Research Article

Anti-Inflammatory Effects of Essential Oils of *Amomum aromaticum* Fruits in Lipopolysaccharide-Stimulated RAW264.7 Cells

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Inflammation is a vital physiologic response of cellular injury, infection, or autoimmune activation. Overproduction of proinflammatory mediators such as cytokines, interleukins, nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX-2) may result in various diseases such as rheumatoid arthritis, asthma, multiple sclerosis, and atherosclerosis. In this study, we assessed for the first time the anti-inflammatory effects of the essential oils of *Amomum aromaticum* fruits (AAE) in RAW264.7 murine macrophage model. As a result, AAE potently inhibited the production of nitric oxide in LPS-induced RAW264.7 cells with the IC50 value of 0.45 ± 0.11 μg/ml. AAE also dose-dependently reduced the expression of two proinflammatory proteins iNOS and COX-2 in the stimulated cells. Phytochemical analysis revealed that major compositions of the volatile oils including 1,8 cineole (48.22%), geranial (9.24%), neral (6.72%), α-pinene (2.43%), and α-terpineol (2.28%) may contribute greatly to the inhibition effects due to their anti-inflammatory properties. The results suggest for the potential uses of AAE in chronic inflammation prevention.

1. Introduction

Chronic inflammation is an undesirable phenomenon of a prolonged inflammatory response. Overproduction of proinflammatory mediators such as cytokines, interleukins, nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX-2) may result in various diseases such as rheumatoid arthritis, asthma, multiple sclerosis, and atherosclerosis [1]. Therefore, control of proinflammatory responses is a wise strategy to prevent the development of inflammatory diseases. Since the ancient time, food was determined as an important source for prevention of diseases. There has been accumulation of evidence that increases consumption of certain foods might lower the risk of cardiovascular disease, cancer, and inflammation [2, 3].

*Amomum aromaticum* Roxb. is a species of the Zingiberaceae family, which is a common spice and food flavoring agent in Vietnam and other Asian countries. The fruits of this plant have been used in traditional medicine for the treatment of cough, abdominal pain, vomiting, diarrhea, and malaria. The oils of seeds have been used in India for benefiting the digestive system, applied to the eyelids to eliminate the inflammation [4, 5]. To date, there has been only few studies about the phytochemicals as well as the biological activities of this plant. Recently, *A. aromaticum* essential oils are shown as promising antileishmanial agent in a screening program of 37 plants of Vietnam flora [6]. The methanolic extract of *A. aromaticum* exhibited significant antimicrobial activity against *Enterococcus faecalis, Staphylococcus aureus, Enterobacter aerogenes, Proteus mirabilis,*
and *Pseudomonas aeruginosa* with the MIC values ranging from 3.41 to 9.63 mg/ml [7].

In this study, we investigated the phytochemical contents of essential oils of the *A. aromaticum* fruits and its anti-inflammatory properties including NO production inhibition assay and inhibitory effects on the expression of two key enzymes of inflammation process: inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW264.7 cells stimulated with LPS.

2. Materials and Methods

2.1. Plant Materials and Essential Oil Preparation. The fruits *A. aromaticum* were freshly collected in Ha Giang province in November 2019. The samples were taxonomically identified by Dr. Nguyen the Cuong, Institute of Ecology and Biological Resources (VAST), and voucher specimens were deposited in the Institute of Marine Biochemistry. The samples (500 g) were hydrodistilled in a Clevenger-type apparatus for 4 h, after which the essential oils were separated and dried with anhydrous Na$_2$SO$_4$. The obtained oils (AAE) were stored at −5°C until used.

