Original Research Article

Linarin attenuates oxaliplatin-induced neuropathic pain by inhibiting NF-κB/NLRP3 signaling pathway

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Abstract

Purpose: *To assess the therapeutic effects of linarin on chemotherapy-induced peripheral neuropathy (CINP) in rats.*

Methods: *A CINP rat model was established using oxaliplatin. The rats were divided into control, CINP, and two linarin treatment groups (20 mg/kg/day and 40 mg/kg/day). Observations were made at various time points, assessing weight gain, mechanical withdrawal thresholds, cold allodynia response, and thermal pain sensitivity. Additionally, the expression levels of various inflammatory factors (IL-1β, IL-6, IL-10, and IL-17), proteins related to glial and neuronal activation (IBA-1, GFAP, c-fos), and proteins linked to NF-κB/NLRP3 signaling (ASC, caspase-1, p65, and p-p65) were evaluated in rat spinal cord tissue.*

Results: *Linarin treatment resulted in improved weight gain, mechanical threshold, decreased withdrawal response, and enhanced paw withdrawal latency (p < 0.001) compared to the CINP group. These improvements or mitigations were more pronounced in the 40 mg/kg/day linarin group. Linarin inhibited the expression of inflammatory factors IL-1β, IL-6, and IL-17 (p < 0.001) but enhanced IL-10 expression (p < 0.001). The activation of microglia, astrocytes, and neurons, as indicated by IBA-1, GFAP, and c-fos (p < 0.001) proteins, was significantly reduced with linarin, especially at the higher dose. Linarin also suppressed the expression of ASC, caspase-1, p65, and p-p65 (p < 0.001) proteins, associated with the NF-κB/NLRP3 signaling pathway.*

Conclusion: *Our study indicates that linarin may serve as a potential therapeutic agent for managing CINP. The beneficial effects of linarin are likely mediated through its immunomodulatory effects and the inhibition of the NF-κB/NLRP3 signaling pathway. Further research is needed to confirm these findings in clinical settings.*

Keywords: *Oxaliplatin, Linarin, NF-kappa B; NLRP3 protein, Neurotoxicity, Neuropathic pain*

INTRODUCTION

The morbidity and mortality of gastrointestinal cancer in China remains high [1]. Although conventional therapeutic drugs are effective, they have serious side effects [2]. One of such drugs, oxaliplatin, causes nerve pain (chemotherapy-induced neuropathic pain, CINP) [3], with the patient's compliance likely to be affected, thus hindering subsequent treatment effect and recovery.

In recent years, with the deepening of research, studies have found that glial cells play an important role in neural pain, especially after nerve damage. During inflammatory response or infection, glial cells are activated and produced in large quantities, leading to the development, maintenance and enhancement of neuropathic pain [4]. Therefore, researchers hope to find drugs that can slow down neurotoxicity and inflammation, especially natural chemical drugs with high safety and low side effects.

Linarin, a natural flavonoid glycoside, is commonly found in plants such as *Asteraceae, chrysanthemum,* and pink [5]. Modern pharmacological investigations have revealed that linarin possesses anti-inflammatory and analgesic properties [6]. Previous studies have also demonstrated that linarin inhibited lipopolysaccharide (LPS)-induced lung injury by regulating TXNIP/NLRP3 and NF-κB signaling pathways, thus suppressing inflammation and oxidative stress [7-9]. Linarin downregulated phagocytosis, production of pro-inflammatory cytokines and expression of activation markers in RAW264.7 macrophages [10]. Another study found that linarin also improved the recovery of zebrafish motor impairment in Alzheimer's disease by inhibiting the activity of acetylcholinesterase [11]. However, the protective effects and underlying mechanisms of linarin against neuropathic pain have not been studied. This study aims to study the protective effect and potential mechanism of action of linarin on nerve pain.

EXPERIMENTAL

Animals and treatment

Sprague-Dawley rats (weighing 200 - 220g) were purchased from Beijing Weitong Lihua Biological Co., Ltd., and were housed under conventional conditions and in a standard animal care facility, at a temperature set at 22 °C in a 12 h/12h light/dark cycle. The rats were allowed unrestricted access to food and water. All experimental procedures were approved by the Ethics Committee of Armed Police Forces Hospital of Sichuan (approval no. 2021021) and were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health [12].

