Environmental Science and Pollution Research (2022) 29:60542–60557
https://doi.org/10.1007/s11356-022-20106-3

RESEARCH ARTICLE

Pharmacological evaluation of *Thuja occidentalis* for the attenuation of neuropathy via AGEs and TNF-α inhibition in diabetic neuropathic rats

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Received: 30 November 2021 / Accepted: 1 April 2022 / Published online: 14 April 2022

Abstract

When diabetes neuropathy occurs, the oxidative stress caused by chronic hyperglycemia may result in chronic neuronal damage. To mitigate the effects of hyperglycemia-induced neuronal damage, it may be beneficial to address oxidative stress and inflammation in the body. The current study evaluated the neuroprotective efficacy of *Thuja occidentalis* in streptozotocin (STZ)-nicotinamide (NAD)-induced diabetic neuropathy in male Wistar rats. A single dose of STZ (65 mg/kg, i.p.) was used to induce diabetic neuropathy in Wistar rats. Serum insulin, glucose, hyperalgesia, oxidative stress, inflammatory markers, and histopathology of the sciatic nerve were evaluated for neuropathy. Wistar rats were treated with varying doses of hydroalcoholic extracts of *Thuja occidentalis* (HAETO) and gabapentin for 30 days. *Thuja occidentalis* considerably corrected the levels of inflammatory markers and oxidative stress caused by hyperglycemia; also, it led to the restoration of neuronal functions, indicating that it is effective in treating diabetic neuropathy. Furthermore, the molecular docking of thujone at the active pockets of various inflammatory mediators (IL-1β, IL-6, TGF-β1, and TNF-α) has shown good interactions with critical amino acid residues. These findings indicate that the hydroalcoholic extract of *Thuja occidentalis* effectively inhibits the development of diabetic neuropathy. The hypoglycemic, antioxidant, anti-hyperalgesia, and anti-inflammatory properties of *Thuja occidentalis* are thought to be responsible for the neuroprotective benefit.

Keywords Diabetes · Neuropathy · Hyperalgesia · *Thuja occidentalis* · Oxidative stress · Inflammatory markers

Introduction

Diabetic neuropathy (DN) is one of the most frequently occurring complications of diabetes, characterized by pain, foot ulcerations, amputations, and mortality. Despite significant feats in managing metabolic disorders, numerous approaches have been yet to explore the various therapeutic strategies for managing pain in diabetes. Additional neuropathy causes include neurotoxic drugs, renal illness, demyelinating neuropathy, vitamin B12 deficiency, hereditary neuropathy, alcoholism, and vasculitis (Alam et al. 2020). Diabetes can result in diabetic neuropathy, a type of nerve damage (Singh et al. 2021a, b, c). Elevated blood sugar (glucose) levels can cause havoc on nerves throughout the body (Kishore et al. 2018). Diabetic neuropathy most frequently affects the nerves in the legs and feet. DM (diabetes mellitus) is a metabolic disorder characterized by hyperglycemia and several associated complications like nephropathy, neuropathy, and retinopathy. (Dewanjee et al. 2018; Singh et al. 2020a, b, c, d, e). This is one of the primary reasons for death globally, affecting nearly 6% of the entire world's population (Ardeleanu et al. 2020). The rapidly rising incidences of diabetes worldwide have a major impact on people's health, life expectancy, quality of life, and healthcare systems (Sharma et al. 2020). According to the International Diabetes Federation (IDF), by 2030, more than 80% of people with diabetes will live in low- and middle-income countries (Saeedi et al. 2019).

Responsible Editor: Mohamed M. Abdel-Daim

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Hyperglycemia is caused by an abnormal hepatic glucose output, a decrease in glucose absorption by skeletal muscles, and a decrease in glycogen synthesis (Simran et al. 2019). Rapidly decreasing body weights, polyphagia, blurred vision, polyuria, and tachycardia are some of the cardinal signs of hyperglycemia (Singh et al. 2020a, b, c, d, e; Rehni et al. 2010). The neuropathy associated with DM is a progressive illness that is frequently asymptomatic. Patients with diabetic neuropathy have been seen to have complications like foot ulcers as the disease progresses (Singh et al. 2020a, b, c, d, e). Although various medications have been successfully researched to treat diabetic neuropathy in the past few decades, there has been a significant prevalence of adverse effects associated with the current pharmacotherapy of DN. Several long non-coding RNAs and micro RNAs (miRNAs) have been developed to improve gene expression, which may facilitate the alleviation of diabetic complications (Fawzy et al. 2020) .

Diabetic-induced oxidative stress results in lipid peroxidation, which damages the cellular organelles and myelin sheath in the sciatic nerve, promoting neuropathy and insulin resistance (Kandeil et al. 2020; Zhang et al. 2020; Singh et al. 2021a, b, c). Excessive free radical production occurs in diabetes due to glucose oxidation, enzymatic degradation of proteins, and other factors (Singh and Singh 2021). A substantial number of studies have confirmed the involvement of numerous inflammatory mediators in developing diabetes complications (Yang et al. 2020). Herbal antioxidants were inversely associated with inflammation markers, implying that inflammation and oxidative stress are related (Kandeil et al. 2011; Hossen et al. 2017; Ekta et al. 2020; Singh et al. 2019). Preclinical and clinical investigations have shown various herbal formulations and phytoconstituents to reduce cytokine and chemokine levels in diabetes (Garg et al. 2022; Grewal et al. 2021). Herbal formulations with anti-inflammatory properties can also be therapeutic agents in diabetic neuropathy. These possible physiological effects of dietary antioxidants have piqued researchers' interest in functional foods and dietary supplements in recent years (Bakht et al. 2020). Moreover, studies have shown that long-term use of herbal medicines appears to be effective in the management of DN (Tiwari et al. 2019).

Arbor vitae or *Thuja occidentalis* Linn. (Cupressaceae) is an herbal drug used widely for a long time in the traditional medicine system for the management of various ailments. *Thuja occidentalis* mainly consists of thujone, thujyl-alcohol, terpinolene, fenchone, limonene, borneol, alpha-terpene, and myricene. Thujone consists of 85% α-thujone and 15% β-thujone is the main compound (Naser et al. 2005; Jasuja et al. 2015; Bagot 2020). Other pharmacological activities represented by *Thuja occidentalis* include antioxidant activity, antibacterial, antifungal, antiinflammatory, antitumoral, anti-diabetic, hypolipidemic, gastroprotective, antiviral, and immunostimulant (Caruntu et al. 2020; Devi and Krishan 2020; Pradhan and Sarangdevot 2020).

Through streptozotocin (STZ)-induced studies, the present paper explores the merits of using *T. occidentalis* Linn. in managing and alleviating diabetic neuropathy due to its potent antioxidant potential. To date, there is no preclinical study on the neuroprotective effect of *T. occidentalis* Linn. on nociceptive behavior in diabetic animals. The present study also explored the mechanistic insight on the role of *T. occidentalis* in STZ induced DN.

Materials and methods

Chemicals

STZ and nicotinamide (NAD) were acquired from M/s Sigma Aldrich, India, and gabapentin from Yarrowchem, India, respectively. Diagnostic kits for biochemical estimates were purchased from Erba Pvt. Ltd., India. All of the other chemicals and solvents utilized were of the highest quality.

Plant material

The aerial part of *Thuja occidentalis* L. was collected from the city Rajpura, District Patiala, Punjab, India, and authenticated from Sri Venkateshwara University in Tirupati, India, under reference number: SVU/SC/48/99/17–18.

Extraction procedure

The dried shade aerial part of *Thuja occidentalis* L. was ground into a coarse powder and passed through a sieve with a mesh size of 40. The coarse powdered materials of the plant were subjected to soxhlet apparatus (Kishore et al. 2018). One kilogram of the *Thuja occidentalis* L. aerial parts was subjected to ethanol in the water ratio of 70:30 (v/v) and was kept at 25 °C for 12 h. The suspension was filtered through Whatman no.4 filter paper. To remove the solvents, the resulting hydroalcoholic solutions were subjected to distillation under a rotary evaporator at 600 °C. After the distillation, the resulting semi-solid was dried in a vacuum desiccator. This extract's nature and yield were noted. Until further use, the plant extract was stored in a refrigerator at 40 °C, and the extracts were named hydroalcoholic extract of *Thuja occidentalis* (HAETO). Furthermore, HAETO extracts were dissolved in water or solvent and used to assess *in vivo* assays.

Fingerprinting of HAETO

Hydroalcoholic extract of *Thuja occidentalis* analyzed with MS/MS for fingerprinting. For the quantification and

detection API 2000 (Applied biosystem/MDS SCIEX, Canada) mass spectrometer in conjunction with electrospray ionization (ESI) source and an LC system has been used (Farooq and Singh 2021).

