Anthocyanin Derived from Purple Sweet Potato Water Extracts Ameliorated Oxidative Stress, Inflammation, Mechanical Allodynia, and Cold Allodynia among Chronic Constriction Injury-Induced Neuropathic Pain in Rats

I. Putu Eka Widyadharma1,2, Thomas Eko Purwata1,2, Dewa Nugrah Suprapta1,3, A. A. Raka Sudewi1,2

1Department of Neurology, Faculty of Medicine, Udayana University, Sanglah Hospital, Denpasar, Bali, Indonesia; 2Doctoral Program of Biomedical Science, Faculty of Medicine, Udayana University, Denpasar, Bali, Indonesia; 3Laboratory of Biopesticide, Faculty of Agriculture, Udayana University, Denpasar, Bali, Indonesia

Abstract

BACKGROUND: Nerve injury leads to multiple events, including oxidative stress, inflammation, and glial cells activation, which all bring about the neuropathic pain condition. Any alternative novel treatment modalities from natural products are thus highly anticipated, given the account that current treatments for neuropathic pain have not generated any satisfactory efficacy.

AIM: This study aims to address the pleiotropic effects of water extracts from the purple sweet potato (PSP) (Ipomoea batatas L.) as an antioxidant, anti-inflammatory, and anti-nociceptive agent to alleviate neuropathic pain behavior.

METHODS: This is a randomized post-test control group design using chronic constriction injury (CCI) rat models. Thirty-two Wistar rats (34 survived until the end of study period) fulfilling the eligible criteria were randomized into either treatment or control group. Treatment group received water extracts of PSP containing 400 mg/kg of body weight/d of anthocyanin for 28 days. Antioxidant activity was evaluated from malondialdehyde (MDA) levels, anti-inflammatory activity was evaluated from prostaglandin E2 (PGE2) levels. Neuropathic pain was assessed from the animal’s behavioral responses toward mechanical and cold allodynia.

RESULTS: The results showed that mean MDA levels of treatment group were significantly lower than control group (0.291 ± 0.046 µg/mL vs. 0.342 ± 0.096 µg/mL; p = 0.004). In addition, neuropathic pain behavior comprising mechanical and cold allodynia were significantly milder among treatment group than in the control group during the observation period (p < 0.05).

CONCLUSION: PSP water extracts had been shown to ameliorate oxidative stress, as well as exerted anti-inflammatory and anti-nociceptive effects and was able to suppress neuropathic pain behavior in Wistar rats with peripheral nerve injury.

Introduction

Pain, as defined by the International Association for the Study of Pain, is an unpleasant sensory and emotional experience resulting from real or potential tissue injury, or the depiction of its injury. Among various types of pain, neuropathic pain has become a major problem. It is highly prevalent due to its association with various diseases, it exerts negative impacts on physical and social function, thus decreasing patient’s quality of life [1].

Neuropathic pain is one of the global challenges. Almost 7–8% of adult general population suffering of chronic pain has neuropathic pain characteristics, while the number is significantly higher in Indonesia, comprising more than 20% from general population [2]. In Netherland, neuropathic pain incidences approximately 8 cases/1000 individual each year. A study conducted in German reported that 37% of patient who comes to visit primary health-care service has suffered low back pain [3]. Neuropathic pain exerts major impact on multiple aspects, affecting not only socio-economically but also emotionally. Chronic neuropathic pain poses a significant correlation with sleep disturbance, emotional, mood, physical function, and social interaction. The negative impact of neuropathic pain of all those aspects will result in depression and low quality of life [4], [5]. Current pharmacologic treatment such as opioids, antidepressants, anticonvulsants, topical analgesics, NMDA receptor antagonists, baclofen, local anesthetics, and clonidine has so far been unable to give satisfactory result [6].

The pathophysiology of neuropathic pain is complex and the entire mechanism is not yet understood [7]. The undertreated peripheral nerve injury...
will induce continuous inflammation and excitation of a primary nociceptive neuron, as a result, spinal cord neuron will be sensitized causing chronic pain. Nerve damage will stimulate the immune cascades as a response to inflammation and oxidative stress that plays an important role in initiating and maintaining acute or chronic neuropathic pain [8]. Glial cells also stimulated as a result of the undertreated nerve damage. Evidence showed that oxidative stress and cyclooxygenase-2 (COX-2) were contributed in the pathophysiology of neuropathic pain [9], [10]. Nerve damage will also initiate the formation of reactive oxygen species (ROS), reactive nitrogen species, and free radicals that induce the oxidative stress. Oxidative stress results from the imbalance between prooxidant and antioxidant. Antioxidants are available endogenously as a cellular defense mechanism or acquired exogenously from the diet [11].

