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Anti-oxidant profile of Divya-Peedantak-Vati abates paclitaxel-induced hyperalgesia and allodynia in CD-1 mice model of neuropathic pain

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ABSTRACT

Background: Paclitaxel (PTX) is a known chemotherapeutic agent, used to treat different types of cancers. However, it leads to neuropathic pain in a sizeable proportion of the patients. Currently, analgesics, opioids, tricyclic antidepressants and anticonvulsants are prescribed for PTX-mediated chemotherapy-induced peripheral neuropathy (CIPN). However, these treatments have acute side-effects, creating a need for safer alternative treatment option.

Method: PTX-mediated CIPN has nociceptive outcomes, like, allodynia and hyperalgesia with an underlying inflammatory and oxidative causes. The herbo-mineral medicine, Divya-Peedantak-Vati (DPV) is known for its effectivity against joint pain and inflammatory disorders in Ayurveda, a traditional Indian system of medicine. Therefore, anti-neuropathic potential of DPV was assessed in PTX-induced neuropathic CD-1 mice, with allodynia and hyperalgesia, as the clinically relevant endpoints. DPV was investigated for its nociception-modulatory and anti-inflammatory effects under *in vivo* and *in vitro* conditions.

Results: DPV treatment exhibited notable anti-allodynic and anti-hyperalgesic effects in a dose dependent manner, and displayed prominent anti-oxidant effects in the sciatic nerve of the study animals. It also moderated, in a dose dependent manner, the levels of cytokine, interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling.

Conclusions: The novelty of this study lies in the demonstration of the anti-neuropathic potential and elucidation of anti-neuropathic mode-of-action of an herbal medicine. Taken together, this study showed that DPV could well be an effective alternative healing option for PTX-mediated CIPN.

Introduction

Chemotherapy prolongs patient's life, but, has several after-effects, neuropathic pain being one of them. Chemotherapy-induced peripheral neuropathy (CIPN) is mostly associated with platinum and taxane-derived anti-cancer drugs and often lead to treatment discontinuity (Quintão et al., 2019). CIPN manifests as loss of sensation, tingling, gait disturbances, pain, paraesthesia, numbness and allodynia (Heuvel et al., 2017). The available options for CIPN treatment are limited, and have adverse side-effects. Usually, patients with CIPN are administered tricyclic anti-depressants and anti-convulsants, despite their suboptimal efficacy and undesirable side-effects (Fukuda et al., 2017; Hou et al., 2018). Duloxetine, recommended by American Society of Clinical Oncology (ASCO) for CIPN, besides, the regular side-effects, exhibits central neurotoxicity with hyporeflexia, mental confusion and catatonia (Quintão et al., 2019). Conventional non-steroidal anti-inflammatory drugs and opioids, despite being anti-allodynic, are undesirable due to their side-effects, like, seizures and respiratory malfunctioning. Reported anti-hyperalgesia and anti-allodynia effects of repurposed anti-diabetic metformin and anti-depressant venlafaxine await confirmation (Yamamoto and Egashira, 2021). Therefore, alternative CIPN treatment options with safer profiles are required. Several independent studies have confirmed the analgesic properties of plants, recognized in traditional systems of medicines, like, *Acorus calamus, Aconiti tuber, Allium sepa, Allium sativum, Artemisia dracunculus, Cannabis sativa, Emblica officinalis, Gingko biloba, Nigella sativa* and *Vochysia divergens*

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(Garg and Adams, 2012). Divya-Peedantak-Vati (DPV) is a herbo-mineral formulation used in traditional Indian medicinal system of Ayurveda for the treatment of joint pain. In a recent pre-clinical study, anti-inflammatory effect of DPV has been ascertained without any observable toxic effects (Balkrishna et al., 2019a). DPV is made of 23 natural ingredients, out of which 19 are herbs, 1 herbo-mineral and 3 minerals. *Dashmool*, a herbal ingredient of DPV, is actually a blend of the roots of ten specific medicinal plants (Balkrishna et al., 2019a) **(supplementary Table S1)**.

Chemotherapy induced peripheral neuropathy, since arise from a scheduled and regimen-driven factor, is preventable (Yamamoto and Egashira, 2021). In this study, the anti-neuropathic potentials of DPV were validated in a paclitaxel (PTX) induced mice model of CIPN. PTX dosage used reproducibly elicited CIPN symptoms as reported earlier (Thangamani et al., 2013). PTX-induced CIPN leads to mitochondrial dysfunction through oxidative stress, and consequently, results in anti-nociceptive effects (Duggett et al., 2017). Mutually affecting pathophysiological processes of oxidative stress and inflammation are found to be simultaneously active in CIPN (Biswas, 2016; Boyette-davis et al., 2015). Chemotherapy induced immunomodulatory effects triggers cytokine driven neuroinflammation during CIPN pathogenesis (Lees et al., 2017). Thus, herbs capable of ameliorating oxidative stress and inflammation with immune-modulatory effects are likely to be potent against CIPN. Two herbs present in DPV, *Tinospora cordifolia* (Ghosh and Saha, 2012; Khan et al., 2016) (an independent ingredient) and *Cedrus deodara* (Gupta et al., 2011) (a part of the classical formulation Yograj Guggulu), have all these three properties. Other herbal components of DPV, *Trachyspermum ammi* (Bairwa et al., 2012; Ranjan et al., 2011) and *Boerhavia diffusa* (Mahesh et al., 2012) are known for their nociception-modulatory effects; the latter having anti-inflammatory and immune-modulatory properties in addition. Besides, *T. ammi* (Bairwa et al., 2012; Ranjan et al., 2011), *Withania somnifera* (Dar et al., 2016), *Vitex negundo* (Gill et al., 2018), *T. cordifolia* (Ghosh and Saha, 2012; Khan et al., 2016) and *Zingiber officinale* (Shakya, 2015), have anti-oxidative property. *Pluchea lanceolata* (Bhagwat et al., 2010; Srivastava and Shanker, 2012) is known to have analgesic property. So, besides evaluating the anti-allodynia, anti-hyperalgesia and nociception-modulatory efficacies of DPV, its anti-oxidant potential was also investigated. Histological analysis of sciatic nerve tissue was undertaken to validate the *in-vivo* efficacy of DPV in ameliorating oxidative stress associated axonal degeneration (Tasnim et al., 2016). *In-vitro* anti-inflammatory effect of DPV was re-validated in THP-1 human macrophagic cell line. The observations from the current study demonstrate that the herbo-mineral medicine, DPV effectively ameliorates CIPN pathophysiology by targeting cytokine-mediated neuroinflammation, triggered by oxidative stress. Altogether, this study provides a compelling evidence for the anti-neuropathic potentials of DPV.

