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Research article

Biological activities supporting development of cosmeceutical skin products from *Wedelia trilobata* (L.) Hitch. flower extract

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Abstract

Importance of the work: This is the first report to demonstrate the potential of *Wedelia trilobata* (L.) Hitch. as an ingredient in cosmeceutical products.

Objectives: To examine the biological activities associated with cosmeceutical products of *W. trilobata* extract.

Materials & Methods: The flowers (WTF), leaves (WTL) and stems (WTS) of *W. trilobata* were separately extracted using 50% ethanol and freeze-drying to obtain WTF, WTL and WTS extracts, respectively. Then, the antioxidant, anti-tyrosinase, antimicrobial, cytoprotective and anti-inflammatory activities of the extracts were investigated.

Results: Screening tests, using the three extracts of the plant showed different positive effects, with WTF having the highest antioxidant, tyrosinase and nitric oxide inhibitory activities. Therefore, WTF was further studied to find the mechanisms involved in anti-inflammatory, antimicrobial and cytoprotective effects. WTF at concentrations of 50–200 µg/mL down-regulated the expression of *cyclooxygenase-2 (COX-2), interleukin-1β* (*IL-1β*) and *tumor necrosis factor-α* (*TNF-α*) genes in macrophages using a semi-quantitative reverse-transcriptase polymerase chain reaction technique and the production of tumor necrosis factor-**α** in keratinocytes. The antimicrobial activity of WTF was demonstrated on many bacteria and *Candida albicans*. WTF prevented H_2O_2-induced cell death of human keratinocyte via down-regulation on the expression of matrix metalloproteinase-1 (*MMP-1*) and *MMP-2* genes and up-regulation on the expression of *procollagen type 1* genes. The gel formulations containing WTF were developed with good stability and inhibited the growth of *Propionibacterium acnes* at statistically significant levels.

Main finding: *W. trilobata* flowers demonstrated a potential source of an active ingredient in skin product development. The plant may have more value-added components for application in cosmeceuticals as well as being useful as a ground cover weed.

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Introduction

Nowadays, there is demand for cosmeceutical products from natural origins that are non-invasive, highly efficacious and cost effective (Saad et al., 2021). Medicinal plants have various biological effects. Antioxidant activity is the first to be mentioned for scavenging free radicals that are harmful to the cells, causing DNA damage and cell membrane destruction, and resulting in cell degeneration or aging (Mishra et al., 2014). Reactive oxygen species (ROS) are produced under various conditions, such as during inflammatory process and as a major second messenger of metabolic oxidative stress (Zuliani et al., 2005). In cells, ROS, including hydrogen peroxide, superoxide and the hydroxyl radical, are manufactured as by-products of mitochondrial respiration (Zuliani et al., 2005). Among a great variety of ROS, hydrogen peroxide (H_2O_2) plays an essential role because it is generated from nearly all sources of oxidative stress and oxygen radicals and has the ability to modulate various signal transduction pathways (Zuliani et al., 2005). In addition, increased free radical levels cause the signal to spread, thus stimulating mitogen-activated protein kinases (MAPKs) which result in activator protein 1 (AP-1) activation and then stimulation of matrix metalloproteinase (MMP) gene expression and inhibition of procollagen type I (procollagen-1) synthesis (Fisher et al., 2002). MMPs have an important role in extracellular matrix degradation. For example, MMP-1, a major metalloproteinase for collagen degradation, instigates degradation of type I and type III fibrillar collagens in the dermis, which are then diminished more by MMP-2 (Quan et al., 2009). Furthermore, MMP-2 potentially degrades the elastic fibers (Berton et al., 2000). Various types of activity by these enzymes are able to completely degrade skin collagen and the elastic network, which leads to wrinkle formation (Kolakul and Sripanidkulchai, 2018). In adolescence, acne is another important facial skin problem, where a major cause is a bacterium, *Propionibacterium acnes*, which is found in the hair follicle or skin sebaceous glands and is the leading cause of acne vulgaris (Hamdy et al., 2017). *P. acnes* can stimulate the secretion of proinflammatory cytokine and induce inflammation in the sebaceous glands and cause ulcers (Hamdy et al., 2017). The inflammatory factors are strongly associated with MMPs. Interleukin-1β (IL-1β) treatment up-regulated secretion of MMP-2 in endometrial fibrosis tissues (Szóstek-Mioduchowska et al., 2019). Tumor necrosis factor-α (TNF-α) augmented collagenolytic activity of MMP-1 through up-regulation of MMP-3 leading to a gradual loss of type I collagen in humans (Ågren et al., 2015).

Wedelia trilobata (L.) Hitch. (*Sphagneticola trilobata* (L.) Pruski), a plant in the family Asteraceae, is native to South America and was later distributed to Myanmar, Laos, Cambodia, Vietnam and Thailand (Ren et al., 2015). It is widely used as a decorative ground cover weed in the gardens (Xuesong et al., 2006). This plant has been used as a traditional herbal medicine for the treatment of backaches, muscle cramps, rheumatism, sores, stubborn wounds, arthritic pain and swelling in the Caribbean and Central America (Ren et al., 2015). In addition, it has been used for the treatment of malaria and fever in Vietnam (Ren et al., 2015). Several other pharmacological activities of *W. trilobata* have been reported, including antioxidant, analgesic, anti-inflammatory, antimicrobial, wound healing, uterine contraction, antitumor, antidiabetic and menstrual pain relief (Balekar et al., 2014). In addition, phytochemical studies have identified some diverse chemicals from this plant, including terpenoids, steroids, flavonoids and phenolics (Balekar et al., 2014; Ren et al., 2017). Many reports did not separate the flower, leaf and stem parts and instead just used the above-ground part or did not use the flower part (Balekar et al., 2014). However, some reports mentioned that sesquiterpene lactones, trilobolide-6-O-isobutyrates A and B, and eudesmanolides were found in *W. trilobata* flowers (Xuesong et al., 2006; Sun et al., 2020). Also important is that non-polar solutions were used as solvents such as hexane, ethyl acetate, chloroform, and methanol. These solvents, if not completely eliminated, will contaminate the finished product, potentially having undesirable side effects (Opuni et al., 2021).

