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D-Limonene ameliorates diabetic neuropathic pain in rats

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Abstract
The present study aimed to explore the role of d-limonene in the management of diabetic neuropathy. Adult Wistar rats (either sex, 200-250 g) were divided into 6 groups (n=6). Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of streptozotocin at a dose of 50 mg/kg body weight freshly dissolved in 0.1 mol/L citrate buffer, pH 4.4. D-limonene was administered in 2 doses (100 and 200 mg/kg; p.o.) to separate groups of rats for 35 successive days daily. Gabapentin (100 mg/kg; i.p) served as standard drug. The neuropathic activity was evaluated in streptozotocin induced diabetic neuropathic rats using Cold and hot water immersion test and tail flick test. After wards, the animals were sacrificed; sciatic nerve is isolated, homogenized and centrifuged for TBARS, GSH, nitrite, catalase and protein estimations. STZ induced diabetic neuropathy caused the decrease in body weight, decrease in tail-flick latency time in radiant heat apparatus, decrease tail-withdrawal latency in tail-immersion (warm water) test, decrease allodynic response in tail-immersion (cold water) test. Furthermore STZ induced diabetic neuropathic rats expressed higher sciatic nerve TBARS and nitrite levels and lower sciatic nerve GSH and catalase levels. D-limonene significantly attenuated (p<0.05) the behavioral and biochemical alterations produced by STZ induced diabetic neuropathy in rats. Catalase, GSH and total protein levels were restored (p< 0.05) in the d-limonene treated animals. D-limonene treated rats showed a significant (p< 0.05) decrease in nitrite and TBARS level. In conclusion, d-limonene may prove to be useful remedy for the management of diabetic neuropathy owing to its possible antioxidant properties.

Keywords: D-Limonene, Streptozotocin, neuropathy, monocyclic monoterpene

Introduction

The word ‘diabetes’ is derived from the Greek word “Diab” (meaning to pass through, referring to cycle of heavy thirst and frequent urination); ‘mellitus’ is the Latin word for “sweetened with honey” (refers to the presence of sugar in the urine) [1]. Diabetes mellitus (DM) is a group of metabolic disorder characterized by a chronic hyperglycemic condition resulting from defects in insulin secretion, insulin action or both. Permanent neonatal diabetes is caused by glucokinase deficiency, and is an inborn error of the glucose-insulin signaling pathway [2]. The prevalence of diabetes is increasing rapidly worldwide and the World Health Organization (2003) has predicted that by 2030 the number of adults with diabetes would have almost doubled worldwide, from 177 million in 2000 to 370 million. Experts project that the incidence of diabetes is set to soar by 64% by 2025, meaning that a staggering 53.1 million citizens will be affected by the disease [3]. The estimated worldwide prevalence of diabetes among adults in 2010 was 285 million (6.4%) and this value is predicted to rise to around 439 million (7.7%) by 2030 [4]. Type 2 diabetes mellitus is the most prevalent form and accounts for 90-95% of these cases. Importantly, 35% of the adult population is estimated to have prediabetes or metabolic syndrome, a condition with higher than normal blood glucose and impaired insulin sensitivity that has yet to reach diagnostic criteria for diabetes mellitus [5]. Although type 1 diabetes mellitus accounts for a far smaller percentage, 5-10% of cases, the incidence has been steadily rising in the past decades to nearly 5% annually in the United States. Hence, both type 1 and type 2 diabetes mellitus remain growing problems throughout the world [6].

Despite the differences in etiology, clinical presentation, and disease prevalence, secondary complications, such as heart disease, stroke, retinopathy, nephropathy, and neuropathy, occur in both type 1 and 2 diabetes mellitus. The long-term manifestation of diabetes can result in the development of some complications, broadly classified as microvascular or macrovascular disease. Microvascular complications include neuropathy (nerve damage), nephropathy (renal disease) and vision disorders (retinopathy, glaucoma, cataract and corneal diseases), while macrovascular complications include heart disease, stroke and peripheral vascular disease, which can lead to ulcers, gangrene and amputation [7]. Diabetic neuropathy (DNP) is the most common complication of diabetes mellitus (DM), which occurs in more than 50% of patients and affects nerve fibers of peripheral nervous system. The patients often present with loss of feeling and numbness in their feet, hands, and legs, which may be accompanied by excessive sensitivity to nociceptive stimuli or may perceive normal stimuli as painful [8, 9]. To date, numerous mechanisms have been proposed to explain the relationship between the severity of hyperglycemia and the development of DNP including increased polyol pathway activity which leads to accumulation of sorbitol and fructose, reduction in Na^+K^+–ATPase activity, abnormal protein kinase C (PKC) activity, formation of advanced glycation end-products and auto-oxidation of glucose leading to the generation of reactive oxygen species [9-11]. In addition, metabolic dysfunction is accompanied by

