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RESEARCH ARTICLE

Anti-Neuropathic Efficacy of Homoeopathic Dilutions of *Toxicodendron pubescens*: A Preclinical Study

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Abstract

Background: *Toxicodendron pubescens* Mill., the botanical name of *Rhus toxicodendron* (*Rhus tox*), also known as 'poison ivy', belongs to the family of *Anacardiaceae* and is widely used in the traditional system of *Homoeopathy* medicine. Its crude form and various dilutions are most commonly used to relieve arthritis symptoms, are beneficial as an anti-inflammatory and are also used for edema-related conditions. **Aim:** The study aims to evaluate the anti-neuropathic efficacy of the crude and diluted forms of *Rhus tox*, a homoeopathic preparation, using the chronic constriction injury (CCI) rat model. **Methods:** In the current study, before conducting the *in vivo* study, the cell viability study on Rhus tox dilutions was determined using the MTT assay with Lipopolysaccharide-treated U-87 glioblastoma cells. Consequently, the effect of the drug on mechanical, cold, and hot allodynia and motor nerve conduction velocity was determined. The levels of antioxidants, pro-inflammatory markers, and histopathological changes were also evaluated. **Results:** The 14-day treatment regimen with *Rhus tox* in the chronic constriction model mitigated behavioural alterations such as mechanical and thermal allodynia with an improvement in the conduction velocity in the sciatic nerve as compared to the corresponding groups. The dilutions were also capable of normalising the altered oxidative and inflammatory markers. The anti-neuropathic efficacy was further confirmed by the improved nerve architecture and reduced infiltration of inflammatory cells in histopathological examination. **Conclusion:** The present observations support the efficacy of ultra-dilutions of *Rhus tox* in the rodent CCI model of neuropathic pain through its antioxidant, anti-inflammatory, and modulatory effects on neuropathic pain.

Major Findings: *Rhus tox* showed a cytoprotective effect on LPS-induced U-87 glioblastoma cells. Treatment with *Rhus tox* in CCI rats attenuated thermal and mechanical allodynia and normalised the nerve conduction. *Rhus tox* normalised oxidative stress and inflammatory levels, improved nerve architecture, and reduced inflammatory cell infiltration.

Keywords: Cytokines, Chronic Constriction Injury, Oxidative Stress, *Rhus tox, Toxicodendron pubescens,* U-87 Glioblastoma

1. Introduction

Any lesion in the somatosensory nervous system enhances the pain sensitivity and produces impulsive pain apart from the loss of function, which can become chronic[1]. Chronic pain, due to its recurring nature, imposes a very high burden not only on the patients and their families but also on the public health system[2]. Among all, the prominent type of chronic pain that impairs the quality of life and is often very challenging for patients is Neuropathic Pain (NP)[3]. The International Association for the Study of Pain defines NP as the pain produced due to a lesion or disease that affects the somatosensory nervous system[4]. Based on the epidemiological studies conducted in specialised centres, the condition is found to be approximately 11-19% of the population, specifically in people above the age of 50 years[5-8]. Clinically, NP is manifested as a combination of symptoms such as dysesthesia, neurological sensory deficits, as well as motor and cognitive deficits[9].

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As the disease encompasses both central and peripheral nervous system disorders, it can be the result of a metabolic disease such as diabetic neuropathy, exposure to toxins, an auto-immune condition, inflammatory disorders, chemotherapy-induced peripheral neuropathies, or hereditary disease[1,10]. The basic pathophysiology of NP remains ambiguous and multifactorial[11,12] as multiple pathways, such as p-38 MAPK, Wnt3a/β catenins, and NF-κB, are activated following a peripheral nerve injury, suggesting their roles in the induction of NP[13-15]. Hence, due to this complexity, pharmacotherapy continues to pose a very big challenge to physicians[16]. Currently, pharmacological options, namely serotonin-noradrenaline reuptake inhibitors, tricyclic antidepressants, gabapentin, and pregabalin, are considered the foremost, whereas medications including Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), opioids, and opiate agents such as tramadol are used as adjuvant agents[17,18]. Apart from these pharmacological approaches, non-pharmacological modalities like interventional, physical, psychological, and internet-based psychological therapies are being explored on a wide scale for the management of NP[16]. With the limited efficacy of the available drugs that target the symptoms rather than the cause, enhancing the age of the population, opioid-related side-effects, and lack of strong evidence regarding alternative therapies, new therapeutic modalities offer great promise in the management of NP[8,19].

The urgency to find new treatment options has led scientists to explore natural sources, as medicinal plants have always been beneficial in healthcare[20] which is mainly because of the low incidence of side effects when compared with synthetic drugs[21]. It has been found that herbal medicines are beneficial and are used all over the world for the management of NP, and an opportunity to develop plant-based drugs[22,23]. Plants like *Lindera angustifolia*, *Cannabis sativa*, *Ocimum sanctum*, *Nigella sativa,* and *Ginkgo biloba* from several preclinical studies are playing a valuable role in NP[24-28].

Over the past several years, many *in vitro* and *in vivo* studies have been carried out to explore the beneficial role of homoeopathic medicines under potentially reproducible conditions[29]. *Toxicodendron pubescens* Mill. also called *Rhus toxicodendron* (*Rhus tox*), belonging to the family of *Anacardiaceae*, is one of the widely used medicines in homoeopathy for the treatment and management of inflammatory conditions[30]. It has been reported to exhibit potent analgesic, immunomodulatory, and anti-inflammatory effects in both crude and diluted forms[31-33]. It has also been concluded and warranted for the anti-inflammatory effect of the homoeopathic dilutions of *Rhus tox* (6cH, 12cH, 30cH, and 200cH) in the carrageenan-induced paw inflammation model, which also exhibited a pro-inflammatory effect at multiple doses[31,32].

To analyse novel analgesics for the treatment of NP, animal models have always played an exponential role in traversing the molecular mechanisms of pain[34]. Given the high clinical relevance of NP originating from the lesions of the sciatic nerve, animal models that depict vital symptoms such as thermal and mechanical hyperalgesia, which are clinically noticed in humans, are widely employed for testing[35]. In the disease processes and treatments investigated, this model has successfully reversed the pain behaviours that are noted clinically[36]. In the surgical models, there are prolonged alterations that occur in neurotransmitter levels; expression of receptors paves the way to central sensitisation in response to the various inflammatory mediators released, thereby elevating the peripheral sensory afferent sensitivity at the injury site as well as the central nervous system[37]. Chronic Constriction Injury (CCI) caused in the sciatic nerve is the most commonly employed surgical lesion method for inducing NP in animals[38], which has a high resemblance to Complex Regional Pain Syndrome-reflex sympathetic dystrophy (CRPS-RSD), commonly observed in humans[39-41] after a total knee arthroplasty, stroke, and fracture[42-45]. The current study explored the anti-nociceptive implication of the crude form (mother tincture) and homoeopathic dilutions (6cH and 30cH) of *Rhus tox* in the CCI-induced NP model. Behavioural studies, such as mechanical and thermal allodynia, were used to evaluate the pain behaviour in rodents. LPS-induced U-87 cell line was used to reveal the cell viability of the drug through an MTT assay.

