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Protective effect of ethyl acetate soluble fraction of ethanolic extract of *Terminalia Chebula* Retz. fruits on diabetic neuropathy in mice

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ABSTRACT

Terminalia chebula Retz. (Combretaceae) fruits have been used traditionally for centuries, especially for treating diabetes and associated complications. Aim of the present study was to evaluate the effect of ethyl acetate soluble fraction of ethanolic extract of fruits of Terminalia chebula Retz. on alloxan induce diabetic neuropathy in mice. Metformin (120 mg/kg), Ethanolic extract (100 mg/kg), Ethyl acetate extract (25, 50mg/kg) were administer orally in alloxan (60mg/kg) induce diabetic mice. After 3 weeks of treatment blood glucose level, nociceptive threshold and motor activity were measured. Diabetic animals with lower pain thresholds compared to the non-diabetic group were considered neuropathic. Significant differences ($p<0.01$) between treated and control groups were observed at different aspects of diabetic neuropathy and also exhibited dose dependent antihyperglycemic activity in diabetic mice. These results suggest that Terminalia chebula Retz. has potent neuroprotective activity against alloxan induced diabetic neuropathy.

Key words: *Terminalia chebula*, diabetic neuropathy, alloxan, flavonoids.

INTRODUCTION

In today's world with increasing capacity of buying, no time for preparing own meals and relying on fast food have increased the incidence of occurrence of Diabetes which increases many folds if tension, anxiety, and mental stress are also present. Prevalence of diabetes mellitus in Indian population is about 35 million with about 13 million of these cases assumed to go undiagnosed, of which around 50% cases are from rural and about 30% cases from urban areas of India [1].

Neuropathy is a common complication of both type 1 (T1DM) and type 2 diabetes (T2DM). The prevalence of neuropathy is estimated to be about 8% in newly diagnosed patients and greater than 50% in patients with long-standing disease [2]. The most commonly used model of diabetic neuropathy are rodents with type 1 diabetes induced by the pancreatic β-cell toxin alloxan. Diabetic animals display physiologic, neurochemical and behavioral changes suggestive of altered pain perception. Behavioral methods can directly distinguish painful (hyperalgesia or allodynia) from non-painful sensation.

Herbal formulations have been used by the majority of Indians since ancient times. In recent years, there has been an increased inclination towards the herbal formulations due to the trend towards the natural sources and a healthy life style. Moreover, the complexity, side effects and costly treatment associated with the allopathic medicines have caused both the health care practitioners and the majority of world populations to turn towards alternative therapies, more likely towards the medicines obtained from natural origins, also these medicines are believed to be affordable [3].

Terminalia chebula Retz. has been introduced to Singapore, where it failed, but it was planted successfully in the botanical garden in Bogor, Java. It was also introduced to Penninsular Malaysia and found throughout India [4]. The fruits seem ovoid or ellipsoid, five ridged, becoming deeply winkled when dry, blackish brown when ripe, 2 - 4.5 x 1.2 x 2.5 mm tall. The flowers appear in May-June and the fruits in July-December. It having a bitter flavor, it possesses laxative, astringent, lubricant, antiparasitical, alterative, antispasmodic and nervine properties. It is therefore used to treat acute and chronic constipation, nervousness, anxiety and feelings of physical heaviness.

The present investigation was, therefore, undertaken to study the effects of ethyl acetate fraction of ethanolic extract of fruits of *Terminalia chebula* on fasting blood glucose and nociceptive threshold in normal as well as alloxan-induced diabetic mice. The results were compared with metformin.

MATERIALS AND METHODS

Collection of plant material

The fruits of *T. chebula* were purchased from the local traders. Sample was authenticated by Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (voucher specimen No. Bot./2010-11/ 001) and has been preserved in same department for future reference.

Preparation of extract

The fruits were dried under shade and powdered by using grinder mixer. The powdered material (150 g) was socked in Petroleum ether (60 – 80^{0C}) to remove lipids, filtered it and the residue was extracted with ethanol using soxhlet apparatus for 72 hr. After extraction the solvent was filtered and evaporated in a vacuum, residue obtained was dissolved in distilled water and extracted with ethyl acetate. The filtrate was evaporated to obtain solid brown colored dry mass of ethyl acetate fraction of ethanolic extct of *Terminalia chebula* (EATC) weighing 12 g (8% w/w) [5].

