PRECLINICAL EVALUATION OF ANTINOCICEPTIVE EFFECT OF *WITHANIA SOMNIFERA* (ASHWAGANDHA) IN DIABETIC PERIPHERAL NEUROPATHIC RAT MODELS

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Summary

The present study was designed to investigate the antinociceptive effect of root extract of *Withania. somnifera* in Streptozotocin (STZ) induced diabetic peripheral neuropathic rat models. Forced swim test was carried out to determine the antidepressant activity. The interference of antidepressant agents with motor activity of rats was determined by open-feild test. Diabetes was induced by a single *i.p.* injection of 50 mg/kg streptozotocin. Tail immersion test (Hot & cold water) and formalin test were performed to determine the extent of neuropathic pain in diabetic rats. Imipramine (10.5mg/kg), flouxetine (14.5mg/kg), Quercetin (10mg/kg) and *W. somnifera* (100mg/kg) was administered orally for 21 consecutive days starting after 4th week in STZ induced diabetic rats. Thiobarbituric acid reactive substance (TBARS), superoxide dismutase (SOD), and Catalase in serum were also performed to access the oxidative stress. Mode of action of *W. somnifera* was determined by administering naloxone (2 mg/kg) prior to its administration. Chronic treatment with *W. somnifera* for 3 weeks starting after 4th week in STZ induced diabetic rat's attenuated hyperalgesia. It also ameliorated diabetic induced raised level of TBARS, and decreased level of SOD and Catalase. Antinociceptive activity of *W. somnifera* was reversed by prior administration of naloxone. It was concluded that antidepressant and antioxidant effect may be responsible for observed antinociceptive effect of *W. somnifera* in STZ induced diabetic peripheral neuropathic rat models.

Keywords: Diabetic peripheral neuropathy, Imipramine, flouxetine, *Withania somniferra*, Quercetin, Streptozotocin.

Running Title: WITHANIA SOMNIFERA AS ANTINOCICEPTIVE

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Introduction

Diabetes is a global health problem and its prevalence is set to increase to more than 360 million worldwide by the year 2025[1]. Diabetes leads to several complications including neuropathy retinopathy, and nephropathy. Diabetic neuropathy is the most common complication affecting more than 50% of the diabetic patients. Etiology of diabetic neuropathy is complex and multifactorial[2]. Diabetic peripheral neuropathy (DPN) is the most common complication of longstanding diabetes mellitus which frequently results in clinically significant morbidities[3]. An internationally agreed simple definition of DPN for clinical practice is "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes"[4]. Pain associated with diabetic neuropathy can occur either spontaneously or as a result of exposure to only mildly painful stimuli (hyperalgesia) or to stimuli not normally perceived as painful (allodynia)[5]. It is known that the pathophysiology of neuropathic pain in the diabetic is accompanied by neurochemical alterations in the nervous system. A number of neurotransmitters likely modulate the ascending and descending pain pathways and norepinephrine and 5-hydroxytriptamine (serotonin, 5-HT) have been implicated as mediators of endogenous analgesia via the descending pain inhibitory pathways[6,7]. In pathological pain states, these endogenous pain inhibitory mechanisms may be dysfunctional, contributing to the central sensitization and hyperexcitability of the spinal and supraspinal pain–transmitting pathways and manifesting as several types of persistent pain, including neuropathic pain[8]. Hyperglycemia is reported to induce oxidative stress through multiple pathways such as redox imbalances secondary to enhanced aldose reductase activity[9] , increased advanced glycation end products[10] , altered protein kinase C activity, especially β-isoforms[11] and mitochondrial overproduction of superoxide[12] hence apart from glycemic control, a corresponding wide range of treatment have been employed to treat patients with neuropathic pain, including tricyclic antidepressants, SSRI, Serotonin-norepinephrine reuptake inhibitors, antiepileptic drugs, opoid analgesics, N-methyl-D-aspartate receptor antagonists, Cholecystokinin receptor antagonists, adenosine, lipoic acids, cannabinoids, capsaicin, protein kinase C inhibitors, aldose reductase inhibitors and VR-1 receptor modulators[13].

W. somnifera is also known as ashwagandha, Studies indicate ashwagandha possess Anxiolytic action[14], Antistress and adaptogenic action[15], Anti-inflammatoryproperties[16], Hypoglycemic, diuretic, and hypocholesterolemic effects[17], Cardiovascularprotection[18]. The antidepressant activity of *W. somnifera* is mediated partly through an adrenoreceptor as well as alteration in the level of central biogenic amines[19]. The active principles of *W. somnifera*, comprising sitoindosides VII–X and Withaferin-A, have been shown to have significant antistress activity against acute models of experimental stress[20].

W. somnifera is also known to modulate the oxidative stress markers of the body. The root extract significantly reduced the lipid peroxidation[21] and increased the superoxide dismutase (SOD) and catalase activity, thus possessing a free radical scavenging property[22]

But there was no scientific report of *W. somnifera* on the effect of diabetic neuropathic pain. Hence the present study was undertaken to investigate the antinociceptive effect of *W. somnifera* aqueous root extract in diabetic Neuropathic rat models.

Materials And Methods

Experimental animals

Male Sprague Dawley rats weighing between 250-290 gm were used. The experimental protocol was approved by Institutional Animal Ethics Committee (KCP/IAE-05/2007) and animals were maintained under standard conditions in the animal house approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

Chemicals: Streptozotocin & Quercetin purchased from Hi Media laboratories Pvt. Ltd., Mumbai. Fluoxetine sample was gifted by Strides acrolabs, Bangalore and, Imipramine was obtained from Torrent pharmaceuticals, Ahmedabad. Standardized aqueous *Withania Somnifera* root extract (Batch no: LOTWS0519) was gifted by Natural remedies Bangalore.

