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Antinociceptive and Neuroprotective Activity of Withania Somnifera (WS) Extract In Rat Model of Vincristine Induced Peripheral Neuropathy

Arunkumar Choulam

ABSTRACT

Peripheral neuropathy is defined as damage to the peripheral nervous system resulting in a syndrome of sensory loss, muscle weakness and atrophy along with vasomotor symptoms, alone or in any combination.
The aim of the present study is to evaluate the Antinociceptive and neuroprotective activity of Withania somnifera (WS) extract in rat model of Vincristine induced peripheral neuropathy.
This study accomplished that the WS extract improved body weights of the rats which were changed during the experiment, nociceptive threshold estimated to the extent of neuropathic pain by assessing the screening procedures; paw and tail cold allodynia, tail immersion, tail flick, paw heat hyperalgesia, motor in-co-ordination and mechanical hyperalgesia. Withania somnifera extract reversed the vincristine induced anemia, leucopenia and thrombocytopenia in rats assessed by estimating the parameters- RBC, WBC, Haemoglobin percentage, hematocrit, platelet count and MCV. Vincristine induced oxidative stress (increased TBARS and decreases gluthathione) was attenuated by WS extract which was estimated in sciatic nerve homogenate. The nerve fiber dearrangement which was seen in the histopathological study of sciatic nerve samples against the vincristine induced peripheral neuropathy was reversed by Withania somnifera extract. Administration of WS extract attenuated the physical, behavioral, hematological, biochemical, histopathological parameters in vincristine induced neuropathy.
Withania somnifera extract administration is claimed to be useful in the treatment of pain associated with peripheral neuropathy which may be attributed to antinociceptive, immunomodulator, antioxidant, neuroprotective activities supports the ethno-pharmacological activity of WS.

Key-words: neuropathy, Antinociceptive, Withania Somnifera,

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INTRODUCTION:-

Peripheral neuropathy is damage to nerves of the peripheral nervous system, which may be caused either by diseases of or trauma to the nerve or the side effects of systemic illness. Peripheral neuropathy is defined as damage to the peripheral nervous system resulting in a syndrome of sensory loss, muscle weakness and atrophy along with vasomotor symptoms, alone or in any combination (Rubin M, 2008).

The control of neuropathic pain is currently a significant clinical challenge. This painful syndrome results from disorders in somatosensory system and the most prominent symptoms include allodynia (pain due to a stimulus which does not normally provoke pain), and hyperalgesia (an increased response to a stimulus which is normally painful) (Torrance N et al., 2006). These sensations can be provoked by both mechanical and thermal (heat or cold) stimulation. Treatment of pain in neuropathies is often unsatisfactory (Bouhassira D et al., 2008). However, this may not only be the result of a lack of adequate drugs. Out of 151 patients with neuropathic pain of diverse origin (55.6% diabetic neuropathies) completing a questionnaire, 72.8% complained of inadequate pain control (Alder AL et al., 1997). In clinical practice, it has been extensively reported that neuropathic pain is difficult to treat, due to an inadequate understanding of the cellular and molecular mechanisms involved in the development and maintenance in this type of pain. The range of therapeutic options for the control of neuropathic pain has increased over the last few years, although the response of neuropathic pain patients too many of the treatments is not satisfactory. Currently, the treatments available in clinical practice include antidepressants (Butler S et al., 1984), anticonvulsants (Thompson M et al., 1985), topical agents, local anaesthetics, non-narcotic (Britell CW et al., 1986) and narcotic analgesics (Rowbotham MC et al., 2003), antiarrhythmics and neurosurgical lesions. In addition to the limited relief of neuropathic pain obtained with these drugs, they often present a low therapeutic window, and the control of pain is frequently associated with the inability of the patient to tolerate the side effects.

Several herbal medicines such as Aconiti tuber (Xu H et al., 2006), Lindera angustifolia, Sesbania sesban (Carlton et al., 2009; Muthuraman et al., 2010), Teucrium polium, Phyllanthus emblica, Vochysia divergens (Bortalanza LB et al., 2002), Cannabis sativa, Tacrolimus (Sood S *et al,* 2010), Nigella sativa, Ocimum sanctum (Muthuraman A, Singh N et al., 2008), and Ginkgo biloba (Shah ZA et al., 2011) are shown to have potential in different types of experimentally induced neuropathic pains. Some clinical reports have also advocated beneficial effect of drugs from plant origin in neuropathic pain conditions. Therefore, ample scope of the new herbal medicine to combat the management of neuropathic pain syndromes is expected. One such popularly used plant that is reported to have antitumor (Chandrashekar A et al., 1996), radiosensitizer (Devi PU et al., 1996), antistressor (Archana R et al., 1999), immunomodulatory (Ziauddin M et al., 1996), anti-inflammatory (Umadevi P 1996) and antibacterial effects is *Withania somnifera Dunal*, which is commonly known as '*Ashwagandha*' (Weiner MA et al., 1994). The present study has been designed to investigate the ameliorative potential of *Withania somnifera Dunal* in rat model of Vincristine induced peripheral neuropathy by assessing behavioral, hematological, biochemical and histo-pathological parameters.

