SUPPLEMENTARY MATERIAL

In vitro Anti-inflammatory activities of Naucleofficaine H as a natural alkaloid from *Nauclea officinalis* Pierrc ex Pitard, through Inhibition of the iNOS pathway in LPS-activated RAW 264.7 macrophages

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ABSTRACT

Naucleoffieine H, a natural indole alkaloid, was isolated and identified from *Nauclea officinalis* Pierrc ex Pitard which is a traditional Chinese medicine used for the treatment of various diseases, such as cold, fever, bronchitis, pneumonia, acute tonsillitis, etc. In the present study, the effect of naucleoffieine H on the anti-inflammatory activities was investigated in LPS-induced RAW 264.7 macrophages. The results showed that naucleoffieine H significantly inhibited the release of nitric oxide (the level of nitrite as a stable biomarker of NO production) and tumor necrosis factor-α (TNF-α). Interestingly, naucleoffieine H down-regulated the overexpression of inflammatory protein induced nitric oxide synthase (iNOS), but no effect on the expression cyclooxygenase-2 (COX-2) protein. In addition, this bioactive alkaloid suppressed enzymatic activity of iNOS activated by LPS. The above results indicated that naucleoffieine H suppress NO and TNF-α overproduction via block the iNOS pathway in LPS-induced RAW 264.7 macrophages.

**Keywords:** *Nauclea officinalis*; indole alkaloids; anti-inflammatory effect; NO; iNOS
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Experimental Materials and Methods

1. Reagents

RPMI 1640 medium and fetal bovine serum were purchased from Invitrogen (NY, USA). Lipopolysaccharide (LPS from E. coli), DMSO, NADPH, DAF-FMDA and MTT were obtained from Sigma-aldrich (USA). Penicillin-streptomycin stock solution, mouse TNF-α ELISA kit, Bradford protein assay kit and nitric oxide synthase assay kit were purchased from Yantai Science & Biotechnology Co., Ltd (Yantai, China). Anti-murine iNOS polyclonal antibody and COX-2 (murine) polyclonal antibody were obtained from Cayman (Michigan, USA). β-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Hydrocortisone Sodium Succinate (HSS, from, Japan) was used as positive control drug in all the experiments.

2. Plant material

The dried stems with bark of N. officinalis were collected in April 2005 from Hainan Province, China, and identified by Dr. Yanyan Zhao of Yantai University. A voucher specimen (No.S200504) was deposited in the Department of Pharmacognosy, Yantai University, China.

3. Extraction, isolation and identification of naucleofficine H

Stems with the bark of N. officinalis (10 kg) were extracted by percolation with methanol and evaporated to dryness in vacuo to yield 750 g of extract. The extract was then suspended in water and partitioned with CHCl₃ (3000 mL × 4), EtOAc (3000 mL × 4), and n-BuOH (3000 mL × 2) to obtain respective fractions (CHCl₃ fraction 190 g, EtOAc fraction 130 g, and n-BuOH fraction 200 g). The CHCl₃ fraction (15 g) was subjected to silica gel column chromatography (300 g) and eluted with a cyclohexane-acetone gradient from 80:20 to 40:60 (v/v); the cyclohexane-acetone (70:30) eluted fraction (2.3 g) was further isolated by a combination of
silica gel column chromatography, Sephadex LH-20 column chromatography, and HPLC to yield naucleofficine H as yellowish powder (\(^1\)H-NMR and \(^{13}\)C-NMR shown in Table S1). The purity was determined by normalization of the peak area in HPLC to be 99.4% (UV detector).

4. Culture of RAW 264.7 cells

Mouse monocyte-macrophage RAW 264.7 cells were maintained in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% heat inactivated fetal bovine serum at 37°C in a humidified incubator with 5% CO\(_2\) and 95% air. The medium was routinely changed every two days. RAW 264.7 cells were passaged until they attained confluence.

5. MTT assay to evaluate the cytotoxicity

RAW 264.7 cells were treated with naucleofficine H (1, 10, 30, 100 µM), respectively. Control group received an equal amount of DMSO, which resulted in a final concentration of 0.2% DMSO in the culture medium. After 24 h incubation, 8 µL of 5 mg/mL MTT solution (final concentration is 200 µg/mL) was added and the cells were incubated for another 4 h at 37°C. After removing the supernatant, 150 µL of DMSO was added to the cells to dissolve the formazan. The absorbance of each group was measured by using a microplate reader at wavelength of 570 nm. The control group consisted of untreated-cells was considered as 100% of viable cells. Results are expressed as percentage of viable cells when compared with control groups.

6. Nitric oxide analysis

The level of nitrite was measured by the Griess assay as an indicator of NO production. \(1 \times 10^6\) cells/mL of RAW 264.7 cells were cultured in a 96-well plant and allowed to attach for 1 h, then
treated by LPS (1 µg/mL) and with or without HSS (100 µM) or naucleofficine H (3-100 µM) for 24 h. 100 µL of the culture supernatant was taken out to determine by measuring the amount of nitrite using 100 µL of Griess reagent (mixture of equal amount of reagent A and reagent B, A: 1% sulphanilamide in 5% H3PO4, B: 0.1% naphthylethylene diamine dihydrochloride). After 10 min incubation at room temperature, the absorbance of mixtures was measured at 540 nm by a microplate reader and the inhibitory rates were calculated by using a standard calibration curve prepared from different concentrations of sodium nitrite.