Material and methods

Chemicals, reagents and cell lines

Divya-Peedantak-Vati (DPV) (Batch # PTV 032, manufactured date: July 2019) was sourced from Divya Pharmacy, Haridwar, Uttarakhand, India. Sodium carboxymethyl cellulose (Na-CMC) (Catalogue # 419,273–100 G) and Lipopolysaccharide (LPS) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium chloride injection I.P. 0.9% w/v manufactured by Infutec Healthcare Ltd. (Indore, Madhya Pradesh, India) was purchased over the counter from local chemist shop. Cell culture media, RPMI-1640 and DMEM, and supplements, fetal bovine serum (FBS), antibiotic/antimycotic mixture and L-glutamine were obtained from Gibco (Amarillo, TX, USA). IL-1β ELISA kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Phorbol 12-myristate 13-acetate (PMA) was from Alfa Aesar (Haverhill, MA, USA), Alamar blue from HiMedia (Mumbai, Maharashtra, India) and Normocin, QB buffer and QB reagent (Quanti-blue™) were from InvivoGen (San Diego, CA, USA). MITOTAX 100 (Paclitaxel injection I.P.), manufactured by Dr Reddy's Laboratories Ltd. (Hyderabad, Andhra Pradesh, India) was purchased from local pharmacy, and Gabapentin (GABA) from TCI Chemical Industry Co. Ltd. (Tokyo, Japan). Human monocytic THP-1 cell line was procured from the ATCC recognized repository at National Center for Cell Science, Pune, Maharashtra, India. HEK-Blue™ TNF-α cells was obtained from Invivogen (San Diego, CA, USA).

In vivo experiments

Animal procurement and housing

Anti-neuropathic validation study was carried according to the study protocol PRIAS/LAF/IAEC-069 which was approved by the Institutional Animal Ethical Committee (IAEC) of Patanjali Research Institute, Haridwar, Uttarakhand, India, on July 17, 2019. Animal experimental plan has been schematically represented in **Fig. 1A**. 6–8-week-old male CD-1 mice (20–25 g) were purchased from Charles River Laboratory–licensed supplier, Hylasco Biotechnology Pvt. Ltd., Hyderabad, India. Animals were housed in a registered animal house (registration number: 1964/PO/RC/S/17/CPCSEA) in polypropylene cages at 22 ± 3 °C and 60–70% relative humidity under 12:12 h light and dark cycle with *ad libitum* access to standard pellet diet (Purina Lab Diet, St. Louis, MO, USA), and sterile filtered RO water.

DPV dose calculation

DPV is a pre-formulated product in tablet dosage form; which was powdered before administration to the experimental animals. The animal equivalent doses of DPV for mice study was calculated from body surface area. The human recommended therapeutic dose of the DPV is 2 tablets (500 mg each) twice a day (2 g per day). Animal equivalent dose (mg/kg) for mice was calculated by multiplying human dose (mg/kg/day) by a factor of 12.3 (Nair and Jacob, 2016). Considering the average human weight of 60 kg, therapeutic equivalent dose for mice was calculated at 410 mg/kg/day, which was taken as the high dose in the *in vivo* assessment of anti-neuropathic pain potential of DPV. One-third of this therapeutic equivalent dose, 137 mg/kg/day was considered as the low dose for the study.

Establishment of CIPN mouse model, allocation of treatment groups for evaluation of anti-neuropathic pain potential of DPV

CIPN was induced in 8–10 weeks old male CD-1 mice using paclitaxel (PTX) according to a previous report (Balkrishna et al., 2020a; Kaur and Muthuraman, 2019; Thangamani et al., 2013). Animals in group G1 received equivalent volumes of normal saline orally and served as normal control (NC). Animals in groups G2 to G5 were intra-peritoneally (i.p.) administered with 2 mg/kg body weight of PTX for 6 consecutive days, and subsequently, screened for neuropathic pain before randomizing into treatment groups G3-G5. Animals in the disease control (DC) group G2 received 0.25% Na-CMC orally. Group G3 was the reference group in which the animals were intra-peritoneally administered the reference drug, gabapentin (GABA) at 100 mg/kg body weight. Animals of groups G4 (DPV 137) and G5 (DPV 410) respectively received 137 and 410 mg/kg oral doses of DPV. Each group has 6 animals and received treatments daily for two weeks.

Evaluation of pain behaviors

Hot Plate Test. The hot plate test was performed on days 0, 6, 11 and 16 to measure latency time to response according to a previous report with minor modifications (Arrau et al., 2011; Balkrishna et al., 2019b). The assay was conducted after a gap of 1 h from dosing. The animals were placed in Perspex cylinder of the hot plate (UgoBasile, Italy) set at 55.0 ± 0.5 °C. Response (licking paws or jumping)/latency time to discomfort was recorded below a cut-off of 20 s to avoid tissue damage. The experimenter was blinded to the treatment conditions.

Tail Flick Test. Tail flick test measures the latency time of response to nociceptive pain. The assay was conducted using a plantar test device

