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ROSMARINIC ACID EXERTS PROTECTIVE EFFECT ON OXALIPLATIN INDUCED PERIPHERAL NEUROPATHY: BEHAVIORAL, NEUROPHYSIOLOGICAL AND HISTOPATHOLOGICAL EVIDANCE

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ABSTRACT

Neuropathic pain is caused by neuron injury in the peripheral or central nerve system. It can be caused by numerous pathological processes. Neuropathy can categorically be caused by the following stimuli: alcohol, chemotherapy drugs like vincristine, platinum, taxanes etc. Peripheral neuropathy is considered as a serious complication of cancer treatment. The study aims to evaluate the effect of Rosmarinic acid on Oxaliplatin induced peripheral neuropathy. Male Sprague dawley rats are used for the whole *In vivo* studies. The following Pharmacological screening methods such as behavioral assessment by thermal hyperalgesia, thermal allodynia, rotarod, von- frey test, static mechanic hyperalgesia and Formalin test. Animals are sacrificed at the end of the study and excised the sciatic nerves for histopathological analysis. The present research work revealed that Rosmarinic acid had a protective role on chemotherapy induced peripheral neuropathy.

KEYWORDS: Neuropathy, Chemotherapy, Oxaliplatin, Cancer, Hyperalgesia, Sciatic nerve.

INTRODUCTION

Peripheral neuropathy is one of the common adverse event happened with chemotherapeutic agents in cancer therapy. Chemotherapy induced peripheral neuropathy (CIPN) was one of the major reason for the noncompliance in cancer patients. Platinum based chemotherapeutic agent's oxaliplatin and cisplatin are listed by World Health Organization (WHO2019) in the essential drug which is used for the treatment of various solid tumors.[1] These drugs having high success rate in the treatment of cancer and thereby increasing the survival rate of cancer patients. But the number of CIPN rate was also increasing in the cancer survivor, 68% of the patents develop CINP in the first month of therapy.[2,3,4] The formation of 1,2-diaminocyclohexylplatinum complex and oxalate by non-enzymatically in the blood plasma is an exceptional features of oxaliplatin over cisplatin. The oxalate formation is one of the mechanism for different clinical presentation (cold – induced neuropathy pain) of oxalipaltin induced neuropathic pain. The formation of additional metabolites like platinum complexes which bind to cellular proteins also contributed to the development of CIPN.[5] The clinical choices for managing CIPN are limited to opioid, tricyclic anti-depressants and anti-convulsants. Even though there are many drugs approved for the management of neuropathic pain, there are currently no analgesics specifically approved for the treatment of CIPN. At present, the clinical choices for CIPN are limited to opioids, tricyclic anti-depressants, and anti-convulsants, but these agents exhibit several adverse effects which diminishes their full clinical utilization.[6] Several herbal medicines such as Phyllanthus emblica Linn. (Family: Phyllanthaceae), Cannabis sativa Linn. (Family: Cannabaceae), Nigella sativa Linn. (Family: Ranunculaceae), Ocimum sanctum Linn. (Family: Lamiaceae), Tribulus terristris Linn. (Family: Zygophyllaceae) and Ginkgo biloba Linn. (Family: Ginkgoaceae) are shown to have potential in different types of experimentally induced neuropathic pain some clinical reports have also advocated beneficial effect of drugs from plant origin in neuropathic pain conditions.[7]

Rosmarinic acid (RA), a phenolic compound found in various labiatae herbs, possesses anti-nociceptive anti-inflammatory, neuroprotective (in different models of chemical induced neurotoxicity, neurodegeneration and neuroinflammation) and potent anti-oxidant properties. Rosmarinic acid is an ester of caffeic acid and 3,4 dihydroxyphenyl lactic acid. Rosmarinic acid is well absorbed from the gastrointestinal tract and as well as from skin.[8]

With the above background, the present study was performed to examine the effect of Rosmarinic acid in suitable rat model of vincristine induced neuropathy.

