

Ameliorative Effect of Green Lipped Mussel Extract on Vincristine-Induced Painful Neuropathy in Rats

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Abstract

Neuropathic pain is a chronic neurodegenerative disease. Neuropathy is well characterized by spontaneous pain, hyperalgesia, hypoesthesia, dysesthesia and allodynia. The present study was designed to investigate the protective effect of Green Lipped mussel (GLM) on vincristine-induced painful neuropathy in rats. Neuropathic pain in experimental animals was induced by the administration of vincristine (50µg/kg intraperitoneally for 14 consecutive days). Vincristine is associated with painful neuropathy due to damages to the peripheral axons. Hot plate, acetone drop, paw pressure and tail immersion tests were performed to assess the degree of thermal hyperalgesia, cold chemical allodynia, mechanical hyperalgesia and allodynia in the hind paw, tail thermal hyperalgesia & muscle grip strength respectively, as an index of peripheral and central pain sensation. Tissue Thiobarbituric Acid Reactive Substances (TBARSs), reduced glutathione (GSH) and total calcium levels were estimated in the sciatic nerve tissue samples. Histopathological changes were also observed in the sciatic nerve tissue as an index of oxidative stress. GLM extract and gabapentin were administered for 14 consecutive days. Vincristine administration resulted in significant reduction in behavioural (i.e. hyperalgesia and allodynic pain sensation) changes along with a rise in the levels of TBARS, total calcium and decrease in GSH levels when compared with the normal control group. Pretreatment with GLM extract significantly attenuated vincristine-induced development of painful behavioral and biochemical changes in a dose-dependent manner, which is similar to that of gabapentin-pretreated group. GLM extract ameliorated vincristine-induced painful neuropathy. It may be due to its potential of antioxidative, neuroprotective and calcium channel inactivation.

Keywords: Mussel extract; Allodynia; Neuropathic pain; Oxidative stress; Vincristine

Introduction

The vinca alkaloids (vincristine and vinblastine) are the most common chemotherapeutic agents used to treat a wide variety of malignancies, including leukemia and lymphoma and prevents tumour cell replication through alteration of cytoskeletal structure and disorientation of microtubules[1]. Clinical application of vincristine is often associated with dose-dependent painful neuropathy due to damages to the

peripheral axons. Painful peripheral neuropathy is the major dose limiting side effect of vincristine and requires discontinuation of treatment, greatly impacting on the survival of cancer patients[2]. Experimental model of peripheral neuropathic pain induced by vincristine has been established in rodents using different systemic dosing schedules of vincristine[1,2]. This model provides consistent and long-lasting neuropathic pain states mimicking vincristine-induced pain conditions in human patients. Therefore, this model is a valuable for studying the mechanisms and pharmacology of vincristine-induced neuropathic pain[3, 4]. Vincristine-induced peripheral neuropathy is characterized by dys-

thesia (abnormal and unpleasant sensation), hyperalgesia (an increased response to painful stimuli) and allodynia (pain in response to a stimulus that does not normally provoke pain) [5].

The analgesic and antineuralgic agents such as tricyclic antidepressants (i.e. amitriptyline, nortriptyline and imipramine), anticonvulsants (i.e. phenytoin, carbamazepine, gabapentin, lamotrigine and topiramate) and opioids have been reported to produce antiallodynic effects in neuropathic pain[6]. However, these drugs are associated with a wide spectrum of adverse effects which limit their full clinical exploitation in amelioration of the neuropathic pain[7].

Numerous herbal medicines (e.g. Cannabis sativa, Ginkgo biloba, Ocimum sanctum, Aconiti tuber, Phyllanthus emblica and Nigella sativa) have been reported for the management of various experimental models of neuropathic pain[8, 9]. Clinical reports have also claimed beneficial effects of herbal medicines in neuropathic pain conditions[10]. Therefore, novel research urges to explore the newer natural medicine in the management of neuropathic pain.

