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Full Length Research Paper

PSidium guajava, a potential resource rich in corosolic acid revealed by high performance liquid chromatography

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Corosolic acid (CA) has become one of the most popular natural compounds in recent years because of its insulin-like and anti-cancer activities. In present study, a simple, precise and accurate high performance liquid chromatography method was developed to determine CA in Psidium guajava. An Agilent SB-C18 (4.6 × 250 mm, 5 µm) column was used for separation and analysis. The separation was performed with a constant mobile phase of acetonitrile and 0.2% formic acid in water (75:25) at a flow rate of 1 min/ml. The analytes were detected at the wavelength of 210 nm. The method was validated in terms of calibration curve, sensitivity, precision, accuracy and stability, and was applied to determine CA in leaves and fruits of P. guajava from different locations. The results showed that CA was detected in leaves with the content almost 1%, but not detectable in fruits of P. guajava. After the leaves were hydrolyzed with hydrochloric acid, the content of CA in leaf samples significantly increased up to 1.54%, and the increasing rates were more than 48.6%, suggesting that the leaf of P. guajava is a potential resource rich in CA, and hydrochloric acid hydrolysis might be a cost-effective approach to produce CA from the leaf of P. guajava.

Key words: Corosolic acid, Psidium guajava, high performance liquid chromatography (HPLC), acidic hydrolysis.

INTRODUCTION

Corosolic acid (CA), a triterpenoid (Figure 1) also named 2α-hydroxyursolic acid, was discovered in *Camptotheca acuminate* (Pasqua et al., 2006), Ugni molinae (Aguirre et al., 2006), *Crataegus pinnatifida* (Ahn et al., 1998), Psidium guajava (Begum et al., 2002; Gutiérrez et al., 2008), *Tiarella polyphylla* (Park et al., 2002), *Eriobotrya japonica* (Li et al., 2009) and *Lagerstroemia speciosa* leaves (Hou et al., 2009), L. speciosa leaves which is called banaba by the local was used as a plant source of CA (Yamada et al., 2008). This compound has attracted interests worldwide recently owing to its activities of anti-diabetes and anticancer (Sivakumar et al., 2009; Xu et al., 2009). In 2004, Miura et al. (2004) found that CA (10 mg/kg) could significantly reduce the level of blood glucose of KK-Ay mice, the mechanism of which involved, at least in part, an increase of glucose transporter isoform 4 translocation in muscle, improving glucose metabolism through reducing insulin resistance (Miura et al., 2006), inhibiting hydrolysis of sucrose (Takagi et al., 2008) and inhibiting alpha-glycosidase (Hou et al., 2009; Benalla et al., 2010). The anti-cancer activities and the mechanisms involved of CA have been extensively investigated since 1998, when Ahn et al. found that CA could dose-dependently inhibit protein kinase C. CA could suppress the M2 polarization of macrophages and tumor cell proliferation by inhibiting both signal transducer and activator of transcription-3 and nuclear factor-kappa B

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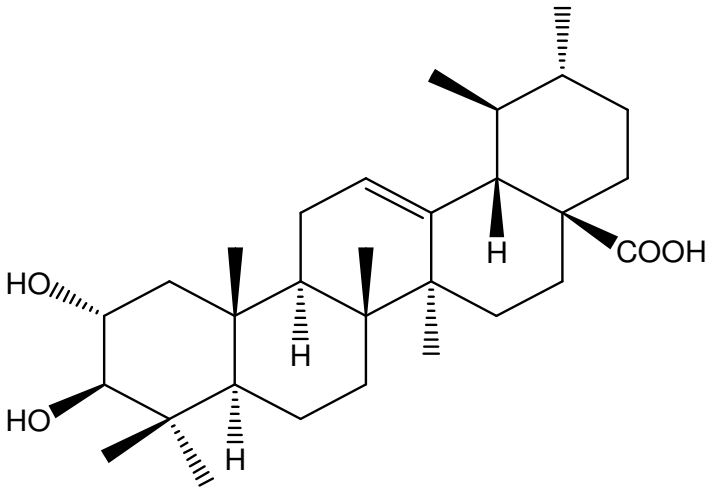

Figure 1. Chemical structure of corosolic acid.

activation (Fujiwara et al., 2011). It could also suppress human epidermal growth factor receptor expression, which in turn promoted cell cycle arrest and apoptotic cell death of gastric cancer cells (Lee et al., 2010), and mediated activated protein kinase activation which lead to inhibition of mammalian target of rapamycin, providing a possible mechanism of inhibition of cancer cell growth and the induction of apoptosis (Lee et al., 2010). Therefore, CA might be a potential lead compound for the treatment of diabetes and cancer.

Although CA has been semi-synthesized (Wen et al., 2005, 2007), the process is too complicated, so CA is still mainly from medicinal herbs. It is very important to find herbal resource rich in CA so as to get more CA for extensive pharmacological research, or even future clinical trials.

