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# Phytochemical and Pharmacological Evaluation of Ethanolic Extract of *Moringa Oleifera* as Neuroprotective Agent in Vincristine Induced Peripheral Neuropathy

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***Abstract:****The objective of this study was to phytochemically analyze the ethanolic extracts of the leaves of Moringa oleifera and thereafter to evaluate its neuroprotective activity in vincristine induced peripheral neuropathy.The fresh leaves of Moringa oleifera were collected, powdered and extracted with ethanol. Thereafter the dried ethanolic extract was subjected to phytochemical analysis using various analytical techniques primarily GC-MS to identify the bioactive phytochemical components. Acute toxicity studies were also carried out on the extract. After overnight fasting peripheral neuropathy was induced in Wistar rats by intraperitoneal injection of vincristine 100µg/kg/day body weight dissolved in saline (0.1ml/kg/day). Group A served as normal control while group B was considered as neuropathy control. Group C was standard receiving methylcobalamin (50µg/kg i. p) and Group D & E neuropathy animals were treated with the extract, dose of 250 and 500 mg/kg/b. w p.o. respectively. During the investigations, studies were carried out by in-vivo models such as tail flick, acetone spray method, pain sensation test, nerve conduction test as well as proinflammatory and antioxidant studies. At the end of study, animals in all groups were sacrificed, the sciatic nerve was dissected and histopathology was performed. The interpretation of the results was done after subjecting the data obtained from various studies to statistical analysis which included descriptive statistics, ANOVA followed by Tukey's test. Ethanolic extract of Moringa oleifera leaves were phytochemically analyzed and showed presence of bioactive chemicals. The ethanolic extracts produced significant neuroprotective activity it was effective in anti-nociceptive activity, and caused changes in in-vivo anti-oxidants enzyme and nerve conduction. Further significant improvements were noted in pro-inflammatory markers. Histopathology studies indicated amelioration of histological features. In conclusion, the ethanolic extracts of Moringa oleifera leaves showed potential neuroprotective activity against vincristine induced peripheral neuropathy.*

***Key Word****:Moringa oleifera, GCMS, Vincristine, Pro-inflammatory, Anti-oxidant enzyme, Nerve Conduction.*

## I. Introduction

Moringa Oleifera is a valuable plant because of its multiple uses such as a food source in the tropics as well as its medicinal uses. The leaves of Moringa are reported to be of utility in the treatment of eye and ear infections, skin diseases, flu, headaches, scurvy, heart burn, asthma, bronchitis, hyper-glycemia, malaria, dyslipidaemia, syphilis,

pneumonia and diarrhoea. It has also been reported to be useful in hypertension and high cholesterol further reports are available in literature which show its antidiabetic, antimicrobial, antioxidant and thrombolytic activity. The leaves also reduce high blood pressure and cholesterol and acts as an anticancer, antimicrobial, antioxidant, anti-diabetic and anti-atherosclerotic agents. Many therapeutically beneficial effects of the Moringa seeds, flowers, pods and root bark are also available in literature [1-3]. Moringa Oleifera leaves has been extensively studied for its utility in diabetic neuropathy but much of these studies are empirical, further the utility of various extracts of these plants for chemotherapy induced peripheral neuropathy (CIPN) is not reported in literature.

This study is focused on phytochemical investigation of the ethanolic extracts of the leaves of Moringa Oleifera and to study and validate the effect of the ethanolic extract of the leaves on CIPN for which various phytochemical evaluations, pharmacological studies and toxicological studies undertaken are reported herein.

## II. Material And Methods

**Extraction and phytochemical analysis of Moringa oleifera leaves:**

Moringa oleifera leaves were collected from Aurangabad, Maharashtra, India. The plant specimen was identified and authenticated by Prof. Arvind S. Dhabe, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad and Reg. No. Herb. /Bot/2019-2020/264. The powdered leaves were Soxhlet extracted with ethanol [4] and were subjected to preliminary phytochemical screening. The sample collected was subjected to HPTLC (TLC Sampler 3 and Scanner III, Camag, Switzerland) on 10 cm×10 cm silica gel 60- GF254 plates (Merck). The bands were visualized by scanning the plate in a UV scanner (Camag TLC Scanner III) at 560 nm. IR spectra were obtained with a Bruker Alpha-E series FT-IR spectrophotometer with ATR. High-resolution mass spectral data were obtained with an Agilent 6500 series Q-TOF mass spectrometer using electrospray ionization [5-8].

**Experimental animals:**

Wistar rats Male (8-10 weeks old) weighing 150–200g and Albino mice Female (8-10 weeks old) weighing 20-25gm were used for the experiment. They were acclimatized for one week prior to experiment. Animals were caged in fully ventilated room, were maintained in 12:12 h light and dark cycle and were housed at temperature of 25 ± 2°C. They had free access to a standard chow diet and water ad libitum. All the experiments conducted on the animals were in accordance with the standards set for the use of the laboratory animal and the experimental protocols were duly approved by the IAEC of Y. B. Chavan College of Pharmacy, Aurangabad, Maharashtra (Reference Number: CPCSEA/IAEC/48/2019-20/164).

**Acute oral toxicity studies for dose fixation:**

The acute oral toxicity study was performed according to the OECD guidelines No. 425 [9] The animals were fasted overnight and limit test was performed as follows;

The limit test was carried out first at 5000mg/kg body weight for one animal and if animal dies, main test is performed. If the animal survives two more animals are dosed, if both survives the test is terminated, the main test is performed with an initial dose of 175mg/kg body weight. Following sequence was followed 175, 550, 1750 and 5000mg/kg body weights.

First one animal is dosed with 175mg/kg body weight. If animal dies a much lower dose is tested. If animal survives, then two more animals are dosed after 48h observation of the first animal. If animals survive then the main test should be terminated. If animal dies, two more animals are dosed and observed. The dosing is stopped when one of the following criteria is met:

1. Three consecutive animals survive at the upper bound.
2. Five reversals occur in any six consecutive animals tested.
3. At least four animals have followed the first reversal and the specified likelihood ratios exceed the critical value.

**Preparation of Dose:** Mortality was not seen in the acute toxicity study up to a dose of 5000 mg/kg/b.w for ethanolic extracts of Moringa oleifera leaves. A dose of 1/10th and 1/20th of 5000mg/kg were considered to be the high dose and low dose respectively; doses were prepared by dissolving in miliQ water.

**Pharmacological effects of ethanolic extracts of the leaves of Moringa oleifera in vincristine induced peripheral neuropathy (VIPN):**

Peripheral neuropathy was induced in rats by intraperitoneal injection of vincristine (100µg/kg/day) body weight dissolved in saline (0.1 ml/kg/day) to induced peripheral neuropathy [10]. All the animals were allowed free access to tap water and normal chow pellet diet and maintained at room temperature in polyethylene cages.

**Group classification:**

Rats were induced vincristine (100µg/kg/day) for 14 consecutive days later on administered standard and test compounds for 21 days. 30 healthy rats were divided into following groups, where N=6 Animals in each group.

