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Anti-inflammatory Effect and Total Flavonoid Content of The Ethanolic Seed Extracts of Three Umbelliferae Species

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ABSTRACT

The seeds of three plants in Umbelliferae, namely *Anethum graveolens*, *Cuminum cyminum* and *Foeniculum vulgare* are used as ingredients in Thai traditional medicine for treating inflammation. Additionally, they are macerated in alcohol and used as a Thai traditional drug. Although *A. graveolensm, C. cyminum* and *F. vulgare* are members of the same family, these plants consist in chemical constituent. There are no studies comparing their anti-inflammatory properties. Our objectives were to investigate and compare the anti-inflammatory activity and total flavonoid content of the ethanolic seed extracts of these three species. Results showed that *F. vulgare* extract had the highest activity in inhibiting nitric oxide, IL-6 production and the highest flavonoid content. The ethanolic extract of *C. cyminum* showed moderate anti-inflammatory activity while *A. graveolens* showed no inhibition of nitric oxide, TNF-α and IL-6 production. *F. vulgare* extract had the best anti-inflammatory properties that were related to total flavonoid content and should be now be assessed in animal models.

Keywords: Anti-inflammation; Umbelliferae; *Anethum graveolens*; *Cuminum cyminum*; *Foeniculum vulgare*

1. Introduction

Inflammation is the immune response when tissue is harmed by trauma, pathogens or chemical reagents [1]. The two steps of the inflammatory process are acute inflammation and chronic inflammation that is induced by many cytokines such as TNF-α, IL-6, IL-1β [2]. Inducible nitric oxide is an enzyme that generates nitric [3]. Inhibition of nitric oxide and proinflammatory cytokine production reduces chronic inflammation that may cause many diseases such as cancer, arthritis and cardiovascular disease [4]. Flavonoids, other phenolic compounds and terpenes that are phytochemicals in food plants and herbs

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can reduce inflammation [5].

The Umbelliferae (parsley family) are used as spices and herbs [6]. In Thailand, the seed of three Umbelliferae species, namely *Anethum graveolens*, *Cuminum cyminum* and *Foeniculum vulgare*, are the ingredients of Thai traditional drugs used for anti-inflammatory and antiflatulence agents [7]. However, there is no report on the comparative anti-inflammatory effects of these plants to see which one has the potential for further development. Thus, the objective of this study was to investigate and compare the *in vitro* anti-inflammatory activity and flavonoid content of *A. graveolens, C. cyminum* and *F. vulgare*.

2. Materials and Methods

2.1 Plant materials

The seed of *A. graveolens*, *C. cyminum* and *F. vulgare* were collected by Thai folk medicine practitioners. Plants were identified by comparison with authentic voucher specimens that were kept in the herbarium of Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkla, Thailand. The voucher specimen numbers are SKP199010701, SKP199062201 and SKP199030301, respectively.

2.2 Chemical and reagents

RAW264.7 cells were purchased from the American Type Tissue Culture Collection (Virginia, US). Dulbecco's modified eagle medium (DMEM), trypsin, penicillin and streptomycin were purchased from Gibco (New York, US). TNF-α and IL-6 ELISA Kit were purchased from Abcam (Cambridge, UK) and lipopolysaccharide (LPS) from Sigma-Aldrich (Missouri, US).

2.3 Preparation of seed extracts

The seed of *A. graveolens*, *C. cyminum* and *F. vulgare* were ground to powder, and each was macerated with 95% ethanol for 3 days. Then, the ethanol phase was filtered through Whatman No.1 paper, evaporated and dried by a lyophilizer. The ethanolic seed extracts of the three plants were weighed and stored at -20°C until used.

