ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Neuroprotective potential of *Phyllanthus amarus* and Esculetin in STZ-induced neuropathy in rats

Srilatha Kota and Pratap Reddy Karnati*

Department of Zoology, Neuroscience lab, Osmania University, Hyderabad-500020, Telangana, India

Article History:

Received on: 24.04.2018
Revised on: 25.07.2018
Accepted on: 27.07.2018

Keywords:

Antioxidant,
Diabetes,
Esculetin,
Neuropathy,
Degeneration of nerve,
Phyllanthus amarus,
Behavioural alterations

ABSTRACT

Hyperglycemia-induced overproduction of free radicals causes the development of diabetic neuropathy (DNP), leading to peripheral nerve degeneration. The current study reports the neuroprotective effects of *Phyllanthus amarus* extract (PAE) and Esculetin (ESC) on Diabetic peripheral neuropathy (DPN) in streptozotocin (STZ)-induced diabetic male rats. Injection of STZ (60mg/kg/b.w, i.p) significantly induced diabetes mellitus (DM), verified by having a blood glucose level of more than 250 mg/dl. Randal-Selitto assessed neuropathic analgesia was assessed by randal-selitto and hot-plate methods and the sciatic nerves were collected and assessed for biochemical parameters at different interval of 7,14 and 21 days. Histopathological changes were also evaluated at the end of the experiment. Treatments with *Phyllanthus amarus* extract (400mg/kg/b.w/d) and esculetin (45mg/kg/b.w/d), Significantly ameliorated the STZ induced diabetic peripheral neuropathy by attenuating, such as decrease in blood glucose level, water intake level, aldose reductase activity (AR), thiobarbituric acid reactive substances (TBARS) and increase in catalase, superoxide dismutase (SOD), GSH, GPx along with body weight. Treatment with *Phyllanthus amarus* and esculetin significantly reversed the pain sensitivity and myelination, degenerative changes of sciatic nerve fibre in both the groups compared to the diabetic control group. Thus, *Phyllanthus amarus* and esculetin possess potential neuroprotective effect in a rat model of diabetic peripheral neuropathy in respect of neuropathic behaviour, improved morphological changes and significant antioxidant activity.

* Corresponding Author

Name: Prof. K. Pratap Reddy
Phone: +91-9573935381
Email: pratapkreddyou@gmail.com

ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v9i4.1681

Production and Hosted by

IJRPS | https://ijrps.com

INTRODUCTION

Diabetic neuropathy is associated with peripheral nerve damage in a length-dependent manner, followed by their structural and functional loss (Said, 1996). Chronic hyperglycemia leads to the progressive development of long-term microvascular complication such as diabetic peripheral neuropathy (DPN) which causes disease and finally leads to death among those affected persons (Said, 1996; Maritim *et al.,* 2003). The prevalence of DPN also increases with time due to poor glycemic control and increased free radical production creating oxidative stress has been involved in related pathogenesis (Maritim *et al.,* 2003). Hyperglycemia mediated oxidative stress is the main cause along with altered polyol pathway, AGE's formation, mitogen-activated protein kinase over activation and PKC etc., (Oyenihi *et al.,* 2015). Although many antioxidant compounds are used and tested on animal models; still hyperglycemia-mediated oxidative stress is an effective target towards diabetic peripheral neuropathy. In the hyperglycemic neuron,

mitochondria are especially most vulnerable, because the production of ROS species can damage their DNA and membranes.

Natural products have been an ultimate source of discovery and development of therapeutic drugs. *Phyllanthus amarus* is commonly known as "Bhui amla", of Euphorbiaceae family, appear as a weed in cultivated and wastelands. It has a powerful antioxidant effect and exhibits numerous pleiotropic properties (Sangeeta *et al.,* 2017) thus, playing a great role for several experimental investigations. Presence of pure triterpenoids named ursolic acid, oleanolic acid and lupeol present in aerial parts of the *P.amarus* were shown to inhibit α-amylase activity (Hasenah, 2013). Besides, the polyphenolic content of *P.amarus* extract shows kinetic inhibition of carbohydrate-hydrolysing enzymes (Mohamad *et al.,* 2014) and reduce hyperglycemia levels in streptozotocin-induced diabetic rats (Owolabi *et al.,* 2011). Molecular docking and pharmacophore modelling studies have shown that phyllanthin (an active lignan present in various *Phyllanthus* species) had an affinity for aldose reductase, which can be used against T2DM68 (Masrur *et al.,* 2015). *Phyllanthus amarus* shows antioxidant and anti-nociceptive effects in sedentary men by improving exercise recovery. It exhibits anti-allodynic and anti-oedematogenic properties in persistent inflammatory and neuropathic pain models. Bhumyamalaki was able to significantly revert the decreased sensory perception and decrease the changes in numbness, tingling and burning pain in lower limbs in human diabetic neuropathy (Patel *et al.,* 2011). On the other hand, *Phyllanthus amarus* found to induce the regeneration of injured sciatic nerve (Panakpaporn, 2012).

Esculetin (6,7–dihydroxy coumarin) is a potent coumarin- derived antioxidant. It occurs in various plants (Anthony *et al.,* 2015), a variety of foods and is the main active ingredient of *Cortex fraxini* (Gennian *et al.,* 2015). Esculetin has broad prospect of developing effective drugs because of its anti-inflammatory, antioxidant, neuroprotective, anticancer properties and so on. It has been reported that esculetin is having aldose reductase inhibitory activity *in vitro* and cataractogenesis (Chan-Sik *et al.,* 2016). Esculetin is showing the antihyperglycemic effect in STZ-induced diabetic rats by modulating carbohydrate metabolic enzymes (Prabakaran *et al.,* 2012). It has anti-proliferating activity by initiating mitochondrial-dependent apoptosis pathway against hepatocellular carcinoma *in vivo* and *in vitro* (Wang *et al.,* 2015). Recent studies have shown the protective effect of esculetin on nociceptive pain of rats in non-inflammatory and inflammatory models (Przemyslaw *et al.,* 2015). Esculetin increases antioxidant activity by inhibiting adipogenesis in 3T3-L1 Cells (Younghwa *et al.,* 2017). Esculetin also protects against neuronal apoptosis caused by cerebral I/R injury by attacking Bcl-2, Bax and caspase-3 proteins (Wang *et al.,* 2012). Besides, it has shown the ability to protect cells against neurodegenerative diseases occur by the death of brain cells and degeneration of axons or dendrites (Kadakol, 2016). Another side, In vitro studies, has shown the protective effect of esculetin on the AD (Alzheimer's disease) by inhibiting BACE1, BChE and AChE enzymes (Ali *et al.,* 2016). Esculetin can restore GSH/GSSG ratio and COX activity in acute restraint stress mice model having the inability of contextual memory (Martin *et al.,* 2016). So far many clinical and exploratory studies have proposed the role of esculetin on vascular proliferative disorders, various skin diseases, hepatotoxicity, diabetes (Atsushi *et al.,* 2008), human bacterial infections, cancer and adipogenesis (Gennian *et al.,* 2015; Kadakol *et al.,* 2015). There is no data about its neuropotential effects against diabetic peripheral neuropathy.

