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EFFECTS OF ETHYL ACETATE FRACTION OF *Z. MAURITIANA*, LAM. LEAVES ON STZ INDUCED DIABETES AND DIABETIC NEUROPATHY IN MICE

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ABSTRACT

Ethanolic extract of leaves of *Ziziphus mauritiana,* Lam. has shown hypoglycemic effect in alloxan induced diabetic rats. Present study was performed to evaluate the phytochemical screening of Ethyl Acetate fraction of leaves of *Z. mauritiana,* Lam. (EAZM) and study its effects on diabetes and diabetic neuropathy in STZ induced diabetic mice. Overnight fasted experimental mice were injected with STZ at a multiple dose of 40 mg/kg body weight for 5 consecutive days. Effects of EAZM on blood glucose were evaluated and action on diabetic neuropathy was studied using behavioral biomarkers of neuropathy as animal models. EAZM revealed the presence of alkaloids, tannins, steroids, triterpenoids, saponins, and flavonoids, indicated dose and time dependent decrease in serum glucose level till 4 weeks of treatment. Diabetic Neuropathy was studied under Thermal Hyperalgesia, Cold Allodynia and Motor In co-ordination. EAZM has increased the tail withdrawal latency in Tail Flick, Tail Immersion and Hot Plate test. It was also found that EAZM has increased the paw withdrawal latency in Cold Allodynia. In Beam Walk test, EAZM decreased the number of foot slips. It can be concluded that EAZM ie. Ethyl Acetate fraction of methanolic extract of *Ziziphus mauritiana,* Lam. leaves has Antidiabetic activity and also protective against diabetic neuropathy in STZ induced diabetic mice.

Keywords: *Ziziphus mauritiana,* STZ, diabetes, Behavioral Biomarkers, Diabetic Neuropathy, Thermal Hyperalgesia, Cold Allodynia, Motor in coordination.

INTRODUCTION

Diabetes mellitus is a wide spread disorder, which has long been in the history of medicine [1]. Report of ethnobotany suggested that about 800 medicinal plants possess antidiabetic potential [2] and the bioactive compounds such as glycosides, alkaloids, terpenoids, carotenoids and flavonoids are effective drugs both in preclinical and clinical studies [3-4]. It has been estimated that, about 1.3 % of the world population suffer from this disease. But, most of the hypoglycemic agents and hypolipidemics used in allopathic practice to treat diabetes mellitus and hyperlipidemia are reported to have side effects in long term use [5]. Hence, there is the need to search for effective and safe drugs for these ailments. However, there are reports that, though numerous traditional medicinal plants are reported to have hypoglycemic properties, many of them proved to be not very effective in lowering blood glucose levels in severe diabetes [6]. Herbal medicine should be investigated as a potential regimen for diabetes and diabetes related complications like neuropathy [7]. The prevalence of neuropathy is estimated to be about 8% in newly diagnosed patients and greater than50% in patients with long-standing disease [8]. Hence, there is need to explore herbal medicines in the context of modern science and validate accordingly. It was found that oral administration of ethanolic extract of *Ziziphus mauritiana* leaves dose dependently reduces the blood glucose level in alloxan induced diabetic rats [9]. Hence, in the present study we

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evaluated the phytochemistry of ethyl acetate extract of *Ziziphus mauritiana* leaves and investigated its effects in diabetes and diabetic neuropathy in STZ induced diabetic mice.

MATERIALS AND METHODS

Drugs and chemicals

Streptozotocin(STZ) Dodal Enterprises, was dissolved in cold 0.01 M citrate buffer, pH 4.5and always prepared freshly for immediate use within30 min. [10], Metformin (Metmin tab.) Jenbur Kt Pharmaceuticals Ltd., Gabapentin (Gabapin cap.) Intas Pharmaceuticals and all other chemicals used in the present study were of analytical grade.

Separation of Ethyl Acetate fraction from methanolic extract of leaves of *Z. mauritiana*, Lam.

