

# New QuEChERS method for quantification of Physalin B and D in *Physalis angulata* L. in Vietnam

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## Abstract

In Vietnam, *Physalis angulata* L. has been widely used as a traditional medicine to treat fever, anti-inflammatory, and expectorant. Currently, there have been studies on the content of chemical composition especially physalin with anti-diabetic, anti-inflammatory, antibacterial, prevent cancer. This study developed a reliable and sensitive method to determine and validate simultaneous Physalin B and Physalin D in *Physalis angulata* L.. The QuEChERS method was used for sample preparation from leaf matrices and quantified by using High-performance liquid chromatography coupled with a diode-array detector. The method of research was validated under AOAC and ICH guidance. Chromatography conditions include Agilent C<sub>18</sub> column (250mm × 4,6mm; 5µm) with a gradient mode using acetonitrile – methanol-water as mobile phase. The recovery of the method ranged from 94.21 – 105.93% and RSD was from 1.20 – 2.31%, the LOD, and LOQ were 0.4 mg/kg – 2.4 mg/kg, respectively. The results of the study showed that the proposed the new QuEChERS method for quantification of Physalin B and D in *Physalis angulata* L. in Vietnam.

## Keywords

QuEChERS, Physalin D, Physalin B, *Physalis angulata*, HPLC-PDA

## Introduction

*Physalis angulata* L. is a wild plant, belonging to the family Solanaceae, occurring in many countries located in tropical and subtropical regions, *Physalis angulata* L. popularly known as ‘campus’, is an annual herb that is distributed widely in the north and northeast regions of Brazil (Ren-

gifo et al. 2013). For a long time, *Physalis angulata* L. has been present in folk medicine remedies, its known ethnopharmacological applications are anti-cancer, diuretic, anti-inflammatory, sedative, depurative, and anti-septic (Rivera et al. 2019). With the development of modern science today, studies have discovered many compounds, the main secondary metabolites of *Physalis angulata* are wit-

hanolides, which are highly variable in chemical structure and exhibit interesting pharmacological activity (He et al. 2007).

Concerning the anti-cancer potential of *P. angulata*, several studies have been performed showing that organic extracts and some purified compounds possess cytotoxic activity against several tumor cell lines (Magalhães et al. 2006; Bouyahya et al. 2018). Physalin B and Physalin D are steroid compounds. Physalin B inhibits the growth of cancer cells. The C5-C6 double bond in Physalin B is the structural feature correlated with the antihepatotoxic action (Chiang et al. 1992). Physalin D exhibits cytotoxicity against several cancer cell lines. A study with crude extracts of the aerial parts of *P. angulata* showed a possible effect of Physalin D in inhibiting the growth of *Mycobacterium tuberculosis* (Januário et al. 2002).

Most previous studies analyze Physalin B and D compounds in different fractions and evaluate biological activities (Soares et al. 2008; Anh et al. 2018; Arruda et al. 2021). To the best of our knowledge, no validated method has been described so far for the quantification of the Physalin B and D compounds present in leaves of *P. angulata*. This study applies the high-performance liquid chromatography (HPLC) with a diode-array detector (DAD) in determining the Physalin content which is a method with high sensitivity, good quantification, and suitable for analyzing natural compounds. Regarding the sample preparation, the QuEChERS approach is helpful for the analysis of chemical compounds, especially herbal matrices. The QuEChERS procedure entails several simple analytical steps and is thus fast and easy to perform. This study is aimed at developing a new QuEChERS method for sample preparation and validating a sensitive, and robust analytical procedure for the determination of Physalin B and D compounds accumulated in leaves of *P. angulata*. The developed procedure was applied to elucidate the Physalin B and D contents in dried leaf samples collected in six provinces of Vietnam.

## Materials and methods

### Chemicals and solvent

All chemicals and reagents used in the experiments had analytical grade purity. Methanol, acetonitrile, ethanol, chloroform, acetone, and ethyl acetate were purchased from Fisher (USA). Ammonium acetate, formic acid, phosphoric acid, and ammonia solution (25%), magnesium sulfate anhydrous ( $\text{MgSO}_4$ ), sodium acetate (NaOAc), and sodium chloride (NaCl) were obtained from Fisher (USA). Primary Secondary amine and Graphitized Carbon Black were purchased from Sigma-Aldrich (USA). Physalin B and D reference standards (assigned purity 98%) were supplied by Wuhan ChemFaces Biochemical Company.

