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Research paper



Optimisation of Gallic Acid and Quercetin Extraction from Phyllanthus Niruri

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Abstract

This paper presents an optimization study of gallic acid and queretin extraction from *Phyllanthis niruri* by using response surface methodology. Gallic acid and quercetin was extracted with the aid of sonication and the polyphenols content was analyzed using Ultra Performance Liquid Chromatography (UPLC). The effect of extraction time, amplitude and ethanol concentration on the yield of gallic acid and quercetin were investigated. The optimum gallic acid yield (10.43 ± 0.28 mg GA/g DW) was found in ethanol concentration of 40.0%, extraction time of 15 minutes and amplitude of 86.85% after solving the regression equation. Meanwhile, the optimum condition to obtain the highest yield of quercetin (17.48 ± 0.21 mg Que/g DW) was found in ethanol concentration of 40.0%, extraction time of 15 minutes and amplitude of 75%. The findings in this work may serve as a useful guide to obtain highest extraction yield of gallic acid and quercetin from *Phyllanthus niruri*.

Keywords: *Phyllanthus niruri; ultrasonic assisted extraction; gallic acid; quercetin; response surface methodology; UPLC.*

1. Introduction

Phyllanthus niruri ver. name Dukung anak (Family: Phyllanthaceae) is a widespread tropical herb that having a smooth bark on the ascending branches with the height of 50-70cm tall. It is found mainly in wet rainforest conditions and spreads rapidly throughout the tropical and subtropical countries such as Malaysia, Indonesia, Thailand, Nigeria, Brazil, and India. *P. niruri* was commonly used as a traditional medicine due to its well-known curative properties. For example, in India, *P. niruri* is a common herb used to heal sickness related to genitourinary system. *P. niruri* extracts had been investigated with the ability to restrict the hepatitis B virus growth, antifungal properties, anti-viral and hypoglycemic action for liver disease treatment [1]. *P. niruri* also has a diuretic property which allowed to be used in urinary tract infections. In addition, the active constituents from *P. niruri* also exhibit anticancer, antioxidant and anti-inflammatory properties by the presence of valuable polyphenols [2]-[3].

The most important factor that affects the yield and recovery of the bioactive components from plant materials is the extraction method. The previous extraction method performed was soxhlet extraction, the extraction time consumed up to 3 hours to obtain 150 ml volume of extract [4]. Another method of extraction performed by [5] was maceration that consumes 10 hours for the extraction process. Both the aforementioned extraction methods were the conventional and traditional method which normally required elevated temperature and longer duration. Furthermore, elevated temperature may cause the thermal degradation of the polyphenol due to the heat exposure for a prolonged period [6]. For instance, vitamin A and E, polyphenols and antioxidant suffer from thermal degradation after exposure to high temperature for a period of time [7]-[8]. Extraction process with a shorter residence time and operating at a mild temperature (< 70 °C) is preferable in order to minimize the thermal degradation of bioactive components during the extraction. Hence, ultrasonic assisted extraction (UAE) was chosen in this work because it operates at a mild temperature besides the sonication enhances both the bulk and inner mass transfer thus enhancing extraction yield and reducing extraction time. To our knowledge, limited study related to optimization of UAE parameters for extraction of *P. niruri* polyphenols, and hence this is the aims of the current work.

2. Material and Method

2.1. Chemicals and Plant Material

Ethanol, HPLC grade dimethyl sulfoxide, aluminium hexachloride, 2,2-diphenyl-1-picrylhydrazyl were obtained from Sigma Aldrich (St Louis, MO). *P. niruri* similar to that with a voucher specimen deposited in the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur (voucher number KLU46618) were purchased from Malaysia Herbal Shop, Selangor, Malaysia. The dried plant was crushed into powder. The powder was kept in an airtight plastic bag in a desiccator at room temperature to prevent moisture absorption prior to the experiment.

2.2. Ultrasonic Assisted Extraction(UAE)

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UAE was carried out using a sonicator Q700 with a probe of 13mm tip diameter. The solvent to plant solid ratio was fixed to 2 wt.%, throughout this work based on our one parameter at time study. The upper and lower bound of all the parameters in the two-level factorial study was set based on the literature and the limitation of the equipment. The two-level factorial (2LF) study was performed with three independent variables, namely, ethanol concentration (X1), time (X2) and amplitude (X3). There are two dependent variables: Gallic acid (Y1) and Quercetin (Y2). A total of 8 experiment points was carried out. Subsequently, optimization of the independent variable was performed according to central composite design (CCD).

