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Original Article

In vitro antioxidant potential of *Mallotus repandus* (Willd.) Muell. Arg stem extract and its active constituent bergenin

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Abstract

Mallotus repandus (MR) is used in Thai traditional medicine recipes for anti-inflammation and adaptive homeostasis. Bergenin is the bioactive constituent in MR stem. This study evaluated the *in vitro* antioxidant activity of bergenin and the methanol, ethanol, and water extracts of MR stem prepared by maceration and Soxhlet extraction by assaying 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$), hydroxyl ($^{\bullet}OH$), superoxide ($^{\bullet}O_2$), and nitric oxide ($NO^{\bullet}$) radical scavenging activities and ferric reducing antioxidant power. Bergenin had the highest ferric reducing power and all the MR extracts showed concentration-dependent ferric reducing power. Methanol and water extracts showed superior radical scavenging activities compared to ethanol extract. The water and methanol Soxhlet extracts demonstrated the highest $ABTS^{\bullet+}$ and $^{\bullet}OH$ radical scavenging activities, respectively, and the macerated water extract had the lowest $^{\bullet}O_2$ and $NO^{\bullet}$ IC_{50} values. Therefore, MR stem and bergenin are promising natural candidates for developing antioxidant supplements.

Keywords: *Mallotus repandus*, bergenin, antioxidant activity

1. Introduction

A free radical is defined as an atom or a molecule possessing an unpaired electron, it is an unstable and reactive molecule (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). Free oxygen radicals (reactive oxygen species, ROS) and free nitrogen radicals (reactive nitrogen species, RNS) are common types of free radicals. Both ROS and RNS are generated by endogenous mechanisms, e.g. immune cell activation, during energy production by mitochondria, by mental stress, and by exogenous sources, e.g. drugs, xenobiotics, heavy metals, and radiation (Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). The overproduction of ROS and RNS disrupts the oxidant-antioxidant balance, leading to oxidative and nitrosative stresses, respectively (Fransen, Nordgren, Wang, & Apanasets, 2012). These phenomena damage cells, biomolecules, tissues, and organs, resulting in diseases such as diabetes, atherosclerosis, cardiovascular disorders, liver diseases, and cancer (Phaniendra, Jestadi, & Periyasamy, 2015). Plants are a natural source of antioxidants, such as ascorbic acid, α-tocopherol, tannins, and polyphenols (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Lambert & Elias, 2010). Consumption of plant antioxidants is a strategy to reduce free radicals and maintain an adequate antioxidant status for body homeostasis.

Mallotus repandus (Willd.) Muell. Arg (MR; family Euphobiaceae) is an herb with potential pharmacological applications due to its anti-inflammatory (Hasan *et al.*, 2014a), analgesic (Hasan *et al.*, 2018), and antioxidant activities (Lin, Lin, Chen, Ujiie, & Takada, 1995). MR leaves have been traditionally used to treat fever, inflammation, and rheumatic arthritis in Taiwan (Shyur, Tsung, Chen, Chiu, & Lo, 2005) and MR stem is a base ingredient in numerous recipes of Thai traditional medicine. It is boiled in water to make a drink for the treatment of tendon inflammation, muscle pain, and for maintenance of homeostasis (Wuttidhammaved, 2007). MR

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stem was registered in the national list of essential medicines of Thailand in 2015 as an herb for bone and muscle pain (Bureau of Drug Control: Food and Drug Administration of Thailand, 2015). Bergenin is the major bioactive constituent in the stem of MR (Rivière *et al.*, 2010) accounting for up to 19% by dry weight (Sriset, Chatuphonprasert, & Jarukamjorn, 2018). Bergenin is a polyphenolic compound and is proposed to be a free radical scavenger with antioxidant action by hydrogen or electron donation (Sumino, Sekine, Ruangrungsi, Igarashi, & Ikegami, 2002; Srinivasan, Chandrasekar, Nanjan, & Suresh, 2007). Hence, bergenin was selected as a chemical marker of MR stem antioxidant activity. While some pharmacological activities of MR have been documented, a study of the antioxidant potential of MR and bergenin is needed to understand their mechanisms against free radicals. Therefore, the present study aimed to examine the antioxidant activities of MR stem extracts and bergenin by *in vitro* anti-oxidative assays including 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$), hydroxyl ($^{\bullet}OH$), superoxide ($^{\bullet}O_2$), and nitric oxide ($NO^{\bullet}$) radical scavenging and ferric reducing antioxidant power.