Antidepressant activity

Antidepressant activity of *W. somnifera* root extract was evaluated by using force swimming test and was compared with standard antidepressants such as fluoxetine and imipramine. The interference of antidepressant agents with motor activity was determined by using gross motor activity of the animals.

Treatment protocol for antidepressant activity (Treatment period-21days)

Groups **A.** Normal control

B. Normal control groups (treated)

(i) Fluoxetine (14.5 mg/kg, p.o.)[23]

(ii) Imipramine (10.5 mg/kg, p.o)[23]

(iii) *W. somnifera* root extarct (100mg/kg, p.o.)[15]

Forced swimming test (FST) [24]

In FST, Two swim sessions were conducted on two consecutive days (one test per day). The FST paradigm includes 2 sections: an initial 15-min pre-test followed by a 5-min test 24 hours later. In the first session (pretest), a rat was placed individually into a bucket (height 31 cm, diameter 29 cm) filled with water (24±1°C) to a depth of 20 cm for 15 min. In the second session, 90 min after drug treatment, rats were again placed in a bucket and time of immobility was determined. The immobility time is an index of depressive-like behavior in the forced swimming test. A rat was defined as immobile when floating motionless or making only those movements necessary to keep its head above water.

Water in the bucket was changed and cleaned thoroughly after each session. At the end of experiment, the rat was wiped with dry towel to maintain body temperature and returned to the home cage.

Gross motor activity[25]

Motor activity was assessed in a chamber having a floor measuring 50×50×37 divided into 16 boxes. The open field was housed in a quiet, darkened testing room and was illuminated by a 25 watt light bulb positioned approximately 90 cm above the center of the floor. Animals were brought into the testing room and gently placed in the center square of the open field facing away from an observer who sat quietly on a stool positioned above one corner of the field so as to be able to view the entire enclosure. The observer then traced the path of the rat's movements on a map of the open field. Total number of squares entered was counted. An animal was considered to have entered a square when it moved all four limbs across the boundary into that square; smaller movements of turning, spinning, or probing with the head or forelimbs were not counted as entering a square. The open-field test lasted 10 min

Induction of diabetes

Diabetes was induced in overnight fasted adult Sprague dawley rats by a single intraperitoneal injection of 50mg/kg STZ[26]. Blood glucose level was measured after 72 h of STZ injection. Blood samples were obtained from tail vein by subjecting the rats to light ether anesthesia. Glucose concentration was measured by using glucometer with glucose-oxidase impregnated strips. Animals with blood glucose levels > 300 mg/dl was considered diabetic and selected for further study.

Treatment protocol

Hypersensitivity to pain stimuli was well developed after 4 week after diabetes induction, treatment was started after 4th week and continued end of upto 7th week

Groups:

A. Normal control (saline)

B. Diabetic control

C. Diabetic groups (treated)

W. somnifera (100mg/kg, p.o.)[15]

Imipramine (10.5 mg/kg, p.o.)[23]

Fluoxetine (14 mg/kg, p.o.) [23]

Quercetin (10 mg/kg, p.o.) [27]

Neuropathic pain models

Tail-immersion (cold[27] & hot water[28])

Spinal thermal sensitivity was assessed by the tail immersion test. Hyperalgesic state towards noxious and non-noxious stimuli was noted the terminal part of the tail (5 cm from the tip of the tail) was immersed in water, maintained at temperatures of 50±0.5°C (noxious) and 10±1°C (non-noxious). The duration of the tail withdrawal latency was recorded, and a cut-off time of 15 s was used to prevent the damage of tail.

Chemical stimuli -Formalin test[26]: Antinociception in non-diabetic and diabetic rats was assessed using the formalin test. The rats were placed in open Plexiglas observation chambers for 30 min to allow them to acclimate to their surroundings; then they were removed for formalin administration. Fifty µl of diluted formalin (0.5% for diabetic rats or 1% for non-diabetic rats) were injected subcutaneously into the dorsal surface[29] of the right hind paw with a 30-gauge needle. The animals were returned to the chambers and nociceptive behavior was observed immediately after formalin injection. Mirrors were placed in each chamber to enable unhindered observation. Nociceptive behavior was quantified as the numbers of flinches of the injected paw during 1-min periods every 5 min, up to 60 min after injection. Flinching was readily discriminated and was characterized as rapid and brief withdrawal, or as flexing of the injected paw. Formalin induced flinching behavior was biphasic. The initial acute phase (0–5 min) was followed by a relatively short quiescent period (10 - 20 min), which was then followed by a prolonged tonic response (25–60 min).

Mechanism of action:

Mechanism of action was evaluated by administering naloxene (2mg/kg)[29] prior to 15min of drug administration.

Biochemical estimation of markers of oxidative stress

After 21 days of treatment blood was withdraw from tail vein and centrifuged at 5000rpm for 20 min and serum was separated

TBARS[30]

Thiobarbituric acid reactive substance (TBARS) in the serum was estimated using the standard protocol. Thiobarbituric acid reacts with malondialdehyde (MDA), a secondary product of lipid peroxidation. The developed color was reddish pink and was estimated at 532 nm.

Estimation of superoxide dismutase[31]

Sod was estimated by method of Erich F. Estimation of SOD is based on detection O_2^- by oxidation of hydroxylamine hydrochloride yielding nitrite, which is measured colorimetrically at 560 nm. O_2^- can also be generated during auto-oxidation of hydroxylamine.

Accompanying the autooxidation of hydroxylamine at pH 10.2, NBT is reduced and nitrite is produced in the presence of EDTA, which can be detected colorimetrically**.**

Catalase[32]

Estimation of Catalase activity was done by determining the decomposition of H_2O_2 at 240 nm in an assay mixture containing phosphate buffer (0.25 M, pH 7).

