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*Research Article*

# The Influence of Ethanolic Extract of Seeds of *Peganum harmala* Linn. on Behavioral and Biochemical Studies in Cognitive Deficit Mice

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**Abstract**

The ethanolic extract of seeds of *Peganum harmala* (PHE) basically screened for its neurotoxic and psychoactive effect and found to be non-neurotoxic and psychoactive by preventing the motor impairment and increasing the locomotion activity of mice in Rota rod and Actophotometer respectively. PHE (5, 2.5 and 1.25 mg/kg p.o) significantly (P<0.001) protected the Sodium nitrite induced memory impairment by decreasing the time required to find the water bottle in spatial water bottle case model. In exteroceptive behavioral memory models of Elevated plus Maze (EPM) and Passive Shock Avoidance paradigm (PSA) the PHE shown improved acquisition and retention memory significantly (P<0.001) by decreasing the transverse latency time (TLT) and increasing the step down latency (SDL) respectively in dose dependent manner. These screening model results were well supported by biochemical parameters, by inhibiting the Acetylcholinestrase (P<0.01) activity, increasing the GSH (P<0.001) level and decreasing the TBARS (P<0.001) level of whole brain. Moreover PHE has exhibited the reduction in the Monoamine oxidase-A (MAO-A) even though a it is insignificant (P<0.05), hence it may delay the metabolism of epinephrine, 5-HT and other monoamines and enhances the action of these neurotransmitters indirectly; this adrenergic system plays an pivotal role in learning and memory. Furthermore PHE (5mg/kg) protected the DNA fragmentation of frontotemporal cortex of the brain from hypoxic effect induced by sodium nitrite in Gel Electrophoresis studies. The characterization of PHE by TLC and HPLC reveals the presence of Harmine and Harmaline alkaloids, might be potential enough to utilize in the management of Alzheimer's diseases.

## 1. INTRODUCTION

Alzheimer's disease affects one person in eight over sixty five and almost half over eighty five years of age, it is the 5th leading cause of death in the elder people. An estimated 4.5 million Americans are suffering from AD currently[1]. The worldwide prevalence of dementia was estimated at 34.4 million in 2009 of which potentially 17-24 million have Alzheimer's disease (based on the 50-70 percent estimate) [2]. Alzheimer's Association estimates the prevalence of Alzheimer's disease in US at 5.3 million. The world prevalence will increase over the next decades as a consequence of the aging Population and the demographic transition in developing countries. A recent report predicted that 66 million people worldwide would be affected by dementia in 2030 and 115 million in 2050 [3].

Therapeutic remedy of cognitive disorders like amnesia, loss of concentration and Alzheimer's disease is still an ordeal in the field of medicine. Memory enhancing drugs like Piracetam [4], pramiracetam, aniracetam[5] and Anticholinesterase drugs like Donepezil[6] are primarily used to enhance memory, mood and behavior. Nonetheless, the following adverse effects affiliated with these agents have narrowed their use [7,8,9].

A number of herbs traditionally employed in the Indian System of Medicine "Ayurveda" have yielded positive results. Ayurveda is the ancient medical science in the Indian subcontinent and is in practice since 12th century BC, that's what it said Indians are much healthier than any other countries. Herbal plants/phytochemicals are being used as a nootropic, improving memory, antiamnesic, neuroprotective and treatment of other cognitive dysfunctions. The use of naturally occurring plant alkaloids which could slow down the Alzheimer's disease: betaβ-carboline alkaloids (hermine) which inhibits the protein known as DYRK1A.

*Peganum harmala* L. occurs at various parts of North-West India and also found in Western Deccan, it belongs to a family Zygophyllaceae and commonly known as Harmala or Syrian rue. The synonym of the plant in difference languages are different in Hindi: Harmal, Kannada: Seeme goranti etc. It is being used as a traditional medicine in the treatment of many more diseases/disorders since ancient centuries; it possesses antioxidative,[10] antinociceptive, analgesic and anti-inflammatory properties[11].

It is used for the treatment of variety of human ailments,[12] a harmal content of this plant enhances the action of epinephrine[13] and β-carboline alkaloids isolated from this plant are reported to possess antidepressant[14]. The different alkaloids of *peganum harmala* have been shown to be monoamino oxidase inhibition (MAO-I)[15], MAO-I can stimulate the central nervous system by inhibiting the metabolism of serotonin and other monoamine. This inhibition of break-down of serotonin in the human body makes MAO-I effective antidepressants. In warm blooded animals, seeds as well as the Harmal alkaloids cause a primary stimulation of motor tracts of the cerebrum, and spinal cord, giving rise to tremors and clonic convulsions. Harmal alkanoids and their derivatives have been suggested for use as protozoicidal agents, coronary dilators, ecbolics and used in the treatment of nervous diseases like post-encephalitic and neuralgia conditions [16]. The seeds of plant contain compounds known as harmal alkaloids; have a long history of use as psychoactive drug, and other purposes. Huge medicinal properties of *Peganum harmala* enforces to reveal the potentiality of *Peganum harmala* in the management of neurodegenerative disorders.

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## 2. MATERIALS AND METHODS

### 2.1 Chemicals

*Peganum harmala* seeds were obtained from Gadghil shop, a traditional herbal product supplier, Belgaum, Karnataka, India. The standard Harmine and Harmaline were obtained from Sd fine chemicals Bangalore . Piracetam was obtained as gift sample from Elite pharmaceuticals, Gujarat. Sodium nitrite purchased from Loba Chemie, Mumbai, India. 5, 5'-dithiobis (2-nitro benzoic acid) (DTNB), Trichloro-Acetic acid (TCA), Thio-Barbituric acid (TBA) and Acetylthiocholine iodide from Himedia and Sigma Aldrich Bangalore, India. RNA later solution and QIAGEN DNeasy Blood & Tissue kit from DNA diagnostics, Karnataka University Dharwad. Other chemicals used were of analytical grade.

### 2.2 Animals

All the experiments were carried out with young Swiss Albino Mice 22-28g (3 month old) after approvals from the Institutional Animal Ethical Committee (IAEC) vide the Approval Number: SETCP/IAEC/ 2008-2009/242. The Procedures are in compliance with the prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA), Government of India. Animals were kept in the animal house of S.E.T's College of Pharmacy, Dharwad, India, under controlled conditions of temperature (23±2ºC), humidity (50±5%) and 12 h light-dark cycle. Animals were fed with rat diet pellet procured from Venkateshwara enterprises, Bangalore and water *ad libitum*. All the animals were acclimatized for seven days before to start the experimental studies.

### 2.3 Plant material

The seeds of *Peganum harmala* were identified and authenticated by qualified taxonomist Dr. S. S. Hebbar, Department of Botany, Karnataka University Dharwad (KUD) Dharwad. A voucher specimen No. SETCPD/Ph.cog/herb/2008/11 has been retained in our department for further reference.

