Original Article

Nitric oxide inhibitory activity of herbal extract formulae for anti-inflammation

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Abstract

Introduction: The objective of this research was to investigate anti-inflammatory activity of herbal extracts formulae from four plants, *Zingiber cassumunar* (ZC), *Centella asiatica* (CA), *Zingiber officinale* (ZO) and *Piper nigrum* (PN) by variable proportion of each plant. The active compound(s) contributing to its anti-inflammatory activity of best formula was verified using high performance liquid chromatography (HPLC) method.

Method: Inhibitory activity against lipopolysaccharide (LPS) induced nitric oxide production in RAW 264.7 cell lines was used as assay for anti-inflammation. Each plant and five formulae (CF1-CF5) were extracted by maceration in 95% ethanol.

Result: The ethanolic extract of CF2 and CF4 showed the highest nitric oxide inhibitory activities with IC_{50} value of 7.83 and 7.87 μg/ml, respectively. Three plant extracts, PN, ZO and ZC also exhibited high nitric oxide inhibitory activities with IC_{50} value of 10.52, 11.93 and 21.33 μg/ml, respectively. The studies on chemical constituents using high performance liquid chromatography (HPLC) showed that piperine and (*E*)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD) were a major compound in the combine formula 2 and 4 extracts. Terpinine-4-ol, 6-gingerol and asiatic acid were a minor compound in the extracts.

Discussion and Conclusion: Combination of herbal extract such as CF2 and CF4 formulae possessed good nitric oxide inhibitory activity which was higher than single plant extract.

Key words: Anti-inflammatory, Nitric oxide, Herbal extract formulae, *Zingiber cassumunar, Centella asiatica, Zingiber officinale, Piper nigrum*

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Introduction

Inflammation is a process involving multiple factors acting in a complex network. The ingress of leukocytes into the site of inflammation is crucial for the pathogenesis of inflammatory conditions. Neutrophils and macrophages are known to recruit and play pivotal roles in acute and chronic inflammation, respectively. At the inflamed site, the recruited cells are activated to release many inflammatory mediators, such as prostaglandins (PGs), kinins serotonin, cyclooxygenase (COXs), tumor necrosis factor alpha (TNF-α) and nitric oxide, which elicit the initiation and maintenance of an inflammatory response, causing a change from the acute phase to the chronic phase of inflammation[1].

There are many previous reports about anti-inflammation activity of *Zingiber cassumunar, Centella asiatica, Zingiber officinale* and *Piper nigrum*[2 - 5]. Thai traditional doctors also used these plants for the treatment of inflammation, such as arthritis, muscle and joint pain and bruised. Some of plants also used as single drug or combination drug as analgesic formulae. Surprisingly, there has not been any analgesic formulae from combination of these plants.

Thus, the objectives of this study were to investigate the anti-inflammatory activity of four plants, *(Zingiber cassumunar, Piper nigrum, Zingiber officinale and Centella asiatica)* by using LPS induced nitric oxide production in RAW 264.7, and create the formulae from combination of these plants by variable proportion to discover the best anti-inflammatory formula. The active compound(s) contributing to its anti-inflammatory activity of best formula was verified using HPLC method.

Method

Chemicals and reagents

RAW 264.7 murine macrophage leukemia cell lines were established and kindly provided by Assoc Prof Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. RPMI Medium 1,640 (RPMI 1,640) powder with L-glutamine, Fetal Bovine Serum (FBS), Penicillin-Streptomycin (P/S), trypsin-EDTA and trypan blue were purchased from Gibco (USA). Phosphate Buffer Saline (PBS) was from Amresco (USA); sodium bicarbonate was from BDH, England; lipopolysaccharide (LPS, from *Escherichia coli*), 3-(4,5- dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were from Sigma. Ninety six well microplates were also purchased from Costar Corning (USA). Standard piperine was purchased from Merck (Germany); 6-gingerol purchased from Wako (Japan); asiatic acid was from Chromadex (USA), Terpinine-4-ol and (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD) were isolated from *Zingiber cassumunar*. HPLC reagents, acetronitrile, methanol and purified water, were purchased from Labscan (Thailand).

