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Experimental study on *Operculina turpethum* in STZ induced diabetic neuropathy, neurodegeneration and cardiovascular complications

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ABSTRACT

Neuropathy and Cardiac complications are the most common trouble in diabetes mellitus with progressive damage due to complex pathogenesis. Many conventional pharmacological agents were withdrawn from clinical studies either due to lack of efficacy or due to side effects on major organs. Over the period of time traditional herbal plants were utilized in the treatment & management of diabetic complications. The aim of the present research work was to investigate efficacy and dynamics of *Operculina turpethum* root (OT) in STZ induced diabetic neuropathy and cardiac complications. Chronic treatment of crude extract of OT (500 mg/kg) showed positive effect in diabetic animals with significant reduction in blood glucose level, serum nitrite, brain homogenate nitrite & nerve homogenate nitrite levels as compared to diabetic control animals. Treatment with OT showed significant rise in body weight compared to Control animals & polyphagia were observed in diabetic animals persisted throughout the period of 8 weeks. Significant improvement was observed by treatment with OT in behavioural parameters like tail flick latency reduction and rise in pain threshold capacity. Nerve conduction velocity measured through BIOPAC system showed significant ($P<0.05$) improvement in diabetic animals, while improvement were observed in ECG profile, R-R interval, R wave amplitude, heart rate & cardiac hypertrophy index in diabetic animals when treated with OT. It was concluded from results that there is definite role of *Operculina turpethum* in the treatment and management of major diabetic complications.

Keywords: Diabetic neuropathy, Nerve conduction velocity, Cardiac complication, Cardiac hypertrophy, *Operculina turpethum*, Streptozotocin.

INTRODUCTION

Diabetic neuropathy (DN) is a heterogenous complication &it is the leading cause of nontraumatic limb amputation and it occurs in 50% of diabetic patients (DPs) with severe pain[1], while 15% of DPs develop foot ulcers[2]. Diabetic animals show many abnormalities that are seen in the diabetic patients with neuropathy, hyperalgesia, allodynia, slow nerve conduction velocity (NCV). The DN depends upon various causative factors including persistent hyperglycemia, oxidative stress, nitrosative stress, defective neurotrophism& autoimmune-mediated nerve destruction [1,3,4]. Many animal studies show promise of pharmacological agents but are withdrawn in clinical study either due to lack of efficacy or due to their side effects on major organs [5]. The occurrence of hyperglycemiaand oxidative stress in diabetes is implicated in the pathogenesis of various cardiovascular complications[6-8]. Various therapeutic strategies, antihyperglycemic, antihyperlipidemic and antioxidant agents can be useful in the prevention of cardiomyopathy in STZ-diabetes [9-11]. In traditional medicine, natural or phytoextracts are considered as alternative medicines to counterbalance the side effects of synthetic medicines [15]. *Operculina turpethum (OT),* commonly known as *trivrit*or *nishot* with immense ethnomedicinal value. Mainly, roots or stem bark of this plant is traditionally used for medicinal purpose. Methanolic extract of stem and roots of OT significantly decreased blood glucose level in healthy rats, glucose loaded and STZ-induced diabetic rats. Aim of the present study was to investigate efficacy and dynamics of *Operculina turpethum* in STZ induced diabetic neuropathy, neurodegeneration and cardiac complications.

MATERIAL & METHODS

Plant material, preparations of extract

The fresh roots of *Operculina turpethum* were collected & authenticated from Directorate of Medicinal and Aromatic Plants Research (DMAPR) at Boriavi, Gujarat, India. Herbal plant samples were deposited

in the department of Pharmacology at the institute. *Operculina turpethum* is a perennial climber with slender, fleshy and branched roots, hard and twisted cord like stem with small ovate leaves [19]. 100 grams of the dried root of *Operculina turpethum* were extracted with 500 ml of water for 4 hrs per day with temperature not exceeding 50 ºC, remaining water from aqueous fraction was removed by heating on water bath till complete drying up & final yield (6.5 %) obtained was stored at room temperature until needed. Suspension of this aqueous extract was prepared in 10 % tween 20 solution.