### 2.4 Ethanolic extract of Seeds of *Peganum harmala* (PHE)

The processed dry seeds (100g) were grinded and were extracted with 80% ethanol for 24 hrs in a continuous extraction using Soxhalet apparatus. The extract was filtered, and ethanol was evaporated on a rotator evaporator under vacuum at a temperature of $45^0$C to a reduced solid state yielding a 10.51 g of extract. Then the PHE was subjected to qualitative chemical tests[11] to confirm the phytochemicals present in it (Table 1).

### 2.5. Preliminary screening of phytoconstituents

The ethanolic extract from *Peganum harmala* (PHE) was used for preliminary screening of phytochemicals such as alkaloids (Wagner and Dragendorff's tests), flavonoids (Shinoda and NaOH tests), glycosides (Keller-Kiliani, conc.$H_2SO_4$ and Molish tests), Lignins (Labat and Lignin tests), phenols (ellagic acid and phenol tests), saponins (foam and haemolysis tests), sterols (Liberman-Burchard, and Salkowski tests), tannins (gelatin test) were carried out [11].

### 2.6. Quantitative estimation of secondary metabolites (Alkaloids)

The presences of alkaloids as secondary metabolites in seeds were quantitatively determined by adopting standard protocol of Harborne method. About 5 g of extract was weighed into a 250ml beaker, and a 200ml of 10% acetic acid in ethanol was added, and allowed to stand for 4hrs. This was filtered using whatmann No.42 filter paper, concentrated in water bath to ¼ (50ml) of the original volume. Then, concentrated $NH_4OH$ was added drop wise to each extract until the precipitate was complete. The suspension was allowed to settle and the precipitate was collected, washed with $NH_4OH$ and then filtered. The residue was dried and weighed. The percentage alkaloid was then calculated[17].

### 2.7. TLC studies

The PHE was dissolved in ethanol and used for chromatographic separation, in which the different spots were separated using the solvent mixture Chloroform, acetone, Diethylamine in the ratio of 50:40:10. The color and $R_f$ values of the separated alkaloids were made visible under the influence of UV light at 254 and 365 nm[18].

### 2.8 HPLC Analysis

#### *2.8.1 Standard Preparation:*

Weigh accurately about 15.00 mg of Harmine & 10.00 mg of Harmaline working Standard were into 25 ml volumetric flask. Add 20 ml Methanol, mixing on cyclo-mixer 2 min after sonicate to dissolve 10 min and make volume with Methanol. Dilute 2 ml of obtained solution into 20 ml of volumetric flask and make volume with Methanol [19].

#### *2.8.2 Sample Preparation:*

Weigh accurately about 100.00 mg of Extract were into 20 ml volumetric flask. Add 20 ml Methanol after sonicate to dissolve 10 min. and make volume with Methanol .Dilute 2 ml of obtained solution into 20 ml of volumetric flask and make volume with Methanol.

#### *2.8.3 Chromatographic conditions*

| | | |
|---|---|---|
| **HPLC** | : | Shimadzu LC- 2010 |
| **Column** | : | Hypersil ODS, C-18 250mm x 4.6mm, 5 μm |
| **Column temperature** | : | 25°C |
| **Mobile Phase** | : | Phosphate Buffer (10 mM pH 6.5): Acetonitrile : Methanol (55:20:25) |
| **Flow rate** | : | 1.2 ml/min |
| **UV Detector** | : | 330 nm |
| **Injection volume** | : | 20µl |
| **Sample compartment temperature** | : | 10°C |
| **Run Time** | : | 30 min |

#### *2.8.4 Evaluation of system suitability*

1) The %RSD for the peak areas of Harmine & Harmaline from five replicate injections of final standard preparation should not be more than 2.0.
2) Tailing factor should NMT 2.0.

#### *2.8.5 Calculation Formula*

$$\text{Assay \%} = \frac{\text{Test Area}}{\text{Std. Area}} \times \frac{\text{Std wt}}{25} \times \frac{2}{20} \times \frac{20}{\text{Spl wt}} \times \frac{}{5} \times \text{Potency}$$

### 2.9. Acute oral toxicity study

Study was performed according to OECD (423) guidelines, Annex 2. Male Swiss mice were selected by random sampling technique. The animals were fasted for 4 hrs with free access to water. Ethanolic extract of *Peganum harmala* (PHE) was administered orally at a dose of 5 mg/kg initially and mortality if any was observed for 72 hrs. If mortality was observed in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one animal out of three animals then the same dose was repeated again to confirm the toxic effect. If no mortality was observed, then only higher (50, 300, 2000mg/kg) dose of PHE extract were employed for further toxicity studies. The animals were observed for toxic symptoms such as behavioral changes, locomotion, convulsions and mortality for 72 hours. Based on the results of acute toxicity studies, it was found that PHE produced severe convulsion, but no mortality were seen at a dose of 300mg/kg b.w. p.o. These severe excitotoxic convulsive effects of PHE were consistent with the previous literature findings, in which the warm blooded animals, seeds cause a primary stimulation of the motor tracts of the cerebrum, and spinal cord, giving rise to tremors and clonic convulsions[16]. Hence 50mg/kg was considered as a safe dose and $1/10^{th}$ of this safe dose was selected as therapeutic dose. To study the dose dependent effect of PHE for its protective action, three doses were selected as a PHE 5, 2.5 and 1.25mg/kg., b.w., p.o.

### 2.10. Preparation of doses

A daily and freshly prepared (in distilled water) doses of Ethanolic extract of *Peganum harmala* (PHE) (5, 2.5 & 1.25 mg/kg b.w p.o) were given to the different groups of experimental animals. A standard drug Piracetam was prepared by dissolving in distilled water, Sodium nitrite 75mg/kg i.p in normal saline and ethanol

(50%) 2.5g/kg i.p in distilled water and was given to their respective groups.

**2.11. Effect of PHE on Motor Coordination and Neurotoxicity: Rota-rod Apparatus**

The motor coordination was assessed using digital Rota rod (Inco-Ambala, India) apparatus. Mice were trained by placing them on a rotating rod (20 rpm), twice daily for three consecutive days before to conduct the experiment, thirty min interval was kept between the two trails. Only those mice which have demonstrated their ability to remain on the rotating rod for at least 2 min were selected for experimental study, motor co-ordination was considered to be impaired if the animal fell-off from the rotating-rod within 9 sec [20]. These selected mice were divided into five groups with 6 animals in each group, and after one hour of administration of PHE 5, 2.5 and 1.25 mg/kg, p.o. and standard drugs, mice were placed on the rotating rod to test for their motor coordination and to record number of falls per 5 minutes of time. The difference between mean numbers of falls between the groups was considered for evaluation of motor impairment. Diazepam (2 mg/ kg i.p.) was used as reference standard [21].

