Available online at **www.joac.info**

ISSN: 2278-1862

Journal of Applicable Chemistry

2021, 10 (1): 62-69

(International Peer Reviewed Journal)

Effect of Tangeretin in STZ Induced Diabetic Neuropathic Pain Model in Rats

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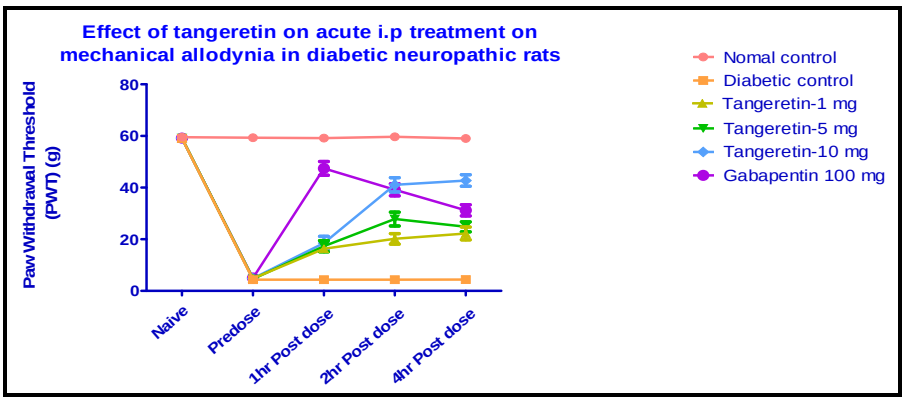
Accepted on 31th December, 2020

ABSTRACT

The objective was to evaluate efficacy of Tangeretin in streptozotocin induced neuropathic pain in rat model. Diabetes mellitus was induced by an injection of streptozotocin at a dose of 45 mg kg^{-1}, i.v. into tail vein of male albino Wistar rats. Tangeretin was dosed at 1, 5 and 10 mg kg^{-1} by intraperitoneal administration in diabetic neuropathic rats. Mechanical hyperalgesia and allodynia was measured using Randalle Selittoanalgesymeter and manual von Frey filaments of increasing weights respectively. Paw withdrawal threshold and percent Paw withdrawal threshold was determined with respect to both hyperalgesia and allodynia. Treatment of Tangeretin at three different levels of 1, 5 and 10 mg kg^{-1} had not significantly altered serum glucose levels throughout the treatment period. In hyperalgesia study, acute treatment with higher dose exhibited 19.93 % reversal of paw withdrawal threshold while with chronic treatment efficacy was raised to 51.56% reversal of Paw withdrawal threshold. In allodynia study, acute treatment reversed Paw withdrawal threshold by 66.69% while with chronic treatment, efficacy was raised to 80.63% reversal of Paw withdrawal threshold. Tangeretin demonstrated better efficacy in reversing mechanical allodynia than mechanical hyperalgesia. Tangeretin could be a good drug candidate for further studies to establish the mechanism of attenuation of neuropathic pain.

Graphical Abstract



Effect of Tangeretin on acute i.p treatment on mechanical allodynia in diabetic rats.

Keywords: Tangeretin, Hyperalgesia, Allodynia, Bioflavonoids, Gabapentin.

INTRODUCTION

Diabetes mellitus is a metabolic disorder manifested by hyper-glycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart and blood vessels [1]. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 [2]. Recently, World Health Organization (WHO) reportedly estimated 1.5 million deaths caused by diabetes [3]. Diabetes prevalence is alarmingly increasing every year with large proportion of patients suffering from neuropathic symptoms. Diabetic neuropathic pain states are really devastating to a patient's quality of life in the long run. There is a growing importance for the treatment of diabetic neuropathic pain for which there are no appropriate treatment strategies. At present, pharmacotherapy of neuropathic pain is largely limited to mainly "off-label" use of drugs approved for other conditions, especially tricyclic anti-depressants and anti-convulsants. Hence, study was planned to address the problem of diabetic neuropathic pain in animal models.

