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ORIGINAL RESEARCH

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The effect of *Pogostemon auricularius* fractions and its compounds on some pro-inflammatory and anti-inflammatory molecules in LPS-stimulated RAW 264.7 macrophages

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ABSTRACT

Background: In Vietnam and in other Asian countries, the above-ground parts of *Pogostemon auricularius* (L.) Hassk. (Lamiaceae), having a different range of ethnopharmacological traditions, are used as a natural cure with antiseptic, analgesic, and anti-inflammatory properties. This study pointed out the anti-inflammatory effects of some fractions and isolated compounds from *P. auricularius*.

Aim: This study aims to examine the anti-inflammatory effects of certain fractions and isolated compounds from *P. auricularius* by measuring the production of various pro-inflammatory factors.

Methods: The anti-inflammatory effects of methanol extract, *n*-hexane, dichloromethane, ethyl acetate, and aqueous fractions, as well as Pogostemins A–C and Pogostemon A–C, isolated from above-ground parts of *P. auricularius*, were ascertained by measuring the quantity of pro-inflammatory factors such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, nitric oxide (NO), and anti-inflammatory IL-10 produced by lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

Results: The fractions of ethyl acetate and dichloromethane displayed a significant activity contrary to the production of NO having IC_{50} values of 25.28 and 28.68 µg/ml, respectively. The new compound, pogostemin C, had the strongest inhibitory effect with an IC_{50} value of 1.36 µg/ml. Besides, pogostemin C displayed the ability to decrease pro-inflammatory TNF-α and increase anti-inflammatory IL-10 in LPS-stimulated RAW 264.7 cells.

Conclusion: This study reveals that the fractions and pogostemin C extracted from the above-ground parts of *P. auricularius* can inhibit the production of NO and TNF-α but activate anti-inflammatory IL-10 production, thereby explaining the ethnopharmacological traditions of this herb.

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Introduction

Pogostemon auricularius (L.) Hassk. (Lamiaceae) is an annual herb that grows in many countries, especially in tropical and subtropical states such as India, Sri Lanka, Bangladesh, China, and Southeast Asia, including Thailand, Indonesia, Cambodia, Myanmar, Vietnam, Laos, Philippines, and Malaysia [1,2]. The above-ground parts of *P. auricularius* have habitually been used to treat rheumatism, diarrhea, fever, and even snakebites [3–5]. This herb has been used as an antiseptic, analgesic, and anti-inflammatory agent. According to the experience of the Tanchangya-Kongmain tribe in Bangladesh, there was an oral administration of the leaf juice of *P. auricularius* in treating tetanus [6]. In Indonesia, numerous people employ the leaves of this herb to relieve children's stomach ache and urinary problems. In Malaysia, the whole of this plant has been used to cure diarrhea and rheumatism [3,4]. Based on the Traditional Chinese Medicine, both children and the elderly with high temperature were treated with *P. auricularius* [1,3].

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Furthermore, many people in India use this herb to treat snakebites. In addition, leaf juice is utilized to treat fever [5].

In Vietnam, *P. auricularius* is known as "Tu hung tai" or "Co co." The recognized Latin name (*Pogostemon auricularius* (L.) Hassk.) has been verified at http://www.theplantlist.org and has various synonyms, including *Dysophylla auricularia* (L.) Blume and *Heliotropium tetrandrum* (L.) Blume. With respect to the Traditional Vietnamese Medicine, this plant is also used for malaria, snakebites, wounds, pharyngitis, itchy skin, eczema, stomach ache, digestive disorders, kidney pain, and rheumatism [2]. A decoction can be used at a dosage of approximately 12–24 g of fresh plant or 10–15 g of dried plant. When it is used as a lotion, the plant is generally cut and extracted or boiled [3].

The previous studies have indicated that the methanol and ethanol extracts of *P. auricularius* possess biological properties, such as antimicrobial, anti-inflammatory, antidiarrheal, and thrombolytic activities, as well as α-amylase inhibition, related to its traditional use [7,8]. Therefore, *P. auricularius* is widely distributed and has been broadly used in the Traditional Vietnamese Medicine. However, so far, there has been only one phytochemical study of this species on the composition of essential oils [9]. Hence, *P. auricularius* should be studied to provide information on the natural compounds of this genus, particularly the clarification of the traditional applications of this herb.

Inflammation is a portion of several pathological circumstances such as atherosclerosis, Alzheimer's disease, cancer, asthma, and infections, such as tuberculosis. At present, nonsteroidal anti-inflammatory drugs are the main drugs used for treating inflammation, which are often associated with gastric and cardiovascular reactions [10].