**2.12. Effect of PHE on Psychoactive and CNS stimulant activity: Actophotometer**

The locomotor activity (horizontal activity) was measured using a digital actophotometer (Inco- Ambala, India). Mice were divided into different experimental groups and gave their respective test/standard drugs. Each mouse was placed individually in the actophotometer for a period of 5 min and basal activity score was obtained after one hour administration of test/standard doses. The results were reported as mean change in the locomotion activity. Diazepam (2 mg/ kg/i.p.) preparation was used as reference standard[22].

**2.13. Effect of PHE on Sodium nitrite intoxication: Spatial two chambered water cage box**

Spatial two-chambered cage was used with the dimension 16 inch length, 11 inch breadth and 5 inch height. A partition placed at a distance of 6 inches from one of the end of cage; divide the cage into a smaller and larger chamber. Mice were randomly divided into 5 groups of 6 each, in which group 1 served as a normal control; group 2, 3 and 4 received PHE (5, 2.5 and 1.25mg/kg p.o); group 5 served as a standard, received Piracetam (400 mg/kg p.o) respectively.

A water feeding bottle was kept in smaller chamber; the animals were water deprivation for 24 hrs. After 90 min of treatment on 15$^{th}$ day, the animals were placed in the larger chamber and allowed to explore the cage. Once the water deprived animal locate the bottle, it was allowed to drink the water for 30 second, the time required to locate the water bottle was noted as 1$^{st}$ retention time. Immediately after 1st retention test before the animal being placed in home cage all the animals except group 1 were injected with sodium nitrite (75 mg/kg i.p). After 24 hrs later the animals were again placed in the larger chamber of two-chamber cage. The time required to locate the water bottle was noted as a day second reading (2nd retention test). But this time the water bottle was kept empty. Immediately after 2$^{nd}$ retention test the whole brain of mice were dissected out and processed for MAO-A assay and estimation [23, 24].

**2.14. Effect of PHE on Ethanol induced Neurodegeneration: EPM & PSA**

In the present investigation the mice were divided into different groups for various interoceptive and exteroceptive behavioural memory models, each group comprised of a minimum of six animals. A group1: served as a young normal received Distilled Water (DW) in the dose of 10 ml/kg p.o. Group 2: as a negative standard control received only ethanol at a dose of 2.5 g/kg, i.p, Group 3: piracetam per se (400 mg/kg, p.o), Group 4: PHE per se 5mg/kg p.o, group 5: received Piracetam and ethanol, group 6, 7 & 8: received daily doses of PHE (5, 2.5 and 1.25mg/kg p.o) and ethanol for 15 days. After 90 minutes of the administration of the different doses of PHE on 15$^{th}$ day, amnesia was induced in young animals by injecting ethanol (2.5 g/kg, i.p) to all the groups of mice except group 1, 3 and 4. The mice were exposed to the training session after 30 minutes of ethanol injection using elevated plus maze and passive shock avoidance apparatus (step down). Retention memory was recorded after 24 hrs on 16$^{th}$ day, immediately after behavioral testing on 16$^{th}$ day whole brain was dissected out and utilized for Acetylcholinestrease, GSH and TBARS estimations.

***2.14.1. Elevated plus maze (EPM)***

EPM served as the exteroceptive behavioral model to evaluate short-term memory in mice. Briefly, EPM for mice consisted of two open arms (16 cm x 5 cm) and two covered arms (16 cm x 5 cm x 12 cm) extended from a central platform (5 cm x 5 cm), and the maze was elevated to a height of 25 cm from the floor. On the first day (i.e. 15$^{th}$day of PHE administration), each mouse was placed at the end of an open arm, facing away from the central platform to measure the acquisition Transfer latency (TL) time. TL was defined as the time (in seconds) taken by the animal to move from the open arm into one of the covered arms with all its four legs. The mouse was allowed to explore the maze for another 2 min and then returned to its home cage. Retention of this learned task (memory) was examined 24 h after the acquisition trial. Significant reduction in TL value indicated improvement of memory [25,26].

***2.14.2. Passive shock avoidance (step down) paradigm.***

Passive avoidance behavior based on negative reinforcement was recorded to examine the long- term memory. The apparatus consisted of a box (27×27×27 cm) having three walls of wood and one wall of Plexiglas, featuring a grid floor (3 mm stainless steel rods set 8 mm apart), with a wooden platform (10×7×1.7 cm) in the centre of the grid floor. The box was illuminated with a 15 W bulb during the experimental period. Electric shock (20 V AC) was delivered to the grid floor. Each mouse was gently placed on the wooden platform set in the centre of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, shocks were delivered for 5 sec and the step-down latency (SDL) was recorded. SDL was defined as the time taken by the mouse to step down from wooden platform to grid floor with its entire paw on the grid floor. Retention was tested after 24 h in a similar manner, except that the electric shocks were not applied to the grid floor, with an upper cut-off time of 180 s [27,28].

**2.15. Effect of PHE on brain acetyl cholinesterase (AchE).**

The estimation of whole brain acetyl cholinesterase activity is carried out based on Ellman's method with slight modifications. The animals were decapitated and brains were dissected out immediately and placed in ice-cold saline. The tissue was weighed and homogenized in 0.1M Phosphate buffer pH 8 (10% w/v), the homogenized tissue was centrifuged to 15,375 x g. for 10 min. 0.4ml aliquot of the supernatant is added to a cuvette containing 2.6 ml phosphate buffer (0.1M, pH 8) and 100µl of DTNB. The contents of the cuvette are mixed thoroughly by bubbling air and absorbance is measured at 412 nm in a Lab India spectrophotometer. When absorbance reaches a stable value, it is recorded as the basal reading. 20µl of substrate i.e., acetylthiocholine is added and change in absorbance is recorded for a period of 10 min. at intervals of 2 min. Change in the absorbance per min. is thus determined. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to 1 x $10^{-7}$ mol of substrate hydrolyzed per minute, using a molar extinction coefficient of 1.36 × $10^{-3}$ $M^{-1}cm^{-1}$. AchE activity is calculated using the formula; [29].

$$R = \frac{\S \text{ O.D. x Volume of Assay (3 ml)}}{E \text{ x mg of protein}}$$

Where,

R = Rate of enzyme activity in 'n' mole of acetyl choline iodide hydrolyzed/min/mg of protein

§ O.D. = Change in absorbance/min,

E = Extinction coefficient 13,600/M/cm

**2.16. Effect of PHE on reduced glutathione (GSH)**

The animals were sacrificed; whole brain was dissected out and homogenized in phosphate buffer pH 8, 10% w/v. The homogenates were centrifuged at 15,375 x g. at 4$^0$C for 20 min. using Remi C-24 high speed cooling centrifuge. Equal volumes of tissue homogenate (supernatant) and 20% trichloroacetic acid were mixed. The precipitated fraction was centrifuged and to 0.25ml of supernatant, 2ml of 0.6mM 5, 5'-dithiobis (2-nitro benzoic acid) reagent was added. The final volume was made up to 3ml with phosphate buffer (0.2M, pH 8.0). The colour developed was read at

412nm against reagent blank. The enzymatic activity is expressed as unit (U) per liter. A U corresponds to one µmol of substrate hydrolyzed per liter, using a molar extinction coefficient of 1.36 × $10^{-3}$ $M^{-1}cm^{-1}$ [30,31].