2. Materials and Methods

2.1 Plant Extract and Extraction Process

The mother tincture used in this study was procured from the commercial batches of Dr. Willmar Schwabe

India Pvt. Ltd. (Schwabe India), which was provided as a gift sample (Product: *Rhus toxicodendron* MT 100ml; Batch No.: 0000390768; Inspection Lot No.: 4000367529; CoA dated 29 January, 2021; Certificate of Analysis as supplementary file). The leaves of *Rhus tox* obtained from the United States were procured through the approved vendor of Schwabe India. Indicatively, 100 g of *Rhus tox* dry leaves were extracted with 200 ml of purified water and 824 ml of strong alcohol (95% v/v ethanol) using the cold percolation method. The extract was prepared as per the method in Homoeopathic Pharmacopoeia of India by using the cold percolation extraction procedure and reported elsewhere[46,47].

A clean, sterilised percolator was used during the preparation, and the bottom was evenly layered with cotton and filter paper. Coarsely powdered dry leaves of *Rhus toxicodendron*, moistened with a small amount of menstruum, were evenly pressed onto the layers, ensuring a compact and void-free mass. The upper surface of the mass was covered with a disc of filter paper. An adequate amount of solvent was poured into the percolator, covering the drug substance fully. Care was taken not to disturb the arrangement while pouring the solvent. The percolator was closed with a lid to prevent contamination by dust and left undisturbed for at least 24 hours. The fluid was allowed to percolate drop by drop into the receiver, controlling the flow with a stop cork and monitoring the level of the menstruum, ensuring it remained above the drug substance. Fresh quantities of menstruum were added periodically without disturbing the arrangement until the required amount had passed through the percolator. After the last drop was received, a sufficient amount of menstruum was added to cover the mass in the percolator. The percolator was then closed with the lid to halt further percolation. This arrangement was left undisturbed for six hours. The stop cork was opened, and the entire fluid percolated into the receiver. The mass was removed and firmly pressed to extract any remaining tincture. Additional menstruum was added to achieve the desired volume of the mother tincture. The qualitative test of the mother tincture was done as per the standard testing procedures at Dr. Willmar Schwabe India Pvt. Ltd. (Schwabe India) and complied with the specifications.

2.2 Drugs and Chemicals

The DuoSet cytokine ELISA kits for rat IL-1β (Cat: DY501-05; Lot: P247538), IL-6 (Cat: DY506-05; Lot: P265082), and TNF-α (Cat: DY510-05; Lot: P263707) were purchased from R&D Systems, USA. Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Product number: L2630; Batch: 0000082452) was obtained from Sigma-Aldrich, USA. The standard drug Gabapentin (Gbt) was purchased from TCI Chemicals, India.

2.3 Preparation of Homeopathic Dilutions

Various dilutions of the mother tincture extract in 70% ethanol were formulated to get the final concentrations of 1×10^{-2}, 1×10^{-4}, 1×10^{-6}, 1×10^{-8}, 1×10^{-10}, 1×10^{-12}, 1×10^{-14}, 1×10^{-16}, 1×10^{-18}, 1×10^{-20}, 1×10^{-22}, 1×10^{-24}, 1×10^{-26}, 1×10^{-28}, 1×10^{-30} represented in Figure 1.

2.4 Cell Culture and Treatment

U-87, a human glioblastoma cell line procured from the National Centre for Cell Science (NCCS), Pune, was maintained as per the standard instructions of NCCS. Briefly, the cells were kept in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1.5 mM of L-Glutamine, and antibiotics including 100 U/ml penicillin and 100µg/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Culture flasks (75 cm^2) were used to culture U-87 cells, and the medium was changed every other day.

2.5 Cell Viability Assay

Cell viability of RT in U-87 cells that contained ROS mediated by LPS was carried out using MTT [3-(4, 5-dimethylthiazol-2yl-)-2, 5-diphenyl tetrazolium bromide] as described earlier[48,49]. Initially, 1×10^4 cells were seeded in triplicate in tissue culture plates of 96-well and were permitted to attain 80% confluence. To induce ROS, the U-87 cells were treated with 500ng/ml of LPS for 20 min. After this step, the media was replaced with a fresh one and kept again for a further 24 hours by treating cells with different concentrations of RT (1×10^{-2} - 1×10^{-30}). The final assay medium was kept with an ethanol concentration of less than 0.1%. Then, to each well, MTT solutions (0.05µg/µl) diluted in PBS were added. The plates were then allowed to form purple formazan crystals by incubating them overnight

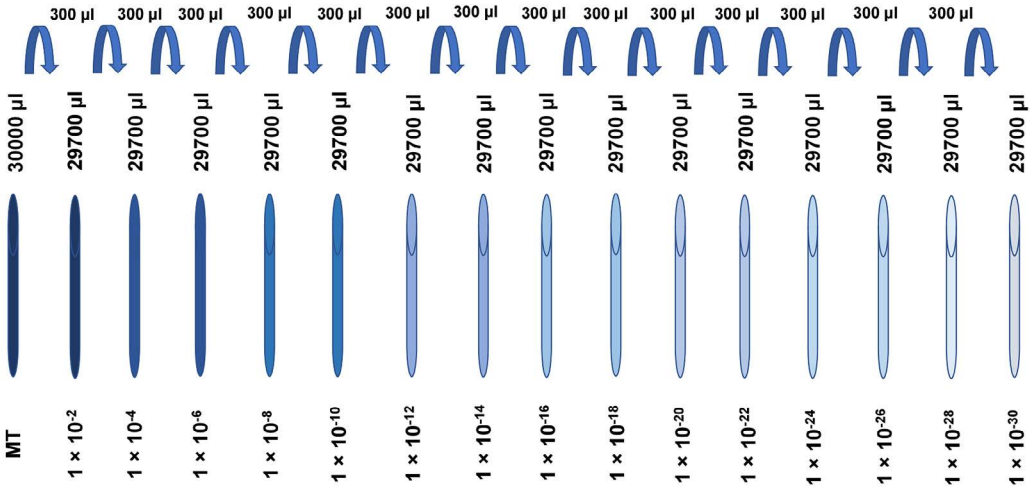


Figure 1. A diagrammatic representation of the preparation of dilutions of RT. The dilutions of the mother tincture were prepared in 70% ethanol to get the final concentrations of 1×10^{-2}, 1×10^{-4}, 1×10^{-6}, 1×10^{-8}, 1×10^{-10}, 1×10^{-12}, 1×10^{-14}, 1×10^{-16}, 1×10^{-18}, 1×10^{-20}, 1×10^{-22}, 1×10^{-24}, 1×10^{-26}, 1×10^{-28}, 1×10^{-30}.

at 37°C. Thereafter, to solubilise the crystals, the detergent solution was added to each well and kept for incubation for around 30 min at 37°C and the intensity was determined by a microplate reader (Synergy HTX, BioTek technologies, USA) at 570 nm. All the data points and assays were performed in quintuplicate. The data were expressed as a percentage (%) of cell viability against the control.