In particular, microglia and astrocyte in the dorsal horn of spinal cord play an important role in chronic pain by several neuronal transmission processes including upregulation of pro-inflammatory factors. Activated microglia will show the increase of P2X4 and P2X7 receptors expression [12], [13]. After nerve damage, adenosine 5'-triphosphate (ATP) is released to extracellular space from undamaged neuron, damaged neuron, or dead neuron which acts as an important extracellular signal molecule. The rapid action of extracellular ATP is mainly mediated by purinergic P2X receptors which also act as a ligand-gated ion channel on the cell surface including microglia [14]. This pathway has a potential to be inhibited and can be considered in the prevention and management of neuropathic pain [12].

Anthocyanin is one of the flavonoids that have been known as the potent antioxidant [15]. Anthocyanin is a heterocyclic flavonoid that is composed of two or three moieties-aglycone anthocyanidins, sugar, and acyl acid. Anthocyanin mainly found in fruits but also available in vegetables, roots, tubers, legumes, and cereals [16]. Anthocyanin effect in pain management has generated satisfactory results. A previous in vitro study found that anthocyanin extracted from cherry tart (Prunus cerasus L.) has the ability as a potent anti-inflammatory and antioxidant compared to other drugs. Anthocyanin that was isolated from tart cherry significantly inhibits lipid peroxidase and shows anti-inflammatory activity [17]. As this previously described evidence of anthocyanin, the investigators want to conduct further study to evaluate the anthocyanin effects on inflammation and free radicals, including the characteristics of neuropathic pain caused by peripheral nerve damage. The aim of this study was to determine the effects of anthocyanin water extracts derived from Balinese cultivar of purple sweet potato (PSP) versus placebo on malondialdehyde (MDA) and prostaglandin E2 (PGE2) levels, and neuropathic pain-associated behavior among male Wistar rats with peripheral nerve injury as a result of chronic constriction injury (CCI).

### Methods

#### Study design

This was a randomized post-test only control group study. All rats initially underwent peripheral nerve injury induction by means of CCI before being randomly selected as subjects followed by random allocation to either the treatment or control group. Rats in the treatment group received 0.9% saline solution administered through nasogastric tube once a day for 28 days, whereas those in the control group received water extracts derived from PSP containing anthocyanin 140 mg/mL with equivalent dose of 400 mg/kg body weight/day also administered through nasogastric tube once a day for 28 days calculated anthocyanin dose was based on previous study by Tall et al. [16]. Neuropathic pain-associated behavior comprising mechanical allodynia and cold allodynia were evaluated before surgery for CCI, day 1, 7, 14, 21, and 28 post-surgery. After treatment period ended, all rats were euthanized by pentobarbital injection and collected for its blood, ligated peripheral nerve, and spinal cord segment L4-6. The samples were then measured for serum MDA and peripheral nerve PGE2 levels. Ultimately, all data were analyzed statistically.

#### Animals

As many as, 18 Wistar rats with the following criteria were allocated for each group: (1) Wistar albino rat, (2) male sex, (3) 8–9 weeks old, (4) weighing 200–300 g, (5) derived from the same parental lineage, and (6) able to eat and drink normally. All rats were derived from the Laboratory of Pharmacology Faculty of Medicine Udayana University. Rats which fulfilled the aforementioned inclusion criteria were randomly allocated to either treatment or control group, preceded by randomization procedure. All rats were placed in an individual cage made of PVC measuring 40 × 30 × 20 cm with dry hay placed as floor mat, maintained on a standard light cycle (lights on between 800 and 2000 h), had free access to food and water. Cage's wall and floor were constantly inspected for its sanitariness and floor mat was replaced at least once a week. All rats were handled with care by professional personnel who were responsible for organizing and documenting job description and schedule, amount of food and liquid consumed, and weighing the rats. Rats were left on the cage for 1 week before CCI procedure was conducted. All experimental procedures were approved by the Ethical Commission and Scientific Coordinator for Healthcare Research Faculty of Medicine Udayana University (ethical approval number: 1619/UN.14.2/KEP/2017) and were conducted in compliance with the WMA Statement on Animal Use in Biomedical Research.
Udayana University by the following steps. Fresh PSPs obtained from farmers were rinsed until clean and peeled. It then was sliced transversely with a thickness of 2–2.5 cm and mixed with clean water with a ratio of 1 kg of PSP to 1 L of water. The mixture was then blended using a juicer and filtered by three-layered cotton filter. The filtered solution was boiled for 30 min for sterilization. The anthocyanin content derived from the corresponding solution was 140.23–147.0 ng/mL as measured using spectrophotometry and stated formally in a letter numbered 145/AN/Lab/FTP/XII/2015 [18]. PSP water extract with anthocyanin dose of 400 mg/kg body weight was given immediately after the CCI surgical procedure.