The leaves of *Z. mauritiana*, Lam. were collected from local area of Aurangabad city, Maharashtra, India. The leaves were dried under shade and powdered by using grinder mixer. The powdered material (150 g) was extracted in Petroleum ether (60 – 80^{0C}) to remove lipids and chlorophyll, filtered and filtrate was discarded, residue extracted with methanol in soxhlet apparatus for 72hr. After extraction the solvent was filtered and evaporated in a vacuum, whatever residue may be obtain was dissolved in distilled water and extracted with Ethyl Acetate using separating funnel. The filtrate obtained was evaporated on water bath to obtain dry mass (EAZM) of 25.66 g (17.10 % w/w) [11-12].

Preparation of test solution

Accurately weighed quantity of Ethyl Acetate fraction (50, 100 and 200 mg/kg) was dissolved in the distilled water to prepare the solution of the extract with appropriate concentration, Span was used as solubility enhancer.

Phytochemical screening

Phytochemical screening and physical properties of Ethyl Acetate fraction from methanolic extract of leaves of Z. *mauritiana* were carried out employing standard procedures and tests [13].

Animals

Swiss albino mice of either sex weighing between (22-30 g) were used. They were maintained at temperature of 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 (Pranav Agro Industries Ltd., Sangli, India) and water. All the experiments were carried out between 9 to 18 hrs. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Y.B. Chavan College of Pharmacy Aurangabad.
(Approval number- CPCSEA/IAEC/P'col/19/2011-12/43).

Acute toxicity study

Acute toxicity study was performed under OECD guideline 425. Individual animals were administered dosage at the interval of 24hours, one at a time. The animal was observed for 24 hours, continuous observation for first 4 hours, followed by occasional observation for every 4 hours up to 24 hours. The next dose is administered according to the mortality of the animal. If the animal survived, a higher dose was given to the next animal. If the first animal died, then the dose is decreased. In case the animal appeared moribund (dying) the animal was sacrificed in a humane way and it is considered to have died because of toxicity. In absence of any information about the substance the starting dose can be 200 or 500 mg/kg body weight. For further dosages a dose progression factor of 1.3 was used. After reaching the reversal of initial direction, four additional animals were dosed using the same up and down procedure. This was the end of the test. The dosages should not exceed 2000 mg/kg. When the first animal dosed with 2000 mg/kg survived, another animal was given the same dose. When a total of 3 animals are dosed with the upper limit (2000 mg/kg) and no deaths occurred, then three animals of other sex are tested at the limit dose level. If mortality was not registered again, the test was terminated.

Neurotoxicity study

In this test, a knurled rod (2.54 cm in diameter) was rotated at a speed of 15 rpm. All animals were trained to remain on the rotating rod for 5 min. In a drug-treated mouse, the neurological deficit was indicated by inability of the mouse to maintain equilibrium for 3 min in each of three trials. EAZM was administered (50, 100, or 200 mg/kg) and the animals were tested for neurological deficit [14].

Induction of Experimental diabetes

Overnight fasted experimental mice were injected with STZ at a multiple dose of 40 mg/kg body weight for 5 consecutive days. The solution was injected intraperitoneally (i.p.) within 5 min after dissolving in citrate buffer pH 4.5 [10]. The mice in group A were injected with distilled water as a vehicle control. The animals were allowed to drink 5% glucose solution overnight to overcome the drug induce hypoglycemia. Fasting blood glucose (FBG) was estimated at the time of induction of diabetes and postprandial glucose (PPG) was checked regularly until stable hyperglycemia was achieved. After a week time for the development of diabetes, the mice with moderate diabetes having glycosuria and hyperglycemia (blood glucose levels of 300 mg/dl) were included in the study as stable hyperglycemic animals.

Experimental groups

Animals were divided into six groups (A-F) each of them with six mice, only group A mice were normal, rest

all groups contain diabetic mice. Group A as normal control received distilled water, group B as diabetic control received distilled water, group C given a standard oral hypoglycemic agent metformin (120 mg/kg), groups D, E and F given EAZM (50 mg/kg), EAZM (100 mg/kg) and EAZM (200 mg/kg) respectively. For the study of diabetic neuropathy seven groups were required (A-G); group A-C were same while group D was given standard gabapentin (100 mg/kg) and groups E-G were given EAZM (50 mg/kg, 100 mg/kg and 200 mg/kg respectively).