MATERIALS AND METHODS

Chemicals and preparation of drug solutions

Rosmarinic acid (Sami Labs, Banglore), Oxaliplatine (Cytocristine, Cipla), Pregabalin (Lyrica),). All the reagents used in the study were of analytical grade. Oxaliplatin and Rosmarinic acid was suspended in 0.3% w/v of Sodium carboxy methyl cellulose solution and were diluted with normal saline.

Experimental Animals

Thirty adult male Sprague dawley rats with a body weight of 200 - 220g were used for this study. Rats were grouped and housed (n=6 per cage) in a room with controlled temperature (21 ± 2 ^{0}C) and 12 hour light-dark cycle was maintained. All the rats had free access to food and water *ad libitum*. All the experimental protocol was approved by the Institutional Animal and Ethical Committee (IAEC), with the approval certificate number 275/2015/IAEC. The experiments were performed in accordance to the committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines for ethical use of animals.

Induction of peripheral neuropathy by Vincristine

Peripheral painful neuropathy was induced in rats by intrapeitonial administration by Oxaliplatin (6µg/kg) once per day for 10 consecutive days as per the method adapted by Muthuraman et.al.[9]

Experimental Design

Group I served as normal control in which rats received 0.3% w/v carboxy methyl cellulose (CMC) per oral (p.o.). vechicle administration and saline(Intraperitonial injection) for 14 days. Group II served as Oxaliplatin control in which rats were administered 0.3% w/v CMC (p.o.). 1 hour before Oxaliplatin injection (6µg/kg) for 10 days for 14 consecutive days. Group III, IV and V rats received Pregabalin (10mg/kg /day) (p.o.); Rosmarinic acid (30 mg/kg/day) (p.o.) and Rosmarinic acid (60 mg/kg/day) (p.o.), respectively 1 hour before Oxaliplatin injection (6µg/kg for 10 days) for 14 Consecutive days. The behavioral tests like thermal hyperalgesia (hot plate), Motor coordination (rotarod), thermal allodynia (Cold plate), tactile mechanical hyperalgesia (Von Frey test), Static mechanical hyperalgesia and sciatic functional index were performed on different days such as 0, 7, 10 and 14. On the day 14th the rats were subjected to formalin test. Thereafter all the rats were sacrificed under deep ether anesthesia and subjected to histopathological analysis was also carried out in sciatic nerve samples.

Behavioral Assessment

Thermal Hyperalgesia (Hot plate test)

In this test, rats were individually placed on a hot plate (Eddy's hot plate) with the temperature adjusted to 55± 1° C. The latency to the first sign of the paw licking or jump response to avoid the heat was taken as the index of the pain threshold: The cut off time was 10seconds in order to avoid the damage to the paw.[10]

Thermal allodynia (Cold plate test)

Rats were placed on ice platform submerged approximately 1 cm below the surface of the cold water (4° C) such the hairy and glabrous skin on the rat fleet were in contact with the cold water. The latency prior to the first reaction was recorded with a cut off time of 30 seconds.[11]

Rota- rod test

Rats were subjected to assess the motor coordination. Rats were placed on the rota- rod treadmill at an accelerating speed of 6 round/minute to 30 round/minute for 3 minutes. The latency to fall was measured and training sessions were given before experiment.[12]

Tactile Mechanical Hyperalgesia (Von Frey Test)

The mechanical hyperalgesia test was evaluated by Von Frey done to the sub plantar surface of the left hind paw. The pointed filament was held at 90º angle with adequate intensity to produce a withdrawal reflex response in rat. The paw withdrawal reflex was recorded in grams.