Plant materials

Four plants, *Zingiber cassumunar* rhizome (ZC), *Piper nigrum* fruit (PN), *Zingiber officinale* rhizome (ZO) and *Centella asiatica* leaf (CA) were cleaned and dried at 50 °C, powdered and extracted by maceration three times with 95% ethanol for 3 days each. The extracts were concentrated under reduced pressure by rotary evaporator. Each plant was combined in variable proportion by weight to give five formulae, combine formula 1 (ZC:PN:ZO:CA – 1:1:1:1), combine formula 2 (ZC:PN:ZO:CA – 2:1:1:1), combine formula 3 (ZC:PN:ZO:CA – 1:2:1:1), combine formula 4 (ZC:PN:ZO:CA – 1:1:2:1), combine formula 5 (ZC:PN:ZO:CA – 1:1:1:2) and extracted by the same method. The extracts were calculated for percentage of yield and dissolved in dimethyl sulfoxide (DMSO) before testing.

Anti-inflammatory activity by determining LPS induced nitric oxide production in RAW 264.7[6]

The murine macrophage cells (RAW 264.7) were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% P/S in 96-wells plate with 1×10^5 cells/well at 37 °C in 5% CO_2 unless otherwise stated. The extracts were prepared at a concentration of 10 mg/ml in DMSO as stock solution and diluted to 3, 10, 30 and 100 μg/ml with complete media. Cells were stimulated

with 5 μg/ml lipopolysaccharide (LPS, Sigma) having test samples at various concentration for 48 hours. Nitric oxide (NO) production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent (0.1% naphthalene diamine dihydrochloride, 1% sulfanilamide in 5% H_2SO_4). Cytotoxicity testing was also tested to ensure that nitric oxide production was not produced by destroying cell membrane (% cytotoxicity less than 30%). This testing used MTT assay or the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method. The absorbance was determined at 540 nm. The IC_{50} was calculated using Prism program. Indomethacin was used as standard compound in this study.

Determination of active constituents in extracts using HPLC

Studies on chemical fingerprint and quantification of active constituents were carried out using a High performance liquid chromatography (HPLC) system (Agilent® LC 1100/1200 system), with photodiode array (PDA) detector (model G1315D) and automatic injector (model G1329A). A reversed-phase column was ZORBAX Eclipse XDB-C18 column (4.6 x 250 mm, 5 micron) protected by Eclipse XDB-C18 analytical guard cartridge (4.6 x 12.5 mm, 5 micron). The mobile phase was mixture of water (A) and acetronitrile (B) with gradient elution as follow: 0 min: 40% B, 30 min: 50% B, 50 min: 90% B and 60 min: 100% B. The flow rate was 1 ml/minute with detection at 206 nm. The operating temperature was maintained at room temperature (25 °C). Data were analyzed by ChemStation® software.

Table 1 Nitric oxide inhibitory activity of ethanolic extracts of each plant and five formulae

Plant	Used part	Code	% Yield	IC_{50} (μg/ml)
Z. cassumunar	Rhizome	ZC	5.35	21.33 ± 2.17
P. nigrum	Fruit	PN	6.46	10.52 ± 0.68
Z. officinale	Rhizome	ZO	5.72	11.93 ± 1.29
C. asiatica	Leaf	CA	11.05	57.28 ± 7.16
Combine formula 1	-	CF1	11.47	18.60 ± 0.12
Combine formula 2	-	CF2	7.90	7.83 ± 0.87
Combine formula 3	-	CF3	10.02	11.82 ± 0.26
Combine formula 4	-	CF4	8.06	7.87 ± 0.05
Combine formula 5	-	CF5	8.45	23.06 ± 2.92
Terpinine-4-ol	-	-	-	> 100
DMPBD	-	-	-	> 100
Asiatic acid	-	-	-	> 100
6-Gingerol	-	-	-	44.57 ± 1.32
Piperine	-	-	-	11.48 ± 1.58
Indomethacin	-	-	-	20.32 ± 3.28