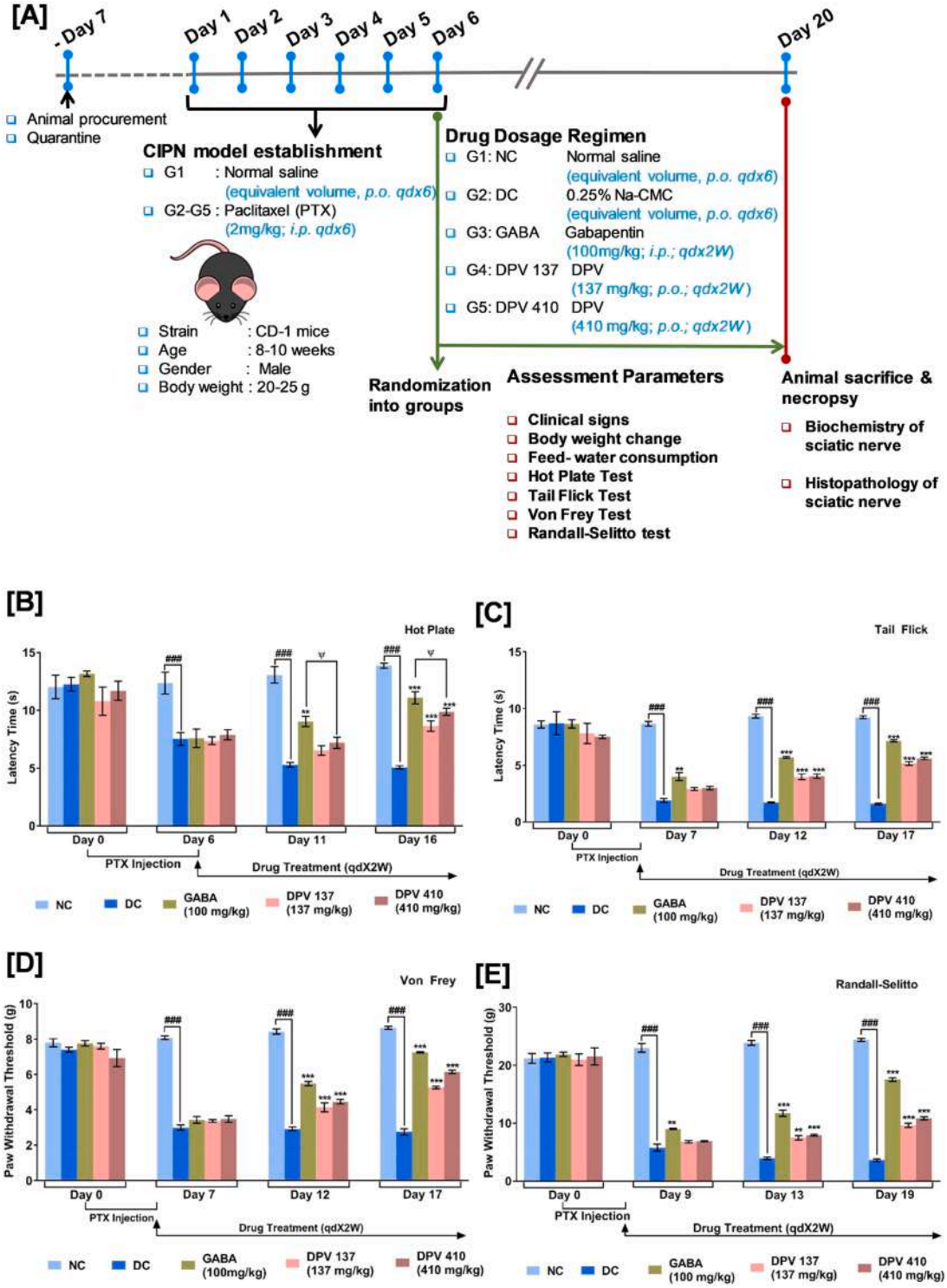


Fig. 1. Evaluation of anti-CIPN potential of DPV. [A] Schematic representing the establishment of PTX-induced murine CIPN model and subsequent plans for evaluation of anti-CIPN effect of DPV. [*qd, quaque die (L)*: once a day; *p.o., per os (L)*: by mouth; *i.p.*, intraperitoneal; × 6: for 6 days; × 2 W: for 2 weeks]. **[B–E]** Graphical representations of the effects of DPV treatment on the CIPN modulated nociceptive behaviors of the experimental animals, as determined through hot plate **(B)**, tail flick **(C)**, Von Frey **(D)** and Randall-Selitto pressure **(E)** tests. Data represented as mean ± SEM and statistical significance denoted as ψ, ** and *** or ### for p values < 0.05, 0.01 and 0.001, respectively. P values were shown with either '*', '#' or 'ψ' depending on whether the comparison was with NC, DC or GABA group.

(7370 plantar test from UgoBasile, Italy) at infrared radiation intensity of 50 with slight modifications of a method reported earlier (Balkrishna et al., 2019b; Keyhanfar et al., 2013). Three readings from each with intermittent gaps of 5 min were recorded below a 15 s cut off. The test was conducted on days 0, 7, 12 and 17 with the experimenter blinded to the treatment conditions.

Von Frey Test. The Von Frey test was conducted on days 0, 7, 12 and 17 to assess responses to mechanical stimuli using Electronic Von Frey (Model: 38,450–001, UgoBasile, VA, Italy) (Donvito et al., 2016; Zanjani et al., 2014). The animals were habituated to the transparent

Perspex cubicles with elevated wire mesh bottom through three 45 min sessions, on consecutive days prior to the experiment. After a 10 min acclimatization, immediately before the experiment, Von Frey filament (0.5 mm diameter) was applied to the plantar surface of the right hind paw of each mouse, with gradual increase in the applied pressure. Lifting, licking or shaking of the paw was monitored and the minimum weight, below the 10 g margin set to avoid tissue damage, that elicited the response was recorded as paw withdrawal threshold (PWT; g). The experimenter, recording a total 3 readings with 3 min gaps in between, was blinded to the treatment conditions.

Randall–Selitto pressure test. The Randall–Selitto pressure test was used to determine static hyperalgesia according to a protocol described previously, with slight modifications (Balkrishna et al., 2019c). The pain response to the minimum mechanical stimulus as applied weight below 25 g, that made the animal vocalize or withdraw the paw, was recorded as paw withdrawal threshold (PWT) using 37,215 Analgesy-meter (UgoBasile, Italy). The test was carried out on days 0, 9, 13 and 19, 1 h after dosing. Data represented is an average of 3 readings recorded with intermittent gaps of 5 min by a researcher oblivious to the treatment conditions.

Histopathological evaluation of sciatic nerves

Sciatic nerves were harvested at the end of the experiment and fixed in neutral buffered formalin before H-E staining according to earlier protocol (Balkrishna et al., 2019b; Balkrishna et al., 2019c). The slides were microscopically visualized and scored for histopathological lesions by a veterinary pathologist blinded to the experimental treatments. The severity of the observed lesions was scored on a scale of 1–5 with 1 conveying minimal aberration (<1%), 2 pertaining to mild anomaly (1–25%), 3 referring to moderate irregularity (26–50%); 4 meaning marked difference (51–75%) and 5 showing severe damage (76 – 100%) of the tissue. Focal, multifocal and diffused distributions of the lesions were recorded at low (10x) and high (40x) magnifications using an Olympus Magnus microscope camera and subsequently, processed with Olympus MagVision image analysis software.

Evaluation of anti-oxidant parameters in sciatic nerve

Reduced (GSH) and oxidized (GSSG) glutathione levels were measured according to an earlier report (Hissin and Hilf, 1976). Briefly, sciatic nerve tissues, homogenized in 0.1 M phosphate buffer (pH 8) containing 25% metaphosphoric acid, were clarified through centrifugation at 10,000 g for 20 min at 4 °C. Clarified homogenate was incubated with o-phthaldialdehyde (OPT) at room temperature for 15 min in dark and fluorescence measured at 350 (excitation) and 420 (emission) nm using Envision microplate reader (Perkin Elmer, USA) to determine the GSH content. GSSG levels were measured by sequentially incubating the clarified tissue homogenates with N-ethylmaleimide (0.04 M) for 30 min in dark, followed by 0.1 N NaOH and eventually, in OPT solution for final 15 min. Fluorescence was measured as above. Malodialdehyde (MDA) levels in tissue homogenates were measured to monitor lipid peroxidation (Heath and Packer, 1968). MDA reacts with thiobarbituric acid (TBA) to form MDA-TBA adduct with a λ_{max} at 532 nm. The reaction was carried out by incubating tissue homogenates with three volumes of 0.5% TBA (diluted in 0.1 M phosphate buffer, pH 8) at 95 °C in a water bath for 25 min, followed by reaction termination on ice. Absorbance measured at 600 nm were subtracted from those at 532 nm using Envision microplate reader (Perkin Elmer, USA). Non-specific absorbance at 600 nm were subtracted from MDA-TBA complex values measured at 532 nm. MDA concentration was calculated using the Lambert-Beer equation using the excitation co-efficient ε^{M} =155 $mM^{-1}cm^{-1}$.

