ABSTRACT

Objective: To evaluate the neuroprotective and neurodegenerative disorder of Aegle marmelos.

Methods: The present study was carried out to evaluate the Alzheimer and Parkinsonism disorders by Actophotometer test. Water maze test and Reserpine induced Parkinsonism models in sodium nitrite induced hypoxic rats.

Results: The Aegle marmelos fruit extracts improved the cognitive defects in hypoxic rats. The inquiry into behavioral studies indicates that sodium nitrite induced neurotoxicity, decreases the locomotor activity and decreases the habituation memory. The study shows a significant reduction (p<0.01) in both doses (200mg/kg and 400mg/kg) of Aegle marmelos extract in water maze test and locomotor activity test. Also the experiment showed the significant reduction in muscular rigidity by decreasing the gripping latency on the rod. The Aegle marmelos fruit extract produced a reduction in the Acetylcholin esterases (AChE) and glutamate levels but significantly increased in dopamine level. Increasing in AChE activity indicates that it ameliorates cholinergic function by inhibiting sodium nitrite which induces increase in AChE activity.

Conclusion: This study strongly suggests that the Aegle marmelos fruit extract attributed its prominent Alzheimer and Parkinsonism activity.

Keywords: Hypoxia, Alzheimer, Parkinsonism, Aegle marmelos, Neuroprotection.

INTRODUCTION

Oxidative stress is to be involved as one of the primary factors that contribute to the development of neurodegenerative diseases like Alzheimer, Parkinsonism and neurological conditions like brain damage, epileptic seizures, stroke, neurotrauma, hypoxia etc. [1]. Cerebral hypoxia relates to the condition in which there is a decrease of oxygen supply to the brain even though there is adequate blood flow, symptoms of mild cerebral hypoxia include inattentiveness, poor judgment, memory loss, decrease in motor co-ordination. Brain cells are extremely sensitive to oxygen lacking and can begin to die within five minutes after an oxygen supply has been cut off, when hypoxia lasts for longer periods of time, it results in coma, seizures and even brain death [2]. Hypoxia can be induced either by decreasing the oxygen level or by an administration of chemicals decreasing the oxygen level in the biological system which leads to oxidative stress in the cells, and may lead to cell mediated dysfunction and then apoptosis [3]. The oxidative stress hypothesis is appealing for Alzheimer disease and other neurodegenerative disorders, since neurons are post mitotic cells and gradual cumulative oxidative damage over time could account for the late life on set and the slowly progressive nature of the disorders [4].

Glutamate is the major excitatory neurotransmitter in the mammalian brain. About 70% of all synapses in the central nervous system utilize glutamate as a transmitter. Glutamate is essential for various physiological processes such as learning and memory perception and executions of motor acts. However, enhanced level of glutamate as observed in several CNS disorder is associated with neurodegeneration [5].

Glutamate is widely and fairly distributed in the CNS and its concentration in the CNS is much higher than the other tissues. It has very important metabolic rate the metabolic neurotransmitter pool is linked by transmitter enzymes that catalyses the inter conversion of glutamate and alpha-oxoglutarate, glutamate in the CNS comes mainly from glucose via the tricarboxylic acid cycle or glutamine which is synthesized by glial cells and taken up by the neurons very little from periphery [6]. Glutamate is stored in the synaptic vesicles and released by calcium dependant excocytosis. The action of glutamate is terminated by carrier mediated reuptake in the nerve terminals and neighboring astrocytes [7].

Aegle marmelos belonging to the family Rutaceae is a fruit bearing tree indigenous to the dry forest in the hills and plains of central and southern India, Sri Lanka, Myanmar. Traditionally, the plant Aegle marmelos is used largely as an analgesic, anti-inflammatory, antiviral and antimicrobial. The unripe dried fruit is used as astringent, digestive, stomachic and use to cure diarrhea and dysentery. The ripe fruit is a good and simple cure for dyspepsia [8].

MATERIALS AND METHODS

Plant material

The dried extract of Aegle marmelos was obtained from Amruta Herbal Pvt Ltd., Indore, through The Himalaya Drug Company Bangalore with Batch No. AHAM 1002, along with the certificate of analysis.

Experimental animals

Colony inbred strains of Wister rats of either sex weighing 200-220g were used for the pharmacological studies. The animals were kept under standard conditions (day/night rhythm) 8:00 am to 8:00 pm, 22 ± 1°C room temperature, in polypropylene cages. The animals were feed on standard pellet diet (Hindustan Lever Pvt Ltd., Bangalore) and water ad libitum.