The green-lipped mussel (*Perna canaliculus*) is native to the New Zealand coast, and is a staple in the diet of the indigenous Maori culture (particularly those who live in coastal regions). GLM preparation has demonstrated strong anti-inflammatory properties and was shown to be as efficient as Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) for reducing inflammation in rats with carrageenan-induced footpad edema[11]. A lipid-rich extract of GLM was shown to prevent the development of adjuvant-induced polyarthritis and collagen-induced auto-allergic arthritis in rats[12]. Zawadzki et al. reported pain relief activity of *Perna canaliculus* Lipid Complex in osteoarthritis Patients[13]. GLM extract reduced osteoarthritis symptoms in patients with knee osteoarthritis[14].

Therefore, the present study was designed to investigate the potential neuroprotective & antioxidative effect of GLM extract as a pretreatment on peripheral neuropathy induced by vincristine.

Materials and Methods

Drugs and chemicals

Vincristine sulfate was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 5,5'-Dithio, bis (2-nitrobenzoic acid), bovine serum albumin and reduced glutathione (GSH) were purchased from S D Fine Chemicals Pvt. Ltd, Mumbai, India. Thiobarbituric acid was purchased from CDH (P) Ltd., New Delhi, India. All other reagents were used in analytical grade. Preparation of Green Lipped Mussel (GLM) Extract

The freeze dried green-lipped mussel powder used in the present study is produced from the entire mussel (minus the shell) and was supplied by Aroma New Zealand Ltd, Christchurch, New Zealand. GLM was extracted with 0.1% Tween-20 overnight & filtered[15].

Animals

Wistar albino rats, weighing 200-250gm, of either sex were

used in the study. The animals were housed under standard laboratory conditions with 12 hr light/dark cycle. Food consists of normal rat chow and water ad libitum. The study was approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) having Registration number and date of registration: 1099/c/07/CPCSEA, dated 27.07.2007. CPCSEA guidelines were followed for animal handling and treatment.

Induction of peripheral neuropathy by vincristine

Painful peripheral neuropathy was induced in rats by the administration of vincristine sulfate (50µg/kg; intraperitoneally (IP) once a day) for 10 consecutive days as described by Siau and Bennett[16].

Experimental protocol

Six groups, each group comprising of six Wistar rats, were used in the present study.

- Group I (Normal control group): Rats were administered with normal saline (1ml/kg 0.9% w/v of sodium chloride; i.p. once a day) for 14 days.
- Group II (Vincristine group): Rats were administered with vincristine (50µg/kg; i.p. once a day) for 14 consecutive days.
- Group III (GLM 50 + VC): Rats were administered with GLM extract (50mg/kg; p.o.) 1 h before each vincristine injection, for 14 days.
- Group IV (GLM 100 + VC): Rats were administered with GLM extract (100mg/kg; p.o.) 1 h before each vincristine injection, for 14 days.
- Groups V (Gabapentin 30 + VC): Rats were administered with gabapentin (30mg/kg; i.p.) 1 h before each vincristine injection, for 14 days.
- Groups VI (GLM per se group): Rats were administered with GLM extract (100mg/kg; p.o.), for 14 days.

Behavioral assessment

Hot plate test: Thermal hyperalgesic pain sensitivity of the rat hind paw was assessed using Eddy's hot plate as described by Eddy et al.[17], with slight modifications, for assessing the degree of noxious thermal sensation. The rats were placed on the top of a controlled preheated ($52.5 \pm 0.5^\circ\text{C}$ for hyperalgesia; $45 \pm 0.5^\circ\text{C}$ for allodynia) and maintained hot plate surface, allowing access to the left hind paw withdrawal response to degree of the nociceptive threshold. The cut-off times of 20 s for hyperalgesia and 30 s for allodynia were maintained.

Acetone drop test: Chemical cold allodynic pain sensitivity of the rat hind paw was assessed using acetone drop application as described by Choi et al.[18], with slight modifications, for assessing the reactivity to non-noxious cold chemical stimuli. The rats were placed on the top of a wire mesh grid, allowing access to the hind paws. Acetone (0.1ml) was sprayed on the plantar surface of left hind paw of rat. Cold chemical sensitive reaction with respect to either paw licking, shaking or rubbing the left hind paw was observed and recorded as paw withdrawal threshold. The cut-off time of 20 sec was maintained.

Paw pressure test: Mechanical hyperalgesic pain sensitivity of the rat hind paw was assessed by raising paw pressure stimulation using Randall–Selitto test apparatus as described by Randall and Selitto[19]. Briefly, mechanical nociceptive threshold, expressed in grams, was measured by applying increasing pressure to the left hind paw. Withdrawal of left hind paw was used to assess the mechanical nociceptive threshold. The cut-off pressure of 450gm was maintained.

