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In vitro antimicrobial and anti-inflammatory effects of herbs against *Propionibacterium acnes*

Tsung-Hsien Tsai [a], Tzung-Hsun Tsai [b], Wen-Huey Wu [c], Jonathon Te-Peng Tseng [a], Po-Jung Tsai [c,*]

[a] *Department of Dermatology, Taipei Medical University–Wanfang Hospital, Taipei, Taiwan, ROC*
[b] *Department of Dentistry, Keelung Chang-Gung Memorial Hospital, Keelung, Taipei, Taiwan, ROC*
[c] *Department of Human Development and Family Studies, National Taiwan Normal University, Taipei 10610, Taiwan, ROC*

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ABSTRACT

Propionibacterium acnes play an important role in the pathogenesis of acne by inducing certain inflammatory mediators and comedogenesis. The objective of this study was to evaluate the antimicrobial and anti-inflammatory effects of herbal extracts against *P. acnes*. Among the ten tested herbs, methanolic extracts of rose (*Rosa damascene*), duzhong (*Eucommia ulmoides* Oliv.), and yerba mate (*Ilex paraguariensis*) were found to inhibit the growth of *P. acnes* with respective minimum inhibitory concentrations of 2, 0.5, and 1 mg/ml. In addition, duzhong and yerba mate extracts reduced the secretion of pro-inflammatory cytokines such as tumour necrosis factor-α, interleukin (IL)-8, and IL-1β by human monocytic THP-1 cells pretreated with heat-killed *P. acnes* at a concentration of 0.1 mg/ml. Our results suggested that duzhong and yerba mate extracts possess both antimicrobial and anti-inflammatory effects against *P. acnes* and can possibly be used as therapeutic agents for acne.

1. Introduction

Acne vulgaris is a common skin disease involving pilosebaceous follicles. The pathogenesis of acne vulgaris is multifactorial, including increased sebum production, comedogenesis, *Propionibacterium acnes* proliferation, and inflammation (Leyden, 2003). *P. acnes* play an important role not only in the process of inflammation but also in the formation of comedones. *P. acnes* contribute to the inflammatory nature of acne by inducing monocytes to secrete pro-inflammatory cytokines including interleukin (IL)-1β, IL-8, and tumour necrosis factor (TNF)-α (Kim, 2005). The major classes of therapeutic agents are topical and systemic retinoids, antimicrobial agents, and systemic hormonal drugs. Bacterial resistance is an ongoing problem in the treatment of acne vulgaris. Recently, new retinoids with additional anti-inflammatory action are being co-administered with antibiotics to reduce the risk of bacterial resistance (Leyden, 2003). Therefore, an agent which can inhibit *P. acnes* growth and suppress the inflammatory response will provide promising benefits to patients with acne vulgaris.

Herbs have been used for many purposes, including medication, nutrition, flavouring, beverages, and fragrance. Much of the early interest in functional foods and nutraceuticals was based on the medicinal uses of herbs. Herbal tea products commonly consumed in Taiwanese daily life are commonly applied in folk medicine or traditional Chinese medicine, like honeysuckle (*Lonicera japonica*), juhua (*Chrysanthemum morifolium*), duzhong (*Eucommia ulmoides* Oliv.), and jiaogulan (*Gynostemma pentaphyllum*). Honeysuckle and juhua are two very popular herbal teas during the summertime. Honeysuckle possesses a wide range of antibacterial properties including action against *Staphylococcus aureus*, streptococci, *Bacillus dysenterii*, and *Salmonella typhi*. In addition, honeysuckle shows hepatoprotective effects against CCl_4-induced hepatic injury (Huang, 1998). Juhua is widely used as a remedy for the common cold, headaches, and hypertension (Huang, 1998). The duzhong extract has antihypertensive, antioxidative, and antigastric ulcer effects, and promotes collagen synthesis (Takeshi, Sanse, & Yoshihisa, 2001). Jiaogulan has been found to have many pharmacological effects, such as hypoglycemic (Norberg et al., 2004), hypolipidemic (Cour, Molgaard, & Yi, 1995), and antiallergic (Huang et al., 2008) functions.