The animals were housed for one week in polypropylene cages prior to the experiments to accustomed to laboratory conditions. It is randomly distributed into five different groups with six animals in each group under identical conditions throughout the experiments.

The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of (688/02/C-Committee for the Purpose of Control and Supervision of Experimental Animals) of Nanadha College of Pharmacy, Erode.

Chemicals

All the chemicals used in the study were of analytical grade.
Preliminary phytochemical study

The preliminary phytochemical screening was performed in accordance with the procedures of Trease and Evans [9].

Acute toxicity test: Acute toxicity of the Aegle marmelos extract has been carried out on 10 rats and 10 mice orally at the rate of 200-2000 mg/kg body weight and was closely observed for 24 hours after treatment of extracts and next ten days for any delayed effect. The Aegle marmelos extract was found to be safe till 2000 mg/kg.

Experimental design

The animals were divided into five groups of six rats each as follows:

- **Group I**: served as control and received normal saline (Normal control)
- **Group II**: served as hypoxic rats and received sodium nitrite water for 14 days (Hypoxic control)
- **Group III**: animals received standard drug Dextromethorphan (10 mg/kg; p.o) and sodium nitrite water for 14 days (Standard drug control)
- **Group IV**: animals received extract (200 mg/kg; p.o) suspended in 1% gum acacia and sodium nitrite water for 14 days.
- **Group V**: animals received extract (400 mg/kg; p.o) suspended in 1% gum acacia and sodium nitrite water for 14 days.

Induction of hypoxia

Hypoxia was induced by allowing the rats to drink sodium nitrite water (30 mg/kg) body weight dissolved in normal water by gavage for 14 days except the control group. Control animals were allowed to drink normal water [10].

Actophotometer test

The locomotor activity was determined by using Actophotometer. Before measuring the cognitive task the animal was placed in Actophotometer to record for 5 min. The locomotor activity was expressed in terms of total photo beam interruption counts / min / animal [11].

Water maze test

The Morris water maze was performed as described. The experimental apparatus consisted of the circular water tank (diameter=100 cm; height=35 cm), containing water at 28°C to a depth of 15 cm and rendered opaque by adding powdered milk. A platform (diameter 4.5 cm; height=14.5 cm) was submerged 0.5 cm below the water surface and placed at the midpoint of one quadrant. After several trials the test was conducted on the 14th day of the sodium nitrite drinking water. The time required to escape on to the platform was recorded [12].

Estimation of neurotransmitters and metabolic enzymes:

(a) Acetyl cholinesterase (ACHE) enzyme determination

20 mg of brain tissue/ml of phosphate buffer (PH 8 & 0.1 M) were homogenized in a potter-Elvehjem homogenizer. A 0.4 ml aliquot of brain homogenate was added to a cuvette containing 2.6 ml of 0.1M phosphate buffer (pH 8). 100 μl of the DTNB reagent was added to the photocell, the absorbance was measured at 412 nm. Acetyl cholinesterase (ACHE) enzyme determination

(b) Estimation of brain glutamate level by UV assay method

In this study, animals were decapitated 3hr after the last behavioral session. The brains were immediately excised and cerebellum discarded. The cortex, striatum and the sub cortical parts were separated and weighed. The sub cortical region of the brain comprised all the remaining parts of the forebrain after dissection of the cerebral cortex and striatum, including the hippocampus, thalamus, hypothalamus, amygdala and other sub thalamic structures [14].

Procedure

Weighted quantity of the brain portion was homogenized with 2 parts by weight of perchloric acid and centrifuged for 10 min at 3,000G. 3 ml of supernatant fluid was adjusted to pH9 with 1 ml phosphate buffer. It was allowed to stand for 10 min in an ice bath and then filtered through fluted filter paper. Absorbance was measured at 340 nm. Similarly a blank reading at 340 nm was measured. The level of glutamate was expressed as μmol/g tissue [14].

(c) Estimation of dopamine level in rat brain

On the day of experiment, rats were sacrificed; whole brain was dissected out and separated the sub cortical region (including the striatum). Weighed a specific quantity of tissue and homogenized in 3 ml HCl and 2 ml Butanol in a cool environment. The sample then centrifuged for 10 min at 2000 rpm. 0.8 ml of the supernatant phase was removed and added to an Eppendorf reagent tube containing 2 ml of N-Heptane and 0.25 ml of 0.1M HCl. After 10 minutes, shaken the tube and centrifuged under same conditions to separate two phases. Upper organic phase was discarded and the aqueous phase was used for dopamine assay.