Von Frey hair test: Mechanical allodynic pain sensation of the rat hind paw was assessed by raising mild touch or pressure stimuli using calibrated Von Frey hair (nylon) filaments as described by Chaplan et al.[20]. Briefly, calibrated nylon filaments, in terms of different bending forces, were applied to the mid plantar surface of left hind paw. The filaments were applied 10 times, starting with the softest and continuing in ascending order of stiffness. A brisk withdrawal of the left hind limb was considered as a positive response. The criterion for the threshold value, in grams, was equal to the filament evoking a withdrawal of the paw 5 times out of 10 trials, that is, 50% response. The cut-off pressure of 30gm was maintained.

Tail immersion test: Spinal thermal hyperalgesic pain sensitivity of the rat tail was assessed by raising heat stimuli with warm water bath as described by Necker and Hellon[21]. Briefly, the terminal part of the tail (1cm) of the rat was immersed in heat noxious temperature ($52 \pm 0.5^\circ\text{C}$), until the tail was withdrawn. The duration of the tail withdrawal reflex was used to assess the thermal heat hyperalgesia. The cut-off time of 10 sec was maintained.

Motor co-ordination test: Motor co-ordination (Muscle grip strength) was evaluated by a rota-rod device. Rats were placed for one minute on the rotating rod (25rpm). The time taken for the falling off from the roller, during five minutes period was recorded[22].

Biochemical estimation: At the end of the study protocol (on 21st day), animals were killed by cervical dislocation and the sciatic nerve was immediately isolated from the body. The sciatic nerve homogenates (10%, w/v) were prepared with 0.1M Tris-HCl buffer (pH 7.4) and the supernatant of the homogenates was used to estimate thiobarbituric acid reactive substances (TBARSs), reduced glutathione (GSH), catalase and total calcium content levels.

Estimation of lipid peroxidation: The levels of lipid peroxidation were estimated by measuring the TBARSs (i.e., malondialdehyde, MDA) concentration levels as described by Okhawa et al.[23]. The concentration of TBARS in tissue homogenate was expressed in terms of nano mole of MDA per milligram of protein. 1,1,3,3-Tetramethoxypropane (1–10nmol) was used as the standard.

Estimation of reduced glutathione (GSH): The level of GSH concentration was estimated as described by Ellman[24]. Equal quantity of sciatic nerve homogenate was mixed with 10% w/v of trichloroacetic acid (TCA) and centrifuged to separate proteins. To 10ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 500ml of 5, 5'-dithio, bis (2-nitrobenzoic acid) and 400ml double distilled water was added. Mixture was vortexed and the absorbance was taken at 412nm within 15 min.

The concentration of GSH was expressed as microgram per milligram of protein in sciatic nerve tissue.

Estimation of catalase: Catalase level was estimated in sciatic nerve tissue as described by Claiborne[25].

Estimation of total calcium content: The level of total calcium content was estimated in sciatic nerve tissue as described by Severinghaus and Ferrebee[26]. Briefly, sciatic nerve tissue homogenate was mixed with 1ml of trichloroacetic acid (4%) in ice cold conditions and centrifuged at 2000rpm for 10min. The clear supernatant was used for the estimation of total calcium ion by atomic emission spectroscopy at 556nm.

Statistical analysis: All the results were expressed as mean \pm SEM. The data from the behavioral & biochemical results were statistically analyzed by using Graph pad prism Version-5.0 software. Groups of data were compared with the analysis of variance (ANOVA) followed by Dunnett's test. Values were considered statistically significant when $p < 0.01$.

Results

Effect of GLM extract on hot plate test (i.e. paw heat hyperalgesia)

Vincristine administration resulted in significant ($p < 0.01$) development of noxious thermal hyperalgesia, by the decrease in hind paw withdrawal threshold, as compared with normal control group. Administration of GLM extract (50 and 100mg/kg; p.o.) significantly attenuated vincristine-induced decrease in the nociceptive threshold for thermal hyperalgesia in a dose dependent manner. Treatment of gabapentin (30mg/kg, i.p.) also produced similar effects. Furthermore, GLM per se (100mg/kg, p.o.) did not show any significant effect on heat hyperalgesic test as compared to the normal control group (Figure 1).