To accomplish this, we investigated the beneficial effects of *Phyllanthus amarus* ethanolic extract and Esculetin in diabetic peripheral neuropathy in relation to traditional and scientifical way with respect to hyperglycemic control and anti-oxidative effects using behavioural, histological and biochemical parameters in type II diabetic rats.

MATERIALS & METHODS

Animals

36 male Wistar rats (Rattus norvegicus) weighs 200±50g were procured from NCLAS, National Institute of Nutrition (NIN), Hyderabad, India. Rats were maintained in standard laboratory conditions, in single rat polypropylene cages, at an ambient temperature of 22±2°C and humidity (45-50%), with a 12 hr light/dark period for minimum 7 days before and during the experimental period. To minimize the animal's suffering, measures were taken as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals) given by animal ethical committee (CPCSEA No: 383/01/a/CPCSEA), Department of Zoology, Osmania University, Hyderabad, India. Animals were provided with drinking water and rat dry food of known weight *ad libitum*.

Plant Extract Preparation

The aerial parts of *Phyllanthus amarus* plant were picked-up from Dendukur Village, Khammam district, Telangana, India during July-august, 2016. They were washed with double distilled water, dried under shade for two weeks then, finely powdered using the blender. The powdered plant material (500g) was then subjected to Soxhlet

extraction using 20% ethanol as the solvent for 40 hrs. Filtration of the extract was done using a 3-4 gauze cloth followed by Whatman No.1paper (32%w/w). Then, the evaporation of filtrate was carried out with the rotary evaporator (DLAB, RE100-Pro) at 50°C (22%w/w). The obtained extract was shade dried and utilized further by keeping in -20°C refrigerator.

Induction of Type 2 diabetes and Experimental design

Non-insulin-dependent (type 2) DM was induced in 18 Wistar male overnight fasted rats by injecting single dose (60 mg/kg b.w, i.p) of streptozotocin mixed in 0.1 M sodium citrate buffer, pH 4.5. Remaining 18 rats were served as non-diabetic. A drop of blood was collected from the tail vein after ~72 h of streptozotocin injection and hyperglycemia was confirmed by using a blood glucose test strip and glucometer (Dr.Morepen Gluco One, Model: BG-03). Only rats with glucose concentration more than 250 mg/dL in the blood were considered diabetic and were taken for the present study (Erbas O *et al.,* 2016). Animals were divided into 6 experimental groups.

1. Group I: Control animals (CT) - provided normal drinking water.

2. Group II: Diabetic control (DC) - received 60mg/kg b.w, i.p STZ in 1ml of buffer.

3. Group III: Diabetic + PAE - received STZ (60 mg/kg b.w, i.p) and then received ethanolic extract of *Phyllanthus amarus* (PAE) plant (400 mg/kg b.w/ml/day) orally from Day1 to 21 after induction of diabetes.

4. Group IV: Diabetic+ESC - received STZ (60mg/kg b.w, i.p) and then received esculetin (45 mg/kg b.w/ml/day), day1 to 21 after induction of diabetes.

5. Group V: PAE - received only ethanolic extract of *Phyllanthus amarus* (400 mg/kg b.w/ml/day) day1 to 21, which are non-diabetic rats.

6. Group VI: ESC - received only esculetin (45 mg/kg b.w/ml/day), day1 to 21, which are non-diabetic rats.

After confirmation of diabetes, from 4th day onwards after STZ injection, PAE and esculetin were administered daily for 21 days. Rats of day 7, 14 and 21 were selected for the experimental studies. Rats were sacrificed and the sciatic nerves were dissected from day 7, 14 and 21 rats and then used for biochemical and histopathological studies. During the experimental period, the gain in body weight of rats was monitored daily. Water intake level was also monitored daily at 10 a.m. High glucose levels were confirmed using a digital glucometer in all rats by taking blood from a tail vein on day 10 and 21 after STZ injection.

Chemicals

Esculetin and Streptozotocin were obtained from Sigma-Aldrich Company, USA. The solvent for plant extraction and all other chemicals used for biochemical analysis and histopathological examination were of analytical grade from Himedia, India.

Behavioural Studies

Thermal and Mechanical nociceptive responses: Thermal pain sensitivity was evaluated by the hot plate test (Gunn *et al.,* 2011). The pain reflexes were evaluated by footpad attachment on the hot plate for each rat. The time between placement of rat on the Remi hot plate (52.0±0.5^{o}C) and the rat showing various behavioural sensations of heat pain (rearing, forepaw and hindpaw licking, face washing, body cleaning, hind leg withdrawal, and jumping off) was noted as a time of response in seconds. Mechanical nociceptive tolerance was assessed by using the Randal-Selitto paw withdrawal test (Randall *et al.,* 1957) with an analgesic meter (TSE systems GmbH, Germany). It generates a linearly increasing limited applied force of up to 250g (calibrated force) to avoid skin damage. The results were expressed based on pressure (gm) maximally exerted by the rats in response to mechanical stimuli induced pain in the paw and the withdrawal of paw was noted. Rats were customized to the devices for 5 days before carrying out the tests. At each time point, rats were tested with five trials, and the values are averaged.

Estimation of aldose reductase activity

Aldose reductase assay was performed in sciatic nerve by Hayman and Kinoshita method (1965). Using 50mM potassium phosphate buffer, the sciatic nerve was homogenized at pH 7.2 followed by centrifugation at 25,000×g / 30 min at 0-4^{0}C and supernatant was used for the assay. At room temperature, the reaction was pre-incubated for 5 min and the further reaction was started by adding NADPH. The decrease in absorbance was recorded for 5 min at 340nm spectrophotometrically. The activity of aldose reductase was indicated in µ moles/mg of protein.

