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## Evaluation of Antioxidant, Cytotoxic Activities and Total Phenolic Content from Leaf Extracts of *Phlogacanthus pulcherrimus*

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Due to functional foods have a potentially beneficial effects on human health, phytochemicals and their biological activities in plants are raising interest. In our previous studies, we have demonstrated the biological activities of the methanolic extract of *Phlogacanthus pulcherrimus* (Acanthaceae) leaves. Therefore, this study was undertaken to evaluate the antioxidant, cytotoxic activities and total phenolic content (TPC) from different solvents of *P. pulcherrimus* leaf extracts. The dried methanolic extract was suspended in distilled water and successively partitioned with dichloromethane, ethyl acetate, n-butanol and residual aqueous portions. The TPC of the extracts were determined by the Folin-Ciocalteu method. The highest TPC was found in the ethyl acetate extract (55.05±3.40 mgGAE/g extract), followed by the butanol, dichloromethane and aqueous extracts. Antioxidant activities of the extracts were evaluated by DPPH and ABTS assays. The ethyl acetate extract showed the highest antioxidant activity both measured by DPPH and ABTS assays with 50% inhibitory concentration (IC<sub>50</sub>) values of 730.28 µg/mL and 628.39 µg/mL, respectively. Additionally, the results showed a positive relationship between antioxidant activity and TPC. MTT assay was employed to evaluate the cytotoxic activity against five different human cancer cell lines, colon cancer (HT29), cervical cancer (HeLa), breast cancer (MCF7), liver cancer (HepG2) and oral cancer (KB) cell lines, and three normal cell lines, monkey kidney (Vero), human keratinocyte (HaCaT) and mouse fibroblast (L929) cell lines. The dichloromethane extract showed the highest cytotoxicity (> 90%) against both cancer and normal cell lines at a concentration of 1000 µg/mL. The current study suggests that the ethyl acetate and dichloromethane extracts of *P. pulcherrimus* have the potential source of natural antioxidant and antiproliferative activity, respectively.

**Keywords:** *Phlogacanthus pulcherrimus*, total phenolic compounds, antioxidant, anticancer

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## Introduction

Thailand is located in the areas that supports a variety of biological resources. Many members of the Acanthaceae family are used for a great many medicinal purposes. Analysis of the bioactive phytochemicals and evaluation of biological activities of plant in this family have been reported such as *Thunbergia laurifolia* (Tangpong and Satarug, 2010), *Andrographis paniculata* (Kumar *et al.*, 2004), *Rhinacanthus nasutus* (Puttarak *et al.*, 2010) and *Barleria strigosa* (Manapradit *et al.*, 2013). *Phlogacanthus* genus is a member of Acanthaceae which are herbs and widely distributed throughout tropical and subtropical Asia. The extracts from some species in this genus have been evaluated for biological activities. For instance, *P. thyrsiflorus* exhibited analgesic, antiinflammatory, antimicrobial, antioxidant, antihyperglycemic and cytotoxic activities (Singh and Singh, 2010; Ahmed *et al.*, 2012; Devi *et al.*, 2012, Chanu *et al.*, 2012; Tassa *et al.*, 2012; Chakravarty and Kalita, 2012; Das *et al.*, 2015). *P. curviflorus* has antioxidant activity (Seal and Chaudhuri, 2015) as well as *P. turgidus* (Thu *et al.*, 2010) and *P. thyrsiformis* (Tiwary *et al.*, 2015) showed cytotoxic activity.

*Phlogacanthus pulcherrimus* T. Anderson (Acanthaceae) is commonly known as Dee Pla Kang and widely distributed in northern and northeastern of Thailand. *P. pulcherrimus* is a shrub which will grow to approximately 2 meters in height. Leaves are edible and has been used as an indigenous vegetable and herbal medicine which is used in several traditional medicines to treat a wide range of illnesses. However, there have been a few reports on biological activity of *P. pulcherrimus*. There are only data for nutrition and mineral content of *P. pulcherrimus* leaves which cultivated in Thailand (Jongrungruangchok *et al.*, 2014). For biological activity, only one published report on the phytochemical screening, antioxidant and anticancer activities of methanolic extract of *P. pulcherrimus* leaves (Lordkhem *et al.*, 2015).

Therefore, the aim of the present study was to evaluate the potential antioxidant, cytotoxic activities and total phenolic content of different extracts obtains dichloromethane, ethyl acetate, n-butanol and residual aqueous portions derived from *P. pulcherrimus*.