2. Materials and Methods

2.1 Chemicals

Bergenin (Cat. No. BP0258, purity > 98%) was a product of Biopurify Phytochemicals (Chengdu, China). 2,2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), nitriloacetic acid, deoxyribose, thiobarbituric acid (TBA), phenazinemethosulphate (PMS), β-nicotinamide adenine dinucleotide (NADH), and N-(1-naphthyl) ethylenediamine dihydrochloride (NED) were products of Sigma-Aldrich Chemical (St. Louis, Missouri, USA). Trichloroacetic acid (TCA) was purchased from RCI-Labscan (Bangkok, Thailand). Nitrobluetetrazolium chloride (NBT) was supplied by Applichem (Darmstadt, Germany). All other laboratory chemicals were of the highest purity and obtained from chemical suppliers.

2.2 Plant material and extraction

The *M. repandus* stem was purchased from Yasothon Panich (Yasothon, Thailand) in May, 2016. The specimen was identified as PANPB-MR 2016-001 by a certified botanist, Dr. Piyarat Itharat, Khon Kaen, University, Thailand. The MR specimen was deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University.

The MR stems were dried at 50°C and ground into fine powder. The dried powder was extracted by two methods, namely by Soxhlet extraction and by maceration. An aliquot of plant powder was extracted with methanol, or ethanol for 3 h, or water for 6 h, using a Soxhlet apparatus, while another aliquot was macerated with methanol, ethanol, or water for 3 days. The methanol and ethanol filtrates were evaporated by rotary evaporation before freeze-drying, while the water filtrates were freeze-dried directly. The MR stem extracts were kept at -20°C for further analysis.

The content of bergenin in the extracts was determined as described previously. The water Soxhlet and macerated extracts contained the highest amounts of bergenin (12.67% and 19.38%, respectively) followed by the methanol (9.28% and 8.73%) and ethanol extracts (2.16% and 1.73%) (Sriset, Chatuphonprasert, & Jarukamjorn, 2018).

2.3 Determination of total phenolic content

The total phenolic content of the MR stem extracts was determined by Folin-Ciocalteu method with some modifications **(**Chatuphonprasert & Jarukamjorn, 2012**)**. In brief, the extract was mixed with Folin-Ciocalteu reagent and kept in the dark for 30 min. Absorbance was measured at a wavelength of 700 nm. The total phenolic content was calculated from a calibration curve for the standard gallic acid and is expressed as mg of gallic acid equivalent per g of dry extract (mg GAE/g).

2.4 Determination of total flavonoid content

The total flavonoid content of the MR stem extracts was determined by the aluminium chloride ($AlCl_3$) colorimetric method with some modifications **(**Chatuphonprasert & Jarukamjorn, 2012**)**. A reaction mixture containing 10% $AlCl_3$ and 1 M sodium acetate (1:1) was added to the extract. After incubation at room temperature for 30 min, the absorbance was measured at a wavelength of 405 nm. The total flavonoid content was calculated equivalent weight of standard quercetin (mg QE/g).

2.5 Determination of percent contribution of tannin

Determination of the percent contribution of tannin was performed based on the principle that polymeric tannin pigments are resistant to bisulfite bleaching (Sinthorn, Chatu phonprasert, Chulasiri, & Jarukamjorn, 2016). At first, the color density of the extract was calculated from the maximal absorbance in the wavelength range 420-700 nm. After determination of the color density, 20% bisulfite solution was added to the extract to measure the remaining polymeric tannin color fraction over the wavelengths 420-700 nm; the percent contribution of tannin = (polymeric color/color density) X 100.

2.6 $ABTS^{\bullet+}$ radical scavenging activity

The total antioxidant capacity of the MR stem extracts and bergenin was determined by $ABTS^{\bullet+}$ radical cation protocol of Sinthorn, Chatuphonprasert, Chulasiri, and Jarukamjorn (2016) with some modifications. Briefly, ABTS was dissolved in 140 mM potassium persulfate and adjusted to achieve an absorbance of 0.70±0.02 at 700 nm. The $ABTS^{\bullet+}$ solution was mixed with bergenin (or an extract) at a ratio of 20:1 and incubated in darkness at room temperature for 6 min. The absorbance was recorded at a wavelength of 700 nm. The antioxidant activity is expressed as the half maximal inhibitory concentration (IC_{50}). The IC_{50} value was defined as the concentration (mg/mL) to inhibit ABTS radicals by 50%, compared with the standard gallic acid.