In our earlier study, we stated the isolation, structural elucidation, and cytotoxicity of three new meroterpenoids, pogostemins A–C (**1–3**) [11]; three new phloroglucinols, pogostemonons A–C (**4–6**) [12], as well as the new triterpene, pogostemon, and the new phloroglucinol derivative, pogostemon D [13] from the above-ground parts of *P. auricularius*. The report shows that a certain fraction and isolated compounds from the above-ground parts of *P. auricularius* can inhibit the production of NO and tumor necrosis factor (TNF)-α but activate anti-inflammatory interleukin (IL)-10 in RAW 264.7 macrophage cells, thus clarifying the ethnopharmacological uses of this herb.

Experimental

General experimental procedures

Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium nitrite, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were acquired from the Grand Island Biological Company (GIBCO, Invitrogen). RAW 264.7, a murine macrophage cell line, was acquired from the University of Perugia, Perugia, Italy. The enzyme-linked immunosorbent assay kits of TNF-α, IL-6, and IL-10 were from BioVision Inc. (Milpitas, CA). Cell culture flasks and 96-well plates were purchased from Corning Inc. (Corning, USA). The ELISA Plate Reader (Bio-Rad, California) was used to measure the absorbance of cells in the MTT cell viability assay.

Plant material and extracts

The fresh above-ground parts of *P. auricularius* (L.) Hassk. were collected from Quang Tri Province, Vietnam (geographical coordinates: N16°44'38.9" E107°14'51.1"). The plant was recognized by Dr. Nguyen T. Cuong, from the Institute of Ecology and Biological Resources, VAST, Vietnam. Fresh materials were dried at ambient temperature for 10 days in an airy area until air-dried and stored before extraction in tightly sealed bags at the room temperature. A voucher specimen (PA01) was deposited at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

The extract of *P. auricularius*, its fractions, and pure compounds were prepared as follows. The dried plant material (2.5 kg) was extracted with MeOH (three times, 10.0 l each) at the room temperature (Fig. 1) as was previously described [11]. The EtOAc fraction was chromatographed, leading to the isolation of six compounds, including three new meroterpenoids, pogostemins A–C (**1–3**) [11], and three new phloroglucinols, pogostemonons A – C (**4–6**) [12] using various forms of open-column chromatography (CC), such as normal-phase, reverse-phase or Sephadex LH-20 columns, and preparative reversed-phase high-performance liquid chromatography.

Cell culture

RAW 264.7 cell lines were cultivated in DMEM augmented using 2 mM L-Glutamine, 10 mM HEPES, 1

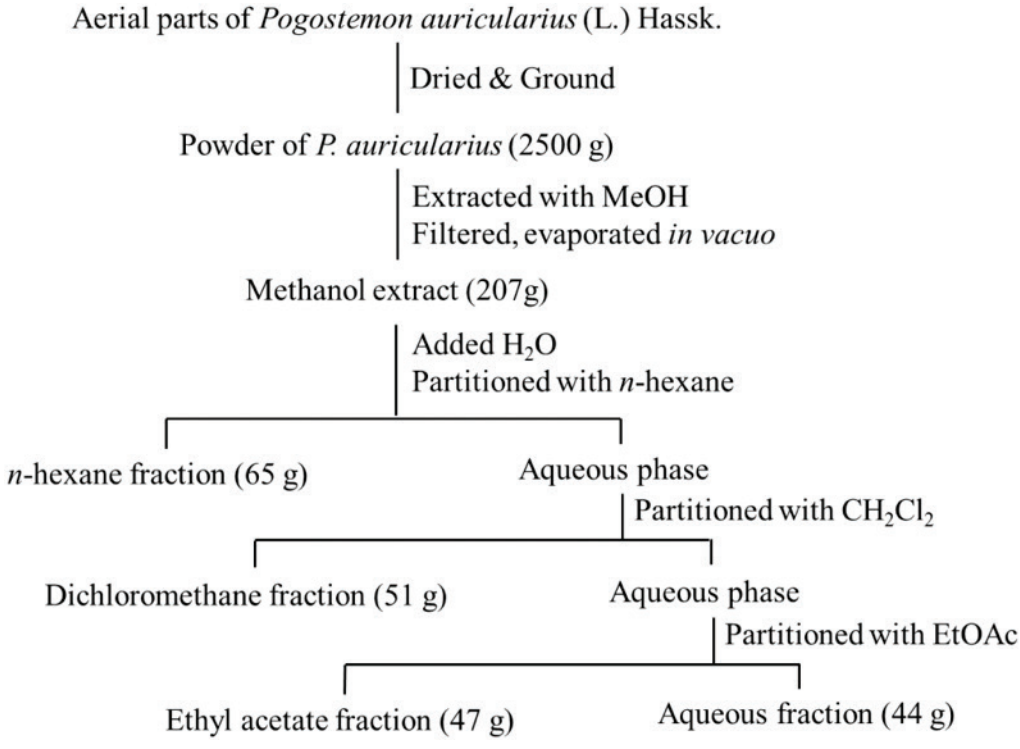

Figure 1. Extraction and fractions carried out with the aerial parts of *P. auricularius.*