In the published literatures, there is no data available regarding the modulation of Vincristine induced peripheral neuropathy by *Withania somnifera Dunal*. Hence the present study is undertaken to validate the model of neuropathic pain induced by Vincristine and the effect of *Withania somnifera* in this model of neuropathic pain.

It is hoped that the innovative information will be delivered about the Antinociceptive and neuroprotective activity of Withania *somnifera Dunal* which may be useful in expansion of novel herbal drug for the treatment of peripheral neuropathy.

The aim of the present study is to evaluate the Antinociceptive and neuroprotective activity of *Withania somnifera* extract in rat model of Vincristine induced peripheral neuropathy.

MATERIALS AND METHODS:-

Experiments were conducted at Jayamukhi College of pharmacy, Warangal. 18 Adult female Wistar rats weighing about (150±10g) were used in the study. The uses of animals in these experiments were authorized and experimental procedures were conducted in conformity with (IAEC) Institutional Animal Ethical Committee (12353P1042/JCP/IAEC/2013) for the care and use of animals were strictly followed throughout the study. Throughout the experiment, these experimental rats were processed in accordance with the CPCSEA guidelines.

VIVARIUM CONDITIONS:

Housing: The animals were housed in groups of three in polypropylene cages. The lids were made of strong steel mesh, and were designed to contain feed hopper and accommodation to hold drinking water bottles. The

rats were acclimatized to the laboratory environment for a week before commencement of the experiment. The animals were handled one time a day for 5 minutes for 1 week previous to testing.

Bedding: The clean paddy husk was used for bedding. Bedding was changed every alternate day by trained personnel to maintain proper hygienic condition.

Feeding: The animals were fed with commercially available standard pellet diet and filtered tap water provided *ad libitum*. Animals were also maintained under controlled temperature at (22±2^{0}C) and relative humidity of 50-60% with an alternating 12hr light-dark cycle (light on 6:00-18:00 hr) was maintained in the animal house.

CHEMICALS AND DRUGS USED:

- 5-5'dithio bis-2-Nitro benzoic acid (DTNB) reagent, Trichloroacetic acid, Thiobarbituric acid, Glutathione, Orthophosphoric acid, Phenol reagent (Folin and Ciocalteau's Reagent) were procured from Himedia Laboratories Ltd, Hyderabad.
- Vincristine was procured from Mahatma Gandhi Memorial hospital, MG road, Warangal.
- Bovine serum albumin was procured from Zeal Chemicals, Hanamkonda, Warangal.
- The extract of *Withania somnifera* gift sample was provided by Sami Labs Limited, Bangalore.

METHODOLOGY:

Drug solution preparation

1 Preparation of Vincristine:

Based on the previous studies for induction of neuropathic activity dose of Vincristine, 50µg/kg b.w., i.p. was selected. As per demand, the drug was prepared further throughout the experimental period in water for injection.

2 Preparation of Withania somnifera extract:

Based on the Acute oral toxicity studies, 100mg/kg b.w., p.o. of withanolides from the extract of W*ithania somnifera* was selected as a dose and i.e., 100mg/kg b.w., p.o. and was dissolved in water for injection as per to get the required dose. The solutions were freshly prepared every day before dosing the animals.

Experimental Work

Acute Oral Toxicity Study:

A preliminary pharmacological study of *Withania somnifera* extract was conducted to assess the gross behavioral effects and safety effects of the drug. The animals were fasted prior to the dosing, food but not water and were with held overnight. Following the period of fasting, the animals were weighed and test substance was administered. After the substance was administered, food was withheld for a further 3-4 hrs. Fixed dose method was adopted as per O.E.C.D. Guideline No 423: (Annexure -2) of CPCSEA.

Three animals were used for each step. The dose level of extracts and formulation to be used as the starting dose was selected from one of the four fixed levels 50, 500, 1000 and 2000mg/kg b.w. p.o. The starting dose level was most likely to produce mortality in some of the dosed animals.

After administration of test sample, the animals were observed continuously for first 4 hrs for behavioral, neurological and autonomic profile changes and the end of 24 hrs for mortality rate during the acute toxicity studies.The therapeutic dose of the drug was considered as 1/10th of effective dose.

METHODS EMPLOYED IN SCREENING OF VINCRISTINE INDUCED PERIPHERAL NEUROPATHY

Vincristine induced peripheral neuropathy:

Antineoplastic agent, vincristine one of the most commonly used chemotherapeutic drugs used to treat a variety of malignant diseases including leukemia and lymphoma, and to prevent tumor cell replication, produces peripheral nociceptive neuropathy after toxic injury of the peripheral nervous system. This model displays all the characteristics of a peripheral sensory neuropathy, i.e. behavioural signs as mechanical and thermal allodynia, mechanical and thermal hyperalgesia, and is associated with significant electrophysiological and histological disorders. Vincristine is known to affect the structure and function of peripheral nerve fibres. It has a high binding affinity to the protein subunit of microtubules, tubulin, causing the dissolution of axonal microtubules and encouraging the proliferation of neuro filaments.

Treatment schedule: For evaluation of Vincristine induced peripheral neuropathy in 18 animals were assigned into three groups of six animals each (n=6).