**2.17. Effect of PHE on Thio Barbituric Acid Reactive Substances (TBARS)**

The animals were sacrificed; whole brain was dissected out and homogenized in phosphate buffer pH 8, 10% w/v. The homogenates were centrifuged at 15,375 X g at $4^0$C for 20 minutes using Remi C-24 high speed cooling centrifuge. 2.0ml of the tissue homogenate (supernatant) was added to 2 ml of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 min. the precipitate was separated by centrifugation and 2.0ml of clear supernatant solution was mixed with 2 ml of freshly prepared 0.67% thiobarbituric acid (TBA). The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The color developed was measured at 532nm against reagent blank. The enzymatic activity is expressed as unit (U) per liter. A U corresponds to µmol of substrate hydrolyzed per liter, using a molar extinction coefficient of 1.56 x $10^5$ $M^{-1}$ $cm^{-1}$ [32,31].

**2.18. Effect of PHE on Monoamine oxidase-A (MAO-A)**

The collected brain samples were washed with cold 0.25 M sucrose, 0.1 M Tris, 0.02 M EDTA buffer (pH 7.4) and weighed. The whole procedure of brain isolation was completed within five minutes. The rat brain mitochondrial fraction was prepared following the procedure. The MAO activity was accessed using spectrophotometer. Briefly, the buffer washed brain sample was homogenized in 9 volumes of cold 0.25 M sucrose, 0.1 M Tris, 0.02 M EDTA buffer (pH7.4) and centrifuged twice at 800 x g for 10 min at 4°C in cooling centrifuge (Remi Instruments, Mumbai). The pellets were discarded and the supernatant was then centrifuged at 12000 x g for 20 min. The precipitates were washed twice with about 100 ml of sucrose-Tris-EDTA buffer and suspended in 9 volumes of cold sodium phosphate buffer (10 mM, pH 7.4, containing 320 mM sucrose) and mingled well at 4°C for 20 min. The mixture was then centrifuged at 15000 x g for 30 min. at 4°C and the pellets were re-suspended in cold sodium phosphate buffer. The protein concentration was estimated by Lowry method using bovine serum albumin.

For estimating MAO-A activity, 2.75 ml sodium phosphate buffer (100 mM, pH 7.4) and 100µl of 4 mM 5-hydroxytryptamine were mixed in a quartz cuvette which was then placed in double beam spectrophotometer (Systronics 2230, Bengaluru, India). This was followed by the addition of 150 µl solution of mitochondrial fraction to initiate the enzymatic reaction and the change in absorbance was recorded at wavelength of 280 nm for 5 min against the blank containing sodium phosphate buffer and 5-hydroxytryptamine. An activity unit was defined as 0.001 absorbance produced by 1mg protein reaction. So the enzyme activity was expressed as follows [33].

$$\text{Enzyme activity} = \frac{\text{Change in absorbance} \times 3}{\text{mg of protein} \times 0.001}$$

**2.19. Effect of PHE on DNA Fragmentation studies of Brain**

The animals were sacrificed; whole brain was dissected out immediately and stored in RNA later solution at -80°C. The samples were brought to room temperature and used. The isolation of DNA was carried out using QIAGEN DNeasy Blood & Tissue kit, which isolates DNA by coloumn elution method. The samples were first treated with Qiagen lysis buffer and protease K and incubated at 55°C until the tissue was completely lysed. Then, RNase (20 mg/mL) was added to each sample and incubated at room temperature for 2 min. Next, 180 µL of ethanol and buffer was added to each sample, and the mixture was transferred to a spin column. The columns were centrifuged at 6000 x g for 1 minute and washed twice. The DNA was then eluted from the spin column by centrifugation. Approximately 4 µl of DNA was loaded in each lane and run at 100 V on a 1 % agarose gel stained with ethidium bromide (0.5 mg/mL). One microgram of DNA standard was run in next lane.

**2.20. Statistical Analysis**

The data were analyzed statistically using analysis of variance (ANOVA) followed by Tukey's post test. Values are expressed as Mean ± Standard errors of mean (S.E.M). P<0.05 is considered as significant and P□0.05 were considered as non-significant. Statistical comparisons were performed by Tukey's post test using Graph Pad Prism version 5.0, U.S.A.

## 3. RESULTS

**3.1. Preliminary screening of secondary metabolites**

The Preliminary screening of the extract of PHE confirms that, there are alkaloids present in the extract, the details are depicted in Table 1.

**3.2. Quantitative estimation of secondary metabolites**

The quantitative estimation of secondary metabolites (alkaloids) present in PHE was found to be 52.38 % as depicted in Table 2.

**3.3. TLC study of alkaloids**

TLC studies of alkaloids of PHE exhibits 5 spots under UV light at 254 and 365 nm. Three spots observed under UV light at 365 nm are fluorescent green in colour with the $R_f$ value of 0.14, 0.32 and 0.47. This $R_f$ value corresponds to the standard alkaloids of harmalol, harmaline and harmine respectively. Furthermore another two spots have been observed under same UV light at 254 nm which are bluish purple in colour with the $R_f$ value of 0.88 and 0.94 respectively, these $R_f$ value are comparable to the $R_f$ values of standard extract [18.] The detailed data obtained were tabulated in Table 3 and Fig. 1.

**3.4 HPLC studies of PHE**

The seeds of *pegamum harmala* are known for the presence of harmine alkaloids. The HPLC chromatogram of two standard alkaloids viz, Harmine and Harmaline have shown the retention time of 3.90 and 4.56 (Rt,min) respectively. The HPLC chromatogram of PHE has shown 2 prominent peaks with significant area. The most abundant peak with 5294783 area is observed at the retention time 3.88 (Rt,min), and another abundant peak with 9830701 area is observed at the retention time 4.59 (Rt,min), which were very closely comparable to HPLC of known standard alkaloids of harmine and harmaline. Hence it may be concluded that PHE contains harmine and harmaline alkaloids. Quantity of these alkaloids in the extract has also been analyzed and found to be 3.26 % and 2.44 % for harmine and harmaline alkaloids respectively as depicted in Table 4. The HPLC spectra of blank, standard alkaloids and PHE have been depicted in Fig. 2, Fig. 3, and Fig. 4.

**3.5 Effect of PHE on motor coordination**

All doses of PHE (5, 2.5 and 1.25 mg/kg) were found to be non-neurotoxic and results were comparable to the normal group of animals, hence these selected doses of PHE do not have any king of impaired motor co-ordination. Diazepam (2 mg/kg) showed significantly (P<0.001) highest number of falls per 5 minutes of time, as indicated in Table 5.

