Original Article

The Effect of Degradation on Biological Activity and Chemical Content of Lom-Am-Ma-Preuk Extract

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Abstract

Introduction: Lom-Am-Ma-Preuk (LAP) remedy is a herbal remedy in Traditional Thai Medicine scripture and is on the National List of Essential Medicine in Thailand. Many herbal ingredients of LAP have significant inhibitory activity against the production of inflammatory mediators. The aim of this current study was to investigate forced degradation of extracts by heat, moisture, oxidation, and acid hydrolysis. The effects of these degradations were determined by anti-inflammatory activity and chemical content analysis of the LAP extract. The identified chemical markers including eugenol, myristicin, and piperine were compared amongst the remedies.

Methods: Antiinflammatory activity was determined by an inhibitory effect on nitric oxide production as assessed through the Griess reaction and content markers were analyzed by high performance liquid chromatography.

Results: The forced degradation conditions which the extracts were treated with included oxidation, heat, moisture, and acid which did not affect the production inhibition of nitric oxide when compared with control (IC_{50} as 29.75, 35.69, 40.50, 48.04, and 40.37 µg/mL, respectively). Eugenol contents in LAP treated with acidic and alkaline treatments were significantly less than control (0.85 ± 0.11, 0.37 ± 0.11, and 2.28 ± 0.11 mg/g extract, respectively). Piperine content in the LAP extract which was treated with oxidation and acid was significantly less than control (24.71 ± 1.02, 25.69 ± 0.96, and 38.48 ± 0.74 mg/g extract, respectively) and myristicin content was not significantly different from controlled content after treatments.

Conclusions: Except under alkaline conditions the LAP ethanolic extract is stable under forced degradation conditions in terms of biological properties. Thus, the preparation of a LAP extract should avoid alkaline conditions in the production process. LAP extract is considered to be a potential candidate for further development into a topical product for analgesic relief.

Keywords: Lom-Am-Ma-Preuk, Forced degradations, Nitric oxide, HPLC

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Introduction

Lom-Am-Ma-Preuk (LAP) remedy is an ancient herbal remedy recorded in Thai scripture.[1] It ameliorates pain relief and is recommended by the National List of Essential Medicine (NELM) in Thailand.[2] The LAP extract has been studied for its anti-inflammatory effects such as production inhibition of inflammatory mediators including nitric oxide (NO), prostaglandin E2 (PGE_2) and TNF-α, which demonstrate significant anti-inflammatory activities through these pathways.[3] Many herbal ingredients of LAP have significant inhibitory activity against production of inflammatory mediators. These include *Alpinia galanga* (L.) Willd. and *Piper nigrum* L. which are active against NO production with IC_{50} values of 21.50 ± 0.09 μg/mL[4] and of 32.0 μg/mL, respectively.[5] *Cleome viscosa* Linn. has been shown to be potent in Fixed oil in doses of 75, 100, and 125 mg/kg body weight and reduced the numbers of writhes in a model of algesia by 91.69, 92.33, and 96.0%, respectively in rodents.[6] *Curcuma zedoaria* (Berg) Roscoe. β-turmeron and α- turmerone can inhibit nitric oxide from macrophages with values of IC_{50} values of 7.3 and 24.0 μg/mL respectively.[7] *Erythrina variegata* L. inhibits the production of (TNF-α), at an IC_{50} value of 9.27 ± 0.72 μg/mL.[3] *Myristica fragrans* Houtt. was effective in the production inhibition of NO, COX-2 and TNF-α, with IC_{50} values of 46.36 ± 1.53, 41.46 ± 1.06, and > 50 μg/mL, respectively. *Plumbago indica* L. was shown to have anti-inflammatory activity through NO and COX-2 production inhibition with IC_{50} values of 36.22 and 31.20 μg/mL, respectively.5 Lastly, *Zingiber cassumunar* Roxb. has been shown to have IC_{50} values of 16.90 ± 3.54 and 3.08 ± 0.34 μg/mL against production inhibition of PGE_2 and TNF-α, respectively.[3] Moreover, eugenol, myristicin, and piperine have been identified as markers for stability control of LAP.[8] The biological activity and chemical contents of LAP ethanolic extract are stable.[1] Eight out of 10 herbal ingredients in LAP showed *in vitro* anti-inflammatory activities. Galangal, nutmeg, and pepper as constituent ingredients in LAP have antiinflammatory activity against NO inhibitory production, so these plants contain active antiinflammatory compounds including eugenol, myristicin, and piperine, respectively. Thus, three compounds were used to be active markers for quality control of LAP remedy. In addition, these three compounds also showed the highest content in analysis determined by HPLC.[8] LAP extract has the potential for the development as a topical product for pain relief. For the force degradation study of the extract it is important and necessary to optimize and find suitable conditions for product development. Therefore, the aim of this study was to examine the effects of degradation on biological activity and chemical content of LAP extract.