Experimental animal

Swiss albino mice of either sex weighing between (25-35 g) were used. They were maintained at 25 ± 2˚C and relative humidity of 45 to 55% and under standard environmental conditions (12 h.

light /12 h. dark cycles). The animals had free access to food and water. All the experiments were carried out between 9 to 17 hrs. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Y. B. Chavan College of Pharmacy, Aurangabad, (CPCSEA/IAEC/P'COL-02/2010-11/20), constituted as per the direction of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under Ministry of Animal Welfare Division, Government of India, New Delhi, India.

Induction Diabetes

Overnight fasted experimental mice were injected with alloxan (Sigma, USA) at a dose of 60 mg/kg body weight [6]. The alloxan was injected intravenously within 10 min after dissolving in saline water. The mice in control group were treated with vehicle. The animals were allowed 5% glucose solution overnight to overcome the drug induce hypoglycemia, 2 hrs after alloxan injection. Fasting blood glucose (FBG) was estimated at the time of induction of diabetes and was checked regularly until stable hyperglycemia was achieved. After a week time for the development of diabetes, the rats with moderate diabetes having polyurea and hyperglycemia (blood glucose levels of 300 mg/dl) were included in the study as stable hyperglycemic animals.

Experimental design

Animals are divided in to six groups (A, B, C, D, E, and F) 6 animals in each. Group A termed as normal control where as group B was diabetic control. Once the stable hyperglycemia was achieved, the mice belonging to group C were treated with an oral dose of metformin (120 mg/kg), Group D with Ethanol extract (ET) (100mg/kg), Group E with Ethyl acetate fraction (EATC) (25mg/kg), Group F with EATC (50 mg/kg) once every day for 21 days while groups A and B mice received vehicle..

Body weight and serum glucose measurement

Body weight and blood glucose level were determined immediately before induction of diabetes and after 1, 2 and 3 weeks. Blood samples from the control and experimental mice were collected from tail vein. The blood samples so collected were analyzed for glucose estimation.

Nociceptive threshold

After completion of dosing period mice were tested for different parameters such as nociceptive thresholds and locomotor activity.

Chemical sensitivity

Formalin (5%) in a volume 0.05 ml was injected (s.c.) into the plantar region of the right hind paw, immediately after the injection, the number of flinches and shakes of the paw was obtained in 5 min interval for 30 min, the data collected between 0 to 10 min. after the formalin injection represent phase I and 10-30 min. represent phase 2. Results were presented as the sum of the total number of flinches/shakes of the formalin injected paw for both the phases [7].

Thermal sensitivity

Hot plate test

Mice were gently restrained and after a few seconds necessary for the struggle to finish, the plantar side of the tested paw was placed on the hot plate surface (55 ± 1°C) [7]. The latency before the first reaction (licking, moving, the paws, and little leaps) was recorded with the cut off time was 30 s in order to prevent paw-tissue damage [8].

Tail flick

The nociceptive response was evaluated by recording the latency to withdrawal of the tail in response to noxious skin heating. The apparatus used is tail flick analgesiometer, the tip of tail of mice is placed on hot metal wire and the latency of withdrawal is calculated manually by stop watch. Cut off time is 20 s to prevent tissue damage [9].

Hot water tail immersion test

Tail of mice was marked at 5 cm from the tip and was immersed in a warm water bath (52.5±0.5°C) until tail withdrawal (flicking response) or signs of struggle were observed (cut-off 30 s). Shortening of the tail withdrawal time indicates hyperalgesia [10].

Cold water tail immersion test

Tail of mice was immersed in a cold water bath (10 ± 0.5°C) until tail withdrawal (flicking response) or signs of struggle were observed (cut-off 30 s). Shortening of the tail withdrawal time indicates hyperalgesia [8, 11]

Motor coordination

Rota rod

Rota rod has been used to evaluate motor coordination by testing the ability of mice to remain on revolving rod. Each mouse was given five trials before the test. The readings were taken at 20 rpm after 21 days of treatment in all groups of mice [12].

Beam walk test

This method offers improved sensitivity over the mouse Rota- rod in determining motor coordination deficits induced by psychotropic agents. Mice were allowed to walk from a start platform along a ruler (80 cm long, 3 cm wide) elevated 30 cm above the bench by metal supports to a goal box (enclosed house). Three trials were performed for each mouse, and were designed such that the mice tested would be aware that there was a goal box that could be reached. A ruler was used because the mouse found this easy to cross, and at the same time, it induced minimum anxiety. Once the mice had been tested on the ruler and they were moved immediately to the beam test. The beam was made of wood, 8 mm in diameter, 60 cm long and elevated 30 cm above the bench by metal support. Mice that fell were returned to the position they fell from, with a maximum time of 60 sec allowed on the beam. The measurements taken were time on beam; the number of foot slips (one or both hind limbs slipped from the beam) and the number of falls [13].

