Quantification of Fractions from Longan Seeds and Their Antimicrobial Activity

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Abstract

The efficacy of herbal drug is determined by quality of the original material because it affects bioavailability, safety, and equality of the product. In this study, simultaneous determination of four maker substances was established for the quality control of longan seed fractions by using high performance liquid chromatography (HPLC). Extracted samples were analyzed with reverse-phase HPLC and eluted with a mixture of methanol and acetic acid aqueous solution, and then were detected at UV 270 nm by a Photo Diode Array Detector. Relative coefficients of variations of intra-day and inter-day analyses were less than 5%, and recovery studies of four marker standards were more than 90%. Thus, this method of simultaneous determination of four marker substances in longan seed extract was reliably established. The sub-fraction of ethyl acetate extract showed antimicrobial activity on several microorganisms, which could be 24 used as natural antimicrobial agents in skin care and human health.

KEYWORDS:longan seed extract, ethyl gallate,ellagic acid, corilagin.

Introduction

Longan (*Dimocarpuslongan*Lour.) is a member of the Sapindaceae family. It is a highly attractive fruit, extensively distributed in Taiwan, China and South East Asia including Vietnam and the Philippines. Some agricultural by-products, which have traditionally been considered as primary waste fractions such as peels, barks, leaves and seed residues, can be potentially exploited as antioxidant agents and nutraceutical since these wastes might contain high amounts of bioactive components.

Fruit seeds are known to contain many phenolic compounds capable of protecting them from oxidative damage and defending them against yeast, fungi and bacteria that might inhibit their germination. Additionally, longan seeds contain high amounts of bioactive compounds, such as phenolic acids, flavonoids, and polysaccharides (Zheng *et al.*, 2009), and exhibit antimicrobial

antioxidant and inflammatory properties (De Assis*et al.*, 2009; Prasad *et al.*, 2010). The previous study demonstrated that water extract of longan fruit contained high levels of polyphenolic compounds such as corilagin, gallic acid and ellagic acid (Song and Barlow, 2005). The analysis of longan fruit from Thailand showed that there was a large variation in the contents of gallic acid, corilagin and ellagic acid ranging from 9.18 to 23.04, from 0 to 50.64, and from 8.13 to 12.65 mg/g respectively, in different longan tissues among cultivars (Rangkadilok*et al.*, 2007).

Corilagin is a member of the tannin family and has strong antioxidant and antimicrobial(Kinoshita *et al.*, 2007) activities. Gallic acid andellagic acid are reported to be potent antioxidants and anticarcinogenic agents (Xu*et al.*, 2000). Although the seed of *Dimocarpuslongan* has been shown to have some physiological effects, there are few studies focusing on Fen Kelongan with respect to its antimicrobial activity. In this study, we evaluated the antimicrobial activity of Fen Kelongan seed extract and itsfractions, and investigate the relationship between the phenolic acids and the antimicrobial activities.

2. Materials and Methods

2.1. Materials and extract

Plant material (*DimocarpuslonganLour.* Fen ke) was obtained from the local markets at commercial maturation season. The dried longan seed was ground into powder and extracted with water (DW) and 50% ethanol (DL). The extract was partitioned with ethyl acetate(DLP), n-butanol and aqueous solvents. The ethyl acetate fraction was further applied to a silica gel column chromatographic separation, resulting in a sub-fraction (DLPS). The obtained fractions were subjected to the antimicrobial assay.

2.2. Chemicals and Reagents

Methanol (HPLC grade) and acetic acid was obtained from Merck. Samples for HPLC were filtered through a 0.45 μm Millipore membrane filter (Bedford, MA, U.S.A.). Gallic acid and ethyl gallate was purchased from Sigma (St. Louis, Mo, U.S.A.). Ellagic acid was purchased from Aldrich. Corilagin was purchased from PIN Chemical Ltd.