2.7 Hydroxyl radical ($^{\bullet}OH$) scavenging activity

Hydroxyl radical ($^{\bullet}OH$) is generated by the ferric (Fe^{3+})-ascorbate-hydrogen peroxide system. The extracts or bergenin were incubated with a freshly prepared reaction mix-

ture of 6.25 µM iron(III) chloride ($FeCl_3$), 25 µM nitriloacetic acid, 700 µM deoxyribose, and 250 µg/mL ascorbic acid at a ratio of 1:1:1:1 and 760 µM hydrogen peroxide at 37°C for 1 h before adding 2.8% TCA and 1% TBA. Then, the mixture was boiled at 100°C for 20 min. The absorbance of thiobarbituric acid reactive substances was measured at a wavelength of 532 nm. The antioxidant activity is expressed as IC_{50} value, defined as the concentration (mg/mL) to inhibit $^{\bullet}OH$ by 50%, compared with the standard gallic acid (Sinthorn, Chatuphon prasert, Chulasiri, & Jarukamjorn, 2016).

2.8 Superoxide radical ($^{\bullet}O_2$) scavenging activity

Superoxide radical ($^{\bullet}O_2$) is produced by the PMS/NADH system. The extracts or bergenin were mixed with the reaction mixture having 73 µM NADH, 50 µM NBT, 15 µM PMS, and 20 mM phosphate buffered saline (PBS) at pH 7.4 before incubation at room temperature for 30 min. The absorbance of the NBT product was measured at a wavelength of 560 nm. The IC_{50} value is defined as the concentration (mg/mL) to inhibit $O_2^{\bullet}$ by 50%, compared with the standard gallic acid (Sinthorn, Chatuphonprasert, Chulasiri, & Jarukam jorn, 2016).

2.9 Nitric oxide radical ($NO^{\bullet}$) scavenging activity

Nitric oxide is generated from sodium nitroprusside and measured by Greiss reaction (Rajeshwar, Kumar, Gupta, & Mazumder, 2005). Nitric oxide was generated from the interaction of sodium nitroprusside in an aqueous solution at physiological pH 7.4 and oxygen to produce nitrite ions (NO_2^-), which were measured by the Griess reagent. The extracts or bergenin were mixed with 10 mM sodium nitroprusside in PBS pH 7.4 at 25°C for 150 min, before adding the Griess reagent (1% sulfanilamide and 0.1% NED in 20% glacial acetic acid; 1:1) at 25°C for 30 min. The absorbance was measured at a wavelength of 540 nm. The IC_{50} value is defined as the concentration (mg/mL) to inhibit $NO^{\bullet}$ by 50%, compared the standard gallic acid.

2.10 Ferric reducing antioxidant power

Reducing power of the extracts and bergenin were measured by the method of Sinthorn, Chatuphonprasert, Chula siri, and Jarukamjorn (2016) with some modifications. The reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) was determined by measuring absorbance of the Perl's Prussian blue complex. In brief, the extracts or bergenin (at concentrations of 0.1-100 µg/mL) were mixed with 0.2 M PBS pH 6.6 and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and then acidified with 10% TCA before centrifugation at 3,000 X *g* at 25°C for 10 min. The supernatant was transferred to a 96-well plate before adding 0.1% $FeCl_3$. Absorbance was measured at a wavelength of 700 nm. An increase in absorbance indicates ferric reducing power capability of the extracts or bergenin, compared with the standard gallic acid.

2.11 Statistical analysis

The results are expressed as the mean ± standard deviation (SD). All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test to determine statistical significance ($p < 0.05$), and the IC_{50} values were calculated by Probit regression with the Statistical Package for Social Studies (SPSS) IBM version 22.0 (Armonk, USA).

3. Results and Discussion

Although water is the most commonly used excipient in Thai traditional medicines incorporating *M. repandus*, pharmacological studies of *M. repandus* have examined its methanol or ethanol extracts (Hasan *et al.*, 2014a, 2018; Lau pattarakasem, Sripa, & Hahnvajanawong, 2010). Hence, we determined the antioxidant activity of methanol, ethanol, and water MR stem extracts from Soxhlet extraction or maceration, which are both widely employed herbal extraction methods (Azwanida, 2015).

