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EVALUATION OF NEUROPROTECTIVE EFFECT OF NEOHESPERIDIN DIHYDROCHALCONE FOLLOWING EXPERIMENTAL PARTIAL SCIATIC NERVE LIGATION IN WISTAR RATS

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ABSTRACT

The bitter orange component Neohesperidin dihydrochalcone (NHDC), which is semi-natural, is a potent sweetener. NHDC shows diverse bioactivities including antioxidant, free radical scavenging activity, ulcer protective activity, anti- adipogenic activity and hepatoprotective activity but it was not evaluated in the chronic neuropathic pain, partial sciatic nerve ligation (pSNL) in rat model by using pregabalin (PREG) as standard. The main objective of the present study is to evaluate the Neuroprotective effect of "Neohesperidin dihydrochalcone" followed experimental partial sciatic nerve ligation in Wistar rats. This study was intended for 20 days, using male Wistar rats. Animals were randomized into six groups (n=6). Normal control, sham control, disease control, treatment group: low dose - NHDC (40 mg/kg, P.O.), high dose –NHDC (80 mg/kg, P.O.), standard - pregabalin (10 mg/kg, P.O.) along with PSNL. During the treatment period, behavioral parameters were measured on 7^{th}, 14^{th} and 22^{nd} day and the animals were subjected to estimation of biochemical parameters (MDA, SOD and Total Protein content) on 7^{th} day and 14^{th} day using plasma and on 22^{nd} day using sciatic nerve tissue homogenate. Administration of NHDC resulted in the dose dependent attenuation in pSNL-induced behavioral and biochemical parameters. Consequently, it can be concluded that neuroprotective effect of NHDC in rats after pSNL may be attributed to various oxidative markers as well as the pro-inflammatory mediators secreted at the injury site. NHDC appears to be a promising

candidate for the development as a novel therapeutic for the patients suffering from the neuropathic pain.

KEYWORDS: Partial sciatic nerve ligation (pSNL), Neohesperidin dihydrochalcone (NHDC), pregabalin, neuroprotective activity hyperalgesia, pro-inflammatory mediators.

1. INTRODUCTION

Pain is a negative sensory and emotional sensation that is connected to, or similar to, existing or potential tissue injury.[31] "Pain is experienced only occasionally in the lives of the healthy, its neural mechanisms lying dormant, but vigilant, ready to be awakened if the tissues of the body are attacked," the English neurologist George Riddoch wrote in a landmark study from 1938.[33] According to one definition, **neuropathic pain** is "the most horrible suffering that may levy on a nerve." It can also be said that the pathology of the neurological system caused the discomfort. It typically involves modifications to the chemistry, structure, and functionality of neurons. The symptoms of neuropathic pain include *hyperalgesia* (increased pain response to a typically painful stimulus), *paraesthesia* (abnormal feeling to a stimulus that is normally not unpleasant), *dysesthesia* (painful abnormal sensation), and allodynia (pain due to a stimulus that does not normally cause pain).[32] According to animal models of neuropathic pain, hyperalgesia is connected to the inflammatory response, and it is characterized by high levels of inflammatory mediators in nerve-damaged rat models.[25]

A number of disorders have been known to be improved by isolated bioactive molecules from the flavonoids class, which are considered to be potential free radical scavengers.[41] Flavonoids can undergo a redox reaction that makes it possible for them to quickly scavenge free radicals attributed to the hydrogen-donating substituent connected to their aromatic ring structures.[28] According to Slimestad and Verheul (2009), flavonoids have been reported to have pharmacological activities and are widely dispersed in higher plants including citrus fruit, buckwheat, and onions. They may have antioxidant, anti-inflammatory, anti-diabetic, and immunomodulatory properties;[26][29][34] anti-inflammatory;[6][14] and immunomodulatory properties,[16] anti-rheumatic[4] and anti-nociceptive[42] medications.

NHDC is not found in nature, the United States Department of Agriculture's Agricultural Research Service employed chemists who created NHDC in the early 1960s.[19] Neohesperidin, its parent chemical, is a naturally occurring flavanone, a subclass of flavonoids, and is present in bitter orange (Citrus aurantium) in levels of 100–200 ppm in its juice and 97,000 ppm in the

peel of its unripe fruit.[45] For the first time about 40 years ago, NHDC was evaluated for use as an intense sweetener in food due to its high sweetness (several hundred times that of sucrose).[8] Posses wide spectrum of activities including sweetening agent,[8] Free radical scavenging activity,[38] Anti ulcer activity,[39] Anti oxidant activity,[38] Inhibition of Hypochlorous Acid-Induced DNA Strand Breakage, Protein Degradation, and Cell Death,[3] Hepatoprotective activity,[9] Anti- inflammatory and anti-apoptotic activity,[36] Anti-adipogenic activity.[17]

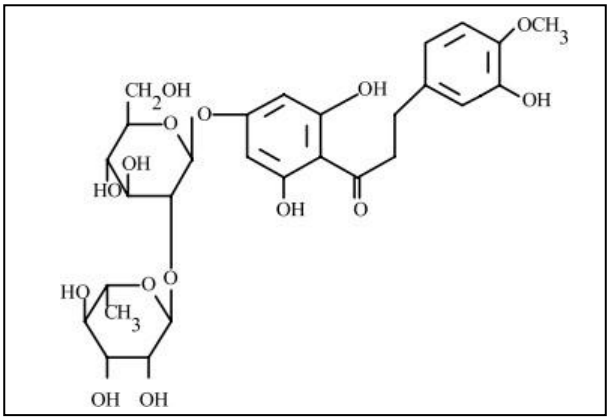


Fig. 1: Chemical structure of Neohesperidin dihydrochalcone.

Neohesperidin dihydrochalcone IUPAC name: 1-[4-[(2*S*,3*R*,4*S*,5*S*,6*R*)-4,5-dihydroxy-6-(hydroxymethyl)-3-[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-2-yl]oxy-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one.

