



Original Article

## Antioxidant activity of mulberry stem extract: A potential used as supplement for oxidative stress-related diseases

Phi Phuong Pham<sup>1</sup>, Noppawan Phumala Morales<sup>1</sup>, Tasana Pitaksuteepong<sup>2</sup>,  
and Warinkarn Hemstapat<sup>1\*</sup>

<sup>1</sup> Department of Pharmacology, Faculty of Science,  
Mahidol University, Ratchathewi, Bangkok, 10400 Thailand

<sup>2</sup> Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences,  
Naresuan University, Mueang, Phitsanulok, 65000 Thailand

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### Abstract

Overproduction of reactive oxygen species is involved in many diseases, including cardiovascular, neurodegenerative diseases, diabetes, cancer, viral and bacterial infections as well as osteoarthritis. Although antioxidant activity of *Morus alba* L. has been investigated in various parts of this plant, a little attention has been paid to the stems of this plant. Therefore, the present study was designed to systematically investigate the antioxidant activity of *M. alba* stem extract using various *in vitro* antioxidant assay systems. The present data showed that the stem extract of *M. alba* exhibited a hydrogen-donating ability, an ability to quench hydroxyl radicals, having superoxide and nitric oxide scavenging activity as well as iron reducing capacity. This study highlights the potential of this plant for further development as a natural source of antioxidant or as an alternative treatment for oxidative stress-related diseases.

**Keywords:** *Morus alba* L., oxyresveratrol, oxidative stress, antioxidant activity, osteoarthritis

### 1. Introduction

Oxidative stress is a condition where the levels of reactive oxygen species (ROS) are significantly overwhelming. Various evidence has suggested that oxidative stress has an essential role in many diseases, including cardiovascular and neurodegenerative diseases, diabetes, cancer, viral and bacterial infections as well as osteoarthritis (OA) (Gospodaryov & Lushchak, 2012). Although currently a full understanding on the pathologic state of cartilage in OA remains unclear, ROS have been implicated as playing a crucial role in the progression of this disease (Aigner & Schmitz, 2011). In inflammatory joint disease, there is an increase in blood flow to the joint, causing an increase in vascular permeability of the joint capsule as well as infiltration

of pro-inflammatory cells into the synovium, leading to an increase in the production of ROS (Anandarajah, 2011). The elevated ROS in cartilage also leads to chondrocyte activation resulting in the production of inflammatory cellular products and chondrocyte death (Anandarajah, 2011). ROS has also been shown to upregulate the production of matrix metalloproteinases (MMPs) and decrease the production of tissue inhibitors of metalloproteinases (TIMPs), causing the direct degradation of proteoglycans and collagen of the cartilage (Rothschild & Woods, 2012). Therefore, an inhibition of ROS production by using an antioxidant therapy may offer an interesting approach for the treatment of OA. Antioxidant can prevent, reduce, or repair the ROS-induced damage (Gospodaryov & Lushchak, 2012; Li, 2011). Natural sources of antioxidants, exogenous compounds derived from dietary plants such as polyphenolic compounds and flavonoids, have also been reported to be an excellent rich source of antioxidants.

\* Corresponding author.

Email address: warinkarn.hem@mahidol.ac.th

*Morus alba* L., commonly known as white mulberry, is cultivated worldwide, in particular in many Asian countries, including China, Vietnam and Thailand. It has long been widely used for different medicinal purposes. Several different parts of white mulberry such as leaves, fruits, root bark, wood and twigs have been shown to exhibit a number of health benefits, including anti-hyperglycemic (Naowaboot, Pannangpetch, Kukongviriyapan, Kongyingoes, & Kukongviriyapan, 2009; Zhang *et al.*, 2009), hypolipidemic (Yang, Yang, & Zheng, 2010), antioxidative effects (Chang *et al.*, 2011), anti-ulcerogenic activity (Abdulla, Ali, Ahmed, Noor, & Ismail, 2009) as well as anti-inflammatory effects (Choi & Hwang, 2005; Chung *et al.*, 2003). These health benefits have been suggested to be attributed to the presence of phenolic constituents such as flavonoids, hydroxystilbenes, anthocyanin, isoquercitrin and etc. (Chang *et al.*, 2011; Doi, Kojima, & Fujimoto, 2000).

