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Isolation of quercetin-3-*O*-sulfate and quantification of major compounds from *Psidium guajava* L. from Vietnam

Phuc-Dam Nguyen [a,b,*], Marie-France Hérent [b], Thi-Bach Le [c], Thi-Buu-Hue Bui [c], Thi-Bich-Hang Bui [d], Thi-Thanh-Huong Do [e], Thanh-Phuong Nguyen [f], Marie-Louise Scippo [g], Patrick Kestemont [h], Joëlle Quetin-Leclercq [b]

[a] *Department of Chemistry Education, School of Education, Can Tho University, 94000 Can Tho, Viet Nam*
[b] *Louvain Drug research Institute (LDRI), Pharmacognosy Research group, Université catholique de Louvain (UCLouvain), avenue E. Mounier, 72, box B1.7203, B-1200 Brussels, Belgium*
[c] *Department of Chemistry, College of Natural Sciences, Can Tho University, 94000 Can Tho, Viet Nam*
[d] *Department of Aquatic Pathology, College of Aquaculture and Fisheries, Can Tho University, 94000 Can Tho, Viet Nam*
[e] *Department of Aquatic Nutrition and Products Processing, College of Aquaculture and Fisheries, Can Tho University, 94000 Can Tho, Viet Nam*
[f] *Department of Coastal Aquaculture, College of Aquaculture and Fisheries, Can Tho University, 94000 Can Tho, Viet Nam*
[g] *Department of Food Sciences, Laboratory of Food Analysis, Faculty of Veterinary Medicine, Fundamental and Applied Research for Animals & Health (FARAH), Veterinary Public Health, University of Liège, bât. B43bis, 10 Avenue de Cureghem, Sart-Tilman, B-4000 Liège, Belgium*
[h] *Research Unit in Environmental and Evolutionary Biology (URBE), Institute of Life, Earth and Environment (ILEE), University of Namur, Rue de Bruxelles 61, B-5000 Namur, Belgium*

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ABSTRACT

The aim of this study is first to identify the major phenolic compound of a crude ethanol extract of *Psidium guajava* leaves collected in Vietnam and to develop validated methods for quantification of phenolic and triterpenic components by HPLC-PDA-HRMS. The major phenolic compound was determined as quercetin-3-*O*-sulfate which is isolated and quantified for the first time in *P. guajava*. Validated HPLC-DAD quantification methods were developed to quantify the major triterpenic and phenolic derivatives of this extract and found to be accurate in the concentration range of 2–50 μg/mL for phenolic, and 5–100 μg/mL for triterpenic compounds. Four crude ethanol extracts of guava leaves collected at different periods of the year were analyzed using the developed methods and were found to be richer in triterpenic than phenolic derivatives. We also observed that the weather or rainfall influenced the richness in bioactive compounds in guava leaves.

1. Introduction

Psidium guajava L., belonging to *Myrtaceae* family, is considered to grow in an area extending from southern Mexico to Central America. Guava is now commonly cultivated in tropical and subtropical regions such as in Asia, Africa, South America, and the Caribbean (Altendorf, 2018). In Vietnam, *P. guajava* is very popular and is planted in many places of both tropical and subtropical areas, mostly for fresh consumption, especially in Mekong Delta where it is cultivated on nearly 4500 ha (Nguyen et al., 2017). From 2015–2017, Vietnam produced an average of 24200 tons of guava fruits each year (Altendorf, 2018). Beside the economic value of guava fruits, many products from guava leaves are commercially available such as “Guava Leaf Tea”, “Guava Leaf Extract” or “Guava Leaf Extract Liquid” (Herbal Goodness, United States), “Orihiro Guava Tea” (Orihiro, Japan), and in Vietnam as “Vietjoy Guava Leaf Tea” (Vietjoy, Vietnam). *P. guajava* leaves displayed a wide range of pharmacological activities such as anti-diabetes, anti-oxidant, anti-microbial, anti-cancer, anti-diarrhea, and anti-inflammatory (Díaz-de-Cerio et al., 2017). A review in 2010 of Deguchi and Miyazaki summarized several effects of “Guava Leaf Tea” (Bansoureicha, Japan) such as inhibition of α-glucosidase enzymes *in-vitro*, reduction of postprandial blood glucose, and improvement of hyperinsulinemia, hypoadiponectinemia, hypertriglycemia and hypercholesterolemia (Deguchi and Miyazaki, 2010). *P. guajava* leaves extract

* Corresponding author at: Department of Chemistry Education, School of Education, Can Tho University, 94000 Can Tho, Viet Nam.
E-mail addresses: npdam@ctu.edu.vn (P.-D. Nguyen), marie-france.herent@uclouvain.be (M.-F. Hérent), ltbach@ctu.edu.vn (T.-B. Le), btbhue@ctu.edu.vn (T.-B.-H. Bui), btbhang@ctu.edu.vn (T.-B.-H. Bui), dtthuong@ctu.edu.vn (T.-T.-H. Do), ntphuong@ctu.edu.vn (T.-P. Nguyen), mlscippo@ulg.ac.be (M.-L. Scippo), patrick.kestemont@unamur.be (P. Kestemont), joelle.leclercq@uclouvain.be (J. Quetin-Leclercq).