Pain or discomfort in the animals was minimized during this study. To establish a chronic neuropathic pain (chemotherapy-induced neuropathic pain, CINP) model, oxaliplatin (2.5 mg/kg/day) was injected intraperitoneally for 4 consecutive days. The experimental groups were

a control group, CINP group, treatment group, CINP + linarin (20 mg/kg) and linarin (40 mg/kg) groups. The treatment group were started on oral administration of linarin 20 mg/kg/day or 40 mg/kg/day, 60 min before the first injection of oxaliplatin, for 21 consecutive days, and the body weights of the rats were measured on days 7, 14, and 21, respectively. After completing a series of behavioral experiments, the rats were sacrificed, and the spinal cord tissues were removed for subsequent experiments.

Von Frey test for mechanical withdrawal threshold

Mechanical allodynia was assessed using von Frey test. The rats were placed in plastic chambers placed on wire mesh racks (rats were acclimatized for 10 - 15 min in advance). The behavior of the rats was examined using a von Frey electronic instrument (BioRad Life Science Inc). The withdrawal threshold was assessed by applying pressure ranging from 0 to 50 g with an accuracy of 0.2 g. Punctate stimuli were administered through the tips of the von Frey fibers beneath the wire mesh floor, targeting the center of each rat's hind paw for 2 seconds. The von Frey instrument automatically recorded the withdrawal threshold. The sensitivity threshold in rats is deemed to be the minimal pressure necessary to evoke a robust and instantaneous withdrawal reflex in the hind paw. Voluntary movements linked to locomotion were not considered withdrawal responses. The hind paw was stimulated every 5 minutes, with the procedure repeated thrice. The mean value of the three measurements was documented as the final result.

Acetone drop test for cold allodynia

Cold allodynia was assessed employing the acetone drop test. Each rat was evaluated individually. Initially, the rats were positioned in a plastic chamber that was set on a wire mesh rack. Subsequently, acetone was delicately dispensed onto the surface of the rat's hind paw using a syringe or pipette. The duration leading to the flinch/lick response was noted over a 40-second period, with acetone drops being applied every 10 minutes during the testing phase. The mean value of the responses was calculated for each measurement.

Hot plate test for thermal hyperalgesia

Thermal hyperalgesia was assessed using a hot plate analgesia apparatus (BioRad). After setting the temperature of the hot plate 53 ± 1 °C, each rat was assessed individually. Their normal foot

lifts were not included in the evaluation. The time it took for the rats to respond by jumping or licking their hind paws on the hot plate was recorded. The heating time of the hot plate was set to 30 s to prevent scalding of the rats, which would affect subsequent experiments. The measurement was repeated 3 times for each rat, at an interval of >10 min between measurements, and then the mean value of the reaction time was calculated as the final result.

Rat weight

All rats were weighed on days 0, 7, 14 and 21 of the study and their body weights recorded.

Enzyme-linked immunosorbent assay (ELISA)

To quantify the expression of inflammatory cytokines in the rat spinal cord tissues, the tissues were washed with PBS and homogenized. This was followed by centrifugation at 1500 g for 15 min at room temperature. The supernatant, which contained inflammatory factors, was carefully collected and the contents of IL-1β, IL-6, IL-17 and IL-10 in the tissues were determined using ELISA detection kit (Biodee, Beijing, China).

Western blotting (WB)

Tissue from the rat spinal cord was lysed using RIPA tissue lysis buffer to generate cell debris and extract proteins. The proteins were mixed with a loading buffer and boiled for 5 min to cause protein denaturation. Following this, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The bands were then transferred onto a polyvinylidene fluoride (PVDF) membrane using constant electricity set at 300 mA for a duration of 60 - 90 min. The PVDF membranes were next incubated with 5 % non-fat dry milk for 2 hours to block nonspecific protein binding. The membranes were then incubated with the primary antibodies IBA-1 (1:1000, Binoway), c-fos (1:1000, Binoway), GFAP (1:1000, Binoway), NLRP3 (1:1000, Binoway), ASC (1:1000, Binoway), caspase-1(1:1000, Binoway), p65(1:1000, Binoway) and p-p65 (1:1000, Binoway). Following an another overnight incubation at 4 °C, horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, Binoway) was added to the membranes. Finally, freshly prepared ECL luminescence solution was applied to the membranes to enable the detection of proteins. The protein bands were visualized and quantified through the analysis of gray values.