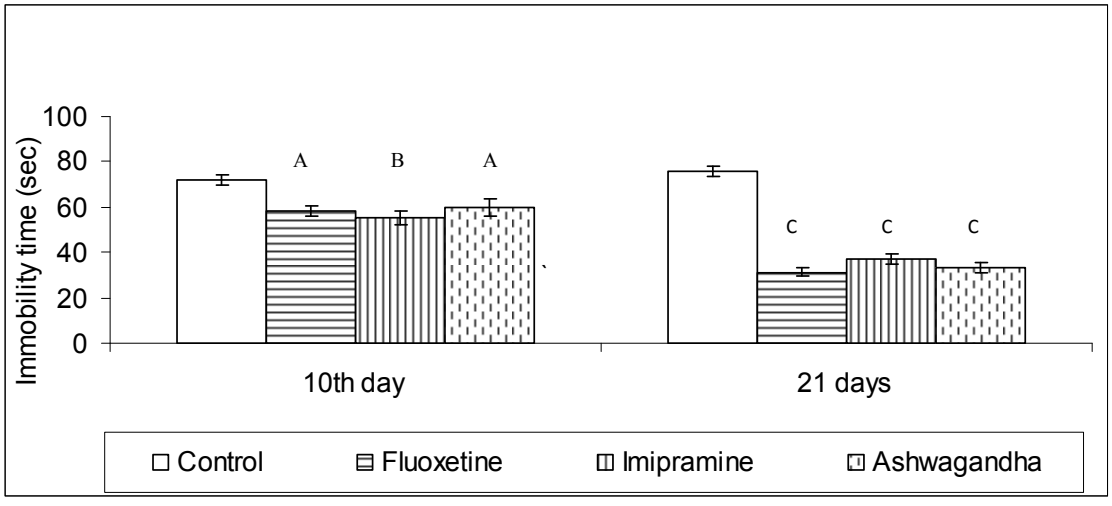
Statistical analysis: Data were expressed as means ±S.E.M. The time course of the pharmacological effect was examined using analysis of variance (ANOVA) followed by Tukey t-test. $P<0.05$ was considered as significant.

Results

Effect of aqueous root extract of *W. somnifera* in Forced swimming test and Gross motor activity

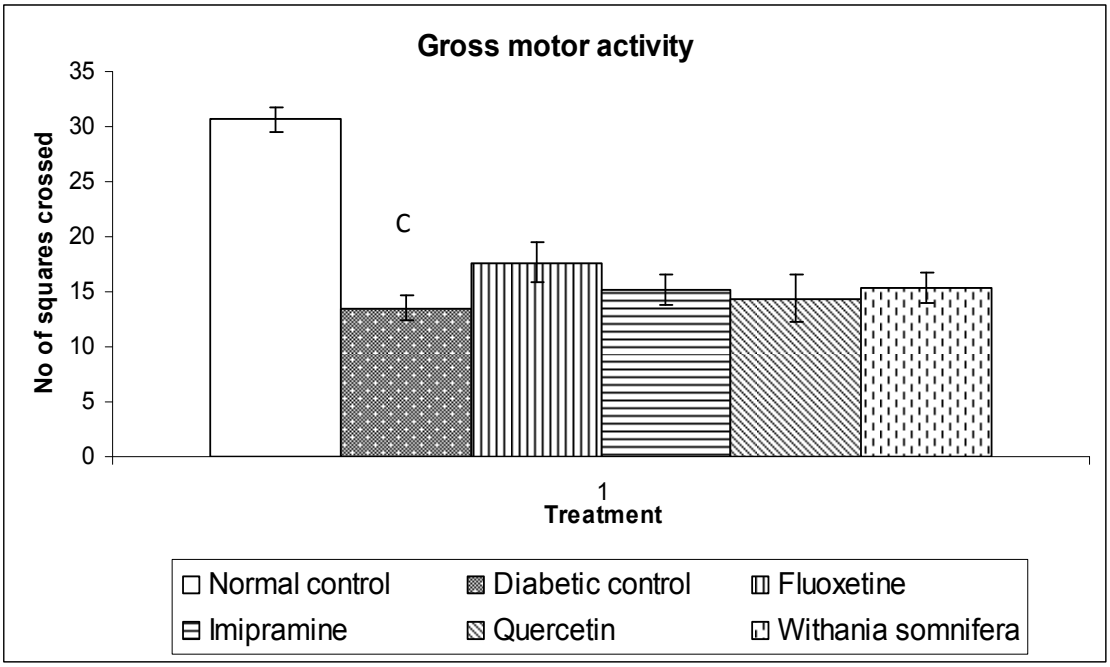
In forced swimming test, the normal rats showed significant decrease in immobility time when treated with fluoxetine ($P<0.05$), imipramine ($P<0.01$), *W. somnifera* ($P<0.05$) for 10days when compared to control. But 21 days of treatment produced a maximum decrease in immobility time in all treated groups. All treated groups showed statistical significance ($P<0.001$) when compared to control as shown in Fig 1. It was observed that there was no significant interference with gross motor activity in all treatment groups when treated for 21 days. (Fig. 2)

Fig-1 Effect of aqueous root extract of *W. somnifera* in Forced swimming test



All values are mean ± SEM, n=6, $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$ when compared to normal control group,

Fig 2: Effect of *W. somnifera* root extract on Gross motor activity



All values are mean ± SEM, n=6, $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$ when compared to control group, $^{a}P<0.05$, $^{b}P<0.01$, $^{d}P<0.001$ when compared to diabetic control group. (Note: Treated groups are not compared with normal control).

