Anti-inflammatory Activities of *Erythrina variegata* Bark Ethanolic Extract

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Background: *The bark of Erythrina variegata Linn.(Ev) is used in Thai traditional medicine for the treatment of many diseases in Thailand and is an ingredient in the Mahanintangthong remedy (antipyretic) and Lomammapruek remedy (analgesic and anti-inflammatory).*
Objective: *To study anti-inflammatory activities of ethanolic extract of E. variegata in vitro.*
Material and Method: *Bark of E. variegata was extracted with 95% ethanol. In this study, Griess reagent was used to measure the anti-inflammatory activity by inhibitory effects of extract on nitric oxide production activated by lipopolysaccharide in RAW 264.7 cell lines, COX-2 and TNF-α were also tested by using ELISA techniques.*
Results: *The ethanolic extract of E. variegata showed potent anti-inflammation properties by inhibiting prostaglandins production through enzyme COX-2 and inhibitory activity against lipopolysaccharide induced nitric oxide production in RAW 264.7 cell lines with an IC_{50} value of 9.27±0.72 and 47.1±0.21 μg/ml, respectively. However it was not effective against TNF-α release.*
Conclusion: *The ethanolic extracts of E. variegata bark showed higher inhibitory effect on PGE_2 as acute inflammation than inhibitory effect on Nitric oxide production and TNF-α release representing chronic inflammation. This study thus supports the use of E. variegata bark for treatment of inflammation-related diseases by Thai traditional medicine.*

Keywords: *Erythrina variegata, Anti-inflammatory activity, Nitric oxide, Tumor necrosis factor-alpha, Cyclooxygenase-2, PGE_2*

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Inflammation is the body's attempt at self-protection; the aim being to remove harmful stimuli, including damaged cells, irritants, or pathogens and begin the healing process. Nitric oxide (NO) and Prostaglandin E_2 (PGE_2) are known to act as secondary mediators of pro-inflammatory cytokines, such as Tumor necrosis factor (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), which are considered to be important initiators of the inflammatory response and mediators of the development of various inflammatory diseases[1]. Among these, TNF-α is implicated as a key cytokine playing an important role in the immune response such as autoimmune reactions, and its production is crucially required for the synergistic induction of NO synthesis in lipopolysaccharide (LPS) stimulated macrophage[2]. TNF-α and NO will reduce production of PGE_2, the major metabolite of the Cyclooxygenase-2 (COX-2) pathway, which plays a critical role in the pathogenesis of acute and chronic inflammatory diseases[3].

Thai traditional medicine used many plants for anti-inflammation *Erythrina variegata* Linn. has long been used to reduce inflammation and its bark is an ingredient in antipyretic and analgesic or anti-inflammation preparation such as Mahanintangthong remedy and Lomammapruek remedy.

The genus *Erythrina* (Leguminoceae) is distributed in the tropical and subtropical regions of the world and encompasses over 100 species. The name "coral tree" is used as a collective term for these plants[4]. *E. variegata* Linn. is typically found on sandy soil in littoral forest, and sometimes in coastal forest up

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to 250 m (800ft) in elevation. It is a fast-growing, 50-60 feet tall and wide deciduous tree with green and yellow-variegated, 6-inch-long leaves. Different parts of the plant have been used as traditional medicine in sedation, opthalmia, asthma, epilepsy, and also as an antiseptic. Its leaves have a cathartic, diuretic, antiseptic and anti-inflammatory action[5]. The leaves are used in fever, inflammation and joint pain. The juice of the leaves is used to relieve earache and toothache[6]. The roots are also used to stimulate lactation and menstruation and is used as a laxative, diuretic and expectorant[7]. In Thailand, the bark is used as an analgesic and antipyretic[8]. Phytochemical investigations on the plant revealed the presence of alkaloids, flavanoids and isoflavanoids, coumarins, lectins, flavones glycosides and steroids[9-12]. However, there has not been any report on anti-inflammation activity of its bark. Thus, in the present study, the anti-inflammation effect of ethanolic extract of *E. variegata* Linn. using in vitro methods was investigated. This result would support the use of this plant as an ingredient in Thai traditional medicine to treat inflammatory-related diseases.

Material and Method

Plant material

The bark of *E. variegata* Linn. was collected from Nakhon Pathom on January 2014. Its barks were dried by hot air oven 50°C and powdered.

Preparation of extract

The crude powder (500g) was macerated in ethanol (95%) for 3 days and extracted for 2 more times. The filtrates were pooled and concentrated to dryness under reduced pressure using an evaporator to obtain a dark brown colored molten mass. The percentage yield was 2.95% w/w.