Bioflavonoids comprise a group of phenolic secondary plant metabolites that are widespread in nature. Among bio-flavonoids, isoflavones are still being extensively studied to indentify therapeutically active constituents in the area of neuropathic pain. Flavonoids like hesperidin have been demonstrated to be beneficial in experimental neuropathic pain [4].

Tangeretin is one of the bitterest citrus flavonoids with an O-polymethoxylated flavone, Tangeretin has been widely found in both the flavedo and albedo of different citrus fruits such as grapefruits, In plants, tangeretin acts as a defensive mechanism against pathogens. Tangeretin possesses a range of known therapeutic effects, including anti-hypercholesterolemic, anti-tumoric and anticarcinogenic activities, as well as neuroprotective effects [5, 6]. With remarkable beneficial evidence of flavones in inflammatory states, it is quite rational to explore possible efficacy of Tangeretin in animal models of neuropathic pain. Hence, our investigation was aimed to evaluate the efficacy of Tangeretin, in streptozotocin (STZ) induced neuropathic pain in rat models.

MATERIALS AND METHODS

Materials: STZ and Tangeretin were purchased from the Sigma Chem-ical Company (St Louis, USA). Thiopentone sodium was supplied by Abbott Lab Ltd (Ankleshwar, India). Gabapentin is the generous gift sample from Sun Pharmaceuticals Ltd, Andheri, and Mumbai. All other chemicals and reagents were used of analytical grade.

Animals: Male albino Wistar rats (Mahaveera Enterprises Pvt Ltd, Hyderabad, India) weighing 200-250 g were selected. Animals were maintained under standard laboratory conditions at 25 ± 2°C, relative humidity 50 ±15% and normal photoperiod (12 h dark/light). Commercial pellet diet (Rayon's Biotechnology Pvt Ltd, India) and water were provided ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee and animal experiments were carried out as per Animal Regulatory Body of the Government (Regd.1769/PO/E/S/14/CPCSEA).

Experimental design: The rats were randomly divided into five groups with six animals each.
Group 1, Normal control group treated with vehicle (5% DMSO+ 0% Tween-80+85% distilled water)
Group 2, Diabetic animals treated with Tangeretin (1 mg kg^{-1}, i.p.)
Group 3, Diabetic animals treated with Tangeretin (5 mg kg^{-1}, i.p.)
Group 4, Diabetic animals treated with Tangeretin (10 mg kg^{-1}, i.p.)
Group 5: Diabetic animals treated with Gabapentin (100 mg kg^{-1}, i.p).

Diabetes induction day was considered as Day 0. Tangeretin acute treatment was given as a single dose on Day 21 of STZ induction and pain parameters were assessed. Chronic treatment was given for seven days following acute treatment and again on 8th day of treatment, pain was assessed.

Induction of diabetes and neuropathic pain: Diabetes was induced by a single intravenous (i.v.) injection of STZ, 45 mg kg^{-1} of body weight, dissolved in citrate buffer (pH 4.5), into the tail vein of animals [7-9]. Diabetes was confirmed after the third day of STZ injection by estimating serum glucose using semi-automatic analyser (Model: Erba Chem 5 plus V2; Transasia Bo-medicals Ltd) and Erba Transasia glucose kit. Animals that developed serum glucose levels of more than 250 mg dL^{-1} were considered diabetic and included into the study. Following induction of diabetes mellitus, animals were allowed for 21 days for the development of neuropathic pain. On 22nd day, animals were evaluated for the development of symptoms of mechanical hyperalgesia and mechanical allodynia. Animals were considered to be neuropathic when the same exhibited mechanical allodynia (i.e., paw with-drawalor flinching behaviour response to the application of a bending force of less than 4 g) and mechanical hyperalgesia (i.e., paw withdrawal response was observed at a paw pressure of less than orequal to 70 g).