In vivo evaluation

Animals

Wistar rats (males) weighing 220–250 g were housed in clean polypropylene cages at 24 ± 2 °C, a humidity of 45 ± 5%, and a 12-h day/night cycle maintained on a standard diet with regular water access. The animal experimental protocol was approved as per the guidelines of the Institutional Animal Ethics Committee (IAEC), Chitkara College of Pharmacy, Chitkara University, Punjab, under the registration number: 1181/PO/ReBi/S/08/CPCSEA, and the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India, were followed.

Induction procedure for diabetic neuropathy (DN)

STZ (65 mg/kg) in citrate buffer was administered intraperitoneally 15 min after NAD (230 mg/kg; i.p.) to induce DN in rats. To confirm the development of diabetes, fasting blood glucose (FBG) levels were assessed 72 h after STZ infusion (Kumar et al. 2022). To examine the course of neuropathic diabetes, blood glucose, body weight, insulin, behavioral markers (mechanical and thermal hyperalgesia), and motor nerve conduction velocity (MNCV) were measured at the end of a 60-day trial (Kishore et al. 2018; Tyagi et al. 2019; Farooq and Singh 2021).

Treatment schedule

During the experiment, 48 rats were randomized into 6 groups comprising 8 rats each. Group I was sham group; group II was positive control with DN induced; group III to V consisted of neuropathic animals administered with 50, 100, and 200 mg/kg, p.o. of HAETO; group VI primarily consisted of neuropathy induced rats receiving 30 mg/kg, intraperitoneal injection of gabapentin. Different concentrations of the extracts (50 mg/kg, 100 mg/kg, and 200 mg/kg, p.o.) were finalized based on oral acute toxicity and pilot studies reported in the literature. Treatment with HAETO and gabapentin was started after 30 days of STZ-NAD administration and continued for the next 30 days. Animals were sacrificed under deep anesthesia at the end of the study, and the sciatic nerve was excised surgically and stored at − 70 °C until further use (Kishore et al. 2018; Tyagi et al. 2019; Farooq and Singh 2021).

Blood glucose, body weight, and serum insulin estimation

At the outset of the study, the bodyweight of each animal was measured, and animals of similar weights were grouped. At the start of the treatment protocol, animals were randomly assigned. Throughout the study, each group's body weight was continually checked. Throughout the study, FBG levels were estimated at a 15-day interval using commercial enzymatic kits purchased from Erba Pvt. Ltd. India. Insulin levels in plasma were measured using an Insulin ELISA kit (Krishgen Biosysytems, India) in blood collected into anticoagulant-coated tubes (Subhasree et al. 2015).

Assessment of behavioral parameters in the form of mechanical and thermal hyperalgesia

Thermal hyperalgesia in DN rats was recorded by performing a tail immersion and hot plate test as the method described by Kishore et al. (2018).

Assessment of mechanical hyperalgesia by Randall-Selitto analgesiometer and allodynia in DN rats

Randall-Selitto analgesiometer was used to determine the hyperalgesia state in diabetic rats, as the method described by Kishore et al. (2018). For assessment of allodynia, Von Frey filaments were used as a method described by Farooq and Singh (2021).

MNCV in DN rats

The MNCV was evaluated in the diabetic animals to assess nerve damage as a method detailed by Kishore et al. (2018).

Estimation of oxidative stress in the sciatic nerve

Homogenate of the sciatic nerve was used to estimate thiobarbituric acid reactive substances (TBARS), catalase, superoxide dismutase (SOD), glutathione (GSH), and nitrite levels in post-mitochondrial supernatant for oxidative stress parameters as methods described by Tekaday et al. (2020).

Estimation of inflammatory mediators in the sciatic nerve

Under deep anesthesia, animals were sacrificed by cervical dislocation, sciatic nerves was extracted, and tissue homogenate was used to determine the presence of various inflammatory mediators such as interleukins (IL-1β, IL-6), transforming growth factor (TGF-β1), and tumor necrosis factor (TNF-α) (Ismail et al. 2018) by ELISA kit protocols (Krishgen Biosystems, India).

Estimation of the advanced glycation end product (AGEs) in sciatic nerve

An AGEs level in the sciatic nerve was determined using a method established by Kishore et al. (2018).

Histopathology of the sciatic nerve

The sciatic nerve of Wistar rats from various treatment groups was used for histopathological examination. It was performed on renal sections of 5 mm thickness that were produced and stained with hematoxylin and eosin (H & E) dye.

Molecular docking studies

To understand the binding interactions chondroitin sulfate at the active pockets of IL-1β, IL-6, TGF-β1, and TNF-α, the molecular docking simulation was carried out with CHARMm-based docking tool, CDOCKER of Discovery Studio Client v20.1.0.19295 software (Wu et al. 2003). The test compound was sketched and cleaned in Discovery Studio Client v20.1.0.19295 workspace, followed by energy minimization in the "Prepare Ligands" program of Discovery Studio Client at pH 7.4. The binding energy of the hits with proteins was estimated as negative of CDOCKER interaction energy (Shih et al. 2011).

Statistical analysis

The study data was reported as mean±SEM ANOVA was employed for statistical analysis, followed by Tukey's post hoc test using sigma stat software. $P<0.05$ was chosen as a statistically significant level.

Results

ESI–MS/MS analysis of the crude extracts

The percentage yield of the HAETO extract was 10.56% w/w. The HAETO extracts were subjected to ESI–MS/MS by flow injection analysis to understand the intensity of ionization response of the various compounds present in them by both positive and negative ionization mode. In positive mode (M+1), seven good intense responses were identified with HAETO (Table 1). The compounds which have identical intense responses and their structures are given.

ESI–MS/MS Fingerprinting of HAETO (Fig. 1)

Table 1 Qualitative studies of HAETO extract by ESI–MS/MS

S. no	Compound name	Precursor ion (M + 1)
1	Catechine	288.03 m/z
2	Gallo Catechine	305.27 m/z
3	Quercetrin	301.23 m/z
4	Kaempferol	435.04 m/z
5	Procyanidin β-3	593.30 m/z
6	Myricitrin	464.21 m/z
7	Thujone	151.23 m/z

The effect of Thuja occidentalis extracts on bodyweight

The effect of *T. occidentalis* hydroalcoholic extract on body weight was determined using an electronic weighing scale. The diabetic control group lost significantly ($p < 0.001$) more weight than the vehicle control group. After induction of diabetes (after the 30th day), therapy with hydroalcoholic extract of *T. occidentalis* (50, 100, and 200 mg/kg p.o.) and gabapentin (30 mg/kg) increased the body weight significantly ($p<0.01$) in comparison to the diabetic control group (Fig. 2).

The effect of Thuja occidentalis extracts on glucose levels

The effect of *T. occidentalis* hydroalcoholic extract on glucose levels was determined using a glucometer. The diabetic control group had significantly ($p < 0.001$) higher glucose levels than the vehicle control group. After induction of diabetes (after the 30th day), treatment with HAETO (50, 100, and 200 mg/kg p.o.) and gabapentin (30 mg/kg) significantly decreased ($p<0.01$) the glucose levels in comparison to the diabetic control group (Fig. 3).

The effect of Thuja occidentalis extracts on insulin levels

The effect of *T. occidentalis* hydroalcoholic extract on insulin levels was determined using an autoanalyzer. Insulin levels were significantly ($p < 0.001$) lower in the diabetic control group than the vehicle control group. After induction of diabetes (after the 30th day), therapy with HAETO (50, 100, and 200 mg/kg p.o.) and gabapentin (30 mg/kg) increased significantly ($p<0.01$) in comparison to the diabetic control group (Fig. 4).

Evaluation of hydroalcoholic extract of T. occidentalis on total AGEs

The diabetic control group had significantly ($p < 0.001$) higher AGEs levels than the vehicle control group. After induction of diabetes (after the 30th day), therapy with HAETO (50, 100, and 200 mg/kg p.o.) and gabapentin (30 mg/kg) significantly decreased AGEs levels ($p<0.01$) compared to the diabetic control group (Fig. 5).