2.6 Animals

The current study utilised female albino Wistar rats of 3-4 months old weighing 190-260 g. Animals were procured from the animal house at Delhi Pharmaceutical Sciences and Research University (DPSRU), New Delhi. Animals at the animal house of the institute were kept in well-ventilated polypropylene cages at a typical setup of temperature at 22 ± 2°C with a corresponding humidity of 30-70% under a 12 h light and dark cycle. Animals were given water *ad libitum* and a standard pellet diet, barring the period where behavioural parameters were evaluated. The animals were also weighed twice a week, and the ones that showed a decrease in body weight by 20% were directly terminated from the study. The study was authorised by the Institutional Animal Ethical Committee (Approval No: IAEC/2019/ I-08) of DPSRU, New Delhi (Reg. No. 215/GO/Reba/S/2000/CPCSEA). Every experimental procedure was carried out as per the regulations of the Committee for Control and Supervision of Experimentation on Animals (CCSEA) under the Prevention of Cruelty to Animals Act, 1960, Ministry of Environment and Forests, Government of India and guidelines of 'Principles of laboratory animal care' (NIH publication number 85-23, revised in 1985). Animal experiments performed in this study were framed in accordance with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments 2.0).

2.7 Chronic Constriction Injury (CCI) Induction in Rats

As per the procedure carried out by Komirishetty *et al.*,[50], surgery was conducted to induce CCI. Initially, the mild anaesthesia was given to animals by intraperitoneal administration of thiopentone sodium (50 mg/kg, *i.p.*)[51]. After the achievement of complete anaesthesia within 15 min, a blunt dissection was done through the biceps femoris in the middle of the left hind limb that exposed the common sciatic nerve. Approximately 5-6 mm of nerve was exposed by freeing the neighbouring tissues that come proximal to the bared tissue, and four ligatures using non-absorbable polyamide surgical sutures (4-0) were tied through the nerve at 1mm apart. Immediately, suturing was done around the muscle and skin layers, and the

povidone-iodine solution was applied externally. The animals were placed in individual cages for their recovery, and the drug administration was done on the following day after the performance of surgery.

2.8 Grouping of Animals and Drug Treatment

Fifty-four animals were divided arbitrarily into 6 groups with 9 rats each (n=9/group/experiment). Group I: Normal control, where rats were provided with vehicle, 0.1 ml in 0.9ml of distilled water. Group II: CCI control, where injuries were induced in animals, and they were given vehicle, 0.1ml in 0.9ml of distilled water. Group III: CCI-induced neuropathic rats were administered Mother tincture, 0.1 ml in 0.9 ml distilled water. Group IV and V: CCI-induced neuropathic rats were administered RT (1×10^{-6} and 1×10^{-30} dilution), 0.1 ml in 0.9ml distilled water, respectively. Group VI: CCI-induced neuropathic rats were administered Gbt (50mg/kg). The treatment regimen was carried out once a day for 14 days by the oral route of administration.

2.9 Experimental Design

As soon as the CCI induction was done, rats were kept accustomed for 3 days. Drug treatment was started on the day after CCI surgery. The two main aspects of anaesthesia, thermal and mechanical allodynia, were measured subsequently on the 3rd, 7th, 11th, and 14th days after the surgery was done[52-54]. The behavioural tests were performed in a randomised order to eliminate bias from 10:00 to 14:00 hr. in the lights-on phase. In the experimental protocol, the Paw Withdrawal Latency (PWL) was noted with 20 sec as the cut-off time. For the estimation of cold and warm allodynia (thermal allodynia), warm water (40 ± 1°C) and cold water (12 ± 1°C) were used for immersing the left hind paw of each rat up to the ankle joint. Mechanical allodynia was recorded using Von-Frey hairs/filaments that consisted of super-tip probes (Exacta, Touch Test Sensory Evaluator, North Coast Medical Incorporation; 2g, 4g, 6g, 8g, 10g, and 15g). The middle of the dorsum of the foot was exposed to Von Frey filaments in increasing order of pressure when the paw touched the mesh floor. Enough force to bend the filaments was applied for 2-3s, and the testing pattern was followed until there was the withdrawal of the ipsilateral paw, which was taken as the beneficial response. If there was no particular response obtained with an initially selected hair (15g), PWL was observed by applying the next higher pressure of 25g. When the response was seen, the next weaker stimulus (below 25g) was taken to record the responses[55]. An average of three readings was recorded for each paw at a gap of 5 min. To avoid any injury to the paw, 25g was set as the cut-off pressure[56,57].

At the end of the treatment, on day 14, animals were given anaesthesia by intraperitoneal administration of thiopentone sodium (50mg/kg) for the estimation of Motor Nerve Conduction Velocity (MNCV). The sciatic nerves of the animals were exposed by maintaining the temperature at 37°C. To measure MNCV, sciatic and tibial nerves were stimulated at the sciatic and tibial notch using bipolar needle electrodes (Power Lab/ML856; AD Instruments, Australia) at 1.5 V amplitude and 0.10Hz frequency for 0.1ms. The stimulating, recording (red and black), and reference (green) electrodes were attached to the animal. An action potential was induced at the proximal end from the stimulating electrode, which was recorded at the distal end of the injury through the recording electrode[58]. Finally, the velocity was calculated using the formula:

2.10 Biochemical Estimation

After recording the MNCV, the animals were euthanized using overdose of thiopentone sodium and the remaining segments of sciatic nerve were collected from every rat and processed as 10% homogenate in ice-chilled phosphate buffer (50mM, pH 7.4) in an ultrasonic processor-Tissue Homogenizer, VCX 130 (Sonics and Materials, Inc., USA). The homogenate was then allowed to centrifuged at 2000g for about 20min at 4°C, and fractions from the supernatant were used to calculate the amount of protein (53), lipid peroxidation measured as Malondialdehyde (MDA)[59-61], reduced Glutathione (GSH)[61,62], catalase[59,63], and Superoxide Dismutase (SOD)[61,64]. The lipid peroxidation (MDA levels) was determined by following the method of Ohkawa *et al.*[60] with slight modifications. The tissue supernatant (100µl) was mixed with 8.1% SDS (0.2ml), 20% acetic acid of pH 3.5 (1.5ml), 0.8% of TBA (1.5ml) and the final volume was made up to 4ml with distilled water. The contents were allowed to boil for 2 h, which resulted in the development of colour. The mixture was allowed to cool, after which

centrifugation was performed at 3000g for 10min. The absorbance of the supernatants obtained was measured at 532nm spectrophotometrically and expressed as µg/mg of protein. The GSH was estimated according to the protocol elaborated in the Glutathione assay kit. The method involves the reduction reaction of reduced glutathione in the samples with a chromophore, resulting in the formation of a stable product measured at 450nm and expressed as nmol/mg of protein. The method explicated by Aebi assessed catalase activity. Approximately 50µl of tissue supernatant was mixed with 100µl of H_2O_2 (30mM) and 1ml of PBS (50Mm, pH-7). The enzymatic reduction was measured spectrophotometrically at every one-minute interval continuously for 3 min at 240nm and stated as U/mg of protein. The SOD activity was estimated as per the instructions in the commercially procured SOD assay kit that recorded the reduction of tetrazolium salt into a formazan dye at 440nm spectrophotometrically. The SOD levels were expressed as U/ml for each sample. All the estimations with standards and samples were performed in triplicate with a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments, Inc., USA). Nitric Oxide (NO) was estimated as per the manufacturer's protocol (Invitrogen-Thermo Fisher Scientific Inc., USA). 150µl of standard sodium nitrite/sample was added to each well-containing 130µl of distilled water. Griess reagent in a volume of 20µl was mixed, and absorbance was measured at 548nm after 30min of incubation. The concentration of NO was represented as µM of NO per mg of protein.

Cytokine levels (IL-1β, IL-6, and TNF-α) were measured in tissue homogenate as per the manufacturer's instructions (R and D Systems, Inc., USA). Briefly, the tissue samples/standards were added to the prepared 96-well plates that were coated with capture antibody. Sandwich ELISA was performed afterwards with the help of a detection antibody to quantify the cytokine levels by using a microplate reader set to 450nm.

2.11 Histopathological Examination

After all the behavioural tests and MNCV estimation on the 14th day, the animals were euthanised with an overdose of thiopentone sodium (intraperitoneal), and the damaged left sciatic nerve was isolated together with 1cm segments from the proximal and distal sides of the ligated portion. A 5mm central portion of the secluded nerve segment was treated with formalin for histopathological examination at the Institute of Liver and Biliary Sciences, New Delhi. The sections of 4µm thickness were acquired and stained with hematoxylin and eosin (H and E) staining. The resulting sections were observed under the Olympus CH20i microscope, and MagVision^{TM} advanced Image Analysis Software was used to examine structural changes such as fibre derangement, bulging of nerve fibres, and appearance of inflammatory cells[54].

2.12 Statistical analysis

For every observed value in each experiment (n=9), the mean ± standard error of the mean (SEM) was determined unless otherwise stated. Statistical analysis was carried out by one-way and two-way Analysis of Variance (ANOVA) and subsequently by Dunnett's multiple comparisons for all the tests. GraphPad Prism9 (GraphPad Software, San Diego, CA) was used to carry out the statistical analysis. The pvalue $p<0.05$ was always taken as statistically significant.

3. Results

3.1 Effect of RT on MTT assay

The result of RT on the percentage cell viability of U-87 cells is depicted in Figure 2 based on one-way ANOVA followed by Dunnett's multiple comparisons test. MTT assay showed that % cell viability in normal cells was found to be 100%, which showed a marked increase on treatment with LPS to about 126.45%. Simultaneously, cell viability induced due to ROS when LPS was used showed a decline with the tested dilutions of RT treatment in a dose-dependent manner, as shown in Figure 2. RT at a dilution of 1 $\times 10^{-30}$ produced a maximum modulatory LPS-triggered percentage cell viability effect on viability and reduced it to 93.41% in comparison to the LPS + control group.

3.2 Effect of RT on Warm Allodynia

Figure 3(a) shows the results of two-way ANOVA followed by Dunnett's multiple comparisons test, where there is a reduced threshold of sensitised nociceptors to lukewarm water (40 ± 1°C) in CCI + vehicle rats in comparison to the normal control, which showed double the values. A 14-day treatment regimen with RT and Gbt produced a prominent paradigm shift ($p<0.05$)

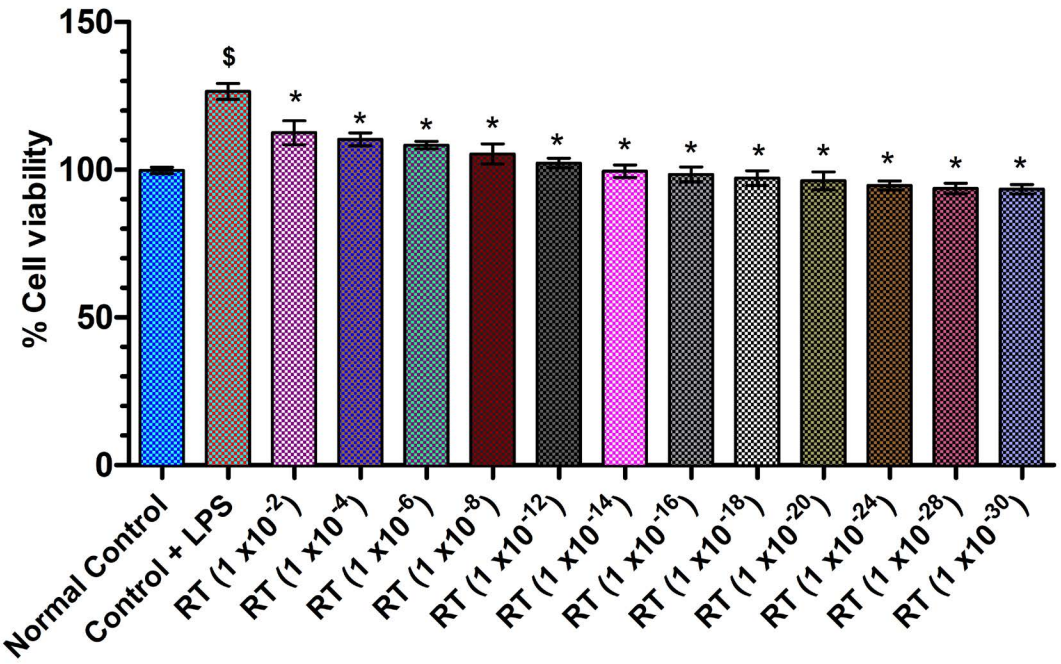


Figure 2. Effect of ultra-dilutions of RT on % cell viability by MTT assay after LPS-induced U-87 cells. Data indicates mean ± S.E.M. of quintuples sample. $=p<0.05 vs. normal cell, *=p<0.05 vs. LPS Control (one-way ANOVA followed by Dunnett's multiple comparisons test).

in the paw withdrawal latencies from the 3rd day itself. Here, RT (30cH) was found to be in congruence with the standard drug Gbt on day 14. Contrary to the previous day's observations, RT (MT), which showed a similar effect to RT (6cH) initially, was surpassed significantly by the same on day 11 and became parallel to RT (30cH).