In particular, oxyresveratrol (2,3',4,5'-tetrahydroxystilbene), hydroxystilbene compound, which has been reported to present at a higher concentration in the stem than in the leaves and twigs (Thongsuk, 2007), exhibited several health benefits similar to its well-known analog, resveratrol. However, it was found to possess stronger antioxidative capacity, but with less cytotoxicity than resveratrol in several *in vitro* model systems (Chung *et al.*, 2003; Lorenz, Roychowdhury, Engelmann, Wolf, & Horn, 2003). Based on our previous study, oxyresveratrol obtained from stem has demonstrated to have the highest antioxidant activity by DPPH assay compared to twigs and leaves, respectively (Thongsuk, 2007). Despite the abundance of active compounds in the stems there has been little focus on this part of the plant, i.e. the stem as a source of medicines. Thus, the present study was designed to systematically investigate the antioxidant activity of an ethanolic extract of *M. alba* obtained from the stem by using various *in vitro* antioxidant assay systems.

## 2. Materials and Methods

### 2.1 Chemicals

Ethanolic *M. alba* stem extract and oxyresveratrol were prepared as described in our previous report (Soonthornsit & Pitaksuteepong, 2012). The content of oxyresveratrol in the stem extract analyzed using HPLC was found to be 17.86% (w/w) (Noi-ang *et al.*, 2015; Soonthornsit & Pitaksuteepong, 2012). Gallic acids (GA), trolox, quercetin (QU), ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), (-) riboflavin, nitroblue tetra-zolium (NBT), superoxide dismutase (SOD), sodium nitro-prusside (SNP), N-1-naphthylethylenediamine dichloride (NED), ethylenediaminetetraacetic acid disodium salts (EDTA), GA, QU, sulfanilamide, phosphoric acid ( $H_3PO_4$ ), thiobarbituric acid (TBA) and sodium nitrite ( $NaNO_2$ ) were purchased from Sigma Aldrich (St. Louis, MO). Folin-Ciocalteu reagent, ethanol, aluminium chloride ( $AlCl_3$ ) and sodium carbonate ( $Na_2CO_3$ ) were obtained from Merck

(Darmstadt, Germany). Trichloro acetic acid (TCA) was purchased from Reiedel-de Haën laboratory chemicals (Seelze, Germany).

### 2.2 Determination of total phenolic content (TPC)

The total phenolic content of the stem extract was determined using the Folin-Ciocalteu method as previously described with slight modifications, in which GA was used as a standard antioxidant (Chumark *et al.*, 2008). Briefly, 100  $\mu$ L of diluted extract solution at various concentrations (5-30  $\mu$ g/mL) or GA solution in 50 % methanol was mixed with 500  $\mu$ L Folin-Ciocalteu reagent (10% v/v). The mixtures were carefully vortexed and incubated at room temperature for 2 min. Then 400  $\mu$ L of 7.5%  $Na_2CO_3$  solution was added to the mixtures and these were further incubated for 60 minutes at room temperature. The absorbance of the reaction mixtures was then measured at 765 nm by using a spectrophotometer (GBC Cintra 10e, Melbourne, Australia). Total phenolic content was expressed as GA equivalents (GAE), which was calculated using the standard antioxidant regression curve. The result was expressed in  $\mu$ mol GAE/mg of dried extract. The experiment was conducted in triplicate with duplicate measurement each time.

### 2.3 Determination of total flavonoid content

The total flavonoid content was determined using aluminium colorimetric method as previously described with slight modifications, in which QU was used as a standard antioxidant (Omoruyi, Bradley, & Afolayan, 2012). All test samples were dissolved in 50% methanol (vehicle). Five hundred microliters of vehicle, QU, or the stem extract at various concentrations (50-800  $\mu$ g/mL) were mixed with 500  $\mu$ L of 2%  $AlCl_3$  and incubated for 60 minutes at room temperature, in which a developing of bright yellow color was observed. The absorbance of the reaction mixtures was measured at 420 nm by using a spectrophotometer. Total flavonoid content was expressed as QU equivalents (QUE), which was calculated using the standard antioxidant regression curve. The result was expressed in  $\mu$ mol QUE/mg of dried extract. The experiment was conducted in triplicate with duplicate measurement each time.