**Group 1:** Administered only vehicle (saline solution) serves as normal control

**Group 2:** Administered vincristine (100µg/kg/day i.p.), serves as neuropathy control

**Group 3:** Neuropathy animals administered reference standard, Methylcobalamin(50µg/kg i.p.)

**Group 4:** Neuropathy animals were treated with Low dose (250 mg/kg/b.w.p.o.) of ethanolic extracts of the leaves of Moringa oleifera dose obtained from acute toxicity.

**Group 5:** Neuropathy animals were treated with High dose (500 mg/ kg/b.w.p.o) ethanolic extracts of the leaves of Moringa oleifera, dose obtained from acute toxicity

After the end of experiment, the parameters expounded below were evaluated.

**Estimation of pain-sensation effect of VIPN in rats by analgesic screening models:**

**Hot plate Method:** In this method heat is used as a source of pain. Animals are individually placed on a hot plate maintained at constant temperature (55°C) and the reaction of animals such as paw licking or jump response is taken as the end point. Normally animals show response in 6-8 sec. A cut off period of 15 sec in observed to avoid damage to the paws. Prior to any treatment, the animals were allowed to familiarize with the test procedure and apparatus, and baseline values were obtained.

**Tail flick method:** Before initiating the test, the tail of each animal was dipped in water at 29°C for 30 mins. Then the whole tail was submerged water at 49°C. The time taken for the animal to show a characteristic tail flick response was recorded. The test was repeated three times for each animal and the average was considered as the withdrawal latency for each animal [11].

**Cold Allodynia-Acetone spray method:** Acetone (100µl) was applied to the plantar surfaces of the left hind paw and withdrawal responses were noted. Testing was repeated 5 times with 3–5 min between each test. The intensity of cold allodynia was expressed as withdrawal frequency: (n paw withdrawal responses/n trials) × 100 [12].

**Measurement of Nerve Conduction Velocity (NCV)**

After anesthesia, rats back were shaved and motor NCV was recorded in a temperature-controlled environment from the left sciatic tibial nerve by a modified non-invasive method. Briefly, the rectal temperature was maintained at 37°C, and the left sciatic nerve was stimulated proximally at the sciatic notch and distally at the knee via bipolar electrodes by AD Instruments (Power lab data acquisition system, New Zealand). The compound muscle action potential (CMAP) was recorded from the ankle by unipolar pin electrodes. NCV was calculated as the ratio of the distance in mm between both sites of stimulation divided by the difference between proximal and distal latencies measured in milliseconds, giving a value for NCV in meters per second (m/s) [13].

**Estimation of pro-inflammatory cytokines viz., IL-6, IL-1beta and TNF-alpha**

The serum samples were used for the estimation of the cytokines viz, IL-1β, IL-6 and TNF-α was done using antigen capture ELISA. The ELISA well plates have been coated with 100µl of IL-6, IL-1β and TNF-α primary antibodies (2.5µg/ml) in carbonate buffer (Na2HPO4 and NaH2PO4, pH 9.6). Primary antibodies of IL-6, IL-1β, & TNF-α were used. The plates have been incubated overnight at 40C to facilitate proper adsorption of antibodies on to the substrate. After 12-14 h of incubation, the plates have been washed thrice with washing buffer i.e., NaCl and Tween 20 in phosphate buffer, pH 7.4 and blocked with 250µl of blocking buffer i.e., BSA in phosphate buffer, pH 7.4 per well. After followed by incubation at 370C for 1h, added standard cytokines for the construction of calibration curve. Remaining wells had been coated with 100µl of diluted cell lysate. The concentration range used for the standards was 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.39 and 0.195ng/ml. The standard cytokines were used, incubated for 1h and washed thrice. Anti-cytokine antibodies such as; anti-IL-6, IL-1β and then anti TNF-α monoclonal antibodies were diluted 1:1000 and added 100 µl per well to the strips containing the respective antigens and incubated at 370C for 1h. After incubation, 100 µl of HRP-conjugate was added. Plate incubated at 370C for 1h. Then 100µl of freshly prepared substrate i.e., TMB in DMSO containing H2O2 was added to all wells. After incubation in dark 370C for 15 mins, color has changed, the reaction has been terminated by adding 50µl of 2.5N

H2SO4 per well and the A450nm was measured by using ELISA reader. A standard calibration graph was plotted (Abs Vs Conc.) and the concentrations of unknown samples have been determined from the graph [14-17].

**Assessment of Anti-oxidant effect:**

**Estimation superoxide dismutase (SOD):** To 2.78 ml sodium carbonate buffer (0.05 mM, pH 10.2), 100 µL of 1 mM EDTA and 20 µL tissue supernatant were added and incubated at 30°C for 45 min. The reaction was initiated by adding 100 µL of adrenaline. The change in the absorbance was recorded at 480 nm for 3 min. Sucrose was used as a blank. The activity of SOD was expressed as units/min/mg of protein

**Estimation Lipid peroxidation (LPO):** The sample mixed with in TCA and the mixed sample was used to estimate MDA. Briefly, LPO was induced by adding ferric chloride (10 µl, 400 mM) and l-ascorbic acid (10 µl, 400 mM) to a mixture containing sample (0.3 ml) in phosphate buffer solution (5 ml, pH 7.4, 0.2 M). After incubation for 1 hr. at 37°C, the reaction was stopped by adding HCl (2 ml, 0.25 N) containing TCA (1 ml, 15% w/v) and TBA (0.5 ml, 0.375% w/v) boiled for 15 min, cooled, and centrifuged, and the absorbance of the supernatant was measured at 532 nm. The results were expressed as. nmoles of MDA/g protein

**Estimation Catalase (CAT):** To 1.9 mL phosphate buffered saline (pH 7.0), 100µl of supernatant was added. To this 1 mL of H2O2 was added and the change in the absorbance was recorded at 240 nm for 3 min. The value was expressed as. nmoles H2O2/min/mg Protein

**Estimation Nitric oxide (NO):** To 20 µl Griess reagent, added 100 µl deionized water and 100 µl samples were added in test tube. Added 100 µl deionized water + 100 µl Griess reagent is use as a blank. Allowed the sample/reagent mixture and blank to develop in the dark for 30 min. (the mixtures were continued to develop past 30 minutes although at a relatively slow rate. Measured the absorbance of sample mixture and blank solutions at 548nm suing the spectrophotometer. The values were expressed as. µmoles/g protein. The assessment of anti-oxidant was performed as given in literature [18-24].

**Histopathological Studies:**

**Preparation of sciatic nerve tissue:**

The animals were euthanized using high dose of pentobarbital and sacrificed. The bilateral sciatic nerves were isolated and 1 cm of sciatic nerve tissue was fixed with 10% formaldehyde. The samples were then dehydrated and embedded in paraffin. After sectioning (5µm thick) with a rotary slicer (LEICA RM2135, Wetzlar, Germany), hematoxylin and eosin stain (H&E) and luxol fast blue staining was performed to evaluate the neuronal damage and myelination status [25].

**STATISTICAL ANALYSIS**

The results are expressed as Mean ± S.E.M. from n=6 rats in each group. Data were analysed using statistical software Graph Pad Prism version 5. The significance of difference among the groups was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test compared between Normal control (Untreated) Vs all groups $p<0.05$ were considered significant.