Enzymatic oxidative stress markers

Estimation of Thio-Barbituric Acid Reactive Substances (TBARS)

The lipids in the sciatic nerve will undergo peroxidation, was estimated according to Bhuyan *et al.* (1981). 1 ml of sciatic nerve homogenate and 1 ml of 20% Tri chloro acetic acid (TCA) were mixed, then heated using water bath at 70°C/10 min and

then cooled at room temperature. After centrifuging the samples at 30,000×g at 25°C for 10 min, 0.4ml of the supernatant was collected, which is devoid of proteins. It was mixed with 0.2ml of 0.5% aqueous solution of thiobarbituric acid (TBA), heated for 10 min then cool at room temperature. The absorbance of the pink coloured condensation Trimethine product determined spectrophotometrically at 533nm. Results were expressed in nanomoles of TBARS/gm tissue (MDA concentrations) and were calculated from the standard curve of tetra ethoxy propane (TEP).

The Sciatic nerve sample was homogenized in 0.05M sodium phosphate buffer, centrifuged at 750×g at 4^0C for 20 min. The supernatant was spun at 12000×g from which 50µl was used for catalase estimation and remaining supernatant was spun at 35000×g at 4^0C for 25min, from which 50µl was used for determining SOD activity.

Estimation of Catalase Activity (CAT)

Catalase activity was measured according to the method of Aebi *et al.* (1984). Catalase activity was measured by following UV-spectrophotometric method. In the ultraviolet range, H_2O_2 undergo decomposition followed by the reduction in extinction per unit time at 240 nm. The reaction mixture (3ml) was prepared by mixing 50µl of the tissue sample, 1.95 ml of sodium-phosphate buffer and 1ml of 30mM H_2O_2 which was added at the time of taking O.D readings. Catalase activity was measured by taking the difference in absorbance per unit time.

Estimation of Superoxide Dismutase Activity (SOD)

The sciatic nerve superoxide dismutase activity was measured by the method of Marklund and Marklund (1974). The method includes inhibition of O_2 dependent autoxidation of pyrogallol by SOD. The assay mixture includes 50 µmoles of Tris HCl buffer at pH 8.2, 0.05 µmole di-ethylene tri-amine Penta-acetic acid (DETAPA), 0.04 µmole pyrogallol and 50 µl of the enzyme. After the addition of pyrogallol, the increased absorbance was noted over 30 seconds at 420 nm, spectrophotometrically and the activity of SOD was expressed as units/mg protein.

Glutathione Peroxidase (GP_X)

GPx activity was estimated by the method of Rotruck *et al*. (1973). 1 ml of DTNB reagent (1% sodium citrate containing 0.04% DTNB) was added to 3 ml of the supernatant obtained after centrifugation of tissue homogenization with Tris-HCl buffer. The developed colour was measured spectrophotometrically at 412 nm. GSH activity was expressed as µ mol of GSH consumed/min/mg protein.

Nonenzymatic Antioxidants

Reduced Glutathione (GSH)

Reduced GSH content in the sciatic nerve was assessed by the Ellman method (1959). The reaction was established on the ability of GSH group to reduce DTNB, (5, 5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent)) to yield a yellow compound 5'-thio-2-nitrobenzoic acid (TNB), measured at 412 nm. The results were expressed as µg/mg protein.

Histopathological Studies

After the experimentation, the sciatic nerve was taken out according to the procedure described by Mizisin (2004). The animals were sacrificed and the sciatic nerve was exposed by removing the skin on the lateral surface and an incision was made on the left thigh. Sciatic nerves were carefully removed and preserved in 10% v/v formalin solution for histopathological analysis. The nerves were paraffin embedded and cut thin (3-5 µm) sections by using microtome and observed under a light microscope with hematoxylin and eosin (H&E) stain (Lillie *et al.,* 1976). Sections were evaluated on their severity of the pathological changes including nerve degeneration and oedema.

Statistical Analysis

The obtained results were expressed as the Mean ± SEM (standard error of the mean) and one-way analysis of variance (ANOVA) followed by t-test to determine the statistical significance between the groups.

RESULTS

The behaviour and health status of all animals were checked throughout the study.

Changes in Blood glucose, body weight and water intake levels

The blood glucose levels, body weights and water intake levels of all groups were shown in tables 1, 2 and 3. STZ-injected rats depicted basic symptoms of diabetes like loss of body weight, polyuria, polydipsia and polyphagia as expected. Significant value differences between the groups revealed the results using one way ANOVA and t-test. Rats exhibited a significant increase ($p < 0.001$) in fasting blood sugar (FBS) level within 48 h after STZ injection and the hyperglycemia was continued till the end of the experiment compared to non-diabetic rats (Table 1). The animals in control, PAE and ESC groups showed normal blood glucose levels throughout the experiment. After inducing diabetes with STZ, predictable loss of body weight ($P<0.001$) with age-matched controls (Table 2)

Table 1: Effect of P.amarus extract and Esculetin on fasting blood glucose (FBS) levels (mg/dL)

Groups	Before STZ injection	After STZ Injection		
		Day 1	Day 10	Day 21
CT	115.36 ± 0.47	118.96 ± 0.92	123.66 ± 4.34	108.5 ± 2.28
DC	116.8 ± 0.45	314.17 ± 19.78#	324.50 ± 23.24#	362.5 ± 34.10#
% of change from control	1.3	178.6	172	259
D+PAE	123.24 ± 0.67	311.83 ± 19.49*	271.50 ± 5.85*	208 ± 3.68*
% of change from control	7	176	117	91.1
D+ESC	123.83 ± 0.79	282.8 ± 13.45**	247.67 ± 7.84**	146 ± 3.44**
% of change from control	7.5	147.1	100	35
PAE	119.11 ± 0.78	135.20 ± 13.1	99.17 ± 4.21	100.17 ± 2.06
% of change from control	3.5	24	-19.2	-8
ESC	100.5 ± 5.04	131.9 ± 11.7	95.83 ± 5.17	96.83 ± 2.46
% of change from control	-13.2	20	-21	-10

(CT: control, DC: Diabetic control, D: Diabetic, PAE: *P.amarus* extract, ESC: Esculetin, STZ: streptozotocin). One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.001 significant against control and *P < 0.01, **P < 0.05 against diabetic group.

Table 2: Effect of *P.amarus* extract (PAE) and Esculetin on body weight in STZ induced diabetic rats.

Expt.Groups	Body Weights			
	Initial	Week 1	Week 2	Week 3
CT	231.33±1.31	242.5±2.47	253.17±0.95	262.17±1.42
DC	235±1.21	222.5±2.20#	205.33±5.42#	191.83±8.02#
D+PAE	230±1.46	220.5±1.73*	215.50±0.76*	219.83±1.14*
D+ESC	232±1.15	210.5±5.85**	212.67±1.99**	222.5±0.76**
PAE	229.83±1.66	234±2.31	241.83±1.17	249.5±0.76
ESC	227.83±4.07	236±1.06	244.67±1.33	253.5±0.76

One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.001 against control group and *P,**P < 0.05 against diabetic group.