2.2. GC/MS Analysis of Essential Oils. GC/MS analysis was performed using an Agilent GC7890A apparatus coupled to a mass selective detector (Agilent 5976C). A HP-5MS fused silica capillary column (60 m x 0.25 mm id. x 0.25 μm film thickness) was used. Helium was the carrier gas with a flow rate of 1.0 ml/min. The inlet temperature was 240°C, and the oven temperature program was as follows: 60°C to 220°C at 4 °C/min and then at 20°C/min to 240°C. The split injection mode was 1:142, the detector temperature was 240°C, and the injection volume was 0.1 μl. The MS interface temperature was 240°C, MS mode, E.I. detector voltage 1300 V, and mass range 40–400 Da at 1.0 scan/s. Identification of components was achieved based on their retention indices and by comparison of their mass spectral fragmentation patterns with those stored on the MS library (NIST08, Wiley09). Component relative contents were calculated based on total ion current without standardization. Data processing was MassFinder4.0.

2.3. Cell Culture. Murine macrophage RAW264.7 cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and penicillin (100 units/ml)-streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA, USA). Cultures were maintained in a CO$_2$ incubator-humidified atmosphere 5% CO$_2$ at 37°C.

2.4. Assay for Inhibition of NO Production. The effects of samples on the NO production in LPS-stimulated RAW264.7 macrophage cells were examined as described previously [8]. The cells were seeded in 96-well plate at 2 x 10$^5$ cells/well and incubated for 18 h. The plates were pretreated with AAE (from 0.1 μg/ml to 100 μg/ml) for 30 min and then incubated for another 24 h with or without 1 μg/ml LPS (Escherichia coli 0111: B4; Sigma Aldrich, USA). 100 μl of the culture supernatant was transferred to other 96-well plates, and 100 μl of Griess reagent was added. The absorbance of the reaction solution was read at 570 nm with a XMark microplate reader (BioRad, USA). The remaining cell solutions in cultured 96-well plate were used to evaluate cell viability by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [9]. Cardamonin, a known NO production inhibitor, was used as a positive control [10].

2.5. Western Blot Analysis. The RAW264.7 cells were harvested and lysed in a lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% NP-40, 5 mM sodium orthovanadate, and protease inhibitors cocktail (BD Biosciences)) and then centrifuged for 10 min at 4°C and 15,000 rpm. An equal amount of protein was separated onto SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to a PVDF membrane (Millipore, Germany). The membranes were blocked in 5% nonfat skim milk for 1 h at room temperature, probed with the appropriate primary antibodies, washed, and then incubated with the corresponding secondary antibodies. α-Tubulin was used as the loading control. The signal was developed using the ECL (enhanced chemiluminescence) system (GE Healthcare, UK) and detected in a gel imaging system Azure c300 (Azure Biosciences, UK). The captured images were analyzed and quantified using ImageJ v. 1.53a (NIH, Maryland, USA).

2.6. Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). Statistical significance was assessed by the two-tailed unpaired Student’s t test, and $P$ values less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Phytochemicals of AAE Analyzed by GC/MS. The essential oils of *A. aromaticum* fruits (AAE) obtained by hydrodistillation yields 1.49% based on a calculation with the dry weight of fruits. A total of 25 compounds were identified by using GC/MS data in combination with the MS library analyses (Table 1). The major chemical group of AAE is monoterpenes with more than 81% of the total contents. Of which, 1,8-cineole (or eucalyptol, 48.22%), geraniol (9.24%), nerol (6.72%), α-pinene (2.43%), α-terpineol (2.28%), and β-pinene (2.18%) are among the most abundance monoterpenes of the fruit oils. Four aliphatic aldehydes was found including n-octanal, 2-octenal, (E,E)-decenal, and (E,E)-dodecanal which comprise about 8.26% of the AAE content. Meanwhile, only one sesquiterpene E-nerolidol (1.69%) was found. Our results were in good agreement with the previous report which shows monoterpenes as the major contents together with the presence of aliphatic aldehyde and sesquiterpene groups [6]. The fruit essential oils contained 55.2% of 1,8-cineole which was slightly higher than that of our findings. The distribution of relative quantities of major
monoterpenes in both studies was found to be similar. The difference of quantities of individual compounds in both samples may be due to the variation of the origin of samples, seasons of collection, or the environmental factors. The presence of high content of 1,8-cineole was not only found in A. aromaticum but also in some other Amomum species including A. tsao-ko (23.87%–45.24%) [11–13] and A. subulatum Roxb (20%–89%) [14–16] depending on parts of the plant used for analysis.

