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Protective effect of ENervin on diabetic neuropathic condition in Schwann cells

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Abstract
Treatment of Diabetic Neuropathy depends on three main approaches: risk factor management and intensive glycaemic control and symptomatic pain management. It's a type of nerve damage caused by long-term high blood sugar levels. ENervin is a polyherbal formulation very effective to rejuvenate nervous system and used for the therapy of Diabetic Neuropathy. Thus, a preliminary study has been planned to observe and evaluate the effect of ENervin on diabetic neuropathy. The ENervin tablet shows the presence of five important compounds like ar-tumerone, stigmasterol, gingerol, lupeol and trilaurin which was confirmed by HPLC analysis. In this experiment, different concentrations of ENervin decreased the Schwann cells in diabetic condition and its beneficial action was compared with Pregabalin, an oral capsule used to treat neuropathic pain. Future preclinical and clinical research of ENervin may help us to strongly support the usage of this polyherbal medicine for the treatment of pain in diabetic neuropathy.

Keywords: ENervin, pregabalin, diabetic neuropathy, phytochemicals

Introduction
Diabetic Neuropathy (DN) is the damage to automatic, motor and/or sensory nerves that result from metabolic or vascular derangement in patients with long standing Diabetes mellitus [1]. The relationships among Schwann cells, axons, and the perineurial barrier emphasize the key role Schwann cells plays normal functions of the nerve. Schwann cells are responsible for action potential velocity through insulation of axons, maintenance of axonal calibre, and correct localization of Na+ channels; immunological and functional integrity of the nerve through the perineurial blood-nerve-barrier; and effective nerve regeneration. In diabetic neuropathy, many of these facets of nerve function are defective. The Schwann cells were selected for experimental DN model, this may be due to the apparent localization of aldose reductase enzyme in Schwann cells. Thus, measurements derived from whole nerve were largely assumed to relate to Schwann cells [2]. Although increasing knowledge of the complexity of the etiology of diabetic neuropathy has highlighted the need for *in vitro* studies, much of this work has yet to be done. The present study attempts to find the role of ENervin on Schwann cells in diabetic neuropathy condition. The most common form of neuropathy in diabetes is known as distal symmetrical polyneuropathy: it can act on all types of neurons, but affects those with longest axons first. Distally innervating neurons must maintain a larger cellular volume (and hence higher metabolic efficiency) [3]. This also implies a greater dependence on Schwann cells and target tissues for mechanical and neurotrophic support. Therefore, these neurons are susceptible to defects in support cells [4]. Patients present with numbness, tingling, and/or pain that typically started in their toes and slowly spreads proximally. Painful diabetic neuropathy (PDN) is a debilitating consequence of diabetes that may be present in as many as one in five patients with diabetes. The objective assessment of PDN is difficult, making it challenging to diagnose and assess in both clinical practice and clinical trials. No single treatment exists to prevent or reverse neuropathic changes or to provide total pain relief [5].

There's no cure for diabetic neuropathy, but studies showed that treatment can slow its progression. Keeping your blood sugar levels within a healthy range is the best way to decrease the likelihood of developing diabetic neuropathy or slow its progression. There is a growing need for studies to evaluate the most potent drugs or combinations for the cure of DN to improve the pain relief and quality of life. Herbal formulations are found to be very effective to rejuvenate nervous system and in the management of diabetic neuropathy [6]. Thus, the present study was conducted to find the protective effect of ENervin on diabetic neuropathic conditions in Schwann cells.

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Materials and Methods
Chemicals
HPLC grade methanol, acetonitrile, acetic acid were purchased from Merck Laboratories. Ultra deionized water utilized in the study was obtained from Milli-Q water purification system. The reference compounds were purchased from Sigma Aldrich company. Their purities were analyzed to be all above 98% by HPLC.

Chromatographic conditions
The separation of phytoconstituents was implemented on a Shimadzu 2695 HPLC system (Shimadzu). The qualitative analysis was carried out on A Zorbax SB-C18 column (250 mm×4.6 mm i.d., 5 μm). The column temperature was set at 35◦C. The mobile phase was composed of acetonitrile (A) and water containing 0.4% (v/v) acetic acid (B).

The gradient program was as follows: 45% A at 0–13 min, 45–56% A at 13–16 min, 56% A at 16–50 min, and 56–100% A at 50–55 min. The re-equilibration duration was 10 min between individual runs. The flow rate was kept at 1.0 mL/min. The injection volume was 10 μL. An online detection wavelength was selected at wavelengths of 240 nm and 430 nm.

Standard solution
The ar-tumerone, stigmasterol, gingerol, lupeol and trilaurin compounds were weighed and dissolved in methanol. The mixed standard solution containing all reference compounds was prepared in a 10 mL volumetric flask, diluting with methanol, and stored at 4◦C. Subsequently, the stock solution was further diluted with methanol to obtain a series of concentrations of working solutions to establish calibration curves.