2.3.Antimicrobial activity assays

2.3.1. Microbial strains

Two strains of gram positive methicillin-resistant *Staphylococcus aureus* TC685 BCRC15211 were selected to compare to a normal strain of *Staphylococcus aureus,* BCRC10451. *Acinetobacterbaumannii*(BCRC10591) was chosen for this study because it is a gram negative bacilli and because it has emerged as the most difficult antimicrobial-resistant bacilli to control and treat. *Propionibacterium acne* (BCRC10723) was chosen because it is a gram-positive bacterium that forms

part of the normal flora of the skin, oral cavity, large intestine, the conjunctiva and the external ear canal. Although primarily recognized for its role in acne, *P. acnes* is an opportunistic pathogen, causing a range of postoperative and device-related infections.

2.3.2. Antimicrobial activity assays- Disc diffusion method

The disc diffusion method(NARMS, 2002) was employed for screening the extract and its fractions for their antimicrobial activities. Briefly, a suspension of the tested microorganism was spread on the Mueller-Hinton Agar (MHA). For the antibacterial test, paper discs (7 mm in diameter) were separately impregnated with 10 µL of the 100 mg/ml plant extracts (1mg/disc) and placed on the inoculated agar. For the positive control, paper discs were impregnated with the same amount of ethyl gallate. The agar plates were incubated at 37°C for 24 hours and the antibacterial activity was determined by measuring diameters (d in mm) of inhibition zone with the following criteria: d<10 mm, less active; 11 < d >15 mm, moderate active; d > 16 mm, very active.

2.4.Calibration Curves.

All marker standards (gallicacid, ellagic acid, corilagin and ethyl gallate) were dissolved in methanol to produce five concentrations (20, 40, 60, 100, 200µg/mL). After filtering through a 0.45 µm membrane filter, 10 µl of each concentration was injected into HPLC column for analysis. The calibration curve was drawn by plotting the peak areas of each marker against the concentrations. Linear regression was used to calculate the parameters of $y= ax+b$, where y is the peak area and x is the concentration of each marker, and a is the slope factor. The correlation coefficient was also calculated.The limits of detection (LOD) and quantification (LOQ) were determined based on the parameters of the analytical curves, considering standard deviation of the response (s) and the slope of the analytical curve (S). Thus, the curves were made in triplicate and values of s and S were used to calculate LOD and LOQ using the equations LOD= 3.3× s/S and LOQ= 10 ×s/S.

2.5. Sample preparation

Longan seed extract was weighed about 50 mg, into 50 mL plastic tubes. Ethanolwas added and vigorously shaken. Then, samples were centrifuged, and the supernatants were transferred into 100 mL volumetric flasks. The residues were then re-extracted with ethanol.All extracts were combined and filtered through 0.45µm PVDF membrane prior to HPLC analysis.

2.6.HPLC Analytical Conditions

HPLC was Hitachi system including pump 5110, photodiode array detector L-5430 and auto-sampler L-5210. A

reversed phase column XBridge C18 (250×4.6mm) was used. A 10 µL of the sample solution prepared as described above was injected into the HPLC column for analysis. The samples were eluted with a gradient system consisting of 1% acetic acid(A) and methanol (B) used as a mobile phase, with a flow rate of 1mL/min. The temperature of the column was maintained at 30 ºC. The gradient system started from 0min (100% A) to 35min (55% A) and then maintained until 50 min. The detection was carried out at 270 nm.

2.7. Method of Validation

The parameters of validation, such as detection and quantification limits for the phenolic compounds in study (LOD and LOQ, respectively), recovery, precision or relative standard deviation (RSD, %), and accuracy, were established(Tavernier*et al.*, 2004).The limit of detection (LOD) and the limit of quantitation (LOQ) were estimated from signal-to-noise ratio of the individual peaks, assuming a minimum detectable signal-to-noise level of 3 and 10, respectively (NARMS, 2002).The accuracy was determined by recovery analyses. Briefly, the extract sample was spiked with the standards of gallic acid, ellagic acid, corilagin and ethyl gallate at low, medium, and high concentration, respectively (Table 1). The recovery (%) was calculated by the equation of $((A-B)/C) \times 100\%$, where C represents the amount of each standard spike, B represents the amount of each marker in ethanol solution of samples, and A represents the total amount of each markers in the solution.Percentage RSD was determined for replicateinjections on each day (intra-day precision) and for mean valuesper day (inter-day precision).

