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Original Article

Antioxidant, antimutagenic and antibacterial activities of extracts from *Phyllanthus emblica* branches

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Abstract

Phyllanthus emblica is an euphorbiaceous plant that has long been used as an ingredient in traditional medicines and functional foods. Although traditional remedies use several parts including the fruit, leaf, flower, stem and roots, the fruit is the most popular. It has been used for health promotion, anti-aging and also for treatment of wide ranges of symptoms and diseases. Because of the broad spectrum of pharmacological activities and high demand for the *P. emblica* fruit, there is a shortage of raw materials. Furthermore, there is a seasonal limitation in which the fruit comes out once a year. The aim of this study is to investigate the bioactive potential and the possibility to use the plant branch. Alcohol based extracts of *P.emblica* branch were analyzed for total phenolic content, antioxidant, antibacterial and antimutagenic activities. *P. emblica* branches were used to prepare the 50% ethanolic extract by maceration (EBE) and methanolic extract by Soxhlet apparatus (MBE). Total phenolic content was determined by Folin-Ciocalteu method and DPPH was used to analyze the antioxidative activity. Antibacterial activity was evaluated by microdilution method and expressed by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Antimutagenicity was performed by preincubation bacterial assay in *Salmonella typhimurium* TA98 and TA100 strains. It was found that both EBE and MBE contained high total phenolic content (643 and 547 TAE mg/g) and strong antioxidative activity (EC_{50} at 30 and 23 mg/ml). The extracts showed antimutagenicity to both direct and indirect-acting mutagens in TA98 and TA100 strains. Both extracts possess antibacterial activity against *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Salmonella* sp. and *Pseudomonas aeruginosa*. In conclusion, the alcoholic extracts of *P. emblica* branch possess high phenolic content with strong antioxidative, antimutagenic and antibacterial activities as previously reported in *P. emblica* fruit. These findings support the traditional uses and may lead to future utilization of *P. emblica* branch in functional foods and skin products.

Keywords: *Phyllanthus emblica* branch, antioxidant, antibacterial, antimutagenic

1. Introduction

Phyllanthus emblica L., emblica or Indian gooseberry or "Makham Pom" in Thai, is an euphorbiaceous plant that is widely distributed in subtropical and tropical areas of China, India, Indonesia, Malaysia and Thailand. The fruit is the most popular part which contains high content of vitamin C and phenolic compounds. It has been used as major constituents for various traditional and Ayurvedic medicines. With previous reports on several pharmacological properties of emblica fruit such as antioxidant (Anila and Vijayalakshmi, 2003; Liu *et al*., 2008; Khopde *et al*., 2001), analgesic and anti-pyretic (Perianayagam *et al*., 2004; Gupta *et al*., 2008), antimicrobial (Ahmad *et al*., 1998; Rahman *et al*., 2009),

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antitumor (Jose *et al*., 2001; Rajeshkumar *et al*., 2003; Sultana *et al*., 2008), anti-inflammatory (Dang *et al*., 2011), immunomodulatory (Liu *et al*., 2012), hypolipidemic (Anila and Vijayalakshmi, 2002), and hypoglycemic activities (Abesundara *et al*., 2004; Akhtar *et al*., 2011; Nain *et al*., 2012), and hepatoprotective (Jeena *et al*., 1999; Achliya *et al*., 2004; Pramyothin *et al*., 2006), and gastroprotective effects (Bandyopadhyay *et al*., 2000; Sairam *et al*., 2002; AL-Rehaily *et al*., 2002), protection of ischemia-reperfusion-induced oxidative stress (Bhattacharya *et al*., 2002), prevention of hyperthyroidism (Panda and Kar, 2003) and protection of UVB-induced photo-aging (Adil *et al*., 2010), the demand of the plant's fruit used for food and medicine, and as an ingredient in cosmetics is high. According to its annual crop, the supply of emblica fruits is limited. It is important to search for other parts as an alternative substitute for the emblica fruit. In the present study, the phenolic content, antioxidant, antimutagenic and antibacterial activities of emblica branch extracts were investigated.

2. Materials and methods

2.1 Materials

Ascorbic acid was from Caro Erba (Italy), 2, 2-diphenyl-1-picrylhydracyl (DPPH), α-tocopherol and tannic acid were from Sigma (USA). Folin-Ciocalteu reagent was from Merck (Germany) and other analytical grade chemicals were used.

