*Available online www.jocpr.com*

**Journal of Chemical and Pharmaceutical Research, 2014, 6(12):137-142**

**Research Article**

**ISSN : 0975-7384**
**CODEN(USA) : JCPRC5**

# Catechins contents and *in vitro* antioxidant activities of *Pentace burmanica* stem bark

**Thidarat Duangyod[1], Chanida Palanuvej[*1] and Nijsiri Ruangrungsi[1, 2]**

[1]*College of Public Health Sciences, Chulalongkorn University, Bangkok, Thailand*
[2]*Faculty of Pharmacy, Rangsit University, Pathumthani, Thailand*

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**ABSTRACT**

*Pentace burmanica Kurz, Thai medicinal plant, is commonly used for treatment of diarrhea. In this study, the antioxidant activities, total phenolic, non-tannin phenolic and total tannin contents of Pentace burmanica stem bark from 12 different Thailand markets were investigated.A simple and reliable method to determine (+)-catechin and (-)-epicatechin contents was performed by high performance liquid chromatography (HPLC). The ethanolic extract of Pentace burmanica stem bark at the concentration of 100 µg/ml showed high antioxidant activities. The percentage of free radical scavenging activity ranged between 71.56 –80.26% in 2, 2-diphenyl-1-picylhydrazyl (DPPH) assay. Ferric reducing antioxidant power (FRAP) values were 0.09 –0.29 mM $FeSO_4$/100 µg crude extract. The percentages of chelating activity were of 4.24 –12.14 in metal ion chelating assay. The antioxidant activities in beta-carotene bleaching assay were of 22.76 –41.06%. The Pentace burmanica extracts contained phenolic, non-tannin phenolic and tannin contents with the range between 35.85 –51.56, 14.08 –40.66, and 10.90 –21.77 µg catechin equivalents /100µg crude extract respectively. Low contents of (+)-catechin were found in the Pentace burmanica extract(< LOQ); whereas the (-)-epicatechin contents were found to be high (10.66-91.55 µg/mg of crude extract). The results demonstrated that greater amount of phenolic contents lead to more potent antioxidant effects and the different sources of Pentace burmanica showed the variation in both antioxidant activities and phenolic contents.*

**Keywords**: *Pentace burmanica*, Antioxidant activity, Non-tannin phenolic, (+)-Catechin, (-)-Epicatechin

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## INTRODUCTION

Medicinal plants have been used to treat human diseases for centuries.Due to fear of side effects from Western medicine, many people are becoming increasingly interested in medicinal plants. The reason for increasing interest towards plant medicines may come from their long-term use and the belief that medicinal plants have no side effects and safe because they are natural [1, 2]. Furthermore, natural antioxidant substances are increasing interested in food and pharmaceutical industry to replace the synthetic antioxidants. The natural antioxidants are believed to play an important role in inferring with the oxidative process [3]. Free radicals, in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS), are involved in pathological conditions such as cancer and Alzheimer's disease. Therefore, excessive production of ROS and RNS can lead to oxidative stress [4-6]. It is known that phenolic compounds involve in reducing the risk of diseases related to oxidative stress. Recent studies have been reported the positive correlation between total phenolic content of plant extracts and the antioxidant activity [7-12]. Hence, it is important to find out the new source of natural antioxidant with is safe and inexpensive.

*Pentace burmanica*Kurzbelonging to the Malvaceae family is commonly known in Thai as Si-Siat-Pleuak. It distributes in the tropical forest of Myanmar, Cambodia, China, Laos, Malaysia, Thailand, and Vietnam. The stem bark of this plant is used for anti-diarrhea in Thai traditional medicine. Moreover, older people in Laos and Northeast Thailand use this stem bark as an ingredient in chewing betel for the strengthening teeth. The water extract and 50% ethanol of *Pentace burmanica* bark extract were found to be an effective antimicrobial agent. This extract was effective in both gram positive (*Staphylococcus aureus* and *Streptococcus mutans*) and gram negative bacteria

(*Escherichia coli*) [13]. Additionally, the *Pentace burmanica* consists of tannin about 9.93% [14]. This medicinal plant has not been accessed for the antioxidant activity. Therefore, this present study is attempted to investigate the antioxidant activities, total phenolic content as well as the (+)-catechin and (-)-epicatechinamounts of *Pentace burmanica* stem bark.