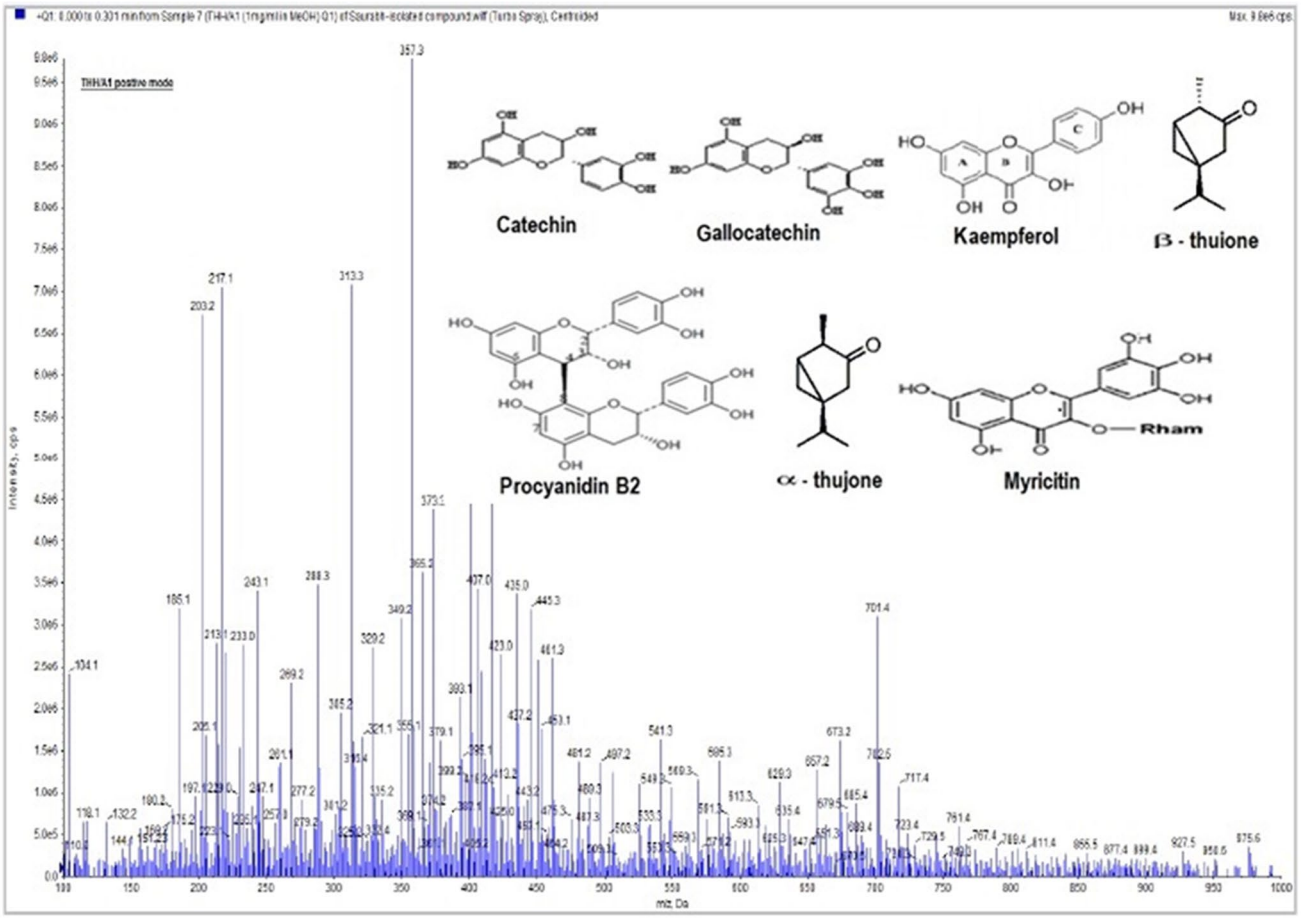

Fig. 1 MS/MS of HAETO extract in the range of 100–1000 m/z (M + H) and identified the structure of compounds

Evaluation of HAETO on mechanical hyperalgesia using randall-selitto analgesiometer

The HAETO was evaluated for hyperalgesia using the randall-selitto analgesiometer. There was a substantial difference between the diabetic control group and the vehicle control group in terms of pain resistance. After induction of diabetes (after the 30th day), therapy with HAETO (50, 100, and 200 mg/kg p.o.) and gabapentin (30 mg/kg) pain resistance increased significantly ($p < 0.01$) in comparison to the diabetic control group (Fig. 6).

Evaluation of HAETO on thermal hyperalgesia using hot plate method

The HAETO on hyperalgesia has been evaluated using the hot plate method. There was a significant increase in the frequency of paw licking, jumping, and rearing in the diabetic control group than the vehicle control group. The treatment with HAETO (50, 100, and 200 mg/kg p.o) and gabapentin (30 mg/kg) after induction of diabetes (after the 30th day) showed dose dependent attenuation in thermal hyperalgesia. There was a decrease in the frequency of paw licking, jumping, and rearing ($p < 0.01$) in comparison to the diabetic control group (Fig. 7).

Evaluation of hydroalcoholic extract of T. occidentalis on hyperalgesia using von Frey hair filaments

The HAETO on hyperalgesia has been evaluated using von Frey hair filaments. A significant decrease in pain resistance in the diabetic control group could be seen than in the vehicle control group. The treatment with HAETO (50, 100, and 200 mg/kg p.o) and gabapentin (30 mg/kg) after induction of diabetes (after the 30th day) showed a marked increase in pain resistance ($p < 0.01$) in comparison to the diabetic control group (Fig. 8).

Effect of Thuja occidentalis extract on oxidative stress parameters in sciatic nerve

The effect of *Thuja occidentalis* hydroalcoholic extract on TBARS in the sciatic nerves has been studied.

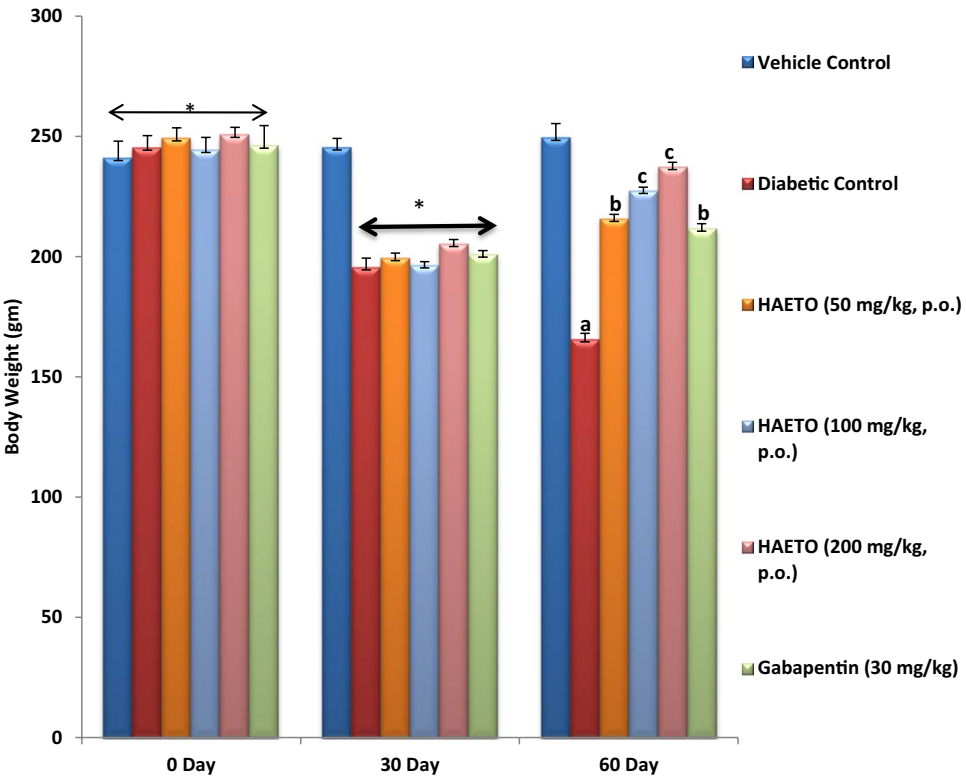

Fig. 2 Effect of hydroalcoholic extract of *Thuja occidentalis* on body weight. Values are represented as mean ± SEM ($n = 8$); *$p < 0.001$ vs. vehicle control on 30th day; [a]$p < 0.001$ vs. vehicle control on 60th day; [b]$p < 0.05$ vs. diabetic control on 60th day; [c]$p < 0.01$ vs. diabetic control on 60th day

Compared to the vehicle control group, there was a significant increase ($p < 0.001$) in the diabetic control group. However, the treatment with HAETO (50 mg/kg, 100 mg/kg, and 200 mg/kg p.o) and gabapentin (30 mg/kg) after induction of diabetes (after the 30th day) decreased the TBARS levels in renal tissue significantly ($p < 0.01$) when compared to the diabetic control group (Table 3). The effect of *T. occidentalis* hydroalcoholic