The current research aimed to promote and increase confidence in the use of *W. trilobata* in cosmeceutical products, by examining the its biological activity of this plant, including its antioxidant, anti-tyrosinase, antibacterial, anti-inflammatory and cytoprotection effects. This research focused on using non-toxic solvents and investigating mechanisms of action at the gene and protein levels.

Materials and Methods

Plant material and extraction

Samples of *Wedelia trilobata* flowers (diameter 2.5 ± 0.12 cm), leaves (length 5.8 ± 0.17 cm) and stems (diameter 0.33 ± 0.05 cm) were collected from Ubon Ratchathani, Thailand during October–November, 2020 and the plant materials were air-dried at 55 °C for 24 h before being pulverized in

an electric grinder. The raw materials were identified and authenticated according to the Flora of Thailand guideline (Volume 13, Part 2). The samples were prepared by maceration with 50% ethanol for 5 d and then were filtered through a thin cloth and centrifuged at 3,000×*g* and 25 °C for 10 min. The clear supernatant was evaporated at 50°C and freeze-dried. The ethanolic extract of flowers (WTF), leaves (WTL) and stems (WTS) were obtained with yields of 18.38%, 9.33% and 8.04%, respectively.

Cell culture

Human keratinocytes (HaCaT)

HaCaT cells were purchased from CLS-cell lines service (Germany) and preserved in a complete medium comprised of Dulbecco's modified eagle medium (DMEM), 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 4.5 g/L glucose, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Murine macrophages (RAW264.7)

The RAW264.7 cells were obtained from PromoCell (Germany). The cells were cultured in DMEM media with 10% calf serum (HyClone; USA) and penicillin (100 U/mL)-streptomycin (100 µg/mL) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Cytotoxicity test

The cells were subjected to different concentrations of extracts and then incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h. MTT assay was used to determine cell survival (Mosmann, 1983) and 570 nm was the recorded absorbance. The outcomes were computed for %cell viability [(Absorbance $_{\text{sample-treated cells}}$ / Absorbance $_{\text{untreated cells}}$) × 100] and shown as the 50% inhibitory concentration (IC_{50}) which was calculated from a linear equation curve plotted between %cell viability and the extract concentration, The IC_{50} value was the extract concentration at which one-half of the cells survived (n = 3).

Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl assay

Anti-oxidant activity was determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (Shimada et al., 1992). Briefly, the extract was diluted and mixed with 1 mM DPPH. After 30 min of incubation, absorbance was measured at 540 nm. The inhibition of radical scavenging was calculated and expressed as the 50% effective concentration (EC_{50}). Ascorbic acid was used as a positive compound

2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) assay

The 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) or ABTS was transformed into free radicals (ABTS +•) by oxidation with potassium persulfate ($K_2S_2O_8$) via dissolving ABTS and $K_2S_2O_8$ in 1 mL of water and leaving it in the dark for 12–16 h at room temperature. After that, the ABTS +• solution was diluted and mixed with the extract; then; absorbance was measured at 734 nm (Hasperué et al., 2016). Trolox was used as a positive compound.

Phenolic content

The total phenolics content was measured using the Folin-Ciocalteu method (Sripanidkulchai and Fangkrathok, 2014). The total phenolic contents were expressed as milligrams gallic acid equivalent (GAE)/g dry basis.

Tyrosinase inhibition assay

The tyrosinase inhibitory effect was determined using an enzymatic test (Momtaz et al., 2008). Briefly, the extract was dissolved in phosphate buffer (pH 6.5). L-tyrosine (substrate) and mushroom tyrosinase were mixed and incubated for 30 min. The absorbance was measured at 492 nm. The inhibition on tyrosinase activity was calculated as the half maximal inhibitory concentration (EC_{50}) value. Kojic acid was used as a positive control.

Anti-inflammatory activity

Nitric oxide production

The nitric oxide (NO) assay was performed according to Yang et al. (2009) with slight modifications. After pre-incubation of RAW264.7 cells (1.5×10^5 cells/mL) with lipopolysaccharides (LPS) at 50 ng/mL for 24 h, the quantity of nitrite (a stable metabolite of NO) was measured. The culture medium was mixed with Griess reagent. The absorbance of the mixture was measured at 540 nm. The NO inhibition was calculated as a percentage and aminoguanidine was used as a positive control.

Inflammatory-related gene expression

The RAW264.7 cells (X,X×10^5 cells/well) were cultured overnight in a 12-well plate and subjected to different concentrations of extract and the positive control. Following incubation at 37 °C in the humidified atmosphere with 5% CO_2 for 24 h, the LPS was added, and the complete sample was further incubated for 24 h. Total RNA was isolated from the treated cells using a GE Healthcare extraction kit (United Kingdom). First-strand cDNA was reverse-transcribed and reverse transcription polymerase chain reaction (RT-PCR) was carried out (Won et al., 2006; Sripanidkulchai et al., 2009). The primer sequences were as follows: *β-actin*, 5'-TCATGAAGTGTGACGTTGACATCCGT-3' (forward) and 5'-CCTAGAAGCATTTGCGGTGCACG ATG-3' (reverse); *COX-2*, 5'-GGAGAGACTATCAAGATAGT-3' (forward) and 5'-ATGGTC AGTAGACTTTTACA-3' (reverse); *IL-1β*, 5'-CAGGATGAGGACATGAGCACC-3' (forward) and 5'-CTCTGCAGACTCAAACTCCAC-3' (reverse); *TNF-α*; and 5'-ATGAGCACAGAAAGCATGATC-3' (forward) and 5'-TACAGGCTTGTCACTCGAATT-3' (reverse). After cDNA synthesis, RT-PCR was performed in a 25 µL reaction mixture composed of 100 ng of the first strand cDNA as the template with 0.20 µM of each primer and 3 mM of MgCl2 for 30 cycles. The thermal profiles were pre-denatured at 94 °C for 3 min followed by denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s. The final extension was carried out at 72 °C for 7 min. Then, the PCR products were examined on 1.5% agarose gel, visualized based on Novel Juice staining; a Gel Documentation and System Analysis machine (Syngene; United Kingdom) was used to assess the RT-PCR product densities. The inflammatory-related gene expressions, consisting of *cyclooxygenase-2 (COX-2), interleukin-1β* (*IL-1β*) and *tumor necrosis factor-α* (*TNF-α*), were measured for the relative mRNA expression level (intensity of targets/intensity of *β-actin*) and statistically tested.

