

Full Length Research Paper

Antioxidant and antibacterial properties of the leaves and stems of *Premna microphylla*

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Premna microphylla is widely used in the Chinese folk medicine for the treatment of different infections diseases. In this study, ascorbic acid and β -carotene contents of the fresh *P. microphylla* leaves and stems were determined by spectrophotometric method. In addition, the antioxidant and antibacterial activities and phenolic contents of the methanol extracts of the dry leaves and stems were evaluated using *in vitro* standard methods. The results showed that ascorbic acid and β -carotene contents of the leaves and stems were both considerable. The antioxidant activities of the stem extract of *P. microphylla* as determined by the total phenolics, flavonoids, and ferrous reducing antioxidant property methods were significantly higher than that of leaves. On the other hand, the leaf extract of the plant had higher level of proanthocyanidins. Moreover, the leaf extract also had higher radical scavenging activity than the stem as shown in 1,1-Diphenyl-2-picrylhydrazyl assay. The antibacterial assay showed that the methanol extracts from the leaves and stems had significant activity against both Gram-positive and Gram-negative bacteria. The activity of the leaf extract was more pronounced on several bacterial species, compared with that of stem extract on three bacterial species with the minimum inhibitory concentrations of 10 mg/ml. The results obtained in this study suggested *P. microphylla* could be a potential good candidate for functional foods as well as pharmaceutical plant-based products.

Key words: *Premna microphylla*, antioxidant, antibacterial, methanol extract.

INTRODUCTION

The genus *Premna*, comprised of 50-200 species, is distributed in tropical and subtropical Asia, Africa, Australia, and the Pacific islands (Yadav et al., 2010). *Premna microphylla* Turcz. (Verbenaceae), distributed mainly in the southern part of China and locally known as

“Doufuchai”, is a deciduous shrub and has been used in Chinese traditional medicine to treat skin cuts and infections, dysentery, appendicitis, swelling, headaches, and viper bites (Zhan et al., 2009; Wang and Xu, 2003). Previous studies have demonstrated that the methanol extract of *P. microphylla* roots possesses anti-inflammatory and antinociceptive effects and a beneficial effect of non-singular immunity in mouse by its antioxidant property (Gao et al., 2003a; Cao et al., 2003a, b). The leaves also possess lipid-lowering effect and immunomodulatory activity in lymphocytes and macrophages (Fang et al., 2004; Gao et al., 2003b).

Phytochemical investigations on *P. microphylla* have resulted in the isolation of ceramide and glyceroglycolipid (Zhan and Yue, 2003). The ceramides are important components in the cell membranes of animals and plants

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Abbreviations: FRAP, Ferrous reducing antioxidant property; DPPH, 1,1-Diphenyl-2-picrylhydrazyl; BHT, butylated hydroxytoluene; 2,6-DCPIP, 2,6-dichloroindophenol; TLC, thin layer chromatography; FW, fresh weight; DW, dry weight; MIC, minimum inhibitory concentrations.

and are emerging as significant second messengers for various cellular processes (Kolter and Sandhoff, 1999). Many extracellular stresses, such as tumor necrosis factor- α and HIV, have been shown to activate sphingomyelinase, which releases ceramides that inhibit cell growth and induce apoptosis (Jayadev et al., 1995). The glyceroglycolipids exist in both terrestrial plants and in plants of marine origin, such as sponges and algae. Some glyceroglycolipids have shown significant cytotoxic activity against several tumor cell lines (Hiraga et al., 2002). Furthermore, a new triterpene glycoside, possessing significant antibacterial and cytotoxic activities, was also isolated from the leaves of *P. microphylla* (Zhan et al., 2009).

Free radicals have been implicated in the causation of several diseases such as liver chirrrosis, atherosclerosis, cancer, diabetes, etc. and some compounds that can scavenge free radicals have great potential in ameliorating these disease processes (Adedapo et al., 2008). Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species (Kaur and Perkins, 1991). Free radicals or Reactive Oxygen Species (ROS) are produced *in vivo* from various biochemical reactions and also from the respiratory chain as a result of occasional leakage. These free radicals are the main culprits in lipid peroxidation (Norhaiza et al., 2009).

Plants are well protected against free radical or ROS damage by enzymatic systems, including superoxide dismutase and catalase (Posmyk et al., 2005), or antioxidant compounds such as ascorbic acid, tocopherols, glutathione, polyphenols, carotenoids and flavonoids which are localized in different cellular compartments (Lisiewska et al., 2006). Furthermore, zeaxanthin (together with lutein and beta-carotene) may also scavenge some of those highly reactive molecules, which are usually overproduced under stress conditions (Niyogi, 1999; Partelli et al., 2010).