Statistical analysis

The experimental data were analyzed and plotted using a GraphPad software version 9.0.0, and expressed as mean ± SD. All data are expressed as the mean ± standard deviation (SD). Prior to conducting any statistical analyses, the data were subjected to a normality test to determine whether they followed a normal distribution. Variance homogeneity tests were then carried out on data that passed the normality test. For data that was found to be normally distributed and demonstrated homogenous variance, one-way ANOVA was used to compare differences amongst multiple groups. Otherwise, Wilcoxon signed-rank test was used. A *p* value of < 0.05 was considered to indicate significant difference between the groups.

RESULTS

Linarin relieved nerve pain caused by oxaliplatin

In the present study, we first established a CINP model (Figure 1 D) using oxaliplatin, and assessed the body weight and behavior of the rats at four distinct time points i.e. day 7, 14 and 21. As time progressed, it was observed that the weight gain in the control group rats was the most rapid, whereas the rats in the CINP group exhibited the slowest weight gain. The weight gain in rats treated with 40 mg/kg/day was greater than that in the group treated with of 20 mg/kg/day, though both treatment groups demonstrated lesser weight gain compared to the control group (Figure 1 A).

Upon evaluating the mechanical withdrawal threshold using von Frey test, we found that the control group rats exhibited no significant alterations at the four time points. In contrast, the mechanical withdrawal thresholds of the rats in the CINP group were significantly reduced compared to day 0, 7, 14 and 21. The rats in the two treatment groups also demonstrated a decrease in mechanical withdrawal thresholds relative to day 0, but their thresholds were higher than those in the CINP group and lower than those in the control group. Notably, the mechanical withdrawal thresholds of the rats treated with 40 mg/kg/day of linarin exceeded those treated with 20 mg/kg/day (Figure 1 B).

Cold allodynia was determined in the four groups using the acetone drop test, with the results expressed as the frequency of withdrawn responses. The results show that control group showed the lowest withdrawn response, while

the reverse case for CINP group. The withdrawal responses of the rats in the two treatment groups were lower than those of CINP group at 3 detection time-points, but the withdrawal responses of the rats treated with 40 mg/kg/day of linarin were higher than that of those treated with 20 mg/kg/day, but higher than in control group (Figure 1 C). Finally, thermal hyperalgesia was determined by hot plate test, expressed as paw withdrawal latency.

The results showed that at the three determination-time points, the control group rats showed the longest time, and the CINP group the shortest time. The time-points of the two treatment groups rats was lower than in the control group but higher than in the CINP group, but the linarin-treated rats received 40 mg/kg/day, which was higher than 20 mg/kg/day (Figure 1 D). The above results showed that CINP led to slow weight gain in rats, a significant decrease in mechanical withdrawal threshold, increase in sensitivity to cold allodynia, and increase in thermal pain sensitivity, but linarin improved the slow weight gain and neurological function decline caused by CINP.
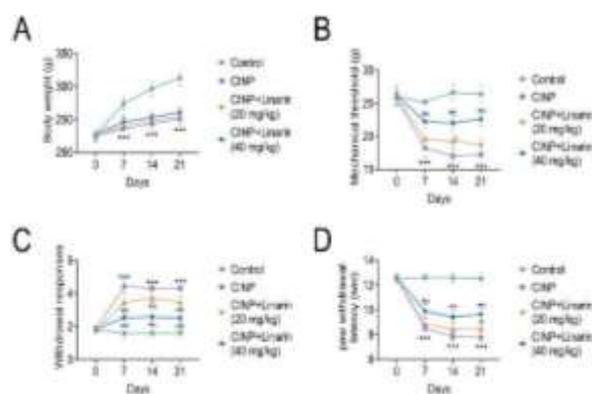

Figure 1: Effect of linarin on CINP-induced body weight change and behavioral neuropathic pain s determined on days 0, 7, 14 and 21. (A) Body weight change of rats (g); (B) Change in mechanical withdrawal threshold of rats; (C) Withdrawal response time of rats; (D) paw withdrawal latency of rats on a hot pad; n = 3, $^{\wedge}p < 0.05$ v.s. day 0; $^{***}p < 0.001$ or $^{\wedge\wedge\wedge}p < 0.001$ v.s. day 0)

Linarin inhibited the expressions of inflammatory factors in rat spinal cord tissue