Chemicals & Instruments

Streptozotocin (STZ), Greiss reagent were purchased from Sigma Aldrich, India. Tween 20 was purchased from Himedia laboratory, India. All other reagents were obtained from S.D Fine chemicals, India. All the reagents were prepared in double distilled water & used freshly. Cooling centrifuge (Remi), Deep freezer, BIOPAC-Data acquisition system (MP36 system, USA), U. V. Visible spectrophotometer (Shimadzu-800), Tissue Homogenizer were utilized for the study.

Animal Protocol

Wistar rats weighing between 250 - 350 grams were used for the study, animals were procured from Zydus Research Centre, Gujarat, India & kept at standard laboratory diet, environmental temperature & humidity with 12:12 hrs light-dark cycle was maintained throughout the study. The animals had free access to standard laboratory diet and water *ad libitum*. The experimental protocol was approved by Institutional animal ethics committee (IAEC) and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) vide protocol no. RPCP/IAEC/2011-2012/R-6.

Study Protocol

A single dose of 45 mg/kg Streptozotocin (STZ) prepared in citrate buffer (pH 7.4, 0.1 M) was injected through IP route to induce a diabetic state. Diabetes was confirmed after 48h of STZ injection, blood glucose level was measured by glucose reagent strips. The rats having plasma glucose level > 230 mg/dl were selected and further used for the study. Body weight, food intake and blood glucose levels were measured before and at the end of the experiment. Diabetic neuropathy & neurodegeneration are late stage complications, so after a basal recording of nociceptive reaction (tail immersion test & hotplate test) at the fifth week of STZ injection diabetic rats were randomly selected and divided in 2 groups of 6 animals in each and another group was on Normal control vehicle (Saline) treated animals. Normal rats, served as Normal control [NC], STZ treated Diabetic rats, served as diabetic control [DC] and Diabetic rats received 500 mg/kg aqueous extracts of the OT[DOT]. Treatment of OT was started after the 5th week of STZ treatment for consecutive 4 weeks.

Parameters assessed

General parameter like body weights & food intake were measured of all the animals (NC, DC & DOT). At the end of the treatment period (after 8th week) animals were sacrificed under deep (50 mg/kg, i.p Ketamine) anaesthesia & blood samples were collected from the retro orbital route. The serum was separated by Centrifugation of blood at 5000 RPM for 10 minutes and stored further for biochemical analysis in deep freeze at -80 to -70°C. Right side of sciatic nerves were rapidly removed and assessed for Nerve conduction velocity (NCV) by using BIOPAC - Data acquisition system, USA (BIOPAC). Left sided sciatic nerve & rat brain (cerebral cortex & cerebrum portion) were removed, washed with ice cold saline & weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (PB) (pH 7.4). Post mitochondrial fraction (PMF) was prepared by treating brain & nerve tissue in PB, centrifuge at 2000 g for 10 minutes, further supernatant were collected centrifuge at 12,000 g for 10 minutes, final supernatant collected considered as PMF. Store these tissue homogenates in deep freeze at - 80 to -70°C till further analysis. Serum, brain homogenate and nerve homogenate were further assessed for Nitrite estimation. Cardiac parameters assessed were ECG recording, heart rate, R-R interval, R wave amplitude, dp/dt max. & cardiac hypertrophy index (Using BIOPAC system).

Statistical Analysis

Data were expressed as Mean ± S.E.M and statistical significance was assessed by using one way analysis of variance (ANOVA). P<0.05 was considered as statistical significance. N =6 (no. of animals).

RESULTS

Body weight & Feed consumption

STZ induced diabetes in 80 % of animals. STZ administration in adult rats produced loss of body weight in DC animals as compared to NC animals as time passes, weekly change in body weight as significantly reduced in diabetic control (DC) animals compared to normal control (NC) (Figure-1), while treatment showed significant rise in body weight which was comparable to NC animals& polyphagia were observed in diabetic animals (at 5th week food intake 96.67 gms & at 8th week food intake reduced to 74.44 gms), while diabetic OT extract treated diabetic animals showed no sign of polyphagia(Similar food intake at 5th week & 8th week).