Neuropathic pain-associated behaviors evaluation

Neuropathic pain-associated behaviors were assessed based on the evaluation of mechanical allodynia and cold allodynia.

Mechanical allodynia

The evaluation was conducted before surgery, days 1, 7, 14, 21, and 28 post-CCI induction. Mechanical allodynia was performed as followed. Rats were placed on a 20 × 20 × 40 cm plexiglass with square pattern metal wire (Figure 1c). Rats were given 15 min free time for the purpose of adaptation and grooming. Mechanical allodynia was evaluated using Von Frey monofilament 4.0, 6.0, 8.0, 10.0, 15.0, and 26.0 g. Monofilament was applied vertically on the rat's plantar surface at the affected CCI limb, beginning with the smallest to the biggest monofilament size until leg withdrawal was observed (Figure 1d). The result was positive if there was a visible leg withdrawal. The test was repeated for 5 times for each of the filament with an interval of 2–3 s. Paw withdrawal threshold was positive if rats responded minimally three out of five examinations. Monofilament use was limited to 60 g to prevent injury) [19].

Cold allodynia

Cold allodynia was evaluated using acetone drops test. The affected CCI limb was sprayed using 0.1 mL acetone by means of a plastic syringe. In rats with no nerve injury, there should be omission of the stimuli or mild and transient paw withdrawal. However, abnormal responses including licking, shivering, or rubbing of the affected limb and the duration between the first time rat’s limb was sprayed and the observable abnormal response(s) (also called paw withdrawal latency) was recorded. Test was limited to 20 s to prevent tissue injury [20].
**Euthanasia**

Rats were euthanized on day 28 post-CCI through lethal dose of pentobarbital (100 mg/kgBW) ip. Subsequently, all samples were collected for its blood and ligated peripheral nerve to be analyzed in the laboratory.

**MDA levels measurement**

MDA levels were measured using TBARS method. Collected blood was initially mixed with EDTA to prevent coagulation. The blood was centrifuged at 2000×g for 10 min on temperature of 4°C. Plasma layer was extracted deliberately and kept on ice if done on the same day or freezed to −70°C if to be kept for up to 30 days. Plasma can be measured without dilution. Each of the specific reagents was added to the glass tube filled with plasma and mixed well, waited for 20 min at room temperature to let the chemical reaction occurs. 1.5 mL of solution was used for reading at 532 nm. Pink color was stable for several hours at room temperature. Total MDA levels were measured by heating the solution at 63°C for 30 min according to standard curve (Oxford Biomedical Research).

**PGE2 levels measurement**

The samples from peripheral nerve was immediately stored at 20°C until examination was performed. PGE2 levels were measured based on ELISA method using PGE2 High Sensitivity Immunoassay Kit from R&D Systems (Minneapolis, MN, USA). All reagents and samples were collected under room temperature before usage. All samples and standards were recommended to be tested under duplication. Total activity tube (TA), non-specific binding (NSB), maximum binding (B0), and empty substrate must be run on every tests as quality controls for every test. All reagents, standard procedure, and samples were prepared. All microplate strips in excess were prepared. All microplate strips in excess were discarded, put into the foil with drying package, and resealed. Tubes for TA and empty substrate were stored. 150 µL of test buffer solution (CCM) was added on NSB tube, 100 µL of test buffer solution (or CCM) was added on zero standard tube (B0), and 100 µL of standard or sample solution was added to the remaining tubes. 50 µL of PGE2 HS conjugate was then added on every tubes (excluding TA and blank substrate tube), followed with 50 µL of PGE2 HS antibody solution on each tube (excluding NSB, TA, and blank substrate tube). All tubes were sealed with the pre-existing paper and incubated for 18–24 h at 2–8°C. The solution was aspirated and tubes were rinsed with rinse buffer solution (400 µL) through sprayed bottle or dispenser manifold, repeated twice with total three rinses. Complete rinse on each step is necessary to ensure good results. After the last rinse, rinse buffer was discarded with aspiration or pouring out. 5 µL of PGE2 HS conjugate was added on TA tube. 200 µL of pNPP substrate was added on all tubes and sealed with pre-existing paper, incubated for an hour with temperature 37°C. 200 µL of stop solution was subsequently added to all tubes. Optical density on each tube was measured using micro template adjusted at 405 nm wavelength with correction between 570 and 590 nm.

