Antioxidant and Anti-inflammatory Activities of Thai Medicinal Plants in Sahasthara Remedy for Muscle Pain Treatment

Narin Kakatum MSc*, Nuanjan Jaiarree PhD**
Sunita Makchucit MSc**, Arunporn Itharat PhD**

** Graduate School, Faculty of Medicine, Thammasat University, Pathumthani, Thailand*
*** Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathumthani, Thailand*

Objective: *Twenty one plants as ingredients of Thai traditional preparation called Sahasthara for relieve muscles pain and distal numbness were studied for their antioxidant and anti-inflammatory activities.*
Material and Method: *The extracts were obtained by maceration with 95% ethanol. They were tested for their antioxidant activity by DPPH scavenging assay and anti-inflammatory activity by determination of inhibitory activity on lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW 264.7 cell lines using Griess reagent.*
Results: *The ethanolic extract of Terminalia chebula Retz. (Gall) showed the highest antioxidant activity (EC_{50} = 3.34 µg/ml). Its fruit extract also exhibited the most potent inhibitory activity on lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW 264.7 cells, followed by Atractylodes lancea and Baliospermum montanum (IC_{50} = 3.3, 9.7 and 12.6 µg/ml, respectively).*
Conclusion: *The results obtained for anti-inflammatory and antioxidant activities of these plants correspond with their traditional use for muscle pain and inflammatory-related diseases.*

Keywords: *Antioxidant, Nitric oxide inhibition assay, Anti-inflammation, Muscle pain, Sahasthara*

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Thai traditional medicine is a popular used for relieve pain for long time ago such as Thai massage, Thai herbal compress balls and herbal drugs. There are many Thai plants and Thai remedy which were used for relieve pain nowadays. They were studied on anti-inflammatory assay for supporting using in Thai traditional medicine. The related assay which was used for determination about anti-inflammation activity *in vitro* screening as the evaluation on the nitric oxide inhibition activity because nitric oxide is an inorganic free radical and also is inflammatory mediator. The high concentration of nitric oxide (NO) play a role in pathogenesis of vasodilatation, acute and chronic inflammation[1,2]. Nitric oxide is produced from L-argenine and chemical reaction which was stimulated by nitric oxide synthase enzyme (iNOS) or bacterial lipopolysaccharide (LPS) in living macrophage cells[3,4]. Thus, evaluation of anti-inflammation by determination on inhibitory NO production is a popularly anti-inflammatory method for screening on plant extracts. In addition, the reactive oxygen species (ROS) is also causes of acute inflammation, because their over production of ROS are also cause of tissue damage and inflammatory diseases[3,4]. Thus, the objectives of this research were to investigate antioxidant and anti-inflammatory activities of plant components of a Thai traditional preparation called Sahasthara which is a popular drug used to treat muscle pain, gout and arthritis in Thailand more than thirty years ago. This preparation is also a drugs in the National List in Essential Medicine 2011 of Thailand. Its formula consists of twenty one plants and pepper (*Piper nigrum* Linn) is a main ingredient[5]. Its plants ingredients were investigated on anti-inflammatory activity by nitric oxide inhibition assay and also tested antioxidant activity by DPPH assay. These results can support for Ministry of Public Heath of Thailand for using each plant ingredient in

Correspondence to:
Itharat A, Department of Applied Thai Traditional Medicine Centre, Faculty of Medicine, Thammasat University, Pathumthani 12120, Thailand.
Phone: 0-2926-9749
E-mail: iarunporn@yahoo.com

this preparation for muscle pain treatment.

Material and Method

Plant materials

Each plant components of Sahasthara remedy were collected and purchased from several regions of Thailand and foreign country. Authentications of plants were carried out and keep at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand for identification, part used of plant ingredients and previously-reported biological activities related with inflammation are shown in Table 1.

Preparation of the plant extracts

Each plant ingredients of Sahasthara was cleaned, dried in an oven at 50°C and grinded to be rough powder. Maceration with 95% ethanol was used for extraction for 3 days per cycle, then filtered and dried the filtrate by evaporator. The residue after maceration was re-extracted with a second lot of ethanol. The filtrates from both extractions were combined and then dried using an evaporator. The percentage yield for each sample is shown in Table 2.