### 2.4 DPPH radical scavenging assay

The free radical scavenging activity of the extracts was assessed by DPPH radical scavenging assay based on the method described previously with some modifications (Roche, Dufour, Mora, & Dangles, 2005). All test samples were dissolved as a stock solution in 100% methanol (vehicle) and further diluted to the following concentration: the stem extract (0.1 mg/mL), trolox (0.1 mM; standard antioxidant) and oxyresveratrol (0.5 mM) solutions. A fixed volume of 1 mM DPPH (100  $\mu$ L) was mixed with varying concentrations of the

stem extract (5-40 µg/mL), different concentrations of trolox or purified oxyresveratrol to obtain a 1 mL of the reaction mixture. The mixtures were then incubated at room temperature for 3 min, and the absorbance was assessed immediately at 515 nm by using a spectrophotometer. A percentage of DPPH radical scavenging activity was calculated according to the following formula: scavenging activity (%) =  $[A - B / A] \times 100\%$ , where: A is the absorbance of a blank sample (vehicle); B is the absorbance of a sample (stem extract, oxyresveratrol or trolox). The percentage of inhibition was plotted against the concentration of the test compounds and the  $IC_{50}$  was obtained. Trolox equivalent (TE) (values were calculated using the standard antioxidant regression curve and the result was expressed in µmol TE/mg of dried extract.

### 2.5 Scavenging of hydroxyl radical by deoxyribose method

The hydroxyl radical scavenging activity of the extract was determined using deoxyribose method based on the method described previously with some modifications (Srinivasan, Chandrasekar, Nanjan, & Suresh, 2007). All test samples were prepared in 50% methanol (vehicle). One hundred microliters of various concentrations of the stem extract (0.785-50 µg/mL), trolox (standard antioxidant) or oxyresveratrol solutions were added to the reaction mixture containing deoxyribose (14 mM, 200 µL), ferrous sulphate (2.5 mM, 100 µL), normal saline (400 µL) and hydrogen peroxide (4 mM, 200 µL) to give a total volume of 1 mL. The mixtures were then incubated for 60 min at 37°C. After incubation, TBA (300 µL, 1% in 50 mM NaOH) and TCA (300 µL, 2.8%) were added to the mixtures. The reaction mixtures were then incubated in a boiling water bath for 10 min and were allowed to cool down at room temperature prior to measuring the absorbance at 532 nm. The  $IC_{50}$  of all test compounds and TE of the extract and oxyresveratrol were calculated from the curves of scavenging activity (%) by natural logarithm of the solvent concentrations.

### 2.6 Superoxide scavenging assay

The superoxide anion ( $O_2^{\cdot-}$ ) scavenging activity of the extract was determined based on the capacity of the samples to enhance the aerobic photochemical reduction of NBT in the presence of riboflavin according to the method described previously with some modifications (Chaabane *et al.*, 2012). All test samples were dissolved in 50% methanol (vehicle). The reaction mixtures were composed of 100 µL EDTA (65 mM), 200 µL riboflavine (40 µM), 200 µL NBT (960 µM) and 400 µL phosphate buffer (50 mM, pH 7.4). The mixtures were then added with 100 µL of the stem extract solution (50-800 mg/mL), oxyresveratrol (reference), quercetin (standard antioxidant) or SOD to get the final volume of the reaction mixtures of 1 mL. Fifty percent methanol was used as a blank, while SOD was used as a positive control. A heated SOD was used to verify the scavenging effect of nonenzymatic-protein,

which was heated for 10 min in boiling water bath prior to use. The mixtures were then incubated for 30 min at 30°C and the absorbance was measured at 560 nm immediately. The  $IC_{50}$  and QUE were calculated from the curves of scavenging activity (%) by natural logarithm of the solvent concentrations.

