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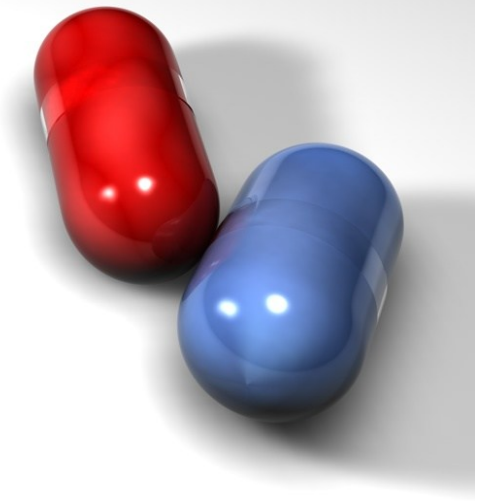

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Letter to the Editor

Murrayafoline A inhibits inflammatory cytokines in RAW264.7 cells stimulated with LPS and poly (I:C) by targeting the aryl hydrocarbon receptor

Dear Editor,

The anti-inflammatory activities of murrayafoline A, an alkaloid isolated from *Glycosmis stenocarpa*, remain poorly understood, particularly in cytokine regulation in macrophages. In this study, the anti-inflammatory activities of murrayafoline A were further evaluated in RAW264.7 cells, which was stimulated with both lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly I:C) to mimic both bacterial and viral infections (Ding et al., 2017). This approach allowed the discovery of whether murrayafoline A, as well as other natural compounds, can inhibit inflammatory responses during both bacterial and viral infections.

The co-stimulation of LPS and poly (I:C) has been used in several previous studies to enhance or limit immune res-ponses (Bogunovic et al., 2020; Ding et al., 2017; Gorskaya et al. 2020). While LPS activates TLR4, moderate activation of TLR3 by poly (I:C) seems to increase cytokine production, whereas excessive TLR3 activation may lead to a reduction in inflammatory cytokine levels (Jiang et al., 2005). In this study, RAW264.7 cells were stimulated with LPS (1 µg/mL) and various concentrations of poly (I:C) (10-50 µg/mL). The results showed that the combination of LPS (1 µg/mL) with poly (I:C) (20 µg/mL) induced peak production of the cytokines TNF-α and IL-6 (data not shown). Next, the cells were pretreated with murrayafoline A (3, 10, 30, or 60 µM) for 1 hour before being stimulated with 1 µg/mL LPS and 20 µg/mL poly (I:C).

The cells were harvested for qRT–PCR analysis at 3, 6, and 24 hours, while the supernatants were collected for ELISA at 24 hours, as described previously (Tran et al., 2022). Murrayafoline A did not affect RAW264.7 cell viability, with viability percentages of 97 ± 5, 100 ± 5, 95 ± 2, and 92 ± 1% at concentrations of 3, 10, 30, and 60 µM, respectively (data not shown).

In a previous study, synthetic murrayafoline A derivatives influenced LPS-stimulated inflammatory cytokine production in bone marrow-derived dendritic cells (Thuy et al., 2013). In the present study, murrayafoline A significantly suppressed the LPS- and poly (I:C)-costimulated production of IL-6, TNF-α, and IL-10 in a concentration-dependent manner in RAW264.7 cells, with IC_{50} values of 2.7 ± 0.7, 3.4 ± 0.5, and 3.7 ± 0.5 µM, respectively (Figure 1). The production of proinflammatory cytokines in activated macrophages is regulated by various intracellular pathways, including the NF-κB pathway, where MyD88 and NF-κB play key roles (Li et al., 2020). As shown in Figure 2, murrayafoline A significantly reduced *Myd88* levels by 40, 61, 70, and 78% and *Nf-κb1* levels by 32, 49, 80, and 84% at concentrations of 3, 10, 30, and 60 µM, respectively, after 6 hours compared with those in the control.

It has been reported that Ahr can regulate inflammatory cytokines such as IL-6 in macrophages under LPS stimulation (Kimura et al., 2009). Recently, the alkaloid