### Plant materials

Fresh *P. angulata* were collected in the Can Tho City, Kien Giang, Ca Mau, Hau Giang provinces, Mekong delta and Binh Dinh province, Central Southern coastal region, Vietnam. The plants were identified at the Department of Biology, Can Tho University (scientific name as *Physalis angulata* L.). The leaf samples were collected separately, dried at room temperature, pulverized, and analyzed individually. All samples were stored in black glass containers and kept at room temperature. Dried samples were collected from traditional drugstores located in these provinces.

### Standard solutions

Individual standard stock solutions of Physalin B and D (1000  $\mu\text{g}/\text{mL}$ ) in methanol were prepared and were stable for approximately 12 months. Working standard solutions were prepared daily by diluting the stock solutions with methanol to provide different concentrations. The standard stock and working solutions were protected from light and stored at 4 °C.

### Optimization of sample preparation

#### Extraction procedure

Accurately weigh approximately 0.5 g of the leaf sample into a 15 mL centrifuge tube. Add about B mL of extraction solvent A, and soak at room temperature for 30 minutes. Then sonicate for C min, centrifuge at 5000 rpm for 5 min. Decant the layer of extract, conduct repeated extraction D again, then combine the extract and blow dry the solvent.

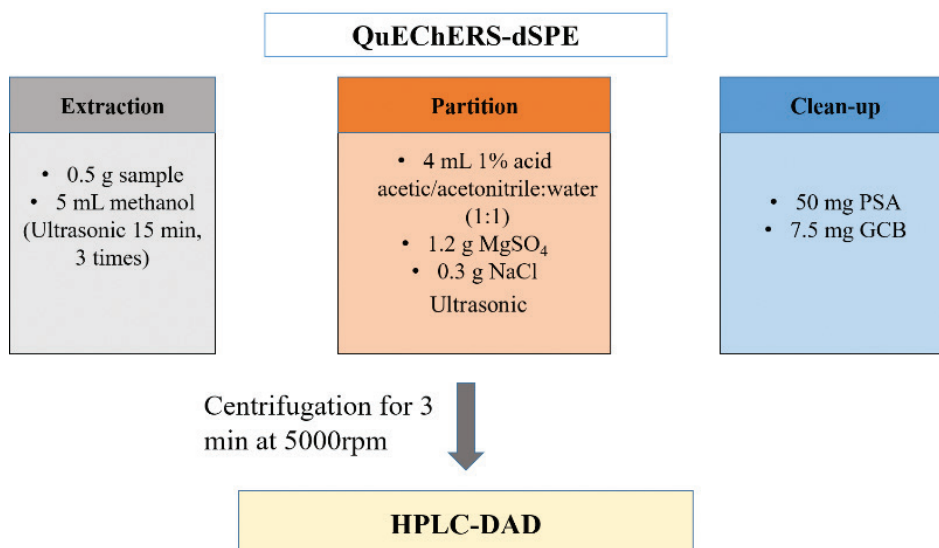
#### QuEChERS procedure

0.5 mL extract blew dry was added into a 15 mL centrifuge tube, then 4 mL of 1% acetic acid/acetonitrile: water (1:1) was added and shaken vigorously for 2 min. After that, 1.2 g  $\text{MgSO}_4$  anhydrous and 0.3 g NaCl was added and the mixture was shaken immediately for one

minute. Centrifugation was carried out at 5000 rpm for 3 min and the clean-up step was done with d-SPE including a mixture of 50 mg Primary Secondary Amine (PSA) and 7.5 mg Graphitized Carbon Black (GCB). The mixture was shaken well and centrifuged at 5000 rpm for 3 min. After removing impurities, the extracts were filtered through a 0.22 $\mu\text{m}$  Nylon filter and transferred into a vial. The final samples were injected into system chromatography (Fig.1).

