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ORIGINAL ARTICLE

Aegle marmelos Ameliorates Epilepsy through Suppression of Oxidative Stress and Inflammation

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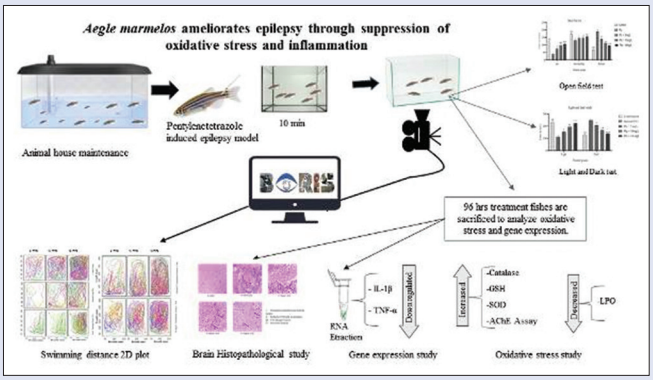
ABSTRACT

Background: Epilepsy remains one of the leading neurological diseases. More than one mechanism exists for epilepsy. Inflammation and oxidative stress are amongst the mechanisms leading to epilepsy. In the current work, we evaluated whether *Aegle marmelos* (AM) is able to reverse epilepsy. **Objectives:** We induced epilepsy in zebrafish through the administration of pentylenetetrazole as a model organism. **Materials and Methods:** To assess the anti-epileptic potency of plant extract, behavioural changes were recorded and analysed by Behavioural Observation Research Interactive Software. Further, oxidative stress, glutathione peroxidase and lipid peroxidation (LPO) levels were estimated. Real-time polymerase chain reaction was performed to quantify the expression of inflammatory markers tumour necrosis factor-alpha (TNFα) and interleukin 1 beta (IL-1β). **Results:** Epilepsy-induced animals displayed a pronounced behavioural change in swimming patterns and preference for darkness and stayed longer periods at the bottom of the tank. On the other hand, plant extract treated animals reversed the behavioural alterations induced by epilepsy. Oxidative stress was seen in animals induced with epilepsy and the same was reversed in animals that were treated with plant extract. LPO was elevated in epilepsy whereas plant extract reduced the same. Inflammatory markers such as TNFα and IL-1β levels were higher in epileptic animals whereas their expression levels were reduced by treatment with the plant extract. Histopathological examination also revealed the inflammatory reaction in the brain induced by epilepsy. **Conclusion:** Treatment with the plant extract restored the tissue architecture to normal. Overall, there was a major reversal of behaviour, oxidative stress and inflammation upon treatment with the plant extract indicating the anti-epileptic potential of AM.

Key words: *A. marmelos*, anti-inflammatory activity, behaviour, epilepsy, oxidative stress

SUMMARY

- The current treatment strategy for epilepsy with modern drugs results in side effects, toxicity and teratogenic effects with approximately 30% of the patients continuing to have seizures. The plant source can be an alternative source of anti-epileptic drug discovery with better safety and efficacy. The present study reported the anti-epileptic activity of hydroethanolic extract of AM in PTZ seizure-induced zebrafish model. There was a major reversal of behaviour, oxidative stress and inflammation upon treatment with the plant extract indicating the anti-epileptic potential of AM. Further studies need to be carried out to study the clinical effectiveness and mechanism of action involved in anti-epileptic activity.



Abbreviations used: BORIS: Behavioural Observation Research Interactive Software; **PCR:** Polymerase chain reaction; **TNF-α:** Tumour Necrosis Factor-alpha; **IL-1β**: Interleukin 1 beta; **PTZ**-Pentylenetetrazole; **qPCR:** Quantitative polymerase chain reaction; **AChE**-acetylcholinesterase; **GSH:** Reduced glutathione; **LPO:** Lipid peroxidation; **SOD:** Superoxide dismutase; **AM:** *Aegle marmelos*; **NMDA receptor:** N-methyl-D-aspartate receptor.