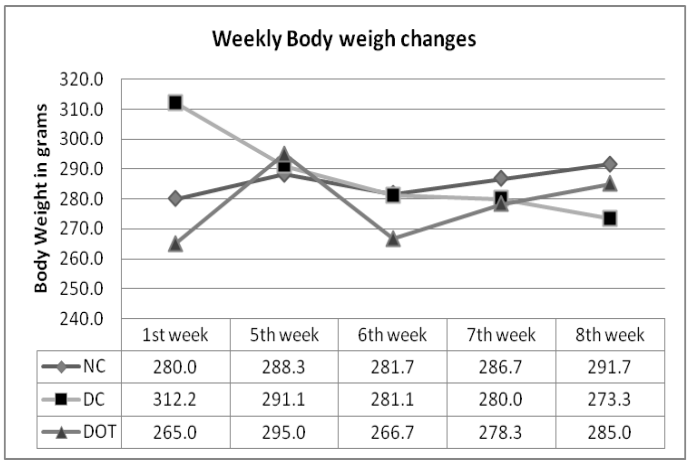


Figure 1: Weekly change in the body weight observed in the NC, DC, DOT groups of animals

Behavioural parameters

Tail Immersion test

Diabetic animals showed thermal hyperalgesia as evidenced by a significant (P < 0.05) reduction in tail flick latency in 80 % of diabetic animals by the end of 8 weeks in comparison to normal animals during hot water tail immersion test. In DOT group tail flick latency of diabetic animals was increased significantly after 4 weeks of treatment (Table-1). An OT treated group of diabetic animals produced a significant reversal of thermal hyperalgesia, which might depend upon the dose of the extract.

Hot Plate Assay:

In OT extract treated diabetic animal (DOT) a pain threshold value was slightly reduced after treatment compare to initial value; while in DC animals showed significant reduction of pain threshold at the 8th week of study, compare to normal control animals (Table-2).

Table 1: Effect of aqueous extract of OT on Tail immersion test

Groups of animals (n=6)	Hot Plate Assay (time in seconds) before treatment	Hot Plate Assay (time in seconds) after treatment
NC	9.21±1.23	7.80±1.14
DC	8.64±1.20	4.63±0.87 *#
DOT	7.77±1.13	7.36±1.107

Table 2: Effect of aqueous extract of OT on Hot Plate Assay

Groups of animals (n=6)	Tail immersion test (by hot water test, time in seconds) before treatment	Tail immersion test (by hot water test, time in seconds) after treatment
NC	4.107±0.827	6.14±1.011
DC	4.41±0.857	4.14±0.830
DOT	4.34±0.850	7.22±1.096 *#

NC – Saline treated Control animal; **DC** – Diabetic animals; **DOT** – OT treated diabetic animals; All Values is expressed as Mean ± S.E.M, (P<0.05); ***** Significantly different from Normal control animals; **#** Significantly different from Diabetic control animals

Biochemical Assessment

Serum glucose estimation:

After 8 weeks of the study period, diabetic animals exhibited significantly higher blood glucose levels (348.16±7.61) as compared to control animals (105.33±4.18), while OT extract treated group (DOT) observed significant reduction in blood glucose level (231.33±6.20) as compared to diabetic animals.

Serum nitrite estimation:

A marked increase in serum nitrite levels was observed in diabetic control (DC) animals (76.68±3.57µg/ml) as compared with normal control animals (56.34±3.06µg/ml). OT treated diabetic animals observed significantly reduction in serum nitrite level (59.78±3.15µg/ml) as compared with DC animals.

Brain nitrite estimation:

Significant rise in brain homogenate nitrite levels was observed in diabetic control animals (89.13±3.85µg/ml) as compared with normal control animals (52.84±2.96µg/ml). Treatment with OT extract in diabetic animals showed significant reduction in the brain nitrite levels (48.15±2.83µg/ml) as compared with diabetic animals.

Sciatic Nerve nitrite estimation:

Nerve nitrite levels were increased in diabetic animals (113.11±4.34µg/ml) as compared with normal control animals (30.29±2.24µg/ml), while OT extract treated diabetic animals found significant reduction in the nerve nitrite levels(52.65±2.96µg/ml) as compared with diabetic animals.