In the present study, a high performance liquid chromatography (HPLC) method was developed and validated for the quantitative determination of CA, and was successfully used to evaluate the content of CA in different parts and collections of *P. guajava*, and the samples derived from the leaves hydrolyzed with hydrochloric acid, the emphasis of which was put on the optimization of hydrolysis conditions.

METHODOLOGY

Materials and reagents

Acetonitrile, formic acid (HPLC grade) and hydrochloric acid (AR grade) from Merck (Darmstadt, Germany), methanol (AR grade) from Kaitong (Tianjin, China), and ethanol and acetone (AR grade) from UNI-CHEM (Hungary) were purchased. The ultra-pure water was purified using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). The reference compound of CA was isolated by one of the authors W.-C. Ye of Jinan University, Guangzhou, China. The samples of *P. guajava* were purchased in local herbal stores or collected in Guangdong province, China (Table 2). The voucher specimens were deposited in Institute of Chinese Medical Sciences, University of Macau, Macao SAR, China.

Instrumentation and chromatographic conditions

A Waters 2695 HPLC system (Waters, Milford, USA) with a quaternary solvent delivery system, online degasser, auto sampler, column compartment and Waters 2996 photodiode array detector were used for quantitative determination. The data was processed by Empower software.

An Agilent SB-C18 (4.6 × 250 mm, 5 µm) column was used for separation and analysis. The separation was performed with a constant mobile phase of acetonitrile and 0.2% formic acid in water (75:25) at a flow rate of 1 ml/min. The chromatogram was monitored with a PAD at the wavelength of 210 nm; the sample injection volume was 10 µl.

Extraction and hydrolysis was performed on a Syncore Polyvap, Analyst and Reactor (BUCHI, Switzerland).

Sample preparation

The dried leaf and fruit powder of *P. guajava* was accurately weighed (0.5 g), fluxed with methanol for 5h under the temperature of 100ºC, then the extract solution was transferred into a 25 ml volumetric flask which was made up to its volume with extraction solvent.

The resultant solution was centrifuged (3000 r/min) for 15 min under 20ºC and the supernatant was filtered through a 0.45 µm Econofilter (Agilent Technologies, USA) prior to injection into the HPLC system.

Hydrolysis and condition optimization

CA was generally isolated from *P. guajava* accompanied with its esters, such as 2α, 3β-dihydroxy-24-p-Z-coumaroyloxyurs-12-en-28-oic acid (guavacoumaric acid) (Begum et al., 2002), 3β-p-E-coumaroyloxy-2α-methoxyurs-12-en-28-oic acid (guajanoic acid)

Table 1. Results of the orthogonal design experiments for sample hydrolysis,

	Temperature (ºC)	Time (h)	Acid concentration (mol/l)	CA content (mg/g)
1	80	3	0.25	5.19
2	80	4	0.5	5.96
3	80	5	1	5.64
4	90	3	0.5	5.58
5	90	4	1	6.37
6	90	5	0.25	5.62
7	100	3	1	6.01
8	100	4	0.25	5.77
9	100	5	0.5	7.11
K1	5.50	5.54	5.48	
K2	5.81	5.98	6.16	
K3	6.24	6.07	5.96	
Range	0.69	0.53	0.68	

Table 2. Content of CA in samples of *P. guajava* with and without hydrolysis (mg/g, n=3).

Sample[a]	Location	Without hydrolysis	With hydrolysis	Increase rate (%)
JG-1	Zhanjiang	5.03 ± 0.05	9.08 ± 0.05	80.60
JG-2	Qingping-1	9.82 ± 0.24	15.38 ± 0.02	56.60
JG-3	Conghua	8.23 ± 0.13	12.76 ± 0.10	55.07
JG-4	Qingping-2	9.94 ± 0.10	14.77 ± 0.49	48.60
JG-5	Shunde	3.84 ± 0.18	8.67 ± 0.03	125.80
JG-6	Gaoming	8.54 ± 0.03	14.71 ± 0.06	72.20
JG-7	Macau-1	-	-	/
JG-8	Macau-2	-	-	/
JG-9	Zhuhai	-	-	/
JG-10	Guangzhou	-	-	/
JG-11	Gaoming	-	-	/

[a] leave sample (JG-1~JG-6), fruit sample (JG-7~JG-11); "-" undetectable; "/" not applicable.

(Begum et al., 2004) and 2α-hydroxy-3β-p-E-coumaroyloxyurs-12, 18-dien-28-oic acid (guajavanoic acid) (Begum et al., 2001). In our preliminary study, guavacoumaric acid, guajanoid acid and guajavanoic acid seemed to be hydrolysable by hydrochloric acid, transformed into CA. In order to determine the total amount of CA in *P. guajava*, the samples of *P. guajava* were hydrolyzed with hydrochloric acid, the conditions of which were optimized using orthogonal design experiment. The factors were temperature (80, 90, and 100ºC), extraction time (3, 4, 5 h) and hydrochloric acid concentration in methanol (0.25, 0.5, 1.0 mol/l), as demonstrated in Table 1.

