RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2013.39.012

ScienceAsia 39 (2013): **12–18**

Antioxidant and anti-matrix metalloproteinases activities of dried longan (*Euphoria longana*) seed extract

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Received 22 Sep 2012
Accepted 20 Dec 2012

ABSTRACT: Longan, a major fruit crop in Thailand, is a rich source of antioxidant polyphenic compounds, mostly gallic acid and ellagic acid. Dried longan seeds contain large amounts of antioxidant polyphenols. In this study, three fractions of dried longan seed extract were collected by Sephadex LH-20 column chromatography to determine the major compounds in each, and the amounts of gallic acid and ellagic acid were quantified. The first fraction was determined to be the major source of gallic acid, while ellagic acid was mainly found in fraction 3. Antioxidant activities were measured using total phenolic content, total flavonoid content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, and metal chelating activity. Fraction 1 had the highest antioxidant activity. In addition, the effect of each fraction on matrix metalloproteinases (MMPs) activity was analysed using a fluorometric assay. Interestingly, fraction 3 had the greatest MMPs inhibitory effect. Notably, dried longan seeds have been established not only as major sources of antioxidants, but also as potent MMPs inhibitors. Since these properties are associated with anti-cancer effects, dried longan seeds could be a novel natural source for compounds used in chemoprevention and chemotherapy.

KEYWORDS: gallic acid, ellagic acid, HPLC, DPPH scavenging activity, metal chelation activity, MMP inhibitor

INTRODUCTION

Longan, *Euphoria longana* Lam. (syn. *Dimocarpus longan* Lour.) is a subtropical fruit widely grown in China and SE Asia, including Thailand, Vietnam, and the Philippines. In Thailand, longan, also known as 'lamyai', is a major fruit export. The fruit appearance includes a brown or light-brown peel with white translucent flesh, while the seeds are round and black with circular white spots at the base. Generally, the flesh is most popular for consumption fresh and in processed products such as canned longan in syrup or dried longan[1]. In Chinese medicine, the flesh of the fruit is used as a stomachic, febrifuge, vermifuge, and as an antidote for poison. The dried longan is also used as a tonic and for the treatment of insomnia and neurasthenic neurosis. While the seeds account for 17% of the fresh weight of whole fruits, these are usually discarded as a waste or burned as fuel. A previous study demonstrated that the aqueous extract of longan contained high levels of polyphenolic compounds such as corilagin, gallic acid, and ellagic acid[2]. The content of these polyphenols was mostly found in dried seed extract, while pulp contained the lowest amounts[1]. Several studies have indicated that gallic acid and ellagic acid have important health benefits. Gallic acid possesses anti-cancer properties via anti-proliferation and apoptosis induction of certain types of cancer cells such as leukaemia, prostate, colon and lung cancer cells[3–6]. Ellagic acid has antioxidant, anticarcinogenic, antifibrosis, antiplasmodial and chemopreventive activity[7–12]. These data indicate that longan seed extract may be a new source of supplementary antioxidants and natural chemopreventive agents. Since the limitation of research on other biological activities such as anti-invasion and anti-metastasis, this present study is first manifestation of the dried longan seed extract action on the major modulator on metastasis processing, especially matrix metalloproteinases (MMPs).

MMPs are a family of secreted and transmembrane Zn^{2+} endopeptidases and are produced by host connective tissue or tumour cells. This family currently includes over 25 members that can be classified according to the organization of MMP domains as well as their specific substrate. The MMPs are multidomain enzymes; generally consisting of : (1) a signal peptide which is essential for secretion;

(2) a propeptide domain that contains a conserve cysteine switch motif of PRCXXPD for maintaining the latency form of MMPs; (3) a catalytic domain that contains one catalytic zinc, one structural zinc, and, generally, three calcium ions and the zinc binding motif HEXGHXXGXXH; and (4) a hemopexin carboxy C-terminal domain[13]. Six groups can be distinguished, including collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), matrilysins (MMP-7 and -26), the membrane-type MMPs (MMP-14 to -17 and -24), and other MMPs[14]. MMPs contribute to tumour growth, invasion, and metastasis by degrading the surrounding basement membrane and extracellular matrix barriers, resulting in migration of tumour cells. Based on our understanding of the characteristics of MMPs and the accumulation of evidence indicating a role for MMPs in cancer extension, considerable effort has been dedicated to developing effective MMP inhibitors (MMPIs) for the treatment of cancer.