2.4 Inhibition of nitric oxide production in LPS-stimulated RAW264.7 cells [8]

RAW264.7 cells were maintained in complete DMEM supplemented with 10% fetal bovine serum 100 U/mL of penicillin and 100 µg/mL of streptomycin. The cells were seeded into a 96-well plate with $1x10^5$ cells/well and incubated at 37°C in an atmosphere containing 5% CO_2 for 24 hr. The cells were replaced with 100 µl of LPS (5 ng/mL) and 100 µl of the seed extracts or prednisolone as a positive control at various concentrations. Then cells were incubated at 37°C in an atmosphere containing 5% CO_2 for 24 hr. The supernatant (100 µl/well) was transferred to another 96-well plate and 100 µl of Griess reagent added into each well. The optical density (OD) of this plate was measured at 570 nm. The percent inhibition of nitric oxide production (NO) was calculated using the equation below.

$$\%\,\text{Inhibiton} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100 \quad (2.1)$$

All sample was screened for nitric oxide production inhibition at 100 µg/ml. The dilution of each sample was performed when the percent inhibition was higher than 50%. After transferring, MTT (10 µl/well) was added into cells to detect the toxicity of the extract to RAW264.7 cells and incubated for 2 hr. Then 0.04 M HCl in isopropanol was substituted to dissolve the formazan. The optical density of these plates was measured at 570 nm. The percent survival of cells was calculated by the equation below. The extract had cytotoxic effect when RAW264.7 cell survival was less than 70%.

$$\%\text{Survival} = \frac{\text{OD sample}}{\text{OD control}} \times 100 \qquad (2.2)$$

2.5 Assessment of IL-6 and TNF-α production

RAW 264.7 cells were treated by various concentrations of seed extracts or prednisolone and LPS (5 ng/ml) following the procedure of NO production inhibition. The supernatant was collected, and cytokine production measured using an IL-6 mouse ELISA kit (ab46100) and the TNF-α Simple Step ELISA kit (ab208348). Briefly, the supernatant was transferred to an ELISA plate that was coated with an antibody for mouse TNF-α or IL-6. Then, the antibody was added to each well and incubated at room temperature. The plate was washed with wash buffer, and TMB substrate (100 µl) was added into each well.

After 20 minutes, 100 µl of stop solution was added to each well and the optical density was read at 450 nm. The percent inhibition of IL-6 and TNF- α production were calculated. All samples were screened for IL-6 and TNF-α production inhibition at 100 µg/ml. The dilution of each sample was performed when the percent inhibition was higher than 50%.

2.6 Determination of total flavonoid content [9]

The seed extracts of *A. graveolens*, *C. cyminum* and *F. vulgare* were prepared at 1,000 µg/mL (500 µl). Then, 5% $NaNO_2$ (75 µl) and 10% $AlCl_3$ (150 µl) were added and the extracts incubated for 5 minutes. 500 µl of 1 M NaOH and 275 µl of deionized water was added next to each tube and incubated for 30 minutes. The optical density was measured at 510 nm. Quercetin was used to plot a standard curve. The total flavonoid content in the extracts is expressed as quercetin equivalents in milligram per gram of dried extract (mg QE/g).

2.7 Statistical analysis

All experiments were performed in triplicate. The results are expressed as mean ± standard error of the mean (mean ± SEM). Data were analyzed using one-way ANOVA with Tukey's test. A p-value < 0.05 denoted statistical significance.

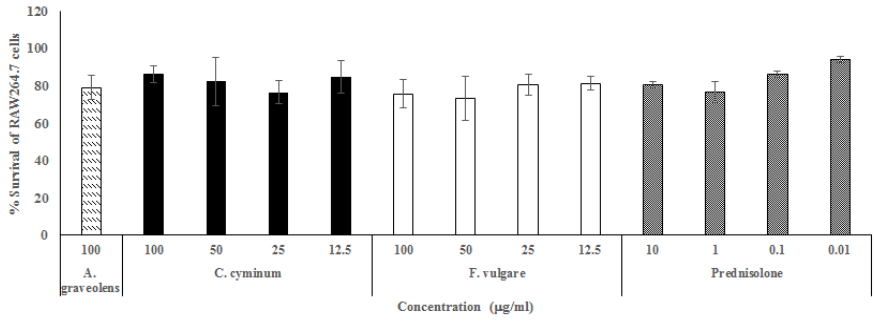


Fig. 1. Effect of the ethanolic seed extracts on survival of RAW264.7 cells.

