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Determining MMP9 activity of diarylheptanoid from *Curcuma comosa* Roxb on macrophage differentiation

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Introduction

Diarylheptanoid consists of two benzene rings conjugated each ring with seven carbon chain. The *Curcuma comosa* Roxb. (Zingiberaceae) has traditionally been used in Thailand as an anti-inflammatory agent, postpartum treatment, uterine bleeding and postmenopausal. The resent studies report that diarylheptanoids are the major substances from *C. comosa* and functions as phytoestrogen, osteoporosis protection and anti-inflammation.

In the early phase of inflammation, the stimulated monocyte in blood circulation increases cell adhesion, migration from vascular into inflamed tissue and changing to macrophage phenotype. The activation of macrophage plays an importance role in inflammation by secretion of proinflammatory mediators, chemokines and proteinase. These substances enhance macrophage to migrate to inflammation site and recruit T lymphocytes. The previous studies showed the anti-inflammation mechanism of diarylheptanoids from *C. comosa* by reduction of human leukocyte cell line (U937) adhesion and decrease the level of TNF-α and IL-1β. Matrix metalloproteinases especially type 2 and 9 are the gelatinase enzyme and have an effect on macrophage invasion through the basement membrane. Although MMP9 is the downstream molecule of TNF-α, there is no data determine the effect of diarylheptanoid on level of MMP9.

Methods

Diarylheptanoid extraction

1 g of *C. comosa* rhizome was extracted by 32.50 ml of ethyl acetate at 65°C for 16 min. The pure diarylheptanoid was isolated from the resulting extract by an opened column chromatography.

Cell culture and differentiation

Human pro-monocyte (U937 cell) was cultured in RPMI-1640 medium supplemented with 10% FBS, 100U/ml of penicillin and 100 ug/ml of streptomycin. To determine the macrophage differentiation, U397 cells were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 and 48 hours.

Cell viability (MTT assay)

$5x10^4$ cells were treated with 5, 10 and 20 ug/ml of diarylheptanoid. At indicate time, the treated and control cell were incubated with 5 mg/ml MTT for 4 h at 37°C. DMSO was used to solubilize formazan crystal and the viability of U937 cells was determined by measuring the absorbance at wavelength 530 nm.

Zymography

To detect matrix metalloproteinase enzyme. The conditioned medium were loaded and separated by gelatin SDS-PAGE. Gels were washed with 2.5% tritonX100 for 1 h and incubated with incubation buffer for 18 h. The molecular weight of MMP-2 and MMP-9 were 72 and 92 kDa when compared to protein marker and enzyme activity was detected by the intensity of cleared band.

Results

Chemical structure of diarylheptanoid extraction

Ethyl acetate extract of the root of *C.comosa* was purified by HPLC and identified by NMR (Fig.1). The compound had molecular weight: 298.38, chemical formula: $C_{19}H_{22}O_3$ and chemical structure was 7-(3,4 dihydroxylphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene.

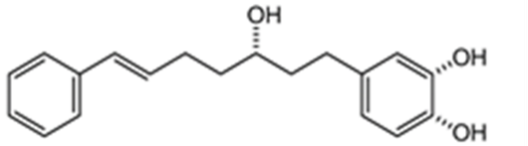

Figure 1. The structure of purified diarylheptanoid

The effect of purified diarylheptanoid extract on cell viability

The survival of U937 cells was not significantly reduced at 5 and 10 µg/ml of diarylheptanoid treatments for 24, 48 or 72 h. However, the concentration at 20 µg/ml statistically significant decreased cell variable at 24 h of the treatment (fig.2A).The cell survival data in co-treatment of diarylheptanoid and PMA were not toxic to the cell in all treatment for 24 -72h when compared to PMA treatment (fig.2B).

Induction of secreted MMP9 production

Effect of dihydroheptanoid was determine by gelatin zymogram. In control U937 cell, MMP9 activity in culture medium was undetectable 24 and slightly increased in 48 h. The MMP9 activity of conditioned medium from 100 nM PMA activated cells was obviously increased. However, dihydroheptanoid in both 5 and 10 µg/ml concentration did not affect to MMP9 activity. MMP2 was not detectable in all conditions (Fig3A, 3B).