mM sodium pyruvate, and 10% FBS, at 37ºC with 5% CO_2. After 3–5 days, the cells were sub-cultured with a ratio of 1:3 and incubated at 37°C in a humid atmosphere of 5% carbon IV oxide (CO_2) [14,15].

MTT cell viability assay

RAW 264.7 cells were sown in 96-well plates in the company of two concentrations (100 and 20 µg/ml) of the fractions or pure compounds. After 24 hours of incubation, 0.5 mg/ml MTT was added, and the cells were incubated for 4 hours at 37°C and 5% CO_2. Subsequently, the supernatant was removed, and the formazan crystals were liquefied in DMSO. Furthermore, the absorbance value of the solution was assessed at 550 nm. The percentage of dead cells was ascertained in relation to the control group [14,15].

Inhibition of nitric oxide (NO) production

The inhibition rates of NO production by *P. auricularius* fractions or pure compounds were evaluated. The RAW 264.7 cells (2×10^5 cells/well) were added to a 96-well plate and cultured as previously described [14]. Thereafter, the cultured medium was removed and substituted with FBS-free DMEM for 3 hours. Next, *P. auricularius* fractions or pure compounds with different concentrations (100, 20, 4, and 0.8 µg/ml) were put in a 96-well plate. The cells were then incubated for 2 hours, and NO was produced by stimulation with 1 µg/ml of LPS for 24 hours. The commercial NO detection kit Griess Reagent System (Promega Corporation, WI) was used to quantify nitrite produced in the cell-cultured medium. Specifically, 100 µl of cell culture medium with 100 µl of Griess reagent (50 µl of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 50 µl 0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride) in a 96-well plate was incubated for 10 minutes at the room temperature, and the absorbance of the solution was measured at 540 nm in a microplate reader (Bio-Rad, California). The quantity of nitrite in the medium was calculated using a sodium nitrite standard curve. FBS-free DMEM medium was used as a blank sample, whereas L-N^G-monomethyl arginine citrate (L-NMMA) was used as a positive control, and macrophages stimulated with LPS at 1 µg/ml and untreated were used as a negative control. The capability of inhibiting NO was measured in doses of 100, 20, 4, and 0.8 µg/ml and was estimated as a half maximal inhibitory concentration (IC_{50}), which was calculated using the TableCurve Version 4.0 program [14–16].

Cytokine assays

To ascertain the effects of pogostemin C on the release of cytokines in cells stimulated with LPS, the production of TNF-α, IL-6, and IL-10 was measured

by ELISA kits at doses of 20, 4, 0.8, and 0.16 µg/ml. The RAW 264.7 cells (5×10^4 cells/well) were cultured in 96-well plates, incubated overnight at 37°C, and tested using several concentrations of pogostemin C for 2 hours. In the next stage, 1 µg/ml of LPS was added at 37°C and 5% CO_2 for 24 hours. Then, the amount of TNF-α, IL-6, and IL-10 in collected supernatants was measured by ELISA kits, based on the manufacturer's instructions [17].

Statistical analysis

All data presented in this study are expressed as mean ± standard deviation of at least three independent parallel testing. A statistical analysis was performed using the SPSS software (version 20.0). Significant differences between the groups were determined by analysis of variance. $p < 0.05$ was considered to be significant.

Results and Discussion

The secretion of the inflammatory mediators, both of pro-inflammatory mediators (IL-6, TNF-α, and NO), and anti-inflammatory mediators (IL-10), is the principal reaction to swelling [18,19]. The cytotoxic assay was utilized in ascertaining the nontoxic concentration of *P. auricularius* fractions and its compounds as follows. The nontoxicity of the analyzed sample was verified through MTT assay, resulting in more than 90% of cell viability. Most of these samples at a concentration of 100 µg/ml were toxic to RAW 264.7 cells. However, at a concentration of 20 µg/ml of these samples tested, the cells were not impaired (viability > 90%), except that compound **3** had a cytotoxic response with a survival rate of 71.34%.

