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Anti-Inflammatory Activity of KNC, a Vietnamese Herbal Medicine Prescription on RAW 264.7 Cells

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ABSTRACT

Kinh-Nghiem-Canh (KNC) is a herbal priscription drug used for the treatment of musculoskeletal pain in the Vietnamese oriental medicine. The anti-inflammatory effect of KNC decoction in lipopolysaccharide (LPS)-treated RAW 264.7 cells was evaluated using ELISA test kits for detection of inflammatory mediators PGE2 and proinflammatory cytokines such as TNF-α, IL-1β, and IL-6. Results showed that anti-inflammatory properties of KNC resulted from the inhibition of nitric oxide (NO) production, prostaglandin E2 (PGE2) release, and other proinflammatory cytokines such as IL-1β, TNF-α, and IL6 in a RAW 264.7 macrophage cell line.

***Keywords*:** Vietnamese traditional medicine, KNC, RAW 264.7 cells, Inflammation.

Introduction

Inflammation is part of the body's immune response to foreign pathogens such as radiation, chemicals, and bacteria.[1] Progression of the inflammatory response is a continuous process from the early stages of an acute inflammatory response to the chronic inflammatory response, followed by healing and repair.[2] Although it plays an essential role in protecting the body, the inflammatory response is also responsible for a variety of chronic diseases such as rheumatoid arthritis, enteritis, and asthma.[1] Inflammation is also one of the most common causes of death worldwide.[1] Currently, there are many anti-inflammatory drugs, especially NSAIDs and glucocorticoids are two classical anti-inflammatory drugs with promising effects on many types of inflammation and widely used, however, these drugs have undesirable effects on many organs.[3,4] In addition, the high cost of these drugs is still a concern when using the medicine every day and for a long time for the patients.[5,6] This point raises the need to find new anti-inflammatory drugs derived from medicinal herbs, which have similar effects, and at the same time limit undesirable effects of the existing drugs.[7,8]

In Vietnam, KNC is an oriental remedy that is effective in the treatment of musculoskeletal pain in the clinic, and is used commonly at local Oriental Hospitals with good results.[9-11] However, there are limited reports on the anti-inflammatory effect of this prescription in cells model. The study evaluated the anti-inflammatory effect of KNC on prostaglandins (PGE2), nitric oxide (NO) productions and proinflammatory cytokines release on macrophage RAW 264.7 cells activated by LPS.

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Materials and Methods

KNC formulation and extraction process

All of the medicinal materials in the prescription (Table 1) were used in the dried form and met the standards in the Vietnamese Pharmacopoeia V,[12] and are processed according to the regulations of traditional medicine. KNC was extracted with hot water at a ratio of 1:1 (1 g medicinal herbs/1 mL water). The extraction was done by decoction machine KTP-EP-25 (Korea Techno Pack, Korea) at the Pharmacy Department - Tue Tinh Hospital, Hanoi, Vietnam. The primary standard for KNC was diluted with water to obtain a range of different concentrations.

Table 1: KNC ingredients

Crude drug local names	Crude drug names	Weight (g)
Độc hoạt	*Radix Angelicae pubescentis*	10
Phòng phong	*Radix Saposhnikoviae divaricatae*	10
Tần giao	*Radix Gentianae macrophyllae*	10
Tang kí sinh	*Herba Loranthi gracilifolii*	10
Ngưu tất	*Radix Achyranthis bidentatae*	10
Bạch thược	*Radix Paeoniae lactiflorae*	05
Thục địa	*Radix Rehmanniae glutinodea praeparata*	05
Khương hoạt	*Rhizoma et Radix Notopterygii*	05
Tế tân	*Radix et Rhizoma Asari*	05
Đảng sâm	*Radix Codonopsis*	10
Đương quy	*Radix Angeliace sinensis*	05
Xuyên khung	*Rhizoma Ligustici wallichii*	05
Đỗ trọng	*Cortex Eucommiae*	05
Cam thảo	*Radix Glycyrrhizae*	02

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Chemicals
The ELISA test kits for inflammatory mediators PGE2 and proinflammatory cytokines TNF-α, IL-1β, and IL-6, RAW macrophage cell 264.7 were purchased from Rockville, MD, USA. Culture medium; Dulbeco's Modified Eagle's Medium and phosphate buffer saline (PBS) were acquired from Sigma Chemical Co. St. Louis, MO, U.S.A.

Cell culture
RAW 264.7 macrophage cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Fetal bovine serum) and 1% penicillin-streptomycin, in a 5% CO_2 incubator at 37°C.

