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*Original Article*

# Antioxidant activities of the standardized water extract from fruit of *Phyllanthus emblica* Linn.

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**Abstract**

*Phyllanthus emblica* Linn. is widely used in Thai traditional medicine for treatment of various diseases. The fruit of *P. emblica* is known as a rich source of vitamin C, and also contains a mixture of phenolic compounds. In this study, the standardized water extract of *P. emblica* fruit was prepared according to Thai Herbal Pharmacopoeia. Total polyphenol contents of the extract were equivalent to 34.22±1.74 g gallic acid/100g extract. Antioxidant activities of the *P. emblica* extract were evaluated by several methods, including DPPH and $ABTS^{\bullet+}$ radical scavenging assays and FRAP assays. The results showed that the extract has an ability of scavenging radicals generated by both DPPH and $ABTS^{\bullet+}$. Similar to Trolox, the water extract of *P. emblica* fruit also had a ferric reducing property. Additionally, the extract effectively inhibited $H_2O_2$-induced free radical production in human myeloleukemic U937 cells as measured by 2,7-DCF-DA. The results imply that the fruits of *P. emblica* are potential sources of natural antioxidants, which have free radical scavenging activity and might be useful for hepato-, cyto-, and radio- protection, as well as reducing oxidative stress in many pathological conditions.

**Keywords:** $ABTS^{\bullet+}$, antioxidant, free radical, DPPH, *Phyllanthus emblica* Linn.

## 1. Introduction

*Phyllanthus emblica* Linn. (synonym: *Emblica officinalis* Gaertn.) is native to the tropics of South and Southeast Asia. In Thailand, it is known as Ma-kham-pom. *P. emblica* is highly nutritious and is an important dietary source of vitamin C, minerals, and amino acids, such as calcium, phosphorus, iron, carotene, thiamine, riboflavin, and niacin (Department of Medical Sciences, 2000). In *P. emblica* fruit, vitamin C is considered to be highly stable due to the presence of tannin and polyphenols (Morton, 1960). Fruits, bark, and leaves of this plant are rich in tannin. Chemical studies on this plant reveal several norbisabolane and

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bisabolane derivatives from the root (Zhang *et al*., 2000; Zhang *et al*., 2001b) as well as new galloyl esters of L-malic acid, mucic acid, and mucic acid 1,4-lactone from the fruit juice (Zhang *et al*., 2001c). Later investigation of the fruit juice results in the isolation of a new ellagitannin and phyllanemblinin A. In addition, phyllanemblinins B-F have been found in the leaves and branches (Zhang *et al*., 2001a).

The ethanol fruit extract shows gastroprotective (Al-Rehaily *et al*., 2002), antiproliferative (Khan *et al*., 2002) and antioxidant activties (Sai Ram *et al.*, 2002). Furthermore, the ethyl acetate fruit extract provokes nitric oxide (NO) scavenging activities (Kumaran and Karunakaran, 2006). The methanol extract shows hepatoprotective activity against $CCl_4$ (Lee *et al*., 2006), and chemopreventive potential for hepatocarcinogenesis (Sultana *et al*., 2008). In addition, the water fruit extract shows anti-carcinogenesis (Jeena *et al*., 1999), hepatoprotective activity against $CCl_4$ (Jose and Kuttan, 2000), anti-tumor (Jose *et al*., 2001), and radioprotective effect against gamma irradiation (Singh *et al*., 2005, 2006).

In the present study, the water extract from fruit of *P. emblica* was prepared according to Thai Herbal Pharmacopoeia (THP). Antioxidant activity of the extract was investigated by several different methods, including DPPH and $ABTS^{\bullet+}$ radical scavenging assays as well as ferric reducing antioxidant power (FRAP) assay. Furthermore, the antioxidant effect of the extract was confirmed by measuring the levels of intracellular peroxides in human myeloleukemic U937 cells.

## 2. Materials and Methods

### 2.1 Plant material

The fruits of *P. emblica* were collected from the forest of Nan Province, Thailand. The voucher specimen (PBM 01402) is kept at the Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

### 2.2 Preparation of plant extract

The water extract of *P. emblica* fruits was prepared as briefly described as follows: 194 kilograms of *P. emblica* dried fruit were boiled with water for 1 h and filtered; the procedure was repeated three times. The extract was spray dried and standardized by high performance liquid chromatography (HPLC) using gallic acid as a reference standard. The percentage yield was 8.76 of raw materials in which a content of gallic acid is 20.5 % w/w of dried extract. The quality control of raw materials and the extract was followed by Thai Herbal Pharmacopoeia (Department of Medical Sciences, 2000).