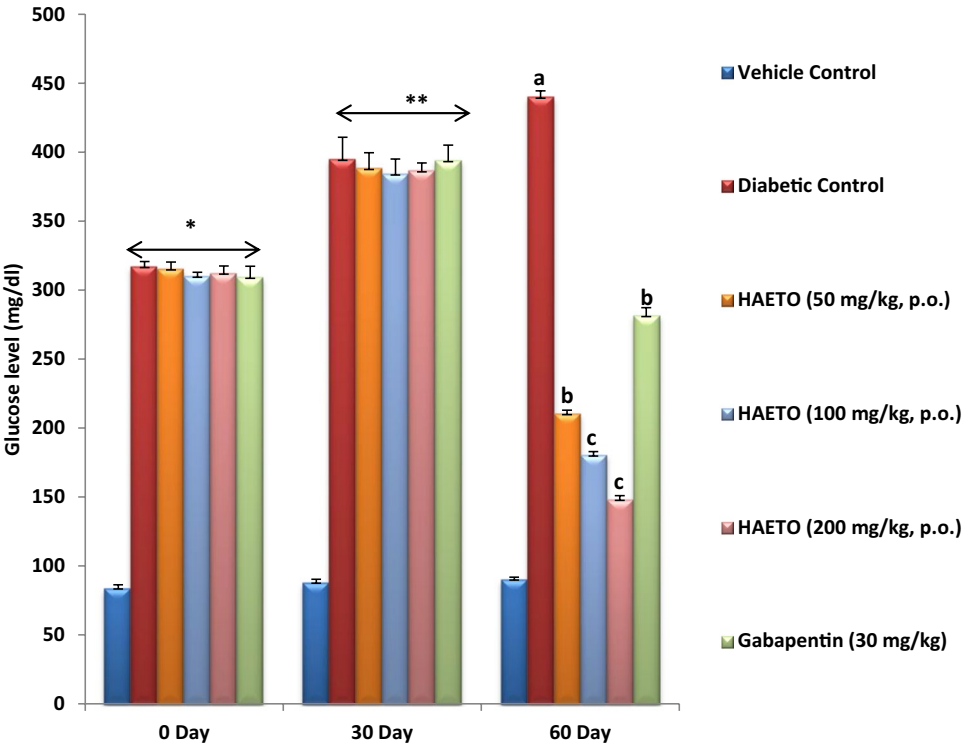

Fig. 3 Effect of hydroalcoholic extract of *Thuja occidentalis* on glucose level (mg/dl). Values are represented as mean ± SEM ($n = 8$); *$p < 0.01$ vs. vehicle control on 0 day. **$p < 0.001$ vs. vehicle control on 30th day; [a]$p < 0.0001$ vs. vehicle control on 60th day; [b]$p < 0.05$ vs. diabetic control on 60th day; [c]$p < 0.01$ vs. diabetic control on 60th day

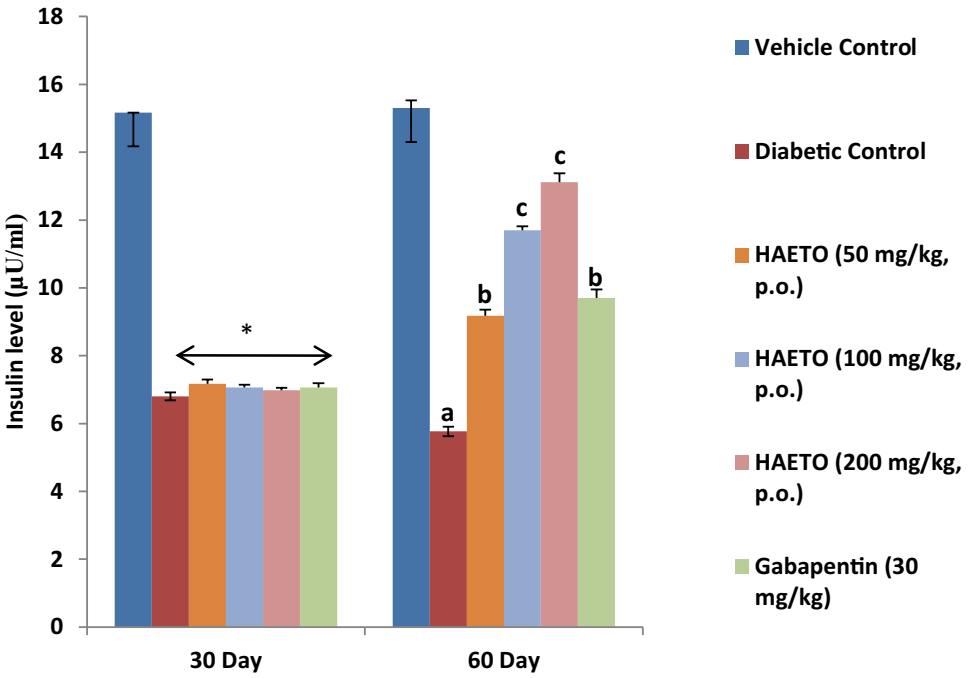

Fig. 4 Effect of hydroalcoholic extract of *Thuja occidentalis* on insulin level (µU/ml). Values are represented as mean ± SEM; *$p < 0.001$ vs. vehicle control on 30th day; [a]$p < 0.001$ vs. vehicle control on 60th day; [b]$p < 0.05$ vs. diabetic control on 60th day; [c]$p < 0.01$ vs. diabetic control on 60th day

extract on catalase, GSH, and SOD levels in the sciatic nerve has been studied. Compared to the vehicle control group, there was a significant decrease in catalase, GSH, and SOD levels ($p < 0.01$) in the diabetic control group. The treatment with HAETO (50 mg/kg, 100 mg/kg, and 200 mg/kg p.o) and gabapentin (30 mg/kg) after induction of diabetes (after the 30th day) increased the catalase, GSH, and SOD levels in sciatic nerve significantly ($p < 0.01$) when compared to the diabetic control group (Table 2).

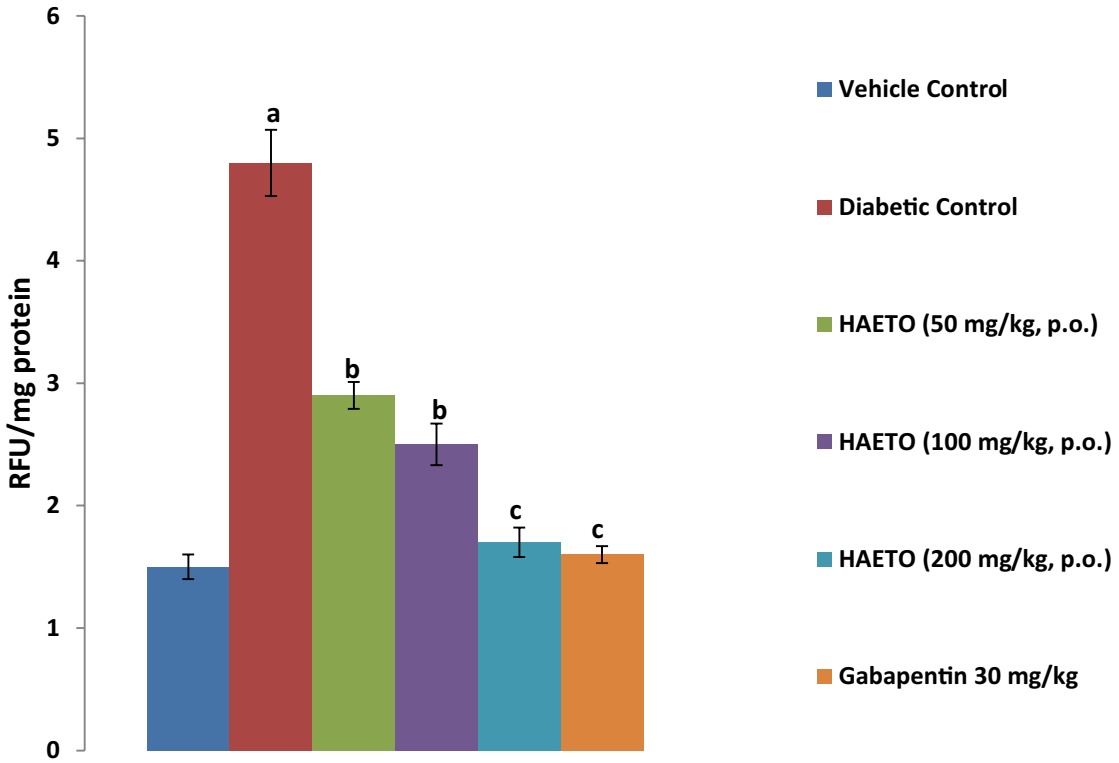

Fig. 5 Effect of hydroalcoholic extract of *Thuja occidentalis* on AGE's level (µU/ml). Values are represented as mean ± SEM; [a]$p < 0.001$ vs. vehicle control; [b]$p < 0.05$ vs. diabetic control; [c]$p < 0.01$ vs. diabetic control