Evaluation of cell viability
The effect of the KNC on cell viability was assessed by the MTT colorimetric test. Cells were suspended in a 96-well plate at a concentration of 1×10^4 cells/well with varying concentrations of the reagent for 24 h. The cells were then treated with MTT solution (2 mg/mL) for 3 h. After the removal of the supernatant, the formazan produced was dissolved in DMSO and the absorbance was measured at 540 nm.

Nitrite test
RAW 264.7 macrophage cells were pretreated with KNC (50–500 μg/mL) for 1 h, then stimulated for 18 h with LPS (1 μg/mL). Every 100 μL of supernatant was reacted with the same volume of Griess reagent [1% sulfanilamide, 0.1% N- (1- naphthyl) -ethylenediamine dihydrochloride, 2.5% phosphoric acid] for 10 min. Nitrite level was assessed by measuring the absorbance at 540 nm.[13] The results were expressed as a concentration of the released of NO from RAW 264.7 cells. Sodium nitrite was used to prepare a standard curve.

Evaluation of prostaglandin (PGE2) and proinflammatory cytokines
RAW 264.7 macrophage cells were pretreated with KNC (50–500 μg/mL) for 1 h, then stimulated for 18 h with LPS (1 μg/mL). The supernatant were collected, and the concentration of proinflammatory cytokines such as IL-1β, TNF-α, and IL6 was determined using ELISA kits. PGE2 concentration was measured with PGE2 test kit according to the manufacturer's instructions.[14,15]

Statistical analysis
The experiments were in triplicates and analysed using ANOVA followed by Tukey test, using GraphPad Prism software (P value < 0.05 was considered statistically significant).

Results and Discussion

Cell viability
The cytotoxic effects of the KNC was evaluated in the presence or absence of LPS by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Results showed that KCN at concentrations of 50-500 µg/mL did not affect the cell viabilities of RAW 264.7 cells in either the presence or absence of LPS, even at a high dose after a period of 24 h (Table 2).

Effects of KNC on NO production
Since KNC showed no cytotoxicity on RAW264.7, KNC was then tested for its ability to inhibit NO production in LPS-activated RAW264.7 macrophages. Compared to the blank group, the LPS stimulation group had a 4-fold higher concentration of NO (equal to 380.32% compared to control 1), the difference was statistically significant with $p < 0.001$. As shown in Table 3, KNC significantly inhibited NO production in LPS-activated RAW264.7 macrophages in a dose dependent manner, all concentrations of KNC had a significant decrease in NO production, the difference was statistically significant with $p < 0.01$ and $p < 0.001$.

Evaluation of prostaglandins (PGE2) and proinflammatory cytokines
The effects of KNC on the production of pro-inflammatory mediators such as PGE_2, IL-1β, TNF-α, and IL-6 in stimulated RAW264.7 cells are presented in Table 4. The results show that the cells treated with 1 µg/mL of LPS significantly ($p < 0.05$) raised the levels of PGE_2, IL-1β, TNF-α, and IL-6 concentrations in the cell culture media as follows: 763.12 ± 55.44, 386.88 ± 35.99, 505.94 ± 34.09, and 434.79 ± 38.04 pg/mL, respectively, compared to the untreated LPS cells (Table 4). However, the concentrations of PGE_2, IL-6, IL-1β, IFN-β, and MCP-1 were decreased in a dose-dependent manner in cells pretreated with 50 - 500 µg/mL of KNC before stimulation with LPS. Compared to the cells with LPS for 18 h (1 µg/mL) only, the concentrations of PGE_2, IL-6, IL-1β, IFN-β, and MCP-1 in the cells pretreated for 1 h with different concentrations of KNC were decreased in all groups, the difference were statistically significant, $p < 0.05$ (at a concentration of 50 µg/mL), $p < 0.01$ (at 100 µg/mL) and $p < 0.001$ (at 300 and 500 µg/mL).
Nitrite oxide (NO) is a product of inflammatory processes that affect the immune system and function of many types of cells.[14,15] Activation of macrophages release many inflammatory mediators such as cytokines and NO. NO has been reported to be one of the most important targets in anti-inflammatory drug development.[15] Inhibition of NO production is one of the mechanisms to further confirm the anti-inflammatory ability of candidate drugs. LPS is the outer membrane component of Gram-negative bacteria, and is also a potent activator of mononuclear cells and macrophages.[15] LPS activates the production of many cytokines including IL-6, IL-1, TNF-α and NO.[14,15] KNC at different concentrations (50 - 500 µg/mL) inhibited the NO production of RAW cells 246.7 after stimulation by LPS.

