Original Article

Potential *in vitro* Anti-inflammatory and Anti-oxidant Activities of Various Extracts of *Etlingera elatior* Inflorescences

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Abstract

Introduction: *Etlingera elatior* (Jack) Smith has been used as folk medicine for earaches and wound cleansing. Although some parts of the plant have been studied for anti-inflammatory properties, there is a lack of research on the inhibition of nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumor necrosis factor α (TNF-α). The findings of this study demonstrated its anti-inflammatory effects and suggested an effective extraction method for further investigations.

Objectives: This study aimed to explore the anti-inflammatory and antioxidant properties of *E. elatior* inflorescence extracts.

Methods: The bracts of *E. elatior* inflorescences were dried and separately extracted by 95%, 70%, and 50% ethanol maceration and decoction. The anti-inflammatory activity was investigated by inhibiting the production of NO, PGE_2 and TNF-α in RAW264.7 cells. Additionally, the anti-oxidant activity was assessed by DPPH scavenging assay.

Results: The 70% ethanolic extract of *E. elatior* exhibited both inhibitory effect on NO production (IC_{50} = 16.36 µg/mL) and DPPH scavenging activity (EC_{50} = 23.78 µg/mL), whereas the 95% ethanolic extract showed comparable inhibitory activity on NO (IC_{50} = 16.78 µg/mL) but not in antioxidant activity. The 95% ethanolic extract also showed moderate inhibition of PGE_2 production (IC_{50} = 45.26 µg/mL).

Conclusion: The ethanolic extracts of *E. elatior* inflorescences, obtained through maceration with 70% ethanol exhibited remarkable anti-inflammatory and anti-oxidant properties. 95% ethanol extracts exhibited anti-inflammation by both inhibition of NO and PGE_2. Consequently, it is imperative to further explore the 70% and 95% ethanolic extracts through in-depth research, including *in vivo* studies, phytochemical analysis, and anti-inflammatory product development.

Keywords: *Etlingera elatior*, Torch ginger flowers, Anti-inflammation, Anti-oxidation, Nitric oxide

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Introduction

Inflammation is a complex biological response that protects the body against harmful stimuli[1]: including infections, chemical exposure, tissue damage, and exposure to bacterial components like lipopolysaccharide (LPS).[2,3] Various cells and mediators, such as neutrophils, mast cells, and macrophages, play crucial roles in the inflammatory process, which produce proinflammatory cytokines and mediators, like nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and prostaglandin E_2 (PGE_2), during inflammation.[4] While these mediators and cells aid in protecting the body, they can also affect normal cells and tissues with the excessive activation leading to cell and tissue damages resulting in inflammatory conditions such as pain, arthritis, including rheumatoid arthritis, diabetes, inflammatory bowel disease, atherosclerosis, and cancer.[5] The management of inflammation and pain typically involves the utilization of non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal medications. Nevertheless, prolonged usage of steroids and NSAIDs can give rise to adverse effects like bleeding, indigestion, cardiac problems, and kidney toxicity.[6] Consequently, certain patients necessitate alternative medications such as herbal or traditional remedies. Hence, there is a growing inclination towards exploring substances derived from natural sources.

Etlingera elatior (Jack) Smith, commonly known as torch ginger, is a plant belonging to the Zingiberaceae family. It is extensively cultivated in Southeast Asia and other tropical regions, including Thailand, Malaysia, Indonesia, and the Boneo Peninsula.[7-9] It is named as "Da-Lah" in Thailand and is consumed as food ingredient or juice. In Malaysian folk medicine, the raw inflorescence is utilized to treat earaches and cleanse wounds[9]. Previous studies have reported various pharmacological activities of *E. elatior*, such as antioxidant properties[10-12], antibacterial effects[13], cytotoxic activity[11], anti-inflammatory properties[14], and anti-aging effects.[15,16] However, these studies mostly report on the rhizome and leaf of this plant. The analysis of phytochemicals in *E. elatior* inflorescences revealed the presence of flavonoids, terpenoids, saponins, and tannins.[10] Furthermore, gallic acid, tannic acid, chlorogenic acid, caffeic acid, quercetin, apigenin, kaempferol, luteolin and myricetin were identified as its chemical constituents.[13] These compounds showed both antioxidant and anti-inflammatory activities.[17] With regard to anti-inflammation, a study conducted on Wistar rats with induced gastric ulceration revealed the anti-inflammatory activity of *E. elatior* flower extract through the modulation of nuclear factor-kappaB-p65 (NF-κB-p65).[14] Another study by Aldi et al. demonstrated the immunomodulatory effects of *E. elatior* extract in allergic male white mice.[18] Nurhayatun and colleagues reported that the methanol extract of the fruit exhibited anti-inflammatory properties by reducing the levels of NF-κB, caspase 3, and IL-1ß in a Mus musculus sepsis model[19]. These findings indicate that the extract derived from various parts of *E. elatior* possesses anti-inflammatory activity. However, there is a lack of research on the inhibition of NO, PGE_2, and TNF-α production of the inflorescences. Therefore, the objective of this study is to explore the anti-inflammatory activity by inhibiting the production of NO, PGE_2, and TNF-α in RAW264.7 cells. Additionally, we evaluated the antioxidant properties using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay. This study not only provides information about the anti-inflammatory effects of *E. elatior* inflorescences extract but also demonstrates an effective extraction method for a further comprehensive investigation of its anti-inflammatory activity, quality control of the extract, and product development.