### Chromatographic conditions

Method development, quantification, and validation studies were performed on UFLC Shimadzu (LC-20AD), detector DAD SPD-M20A. Chromatography separation was performed on an Agilent  $\text{C}_{18}$  column (250 mm  $\times$  4,6 mm; 5  $\mu\text{m}$ ) and monitored at 225 nm with the column temperature at 30 °C. The gradient elution program was shown



**Figure 1.** Schematic representation of the QuEChERS-dSPE extraction procedure.

**Table 1.** Gradient elution program.

Time	Mobile phases ratio		
	A (%)	B (%)	D (%)
0.01	0	10	90
1.5	0	10	90
2.0	22	10	68
13.0	35	10	55
14.0	40	10	50
26.0	55	10	35
27.0	80	10	10
28.0	80	10	10
29.0	0	10	90
35.0	0	10	90

in Table 1. The mobile phase consisted of acetonitrile (A), methanol (B), and water (D) and pumped at a flow rate of 1.0 mL/min.

## Method validation

The proposed method was validated for selectivity, linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, and accuracy according to the Association of Official Analytical Chemists (AOAC) and ICH guidelines.

## Results and discussion

### Optimization of sample preparation

#### Extraction method

When employed as a source of natural chemicals, ultrasound-assisted extraction (UAE) is an intriguing approach for obtaining highly valuable compounds that might contribute to the increased value of some food by-products. The major advantages will be more efficient extraction, which will save energy, and the use of moderate temperatures, which will assist heat-sensitive chemicals. To successfully

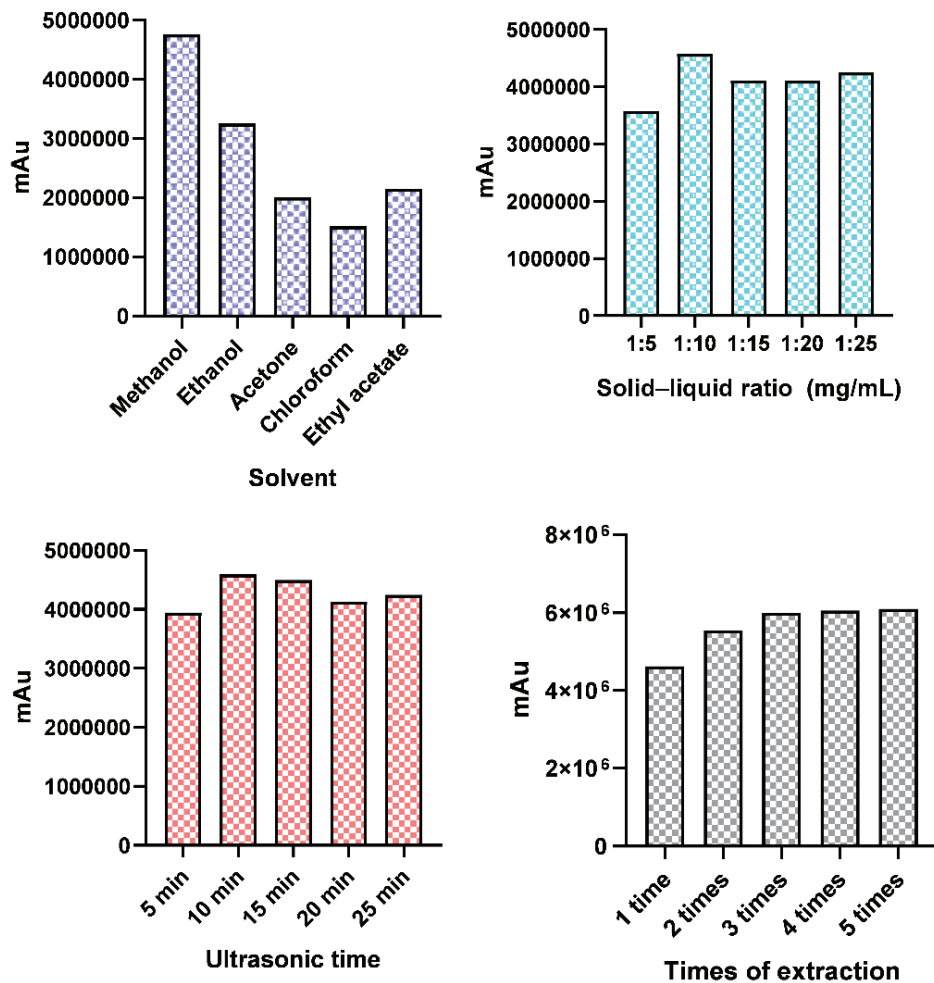
use the UAE, it is required to examine the impact of numerous process factors, including the most important ones are the applied ultrasonic power, frequency, extraction temperature, reactor parameters, and solvent-sample interaction.