The search for effective MMPIs has passed through several stages over the past 20 years[15]. The goal is to develop safe and effective MMPIs for cancer patients. Due to the varied mechanisms of diminished MMPs activity, this could be carried out in many ways, including direct inhibition of the enzymes, blocking the activation of proenzyme MMPs, enhancing of MMPs inhibitor production, or suppressing MMPs expression. Both synthetic and natural MMPIs are increasingly sought for use in clinical applications. Besides, MMPIs derived from natural products should display fewer side effects[16]. Interestingly, oxidants can convert pro-MMP to active MMP through binding to the thiol group of a cysteine in the prodomain[17,18]. Hence, this pro-MMP activation could be abrogated by the action of antioxidant agents[19,20] such as herbs, plants, fruits, and other agriculture products. In addition, flavonoid and polyphenolic compounds also disrupt the pathway of oxidative-stress induced MMPs expression. Their inhibition of MMPs at the levels of both expression and activity was suggested as the mechanism by which flavonoids and polyphenols may impact the extracellular matrix degradation and remodelling leading to the anti-invasion and anti-metastatic effects[21].

In this study, the biological activities of dried longan seed extract were analysed for antioxidant activity and anti MMPs activity. This is the first such screening of dried longan seeds on MMP activity, and is a necessary step for further investigation of the effect of dried longan seed extract on MMPs expression and activity in vitro as well.

MATERIALS AND METHODS

Chemicals

Standard gallic acid, catechin, ellagic acid, corilagin, ascorbic acid, α-tocopherol and ferene were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Ammonium iron(II) sulphate was obtained from Merck (Darmstadt, FR Germany). Acetone (AR grade) and methanol (HPLC grade) were obtained from LabScan. Sephadex LH-20 was purchased from GE Healthcare.

Plant materials

Fresh longan fruits (*E. longana* cv. Biew Kiew) were purchased from local markets in Chiang Mai, Thailand. Removed seeds were collected and dried in a hot air oven at 50 °C for 48 h and then ground to a fine powder.

Extraction and fractionation

Dried longan seed powders were extracted with 80% acetone in ratio 1:10 (w/v) at room temperature for 24 h. The resulting slurries were filtered through a Whatman No.1 filter paper. The filtrate was evaporated and lyophilized to yield dried longan seed extracts or crude extracts. The crude extracts were dissolved in methanol at 20 mg/ml and subjected to Sephadex LH-20 column chromatography. The eluate was consecutively collected in 3 ml/tube and the absorbance at 360 nm measured. Each pooled fraction was collected according to the distinct peak at 360 nm. Each fraction was evaporated and separated for chemical analysis by high performance liquid chromatography (HPLC).

High performance liquid chromatography

Each fraction at 10 mg/ml in methanol (MeOH) was separated on a Hypersil ODS-2, 5 µM based on type A silica (Thermo Scientific) column (250×4.6 mm; Thermo-Hypersil Co.). The mobile phases were 80% MeOH (solvent A) and 0.4% formic acid (solvent B). The flow rate of the mobile phase in the HPLC column was set at 1 ml/min by using the gradient of solvent A and solvent B. The separation processes were started from 0–40 min and detected via a UV detector set at 270 nm. The HPLC column temperature was maintained at 25 °C. The HPLC chromatogram was recorded and the peaks of active compounds identified by comparison with standard gallic acid (GA) and ellagic acid (EA).