### 2.7 Nitric oxide scavenging assay

The determination of nitric oxide (NO) scavenging activity of the stem extract was based on the previous studies with some modifications (Omoruyi *et al.*, 2012; Rabelo *et al.*, 2013). All test samples were prepared in 50% methanol (vehicle). In brief, one milliliter of 10 mM SNP that was freshly prepared in distilled water was first mixed with 250 µL of phosphate buffer saline (pH 7.4) and 250 µL of the stem extract solution (0.17-166.67 mg/mL) or oxyresveratrol at various concentrations, and incubated further at room temperature for 60 min (incubation 1). An aliquot of 500 µL of the solution was mixed with 500 µL of 1% sulfanilamide in 2%  $H_3PO_4$ , and incubated at room temperature for 5 min (incubation 2). Following the second incubation, 500 µL of 0.1% NED was added to the mixture and incubated for 10 min at room temperature (final incubation). The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with NED was measured at 546 nm as indicated by a color-stable compound (purple color). Sodium nitrite at various concentrations was used as a standard antioxidant for nitrite ions ( $NO_2^-$ ) to construct the curve for calculating of amount of NO. The  $IC_{50}$  of the extract and oxyresveratrol were calculated from the curves of scavenging activity (%) by natural logarithm of the solvent concentrations.

### 2.8 Ferric ion reducing antioxidant power (FRAP) assay

The ferric ion reducing capacity of the extract was evaluated using FRAP assay, which was performed according to the method described previously with some modifications (Benzie & Strain, 1996). In brief, one milliliter of FRAP reagent was mixed with 50 µL of various concentrations of ferrous sulfate ( $FeSO_4 \cdot 7H_2O$  solution; standard antioxidant) and the stem extract solution (50-300 µg/mL), or oxyresveratrol. The reaction mixtures were then incubated at room temperature for 3 min and the absorbance of reaction mixtures was measured at 593 nm by using a spectrophotometer. The regression equation of the standard antioxidant was used to calculate the FRAP values (µmol Fe (II) per mg) of the dry stem extract.

### 2.9 Statistical data analysis

The results in this study are presented as descriptive data and therefore all the data values are expressed as the mean ± standard error of the mean (SEM). Analysis was implemented in GraphPad Prism (v.6.0).

### 3. Results

#### 3.1 Total phenolic content and total flavonoids content:

The amounts of total phenolic and total flavonoid of the *M. alba* stem extract are shown in Table 1. GA and QU were used as the standard antioxidant, in which the total phenolic content and total flavonoid content of the extract were expressed in micromole (mmol) of GAE per milligram of dried extract (mmol GAE/mg extract) and  $\mu\text{mol}$  of QUE per milligram of dried extract (mmol QUE/mg extract), respectively. The total phenolic content was  $34.87 \pm 1.12 \mu\text{mol GAE/mg extract}$  and the total flavonoid content was  $0.13 \pm 0.00 \mu\text{mol QUE/mg extract}$ .

#### 3.2 *In vitro* antioxidant capacity assays:

Several radical scavenging assays were conducted in this study, including DPPH radical, hydroxyl radical, superoxide radical, nitric oxide radical as well as FRAP assays. The free radical scavenging activities of standard antioxidant (trolox or QU), oxyresveratrol and extract increased dose-dependently in various assays and their  $\text{IC}_{50}$  values are summarized in Table 2. The  $\text{IC}_{50}$  values of the standard antioxidant, extract and oxyresveratrol were  $6.46 \pm 0.05 \mu\text{g/mL}$ ,  $26.62 \pm 0.96 \mu\text{g/mL}$  and  $13.5 \pm 0.48 \mu\text{g/mL}$ , respectively for DPPH radical assay;  $1.49 \pm 0.03 \mu\text{g/mL}$ ,  $5.16 \pm 0.28 \mu\text{g/mL}$  and  $3.34 \pm 0.16 \mu\text{g/mL}$ , respectively for hydroxyl radical assay;  $65.30 \pm 7.03 \mu\text{g/mL}$ ,  $173.73 \pm 12.66 \mu\text{g/mL}$  and no scavenging activity, respectively for superoxide radical, and no scavenging activity,  $12.19 \pm 1.03 \mu\text{g/mL}$  and  $8.06 \pm 0.30 \mu\text{g/mL}$ , respectively for NO radical assay.

Table 1. Total phenolic content and total flavonoid content of the ethanolic stem extract of the *M. alba*

Assays	Content
Total Phenolic Content ( $\mu\text{mol GAE/mg extract}$ )	$34.87 \pm 1.12$
Total Flavonoid Content ( $\mu\text{mol QUE/mg extract}$ )	$0.13 \pm 0.00$

In addition, the free radical scavenging activities of the extract were also expressed in mmol TE/mg dried extract for DPPH and  $\text{HO}^\bullet$  scavenging assays, in mmol QUE/mg dried extract for  $\text{O}_2^{\bullet-}$  scavenging assay, or in mmol ascorbic acid equivalent (AAE)/mg dried extract for FRAP assay, depending on the standard antioxidant used in each assay (Table 3). However, for the NO radical scavenging activity the data were expressed as nmol of scavenged NO by 1 mg of a test compound.