A well-known and widely accepted animal model for inducing neuropathic pain in lab animals is **partial sciatic nerve ligation (PSNL)**. The sciatic nerve is partially ligated in PSNL.[35] The current study was designed to examine the potential therapeutic benefit of NHDC (40 and 80 mg/kg, p.o.) in PSNL-induced neuropathic pain in rats by evaluating behavioral, biochemical, and histopathological parameters. This was done in light of the reported antioxidant, anti-inflammatory, and anti-ulcer activities of NHDC.

2. MATERIAL AND METHODS

2.1.Animals

Adult male Wistar rats, weighing 200–300 g were used in the present study. Animals were procured from the Vab Biosciences, Hyderabad, Telangana, India. Animals were maintained at 25 ± 5°C, with relative humidity of 55 ± 10% and 12:12 h dark/light cycle and given free access to the food and drinking water *ad libitum*.

All experiments were carried out between 10:00 and 17.00 h. All experimental procedures were done following the "Committee for Purpose of Control and Supervision of Experimental on Animal" [CPCSEA] guidelines. The study was reviewed and approved by the Institutional Animal Ethical Committee (GPRCP/IAEC- 02/29/12/2021/PCL-3), G. Pulla Reddy College of Pharmacy, Hyderabad, India.

2.2.Drugs and chemicals

NHDC was procured from CARBANIO, India. Pregabalin was procured from Cipla. Liquid Gold Total protein assay kit (AUTOSPAN), India. Ketamine, Xylazine, Sodium CMC, 10% formalin, Disodium EDTA solution, Pyrogallol solution, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), and Hydrochloric acid were procured from SD FINE CHEMICALS, Hyderabad. All the reagents used in the present study were of analytical grade.

2.3.Induction of peripheral neuropathic pain

The rats were anesthetized using ketamine (60 mg/kg) and xylazine (8 mg/kg) and half of the sciatic nerve was ligated at the upper thigh level using an 8-0 nylon suture. Sham surgery was done by exposing the sciatic nerve without ligation.[35] Behavioral and were conducted on 7^{th}, 14^{th} and 22^{nd} day. Biochemical parameters were performed on 7^{th} and 14^{th} day using plasma and on 22^{nd} day using sciatic nerve tissue homogenate.

2.4.Experimental protocol

Animals were divided into following six groups (n= 6).

Group-I (Normal): No treatment/ligation.

Group-II (Sham): Vehicle (1 ml/kg, p.o.) for 15 days. Sciatic nerve was exposed without ligation.

Group-III (Disease Control): Surgical exposure and ligation of sciatic nerve.

Group IV (Low Dose): NHDC 40 mg/kg, p.o.

Group V (High Dose): NHDC 80 mg/kg, p.o.

Group-VI (Standard): Pregabalin 10 mg/kg, p.o.

All drugs were freshly prepared before administration. Test and Standard drugs were administered for 20 days after ligation. During the treatment period, behavioral parameters were measured on 7^{th}, 14^{th} and 22^{nd} day and, the animals from all groups were subjected to estimation of biochemical parameters (MDA, SOD and TP) on 7^{th} day and 14^{th} day using plasma from the blood obtained from retro- orbital plexus. At the end of the treatment period

all the animals were sacrificed under euthanasia and the sciatic nerve tissue homogenates were excised for biochemical estimations of MDA, SOD and Total Protein Content on 22nd day.

2.5. Assessment of behavioral parameters

2.5.1. Thermal hyperalgesia test: The thermal hyperalgesia index was determined using Eddy's hot plate method and the thermal nociceptive threshold. The hot plate was kept at a temperature of 55^0 C. The rat was then set on the plate, and the nociceptive threshold was measured in terms of the rat's paw- licking, jumping, and other reflexes. The animal typically has a baseline reaction time of 6 to 8 seconds. By keeping a 15- second cutoff time and recording the data three times, the paw withdrawal latency was measured in seconds. The mean of three results was considered.[7]

2.5.2. Motor coordination test: Using a rota-rod device, motor coordination (grip muscle strength) was assessed (Model KI- 9616-4, India). Rats were individually placed for a minute on the rotating rod (25 RPM). During the one-minute observation, the fall off time from the rotating rod was noted. A rat was retrieved from the equipment and put back in its cage after it fell from the beam. The time at which the rat dropped off the beam were noted as the latencies (lengths of time the rat spends on the beam). Usually, it is preferable to conduct multiple trials. After at least a 15-minute interval, the test was done two or three more times. The mean of the latencies of the 3 trials was calculated.[11]

2.5.3. Locomotory activity test: To evaluate how drug therapy affected spontaneous motor (exploratory) activity, a photoactometer test was used. Rats were placed in one of the maze's four quadrants, facing the maze's centre. Five minutes were spent observing each animal in a square closed field arena (30 x 30 x 30 cm) that had six photocells installed in the outer wall. A six-digit counter was used to keep track of photocell beam interruptions caused by locomotor and exploratory motion. The number of counts on the counter was considered during the study. Between trials, maze surfaces were cleaned with a moderate detergent and lukewarm to get rid of any odour residues.[5]

2.6. Assessment of biochemical parameters

2.6.1. Determination of malondialdehyde (MDA) levels: The MDA level was estimated using Buege et al., 1978.[2] 1ml of 2% tissue homogenate in 2ml of TCA-TBA-HCl solution was mixed and heated for 15 min in boiling water bath (100^o C). After cooling, the precipitate was removed by centrifugation at 3000 rpm for 10 mins. The resultant colored layer was

separated and absorbance was measured at 535nm against blank using spectrophotometer. Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95ºC for 30 min to form thiobarbituric acid reactive product the absorbance of the resultant pink product can be measured at 535nm.

