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Phillygenin Ameliorates Neuropathic Pain by Inhibiting TLR4/MyD88/NF-κB Pathway in Rats

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WZ conceived and designed experiments. LH analysed data, performed experiments and wrote the manuscript. LH, WZ, ML and RL provided technical support, data collection and analysis. All authors approved the final manuscript.

Key words
Neuropathic pain, Phillygenin, Pro-inflammatory cytokines, TLR4, MyD88

ABSTRACT

The objective was to elucidate the effects of phillygenin (PHI) and its potential mechanism involving TLR4 and MyD88/NF-κB signaling in neuropathic pain. Male Sprague-Dawley rats were obtained for animal studies and neuropathic pain was induced by constructing chronic constriction injury (CCI) models. PHI (20 mg/kg) was treated through intragastric administration. Von Frey Test and hot plate assay were implemented for determining 50% paw-withdrawal threshold (PWT) and paw-withdrawal latency (PWL). Nitric oxide (NO) levels were detected through the NO assay, and pro-inflammatory cytokine expression was measured using ELISA. Western blotting and RT-qPCR was conducted for protein and mRNA level detection, respectively. Treatment with PHI dramatically enhanced 50% PWT and PWL. Moreover, PHI demonstrated a considerable reduction in NO levels and decreased the expression of TNF-α, IL-1β, and IL-6, which are pro-inflammatory cytokines. PHI also downregulated TLR4 and MyD88 expression, and inhibited the phosphorylation of NF-κB. To conclude PHI ameliorated inflammatory status and alleviated neuropathic pain in CCI rats through downregulating targeting TLR4 and suppressed MyD88/NF-κB signaling.

INTRODUCTION

Neuropathic pain is a chronic condition that affects a significant number of individuals worldwide, causing physical, emotional, and economic burdens (Finnerup *et al*. 2021). It arises due to the dysfunction occurred in nervous system, causing abnormal signaling and processing of pain signals (Rosenberger *et al*., 2020). Patients with neuropathic pain experience various symptoms such as shooting or burning pain, numbness, tingling, and hypersensitivity to stimuli (St John Smith, 2018). Various factors can cause neuropathic pain, including injury, infection, diabetes, or chemotherapy. This condition can bring heavy burdens to the patients, and the current treatments for neuropathic pain are often limited in efficacy and associated with adverse effects (Park and Park, 2017). Therefore, there is an urgent need for the development of alternative treatment options for neuropathic pain.

Toll-like receptor 4 (TLR4) belongs to the TLR family and plays a role in initiating the innate immune response (Liu *et al*., 2022a). TLR4 has been found in various cells, which included immune cells and neurons. It was reported that TLR4 could be activated by lipopolysaccharides (LPS) and damage-associated molecular patterns (DAMPs) (Bolourani *et al*., 2021). Activation of TLR4 leads to the upregulation on myeloid differentiation factor 88 (MyD88). Furthermore, activated TLR4 can activate the nuclear factor-κB (NF-κB) pathway, resulting in the upregulation of various inflammatory factors and the production of chemokines (Zhou *et al*., 2017). TLR4 participates during the occurrence and progression of neuropathic pain. Publications have shown that TLR4 was increased in the spinal cord when neuropathic pain was induced in animal models (Xu *et al*., 2020), and mice deficient in TLR4 exhibit reduced pain behavior in various neuropathic pain models (Cao *et al*., 2009). Therefore, implementation of TLR4 antagonists or inhibitors might have analgesic effects in neuropathic pain treatment.

Phillygenin (PHI) is a bioactive compound found in Forsythia suspensa, and traditionally used for the treatment of inflammatory and infectious diseases (Liu *et al*., 2022b). PHI possesses a wide range of bioactive properties, including improving inflammatory status, alleviating cancer progression, and protecting neuron

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injury (Wang *et al*., 2021). These diverse capabilities are attributed to the modulation of various cellular processes, such as NF-κB-related inflammation and PI3K/Akt-mediated proliferation of tumor cells (Wang *et al*., 2022). Recent research indicated that PHI could suppress TLR4 activation, which further inhibited downstream NF-κB signaling pathway, suggesting its potential as a treatment target for the therapy of inflammatory and neuropathic conditions (Xue *et al*., 2023). However, the mechanism of action underlying the potential use of PHI in treating neuropathic pain remains unclear.