## III. Result

**Extraction and phytochemical analysis of Moringa oleifera leaves:**

Yield of ethanolic extract of Moringa oleifera leaves was 17.51%. The preliminary phytochemical screening showed presence of alkaloids, saponins, flavonoids, tannins, carbohydrates, cardiac glycosides, proteins and amino acids as given in Table 1.

**Table 1:** Phytochemical screening of ethanolic extracts*Moringa oleifera* leaves

| ***Phytoconstituents*** | ***Ethanolic Extract of Moringa oleifera*** |
|---|---|
| *Alkaloids* | + |
| *Triterpenes* | - |
| *Phytoseterol* | - |
| *Saponins* | + |
| *Flavonoids* | + |
| *Tannins* | + |
| *Fixed oils and Fats* | - |
| *Gums and Mucilage's* | - |
| *Carbohydrates* | + |
| *Cardiac Glycosides* | + |

| | |
|---|---|
| *Proteins and amino acids* | + |

*(+) Indicates respective constituent present and (-) Indicates absence of phytochemical*

**FT-IR spectra of ethanolic extract of the leaves of *Moringa oleifera.***

The results obtain for the FTIR spectra of ethanolic extracts of Moringa oleifera leaves is given in Figure 1 below;

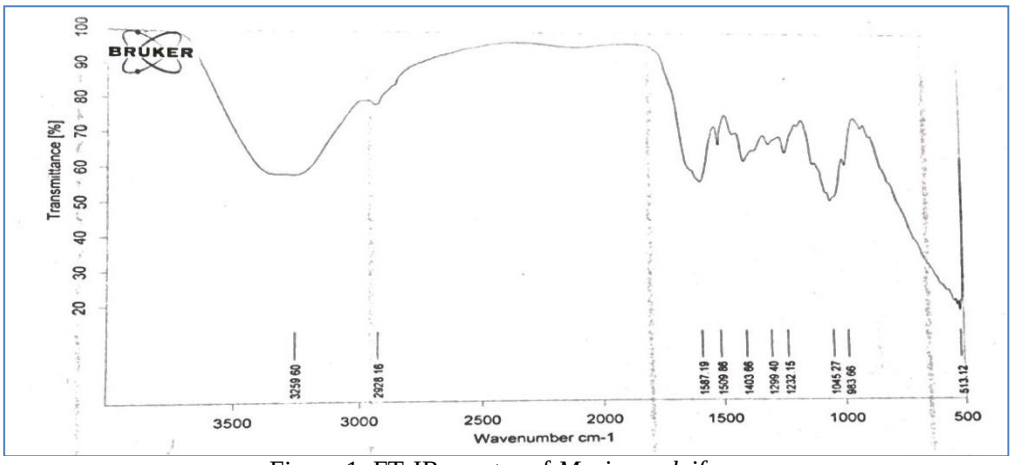



Figure 1: FT-IR spectra of *Moringa oleifera*

[FT-IR (Zn-Se, $cm^{-1}$): 3259.60 (O-H), 2928.16 (C-H), 1587.19 (C-C), 1509.86 (N-O nitro compound), 1403.66 (C-C), 1299.40 (C-H alkyl halides), 1232.16 (C-N aliphatic amines), 1045.27 (C-N), 983.66 (=C-H alkenes)]. (Source: Bruker Alpha series, Instrumentation Lab, Karnataka College of Pharmacy, Bangalore).

**HPTLC of ethanolic extract of the leaves of *Moringa oleifera:***

HPTLC photo documentation revealed presence of phytoconstituents with different Rf values (Table 2). Densitometry scan of the plates showed a total of 11 spots were visible at 575 nm (Figure 2).

**Table 2:** Rf values and color of spots of ethanolic extracts of *Moringa oleifera* leaves detected *at 575 nm*

| Sr. No. | Color | Rf value |
|---|---|---|
| 1 | Blue | 0.06 |
| 2 | Yellow | 0.17 |
| 3 | Blue | 0.34 |
| 4 | Yellow | 0.43 |
| 59 | Pink | 0.51 |
| 6 | Ash | 0.60 |
| 7 | Ash | 0.65 |
| 8 | Violet | 0.73 |
| 9 | Blue | 0.82 |
| 10 | Blue | 0.85 |
| 11 | Dark Blue | 0.97 |

three months had also gone down by(-11.00%). The desirable alterations in respect of all the above parameters which were attributable to the abovemedication, were statistically significant, P<0.001---0.033.

**Table no3 :** Shows Percent Change in Lipids,( mg/dL) on a regular dose of Rosuvastatin 20mg for 6 weeks**.**

| | Rosuvastatin 20mg (before) | Rosuvastatin 20mg (After) | Percentage Change | P value |
|---|---|---|---|---|
| Lipids, mg/dL | | | | |
| Total Cholesterol (TC) | 226.1±35.4 | 166.2±25.7 | -26.49% | <0.001 |
| LDL-C | 156.1±27.8 | 97.9±14.7 | -37.28% | <0.001 |
| HDL-C | 35.5±2.21 | 38.4±3.6 | +8.17% | <0.001 |
| Triglyceride | 164.6±28.2 | 136.2±23.4 | -17.3% | <0.001 |
| Non-HDL-C | 182.4±29.2 | 128.2±20.5 | -29.71% | <0.001 |

| | | | | |
|---|---|---|---|---|
| Glucose and HbA1C | | | | |
| FBG, mg/dL | 148.2±26.9 | 91.95±8.8 | -37.95% | <0.001 |
| HbA1c, % | 5.62±0.4 | 5.5±0.2 | -2.13% | 0.187 |