3. Results and Discussion

The standard compounds, gallic acid, corilagin, ethyl gallate and ellagic acid were all well separated as indicated by HPLC chromatogram shown in Figure 1 and their respective retention times were 6.8 min, 18.6 min, 22.6 min and 27.9 min. The peak values reflect the concentrations of each standard compounds and a calibration curve was established for each compound. The linear regression equation was used to fit the data, high correlation coefficient ($R^2 \geq 0.997$) was obtained which are listed in Table 2, using the standard solutions with various concentrations shown in Table 3, an intra-day test, and an inter-day tests were used to check for reproducibility. The relative standard deviations (RSDs) of the intra-day test were less than 1 % and those of the inter-day were less than 2.1%. These results suggest that this method has very good reproducibility.

Recoveries of the standard compounds were analyzed as shown in Table 1. All of the recoveries are in the range of 90-110%. According to the methods used to determine the LOD and

LOQ, the limits of quantification (LOQ) were 0.06, 0.81, 0.3 and 0.39μg/ml for gallic acid, corilagin, ethyl gallate and ellagic acid, respectively. Thus this method is sufficiently sensitive for the present purposes.

The contents of gallic acid, corilagin, ethyl gallate and ellagic acid in longan seeds depended on the methods of extraction (Table 4). The crude water extract (DW) contained the least amount of these compounds, which were slightly increased by the ethanol extract (DL). The ethyl acetate extract (DLP) dramatically increased all contents and its subfraction (DLPS) exhibited the highest contents than other fractions.

The antimicrobial activities of various extracts were tested against five pathogenic microorganisms following the disc diffusion method. The results are shown in Table 5. In this case, clear zones of more than 16 mm diameters were considered as highly susceptible, 11 to 15 moderately susceptible and less than 10 as resistant. The DLPS fraction was found to exhibit the highest antimicrobial activity. The highest activity was found against Methicillin-resistant *Staphylococcus aureus*. From above result, it is evident that the DLPS fraction had a stronger antimicrobial activity than other extract and fractions.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first discovered 1961 in the Great Brittan and 1968 in US. Since then it has posed a serious threat to public health either via healthcare-associated MRSA (HA-MRSA) or community-associated MRSA (CA-MRSA). It is very important to point out in this study that two strains of methicillin-resistant *Staphylococcus aureus* (MRSA) are equally susceptible to the DLPS fraction as the methicillin-sensitive *Staphylococcus aureus* (MSSA). Thus, the DLPS fraction could potentially be used as disinfectants and antiseptics.

Acineobacterbaumannii has emerged to be a multi-drug resistance and is more easily transmitted than MRSA or vancomycin-resistant Enterococcus. The DLPS fraction is effective against this microbe and could potentially be used to control infections caused by MDR *A. bsumannii*. *Propionibacterium acne* is the pathogen of acne and is sensitive to the DLPS fraction. Thus, the DLPS fraction could be used as skin care to control acne.

In the last decades, there has been particular interest in the use of abundant naturally occurring antimicrobials (herbs, spices and plants) (Burt, 2004). Water extracts of the seed in Fen Kelongan showed no activity but ethyl acetate sub-fractions showed good inhibition activity.Considering the results obtained, the activity fraction of Fen Kelongan seed has good antimicrobial activities, and its bioactivity may be partly due to the phenolic compounds. Higher contents of ethyl gallate, gallic acid, ellegic acid and corilagin present in

DLPS fraction may result in higher antimicrobial activities than other extract and fractions. The efficiency of natural drugs might be several phytochemical rather than single compound. It remains to be determined which one of the four ingredients exhibits the most potent antimicrobial activities.

