Research Paper

Ethanol Extracts from *Toona sinensis* Seeds Alleviate Diabetic Peripheral Neuropathy through Inhibiting Oxidative Stress and Regulating Growth Factor

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Wang, *et al.*: Effects of *Toona sinensis* Seeds on Diabetic Neuropathy

***Toona* sinensis (A. Juss.) Roem has long been used in traditional Chinese medicine for the treatment of diabetic mellitus. The aim of this study was to investigate the effect of ethanol extracts of *T. sinensis* seeds on diabetic peripheral neuropathy and the preliminary mechanism. Male Wistar rats were used for inducing diabetic peripheral neuropathy model, the variation of body weight; blood glucose, general status, thermal perception thresholds and sciatic nerve conduction velocity were used to confirm the diagnosis of diabetic peripheral neuropathy. After the treatment, the sciatic nerve conduction velocity was accelerated, the tail-flick latency was reduced, the hyperglycemia and oxidative stress injury in sciatic nerve were ameliorated, contents of serum nerve growth factor β were up-regulated and tumor necrosis factor α, interleukin-6 were down-regulated significantly (P<0.05) compared with the model rats. This research demonstrates that ethanol extracts of *T. sinensis* seeds offers potential for intervening diabetic peripheral neuropathy and the protective mechanisms may be alleviating hyperglycemia, inhibiting oxidative stress and regulating growth factor and inflammatory cytokines.**

Key words: *Toona sinensis*, diabetic peripheral neuropathy, oxidative stress, NGF-β, TNF-α

Diabetic peripheral neuropathy (DPN) is one of the most common chronic complications in patients with diabetes mellitus (DM)[1]. Nearly 60% of the patients suffer from peripheral neuropathy[2]. The pathogenesis of DPN includes many factors such as metabolic, vascular, autoimmune, oxidative stress and neurohormonal growth factor deficiency[3,4]. Among them, oxidative stress[5,6] plays an important role and a number of neurohormonal cytokines were involved such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 (TGF-β1) and nerve growth factor beta (NGF-β)[7-9]. Thus, the pathophysiology and therapeutic development of DPN are active areas of research.

Toona sinensis (A. Juss.) Roem is widely used as a delicious vegetable in China. *T. sinensis* is also a well-known Chinese herb and all parts of it, including root, bark, petioles, leaves, fruits, and seeds, have been used for medicinal purposes. The aqueous extracts of *T. sinensis* leaves have revealed anticancer, antioxidant, antidiabetic and antivirus antiviral[10-12]. The chemical composition included polyphenols, saponins, sesquiterpenes, alkaloids and volatile oil[13,14]. In the present research, ethanol extracts from *T. sinensis* seeds (ETS) were used to observe the protective effects on peripheral neuropathy in rats with type 2 DM by inhibiting oxidative stress and regulating growth factor in an attempt to investigate the protective mechanism and provide evidence for the clinical treatment of DPN.

MATERIALS AND METHODS

T. sinensis seeds were purchased from Jinan Shengke Technology Development Co., Ltd. China, and authenticated at Department of Pharmacognosy of Weifang Medical University. The powdered seeds (10 kg) were extracted with 85% ethanol and 75% ethanol (2×100 l, each 3 h), respectively. The combined ethanol extracts were concentrated to yield crude

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extract (1064 g) under reduced pressure. Male Wistar albino rats weighing between 180-220 g were provided by Weifang Medical Experimental Animal Center and bred in standard animal facility. The animals were kept in controlled conditions and fed with standard pellet diet and water *ad libitum*. The current study protocol was approved by Ethics Committee of Weifang Medical University for animal studies.

Streptozotocin (STZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, St. Louis, MO, USA: Rat NGF-β (1909871108), TNF-α (R131029-12b) and IL-6(R130926-06b) ELISA kit were purchased from NEO Bioscience technology, Beijing, China; total antioxidant capability (T-AOC, 20130726), malondialdehyde (MDA, 20130726), superoxide dismutase (SOD, 20131218), glutathione peroxidase (GSH-PX, 20130723) assay kits were purchased from Jiancheng Bioengineering Institute, Nanjing, China. All other chemicals and reagents used were of analytical grade.

Antioxidant activities *in vitro*:

The total antioxidant capability (T-AOC) kit and microplates were used for quantitative assay. The reaction principle is antioxidants reduce Fe^{3+} to be Fe^{2+}, while Fe^{2+} and Fehling's substances form stable complex compounds. The ETS were dissolved with 80% alcohol; with the concentration (mg/ml) as horizontal coordinate and the absorbance as vertical coordinate, a reducing power curve was drawn. Ascorbic acid was used for positive control.

