EVALUATION OF ETHONOLIC EXTRACT OF OTTELIA ALISMOIDES (L.) PERS ON THE PAIN THRESHOLD RESPONSE IN STZ INDUCED DIABETIC NEUROPATHIC PAIN MODEL IN RATS

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Abstract
The present study was designed to evaluate the Ethonolic extract of Ottelia alismoides on the pain threshold response in STZ induced diabetic neuropathic pain model in rats. To the overnight fasted rats, diabetes was induced by single dose of STZ (55mg/kg, b.w., i.p) injection dissolved in 0.01 M citrate buffer at pH 4.5. Before STZ injection the basal reaction time were taken in different behavioural models. After STZ injection the drug treatment was started from week 4 onwards and continued upto 8th week. All the group were treated with insulin except vehicle treated group to maintain plasma glucose levels. Behavioural assessments like thermal hyperalgesia and allodynia were performed at 4th, 6th & 8th week. At end of the study period all the experimental animals were sacrificed followed by the biochemical and oxidative stress were evaluated in sciatic nerve tissues. Animals treated with plant extract of EEOA significantly decreased blood glucose level and restore the reduced body weight and organ weights. The plant extract exhibited significant decrease in oxidative stress and increase in endogenous antioxidant enzyme levels. After 8th weeks of treatment of EEOA produced more significant antinociceptive activity as compare to pregabalin. Treatment of insulin did not alter behavioural parameters. Histopathological analysis indicated that plant extract of EEOA corrected the sciatic tissue in the diabetic rats. Thus, from this study we concluded that the plant extract exhibits significant antidiabetic, antioxidant and neuroprotective activity against STZ induced diabetic neuropathy in rats.

Keywords: Diabetic neuropathy, Streptozotocin, Pregabalin, Ottelia alismoides

Received on- 10.10.2017; Revised and accepted on- 25.10.2017; Available online- 31.10.2017

Introduction
Diabetes is a serious, chronic disease that occurs either when the pancreas does not produce enough insulin (a hormone that regulates blood glucose), or when the body cannot effectively use the insulin it produces. More than 400 million people live with diabetes. Globally, an estimated 422 million adults are living with diabetes mellitus, according to the latest 2016 data from the World Health Organization(WHO). Diabetes prevalence is increasing rapidly; previous 2013 estimates from the International Diabetes Federation put the number at 381 million people having diabetes. The number is projected to almost double by 2030. Type 2 diabetes makes up about 85-90% of all cases. Increases in the overall diabetes prevalence rates largely reflect an increase in risk factors for type 2, notably greater longevity and being overweight or obese. Long standing diabetes mellitus leads to multiple organ damage and it is associated with an increased prevalence of
microvascular disease (Nephropathy, Neuropathy & Retinopathy) and macro vascular diseases (Peripheral vascular disease, Ischemic heart disease & Stroke). Poor glycemic control, a factor that has been observed in the Indian population with diabetes put them at risk of complication including neuropathy-24.6%, cardiovascular disease-23.6%, kidney problem-21.1%, retinopathy-16.6% and foot ulcer-5.5%\textsuperscript{4}.

Globally diabetic neuropathy affects approximately 132 million people as of 2010 (1.9% of the population). Diabetes is the leading known cause of neuropathy in developed countries, and neuropathy is the most common complication and greatest source of morbidity and mortality in diabetes\textsuperscript{4}. Several medications are used to relieve nerve pain, but they did not work for everyone and most have side effects that must be weighed against the benefits they offer. There are also a number of alternative therapies, such as capsaicin cream (made from chili peppers), physical therapy or acupuncture, that may help with pain relief medications. Anti-seizure medications drugs such as gabapentin (Gralise, Neurontin), pregabalin (Lyrica) and carbamazepine (Carbatrol, Tegretol) Tricyclic antidepressant drugs such as amitriptyline, desipramine (Norpramin) and imipramine (Tofranil), serotonin and nor epinephrine reuptake inhibitors (SNRIs), such as duloxetine (Cymbalta), can relieve pain with fewer side effects\textsuperscript{5}.

Various herbal remedies for diabetic neuropathy described in the ancient healthcare system of India. The herbs like Ashwagandha, Chandraprabha Vati, Shilajit, Indian gooseberry, Turmeric, Flaxseed oil, Ginger, Fenam seed, Castor oil, Holy basil, Jamun and Fenugreek, Bitter melon, Ginseng, Cayenne Pepper, cinnamon, Bilberry leaves & Plecospermum spinosum\textsuperscript{6}. Above herbs for diabetic neuropathy may be alleviate or decrease the symptoms of neuropathy.

Ottelia alismoides (L.) pers belongs to the family hydrochritaceae which is commonly known as duck lettuce. It is Traditional aquatic plant and Widely distributed in northeastern Africa, South Korea, eastern and southeast Asia and the tropical regions of Australia, Bangladesh\textsuperscript{7} and the whole plant is used as Traditional medicine for the treatment of Haemorrhoids, pneumonia, asthma, breast cancer & tuberculosis\textsuperscript{8}. It is found throughout India in tanks, ponds, streams and ditches. Earlier two diastereomeric 4-methylene-2-cyclohexenones, otteliones A and B, ten new diarylheptanoids (2,3,4,5a,6,7&8) together with a hydroxylated analog of ottelione A & B and 3a hydroxylorettellone have been isolated from this plant. Phytochemical present in this genus contain different natural compounds like Glycosides, Alkaloids, Flavonoids, Terpenoids, Tannins, Phenolic compounds. The alkaloids and glycosides present in the herbal drugs are proved to be potent antioxidants activity. The plant extract has also been reported to have anti-tuberculosis\textsuperscript{9}, cytotoxicity\textsuperscript{10} and anti proliferative activity\textsuperscript{11}. Research in this area is still unexplored, therefore in our present study, we have investigated the effect of neuroprotective activity of Ottelia alismoides against diabetic neuropathy.