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has also been widely used for the treatment of diabetes in Korea (Park and Lee, 2013) or in China (Wang and Chiang, 2012). Guava leaves extracts were also proven to improve the immune systems of different fishes: Cyprinus carpio var. koi L., Labeo rohita, Oreochromis niloticus and Pangasianodon hypophthalmus (David et al., 2017; Giri et al., 2015; Gobi et al., 2016; Omitoyin et al., 2019; Truong et al., 2020).

Phytochemical analysis of guava leaves extracts showed that they contained phenolic compounds such as ellagic acid (Bezerra et al., 2018; Díaz-de-Cerio et al., 2016a), hyperin (Díaz-de-Cerio et al., 2016a), isoquercitrin, guajaverin, avicularin (Bezerra et al., 2018; Díaz-de-Cerio et al., 2016a; Wang et al., 2017), reynoutrin, rutin, genistin, quercitrin, quercetin and kaempferol (Wang et al., 2017). In addition, terpenoids have been isolated and identified from *P. guajava* leaves as asiatic acid (Begum et al., 2002; Chao et al., 2020), maslinic acid (Chao et al., 2020), corosolic acid (Begum et al., 2002; Chao et al., 2020), triterpenic esters called here 4TTE (3*β*-O-(*cis-p*-coumaroyl) corosolic acid, 3*β*-O-(*trans-p*-coumaroyl) corosolic acid, 3*β*-O-(*cis-p*-coumaroyl) maslinic acid, and 3*β*-O-(*trans-p*-coumaroyl) maslinic acid) (Chao et al., 2020), oleanolic acid and ursolic acid (Begum et al., 2004; Chao et al., 2020). Our previous works revealed that the crude ethanol extract of guava leaves from a sample collected in June 2018 in Vietnam contained triterpenic derivatives and phenolic compounds with corosolic acid, maslinic acid, 4TTE, avicularin, guajaverin and one unknown compound as main component (Truong et al., 2020).

Because of the various positive effects of these compounds (Chao et al., 2020; Díaz-de-Cerio et al., 2016b; Park and Lee, 2019; Wang et al., 2010) and P. guajava leaves ethanol extract, quantification of these bioactive component is a necessary requirement to assess the part of each of them in the bioactivity of this specific crude ethanol extract and to standardize extracts that could be commercialized. There are several publications reporting the quantification of flavonoids from the leaves of P. guajava by HPLC-UV (Bezerra et al., 2018; Díaz-de-Cerio et al., 2016a; Díaz-de-Cerio et al., 2016b; Irondi et al., 2016; Rahman et al., 2018; Santos et al., 2017; Wang et al., 2017), but limited publications are related to quantification of triterpenic compounds from this plant. In 2011, an article reported the quantification of corosolic acid in the leaves of P. guajava by using HPLC-DAD at 210 nm and a very recent publication in 2020 of these authors described the quantification of nine triterpenic components by HPLC but using two different detectors: DAD at 310 nm for triterpenic aromatic esters and ELSD for other triterpenic acids (Chao et al., 2020; Chen et al., 2011).

This paper presents the development and validation of a method for quantification of phenolic and triterpenic compounds from a crude ethanolic extract of guava leaves from Vietnam by using HPLC-DAD system. This work also presents the isolation for the first time in P. guajava quercetin-3-O-sulfate as a major flavonoid whose structure was not identified in this crude guava leaves extract nor its ethyl acetate fraction in our previous publication (Truong et al., 2020). Finally, the content of these bioactive compounds in ethanol extracts of guava leaves collected at 4 different harvest periods was compared to provide some useful information about the relationship between the weather and the biosynthesis of phenolic and triterpenic derivatives and the more suitable period to collect the guava leaves to be used for health effects.

2. Experimental

2.1. Chemicals and instruments

Ethanol (96%, of analytical grade) used for sample extraction was purchased from Chemsol, Vietnam. Methanol, acetonitrile, dichloromethane, ethyl acetate, hydrochloric acid, acetic acid, and formic acid (VWR, France) were HPLC grade and water was purified by a MilliQ system (Milllipore Corporation, Bedford, MA, USA). The standards of ellagic acid (95%), guajaverin (95%) and avicularin (90%) were purchased from Sigma-Aldrich, Germany. Hyperin (98%), isoquercitrin (99%) and quercetin (99%) were purchased from Extrasynthese, France. Corosolic acid (99%) and maslinic acid (98%) were purchased from Avachem, San Antonio, USA while the mixture of the four triterpenic ester (4TTE) including 3*β*-O-(*cis-p*-coumaroyl) corosolic acid, 3*β*-O-(*trans-p*-coumaroyl) corosolic acid, 3*β*-O-(*cis-p*-coumaroyl) maslinic acid, and 3*β*-O-(*trans-p*-coumaroyl) maslinic acid were isolated as explained in (Catteau et al., 2021). The purity of this mixture was measured as 93% using an Accela HPLC system (Thermo Fisher Scientific) using the same system as explained below. These esters were considered as one compound for quantification as *cis/trans* forms transform to each other in solution. Filter-paper (Whatman™ 1001–400 Grade 1 Qualitative Filter Paper, Diameter: 40 cm, Pore Size: 11 μm) was used for the preparation of plant extracts. PTFE membranes (0.45 μm) for preparation of samples before HPLC injection were purchased from Whatman™, UK. Column chromatography and vacuum liquid chromatography (VLC) were realized on Kieselgel 60 (63–200 mesh), Merck, and Silica gel C18 LiChroprep® RP-18 (40–63 μm) Merck. TLC was performed on pre-coated Silica gel 60F_{254}, plastic sheets 20 × 20 cm, Merck. Preparative TLC was conducted on TLC Silica gel 60, glass plates 20 × 20 cm, Merck.