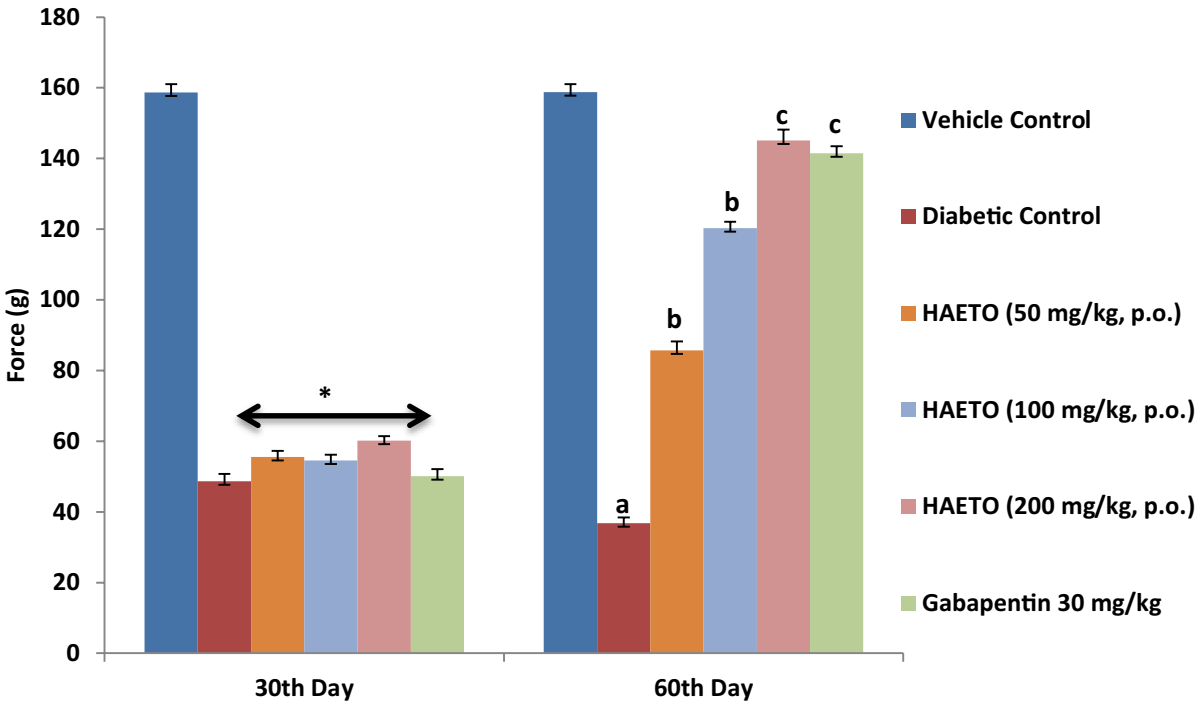

Fig. 6 Effect of hydroalcoholic extract of *Thuja occidentalis* on hyper algesia level (µU/ml). Values are represented as mean ± SEM; *p < 0.001 vs. vehicle control on 30th day; [a]p < 0.001 vs. vehicle control on 60th day; [b]p < 0.05 vs. diabetic control on 60th day; [c]p < 0.01 vs. diabetic control on 60th day

Evaluation of HAETO on nitrite level in the sciatic nerve

The nitrite content of a HAETO was determined. A considerable increase was observed in the diabetic control group to that in the vehicle control group. After induction of diabetes (after the 30th day), therapy with HAETO (50, 100, and 200 mg/kg p.o.) and gabapentin (30 mg/kg) significantly lowered the nitrate levels ($p < 0.01$) in comparison to the diabetic control group (Fig. 9).

Evaluation of HAETO on inflammatory mediators level in sciatic nerve

The evaluation of hydroalcoholic extract of *T. occidentalis* on inflammatory mediators has been evaluated. A significant

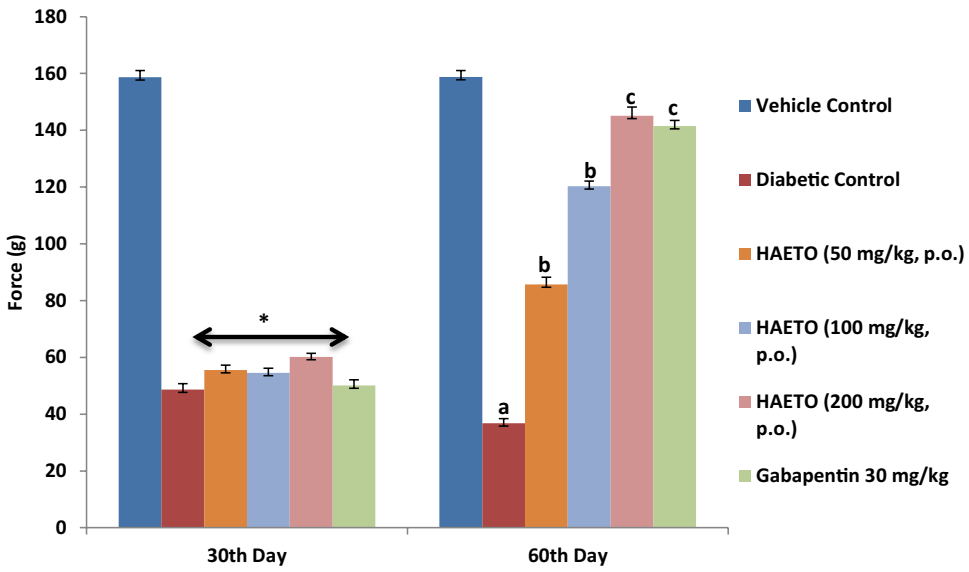

Fig. 7 Effect of hydroalcoholic extract of *Thuja occidentalis* on thermal hyperalgesia. Values are represented as mean ± SEM ($n = 8$); *p < 0.001 vs. vehicle control on 30th day; [a]p < 0.001 vs. vehicle control on 60th day; [b]p < 0.05 vs. diabetic control on 60th day; [c]p < 0.01 vs. diabetic control on 60th day

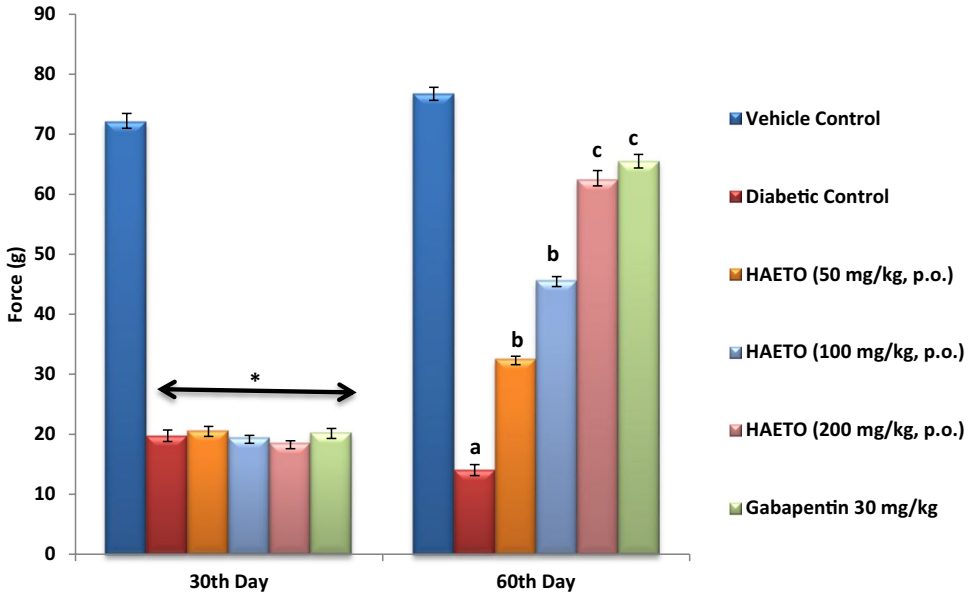

Fig. 8 Effect of hydroalcoholic extract of *Thuja occidentalis* on hyper algesia level (µU/ml). Values are represented as mean ± SEM ($n = 8$); *$p < 0.001$ vs. vehicle control on 30th day; [a]$p < 0.001$ vs. vehicle control on 60th day; [b]$p < 0.05$ vs. diabetic control on 60th day; [c]$p < 0.01$ vs. diabetic control on 60th day