Methods

Plant materials and extract preparation

The LAP remedy contains 10 herbs and 2 elements as shown in Table 1. All plant materials were purchased from various herbal shops in Bangkok. After washing, the plant materials were oven dried at 60°C for 24 hours, then ground to powder and mixed with the 2 elements according to the remedy ratio. The remedy powder was macerated with 95% ethanol with a 5:1 ratio. The extract was evaporated to dryness by a rotary evaporator (Table 1).

Stress test (Force degradation)

The LAP extract was exposed to 5 treatments including acid, alkaline, oxidation, moisture, and temperature. A 50 mg extract was placed in each test tube and the stress agent was added, and placed on a water bath at 80°C for 30 minutes (Table 2). Then, each degraded extract was tested for bioactivity and its chemical contents were measured by high performance liquid chromatography (HPLC).

Inhibitory effect on NO production in LPS induced RAW 264.7 cells

The RAW 264.7 cells (ATCC® TIB-71™) were grown on Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin, and incubated in an atmosphere of 95% air, and 5% carbon dioxide (CO_2), at 37°C. The cells were seeded at 1×10^6 cells per well in 96 well plate, and incubated for 24 hrs. The extract was diluted to give 4 dilutions (1, 10, 30, and 50 μg/mL) with 0.2% DMSO for testing. The old medium was removed, then added 100 μL of tested sample and 100 μL of 5 ng/mL LPS per well, incubated for 24 hrs. The measurement of NO production inhibition

was determined by Griess reagent, a 100 µL of supernatant was transferred to a new 96 well plate and 100 µL of Griess reagent was added. The developed color from the reaction was measured by a microplate reader at 570 nm.[9] The data were calculated as % inhibition as follow:

$$\% \text{ Inhibition} = \left(\frac{\text{A-B}}{\text{A}}\right) \times 100$$

A = O.D. control
B = O.D. sample
O.D. = optical density

Cell viability by MTT assay

This method was used to confirm that the production inhibition of No was not the result of cytotoxic activity of the tested samples. The cell culture protocol was similar to the above. The cells were seeded at 1×10^5 cell per well in a 96 well plate, and incubated for 24 hrs. The extract was diluted to give 4 dilutions (1, 10, 30, and 50 µg/mL) with 0.2% DMSO for testing. The old medium was removed, then added 100 µL of tested sample per well, incubated for 24 hrs. The measurement of cytotoxicity was determined by adding 10 µL MTT reagent, incubated for 2 hrs, then color measured from the reaction by a microplate reader at 570 nm.[10] The data was calculated as % viability as followed.

$$\% \text{ Viability} = \left[100-\left(\frac{\text{A-B}}{\text{A}}\right)\right] \times 100$$

A = O.D. control
B = O.D. sample

Determination of marker contents by HPLC

The mobile phase system was water-acetonitrile using gradient elution as follows: 0 minute, 95:5; 5 minutes, 95:5; 50 minutes, 50:50; 60 minutes 5:95; 65 minutes, 0:100; 65.10 minutes, 95:5; 70 minutes, 95:5. A 10 mg LAP extract that had undergone stress tests was dissolved in 1 ml of methanol and filtered through a 0.45 µm Nylon filter. The HPLC column was a Phenomenex Luna 5 µ C-18 100A analytical column (250 x 4.60 mm). The contents of anti-inflammatory markers i.e. eugenol, myristicin and piperine were determined within the range of concentration as follows; eugenol 5 - 50 µg/mL, myristicin 40 - 200 µg/mL, and piperine 200 - 900 µg/mL.[8]

Statistical analysis

The values of % inhibition, IC_{50} and contents were represented as the mean ± standard error of mean. The % inhibition values were calculated as IC_{50} in statistical software. The mean values were measured and analysed by the analysis of variance (one-way ANOVA). Analysis was performed for comparison between the control groups and the others. The $P < 0.05$ was considered as statistical significant.