We determined the amounts of antioxidant constituents, including phenolics, flavonoids, and tannins present in the MR stem extracts, and they are summarized in Table 1. The total phenolics content was the highest in the water Soxhlet extract (70.55 ± 3.36 mg GAE/g), followed by the methanol Soxhlet (64.85 ± 2.35), the ethanol macerated (58.56 ± 3.33), the methanol macerated (55.50 ± 2.20), the ethanol Soxhlet (52.06 ± 3.37), and the water macerated extracts (40.65 ± 1.27). The ethanol macerated extract showed the highest content of flavonoids (8.57 ± 1.59 mg QE/g), followed by the ethanol Soxhlet (6.30 ± 0.22), the methanol macerated (5.23 ± 0.14), the methanol Soxhlet (2.29 ± 0.06), and the water Soxhlet extracts (1.03 ± 0.16). Flavonoids were not detected in the water macerated extract. The percent contribution of tannin in the extracts varied from 70.66 to 83.25%. The highest percent contribution of tannin was found in the methanol macerated extract, followed by the ethanol macerated, the water Soxhlet, the water macerated, the methanol Soxhlet, and the ethanol Soxhlet extracts, in this rank order (Table 1). Noticeably, for extracts prepared by Soxhlet extraction, both the total phenolic and total flavonoid contents of the ethanol extract were significantly different from the methanol and water extracts ($p < 0.05$), while the percent contributions of tannin in the methanol, ethanol, and water extracts were similar. For extracts prepared by maceration, the total phenolic content of the water extract was significantly different from the methanol and ethanol extracts ($p < 0.05$), while the total flavonoid contents of the methanol and ethanol extracts were significantly different ($p < 0.05$). The methanol and water extracts were significantly different in percent contribution of tannin ($p < 0.05$). In a comparison between Soxhlet extraction and maceration, the total phenolic contents of methanol and water extracts were significantly different ($p < 0.05$). Significant difference of methanol and ethanol extracts was seen in total flavonoid content and in percent contribution of tannin ($p < 0.05$). Maceration is a cold extraction method that is simple to carry out and preserves thermolabile compounds, but it is time-consuming compared to Soxhlet extraction (Selvamuthukumaran & Shi, 2017). In contrast, Soxhlet extraction is a hot extraction method that is faster and is suitable for isolation of thermostable compounds, but it is comparatively complicated and requires specialized equipment. (Annegowda, Mordi, Ramanathan, Hamdan, & Mansor, 2012; Azwanida, 2015). Our results demonstrate that the contents of phenolics, flavonoids, and tannin in the MR stem extracts depended on both the type of solvent and the

extraction technique. This was seen particularly for flavonoids and tannin, where maceration provided higher phytochemical contents than Soxhlet extraction. This might be due to thermal degradation of these phytochemicals during the hot extraction, and is consistent with previous studies showing decreases in the contents of flavonoids and tannin in vegetables after heating to 80°C (Sharma *et al.*, 2015; Somsub, Kongkachui chai, Sungpuag, & Charoensiri, 2008; Srisawa *et al.*, 2010). Our quantitative determination of phenolics, flavonoids, and tannin in the MR stem extracts confirms and extends a previous qualitative analysis of phytochemical constituents in ethyl acetate extract of MR stem (Hasan *et al.*, 2014b).