Static Mechanical Hyperalgesia (Randall and Salitto Test)

The nociceptive threshold was an expression of mechano-hyperalgesia index due to pressure stimulation as it is documented by Randall and Salitto (1957) was done in which nociceptive threshold in grams was measured by applying the pressure to the hind paw. The withdrawal of the paw serves as the end point to evaluate the nociceptive threshold. The cut off pressure was maintained at 200grams.[13]

Determination of Sciatic Functional Index (SFI)

The rats were subjected to toes print analysis to measure the SFI. The tests was carried out in an 8.2× 42 cm corridor with darkness in one end and covered with a with sheet of paper. The hind paw of rats was dipped in black Indian ink and the animal allowed to walking freely in the corridor. The analysis of the foot prints was done by considering the toes lengths from each other. Thus the distance from the heel to the third toe - print length (PL), Distance from the first toe to the fifth toe-toe spread (TS), and distance from the second toe to the fourth-intermediary toe spread (ITS) were measured.[14]

Formalin Test

This test was performed at the end of other behavioral assessment. The formalin test was done in each rat after acclimatization within a period of 15minute in an observation box. Then the animals were administered with the control and test drug prior to formalin injection. The 2.5% formalin of 0.1ml was injected to the sub plantar region. The nociception evaluation was done by quantification of the paw licking and paw elevation time

parameters. The earlier (acute) phase was recorded at between 0-10 minutes, the delayed phase was recorded at between 20-40 minutes and with a resting period being noted between 10-20 minutes in between the acute phase and the delayed phase.

Statistical Analysis

All the results were expressed as mean ± standard deviation. The data were statistically analyzed by one way analysis of variance (ANOVA) followed by post hoc tukey's multiple comparison test for formalin test. Two way ANOVA (treatment Vs duration) followed by Bonferroni's post test was used for hot plate test, cold plate test, Randall and selitto test and sciatic functional index, Probability values of less than 0.05 was considered as significant. The analysis was carried out using Graph pad prisom software.

RESULTS AND DISCUSSION

Effect of Rosmarinic acid on hot plate test

The induction of neuropathy by administration of Oxaliplatin demonstrated a significance ($p<0.001$) in development of thermal hyperalgesia as it is demonstrated with increase of hind paw licking, lifting or jumping from the hot plate surface. The pain perception elevation is a sign of neurotoxicity. Rosmarinic acid administration had a significance ($p<0.001$) attenuation of Oxaliplatin induced nociception threshold which is dose and time dependent. The administration of Pregabalin served as the positive control gave significant($p<0.001$) result.

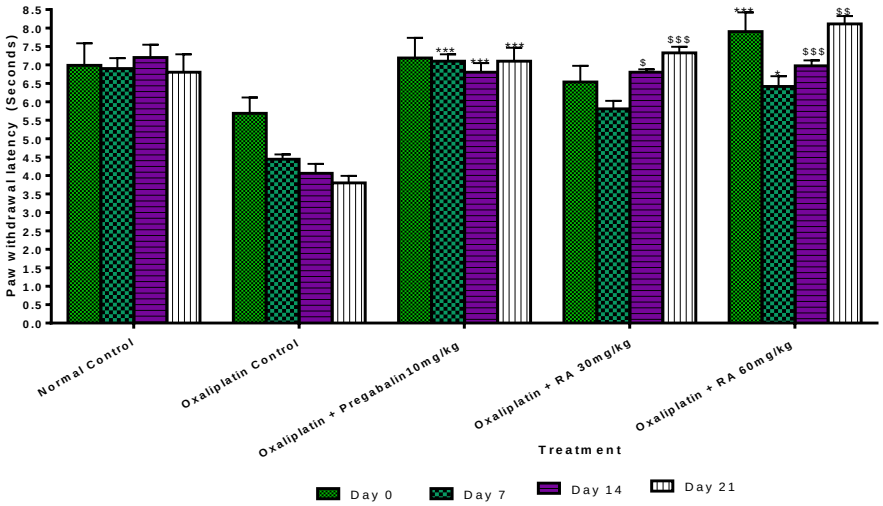

Figure 1: **Effect of Rosmarinic acid on hot plate test.**

Results expressed as mean±SEM, n=6 rats per group. Two- way ANOVA was done followed by Bonferonni's test, this indicated significance at $P<0.001$ in comparison with the normal rats.* indicates $P<0.05$, *** indicates $P< 0.001$ significance with comparison to normal and $ indicates $P< 0.05$, $$ indicates $P<0.01$ and $$$ indicates $P<0.001$ significance in comparison with the Oxaliplatin and Pregabalin.