Calculation

$$\text{The concentration of MDA} = \frac{\text{Absorbance x D}}{\text{L x a}}$$

Where,

L: Light bath (1 cm)

a: $1.56 \times 10^5\ M^{-1}\ cm^{-1}$

D: Dilution factor

Units: MDA in plasma (µmol/mL), MDA in tissue (µmol/mg of protein)

2.6.2. Determination of superoxide dismutase (SOD) levels: SOD level was determined by Marklund et al. 1974.[21] 0.1 mol/L Tris-HCl buffer solution with 1 mM/L EDTA, 2.35 ml of solution A, 0.02 ml of tissue homogenate (sample), and 0.15 ml of solution B (4.5 mmol/L pyrogallol solution in HCl) were combined to prepare the solution. The solution was immediately vortexed, and an aliquot's absorbance was determined at 325 nm. After one minute, another aliquot value was determined. The rate of pyrogallol auto-oxidation is indicated by the difference in absorbance. The same procedure as in step I was used; 2 ml of the water was added to solution A before solution B to prepare blank. The sample was properly diluted to give 50% reduction in auto-oxidation rate i.e., $\Delta A^{325} = 0.030$.

Table 1: Volume of sample and reagent.

S. No	Sample/ reagent volume	Blank	Test
1	Solution A (ml)	2.35	2.35
2	Distilled water (ml)	2.00	1.80
3	Sample solution (ml)	-	0.02
4	Solution B (ml)	0.15	0.15

Calculations

$$\% \text{ inhibition of pyrogallol auto-oxidation} = \frac{\Delta A \text{ test}}{\Delta A \text{ Control}} \times 100$$

$$\text{SOD activity} = \frac{\%\ \text{inhibition of pyrogallol}}{50}$$

Where:

ΔA = Final - initial absorbance

Units: SOD in plasma (U/mL), SOD in tissue (U/mg of protein)

2.6.3. Determination of Total protein levels

The level of Total Protein was determined by Modified Biuret, End Point Assay, 1974.

- Reagent 1 - Biuret reagent
- Reagent 2 - Total protein standard (Bovine Serum Albumin)

Table 2: Volume of sample and reagent.

Pipette into tube marked	Reagent blank	Standard	Test
Serum/plasma	-	-	0.01 ml
Reagent 2	-	0.01 ml	-
Reagent 1	1 ml	1 ml	1 ml

- Mix well and incubate at 37^{o} C for 5 minutes.
- Measure the absorbance at 578 nm (550-580 nm).

Calculation

$$\text{Total protein concentration} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 6.5$$

Units: g/mg in tissue**,** g/dL of in plasma**.**

2.7.Histopathology

Sciatic nerves close to the ligation were taken from all six groups on the 22nd day of the investigation, placed in a container with a 10% neutralised formalin buffer solution, and given a code number. The samples were placed in the fixative solution for around 24 hours, washed with running tap water for 4-6 hours, and then rinsed with alcohol and xylene in increasing concentrations. Following automatic sample preparation, the tissues were embedded in liquid paraffin. The tissues were subsequently cut into slices that were 4-6 µm thick and stained with hematoxylin and eosin. Under a light microscope, specific regions were investigated, and pictures were taken.[12]

Statistical analysis

Using GraphPad Prism software (8.0.1), one-way analysis of variance (ANOVA) and Tukey's multiple comparison test was used to statistically assess all results, which are shown as Mean ± SEM.

3. RESULTS

3.1. BEHAVIORAL PARAMETERS

3.1.1. Impact of NHDC (40 and 80 mg/kg p.o.) on paw withdrawal latency in sciatic nerve ligation-induced neuropathic pain in Wistar rats

Thermal hyperalgesia was performed to all groups on days 7, 14, and 22 of the trial. In rats with pSNL, the control group demonstrated a significant decrease in paw withdrawal latency on day seven and continued on day twenty-two after surgery, indicating the induction of thermal hyperalgesia. The paw withdrawal latency of disease control considerably increased ($p< 0.0001$) on the 22nd post-operative day (Table 3). When NHDC (40 and 80 mg/kg p.o.) was administered, the paw withdrawal latency significantly increased on day 22 compared to day 7 ($p<0.0001$) (Figure 2).

3.1.2. Impact of NHDC (40 and 80 mg/kg p.o.) on motor coordination in sciatic nerve ligation-induced neuropathic pain in Wistar rats

Motor co-ordination test was performed to all groups on days 7, 14, and 22 of the trial. In rats with pSNL, the control group demonstrated a significant decrease in retention time on rota-rod on day seven and continued on day twenty-two after surgery, indicating the induction of motor incordination. The retention time of disease control considerably decreased ($p< 0.0001$) on the 22nd post-operative day (Table 4). When NHDC (40 and 80 mg/kg p.o.) was administered, the retention time on rota-rod significantly increased on day 22 compared to day 7 ($p<0.0001$) (Figure 3).

3.1.3. Impact of NHDC (40 and 80 mg/kg p.o.) on locomotory activity in sciatic nerve ligation-induced neuropathic pain in Wistar rats

Motor co-ordination test was performed to all groups on days 7, 14, and 22 of the trial. In rats with pSNL, the control group demonstrated a significant decrease in locomotory activity on day seven and continued on 22nd day after surgery, indicating the induction of neuropathic pain. The locomotory activity of disease control considerably decreased ($p< 0.0001$) on the 22nd post-operative day (Table 5). When NHDC (40 and 80 mg/kg p.o.) was administered, the locomotory activity significantly increased on day 22 compared to day 7 ($p<0.0001$) (Figure4).

3.2 BIOCHEMICAL PARAMETERS

3.2.1. Impact of NHDC (40 and 80 mg/kg p.o.) on variations in MDA levels (lipid peroxidation assay) in sciatic nerve ligation-induced neuropathic pain in Wistar rats

Increase in MDA levels due to oxidative stress in tissue at the site of ligation is seen in animal. Lipid peroxidation assay was performed to all groups on days 7, 14 using plasma and 22 day using tissue homogenate. In rats with pSNL, the control group demonstrated a significant increase ($p < 0.0001$) in MDA levels on day 14 indicating the induction of neuropathic pain (Table 6). When NHDC (40 and 80 mg/kg p.o.) was administered, the MDA levels significantly decreased compared to the disease control in high dose ($p < 0.0001$) and low dose ($p < 0.05$) (Table 6). MDA levels in treatment group significantly decreased compared to disease control group on the 22nd post-operative day ($p < 0.0001$) (Figure 5).