Materials and methods

Plant Material

The whole plant Ottelia alismoides (L) PERS., were collected in the month of February from Agaram Village Kudapakkam Villianur Pondicherry. The plant material was taxonomically identified and authenticated by Dr.N.Ayyapan Researcher, French Institute of Pondicherry, Pondicherry.

Preparation of Crude Extract

The whole plant of Ottelia alismoides (L) PERS. were dried under shade and then powdered with a mechanical grinder. The powder was passed through sieve No:40 and stored in an airtight container for further use. The fine powdered whole plants were kept with 90% alcohol in soxhlet apparatus to get the crude drugs\textsuperscript{12}.

Phytochemical Screening

Various phytochemical tests were performed to determine the presence of Glycosides, Alkaloids, Flavonoids, Terpenoids, Tannins, Phenolic compounds in extracts\textsuperscript{13}.

Acute toxicity study

The Acute toxicity study of ethanolic extract of the whole plant of Ottelia alismoides were performed in swiss albino mice (20-25gm) according to Organization for Economic Cooperation And Development (OECD) guidelines No:423. Institutional Animal Ethics Committee approval
was obtained for animal experiment (IAEC No: NCP/IAEC 2016-17-18/CPCSEA). The plant extract was dissolved in water and administered orally to overnight fasted animals at the doses of 5, 50, 300, 2000 mg/kg of body weight in mice. After administration of the extract, the animals were observed continuously for 14 days for mortality and general behavior. Based on performed toxicity tests, the LD₅₀ dose was selected as 200 & 400 mg/kg, p.o.

Chemicals Used
Streptozotocin was obtained from Sisco research laboratories Pvt. Ltd, Mumbai, India and Pregabalin was purchased from Swapnaroop drugs & pharmaceuticals, Aurangabad, Maharashtra, India. All other chemicals and reagents used were of analytical grade.

Animals & Induction of Diabetes
Wistar albino rats of male sex weighing between 180-250 gm were gathered from the Nandha college of pharmacy, Erode. The rats were housed in cage under standard laboratory conditions 23±2 °C with 2h light and dark cycle and hand free access to water with standard chow diet. A freshly prepared solution of Streptozotocin (STZ 55 mg/kg in 0.01 M citrate buffer, pH 4.5) was injected intraperitoneally to overnight-fasted rats. The rats exhibited hyperglycaemia within 72 h of STZ administration. The rats having fasting blood glucose (FBG) values of 300 mg/dl or above were considered for the study. The study of test compound and standard drugs were dissolved in 0.5% CMC and administered orally with the help of gastric tube.

Experimental Design
Diabetic rats were randomly divided into five groups and each group consists of 6 animals. The basal reaction time of the rats before administration of STZ was taken in different analgesic models at week 0.

Group I- STZ dissolved in freshly prepared citrate buffer + Vehicle (0.5% CMC).

Group II- STZ + Insulin (5 IU/kg, i.m) dissolved in 0.5% CMC.

Group III- STZ + Insulin + Standard drug Pregabalin (15mg/kg) dissolved in 0.5% CMC.

Group IV- STZ + Insulin + EEOA (200mg/kg, b.wt; p.o) suspended in 0.5% CMC.

Group V- STZ + Insulin + EEOA (400mg/kg, b.wt; p.o) suspended in 0.5% CMC.

In the first four weeks of the study, all the groups were left untreated. From week 4 onwards the drug treatment started and continued up to the week 8 after STZ injection. Behavioural assessments like thermal hyperalgesia (hot plate, tail flick & Tail immersion), Mechanical hyperalgesia (Tail clip) thermal allodynia (cold plate) & (chemical hyperalgesia) formalin were performed on week 4th, 6th & 8th of the study. At the end of 8th week the Body weight and the blood glucose were measured. All the treated animals were sacrificed the liver & kidney was collected for its weights and sciatic nerve was isolated for Biochemical and histopathological studies.

Evaluation of parameters
General parameters
Body weight and Organ weight
The Body weight of rats was checked using a top loader weighing balance. The body weight is expressed in grams. The vital organs such as kidney and liver were collected. Blood was removed from the organs with the filter paper and their weights were assessed with an electric weighing balance. The organ weights were expressed in grams or milligrams.

Evaluation of biochemical parameters
Estimation of Blood glucose level
The Blood glucose levels were estimated by Hexokinase method. 150 μl of reagent 1 was added with 30 μl of reagent 1A and to this 20 μl of suitable diluents was added and the contents were mixed thoroughly. To this mixture, 2 μl of serum sample was added. Then the contents were mixed and incubated at 37°C for 10 seconds. After zeroing the instrument with blank the absorbance of standard followed by the test sample was measured at 340 nm. The values were expressed as mg/dl.

