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Anti-inflammatory effect of volatile oils of *Piper nigrum* Linn. in RAW 264.7 cells

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Introduction

Piper nigrum Linn., namely "Phrik Thai" in Thai, belongs to the Piperaceae family, is widely cultivated in the world. This plant is an aromatic woody perennial climber, trailing and rooting at the nodes. The leaves are variable in length from 12.5 to 17.5 cm and width from 5.0 to 12.5 cm. The flowers are dioecious but often the female bears 2 anthers and the male a pistillode, anther 2-called. The fruits are orange red to reddish when ripe[1]. The volatile oil and pungent compounds are components of *Piper nigrum*. Piperine is the major contribution to pungency whereas essential oil constituents such as α- and β-pinene, limonene, sabinene, etc., are the major aroma and flavor compounds of this plant[2]. Another important volatile component of *Piper nigrum* is pipene, which is famous odorants[3]**.** Medicinally, *Piper nigrum* is used as preservatives, biocontrol agent, digestive disorders, diarrhea, indigestion and can be used against respiratory disorders including cold, fever and asthma[4]**.** Related activities including antibacterial, antioxidant and anti-inflammatory[5,6] have been reported.

Nitric oxide (NO) is one of the critical mediators released in response to pathogenic infections which is synthesized universally from L-arginine by nitric oxide synthase (NOS)[7]. Inducible nitric oxide synthase (iNOS) is responsible for the over-production of NO which can cause the vasodilation and hypotension observed during septic shock and inflammation[8]. Thus, inhibitors of iNOS may be useful candidates for the treatment of inflammatory diseases accompanied by the overproduction of NO. Therefore, the objective of this study was to investigate the inhibitory activity of volatile oils of *Piper nigrum* from six different sources throughout Thailand on LPS-induced NO release using RAW 264.7 cells.

Methods

Collection of plant materials

The dried fruits of *Piper nigrum* were obtained from six different sources throughout Thailand including Phuket, Roi Et, Ratchaburi, Chiang Rai, and Chanthaburi (2 locations). They were identified by Dr. Nijsiri Ruangrungsi.

Determination of volatile oil content

The ground samples (50 g) were added with distilled water (500 ml) in round bottom flask. They were distilled in Clevenger apparatus for 5 hours, stop the heating after 30 minutes, wait at least 10 minute and read volume of oils that were separated in the receiving tube.

Anti-nitric oxide production in RAW264.7 cells

The volatile oils of *Piper nigrum* were evaluated for their inhibitory effect on NO production in murine macrophage like RAW 264.7 cells by a modified method[9]. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent according to griess reaction. The % inhibition of NO was calculated by the following equation, and IC_{50} values were determined graphically (n=4):

$$\% \text{ Inhibition of NO} = \frac{[(\text{control-blank of control})-(\text{sample-blank of sample})]}{[(\text{control-blank of control})]} \times 100$$

Assay of cell viability

Since the observed on the inhibitory effect of the samples on NO production may be due to cell death. Viability of cell was tested by MTT assay[10]. This assay requires active mitochondria of living cells to reduce MTT, a pale yellow substrate, to dark blue formazan. The tested volatile oils were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of the control (vehicle-treated) group.

Results

Collection of plant materials

The fruits of *Piper nigrum* from six sources were globose and ovoid. They were 4 to 5 mm in diameter, with brown to black colour with thin testa, globon seeds (Figure 1).

Figure 1. Black fruits of *Piper nigrum*

Determination of volatile oil content

The dried powdered of *Piper nigrum* fruits from six sources were distilled for volatile oils in a Clevenger apparatus (Figure 2). The present study showed that the Chanthaburi sample (CHA1) possessed the highest percentage of volatile oils content (2.27±0.23 %), followed by the Chiang Rai sample (CHI) (2.13±0.23 %) and the Phuket sample (PHU) (2.07±0.12 %), respectively. (Table 1)

Figure 2. Clevenger apparatus for determination of volatile oil content in *Piper nigrum*

Table 1. The volatile oil content of Piper nigrum from six sources throughout Thailand

Samples	Volatile oils content (% by weight) (n=3)
PHU	2.07±0.12
ROI	1.93±0.12
RAT	1.86±0.23
CHI	2.13±0.23
CHA (1)	2.27±0.23
CHA (2)	1.73±0.23

PHU = Phuket; ROI = Roi Et; RAT = Ratchaburi; CHI = Chiang Rai; CHA = Chanthaburi

Anti-nitric oxide production in RAW264.7 cells

The volatile oils were investigated for their inhibitory effect on NO production using murine macrophage-like RAW264.7 cells. The result indicated that these volatile oils showed activity against NO release (Table 2).

Table 2. Anti-nitric oxide production in RAW264.7 cells of Piper nigrum oils from six sources throughout Thailand

Samples	% Inhibition at different concentrations (µg/ml)					IC_{50} (µg/ml)
	0	3	10	30	100	
PHU	0.0±2.6	6.1±1.1	15.4±1.1	73.5±4.3	101.0±0.9 [b]	21.7
ROI	0.0±2.6	7.3±0.9	13.8±1.6	70.3±3.4	100.0±1.4 [b]	22.9
RAT	0.0±2.6	5.9±2.0	22.1±1.0	88.6±3.8 [b]	101.0±0.9 [b]	16.9
CHI	0.0±2.6	8.0±3.1	21.0±2.6	90.1±4.0 [b]	100.7±1.2 [b]	12.9
CHA (1)	0.0±2.6	4.5±0.9	17.3±2.7	67.1±1.8	100.6±0.6 [b]	22.4
CHA (2)	0.0±2.6	7.2±2.0	14.0±2.0	80.0±3.5	101.1±0.8 [b]	20.8
Indomethacin	0.0±1.2	2.8±0.9	21.2±1.4	61.0±2.6	79.8±1.4	34.9

[a] Each value represents mean± S.E.M. of four determinations.

[b] Cytotoxic effect was observed

Discussion

The content of volatile oils in *Piper nigrum* from six sources throughout Thailand was found to be more than 1% v/w, which is in accord with official standard in Thai Herbal Pharmacopiea (THP, 2016). Therefore, the content of volatile oils in *Piper nigrum* indicates the quality of the herb. The anti-inflammatory activity of volatile oils were tested based on their NO inhibitory effect. The result indicated that the sample from Chiang Rai possessed the highest activity with an IC_{50} value of 12.9 µg/ml, followed by the ones from Ratchaburi (IC_{50} = 16.9 µg/ml) and Chanthaburi (2) (IC_{50} = 20.8 µg/ml), respectively. All of these, they can be exhibited higher effect than Indomethacin (IC_{50} = 34.9 µg/ml). Thus, searching for the inhibitory effect of NO production using murine macrophage-like RAW264.7 cells of volatile oils from *Piper nigrum* from six sources throughout Thailand had mighty to target for anti-inflammation. They seem likely that mechanisms underlying the action of these volatile oils are related to the inhibition of NO production. Thus, we are interested structure elucidation of volatile oils from *Piper nigrum using* gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS), respectively.

Conclusions

Results obtained from this study showed that the content of volatile oils of *Piper nigrum* from six sources throughout Thailand indicated the quality of the herb. They are mainly responsible for anti-inflammatory effect through the inhibition of NO release. The results suggest that the volatile oils from this plant can be developed as an alternative formulation for the treatment of inflammation.

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