Scented teas are popular not only in Western countries but also in Taiwan, like jasmine (*Jasminum sambac*), lavender (*Lavandula angustifolia*, formerly *Lavandula officinalis*), osmanthus (*Osmanthus fragrans* Lour), and rose (*Rosa damascene*). They are usually used alone or mixed with other herbal teas to provide fragrance and a pleasant taste. In addition to the role of fragrance or pleasant smells, they are thought to be functional for human health. For example, lavender is approved for balneotherapy for circulatory disorders. Lavender oil inhibits mast cell degranulation and has

* Corresponding author. Address: Department of Human Development and Family Studies, National Taiwan Normal University, 162 Hoping E. Rd., Sec. 1, Taipei 10610, Taiwan, ROC. Tel.: +886 2 23636425x55; fax: +886 2 23639635.
E-mail address: pjtsai@ntnu.edu.tw (P.-J. Tsai).

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antimicrobial and antiphlogistic effects (Thornfeldt, 2006). Furthermore, lemongrass (*Cymbopogon citrates*; Cheel, Theoduloz, Rodrigues, & Schmeda-Hirschmann, 2005) and yerba mate (*Ilex paraguariensis*; Gugliucci, 1996) exhibit antioxidant activities and are becoming more popular in Taiwan.

Our previous study showed that herbal extracts from jasmine, jiaogulan, lemongrass, honeysuckle, duzhong, and yerba mate possess antimicrobial activity against cariogenic *Streptococcus sanguinis* (Tsai, Tsai, Chien, Lee, & Tsai, 2008). However, until now, their antimicrobial activities against *P. acnes* have not been reported. Moreover, aqueous extracts of lavender, sweet osmanthus, lemongrass, rose, and juhua showed anti-inflammatory effects which were attributed to the moderate inhibitory activity of nitric oxide production in LPS-stimulated Raw 264.7 macrophages (Tsai, Tsai, Yu, & Ho, 2007). Since the *P. acnes*-mediated inflammatory response and comedogenesis are known to be involved in the pathogenesis of acne vulgaris, this spurred our interest in examining possible anti-inflammatory effects of herbal extracts on the inflammatory reaction specifically incited by *P. acnes*. In the present study, the ability of ten herbal extracts to inhibit the growth of *P. acnes* was examined, and the anti-inflammatory effects of these herbal extracts were further evaluated.

2. Materials and methods

2.1. Materials

The strain of *P. acnes* (BCRC10723, isolated from facial acne) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). *P. acnes* were cultured in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) with 1% glucose. The bacteria were cultured in an anaerobic atmosphere using BBL GasPak systems (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA).

The human monocytic THP-1 cell line (BCRC 60430) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% heated-inactivated fetal bovine serum (FBS, Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml). The human fibroblast Hs68 cell line (BCRC 60038) was obtained from the Bioresource Collection and Research Center. The immortalised human HaCaT keratinocyte cell line was developed by Boukamp et al. (1988). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heated-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). These cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

The assay kits for TNFα, IL-1β, and monocyte chemoattractant protein (MCP)-1 were purchased from eBioscience (San Diego, CA, USA). The IL-8 assay kit was purchased from R&D (Minneapolis, MN, USA). All chemicals were of analytical-grade purity.

2.2. Preparation of extracts

Ten dried herbs including juhua, honeysuckle, jasmine, lavender, rose, osmanthus, duzhong, jiaogulan, lemongrass, and yerba mate were purchased from a local supermarket in Taipei, Taiwan. The dried herbs were ground up and then extracted with methanol according to our previous study (Tsai et al., 2008). Briefly, 10 g of each dried herb was extracted with 50 ml of methanol at room temperature for 3 h. After extraction, the mixture was filtered, and the residue was re-extracted with 50 ml of fresh methanol overnight. The combined methanolic solution was centrifuged at 12,000*g* for 10 min and evaporated on a rotary evaporator. The methanolic extract was reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 400 mg/ml for the subsequent experiments.

2.3. Determination of antimicrobial activity

The herbal extracts were tested against *P. acnes* by determining the minimum inhibitory concentration (MIC) values obtained by a microdilution broth method as previously described (Tsai et al., 2008). Briefly, *P. acnes* was incubated in BHI broth with 1% glucose for 72 h under anaerobic conditions and adjusted to yield approximately 1×10^8 colony-forming units (CFU)/ml. In sterile 96-well microtiter plates, 100 μl of a plant extract was diluted with broth and added to wells containing 100 μl of the bacterial suspension in broth. Twofold serial dilutions were made in broth over a range to give concentrations of 0.06–8 mg/ml of the methanolic extracts. To adjust the interference by plant pigments, a parallel series of mixtures with un-inoculated broth was prepared. Triplicate samples were performed for each test concentration. After incubation for 72 h at 37 °C under an anaerobic condition, microbial growth was determined by absorbance at 600 nm using a microplate reader (Biotek Instruments, Winooski, VT, USA). The MIC was defined as the lowest concentration of a test compound which inhibited the growth of *P. acnes*. The experiments were performed in triplicate.