vascular deficiency and nerve hypoxia, which may contribute to nerve fiber loss and injury in diabetes. Based on these studies, various therapeutic agents including aldose reductase inhibitors (ARIs), anti-oxidants, selective PKC inhibitors, and neurotrophic factors have been used to improve peripheral nerve dysfunction in diabetic animals and patients [10].

D-limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a monocyclic monoterpene that is mainly present in the citrus essential oils with lemon-like odors [12]. d-limonene is listed in the Code of Federal Regulations as generally recognized as safe (GRAS) as a flavouring agent and is used in common foods such as fruit juices, soft drinks, baked foods, ice-cream and desserts. In humans, d-limonene has demonstrated low toxicity after repeated dosing for upto1 year[12]. It is found in essential oils of many plants including lemons, oranges, grapefruit, caraway, dill, bergamot, peppermint, spearmint, grasses and tomatoes [13]. D-limonene is reported to have a number of pharmacological effects including antioxidant [14], chemopreventive [15], anti-carcinogenic properties [16], anti-hyperglycemic [17], hypolipidemic [18], anti- inflammatory [19] and antimicrobial efficacies [20]. However, no sufficient studies have been carried out to explore the role of d-limonene as in the treatment of diabetic neuropathy, to best of our knowledge. Therefore, the present study aims at investigating the potential of monocyclic monoterpene d-limonene in experimental diabetic neuropathy in rats.

Materials and methods

Adult Wistar rats (either sex), weighing between 200-250 g, were procured from the CPCSEA registered approved breeder. The animals were kept in quarantine section till monitoring of health status of animals and subsequently transferred to the housing area. Animals were housed in polypropylene cages with dust free rice husk as a bedding material and maintained under standard laboratory conditions with controlled temperature (23 ± 2°C), humidity (40 ± 10%) and natural (12 h each) light-dark cycle. The animals were fed with standard rodent pellet diet (Ashirwad Industries, Mohali) and water *ad libitum.* The experiment was carried out between 09:00 and 18:00 h. The care of laboratory animals was done following the guidelines of CPCSEA, Ministry of Forests & Environment, Government of India.

Drugs and chemicals

D-limonene was procured from Himedia. Streptozotocin was purchased from SRL, Mumbai. Gabapentin was purchased from Alaina Pharma Company. DTNB, thiobarbituric acid purchased was from Himedia laboratories, Mumbai. Reduced glutathione, 5, 5′-dithiobis (2-nitrobenzoic acid), bovine serum albumin, tris-buffer, sucrose, trichloroacetic acid, citric acid monohydrate, sodium nitrate, copper sulfate, sodium potassium tartarate, tri-sodium citrate, sodium hydroxide procured from Hi-media.

Induction of diabetic neuropathy

Diabetes was induced in overnight fasted rats by intraperitoneal injection of streptozotocin (SRL, Mumbai) at a dose of 50 mg/kg body weight freshly dissolved in 0.1 mol/L citrate buffer, pH 4.4. The plasma glucose was measured after 4 days of streptozotocin injection, the blood samples were collected via retro-orbital plexus technique using heparinized capillary glass tubes. Animals showing plasma glucose more than 250 mg/dl were included in the study and were maintained for 7 weeks (49 days) for induction of diabetic neuropathy studies. Control rats received equal volume of citrate buffer [21].

Experimental design

Preventive treatment was started 14 days after the streptozotocin injection with d-limonene (100 and 200 mg/kg; p.o. for 5 weeks). The doses of d- limonene were selected on the basis of literature reports and terminated at 49th day (7th week) [21, 22].

Group 1- (Normal Control): Rats were handled gently without any stress for 14 days. After that on 15th day saline was given orally to rats for 35 successive days. On 49th day behavioural studies were carried out.

Group 2- (DL200 per se): Rats were handled gently without any stress for 2 weeks (14 days). D-limonene (200 mg/kg; p.o.) was started at 3rd week (15th day) upto 7th week (49th day). On 49th day behavioural studies were carried out.