## Materials and methods

### *Plant material*

*P. pulcherrimus* are shrubs and small trees. The leaves are opposite decussate and large about 20 cm long and 4-5 cm wide, inflorescence terminal thyrse and corolla dark red which characteristics of *P. pulcherrimus* are shown

in Fig. 1A-1B. The leaves of *P. pulcherrimus* were collected in April 2014, at Non Mueang, Na Klang District, Nong Bua Lamphu Province, Thailand. The plant was identified by Mr. Winai Somprasong, Plant Variety Protection Division, Department of Agriculture, Bangkok, and comparison with herbarium specimens in Bangkok herbarium, Thailand.



**Fig.1** Characteristics of *P. pulcherrimus* (A) plant and leaves (B) inflorescence

#### ***Extraction of Plant Materials***

The fresh leaves were dried in hot air oven (50 °C) for 3-5 days and then ground to coarse powder (1580 g). The plant extract was macerated separately with methanol, at room temperature, in shaking condition for seven days. The extract was filtered through Whatman filter paper (No. 1). The methanolic extracts were concentrated to dryness at reduced pressure using rotary evaporator. After solvent removal, the methanolic extracts (310.75 g) was then suspended in water and successively suspended in water, ethyl acetate, n-butanol in a sequential order of increasing polarity and residual aqueous portions also coded DE, EE, BE and AE, respectively and then evaporated to dryness and stored in refrigerator until use.

#### ***Determination of total phenolic content***

The total phenolic content (TPC) was determined by the spectrophotometric using Folin-Ciocalteu method (Armania *et al.*, 2013). In brief, a volume of 0.5 ml of the plant extract (1-2 mg/ml) was mixed with 2.5 ml of 10% Folin-Ciocalteu reagent. After 5 min, 2.0 ml of a 7.5% of sodium

carbonate ( $\text{Na}_2\text{CO}_3$ ) solution was added to the mixture and mixed thoroughly. The mixture was kept in the dark for 60 min at 40 °C. The absorbance was measured at 765 nm using UV-visible spectrophotometer (UV-1601, Shimadzu, Japan). The calibration line was constructed using a standard curve range from 20-100  $\mu\text{g}/\text{ml}$  of gallic acid. The TPC was expressed as milligrams of gallic acid equivalents per gram of extract (mgGAE/g of extract). The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained.

### ***Determination of antioxidant activity***

#### **DPPH radical scavenging activity**

The measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was carried out according to the method of Armania *et al.*, (2013) with some slight modifications. Briefly, the 50  $\mu\text{l}$  of various concentrations (250-2000  $\mu\text{g}/\text{ml}$ ) of test sample was added to 195  $\mu\text{l}$  of 0.1 mM methanolic DPPH solution on a 96-well plate. After incubation at room temperature for 30 min in the dark, the absorbance was measured at 492 nm using the Biochrom Anthos MultiRead 400 microplate reader (Biochrom, UK). Different concentrations (10-50  $\mu\text{g}/\text{ml}$ ) of trolox was used to make the standard curve and used as a positive control. The 50% inhibition concentration ( $\text{IC}_{50}$ ) was calculated using GraphPad Prism 5 software and expressed as milligrams trolox equivalent antioxidant capacity (mgTEAC)/g extract.

#### **ABTS radical scavenging activity**

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of extract was conducted according to the method of Armania *et al.*, (2013) with some slight modifications. Firstly, the ABTS radical cation was prepared by mixing a 7 mM ABTS with 2.45 mM potassium persulfate (1: 1). The mixture was incubated in the dark at room temperature for 16-24 h until the reaction was turned to blue-green colors. The ABTS radical solution was adjusted with methanol to obtain an absorbance of working solution  $0.700 \pm 0.020$  (mean  $\pm$  SD) at 734 nm. Afterward, 0.1 ml of extract solution was mixed with 0.9 ml of the ABTS working solution and incubated at room temperature for 6 min. After reaction, the reduction in absorbance at 734 nm was determined using a UV-Visible spectrophotometer (UV-1601, Shimadzu, Japan). The 50% inhibition concentration ( $\text{IC}_{50}$ ) was calculated and expressed as mgTEAC/g extract.

