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ORIGINAL ARTICLE

Neuroprotective Efficacy of Polyphenols of Marine Brown Macroalga *Ecklonia cava* in Diabetic Peripheral Neuropathy

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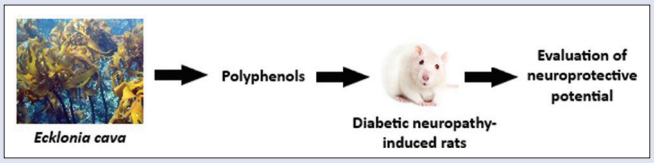
ABSTRACT

Objectives: In this study, the neuroprotective effect of polyphenols isolated from the brown marine macroalga *Ecklonia cava* (EC) was evaluated in experimental diabetic peripheral neuropathy (DPN). **Materials and Methods:** The polyphenolic fraction from EC was isolated. DPN was induced in animals by intraperitoneal injection of streptozotocin (45 mg/kg, b. w) and maintained for 6 weeks followed by treatment with EC polyphenols (ECPP) or epalrestat for 30 days. Nerve conduction velocity (NCV) of sciatic nerves and the compound muscle action potential (CMAP) of the gastrocnemius muscle were measured using a non-invasive method followed by neuropathic thermal analgesia and muscular grip strength. Sciatic nerve aldose reductase (AR) activity, intraneural sorbitol accumulation, Na^+K^+-ATPase activity, production of proinflammatory cytokines (interleukin-6 [IL-6], IL-1 β, and tumor necrosis factor alpha [TNF-α]), and expression of AR and protein kinase C (PKC) were assessed. **Results:** The ECPP were found to inhibit AR activity as well as their expression in diabetic animals, thereby improving the NCV, CMAP, muscle grip strength, hot plate, and tail-flick response time. Improvements in the sciatic nerve Na^+K^+-ATPase activity and intraneural accumulation of sorbitol, an index of AR overactivity, were evident with ECPP treatment. The production of proinflammatory cytokines (IL-6, IL-1 β, and TNF-α) and expression of PKC were also diminished. **Conclusion:** The data suggest that the polyphenols of EC have neuroprotective potential against experimental DPN.

Key words: Aldose reductase, antidiabetic activity, *Ecklonia cava*, macroalgae, peripheral neuropathy, polyphenols

SUMMARY

The *Ecklonia cava* polyphenols inhibited aldose reductase activity as well as their expression in diabetic animals. They also improved the nerve conduction velocity, compound muscle action potential, muscle grip strength, hot plate, and tail-flick response time. *Ecklonia cava* polyphenols also improved the sciatic nerve Na^+K^+-ATPase activity and intraneural accumulation of sorbitol. They also reduced the production of proinflammatory cytokines (interleukin-6, interleukin-1 β, and tumor necrosis factor-alpha) and expression of protein kinase C in the sciatic nerves of diabetic animals.



Abbreviations used: ECPP: *Ecklonia cava* polyphenols; NCV: Nerve conduction velocity; DPN: Diabetic peripheral neuropathy; CMAP: Compound muscle action potential; STZ: Streptozotocin; AR: Aldose reductase; PKC: Protein kinase C.

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INTRODUCTION

Diabetic peripheral neuropathy (DPN), a complication of uncontrolled type I diabetes, is a spectrum of clinical or subclinical manifestations affecting the peripheral nervous system.[1] Around 30 million people worldwide are affected and is a prominent source of mortality and morbidity.[2] Neurons have a consistently elevated demand of glucose and uptake depends primarily on its extracellular concentration. Hyperglycemia in diabetes causes neuronal glucose to rise up to four-fold. Such events, if persistent or frequent, may lead to neuronal damage owing to intracellular metabolism of glucose, an incidence commonly called glucose neurotoxicity.[3] Schwann cells are known to conduct the action potential through the insulation of axons, the maintenance of axonal caliber, effective nerve regeneration after axonal injury, and other neural functions in the peripheral nervous system.[4] Therefore, nerve dysfunction, such as reduced nerve conduction velocity (NCV), axonal atrophy, and impaired axonal regeneration can occur due to Schwann cell abnormalities, as a result of hyperglycemia.[5-7]

The polyol pathway of glucose metabolism plays crucial function in developing neuropathy.[8] Aldose reductase (AR; EC 1.1.1.21) is the first enzyme in the polyol pathway that catalyzes the reduction of glucose to sorbitol using NADPH as co-factor, then to fructose by sorbitol dehydrogenase, the second enzyme using NAD+ co-factor. As AR is localized to Schwann cells in the peripheral nerves,[9] its hyperglycemic activation is believed to affect nerve functions. During hyperglycemia, sorbitol accumulates in AR-containing tissues as it is impermeable to the cell membranes and cannot diffuse out and hence, creates hyperosmotic stress on the cell, thereby inducing neuropathic pain.[10] Accumulation of intracellular sorbitol and fructose leads to insufficiency of other organic electrolytes such as taurine and myo-inositol that regulate cellular osmolality.[11] Lessening of myo-inositol in the peripheral nerves interferes with the production