Table 1. Total flavonoid content and the IC_{50} values of the ethanolic seed extract of three plant species on anti-inflammatory activity.

Extract	Total flavonoid content (mg QE/g of dried extract)	IC_{50} (μg/mL)		
		Nitric oxide	TNF-α	IL-6
A. graveolens	37.67 ± 10.74	> 100	> 100	>100
C. cyminum	92.50 ± 6.66	65.30 ± 7.35	> 100	>100
F. vulgare	147.17 ± 11.15	47.91 ± 6.93	> 100	77.68 ± 4.43
Prednisolone	-	0.14 ± 0.05	0.08±0.01	0.1 ± 0.01

3. Results and Discussion

3.1 Cytotoxicity of the ethanolic seed extracts of *A. graveolens*, *C. cyminum* and *F. vulgare* on RAW264.7 cells

All extracts were investigated for toxicity on RAW264.7 cells. The results showed that all ethanolic seed extracts and LPS (5 ng/ml) were not toxic to RAW264.7 cells and the percent survival > 70% and were not significantly different when compared with normal cells, as shown in Fig. 1.

3.2 Effect of the ethanolic seed extracts of *A. graveolens*, *C. cyminum* and *F. vulgare* on nitric oxide production.

The ethanolic extract of *C. cyminum* and *F. vulgare* showed inhibition of nitric oxide production at concentrations of 100 μg/ml with percent inhibitions of 69.81± 3.20 and 78.70 ± 6.81 %, respectively, whilst *A. graveolens* (100 μg/mL) showed the least inhibitory effect 34.55±1.29%. The ethanolic extracts of *C. cyminum* and *F. vulgare* and their effect on nitric oxide production as a function of concentration is shown in Fig. 2.

Half of the maximal inhibitory concentration (IC_{50}) of *F. vulgare* extract was lower vs. *C. cyminum* with IC_{50} values of 47.91 vs. 65.30 μg/mL (Table 1) suggesting a better inhibitory effect.

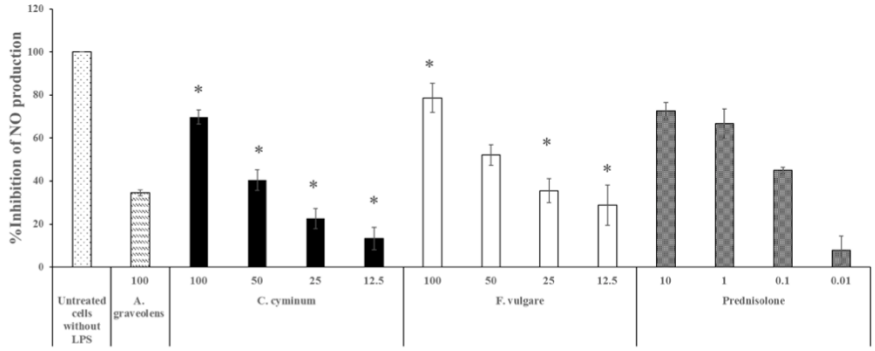
3.3 Effect of the ethanolic seed extracts of *A. graveolens*, *C. cyminum* and *F. vulgare* on proinflammatory cytokine production.