Compared with the control group, the protein expression levels of IL-1β, IL-6, IL-10 and IL-17 were significantly higher than in the control group, while the expression of these three inflammatory factors in the treatment group decreased significantly; however, the expression levels of inflammatory factors in the spinal cord tissue of rats treated with 40 mg/kg/day of linarin was lower. With regard to IL-10, the CINP

treatment significantly elevated its level. Among the rats treated with 40 mg/kg/day of linarin, there was a significant increase in IL-10 expression compared to the CINP group. However, there was no significant change in IL-10 expression among rats receiving a 20 mg/kg/day linarin dose (Figure 2). The above results indicate that linarin treatment inhibited the expression of inflammatory factors in rat spinal cord tissue.
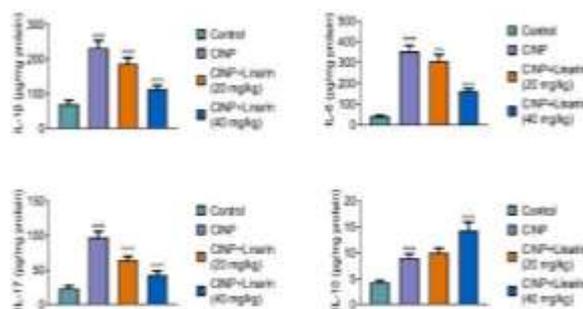

Figure 2: Effect of linarin on CINP-induced expressions of IL-1β, IL-6, IL-17, and IL-10; n = 3, $^{\wedge\wedge}p < 0.01$ or $^{\wedge\wedge\wedge}p < 0.001$ v.s. CINP group; $^{***}p < 0.001$ *vs.* control group

Linarin inhibited the activation of glial cells and neurons in spinal dorsal horn of rats

The expression levels of IBA-1, GFAP, and c-fos in the dorsal horn of the spinal cord were evaluated via WB, and these three proteins were used as indicators of the activation of microglia, astrocytes, and neurons. The expression levels of the three proteins in the control group were the lowest, but CINP significantly increased their expression. Compared with CINP group, the expression levels of the three proteins in the treatment group were significantly reduced; an increase in the treatment dosage corresponded to a more pronounced reduction in protein expression levels. (Figure 3). Thus, linarin treatment inhibits glial and neuronal activation in the dorsal horn of the rat spinal cord.

Linarin inhibited NF-κB/NLRP3 signal transduction

The expression levels of the proteins - ASC, caspase-1, p65, and p-p65 - were at the lowest level in the control group, but CINP treatment significantly increased the expression levels of these proteins. Compared with CINP group, the group treated with linarin decreased the expression of these proteins, and the reduction was greater in rats treated with the higher Linarin dose (40 mg/kg/day, as shown in Figure 4. Thus, linarin inhibited NF-κB/NLRP3 signal transduction.
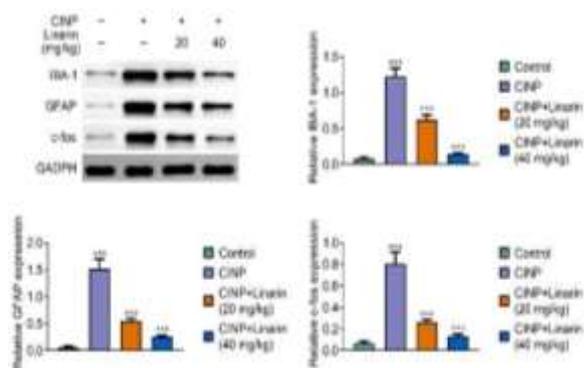
Figure 3: Effect of linarin on CINP-induced expressions of IBA-1, GFAP, and c-fos; n = 3, $^{\wedge\wedge\wedge}p < 0.001$ v.s. CINP group; $^{***}p < 0.001$ *vs.* control group
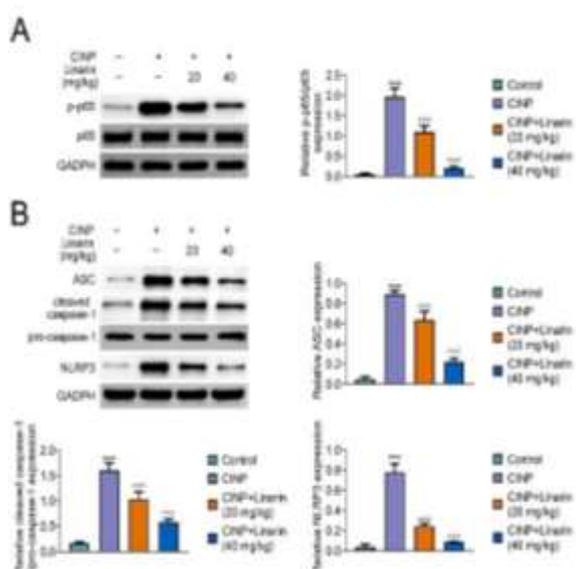