DPPH was dissolved and diluted with absolute alcohol[15] to attain a concentration of 0.2 mmol/l. The ETS were dissolved with 80% alcohol at different concentrations and the same volume of DPPH solution was added, and the mixtures were placed away from light for 15 min. The absorbance was immediately detected at 515 nm wavelength with ascorbic acid as positive control.

Fresh liver was separated from healthy Wistar rat and 10% homogenate was made in ice bath. Different concentrations of ETS were added into 1.5 ml of homogenate, then 0.1 ml of H_2O_2 was added and the mixture was reacted in 37° for 30 min. MDA contents were assessed using thiobarbituric acid reactive substances assay by measuring the absorbance value at wavelength of 532 nm.

Induction of experimental DPN and treatments:

Twenty Wistar rats were selected as the control group (group 1). The remaining rats were fed with a high-fat diet (HFD) for 6 w and were injected intraperitoneally with a dose of 35 mg/kg STZ in citrate buffer (pH=4.5). Blood was drawn from the tail vein after 72 h and the rats with blood glucose greater than 16.7 mmol/l were treated as DM rats and were used for further experiments. 6 w later, tail-flick tests were conducted and eight control rats and eight DM rats were selected randomly for detection of DPN by means of sciatic nerve conduction velocity (SNCV) and determination of NGF-β. The abnormalities of these parameters were used to confirm the diagnosis of DPN. The DPN rats were then divided into four groups according to their blood glucose gradient, each 12 as follows: group 2 (DPN model); groups 3 to 5 (DPN rats treated with ETS 0.10, 0.15, 0.2 g/kg/day, respectively). The rats in groups 1 and 2 were treated with an equal volume of distilled water. Each group was administered by gavage once a day for 8w. General status and food intake were observed every day, the values of body weight and blood glucose were determined regularly.

Tail-flick tests:

Tail-flick tests were conducted before and after administration at the 6th, 12th and 20th weeks. Tail-flick tests were assessed by tail-flick latency evoked by a noxious heat stimulus by tail-flick instrument (YLS-12A, Yiyan Technology Development Co., Ltd., Shandong, China.). In the test, radiant heat was focused on the distal part of animal’s tail. The cut-off time for the tail-flick reaction was set to 16 s to avoid damage to the tail[16]. Prior to any treatment, the rats were allowed to acclimatize to the test procedure and apparatus for at least 20 min. The test was repeated three times for each rat and mean of three measures was reported. The interval of three measurements was 5 min.

SNCV detection and biomarker analysis:

The experiment animals were anesthetized with 10% chloral hydrate (0.4 ml/100 g) after overnight fasting. With the subjects prone, right sciatic nerves were dissected with a glass needle. The stimulating and recording electrodes were placed directly under the sciatic nerve and the distance between the stimulation electrode and recording electrode was recorded. Then the nerve conductions were made and recorded using the Functional Experiment System (BL-420s, Taimeng, Sichuan, China). SNCV was calculated using the following equations: SNCV: s/t (m/s)=distance between stimulating and recording electrode/ latency[17]. After SNCV was detected, blood samples

from heart were collected and the serum biomarkers including NGF-β, TNF-α and IL-6 were determined by commercial ELISA kits using a BioTek microplate reader (Gene Co., Ltd. USA.). The left sciatic nerve was immediately made into 10% homogenate (tissue gram/saline volume in milliliter=1:9) used for analysis of MDA, SOD, and GSP-PX using the commercial kits.

Statistical analysis:

The experimental results were subjected to analysis of variance using SPSS 16.0 and expressed as mean±SD.

RESULTS AND DISCUSSION

Antioxidant activities *in vitro* are shown in fig. 1. The absorbance at 510 nm showed the antioxidant capability of ETS in spite of the inferiority compared with ascorbic acid. The IC_{50} values of ETS and positive control drug on DPPH radical and lipid peroxidation were detected and the results are listed in Table 1. They indicate that the extracts could well scavenge DPPH radicals and inhibited lipid peroxidation.