**Statistical analysis**

Data analyses were performed by the following steps. Descriptive statistics was used to describe baseline characteristics of the two groups and its frequency distribution for every variable. Normality test was done using Shapiro–Wilk test due to the relatively small sample size. p value of more than 0.05 was defined as normally distributed, and vice versa. The difference between MDA and PGE2 levels, and neuropathic pain behavior on both groups was tested using independent t-test when data were normally distributed or Mann–Whitney test on abnormally distributed data. p<0.05 was deemed statistically significant. Data were gathered from 34 samples which survived until the end of observation period (day 28). One sample from each group was excluded because of outlier data. All data from 32 samples were statistically analyzed.

**Results**

**Surgical procedure for CCI induction**

This study was conducted between September 2017 and May 2018 at the Laboratory of Pharmacology, Integrated Biomedical Laboratory, and Anatomical Pathology Department from Faculty of Medicine Udayana University, Denpasar. All rats underwent surgical procedure for CCI induction as a form of peripheral nerve injury. Four loose ligations were done on the sciatic nerve (Figure 1a). CCI was deemed successful if the affected limb demonstrated genu articular flexion with toes abduction (Figure 1b).

**Rats follow-up**

As many as, 18 rats were allocated to each group (treatment and control) and each rat was placed on an individual case as previously described. One rat from treatment and control group died 10 and 6 days after CCI, respectively, leaving a total of 34 samples from treatment and control group. Data were gathered from 34 samples which survived until the end of observation period (day 28). One sample from each group was excluded because of outlier data. All data from 32 samples were statistically analyzed.
mechanical allodynia from both treatment and control group showed abnormal data distribution (p < 0.05), while cold allodynia in treatment and control group showed normal data distribution (p > 0.05).

**MDA levels were significantly lower in the treatment versus control group**

In the treatment group, the mean serum MDA levels were $0.110 \pm 0.042 \, \mu\text{mol}$ lower than in the control group, i.e., $0.226 \pm 0.032 \, \mu\text{mol/L}$. There was a significant difference in mean MDA content of $0.116 \, \mu\text{mol}$ in both groups (p < 0.001) (Table 1). These results indicated that PSP water extracts lowered oxidative stress in the treatment group compared to the control group after peripheral nerve injury.

**PGE2 levels were significantly lower in the treatment versus control group**

PGE2 levels on the ligated sciatic nerve of treatment group were lower than that of control. In the treatment group, mean PGE2 levels were $0.342 \pm 0.096 \, \text{ng/mL}$ lower than control group, i.e., $0.431 \pm 0.061 \, \text{ng/mL}$. There was a significant difference of $0.089 \, \text{ng/mL}$ in both groups (p < 0.05) (Table 1). These results indicated that sweet potato water extracts lowered the degree of inflammation among treatment group as opposed to control group after peripheral nerve injury.

**Mechanical allodynia was significantly ameliorated among treatment versus control group on day 7 thereafter**

The observations of mechanical allodynia behavior were performed before the surgical procedure for CCI induction, on days 1, 7, 14, 21, and 28 post-surgery. Normality test showed that mechanical allodynia was not normally distributed, thus Mann–Whitney test was used. Mechanical allodynia did not differ significantly before CCI procedure and shortly thereafter. However, treatment group was shown to have milder symptom after day 7 as marked by the ability to tolerate larger Von Frey monofilament when compared with control group (26 g vs. 10 g) with statistical significance (p < 0.001). This was also seen on day 14, wherein median von frey monofilament on treatment group was significantly larger than control (15 g vs. 10 g) with statistical significance (p < 0.001). On day 21, rats in the treatment group still able to tolerate larger von frey monofilament when compared with control group (10 g vs. 8 g) with statistical significance (p < 0.005). The effect persisted even until day 28, in which treatment group was able to tolerate larger monofilament (15 g vs. 10 g) with statistical significance (p < 0.001).