Table 2: Percentage of cell viability

KNC concentration (µg/mL)	OD 540 nm	Cell viability (%)	P values
0	0.218 ± 0.009	100.00	-
50	0.231 ± 0.017	105.95 ± 8.21	> 0.05
100	0.226 ± 0.011	103.67 ± 8.92	> 0.05
300	0.215 ± 0.012	98.62 ± 10.06	> 0.05
500	0.209 ± 0.008	95.87 ± 9.84	> 0.05

Values are Mean + SEM, n = 3, P < 0.05, considered statistically significant.

Table 3: Inhibition of LPS-induced NO production in RAW264.7 mouse macrophages

KCN concentration (µg/mL)	LPS (1µg/mL)	NO (µM)	% NO with a)	*P* with a)	*P* with b)
Normal group [a]	-	3.096 ± 0.39		-	< 0.001
0 [b]	+	11.77 ± 0.92	380.32 %	< 0.001	-
50	+	7.39 ± 0.64	238.71 %	< 0.001	< 0.01
100	+	6.52 ± 0.71	210.75 %	< 0.01	< 0.01
300	+	5.23 ± 0.60	168.82 %	< 0.01	< 0.001
500	+	4.23 ± 0.52	136.56 %	< 0.05	< 0.001
Quercetin[c]	+	4.51 ± 0.48	146.95 %	< 0.05	< 0.001

[a]Normal: normal group without LPS. [b]Cells were treated with LPS (1 µg/mL). [c]Positive control (Quercetin) at concentration of 10 µM. Values represent the mean ± SEM of three determinations. * $p < 0.05$; ** $p < 0.01$ (between KNC-treated group and the control group). ## $p < 0.01$ (between LPS-treated group and the control group).

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The NO production inhibition could be attributed to the constituents of KNC. Tai and Cheung reported that the extract of *Saphoshnikovia divaricata* (Phòng phong) did not show cytotoxicity on RAW 264.7 cells, and significantly decreased NO production by lipopolysaccharides (LPS) activated RAW macrophages 264.7.[16,17]

With chemical components such as avicularin, and quercetin, *Herba Loranthi gracilifolii* (Tang kí sinh) has been reported to be effective in treating osteoarthritis pain.[18] Avicularin exhibited anti-inflammatory activity by inhibiting the production of NO, PGE2 and inhibiting the release of cytokines in RAW 264.7 cells.[18] In addition, the anti-inflammatory effect of *Herba Loranthi gracilifolii* was also demonstrated by Taguchi *et al*. who showed that quercetin isolated from *Herba Loranthi gracilifolii* exhibited anti-inflammatory effect on many different experimental models in guinea pigs, rats caused by various agents such as carrageenan, dextran, histamine, serotonin and bradykinin.[19]

Flavonoids found in *Glycyrrhiza uralensis* (Cam thảo) also inhibited NO release.[20] Flavonoids have an important enzyme inhibitory effect in NO synthesis, thereby inhibiting NO release from macrophages during inflammation.[21] Shin *et al* investigated a flavonoid from *Glycyrrhiza uralensis*, glycyrol, in LPS-stimulated RAW cells. Glycyrol increased NO concentration through the decrease in nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) of LPS-stimulated RAW cells.[20,21] Tang *et al*. evaluated the anti-inflammatory activity of extract of *Radix Achyranthis bidentatae* (Ngưu tất) against lipopolysaccharide NO production in mouse mononuclear macrophage cell line. This extract significantly inhibited NO production, inhibited vasodilator effects, reduced vascular permeability, and production of PG, in inflammatory responses.[22]