Total phenolic contents

The Folin-Ciocalteu method was used for the determination of total phenolic compounds with minor modification from a previous method[22]. Briefly, 125 µl of standard gallic acid solution (0–0.32 mg/ml) or 125 µl of the fractions at 1 mg/ml in dimethylsulphoxide (DMSO) was added to 0.5 ml of distilled water and 125 µl of Folin-Ciocalteu reagent and then incubated for 10 min. Thereafter, 1.25 ml of 7% Na_2CO_3 and 1 ml of distilled water were added into each sample. Then, all samples were warmed at 45 °C for 15 min. The absorbance at 765 nm was measured using a spectrophotometer. The concentration of total phenolic compounds was calculated from gallic acid standard curve and the data was expressed as mg of the gallic acid equivalent (GAE) per 1 g of dry weight sample.

Total flavonoid contents

Total flavonoid content was evaluated according to a colorimetric assay with $AlCl_3$ with minor alteration[23]. Briefly, 250 µl of standard catechin solution (0–0.32 mg/ml) or 250 µl of the fractions at 1 mg/ml in DMSO was mixed with 1.25 ml of distilled water and then 75 µl of 5% $NaNO_2$ was added. After 10 min, 150 µl of 10% $AlCl_3 \cdot 6\,H_2O$ was added and the mixture stood at room temperature for 10 min. Next, 500 µl of 1 M NaOH and 275 µl of distilled water were added. Five minutes later, each sample was mixed and its absorbance at 532 nm measured using a spectrophotometer. The concentration of total flavonoids was obtained from a catechin standard curve and values were shown as mg of catechin equivalents (CAE) per 1 g of dry weight sample.

DPPH-radical scavenging activity

The scavenging of free radicals was assessed by using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical with minor modifications from previously published methods[24,25]. Briefly, 0.1 ml of each fraction at different concentrations (25–100 µg/ml in MeOH) or positive control (5 µl/ml in MeOH α-tocopherol and 1 mg/ml in MeOH ascorbic acid) was added into 0.1 ml of DPPH solution (0.2 mM in MeOH) to obtain a final concentrations of each fraction of 12.5–50 µg/ml. After 30 min at 25 °C in darkness, the absorbance of the mixture was measured at 517 nm. The inhibition of DPPH by the samples was calculated as: DPPH-scavenging activity = (Absorbance of sample − Absorbance of sample blank)/(Absorbance of DPPH control − Absorbance of DPPH control blank).

Sample blank was contained MeOH instead of DPPH solution. DPPH control included DPPH solution and MeOH, while DPPH control blank was contained only MeOH. Then, the concentration at which 50% of DPPH scavenging occurs (SC_{50}) was read and used for comparative analysis among the fractions.

Metal ion chelating activity

The chelation of metal ions of the fractions was determined by a modification of a previously published method[26]. Briefly, 4 µl of 2 mM ammonium iron(II) sulphate solution was mixed with 80 µl of each fraction at different concentrations (0, 400, and 800 µg/ml in MeOH). The reaction was initiated by the addition of 16 µl of 5 mM ferene. The mixture was shaken vigorously and left at room temperature for 10 min. The absorbance of the solution was measured at 593 nm. Methanol was replaced for sample in the control, while the blank of sample or control contained methanol in place of ferene solution. The decrease in ferrous ion oxidation was calculated as: Metal chelating activity = 1 − (absorbance of sample − absorbance of sample blank)/(absorbance of control − absorbance of control blank).

The results were expressed as the concentration of the extracts that decreased ferrous ions by 50% (EC_{50}).