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INTRODUCTION

Epilepsy is one of the leading neurological disorders that affects 50,000,000 people across the world.[1] The international league against epilepsy defines epileptic seizure as a 'transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain'.[2] Based on the aetiology, epilepsy has been classified as infectious, genetic structural, metabolic, immune and unknown.[3]

Oxidative stress has been shown to be associated with epilepsy. The extended seizure-mediated excitation of neurons results in oxidative stress. This further aggravates the condition when the unpaired electrons escape from the electron transport chain and cause superoxide molecules by interacting with molecular oxygen. Superoxide molecules in turn cause brain damage due to seizures.[4] In addition, reactive oxygen species can cause disruption of intracellular calcium homeostasis and potentially exacerbates the epileptic condition. Loss of calcium homeostasis can even influence the extent of excitability of neurons, negatively alter the cellular energetics and ultimately cause apoptosis of neurons.[5]

In addition to oxidative stress, inflammation has also been shown to be one of the causes of epilepsy. Astrocytes and glial cells are known to

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trigger inflammatory cascade in the neuronal system. A variety of insults to the brain potentiates seizures and epilepsy during cases of chronic inflammation. Microglia play a vital role in brain immunity. Activation of microglial cells induces inflammation and subsequently seizures.[6] Most importantly, the expression of interleukin 1 beta (IL-1β) by microglia is in excess after the seizures.[7]

In the current work, we evaluated the therapeutic potential of *Aegle marmelos* (AM) plant extract using the epileptic model in zebrafish. AM are widely known as Bengal quince distributed in South East Asia. A wide variety of bioactive compounds have been extracted from AM which have medicinal values.[8] It has been reported to possess antioxidant, hepatoprotective and neuroprotective activities.[9] The plant has been shown to possess strong anti-inflammatory activity.[10] Since the plant extract has both antioxidant and anti-inflammatory potentials, we evaluated its effect on epilepsy.

MATERIALS AND METHODS

Collection and maintenance

Danio rerio (zebrafish) was obtained from the aquarium. For acclimatising the fish to laboratory conditions, they were maintained in a 55 L glass tank with deionized water. Continuous aeration was done by an air compressor. The temperature of the water was maintained between 26 and 27°C and the pH of the water was maintained between 7.3 and 7.5. Commercially available food pellet was provided to the animals twice a day. The care and husbandry of the zebrafish used in this study were in accordance with the guidelines that regulate the care and use of laboratory animals by humans for research purposes.

AM extraction method

AM plant leaf sample was collected from the local area of Yunnan province. Collected leaves were washed with tap water and washed thrice with distilled water. Leaves were hot air-dried for 3 days at 40°C. Around 20 g of dried leaf sample was weighed and grounded using a motor and pestle. The extract is prepared by the hydro-alcoholic method as 1:1 ratio. That is, to the sample 100 mL of distilled water and 100 mL of ethanol were added and stirred with heating at 50–60°C for 10 min. Then, the sample was filtered using Whatman no. 1 filter paper and the filtered sample was dried in Petri plates for 24 h at 40 – 50°C under a hot air oven.

Toxicity assess and dose fixing

Fishes weighed between 0.5 and 0.6 g were selected and randomly separated as n = 5/tank. The AM leaf extract concentration was taken for LC_{50} as 20, 40, 60, 80 and 100 mg/L in the tanks. The control group was maintained in a separate tank and the fishes were maintained under treatment for 7 days. No toxicity occurs up to 100 mg/L and 5, 10 and 15 mg/L dose range selected for the treatment.

Induction of epilepsy

Epilepsy was induced in fishes by administering pentylenetetrazole (PTZ) by following the method described by,[11] with slight modifications.

Software analysis

Video recordings of swimming fishes were subjected to analysis by Behavioural Observation Research Interactive Software (BORIS) version 8.0.8 to evaluate the behaviour of fishes. GraphPad Prism 8.0 software was used for the statistical analysis of the open field tests, light and dark.

Swim patterns

Analysis of the swimming patterns of the fish was done by subjecting the video recordings of the fish to BORIS software.[12] Swimming patterns of various groups were compared to evaluate the effect of plant extract.

Freezing Time

The test estimates the time duration spent by the fish without any physical movements. The test aims at determining the impact of epilepsy on the physical movement of fish which is also an indication of physiological stress caused.

Open field test

This study was done to identify the time duration spent by fish in the top, middle, and bottom of the tank.[13]

Aquatic dark and light test

This test is done in a tank that is half dark and the other half light transparent. Various treatment groups of animals were tested for their preference towards light/darkness.[14]

Catalase assay

For assessing the catalase activity in tissues, the method explained by,[15] was followed with slight modifications.

Lipid peroxidation (LPO) assay

LPO was quantified using the thiobarbituric acid-reactive substances assay. Malondialdehyde makes a complex with thiobarbituric acid, which is quantified via an LPO assay kit (Sigma-Aldrich MAK085) spectrophotometrically.

Reduced glutathione (GSH) assay

GSH was estimated using the GSH assay kit (Sigma-Aldrich CS0260) following the protocol given by the manufacturer.