Behavioral assessment
Hot Plate Method
The method was described by Eddy’s. In this hot plate method animals from the each group were placed on the hot plate (Eddy’s hot plate) which is commercially available consists of an electrically heated surface. Temperature of this hot plate is maintained at 55 C- 56 C. This can be a copper
plate or a heated glass surface. The observation is done up to the time until paw licking or jumping was noted the cut-off time was 10 s. The reaction time was noted after the oral administration of the drugs and test compounds.

**Tail Flick Method**
The nociceptive response was evaluated regarding the latency to withdrawal of the tail in response to noxious radiant heating. Animals were placed into individual restraining cages leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The apparatus used is tail flick analgesiometer, the tip of tail of rat is placed on hot metal wire and latency of withdrawal is calculated manually by stop watch (D’Amour and Smith in 1941)19,20.

**Tail Immersion Method**
**Hot / cold water Tail Immersion Test**
The procedure was based on the reaction time of the typical tail-withdrawal reflex in rats induced by immersing the end of the tail in warm water of 52.5±1ºC or cold water of 10±0.5ºC. The lower portion of the tail, nearly 5 cm from tip, was marked and immersed in a beaker of freshly filled warm water or cold water. The rats reacted by withdrawing the tail in a few seconds. This reaction was measured two to three times, with an interval of at least 15 min, in order to obtain two consecutive values that differed no more than 10%. After each determination, the tail was carefully dried. The cut-off time for warm water tail immersion was 15 s and cold water tail immersion 30 s Sharma et al., 2006 21.

**Cold Plate Method**
Cold-plate tests were conducted to evaluate the hyperalgesic responses to cold stimuli. The rats were placed in the Plexiglas compartment. The time that elapsed between placing the animals in the device and the time point that they quickly withdraw their paws was recorded . The steps taken by the animals when walking or the slow paw-withdrawal behavior related to locomotion were not considered (Ortega-Alvaro et al., 2012). Measurements were repeated three times for each rat, and the average of these three values was calculated. To avoid paw damage, the experiment was not conducted for more than 30 s. (Rosellini et al., 2012) 22.

**Formalin Test**
At the end of the study, all the groups were subjected to the formalin test. Briefly, each rat was acclimatized to the observation box before the formalin test. After an adaptation period of 15 min, the right hind foot paw was injected with 50 mL of 2.5% formalin in the intraplantar region. Nociception was evaluated by quantifying paw licking time during the first 10 min (acute phase) and at 20– 40 min (delayed phase) (Khalilzadeh et al., 2008; Luiz et al., 2007) 24,25.

**Evaluation of antioxidants parameters**
**Nerve collection and preparation of Homogenate**
After 8 weeks of experiment, sciatic nerve was removed bilaterally from the inguinal ligament to its trifurcation and incubated in triton x 100 for 20 min to remove blood from sciatic nerve. Sciatic nerves were chopped into small pieces and then homogenized with phosphate buffer solution (pH 7.0) by using homogenizer. The homogenate was then centrifuged in a cold centrifuge (Thermo Scientific) at 4ºC, 4000 rpm for 10 min. The resulting homogenate was passed through a cellulose filter to remove impurities and was aliquoted for the measurement of SOD, GSH, CAT, GPx, LPO & Na⁺, K⁺, ATPase activity 26.

**Superoxide dismutase(SOD)**
SOD activity was assayed in the sciatic nerve homogenate according to the method of Misra and Fridovich 27. The enzyme catalyzes the dismutation of superoxide anion (O2⁻) to hydrogen peroxide and molecular oxygen. The absorbance was recorded at 480 nm. The activity was expressed as units/mg protein.

**Catalase (CAT)**
The enzyme activity was assayed by the method of Sinha 28. Hydrogen peroxide (H2O2) decomposition
by CAT was monitored spectrophotometrically by following the decrease in absorbance at 590 nm. The activity of enzymes was expressed as units/mg protein.

**Reduced glutathione (GSH)**

The total reduced glutathione was measured according to the method of Ellman.\(^{29}\) In this assay, GSH reduced 5, 5'-dithiobis (2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid. The absorbance was measured spectrophotometrically at 412 nm. The amount of GSH was expressed in units/mg protein.

**Glutathione peroxide (GPx)**

GPx activity was assessed by modified method of Flohe and Gunzler (1984).\(^ {30}\) In this assay GPx were determined by inhibit cytochrome C. The absorbance was measured at 412 nm. The rate of decrease in H₂O₂ and the amount of reduced Nicotinamide Adenine Dinucleotide phosphate (NADPH) oxidized. The amount of GPx was expressed in units/mg protein.

**Evaluation of oxidative stress parameter**

**Lipid Peroxidation (LPO)**

Lipid peroxidation (LPO) was assayed by the method of Ohkawa et al.\(^ {31}\) The malondialdehyde (MDA) was estimated by determining the accumulation of thioarbituric acid reactive substance (TBARS) in the nerve homogenate. The absorbance was measured by a UV spectrophotometer at 532nm. The level of lipid peroxides was expressed as units/mg protein.