In vitro anti-inflammatory, cell viability and AP-1/NF-κB Reporter assays

Cytosafety and anti-inflammatory effect of DPV was evaluated in THP-1 cells, cultured in RPMI-1640 media, supplemented with 10% heat-inactivated fetal bovine serum in the presence of penicillin–streptomycin (100 U/ml) and L-glutamine (4 mM) cells, as described earlier (Balkrishna et al., 2020a). Briefly, 10^3 cells/well were differentiated into macrophages with 20 ng/ml phorbol 12-myristate 13- acetate (PMA) for 24 h before inducing inflammation with LPS (500 ng/ml) and simultaneously treating with different concentrations (1, 3, 10, 30 and 100 μg/ml) of DPV. Levels of secreted interleukin-1β (IL-1β) were measured in the cell supernatants after 24 h using ELISA kits from BD Biosciences following the manufacturer's protocol. Absorbance was recorded at 450 nm using the Envision microplate reader (Perkin Elmer, USA). Viability of the same cells was determined through fluorescence at 530 nm (excitation) and 590 nm (emission) from Alamar blue (15 μg/ml) staining. HEK-Blue™ TNF-α cells were grown in DMEM containing L-Glutamine (2 mM), 100 U/ml Penicillin, 100 μg/ml Streptomycin, 100 μg/ml Normocin and 10% heat inactivated FBS. SEAP reporter assay was done according to an earlier report (Balkrishna et al., 2020b). Briefly, 2.8×10^5 cells/ ml of HEK-Blue™ TNFα cells were treated with different concentrations of DPV (1, 3, 10, 30 and 100 μg/ml) for 24 h before inducing with 1 ng/ml of human TNF-α for another 24 h. Secreted SEAP levels in the supernatant were measured using QUANTI-Blue™ solution [Invivogen (San Diego, CA, USA)] as per manufacturer's protocol. All cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

Data representation and statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) for each group using GraphPad Prism version 7.0 software (San Diego, CA, USA). Statistical analysis was done using in-built 'analysis' option of GraphPad Prism. One-way analysis of variance (one-way ANOVA) followed by Dunnett's multiple comparison test was used to determine the statistical significance of the difference observed in more than two groups. p-values < 0.05 were considered statistically significant.

Results

DPV alleviated pain responses

PTX-induced neuropathy causes hyperalgesia and allodynia.(Scripture et al., 2006) Therefore, effect of DPV on these pain responses was evaluated to establish its ability to clinically attenuate CIPN. Heat-hyperalgesia ameliorative efficacy of DPV was evaluated through hot plate test **(Fig. 1B)**. Latency times were reduced to almost half from an average of 11.97 ± 0.50 s on day 0 to 7.59 ± 0.12 s by day 6 across all the experimental groups, except NC with no chemotherapy, thereby, confirming the CIPN model. The untreated chemotherapy exposed DC group over time experienced further reductions in latency times. GABA group receiving the standard anti-CIPN drug gabapentin experienced a statistically significant increase in latency time from 7.58 ± 0.80 s on day 6 to 9.03 ± 0.45 s by day 11. DPV treated DPV 137 and DPV 410 groups, demonstrated apparent upward trends in latency times. Encouragingly, the latency time of DPV 410 group became comparable to the GABA group **(Fig. 1B)**. By day 16, the average latency time of GABA group reached 11.08 ± 0.53 s, almost getting restored to the original average before PTX treatment. With average latency times of 8.64 ± 0.45 s and 9.87 ± 0.30 s, respectively, DPV 137 and DPV 410 groups also exhibited significant increase relative to the DC group. Through tail flick test, nociception modulatory activity of DPV was evaluated **(Fig. 1C)**. The NC group maintained a threshold latency around 8.96 (± 0.19) s. By day 7, CIPN induction reduced this threshold to almost one-third, to an average of 3.30 (± 0.35) s in the treatment groups. This reduction of latency threshold confirmed allodynia in the model. GABA group experienced an increase in the average latency time to 5.69 ± 0.09 and 7.15 ± 0.09 s, respectively, by days 12 and 17. The latency times of DPV treated groups improved significantly compared to DC group by day 12 onwards. Mechano-allodynia is one of the common

manifestations of CIPN. Therefore, DPV efficacy in alleviating this pain behavior was evaluated through Von Frey test (**Fig. 1D**). An average paw withdrawal threshold around 7.83 ± 0.19 g was maintained across all the experimental groups before CIPN induction. CIPN reduced this average to less than half (2.36 ± 0.42 g) across the PTX treated groups by day 7. However, anti-CIPN treatments with gabapentin or DPV rapidly assuaged this allodynia. By day 12, gabapentin almost doubled the PWT to 5.49 ± 0.12 g from 3.43 ± 0.19 g on day 7 in the GABA group. The average further increased to 7.24 ± 0.04 g by day 17. The DPV treated groups also experienced a statistically significant increase in their average PWT values over DC group by days 12 and 17. The two DPV treated groups, DPV 137 (4.14 ± 0.26 g) and DPV 410 (4.46 ± 0.13 g) had comparable average PWT values on day 12. By day 17, the increase in the average PWT value of DPV 410 group was slightly more than that of DPV 137 group (6.15 ± 0.10 g *versus* 5.27 ± 0.07 g). Nevertheless, the increase in the average PWT values in all the treatment groups by day 17 when compared to that of DC group clearly revealed a time-dependent relief of mechano-allodynia. Randall-Selitto test that assesses neuroinflammation associated allodynia was used to obtain the preliminary indication of efficacy of DPV in moderating this pathology of CIPN (**Fig. 1E**) (Ji et al., 2018). The normal PWT across all groups before CIPN induction was observed to be 21.38 ± 0.17 g, which reduced to around 6.47 ± 0.36 g by day 9 after PTX treatment. However, the average PWT (8.99 ± 0.09 g) of GABA group was still significantly higher than that of the DC group (5,75 ± 0.57) on day 9. On subsequent time points of days 13 and 19, average PWT of GABA group rose to 11.69 ± 0.51 g and 17.54 ± 0.31 g, respectively. The increases in DPV treated groups on day 13 leading to overall averages of 7.47 ± 0.38 g in DPV 137 and 7.90 ± 0.12 g DPV 410 were statistically significant against DC group. Likewise, with average PWT values of 9.61 ± 0.35 g and 10.83 ± 0.28 g for DPV 137 and DPV 410 groups, respectively, the increase was found to be substantial. During the entire duration of the