Group 3- (STZ Control): Streptozotocin (50 mg/kg; i.p) dissolved in citrate buffer pH 4.4, was administered. After 2 weeks, saline was given daily to rats for 35 days. On 49th day behavioural studies were carried out.

Group 4- (STZ + DL100): Streptozotocin (50 mg/kg; i.p) was administered to rats. D-limonene (100 mg/kg; p.o.) was started at 3rd week (15th day) of STZ administration upto 7th week (49th day). On 49th day behavioural studies were carried out.

Group 5- (STZ + DL200): Streptozotocin (50 mg/kg; i.p) was administered to rats. D-limonene (200 mg/kg; p.o.) was started at 3rd week (15th day) of STZ administration upto 7th week (49th day). On 49th day behavioural studies were carried out.

Group 6- (STZ + Gaba): Streptozotocin (50 mg/kg; i.p) was administered to rats. Gabapentin (100 mg/kg) was injected i.p. daily to rats for 35 successive days started from 3rd week (15th day) of STZ administration upto 7th week (49th day). On 49th day behavioural studies were carried out.

On 49th day, the animals were subjected to tail-flick test [23], tail-immersion (warm water) test [24], and tail-immersion (cold water) test [25]. The animals were sacrificed by decapitation on 49th day sciatic nerve was isolated for estimating TBARS [26]. GSH [27]. Catalase [28] and nitrite levels [29].

Estimation of blood glucose level

Blood was collected from retro-orbital of rats for the determination of the blood glucose levels. After the sample collection the blood glucose was determined by Accu-Check Active Strips.

Tail flick test

Acute nociception was induced by tail flick apparatus. Briefly, each rat placed in a restrainer and the tail flick latency was determined by placing the tail of rats near to red hot wire and the time taken to remove the tail from the noxious thermal stimulus. Cut off time was kept at 10-12 sec for each animal, 2 to 3 recordings were made at an interval of 15 min; the mean value was used for statistical analysis [23].

Tail-immersion (warm water) test

The animals were trained for 3 days prior to test. Rat tail was immersed in hot (52 ± 0.5ºC) water and the tail flick response latency (withdrawal response) or any signs of struggle were observed as the end point response. Cut off time was kept at 15 sec. The shortening time of withdrawal of tail indicates hyperalgesia. The test was repeated 3 times within 30 min and mean is taken as final response [24].

Tail-immersion (cold water) test

Rat tail was immersed in cold (10 ± 0.5ºc) water and the tail flick response latency (withdrawal response) or any signs of struggle were observed as the end point response. Cut off time was kept at 15 sec. The shortening of tail withdrawal time indicates hyperalgesia. The test was repeated 3 times within 30 min and mean is taken as final response [25].

Statistical analysis

All the results are expressed as Mean ± SEM. The data of all the groups were analyzed by one-way ANOVA followed by Tukey's test using software Graph Pad Prism 6 (Graph Pad Software Inc., USA). A value of P<0.05 was considered to be significant.

Results

Effect of d-limonene on Blood Glucose level

Blood glucose level was significantly (p< 0.05) high in 7 weeks diabetic rats (STZ control) as compared to normal control. Administration of DL100 and DL200 for 35 days significantly (p< 0.05) decreases the plasma glucose level of diabetic rats. DL when administered at a dose of 200 mg/kg; p.o. to normal rats showed no statistical significant effect on blood glucose level. (Table no.1)

Table 1: Effect of d-limonene on Blood Glucose level of rats

Groups	Treatment	Conc. (mg/dL)
Normal Control	Vehicle treated	83.166 ± 9.537
DL200 per se	200 mg/kg; p.o. for 35 days	88.333 ± 1.966
STZ Control	50 mg/kg; i.p.	492.33 ± 11.201[a]
STZ + DL100	50 mg/kg; i.p. + 100 mg/kg; p.o. for 35 days	419 ± 19.718[b]
STZ + DL200	50 mg/kg; i.p. + 200 mg/kg; p.o. for 35 days	459 ± 17.0293[b]
STZ + Gaba	50 mg/kg; i.p. + 100 mg/kg; i.p. for 35 days	509.8333 ± 15.393[b]

Effect of d-limonene on Tail flick latency in rats

Administration of STZ caused the induction of diabetic neuropathy which resulted in reduction in nociceptive threshold when compared to normal control group (p< 0.05). This deficit in tail flick response latency was significantly (p<0.05) reversed on treatment with DL100 and DL200 for 35 days (started 15 days after STZ injection) as compared to STZ control group. The administration of gabapentin significantly lessened the STZ induced nociceptive threshold. However d-limonene when administered at a dose of 200 mg/kg; p.o. to normal rats showed no statistical significant effect on tail flick latency. (Figure 1)

