Songklanakarin J. Sci. Technol.
42 (6), 1319-1325, Nov. - Dec. 2020

http://www.sjst.psu.ac.th

Original Article

Investigation on the potential application of *Morus alba* stem extract for inflammatory acne vulgaris

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Received: 1 July 2019; Revised: 19 August 2019; Accepted: 17 September 2019

Abstract

This study was aimed to investigate the potential use of *Morus alba* stem extract for treatment of inflammatory acne through its inhibitory activities against acne-causing bacteria, free radicals, prostaglandin (PG)-E_2 and tyrosinase enzyme. The antibacterial studies found that among *Propionibacterium acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus*, the extract specifically inhibited *P. acnes* with MIC and MBC of 3.125 mg/ml and 12.5 mg/ml, respectively. It also exhibited the ability to scavenge free radicals including DPPH, superoxide and nitric oxide radicals with IC_{50} values of 32.07, 67.29 and 12.12 µg/ml, respectively. Moreover, the extract could significantly inhibit the production of PGE_2, a potent inflammatory mediator. Additionally, it presented potent anti-tyrosinase activity (5.76 ± 1.58 µg/ml) and was significantly more effective than kojic acid (30.61 ± 13.35 µg/ml), offering great benefits to reduce post inflammatory hyperpigmentation. These findings suggest potential application of the extract in cosmeceutical formulations for inflammatory acne.

Keywords: *Morus alba*, stem extract, acne, antibacterial, antioxidant, anti-inflammation

1. Introduction

Acne vulgaris is the most prevalent skin disease worldwide (Tan & Bhate, 2015). It is a chronic inflammatory skin disease of the pilosebaceous unit consisting of hair, hair follicle, arrector pili muscle and sebaceous gland. Acne is caused by an increased rate of sebum production due to the activation of the androgen hormone (i.e. testosterone). The conversion of testosterone by 5α-reductase enzyme results in a more potent androgen, namely dihydrotestosterone (DHT). DHT causes thickening of the follicular epithelium, resulting in the blocking of the duct orifices, causing an accumulation of sebum in the lower part. Consequently, the formation of comedone occurs. Anaerobic environment of the blocked follicles is the optimum condition for the overgrowth of *Propionibacterium acnes,* which plays a major role in the etiology of inflammatory acne (Dessinioti & Katsambas, 2010). *P. acnes* can secrete lytic and lipase enzymes, which lead to a disruption of the follicular epithelium and inflammatory reactions (Grange, Weill, Dupin, & Batteux, 2010). *P. acnes* is able to produce chemotactic factors (Leyden, 1997), which attract either neutrophils or macrophages to the acne lesions and consequently produce proinflammatory cytokines and chemokines (Nagy *et al*., 2005). It can also activate neutrophils, macrophages and keratinocytes to produce reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) as well as reactive nitrogen species (RNS) such as nitric oxide (•NO) and peroxynitrite (ONOO•) (Grange, Weill, Dupin, & Batteux, 2010). These molecules cause apoptosis, necrotic cell death, cell proliferation as well as inflammatory reactions (Nicco,

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Laurent, Chereau, Weill, & Batteux, 2005). *Staphylococcus epidermidis* was reported to be an indirect manner of acne pathogenesis. It can produce a biofilm, which causes favorable anaerobic conditions for the overgrowth of *P. acnes* (Heilmann *et al*., 1996). Like *S. epidermidis*, *Staphylococcus aureus* is skin commensal microbiota. At some point, it may become a pathogen causing skin infections such as pimples, folliculitis and abscesses.

First line treatments for inflammatory acne are topical retinoid and/or topical benzoyl peroxide. These medications are known to cause various side effects, such as peeling, itching, redness and excessive dryness (Spellman & Pincus, 1998). In rare severe cases, side effects such as skin discoloration, photosensitivity to UV light, blistering etc. may also be observed. Most importantly, the use of oral and topical antibiotics to treat acne is reported to increase drug-resistance in many countries, and more than 50% of *P. acnes* strains show resistance to especially topical macrolides (Walsh, Efthimiou, & Dréno, 2016). Therefore, the use of medicinal plants is gaining attention as an alternative treatment.