Determination of mechanical hyperalgesia: Mechanical nociceptive threshold or Paw withdrawal threshold (PWT), an index of mechanical hyperalgesia, was assessed by previously described method [10]. The paw withdrawal threshold was quantified using the RandalleSelitto paw pressure analge symeter (model, 37215; UGO Basile, Italy). Increasing pressure at a linear rate of 10 g s^{-1} was applied to the center of the hind paw. Pressure at which animal withdraws its paw was recorded and expressed in mass units (g), with a cutoff of 150 g to avoid potential tissue injury. PWT was recorded for the left hind paw before and up to 4 h after treatment. Percent reversal of PWT was determined using the formula: [(Post-dose threshold - Pre-dose)/(Naive threshold-pre-dose threshold)] X100.

Determination of mechanical allodynia: The rats were placed individually in plastic cages with a plastic mesh floor to determine withdrawal threshold. The animals were tested after acclimatization to the environment, typically 20-30 min after placement in the cage. The paw withdrawal threshold in response to mechanical stimulation was measured using the up and down method [11] by applying calibrated von Frey filaments (Aesthesio®; Ugo basile, Italy) to the hind paw from underneath the cage through openings in the mesh floor. A series of von Frey filaments (0.4, 0.7, 0.16, 0.40, 0.60, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10, 15, 26 and 60 g) were applied vertically to the plantar surface of the hind paw for 5 s while the hair was bent. Brisk withdrawal of paw or paw flinching was considered a positive response. The absence of a response in the animals at a pressure of 60 g was considered the cut off value. The stimulation with one filament was repeated five times at 10-15 s intervals, when lack of a response, the next filament with greater bending force was applied. The lowest force required to elicit a paw withdrawal response was recorded as the PWT (g). The animals that exhibited paw withdrawal response or flinching response at less than 4 g were considered to have developed the allodynia. Percent reversal of PWT was determined using the formula: [(Post-dose threshold- Pre-dose)/(Naive threshold-pre-dose threshold)] X 100.

Statistical analysis: The results were expressed as mean ± standard deviation (S.D). Differences in PWT (both in hyperalgesia and allodynia) were determined by two-way analysis of variance followed by Bonferroni post hoc test. Differences at *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of Tangeretin on serum glucose in control and treated rats: Serum fasting glucose levels were estimated on Day 0, Day 7, Day 14, Day 21 and Day 28. Figure 1 shows serum fasting glucose levels in normal control, diabetic control and treatment groups. i.p. administration of Tangeretin at three different dose levels of 1, 5 and 10 mg kg^{-1} has not significantly altered serum glucose levels

throughout the treatment period.