To 0.02 ml of the HCl phase, 0.4 ml HCl and 0.01 ml EDTA/Sodium acetate buffer (pH 6.9) were added, followed by 0.01 ml iodine solution for oxidation. The reaction was stopped after 2 min by the addition of 0.1 ml Sodium Thiosulphate in 5M Sodium hydroxide. 10M acetic acid was added 1.5 min later. Then this solution was heated to 100°C for 6 min. When the sample again reaches room temperature excitation and emission spectra were read (330 to 375 nm) in a Spectrofluorimeter.

Then compared the tissue values (fluorescence of tissue extract fluorescence of tissue blank) with an internal reagent standard (fluorescence of internal reagent standard fluorescence of internal reagent blank). Tissue blanks for the assay were prepared by adding the reagents of the oxidation step in reversed order (Sodium thiosulphate before iodine). Internal reagent standards were obtained by adding 0.005 ml distilled water and 0.1 ml Butanol to 20 ng of dopamine standard [15].

Resperine induced Parkinsonism

Risperine is an alkaloid isolated from the dried roots of Rauwolfia serpentina belonging to the family Apocynaceae. Intraperitoneal (2.5 mg/kg) injections of Risperine in rats produced the signs and symptoms of parkinsonism. After 20-30 min of drug administration motor disorders are apparent. The animals were sedated and markedly hypo kinetic with poor movement coordination. Hind limb rigidity, arched body position, fixed facial expression and proad on the other typical effects of Reserpine. The effects peak at 1-2 h post administrations and subside within 24 h.

The animals were divided into five groups, namely vehicle control, negative control, positive control and two doses of test drug at 200mg/kg and 400mg/kg respectively. About 30 min after Reserpine administration, the test or standard drug was administered and percentage inhibition of the reserpine effect was evaluated [16]. A simple grasping test helps to assess the muscular rigidity of the animal. A metal rod (id 0.5 cm) was held at a height of 50 cm above table. The animal was made to grasp the rod with its forelimbs and the total time for which it remains on the bar was noted [17].

RESULT S

Behavioral studies

Standard drug (Dextromethorphan) and the Aegle marmelos dried fruit extract improve the cognitive defects in hypoxia treated rats.

Effect of standard drug (Dextromethorphan) and Aegle marmelos dried fruit extract on water maze

There is an increase in escape latency in hypoxic control group when compared with the control group (P<0.01). The group treated with Standard drug (Dextromethorphan) 10 mg/kg; p.o showed the significance of (p < 0.01). The groups treated with 200mg and 400mg of Aegle marmelos dried fruit extract showed the significance of (p < 0.01) as shown in Table 1.
Table 1: Effect of Aegle marmelos dried fruit extract on water maze

<table>
<thead>
<tr>
<th>Groups</th>
<th>Escape latency (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Normal control) normal saline 5 ml/kg p. o</td>
<td>30.16 ± 0.60</td>
</tr>
<tr>
<td>II (Hypoxic group) NANO ; 30mg/kg p. o</td>
<td>59.33 ± 0.72</td>
</tr>
<tr>
<td>III (Standard drug) 10 mg/kg + NANO ; p. o</td>
<td>29.66 ± 0.76</td>
</tr>
<tr>
<td>IV (AGMF Extract) 200mg/kg + NANO ; p. o</td>
<td>37.75 ± 0.70</td>
</tr>
<tr>
<td>V (AGMF Extract) 400mg/kg + NANO : p. o</td>
<td>30.30 ± 0.77</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM (n=6), a= **P<0.01 when compared with control group, b= **P<0.01 when compared with hypoxic control group. Symbol represents the statistical significance done by ANOVA, followed by Dunnet’s “t” test.

