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Inhibitory effects of *Piper betle* on production of allergic mediators by bone marrow-derived mast cells and lung epithelial cells

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Abstract

The leaves of the *Piper betle* Linn. (Piperaceae) are used in traditional medicine and possess anti-oxidant, anti-bacterial, anti-fungal, anti-diabetic and radioprotective activities. However, little is known about their anti-allergic activity. Therefore, the effects of *P. betle* ethanolic extract (PE) on the production of histamine and granulocyte macrophage-colony-stimulating factor (GM-CSF) by murine bone marrow mast cells (BMMCs) and on the secretion of eotaxin and IL-8 by the human lung epithelial cell line, BEAS-2B, were investigated *in vitro*. PE significantly decreased histamine and GM-CSF produced by an IgE-mediated hypersensitive reaction, and inhibited eotaxin and IL-8 secretion in a TNF-α and IL-4-induced allergic reaction. The results suggest that *P. betle* may offer a new therapeutic approach for the control of allergic diseases through inhibition of production of allergic mediators.

1. Introduction

Piper betle Linn. is an edible plant with leaves that have been traditionally used in India, China and Thailand for prevention of oral malodor, since it has anti-bacterial activity against obligate oral anaerobes responsible for halitosis [1]. Aqueous extracts of *P. betle* have also been shown to reduce the adherence of early dental plaque bacteria [2]. *P. betle* is a member of the Piperaceae family and its leaves have a strong pungent and aromatic flavor. As well as use as a mouth freshener, the leaves are used for wound healing [3] and digestive and pancreatic lipase stimulant activities in traditional medicine [4]. Anti-oxidant [5,6], anti-bacterial and anti-fungal [1,7–9], anti-inflammatory [10], anti-diabetic [11] and radioprotective [10] activities of *P. betle* have also been reported.

In Thailand, *P. betle* is commonly known by its Thai name of "Phlu". Several studies have examined the antimicrobial and anti-itching activities of *P. betle*. Tappayuthpijarn et al. [7] reported an inhibitory effect of *P. betle* extract on the growth of clinical isolates of *Staphylococcus aureus* (122 strains), *Escherichia coli* (31 strains) and *Pseudomonas aeruginosa* (57 strains); and Boonyaratanakornkit et al. [8]

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demonstrated *in vitro* and *in vivo* antimicrobial activities of ointment of the *P. betle* leaf extract, with the ointment preventing growth of *S. aureus*, β-hemolytic streptococcus group A and dermatophytes, which are all microorganisms that can cause skin infection. In a clinical trial, *P. betle* ointment cured and improved ringworm skin lesions at rates of 40% and 26%, respectively. Pongpech and Prasertsilpe [9] found that *P. betle* gel inhibited growth of dermatophytes that cause ringworm and growth of *Candida* spp. more effectively than tolnaftate and with a similar inhibitory effect to that of clotrimazole. However, the effect of *S. aureus* inhibition was less than those of gentamicin and oxytetracyclin/polymyxin B ointment. A comparative study of *P. betle* gel and two anti-pruritic drugs (calamine lotion and 0.1% betamethasone valerate) was conducted by Wongsiri-amnuoy [12] in patients with chronic dermatitis in Sanpatong Hospital, Chiang Mai, Thailand. This study showed that the anti-pruritic effect of *P. betle* gel is significantly greater than that of calamine lotion and similar to that of 0.1% betamethasone. Subsequently, the Thai Government Pharmaceutical Organization has approved *P. betle* gel as a commercial pharmaceutical product for alleviation of itching.

The main goal of the current study was to investigate the effects of *P. betle* ethanolic extract (PE) on histamine and granulocyte macrophage-colony-stimulating factor (GM-CSF), which are allergic mediators produced by bone marrow mast cells (BMMCs), and on eotaxin and interleukin-8 (IL-8), which are associated with an asthmatic response and secreted by BEAS-2B lung epithelial cells.

2. Materials and methods

2.1. Plant extract preparation

Leaves of *P. betle* were collected from the faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Three hundred grams of fresh leaves were chopped and extracted by maceration with 98% ethanol for 3 days. The ethanolic extract was dried under reduced pressure, dissolved in DMSO (10 mg/ml) and stored at −20 °C until use. The final concentration of DMSO is not more than 0.1% in each test sample.

2.2. BMMCs

BMMCs were generated from femoral bone marrow cells of 6-week-old C57BL/6 mice. The cells were grown in BMMC medium containing RPMI 1640 (Gibco), with 10% heat-inactivated fetal bovine serum (MultiSer, Lot No B12048-500), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM MEM non-essential amino acid solution (Gibco), 50 μM 2-ME and 0.01 μg/ml recombinant murine IL-3 as a source of mast cell growth factor; Peprotech EC, London) in a 5% CO_2 incubator at 37 °C. Non-adherent cells were recovered every week and further expanded in fresh medium at 5×10^5 cells/ml for 4 weeks. The BMMCs were identified by staining with 0.05% toluidine blue (pH 4.1).