3.3 Effect of RT on Cold Allodynia

Figure 3(b) represents a notable decline ($p<0.05$) in paw withdrawal due to the allodynia produced in CCI + Vehicle rats in response to the cold stimulus (cold water; 12 ± 1°C) as analysed by two-way ANOVA followed by Dunnett's multiple comparisons test. This significant reduction was observed for the normal control group, where the latency was recorded to be almost 20s. RT (6cH and 30cH) and Gbt were found to be successful in enhancing the reduced latency to normal when the statistical test was applied. These significant observations were seen right after the surgery on the 3rd day. However, the RT (MT) could show these noteworthy effects only from day 7 onwards of the 14-day treatment paradigm.

3.4 Effect of RT on Mechanical Allodynia

The paw withdrawal latency in rats, when 15 g of Von Frey filament touched the middle of the dorsum of the foot in contact with the mesh floor, was shown in Figure 3(c). Here, it was revealed that rats in the CCI + vehicle group showed less latency to withdraw their paw when the mechanical pressure was applied. This latency time was significantly less ($p<0.05$) compared to the normal control group right from day 3. Two-way ANOVA with subsequent Dunnett's multiple comparison test demonstrated that this trend of reduced paw withdrawal latency was brought back to normal significantly by treatment with RT (6cH and 30cH) and standard drug Gbt from day 3 itself, whereas RT (MT) took 7 days to show this significant effect. The result of RT (30cH) was almost complementary with Gbt on day 11, confirming its notable effect on allodynia.

3.5 Effect of RT on MNCV

The results depicted in Figure 4 comparison test showed that CCI produced a pronounced reduction in the nerve conduction velocity in comparison to the normal control group due to severe nerve damage in

rats. Statistical analysis (One-way ANOVA followed by Dunnett's multiple comparisons test) revealed that administration of RT (ultra-dilutions) showed a remarkable enhancement in the velocity ($p<0.05$) in agreement with the standard group Gbt (50mg/kg). The significant upward trend in the motor nerve velocity with RT was found to be concentration-dependent. The more diluted 30cH had better MNCV compared to the less diluted 6cH and even the mother tincture.

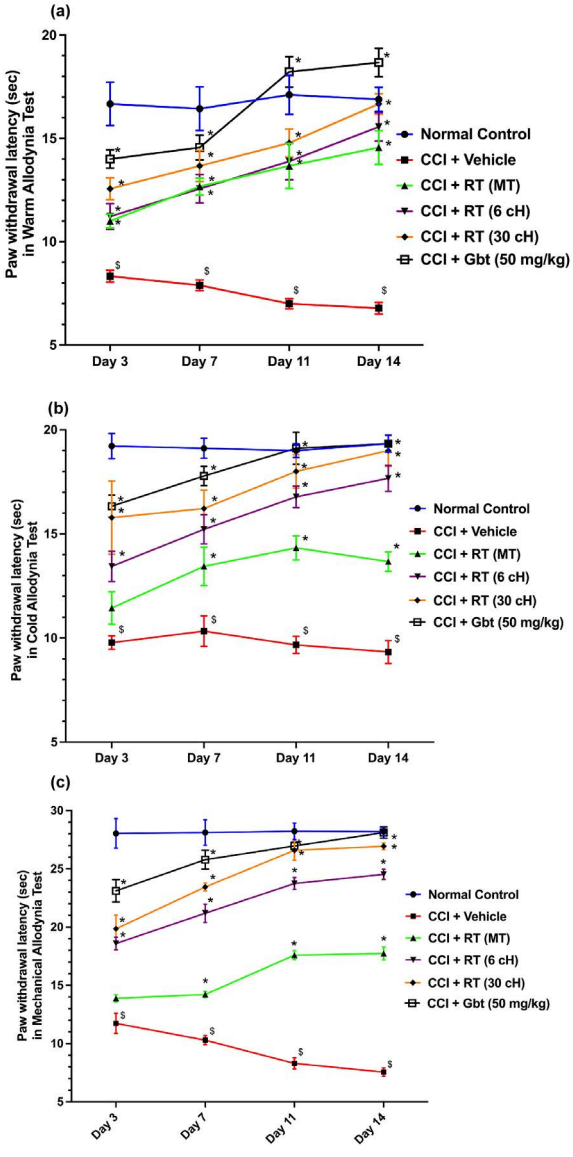


Figure 3. Effect of RT on rat paw withdrawal latency in seconds during - **(a).** Warm Allodynia, **(b).** Cold Allodynia, and **(c).** Mechanical Allodynia test in CCI-induced neuropathic pain. Data represent mean ± S.E.M. (N=9). $=p<0.05 vs. normal control, *=p<0.05 vs. CCI Control (two-way ANOVA followed by Dunnett's multiple comparisons test).

3.6 Effect of RT on Biochemical Parameters

The implication of RT treatment on oxidative and nitrosative stress and biochemical parameters was analysed between different groups by one-way ANOVA, followed by Dunnett's multiple comparisons test, and represented in Figure 5. The MDA (a marker of lipid peroxidation) and nitric oxide levels were observed to be notably elevated ($p<0.05$) in the CCI + vehicle group in comparison to the normal control, which indicated severe oxidative stress that occurs soon after the sciatic nerve injury. This augmentation in the LPO and NO status was notably reversed by the crude and diluted forms of RT (MT, 6cH, and 30cH). Enzymatic and non-enzymatic markers of the antioxidant defense system including catalase and SOD activities and reduced glutathione (GSH) were remarkably on a lower level in the sciatic nerve homogenate of CCI control compared to the normal control group. Treatment with RT for 14 days appreciably restored the GSH, catalase, and SOD levels ($p<0.05$) starting with the crude form at best compared to CCI Control. All these results were in congruence with the standard group of Gbt, which significantly reduced the oxidative damage produced in the injured animals.

