ABSTRACT

Objective: The natural product Momordica cymbalaria Fenzl has been in use considerably because of its antidiabetic activity. In this work, we have investigated the protective action of one of its phytoconstituent, a saponin, in peripheral neuropathy—a diabetic complication.

Methods: The saponin was isolated and identified by preparative high-performance liquid chromatography (preparative HPLC) and thin layer chromatography TLC). High glucose (56 mM)-mediated neuropathy was induced in NB-41A3 mouse neuroblastoma cells and the neuroprotective activity of the saponin was assessed by evaluating its effect on the two polyol pathway enzymes—aldose reductase (AR) and sorbitol dehydrogenase (SDH). The accumulation of intracellular sorbitol, activity of Na+K+-ATPase and production of pro-inflammatory cytokines IL-6, IL-1β and TNF-α were also investigated.

Results: The results showed a significant reduction in AR activity and intracellular accumulation of sorbitol on saponin treatment. Improvement in Na+K+-ATPase activity was evident with a noteworthy reduction in IL-6, IL-1β and TNF-α production.

Conclusion: These results suggest that the saponin possesses neuroprotective activity in diabetic peripheral neuropathy.

Keywords: Diabetic peripheral neuropathy (DPN), Saponin of Momordica cymbalaria (SMC), Neuroblastoma cells, Aldose reductase, Sorbitol dehydrogenase, Na+K+-ATPase

INTRODUCTION

Diabetic peripheral neuropathy (DPN) is a multifactorial disorder arising from hyperglycemia and/or insulin deficiency. It is characterized by a complex pathogenic network of interrelated metabolic, neurotrophic, and vascular defects [1]. Diabetic neuropathy influences all peripheral nerves including sensory and motor neurons and the autonomic nervous system. As all the organs and systems are innervated, it can affect all. It usually results in sensory abnormalities such as burning sensations, hyperalgesia, allodynia and dysesthesia, leading to alteration in patient’s quality of life [2].

The polyol pathway of glucose metabolism plays a crucial role in the development of neuropathy [3]. Polyol pathway overactivity [4, 5] and increased non-enzymatic glycation [6, 7] has been implicated in diabetic neuropathy. It is a two-step metabolic pathway in which glucose is reduced to sorbitol by the enzyme aldose reductase, which is then converted to fructose by sorbitol dehydrogenase. The first enzyme, aldose reductase (AR), requires NADPH as a cofactor, and the second enzyme sorbitol dehydrogenase (SDH) needs NAD+. During hyperglycemia, sorbitol accumulates in AR-containing tissues, presumably because oxidation by SDH is rate limiting and because polyols do not readily diffuse across cell membranes it was thought that accumulation of sorbitol produces osmotic that leads to diabetic lesions [8, 9]. Treatment with AR inhibitors in animal models has shown prevention of various diabetic complications including cataract, neuropathy, and nephropathy [10]. It is reported that AR inhibitor epalrestat prevents high glucose-induced smooth muscle cell proliferation and hypertrophy [11] thereby preventing their dysfunction and remodeling [12].

Na+K+-ATPase is an integral membrane protein which is responsible for providing and preserving the electrochemical gradient of Na and K ions between two sides of the plasma membrane. Decrease in Na+K+-ATPase activity in peripheral nerves plays an important role in the development of diabetic neuropathy [13]. Diabetic conditions are associated with chronic low-grade inflammation, evidenced by raised pro-inflammatory cytokines which include IL-1β, IL-6 and TNF-α which contribute to the pathogenesis of diabetic neuropathy [14].

Plants constitute a rich source of bioactive chemicals. Since many plants are largely free from adverse effects and have excellent pharmacological actions, they could possibly lead to the development of new classes of safer antidiabetic agents or diabetic complication resolving agents. Momordica cymbalaria (Cucurbitaceae) has been found to possess Type I [15] and Type II [16] anti-diabetic activities. The current study was conducted to investigate into the in vitro effects of the saponin of Momordica cymbalaria (SMC) on polyol pathway, Na+K+-ATPase activity and IL-1β, IL-6 and TNF-α in NB-41A3 mouse neuroblastoma cells.