## EXPERIMENTAL SECTION

Sample collection

Twelve samples of *Pentace burmanica* stem bark were collected from markets in 10 provinces as Buriram, Chaiyaphum, Chiang Rai, NakhonNayok, NakhonPhanom, Phetchabun, Sa Kaeo, Sisaket, Surin, and UbonRatchathani. All sets of crude drugs were authenticated by Associate Professor Dr. NijsiriRuangrungsi. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Sample extraction

Ground sample of *Pentace burmanica* stem bark (5 g) was exhaustively extracted with 95% ethanol using a Soxhlet apparatus. The 95% ethanol extract was filtered through Whatman No. 4 and evaporated under vacuum. The extract yield was weighed, recorded and stored at -20 °C to avoid the possibility of degradation of active compound. The extract at concentration of 100 µg/ml in methanol was used to evaluate the antioxidant activities, total phenolic and total tannin contents. For HPLC analysis, the concentration of the extract at 1 mg/ml was used.

Chemicals and materials

(+)-Catechin hydrate (CAS no. 225937-10-0, purity ≥98 %), (+)-catechin (CAS no. 154-23-4, purity ≥99 %), (-)-epicatechin (CAS no. 490-46-0, purity ≥98 %), buthylated hydroxyl toluene (BHT),2, 2-diphenyl-1-picylhydrazyl (DPPH), hide powder, iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), linoleic acid, sodium carbonate ($Na_2CO_3$), sodium acetate ($C_2H_3NaO_2$), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron (III) chloride ($FeCl_3 \cdot 6H_2O$) was purchased from Ajax Finechem (New Zealand) Beta-carotene and ferrozine were from Fulka (USA). Ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu reagent, and Tween 20 were obtained from Merk (Darmstadt, Germarny). HPLC grade methanol and acetonitrile were obtained from RCI Labscan, Thailand. Formic acid was purchased from Fisher Scientific (Leicestershire, UK). Ultra-pure water used for aqueous solution was prepared by SNW ultra-pure water system (NW20VF, Heal Force). The filters were 46 mm x 0.45 µm nylon membrane filters (National Scientific, TN) and 13 mm x 0.45 µm PTFE membrane syringe filters (ANPEL Scientific Instrument, China).

Antioxidant activities

2, 2-diphenyl-1-picylhydrazyl (DPPH) assay

Five hundred microliters of *Pentace burmanica* extract (100 µl/ml) was mixed with 500 µl of 120 µM DPPH solution in methanol. The incubation was performed in the dark at room temperature for 30 min. The reduction of the DPPH radical was determined by measuring the absorbance at 517 nm using a spectrophotometer (UV-1800 model, Shimadzu, Kyoto, Japan). A blank sample contained the same amount of methanol and DPPH solution. (+)-Catechin hydrate was used as a positive control. Triplicate measurements were carried out. Percent scavenging activity was calculated from the following equation:

$$\text{Scavenging activity (\%)} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{control}}] \times 100$$

Ferric reducing antioxidant power (FRAP) assay

FRAP reagent was prepared according to the method of Benzie and Strain [15]. Briefly, the FRAP reagent was prepared by mixing 100 ml of 300 mM acetate buffer pH 3.6 with 10 ml of 10 mM 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ) dissolved in 40 mMHCl and 10 ml of 20 mM $FeCl_3 \cdot 6H_2O$. Freshly prepared reagent was warmed at 37 °C for 15 min before used. Twenty five microliters of each sample (100 µg/ml) was mixed with 175 µl of the FRAP reagent and then left for 30 min under the dark conditions at room temperature. The absorbance was measured at 593 nm using a microplate reader (BiochromAsys UVM 340). $FeSO_4 \cdot 7H_2O$ was used as standard reference and different concentrations in the range of 0.1-1.0 mM were used for calibration curve. Results were expressed in mM Fe (II)/100 µg of crude extract. In order to make comparison, (+)-catechin hydrate was also tested under the same conditions as standard antioxidant compound. All samples were performed in triplicate.

