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Phyllanthus urinaria Inhibits Drug-Resistant *Propionibacterium acnes* Growth and *P. acnes*-Induced IL-8 Production in THP-1 cells

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Acne treatments are usually employed to inhibit inflammation or kill skin pathogens. However, these remedies can induce side effects such as xerosis cutis and skin irritation if they are used excessively. Therefore, natural products garnered the attention as alternatives to synthetic drugs, particularly those presenting high antibacterial and anti-inflammatory activities with no side effects. The antibacterial and anti-inflammatory activities of *Phyllanthus urinaria* extract (PUE) against drug-susceptible and drug-resistant skin pathogens have not been reported. In the present study, we evaluated the anti-bacterial and anti-inflammatory activities of PUE. We determined the anti-bacterial activities of PUE using the disk diffusion method. Various PUEs (water, 20%, 50%, and 100% ethanolic extracts) showed excellent antibacterial activities against *Propionibacterium acnes (P. acnes)*, which are acne-causing bacteria. In addition, PUE inhibited the production of interleukin (IL)-8 in a dose-dependent manner in *P. acnes*-induced THP-1 monocytic cells, an indication of its anti-inflammatory effects. In order to determine whether PUE can safely be applied to the human skin, the cytotoxic effects of PUEs were determined by MTT assay in human keratinocyte HaCaT cells. PUE exhibited a low cytotoxicity at 10 ìg/mL. The presence of rutin in PUE was determined using HPLC fingerprint. The content of rutin in water, 20%, 50%, and 100% ethanolic extracts was 5.2 mg/g, 1.5 mg/g, 0.7 mg/g, and 0.25 mg/g, respectively. Taken together, our results suggest that PUEs are attractive acne-mitigating candidates for topical application.

Key words: Acne, drug-resistant skin pathogen, inflammation, *Phyllanthus urinaria*, *Propionibacterium acnes*.

Acne vulgaris is the most common human skin multifactorial disorder of the pilosebaceous unit. Its widely recognized pathophysiology results from the interplay of the following four factors: follicular epidermal hyperproliferation with subsequent plugging of the follicle, excessive sebum production, which may serve as a nutrient source for *Propionibacterium acnes* (*P. acnes*), overproliferation of *P. acnes*, and inflammation[1, 2]. In particular, *P. acnes* in the anaerobic and sebum rich environment of the follicle contributes to the inflammatory reaction of acne by inducing monocytes to produce pro-inflammatory cytokines, including tumor necrosis factor (TNF)-β and interleukin (IL)-8[3, 4]. The usual drugs, namely, benzoyl peroxide, adapalene, clindamycin, and

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tetracycline, used in the treatment of acne are effective against inflammatory acne[5-7], but may lead to the emergence of resistant pathogens and various side effects, such as dryness, redness, irritation of the skin, and hypopigmentation[8]. In addition, some oral antibiotics can cause gastro-intestinal disorders and increase the risk of venous thromboembolism[9-11]. Thus, there is a need for alternative, non-antimicrobial compounds for the treatment of acne vulgaris. Natural essential oils such as *Thymus quinquecostatus* oil, *Abies koreana* oil, citrus oils, and natural extracts such as those from *Mollugo pentaphylla*, *Angelica anomala*, *Matteuccia orientalis*, and *Orixa japonica* have been reported to be efficacious anti-acne materials[12-15].

Phyllanthus urinaria (*P. urinaria*) is one of the herbal plants belonging to the genus *Phyllanthus* (Euphorbiaceae), which are widely distributed in Eastern Asia, including Korea and China. It has been reported to have pharmacological effects such as anti-oxidant activity[16], anti-*Helicobacter pylori*-induced inflammatory activity[17], anti-hypertensive activities[18], phagocytic activity[19], and antiviral activity against hepatitis B and related hepatitis viruses[20-22] as well as anti-cancer activity[23, 24] and anti-angiogenic activity[25]. In addition, it also exhibits á-amylase inhibitory activity[26]. However, the anti-bacterial activities of *P. urinaria* against drug-resistant skin pathogens have not been described. In this study, we demonstrate that *P. urinaria* has antibacterial effects against acne pathogens and anti-inflammatory activities in human monocytic THP-1 cells. To the best of our knowledge, this is the first report demonstrating the *in vitro* anti-acne activities of *P. urinaria*, providing a scientific basis for their use for human skin health

