefore measured by Morris Water Maze test in the present study. I/R induced a failure in the
spatial memory function of the rats when they were tested on day15 following the surgery and reperfusion. This result demonstrated that HASM pretreated animals shortened the escape latency compared with Disease control group.

In the present study, Disease control rats exhibited an up regulation in TNF-α expression in ischemic brain tissue. These results are collaborated by previous studies, which reported that exacerbated TNF-α levels may be important factor in reperfusion injury following transient brain ischemia [38].

In the present study, a significant increase in the inflammatory cytokines (TNF-α and IL-6) was found in Disease control group as compared with the sham group. The researchers [39] showed that transient global cerebral I/R resulted in a substantial increase in the mRNA expression levels of TNF-α and IL-6 in the rat frontal cortex. The previous data indicate that inflammatory response was initiated after transient cerebral ischemia and the release of inflammatory cytokines such as IL-6 and TNF-α occurred in the brain [40]. In pretreatment groups of HASM a significant decrease in these levels were found at both doses of 200 mg/kg, b.wt., and 400 mg/kg, b.wt. respectively.

As a crucial catalyst of COX-2, PGE2 is a marker of COX-2 activity [41]. A previous study reported that changes in PGE2 levels in the frontal cortex were correlated with changes in the expression pattern of COX-2 in the ischemic hemisphere of a rat model of focal I/R injury [42]; PGE2 may therefore be used as a marker of COX-2 activity. COX-2 inhibitor is able to suppress the metabolism of arachidonic acid via inhibiting COX-2 activity [43], thereby reducing the production of PGE2 and alleviating inflammatory response injuries. It may also relieve the degree of brain tissue injury and encephaledema to protect brain tissues [43]. In present study, test groups of HASM-I & II (200mg/kg, b.wt., 400 mg/kg, b.wt.) has shown a significant reduction in the elevated levels of PGE2 when compared to that of disease control.

Oxidative stress is the first event in a sequence leading to neuronal loss following cerebral ischemia reperfusion [44]. Essential cell components, including DNA, protein and lipids, are subject to excessive oxidative assault, leading to cell injury. Antioxidant defensive molecules, such as superoxide dismutase, glutathione and catalase are able to ameliorate the elevation of oxidants and therefore reduce damage to tissues [45].

The incidence of post-reperfusion lesions and oxidative stress refers to elevated intracellular levels of ROS, which may result in damage to tissue, lipids, proteins, and DNA. ROS directly damages cellular membranes through lipid peroxidation [46-47]. In our present study, rats subjected to I/R presented high levels of MDA in the brain tissue. However, the HASM-I &II groups (200 mg/kg and 400 mg/kg) attenuated the elevated levels of MDA suggesting that HASM protects from brain damage caused by I/R induced oxidative stress.

GSH, a vital intracellular non-protein thiol, acts as a scavenger of free radicals [48]. Previous studies demonstrated that cerebral ischemia mediated lipid peroxidation is concurrent with GSH depletion in brain tissue [44, 49-50]. Furthermore, it was suggested that antioxidant enzymes such as GPx, SOD and CAT may serve a key role in the regulation of redox homeostasis in tissues [51, 52]. In the present study, disease control animals displayed significant reductions in GSH, GPx, SOD and CAT levels in the brain ischemic tissue, compared to sham group.

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Test groups of HASM at both dose levels (200 mg/kg, 400 mg/kg) was able to restore depleted antioxidant levels due to its potential antioxidant activity.

Histopathological changes in neuron after ischemia/reperfusion injury were investigated by hematoxylin-eosin staining. The sections of the sham group showed normal architecture with no pathologic changes, whereas the sections of Disease control group showed a focus of brain damage with neuronal loss and presence of numerous vacuolated spaces. The corresponding area in the sections from HASM-I group showed partial neuronal loss with presence of intact neurons in between the vacuolated spaces. Moreover, test groups of HASM-I & II (200 mg/kg, b.wt., 400 mg/kg, b.wt.) has ameliorated the neuronal abnormalities as compared with the Disease control animals.

SUMMARY AND CONCLUSION

Ischemic stroke is one of the leading causes of disability and mortality. On the one hand, stroke leads to complex processes, including intracellular calcium overload, free radicals mediated toxicity and disruption of the blood brain barrier, which lead to acute neurological deficit and many plant constituents are being proven to have protective effects in various neurodegenerative disorders. The present investigation demonstrated the antioxidant and anti-inflammatory effect of hydroalcoholic extract from Syzygium malaccense L. on different free radicals and inflammatory cytokines was tested with cerebral ischemia and reperfusion induced oxidative stress. The findings of the study suggested that the HASM is protective against ischemia induced oxidative stress by mechanisms involving inhibition of free radical generation, reactive oxygen species scavenging and accelerating the antioxidant defence mechanism against ischemia reperfusion injury as evidenced by histopathological observation of brain tissue. The potential antioxidant effect of HASM may be due to higher phenolics and flavonoids contents available in the extract. Further work is required to isolate and characterize the responsible bioactive constituents from Syzygium malaccense L. that are responsible for its antioxidant and anti-inflammatory properties.

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