The activity of the inhibition of NO

In this study, methanol extract and four fractions were assessed, including *n*-hexane, dichloromethane, ethyl acetate, and aqueous fractions from *P. auricularius*, which inhibited NO production in the LPS-stimulated RAW 264.7 cells. The ethyl acetate and dichloromethane fractions considerably impeded nitrite with IC_{50} values of 25.28 and 28.68 µg/ml, respectively. Moreover, the *n*-hexane fraction was idle in the NO inhibition testing, while the methanol extract and the aqueous fraction revealed activity having the IC_{50} values of 60.54 and 64.73 µg/ml, respectively.

Since the activity of the NO inhibition testing was the strongest, the ethyl acetate fraction was secluded by column chromatography to obtain six new compounds (Fig. 2), comprising pogostemins A–C (**1**–**3**) [11], and three new phloroglucinols, pogostemonons A–C (**4**–**6**) [12]. Subsequently, the effects of these compounds on NO production were investigated. The inhibitory activity of compound **3** was the strongest with the IC_{50} values of 1.36 µg/ml. Compound **5** was idle in the NO inhibition testing, whereas the other compounds showed activity with IC_{50} values ranging from 9.05 to 24.24 µg/ml. The potency of compound **3** was four times greater than that of L-NMMA, which was a positive control (IC_{50} = 6.22 µg/ml) (Table 1).

Based on the inhibition of NO production and the outcome of the MTT cell viability test, compound **3** exhibited a significant anti-inflammatory activity and it would be worthy to continue researching on its effects on cytokines. In fact, the inhibitory activity of compound **3** was the strongest with an IC_{50} value of 1.36 µg/ml.

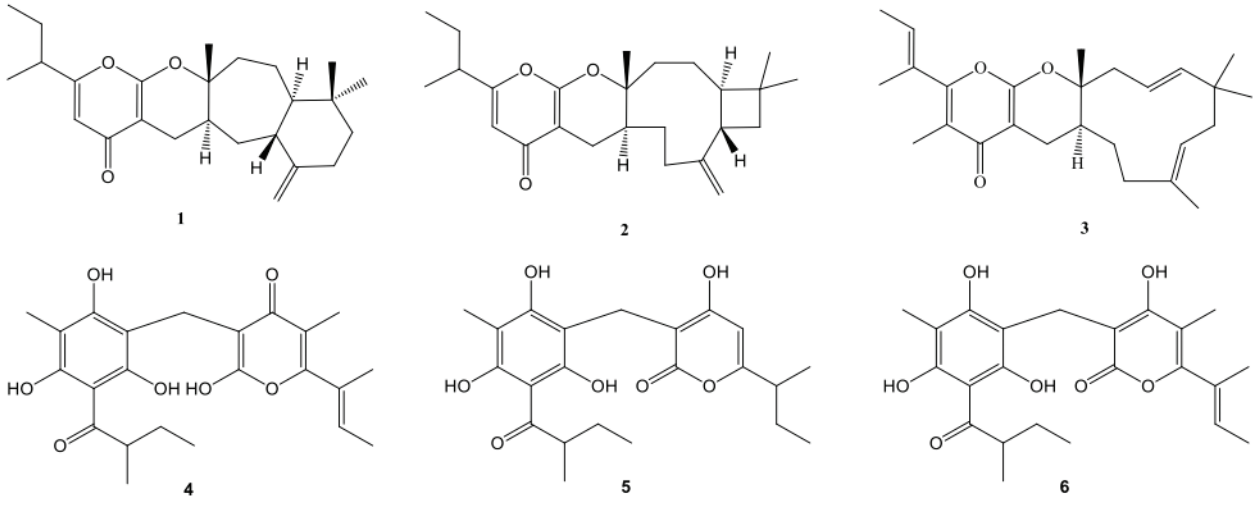

Figure 2. Structure of pogostemins A–C (**1–3**) and pogostemonons A–C (**4–6**).

Table 1. Biological activity of tested extract, fractions, or compounds on the NO inhibitory activity.