Metal ion chelation activity

The chelating activity of sample on $Fe^{2+}$ was measured according to the method of Gupta *et al*. [16]. Briefly, 150 µl of each *Pentace burmanica* extract at concentration of 100 µl/ml in methanol was incubated with 7.5 µl of 2 mM $FeCl_2$ for 5 min. Then 30 µl of 5 mMferrozine was added to the mixture. After 10 min, the absorbance of ferrous ion-ferrozine complex at 562 nm was read using a microplate reader. EDTA was served as positive control. All determinations were performed in triplicate. The ability of the sample to chelate ferrous ion was calculated using the following equation:

Chelating activity (%) = [(Absorbance $_{control}$–Absorbance $_{sample}$)/ Absorbance $_{control}$] x 100

Beta-carotene bleaching assay

Beta-carotene bleaching assay was performed in cuvette to investigate the lipid peroxidation activity. Briefly, 1 mg of beta-carotene, 40 mg of linoleic acid, and 400 mg of Tween 20 were mixed in 4 ml of chloroform. Then chloroform was removed at 40 °C under vacuum. The mixture was immediately diluted with 100 ml of water then the mixture was vigorous agitated for 5 min using ultrasonic bath to form an emulsion. Aliquots of the emulsion (1 ml) were transferred into different cuvettes which contained 250 µl of sample (100 µg/ml). The mixture was then gently mixed and placed in a water bath at 50 °C for 180 min. Absorbance of the sample was recorded at 0 min and 180 min at 470 nm using a spectrophotometer. All determinations were performed in triplicate. (+)-Catechin hydrate and BHT were used as positive controls. The negative control was methanol. The degradation bleaching rates of beta-carotene was evaluated as the percent of antioxidant capacity using the following equation:

Antioxidant capacity (%) = $[1-(A_0-A_{180})/(C_0-C_{180})] \times 100$

$A_0$, $A_{180}$ : absorbance at zero time and end time of incubation for test sample respectively
$C_0$, $C_{180}$ : absorbance at zero time and end time of incubation for test control respectively

Total phenolic content

The total phenolic content of sample was determined using the Folin-Ciocalteu assay. Eight hundreds microliters of sample extracts (100 µg/ml) and 200 µl of 15% Folin- Ciocalteu reagent were added in the test tube then adjusted the volume to 2.0 ml with water. After 5 min, 1.0 ml of $Na_2CO_3$ (0.106 g/ml) was added. After 60 min of incubation in the dark at room temperature, the absorbance at 756 nm was measured using a spectrophotometer. The total phenolic contents in all sample extracts were expressed as micrograms of catechin equivalents (CE) per 100 µg crude extract. Triplicate measurements were carried out.

Total tannin content and non-tannin phenolic content

The total tannin content was estimated by Folin-Ciocalteu assay. Briefly, 3.5 mg of hide powder was weighed, and then 5 ml of sample (100 µg/ml) was added in the test tube. The mixture was shaken for 60 min afterwards centrifuged for 10 min at room temperature and finally the supernatant was collected. The supernatant had only simple phenolic compounds other than tannins. The tannins would have been precipitated along with the hide powder. The phenolic content of the supernatant was then measured following the same procedure describe above. The content of non-tannin phenols was expressed as micrograms of CE per 100 µg crude extract. Total tannin content was determined by subtraction of non-tannin phenolic content from total phenolic content. All samples were performed in triplicates.

(+)-Catechin and (-)-epicatechin analysis by HPLC

The determination of (+)-catechin and (-)-epicatechin contents were performed by HPLC analysis. (+)-Catechin and (-)-epicatechin were identified by comparing the retention time and UV spectrum of each peak with those of standard compounds. The quantitation of catechins was evaluated by comparing the area under peak with the calibration curve.

Preparation of standard solution

The stock solution of (+)-catechin and (-)-epicatechin were prepared by dissolving 1 mg of each compound in 1 ml of methanol. The solution was filtered through a 0.45 µm PTFE membrane syringe filter.

Preparation of sample solution

One miligram of *Pentace burmanica* stem bark extract was dissolved in 1 ml of methanol and vortex for 1 min. Then the solution was filtered through a 0.45 µm PTFE membrane syringe filter before chromatographic analysis.