MATERIALS AND METHODS

Plant material and extraction

P. urinaria plants were collected in September 2014 from Jeju Island, Korea. Voucher specimen number CSC-021 has been deposited at the herbarium of Cosmetic Sciences Center, Department of Chemistry and Cosmetics, Jeju National University. The materials for extraction were cleaned, dried at room temperature for 2 weeks, and ground into a fine powder using a blender. The dried powder (whole plants, 450 g) was extracted with 20% ethanol (EtOH), 50% EtOH, and 100% EtOH at room temperature for 24 h and then evaporated under a vacuum. In the case of water extracts, the dried power was extracted twice with distilled water at 60°C for 6 h. The yield and recovery of these four fractions were as follows: water (58.3 g, 38.8%), 20% EtOH (88.5 g, 19.6%), 50% EtOH (83.4 g, 18.5%), and 100% EtOH (86.2 g, 19.2%).

Microorganisms

P. acnes CCARM 0081, *P. acnes* CCARM 9009, *P. acnes* CCARM 9010, and *P. acnes* CCARM 9089, which are involved in acne, were selected as test microorganisms according to their pathological capacity. They were obtained from the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Seoul, Korea). *Propionibacterium* strains were cultured at 37°C for 48 h in GAM agar medium (Nissui Pharmaceutical Co., Tokyo, Japan) under anaerobic conditions before the assay.

Disk diffusion assay

The antibacterial activity was determined using the agar diffusion technique as described previously by Yoon *et al*[15]. Agar diffusion susceptibility testing was performed using the disc method. A disc of blotting paper (8 mm) was impregnated with 20 µL of a 100 mg/mL solution of each PUE dissolved in dimethylsulfoxide (DMSO). Thus, the disc potencies were 0.5 mg for the crude extract. Erythromycin (Sigma, St. Louis, MO, USA) was used as the standard drug. After drying, the disc was placed on a plate of sensitivity testing agar inoculated with the test *Propionibacterium* sp. Petri dishes were left at room temperature for approximately 1 h to allow the extract from the disc to infuse into the medium and were then incubated at 37°C for 48 h under anaerobic conditions for *Propionibacterium* sp. (BBL GasPak System). The zones showing no growth were then noted and their diameter was recorded as the zone of inhibition. The diameter of the inhibition zone was measured with calipers.

Cell culture and cytotoxicity assay

Human keratinocyte HaCaT and monocytic THP-1 cells were cultured in DMEM and RPMI medium containing 10% fetal bovine serum and penicillin streptomycin at 37°C in a humidified 95% air, 5% CO_2 atmosphere. The cells

were seeded on 24-well plates and PUE treatment was initiated 24 h after seeding. The general viability of the cultured cells was determined by the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan. MTT was added to each well in a 1/10 volume of media. Cells were incubated at 37°C for 3 h and DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a spectrophotometer (Molecular devices, SpectraMax M2, Sunnyvale, CA, USA). The entire experiment was performed in triplicate and results were confirmed by three independent experiments.

Measurement of cytokine production

Human monocytic THP-1 cells (1×10^5) in serum-free medium were stimulated with 100 µg/mL of *P. acnes* alone or in combination with the indicated concentrations of PUE, and incubated for 48 h. The culture supernatants were then harvested to determine IL-8 concentrations. The IL-8 concentration in the culture supernatant was measured by using an enzyme-linked immunosorbent assay (ELISA) kit (BioSource, Camarillo, CA, USA).

High-performance liquid chromatography (HPLC) fingerprinting

Since rutin was reported as an ingredient of *Phyllanthus* spp. and presents anti-inflammation properties, we investigated its presence in PUE[27] using HPLC with a Waters e2695 separation module (Waters, Milford, MA, USA) coupled to a Waters 2489 UV visible detector, utilizing a YMC Pro C_{18} RS column (250 ×4.6 mm; YMC Co., Kyoto, Japan) at a flow rate of 0.6 mL/min. The column was placed in a column oven at 40°C.