2.3. UPLC Quantification of Polyphenols

Polyphenols from *P. niruri* extract such as quercetion and gallic acid was determined and quantified by Waters Acquity UPLC H-Class (Milford, MA) fitted with UPLC C18 Column (2.1 × 75mm, 1.8µm) and UPLC C18 column guard (2.1x5 mm, 1.8µm). The UPLC system is equipped with photodiode array detector and connected to a computer running Water Empower 2 software. The eluent system consists of A (0.1% formic acid in H2O) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.3 ml/min. The gradient elution: 0-5 min, 30% B; 5-10 min, 30-40% B; 10-15min, 40-50% B; 15-35min, 50-95% B; 35-45 min, 95-5% B. The column is maintained at room temperature and he injection volume of 2 µl was set for each sample. The sample is filtered using the 0.2 µm nylon membrane filter before injection to UPLC system. The peak of quercetin and gallic acid was detected at λ = 350 nm.

2.4. Validation Experiment

The optimum condition that gave the highest yield of gallic acid and quercetin within the range of the factor studied was obtained from the optimization study via response surface methodology (RSM). Verification was performed by running experiment according to the optimum conditions obtained from RSM and the actual response is compared to the predicted response from the model.

3. Results and Discussion

3.1. UPLC Quantification of Gallic Acid and Quercetin

Previous method employed to analyze active components from *P. niruri* was HPLC [9 - 11], with an analysis time to achieve active component separation ranging from 32 to 45 minutes. A faster quantification method is always desired to reduce the overall analysis time. In this work, UPLC which offers a faster separation was employed to *P. niruri* polyphenols for the first time. Figure 1 shows the UPLC chromatogram of *P. niruri* extract. The chromatogram showed a good separation for both gallic acid and quercetin. Active components were identified by comparing both retention time and UV spectra of the authentic standard and extract as shown in (Figs. 1 to 3). The retention time and UV spectra of both the standard and plant extracts show a good match, thus confirming the presence of gallic acid and quercetin. Quantification of active components was measured by comparing the peak areas from the extract and with the calibration curve developed using an authentic standard. The calibration curve of quercetin and gallic acid showed good linearity (r2 = 0.998) for the concentration range from 0.005 to 0.5 g/ml. The analysis time using UPLC method in this work is about 20 minutes, which is faster than other reported methods such as 32 minutes [12], 40 minutes [10] and 45 minutes [13].

3.2. Effect of Solvent Type to the Polyphenols Extraction

Solvent type is the one of the factors affecting the extraction of the polyphenol. The yield of polyphenol extraction using solvent of different polarities such as water, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, 100% ethanol, 20% isopropanol, 40% isopropanol, 60% isopropanol, 80% isopropanol and 100% isopropanol was studied for UAE. In order to get a fair comparison, all extractions were carried out at the equivalent solid to solvent ratio. Solvent polarity and its structural characteristic affect the solubility of bioactive components in a different solvent. It was found that highly methoxylated compounds such as quercetin, which is a lipophilic compound shows good stability in lower polarity solvent [5], [14]. Similarly, [15] reported that the amount of sinensetin and eupatorin, which is highly methoxylated compounds, found to be extracted at higher extracted amount at lower polarity solvent, isopropanol extract.

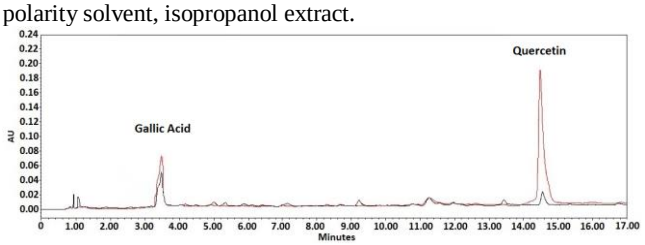


Fig. 1: Identification of active compound by comparing retention time.