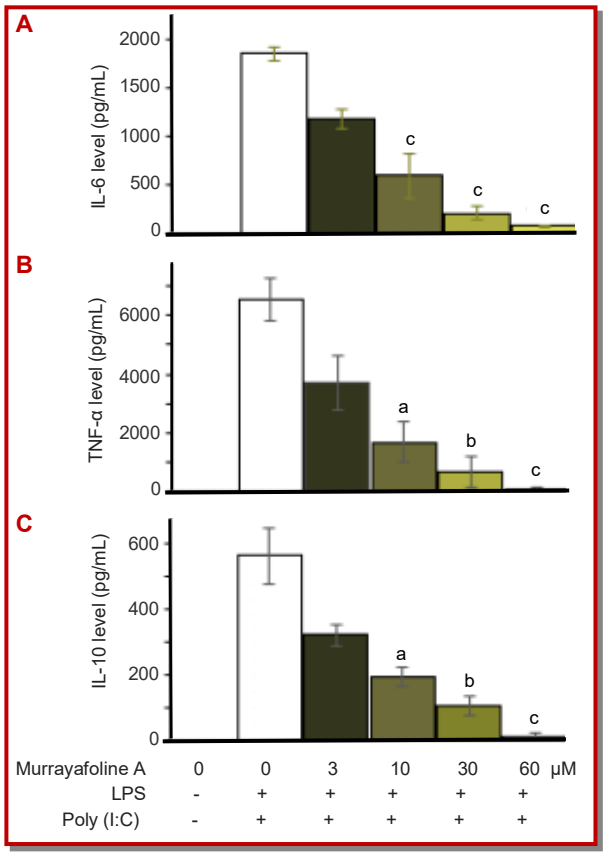

Figure 1: Murrayafoline A inhibited IL-6, TNF-α, and IL-10 production in LPS- and poly (I:C)-stimulated RAW264.7 cells. The cells were pretreated with various concentrations of murrayafoline A followed by the presence of LPS (1 µg/mL) and poly (I:C) (20 µg/mL) for 24 hours. Data are the mean ± SD of 3 replicate experiments. [a]p<0.05; [b]p<0.01; [c]p<0.005 vs the samples stimulated with only LPS

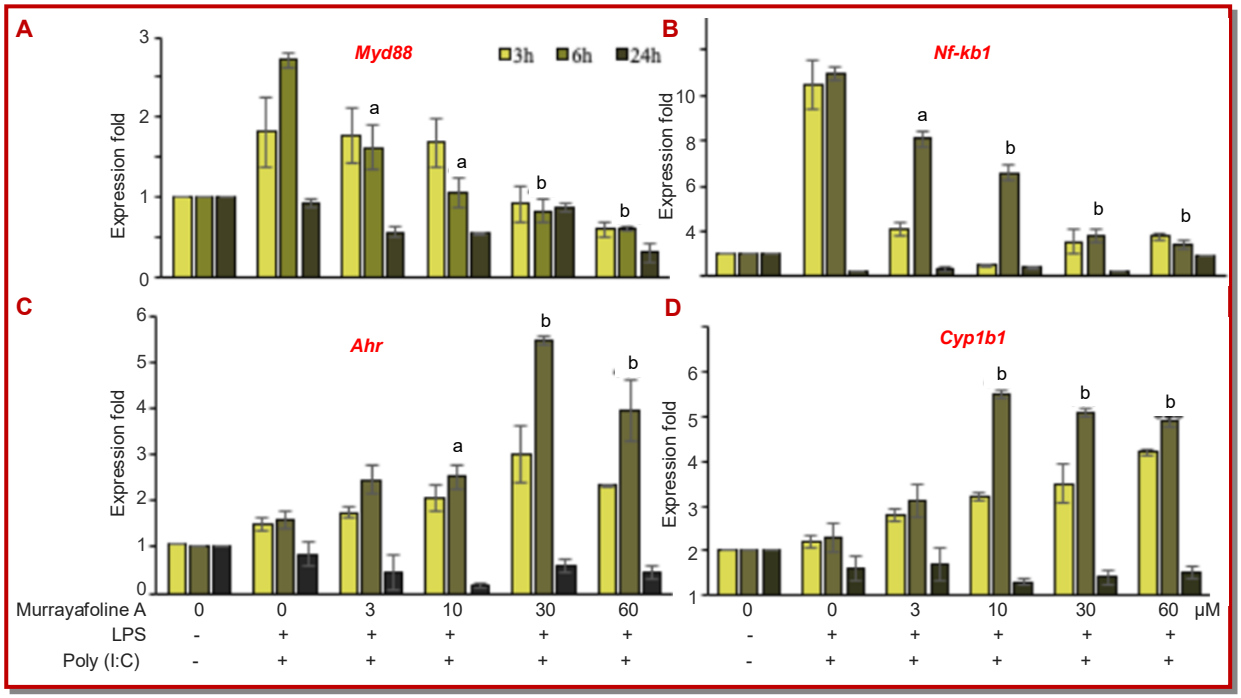

Figure 2: Murrayafoline A suppressed the expression of *Myd88*, *Nf-κb1*, and enhanced the expression of Ahr in LPS- and poly (I:C)-costimulated RAW264.7 cells after 6 hours. The gene expression of *Myd88*, *Nf-κb1*, Ahr and *Cyp1b1* was detected via real-time quantitative PCR. Data are presented as the mean ± SD of 3 replicate experiments. [a]p<0.01; [b]p<0.005