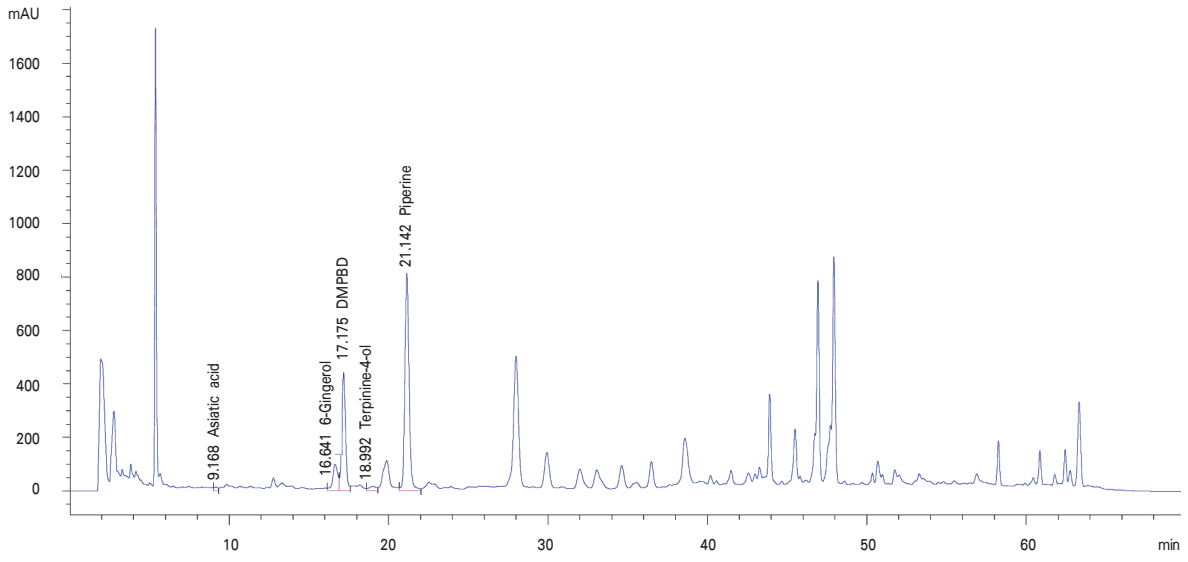

Figure 1 HPLC chromatogram of combine formula 2 (CF2)

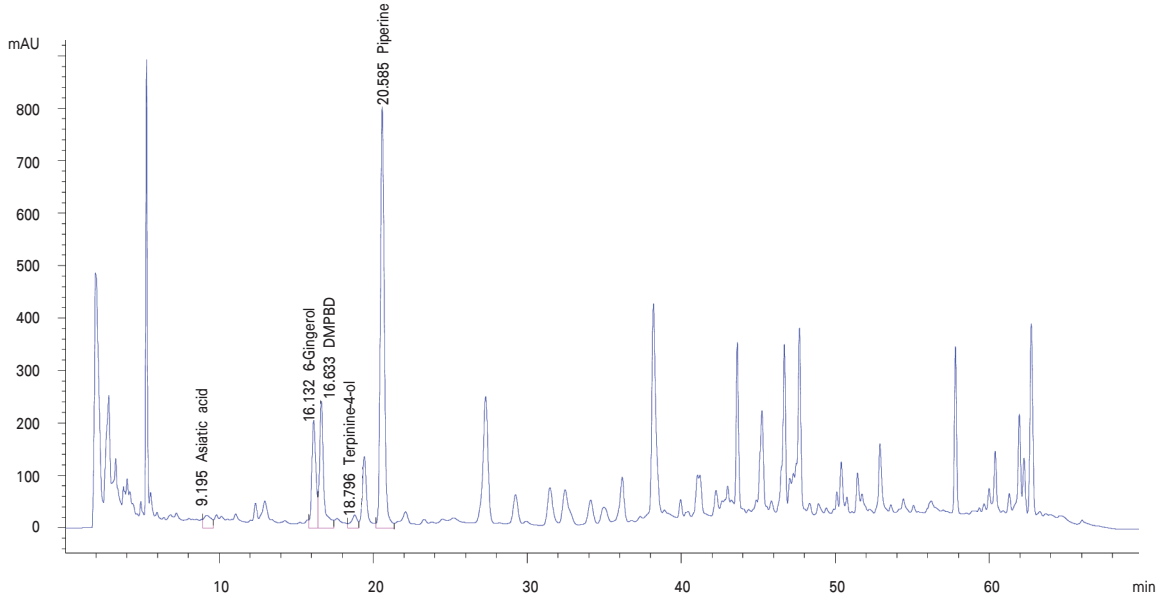

Figure 2 HPLC chromatogram of combine formula 4 (CF4)