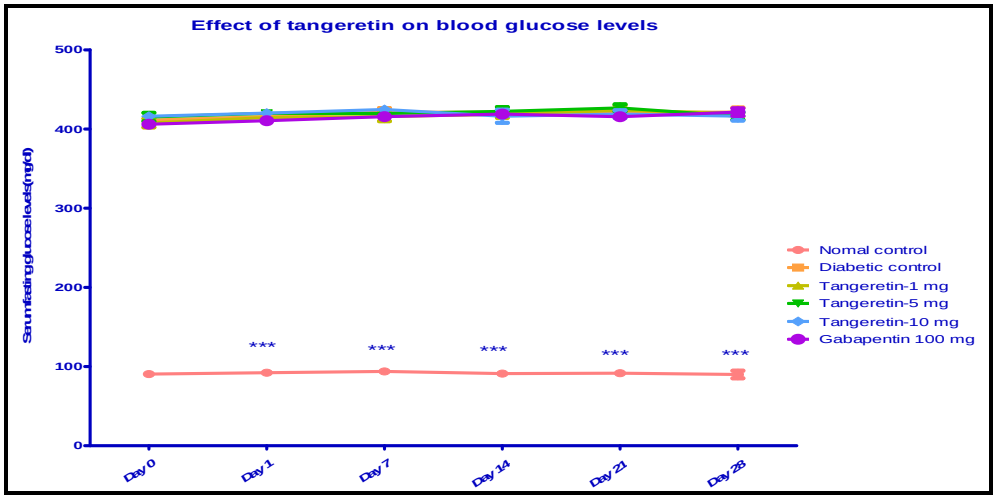


Figure 1. Effect of Tangeretin on serum glucose in control and treated rats

Effect of acute treatment of Tangeretin on mechanical hyperalgesia: In all the animals, cut off PWT was 150 g. In normal control rats, PWT was not significantly altered. In diabetic control rats, PWT declined from 150 g (naive) to 30 g (pre-dose). In Tangeretin(1 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 141.50 ± 1.26, 35.33 ± 5.53, 50.17 ± 8.71, 48.50 ± 9.03 and 51.33 ± 4.53 respectively. In Tangeretin(5 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 142.83 ± 1.21, 41.83 ± 4.88, 61.17 ± 8.11, 49.92 ± 5.52 and 53.00 ± 3.79 respectively. In Tangeretin(10 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 142.00 ± 0.82, 43.75 ± 2.04, 71.61 ± 7.22, 63.33 ± 7.10 and 55.67 ± 7.40 respectively. The effect observed with Tangeretin in reversing mechanical hyperalgesia was almost negligible though values were statistically significant ($p < 0.001$) at 1 h and 2 h post-dose. The effect was neither dose dependent nor time dependent with Tangeretin whereas Gabapentin (100 mg kg^{-1}, i.p) had shown remarkable ($p < 0.001$) reversal of PWT at 1 h, 2 h and 4 h post-dose when compared to pre-dose PWT. Peak effect was observed at 1 h post-dose with Gabapentin (Figure 2).

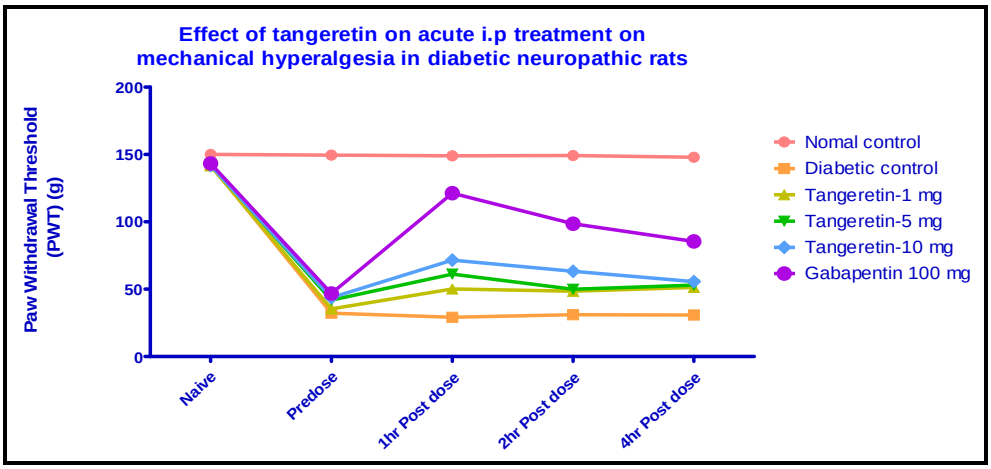


Figure 2. Effect of Tangeretin on acute i.p treatment on mechanical hyperalgesia in diabetic rats.

Effect of chronic treatment of Tangeretin on mechanical hyperalgesia: Figure 3shows PWT of animals in control and chronic treatmentgroups. In all the animals, cut off PWT was 150 g. In normal control rats, PWT was not significantly altered. In diabetic control rats, PWT declined from 150 g

