RESEARCH ARTICLE

Inhibition of Neutrophil Chemotaxis and Superoxide anion Generation by a Pure Compound from *Artocarpus lakoocha* Roxb.

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Abstract

Artocarpus lakoocha Roxb., belonging to the family Moraceae and called Ma-Haad in Thailand, has been used in traditional folk medicine for the treatment of various diseases. A pure compound from *A. lakoocha* (Compound A) was demonstrated to possess strong anti-inflammatory activity in the EPP-induced mouse ear edema model. Neutrophil has been known to play an important role in acute inflammation. The aims of this study were to investigate the anti-inflamatory effects of Compound A *in vitro* based on human neutrophil chemotaxis and superoxide anion generation. Neutrophils were isolated from the peripheral blood of healthy donors by discontinuous Percoll density gradient centrifugation and viability was assessed by trypan blue exclusion. The cytotoxic effects of Compound A were primarily investigated by XTT assay. Its direct free-radical scavenging activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The effects of Compound A on fMLP-induced human neutrophil chemotaxis and superoxide anion generation (SAG) were determined spectrophotometrically. The results showed that fMLP-induced human neutrophil chemotaxis and SAG were inhibited in a concentration-dependent manner by Compound A. In addition, compound A exhibited strong radical-scavenging activity when compared with trolox. Slight cytotoxic effects of compound A were observed at the concentrations used These findings suggest that inhibition of fMLP-induced chemotaxis and superoxide anion generation of activated human neutrophils might account, at least in part, for the anti-inflammatory activity of Compound A.

Keyword: *Artocarpus lakoocha* Roxb., neutrophil chemotaxis, neutrophil superoxide anion generation, DPPH scavenging

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การยับยั้งการเคลื่อนที่แบบ chemotaxis และการสร้าง superoxide anion จากเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิล (neutrophils) โดยสารบริสุทธิ์จากต้นมะหาด (*Artocarpus lakoocha* Roxb.)

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บทคัดย่อ

มะหาด (*Artocarpus lakoocha* Roxb.) เป็นพืชสมุนไพรในวงศ์ Moraceae และถูกใช้ในการรักษาโรคต่าง ๆ ในทางการแพทย์พื้นบ้านเป็นระยะเวลายาวนาน จากการศึกษาฤทธิ์ต้านการอักเสบในสัตว์ทดลอง (*in vivo*) พบว่าสารA ซึ่งเป็นสารบริสุทธิ์ที่สกัดจากต้นมะหาดมีฤทธิ์ยับยั้งการอักเสบแบบเฉียบพลันของใบหูหนูที่ถูกเหนี่ยวนำด้วย ethyl phenylpropiolate (EPP) เซลล์เม็ดเลือดขาวชนิดนิวโทรฟิลมีบทบาทที่สำคัญต่อการอักเสบแบบเฉียบพลัน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษากลไกต้านการอักเสบในหลอดทดลอง (*in vitro*) ของสาร A ต่อการทำงานของนิวโทรฟิลของคนที่ถูกกระตุ้นด้วย fMLP โดยดูผลต่อการเคลื่อนที่แบบ chemotaxis และการสร้าง superoxide anion นิวโทรฟิลได้จากการแยกเลือดของอาสาสมัครด้วยวิธี discontinuous Percoll density gradient centrifugation และตรวจสอบ viability โดยใช้ trypan blue exclusion ผลความเป็นพิษต่อนิวโทรฟิลของสาร A ทดสอบโดย colorimetric XTT assay ผลโดยตรงในการกำจัดอนุมูลอิสระของสาร A ทดสอบด้วยวิธี DPPH scavenging assay ผลของสาร A ต่อการเคลื่อนที่แบบ chemotaxis และการสร้าง superoxide anion ของนิวโทรฟิลจากคน วัดโดยใช้เทคนิค spectrophotometry ผลการทดลอง พบว่าสาร A สามารถยับยั้งการเคลื่อนที่แบบ chemotaxis และการสร้าง superoxide anion ของนิวโทรฟิลในลักษณะที่ขึ้นกับความเข้มข้น และมีผลเป็นพิษต่อเซลล์เล็กน้อยในความเข้มข้นที่ใช้ศึกษา อีกทั้งยังสามารถกำจัดอนุมูลอิสระได้โดยตรง ผลจากการศึกษานี้อาจกล่าวได้ว่า ฤทธิ์ของสาร A ในการยับยั้งการเคลื่อนที่แบบ chemotaxis และการสร้าง superoxide anion ของนิวโทรฟิลที่ถูกกระตุ้น รวมทั้งฤทธิ์ในการกำจัดอนุมูลอิสสระโดยตรง อาจเป็นกลไกของฤทธิ์ต้านการอักเสบแบบเฉียบพลันของสาร A

