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ANTI-INFLAMMATORY PROPERTIES OF *AMOMUM MAXIMUM* ROXB AND *AMOMUM MURICARPUM* ELMER IN THE NORTH OF VIETNAM

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SUMMARY

Inflammation is the body's homeostatic defense mechanism in which the immune system reacts to remove foreign bodies. Chronic inflammation can increase the risk for additional damage like autoimmune diseases, arthritis, diabetes and can result in death. *Amomum maximum* Roxb and *Amomum muricarpum* Elmer distributed widely in Vietnam have been used in traditional medicine for treatment of some gastrointestinal diseases. This study aimed to investigate the anti-inflammatory effects of the methanol extracts of *A. maximum* (AMM) and *A. muricarpum* Elmer (AMC) in murine macrophage RAW 264.7 cell line. The total extracts showed that the extracts exhibited low cytotoxicity and potent anti-inflammatory activities by suppressing excessive nitric oxide (NO). The IC_{50} values of AMC and AMM were found to be 12.67 ± 1.7 µg/mL and 42.7 ± 2.5 µg/mL, respectively. To elucidate the underlying mechanism, the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were evaluated using Western blot analysis. Our data demonstrated that AMC reduced the inflammatory response in a lipopolysaccharide (LPS)-induced RAW264.7 cell model via inhibition of iNOS and COX-2 while AMM seemed to modulate the inflammatory effect through the iNOS pathway only. In conclusion, AMM and AMC root extracts might be potential candidates for a study of naturally alternative anti-inflammatory drugs.

Keywords: *Amomum muricarpum*, *Amomum maximum*, anti-inflammation, nitric oxide synthase, cyclooxygenase

INTRODUCTION

Inflammation, which is classified into acute and chronic stage, is a vital biological process responding to eliminate detrimental agents such as radiation, pathogens, and damaged cells. Cell membranes, in the presence of inflammatory stimuli, cause the release of phospholipase A_2, arachidonic acid, and other inflammatory mediators such as cytokines, prostaglandins, and leukotrienes, inducing the migration of leukocytes to inflammation (Mollace *et al.,* 2005).

Acute inflammation which is characterized by the increasing movement from blood to particular damaged sites of certain immune cells, for instance, neutrophils and macrophages. In the innate immune response, macrophages use several potent mechanisms such as NO signaling to tackle the situation. The period of this phase is dependent on the balance between the surviving capacity of the pathogens and the ability of the macrophages to eliminate them. If macrophages are able to control the infection and remove the nonself elements in this duration, macrophages, then, exert anti-inflammatory and healing function. Otherwise, it leads to the chronic stage which simultaneously destroys and heals the injured tissues. It is also commonly associated with severe diseases such as cancer and diabetes (Valledor *et al.,* 2010).

Being distributed in China, Indian subcontinent, and Southeast Asia, *Amomum*, which belongs to the Zingiberaceae family, contains 170 species. As listed as spice ginger, it is valuable as a condiment and flavoring agent in Asia countries. In folklore medicines, the *Amomum* flowers, fruit, root, stem, leaf extracts have a wide range of pharmacological activities such as analgesic, anti-inflammatory activities and improve the problem related to the digestive system (Lee *et al.,* 2012, Dang *et al.,* 2020).

Amomum muricarpum Elmer is a member of *Amomum* genus. In Vietnam, it is distributed in Hanoi, Lam Dong, Lao Cai, Ninh Binh, and Yen Bai. Several types of phytochemicals have been found in *A. muricarpum* including diarylheptanoids, flavonoids, tannins, saponins (Barbosa *et al.,* 2016). *A. maximum* Roxb is a perennial plant, growing abundant in tropical forests in South China and Southeast Asia including Vietnam. *A. maximum* fruits have been occasionally used as a traditional medicine to treat stomach diseases, and digestive disorder. A recent research showed that *A. maximum* extracts exhibited the cytotoxic effect in several cancer cell lines (Luo *et al.,* 2014); nevertheless, there is no available data showing their anti-inflammatory activities in murine macrophage RAW264.7. Considering that many *Amomum* species have been found to have pharmacological effects, *A. muricarpum* and *A. maxium* are expected to have potential biological activity. In this study, we investigated the anti-inflammatory activities of two methanolic extracts of these species.

MATERIALS AND METHODS

Plant materials

The stems of *A. muricarpum* Elmer and roots of *A. maximum* Roxb collected from Me Linh biodiversity station, Vinh Phuc in May 2019 were identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources. A voucher of specimens was deposited at University of Science and Technology of Hanoi.