Table 2: Effect of Aegle marmelos dried fruit extract on locomotor activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
<th>150 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Normal control) normal saline 5 ml/kg p. o</td>
<td>370.83 ± 5.83</td>
<td>404.00± 2.13</td>
<td>413.83 ± 2.21</td>
<td>421.00± 4.05</td>
<td>431.16± 6.74</td>
<td>423.33± 7.20</td>
</tr>
<tr>
<td>II (Hypoxic group) NANO ; 30mg/kg p. o</td>
<td>199.66 ± 2.29</td>
<td>224.83± 2.21</td>
<td>244.83 ± 2.21</td>
<td>256.83± 2.97</td>
<td>264.83± 4.01</td>
<td>276.33± 8.36</td>
</tr>
<tr>
<td>III (Standard drug)</td>
<td>369.33 ± 2.17</td>
<td>401.50± 1.66</td>
<td>410.50 ± 1.76</td>
<td>416.33± 2.97</td>
<td>427.00± 4.01</td>
<td>426.16± 8.36</td>
</tr>
<tr>
<td>IV (AGMF Extract) 200mg/kg + NANO ; p. o</td>
<td>346.16 ± 2.13</td>
<td>386.50± 2.29</td>
<td>400.00±5.91b</td>
<td>405.16± 4.64b</td>
<td>415.83± 5.97b</td>
<td>421.33± 10.22b</td>
</tr>
<tr>
<td>V (AGMF Extract) 400mg/kg + NANO ;</td>
<td>365.00 ± 1.41b</td>
<td>395.50± 1.66b</td>
<td>405.66 ± 2.36b</td>
<td>412.66± 4.66b</td>
<td>423.00± 5.02b</td>
<td>425.00± 8.95b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM (n=6), a= **P<0.01 when compared with control group, b= **P<0.01 when compared with hypoxic control group. Symbol represents the statistical significance done by ANOVA, followed by Dunnet’s “t” test.

Effect of standard drug (Dextromethorphan) and Aegle marmelos dried fruit extract on locomotor activity

The hypoxia induced group (hypoxic control) indicated decrease in locomotor activity and showed a significance of (P<0.01) in comparison with the control group. The group treated with Standard drug (Dextromethorphan) 10 mg/kg; p. o showed the significance of (p < 0.01) in comparison with the locomotor activity. The groups treated with 200 mg and 400 mg of Aegle marmelos dried fruit extract showed the significant increase in the locomotor activity of (p < 0.01) as shown in table 2.

Effect of standard drug (Dextromethorphan) and Aegle marmelos dried fruit extract on Acetyl cholinesterase activity (ACHE)

Induction of hypoxia significantly (P<0.01) increased the ACHE activity when compared to normal control group. In the treated group there was a significant (P<0.01) reduction in enzyme levels on both standard drug (Dextromethorphan) and with 200 mg and 400 mg of Aegle marmelos fruit extract as shown in table 3.

Effect of standard drug (Dextromethorphan) and Aegle marmelos dried fruit extract on brain glutamate levels

Induction of hypoxia significantly (P<0.01) increased the glutamate levels when compared with normal control group. In the standard drug, Dextromethorphan treated group there was a significance (P<0.01) reduction in glutamate levels as well as on 200 mg/kg and 400 mg/kg of Aegle marmelos dried fruit extract treated rats as shown in the table 4.

Effect of standard drug (Dextromethorphan) and Aegle marmelos dried fruit extract on dopamine levels

The dopamine levels in negative control group had significantly decreased (P<0.01) when compared with the normal control group. The levels of dopamine in the standard drug Dextromethorphan treated animals showed a significant increase (P<0.01). The levels of dopamine in 200 mg/kg and 400 mg/kg of Aegle marmelos dried fruit extract treated groups increases significantly (P<0.01 and P<0.01) respectively in comparison with the negative control group as showed in the table 5.

Table 3: Effect of Aegle marmelos dried fruit extract on Acetyl cholinesterase activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Micromole/min/mg protein sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Normal control) normal saline 5 ml/kg p. o</td>
<td>16.05±0.20</td>
</tr>
<tr>
<td>II (Hypoxic group) NANO ; 30mg/kg p. o</td>
<td>20.21 ± 0.27b</td>
</tr>
<tr>
<td>III (Standard drug) 10 mg/kg + NANO ; p. o</td>
<td>15.10 ± 0.21b</td>
</tr>
<tr>
<td>IV (AGMF Extract) 200mg/kg + NANO ; p. o</td>
<td>13.18 ± 0.19b</td>
</tr>
<tr>
<td>V (AGMF Extract) 400mg/kg + NANO ; p. o</td>
<td>14.25 ± 0.16b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM (n=6), a= **P<0.01 when compared with control group, b= **P<0.01 when compared with hypoxic control group. Symbol represents the statistical significance done by ANOVA, followed by Dunnet’s “t” test.