3.2.2. Impact of NHDC (40 and 80 mg/kg p.o.) on variations in SOD levels in sciatic nerve ligation-induced neuropathic pain in Wistar rat

Decrease in SOD levels due to oxidative stress of the animals in tissue at the site of ligation is seen. Estimation of SOD was performed to all groups on days 7, 14 using plasma and 22 day using tissue homogenate. In rats with pSNL, the control group demonstrated a significant decrease ($p < 0.0001$) in SOD levels on day 7 continued to decrease till day 14 indicating the induction of neuropathic pain (Table 7). When NHDC (40 and 80 mg/kg p.o.) was administered, the SOD levels significantly increased compared to the disease control in high dose and low dose ($p < 0.0001$) (Table 7). SOD levels in treatment group significantly increased compared to disease control group on the 22nd post-operative day ($p < 0.01$) (Figure 6).

3.2.3. Impact of NHDC (40 and 80 mg/kg p.o.) on variations in total protein content in sciatic nerve ligation-induced neuropathic pain in Wistar rats

Increase in Total protein levels due to oxidative stress of the animals in tissue at the site of ligation. Modified Biuret test was performed to all groups on days 7, 14 using plasma and 22 day using tissue homogenate. In rats with pSNL, the control group demonstrated a significant increase ($p < 0.0001$) in total protein content on day 7 and continues to increase till day 14 indicating the induction of neuropathic pain (Table 8). When NHDC (40 and 80 mg/kg p.o.) was administered, the total protein content significantly decreased compared to the disease control in high dose ($p < 0.0001$) and low dose ($p < 0.05$) (Table 8). Total protein content in treatment group significantly decreased compared to disease control group on the

22nd post-operative day ($p < 0.0001$) (Figure 7).

Table 3: Impact of NHDC (40 and 80 mg/kg p.o.) on paw withdrawal latency in sciatic nerve ligation-induced neuropathic pain in rats in Wistar rats.

Paw withdrawal latency (sec)					
Days	**Normal Control**	**Disease Control**	**Low Dose**	**High Dose**	**Standard**
7	5.267 ± 0.08433	1.95 ± 0.0223 (α)	2.250 ± 0.04282	2.600 ± 0.03651 (μ)	2.330 ±0.04944
14	5.467 ± 0.1453	2.000 ± 0.3651(α)	2.533 ± 0.04944(*)	2.900 ± 0.03651(*)	2.650 ± 0.04282(*)
22	5.333 ± 0.1333	2.050 ± 0.2236(α)	2.967 ± 0.04944 (μ)	3.467 ± 0.08433 (μ, Δ, κ)	3.033 ± 0.04944 (μ)

Data is expressed as Mean±SEM and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

α $p < 0.0001$ vs Normal Control

μ $p < 0.0001$, * $p < 0.01$ vs Disease Control Δ $p < 0.01$ vs Low dose

κ $p < 0.05$ vs Standard

Table 4: Impact of NHDC (40 and 80 mg/kg p.o.) on motor coordination in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Retention time (sec)					
Days	**Normal Control**	**Disease Control**	**Low Dose**	**High Dose**	**Standard**
7	75 ± 2.910	17 ± 0.8563 (&)	24.33 ± 1.282	36.500 ± 1.0570 (@, Δ)	26.67 ± 1.282
14	76.33 ± 3.106	19.33 ± 1.4760 (&)	29.33 ± 1.282	42.67 ± 1.3820 (@, *,μ)	31.00 ± 1.626 ($)
22	79.83 ± 2.982	19.33 ± 1.4760 (&)	35.00 ± 1.125 (#)	44.83 ± 0.6009	37.33 ± 1.626

Data is expressed as Mean±SEM and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

& $p < 0.0001$ vs Normal Control

@ $p < 0.0001$, $ $p < 0.05$, # $p < 0.001$ vs Disease Control Δ $p < 0.05$, * $p < 0.01$ vs Low Dose

μ $p < 0.05$ vs Standard

Table 5: Impact of NHDC (40 and 80 mg/kg p.o.) on motor coordination in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Number of counts per 5 minutes					
Days	**Normal Control**	**Disease Control**	**Low Dose**	**High Dose**	**Standard**
7	117.5 ± 3.658	35.330 ± 1.626 (@)	43.17 ± 0.7923	49.33 ± 1.6670 (#)	43.33 ± 0.8819
14	123.2 ± 3.114	39.170 ± 2.949 (@)	50.67 ± 2.4590	57.17 ± 3.0270 (β)	61.33 ± 1.8740 ($)
22	117.2 ± 2.600	34.500 ± 3.566 (@)	82.00 ± 3.3670 ($)	94.17 ± 3.9110 ($, μ)	76.00 ± 2.9100

Data is expressed as Mean±SEM and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

@ $p < 0.0001$ vs Normal Control

$ $p < 0.0001$, # $p < 0.05$, β $p < 0.01$ vs Disease Control μ $p < 0.01$ vs Standard

Table 6: Impact of NHDC (40 and 80 mg/kg p.o.) on variations in MDA levels in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

MDA content in plasma (μmol/mL of protein)					
Days	**Normal Control**	**Disease Control**	**Low Dose**	**High Dose**	**Standard**
7	0.2529 ± 0.0192	0.5722 ± 0.0898	0.5957 ± 0.0519	0.5207 ± 0.0292	0.5983 ± 0.0681
14	0.2888 ± 0.0333	1.0590 ± 0.1828 (@)	0.6047 ± 0.0246 ($)	0.2658 ± 0.0162 (#)	0.3722 ± 0.0162 (#)
MDA content in tissue (μmol/mg)					
22	0.2217 ± 0.0147	1.4840 ± 0.2266 (@)	0.5013 ± 0.0279 (#)	0.2038 ± 0.0180 (#)	0.2038 ± 0.0180 (#)