Chromatographic conditions

Shimadzu DGU-20A3 HPLC (Shimadzu, Japan) consisted of a binary solvent delivery system, an auto-sampler, a column temperature controller, and a photo diode array detector (Shimadzu SPD-M20A, Shimadzu, Japan). System control and data analysis were processed with Shimadzu LC Solution software. The chromatographic separation was accomplished with an Inersil ODS-3 column (5 µm x 4.6 x 250 mm) and an Inertsil ODS-3 HPLC guard column (5 µm x 4.0 x 10 mm) using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as mobile phase at a flow rate of 1 ml/min. The isocratic program was set at 20% B for 15 min. The mobile phases were filtered through 0.45 µm nylon membrane filters and degassed using an ultrasonic bath before analysis. The column temperature was maintained at 40 °C and the injection volume was 1 µl. The wavelength was set at 280 nm.

## RESULTS AND DISCUSSION

As previously reported, the antioxidant activity depends on the chosen method, on the concentration and on the nature and physicochemical properties of studied antioxidants. The antioxidant capacities are influenced by many factors which cannot be fully described by a single method. It is necessary to perform more than one type of antioxidant activity measurement to take into account the various mechanisms of antioxidant actions [8]. Consequently, the ethanolic extracts of*Pentace burmanica* stem bark from 12 different sources throughout Thailand at the concentration of 100 µg/ml were investigated for their antioxidant activities by four different methods: DPPH assay, FRAP assay, metal ion chelating assay, and beta-carotent bleaching assay. The results were showed in table 1. The ethanolic extracts of*Pentace burmanica* stem bark showed as good free radical scavenger with 71.56 –80.26% inhibition in DPPH assay. These extracts reduced the most of $Fe^{3+}$ ions in the ferric reducing power investigation with the FRAP values ranged between 0.09 –0.29 mM $FeSO_4$/100 µg crude extract. The reducing power (FRAP values) of the extracts were increased with the quantity of phenol in almost samples except the sample no.9 and 10. The results were consistent with the finding of various researches that showed positive correlations between total phenolic content and antioxidant activity [7-12]. The percentages of chelating activity of *Pentace burmanica* stem bark ethanolicextracts were of 4.24 –12.14%. The chelating activity of EDTA standard was of 98.39%. The results demonstrated that the extracts at the concentration of 100 µg/ml had the ability to chelate iron but the percent chelating activities were quite low when compared with EDTA. These results might indicate that catechins or phenolic compound presenting in *Pentace burmanica* extract might not be the main chelators of ferrous ions. Hider *et.al* stated that a sample containing high polyphenols might not chelate metal if the polyphenols present did not have suitable groups that could chelate the cations [17]. The antioxidant activities in beta-carotene bleaching assay were of 22.76 –41.06%. The results suggested that the sample extracts had the ability to inhibit oxidation. In this present study, it was observed that the samples from different sources exhibited a variation in antioxidant activities.

**Table 1 The antioxidant activities of *Pentace burmanica* stem bark extract from 12 different sources throughout Thailand**

| No. of sample | DPPH Inhibition (%) | FRAP value[a] | Ferrous ion chelation (%) | Beta-carotene bleaching inhibition(%) |
|---|---|---|---|---|
| 1 | 78.63 ± 1.64 | 0.18 ± 0.01 | 19.64 ± 4.88 | 22.76 ± 12.07 |
| 2 | 79.76 ± 0.50 | 0.16 ± 0.02 | 13.94 ± 1.53 | 32.59 ± 5.63 |
| 3 | 77.49 ± 1.24 | 0.14 ± 0.03 | 15.50 ± 1.00 | 33.27 ± 9.29 |
| 4 | 80.01 ± 1.63 | 0.18 ± 0.03 | 13.71 ± 1.63 | 31.66 ± 4.78 |
| 5 | 71.56 ± 1.04 | 0.10 ± 0.03 | 12.10 ± 2.42 | 41.06 ± 7.61 |
| 6 | 80.26 ± 0.48 | 0.22 ± 0.05 | 15.40 ± 0.95 | 28.11 ± 5.80 |
| 7 | 79.45 ± 1.33 | 0.19 ± 0.01 | 13.19 ± 5.59 | 32.28 ± 7.36 |
| 8 | 80.08 ± 0.22 | 0.19 ± 0.01 | 17.61 ± 5.55 | 39.09 ± 9.59 |
| 9 | 78.88 ± 0.58 | 0.29 ± 0.03 | 13.14 ± 2.12 | 32.13 ± 4.40 |
| 10 | 79.38 ± 0.82 | 0.09 ± 0.02 | 11.86 ± 1.17 | 32.96 ± 2.69 |
| 11 | 76.48 ± 1.61 | 0.21 ± 0.05 | 14.80 ± 2.95 | 27.40 ± 8.88 |
| 12 | 79.45 ± 1.84 | 0.21 ± 0.04 | 13.06 ± 4.65 | 34.45 ± 12.24 |
| (+)-Catechin hydrate | 82.66 ± 0.24 | 0.542 ± 0.003 | 2.59 ± 1.87 | 21.67 ± 5.01 |
| BHT | - | - | - | 44.50 ± 6.62 |
| EDTA | - | - | 98.39 ± 0.16 | |