### ***Determination of cytotoxic activity***

#### **Cell cultures**

The five cancer cell lines including human colon adenocarcinoma (HT29), human cervical carcinoma (HeLa), human breast adenocarcinoma (MCF7), human hepatocellular carcinoma (HepG2) and human oral epidermoid carcinoma (KB) as well as three normal cell lines including african green monkey kidney (Vero), human keratinocyte (HaCaT) and mouse subcutaneous connective tissue (L929) cell lines were used for cytotoxicity assay. The HT29, MCF7, HepG2, and Vero cell lines were obtained from Dr. Porntipa Picha, Research Division, National Cancer Institute, Bangkok, the HeLa, KB and L929 cell lines were obtained from Scientific Instruments Centre, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok. Including, the HaCaT cell line was obtained from Dr. Amornpan Sereemasapun, Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok. The cells were cultured in RPMI 1640 medium with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 0.05 µg/ml gentamycin in a humidified incubator containing 5% CO<sub>2</sub> at 37 °C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

In vitro cytotoxic activity was conducted according to the MTT colorimetric assay of Mosmann (1983) with slight modifications. Briefly, the cells were trypsinized and diluted with RPMI 1640 medium with 5% v/v FBS. The cell lines were seeded into each well of 96-well plates at densities of  $1-1.8 \times 10^5$  cells/ml in 100 µl cell culture and incubated for 24 h at 37 °C. After incubation, the cells were treated with different extracts (dichloromethane, ethyl acetate, n-butanol and aqueous portions) at 125-1000 µg/ml (final concentration in each well) and incubated for 20 h. The 0.2% (v/v) dimethyl sulphoxide (DMSO) and 0.5% anticancer drug mitomycin C (MMC) were used as negative and positive control, respectively. After treatment, 50 µl of MTT working solution (2 mg/ml in phosphate buffered saline) was added in each 96-well plates and incubated continuously for 4 h. The supernatant was removed from each well and replaced with 100 µl dimethyl sulfoxide: absolute ethanol (1:1) to solubilized formazan crystals. After the blue-violet crystals were dissolved, the absorbance of each well was measured at 570 nm using a microplate reader (Anthos MultiRead 400, Biochrom, UK). The percentage of inhibition of cell growth was calculated and the 50% inhibitory concentration (IC<sub>50</sub>) was estimated using GraphPad Prism 5 software.

### Statistical analysis

All experimental measurements were carried out in triplicate and expressed as mean  $\pm$  standard deviation (SD). The data were analyzed by Statistical Package for the Social Science (SPSS) version 17.0. One-way analysis of variance (ANOVA) were used to show the mean differences between all samples. Duncan's multiple range tests (DMRT) were used to determine the significant differences between groups.  $P \leq 0.05$  was considered statistically significant.

### Results and Discussion

#### Extraction yields and Total phenolic content (TPC)

The yield of methanolic extracts have 345.81 g from 1580.00 g of powdered plant material. The results in Table 1 shows the yield of extracts and their respective total phenolic content. The yield of extract obtained from 310.75 g of dry methanolic crude was measured for each extract. The highest yield of solid residue (209.95 g) was obtained using the dichloromethane as extraction solvent. It has been reported that the efficiency of the extraction depends on many parameters such as the diameter of the powder and the volume and type of the solvents used, including multisolvent extraction. The total phenolic contents in the examined extracts ranged from  $13.77 \pm 0.57$  to  $55.05 \pm 3.40$  mg GAE/g extract. The highest amount of phenolic compounds was found in the ethyl acetate extract which was  $55.05 \pm 3.40$  mg GAE/g extract, followed by the n-butanol extract ( $25.02 \pm 5.60$  mg GAE/g extract), dichloromethane extract ( $15.16 \pm 0.82$  mg GAE/g extract) and aqueous residual extract ( $13.77 \pm 0.57$  mg GAE/g extract). Phenolic compounds are secondary metabolic products from plants which have been highly positive relationship between total phenolics and antioxidant capacity (Saeed *et al.*, 2012).

**Table 1** Extraction yields and total phenolic content in the crude extracts of *Phlogacanthus pulcherrimus*

Extract	Weight of extracts (g)	Total phenolic content (mgGAE/g extract)*
Dichloromethane extract (DE)	209.95	$15.16^c \pm 0.82$
Ethyl acetate extract (EE)	3.89	$55.05^a \pm 3.40$
n-butanol extract (BE)	17.67	$25.02^b \pm 5.60$
Aqueous extract (AE)	67.72	$13.77^c \pm 0.57$

\*Values expressed are mean  $\pm$ SD of triplicate measurements

\*mgGAE/g of extract: milligrams of gallic acid equivalents per gram of extract

a-c means with the different letters in the same column were significant at  $p \leq 0.05$

### ***Antioxidant activity***

In our body as a result of biological oxidation, free radicals and other reactive oxygen species (ROS) are formed which can lead to cellular and DNA damage as well as homeostatic disruption. The human body has defense this process by producing antioxidants which are either naturally within the body or externally supplied through food. Currently, the highly significant correlation between consumption of potent dietary antioxidants and lower the effects of oxidative stress. Therefore, potential dietary antioxidants can be screened with in vitro antioxidant. The DPPH and ABTS radical scavenging activities have been widely used to evaluate the antioxidative activity of plant extracts and foods. In this study, antioxidant capacity was examined using those different assays.