Grouping and treatment protocol: Three groups of animals were made, each group consisting of six Rats. The following were the groups.

Table 4.1: Grouping and treatment protocol

Group No	Groups	Drug	Dose	Route of administration
Group 1	Control	Water for injection	5ml/kg	i.p
Group 2	Neuropathy	Vincristine	50µg/kg	i.p
Group 3	Neuropathy+ Withania	Withania somnifera + Vincristine	100mg/kg+50µg/kg	p.o.+ i.p

PHYSICAL EXAMINATION

Determination of Body Weights: Animal were taken and they were marked accordingly. These animals were then placed on the weighing balance and the individual weights were noted down. Body weights of all the animals in each group were determined on the 0 day, 7th day, 14th day of the experimental period and differences in weights were observed.

BEHAVIORAL EXAMINATION

1. On the 14th day of the experiment the rats were placed on the top of a wire mesh floor, allowing access to the hind paws of animals one by one and allowed to habituate for 30min prior to testing.
2. Acetone 100ul (0.1 ml) was applied with a glass syringe fitted with a blunted needle at the centre of the plantar face of a hind paw of rat, without touching the skin.
3. The time taken to appear the cold sensitive reaction with respect to either paw licking, shaking or rubbing the hind paw was recorded within 20 seconds.

Tail cold allodynia:

1. On the 14th day the experiment tail cold allodynia test was performed.
2. The rat was gently held and the terminal part of the tail (1 cm) was immersed in a rectangular Plexiglas container filled with water which was maintained at the right temperature 0-4°C by the addition of ice cubes.
3. The duration of the tail withdrawal reflex was recorded.
4. A cut of latency of 40 seconds was kept to avoid tail injury.

Motor co-ordination (Rota-rod test):

1. Motor co-ordination test was performed on 14th day of the experiment.
2. The rats were placed one by one on the rotating rod that was set to 20-25 rpm and the performance time that each rat was able to remain on the rota-rod i.e., the 'fall of time' when the animal falls from the rotating rod was recorded.
3. The cut-off time of 60 seconds was maintained

Tail Immersion Test:

1. Tail immersion test was performed on the 14th day of the experiment.
2. Rats were placed into individual cylindrical rat holders or handled normally leaving the tail hanging out freely.
3. Lower 5 cm portion of the tail was marked and the terminal part of the tail (1cm) was immersed in a beaker of freshly filled water maintained at exactly a temperature of 50ºC ± 0.5ºC.
4. The duration of tail withdrawal reflex or signs of struggle was recorded as response of heat sensation.
5. A cut off time of 20 seconds was observed for tail immersion so as to avoid the damage to tail of an animal.

Mechanical hyperalgesia (Pin prick test):

1. On the 14th day of the experiment mechanical hyperalgesia test using pin prick was performed.
2. The plantar surface of the left hind paw was touched with the point of the bent 18 gauge needle (at 90^0 angles) at intensity sufficient to produce a reflex withdrawal response in rats, but at an intensity which was insufficient to penetrate the skin.
3. The duration of the paw withdrawal was recorded in seconds for all the animals.
4. A cut-off time of 20 seconds was maintained to avoid the paw damage.

Paw Heat-Hyperalgesia (Hot plate):

1. Paw heat hyperalgesia using Eddy's hot plate method was carried out on the 14th day of the experiment.
2. The rats were placed one after another on the top of a controlled preheated (52.5 ± 0.5ºC for thermal hyperalgesia) and maintained hot plate surface, allowing access to the hind paw withdrawal response to degree of the nociceptive threshold.
3. The reaction time was taken by observing hind paw licking or jump response (which ever appears first) in animals when placed on the hot plate maintained at constant temperature.
4. A cut off period of 20 seconds was observed to avoid damage to paws.

Tail Flick Method:

1. Tail flick method on 14th day of experiment was executed using Analgesiometer.
2. Rats were placed in cylindrical rat holders leaving the tail hanging out.
3. The reaction time to radiant heat was taken by placing the terminal (junction between proximal 2/3 and distal 1/3 i.e last 1-2 cm) portion of the tail on the radiant heat source which is produced by heating the tantalums wire.
4. The tail-withdrawal from the heat (flicking response) was taken as the end point and time was noted.
5. A cut off period of 20 seconds was observed then tail was removed from the source of heat to avoid damage to avoid tissue damage.

HEMOGRAM

1. The cover slip, counting chamber and the lenses of microscope were cleaned first with the help of xylol and then with absorbent cotton.
2. The counting chamber was adjusted and observed or RBC squares under low power microscope, keeping the cover slip resting on the platform of the slide.
3. The objective of microscope was raised and then it was adjusted for high power and the chamber of RBC square was adjusted under high power. The RBC pipette was then cleaned and dried.
4. The blood drops were sucked in the RBC pipette up to the mark 0.5 immediately the RBC dilution fluid was sucked up to the mark 101.
5. The pipette was brought to a horizontal position and the tail was placed over the tip of the pipette. A simple knot was given to the rubber tube. The pipette was rolled over the palms to mix the blood with the dilution fluid for one minute.
6. Few drops (2-3) are discarded and then pipette was held at the angle of 45º to the surface of the counting chamber and it was applied to the narrow slit between the counting chamber and the cover slip. A drop was allowed to come out from the pipette. The fluid would run into the capillary space because of the capillary action and it was filled.
7. The fluid was allowed to settle for 2-3 minutes on the stage of microscope.
8. The RBC chamber was located and the RBCs are counted in smaller squares. This was repeated in another four such chambers