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of phosphoinositide producing inadequate diacylglycerol (DAG) to sustain the content of protein kinase C (PKC) essential for Na+/K+-ATPase activation.[12,13] Amendments in PKC activation also interfere with an important myelin protein's (PO) phosphorylation of peripheral nerve and deliberate an important pathogenetic role in primary segmental demyelination.[14]

Polyphenols isolated from various marine macroalgae have been found to possess anti-diabetic activities.[15-17] The brown macroalga *Ecklonia cava* (EC) Kjellman of the family Lessoniaceae has been reported to exhibit antidiabetic activity by mechanisms such as inhibiting glucose absorption, stimulating insulin secretion in streptozotocin (STZ)-diabetic mice,[18] and activating AMPK and Akt signaling pathways.[19] The polyphenols of EC were isolated, characterized, and reported by various researchers. The polyphenols found were phloroglucinol, triphlorethol A, eckol, eckstolonol, phlorofucofuroeckol A, dieckol, 6,6'-bieckol, 8,8'-bieckol, and fucofuroeckol A and were found to reduce glucose[20] and lipid[21] levels in high-fat diet-induced obese mice. However, the neuroprotective activity of EC polyphenols (ECPP) in DPN is not explored yet. Hence, in this study, we investigated the effects of ECPP on NCV, compound muscle action potential (CMAP), AR activity, and intraneural sorbitol accumulation in peripheral nerves (sciatic nerve). The expressions of AR, PKC, and proinflammatory cytokines were also studied.

MATERIALS AND METHODS

Chemicals and reagents

STZ, RIPA buffer and protease inhibitor cocktail tablets (SigmaFAST™) were procured from Sigma-Aldrich (USA). DL-glyceraldehyde and NADPH were obtained from Himedia, Mumbai, India. Primary antibodies for interleukin-6 (IL-6), IL-1 β, tumor necrosis factor alpha (TNF-α), and rabbit anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology, USA. Primary antibodies for AR, PKC, and Goat Anti-Rabbit IgG-HRP were procured from Abcam, USA. Cell culture media and reagents were obtained from Gibco, Thermo Scientific. Epalrestat was received as a generous gift from Zydus Cadila, India.

Experimental animals

Wistar rats (160–220 g) were maintained at room temperature (25°C ± 2°C) with a 12-h light/12-h dark cycle. The animals were given pellet chow and water *ad libitum* except during experimentation. The protocol was approved by the Institutional Animal Ethics Committee registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, bearing registration number 1564/PO/Re/S/11/CPCSEA.

Isolation of *Ecklonia cava* polyphenols

EC was procured from south China coastline through a reputed commercial dealer and authenticated. Dried, fine powders of EC were subjected to continuous hot extraction with 70% methanol for 3 h with reflux at 70-75°C three times successively. The extract was concentrated to half its volume and partitioned with n-hexane (×5) to remove pigments and lipids. Aqueous fraction contained soluble polyphenols (positive with the Folin–Ciocaulteu's phenol reagent) that were precipitated with ethylacetate (1:1), concentrated in a rotary evaporator, and lyophilized to obtain buff crystals. The polyphenol fraction was designated ECPP.

Determination of polyphenolic concentration

The concentration of polyphenol was determined using the Folin–Ciocaulteu's method.[22] Tannic acid standard was used to construct the calibration curve. The absorbance against a blank was measured at 750 nm.

Induction of peripheral neuropathy

Wistar rats were rendered diabetic with STZ injection (45 mg/kg, i. p.) and maintained for 6 weeks. The animals were grouped as: Group I: Normal control (untreated); Group II: DPN control– STZ (45 mg/kg b. w; i. p); Group III: DPN control + ECPP (100 mg/kg; oral); Group IV: DPN control + ECPP (200 mg/kg; oral); and Group V: DPN control + Epalrestat (AR inhibitor; 100 mg/kg).[23] The treatment groups were treated with ECPP or epalrestat for 30 consecutive days.

Measurement of Nerve Conduction Velocity and Compound Muscle Action Potential

NCV is used to assess the function, especially the electrical conductance of the sensory and motor nerves. In anesthetized rats, motor NCV (MNCV) (in milliseconds) was recorded from the sciatic nerve of the left tibia through a non-invasive modified method.[24,25] The nerve was stimulated at the sciatic notch proximally and at the knee distally by bipolar electrodes by AD Instruments (Powerlab data acquisition system, New Zealand). Using unipolar pin electrodes, the CMAP of the gastrocnemius muscle was recorded from the ankle.