The ethanolic extract of *A. graveolens* (100 μg/mL) had no inhibitory effect on TNF-α production (%Inhibition = 0.01%) but it inhibited IL-6 production with a percent inhibition of 40.81 ± 2.06 %. *C. cyminum* extract showed moderate activity on TNF-α and IL-6 inhibition with percent inhibitions of 38.72 ± 0.74 and 25.45 ± 1.48%, respectively, at concentrations of 100 μg/mL. The ethanolic extract of *F. vulgare* showed the highest activity when compared with the other seed extracts. The *F. vulgare* extract inhibited TNF-α with a percent inhibition of 42.21 ± 0.42% that was lower than 50%. However, its inhibitory effect on IL-6 production was highest, 63.20 ± 1.04% at 100 μg/mL.

When *F. vulgare* extracts were diluted in various concentrations (12.5-100 μg/mL), they showed dose-independent IL-6 inhibition. The IC_{50} of *F. vulgare* extract on IL-6 inhibition was 77.68 ± 4.43 μg/mL. The results of the three seed extracts on TNF-α and IL-6 production are shown in Figs. 3 and 4 and Table 1.

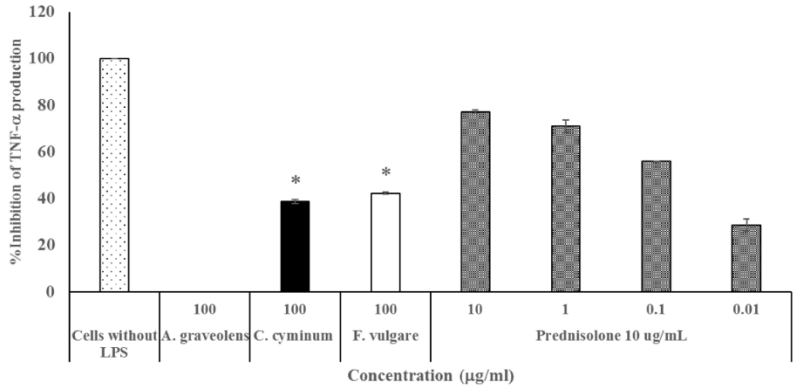
3.4 Total flavonoid content of the ethanolic seed extracts of *A. graveolens*, *C. cyminum* and *F. vulgare*.

The ethanolic contents of the seed extracts of the three plant species were analyzed for total flavonoid content, as shown in Table 1. The ethanolic extract of *F. vulgare* had the highest flavonoid content followed by *C. cyminum* and *A. graveolens*.



* p-value < 0.05 when compared with the LPS-treated RAW264.7 cells

Fig. 2. The percent inhibition of the ethanolic seed extracts on nitric oxide production.



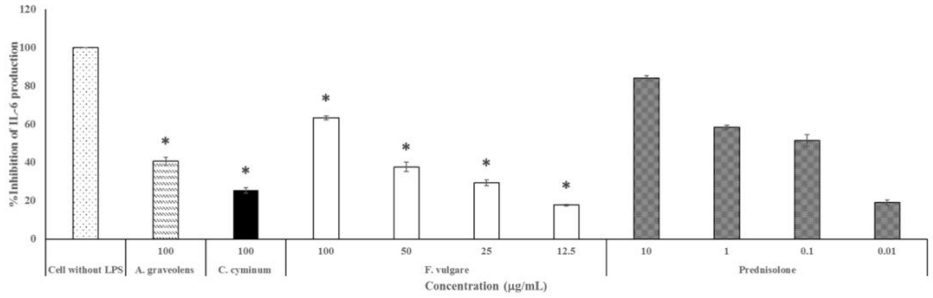
* p-value < 0.05 when compared with the LPS-treated RAW264.7 cells

Fig. 3. The percent inhibition of the ethanolic seed extracts at 100 µg/mL on TNF-α production.

Umbelliferae are used as spices that contain volatile oil [6]. *A. graveolens*, *C. cyminum* and *F. vulgare* are macerated with alcohol and used in Thai traditional medicine for the treatment of pain, inflammation and flatulence [7].There are no scientific reports on the anti-inflammatory effect of these ethanolic extracts. Our study investigated and compared the anti-inflammatory effect and flavonoid content of the ethanolic extracts of *A. graveolens*, *C. cyminum* and *F. vulgare*.