On day 7 post-surgery, monofilament size in both groups was similarly decreased, whereas median monofilament size in treatment group was significantly greater than control. This phenomenon continued until day 14 (p < 0.001) (Figure 2a). On day 21, decrease in mean monofilament size still occurred in both groups with significant difference although the mean size reduction was not as high as observed on day 7 and day 14 (p < 0.05). In addition, there was no observable decrease of monofilament size on day 28 in the treatment group, while in the control group there was a decrease in mean monofilament size and this difference was statistically significant (p < 0.001).

| Parameter                      | Groups   | Mean ± SD       | Range (min–max) | Mean difference | 95% CI (min–max) | p value  |
|-------------------------------|----------|-----------------|-----------------|-----------------|-----------------|----------|
| MDA serum levels (µmol)       | Treatment| 0.291 ± 0.046   | 0.206 – 0.365   | 0.102           | 0.065 – 0.140   | < 0.001* |
|                              | Control  | 0.394 ± 0.057   | 0.325 – 0.515   |                 |                 |          |
| Peripheral nerve’s PGE2 levels (ng/mL) | Treatment | 0.342 ± 0.096 | 0.141 – 0.466 | 0.089           | 0.031 – 0.147   | 0.004*   |
|                              | Control  | 0.431 ± 0.061   | 0.278 – 0.528   |                 |                 |          |

*Significant at p<0.05. MDA: Malondialdehyde, PGE2: Prostaglandin E2, CI: Confidence interval.

All of these results indicated that mechanical allodynia behavior can readily be assessed since day 7 post-CCI induction and persisted until day 28. PSP water extracts administration was proven to ameliorate mechanical allodynia behavior characterized by greater reduction of average monofilament size than control group.
Cold allodynia was significantly ameliorated among treatment versus control group on day 1 thereafter

Before CCI procedure, there was no significant difference of paw withdrawal latency between treatment and control group. However, cold allodynia was significantly ameliorated as early as day 1 among treatment when compared with control group as shown by greater paw withdrawal latency among the former group (6.51 ± 0.15 vs. 6.11 ± 0.18, respectively), thus indicating that treatment subjects were able to better withstand cold exposure than of control group (Figure 2b). The difference persisted to days 7, 14, 21, and 28 after CCI with treatment group demonstrating consistently greater latency time than those of control group with statistical significance.

The average paw withdrawal latency on day 1 after CCI in both groups altogether showed a decrease value, although the amount was greater significantly on the treatment group than control. The difference persisted on days 7, 14, 21, and 28 with statistical significance (p < 0.001). Paw withdrawal latency in both groups was similarly decreased, where the mean time of withdrawal in the treatment group was significantly greater than the control. These results indicated that cold allodynia behavior was readily observable from day 1 to day 28 post-CCI. It can be concluded, therefore, that the administration of PSP water extracts among treatment group ameliorated cold allodynia behavior to a greater extent than the placebo.

Discussion

Anthocyanin had been extensively studied for its antioxidant properties. However, to the best of our knowledge, there has been no study using anthocyanin derived from PSP to suppress oxidative stress after peripheral nerve injury. Among the neuropathic pain models, the CCI model has been widely used because it produces tactile alodinia that resembles observable conditions in patients with neuropathic pain [21]. CCI caused significant oxidative damage to the sciatic nerve with indicators of increased MDA and nitric oxide (NO) concentrations and reduction in GSH levels, besides inciting inflammation through increasing pro-inflammatory cytokines production and secretion (including interleukin [IL]-6, IL-1β, and tumor necrosis factor-alpha) [22].