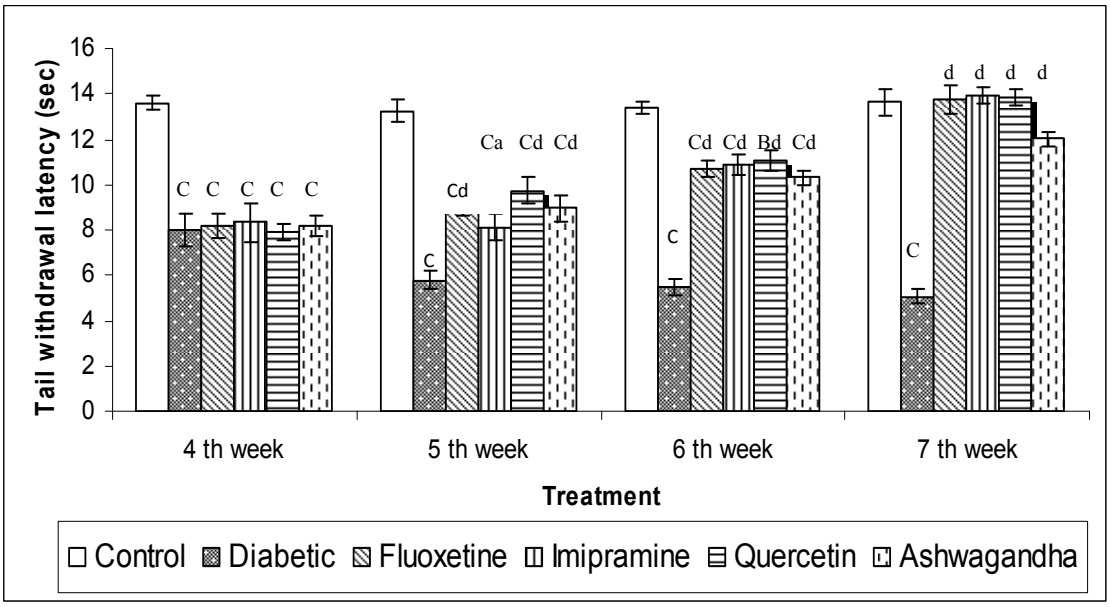
Effect of *W. somnifera* root extract in Tail immersion test (Cold water)

Tail flick latencies of diabetic rats were significantly (P<0.001) less than non-diabetic rats indicative of hyperalgesia. After three weeks of treatment, Tail withdrawal latency of groups treated with fluoxetine, imipramine, quercetin, *W. somnifera* were increased significantly (P<0.001) when compared to diabetic control. (Fig 3)

Effect of *W. somnifera* root extract in Tail immersion test (Hot water)

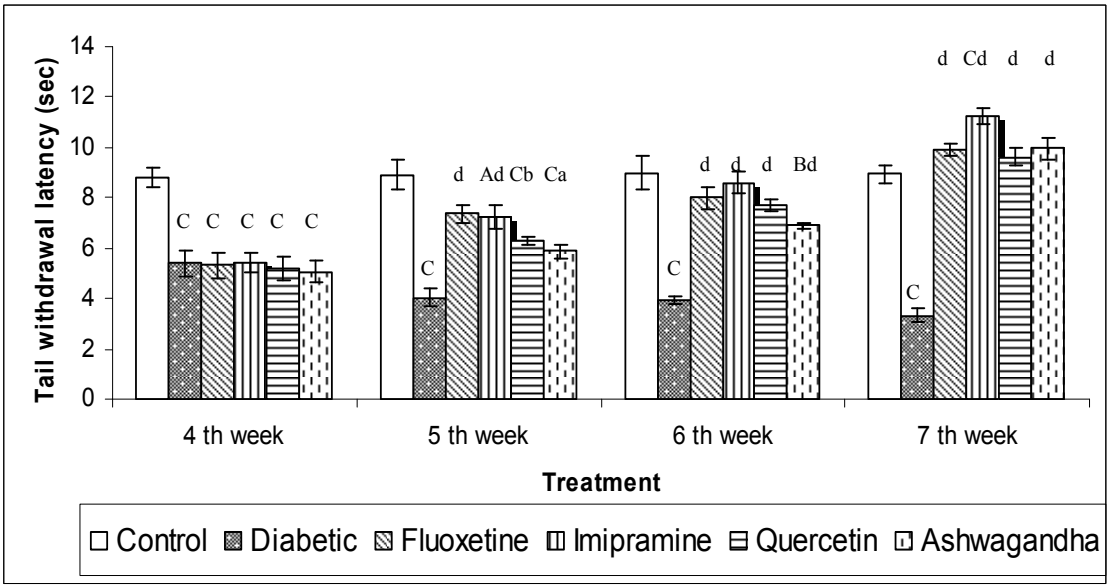
After three weeks of treatment, fluoxetine, imipramine, quercetin, and *W. somnifera* root extract treated groups significantly (P<0.001) increased latency period when compared to diabetic control. In group treated with imipramine, latency period was significantly (P<0.01) increased when compared to normal control. Comparative analysis revealed that there was no significant difference found in latency period between *W. somnifera* and standard drug treated groups. (Fig. 4)

Fig-3 Effect of *W. somnifera* root extract in Tail immersion test (Cold water)



All values are mean ± SEM, n=6, $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$ when compared to normal control group, $^{a}P<0.05$, $^{b}P<0.01$, $^{d}P<0.001$ when compared to diabetic control.

Fig-4 Effect of *W. somnifera* root extract in Tail immersion test (hot water)



All values are mean ± SEM, n=6, $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$ when compared to normal control group, $^{a}P<0.05$, $^{b}P<0.01$, $^{d}P<0.001$ when compared to diabetic control.