Table 3: Effect of *P.amarus* extract (PAE) and Esculetin on daily water intake level (ml).

Expt.Groups	Water Intake(ml)		
	Week 1	Week 2	Week 3
CT	82.33±1.84	83.33±2.95	85.33±3.17
DC	361.17±29.69#	386.67±33.13#	403.33±31.69#
D+PAE	263.67±4.45*	204.33±1.89*	122.83±1.92*
D+ESC	241.17±3.20**	188.5±1.26**	110.33±1.28**
PAE	82.17±2.18	83.33±0.88	86.67±3.92
ESC	85±3.04	87.50±1.48	91.50±2.55

One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.001 against control group and *P, **P < 0.001 against diabetic group.

was observed. The water intake levels and final body weight were normal in the control, PAE and ESC groups. Diabetic rats were showing high water intake levels (P<0.001) and there is no gain of body weight till the end of 3 weeks (Table 3).

These symptoms are common for type 2 diabetes in humans as well as in animal models. However, daily administration of the *P.amarus* extract and esculetin to diabetic rats after 21 days generated a significant gain of body weight and restoration of water capacity to some extent (P<0.05), which indicates proper utilization of glucose in the rats. Whereas, the results obtained by the esculetin were significantly higher than the plant extract treated group. The noticed results could be assigned to the improved glycemic control due to the ameliorative effect of the *Phyllanthus amarus* extract and esculetin in managing glucotoxicity, lipotoxicity, induced adipogenesis, oxidative stress and inflammation.

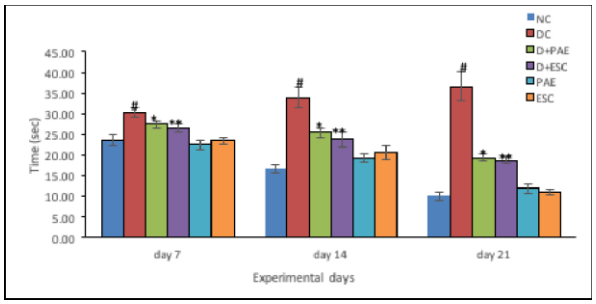

Figure 1a: Protective Effect of P.amarus extract and Esculetin on temperature-induced nociceptive pain of diabetic rats.

(CT: control, DC: Diabetic control, D: diabetic, PAE: *P.amarus* extract, ESC: Esculetin) One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.001 against control group and *P<0.01, **P < 0.01 against diabetic group.

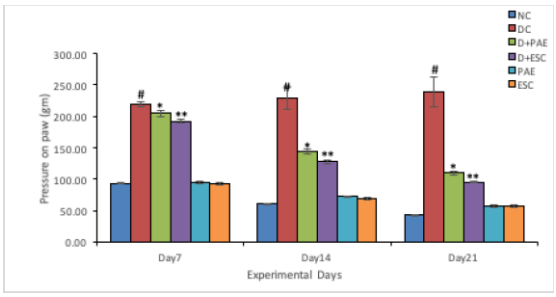

Figure 1b: Protective Effect of P.amarus extract and Esculetin on mechanically-induced nociceptive pain of diabetic rats.

(CT: control, DC: Diabetic control, D: diabetic, PAE: *P.amarus* extract, ESC: Esculetin) One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.001 against control group and *P<0.01, **P < 0.01 against diabetic group.

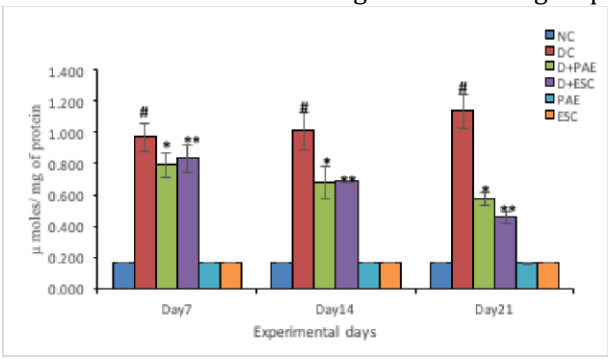

Figure 2: Protective Effect of *P.amarus* extract and Esculetin on aldose reductase activity in diabetic rats. One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6).#P < 0.001 against control group and *P<0.05,**P < 0.05 against diabetic group.

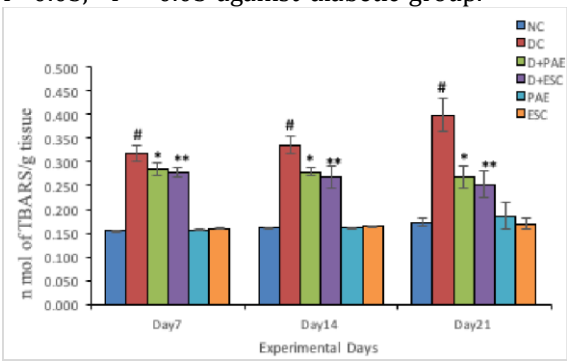

Figure 3a: Protective Effect of *P.amarus* extract and Esculetin on TBARS assay in diabetic rats.

One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.001against control group and *P<0.05,**P < 0.05 against diabetic group.

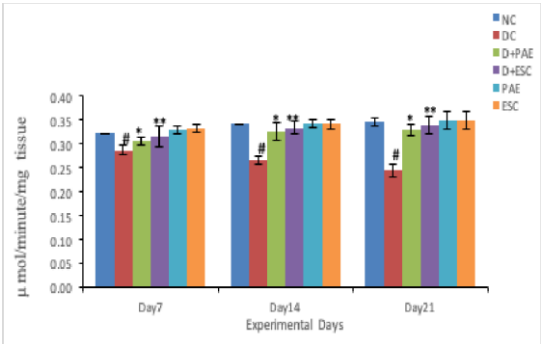

Figure 3b: Protective Effect of *P.amarus* extract and Esculetin on catalase activity in diabetic rats.

One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.01 against control group and *P<0.05, **P < 0.05against diabetic group.

Nociceptive responses: Diabetic rats also showed significantly reduced heat sensitivity on a hot plate as measured to the control group (p<0.001). *P.amarus* extract and esculetin administered rats showed reversal (p<0.01) of thermal nociceptive pain response when compared to the diabetic animals. The paw removal threshold response in diabetic rats were significantly lowered when compared to the control group (p<0.001). Upon usage with *P.amarus* extract (p<0.01) and esculetin

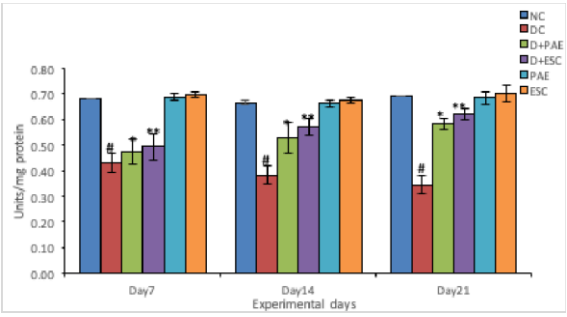

Figure 3c: Protective Effect of *P.amarus* extract and Esculetin on superoxide dismutase activity in diabetic rats.