Neurophysiological assessment

Nerve Conduction Velocity (NCV) recording in Sciatic nerve:

A significant decrease in the NCV was recorded in diabetic control (17.70±1.71) animals compared to Normal control animals (80.38±3.66), due to hyperglycaemic effect in the diabetic animals it leads to slow firing of action potential which generates slow conduction velocity in sciatic nerve. Treatment with OT extract showed significant rise in the NCV (145.94±4.93). Here diabetic animals when treated with OT extract showed significant improvement in NCV value as we increase trigger value in mv compared to Normal control animals (Figure-2), while diabetic control animals was found significant low conduction velocity of sciatic nerve.

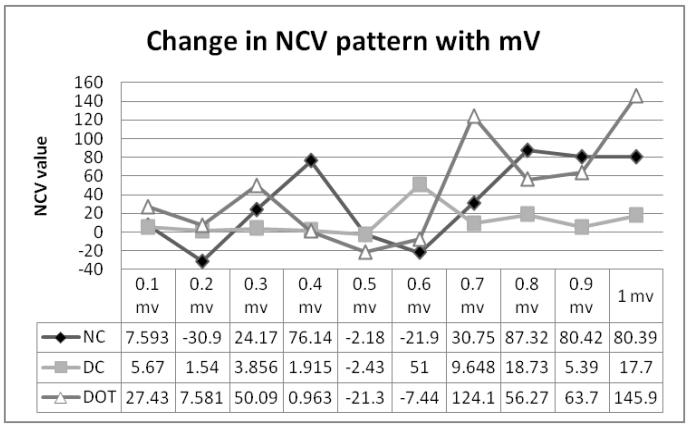


Figure 2: NCV (cm/msec) recording of sciatic nerve in the NC, DC, DOT groups of animals

Cardiac parameter assessment

ECG Profile, Heart rate and dp/dt max.:

Our study showed that there was significant decrease in the ECG profile of diabetic rats compared to normal control animals, while the OT treated group of diabetic animals (DOT) showed improvement in ECG profile compared to diabetic control animals. Heart rate was drastically reduced in diabetic animals compared to normal animals, while treatment with OT showed an improved heart rate. Left ventricular function was determined by the mean of dp/dt max. Profile which was derived during recording of ECG. In diabetic animal's dp/dt max value was significantly decreased compared to a normal control animals (NC), while treatment with OT showed significant improvement in left ventricular functioning by a significant rise in dp/dt max. value (Table-3).

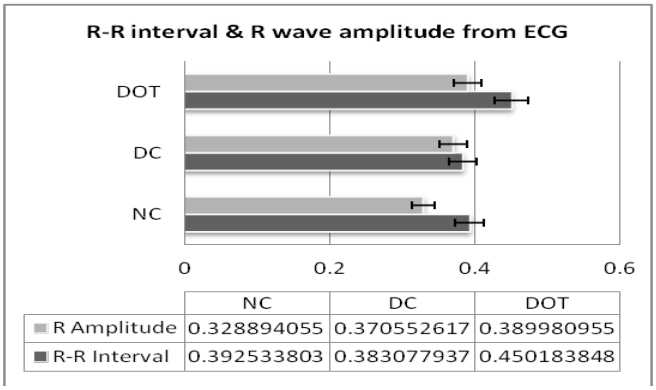
Table 3: Comparison of ECG profile, Heart rate & dp/dt max of different groups of animals

Groups of animals (n=6)	ECG (Hz)	Heart rate (beats/min)	dp/dt max.
NC	0.005981 ± 0.00209	361.34 ± 16.90	0.3434 ± 0.042
DC	0.001518 ± 0.00276 *	291 ± 39.14 *	0.277 ± 0.084 *
DOT	0.002495 ± 0.000767 #*	324.21 ± 33.80 #*	0.392 ± 0.056 #*

NC – Saline treated Control animal; **DC** – Diabetic animals; **DOT** – OT treated diabetic animals; All Values is expressed as Mean ± S.E.M, (P<0.05); ***** Significantly different from Normal control animals; **#** Significantly different from Diabetic control animals

R-R interval & R wave amplitude changes:

In this study from the ECG profile R-R interval revealed which was low in the diabetic animals but not significantly different from normal control rats, while treatment with OT had significantly altered the range of R-R interval (Hz) which was higher than remaining two groups of animals. R wave amplitude (Hz) was not significantly affected in diabetic control group of animals, while treated group (DOT) showed a rise in R wave amplitude compare to DC and NC groups (Figure-3).



