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A lipid extract of *Perna canaliculus* affects the expression of pro-inflammatory cytokines in a rat adjuvant-induced arthritis model

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Key words

Lyprinol®, NSAID, adjuvant-induced arthritis, pro-inflammatory cytokines

Summary

As published initially in this same journal in 2000 (1), the lipid extract of Perna canaliculus (New Zealand green-lipped mussel; Lyprinol®) is known for its anti-inflammatory effects in animal models and in human controlled studies (arthritis; asthma). As a follow-up of its effects on pain in a rat model of adjuvant-induced arthritis (AIA) (2), we studied its effects on the production of cytokines known to be associated with inflammation (IL-6, IL-1α TNF-α, IFN-γ). Feeding with Lyprinol was associated with significantly decreased expression levels of TNF-α and IFN-γ when compared to Naproxen (positive control) and, even more when compared with sham and extra-virgin olive oil (negative control). When compared to Naproxen, sham and extra-virgin olive oil, the levels of IL-6 and IL-1α were also marginally decreased in rats fed with Lyprinol. This study demonstrates that AIA rats fed with Lyprinol had decreased production of cytokines associated with inflammation.

Introduction

When given to animals and humans, a lipid-rich extract prepared by supercritical fluid carbon dioxide extraction of freeze-dried stabilized New Zealand green-lipped mussel *Perna canaliculus* powder (Lyprinol®), has shown significant anti-inflammatory (AI) activity (1-7); this was described extensively for the first time in a special issue of this journal (1). According to Whitehouse and coworkers (8), when treated per os (p.o.) with this lipid extract, Wistar and Dark Agouti rats developed neither adjuvant-induced polyarthritis nor collagen (II)-induced auto-allergic arthritis. This was achieved with doses 200 times lower than other seeds or fish oils (8). In contrast to non-steroidal- anti-inflammatory drugs (NSAIDs), whole mussel extract of *Perna candaliculus* is non-gastrotoxic in disease-stressed rats at 300 mg/kg p.o. (7). Further, Lypri-

nol does not affect platelet aggregation in both humans and rats (5,8). Clinical studies, either controlled or randomized, have demonstrated very significant AI activity in patients with osteoarthritis (OA) (3,4,6,9), asthma (10), and other inflammatory conditions (11). There are no reported side-effects, even at doses up to 2,500 mg/day in patients. The lipid extract of *Perna canaliculus* seems to be a reproducible, stable source of bioactive lipids with much greater potency than plant/marine oils currently used as nutritional supplements to ameliorate signs of inflammation (5,8,12). This lipid extract's subfractions were also found to inhibit LTB4 biosynthesis by polynuclear white blood cells in vitro, and PGE2 production by activated macrophages (13). Much of this AI activity was found to be associated with omega-3 PUFAs and natural antioxidants, e.g. carotenoids. However, the exact mechanisms of its actions are not clear.

We conducted a series of experiments to understand the anti-inflammatory mechanism of action of this lipid extract and its effects on pain control (2). In this article, we present the effects observed on the production of IL-6, IL-1α, TNF-α, IFN-γ, the four cytokines considered to be associated with inflammation in the AIA rat model. When compared to Naproxen, sham and extra-virgin olive oil, we found that Lyprinol is effective in reducing the production of TNF-α and IFN-γ. The levels of IL-6 and IL-1α were also marginally decreased in AIA induced rats fed with Lyprinol.

Materials and Methods

Induction of inflammation in Sprague-Dawley (SD) rats

Four groups of six 6-week-old male SD rats were purchased from the Central Animal Facility (CAF) of Hong Kong Polytechnic University (HKPU). All the rats were kept and cared under conditions that fully met the requirements of the Procedures for the Care of Laboratory Animals or Animals (Control of Experiments) Regulations Chapter 340 of the Hong Kong SAR government. Ethics approval (ASESC No.04/9) had been obtained from The Animal Subjects Ethics subcommittee of the HKPU. Arthritis was induced in anesthetized animals by administration of adjuvant according to the method previously described (8) with minor modifications. Briefly, at day 0, each rat was injected in the paw of the right hind limb with 100 μl of Freund's complete adjuvant (Sigma, St. Louis, MO, USA) containing 10 mg/ml of Mycobacterium butyricum (Difco, Livonia, MI, USA). Another six rats without arthritis induction were observed as normal group.