Morus alba L. is commonly known as white mulberry and belongs to the Moraceae family. *M. alba* has long been widely used in traditional medicine (Sánchez-Salcedo, Amorós, Hernández, & Martínez, 2017). It was demonstrated to inhibit 5α-reductase enzyme which converts testosterone to DHT, and is one of the main causes of acne (Jang *et al*., 2007). In addition, our previous study found that the extract prepared from mulberry stems possessed antioxidant activity stronger than extracts from twigs or from leaves (Thongsuk, 2007). Moreover, we also investigated its anti-inflammatory activity using lipopolysaccharide-stimulated RAW 264.7 cell model (Soonthornsit, Pitaksutheepong, Hemstapat, Utaisincharoen, & Pitaksuteepong, 2017). It was observed that *M. alba* stem extract could inhibit inducible nitric oxide synthase (iNOS), nitric oxide and cyclooxygenase (COX)-2. However, its antibacterial activity against acne causing bacteria has not been investigated. Therefore, this study aimed to investigate the potential application of *M. alba* stem extract through the inhibitory activities against acne-causing bacteria as well as against various free radicals. The anti-inflammatory activity of the stem extract was also confirmed through the inhibition of PGE_2. In addition, anti-tyrosinase activity of the extract, with the expectation to prevent or reduce hyperpigmentation caused by acne, was also investigated.

2. Materials and Methods

2.1 Plant material and preparation of *M. alba* stem extract

The stems of *M. alba*, variety “Buriram 60” were received from the Queen Sirikit Sericulture Center (Tak, Thailand) and authenticated by Dr. Pranee Nangngam of the Faculty of Science, Naresuan University (Voucher No: 004069).

The ethanolic extracts were prepared by maceration technique as described by Wongwat *et al.* High-performance liquid chromatography (HPLC) analysis was performed to quantify a marker in the extract named oxyresveratrol (2,4,3’,5’-tetrahydroxystilbene). The HPLC unit (Shimadzu, Kyoto, Japan) was equipped with a pump (LC-10ATVP), an auto-sampler (SIL-20ACHT), a column oven (CTO-10ASVP) and a UV-Vis detector (SPD-10AVP). Analysis was performed using C18 bonded-silica gel (Gemini, 5 µm, 150 x 4.6 mm, Phenomenax, Torrance, USA). The mobile phase consisted of acetonitrile and 0.0125 M phosphate buffer at pH 3 (1:3). The parameters of the analytical condition were set, including flow rate of 1 ml/min, column oven temperature of 30°C, and the detector wavelength of 320 nm. The injection volume was 20 µl, and the running time was set at 13 min.

2.2 Antibacterial assays

The study protocol for antibacterial activity was approved by the Naresuan University Institutional Biosafety Committee (NUIBC MI 59-06-20). *P. acnes* DMST 14916, *S. epidermidis* ATCC 12228 and *S. aureus* ATCC 25923 were used in this study. They were obtained from the culture collection of the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand.

2.2.1 Disc diffusion method

As recommended by the 2013 Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute [CLSI], 2013), the concentration of the strain was adjusted to yield approximately 1×10^8 CFU/ml by direct colony suspension method. Then, the bacterial suspension was spread evenly onto agar surface. After that, 20 µl of the stock solution of *M. alba* stem extract (100 mg/ml) was pipetted onto a sterile paper disc (the amount of test material was 2 mg/disc). After drying, the disc was placed on the agar surface. For *P. acnes*, the inoculated Brain Heart Infusion (BHI) agar plate was incubated at 37 °C under anaerobic conditions for 72 h. On the other hand, the inoculated Mueller Hinton agar (MHA) plates of *S. epidermidis* and *S. aureus* were incubated at 37 °C under aerobic conditions for 24 h. A clindamycin disc (2 µg/disc) was used as a positive control for *P. acnes*, and a gentamicin disc (10 µg/disc) was used as a positive control for *S. epidermidis* and *S. aureus*. In order to eliminate the effects of the solvent used to dissolve the extract, a sterile paper disc containing 20 µl of DMSO was used as a negative control.

2.2.2 Broth macrodilution method

Serial doubling dilutions of the *M. alba* stem extract solution were prepared to obtain a final concentration range of 0.2-25.0 mg/ml. For each dilution, 2 ml of that dilution was placed into a test tube and combined with 200 µl of *P. acnes* suspension. The final concentration of *P. acnes* suspension was 1×10^5 CFU/ml. The tubes were incubated at 37 °C under anaerobic conditions for 72 h. These tubes were then used to determine the minimum inhibitory concentration (MIC) of the extract. The MIC is defined as the lowest concentration of the extract that inhibits at least 90% of bacterial growth.

The minimum bactericidal concentration (MBC) is the lowest concentration of the extract that completely kills the observed bacteria. In order to determine the MBC, 10 µl of the final contents were taken from each tube used during the MIC procedure and transferred to BHI agar plate. The plates were then incubated at 37 °C under anaerobic conditions for 72 h. *P. acnes* suspension combined with medium was used as a control.