Plant extraction

The samples (10 g) were cut into pieces then suspended in methanol 100% (Fisher Scientific Ltd.) with the ratio of 1:3 (w:v). The mixtures were then sonicated three times in an ultrasonic bath in 30 minutes at 40°C each to accelerate the extraction process. The obtained extracts were subjected to a rotary evaporator which removes the solvent by evaporation to obtain the crude extract of *A. maximum* Roxb - AMM (720 mg) and *A. muricarpum* Elmer - AMC (940 mg). The extracts were then dissolved in DMSO to acquire the desirable concentration and then stored at low temperatures for further analysis.

Cell culture

Murine macrophage RAW 264.7 cell line was kindly provided by Prof. Lee Jeong Hyung, Kangwon National University, Gangwon-do, Korea. The cells, cultured with DMEM supplemented with 10% FBS (Gibco, Invitrogen, USA), 1% Pen Strep solution (penicillin 10000 units/mL and streptomycin 10000 µg/mL, Gibco, Invitrogen, USA), were maintained in the humidified incubator at 37°C with 5% CO_2. The medium was changed every 1 - 2 days and daily checked for contamination under the microscope CKX-53 (Olympus, Japan).

Assay for inhibition of NO production

The effects of samples on the NO production in LPS-stimulated RAW 264.7 macrophage cells, based on the Griess reaction, were examined. The cells were seeded in a 96-well plate at the concentration of 0.5 x 10^5 cells per well and incubated in the humidified incubator at 37°C, 5% CO_2 for 22 hours. After 22 hours of incubation, cells were pre-treated with AMC sample with four concentrations 3.125, 6.25, 12.5 and 25 µg/mL (DMSO ≤1.25%, v/v) and AMM with 10, 25, 50, 100 µg/mL then after 30 mins added 0.1 mg/mL LPS (*Escherichia coli* 0111: B4; Sigma Aldrich, USA). The cells were incubated for the next 24 hours. 100 µL of the culture supernatant was transferred to a different 96-well plate, adding 100 µL of Griess reagent. The absorbance of the reaction solution was read

at 570 nm with an iMark microplate reader (BioRad, USA). The remaining cell from the original 96-well plate was further used for cell viability assay (MTT assay). The assay is based on the cleaving action of dehydrogenases in functioning mitochondria of living cells on the tetrazolium ring of MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide), estimating viable cell number. Cardamonin, which is a well-known NO production inhibitor, was used as a positive control.

Western Blot analysis

Proteins from RAW 264.7 cells were collected using 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 Bio- sciences) and then centrifuged at 15,000 rpm in 5 mins. An equal amount of denatured proteins, after being quantified by Bradford assay, was separated onto SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) then transferred to PVDF membranes (Millipore, Germany). The membranes were blocked in 3% BSA in TBS-Tween 20 for 1 hour under gentle agitation, at room temperature. They were incubated overnight for 16 hours at 4°C with either primary anti-COX-2, iNOS (Thermo Fisher, USA), β-Tubulin (Santa Cruz, USA), washed and further incubated with the corresponding secondary antibodies. The signals were revealed using the ECL (enhanced chemiluminescence) system (GE Healthcare, UK) and detected by ImageQuant LAS 400, GE Healthcare, UK. The captured images were analyzed and quantified using ImageJ v. 1.53a (NIH, Maryland, USA).

Statistical analysis

Most statistical results were analyzed from at least three independent experiments. Data were presented as the mean ± standard deviation (SD) using Graphpad Prism software. Student's t-test and ANOVA test were used for statistical analysis. Immunoblotting images were analyzed by ImageJ software.

RESULTS AND DISCUSSION

Evaluation of NO production and cell viability

Nitric oxide (NO) is a well-known mediator, which associates inflammation in both physiological and pathological processes. It is a short-lived second messenger which is normally produced by immunocompetent cells, for instance, macrophages (Coleman 2001), and becomes detrimental to the body in excessive amounts. The evaluation of NO production was determined by Griess reaction and carried out simultaneously with MTT assay. Our results demonstrated the significant NO inhibition of AMC at 12.5 and 25 µg/mL with no cytotoxicity (Fig. 1). Meanwhile, even though AMM showed significant inhibition at 50 and 100 µg/mL, the latter caused the toxicity, reducing the viability to 79.5%. These results excluded the potential cytotoxic effect of AMC (6.25, 12.5, and 25 µg/mL) and AMM (10, 25, 50 µg/mL) indicating that they could be good subjects for further study.