Products/drug tested fed to the treatment and control groups of rats

Rats in the lipid extract of *Perna canaliculus* (Lyprinol®, Pharmalink International Ltd., Burleigh Heads, QLD, Australia) study group were fed by oral gavage at a dosage of 25 mg Lyprinol / kg body weight. Typically, the required amount of Lyprinol was made up with olive oil (Virgin®, Bertolli, Italy) to 300 μl before being force-fed to the rats with a stainless steel stomach tube. 300 μl of olive oil, and 20 mg/ kg body weight of Naproxen were fed as vehicle and positive control respectively. Naproxen

is a NSAID that is routinely being used to treat inflammation and known to have gastro-toxic side effects. Normal chow was provided to all the animals.

Splenocyte preparation

At day 7 and 14 after arthritis induction, splenocytes of each rat in the Lyprinol®, Naproxen and olive oil were prepared as described previously (2,14). Briefly, spleens were aseptically cut off and minced into tiny pieces. Single cell suspension was prepared by gentle grinding of spleen pieces in RPMI 1640 medium (Life Technologies, Invitrogen, Carlsbad, CA, USA). Splenocytes (mostly B and T cells) from the crude spleen cell suspension were recovered by using Ficoll-Hypaque® Plus lymphocyte isolation kit (Pharmacia Biotech, Piscataway, NJ, USA) as described in the manufacturer's manual. Recovered splenocytes (1.5 ml) were transfered into a sterile centrifuge tube and 4 volumes of pre-cold 0.83% ammonium chloride (NH$_4$Cl) were added and incubated for 10 minutes for the lysis of residual erythrocytes (2). Splenocytes were recovered by centrifugation at 1000 x g for 5 minutes, washed with 8 ml pre-warm (37°C) sterile PBS buffer (137mM NaCl, 2.7mM KCl, 4.3mM Na$_2$HPO$_4$–7H$_2$O, 1.4mM KH$_2$PO$_4$, pH 7.4) and finally re-suspended in appropriate volume of complete RPMI 1640 medium (10% fetal bovine serum, 100units/ml of penicillin and 100μg/ml of streptomycin, supplemented with L-glutamine and 25 mM HEPES).

Cell count and viability staining

Re-suspended splenocytes were stained with 0.4% Trypan blue exclusion dye (0.4 g Trypan blue in 100ml PBS buffer) at ratio 1:1. The number of splenocytes was counted and calculated with the aid of a hemocytometer. More than 99% of splenocytes were viable. Except stated otherwise, splenocytes were diluted to a working population of 5 x 106 cells/ ml in the following experiments.

ELISA assay for cytokines

5 x 106 splenocytes/ ml were seeded in 24-well plate; a suboptimal concentration of 1.25 μg/ ml of lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA) was used to prime the splenocyte culture. Cell culture was incubated in 37? incubator at 80% humidity and 5% CO$_2$ atmosphere

condition. After incubating with LPS, supernatants were collected either at 10 hours or at a specific time wherever indicated in the text, before being stored at -80 °C until use. IL-6, IL-1α, TNF-α and IFN-γ were measured by an enzyme-linked immunosorbent assay (ELISA) sandwich type assay (BioSource, Camarillo, CA, USA) as described in the user's manual. Samples used for measurements of IL-6, TNF-α and IFN-γ were supernatants of splenocytes that had been incubated with LPS for 10 hours. On the other hand, because of the low levels of expression of IL-1α, samples used for measurements of IL-1α were supernatants of splenocytes that had been incubated with LPS for 24 hours. Data obtained were compared to those of the control group and analyzed by Student's t-test.