The UAE procedure was optimized about the solvent (chloroform, ethyl acetate, acetone, methanol, ethanol), solid-liquid ratio (1:5, 1:10, 1:15, 1:20, 1:25 g/mL), sonication time (5, 10, 15, 20 and 25 min), and extraction times (once, twice, third, fourth, and fifth) under investigation. The sum of the Physalin B and D peak areas was used to evaluate the extraction efficiency.

The results of the optimization of the sample preparation procedure are shown in Fig. 2. Based on the solubility of the analytes and referring to some related references, five extraction solvents were selected: methanol, ethanol, acetone, chloroform, and ethyl acetate. The survey results showed that the extraction efficiency of Physalin from the sample matrix was highest with methanol solvent. Therefore, methanol was selected as the extraction solvent.

A higher volume of extraction solvent can dissolve analytes more effectively. Thus, the liquid-to-solid ratio is also an important factor during extraction. To evaluate the effect of this factor on the extraction yields, we examined different ratios ranging from 1:5 to 1:25 g/mL. It was found that the Physalin B and D contents increased with an increase in the solid-liquid ratio from 1:5 to 1:10 g/mL. From ratio 1:15 to 1:25 g/mL, the contents of analytes decreased.

For the ultrasonic-assisted extraction method, the ultrasonic time factor addition to having a great influence on the extraction efficiency. Specifically, when increasing the ultrasonic time from 5 minutes to 10 minutes, the extraction efficiency increased significantly before stabilizing at 15 minutes. However, when the ultrasonic time was selected at 10 minutes, the extraction times were investigated. After 3 times of extraction, the extract contained active ingredients. It is recommended to choose



**Figure 2.** Results of the optimization of the sample preparation procedure.

an ultrasonic time of 15 minutes for follow-up surveys. The data shown in Fig. 1 illustrate that the ideal number of extractions was three in order to extract more than 99% of the analytes from the *P. angulata* matrices.

### QuEChERS procedure

QuEChERS method was a sample preparation approach developed by Anastasiades et al. as a simple, rapid, effective, yet inexpensive way to extract analytes from the matrix, followed by a dispersive solid-phase extraction (d-SPE) cleanup of the extract (Bruzzoniti et al. 2014). QuEChERS method involves an acetonitrile salting-out extraction of a solid sample in an aqueous environment

followed by dispersive solid phase extraction (d-SPE) to remove a majority of the remaining matrix interferences.

For the matrices of herbal, the removing impurities step in the sample preparation is really necessary. Because this step avoids a decrease in column efficiency when injecting samples into the chromatography system. The extraction solvent of choice was methanol - a strong soluble solvent, dissolving the majority of polar metabolites along with intermediate and low polar compounds. Therefore, when extracted with methanol, these substances, especially polar impurities, are dissolved simultaneously. This poses a requirement that a suitable impurities removal method be developed that can reduce impurities while still retaining the majority of the analyte. Among the extraction methods that can be used

to extract organic compounds from medicinal and plant samples, the QuEChERS method is becoming increasingly popular (Malysheva et al. 2020; Xu et al. 2017). This method is based on the decrease in solubility of organic substances dissolved in water as the salt concentration in the aqueous solution increases.

Acetonitrile (MeCN) is evaluated as a solvent that can extract many active compounds with higher efficiency and selectivity than acetone or ethyl acetate. Furthermore, proper miscibility with water allows good penetration into the aqueous fraction of the sample, also allowing relatively easy separation of the different phases by the addition of salts. Commonly used salt-forming agents are inorganic salts such as anhydrous  $MgSO_4$ , NaCl, and NaOAc to create separation between the water and MeCN layer and remove the residual water layer in the sample, these salts promote the transfer of compounds.  $MgSO_4$  completely separates the liquid-liquid phase and is better able to bind large amounts of water, while NaCl can reduce the co-extraction of other components in the matrix and thus produce good chromatographic peaks.