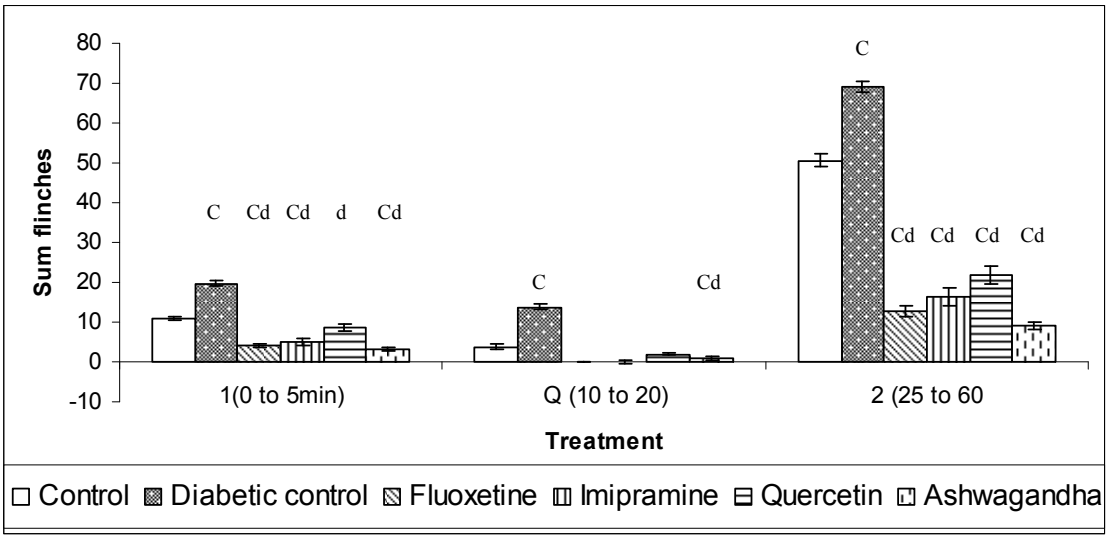
Effect of *W. somnifera* root extract in Formalin test

Diabetic animals shown hyperalgesia to chemical stimuli in all three phases. Groups treated with fluoxetine, imipramine, quercetin, *W. somnifera* significantly (P<0.001) decreased sum of flinches in 1st phase flinches when compared to diabetic control. Fluoxetine (P<0.001), imipramine (P<0.001), and *withania somnifera* (P<0.001), were significantly decreased flinches when compared to normal control. *W. somnifera* was significantly (P<0.001) more active than quercetin.

All treatments were significantly (p<0.001) decreased sum of flinches in Q phase when compared to diabetic control and normal control.

In 3rd phase groups treated with fluoxetine (P<0.001), imipramine (P<0.001), quercetin (P<0.001), *W. somnifera* (P<0.001), were significantly decreased flinches when compared to diabetic as well as normal control groups. *W. somnifera* was significantly (P<0.001) more active than quercetin (Fig. 5)

Fig-5 Effect of *W. somnifera* root extract in Formalin test



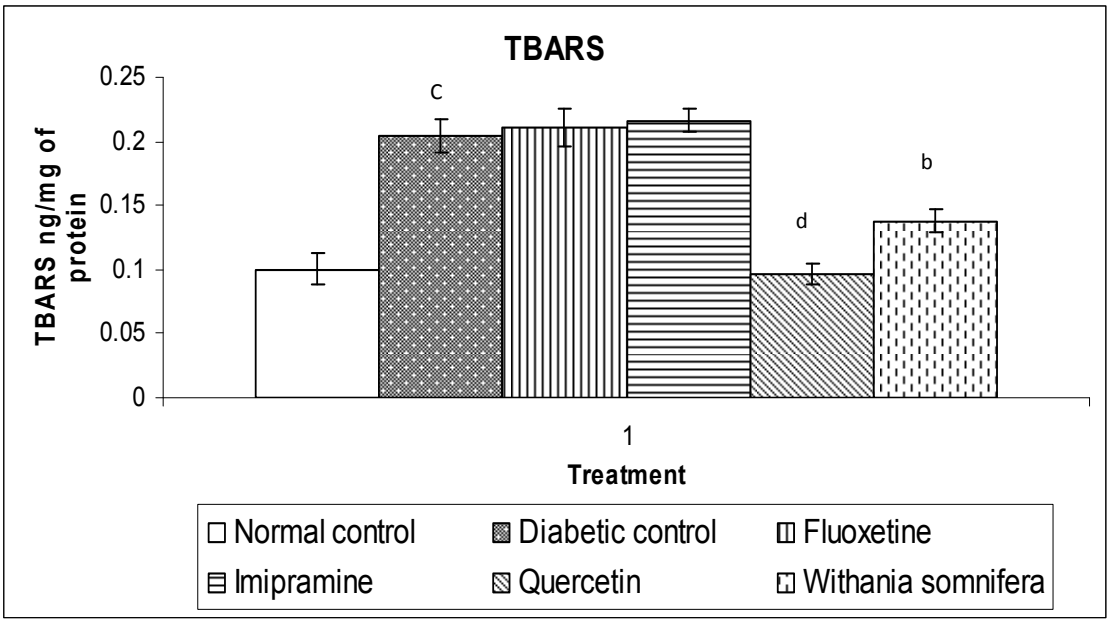
All values are mean ± SEM, n=6, $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$ when compared to normal control group, $^{a}P<0.05$, $^{b}P<0.01$, $^{d}P<0.001$ when compared to diabetic control

Effect of *W. somnifera* root extract on biochemical parameters

Thiobarbituric acid reactive substance (TBARS)

W. somnifera (P<0.01), Quercetin (P<0.001) treated groups significantly shown decrease in TBARS when compared to diabetic control. Imipramine, fluoxetine treated groups and diabetic group shown significant (P<0.001) increase in TBARS when compared to normal control. (Fig. 6)

Fig 6: Effect of *W. somnifera* root extract on serum TBARS



All values are mean ± SEM, n=6, $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$ when compared to control group, $^{a}P<0.05$, $^{b}P<0.01$, $^{d}P<0.001$ when compared to diabetic control group.(**Note**: Treated groups are not compared with normal control).