BMMCs were sensitized by incubation with mouse monoclonal anti-DNP IgE (MMCE) produced by EC1 cells at a BMMC:MMCE ratio of 10:1 overnight in a 5% CO_2 incubator at 37 °C. Viable BMMCs at 2.5×10^6 and 6.25×10^5 cells/ml were used for the histamine and GM-CSF tests, respectively. After sensitization, BMMCs were washed once and counted to adjust for the amount of cells in Tyrode's solution for the histamine test and in the BMMC medium without IL-3 for the GM-CSF test. Viable cells were counted using a trypan blue exclusion test.

For the histamine inhibition test, BMMCs (400 μl) were incubated at 37 °C for 5 min, and then 50 μl of plant extract (at a final concentration of 10, 25 or 50 μg/ml) or solvent control (at a final concentration of 0.1% DMSO in Tyrode's solution in spontaneous tubes and control tubes) was added at room temperature for 10 min. The BMMC culture was then challenged with 50 μl of DNP-BSA (final concentration of DNP 30 ng/ml) or Tyrode's solution (spontaneous tubes). The reaction was stopped after 30 min by placing the treated cells on ice for 15 min. The culture supernatant was collected after centrifugation at 1500 rpm at 4 °C for 5 min. The supernatant was used to determine histamine release and the precipitated cells were collected for evaluation of cytotoxicity. Total cell-associated histamine was extracted in the presence of perchloric acid. The sample was diluted with distilled water (1:1) and measured by HPLC using histamine (100 ng/ml) as a standard. Cell cytotoxicity was determined using the trypan blue exclusion test.

For the GM-CSF inhibition test, BMMCs (400 μl) were pre-incubated with 50 μl of plant extract (at a final concentration of 10 or 25 or 50 μg/ml) or solvent control (at a final concentration of 0.1% DMSO in BMMC medium without IL-3 in spontaneous tubes and control tubes) at room temperature for 10 min. Then the BMMC culture was challenged with 50 μl of DNP-BSA (final concentration of DNP 30 ng/ml) or BMMC medium without IL-3 (spontaneous tubes). The cells were incubated in a 5% CO_2 incubator at 37 °C for 6 h. The culture supernatant was collected after centrifugation at 1500 rpm at 4 °C for 5 min. The supernatant was assayed for mouse GM-CSF using a Quantikine ELISA kit (R & D Systems, Minneapolis, MN, USA).

2.3. BEAS-2B cells

BEAS-2B were grown until confluence in DMEM/F12 culture medium (Gibco) with 5% FBS, 1% L-glutamine, 100 unit/ml penicillin and 100 mg/ml streptomycin in a 5% CO_2 incubator at 37 °C. The confluent cells were trypsinized and counted for viable cells. Viable BEAS-2B cells at 1×10^5 cells/ml were grown in culture medium without L-glutamine and antibiotics in a 6-well plate (1.5 ml/well) for 3 days. The culture plate was used for the eotaxin and IL-8 inhibition tests.

For the eotaxin inhibition test, the culture supernatant of BEAS-2B cells was removed, and the cells were washed once and maintained in DMEM/F12 medium (1 ml/well). The cells were pre-incubated with plant extract (at a final concentration of 5, 10, 25, or 50 μg/ml) or solvent control (at a final concentration of 0.1% DMSO in DMEM/F12 medium in spontaneous tubes and control tubes) at 37 °C for 1 h. Human recombinant TNF-α (PeproTech EC, London) and human recombinant IL-4 (both at final concentrations of 100 μg/ml) or DMEM/F12 medium (spontaneous tubes) were then added to each well, and the culture plate was incubated for a further 18 h. The culture medium was then centrifuged at 1500 rpm at 4 °C for 5 min, and the supernatant was collected for measurement of eotaxin using a human eotaxin ELISA set (OptEIA, BD Biosciences). The culture cells were trypsinized and the cytotoxicity of plant extracts was evaluated using the trypan blue exclusion test.

For the IL-8 inhibition test, the culture supernatant of BEAS-2B cells was removed, and the cells were washed once and maintained in DMEM/F12 medium. The cells were pre-incubated with plant extract (at a final concentration of 6.3, 12.5, 25, or 50 μg/ml) or solvent control (0.1% DMSO in DMEM/F12 medium) at 37 °C for 1 h. After addition of peptidoglycan (PGN) (at a final concentration 10 μg/ml in PBS) to each well, the culture plate was incubated for a further 18 h. The culture medium was then centrifuged at 1500 rpm at 4 °C for 5 min and the supernatant was collected for measurement of IL-8 using a Quantikine human IL-8 ELISA kit (R&D Systems). The cultured cells were trypsinized and counted for viable and dead cells by the trypan blue exclusion test.

2.4. Statistical analysis

Data are reported as means ± S.E.M. and were analyzed by one-way ANOVA. For comparison between groups, the difference in means