ХИМИЯ И ТЕХНОЛОГИЯ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ И БИОЛОГИЧЕСКИ АКТИВНЫХ СОЕДИНЕНИЙ
CHEMISTRY AND TECHNOLOGY OF MEDICINAL COMPOUNDS AND BIOLOGICALLY ACTIVE SUBSTANCES

RESEARCH ARTICLE

Optimization of total flavonoid content of ethanolic extract of *Persicaria pulchra* (Bl.) Soják for the inhibition of α-glucosidase enzyme

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Objectives. *There has been a rapid increase in the number of diabetic patients since the past few decades in developed and developing countries. This rapid increase is accompanied by alarming costs of treatment. α-Glucosidase inhibitors are one of the most effective drugs employed for the reduction of postprandial hyperglycemia to manage Type 2 diabetes mellitus. Additionally, flavonoids, a group of natural substances, which are widely distributed in plants and possess variable phenolic structures, exhibit outstanding hypoglycemic activity and are considered as potential α-glucosidase inhibitors. In Vietnam, Persicaria pulchra (Bl.) Soják (P. pulchra) is employed in traditional medications. It possesses high flavonoid contents and its anti-diabetes ability has been hypothesized, although it has attracted less attention for investigation. Hence, the aim of this study is to optimize the condition of the P. pulchra extract to obtain the highest total flavonoid content and measure the bioactivities of P. pulchra, such as the anti-α-glucosidase and antioxidant activities.*
Methods. *The effects of the extracting conditions, including the temperature, extraction time, liquid-to-solid ratio (LSR), and ethanol (C_2H_5OH) concentration, on the total flavonoid content are investigated via experiments and analyzed by the response surface methodology (RSM). Concurrently, the optimal extraction also determines the anti-α-glucosidase and antioxidant activities.*
Results. *The optimal extraction condition for the highest flavonoid content (530 mg quercetin/g) is determined in 60 min, at 53°C, with LSR of 9.46 g/g and C_2H_5OH concentration of 62%. Moreover, the optimal plant extract exhibits good α-glucosidase inhibition with a half-maximal inhibitory concentration (IC_{50}) of 22.67 mg/mL, compared to the positive control (acarbose −7.77 g/mL). Additionally, P. pulchra is proposed to be a potential antioxidant with an IC_{50} of ~12.68 µg/mL.*

Conclusions. *The study confirmed the optimal extraction condition of P. pulchra that will obtain the highest total flavonoid content and revealed the potentials of P. pulchra in α-glucosidase inhibition and antioxidation.*

Keywords: *flavonoid, plant extraction, optimization, anti-diabetes, response surface methodology.*

ОРИГИНАЛЬНАЯ СТАТЬЯ

Оптимизация общего содержания флавоноидов в спиртовом экстракте *Persicaria pulchra* (Bl.) Soják для ингибирования фермента α-глюкозидазы

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Цели. *В последние десятилетия в развитых и развивающихся странах наблюдается быстрый рост числа больных диабетом, который сопровождается ростом стоимости лечения. Ингибиторы α-глюкозидазы являются одним из наиболее эффективных препаратов, применяемых для снижения постпрандиальной гипергликемии при лечении сахарного диабета 2 типа. Кроме того, флавоноиды, группа природных веществ, широко распространенных в растениях и содержащих различные производные фенола, проявляют значительную гипогликемическую активность и могут служить потенциальными ингибиторами α-глюкозидазы. Во Вьетнаме Persicaria pulchra (Bl.) Soják (P. pulchra) используется в народной медицине. Он обладает высоким содержанием флавоноидов и, предположительно, противодиабетическими свойствами, хотя исследован мало. Таким образом, целью настоящего исследования является оптимизация экстрагирования P. pulchra для получения наиболее высокого общего содержания флавоноидов, а также определение его биологической активности – анти-α-глюкозидазной и антиоксидантной.*

Методы. *Экспериментально исследовано влияние условий экстрагирования, а именно температуры, времени экстракции, соотношения жидкость : твердое вещество и концентрации этанола, на общее содержание флавоноидов с помощью методологии анализа поверхности отклика. Показано, что оптимальные условия экстракции определяют анти-α-глюкозидазную и антиоксидантную активность.*

Результаты. *Найдены оптимальные условия экстракции для получения максимального содержания флавоноидов (530 мг кверцетина/г): время экстракции 60 мин, температура 53 °C, отношение жидкость : твердое вещество 9.46 г/г и концентрация этанола 62%. Растительный экстракт, полученный в оптимальных условиях, проявляет хорошее ингибирование α-глюкозидазы с концентрацией полумаксимального ингибирования (IC$_{50}$) 22.67 мг/мл по сравнению с положительным контролем (акарбоза – 7.77 г/мл).*

Выводы. *Исследование выявило оптимальные условия экстракции P. pulchra, позволяющие получить наиболее высокое общее содержание флавоноидов, и подтвердило перспективы применения P. pulchra для ингибировании α-глюкозидазы и антиоксидантного окисления.*

Ключевые слова: *флавоноид, экстракция растений, оптимизация, противодиабетические средства, методология поверхности отклика.*