The results showed that the *F. vulgare* extract had the highest flavonoid content and the highest anti-inflammatory activity in terms of inhibiting nitric oxide and IL-6 production. Nitric oxide is produced by macrophages activated by pathogens, which then induce the production of proinflammatory cytokines secretion such as IL-6, TNF-α, IL-1 and IFN-γ [3, 10] and subsequent tissue inflammation. Moreover, TNF-α is produced in large amounts and can also stimulate IL-6 synthesis, which, in turn, enhances acute inflammation [11]. The ethanolic extract of *F. vulgare* can inhibit IL-6 and nitric oxide production and may also reduce chronic inflammation.

Our data suggest that the anti-inflammatory effects of *A. graveolens*, *C. cyminum* and *F. vulgare* could be related to their flavonoid content. The *F. vulgare* extract showed the highest anti-inflammation and had the highest



* p-value < 0.05 when compared with the LPS-treated RAW264.7 cells
Fig. 4. The percent inhibition of the ethanolic extracts on IL-6 production.

flavonoid content, followed by *C. cyminum*, which only inhibited nitric oxide production and *A. graveolens*, which had no in vitro effect on nitric oxide and IL-6 production. A previous report indicated that the seed oil of *C. cyminum* and *F. vulgare* showed anti-inflammatory activity that involved the NF-κB signaling pathway [12-14]. The main compound of the seed oils of *A. graveolens*, *C. cyminum* and *F. vulgare* are terpenes compound [15-17] but phenolic compounds including flavonoids are also present [18, 19].

F. vulgare has been reported to contain quercetin and kaempferol that inhibit inflammation via decreased activation of NF-κB and STAT-1 that are involved in the upregulation of inflammatory cytokines [20, 21]. Our results showed that the ethanolic extract of *F. vulgare* had the highest flavonoid content, and the highest anti-inflammatory activity. However, the ethanolic extract of *F. vulgare* may have also contained terpenes which would have contributed to the observed anti-inflammatory effect. The *C. cyminum* extract showed moderate anti-inflammatory activity, but other research has showed that the ethanolic extract of *C. cyminum* has analgesic properties and has been used to treat inflammation in animals [22]. The ethanolic extract of *C. cyminum* may contain volatile oils that decrease inflammation. The ethanolic extract of *A. graveolens* showed the lowest anti-inflammatory activity and the least amount of flavonoid. Again, in contrast to our finding, others report an anti-inflammatory effect of *A. graveolens* seed oil or non-polar extract [15, 23]. The petroleum ether extract of *A. graveolens* showed significant effect on inhibition of nitric oxide production, IL-6 and IL-1 in RAW264.7 cells, while the butanolic extract had no effect on nitric oxide production [23]. Similarly, our ethanolic extract of *A. graveolens* showed no inhibition effect on nitric oxide, TNF-α and IL-6 production. One possibility for the discrepant finding is that the chemical constituents of *A. graveolens* that inhibit the inflammatory process may be a non-polar group, so its ethanolic extract showed less in vitro anti-inflammatory effect than *F. vulgare* and *C. cyminum*. However, an exogenous substance is modified by the liver to structure changing and uptake to blood flow [24]. Thus, the ethanolic extract of *A. graveolens* may show anti-inflammatory effect in animal models.

4. Conclusion

The results of this study showed that anti-inflammatory activity might relate to the flavonoid content of the ethanolic seed extracts of *A. graveolens*, *C. cyminum* and *F. vulgare*. *F. vulgare* extract showed the

best anti-inflammatory agent and contained the most flavonoids followed by *C. cyminum* while *A. graveolens* extract showed no in vitro anti-inflammatory activity and contained the least flavonoids. These findings suggest that the ethanolic extract of *F. vulgare* should be investigated for the mechanism of its anti-inflammatory potency for possible medicinal applications.

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