Calculation of red blood cells:

$$\text{Total RBC/mm}^3 = \text{Number of RBC counted} \times \text{Dilution factor} \times \frac{\text{Depth factor}}{\text{No. of chambers counted}}$$

White blood cell count (WBC):

1. The cover slip, counting chamber and the lenses of microscope were cleaned first with the help of xylol and then with absorbent cotton.
2. The counting chamber was adjusted and observed or WBC squares under low power microscope, keeping the cover slip resting on the platform of the slide.
3. The objective of microscope was raised and then it was adjusted for high power and the chamber of WBC square was adjusted under high power. The WBC pipette was then cleaned and dried.
4. The blood drops were sucked in the WBC pipette up to the mark 0.5 immediately the WBC dilution fluid was sucked up to the mark 11.
5. The pipette was brought to a horizontal position and the tail was placed over the tip of the pipette. A simple knot was given to the rubber tube. The pipette was rolled over the palms to mix the blood with the dilution fluid for one minute.

6. Few drops (2-3) are discarded and then pipette was held at the angle of 45° to the surface of the counting chamber and it was applied to the narrow slit between the counting chamber and the cover slip. A drop was allowed to come out from the pipette. The fluid would run into the capillary space because of the capillary action and it was filled.
7. The fluid was allowed to settle for 2-3 minutes on the stage of microscope.
8. The WBC chamber was located and the WBCs in 16 small squares of WBC chamber are counted. This was repeated in another four such chambers.

Calculation of white blood cells:

$$\text{Total WBC/mm}^3 = \frac{\text{Number of WBC counted} \times \text{dilution factor} \times \text{depth factor}}{\text{No. of chambers counted}}$$

Haemoglobin estimation (Hb %):

1. The graduated diluting tube and the micropipette were cleaned thoroughly and dried.
2. The graduated tube was filled with 0.1N HCl up to the mark or till the micropipette touches the level of acid in the tube.
3. The blood is sucked in the haemometer pipette exactly up to 20 cm mark.
4. The blood was immediately deposited at the bottom of the graduated tube. The pipette was rinsed two to three times in HCl.
5. The blood was mixed with the help of stirrer and the solution was allowed to stand for 10-15 minutes, so that the haemoglobin was converted into acid haematin.
6. The mixture was then diluted with distilled water drop by drop each time mixing the solution with the stirring rod until the colour of the solution matches the standards on either side.
7. Stirring rod was removed before comparing the tubes. Matching should be done against natural light or fluorescent tube light.
8. The level of the fluid at its lower meniscus was noted and the reading on the scale corresponding to this level was read as gram/dl.

Hematocrit (Hct):

1. Platelets were counted by the direct method using Rees Ecker diluting fluid.
2. The tip of the tail was shaved, and then scrubbed cleanly.
3. Using scissors about 1 cm of the tail was clipped off, the skin on the stump pushed forward and the bared tip quickly immersed in the solution and a single drop of blood allowed flowing into the diluting fluid.
4. The diluting fluid was drawn in a red cell pipette to the 0.5 mark, and a freely flowing drop of blood from the tail was drawn to the 1.0 mark.
5. The rest of the pipette was then filled to the 101 mark with diluting fluid.
6. After shaking for approximately three minutes a drop was mounted in a standard haemocytometer where the platelets were allowed about 15 minutes to settle and then counted as for a red cell count.
7. The platelets were easily recognized as small dark refractile objects exhibiting Brownian movement.

Mean cell volume (MCV):

All the groups of rats after 14th day were euthanized using ketamine followed by cervical dislocation. The death was confirmed by palpating for lack of heartbeat prior to beginning dissection. The dissection area was swapped with 70% ethanol and the hairs were neatly shaved. The skin was cut and right sciatic nerve was exposed through a gluteus muscle splitting incision (Jain V et al., 2009). The sciatic nerve was exposed from its origin in the lumbar spine to distal to the trifurcation at knee level, to acquire a maximum length. The uniformity among the different nerve samples was maintained by taking the constant weight of the respective samples (Wang HD et al., 1998). The excised sciatic nerve homogenate (10% w/v) was prepared with 0.1 M Tris-HCl buffer (pH 7.4). The tubes with homogenate were kept in ice water for 30 minutes and centrifuged at 4°C (2500 rpm, 10 min). The supernatant of homogenate thus obtained was separated, used to assay and estimate total protein content, Thio-barbituric acid reactive substances (TBARS), reduced glutathione.

ESTIMATION OF PROTEIN CONTENT:

To 0.1 ml of serum/pleural fluid (tissue homogenate) 1 ml of alkaline mixture reagent was added and kept for 10 minutes, then 4 ml of phenol reagent was added, heated at 55°C for 5 minutes for color development and then cooled for 1 minute. Reading was taken against blank at 610 nm using spectrophotometer. The protein

content was calculated from standard curve prepared with bovine serum albumin and expressed in terms of mg protein/gm of wet tissue.