Table 2 Effect of hydroalcoholic extract of *Thuja occidentalis* on oxidative stress parameters in the sciatic nerve. Values are represented as mean ± SEM ($n = 8$); [a]$p < 0.001$ vs. vehicle control; [b]$p < 0.05$ vs. diabetic control; [c]$p < 0.01$ vs.;diabetic control

Groups	CAT (U/mg protein)	GSH (µM/mg protein)	SOD (U/mg protein)	TBARS (nmol/mg protein)
Vehicle control	7.59 ± 0.21	18.51 ± 0.36	22.87 ± 0.58	1.47 ± 0.08
Diabetic control	2.74 ± 0.20[a]	6.67 ± 0.24[a]	8.58 ± 0.42[a]	7.74 ± 0.23[a]
HAETO (50 mg/kg, p.o.)	4.39 ± 0.20	10.56 ± 0.23	13.89 ± 0.25	4.39 ± 0.19
HAETO (100 mg/kg, p.o.)	5.55 ± 0.17[b]	13.77 ± 0.20[b]	16.98 ± 0.29[b]	3.17 ± 0.04[b]
HAETO (200 mg/kg, p.o.)	6.26 ± 0.15[c]	17.18 ± 0.17[c]	20.98 ± 0.26[c]	2.21 ± 0.06[c]
Gabapentin (30 mg/kg)	6.41 ± 0.25[c]	16.06 ± 0.17[c]	19.91 ± 0.41[c]	2.41 ± 0.12[c]

increase ($p < 0.001$) in levels of IL-1β, IL-6, TGF-β, and TNF-α could be seen in the diabetic control group compared to the vehicle control group. The treatment with HAETO (50, 100 and 200 mg/kg, p.o.) and gabapentin (30 mg/kg) after induction of diabetes (after 30th day) decreased the levels of IL-1β, IL-6, TGF-β, and TNF-α significantly ($p < 0.01$) in comparison to the diabetic control group (Table 3).

Effect of Thuja occidentalis on histopathological changes in sciatic nerve

In-vehicle control group, the nerve myelin sheath is intact without any degeneration in Schwann cells and edema in endoneural spaces. In the diabetic control group, there is degeneration of myelin sheath and Schwann cells and axonal swelling. In *Thuja occidentalis* (50, 100, and 200 mg/kg) and gabapentin treatment groups, there is reduced inflammation, accumulation of neutrophils and edema in neural vessels, and normal texture of myelinated fibers without any necrotic changes have seen. A bar scale of 200 µm was used for microscopic examinations (Fig. 10).

Molecular docking studies

In order to discover the interaction patterns of thujone with IL-1β, IL-6, TGF-β1, and TNF-α, docking studies were carried out using PDB IDs 1ITB, 1ALU, 3TZM, and 2AZ5 crystal structures, respectively. The thujone at the active pocket of IL-1β, IL-6, TGF-β1, and TNF-α forms hydrogen bonds, Pi-alkyl, and Van der Waals interactions with the crucial amino acid residues (Table 4).

Discussion

Animal models have been used to answer a wide range of scientific questions, from basic science to the development and testing of new vaccines and therapies. It is acknowledged that major medical breakthroughs such as blood circulation,

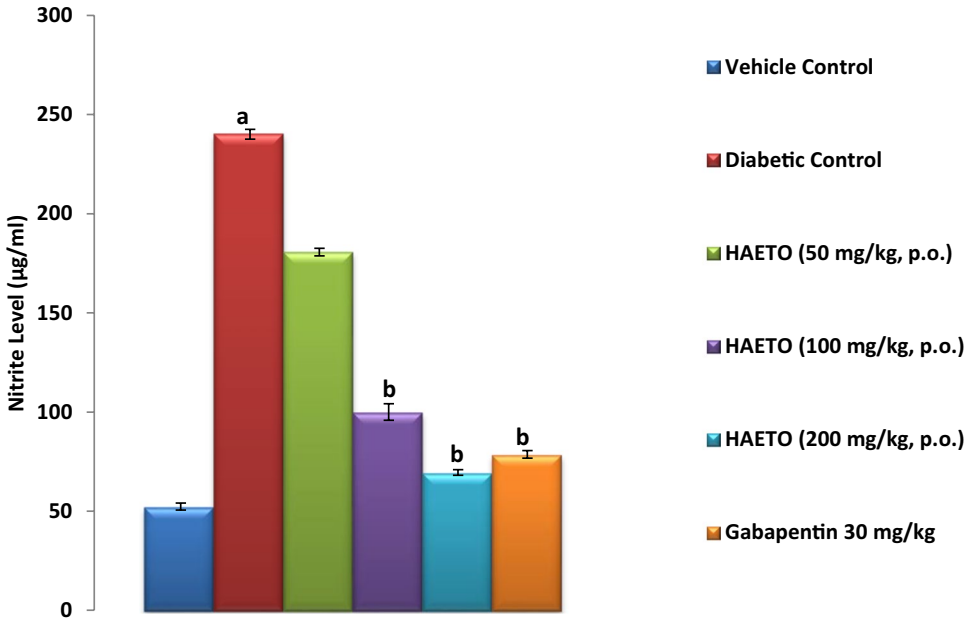

Fig. 9 Effect of hydroalcoholic extract of *Thuja occidentalis* on nitrile level (µU/ml). Values are represented as mean ± SEM ($n = 8$); [a]$p < 0.001$ vs. vehicle control; [b]$p < 0.01$ vs. diabetic control

Table 3 Effect of HAETO on various inflammorty markers **a** IL-1β level (pg/mg protein), **b** IL-6 (pg/mg protein), **c** TGF-β (pg/mg protein), and **d** TNF-α (pg/mg protein). Values are represented as mean ± SEM ($n = 8$); [a]$p < 0.001$ vs. vehicle control; [b]$p < 0.05$ vs. diabetic control; [c]$p < 0.01$ vs. diabetic control

Groups	IL-1β (pg/mg protein)	IL-6 (pg/mg protein)	TGF-β (pg/mg protein)	TNF-α (pg/mg protein)
Vehicle control	38.06 ± 0.54	12.23 ± 1.091	40.5 ± 1.20	56.66 ± 2.17
Diabetic control	106.245 ± 2.09[a]	82.74 ± 1.18[a]	200.17 ± 4.92[a]	335.5 ± 5.22[a]
HAETO (50 mg/kg, p.o.)	72.66 ± 1.53	52.66 ± 1.032	149.2 ± 2.55	220.83 ± 2.44
HAETO (100 mg/kg, p.o.)	52.15 ± 0.96[b]	33.48 ± 0.86[b]	91.83 ± 2.27[b]	141.83 ± 2.68[b]
HAETO (200 mg/kg, p.o.)	43.05 ± 0.70[c]	21.71 ± 0.81[c]	66.33 ± 1.99[c]	80.83 ± 1.24[c]
Gabapentin (30 mg/kg)	46.2 ± 0.67[c]	23.78 ± 0.76[c]	71.83 ± 2.32[c]	83.83 ± 1.4[c]

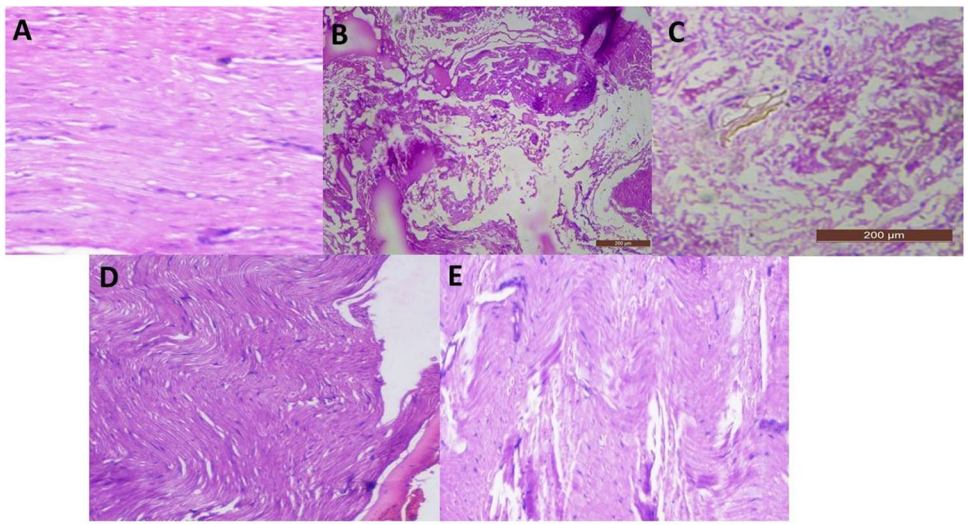

Fig. 10 Effect of various treatments on the histopathological changes in sciatic nerve (H&E × 100). **A** Vehicle control, **B** DN control, **C** HAETO (100 mg/kg, p.o.), **D** HAETO (200 mg/kg, p.o.), **E** gabapentin (30 mg/kg)