Statistical analysis

All observations are given mean ± SEM and data were analyzed using One way ANOVA followed by *Dunnett's- test* and compare with respective control group. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Mice with diabetes (blood glucose concentration >300mg/dl) developed increased sensitivity to thermal stimuli as well as motor incoordination compared to the nondiabetic control group. These animals were included in experiments.

Diabetic mice in showed significant ($p < 0.001$) decrease in body weight as compare to control animals (36.83 ± 1.302). Vehicle treated diabetic control group showed significant ($p < 0.001$) decrease in body weight at the end of 21 days treatment compare to normal control animals.

Table 1. Effect of EATC on fasting blood glucose.

Animal: mice

Group	Treatment	Fasting blood glucose level (mg/dl)			
		Initial	1st week	2nd week	3rd week
A	Control	71.17±3.016	77.00±2.422	79.67±1.626	71.67±7.504
B	Diabetic control	359.0±10.56	351.3±9.932**	345.3±8.417**	341.2±8.807**
C	D + Metformin (120mg/kg)	345.3 ± 11.46	127.3±8.07**	105.5±5.271**	93.17±4.393**
D	D + ETC (100mg/kg)	353.7 ± 8.697	158.2 ±6.263**	107.2 ±3.591**	91.33 ±3.602*
E	D + EATC (25gm)	357.7 ± 12.7	228.3 ± 6.776**	157.0 ± 4.761**	123.5 ± 4.169**
F	D + EATC (50gm)	349.0 ± 12.09	157.0 ± 14.29**	112.2 ± 3.673**	98.83 ± 3.331**

Data is presented as Mean ± SEM (n=6), One-way ANOVA followed by Dunnett's- test , vs. Respective Control
***P < 0.01*

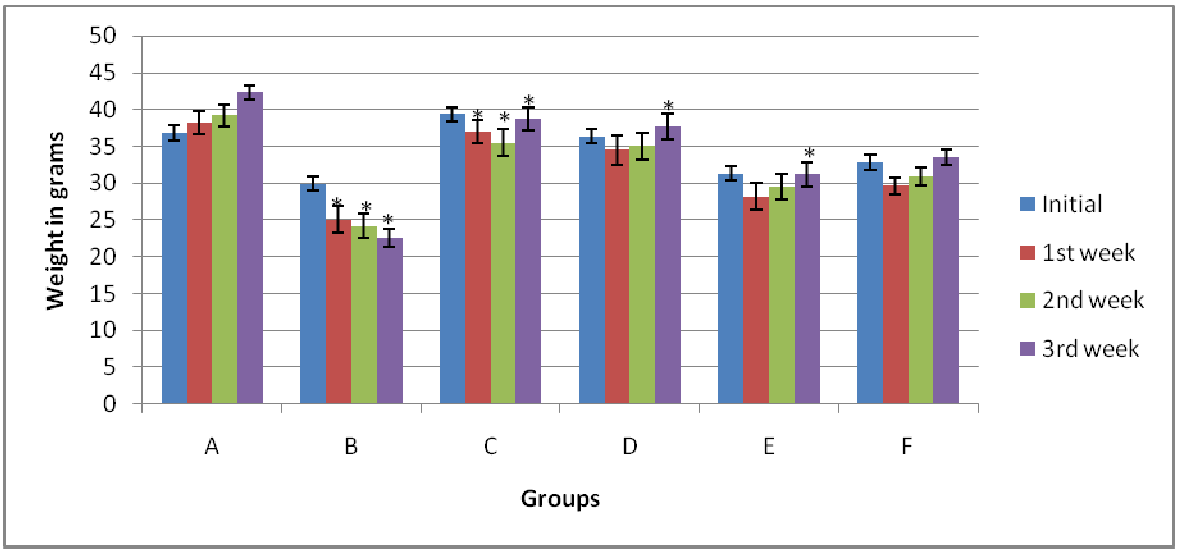

Fig. 1. Effect of EATC on body weight.
*Data is presented as Mean ± SEM (n=6). One-way ANOVA followed by Dunnett's- test. **P < 0.05*