ESTIMATION OF GLUTATHIONE CONTENT:

Reduced glutathione was measured according to the method of Beutler et al. 1963. To 0.1 ml of supernatant, 1 ml of sodium phosphate buffer and 0.5 ml of DTNB reagent were added. The absorbance of the yellow color developed was measured at 412 nm within 15 min. The concentration of reduced glutathione was expressed as µg/mg of protein. The glutathione content was determined from standard graph by using pure glutathione.

ESTIMATION OF THIO-BARBITURIC ACID REACTIVE SUBSTANCES

To 0.5 ml of brain homogenate 0.5 ml of 30% trichloro acetic acid (TCA) was added to precipitate the proteins and vortexed for 30 sec. Clear supernatant was taken after centrifuging at 3000 rpm for 10 min. To the supernatant, 500µl of 1%TBA solution and 500µl of water was added and this solution was heated for 1hr at 98°C. Cool the solutions to room temperature and kept them in ice for 5 minutes. Then read the pink color at 532 nm using spectrophotometer. Standard graph was plotted using TEP (1,1,3,3-tetra ethoxy propane).

HISTOPATHOLOGY OF SCIATIC NERVE

Samples of sciatic nerve stored in the fixative solution 10% formalin and the samples of sciatic nerve tissue were paraffin embedded, cut into thin sections with 4 µm thickness size. Staining was done by using hematoxylin and eosin (Yukari et al., 2004) as before examination using light microscopy. Nerve sections were examined under light microscope (100×) for the observation of axonal degeneration.

STATISTICAL ANALYSIS:

All the results were expressed as mean ± standard deviation. The data from the physical, behavioral, hematological results were statistically analyzed by one-way analysis of variance followed by Bonferonni's post hoc test by using Graph pad prism Version-5.0 software.

The data from the biochemical results were statistically analyzed by one-way ANOVA followed by Bonferonni's post hoc tests. The p-value <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION:-

PHYSICAL EXAMINATION

Whenever the chemotherapeuthic agent such as Vincristine was used used in management of cancers especially Hodgkins lymphoma, non-Hodgkins lymphoma and leukemia it caused loss of body weight. This parameter was attenuated by concurrent administration of *withania*. During the experimental period the hair loss was scrutinized this alopecic effect of vincristine was invalidated by WS extract.

Effect of *Withania somnifera* extract on body weights of rats

Table 5.1: Effect of *Withania somnifera* on body weights of rats in vincristine induced peripheral neuropathy

S.no	Treatments	Dose	Animal body weights(g) on different days		
			0 day	7th day	14th day
1	Control (water for injection)	5ml/kg, i.p	148.5 ± 3.27	152.2 ± 2.78	155.7 ± 2.16
2	Neuropathy	50 µg/kg, i.p	149.7 ± 2.16ns	145.7 ± 2.16a1,ns	142.2 ± 2.31a,ns
3	Neuropathy + Withania somnifera	50 µg/kg, i.p + 100 mg/kg, p.o	150.8 ± 3.65 ns	149.8 ± 1.72ns,b1	151.5 ± 2.42a2,b

BEHAVIORAL EXAMINATION:

It has been hypothesized that vincristine-induced neuropathic pain is due to neuronal toxicity and/or neurological disorder, the exact mechanism responsible is still unknown. Recently, (Weng HR et al., 2003)

suggested a state of central sensitization develops in spinal wide dynamic range neurons with repeated vincristine treatment that contributes to the neuropathic pain.

Withania somnifera was also highly effective against static, cold and dynamic mechanical allodynia. It is generally admitted that small diameter C- and Aδ-fibres are mainly involved in the response to cold stimuli and in the response to intense mechanical stimuli whereas large Aβ-fibres (low threshold fibres) respond to tactile stimuli. Vincristine treatment-induced neuropathy involved degeneration of myelinated fibres; but unmyelinated fibres also degenerate since occasionally swollen nonmyelinated fibres (Sahley TL et al., 1979) and a significant decrease in microtubule density suggesting swelling unmyelinated axons have been described (Shibata M et al., 1989). The present results showed that enkephalins protected from degradation by *Withania somnifera* could bind to opioid receptors expressed only on C- and Aδ-mechanonociceptors and also on cold thermonociceptors. The fact that *Withania somnifera* is also active on small intensity mechanical stimulus (pin prick) could reveal an expression of opioid receptors on large Aβ-fibres (low threshold mechanoreceptors) in the pathological conditions resulting from vincristine treatment. In fact it has been shown that opioid analgesics can act outside the central nervous system, targeting opioid receptors expressed at sites of peripheral damaged tissue. Besides this enkephalins were reported to be released from leukocytes recruited in injured tissue. In neuro inflammatory and neuropathic conditions the phasic increase of locally secreted enkephalins could interact with opioid receptors located on sensory endings. As in the central nervous system, the dual inhibitors could enhance the amounts of enkephalins at the peripheral receptor level thus potentiating the antinociceptive response.