INTRODUCTION

Diabetes is rapidly becoming one of the most severe diseases of the 21st century largely because of current unhealthy lifestyles, like the consumption of greasy food and lack of exercise [1]. Particularly, this disease accounted for the deaths of about 4 mln people in 2017, along with its accompanying huge cost of treatment (about USD 850 mln, which is expected to increase significantly by the year 2030 [2]. Moreover, the prevalence of diabetes has increased sharply in recent decades. In 1980, the number of diabetic patients was about 108 mln, which increased to 425 mln in 2017 and is predicted to be nearly 630 mln by 2045 [2, 3]. The disease is mainly divided into three groups: Type 1, Type 2, and gestational diabetes [4]. Type 1 diabetes is an autoimmune disease that is caused by the self-destruction of β-pancreatic cells, which are key to insulin production while Type 2 diabetes is caused by insufficient insulin secretion or insulin resistance of cells [5, 6]. Actually, Type 2 diabetes accounts for up to 95% of the cases, thereby demonstrating its widespread prevalence. The third form of diabetes is common with pregnant women because of significant changes in their hormones during pregnancy, which could manifest in an increase in the blood glucose level [4]. According to a previous study, diabetes mellitus is risky and can cause death because of its life-threatening complications, such as coronary artery disease, heart failure, retinopathy, nephropathy, and atherosclerosis [4, 7].

Diabetes mellitus is generally caused by unhealthy lifestyles and can be effectively treated by proper diets and exercises. However, the utilization of medicine is still the main therapy [8]. Some typical drugs that are employed presently to treat the disease are sulphonylurea, biguanide, thiazolidinediones, and α-glucosidase inhibitors [9–12]. Nevertheless, these drugs exhibit side effects on the health of patients. For instance, biguanide negatively affects the digestive system, kidneys, and increases the risk of contracting ketone infection [13]. Thiazolidinediones are a new approach to the treatment of insulin resistance but can cause heart failure, edema, and weight gain [13]. Alternative therapy has been developed so far and involves the utilization of bioactive compounds, which are extracted from natural plants, as a dietary supplement for the treatment of diabetes mellitus. Some natural substances, such as polyphenols, flavonoids, alkaloids, glycosides, and saponins, have been demonstrated to cure the disease through their structure–activity relationship (SAR) [14]. Therein, researchers have invested much attention to flavonoids because they exhibit diverse biological activities, especially their ability to promote the treatment of Type 2 diabetes and its resulting complications [15, 16]. Flavonoids have demonstrated a wide range of hypoglycemic functions and counteracted hyperglycemia by stimulating insulin production and enhancing its absorption into cells and inhibiting the enzyme, α-glucosidase, which is mainly responsible for the breakdown of oligosaccharides, trisaccharides, and disaccharides into glucose in the mucosa [17, 18]. The enzyme is inhibited by flavonoids via either competitive or non-competitive inhibition [17].

Amongst the anti-diabetic herbals, *Persicaria* plants demonstrated their ability to combat diabetes with an inhibitory capacity of ~70%, compared to rutin (86%) [19]. Many species belonging to the *Persicaria* genus, like *Persicaria hydropiper* and *Polygonum perfoliatum* L. are biologically active and are employed in traditional medicines because they include phenolics, flavonoids, phenylpropanoids, steroids, and benzoquinones [20, 21]. Nevertheless, a lot of other *Persicaria* plants, e.g., *P. pulchra*, which is utilized frequently in Vietnamese traditional medicine, have not been investigated for the treatment of diabetes. Based on previous studies on the bioactive compounds of other *Persicaria* plants, *P. pulchra* is well expected to contain high flavonoid contents to cure diabetes mellitus via α-glucosidase inhibition [22].

In this study, the optimal condition for the one-time ethanolic extraction of *P. pulchra* to obtain the highest flavonoid contents was determined by the response surface methodology (RSM). The method was introduced by Box and Wilson, in 1951, and has been applied to the determinations of the best conditions of extraction ever since [23]. The effects of the concentration of ethanol (C_2H_5OH), the extraction temperature, extraction time, and liquid-to-solid ratio (LSR) on the flavonoid contents were comprehensively investigated. Finally, the α-glucosidase inhibition and antioxidation of the optimal plant extract were evaluated.

MATERIALS AND METHOD

Materials

P. pulchra leaves were harvested from Binh Chanh District, Ho Chi Minh City, Vietnam. The plant was authenticated by the Department of Ecology and Evolutionary Biology of the Faculty of Biology and Biotechnology, Ho Chi Minh City University of Science, Vietnam National University. The following reagents were purchased from commercial suppliers in pure grade: C_2H_5OH, methanol (CH_3OH), distilled water, sodium nitrite ($NaNO_2$), sodium carbonate (Na_2CO_3), sodium hydroxide ($NaOH$), aluminum chloride ($AlCl_3$), and dimethyl sulfoxide (DMSO). The Folin–Ciocalteau reagent, quercetin (QUE), gallic acid (GA), *para*-nitrophenyl-α-D-glucopyranoside (*p*-NPG), α-glucosidase, and 1,1-diphenyl-2-picryhydrazyl (DPPH) were obtained from *Merck* (Germany).

Preparation of the ethanolic extract

Freshly harvested leaves of *P. pulchra* were rinsed with tap water to remove dirt and impurities. Thereafter, the leaves were air-dried, pulverized, and stored in a

sealed container for the experiment. Before the extraction, the moisture content of the sample was determined by a moisture analyzer Sartorius MB45 (*Sartorius*, Germany) to be ~11%.