(naive) to 30 g (pre-dose). In Tangeretin(1 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 142.67 ± 5.85, 44.33 ± 7.27, 52.67 ± 9.16, 55.17 ± 6.31 and 55.83 ± 3.85 respectively. In Tangeretin(5 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 141.83 ± 1.57, 52.50 ± 4.79, 80.17 ± 5.40, 86.83 ± 5.81 and 69.08 ± 5.26 respectively. In Tangeretin(10 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 143.33 ± 2.62, 58.52 ± 6.49, 72.73 ± 10.15, 102.25 ± 8.03 and 86.50 ± 8.36 respectively. The effect observed with Tangeretin was statistically significant ($p < 0.001$) at 1 h 2 h and 4 h post-dose whereas Gabapentin (100 mg kg^{-1}, i.p) had shown remarkable ($p < 0.001$) reversal of PWT at 1 h, 2 h and 4 h post-dose when compared to pre-dose PWT. Peak effect was observed at 1 h post-dose with Gabapentin.

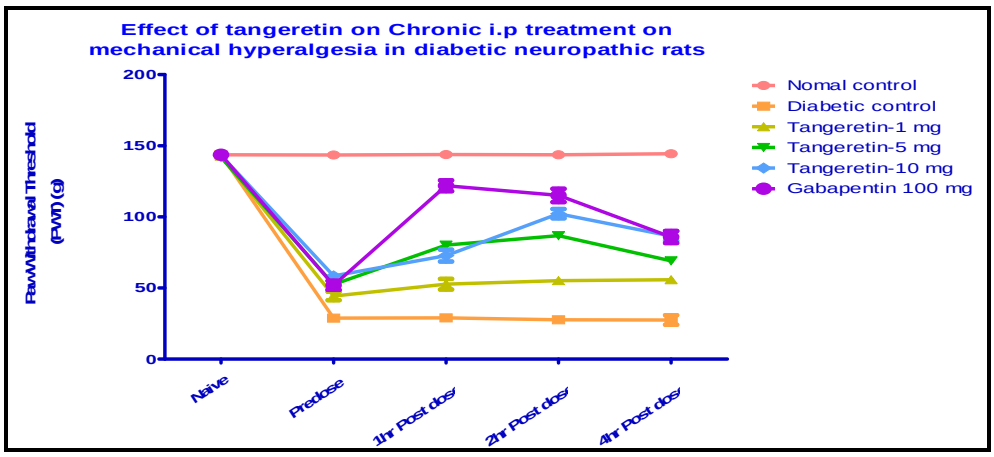


Figure 3. Effect of Tangeretin on chronic i.p treatment on mechanical hyperalgesia in diabetic rats.

Effect of acute treatment of Tangeretin on mechanical allodynia: PWT of animals in allodynia test is presented in figure 4. In all animals, cut off PWT was 60 g. In diabetic control rats, PWT declined from 60 g (naive) to 4.3 g (pre-dose). In Tangeretin (1 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 59.17 ± 0.90, 4.78 ± 0.49, 16.31 ± 2.62, 20.17 ± 4.96 and 22.25 ± 6.07 respectively. In Tangeretin (5 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 59.17 ± 0.90, 4.59 ± 0.32, 17.33 ± 5.25, 27.83 ± 6.59 and 24.83 ± 4.71 respectively. In Tangeretin (10 mg kg^{-1}) treatment group, naive PWT, pre-