**Other parameter**

**Na⁺, K⁺, ATPase activity**

Assay mixture was incubated for 30 min at 37 °C and the reaction was stopped by the addition of 3.5 mL of a solution-A (0.5% ammonium molybdate, 0.5 mol/L H₂SO₄, and 2% sodium dodecyl sulphate). The amount of liberated phosphate (Pi) was estimated and the values are recorded (Fiske and Subbarow(1925)).\(^{32,33}\) Further by adding tissue extract to the above same assay mixture and incubated for 30 min at 37 °C and the Na⁺/K⁺-ATPase activity was measured in a spectrophotometer at 700nm and the activity expressed as n mol pi released/mg protein per hour at 37 °C.

**Statistical analysis**

The data of all the results were represented as mean ± S.E.M and statistically analysed by one-way ANOVA followed by post hoc Tukey's multiple comparison test for antioxidant parameters and formalin test. Two-way ANOVA followed by Bonferroni post test was used for Body weight, Fasting Blood Glucose, Hot plate, Cold plate, Tail flick, Tail clip & Tail immersion. Statistical analysis p<0.05 were considered as significant. The analysis was carried out using Graph pad prism software (version 5).

**Results**

**Estimation of EEOA on Plasma Glucose level in diabetic rats**

Administration of STZ (55mg/kg) significantly elevated (p<0.001) the plasma glucose level in all groups. Treatments (p<0.001) significantly decreased the plasma glucose levels. Treatment of insulin significantly (p<0.001) reduced the plasma glucose levels in comparison to vehicle treated STZ rats (Group I). Drug treatment groups (Groups III & IV) did not influence the plasma blood glucose levels in comparison to insulin treated rats (Group II). Group V along with insulin significantly decreased (p<0.05) the plasma glucose levels in comparison to Insulin treated rats. (Table no:1)

**Estimation of EEOA on Body weight in Diabetic rats**

Administration of STZ (55mg/kg) significantly decreased p<0.001) the Body weight in vehicle treated diabetic groups. Treatments (p<0.001) significantly increased the body weight. Treatment of Insulin significantly (p<0.001) increased the Body weight in comparison to vehicle treated STZ rats. Co-administration of Drug treatment Groups (III, IV & V) along with Insulin did not influence in the Body weight in comparison to Insulin alone treated rats.

**Estimation of EEOA on Organ weight in Diabetic rats**

Administration of STZ (55mg/kg) significantly increased the liver & kidney weights in vehicle treated groups. Treatments (p<0.01) significantly decreased the liver & kidney weights. Co-administration of Group III, IV & V along with Insulin significantly decreased the liver & kidney weights in comparison to Insulin treated group.
Table No. 01: Effect of EEOA on Plasma Glucose level in experimentally induced Diabetic rat model

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma Glucose Level (mg/dl)</th>
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<tr>
<td></td>
<td>0 week</td>
</tr>
<tr>
<td>Group I</td>
<td></td>
</tr>
<tr>
<td>STZ(55mg/kg)+Vehicle</td>
<td>325.15±17.76</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
</tr>
<tr>
<td>STZ + Insulin (5 IU/kg)</td>
<td>322.10±22.31</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
</tr>
<tr>
<td>STZ+ Insulin+ Pregabalin (15mg/kg)</td>
<td>326.15±13.18</td>
</tr>
<tr>
<td>Group IV</td>
<td></td>
</tr>
<tr>
<td>STZ+ Insulin+ EEOA (200mg/kg)</td>
<td>321.10±15.46</td>
</tr>
<tr>
<td>Group V</td>
<td></td>
</tr>
<tr>
<td>STZ+ Insulin+ EEOA (400mg/kg)</td>
<td>324.10±12.18</td>
</tr>
</tbody>
</table>

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)
***, indicates significance P <0.001 when compared to vehicle treated Diabetic groups.
** indicates significance P <0.05 when compared to Insulin treated diabetic groups.
Data was analysed by Two-way ANOVA followed by Bonferroni post test.

Table No. 02: Effect of EEOA on Body Weight and Organ Weight in Experimentally induced Diabetic rat model

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight</th>
<th>Organ weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
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<td></td>
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<td></td>
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<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ(55mg/kg)+Vehicle</td>
<td>204.16±6.93</td>
<td>4.55±0.45</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ + Insulin (5 IU/kg)</td>
<td>245.06±5.31***</td>
<td>4.98±0.34**</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ+ Insulin+ Pregabalin (15mg/kg)</td>
<td>248.31±5.30***</td>
<td>2.84±0.31**</td>
</tr>
<tr>
<td>Group IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ+ Insulin+ EEOA (200mg/kg)</td>
<td>240.56±5.29***</td>
<td>2.30±0.30**</td>
</tr>
<tr>
<td>Group V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ+ Insulin+ EEOA (400mg/kg)</td>
<td>249.10±5.20***</td>
<td>2.96±0.50**</td>
</tr>
</tbody>
</table>

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)
**,*** indicates significance P<0.01 & P <0.001 respectively when compared to vehicle treated Diabetic groups.

Estimation of EEOA on antioxidant enzymes levels

Effect of EEOA on SOD
The SOD level were measured and showed Fig. no.- (1). Treatment groups (II, III, IV, V) significant increase (p<0.05 & p<0.01) in the SOD activity in the sciatic nerve homogenate in comparison to vehicle treated diabetic groups. Group IV did not alter the SOD activity in comparison to Insulin alone treated diabetic rats. Both Groups III & V along with Insulin treatment rats showed significant increase (p<0.01) the SOD level in comparison to Insulin alone treated rats.
Fig. No. 01: Effect of **EEOA** on SOD in experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

"**" Indicates significance p<0.05 & p<0.01 respectively compared to vehicle treated diabetic groups.