The IC_{50} values of AMC and AMM were found to be 12.67 ± 1.7 µg/mL and 42.7 ± 2.5 µg/mL which were both noticeably lower than that of *A. testaceum* Ridl in ethanol extract (81.42 ± 3.48 µg/mL) (Makchuchi et al. 2010). These represent the potential of the plants against inflammation. Moreover, a recent study showed that diarylheptanoids of AMC (muricarpone B) displayed its inhibitory activity with an IC_{50} of 6.57 ± 1.18 µM (Le *et al.,* 2017), which complement the effect of AMC extract.

Inhibitory effect of the extracts on iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells

To ascertain the anti-inflammatory activity of AMC and AMM in RAW 264.7, the regulation of COX-2 and iNOS protein was tested by immunoblotting. There have been various investigations in the concomitant biosynthesis and release of both NO and prostaglandins (PGs). Stimuli that promote iNOS and NO formation also have the potential to enhance COX2 expression, but with varying time courses for induction (J. B. Weinberg 2000).

Since both mediators are involved in the underlying mechanism of several inflammatory disease states, the correlation between NO and PG biosynthesis has been studied to demonstrate a possible combined approach in the treatment of many conditions.

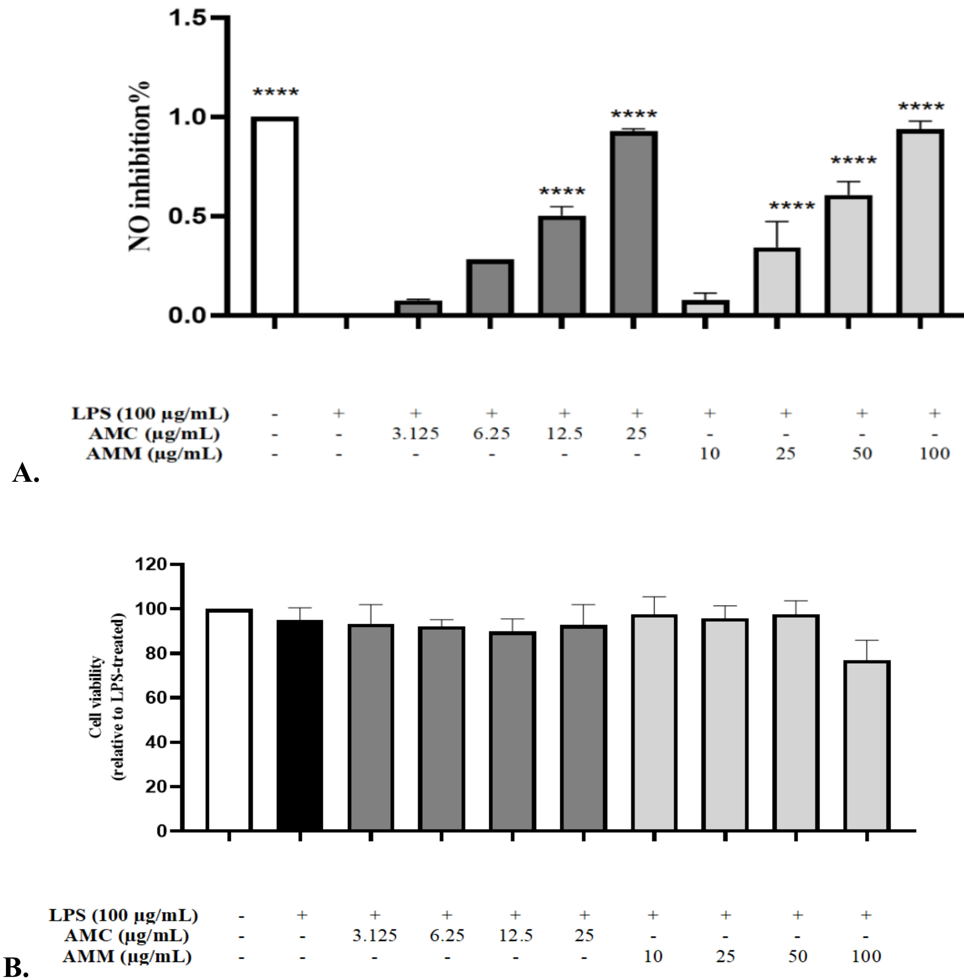

Figure 1. A. Effect of AMC and AMM on NO production in RAW 264.7 cells. Data represent the means ± SD from three separate experiments. **B**. Cell viability of AMC and AMM in RAW 264.7 cells. The cells were treated with both samples for 30 minutes and then stimulated with LPS (0.1 mg/mL) for 24 h. *** $P < 0.001$ versus untreated group by one-way ANOVA with Dunnett's test.