Apart from this *Withania somnifera* may interact with other ion channels expressed in sensory neurons, and that the cold sensation appears to involve potassium channels in the transduction and modulation of temperature information. In this regard, it is important to mention that *Withania somnifera* induced antinociception also involves the opening of voltage- and small-conductance Ca^{2+}-gated K^{+}channels (Lehning EJ et al., 1996). *Withania somnifera* reduces the ongoing mechanical allodynia and heat hyperalgesia in response to the vincristine induced peripheral neuropathy in rats which may be due to its interference with calcium concentration through inhibition of voltage activated $Ca^{2+}$and/or K^{+} channels in a PKC-dependent fashion, which suggests that *WS* could modulate other ion channels involved in painful conditions. The present data advocates that *WS* promotes antinociceptive and anti-allodynic actions could reflect a modulation of $Ca^{2+}$and/or K^{+} ion channels as well as an indirect or direct inhibition of the PKC signalling mechanisms.

Effect of *Withania somnifera* extract on paw cold allodynia (Acetone drop test)

Table 5.2: Effect of *Withania somnifera* on paw cold allodynia test in rats in vincristine induced peripheral neuropathy

S.no	Treatments	Dose	Latency of withdrawal response (Sec)
1	Control (water for injection)	5ml/kg, i.p	6.13 ± 0.57
2	Neuropathy	50 µg/kg, i.p	2.76 ± 0.57[a]
3	Neuropathy + *Withania somnifera*	50 µg/kg, i.p + 100 mg/kg, p.o	12.2 ± 0.59[a,b]

Effect of *Withania somnifera* extract on tail cold-allodynia:

Table 5.3: Effect of *Withania somnifera* on tail cold allodynia in rats in vincristine induced peripheral neuropathy

S.no	Treatments	Dose	Latency of withdrawal response (Sec)
1	Control (water for injection)	5ml/kg, i.p	29.2 ± 0.79
2	Neuropathy	50 µg/kg, i.p	17.1 ± 0.59[a]
3	Neuropathy + *Withania somnifera*	50 µg/kg, i.p + 100 mg/kg, p.o	37.3 ± 0.58[a,b]

Effect of *Withania somnifera* extract on motor co-ordination (Rota-rod test)

Table 5.4: Effect of *Withania somnifera* on motor co-ordination test in rats in vincristine induced peripheral neuropathy

S.no	Treatments	Dose	Latency of fall off time (Sec)
1	Control (water for injection)	5ml/kg, i.p	37.3 ± 0.70
2	Neuropathy	50 µg/kg, i.p	17.5 ± 0.47[a]
3	Neuropathy + *Withania somnifera*	50 µg/kg, i.p + 100 mg/kg, p.o	39.1 ± 0.70[a,b]

Effect of *Withania somnifera* extract on tail immersion test (tail heat hyperalgesia)

Table 5.5: Effect of *Withania somnifera* on tail immersion test in rats in vincristine induced peripheral neuropathy

S.no	Treatments	Dose	Tail withdrawal latency (Sec)
1	Control (water for injection)	5ml/kg, i.p	6.92 ± 0.70
2	Neuropathy	50 µg/kg, i.p	3.04 ± 0.42[a]
3	Neuropathy + *Withania somnifera*	50 µg/kg, i.p + 100 mg/kg, p.o	5.28 ± 0.76[a1,b]

Effect of *Withania somnifera* extract on mechanical hyperalgesia (Pin prick test)

Table 5.6: Effect of *Withania somnifera* on mechanical hyperalgesia in rats in vincristine induced peripheral neuropathy

S.no	Treatments	Dose	Latency of paw withdrawal (Sec)
1	Control (water for injection)	5ml/kg, i.p	10.7 ± 0.48
2	Neuropathy	50 µg/kg, i.p	2.31 ± 0.48[a]
3	Neuropathy + *Withania somnifera*	50 µg/kg, i.p + 100 mg/kg, p.o	9.34 ± 0.83[a1,b]

Effect of *Withania somnifera* extract on paw heat-hyperalgesia (hot plat test)

Table 5.7: Effect of *Withania somnifera* on paw heat-hyperalgesia in rats in vincristine induced peripheral neuropathy

S.no	Treatments	Dose	Latency of paw licking (Sec)
1	Control (water for injection)	5ml/kg, i.p	10.80 ± 0.398
2	Neuropathy	50 µg/kg, i.p	4.12 ± 0.651[a]
3	Neuropathy + *Withania somnifera*	50 µg/kg, i.p + 100 mg/kg, p.o	15.64 ± 0.379[a,b]

Effect of *Withania somnifera* extract on tail flick test

Table 5.8: Effect of *Withania somnifera* on tail flick test in rats in vincristine induced peripheral neuropathy

S.no	Treatments	Dose	Latency of tail withdrawal (Sec)
1	Control (water for injection)	5ml/kg, i.p	6.27 ± 0.48
2	Neuropathy	50 µg/kg, i.p	2.99 ± 0.49[a]
3	Neuropathy + *Withania somnifera*	50 µg/kg, i.p + 100 mg/kg, p.o	5.11 ± 0.31[a,b]

HAEMOGRAM:

Chemotherapy for malignancy results in depletion of hematopoietic stem and progenitor cells and the bone marrow stroma (myeloablation), with depression of blood formation until the hematopoietic tissue bed repairs and reconstitutes.