Introduction

The neutrophil granulocyte is a key factor in cellular innate defence mechanism against infection or tissue damage. Neutrophils or polymorphonuclear neutrophilic leukocytes (PMNs) play a crucial role in acute inflammation. Neutrophils migrate to the site of inflammation along a chemotactic concentration gradient mediated by cell-adhesion molecules. Micro-organisms are phagocytosed by activated neutrophils, and will be killed either by oxygen dependent or oxygen independent mechanisms. The oxygen dependent activity depends on a complex enzyme, NADPH oxidase, which converts molecular oxygen (O_2) into superoxide anion (O_2^-) whereas the oxygen independent mechanism depends on degranulation of microbicidal substances (Tosi 2005). However, excessive neutrophil activation also causes tissue injury which can contribute to the development of various inflammatory diseases such as lung disease, glomerulonephritis, and arthritis (Korkmaz et al. 2008).

Artocarpus lakoocha Roxb., known in Thai as Ma-Haad which is a plant belonging to the family of Moraceae, has been known for its high content of stilbenoids such as pinostilbene, desoxyrhapontigenin, pterostilbene, resveratrol and oxyresveratrol. Apart from stilbene, lectins and a flavonol glycoside have been found as constituents of this plant (Chatterjee et al.1988, Wongkham. et al. 1995). The extract of *A.lakoocha* Roxb has been showed to have anti- herpes simplex virus (HSV) (Jensen et al. 1977), anthelmintic activity (Charoenlarp et al. 1981, Salguero 2003, Saowakon et al. 2009), and antimicrobial activity (Pandey A and Bhatnagar SP 2009). The heartwood extract and the isolated phytochemicals of *A. lakoocha* Roxb. have been found to have melanogenesis- and tyrosinase-inhibitory activities (Tengamnuay et al. 2006, Donsing et al. 2008). *A. lakoocha* is one of many plants that contain many phenolics (flavonoids and phenolic acids), generally known as strong antioxidants (Jasprica et al. 2007) and the ethanolic extract of *A. lakoocha* was found to contain important antioxidants (Singhatong et al. 2010). Oxyresveratrol (trans-2,4,3',5'-tetra-hydroxystilbene), a major constituent purified from the heartwood of *A. lakoocha* Roxburgh (Moraceae), has been shown to possess several pharmacological effects including *in vitro* anti-HSV potential (Sritulaluk et al. 1998, Likhitwitayawuid et al. 2005, Chuanasa et al. 2008), anti-VZV activity (Sasivimolphan et al. 2009) anti-HIV (Jagtap and Bapat 2010), potent tyrosinase inhibitory and antioxidant activities (Sritulaluk et al. 1998, Kim et al. 2002, Lorenz et al. 2003, Tengamnuay et al. 2004; 2006, Likhitwitayawuid et al. 2006, 2008, Jagtap and Bapat 2010) and antiglycation (Jagtap and Bapat 2010). It was also suggested to be neuroprotective and inhibit the apoptotic cell death in transient ischemia in a rat model (Andrabi et al. 2004). Our previous study has demonstrated that a pure compound (Compound A) from *A. lakoocha* possesses a strong inhibitory effect on the acute phase of inflammation *in vivo* as seen in ethyl phenylpropiolate (EPP)-induced ear edema in mouse (unpublished observations). Therefore, the aims of this study were to investigate possible anti-inflammatory actions *in vitro* of Compound A from *A. lakoocha* extract based on human neutrophil chemotaxis and superoxide anion generation; where indomethacin was used as a reference compound. The

cytotoxic effect and free-radical scavenging activity of Compound A were also investigated.