One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.001 against control group and *P<0.05,**P < 0.05 against diabetic group.

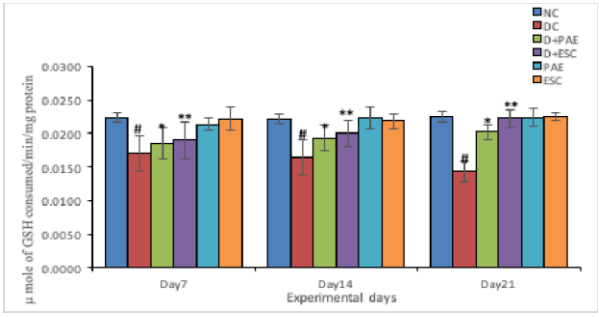

Figure 3d: Protective Effect of *P.amarus* extract and Esculetin on glutathione peroxidase activity in diabetic rats.

One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.05 against control group and *P<0.05, **P < 0.05 against diabetic group.

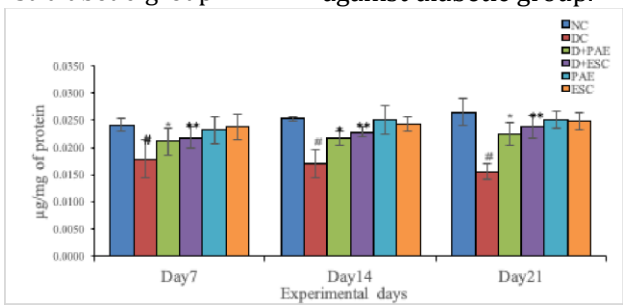

Figure 3e: Protective Effect of *P.amarus* extract and Esculetin on reduced glutathione content in diabetic rats. One-way ANOVA followed by t-test demonstrated significant differences between the groups. All values are presented as Mean±SEM, (n = 6). #P < 0.05 against control group and *P<0.05, **P < 0.05 against diabetic group.

(p<0.001) for three weeks daily, the pain threshold values on Randall Selitto were significantly got normalized. The percent of change in nociceptive responses (Hotplate and Randall Selitto tests) of Day 21 rats is more than Day 7 and day 14 rats of similar treatment. The results of PAE and ESC separate groups were found to be nearer to that of the control group.

Besides, the esculetin treated group is showing more improvement than the PAE treated group (Fig.1a and b).

Aldose reductase assay

Aldose reductase activity was significantly greater in diabetic rats when compared to the control rats (p<0.001) in sciatic nerve tissue confirmed the activation of the polyol pathway and progression of diabetic peripheral neuropathy. On treatment with PAE and ESC, there is a significant decrease in AR activity (p<0.05) may be due to their inhibitory effect (Fig.2).

Oxidative stress biomarkers

1. Lipid peroxidation (LPO), 2. Catalase (CAT), 3. Superoxide dismutase (SOD), 4. Glutathione peroxidase (GPx), 5. Reduced Glutathione (GSH).

LPO, CAT, SOD, GPx, GSH levels for all groups (n=6) on Day 7, 14 and at the end of the experiment (Day 21) are shown in fig (3). The initiation of diabetes in the rats resulted in significantly (P<0.001) enhanced levels of TBARS in the sciatic nerve compared to the control group. The treatment of diabetic rats daily for three weeks with 400mg/kg b.w of PAE extract and 45mg/kg b.w of ESC exhibited significant impediment of diabetes-induced increase of TBARS (Fig.3a) in the sciatic nerve(P<0.05). Furthermore, the diabetic control rats exhibited lower enzymatic marker levels of CAT (P<0.01), SOD (P<0.001), GPx (P<0.05) and reduced levels of GSH (P<0.05) when compared with the control rats (Fig. 3b, 3c, 3d, 3e respectively). The decreased activity of antioxidants GSH, SOD, CAT, GPx levels in the sciatic nerve were gradually improved (P<0.05) in the preventive groups (D+PAE, D+ESC) than that in the STZ group, while those in PAE and ESC separate groups, no change is observed (p < 0.05). *Phyllanthus amarus* etha-

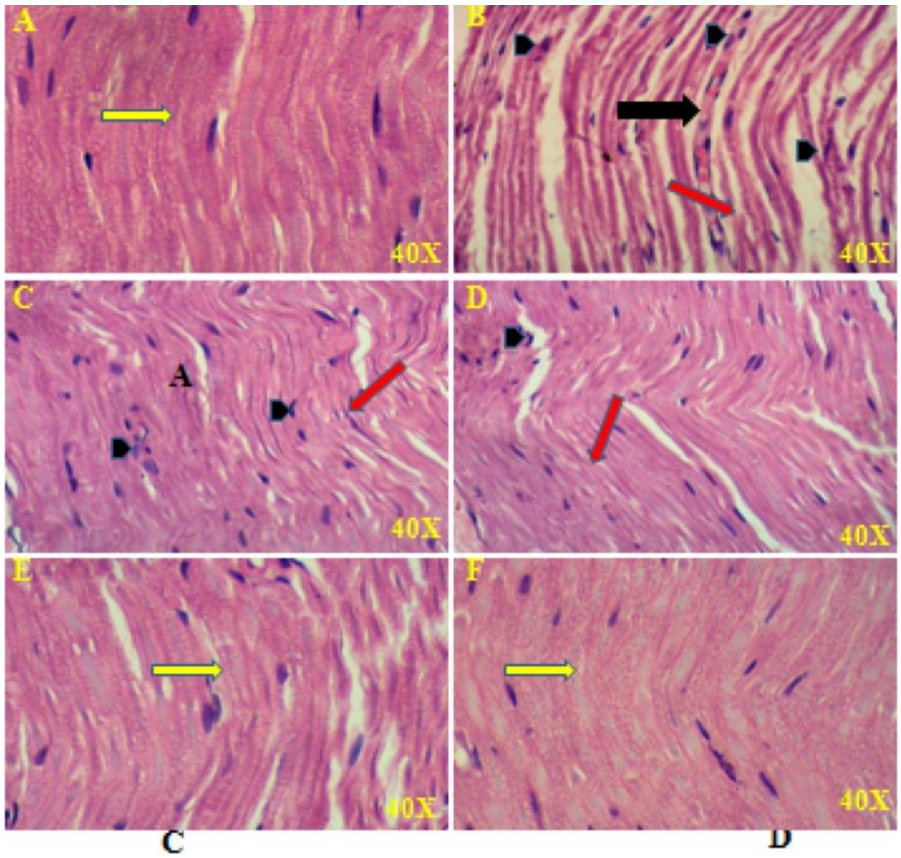

Figure 4: Effect of *Phyllanthus amarus* extract (PAE) and Esculetin (ESC) on the histopathological study of rat sciatic nerve after 3 weeks.