All tests were performed in triplicate.

2.2.3 Broth microdilution method

The broth microdilution method was set up in almost the same way as the broth macrodilution method. However, the broth microdilution method was prepared in a 96-well micro-plate. Serial doubling dilutions of the extract solution were prepared in the plate to obtain a final concentration range of 0.2-25.0 mg/ml. Each dilution (100 µl) was placed into its own well and combined with 10 µl of *S. epidermidis* or *S. aureus* suspension for a final concentration of 1×10^5 CFU/ml. The MBC was determined by seeding 10 µl from each well onto a MHA plate and the plates were incubated at 37 °C for 24 h. *S. epidermidis* or *S. aureus* suspension combined with medium was used as a control. All tests were performed in triplicate.

2.3 Antioxidant assays

2.3.1 DPPH radical scavenging assay

The free radical scavenging capacity of the stem extract and its marker, oxyresveratrol, was determined using DPPH assay. The stem extract and oxyresveratrol were prepared in methanol to various concentrations. Each of these sample solution (75 µl) was mixed separately with 150 µl of 0.2 mM DPPH solution. Butylated hydroxytoluene (BHT) was used as a positive control. The reaction mixture was incubated at room temperature for 30 min in the dark, and the absorbance was measured at 515 nm using a microplate reader. Methanol (75 µl) in the place of the sample solution was used as the blank. A percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [1-(\text{Sample OD}/\text{Blank OD})] \times 100\%$$

where the sample OD is the absorbance of DPPH solution mixed with the test sample (the stem extract, oxyresveratrol or BHT) and the blank OD is the absorbance of DPPH solution mixed with methanol (vehicle). The concentration yielding 50% radical scavenging (IC_{50}) is reported.

2.3.2 Superoxide radical scavenging assay

The superoxide anion scavenging activity was determined based on the capacity of the samples to enhance the aerobic photochemical reduction of nitrotetrazolium blue chloride (NBT) in the presence of riboflavin. The *M. alba* stem extract or oxyresveratrol solution was prepared in methanol, while L-ascorbic acid, a positive control, was dissolved in water.

Briefly, 20 µl of 65 mM Ethylenediaminetetraacetic acid (EDTA) solution was mixed with 40 µl of 40 µM riboflavin, 40 µl of 960 µM NBT, 80 µl of 50 mM phosphate buffer (pH 7.4) and 20 µl of the sample solution. The mixture was illuminated under a fluorescent lamp for 10 min. The absorbance was measured at 560 nm using a microplate reader. Methanol (20 µl) in the place of the sample solution was used as the blank. The percentage of superoxide radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [1-(\text{Sample OD}/\text{Blank OD})] \times 100\%$$

where sample OD is the absorbance of all reagents in the presence of the sample (the stem extract, oxyresveratrol or L-ascorbic acid) and the blank OD is the absorbance of all reagents without the sample.

2.3.3 Nitric oxide radical scavenging assay

Various solution concentrations of the extract and oxyresveratrol were prepared by dissolving in DMSO and methanol, respectively. L-ascorbic acid, the positive control, was dissolved in water. Briefly, 10 mM sodium nitroprusside (SNP) solution (1 ml) was freshly prepared by dissolving SNP in deionized water and then adding 250 µl of phosphate buffer saline (pH 7.4) as well as 250 µl of one of the various concentrations of the extract solution, oxyresveratrol or L-ascorbic acid. The mixture was incubated at room temperature for 150 min (incubation 1). Then the incubated solution (110 µl) was mixed with 55 µl of 1% sulfanilamide in 2% H_3PO_4 and incubated at room temperature for 5 min (incubation 2). After that, 55 µl of 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) was added to the solution, which was incubated again at room temperature for 10 min (final incubation). The thrice incubated solution was measured for absorbance at 546 nm using a microplate reader. The percentage of nitric oxide radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [1-(\text{Sample OD}/\text{Blank OD})] \times 100\%$$

where the sample OD is the absorbance in the presence of the sample (the stem extract, oxyresveratrol or L-ascorbic acid) and the blank OD is the absorbance of all reagents without the sample.