Data is expressed as Mean±SEM and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

@ $p < 0.0001$ vs Normal Control

# $p < 0.0001$ vs Disease Control, $ $p < 0.05$ vs Disease Control

Table 7: Impact of NHDC (40 and 80 mg/kg p.o.) on variations in SOD levels in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

SOD in plasma (U/mL of protein)					
Days	**Normal Control**	**Disease Control**	**Low Dose**	**High Dose**	**Standard**
7	6.658 ± 0.142	0.8250 ± 0.27490 @	2.422 ± 0.40120 β	4.335 ± 0.03695 β, Δ	5.257 ± 0.1313β
14	6.67 ± 0.162	1.4920 ± 0.09325 @	3.422 ± 0.04012β	5.337 ± 0.037 β, Δ	4.257 ± 0.13β

SOD in tissue (U/mg)					
22	6.318 ± 0.1848	2.7200 ± 0.26270 @	4.540 ± 0.09719 β, z	6.530 ± 0.11050 β, Δ, γ	5.417 ± 0.1545 β

Data is expressed as Mean±SEM and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

@ $p < 0.0001$ vs Normal control β $p < 0.0001$ vs Disease Control Δ $p < 0.0001$ vs low dose

z $p < 0.05$ vs Standard

γ $p < 0.0001$ vs Standard

Table 8: Impact of NHDC (40 and 80 mg/kg p.o.) on variations in Total protein content in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Total protein content in plasma (g/dL)					
Days	**Normal Control**	**Disease Control**	**Low Dose**	**High Dose**	**Standard**
7	6.723 ± 0.1588	10.78 ± 0.5148 (@)	9.254 ± 0.168 ($)	7.638 ± 0.356 (#, €)	8.178 ± 0.216 (#)
14	6.753 ± 0.1035	12.93 ± 0.2177 (@)	10.040 ± 0.317 (#, ¥)	6.637 ± 0.306 (#, Δ, ¥)	8.137 ± 0.153 (#)
Total protein content in tissue (g/mg)					
22	7.024 ± 0.1996	15.28 ± 0.2803 (@)	10.6 ± 0.473 (#, ¥)	6.790 ± 0.283 (#, β)	7.723 ± 0.220 (#)

Data is expressed as Mean±SEM and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

@ $p < 0.0001$ vs Normal Control

# $p < 0.0001$, $ $p < 0.05$ vs Disease Control β < 0.0001, Δ $p < 0.001$ vs Low Dose

¥ $p < 0.0001$, € $p < 0.05$ vs Standard

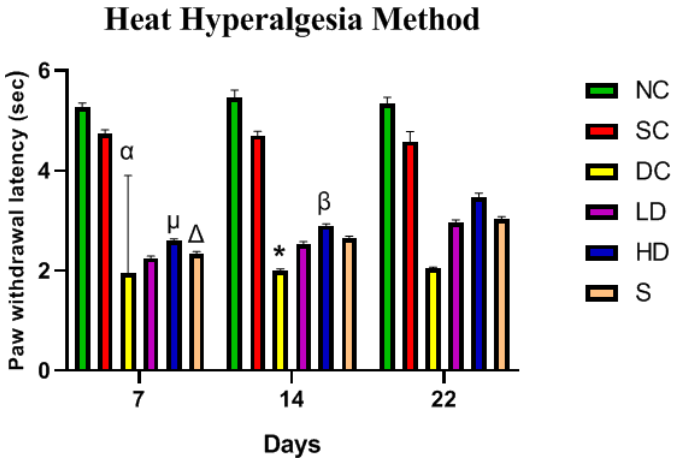


Figure 2: Impact of NHDC (40 and 80 mg/kg p.o.) on paw withdrawal latency in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Values are expressed as mean±SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

α p < 0.0001, *p < 0.05 vs Low dose (LD) (week 3) µ p< 0.0001, β p < 0.001 vs High dose (HD) (week 3) Δ p < 0.0001 vs Standard (S) (week 3).

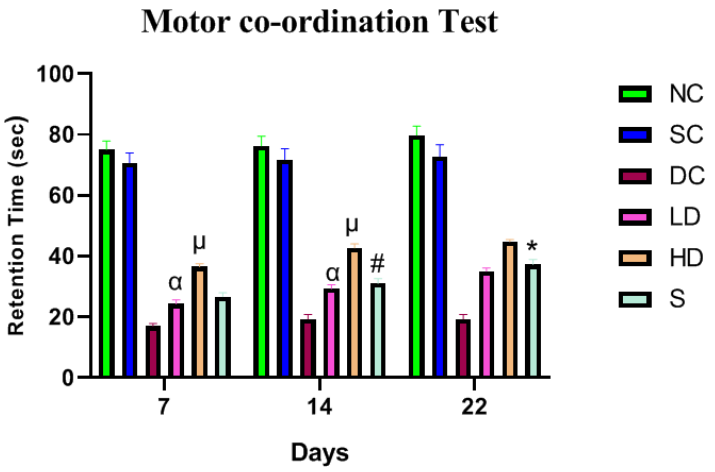


Figure 3: Impact of NHDC (40 and 80 mg/kg p.o.) on motor coordination in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Values are expressed as mean±SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

α p < 0.0001 vs Low dose (LD) (week 3) µ p< 0.0001 vs High dose (HD) (week 3)