HPLC-DAD was performed on an Accela HPLC system (Thermo Fisher Scientific) consisting of an Autosampler 60057–60020, a DAD detector 60057–60050, and an Pump 60057–60010, all piloted by ChromQuest software.

HR-MS detections were carried out after the same HPLC system, by a ThermoScientific LTQ Orbitrap XL mass spectrometer from the UCLouvain Massmet platform. HR-MS were measured with APCI source or ESI source in the negative mode using full-scan MS with a mass range of 100–2000 *m/z*. The following (-) APCI conditions were applied: vaporizer temperature, 400 °C; sheath gas (N_2) flow rate, 20 a.u.; auxiliary gas (N_2) flow rate, 5 a.u.; sweep gas (N_2) flow rate, 10 a.u.; capillary temperature, 250 °C; capillary voltage, − 25 V; tube lens, − 103.27 V. The following (-) ESI conditions were applied: flow rate, 1000 μL/min with a split of 50/50 before reaching mass detector; spray voltage, 2.5 kV; sheath gas (N_2) flow rate, 15 a.u.; auxiliary gas (N_2) flow rate, 15 a.u.; sheath gas (N_2) flow rate, 15 a.u capillary temperature, 275 °C; capillary voltage, − 23 V; tube lens, − 163.27 V. Data acquisition and processing were performed with Xcalibur software.

NMR spectra were acquired in CD_3OD on a Bruker Ascend-600 instrument using standard pulse sequences and parameters from the UCLouvain NEST platform.

2.2. Plant material

Leaves of *Psidium guajava* L. were collected at Binh Minh district, Vinh Long province, Vietnam in four different periods (06/2018, average temperature: 24 °C, average rainfall amout 462 mm; 09/2018, average temperature: 25 °C, average rainfall amout 777 mm; 12/2018, average temperature: 24 °C, average rainfall amout 45 mm; and 03/2019, average temperature: 28 °C, average rainfall amout 65 mm) and identified by PhD. DANG Minh Quan (Department of Biology Education, School of Education, Can Tho University). Voucher specimens of Psig2018.06, Psig2018.09, Psig2018.12, and Psig2019.03 respectively were deposited at the Department of Chemistry, College of Natural Sciences, Can Tho University.

2.3. Preparation of plant extracts

Fresh guava leaves (5 kg) were dried in an oven at 50 °C for 24 h to obtain the dry leaves, then these leaves were ground by a blender to have 1 kg samples of dry powder. The dry powders of *P. guajava* leaves (1 kg) collected in four different months were macerated with 5 L of ethanol at room temperature for 24 h, then filtered by filter-paper (this extraction was repeated 3 times). The solutions were concentrated under reduced pressure with a rotatory evaporator at 45 °C until obtaining a dark syrup which was then lyophilized to give the crude extracts: 88 g (Pg2018.06, 8.8% leaves dry weight), 90 g (Pg2018.09, 9.0% leaves dry

weight), 80 g (Pg2018.12, 8.0% leaves dry weight), 85 g (Pg2019.03, 8.5% leaves dry weight) respectively. All extracts were stored at 4 °C until analysis.

The extract solutions at a concentration of 1 mg/mL were prepared in methanol for identification and quantification of phenolic and triterpenic derivatives by HPLC-DAD. The results are reported as the average of three injections of these samples on 2 different days (n = 3, k = 2).

2.4. Isolation of unidentified major flavonoid

The crude ethanol extract (7.4 g) collected in June 2018 was subjected to silica gel VLC (5 × 10 cm) using a gradient of solvent dichloromethane – ethyl acetate – methanol (100:0:0 – 0:0:100) to give 10 fractions. VLC-fraction 7 [1460 mg, eluted with ethyl acetate – methanol (80:20)] was separated by C-18 column chromatography using a gradient of MeOH – H_2O (20:80 –100:0); sub-fraction 7.4 [15 mg, eluted with MeOH – H_2O (70:30)] was purified by preparative TLC eluting with the mixture of solvents ethyl acetate – formic acid – acetic acid – water – methanol (50: 2: 2: 5: 2) to obtain quercetin-3-*O*-sulfate (compound (**7**), 7 mg, $R_f = 0.53$).

Acid hydrolysis of quercetin-3-*O*-sulfate (**7**): 1 mg of compound (**7**) was hydrolyzed with 10 mL of 0.1 N HCl in a glass flask at 100° for 30 min. The solution after acid hydrolysis (C7-AH) was extracted with EtOAc (3 ×10 mL). The EtOAc layer was evaporated to give the fraction C7-EtOAc which contained as major compound quercetin whose identification was confirmed by HPLC-DAD in comparison with the authentic standard.