Materials and methods

Isolation of human neutrophils

Neutrophils were isolated from heparinized venous blood from healthy volunteers by dextran sedimentation followed by centrifugation through discontinuous plasma-percoll density (Wanikiat et al. 2008). Venous blood obtained from healthy donors, was mixed with an equal volume of 6% dextran and left to stand for 30 min. The leukocyte-rich plasma was removed and centrifuged at 280*g* for 10 min. and the resultant pellet resuspended in 50% Percoll and layered on top of a discontinuous Percoll gradient (65% Percoll and 75 % Percoll). After centrifugation, PMN were harvested and washed. Any contaminating red cells were removed by hypotonic lysis. Cells were then counted in an improved Neubauer counting chamber, the cells were > 95% viable as determined by trypan blue exclusion and were resuspended in phosphate buffered saline as required.

Cytotoxic assay

Neutrophils (1×10^6 cells/ml in RPMI 1640) were pre-incubated with 1-1,000 µM of Compound A from *A.lakoocha* extract for 1 hour. XTT was then added and incubated at 37 °C for an additional 3 hours. The XTT formazan product was measured spectrophotometrically at 450 nm (Mosmann 1983).

DPPH radical scavenging assay

The free radical scavenging activity of Compound A (6.25-200 µM) was determined using the reduction of the stable free radical, 2,2-diphenyl-1-pycryl-hydrazyl (DPPH), (2 mM in methanol). Absorbance of a stable nonradical form of DPPH was measured spectrophotometrically at 515 nm for 10 min (Cavin et al. 1998). The radical scavenging activity was calculated as a percentage of radical reduction. Trolox, a known radical scavenger, was used as a reference compound. Ethanol was used as the blank solution. The scavenging activity of the DPPH radicals was calculated according to the following formula

$$\%\ \text{DPPH radical scavenging activity} = \frac{[(\text{absorbance of control} - \text{absorbance of sample}) \times 100]}{\text{absorbance of control}}$$

Determination of chemotaxis

An in vitro assay for chemotaxis of neutrophils *was performed using* a 96 well chemotaxis chamber. The bottom wells of the chamber were filled with fMLP. The upper wells with the installed filter were filled with neutrophils (3×10^6 cells/ml) which had been treated with 1-100 µM of Compound A or indomethacin (a reference compound, 0.01–100 µM). The chamber was incubated for 45 minutes at 37 °C. The filter was then removed, washed, fixed and stained with Diff-Quik solution. Chemotaxis was quantified spectrophotometrically measuring absorbance at 550 nm and the magnitude of the absorbance taken as being directly proportional to the number of cells which migrated and were trapped in the filter. Basal absorbance was taken as cells without fMLP. (Wanikiat et al. 2008).

Determination of superoxide anion generation (SAG)

Neutrophil superoxide anion generation was determined by spectrophotometric evaluation of the SOD-inhibitable reduction of ferricytochrome C to ferrocytochrome C in the presence of cytochalasin B. Briefly, neutrophils (1×10^6 cells/mL) re-suspended in PBS containing cytochrome C and cytochalasin B were incubated with 0.1-100 µM of Compound A or the vehicle for 10 min before stimulating with fMLP (10 nM) for 10 min. SAG was measured by the reduction of cytochrome C at 550 nm.. Basal absorbance was taken as cells without fMLP. Indomethacin was used as a standard reference compound (Wanikiat et al. 2008).

Statistically Analysis

The results were presented as mean ± S.E.M.; n represented the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four concentrations (n=6). Statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons. P values < 0.05 were considered significant. Percentage of inhibition was calculated from the difference between drug treated group and control group.

Results

Effect of Compound A on cell viability

Compound A was assessed for its cytotoxicity on human neutrophils prior to the determination of its effects on human neutrophil functional responsiveness. Incubation of neutrophils with Compound A at the concentration of 1-100 µM for 4 hours caused slight cytotoxic effect, but its strong cytotoxic effect was observed at the concentration of 1000 µM (CC_{50} > 3,000 µM) (Figure 1).

Effect of Compound A on DPPH radical scavenging

Compound A (6.25-200 µM) exhibited strong radical scavenging activity (EC_{50} = 10.2 ± 3.7 µM, n=5) and comparable with Trolox, a reference compound, which showed high radical scavenging activity (EC_{50} = 7.1± 1.5 µM, n=5) (Figure 2).