MMP inhibitor profiling; fluorometric

The screening of longan seed extract fractions on MMPs activity was carried out using an MMP inhibitor profiling kit (Enzo Life Sciences AK016). Each fraction was prepared at 100 µg/ml in DMSO for interaction with recombinant MMPs (MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, and MMP-14) following the manufacturer's instructions. Moreover, in this kit also included broad inhibitor (NNGH) which was used for control inhibitor. The mixture was placed in an incubator 37 °C for 30–60 min. The MMPs activity was measured using an Omni-MMP fluorogenic substrate (BioMol Research) or broad substrate for MMPs and detected the fluorescence signal at Ex/Em 328/420 nm. The MMPs activity was calculated as: MMPs activity = (ABU of sample/ABU of MMP control).

The samples were compared to a control that contained assay buffer instead of the samples (100% of MMP activity). Ellagic acid (EA) and gallic acid (GA) at 15 µM were used to compare the attenuated capacities on MMPs activity to each fraction.

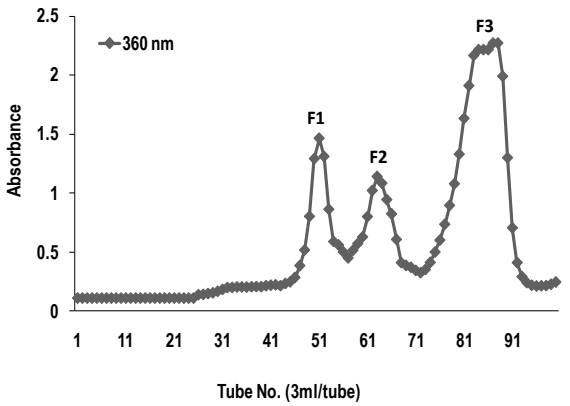

Fig. 1 Isolation of dried longan seed acetone extract by using Sephadex LH-20 column chromatography. Crude acetone extract at 20 mg/ml in methanol was subjected into Sephadex LH-20 column chromatography which eluted with methanol at flow rate 1 ml/min. The eluates were collected at 3 ml/tube and measured the absorbance at 360 nm. Then, the 3 major fractions were collected by following this graph. F1, fraction 1; F2, fraction 2; and F3, fraction 3.

Statistical analysis

The results are presented as mean $\pm$ SD of at least three independent experiments. All data were analysed via one-way ANOVA using the SPSS software. Differences were considered significant at $p \leqslant 0.05$.

RESULTS AND DISCUSSION

Isolation and identification of gallic acid and ellagic acid from dried longan seed fractions

The acetone extract of dried longan seed extract was further isolated using Sephadex LH-20 column chromatography with methanol, resulting in 3 major fractions (fraction 1, fraction 2, and fraction 3) as illustrated in Fig. 1. The yields of each fraction were 3.1, 0.8, and 4.1 g/200 g DW, respectively. HPLC analysis was used for determination of the major compounds in each fraction which was compared with the retention time (RT) of gallic acid and ellagic acid standard (RT of gallic acid = 11.3 min, and ellagic acid = 25.6 min). Our HPLC chromatogram indicated that gallic acid was found mostly in fraction 1, while ellagic acid was dominant in fraction 3. However, fraction 2 contained small amounts of these two compounds mixed with unidentified components. Table 1 shows the quantification of total gallic acid was significantly higher in fraction 1 ($p \leqslant 0.05$) as compared to the others, the amount of ellagic acid was significantly ($p \leqslant 0.05$) enriched in fraction 3. These compounds were clearly separated in each fraction included GA-enriched fraction 1 and EA-plentiful fraction 3. Moreover, both of them have been reported to be good scavengers of free radicals and possess the potential of anti-cancer properties[27–30]. Thus their antioxidant capacity might be inherent to be the potent antioxidant of dried longan seed extract. Their structures are shown in Fig. 2.

Table 1 The gallic acid and ellagic acid contents of dried longan seed fractions.

	F1	F2	F3
Gallic acid (μg/ml)	$206 \pm 34^{*}$	32 ± 7	7.7 ± 3.5
Ellagic acid (μg/ml)	22 ± 6	30 ± 12	$217 \pm 76^{*}$

* Significantly different value at $p \leqslant 0.05$ as compared to the others.