Superoxide dismutase (SOD) assay

SOD activity was quantified using the SOD assay kit (Sigma-Aldrich MAK379) and the protocol provided by the manufacturer was adopted.

Acetylcholinesterase (AChE) assay

The assessing the AChE activity in tissues by,[16] the method explained by was followed with slight modifications.

Quantitative real-time PCR

Real-time quantitative polymerase chain reaction (qPCR) was performed on the converted cDNAs using the Roche SYBR® Green RT-PCR kit. The PCR reactions were carried out in a Roche LightCycler Nano instrument with a final reaction volume of 20 µl having a final concentration of 0.5 M forward and reverse primers. LightCycler Nano software was used to analyse the quantification cycle (Cq) results. The Cq technique was used to compute relative gene expression values based on the ΔΔCq results.

Histopathology

Histopathological sections of the zebrafish brain were prepared by following the method explained by,[17] with slight modifications.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine significant difference between the values (mean ± SD with n = 3) and subsequently followed by One way ANOVA, Tukey's multiple

comparisons test, at *P* value <0.05 was considered as the minimum value to consider a statistically significant difference.

RESULTS

Open field test

Administration of PTZ at a concentration of 170 mg/kg successfully established epilepsy in zebrafish as evidenced by the behavioural changes. In the open field test, fishes preferred to stay at the bottom for a relatively longer duration than the control group. The same pattern was observed in the duration of time spent by the animals in the intermediate region. Control animals stayed in the intermediate range for a period of 172.666 ± 5.249 sec, whereas induced animals spent a relatively shorter duration of time in the intermediate level of the tank. Contrary to the top and intermediate levels the duration of time spent in the bottom layer increased significantly. The control fishes stayed for a period of 67 ± 5.099 sec, while the epilepsy-induced fishes displayed a preference for a drastically longer period of time 189.666 ± 11.115 sec (*P*-value < 0.0001) [Table 1]. Overall, there was a total change in the behaviour of fishes induced for epilepsy with respect to the open field test. By contrast, treatment with the extract of AM restored the behavioural pattern of zebrafish back to normal. A higher dose of the extract increased the duration spent in the top layer of the tank (From 40 ± 6.480 in epilepsy to 106.666 ± 8.178 in 15 mg/L of extract) [Table 1, Figures 1 and 2].

Light and dark test

When fishes were given dark and light areas in the same tank [Figure 3] epilepsy-induced fishes preferentially stayed in the dark areas compared to control (248 ± 8.981 sec compared to control 127.666 ± 7.760 sec). In contrast, the light area preference declined from 232.333 ± 7.760 sec to 112 ± 8.981 sec in control and epilepsy-induced fishes, respectively. The treatment with the extract gradually increased the light preference in a dose-dependent manner [Table 2 and Figure 4]. Similarly, the time spent in the dark area also decreased in a dose-dependent manner.

Behavioural parameters analysis

Analysing various parameters of the behaviour of fishes revealed the beneficial effect of AM against epilepsy. In the epilepsy-induced fishes, sudden freezing (bouts of inaction in locomotion) was observed, whereas treatment with AM restored the behaviour. This kind of freezing was not evidenced in the control group. A dose of 5 mg/L of the extract itself greatly diminished the freezing duration [Table 3]. Further with 10 and 15 mg/L doses of the extract freezing behaviour was completely absent. The activity was reduced to a greater extent due to epilepsy induction which was restored to normal by the plant extract in a dose-dependent manner [Figure 5].

Table 1: Open field test

Sl. No	Time in seconds		
	Top	Intermediate	Bottom
Control group	120.333±6.847$^{\#\#}$	172.666±5.249$^{\#\#}$	67±5.099$^{\#\#\#}$
Induced (PTZ)	40±6.480	130.333±12.229	189.666±11.115
PTZ+5 mg/L	74±8.831*	145.333±6.548*	140.666±8.993*
PTZ+10 mg/L	91±7.348**	151.666±6.236**	117.333±3.399**
PTZ+15 mg/L	106.666±8.178***	157.666±6.236**	95.666±2.624***

The treated and untreated groups in the open field tests were asseverated using Mean±SD and one-way ANOVA. The values are highly significant between treated and untreated groups (*P*-value<0.0001). Data are represented as the means±SD. #=Control group vs Induced group; *=Induced group vs Treated groups. $^{\#}P$<0.05, $^{\#\#}P$<0.001, $^{\#\#\#}P$<0.0001, $^{*}P$<0.05, $^{**}P$<0.001, $^{***}P$<0.0001