Therefore, the combined use of  $MgSO_4$  and NaCl has been studied for better results than using each salt separately. A ratio of  $MgSO_4$ :NaCl (4:1) has been shown to be the most effective in terms of selectivity and separation of aqueous and organic phases, maintaining high recovery. Table 2 shows the ingredients for surveying the process of removing polar impurities. To evaluate the purification efficiency of each procedure, the total peak of impurities and Physalin of the tested samples were compared. The result in Table 2 illustrated that using 1.2 g  $MgSO_4$  and

0.3 g NaCl then cleaned with 50 mg PSA and 7.5 mg GCB gave the highest Physalin content.

The impurity peak on the chromatogram after applying the QuEChERS method was significantly reduced. The results are shown in the area of the impurity peak and the Physalin peak (Fig. 3).

## Optimization of chromatographic conditions

Most of the studies focused on the quantification of Physalin B and Physalin D in herbals of the same genus *Physalis* such as *Physalis alkekengi* L., *Physalis franchetii* (Zheng et al. 2012; Laczkó et al. 2017) with different matrices, so the chromatographic conditions are also different. After referring to previous research, it has been shown that common mobile phase solvents for the analysis of physalins include Physalin B and Physalin D in *P.angulata* by liquid chromatography method and based on the nature of physalin chemistry and solubility of two analytes, choose to investigate mobile phase with the following components: Acetonitrile, methanol, and water.

In the studies of Arruda et al. (Arruda et al. 2021) and Manuela Oliveira de Souza et al. (De Souza et al. 2013), the optimal chromatographic condition was a gradient elution program with acetonitrile composition and 0.05% TFA/water. However, our research used water and acetonitrile as mobile phases, the peak parameters were still satisfactory. Our studies used an aqueous unbuffered mobile phase, which is both friendly to the chromatographic system and to the environment. Because of the problem of unstable baselines and overlapping peaks, a gradient program incorporating 3 solvent channels should be investigated. As a result, the mobile phase used consisted of acetonitrile (A), methanol (B), and water (D). The gradient elution program was shown in Table 1.

The best detection wavelength is when the analytes give the highest absorption and must be different from the absorption wavelength of the solvent to avoid baseline noise. According to studies analyzing Physalin B and Physalin D by HPLC/PDA method (De Souza et al. 2013; Arruda et al. 2021) chose to investigate wavelengths from 225 nm and 310 nm. Scanning the spectrum in the UV 190 - 400 nm region, the results showed that at 225 nm, the analytes gave good signals. So that 225 nm was selected as the detection wavelength of the analyte process.

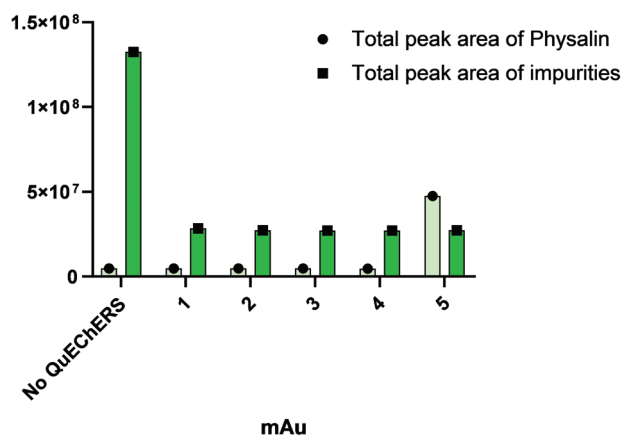
## Method validation

### System suitability

The system stability was tested by carrying out six replicate injections of a mixed standard solution (10  $\mu\text{g/mL}$ ) and determining the theoretical plate number (N) and resolution (Rs), symmetry factor (As), and repeatability [relative standard deviation (RSD) of RT and area] of the analytes (Table 3). The %RSD values of the peak area and RT of