PGE2 is considered to be one of the most potent inflammatory mediators in the inflammatory response and plays a major role in the pathogenesis of various inflammatory diseases, tumour, oedema, growth and invasion.[23,24] PGE2 is transformed from arachidonic acid through a catalytic reaction of COX-2.[24] Nonsteroidal anti-inflammatory drugs (NSAIDs) have antipyretic, anti-inflammatory and analgesic effects through inhibiting COX activity and reducing the production of inflammatory mediators such as PGE2.[25] In our experiment, KNC extract inhibited the PGE2 production of RAW stimulated by LPS significantly in a dose-dependent manner. Cytokines play vital role in regulating inflammation. TNF-α, IL-1β and IL-6 are multifunctional proinflammatory cytokines and exhibit various inflammatory effects in chronic inflammatory diseases such as rheumatoid arthritis and atherosclerosis.[25,26] TNF-α has long been considered a major mediator for the induction of apoptosis and the development of humoral immune responses. However, high concentrations of TNF-α caused adverse effects, such as tissue damage and intense septic shock.[26] The other cytokine such as IL-1β is mainly produced by macrophages, monocytes and T cells and is also involved in immune defense against infection. Both cytokines TNF-α and IL-1β also produce IL-6 so that the three molecules (TNF-α, IL-1β, and IL-6) simultaneously act and cause many changes in damaged tissues. Interleukine-1β is a cytokines of an acute inflammatory response, released in response to infection or damage to cells caused by the innate immune system.[27] IL-1β is secreted from monocytes/macrophages, B cells, fibroblasts, most epithelial cells and endothelial cells, and stored in keratin cells.[27] IL-1β increased expression of adhesion molecules, increased migration of neutrophils and macrophages, caused sock-like physiology, increased liver protein generation, fever, increased protein production and erythrocyte renewal, cartilage degeneration, iNOS activation, acute inflammatory response.[28] KNC (50 - 500 µg/mL) resulted in the gradual decrease in the concentration of IL-1. Compared to LPS treated only, KNC condensate had a marked decrease in IL-1β production, the difference was statistically significant ($p < 0.01$ and $p < 0.001$). In the immune system, increasing levels of TNF-α is associated with chronic diseases such as osteoarthritis, and rheumatoid arthritis. Activated macrophages and fibroblasts release other cytokines including TNF-α, a central component of the cytokines chain, stimulates the production of other inflammatory secondary mediators. TNF-α activates and proliferates synovial membrane cells to create Pannus organization, release collagen fibrinolytic enzymes; at the same time activating cartilage cells, destroying cartilage, inhibiting proteoglycan synthesis.[26] Therefore, inhibition of TNF-α is one of the important targets for osteoarthritis treatment. In addition, IL-6 also plays an essential role in host defense, acute phase response, immune response and neural cell function.[27,28] It has been shown that high IL-6 levels have been reported in a bacterial and viral infections, trauma, autoimmune diseases and inflammation.[29]

To have more insight to the anti-inflammatory property of KNC, the study further evaluated the effect of KNC on the TNF-α and IL-6 release inhibition. At the different concentrations of KNC, the TNF- production decreased significantly, the differences were statistically significant at $p < 0.01$ and $p < 0.001$. When the concentration increased from 50 - 500 µg/mL, the amount of TNF-α released decreased gradually. Similarly, the cells treated with KNC decreased IL-6 concentration significantly compared to LPS treated cell only, the difference was statistically significant with $p < 0.05$ (at 50 µg/mL), $p < 0.01$ (at 100 µg/mL) and $p < 0.001$ (at 300 and 500 µg/mL). It is important to note that, the KNC extract is composed of *Radix angeliace sinensis* (Đương quy) and *Radix glycyrrhizae* (Cam thảo). These ingredients are rich source of flavonoids that inhibit TNF-α, inhibiting NO release through NO-synthase (iNOS). These flavonoids also inhibited cytokines secreted from macrophages such as TNF-α, IL-1, and IL-6, helping to reduce the progression of diseases related to inflammation.[30]

Table 4: Effects of KNC on production of prostaglandins (PGE2) and proinflammatory cytokines of RAW cells stimulated by LPS.

KNC concentration (µg/mL)	LPS (1 µg/mL)	Proinflammatory cytokines (pg/mL)			
		PGE2	IL-1β	TNF-α	IL-6
Normal group[a]	-	186.54 ± 11.13	32.52 ± 3.48	38.12 ± 4.11	34.69 ± 3.55
0 [b]	+	763.12 ± 55.44	386.88 ± 35.99	505.94 ± 34.09	434.79 ± 38.04
50	+	484.39 ± 42.06*	264.46 ± 21.98	356.28 ± 31.10	342.03 ± 24.17
100	+	366.94 ± 29.95**	204.94 ± 20.70	283.38 ± 22.20	294.94 ± 27.47
300	+	302.88 ± 24.50	159.34 ± 15.91	195.38 ± 13.79	215.80 ± 17.93
500	+	224.64 ± 22.38	74.33 ± 7.70	112.86 ± 8.61	166.51 ± 15.88
Quercetin[c]	+	236.37 ± 23.59$^{\#\#}$	69.82 ± 6.85	130.21 ± 9.46	173.62 ± 16.96

[a]Normal: normal group without LPS. [b] Cells were treated with LPS (1 µg/mL). [c]Positive control at concentration of 10 µM. Values represent the mean ± SEM of three determinations. $^{*}p < 0.05$; $^{**}p < 0.01$ (between KNC-treated group and control group). $^{\#\#}p < 0.01$ (between LPS-treated group and control group).

Conclusion

In conclusion, KNC extract showed inhibitory effect on NO production, prostaglandins PGE2, and proinflammatory cytokines on RAW cells activated by LPS. This is the first investigation of KNC anti-inflammatory activity *in vitro*. Therefore, KNC has a potential anti-inflammatory application.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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