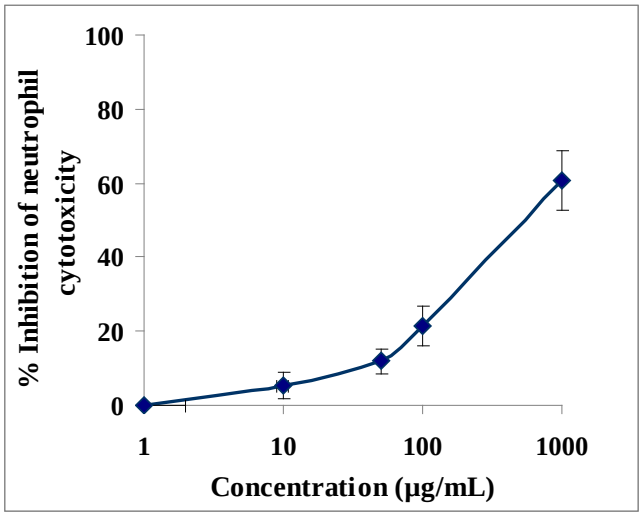

Figure 1 Cytotoxic effects of Compound A on human neutrophils. Results are mean ± S.E.M using cells from different donors (n=5).

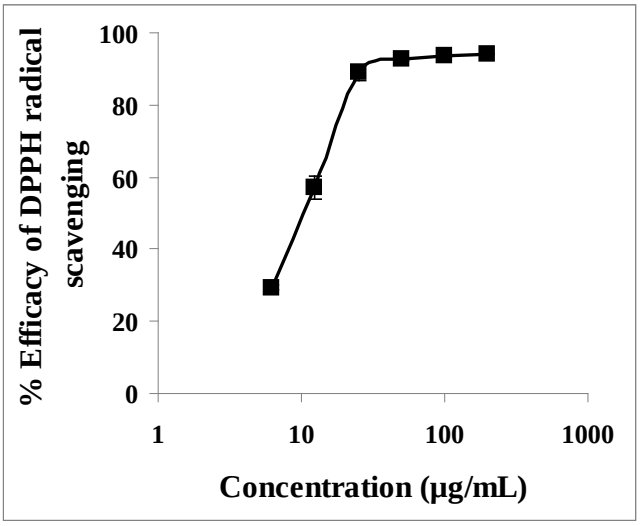

Figure 2 DPPH radical scavenging activity of Compound A. Results are mean ± S.E.M (n=5).

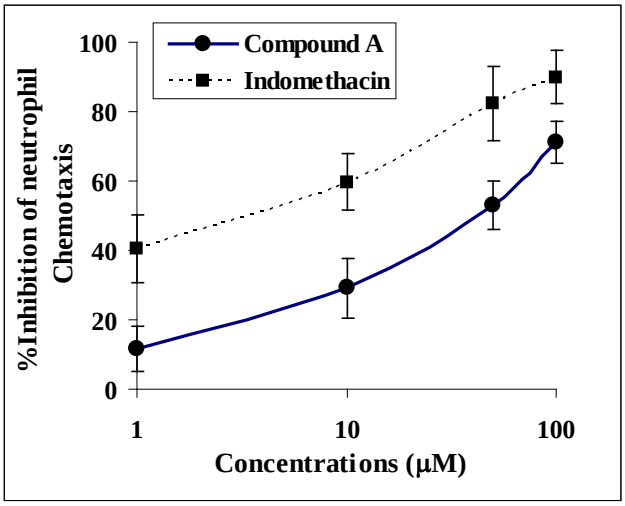

Figure 3 Inhibitory effects of Compound A on fMLP-induced chemotaxis in human neutrophils. Results are mean ± S.E.M using cells from different donors (n=6).

Effect of Compound A on chemotaxis

Pre-incubation of human neutrophils with Compound A (1-100 µM) for 10 min at 37◦C, significantly suppressed fMLP-induced human neutrophil chemotaxis in a concentration-dependent manner ($P < 0.05$, ANOVA) with an IC_{50} of 53.6±5.8 µM (n=6). Indomethacin, a reference compound, more potently inhibited fMLP-induced human neutrophil chemotaxis with IC_{50} of 4.5±2.3 µM, n=6 (Figure 3).