DPPH radical scavenging assay

The method of DPPH radical scavenging assay was evaluated by using a modification of a published method[4,42]. The briefly explanation, the plant extracts were dissolved in absolute ethanol and also diluted as 5 concentration (100, 50, 10, 5 and 1 µg/ml). Sample solution in each concentration (100 µl) was mixed with an equal volume of 6×10^{-5} M DPPH (in absolute ethanol) in 96 well plate and allowed to stand at room temperature for 30 min. After that they were measured by spectrophotometer at 520 nm. Butylated hydroxytoluene (BHT), a well known synthetic antioxidant, was used as a positive control.

Anti-inflammatory activity on NO inhibition assay

The anti-inflammatory assay followed by the previously publication[4]. The determination on inhibitory effect of NO production by murine macrophage (RAW 264.7 cells). In briefly, the cells were cultured in RPMI 1640 medium supplemented with 10% heated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C in 5% CO_2 atmosphere with 95% humidity. The viable cells were counted and diluted with medium to give a final concentration as 1×10^6 cells/ml. These cell suspensions (100 µl/well) were seeded in each well of a 96-well plate and allowed to adhere for 1 h at 37°C under 5% CO_2. After that, the medium was replaced with fresh medium containing 5 mg/ml of LPS and followed by adding sample solution at various concentrations. The cells in 96 well plate was incubated for 48 h. After exposure time, the culture media were determined the NO production by using the Griess reagent. In the same time, cells on 96 well plant were tested on cytotoxic assay by using MTT assay. The concentration which showed more 70-80% viable cells comparing with control can be determined NO production. IC_{50} values was calculated from the Prism program. Indomethacin was used as a positive control.

Results and Discussion

The results of DPPH radical scavenging activity and the inhibitory activity exerted by all plants extracts against LPS-induced NO production in RAW 264.7 cell lines are shown in Table 2.

For DPPH radical scavenging activity, the ethanolic extract of *Terminalia chebula* Retz (gall), *Terminalia chebula* Retz (fruit), *Myristica fragrans* Houtt (seed), (EC_{50} = 3.77, 4.98 and 6.14 µg/ml, respectively) exhibited higher activity than BHT (EC_{50} = 12.89 µg/ml). The previous phytochemical studies on these plants, especially *Terminalia chebula* Retz (gall), *Terminalia chebula* Retz (fruit) indicated the presence of phenolic compounds[37,38]. It is likely that the phenolic compounds in *Terminalia chebula* are largely responsible for the strong antioxidant properties.

For nitric oxide inhibitory activity, the results showed that the ethanolic extract of *Terminalia chebula* Retz (fruit), *Atractylodes lancea* and *Baliospermum montanum* showed high NO inhibition activity (IC_{50} = 3.3, 9.7 and 12.6 µg/ml, respectively). Interestingly, these plant extracts exhibited higher NO production inhibitory effect than that of the positive control indomethacin (IC_{50} = 56.8 µM or 20.3 µg/ml). The ethanolic extracts of *Piper retrofractum, Terminalia chebula* Retz (gall) and *Kleinhovia hospita* also showed strong inhibitory activity (IC_{50} < 30 µg/ml). *Atractylodes lancea* was previously reported to inhibit NO production in a dose-dependent manner, but as the percentage inhibition at 500 µg/ml was only 41.1%[15], so these results report a much higher activity.

Conclusion

These results indicate that many of the Thai medicinal plants, which are used for relief of muscle pain and distal numbness, possess a strong antioxidant