Effect of Rosmarinic acid on cold plate test

The administration of Oxaliplatin as neuropathy inducer, reflected thermal allodynia, with significance ($p<0.001$) development, this was indicated with an increase of hind paw licking, lifting, or jumping from the cold plate surface as shown.

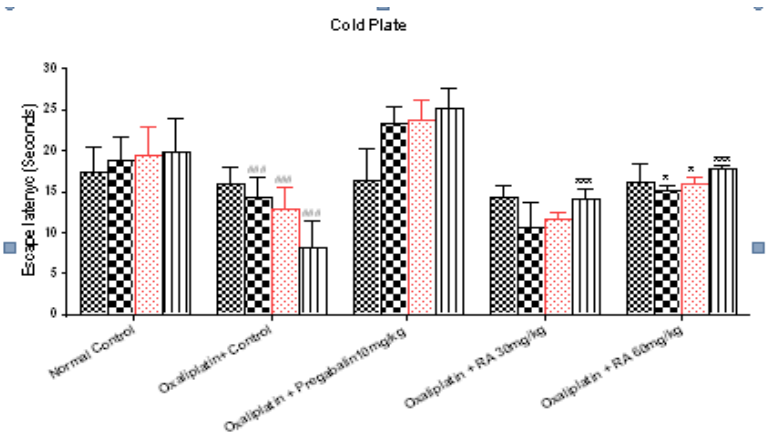

Figure 2: Effect of Rosmarinic acid on cold plate.

Results are expressed as mean±SEM, n=6 rats per group. Two- way ANOVA was followed by Bonferonni's test. This indicates significance at P<0.001 in comparison with normal in corresponding days where * indicates significance at P<0.05, ** indicates significance at P<0.01 and *** indicates significance at p<0.001 in Oxaliplatin treated rats in corresponding days.

Effect of Rosmarinic acid on Motor performance

Oxaliplatin treatment did not show any significant difference in the performance of rats on the accelerating rota rod from that of normal control group on day 0,7 and 14 (30 and 60 mg/kg doses) and Pregabalin treatment did not show any significant difference in comparison to oxaliplatin treated rats (data not shown).

Effect of Rosmarinic acid on static mechanical hyperalgesia

The administration of Oxaliplatin, resulted in a significance (P<0.001) development of machano-hyperalgesia which is reflected in the increase in paw withdrawal due to the administration of Rosmarinic acid reduced the nociceptive threshold significantly (p<0.01) thus dose dependent. Pregabalin reflected similar effect of reducing the nociceptive threshold significantly (p<0.001).

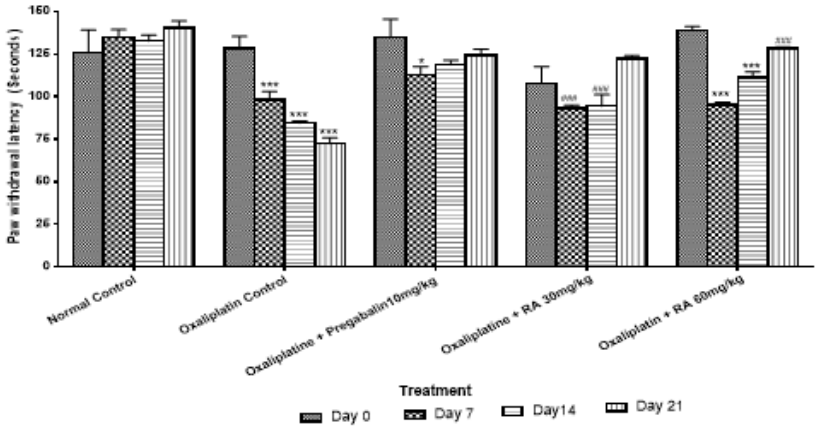

Figure 3: Effect of Rosmarinic acid on static mechanical hyperalgesia.

Results are expressed as mean±SEM, n=6 rats per group. Two- way ANOVA was done followed by Bonferonni's test and the result indicates significance at P<0.001 in comparison with normal in corresponding days where * indicates significance at P<0.05, ** indicates significance at P<0.01 and *** indicates significance at p<0.001 in treated rats in corresponding days. ### indicates significance at p<0.001 in comparison with Oxaliplatin control.