Methods

Plant materials and preparation of extracts

E. elatior was collected from Yala province, Thailand, in December 2020 and authenticated by Mr. Sakwichai Onthong, the Medical Plant Research Institute, Department of Medical Science, Thailand. The plant specimen (voucher number DMSC 5256) was deposited at the herbarium of Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand.

The inflorescences of *E. elatior* were washed and the floral bract, involucral bract, and true flower were pulled out from the receptacle. The bracts were combined and subjected to drying in a hot air oven at a temperature of 45 ºC. The dried crude was then separately extracted through maceration and decoction methods. For maceration, the crude was separately macerated with ethyl

acetate, 95% ethanol, 70% ethanol, and 50% ethanol (3 days x 3 times). Each extract was filtered and evaporated under reduced pressure at a temperature below 45ºC. For decoction, the crude was boiled in distilled water for 15 minutes, followed by filtration using filter paper. The residue underwent two additional extractions using the same procedure, and the resulting extracts were combined and dried using a freeze dryer to obtain the aqueous extract. All samples were stored at -20 ºC until used.

Chemicals and reagents

Murine macrophage cell line (RAW 264.7: ATCC® TIB-71™) were purchased from American Type Culture Collection (ATCC®, VA, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories, Inc (UT, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin (P/S), phosphate buffer saline (PBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (MO, USA). Prostaglandin E_2 and Tumor necrosis factor-a immunoassay kits were purchased from Enzo Life Sciences, Inc. (NY, USA).

Cell culture

RAW 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 µg/L streptomycin, and 100 IU/mL penicillin at 37 °C with 95% humidity in a 5% CO_2 atmosphere.

Cell viability assay

Cell viability was measured by the mitochondria-dependent reduction of MTT (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution to formazan. RAW 264.7 cells were seeded in 48-well plates at the density of 1.2 x 10^5 cells per well and then cultured for 24 hours. The cells were treated with various concentrations of *E. elatior* extracts (1-100 κg/mL) for 24 hours. Subsequently, cells were incubated with MTT solution at final concentration of 0.1 mg/mL for 1 h at 37 °C. The medium was then removed and the formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm with correction at 650 nm using microplate reader (Biotek, VT, USA). The percentage of cell viability was calculated by comparing the treated cell to the control (100%). The cell viability of more than 85% indicated that the inhibitory effect was not resulting from cell death.

Measurement of nitric oxide

The determination of anti-inflammatory activity by inhibiting the NO production followed the method outlined in the study conducted by Miranda *et al.*[20] The cells were seeded into 48-well plate at a density of 1.2 x 10^5 cells per well and cultured for 24 hours. Subsequently, the cells were treated with various concentrations of *E. elatior* extracts (1-100 µg/mL). N omega-Nitro-L-arginine methyl ester hydrochloride (L-NAME; NOS inhibitor) was used as positive control. After 1 hour treatment, the cells were stimulated with 0.2 µg/mL of LPS and cultured for 24 hours. Nitric oxide production was determined by measuring the concentration of nitrite in culture medium using Griess's reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl in water). Briefly, the culture medium in each well (100 µL) was transferred to another 96-well plate and then 100 µL of Griess's reagent was added and incubated for 10 minutes. The absorbance was measured at 520 nm, with correction at 665 nm. Nitrite concentrations were calculated from sodium nitrite ($NaNO_2$) standard curve. The inhibitory activity (%) of nitric oxide production was calculated by the following equation:

$$\text{Inhibition (\%)} = \left[\frac{N_{control} - N_{sample}}{N_{control}}\right] \times 100$$

Where $N_{control}$ was the nitrite concentration of the control and N_{sample} was the nitrite concentration of sample. The IC_{50} values were calculated from the GraphPad Prism software (GraphPad®, USA).