"**a" indicates significance p<0.01 when compared to Insulin treated diabetic groups.

Fig. No. 02: Effect of **EEOA** on CAT level in experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

"*" **" Indicates significance p<0.05 & p<0.01 respectively when compared to vehicle treated diabetic groups.

*" Indicates significance p<0.05 when compared to Insulin treated diabetic groups.
Fig. No. 03: Effect of EEOA on GPx in experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

**,** indicates significance p<0.01 & p<0.001 Respectively when compared to vehicle treated diabetic groups.

*.** indicates significance p<0.05 & p<0.01 respectively when compared to insulin treated diabetic groups.

Fig. No. 04: Effect of EEOA on GSH in experimentally induced Diabetic rat model
The values were expressed as Mean ± S.E.M (n=6 animals in each group).  

*** indicates significance $p<0.001$ when compared to vehicle treated diabetic groups.  
aaa indicates significance $P<0.05$ & $p<0.01$ respectively when compared to Insulin treated diabetic groups.

**Effect of EEOA on CAT**

The CAT level were measured and showed in Fig. no-(2). The Groups III, IV & V showed significant increase ($p<0.05$ & $p<0.01$) in CAT activity when compared to vehicle treated group. Insulin treated diabetic rats did not increase the CAT activity in comparison to vehicle treated diabetic rats. CAT levels were significantly ($p<0.05$) increased with Extract treated group in comparison to insulin alone treated diabetic group.

**Effect of EEOA on GPx**

The GPx level were measured and showed in Fig. no-(4). GPx activity was significantly increase in treatment Groups (II, III, IV & V) ($p<0.01$ & $p<0.001$) in comparison to vehicle treated diabetic group. Groups III, IV & V along with Insulin, showed significant increase ($p<0.05$ & $p<0.01$) the GPx levels in comparison to insulin treatment rats.

**Effect of EEOA on GSH**

The GSH level were measured and showed in Fig. no-(5). Group II, III, IV & V exhibited a significant increase ($p<0.001$) in the GSH levels in comparison to vehicle treated diabetic groups. Extract groups showed significant increase ($p<0.05$ & $p<0.01$) the GSH levels in comparison to insulin treated groups.

**Estimation of oxidative stress parameter**

**Effect of EEOA on LPO**

The Liver Malondialdehyde (MDA) levels were measured and showed in Fig. no-(5). Treated Groups (II, III, IV & V) significantly decreased ($p<0.05$, $p<0.01$ & $p<0.001$) the LPO levels in comparison to Group I. Group III along with Insulin significantly did not alter the LPO levels in comparison to Insulin alone treated rats. Group IV & V along with insulin significantly decreased ($p<0.01$ & $p<0.001$) the LPO levels in comparison to Insulin alone treated rats.
Fig. No. 06: Effect of EEOA on Na+ K+ ATPase Activity experimentally induced Diabetic rat model
The values were expressed as Mean ± S.E.M (n= 6 animals in each group)
*** indicates significance P <0.001 when vehicle treated diabetic groups
** and *** indicates Significance P < 0.01 and P < 0.001 respectively when compared to Insulin treated diabetic groups.

Fig. No. 07: Effect of EEOA on Hot plate test (Thermal Hyperalgesia) in experimentally induced Diabetic rat model
The values were expressed as Mean ± S.E.M (n= 6 animals in each group)
*** Indicates significance P <0.001 when compared to 0 week
**Effect of EEOA on Na⁺ K⁺ ATPase activity**

The Na⁺, K⁺, ATPase activity were measured and showed in Fig. no-(6). Treated Groups (II, III, IV & V) significantly increased (p<0.001) the Na⁺, K⁺, ATPase levels in comparison to vehicle treated diabetic rats. Extract treated Groups (III, IV & V) along with insulin significantly increased (p<0.01 & p<0.001) the Na⁺, K⁺, ATPase levels in comparison to Insulin alone treated rats.

**Behavioral assessment**

**Effect of EEOA on Hot plate test (Thermal hyperalgesia)**

The Paw Withdrawal latency were measured and showed in Fig. no-(7). Vehicle treated diabetic rats. significantly decrease (p<0.001) in Paw withdrawal latency at week 4th when compared to week 0. Insulin in alone treated rats from week 4 onwards did not alter the withdrawal latency at week-6and 8. Treatment of Groups (III, IV & V) along with Insulin significantly increased (P <0.01& p<0.001) the Pain thresholds from week 6 and 8 onwards in comparison to week 4. Insulin alone treated rats in comparison to Group III, IV & V along with insulin treated rats exhibited significant increased in Pain thresholds when compared to corresponding weeks.

**Effect of EEOA on Tail Flick test (Thermal hyperalgesia)**

The escape latency were measured and showed in Fig. no-(8). Vehicle treated diabetic rats exhibited a significant decrease (P <0.001) in the escape latency period at week 4 in comparison to week 0. Insulin treatment group from week 4 onwards did not alter the withdrawal latency from week 4 to 6 & 8 week. Drug treatment Groups III, IV & V along with insulin significantly increase (p<0.05 & P <0.001) the Pain thresholds from week 6 &week 8 onwards in comparison to week 4. Insulin treated rats in comparison to Groups III, IV & V exhibited significant increase (p<0.05, p< 0.01 & P < 0.001) in pain thresholds at corresponding weeks.