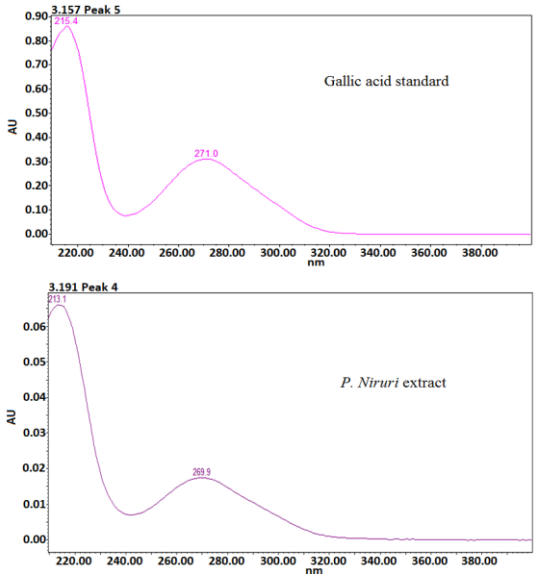


Fig. 2: Identification of gallic acid by matching UV spectra of sample to standard in Empower software library.

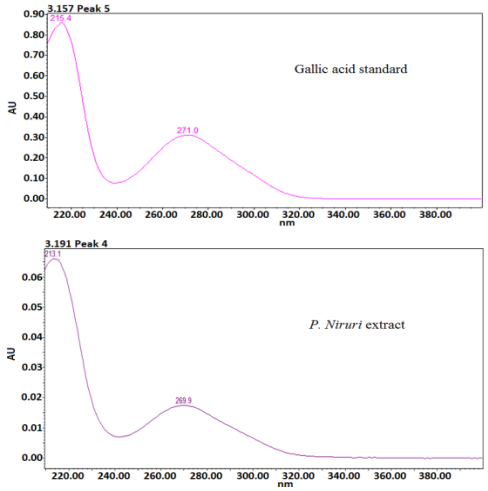


Fig. 3: Identification of quercetin by matching UV spectra of sample to standard in Empower software library.

On the other hand, a highly hydroxylated compound such as gallic acid, which is hydrophilic is much easily soluble in water. Result from this work shows that gallic acid has the higher extraction yield in water compared to quercetin. The result in Table I shows that aqueous alcoholic solvent (20% of either ethanol and isopropanol) has a higher simultaneous extraction yield of both gallic acid (13.19 mg GA/g DW) and quercetin (10.14mg Que/g DW) compared to pure solvent such as 100% ethanol, 100% isopropanol and pure water. The results suggest that the polarity of the solvents used affect the efficiency of the polyphenol extraction. The result shows that, a mixture of lower and high polarity solvent produced a higher extraction yield of both target components. For instance, solvent with a lower polarity index such as the isopropyl alcohol has a better efficiency to extract a wider range of phenolic content [16]. The major components present in Phyllanthus species is active hydrolyzable tannins that can be extracted using the ethanol-water mixture as the components are semipolar compounds such as ellagitannins and gallotannins [17], which is in good agreement to the result obtained in this work.

Table 1: Effect of Solvent on Polyphenols Extraction from *Phyllanthus niruri*

Solvent	Gallic Acid (mg GA/g DW)	Quercetin (mg Que/g DW)
Ethanol	0.99 ± 0.088	7.34 ± 0.730
Isopropanol	1.26 ± 0.011	8.55 ± 0.843
Water	15.44 ± 2.436	3.19 ± 0.539
20% Ethanol	13.19 ± 0.368	10.14 ± 4.519
40% Ethanol	9.86 ± 0.125	5.48 ± 0.314
60% Ethanol	9.20 ± 0.102	6.12 ± 0.206
80% Ethanol	4.42 ± 0.221	6.74 ± 1.148
20% Isopropanol	3.06 ± 0.336	9.10 ± 2.545
40% Isopropanol	2.67 ± 0.323	8.50 ± 1.437
60% Isopropanol	2.34 ± 1.190	9.63 ± 1.128
80% Isopropanol	2.21 ± 0.139	8.45 ± 0.455

3.3. Factorial Analysis of UAE

A 2^{5-1} factorial design with three parameters were studied for UAE. 8 experiments were tabulated for UAE factorial design. Fractional factorial experimental design and the result for UAE was tabulated in Table 2. Response was analysed by examining the model fitting, interpreting the model graphically, finding the optimum point, and performing a model validation.