3.7 Effect of RT on Cytokine Levels in Nerve Tissue Homogenate

From the sciatic nerve homogenate collected towards the end of the regimen on day 14, cytokine assays were carried out for IL-1β, IL-6, and TNF-α, depicted in Figure 6, based on one-way ANOVA followed by Dunnett's multiple comparisons test. Pro-inflammatory cytokine levels showed a notable upward trend in the CCI + vehicle group when compared to the normal control group. Except for IL-Iβ, IL-6, and TNF-α showed a noteworthy reduction ($p<0.05$) with the treatment of all forms of RT, *i.e.*, crude and diluted forms. At the same time, IL-Iβ levels were normalised with the ultra-dilutions of the drug (6cH and 30cH) rather than its crude form. These results were parallel to the effects of the standard drug Gbt (50mg/kg), which always showed an excellent and significant effect ($p<0.05$) on the altered cytokine levels in bringing them towards the normal level.

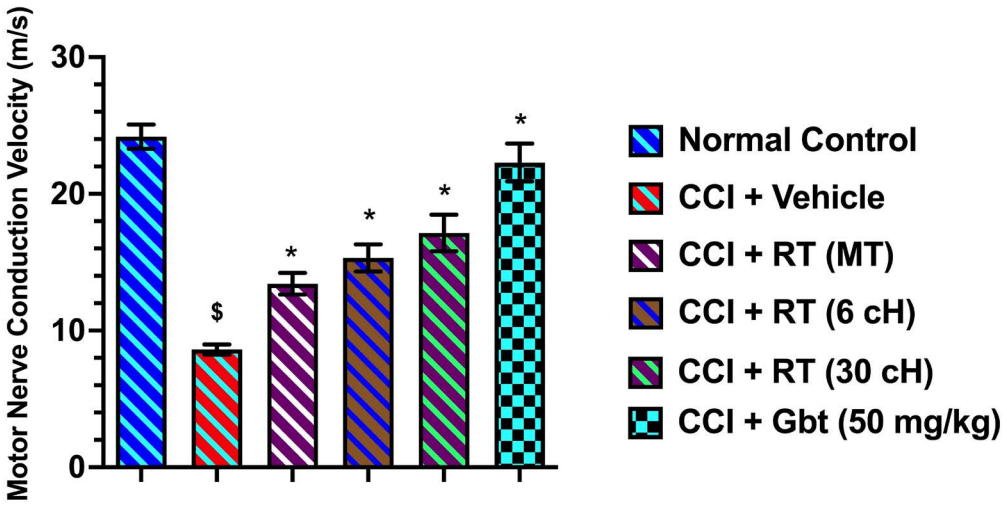


Figure 4. Effect of RT on Motor Nerve Conduction (in m/s) in CCI-induced neuropathic pain. Data represent mean ± S.E.M. (N=9). $=p<0.05 vs. normal control, *=p<0.05 vs. CCI Control (one-way ANOVA followed by Dunnett's multiple comparisons test; n=9).

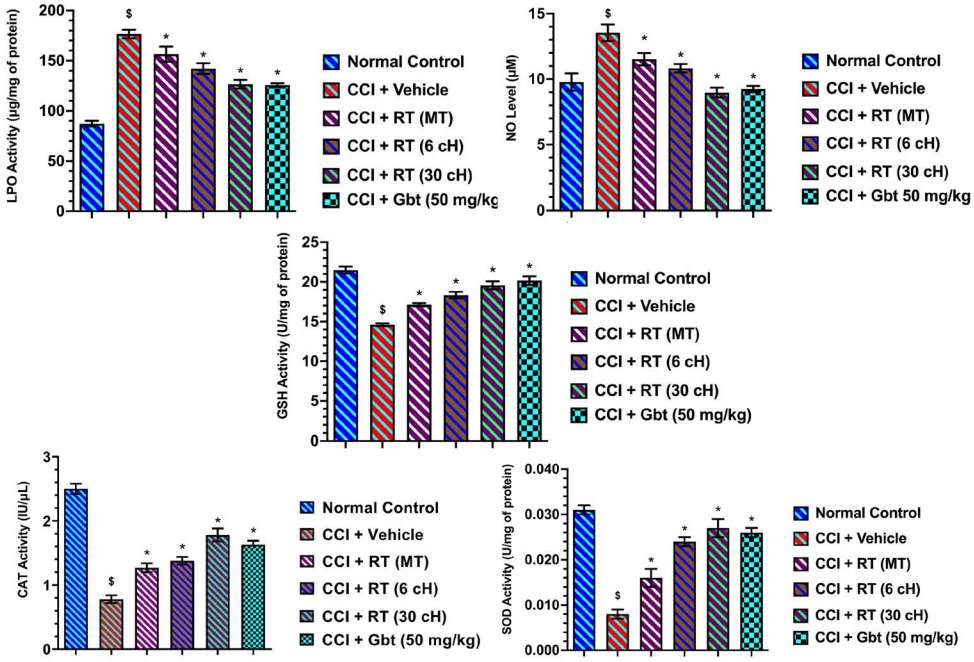


Figure 5. Effect of RT on biochemical parameters; LPO (µg/mg of protein), NO (µM), GSH (U/mg of protein), CAT (IU/µL), and SOD (U/mg of protein) in CCI-induced neuropathic pain. Data represent mean ± S.E.M. (N=9). $=p<0.05 vs. normal control, *=p<0.05 vs. CCI Control (one-way ANOVA followed by Dunnett's multiple comparisons test; n=9).

3.8 Effect of RT in CCI-Induced Histopathological Changes in Sciatic Nerve

The observations, as shown in Figure 7, indicated that the normal control group showed a usual morphological view of the sciatic nerve, whereas the CCI control group with the sciatic nerve injury demonstrated swollen nerve fibres, altered fibre architecture, enhanced inflammatory cell infiltration, and augmented neuronal gaps. It was observed that