*[a]mM $FeSO_4$/100 µg crude extract*

LOQ: Limit of quantitation

The ethanolic extract of *Pentace burmanica* stem bark contained phenolic, non-tannin phenolic and tannin contents with the range between 35.85 –51.56, 14.08 –40.66, and 10.90 –21.77 µg CE/100 µg crude extract respectively as shown in table 2. The extract yields of 12 different sources of *Pentace burmanica* were depicted in table 2. All *Pentace burmanica* stem bark samples showed high antioxidant activities, total phenolic, and non-phenolic contents whereas total tannin contents were quite low. Nevertheless, the total tannin contents of *Pentace burmanica* extract agreed with previous report[14]. The values of antioxidant activities, total phenolics, non-tannin phenolics, and total tannin were found to be different for different sources of *Pentace burmanica*. The findings demonstrated high antioxidant activities related to non-tannin phenolic content that were in accordant with previous studies [18, 19].

**Table 2The total phenolic, non-tannin phenolic, total tannin, (+)-catechin, (-)-epicatechin contents and the extract yields of *Pentace burmanica* stem bark extract from 12 different sources throughout Thailand**

| No. of sample | Total phenolics[a] | Non-tannin phenolics[a] | Total tannin[a] | (+)-Catechin[b] | (-)-Epicatechin[b] | Extract yield (% w/w) |
|---|---|---|---|---|---|---|
| 1 | 42.20 ± 0.12 | 28.55 ± 0.61 | 13.65 | 9.06 ± 0.06(<LOQ) | 43.60 ± 1.27 | 33.65 |
| 2 | 45.58 ± 0.47 | 27.20 ± 0.21 | 18.38 | 13.02 ± 0.68(<LOQ) | 66.89 ± 2.24 | 42.36 |
| 3 | 39.80 ± 0.22 | 18.56 ± 0.20 | 21.24 | 8.81 ± 0.41(<LOQ) | 21.37 ± 0.04 | 18.15 |
| 4 | 46.66 ± 0.17 | 29.32 ± 0.37 | 17.34 | 9.63 ± 0.15(<LOQ) | 57.87 ± 1.86 | 32.49 |
| 5 | 35.85 ± 0.16 | 14.08 ± 0.08 | 21.77 | 8.05 ± 0.10(<LOQ) | 10.65 ± 0.10 | 22.13 |
| 6 | 46.85 ± 0.10 | 32.31 ± 0.53 | 14.54 | 17.50 ± 0.31 | 89.84 ± 0.84 | 34.90 |
| 7 | 47.16 ± 0.09 | 32.81 ± 0.07 | 14.35 | 14.08 ± 0.14 (<LOQ) | 62.35 ± 2.81 | 34.26 |
| 8 | 44.84 ± 0.18 | 28.41 ± 1.09 | 16.43 | 18.09 ± 0.16 | 71.17 ± 2.07 | 32.54 |
| 9 | 45.41 ± 0.07 | 31.89 ± 0.25 | 13.51 | 13.91 ± 0.71 (<LOQ) | 83.23 ± 1.73 | 37.80 |
| 10 | 51.56 ± 0.18 | 40.66 ± 0.92 | 10.90 | 16.35 ± 0.13 | 91.55 ± 1.99 | 35.89 |
| 11 | 50.38 ± 0.46 | 35.59 ± 0.02 | 14.80 | 11.75 ± 0.72 (<LOQ) | 51.70 ± 2.02 | 33.37 |
| 12 | 47.67 ± 0.34 | 29.97 ± 0.06 | 17.70 | 15.88 ± 0.77 | 66.65 ± 0.57 | 34.90 |