In previous study, the crude methanol extract of from *P. pulcherrimus* leaves showed promising antioxidant activity using DPPH and ABTS radical scavenging activity which is related to its total phenolic content (Lordkhem *et al.*, 2015). According to results of antioxidant activity by DPPH methods, *P. curviflorus* (Wall.) Nees var. *curviflorus* (Panyaphu *et al.*, 2012) and *P. thyriflorus* (Chanu *et al.*, 2012) showed that the methanolic extract of leaves had the antioxidant capacity. Then, the methanolic extract from *P. pulcherrimus* was partitioned with solvents of increasing polarities: dichloromethane (DE), ethyl acetate (EE), n-butanol (BE) and residual aqueous (AE), respectively. The antioxidant activities of four different extracts were expressed in terms of antioxidant capacity (mgTEAC/g extract) that compared with trolox as standards and IC<sub>50</sub> values (µg/ml) (Table 2). The examination of antioxidant capacity of extracts showed different values. The obtained values varied from 8.25±1.07 to 33.27±5.81 mgTEAC/g extract for DPPH assay and 15.45±1.76 to 45.50±3.85 mgTEAC/g extract for ABTS assay. The largest capacity to neutralize DPPH and ABTS radicals were found for ethyl acetate extract which inhibited 50% of free radicals at the concentration of 730.28 and 628.39 µg/ml, respectively. The minutest capacity to inhibit radicals was determined for dichloromethane extract. Due to low activity of dichloromethane extract, IC<sub>50</sub> are not calculated for it. From this data, the extracts containing high levels of phenolic content generally exhibit high antioxidant activities. It demonstrated that phenolic compounds are very important plant constituents because their phenolic hydroxyl groups are plays an important role in controlling antioxidants and scavenging ability (Chanu *et al.*, 2012).

**Table 2** Antioxidant activity of the various extracts from *P. pulcherrimus* in DPPH and ABTS assay

Extracts	DPPH assay		ABTS assay	
	IC <sub>50</sub> ( $\mu\text{g/ml}$ )	Antioxidant capacity* (mgTEAC/g extract)	IC <sub>50</sub> ( $\mu\text{g/ml}$ )	Antioxidant capacity* (mgTEAC/g extract)
DE	>2000	8.25 <sup>c</sup> ±1.07	>2000	15.45 <sup>c</sup> ±1.76
EE	730.28	33.27 <sup>a</sup> ±5.81	628.39	45.50 <sup>a</sup> ±3.85
BE	1422.93	18.53 <sup>b</sup> ±1.62	1160.41	26.44 <sup>b</sup> ±2.39
AE	>2000	9.87 <sup>c</sup> ±0.26	1065.33	23.06 <sup>b</sup> ±2.04
Trolox	26.86	-	31.59	-

\*Values expressed are mean  $\pm$  SD of triplicate measurements

\*mgGAE/g of extract: milligrams of gallic acid equivalents per gram of extract

a-c means with the different letters in the same column were significant at  $p \leq 0.05$

### *Cytotoxic activity*

During the last decade, in vitro cytotoxicity assays are commonly used for product development, drug discovery, drug delivery and evaluate the biological effects of chemicals on mammalian cells. In our previous publication (Lordkhem *et al.*, 2015), the methanolic extract of *P. pulcherrimus* leaves exhibited cytotoxic activity against three cancer cell lines (HT-29, HepG2 and KB) and Vero normal cell line with IC<sub>50</sub> value 308.00, 642.04, 259.88 and 84.27  $\mu\text{g/ml}$ , respectively. Therefore, the other cell lines and solvents should be additionally investigated. In this study, the cytotoxicity of the four different extracts (DE, EE, BE and AE) from leaves of *P. pulcherrimus* were evaluated in the five cancer cell lines (HT29, HeLa, MCF7, HepG2 and KB) and three normal cell lines (Vero, HaCaT and L929) by the MTT colorimetric assay. For screening of cytotoxicity evaluation of all extracts at 1000  $\mu\text{g/ml}$  concentration and exposure time 24 hours, the results showed that the dichloromethane extract (DE) showed the highest cytotoxic activity more than 90% cytotoxic against all cell lines. The ethyl acetate (EE) and n-butanol (BE) extracts showed moderated cytotoxicity and the residual aqueous exhibited no cytotoxic activity against both the cancer and normal cell lines (data not shown).