**3.6 Effect of PHE on Locomotion activity**

The depressant standard drug Diazepam (2mg//kg, i.p) significantly (p<0.001) reduces the locomotion of animals comparative to normal group of animals. Whereas different doses of PHE; 5, 2.5 and 1.25 mg/kg. p.o., significantly (p<0.001 p<0.05 and p□0.05) increases the locomotion activity of animals comparative to normal group of animals indicating that these does have dose dependant psychoactive/CNS stimulant activity respectively as indicated in Table 5.

**3.7 Effect of PHE on Sodium Nitrite induced intoxication (Hypoxia)**

In before administration of test/standard drugs of 1st retention test on 15th day all the groups of animal did not show any significant differences in finding the water bottle except hypoxia induced group2, administered with Sodium nitrite alone, which enhances the time require to find the water bottle significantly (P<0.05) compare to normal group 1. After administration of drugs in 2nd retention test, the hypoxia group 2, significant (P<0.001) increase the time compare to normal group 1. Piracetam 400 mg.kg, p.o, and all the

doses of PHE; 5, 2.5, 1.25 mg/kg showed significant P<0.001, P<0.001, P<0.001, and P<0.05 reduction in time to locate the water bottle compare to hypoxia-induced negative group 2 respectively. Group 3 Piracetam per se significantly P<0.01 decreases the time, whereas group 4, PHE 5mg/kg. p.o per se, although it reduce the time, was found to be insignificant (P□0.05) compared to normal group, as indicated in Table 6.

**3.8 Effect of PHE on Ethanol induced Neurodegenaration**

The effect of Ethanol (Eth) (2.5 g/kg; i.p.) induced amnesia in mice before training session for elevated plus maze (EPM) and passive shock avoidance (PSA) are represented in Table 7 and 8. Furthermore, Ethanol administered mice significantly (P<0.001) increased transfer latency (TL) and profoundly decreased the step down latency (SDL) when compared to normal group of animals in EPM and PSA. These observations suggested that Ethanol has produced impaired learning as well as memory. However, per se group of Piracetam and PHE (5mg/kg) improve memory in EPM model significantly (P<0.01), in PSA model, these two groups significantly increase memory (P<0.001) and (P<0.001) respectively. Ethanol induced memory deficits were successfully reversed by PHE (5mg/kg) (P<0.001), PHE 2.5mg/kg (P<0.001), PHE 1.25mg/kg (P<0.05) as indicated by decreased TL and increased SDL, as indicated in Table 7 & 8 respectively. Pre-treatment with Piracetam (400 mg/kg, p.o) reversed ethanol induced memory deficits (P<0.001) as expected in both the models, as indicated in Table 7 & 8 respectively.

**3.9. Effect on Whole Brain Acetylcholinesterase (AchE) activity**

Ethanol per se significantly (P<0.001) increased AchE activity compared to normal. Whereas Piracetam per se reduced the activity significantly (P<0.01) and PHE (5mg/kg) alone reduced the AchE activity but it was insignificant (P□0.05) compared to normal control. On the other hand, administration of Ethanol (2.5 g/kg, i.p.) increased the brain AchE activity, which was reversed by PHE 5mg/kg (P<0.01), PHE 2.5mg/kg (P<0.05) and PHE 1.25mg/kg (P>0.05), when administered for a period of 14 days. Piracetam (400 mg/kg; p.o) being a standard drug, reversed increased AchE (P<0.001) induced by Ethanol compared to ethanol group of animals, as indicated in Fig. 5.

**3.10. Whole brain Antioxidant studies (GSH and TBARS)**

Ethanol per se group significantly (P<0.001) decreases GSH and increases (P<0.001) TBARS levels compared to normal group. Per se group of Piracetam and PHE (5mg/kg) increases GSH level significantly (P<0.001) and insignificantly (P□0.05) respectively, whereas in TBARS level these two groups are significantly decreases (P<0.001) and (P<0.01) respectively. Pretreated groups such as piracetam and PHE of all the doses were successfully reverses (P<0.001) and (P<0.001) respectively, except PHE 2.5 mg/kg and PHE 1.25 were having (P<0.01) & (P<0.05) in GSH, as indicated in Fig. 6 & 7 respetively.

**3.11. Whole brain Monoamine oxidase-A (MAO-A) Assay**

Sodium nitrite induced Hypoxia group increases the MAO-A activity significantly (P<0.001) compared to the normal group of animals. Whereas pretreated doses of PHE (5, 2,5 and 1.25 mg/kg, p.o) do not reverse the elevated MAO-A activity induced by Sodium nitrite, as indicated in Table 9.

**3.12. DNA fragmentation studies of frontotemporal cortex of brain by Gel Electrophoresis**

The isolation of DNA from frontotemporal cortex of different experimental group of animals was subjected to 1 % agarose Gel electrophoresis. Lane 1 shows the laddering pattern of standard 100 bp DNA. Lane 2 indicates normal laddering pattern of DNA from Normal group of animal. Lane 3 shows oligointranucleosomal DNA fragmentation and distinct laddering pattern in hypoxia induced frontotemporal cortex. Lane 4 shows decreased intranucleosomal DNA fragmentation in Hypoxia + PHE (5mg/kg) treated animals. Lane 5 shows standard Bam H fragmented DNA, to compare the presence of Genomic DNA. In hypoxia induced neurodegenerative disorder, prominent apoptosis and DNA fragmentation occurs. This study reveals the information regarding the effect of sodium nitrite induced Hypoxia on fragmentation of DNA and laddering pattern in frontotemporal cortex, as indicated in Fig. 8.

**4. DISCUSSION**

The data generated from the characterization of the PHE by preliminary phytochemical screening, quantification of alkaloids, TLC and HPLC has provided the chemical basis for the wide distribution of alkaloids present in the seeds of *Peganum harmala*, which may be responsible for reversal effect of sodium nitrite and ethanol induced neurotoxicity of the present study.

Muscular impairment/incordination is a reflect of neurotoxicity of a compound, a Rota rod appparus is being recommended to study the impaired motor coordination. In this present study all the doses of PHE 5, 2.5 and 1.25 mg/kg did not produced any kind of impaired motor coordination by reduction in the number of falls, hence ruled out the possible neurotoxic effect of ethanolic extract of *Peganum harmala*.

The psycho-activeness of PHE 5, 2.5 and 1.25 mg/kg might be due to its antidepressant effect, which was in seen in actophotometer model by increasing the locomotion activities of animals, hence this psychoactive/antidepressant effect might be due to its MAO-A inhibition effect of PHE [15].

Sodium nitrite impairs memory in similar fashion to memory impairment in aged mice[23,24] which involve the impairment of Ach synthesis in brain[34]. The evidences can be obtained from the various experiments conducted on animals that were exposed to a learning paradigm in hypoxic condition [35,35]. Hence learning impairment of mice in sodium nitrite induced hypoxia may be due to loss of Ach in central cholinergic system, which was reversed by anticholinestrase action of PHE as proved in the anticholinestrease model of the present study.