Anemia is a common complication of myelosuppressive chemotherapy that results in a decreased functional capacity and quality of life (QOL) for cancer patients. Repeated cycles of chemotherapy may impair erythropoiesis cumulatively which causes decreases the blood cell count.

Chemotherapeutic agents such as vincristine damage both dividing cells and resting cells, and cause myelosuppression (Kaley TJ and Deangelis LM, 2009) which can result in reduction of erythropoietin, and in turn consequently interferes with erythropoiesis and may cause anemia... This effect of vincristine was subsequently vitiated by *withania* which increased the RBC count, hemoglobin percentage and PVC.

Leucopenia is mainly caused by bone marrow depression with Chemotherapeutic agents such as vincristine it causes damage to pluripotent myeloid stem cells and committed myeloid progenitor cells (Abrey LE et al., 2005), which cause decreased neutrophil production and this may lead to leucopenia or neutropenia. This chemotherapy aggressiveness was abated by the use of *Withania somnifera.*

The apoptosis of megakaryocyte progenitors is the major cause of chemotherapy-induced thrombocytopenia and our study suggest that *withania* was efficacious to prevent platelet loss may be by expedite recovery of these bone marrow cells.

The major activity of *Withania* may be the stimulation of stem cell proliferation by which it is dissipating, the effect of vincristine induced anemia, leucopenia and thrombocytopenia.

Table 5.9 : Effect of *Withania somnifera* on haematological tests in rats in vincristine induced peripheral neuropathy

Groups	RBC(x10^6 cell/mm^3)	WBC(x10^3 cells/mm^3)	Haemoglobin (%)	Platelet count (x10^5cells/mm^3)	Hematocrit (HC)	Mean cell volume
Control	5.75 ± 0.18	11.0 ± 0.31	14.8 ± 0.40	68.10 ± 0.56	43.3 ± 0.45	53.2 ± 1.47
Neuro-pathy	4.82 ± 0.23^a	9.20 ± 0.28^a	13.4 ± 0.37^a	57.10 ± 0.61^a	38.0 ± 0.73^a	45.5 ± 1.05^a
Neuro-pathy + *Withania somnifera*	6.15 ± 0.21^{a_1}	10.50 ± 0.45^{a_2,b}	14.2 ± 0.25^{a_1,b_1}	63.40 ± 0.52^{a,b}	41.1 ± 0.72^{a,b}	48.3 ± 1.37^{a,b_1}

BIOCHEMICAL ESTIMATION:

Chemotherapeutic agents induce mitochondrial changes and apoptosis through mechanisms associated with lipid peroxidation/ROS production (Dario S et al., 2007). Anticancer agents can cause mitochondrial permeabilization through enhanced generation of lipid peroxidation, and once the mitochondrial membrane barrier function is lost, several factors contribute to cell death. Whereas lipid peroxidation, among other factors, induces or facilitates mitochondrial permeabilization, glutathione and antioxidant enzymes inhibit it.

If generation of ROS by a cancer chemotherapeutic agent or a free radical intermediate of the drug plays a role in its cytotoxicity, antioxidants may interfere with the drug's antineoplastic activity (Wang HD et al., 1998). However, if the reactive species are responsible only for the drug's adverse effects, antioxidants may actually reduce the severity of such effects without interfering with the drug's antineoplastic activity. Glutathione is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals (Feresin GE et al., 2002) and peroxides. It is the major endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds. Antineoplastic agent vincristine generates a high level of oxidative stress in biological systems. This oxidative stress was decreased by increasing the glutathione levels when *WS* was administered.

During cancer chemotherapy, oxidative stress-induced lipid peroxidation generates numerous electrophilic aldehydes that can attack many cellular targets**.** As an index of free radical generation (Kamei et al., 2005) Thiobarbituric acid reactive substances - TBARS – which is formed as a byproduct of lipid peroxidation (Balanehru S and Nagarajan B, 1991) is used as markers for oxidative stress. These products of oxidative stress can slow cell cycle progression of cancer cells and cause cell cycle checkpoint arrest, effects that may interfere with the ability of anticancer drugs to kill cancer cells. The aldehydes may also inhibit drug-induced apoptosis

by inactivating death receptors and inhibiting caspase activity. These effects were diminished by *Withania somnifera*. It may enhance therapy by reducing the generation of oxidative stress-induced aldehydes.

Histopathology of sciatic nerve:

Vincristine caused localized axonal toxicity as a cause of distal axonal degeneration as it is consistent with the largely predominant axonal damage. It caused the degeneration of myelinated and unmyelinated fibers which was pathologically correlated with neuropathic pain assessed with behavioral tests. The axonal swelling and the dearrangement of nerve fibres due to oxidative stress or metabolic derangement seen with the vincristine was condensed by *withania somnifera*. Thus *withania* offered resistance against the axonopathy caused by vincristine by its neuroprotective or antioxidant effect.