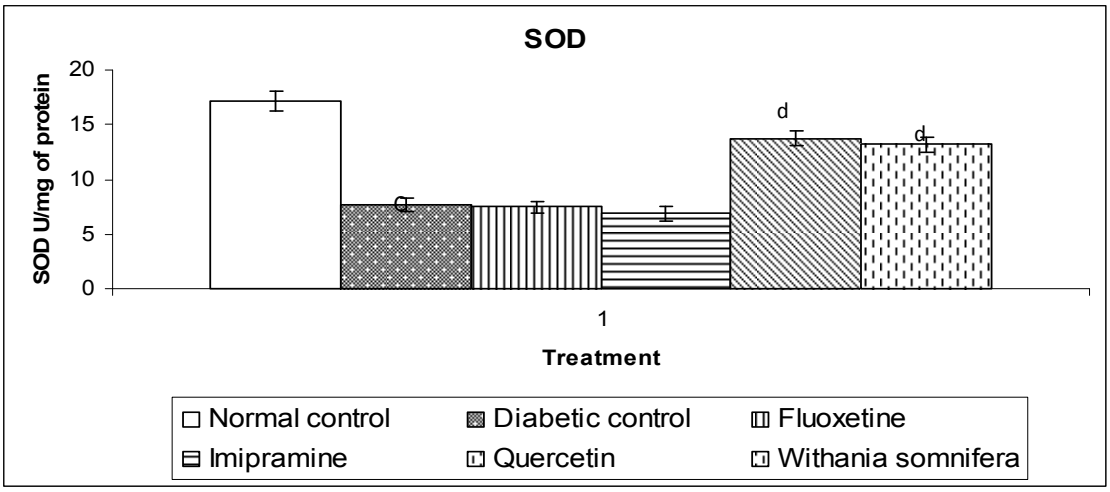
Superoxide dismutase (SOD)

W. somnifera (P<0.001), Quercetin (P<0.001) treated groups were significantly shown increase in serum sod when compared to diabetic control. *W. somnifera* (P<0.01), Quercetin (P<0.05), Imipramine (P<0.001), fluoxetine (P<0.001) treated groups shown significant decrease in sod when compared to normal control. (Fig. 7)

Catalase

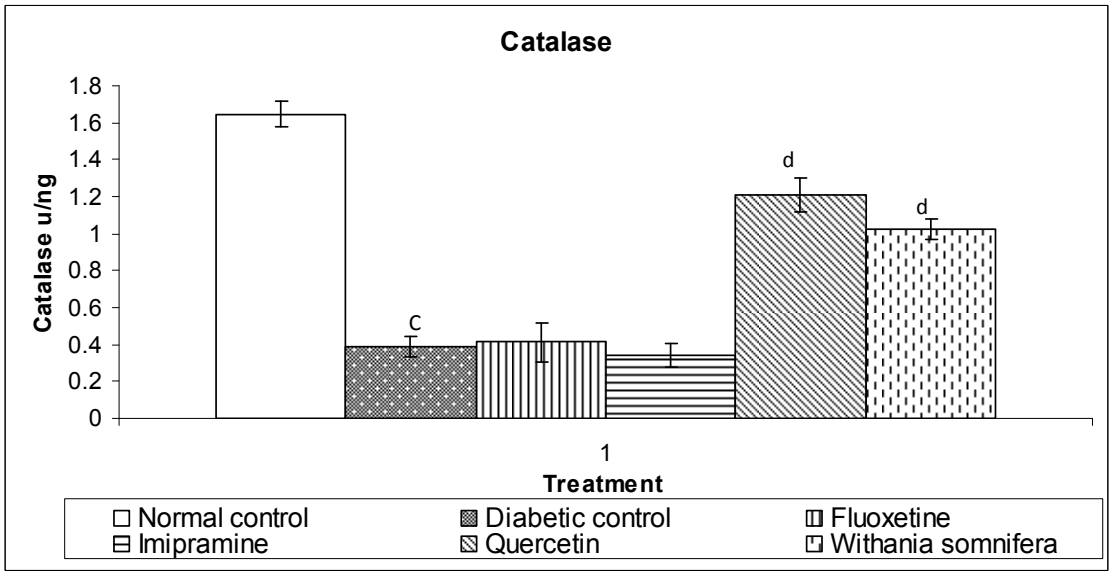
W. somnifera (P<0.001), Quercetin (P<0.001) treated groups were significantly shown increase in serum catalase when compared to diabetic control. *W. somnifera* (P<0.001), Quercetin (P<0.01), Imipramine (P<0.001), and fluoxetine (P<0.001) treated groups shown significant (P<0.001) decrease in catalase when compared to normal control. (Fig. 8)

Fig 7: Effect of *W. somnifera* root extract on serum SOD



All values are mean ± SEM, n=6, $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$ when compared to control group, $^{a}P<0.05$, $^{b}P<0.01$, $^{d}P<0.001$ when compared to diabetic control group.(**Note**: Treated groups are not compared with normal control).

Fig 8: Effect of *W. somnifera* root extract on serum catalase



All values are mean ± SEM, n=6, $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$ when compared to control group, $^{a}P<0.05$, $^{b}P<0.01$, $^{d}P<0.001$ when compared to diabetic control group. (Note: Treated groups are not compared with normal control).

Discussion and Conclusion

In diabetic neuropathy, symptomatic treatment involves use of antidepressants and anticonvulsants. Antidepressants are effective in relieving neuropathic pain. Many clinicians prescribe antidepressants rather than anticonvulsants as first-line in neuropathic pain, either because of perceived greater chance of benefit or lower chance of adverse effects[33].