2.4. Preparation of heat-killed P. acnes

P. acnes were cultured in BHI broth with 1% glucose for 72 h at 37 °C under an anaerobic condition. The log-phase bacterial culture was harvested, washed three times with PBS, and incubated at 80 °C for 30 min to kill the bacteria. The heat-killed *P. acnes* were stored at 4 °C until use.

2.5. Measurement of cytokine production in human monocytic cells

Human monocytic THP-1 cells were seeded at 1×10^6 cells/ml in 24-well plates with serum-free medium, and were stimulated with heat-killed *P. acnes* (wet weight 100 μg/ml) alone or in combination with different concentrations (0.01, 0.05, and 0.1 mg/ml) of herbal extracts for an 18-h incubation. Cell-free supernatants were collected, and concentrations of MCP-1, TNFα, IL-1β, and IL-8 were analysed with respective enzyme immunoassay kits.

2.6. In vitro cytotoxicity assay

HaCaT and HS68 cells were cultured in DMEM containing 10% FBS and penicillin–streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HaCaT (1×10^5 cells/ml) and HS68 (1×10^4 cells/ml) were seeded on 96-well plates, and extract treatment began 24 h after seeding. Cell viability was evaluated by conventional 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assays. After a 24-h incubation with various concentrations of herbal extracts, 0.1 ml of MTT (5 μg/ml) was added to each well. After 2 h of incubation, the supernatant was removed, and the precipitate was dissolved in 100 ml of acidic isopropanol. The optical density (OD) of the resulting solution was measured spectrophotometrically at 540 nm. The concentration which reduced the cell viability by 50% (TC_{50}) for each extract was calculated from fitted dose–response curves. The experiments were performed in triplicate.

2.7. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using the SPSS 13.0 statistical package (Chicago, IL, USA). The Mann–Whitney *U*-test was used to

compare differences between the DMSO vehicle and herbal treatments. A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Antimicrobial activity of herbal extracts against P. acnes

We tested the antimicrobial activities of ten herbal extracts against *P. acnes* (Table 1). Extracts of rose, duzhong, and yerba mate exhibited notable antimicrobial activity against *P. acnes*. The duzhong extract showed the greatest antimicrobial activity against *P. acnes* with an MIC of 0.5 mg/ml. The yerba mate extract showed moderate antibacterial activity against *P. acnes* at an MIC of 1 mg/ml. The rose extract was less effective against *P. acnes* with an MIC of 2 mg/ml. *In vitro* antimicrobial activities of essential oils, medicinal plants, and chemicals against *P. acnes* have been reported (Chomnawang, Surassmo, Nukoolkarn, & Gritsanapan, 2005; Docherty, McEwen, Sweet, Bailey, & Booth, 2007; Kim, Kim, Lee, & Hyun, 2008; Kim et al., 2008). However, a limited number of studies have been performed to assess the anti-*P. acnes* activities of herbal tea extracts. The essential oil of rosemary (*Rosmarinus officinalis* L.) had antibacterial activity against *P. acnes* with an MIC value of 0.56 mg/ml (Fu et al., 2007). Guava (*Psidium guajava*) and walnut (*Juglans regia*) leaf extracts also showed anti-*P. acnes* activity (Qadan et al., 2005). Anti-*P. acnes* activities of extracts from rose, duzhong, and yerba mate were demonstrated herein. However, the beneficial effect of consuming these herbal extracts in the treatment of *P. acnes* infection remains to be evaluated in further studies.

3.2. Anti-inflammatory activities of the herbal extracts

We describe here for the first time the antimicrobial activities of extracts of rose, duzhong, and yerba mate against *P. acnes*. To further ascertain whether these selected extracts possess biological properties against inflammatory acne, subsequent experiments were conducted to determine their inhibitory effects on the pro-inflammatory mediator secretion in co-culture of THP-1 cells with heat-killed *P. acnes*.