2.3.4 ABTS radical cation decolorization assay (ABTS assay)

2, 2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS is not a free radical, and thus, it must be first changed to the ABTS radical cation ($ABTS^{\bullet+}$) by reacting with a strong oxidizing agent. Briefly, ABTS was dissolved in water to a concentration of 7 mM. The ABTS solution was mixed with 2.45 mM potassium persulfate and kept at room temperature for 12 h in the dark to generate $ABTS^{\bullet+}$. Then the solution was diluted with absolute ethanol to an absorbance of 0.70 ± 0.02 at 732 nm. A stock solution of the extract was prepared in absolute methanol. Various concentrations of the standard trolox were prepared in 70% methanol. The ABTS assay was conducted by mixing 180 µl of the diluted $ABTS^{\bullet+}$ solution with 20 µl of the extract solution or the standard trolox solution. This reaction mixture was incubated at room temperature for 6 min, and the absorbance was assessed at 732 nm using a microplate reader. TEAC values were calculated from the trolox standard curve and are expressed as trolox equivalent (mg of trolox equivalent per mg of the extract).

2.3.5 Ferric reducing antioxidant power assay (FRAP assay)

This method measures the ability of an antioxidant to reduce ferric iron by donating electrons. The method is based on the reduction of a complex of ferric iron and 2,4,6-tri-2-pyridyl-2-triazine (TPTZ) at a low pH to the ferrous form. The *M. alba* stem extract was dissolved in absolute methanol, and standard ferrous sulfate was dissolved in water. The FRAP solution was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM Ferric chloride solution at a ratio of 10:1:1. The FRAP solution (180 µl) was mixed with 20 µl of the extract solution, or various concentrations of the standard ferrous sulfate solution. The reaction mixture was incubated at room temperature for 30 min, and the absorbance was assessed immediately at 595 nm using a microplate reader. Ferric reducing antioxidant power was calculated from the ferrous sulfate standard curve and expressed as µg of Fe^{2+} equivalent per mg of the extract.

2.4 Anti-inflammatory activity assay

Anti-inflammatory activities of *M. alba* stem extract and oxyresveratrol were investigated through the inhibition of PGE_2 production in LPS-stimulated RAW 264.7 cells. The RAW 264.7 macrophage cells were seeded at 3×10^4 cells/well in a 6-well plate and incubated overnight. Then the cells were pretreated with *M. alba* stem extract (50 µg/ml) or oxy-resveratrol (7.5 µg/ml) for 2 h before stimulation with 5 µg/ml of lipopolysaccharide (LPS) for 22 h. After that, the cell culture supernatant was collected and the PGE_2 production was measured with a prostaglandin E_2 assay kit according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, USA). Diclofenac (DCS), a well-known nonsteroidal anti-inflammatory drug, at 1 µg/ml was used as a control. Untreated and LPS-induced RAW 264.7 cells without treatment were used as negative and positive controls, respectively.

2.5 Anti-tyrosinase activity (Dopachrome) assay

M. alba stem extract, oxyresveratrol and kojic acid (positive control) were dissolved and diluted to various concentrations in the range 0.004-0.25 mg/ml. Forty µl of these samples were added to the wells of 96-well plate. Then, 80 µl of 67 mM phosphate buffer (pH 6.8) and 40 µl of 15 mM L-DOPA were added. The background absorbance of mixed solution was measured at 490 nm (Sample OD_{A0}) by using a microplate reader. Then, 40 µl of mushroom tyrosinase (125 U/ml) was added. The solution was mixed and incubated at room temperature for 20 min. The absorbance was again measured (Sample OD_{A1}). Phosphate buffer (67 mM, pH 6.8) (120 µl) in place of the sample solution was used as the blank. The experiments were performed in triplicate. Tyrosinase inhibitory activity was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = 1 - \frac{(\text{Sample OD}_{A1}) - (\text{Sample OD}_{A0})}{(\text{Blank OD}_{A1}) - (\text{Blank OD}_{A0})} \times 100$$

2.6 Statistical analysis

All data are expressed as mean ± standard deviation (S.D.). Statistical analysis used one-way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons (Graphpad Prism 6.0, Graphpad Software Inc., San Diego, USA). *P*-values less than or equal to 0.05 were considered significant.

3. Results and Discussion

3.1 *M. alba* stem extract and oxyresveratrol content

The *M. alba* stem extract showed a yield of 4.08% with oxyresveratrol content of 15.06 ± 0.36% w/w in the dried extract.

3.2 Antibacterial activity

M. alba stem extract is gaining attention as an alternative treatment for inflammatory acne because it possesses anti-androgenic (Jang *et al*., 2007) and anti-inflammatory (Soonthornsit, Pitaksutheepong, Hemstapat, Utaisincharoen, & Pitaksuteepong, 2017) activities. However, the inhibitory activities against acne-causing bacteria, especially *P. acnes* which plays a major role in the etiology of inflammatory acne (Dessinioti & Katsambas, 2010), have not yet been investigated.