The supraspinal and spinal serotonin (5–HT) pathways are involved in pain perception[34]. Tail flick test is a spinally mediated reflex to noxious stimuli. The fast raising pain in the tail flick gives rise to rapid tail withdrawal at the lowest possible threshold for pain before the pain reaches the supraspinal region. Where as in hot plate test, paw licking and jump response is mediated through supraspinal centres. In the last two decades a number of reports have indicated the direct role of the opioidergic system in antidepressant induced antinociception[35,36]. But the mode of action of antidepressants is based on its interference with reuptake of monoamines (Norepinephrine and 5 – HT).

An attempt was made to explore the usefulness of antidepressant activity of aqueous root extract of *W. somnifera* in alleviating the diabetic induced neuropathic pain and to elucidate the possible mechanism of action of their antinociception.

In the present study initially antidepressant activity of *W. somnifera* was carried out. Treatment for 10 days produced a less significant antidepressant activity when compared to 21 days treatment. But it has been reported that *W. somnifera* root powder (100 mg/kg) orally as an aqueous suspension daily for seven days given before the swimming test in water at 10ºC also increased total swimming time, indicating better stress tolerance in rats[37]. Hence in the present study the diabetic rats were treated for 21 days instead of 7 days.

W. somnifera (100mg/kg) exhibited significant antidepressant like effect in rats by interacting with adrenergic system because prazosin (α1 adrenergic antagonist) partially antagonized the immobility reducing action of *W. somnifera*. This observation suggests the possible involvement of α1 adrenergic receptors in the immobility reducing action of *W. somnifera* and also reserpine (vesicular re-uptake blocker which depletes catecholamines or lowers noradrenaline) induced increase in mean immobility time was significantly reversed by treatment with *W. somnifera* (100 mg/kg, i.p.) tempting to suggest the involvement of biogenic amines in antidepressant action of *W. somnifera* [38].

Monoamines, opioid and 5HT receptors are believed to be involved in antidepressant-induced antinociception. Studies have provided evidence of interaction of tricyclic antidepressants with opioid receptors, which could account for their analgesic action[39,40] Tricyclic antidepressants activate opioid system, through both, a direct opioid receptor interaction and an indirect action through enhanced release of opioid peptide[39]. In our study imipramine, fluoxetine and *W. somnifera* increased the latency of tail-flick response in rats and was attenuated by prior treatment with naloxone, suggesting implication of an

opioid antagonist reversible nature to imipramine, flouxetine and *W. somnifera* induced antinociception and an opioid system involvement

Amitriptyline & desipramine blocked tonic flinch responses in the formaldehyde solution test[41]. Our present study has also shown that administration of imipramine, fluoxetine and *W. somnifera* blocked the tonic flinch responses.

Treatment of streptozotocin induced diabetic rats with antioxidants has attenuated oxidative stress and vascular dysfunction, which may be a major factor in the development of diabetic neuropathy. Diabetic rats exhibited a significant increase in TBARS, an index of lipid peroxidation and reduction in antioxidant enzyme activity. These parameters regained to normal levels when treated with *W. somnifera* and quercetin. Quercetin has proven to protect against the development of diabetic neuropathy by inhibition of lipid peroxidation and restoration of antioxidant enzymes in diabetic rats. Thus reverses the oxidative stress induced changes in nerve physiology of diabetic rats as reported earlier[42].

W. somnifera is known to modulate the oxidative stress markers of the body. The root extract significantly reduced the lipid peroxidation and increased the superoxide dismutase (SOD) and catalase activity, thus possessing a free radical scavenging property[21,22].

The results of this pre-clinical study on rats display potential antinociceptive effect of *W. somnifera*. Data here obtained allows us to propose this plant species as an excellent candidate for isolating new substances with potential antinociceptive effect.

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References

1. Wild, S., Roglic, G., Green, A., Sicree, R., King, H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27: 1047–1053.
2. Van Dam, P.S. Oxidative stress and diabetic neuropathy: pathophysiological mechanisms and treatment perspectives, Diabetes/Metab. Res. Rev 2002; 18: 176–184.
3. Pop-busui R,Sima A,Stevens M. Diabetic neuropathy and oxidative stress .Diabetic Metab Res Rev 2006; 22:257-73.
4. Boulton AJM, Gries FA, Jervell JA: Guidelines for the diagnosis and outpatient management diabetic peripheral neuropathy. Diabet Med 1998.15:508 –514.
5. Brown MJ and Asbury AK. Diabetic neuropathy. Ann Neurol 1984; 15: 2-12.

6. Basbaum, A.I., Fields, H.L. Endogenous pain control systems: brainstem spinalpathways and endorphin circuitry. Annu. Rev. Neurosci. 1984; 7: 309–338.

7. Yaksh, T.L. Pharmacology of spinal adrenergic systems which modulate spinal nociceptive processing. Pharmacol. Biochem. Behav 1985; 22: 845–858

8. Coderre TJ, Katz J: Peripheral and central hyperexcitability: differential signs and symptoms in persistent pain. Behav Brain Sci 1997; 20: 404–419.

9. Yagihashi S, Yamaghasi SI, Wada Ri R, Baba M, Hohman TC,Yabe Nishimura C,Neuropathy in diabetic mice overexpressing human aldose reductase and effects of aldose reductase inhibitor.Brain 2001;124:2448-58.

10. Brownlee M, Cerami A,Vlassara H.Advanced products of non enzymatic glycolysation and the pathogenesis of diabetic vascular disease.Diabetes Metab Rev 1988;4:437-51.

11. Cameron NE,Cotter MA,Jack AM,Basso MD,Hohman TC. Protein kinase C effects on nerve function, perfusion, Na (+) K (+)-ATPase activity and glutathione content in diabetic rats.Diabetolgia 1999; 42:1120-30.