Table 2 Determination of active constituents in each plant and combine formula 2 and 4 extracts using HPLC

Extracts	Contents (mg/g of extract)				
	Terpinine-4-ol	DMPBD	Piperine	6-Gingerol	Asiatic acid
Z. cassumunar	22.92 ± 0.08	93.12 ± 0.04	-	-	-
P. nigrum	-	-	264.80 ± 0.44	-	-
Z. officinale	-	-	-	21.60 ± 0.93	-
C. asiatica	-	-	-	-	5.61 ± 0.12
CF2	6.68 ± 0.97	26.11 ± 0.28	55.88 ± 0.37	4.22 ± 0.14	12.10 ± 0.25
CF4	8.05 ± 0.54	16.68 ± 0.35	53.37 ± 0.21	7.29 ± 0.04	16.19 ± 0.41

Result

The results of nitric oxide inhibitory activity showed in Table 1. It was found that the ethanolic extract of CF2 and CF4 showed the highest nitric oxide inhibitory activities (IC_{50} value of 7.83 and 7.87 μg/ml, respectively) which was better than indomethacin (IC_{50} = 20.32 μg/ml). Three plant extracts in formula, PN, ZO and ZC also exhibited high nitric oxide inhibitory activities with IC_{50} value of 10.52, 11.93 and 21.33 μg/ml, respectively. The results revealed that combination of herbal extract such as CF2 and CF4 formulae possessed higher nitric oxide inhibitory activity than single plant extract. Piperine, a major compound in *Piper nigrum*, showed highest (IC_{50} = 11.48 μg/ml) and 6-gingerol, a major pungent compound in *Zingiber officinale*, showed moderate (IC_{50} = 44.57 μg/ml) nitric oxide inhibitory activity, but terpinine-4-ol, DMPBD (found in *Zingiber cassumunar*) and asiatic acid (found in *Zingiber cassumunar*) were apparently inactive (IC_{50} > 100 μg/ml).

Studies on chemical fingerprint and quantification of active constituents using a HPLC showed in figure 1, 2 and table 2. It was apparent that piperine and DMPBD were major compounds in the combine formula 2 and 4 extracts. Terpinine-4-ol, 6-gingerol and asiatic acid were minor compounds in the extracts.

From these results, piperine, 6-gingerol and DMPBD should be used as markers for standardization of extract because piperine was present in high amount and with high nitric oxide inhibitory activity, while 6-gingerol had moderate activity. The previous report of DMPBD, a major compound in *Zingiber cassumunar*, also showed anti-inflammatory activity by inhibiting COX-I and COX-II enzyme so that it should be selected to use as marker too[7].

Discussion and Conclusion

In summary, the results indicated that the combination of herbal formulae form these plants could inhibit nitric oxide production as one of inflammation pathway. These results supported use of these preparation to relief pain from inflammation. However, it should be confirmed by another anti-inflammation assay such as COX-I, COX-II, LOX-5 and TNF-α inhibitor and test all of these plants and formulae *in vivo*.