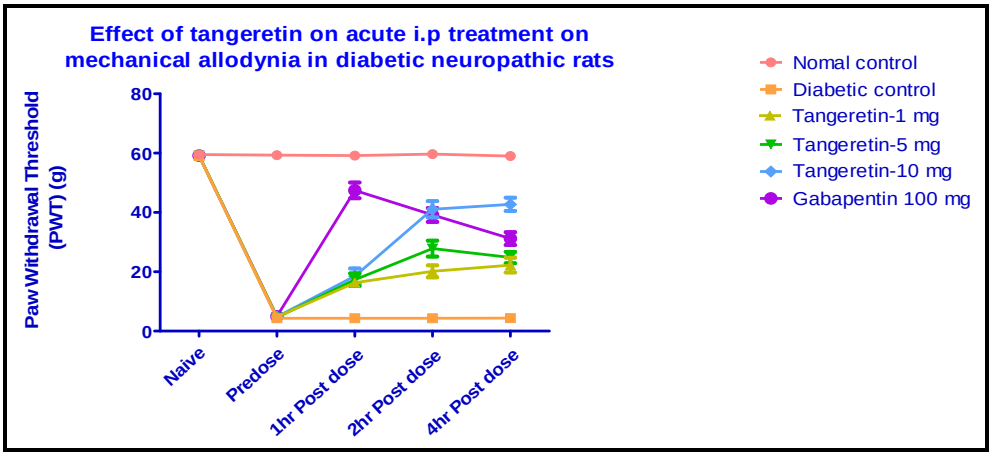


Figure 4. Effect of Tangeretin on acute i.p treatment on mechanical allodynia in diabetic rats.

dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 59.17 ± 0.90, 4.83 ± 0.27, 18.50 ± 6.47, 41.07 ± 6.76 and 42.75 ± 5.49 respectively. Tangeretin had significantly ($p < 0.001$) reversed PWT at

2 h and 4 h post-treatment. At all three doses, there was no significant reversal of PWT at 1 h post-treatment. The maximum reversal was observed at 2 h post-dose at all doses but effect was almost maintained at 4 h post-dose at higher dose whereas, the effect declined 4 h post-dose with Tangeretin0.1 mg/kg and 1 mg kg^{-1} doses. Dose-dependent increase in the effect was observed with increasing dose. Tangeretin had reversed mechanical allodynia on par with the standard drug, Gabapentin but the only difference was that effect was delayed with Tangeretin.

Effect of chronic treatment of Tangeretin on mechanical allodynia: In all animals, cut off PWT was 60 g. In diabetic control rats, PWT declined from 60 g (naive) to 4.33 g (pre-dose). In Tangeretin (1 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 60.0 ± 0.0, 12.71 ± 2.41, 23.69 ± 5.85, 35.24 ± 6.27 and 32.10 ± 5.68 respectively. In Tangeretin (5 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 60.0 ± 0.0, 15.86 ± 2.18, 27.13 ± 5.67, 40.74 ± 9.65 and 46.11 ± 7.83 respectively. In Tangeretin (10 mg kg^{-1}) treatment group, naive PWT, pre-dose PWT, 1 h, 2 h, and 4 h post-dose PWT were found to be 60.0 ± 0.0, 19.65 ± 3.24, 31.34 ± 7.16, 55.24 ± 10.28 and 56.81 ± 9.81 respectively. Tangeretin had significantly ($p < 0.001$) reversed PWT at 1 h, 2 h and 4 h post-treatment at all dose levels. The maximum reversal was observed at 2 h post-dose at all doses but effect was almost maintained at 4 h post-dose at higher dose whereas, the effect was not maintained and declined 4 h post-dose with Tangeretin at 0.1 and 1 mg. Dose-dependent increase in the effect was observed with increasing dose. Our results reveal that chronic treatment exhibited superior anti-allodynic effect when compared to acute treatment (Figure 5).