The optimal hydrolysis conditions were determined by the K values comparison. For sample preparation, the dried sample powder of *P. guajava* was accurately weighed (0.5 g) and extracted under the conditions described in Table 1. The extracts were cooled down to room temperature and transferred into 25 ml volumetric flask which were made up to its volume with extraction solvent, then the solutions were centrifuged (3000 r/min) for 15 min under 20ºC, the supernatants were filtered through a Econofilter (0.45 μm) for HPLC analysis.

Method validation

The developed method was validated in terms of calibration curve, sensitivity, precision, accuracy and stability.

For calibration curve construction, the methanol stock solution (0.90 mg/ml) was prepared and diluted with methanol to give five concentrations. The calibration equation was calculated by plotting the peak area versus the concentration of CA.

The sensitivity study was completed by analyzing the limit of detection (LOD) and limit of quantification (LOQ) which were determined at a signal-to-noise (S/N) ratio of about 3 and 10, respectively.

The precision of the method was expressed by intra-day and inter-day repeatability. The intra-day repeatability was evaluated by analyzing one sample (JG-3) under the optimized chromatographic conditions in three duplicates a day. For inter-day repeatability, the measurement was conducted once a day for three consecutive days.

The accuracy of the assay was evaluated by spiking recovery test. The recovery of CA was determined by spiking known

amount of reference CA to 0.25 g of leaf sample of JG-2. The spiked samples were then treated as described in the Sample preparation section. The recovery was calculated with the following equation:

Recovery (%) = {(Amount detected- Amount original)/amount spiked} ×100%.

The stability was tested by analyzing the sample of JG-2 at 0, 2, 4, 6, 8, 10, 12, and 24 h, peak areas of CA ware recorded and compared.

RESULTS AND DISCUSSION

Optimization of chromatography

In the previous publications on the analysis of *P. guajava*, different kinds of acid with different concentrations (0.1-1%) were used as modifier to reduce peak tailing of the analytes, thus leading to the improvement of resolution (Olszewska, 2008; Chen et al., 2008; Ho et al., 2008).

In the present study, formic acid and acetic acid as modifier, and methanol and acetonitrile as organic phase were tested. It was found that the baseline separation of CA from other analytes could be achieved within 11 min, when the mobile phase composed of acetonitrile and 0.2% formic acid in water with the proportion of 75: 25 at the flow rate of 1 ml/min.

The resolution of several columns of C18 and C8 from different companies were also compared, it was found that Agilent SB-C18 (4.6 × 250 mm, 5 µm) column was suitable for the analysis of CA (Figure 2).

Method validation

The calibration curve was figured out to be $y = 4.36\times10^6 x + 1.04\times10^4$ with $R^2 = 0.9999$. The LOQ and LOD were 221.0 and 66.3 ng, respectively. The relative standard deviations (RSDs) of intra-day and inter-day repeatability were 1.8 and 1.5%, respectively.

The average spike recovery (n = 6) was 100.8%. And the stability study showed that the sample was stable within at least 24 h. All above data indicated that the established method is rapid, precise and accurate, and was sensitive enough for the quantification of CA in samples of *P. guajava*.

Optimal hydrolysis conditions

The results of orthogonal design experiments for hydrochloric acid hydrolysis were summarized in Table 1. By comparing the range of these three factors, it could be found that these three factors had different effects on the hydrolysis of CA esters. From the K values, the best hydrolysis condition for CA esters could be figured out, that is, samples were treated with 0.5 mol/l hydrochloric acid for 5 h under the temperature of 100ºC.

Sample determination

The established method was used to determine six leaf samples of *P. guajava* with or without hydrochloric acid hydrolysis, the representative chromatograms were shown in Figure 2, the results were summarized in Table 2. From Table 2, it was found that CA could be detected in all six leaf samples with the range from 3.84 to 9.94 mg/g of the dried leaves.

The highest content was found in the samples from Qingping and Gaoming China, which is up to almost 1.0%, much more higher than that in banaba (Mallavadhani et al., 2008) and other herbs (Li et al., 2009; Olszewska, 2008), although more samples should be analyzed to find out the best location.

It was also excited to found that hydrochloric acid hydrolysis could significantly increase the content of CA, the increasing rates were from 48 to 125% among all six leaf samples determined, suggesting that CA co-exist with its esters in the leaves of *P. guajava*. Hydrochloric acid hydrolysis might be a cost-effective approach to produce CA from the leaf of *P. guajava*.