The swimming pattern was impaired with circular and erratic fashion with scrolling in the epilepsy-induced fishes. In a dose-dependent manner, the plant extract restored the swimming pattern in epilepsy-induced fishes. The extract of 5 mg/L induced a circular motion and 10 mg/L caused a circular with a zig-zag pattern of swimming, whereas a dose of 15 mg/L extracts induced all kinds of swimming such as straight, zig-zag, and circular motions [Table 3]. Most importantly, aggressive behaviour was witnessed in epilepsy-induced fishes which are absent in control fishes. The aggression was reverted by the plant extract at a dose as low as 5 mg/L. A change in tail colour is a feature seen in zebrafish which are under stress. A light-reddish colour was seen in fishes treated with PTZ for the induction of epilepsy. A dose of 15 mg/L AM extract reverted the colour to normal (though in some fishes in the same group light-reddish colour was seen) [Figure 6].

Antioxidant activity

Further to evaluate the inherent antioxidant activity, a catalase assay was performed. In the epilepsy-induced group, catalase activity significantly diminished compared to the control. However, the catalase activity was enhanced greatly in the AM extract treated groups. A significant increase in catalase activity was seen in the doses 5 mg/L, 10 mg/L and 15 mg/L in a dose-dependent manner (*P*-value <0.0001) [Figure 7]. GSH activity was also elevated in all the treatment groups in a dose-dependent manner. Especially, the activity was restored greater than the control group upon treatment with 15 mg/L AM extract (*P*-value <0.0001) [Figure 8].

Figure 1: Open field test

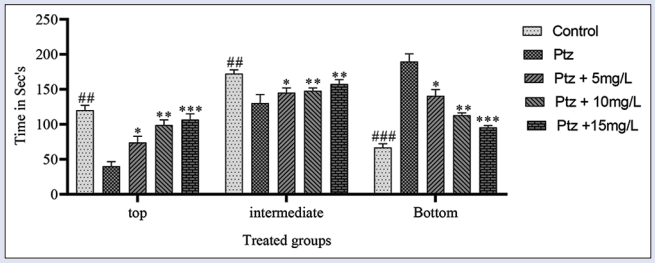

Figure 2: Open field test

Figure 3: Light and dark test

The extent of LPO was also quantified. In the epilepsy-induced group, the LPO levels were significantly increased than in the control. A dose-dependent decrease in LPO activity was seen in the treatment groups (*P*-value <0.0001) [Figure 9]. Similar to the other parameters, SOD activity was significantly decreased in the epilepsy-induced group. In contrast, SOD activity was also greatly enhanced in a dose-dependent manner in treatment groups (*P*-value <0.0001) [Figure 10].

To evaluate the neurotransmitter activity acetylcholine esterase assay was performed. The enzyme acetylcholine esterase is required for the synthesis of a variety of neurotransmitters. The enzyme activity assay revealed that induction of epilepsy suppressed the acetylcholine esterase activity compared to control. The plant extract is potent in enhancing the activity of the enzyme (*P*-value <0.0001) [Figure 11]. A dose-dependent increase in the activity of the enzyme is seen in the treatment groups.

The effect of AM in modulating inflammation was evaluated by gene expression analysis by qPCR. The expression of tumour necrosis factor-alpha (TNF-α) and IL-1β were evaluated and β-actin was used as the internal control. The expression level of TNF-α was greatly increased in response to epilepsy induction and treatment with the plant extract reduced the expression in a dose-dependent manner (*P*-value <0.0001) [Figure 12]. Similar results were observed in the expression pattern of IL-1β. The treatment with plant extract decreased the expression (*P*-value <0.0001) [Figure 12].

Histopathological analysis

Histopathological examination of the brain revealed the infiltration of brain tissue with more neutrophils and pyknotic nucleus indicative of ongoing inflammation with induction of apoptosis in epilepsy-induced animals [Figure 13]. On the other hand, treatment with AM gradually reduced the neutrophil infiltration with a reduced number of the pyknotic nucleus. With an increasing dose of AM reduction in neutrophils and a reduced number of the pyknotic nucleus were observable. With the highest dose (15 mg/mL) of AM the histopathological sections were almost near to control.