Natural products from microorganisms have been the primary source of antibiotics, but with the increasing acceptance of herbal medicine as an alternative from of health care, the screening of medicinal plants for active compounds has become very important because these may serve as promising source of novel antibiotic prototypes (Rabe and van Staden, 1997; Koduru et al., 2006). It has been shown that *in vivo* screening methods could provide the needed preliminary observations necessary to select crude plant extracts with potentially useful properties for further chemical and pharmacological investigations (Seddik et al., 2010).

However, the antioxidative capacities and antibacterial properties of *P. microphylla* have not been reported. The present study, therefore, investigated the antioxidant and antibacterial properties of the leaves and stems of *P. microphylla* using *in vitro* antioxidant methods and against some selected species of bacteria. The findings from this work may add to the overall value of the medicinal potential of the herb.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid, potassium ferricyanide, butylated hydroxytoluene (BHT), Folin-Ciocalteu's phenol reagent, ascorbic acid, iron chloride (FeCl_3), sodium carbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Catechin, tannic acid, β -carotene, quercetin and vanillin were purchased from Herbfine (Nanchang, China). All the other chemicals used including the solvents, were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

Plant material

The leaves and young stems of *P. microphylla* were collected from Wudang Mountain, Shiyan, Hubei province, China, in June, 2008. The plant was identified at the laboratory of Botany, Yangtze University by Ming Qin Zhou and voucher specimens (Pm-LT 2008/7/15) has been deposited in the Herbarium at College of Horticulture and Gardening, Yangtze University.

Determination of ascorbic acid

The ascorbic acid content was measured using a modified method of Norhaiza et al. (2009). The leaf or stem fresh samples were ground into powder and extracted in 1% of phosphate-citrate buffer, pH 3.5 using chilled pestle and mortar. The homogenate was filtered by Whatman No 1 filter paper. The filtrate was added to the 1 ml of 1.7 mM 2,6-dichloroindophenol (2, 6-DCPIP) in 3 ml cuvette. The absorbance at 520 nm was read within 10 min of mixing the reagents with the extraction buffer as a blank. The content of ascorbic acid was determined as mg per g Fresh Weight (FW).

Determination of β -carotene

β -carotene content was determined using the method described by Harborne (1973). Fresh leaves or stems were ground in the presence of cold acetone and light petroleum. The extract was applied on to Thin Layer Chromatography (TLC) plate silica gel 60 F₂₅₄ plate (20 × 20 cm, Sigma-Aldrich). The plate was developed with hexane: acetone (1:1). The spot was scraped and diluted back to the extraction solvent. The absorbance was read at 451 nm. The content of β -carotene was determined as mg per g FW by using an equation obtained from the standard curve of β -carotene.

Extract preparation

Both leaves and stems were air dried at room temperature to constant weights. The dried plant materials were separately ground to powders. Two hundred grams of powdered leaves or stems were soaked in 1 L of methanol separately for 48 h on an orbital shaker. Extracts were filtered using a Buckner funnel and Whatman No 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at 40°C using a rotary evaporator. The percentage yield for the leaves was 6.5% while that of the stems was 5.8%. Each extract was resuspended in methanol to make a 50 mg/ml stock solution.

Determination of total phenolics

The amount of total phenolics in the extracts were determined by

the modified Folin-Ciocalteu method (Jimoh et al., 2007). Samples (1 ml) were mixed with 4 ml of tenfold diluted Folin-Ciocalteu reagent and 5 mL sodium carbonate solution (75 g/l) in a tube. The tubes were vortexed for 20 s and allowed to stand for 30 min at 40°C from color development. The absorbance was measured at 765 nm. The content of total phenolics was expressed as mg of gallic acid equivalents per g Dry Weight (DW).

Determination of total flavonoids

Total flavonoids were determined using the method of Aliero et al. (2008). To 1 ml of the sample, 1 ml of 2% AlCl₃-ethanol solution was added. After 1 h at 25°C, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as quercetin equivalent (mg/g DW) from a calibration curve.

Determination of proanthocyanidin

Determination of proanthocyanidin was based on the procedure by Adedapo et al. (2008). A volume of 1 ml of 0.1 mg/ml of extract solution was mixed with 6 ml of 4% vanillin solution in methanol (w/v) and 3 ml 2% hydrochloric acid; the mixture was allowed to stand for 15 min at 25°C. The absorbance was measured at 500 nm and the result expressed as catechin equivalent (mg/g DW).