P. acnes contribute to the inflammatory nature of acne by inducing monocytes to secrete pro-inflammatory cytokines including TNFα, IL-1β, and IL-8 (Vowels, Yang, & Leyden, 1995). MCP-1 is associated with modulating monocyte migration in response to inflammation (Graves & Jiang, 1995). Therefore, to investigate the anti-inflammatory potential of these herbal extracts, we performed an ELISA for TNFα, IL-1β, IL-8, and MCP-1 in supernatants of heat-killed *P. acnes*-stimulated THP-1 monocytes. To account for any reduction in pro-inflammatory cytokines resulting from cytotoxic effects of the extracts, the cytotoxicity induced by these extracts was determined by MTT assays in THP-1 cells. Methanolic extracts of rose, duzhong, and yerba mate had low cytotoxic effects at a concentration of 0.1 mg/ml (data not shown). In addition, following an 18-h incubation period, methanolic extracts from rose, duzhong, and yerba mate (up to 0.2 mg/ml) did not increase the secretion of either TNF-α, IL-8, IL-1β, or MCP-1 by THP-1 cells in the absence of heat-killed *P. acnes* (data not shown).

As shown in Figs. 1 and 2, THP-1 cells treated with heat-killed *P. acnes* showed increases in TNFα, IL-1β, IL-8, and MCP-1 secretion. These results confirmed that *P. acnes* can stimulate pro-inflammatory mediators and also that it plays an important role in the pathogenesis of inflammatory acne. All three tested extracts suppressed the secretion of TNFα, one of the most important pro-inflammatory cytokines, in dose-dependent manners (Fig. 1A). Furthermore, duzhong and yerba mate extracts also inhibited IL-1β (Fig. 1B) and IL-8 (Fig. 2A) secretion. The duzhong extract also decreased MCP-1 production, while neither rose nor yerba mate had an effect on MCP-1 release (Fig. 2B). It is worth noting that rose extract inhibited TNFα (Fig. 1A), but increased IL-1β (Fig. 1B) and IL-8 (Fig. 2A), secretion. In acne, the host response to *P. acnes* can result in the production of pro-inflammatory cytokines and contribute to the clinical manifestations of the disease. TNFα is a pleiotropic cytokine produced by activated macrophages. IL-8 along with other *P. acnes*-induced chemotactic factors may play important roles in attracting neutrophils to the pilosebaceous unit (Kim et al., 2002). Due to the functional redundancy and pleiotropic effects of inflammatory mediators, it is difficult to identify a single molecule as the best candidate for anti-inflammatory therapeutics. It is a better strategy to inactivate a range of inflammatory mediators not just a single cytokine

Table 1
Minimal inhibition concentrations (MICs) of the methanolic extracts of herbs against *Propionibacterium acnes*.

Common name	Botanical name	Part examined	MIC (mg/ml)
Honeysuckle	*Lonicera japonica*	Flowers	>4
Jasmine	*Jasminum sambac*	Flowers	>4
Juhua	*Chrysanthemum morifolium*	Flowers	>4
Lavender	*Lavandula officinalis*	Flowers	>4
Osmanthus	*Osmanthus fragrans Lour*	Flowers	>4
Rose	*Rosa damascene*	Flowers	2
Duzhong	*Eucommia ulmoides* Oliv.	Leaves	0.5
Jiaogulan	*Gynostemma pentaphyllum*	Leaves	>4
Lemongrass	*Cymbopogon citrates*	Leaves	>4
Yerba mate	*Ilex paraguariensis*	Leaves	1