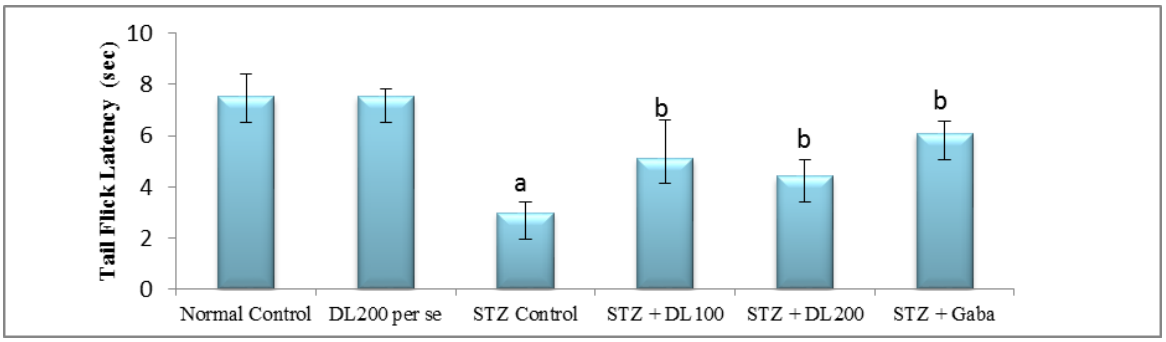

Fig 1: Effect of d-limonene on Radiant Tail flick latency in rats

Values are represented as mean ± S.E.M. [a] denotes p< 0.05 compared to Normal Control and STZ control; [b] denotes p< 0.05 compared to STZ Control (one way ANOVA followed by Tukey's test).

Effect of d-limonene on Tail-immersion (warm water) test in rats

STZ induced diabetic neuropathy resulted in significant development of hot allodynia after 49 days as compared to normal control group in a significant manner (p< 0.05). DL100 and DL200 treatment significantly (p< 0.05) increased the tail-withdrawal latency as compared to STZ control group. The administration of gabapentin significantly lessened the development of STZ induced Hot Allodynia. However d-limonene when administered at a dose of 200 mg/kg; p.o. to normal rats showed no statistical significant effect on tail-withdrawal latency. (Figure 2).

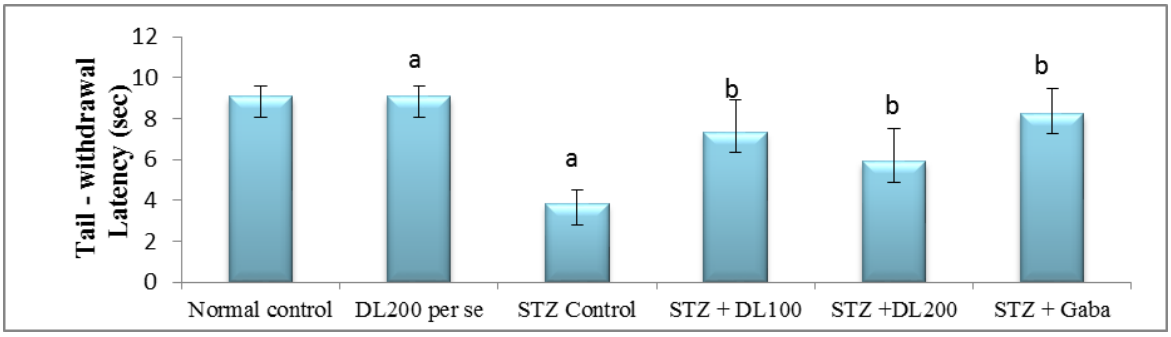

Fig 2: Effect of d-limonene on Tail-immersion (warm water) test in rats

Values are represented as mean ± S.E.M. [a] denotes p< 0.05 compared to Normal Control and STZ control; [b] denotes p< 0.05 compared to STZ Control (one way ANOVA followed by Tukey's test).