The extracts were prepared, at controlled temperatures, extraction times, C_2H_5OH concentrations, and LSR (Table 1). Next, 5.00 g of the dried sample was extracted by aqueous C_2H_5OH in a 100 mL beaker, which was assisted by an agitator (300–400 rpm). The primary extract was filtered afterward, under vacuum condition, followed by rotary vacuum evaporation, until the excess solvent was completely removed. The extraction yield was determined by Eq. (1):

$$H = \frac{m_{extract}}{m_{sample}} \times 100\%, \qquad (1)$$

where $m_{extract}$ is the weight of dry extract (g) and m_{sample} is the weight of the dry leaf powder (g).

Determination of the total polyphenol content (TPC)

TPC in the extracts was determined, utilizing the Folin–Ciocalteu colorimetric method, according to Sánchez-Rangel [24]. Briefly, ~10 mg of the primary ethanolic extract was dissolved in 1 mL of pure DMSO to form a concentrated sample solution, which was diluted to different concentrations before the measurement. Next, 200 µL of the Folin–Ciocalteau reagent was mixed with 40 µL of the sample solution. The mixture was thereafter homogenized in a sonication bath for 5 min, at room temperature. Afterward, 600 µL of 20% Na_2CO_3 and 3.16×10^3 µL of distilled water were added to the mixture. A homogenous solution was obtained in the sonication bath after 30 min. Thereafter, the absorbance of the mixture was measured, at a wavelength of 760 nm. Moreover, GA was employed as a standard to depict the calibration graph.

Determination of the total flavonoid content (TFC)

A colorimetric method was applied to measure TFC [25]. Therein, ~10 mg of the extract was dissolved in 1 mL of CH_3OH to yield the mother liquor. To measure the sample absorbance, it was diluted to appropriate concentrations to obtain various sample solutions. Further, 2 mL of distilled water, 0.5 mL of the sample, and 0.15 mL of 5% $NaNO_2$ were mixed. After 5 min, 0.15 mL of 10% $AlCl_3$ was added to the mixture. Within 1 min, 1 mL of 1 M NaOH and 1.2 mL of distilled water were added. The absorbance of the mixture was measured, at a wavelength of 425 nm. QUE was utilized as a standard to illustrate the calibration graph.

Experimental design

The experiments adopted the rotatable central composite design (CCD) to determine the most suitable regression equation [23]. Compared to a single-factor optimization, this highly effective mathematical and statistical technique exploits higher accuracy by considering the interactions between variables. The objective is to determine the relationship between the factors and response values to determine optimal conditions. The Design Expert® 11 software (*Stat-Ease Inc.*, USA) was applied to this study.

The design consisted of 36 experiments, including 12 replicates, at the center points. The center points defined the experimental error and reproducibility of the data. The independent variables in this study are temperature (X_1: 45–65°C), time (X_2: 40–100 min), LSR (X_3: 8–12 g/g), and C_2H_5OH concentration (X_4: 50–80%). The coded and uncoded levels of the independent variables are described in Table 1. The values of the independent variables are expressed in codes, as −1, 0, and +1 interval, corresponding to the lower, center, and upper levels of each variable, respectively. Each experiment was performed three times to obtain the average TFC value, which was expressed as a response, Y, variable.

***In vitro* α-glucosidase inhibitory assay**

The investigation of the α-glucosidase enzyme inhibitory activity of the extract was conducted following Mahomoodally's method [26]. The test was performed on 96-well plates. The extract was dissolved in DMSO before the test. Additionally, 40 and 20 µL of the sample solution and α-glucosidase enzyme (1 U/mL) were added to the well, respectively. Next, 100 µL of a phosphate buffer (pH 6.8) was added to the mixture. Thereafter, the plate was incubated for 5 min, at 37°C. After that, 40 µL of 0.1 mM *p*-NPG was added to the reacting mixture. Incubation proceeded for 30 min, at 37°C. Subsequently, ~100 µL of 0.1 M Na_2CO_3 was added to terminate the reaction, and the absorbance of the sample was measured, at 405 nm. Acarbose was employed as a positive control. The percent inhibition of the α-glucosidase reaction was calculated as follows:

$$I\% = \left(\frac{A-B}{A}\right) \times 100\%, \qquad (2)$$

A: absorbance, at 405 nm of the blank (α-glucosidase and the substrate),
B: absorbance, at 405 nm of the subject (α-glucosidase, the substrate, and the sample).

***In vitro* antioxidant assay**

The antioxidant activity of the plant extract was investigated according to Sharma's method [27]. The antioxidative substances in the extract neutralized the DPPH radical by donating hydrogen. Thus, the reactive solution changed from violet to light-yellow, and its absorbance was measured, at 517 nm. Next, 180 µL of DPPH, which was dissolved in 80% CH_3OH, was added to 120 µL of the sample. The mixture was incubated for 30 min, at 30°C, in the dark. This was followed by the measurement of its absorbance, at 517 nm. Ascorbic acid

Table 1. Rotatable CCD with four factors and TFC (Y)