Quercetin-3-*O*-sulfate: Yellow amorphous powder; ^{1}H NMR (CD_3OD, 600 MHz) δ ppm: 6.19 (1 H, d, $J = 2.4$ Hz, H-6), 6.40 (1 H, d, $J = 2.4$ Hz, H-8), 7.69 (1 H, d, $J = 2.4$ Hz, H-2′), 7.88 (1 H, d, $J = 8.4$ Hz, H-5′), 7.66 (1 H, dd, $J = 8.4$, 2.4 Hz, H-6′); ^{13}C NMR (CD_3OD, 125 MHz) δ ppm: 159.4 (C-2), 133.4 (C-3), 179.2 (C-4), 163.0 (C-5), 100.3 (C-6), 167.1 (C-7), 95.0 (C-8), 158.4 (C-9), 105.5 (C-10), 123.0 (C-1′), 117.1 (C-2′), 146.1 (C-3′), 150.1 (C-4′), 116.2 (C-5′), 123.4 (C-6′); negative mode HR-ESI-MS *m/z* 380.99106 $[M-H]^-$ (calcd. for $C_{15}H_9O_{10}S$ 380.99109), 301.03470 $[M-SO_3H]^-$.

2.5. Analysis of samples by HPLC-DAD

Identification and quantitative determination of phenolic and triterpenic derivatives in the crude ethanol extracts were carried out by an Accela HPLC system (Thermo Fisher Scientific) consisting of a DAD detector, an autosampler, an injection system, and a quaternary pump, all piloted by ChromQuest software. This HPLC system was used very effectively in the quantification of phenolic compounds (Sasmaz et al., 2020). The quantification of phenolic compounds was conducted on a Phenomenex® Lichrospher C18, 4.6 × 250 mm column packed with 5 μm particles. 10 μL of samples were injected in the full loop injection mode. The column was eluted at a constant flow rate of 0.8 mL/min using a binary solvent system: solvent A, MilliQ water 0.1% formic acid and solvent B, acetonitrile HPLC grade (0–10 min, 17% B; 28 min, 28% B; 38 min, 38% B; 39–49 min, 100% B; 50–60 min, 17% B). DAD detector was set at 254 nm.

The Agilent® Poroshell 120 EC-C18 column (4.6 ×100 mm packed with 2.7 μm particles) was chosen for analysis of triterpenic component. 20 μL of samples were injected in the full loop injection mode. A flow rate of 0.4 mL/min was applied for this method and the mobile phase consisted of a gradient of solvent A- MilliQ water; solvent B-acetonitrile HPLC grade and solvent C-methanol HPLC grade (0–3 min, 35% A, 30% B, and 35% C; 10 min, 25%A, 40%B and 35% C; 30–49 min, 0% A, 65% B, and 35% C; 50–60 min, 35% A, 30% B, and 35% C). DAD detector was set at 210 nm.

2.6. Standard solution

Stock solutions of guajaverin, corosolic acid, and 4TTE were prepared in methanol at 500 μg/mL and stored at 4 °C. Then, they were diluted in methanol to obtain five concentration levels (2, 5, 10, 25, 50 μg/mL for guajaverin; and 5, 10, 20, 50, and 100 μg/mL for corosolic acid and 4TTE). Calibration standards were analyzed three times (n = 3) with three series of experiments (k = 3) at these five concentrations (m=5). The peak areas and concentrations of each standard were fitted to linear regression and to linear regression after square root transformation to choose the most suitable regression model.

2.7. Validation method

Validation of the method was performed with three independent series of experiments to analyze the following criteria: selectivity, stability, linearity, trueness, precision and accuracy, the limit of detection (LOD), the limit of quantification (LOQ), and uncertainty measurement.

The selectivity was examined by checking MS spectra at the beginning, the middle, and the end of peaks from the HPLC-HRMS chromatograms of crude extracts and corresponding standards (Beaufay et al., 2019).

The stability was verified by comparing the retention times and peak areas of the stock solutions of standards and plant extract solutions after 30 days at 4 °C of storage (Stevigny et al., 2004; Wang et al., 2017).

A signal-to-noise ratio (S/N) of 3 was used for estimating the LOD, and the LOQ is the lowest limit of the validated concentration range that can be quantified with acceptable precision and accuracy in our experiments (Beaufay et al., 2019; EDQM, 2011).

The trueness and precision were expressed by the relative bias (RB) and the relative standard deviation (RSD) respectively with the limits of 15% according to the EMA guidelines. The accuracy evaluation was based on the total error (sum of systematic and random error). The acceptance limits (λ) were set at ± 20% and the relative β-expectation was set at 95% (Beaufay et al., 2019).

2.8. Applications

Four crude ethanol extracts of guava leaves were collected in different periods of the year at the same farm in Vinh Long province, Vietnam. The leaves were harvested from many different guava trees to obtain 1 kg dry leaves samples. Four crude ethanol extracts were analyzed with the validated methods for their concentrations of ellagic acid, hyperin, isoquercitrin, reynoutrin, guajaverin, avicularin, quercetin-3-*O*-sulfate, corosolic acid, maslinic acid, and 4TTE to analyze variations according to the collection period and determine the richest one.