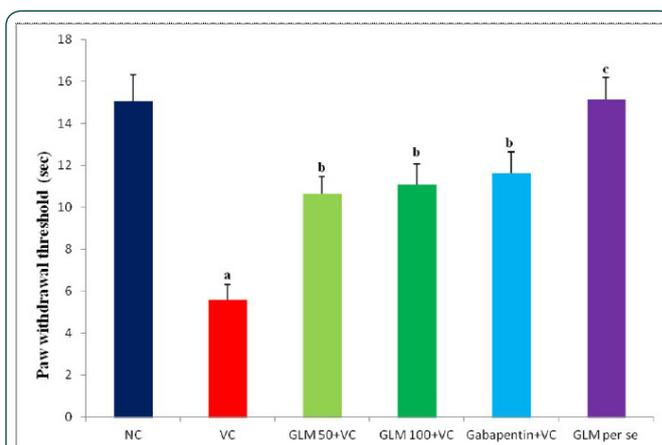
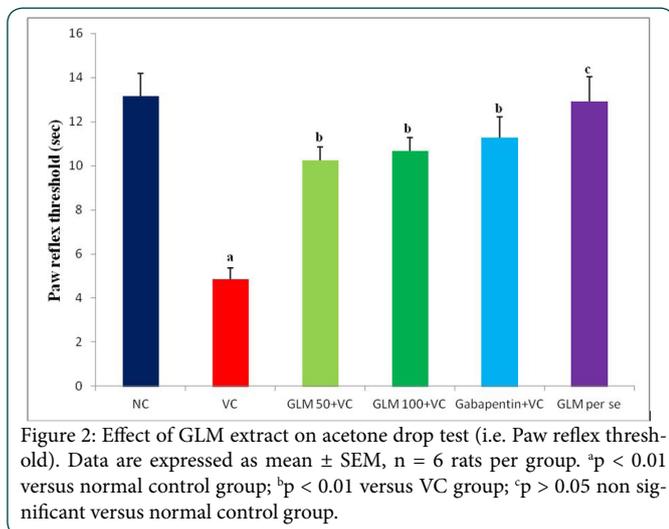


Figure 1: Effect of GLM extract on hot plate test (i.e. Paw withdrawal threshold). Data are expressed as mean \pm SEM, n = 6 rats per group. * $p < 0.01$ versus normal control group; ^b $p < 0.01$ versus VC group; ^c $p > 0.05$ non significant versus normal control group.

Effect of GLM extract on acetone drop test (i.e. paw cold allodynia)

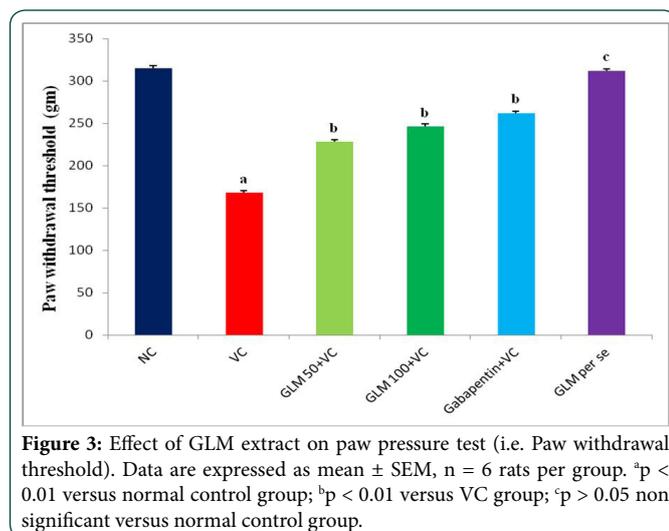
Vincristine administration resulted in significant ($p < 0.01$) development of non-noxious cold chemical allodynia, by the decrease in the left hind paw withdrawal threshold as compared with normal control group. Administration of GLM extract

(50 and 100mg/kg; p.o.) significantly attenuated vincristine-induced decrease in the nociceptive threshold for thermal allodynia in a dose-dependent manner. Treatment of gabapentin (30mg/kg, i.p.) also produced similar effects. Furthermore, GLM per se (100mg/kg, p.o.) did not show any significant effect on cold chemical allodynic test as compared to the normal control group (Figure 2).