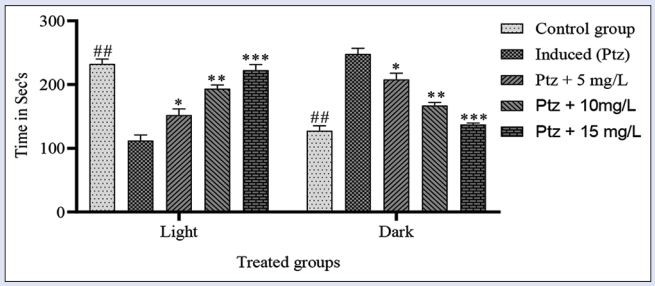

Figure 4: Light and dark test

Table 2: Light and dark Test

Sl. No	Time in seconds	
	Dark	Light
Control group	127.666±7.760$^{\#\#}$	232.333±7.760$^{\#\#}$
Induced (PTZ)	248±8.981	112±8.981
PTZ+5 mg/L	208±9.899*	152±9.899*
PTZ+10 mg/L	167±4.898**	193.666±5.734**
PTZ+15 mg/L	137.333±2.175***	222.666±8.653***

The treated and untreated groups in the light/dark tests were asseverated using mean±SD, one-way ANOVA. The values are highly significant between treated and untreated groups (*P*-value<0.0001). Data are represented as the means±SD. # = Control group vs Induced group, *=Induced group vs Treated groups. $^{\#}P<0.05$, $^{\#\#}P<0.001$, $^{\#\#\#}P<0.0001$, $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$

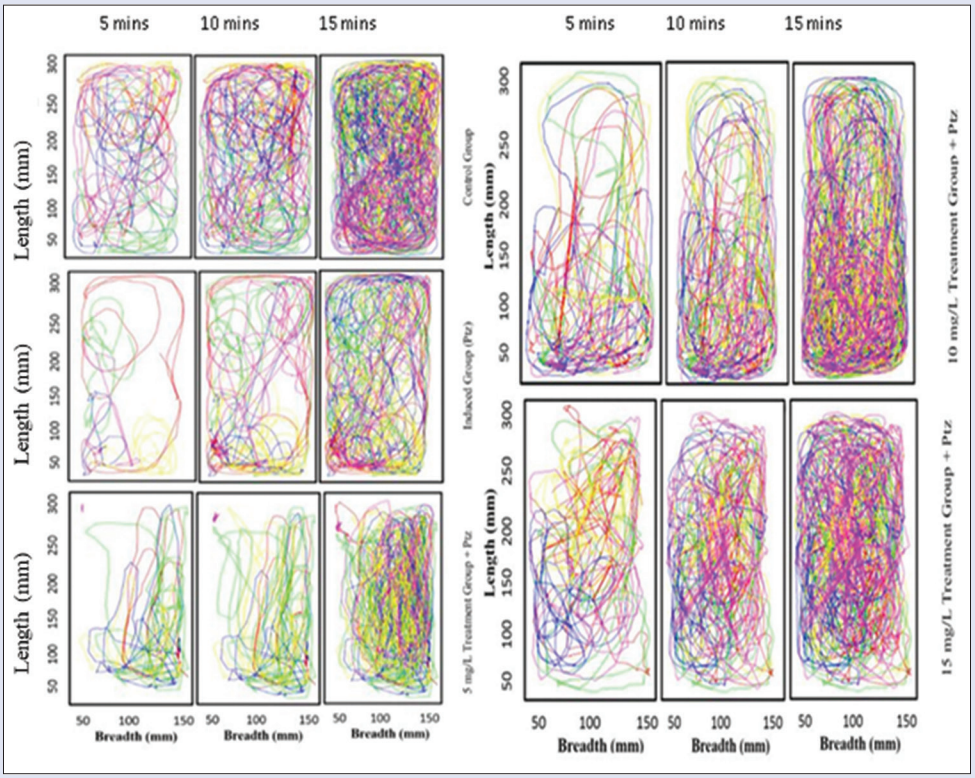

Figure 5: Swimming distance and pattern (2D-plot)

Table 3: Behavioural parameters analysis

Behavioural parameters analysis	Control group	Induced (PTZ)	PTZ+5 mg/L (AM)	PTZ+10 mg/L (AM)	PTZ+15 mg/L (AM)
Freezing	None	3 min	1 min	None	None
Active condition	Normal	Less	Less active	Less active	Active equal to control
Swimming pattern	Circular, zig-zag and straight	Circular and erratic, scrolling	Circular	Zig-zag and circular	Straight, circular and zig-zag
Aggression	None	yes	None	None	None
Colour change	Normal	Tail (light reddish) colour change	Tail (light reddish) colour change	Tail (light reddish) colour change	Tail (light reddish to normal) colour change