Collection of blood and determination of blood glucose

Blood samples from the control and experimental mice were collected from tail vein. The samples so collected were analyzed for glucose estimation.

Diabetic Neuropathy

Mice may develop hyperglycemia and other clinical diabetic symptoms within 3 days of STZ injection. After completion of dosing period mice were tested for different parameters of Neuropathy.

Thermal Hyperalgesia

Tail flick test

The nociceptive response was evaluated by recording the latency to withdrawal of the tail in response to noxious radiant 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 25 s to prevent tissue damage [15].

Tail immersion test

The lower 5 cm portion of the animal tail is marked. This part of the tail is immersed in a cup of freshly filled water of exactly 55 °C. Within a few seconds the animal reacts by withdrawing the tail. The reaction time is recorded in 0.5 s units by a stopwatch. After each determination the tail is carefully dried. The cut off time of the immersion is 12 s [16-17].

Hot Plate Method

In this test, animals were individually placed on a hot-plate (Eddy's hot-plate) with the temperature adjusted to 55 ± 1 ˚C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold; the cut-off time was 10 s in order to avoid damage to the paw [18].

Cold Allodynia

Cold sensitivity was measured as the number of foot withdrawal responses after application of acetone to the plantar surface of the paw. A drop of acetone was gently applied to the heel of the rat with a syringe connected to a thin polyethylene tube while the rats were standing on a metal mesh. A brisk foot withdrawal response, after the spread of acetone over the plantar surface of the paw, was considered as a sign of cold allodynia [18].

Motor Inco-ordination (Beam Walk test)

In this method, the number of hind paw slips as mice crossed the beam-walk apparatus, was used to access the sensorimotor ability. For a slip to be counted, the foot had to lose during walking on the beam-walk apparatus [16].

Statistical analysis

All observations are given in Mean ± SEM (n=6) and data were analysed using One way ANOVA followed by *Dunnett's- test* using INSTAT GraphPad.

RESULTS

Phytochemical screening and pharmacognostical evaluation of EAZM

Phytochemical screening

The preliminary phytochemical screening of EAZM revealed the presence of alkaloids, tannins, steroids, triterpenoids, saponins, and flavonoids.

Acute toxicity

Animals treated with EAZM were free of any toxicity as per acceptable range given by the OECD guidelines no. 425 and no mortality was found up to 2000 mg/kg (Table 2.). Hence the doses 50, 100 and 200 mg/kg were selected for present study.

Neurotoxicity study

Mice treated with doses of EAZM (50, 100 and 200 mg/kg) were able to maintain equilibrium on the rotarod apparatus for complete duration of 5 min.

Effect of EAZM on blood glucose of diabetic mice

After 5 consecutive days of administration of STZ on 7^{th} day (1^{st} week) no stable hyperglycemia was observed. But, after 14^{th} day stable hyperglycemia was observed. Diabetic mice in all groups showed very significant ($p<0.05$, $p<0.001$) increase in serum glucose level as compare to control animals. Vehicle treated diabetic control group showed significant ($p<0.001$) increase in serum glucose level on treatment as compare to normal control animals. Metformin, EAZM100 mg/kg and 200 mg/kg treated animals showed significant ($p<0.001$) reduction in serum blood glucose level after treatment as compare to control. Dose and time dependent decrease in serum glucose level was observed till 4 weeks of treatment (Table 3, Fig. 1.).

Diabetic Neuropathy

After 3 week of diabetic induction, diabetic control animals have developed Neuropathy with blood glucose level 425±30.4 and 510.3±23.9 after 4 week.

Diabetic Neuropathy was studied under Thermal Hyperalgesia, Cold Allodynia and Motor In co-ordination.

Thermal Hyperalgesia

Thermal hyperalgesia was evident in STZ induced diabetic mice as the diabetic control group shows significantly ($p<0.0001$) decreased tail and paw withdrawal latency in the animal models of behavioral biomarkers of neuropathy.

Tail flick test

EAZM has significantly ($p<0.0001$) increased the tail withdrawal latency of STZ induced diabetic mice dose dependently, as compared to normal control and diabetic control in tail flick test (Table 4., Fig. 2.).