Ferric reducing antioxidant potential (FRAP) assay

A modified method of Wong et al. (2006) was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer (3.1 g C₂H₃NaO₂·3H₂O and 16 ml C₂H₄O₂), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl₃·6H₂O aqueous solution. The fresh working solution was prepared by mixing 25 acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl₃·6H₂O aqueous solution. The temperature of the solution was raised to 37°C before using. Plant extracts (150 µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM FeSO₄. Results were expressed in µM Fe (II)/g DW and compared with that of BHT, ascorbic acid and quercetin.

DPPH free radical scavenging activity

The effect of extracts on DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi (2005). A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02 - 0.1 mg of the extract. The reaction mixture was vortexed thoroughly and left in the dark at 25°C for 30 min. The absorbance of the mixture was measured at 517 nm. Ascorbic acid and BHT were used as references. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs_{control} - Abs_{sample}) / Abs_{control}] × 100 where Abs_{control} is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract/standard.

Antibacterial assay

Antibacterial assay was measured using the method of Mokbel and Hashinaga (2005). Nine bacterial species which included four Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Micrococcus kristinae*) and five Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella*

typhi, *Vibrio mimicus*, *Shigella dysenteriae*) was collected as pure cultures from the Laboratory of Microbiology, College of Agronomy, Yangtze University.

The bacteria stock culture media was maintained on nutrient hard agar (peptone 1 g, meat extract 0.5 g, sodium chloride 0.25 g and agar 1 g per 100 ml H₂O) at 4°C. The bacterial culture medium was prepared as following, nutrient broth (peptone 0.5 g, meat extracts 0.25 g, sodium chloride 0.25 g per 50 ml H₂O) and soft agar medium (peptone 0.5 g, meat extracts 0.25 g, sodium chloride 0.125 g and agar 0.2 g per 50 ml H₂O) adjusted to pH 6.6 and autoclaved at 120°C for 20 min.

The Minimum Inhibitory Concentrations (MIC) were defined as the lowest concentration (mg/ml) of the methanol extract of leaf or stem in agar plates showing no visible bacterial growth. The soft nutrient agar was then added onto a Petri dish containing 15 ml hard agar. The plant methanol extracts with 50 ml were then individually added at different concentrations (0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/L) to soft agar and mixed well before poured into sterile Petri dishes containing 15 ml hard agar. The cultures (5 µl) were taken from nutrient broth and added to three places on the medium surface and incubated at 37°C. To compare the activity with standard antibiotics, chloramphenicol and kanamycin served as standards.

Statistical analysis

The experimental results were expressed as mean ± standard error (SE) of three replicates. The data were analyzed using ANOVA (SAS, Version 8.1) at P < 0.05 (Fisher's protected least significant difference).

RESULTS

Ascorbic acid and β-carotene contents

The results indicated that ascorbic acid content was significant higher in leaves than in stems, while a slight difference in β-carotene content between leaves and stems (Table 1).

Total phenolic, flavonoid and proanthocyanidin contents

Results obtained in the present study showed that the level of polyphenol compounds in the methanol extracts of the leaves and stems of *P. microphylla* were considerable (Table 2). The stem extract possessed significantly higher contents of total phenolics and flavonoids than the leaf extract. On the other hand, the leaf extract possessed significantly higher content of proanthocyanidin than the stem extract.

Total antioxidant activity

The total antioxidant activity of the extracts of *P. microphylla* was in the range of 362.18 -1806.53 µm Fe (II)/g DW (Table 3). The FRAP values for the extracts of leaves were significantly lower than that of ascorbic acid

Table 1. Ascorbic acid and β -carotene contents (mg/g FW) of leaves and stems of *P. microphylla*. Values are means \pm SE (n = 3).

Compounds	Leaves	Stems
Ascorbic acid content	3.17 \pm 0.21*	0.47 \pm 0.05
β -carotene content	0.23 \pm 0.06	0.21 \pm 0.02

* indicated that this value is significantly different from the other at P < 0.05.

Table 2. Polyphenol contents of the methanol extracts of the leaves and stems of *Premna microphylla*. Values are means \pm SE (n =3).

Phenolics	Leaves	Stems
Total phenolics (mg gallic acid/g DW)	7.84 \pm 0.47	12.72 \pm 0.21*
Flavonoids (mg quercetin/g DW)	0.65 \pm 0.02	1.08 \pm 0.09*
Proanthocyanidins (mg catechin/g DW)	1.38 \pm 0.13*	0.81 \pm 0.05

* indicated that this value is significantly different from the other at P < 0.05.