Effect of d-limonene on Tail-immersion (cold water) test on rats

STZ administration to rats resulted in development (p< 0.05) of cold allodynia (decrease the allodynic response) as compared to control group after. DL100 and DL200 administration for 35 days (started 15 days after STZ injection) significantly (p<0.05) attenuated the development of cold allodynia as compared to STZ control group. The administration of gabapentin significantly lessened the STZ induced cold allodynia. However d-limonene when administered at a dose of 200 mg/kg; p.o. to normal rats showed no statistical significant effect. (Figure 3)

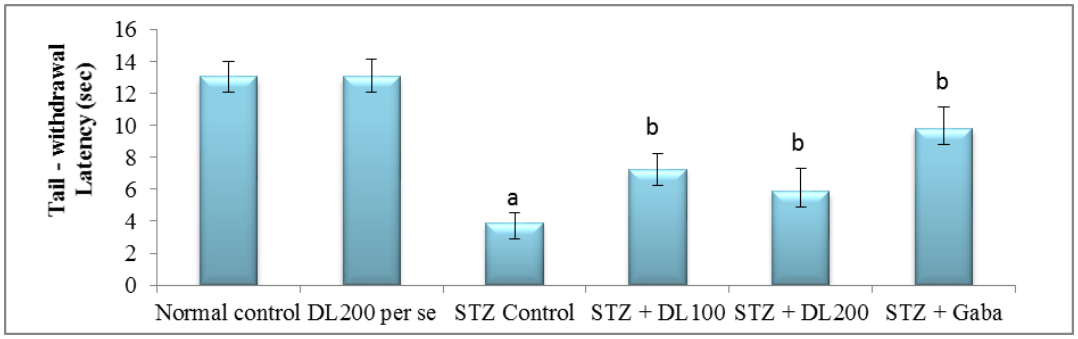

Fig 3: Effect of d-limonene on Tail-immersion (cold water) test in rats

Values are represented as mean ± S.E.M. [a] denotes p< 0.05 compared to Normal Control and STZ control; [b] denotes p< 0.05 compared to STZ Control (one way ANOVA followed by Tukey's test).

Effect of d-limonene on Loco-motor Activity in rats

STZ administration in rats resulted in significant decrease in loco-motor activity after 49 days as compared to normal control group (p< 0.05). However, both DL100 and DL200 treatments for 35 days (started 15 days after STZ injection) to STZ treated rats significantly (p< 0.05) improves the reduction in loco-motor activity when compared to STZ control group. The administration of gabapentin significantly improves STZ induced decrease in loco-motor activity. However d-limonene when administered at a dose of 200 mg/kg; p.o. to normal rats showed no statistical significant effect on actophotometer scoring. (Figure 4)

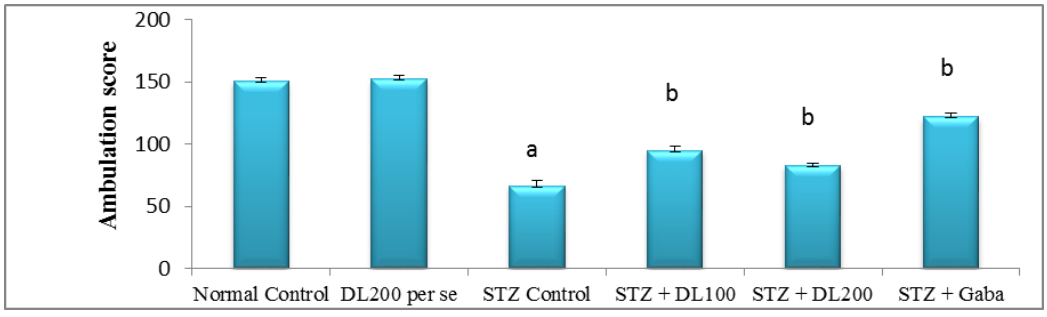

Fig 4: Effect of d-limonene on Loco-motor activity in rats

Values are represented as mean ± S.E.M. [a] denotes p< 0.05 compared to Normal Control and STZ control; [b] denotes p< 0.05 compared to STZ Control (one way ANOVA followed by Tukey's test).

Effect of d-limonene on sciatic nerve TBARS, Nitrite, GSH and Catalase levels

The induction of diabetic neuropathy in rats resulted in significantly ($p<0.05$) higher levels of TBARS and nitrite however lower level of GSH and catalase in sciatic nerve in 7 weeks diabetic rats (STZ control) as compared to normal control. Both DL100 and DL200 treatment for 35 days (started 15 days after STZ injection) significantly decreases ($p< 0.05$) the elevated level of TBARS and nitrite however increases the level of GSH and catalase as compared to STZ control group. However, administration of gabapentin for 35 days has significantly decreased ($p< 0.05$) the TBARS and nitrite level however increases the GSH level and catalase activity in STZ induced diabetic neuropathy.