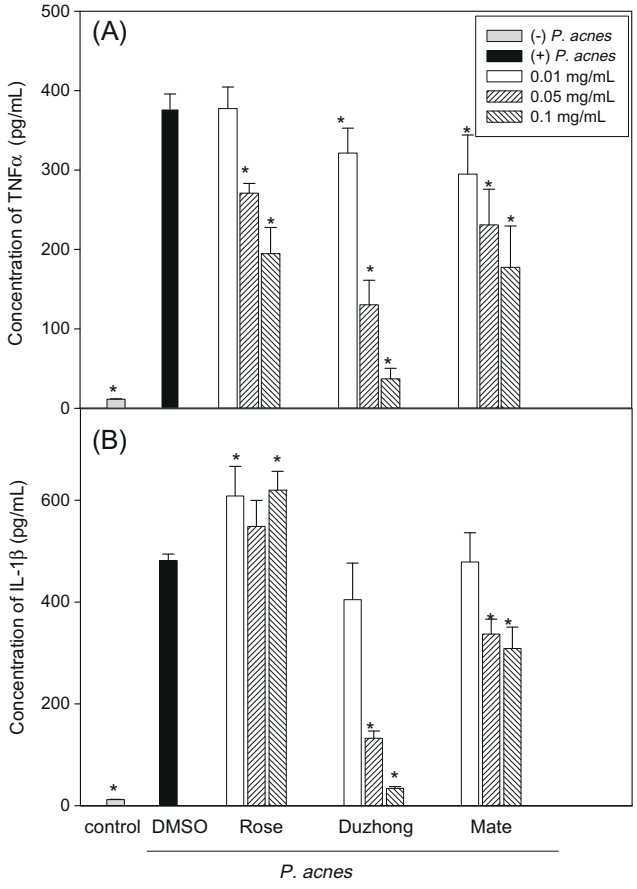

Fig. 1. Effects of herbal extracts on the production of pro-inflammatory cytokines such as TNFα (A) and IL-1β (B) in heat-killed *Propionibacterium acnes*-treated THP-1 cells. Data are expressed as the mean ± SD. *$p < 0.05$ compared to the DMSO vehicle; (−) control, no treatment of heat-killed *P. acnes*.

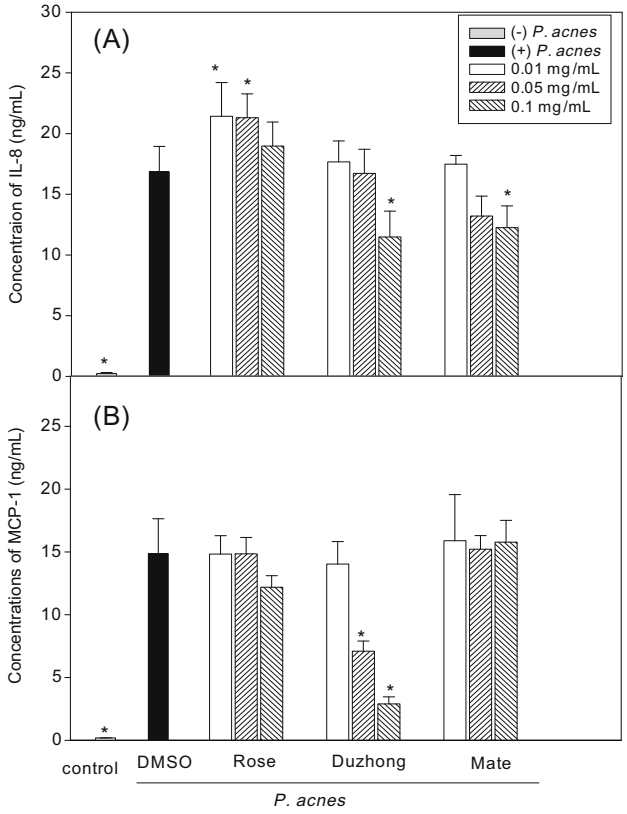

Fig. 2. Effects of herbal extracts on the production of pro-inflammatory cytokines such as IL-8 (A) and MCP-1 (B) in heat-killed *Propionibacterium acnes*-treated THP-1 cells. Data are expressed as the mean ± SD. $^{*}p < 0.05$ compared to the DMSO vehicle; (−) control, no treatment of heat-killed *P. acnes*.

through blocking activation of common transcription factors such as NF-κB involved in their induction. Thus our data suggest that duzhong and yerba mate exhibited anti-inflammatory properties and may be regarded as health-benefiting substances.

Duzhong leaves contain many phytochemicals, such as polyphenolics, flavonoids, and triterpines (Kawasaki, Uezono, & Nakazawa, 2000; Tsai et al., 2008). Aucubin isolated from duzhong showed a protective effect in preventing ultraviolet B (UVB)-induced oxidative stress (Ho et al., 2005a) and had an inhibitory effect on matrix metalloproteinase (MMP)-1 production in UVB-irradiated human fibroblasts (Ho et al., 2005b). The methanol extract of duzhong leaves also stimulated collagen synthesis in an aged-rat model (Li et al., 1998). Natural plant extracts are often added to skin care and cosmetic products. Therefore, duzhong extract or its component might be considered potential agents to use in anti-acne, anti-ageing, and skin care formulae.

Yerba mate tea reduced lipid peroxidation and TNFα in mice exposed to cigarette smoke and is considered a potential anti-inflammatory and nutritional resource against cigarette smoke-induced inflammation (Lanzetti et al., 2008). Yerba mate tea-derived compounds inhibit proteasome activity, suggesting that it may be used in psoriasis and inflammatory disorders (Arbiser et al., 2005). Although an anti-inflammatory property of yerba mate against *P. acnes* was identified, its dermatologic benefits still need to be investigated.