*Tumor necrosis factor-**α** production*

The tumor necrosis factor-α (TNF-α) levels in human keratinocytes (HaCaT cells) were measured using an immunoassay (Human TNF-α ELISA kit, Cat.No. BMS223-4; Invitrogen; Austria) as stated in the manufacturer's specifications. The cells (1.5×10^5 cells/well) were cultured overnight in a 12-well plate and subjected to different concentrations of extract and a positive control. Following incubation at 37 °C in the humidified atmosphere with 5% CO_2 for 24 h, the LPS (50 ng/mL) was added after which the complete sample was incubated for 24 h. Cell culture supernatants were removed using centrifugation. Samples (each 100 µL) of each supernatant were placed in the wells. After that, 50 µL of TNF-**α** conjugate was added into every well and incubated at room temperature for 2 h on a shaker. Wash buffer was added into and removed four times. Then, the remaining wash buffer was removed out via aspirating, inverting the plate and blotting it on clean paper towels. Then, 100 µL of substrate solution was added and the sample was incubated for 1 h. Next optical density of 450 nm was calculated using a microplate reader (Shimadzu, Japan). The sample absorbance was calculated by comparison with a TNF-**α** standard curve.

Anti-microbial activity

The bacteria used in this study were four Gram-positive strains (*Staphylococcus aureus* TISTR 1466, *S. epidermidis* TISTR 518*, Bacillus subtilis* TISTR 008, *Propionibacterium acnes*) and three Gram-negative strains (*Escherichia coli* TISTR 780*, Salmonella enterica* TISTR 1529, *Pseudomonas aeroginosa* TISTR 781). These bacteria were grown in Mueller Hinton broth. In addition, *Candida albicans*, an opportunistic pathogenic yeast, was grown in Sabouraud dextrose broth. Microbial strains were grown in the broth at 37 °C for 18–20 h and diluted to 1:100 in a normal saline solution of 1×10^6 colony forming units (cfu)/mL. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and inhibition zone were determined. Clindamycin was used as the reference antibiotic. The antibiotic sensitivity of the WTF gel was determined using the disc diffusion method. The diameter of the inhibition zone was measured in millimeters and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2011). The antibiotic discs used were ampicillin (1 µg), amphotericin B (1 µg) and clindamycin (2 µg), according to Chuangchot et al. (2017).

Cytoprotective effect

HaCaT cells (2 × 10^5 cells/well) were cultured, seeded on a 96-well plate and incubated for 24 h. After that, the cells were subjected to various concentrations of WTF and 400 µM H_2O_2 for 24 h at 37 °C with 5% CO_2. MTT assay was used to determine the extract's cytoprotective effect against H_2O_2 and shown as %cytoprotection compared to the untreated cells and H_2O_2-treated cells (Kolakul and Sripanidkulchai, 2018).

Expression of MMP-1, MMP-2 and procollagen-1 genes

Human HaCaT keratinocyte cells (1.5×10^5 cells/well) were seeded in a 12-well plate and grown to 80% confluence. These cells were subjected to various concentrations of WTF and 400 µM H_2O_2 for 24 h at 37 °C with 5% CO_2. The expressions of the *MMP-1*, *MMP-2* and *procollagen-1* genes were evaluated using a semi-quantitative RT-PCR technique with slight modifications from Kolakul and Sripanidkulchai (2018). Briefly, after cell harvesting and RNA isolation, total RNA (40 ng) was reverse-transcribed and RT-PCR was performed using an RT-PCR two-step kit (Qiagen, Germany). The PCR profiles were pre-denatured at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s. The final extension was carried out at 72 °C for 7 min. The primer sequences were: *β-actin*, 5'- GGCACCACACCTTCTACAATGAG-3' (forward) and 5'-CGTCATACTCCTGCTTGCTGATC-3' (reverse); *MMP-1*, 5'-ATTCTACTGATATCGGGGCTTTGA-3' (forward) and 5'-ATGTCCTTG GGGTATCCGTGTAG-3' (backward); *MMP-2*, 5'-ACTGAGTGGCCGTGTTTGC-3' (forward) and 5'-CGCTTCTGGCTGGGTCTGT-3' (backward reverse); and *procollagen-1*, 5'-CTCGAGGTGGACACCACCCT-3' (forward) and 5'-CAGCTGGATGGCCACATCGG-3' (reverse). The PCR products were examined on 1.5% agarose gel, visualized based on Novel Juice staining; a Gel Documentation and System Analysis machine was used to assess the RT-PCR product densities. The gene expressions were measured for the relative mRNA expression level to compare with *β-actin*.

Development of prototype cosmeceutical product

The WTF gels were prepared with concentrations of 0.25% (F1), 0.5% (F2), 1% (F3) and 5% (F4). Hydroxyethyl cellulose was used as a gelling agent. Then, the stability of the gel prepared under accelerated conditions was tested using the heating/cooling method by storing the gel at 4 °C and 45 °C every 48 h for six cycles. Then, the color, odor and gel characteristics were observed. In addition, the ability of the gel to inhibit *P. acnes* was tested using the disc diffusion technique (Chuangchot et al., 2017) and was compared to before the stability test.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± SD. The data were analyzed using one-way analysis of variance and multiple comparisons were performed using the least significant differences. All tests were considered significant at $p < 0.05$.

Results

Screening tests of extracts

The extracts of the flowers (WTF), leaves (WTL) and stems (WTS) of *W. trilobata* were preliminarily tested for bioactivity to select the best extracts before further study. It was found that the WTF produced the highest yield of extracts and had better biological activity than those of the WTL and WTS, respectively (Table 1). The WTF had higher antioxidant activity than for the other extracts when tested based on both the DPPH and ABTS methods. In addition, WTF had anti-tyrosinase activity in enzymatic assays and anti-inflammatory activity through inhibition of nitric oxide production in the RAW264.7 macrophages. WTF was selected for further study based on the results of all biological activity examination which were related to the development of herbal extracts into cosmeceuticals.