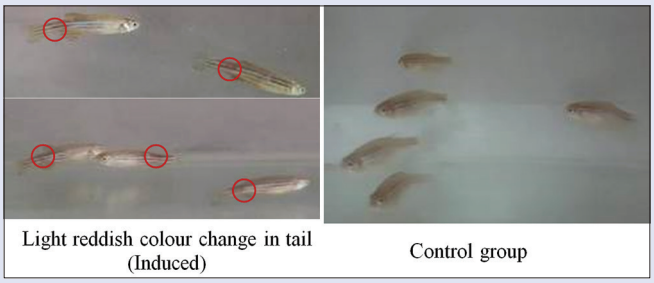

Figure 6: Fish tail colour appearance

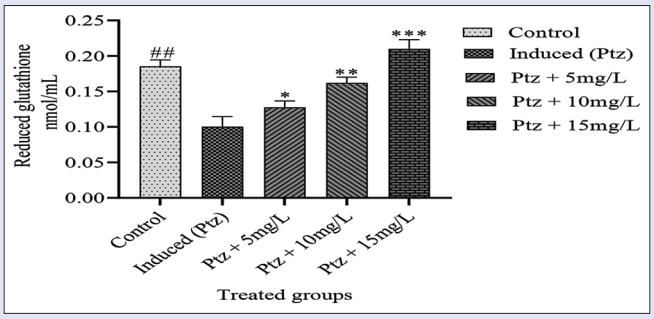

Figure 8: GSH *activity*

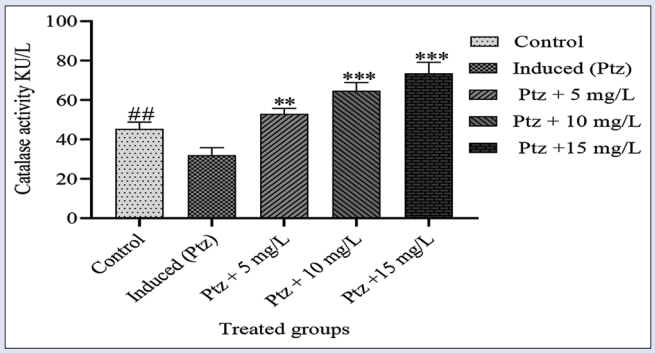

Figure 7: Catalase activity

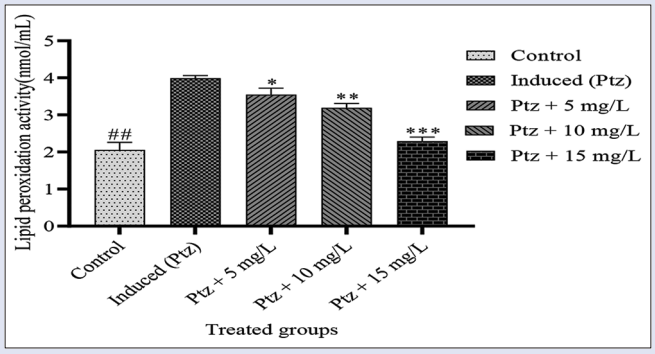

Figure 9: LPO assay

DISCUSSION

Epilepsy being one of the highly prevalent disorders of the brain, we evaluated the effect of AM in ameliorating epilepsy. Epilepsy was induced by administering PTZ in the tail in the zebrafish model. In the current work, we followed the strategy used to evaluate epilepsy-related behaviour in *Danio rerio*.[18] Various behavioural changes have been associated with epilepsy in zebrafish. Sudden freezing, erratic turning and twitching are some of the parameters related to epilepsy. We evaluated the behaviour of animals to evaluate the anti-epileptic activity of AM plant extract.

The behavioural analysis revealed that the extract was effective in restoring their activity to normal. Open field test is effective in revealing the behaviour of fishes. Thigmotaxis is the behaviour of animals including fishes that have anxiety. Thigmotaxis is characterised by the movement of animals near the wall and shying away from the centre.[19,20] In the rodent model, animals with epilepsy display an increased thigmotaxis.[21] From Table 1 and Figure 2, it is apparent that AM extract has a profound effect on reversing the effect of PTZ in an efficient manner. Incursion, on the other hand, is a type of behaviour in animals in which the animals tend to move to the centre.[22] While the administration of the extract AM reduced thigmotaxis, it increased the occurrence of incursion [Figure 2].

Scototaxis is a behaviour of animals wherein the animals prefer to stay away from the light. In the current experiment, epilepsy-induced animals showed a preference for darkness [Figure 4 and Table 2]. Scototaxis is a behaviour associated with anxiety.[23] Anxiety and anxiety-like behaviour have been well observed in individuals with epilepsy.[24] Upon treatment with the AM plant extract, the scototaxis behaviour is ameliorated in a dose-dependent manner.