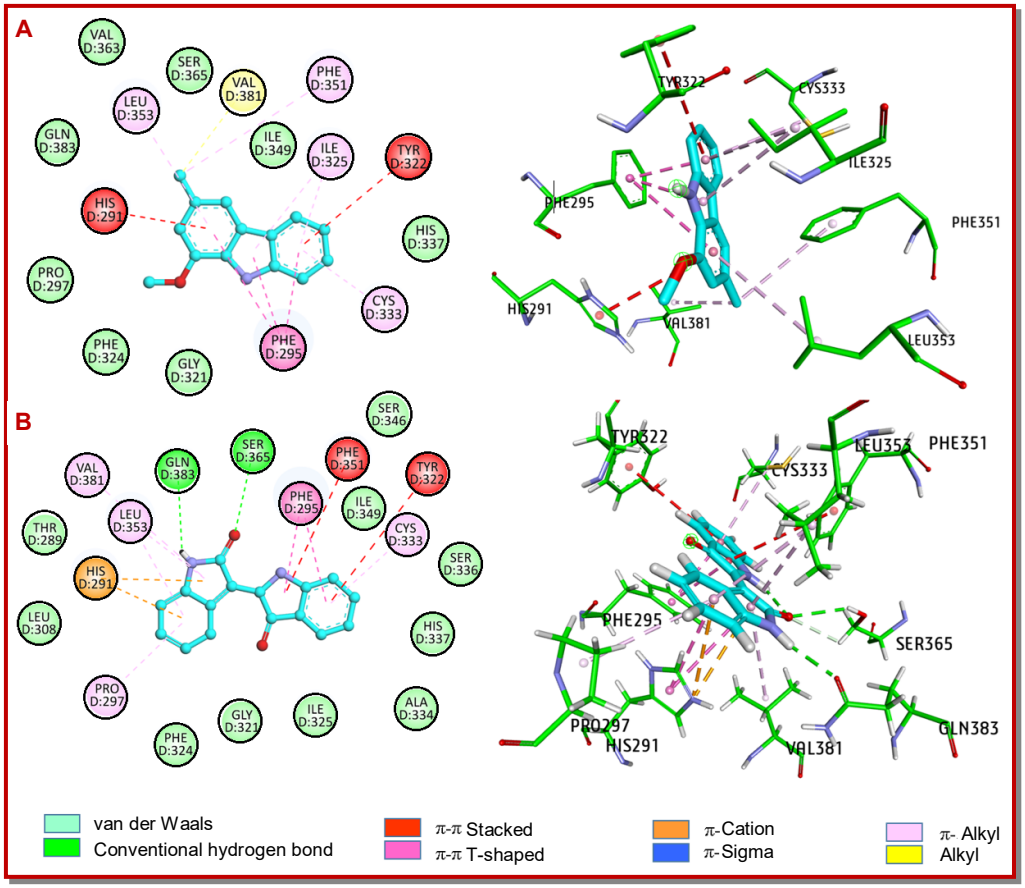

Figure 3: 2D and 3D interactions of murrayafoline A (A) and the reference compound indirubin (B), FICZ (C) and TCDD (D) within the active site pocket of Ahr (PDB ID: 7ZUB) **Cont..**