Table 1. The ethnobotanical data and biological activities of plants

Species	Places of specimen collection	Voucher specimen number	Thai name	Plant part	Biological activities
Anacyclus pyrethrum (L.) DC. Compositae	India	SKP051011601	Kot Krukkra	rhizome	Antioxidant[6]
Anethum graveolens L. Umbelliferae	India	SKP199010701	Thian ta takkataen	fruit	Antioxidant[7-9]
Arorus calamus L. Araceae	Songkla	SKP015010301	Vannam	rhizome	Antioxidant[10] Anti-inflammation[11,12]
Atractylodes lancea (Thunb.) DC. Compositae	China	SKP051011201	Kot kamao	root	Antioxidant[13] Anti-inflammation[14-16]
Baliospermum montanum (Willd.) Muell. Arg. Euphorbiaecae	Pathumthani	SKP121021301	Tongtang	root	Antioxidant, Anti-inflammation [17]
Cinamomum camphora (L.) Sieb Lauraceae	Bangkok	SKP096030301	Karaboon	crystal	Antioxidant, Anti-inflammation[18,19]
Cuminum cyminum L. Umbelliferae	India	SKP199030301	Thian khao	fruit	Antioxidant[20,21]
Ferula assafoetida L. Umbelliferae	India	SKP199060101	Mahahing	oleogum resin	Anti-inflammation[22]
Kleinhovia hospita L. Sterculiaceae	Chantaburee	SKP183110801	Hatsakunthet	root	n/a
Lepidium sativum L. Cruciferae	India	SKP019121901	Thian daeng	seed	Antioxidant[23]
Merremia vitifolia (Burm.f.) Hall. f. Convolvulaceae	Bangkok	SKP054132201	Jingjoa	root	n/a
Myristica fragrans Houtt. Myristicaceae	Chumporn	SKP121130601	Lokchan	seed	Anti-inflammation[24]
Myristica fragrans Houtt. Myristicaceae	Chumporn	SKP121130601	Dokchan	aril of seed	Antioxidant[25]
Nigella sativa L. Ranunculaceae	India	SKP160141901	Thiandam	seed	Antioxidant[26] Anti-inflammation[27]
Picrorrhiza kurroa. (Kutaki) Royle ex Benth Scrophulariaceae	India	SKP177161101	Kot kanpraw	root	Antioxidant[28] Anti-inflammation[29,30]
Pimpinella anisum L. Umbelliferae	India	SKP199160101	Thian sattabud	seed	Antioxidant[31] Anti-inflammation[32]
Piper nigrum L. Piperaceae	Jantaburee	SKP146161401	Pikthai	fruit	Antioxidant[33] Anti-inflammation[34]
Piper retrofractum Vahl. Piperaceae	Jantaburee	SKP146161801	Dee pree	fruit	Antioxidant[35]
Plumbago indica L. Plumbaginaceae	Bangkok	SKP148160901	Jattamoonpleng dang	root	Anti-inflammation[14,36]
Terminalia chebula Retz. Combretaceae	India	SKP019200301	Kot pung pla	gall	Antioxidant[37,38] Anti-inflammation[38]
Terminalia chebula Retz. Combretaceae	Rayong	SKP049200301	Samao thai	fruit	Antioxidant[39] Anti-inflammation[40,41]

n/a = not applicable

activity and are active against LPS-induced NO production in RAW 264.7 cell lines. The ethanolic extract of *Terminalia chebula* Retz (gall and fruit) showed strong activities for both effects measured inhibition so this species should be further examined to determine the compounds responsible. The ethanolic extracts of *Atractylodes lancea, Baliospermum montanum, Piper retrofractum, Kleinhovia hospita* also exhibited strong NO-inhibitory activity.

These results support the use of these plants

Table 2. Percentage of yield of the ethanolic extracts of plants, antioxidant activity (EC_{50} µg/ml ± SEM) and inhibition of NO production (IC_{50} µg/ml ± SEM) of plant extracts (n = 3)