Table 1 Total phenolic content and biological activities (mean ± SD, $n = 3$) of leaves (WTL), stems (WTS) and flowers (WTF) of *Wedelia trilobata*

Sample	Total phenolic (µg GAE/mg)	DPPH (EC_{50}, µg/mL)	ABTS (EC_{50}, µg/mL)	Tyrosinase inhibition (EC_{50}, mg/mL)	Nitric oxide inhibition (EC_{50}, mg/mL)
WTF	622.54±71.24	5.50±0.10	64.81±4.69	0.752±0.09	159.14±6.39
WTL	572.45±35.39	11.41±0.16	89.68±6.48	1.039±0.45	189.56±8.45
WTS	183.24±28.16	21.36±0.33	112.15±8.46	4.726±0.62	254.18±10.15
Vitamin C	ND	3.21±0.10	ND	ND	ND
Trolox	ND	ND	5.95±0.37	ND	ND
Kojic acid	ND	ND	ND	0.01±0.00	ND
Aminoguanidine	ND	ND	ND	ND	45.32 ± 0.72

GAE = gallic acid equivalents; DPPH = 2,2-diphenyl-1-picrylhydrazyl; ABTS = 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); ND = not determined; EC_{50} = half maximal effective concentration.

Effect of flower extract on cell viability

Human keratinocytes (HaCaT cells) and murine macrophages (RAW264.7 cells) were used as models to study the cytoprotective and anti-inflammatory effects of WTF extracts, respectively. Both types of cells were tested for the effect of WTF on cell viability using MTT assay. After 24 h of incubation with various concentrations of the extracts, WTF were found to inhibit cell growth. The half IC_{50} values were 1,646.70 and 487.25 µg/mL in HaCaT and RAW264.7 cells, respectively. The effect of WTF on cell viability is shown in Fig. 1. In the HaCaT cytotoxicity effect test, the maximum WTF concentration was 5 mg/mL and the IC_{50} was determined (data not shown). Based on these results, the extracts at doses in the range 50–200 µg/mL were used in the evaluation of further biological effects.

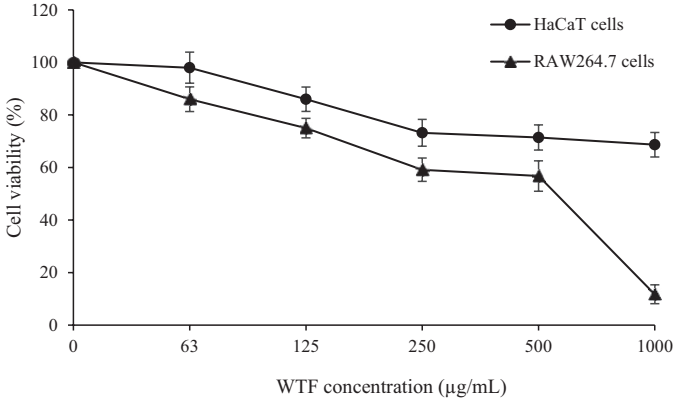

Fig. 1 Effect of *Wedelia trilobata* flowers (WTF) on viability of HaCaT and RAW264.7 cells, where cells treated for 24 h and values expressed as mean ± SD (n = 3)

Anti-inflammatory effect of flower extract

The expressions of the inflammatory-related genes *COX-2*, *IL-1β* and *TNF-α* in macrophages (RAW264.7) were not changed when treated with the extracts alone but were up-regulated after treatment with LPS. After incubation for 24 h with WTF at 50–200 µg/mL concentrations, the WTF seemed to inhibit the expressions of these genes in a dose-dependent manner. In particular, at a concentration of 200 µg/mL, WTF produced a significantly higher suppression than indomethacin (Fig. 2). WTF (10–50 µg/mL) inhibited the production of tumor necrosis factor-**α** (TNF-**α**) in *E. coli* LPS-stimulated keratinocytes (HaCaT cells), as shown in Fig. 3.

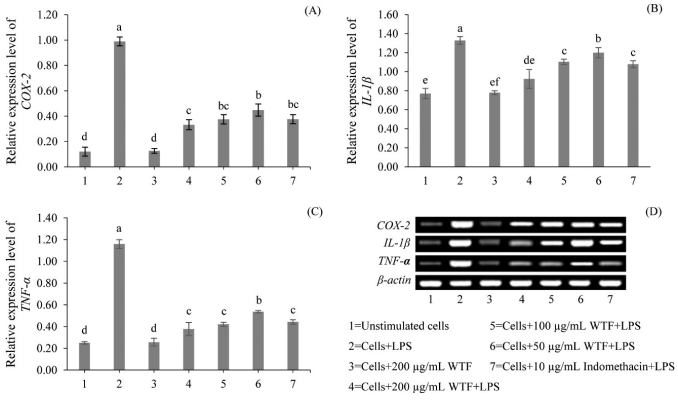

Fig. 2 Effects of *Wedelia trilobata* flowers (WTF) on: (A) mRNA expression of *COX-2*; (B) *IL-1β*; (C) *TNF-α* and (D) amplified bands of *COX-2*, *IL-1β*, *TNF-α* and *β-actin* in macrophages (264.7 cells), where different lowercase letters above histograms indicate significant ($p < 0.05$) differences and error bars represent ± SD

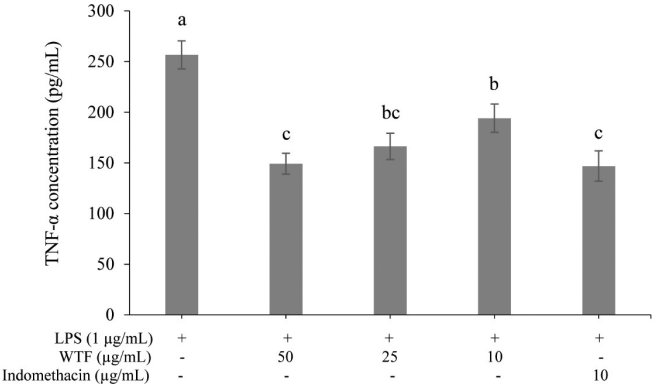

Fig. 3 Effect of *Wedelia trilobata* flowers (WTF) on tumor necrosis factor-**α** (TNF-**α**) production in lipopolysaccharide (LPS)-induced HaCaT cells, where different lowercase letters above histograms indicate significant ($p < 0.05$) differences and error bars represent ± SD

Anti-microbial effect of flower extract

The antimicrobial activity showed that WTF was effective against both Gram-positive bacteria (*S. aureus*, *S. epidermidis, B. subtilis*, *P. acnes*) and Gram-negative bacteria (*E. coli, S. enterica*, *P. aeroginosa*) and also inhibited an opportunistic pathogenic yeast (*C. albicans*). Among the microorganisms tested, WTF produced the greatest inhibition effect of *B. subtilis* (Tables 2 and 3).