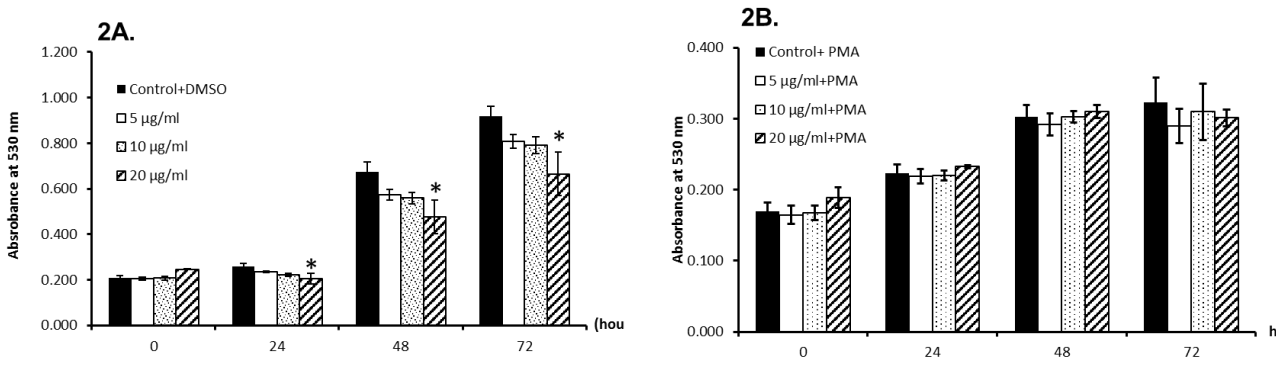

Figure 2. The effects of diarylheptanoid on cell. U937 cells were treated with 5, 10 and 20 ug/ml of diarylheptanoid for 0, 24, 48 and 72 h (2A). To measure the cell toxicity in activated macrophage condition, U937 cells were treated with 5, 10 and 20 ug/ml of diarylheptanoid contained with 100 nM PMA for 0, 24, 48 and 72 h (2B). Number of viable cell was determine using MTT assay at 530 nm. Data represent as mean±SEM of three times independent. **P< 0.05,* compared to control+DMSO at 0 h of treatment.

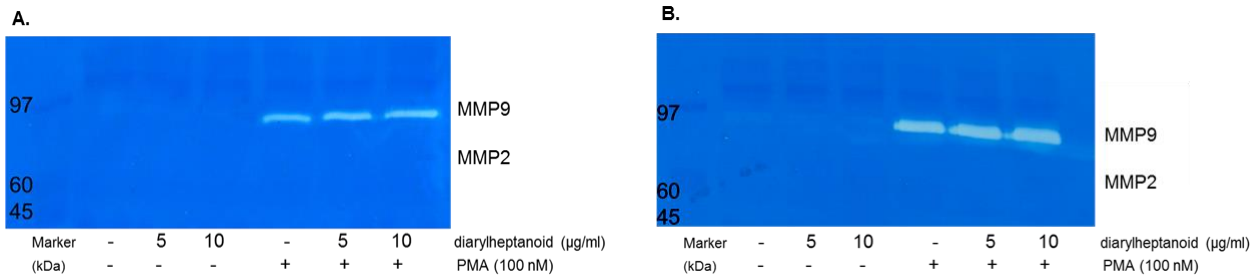

Figure 3. Gelatinolytic activities by zymography on U937 cell. Conditioned media from U937 cells, 5 and 10 μg/ml of diarylheptanoid and 100 nM PMA treatments were subjected to gelatin-SDS gel. After separated by electrophoresis, washed and incubated as described in the method, the MMPs activity was present in cleared band for 24h incubation (A) and 48 h incubation (B). MMP2,9 are known as 72 kDa and 97 kDa gelatinase.

Discussion

It is well establish that diarylheptanoid from *C. comosa* rhizome has an active function on anti-inflammation. Several inhibition mechanisms were revealed in various types of cells. In microglia cell, the extract from *C. comosa* reduces NO production by the expression of interferon regulatory factor-1 transcription factor, decreases mRNA of monocyte chemoattractant protein-1 (MCP-1)[1]. Emphasizing on immune response, the actions of *C. comosa* extract on monocyte to macrophage differentiation are investigated. In human monocyte cell line (U937) was used determine the expression of cell surface molecules to identify the action of diarylheptanoid from *C. comosa* on monocyte adhesion [2]. Moreover, diarylheptanoid showed the suppression of pro-inflammatory cytokine, TNF-α and IL-1β, in PMA induced U937 cell [3] .In our study, the *C. comosa* rhizome was extracted with ethyl acetate, purified and elucidated chemical structure. Cell cytotoxicity was investigated MTT assay and the result showed that 5-10 µg/ml of diarylheptanoid did not affect to cell viability whereas the higher dose (20 µg/ml) decreased cell survival. This result was similar as previous study which had the non-toxic dose at the concentration from 0.01 to 10 ug/ml [3]. Because of MMP9 is the gelatinase enzyme, the level of MMP9 have been used to determine the cell migration. After activation with phorbol 12-myristate 13-acetate (PMA), U937 cell also increased in both MMP9 mRNA and protein level [4, 5]. Thus, MMP9 might play an important role on macrophage migration and inflammation activation. TNF- α is enhanced in activated macrophage that can increase MMP9 expression. Therefore, diarylheptanoid treatment, which attenuated TNF- α level in U937 cell, could be have the reduction effect on MMP9. In our result, treatment of 100 nM PMA for 24 and 48 h can increase of MMP9 activity. This supported that PMA activation condition was succeed. Unfortunately, our results did not detect the MMP9 reduction on zymography assay. The activity of MMP9 might be regulated by other inflammatory cytokine which markedly related with activated macrophage. For example, TGF-β, IL-1 have been shown to induce MMP9 expression in mouse macrophages and mouse mouse osteoblastic cells respectively [6, 7].

In summary, diarylheptanoid (7-(3,4 dihydroxylphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene) which was extracted from C. comosa has no effect on MMP9 secretion.

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