The developed method was also applied to analyze five fruit samples of *P. guajava*. However, CA was undetectable in all five fruit samples with or without hydrochloric acid hydrolysis under the present chromatographic conditions, indicating that CA is mainly contained in leaf, not in fruit of *P. guajava*.

Conclusion

A rapid, precise and accurate HPLC method was developed and validated, and was successfully applied for quantitative analysis of CA in *P. guajava*. CA is mainly contained in leaf not fruit of *P. guajava*. The leaf of *P. guajava* is the potential resource rich in CA. Hydrochloric acid hydrolysis could significantly increase the content of CA in leaf samples, and might be a cost-effective way to produce CA from the leaf of *P. guajava*.

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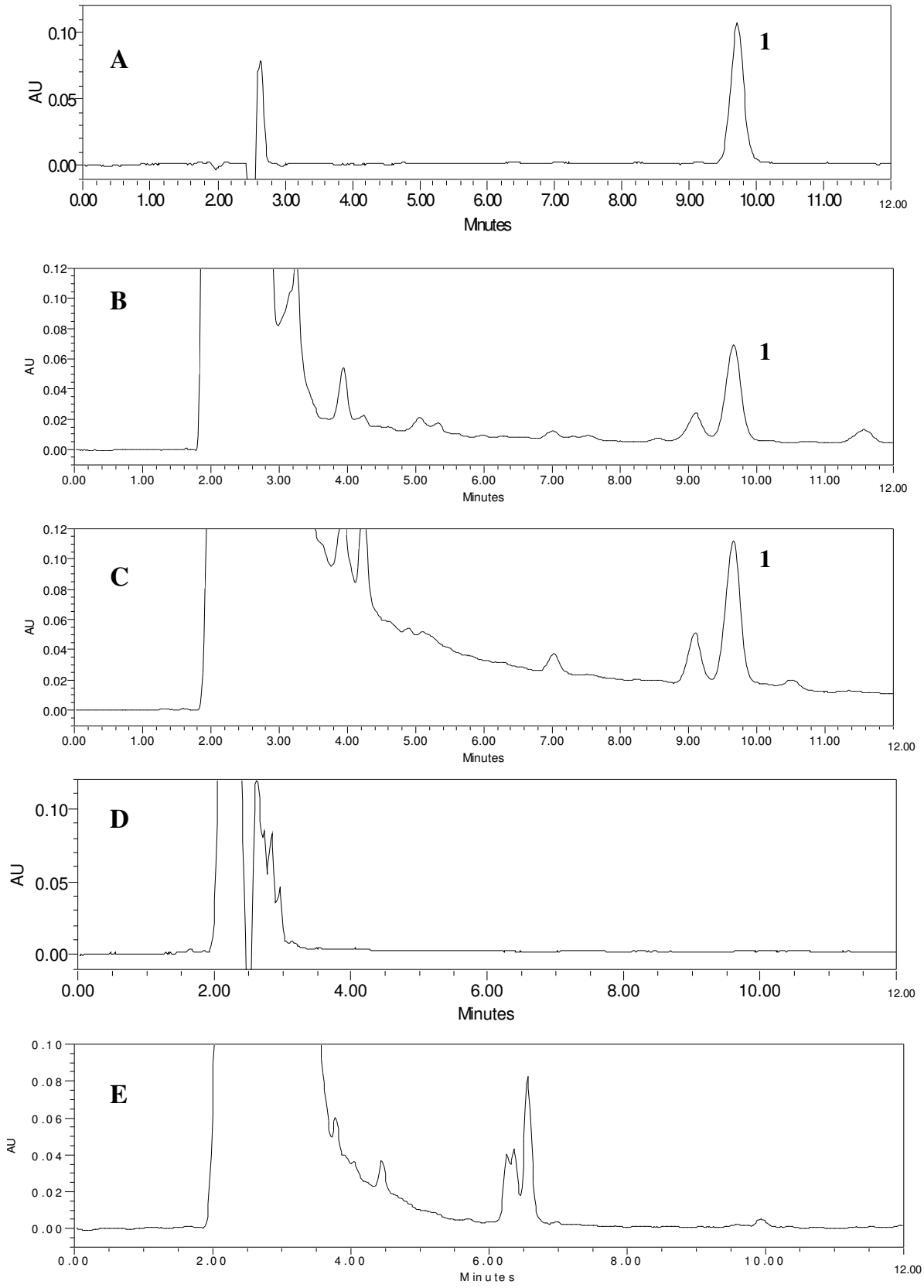

Figure 2. Typical chromatograms of A: Reference of Corosolic acid; B: Leaf sample (JG-2) without hydrolysis; C: Leaf sample (JG-2) with hydrolysis. D: Fruit sample (JG-7) without hydrolysis; E: Fruit sample (JG-7) with hydrolysis; 1: Corosolic acid.

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