Results

Inhibitory effect on NO production in LPS induced RAW 264.7 cells

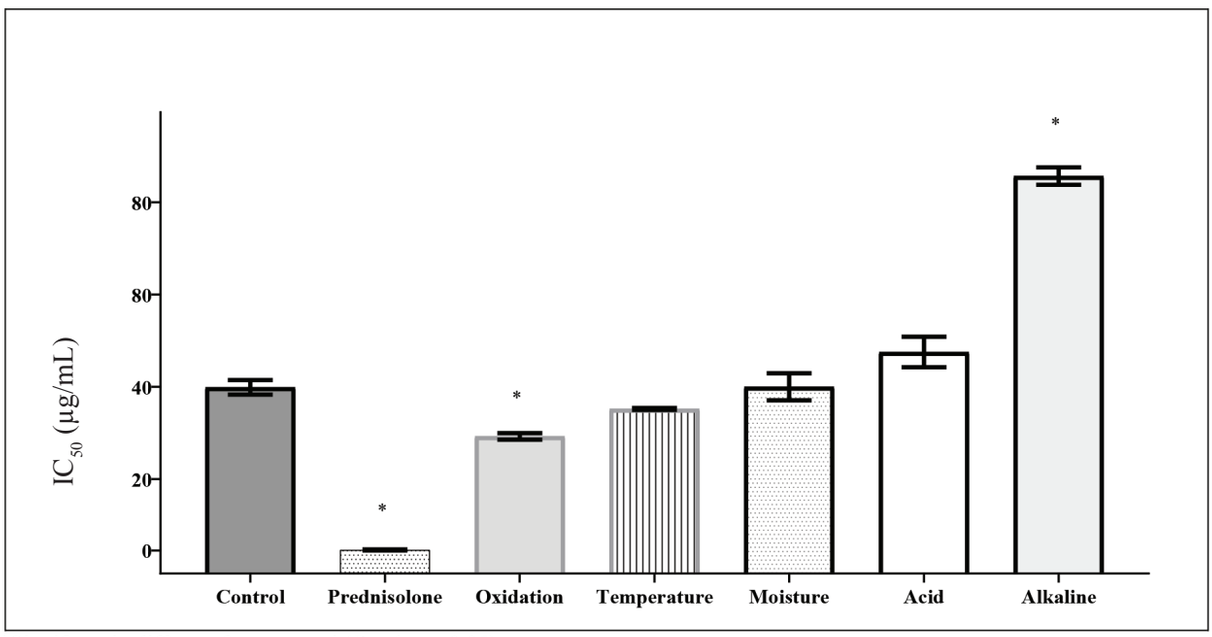
The control of the LAP extract and those undergoing forced degradation treatments at all tested concentrations showed > 70% of cell viability. LAP extract at concentration of 100 µg/mL showed a 91.05% reduction of NO production. The treatments with heat, moisture, oxidation, and acid conditions did not affect the inhibitory production of NO with percentage of 97.80, 95.94, 99.00, and 89.16, respectively, and were not significantly different from control. Only alkaline treatments rendered LAP extract to be less active with reduced percentage of NO production inhibition and increased IC_{50} value. Oxidation treatments gave significantly lower IC_{50} value than control (*P*-value < 0.05) (Table 4 and Figure 1).

Determination of chemical content by HPLC

The content of three markers in LAP were determined by a HPLC analytical method[8] which showed a linear relationship within the specified ranges for each marker (Table 3). The contents of eugenol, myristicin, and piperine in control LAP extract were 2.28 ± 0.11, 6.21 ± 0.34, and 38.48 ± 0.74 mg/g extract, respectively (Table 5).