### 2.3 Determination of total polyphenols

Total phenolic content of the *P. emblica* extracts was determined by Folin-Ciocalteu reagent (Kumazawa *et al*., 2002). The assay reaction contained the mixture of 10 mg/ml extract and the Folin-Ciocalteu reagent (Merck Chemical Supplies, Darmstadt, Germany) and 10% $Na_2CO_3$ in each 0.5 ml, which were incubated at room temperature for 1 h. The absorbance was measured by spectrophotometer at 760 nm. The calibration curve of polyphenols was prepared using gallic acid as standard in concentration range 2-8 mg/L. The phenolic content was expressed as grams of gallic acid in 100g of crude extract.

### 2.4 DPPH radical scavenging assay

The free radical scavenging of the extract was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay (Brand–Williams *et al.*, 1995). All extracts were prepared in various concentrations from 10-200 µg/mL in 50% methanol solution. The reaction assay contained 3 ml of 0.060 mM DPPH (Sigma Chemical Co. St. Louis, MO, USA) in methanol and 200 µL of extract solution. The reaction mixture was incubated in the dark at room temperature for 30 min. The absorbance of the mixture was measured by spectrophotometer at wavelength 517 nm. Gallic acid was used as references and 50% methanol solution was used as vehicle control. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100.$$

### 2.5 $ABTS^{\bullet+}$ radical scavenging assay (Re *et al*., 1999)

The $ABTS^{+}$ radical was generated by a reaction between 7 mM ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) (Sigma Chemical Co. St. Louis, MO, USA) and 4.9 mM potassium persulfate in water. Sample solution 50 mL with various concentrations was mixed with 3 ml $ABTS^{\bullet+}$ solution and the absorbance was measured at 734 nm at 6 min. The % reduction of $ABTS^{\bullet+}$ content represented as the inhibitory effect of sample. Trolox was used as standard and 50% methanol solution was used as vehicle control. The ability to scavenge $ABTS^{\bullet+}$ radical was calculated by the following equation:

$$\text{ABTS}^{\bullet+}\text{ radical scavenging activity (\%)} = [(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100.$$

The antioxidant activity of extract was also expressed as trolox equivalent antioxidant capacity (TEAC), which was the ratio of % inhibition of sample to that of Trolox at the same concentration. All experiments were done in triplicate for each concentration.

### 2.6 Ferric reducing antioxidant power (FRAP) assay

The reducing ability of the plant extract using ferric ion was modified from Benzie and Strain (1996). The assay

was preformed by the mixture of 4.5 mL FRAP reagent (Sigma Chemical Co. St. Louis, MO, USA), 450 mL double distilled water and 150 mL sample or Trolox as standard positive control. Then, the reaction assay was incubated at room temperature in the dark for 30 min. Absorbance was monitored at 595 nm. All experiments were done in triplicate for each concentration. The production of ferrous sulfate by the extract was calculated using a standard curve of ferrous sulfate. The antioxidant activity of extract was expressed as μmole of $FeSO_4$ per mg of the extract and compared with that of Trolox.

### 2.7 Determination of cellular antioxidant status

Human myeloleukemic U937 cells were maintained in RPMI (Gibco, Invitrogen, USA) containing 10% fetal bovine serum, penicillin G (100 IU/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) and incubated at 37°C in a 5% $CO_2$ atmosphere. Various concentrations of the extract were added to $5x10^5$ cells/plate in a serum-free RPMI medium and incubated for 24 hrs. The cells were washed twice with phenol red-free HBSS.

The production of intracellular peroxides was determined by 2,7-DCF-DA (Molecular Probe, Invitrogen, USA) which indicates the antioxidant activity of the extract. Cells were loaded with 10 μM $H_2$DCF-DA in phenol red-free HBSS and incubated for 45 min. After cells were washed once by phenol red-free HBSS and only $4x10^4$ cells in 100 mL HBSS were added into 96-well plate. Hydrogen peroxide (2mM) was used to induce oxidative stress for 30 min. The fluorescence values before and after addition of hydrogen peroxide were measured using a fluorescence plate reader (485-nm excitation and 530-nm emission, Packard Fusion™) and referred as the stress and background fluorescence level. Phenol red-free HBSS was used as control. The antioxidant activity was calculated by the following equation:

$$\text{Antioxidative capacity (\%)} = [(FL_{stress} - FL_{background})_{control}] / [(FL_{stress} - FL_{background})_{test}]$$