Effect of GLM extract on paw pressure test (i.e. paw mechanical hyperalgesia)

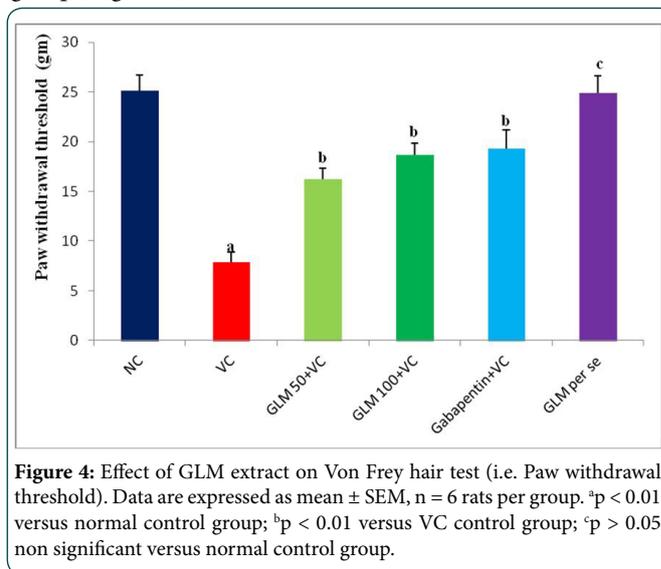
Administration of vincristine resulted in significant (p<0.01) mechanical hyperalgesia by the decrease in the left hind paw withdrawal threshold as compared with normal control group. GLM extract (50 and 100mg/kg; p.o.) administration significantly attenuated vincristine-induced decrease in the nociceptive threshold for mechanical hyperalgesia in a dose-dependent manner. Treatment with gabapentin (30mg/kg, i.p.) also produced similar effects. Furthermore, GLM per se (100mg/kg, p.o.) did not show any significant effect on mechanical hyperalgesic test as compared to the normal control group (Figure 3).



Effect of GLM extract on Von Frey hair test (i.e. paw mechanical allodynia)

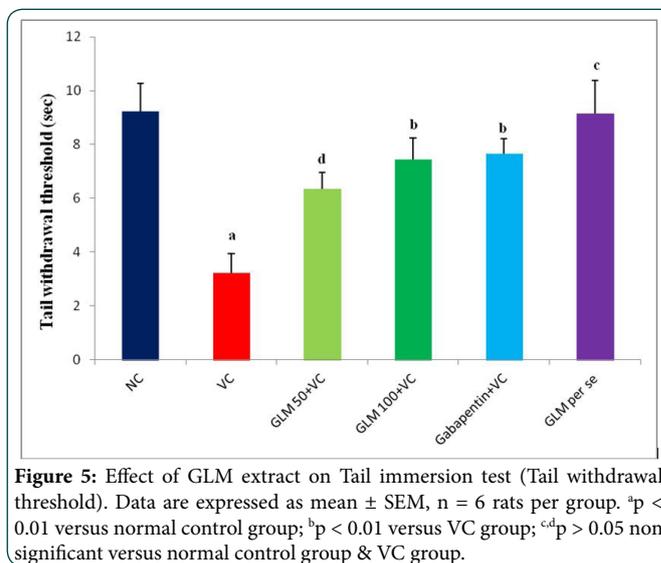
Administration of vincristine resulted in significant non-nox-

ious tactile mechanical allodynia, noted by the decrease in the left hind paw withdrawal threshold as compared with normal control group. GLM extract (50 and 100mg/kg; p.o.) administration significantly attenuated vincristine-induced decrease in the nociceptive threshold for mechanical allodynia in a dose-dependent manner. Treatment with gabapentin (30mg/kg, i.p.) also produced similar effects. Furthermore, GLM per se (100mg/kg, p.o.) did not show any significant effect on mechanical allodynic test as compared to the normal control group (Figure 4).