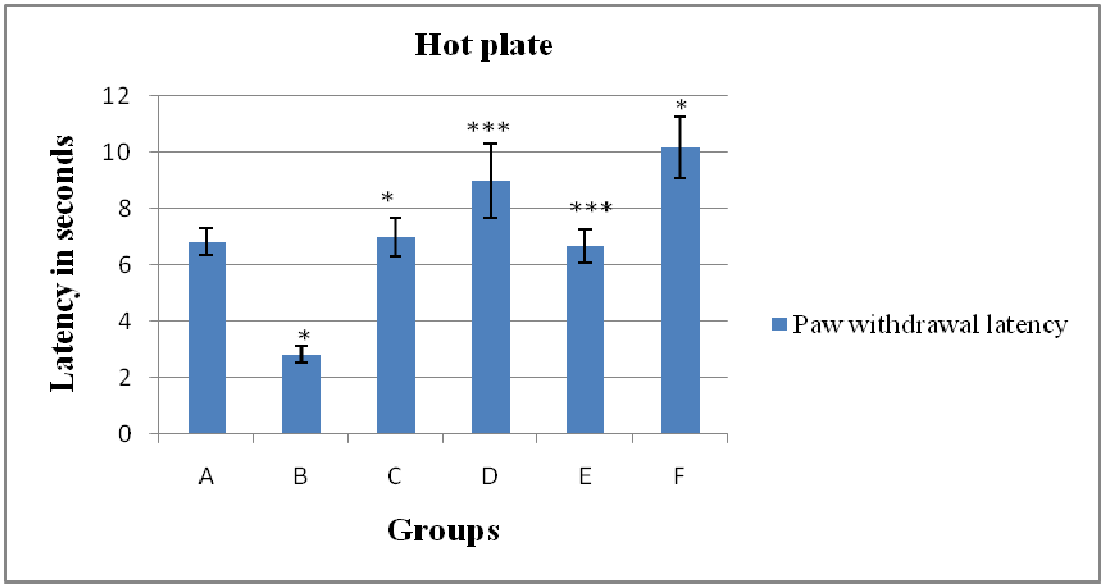

Fig. 2. Effect of EATC treatment on paw withdrawal latency in hot-plate test
*One-way ANOVA followed by Dunnett's- test. *P < 0.05, ***P < 0.001*

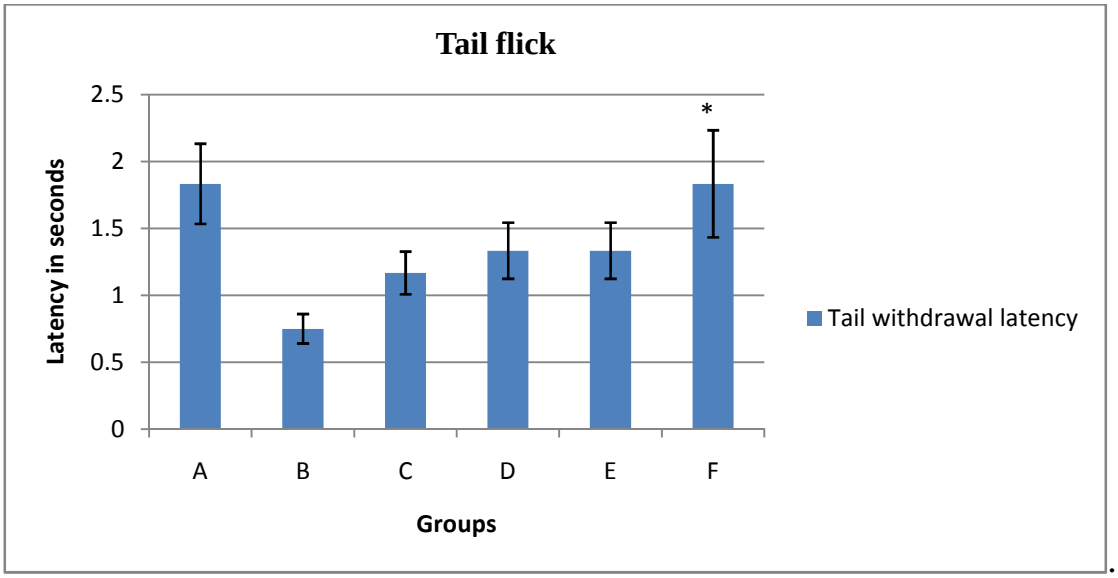

Fig. 3. Effect of EATC treatment on tail withdrawal latency in tail flick test
*One-way ANOVA followed by Dunnett's- test. *P < 0.05 vs respective control*