Table 3. Total antioxidant activity (μ m Fe (II)/g DW) of the methanol extracts of the leaf and stem of *Premna microphylla*. Values are means \pm SE (n =3).

Extracts	FRAP
Leaves	362.18 \pm 29.25
Stems	1806.53 \pm 15.44
Ascorbic acid	1709.24 \pm 36.81
BHT	65.78 \pm 3.18
Quercetin	3018.37 \pm 86.29

and quercetin, but significantly higher than that of BHT.

concentration (Table 4).

DPPH radical scavenging activity

It was observed that DPPH radical scavenging activity of the extracts of the leaves had higher activity than that of the stems (Figure 1). At a concentration of 0.1 mg/ml, the scavenging activity of the leaves reached 87.3%, while at the same concentration, that of stems was 71.9% (Figure 1). The methanol extracts of leaves and stems were fast and effective scavengers of the DPPH radical and this activity was higher than that of BHT (55.8% at 0.1 mg/ml), however lower than that of ascorbic acid (98.6% at 0.1 mg/ml).

Antibacterial activity

The antibacterial activity of the leaf extract of *P. microphylla* was much higher than that of the stem extract. The leaf extract was active against all tested bacterial species except *V. mimicus* and *S. dysenteria* at MIC of 10 mg/mL while the stem extract was only active against, *S. pyogenes*, *E. coli* and *P. aeruginosa* at similar

DISCUSSION

Ascorbic acid and β -carotene contents

Ascorbic acid also known as vitamin C is one of the most abundant antioxidants in plant where the role of ascorbate is to protect plant against oxidative stress (Smirnoff, 2000). It is a powerful water soluble antioxidant and its established role is to prevent scurvy (Padayatty et al., 2003). Likewise, carotenoids are also classified among the basic constituents of the antioxidative effect (Norhaiza et al., 2009) and β -carotene is a precursor of vitamin A, which is important in human vision and to prevent certain types of cancer (Craft et al., 1993). In South of China, the aqueous extracts of fresh young leaves and stems were often processed into 'Guanyindoufu', a folk functional food, which possessed febrifuge, stomachic and anti-inflammatory activities (Wang et al., 2007). The beneficial properties for human health of 'Guanyindoufu' might be attributed to the strong antioxidant activity of the considerable ascorbic acid and β -carotene contents in young leaves and stems of *P. microphylla*.

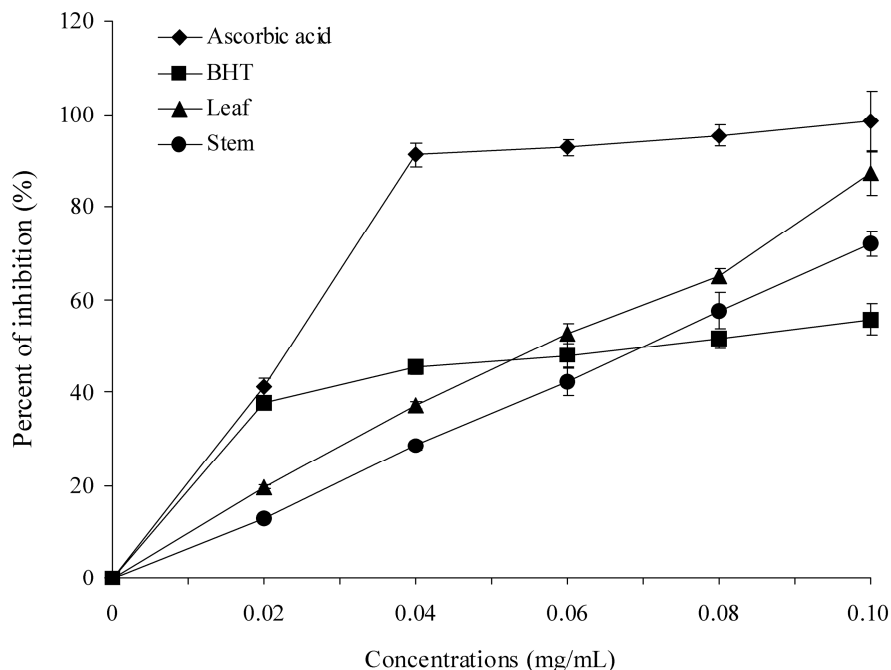


Figure 1. DPPH scavenging activities of the methanol extracts of the leaves and stems of *Premna microphylla*. Values are means \pm SE (n = 3).