2.9. Statistical analysis

All statistical analyses were performed by using Microsoft Excel, Minitab 16 and Graphpad Prism 5 Software. One-way analysis of variance (ANOVA) was performed on the quantitative data generated from the analysis in order to determine variation of the phenolic and triterpenic derivatives concentrations between different samples of guava leaves. When significant differences were found, multiple pair-wise comparisons were determined using the Tukey's Studentized Range HSD test at a level of significance of $p \leq 0.05$.

3. Results and discussion

3.1. Identification of major component from P. guajava

In our previous publication, the phenolic and triterpenic derivatives of the striped catfish bioactive crude ethanol extract of leaves of *P. guajava* collected in Vietnam were identified by HPLC-DAD-Orbitrap-

MS methods. The main triterpenic compounds were 4TTE, corosolic acid, and maslinic acid. The major phenolic component were guajaverin, avicularin and a compound that we could not identify (Truong et al., 2020). The chromatograms of standards and crude extracts are shown in Figures 1–2s respectively and Figures 3–4s show the structure of these component.

The unidentified compound (**7**) was isolated from the crude ethanol extract as mentioned above (part 2.4) to obtain 7 mg pure compound. Its negative HR-ESI-MS (Figure 5s) gives a molecular ion peak $[M-H]^-$ at *m/z* 380.99106 (calcd. for $C_{15}H_9O_{10}S$ 380.99109) corresponding to a molecular formula $C_{15}H_{10}O_{10}S$. An ion peak $[M-SO_3H]^-$ at *m/z* 301.03470 shows the presence of a quercetin moiety in the structure of this compound. Acid hydrolysis yielded fraction C7-EtOAc which was identified as quercetin by comparison with a quercetin standard by the developed HPLC method (Figure 6s). These evidences revealed the structure of this compound as quercetin sulfate. NMR experiments allowed to determine the exact position of the sulfate group. The data from ^{13}C NMR spectrum showed only one upfield shift of carbon 3, and downfield shifts of carbons 2, 4 and 10 in comparison with the reference quercetin (Dueñas et al., 2012) indicating the sulfation at position 3 (Barron et al., 1986). Thus, the structure of this compound was elucidated as quercetin-3-O-sulfate which was identified by LC-HRMS-MSQtof (Lorena et al., 2022). However, this is the first report of the isolation of quercetin-3-O-sulfate in P. guajava. This information is important as quercetin was only identified as a minor compound in our extracts, with a very similar (in ESI) or completely similar (in APCI) MS spectra but a different retention time (which can only be observed by co-injection). The presence of quercetin-3-*O*-sulfate should be searched in samples from other origins.

Some quercetin sulfates (quercetin-3′-*O*-sulfate, quercetin-4′-*O*-sulfate, quercetin-7-*O*-sulfate, quercetin-3,3′-*O*-disulfate, quercetin-4′,7-*O*-disulfate, quercetin-3,3′,4′-*O*-trisulfate) showed antioxidant activities but they were usually less active than quercetin, expect quercetin-3-*O*-sulfate which was more active than quercetin (Correia-da-Silva et al., 2014; Sak, 2017; Valentová et al., 2017). The research of Chao in 2009 showed that a quercetin sulfate (but the position of the sulfate is not mentioned) was effective in the prevention of high glucose-induced apoptosis (C. L. Chao et al., 2009). Moreover, quercetin-7-O-sulfate, quercetin-4′,7-O-disulfate, quercetin-3,3′,4′,7-O-tetrasulfate, and quercetin persulfate showed antiaggregant, anticoagulant, and anticancer activities (Correia-da-Silva et al., 2014). Quercetin-7-O-sulfate and quercetin-4′-O-sulfate were less active than quercetin in the growth inhibition activity of some cancer cells (Sak, 2017). Flavonoid sulfates, which are more water soluble than non sulphated ones, are much less common in plants than non conjugated flavonoids. They are found mainly in species occurring in coastal and swampy areas rich in mineral salts. In plants, binding of inorganic sulphate with polyphenolics, like flavonoids, is probably connected with biochemical adaptation of species to environment (Barron et al., 1988; Bylka et al., 2001). Their biosynthesis in plant and their biological activities were reviewed recently (Teles et al., 2018).

3.2. Validation of method

The major triterpenic derivatives in the leaves of *Psidium guajava* are 4TTE, corosolic acid, and maslinic acid. 4TTE (a mixture of 4 triterpenic aromatic esters) was purified by semi-preparative HPLC, but the esters could not be isolated from each other due to the conversion of the *Z* and *E* diastereomers in solution. So we used this mixture as a standard for the quantification of these esters from the crude ethanol extract. Maslinic acid is an isomer of corosolic acid and has similar UV absorption on HPLC-DAD, so we used corosolic acid as the reference to quantify the presence of corosolic acid and maslinic acid in the crude ethanol extracts of guava leaves.

The major phenolic components in the crude ethanol extracts were guajaverin, avicularin, quercetin-3-*O*-sulfate, reynoutrin, hyperin, isoquercitrin, and ellagic acid. The structures of guajaverin, avicularin, reynoutrin, hyperin, isoquercitrin are similar with the same aglycone (quercetin), the difference being the sugar moiety. The HPLC-DAD chromatograms showed that guajaverin displayed similar UV absorption than avicularin, hyperin, and isoquercitrin. As no standard of reynoutrin was available, we also considered its response factor as the same. Thus all these flavonoids were quantified using the standard of guajaverin. Quercetin-3-*O*-sulfate showed a lower absorbance (64%) than guajaverin at the same concentration at 254 nm. A correction factor was then applied for the quantification based on guajaverin standard. Ellagic acid has a different UV spectrum than guajaverin with a peak area equal to 93% of that of guajaverin at the same concentration at 254 nm. This was also taken into account for ellagic acid content calculations.