The present study aimed to investigate the potential effects of PHI on neuropathic pain progression and elucidate its underlying mechanism. To accomplish this, we utilized rat models of chronic constriction injury (CCI) to simulate neuropathic pain in vivo. Our hypothesis posited that treatment with PHI would significantly reduce TLR4 expression and suppress the MyD88/NF-κB signaling pathway, thereby mitigating neuropathic pain behaviors in CCI rats. Through this research, we aim to provide novel insights into the potential clinical application of PHI for the treatment of neuropathic pain.

MATERIALS AND METHODS

Animal models

Male Sprague-Dawley rats (210-250 g) were purchased from the XXXXXX. All rats were kept in individual cages with a 12 h light/dark cycle, constant temperature (22±2°C) and humidity (50±10%), and food and water were free available. The acclimatization of animals (at least 7 days) was performed before they underwent various treatments and experiments. All animal experiments were reviewed and authorized by the Animal Care and Use Committee of the XXXXXX.

Then CCI model was established as per a previous study (Bennett and Xie, 1988). During surgery, the rats received continuous anesthesia with 2% isoflurane in oxygen, and the explosion of left sciatic nerve was made at the mid-thigh level. Subsequently, four 4-0 chromic gut was used as loose ligatures for making ties around the nerve with a 1-mm interval. The tightened ligatures were further made to produce a mild nerve constriction, which induced the CCI condition. Layers of 4-0 silk sutures were used to close the wound, and all rats were then kept in a warm environment for recovering. Rats that exhibited motor dysfunction or showed signs of infection were excluded from the study. A total of 40 rats were then randomly allocated into 4 groups (10 rats in each): (1) Sham group: rats underwent the surgical processes as the CCI group but nerve ligation was not made; (2) CCI group, where CCI surgery were implemented; (3) CCI+PHI group, where CCI surgery were implemented and rats received daily intragastric administration of PHI (20 mg/kg) for 14 consecutive days; (4) CCI+NC group, where CCI surgery were implemented and rats were treated with the same volume of normal saline. After model establishment, the rats were collected for subsequent experiments.

Von frey test

Von Frey assay (Ugo Basile, Italy) were used to assess mechanical allodynia before surgery and on postoperative days 0, 2, 4, 6, 8 10, 12 and 14. All rats were maintained in boxes that contained a wire mesh bottom and acclimatized for 30 min. After acclimation, and then the von Frey test was conducted to the midplantar surface of the ipsilateral and contralateral hind paw. Cut-off was set at 26 g and the data were collected automatically. Pain-like responses, such as an abrupt withdrawal of the paw, licking, or vigorously shaking, were noted. The 50% paw withdrawal threshold (PWT) was determined through the up down approach as described in a previous study (Li *et al*., 2019).

Hot plate assay

To assess thermal hyperalgesia, hot plate assay was performed on rats before surgery and on postoperative days 0, 2, 4, 6, 8, 10, 12, and 14. The Plantar Test Apparatus (Ugo Basile, Italy) was obtained for this purpose. Totally 30 min of acclimatization were firstly made. The mid-plantar surface of the right hind paw was treated by a radiant heat source, and the paw withdrawal latency (PWL) was recorded. To prevent injury, a 20-second cutoff threshold was set for the heat stimulation test. A decrease in PWL was considered a sign of heat hyperalgesia. The behavioral tests were accomplished by experimenters who were blinded to the group assignments (Li *et al*., 2019).

Nitric oxide (NO) assay

The expression of NO was detected using a 2,3-diaminonaphthalene (DAN) assay kit. First, the tissue samples were isolated and homogenized in PBS at 4 °C, and then underwent centrifugation at 4 °C for 15 min (12,000 rpm). Next, the supernatant was obtained, and 50 μL of the sample and 50 μL of DAN solution (5 mM in 0.62 M HCl) were added and maintained for 15 min at room temperature. Afterward, NaOH (100 μL, 2 M) was used to terminate the reaction. Using a fluorescence spectrophotometer, the intensity was measured (excitation wavelength: 365 nm, emission wavelength: 450 nm).