Validation
The HPLC method described in this article was validated interms of linearity, sensitivity, precision, stability, and accuracy. Six different concentrations of working solutions were analysed in triplicate to establish calibration curves. The calibration curves of various analytes were constructed by plotting the mean peak areas vs. the concentration of the reference compounds. The limits of detection (LOD) and quantification (LOQ) were determined by injecting a series of dilute standard solutions until a signal-to-noise ratio (S/N) of 3 and 10 was obtained, respectively. The precision test was performed by the measurements of intra- and inter-day variability. For the intra-day precision test, the test samples were analyzed for replicates within one day, while, for the inter-day precision test, the samples were examined in duplicates for three consecutive days. Quantities of the analytes were calculated from their corresponding calibration curves. The recovery was calculated with the following equation: Recovery (%) = (amount determined−amount original)/amount spiked×100%.

Cell culture
Schwann cells (ATCC: CRL-2765) were purchased and cultured in T75 flasks with DMEM (containing 4 mM 1-glutamine, 1.5 g/L sodium bicarbonate, and 5.5 mM glucose), supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum in an incubator. Schwann cells were cultured for 48 h under hyperglcemic conditions with or without ENervin treatment and then collected for further analysis.

Cell viability by MTT assay
The Schwann cells were cultured in 96-well tissue culture plates for the assay. The microplates filled with cells (100 μl) with a density of $3×10^5$ were taken as negative control. The cells were observed for 24 hours, and the growth medium by means of micropipette and the monolayer of cells rinsed twice with Minimum Essential Media (MEM) devoid of Fetal Bovine Serum (FBS) for removing dead cells and extra FBS. 1ml of medium (without FBS) having various concentration of ENervin solution (1-5.0mM/ml) were added on to respective wells; 20 μl of MTT (5 mg/ml in PBS) were added on to every well, and the cells kept in 5% CO_2 incubator for 6-7 hrs. After removing the medium, DMSO (1ml) was added on to every well and Pregabalin (1mg/ml) was tested as a comparison standard drug. Propanol (50 μl) was added to the pellet and the plates were mixed gently to solubilize the molded formazan product. The plates were kept on a shaker for 15 min and the absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) (MINDRAY90) reader at 570 nm. Each experiment was done in triplicate and the IC_{50} of the test samples as the percentage survival of the cells was calculated.

Statistical analysis
Results were measured as mean ± S.E.M. Statistical significance was resolute by one-way analysis of variance (ANOVA) and post hoc least-significant difference test by SPSS software (version 22.0). P values less than 0.05 was considered significant

Results and discussions
DN is associated with significant impairment in the nervous system of diabetic patients [7]. For the cure of DN, the ENervin tablets can be given as a therapeutic drug for patients. So therefore, there is a need for the scientific justification for the usage of this drug clinically. The ENervin tablet shows the presence of five important compounds such as ar-tumerone, stigmasterol, gingerol, lupeol and trilaurin along with few more compounds which was confirmed by HPLC technique (Figure 1; Table 1) and these phytoconstituents are very important for the cure of diabetic neuropathic condition [8]. The early reports demonstrated that morphological changes in Schwann cells in human diabetic neuropathy are now supported by an increased awareness of molecular alterations in Schwann cells during diabetes. Schwann cells express a wide range of receptors and, when they sense insults or danger signals, they increase the synthesis and secretion of factors that stimulate neuroprotection, regrowth and remyelination, or factors that aggravate disease phenotypes [9].
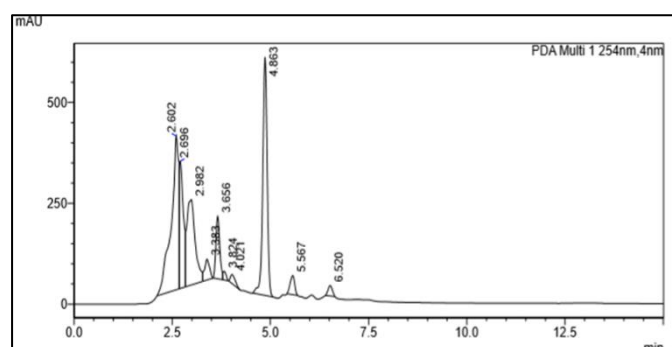

Fig 1: HPLC analysis of ENervin

Table 1: Various phytoconstituents present in the ENervin

PDA Ch1 254nm							
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.602	5.91225	381166	29.365		M	Beta-sitosterol
2	2.696	2182441	315547	12.588		V M	Trilaurin
3	2.982	3125403	211291	18.027		V M	Lupeol
4	3.383	444366	51519	2.563		V M	Gingerol
5	3.656	1068365	155033	6.162		M	Difenoxin
6	3.824	187589	21625	0.630		V M	Stigmasterol
7	4.021	4558567	22419	1.082		M	Difenoxin
8	4.863	375251	591115	26.293		M	n-hexadecanoic acid
9	5.5567	975251	47326	2.164		M	ar-tumerone
10	6.520	195201	25818	1.126		M	Phthalic acid esters
Total		17337647	1822860				

The most recent studies have demonstrated that Schwann cells regulate many aspects of axonal function, so that disruption of their metabolism by diabetes results in the accumulation of neurotoxic intermediates and compromises production of neuronal support factors, contributing to axonal degeneration, endothelial dysfunction and diabetic neuropathy. The earliest descriptions of pathology in diabetic neuropathy indicated that schwann cell neuropathy accompanied axonal degeneration.