Neuropathic thermal analgesia – Hot plate and tail flick methods

In the hot plate method, each rat was placed on the hot plate at 55°C–56°C and the time taken for the response to occur (either licking of paw or jumping) was recorded. A cutoff time of 10 s was kept to avoid damage to the paw of the animal.[26] In the tail-flick method, the tail of each rat was immersed in water at 29°C for 30 min before beginning the test. Then, it was re-immersed in water at 49°C. The time taken for the animal to flick its tail was recorded.[27]

Muscular grip strength test

Muscle relaxation is indicated by a loss in muscle grip which occurs in full blown peripheral neuropathy. This effect was studied in animals using a rotating rod (Rotarod, INCO Instruments, India). The difference in time to fall off from the rotating rod between the control and treated animal is taken as an indicator of muscle weakness. The rate of rotation of the rod (20 rpm) was tuned in a way that a normal animal can endure on it for a substantial period (3–5 min).[28]

Aldose reductase activity

The sciatic nerves of euthanized animals were exposed through a dorsal incision of the thigh and the nerve of full length was removed and transferred to a petri dish-containing DMEM supplemented with 10% FBS, penicillin G (100 IU/mL), streptomycin sulfate (100 µg/mL), and amphotericin B (2.5 µg/mL). After rinsing thoroughly, the nerves were homogenized with a Polytron homogenizer using a lysis buffer at 0°C–4°C containing 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, 1% NP40, and a protease inhibitor cocktail (SigmaFAST™, Sigma, USA). The lysate was centrifuged, and the supernatant was stored at −80°C till further use. For the determination of the sciatic nerve AR activity, 0.7 mL of phosphate buffer (67 mM), 0.1 mL of NADPH (0.25 mM), 0.1 mL of homogenate supernatant, and 0.1 mL of DL-glyceraldehyde (substrate) (0.5 mM) were taken in a cuvette. Absorbance of the final solution was taken against a

reference cuvette containing all components except the substrate, DL-glyceraldehyde. The enzymatic reaction was started by the addition of the substrate and the absorbance (OD) was recorded at 340 nm for 3 min at 30 s interval. The AR activity was expressed as μmoles/min/mL and calculated as per the following equation.[29]

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank}) \times \text{Total assay volume} \times \text{DF}}{\text{Millimolar extinction coefficient of NADPH at 340 nm} \times \text{supernatant volume}}$$

Where, DF = Dilution factor; Total volume (in ml) of assay = 3; Volume (in ml) of homogenate supernatant = 0.1; Millimolar extinction coefficient of β-NADPH at 340 nm = 6.22

Intraneural sorbitol accumulation

The estimation of sorbitol in the TCA-precipitated de-proteinized supernatant was done using an Agilent 1120 HPLC (EZChrome® software) on a Waters Sunfire® C_{18} RP column (250 mm × 4.6 mm, 5 μm, Milford, MA), and peak detection was performed at 231 nm. A gradient elution was performed with H_2O and acetonitrile (ACN) with flow rate 1.0 mL/min; analyte injection volume was 25 μL. Sorbitol reference standard (Sigma-Aldrich, India) was used at a concentration of 100 μg/mL. The gradient program followed was: 0–2 min-H_2O: ACN: 30:70; 2–6 min-H_2O: ACN: 12.5:87.5; 6–8 min-H_2O: ACN: 05:95; 8–9 min-H_2O: ACN: 12.5:87.5; 9–10 min-H_2O: ACN: 20:80; 10–11 min-H_2O: ACN: 30:70.[30] Sorbitol concentration was determined by quantifying the area under the curve of sorbitol.

Measurement of Na^+K^+-ATPase activity

The Na^+K^+-ATPase activity was assayed in the sciatic nerve lysate by the spectrophotometric determination of inorganic phosphate (Pi) released from ATP, in the presence and absence of ouabain, a specific Na^+K^+-ATPase antagonist. The lysate was incubated at 37°C in a reaction mixture containing Tris-HCl (30 mM pH 7.4), EDTA (0.1 mM), NaCl (50 mM), KCl (5 mM), $MgCl_2$ (6 mM), and ATP (1 mM) in the presence or absence of 0.5 mM ouabain.[31] After preincubating the homogenate for 10 min at 37°C, the reaction was started by the addition of ATP and stopped with 50 μL of TCA (30%) after 20 min. To determine inorganic phosphate (Pi) in the supernatant, 750 μL of a reducing solution containing 3.5% ferrous ammonium sulfate, 1.0% thiourea and 1.0% H_2SO_4 and 150 μL of an ammonium molybdate solution containing 4.4% ammonium molybdate, and 9% of H_2SO_4 were added to 750 μL of the solution to be assayed. After 10 min incubation at room temperature, the A_{750} nm was measured and Na^+K^+-ATPase activity was calculated as the difference between the presence or absence of ouabain-sensitive Na^+K^+-ATPase activity.[32] Total protein was estimated by bicinchoninic acid (BCA) protein assay kit (BCA-1, Sigma, USA).

Cytokine ELISA

The production of proinflammatory cytokines (IL-6, IL-1 β, and TNF-α) was assessed in the sciatic nerve lysate supernatant samples by indirect sandwich ELISA. Primary (capturing) anti-IL-6, IL-1 β, and TNF-α (Santa Cruz Biotech, USA) were used to coat ELISA plates. Standard IL-6, IL-1 β, and TNF-α (SCBT, USA) were added for constructing the calibration curve. Detecting anti-IL-6, anti-IL-1 β, and anti-TNF-α monoclonal antibodies (1:1000 dilution; SCBT, USA) and anti-mouse IgG-HRP (monoclonal, 1:5000, SCBT, USA) were used.