The antibacterial activities of *M. alba* stem extract measured using the disc diffusion method are given in Table 1. It was found that the extract showed the strongest inhibitory effect against *P. acnes*, followed by *S. epidermidis* and *S. aureus*, respectively. The positive control showed inhibition zones against *P. acnes*, *S. epidermidis* and *S. aureus* of 48.39 mm, 30.56 and 27.55, respectively. The control disc (DMSO) showed no zone of inhibition against any of the three micro-organisms. Further experiments were conducted to determine the MIC and the MBC of the extract.

The MIC and the MBC of *M. alba* stem extract were further evaluated using broth dilution method to determine their bacteriostatic and bactericidal properties. It was found that the MIC against *P. acnes* was 3.125 mg/ml while the MBC against *P. acne* was 12.5 mg/ml (Table 2). At these concentrations, the extract has no effect on *S. epidermidis* and *S. aureus*. On increasing concentration of the extract to 25 mg/ml, the observed inhibition of *S. epidermidis* and *S. aureus* was approximately 50%.

It has been reported that *P. acnes*, *S. epidermidis* and other skin flora co-exist in acne lesions (Nishijima, Kurokawa, Katoh, & Watanabe, 2000). Wang *et al*. (2014) further investigated and suggested that *P. acnes* and *S. epidermidis* co-exist on the skin surface without counteracting each other. Once *P. acnes* overgrew and a comedone developed, *S. epidermidis* entered the acne lesions. It mediated

Table 1. Antibacterial activities of *M. alba* stem extract from the disc diffusion method.

Microorganism	Diameter of inhibition zone ± S.D. (mm)		
	M .alba stem extract	Clindamycin	Gentamicin
P .acnes	20.44 ± 1.74	48.39 ± 2.23	-
S .epidermidis	14.78 ± 0.83	-	30.56 ± 0.53
S. aureus	11.89 ± 0.22	-	27.55 ± 0.46

Table 2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the *M. alba* stem extract against *P. acnes*, *S. epidermidis* and *S. aureus*.

Microorganism	*M .alba* stem extract	
	MIC (mg/ml)	MBC (mg/ml)
P .acnes	3.125	12.5
S .epidermidis	> 25.0	> 25.0
S. aureus	> 25.0	> 25.0

fermentation of glycerol in the anaerobic environment of acne lesion, inhibiting overgrowth of *P. acnes* (Wang *et al*., 2014). In addition, it was reported that *S. epidermidis* could counteract *S. aureus* infection by secreting a serine protease to combat colonization by *S. aureus* (Iwase *et al*., 2010). As detailed above, *S. aureus* is skin commensal microbiota and at some point it becomes a pathogen causing skin infections, such as pimples, folliculitis and abscesses. Therefore, the *M. alba* stem extract, which can inhibit *P. acnes* but not *S. Epidermidis,* may provide benefits in treatment of inflammatory acne and prevent development of abscesses. However, it must be used at an early stage before abscesses are evident.

Phenolic compounds are secondary metabolites most commonly found in natural sources, and contain benzene rings with one or more hydroxyl substituent. These compounds can be classified as flavonoids (e.g. flavones, flavanols, flavanones, anthocyanidins) and non-flavonoids (e.g. stilbenoids, tannins, lignans, lignins, phenolic acids) compounds.

In our parallel study, it was found that the total phenolic content in *M. alba* stem extract was 203.35 ± 7.10 µg GA/mg extract. On the other hand, the total flavonoid content in the extract was found to be 11.63 ± 1.64 µg QUE/mg extract. From the HPLC analysis detailed above, the content of oxyresveratrol was 15.06 ± 0.36% w/w in the dry extract (i.e. 150 µg/mg extract). Therefore, it can be concluded that oxyresveratrol, classified as stilbenoid compound, is the major phenolic compound in the extract. These results are in agreement with findings reported earlier (Chan, Lye, & Wong, 2016). They suggested that the stems of *M. alba* contained various polyphenol compounds including dihydromorin, 2,3-trans-dihydromorin-7-*O*-β-glucoside, moracin M, mulberroside A, oxyresveratrol, resveratrol and steppogenin, which were classified as stilbenoid compounds. Among these, oxyresveratrol was reported to be the major compound (Chan, Lye, & Wong, 2016). These compounds (e.g. flavonoids, stilbenoids, etc.) are involved in inhibiting the growth of bacteria (Choi, Jang, Lee, Leem, & Kim, 2013). They can diffuse through the bacterial cell wall and form hydrogen bonds with bacterial cell proteins, causing denaturation of the protein and cell lysis (Lawrence & Block, 1986; Ngajow, Abidjulu, & Kamu, 2013). Thus, the antibacterial activity against *P. acne* in this study may be due to oxyresveratrol as well as other stilbenoid compounds, and, to a lesser extent flavonoids.