**Figure. 2:** Spots of ethanolic extracts of *Moringa oleifera* leaves

**GCMS of ethanolic extract of Moringa oleiefera leaves:**

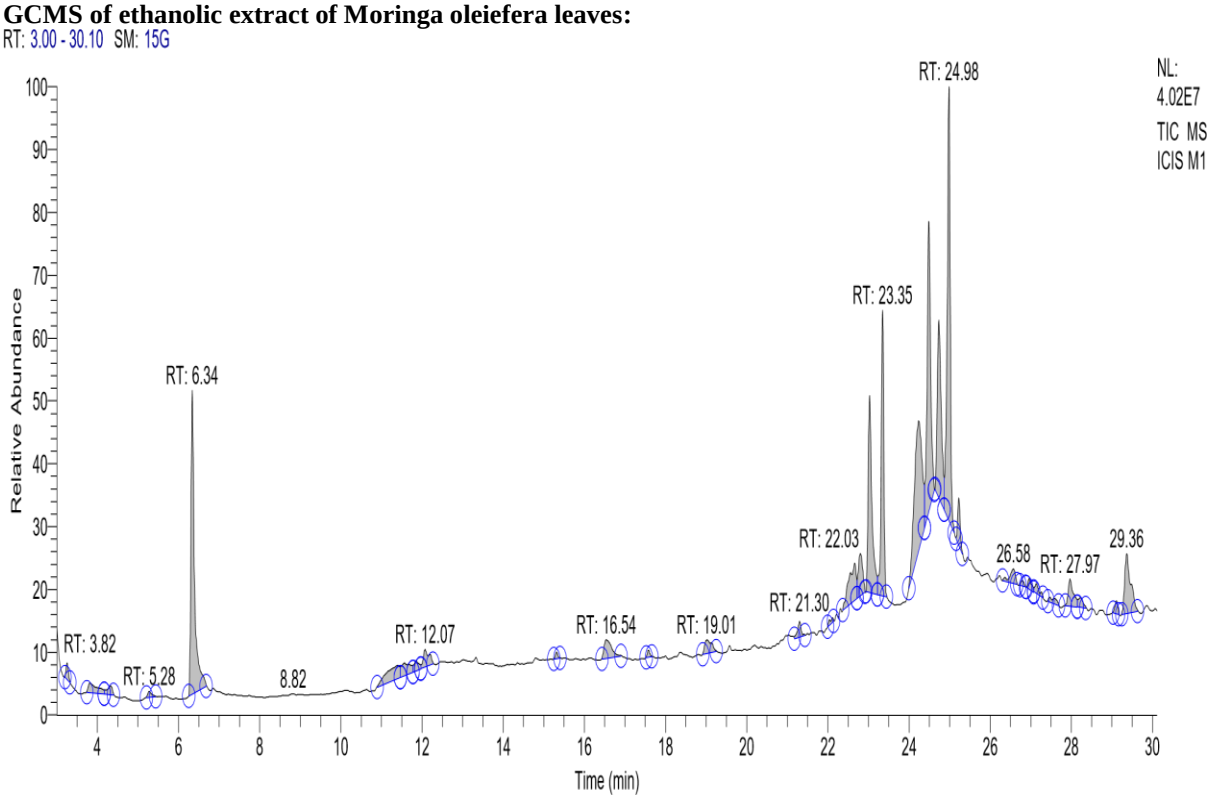



**Figure 3:** GC-MS of ethanolic extract of *Moringa oleiefera* leaves

The Library Search Results of ethanolic extract of moringa oleifera leaves on subjecting to Gas Chromatography Mass spectroscopy presented the following bioactive chemicals as shown in Table 3.

**Table 3:** Library Search Results after GCMS and bioactivity of chemical constituents of ethanolicextracts of *Moringa oleifera* leaves.

| Name | Molecular Formula | RT | Area % | Bioactivity |
|---|---|---|---|---|
| Ethan amine, 2 (methylthio) | $C_3H_9NS$ | 3.25 | 0.44 | Antibacterial [26] |
| 1,3,5,7-Tetroxane | $C_4H_8O_4$ | 3.25 | 0.44 | Antimalarial, antipyretic, or anti-inflammatory agents[27] |
| 2,2Dimethoxybutane | $C_6H_{14}O_2$ | 3.82 | 1.05 | Fixed oil ,antibacterial and antioxidant [28] |
| Guanidine, N, N-dimethyl | $C_3H_9N_3$ | 4.33 | 0.50 | Antagonized or prevented tubocurarine[29] |
| 3-Hexen2one | $C_6H_{10}O$ | 5.28 | 0.25 | Volatile flavor compound[30] |
| 1,3-Dioxolane, 2,2,4-trimethyl | $C_6H_{12}O_2$ | 6.34 | 12 | Volatile oil ,antioxidant.[31] |
| 1,3-Dioxolane-2-methanol,2,4-dimethyl | $C_6H_{12}O_3$ | 6.34 | 12 | Antimicrobial agent[32] |
| Erythritol | $C_4H_{10}O_4$ | 11.39 | 2.16 | Strong antimicrobial and antibiofilm activity[33] |
| dl Threitol | $C_4H_{10}O_4$ | 11.39 | 2.16 | Antibacterial[34] |
| 1,2,3,4Butanetetrol, [S(R*,R*)] | $C_4H_{10}O_4$ | 11.39 | 2.16 | Antibacterial and antifungal[35] |
| Propanal, 2,3dihydroxy, (S) | $C_3H_6O_3$ | 12.07 | 1.13 | Antimicrobial potential[36] |
| 3',4',5,7-Tetramethoxy flavone | $C_{19}H_{18}O_6$ | 15.32 | 0.18 | Effective Dipeptidyl Peptidase-4 ( DPP-4 ) and α-Amylase inhibitors (anti-diabetic)[37] |
| 2H Pyran3ol, 6ethenyltetrahydro2,2,6trimethyl(**(+)-cis-Linalool 3,7-oxide)** | $C_{10}H_{18}O_2$ | 16.54 | 1.56 | Antioxidant and antibacterial[38] |
| S [ 2Aminoethyl]dl cysteine | $C_5H_{12}N_2O_2S$ | 17.58 | 0.20 | Antibacterial[39] |
| Pyrrole, 2-methyl-5-phenyl | $C_{11}H_{11}N$ | 19.01 | 1.05 | Antispasmodic activity[40] |
| 10Octadecenal | $C_{18}H_{34}O$ | 21.30 | 0.62 | Antiproliferative effect and antibacterial[41] |
| Cyclopropane tetradecanoic acid, 2-octyl, methyl ester | $C_{26}H_{50}O_2$ | 21.30 | 0.62 | Antimicrobial[42] |
| 9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1- [(trimethylsilyl) oxy] methyl-ethyl ester, (Z, Z, Z) | $C_{27}H_{52}O_4Si_2$ | 21.30 | 0.62 | Glycolipid biosurfactant[43] |
| Cyclopentaneundecanoic acid, methyl ester | $C_{17}H_{32}O_2$ | 22.03 | 0.21 | Antimicrobial[44] |
| L (+) Ascorbic Acid 2,6 -dihexadecanoate | $C_{38}H_{68}O_8$ | 22.66 | 3.10 | Antitumor and antibacterial properties[45] |
| Pentadecanoic acid, 14-methyl, methyl ester | $C_{17}H_{34}O_2$ | 22.66 | 3.10 | Antioxidant[46] |
| Methyl 13-methyltetradecanoate | $C_{16}H_{32}O_2$ | 22.66 | 3.10 | Antioxidant, Cancer-preventive, Hypercholesterolemi, Lubricant, Nematicide[47] |
| Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ | 22.66 | 3.10 | Antibacterial activities, Antifungal, Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic flavour, hemolytic, 5-Alpha reductase inhibitor, potent antimicrobial activity[48,49] |
| n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 22.79 | 2.06 | Anti-oxidant, Hypocholesterolemic, Nematicide, Anti-androgenic, Hemolytic, Pesticide, Lubricant, 5-Alpha reductase inhibitor, antipsychotic[50] |
| Tridecanoic acid | $C_{13}H_{26}O_2$ | 22.79 | 2.06 | Anthelminthic, anti-inflammatory and antimicrobial activities and anti-cancerous activity, antimicrobial [51] |
| Myristic acid | $C_{14}H_{28}O_2$ | 22.79 | 2.06 | Antioxidant, cancer preventive, nematocide, hypercholesterolemic, lubricant[52] |
| Pentadecanoic acid | $C_{15}H_{30}O_2$ | 23.03 | 8.30 | Active dietary fatty acid that attenuates inflammation, anemia, dyslipidemia, and fibrosis[53] |
| Decanoic acid, ethyl ester | $C_{12}H_{24}O_2$ | 23.35 | 8.26 | Reduce the secondary neuronal damage Food additive, favoring agent, defensive secretion ester [54, 55] |
| Hexadecanoic acid, ethyl ester (Stearic acid) | $C_{18}H_{36}O_2$ | 23.35 | 8.26 | Antioxidant, Anti-inflammatory, Anthelmintic, Antibacterial, Antiallergic, Hypocholesterolemic, Hemolytic, Pesticide, Nematicide, Lubricant, Flavour, 5 alpha-reductase inhibitors, Antiandrogenic [56] |
| 9-Octadecenoicacid (Z),2-hydroxy1 (hydroxymethyl) ethyl ester | $C_{21}H_{40}O_4$ | 24.23 | 11.26 | Inhibition of proliferative effect in keloid fibroblasts [57] |