The present study evaluated the *in vitro* antioxidant activities of the MR stem extracts and bergenin based on their capability to eliminate either non-biological ($ABTS^{\bullet+}$) or biological free radicals ($^{\bullet}OH$, $^{\bullet}O_2$, and $NO^{\bullet}$), and their reducing ability toward ferric ions. The blue-green $ABTS^{\bullet+}$ chromophore is decolorized in the presence of antioxidant (Schlesier, Harwat, Bohm, & Bitsch, 2002). The $ABTS^{\bullet+}$ IC_{50} values of all MR stem extracts and bergenin are shown in Table 2. Among the extracts prepared by Soxhlet extraction, the water extract showed the highest $ABTS^{\bullet+}$ radical scavenging activity with an IC_{50} value of 0.27 ± 0.04 mg/mL, followed by the methanol extract (0.32 ± 0.01 mg/mL). The ethanol extract had significantly less $ABTS^{\bullet+}$ radical scavenging activity than the methanol or water extracts (IC_{50} 0.69 ± 0.06 mg/mL, $p < 0.05$, both). In contrast, the methanol macerated extract showed an $ABTS^{\bullet+}$ radical scavenging activity (IC_{50} 0.29 ± 0.02 mg/mL) that was comparable with the ethanol macerated extract (IC_{50} 0.38 ± 0.05 mg/mL) but the water macerated extract was a weaker $ABTS^{\bullet+}$ radical scavenger (IC_{50} 0.42 ± 0.02 mg/mL) than the methanol and ethanol macerated extracts. Bergenin showed extensive $ABTS^{\bullet+}$ radical scavenging activity with an IC_{50} value of 0.08 mg/mL, significantly lower than all MR stem extracts ($p < 0.05$). However, gallic acid exhibited significantly more scavenging potential against $ABTS^{\bullet+}$ radicals ($p < 0.05$).

Table 1. Total phenolic and flavonoid contents and percent contribution of tannin of the *M. repandus* stem extract.

The extracts of *M. repandus* stem	Total phenolics[a] (mg GAE/g)	Total flavonoids[b] (mg QE/g)	% Contribution of tannin
Soxhlet extraction			
Methanol extract	64.85 ± 2.35	2.29 ± 0.06	73.94 ± 3.32
Ethanol extract	52.06 ± 3.37	6.30 ± 0.22	70.66 ± 1.87
Water extract	70.55 ± 3.36	1.03 ± 0.16	75.10 ± 3.40
Maceration			
Methanol extract	55.50 ± 2.20	5.23 ± 0.14	83.25 ± 1.51
Ethanol extract	58.56 ± 3.33	8.57 ± 1.59	79.74 ± 3.72
Water extract	40.65 ± 1.27	Not detected	74.24 ± 0.37

Note: The results are expressed as mean ± SD (n=3-4) from 3 independent experiments.
[a] equivalent to gallic acid (GA); [b] equivalent to quercetin (Q). [$] $p < 0.05$.

Table 2. The IC_{50} values of the *M. repandus* stem extract and bergenin.

Tested materials	IC_{50} (mg/mL)[a]			
	$ABTS^{\bullet+}$	$^{\bullet}OH$	$^{\bullet}O_2$	$NO^{\bullet}$
Gallic acid[b]	0.004 ± 0.00	0.038 ± 0.00	0.003 ± 0.00	0.025 ± 0.00
Bergenin	0.08 ± 0.00[*]	0.12 ± 0.00	0.25 ± 0.01	0.35 ± 0.01
M. repandus stem extract				
Soxhlet extraction				
Methanol extract	0.32 ± 0.01[*#]	1.73 ± 0.05[*#]	6.14 ± 0.61[*#]	16.29 ± 0.66[*#]
Ethanol extract	0.69 ± 0.06[*#]	1.91 ± 0.18[*#]	7.87 ± 0.59[*#]	16.70 ± 0.39[*#]
Water extract	0.27 ± 0.04[*#]	2.14 ± 0.37[*#]	7.75 ± 0.32[*#]	31.90 ± 1.37[*#]
Maceration				
Methanol extract	0.29 ± 0.02[*#]	2.70 ± 0.18[*#]	5.70 ± 0.23[*#]	33.65 ± 0.67[*#]
Ethanol extract	0.38 ± 0.05[*#]	2.18 ± 0.12[*#]	9.49 ± 0.94[*#]	32.70 ± 0.50[*#]
Water extract	0.42 ± 0.02[*#]	2.68 ± 0.10[*#]	5.03 ± 0.36[*#]	13.44 ± 0.90[*#]

Note: The results are expressed as mean ± SD (n=3-4) from 3 independent experiments.
[a] the concentration to inhibit the radical, $ABTS^{\bullet+}$, $^{\bullet}OH$, $^{\bullet}O_2$, or $NO^{\bullet}$, by 50%; [b] the standard gallic acid.
[*] $p < 0.05$ versus Gallic acid; [#] $p < 0.05$ versus Bergenin; [$] $p < 0.05$.