During swimming, certain fishes undergo sudden freezing behaviour. This kind of sudden freezing has been linked to decreased AChE activity.[25] The accumulation of AChE at the synapses leads to alterations in the muscular activity in animals.[26-29] Since the AM plant extract reduces the incidence of freezing behaviour in epilepsy-induced fishes, it can be concluded that the plant extract activates the AChE activity.

An erratic swimming pattern is one of the signs of epilepsy in zebrafish. The fishes tend to make sudden twists with scrolling of the body. This could be due to the ongoing neurological changes associated with epilepsy.[30] Zig-zag and circular patterns of swimming are associated with anxiety. Anxiety and anxiety-like behaviour are symptoms associated with epilepsy.[31] The treatment with AM plant extracts successfully restored all the symptoms associated with epilepsy and anxiety-related abnormal

swimming behaviour. Open field tests, erratic swimming patterns and colour changes [Figure 3] collectively indicate the physiological stress induced by epilepsy.

Oxidative stress has been suggested as one of the reasons for the development of epilepsy.[3] A growing body of evidence suggests that oxidative stress leads to mitochondrial dysfunction during the development of epilepsy. We evaluated the catalase activity in the zebrafish. Surprisingly, we observed a dose-dependent increase in catalase activity upon treatment with AM plant extract. The elevated antioxidant activity was further confirmed by GSHactivity. Further, as a result of oxidative stress increased LPO has been observed in the epilepsy model.[32] To our surprise, the extent of LPO in epilepsy-induced zebrafish was significantly reduced by exposure to AM plant extract. Oxidative stress was further impaired by the elevated activity of SOD.

AChE is one of the precursors of a variety of neurotransmitters. In our epilepsy model, there was a strong inhibition of this enzyme. A decline in AchE activity can be correlated with neuronal death.[33-35] This correlates well with the freezing behaviour of fishes. Due to the lack of neurotransmitters, the fishes undergo sudden freezing behaviour. At neuromuscular junctions, due to the lack of neurotransmitters, there is likely to be a lag in muscular coordination.

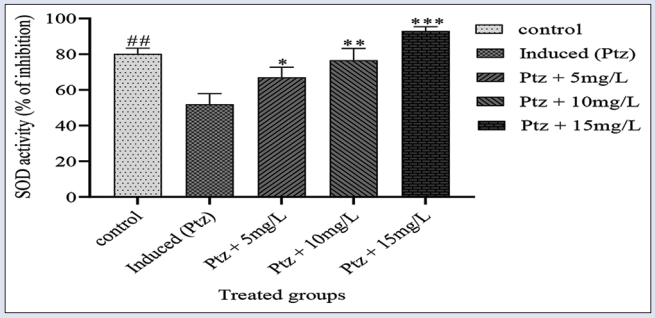

Figure 10: SOD assay

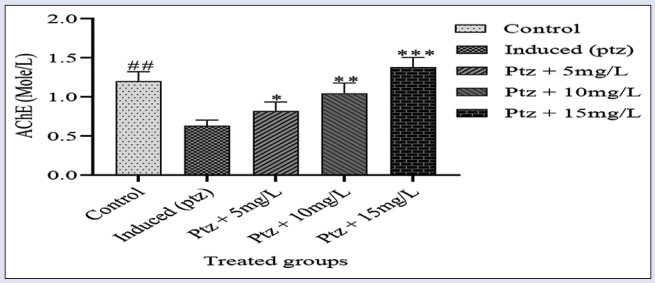

Figure 11: AChE assay

Estimation of the expression of proinflammatory cytokines TNF-α and IL-1β revealed that both are elevated in the cases of epilepsy. TNF-α from the glial cells tends to cause neuronal excitatory input.[36] Inflammation has been shown to be one of the causative agents of epilepsy.[37] IL-1β is also an inflammatory cytokine secreted by the microglia and astrocytes and has been shown to cause neuronal hyper-excitability through the upregulation of N-methyl-D-aspartate receptors.[38] AM plant extract has been observed to impair both TNF-α and IL-1β to result in controlling inflammation during epilepsy.