Measurement of PGE_2 and TNF-α

The cells were seeded into 48-well plate (1.2 x 10^5 cells per well) and cultured for 24 hours. The cells were treated with various concentrations of *E. elatior* extracts (1-100 µg/mL). Indomethacin was used as positive control of PGE_2 assay, whereas, dexamethasone was used for TNF-α assay. After 1 hour treatment, cells were stimulated by 0.2 µg/mL of LPS and cultured for 24 hours. The culture medium in each well was collected and kept in -80 ºC until being tested for PGE_2 and TNF-α. The quantity of PGE_2 and TNF-α in the cultured medium

were quantified by EIA kits according to the manufacturer's instructions (Enzo Life Sciences, USA). The inhibition (%) of PGE_2 and TNF-α production was calculated by the following equation:

$$\% \text{ Inhibition} = \left[\frac{OD_{control} - OD_{sample}}{OD_{control}} \right] \times 100$$

Where $OD_{control}$ was the absorbance of the control and OD_{sample} was the absorbance of sample. The IC_{50} values were calculated from the GraphPad Prism software (GraphPad®, USA).

DPPH Radical Scavenging Activity

DPPH scavenging activity as a model of antioxidant activity was conducted following the modified method of Yamasaki et al.[21] The extract was dissolved in absolute ethanol or distilled water to achieve a concentration of 2000 µg/ml as a sample stock solution. Subsequently, each extract was further diluted to obtain working solution at concentrations of 200, 100, 60, 20, and 2 µg/ml. The DPPH scavenging reaction started by adding 100 µl of $6x10^{-5}$ M DPPH in 100 µl aliquot of the working solution and stored in darkness at room temperature for 30 minutes. The absorbance was subsequently measured at 520 nm. Butylated hydroxytoluene (BHT) was used as a positive control. Inhibition (%) was calculated using the following equation.

$$\% \text{ Inhibition} = \left[\frac{OD_{control} - OD_{sample}}{OD_{control}} \right] \times 100$$

Where $OD_{control}$ was the absorbance of the control and OD_{sample} was the absorbance of sample. The IC_{50} values were calculated from the GraphPad Prism software (GraphPad®, USA).

Statistical analysis

The experiments were performed in triplicate, and the results were presented as the mean ± standard deviation (SD). The EC_{50} and IC_{50} values were determined by regression analysis, and for multiple comparisons of dataset, ANOVA and Dunnett's post-hoc test was conducted. All statistical analyses were calculated by using GraphPad Prism software (GraphPad®, USA).

Results

Plant material extractions

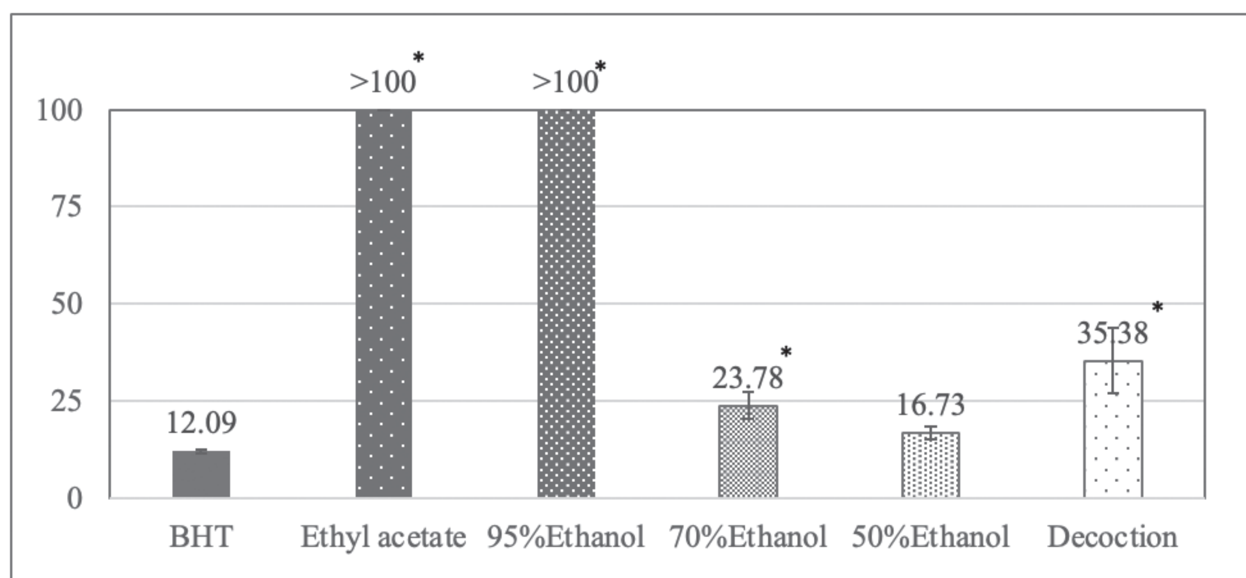
The percentage yields of the plant extracts are shown in Table 1. The extraction yield obtained from 95% ethanol maceration demonstrated the highest value of 19.96%, followed by the 70% ethanol extract. The ethyl acetate extract demonstrated the lowest yield, with a value of 9.82%.