No.	Coded variables (Real variables)				TFC Y
	X_1	X_2	X_3	X_4	
1	−1 (45)	−1 (40)	−1 (8)	−1 (50)	469 ± 14
2	1 (65)	−1 (40)	−1 (8)	−1 (50)	478 ± 15
3	−1 (45)	1 (100)	−1 (8)	−1 (50)	460 ± 12
4	1 (65)	1 (100)	−1 (8)	−1 (50)	467 ± 7
5	−1 (45)	−1 (40)	1 (12)	−1 (50)	472 ± 14
6	1 (65)	−1 (40)	1 (12)	−1 (50)	491 ± 11
7	−1 (45)	1 (100)	1 (12)	−1 (50)	441 ± 11
8	1 (65)	1 (100)	1 (12)	−1 (50)	444 ± 11
9	−1 (45)	−1 (40)	−1 (8)	1 (80)	457 ± 21
10	1 (65)	−1 (40)	−1 (8)	1 (80)	461 ± 22
11	−1 (45)	1 (100)	−1 (8)	1 (80)	459 ± 12
12	1 (65)	1 (100)	−1 (8)	1 (80)	456 ± 16
13	−1 (45)	−1 (40)	1 (12)	1 (80)	444 ± 15
14	1 (65)	−1 (40)	1 (12)	1 (80)	453 ± 11
15	−1 (45)	1 (100)	1 (12)	1 (80)	424 ± 13
16	1 (65)	1 (100)	1 (12)	1 (80)	431 ± 15
17	−2 (35)	0 (70)	0 (10)	0 (65)	437 ± 11
18	2 (75)	0 (70)	0 (10)	0 (65)	440 ± 9
19	0 (55)	−2 (10)	0 (10)	0 (65)	449 ± 4
20	0 (55)	2 (130)	0 (10)	0 (65)	426 ± 14
21	0 (55)	0 (70)	−2 (6)	0 (65)	504 ± 10
22	0 (55)	0 (70)	2 (14)	0 (65)	474 ± 14
23	0 (55)	0 (70)	0 (10)	−2 (35)	461 ± 12
24	0 (55)	0 (70)	0 (10)	2 (95)	456 ± 13
25	0 (55)	0 (70)	0 (10)	0 (65)	534 ± 10
26	0 (55)	0 (70)	0 (10)	0 (65)	529 ± 9
27	0 (55)	0 (70)	0 (10)	0 (65)	536 ± 12
28	0 (55)	0 (70)	0 (10)	0 (65)	521 ± 14
29	0 (55)	0 (70)	0 (10)	0 (65)	529 ± 7
30	0 (55)	0 (70)	0 (10)	0 (65)	521 ± 12
31	0 (55)	0 (70)	0 (10)	0 (65)	528 ± 8
32	0 (55)	0 (70)	0 (10)	0 (65)	530 ± 7
33	0 (55)	0 (70)	0 (10)	0 (65)	532 ± 15
34	0 (55)	0 (70)	0 (10)	0 (65)	536 ± 7
35	0 (55)	0 (70)	0 (10)	0 (65)	522 ± 10
36	0 (55)	0 (70)	0 (10)	0 (65)	529 ± 9

was employed as a positive standard. The percentage inhibition can be calculated by the following equation:

$$I\% = \left(\frac{A-B}{A}\right) \times 100\% \qquad (3)$$

A: Absorbance, at 517 nm, of the DPPH radical in CH_3OH,
B: Absorbance, at 517 nm, of the DPPH radical solution, mixed with the sample.

RESULTS AND DISCUSSION

### Extraction efficiency, TPC, TFC, and α-glucosidase inhibition

Table 2 presents the bioactive compound contents in the highly concentrated ethanolic (96%) extract with LSR of 10 g/g, obtained after 60 min, at 50°C. TPC and TFC of the plant are 388 mg GA/g and 423 mg QUE/g, respectively. Interestingly, TPC and TFC of *P. pulchra* were much higher

Table 2. Total polyphenol content, total poly flavonoids content and α-glucosidase inhibition of *P. pulchra* compared to some bioactive plant extracts

Plant	TPC, mg GA/g	TFC, mg QUE/g	IC$_{50}$ of α-glucosidase inhibition, µg/mL
P. pulchra	388 ± 16	423 ± 16	33.53 ± 1.25
P. odoratum	52.59	19.97	–
P. minus	174	53.19	–
N. mirabilis	–	–	32.7
P. urinaria	–	–	39.7
K. candel	–	–	35.4

than those of other plants in the *Persicaria* family, such as *P. odoratum* and *P. minus* [28, 29]. The crude ethanolic extract of *P. pulchra* exhibited good inhibition of α-glucosidase with IC$_{50}$ of 33.53 ± 1.25 µg/mL, which is comparable to those of some anti-diabetic Vietnamese plants, like *N. mirabilis*, *P. urinaria*, and *K. candel* [30].

The extraction of bioactive compounds from dried P. pulchra, employing 96% C$_2$H$_5$OH obtained the highest yield, ~12%, in the one-time extraction. Figure 1 indicates that the extract recoveries, at the 2nd and 3rd extractions, decreased three to six times, compared to the 1st extraction, along with a decrease in the TPC and TFC contents. This
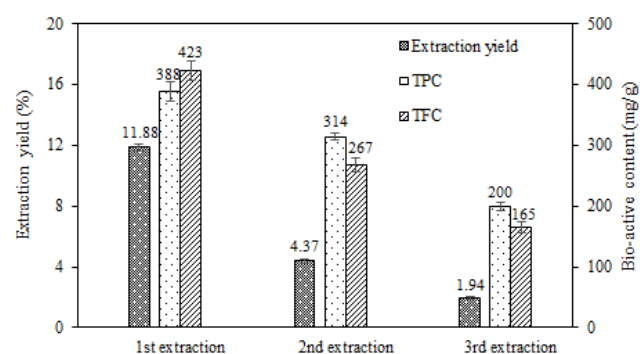
Fig. 1. Extraction yield, TPC, and TFC content of the ethanolic extract, at different extraction times.