(A) Normal rat; (B)Diabetic control(DC); (C) STZ+ Phyllanthus amarus(D+PAE); (D) STZ+Esculetin (D+ESC) (E)PAE alone (400mg/kg B.W/day) (F) ESC alone (45mg/kg B.W/day) respectively. A, E and F: Yellow arrow exhibits normal fibre arrangement; B, C, D: Red arrows exhibit fibre derangement, swelling of nerve fibre shown by bold arrow and arrowhead represents presence of activated Schwann cells; C, D: reduction of oedema and degenerative changes were seen in *Phyllanthus amarus* (400mg/kgB.W/day), Esculetin (45mg/kgB.W/day) treated diabetic rats. C: Normal structure of sciatic nerve is partially attained D: A normal structure of sciatic nerve is completely evident. (H&E × 400).

nolic extract and Esculetin are thus significantly effective in lowering the free radical or oxidative stress level at 400mg/kg b.w and 45mg/kg b.w doses respectively. While, enhanced levels of these assays ($P<0.05$), were observed in D+ESC group rats in relation to D+PAE rats.

Histopathology

At the end of the experiment, morphological changes were examined in the sciatic nerve section for all the groups. Examination of sciatic nerve sections under the light microscope, in the control group

exhibited normal structure, myelination and morphology (Fig. 5A) while diabetic control group showed drastic changes including reduction in the number of myelinated fibres and appeared to be more small fibres, axonal degeneration, necrosis, swelling, thin, loosely formed and disorganized myelin sheath and many Schwann cells (Fig. 5B) compared to non-diabetic rats. Treatment with *P.amarus* extract and esculetin (Fig.5C, 5D) reduced the changes so that axonal regeneration and a few dispersed Schwann cells were observed in these preventive groups. No detectable injury was shown in PAE and ESC separate groups (Fig.5E, 5F). D+ESC at 45mg/kg b.w was found to be more effective than D+PAE at400 mg/kg b.w.

Discussion

In the present study, the neuroprotective effects of ethanolic extract of *Phyllanthus amarus* (PAE) aerial parts and Esculetin (ESC, a coumarin derivative) on the development of diabetic peripheral neuropathy in streptozotocin (STZ) induced diabetic rats were evaluated. The experimental data has shown, sustained increase in blood glucose levels will cause diabetes mellitus (DM) and its complications, mainly causing peripheral nervous system malfunctions. In the present study, STZ-injected rats depicted common symptoms of diabetes like elevation of FBS levels, decrease in body weight, polydipsia, polyphagia and excessive passage of

urine after STZ injection and these symptoms were continued till the end of the experiment compared to non diabetic rats and these results were in agreement with the previous reports (Wu and Yan, 2015; Javidanpour *et al.,* 2012; Pellegrino *et al.,* 1998). Besides, STZ induced diabetic neuropathy is well studied in the rat model (Bina *et al.,* 2017). Our results confirmed that *P.amarus* extract and esculetin with their respective doses caused a significant decrease in the blood glucose concentration, able to gain the body weight and lessen the water intake levels in relation to diabetic control (DC) rats. In this regard, the hypoglycemic effect of *Phyllanthus amarus* is may due to the presence of phyllanthin, chief component present in the plant aerial parts. Esculetin may lower the glucose levels from the existing β-cells of the pancreas by stimulating the insulin production. As per the earlier studies, diabetic rats have shown significantly increased aldose reductase (AR) activity when compared to the normal rats, which is the predominant polyol-pathway linked pathogenic factor in the diabetic peripheral nerve (Bhaskar and Pratap Reddy, 2014; Jung *et al.,* 2011). *Phyllanthus amarus* and esculetin have significantly inhibited the AR activity, such that sorbitol accumulation may be decreased in the sciatic nerve, leads to improvement in the motor and sensory nerve conduction.

Although the proper mechanism of the nociceptive reaction is unsure, in existence, it is found that sensitized peripheral nerve nociceptive response is a common symptom related with diabetic peripheral neuropathy (Ristagno *et al.,* 2012). Diabetic rats showed significantly reduced heat sensitivity and paw withdrawal threshold response on a hot plate and Randall Selitto respectively when compared to the normal group confirmed the pathophysiology of nerve damage, where the behaviour of rats was changed with diabetes-induced neurodegeneration (Bhaskar and Pratap Reddy, 2014). There is an extending aid for the neuroprotective effects of traditional, as well as recently found, present antioxidant agents in animal models of diabetic peripheral neuropathy (Ali *et al.,* 2016; Erbas *et al.,* 2016; Jameel, *et al.,* 2016; Kadakol *et al.,* 2016; Wang *et al.,* 2012). In the current study, PAE and ESC treated diabetic rats could successfully improve the nociceptive responses on a hot plate and Randall Selitto by increasing the pain threshold values. The diabetic rats treated with *P.amarus* and esculetin significantly ameliorated the nociceptive responces, attributes to their anti-nociceptive, anti-inflammatory and neuroptrotective activity in STZ-induced DPN.