Table 2: Effect of d-limonene on levels oxidative stress parameters

Treatment	TBARS (µM/ml)	GSH (µM/ml)	Nitrite (µM/ml)	Catalase (units/mg of protein
Normal Control	10.614 ± 1.325	95.798 ± 2.229	17.88 ± 2.453	0.7566 ± 0.0136
DL200 per se	9.843 ± 1.143	96.355 ± 2.281	16.13 ± 2.513	0.82 ± 0.01095[a]
STZ Control	29.13 ± 1.44[a]	40.64 ± 1.737[a]	29.71 ± 3.41[a]	0.351 ± 0.0231[a]
STZ + DL100	18.635 ± 2.670[b]	79.26 ± 1.493[b]	23.89 ± 3.02[b]	0.528 ±0.01169[b]
STZ + DL200	21.73 ± 2.025[b]	69.232 ±2.0713[b]	24.10 ± 1.76[b]	0.435 ±0.01643[b]
STZ + Gaba	12.166 ± 1.192[b]	80.49 ± 0.2578[b]	18.77 ± 0.29[b]	0.67 ± 0.0252[b]

Values are expressed as mean ± S.E.M by one way ANOVA followed by Tukey's Test.

Discussion

Diabetic neuropathy was successfully induced in Wistar rats, 49 days after administration of streptozotocin. These diabetic rats showed development of thermal and cold hyperalgesia as indicated by decrease in tail withdrawal latency, allodynic response and reduction in nociceptive threshold as compared to normal rats. This was accompanied by decrease in loco-motor activity and tail flick latency assessed by performance on actophotometer and radiant heat tail flick apparatus respectively in STZ control as compared to treatment group. These findings are in agreement with the previous studies which reported the induction of neuropathic pain by intraperitoneal administration of streptozotocin [21, 30]. The administration of DL100 and DL200 to rats for 35 days prevented the development of thermal and cold hyperalgesia. This is observable from reduced tail flick latency (in analgesiometer test), tail-withdrawal latency (in warm water and cold water tests) compared to the diabetic neuropathy control rats. In the present study, the diabetic rats showed increased oxidative damage in the sciatic nerve due to excessive hyperglycaemia which is indicated by higher TBARS, nitrite levels and lower reduced glutathione levels and catalase activity. Previous studies also elucidated a clear correlation between prolonged chronic hyperglycaemia and oxidative stress in diabetic rats [31, 32]. This enhanced oxidative stress causes lipid peroxidation during diabetes and endogenous defences rendered insufficient to neutralize the reactive oxidative species. The same has been observed in the present study as higher TBARS and nitrite levels indicates enhanced lipid peroxidation, lower GSH levels and catalase activity indicates limited endogenous antioxidant defence substances to arrest the oxidative damage. DL100 and DL200 administration for 35 days ameliorated the rise of TBARS and nitrite levels. Furthermore, DL treated groups expressed higher levels of GSH and catalase activity as compared to diabetic control rats. Previous studies have also demonstrated that the effect of d-limonene reduces the oxidative stress in STZ-induced diabetic rats by decreasing lipid peroxidation and sparing the activities of antioxidant enzymes [22]. Furthermore, it is also demonstrated that limonene protected the cells to the oxidative stress induced by exogenous addition of H_2O_2 [33].

The important roles played by oxidative stress in mediating diabetic neuropathy (DN) cannot be overemphasized and occupied the mainstream in the search for an efficient and efficacious treatment of nerve dysfunction in diabetes within the past decade [34] increasingly large number of antioxidants viz. shown vitamin A, C, and E, curcumin, α-lipoic acid, melatonin, acetyl-L-carnitine, and flavonoids have shown benefits in experimental animal models of DN [35-36]. At present, no antioxidant treatment has been approved by the United States Food and Drug Administration for DN although α-lipoic acid, which seems to be the leading antioxidant in clinical trials, has been approved in some European countries [37]. Generally, antioxidants work to achieve two main goals: reduce the harmful effects of free radicals either by preventing their formation or by scavenging and inactivating them or boost the natural defense systems by inducing the activities of antioxidant enzymes and regenerating other proteins involved in antioxidant pathways [37]. On the basis of above discussion, it may be concluded that d-limonene may have exert its effect in diabetic neuropathy rats by acting as free radical scavenger and/or reduces oxidative stress by strengthening the antioxidant defense system.

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