3.2. AAE Reduced the NO Production in LPS-Induced RAW264.7 Cells by Inhibiting the Expressions of iNOS and COX-2. NO is an important signaling molecule in various physiological and pathophysiological responses [17]. Searching for inhibitors of NO production in LPS-stimulated macrophages has been a worldwide effort for the development of anti-inflammatory agents. The in vitro anti-inflammatory activity of AAE was investigated by determining its NO production inhibitory effect in LPS-stimulated RAW264.7 cells. The primary screening results showed that AAE inhibited potently the NO production (about 100%) in the stimulated cells at concentration of 100 μg/ml. We further evaluated the potency of the inhibitory activity of AAE by determining its IC50 value. As the results, the IC50 value of AAE was determined as 0.45 ± 0.11 μg/ml which was slightly higher than the positive control, cardamonin (0.59 ± 0.18 μg/ml). Treatment of AAE at the screening concentration after 24 h had no impact on the cell viability (data not shown). Next, we investigated the effects of AAE on the two key enzymes of inflammation process: iNOS (inducible nitric oxide synthase), mainly responsible for the production of NO and COX-2 (cyclooxygenase-2), in charge of production inflammatory mediators such as PGE2 (prostaglandin E2). The western blot analysis revealed that AAE dose-dependently inhibited the expression of both enzymes. Remarkably, at a concentration of 0.3 μg/ml, the inhibitory effects of AAE against iNOS and COX-2 expressions were still observed significantly (Figure 1(b)). To our knowledge, this is the first report of this potent anti-inflammatory activity of the A. aromaticum essential oils.

The phytochemicals are considered as the major contributors to the biological activity of a plant sample. In our study, we found that the fruit essential oils showed remarkable anti-inflammatory effects. The major composition of AAE, as indicated, is 1,8-cineole which comprises about 48% of the total oil content. Interestingly, 1,8-cineole was demonstrated as a very promising anti-inflammatory agent. Molecular mechanism studies indicated that 1,8-cineole effectively reduced the expression of proinflammatory cytokines such as TNF-IL-1β and IL-6 with the IC50 values ranging from 0.2 to 7.0 μM. It was found to be a potent inhibitor of NF-κB activation [18], 1,8-cineole also displayed its anti-inflammatory properties in various animal models. This compound was advanced to clinical trials for bronchial asthma. When administered as an adjunct therapy with prednisolone, 1,8-cineole showed a significant improvement in respiratory volume and quality of asthma. The effect was still maintained when the dosage of prednisolone was decreased by 36% [19]. Other major compositions of AAE such as α-pinene [20], α-terpineol [21], geraniol [22], neral, and geranial [23] also exhibited their effects of anti-inflammation. It is demonstrated that the chief monoterpenes of AAE seem to greatly contribute to the anti-inflammatory activity of the fruit oils.

The anti-inflammatory properties of essential oils of some other Amomum species were reported. The fruit extract of A. tsao-ko displayed potent anti-inflammatory effects in RAW264.7 cells stimulated with LPS [24]. Further studies showed that the effects were achieved because this extract induced the expression of heme oxygenase-1 which consequently increased the Nrf-2 activation. The similar effects were also obtained from different extracts and isolated compounds from A. tsao-ko [25–27]. Agnihotri et al. investigated the topical anti-inflammatory effect of the fruit essential oils of A. subulatum. The results showed that the volatile oils exhibited moderate activities compared with standard drug, diclofenac [14]. The extracts of A. compactum, A. xanthoides, and A. vilosum also demonstrated their anti-inflammatory activities in vitro and in vivo [28–30]. Interestingly, there have been very few studies on the Amomum essential oils with anti-inflammation. In our study, we have reported that AAE is a promising anti-inflammatory agent by potently inhibiting the production of nitric oxide, the expressions of iNOS and COX-2 in LPS-induced RAW264.7 murine macrophages. Notably, A. aromaticum has been traditionally used as a common spice suggesting its safety effects in therapeutic use.