The majority of clinical and basic research in diabetic neuropathy since then has focused on the effects on neurons. However, accumulating data from research into the development and regeneration of the PNS has identified Schwann cells as equally indispensable components that maintain neuronal structure and function, nourish axons and promote survival and growth upon injury [10].

Treatment which repairs nerves has yet to be found and translated into clinical trials and eventually approved therapy in clinical practice. Whilst a number of treatment options exist and various guidelines and algorithms have been formulated, none are satisfactory.

Various symptomatic treatments have been proposed to manage neuropathic pain but few have been found to be effective, with only three medications currently FDA approved for DN [11]. The effect of ENervin on Schwann cells (cell line for diabetic neuropathy research) was done in comparison with the standard pharmacological drug Pregabalin.

High glucose can induce oxidative stress and inflammatory responses to activate the Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-KB) signal pathway, which plays an important pathogenic process in DN. The vascular mechanism of the neuropathy involves impaired endoneurial blood flow to peripheral nerves leading to the destruction of neuronal and Schwann cells (SCs).

Actually the destructed Schwann cells in neuropathic condition were reduced after ENervin treatment similar to that of standard control, which proves that ENervin formulation showed a positive effect on injured nerve cells in DN patients [12]. In this experiment, the schwann cells treated with ENervin concentration at 10mM showed 35.6% glucose destructed nerve cells viability whereas the schwann cells cultured in glucose condition treated with pregabalin showed 28.7% cell viability (Table 2, Figure 2).

The diabetic injured schwann cells without treatment showed 100% viability. Therefore ENervin showed 64.4% protection whereas pregabalin showed 71.3% against diabetic neuropathy condition.

Table 2: The MTT assay results of Schwann cells treated with ENervin

Treatment	Conc (mM)	Cell viability (%)
SC untreated cells	-	100.0 ± 8.4
SC + EN	1	74.1 ± 5.8
SC + EN	2.5	60.8 ± 4.1
SC + EN	5.0	51.4 ± 4.5
SC + EN	10.0	35.6 ± 2.7
SC + PG	1mg	28.7 ± 1.4

Values are expressed as Mean ± SEM (n=3).SC-Schwann cells; SC+EN – Schwann cells +ENervin; SC+PG - Schwann cells + Pregabalin, The IC_{50} of the ENervin test product against SC cells is 4.9mM.
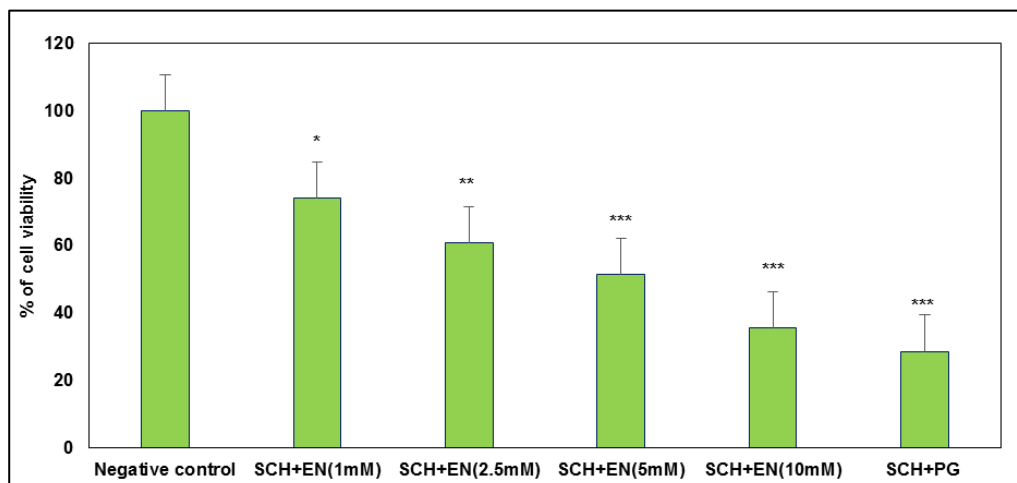
Fig 2: Effect of various concentration of ENervin on Schwann cells in comparison with pregabalin Results were expressed as Mean ± SEM (n=3). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ statistically significant as compared with SC untreated group.

The inhibition of destructed Schwann cells by Enervin Tablets, one among the herbal formulation is found to be very effective to rejuvenate nervous system and in the treatment of Diabetic Neuropathy. This curative action may be due to the important phytoconstituents which plays an important role for the diabetic neuropathy cure which was already proved by the researchers.

Conclusion

This polyherbal formulation showed a significant protection against diabetic neuropathy condition and this effect was comparable with that of Pregabalin. Therefore, further preclinical and clinical studies required to support the ongoing trial of ENervin on diabetic neuropathic patient.

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