3.3 Antioxidant activity

P. acnes in an acne lesion produces chemotactic factors and enzymes, leading to the accumulation of neutrophils (Wong *et al*., 2016). The neutrophils generate reactive species including ROS and RNS, causing inflammation. It has been reported that acne patients have low levels of antioxidants and thus reactive species could not be removed (Arican, Kurutas, & Sasmaz, 2005). The antioxidant activities of *M. alba* stem extract and its main bioactive component, namely oxyresveratrol, were investigated.

The concentrations of the *M. alba* stem extract, oxyresveratrol and positive controls (BHT or L-ascorbic acid) required to scavenge the DPPH, superoxide and nitric oxide by 50% (IC_{50}) are shown in Table 3. The *M. alba* stem extract and oxyresveratrol showed hydrogen donating capability in the DPPH radical scavenging assay. However, the stem extract and oxyresveratrol exhibited a weaker superoxide radical scavenging ability than that of L-ascorbic acid. Interestingly, the extract and oxyresveratrol exhibited potent nitric oxide radical scavenging activities and were significantly more effective that L- ascorbic acid, the positive control. These results are in agreement with our previous study, which showed that the ethanolic extract prepared from *M. alba* stems and oxyresveratrol possessed anti-inflammatory activity in LPS-induced RAW 264.7 cells via the inhibition of various mediators, including •NO (Soonthornsit, Pitaksutheepong, Hemstapat, Utaisincharoen, & Pitaksuteepong, 2017).

Free radical scavenging activity of the extract was quantified using the TEAC method/ABTS assay. The trolox equivalent antioxidant capacity of the *M. alba* stem extract was 1.11 ± 0.05 mg TE/mg extract. In the FRAP assay, the reducing capacity of the *M. alba* stem extract was measured by reacting with ferric tripyridyltriazine (Fe^{3+} TPTZ) complex and this produced a colored product, ferrous tripyridyltriazine (Fe^{2+} TPTZ) (Li *et al*., 2006). The ferric reducing-antioxidant power of the extract was 0.63 ± 0.10 µg Fe^{2+}/mg extract, suggesting that the extract has a reducing activity which could protect against tendency for oxidation.

3.4 Anti-inflammatory activity assay

P. acnes triggers an inflammatory reaction via various pathways, such as upregulation of toll-like receptor (TLR)-2, Janus kinase (JNK), Nuclear factor (NF)-κB etc. Various pro-inflammatory mediators and enzymes are in-

Table 3. Concentrations (IC_{50}) of the *M. alba* stem extract, oxyresveratrol and positive controls providing 50% radical scavenging.

Sample	IC_{50} ± S.D. (µg/ml)		
	DPPH	Superoxide	Nitric oxide
M. alba stem extract	32.07 ± 0.93*	67.29 ± 5.90*	12.12 ± 1.71*
Oxyresveratrol	7.12 ± 0.33	194.30 ± 3.16*	2.39 ± 0.33*
Butylated hydroxytoluene†	9.74 ± 2.68	-	-
L-ascorbic acid††	-	17.88 ± 5.49	85.10 ± 2.18

* Mean is significantly different ($p \leq 0.05$) from the positive control based on one-way analysis of variance (ANOVA) and Tukey's test.
† Positive control of DPPH radical scavenging assay.
†† Positive control of superoxide and nitric oxide radical scavenging assays.

volved in the cascade of inflammation in acne, including PGE_2. In this study, the anti-inflammatory activities of *M. alba* stem extract and its bioactive component, oxyresveratrol, were confirmed through the inhibition of PGE_2 production. The results are shown in Figure 1. It is clear that the stem extract as well as oxyresveratrol could significantly inhibit PGE_2 production compared to LPS-induced RAW 264.7 cells without treatment. These results match our previous study in which the extract inhibited COX-2, an inducible enzyme that mediates the conversion of arachidonic acid to prostaglandins (Soonthornsit, Pitaksutheepong, Hemstapat, Utaisincharoen, & Pitaksuteepong, 2017). The higher inhibitory effect of the stem extract compared to oxyresveratrol may be due to other flavonoid and stilbenoid compounds present in the extract, as discussed above.

3.5 Anti-tyrosinase activity

Hyperpigmentation is a common consequence, occurring after a skin injury or inflammation from acne. Niacinamide, arbutin and kojic acid are well-known skin lighteners. In order to evaluate the skin lightening effect of *M. alba* stem extract and its major compound (i.e oxyresveratrol), mushroom tyrosinase inhibition assay was performed. Interestingly, the extract and oxyresveratrol exhibited potent anti-tyrosinase activities and were significantly more effective than kojic acid, a well-known tyrosinase inhibitor (Figure 2).