Antioxidant capacities of the extract and oxyresveratrol were  $0.98 \pm 0.05$  and  $2.04 \pm 0.09 \mu\text{mol TE/mg}$  of test compound for DPPH scavenging assay and  $1.11 \pm 0.08$  and  $1.63 \pm 0.03 \mu\text{mol TE/mg}$  of test compound for  $\text{HO}^\bullet$  scavenging assays, respectively. In addition, antioxidant capacity of the extract was  $1.13 \pm 0.16 \mu\text{mol QUE/mg}$  of test compound, but undetectable for oxyresveratrol in  $\text{O}_2^{\bullet-}$  scavenging assay. These results indicate that the extract has a hydrogen-donating ability and the ability to quench hydroxyl radicals as well as having superoxide scavenging activity. Similar to the extract, oxyresveratrol also demonstrated antioxidant capacity albeit superoxide scavenging activity was undetectable. In the FRAP assay, the reducing capacity of the extract and oxyresveratrol were  $1.29 \pm 0.15$  and  $4.27 \pm 0.28 \mu\text{mol AAE/mg}$  of test compound, suggesting that the stem extract and

Table 2. The  $\text{IC}_{50}$  of the *M. alba* stem extract and oxyresveratrol in scavenging assays

Scavenging assays	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )		
	Standard antioxidant	Extract	Oxyresveratrol
<sup>a</sup> DPPH	$6.46 \pm 0.05$	$26.62 \pm 0.96$	$13.5 \pm 0.48$
<sup>a</sup> $\text{HO}^\bullet$	$1.49 \pm 0.03$	$5.16 \pm 0.28$	$3.34 \pm 0.16$
<sup>b</sup> $\text{O}_2^{\bullet-}$	$65.30 \pm 7.03$	$173.73 \pm 12.66$	N/A
NO	N/A	$12.19 \pm 1.03$	$8.06 \pm 0.30$

<sup>a</sup>Trolox was used as a standard antioxidant;

<sup>b</sup>Quercetin was used as a standard antioxidant.

Table 3. Antioxidant capacities of the *M. alba* stem extract and oxyresveratrol

Scavenging Assays	Antioxidant capacities	
	Extract	Oxyresveratrol
DPPH ( $\mu\text{mol TE/mg}$ of test compound)	$0.98 \pm 0.05$	$2.04 \pm 0.09$
$\text{HO}^\bullet$ ( $\mu\text{mol TE/mg}$ of test compound)	$1.11 \pm 0.08$	$1.63 \pm 0.03$
$\text{O}_2^{\bullet-}$ ( $\mu\text{mol QUE/mg}$ of test compound)	$1.13 \pm 0.16$	N/A
FRAP ( $\mu\text{mol AAE/mg}$ of test compound)	$1.29 \pm 0.15$	$4.27 \pm 0.28$
NO (nmol of scavenged NO/ $\mu\text{g}$ of test compound)	$1.88 \pm 0.36$	$2.23 \pm 0.03$

oxyresveratrol have a reducing activity which can prevent the tendency of oxidation reaction in the *in vitro* system. Moreover, the ability of the extract and oxyresveratrol for nitric oxide radical scavenging activity were  $1.88 \pm 0.36$  and  $2.23 \pm 0.03$  nmol of scavenged NO/ $\mu\text{g}$  of test compound, respectively.