In line with the previous reports, in diabetic control rats, we noticed a significant increase in lipid peroxidation (LPO), a significant decrease of catalase (CAT) and superoxide dismutase activity (SOD), and a significant decrease of reduced glutathione (GSH), glutathione peroxidase (GPx) content in the sciatic nerve. Hyperglycemia-induced oxidative damage and a further increase in free radicals lead to cellular dysfunction with damaged mitochondria, increased production of ROS, slow down of NCV, followed by axonal loss of nerve fibres via activation of programmed cell death or necrosis (Wu *et al.,* 2015). The estimation of the oxidative and anti-oxidative position of study groups disclosed that *P.amarus* extract and esculetin were able to reduce the lipid peroxides and will enhance the SOD, CAT, GSH, GPx levels in the sciatic nerve tissue of diabetic rats. This study has revealed that, in addition to the decrease of glucose level in the blood, the results have shown the use of *P.amarus* extract and esculetin had antioxidative nature even after the start of neuropathy and continued after that, considering it is a progressive process. Studies by Mohamad *et al.,* 2014 reported that *P.amarus* extract was able to scavenge the free radicals in diabetes milletus, but there was no study on the role of *P.amarus* and its possible application in the alleviation of hyperglycaemia-bringing oxidative stress in sciatic nerve. Prabakaran *et al.,* (2012) proved that treatment of STZ induced diabetic rat with esculetin exerts a protective effect against hepatic and renal tissues, but there is no study on its role against DPN by attenuating hyperglycemia-mediated oxidative stress, which plays a critical role in diabetic complications. Reactive oxygen and nitrogen species (ROS&RNS) are the potential sources to the pathogenesis of diabetic neuropathy (Oyenihi *et al.,* 2015). In this regard, the evaluation of antioxidant activity of *P.amarus* extract and esculetin by enzymatic and non-enzymatic anti-oxidant markers on free radicals revealed their good anti-oxidant nature and our results were indicating that esculetin have high antioxidant properties. So, the herbal drugs with antihyperglycemic, anti-oxidant activities are yet to be commercially obtained as modern medicines, even though they are traditionally in use as therapeutics.

The results include fibre degeneration, endoneurial swelling, partially separated myelinated nerve fibres, axonal atrophy of sciatic nerve tissue were reported in STZ induced DPN rats were in parallel with the findings of Omran (2012). If treatment is not established, these effects will cause degenerative changes in the peripheral nervous system. Morphological observation of sciatic nerve showed a significant decrease of oedema and caused regenerative changes and normal fibre arrangement were observed in *P.amarus* and esculetin treated diabetic rats. Behavioural, histological and biochemical results established that *P.amarus* extract and esculetin could decrease diabetes-induced

neurodegeneration in peripheral nerves. The results obtained in PAE and ESC alone treated groups, were in similar with the control group. Overall, these findings suggest clear evidence of the neuroprotective potential of *Phyllanthus amarus* extract and esculetin against hyperglycemia, oxidative stress and apoptotic cell death in diabetic peripheral neuropathy in rats.

CONCLUSION

The results of the present study along with the hypoglycemic effect showed neuroprotective effects of *Phyllanthus amarus* ethanolic extract and Esculetin treatments in attenuating STZ-induced diabetic peripheral neuropathy possibly through anti-hyperglycemic, anti-nociceptive, antioxidant and neuro-regeneration developing mechanisms. Our results proved that *Phyllanthus amarus* extract and esculetin administration lowered the glucose levels, water-intake level and improved the metabolic pathways including antioxidant defence system, body weight and morphology of sciatic nerve. Esculetin seemed to be more potent than the *Phyllanthus amarus* extract in affecting most of these aspects. However, more advanced clinical studies are required to assess the efficiency and long-term protection of *Phyllanthus amarus* extract and esculetin which can be used as a treatment modality for diabetic neuropathy.

Conflict of Interest

The authors declare no conflict of interest

Acknowledgement

Authors would like to thank for funding and facilities from UGC-DSA-I SAP-II. Lr.No. F.5-26/2015/DSA-1 (SAP-II) programme.

REFERENCES

Aebi, H. 1984. Catalase in vitro. *Methods Enzymol*, 105, 121–6.

Ali, M.Y, Jannat, S, Jung, H.A, Choi, R.J, Roy, A, Choi, J.S. 2016. Anti-Alzheimer's disease potential of coumarins from Angelica decursiva and Artemisia capillaries and structure-activity analysis. *Asian Pac. J. Trop. Med*, 9, 103–111.

Anthony, Cemaluk C. Egbuonu, Ambrose, E. Ogbu, Ifeoma I. Ijeh and Lawrence, U.S. Ezeanyika. 2015. Sub-Chronic Esculetin (6,7-Dihydroxy-Coumarin)-Induced Alteration in Some Haematological and Serum Parameters in Normal Male Wistar Rats. *Asian Journal of Biochemistry*, 10 (6), 306-311.

Atsushi, K, Minoshima, Y, Yamamoto, J, Adachi, I, Alison a Watson, and Robert j. Nash. 2008. Protective Effects of Dietary Chamomile Tea on Diabetic Complications *J. Agric. Food Chem, 56,* 8206–8211.

Bhaskar N, Pratap Reddy K. 2014. Neuroprotective and antinociceptive effect of curcumin in diabetic neuropathy in rats. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6(5), 131-138.

Bhuyan KC, Bhuyan DK, Johansen N. 1981. Estimation of malondialdehyde. *IRCS Med Sci*, 9, 126-7.

Bina Kumari Mehta, Damini Nerkar. 2017. Sugato Banerjee Characterization of Peripheral Neuropathy in Rat Model of Type 2 Diabetes. *Indian Journal of Pharmaceutical Education and Research*, 51(1), 92-101.

Chan-Sik Kim, Junghyun Kim, Yun Mi Lee, Eunjin Sohn and Jin Sook Kim. 2016. Esculetin, a Coumarin Derivative, Inhibits Aldose Reductase Activity *in vitro* and Cataractogenesis in Galactose-Fed Rats. *Biomol Ther,* 24(2), 178-183.

Ellman GL. 1959. Tissue sulfhydryl groups. *Arch Biochem Biophys*, 82(1), 70-77.

Erbaş O, Oltulu F, Yılmaz M, Yavaşoğlu A, Taşkıran D. 2016. Neuroprotective effects of chronic administration of levetiracetam in a rat model of diabetic neuropathy. *Diabetes Res Clin Pract,* 114:106.

Gennian Mao, Shiyun Zhang, Huihui Song, Shujun Ding, Peihai Zhu, Xuechuan Wang and Chengyuan Liang. 2015. Synthesis, biological activities and therapeutic properties of esculetin and its derivatives. *Journal of Chemical and Pharmaceutical Research*, 7(4), 122-130

Gunn, A, Bobeck, EN, Weber, C, Morgan, MM. 2011. The influence of non-nociceptive factors on hotplate latency in rats. *J Pain*, 12(2), 222-7.

Hasenah Ali, Houghton, P.J, Amala Soumyanath. 2013. The α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus. Journal of Ethnopharmacology, 107(3*), *449-455.*

Haymann, Kinoshita. 1965. Isolation and properties of A.R. *j.b.c*, 240, 877-882.

Jameel, T. Umar, Kumar, J, Hoda, N. 2016. Coumarin: a privileged scaffold for the design and development of antineurodegenerative agents. *Chem. Biol. Drug Des,* 87, 21–38.