Table 1: Chemical compositions of essential oils of A. aromaticum fruits.

| No. | Compounds            | RI   | Relative percentage (%) |
|-----|----------------------|------|-------------------------|
| 1   | α-Thujene            | 930  | 0.16                    |
| 2   | α-Pinene             | 939  | 2.43                    |
| 3   | Sabinene             | 978  | 0.54                    |
| 4   | β-Pinene             | 984  | 2.18                    |
| 5   | Myrcene              | 991  | 0.46                    |
| 6   | n-Octanal            | 1003 | 0.47                    |
| 7   | α-Phellandrene       | 1010 | 1.43                    |
| 8   | O-Cymene             | 1029 | 0.51                    |
| 9   | Limonene             | 1034 | 2.9                     |
| 10  | 1,8-Cineole          | 1038 | 48.22                   |
| 11  | (E)-β-cymene         | 1048 | 0.83                    |
| 12  | 2-Octenal            | 1058 | 0.95                    |
| 13  | γ-Terpinenate        | 1063 | 0.3                     |
| 14  | Linalool             | 1101 | 0.37                    |
| 15  | Isoneral             | 1166 | 0.19                    |
| 16  | δ-Terpinone          | 1174 | 0.21                    |
| 17  | Isogeranial          | 1184 | 0.3                     |
| 18  | Terpinen-4-ol        | 1185 | 0.92                    |
| 19  | α-Terpinol           | 1198 | 2.28                    |
| 20  | Neral                | 1246 | 6.72                    |
| 21  | Geraniol             | 1256 | 1.33                    |
| 22  | (E,E)-Decanal        | 1264 | 4.9                     |
| 23  | Geranial             | 1275 | 9.24                    |
| 24  | (E,E)-Dodecanal      | 1470 | 1.94                    |
| 25  | E-Nerolidol          | 1570 | 1.69                    |

Total identified (%) 91.47
Yield* (%) 1.49

*a Yield calculated based on the fresh materials; RI: retention index.
For the first time, the anti-inflammatory properties of the fruit essential oils of *Amomum aromaticum* Roxb. were investigated. The volatile oils displayed potent inhibitory effects against the production of nitric oxide; the expression of two proinflammatory enzymes iNOS and COX-2 in RAW264.7 macrophages was stimulated with LPS. Phytochemical investigation revealed that the essential oils contain various anti-inflammatory compositions including 1,8 cineole (48.22%), geranial (9.24%), neral (6.72%), α-pinene (2.43%), and α-terpineol (2.28%). These findings suggest that essential oils of *A. aromaticum* fruits can be an alternative natural source for prevention of chronic inflammation. Further studies are necessary for evaluation of anti-inflammation mechanisms of action and in vivo assessments of the very promising essential oils.

### Data Availability

The data used to support the findings of this study are included within the article.

### 4. Conclusions

For the first time, the anti-inflammatory properties of the fruit essential oils of *Amomum aromaticum* Roxb. were investigated. The volatile oils displayed potent inhibitory effects against the production of nitric oxide; the expression of two proinflammatory enzymes iNOS and COX-2 in RAW264.7 macrophages was stimulated with LPS. Phytochemical investigation revealed that the essential oils contain various anti-inflammatory compositions including 1,8 cineole (48.22%), geranial (9.24%), neral (6.72%), α-pinene (2.43%), and α-terpineol (2.28%). These findings suggest that essential oils of *A. aromaticum* fruits can be an alternative natural source for prevention of chronic inflammation. Further studies are necessary for evaluation of anti-inflammation mechanisms of action and in vivo assessments of the very promising essential oils.

### Conflicts of Interest

All authors declare that they have no conflicts of interest.

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