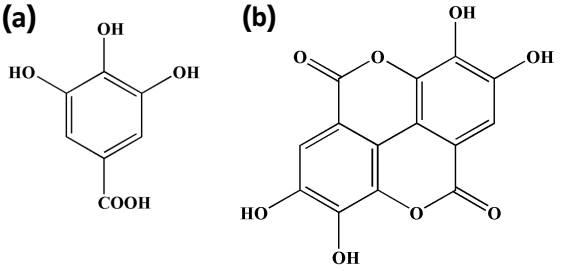

Fig. 2 The structures of (a) gallic acid and (b) ellagic acid.

Antioxidant activities of dried longan seed fractions

The antioxidant assays included total phenolic acid contents, total flavonoid contents, DPPH scavenging activity and metal ion chelating activity were used to determine the antioxidant capacity of each fraction. Fraction 1 had a significantly higher ($p \leqslant 0.05$) quantity of total phenolic and flavonoid contents (Table 2). In addition, the SC_{50} of DPPH scavenging and EC_{50} of metal chelating activity were correlated with the amount of phenolic compounds and flavonoids. The assays showed that GA-rich fraction 1 was the most effective in regard to antioxidant activity. Interestingly, other phenolic compounds in longan seeds have been also reported by HPLC-ESIMS analysis such as ethyl gallate, methyl brevifolin carboxylate, brevifolin, 4-*O*-α-L-rhamnopyranosylellagic acid and 1-*O*-galloyl-β-D-glucopyranoside[31]. Later, Zhang et al[16] reported that these compounds might be the important contributors to the high antioxidant activity in longan seeds other than the predominant gallic acid and ellagic acid. Their results also indicated the decrease in antioxidant by DPPH scavenging as-

Table 2 The antioxidant activities of dried longan seed fractions.

	F1	F2	F3
Total phenolic acid (mg GAE/g DW)	$203 \pm 13^{*}$	123 ± 13	121 ± 4
Total flavonoid (mg CAE/g DW)	$89 \pm 6^{\#}$	80 ± 4	53 ± 6
DPPH scavenging activity (SC_{50}; μg/ml)[a]	$14 \pm 2^{\#}$	20 ± 3	41 ± 7
Metal chelating activity (EC_{50}; μg/ml)[b]	563 ± 64	603 ± 54	615 ± 16

[*] Significantly different at $p \leqslant 0.05$ as compared to F2 and F3.

[#] Significantly different at $p \leqslant 0.05$ as compared to F3.

Note: Percent of DPPH scavenging ability of 5 μg/ml α-tocopherol and 1 mg/ml ascorbic acid (positive controls) was 96.7 ± 0.5 and 95.9 ± 1.9, respectively.

[a] SC_{50} = The concentration of fraction that scavenges 50% of DPPH radicals.

[b] EC_{50} = The concentration of fraction that chelates 50% of ferrous ion.

All data are expressed as mean ± SD from three different experiments conducted in duplicate.

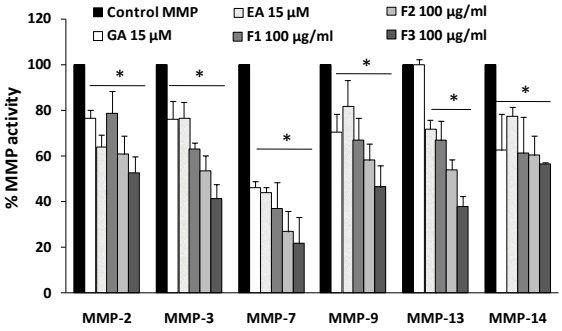

Fig. 3 The effect of the fractions from dried longan seed extract on MMPs activity by using fluorometric assay. Six recombinants MMPs were represented for the major MMP groups consist of MMP-3, stromelysin; MMP-7, matrilysin; MMP-2 and -9, gelatinases; MMP-13, collagenase; and MMP-14, membrane type MMP. Each fraction at 100 μg/ml or ellagic acid and gallic acid at 15 μM was reacted with each MMP in 96 wells plate to form sample (inhibitor)/MMP complexes at 37 °C for 30–60 min. Thereafter, Omni-MMP fluorescence substrate was added into the reaction and then measured the fluorescence light at Ex/Em 328/420 nm. (* significantly different at $p \leqslant 0.05$ as compared with control MMP).