*[a] µg CE/100 µg crude extract*
*[b] µg/mg of crude extract*

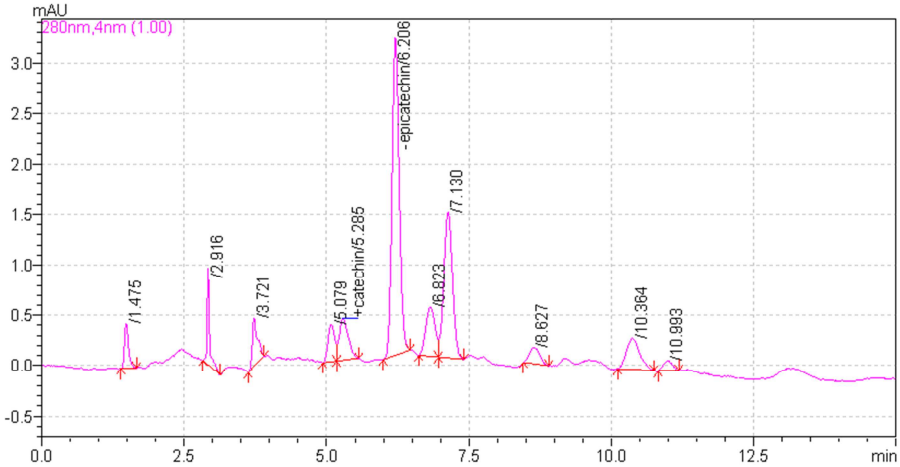


**Figure 1 HPLC chromatogram of *Pentace burmanica* stem bark extract**

**Table 3 The method validation parameters of (+)-catechin and (-)-epicatechin**

| Parameter | (+)-Catechin | (-)-Epicatechin |
|---|---|---|
| **Linearity** | $y$ = 746.29$x$-2203.3 | $y$ = 517.61$x$-652.07 |
| **$R^2$** | 0.9990 | 0.9989 |
| **Range** | 5 - 200µg/ml | 5-200µg/ml |
| **Peak purity index** | 0.999 | 0.999 |
| **Accuracy: % Recovery** | 91.11 - 97.02 % | 87.12 - 93.78% |
| **Precision** | | |
| **- Repeatability** | 0.27 – 0.42%RSD | 0.31 – 0.62%RSD |
| **- Intermediate precision** | 1.66 – 2.93 %RSD | 0.76 – 1.13 %RSD |
| **Limit of detection (LOD)** | 4.80µg/ml | 5.14 µg/ml |
| **Limit of quantitation (LOQ)** | 14.54 µg/ml | 15.57 µg/ml |
| **Robustness** | | |
| **- Retention time** | 0.58 – 0.96 %RSD | 0.58 – 1.09 %RSD |
| **- Peak area** | 4.27 – 4.58 %RSD | 1.24 – 1.65 %RSD |

HPLC chromatogram of *Pentace burmanica* stem bark extract showed several chemical compounds containing in the extract (Figure 1). Both (+)-catechin and (-)-epicatechin peaks were found in the chromatogram. Low concentration of (+)-catechinin was detected in *Pentace burmanica*; whereas (-)-epicatechin was found to be high in content (Table 2). The maximum content of (-)-epicatechin was 91.55 µg/mg of crude extract; while the minimum was 10.66 µg/mg of crude extract. Varied concentration of(-)-epicatechin might be due to the difference of geographical areas and the age of *Pentace burmanica*. Suwannakood reported that age and height of *Pentace*

*burmanica* had arelationship with a quantity of tannin extract [13]. Validity of the method was previously reported on its reliability for catechins analyses in *Pentace burmanica*stem bark [20].The summary was shown in table 3.

## CONCLUSION

This present study represents the first report on the antioxidant activities, total phenolic contents as well as (-)-epicatechin contents of *Pentace burmanica* stem bark.High antioxidant activities of *Pentace burmanica* extracts were related to phenolic contents especially non-tannin phenolic compounds. In addition, the different sources of *Pentace burmanica* showed the variation in antioxidant activities and phenolic contents.

**Acknowledgements**
The authors are gratitude to the Herbal Remedies and Alternative Medicine Task Force of STAR: Special Task Force for Activating Research under 100 Years Chulalongkorn University Fund and thankful to College of Public Health Sciences, Chulalongkorn University for necessary assistance and instrumental support.

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