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References

1. Laupattarakasem P, Wangsrimongkol P, Surarit R, Hahnvajanawon C. In vitro and in vivo anti-inflammatory potential of *Cryptolepis buchanani*. J Ethnopharmacol 2006;108:349-54.
2. Jeenapongsa R, Yoovathaworn K, Sriwatanakul KM, Pongprayoon U, Sriwatanakul K. Anti-inflammatory activity of (E)-1-(3,4-dimethoxyphenyl) butadiene from *Zingiber cassumunar* Roxb. J Ethnopharmacol 2003;87:143-8.
3. Dunstan CA, Noreen Y, Serrano G, Cox PA, Perera P, Bohlin L. Evaluation of some Samoan and Peruvian medicinal plants by prostaglandin biosynthesis and rat ear oedema assays. J Ethnopharmacol 1997;57:35-56.
4. Shen CL, Hong KJ, Kim SW. Effects of ginger (*Zingiber officinale* Rosc.) on decreasing the production of inflammatory mediators in sow osteoarthritic cartilage explants. J Med Food 2003;6:323-8.
5. Pooja S, Agrawal R, Nyati P, Savita V, Phadnis P. Analgesic activity of *Piper Nigrum* extract per se and its interaction with diclofenac sodium and pentazocine in albino mice. The Int J Pharmacol 2007;5:30.
6. Tewtrakul S, Itharat A. Nitric oxide inhibitory substances from the rhizomes of *Dioscorea membranacea*. J Ethnopharmacol 2007;109:412–6.
7. Tangyuenyongwatana P, Jongkon N, Sangma C, Gritsanapan W. Cyclooxygenase assays and molecular modeling study of some chemical constituents from *Zingiber cassumunar*. J Thai Trade & Alt Med 2009; 7:139.

บทคัดย่อ

การทดสอบฤทธิ์ยับยั้งการหลั่งไนตริกออกไซด์ของสารสกัดสมุนไพรผสม

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สาขาการแพทย์แผนไทยประยุกต์ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์ จังหวัดปทุมธานี

บทนำ: การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านการอักเสบของสารสกัดเอทานอลของสมุนไพร ๔ ชนิด ได้แก่ เหง้าไพล *(Zingiber cassumunar)*, ใบบัวบก *(Centella asiatica)*, เหง้าขิง *(Zingiber officinale)* และเมล็ดพริกไทย *(Piper nigrum)*

วิธีการศึกษา: ใช้วิธีการทดสอบฤทธิ์ยับยั้งการหลั่งไนตริกออกไซด์จากเซลล์แมคโครฟาจของหนู ซึ่งถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์ (LPS) ทั้งแบบที่เป็นสมุนไพรเดี่ยวและแบบที่นำสมุนไพรทั้ง ๔ ชนิดมาผสมในอัตราส่วนที่แตกต่างกัน ๕ สูตร (CF1-CF5)

ผลการศึกษา: ผลการทดลองพบว่า สารสกัดสมุนไพรผสมสูตรที่ ๒ และ ๔ สามารถยับยั้งการหลั่งไนตริกออกไซด์ได้สูงที่สุด โดยมีค่าความสามารถในการยับยั้งร้อยละ ๕๐ (IC_{50}) เท่ากับ ๗.๘๓ และ ๗.๘๗ ไมโครกรัม/มิลลิลิตร ตามลำดับ นอกจากนั้นสมุนไพรเดี่ยวที่เป็นองค์ประกอบของตำรับ ได้แก่ เมล็ดพริกไทย เหง้าขิง และเหง้าไพล พบฤทธิ์ยับยั้งการหลั่งไนตริกออกไซด์ที่สูงเช่นกัน โดยมีค่าความสามารถในการยับยั้งร้อยละ ๕๐ (IC_{50}) เท่ากับ ๑๐.๕๒, ๑๑.๙๓ และ ๒๑.๓๓ ไมโครกรัม/มิลลิลิตร ตามลำดับ จากการศึกษาองค์ประกอบทางเคมีพบว่าสารสกัดสมุนไพรผสมสูตรที่ ๒ และ ๔ มีสาร piperine และ DMPBD เป็นองค์ประกอบหลัก และพบสาร terpinine-4-ol, 6-gingerol และ asiatic acid ในปริมาณเล็กน้อย

วิจารณ์ และสรุปผลการศึกษา: ผลการทดสอบแสดงให้เห็นว่า สารสกัดสมุนไพรในรูปแบบผสม เช่น สูตร CF2 และ CF4 มีฤทธิ์ยับยั้งการหลั่งไนตริกออกไซด์ดีกว่าสมุนไพรเดี่ยว

คำสำคัญ: ฤทธิ์ต้านการอักเสบ, ไนตริกออกไซด์, สารสกัดสมุนไพรผสม, เหง้าไพล, ใบบัวบก, เหง้าขิง, เมล็ดพริกไทย