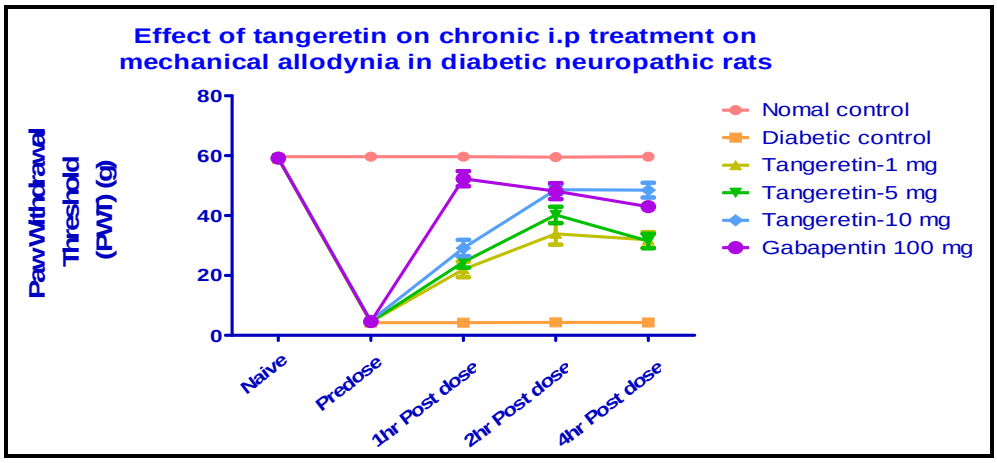


Figure 5. Effect of Tangeretin on chronic i.p treatment on mechanical allodynia in diabetic rats.

Effect of Tangeretin on percent reversal of hyperalgesia and allodynia: Percent reversal was represented at 2 h post-dose following treatment of Tangeretin. In hyperalgesia study, acute treatment with Tangeretin at three different dose levels of 1, 5 and 10 mg kg^{-1} had shown percent reversals of PWT as 12.4, 8 and 19.93 respectively while chronic treatment with Tangeretin at three different dose levels of 1, 5 and 10 mg kg^{-1} had shown percent reversals of PWT as 11.02, 38.43 and 51.56 respectively. In allodynia study, acute treatment with Tangeretin at three different dose levels of 1, 5 and 10 mg/kg had shown percent reversals of PWT as 28.30, 42.59 and 66.69 respectively while chronic treatment with Tangeretin at three different dose levels of 1, 5 and 10 mg kg^{-1} had shown percent reversals of PWT as 53.80, 63.35 and 80.63 respectively. The result clearly indicates that Tangeretin remarkably exhibiting higher degree of efficacy in the reversal of allodynia in comparison to moderate efficacy observed in reversing hyperalgesia.

Streptozotocin is well reported for its selective pancreatic islet b-cell cytotoxicity [12] and has been widely used to induce diabetes mellitus in animals. Our results reveal that STZ injected rats exhibited significantly increased blood glucose levels as compared with control rats.

The STZ induced diabetic rat and mice [**13-15**] have commonly been used as model of neuropathic pain with signs of hyperalgesia and allodynia that may reflect signs observed in diabetic patients. In our study, i.v. administration of STZ (45 mg kg^{-1} body weight) significantly resulted in hyperglycemia. Altered pattern of nociception may not be due to inherent neurotoxicity of STZ [**16**], but STZ induced hyperalgesia clearly contributes to a wide array of pathophysiological symptoms that can lead to altered nociceptive responses tested in various animal models [**15, 17**].

Our study results report that i.p. administration of Tangeretin at three different dose levels of 1, 5 and 10 mg kg^{-1} did not significantly alter serum glucose levels throughout the treatment period. Our result is in contrast to the previous finding of anti-hyperglycemic effect of Tangeretin in terms of reduction in glucose levels. The discrepancy between these two studies could possibly be attributed to variation in experimental design, dose and route of administration. However, given that our aim was mainly to assess the mechanical hyperalgesia and mechanical allodynia, we have estimated only serum glucose levels but not focused on HbA1C and insulin.

Tangeretin exhibited moderate effect in reversing mechanical hyperalgesia while it elicited high degree of efficacy in reversing mechanical allodynia upon both acute and chronic treatment. Previous in-vivo study reported that Tangeretin has anti- inflammatory and anti- oxidant potential [**17**]. However, the efficacy of Tangeretin is higher at 2 h post-dose in both hyperalgesia and allodynia. In our study, Tangeretin was found to be effective even at 4 h post-treatment. This indicates the results are possibly correlating with the earlier pharmacokinetic studies.

CONCLUSION

Tangeretin demonstrates better efficacy in reversing mechanical allodynia than in mechanical hyperalgesia. Tangeretin could be a deserved candidate for further studies to establish the mechanisms in the area of neuropathic pain.

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