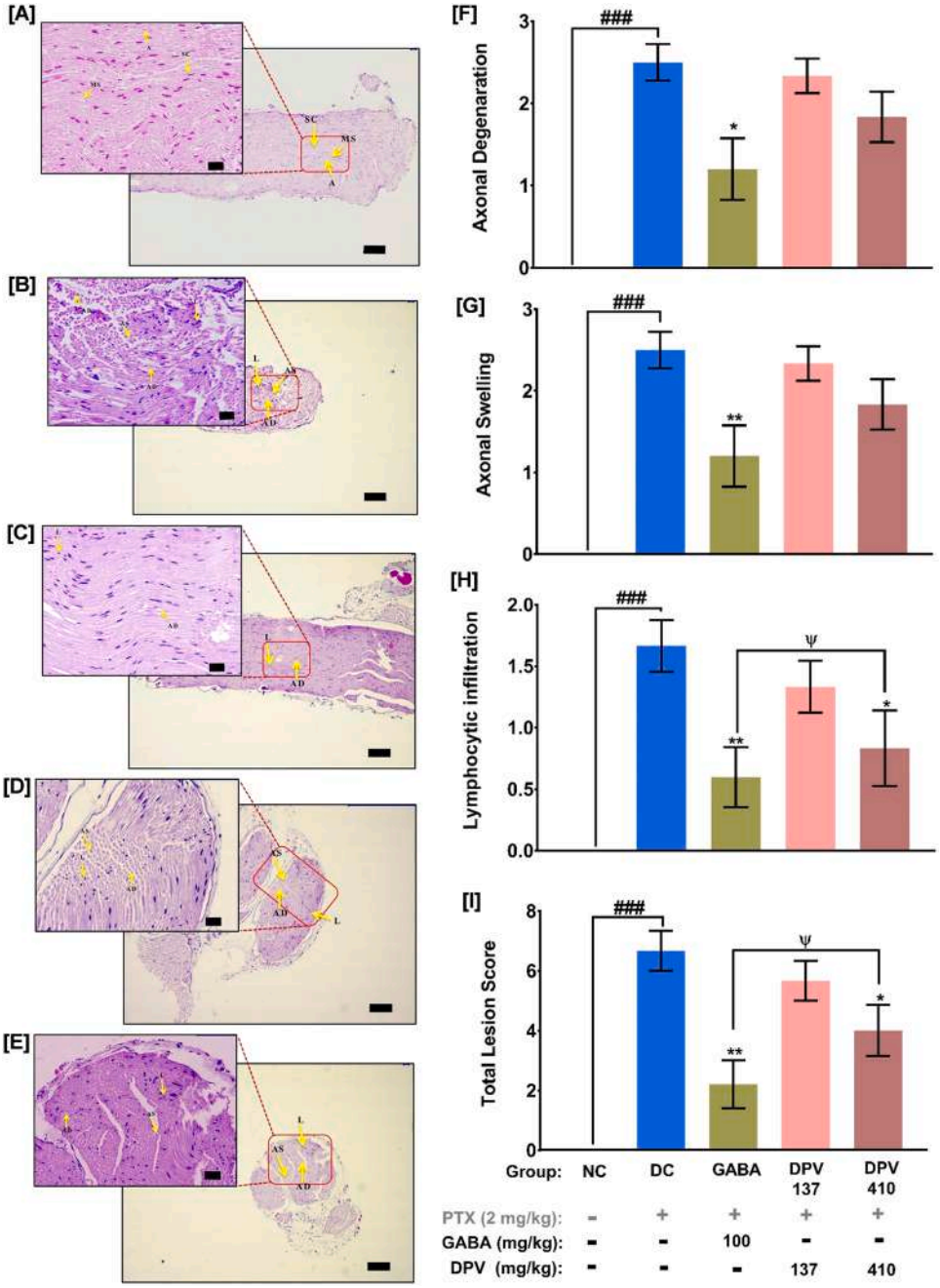


Fig. 2. DPV restores CIPN-associated histopathological profile to normalcy. [A–E] Representative micrographs showing the H and E stained sciatic nerve sections from NC **(A)**, DC **(B)**, GABA **(C)**, DPV 137 **(D)** and DPV 410 **(E)** groups at low (10X; scale bar: 100 μm) and high (40X; scale bar: 20 μm) magnifications. A: Intact axons, SC: Schwann cells, MS: myelin sheath, AD: axonal degeneration, AS: axonal swelling, L: lymphocytic infiltration. **[F–I]** Quantitative representation of scorings for axonal degeneration **(F),** axonal swelling **(G)**, lymphocytic infiltration **(H)** and overall lesions **(I)**. Data represented as mean ± SEM, for $N = 6$ in each experimental group. Statistical significance of the observation was determined through one-way ANOVA followed by Dunnett's multiple comparison test and represented as ### for $p < 0.001$, when significantly different in comparison to NC; * for $p < 0.05$ and ** for $p < 0.01$, when significantly different in comparison to DC, and ψ, when the observation was comparable to the GABA group.

experiment, no significant changes in body weight gain, and/or feed habits of the animals were observed (data not shown). Taken together, all of these observations confirmed that DPV could effectively reduce PTX induced pain sensitivity.

DPV treatment moderated PTX induced neuropathic degeneration of sciatic nerve

CIPN causes degeneration of peripheral nerves (Fukuda et al., 2017; Hou et al., 2018). Hence, the contextually pertinent anti-neurodegenerative ability of DPV was explored through histopathological approach **(Fig. 2)**. Micrograph of H-E stained sciatic nerve section from NC group had intact axons (A), Schwann cells (SC) and myelin sheath (MS) **(Fig. 2A)**, with no lesion score to report **(Fig. 2F–I)**. CIPN induction resulted in degenerated (AD) and swollen axons (AS), with lymphocyte infiltration (L) **(Fig. 2B)**. Reduction in these pathological features were noticed after gabapentin and DPV treatments **(Fig. 2C, D and E)**. DC group with untreated CIPN showed highest total lesion score and also maximum scores in individual categories of axonal degeneration, swelling and lymphocytic infiltration. Gabapentin treatment (GABA) could reduce the lesion score for axonal degeneration significantly ($p < 0.05$). Although, DPV treatment had similar effects on axonal degeneration but the observed changes were not statistically significant **(Fig. 2F)**. Likewise, GABA group experienced noticeable reduction in axonal swelling ($p < 0.05$), although, DPV treated groups followed the trend, but, the reductions were not statistically significant **(Fig. 2G)**. Lymphocytic infiltration in gabapentin treated GABA group was almost reduced to one-third of that found in DC group ($p < 0.01$). DPV treatment at 410 mg/kg body weight was also effective in reducing the lymphocytic infiltration ($p < 0.01$) to levels comparable to GABA group **(Fig. 2H)**. Although, DPV treatment at 137 mg/kg body weight trended towards a decrease in lymphocytic infiltration, the observation was not found to be statistically significant. The overall pattern of the total lesion score corroborated with the trend observed in each individual category. Overall, DPV treatment at 410 mg/kg body weight was as effective as gabapentin in reducing the total lesions **(Fig. 2I)**. Sciatic nerve sections from normal animals belonging to NC group did not exhibit any of these histopathological anomalies. Altogether these observations showed that DPV treatment, can alleviate the of PTX induced neuropathic etiology at the determined therapeutic dose.

DPV assuaged oxidative stress in vivo and inflammation in vitro

PTX-induced CIPN pathology generates oxidative stress in the peripheral nerves, resulting in elevated intra and extra-cellular levels of reactive oxygen species (ROS) in the tissues (Duggett et al., 2017). Consequently, the inherent ROS scavenging machinery of the body gets activated wherein, enzymes, like, glutathione peroxidases and peroxiredoxins scavenge ROS while converting reduced glutathione (GSH) to its oxidized form (GSSG) (Deneke and Fanburg, 1989; Meister, 1988). So, tissue levels of GSSG, GSH and GSH: GSSG ratio are used as bio-indicators of oxidative stress. Lipid peroxidation is an unavoidable spin-off of oxidative stress because of the free radical generation (Barrera, 2012). Lipid peroxidation can be monitored through the biomarker, Malondialdehyde (MDA) (Naik et al., 2006; Niki, 2008). So, the levels of MDA, GSSG and GSH and GSH:GSSG ratio in sciatic nerve tissues were estimated to assess the effect of DPV on CIPN-associated tissue oxidative stress **(Fig. 3A–D)**. The increased oxidative stress, and concomitant enhanced lipid peroxidation in DC group due to PTX treatment manifested as significantly high MDA levels ($p < 0.05$) than NC group. With gabapentin and DPV treatments, the MDA levels were effectively reduced ($p < 0.01$) in the respective treatment groups as compared to the DC group **(Fig. 3A)**. MDA levels in DPV treatment groups were comparable to that of GABA group **(Fig. 3A)**. CIPN induction almost doubled the GSSG level in the DC group from 51.46 ± 7.72 to 117.34 ± 10.61 μM/mg of tissue ($p < 0.05$), confirming the ensuing oxidative stress **(Fig. 3B)**. Gabapentin treatment apparently reduced the GSSG level but no statistically significance was observed for this. But, DPV treatments at both low (137 mg/kg body weight) and high (410 mg/kg body weight) doses reduced the GSSG levels in the sciatic nerve tissues, significantly ($p < 0.05$). Likewise, as expected a trend just opposite to that of GSSG was observed for tissue GSH levels **(Fig. 3C)**. The significantly low ($p < 0.05$) GSH in the DC group with PTX-induced CIPN once again conformed tissue oxidative stress. Both gabapentin and DPV treatments were effective in raising the GSH levels in sciatic nerve tissues. However, the effect was statistically significant in GABA group only. Corroborating with all these observations, a significantly reduced ($p < 0.05$) GSH:GSSG ratio in the DC group compared to NC, attested to the existing oxidative stress **(Fig. 3D)**. Gabapentin ($p < 0.01$) and DPV ($p < 0.05$) treatments increased this ratio significantly, and the increase was comparable to each other **(Fig. 3D)**. Taken together, these observations demonstrated that DPV treatment could rescue sciatic nerves