Results

Levels of pro-inflammatory cytokine interleukin-6 (IL-6)

Figure 1 shows that the level of the pro-inflammatory cytokine IL-6 was decreased at day 7. The level of IL-6 in the Lyprinol® group was significantly lower than the one of the control and olive oil groups. The level of IL-6 in the Lyprinol® group is close to the one observed in the NSAID Naproxen group. Our results clearly demonstrated that Lyprinol® can decrease the production of the pro-inflammatory cytokine IL-6 in the early phase of AIA.

Figure 1 - Amount of pro-inflammatory cytokine interleukin-6 (IL-6) produced by LPS-stimulated splenocytes after 7 days of arthritis induction. Although not statistically significant, Lyprinol lowered the amount of IL-6 production when compared to control and olive oil groups, though not as effectively as the NSAID Naproxen. Data shown are mean + S.E.M. (n=6)
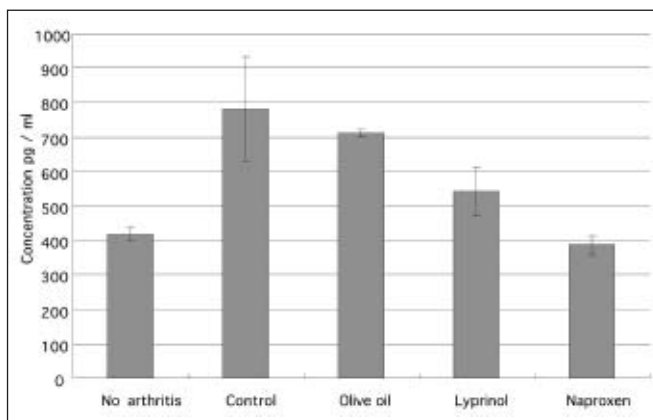
Levels of pro-inflammatory cytokine interleukin-1α (IL-1α)

IL-1α level of AIA rat splenocytes at days 7 and 14 was measured. Figure 2 shows that the level of IL-1α at day 7 and especially at day 14 was reduced significantly in the Lyprinol® group when compared to the control and olive oil groups. This effect on IL-1α production was not seen in rats fed with the NSAID Naproxen. The results demonstrated that Lyprinol can reduce the production of the pro-inflammatory cytokine IL-1α in AIA rats.

Levels of cytokines tumor necrosis factor-α (TNF-α) and interferon-gamma (IFN-γ)

As shown in figure 3, levels of TNF-α in the Lyprinol® group on day 14 were greatly decreased when compared to those of the control group. Indeed, the level of TNF-α de-