was mainly caused by the reduced diffusivities of the bioactive compounds with several extraction rounds. In the one-time extraction, the substances in the plant readily diffused into C$_2$H$_5$OH because of the large concentration difference between the plant and the solvent. During subsequent extractions, those compounds were hardly extracted owing to their low concentrations in the plant. Consequently, more extractions, resulting in the utilization of more solvent and increase in the consumption of energy and time, were conducted. Therefore, the objective of this work was to investigate an optimal condition that would obtain the highest TFC content in the first extraction. Moreover, the α-glucosidase inhibition of the optimal extract was evaluated based on SAR between the flavonoids and diabetes treatment [31].

Effect of the extraction parameters on TFC

Some extraction conditions, such as the solvent concentration (%), temperature (°C), time (min), and LSR (g/g) exert significant impacts on flavonoid yields of natural plants. The effects of the extraction parameters on TFC are shown in Fig. 2. One of the important steps to optimize the bioactive compounds obtained from the plant is to select an appropriate solvent. CH$_3$OH and C$_2$H$_5$OH are "universal" solvents that could penetrate cell membranes and extract compounds via hydrogen bonds [32]. However, in the pharmaceutical industry, C$_2$H$_5$OH is mostly utilized, compared to CH$_3$OH, because it is less toxic.

Employing different solvent concentrations, from 35 to 95% (Fig. 2a), TFC of the plant extract peaked at 536 mg QUE/g with 65% C$_2$H$_5$OH, which is consistent with the result of previous studies [33]. It was predicted that the flavonoids in the plant were highly polar while higher concentrations of C$_2$H$_5$OH are averagely polar, thereby reducing its extraction capacity. The 65% C$_2$H$_5$OH was utilized afterward for the next experiments. As could be observed from Fig. 2b, the temperature greatly influenced the extraction of flavonoids from the plant. When the extraction temperature increased from 35 to 55°C, a corresponding increase in TFC, from 437 to 529 mg QUE/g, was observed and followed by a decrease in TFC, at a higher temperature, due to the thermal degradation of the flavonoids. According to the previous study, some thermally susceptible flavonoids, such as myricetin, kaempferol, rhamnetin, and QUE might be present in the plant extract [34]. A similar phenomenon could be observed in Fig. 2c in which TFC approached a peak of 534 mg QUE/g after 70 min. The temperature, at 55°C, and time, 70 min, were applied to the next experiments to investigate the effect of LSR on TFC. However, this factor did not affect TFC as significantly as the others. TFC of the extract was low at low ratios (6 : 1 and 8 : 1) because the difference between the concentration of the plant and the environment was not enough to induce the diffusion of the flavonoids into the solvent. The TFC value reached a peak of 532 mg QUE/g with a 10 : 1 ratio and decreased with increasing solvent. Other compounds, like tannins and saponins, in the plant could be extracted along with the flavonoids when a higher amount of the solvent was utilized. Therefore, TFC of the extract was reduced.
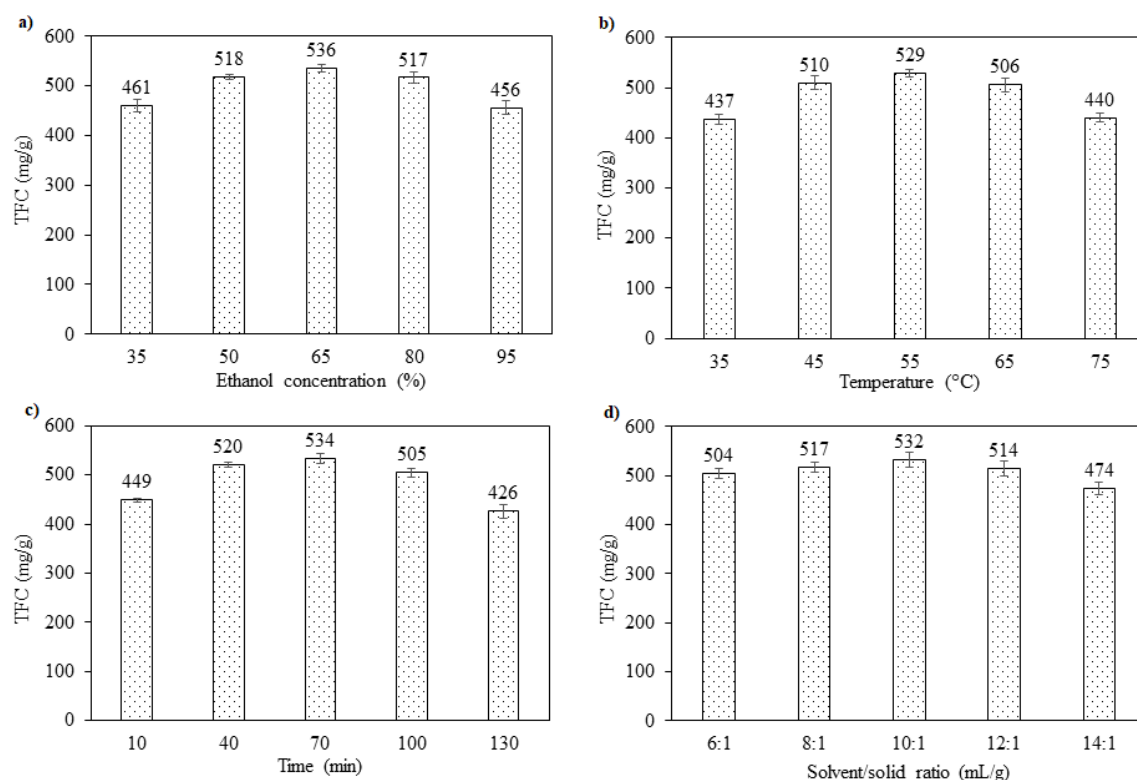
Fig. 2. Effects of (a) C_2H_5OH concentration, (b) temperature, (c) time, and (d) LSR on TFC.