Table 1 Yield (%) of the extracts obtained from various extraction method

Extraction solvent	Extraction method	Code	Yield (%)
Ethyl acetate	Maceration	EEE	9.82
95% Ethanol	Maceration	EE95	19.96
70% Ethanol	Maceration	EE70	18.93
50% Ethanol	Maceration	EE50	16.66
Water	Decoction	EEDec	15.76

DPPH scavenging activity

As shown in Figure 1, the 50% ethanolic extract (EE50) exhibited the highest DPPH scavenging activity, with an EC_{50} value of 16.73 ± 1.58 µg/mL, which was not significantly different from BHT (12.09 ± 0.50 µg/mL). The EEE and EE95 demonstrated no activity in DPPH scavenging (EC_{50} > 100 µg/mL). The EE70 and EEDec showed moderate DPPH scavenging activity, with EC_{50} values of 25.12 ± 2.88 µg/mL and 35.38 ± 8.35 µg/mL, respectively, which were significantly higher than that of BHT.



*Significant different from the positive control, BHT, analyzed by ANOVA with Dunnett's analysis (p-value<0.05)

Figure 1 DPPH scavenging activity of *E. elatior* extracts obtained from various extraction solvent.

Inhibitory effects on nitric oxide production

The inhibitory activity of *E. elatior* extracts on the production of nitric oxide from the *LPS*-induced RAW264.7 cells are showed in Table 2. Among the extracts tested, EE70 exhibited the most potent inhibitory effect on nitric oxide production, with an IC_{50} value of 16.36 ± 4.12 µg/mL. EE95 and EEE extracts exhibited IC_{50} values of 16.78 ± 7.21 and 22.59 ± 3.33 µg/mL, respectively. Both EE50 and EEDec demonstrated no activity (IC_{50} > 100 µg/mL). However, all extracts possessed inhibitory effect less than the NOS inhibitor, L-NAME. Moreover, the extracts did not exhibit cytotoxic activity, as showed by the percentage survival exceeding 80%. This indicates that the inhibitory activity of the extracts is not a result of cell death.

Table 2 Inhibitory effects of *E. elatior* extracts on nitric oxide, PGE_2 and TNF-α production from RAW 264.7 cells.

Sample	Inhibitory effects (IC_{50}; µg/mL)		
	Nitric oxide	PGE_2	TNF-α
EEE	22.59 ± 3.33*	66.56 ± 14.59*	>100*
EE95	16.78 ± 7.21*	45.26 ± 9.28*	>100*
EE70	16.36 ± 4.19*	>100*	>100*
EE50	96.09 ± 12.1*	>100*	>100*
EEDec	>100*	>100*	>100*
L-NAME	6.69 ± 1.95 (0.029 ± 0.008 µM)	NA	NA
Indomethacin	NA	9.31 ± 3.23 (0.026 ± 0.009 µM)	NA
Dexamethasone	NA	NA	35.73 ± 15.31 (0.091 ± 0.039 µM)

*Significant difference from the positive control (*p*-value <0.05) analyzed by ANOVA with Dunnett post-hoc analysis.

Inhibitory effects on PGE_2 and TNF-α production

As shown in Table 2, EE95 and EEE are only two extracts exhibiting the production of PGE_2 of the *LPS*-induced RAW264.7 cells with IC_{50} values of 45.26 ± 9.28 and 66.56 ± 14.59 µg/mL, respectively, other extracts possessed no inhibitory activity. The positive control, indomethacin showed potent inhibitory effect significantly more than the extracts (IC_{50} = 9.31 ± 3.23 µg/mL; 0.026 ± 0.009 µM). With regard to inhibitory effect on TNF-α production, none of the extracts exhibited inhibitory activity, while the positive drug, dexamethasone, exhibited potent activity with IC_{50} value of 35.73 ± 15.31 µg/mL (0.091 ± 0.039 µM).