ELISA assay

The expression of TNF-α, IL-1β, and IL-6 were monitored using commercial ELISA kit (R and D Systems, USA), following the protocol of manufacturer. Briefly,

the spinal cord tissue samples were obtained as described above. Then, totally 200 μL of detection reagent was added and maintained for 2 h, after which 200 μL of substrate solution was added and maintained for 1 h in the dark. For terminating the reaction, a total of 50 μL stop solution was added, and the absorbance was monitored through a microplate reader (450 nm).

RT-qPCR

TRIzol reagent (Invitrogen, USA) was obtained to isolate total RNA from the spinal cord tissues, following the protocol of manufacturer. Then, total RNA was reverse transcribed into cDNA using a Prime Script RT Reagent Kit (Takara Bio, Japan). The expression levels of mRNAs were quantified using a SYBR Premix kit (Takara Bio, Japan) and a Step One Plus Real-Time PCR system (Applied Biosystems, USA). The mRNA levels were normalized to that of GAPDH, and the calculation was performed as per the $2^{-\Delta\Delta Ct}$ method. The primers used are listed as follows:

TLR4-F: 5'-GAATGCTAAGGTTGGCACTCTC -3'
5'-CTCAGGCAGGAAAGGAACAATG-3'
MyD88-F: 5'-GCTGAGAGGAAGAGTTCTAC-3'
5'-CAGTGATAACCCTGGACTAC -3'
NF-κB: 5'-AGACCTGGAGCAAGCCATTAG -3'
5'-CGGACCGCATTCAAGTCATAG-3'
GAPDH: 5'-TTCAACGGCACAGTCAAGG-3'
5'- GTCTTCTGAGTGGCAGTGATG -3'

Western blotting

For western blot analysis, spinal cord tissue samples underwent homogenization in RIPA buffer (Roche, Switzerland), which was added with protease inhibitor cocktail. Then quantification was performed via BCA Protein Assay kit (Thermo Scientific, USA). After separation on 10% SDS-PAGE, proteins were transferred to PVDF membranes (Millipore, USA). Then 5% non-fat milk was obtained for blocking. Subsequently, the membranes were incubated with primary antibodies against TLR4 (ab22048, Abcam, 1:1000), MyD88 (ab219413, Abcam, 1:1000), p-NF-κB (#3039, CST, 1:1000), NF-κB (#6956, CST, 1:1000), and β-actin (ab8226, Abcam, 1:1000) overnight at 4°C. The membranes were further incubated with secondary antibodies (1:1000) for 2 h at room temperature. The protein levels of target genes were quantified by visualizing protein bands using enhanced chemiluminescence reagents (Millipore, USA) and analyzed through ImageJ (National Institutes of Health, USA). Normalization to β-actin expression levels was performed.

Statistical analysis

All data were presented as means ± standard deviation (SD) and analyzed using GraphPad Prism 8. One-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used to determine the statistical significance of differences among multiple groups. $P < 0.05$ was considered statistically significant.

RESULTS

The behavioral test data presented in Figure 1A, B demonstrated that rats in the CCI model group exhibited significantly lower 50% PWT and PWL compared to the Sham group. However, when compared with CCI rats, the additional administration of PHI resulted in a significant improvement in both the 50% PWT and PWL. Moreover, implementing NO assay and ELISA assay (Fig. 2) revealed that the levels of NO, and inflammatory cytokines including TNF-α, IL-1β, and IL-6 were markedly elevated after the establishment of the CCI model, whereas usage of PHI significantly reduced these expression levels. Western blotting and RT-qPCR (Fig. 3A, B) results showed that the levels of TLR4, MyD88, and p-NF-κB were dramatically increased by CCI induction, while treatment with PHI significantly reversed the change in these expression levels. Overall, the above results suggested that PHI administration might ameliorate neuropathic pain in rats induced by CCI, potentially through downregulation of the TLR4, inhibition of MyD88/NF-κB signaling and reduction of pro-inflammatory cytokines.