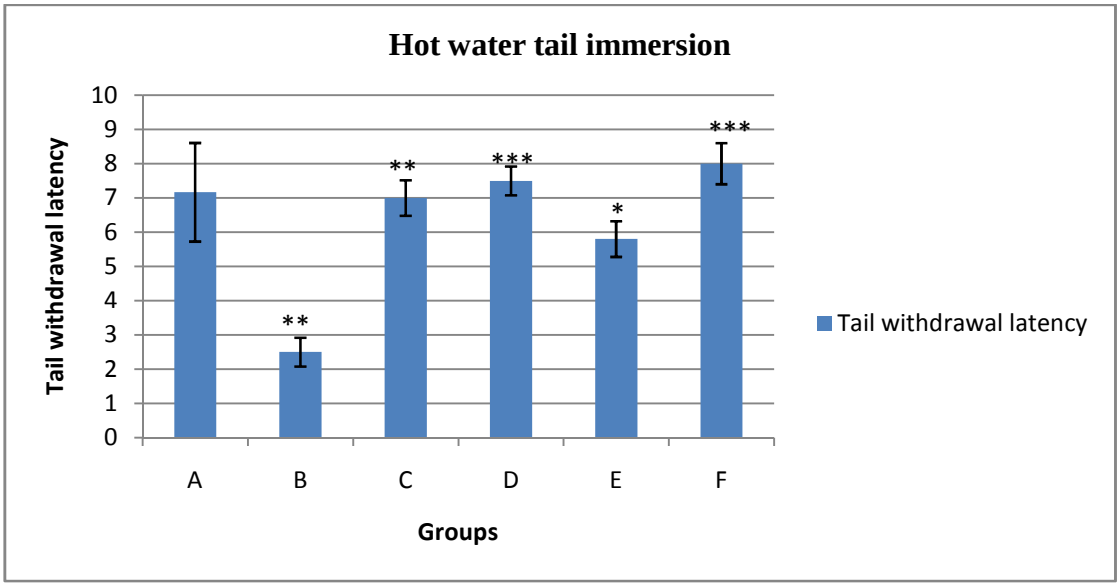

Fig. 4. Effect of EATC treatment on tail withdrawal latency in hot water tail immersion test
*One-way ANOVA followed by Dunnett's- test. **P < 0.01 and ***P < 0.001 vs respective control*

Metformin 120, Ethanol 100, EATC 25, 50 mg/kg treated animals showed significant ($p < 0.001$) increase in body weight at the end of 21 days. Dose and time dependent increase in body weight was observed till 3 weeks of treatment (Fig. 1).

Similarly, diabetic mice in all groups showed significant ($p < 0.001$) increase in serum glucose level as compare to control animals (71.17±3.016). Vehicle treated diabetic control group showed significant ($p < 0.001$) increase in serum glucose level on treatment as compare to normal control animals. Metformin 120, ETC 100, EATC 25, 50 mg/kg treated animals showed significant ($p < 0.001$) reduction in serum blood glucose level after treatment as compare to control. (Table 1).

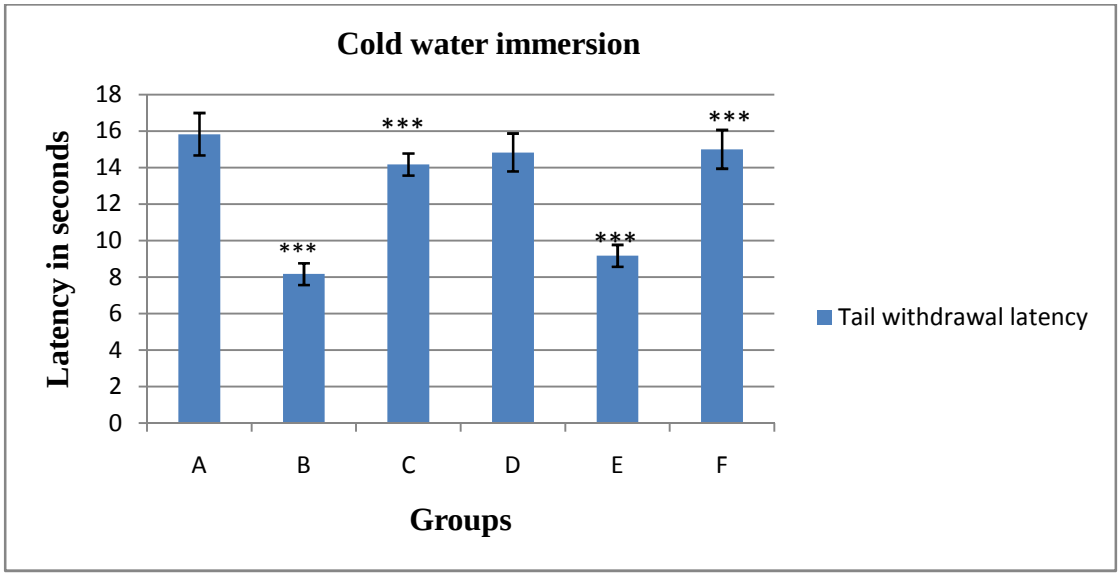

Fig. 5. Effect of EATC treatment on tail withdrawal latency in cold water tail immersion test
*One-way ANOVA followed by Dunnett's- test. ***P < 0.001 vs respective control*