	NC	DC	DOT
R Amplitude	0.328894055	0.370552617	0.389980955
R-R Interval	0.392533803	0.383077937	0.450183848

Figure 3: R-R interval & R-Wave amplitude recorded from ECG in NC, DC, DOT group of animals

All Values is expressed as Mean ± S.E.M, (P<0.05); * Significantly different from Normal control animals; # Significantly different from Diabetic control animals

Cardiac Hypertrophy Index:

Our study showed that STZ diabetic rats observed with rise in Cardiac Hypertrophy Index (CHI) (0.0037 ± 0.000196) in diabetic control animals compare to normal control animals (0.003604 ± 0.000136); while OT treated diabetic animals observed reduction in CHI (0.003429 ± 0.000174) compared to diabetic control animals.

DISCUSSION

Hyperglycemia and inflammation unleash a cascade of events that affects cellular proteins, gene expression and cell surface receptor expression, ultimately resulting in progressive pathologic changes and subsequent diabetic complications [16]. In the present study, streptozotocin-injected rats has significantly higher blood glucose levels, body weights which compared to control animals supported by experimental work [30,31] but weekly changes in body weight in diabetic animals were not much significantly reduced compare to control animals. Treatment with aqueous extracts of *Operculina turpethum* (OT) significantly reduced blood glucose level which again reveals their hypoglycaemic and antidiabetic activity with support of earlier literature. The nociceptive threshold was significantly lower in diabetic animals than nondiabetic animals, indicating that diabetic rats exhibit thermal hyperalgesia. In current study tail immersion test revealed that treatment with aqueous extract of OT prevents allodynia which further reduces neuropathic pain in diabetic rats. Decrease in pain threshold was observed with noxious stimulus (heat) in hot plate assay which was failed to induce paw-withdrawal in normal rats before the cut off time (10s) indicating that this force is noxious. However, diabetic rats showed a significant reduction in paw withdrawal threshold which indicates the development of hyperalgesia. Similar models of mechanical allodynia and thermal-hyperalgesia in streptozotocin-induced rats have been studied previously[20,30],While OT treated diabetic animals had produced protection compared to diabetic control animal, which revealed that higher dose may be required to produce significant prevention in neuropathic pain. The mechanism responsible for the antinociceptive action of the extract is partly related to the modulated release or action of pro-inflammatory mediators involved in the models of pain used. Clinical studies have reported laxative, anti-inflammatory, analgesic, antiarthritic and anti-helminthic effects of root powder of OT[21,22] and which phytochemical is responsible for protection in neuropathic pain & neuroprotection still need to be explored. The exact mechanisms responsible for the decreases in pain threshold level in treated diabetic rats with OT is not yet completely established & may involve a combination of central and peripheral activity. The pathogenesis of diabetic neuropathy is complex and involves multiple pathways. Studies have demonstrated that even with stringent blood glucose control, the prevention of neuropathy is not successful which suggests that there may be a release of early mediators between hyperglycaemia-induced metabolic and enzymatic changes and the nerve damage. The release of mediators might modulate neuronal homeostasis independently of the initial metabolic stimulus. These mediators have been proposed to be neuropoietic cytokines such as IL-1, IL-6 and TNF-α [23]. Hyperglycaemia is reported to induce oxidative stress through multiple mechanisms such as redox imbalances, increased advanced glycation end product [24]. In addition, other key mediators of hyperglycaemia induced oxidative injury are peroxynitrite, which is formed by combination of superoxide with nitric oxide that exerts detrimental effects on the nerve tissue leading to neuropathic pain [25]. In DN antioxidant treatment has been tried to combat oxidative stress in both animals and diabetic patients, vitamin C or α-lipoic acid, free amino acids also improve responses to insulin and thus can provide additional benefit to the proposed reduction of oxidative stress in tissues [16]. Our study observed marked elevation of serum, brain homogenate & nerve homogenate nitrite levels in diabetic animals in the present study, indicating nitrosative stress [26]& impaired neurochemical-structural abnormalities which leads to neuronal damage or degeneration in diabetic rats. Our study showed that treatment with an aqueous extract of OT at a dose of 500 mg/kg/day was observed highly significant reduction in nitrite level of serum, brain homogenate and nerve homogenate which explains its role in nitrosative stress, neuroprotection& neuropathic pain. A single dose of STZ can produce diabetes in animal models as a result of direct toxic effect on β-cell by alkylation. Early symptoms of neuropathy seen in STZ diabetic rodents include hyperalgesia, allodynia and slow NCV. PARP activation executes an imperative role in peripheral sensory nerve fiber dysfunction and degeneration in DN. Studies conducted with PARP inhibitors have shown to counteract small sensory nerve fiber dysfunction and degeneration [27]. Pathogenetic mechanisms underlying the progressive nerve fiber loss seem to be multifactorial, including polyol pathway, glycation, reactive oxygen species, and altered protein kinase C activity [28]. In the present study Diabetic control animals show significant reduction in NCV compare to normal control animals. Treatment with OT showed a significant rise in the NCV compared to diabetic control animals. In another study botanical extract PMI-5011 essentially reversed NCV deficit associated with pre diabetic neuropathy. It has also reduced thermal and mechanical hypoalgesia, that is, sensory loss, which is a major cause of foot ulceration and amputation in human subjects with diabetes mellitus [4]. Several evidence suggests that altered glucose supply and utilization by cardiac myocytes could be responsible to cause primary injury in the pathogenesis of this specific heart muscle disease [9]. So, it is necessary to increase glucose utilization or increase the rate of glucose transport in the diabetic heart. In our study, STZ produced a significant increase in glucose levels associated and decreased heart rate was observed in diabetic control animals as compared to normal control (NC) animals, while treatment with OTshowed significant rise in heart rate compared to diabetic control animals, this indicates OT has beneficial effects in diabetic cardiac complication. Treatment with OT also created significant effect on ECG profile compare to diabetic control animals, while treatment with OT had also improved in dp/dt max. value, a marker of left ventricular function compared to diabetic control animals, supported by experimental work which tells that myocardial fibrosis, cardiac hypertrophy index & left ventricular hypertrophy index are the most frequently proposed mechanisms & markers to explain cardiac changes in diabetic cardiomyopathy.