Tail Immersion Test

Similarly EAZM has significantly ($p<0.0001$) increased the tail withdrawal latency of STZ induced diabetic mice dose dependently, as compared to normal control and diabetic control in tail immersion test (Table 5., Fig. 3.).

Hot Plate Test

Again EAZM has significantly ($p<0.0001$) increased the paw withdrawal latency of STZ induced diabetic mice dose dependently, as compared to normal control and diabetic control in hot plate test (Table 6., Fig. 4.).

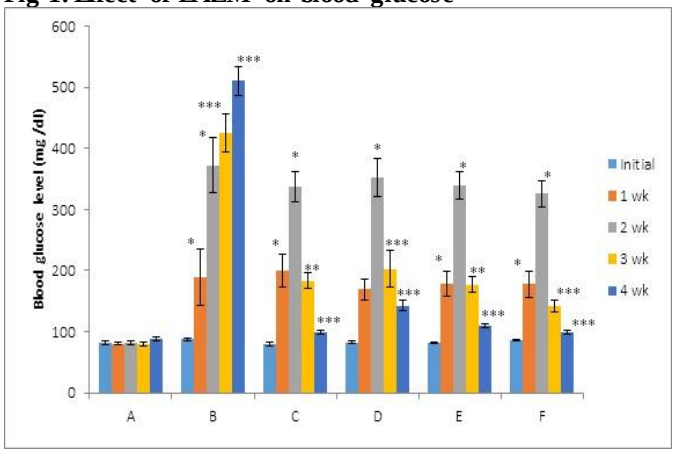
Cold Allodynia

EAZM has significantly ($p<0.0001$) increased the paw withdrawal latency of STZ induced diabetic mice dose dependently, as compared to normal control and diabetic control in cold allodynia (Table 7., Fig. 5.).

Motor Inco-ordination (Beam Walk Test)

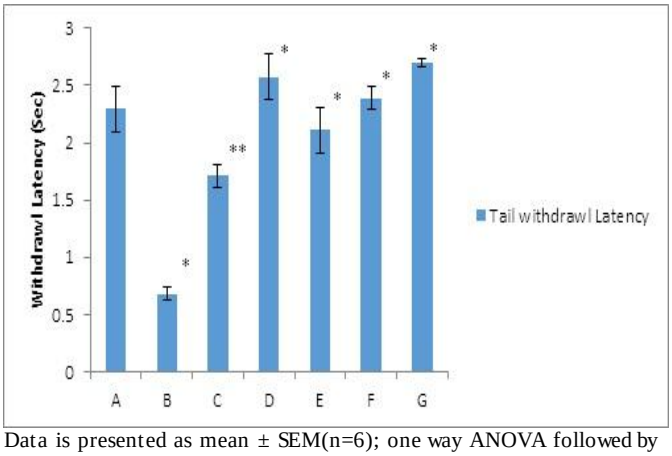
EAZM has significantly ($p<0.0001$)decreased the number of foot slips of STZ induced diabetic mice dose dependently, as compared to normal control and diabetic control in beam walk test (Table 8., Fig. 6.).

Fig 1. Effect of EAZM on blood glucose



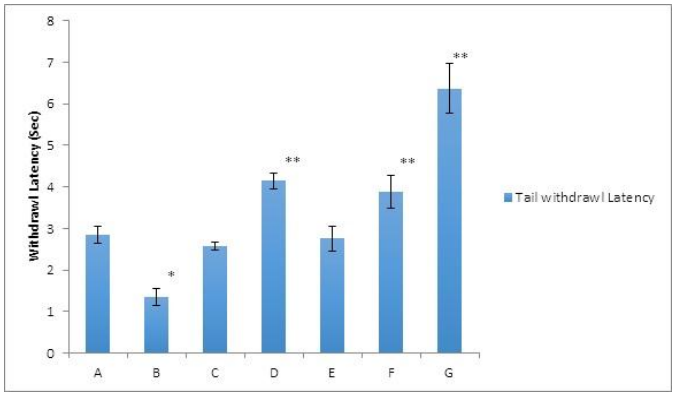
Data is presented as Mean ± SEM (n=6); One-way Analysis of Variance (ANOVA) followed by *Dunnett's- test*. *P < 0.05,**p < 0.01, ***p < 0.001 vs Normal Control and Diabetic Control.