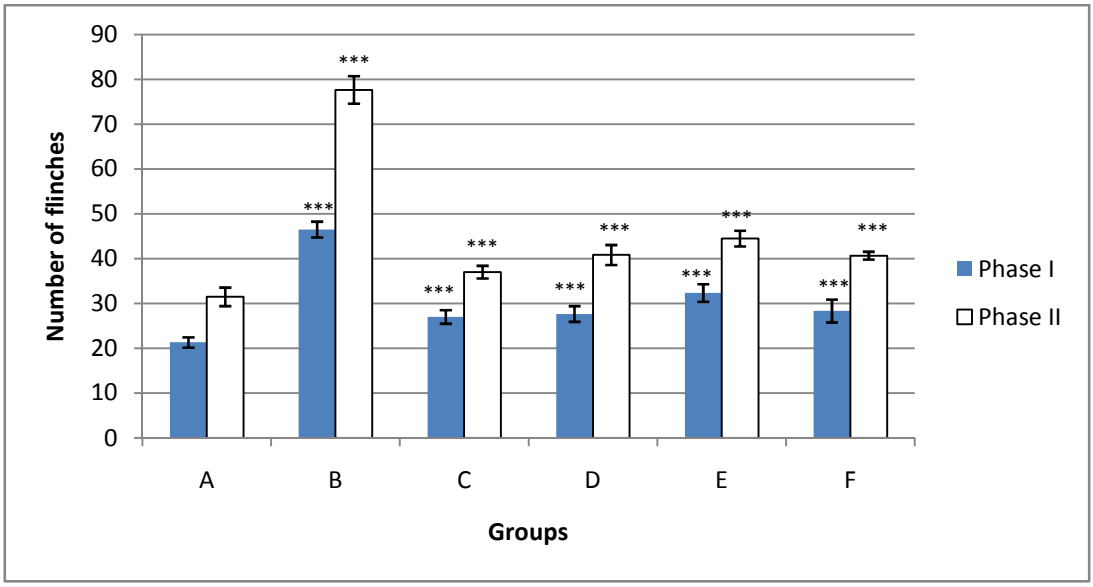

Fig. 6. Effect of EATC treatment on number of flinches in formalin induce pain
*One-way ANOVA followed by Dunnett's- test. ***P < 0.001 vs respective control*

The nociceptive threshold was significantly ($p < 0.05$) lower in diabetic mice in all groups as compared with control animals (6.833 ± 0.4773). Thermal hyperalgesia was evident in alloxan treated animals since the paw withdrawal latency on hot plate was significantly shorter than that of normal control animals after the third week of diabetes. At the end of 21 days of respective treatment of Metformin 120, ETC 100, EATC 25, 50 mg/kg animals showed significant increase in reaction time (Fig. 2).

Similarly, in tail flick experiment nociceptive threshold was lower in diabetic mice in all groups as compared with control animals. Thermal hyperalgesia was evident in alloxan treated animals since the tail withdrawal latency was shorter than that of control animals after the end of third week. Metformin 120, ETC 100, EATC 25, 50 mg/kg treatment showed increase in reaction time at the end of 21 days. (Fig. 3).