The most powerful free radicals associated with human diseases are $^{\bullet}OH$, $^{\bullet}O_2$, and $NO^{\bullet}$ (Fransen, Nordgren, Wang, & Apanasets, 2012). All the Soxhlet extracts showed comparable IC_{50} values (1.73-2.14 mg/mL) against the $^{\bullet}OH$ radicals, while the lowest IC_{50} values against $^{\bullet}O_2$ (6.14 ± 0.61 mg/mL) and $NO^{\bullet}$ (16.29 ± 0.66 mg/mL) were for the methanol Soxhlet extract. Interestingly, the ethanol Soxhlet extract exhibited comparable activity to that of methanol Soxhlet extract against $NO^{\bullet}$ (16.70 ± 0.39 mg/mL). Regarding maceration, the IC_{50} value for $^{\bullet}OH$ scavenging activity of ethanol extract was the lowest (2.18 ± 0.12 mg/mL), while water extract showed the lowest IC_{50} values against $^{\bullet}O_2$ (5.03 ± 0.36 mg/mL) and $NO^{\bullet}$ (13.44 ± 0.99 mg/mL) radicals. These results indicate by the high IC_{50} values of all MR stem extracts, especially the IC_{50} values for $^{\bullet}O_2$ and $^{\bullet}OH$ scavenging activities, only low antioxidative activity *in vitro* compared with the gallic acid standard. Corresponding to the $ABTS^{\bullet+}$ scavenging activity, bergenin demonstrated far more activity against $^{\bullet}OH$, $^{\bullet}O_2$, and $NO^{\bullet}$ radicals than any MR stem extract, and gallic acid possessed the greatest scavenging activity against these free radicals. Collectively, the water and methanol Soxhlet extracts possessed the highest $ABTS^{\bullet+}$ and $^{\bullet}OH$ radical scavenging activities, respectively, while the water macerated extract had the lowest IC_{50} values toward both $^{\bullet}O_2$ and $NO^{\bullet}$ radicals. The highest bergenin content was found in the water macerated extract of MR stem, which correlated well with its scavenging activity against some free radicals under *in vitro* conditions (Sriset, Chatuphonprasert, & Jarukamjorn, 2018). Both water and methanol Soxhlet extracts exhibited antiradical activity; however, there was no parallel relationship between scavenging activity, as determined by IC_{50} value, and bergenin content in the extracts. This might be explained by the presence of other phytochemical compounds with antioxidant activity, such as lupeol and friedelin, in the MR stem extracts (Rivière *et al.*, 2010; Santiago & Mayor, 2014; Sunil, Duraipandiyan, Ignacimuthu, & Al-Dhabi, 2013).

All MR stem extracts and bergenin showed the ability to transform Fe^{3+} to a stable Fe^{2+} product. The reducing power of the Soxhlet and macerated extracts and bergenin was measured through absorbance at 700 nm and compared to the standard antioxidant gallic acid, and all increased in a concentration-dependent manner (Figure 1). Noticeably, the Fe^{3+} reducing potential of bergenin was higher than that of any MR stem extract. However, the gallic acid standard showed a larger increase in the absorbance at the highest concentration.

The antioxidant activity of the MR stem extracts correlated well with the phenolic, tannin, and bergenin contents, but not that of flavonoids. This might be due to the very small amounts of flavonoids present in the MR stem extracts. The highest phenolic content was in the water Soxhlet extract of MR stem, which reduced $ABTS^{\bullet+}$ radicals, and the high phenolic and tannin content in the methanol Soxhlet extract of MR stem showed inhibition of $^{\bullet}OH$ radicals. Moreover, high scavenging capability against $^{\bullet}O_2$, and $NO^{\bullet}$ was related to the high amount of bergenin in the water macerated extract of MR stem. These observations imply that phenolics might be responsible for scavenging $ABTS^{\bullet+}$, $^{\bullet}OH$, $^{\bullet}O_2$, and $NO^{\bullet}$ radicals (Tsao & Deng, 2004) while tannins might play a specialized role in the antioxidant effect against $^{\bullet}OH$ radicals (Fawole *et al.*, 2010). Thus, the free radical scavenging activity of the MR stem extracts was chiefly due to the amount and variety of phenolic compounds (Tsao & Deng, 2004; Zheng & Wang, 2001). A previous study reported ethyl acetate and *n*-hexane fractions of MR stem as $^{\bullet}O_2$ and $^{\bullet}OH$ scavengers, determined by electron spin resonance technique (Lin, Lin, Chen, Ujiie, & Takada, 1995). Bergenin is a C-glucoside derivative of 4-*O*-methylgallic acid with the hydroxyl rich structure able to engage free radicals by an electron donating mechanism (Badhani, Sharma, & Kak kar, 2015; Bajracharya, 2015; Song *et al.*, 2013). In the present study, bergenin showed effective free radical scavenging activity. In accordance with previous studies, bergenin exhibited good scavenging activities toward 1,1-diphenyl-2-picrylhydrazyl (DPPH), $ABTS^{\bullet+}$, and H_2O_2 with IC_{50} values of 0.043, 0.075, and 0.033 mg/mL, respectively, and antioxidant activity against lipid peroxidation with an IC_{50} value of 0.365 mg/mL (Srinivasan, Chandrasekar, Nanjan, & Suresh, 2007; Sumino, Sekine, Ruangrungsi, Igarashi, & Ikegami, 2002). Hence, bergenin might be the ultimate antioxidant derived from the MR stem.