4. Conclusions

This is the first study reporting the specific anti-*P. acnes* activity of *M. alba* stem extract. Oxyresveratrol, a major component in the extract, may contribute to this effect. Both the extract and oxyresveratrol were shown to possess antioxidant effects, especially on NO production. Their anti-inflammatory activities were observed through the inhibition of PGE_2, a potent inflammatory mediator. In addition, both the extract and oxyresveratrol showed anti-tyrosinase activities, which is beneficial for reducing post-inflammatory hyper-pigmentation. These findings suggest that the *M. alba* stem extract has potential for use in topical cosmeceutical formulations for treatment of inflammatory acne, and is recommended for use at early stages before abscesses are evident.

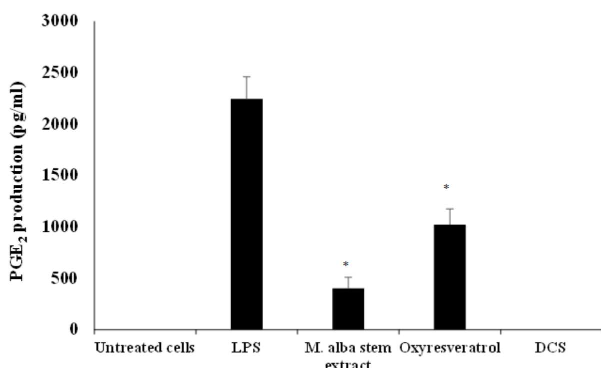

Figure 1. Inhibition of PGE_2 production in LPS stimulated RAW 264.7 cells by the *M. alba* stem extract (50 µg/ml), oxyresveratrol (7.5 µg/ml) and diclofenac (DCS; 1 µg/ml). * significantly different ($p<0.05$) from LPS-stimulated cells without treatment.

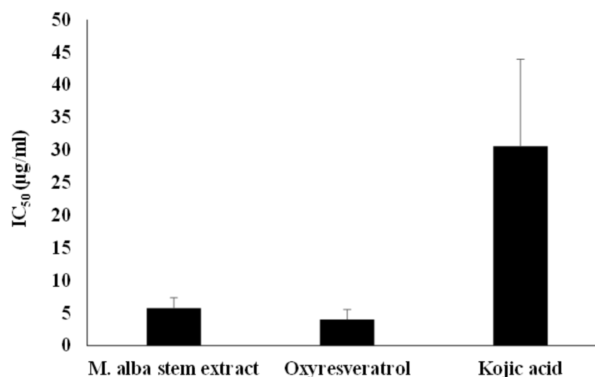

Figure 2. Mushroom tyrosinase inhibitions (IC_{50}) by the *M. alba* stem extract, oxyresveratrol and kojic acid.

Acknowledgements

This research was funded by the Agricultural Research Development Agency (Public Organization) (ARDA), Bangkok, Thailand, grant number CRP6105020880. We are also grateful to the Naresuan University, Phitsanulok, Thailand for the annual revenue (grant number R2561B003) and the Graduate student scholarship academic year 2015 for Ms Srihaphon and Ms Wongwat. Many thanks are due to the Queen Sirikit Sericulture Center (Tak, Thailand) for supplying the plant materials and to the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation for facility support.

References

Arican, O., Kurutas, E. B., & Sasmaz, S. (2005). Oxidative stress in patients with acne vulgaris. *Mediators of Inflammation, 2005*(6), 380-384. doi:10.1155/MI. 2005.380

Chan, E. W. C., Lye, P. Y., & Wong, S. K. (2016). Phytochemistry, pharmacology, and clinical trials of *Morus alba*. *Chinese Journal of Natural Medicines, 14*(1), 17-30.

Choi, S. W., Jang, Y. J., Lee, Y. J., Leem, H. H., & Kim, E. O. (2013). Analysis of functional constituents in mulberry (*Morus alba* L.) twigs by different cultivars, producing areas, and heat processings. *Preventive Nutrition and Food Science, 18*(4), 256-262.

Clinical and Laboratory Standards Institute. (2013). *Performance standards for antimicrobial susceptibility testing, twenty-third informational supplement, M100-S23*. Wayne, PA: Clinical and Laboratory Standards Institute.

Dessinioti, C., & Katsambas, A. D. (2010). The role of *Propionibacterium acnes* in acne pathogenesis: facts and controversies. *Clinical in Dermatology, 28*(1), 2-7.