Here, we have evaluated the 50% inhibitory concentration (IC<sub>50</sub>) only the dichloromethane extract (DE). All obtained results for cytotoxicity are summarized in Table 3. According to US NCI plant screening program, the extract that gave IC<sub>50</sub> of 20  $\mu\text{g/ml}$  or less is considered active while it is 4  $\mu\text{g/ml}$  or less for pure compound (Lee and Houghton, 2005). Based on IC<sub>50</sub> values, this extraction showed the moderate cytotoxic activity against both cancer and normal cell lines. However, the extraction showed the high cytotoxic activity with IC<sub>50</sub> less than 100  $\mu\text{g/ml}$  against HeLa and MCF7 with IC<sub>50</sub> value of 81.46 and 45.34  $\mu\text{g/ml}$ , respectively. Including, it also showed cytotoxic effect against the normal cell, Vero and L929 with IC<sub>50</sub> value of 53.41 and 88.47  $\mu\text{g/ml}$ ,



respectively. In digestive cell lines, the extract did not show cytotoxic effect against HT29, HepG2 and KB cancer cell lines (IC<sub>50</sub> more than 100 µg/ml).

**Table 3** In vitro cytotoxic activity of the dichloromethane extract of *P. pulcherrimus* leaves on the eight cell lines measured by the MTT assay

Cytotoxic activity	Cancer cell lines					Normal cell lines			
	IC <sub>50</sub> (µg/ml)	HT29	HeLa	MCF7	HepG2	KB	Vero	HaCaT	L929
		271.66	81.46	45.34	124.98	105.07	53.41	102.81	88.47

To the best of our knowledge, this study was conducted of the cytotoxic effects on the human cell lines for extracts of *P. pulcherrimus* leaves. Our result was supported with findings of Tiwary *et al.*, (2015) who reported the ethanolic extract from leaves of *P. thyriformis* at 50 µg/ml concentration showed no cytotoxic activity for HepG2. However, *P. thyriformis* could inhibit the growth of HeLa and MCF7 cells with 58.03±4.2 and 52.34±1.8%, respectively. In contrast, the methanolic extract of *P. turgidus* displayed cytotoxic activity against HepG2 with IC<sub>50</sub> value of 9.4±0.2 µg/ml (Thu *et al.*, 2010). Moreover, both methanolic extract of *P. turgidus* and ethanolic extract *P. thyriformis* leaves have an effect on MCF-7; human breast cancer cell line (Thu *et al.*, 2010; Tiwary *et al.*, 2015). Nevertheless, the extract also showed significant cytotoxicity towards the Vero and L929 normal cell lines with IC<sub>50</sub> value of 53.41 and 88.47 µg/ml, respectively. It is well known that the different cell lines might exhibit different sensitivities which were treated with different extracts. So, the use of more than one cell line both normal and cancer cell lines seems necessary for cytotoxic screening. Furthermore, the dichloromethane extract showed the presence of three major components such as 3,5-Dimethylbenzaldehyde (28.61%), 1-Dodecanol (23.82%) and Squalene (18.58%) using the GC-MS analysis (data not shown). Therefore, the further detailed investigations of biological activity and chemical composition of medicinal plants as a potential source of natural antioxidants and cytotoxicity are necessary.

## Conclusion

This is perhaps the first study that deals with focused on antioxidant and cytotoxic potential of various crude extracts of *Phlogacanthus pulcherrimus* and determinations of their chemical composition in terms of phenolic content. The ethyl acetate extract was the most potent antioxidants both measured by DPPH and ABTS assays and also have the highest total phenolic content. Our finding support positive relationship between antioxidant activity and total phenol content indicated that these compounds contribute to the strong

antioxidant activity. MTT assay was employed to evaluate for their cytotoxic activity against eight different cell lines. The dichloromethane extract showed the high cytotoxicity with IC<sub>50</sub> less than 100 µg/ml against both cancerous (HeLa and MCF7) and normal (Vero and L929) cell lines. Further studies of this plant should be directed isolation of pure chemical constituents and in vivo biological activities evaluation in order to prepare high value of natural pharmaceutical products.

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