Optimization of TFC in the first-time extract by RSM

Because of the effects of the C_2H_5OH concentration, time, temperature, and LSR on the TFC, those four factors were selected, as variables, to determine the optimal extraction condition. Generally, TFC of the ethanolic extract ranged significantly, from 424 to 536 mg QUE/g. Therein, the experiments at the center point resulted in the highest TFC, thus strongly indicating that the optimal point was close to the center (Fig. 3). Conversely, the two experiments that obtained the lowest TFC were those at the upper point (65°C, 100 min, and LSR of 12 : 1) because of a combination of disadvantageous factors against the extraction.

By applying the orthogonal planning method and employing the Design Expert® software, the regression

coefficients of the objective functions were calculated. The compatibility of the build model with the experiment was validated via the coefficient of determination (R^2 value). Table 3 presents the ANOVA analysis of the response surface model, which was extracted from the software. The F-value of the model, 106.10, implied the quadratic model whose *p*-value of <0.0001 was adequate to predict the TFC value. R^2 of 0.9861 indicated that the regression model accounted for 98.61% of the total variability.

The coefficients of the regression equation were derived from the formula of orthogonal matrices, after which they are tested to determine their significance level. There was only a 0.01% chance that an F-value, this large, could occur due to noise. *p*-Values, <0.0500,
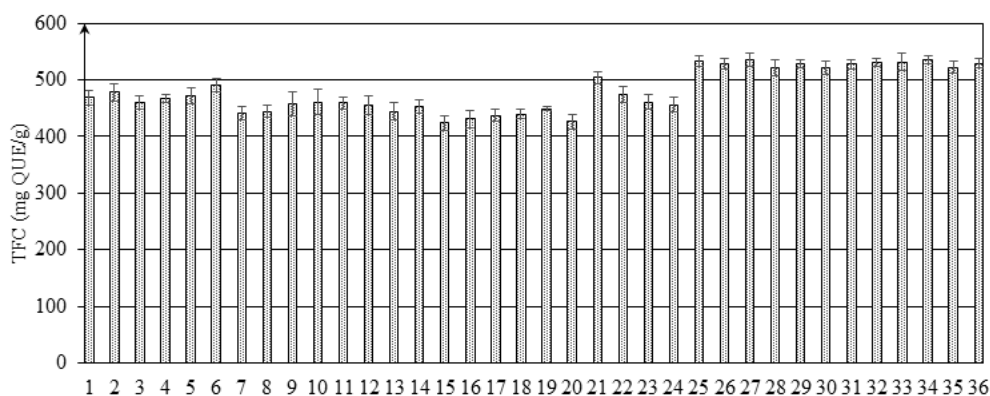

Fig. 3. TFC values of the plant extract, at 36 planning points.

Table 3. ANOVA table for the effects of the extraction parameters on TFC in the *P. pulchra* extracts

Source	DF (Degree of Freedom)	SS (Sum of Squares)	Mean square	F-value	p-value
Model	14	50341.02	3595.79	106.10	<0.0001
X_1	1	168.13	168.13	4.96	0.0370
X_2	1	1947.97	1947.97	57.48	<0.0001
X_3	1	1162.04	1162.04	34.29	<0.0001
X_4	1	900.38	900.38	26.57	<0.0001
X_1X_2	1	66.53	66.53	1.96	0.1758
X_1X_3	1	27.56	27.56	0.8132	0.3774
X_1X_4	1	27.56	27.56	0.8132	0.3774
X_2X_3	1	588.06	588.06	17.35	0.0004
X_2X_4	1	175.56	175.56	5.18	0.0334
X_3X_4	1	189.06	189.06	5.58	0.0279
X_1^2	1	16078.86	16078.86	474.41	<0.0001
X_2^2	1	12832.51	12832.51	378.63	<0.0001
X_3^2	1	3304.03	3304.03	97.49	<0.0001
X_4^2	1	10105.48	10105.48	298.17	<0.0001
Residual	21	711.73	33.89	–	–
Lack of fit	10	400.82	40.08	1.42	0.2870
Linear	0.6789				
Quadratic	<0.0001				
Cubic	0.2477				
R^2	0.9861				
Adjusted R^2	0.9768				

indicated that the terms of the model were significant. In this case, X_1, X_2, X_3, X_4, X_2X_3, X_2X_4, X_3X_4, X_1^2, X_2^2, X_3^2, X_4^2, were significant model terms. After the analysis, the Design Expert® software results of the second-degree regression equation for the virtual variables are as described in Eq. (4).