Table 4: Effect of Aegle marmelos dried fruit extract on glutamate levels

<table>
<thead>
<tr>
<th>Groups</th>
<th>Micromoles/gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Normal control) normal saline 5 ml/kg p. o</td>
<td>74.01±0.49</td>
</tr>
<tr>
<td>II (Hypoxic group) NANO ; 30mg/kg p. o</td>
<td>86.13±0.32b</td>
</tr>
<tr>
<td>III (Standard drug) 10 mg/kg + NANO ; p. o</td>
<td>72.30 ± 0.59</td>
</tr>
<tr>
<td>IV (AGMF Extract) 200mg/kg + NANO ; p. o</td>
<td>68.06 ± 0.85b</td>
</tr>
<tr>
<td>V (AGMF Extract) 400mg/kg + NANO ; p. o</td>
<td>70.03±0.47b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM (n=6), a= **P<0.01 when compared with control group, b= **P<0.01 when compared with hypoxic control group. Symbol represents the statistical significance done by ANOVA, followed by Dunnet’s “t” test.
DISCUSSION

The present study has revealed that the neuroprotective effect of the standard drug (Dextromethorphan) and Aegle marmelos dried fruit extract on muscular rigidity (Parkinsonism).

Effect of standard drug (Dextromethorphan) and Aegle marmelos dried fruit extract on muscular rigidity (Parkinsonism)

Induction of hypoxia significantly (P<0.05) increased the muscular rigidity by being on the rod when compared with normal control group. In the standard drug Dextromethorphan treated group there was a significance (P<0.05) reduction in muscular rigidity by decreasing the gripping latency on the rod, as well as on 200 mg/kg and 400 mg/kg of Aegle marmelos dried fruit extract treated rats with significance (P<0.01 and P<0.001) respectively in comparison with negative control group, as shown in the table 6.

**Explanation of the table 6**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Gripping latency in seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Normal control) normal saline 5 mL/kg p. o</td>
<td>26.16 ±2.31</td>
</tr>
<tr>
<td>II (Hypoxic group) NANO 2; 30 mg/kg; p. o</td>
<td>34.00 ±1.46*</td>
</tr>
<tr>
<td>III (Standard drug) 10 mg/kg + NANO 2; p. o</td>
<td>25.00 ±2.50**</td>
</tr>
<tr>
<td>IV (AGMF Extract) 200 mg/kg + NANO 2; p. o</td>
<td>24.16 ±1.24**</td>
</tr>
<tr>
<td>V (AGMF Extract) 400 mg/kg + NANO 2; p. o</td>
<td>20.16 ±2.15**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM (n=6), a= **P<0.01 when compared with control group, b= **P<0.01 when compared with hypoxic control group. Symbol represents the statistical significance done by ANOVA, followed by Dunnet’s ‘t’ test.

The AChE activity has been shown to be increased within and around hypoxic brain. The calcium influx followed by oxidative stress is involved in the increase in activity of AChE induced by sodium nitrite, decreasing cell membrane order and ultimately leading to the exposure of more active sites of the enzyme. Increasing in AChE activity and ROS production indicates that it is possible to ameliorate cholinergic function by inhibiting sodium nitrite which increases in AChE activity.

The AChE activity in brain was increased in rats which were treated with sodium nitrite when compared with the normal. The sodium nitrite induces increase in AChE was attenuated by standard drug (Dextromethorphan) and Aegle marmelos dry fruit extract treatment.

Standard drug (Dextromethorphan) and Aegle marmelos dry fruit extract at 200 mg/kg and 400 mg/kg had shown the significant reduction on the gripping latency due to induced muscular rigidity by Reserpine.

CONCLUSION

In conclusion, we suggest that noticeably standard drug (Dextromethorphan) and Aegle marmelos dry fruit extract improves anti hypoxic effects induced by sodium nitrite and this effect mediated by the antioxidant properties. Further studies may be carried out to know the actual mechanism.

CONFLICT OF INTERESTS

Declared None

ACKNOWLEDGEMENT

The authors are very much thankful to Mr. S. Sengottuvelu of Nandha College of Pharmacy and Research Institute, Erode, TN for his continuous help during the project and also to the Management
of Nandha Institutions, Erode for supplying the facilities, reagents and chemicals required for the work.

REFERENCES