![Graph showing effect of EEOA on tail flick test](image_url)

**Fig. No. 08: Effect of EEOA on Tail Flick test (Thermal Hyperalgesia) in experimentally induced Diabetic rat model**

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

### Indicates significance P <0.01 when compared to 0 week

**Indicates significance P <0.05 & P <0.001 respectively when compared to 4th week

Indicates Significance P < 0.01 when compared to Insulin treated diabetic group at corresponding weeks.
Fig. No. 09: Effect of EEOA on Hot water Tail Immersion test (Thermal Hyperalgesia) in experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

### Indicates significance P<0.001 when compared to 0 week

**, *** Indicates significance P<0.01 & P<0.001 respectively when compared to 4th week

* Indicates Significance P<0.05 when compared to Insulin treated diabetic group at corresponding weeks.

Fig. No. 10: Effect of EEOA on cold water Tail Immersion test (Cold allodynia) in experimentally induced Diabetic rat model.

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

### Indicates significance P<0.001 when compared to 0 week

*, ***, **** Indicates significance P<0.05, P<0.01 & P<0.001 respectively when compared to 4th week
Effect of *EEOA* on Hot water Tail Immersion test (Thermal hyperalgesia)

The escape latency were measured and showed in Fig. no-(9). Vehicle treated diabetic rats (Group I) exhibited a significant decrease (P <0.001) in the escape latency period at week 4 in comparison to week 0. Insulin treatment group from week 4 onwards did not alter the escape latency from week 4 to 6 & 8 week. Treatment of Groups III, IV & V significantly decrease (P <0.01 & P <0.001) the pain perception from week 6 & week 8 onwards in comparison to week 4. Insulin treatment group in comparison to drug treatment groups exhibited significant decrease (P < 0.05) in pain perception at corresponding weeks.

Effect of *EEOA* on Cold water Tail Immersion test (Cold allodynia)

The escape latency were measured and showed in Fig. no-(10). Group I of diabetic rats exhibited a significant decrease (P <0.001) in the escape latency period at week 4 in comparison to week 0. Insulin treatment group significantly (p<0.05) improved the escape latency at week 6 and 8. Drug treated groups III, IV & V significantly increased (P <0.05, P <0.01 & P<0.001) the pain threshold from week 6 &8 onwards in comparison to week 4. Insulin treated rats in comparison to Group III, IV & V exhibited significant decrease (P < 0.05, P < 0.01 & P < 0.001) in pain perception at corresponding weeks.

**Fig. No. 11: Effect of *EEOA* on Cold plate test (cold allodynia) in experimentally induced Diabetic rat model**

The values were expressed as Mean ± S.E.M (n=6 animals in each group)

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**Table:**

<table>
<thead>
<tr>
<th>Condition</th>
<th>0th week</th>
<th>4th week</th>
<th>6th week</th>
<th>8th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (STZ+vehicle)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group II (STZ)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Group III (Pregabalin)</td>
<td></td>
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<tr>
<td>Group IV (EEOA 200mg/kg)</td>
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<td></td>
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<tr>
<td>Group V (EEOA 400mg/kg)</td>
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<td></td>
</tr>
</tbody>
</table>

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a Indicates Significance P<0.05 when compared to Insulin treated diabetic group at corresponding weeks.

**Insulin (5 IU/kg)**

The values were expressed as Mean ± S.E.M (n=6 animals in each group)

### Indicates significance P <0.001 when compared to 0 week

**, ** Indicates significance P <0.01 & P <0.001 respectively when compared to 4th week

a Indicates Significance P <0.05 when compared to Insulin treated diabetic group at corresponding weeks.
Fig. No. 12: Effect of EEOA on Tail Clip test (Mechanical Hyperalgesia) in experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

### Indicates significance P <0.001 when compared to 0 week

*** Indicates significance P < 0.05, P < 0.01 & P < 0.001 respectively when compared to 4th week

a,aa Indicates Significance P < 0.05 & P < 0.01 respectively when compared to Insulin treated diabetic group at corresponding weeks.

Fig. No. 13: Effect of EEOA on Formalin test (Chemical Hyperalgesia) in experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

**** Indicates significance P < 0.01 & P <0.001 respectively when compared to vehicle treated diabetic groups.

aa,aaa Indicates P < 0.01 & P < 0.001 respectively when compared to Insulin treated diabetic groups.
Effect of EEOA on Tail clip test (Mechanical hyperalgesia)
The Tail withdrawal latency were measured and showed in Fig. no-(12). Vehicle treated diabetic rats exhibited a significant decrease (P <0.001) in Tail withdrawal latency period at week 4 in comparison to week 0. Treatment of Insulin in diabetic rats from week 4 onwards did not alter the escape latency at week-6 and 8. Treatment of Groups III, IV & V along with insulin significantly increased (P <0.05 , P <0.01 & P <0.001) the Tail withdrawal latency from week 6 & 8 onwards in comparison to week 4. Insulin alone treated rats in comparison to Group IV & V significant increased (P < 0.05 P < 0.01) the Tail withdrawal latency at corresponding weeks.