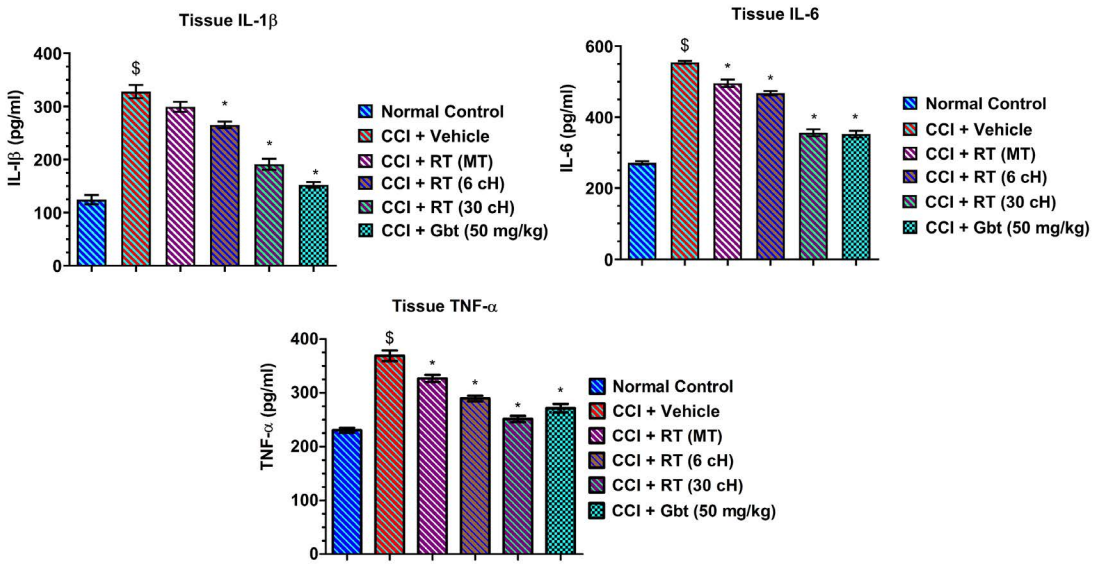


Figure 6. Effect of RT on pro-inflammatory cytokines in sciatic nerve homogenate in CCI-induced neuropathic pain. Data represent mean ± S.E.M. (N=9). $=p<0.05 vs. normal control, *=p<0.05 vs. CCI Control (one-way ANOVA followed by Dunnett's multiple comparisons test; n=9).

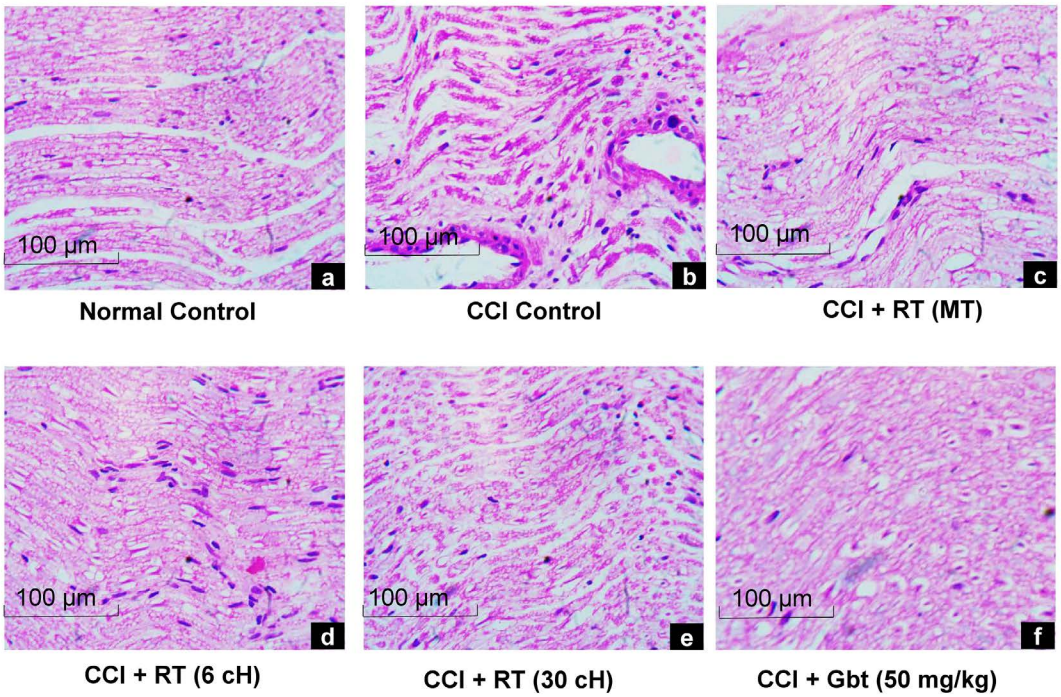


Figure 7. Histopathological changes in sciatic nerve (Hematoxylin and Eosin 400X). **(a).** The normal control group showed a normal morphological view of the sciatic nerve. **(b).** The CCI control group demonstrated swollen nerve fibres, altered fibre architecture, and enhanced inflammatory cell infiltration. **(c).** The mother tincture-treated group showed less pronounced inflammation but mild infiltration. **(d).** Treatment with RT (1 x 10^{-6}) showed negligible inflammation with mild inflammatory cell infiltration. **(e).** Treatment with RT (1 x 10^{-30}) completely inhibited inflammation and reduced infiltration of cells. **(f).** Treatment with Gbt normalised the fibre derangement observed in the CCI control group.

with 14-day treatment, Mother tincture showed less pronounced inflammation but mild infiltration, and treatment with RT (1 x 10^{-6}) produced negligible inflammation with mild inflammatory cell infiltration. Treatment with RT (1 x 10^{-30}), on the other hand, almost completely inhibited inflammation and reduced infiltration of cells. In animals that were treated with Gbt, the fibre derangement that was observed in the CCI control group was normalised, reversing the negative effects of the peripheral nerve injury. The results are depicted in the Figure 7 as: (a) normal control group showed a normal morphological view of the sciatic nerve; (b) CCI control group demonstrated swollen nerve fibers, altered fiber architecture, and enhanced inflammatory cell infiltration; (c) mother tincture-treated group showed less pronounced inflammation but mild infiltration; (d) treatment with RT (1 x 10^{-6}) showed negligible inflammation with mild inflammatory cell infiltration; (e) treatment with RT (1 x 10^{-30}) completely inhibited inflammation and reduced infiltration of cells; and (f) treatment with Gbt normalised the fiber derangement observed in the CCI control group.

4. Discussion

To solve the challenges in drug discovery in the area of neuropathic pain, researchers are in a constant quest to find new chemical entities through the employment of surgical methods in rats and mice, as surgical models have a preponderant role in inducing pain states[65]. Animal models that involve peripheral nerve injury, such as chronic constriction injury in the sciatic nerve, emulate human indications of neuropathic pain, such as hyperalgesia, spontaneous pain, and allodynia, making them a principal model in NP experiments[38]. The animals with chronic constriction injury show unsolicited behaviour which remains in consonance with the pain, reduced thresholds, and magnified reaction in response to mechanical and thermal stimuli[38,66,67]. These responses appear two days after the surgery and peak at 10-18 days, which can last up to two months[38,68-70].