Statistical analysis

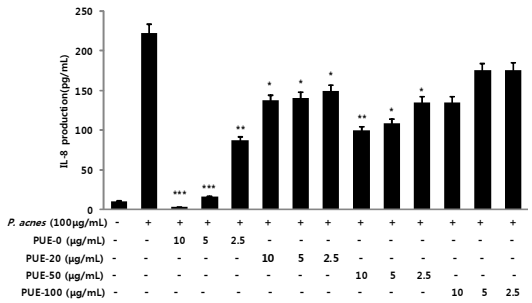
All data are expressed as means ± standard deviation (S.D.). Significant differences among the groups were determined using the unpaired Student's t-test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Multiple factors are involved in acne pathogenesis, in which the excessive sebum production may serve as a nutrient source for *P. acnes*, leading to overgrowth and colonization of *P. acnes*, and *P. acnes*-induced inflammation. Therefore, inhibitors of *P. acnes* growth and *P. acnes*-induced inflammation have been proposed as promising targets for the development of clinical and cosmetic anti-acne agents. As part of our ongoing alternative medicine program for anti-acne agents, we directed our attention toward the identification of natural products that combine a relatively narrow spectrum of activity against *P. acnes*, including tetracycline-, erythromycin-, and clindamycin-resistant strains, for potential topical applications in patients with mild to moderate acne vulgaris.

Herein, we report the anti-bacterial and anti-inflammatory activities of PUEs against drug-susceptible and -resistant *P. acnes*. First, we prepared aqueous (PUE-0) as well as 20% (PUE-20), 50% (PUE-50), and 100% ethanolic extracts (PUE-100) of *P. urinaria* as tested materials. The *in vitro* antimicrobial potential of PUEs against the tested drug-susceptible and -resistant *P. acnes* was quantitatively assessed by the presence or absence of inhibition zones using the agar diffusion method. According to the results presented in Table 1, PUE presented a great antimicrobial activity against all investigated skin pathogens. The diameter of the growth inhibition zones ranged from 11 to 15 mm (including the diameter of the disc, 8 mm) with the highest inhibition zone values observed against *P. acnes* CCARM 9089 for PUE-0 and PUE-50 (15 mm).

Since *P. acnes* contributes to the inflammatory reaction of acne by inducing monocytes to produce pro-inflammatory cytokines, including tumor necrosis factor (TNF)-β and interleukin (IL)-8[28, 29], we next examined the effects of our four PUEs (PUE-0, PUE-20, PUE-50, and PUE-100) on IL-8 production in human monocytic THP-1 cells. To determine the inhibitory effect of PUE-0, PUE-20, PUE-50, and PUE-100 on IL-8 production, an IL-8 ELISA was used to assess IL-8 production by heat killed *P. acnes*-stimulated human monocytic THP-1 cells. When THP-1 cells were stimulated with heat killed P. *acnes* (100 µg/mL) for 24 h, IL-8 levels increased dramatically in the culture medium. As shown in Figure 1, all PUEs significantly inhibited the IL-8 production induced by heat killed *P. acnes*, in a concentration dependent manner. The highest inhibitory values were observed when using the aqueous extract (PUE-0) with an IC_{50}

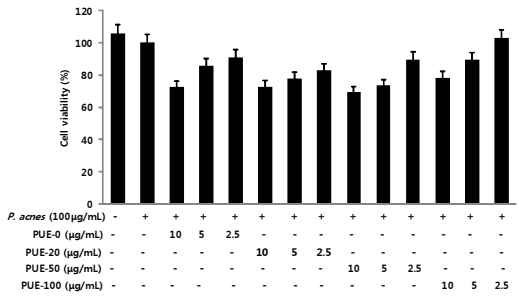


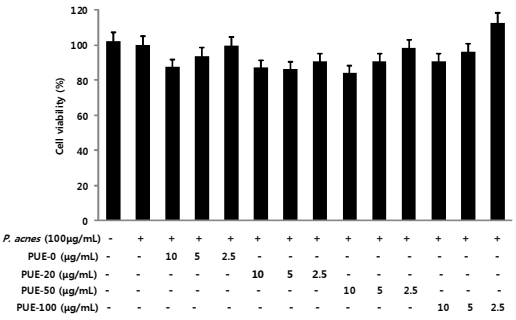
Cells (1.0×10^5 cells/mL) were stimulated by heat-killed *P. acnes* (100 μg/mL) for 48 h in the presence of PUE-0, PUE-20, PUE-50, and PUE-100. Supernatants were collected, and the concentration of IL-8 was determined by ELISA. Values are the mean ± SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$