3.3.1 Effect of Solvent concentration, Time and Amplitude

The effect of solvent concentration, time and amplitude on the polyphenol extraction yield is summarized in Table 2. The solvent concentration ranged from 20% to 80%, time ranged from 3 minutes to 9 minutes and amplitude ranged from 20% to 90% were studied in the factorial design.

Table 2: Experimental Design and Response for Factorial Analysis of UAE.

Run	Factors			Responses	
	EtOH concentration (%)	Time (min)	Amplitude (%)	Gallic Acid (mg GA/g DW)	Quercetin (mg Que/g DW)
1	80	9	90	4.009	97.825
2	80	9	20	2.803	84.179
3	20	3	90	3.784	88.273
4	20	3	20	2.672	78.72
5	20	9	20	3.624	93.731
6	20	9	90	4.62	101.919
7	80	3	20	1.705	69.168
8	80	3	90	3.188	84.179

Table 3 and Table 4 show the percentage contributions of each factor on the yield of extraction for gallic acid and quercetin. The main factor A, B and C played the major contribution in the gallic acid and quercetin extraction, which contributes more than 90% compared to the interactive factors. The suggested best condition for main factor in UAE to maximize gallic acid and quercetin yield are at 20% ethanol concentration, 9 minutes extraction time and 90% amplitude. The optimum condition was achieved by setting the ethanol concentration, time and amplitude in range, whereas maximising the gallic acid and quercetin yield. The desirability of the optimum solution is 0.998, which is closer to the maximum value of 1.0, indicating that the solution is close to the optimum condition for both gallic acid and quercetin extraction. Three experimental runs were performed on the optimum point obtained from the two-level factorial study. It was found that the predicted (from two-level factorial) and actual response (experiment) are in good agreement with deviation of less than 10%. The result indicates that the optimisation model based on two-level factorial study is sufficiently accurate to predict the gallic acid and quercetin extraction yield. It was found from 2LF analysis that, none of the factors A, B, and C has a combined interactive effect on the yield of gallic acid and quercetin.

Table 3: Sum of Squares and The Percent Contribution Of Each Factor For Gallic acid.

Factor	Effect Estimate	Sum of Squares	% Contribution
A-EtOH Concentration	-2.360	11.140	43.440
B-Time	1.890	7.160	27.830
C-Amplitude	1.840	6.840	26.590
AB	-0.240	0.110	0.440
AC	-0.480	0.460	1.780
BC	0.056	0.006	0.024

Table 4: Sum of Squares and the Percent Contribution of Each Term for Quercetin.

Factor	Effect Estimate	Sum of Squares	% Contribution
A-EtOH Concentration	-15.33	469.82	14.48
B-Time	21.76	946.94	29.18
C-Amplitude	28.27	1597.99	49.23
AB	-3.28	21.54	0.66
AC	3.37	22.77	0.70
BC	-9.03	162.99	5.02

Table 5: Comparison between the Predicted and Experimental Value for Optimum Condition from 2LF.

Response	Run	Predicted Value	Experimental Value	Error (%)
Gallic Acid (mg GA/g DW)	Run 1	8.231	8.215	0.195
	Run 2	8.231	7.596	8.36
	Run 3	8.231	7.574	8.674
Quercetin (mg Que/g DW)	Run 1	90.926	91.085	0.175
	Run 2	90.926	89.448	1.652
	Run 3	90.926	92.891	2.115

3.4. Experimental Design of UAE optimization

A CCD with a total of 20 experiments which include 7 runs for factorial design, 7 runs for axial points and 6 repetitive runs at the central point were performed. The CCD experimental design and responses is shown in Table 6. The values of regression coefficients were calculated, the response variable and the test variables were fitted to the second-order polynomial equation. The model equation in coded form is given as follows:

$$\text{Gallic acid} = 7.75 + 0.65A + 1.11B + 0.30C + 0.33AB - 0.18AC + 0.26BC + 0.25A^2 + 0.29B^2 - 0.34C^2 \quad (1)$$

$$\text{Gallic acid} = 13.59 + 1.60A + 0.44B - 0.25C + 0.43AB - 0.53AC - 0.67BC - 0.10A^2 - 0.25B^2 + 0.06C^2 \quad (2)$$