Effect of GLM extract on Tail immersion test (i.e. tail heat hyperalgesia)

Administration of vincristine resulted in significant (p<0.01) thermal hyperalgesia by the decrease in the tail withdrawal threshold as compared with normal control group. GLM extract (50 and 100mg/kg; p.o.) administration significantly attenuated vincristine-induced decrease in the nociceptive threshold for mechanical hyperalgesia in a dose dependent manner. Treatment with gabapentin (30mg/kg, i.p.) also produced similar effects. Furthermore, GLM per se (100mg/kg, p.o.) did not show any significant effect on tail heat hyperalgesic test as compared to the normal control group (Figure 5).



Effect of GLM extract on muscle grip strength

Administration of vincristine to Wistar rats resulted in decreased muscle grip strength noted by the decrease in the fall off time from rota rod as compared to the normal control group. While mussel extract (50mg/kg & 100mg/kg) as well as gabapentine significantly ($P < 0.01$) attenuated vincristine-induced decrease in fall off time. However, GLM per se 100mg/kg, p.o.) did not produce any significant change in fall off time as compared to the normal control group (Figure 6).

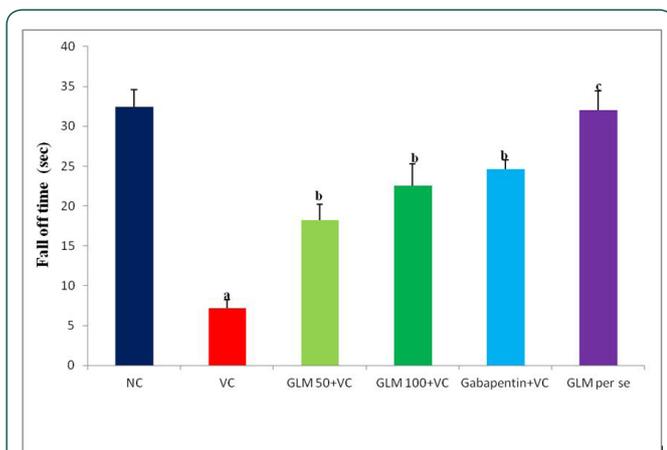


Figure 6: Effect of GLM extract on muscle grip strength (i.e. Fall off time). Data are expressed as mean \pm SEM, $n = 6$ rats per group. ^a $p < 0.01$ versus normal control group; ^b $p < 0.01$ versus VC group; ^c $p > 0.05$ non significant versus normal control group.

Effect of GLM extracts on tissue oxidative stress biomarkers

Administration of vincristine resulted in significant ($p < 0.01$) increase in TBARS, total calcium levels and decreased in the levels of reduced GSH & catalase as compared to the normal control group. GLM extract (50 and 100mg/kg; p.o.) administration significantly reduced vincristine-induced increase in sciatic nerve tissue MDA, total calcium and decrease in reduced GSH & catalase levels in a dose-dependent manner. Treatment with gabapentin (30mg/kg, i.p.) also produced similar effects. Furthermore, GLM per se (100mg/kg, p.o.) did not show any significant effect on oxidative stress markers (Table 1).

Groups	TBARS (nmols MDA/mg of protein)	GSH (mg/mg of protein)	CAT (Units/mg of protein)	Total calcium (ppm/mg of protein)
Normal control group	5.97 \pm 0.26	73.25 \pm 2.14	0.194 \pm 0.002	2.98 \pm 0.25
Vincristine group	8.28 \pm 0.24 ^a	45.93 \pm 2.92 ^a	0.111 \pm 0.003 ^a	22.72 \pm 0.41 ^a
GLM extract (50 mg/kg; p.o.) + Vincristine group	6.86 \pm 0.16 ^b	54.91 \pm 1.48 ^b	0.156 \pm 0.007 ^b	11.64 \pm 0.27 ^b
GLM extract (100 mg/kg; p.o.) + Vincristine group	6.58 \pm 0.14 ^b	64.85 \pm 1.78 ^b	0.178 \pm 0.008 ^b	8.06 \pm 0.38 ^b
Gabapentin+ Vincristine group	6.46 \pm 0.19 ^b	65.93 \pm 1.76 ^b	0.187 \pm 0.015 ^b	7.26 \pm 0.42 ^b
Per se (GLM, 100 mg/kg; p.o.) group	6.01 \pm 0.28 ^c	73.48 \pm 1.45 ^c	0.183 \pm 0.009 ^c	3.05 \pm 0.54 ^c

Table 1: Data were expressed as mean \pm S.E.M., $n = 6$ rats per group ^a $p < 0.01$ versus normal control group, ^b $p < 0.01$ versus Vincristine control group. ^c $p > 0.05$ non-significant versus normal control group.