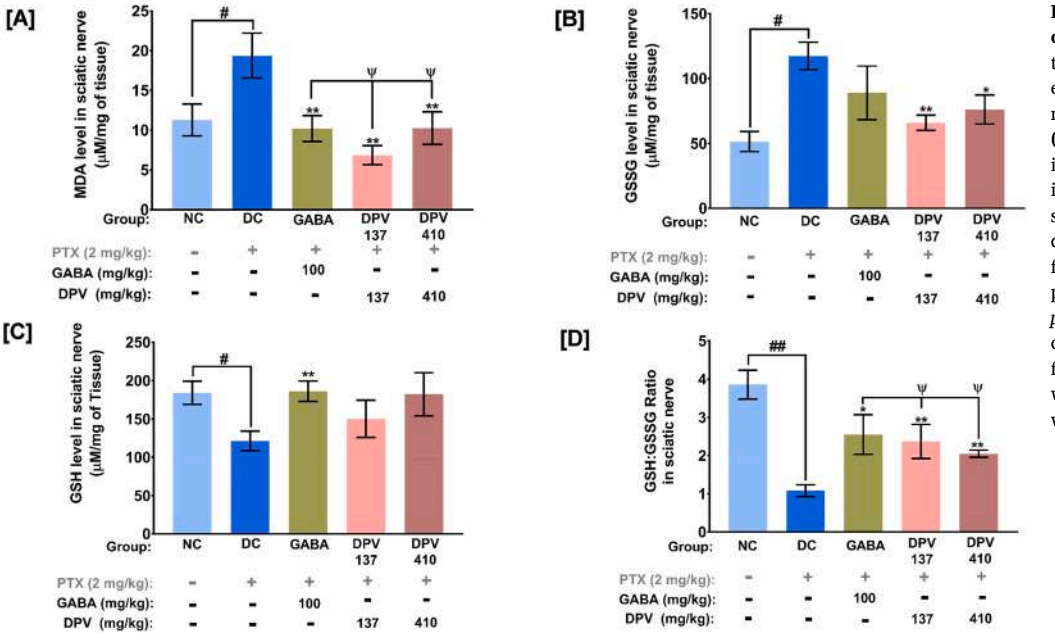


Fig. 3. DPV exhibits *in vivo* anti-oxidative property. [A–D] Quantitative representations of the anti-oxidant effect of DPV on neuropathic sciatic nerves as MDA (**A**), GSSG (**B**) and GSH (**C**) levels and GSH:GSSG ratio (**D**). Data is represented as mean ± SEM, for $N = 6$ in each experimental group. Statistically significance of the observation was determined through one-way ANOVA followed by Dunnett's multiple comparison test and represented as # for $p<0.05$ and ## for $p<0.01$, when compared to NC; * for $p < 0.05$ and ** for $p < 0.01$, when the comparison was with DC, and ψ, when the observation was comparable to the GABA group.

from PTX induced CIPN associated oxidative stress.

Oxidative stress and inflammation can induce each another and often co-exist in many pathological conditions, including CIPN (Biswas, 2016; Boyette-davis et al., 2015). Oxidative stress leads to neuroinflammation in CIPN (Hussain et al., 2016). Therefore, anti-inflammatory effect of DPV was evaluated *in vitro* on LPS-induced inflammation in differentiated human monocytic THP-1 macrophages. *In vitro* doses of DPV were decided based on cytotoxicity assay. A range from 1 to 100 μg/ml (1, 2, 10, 30 and 100 μg/ml) of DPV was found to be safe on these inflamed macrophages **(Fig. 4A)**. Inflammation in THP-1 macrophages was efficiently subdued by DPV at 30 and 100 μg/ml concentrations, as evident from statistically significant reduction in the levels of secreted pro-inflammatory cytokine, IL-1β **(Fig. 4B)**. The other pro-inflammatory cytokine, besides IL-1β, which is implicated in neuroinflammation and neuropathic pain is tumor necrosis factor-alpha (TNF-α) (Hung et al., 2017). DPV has already been associated with moderation of pro-inflammatory cytokines, like, TNF-α and IL-6 in the generic carrageenan induced paw edema model of inflammation (Balkrishna et al., 2019a). NF-κB signaling is associated with inflammatory pathologies, CIPN being no exception (Fumagalli et al., 2021). TNF-α induced activation of NF-κB signaling is implicated in CIPN associated inflammation (Zhang et al., 2020). Therefore, effect of DPV on TNF-α induced NF-κB/AP1 transcriptional activity was assessed through a reporter assay, using a genetically engineered HEK-293 cell line (HEK-Blue™ TNF-α cells) with a TNF-α inducible SEAP reporter under AP1/NF-κB promoter elements. NF-κB/AP1 transcriptional activity in DPV pre-treated HEK-Blue™ cells were induced through TNF-α. Colorimetrically, SEAP transcription was found to decrease dose dependently upon DPV treatment of HEK-Blue™ TNF-α cells; the decline was statistically significant for 30 and 100 μg/ml doses **(Fig. 4C)**. As expected, in the absence of TNF-α, the cells did not exhibit any SEAP expression, thereby, indicating absence of NF-κB/AP1 transcriptional activity. This observation demonstrated that DPV is capable of targeting the neuroinflammation causing TNF-α/ NF-κB signaling pathway in CIPN. Taken together, these observations provided robust preliminary evidences for CIPN pathophysiology specific *modus operandi* of DPV.

Discussion

Severe side-effects are associated with available CIPN treatments. Therefore, the herbo-mineral formulation, DPV, was tested as an alternative treatment option, because of its already reported anti-inflammatory and analgesic effects in carrageenan induced paw edema rodent model (Balkrishna et al., 2019a). CIPN pathology is the combined outcome of several interlinked signaling pathways involved in oxidative stress, inflammation, neurodegeneration and microtubule assembly dynamics (Duggett et al., 2017; Fukuda et al., 2017; Hou et al., 2018).