Figure 2 - Amount of pro-inflammatory cytokine IL-1-alpha produced by LPS-stimulated splenocytes at (A) day 7 and (B) Day 14 after induction of arthritis in rats
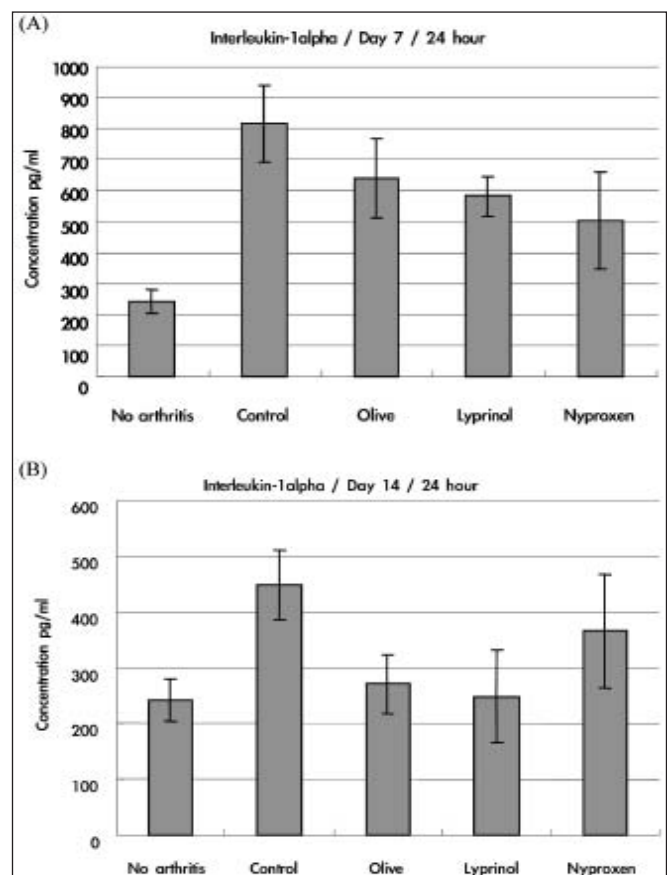
Figure 3 – Amount of pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) produced by post 10 hours LPS-stimulated splenocytes collected after 14 days of arthritis induction. Lyprinol effectively controlled the amount of TNF-α produced when compared to control and olive oil groups. More importantly, Lyprinol was more effective than the NSAID Naproxen. Data are mean + S.E.M. (n=6). * p < 0.05
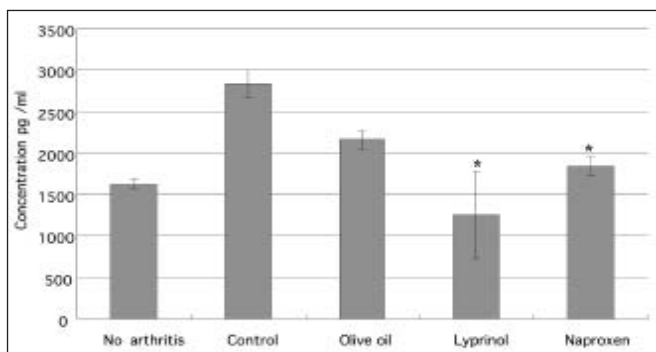

Figure 4 – Amount of interferon-gamma (IFN-γ) produced by post 10 hours LPS-stimulated splenocytes collected after 14 days of arthritis induction. As can be seen, Lyprinol effectively lowered the amount of IFN-γ produced when compared to control and olive oil groups. Strikingly, Lyprinol controlled the IFN-γ production even more effectively than the NSAID Naproxen. Values shown are mean + S.E.M. (n=6). * p < 0.05
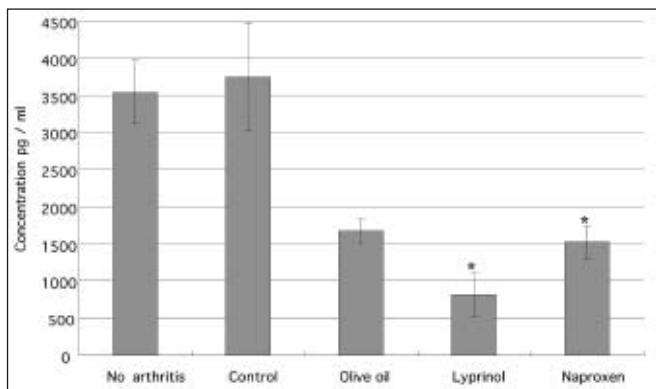
tected was lower than the one found in AIA rats treated with the NSAID Naproxen. Besides, the production of another pro-inflammatory cytokine, IFN-γ, was also found to be significantly decreased. As shown in figure 4, IFN-γ level of Lyprinol® group was dramatically decreased when compared to the control and olive oil-fed groups. Again, the level of INF-γ in the Lyprinol® group was even lower than the one observed in the AIA rats treated with the NSAID Naproxen. Our results showed that Lyprinol® can decrease the production of IL-6, TNF-α, IL-1α and IFN-γ in AIA rats.