Even though we identified antibacterial and anti-inflammatory effects of duzhong and yerba mate extracts against *P. acnes*, we did not determine their mechanisms of action. *P. acnes* lipase is recognised as one of the major factors in the pathogenesis of acne, being responsible for the hydrolysis of sebum and release of inflammatory compounds (Higaki, 2003). In addition, *P. acnes* has three separate clusters of genes that encode enzymes involved in extracellular polysaccharide biosynthesis, suggesting that it can form an extracellular bioflim matrix (Brüggemann et al., 2004). New data are needed to clarify the antimicrobial and anti-inflammatory properties found in this study by analysing the effects of these selected herbal extracts on bacterial morphology, bacterial membrane integrity, biofilm formation, and lipase activity of *P. acnes*.

3.3. In vitro cytotoxicity of herbal extracts using human skin cells

Due to the possibility of introducing herbal ingredients as topical agents for acne, the cytotoxicity of these selected extracts was examined in human skin keratinocytes and fibroblasts using *in vitro* MTT assays (Fig. 3). The respective TC_{50} values of rose, duzhong, and mate against HaCaT keratinocytes were 2.54, 1.74, and 0.82 mg/ml. The respective TC_{50} values of rose, duzhong, and yerba mate against HS68 fibroblasts were >2.5, 2.49, and 0.80 mg/ml. Rose extracts showed the relatively lowest cytotoxic effects on both skin cells compared to the other tested compounds. Cell viability was significantly reduced after exposure of both cell lines to the yerba mate extract at a concentration of 1 mg/ml. Although the two cell types responded similarly, HaCaT keratinocytes were more sensitive than HS68 fibroblasts to these compounds. Toxicity data reported by this study can potentially be used to assess the human topical risk exposure to these selected herbal extracts. Further studies are needed to clarify the risk of these materials as well as the suitability of their applications for human use.

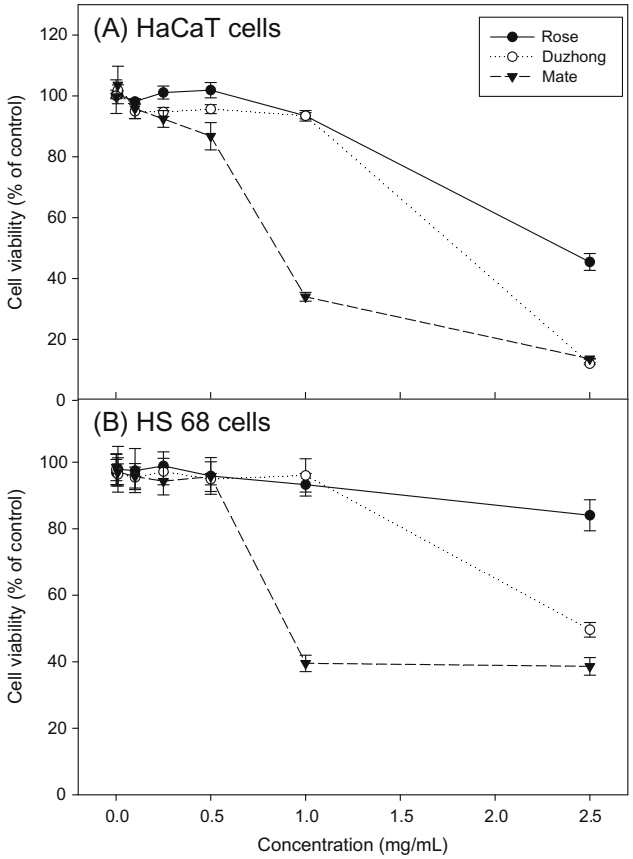

Fig. 3. *In vitro* cytotoxicity of herbal extracts against human HaCaT keratinocytes (A) and HS68 fibroblasts (B). HaCaT or HS68 cells were cultured for 24 h in medium with the indicated contractions of herbal extracts. The cellular cytotoxicity was determined according to an MTT assay, and results are expressed as the mean ± SD from three independent experiments.

4. Conclusions

The above results suggest that duzhong and yerba mate may be useful in the treatment of acne vulgaris. Although the antibacterial and anti-inflammatory effects of duzhong and yerba mate extracts against *P. acnes* were demonstrated, their mechanism remains unknown. Further study is needed to clarify the active constituents and their possible inhibitory mechanisms against pro-inflammatory cytokines. Cosmeceuticals and nutraceuticals are growing areas of interest and controversy. Developing new active botanical extracts and compounds to provide dietary supplements and cosmetics as anti-acne agents is still a field with great potential.

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