Our study demonstrated that AMC simultaneously attenuated the expression of iNOS and COX-2 at concentration-dependent manner of 6.25, 12.5, and 25 μg/mL (Fig. 2); tubulin was used as loading control. COX-2 inhibition was observed to be significant. At the concentration of 25 μg/mL, AMC suppressed the generation of COX-2 to a greater extent than in control, as a result of previously activated macrophages in the control sample. Similarly, the expression of iNOS was decreased in the same trend as seen for COX-2. These results suggested that AMC may follow the mechanism of iNOS and COX-2 co-regulation.

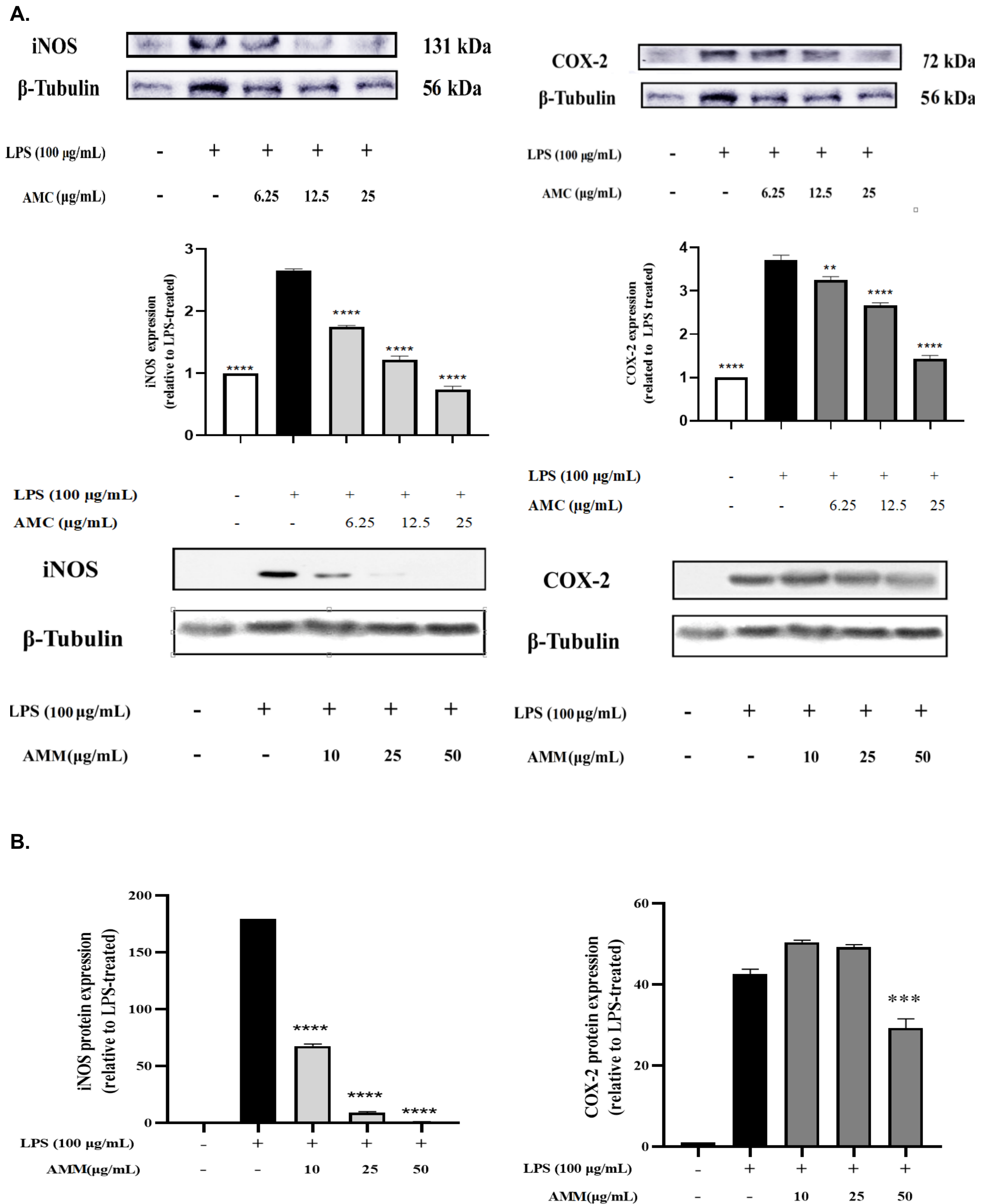

Figure 2. Evaluation of inflammatory protein expression in LPS-induced RAW 264.7 cells using western blotting. RAW264.7 cells were treated with AMC (6.25, 12.5 and 25 µg/mL) (**A**) and AMM (10, 25, 50 µg/mL) (**B**), respectively. Relative protein expression of COX-2 and iNOS by each concentration versus LPS. These data represent one replicate, measured by ImageJ software. $P < 0.001$.