Cytoprotective effect of extracts against H_2O_2-induced cells

H_2O_2-induced cell death in HaCaT was prevented in a dose-dependent manner by a pretreatment with WTF for 24 h. Compared to the control, the addition of WTF concentrations of 6.25, 12.50, 25.00 and 50.00 µg/mL continuously raised the cytoprotection to 9.23%, 10.27%, 17.93% and 19.94%, respectively (Fig. 4).

Table 2 Anti-microbial activity (mean ± SD, n = 3) of *Wedelia trilobata* flowers (WTF)

Microorganism	Inhibition zone (mm)				
	Ampicillin (1 µg)	Clindamycin (2 µg)	Amphotericin B (1 µg)	DMSO	WTF (0.4 mg)
Bacteria					
S. aureus	51±1.7	31.7±1.5	ND	NA	13.7±1.2
S. epidermidis	86.3±1.5	39.3±1.2	ND	NA	14.3±1.2
S. enterica	15.0±1.0	ND	ND	NA	11.0±0.0
E. coli	37.7±0.6	ND	ND	NA	12.0±1.0
P. aeruginosa	NA	ND	ND	NA	11.3±1.2
B. subtilis	39.7±2.5	ND	ND	NA	16.2±1.2
Yeast					
C. albicans	ND	ND	28.3±0.6	10.0±0.0	13.7±1.2

DMSO = dimethyl sulfoxide; NA = no activity; ND = not determined

Table 3 Anti-microbial activity of *Wedelia trilobata* flowers (WTF)

Microorganism	Anti-microbial activity					
	MIC	MBC	MIC	MBC	MIC	MBC
Bacteria	Ampicilin (µg/mL)		WTF (mg/mL)		Clindamycin (µg/mL)	
S. aureus	0.13	0.25	2.00	4.00	ND	ND
S. epidermidis	0.06	0.13	2.00	4.00	ND	ND
S. enterica	8.00	8.00	4.00	>4.00	ND	ND
E. coli	4.00	8.00	4.00	>4.00	ND	ND
P. aeruginosa	8.00	16.00	>4.00	>4.00	ND	ND
B. subtilis	4.00	8.00	1.00	1.00	ND	ND
P. acnes	ND	ND	2.00	4.00	0.16	5.00
Yeast	Amphotericin B (µg/mL)				WTF (mg/mL)	
C. albicans	0.50		1.00		2.00	4.00

MIC = Minimum inhibitory concentration; MBC = minimum bactericidal concentration; ND = not determined

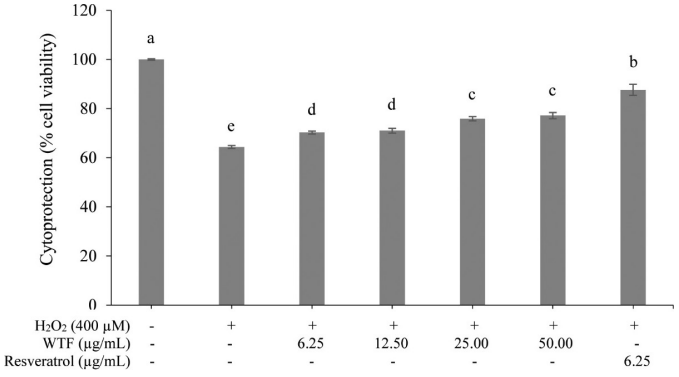

Fig. 4 Protective effects of *Wedelia trilobata* flowers (WTF) on H_2O_2-induced cell death in human HaCaT keratinocytes, where different lowercase letters above histograms indicate significant ($p < 0.05$) differences and error bars represent ± SD

Effects of flower extract on mRNA expressions of MMP-1, MMP-2, and procollagen-1 in H_2O_2-induced HaCaT cells

RT-PCR was used to assess the WTF effects on *MMP-1*, *MMP-2*, and *procollagen-1* expressions. At 24 h after H_2O_2 induction, as displayed in Fig. 5A-B, the mRNA expression of *MMP-1* and *MMP-2* was stimulated by H_2O_2 induction compared to the untreated cells. However, increased *MMP-1*, and *MMP-2* expression was notably lowered due to the WTF pretreatments at 5, 10 and 25 µg/mL. On the other hand, mRNA expression of *procollagen-1* greatly diminished compared to the untreated cells. WTF Pretreatment of concentrations at 5, 10, and 25 µg/mL resulted in the reduced expression of *procollagen-1* compared to the untreated cells and H_2O_2-treated cells (Fig. 5C). Thus, through the transcriptional modulation of keratinocytes genetic expression, WTF reduced *MMP-1*, *MMP-2* and enhanced *procollagen-1* production.

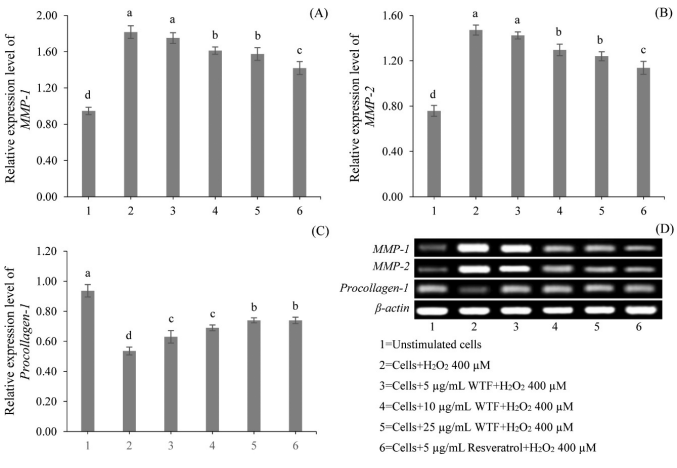

Fig. 5 Effects of *Wedelia trilobata* flowers (WTF) on mRNA expression of: (A) *MMP-1*; (B) *MMP-2*; (C) *procollagen-1*; (D) amplified bands of *MMP-1*, *MMP-2*, *procollagen-1* and *β-actin* in H_2O_2-induced HaCaT keratinocytes, where different lowercase letters above histograms indicate significant ($p < 0.05$) differences and error bars represent ± SD

Effect of prototype WTF gel on Propionibacterium acnes

The WTF gel, was clear, yellow, non-clumping, non-greasy and non-sticky when applied to the skin, as shown in Fig. 6A, Four WTF gel formulations were produced at concentrations of 0.25% (F1), 0.5% (F2), 1% (F3) and 5% (F4). After testing the stability of the WTF gel, it was found that the gel texture, color, and odor did not change. In addition, from the study of antibacterial activity against *P. acnes* of the WTF gels, based on before and after the stability test, it was found that WTF gel had a concentration-dependent antibacterial activity, with the activity against *P. acnes* before and after the stability test being slightly different but not significantly so, as shown in Fig. 6B.