Fig. 1. Inhibitory effects of PUE-0, PUE-20, PUE-50, and PUE-100 on IL-8 production in RAW 264.7 cells

value of 1.64 μg/mL. The cytotoxic effects of all PUEs were assessed in the presence or absence of *P. acnes* via an MTT assay (Fig. 2A). PUEs were only slightly cytotoxic for THP-1 cells at the concentrations (2.5, 5.0, and 10 μg/mL) employed to inhibit IL-8 production. However, no significance was observed. Thus, the inhibitory effects cannot be the results of PUE cytotoxic effects. Furthermore, we also examined the cytotoxic effects of PUEs in HaCaT human keratinocyte cells to assess PUE suitability for use as a therapeutic agent on the human skin. As shown in Figure 2B, cell viability remained above 80% following treatment with PUEs at concentrations up to the effective dose of 10 μg/mL. These data suggest that PUE presents a low cytotoxicity against mammalian cell lines.

Finally, rutin has been reported as an effective anti-inflammatory ingredient in *Phyllanthus sp*[27]. Thus, HPLC fingerprinting was





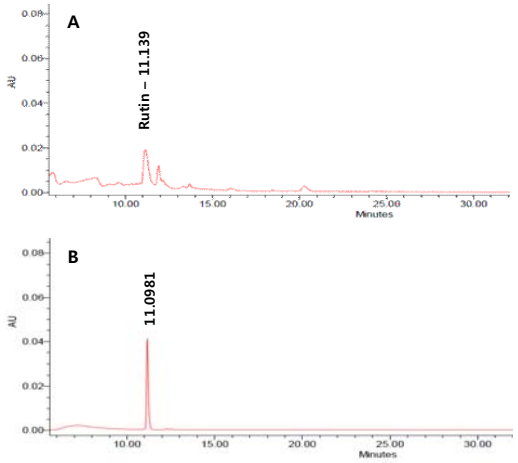
MTT assays were performed after incubation of THP1 and HaCaT cells with various concentrations of PUE-0, PUE-20, PUE-50, and PUE-100 (2.5, 5.0, 10 μg/mL) for 24 h at 37°C in 5% CO_2 atmosphere. MTT (1 mg/mL) was added to each well in a 1/10 volume of media. Cells were incubated at 37°C for 3 h, and DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm with a spectrophotometer.

Fig. 2. Cell viability of human monocytic THP-1 (A) and keratinocyte HaCaT (B) cells treated with PUE-0, PUE-20, PUE-50, and PUE-100

Table 1. Antimicrobial activity of *P. urinaria* extracts against drug-susceptible and -resistant *P. acnes*.

Strains	Drug-resistance) pattern (MIC; μg/mL	Paper Disc diffusion (mm) PUE-0	PUE-20	PUE-50	PUE-100
P. acnes CCARM 0081	Susceptible	14	11	14.5	12
P. acnes CCARM 9009	Clindamycin (64)	13	11	13.5	11
P. acnes CCARM 9010	Clindamycin (64)	12.5	11	13.5	11
P. acnes CCARM 9089	Clindamycin (128)	15	10	15	12

The inhibitory effect of *P. urinaria* on test bacteria was determined by the disk (ϕ 8.0 mm) diffusion method. *Propionibacterium* species were grown at 37°C for 48 h in media. The same volume of ethanol (20 μL) and erythromycin were used as controls.



The upper side (A) and lower side (B) represent PUE-0 (aqueous extract of *P. urinaria*) and standard rutin, respectively. The wavelength of rutin absorbance is 280 nm.

Fig. 3. HPLC fingerprinting analysis of PUE-0, PUE-20, PUE-50, and PUE-100

developed in this study to identify PUE ingredients. Rutin was used as the standard substance. Using the conditions described in the experimental section, rutin was well resolved from PUEs with excellent peak shapes. The rutin content in PUE-0, PUE-20, PUE-50, and PUE-100 was 5.2 mg/g, 1.5 mg/g, 0.7 mg/g, and 0.25 mg/g, respectively (Fig. 3). Taken together, our results suggest that PUEs are attractive acne-mitigating candidates for topical application.

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