Meanwhile, the results were different with AMM extract. It profoundly inhibited iNOS expression at the concentration from 10 to 50 µg/mL, demonstrating that the AMM modulates the inflammatory effect through the iNOS pathway. The same result cannot be obtained with COX-2 enzyme. Only the concentration of 50 µg/mL shows the inhibitory effect whereas the COX-2 expression of the lower concentrations were similar to the control treated with LPS. This result might be explained by two possibilities. The AMM may regulate mainly the iNOS pathway but does not follow the arachidonic acid pathway. A similar effect was found in the anti-inflammatory investigation of *Actinidia argutana* extract which it only suppressed the level of iNOS and production of NO with no impact on prostaglandin E2 (PGE2) and COX-2 expression (Kim *et al.,* 2014). Another explanation is the inflammatory inhibition via the nuclear factor-kB pathway in LPS-induced macrophage (Kim *et al.,* 2014).

CONCLUSION

The investigation of anti-inflammation effects of the methanol extracts from *A. maximum* Roxb (AMM) and *A. muricarpum* Elmer (AMC) demonstrated that they inhibited the production of pro-inflammatory mediator NO in RAW 264.7 model. No cytotoxicity was recorded at the concentration range up to 25 µg/mL with AMC and 50 µg/mL with AMM. Western blot analyses revealed that AMC inhibited the expression of two key enzymes of inflammation process including iNOS and COX-2 in a dose-dependent manner. AMM reduced the iNOS expression dose dependently but had significant inhibitory effects against COX-2 only at 50 µg/mL. This study is a good suggestion for further investigation of the bioactive inflammators of these two cardamom plants.

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NGHIÊN CỨU HOẠT TÍNH KHÁNG VIÊM CỦA *AMOMUM MAXIMUM* ROXB VÀ *AMOMUM MURICARPUM* ELMER TẠI MIỀN BẮC VIỆT NAM

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TÓM TẮT

Viêm là cơ chế bảo vệ nội môi của cơ thể, trong đó hệ thống miễn dịch phản ứng để loại bỏ các dị vật. Viêm mãn tính có thể làm tăng nguy cơ bị thêm các tổn thương như các bệnh tự miễn, viêm khớp, tiểu đường và có thể dẫn đến tử vong. Cây Đậu khấu chín cánh *Amomum maximum* Roxb và cây Sa nhân quả có mỏ *Amomum muricarpum* Elmer phân bố rộng rãi ở Việt Nam và đã được sử dụng trong y học cổ truyền để điều trị một số bệnh đường tiêu hóa. Nghiên cứu này nhằm đánh giá tác dụng kháng viêm của cặn chiết methanol từ rễ của Đậu khấu chín cánh (AMM) và Sa nhân quả có mỏ (AMC) trên dòng tế bào đại thực bào của chuột RAW 264.7. Kết quả cho thấy hai cặn chiết nghiên cứu có hoạt tính kháng viêm mạnh thông qua ức chế việc ức chế sản sinh oxit nitric (NO) đồng thời thể hiện độc tính tế bào thấp ở nồng độ thử nghiệm. Giá trị IC_{50} của AMC và AMM được xác định lần lượt là 12,67 ± 1,7 µg/mL và 42,7 ± 2,5 µg/mL. Nghiên cứu sâu hơn về cơ chế bằng phương pháp Western blot đã chứng minh AMC làm giảm phản ứng viêm trong mô hình tế bào RAW264.7 do lipopolysaccharide (LPS) gây ra thông qua ức chế nitric oxide (iNOS) và cyclooxygenase-2 (COX-2) trong khi AMM dường như chỉ điều chỉnh tác dụng viêm thông qua con đường iNOS là chủ yếu. Như vậy, hai cặn chiết AMM và AMC có thể là những ứng cử viên tiềm năng để nghiên cứu về các loại liệu pháp kháng viêm thay thế từ tự nhiên.

Từ khoá: *Amomum muricarpum*, *Amomum maximum*, kháng viêm, nitric oxide synthase, cyclooxygenase