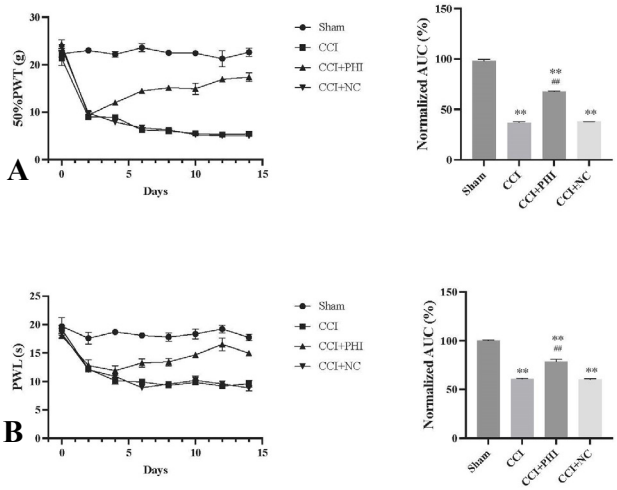

Fig. 1. PHI attenuated mechanical allodynia and thermal hyperalgesia in CCI rats. (A) Time course effect of the PHI treatment on mechanical allodynia of CCI rats and 50% PWT was determined. (B) Time course effect of the PHI treatment on thermal hyperalgesia of CCI rats and PWL was determined. **P<0.01 vs Sham group. ##P<0.01 vs CCI group.

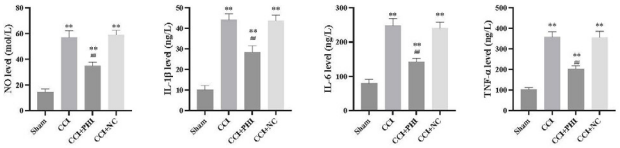

Fig. 2. PHI reduced inflammation status in CCI rats. The NO, TNF-α, IL-1β, and IL-6 expression measured after PHI treatments in CCI rats. **P<0.01 vs Sham group. ##P<0.01 vs CCI group.

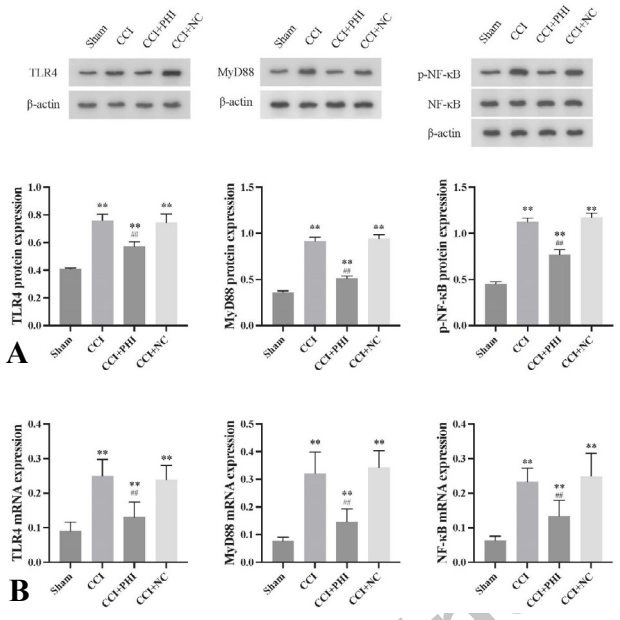

Fig. 3. PHI inhibited TLR4/MyD88/NF-κB pathway in CCI rats. (A) The protein expression of TLR4, MyD88, and p-NF-κB/NF-κB determined by western blotting. (B) The mRNA expression of TLR4 and MyD88 detected by RT-qPCR. **P<0.01 vs Sham group. ##P<0.01 vs CCI group.