# p< 0.01 vs S1, *p < 0.0001 vs Standard (S) (week 1)

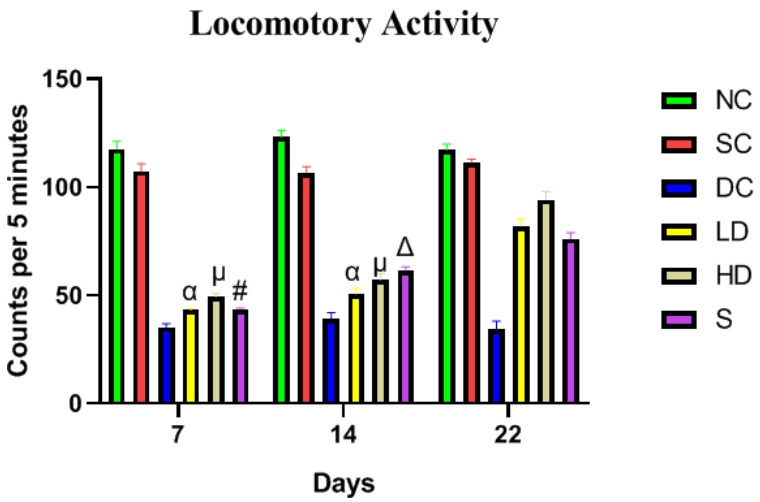


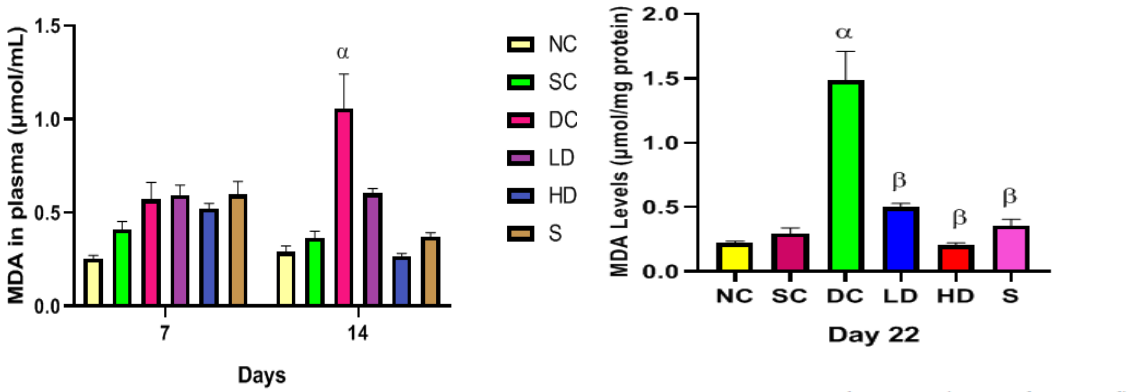
Figure 4: Impact of NHDC (40 and 80 mg/kg p.o.) on locomotory activity in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Values are expressed as mean±SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

α p < 0.0001 vs Low dose (LD) (week 3) μ p< 0.0001 vs High dose (HD) (week 3)

Δ p < 0.05, # p < 0.0001 vs Standard (S) (week 3)

Lipid peroxidation Assay



α p< 0.01 vs Disease Control (DC) (Day 7)

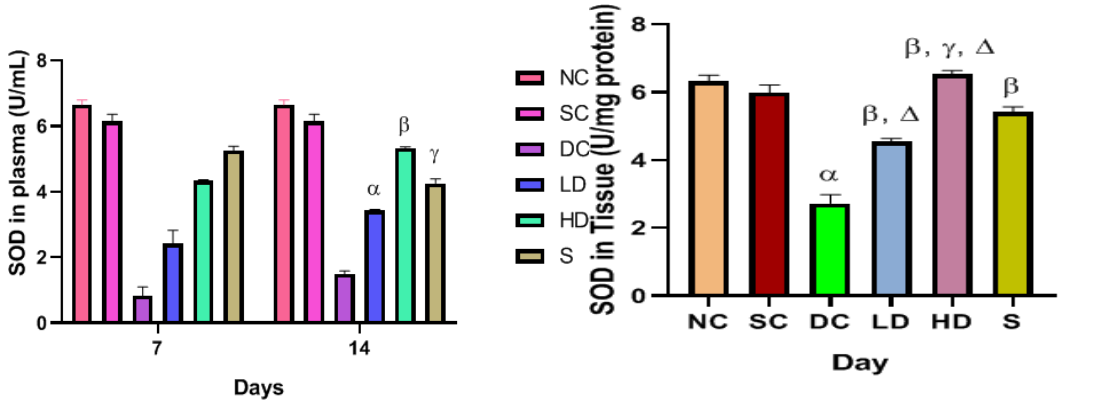
α p < 0.0001 compared to NC (normal control)

β p < 0.0001 compared to DC (disease control)

Figure 4: Impact of NHDC (40 and 80 mg/kg p.o.) on variations in MDA levels in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Values are expressed as mean±SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

SOD levels



α p < 0.01 vs Low dose (LD) (day 7)

β p < 0.01 vs High dose (HD) (day 7)

γ p < 0.01 vs Standard (day 4)

α p < 0.0001 vs Normal control (NC)

β p < 0.01 vs Disease control (DC)

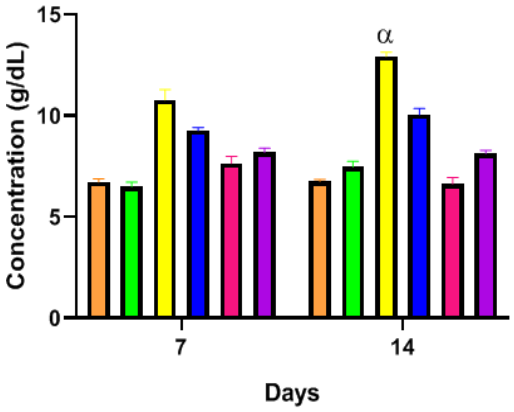
γ p < 0.01 vs Low dose (LD)

Δ p < 0.05 vs Standard (S)

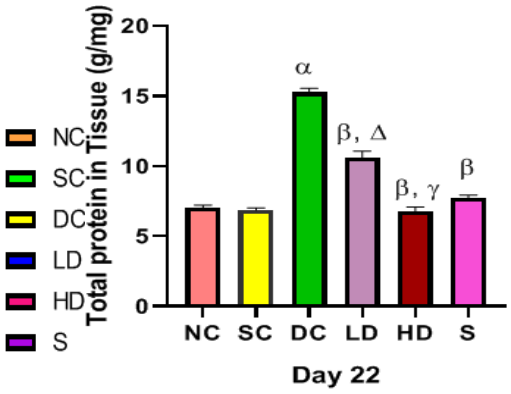
Figure 6: Impact of NHDC (40 and 80 mg/kg p.o.) on variations in SOD levels in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Values are expressed as mean ± SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for comparison of means.