Even though there are several reports verifying the efficacy of ultra-molecular dilutions of homoeopathic medicines[31,71-74], only a few pre-clinical studies exist that verify and replicate the same under standard conditions[29,75-81]. Increasing controversy in the efficiency of ultramolecular dilutions of homoeopathic medicines necessitates the need for systematic data in the field of preclinical and clinical research[82]. A work reported by Chikramane *et al.*[83] confirms the existence of nanoparticles of mother tincture to the lowest of 200cH dilution, and multiple studies have further established this for the plant *Rhus tox* and the efficacy of its homoeopathic dilutions, such as in arthritis pain[82-84]. The results of our present study show that RT in its crude and diluted forms ameliorates the allodynia and molecular alterations produced due to severe damage to the sciatic nerve caused by chronic nerve constriction. This was based on the initial investigation of *Rhus tox* on the *in vitro* model of the LPS-treated U-87 glioblastoma cell line. Different concentrations were selected that showed a reduction in percentage cell viability induced by LPS and were further tested for the *in vivo* study of the anti-neuropathic efficacy of RT in CCI-induced sciatic nerve injury. Previous studies have also demonstrated the role of *Rhus tox* in inflammation, which plays a vital role in inflammatory disorders[85].

A cell viability study of RT on the U-87 cells was carried out by MTT assay. The assay estimates the metabolic reduction of soluble MTT salt to a coloured insoluble formazan crystal, carried out by mitochondrial dehydrogenase enzyme, reflecting the cell's viability. Thus, the amount of formazan dye formed corresponds to the number of viable cells in the culture[86]. LPS, which forms a major constituent in the outer membrane of Gram-negative bacteria[87] produced a hike in the percentage of cell viability to 126.45% from 100% in the normal control group. This hike in cell viability measured by the MTT assay showed a decline in varying concentrations of RT. 1 × 10^{-30} dilution produced the maximum inhibitory effect on cell viability and brought down the value to 93.41%.

CCI instigated the neuropathic pain model, which is one of the most validated models that demonstrated anomalous posture in the injured hind-paw, where the toes are seen to be held together, flexed plantar region and everted paws[88]. They repeatedly shook, shielded, and licked the injured paw, indicating spontaneous pain[66]. In line with these observations, animals exhibited elevated levels of pain threshold and showed less paw withdrawal latency due to the allodynia

induced in them. Various studies reported that there was a reduction in both non-myelinated and myelinated fibres, with a more specific effect on myelinated neurons, which led to the behavioural alterations observed in this model[89-92]. Reports suggested that the surviving fibres accounted for the behavioural changes observed in the animals[91,93]. This was confirmed by the finding that there is a decline in the conduction velocities in remaining myelinated neurons in CCI, which is likely due to the segmental demyelination in or around the lesion site[94,95]. With a 14-day treatment regimen, RT (crude and ultra-dilutions) showed immense potency in alleviating these behavioural as well as conduction anomalies. The 30cH potency of RT was observed to be the most effective of all, irrespective of the kind of stimulus applied to animals. This group of animals was also successful in attenuating the histopathological changes induced due to the injury. It has been observed that many underlying mechanisms, including oxidative stress and inflammation, pave the way to the progression of nociceptor sensitisation and successive neuropathic pain because of nerve injury[49,96-99].

Reactive oxygen and nitrogen species have been registered to be the contributing factors to pathophysiological changes in neuropathic pain[100]. They diminish the antioxidant defence system and cause DNA damage, activating the PARP enzyme, which in turn augments the inflammatory cascade in the nerve[101-103]. The reactive oxygen species level is indirectly measured from the concentration of MDA and NO formed in the tissue, which is an indicator of the oxidative/nitrosative stress[96]. Various reports have stated the role of nitric oxide and its elevation in the initiation and progression of peripheral neuropathic pain[104,105]. These studies were corroborated by the elevated levels of MDA and NO, which indicated severe oxidative damage in the tissue. The treatment of RT for 14 days produced an immense enhancement of the values, demonstrating the antioxidant capacity of the plant. Similarly, endogenous antioxidant enzymes namely SOD and catalase primarily form the antioxidant defense that fights against the free radicals[104,106] were found to be reduced, confirming impairment in the antioxidant defence system in neuropathic pain. In congruence with these observations, reduced glutathione, which is a low molecular weight non-enzymatic antioxidant, was also observed at a lower level in the CCI group in comparison to the normal control. This is mainly because the loss of glutathione, especially in the mitochondria, leads to enhanced vulnerability of neurons to toxic stimuli and further oxidative stress[107-109]. Oxidative stress caused by a decline in these antioxidant defence systems was also normalised by treatment with RT, showcasing its effectiveness in combating oxidative stress, which is a prominent mechanism underlying neuropathic pain.

The work also demonstrated a decline in the pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α, which point to the involvement of the inflammatory cascade in the peripheral nerve injury. This is because the consequence of CCI injury, glial cells get triggered and lead to the emanation of interleukins (IL-6, IL-1β, and TNF-α) that further facilitate the pain process[110]. TNF-α acts as the initiator, which triggers the secretion of IL-1β and IL-6, and acts as a potent mediator of inflammation[111,112]. Previous studies have confirmed its role in mitigating allodynia and hyperalgesia in various animal models[113-116]. Results from these cytokine studies confirmed the overall protective role of RT in the inflammatory cascade and were supported by histopathological examinations specifying inhibited inflammation and reduced infiltration of cells around the sciatic nerve fibers. Hence, from these findings, RT is found to have an influential part in the mechanism of oxidative stress and inflammation and is thus bound to have anti-neuropathic efficacy.

5. Conclusion

Our study concluded that homoeopathic dilutions of RT (MT, 6cH, and 30cH) have notable effects on the surgical model of CCI-induced neuropathic pain. It was observed that the drug reversed the allodynia observed with various stimuli, including temperature and touch, *i.e.,* thermal and mechanical allodynia. This is based on its effect on a molecular level, where it acts on the oxidative/nitrosative stress and inflammatory pathways by acting on pro-inflammatory cytokines. Both are predominant factors in aggravating the neuropathic pain caused by damage to the sciatic nerve. Even though the results are supportive, further pre-clinical and standardised clinical studies are needed to elucidate these effects or any other mechanism of action of RT, suggesting its anti-nociceptive effect.

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7. Author Contribution

Nikhila Shekhar: Investigation, Data curation, Formal analysis, Visualisation, Writing- original draft. Anil Khurana: Conceptualisation, Project administration, Funding acquisition, Writing- review and editing. Debadatta Nayak: Conceptualisation, Project administration, Funding acquisition, writing, review and editing. Ajit Kumar Thakur: Conceptualisation, Methodology, Supervision, Writing- review and editing, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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