It is clear that ROS is involved in the formation and maintenance of neuropathic pain [23]. It was demonstrated herein that the administration of anthocyanin after peripheral nerve injury was able to reduce oxidative stress as indicated by marked reduction of MDA levels. This result was similar to previous study which linked the use of anthocyanin from various natural sources to suppress oxidative stress (Jawi, 2008; Bakar, 2010) [24], [25]. A study by Kaulaskar et al. [23] showed that the administration of naringenin with doses of 25 and 50 mg/kg on rats induced with CCI was able to reduce MDA levels and increase antioxidant GSH. The exerts its antioxidant effects through several mechanisms, i.e.: (1) To prevent the formation of free radicals by inhibiting the enzyme xanthine oxidase and metal transition; (2) inhibits free radicals which damage target cells (donates electrons and captures free radicals); (3) inhibit the propagation of oxidative reactions (chain breaking antioxidant); and (4) reinforces the cell's antioxidant capacity (sparing effect) and induces the expression of endogenous antioxidants [26].

We also found that anthocyanin markedly lower PGE2 levels as one of the important indicators for inflammation. Anthocyanins have consistently been shown to have anti-inflammatory properties, proven by their ability to: (1) Decrease concentration and expression of pro-inflammatory mediators while increasing anti-inflammatory molecules; (2) diluting inducible nitric oxide synthase (iNOS) activity and thus decrease the expression of NO; and (3) reduces COX-2 activity and thus decreases PGE2 expression. Most of these effects were related to inhibition of translocation and activation of transcription factors which play a key role in the initiation and maintenance of inflammation (Vendrame and Klimis-Zacas, 2015) [27]. In fact, the previous study has robustly demonstrated the anti-inflammatory effects modulated by anthocyanin, including inhibition of pro-inflammatory enzymes (e.g., COX-2, lipoxygenase, and iNOS) as well as inhibition of various transcription factors (mitogen-activated protein kinase, protein kinase C, and nuclear factor kappa B) [28].

PGE2 is well-associated in the pathogenesis of peripheral nerve injury and neuropathic pain. For example, PGE2 and its receptors (EP1 and EP4) were shown to be upregulated 2–4 weeks after peripheral nerve injury in animal model [29]. It was also able to increase peripheral nociceptor response through binding with TRPV1 and SCN10A receptors, those of which activated leads to an increase of peripheral nociceptors sensitization and triggers nociceptive impulse propagation along the peripheral nerve [30]. The reduction of PGE2 levels by means of anthocyanin could, therefore, serve as a potential treatment avenue to inhibit peripheral nerve sensitization and ultimately minimizing central sensitization.

The administration of anthocyanin was also proven to significantly ameliorate peripheral nerve injury-induced neuropathic pain behavior of all qualities, i.e., mechanical alldynia and cold alldynia. Indeed, this study confirmed the previous findings in which anthocyanin derived from tart cherry with dose of 400 mg/kg body weight was able to suppress inflammation-induced mechanical hyperalgesia [16].
The exact mechanisms underlying these findings have not been fully elucidated, but it is prudent to assume that it was at least in part due to its antioxidant and anti-inflammatory properties. It is congruent with current knowledge of the pathogenesis of neuropathic pain, in which inflammation, nerve growth factors, and other algogenic substances on the damaged nerves contribute to synaptic and neuronal plasticity, all of which leads to neuronal hyperexcitability and neuropathic pain [31].

Cold allodynia is a frequent clinical complaint in patients with neuropathic pain. The mechanism underlying the occurrence of cold alodinia is still not fully known. A study showed a significant decrease in foot withdrawal time on cold metal plate. Activation of C nerve fibers mediates the occurrence of cold alodinia [32]. A recent study by Lippoldt et al. [33] proves that cold allodynia induced by inflammation, neurological injury, and chemotherapy was exclusively mediated by a molecular pathway that neutralizes antibodies against the glial cell line-derived neurotrophic factor family of receptors (GFRα3).

This study proved that the water extracts of Balinese cultivar of PSP with anthocyanin content of 400 mg/kg BW/day can suppress the neuropathic pain behavior compared to control in CCI-induced neuropathic pain model in rats.

Conclusion

Anthocyanin derived from water extracts of Balinese cultivar of PSP in a dose of 400 mg/kg BW/day was significantly able to suppress free radicals (reduced MDA levels), inflammation (PGE2 levels), and peripheral nerve injury-induced neuropathic pain behaviors (mechanical allodynia and cold allodynia). This study merits further investigation in the use of anthocyanin for ameliorating neuropathic pain, potentially as an adjunctive treatment incorporated into a clinical trial.

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