3.5. Effect of Ethanol Concentration, Extraction Time and Amplitude in Polyphenol Extraction

The effect of the three factors, i.e., ethanol concentration, extraction time and amplitude on gallic acid and quercetin extraction were analysed using RSM. Result from the experiment is shown tin Table 6. Three-dimensional response surface and contour plot were generated to study the interactive effect of the variables to the response. The effect of non-interaction factors ethanol concentration (A), time (B) and amplitude (C) on polyphenol extraction is depicted in Figure 4 for both gallic acid and quercetin. The interactive effects have p-value higher than 0.10 indicating that interaction between the factors is not significant to the response. The result obtained from CCD study agrees to the two-level factorial analysis.

Table 6: Experimental Design and Response for UAE Optimization.

Run	Ethanol concentration (%)	Time (min)	Amplitude (%)	Gallic Acid (mg GA/g DW)	Quercetin (mg Que/g DW)
1	48.5	11.00	82.50	8.207	14.996
2	15.0	15.00	75.00	7.214	14.569
3	27.5	11.00	95.11	6.114	14.043
4	27.5	11.00	82.50	7.524	19.092
5	27.5	17.73	82.50	10.254	16.139
6	40.0	7.00	75.00	8.125	14.954
7	27.5	11.00	82.50	7.414	16.994
8	27.5	11.00	82.50	8.820	18.645
9	27.5	11.00	69.89	6.365	14.741
10	15.0	15.00	90.00	8.781	14.269
11	27.5	4.27	82.50	5.786	15.053
12	27.5	11.00	82.50	8.082	19.616
13	27.5	11.00	82.50	7.937	17.875
14	40.0	7.00	90.00	7.948	18.433
15	15.0	7.00	75.00	6.044	14.635
16	6.5	11.00	82.50	7.598	18.422
17	27.5	11.00	82.50	6.898	18.388
18	15.0	7.00	90.00	7.465	12.931
19	40.0	15.00	75.00	9.737	18.113
20	40.0	15.00	90.00	11.500	15.711

Table 7: Condition for factors for optimum polyphenol extraction

Factor	Gallic Acid	Quercetin
Ethanol concentration (%)	40	40
Extraction time (min)	15	15
Amplitude (%)	86.85	75
Extraction yield (mg/g DW)	10.07	17.78
Desirability	0.825	0.888

The relationship between the response and experimental variables is shown in Figure 4. It can be observed that longer extraction time (>13 min) and higher sonication amplitude (ranging from 80 to 87%) gave a higher yield of gallic acid. Higher ethanol concentration (40%) is also favorable for gallic acid extraction. Quercetin extraction also favors longer extraction time (>13 min) and higher ethanol concentration (40%), in a similar manner to that of gallic acid. However, the best sonication amplitude for quercetin extraction is lower (75 to 78%) than that of gallic acid. Earlier, Nguang et al. [18] also found that 40% aqueous ethanol yielded the highest yield of total polyphenol and total flavonoid from *P. niruri.*

The optimum parameter to obtain the highest yield of gallic acid and quercetin was determined from the model equation with the condition of all the parameters is kept in range. Only one response is optimized at a time. The optimum condition for gallic acid extraction was found in ethanol concentration of 40%, extraction time of 15 min and sonication amplitude of 86.85% with the yield of gallic acid of 10.07 GA/g DW as shown in Table 6. Meanwhile, the optimum condition for quercetin extraction was found in ethanol concentration of 40%, extraction time of 15 min and sonication amplitude of 75% with the yield of gallic acid of 17.78 GA/g DW. The desirability for gallic acid and quercetin optimization is close to unity with the value of 0.825 and 0.888, respectively (Figure 5). The model is considered good since the desirability exceeds 0.8.

The suitability of the model equation to predict the desired response (gallic acid and quercetin yield) is tested experimentally using the optimum conditions described in Table 7. The result of the verification using triplicate run is presented in Table 8. The deviation between the predicted and measured responses was ranging from 0.14% to 4.74%. The experimental values were in good agreement with the predicted values of the model with an error less than 5%, which proved adequacy of the model for predicting the optimum yield of gallic acid and quercetin from UAE.