Impairment of learning and memory by ethanol is well known, however the neurobiological mechanisms underlying these effects are yet to be elucidated. It has been proposed that acute ethanol administration impairs specific types of learning and memory based upon research results from a variety of fields including molecular biology, neuropharmacology and behavioral pharmacology. The majority of these investigations have demonstrated that ethanol interacts with specific brain regions thereby impairing specific types of learning and memory. Mounting evidence suggests that cognitive processes dependent on the hippocampus and related brain structures are particularly vulnerable to the deleterious effects of ethanol [36]. With respect to above mechanism the ethanol impairs the learning capabilities and retention of memory of mice in behavioral memory such as EPM and PSA, this impaired cognition effect were reversed by standard Piracetam and PHE of all the doses in dose dependant manner.

Animal studies has revealed that alcohol has been found to reduce the number of cholinergic neurons in the basal forebrain leading to reduced hippocampal function, a structure heavily implicated in memory consolidation[37]. Central cholinergic conformity is recognized as the pivotal neurotransmitter associated in governing cognitive functions. Cholinergic neuronal death in hippocampus is the considerable feature of Alzheimer's disease and improvement of cardinal cholinergic activity with the help of anticholinesterase is currently the backbone of the pharmacotherapy of dementia in Alzheimer;s disease [38,39.] PHE and Piracetam successfully reversed the elevated cholinesterase activity of ethanol; hence PHE may be an effective anticholinesterase agent which increases the activities of cholinergic system of brain responsible for cognitive performance.

One of primary factor involved in amelioration of Neurodegenerative disorders like, Alzheimer's disease, Parkinson's disease and various neurological phases such as epileptic seizures, stroke, brain damage, neurotrauma, and cerebral ischemia/hypoxia is oxidative stress[40]. Oxidative stress is indicated by increased in TBARS and decreased in GSH levels as seen in ethanol group of animals comparative to normal group of animals indicating that decrease in the antioxidant enzymatic activity[41,42]. This decreased antioxidant activity was significantly increased by standard piracetam and PHE in dose dependant manner by increasing the level of GSH and decreasing the level of TBARS.

The different alkaloids of *Peganum harmala* have been shown to be inhibit monoamine oxidase (MAO-I). MAO-I can stimulate the central nervous system by inhibiting the metabolism of epinephrine, serotonin and other monoamine. This inhibition of the break-down of serotonin in the human body makes MAO-I effective antidepressants and psychoactive[15]. Harmal content of *Peganum harmala* plant enhances the action of epinephrine. The PHE found to be reduced MAO-A activity insignificantly, this insignificant effect

might be due to presence of mixed and reduced concentration of harmine alkaloids in a extract. Whereas, in isolated harmal alkaloids from the seeds of *Peganum harmala* (PHE) found to be significant MAO-A inhibition action (other part of the study), hence stimulate the central nervous system and enhances the activities of epinephrine and other monoamines. In the CNS, adrenergic system is comprised of several brainstem nuclei that, when activated, release nor epinephrine and epinephrine in many regions of the brain, supporting the hypothesis suggesting that the adrenergic system plays a role in learning and memory [43].

DNA fragmentation (oligointernucleosomal) can be demonstrated biochemically with the help of Gel Electrophoresis, when apoptotic bodies are detectable in only a small fraction of cells [44]. DNA electrophoresis may provide biochemical evidence of oligointernucleosomal DNA fragmentation but is subject to problems of sensitivity. A number of factors may affect the results of studies of apoptosis. Recent evidence shows that genetic differences among strains of animals alter the susceptibility to excitotoxic insult [45]. One possibility is that younger animals display apoptosis more readily than mature animals. This may be a feature of the developing brain because many components of the cell cycle that are highly expressed in developing organs participate in apoptosis. In fact, experimental evidence suggests that the stage of the cell cycle may influence apoptosis. Features of apoptosis are present in the described model of cerebral hypoxia-ischemia; apoptosis may represent a mode of ischemic cell death that could be the target of novel treatments that could potentially expand the therapeutic window for the management of neurodegeration process[46,47].

**Table 1:** Qualitative chemical analysis of ethanolic extracts of seeds of *Peganum harmala*

| Tests | Ethanolic extract |
|---|---|
| Alkaloids | +ve |
| Steroids | - ve |
| Flavonoid | - ve |
| Proteins | - ve |
| Carbohydrates | +ve |
| Saponin | - ve |
| Amino acid | - ve |
| Tannins | - ve |

Key words: +ve (present); -ve (absent)

**Table 2:** Quantitative estimation of secondary metabolites

| Sl. No | Extract | Secondary metabolite | % |
|---|---|---|---|
| 1. | PHE | Alkaloids | 52.38 |

**Table 3:** TLC study of alkaloids

| Sl. No | UV (nm) | Spots | Color | $R_f$ value |
|---|---|---|---|---|
| 1. | 365 | Spot 1 | Fluorescent green | 0.14 |
| 2. | 365 | Spot 2 | Fluorescent green | 0.32 |
| 3. | 365 | Spot 3 | Fluorescent green | 0.47 |
| 4. | 254 | Spot 4 | Bluish purple | 0.88 |
| 5 | 254 | Spot 5 | Bluish purple | 0.94 |

**Table 4:** HPLC data of alkaloids

| Sl. No | Alkaloids | Retention time | Harmine Assay (%) | Harmaline Assay (%) |
|---|---|---|---|---|
| 1. | Harmine (standard) | 3.90 | 98.90 | - |
| 2. | Harmaline (standard) | 4.56 | - | 99.90 |
| 3. | PHE (Peak 1) | 3.88 | 3.26 | - |
| 4. | PHE (Peak 2) | 4.59 | - | 2.44 |

**Table 5:** Effect of *Peganum harmala* ethanol extract on Rota rod apparatus and Actophotometer

| Sl. No. | Groups | Dose | Rota Rod | Actophotometer |
|---|---|---|---|---|
| | | | Number of falls/ 5 min | Locomotion / 5 min |
| 1 | Normal | 10ml/kg. p.o | 1.333 ± 0.21 | 546.5 ± 11.07 |
| 2 | Diazepam | 2mg/kg, i.p | 5.500 ± 0.67 $^{a***}$ | 136.0 ± 15.16 *** |
| 3 | PHE | 5mg/kg. p.o | 0.3333 ± 0.21 | 718.0 ± 21.05 *** |
| 4 | PHE | 2.5mg/kg. p.o | 0.8333 ± 0.30 | 625.2 ± 23.25 * |
| 5 | PHE | 1.25mg/kg. p.o | 1.167 ± 0.30 | 600.5 ± 14.05 |

*Each group consists of 6 animals (n=6). Values are Mean±S.E.M. P<0.05 is considered as significant, ***P<0.001, *P<0.05 as compared to normal group.*