### 3.3 Contribution of oxyresveratrol in the total antioxidant capacity of the extract:

The present study was also designed to evaluate how much extent oxyresveratrol contributes to the total antioxidant capacity of this plant extract. The antioxidant capacities of oxyresveratrol equivalent (OXE) of the extract in each assay were expressed as the amount of  $\mu\text{g}$  of oxyresveratrol in one  $\mu\text{g}$  of the dried extract (Table 4). Since the percent yield of oxyresveratrol obtained from the stem extract is approximately 17.86% (w/w), it plausible to estimate that one microgram of the extract contains approximately 0.18  $\mu\text{g}$  of oxyresveratrol. However, in the present finding, it was found that the antioxidant capacities of OXE of the extract from all the assays, excepted for  $\text{O}_2^{\cdot-}$  scavenging assay, were higher than expected, which were  $0.50 \pm 0.02$ ,  $0.69 \pm 0.06$ ,  $0.36 \pm 0.00$  and  $0.67 \pm 0.05$   $\mu\text{g}$  OXE/ $\mu\text{g}$  dried extract for DPPH scavenging,  $\text{HO}^{\cdot}$  scavenging, FRAP and NO scavenging assays, respectively.

## 4. Discussion

The health benefits of using plant-based dietary supplements have recently become the focus of attention in OA research (Mobasheri, 2012; Shen *et al.*, 2012). Hydroxystilbenes, naturally occurring polyphenolic compounds, are known for their free radical scavenging capacities (Lorenz *et al.*, 2003). For instance, resveratrol, a well-known representative of this group found in the grape skin, berries and red wine, is a potent antioxidant and free radical scavenger that has been shown to possess several pharmacological properties, including anti-inflammatory, anti-apoptotic and antioxidant activities (Elmali *et al.*, 2005; Lei *et al.*, 2012; Wang, Gao, Chen, Li, & Tian, 2012). These properties are thought to contribute to its potential OA-protective effects (Shen *et al.*, 2012). Therefore, it was suggested that dietary polyphenols may be used as dietary supplement to alleviate the associated

symptom of OA (Shen *et al.*, 2012).

Oxyresveratrol is structurally analogous to resveratrol, but with an additional hydroxyl group compared to resveratrol (Lorenz *et al.*, 2003). The stem extract of *M.alba* has been reported to contain oxyresveratrol at the highest amount in comparison to twig and leave extracts, respectively (Thongsuk, 2007). In addition, oxyresveratrol was found to exhibit stronger antioxidative capacity than resveratrol in several *in vitro* model systems, including DPPH and NO radical scavenging assays and in  $\text{FeSO}_4/\text{H}_2\text{O}_2$ -induced lipid peroxidation in rat liver microsomes model (Chung *et al.*, 2003; Lorenz *et al.*, 2003). An additional hydroxyl group of oxyresveratrol was postulated to be responsible for its higher potency (Lorenz *et al.*, 2003). Furthermore, oxyresveratrol is less toxic and more soluble in aqueous solution than resveratrol (Lorenz *et al.*, 2003). Based on these findings, the present study was focused on determining the antioxidative properties of the stem extract of *M.alba* as well as oxyresveratrol.

It has been suggested that medicinal plants containing phenolic compounds may offer the protective effects on the progression of OA as well as alleviating OA pain (Mobasheri, 2012; Shen *et al.*, 2012). Consistent with this, the recent result from our group has shown that the stem extract of *M. alba*, a plant containing oxyresveratrol exhibited pain-relieving effect in the rat model of OA (Khunakornvichaya *et al.*, 2016). Although, the precise mechanisms involving its analgesic effect remain unclear, the presence of the polyphenolic hydroxystilbene, oxyresveratrol, may contribute to this efficacy in OA. Albeit a number of studies have been reported around the free radical scavenging activity from different parts of *M. alba*, the antioxidant capacity of the stem extract of this plant has not been reported. In addition, variability in different environmental conditions and regions in the world has the potential to yield different chemical compositions and as such altered biological activities. Therefore, the present study was conducted to confirm its antioxidant capacity of oxyresveratrol and stem extract of *M. alba* obtained from the Queen Sirikit Sericulture Center, Tak Province, in the Northern part of Thailand.