Effect of EEOA on Formalin (Chemical hyperalgesia)
The acute phase were measured and showed in Fig. no-(13). In acute phase the groups III, IV & V treated rats spent significantly less time in paw licking in comparison to vehicle treated diabetic rats. Insulin treated group did not significantly alter the paw licking response when compared to group I. Groups III, IV & V along with insulin treated rats exhibited significant decrease in paw licking (p<0.01 & p<0.001) time when compared to Insulin alone treated rats. In Delayed phase Group III, IV & V treated rats spent significantly decreased in licking the paw in comparison to vehicle treated diabetic rats. Insulin alone treated groups did not alter the paw licking response in comparison to vehicle treated rats. Group III, IV & V along with insulin treated rats exhibited significant decrease in paw licking (p<0.01 & p<0.001) time when compared to Insulin alone treated rats.

Histopathology of the sciatic nerve
Morphological changes were examined in the sciatic nerve of all the groups of the experiment. In the peripheral nerves of the diabetic rat there was the nerve fascicle is surrounded by relatively thick perineurium and nerve axons are shrunken. Myelin sheath appears fragmented and separated in some fibers. Edema is also noticed in between the nerve fibers other field shows marked thickening of less dense myelin sheath in large myelinated fibers with loss of their axons in the sciatic nerve of vehicle treated group (Fig. No: 14). When compared to insulin treated group (Fig. No: 15). Both pregabalin (Fig. No: 16) & EEOA (200mg/kg & 400mg/kg) (Fig. No: 17&18) treated group was found to be more effective than insulin treated group.

Discussion
Hyperglycemia must be the originator in the pathogenesis of diabetic complications. Diabetes is a heterogeneous set of disorders with a range of pathologies and one of the most frequent complications of diabetes is peripheral neuropathy.45. Diabetic neuropathy is characterized by progressive chronic neuropathic pain that is tingling and burning in nature with hyperesthesia (excessive physical sensitivity, especially of the skin) and paresthesia (an abnormal sensation, typically tingling or pricking (pins and needles), caused chiefly by pressure on or damage to peripheral nerves) with deep aching and it is increased by touch.36. Neuropathic pain is defined as a form of chronic pain that results from damage or abnormal function of central or peripheral nervous system. The clinical presentation of neuropathic pain induces hyperalgesia, allodynia and spontaneous pain.36. Hyperglycemia leads to the toxicity of neurons due to increased glucose oxidation, leading to increased reactive oxygen species that may be controlled by the treatment with antioxidant.38-40.

In this study, Ottelia alismoides (L) PERS., extract was given for the treatment of neuropathic pain in STZ induced diabetic rats. The preliminary phytochemical analysis of EEOA revealed the presence of Glycosides, Alkaloids, Flavonoids, Tannins, Terpenoids, phenolic compounds. The tannins, phenols and flavonoids present in the plant Ottelia alismoides shows anti-diabetic action. The alkaloids and glycosides present in the herbal drugs are proved to be potent antioxidants as well as nephroprotective agent.41.

Acute toxicity studies revealed the non-toxic nature of the EEOA there was no lethality or any toxic reactions found with high dose (2000 mg/kg body weight) till the end of the study. According to the OECD 423 guidelines (Acute Oral Toxicity: Acute Toxic Classic Method), an LD50 dose of 2000 mg/kg and above was considered as unclassified so the EEOA was found to be safe.
In the present study STZ was used for induction of diabetes neuropathy in rats. The development of neuropathy was observed at 0 week after STZ induction, which was consistent with previous reports. STZ induced diabetic animal models are most accepted animal models of diabetic neuropathy owing to their reproducibility and reversible diabetes. STZ action involved pancreatic DNA alkylation through GLUT2 transporter mechanism. Which intern triggers multiple biochemical pathways such as polyol pathway, hexosamine pathway, protein kinase C pathway (PKC), advance glycation end (AGE) product and poly adipose ribose polymerase (PARP) pathway all of these pathways contribute towards oxidative stress by generating ROS in a mitochondria results in nerve damage and neuropathy. Rats injected with 55mg/kg showed significant increase in blood glucose levels and decreased nociceptive thresholds. Similar thermal hyperalgesia, mechanical and formalin evoked pain in STZ induced rats have been demonstrated earlier. Pregabalin a selective Ca2.2 (α2-δ subunit) channel antagonist and it has anti neuropathic pain, anticoagulant and anxiolytic activities. Treatment with EEOA extract along with insulin in diabetic rats significantly increased the nociceptive threshold, decreased oxidative stress in a dose dependent manner.

Before recording the findings on the kidney and liver the effects of STZ on the plasma glucose levels and body weights were noted. The observations and results of the present study demonstrated that STZ was effective in producing severe hyperglycaemia in experimental animals. The animals Treated groups II, III & IV appeared ill-looking with loss of their body weights because of injurious effects of STZ which caused alkylation of DNA and produced hyperglycaemia and necrotic lesions. Our invivo results showed that EEOA extract (200 and 400 mg/kg) treatment had an attenuating effect on the serum glucose of the diabetic animals with significant improvement in body weight it may be due to EEOA persisting insulin secretoroge action like sulfonlureas.

Increase in the weight of kidney (hypertrophy) in STZ treated rats. The mechanism of renal hypertrophy is unknown, evidence suggest that local alterations in the production of one or more growth factors and/or their receptors are crucial to this process. An increase (hypertrophy) in the weight of liver was compared with animals in treated groups. It could be attributed to increased triglyceride accumulation leading to enlarged liver which could be due to the increased influx of fatty acids into the liver induced by hypoinsulinemia and the low capacity of excretion of lipoprotein secretion from liver resulting from a deficiency of apolipoprotein B synthesis. The EEOA treated animals were found to have significant decreased in organ weights when compared with that of animals with diabetes induced neuropathy.