Effect of Rosmarinic acid on formalin test

Oxaliplatin induced peripheral neuropathy, the administration of Rosmarinic acid in 30mg/kg and 60mg/kg respectively showed a significance (P<0.01) reduction in paw licking and paw elevation in acute phase(0-10min), while in delayed phase(30-40min) it showed a significant reduction in paw licking and elevation at p<0.001as shown in (figure 4) and (Figure 5**)** respectively. Meaning, Rosmarinic acid acts both centrally and peripheral in attenuation of neuropathic pain.

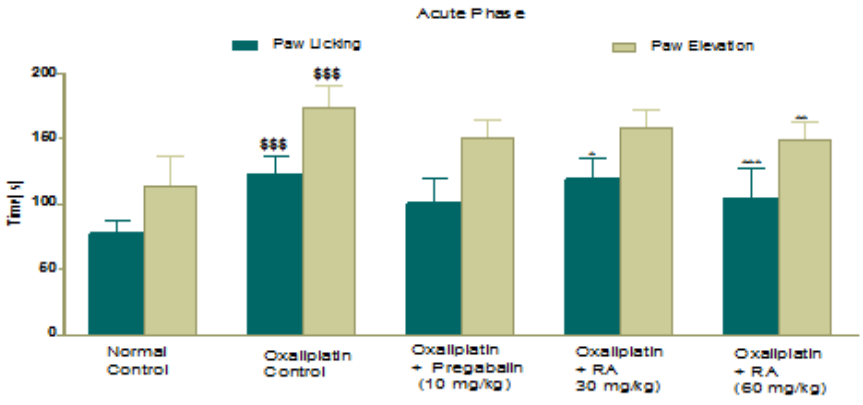

Figure 4: Effect of Rosmarinic acid on formalin test in acute phase.

Results are expressed as mean±SEM, n=6 rats per group. One-way ANOVA was done followed by post hoc tukey's multiple comparison test and the result indicated significance at P<0.001 in comparison with oxaliplatin control in corresponding groups, where * indicates significance at P<0.05, ** indicates significance at P<.01, *** indicates significance at p<0.001 and $$$ indicates significance p<0.001 in Oxaliplatin Vs Normal.

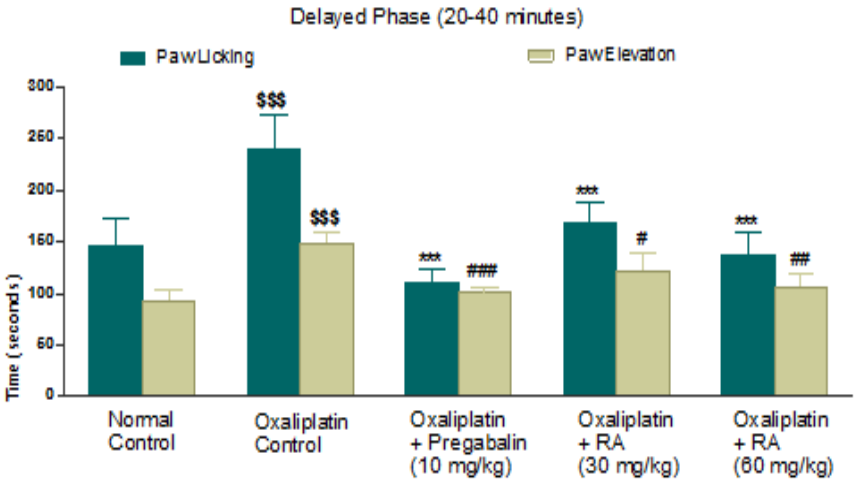

Figure 5: Effect of Rosmarinic acid on Formalin test in Delayed phase.