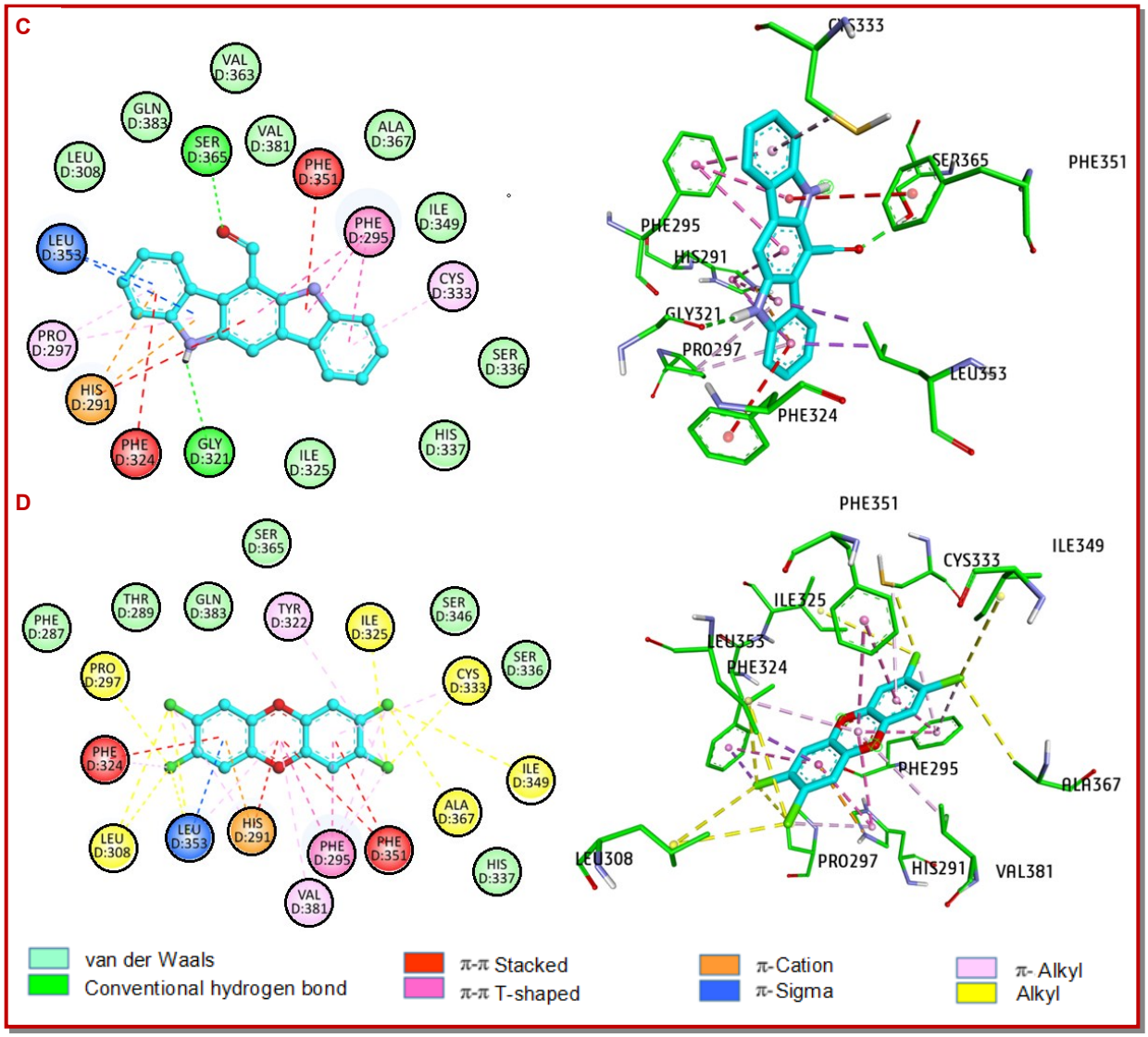

Figure 3: 2D and 3D interactions of murrayafoline A (A) and the reference compound indirubin (B), FICZ (C) and TCDD (D) within the active site pocket of Ahr (PDB ID: 7ZUB)

9-hydroxy-canthin-6-one from *Eurycoma longifolia* was reported to inhibit the production of inflammatory cytokines, including IL-6, TNF-α, and IL-10, in LPS-induced RAW264.7 cells, possibly via Ahr activation (Tran et al., 2022). Interestingly, in the present study, murrayafoline A at concentrations of 3, 10, 30, and 60 µM increased Ahr expression by 2.4-, 2.5-, 5.5-, and 4.0-fold, respectively, compared with the control. In addition, murrayafoline A at concentrations of 3, 10, 30, and 60 µM also increased the expression of *Cyp1b1,* a downstream gene induced by Ahr activation by 2.1-, 4.5-, 4.1-, and 3.9-fold, respectively, compared with the control. These findings suggest that murrayafoline A may suppress the inflammatory response through Ahr activation in macrophages. To better understand the molecular mechanisms governing Ahr regulation and whether murrayafoline A interacts with Ahr and how this interaction affects Ahr expression, an *in silico* analysis was conducted and the results were compared with those of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 6-formylindolo [3,2-b] carbazole (FICZ), two high-affinity Ahr ligands (Ehrlich et al., 2018). Docking simulations revealed that murrayafoline A binds to Ahr (PDB ID: 7ZUB) with a binding affinity of -9.663 kcal/mol, which is stronger than that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (-9.574 kcal/mol) but weaker than that of 6-formylindolo [3,2-b] carbazole (FICZ) (-12.82 kcal/mol) and indirubin (-12.52 kcal/mol), an Ahr cocrystallized compound. The primary interactions observed for murrayafoline A were hydrophobic, including alkyl and -π alkyl interactions with Cys333, Ile325, Phe351, Val381, and Leu353, as well as π-π stacking interactions with Tyr322, Phe295, and His291 (Figure 3A). Notably, the key residues Cys333, Phe351, Tyr322, Val381, Leu353, and Phe295 are also involved in interactions with indirubin (Gruszczyk et al., 2022; Figure 3B). Similar to FICZ (Figure 3C) and TCDD (Figure 3D),