**Table 2.** Experiment to investigate the process of removing polar impurities by QuEChERS method.

No.	Ingredients for sample extraction step (mg)			Ingredients for clean-up steps (mg)	
	$MgSO_4$	NaCl	NaOAc	PSA	GCB
1	1.2		0.3	50	7.5
2	1.2	0.3		50	7.5
3	2.4	0.6		50	7.5
4	1.2	0.3		50	10
5	1.2	0.3		50	7.5



**Figure 3.** The results of removing polar impurities and clean-up samples.

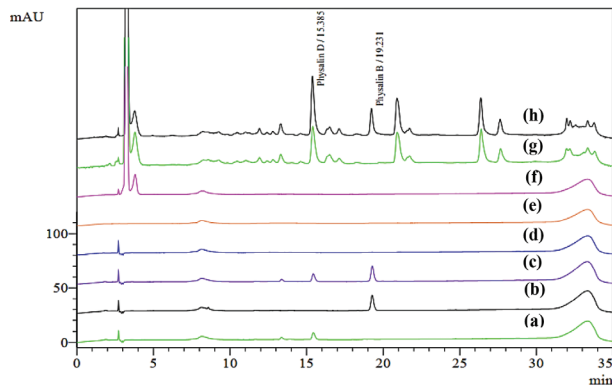
all analytes were less than 2.0%. Therefore, the proposed method met this requirement.

### Specificity

The specificity was tested by employing the HPLC method

**Table 3.** System suitability of the HPLC-PDA method.

	$t_r$ (min)	S (mAU)	$A_s$	$R_s$	$k'$	N	
Physalin D	Mean	15.446	309916	1.163	6.445	5.781	28115
	SD	0.01	1.394	0.007	0.102	0.004	301
	RSD%	0.07	0.45	0.62	1.59	0.073	1.07
Physalin B	Mean	19.296	639863	1.0	10.365	7.471	42471
	SD	0.009	3.179	0.006	0.057	0.004	587
	RSD%	0.05	0.5	0.6	0.55	0.05	1.38



**Figure 4.** HPLC chromatograms for specificity (a: Standard solution of Physalin D; b: Standard solution of Physalin B; c: Mixed standards solution; d: Extraction solvent; e: Mobile phase solvent; f: Solvent; g: Spiked sample; h: Standard addition).

to analyze the extracts of the leaf of *P. angulata*. It was evaluated by comparing the RT and UV absorption spectrum of each component in standard solutions with those of the peaks obtained by analyzing the extracts. As shown in Fig. 4, the HPLC method could distinguish Physalin B and D from other components of the leaf matrices. The peak purity of the seven compounds was > 99.9%, as obtained from the spectrum overlaying the graphs of three-point purity detection.

### Linearity, the limit of detection, and the limit of quantification

The stock solutions were diluted and mixed to six different concentrations ranging from 10 to 250  $\mu\text{g}/\text{mL}$  of Physalin D, and from 2.0 to 50  $\mu\text{g}/\text{mL}$  of Physalin B. To evaluate the linearity, each mixed standard sample was injected in triplicates into the HPLC system, and calibration curves were obtained by plotting the average of the peak area responses versus concentration for each

sample. Squared correlation coefficients ( $R^2$ ) were higher than 0.997 for Physalin B and D. The LOD and LOQ were shown in Table 4.

### Precision

The precision of the method was verified by evaluating the intra-day and inter-day precisions. The relative standard deviation (%RSD) was selected as a measure of precision. The intra-day precision was examined by analyzing six samples of each substance in a single day, while the inter-day precision was determined by analyzing six samples each day for three days. The precision results shown in Table 4, indicate that the overall intra-day and inter-day variations (%RSD) were below 6%, suitable with AOAC guidelines.

### Accuracy

The accuracy of the method was investigated by performing recovery studies. Three different concentrations, including low (50%), medium (100%), and high amounts (150%) of reference compounds, were added to the blank samples. Then, the spiked samples were added and quantified according to the methods mentioned above. The results indicated that the developed method exhibited good accuracy, with an overall recovery ranging from 94.21% to 105.93%. Considering the results of the recovery test, the method was deemed accurate.