Results is expressed as mean±SEM, n=6 rats per group. One-way ANOVA was done and the result indicated significance at P<0.001 in comparison with oxaliplatin control in corresponding groups, where *** indicates significance at p<0.001, $$$ indicates significance p<0.001 in Oxaliplatin vs normal and ##, ### indicates significance at P<0.01 and P<0.001 in Oxaliplatin vs Rosmarinic acid treated rats.

Effect of Rosmarinic acid on sciatic nerve function index

Oxaliplatin administered rats resulted in sciatic functional loss as reflected by a significant rise (p< 0.001) in sciatic functional index level. Administration of Rosmarinic acid (30 and 60mg/kg) significantly attenuated (p<0.001) oxaliplatin induced rise in sciatic functional index in a dose dependent manner. However, Rosmarinic acid treatment at all the dose level was unable to restore the sciatic functional index to a baseline score. Similar effect was seen with pregabalin administration (Table 1).Rosmarinic acid had significance in amelioration by increasing the sciatic functional index.

Table 1: Effect of Rosmarinic acid on sciatic nerve functional index.

Group	Sciatic Nerve Index		
	0 day vs 7th	7th vs 10 day	10th vs 14 day
OX+CMC	-58.1267	-25.6724***	-18.6781***
OX+Pregabalin	-38.6732	-12.7892**	-2.345***
RA 30mg/kg+OX	-42.7341	-19.2344**	-9.7224***
RA 60mg/kg+OX	-46.2745	-13.2376**	-6.4327***

The values are expressed in mean±SEM (n=6), ** indicates P<0.001 and *** indicates P<0.001 compared to control. Where, OX: Oxaliplatin, CMC: Carboxy methyl cellulose, RA: Rosmarinic acid

Histopathology

The administration of Oxaliplatin showed significant histopathological changes, as the sciatic nerve exhibited axonal degeneration and fibers derangements. The administration of Rosmarinic acid significantly attenuated Oxaliplatin induced axonal degeneration in 30mg/kg and 60mg/kg. Pregabalin groups exhibited similar attenuation of sciatic nerve degeneration. A-Exhibited normal sciatic nerve, B-Exhibited sciatic nerve degeneration in Oxaliplatin treated rat, C-shows sciatic nerve axonal recovery in pregabalin treated rat, D-shows sciatic nerve axonal regeneration in 30mg/kg Rosmarinic acid treated rat and E-shows more Sciatic nerve axonal regeneration in 60mg/kg in Rosmarinic acid treated rat in Oxaliplatin induced Neuropathy in comparison to normal control and negative control. Thus the protective effect of Rosmarinic acid was note