respiration physiology, and the hormonal system have been used for research purposes on various species of animals resembling human physiology and biology (King 2012). In our study chemically (STZ)-induced diabetic animal model is used to study the effect of HAETO in DN. In chemically induced models of type 1 diabetes, a high percentage of endogenous beta cells are destroyed, resulting in low endogenous insulin production and hyperglycemia (Singh et al. 2022). Chemically induced diabetes is not only a simple and low-cost model of diabetes in rodents, but it can also be used in higher animals. Because of its structural similarity to glucose, glucose can compete with STZ, making fasting animals more vulnerable to STZ (Barré-Sinoussi & Montagutelli 2015). STZ causes clinical features in animals that are similar to those seen in people with diabetes. As a result, STZ-treated animals have been used to investigate diabetogenic mechanisms and for preclinical testing of novel antidiabetic therapies. STZ-induced type 1 diabetes in rodents is a well-established and widely accepted method for studying diabetes pathogenesis and complications (Singh et al., 2020a, b, c, d, e). The single STZ injection model should continue to be a cost-effective, time-saving, and convenient platform for studying the pathophysiological mitochondrial mechanisms of cell derangement caused by diabetic glucotoxicity in various rodent models (Singh et al. 2022).

Allodynia and hyperalgesia are the clinical features by which DN has been characterized (Rosenberger et al. 2020). Additionally, it is characterized by an increased nociceptive response, decreased neuronal hypoxia, motor nerve condition velocity, and a decreased threshold for painful stimuli. The pathophysiology of progressive nerve fiber loss appears multifaceted, involving the polyol pathway, reactive oxygen species, glycation, and altered protein kinase C activity (Callaghan et al. 2020; Pang et al. 2020). Hyperglycemia, oxidative stress, and inflammation unleash a cascade of events that affect cellular proteins, gene expression, and cell surface receptor expression, ultimately resulting in progressive pathologic changes and subsequent diabetic complications (Tekaday et al. 2020; Bignold and Johnson 2021; Khan et al. 2021). In the present study, STZ-injected rats had significantly higher blood glucose levels, increased food, and water intake, and decreased body weight. The nociceptive threshold was significantly lower than non-diabetic rats, indicated by tactile allodynia thermal and mechanical hyperalgesia in diabetic rats, and results are in line with previous studies (Kishore et al. 2018; Kumar et al. 2022; Singh et al., 2020a, b, c, d, e).

Thuja occidentalis L. is a plant rich in flavonoids, glycosides, and triterpenoids (Bagot 2020) and hence arouses tremendous interest in antidiabetic potential (Bhargava et al. 2022), which could be considered a lead to further study effect of this part of the plant on diabetic complications such as neuropathy and nephropathy. *T. occidentalis* extracts revealed the presence of triterpenoids and flavonoids on further chemical analysis. In the present study, the results evidenced the previous literature reviews that revealed the presence of terpenoids, flavonoids, tannins, and carbohydrates as the major constituents of the plant (Caruntu et al. 2020). Furthermore, MS–MS analysis revealed the presence of different compounds such as catechine, gallocatechin, thujone, and kaempferol in HAETO. Previous literature showed that these compounds mentioned above are an effective dietary strategy for decreasing postprandial glucose responses (Jasuja et al. 2015).

STZ is a nitrosourea analog widely used in experimental animals to induce DM. The STZ action is thought to be a result of its alkylating ability. STZ causes selective pancreatic islet of cell cytotoxicity in experimental animals due to its DNA-alkylating activity mediated by the methyl nitrosourea moiety (Kishore et al. 2018). STZ impairs glucose-stimulated insulin release and increases insulin resistance in rats by rapidly destroying pancreatic

Table 4 Molecular docking of thujone at the active pocket of protein molecules

Protein	Binding interaction energy	Type of interaction	Bonding amino acids
IL-1β	− 13.5641	H-bond	Gly136, Gly135
		Pi-Alkyl	Phe133
		Van der Waals	Asp142, Trp120, Leu134
IL-6	− 23.0527	H-bond	Gln175
		Pi-Alkyl	Phe74
		Van der Waals	Lys66, Met67, Glu172, Ser176, Arg 179
TGF-β1	− 13.477	H-bond	Lys232
		Pi-Alkyl	Val219, Ala350
		Van der Waals	Leu260, Asn338, Ala230, Asp351, Lys213, Gly214, Lys337, Gly212, Ser287
TNF-α	− 10.8763	H-bond	Gly121, Tyr151
		Pi-Alkyl	Leu120, Tyr119, Tyr59
		Van der Waals	Tyr119, Gly121, Gln61, Ser60, Leu 120

cells. STZ impairs glucose-stimulated insulin release and increases insulin resistance in rats by rapidly destroying pancreatic cells (Sharma et al. 2021). This results in a decrease in glucose entry into peripheral tissues, muscle, and adipose tissue, an increase in gluconeogenesis and hepatic glucose synthesis, and a higher blood glucose level (Baeza-Flores et al. 2020). Hyperglycemia has been shown to cause oxidative stress via various mechanisms, including redox imbalances caused by increased aldose reductase activity, increased advanced glycation end products, altered protein kinase C activity, particularly the isoforms, prostanoid imbalances, and mitochondrial superoxide over-production (Nádró et al. 2021). All of these pathways combine to generate oxidative stress, which results in the activation of NF-κβ, TGF-β1, and TNF-α, as well as the stimulation and expression of COX-2 mRNA and gene (Stascheit et al. 2021). In the current study, administration of *Thuja occidentalis* dose-dependently ($p < 0.01$) and significantly attenuates the production and release of TNF-α, TGF-β, IL1-β, and IL-6 in diabetic rats and inhibits the growth and exacerbation of chronic DN when compared with diabetic control Wistar rats, and results are in line various studies (Kishore et al. 2018; Singh et al., 2020a, b, c, d, e). These results demonstrated the anti-inflammatory potential of *Thuja occidentalis* by inhibiting the expression of cytokines.

Hyperglycemia-induced allodynia and hyperalgesia have been linked to functional alterations in sensory and motor neurons (Zeidman 2021). Hyperalgesia, allodynia, enhanced pain perception, and decreased MNCV were seen in the current study. According to previous research, thermal hyperalgesia develops gradually due to persistent hyperglycemia-induced damage and necrotic alterations in myelinated axons (Kishore et al. 2018; Farooq and Singh 2021). In the current study, the treatment of diabetic rats with *Thuja occidentalis* and gabapentin effectively attenuated DN's behavioral and functional signs. *Thuja occidentalis* treatment for 30 days resulted in a significant restoration in the levels of the parameters mentioned above.

The production of AGEs under oxidative stress conditions may result from an interaction between the carbohydrate molecule and the free amino group of proteins (Singh et al., 2020a, b, c, d, e). AGEs are widely implicated in DN via cytokines and growth factors, causing significant diabetes consequences (Yu et al. 2021). The HAETO reduced STZ-induced oxidative stress-induced sciatic nerve injury by increasing antioxidant enzyme levels and decreasing AGE levels, supporting its potential role in reducing hyperglycemia. Increase the antioxidant capacity in chronic oxidative stress. Triterpenoids and flavonoids are well-known antioxidant phytochemicals. Triterpenoids and flavonoids alleviate oxidative stress by inhibiting the production of free radicals, slowing the degradation of GSH, and detoxifying LPO's mediated reactive products (Garg et al. 2022). In diabetic rats, a considerable rise in lipid peroxides and decreased antioxidant enzyme activity were reported. *Thuja occidentalis* L. is shown in this study to considerably repair biochemical markers such as lipid peroxidation, GSH, catalase, and SOD activity in the sciatic nerves of diabetic rats. *Thuja occidentalis*' antioxidant properties are generally documented, corroborating our current investigation (Kishore et al. 2018).