Western blot analysis

Initially, total protein in the sciatic nerve lysate supernatant was estimated by BCA protein assay kit. Aliquots of the lysates containing 40 μg of protein were subjected to denatured SDS-PAGE on polyacrylamide gels (MiniProtean TGX precast gels, BioRad). Western blot analyses were performed with AR and PKC primary antibodies (1:500 dilution, abcam, USA). Beta-actin mAb (1:500 dilution, abcam, USA) was used as a loading control, then labeled with HRP-conjugated secondary antibody (1:2000 dilution, abcam, USA). The antibody-reactive bands were visualized by enhanced chemiluminescence detection kit (Pierce, Thermo Scientific).

Statistical analysis

The results are expressed as a mean ± standard error of the mean, and one-way analysis of variance followed by Dunnett's test was used to determine statistical significance. Values of $P < 0.001$ were considered statistically significant.

RESULTS

Effect of *Ecklonia cava* polyphenols on nerve conduction velocity and compound muscle action potential

The concentration of polyphenols extracted from EC was found to be 2.516 mg/mL. Peripheral neuropathy is characterized by lowering of NCV. Sciatic NCV measured 6 weeks after STZ injection showed a significant 2.5-fold reduction ($P < 0.001$) when compared to the normal controls. With ECPP treatment (100 and 200 mg/kg), the NCV in diabetic group improved by 1.8- and 2.23-fold, respectively. Epalrestat treatment produced 2.34-fold increase in NCV [Figure 1a]. Furthermore, a 3.34-fold reduction in CMAP was observed in the DPN control animals as compared to the normal controls ($P < 0.001$). With ECPP treatment, a dose-dependent restoration of CMAP by 1.91- and 2.71-fold was witnessed ($P < 0.001$). With epalrestat treatment, a 2.94-fold increment in the CMAP was observed [Figure 1b].

Effect of *Ecklonia cava* polyphenols on neuropathic thermal analgesia

Diabetic rats exhibited 3.63-fold increase in the tail-flick latency time as compared to normal rats ($P < 0.001$). Treatment with ECPP reduced the response time in a dose-dependent manner by 2.17- and 3.29-fold when compared to the DPN controls [$P < 0.001$; Figure 2a]. Furthermore, the reaction time in hot plate method that was increased by 8.21-fold in DPN rats was decreased by 3.41- and 5.06-fold after treatment with ECPP [Figure 2b].

Effect of *Ecklonia cava* polyphenols on muscular grip strength

The time of residence was significantly reduced by 3.28-fold in the DPN control group against normal controls ($P < 0.001$). After ECPP treatment, the animals demonstrated a significant improvement of residence time by 1.98- and 2.55-fold on the rotating rod. A similar response (2.3-fold increase, $P < 0.001$) was observed within the group administered with the standard drug epalrestat [Figure 3].

Effect of *Ecklonia cava* polyphenols on sciatic nerve aldose reductase activity

Figure 4 shows 72.76-fold increment in the sciatic nerve AR activity in the DPN control group when compared to the normal animals ($P < 0.001$). However, this elevated AR activity was attenuated

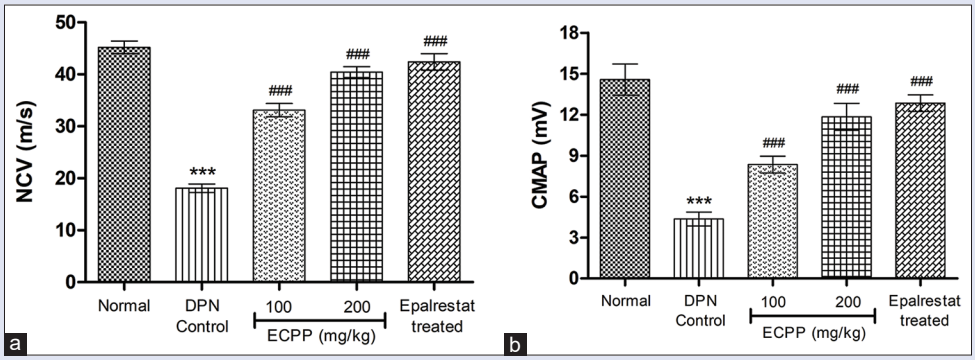

Figure 1: Effect of *Ecklonia cava* polyphenols on the neuro-muscular electrophysiology of normal and diabetic animals. The nerve conduction velocity of the left tibial sciatic nerve (a) and compound muscle action potential of the gastrocnemius muscle (b) were measured after treatment for 30 days. Epalrestat, an aldose reductase inhibitor, was used as a positive control. Values are expressed as mean ± standard error of the mean ($n = 6$). ***$P < 0.001$ compared with normal controls, ###$P < 0.001$ compared with diabetic peripheral neuropathy control