$$Y = 528.87 + 2.63X_1 - 9.23X_2 - 6.96X_3 -$$
$$6.13X_4 - 6.06X_2X_3 + 3.31X_2X_4 - 3.44X_3X_4 - \qquad (4)$$
$$-22.74X_1^2 - 21.03X_2^2 - 10.18X_3^2 - 17.81X_4^2$$

As could be seen in Eq. (4), all the four variables were captured in the equation, thus confirming the influence of the investigated factors on the extraction process. Additionally, the second-order regression coefficients were negative, indicating that the response surface was convex, and presented the maximum point. The temperature and time variables possessed the highest coefficients (22.74 and 21.03, respectively), proving that they exerted significant impacts on TFC. Moreover, the coefficient of the slope was close to the TFC value that was measured in the experiments, at the center point, and was significantly higher than the other coefficients in the resulting equation. Therefore, the predicted maximum point was around the center point and was approximately the value of the slope. The response surfaces were plotted from Eq. (2) to determine the optimal point and visually evaluate the interactions of variables with TFC. Due to the great impacts of the temperature and time parameters on the response variable, they were selected, at different LSR and C_2H_5OH concentrations, as illustrated in Fig. 4. Generally, the peaks of the response surfaces were near the center point of the temperature and time variables. At an LSR of 8 g/g (Figs. 4a, 4b, and 4c), the highest TFC was ~526 mg QUE/g with 65% C_2H_5OH, and the lowest value was 505 mg QUE/g with 80% C_2H_5OH. It was predicted that the optimal point of solvent concentration was near the 65% C_2H_5OH concentration. Additionally, the peaks of the response surfaces with LSR of 10 g/g were all higher than those with LSR of 8 g/g (Figs. 4d, 4e, and 4f). The TFC value simultaneously reached a peak with 65% C_2H_5OH and hit the low with 80% ethanol. With a higher LSR of 12 g/g (Figs. 4g, 4h, and 4i), a decrease in TFC was observed, along with an increase in the C_2H_5OH concentration. Therefore, it is possible to predict that the optimal conditions were close to LSR of 10 g/g and 65% C_2H_5OH. Summarily, the optimal conditions were close to the center points of the temperature (55°C), time (70 min), and C_2H_5OH concentration (65%) with LSR of ~10 g/g.
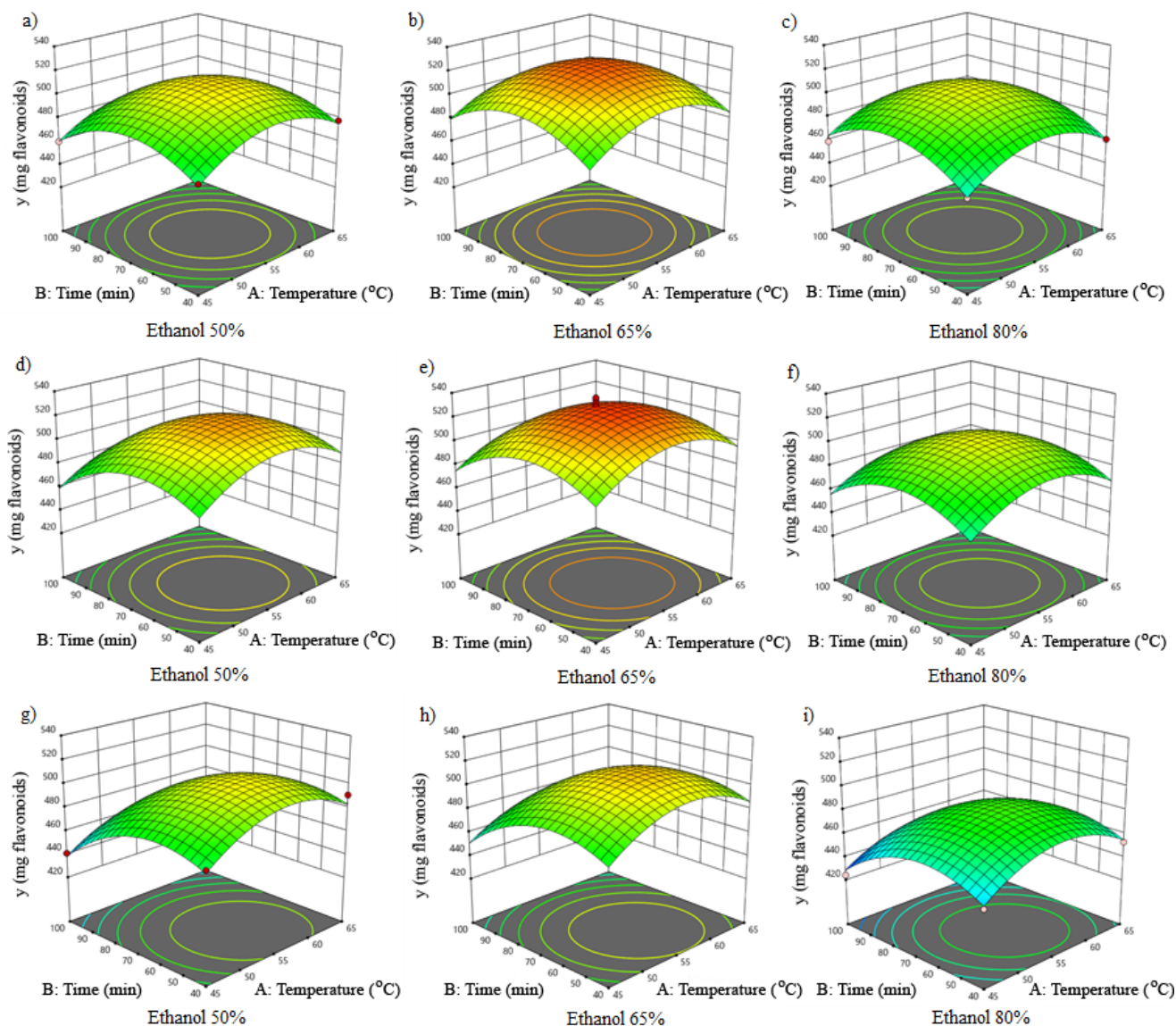
Fig 4. 3D response surfaces, plotted at LSRs of (a, b, c) 8 g/g, (d, e, f) 10 g/g, and (g, h, i) 12 g/g.