7. Measurement of TNF-α

The TNF-α concentration was measured by a mouse TNF-α ELISA kit. 5×10^5 cells/mL of RAW 264.7 cells were treated by LPS (1 µg/mL) with or without naucleofficine H (3-100 µM) for 6 h. 100 µL of the culture supernatant was taken out to determine the level of TNF-α by using ELISA assay kits according to the manufacturer’s recommendations.

8. Assay of iNOS enzymatic activity

The iNOS enzymatic activity was determined by a Nitric Oxide synthase Assay Kit. RAW 264.7 cells were cultured in a 96-well plate and treated by LPS and with or without naucleofficine H (3-100 µM) for 2 h. The assays were performed according to the manufacturer’s instructions (Zhao et al. 2013).

9. Detection of iNOS, COX-2 and β-actin expression

RAW 264.7 cells were prepared for 1 h and treated with LPS (1 µg/mL) with or without naucleofficine H (3-30 µM) for 24 h. The cells were collected and the total protein concentration was measured using a Bradford protein assay kit. The equal quantities (30 µg) of proteins were loaded onto 8% sodium dodecyl sulfate polyacrylamide gels. And then target proteins were
transferred onto nitrocellulose membranes. The membranes were blocked with 7% skimmed milk in TBS-T (1×TBS with 0.05% Tween 20) at room temperature for 4 h. Washed three time by TBS-T, the membranes was incubated in primary antibody solutions overnight at 4°C. After washed with TBS-T, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibody solutions. The bands were detected by ECL and the bands representing iNOS, COX-2 and β-actin were quantitated by DigDoc100 program. The levels of corresponding iNOS and COX-2 were normalized on the basis of the corresponding β-actin levels.

10. Statistical analysis

All results are expressed as means ± SD (n=3). Statistical comparison was conducted using Student’s t-test after ANOVA. The results are considered to be significant when p < 0.05.

11. References

Zhao F, Chen L, Zhang ML, Bi CC, Li LC, Zhang QZ, Shi CC, Li M, Zhou SS, Kong LH. 2013.  Indole Alkaloid, 3-(Hydroxy-methyl)-6,7-Dihydroindolo [2,3-a] Quinolizin-(12H) -one, via NF-κB Inactivation in RAW 264.7 Macrophages. Planta Med. 79: 782–787.
| Position | δ_H  | δ_C  | Position | δ_H  | δ_C  |
|----------|------|------|----------|------|------|
| 1        | 11.02 (1H, s) |      | 13       |      | 136.0 |
| 2        |      | 135.2 | 14a      | 2.92 (1H, m) | 28.2 |
| 3        | 4.89 (1H, d, 5.7) | 52.2 | 14b      | 2.07 (1H, dt, 13.7, 3.9) |      |
| 5a       | 4.75 (1H, m) | 41.1 | 15       | 2.72 (1H, m) | 28.7 |
| 5b       | 2.93 (1H, m) |      | 16       | 2.46 (1H, dd, 5.9) | 47.6 |
| 6a       | 2.80 (1H, m) | 20.2 | 17       | 5.30 (1H, t, 5.4) | 92.4 |
| 6b       | 2.60 (1H, m) |      | 17-OH   | 6.33 (1H, d, 4.6) |      |
| 7        |      | 108.0 | 18       | 1.60 (3H, d, 6.7) | 11.5 |
| 8        |      | 126.6 | 19       | 5.43 (1H, q, 6.7) | 119.9 |
| 9        | 7.39 (1H, d, 7.9) | 117.6 | 20       |      | 134.5 |
| 10       | 6.97 (1H, d, 7.9) | 118.6 | 21a      | 4.16 (1H, d, 13.0) | 66.3 |
| 11       | 7.04 (1H, d, 7.9) | 120.9 | 21b      | 4.03 (1H, d, 13.0) |      |
| 12       | 7.33 (1H, d, 7.9) | 111.1 | 22       |      | 167.8 |

Note: ^1H-NMR and ^13C-NMR were obtained at 400 and 100 MHz, respectively.

**Table S1.** ^1H-NMR and ^13C-NMR spectroscopic data of naucleofficine H (DMSO-d$_6$, δ)
Figure S1. Cytotoxicity of nucleofieine H on RAW 264.7 cells.

The cells were treated with nucleofieine H (1-100 μM) for 24h. Cell viability was examined using an MTT assay. Data are means ± SD of three independent experiments.
Figure S2. Effect of naucleoffine H (100, 30, 10 and 3 μM) on production of TNF-α in LPS-induced RAW 264.7 cells. HSS (100 μM) represented the positive control. Data are means ± SD of three independent experiments. **p < 0.01 vs. LPS-treated control group; ##p < 0.01 vs. untreated group.