Fig 2. Effect of EAZM on tail withdrawal latency in Tail Flick test



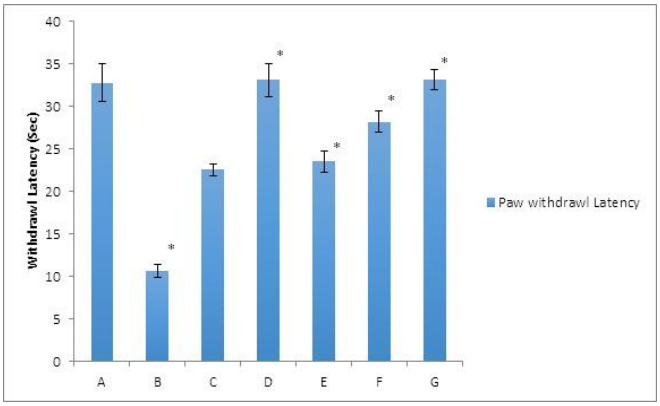
Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*.*p < 0.0001, **p < 0.01 vs Normal Control and Diabetic Control.

Fig 3. Effect of EAZM on tail withdrawal latency in Tail Immersion test



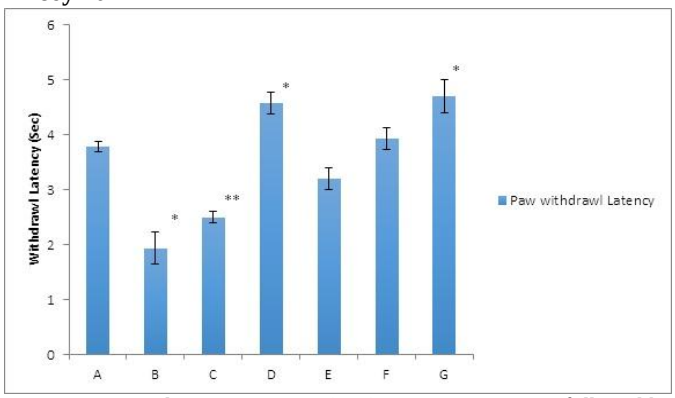
Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*.*p < 0.05, **p < 0.0001 vs Normal Control and Diabetic Control.

Fig 4. Effect of EAZM on paw withdrawal latency in Hot Plate test



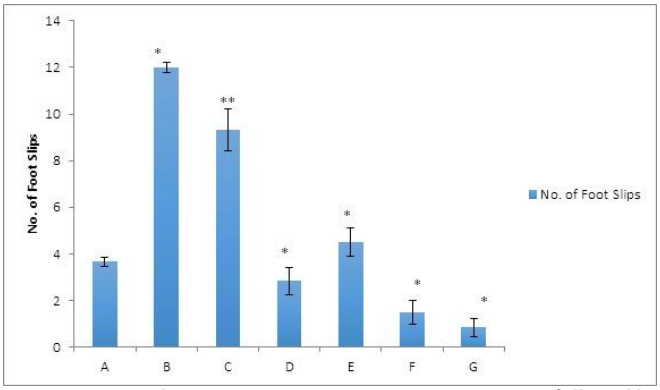
Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*.*p < 0.0001 vs Normal Control and Diabetic Control.

Fig 5. Effect of EAZM on paw withdrawal latency in Cold Allodynia



Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*.*p < 0.0001, **p < 0.01 vs Normal Control and Diabetic Control.

Fig 6. Effect of EAZM on no. of Foot slips in Beam Walk test.



Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*.*p < 0.0001, **p < 0.05 vs Normal Control and Diabetic Control.