Cell culture

RAW 264.7 murine macrophage cell line was cultured in RPMI 1640 Medium (Sigma, St. Louis, MO, USA) which was supplemented with 10% heated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and incubated at a temperature of 37°C, 95% humidity in 5% CO_2 atmosphere. Cell lines were subcultured every 3 days.

Determination of nitric oxide (NO) production[13,14]

The RAW 264.7 cell line was cultured in RPMI 1640 (BIOCHROM^{AG}) supplement with 10% heated fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were grown at 37°C and 5% CO_2 in humidified air. Cells were seeded in 96-well plates, $1x10^5$ cells/well, and allowed to adhere for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing 100 µg/ml of lipopolysaccharide (LPS) together with test sample at various concentrations and then incubated for 24 hours. NO production was determined by measuring the accumulation of nitrite in the supernatant using the Griess's reagent (100 µl) which was added to 96-well plates and absorbance was read using a microplate reader at 570 nm.

The inhibition of NO production was calculated and IC_{50} values were calculated using the Prism program.

MTT assay[13,14]

Briefly, after 24 hours incubation with test samples, MTT solution (10 µl, 5 mg/ml in PBS) was added to the wells and then incubated at 37°C at 5% CO_2 atmosphere with 95% humidity for 2 hours. After that the medium was removed and isopropanol containing 0.04 M HCl was added to dissolve the formazan solution and the absorbance was read using a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample treated group was less than 70%.

Inhibitory effect on LPS-induced TNF-α release from RAW 264.7 cells line[13,14]

Inhibitory effects on the release of TNF-α from RAW 264.7 cells were evaluated using Quantikine mouse TNF-α ELISA test kit. Cells were seeded in 96-well plates, $1x10^5$ cells/well, and allowed to adhere for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing 100 µg/ml of LPS together with test sample at various concentrations and then incubated for 24 hours. The supernatant (50 µl) was then transferred into 96-well ELISA plate and TNF-α concentrations were determined. The inhibition of TNF-α production was calculated and IC_{50} values were calculated using the Prism program.

Inhibitory effect on LPS-induced PGE_2 release from RAW 264.7 cells line (COX-2) [13-15]

The RAW264.7 cells were seeded in 96-well plates, $1x10^5$ cells/well, and 5 µg/ml of LPS added to stimulate macrophage and allowed to adhere for 24 hours at 37°C in a humidified atmosphere containing

5% CO_2. After incubation, supernatant was collected, and amount of PGE_2 determined using a PGE_2 Enzyme Immuno-Assay Kit (Cayman Chemical Company). Amount of PGE_2 was measured relative to that of positive control.

Statistical analysis

Results were expressed as mean ± SEM of four determinations at each concentration for each sample. The IC_{50} values were calculated using the Prism program.

Results

Inhibitory effect on NO production

Effect of *E. variegata* extract on the pro-inflammatory mediator (NO) in activated murine macrophage cell lines was measured as anti-inflammatory properties compared with positive control (Prednisolone). The result of inhibitory activity against LPS induced NO production are shown in Table 1 and Table 4. The *E. variegata* ethanolic extract exhibited moderate inhibitory activity (IC_{50} value of 47.1±0.21 µg/ml). However, *E. variegata* exhibited less anti-inflammatory activity than Prednisolone (IC_{50} value of 1.31±0.05 µg/ml) which is a positive control of anti-inflammatory drug. The cytotoxic effect of *E. variegata* extract was also determined using the MTT assay. *E. variegata* extract up to concentration of 100 µg/ml showed no cytotoxicity (*i.e.* less than 30% cells were affected).

Inhibitory effect on LPS-stimulated PGE_2 release from RAW 264.7 cells (COX-2)

Results of assay determining inhibitory effect of *E. variegata* on LPS-stimulated PGE_2 (COX-2) release from RAW 264.7 cell lines using PGE_2 Enzyme Immuno-Assay Kit are shown in Table 2 and Table 4. The Ev extract exhibited strong potency with IC_{50} value of 9.27±0.72 µg/ml. However, *E. variegata* exhibited less anti-inflammatory activity than prednisolone (IC_{50} of 0.96±0.01 µg/ml) which is a positive anti-inflammatory drug.

Inhibitory effect on LPS-induced TNF-α release from RAW 264.7 cells

Results of the assay are shown in Table 3. and Table 4. The Ev extract has no inhibitory effect which having IC_{50} of more than 100 µg/ml. However, prednisolone, the standard positive control showed strong anti-inflammatory activity against TNF-α with IC_{50} of 0.95±0.19 µg/ml.