Consistent with previous studies, in which the extract from different parts of *M.alba* have been shown to contain phenolic compounds (Chang *et al.*, 2011; Khan *et al.*, 2013),

Table 4. The antioxidant capacities of the *M.alba* stem extract in scavenging assays compared to that of oxyresveratrol

Assays	Oxyresveratrol equivalent of extract ( $\mu\text{g}$ OXE/ $\mu\text{g}$ dry extract)	Estimated oxyresveratrol fractions by antioxidant activities (% w/w)	Yielded fraction of oxyresveratrol (% w/w)
DPPH scavenging	$0.50 \pm 0.02$	50	
$\text{HO}^{\cdot}$ scavenging	$0.69 \pm 0.06$	69	
$\text{O}_2^{\cdot-}$ scavenging	Undetectable	Undetectable	17.86
FRAP	$0.36 \pm 0.00$	36	
NO scavenging	$0.67 \pm 0.05$	67	

the current study also observed the presence of phenolic components in the stem extract of *M. alba*. In addition, our results are in agreement with previous findings (Chang *et al.*, 2011; Khan *et al.*, 2013), in which the stem extract of *M. alba* exhibited positive results in all the antioxidant assay systems, suggesting that *M. alba* stem extract may exhibit its antioxidant capacity through multiple mechanisms.

The main ROS produced by chondrocytes such as NO and superoxide has been implicated in the pathogenesis of OA as well as OA pain (Abramson, 2008; Henrotin, Bruckner, & Pujol, 2003). In addition, ROS has also been involved in the inflammation and hyperalgesia associated with intraplantar injection of carrageenan in rats (Wang *et al.*, 2004). Wang *et al.* (2004) reported that inflammation and hyperalgesia as well as the formation of peroxynitrite were attenuated by injection of SOD mimetic. Therefore, it was postulated that the SOD mimetic mediated its anti-inflammatory and anti-hyperalgesic effects by removing  $O_2^{\cdot-}$  prior to it interacting with NO, thereby reducing peroxynitrite formation (Wang *et al.*, 2004). It is likely that the analgesic effect of the stem extract of *M. alba* that was previously observed by our group (Khunakornvichaya *et al.*, 2016) may be attributed to its ability to scavenge the main ROS produced by chondrocytes.

The present study also determined the extent of oxyresveratrol that contributed to the total antioxidant capacity in comparison to the stem extract. Since the content of oxyresveratrol in the stem extract is 17.86% (w/w), therefore it could be estimated that in 1  $\mu$ g of the extract contains approximately 0.18  $\mu$ g of oxyresveratrol. However, this was not the case, as it was found that the antioxidant capacities of oxyresveratrol relative to the stem extract were higher than expected ( $> 0.18 \mu$ g), suggesting that oxyresveratrol contributed, at least in part, to the total antioxidant activity. In addition, it is plausible that other phenolic constituents such as flavonoids present in the stem extract may contribute to the remaining activity.

It has been reported that the number and the position of phenolic hydroxyl groups on the aromatic ring are crucial for their free radical scavenging activity (Cai, Mei, Jie, Luo, & Corke, 2006; Heim, Tagliaferro, & Bobilya, 2002; Lorenz *et al.*, 2003). For instance, Lorenz and colleagues (2003) suggested that phenolic hydroxyl groups are essential for the aromatic stilbene system activation, and that at least one OH group is required for a radical scavenging reaction of the stilbenes. These authors also suggested that the scavenging capacity increases with the number of hydroxyl groups in the molecule. The present study found that, although oxyresveratrol contains 4 hydroxyl groups on the aromatic ring, its antioxidant capacity was approximately 2-fold lower than that of trolox (Table 5). Since one molecule of trolox can scavenge approximately two molecules of peroxy radical (Liegeois, Lermusieau, & Collin, 2000), this suggests that not all of hydroxyl groups in oxyresveratrol molecule exhibited the same scavenging activity. Moreover, there may be other factors that contribute to the antioxidant capacity of the compound.

Table 5. Antioxidant capacities of oxyresveratrol when compared with trolox

Assays	Trolox equivalent of oxyresveratrol ( $\mu$ mol TE/ $\mu$ mol)
DPPH scavenging	0.50 $\pm$ 0.04
HO <sup>•</sup> scavenging	0.40 $\pm$ 0.01

The present study was the first to investigate the antioxidant capacities of the ethanolic stem extract of *M. alba* by using various antioxidant assay systems. In addition, this finding may provide a promising candidate to be used as dietary supplements for reducing OA pain or an alternative treatment for oxidative stress-related diseases. Although the precise mechanisms responsible for the efficacy of this plant extract cannot be explained in the present study, it is possible that its analgesic effect could be attributed to its antioxidant properties. However, further study is required to evaluate the relationship between antioxidant capacity and pain relieving effect of this plant extract in animal models as well as to fully define the precise mechanisms involved.

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