## 3. Result and Discussion

Previous studies have reported the fruits of *P. emblica* contain phenolic compounds and hydrolysable tannins. In our study, total polyphenol contents of the water extract of *P. emblica* were equivalent to 34.22±1.74 g gallic acid/100g extract. Similarly, Naik *et al.* (2005) reported 33% gallic acid equivalents of the total phenolic content present in the aqueous extract of this fruit. A variety of phytochemicals in *P. emblica* fruit has been identified including hydrolysable tannins (such as emblicanin-A, emblicanin-B, punigluconin, and pedunculagin), gallo-ellagitannoids, flavonoid (rutin), trigalloyl glucose, and phyllemblic acid (Ghosal *et al*., 1996, Pillay *et al.,* 1988). In addition, this fruit has been long claimed to be a rich source of ascorbic acid. However, it is still inconclusive whether the fruit of *P. emblica* does contain ascorbic acid since various methods of estimation were used in each study. Ghosal *et al.* (1996) reported that the strong antioxidant action of *P. emblica* does not result from either the free or the conjugated form of ascorbic acid, but rather the presence of the hydrolysable tannins. In contrast, the earlier reported antioxidant hydrolysable tannins, emblicanins A and B were validated in fact corresponding to β-glucogallin and mucic acid 1,4-lactone 5-O-gallate, respectively (Pozharitskava *et al.* 2007). Moreover, several studies asserted that *P. emblica* in deed contains a high content of ascorbic acid; 0.34-0.38% (Raghu *et al*., 2007) and 0.40% (Scartezzini *et al*., 2006) w/w of the fruit which accounts for 45-70% of the antioxidant activity. Recently, a study using multiple HPLC methods found small amounts of free ascorbic acid in *P. emblica* fruit (Majeed *et al.* 2009). Nonetheless, the fruit of *P. emblica* has been proved as a good source of natural antioxidants used for scavenging free radicals and reducing oxidative stress.

Antioxidant activities of the water extract of *P. emblica* fruit were evaluated herein by measuring its ability of scavenging free radical generated by DPPH and $ABTS^{\bullet+}$ and reducing ferric to ferrous (FRAP assay). As shown in Figure 1, 2 and Table 1, the inhibition of DPPH and $ABTS^{\bullet+}$ as well as the reduction of ferric ions to ferrous sulfate by the extract were dose-dependent. Fifty percent inhibition of DPPH and

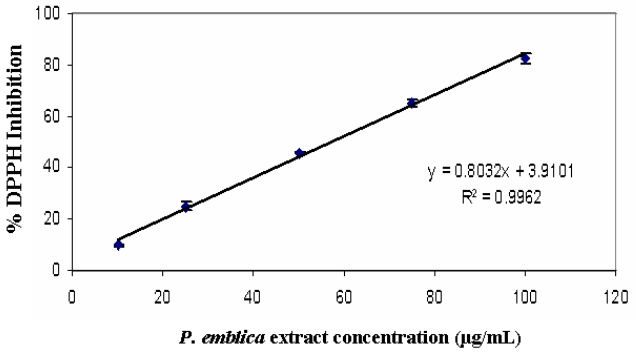


Figure 1. Antioxidant activity measured by DPPH assay of water extract of *P. emblica.*

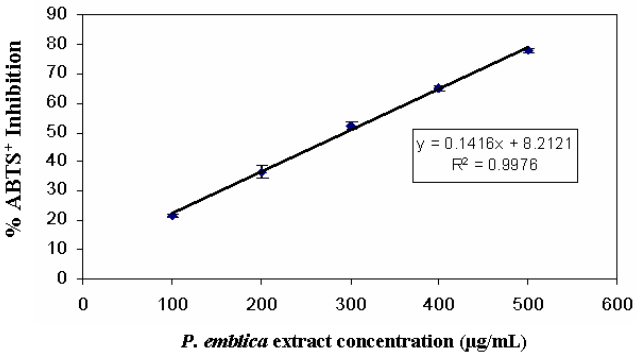


Figure 2. Antioxidant activity measured by $ABTS^{\bullet+}$ assay of water extract of *P. emblica.*

Table 1. Effect of *in vitro* antioxidant activity of *P. emblica*.

| Assay | IC50 (ug/mL) | | Relative to standard |
|---|---|---|---|
| | *P. emblica* | Standard(*gallic acid, **Trolox) | |
| DPPH | 51.3±16.5 | 8.5±0.7* | 0.182±0.070* |
| $ABTS^{\bullet+}$ | 295±5.4 | 177±3.22** | 0.600±0.011** |
| DCF | 0.65±0.04 | 4.48 ±1.32* | 6.90±1.15* |

(n=3; values are mean ± S.D.)