Table 4. Antibacterial activity of the methanol extracts of the leaf and stem of *P. microphylla*.

Bacterial species	Minimum inhibitory concentration (mg/ml)			
	Leaves	Stems	Chloramphenicol (μ g/ml)	Kanamycin (μ g/ml)
Gram positive bacteria				
<i>S. aureus</i>	10.0	Na	<2	<2
<i>B. subtilis</i>	10.0	10.0	<2	<2
<i>S. pyogenes</i>	10.0	10.0	<2	<2
<i>M. kristinae</i>	10.0	Na	<2	<2
Gram negative bacteria				
<i>E. coli</i>	10.0	10.0	<2	<2
<i>P. aeruginosa</i>	Na	Na	<20	<10
<i>S. typhi</i>	10.0	Na	<2	<2
<i>V. mimicus</i>	10.0	Na	<5	<2
<i>S. dysenteriae</i>	Na	Na	<2	<2

Na = not active at 10 mg/ml.

Total phenolic, flavonoid and proanthocyanidin contents

Phenolics are one of the most numerous groups of substances in plant kingdom ranging from simple molecules, such as phenolic acids, to complex compounds, such as flavonoids, flavonols, proanthocyanidins. Phenolic compounds in plants constitute a major class of secondary plant metabolites with bioactive potential attributed to antioxidant and antibacterial activities. These activities is believed to be mainly due to their redox properties (Zheng and Wang, 2001), which play an important role in adsorbing and neutralizing free radicals, quenching

singlet and triplet oxygen, or decomposing peroxides. Results obtained in the present study revealed that the level of these phenolic compounds in the methanol extracts of the leaves and stems of *P. microphylla* were considerable (Table 2). The results strongly suggested that phenolics are important components of this plant, and some of its pharmacological effects could be attributed to the presence of these valuable constituents.

Total antioxidant activity

The antioxidant potentials of the methanol extracts of the

leaves and stems of *P. microphylla* were estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). The reducing ability of the extract of stems was significantly higher than that of leaves (Table 3). Antioxidant activity increased proportionally with the polyphenol content. According to recent reports, a highly positive relationship between total phenols and antioxidant activity appeared to be the trend in many plant species (Adedapo et al., 2008). Similarly, there was a positive correlation between total antioxidant activity (FRAP) and total phenolics with a coefficient of $R^2 = 0.76$ in the present study, implying the antioxidative activity of the extracts of *P. microphylla* primarily due to the presence of phenolic compounds in the extract.

DPPH radical scavenging activity

The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability (Liu et al., 2008). Though the DPPH radical scavenging abilities of the extracts were significantly less than that of ascorbic acid (Figure 1), the study revealed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. It is therefore, indicated that the extracts of *P. microphylla* might be useful therapeutic agents for treating radical-related pathological damage.

Antibacterial activity

The antibacterial activity of the methanol extracts of the leaves of *P. microphylla* is much higher than that of the stems. Though the MIC of 10 mg/ml is very high, nevertheless it showed that the plant extracts under *in vitro* study have broad spectrum antibacterial activity. It is known that, in general, the Gram-negative bacteria are more resistant than the Gram-positive ones (Afolayan, 2003). However, the study showed that three of the Gram-negative organisms used in this study were sensitive to this extract even then at high MIC of 10 mg/ml. *S. pyogenes* and *P. aeruginosa* are known pathogens of respiratory infections (Rojas et al., 2001). Its inhibition by the two extracts suggested their possible use in the treatment of chest and respiratory infections. Furthermore, *E. coli* was also inhibited by the extracts of the leaves and stems. Although it belongs to the normal flora of humans, an enterohemorrhagic strain of *E. coli* has caused serious food poisoning, and preservatives to eliminate its growth are needed (Buchanan and Doyle, 1997; Gulcin et al., 2003). The extracts of *P. microphylla* might therefore be of use.

Conclusion

The results from this study indicated that the leaf and stem extracts of *P. microphylla* possessed antioxidant

properties and could serve as free radical inhibitors or scavenger, or acting possibly as primary antioxidants. The methanol extracts of *P. microphylla* are not as effective as the standard drugs - chloramphenicol and knamycin, but possessed broad spectrum antibacterial activity. In addition, microorganisms become resistant to antibiotics over time. Since major attention has been recently devoted to natural sources of antioxidant and antibacterial materials, the data obtained in this study suggest a possible use of *P. microphylla* as a source of natural antioxidant and a broad spectrum bactericidal agent.

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