Eugenol contents in LAP treated with acid and alkaline demonstrated significantly less anti-inflammatory activity than that of control (0.85 ± 0.11, 0.37 ± 0.11, and 2.28 ± 0.11 mg/g extract, respectively) (*P*-value < 0.05). The treatments with heat, oxidation, and moisture did not significantly reduce eugenol content. While piperine contents in LAP treated with oxidation and acid had significantly less content than that of control (24.71 ± 1.02, 25.69 ± 0.96, and 38.48 ± 0.74 mg/g extract, respectively) (*P*-value < 0.05). Other treatments did

not significantly alter piperine content from control (38.48 ± 0.74 mg/g extract) with less influence from temperature and moisture (36.28 ± 5.04 and 36.74 ± 1.86 mg/g extract, respectively). However, myristicin content was not significantly different from control in every treatment (Table 5).



*Significantly different from control (*P*-value < 0.05)

Figure 1 NO production inhibition activity of Lom-Am-Ma-Preuk extract after forced degradation (N = 3).

Table 1 Herbal ingredients in Lom-Am-Ma-Preuk remedy, and voucher specimen numbers

Species	Plant part	Voucher specimen number[(a)]	Traditional use[11]
Allium sativum L.	Bulbs	SKP 006 01 19 01	Antipyretic, Carminative
Alpinia galanga (L.) Willd.	Rhizome	SKP 206 01 07 01	Analeptic, Gynecology
Cleome viscosa L.	All of trunk	SKP 039-1 03 22 01	Promote blood circulation
Crateva adansonii DC.	Bark	SKP 039-1 03 01 01	Analgesic, Analeptic
Crateva religiosa G.Forst.	Bark	SKP 039-1 03 18 01	Analeptic, Promote blood circulation
Curcuma zedoaria (Berg) Roscoe.	Rhizome	SKP 206 03 26 01	Analgesic, Carminative
Erythrina variegata L.	Bark	SKP 206 01 07 01	Analeptic, Gynecology
Myristica fragrans Houtt.	Nutmeg	SKP 121 13 06 01	Cardio tonic, Carminative
Myristica fragrans Houtt.	Mace	SKP 121 13 06 01	Cardio tonic, Carminative
Piper nigrum L.	Seed	SKP 146 16 14 01	Carminative, Tonic for longevity
Plumbago indica L.	Root	SKP 148 16 09 01	Gynecology, Carminative
Zingiber cassumunar Roxb.	Rhizome	SKP 206 26 03 01	Analgesic, Gynecology

[(a)]Voucher specimen number were shown at Prince of Songkla University Herbarium (PSU)

Note: There were two natural chemicals in the formula such as camphor and rock salt in the same proportion.

Table 2 Forced degradation treatments by acid, alkaline, oxidation and moisture

Treatment*	Volume (mL) of each reagent
Acid (3 N HCl)	0.15 mL
Alkaline (3 N NaOH)	0.15 mL
Oxidation (3 N H_2O_2)	0.15 mL
Moisture (Distilled water)	0.15 mL

*Each treatment conditions was at 80°C for 3 hours.

Table 3 The linear equations for determination of markers

Standard markers	Range of concentrations (μg/mL)	Linear equations
Eugenol	5 - 50	$y = 53.336x - 25.999$ ($R^2 = 0.9998$)
Myristicin	40 - 200	$y = 139.02x - 790.75$ ($R^2 = 0.9997$)
Piperine	200 - 900	$y = 22.935x - 903.99$ ($R^2 = 0.9997$)

Table 4 The production inhibition (%) of NO production from RAW 264.7 cells at various concentrations of LAP extract under forced degradation treatments, IC_{50} (µg/mL) and % cell viability (N = 3)