Javidanpour, S, Fatemi, Tabtabaei SR, Siahpoosh, A, Morovati, H, Shahriari, A. 2012. Comparison of the effects of fresh leaf and peel extracts of walnut (Juglans regia L.) on blood glucose and β-cells of streptozotocin-induced diabetic rats. *Vet Res forum,* 3,251–5.

Jung, H. A, Islam, M. D, Kwon, Y. S, Jin, S. E, Son, Y. K, Park, J. J, Sohn, H. S, Choi, J. S. 2011. Extraction and identification of three major aldose reductase inhibitors from Artemisia Montana. *Food. Chem. Toxicol,* 49, 376-384.

Kadakol, A, Malek, V, Goru, S.K, Pandey, A, Gaikwad, A.B. 2015. Esculetin reverses histone H2A/H2B ubiquitination H3 methylation, acetylation and phosphorylation in preventing type 2 diabetic cardiomyopathy. *J. Funct. Food*, 17, 127.

Kadakol, A, Sharma, N, Kulkarni, YA, Gaikwad AB. 2016. Esculetin: A phytochemical endeavour fortifying effect against non-communicable diseases. *Biomed Pharmacother*, 84, 1442-1448.

Lillie, RD, Pizzolato, P, Donaldson, PT. 1976. Nuclear stains with soluble metachrome mordant lake dyes. The effect of chemical end group blocking reactions and the artificial introduction of acid groups into tissues. *Histochemistry*, 49, 23-35.

Maritim AC, Sanders RA, Watkins 3rd JB. 2003. Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol*, 17(1), 24–38.

Marklund S, Marklund G. 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem*, 47(3), 469-74.

Martín Aragón, S.M, Villar, A, Benedí, J. 2016. Age-dependent effects of esculetin on mood-related behaviour and cognition from stressed mice are associated with restoring brain antioxidant status. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, 65, 1–16.

Masrur, H, Ulfa, A, Ardiansyah, R. 2015. Pharmacophore modelling and molecular docking studies on Phyllanthus niruri as a target for diabetes mellitus. *Australian Journal of Basic and Applied Sciences*, 9(31), 389-39.

Mizisin A. 2004. Schwann cells in diabetic neuropathy. *J Mol Cell Biol*, 31, 1105-16

Mohamad F. Mahomoodally, Devany Devi Muthoora. 2014. Kinetics of inhibition of carbohydrate-hydrolysing enzymes, antioxidant activity and polyphenolic content of *Phyllanthus amarus* Schum. & Thonn. (Phyllanthaceae). *journal of herbal medicine*, 208–223.

Omran OM. 2012. Histopathological study of evening primrose oil effects on experimental diabetic neuropathy. *Ultrastrict Pathol*, 36, 222-227.

Owolabi, O.A, James, D.B, Anigo, K.M, Ironmonger, G.W and Olaiya, I.I. 2011. Combined Effect of Aqueous Extracts of *Phyllanthus amarus* and *Vitex doniana* Stem Bark on Blood Glucose of Streptozotocin (STZ) Induced Diabetes Rats and Some Liver Biochemical Parameters. *British Journal of Pharmacology and Toxicology,* 2(3) 143-147.

Oyenihi AB, Ayeleso AO, Mukwevho E, Masola B. 2015. Antioxidant strategies in the management of diabetic neuropathy. *Biomed Res Int,* 515042.

Panakpaporn Wannannond. 2012. Phyllanthus Amarus Facilitates the Recovery of Peripheral Nerve after Injury. *Am. J. Applied Sci*, 9 (7), 1000-1007.

Patel, K, Patel, M, Gupta, SN. 2011. Effect of *Atibalamula* and *Bhumyamalaki* on diabetic neuropathy. *AYU,* 32(3), 353-6.

Pellegrino M, Christophe B, Rene G, Michele R, Michele M, Dominique HB, Michela N and Gerard R. 1998. Development of a new model of type II diabetes in adult rats administered with streptozotocin and nicotinamide. *Diabetes,* 47, 224-229.

Prabakaran, D, Ashokkumar, N. 2012. Antihyperglycemic effect of esculetin modulated carbohydrate metabolic enzymes activities in streptozotocin-induced diabetic rats. *J. Funct. Food,* 4, 776–783.

Przemyslaw Rzodkiewicz, Emilia Gasinska, Slawomir Maslinski and Magdalena Bujalska-Zadrozny. 2015. Antinociceptive properties of esculetin in non-inflammatory and inflammatory models of pain in rats. *Clinical and Experimental Pharmacology and Physiology*, 42, 213–219

Randall, L.O, and Selitto, J.J. 1957. A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn. Ther,* 111, 409-419.

Ristagno, G, Fumagalli, F, Porretta-Serapiglia, C, Orru, A, Cassina, C, Pesaresi, M, Masson, S, Villanova, L, Merendino, A, Villanova, A. 2012. Hydroxytyrosol Attenuates Peripheral Neuropathy in Streptozotocin-Induced Diabetes in Rats. *J. Agric. Food Chem,* 60, 5859–5865.

Rotruck, JT, Pope, AL, Ganther, HE, Swanson, AB, Hafeman, DG, Hoekstra, WG. 1973. Selenium: Biochemical role as a component of glutathione peroxidase. *Science*, 179(4073), 588-90.

Said G. 1996. Diabetic neuropathy: an update. *J Neurol*, 243, 431-440.

Sangeeta Devi, Romana Rashid and Muneesh Kumar. 2017. Phytochemistry and pharmacological properties of *Phyllanthus amarus* Schum: A review.*The Pharma Innovation Journal*, 6(12), 169-172.

Wang, C, Pei, A, Chen, J, Yu, H. Sun, M.L, Liu, C.F, Xu, X. 2012. A natural coumarin derivative esculetin

offers neuroprotection on cerebral ischemia/reperfusion injury in mice. *J. Neurochem,* 121(59)E, 1007–1013.

Wang, J, Lu, M.L, Dai, H.L, Zhang, S.P, Wang, H.X, Wei, N. 2015. Esculetin, a coumarin derivative, exerts in vitro and in vivo antiproliferative activity against hepatocellular carcinoma by initiating a mitochondrial-dependent apoptosis pathway. *Brazilian Journal of Medical and Biological Research*, 48(3), 245-253,

Wu J, Yan LJ. 2015. Streptozotocin-induced type 1 diabetes in rodents as a model for studying mitochondrial mechanisms of diabetic β cell glucotoxicity. *Diabetes Metab Syndr Obes*, 8, 181-188.

Younghwa Kim and Junsoo Lee. 2017. Esculetin Inhibits Adipogenesis and Increases Antioxidant Activity during Adipocyte Differentiation in 3T3-L1 Cells. *Prev. Nutr. Food Sci,* 22(2), 118-123.