### Application

In 2002, Januário et al. in Brazil, for the first time isolated Physalin D in *Physalis angulata* (Januário et al. 2002). Fractions containing physalins B, F and D exhibited a minimum inhibitory concentration value (MIC) against *Mycobacterium tuberculosis* H37Rv strain of 32  $\mu\text{g}/\text{mL}$ . Purified physalin B and physalin D were also tested showing MIC values against *Mycobacterium tuberculosis* H37Rv strain of > 128  $\mu\text{g}/\text{mL}$  and 32  $\mu\text{g}/\text{mL}$  respectively, suggesting that physalin D plays a relevant role in the antimycobacterial activity displayed. In the research of Souza et al. (De Souza et al. 2013), Physalin B and Physalin D content were respectively 13.2 mg/g and 11 mg/g in dried ethanol extracts of *P. angulata* leaves in Brazil. In Vietnam, studies on *Physalis angulata* L. have mainly isolated Physalin compounds, there is almost no quantitative research on these compounds in plants. Research by Ton Nu Lien Huong et al. on *P. angulata* collected in Dong Thap province which is also in the South region, of Vietnam has detected Physalin B and G components by NMR technique (Huong et al. 2017).

Quantitative results of 12 samples of *P. angulata* leaves were collected in 6 provinces in South region including Can Tho City, Hau Giang, Kien Giang, An Giang, Ca Mau Province, and Binh Dinh Province in the Central Southern

**Table 4.** Precision, recovery, calibration parameters, LOD and LOQ.

Substance	Calibration curve		Precision (n = 6)		Recovery (%)			LOD ( $\mu\text{g}/\text{mL}$ )	LOQ ( $\mu\text{g}/\text{mL}$ )
	Regression equation	$R^2$	Intra-day RSD (%)	Inter-day RSD (%)	Low-level	Mid-level	High-level		
Physalin D	$y = 5014.6x - 3167.1$	0.9998	1.52	1.92	105.93	105.36	102.43	0.4	1.35
Physalin B	$y = 9921.4x - 471.62$	0.9997	1.76	1.50	94.21	102.55	104.08	0.5	2.4

**Table 5.** Content of Physalin B and D in leaves of *P. angulata* collected in Vietnam.

No.	Location	Code sample	Physalin D (%)*	Physalin B (%)*
<b>South Region, Vietnam</b>				
1	Can Tho City	CT	0.566	0.114
2	Hau Giang	HG1	0.121	ND
3	Province	HG2	0.853	0.039
4	Kien Giang	KG1	0.770	ND**
5	Province	KG2	0.673	ND
6		KG3	1.187	0.0007
7		KG4	0.865	ND
8	An Giang	AG	0.617	ND
	Province			
9	Ca Mau	CM1	0.679	0.0003
10	Province	CM2	0.824	ND
11		CM3	1.376	0.040
<b>Central Southern Coastal Region, Vietnam</b>				
12	Binh Dinh	BD	0.042	0.618
	Province			

(\*) g/100 g dried leaf sample; (\*\*) ND: not detected

coastal region, Vietnam (Table 5). All leaf samples had Physalin D, Physalin D content was highest in the Ca Mau province leaf sample (CM3 sample) and the lowest in Binh Dinh Province, respectively 1.376% and 0.042%. Ca Mau and Kien Giang provinces were two locations that significantly Physalin D content in *P. angulata*. Besides, Physalin B was only detected in 6/12 leaf samples with concentrations from 0.001 to 0.618%. Physalin B content in *P. angulata* collected in the Mekong Delta was low. In contrast, leaf samples in Binh Dinh province in Central Southern Coastal Region,

Vietnam was significantly high in Physalin B concentration. This difference can be explained by the different geographical and climatic conditions of the collected location.

## Conclusion

In this study, the new QuEChERS method and high-performance liquid chromatography (HPLC) with a diode-array detector (DAD) were developed for determining Physalin B and D compounds in the leaves of *P. angulata*. The optimum extraction procedure and QuEChERS method allowed for good efficiency and extraction yields. In addition, the HPLC protocol permitted a qualitative separation of the compounds and proved to be efficient, precise, and accurate. Therefore, it could be used for the simultaneous determination of Physalin B and D present of *P. angulata*. The experimental results of fresh samples collected from provinces of Vietnam indicated important sources of Physalin which there are specific applications from the leaves of *Physalis angulata* L. in the care of human health.

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