Discussion

The determination of anti-inflammatory activities of 95% ethanolic extract of *E. variegate* bark through the three pathways revealed that the extract has anti-inflammatory properties only through two pathways. It could inhibit NO production and releasing of PGE_2 although not as good as prednisolone, a positive control ($p<0.01$). However, the extract did not effect the TNF-α release (IC_{50} of greater than 100 µg/ml). This plant extract showed moderate inhibitory activity (IC_{50} of 47.1±0.21 µg/ml) against LPS-induced NO production. As for release of PGE_2, it responds in an acute phase of inflammation by sensitizing spinal neurons to pain, produces fever, increases vasopermeability and extracellular substances[16]. *E. variegata* extract showed high anti-inflammatory effect through LPS-stimulated PGE_2 release (COX-2) relative to prednisolone (IC_{50} value 9.27±0.72 and 0.96±0.01 µg/ml, respectively). It is reported that the secondary metabolites from this plant was a good source for NSAID drug development[17]. The alkaloids extracted from its leaves are reported to have anti-inflammatory

Table 1. Inhibitory effect of the ethanolic extract from *E. variegata* on LPS induced NO production, cytotoxicity and IC_{50} in RAW 264.7 cells

Plants and positive control	% inhibition of nitric oxide production (% cytotoxicity)						IC_{50}(µg/ml)
	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
E. variegata Linn.	-	2.87±0.72 (-29.34±2.42)	10.75±1.59 (-17.56±3.29)	30.65±0.44 (-15.54±1.76)	53.02±0.26 (7.33±4.43)	85.24±2.27 (16.19±1.2)	47.1±0.21
Prednisolone	32.35±1.87 (3.97±1.29)	46.11±0.48 (7.44±3.18)	58.45±1.17 (8.71±1.8)	64.86±2.03 (11.79±1.07)	74.03±1.17 (6.57±6.65)	-	1.31±0.05

Mean of triplicate ± SEM (n = 3), "-": not done

Table 2. Inhibitory effect of the ethanolic extract from *E. variegate* bark on LPS-stimulated PGE_2 release (COX-2) in RAW 264.7 cells (IC_{50} and percentage of inhibition on PGE_2 release at various concentrations)

Plants and positive control	% inhibition effect of PGE_2 release (COX-2)						IC_{50} (µg/ml)
	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	
E. variegata Linn.	-	-	-7.55±0.19	54.62±4.57	75.02±2.05	81.55±0.19	9.27±0.72
Prednisolone	-3.45±0.19	-3.73±0.28	53.31±0.09	81.83±0.84	-	-	0.96±0.01

Mean of duplicate ± SEM (n = 2), "-": not done

Table 3. Inhibitory effect of the ethanolic extract from *E. variegate* bark on LPS-induced tumor necrosis factor-alpha (TNF-α) release from RAW 264.7 cells (percentage of inhibition on TNF-α release at various concentrations IC_{50} and)

Plants and positive control	% inhibition effect of TNF-α release					IC_{50} (µg/ml)
	0.1 µg/ml	1 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	
E. variegata Linn.	-	-	-	-	9.92±2.1	>100
Prednisolone	34±4.09	50.93±3.14	70.5±4.07	86.83±2.21	-	0.95±0.19

Mean of duplicate ± SEM (n = 2), "-": not done

Table 4. Anti-inflammatory activities of *Erythrina variegata* extract through three path ways (inhibitory effect on NO production, PGE_2 release, TNF-α release)

Plant extract and positive control	IC_{50} of inhibitory effects stimulated by LPS (µl/ml)		
	NO production	PGE_2 release	TNF-α release
E. variegata Linn.	47.10±0.21	9.27±0.72	>100
Prednisolone	1.31±0.05	0.96±0.01	0.95±0.19

activity[18].

In summary

This plant extract has inhibitory effect on inflammation process. Many reports on animal model or in vivo study have shown that all part of this plant had anti-inflammatory activitity[19,20]. However, this is the first report of testing in vitro or in cells of the activity of its bark extract through the three pathways of anti-inflammation. Yet, *E. variegata* bark extract showed weak activity against TNF-α release which represents chronic inflammation and involves an increase in many mediators (IC_{50} of greater than 100 µg/ml). The cell viability or cytotoxicity test of this extract using MTT assay showed no toxicity. These results confirm and support on-going use of *E. variegata* bark in Thai traditional medicine for treatments of inflammation. In addition, *E. variegate* bark is also confirmed as an ingredient in the Mahanintangthong remedy which is used as antipyretic. It can reduce acute inflammation because its effect is to inhibit PGE_2 release, so it can reduce fever. This confirmed the use of its bark as ingredient in Lomammapruek remedy which is used for reducing pain and inflammation. Its bark showed better acute anti-inflammatory activity than for chronic inflammation because the ethanolic extract of its bark showed higher inhibitory effect on COX-2 as acute inflammation than on NO release and has no inhibitory effect through TNF-α release which represents the chronic inflammation.