Employing the Design Expert® software, the optimal extraction conditions to obtain the maximum TFC were 52.25°C, 59.76 min, LSR of 9.201, and with 61.17% C_2H_5OH. The predicted TFC was 528 ± 11 mg QUE/g. To verify the obtained result, three experiments were performed, under the optimal conditions. As presented in Table 4, the plant extract obtained TFC of 531 ± 7, under the conditions of 53°C, 60 min, LSR of 9.46, and 62% C_2H_5OH, which is close to the predicted value. Generally, the error between the experimental and calculated values

was <5%, indicating that the obtained model was reliable and meaningful, in practice.

α-Glucosidase inhibition and the antioxidant activity of the optimal extract

The crude extract of *P. pulchra* exhibited good α-glucosidase inhibition with IC_{50} of 33.53 µg/mL. To determine the effect of the flavonoid content on this bioactivity, the relationship between TFC and IC_{50} should be investigated. As shown in Fig. 5, when TFC of the extract increased (from 423 to 526 mg QUE/g),

Table 4. Comparison of the experimental and predicted values, under the optimal condition.

Temperature, °C	Time, min	LSR, g/g	C_2H_5OH concentration, %	Predicted TFC, mg QUE/g	Experimental TFC, mg QUE/g	Error with the model, %
					526	0.38
53 ± 2	60	9.46	62 ± 0.5	528 ± 11	539	2.08
					529	0.20

IC$_{50}$ decreased (from 33.53 to 22.67 μg/mL), indicating an increase in the α-glucosidase inhibition. Because of the positive correlation between TFC and the enzyme inhibition, the optimal extraction condition for the highest TFC resulted in the best inhibitory capacity without the optimization of the IC$_{50}$ value, thus reducing the time and cost of the analysis.
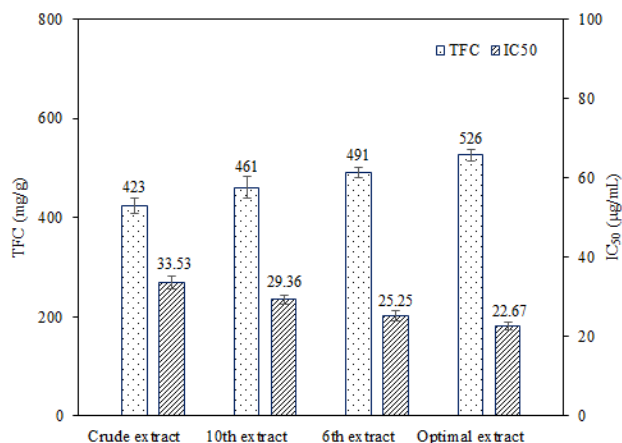

Fig. 5. Effect of TFC on the α-glucosidase inhibition of the plant extract.

Moreover, the IC$_{50}$ value of the optimal extract (22.67 ± 1.00 μg/mL) is only triple of that of acarbose (7.77 ± 0.36 μg/mL), which is a common α-glucosidase inhibitor for Type 2 diabetes. It was safely claimed that an optimal extract could be utilized as an alternative to acarbose to reduce its adverse effects [35, 36]. Flavonoids are not only α-glucosidase inhibitors; they are also great antioxidants, which neutralize free radicals, produce complexes with several metal ions or induce antioxidative enzymes in the body [37]. The antioxidant assay exhibited an IC$_{50}$ value of the optimal extract to be 12.68 ± 0.10 μg/mL, which is three times greater than that of ascorbic acid (2.82 ± 0.14 μg/mL). These results could broaden the knowledge of and avail new insights on the bioactivity of *P. pulchra*, particularly its potentials to inhibit α-glucosidase and its potential antioxidant activity for the treatment of diabetes.

CONCLUSIONS

Extraction factors, such as temperature, time, C$_2$H$_5$OH concentration, and LSR, were proven to exert significant effects on TFC in the one-time extraction of *P. pulchra*. The highest TFC was ~526 mg QUE/g of the sample, under the 53°C, 60 min, 62% C$_2$H$_5$OH, and LSR of 9.46 g/g conditions. Moreover, the relationship between TFC and the α-glucosidase inhibition of the plant extract was determined therein the optimal extract exhibited the lowest IC$_{50}$ value of 22.67 μg/mL.

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