In summary, three standards: guajaverin, corosolic acid, and 4TTE, were selected to quantify the major phenolic and triterpenic derivatives from the crude ethanol extracts of guava leaves from Vietnam by the validated method we developed.

3.2.1. Selectivity

Selectivity was validated by the comparison of retention times and MS spectra (-)APCI or (-)ESI of peaks of the crude extract and standards of guajaverin, ellagic acid, hyperine, isoquercetin, avicularin, isolated quercetine-3-*O*-sulfate, maslinic acid, corosolic acid, and 4TTE at retention times corresponding to the beginning, the middle, and the end of these peaks. The similar data indicated the selectivity of the method (supporting information Figure 7s-10s).

3.2.2. Stability

Stability was checked by analysis of the retention times and peak areas of crude extracts and stock solutions in methanol after 30 days storage at 4 °C. RSD (%) of retention times and peak areas, less than 5%, indicated that there was no significant degradation of these solutions (Table 1).

3.2.3. Response function

Calibration standards of guajaverin, corosolic acid, and 4TTE were prepared in methanol without matrix (m=5, n = 3, k = 3). Two different regression models were applied: linear regression and linear regression after square root transformation to choose the most suitable regression model using the accuracy profiles method (Beaufay et al., 2019; Rafamantanana et al., 2009). The accuracy profile obtained with the square root transformation regression was chosen as the response function for corosolic acid. For the mixture of 4TTE and guajaverin, the most adequate model was selected as the linear regression (Figure 11s).

3.2.4. Trueness, precision and accuracy

The trueness (Hubert et al., 2004) was analyzed for each

Table 1
Stability test of standards and crude extract (n = 3, k = 2).

Compounds	References		Crude extract	
	RSD (%) of Retention time	RSD (%) of Peak areas	RSD (%) of Retention time	RSD (%) of Peak areas
Ellagic acid			0.28	4.50
Hyperin			0.21	3.48
Isoquercitrin			0.19	3.02
Reynoutrin			0.16	2.76
Guajaverin	0.13	3.73	0.18	2.39
Avicularin			0.18	2.10
Quercetin-3-*O*-sulfate			0.20	1.67
Maslinic acid			0.12	4.90
Corosolic acid	0.29	1.22	0.13	3.91
4TTE	0.50	1.53	0.11	3.23

concentration solution of the validation standards and presented as relative bias (RB). The relative bias of corosolic was less than 5% and it was less than 10% for the mixture of 4TTE and guajaverin (Table 2) indicating the excellent trueness of the method (Rafamantanana et al., 2009).

The precision was expressed in relative standard deviation (RSD %) values to evaluate the repeatability (intra-day) and intermediate precision (inter-day) (Beaufay, et al., 2019; Rafamantanana, et al., 2009). The results show that the repeatability and the intermediate precision were less than 5% for corosolic acid and less than 7% for the standards of guajaverin and 4TTE (Table 2). The trueness and precision values ($\leq$ 15%) are in agreement with EMA guidelines criteria (Agency, 21 July 2011; Beaufay et al., 2019).

According to ISO 5725–1 (ISO-5725–1, 1994(en)), the accuracy is used to describe the closeness of agreement between a test result and the accepted reference value. Figure 11s shows that the relative upper and lower 95% β-expectation tolerance limits are inside the acceptance limits set at ± 20% for all standards of guajaverin, corosolic acid, and 4TTE. These results proved the accuracy of the method for the range of concentrations from 2 to 50 μg/mL of guajaverin, from 5 to 100 μg/mL of corosolic acid, and 4TTE standards.

3.2.5. Limit of detection and limit of quantification

The limit of detection (LOD) is estimated as 0.1 μg/mL for guajaverin, 0.8 μg/mL for corosolic acid, and 1.2 μg/mL for 4TTE by the signal-to-noise ratio of three as mentioned before in part 2.7. The limit of quantification (LOQ) is 2 μg/mL for guajaverin, 5 μg/mL for both corosolic acid and 4TTE standards. It was determined as the lowest concentration level of the test in agreement with the accuracy profiles (Beaufay et al., 2019; Rafamantanana et al., 2009).

3.2.6. Uncertainty of measurement

According to ISO/IEC Guide 98–3, the uncertainty of measurement characterizes the dispersion of the values that could reasonably be attributed to the measurement (ISO/IEC-GUIDE-98–3, 2008). The expanded uncertainty was evaluated by using a coverage factor of k = 2 with a confidence level of 95% (Beaufay et al., 2019; Rafamantanana et al., 2009). The results (Table 3) show that relative expanded uncertainty was less than 10% for guajaverin and corosolic acid, and less than 15% for 4TTE located inside the ± 20% acceptance limits.

Table 3
Uncertainty estimations of guajaverin, corosolic acid and 4TTE mixture at each concentration level investigated during the method validation using the selected regression model. The expanded uncertainty was calculated with a coverage factor of 2.