Discussion

E. elatior is an edible plant which commonly consumed in south-east Asia particularly in Thailand and Malaysia. It has been used as a folk medicine in Malaysia for the treatment of earache and as a wound cleaner[9]. This study aimed to investigate anti-inflammatory activity by inhibiting NO, PGE_2 and TNF- α production in RAW264.7 cells. Additionally, the anti-oxidant by DPPH scavenging assay were also evaluated.

For anti-oxidant activity, our study revealed that the 50% ethanolic extract exhibited potent antioxidant properties as demonstrated by the DPPH scavenging assay, which were comparable to the positive control, BHT. A previous study conducted by Lachumy and colleagues also reported potent DPPH scavenging activity comparable to BHT for the 80% methanolic extract[12]. These findings support the result that *E. elatior* flower possesses DPPH scavenging activity, thus suggesting its potential utilization as an anti-oxidant product.

With regard to the inflammatory activity, *E. elatior* extract demonstrated the most inhibitory effect on NO production in the induced RAW264.7 cell. Interestingly, the EE70 and EE95 showed potent inhibition with IC_{50} values less than 20 µg/mL. EE95 also inhibited the PGE_2 production. The results of our study related to a previous study of Juwita and colleague, which demonstrated that the 95% ethanolic extract obtained through the soxhlet method effectively suppressed the development of gastric ulceration by down-regulating the expression of NF-kB-p65[14]. These results indicate that *E. elatior* 95% ethanolic extract possessed inhibitory effect on nitric oxide. Moreover, our study showed that the EE70 demonstrated potent NO inhibition as well as the EE95 and EEE extracts. In contrast, the aqueous extract and 50% ethanol extract displayed no effects in all anti-inflammatory activities. These findings suggest that compounds found in *E. elatior*, which were more likely to dissolve in ethanol, tend to inhibit the production of NO and PGE_2. Nevertheless, it is important to note that the phytochemical analysis conducted in this study had limitations, and further research is needed to explore this aspect comprehensively, including assessments of total phenolics, total flavonoids and chemical constituents.

Nitric oxide (NO) plays a dual role intrinsically. It serves as a vital physiological signaling molecule, facilitating diverse cellular functions. However, in contrast, it elicits cytotoxic and mutagenic effects when present in abundance, particularly under conditions of oxidative stress. It plays a significant role in the prolongation of inflammation and immunological responses[22]. Excessive NO production from inflammatory cells lead to several diseases by several mechanisms. As a pro-inflammatory cytokine NO can be produced by various cell types, including immune cells such as macrophages and neutrophils, during chronic inflammation. It can contribute to the inflammatory response through several mechanisms particularly by the induction of cytokines. NO can stimulate the production of other pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6). These cytokines contribute to the amplification of the inflammatory response[23]. Therefore, inhibiting the excessive production of NO can help reduce these inflammatory responses.

In conclusion, our study presents the initial findings that the ethanolic extracts of *E. elatior* inflorescences, obtained through maceration with 70% ethanol and 95% ethanol, exhibited remarkable anti-inflammatory activity by inhibiting the production of NO. In addition, EE95 exhibited moderate anti-inflammatory activity in suppressing the production of PGE_2 and the 70% ethanolic extract demonstrated high DPPH scavenging activity. Consequently, the 95% and 70% ethanolic extracts should be investigated in in-depth research, including *in vivo* studies, phytochemical analysis, and quality control of extract for further anti-inflammatory product development.

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Compliance with Ethics Requirements. Not applicable.

Conflict of interest. The authors declare that there is no conflict of interest regarding the publication of this paper.

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Author Contributions. Weerachai Pipatrattanaseree designed all experiments, collected the plant materials, prepared the plant material and extracts, performed anti-oxidant experiment and wrote the original draft manuscript. Thitiporn Thaptimthong conducted anti-inflammatory experiments and assisted in writing the original draft manuscript. Narumon Boonrasri helped preparation of extracts. Sadudee Rattanajarasroj advised and supported Thitiporn Thaptimthong for anti-inflammatory experiments. Sakwichai Onthong was responsible for identifying the plant specimen. Siriwan Chaisomboonpan provided valuable supported and commented throughout the entire project. All authors read and approved the final version of the manuscript.

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