MATERIALS AND METHODS

Plant material

The fresh roots of Momordica cymbalaria Fenzl were collected from Gadag district of Karnataka, India, and was identified and authenticated by the Department of Botany, Bangalore University, Bangalore, India (voucher No. 18122003).

Reagents

Fetal Bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Phosphate Buffered Saline (PBS), Ham’s F12 medium, glycine, EDTA, Glucose, antibiotics, D-fructose, Triethanolamine, β-Nicotinamide Adenine Dinucleotide, reduced form [β-NADH], Bovine Serum Albumin (BSA), Zymel®-® NADPH, Lithium sulphate, DL-glyceraldehyde, sorbitol, acetonicitrile, adenosine triphosphate (ATP) ferrous ammonium sulphate, thioura and ammonium molybdate were obtained from Hi-Media Laboratories Ltd., Mumbai, India. Protease inhibitor cocktail was obtained from (Sigma-Aldrich, Bangalore, India). IL-1β, IL-6 and TNF-α standard, IL-1β, IL-6 and TNF-α primary antibodies and HRP-conjugated secondary antibody were obtained from Santa Cruz Biotechnology, USA.
Extraction and isolation

The powdered roots were Soxhlet extracted with methanol. Methanolic extract was saponified and hydrolyzed with 0.5 N KOH in distilled water for 1-2 h. The unsaponifiable fraction was then extracted with diethyl ether. The upper organic layer was extracted with diethyl ether for complete separation of sterols. Ether and 1 % aqueous KOH was added, and the ether layer was separated. The ether was distilled off and 6 ml acetone was added and shaken. The solvent was completely removed, and the residue was dried to a constant mass. To confirm the presence of triterpenoid saponins, high-performance thin layer chromatography (HPTLC) was performed on Silica gel 60 F254 using chloroform: glacial acetic acid: methanol: water (16:8:3:2) as mobile phase and spraying with the anisaldehyde-sulfuric acid reagent. For further purification, the saponins so obtained were chromatographed on preparative HPLC (Agilent 1260 Infinity) using a C18 column (5 mm) and UV detector; flow rate was 5 ml/min. The mobile phase used was acetonitrile:10 mM K2HPO4 buffer (pH 5.8):70:30 for the first 10 min, changed to 90:10 for the next 10 min. Pure diosgenin (Yarrow chem, Mumbai) was used as a standard.

Cell culture and cytotoxicity assessment of the saponin

NB-41A3 mouse neuroblastoma cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Ham's F12 medium supplemented with 10 % fetal bovine serum, 50 IU/ml penicillin, 25 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The assessment of cytotoxicity of SMC on NB-41A3 cell line was done by MTT assay [17]. Cells plated onto 12-well plate, each well containing 5×10^5 cells, were incubated for 72 h. After incubation, medium from each well was removed, the formazan crystals were dissolved in 200 µl of DMSO and 25 µl of glycine buffer (pH 10.5), and the absorbance was measured at 570 nm in a microplate reader. The percentage cell viability was calculated.

High glucose-induced neurotoxicity

For neurotoxicity assay, cells were seeded in a 96-well plate (5-10×10^3 cells/well) and left to attach on the substrate overnight before being exposed to SMC, and incubated at 37 °C for 24 h. The cells were then treated with various concentrations of SMC (500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906 & 1.953 µg/ml) and incubated for 36 h. After 36 h 50 µl MTT (5 mg/ml) solution was added to each well and the cells were incubated in the dark at 37 °C for an additional 4 h. Thereafter, the medium was removed, the formazan crystals were dissolved in 200 µl of DMSO and 25 µl of glycine buffer (pH 10.5), and the absorbance was measured at 570 nm in a microplate reader. The percentage cell viability was calculated.