Table 1. Physical properties of EAZM

Sr.no.	Property	Inference
1.	Colour	Dark green
2.	Odour	Slightly aromatic
3.	Taste	Bitter
4.	Appearance	Solid
5.	Total ash value	8.02%
6.	Acid insoluble ash	2.72%
7.	Alcohol soluble extractive value	25.6%
8.	Water soluble extractive value	67%

Table 2. Acute toxicity study

Drug	Code	Toxicity			Additional observation								Behavioural observation										
						ANS/CNS																	
		ON SET	STOP	No. of death	Skin and fur	Eyes Lacri	Salivation	Diarrhoea	Respiration	Straub tail	Pilo erection	Convulsions	Motor activity	Stereotypy	Tremors	Catalepsy	Sedation	Hypnosis	Writhing	Muscle spasm	Analgesia	Arching & rolling	Writhing
EAZM	H	Nil	Nil	Nil	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N
	B	Nil	Nil	Nil	N	N	N	N	N	N	Y	N	Y	N	N	N	N	N	N	N	N	N	N
	T	Nil	Nil	Nil	N	N	N	N	N	N	Y	N	Y	N	N	N	N	N	N	N	N	N	N

Lacri-lacrimation, Y – yes, N – no.

Table 3. Effect of EAZM on fasting blood glucose

Group	Fasting blood glucose level (mg/dl)				
	Initial	1st week	2nd week	3rd week	4th week
A	81.50±3.25	80.33±2.92	81.83±2.62	79.33±2.43	88.33±3.0
B	87.33±2.49	189.2±45.86*	372.7±45.07*	425±30.4***	510.3±23.9
C	79.00±2.92	199.8±26.09*	337.8±24.41*	183.0±12.97**	98.67±3.01***
D	82.67±2.43	168.7±17.64	352±31.70*	203±30.55***	142.7±8.67***
E	80.83±1.27	177.7±20.22*	339.7±22.39*	177.0±12.68**	109.8±3.14***
F	85.83±1.53	177.3±20.57*	325.5±21.28*	141.5±9.94***	98.33±3.08***

Data is presented as Mean ± SEM (n=6); One-way Analysis of Variance (ANOVA) followed by *Dunnett's- test*. *P < 0.05, **p < 0.01, ***p < 0.001 vs Normal Control and Diabetic Control.

Table 4. Effect of EAZM on tail withdrawal latency in Tail Flick test

Group	Tail Withdrawal Latency (Sec)
A	2.29±0.26
B	0.68±0.06*
C	1.71±0.14**
D	2.57±0.22*
E	2.11±0.22*
F	2.38±0.16*
G	2.69±0.03*

Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*. *p < 0.0001, **p < 0.01 vs Normal Control and Diabetic Control.

Table 5. Effect of EAZM on tail withdrawal latency in Tail Immersion test

Group	Tail Withdrawal Latency (Sec)
A	2.84±0.208
B	1.34±0.26*
C	2.57±0.184
D	4.14±0.29**
E	2.75±0.37
F	3.88±0.49**
G	6.36±0.66**

Data is presented as mean ± SEM (n=6); one way ANOVA followed by *Dunnett's test*. *p < 0.05, **p < 0.0001 vs Normal Control and Diabetic Control.

Table 6. Effect of EAZM on Paw withdrawal latency in Hot Plate test

Group	Paw Withdrawal Latency (Sec)
A	32.79±2.24
B	10.67±0.88*
C	22.55±0.74
D	33.10±1.9*
E	23.51±1.22*
F	28.21±1.33*
G	33.18±1.26*

Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*. *p < 0.0001 vs Normal Control and Diabetic Control.

Table 7. Effect of EAZM on Paw withdrawal latency in Cold Allodynia

Group	Paw Withdrawal Latency (Sec)
A	3.79±0.13
B	1.94±0.30*
C	2.49±0.10**
D	4.57±0.29*
E	3.20±0.21
F	3.93±0.20
G	4.70±0.38*

Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*. *p < 0.0001, ** p < 0.01 vs Normal Control and Diabetic Control.

Table 8. Effect of EAZM on no. of Foot Slips in Beam Walk test

Group	Number of Foot Slips
A	3.66±0.21
B	12.00±0.25*
C	9.33±0.95**
D	2.83±0.65*
E	4.50±0.67*
F	1.50±0.50*
G	0.83±0.40*

Data is presented as mean ± SEM(n=6); one way ANOVA followed by *Dunnett's test*. *p < 0.0001, **p < 0.05 vs Normal Control and Diabetic Control.