say in the order: gallic acid, ethyl gallate, methyl brevifolin carboxylate, corilagin, ellagic acid, brevifolin, 1-*O*-galloyl-β-D-glucopyranoside, and 4-*O*-α-L-rhamnopyranosylellagic acid. Fraction 1 belonged to GA-rich which might be the main compound contributing to high antioxidant capacity.

Anti-MMPs activity of dried longan seed fractions

Several natural compounds have been identified as MMPIs, such as the extract of baicalin, cinnamon, euonymus, and magnolia[32–34]. Six recombinant MMPs were used for determination of inhibitory effects of each fraction, including MMP-2 and MMP-9 for gelatinases, MMP-3 for stromelysin, MMP-7 for matrilysin, MMP-13 for collagenase, and MMP-14 for membrane type MMP. The inhibitory profiling of dried longan seed fractions is shown in Fig. 3. The present results indicate that each fraction significantly inhibits a broad range of MMP family. Among of fractions, EA-enriched fraction 3 was possessed as the strongest MMPs inhibitor and seemed to have more inhibitory potency than EA and GA control. In the other words, the synergistic effect of the mixture compounds in dried longan seed fractions might be manipulated to enhance the anti-MMP activity effect, particularly in EA-dominant fraction 3.

Shortcomings of the first generation of MMPIs development include unsatisfying effects on some physiological functions and on some metalloproteinases such as ADAMs[35,36]. A new generation of MMPIs should be based on either selective MMP inhibition or on different inhibitory mechanisms to increase the specificity[37–40]. In this case, a limitation of MMPs kit is that it contains all recombinant MMPs where only the catalytic domain and not the full length MMP structure is present. Therefore, their non-selective inhibition effect could be explained that Zn^{2+} containing in catalytic domain of all MMPs might be the target for the action of each, in other word each fraction might be playing a role in Zn^{2+} chelation activity. Since Zn^{2+} is essential for MMPs proteolytic activity, the possible effect of each fraction on Zn^{2+} chelation activity leads to undistinguishable effect of the different MMPs. In addition, Ende et al[41] reported that a numerous flavonoid compounds inhibit recombinant MMP-2 and MMP-9[41]. Although the definite inhibitory mechanism is unclear, it is believed that the present of hydroxyl groups or carbonyl groups on their phenolic rings can chelate active Zn^{2+} ions and inhibit MMPs activity[42,43]. In fact, not only Zn^{2+} is important for proteolytic activity of MMPs, but also calcium ion (Ca^{2+}) are also needed for activity and to maintain the conformation of MMPs[44]. Hence, both of essential metals might be synergistically chelated

by the flavonoid compounds in fraction 3.

Although this study did not determine the action of dried longan seed fractions on MMP-activity unequivocally, there is evidence indicating a correlation between antioxidant and anti-MMP activity via a decrease in oxidative stress and free radicals by antioxidant agents which diminish pro-MMPs activation[19,20]. Oxidants can convert pro-MMP to active MMP by binding to the thiol group of a cysteine in the prodomain[17,18] and interrupt Cys-Zn^{2+} interaction. This study is first to report the anti-MMPs activity of dried longan seed extract, and the development of MMPI derived from natural product may be possible in a near future. Even though dried longan seed fractions could not be used as MMPI or drugs in clinical trials yet, activities of dried longan seed extract other than the well known antioxidant capacity are interesting and should be further investigated in the future.

Acknowledgements: This study was supported by a grant of the Royal Golden Jubilee PhD programme.

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