AR and SDH expression analysis by ELISA

The effect of SMC on the expression of AR and SDH in a hyperglycemic state (56 mM glucose) was evaluated by enzyme-linked immunosorbent assay (ELISA). The cell lysate was incubated with anti-AR antibody (H-110) 1:2000; Santa Cruz, CA, USA) & anti-SDH antibody [(H-284) 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA] followed by rabbit anti-mouse IgG-HRP (1:5000; sc-358914, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

NaK'-ATPase activity

Cells were incubated for 72 h. After incubation, cells adhering were lysed using ice-cold cell lysis buffer containing 1x Triton-X-100 (1 %), 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl and a protease inhibitor cocktail of aprotinin, benzamidine, leupeptin, pepstatin A, and PMSF. The homogenate was used for further enrichment. To confirm the presence of triterpenoid saponins, high-performance thin layer chromatography (HPTLC) was performed on Silica gel 60 F254 using chloroform: glacial acetic acid: methanol: water (16:8:3:2) as mobile phase and spraying with the anisaldehyde-sulfuric acid reagent. For further purification, the saponins so obtained were chromatographed on preparative HPLC (Agilent 1260 Infinity) using a C18 column (5 mm) and UV detector; flow rate was 5 ml/min. The mobile phase used was acetonitrile:10 mM K2HPO4 buffer (pH 5.8):70:30 for the first 10 min, changed to 90:10 for the next 10 min. Pure diosgenin (Yarrow chem, Mumbai) was used as a standard.

The separation and quantification of samples and standards were performed using an Agilent 1120 binary HPLC pump equipped with a dual wavelength absorbance detector, and an EZchrom software. Separations were performed on a Waters Sunfire® C18 reversed-phase column (250 mm×4.6 mm, 5 µm, Mőförd, MA) and peak detection was performed at 231 nm. A gradient elution using H2O and acetone/tert-butanol and a flow rate of 1.0 ml/min was maintained throughout the separation. Injection volume used was 25 µl. Sorbitol reference standard (Sigma-Aldrich, India) was used to determine concentration 100 µg/ml. An initial solvent composition of 30 % H2O and 70 % tert-butanol was maintained at injection and held for 2 min. At 2 min the proportion of tert-butanol was increased linearly to 87.5 % over a 2 min period then held for 2 min. At 4 min the proportion of acetone/tert-butanol was increased linearly to 95 % over 1 min and held for 1 min. At 5 min, the elution solvent was returned to 87.5 % over a 1 min period followed by to 80 % for the next 1 min and then to 70 % in the next one min. The system was allowed to equilibrate for 5 min prior to starting the next injection [20].

AR and SDH expression analysis by ELISA

The effect of SMC on the expression of AR and SDH in a hyperglycemic state (56 mM glucose) was evaluated by enzyme-linked immunosorbent assay (ELISA). The cell lysate was incubated with anti-AR antibody (H-110) 1:2000; Santa Cruz, CA, USA] followed by rabbit anti-mouse IgG-HRP (1:5000; sc-358914, Santa Cruz Biotechnology, Santa Cruz, CA, USA).
Measurement of pro-inflammatory cytokines IL-6, IL-1β and TNF-α

The concentration of the cytokines in the culture supernatant was determined by ELISA. The primary detecting antibodies for IL-6 [(R-19) 1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA], IL-1β [(H-153) 1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA] and TNF-α [(M-18) 1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA] were used. Rabbit anti-mouse IgG-HRP (1:5000; sc-358914, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for final detection.

Statistical analysis

The results are expressed as mean ± standard error of mean (SEM) and one-way analysis of variance (ANOVA) followed by Dunnett’s test was used to determine statistical significance. Values of p<0.001 were considered to be significantly different from each control.

RESULTS

Extraction and isolation of SMC

High-performance thin layer chromatography (HPTLC) of the extract indicated the presence of triterpenoid saponin. High-performance liquid chromatogram of the extract yielded two peak signals at 2.5 and 3.0 min respectively against the Standard diosgenin at 3.5 min (fig. 1). The second component which corresponds to the standard signal (at 3.5 min) was eluted out and collected.