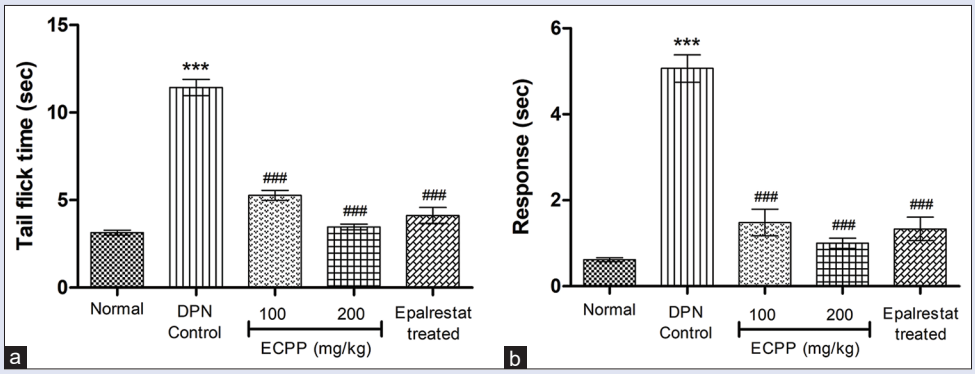

Figure 2: Effect of *Ecklonia cava* polyphenols on tail-flick latency (a) and hot-plate response time (b) in animals with diabetic peripheral neuropathy. Values are expressed as a mean ± standard error of the mean ($n = 6$). ***$P < 0.001$ compared with normal controls, ###$P < 0.001$ compared with diabetic peripheral neuropathy control

by 1.57-fold ($P < 0.05$) and 2.46-fold ($P < 0.001$) after treatment with ECPP of dose 100 and 200 mg/kg, when compared to the DPN control. Furthermore, treatment with epalrestat exhibited 2.95-fold reduction in AR activity [Figure 4].

Effect of *Ecklonia cava* polyphenols on the intraneural accumulation of sorbitol

The DPN control rats displayed an exorbitant 1300-fold increase in the accumulation of intraneural sorbitol when compared to nondiabetic mice ($P < 0.001$). After treatment with ECPP (100 and 200 mg/kg), intraneural sorbitol accumulation was reduced significantly by 1.45- and 2.13-fold, respectively. Epalrestat reduced sorbitol accumulation by 2.68-fold ($P < 0.001$) [Figure 5].

Effect of *Ecklonia cava* polyphenols on sciatic nerve Na^+K^+-ATPase activity

Na^+K^+-ATPase activity was decreased by 2.27-fold in the sciatic nerves of DPN control animals as compared to those of the normal control animals ($P < 0.001$). After treatment with ECPP (100 and 200 mg/kg), the Na^+K^+-ATPase activity in the DPN control animals was increased by 1.54-fold ($P < 0.05$) and 2.04-fold ($P < 0.001$), respectively. With epalrestat treatment, the enzymatic activity was increased by 1.96-fold [Figure 6].

Cytokine ELISA

The production of all three proinflammatory cytokines, namely, IL-6, IL-1 β, and TNF-α was increased by 6.93-, 7.64- and 7.5-fold in the nerves of DPN control animals compared to those of the normal controls ($P < 0.001$). While a significant reduction ($P < 0.001$) was observed post-ECPP treatment, reduction in the cytokine production after epalrestat treatment, however, was less significant ($P < 0.05$ and $P < 0.5$) compared to the DPN control [Figure 7].

Western blot analysis

As shown by Western blot analysis, expressions of AR and PKC were significantly increased in the DPN control animals by 36.68- and 12.93-fold when compared to the normal animals ($P < 0.001$). However, this expression was markedly attenuated in animals treated with ECPP (100 and 200 mg/kg) where 1.84- and 2.38-fold reduction in AR expression [Figure 8a] and 1.23- and 3.27-fold reduction in PKC expression was observed [Figure 8b].

DISCUSSION

DPN represents a state of complication associated with chronic hyperglycemia affecting all peripheral nerves, including sensory and motor neurons characterized by pain, paresthesia, hyperesthesia, dysesthesia, proprioceptive defect, loss of sensation, muscle weakness, and atrophy.[33] The polyol pathway has been identified as a major

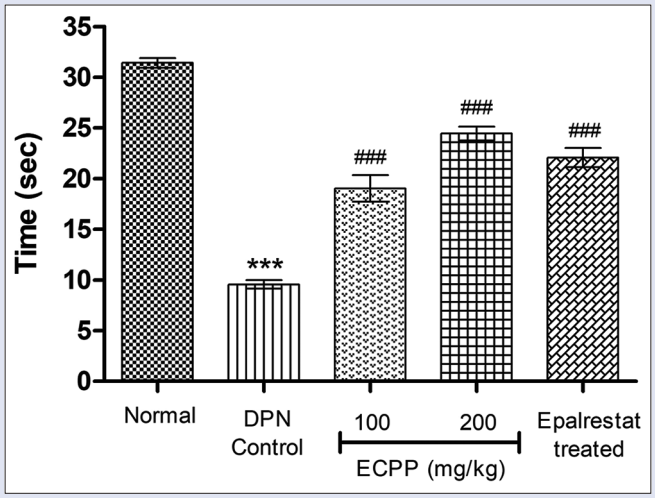

Figure 3: Effect of ECPP on muscular grip strength of diabetes-induced neuropathic animals. The animals were placed on a rotating rod (20 rpm) and their residence time on it is considered as an index of muscle weakness/strength. Epalrestat was used as a positive control. Values are expressed as a mean ± standard error of the mean (n = 6). ***P < 0.001 compared with normal controls, ###P < 0.001 compared with diabetic peripheral neuropathy control