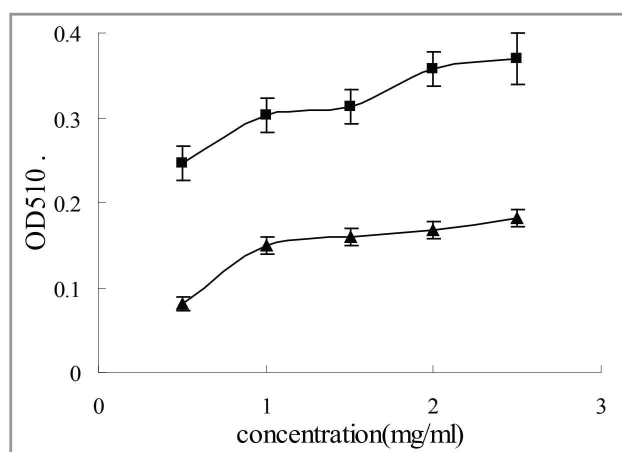

Fig. 1: Total antioxidant activities of the ETS.
■ ETS; ▲ ascorbic acid. All values are expressed in mean±SD; *n*=3.

During the experiment period, 4 rats died from intubations problems during drug administration including 2 in group 2, 1 in group 3 and 1 in group 5. The animals were immediately necropsied after death, and no abnormality was observed in gross examination. Rats in DPN group showed depressive spirit, less activity, increase of feeding, drinking, urinating and other signs of DM. Treatment groups improved these general features. The blood glucose and body weight of the experimental rats are shown in Table 2. The body weight of normal rats markedly increased from 200±20 g at the baseline to 358±32 g at the end of the experiment (P<0.05). By contrast, the values of DPN rats were decreased after a period of increase caused by the HFD. Treated groups prevented the body weight loss to varying degrees. The blood glucose levels of DPN rats were significantly higher than that of normal rats in group 1 (P<0.05); however, the blood glucose levels of rats in groups 3 to 5 were significantly decreased at the end.

The results of effects on oxidative stress in sciatic nerve of DPN rats are listed in Table 3. Compared with that of rats in normal control, the MDA contents in sciatic nerve of rats in DPN group were significantly increased while the SOD and GSH–PX activities were significantly decreased (P<0.05). The groups treated ETS apparently prevented these abnormities (P<0.05).

DPN, one of the most common chronic complications

TABLE 1: IC_{50} OF POLYPHENOLS ON REACTIVE OXYGEN RADICALS (*n*=5)

	ETS (mg/ml)	Ascorbic acid (μg/ml)
DPPH	0.33±0.02	9.51±0.75
Lipid peroxidation	8.19 ±0.71	61.50±4.19

All values are expressed in mean±SD.

TABLE 2: EFFECTS ON BODY WEIGHT AND BLOOD GLUCOSE

Group	*n*	Body weight (g)				Blood glucose (mM)			
		0 w	6 w	12 w	20 w	0 w	6 w	12 w	20 w
1	12	200±20	251±23[b]	284±23[b]	358±32[b]	3.3±0.3	3.4±0.4[b]	3.2±0.3[b]	3.8±0.6[b]
2	10	200±20	438±36[a]	508±49[a]	434±41[a]	3.3±0.3	20.9±3.5[a]	21.3±3.7[a]	23.1±5.0[a]
3	11	200±20	441±38[a]	471±40[a]	481±39[a]	3.3±0.3	21.2±2.2[a]	21.9±2.9[a]	9.5±1.6[b]
4	12	200±20	439±32[a]	462±36[a]	499±37[a]	3.3±0.3	19.5±2.9[a]	20.6±2.6[a]	10.3±0.9[b]
5	11	200±20	424±33[a]	453±37[a]	484±40[a]	3.3±0.3	20.7±2.9[a]	19.3±2.9[a]	8.4±1.2[b]

All values are expressed in mean±SD. [a]*P*<0.05 compared with the normal control group; [b]*P*<0.05 compared with the DPN group.

TABLE 3: EFFECTS ON OXIDATIVE STRESS INDICATORS IN SCIATIC NERVE OF DPN RATS

Group	1	2	3	4	5
n	12	10	11	12	11
SOD (U/mg prot)	95.14±8.33[b]	68.47±6.90[a]	76.13±7.41[b]	84.01±8.44[b]	82.37±8.05[b]
MDA (nmol/mg prot)	2.82±0.28[b]	7.93±0.68[a]	6.03±0.59[b]	4.96±0.45[b]	4.52±0.42[b]
GSH-PX (U)	129.11±10.34[b]	79.13±7.83[a]	89.76±8.71[b]	96.45±9.23[b]	101.72±9.55[b]

All values are expressed in mean±SD. [a]P<0.05 compared with the normal control group; [b]P<0.05 compared with the DPN group.