Conclusion

The ethanolic extract of *E. variegata* bark exhibited higher inhibitory effect on PGE_2 as an acute

inflammatory effect than on NO production and has no effect on TNF-α release as chronic inflammation. These results are relevant to the use of *E. variegata* bark in Thai traditional medicine for treatment of inflammation-related diseases. In addition, *E. variegata* bark is also used as an ingredient in the Mahanintangthong remedy (antipyretic) and Lomammapruek remedy (analgesic and anti-inflammation). This study supports the use of *E. variegata* bark in Thai traditional medicine remedies for inflammatory treatment. The anti-inflammatory compounds should be further isolated from the ethanolic extract of its bark and studied.

What is already known on this topic?

Inflammation is defined as the local response of living mammalian tissue to injury due to any agent. The body's defense mechanism acts to eliminate the spread of the injurious agent, which may be due to heat, cold, radiation, trauma, organic and inorganic poisons, bacteria, fungi, parasites, antigen anti-body reaction and cell mediated reactions. Many Thai traditional medicinal plants are used in treatment of inflammation-related diseases. However, these plants are the subject of little scientific reporting in support of using them in treatment of inflammation-related diseases.

What this study adds?

Knowledge about the anti-inflammatory activities of ethanolic extract of *E. variegata* in vitro regarding inhibitory activity against lipopoly-saccharide induced NO production, inhibition of PGE_2 reduction by enzyme COX-2 and TNF-α in RAW 264.7 cell lines. It was shown that the ethanolic extract of *E. variegata* showed potent anti-inflammation as inhibitory activity against lipopolysaccharide induced nitric oxide production and inhibition of prostaglandins release by enzyme COX-2, but was not effective against TNF-α. These results appear to support the use of *E. variegata* for the treatment inflammation-related diseases and these results related the on-going future use of the plant in Thai traditional medicine.

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Potential conflicts of interest

None.

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ฤทธิ์ต้านการอักเสบของสารสกัดเปลือกทองหลาง

ปัญญ์ ทองมี, อรุณพร อิฐรัตน์

ภูมิหลัง: *เปลือกของต้นทองหลางในประเทศไทยถูกนำมาใช้ในการแพทย์แผนไทยในการรักษาโรคต่าง ๆ มากมาย และยังเป็นส่วนประกอบของสมุนไพรในตำรับต่าง ๆ เช่น ตำรับยามหานิลแท่งทอง (แก้ไข้) และตำรับยาแก้ลมอัมพฤกษ์ (แก้ปวด แก้กล้ามเนื้อและเส้นเอ็นอักเสบ)*

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์ต้านการอักเสบแบบภายนอกกายของสารสกัดทองหลางชั้นแอทธานอล*

วัสดุและวิธีการ: *นำเปลือกของต้นทองหลางมาหมักด้วย 95% แอทธานอล นำสารสกัดที่ได้ไปศึกษาฤทธิ์ต้านการอักเสบโดยดูการยับยั้งการสร้างไนตริกออกไซด์โดยใช้วิธี griess reagent, ฤทธิ์ยับยั้งการหลั่ง enzyme cyclooxygenase-2 (COX-2) และการหลั่ง tumor necrosis factor-alpha (TNF-α) โดยใช้ ELISA kits ในเซลล์ RAW 264.7 เมื่อถูกกระตุ้นด้วย lipopolysaccharide (LPS)*

ผลการศึกษา: *สารสกัดชั้นเอทธานอลของเปลือกต้นทองหลางออกฤทธิ์ต้านการอักเสบที่ดี โดยออกฤทธิ์ยับยั้งเอ็นไซม์ COX-2 และยับยั้งการหลั่งไนตริกออกไซด์ในเซลล์ RAW 264.7 (ค่า IC_{50} เท่ากับ 9.27±0.72 และ 47.1±0.21 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ) แต่ไม่มีผลในการยับยั้งการหลั่ง TNF-α*

สรุป: *สารสกัดชั้นเอทธานอลของเปลือกต้นทองหลาง แสดงฤทธิ์ต้านการอักเสบโดยออกฤทธิ์ยับยั้งเอ็นไซม์ COX-2 ที่เป็นผลของการอักเสบแบบเฉียบพลันดีกว่าฤทธิ์ในการยับยั้งการหลั่งไนตริกออกไซด์และการหลั่ง TNF-α ซึ่งเกิดในการอักเสบแบบเรื้อรัง ผลการวิจัยนี้สนับสนุนการใช้เปลือกต้นทองหลางทางการแพทย์แผนไทยในการรักษาอาการอักเสบ*