Cytotoxicity assessment and high glucose-induced neurotoxicity

To evaluate the cytotoxicity of SMC, NB-41A3 cells were incubated in the presence of a series of SMC concentrations. It was found that the saponin did not exert significant cytotoxicity on the cells. Even at the highest test concentration of 500 µg/ml, 81.31 % viability of cells was evident (fig. 2). When the cells were exposed to 56 mM glucose for 36 h decrease in cell viability was evident as a result of cell death. However, cell survival increased when treated with SMC in a dose-dependent fashion when compared with the diabetic control (fig. 3). This reveals the ability of SMC to protect the cells from glucose neurotoxicity and impart normal viability & growth.

AR and SDH activities and sorbitol accumulation

Neuroblastoma cells were cultured in the presence of high glucose and SMC to evaluate its effect on the activities of AR and SDH, and also on sorbitol accumulation. Saponin of Momordica cymbalaria at 50 µg/ml has shown significant AR inhibition activity (p<0.001) when compared to high glucose control (group 2). The AR inhibition activity of SMC is, in fact, comparable to the activity of epalrestat, the standard AR inhibitor. SMC also inhibited SDH activity as compared to the controls.

However, this inhibitory activity was not found to be significant. Epalrestat, too, did not show any significant SDH inhibition activity (fig. 4). Aldose reductase and SDH activities for the normal control without glucose were not detected.

Saponin of Momordica cymbalaria has also been found to significantly reduced the formation of sorbitol (p<0.001) under hyperglycemic state when compared to high glucose control group. The activity has been found to comparable to that of the standard drug epalrestat. However, sorbitol remained undetected in the normal control group without glucose (fig. 5).

The results indicate over-expression of AR and SDH in a hyperglycemic condition while treatment with SMC significantly reduced AR expression as compared to the high glucose control (p<0.001).

However, reduction in SDH expression was not identified significantly. Reduction in AR expression was found to be profound for epalrestat while no significant effect was seen in the inhibition of SDH expression (fig. 6). These results in conjunction with those of the AR and SDH activities are indicative of the AR inhibiting property of SMC.

Fig. 1: HPLC chromatograms of standard diosgenin (A) and saponin of Momordica cymbalaria (B). The retention times (RT) of both were same (3.6 min)

Fig. 2: Cytotoxicity assessment of SMC by MTT assay. Values are expressed as mean±SEM (n=6). *** p<0.001 compared with the normal control

Fig. 3: Effect of SMC in high glucose-induced neurotoxicity in NB-41A3 cells. A dose-dependent survival mediated by the SMC is seen. Values are expressed as mean±SEM (n=6). *** p<0.001 compared with the normal control, ### p<0.001 compared with the high glucose control
Fig. 4: Effect of SMC of aldose reductase (A) and sorbitol dehydrogenase (B) activities. Values are expressed as mean±SEM (n=6). *** p<0.001 compared with the normal control; ### p<0.001 compared with the high glucose control; ns = statistically not significant when compared with high glucose control.

Fig. 5: Effect of SMC on the intraneural accumulation of sorbitol under high glucose condition. *** p<0.001 compared with the normal control; ### p<0.001 compared with the high glucose control. AR and SDH expression

The membrane Na⁺K⁺-ATPase activity was found to be significantly less for cells maintained in a high glucose environment as compared to the normal control (p<0.001).

Fig. 6: Effect of SMC on the expression of aldose reductase (A) and sorbitol dehydrogenase (B) by NB-41A3 cells maintained in high glucose (56 mM). Values are expressed as mean±SEM (n=6). *** p<0.001 compared with the normal control; ### p<0.001 compared with the high glucose control; p<0.05 compared with the high glucose control, ns = statistically not significant when compared with high glucose control. Na⁺K⁺-ATPase activity

Improvement in the activity was evident (fig. 7) significantly in cells treated with SMC (50 µg/ml) while epalrestat was not found to restore the activity, leaving an insight that epalrestat may not have any effect on Na⁺K⁺-ATPase.