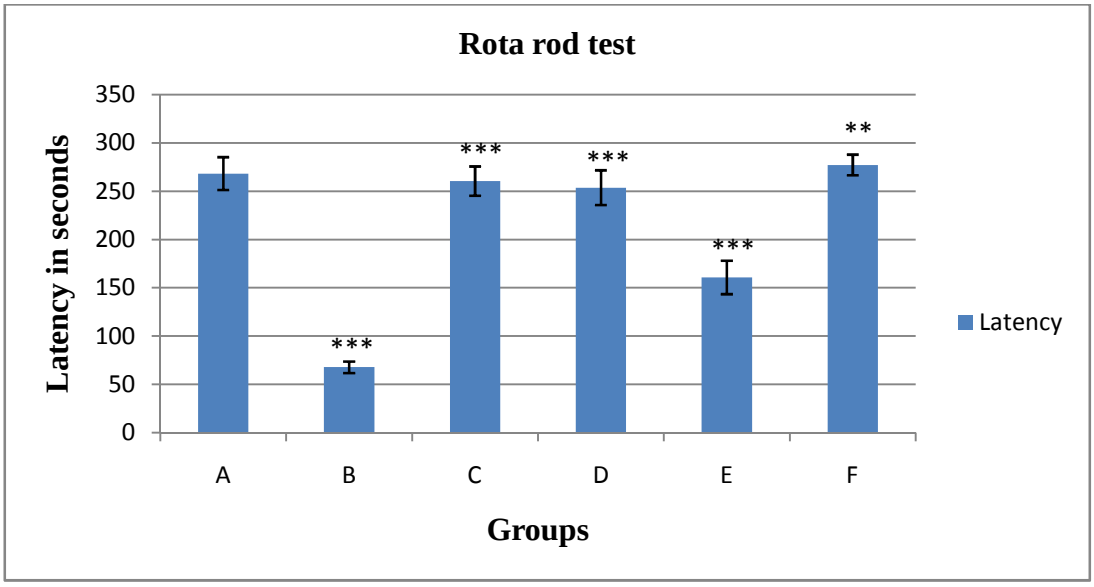

Fig. 7. Effect of EATC treatment on fall down latency in rota rod test
*One-way ANOVA followed by Dunnett's- test. **P < 0.01 and ***P < 0.001 vs respective control*

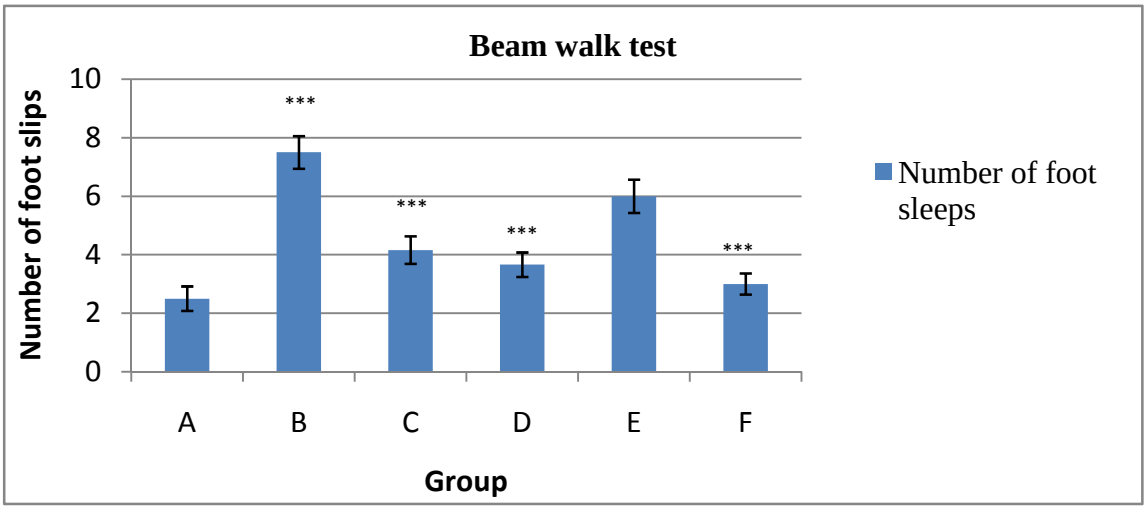

Fig. 8. Effect of EATC treatment on number of foot slips in beam walk test
*One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. ***P < 0.001 vs respective control*

In the experiment of hot water tail immersion nociceptive threshold was significantly ($p < 0.01$) lower in diabetic mice as compared to normal control animals. Thermal hyperalgesia was evident in alloxan treated animals since the tail withdrawal latency was shorter than that of control animals. After 3 weeks of respective treatment of Metformin 120, ETC 100, EATC 25, 50 mg/kg animals showed significant ($p < 0.001$) increase in reaction time (Fig. 4).

Thermal hyperalgesia was evident in alloxan treated animals since the tail withdrawal latency in cold water was shorter significantly ($p < 0.001$) than that of control animals after 21 days of diabetes. Treatment with Metformin 120, ETC 100, EATC 25, 50 mg/kg animals showed significant ($p < 0.001$) increase in reaction time (Fig. 5).