2.2 Preparation of plant extracts

Fresh emblica branches were collected in Khon Kaen province in January, 2011. After identification, a voucher specimen (KP 021) are deposited in the Faculty of Pharmaceutical Sciences, Khon Kaen University herbarium. The ethanolic extract of emblica branches (EBE) was produced, according to the petty patent (no. 4599, Thailand). The plant powder was macerated in 50% ethanol at a ratio of 1:5 for 7 days then filtered through Whatman® No.1 paper and centrifugated at 500 g for 10 min. The supernatant was evaporated under rotary evaporator and then freeze-dried in lyophilizer (Christ®, German) with %yield of 5.4. In parallel, the methanolic extract of emblica branches (MBE) was prepared using Soxhlet apparatus at 50°C for 3 h (at a ratio of 1:10), then the filtrate was similarly dried with %yield of 18.83. Both extracts were kept at-20°C until used.

2.3 Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu method (Singleton *et al*., 1999). The extracts were dissolved in methanol at various concentrations (0.1-5.0 mg/ml), then the extract solution (0.5 ml) was mixed with the Folin-Ciocalteu reagent (0.25 ml) and 20% sodium carbonate (1.25 ml). After mixing and standing at room temperature for 40 min, the absorbance was measured at 725 nm. The total phenolic content was expressed as mg tannic acid equivalent (TAE)/g dried extract.

2.4 Determination of DPPH radical scavenging activity

The free radical scavenging activity was determined by the method described by Shimada *et al*. (1992). The extract was dissolved in methanol at various concentrations (0.1-5 mg/ml), then 2.8 ml of each extract solution was mixed with 0.2 ml of DPPH solution (1 mM in methanol). After incubation at room temperature for 15 min, the absorbance was measured at 515 nm. The negative (methanol) and positive (vitamin C) controls were run in parallel. The scavenging activity was calculated using the formula, % scavenging = $[(A515_{control} - A515_{sample})/A515_{control}] \times 100$.

2.5 Antimutagenic test

The preincubation mutation method as originally described by Araki *et al* (1984) and further modified for plant extract test by Sripanidkulchai *et al*. (2002) was carried out in both the presence and absence of the rat hepatic microsomal fraction (S-9) mixture in order to detect indirect and direct antimutagenic, respectively. Two standard test strains, *Salmonella typhimurium* TA98 and TA100 were used. The dried plant extract was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mg/ml. The mixture of extract solution (0-0.1 ml) with 0.5 ml of S-9 mixture or 0.1 M phosphate buffer (pH 7.4) and 0.1 ml of the test bacterial solution was incubated at 30°C for 30 min in the presence of positive mutagens. Three standard positive mutagens were used, including two direct-acting (2-aminofluorene, AF-2; and 4-nitroquinoline-1-oxide, 4-NQO) and one indirect-acting (2-aminoanthracene, 2-AA). After incubation, the mixture was rapidly mixed with 2 ml of molten top agar containing 0.1 mmol of histidine and biotin, and poured rapidly into 30-ml Vogal-Bonner minimal agar plate and incubated at 37°C for 48 h. The background (negative) control (using DMSO) and positive control (using mutagens) were parallel conducted. The revertant colonies were counted, and the toxic effect was determined by viewing the background lawn under a stereo microscope. The % inhibition of plant extracts on number of revertant colonies was calculated.

2.6 Antibacterial test

Clinical isolated strains of five bacteria, including *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Salmonella* sp and *Pseudomonas aeruginosa* obtained from Srinagarind hospital, Khon Kaen University, Thailand, were used. The plant extract was dissolved in DMSO at a concentration of 50 mg/ml, then filtered through a 0.45 μm membrane filter and further tested by microbroth dilution method (Buwa and Van Staden, 2006). The extract solution was serial diluted with Mueller Hilton broth and put into a 96-well microplate

containing 100 µl of test bacteria at concentration of McFarland No 0.5 ($1x10^8$ cell/ml or $1x10^7$ cell/well). DMSO was used as a positive growth control, and standard antibiotics were used as inhibitors of bacterial growth. After incubation at 37°C for 18-24 h, the minimal inhibitory concentration (MIC) was determined as the lowest concentration of each extract that completely inhibited growth of microorganism. Then each 5 µl of the mixture broth was further cultured on an agar plate at 37°C for 18-24 h and the lowest concentration at which no microbial growth was observed as the minimal bactericidal concentrate (MBC) of the extract.