Discussion

AIA is a loco-regional highly inflammatory experimental condition, with systemic repercussions. AIA in the rats is a standard model accepted for the study of inflammation, and its control by medications or supplements (2,8,15). The lipid extract of *Perna canaliculus* (the green-lipped mussel of New Zealand; Lyprinol®) is known as a powerful anti-inflammatory product in animal models and human diseases (asthma, arthritis). We used the AIA rat model in order to get a better understanding of the mechanisms resulting in the anti-inflammatory effects of Lyprinol®. We measured the levels of cytokines that are known to be pro-inflammatory: IL-1α, IL-6, TNF-α and IFN-γ. We compared results observed when using Lyprinol with the ones observed with sham, olive oil (negative control), and the non-steroid anti-inflammatory drug (NSAID) Naproxen (positive control); and we observed a group of rats that had no AIA as reference. We found that the group of rats given Lyprinol had a significantly decreased production of some of pro-inflammatory cytokines; this provides a partial explanation on how Lyprinol can help to control the symptoms related to inflammation (2, 4-7).

Lyprinol® did control the production of pro-inflammatory cytokines better than Naproxen in AIA rats. Extra-virgin olive oil was ineffective.

The AIA rat model has been extensively studied, both to assess the efficacy of medications and monitor inflammation-associated cytokines. For example, Anderson (16) found that SC-58125, a selective COX-2 inhibitor, inhibited IL-6 and IL-6 mRNA. Avramidis (17) found that grape melanin normalized elevated levels of IL-6 and TNF-α. Badger (18) found that idoxifene, a selective estrogen receptor modulator, reduced serum IL-6 levels in animals treated with 10 mg idoxifene/kg body weight/day. D-43787, a cyclosporine receptor-binding immunomodulator was found to inhibit LPS-induced IL-6 and TNF-α production (19). Bindarit, an inhibitor of MCP-1, was found to decrease TNF-α production after LPS induction (20). Kim et al. (21) described the antinociceptive and anti-inflammatory effects of ethylacetate extracts from Bang-Poon (*Radix lebouriellae*) on IL-6. Prophylactic and 6-day therapeutic treatment with FK506 (tacrolimus) was found to reduce the levels of IL-6 and TNF-α (22). An extract from an Indian plant, *Swertia chirayita*, was also found to reduce in a dose-dependent fashion, the levels of TNF-α, IFN-γ and IL-1, while IL-6 was only affected when higher doses (23.72 and 35.58 mg/kg) were administered (23). Magari and coworkers found that leflunomide inhibited anti-

CD3/CD28 induced production of TNF-α, IL-6 and IL-1, (24). Barsante et al. found that atorvastatin significantly decreased the concentrations of IL-6, TNF-α and IL-1, (25). The Chinese herbal preparation QFGJS was also reported to decrease significantly the serum levels of IL-6, TNF-α, IL-1α (26). A targeted DNA vaccine using naked DNA which encodes for TNF-α resulted in the generation of immunological memory to its gene product which effectively inhibits the development of AIA (27). Other studies used different assays to evaluate the control of production of these pro-inflammatory cytokines: for instance a novel inhibitor of p38 MAP kinase, TAK-715 (28) inhibited LPS-stimulated release of TNF-α from human monocytic THP-1 cells in vitro. The benefits observed in animal models associated with the reduction in the production of TNF-α resulted in the suggestion (29) of a beneficial association between pentoxiphylline and Lyprinol®, instead of low-dose prednisolone. All these previous studies on the same AIA animal model have been using substances that lack the impressive clinical baggage that Lyprinol carries, both in terms of efficacy, and safety.

Nevertheless, it should be stressed that AIA rats receiving Naproxen experienced multiple hemorrhagic ulcerations of the gastro-intestinal tract on post-mortem examinations (after harvest of the splenocytes) while the ones receiving Lyprinol® fared very well. Further, in our previous studies, rats taking Lyprinol recovered from AIA after one year of administration (14).

Conclusion

Administration of Lyprinol, the lipid complex of *Perna canaliculus*, to rats with adjuvant-induced arthritis resulted in a diminution of production of some cytokines (i.e. IL-6, IL-1α, TNF-α, and IFN-γ) known to be associated with inflammation. This effect is more pronounced than the one Fed with Naproxen (positive control), and much stronger than the one Fed with extra-virgin olive oil (other control). Our results suggest that further investigations on Lyprinol as a treatment of arthritis should be considered.