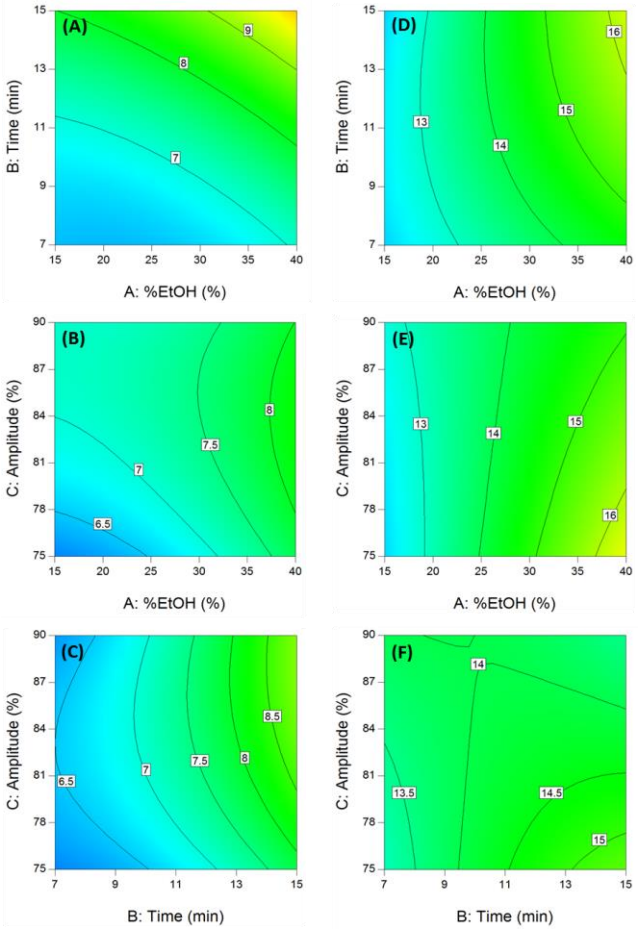


Fig. 4: Contour plot for gallic acid and quercetin extraction from P. niruri. Gallic acid (A, B, C). Quercetin (D, E, F).

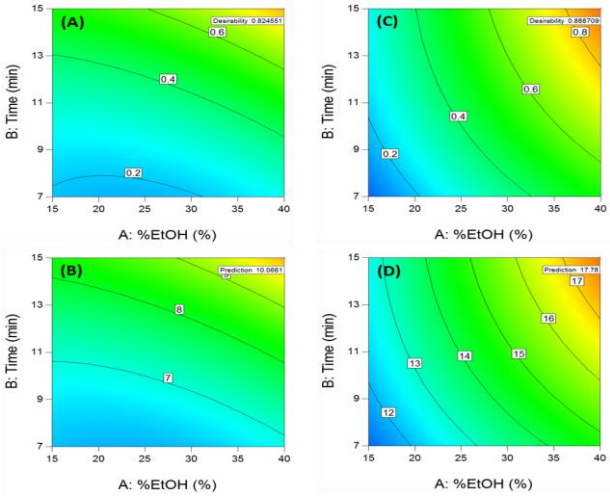


Fig. 5: Optimum extraction point for gallic acid (A, B) and quercetin (C, D)

Table 8: Validation of the predicted optimum point

Response	Run	Predicted Value	Experimental Value	Error (%)
Gallic Acid	**Run 1**	10.498	10.103	3.91
	Run 2	10.498	10.706	1.943
	Run 3	10.498	10.468	0.287
Quercetin	**Run 1**	17.212	17.524	1.78
	Run 2	17.212	17.692	2.713
	Run 3	17.212	17.236	0.139

4. Conclusion

The optimum condition for gallic acid extraction can be obtained using ethanol concentration 40%, extraction time of 15 minutes and amplitude of 86.85% after solving the regression equation. Under the optimum condition, the gallic acid yield was 10.426 ± 0.28 mg GA/g DW. Meanwhile, the optimum condition for quercetin extraction was found at ethanol concentration 40%, extraction time 15 minutes and amplitude 75%, which gave a quercetin yield of 17.484 ± 0.208 mg Que/g DW.

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