**Table 6:** Effect of PHE on Spatial water case box of Sodium Nitrite induced Hypoxia in young mice.

| Sl. No. | Groups | Dose | TL in Seconds | |
|---|---|---|---|---|
| | | | Before | After |
| 1 | Normal control | 10ml/kg | 129.20 ± 4.31 | 28.83±2.3 |
| 2 | Hypoxia control | 75 mg/kg | 167.8 ± 5.9$^{a}$ | 81.90±4.8$^{c}$ |
| 3 | Young + Pira | 400 mg/kg | 134.3±7.4 | 12.75±1.0$^{b}$ |
| 4 | Young + PHE | 5 mg/kg | 141.5±9.8 | 20.82±2.0 |
| 5 | Hypoxia + Pira | 400 mg/kg | 125.2+7.8 | 35.95±1.9$^{d}$ |
| 6 | Hypoxia + PHE | 5 mg/kg | 150.3 ± 6.9 | 40.78±3.1$^{d}$ |
| 7 | Hypoxia + PHE | 2.5 mg/kg | 155.3± 8.6 | 64.52±1.9$^{d}$ |
| 8 | Hypoxia + PHE | 1.25 mg/kg | 170.3±4.8 | 68.88±1.9$^{e}$ |

Each group consists of 6 animals (n=6). Values are Mean±S.E.M. P<0.05 is considered as significant, $^{a}$P<0.05, $^{b}$P<0.01, $^{c}$P<0.001, as compared to normal group. $^{d}$P<0.001, $^{e}$P<0.05 as compared to Hypoxia group.

**Table 7:** Effect of *Peganum harmala* ethanol extract on transfer latencies (TL) of young mice on Elevated plus maze (EPM).

| Sl. No. | Groups | Dose | TL in Seconds | |
|---|---|---|---|---|
| | | | Before | After |
| 1 | Normal control | 10ml/kg | 37.50 ±1.5 | 34.67±0.8 |
| 2 | Ethanol control | 75 mg/kg | 72.17 ± 2.4$^{a}$ | 71.67±2.8$^{a}$ |
| 3 | Young + Pira | 400 mg/kg | 27.17±0.8$^{b}$ | 23.67±0.9$^{b}$ |
| 4 | Young + PHE | 5 mg/kg | 32.33±0.4 | 27.17±1.4 |
| 5 | Ethanol + Pira | 400 mg/kg | 47.67+1.7$^{c}$ | 44.67±1.5$^{c}$ |
| 6 | Ethanol + PHE | 5 mg/kg | 57.50±1.8$^{c}$ | 50.50±1.3$^{c}$ |
| 7 | Ethanol + PHE | 2.5 mg/kg | 63.33±2.4$^{d}$ | 63.17±2.1$^{d}$ |
| 8 | Ethanol + PHE | 1.25 mg/kg | 71.67±2.1 | 66.50±1.8 |

Each group consists of 6 animals (n=6). Values are Mean±S.E.M. P<0.05 is considered as significant, $^{a}$P<0.001, $^{b}$P<0.01, as compared to normal group. $^{c}$P<0.001, $^{d}$P<0.05 as compared to Ethanol group.

**Table 8:** Effect of *Peganum harmala* ethanol extract on Step-down-latencies (SDL) of young mice on Passive shock avoidance.

| Sl. No. | Groups | Dose | SDL in Seconds | |
|---|---|---|---|---|
| | | | Before | After |
| 1 | Normal control | 10ml/kg | 61.83±0.5 | 81.83±2.6 |
| 2 | Ethanol control | 75 mg/kg | 36.67±1.6$^{a}$ | 35.67±1.9$^{a}$ |
| 3 | Young + Pira | 400 mg/kg | 106.5±2.8$^{a}$ | 125.5±2.7$^{a}$ |
| 4 | Young + PHE | 5 mg/kg | 77.33±2.9$^{a}$ | 105.7±3.8$^{a}$ |
| 5 | Ethanol + Pira | 400 mg/kg | 74.83+2.7$^{b}$ | 101.3±4.1$^{b}$ |
| 6 | Ethanol + PHE | 5 mg/kg | 60.83±1.4$^{b}$ | 77.33±3.2$^{b}$ |
| 7 | Ethanol + PHE | 2.5 mg/kg | 50.50±2.0 | 59.17±1.4$^{c}$ |
| 8 | Ethanol + PHE | 1.25 mg/kg | 47.00±1.7$^{d}$ | 50.67±2.2$^{d}$ |

Each group consists of 6 animals (n=6). Values are Mean±S.E.M. P<0.05 is considered as significant, $^{a}$P<0.001, as compared to normal group. $^{b}$P<0.001, $^{c}$P<0.01, $^{d}$P<0.05, as compared to Ethanol group.

**Table 9:** Effect of *Peganum harmala* ethanol extract on Monoamine oxidase-A (MAO-A) inhibition

| Sl. No. | Groups | Dose | MAO –A activity U/g protein | % Inhibition |
|---|---|---|---|---|
| 1 | Normal control | 10ml/kg | 7.883 ± 0.9457 | - |
| 2 | Hypoxia control | 75 mg/kg | 31.30 ± 1.264 *** | 0 |
| 3 | Hypoxia + PHE | 5 mg/kg | 27.57 ± 0.8480 | 11.91 |
| 4 | Hypoxia + PHE | 2.5 mg/kg | 28.73 ± 0.9834 | 8.21 |
| 5 | Hypoxia + PHE | 1.25 mg/kg | 30.40 ± 1.876 | 2.87 |

Each group consists of 6 animals (n=6). Values are Mean±S.E.M. P<0.05 is considered as significant, ***P<0.001 as compared to Normal group.