Grange, P. A., Weill, B., Dupin, N., & Batteux, F. (2010). Does inflammatory acne result from imbalance in the keratinocyte innate immune response? *Microbes and Infection, 12*(14-15), 1085–1090.

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., & Götz, F. (1996). Molecular basis of

intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Molecular Microbiology, 20*(5), 1083-1091.

Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., . . . Mizunoe, Y. (2010). *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. *Nature, 465*(7296), 346-349. doi:10.1038/nature09074

Jang, S., Lee, Y., Hwang, S. L., Lee, M. H., Park, S. J., Lee, I. H., . . . Kim, C. D. (2007). Establishment of type II 5α-reductase over-expressing cell line as an inhibitor screening model. *The Journal of Steroid Biochemistry and Molecular Biology, 107*(3-5), 245-252. doi:10.1016/j.jsbmb.2007.03.039

Lawrence, C. A., & Block, S. S. (1986). Disinfection, sterilization and preservation. Philadelphia, PA: Lea and Febiger.

Leyden, J. J. (1997). Therapy for acne vulgaris. *The New England Journal of Medicine, 336,* 1156-1162.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J., & Cheng, S. (2006). Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chemistry, 96*(2), 254-260. doi:10.1016/j.foodchem.2005.02.033

Nagy, I., Pivarcsi, A., Koreck, A., Széll, M., Urbán, E., & Kemény, L. (2005). Distinct strains of *Propionibacterium acnes* induce selective human beta-defensin-2 and interleukin-8 expression in human keratinocytes through toll-like receptors. *Journal of Investigative Dermatology, 124*(5), 931-938.

Ngajow, M., Abidjulu, J., & Kamu, V. S. (2013). The antibacterial activity of matoa bark extract (*Pometia pinnata*) againts *Staphylococcus aureus*. *Jurnal MIPA Unsrat Online, 2,* 128-132.

Nicco, C., Laurent, A., Chereau, C., Weill, B., & Batteux, F. (2005). Differential modulation of normal and tumor cell proliferation by reactive oxygen species. *Biomedicine and Pharmacotherapy, 59*(4), 169-174.

Nishijima, S., Kurokawa, I., Katoh, N., & Watanabe, K. (2000). The bacteriology of acne vulgaris and anti-microbial susceptibility of *Propionibacterium acnes* and *Staphylococcus epidermidis* isolated from acne lesions. *Journal of Dermatology, 27*(5), 318-323. doi:10.1111/j.1346-8138.2000.tb02174.x

Sánchez-Salcedo, E. M., Amorós, A., Hernández, F., & Martínez, J. J. (2017). Physicochemical properties of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves, a new food supplement. *Journal of Food and Nutrition Research, 5*(4), 253-261.

Soonthornsit, N., Pitaksutheepong, C., Hemstapat, W., Utaisincharoen, P., & Pitaksuteepong, T. (2017). *In vitro* anti-inflammation activity of *Morus alba* L. stem extract in LPS-stimulated RAW 264.7 cells. *Evidence-Based Complementary and Alternative Medicine.* doi:10.1155/2017/3928956

Spellman, M. C., & Pincus, S. H. (1998). Efficacy and safety of azelaic acid and glycolic acid combination therapy compared with tretinoin therapy for acne. *Clinical Therapeutics, 20*(4), 711–721.

Tan, J. K. L., & Bhate, K. (2015). A global perspective on the epidemiology of acne. *British Journal of Dermatology, 172*(S1), 3-12.

Thongsuk, P. (2007). *In-vitro and clinical study of mulberry extract for skin whitening product* (Master's thesis, Naresuan University, Phitsanulok, Thailand).

Walsh, T. R., Efthimiou, J., & Dréno, B (2016). Systematic review of antibiotic resistance in acne: an increasing topical and oral threat. *Lancet Infectious Diseases, 16*(3), e22-32. doi:10.1016/S1473-3099(15)00527-7

Wang, Y., Kuo, S., Shu, M., Yu, J., Huang, S., Dai, A., . . . Huang, C. M. (2014). *Staphylococcus epidermidis* in the human skin microbiome mediates fermentation to inhibit the growth of *Propionibacterium acnes*: Implications of probiotics in acne vulgaris. *Applied Microbiology and Biotechnology*, *98*(1), 411-424. doi:10.1007/s00253-013-5394-8

Wong, A., Zhang, B., Jiang, M., Gong, E., Zhang, Y., & Lee, S. W. (2016). Oxidative stress in acne vulgaris. *HSOA Journal of Clinical Dermatology and Therapy, 3,* 020. doi:10.24966/CDT-8771/100020