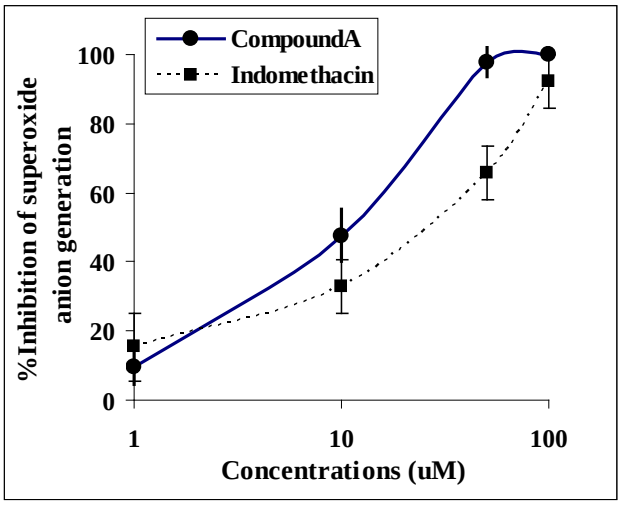

Figure 4 Inhibitory effects of Compound A on fMLP-induced superoxide anion generation in human neutrophils. Results are mean ± S.E.M. using cells from different donors (n=6).

Effect of Compound A on superoxide anion generation (SAG)

Upon stimulation with fMLP (10 nM), neutrophils released O_2^- into the extracellular medium, as determined by SOD-inhibitable cytochrome C reduction. Pre-incubation of the cells with Compound A at the concentration of 1-100 µM for 10 min at 37°C, fMLP-induced SAG by human neutrophils was significantly suppressed in a concentration-dependent manner ($P < 0.05$, ANOVA), with an IC_{50} = 7.8 ± 3.8 µM, n=6), and comparable with that of Indomethacin (1–100 µM) which caused strong inhibition of fMLP-induced SAG in human neutrophils, with IC_{50} =13.5 ± 2.9 µM, n=6 (Figure 4).

Discussion and Conclusion

PMNs serve as the primary line of host defense during inflammation (Hotchkiss and Karl 2003). The normal inflammatory response to infections requires the peripheral leukocytes to migrate across the blood vessels to the site of infection in response to chemotactic factors released in the site. The emigration of leukocytes such as neutrophils into inflammatory sites requires adhesion to the endothelium of small venules. The initial adhesive event is margination characterized by rolling of neutrophils along the luminal surface of the endothelium**.** Each member of the selectin family of adhesion molecules has been shown to support neutrophil rolling under conditions of flow. Each selectin functions primarily as a lectin, recognizing carbohydrate structures on the leukocyte or endothelial cell surface. Once the marginated neutrophil forms a stationary adhesion with endothelial cells, it is stimulated by chemotactic factors to downregulate the selectin-based adhesion and upregulate adherence dependent on beta 2-integrins, principally CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1). These adhesion molecules interact with intercellular adhesion molecule 1 (ICAM-1) and possibly other structures on the endothelial cell, and the leukocyte rapidly migrates into surrounding tissue. Chemotaxis is one of the most important neutrophil functions. Our

previous study has shown that Compound A, a pure compound from *A. lakoocha* extract, possessed strong anti-inflammatory activity in the EPP-induced mouse ear edemamodel (unpublished observation). .In this study, we found that Compound A inhibited fMLP-induced chemotaxis in human neutrophils in a concentration-dependent manner. *A. lakoocha* is one of numerous plants that contain many phenolics, generally considered to be strong antioxidants (Jasprica et al, 2007). The Study by Singhatong et al. 2010 showed that the ethanolic extract of *A. lakoocha* exhibited antioxidant activity measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.(Singhatong et al. 2010). The results from our study also showed that compound A possessed strong antioxidant activity as shown by DPPH radical scavenging assay. Moreover, compound A was shown to strongly inhibit the generation of superoxide anion in activated human neutrophils, in a concentration-dependent manner. Interestingly, this compound at the effective concentrations exerted only slight cytotoxic effects on neutrophils.

In conclusion, the present study is the first to show that compound A from ethanolic extract of *A. lakoocha* significantly inhibited chemotaxis and superoxide anion generation in a concentration-dependent manner in activated human neutrophils, and also exerted a strong direct radical scavenging activity. These effects might attribute, at least in part, to its anti-inflammatory activity.

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