The herbal components of DPV are known for their anti-inflammatory, anti-oxidative, anti-nociceptive and analgesic properties (Balkrishna et al., 2020a). High performance liquid chromatographic analysis identified withaferin A, rutin, curcumin, colchicine and caffeic acid as the marker phytocompounds of DPV **(Table 1, supplementary Fig. S1)** (Balkrishna et al., 2019a). Neuroprotective effect of withaferin A is well documented (Tiwari et al., 2018). In fact, it modulates several pathways in a disease-specific manner to impart neuroprotection (Ram et al., 2021). Interestingly, withaferin A was found to be effective against breast cancer, alone or in combination with cisplatin, suggesting that a co-treatment chemotherapeutic CIPN-free regime is achievable (Kakar et al., 2014). p38/MAPK (mitogen activated protein kinase) signaling inhibition-mediated anti-oxidant, anti-inflammatory and immune-modulating activities of rutin make it an excellent neuroprotector (da Silva et al., 2020). Similarly, neuroprotective role of curcumin is also reported against CIPN (Cheng et al., 2015). Caffeic acid can abate cisplatin induced neurotoxicity by modulating MAPK/Erk, PI3k/Akt and AMPK/SIRT1 pathways (Ferreira et al., 2019). Colchicine

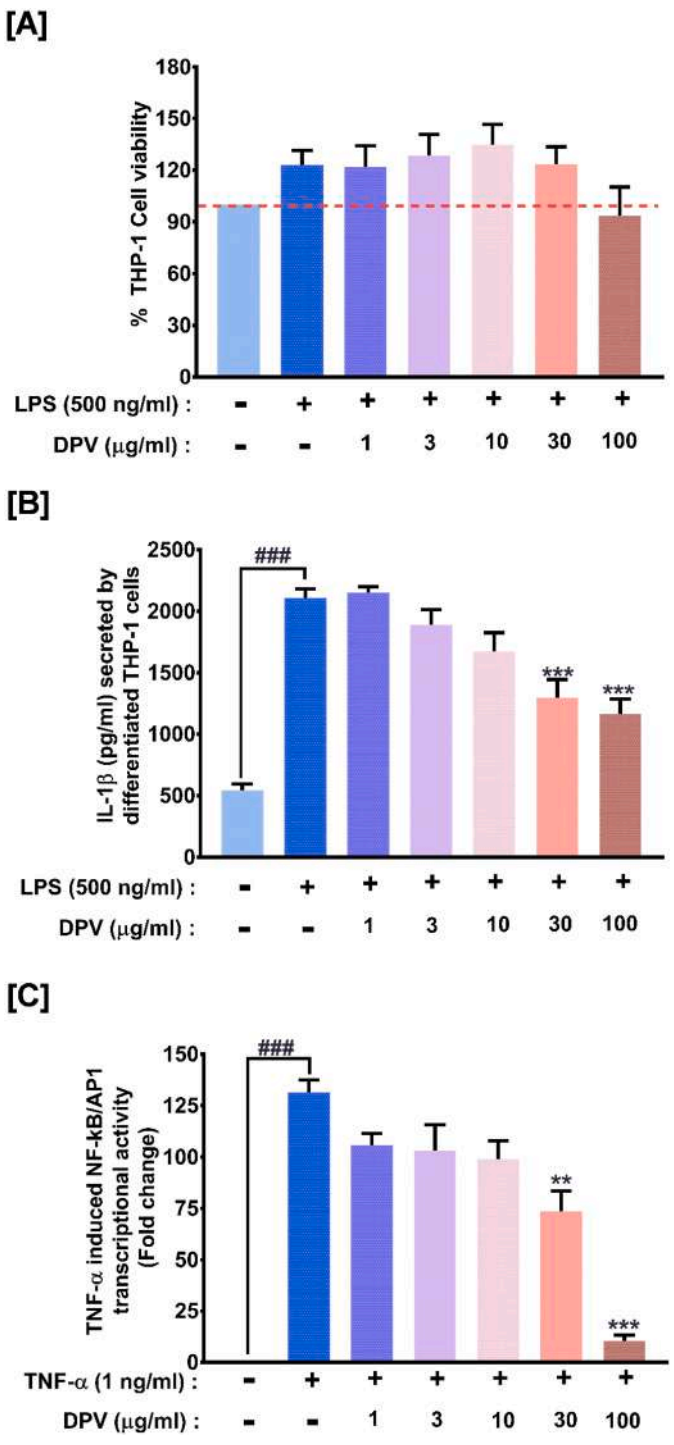
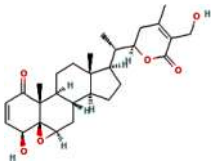
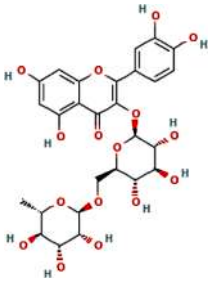
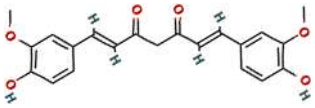
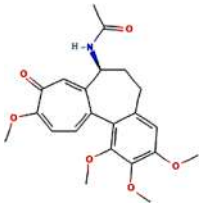
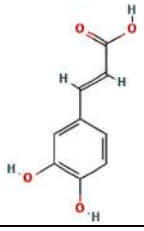


Fig. 4. DPV demonstrates *in vitro* anti-inflammatory property. [A] Graphical representation of the cytosafety of different concentrations DPV on THP-1 cells and suitability of the LPS-induced *in vitro* inflammatory model for subsequent experiments. **[B]** Quantitative representation of the levels of IL-1β secreted by differentiated THP-1 cells in response to LPS-induced inflammation and corresponding reductions in the same due to DPV treatments. **[C]** Quantitative representation of the TNF-α-induced AP1/NF-κB signaling measured through SEAP reporter activity and its dose-dependent alleviation thereof by DPV treatments. Data is represented as mean ± SEM from triplicate data of three independent experiments. Statistical significance was analyzed through one-way ANOVA followed by Dunnett's multiple comparison test and represented as *###* for $p < 0.001$, when significantly different from the normal cells, untreated with LPS, DPV or TNF-α, or as ** and *** for $p < 0.01$ and <0.001, respectively, when the comparison was with the DPV untreated by LPS or TNF-α treated group.

Table 1
*, # Phyto-constituents present in DPV as determined through HPLC.

S. No.	Name	Chemical Structure	Quantity (µg/mg)
1.	Withaferin A		39.19
2.	Rutin		1.20
3.	Curcumin		0.36
4.	Colchicine		0.25
5.	Caffeic acid		0.15

* Information provided in this table is excerpted from Table 1 of the reference, (Balkrishna et al., 2019a).

HPLC chromatogram of DPV is provided as supplementary Fig. S1.

is neuroprotective in gerbil model of ischemia (Pratt et al., 1994). Microtubule destabilizing effect of colchicine is also reported (Kapoor et al., 2018). Therefore, taken together, the phytochemical constitution of DPV can be clearly attributed to the its observed anti-CIPN effect.