The role of inflammatory pathways in the development of epilepsy has well been elucidated.[37] Neurodegeneration is promoted by infiltrating neutrophils.[39,40] In the fishes treated with PTZ for the induction of epilepsy, there were more neutrophils in the brain sections indicating the onset of inflammation. Inflammatory reactions in the brain are also a sign of damage to the blood-brain barrier. The treatment with increasing doses of plant extract reduced the neutrophil accumulation and subsided the apoptosis of neuronal cells which can be correlated with the anti-inflammatory activity of the plant extract. Further, it also indicates the anti-apoptotic activity of the plant extract.

CONCLUSION

In the current study, AM plant extract effectively reduces epilepsy as suggested by the behavioural assays. The thigmotaxis and scototaxis behaviour are reduced while incursion is enhanced which are some of the signs of epilepsy. Sudden freezing behaviour was also reduced by the plant extract. Oxidative stress is one of the causes of epilepsy and AM plant extract effectively reduced the oxidative stress. The antioxidant property of the plant originates from the wide array of phytoconstituents including phenolics, flavonoids, linolenic acid, vitamin E, etc.[41] These phytoconstituents could have contributed to the antioxidant activity of the plant extract. Further, inflammation is also one of the reasons for the development of epilepsy and the plant extract reduced the inflammation. Overall, the plant extract is effective in reversing epilepsy through multiple mechanisms.

Acknowledgements

The authors would like to thank the BORIS software and the development team for making the software available as open-source.

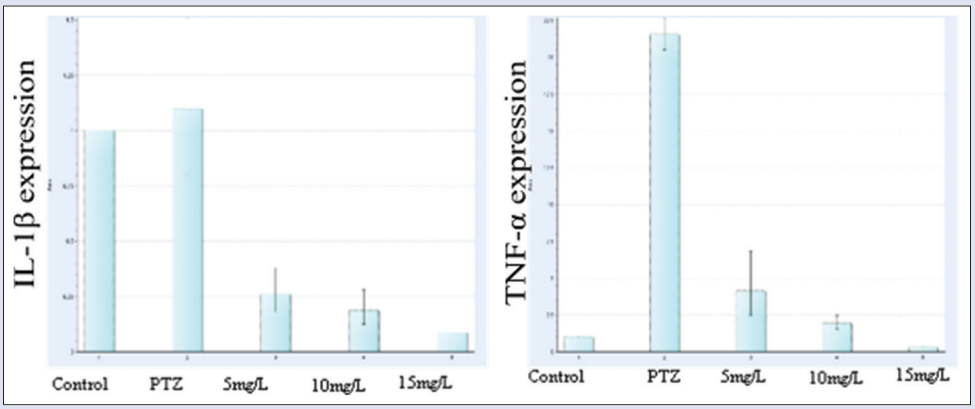

Figure 12: IL-1β and TNF-α

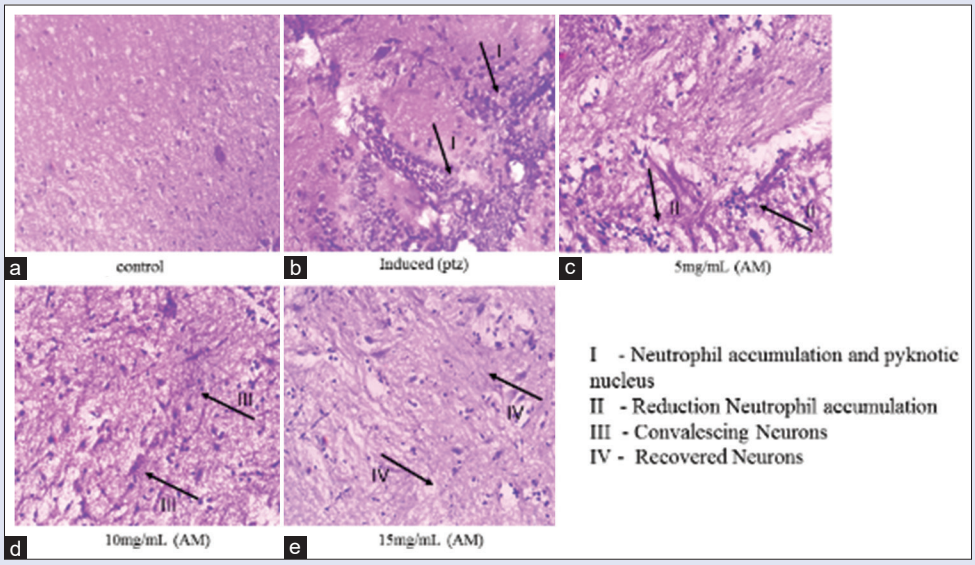

Figure 13: Histopathological analysis

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Nil.

Conflicts of interest

There are no conflicts of interest.

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