of DM, is a heterogeneous group of disorders with various pathologies and hyperglycemia must be the initiator. To diabetic, affected by the hyperglycemia *in vivo*, carbohydrate oxidation increases and a series of pathways are activated, which lead to the unbalanced oxidation and anti-oxidization of organisms and the excessive active oxygen radicals, causing the damage of tissues and cells, finally resulting in many DM complications. Oxygen free radical damage is the main contributor to DPN and is regarded as the direct or indirect common pathway in which DPN progresses[18]. Biomarkers for oxidative damage such as MDA were demonstrated to increase in diabetes. Oxidative stress also diminishes endogenous antioxidant enzyme defenses such as SOD and GSH-PX activities[19], which are very important to the regulation of oxidative status in diabetes. The research shows that the ETS possesses certain antioxidant activity *in vitro* and *in vivo*. The changes before and after administration suggest that one of the mechanisms of the protective effect may be anti-oxidative stress.

Tail-flick is one of the standard tests for measuring the rate of nociception. In this study, a significant increase in the tail-flick latency was observed after 6 w of STZ induction (P<0.05). These deficits were significantly reversed after treating with ETS for 8 w (P<0.05, fig. 2).

The results of effects on SNCV in DPN rats are shown in fig 3. Influenced by DM, the SNCV of DPN rats in group 2 was much slowed compared with the normal subjects in Group 1 (P<0.05). However, groups 3, 4 and 5 were notably improved than group 2 (P<0.05).

In clinical examination, the lower limbs usually reveal abnormal sensations of vibration, pressure, pain, and temperature perception[20], and DPN is manifested by deficit in nerve conduction velocity as well as thermal perception thresholds[21]. In the present study, we successfully induced DPN models by feeding them HFD for 12 w accompanied by an injection of low dose STZ. 6 w after modeling, early functional abnormalities in the peripheral nerves were observed, including thermal hyperalgesia and reduction in SNCV

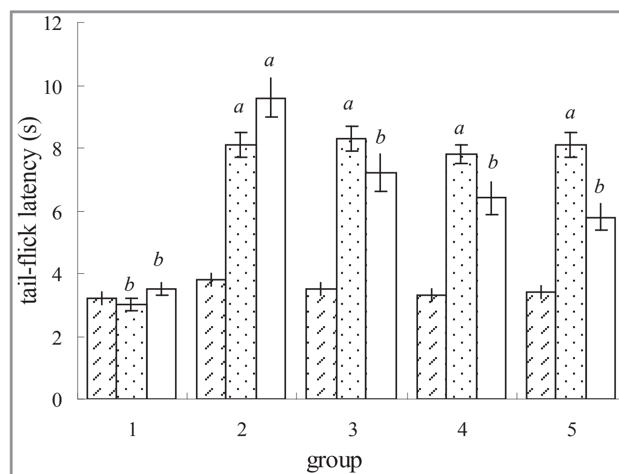

Fig.2: Effect on tail-flick latency.
▨ 6 w; ⬚ 12 w; □ 20 w. All values are expressed in mean±SD. [a]P<0.05 compared with the normal control group; [b]P<0.05 compared with the DPN group.

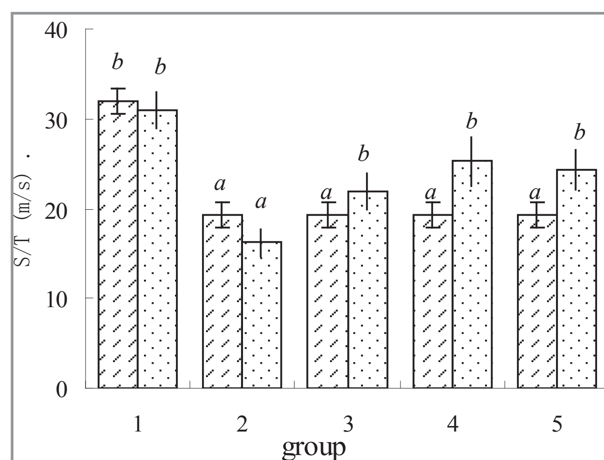

Fig. 3: Sciatic nerve conduction velocity in experiment rats.
▨ Before treatment; ⬚ After treatment. All values are expressed in mean±SD.[a]P<0.05 compared with the normal control group; [b]P<0.05 compared with the DPN group.

and by the end of the experiment, these abnormalities became more noticeable and accompanied with disordered growth factor and oxidative stress injury. The alleviation of these parameters by ETS showed the protective effect on DPN.

Fig. 4 shows the effect of ETS on contents of serum NGF-β before and after treatment. The concentrations

of NGF-β were significantly decreased ($P<0.05$) before treatment compared with normal controls, which can help to manifest the neurologic damage. The NGF-β contents were apparently increased in groups treated with ETS ($P<0.05$).