All the tested phenolic acids and extract mixtures had antibacterial activity that was determined against different kinds of bacteria. A relationship between antimicrobial activity and chemical composition was previously demonstrated (Klančnik*et al.*, 2009). The investigated Fen Kelongan seed extracts may be used for the preservation of processed foods as well as pharmaceutical and natural therapies of the treatment of infectious diseases in humans.

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Figure Caption

Figure 1. HPLC chromatographic profiles by HPLC: (a) standard compounds (b) Water extract of longan seed (c) ethanol extract of longan seed (d) Ethyl acetate fraction of longan seed extract (e) Sub-fractions of ethyl acetate of longan seed extract. (1) gallic acid (2) corilagin (3) ethyl gallate (4) ellagic acid

Table 1. Recovery of marker substance in longan seed extract

Compound	Concentration (μg/ml)	Recovery (%)
Gallic acid	20	91
	100	95
	200	92
Corilagin	20	102
	100	97
	200	105
Ethyl gallate	20	101
	100	90
	200	90
Ellagic acid	20	95
	100	102
	200	96

Table 2. Calibration curves of marker substances

Compound	Concentration	Regression equation	Linear range
Gallic acid	Y=18256x-39679	r^2=0.9996	20-200μg/ml
Corilagin	Y=7756x-1977	r^2=0.9990	20-200μg/ml
Ethyl gallate	Y=17226x-17863	r^2=0.9997	20-200μg/ml
Ellagic acid	Y=23051x-61569	r^2=0.9970	20-200μg/ml

Table 3. Precision of intra-day and inter-day analysis of marker substances

Compound	Concentration (μg/ml)	Intra-day(n=3) RSD(%)	Inter-day(n=3) RSD(%)
Gallic acid	10	0.30±0.02	1.60±0.03
	60	0.40±0.01	0.10±0.05
	100	0.20±0.01	0.40±0.07
Corilagin	10	0.40±0.09	2.10±0.05
	60	0.87±0.05	1.60±0.12
	100	0.33±0.07	2.10±0.03
Ethyl gallate	10	0.80±0.07	1.20±0.08
	60	0.40±0.02	0.40±0.02
	100	0.10±0.08	0.90±0.04
Ellagic acid	10	0.30±0.02	1.40±0.37
	60	0.10±0.01	1.50±0.09
	100	0.10±0.02	1.70±0.05

Table 4. Content of marker substance in longan seed extract

samples	Gallic acid （mg/g）	Corilagin. （mg/g）	Ethyl gallate （mg/g）	Ellagic acid （mg/g）
DW	4.9	10.5	-	5.7
DL	5.7	20.8	2.4	8.9
DLP	72.8	159.2	24.8	73.6
DLPS	112.2	245.2	34.3	63.0

[a] The tested samples were DW: crude water extract; DL: crude 50%ethanol extract; fractions: DLP: ethyl acetate; DLPS: sub-fractions.

Table 5. Antimicrobial activity of longan seed extract and fractions

Tested Microorganisms	Inhibition zone diameter (mm) of tested sample[a]			
	DW	DL	DLP	DLPS
Methicillin-resistant *Staphylococcus aureus*(TC685)	-	-	16.0±0.15	16.0±0.10
Methicillin-resistant *Staphylococcus aureus* (BCRC15211)	-	-	14.0±0.14	15.0±0.00
Staphylococcus aureus (BCRC10451)	-	-	15.0±0.14	15.0±0.07
Acinetobacterbaumannii (BCRC10591)	-	11.0±0.00	15.0±0.10	13.0±0.14
Propionibacterium acne (BCRC10723)	-	12.0±0.10	12.0±0.00	13.5±0.07

(-) no growth inhibition zone observed.DW, DL 4 mg/disc; DLP 2 mg/disc; DLPS 1 mg/disc (n=2).

[a]The tested samples were DW: crude water extract; DL: 50% ethanol extract ; DLP: ethyl acetate; sub-fractions: DLP