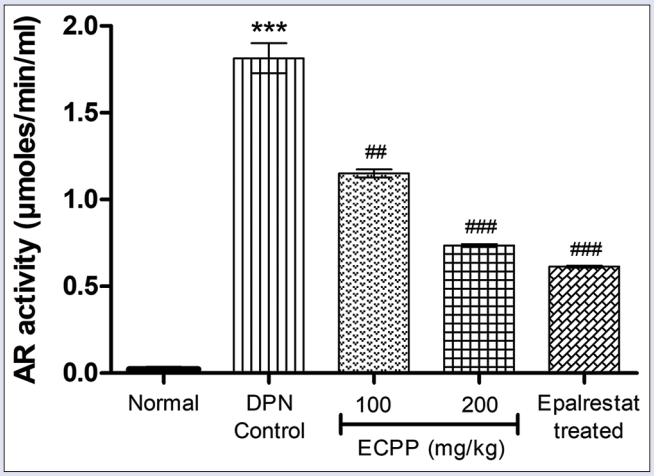

Figure 4: Effect of *Ecklonia cava* polyphenols on aldose reductase activity in the sciatic nerve of diabetic animals. Full length tibial sciatic nerves were isolated from the respective animal groups and homogenized. The aldose reductase enzymatic activity in the homogenates of respective groups was determined. Epalrestat, an AR inhibitor, was used as a positive control. Values are expressed as a mean ± standard error of the mean (n = 6). ***P < 0.001 compared with normal controls, ###P < 0.001, ##P < 0.01 compared with diabetic peripheral neuropathy control

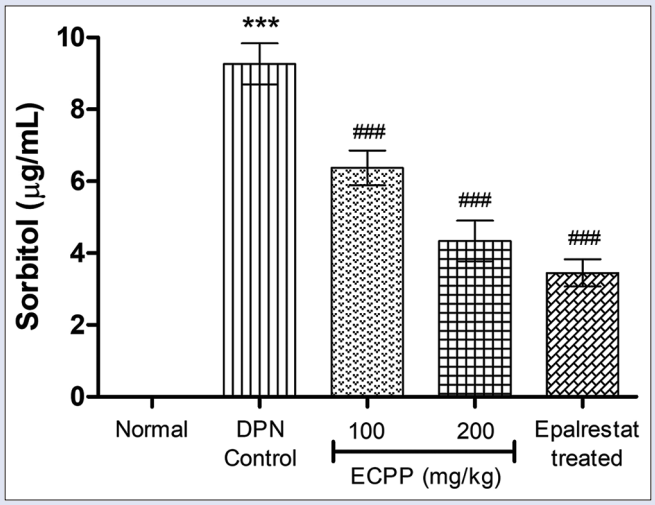

Figure 5: Effect of *Ecklonia cava* polyphenols on intraneural accumulation of sorbitol in the sciatic nerves of diabetic animals. Sorbitol concentration in the nerve homogenates was estimated by analytical high-performance liquid chromatography employing a gradient program of mobile phase (CH_3CN and H_2O) with a flow of 1.0 mL/min against a standard sorbitol solution (100 µg/mL). Sorbitol concentration was determined by quantifying the area under the curve of sorbitol peak. Values are expressed as mean ± standard error of the mean (n = 6). ***P < 0.001 compared with normal controls, ###P < 0.001 compared with diabetic peripheral neuropathy control

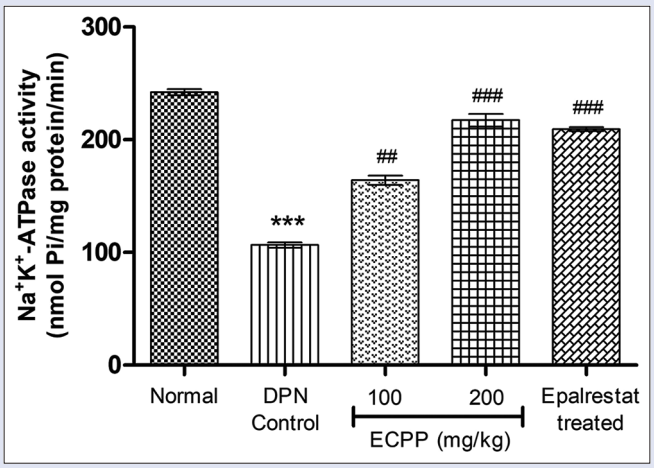

Figure 6: Effect of *Ecklonia cava* polyphenols on the Na^+K^+-ATPase activity in the sciatic nerves of diabetic animals. The enzymatic activity in the nerve homogenate was assayed spectrophotometrically by determining the inorganic phosphate released from ATP, in the presence and absence of ouabain, a specific Na^+K^+-ATPase antagonist. Values are expressed as mean ± standard error of the mean (n = 6). ***P < 0.001 compared with normal controls, ###P < 0.001, ##P < 0.01 compared with diabetic peripheral neuropathy control

contributor to the development of neuropathy. Reduced conduction velocity has been found to develop in motor and sensory nerves in diabetic animals[34] that have been prevented or reversed by treatment with an AR inhibitor.[35]