Total Protein Content Assay



α p < 0.0001 compared to Disease control (DC) (day 7)



α p < 0.0001 vs Normal control (NC)

β p < 0.0001 vs Disease control (DC)

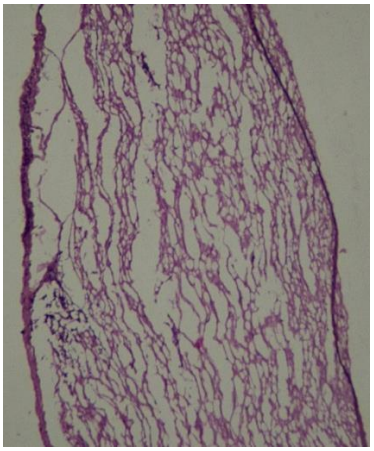
γ p < 0.0001 vs Low dose (LD)

Δ p < 0.0001 vs Standard (S)

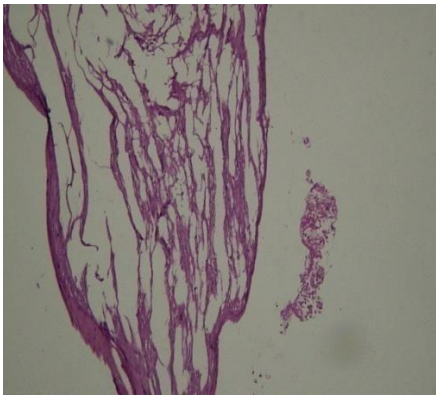
Figure 7: Impact of NHDC (40 and 80 mg/kg p.o.) on variations in Total protein content in sciatic nerve ligation-induced neuropathic pain in Wistar rats.

Values are expressed as mean±SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for comparison of means.

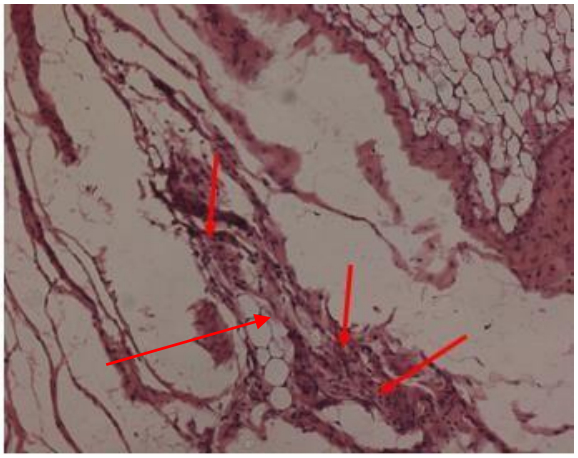
3.3 HISTOPATHOLOGICAL STUDIES



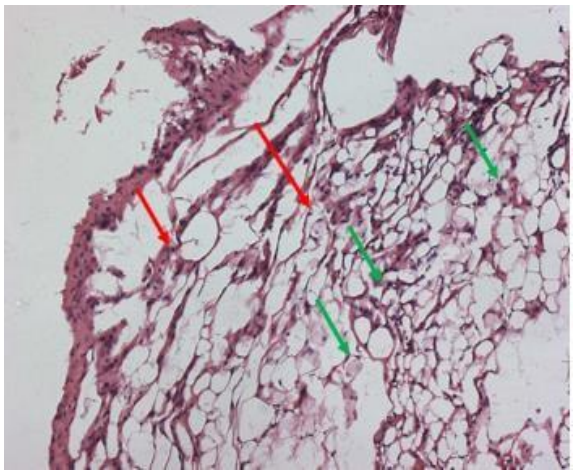
Normal Control (A)



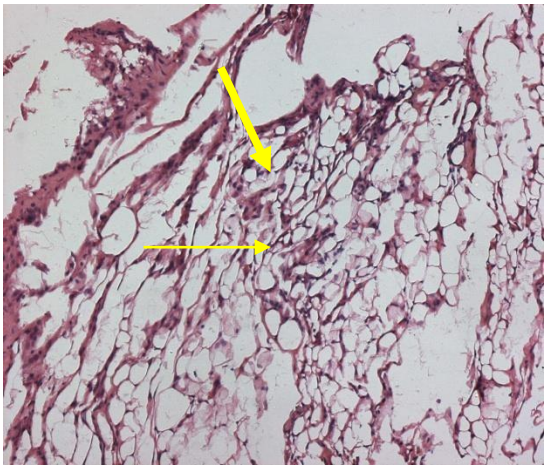
Sham Control (B)



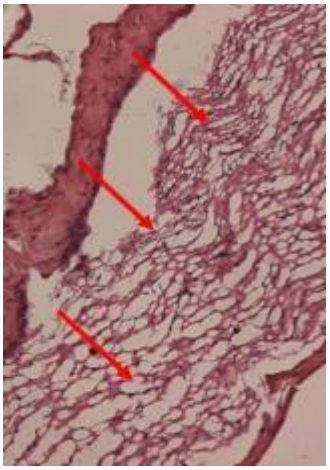
Disease Control (C)



Low Dose (D)



High Dose (E)



Standard (F)

Figure 8: Effect of NHDC on PSNL-induced alterations in the histopathology of the sciatic nerve. Photomicrographs of sections of sciatic nerve from rats stained with H & E.