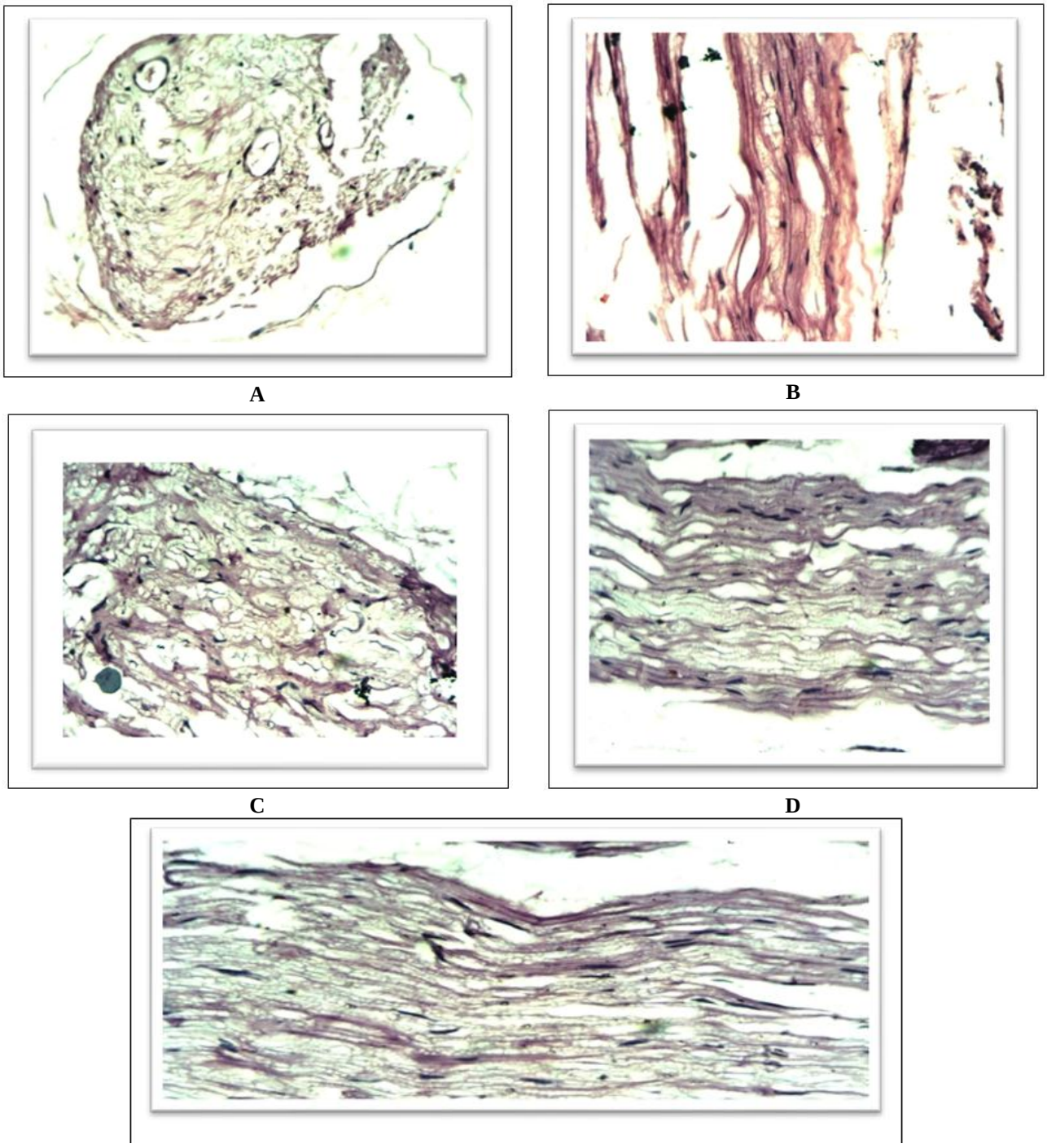

Figure 6: Histopathological examination of Rosmarinic acid on Oxaliplatin induced Neuropathy, A- Normal control, B-Oxaliplatin control, C-Oxaliplatin + pregabalin, D-Oxaliplatin +Rosmarinic acid (30mg/kg), E-Oxaliplatin+Rosmarinic acid (60mg/kg).

CONCLUSION

Cancer therapy utilizes Oxaliplatin as the major first line drug that can be either administered as a single or in combination with other compatible cytotoxic therapeutic agents. However, their utility has been limited due to the neurotoxicity (CIPN) effects they cause.[15]

Presently, there is no standard regimen that is available in management of CIPN, hence, for many patients who develops neurotoxicity are forced to decrease the dose or discontinue with chemotherapy with Oxaliplatin, Vincristine, Cisplatin and Paclitaxel.[16] Rosmarinic acid evidently and significantly decreased nociceptive threshold in hyperalgesia, thermal allodynia from chemotherapy induced peripheral neuropathy. The treatment with RA in rat significantly decrease the nociceptive threshold, decreased oxidative stress and improved both sciatic functional index (SFI) in dose and

time dependent manner thus, Rosmarinic acid, has a significant protective effect in nerve degeneration.

The nociceptive pain induced by 0.2% w/v formalin is associated with the injury of the tissue. In this the acute phase (0-10min.) is short lived and associated with c-fiber activation in response to peripheral stimulation. The delayed phase (20-40 min.) is long lived and is more characterized with tissue damage due to inflammation and functional alteration in the dorsal horn. Thus, it can be termed as inflammatory phase.[17] The study revealed that Romarinic acid had a significant protective effect on chemotherappy induced peripheral neuropathy (CPIN).

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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