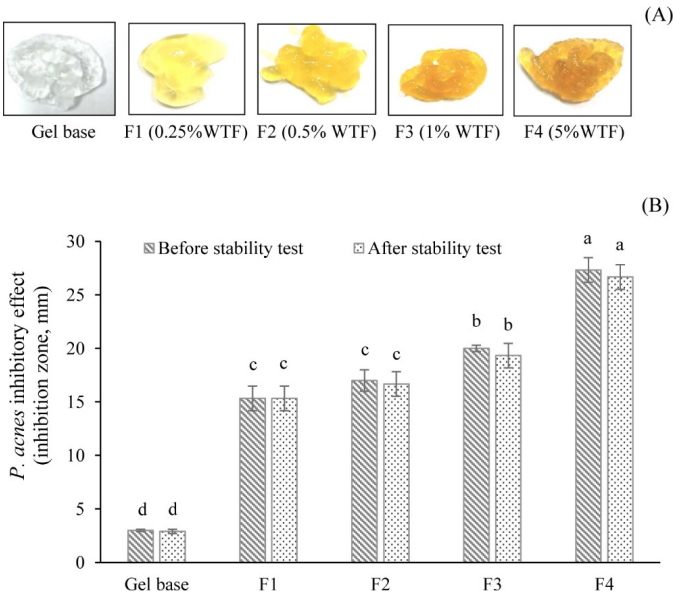

Fig. 6 Physical characteristics of *Wedelia trilobata* flowers (WTF): (A) gel; (B) *P. acnes* inhibitory effect (before-after stability test of WTF gel), where values expressed as mean ± SD (n = 3) and different lowercase letters above histograms indicate significant ($p < 0.05$) differences and error bars represent ± SD

Discussion

The current research showed that the ethanolic *Wedelia trilobata* extract had potential to be further developed into a skin-applied product to reduce the appearance of wrinkles, improve radiance or inhibit bacterial acne. *W. trilobata* extract, especially the flower part (WTF), had antioxidant activity and a high total phenolic content. The antioxidant activity of WTF had an EC_{50} value of 5.50±0.10 µg/mL and when compared to vitamin C (EC_{50} of 3.21±0.10 µg/mL), this could be considered to be a high level. In addition, in the flower part, the total phenolic content, an essential plant bioactive compound, was 622.4 ± 71 mg/g, and the extract content (%yield) was higher than for the leaves and stems. Antioxidant activity is strongly associated with skin health; for example, Mishra et al. (2014) suggested that exposure to ultraviolet (UV) light oxidizes biomolecules in cells, which can be prevented by the use of skin care products with antioxidant activity. In the current study, WTF had antioxidant and anti-tyrosinase activities, which are factors involved in reducing skin dullness. Reports show that herbs with strong antioxidant activity tend to have strong anti-tyrosinase activity. For example, in the melanin production process, UV light induced skin pigmentation by inducing oxidative stress in melanocytes (Choi et al., 2005). Therefore, herbs that have high anti-oxidation effects are often important in cosmeceuticals for skin whitening. In addition, the WTF extract had a high content of phenolic compounds, which are reported to have antioxidant and anti-melanin activity (Sripanidkulchai and Fangkrathok, 2014; Chaiprasongsuk et al., 2016). In the current study, the extracts of the *W. trilobata* flower, leaves, and stems had anti-inflammatory effects. They were able to inhibit the secretion of nitric oxide (NO) in *E. coli* LPS-stimulated macrophages, especially the flower parts that had the greatest inhibitory effect on NO secretion. Therefore, the WTF was selected for further study on the mechanism of action through the expression of three genes (COX-2, IL-1β and TNF-α), all of which play a crucial role in inflammation. The results of the study found that WTF at concentrations of 50–200 µg/mL was able to inhibit the expression of all three genes in macrophages (RAW264.7 cells). Furthermore, WTF inhibited the secretion of tumor necrosis factor-**α** protein in keratinocytes (HaCaT cells). Regarding the antimicrobial activity, the WTF inhibited the growth of both Gram-positive and Gram-negative bacteria. In addition, the WTF inhibited the growth of the yeast, *Candida albicans*. However, the antimicrobial effect in this study compared to the positive control showed that the antibacterial activity of WTF was not very high.

In addition, WTF at concentrations of 6.25–50.00 µg/mL had a cytoprotective effect based on testing using H_2O_2-induced keratinocytes. Compared to the H_2O_2-induced group, the WTF -treated cells had an increased survival rate. When tested for the effect on the expression of *MMP-1*, *MMP-2*, and *procollagen-1* genes in H_2O_2-induced keratinocytes, the WTF increased *procollagen-1* gene expression and decreased the expression of the *MMP-1* and *MMP-2* genes. From these results, WTF had an anti-aging effect, since these three genes