Nitric oxide (NO) causes protein nitrosylation, lipid peroxidation, protein nitration, and DNA damage that promote cell death, contributing to neuropathy (Singh et al. 2021a, b, c). The presence of various phytoconstituents in HAETO may contribute to its antioxidant property, thus showing inhibition of neurodegeneration in DN (Pang et al. 2020). In diabetes, enhanced levels of TNF-α and interleukins cause microvascular permeability and nerve damage due to phosphorylation of p38, thus promoting the development of micro-angiopathy and polyneuropathy (Dewanjee et al. 2018). In the current study, administration of *Thuja occidentalis* dose-dependently and significantly attenuates the production and release of TNF-α, TNF-α, TGF-β, and IL-1β in diabetic rats and inhibits the growth and exacerbation of chronic DN when compared with diabetic control Wistar rats (Yang et al. 2020). These results demonstrated the anti-inflammatory potential of *Thuja occidentalis* by inhibiting the expression of cytokines.

DN causes structural alterations in peripheral nerves. Axonal degeneration causes endoneural edema, decreased blood supply to nerves, and consequently reduced MNCV (Sharma et al. 2020). Administration of hydroalcoholic extract of *Thuja occidentalis* markedly ($p < 0.01$) increased MNCV compared to diabetic control rats. Results obtained by treatment of DN rats with hydroalcoholic extract of *Thuja occidentalis* (50 mg/kg, 100 mg/kg, and 200 mg/kg, p.o.) were prominent compared to the effect of gabapentin (30 mg/kg). These effects may be related to the anti-hyperglycemic action of *Thuja occidentalis*, which may be mediated through the alleviation of oxidative/nitrosative stress and the reduction of the production of AGEs (Rosenberger et al. 2020).

Furthermore, we compared various behavioral and biochemical effects of hydroalcoholic extract of *Thuja occidentalis* with gabapentin to improve the clinical rationality of the current report. By modulating oxidative-nitrosative stress and inflammatory cytokine release in diabetic rats, HAETO reverses neuropathic pain in them.

The molecular docking studies were further carried out to see the interaction pattern of thujone with different protein targets and were found that the test molecule has good interactions with inflammatory markers, i.e., IL-1β, IL-6, TGF-β1, and TNF-α with good binding energies (Dong et al. 2018). The position of thujone with respect

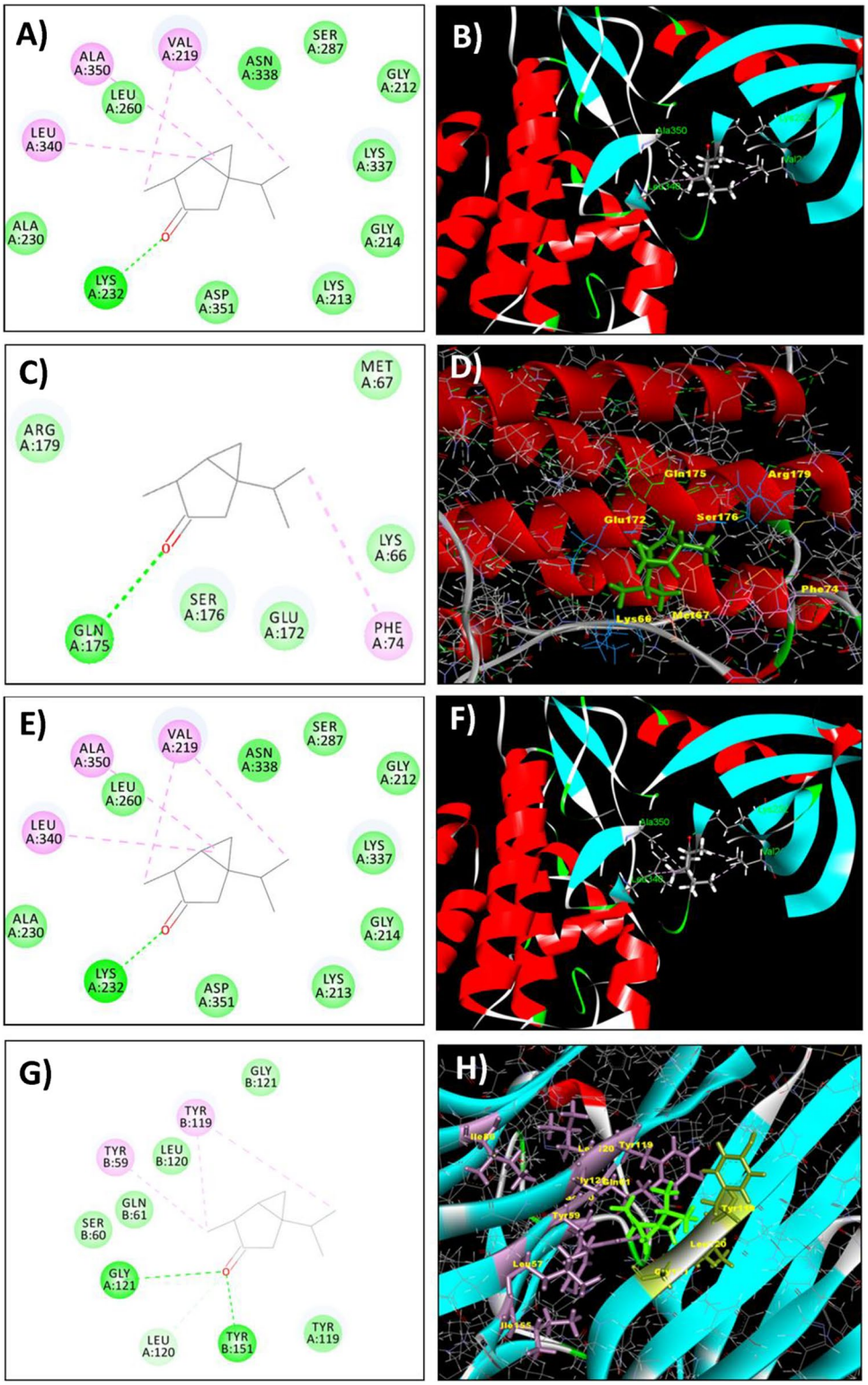

Fig. 11 The Interactions of thujone with the active site of IL-1β (**A** and **B**), IL-6 (**C** and **D**), TGF-β1 (**E** and **F**), TNF-alpha (**G** and **H**) pocket. The hydrogen bonds are indicated with green dashed lines, and the van der Waal forces are white. Thus, *Thuja occidentalis* may find a therapeutic application in treating diabetic patients with neuropathic pain. However, additional research is necessary to elucidate the precise mechanism of *Thuja occidentalis* antinociceptive potential. To our knowledge, there is no evidence to support the efficacy and potency of *Thuja occidentalis* in animal models of peripheral DN

to the key residues in the binding site of TNF-alpha, IL-1β, and IL-6 is shown in Fig. 11. The binding of the thujone with some of the essential residues of these target sites may describe one of its anti-inflammatory mechanisms of action.

Conclusion

T. occidentalis probably exerts its benefit by alleviating inflammatory stress. It is plausible to believe that *T. occidentalis*' neuroprotective impact is due, at least in part, to

its effect on AGE production and inflammatory condition. *T. occidentalis* may be advantageous by protecting against diabetic neuronal problems. Taken together, our findings suggest a beneficial role for *T. occidentalis* hydroalcoholic extract in reversing the progression of neuronal damage, and additional research elucidating the role of *T. occidentalis* or its bioactive components in molecular pathways may provide insight into the cellular mechanisms underlying diabetic complications and the development of neuropathy. As a result, the presented studies can be generalized to other species and can be followed up with clinical studies to assess *Thuja occidentalis* efficacy in treating DN in humans as well.

Abbreviations Ages: Advanced glycation end products; DM: Diabetes mellitus; DN: Diabetic neuropathy; ESI: Electrospray ionization; FBG: Fasting blood glucose; GSH: Glutathione; H & E: Hematoxylin and eosin; HAETO: Hydroalcoholic extracts of *Thuja occidentalis*; IAEC: Institutional Animal Ethics Committee; IDF: International Diabetes Federation; IL: Interleukins; MNCV: Motor nerve conduction velocity; NAD: Nicotinamide; NO: Nitric oxide; SOD: Superoxide dismutase; STZ: Streptozotocin; TBARS: Thiobarbituric acid reactive substances; TGF-β1: Transforming growth factor-β1; TNF-α: Tumor necrosis factor-α

Acknowledgements The authors are grateful to the Chitkara College of Pharmacy, Chitkara University, Rajpura, Patiala, Punjab, India, for providing the necessary facilities to carry out the research work.

Author contribution Conceptualization, conceived, and designed the experiments: TGS. Analyzed the data: TGS and SG. Wrote the manuscript: SKB, SS, and AM. Software: MS. Editing of the manuscript: SKB and SG. Critically reviewed the article: TGS. Supervision: TGS.

Data availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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