3. Results

3.1 Phenolic content and antioxidative activity of *P. emblica* branch extracts

Total phenolic content and DPPH radical scavenging activity of alcoholic extracts of *P. emblica* branches are shown in Table 1. Both 50% ethanolic extract of *emblica* branches (EBE) and methanolic extract of *emblica* branches (MBE) possess similar high phenolic contents (643.07±24.34 and 547.41±20.46 TAE mg/g dried extract) and strong antioxidative activity (EC_{50} of DPPH at 29.93±2.15 and 22.89±3.60 mg/ml, respectively.

3.2 Antimutagenicity of *P. emblica* branch extracts

Using bacterial mutation assay, neither EBE and MBE up to 100 mg/plate showed mutagenicity to TA98 and TA100 strains of *Salmonella typhimurium* in the presence and absence of S-9 activation (data not shown), suggesting the safety of these *P. emblica* branch extracts. For antimutagenic test in the absence of S-9 activation, EBE and MBE showed antimutagenicity to AF-2 with IC_{50} at 9.2±0.3 and 6.2±0.05; >10 and 4.7±0.05 mg/plate for TA98 and TA100, respectively. Similar effect was also observed when testing with another direct mutagen, 4-NQO, that gave the IC_{50} at 6.1±0.2 and 9.7±0.2; >10 and 8.7±0.2 mg/plate for TA98 and TA100, respectively. The results on indirect mutagen 2AA revealed the strong antimutagenicity of both EBE and MBE, giving the IC_{50} at 0.7±0.1 and 0.9±0.1 for TA98 and 0.6±0.1 and 0.6± 0.1 mg/plate for TA100, respectively (Figure 1).

3.3 Antibacterial activity of *P. emblica* branch extracts

The antibacterial activity of alcoholic extracts of *P. emblica* branch on five clinical strains was assessed by MIC and MBC values. Both EBE and MBE similarly inhibited *S. epidermidis*, *E. coli*, *Salmonella* sp and *P. aeruginosa*. However, EBE inhibited *S. aureus* slightly more than MBE did. Among these five bacteria tested, *S. epidermidis* was the most sensitive to EBE and MBE (Table 2).

4. Discussion and Conclusions

According to traditional uses of all parts of the plant including fruits, seeds, leaves, branches, bark, flowers and roots (Habib-ur-Rehman *et al*., 2007) and to encounter the fruit's seasonal limitation, this study selected the branch as a substitute candidate with the reason that branches can be obtained at anytime of the year. To our knowledge, besides the fruit, there are some phytochemical and pharmacological studies of the plant leaves (Srirama *et al*., 2012; Jeyasankar *et al*., 2012), but there are very few reports on the plant's

Table 1. Total phenolic content and antioxidative activity of ethanolic and methanolic extracts of *P. emblica* branches.

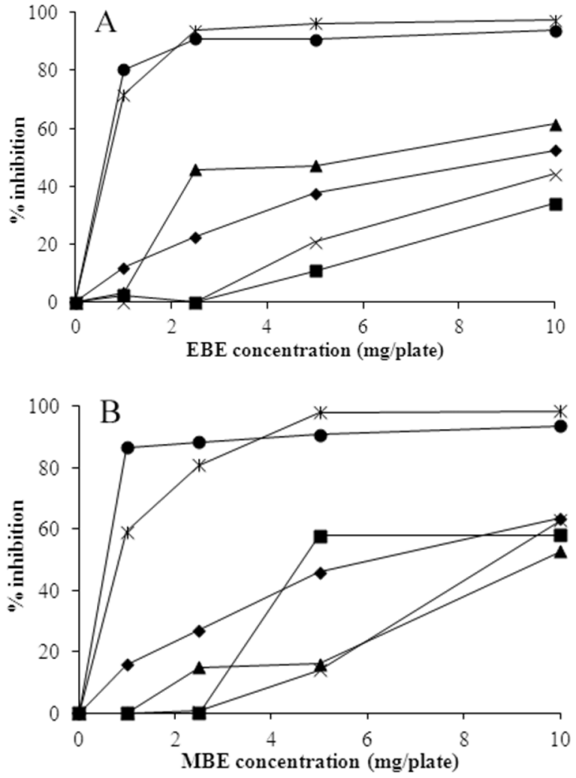
Extract	Phenolic Content[1] (eq. tannic acid, mg/g)	DPPH[2] (EC_{50} mg/ml)
EBE	643.07±24.34	29.93±2.15 (0.99±0.01)
MBE	547.41±20.46	22.89±3.60 (0.99±0.01)
Vit. C	-	9.58±1.89 (0.99±0.01)

[1] expressed as mean±SD (n=3)
[2] expressed as effective concentration at 50% inhibition (EC_{50}) and (r^2), n=3
EBE = ethanolic extract of *P. emblica* branches
MBE = methanolic extract of *P. emblica* branches

Table 2. Antibacterial activity of alcoholic extracts of *P. emblica* branch.