Treatment	%NO production inhibition %cell viability							IC_{50} ± SEM (µg/mL)
	0.01 (µg/mL)	0.1 (µg/mL)	1 (µg/mL)	10 (µg/mL)	30 (µg/mL)	50 (µg/mL)	100 (µg/mL)	
Control	-	-	-37.75 ± 3.90 (91.07 ± 6.67)	-4.87 ± 2.09 (93.54 ± 12.28)	33.90 ± 2.23 (82.68 ± 3.53)	64.37 ± 3.39 (89.59 ± 1.62)	91.05 ± 0.86 (90.19 ± 4.20)	40.37 ± 1.59
Heat	-	-	-2.45 ± 1.01 (98.35 ± 2.93)	6.04 ± 1.51 (99.47 ± 5.18)	38.84 ± 0.40 (88.66 ± 8.44)	75.37 ± 1.28 (98.01 ± 13.25)	97.80 ± 0.50 (92.08 ± 5.46)	35.69 ± 0.22
Moisture	-	-	-2.51 ± 1.26 (99.89 ± 3.49)	3.36 ± 2.28 (99.16 ± 0.43)	32.13 ± 4.63 (91.06 ± 1.64)	64.51 ± 4.53 (99.87 ± 2.15)	95.94 ± 1.94 (97.52 ± 9.06)	40.50 ± 2.94
Oxidation	-	-	0.09 ± 1.67 (94.28 ± 7.97)	6.86 ± 0.35 (96.35 ± 4.06)	50.71 ± 1.80 (89.07 ± 1.16)	87.39 ± 1.46 (93.26 ± 10.46)	99.00 ± 0.16 (78.92 ± 4.93)	29.75 ± 0.71*
Acid	-	-	-6.94 ± 2.43 (90.04 ± 2.39)	0.45 ± 2.69 (87.92 ± 4.57)	24.75 ± 3.13 (89.92 ± 4.15)	53.30 ± 4.84 (95.27 ± 1.44)	89.16 ± 3.63 (92.74 ± 1.02)	48.04 ± 3.30
Alkaline	-	-	-11.91 ± 3.86 (90.51 ± 1.67)	-5.87 ± 4.96 (98.78 ± 6.51)	12.39 ± 1.14 (91.47 ± 9.77)	27.61 ± 0.59 (86.76 ± 4.86)	58.47 ± 2.32 (86.36 ± 5.29)	86.20 ± 1.89*
prednisolone	3.17 ± 3.08 (92.37 ± 1.82)	42.48 ± 1.59 (76.11 ± 1.36)	70.75 ± 0.88 (77.17 ± 2.62)	73.89 ± 0.51 (71.89 ± 1.16)	-	90.81 ± 4.32 (71.20 ± 0.55)	-	0.12 ± 0.01*

*Significantly different from control (*P*-value < 0.05)

Table 5 Determination of markers in Lom-Am-Ma-Preuk extracts after forced degradations analyzed by HPLC

Treatments	Content of markers (mg/g extract)[a]		
	Eugenol[b]	Myristicin[b]	Piperine[c]
Control	2.28 ± 0.11	6.21 ± 0.34	38.48 ± 0.74
Acid	0.85 ± 0.11*	4.44 ± 0.52	25.69 ± 0.96*
Alkaline	0.37 ± 0.11*	4.61 ± 0.65	27.49 ± 2.37
Temperature	1.67 ± 0.36	5.31 ± 0.48	36.28 ± 5.04
Oxidation	1.32 ± 0.05	4.76 ± 0.21	24.71 ± 1.02*
Moisture	1.81 ± 0.22	5.69 ± 0.05	36.74 ± 1.86

[a] Mean ± S.E.M
[b] Determination at 210 nm
[c] Determination at 256 nm
*Significantly different from control (*P*-value < 0.05)

Discussion

Lom-Am-Ma-Preuk (LAP) remedy is listed in the National List of Essential Medicine (NELM), and has been traditionally utilized as a therapy for pain relief. The LAP extract has been demonstrated its anti-inflammatory activity by *in vitro* production inhibition of NO, PGE_2, COX-2 and TNF-α.[3] Eight out of ten herbal ingredients of LAP had significant inhibitory activity against production of inflammatory mediators. Therefore, LAP extract was considered as a potential candidate for the development of a topical pain relief product. Forced degradation is a powerful tool used routinely in pharmaceutical development in order to develop stability indicating methods that lead to quality stability data and to understand the degradation pathways of the drug substances and drug products. The results of this study demonstrate that alkaline conditions have a detrimental effect on anti-inflammatory activity of LAP extract and should be avoided during formulation and storage. Oxidation appeared to have beneficial effects as to anti-inflammatory activity as shown by a significantly reduced IC_{50} value and higher antiinflammatory effect under oxidation conditions. The contents of eugenol and piperine were affected by certain forced degradation treatments, eugenol content was lowered by acid and alkaline conditions, while piperine content was reduced significantly by acid and oxidation. In the previous study, eugenol decreased inducible nitric oxide synthase (iNOS), which is pathologically generated from NO in inflammatory process.[12] Eugenol is involved in NO production inhibition. Acid and alkaline treatments showed reduced eugenol content compared to controls which significantly decreases the effect of NO production inhibition. It might be assumed that Eugenol is a suitable biological and chemical marker for preparation and development of LAP extracts especially in NO inhibition activity. Piperine inhibited nitric oxide with an IC_{50} value of 70.25 μg/mL[3] and demonstrated efficacy in reducing TNF-α levels in animal studies.[13] Piperine exerted enhanced production inhibition of TNF- α than NO. Acid and oxidation treatments reduced piperine content compared to control. However, the decrease in piperine content did not affect NO production inhibition activity. The IC_{50} value of control and acid treatment were not significantly different which is consistent with previous findings. Myristicin appeared to be relatively more stable and an appropriate marker for LAP extracts. The results of *in vitro* evaluation of anti-inflammatory activity also suggested that this activity was due to these 3 active markers and other compounds as evident by its stability in biological activity of LAP extract except under alkaline condition. Further studies on the chemical composition of LAP extract under oxidation conditions are an avenue of investigation that requires greater scientific scrutiny and studies.