DISCUSSION

Streptozotocin induced diabetes is a well-documented model of experimental diabetes. Previous reported literature indicates that the type of diabetes and characteristics differ with the employed dose of STZ and animal and species used. A single diabetogenic dose of STZ (70-250mg/kg, body weight) has been demonstrated to induce complete destruction of β cells in most species within24 hour, multiple sub-diabetogenic doses of STZ partially damage islets, thereby triggering an inflammatory process leading to macrophage and subsequent lymphocyte infiltration, which is followed by the onset of insulin deficiency [10]. Diabetes mellitus (DM) is an endocrine disorder that is characterized by hyperglycemia. It is caused by inherited and/or acquired deficiency in production of insulin by beta cells of pancreas or by the ineffectiveness of the insulin produced, which leads to hyperglycemia [19]. We have followed the multiple dosing type of induction of diabetes by administration of STZ (40 mg/kg, 5 consecutive days). Observations indicate that diabetes was successfully induced in mice under study.

Herbal formulations have been used by the majority of Indians since ancient times [20]. Our own pharmacognostical evaluation of EAZM revealed the presence of alkaloids, tannins, steroids, triterpenoids, saponins, and flavonoids. As stated earlier that ethanolic extract of *Ziziphus mauritiana* leaves dose dependently reduces the blood glucose level in alloxan induced diabetic rats, we have studied antidiabetic activity of Ethyl Acetate fraction of methanolic extract *Ziziphus mauritiana*, Lam. leaves (EAZM) in STZ induced diabetic mice. Results show that EAZM has potential antidiabetic activity. All forms of diabetes are characterized by chronic

hyperglycaemia and the development of diabetes-specific microvascular pathology in the retina, renal glomerulus and peripheralnerve. As a consequence of its microvascular pathology, diabetes is a leading cause of blindness, end stage renal disease and a variety of debilitating neuropathies [21]. Of the secondary complications of diabetes, neuropathy is the most frequently encountered and can be expected to show some manifestation in over half of all diabetic patients during their life span [21]. The most useful model of diabetic neuropathy should exhibit the key feature present in human pathology. Diabetic rodents show behavioral, functional, structural and molecular biomarkers and they are widely used as models to investigate the etiology as well as to screen the efficacy of the potential therapeutic interventions [16]. Behavioral studies in diabetic rats often focus on the response to a painful or non-painful sensory stimulus, thereby measuring hyperalgesia and allodynia, respectively. The simplest of such tests measures the time to withdrawal of a limb such as the tail or a paw from a noxious heat source, with a faster withdrawal time being interpreted as hyperalgesia and a slower one as hypoalgesia [22]. We have studied diabetic neuropathy using animal models such as thermal hyperalgesia (tail flick test, tail immersion and hot plate test), cold allodynia and motor inco-ordination (beam walk test). These models are supposed to be Behavioral Biomarkers of diabetic neuropathy [16]. After 4th week diabetic control group has shown signs of neuropathy while treated group has shown improvement in their condition. EAZM has dose dependently shown protective action in thermal hyperalgesia, cold allodynia and motor in coordination. Thus, EAZM has reduced diabetic neuropathy.

Reports of earlier studies suggested that various plants was proved to possessing wide variety of natural antioxidant constituents such as tannins, saponosides, alkaloids, flavonoids, phenolic acids and poly phenols etc. which enhances free radical scavenging activities and responsible to ameliorate change in antioxidant enzymes which may be helpful for treatment of diabetic related complications [23]. The tannins, polyphenols and flavonoids present in herbal drugs are proved to be effective in diabetes treatment [24]. EAZM shows presence of these phytochemicals. The present study indicates that STZ induced diabetic mice shows elevated blood glucose level and reduced pain threshold in thermal hyperalgesia and cold allodynia, and also reduced motor inco-ordination. It can be concluded that EAZM ie. Ethyl Acetate fraction of methanolic extract of *Ziziphus mauritiana*, Lam. leaves has Antidiabetic activity and also protective against diabetic neuropathy in STZ induced diabetic mice.

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