Figure 4: Effect of linarin on CINP-induced expression of NLRP3, ASC, caspase-1, p65, and p-p65. (A) Protein expression of p65 and p-p65. (B) Protein expression of NLRP3, ASC, and caspase-1; n = 3, $^{\wedge\wedge\wedge}p < 0.001$ v.s. CINP group; $^{***}p < 0.001$ *vs.* control group

DISCUSSION

Finding cancer drugs with high safety and few side effects has always been one of the difficult problems in basic drug research. Today's cancer treatment drugs have several side effects including neurotoxicity. As a natural plant ingredient, linarin has the advantage of high safety, but its specific mechanism of action still needs to be investigated prior to use in clinical trials.

Treatment with CINP slowed down the weight gain of the rats and caused them nerve pain, but treatment with linarin led to some improvement and produced therapeutic effect [13, 14]. In other studies, it was discovered that a derivative of linarin ameliorated ischemia-mediated nerve damage in rats [15]. The current findings were consistent with previous studies, but due to unclear biological mechanism, further research needs to be carried out.

CINP significantly increased the expressions of IL-1β, IL-6, IL-17 and IL-10. However, although linarin treatment dose was significantly reduced, the expressions of IL-1β, IL-6 and IL-17 were relatively low, IL-10 expression significantly increased in rats treated with 40mg/kg/day of linarin. These factors play a regulatory role in inflammatory response, and IL-10 plays a role in limiting the immune response, preventing pathogens from causing damage to the body and maintaining homeostasis. It is generally believed that imbalance of IL-10 will lead to an increase in the risk to some diseases [16]. Another study found that linarin significantly suppressed the levels of pro-inflammatory cytokines (IL-6, TNF-α, IFN-γ, and IL-1β) and enhanced the levels of anti-inflammatory cytokines (IL-10) in colitis mRNA levels [17]. This aligns more closely with our findings. Although in this study, inflammation was induced by CINP.

The expressions of the three proteins, *viz,* IBA-1, GFAP, c-fos related to glial cell and neuron activation were also investigated. Linarin mitigated the heightened protein expression of CINP, indicating that it suppressed the activation of glial cells and neurons in the dorsal horn of the rat spinal cord. Another study showed that linarin inhibited the activation of IBA-1, which in turn regulates microglial activation and enhances remyelination and repair [15].

NLRP3, ASC, caspase-1, p65, and p-p65 are the key proteins of NF-κB/NLRP3 signal transduction, usually with low expression, and their activation indicates inflammation. Linarin significantly suppressed the increased protein expression induced by CINP, suggesting that it suppresses NF-κB/NLRP3 signaling. Several other studies have also shown that flavonoids regulated NF-κB and NLRP3 inflammatory signaling pathways in porcine aortic endothelial cells with Glässer disease [18]. Yi *et al* reviewed the role of flavonoids, one of which is to target NF-κB and NLRP3 thereby alleviating inflammation [19].

Limitations of this study

There are limitations in this study. First, the safety and toxicity of linarin were not evaluated. Second, molecular cell experiments which would have provided some insights into mechanism of action of linarin at the *in vitro* level, were not included in this work. Nevertheless, this study

paves the way for the potential application of linarin in the treatment of neuropathic pain.

CONCLUSION

Linarin attenuates oxaliplatin-induced neuropathic pain by inhibiting NF-κB/NLRP3 signaling pathway, providing a potential strategy for its clinical application.

DECLARATIONS

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None provided.

Ethical approval

This study was approved by the Ethics Committee of Armed Police Forces Hospital of Sichuan (approval no. 2021021).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Siyu Zeng, Chenming Ling, Hao Chen and Yu Wang designed the study and carried them out, supervised the data collection, analyzed the data, interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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REFERENCES

1. Zhang YQ, Wu D, Deng MQ, Chang XY, XXX, Miao JW, Wu YM. Individual management of cervical cancer during pregnancy. Eur J Gynaecol Oncol 2020; 41(5): 833-837.

2. Karasawa T, Steyger PS. An integrated view of cisplatin-induced nephrotoxicity and ototoxicity. Toxicol Lett 2015; 237(3): 219-227.