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References

1. Foundation of Thai Traditional Medicine and Ayuvedathamrong school. Tamrakarnphaetthaideim (Phaetthayasat songkhro conservation issue). Bangkok, Thailand: Supawanichkarnpimm; 2007.
2. The Thai National List of Essential Committee. *The Thai National List of Essential Herbal Medicine.* Nonthaburi, Thailand: Ministry of Public Health; 2007.
3. Thongmee P. Anti-inflammatory and antioxidant activity of Thai traditional remedy for stroke called Lom-Am-Ma-Pruek and its plant ingredients. [Master's thesis]. Thammasat University, Pathum Thani, Thailand: Faculty of Medicine; 2015.
4. Anuthakoengkun A, Makchuchit S, Itharat A. Inhibitory effect on nitric oxide production and antioxidant activity of Thai medicinal plants of Zingiberaceae Family for osteoarthritis treatment. Poster presented at: Thammasat national seminar academic, 2012; Pathum Thani, Thailand.
5. Kakatum, N. Anti-inflammatory activity of Thai traditional remedy extract for muscle pain treatment called Sahastara and its plant ingredients. [Master's thesis]. Thammasat University, Pathum Thani, Thailand: Faculty of Medicine; 2011.
6. Gene RM, L. Segura, T Adzet, E Marin, J Iglesias. *Heterotheca inuloides*: Anti-inflammatory and analgesic effects. *J Ethnopharmacol*. 1998;60:157-162.
7. Kim KI, Shin KS, Jun WJ, et al. Effects of Polysaccharides from rhizomes of *Curcuma zedoaria* on macrophage functions. *Biosci Biotechnol Biochem.* 2014;65(11):2369-2377.
8. Piwngam K, Kanokkangsadal P, Pipatrattanaseree W, et al. Validated High Performance Liquid Chromatographic (HPLC) Method for anti-inflammation activity of Lom-Am-Ma-Preuk Remedy. *STA*. 2020;25(3):60-67.
9. Anuthakoengkun A, Itharat A. Inhibitory effect on nitric oxide production and free radical scavenging activity of Thai medicinal plants in osteoarthritic knee treatment. *J Med Assoc Thai.* 2014;97(S1):16-24.
10. Ongthanasup T, Kanokkangsadal P, Panthong S, Itharat A. Anti-inflammatory activity of extracts from Thai herbal compress ball and its plant ingredients. *STA*. 2020;25(3):68-77.
11. Foundation of Thai Traditional Medicine and Ayuvedathamrong school (Chivokkomaraphat). Thai Pharmaceutical book (Tam ra p̣hesạtchakarm thai). Bangkok, Thailand: Supawanichkarnpimm; 2016.
12. Li W, Tsubouchi R, Qiao S, Haneda M, Murakami K, Yoshino M. Inhibitory action of eugenol compounds on the production of nitric oxide in RAW264.7 macrophages. *BioMed Res. Int.* 2006;27(2):69-74.
13. Pradeep CR, Kuttan G. Effect of piperine on the inhibition of nitric oxide (NO) and TNF-alpha production. *Immunopharmacol Immunotoxicol*. 2003;25(3):337-346.