Marine algae are one of the richest sources of structurally diverse natural products. In recent years, an increasing number of novel compounds have been isolated from marine algae and many of them have been reported to possess different biological activities.[36-39] The antidiabetic potential of brown marine alga EC has been reported by many researchers,[18-21] but its neuroprotective activity in diabetes is not explored.

In the present study, the protective role of ECPP in experimental diabetic neuropathy was explored. To accomplish this, first, we studied the ability of ECPP to improve the neuromuscular electrophysiology (NCV of the sciatic nerve and CMAP of the gastrocnemius muscle) and muscular grip strength of STZ-induced diabetic animals. The results show significant restoration of NCV and CMAP and muscle grip strength in animals

treated with ECPP indicating its ameliorating effect. Consequently, a significant improvement in neuropathic thermal analgesia was achieved as the response time in hot plate and tail-flick experiments was significantly reduced. In experimental diabetic neuropathy, dramatic decrease in NCV has been widely reported.[40,41] The deficit in Na^+K^+-ATPase activity has serious implications in nerve physiology. Decreased sciatic nerve Na^+K^+-ATPase activity has been found to alter the normal membrane axon repolarization after the repolarization induced by an action potential, resulting in decreased NCV.[42,43] Treatment with ECPP, in the present study, has significantly restored the sciatic nerve Na^+K^+-ATPase activity. Similar results were obtained reduced Na^+K^+-ATPase activity was normalized in STZ-induced diabetic rats treated with pre-germinated brown rice.[44]

AR overactivity has been implicated in hyperglycemia that leads to the accumulation of sorbitol in the peripheral nerves that eventually causes neuropathic pain, a characteristic in diabetic neuropathy.[10,45]

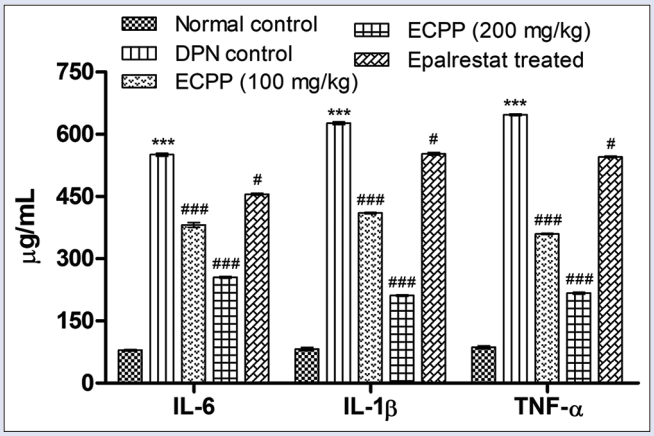

Figure 7: Effect of *Ecklonia cava* polyphenols on the expression of the proinflammatory cytokines (interleukin-6, interleukin-1β , and tumor necrosis factor alpha) in the sciatic nerves of diabetic animals. Production of cytokines in the diabetic animals before and after treatment with SLPP was measured using ELISA. Values are expressed as mean ± standard error of the mean ($n = 6$). $^{***}P < 0.001$ compared with normal controls, $^{\#\#\#}P < 0.001$, $^{\#}P < 0.05$ compared with diabetic peripheral neuropathy control

This overactivity of AR was also been found to be in correlation with the decreased Na^+K^+-ATPase activity and MNCV of the caudal nerves in STZ-diabetic rats. Strategic treatment with an AR inhibitor elevated Na^+K^+-ATPase activity and normalized the reduced MNCV.[46] The study revealed a significant reduction in the sciatic nerve AR activity after the treatment of neuropathic rats with ECPP. Sorbitol accumulation, a consequence of AR overactivity, was also lowered considerably in the sciatic nerves of animals treated with ECPP. The attenuation of these two polyol pathway overactivity of ECPP was found to be comparable to that of epalrestat which suggests that ECPP is a potential AR inhibitor.

The overexpression of proinflammatory cytokines (IL-6, IL-1 β, and TNF-α) in diabetic neuropathy directly increases nerve excitability and damage myelin leading to edema and further infiltration by immune cells.[47] Production of IL-1 β in injured nerves has directly been found to sensitize nociceptors in primary afferent neurons.[48] IL-6 induces pain directly by increasing the sensitivity of nerve endings[49] and can also enhance neuropathic pain in the dorsal horn through the activation of STAT3 signaling pathway as it is the key mediator of signal transduction of pain in glial cells after peripheral injury.[50] Evidence exists that inhibition of cytokine production has proven beneficial in the management of neuropathic pain and neuroinflammation.[51] Our results demonstrated significant reduction in the production of all the three proinflammatory cytokines (IL-6, IL-1 β, and TNF-α) in the sciatic nerve of neuropathic rats as compared to the diseased animals in a dose-dependent manner.