Fig. 7: Effect of SMC on Na⁺K⁺-ATPase activity in NB-41A3 cells maintained in high glucose (56 mM). Values are expressed as mean±SEM (n=6). ### p<0.001 compared with the normal control; ns = statistically not significant when compared with high glucose control.
Measurement of IL-6, IL-1β and TNF-α

Assessment of the anti-inflammatory activity of SMC was done on NB-41A3 cells maintained in high glucose (56 mM) environment. It was found that the production of all three pro-inflammatory cytokines viz., IL-6, IL-1β and TNF-α was increased considerably by the cells in high glucose as compared to those in a normoglycemic (5.5 mM) state. However, treatment with SMC has significantly suppressed the production of the cytokines as compared to the high glucose control (p<0.001) and the standard, epalrestat (fig. 8).

DISCUSSION

Diabetic peripheral neuropathy represents a state of complication associated with chronic hyperglycemia influencing all peripheral nerves including sensory and motor neurons and the autonomic nervous system characterized by pain, paresthesia, hyperesthesia, dysesthesia, proprioceptive defect, loss of sensation, muscle weakness and atrophy [23]. The polyol pathway has been found to be a major contributor in the development of neuropathy. Reduced conduction velocity has been found to develop in motor and sensory nerves in diabetic animals [24]. The deficit develops between 2 and 3 w after diabetes induction in rats, has no visible structural correlates and is readily reversible on the establishment of normoglycemia with insulin [25]. The conduction deficit is also prevented or reversed by treatment with an aldose reductase inhibitor [26]. The current study has been undertaken to establish the neuroprotective effect of a saponin isolated from Momordica cymbalaria. Even though the general anti-hyperglycemic activity of Momordica cymbalaria has been studied by few [27, 28] but the neuroprotective activity, in specific, has never been attempted but only by us [29]. Neurons have a constantly high glucose demand, cannot afford anaerobic and glycolytic bursts and have a physiology that cannot accommodate episodic glucose uptake under the influence of insulin. Hyperglycemia in diabetes causes up to four-fold increase in neuronal glucose levels. If this is persistent, or if such episodes are regular events, then intracellular glucose metabolism leads to neuronal damage, or glucose neurotoxicity [30]. The SMC has shown to protect the cells in the presence of high glucose as a dose-dependent viability of cells was observed. Ninety-three percent cells were viable at 100 µg/ml of SMC against a meager 21.5 % of diabetic control. Chronic hyperglycemia offers a plethora of pathophysiological mechanisms that give rise to neuropathy. Polyol pathway activation due to increased glucose is one such that leads to overexpression of aldose reductase [31] which in turn leads to accumulation of sorbitol in the nerve tissue thereby causing neuropathic pain [32].

Many studies with experimental animals suggest that inhibition of AR could be effective in the prevention of neuropathy [4, 5]. Our findings of experiments with AR & SDH activities suggest that SMC significantly inhibited AR activity as compared to the diabetic control. Expression analysis of AR and SDH by ELISA also showed a significant drop in AR concentration in cells treated with SMC as compared with the diabetic control. Elevated expression of AR was found in IMS32 Schwann cells in the presence of high glucose (30 & 56 mM) when measured by Western blotting [31]. However, neither an attenuation of SDH activity nor any reduction in SDH expression was observed upon SMC treatment as compared to the AR. This behavior gives us an impression that the SDH inhibition property of SMC is probably insignificant. In fact, studies involving the evaluation of a SDH inhibitor have found that SDH inhibition does not offer an effective approach for the treatment of diabetic neuropathy and is adverse rather than beneficial [33]. Aldose reductase overexpression results in accumulation of intracellular sorbitol which being impermeable causes hyperosmotic stress. Our study involving measurement of intracellular accumulation of sorbitol using HPLC showed a significant reduction in sorbitol accumulation in the cells treated with SMC as compared to the diabetic control. This activity, which is comparable to that of epalrestat, suggests the possibility of SMC to be considered as a therapeutic candidate in neuropathic pain, calling for further investigations to be done.