3. Pasetto LM, D'Andrea MR, Rossi E, Monfardini S. Oxaliplatin-related neurotoxicity: how and why? Crit Rev Oncol Hematol 2006; 59(2): 159-168.

4. Lee JH, Kim W. The Role of Satellite Glial Cells, Astrocytes, and Microglia in Oxaliplatin-Induced Neuropathic Pain. Biomedicines 2020; 8(9): 324.

5. Abbet C, Slacanin I, Corradi E, De Mieri M, Hamburger M, Potterat O. Comprehensive analysis of Cirsium spinosissimum Scop., a wild alpine food plant. Food Chem 2014; 160: 165-170.

6. Qi W, Chen Y, Sun S, Xu X, Zhan J, Yan Z, Shang P, Pan X, Liu H. Inhibiting TLR4 signaling by linarin for preventing inflammatory response in osteoarthritis. Aging (Albany NY) 2021; 13(4): 5369-5382.

7. Zhong H, Hao L, Li X, Wang C, Wu X. Anti-inflammatory Role of Trilobatin on Lipopolysaccharide-induced Acute Lung Injury through Activation of AMPK/GSK3β-Nrf2 Pathway. Signa Vitae 2020; 16(2): 160-166.

8. López AL, Paolini MV, Fernández Romero DS. Lung disease in patients with common variable immunodeficiency. Allergologia et Immunopathologia 2020; 48(6): 720-728.

9. Zhu W, Zhao Z, Liu X, Ni X, Lei X, Li X, Deng R, Zhao L. Ginkgolide K potentiates the protective effect of ketamine against intestinal ischemia/reperfusion injury by modulating NF-κB/ERK/JNK signaling pathway. Trop J Pharm Res 2021; 20(1): 11-16.

10. Kim B, Lee JH, Seo MJ, Eom SH, Kim W. Linarin down-regulates phagocytosis, pro-inflammatory cytokine production, and activation marker expression in RAW264.7 macrophages. Food Sci Biotechnol 2016; 25(5): 1437-1442.

11. Pan H, Zhang J, Wang Y, Cui K, Cao Y, Wang L, Wu Y. Linarin improves the dyskinesia recovery in Alzheimer's disease zebrafish by inhibiting the acetylcholinesterase activity. Life Sci 2019; 222(112-116.

12. Ni W, Zheng X, Hu L, Kong C, Xu Q. Preventing oxaliplatin-induced neuropathic pain: Using berberine to inhibit the activation of NF-κB and release of pro-inflammatory cytokines in dorsal root ganglions in rats. Exp Ther Med 2021; 21(2): 135.

13. Rahman B, Goswami M. Comparative Evaluation of Indirect Pulp Therapy in Young Permanent Teeth using

Biodentine and Theracal: A Randomized Clinical J Clin Pediatr Dent 2021; 45(3): 158-164.

14. *Li V, Vigneswaran WT. Molecular Markers in Guiding Lung Cancer Diagnosis and Treatment. J Mens Health 2022; 18(8): 164.*

15. *Xie C, Yue S, Li X, Li Z, Li W, Huang G, Ma G, Liu W, Wang Y, Zhang Y. A Linarin Derivative Protects against Ischemia-Induced Neuronal Injury in Mice by Promoting Cerebral Blood Flow Recovery via KDELR-Dependent CSPG4 Activation. Oxid Med Cell Longev 2022; 2022: 6434086.*

16. *Jin C, Liu J, Jin R, Yao Y, He S, Lei M, Peng X. Linarin ameliorates dextran sulfate sodium-induced colitis in C57BL/6J mice via the improvement of intestinal barrier, suppression of inflammatory responses and modulation of gut microbiota. Food Funct 2022; 13(20): 10574-10586.*

17. *Islam H, Chamberlain TC, Mui AL, Little JP. Elevated interleukin-10 levels in COVID-19: potentiation of pro-inflammatory responses or impaired anti-inflammatory action? Frontiers in Immunology 2021; 12: 677008.*

18. *Fu S, Liu H, Xu L, Qiu Y, Liu Y, Wu Z, Ye C, Hou Y, Hu CA. Baicalin modulates NF-κB and NLRP3 inflammasome signaling in porcine aortic vascular endothelial cells Infected by Haemophilus parasuis Causing Glässer's disease. Sci Rep 2018; 8(1): 807.*

19. *Yi YS. Flavonoids: Nutraceuticals for Rheumatic Diseases via Targeting of Inflammasome Activation. Int J Mol Sci 2021; 22(2).*