The present study reveals that the free radical scavenging activity of MR stem extracts against both ROS and RNS is due to a variety of phytochemical compounds present in MR (Fawole *et al.*, 2010; Jaberian, Piri, & Nazari, 2013). These findings lead to an idea of the protective efficacy of the MR stem extracts through a mechanism of free radical - breaking by hydrogen or electron donation. Importantly, the MR stem contains many natural constituents other than bergenin, including tannins, steroids and terpenoids, which are also considered to possess antioxidant and anti-inflammatory

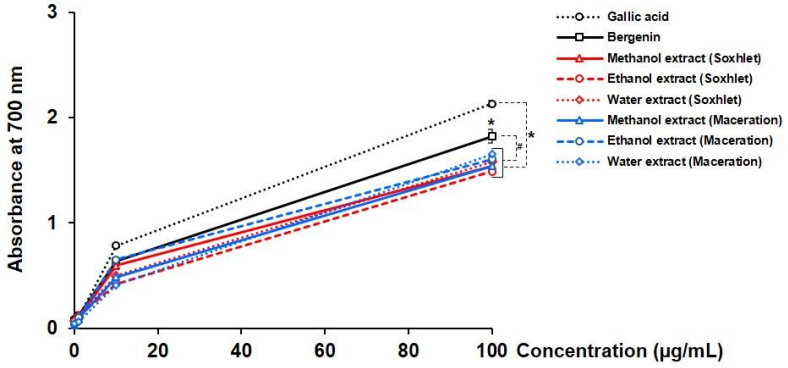

Figure 1. Ferric reducing antioxidant power of bergenin and *M. repandus* stem extract, compared with gallic acid. $^{*}p < 0.05$ versus Gallic acid; $^{\#}p < 0.05$ versus Bergenin.

activities (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010; Rivière *et al.*, 2010; Sutthivaiyakit, Thongtan, Pisutjaroen pong, Jiaranantanont, & Kongsaeree, 2001). Stem and leaf of MR were revealed to have a predominantly anti-inflammatory effect in Thai traditional use (Hasan *et al.*, 2014a, 2018). These observations suggest that MR extract might bring more benefits than a pure compound due to simultaneous antioxidant and anti-inflammatory activities of various natural phytochemicals. In addition, preparation of crude MR extract in the current study was simpler than the purification/ identification of pure bergenin.

4. Conclusions

The phenolic, flavonoid and tannin phytochemical contents of extracts of MR stem were obtained for both Soxhlet and maceration extracts, each done with methanol, ethanol, and water. The *in vitro* antioxidant capabilities of the MR stem extracts and bergenin to scavenge either non-biological ($ABTS^{\bullet+}$) or biological free radicals ($^{\bullet}OH$, $^{\bullet}O_2$, and $NO^{\bullet}$) were determined. The water and methanol Soxhlet extracts were superior to all other MR stem extracts against $ABTS^{\bullet+}$ and $^{\bullet}OH$, respectively, while the water macerated extract had the greatest activities against $^{\bullet}O_2$ and $NO^{\bullet}$. The ferric reducing antioxidant power was comparable and concentration-dependent among all the MR stem extracts. Bergenin possessed efficient antioxidant activity with the largest reducing power. Therefore, the MR stem and bergenin are promising candidates for natural antioxidant supplements. However, a further study is required to demonstrate the cellular mechanism(s) behind the antioxidant potential of the MR stem and bergenin.

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