$ABTS^{\bullet+}$ were achieved at approximately 50 and 300 µg/mL of the extract, respectively. Noticeably, as the gallic acid content in our extract was 20% w/w, the 50% DPPH inhibitory dose (IC50) of the extract should be equivalent to approximately 10 µg/mL of gallic acid. This is similar to IC50 of the standard gallic acid (8.5 µg/mL) for DPPH activity as shown in Table 1. Therefore, it is possible that gallic acid in the extract plays a major role in DPPH radical scavenging activity. In addition, the extract of *P. emblica* was able to reduce ferric ion to $FeSO_4$ (7.46±0.56 µmole of $FeSO_4$/mg) which is about 0.89 fold compared to Trolox (8.35±0.16 µmole of $FeSO_4$/mg) in Figure 3 and Table 2.

Cellular antioxidant effect of the water extract of *P. emblica* fruit was further evaluated in human myeloleukemic U937 cells by DCF assay measuring the levels of intracellular reactive oxygen species (ROS) induced by $H_2O_2$. The result showed that the fruit extract can inhibit the production of intracellular ROS in a dose dependent manner (Figure 4). The concentration of the extract for 50% ROS inhibition was 0.62 µg/mL, which is higher than that of gallic acid (IC50 = 4.28 µg/mL). The results thus indicated that *P. emblica* fruit extract not only has radical scavenging activity, but also shows efficacy as a strong inhibitor against $H_2O_2$-induced cellular oxidative stress.

The results of these assays suggested that the antioxidant activity of extract does not depend upon only one particular compound. An earlier study by Scartezzini *et al.* (2006) showed that the antioxidant activity of aqueous extract of processed fruit is due to ascorbic acid for only 60% or less. Furthermore, hydrolysable tannins can also exhibit a very strong antioxidant activity *in vitro* and *in vivo* (Ghosal *et al.*, 1996, Bhattacharya *et al.*, 2000). The fruit extract of *P. emblica* also significantly inhibited chromium-induced free radical production and restored the antioxidant status in lymphocytes back to normal level (Sai Ram *et al.*, 2002). Thus, the antioxidant activity of *P. emblica* is likely attributed to its cytoprotective property.

In conclusion, the water extract of *P. emblica* fruit prepared according to Thai Herbal Pharmacopoeia has a strong potential for free radical scavenging, ferric reducing as well as inhibiting ROS production. The results imply that the fruits of *P. emblica* are potential sources of natural antioxidants useful for hepato-, cyto- and radio- protection as well as reducing oxidative stress in many pathological conditions.

Table 2. Effect of ferric reducing activity of *P. emblica*.

| Treatment | $FeSO_4$ (umole/mg) | Relative to Trolox |
|---|---|---|
| *P. emblica* | 7.46±0.56 | 0.893 ± 0.067 |
| Trolox | 8.35 ±0.16 | 1.000 ± 0.019 |

(n=3; values are mean ± S.D.)

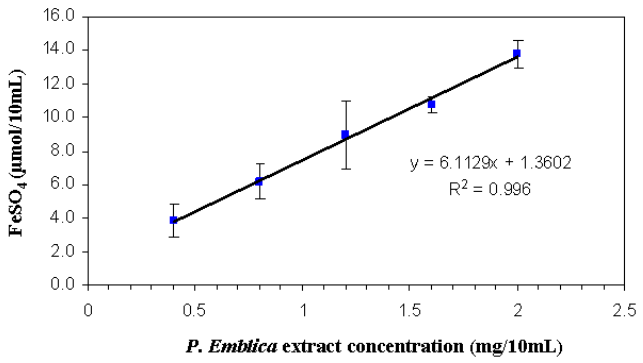


Figure 3. Antioxidant activity measured by FRAP assay (expressed as µmole of $FeSO_4$) of water extract of *P. emblica*.

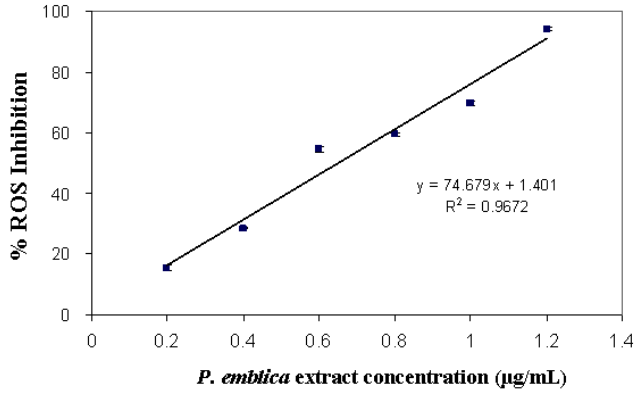


Figure 4. Antioxidant activity measured by DCF assay (expressed as % ROS inhibition) of water extract of *P. emblica*.

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