| | | | | |
|---|---|---|---|---|
| 10Octadecenoicacid, methyl ester | $C_{19}H_{36}O_2$ | 24.23 | 11.26 | Enhances the immunity of hydroxy unsaturated fatty acid[58] |
| Phytol | $C_{20}H_{40}O$ | 24.48 | 11.38 | Antimicrobial, Anticancer, Anti-inflammatory, Diuretic. Antinociceptive and Antioxidant Activities[59-61] |
| 3,7,11,15Tetramethyl2hexadecen1ol | $C_{20}H_{40}O$ | 24.48 | 11.38 | Antimicrobial, anti-inflammatory, anticancer, diuretic, antifungal against S. typhi, resistant gonorrhea, joint dislocation, hernia headache, stimulant, anti-malarial, anti-diabetic[62] |
| Phytol, acetate(Diterpene) | $C_{22}H_{42}O_2$ | 24.48 | 11.38 | Antimicrobial, anticancer, anti- inflammatory, diuretic, hypocholesterolemic, nematicide, anti-coronary, antiarthritic, hepatoprotective, anti-androgenic[63] |
| 7 Hexadecyn1ol | $C_{16}H_{30}O$ | 24.74 | 7.58 | Antioxidant[64] |
| Cis, cis7,10, Hexadecadienal | $C_{16}H_{28}O$ | 24.74 | 7.58 | Anti-inflammatory[65] |
| Dichloroacetic acid, tridec2ynylEster | $C_{15}H_{24}C_{l2}O_2$ | 24.74 | 7.58 | Dichloroacetic acid (DCA) are used for cosmetic treatments (such as chemical peels and tattoo removal) and as topical medication for the chemoablation of warts, including genital warts. It can kill normal cells as well. Salts of DCA are used as drugs since they inhibit the enzyme pyruvate dehydrogenase kinase [66] |
| Cis, cis, cis7,10,13-Hexadecatrienal | $C_{16}H_{26}O$ | 24.98 | 14.63 | Antioxidant activity[67] |
| 9,12,15Octadecatrienoicacid, (Z,Z,Z) | $C_{18}H_{30}O_2$ | 24.98 | 14.63 | Antibacterial[68] |
| 9,12,15Octadecatrien1ol,(Z,Z,Z) | $C_{18}H_{32}O$ | 24.98 | 14.63 | Antioxidant and anticancer[69] |
| Ethyl 9,12,15octadecatrienoate | $C_{20}H_{34}O_2$ | 24.98 | 14.63 | Antimicrobial and pesticide[70] |
| Docosanoic acid, ethyl ester | $C_{24}H_{48}O_2$ | 25.23 | 1.27 | Anticancer[71] |
| Nonanoic acid, 5methyl,ethyl ester (lauric acid ) | $C_{12}H_{24}O_2$ | 25.23 | 1.27 | Antibacterial[72] |
| Pterin 6-carboxylicAcid | $C_7H_5N_5O_3$ | 26.58 | 0.87 | Anti-tumor anti-viral, anti-. HIV, antiprotozoal[73,74] |
| Diazo progesterone | $C_{21}H_{30}N_4$ | 26.80 | 0.19 | Antibacterial and antioxidant activity [75] |
| Cyclohexanol, 5methyl2(1methylethenyl) | $C_{10}H_{18}O$ | 26.96 | 0.46 | Antimicrobial, anticancer, anti-tumor, analgesic, anti-inflammatory, sedative, antifungal, hypocholesterolemic, insecticide, insectifuge chemo preventive and pesticidal[76] |
| Nerolidyl acetate | $C_{17}H_{28}O_2$ | 26.96 | 0.46 | Antibacterial and antifouling activities[77] |
| Eicosanoic acid, phenylmethyl ester | $C_{27}H_{46}O_2$ | 27.14 | 0.47 | antibacterial activity [74] |
| 9 Octadecenamide,(Z) | $C_{18}H_{35}NO$ | 27.97 | 1.53 | Anti-inflammatory activity and antibacterial activity [73] |
| 6,9,12Octadecatrienoicacid, phenylmethyl ester,(Z,Z,Z) | $C_{25}H_{36}O_2$ | 27.97 | 1.53 | Antioxidant, anti-inflammatory, antimicrobial, pesticide and cancer preventive[78] |
| 8 Methyl 6-nonenamide | $C_{10}H_{19}NO$ | 28.22 | 0.52 | Antimicrobial, anti-inflammatory [62] |
| Tricyclo [4.3.1.1(3,8)] undecan1amine | $C_{11}H_{19}N$ | 28.22 | 0.52 | Anti-viral activity[74] |
| Oxirane, [(hexadecyloxy) methyl] | $C_{19}H_{38}O_2$ | 29.11 | 0.40 | Antimicrobial activity[79] |
| Octadecane, 1(ethenyloxy) | $C_{20}H_{40}O$ | 29.11 | 0.40 | Recommended as an ether and acts as an antisepsis[80] |
| Squalene (triterpene) | $C_{30}H_{50}$ | 29.36 | 3.95 | Antiaging, analgesic, antidiabetic, anti-inflammatory, antioxidant, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective, hypocholesterolemic, antiulcerogenic, vasodilator, antispasmodic, anti-bronchitis, anti-coronary activities[60] |
| 1,5,9Undecatriene,2,6,10trimethyl,(Z) | $C_{14}H_{24}$ | 29.36 | 3.95 | As an oviposition deterrent against different species of mosquitoes[81] |
| Farnesol | $C_{15}H_{26}O$ | 29.36 | 3.95 | Antimicrobial and insecticidal properties ,potent antitumor effects in HeLa human cervical cancer cells[82] |
| 2 Methyl 3 (3 methyl but2enyl) 2 (4 methylpent3enyl) Oxetane | $C_{15}H_{26}O$ | 29.36 | 3.95 | Catechol-O-methyltransferase inhibitor, methyl donor, methyl guanidine inhibitor[83] |
| Oxirane, [(hexadecyloxy) methyl] | $C_{19}H_{38}O_2$ | 29.11 | 0.40 | Antimicrobial activity[79] |
| Octadecane, 1(ethenyloxy) | $C_{20}H_{40}O$ | 29.11 | 0.40 | Recommended as an ether and acts as an antisepsis[80] |
| Squalene(triterpene) | $C_{30}H_{50}$ | 29.36 | 3.95 | Antiaging, analgesic, antidiabetic, anti-inflammatory, antioxidant, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective, hypocholesterolemic, antiulcerogenic, vasodilator, antispasmodic, anti-bronchitis, anti-coronary activities[60] |

**Acute Toxicity Study:**

Mortality was not seen in the acute toxicity study up to a dose of 5000 mg/kg for ethanolic extract of *Moringa oleifera* leaves and a dose of 1/20th and 1/10th of 5000 mg/kg (250 mg/kg and 500 mg/kg) were considered as low and high dose for pharmacologic screening. The drugs were prepared by dissolving in miliQ water and further studies were carried out.