	Concentration level (μg/mL)	Uncertainty (μg/mL)	Expanded uncertainty (μg/mL)	Relative expanded uncertainty (%)
Guajaverin	2	0.05	0.11	5.41
	5	0.25	0.50	9.95
	10	0.38	0.76	7.61
	25	0.53	1.07	4.27
	50	1.03	2.06	4.12
Corosolic acid	5	0.17	0.33	6.66
	10	0.37	0.75	7.47
	20	0.69	1.38	6.91
	50	1.53	3.06	6.13
	100	3.74	7.47	7.47
4TTE	5	0.20	0.39	7.87
	10	0.41	0.81	8.13
	20	1.22	2.44	12.2
	50	3.50	7.00	14.0
	100	3.59	7.11	7.17

3.2.7. Linearity

The linearity is described as the relationship between introduced and calculated concentrations. The concentrations of validation standards were back-calculated following the above accuracy profiles and compared to introduced concentrations to build the regression line (Figure 12s) (Beaufay et al., 2019). The slope values close to 1 with 1.0198 for guajaverin, 1.0170 for corosolic acid, and 1.0216 for 4TTE which demonstrated the good linearity of the method.

3.3. Application to samples of P. guajava ethanol extracts

The guava leaves used in our research were collected at four different periods in Vinh Long province, Vietnam. The average temperature in Mekong Delta in general and Vinh Long in particular ranges from 26.5°

Table 2
Validation results obtained for quantification methods of guajaverin, corosolic acid and 4TTE.

Validation criteria		Guajaverin concentration level (μg/mL)									
		2	5	10	25	50					
Response function		Linear regression Calibration range (5 points) 2 – 50 μg/mL									
Trueness	Relative bias (%)	6.88	8.37	1.09	-3.34	2.86					
Precision	Repeatability (RSD%)	1.28	5.14	3.42	0.92	0.62					
	Intermediate precision (RSD%)	2.25	4.50	3.55	1.95	1.75					
Accuracy (95% relative β-expectation lower and upper tolerance limits in %)		-1.17	-2.50	-7.81	-10.4	-5.76					
		14.9	19.2	10.0	3.70	11.5					
Linearity	Slope	1.02									
	Intercept	-0.11									
	R^2	0.99									
		Corosolic acid concentration level (μg/mL)					4TTE concentration level (μg/mL)				
		5	10	20	50	100	5	10	20	50	100
Response function		Linear regression after square root transformation Calibration range (5 points) 5 – 100 μg/mL					Linear regression Calibration range (5 points) 5 – 100 μg/mL				
Trueness	Relative bias (%)	-1.84	3.08	-0.20	4.28	1.31	4.10	1.34	5.22	3.06	2.04
Precision	Repeatability (RSD%)	2.43	1.58	0.76	1.27	1.33	4.33	3.55	6.88	5.55	2.18
	Intermediate precision (RSD%)	3.09	3.20	3.01	2.60	3.24	3.97	3.76	5.76	6.30	3.17
Accuracy (95% relative β-expectation lower and upper tolerance limits in %)		-10.2	-8.45	-15.1	-5.08	-10.4	-13.6	-8.14	-8.51	-13.6	-7.72
		7.20	14.6	14.7	13.6	13.1	5.35	10.8	18.9	19.7	11.8
Linearity	Slope	1.02					1.02				
	Intercept	0.10					0.11				
	R^2	0.99					0.99				

to 27.4 °C. January is the coldest month with an average temperature from 24.9° to 25.2 °C, and April is the hottest month with an average temperature from 27.6° to 28.6 °C, the highest temperature may reach 38 °C. The weather in Mekong Delta or in Vinh Long could be divided into two seasons: the dry season and the rainy season with approximately 103–127 rainy days per year. The rainy season normally starts from May to the end of November accounting for 93–96% of annual rainfall, in which the highest level is in September-October (215–329 mm). The dry season begins in December and ends in April of the next year which has 1–8 rainy days every month with rainfall around 10–50 mm (Nguyen, 2017).

Four samples of guava leaves were harvested at 4 different periods with two samples in the rainy season (Pg2018.06 and Pg2018.09) and two others in the dry season (Pg2018.12 and Pg2019.03). Their crude ethanol extracts were analyzed with the developed methods. Figure 1–2s show the HPLC-DAD chromatograms of these extracts and the standards: ellagic acid, hyperin, isoquercitrin, guajaverin, avicularin, quercetin-3-*O*-sulfate, maslinic acid, corosolic acid, and 4TTE.

Quantification results (Table 4) show that the amount of phenolic and triterpenic derivatives from the four samples is variable from 77.1 to 128 mg/g of ethanol extract. The total content of bioactive component in Pg2018.09 is higher by about 64%, 10%, and 39% of that in Pg2018.06, Pg2018.12, and Pg2019.03, respectively.

The relative abundance of the major phenolic component is as follows quercetin-3-*O*-sulfate > avicularin > guajaverin, except for sample Pg2018.12 where avicularin is the major phenolic derivative. Quercetin-3-*O*-sulfate reaches 13.3 mg/g of crude extract in sample Pg2018.09, while the highest content in avicularin (13.2 mg/g of crude extract) was found in sample Pg2018.12. The highest total content in phenolic compounds was found in sample Pg2018.12 with 48.2 mg/g of crude extract, slightly higher than sample Pg2018.09 (41.9 mg/g of crude extract).