**(a)** TLC at 254 nm **(b)** TLC at 365 nm

**Figure 1:** TLC of alkaloids

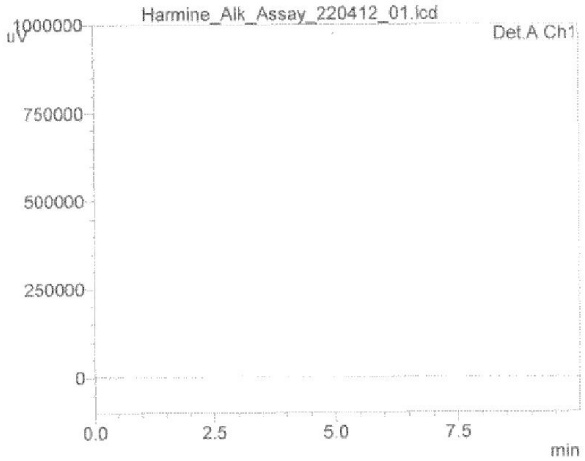


**Figure 2:** HPLC chromatogram of blank (without sample)

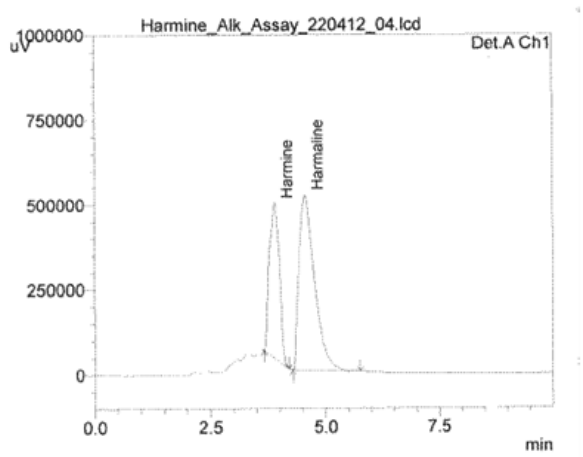


**Figure 3:** HPLC chromatogram for Standard alkaloids of Harmine and Harmaline.

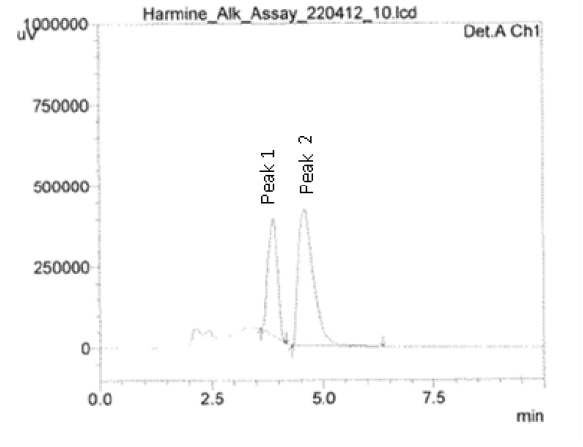


**Figure 4:** HPLC chromatogram for PHE

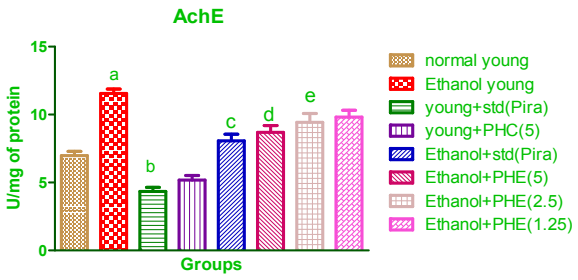


**Figure 5.** Effect of *Peganum harmala* ethanol extract on whole brain Acetylcholinesterase (AchE) in young Mice.

*Each group consists of 6 animals (n=6). Values are Mean±S.E.M. P<0.05 is considered as significant, [a]P<0.001, [b]P<0.01, as compared to normal group. [c]P<0.001, [d]P<0.01, [e]P<0.05, as compared to Ethanol group.*

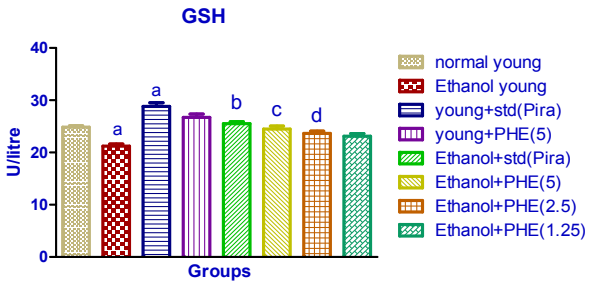


**Figure 6.** Effect of *Peganum harmala* ethanol extract on whole brain reduced Glutathione (GSH) in young mice.

*Each group consists of 6 animals (n=6). Values are Mean±S.E.M. P<0.05 is considered as significant, [a]P<0.001, as compared to normal group. [b]P<0.001, [c]P<0.01, [d]P<0.05, as compared to Ethanol group.*

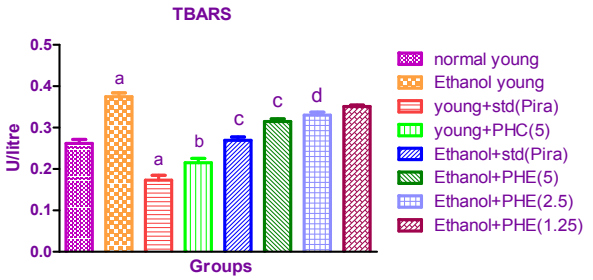


**Figure 7.** Effect of *Peganum harmala* ethanol extract on whole brain ThioBarbituric Acid Reactive Substances (TBARS) in young mice.

*Each group consists of 6 animals (n=6). Values are Mean±S.E.M. P<0.05 is considered as significant, [a]P<0.001, [b]P<0.01, as compared to normal group. [c]P<0.001, [d]P<0.05, as compared to Ethanol group.*

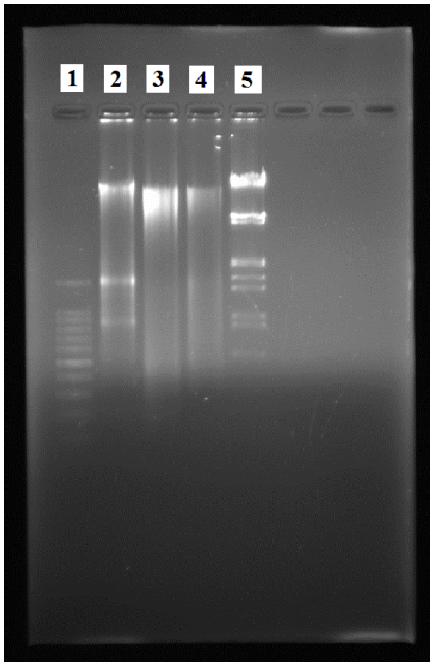


**Figure 8:** DNA Fragmentation studies of PHE on frontotemporal cortex of Brain by Gel Electrophoresis.

Gel electrophoresis of isolated DNA of amnesic Frontotemporal cortex from various experimental groups of animals. Lane 1, Standard 100bp DNA ladder, Lane 2, Normal frontotemporal cortex which shows normal DNA fragments; lane 3, frontotemporal cortex of the Hypoxic brain, shows prominent oligointranucleosomal DNA fragmentation and prominent laddering pattern, indication of DNA apoptosis; lane 4, frontotemporal cortex of the Hypoxic brain treated with PHE (5mg/kg, b.w), shows less intranucleosomal DNA fragmentation compared to hypoxic brain, indicates prevention of DNA apoptosis. Lane 5 shows Standard DNA fragmented by Bam H. It is used as standard DNA to compare the fragments from Genomic DNA.

## 5. CONCLUSION

The present study reveals the influence of ethanolic extract of *Peganum harmala* (PHE) as antioxidant in ethanol influenced oxidation in whole brain which delays the neurodegenerative process. It acts as a good anticholinestease agent, which influences the activity of Ach in the brain and enhances the cognitive performance of mice in EPM and PSA paradigm. The neuroprotective effect of PHE was further supported by its mild MAO-A inhibitory action, by which it may slightly influences the action of epinephrine and other monoamines. PHE also prevented the DNA fragmentation of frontotemporal cortex of the brain by decreasing in the intranucleosomal DNA fragmentation and lowering the laddering pattern. Hence all these preventive measure of ethanolic extract of seeds of *Peganum harmala* (PHE) might be due to presence of Harmine and Harmaline alkaloids and potential enough in the management of Neurodegenerative disorders.

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