Na+K+-ATPase is critical for the membrane potential, and many transports, a change in its activity would have profound consequence in these tissues. Streptozotocin-treated diabetic rodents are animal models used to assess metabolic and physiological changes induced by insulin-dependent diabetes. Among the diabetes-induced metabolic changes, disturbances of Na+-K+-ATPase activity have been widely reported [34]. Hyperglycemia, through the activation of PKC, is thought to reduce Na+-K+-ATPase activity, resulting in decreased nerve conduction and nerve regeneration [35]. Interestingly, it has been found that Na+-ATPase gene (ATP1 A1) polymorphism allows diabetes to induce a greater impairment of Na+-K+-ATPase activity which could in turn
favour the development of neuropathy [36]. The saponin of *Momordica cymbalaria* has been found to increase the Na⁺K⁺-ATPase activity as compared to the diabetic control. Other authors have also reported reversal of Na⁺K⁺-ATPase activity reduction in diabetic animals after treatment with curcumin [37].

All the characterized classical pathways like poloy path, PKC pathway, MAPK pathway, and increased production of AGES could directly or indirectly initiate and progress the production of inflammatory mediators [38]. Hyperglycemia leads to the formation of Advanced Glycated End (AGES) products due to non-enzymatic glycation of proteins that act on various receptors present on microglia and macrophages stimulate production of cytokines like IL-1, IL-6, IL-1β, TNF-α, chemotactic protein-1, C-reactive protein, and chemokines like CCL-2, CXC, and so forth [39]. We have evaluated the ability of SMC to suppress neuroinflammation by looking into its effect on the production of inflammatory cytokines viz., IL-6, IL-1β and TNF-α by neuroblastoma cells maintained in high glucose. The diabetic control showed increased production of all the three cytokines than the normal control whereas SMC treatment has been found to reduce the production of all cytokines significantly as compared to the diabetic control. It can be clearly concluded that SMC possesses ability to reduce neuroinflammation and hence, neuropathic pain. Neuroinflammation has also been reported to cause nerve damage due to apoptosis induced by MAPK signalling leading to structural damage of peripheral nerves [40]. The dose-dependent reversal of glucose neurotoxicity of high glucose in neuroblastoma cells by SMC may find its explanation in the reduced formation of cytokines that might have prevented induction of apoptosis via the MAPK signalling pathway. However, the action of SMC on MAPK activation is yet to be revealed and is under investigation by us.

The main objective of the study was to investigate into the neuroprotective effect of saponin of *Momordica cymbalaria* in NB-41A3 mouse neuroblastoma cells. The saponin was found to reduce cells maintained in high glucose. Intracellular sorbitol accumulation ameliorated the neuropathic pain. The Na⁺K⁺-ATPase activity was also found to be reduced which is a positive indication towards the reduced formation of cytokines that might have prevented induction of apoptosis that the MAPK signalling pathway. However, the action of SMC on MAPK activation is yet to be revealed and is under investigation by us.

**CONCLUSION**

In this study, we find that SMC possesses significant aldose reductase inhibitory activity which can prove beneficial in the management of neuropathy along with proper glycemic control. The Na⁺K⁺-ATPase activity improved in the presence of SMC which is beneficial for the proper membrane potential and nerve conduction. SMC was also found to have a prominent effect in the reduction of pro-inflammatory cytokines formation in the presence of high glucose that explains its anti-neuro inflammatory activity.

**ACKNOWLEDGEMENT**

The authors deeply express their gratitude to Karnataka College of Pharmacy, Bangalore, India, for the experimental infrastructure and financial support.

**CONFLICT OF INTERESTS**

The authors have declared that there is no conflict of interest.

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