**Pharmacological effects of ethanolic extracts *Moringa oleifera*in vincristine induced peripheral neuropathy (VIPN):**

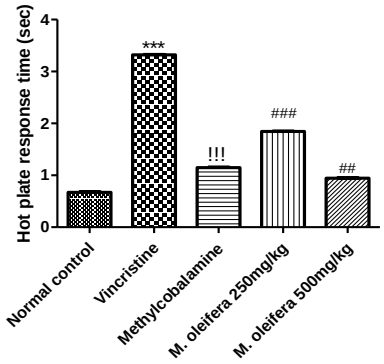
Pharmacological effects of different drugs were studied after administration of Vincristine (100µg/kg/day i.p.), Methylcobalamin (50µg/kg i.p.), ethanolic extracts of *Moringa oleifera* leaves (250mg/kg; P.O and 500mg/kg; P.O) in rat neuroprotective models.

**Pain-sensation effect of VIPN in rats by analgesic screening model:**

The pain sensation effects of VIPN in rats using hot plate is shown in Figure 4, the treated neuropathy control Wistar rats displayed a significant increase ($p<0.001$) in hotplate response time in comparison to the normal control. Whereas the group receiving ethanolic extracts of *Moringa oleifera* higher and lower dose displayed a significant change in hotplate response time $p< 0.001$, $p< 0.01$ respectively, when compared with standard, Methylcobalamincontrol.

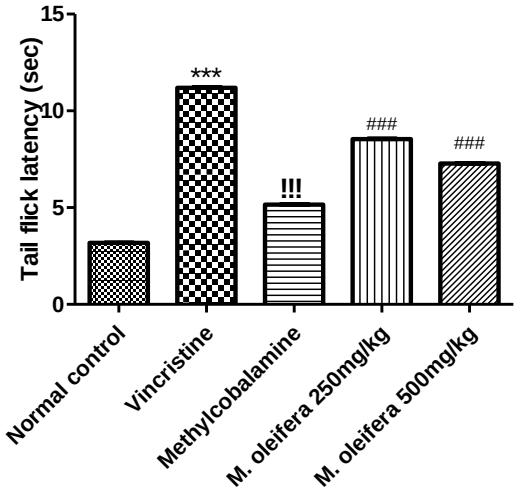
The tail flick latency was significantly higher for vincristine neuropathy control rats when compared to normal control ($p<0.001$)whereas the tail flick latency time was significantly lower in Methylcobalamin control rats ($p<0.001$) when compared with neuropathy control,the group receiving ethanolic extracts of *Moringa oleifera* higher and lower dose displayed significant change $p< 0.001$, $p< 0.01$ respectively when compared with Standard, Methylcobalamincontrol as depicted in Figure 5.

Results obtained for cold allodynia-acetone spray test shown in Figure 6 showed similar results wherein the increase in discharge rate was significantly higher in vincristine neuropathy control when compared to normal control and the ethanolic extracts of *Moringa oleifera* showed lower discharge rate when compared to vincristine induced neuropathy control group.

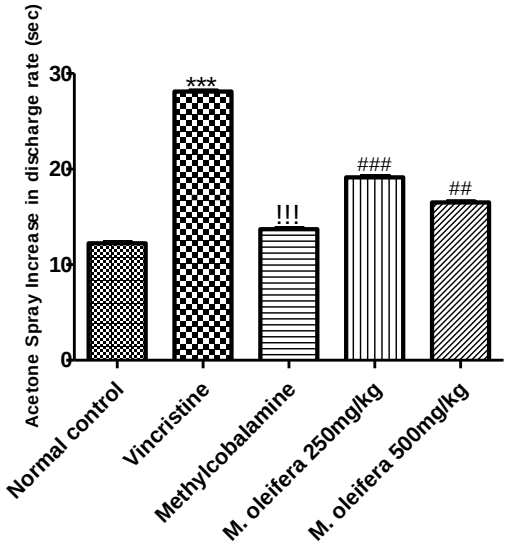



*Values are expressed as Mean ± S.E.M (n=6).* $^{***}p<0.001$ *when compared with normal control,* $^{!!!}p<0.001$ *when compared with Vincristine,* $^{###}p< 0.001$, $^{##}p< 0.01$ *when compared with Standard, Methylcobalaminecontrol.*

**Figure 4:** Pain-Sensation Effect of VIPN in rats using Hot-Plate Method

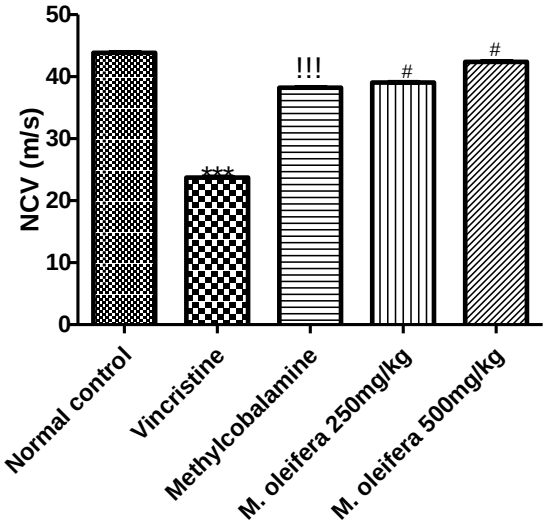



*Values are expressed as Mean ± S.E.M (n=6). $^{***}p<0.001$ when compared with normal control, $^{!!!}p<0.001$ when compared with Vincristine, $^{###}p< 0.001$, $^{###}p< 0.001$ when compared with Standard, Methylcobalaminecontrol.*
**Figure 5:** Pain-Sensation Effect of VPN In rats using Tail-Flick Method.




*Values are expressed as Mean ± S.E.M (n=6). $^{***}p<0.001$ when compared with normal control, $^{!!!}p<0.001$ when compared with Vincristine, $^{###}p< 0.001$, $^{##}p< 0.01$ when compared with Standard, Methylcobalaminecontrol.*
**Figure 6:** Cold Allodynia -Acetone Spray Test.

**Measurement of Nerve Conduction Velocity (NCV):**
Peripheral neuropathy is characterized by lowering of NCV. The NCV of different groups were measured after treatment with Methylcobalamin and lower and high dose of ethanolic extracts of *Moringa oleifera*, Figure 7. It was observed that animals with neuropathy had significantly lower NCV compared with normal control (*p<0.001*). Methylcobalamin and *Moringa oleifera*ethanolic extracttreated groups showed significant amelioration in NCV when compared with the neuropathic group (*p<0.001*).