Oxidative stress is believed to be a biochemical trigger for sciatic nerve dysfunction and reduced endoneurial blood flow in diabetic rats. Persistent hyperglycemia disturbs the endogenous antioxidant defense mechanism and prevents the free radicals scavenging activity. In this study the activities of various antioxidant enzymes SOD, CAT, GPx, GSH & LPO had been found to be modulated in diabetic animals. The significant different in the activities of various biomarkers in diabetic versus treated animals indicated the augmented oxidative stress and tissue response during diabetes. Moreover nervous tissue is considered to be rich in polyunsaturated fatty acids mainly arachidonic acid and docosahexaenoic acids which are highly sensitive to LPO and depleted antioxidants status. The diabetic animals exhibited the similar biochemical imbalance in term of increased LPO levels and decreased endogenous antioxidant enzyme levels. Hence, these levels revealed the magnitude of oxidative stress and extent of structural and functional damage to the nervous system.

Diabetes causes increases in the LPO products such as malondialdehyde (MDA) or conjugated dienes in sciatic nerves. Scavenging free radicals reduced the above effects of sciatic pain models. In the present study EEOA exhibited significant increase in endogenous antioxidant enzyme and significantly decreased LPO levels. This antioxidant activity of the Ottelia alismoides extract could also have influenced its activity in diabetic neuropathic pain.

Acute hyperglycemia induced by STZ elicits reduction of Na+, K+, ATPase activity. Decrease in sciatic nerve Na+, K+, ATPase activity could alter
the normal membrane axon repolarisation after the depolarization induced by an action potential. EEOA treatment restored Na\(^+\) K\(^+\) ATPase activity. It might be possibly improved by inhibition of oxidative stress and also by amelioration of vascular function\(^{58}\).

Behavioral studies in diabetic rats often focus on the response to a painful or non-painful sensory stimulus, there by 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 hypalgesia\(^{59}\). In the present study diabetic neuropathy using animal models such as thermal hyperalgesia (tail flick, hot water tail immersion and hot plate) mechanical hyperalgesia (tail clip method) cold allodynia (cold plate, cold water tail immersion) and chemical hyperalgesia (formalin test)\(^{60}\).

These models are supposed to be behavioural biomarkers of diabetic neuropathy. The hot plate and test involves two types of responses paw licking and jumping. Both responses integrate at supraspinal structures with the C and A\(\delta\) type I and II sensitive fibres participating in this model. The tail withdrawal (tail flick, tail clip and tail immersion) response after thermal stimuli is elicited by the spinal motor reflex most probably via endogenous release of substance P in the spinal cord\(^{61}\). EEOA extract showed antinociceptive effect in the diabetic rats in both hot plate and tail immersion models, which indicate that EEOA may act via central mechanism. Cold allodynia (cold plate) might be caused by disproportionate loss of A\(\delta\) fibers and sensitization of cold receptors in peripheral neuropathic pain. Impaired blood flow also seems to contribute to allodynia\(^{62}\). Vasodilator treatment has been demonstrated to reduce allodynia in diabetic rats. EEOA may act on improved neuronal blood flow by its direct vasorelaxant properties.

The nociception induced by formalin is associated with injured tissue. It characterised by two phases acute & delayed. The acute phase(0-10min) is short lived and initiates immediately after injection and is characterised by C-fibre activation due to peripheral stimuli. The delayed phase(20-40min) is a longer, persistent period caused by local tissue inflammation and also by functional changes in the dorsal of the spinal cord. Therefore, this phase is inhibited both both by opioids and analgesic agents. Substances that act primarily as central analgesic inhibit both phases while peripherally acting drugs inhibit only the delayed phase. In the present study the EEOA extract exhibited analgesia only in the delayed phase which suggests that the extract would possibly prevent the inflammatory markers induced pain perception.

The H&E stained lateral section of vehicle treated diabetic groups sciatic nerve (A) showed nerve fascicle is surrounded by relatively thick perineurium. The nerve axons are shrunken. Myelin sheath appears fragmented and separated in some fibers. Edema is also noticed in between the nerve fibers Other field shows marked thickening of less dense myelin sheath in large myelinated fibers with loss of their axons compared to insulin treated diabetic groups (B). Treatment of Pregabalin and Ottelia alismoides (400 mg/kg body weight) in diabetic rats was shown to improve the thick myelinated nerve fibers with intact axons vehicle treated diabetic groups (A) surrounded by hypertrophied Schwann cells . The thin myelinated nerve fibers have normal appearance, edema partly reduced in the endoneurium in dose dependent manner.

**Conclusion**

The present study has shown that the ameliorative properties of Ottelia alismoides extract against STZ induced diabetic neuropathy in rats via its antihyperglycemic and antioxidative properties. Therefore, Ottelia alismoides application useful for the treatment of neuropathic pain induced by chronic diabetes.
Reference

Sumithira G. et al., Int. J. Pharm. & H. Care Res., Vol.–05 (04) 2017 [103 - 123]

30. Rotruck. Glutathione peroxidase was assayed by the method of .1973