In chemically induce pain method, alloxan treated animals shows significant increase in number of flinches than that of control animals at the end of 21 days. Treatment with Metformin 120,

ETC 100, EATC 25 and 50 mg/kg animals showed significant ($p < 0.001$) decrease in number of flinches (Fig. 6).

Increase glucose level may damage muscle spindle and can lead to deficits such as motor incoordination. Fall down latency of animal on rotating rod is calculated to evaluate motor incoordination. In the present study significant shortening of fall down latency was observed with alloxan treated animals. Significant ($p < 0.001$) increase in fall down latency was also observed with Metformin 120, ETC 100, EATC 25, 50 mg/kg treatment (Fig. 7)

The beam walk test showed increase in number of foot (hind paw) slips in alloxan treated animals as compare to control group. Metformin 120, ETC 100, EATC 25, 50 mg/kg treated animals showed significant ($p < 0.001$) decrease in number of hind paw slips at the end of 21 days. (Fig. 8)
The extensive literature survey for various traditional claims and scientific documentation indicated that aerial parts of *Terminalia chebula* Retz. have been claimed for their use in treatment of diabetes, pain and as nervine tonic [5, 14]. But it has not been documented so far for its effect in case of diabetic neuropathy. In light of which the present investigation was carried out.

Preliminary phytochemical investigation of the ethyl acetate fraction of ethanolic extract of fruits of the *Terminalia chebula* showed the presence of alkaloids, glycosides, steroids, saponins, flavonoids, triterpenoids and tannins. The tannins, polyphenols and flavonoids present in the herbal drugs are proved to be effective in diabetes treatment. The alkaloids and glycosides present in the herbal drugs are proved to be potent antioxidants as well as nephroprotective agents [15]. The steroids such as β-sitosterol are scientifically documented for their antioxidant potential.

The unique capability of alloxan to selectively destroy the pancreatic beta cells and cause hyperglycemia. Several researchers have proposed that free radicals take part in the pancreatic β-cell damage produced by alloxan [16, 17]. In the present study, the alloxan-induced diabetic mice showed high blood glucose level. Hyperglycemia was evident throughout the entire experimental period indicating state of diabetes.

In the present study we have established the alloxan-induced diabetic mice model and have shown that these animals exhibit a profound hyperalgesia and motor incoordination. In agreement with previous reports, this hyperalgesia was evident within 2 weeks of alloxan injection and lasted for at least 4 weeks.

Thermal hyperalgesia observed in diabetic animals [18]. It is a well known that diabetic mice display exaggerated hyperalgesic behavior in response to noxious stimuli that may model aspects of painful diabetic neuropathy [19]. Although evaluation of mechanisms causing these symptoms is complicated because of the overlap between the systemic effects of hyperglycemia and its toxic effects within the peripheral nervous system, direct functional toxicity of hyperglycemia in the peripheral nervous system, an increased activity of primary afferent fibers leading to an increased excitatory tone within the spinal cord, increased release of glutamate and activation of the NMDA receptor, reduced activity of both opioidergic and GABAergic inhibitory systems [20], decreased activity of nNOS–cGMP system in neurons of dorsal root ganglion, altered sensitivity of the dopaminergic receptors and altered responsiveness of the dopaminergic system, possibly through the enhancement and/or deactivation of the endogenous Met-enkephalinergic

system, and alterations in L-type Ca^{2+} channel could be involved in the modulation of nociception in diabetic mice [21].

In the present study, alloxan-injected mice had significantly higher blood glucose level, decreased body weight and the nociceptive threshold was significantly lower, indicating that diabetic animals exhibit thermal hyperalgesia. Study on Rota rod and beam walk test indicates motor incoordination in diabetic animal.

Chemical hyperalgesia during phase 2 of the formalin test in diabetic mice is associated with increased cyclooxygenase-2 expression and prostaglandin E-2 release in the spinal cord. Formalin also increased the expression of postsynaptic NMDA and AMPA receptors for glutamate and enhanced electrophysiological activity in the dorsal horn neurons spinal amplification of pain signals with paradoxical reduction in ongoing signals from the periphery and decreased spinal release of glutamate suggest significant role of central sensitization in the formalin-induced hypersensitivity in experimental diabetes. Although the investigation of neuropathic pain in diabetes has primarily focused on the peripheral nerves, the growing body of evidence suggests spinal cord and higher CNS as generators and amplifiers of pain [7, 22].

In the present study, diabetic animals shows elevated blood glucose level, reduced pain threshold and motor incoordination. EATC treatment restored body weight, blood glucose, along with pain threshold and motor coordination in diabetic mice.

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