*Values are expressed as Mean ± S.E.M (n=6). $^{***}p<0.001$ when compared with normal control, $^{!!!}p<0.001$ whencompared with Vincristine, $^{\#}p< 0.05$ when compared with Standard, Methylcobalaminecontrol.*

**Figure 7:** Measurement of Nerve Conduction Velocity (NCV)

**Estimation of pro-inflammatory cytokines viz., IL-6, IL-1beta and TNF-alpha:**

Assessment of the serum anti-inflammatory activity of ethanolic extracts of *Moringa oleifera*leaves and Methylcobalamin was done. It was found that the production of all three pro-inflammatory cytokines viz**.,** IL-6, IL-1β and TNF-α increased considerably in vincristine treated (neuropathy control) group when compared to the normal control. Hower treatment with ethanolic extracts of *Moringa oleifera* leaves and Methylcobalamin significantly suppressed the production of the cytokines when compared to the disease control (neuropathy control) as shown in Table 4.

**Table 4:** Estimation of pro-inflammatory cytokines viz., IL-6, IL-1β and TNF-α

| ***Sl. No.*** | ***Group*** | ***IL-6 (µg/ml)*** | ***IL-1beta (µg/ml)*** | ***TNF-alpha (µg/ml)*** |
|---|---|---|---|---|
| 1 | *Normal control* | 231.6±2.27 | 138.2±2.32 | 187.6±2.86 |
| 2 | *Vincristine 100µg/kg/day i.p.* | 619.4±2.15$^{***}$ | 489.4±2.17$^{***}$ | 647.8±2.55$^{***}$ |
| 3 | *Methylcobalamine 50µg/kg i.p.* | 441.7±2.134$^{!!!}$ | 388.3±5.226$^{!!!}$ | 463.5±2.593$^{!!!}$ |
| 4 | *Moringa oleifera 250mg/kg; P.O.* | 538.1±4.60$^{\#\#\#}$ | 459.4±4.31$^{\#\#\#}$ | 549.1±2.16$^{\#\#\#}$ |
| 5 | *Moringa oleifera 500mg/kg; P.O.* | 351.7±2.13$^{\#\#\#}$ | 291.3±5.22$^{\#\#\#}$ | 373.5±2.59$^{\#\#\#}$ |

*Values are expressed as Mean ± S.E.M (n=6). $^{***}p<0.001$ when compared with normal control, $^{!!!}p<0.001$ when compared with Vincristine, $^{\#\#\#}p< 0.001$ when compared with Standard, Methylcobalaminecontrol.*

**Assessment of Anti-oxidant effect viz., Superoxide dismutase (SOD), Lipid peroxidation (LPO), Catalase (CAT), Nitric oxide (NO):**

LPO and NO are upregulated in vincristine induced neuropathy whereas there is a significant decrease in the production in SOD and CAT when compared to the normal control (p*<0.001*). On the treatment with ethanolic extracts of *Moringa oleifera* leaves and Methylcobalamin there is a significant reduction in the production of LPO and NO (p*<0.001*), and an increase in SOD and CAT when compared to the disease control group as shown in Table 5.

**Table 5:** Assessment of Anti-oxidant effect viz., Superoxide dismutase (SOD), Lipid peroxidation (LPO), Catalase (CAT), Nitric oxide (NO)

| ***Sl. No.*** | ***Group*** | ***Serum SOD (units/min/mg of protein)*** | ***Serum LPO (nMoles of MDA/g protein)*** | ***Serum CAT (nmoles $H_2O_2$/min/mg Protein*** | ***Serum NO (µmols/g protein)*** |
|---|---|---|---|---|---|
| 1 | *Normal control* | 23.5±0.72 | 1.09±0.27 | 10.7±0.21 | 13.58±0.28 |
| 2 | *Vincristine 100µg/kg/day i.p.* | 12.73±0.35$^{***}$ | 2.06±0.35$^{***}$ | 5.04±0.26$^{***}$ | 65.44±0.26$^{***}$ |
| 3 | *Methylcobalamine 50µg/kg i.p.* | 19.10±0.29$^{!!!}$ | 1.53±0.98$^{!!!}$ | 10.07±0.34$^{!!!}$ | 39.76±0.74$^{!!!}$ |
| 4 | *Moringa oleifera 250mg/kg; P.O.* | 16.54±0.38$^{\#\#}$ | 1.87±0.64$^{\#\#}$ | 16.24±0.49$^{\#\#\#}$ | 46.21±0.36$^{\#\#}$ |
| 5 | *Moringa oleifera 500mg/kg; P.O.* | 21.49±0.84$^{\#\#}$ | 1.22±0.28$^{\#\#}$ | 13.57±0.94$^{\#\#}$ | 39.27±0.48$^{\#\#}$ |

*Values are expressed as Mean ± S.E.M (n=6). $^{***}p<0.001$ when compared with normal control, $^{!!!}p<0.001$ when compared with Vincristine, $^{\#\#\#}p< 0.001$, $^{\#\#}p< 0.01$ when compared with Standard, Methylcobalaminecontrol.*

**Effect of drugs in Sciatic nerve – Histopathology**

Normal control Figure 5 (a) shows normal appearance of sciatic nerve surrounded by perineurium (arrow). Nerve showed numerous nerve fibers containing centrally placed axon which is surrounded by remnants of myelin sheath (thick arrow), along with the normal appearance of endoneurium and Schwann cells. Histology of the disease group treated with Vincristine 100µg/kg/day i.e., Figure 5 (b) shows loss of architecture of nerve and perineurium, loss of nerve fibers and the space replaced with fluid accumulation-stained pink in color (arrow), sparsely arranged nerve fibers was observed. Histology of the sciatic nerve for rats treated with Methylcobalamin 50µg/kg i.p. Figure 5 (c) shows appearance of perineurium and the nerve fibers with axon surrounded by myelin sheath and appearance of endoneurium and Schwann cells (arrow). The histology of sciatic nerve of rats treated with ethanolic extract of *Moringa oleifera* 250mg/kg, p.o. Figure 5 (d) shows appearance of perineurium and the nerve fibers with axon surrounded by myelin sheath and appearance of endoneurium and Schwann cells with characteristic wavy appearance of nerve fibers (arrow). Finally, the histology of sciatic nerves of the group receiving ethanolic extract of *Moringa oleifera* 500mg/kg, p.o. Figure 5 (e) shows nerves with normal appearance of perineurium and the nerve fibers with axon surrounded by myelin sheath and with endoneurium and Schwann cells and dense nerve fibers (arrow) similar to that of normal control group.