are involved in skin health. Procollagen-1 plays a role in the formation of collagen type I, which is the most common collagen and has the most anti-aging properties among all types of collagens, whereas in contrast, MMP-1 (collagenase) acts as a degrading enzyme of collagen types I and III and likewise, MMP-2 (gelatinase-A) acts as a collagen-digesting enzyme distributed in the dermal-epidermal junction (Ruangpanit et al., 2001; Hayami et al., 2008). From the results of all studies, *W. trilobata* can be considered as a medicinal herb with potential to be used as a pharmaceutical, especially its flower. This may be due to the important substances in the flower, in the group of terpenoids, flavonoids and polyacetylenes (Quang et al., 2007). The flowers have been reported to contain sterols, flavonoids and benzene derivatives (Qiang et al., 2011). These active compounds have been reported to have various biological effects such as anti-inflammatory, wound-healing, antioxidant and anti-melanin activity (Eom et al., 2016; Rajan and Muraleedharan, 2017). Therefore, WTF was selected for the development of a prototype gel. The gel was prepared in four concentrations (0.25%, 0.5%, 1% and 5% of WTF). After testing the stability under accelerated conditions of heating-cooling cycles, the prototype gels were examined for their physical characteristics and efficacy in inhibiting *P. acnes*. This prototype product was extremely stable because the physical characteristics (texture, color and smell) of the gels mixed with WTF were unchanged. However, the inhibitory activity of *P. acnes* was slightly altered but not significantly different. F3 and F4 were significantly different in activity against *P. acnes* both before and after the stability studies. The WTF product inhibited microorganisms, especially the group related to acne and other dermatological diseases. Therefore, the extract is of interest for the development of cosmetic products, since the pathogenesis of acne includes induction of inflammatory responses, production of sebum and hyperplasia of sebaceous glands (Jin and Lee, 2018). In addition to *W. trilobata*, there are several other interesting and potent plants belonging to the Asteraceae family that should be developed into cosmetic products, such as *Tagetes erecta* Linn and *Carthamus tinctorius* L. *T. erecta* flowers that showed effective inhibition of hyaluronidase, elastase and MMP-1, which may be useful as an anti-wrinkle agent (Maity et al., 2011). *C. tinctorius* should be developed as a hair cosmeceutical product because it enhanced hair growth at both *in vitro* and *in vivo* levels (Junlatat and Sripanidkulchai, 2014).

The information obtained from the current research should be particularly useful in supporting the use of *W. trilobata*, especially the flowers, as an ingredient in cosmeceutical products.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Ågren, M.S., Schnabel, R., Christensen, L.H., Mirastschijski, U. 2015. Tumor necrosis factor-α-accelerated degradation of type I collagen in human skin is associated with elevated matrix metalloproteinase (MMP)-1 and MMP-3 *ex vivo*. Eur. J. Cell Biol. 94: 12–21. doi.org/10.1016/j.ejcb.2014.10.001

Balekar, N., Nakpheng, T., Srichana, T. 2014. *Wedelia trilobata* L. A phytochemical and pharmacological review. Chiang Mai J. Sci. 41: 590–605.

Berton, A., Godeau, G., Emonard, H., Baba, K., Bellon, P., Hornebeck, W., Bellon, G. 2000. Analysis of the *ex vivo* specificity of human gelatinases A and B towards skin collagen and elastic fibers by computerized morphometry. Matrix Biol. 19: 139–148. doi.org/10.1016/S0945-053X(00)00057-3

Chaiprasongsuk, A., Onkoksoong, T., Pluemsamran, T., Limsaengurai, S., Panich, U. 2016. Photoprotection by dietary phenolics against melanogenesis induced by UVA through Nrf2-dependent antioxidant responses. Redox Biol. 8: 79–90. doi.org/10.1016/j.redox.2015.12.006

Choi, H., Ahn, S., Lee, B.G., Chang, I., Hwang, J.S. 2005. Inhibition of skin pigmentation by an extract of *Lepidium apetalum* and its possible implication in IL-6 mediated signaling. Pigment Cell Res. 18: 439–446. doi.org/10.1111/j.1600-0749.2005.00266.x

Chuangchot, C., Tattawasart, U., Sripanidkulchai, B., Junlatat, J., Fangkrathok, N. 2017. Antibacterial and antioxidant activity of *Rafflesia kerrii* extract against multidrug-resistant bacteria. Songklanakarin J. Sci. Technol. 39: 163–170.

Eom, H.J., Kang, H.R., Kim, H.K., Jung, E.B., Park, H.B., Kang, K.S., Kim, K.H. 2016. Bioactivity-guided isolation of antioxidant triterpenoids from *Betula platyphylla* var. *japonica* bark. Bioorg. Chem. 66: 97–101. doi.org/10.1016/j.bioorg.2016.04.001

Fisher, G.J., Kang, S., Varani, J., Bata-Csorgo, Z., Wan, Y., Datta, S., Voorhees, J.J. 2002. Mechanisms of photoaging and chronological skin aging. Arch. Dermatol. 138: 1462–1470. doi: 10.1001/archderm.138.11.1462

Hamdy, A.A., Kassem, H.A., Awad, G.E.A., et al. 2017. *In-vitro* evaluation of certain Egyptian traditional medicinal plants against *Propionibacterium acnes*. S. Afr. J. Bot. 109: 90–95. doi.org/10.1016/j.sajb.2016.12.026

Hasperué, J.H., Rodoni, L.M., Guardianelli, L.M., Chaves, A.R., Martínez, G.A. 2016. Use of LED light for Brussels sprouts postharvest conservation. Sci. Hortic. 213: 281–286. doi.org/10.1016/j.scienta.2016.11.004

Hayami, T., Kapila, Y.L., Kapila, S. 2008. MMP-1 (collagenase-1) and MMP-13 (collagenase-3) differentially regulate markers of osteoblastic differentiation in osteogenic cells. Matrix Biol. 27: 682–692. doi.org/10.1016/j.matbio.2008.07.005

Jin, S., Lee, M.Y. 2018. *Kaempferia parviflora* extract as a potential anti-acne agent with anti-inflammatory, sebostatic and anti-*Propionibacterium acnes* activity. Int. J. Mol. Sci. 19: 3457. doi.org/10.3390/ijms19113457

Junlatat, J., Sripanidkulchai, B. 2014. Hair growth-promoting effect of *Carthamus tinctorius* floret extract. Phytother. Res. 28: 1030–1036. doi.org/10.1002/ptr.5100

Kolakul, P., Sripanidkulchai, B. 2018. Protective effects of *Lagerstroemia speciosa* extract against UV-A damage on skin cells. Ind. Crops. Prod. 124: 9–19. doi.org/10.1016/j.indcrop.2018.07.047

Maity, N., Nema, N.K., Abedy, M.K., Sarkar, B.K., Mukherjee, P.K. 2011. Exploring *Tagetes erecta* Linn flower for the elastase, hyaluronidase and MMP-1 inhibitory activity. J. Ethnopharmacol. 137: 1300–1305. doi.org/10.1016/j.jep.2011.07.064