Although, this study did not explore the anti-cancer properties of DPV, nevertheless, there are reports associating individual marker compounds with anti-cancer effect. Withaferin A is effective against a variety of cancers, and acts by activating tumor suppressor TRIM16 at least in case of melanoma (Nagy et al., 2020). Rutin modulates several signaling pathways like, Wnt/β-catenin, PI3K/Akt, JAK/STAT, MAPK, NF-κB involved in cancer pathogenesis (Imani et al., 2021). Anti-cancer effects of curcumin and its derivatives are well established (Mansouri et al., 2020). Anti-proliferative effect of colchicine due to its microtubule-destabilize property causing G2/M arrest and consequently, apoptosis is well documented (Kumar et al., 2016). The anti-cancer property of caffeic acid comes from its anti-oxidant potential and ability to inhibit angiogenesis through modulating MMP-2 and MMP-9 expressions (Monteiro Espíndola et al., 2019). Thus, presumably, DPV has dual effectivity against cancer and neuropathy induced by cancer chemotherapy, suggesting excellent potential for this formulation in combinatorial chemotherapy.

The marker phytocompounds of DPV are also implicated in modulation of inflammatory signaling pathways through TNF-α and NF-κB. Withaferin A inhibits NF-κB activation and TNF-α mediated inflammatory manifestations (Grunz-Borgmann et al., 2015). Withaferin A has been shown to target the active site of IKKβ, a kinase indispensable for NF-κB activation (Heyninck et al., 2014). Rutin modulates inflammatory immune responses by preventing NF-κB and inducing Nrf2 activations, and reducing TNF-α production in immune cells. It can alleviate pain in neuropathy by cGMP/PKG/ATP-sensitive potassium channels activation in neurons (Ferraz et al., 2020). Curcumin attenuates NF-κB signaling either by blocking TNF-α –induced IκB-degradation-mediated NF-κB activation or by inhibiting IL-6 and TNF-α-induced DNA binding activities of AP-1/NF-κB complex (Jobin et al., 1999; Xu et al., 1998). Microtubule stabilization by taxols, like paclitaxel, induce TNF-α dependent and independent IκB degradation and subsequent NF-κB activation. When colchicine destabilizes the microtubules, NF-κB activation has been shown to be reversed (Jackman et al., 2009). Therefore, colchicine present in DPV could inhibit both TNF-α dependent and independent activation of NF-κB. Similar effect of caffeic acid present in DPV on NF-κB activation could be presumed based on available literature (Natarajan et al., 1996). So, it is inevitable that DPV would efficiently target the signaling pathways behind CIPN pathology.

One of the mineral component of DPV, *Godanti Bhasma (GB)*, is anhydrous Calcium sulfate, prepared according to the specific classical Ayurvedic process of incinerating Gypsum (Das et al., 2020). Another mineral component of DPV, *Mukta Shukti Bhasma (MSB),* is prepared by detoxification/purification of pearls with citric acid from lemon juice at 95–102 °C temperature, and repeated calcination of the aragonite present within to yield calcite (Sharma et al., 2016). *Praval Pishti (PP)* is the third mineral component of DPV and like MSB, it is also prepared through purification and calcination but without heat. Purification is done with rose water and calcination in *Aloe vera* gel (Satpute et al., 2021). Preparation procedures of *GB, MSB* and *PP* yield nanoparticles of minerals, thereby, most likely enhancing their bioavailability (Charoo et al., 2019). Putatively, the underlying commonality between *GB, MSB* and *PP* is to provide calcium to the body in a bioavailable form. Dysregulation of cellular calcium homeostasis is a causality of allodynic and hyperalgesic outcomes of CIPN (Siau and Bennett, 2006). Platinum-based chemotherapeutic drugs induce mitochondrial damage that leads to intracellular oxidative stress eventually, causing dysregulation of calcium homeostasis and concomitant undesirable alterations in axonal excitability (Salat, 2020). Plausibly, calcium ions supplied by DPV, through *GB, MSB* and *PP*, ensure restoration of calcium homeostasis in the neuropathic nerves. The herbo-mineral component of DPV, *Shilajit* is a gluey exudate from mountain rocks of the Himalayas, containing humus, organic plant materials, and fulvic acid (Meena et al., 2010), the last component being known for its anti-oxidative and anti-inflammatory properties (Winkler and Ghosh, 2018). DPV treatment did not elicit any clinical signs, neither did it affect the regular metabolism as evident from absence of change in body weights and food and water consumptions of the experimental animals (data not shown). Therefore, taken together, DPV could well be a safe alternative treatment option for CIPN.

The current study has experimentally established the potency of the herbo-mineral medicine, DPV in alleviating the pathophysiology of paclitaxel-mediated chemotherapy induced peripheral neuropathy using a mouse model of neuropathic pain. This study innovatively worked out the mode-of-action of DPV using promoter transactivation approach to reveal that this herbal medicine targets oxidative stress mediated neuroinflammation through cytokine modulation in assuaging neuropathic pathogenesis. Besides, its analgesic property ensures relief from neuropathy associated allodynia and hyperalgesia. While, chemotherapy has become an indispensable course of treatment for cancer, this alternative treatment in ameliorating the after-effects of chemotherapy without its own undesirable effects is quite promising.

Conclusion

In conclusion, the herbo-mineral formulation, DPV effectively relieved nociceptive outcomes of CIPN, restored tissue structure of neuropathic peripheral nerves to normal by ameliorating oxidative stress and underlying cytokine responses. DPV is a combination of several herbs conferring it the ability to target multiple pathways. The unique composition of this formulation is attributable for its excellent anti-neuropathic effects. Taken together, this study has provided the evidence for DPV as a potent anti-CIPN agent and ushers the requirement for further evaluation of its suitability in CIPN treatment through controlled clinical studies.

Author agreement statement

Manuscript title: "**Anti-oxidant Profile of Divya-Peedantak-Vati Abates Paclitaxel-Induced Hyperalgesia and Allodynia in CD-1 Mice Model of Neuropathic Pain**". We, the undersigning authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons, who satisfied the criteria for authorship, but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the editorial process. He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

CRediT authorship contribution statement

Acharya Balkrishna: Conceptualization, Resources, Project administration. **Shadrak Karumuri:** Investigation, Methodology, Visualization, Data curation, Software, Formal analysis. **Sachin S Sakat:** Investigation, Validation. **Swati Haldar:** Writing – original draft. **Anurag Varshney:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The test article, Divya-Peedantak-Vati was manufactured by Divya Pharmacy, Haridwar, Uttarakhand, India. Besides, providing the test article, Divya Pharmacy was not involved in any aspect of this study. Acharya Balkrishna is a trustee in Divya Yog Mandir Trust, Haridwar, India that governs Divya Pharmacy, Haridwar. In addition, Acharya Balkrishna is one of the founding promoter and holds an honorary managerial position in Patanjali Ayurved Ltd, Haridwar, India. Divya Pharmacy and Patanjali Ayurveda Ltd commercially manufacture and sell several ayurvedic products. All other authors, Shadrak Karumuri, Sachin S Sakat, Swati Haldar and Anurag Varshney, declare no conflict of interest, and, have been employed at Patanjali Research Institute which is governed by Patanjali Research Foundation Trust (PRFT), Haridwar, Uttarakhand, India. PRFT is an independent not-for-profit organization. In addition, Anurag Varshney is an Adjunct Professor in Department of Allied and Applied Sciences, University of Patanjali, Haridwar, India; and in Special center for Systems Medicine, Jawaharlal Nehru University, New Delhi, India.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2022.100229.

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