Extracts/Compounds	NO (IC50, µM)	% Survival/Cytotoxic activity	
		at 100 µg/ml	at 20 µg/ml
Methanol extract	60.54 ± 2.66	49.61	>100
n-Hexane fraction	N/A	46.34	>100
Dichloromethane fraction	28.68 ± 1.49	31.18	98.79
Ethyl acetate fraction	25.28 ± 1.52	92.77	>100
Aqueous fraction	64.73 ± 1.84	63.09	86.86
Pogostemin A (**1**)	24.24 ± 2.92	21.55	42,94
Pogostemin B (**2**)	20.84 ± 2.63	95.40	99.11
Pogostemin C (**3**)	**1.36 ± 0.13**	28.90	71.34
Pogostemonon A (**4**)	11.89 ± 1.73	37.69	94.22
Pogostemonon B (**5**)	>100	>100	>100
Pogostemonon C (**6**)	9.05 ± 0.71	32.98	>100
L-NMMA	6.22 ± 0.84	89.77	95.72

Table 2. Effects of pogostemin C toward IL-6, IL-10, and TNF-α concentrations in RAW 264.7 cells.

Cytokine Concentration (pg/ml)	Samples Concentration (µg/ml)				
	Pogostemin C				LPS
	20	4	0.8	0.16	
IL-6	130.97 ± 17.31	121.35 ± 18.02	111.06 ± 14.99	141.06 ± 18.36	136.74 ± 8.65
IL-10	384.85* ± 30.94	232.77 ± 14.81	214.15 ± 13.08	209.97 ± 9.44	218.81 ± 32.67
TNF-α	428.44* ± 51.09	534.07 ± 37.99	609.82 ± 56.38	632.17 ±49.84	714.09 ± 57.29

*Indicates data statistically significantly different in comparison with the control (no treated cells) at $p < 0.05$.

The activity of decreasing pro-inflammatory increasing IL-10

This study assessed the additional effects of compound **3** on LPS-induced cytokine production. The amount of TNF-α, IL-6, and IL-10 produced in the culture supernatants of RAW 264.7 cells was measured. According to the cytotoxic result in Table 1, the doses of compound **3** should be reduced in the cytokine experiment. Thus, four concentrations of compound **3** (including 20, 4.0, 0.8, and 0.16 µg/ml) were used for this test. The result showed that 20 µg/ml of compound **3** had an anti-inflammatory effect by decreasing TNF-α and increasing IL-10 production in stimulated LPS-induced RAW 264.7 compared to unstimulated cells ($p < 0.05$). In addition, compound **3** was idle in the IL-6 inhibition testing at the entire concentration tested (Table 2).

Compound **3** is a meroterpenoid having γ-pyrone and humulene skeleton. Terpene humulene is a natural compound found in various plants. Besides, the anti-inflammatory properties of humulene were assessed and compared with dexamethasone, which is a steroidal anti-inflammatory [20]. Hence, the relative nature of the structural activity of compound **3** should be studied in the subsequent research.

Consequently, pogostemin C (compound **3**) may be helpful for future analysis in averting inflammatory diseases, and *P. auricularius* has colossal prospective to offer new active ingredients.

Furthermore, other biologically active compounds isolated from the aerial parts of Pogostemon species [*Pogostemon cablin* (Blanco) Benth.], such as a tricyclic sesquiterpene patchouli alcohol, have medicinal interest; the anti-inflammatory effect of on LPS-stimulated RAW264.7 cells was studied by Xian et al. [21]. Pretreatment with different concentrations dose dependently decreased the production of TNF-α, IL-1β, IL-6, NO, and prostaglandin E2 in LPS-stimulated RAW264.7 cells. The authors concluded that patchouli acid is an important anti-inflammatory constituent of *P. cablin,* in which effect may be mediated by downregulation of the mRNA expression of a panel of inflammatory mediators, such as TNF-α, IL-1β, IL-6, iNOS, and COX-2 [21].

Therefore, our investigation on the phytochemical and biological studies of *P. auricularius* will be helpful in understanding the natural compounds of this genus and clarifying the effect of this herb used in traditional Vietnam medicine. It was earlier demonstrated [22] that *Sarcosperma affinis*, known in Vietnamese Ethnomedicine, actually has a predictable pharmacological activity. The needles of *Pinus sylvestris* which are used as the anticancer folk therapies in ethnopharmacology in Estonia [23] were confirmed to have a cytotoxic effect on breast cancer cells *in vitro* [24]. Therefore, based on our current and previous experiences, the data of ethnomedicine may be pivotal.

Conclusion

This study has shown that the fractions and pogostemin C isolated from the above-ground parts of *P. auricularius* can inhibit the production of NO and TNF-α but activate anti-inflammatory IL-10, thus explaining the ethnopharmacological traditions of this herb.

Declaration of interest

The authors declare that there is no conflict of interest. The authors are solely responsible for the content and writing of this article. All listed authors have read and approved the submitted manuscript.

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