Mishra, A.P., Saklani, S., Milella, L., Tiwari, P. 2014. Formulation and evaluation of herbal antioxidant face cream of *Nardostachys jatamansi* collected from Indian Himalayan region. Asian Pac. J. Trop. Biomed. 4: S679–S682. doi.org/10.12980/APJTB.4.2014APJTB-2014-0223

Momtaz, S., Mapunya, B.M., Houghton, P.J., Edgerly, C., Hussein, A., Naidoo, S., Lall, N. 2008. Tyrosinase inhibition by extracts and constituents of *Sideroxylon inerme* L. stem bark, used in South Africa for skin lightening. J. Ethnopharmacol. 119: 507–512. doi.org/10.1016/j.jep.2008.06.006

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65: 55–63. doi.org/10.1016/0022-1759(83)90303-4

Opuni, K.F.M., Togoh, G., Frimpong-Manso, S., Adu-Amoah, D., Alkanji, O, Boateng, K.P. 2021. Monitoring of residual solvent contamination in herbal medicinal products in Ghana: A pilot study. Sci. Afr. 13: e00825. doi.org/10.1016/j.sciaf.2021.e00825

Qiang, Y., Du, D.L., Chen, Y.J., Gao, K. 2011. *ent*-Kaurane diterpenes and further constituents from *Wedelia trilobata*. Helv. Chim. Acta. 94: 817–823. doi.org/10.1002/hlca.201000301

Quan, T., Qin, Z., Xia, W., Shao, Y., Voorhees, J.J., Fisher, G.J. 2009. Matrix-degrading metalloproteinases in photoaging. J. Investig. Dermatol. Symp. Proc. 14: 20–24. doi.org/10.1038/jidsymp.2009.8

Quang, T.T., Jossang, J., Jossang, A., Kim, P.P.N., Jaureguiberry, G. 2007. Wedelolides A and B: Novel sesquiterpene δ-lactones, (9*R*)-Eudesman-9,12-olides, from *Wedelia trilobata*. J. Org. Chem. 72: 7102–7105. doi.org/10.1021/jo070771m

Rajan, V.K., Muraleedharan, K. 2017. A computational investigation on the structure, global parameters and antioxidant capacity of a polyphenol, gallic acid. Food Chem. 220: 93–99. doi.org/10.1016/j.foodchem.2016.09.178

Ren, H., Xu, Q.L., Luo, Y., Zhang, M., Zhou, Z.Y., Dong, L.M., Tan, J.W. 2015. Two new ent-kaurane diterpenoids from *Wedelia trilobata* (L.) Hitchc. Phytochem. Lett. 11: 260–263. doi.org/10.1016/j.phytol.2015.01.004

Ren, H., Xu, Q.L., Zhang, M., Dong, L.M., Zhang, Q., Luo, B., Luo, Q.W., Tan, J.W. 2017. Bioactive caffeic acid derivatives from *Wedelia trilobata*. Phytochem. Lett. 19: 18–22. doi.org/10.1016/j.phytol.2016.11.001

Ruangpanit, N., Chan, D., Holmbeck, K., Birkedal-Hansen, H., Polarek, J., Yang, C., Bateman, J.F., Thompson, E.W. 2001. Gelatinase A (MMP-2) activation by skin fibroblasts: Dependence on MT1-MMP expression and fibrillar collagen form. Matrix Biol. 20: 193–203. doi.org/10.1016/S0945-053X(01)00135-4

Saad, H.M., Tan, C.H., Lim, S.H., Manickam, S., Sim, K.S. 2021. Evaluation of anti-melanogenesis and free radical scavenging activities of five *Artocarpus species* for cosmeceutical applications. Ind. Crop. Prod. 161: 113184. doi.org/10.1016/j.indcrop.2020.113184

Shimada, K., Fujikawa, K., Yahara, K., Nakamura, T. 1992. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J. Agric. Food Chem. 40: 945–948.

Sripanidkulchai, B., Fangkrathok, N. 2014. Antioxidant, antimutagenic and antibacterial activities of extracts from *Phyllanthus emblica* branches. Songklanakarin J. Sci. Technol. 36: 669–674.

Sripanidkulchai, B., Junlatat, J., Wara-aswapati, N., Hormdee, D. 2009. Anti-inflammatory effect of *Streblus asper* leaf extract in rats and its modulation on inflammation-associated genes expression in RAW 264.7 macrophage cells. J. Ethnopharmacol. 124: 566–570. doi.org/10.1016/j.jep.2009.04.061

Sun, L., Wang, Z., Wang, Y., Xu, J., He, X. 2020. Anti-proliferative and anti-neuroinflammatory eudesmanolides from Wedelia (*Sphagneticola trilobata* (L.) Pruski). Fitoterapia 142: 104452. doi.org/10.1016/j.fitote.2019.104452

Szóstek-Mioduchowska, A.Z., Baclawska, A., Okuda, K., Skarzynski, D.J. 2019. Effect of proinflammatory cytokines on endometrial collagen and metallopeptidase expression during the course of equine endometrosis. Cytokine 123: 154767. doi.org/10.1016/j.cyto.2019.154767

Won, J.H., Im, H.T., Kim, Y.H., Yun, K.J., Park, H.J., Choi, J.W., Lee, K.T. 2006. Anti-inflammatory effect of buddlejasaponin IV through the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages *via* the NF-κB inactivation. Br. J. Pharmacol. 148: 216–225. doi.org/10.1038/sj.bjp.0706718

Xuesong, H., Shiyi, O., Shuze, T., Liang, F., Jianzhong, W. 2006. Simultaneous determination of trilobolide-6-*O*-isobutyrates A and B in *Wedelia trilobata* by gas chromatography. Chin. J. Chromatogr. 24: 499–502. doi.org/10.1016/S1872-2059(06)60021-7

Yang, E.J., Yim, E.Y., Song, G., Kim, G.O., Hyun, C.G. 2009. Inhibition of nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages by Jeju plant extracts. Interdiscip. Toxicol. 2: 245–249. doi.org/10.2478/v10102-009-0022-2

Zuliani, T., Denis, V., Noblesse, E., Schnebert, S., Andre, P., Dumas, M., Ratinaud, M.H. 2005. Hydrogen peroxide-induced cell death in normal human keratinocytes is differentiation dependent. Free Radic. Biol. Med. 38: 307–316. doi.org/10.1016/j.freeradbiomed.2004.09.021