The results in fig. 5 showed that serum levels of pro-inflammatory cytokines TNF-α and IL-6 were significantly ($P<0.05$) increased in DPN rats compared with the normal controls. Treated with ETS significantly decreased the contents of IL-6 ($P<0.05$) and the effects on TNF-α showed significantly decreased only in the group treated with high dose ETS.

A series of studies have shown that, NGF-β and proinflammatory cytokines IL-6 and TNF-α are all important biomarkers of neuropathy, they might have vital implications clinically. The role of TNF-α is still controversial and Magrinelli's clinical research showed it was not found to be associated with DPN. Data from animal models documented that TNF-α was down regulated in the dorsal root ganglion in early stages of experimental diabetes, while the opposite occurred in later stage[22,23]. In our research mean value of serum TNF-α level was found significantly increased in DPN rats and decreased in high dose ETS group compared with DPN model rats. It might thus be hypothesized that the model rats were in later stage of DPN after the whole experiment. The pro-inflammatory cytokines IL-6 has been linked to various diabetic complications[24]. In our research mean value of serum IL-6 level was found increased in DPN rats and decreased in three ETS groups significantly. NGF performs a vital neuroprotective role with the ability to potentiate axonal growth. A low serum NGF-β level is correlated with neural damage in type 2 DM. Evidence showed that patients with more severe symptoms had lower NGF levels[25]. Our findings suggested that ETS could significantly decrease TNF-α and increase NGF-β level, which might be important protective mechanisms against DPN.

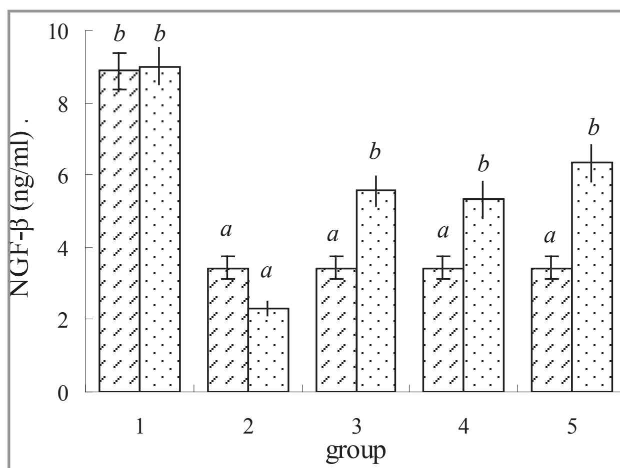

Fig. 4: Contents of NGF-*β* in diabetic peripheral neuropathy rats. **Before treatment; After treatment. All values are expressed in mean±SD. [a]P<0.05 compared with the normal control group; [b]P<0.05 compared with the DPN group.**

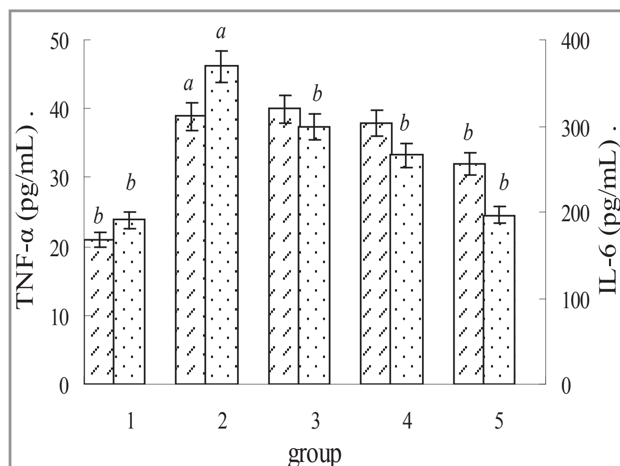

Fig. 5: Contents of IL-6 and TNF-*α* in diabetic peripheral neuropathy rats.
TNF-*α*; IL-6. All values are expressed in mean±SD. [a]P<0.05 compared with the normal control group; [b]P<0.05 compared with the DPN group.

In summary, alleviation of DPN in rats by early intervention with ETS is a result of the suppression of multiple pathogenic mechanisms, including alleviating hyperglycemia, inhibiting oxidative stress and regulating growth factor, etcetera. Nevertheless, we still do not know the underlying molecular mechanisms that ETS used to regulate these changes. In our further studies, we will separate the active components for investigating the further molecular mechanisms and contribute to adjuvant treatment for DPN.

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Conflicts of interest:

There are no conflicts of interest.

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