Plant name	Code	% Yield of extract	Antioxidant activity EC_{50} ± SEM (µg/ml)	Inhibition of NO production	
				% Inhibition at conc 100 µg/ml (% cytotoxicity)	IC_{50} ± SEM (µg/ml)
A. pyrethrum	Ap	11.5	24.00 ± 1.37	48.6 ± 1.4 (5.1 ± 5.6)	> 100
A. graveolens	Ag	5.7	> 100	40.5 ± 2.5 (29.9 ± 3.1)	> 100
A. calamus	Ac	5.1	> 100	90.6 ± 3.5* (40.30 ± 4.50)	30.1 ± 4.1
A. lancea	Al	16.4	> 100	94.0 ± 3.2*(87.5 ± 2.7)	9.7 ± 0.5
B. montanum	Bm	1.1	38.1 ± 2.1	95.6 ± 1.4 (27.8 ± 2.0)	12.6 ± 1.6
*C. camphor*a	C	No extract	> 100	45.9 ± 1.7 (24.66 ± 1.35)	> 100
C. cyminum	Cc	6.6	67.1 ± 2.4	95.6 ± 1.4 (27.8 ± 2.0)	12.6 ± 1.6
F. assafoetida	Fa	No extract	> 100	45.9 ± 1.7 (24.7 ± 1.4)	> 100
K. hospita	Kh	1.2	> 100	79.6 ± 3.7 (15.4 ± 4.2)	74.7 ± 4.4
L. sativum	Ls	5.4	30.5 ± 1.5	30.3 ± 2.3 (3.3 ± 2.5)	> 100
M. vitifolia	Mv	4.9	28.4 ± 3.5	94.2 ± 2.5* (29.0 ± 4.3)	28.95 ± 3.86
M. fragrans	Mf (s)	13.56	6.1 ± 0.4	8.6 ± 5.5 (22.9 ± 9.2)	> 100
M. fragrans Houtt.	Mf (a)	22.2	14.9 ± 0.7	68.0 ± 8.7** (41.4 ± 1.5)	> 100
N. sativa	Ns	21.9	> 100	36.9 ± 6.4 (44.9 ± 5.1)	> 100
P. kurroa	Pk	18.2	31.0 ± 0.8	28.8 ± 4.8 (13.6 ± 4.1)	> 100
P. anisum	Pa	3.0	> 100	89.9 ± 3.3* (53.4 ± 2.4)	48.62 ± 4.2
P. nigrum	Pn	6.5	> 100	95.8 ± 3.0** (31.0 ± 0.5)	32.0 ± 3.1
P. retrofractum	Pr	10.9	> 100	91.1 ± 3.4 (19.9 ± 12.0)	25.9 ± 2.5
P. indica.	Pi	8.5	> 100	79.5 ± 2.3* (36.6 ± 3.6)	> 100
T. chebula	Tc (g)	25.8	3.8 ± 0.5	88.3 ± 5.6* (40.9 ± 2.2)	28.7 ± 3.4
T. chebula	Tc	41.7	5.0 ± 0.1	89.3 ± 4.5* (32.7 ± 7.4)	3.3 ± 3.6

*Cytotoxic effect was observed , ** Concentration at 50 µg/ml

in Thai folk medicine for the relief of muscle pain, distal numbness and inflammatory-related diseases and suggest that their ability is through the inhibition of NO release. It might be worth while to investigate some of these species further as a means of identifying novel leads for anti-inflammatory drugs.

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Potential conflicts of interest

None.

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ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านการอักเสบของสมุนไพรไทยในตำรับสหัสธาราที่ใช้รักษาอาการปวดกล้ามเนื้อ

นรินทร์ กากะทุ่ม นวลจันทร์ ใจอารีย์ ศุณิตา มากชูชิต อรุณพร อิฐรัตน์

***วัตถุประสงค์**: พืชสมุนไพรไทยจำนวน 21 ชนิด ในตำรับยาไทยชื่อ สหัสธาราที่ใช้ระงับอาการปวดกล้ามเนื้อและอาการชา ถูกนำมาศึกษาฤทธิ์ต้านอนุมูลอิสระ และฤทธิ์ต้านการอักเสบ*

***วัสดุและวิธีการ**: การสกัดใช้วิธีการหมักพืชด้วย เอทธานอล 95% สารสกัดถูกทดสอบฤทธิ์ต้านอนุมูลอิสระด้วย วิธี DPPH scavenging assay และ ฤทธิ์ต้านการอักเสบด้วย การยับยั้งการสร้าง ไนตริกออกไซด์ ในเซลล์ RAW 264.7 เมื่อถูกกระตุ้นด้วย lipopolysaccharide (LPS)โดยการใช้ Griess reagent*

***ผลการศึกษา**: สารสกัดเอทธานอลของโกฐพุงปลา แสดงฤทธิ์ต้านอนุมูลอิสระดีที่สุด (EC_{50} เท่ากับ 3.34 ไมโครกรัมต่อมิลลิลิตร) สารสกัดสมอไทยแสดงฤทธิ์ต้านการอักเสบดีที่สุด รองลงมาคือ โกฐเขมา และตองแตก (IC_{50} เท่ากับ 3.3, 9.7 และ 12.6 ไมโครกรัมต่อมิลลิลิตร)*

***สรุป**: ผลการออกฤทธิ์ต้านการอักเสบและฤทธิ์ต้านอนุมูลอิสระของพืชสนับสนุนการใช้สมุนไพรในตำรับยาไทยที่ใช้แก้ปวดกล้ามเนื้อและโรคที่เกิดจากการอักเสบ*