12. Brownlee M. A radical explanation for glucose-induced beta cell dysfunction. J Clin Invest 2003; 112:1788-90

13. Gidal BE,Billington R New and emerging treatment options for neuropathic pain.Am J Manage Care 2006;12: 269-78.

14. Bhattacharya SK, Bhattacharya A, Sairam K, Ghosal S. Anxiolytic-antidepressant activity of Withania somnifera glycowithanolides: an experimental study. Phytomedicine 2000; 7:463-469

15. Archana R, Namasivayan A. Antistressor effect of Withania somnifera. *J* Ethnopharmacol1999; 64:91-93.

16. Anbalagan K, Sadique J. Influence of an Indian medicine (Ashwagandha) on acute phase reactants in inflammation. Indian J ExpBiol 1981; 19:245-249.

17. Andallu B, Radhika B. Hypoglycemic, diuretic and hypocholesterolemic effect of winter cherry (*Withania somnifera*) root. Indian J Exp Biol 2000; 38:607-609.

18. Mohanty D.S. Arya, A. Dinda, K.K. Talwar, S. Joshi, S.K. Gupta. Mechanisms of cardioprotective effect of *Withania somnifera* in experimentally induced myocardial infarction. *Basic Clin. Pharmacol. Toxico* 2004; 94(4): 184-190.

19. Shah PC, Trivedi NA, Bhatt JD, Hemavathi KG. Effect of Withania somnifera on forced swimming test induced immobility in mice and its interaction with various drugs. Indian J Physiol Pharmacol 2006; 50(4):409-15.

20. Bhattacharya SK, Goel RK, Kaur R, Ghosal S. Antistress activities of sitoindosidesVII and VIII, new acyl steryl glucosides from Withaniasomnifera. Phytother Res 1987; 1:32– 3.

21. Dhuley JN. Effect of ashwagandha on lipid peroxidation in stress-induced animals. J Ethnopharmacol 1998; 60:173–8.

22. Panda S, Kar A. Evidence for free radical scavenging activity of Ashwagandha root powder in mice. Indian J Physiol Pharmacol 1997; 41:424–6.

23. Dhingra D, Valecha R. Evaluation of antidepressant-like effect of aqueous and ethanolic extracts of terminalia bellirica Roxb fruits in mice. Indian J Exp Biol 2007; 45(7):610.
24. Tomonaga S, Yamane H, Onitsuka E, Yamada S, Sato M, Takahata Y et.al. Carnosine-induced antidepressant-like activity in rats. Pharmacology, Biochemistry and Behavior 2008; 89: 627–632.
25. Weiss J M, Cierpial M A, West C H K. Selective Breeding of Rats for High and Low Motor Activity in a Swim Test: Toward a New Animal Model of Depression. Pharmacology Biochemistry and Behavior 1998; 61(1): 49–66.
26. Calcutt NA, Jorge MC, Yaksh TL, Chaplan SR. Tactile allodynia and formalin hyperalgesia in streptozotocin-diabetic rats: Effects of insulin, aldose reductase inhibition and lidocaine. Pain 1996; 68:293.
27. Anjaneyulu M, Chopra K. Quercetin attenuates thermal hyperalgesia and cold allodynia in STZ-induced diabetic rats. Indian Journal of Experimental Biology 2004; 42(8): 766-769.
28. Wang YX, Bowersox SS, Pettus M, Gao D. Antinociceptive properties of fenfluramine, a serotonin reuptake inhibitor, in a rat model of neuropathy. The Journal of Pharmacology and Experimental Therapeutics 1999; 291:1008-1016
29. Anjaneyulu M, Chopra K. Possible involvement of cholinergic and opioid receptor mechanisms in fluoxetine mediated antinociception response in streptozotocin-induced diabetic mice. Eur J Pharmacol 2006; 538(1-3):80-84
30. Gelvan D, Saltman P. Different cellular targets for Cu- and Fe-catalyzed oxidation observed using a Cu-compatible thiobarbituric acid assay. Biochim Biophys Acta. 1990; 1035:353-60.
31. Erich F, Elastner. Inhibition of nitrite formation from hydroxyl ammonium chloride. A simple assay for super oxide dismutase. Anal Chem 1976; 70:616-20.
32. Eva ML. Mechanism of pH dependent hydrogen per oxide cytotoxicity *invitro*. Arch Biochem Biophyi 1988; 365(2):362-72.
33. Leijon, G. and Boivie, J., Central post-stroke pain: a controlled trial of amitriptyline and carbamazepine, Pain. 1989; 36 27–36.
34. R.B. Messing, L.D. Lytle, Serotonin containing neurons: their possible role in pain and analgesia, Pain. 1977; 4: 1–21
35. D.A. Brase, Roles of serotonin and g-aminobutyric acid in opioid effect, Adv. Biochem. Psychopharmacol 1979; 20: 409–428.

36. Cameron NE, Cotter MA: Effects of antioxidants on nerve and vascular dysfunction in experimental diabetes. Diabetes Res Clin Pract. 1999; 45:137–146.
37. Archana R, Namasivayan A. Antistressor effect of Withania somnifera. *J* Ethnopharmacol1999; 64:91-93.
38. Shah PC, Trivedi NA, Bhatt JD, Hemavathi KG. Effect of Withania somnifera on forced swimming test induced immobility in mice and its interaction with various drugs. Indian J Physiol Pharmacol 2006; 50(4):409-15.
39. Gray AM, Spencer PSJ, Sewell RDE. The involvement of the opioidergic system in the antinociceptive mechanism of action of antidepressant compounds. Br J Pharmacol 1998; 124:669-74.
40. Biegon A, Samuel D. Interaction of tricyclic antidepressants with opioid receptors. *Biochem Pharmacol* 1980**;** 29:460-2.
41. Acton J, McKenna JE and Melzack R, Amitriptyline produces analgesics in the formalin test, Exp Neurol 1992; 117:94-96
42. Cameron NE, Cotter MA, and Maxfield EK: Anti-oxidant treatment prevents the development of peripheral nerve dysfunction in streptozotocin-diabetic rats. *Diabetologia* 36:299 –304, 1993