Acknowledgements

References

1. Allergie & Immunologie (Paris), 2000; 32: 259-294.
2. Lee CH, Lum JHK, Ng CKC, McKay J, Butt YKC, Wong MS, Lo SCL. Pain controlling and cytokine-regulating effects of Lyprinol, a lipid extract of *Perna canaliculus*, in a rat adjuvant-induced arthritis model. eCAM 2007;doi:10.1093/ ecam/nem100 Advance access published September 26, 2007.
3. Cho SH, Jung YB, Seong SC, Park HB, Byun KY, Lee DC, Song EK, Son JH. Clinical efficacy and safety of Lyprinol, a patented extract from New Zealand green-lipped mussel (*Perna canaliculus*) in patients with osteoarthritis of the hip and knee: A multicenter 2-month clinical trial. Allergie & Immunologie (Paris) 2003;35:212–6.
4. Gibson SLM, Gibson RG. The treatment of arthritis with a lipid extract of *Perna canaliculus*: a randomized trial. Complementary Therapies in Medicine 1998;6: 122.
5. Halpern GM. Anti-inflammatory effects of a stabilized lipid extract of *Perna canaliculus* (Lyprinol®). Allergie & Immunologie (Paris) 2000;32:272–8.
6. Lau CS, Chiu PKY, Chu EMY, Cheng IYW, Tang WM, Man RYK, Halpern GM. Treatment of knee osteoarthritis with Lyprinol®, lipid extract of the green-lipped mussel -a double-blind placebo-controlled study. Progress in Nutrition 2004;6: 17–31.
7. Rainsford KD, Whitehouse MW. Gastroprotective and anti-inflammatory properties of green-lipped mussel (*Perna canaliculus*) preparation. Arzneimittelforschung 1980;30:2128–32.
8. Whitehouse MW, Macrides TA, Kalafatis N. Anti-inflammatory activity of a lipid fraction (Lyprinol®) from the N. Z. green-lipped mussel. Inflammopharmacology 1997;5:237–246.
9. Gruenwald J, Graubaum HJ, Hansen K, Grube B. Efficacy and tolerability of a combination of Lyprinol and high concentrations of EPA and DHA in inflammatory rheumatoid disorders. Advances in Therapy 2004;21:197–201.
10. Emelyanov A, Fedoseev G, Krasnoschekova O, Abulimity A, Trendeleva T, Barnes PJ. Treatment of asthma with lipid extract of New Zealand green-lipped mussel: a randomised clinical trial. European Respiratory Journal 2002;20: 596–600.
11. Halpern GM. The Inflammation Revolution. Square One Publishers, Garden City, NY., USA. 2005.
12. Sinclair AJ, Murphy KJ, Li D. Marine lipids: overview: new insights, and lipid composition of Lyprinol®. Allergie & Immunologie (Paris) 2000;32:261–271.
13. Dugas B. Lyprinol inhibits LTB4 production by human monocytes. Allergie & Immunologie 2000;32:284–9.
14. Zimecki M, Weber-Dabrowska B, Lusiak-Szelachowska M, Mulczyk M, Boratynski J, Pozniak G, Syper D, Gorski A. Bacteriophages provide regulatory signals in mitogen-induced murine splenocyte proliferation. Cellular & Molecular Biology Letters

2003;8:699-711.

15. Whitehouse MW. Adjuvant-induced polyarthritis in rats. In: Greenwald RA, Diamond HS (Eds.) CRC Handbook of Animal Models for the Rheumatic Diseases Vol. 1, CRC Press, Miami, FL. 1996.

16. Anderson GD, Hauser SD, McGarity KL, Bremer ME, Isakson PC, Gregory SA. Selective inhibition of cyclooxygenase (COX)-2 reverses inflammation and expression of COX-2 and interleukin-6 in rat adjuvant arthritis. Journal of Clinical Investigation 1996;97:3672-9.

17. Avramidis N, Kourounakis A, Hadjipetrou L, Senchuk V. Anti-inflammatory and immunomodulating properties of grape melanin. Inhibitory effects on paw edema and adjuvant-induced disease. Arzneimittelforschung 1998;48:764-71.