Effect of *Withania somnifera* extract on oxidative stress markers and reduced glutathione in rats in vincristine induced peripheral neuropathy

Table 5.10 : Effect of *Withania somnifera* on biochemical tests in rats in vincristine induced peripheral neuropathy

Groups	Total Protein (mg/gm of tissue)	GSH (µg/mg of protein)	TBARS(ng of MDA/mg protein)
Control	5.55 ± 0.148	73.95 ± 1.520	4.97 ± 0.160
Neuropathy	6.11 ± 0.090[a]	39.20 ± 1.413[a]	8.08 ± 0.315[a]
Neuropathy + *Withania somnifera*	5.82 ± 0.058[a1,b]	61.18 ± 1.166[a,b]	5.74 ± 0.234[a,b]

HISTOPATHOLOGY OF SCIATIC NERVE

Effect of *Withania somnifera* extract on histopathological changes of sciatic nerve in vincristine induced peripheral neuropathy

Vincristine treatment (50µg/kg i.p.) produced peripheral neuropathy and histopathological changes assesed in cross section as well as transverse sections of sciatic nerve. These histopathological changes in sciatic nerve noted by its axonal swelling, axonal degeneration and fibers derangements. However, concurrent administration of extract of *Withania somnifera* (100 mg/kg p.o.) attenuated vincristine induced nerve fiber dearangement as marker of histopathological alterations in sciatic nerve tissue sections.

A **B** **C**

Figure 5.1: Effect of *Withania somnifera* on histopathological changes in cross sections of sciatic nerve in vincristine induced neuropathy

A, B and C are the cross-sections of the sciatic nerve of normal rat, Control, Vincristine treated and Vincristine + *Withania somnifera* treated groups respectively. In figure A shows axonal arangement; in figure B shows axonal degeneration and sweling, while in figure C show attenuation of vincristine induced axonal degeneration.

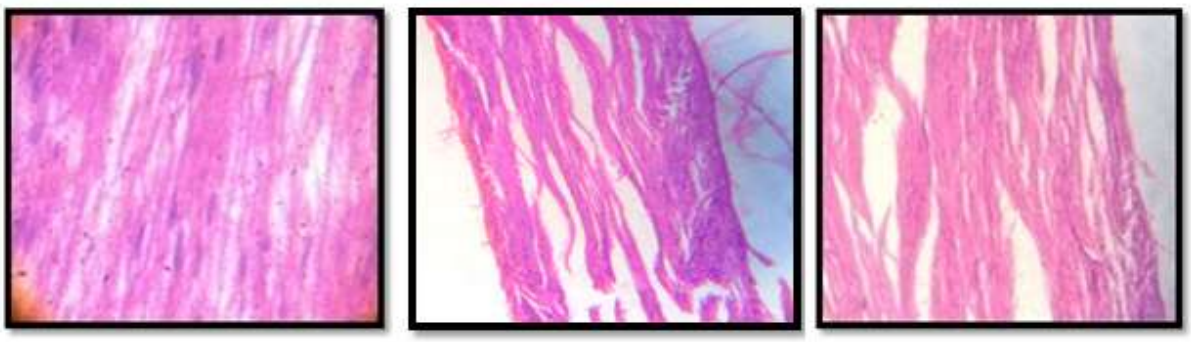

Figure 5.2: Effect of *Withania somnifera* on histopathological changes in longitudinal-sections of sciatic nerve in vincristine induced neuropathy

A, B and C are the longitudinal-sections of the sciatic nerve of normal rat, Control, Vincristine treated and Vincristine + *Withania somnifera* treated groups respectively. In figure A shows nerve fiber arrangement; in figure B shows nerve fiber derangement, while in figure C show attenuation of vincristine induced nerve fiber derangement.

CONCLUSION:-

The present study evaluated the antinociceptive, immunomodulator, anti-oxidative, neuroprotective potential of extract of *Withania somnifera* in rat model of vincristine induced peripheral neuropathy by assessing physical, behavioral, hematological, apart from this biochemical and neuro-physiological parameters in sciatic nerve samples also revealed.

This study accomplished that the *WS* extract improved body weights of the rats which were changed during the experiment, nociceptive threshold estimated to the extent of neuropathic pain by assessing the screening procedures; paw and tail cold allodynia, tail immersion, tail flick, paw heat hyperalgesia, motor in-co-ordination and mechanical hyperalgesia.

Withania somnifera extract reversed the vincristine induced anemia, leucopenia and thrombocytopenia in rats assessed by estimating the parameters- RBC, WBC, Haemoglobin percentage, hematocrit, platelet count and MCV.

Vincristine induced oxidative stress (increased TBARS and decreases gluthathione) was attenuated by *WS* extract which was estimated in sciatic nerve homogenate.

The nerve fiber dearrangement which was seen in the histopathological study of sciatic nerve samples against the vincristine induced peripheral neuropathy was reversed by *Withania somnifera* extract.

Administration of WS extract attenuated the physical, behavioral, hematological, biochemical, histopathological parameters in vincristine induced neuropathy.

Withania somnifera extract administration is claimed to be useful in the treatment of pain associated with peripheral neuropathy which may be attributed to antinociceptive, immunomodulator, antioxidant, neuroprotective activities supports the ethno-pharmacological activity of WS.

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