Sample	*S. aureus*		*S. epidermidis*		*E. coli*		*Salmonella* sp		*P. aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
EBE	3.2	>3.2	1.6	3.2	>3.2	>3.2	3.2	3.2	>3.2	>3.2
MBE	>3.2	>3.2	1.6	3.2	>3.2	>3.2	3.2	3.2	>3.2	>3.2
ampicillin	0.15	0.25	8.00	8.00	0.50	1.00	1	2	-	-
gentamicin	2	4	0.5	0.5	16	16	1	2	0.5	1

Values are expressed as mean of triplicate experiments in term of mg/ml, mg/ml for the plant extracts and standard antibiotics, respectively.



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mutagens	IC_{50} (mg/plate)			
	EBE		MBE	
	TA98	TA100	TA98	TA100
AF2	9.2±0.3	>10	6.2±0.05	4.7±0.05
4NQO	6.1±0.2	>10	9.7±0.2	8.7±0.2
2AA	0.7±0.1	0.6±0.1	0.9±0.1	0.6±.01

Figure 1. %inhibition of alcoholic extracts of *P. emblica* branches on *Salmonella typhimurium* TA98 and TA100 in the presence and absence of metabolic activation A: EBE and B: MBE, C: IC_{50} values. (AF2 = 2-aminofluorene; 4-NQO = 4-nitroquinoline-1-oxide; 2AA = 2-aminoanthracene, —◆— = AF2 (-S9) TA98; —■— = AF2 (-S9) TA100; —▲— = 4NQO (-S9) TA98; —✳— = 4-NQO (-S9) TA100; —✳— = 2AA (+S9) TA98; —●— = 2AA (+S9) TA100. Values are average from triplicate experiments.

branches. Our results revealed antioxidant, antimutagenic and antibacterial activities of *P. emblica* branch extracts. The plant extraction process included the cold extraction with 50% ethanol and Soxhlet extraction with methanol to obtain two crude alcoholic extracts namely EBE and MBE, respectively. Both plant extracts showed high phenolic contents with strong antioxidative activity as observed in the fruit part (Sripanidkulchai and Junlatat, 2014). The finding that EBE and MBE possess high antioxidative activity, suggests that alcoholic extracts of *P. emblica* branches has potential to be used. Moreover, the absence of mutagenicity of the plant branch extracts suggests the safety of the branch part. However, acute and chronic toxicities of the branch extract should be further confirmed in animal model. The antimutagenesis of both extracts support the anticancer effect of *P. emblica* fruit (Jose *et al*., 2001). The antibacterial activity of the alcoholic extracts of the plant branch was found to be similar to that previously reported in the plant fruit (Ahmed *et al*., 1998). Moreover, our recent study also demonstrated anti-inflammatory and antimelanogenesis effects of the plant branch extract, which contained at least seven phenolic compounds, i.e., gallic acid, vanillic acid, epigallocatechin gallate, vanillin, coumuric acid and ellagic acid, as identified by HPLC analysis (Sripanidkulchai and Junlatat, 2014). Based on the clinical efficacy of the fruit part of *P. emblica* to promote health and longevity, anti-aging and revitalizing, the plant alone or used as an ingredient of polyherbal formulations has been used in skin and beauty products. The fruit extract was also reported to promote collagen synthesis, inhibit the collagen degradation (Fujii *et al*., 2008; Chanvorachote *et al*., 2009) and act against UVB-induced photo-aging in human skin fibroblasts (Adil *et al*., 2010). Therefore further studies on these activities of the branch extracts should be done.

In conclusion, the present study demonstrates high phenolic content and strong antioxidative activity of ethanolic and methanolic extracts of *P. emblica* branches as previously observed in the plant fruit. The studies on selected pharmacological activities showed the potential effects of the alcoholic extracts of *P. emblica* branch as antibacterial and antimutagenic activities. These results suggested that *P. emblica* branch can be a substitute of *P. emblica* fruit, which is highly demanded for the utilization as an ingredient in traditional medicine and cosmetics.

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