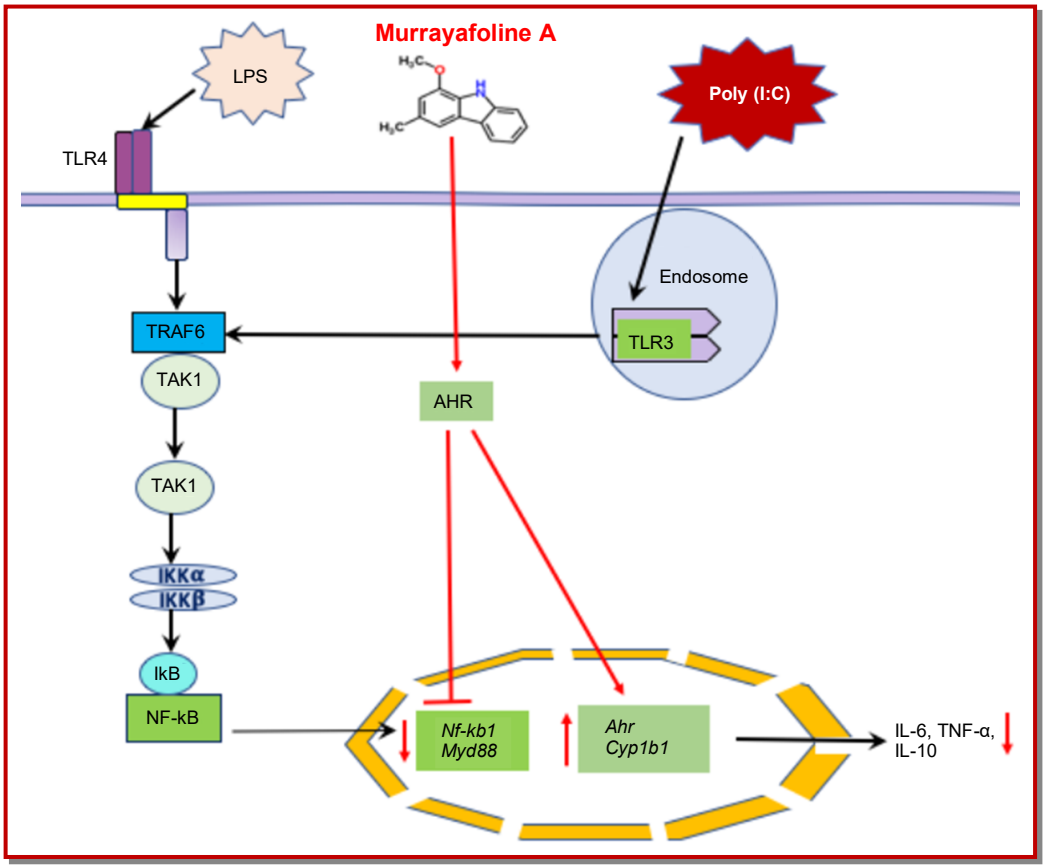

Figure 4: The hypothesis of the anti-inflammatory activity of murrayafoline A in LPS- and poly (I:C)-costimulated RAW264.7 cells

murrayfoline A interacts with Phe351, Phe295, Cys333, Leu353, Phe324, and His291 (Hirano et al., 2015). Interestingly, these key residues are located within the ligand-binding domain of Ahr, suggesting their crucial role in ligand recognition and binding specificity. These findings support the potential of murrayafoline A as an Ahr ligand (Figure 4). Nevertheless, to confirm that murrayafoline A is an Ahr ligand, further investigation is needed to assess its effects in Ahr-deficient cells.

In conclusion, inflammatory cytokine production in RAW264.7 cells induced by the costimulation of the TLR4 agonist LPS and the TLR3 agonist poly (I:C) can be inhibited by murrayafoline A from *Glycosmis stenocarpa*, potentially by reducing the expression of *Myd88* and *NF-κb1* through an Ahr-related mechanism.

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Conflict of interest: The authors declare that they have no conflicts of interest.

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