DISCUSSION

Recent studies have shown that neuropathic pain is linked to an increase in inflammatory factors and TLR4 expression (Li *et al*., 2019). TLR4 signaling pathway activation was reported to mediate neuropathic pain and increase inflammatory response (Navia-Pelaez *et al*., 2021). Another study suggested that the increased TLR4 level was linked to partial sciatic nerve ligation mediated neuropathic pain (Jin *et al*., 2021). Furthermore, Zhang *et al*. (2020) found that opioid receptor agonists non-stereo-selectively activated the TLR4 expression and raised the levels of TNF-α, IL-1β, and IL-6, leading to neuroinflammation. Li *et al*. (2019) reported that TLR4 and MyD88 levels were enhanced in the ganglion of rats when treated with paclitaxel, resulting in significant reductions in their 50% PWT and PWL. Similarly, we showed that the 50% PWT and PWL in CCI rats were dramatically increased. Our results suggested that the level of TLR4 and inflammatory response in CCI rats was upregulated, indicating that targeting TLR4 might alleviate neuropathic pain. Regarding non-surgical treatments for neuropathic pain, the pharmacological guidelines primarily focus on symptom management. First-line therapy typically involves the use of drugs such as tricyclic antidepressants or serotonin-norepinephrine reuptake inhibitors. For second-line therapy, opioids or lidocaine are commonly prescribed. However, it is worth noting that adverse effects such as vomiting, constipation, and ataxia are frequently reported (Cavalli *et al*., 2019). Therefore, there is an urgent need for new drugs that possess fewer adverse effects to effectively address neuropathic pain.

PHI has been found to participated in various aspects of inflammatory status relief and in reducing TLR4 expression. For instance, a study showed that administration of PHI inhibited the LPS-induced inflammatory status and apoptosis in BEAS-2B cells, which subsequently activated PPAR-γ signaling via downregulating MMP8 and alleviated acute lung injury (Lin and Yang, 2021). Additionally, a previous study found that dietary PHI supplementation reduced malondialdehyde and inflammatory mediator production, increased antioxidant enzyme contents and Bcl-2 level, and ameliorated aflatoxin B1-induced liver damage (Guo *et al*., 2022). Xue *et al*. (2023) found that PHI reversed the expression of SOD, and MDA and downregulated the levels of TNF-α, IL-1β, IL-6, and IL-10 in colitis mice, and reduced the proportion of tyrosine kinase Src activated by TLR4, suggesting that PHI might be a potential drug candidate that could effectively safeguard against colitis. Similarly, our study newly demonstrated that PHI played a beneficial role in reducing the inflammation in CCI rats and might be an alternative therapy strategy for neuropathic pain.

Previous publications have investigated the role of the TLR4 and its down streaming MyD88/NF-κB signaling in inflammation and neuropathic pain. Wang *et al*. (2020) indicated that the upregulation of the TLR4 and MyD88/NF-κB signaling was participated in the development of inflammation and contributed to vascular dementia. MicroRNA-27a was found to modulate the TLR4 and MyD88/NF-κB signaling, which further decreased pro-inflammatory cytokines levels and ameliorated acute lung injury (Ju *et al*., 2018). Liu *et al*. (2018) suggested that activating the GABA receptor might inhibit the TLR4 and MyD88/NF-κB signaling and ameliorate the progression of diabetic neuropathic pain. Our results indicate that PHI influences the TLR4 expression and inhibits MyD88/NF-

κB signaling, highlighting its crucial role in neuropathic pain.

In summary, our study demonstrated that PHI treatment could reduce TLR4 level and attenuated the inflammatory status in CCI rats, indicating that targeting the TLR4 and its down streaming MyD88/NF-κB signaling might be a viable therapeutic strategy for neuropathic pain. However, it is important to acknowledge the limitations of our study. Firstly, the experiment was conducted solely on CCI rats, and the results may not directly translate to other animal models or human patients. Further studies involving diverse models and human subjects are necessary to validate the effectiveness of PHI treatment. Secondly, our study primarily focused on the TLR4 pathway and its associated signaling cascade. The complex nature of neuropathic pain involves multiple pathways and molecular mechanisms. Therefore, it is crucial to explore other inflammatory factors and signaling pathways involved in neuropathic pain to obtain a more comprehensive understanding of the therapeutic effects of PHI.

DECLARATIONS

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IRB approval

All animal experiments were reviewed and authorized by the Animal Care and Use Committee of the Wuhan Fourth Hospital

Ethics statement

The ethic approval was obtained from the Ethic Committee of Wuhan Fouth Hospital.

Statement of conflicts of interest

The authors have declared no conflict of interest.

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