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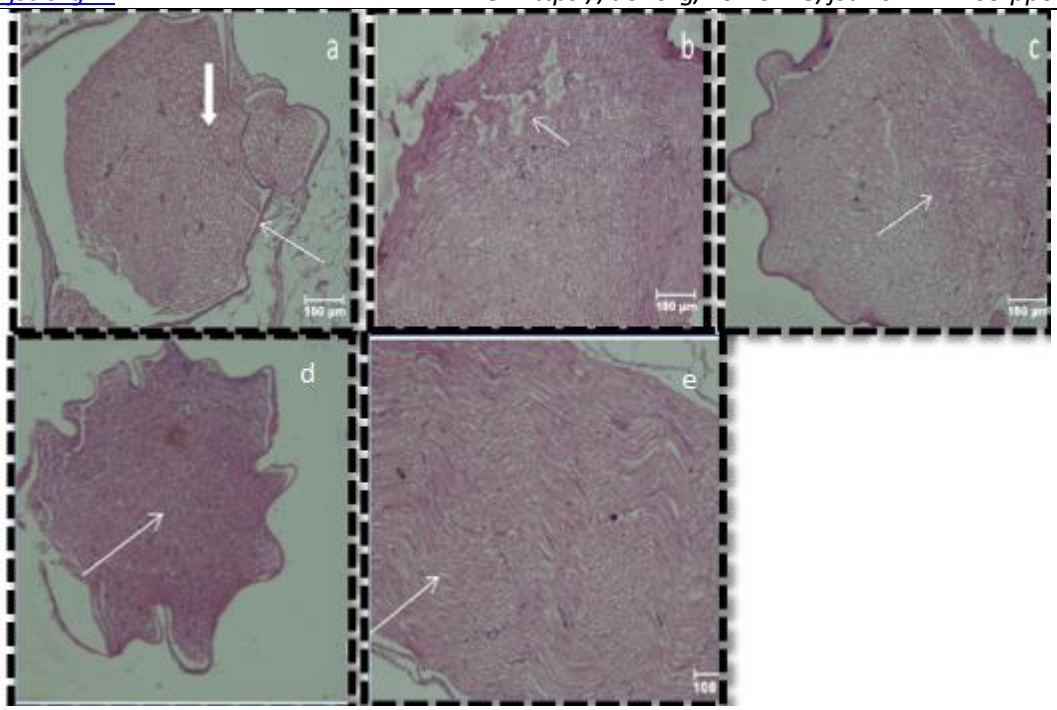


**Figure 5:** Sciatic Nerve tissue – Histology H&E Staining (Scale bar = 100µm). (a) Normal control, (b) Vincristine 100µg/kg/day i.p., (c) Methylcobalamin 50µg/kg i.p., (d) Ethanolic extract of *Moringa oleifera* 250mg/kg p.o., (e) *Moringa oleifera* 500mg/kg p.o.

## IV. Discussion

In recent times there has been an increasing interest towards natural sources for treatment, in particular the use of herbal products [84]. From the phytochemical analysis results, particularly the GCMS and library search data it is evident that various bioactive chemical components are present in the ethanolic extract of Moringa oleifera leaves. From the neuroprotective point of view the presence of antioxidant and anti-inflammatory phytoconstituents play an important role. The library search data suggests the presence of antioxidants for example Cis, cis, cis7,10,13-Hexadecatrienal, 7 Hexadecyn1ol, Hexadecanoic acid, ethyl ester, Myristic acid, n-Hexadecanoic acid. Further the results also indicate the presence of anti-inflammatory components for example Cis, cis 7,10, hexadecadienal, Phytol, acetate (Diterpene), Squalene (triterpene).

Taking these considerations, the neuroprotective effect of ethanolic extracts of Moringa oleifera in vincristine induced neuropathy was studied, methylcobalamin has potential neuroprotective benefits and was used as a standard [85]. This study revealed that, the vincristine induced behavioral (thermal hyperalgesia pain sensation i.e., hot plate, tail flick methods, and allodynia using Cold Allodynia-Acetone spray method), biochemical (IL-6, IL-1β, TNF-α, SOD, LPO, CAT and NO levels) alteration as well as the nerve conduction velocity test (NCV) and histopathological changes in the sciatic nerve were reverted to normal after the administration of the ethanolic extracts of Moringa oleifera leaves.

Peak behavioral alterations occur after two to three weeks in vincristine induced neuropathic model [86,87] thus pain sensation assessment studies were conducted on 21st days after vincristine administration. It is evident from the recent studies that the drugs which inhibit TNF-α, IL-1β and IL-6 synthesis aid in the recovery of neuropathic pain condition in animals [88] as well as in human beings [89], and since vincristine mediates various neurotoxic effects such as the generation of free radical and cytokines (TNF-α, IL-1β and IL-6) thus it is imperative that the levels of TNF-α, IL-1β and IL 6 levels have to be particularly investigated.

The results showed that the treatment with ethanolic extracts of Moringa oleifera leaves ameliorated VIPN as there is a decrease in the increased levels of TNF-α, IL-1β and IL-6 these findings are supported by such findings in literature [90-92]. The neuroprotective effects of ethanolic extracts of Moringa oleifera leaves may be attributed to the presence of bioactive compounds as mentioned earlier, which may be responsible for quenching of free radicals, or otherwise, it may be due to the up-regulation of antioxidant and anti-inflammatory systems.

The results indicate that vincristine increased nitric oxide level which is supported by earlier finding [93]. Evidence in prior experimentation proposes a contentious role of Nitric oxide synthase (NOS system) in neuropathy, scientific reports suggest involvement of both inducible NOS (iNOS) and neuronal NOS (nNOS), in the development of chemotherapy induced peripheral neuropathy. Studies also report that the vincristine induced thermal hyperalgesia might be a consequence of the dysfunction of L-arginine/NO/cGMP cascade in the spinal cord [94].

Studies have revealed a marked reduction in the amplitude of sensory potentials and slowing of nerve conduction with administration of vincristine further peripheral neuropathy is characterized by lowering of NCV [95,96]. Whereas in our study standard Methylcobalamin and ethanolic extract Moringa oleifera treated groups showed significant amelioration in NCV when compared with the neuropathic group.

Histopathological changes in the sciatic nerve, revealed sciatic nerve fiber derangement i.e., loss of architecture of nerve and perineurium, loss of nerve fibers and the space replaced with fluid accumulation-stained sparsely arranged nerve fibers was observed these observations are axonal swelling and an increase in the number of Schwann and satellite cells in VIPN these are in line with the previous findings [97,98]. Ethanolic extracts of Moringa oleifera leaves ameliorated histological alterations caused by vincristine.

## V. Conclusion

The phytochemical analysis ethanolic extract of Moringa oleifera leaves revealed the presence of bioactive chemical constituents. The ethanolic extract of Moringa oleifera was subjected to acute oral toxicity studies and was found to be safe up to 5000mg/kg body weight. Ethanolic extracts of Moringa oleifera were studied at two dose levels i.e., 250mg/kg and 500mg/kg. The higher dose 500 mg/kg dose was found to be better to the 250 mg/kg dose in terms of behavioral (thermal hyperalgesia i.e., hot plate, tail flick methods, and allodynia using Cold Allodynia-Acetone spray method), and biochemical (IL-6, IL-1β, TNF-α, SOD, LPO, CAT and NO levels) alteration along with nerve conduction velocity test (NCV) and histopathological change (sciatic nerve) evaluations.

To conclude, ethanolic extract of Moringa oleifera leaves showed potential neuroprotective activity as they have effective inducible NOS (iNOS) and neuronal NOS (nNOS) inhibiting property, antioxidative and anti-inflammatory activity.

**CONFLICT OF INTEREST STATEMENT:**

We declare that we have no conflict of interest.

**ACKNOWLEDGEMENTS**

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## VI. ABBREVATIONS

VIPN: Vincristine induced peripheral neuropathy; SOD: Superoxide dismutase; LOP: Lipid peroxidation; CAT: Catalase; NO: Nitric Oxide; NCV: nerve conduction velocity test; NOS: Nitric oxide synthase iNOS: inducible NOS; nNOS: neuronal NOS; TNF-α: Tumor necrosis factor alpha; IL-1β and IL-6: proinflammatory cytokines interleukin-1beta and interleukin-6

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