Discussion

Vincristine common chemotherapeutic agent has been widely used for the management of various life-threatening cancer disorders including Hodgkin's disease. However, the clinical application of vincristine has been limited due to unavoidable painful 'dying-back' neuropathy. It has the property of high binding affinity towards β -tubulin of microtubules of peripheral nervous system. Microtubules are key components of the cytoskeleton and are composed of heterodimers of α -tubulin and β -tubulin, which assemble into linear, hollow, cytoplasmic filaments[27].

Vincristine has been established to cause disruption of microtubule polymerizations leading to destabilize microtubules, block proliferation by cell cycle arrest and cause cell death via the induction of apoptosis for its chemotherapeutic as well as neurotoxic actions[28].

Furthermore, Vincristine is also responsible to alter the cellular Ca^{2+} and free radical levels[29], which play a key role in the pathogenesis of painful neuropathy associated with vincristine. Free radical generation induced by calcium has been implicated to potential neuronal injury[30, 31]. Calcium accumulation has been triggered as a self-destructive cascade via calcium-binding protein such as calmodulin and calpain leading to neuronal hyper excitability, adenosine triphosphate depletion, free radical generation and activation of cytosolic phospholipases and proteases[30, 32]. Calcium-induced activation of calpains has also been reported to degrade axonal cytoskeleton resulting in the axonal degeneration via β -tubulin polymerization[33].

In the present study, vincristine (50 μ g/kg; i.p., once a day, for 10 consecutive days) administration produced significantly ($p < 0.01$) peripheral neuropathic pain in Wistar rats. The results of the present study are similar with the earlier studies[34]. Whereas administration of GLM extract significantly ($p < 0.01$) attenuated vincristine-induced behavioural (i.e. paw and tail heat hyperalgesia, cold allodynia, mechanical hyperalgesia and mechanical allodynia) & muscle grip strength.

In the present study, vincristine treatment resulted in the rise in the levels of TBARS (an index of lipid peroxidation) and total calcium and fall in the reduced GSH & catalase (an endog-

enous antioxidant molecule), thus supporting the contention that free radicals may contribute in pathogenesis of neuropathy. These observations are similar with the earlier findings[30,34]. Free radicals are established to induce potential tissue damage and painful neuropathy[1,4]. Moreover, calcium accumulation and free radical generations have been reported as a major culprit event in different types of neuropathic pain disorders like posttraumatic, anti-HIV drugs, tibial sural transection, chronic constriction injury, ischemic-reperfusion injury and vincristine-induced neuropathy[30].

The administration of GLM extract significantly attenuated the vincristine-induced alterations of peripheral and central behavioural and oxidative stress marker changes. Neuroprotective effect of GLM extract is due to its anti-inflammatory action. GLM extract has anti-inflammatory activity[35]. Mussel extract is a rich source of Eicosatetraenoic Acid (ETA), α -Linolenic Acid (ALA), Eicosapentanoic Acid (EPA), Docosahexaenoic Acid (DHA) and chondroitin sulphate. ETA is Omega-3 fatty acid and responsible for the anti inflammatory action of GLM extract. This anti-inflammatory effect has been attributed to several factors, one of which is a reduction in the biosynthesis of the proinflammatory prostaglandins[36]. The antioxidant effect of GLM extract may be due to its free radical scavenging and calcium channel modulatory actions.

However, data of the present study is still insufficient to elaborate the precise mechanism of GLM extract in neuropathic pain. Nevertheless, further studies are needed to substantiate these findings.

Conclusion

In conclusion, GLM extract has a neuroprotective, anti-inflammatory, antioxidative and calcium channel inactivating potential on vincristine induced peripheral neuropathy in Wistar rats. Therefore, GLM extract could be an alternative approach for the management of neuropathic pain. However, similar studies on various animal models are required to obtain a reliable oversight of the effect of GLM extract on vincristine-induced neuropathic pain in a clinical setting.

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Conflict of interest

The authors declared no conflicts of interest.

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