Previously electrocardiogram (ECG) was considered as the most common mean for evaluating cardiac disease, several ECG criteria have been proposed for the detection of left ventricular hypertrophy (LVH) both in clinical practice and in epidemiological studies, but doubts have been raised about their predictive value, particularly in the elderly, where cardiac fibrosis are highly represented [12,13]. Left ventricular mass (LVM) tends to increase with age, mainly due to increase in electrically-inactive fibrous tissue [14]. After treatment with OT produced improvement in wet heart weight (HW) / Body weight (BW) may be due to an increased reduction in the collagen level particularly in the left ventricular region. Present study also showed

significant rise in the serum nitrite level in diabetic animals compare normal control animals, which was supported by literature that increased Reactive oxygen species (ROS) generation may activate maladaptive signaling pathways, which may lead to cell death, which ultimately promote abnormal cardiac remodelling & contribute to the characteristic morphological and functional abnormalities that are associated with diabetic cardiomyopathy [29].Treatment with OT found to be use full in reducing serum nitrite level in diabetic animals which proves the antioxidant property of OT by reducing ROS or augment myocardial antioxidant defence mechanisms & might have therapeutic efficacy in improving myocardial function in diabetes mellitus, backened by literature that some phytoconstituents like galootannins found to possess multiple biological activities including anticancer, antioxidant, antimicrobial activities, and cardio protective effects[17]. In one study treatment with Gallic acid was found to possess antihyperglycemic, antihyperlipidemic potential as well as improvement in cardiac dysfunction associated with STZ-diabetes [18].

CONCLUSION

These studies have concluded that there is definite role of OT in the reversal of major diabetic complications especially neuropathy and cardiovascular complication. In this study number of animals utilized was limited as per ethical approval, so further extrapolation of this study need to be done by using other species of animals.

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