The concentration of triterpenic compounds is higher than phenolic derivatives in all four crude extracts. Corosolic acid is the major compound of all samples (as 4TTE is a mixture of four different compounds). Sample Pg2019.03 has the highest content in triterpenic aromatic esters with 37.9 mg/g of crude extract. In all samples, the concentration of maslinic acid is lower than corosolic acid (from 0.29 to 0.71 mg/g) of crude extract.

The contents of bioactive compounds in two samples Pg2018.09 and Pg2018.12 are higher than those of the two other samples. The richest Pg2018.09 extract (126 mg/g) was collected in September which is one of the highest rainfall months of the year, and Pg2018.12, containing 115 mg/g bioactive component, was harvested at the end of the rainfall season in December. Pg2018.09 extract is the richest in total bioactive metabolites and contained the highest concentration of triterpenic derivatives as well as corosolic acid. These results indicate that the weather or rainfall may influence the biosynthesis of bioactive component in guava leaves. It seems that during the rainfall season the guava plants can produce more active compounds. Therefore, we should collect the leaves in rainy months as September to obtain the highest concentration of these metabolites, but this has to be confirmed in more samples.

4. Conclusions

A major compound from the *P. guajava* ethanol extract was identified as quercetin-3-*O*-sulfate which also identified by LC-HRMS-MS-Qtof in a previous publication (Lorena et al., 2022), but this compound is isolated from this plant for the first time. Furthermore, selective and accurate methods for quantification of phenolic and triterpenic derivatives was developed and validated in the range of 2–50 μg/mL for phenolic compounds and of 5–100 μg/mL for triterpenic acids and aromatic esters. These methods were applied to quantify phenolic and triterpenic compounds from four ethanol extracts of guava leaves collected at different periods in the same farm. The results show the concentrations of these derivatives vary from 77.1 to 126 mg/g of ethanol extract.

Table 4
Phenolic and triterpenic derivatives content in four samples of guava leaves collected in different periods by the validated methods (n = 3, k = 2, mean = ± SD) in mg/g ethanol extract.

Samples	Pg2018.06	Pg2018.09	Pg2018.12	Pg2019.03
Ellagic acid	3.51 ± 0.09	5.07 ± 0.21	2.40 ± 0.07	5.76 ± 0.56
Hyperin	3.06 ± 0.04	4.77 ± 0.13	4.82 ± 0.10	2.68 ± 0.08
Isoquercitrin	2.05 ± 0.98	2.61 ± 0.05	3.35 ± 0.05	1.75 ± 0.04
Reynoutrin	2.28 ± 0.03	2.80 ± 0.05	3.94 ± 0.10	1.90 ± 0.03
Guajaverin	4.47 ± 0.06	6.20 ± 0.13	8.78 ± 0.22	3.45 ± 0.11
Avicularin	6.24 ± 0.09	7.20 ± 0.13	13.2 ± 0.36	4.28 ± 0.11
Quercetin-3-*O*-sulfate	6.87 ± 0.07	13.3 ± 0.20	11.7 ± 0.22	7.77 ± 0.34
Total phenolic content (a)	**28.5 ± 0.2**	**41.9 ± 0.5**	**48.2 ± 0.9**	**27.6 ± 1.0**
Maslinic acid	5.07 ± 0.22	15.0 ± 0.42	8.99 ± 0.42	3.40 ± 0.21
Corosolic acid	19.7 ± 0.93	41.0 ± 0.84	24.3 ± 1.75	21.8 ± 1.27
4TTE	23.8 ± 0.98	28.2 ± 0.59	33.3 ± 1.67	37.9 ± 2.64
Total triterpenic content (b)	**48.6 ± 2.0**	**84.2 ± 1.4**	**66.6 ± 3.5**[a]	**63.1 ± 4.0**[a]
Total (a + b)	**77.1 ± 1.9**	**126 ± 1**	**115 ± 3**	**90.7 ± 3.5**

[a] Means are not significantly different.

Pg2018.09 extract is the richest in total bioactive metabolites, and contained the highest concentration of triterpenic derivatives as well as corosolic acid. Pg2018.12 extract possessed the highest phenolic content compared to those of the three other extracts. This research shows that season of collection greatly affects the content of guava leaves, and indicates that collection in the rainy months as September seems to be better to obtain the highest contents of phenolic and triterpenic derivatives.

Author contributions

Phuc-Dam Nguyen: Investigation, Formal analysis, Methodology, Validation, Writing - original draft. **Marie-France Hérent:** Formal analysis, Validation. **Thi-Bach Le:** Resources. Methodology. **Thi-Buu-Hue Bui:** Conceptualization, Writing - review & editing. **Thi-Bich-Hang Bui:** Conceptualization, Project administration. **Thi-Thanh-Huong Do:** Conceptualization, Writing - review & editing. **Thanh-Phuong Nguyen:** Conceptualization, Writing - review & editing. **Marie-Louise Scippo:** Conceptualization, Writing - review & editing. **Patrick Kestemont:** Conceptualization, Funding acquisition, Writing - review & editing, Visualization. **Joëlle Quetin-Leclercq:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2022.104928.

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