18. Badger AM, Blake SM, Dodds RA, Griswold DE, Swift BA, Rieman DJ, Stroup GB, Hoffman SJ, Gowen M. Idoxifene, a novel selective estrogen receptor modulator, is effective in a rat model of adjuvant-induced arthritis. Journal of Pharmacology and Experimental Therapeutics 1999;291:1380-6.

19. Pahl A, Zhang M, Torok K, Kuss H, Friedrich U, Magyar Z, Szekely J, Horvath K, Brune K, Szlenyi I. Anti-inflammatory effects of a cyclosporine receptor-binding compound, D-43787. Journal of Pharmacology and Experimental Therapeutics 2002;301:738-46.

20. Guglielmotti A, D'Onofrio E, Coletta I, Aquilini L, Milanese C, Pinza M. Amelioration of rat adjuvant arthritis by therapeutic treatment with bindarit, an inhibitor of MCP-1 and TNF-alpha production. Inflammation Research 2002;51:252-8.

21. Kim HW, Kwon YB, Ham TW, Roh DH, Yoon SY, Han HJ, Kang SK, Lee HJ, Mar WC, Yang IS, Beitz AJ, Lee JH. The antinociceptive and anti-inflammatory effect of ethylacetate extracts from Bang-Poong (Radix lebouriellae) on the Freund's adjuvant-induced arthritis in rats. Journal of Veterinary Science 2002;3:343-9.

22. Magari K, Miyata S, Nishigaki F, Ohkubo Y, Mutoh S, Goto T. Differential effects of FK506 and methotrexate on inflammatory cytokine levels in rat adjuvant-induced arthritis. Journal of Rheumatology 2003;30:2193-200.

23. Kumar IV, Paul BN, Asthana R, Saxena A, Mehrotra S, Rajan G. Swertia chrayita mediated modulation of interleukin-1beta, interleukin-6, interleukin-10, interferon-gamma, and tumor necrosis factor-alpha in arthritic mice. Immunopharmacology & Immunotoxicology 2003;25:573-83.

24. Magari K, Miyata S, Nishigaki F, Ohkubo Y, Mutoh S. Comparison of anti-arthritic properties of leflunomide with methotrexate and FK506: effect on T cell activation-induced inflammatory cytokine production in vitro and rat adjuvant-induced arthritis. Inflammation Research 2004;53:544-50.

25. Barsante MM, Roffe E, Yokoro CM, Tafuri WL, Souza DG, Pinho V, Castro MS, Teixeira MM. Anti-inflammatory and analgesic effects of atorvastatin in a rat model of adjuvant-induced arthritis. European Journal of Pharmacology 2005;516:282-6.

26. Cai X, Zhou H, Wong YF, Xie Y, Liu ZQ, Jiang ZH, Bian ZX, Xu HX, Liu L. Suppressive effects of QFGJS, a preparation from an anti-arthritic herbal formula, on rat experimental adjuvant-induced arthritis. Biochemical and Biophysical Research Communications 2005;337:586-94.

27. Wildbaum G, Youssef S, Karin N. A targeted DNA vaccine augments the natural immune response to TNF-alpha and suppresses ongoing adjuvant arthritis. Journal of Immunology 2000;165:5860-6.

28. Miwatashi S, Arikawa Y, Kotani E, Miyamoto M, Naruo K, Kimura H, Tanaka T, Asahi S, Ohkawa S. Novel inhibitor of p38 MAP kinase as an anti-TNF-alpha drug: discovery of N-[4-[2-ethyl-(3-methylphenyl)-1,3- thiazol-5yl]-2- pyridyl]benzamide (TAK-715) as a potent and orally active anti-rheumatoid arthritis agent. Journal of Medicinal Chemistry 2005;48:5966-79.

29. Whitehouse MW. Anti-TNF-alpha therapy for chronic inflammation: reconsidering pentoxiphylline as an alternative to therapeutic protein drugs. Inflammopharmacology 2004;12:223-7.