Hyperglycemia serves as a key signaling event in the activation of the PKC family of protein kinases.[52] The contribution of PKC to diabetic neuropathy is through neurovascular mechanisms such as blood flow and conduction velocity. There are immunochemical evidence for the presence of PKC-α,-β1,-β2,-γ,-δ, and-ε isoforms in nerve.[53,54] It was found that overexpression of PKC was involved in the reduction of Na^+-K^+-ATPase activity, leading to decreased nerve conduction and regeneration. The manifestations were normalized on treatment with a nonselective PKC inhibitor.[55,56] The contribution of polyol pathway to high glucose-induced PKC activation has been studied by investigators. AR overactivity has been implicated in hyperglycemic activation of PKC.[57] Inhibition of AR by tolrestat (AR inhibitor) prevented high glucose-induced activation of PKC in cultured vascular smooth muscle cells (VSMCs) isolated from rat aorta. Furthermore, ablation of AR gene using RNA interference, to exclude the nonspecific effects of AR

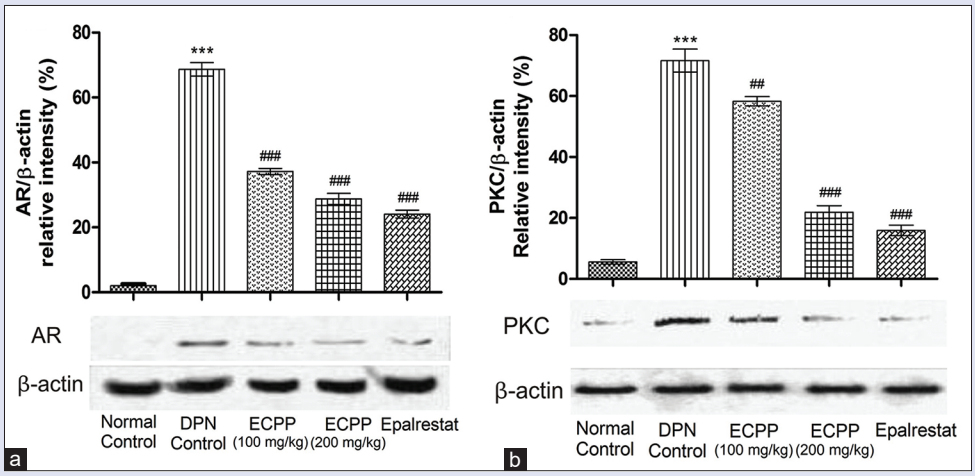

Figure 8: Effect of *Ecklonia cava* polyphenols on the expression of aldose reductase (a) and protein kinase C (b) in the sciatic nerves of diabetic animals. The levels of expression of aldose reductase and protein kinase C were detected by Western blot analysis and normalized to β-actin. Values are expressed as mean ± standard error of the mean ($n = 6$). $^{***}P < 0.001$ compared with normal controls, $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$ compared with diabetic peripheral neuropathy control

inhibitors,[58] reduced AR protein to undetectable levels and consequently, and prevented high glucose-induced activation of PKC. High glucose has been found to stimulate the membrane translocation of conventional (α, β1, β2, and γ) and novel (δ and ε) isoforms of PKC, the most significant being the PKC-β (β1 and β2) and-δ isoforms followed by enhancement in their phosphorylation. Treatment with AR inhibitors prevented both high glucose-induced membrane translocation and phosphorylation of the PKC isoforms. The AR inhibitors also prevented the increase in hyperglycemia induced-DAG synthesis from phospholipids and also abrogated phospholipase C (PLC) phosphorylation, an event essential for DAG synthesis.[59] However, AR inhibition did not inhibit phorbol-12-myristate-13-acetate (PMA)-induced membrane translocation of PKC, suggesting that inhibition of AR does not prevent PKC activation directly, but prevents DAG synthesis through inhibition of PLC phosphorylation.[60,61] Our Western blotting results, in absolute harmony with the preceding discussion, reveal significant reduction in the expression of PKC and AR proteins in the sciatic nerves of diabetic animals treated with ECPP as compared to the diabetic animals, thus clearly indicating its decisive role of AR inhibition.

CONCLUSION

The neuroprotective role of the polyphenols of the brown alga EC was evaluated in experimental DPN. We found that ECPP improved the NCV, CMAP, hot plate and tail-flick latencies and muscular grip strength of animals with diabetic neuropathy. It also reduced AR activity and its expression and consequently, prevented the accumulation of sorbitol in the sciatic nerves of diabetic animals. The Na^+K^+-ATPase activity was restored significantly, and the production of proinflammatory cytokines (IL-6, IL-1 β, and TNF-α) was reduced as well. Finally, the expression of PKC was also attenuated. These findings suggest that ECPP may be promising in ameliorating peripheral neuropathy in diabetes mellitus.

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Conflicts of interest

There are no conflicts of interest.

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