Sciatic nerve microscopic image of

(A) Normal - Normal morphology of sciatic nerve and its bundles were observed.

(B) Sham Control - Normal morphology of sciatic nerve and its bundles were observed.

(C) Disease Control - Multi focal inflammation in sciatic nerve bundles with infiltration of inflammatory cells were observed - Red arrow

(D) Low dose (NHDC 40 mg/kg, p.o.) treated - Few foci of inflammation with infiltration of inflammatory cells were observed in sciatic nerve bundles – yellow arrow.

(E) High dose (NHDC 80 mg/kg, p.o.) treated - Normal morphology of sciatic nerve [red arrow] with fatty tissues [green arrow]

(F) Standard (Pregabalin 10 mg/kg, p.o.) treated - Normal morphology of sciatic nerve and its bundles were observed.

4. DISCUSSION

In the current work, we looked at the possible effectiveness of orally administering neohesperidin dihydrochalcone (NHDC) to treat rats with neuropathic pain brought on by partial sciatic nerve ligation (pSNL). Because it closely reflects the bulk of the main defining elements of neurogenic pain in patients after peripheral nerve injury, the partial sciatic nerve ligation (pSNL) model of peripheral neuropathic pain is a well acknowledged and used model of this disorder.[9][22][1] Unilateral sciatic nerve ligation caused significant behavioural alterations in motor coordination and thermal hyperalgesia. Primary afferent nerve sensitization has been shown to be the mechanism causing and maintaining hyperalgesia.[13] Potassium ions and substance P levels are elevated in spinal C-fibers suffering persistent neuropathic pain, which reduces the activation threshold of thermal and physical sensors.[18] The ipsilateral mechanical allodynia and thermal hyperalgesia, which emerged right away after surgery and continued for months, were lessened by unilateral pSNL. The findings of this investigation confirm earlier findings that unilateral pSNL caused inflammation.[13] Inflammatory mediators may have been released, leading to this inflammation. The new study's findings were made in the same way as those of prior investigations that backed up NHDC's anti-inflammatory and antioxidant effects.[36][39] The findings of this study point to NHDC's substantial role in the modulation of peripheral pain. The pSNL causes injury to the motor and sensory fibres, sensitising the neurons, and reducing the latency of paw withdrawal. A useful technique for evaluating analgesics with peripheral effect is the hot plate approach.[22] We were able to validate this thermal hyperalgesia using an independent measurement of the latency of paw withdrawal caused by heat stimulation in the current experiment. Treatment with NHDC significantly lengthened the paw withdrawal latency in rats that had pSNL. This study discovered that tissue levels of MDA, SOD, and total protein rose following nerve injury. The increase in MDA, total protein and decrease in SOD levels is evidence of oxidative damage. According to a different study, nerve ligation causes more oxidative stress, as seen by increased protein and MDA levels.[43] In the current study, rats given pSNL displayed low motor coordination, a considerably shorter time needed to remove paw, and lower locomotor activity, all of which indicate that neuropathic pain was induced. Pregabalin and NHDC (40 mg/kg and 80 mg/kg) significantly enhanced thermal paw withdrawal latency, motor coordination, and locomotor activity, showing that NHDC is beneficial as a neuroprotective. Treatment with either the experimental drug NHDC (40 and 80 mg/kg p.o.) or the standard pregabalin (PREG, 10 mg/kg p.o.) gradually reduced the levels of malondialdehyde (MDA), total protein and increase SOD levels. On the other hand, there was no discernible impact on

motor coordination. Pregabalin is an analogue of GABA in structure but not in function. Its chemical name is [(S)-3-(amino methyl)-5-methylhexanoic acid] (Owen, 2007). Its analgesic, anti-inflammatory, anti-oxidant, and anxiolytic effects have been demonstrated in rats.[24][30][37] In animal models of neuropathic pain, pregabalin has been demonstrated to exhibit anti-hyperalgesic and anti-allodyniac effects.[15][27] Free radical synthesis and oxidative stress are thought to have a role in the aetiology of neuropathic pain.[23] The attenuating effect on pSNL via lowering the inflammatory mediators and free radicals may be responsible for NHDC's therapeutic effects in pSNL- induced neuropathic pain, according to the observations. Based on the data gathered and the literature, we may claim that the citrus-rich semi- synthetic flavonoid, NHDC, lowers PSNL-induced neuropathic pain in rats, which is supported by its anti-inflammatory and antioxidative effects. On the 22nd day after surgery, histopathological results revealed a rise in the infiltration of inflammatory cells in the control group. In comparison to disease control groups, the infiltration of inflammatory cells was reduced in the treatment groups. The reduction in pSNL-induced behavioural hyperalgesia, locomotory activity, and biochemical (MDA, SOD, and total protein content) variance is evidence of the neuroprotection found with NHDC.

5. CONCLUSION

In order to report the neuroprotective impact of NHDC, numerous evaluation parameters have been carried out in this study. Treatment with NHDC (40 and 80 mg/kg P.O) has shown significant effect on partial sciatic ligation induced neuropathic pain characterized by increased paw withdrawal latency, increased locomotory activity, decreased MDA content, increased SOD content and decreased total protein content.

The chronic neuropathic pain brought on by pSNL was reduced by NHDC. This attenuation is related to NHDC's anti-inflammatory and antioxidant properties. This shows that the therapeutic strategy for treating chronic peripheral neuropathy involves the antioxidant impact. The effect was improved by NHDC at greater doses. This suggests that the pain will be better relieved with an increase in NHDC dose. More pharmacokinetic studies are advised to better understand the mechanism of action and to identify a potential therapeutically relevant drug of interest for the treatment of chronic neuropathic pain.

As a result of its neuroprotective properties, NHDC administration (40 and 80 mg/kg P.O.) has demonstrated a substantial effect on partial sciatic nerve ligation-induced neuropathic pain in rats, according to the current study.

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